Unravelling the malaria mosquito's sense of smell:

Neural and behavioural responses to human-derived compounds

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Contents

Propositions

1) Bacterial volatiles emanating from the human feet affect the detection of carbon dioxide. (This thesis)

2) The current degree of correlation between the *in vivo* electrophysiological responses of olfactory receptor neurons of *Anopheles gambiae* and the responses obtained from olfactory receptor proteins expressed in the *Drosophila melanogaster* heterologous expression system is not strong and necessitates *in vivo* validation of heterologous results in all cases. (This thesis)

3) Instead of mimicking the animal nose to create a portable diagnostic device, more attention should be given to actually use real animal noses in the detection of deadly diseases.

   Glatz, R., and K. Bailey-Hill. 2011. Mimicking nature's noses: From receptor deorphaning to olfactory biosensing. Progress in Neurobiology 93: 270-296

   Horvath *et al.* 2008. Human ovarian carcinomas detected by specific odor. Integrative Cancer Therapies 7: 2: 76-80

4) Cooperation between universities, research institutes, funding agencies and publishers is necessary to develop a new business model for publishing articles which will allow truly free access to scientific knowledge.

5) The preference of scientists for high impact factors slows down the fight against malaria.

   Insall, R. 2003. Impact factors: target the funding bodies. Nature 423, 585.

6) If suddenly all the annual malaria deaths in Africa would occur on one day, like a tsunami or an earthquake, the public would finally grasp the severity of the malaria problem.

   World Health Organisation. 2010. World Malaria Report 2010. Geneva.

7) Entrepreneurs that have failed in starting up their own business are seen as more experienced, scientists that have failed in reaching their research objectives are seen as not good enough.

Propositions belonging to the thesis, entitled

"Unravelling the malaria mosquito's sense of smell:
Neural and behavioural responses to human-derived compounds"

Remco A. Suer
Wageningen, 9 Mei 2011

Abbreviations

AeOR	*Aedes aegypti* olfactory receptor
AgGR	*Anopheles gambiae* gustatory receptor
AgIR	*Anopheles gambiae* ionotropic receptor
AgOR	*Anopheles gambiae* olfactory receptor
DmHES	*Drosophila melanogaster* heterologous expression system
EAG	electroantennogram
ESR	extensively sustained response
GLM	generalised linear model
GP	grooved peg
GR	gustatory receptor
IR	ionotropic receptors
lst	long sharp-tipped
OBP	odorant binding protein
OR	olfactory receptor
ORN	olfactory receptor neuron
PCA	principle component analysis
PLS	projections to latent structures by means of partial least squares
sbt	short blunt-tipped
SSR	single sensillum recording
sst	short sharp-tipped
TS (TSA-E)	trichoid sensilla (type A-E)
VIP	variable importance on the projection
w/v	weight/volume
w/w	weight/weight

Abstract

Malaria still affects the lives of more than a quarter billion people, killing more than 780,000 persons, mostly children, every year. With resistance to both drugs and insecticides occurring and growing, olfactory-based surveillance and attract-and-kill strategies are becoming more important for integrated vector management, which can be used in the fight against malaria and its principal vector, the mosquito *Anopheles gambiae*. Odours are involved in almost all behaviours of these mosquitoes, providing the opportunity for behavioural manipulation using olfactory cues. Knowledge on the peripheral olfactory system of the malaria mosquito is essential to realise such behavioural manipulation.

Within a large multi-national project that aimed for the discovery and utilisation of infochemicals to disrupt host-seeking behaviour of the malaria mosquito *An. gambiae*, the work presented in this thesis focused on the identification of chemical components, most of which are present in human emanations, that can be perceived by the peripheral olfactory system of the mosquito and the role of their possible involvement in host seeking.

Previous research had characterised the response spectra of the olfactory receptor neurons (ORNs) associated with grooved pegs and two of the five morphological types of trichoid sensilla; types C and E. The first part of this thesis represents a systematic study on the ORNs innervating the three remaining trichoid sensilla, types A, B and D. Stimulation of the ORNs using an odour panel of 132 compounds, 80 of which are found in human skin emanations, led to the identification of six response types for neurons located in long sharp-tipped trichoid sensilla (TSB) and five response types for neurons associated with short blunt-tipped trichoid sensilla (TSD). Some of the best ligands of the identified response types were human-derived compounds. The neurons located in the longest sharp-tipped trichoid sensilla (TSA) did not show responses to any of the odours tested.

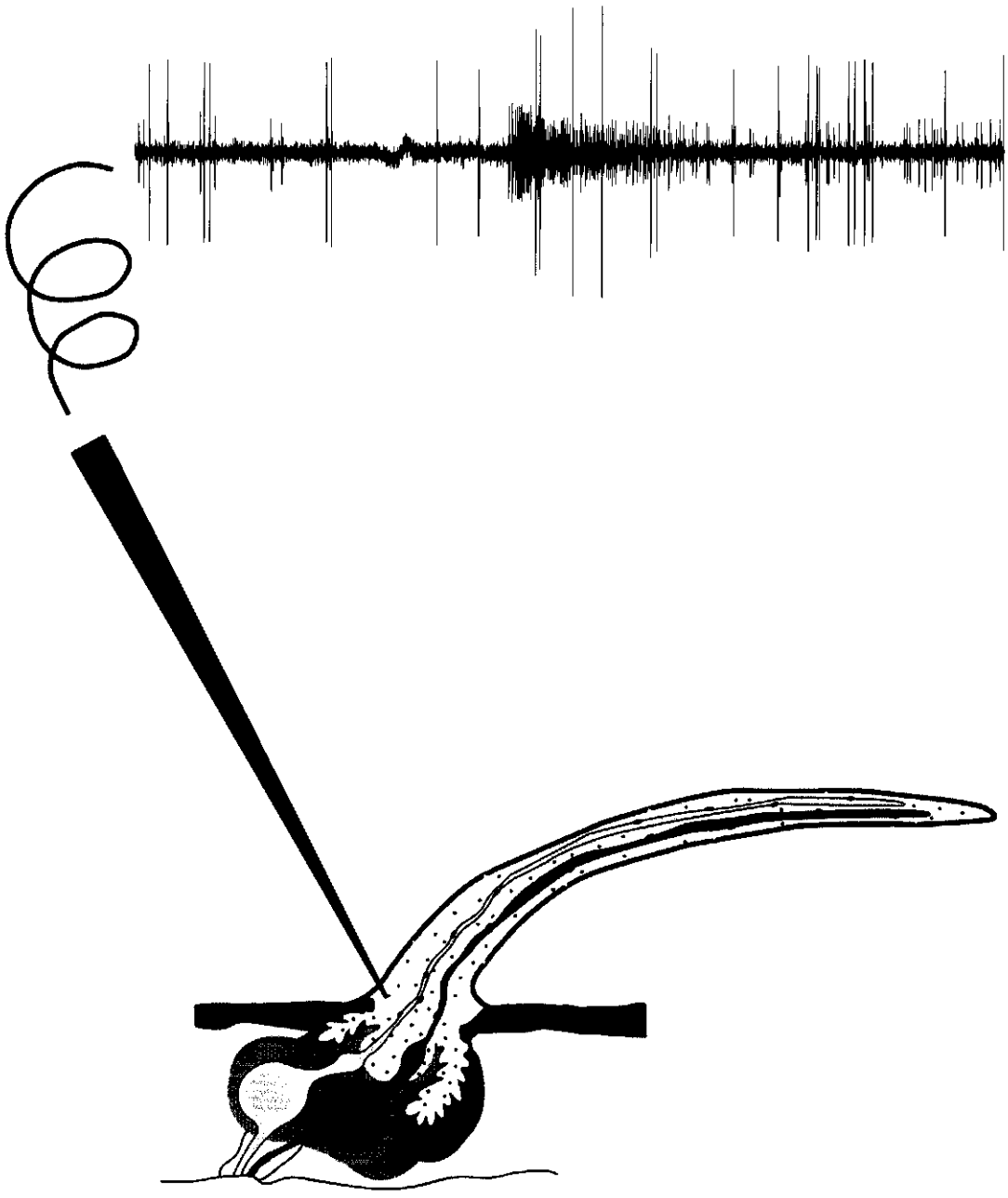
The search for novel repellents and attractants has been facilitated by previous work done by other members of the large multinational research consortium encompassing this thesis. They had identified 79 olfactory receptors (AgORs), 50 of which have been functionally characterised via the *Drosophila melanogaster* heterologous expression system (DmHES). The work presented in this thesis has built on these data and found that the profiles of seven of the 11 identified response types of the trichoid sensilla types B and D were significantly correlated with profiles of single *An. gambiae* olfactory receptors (AgORs) as obtained via the DmHES. Four of the profiles obtained *in vivo* could not be matched with profiles from individual, heterologously expressed AgORs. A direct comparison carried out in this thesis between AgOR8 expressed in the DmHES and *in vivo* electrophysiological recordings from the neuron expressing AgOR8 showed some dissimilarity between both. Therefore caution should be taken when extrapolating data from the heterologous system to the *in vivo* situation as differences in the responses and response spectra between both systems do exist.

The second part of this thesis shows that the physiological state of the mosquito influences odour perception and that ORNs of female *An. gambiae* present in the capitate peg of the maxillary palp are even more sensitive to human-derived odorants immediately after completing the first gonotrophic cycle compared to naïve unfed mosquitoes. Within the large project encompassing this PhD, it was discovered that bacteria present on human feet produce odours that were attractive to host-seeking *An. gambiae* females. Nine odours that were more abundant in the headspace of attractive bacterial samples are detected by the three ORNs associated with the capitate peg sensilla on the maxillary palps. The responses of these three palpal ORNs to the same odours varied with different physiological stages of the mosquitoes. It is known that host-seeking behaviour is suppressed during the first 48h following a blood meal. One ORN showed lower responses to several bacterial volatiles after a blood meal, while another ORN produced stronger responses to some of these volatiles produced by human skin microbiota after oviposition compared to before a blood meal. This is the first report of such increased sensitivity after completion of a gonotrophic cycle.

In addition it was found that five of these bacterial volatiles can completely suppress the response to CO_2, an important mosquito attractant. These five bacterial volatiles all elicited responses from other palpal ORNs and/or from other antennal ORNs as well. This suggests that volatiles produced by human feet microbiota can be used to manipulate mosquito host-seeking behaviour.

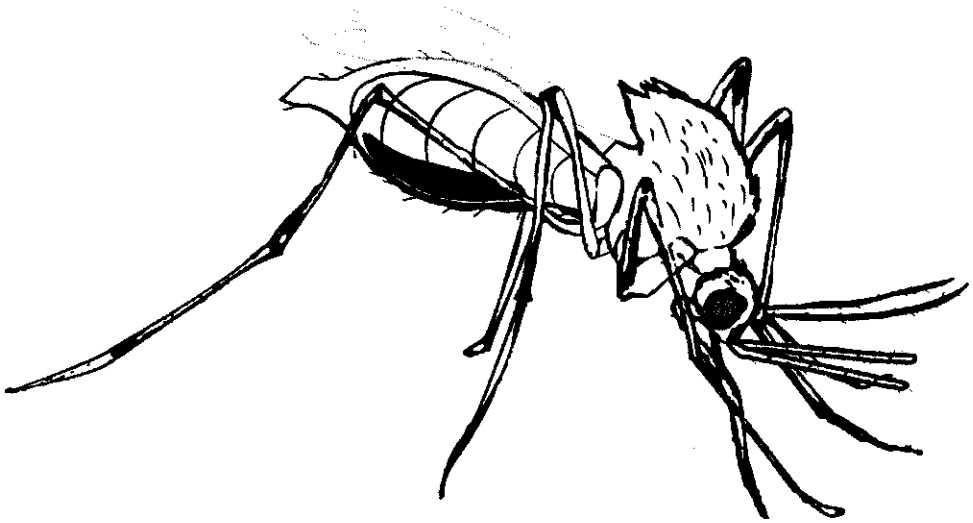
One of the bacterial volatiles (2-phenylethanol) as well as another compound of human origin (linalool oxide) that represents the best ligand of one of an earlier identified response type of trichoid sensilla B elicited an extreme, strong response with a unique temporal pattern from one of the two ORNs associated with trichoid sensilla E (TSE) of *An. gambiae*. These responses were named extensively sustained responses (ESRs), defined as an excitation response after a short 200 ms stimulus lasting for 10 minutes or more. This unusual temporal pattern has been described in detail and linked to behavioural changes that may be used for manipulation of mosquito behaviour. Such a direct link between particular temporal characteristics of electrophysiological activity of an insect ORN and ensuing changes in elements of host-seeking behaviour has not been described before. These findings highlight the importance of temporal features of chemosensory activity in modulating behaviour.

The results presented in this thesis increase our knowledge on the functioning of the peripheral olfactory system of the malaria mosquito *An. gambiae*. This provides a firm basis for further electrophysiological research and at the same time offers some promising leads for olfactory manipulation of host-seeking behaviour of mosquitoes, which might lead to more optimal blends for use in monitoring systems and attract-and-kill strategies necessary to decrease mosquito-human interactions and thereby lowering malaria transmission.



Chapter 1

General introduction



Remco A. Suer

1 General background information

1.1 Malaria

Malaria is one of the most common infectious diseases and the most important parasitic infection affecting humans. Malaria is widespread in tropical and subtropical regions (Greenwood 2005, Snow 2005). In 2009 around 243 million clinical cases were reported worldwide resulting in around 781.000 deaths, 91% of which were reported in Africa and most of them (85%) are young children below the age of 5 (WHO 2010).

Malaria is caused by protozoan parasites of the genus *Plasmodium*. The most serious forms of the disease for humans are caused by *Plasmodium falciparum* and *Plasmodium vivax*, but other related species (*Plasmodium ovale*, *Plasmodium malariae*, and sometimes *Plasmodium knowlesi*) can also infect humans (Hay *et al.* 2004, Greenwood 2005, WHO 2009). This group of human-pathogenic *Plasmodium* species are usually referred to as *malaria parasites*. The life cycle of these parasites consists of two different phases taking place in a mosquito and a human as hosts. When a mosquito carrying *Plasmodium* parasites takes a blood meal from a healthy human being, it injects the parasites together with saliva. In the human, sporozoites will enter the bloodstream, from where they are carried to the liver. After an incubation time in the liver cells, during which they are transformed to merozoites, the latter enter the bloodstream to invade red blood cells where they undergo asexual development. At this stage some parasites are converted to the sexual form (gametocytes). When a mosquito bites an infected human it takes up the sexual forms of the *Plasmodium* parasites that will continue their life cycle in the mosquito. In the mosquito the gametocytes are transported to the midgut where they undergo several changes, multiply and disperse to the salivary glands ready to be injected into another human.

Human malaria parasites are transmitted by female *Anopheles* mosquitoes. Only a small portion of all *Anopheles* species are capable of transmitting human Plasmodia. The most important vectors of human malaria belong to the *Anopheles gambiae* Giles *sensu lato* complex, with *Anopheles gambiae* Giles *sensu stricto* as the most effective.

1.2 Malaria vectors

Mosquitoes belong to the dipteran family of the Culicidae. The Culicidae comprises about 3500 species in three subfamilies: the Anophelinae (3 genera), the Culicinae and the Toxorhynchitinae (1 genus). The Culicinae consists of at least 37 genera which make up >80% of all mosquito species. Other major disease vectors belonging to the Culicinae are *Aedes aegypti*, capable of transmitting dengue fever, Chikungunya and yellow fever, and *Culex quinquefasciatus* which transmits West Nile virus and lymphatic filariasus. Malaria is only transmitted by anophelines. The genus *Anopheles* falls within the subfamily Anophelinae. The genus *Anopheles* consists of about 420 mosquito species of which around 70 species are known vectors of different species of *Plasmodium*. About 40 of these mosquito species are considered

important malaria vectors (Service 1993). Of which the most important vector species are *An. fluviatilis, An. culicifacies, An. quadrimaculatus, An. maculipennis atroparvus, An. funestus* and, as mentioned above, especially mosquitoes from the *An. gambiae* complex.

Anopheles gambiae is actually a complex of 7 sibling species. Midway in the 20th century it was established that *An. gambiae* mosquitoes in different parts of Africa showed different levels of vectorial capacity; some populations being much more efficient in spreading disease than others. When insecticides were introduced for malaria control, the differences in the responses of different *Anopheles* mosquitoes to house spraying with DDT became visible. Eventually the difference between species in their response to insecticide combined with their preference for either salt water, fresh water or mineral water for breeding has led to the identification of 7 sibling species using various methods, ranging from cross mating techniques (White 1974), to chromosomal differentiation (Coluzzi 1979, Coluzzi *et al.* 2002), to DNA studies using PCR (Scott *et al.* 1993). The seven species are two salt water species: *An. melas* at the African west coast (Theobald, 1903) and *An. merus* at the African east coast (Donitz, 1902), one mineral water species: *An. bwambae* found only in mineral springs in Uganda and three fresh water species: *An. gambiae* Giles *sensu stricto* (1902), *An. arabiensis* Patton (1905) and *An. quadriannulatus* A and *An. quadriannulatus* B (Theobald, 1911) (White 1974, Gillies and Coetzee 1987, Hunt *et al.* 1998, Coetzee *et al.* 2000). *Anopheles gambiae s.s.* is abundant throughout the continent, but generally more in humid areas. *Anopheles quadriannulatus* is found in certain parts of South Africa and Ethiopia. *Anopheles arabiensis* lives in sympatry with *An. gambiae s.s.* but with a better adaptation to drier areas (Gillies and Coetzee 1987, Coetzee *et al.* 2000, Coetzee 2004).

Anopheles gambiae s.s. is considered the most important malaria vector within this complex because it has a wide distribution, is anthropophilic (prefers humans), endophagic and endophilic (feeds and rests indoors), has a high susceptibility to infection by *Plasmodium falciparum*, which is responsible for the deadliest form of malaria, and *An. gambiae* has a relatively high survival rate, longevity and adaptability to breeding sites (Curtis 1996, Takken and Knols 1999, Coetzee *et al.* 2000, Coetzee 2004, WHO 2009).

1.3 Role of olfaction in mosquitoes

Olfaction is involved in almost all behaviours carried out by mosquitoes. Behaviours such as foraging (for blood and sugar), oviposition and, possibly, mating are all partly regulated by semiochemicals (Takken and Knols 1999). Other cues important for localising food, mates and oviposition sites are physical and visual cues. Physical cues, like heat and moisture (Laarman 1955, Takken *et al.* 1997, Olanga *et al.* 2010), are known to increase behavioural responses of mosquitoes and visual cues are used in upwind flight at low light conditions (Gibson 1995). However, the most important cues to a nocturnal species like *An. gambiae* are olfactory (Takken and Knols 1999).

1.3.1 Sugar feeding behaviour

Both male and female mosquitoes are known to use sugar as an energy source. Natural sugar sources include nectar and honeydew which the insect locates through both visual and olfactory cues (Foster 1995). The mosquito starts life as an egg which transforms into a larva that goes through four larval stages, then transforms into a pupa from which it emerges as an adult mosquito. After emergence mosquitoes have very low energy reserves which will create a lethal energy deficit if they do not feed within the first 36 h (Takken *et al.* 1998). Even female malaria mosquitoes need sugar before using blood as food source. Sugar feeding behaviour seems to be largely odour-mediated. When 1-day old females and males were given the choice between honey and human skin volatiles (as replacements for sugar-related and human-related volatiles) both preferred the honey. After five days of access to sugar, males continued to prefer honey but the females changed their preference in favour of human volatiles (Foster and Takken 2004).

1.3.2 Mating behaviour

In many mosquito species, mating starts with a female reaching a swarm of males usually during twilight above sites with distinct visual cues (Marchand 1984). Male mosquitoes recognize females reaching a swarm by their lower wing beat frequency compared to males (Clements 1999). *Anopheles gambiae* mosquitoes, however, are known to form mixed swarms with greatly overlapping wing beat frequencies (Tripet 2004), which suggests that other recognition mechanisms must be involved. Recent evidence has indicated that wing beat frequencies are important for mate recognition (Cator *et al.* 2010, Pennetier *et al.* 2010). Mosquito olfactory cues might also be of importance during mating. Cues like sex pheromones and species-specific contact pheromones. have been described for several mosquito species (Kliewer *et al.* 1966, Nijhout and Graig 1971) The proportions of several cuticular hydrocarbons of female *An. gambiae* changes after mating (Polerstock 2002) indicative of a possible involvement in the chemical communication between males and females. Attraction to host odours may also be a mating cue. It is known that certain species intercept females and mate at the host (McIver 1968, Takken and Knols 1999, Yuval 2006). Mating in *Anopheles* species occurs in swarms and it has been hypothesised that these swarms produce several volatiles that might act as sex and/or aggregation pheromones (B.G.J. Knols, pers. comm.).

1.3.3 Oviposition behaviour

Because mosquitoes need to find suitable water bodies, the selection of a suitable habitat for oviposition is of high important for the fitness of organisms like mosquitoes that do not show any parental care as is the case for most mammals for example. For selection of oviposition sites mosquitoes depend on and make use of tactile, chemotactile, olfactory, or visual cues to

assess the characteristics of that breeding site (Bentley and Day 1989, Millar *et al.* 1992, Takken and Knols 1999, Blackwell and Johnson 2000).

Mosquitoes can use several chemical odour cues to locate potential breeding sites, such as substances emitted from mosquito larvae, pupae and eggs, or from organic materials in the water such as soakage pits and hay and grass infusions (Millar *et al.* 1992, Allan and Kline 1995, Sumba *et al.* 2004, Rejmankova 2005). Miller (1992) reported that *Culex quinquefasciatus* was attracted to several chemicals found in hay infusions, like phenol, 4-methylphenol, 4-ethylphenol, 3-methylindole and indole.

Information on chemical compounds possibly used for oviposition site selection is available for several *Culex* and *Aedes* mosquito species, which transmit diseases like dengue and West-Nile virus, but remains rather scarce for *An. gambiae*. Several compounds, such as 3-methylindole, *m*-cresol, 4-methylcyclohexanol and indole, were found to be potential oviposition attractants for *An. gambiae* Giles (Blackwell and Johnson 2000). Lindh *et al.* (2008b) showed that gravid *An. gambiae* females were attracted to odours produced by bacterial species isolated from known oviposition sites. These authors reported 13 putative oviposition attractants among which 3-methylbutanoic acid, 3-methyl-1-butanol, 2-phenylethanol, 2-tridecanone and indole.

1.3.4 Host-seeking behaviour

As mentioned above, during their lifetime male mosquitoes will only feed on sugar sources. With exception of a few autogenous species, female mosquitoes need a blood meal to complete their gonotrophic cycle and to build up sufficient energy reserves before egg development can occur (Takken *et al.* 1998).

During host-seeking, female mosquitoes use physical and chemical cues emanating from host organisms to detect and locate them. Heat, moisture and visual cues help mosquitoes in detecting hosts at close range (Laarman 1955, Gibson 1995). Olfactory cues are thought to be more important at intermediate and larger distances. Carbon dioxide (henceforth abbreviated as CO_2) is one of the best known kairomones for mosquitoes and has been shown to attract mosquitoes up to distances of almost 40 m (Gillies and Wilkes 1968, Takken 1991). Next to carbon dioxide, several chemical compounds emitted by cattle and humans were found to attract mosquitoes, such as 1-octen-3-ol, ammonia and lactic acid (Gillies and Wilkes 1968, Kline *et al.* 1991b, Kline *et al.* 1991a, Takken 1991, Cork and Park 1996, Takken and Knols 1999, Meijerink *et al.* 2000, Braks *et al.* 2001, Zwiebel and Takken 2004, Smallegange *et al.* 2005b). Human skin emanations are also involved in host-seeking behaviour.

Human skin odour is a complex blend of a multitude of volatiles. GC-MS analysis revealed 346 compound peaks (Bernier *et al.* 1999, Bernier *et al.* 2000), most of them have been identified and several compounds are known to attract *An. gambiae* (Takken and Knols 1999, Meijerink *et al.* 2000, Meijerink *et al.* 2001, Qiu *et al.* 2006, Okumu *et al.* 2010). A recent study by Verhulst *et al.* (2009) showed that bacterial samples collected from human feet and subsequently grown on agar were attractive to mosquitoes. In these bacterial samples 14

volatile compounds were more abundant in the headspace from the agar plates with higher concentrations of bacteria. A synthetic blend composed of 10 of the 14 compounds was attractive to female *An. gambiae* mosquitoes in olfactometer assays and semi-field experiments.

After ingesting a blood meal, female mosquitoes stop host seeking and start to search for an oviposition site. The yellow fever mosquito *Aedes aegypti* (L.) stopped responding to human odours after having had a blood meal (Klowden and Lea 1978) and females that had taken a blood meal but retained eggs did not respond to host cues until 24 h after oviposition (Klowden and Lea 1979). Takken *et al.* (2001) showed similar responses for *An. gambiae*; the first 24 h after a blood meal *An. gambiae* did not respond to the odours of a human hand anymore but gradually they started to respond to host cues again until 72 h later they reached the same level of responsiveness as before a blood meal (Takken *et al.* 2001). This suggests that host-seeking behaviour is inhibited for approximately two days after blood feeding to repletion (Takken *et al.* 2001), which is supported by the down-regulation of olfactory genes following a blood meal (Fox *et al.* 2001).

Research is now focussing on the chemical identity of the volatiles to which malaria mosquitoes respond and what their role is in host detection and localisation. These odours may be used for the manipulation of these behaviours in order to monitor and control the mosquito population and thereby prevent malaria transmission.

2 Morphology of mosquito olfactory system

2.1 Olfactory organs

Insects have various organs involved in the perception of chemical or physical stimuli. On these organs small cuticular extensions of various forms, called sensilla, are present. The sensilla are involved in stimulus perception and can be found on various body parts of insects, including for example the head appendages but also the legs (McIver 1982, Hansson 1999). In mosquitoes, the perception of olfactory cues is made possible through specific sensilla that are mostly found on the head appendages (Fig. 1). Until very recently it was thought that only the mosquito antennae and maxillary palps contained olfactory sensilla (McIver 1982). Molecular studies on candidate chemoreceptors in *Ae. aegypti*, *An. gambiae* and *Cx. quinquefasciatus* found olfactory receptor genes expressed in tissue of the proboscis, suggesting that also this organ might have an olfactory function (Melo *et al.* 2004, Pitts *et al.* 2004, Xia and Zwiebel 2006). This was proven by Kwon *et al.* (2006) who showed electrophysiological activity of sensilla on the proboscis of *An. gambiae* in response to stimulation with odours.

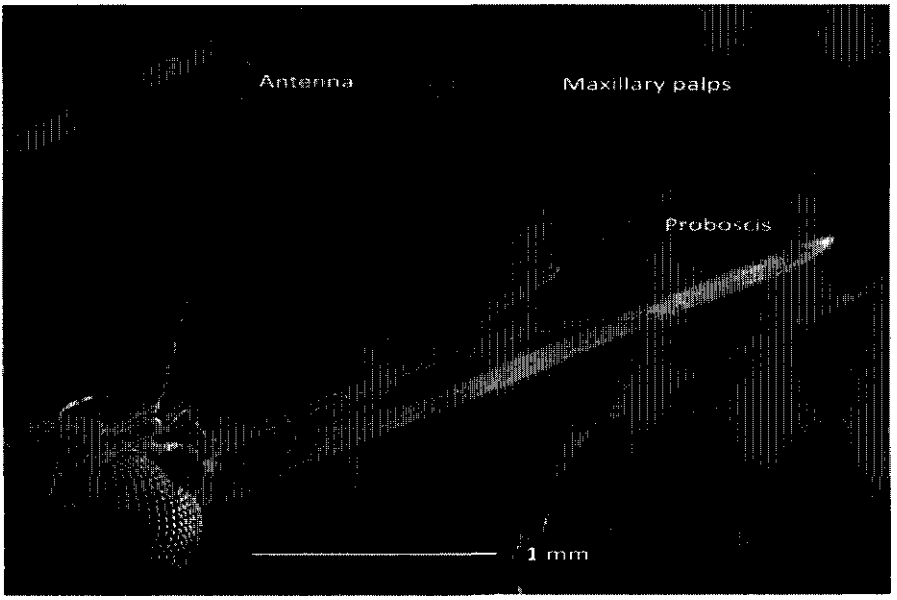


Figure 1: Scanning electron micrograph of the head of a female *Anopheles gambiae s.s.* showing the three olfactory organs; the antenna, maxillary palp and the proboscis. Photograph by H. Smid.

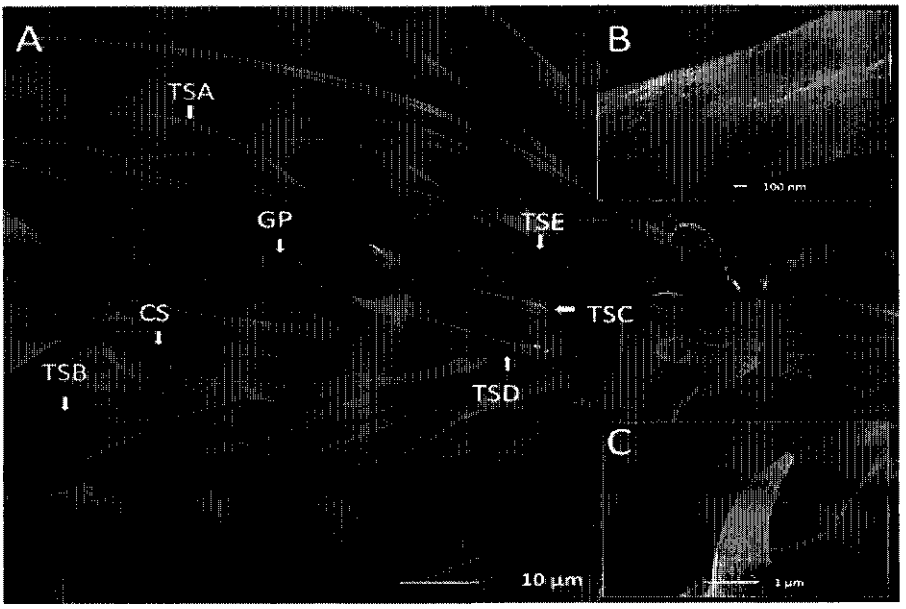


Figure 2: Scanning electron micrograph of antennal olfactory sensilla of *Anopheles gambiae*. **A)** An antennal segment containing different types of olfactory sensilla. **B)** A close-up of a trichoid sensilla showing the single-walled outer structure with pore channels through which odour molecules enter the inner sensillum. **C)** Close-up of a grooved peg, clearly showing the double-walled finger like structure. Spoke channels between these fingers lead into the inner sensillum. TSA-E: trichoid sensilla type A-E; GP: grooved peg; CS: coeloconic sensilla. Photograph by H. Smid.

2.2 Olfactory sensilla

Several types of olfactory sensilla are present on the olfactory organs of mosquitoes; sensilla trichodea, grooved peg sensilla, capitate peg sensilla and coeloconic sensilla (Fig. 2). A standard insect olfactory sensillum contains one to several bipolar olfactory receptor neurons (ORNs) that send their branched or unbranched sensory dendrites into the sensillum lumen (Fig. 3). On these dendrites the olfactory receptor molecules can be found that bind the odour molecules.

There are roughly two distinct categories of olfactory sensilla in relation to differences in structural properties; single-walled and double-walled sensilla. Single-walled sensilla contain walls with pore channels through which transportation of the odour molecules occur (Steinbrecht 1997; Fig. 3C). Conversely double-walled sensilla have so-called spoke channels through which molecule transportation probably occurs. These spoke channels are formed at the contact points where the hollow cuticular finger-like structures, of which the double-walled sensilla is composed, are fused with each other (Fig. 2B). Double-walled sensilla generally seem to be innervated by more ORN than single-walled sensilla. Of the olfactory sensilla the trichoid sensilla on the antenna, capitate peg sensilla on the maxillary palps and the T2 chemosensilla on the proboscis are described as single-walled sensilla (Ismail 1964, Boo 1980, McIver 1982). The grooved pegs and coeloconic sensilla on the antennae have a double-walled structure (Ismail 1964, Boo and McIver 1976, Pappas and Larsen 1976, McIver 1982).

2.2.1 Antenna

Trichoid sensilla (TS) are the most abundant olfactory sensilla on the antennae of female mosquitoes ranging from 200-1200 per antenna depending on the mosquito species (Ismail 1964, Boo 1980, McIver 1982, van den Broek and den Otter 2000, Pitts and Zwiebel 2006). Trichoid sensilla are single-walled hair-like cuticular extensions containing dendrites of one to two ORNs. Odour molecules can reach the olfactory receptor (OR) molecules on these dendrites via a stimulus-conducting system of pores, pore kettles, and pore tubules for single walled sensilla like the trichoid (Muir and Cribb 1994, Steinbrecht 1997). These trichoid sensilla can be divided into either four (*Aedes* and *Culex*) or five sub-types (*Anopheles*) based on length (between 20 µm and 60 µm), shape (sharp-tipped or blunt-tipped) and the thickness (thick or thin walled) of the walls (Boo 1980, McIver 1982). The four subtypes of *Aedes* and *Culex* species are the short sharp-tipped (sst), the long sharp-tipped (lst) and the short blunt-tipped type I (sbtI) and type II (sbtII), type II being the shortest of all. The sharp-tipped trichoid sensilla have thicker walls than the blunt-tipped trichoid sensilla (McIver 1982). The classification for the trichoid sensilla (TS) of *Anopheles* species is different. For *Anopheles* five different subtypes have been named, called type A-E (Boo 1980). Trichoid sensillum A is the longest type with the thickest wall and a sharp tip bent towards the flagellum. Trichoid sensillum B has about 85% of the length of TS A, has a thinner wall, and a wider base diameter than TSA. Trichoid sensillum C is intermediate in all characteristics. It is shorter than TSB but longer than TSE. Wall thickness is intermediate as well. So is the tip shape, more blunt than B but sharper than TSD.

TSD is the easiest to recognise. It is a short (65% of the length of TSA) blunt-tipped sensillum with a thin wall. TSD resembles most the sbtII type in *Aedes* and *Culex* species. The last subtype is TSE. This is the shortest TS with a sharp tip (Boo 1980, McIver 1982).

A completely different type of olfactory sensillum is the grooved peg sensillum (Fig. 2C). These sensilla are short, double-walled peg-like structures containing dendrites of three to five olfactory receptor neurons. Depending on the mosquito species 10 to approximately 350 grooved pegs of 7 - 15 µm length are found per female antenna. Several studies reported two morphologically and electrophysiologically distinct subtypes. A long and short form of grooved peg sensilla has been found in several *Aedes* and *Culex* species (McIver 1974, 1982, Bowen 1995). Two subtypes have also been described for *An. stephensi* (Boo and McIver 1976) but a recent comparison between *An. gambiae* and *An. quadriannulatus* could not make such a division (Pitts and Zwiebel 2006).

The last type of olfactory sensillum on the antenna is the large coeloconic sensillum, also referred to as pitted pegs. This type of olfactory sensillum is only found in anophelines (McIver 1982, Sutcliffe 1994). Large coeloconic sensilla are double-walled peg-like structures found in round openings in the cuticle. The number of large coeloconic sensilla varies from 28-50 per antenna depending on the species. Each sensillum is innervated by 4-5 ORNs (Boo and McIver 1975, 1976, McIver 1982, Pitts and Zwiebel 2006).

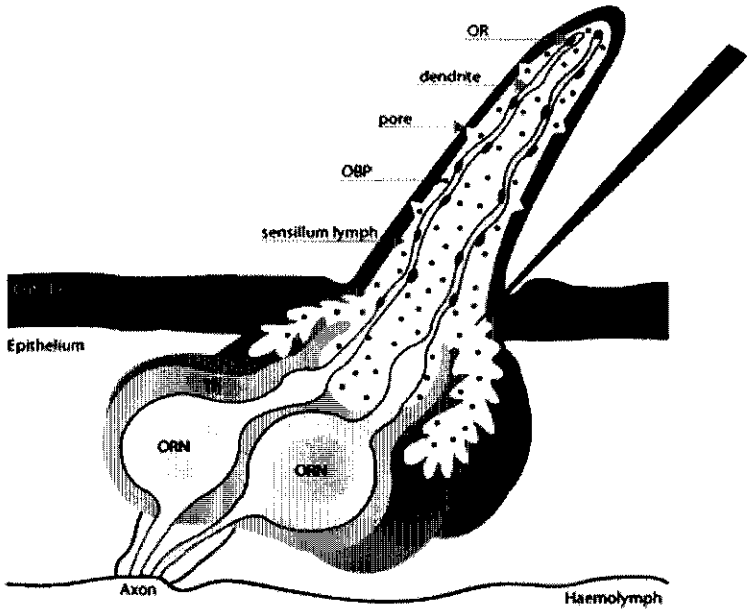


Figure 3: Schematic drawing of the structure of a typical olfactory sensillum. The black needle represents the tungsten electrode used to record the action potentials generated by the olfactory receptors on the dendrites of the olfactory receptor neurons (ORNs).OR: olfactory receptor; OBP: odour binding protein; and the accessory cells: tormogen (To), thecogen (Th) and trichogen cells (Tr). Modified after Jacquin-Joly and Merlin 2004.

2.2.2 Maxillary palp

On the maxillary palps of female mosquitoes only one type of olfactory sensillum is found, the capitate peg sensillum (Fig. 4A). This is a thin single-walled club-shaped sensillum innervated by three ORNs. The capitate peg is between 20-25 µm in length and contains numerous pores (McIver 1972, McIver and Siemicki 1975).

2.2.3 Proboscis

The type 2 (T2) chemosensilla (Fig. 4B) present on the proboscis of anopheline and culicine mosquitoes appear to have an olfactory function (Melo *et al.* 2004, Pitts *et al.* 2004, Kwon *et al.* 2006, Xia and Zwiebel 2006). This T2 chemosensillum is a short (5-7 µm) hair-like structure placed on a socket with longitudinal grooves (Pappas and Larsen 1976). This chemosensillum appears to be innervated by 2 ORNs both of which can detect odorants (Kwon *et al.* 2006).

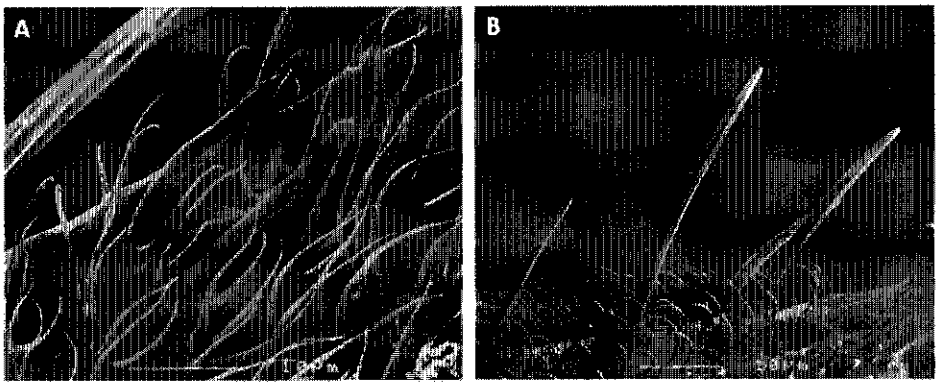


Figure 4: Scanning electron micrographs of olfactory sensilla on maxillary palps and proboscis of *Anopheles gambiae*. **A)** the club-shaped capitate peg sensilla of the maxillary palp. **B)** the T2 chemosensilla on the proboscis. Photographs by H. Smid.

2.3 Biochemistry and molecular biology

2.3.1 Olfactory receptors

The response spectra of a particular ORN depends on which olfactory receptor (OR) or receptors (ORs) are present on the dendrites of that neuron. Until 2005 the general thought was that each ORN had only one type of OR. However Goldmann (2005) showed the expression of two functional odorant receptors in one ORN and Lu *et al.* (2007) showed that three *Anopheles gambiae* gustatory receptors (AgGRs) were expressed in the same ORN on the maxillary palp of anopheline mosquitoes. Though these three AgGRs are classified as gustatory receptors (GRs), they clearly exhibited olfactory properties.

ORNs evoked by carboxylic acids, components emanating from the human skin (Zeng *et al.* 1991, Cork and Park 1996, Zeng *et al.* 1996, Braks *et al.* 1999, Braks and Takken 1999, Bernier *et al.* 2000).

3.1.2 Antenna: Responses from grooved peg sensilla

The olfactory function of the double-walled grooved peg sensilla were studied in *An. gambiae*, *Ae. aegypti* and *Cx. quinquefasciatus*, though less extensively than the trichoid sensilla (Appendix 1). The most extensive study was conducted by Qiu *et al.* (2006), and described five functional types of grooved pegs in *An. gambiae* responding mostly to ammonia, some amines and carboxylic acids. Several of these compounds, in synthetic or natural blends, have been shown to attract *An. gambiae* (van den Broek and den Otter 2000, Meijerink *et al.* 2001, Smallegange *et al.* 2005b, Smallegange *et al.* 2009).

Electrophysiological studies on *Aedes* and *Culex* mosquitoes have recorded the responses of grooved pegs but never have they been classified according to response spectra. Grooved pegs in all mosquito species respond to ammonia and several short chained carboxylic acids. Whereas all grooved pegs recorded from in *Anopheles* and *Culex* species showed responses towards ammonia, not all grooved pegs in *Ae. aegypti* were excited by ammonia. Moreover, lactic acid, a known attractant (Smith *et al.* 1970, Geier *et al.* 1996), evoked strong responses in the grooved pegs of *Ae. aegypti* but only mild responses in either *An. gambiae* or *Cx. quinquefasciatus*.

3.1.3 Antenna: Responses from coeloconic sensilla

The large coeloconic sensilla in *An. gambiae* have a structure similar to the grooved pegs indicative of an olfactory function as mentioned above (Boo and McIver 1975, 1976a, McIver 1982b). Still in none of the three most important vector mosquito species electrophysiological studies have been performed on this type of olfactory sensilla.

3.1.4 Responses from palpal capitate peg sensilla

The maxillary palp of mosquitoes represents an intriguing olfactory organ. As mentioned above, it only has one type of olfactory sensillum, the capitate peg, and all capitate peg sensilla are similarly being innervated by three ORNs. For all three mosquito species *An. gambiae*, *Ae. aegypti* and *Cx. quinquefasciatus* it is known which ORs are expressed in the ORNs of the capitate peg sensilla (Bohbot *et al.* 2007, Lu *et al.* 2007, Arensburger *et al.* 2010). All capitate pegs contain three ORNs, one of which responds to CO_2. This ORN, termed the A-neuron, expresses three GRs in each species that are close homologues of each other (Jones *et al.* 2007, Lu *et al.* 2007, Kent 2008, Arensburger *et al.* 2010). The same is true for the ORs expressed in the ORN that responds with a high sensitivity to 1-octen-3-ol; the B-neuron. It contains the non-specific OR7 co-receptor and OR8 which display a high sequence homology between species

(Bohbot *et al.* 2007, Lu *et al.* 2007, Arensburger *et al.* 2010). Such high homologies were not found for the third ORN, the C-neuron. In *An. gambiae* AgOR28 is expressed in the C-neuron and the third ORN in *Ae. aegypti* expresses AaOR49, which is not closely related to AgOR28 (Bohbot *et al.* 2007). As can be seen in Appendix 1 the three capitate peg ORNs of *An. gambiae* and *Cx. quinquefasciatus* have been characterised in great detail (Lu *et al.* 2007, Syed and Leal 2007). Both studies show only a small overlap in the response spectra. In *An. gambiae* the A-neuron has been found to respond to several odours as well as to CO_2. The A-neuron in *Cx. quinquefasciatus* was only excited by CO_2 and not by any of the other odours tested. The B-neuron, next to its high sensitivity to 1-octen-3-ol, has a broader response spectrum in *Cx. quinquefasciatus* than in *An. gambiae*. The C-neuron of *An. gambiae* is a very broadly tuned ORN, responding to over 20 different odorants, while the C-neuron in *Cx. quinquefasciatus* is unaffected by all tested odours. To date no characterisation has been made from the capitate peg sensilla of *Ae. aegypti*.

3.1.5 Responses from the T2 chemosensilla of the proboscis

Studies showed that multiple AgORs, including the highly conserved OR7, were expressed in the T2 chemosensilla on the proboscis of *An. gambiae* mosquitoes, which suggested an olfactory function (Kwon *et al.* 2006). Among the identified AgORs, AgOR6 was consistently found in several preparations. Through single sensillum recording (SSR) of the T2 chemosensilla Kwon *et al.* (2006) recorded olfactory responses to several human-related odorants, such as 4-methylbutanoic acid, butylamine and several oxocarboxylic acids and ketones. A functional classification has yet to be performed on the *Anopheles* T2 chemosensilla. Until today no articles have been published on the olfactory sensilla of the proboscis of *Aedes* and *Culex* species.

3.1.6 Temporal response properties

Little attention has been given to the temporal properties of ORN responses, though temporal characteristics have been suggested to provide the peripheral nervous system with an extra dimension in odour coding (Yao *et al.* 2005, Carey *et al.* 2010). Raman *et al.* (2010) showed that the temporal characteristics of neural odour coding recorded in the antennal lobe (AL) are formed by the temporal characteristics of ORN activity. Recordings from locust (*Schistocerca americana*) antennae revealed that variation in the temporal properties of the odour-elicited firing patterns of the peripheral ORNs contained information on identity, concentration and duration of odours (Raman *et al.* 2010).

Several SSR-studies on mosquitoes reported that a single odour can evoke different temporal responses depending on the receptor it activates and that a receptor can show different temporal responses for different odours (Qiu *et al.* 2006, Ghaninia *et al.* 2007b, Hill *et al.* 2009, Siju *et al.* 2010). Two studies also reported the occurrence of post-stimulatory

inhibition (Qiu *et al.* 2006, Hill *et al.* 2009), which might provide an extra identifying property of the particular odour to the central nervous system.

Olfactory responses of neurons can be subdivided into three types of responses: phasic (fast adapting), tonic (slow adapting) or phasic-tonic responses (fast increase, slow return to baseline level). Phasic responses have been put forward as a means of informing insects about odour concentrations and a means to assess the rapid changes in concentrations encountered when crossing the filamentous odour plume (Almaas *et al.* 1991, den Otter and van der Goes van Naters 1992, de Bruyne *et al.* 2001, Olsson *et al.* 2006). Tonic responses have been proposed as signals to sustain upwind flight when loosing contact with an odour plume (Almaas *et al.* 1991, den Otter and van der Goes van Naters 1992, de Bruyne *et al.* 2001). To date, at least to our knowledge, no direct correlation between a temporal response pattern from peripheral ORNs and the occurrence of specific behaviours has been shown for mosquitoes and insects in general.

3.1.7 The influence of physiological state on the responsiveness of ORNs

The physiological state of the mosquito can have an influence on its olfactory responsiveness. As mentioned in paragraph 1.3.4, during 48 h after a blood meal female mosquitoes do not exhibit host-seeking behaviour or the frequency of this behaviour is reduced. During this period females are probably more tuned to oviposition cues. Qiu *et al.* (2006) studied the effect of a blood meal on the response spectra of sensilla trichodea subtype E and the grooved peg sensilla. They discovered a third functional type following ingestion of a blood meal that displayed a high sensitivity to indole, carboxylic acids C6-C9 and 3-methylindole (an oviposition stimulant). All of these compounds have been found in human sweat or skin emanations (Cork and Park 1996, Bernier *et al.* 2000, Meijerink *et al.* 2000). Some of the compounds identified from human emanations, like phenols, indole and some carboxylic acids, have also been shown to affect the oviposition behaviour of mosquitoes (Bentley and Day 1989, Millar *et al.* 1992, Blackwell and Johnson 2000). Two functional types of TSE responded after a blood meal to 3-methylindole (Qiu *et al.* 2006) while none of the neurons associated with TSE in *An. gambiae* showed responses to this compound before a blood meal. The odorant 3-methylindole has been found in larval water that elicited EAG responses from *An. gambiae* (Blackwell and Johnson 2000) and the same odorant stimulates oviposition in *Cx. tarsalis* and *Cx. quinquefasciatus* (Du and Millar 1999). These up-regulated responses might aid the mosquito in the selection of an oviposition site.

Qiu *et al.* (2006) also reported a reduction in response of TSE I to ammonia, several carboxylic acids and alcohols post-blood meal indicating a possible involvement in the inhibition of host-seeking behaviour. The second E-type, TSE II, also revealed changed responses after a blood meal. After a blood meal, one TSE II-ORN stopped responding to 4-methylphenol and 4-ethylphenol, compounds known to be present in human sweat (Cork and Park 1996). This down-regulation of the sensitivity to odours after a blood meal might affect host-seeking behaviour.

Fox *et al.* (2001) reported the down-regulation of a female-specific putative olfactory receptor gene (AgOR1) after a blood meal, which coincides with a complete inhibition of a behavioural response following a blood meal (see above). When this receptor gene was expressed in the heterologous empty neuron of *D. melanogaster,* this neuron showed specific responses to 4-methylphenol and 4-ethylphenol (Fox *et al.* 2001, Hallem *et al.* 2004).

A study in *Ae. aegypti* on changes in responsiveness after a blood meal revealed that most sensilla, including the short sharp-tipped trichoid sensilla (equivalent to TSE in *An. gambiae*), did not show changes in responsiveness after a blood meal (Siju *et al.* 2010). Only neurons housed within four of the five functional types of short blunt-tipped II trichoid sensilla showed increased response intensity 24 h post blood meal (PBM) to indole and phenolic compounds (Siju *et al.* 2010). All these compounds have been linked to oviposition site- related odour cues (Davis 1976, Bentley *et al.* 1979, Bentley 1981, Bentley *et al.* 1982, Millar *et al.* 1992, Beehler *et al.* 1994, Lindh *et al.* 2008b). The increases were still visible at 72 h PBM although less pronounced. The test panel Siju *et al.* (2010) used contained several putative host-seeking related odours but no decreases in response intensity in TS to these compounds were observed after a blood meal.

In addition to down- and up-regulation of the sensitivity to odorants in TSE after a blood meal, Qiu *et al.* (2006) also found down-regulation of sensitivity of GP-neurons after a blood meal. Decreased sensitivity to ammonia was found in neurons innervating GP-sensilla of blood-fed *An. gambiae* (Qiu *et al.* 2006). Ammonia has been proven to attract mosquitoes including *An. gambiae* (Braks *et al.* 2001, Meijerink *et al.* 2001, Smallegange *et al.* 2005b) both on its own and in combination with lactic acid and carboxylic acids. Reduced sensitivity to ammonia following a blood meal again might indicate involvement in the suppression of host-seeking behaviour.

In *Ae. aegypti* reduced sensitivity of grooved peg neurons following a blood meal has been reported as well. It was found that the sensitivity of lactic acid-sensitive neurons of antennal GP-sensilla was suppressed after a blood meal (Davis 1984). However, no complete study on the effects a blood meal has on the sensitivity of GP-ORNs has been performed for *Ae. aegypti* and *Cx. quinquefasciatus*.

3.2 Electrophysiology of heterologous OR-expression systems

With the identification of putative olfactory receptor genes and several heterologous systems, like the DmHES, capable of expressing ORs, efforts to characterise the ORs of *An. gambiae* have been intensified. To-date, two studies have functionally characterised a large part of the AgOR candidate repertoire. Seventy-two AgOR candidates have been cloned and successfully expressed in the DmHES (Dobritsa *et al.* 2003) and 50 of these AgORs were functional (Carey *et al.* 2010). All of them have been characterised with a panel of over 90 different odorants. Carey *et al.* (2010) showed response spectra going from very narrow (responding to only a few odours) to extremely broad (responding to more than 60 different odours). Key odorants indicating the

presence of hosts are thought to be detected by narrowly tuned ORs or specialist ORs. While the more generalistic, broadly tuned, ORs might function as background detectors. It was also reported that some odours only evoked responses from very few receptors while others, for example most esters, evoked strong responses from many broadly tuned receptors. Wang *et al.* (2010) functionally characterised 37 of the 79 putative AgORs using the *Xenopus* heterologous expression system and a panel of 88 odorants. They too found specialists and generalists among the AgORs tested.

Both studies expressing the AgORs in their heterologous expression system reported that no responses were found to several known mosquito attractants like ammonia, amines and carboxylic acids, all of which are compounds that elicit electrophysiological responses from grooved pegs *in vivo*. Benton *et al.* (2009) discovered a new family of olfactory receptors, ionotropic receptors (IRs) that never occurred together with the classic ORs except for DmOR35 in the coeloconic sensilla (Couto *et al.* 2005, Yao *et al.* 2005). In *Drosophila* the grooved pegs are called coeloconic sensilla (which translates as pegs in pits). The grooved pegs and coeloconic sensilla in other insects have almost identical structures but early studies thought that the grooved pegs of *Drosophila* were located in pegs, hence the name coeloconic sensilla. It is now known that these sensilla are not true pitted pegs (coeloconic sensilla). Because this name has been used in numerous studies it was decided not to change the nomenclature in this thesis. Benton *et al.* (2009) further identified nine IRs that were expressed in the coeloconic sensilla. Orthologs to the IRs in *Drosophila* have been identified for *An. gambiae* and when heterologously expressed they conferred responses to human-related odorants that did not elicit responses in any AgORs (Liu *et al.* 2010). It is highly probable that these IRs are expressed in the grooved pegs of mosquitoes, possibly also in the coeloconic sensilla, which makes them interesting targets for further electrophysiological studies.

Lu *et al.* (2007) compared the functional characteristics of heterologously expressed AgORs with the corresponding ORNs on the maxillary palp expressing the same AgORs, as confirmed by *in-situ* hybridisation (Lu *et al.* 2007). The response spectra of all three AgORs expressed in either the heterologous systems or *in vivo* were very similar, indicating that heterologous systems are useful in the search for odours that are detected by mosquitoes and that might be used for the manipulation of olfactory behaviour of *An. gambiae.*

4 Research objectives

This thesis has been carried out as part of the "Grand Challenges in Global Health" project #121 entitled: 'Disruption of malaria transmission by chemical manipulation of anopheline olfactory responses'. The overall goal of this project is the discovery and utilisation of infochemicals to disrupt host-seeking behaviour of the malaria mosquito *An. gambiae.*

The work presented in this thesis focused on investigating the chemical components in human emanations that play a role in the host-seeking behaviour of *An. gambiae* and how these human odours are perceived by the olfactory system of the mosquito.

The specific research objectives for this thesis were:

- To classify and characterise the response spectra of the ORNs innervating the three trichoid sensilla types A, B and D that had not yet been studied previously.
- To evaluate whether AgORs expressed in the *Drosophila melanogaster* heterologous expression system, can be used in the search for inhibitors of AgOR responses.
- To investigate the role of the maxillary palp capitate peg ORNs in host-seeking.
- To examine the possible effect that a transition of one physiological state to another might have on the responsiveness of capitate peg ORNs.

5 Outline of this thesis

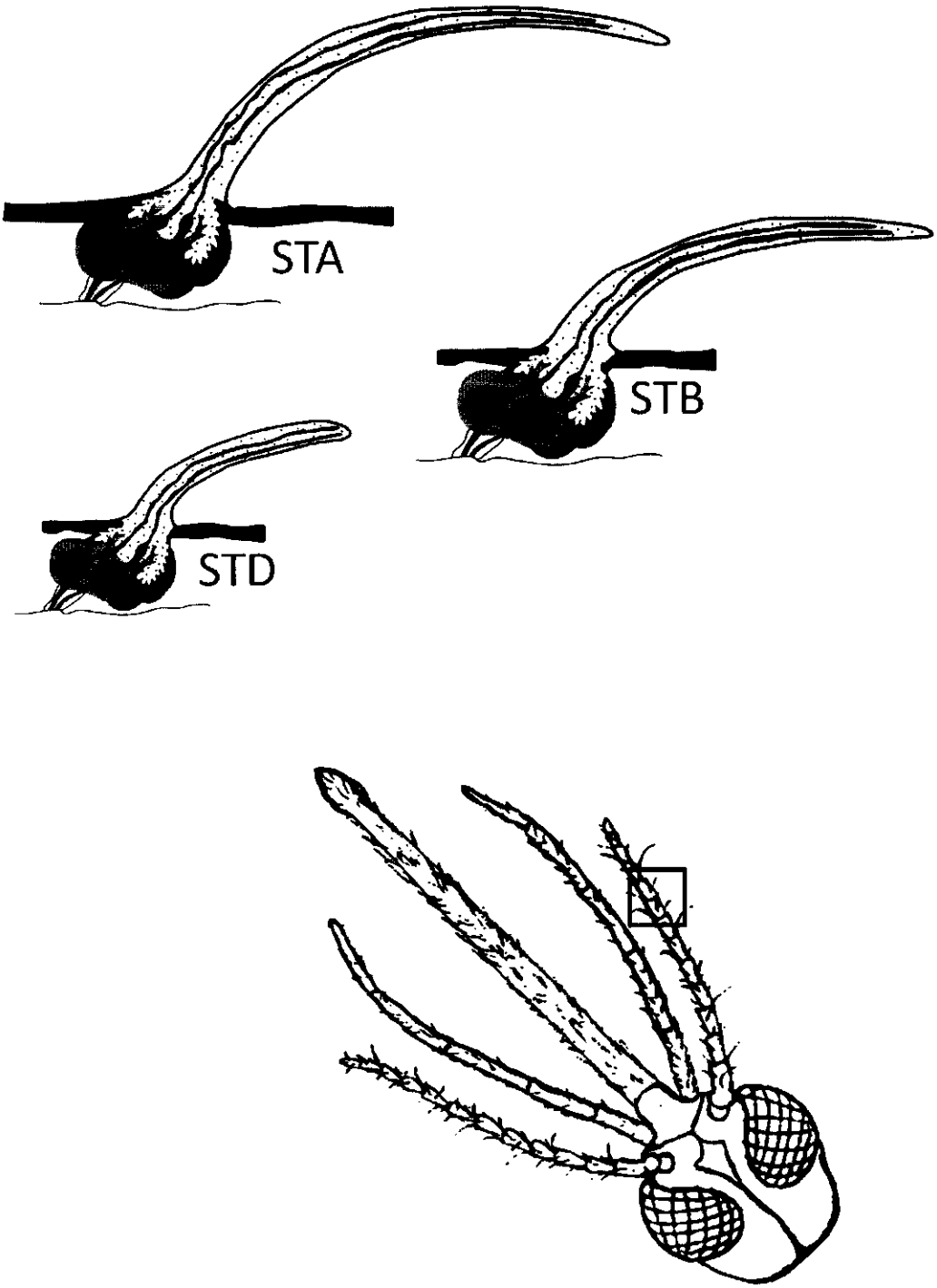
Chapter 2 provides the missing information on the olfactory responses of the antennal trichoid sensilla via an extensive characterisation of the not yet studied trichoid sensilla types of *An. gambiae* according to the responses of the ORNs to a panel of 132 compounds belonging to different chemical groups. The response types identified are matched with heterologously expressed candidate AgORs.

Previously performed experiments within the GCGH project on binary blends done on AgOR8 expressed in the *Drosophila melanogaster* heterologous expression system revealed several potential inhibitors for the best ligand of AgOR8, 1-octen-3-ol. In **Chapter 3** the responses to the same binary blends obtained from the heterologous system are compared with the responses obtained by *in vivo* electrophysiology on the capitate peg B-neuron expressing AgOR8.

In **Chapter 4** the role of the maxillary palp in host detection is examined by studying the responses of the capitate peg ORNs to 11 compounds identified in the headspace of human skin bacteria that have shown to be attractive to female *An. gambiae*. A detailed description is given of the effect of a blood meal and of oviposition on the olfactory responses of the three ORNs innervating the capitate peg sensilla as well as the inhibitory effects some of the compounds produced by human skin bacteria have on the neuron sensitive to CO_2.

Chapter 5 provides a detailed study on the temporal characteristics of an ORN innervating the trichoid sensilla type E. This ORN showed a prolonged response in reaction to stimulation by two human-related compounds. This prolonged response is studied in further detail together with the possible behavioural effects of such stimuli.

In **Chapter 6** the contribution of the research reported in this PhD thesis to the overall knowledge on mosquito olfaction is discussed together with the improvements that can be made and future perspectives.


STA
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Chapter 2

Electrophysiological characterisation of olfactory receptor neurons innervating antennal trichoid sensilla B and D in *Anopheles gambiae* s.s.

Remco A. Suer, Yu Tong Qiu and Joop J.A. van Loon

Female *Anopheles gambiae* locate their sugar meals, mates, human blood hosts and oviposition sites primarily through odour detection. These odours are detected by olfactory receptor neurons (ORNs) associated with different types of sensilla on the antennae and maxillary palps. This paper describes a systematic study of the electrophysiological responses of two types of antennal trichoid sensilla of female *An. gambiae* mosquitoes. Using a panel of 132 chemical compounds of which 80 were previously reported to be present in human-derived odours, we identified six response types for neurons located in long sharp-tipped trichoid sensilla (TSB) and five response types for neurons associated with short blunt-tipped trichoid sensilla (TSD). ORNs associated with TSB or TSD respond to many different odours from ten distinct chemical groups. Of the 80 human-derived compounds tested, 44 elicited responses in either TSB or TSD and 13 odorants elicited responses in both. Two of these represented the best ligands of one or more response types. The profiles of seven of the 11 identified response types were significantly correlated with profiles of single AgORs as identified via the *Drosophila* empty neuron heterologous expression system.

Introduction

Olfactory information plays an important role in mosquito behaviour. Female mosquitoes find their sugar meals, mates, blood hosts and oviposition sites primarily based on responsiveness to odours (Takken 1991, Takken and Knols 1999, Zwiebel and Takken 2004). Odours released from human emanations like sweat have been shown to influence mosquito host-seeking behaviour, (Meijerink *et al.* 2000, Braks *et al.* 2001), especially for *Anopheles gambiae* Giles *sensu stricto* which is strongly anthropophilic. Human feet were found to be the preferred biting site of female *An. gambiae* (de Jong and Knols 1995b). As the smell of Limburger cheese to the human nose bears remarkable resemblance to that of human feet, Knols *et al.* (1997) investigated whether *An. gambiae* was attracted to the carboxylic acids emanating from the cheese. This indeed was the case. Many studies have shown the presence of the same carboxylic acids in emanations from the human skin (Bernier *et al.* 1999, Bernier *et al.* 2000, Healy and Copland 2000, Bernier *et al.* 2002, Gallagher *et al.* 2008). Some of the carboxylic acids influenced the host-seeking behaviour of *An. gambiae* either alone or in a blend (Smallegange *et al.* 2005b, Okumu 2008, Okumu *et al.* 2009, Smallegange *et al.* 2009). Recently Verhulst *et al.* (2009) found that the cultured skin microflora present on human feet produced several compounds, including two carboxylic acids that were attractive to *An. gambiae*.

Odours are detected by the peripheral olfactory system of the mosquito that consists of several olfactory sensilla located on the antennae (trichoid sensilla, coeloconic sensilla and grooved pegs), maxillary palps (capitate peg sensilla) and proboscis (T2-chemosensory sensilla) (McIver 1982, Clements 1999, Fox *et al.* 2001, Pitts *et al.* 2004, Kwon *et al.* 2006, Pitts and Zwiebel 2006). Two to five olfactory receptor neurons (ORNs) innervate these sensilla, each expressing two or more olfactory receptors (ORs) (Boo and McIver 1976, McIver 1978, Boo 1980, Zwiebel and Takken 2004). Trichoid sensilla represent the most abundant type of olfactory sensilla comprising two-thirds of all sensilla on the antenna of *An. gambiae* (McIver 1982, Pitts and Zwiebel 2006). Anophelines have five different morphological subtypes of trichoid sensilla whereas *Aedes* and *Culex* mosquitoes have four subtypes (McIver 1982). The five subtypes of trichoid sensilla (TS) of anopheline mosquitoes have been designated as A, B, C, D and E (Boo 1980), whereas the designations in culicine mosquitoes are sharp-tipped trichoid sensilla (long and short) and short blunt-tipped (I and II) trichoid sensilla (McIver 1978, McIver 1982).

In the last four years several studies on three mosquito species have been conducted to characterise the response spectra of the olfactory receptor neurons (ORNs) innervating the trichoid sensilla with the aim of providing further insight in olfactory coding and to identify potential attractants or repellents. Next to the grooved pegs, Qiu *et al.* (2006) characterised two morphological types of trichoid sensilla, TSC and TSE of *An. gambiae* s.s., using a panel of 44 odorants. They classified in total six different response types for these two trichoid sensilla: two response types of TSE and four types of TSC. In *Ae. aegypti* (L.) 11 different response types were found for all four morphological types of its trichoid sensilla, based on an electrophysiological study performed with a panel of 16 odours (Ghaninia *et al.* 2007b). Using a

panel of 44 odorants, Hill *et al.* (2009) characterised all four types of trichoid sensilla of *Culex quinquefasciatus* Say and reported 18 different response types in *Cx. quinquefasciatus.* All three studies reported strong responses to several alcohols like 1-octen-3-ol, a known attractant for several Diptera (Vale and Hall 1985, Cork and Park 1996, Mboera 2000), and 4-methylcyclohexanol and indole, which are known as oviposition stimulants/attractants (Bentley *et al.* 1982, Beehler *et al.* 1994, Allan and Kline 1995, Du and Millar 1999). All three single sensillum studies described above also reported activity of several ORNs evoked by carboxylic acids, components of human skin odour (Qiu *et al.* 2006, Ghaninia *et al.* 2007b, Hill *et al.* 2009). Hill *et al.* (2002) discovered 79 putative olfactory receptors (AgORs) in the malaria mosquito *Anopheles gambiae* Giles *sensu stricto* using a bioinformatics approach based on OR-genes of *Drosophila melanogaster* Meigen. Using the *Drosophila melanogaster* heterologous expression system (DmHES, Dobritsa *et al.* 2003), Carey *et al.* (2010) were able to express 50 of these 79 AgORs in this heterologous expression system and characterised the responsiveness of the AgORs by employing a panel of 110 odorants. Wang *et al.* (2010) characterised 72 AgORs in a heterologous *Xenopus* oocytes expression system using 88 different odours. Lu *et al.* (2007) identified the AgORs present on the ORNs associated with the capitate peg sensilla on the maxillary palps of *An. gambiae* and showed that the response pattern of the AgORs expressed in the heterologous system were similar to the response patterns of the AgORs *in vivo.* It is expected that many of these AgORs are expressed in ORNs associated with the different morphological types of trichoid sensilla as they are the most abundant type of olfactory sensilla.

The present study focuses on the electrophysiological characterisation of the ORNs innervating trichoid sensilla types A, B and D of *An. gambiae* Giles *sensu stricto* females. A panel of 132 volatile compounds belonging to different chemical classes is used. With a focus on host seeking, 80 compounds reported to be of human origin are included in the panel. An attempt is made to link the obtained response patterns with those of single AgORs when expressed in the DmHES.

Materials and Methods

Rearing

A colony of *Anopheles gambiae* Giles *sensu stricto* (hereafter *An. gambiae*), originating from Suakoko, Liberia (courtesy Prof. M. Coluzzi), has been cultured at Wageningen University, The Netherlands, since 1988. The mosquito colony was kept at standard insectary conditions of 27 ± 1°C, 80 ± 5% RH and at an adjusted 12:12 day-night rhythm, with light phase starting at 12:00 am with a gradual transition of 30 minutes from complete darkness to full light and vice-versa. Adults were kept at 30×30×30 cm gauzed cages with access to a 6% (w/v) glucose solution on filter paper. Female mosquitoes received blood meals from a human arm twice a week. Eggs were laid on damp filter paper that was placed in plastic cups with tap water. Larvae were fed

daily with Tetramin® baby fish food (Melle, Germany). Pupae were collected daily and allowed to emerge in the aforementioned gauze cages.

For the experiments, 5-8 d old females that had not received a blood meal were used. Females that are attracted towards a hand held next to the cage were selected to ensure the collection of behaviourally responsive mosquitoes. Females were collected by means of an aspirator and placed in a small cage with access to water via damp cotton wool placed on top of the cage.

Electrophysiology

Nomenclature

To avoid confusion on the names that have been used in this study, a small overview is given with the different names for the different parts of the olfactory system. On the antennae 5 different types of structures (sensilla) can be found: sensilla chaetica (bristles), grooved peg sensilla, coeloconic sensilla (pitted pegs), sensilla ampullacea and trichoid sensilla (hairs) (Ismail 1964, McIver 1982, Pitts and Zwiebel 2006). The grooved pegs, coeloconic sensilla and trichoid sensilla have an olfactory function. Boo (1980) studied the fine structure of the trichoid sensilla by use of an electron microscope and subdivided the trichoid sensilla based on morphological differences in 5 different types: trichoid sensilla types A-E. Located at the base of these sensilla we can find different neurons. In electrophysiology, by convention, the neuron generating the largest spike amplitude is called neuron A, the second largest: neuron B, etc.

In this study we characterised the A and B neurons associated with trichoid sensilla type A (TSA), trichoid sensilla type B (TSB) and trichoid sensilla type D (TSD) based on their response spectra. The A neurons of different trichoid sensilla could respond differently to the same odour and B neurons of different trichoid sensilla could also respond differently to the same odour. Based on their activity spectra we found specific response types for the different neurons. Response types were given the letter of the neuron, thus A or B, and a number to represent a specific response spectrum.

Preparation

A female mosquito was fixated on a Perspex block (1.1x1.1x1.5cm) using transparent double-sided sticky tape (Fisher Scientific) as described by Qiu *et al.* (2006). Legs were removed to increase stability and to restrict further movement of the mosquito. The wings, mouthparts and abdomen of the mosquito were pressed against the sticky tape. Maxillary palps were fixated on the tape at an angle of 45 degrees with the proboscis to minimize movement of the head. The antennae were immobilised and fixed at an angle of 90 degrees. Once mounted, the mosquito was placed in the electrophysiology rig under an Olympus XI51 inverted microscope (15x50). Recordings were made from 10 preparations for trichoid sensilla A found on antennal segments 5-11, from 88 preparations for trichoid sensilla type B located on segments 4-9 and from 88

preparations for trichoid sensilla type D found on antennal segments 4-10. Sensilla located on the first three flagella could not be studied due to our immobilisation method.

Single sensillum recording (SSR)

Two tungsten electrodes were used to contact the mosquito preparation. These electrodes were electrolytically sharpened by repeated dipping in a saturated KNO_2 solution at a voltage of 4-9V until a tip diameter of 1-2 µm was reached. The indifferent electrode was grounded and placed in the eye of the mosquito using a mechanical micromanipulator. A motorised micromanipulator (MX7500L, Siskiyou, Grants Pass, Oregon, USA) was used to position the recording electrode at the basis of a trichoid sensillum and pierce the cuticle of the sensillum until neural action potentials were registered. The analogous signals were augmented by a preamplifier connected to the recording electrode and digitised by an analogue–digital conversion interface (IDAC4, Syntech, Hilversum, The Netherlands) at a sample rate of 10666,7/s. The digital signals were recorded and visualised using Autospike 3.9 software (Syntech) running on a Windows PC (Acer Aspire).

Chemical stimuli and stimulus delivery

A panel of 132 chemical compounds was used for the characterisation of response profiles of ORNs. Compounds were selected based on published information on (putative) behavioural relevance (Appendix 2). These compounds belong to ten chemical groups: carboxylic acids, amines, alcohols, ketones, phenols, esters, heterocyclics, alkanes, aldehydes and terpenes. One exception is Henkel 100, a commercially available blend of 100 compounds, each at a 1% concentration. All other chemicals were of the highest purity grade commercially available (between 95% to >99% pure; Appendix 2). Chemicals were diluted in paraffin oil except for ammonia and lactic acid, which were diluted in distilled water and for 7-octenoic acid and 3-methyl-2-hexenoic acid, which were diluted in tert-butyl methyl ether (Qiu *et al.* 2006).

The concentration of 1% (w/w) was chosen to allow comparison of response profiles with previous studies in *An. gambiae* (Qiu *et al.* 2006). To establish dose-response relations, series of five dilutions (0.001, 0.01, 0.1, 1 and 10% w/w) were tested. Of each solution, 10 µl was applied to a strip of filter paper (0.5 x 1.5 cm) inserted into a Pasteur pipette serving as an odour cartridge that was closed with a 1 ml pipette-point and Parafilm® and kept at -25 °C. These cartridges were used up to 10 times except for some of the most volatile compounds (e.g. ammonia and acetone), dilutions of which were freshly prepared at the beginning of each experimental day and used a maximum of 3 times.

A constant, charcoal filtered and humidified airstream of 0.5 l/min was delivered to the antenna via a glass tube. The vapour of test compounds in odour cartridges was injected into the airstream at 0.5 l/min by using a stimulus controller (CS-55, Syntech). A synchronised airstream generated by the stimulus controller was injected into the main airstream in order to keep the total airflow constant. Stimulus duration was set at 200 ms.

Analysis

Single sensillum recordings were automatically filtered to increase signal-to-noise ratio using Autospike software (Filter: 20 Hz - 1694 Hz) in combination with Automacrorecorder (ReadmeSoft) software. All recordings from trichoid sensilla showed activities from two neurons. Action potentials from different neurons could be distinguished by spike amplitude, spike shape and by accounting for doublet wave forms present in a recording (Fig. 1). The effect of an odour was quantified by the change in action potential firing frequency, calculated as the number of action potentials during the 500 ms after the onset of the stimulus minus the average firing frequency during four intervals of 500 ms preceding the onset of the stimulus. The resulting count was doubled to obtain the number of spikes per second. A significant increase in firing frequency is described as excitation and a decrease as inhibition.

Several different electrophysiology rigs were used during this experiment and the time it took for the injected stimulus to travel down the glass tube to reach the antenna varied slightly between different setups. Therefore, this delay was added to the onset of stimulus time. The calculated delay matched closely with the observed delay between stimulus onset and response onset.

Responses were classified as excitatory if the increase of firing frequency was higher than two times the standard deviation of the mean spontaneous firing frequency for A- or B-neurons of TSB or TSD. Because the overall spontaneous firing frequency was lower than twice the standard deviation of those neurons, our criterion for inhibition was set as a decrease of firing frequency greater than once, instead of twice, the standard deviation of the overall mean spontaneous firing frequency for A- or B-neurons of TSB or TSD (see Table 1).

Cluster analysis

A hierarchical cluster analysis was performed using SPSS software (SPSS 16.0, SPSS Inc., Chicago) to categorise the ORNs into different response types. Because of incomplete recording series and the fact that a cluster analysis does not permit missing values, a limited number of sensilla (n=12 for TSB, n=14 for TSD) and odours could be used for a cluster analysis. Not all 132 compounds in the panel elicited responses; therefore a hierarchical cluster analysis (squared Euclidian distances, Ward's method) was performed only on compounds eliciting responses in at least one ORN. For trichoid sensilla type D (TSD) 18 odours were used, for TSB 40 odours could be used for the hierarchical cluster analysis. The hierarchical cluster analysis organised the data according to the strength of the responses of the different ORNs towards the 18 or 40 odorants. Each ORN is assigned a coordinate in this 18 or 40-dimensional space. An iterative process then links the ORNs, based on the distances between different ORNs in the multidimensional space. The distances between these clusters were calculated using Ward's method, which uses an analysis of variance (minimizing the variance) approach to evaluate the squared Euclidian distances between clusters. The Scree procedure was used to intersect the dendrogram in order to decide which number of clusters best represents our dataset (Bieber

and Smith 1986). In the Scree procedure, a sharp increase in distance among clusters is used to identify a change in the nature of the clustering steps.

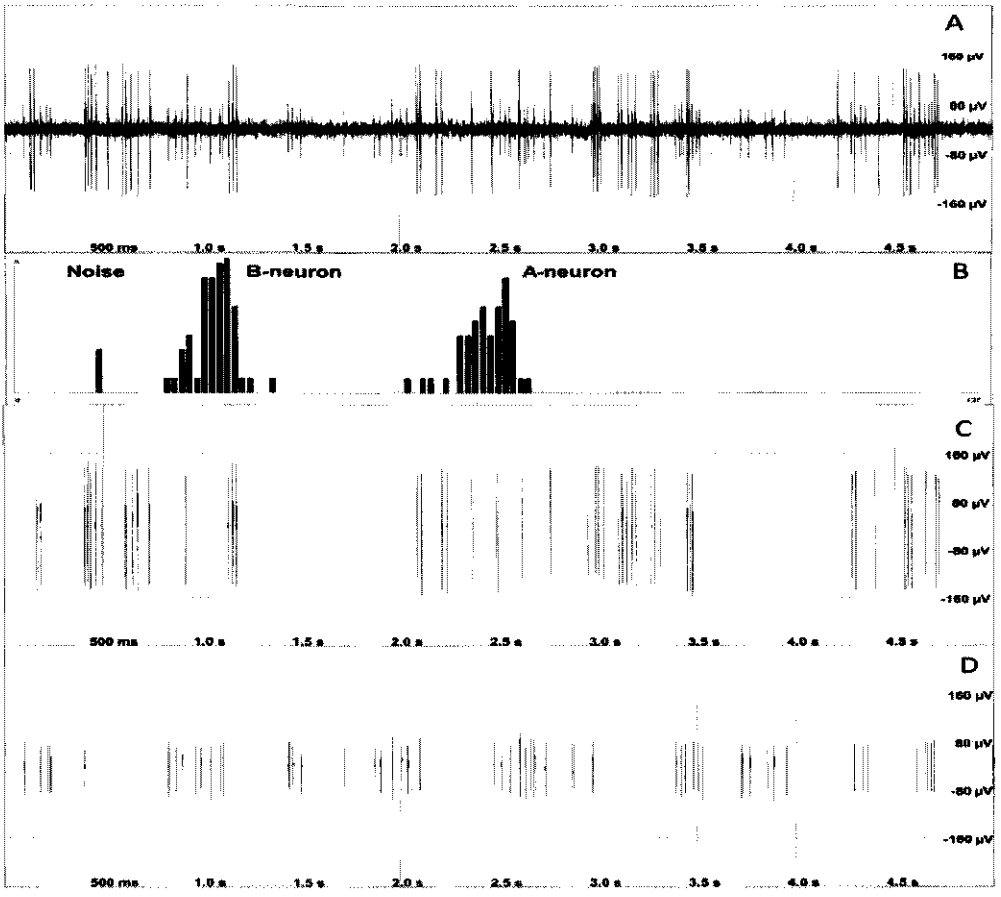

Figure 1: Differentiation of two ORNs associated with a trichoid sensillum type B; **A)** shows a typical recording from a TSB. The spike amplitudes originating from two ORNs can be seen. **B)** spike amplitude histogram for the recording shown in 2A, clearly separating A-neuron and B-neuron based on their spike amplitudes. **C)** spikes originating from the A-neuron and **D)** spikes originating from the B-neuron.

Statistical analysis

Statistical analysis of the electrophysiological data for the dose response relations was done using Genstat software (Genstat 14, VSN International Ltd, UK). Group differences were calculated using a Generalized Linear Model with Poisson distribution with a log function. To analyse whether the firing rate of the neuron in response to one concentration of odorant differed from the firing rate elicited by another concentration, two-sided t-probabilities were

calculated to test pairwise differences between the means. Effects were considered to be significant at $P < 0.05$.

Tuning width

To visualize the differences in specificity of ORNs belonging to different response types, tuning curves were generated for each type by assigning the odorant that elicited the strongest response the central position and arranging odorants that elicited weaker responses at the left or right side of the graph, ranked according to decreasing effectiveness. To compare the tuning curves of the ORNs with each other, the kurtosis values were used, calculated by taking the 4th central moment divided by the 4th power of the standard deviation, minus 3, representing an index of the shape of a distribution compared to the normal distribution. Kurtosis values are positive if the tails are overrepresented, compared to a normal distribution and negative if the tails are underrepresented. The normal distribution has zero kurtosis.

Comparison of in vivo *data with* DmHES *characterised AgORs*

The activity profiles of the *in vivo* response types and the response profiles of AgORs to odorants as characterised by expression in the *Drosophila melanogaster* heterologous expression system (DmHES) by Carey et al. (2010) were visualised using a Principle Component Analysis (PCA) in the software program SIMCA-P (12^{th} edition, Umetrics, Umeå, Sweden). A PCA is a method to represent a multivariate data set as a low dimensional plane of which the axes are called principal components. Significant principal components are determined by cross validation (Eriksson *et al.* 1996). For TSB 52 odours could be used for the PCA and 25 odours for TSD.

To find relationships between the *in vivo* and DmHES datasets, a PLS was carried out in SIMCA-P to explain the *in vivo* response types (Y) by the DmHES characterised AgORs. PLS stands for "projections to latent structures by means of partial least squares" and is a method for regression analysis used to link two data matrices X and Y by a multivariate model (Eriksson *et al.* 1996). The total variation in X (R^2X_{cum}) and Y (R^2Y_{cum}) that is explained by all significant principal components as well as the predictive power of the model (Q^2_{cum}: Goodness of prediction) are given as a 'goodness of fit'.

The strength of the relationship between an AgOR and a specific response type can be given by the regression coefficient. These regression coefficients are the PLS model parameters that are used to re-express the Y variables, the response types, as a multiple regression models of the X-variables (the AgORs).

Another valuable parameter is the variable importance on the projection (VIP). VIP is the summed influence on Y of every term (X_k) in the model. Variables with VIP values larger than one, are most relevant for explaining Y (Eriksson *et al.* 1996).

Results

According to the recordings all 3 types of trichoid sensilla (TS) housed two olfactory receptor neurons (ORNs). Ten preparations were studied to establish the response spectra of the two ORNs associated with the TSA, the longest sharp-tipped trichoid sensilla. None of the 132 odours tested, evoked responses from these ORNs.

Responses to our panel were found from the ORNs of trichoid sensilla types B and D. The two ORNs, the A and B neurons, of TSB and TSD exhibited different spontaneous firing frequencies. The spontaneous firing frequency per second of A-neurons (12.9±0.2 for TSB, 16.9±0.4 for TSD) was significantly higher than that of B-neurons (6.9±0.2 and 4.9±0.2 for TSB and TSD respectively; t-test, $P < 0.001$ and $P < 0.001$; table 1).

Table 1: Spontaneous firing frequencies of olfactory neurons in trichoid sensilla B and D. The criteria for a response were based on the standard deviation of the spontaneous firing rates of the different neuron types. Excitation was noted when the increase in firing frequency was greater than twice the standard deviation of the spontaneous activity of the ORN. Inhibition was defined as a decline greater than one standard deviation of the spontaneous activity.

Trichoid Sensilla	Neuron	Number of recordings	Mean spontaneous firing rate (spikes/s)	SD	excitation criterion (increase of firing rate (spike/s))	Inhibition criterion (decrease of firing rate (spike/s))
B	A	788	12.9	6.8	> 13.6	< 6.8
	B	715	6.9	5.5	> 11.0	< 5.5
D	A	468	16.9	8.9	> 17.8	< 8.9
	B	468	4.9	4.1	> 8.2	< 4.1

Trichoid sensilla morphological type B: the intermediate-long sharp-tipped sensillum

Trichoid sensilla B (TSB) were longer than both TSC and TSE but shorter than TSA. The different sizes are consistent with the relative lengths as described by Boo *et al.* (1980) TSB has a sharp tip which bends toward the segment and its diameter at the base is wider than that of other trichoid sensilla (Fig. 2).

The electrophysiological responses of 88 TSB neurons to the panel of 132 odorants were recorded (Appendix 2). Out of the 132 odorants tested, 71 elicited responses. The majority of responses were excitatory (Figs. 4 and 7 below) and only few of the responses were inhibitory. The overall amplitude of the action potentials for type B was between 0.12 mV and 0.20 mV with a signal to noise ratio better than 2 to 1.

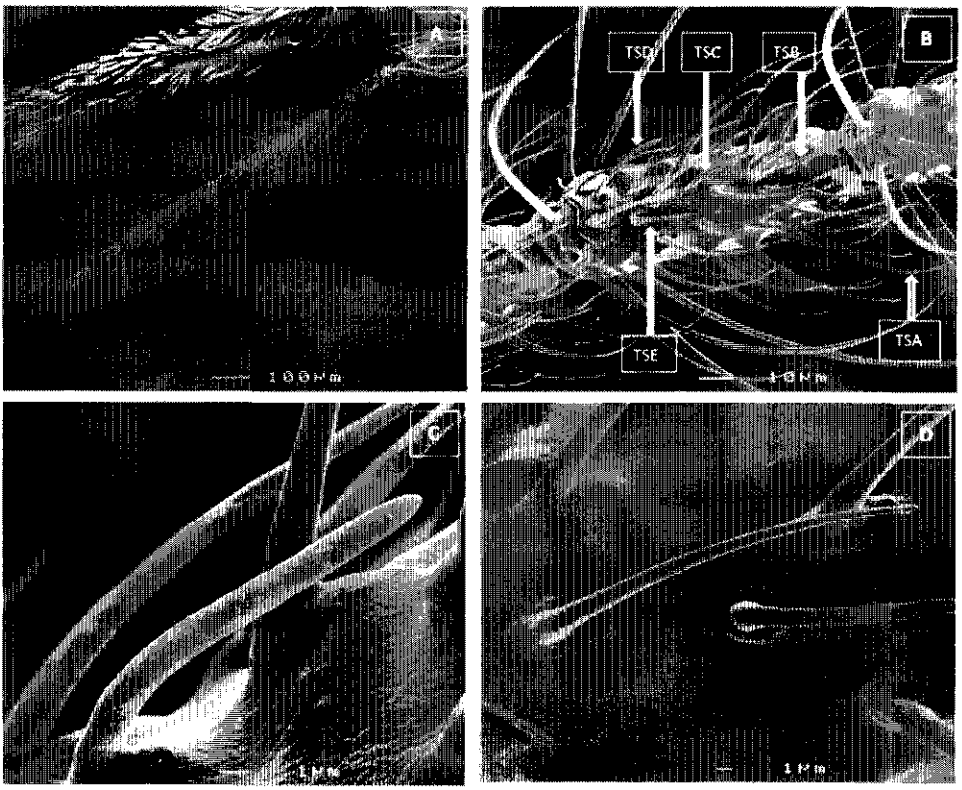

Figure 2: The olfactory sensilla of *Anopheles gambiae* s.s. **A)** scanning electron micrograph (SEM) of the whole antenna of a female *Anopheles gambiae* consisting of 13 segments. **B)** SEM of one segment of the antenna showing different morphological types of trichoid sensilla (TS). **C)** SEM of a trichoid sensillum type D (TSD); the short blunt-tipped type. **D)** SEM of trichoid sensilla type B (TSB), the sharp-tipped sensilla with intermediate length. Photographs by H. Smid.

Cluster Analysis

Cluster analysis was performed on the responses of 12 sensilla to 40 of the 71 active odorants (indicated with * in Figure 4) resulting in four distinct response types for neuron A and two distinct types for neuron B (Fig. 3). Most of the A neurons in TSB we recorded from belonged to type A1 (n=5). Only one sensillum belonged to type A2, two other sensilla were classified as a third type, A3, and the four remaining sensilla were classified as type A4. For the two B neuron types the numbers of sensilla allocated were six for B1 and five for B2.

The cluster analysis also revealed that response type A1 was always associated with response type B1 and response type A4 always with B2. The B-type sensilla containing an A3 neuron were found to be paired with either B1 or B2 neurons. The sensilla with only the A2 type ORN had a B1 type neuron co-localised with it. Additional measurements revealed that this A2 type could also be co-compartmentalized with B2 type neurons (data not shown).

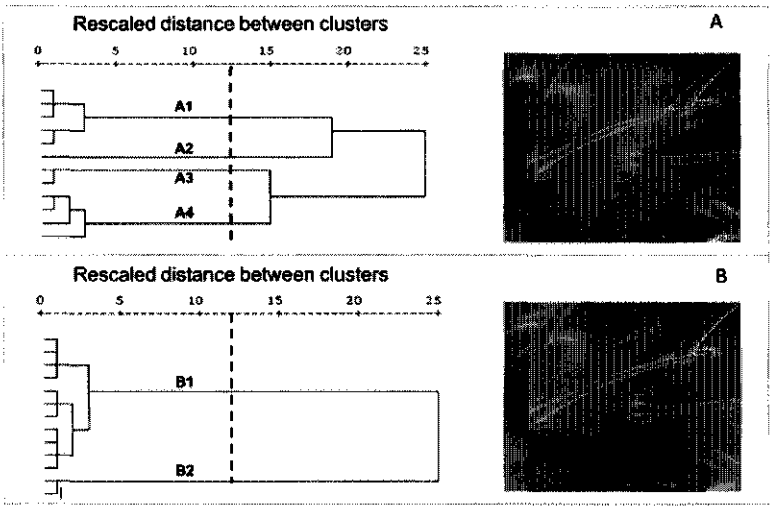

Figure 3: Dendrograms obtained by a hierarchical cluster analysis (Squared Euclidian distance, Ward's method) on responses from 12 A-neurons and 11 B-neurons associated with trichoid sensilla type B using their responses to a panel of 40 compounds. The Scree procedure, which entails that a sharp increase in distance among clusters identifies a change in the nature of the clustering steps, was used to define the intersect (dashed line) that best represents the number of clusters for our dataset. **A)** dendrogram for the TSB A-neurons. **B)** dendrogram for the TSB B-neurons.

Characterisation of the response spectra of ORNs associated with TSB

The complete response profiles for all six different neuron response types of TSB were constructed using the 71 odorants that elicited responses in at least one neuron of TSB (Fig. 4). Both A neurons and B neurons exhibited response types (A3 and B1) that did not respond (< 25 spikes/s) to any of the tested odorants. Next to these clear difference in response profiles, B1 and B2 type ORNs also differed in that B2 neurons had a spike amplitude clearly smaller than any A neuron it was co-occurring within the same sensillum, while the B1 neurons had a spike amplitude close to that of the A neuron housed in the same sensilla.

Terpenes elicited responses from three out of the four strong responding ORN types, exception from type B2. This was exactly the opposite for the phenols that elicited responses only from type B2. Ketones, esters and aldehydes elicited variable responses from each type. The heterocyclics only produced strong responses in types A2 and B2. Alcohols elicited more and stronger responses from types A2 and A4.

As for individual types, response type A1 (Fig. 4A) was a type that did not exhibit very strong responses (< 50 spikes/s) compared to the other types found in TSB sensilla, except the non-responsive A3 type. The best ligand for response type A1 (linalool oxide) elicited an increase of 50 spikes/s (Fig. 4A). Except for the response to Henkel-100, a commercially available mixture that contains 100 chemical compounds in a 1% concentration and linalool oxide, no responses were found higher than 50 spikes/s in response type A1. The strongest stimulants for response

type TSB A1 were linalool oxide, followed by 2-ethyl-1-hexenol and isoamyl acetate. Other odorants with intermediate responses were (+)- and (-)-carvone, 6-methyl-5-hepten-2-one, isobutylthiazole, 1-octen-3-ol, octanol, 3-octanone and 2-nonanone. This type can be distinguished from other types by a strong response to linalool oxide, no response to 4-methylcyclohexanol and its co-localisation with B1-neurons that did not respond to any of the tested odorants.

Even though in the hierarchical cluster analysis only one sensillum was attributed to type A2, using a diagnostic panel this type was found several times and recordings were similar to the response spectra shown in Fig. 4B (data not shown). The best ligand of the A2 type was isoamyl acetate, which did not elicited strong responses from other response types. The A2 was the only type that responded strongly to (-)-α-thujone, 4-methylcyclohexanol and ethyl butyrate. Other good ligands of A2 were (+)- and (-)-carvone, benzaldehyde, 2-isobutyl thiazole and the alcohols 3- and 2-methyl-1-butanol, 3-methyl-2-cyclohexen-1-ol, 1-hexen-3-ol and 1-hexanol. This type can be identified by a strong response to isoamyl acetate and 4-methylcyclohexanol.

ORNs of response type A3 (Fig. 4C) produced weak but some significant responses; none of the 132 odorants elicited an increase in spike frequency higher than 25 spikes/s. A3-ORNs were housed together with either type of B neurons. This type is best recognized by a lack of response of the A neuron towards each of the following four odorants in our diagnostic panel: linalool oxide, isoamyl acetate, 4-methylcyclohexanol and decanal.

Response type A4 ORNs (Fig. 4D) were responsive to aldehydes (decanal, octanal and nonanal), ketones (3-octanone, 2-nonanone and 2-decanone) and the alcohol 1-octen-3-ol. All these active ligands had a chain length of 8-10 carbons with the strongest ligand being decanal. Other odorants that elicited good responses were 1-hexanol, and several esters; ethyl hexanoate, phenethyl acetate and dimethyl adipate. This type could be distinguished from other types by its strong responses to decanal and octanal and a lack of response to linalool oxide. It always co-occurred together with a type B2 neuron.

The B neurons can be divided into two types; B1: an unresponsive type and B2 a highly responsive type. B1 neurons showed some excitation responses but no increases higher than 25 spikes/s to any of the 132 odorants tested (Fig. 4E). B2 neurons responded to many different odorants, and responses to seven of the ligands (eugenol, acetophenone, 2-acetylpyridine, 2-acetylthiophene, 4,5-dimethylthiazole, 2,4,5-trimethylthiazole and benzaldehyde) were close to or more than 80 spikes/s. B2 neurons (Fig. 4F) were also the only neurons in trichoid sensilla B responding to phenols such as 2-methylphenol, 3-methylphenol, 4-methylphenol, 2-ethylphenol and 4-ethylphenol. As can be seen in Fig. 4F several thiazole compounds and esters also elicited good responses. B2 neurons can be distinguished from other types by a spike amplitude clearly smaller than that of the A-neuron co-localised in the same trichoid sensilla and they can be distinguished via their strong responses to acetophenone and benzaldehyde.

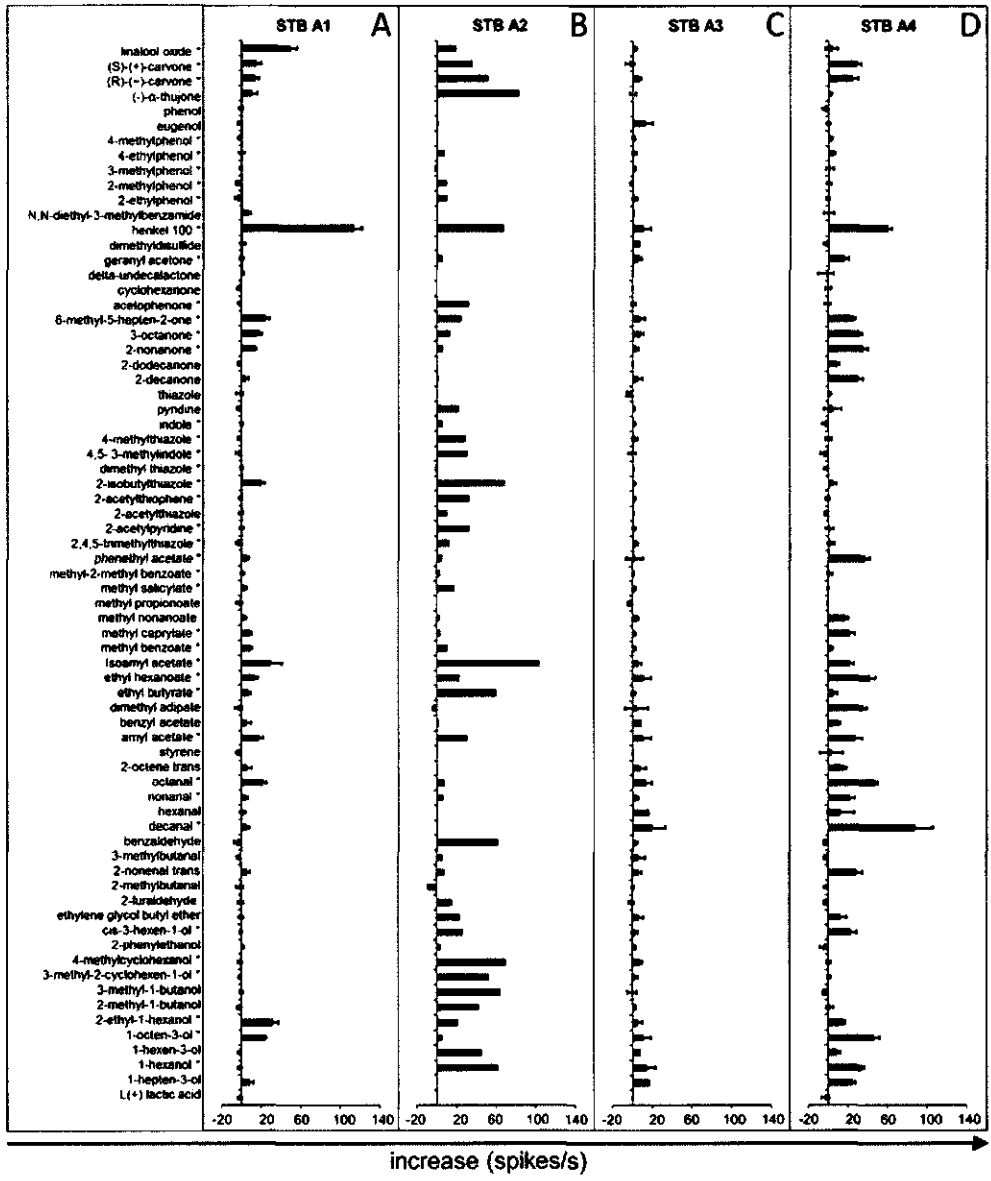

Figure 4A-D: The complete response profiles of the response types of TSB. A panel of 132 odorants at a 1% dilution was used for the characterisation. Shown are the responses to 71 odours; 41 compounds did not meet the response criterion. Odorants marked with asterisks were used for the cluster analysis (Fig. 3). Error bars represent standard error of the mean. Number of replicates for each type was: A1: n=5; A2: n=1; A3: n=2; A4: n=4. The different chemical groups have different colours; green: terpenes; dark blue: phenols; black: other compounds; yellow: ketones; purple: heterocyclics; pink: esters; light blue: alkanes; brown: aldehydes; red: alcohols.

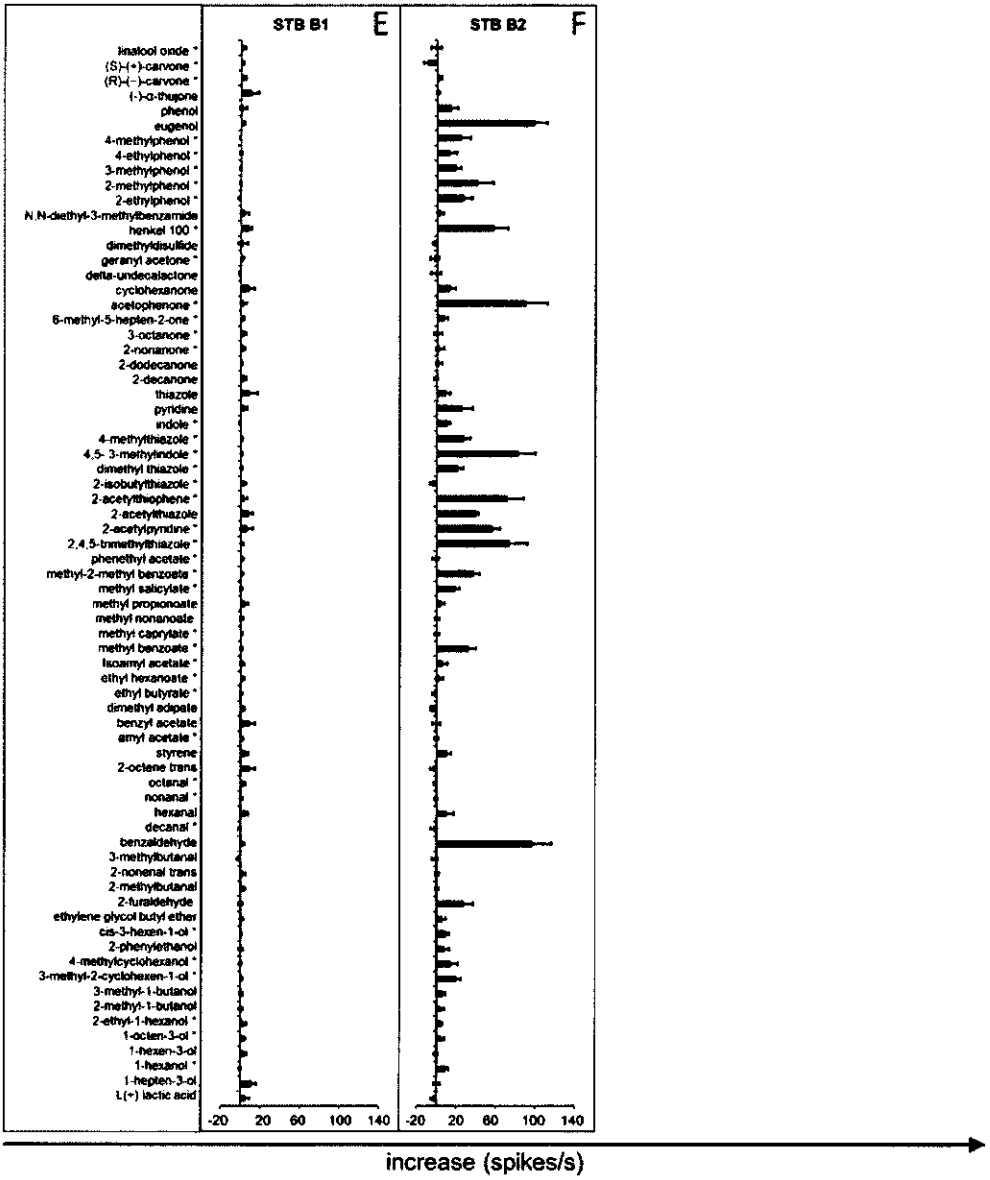

Figure 4E-F: The complete response profiles of the response types of TSB. A panel of 132 odorants at a 1% dilution was used for the characterisation. Shown are the responses to 71 odours; 41 compounds did not meet the response criterion. Odorants marked with asterisks were used for the cluster analysis (Fig. 3). Error bars represent standard error of the mean. Number of replicates for each type was: B1=6 and B2=6. The different chemical groups have different colours; green: terpenes; dark blue: phenols; black: other compounds; yellow: ketones; purple: heterocyclics; pink: esters; light blue: alkanes; brown: aldehydes; red: alcohols.

Dose-response relations

The dose-response relations were measured for several of the best ligands of the different response types. It is clear that the strength of the responses increased with increasing concentrations (Fig. 5). Typically for odorant a dose-response curve is a sigmoid curve where at a certain concentration a response plateau is reached. Of all ligands for TSB only the dose-response curve of benzaldehyde (Fig. 5l) seems to reach a plateau. For all other curves the 10% dose elicited a significantly higher response than at 1% dose (paired sample t-test, $P < 0.01$).

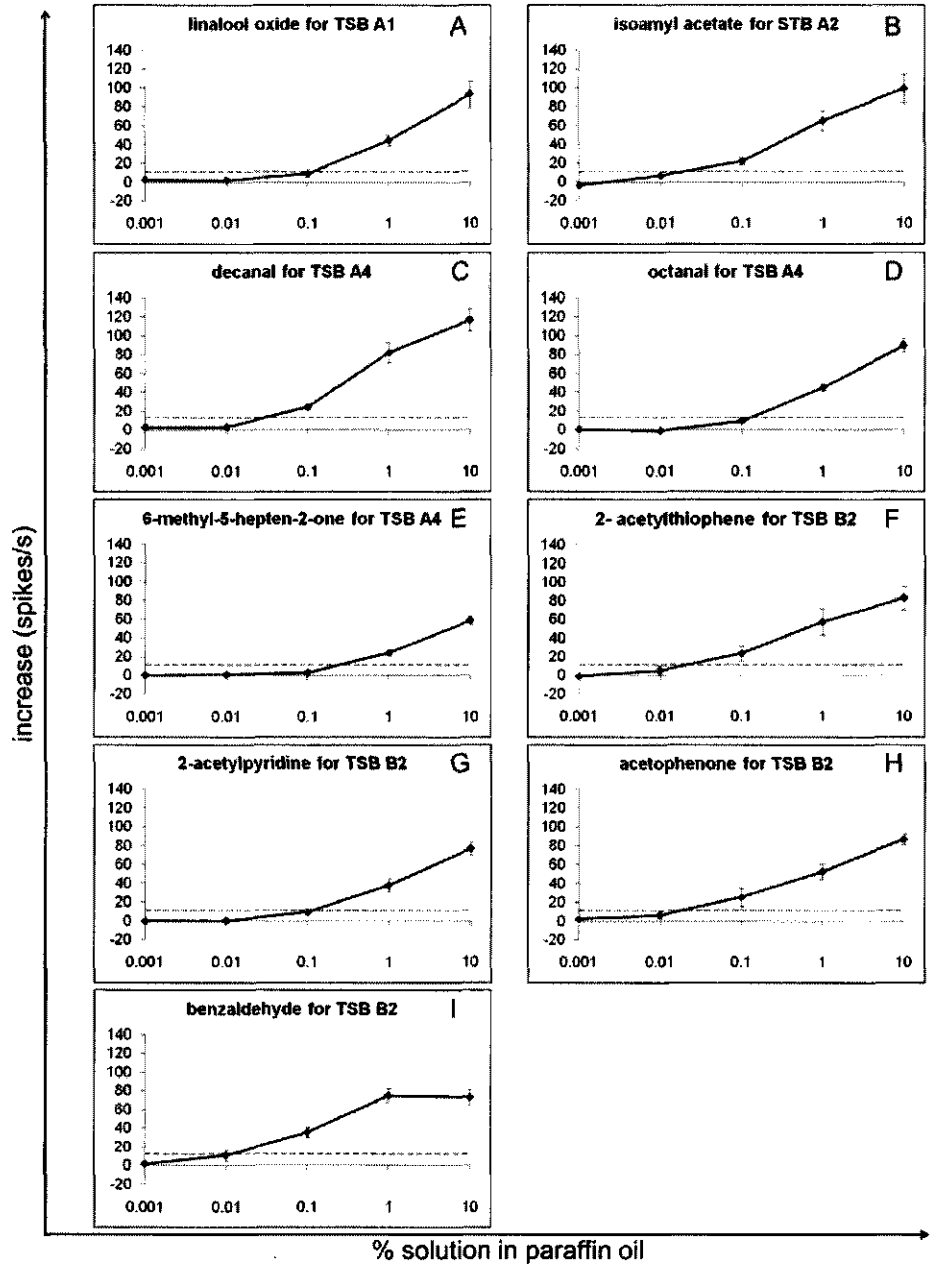

Figure 5: Dose-response relations of the best ligands for ORNs innervating trichoid sensilla type B. The X-axis shows the dilution percentage of these odorants in paraffin oil. The Y-axis shows the response in spikes/s. **A)** dose-response curve for the best ligand of A1: linalool oxide. **B)** dose response curve for the best ligand of A2: isoamyl acetate. **C-E)** dose response curves for the best ligands of type A4: decanal, octanal and 6-methyl-5-hepten-2-one. **F-I)** dose response curves for the best ligands for B2; 2-acetylthiophene, 2-acetylpyridine, acetophenone and benzaldehyde. The dashed line signifies the response criterion for excitation, i.e. twice the standard deviation of the spontaneous activity. Following this criterion the intersection between the dashed line and the dose response curve is the threshold value.

Trichoid sensilla morphological type D: the short blunt-tipped sensillum

Trichoid sensillum type D (TSD) was easily recognised as it is one of the shortest trichoid sensilla. The diameter of the hair does not change much from base to tip and it is the only trichoid sensillum type with a blunt tip. The electrophysiological responses of 88 TSDs located on antennal segments 4 to 10 to a panel of 132 odorants were recorded (Appendix 2).

Only 35 of the 132 compounds elicited responses from ORNs innervating TSDs. The ORNs associated with TSD had a much narrower responsive range (Fig. 7) than the ORNs associated with TSB (see above).

Similar as for TSB, most responses of TSD ORNs were excitatory and few inhibitory. The average responses for TSD ORNs seemed to be lower than for TSB. The amplitude of the action potentials recorded from TSD ORNs was between 0.08 mV and 0.12 mV. This is significantly lower than the amplitude recorded from TSB ORNs (between 0.12mV and 0.20mV). The lower amplitude led to a smaller signal to noise ratio for TSD ORNs.

Cluster analysis

We applied the hierarchical cluster analysis separately for A and B neurons. This resulted in three response types for neuron A (A1, A2 and A3) (Fig. 6A) and two response types for neuron B (B1 and B2) (Fig. 6B). Of the 13 sensilla used for the hierarchical cluster analysis most belonged to type A1 (8 of 13). Three sensilla were assigned to type A2 and two to type A3.

Characterisation of the response spectra of ORNs associated with TSD

The response profiles of the five response types that resulted from the cluster analysis were visualised using all 35 odorants that elicited responses (Fig. 7). Response type A2 had overall the weakest, A1 intermediate and A3 has the highest responses to its ligands. Responses of A3 were almost twice as strong as the responses of A2, although they showed similar response profiles (Fig. 7A-C). The two response types of the B neurons of TSD looked similar but had different firing frequencies like the response types for the A-neuron, response type B1 having the lowest frequency.

The response types of A and B neurons showed different response profiles. The three A types responded to more odorants and odorants from more chemical groups than did the B neurons. All the response types of the A-neuron responded best towards alcohols; 10 of the 35 response-eliciting compounds were alcohols. The best ligand was the alcohol 4-methylcyclohexanol. Other alcohols with similar carbon chain lengths eliciting excitatory responses were 3-methyl-2-cyclohexen-1-ol, 1-hexanol, 1-hexen-3-ol, ethyl-1-hexanol and 1-hepten-3-ol. Other alcohols eliciting responses were 2-methyl-1-butanol, 3-methyl-1-butanol, 2-phenylethanol and 2-butoxyethanol. The odorants 4-methylcyclohexanol and 2-ethyltoluene elicited the strongest responses from all three A-neuron response types. Among the odorants

eliciting responses of lower strength are 2-ethylphenol and 2-methylphenol, (+)- and (-)-fenchone, styrene, benzaldehyde and 4,5-dimethylthiazole.

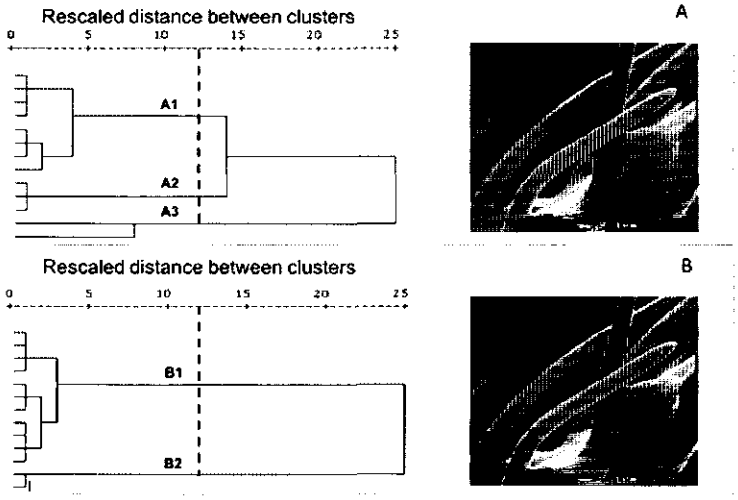

Figure 6: Dendrograms obtained by a hierarchical cluster analysis (Squared Euclidian distance, Ward's method) on 13 A-neurons and 13 B-neurons associated with TSD according to their responses to a panel of 18 compounds (indicated with an * in Fig.7). The Scree procedure, which entails that a sharp increase in distance among clusters identifies a change in the nature of the clustering steps, was used to define the intersect (dashed line) that best represents the number of clusters for our dataset. **A)** dendrogram of the TSD A-neurons. **B)** dendrogram of the TSD B-neurons.

The response profiles of response types B1 and B2 are similar to each other but different from the three A-neuron response types. Of the 13 sensilla used for the cluster analysis, 11 were attributed to response type B1 and only two to type B2. The two A-neurons that co-compartmentalised with these two type B2 ORNs also formed a separate cluster, i.e. type A3. TSD response type B1 neurons were thus co-compartmentalised with both A1 and A2 response type ORNs.

In both response types for neuron B the heterocyclics formed the group eliciting the strongest responses (Fig. 7D-E). Two of the heterocyclic compounds, 2-acetylthiophene and 2-acetylpyridine, were amongst the three ligands eliciting the strongest responses. The third best ligand is a ketone, acetophenone. Henkel 100 also elicited strong excitatory responses. Other odorants eliciting significant responses were several thiazoles (4-methylthiazole, 2-acetylthiazole, 2,4,5-trimethylthiazole), a ketone (cyclohexanone), an aldehyde (benzaldehyde), an ester (methyl benzoate) and several alcohols (ethylene glucol butyl ether, 3-methyl-2-cyclohexen-1-ol and 4-methylcyclohexanol).

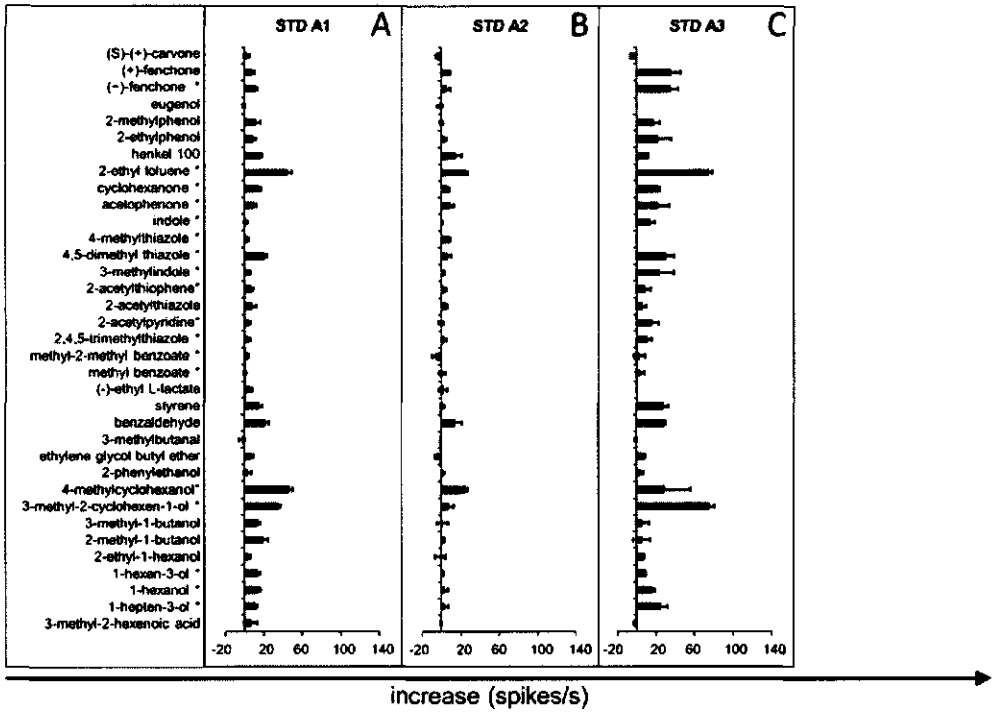

Figure 7A-C: The complete response profiles of the response types of TSD. A panel of 132 odorants at a 1% dilution was used for the characterisation. Graphically represented are responses (in spikes/s) of the three response types according to the cluster analysis, to 35 of the 132 odorants to which at least one neuron showed a response. Odorants with asterisks were used for the cluster analysis (Fig. 6). Error bars represent standard error of the mean. Number of replicates for each type was: A1: n=8; A2: n=3; A3: n=2. Responses to compounds from different chemical groups are given different colours; terpenes in green, phenols in dark blue, ketones in yellow, heterocyclics in purple, esters in pink, alkanes in light blue, aldehydes in brown, alcohols in red and other compounds in black.

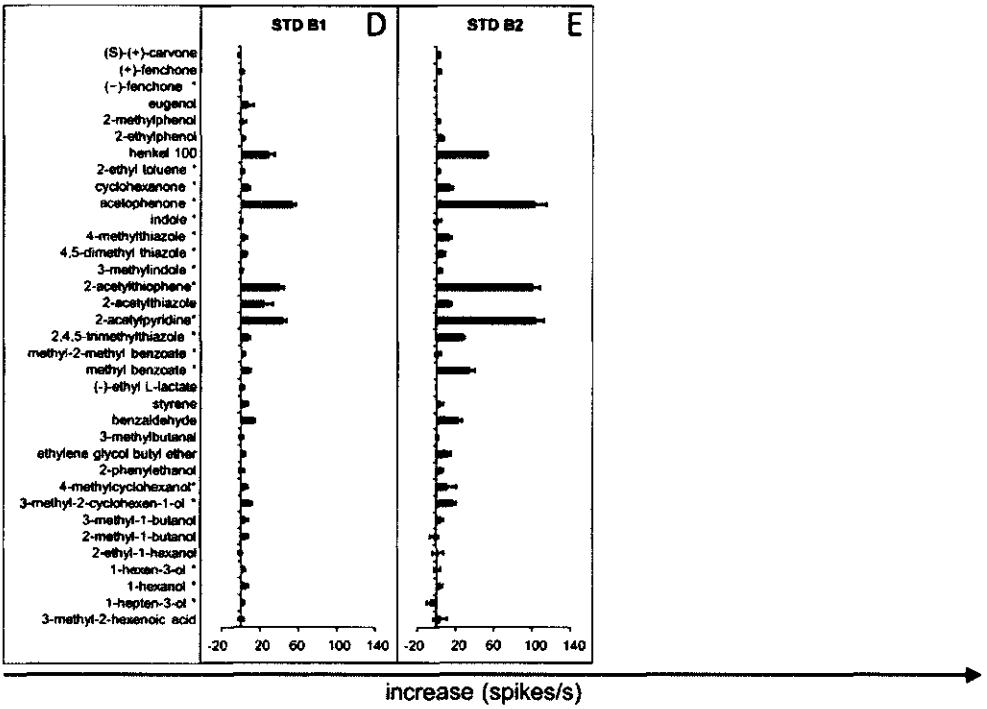

Figure 7D-E: The complete response profiles of the response types of TSD. A panel of 132 odorants at a 1% dilution was used for the characterisation. Graphically represented are responses (in spikes/s) of the three response types according to the cluster analysis, to 35 of the 132 odorants to which at least one neuron showed a response. Odorants with asterisks were used for the cluster analysis (Fig. 6). Error bars represent standard error of the mean. Number of replicates for each type was: B1: n=11 and B2: n=2. Responses to compounds from different chemical groups are given different colours; terpenes in green, phenols in dark blue, ketones in yellow, heterocyclics in purple, esters in pink, alkanes in light blue, aldehydes in brown, alcohols in red and other compounds in black.

Dose-response relations for TSD ORNs

Identifying the specific response types that were recorded by using a diagnostic panel as described for TSB was not feasible for the different types of TSD. As can be seen from Fig. 7A-C and 7D-E the response profiles look rather similar between the three A neuron response types and between the two B neurons response types. This also means that the best ligands for all three response types are the same, 4-methylcyclohexanol and 2-ethyltoluene. The two response types for neuron B also have the same best ligands: 2-acetylthiophene, 2-acetylpyridine and acetophenone. For this reason we investigated the dose-response relationships for these best ligands without looking at the separate response types. No significant differences in threshold values were found between different ligands.

Figures 8A and 8C show the dose-response relations to 4-methylcyclohexanol and 2-ethyltoluene, the best ligands for response types A1, A2 and A3 to a series of concentrations ranging from 0.001% to 10% dilutions. The threshold concentration for 2-ethyltoluene and 4-methylcyclohexanol was between 0.1% and 1%, while the threshold concentration of the ligands of the B neuron was between 0.01% and 0.1% (Fig. 8D-F).

The dose-response curve for 4-methylcyclohexanol reaches a plateau at a dose of about 1% (Fig. 8A), while 2-ethyltoluene shows a clear increase in response intensity (paired sample t-test; $P = 0.008$) from 1% to 10% dilution (Fig 8C). A more detailed dose-response study for 4-methylcyclohexanol with four extra concentrations between 0.1% and 10% dilution (Fig. 8B) showed that the four highest concentrations did not significantly differ from each other (GLM; $P > 0.05$), indicating a plateau that was reached at 1% for 4-methylcyclohexanol.

For response types B1 and B2 of the TSDs, dose-response relations were obtained for 2-acetylthiophene, 2-acetylpyridine and acetophenone, and all three were of similar shape (Fig. 8D-F). The response threshold was between 0.01% and 0.1% dilution and for all three odorants the 10% dilution elicited significantly higher responses than the 1% dilution (paired sample t-test, $P = 0.019$; $P = 0.003$; $P = 0.049$ respectively).

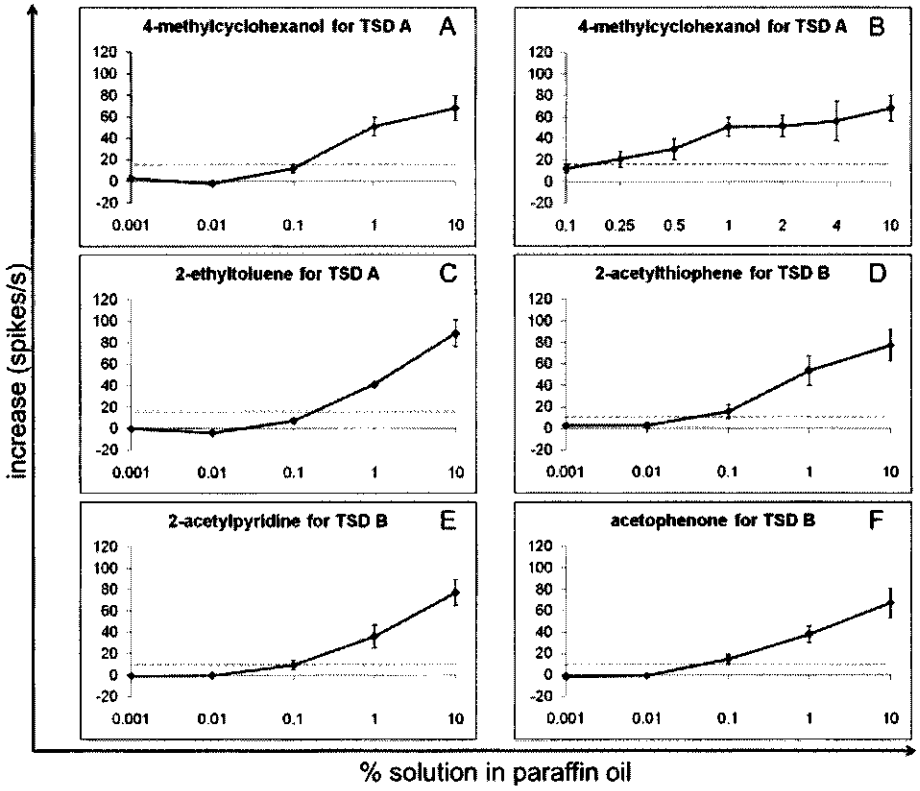

Figure 8: Dose-response relations for the best ligands for TSD-ORNs. X-axis shows the dilution percentage, except for 8B. Y-axis shows the response to an odorant in spikes/s. **A)** dose-response curves for the A-neurons of TSD in response to 4-methylcyclohexanol. **B)** dose-response curve of the A-neurons of TSD in response to 4-methylcyclohexanol for the dose range 0.1 – 10%. **C)** dose-response curve for TSD A-neurons in response to 2-ethyl toluene. **D-F)** dose-response curves for the B-neurons of TSD to 2-acetylthiophene **(D)**, to 2-acetylpyridine **(E)** and to acetophenone **(F)**. For all measurements: n > 6. Error bars represent standard error of the mean. The dashed line indicates a response level of the spontaneous activity level plus twice the standard deviation. The intersection between the dashed line and the dose-response curve is the threshold dose.

Temporal aspects

Typical temporal responses of ORNs are the phasic response (Fig. 9A-B) and the prolonged phasic-tonic response (Fig. 9C), both response types were found in A and B neurons from TSB as well as TSD.

Within the phasic responses, differences can be noted. Some responses stopped right after the stimulus was terminated. A stimulus of 0.2 s would result in a higher firing frequency that dropped to normal spontaneous activity levels right after the 0.2 s stimulus duration. However, some odours, like 6-methyl-5-hepten-2-one, also elicited higher firing frequencies that stopped suddenly but their firing frequency remained high a bit (100 ms) longer than the stimulus duration (Fig. 9A). The response of TSB type A4 neurons demonstrated another feature of odour coding: a post-excitatory inhibition (Fig. 9B). Several aldehydes showed such an inhibition of the spontaneous activity after an excitation. When stimulated with a higher concentration the phasic response pattern changed into phasic-tonic pattern. The post-excitatory inhibition found for decanal disappeared when stimulated with a 10-fold higher concentration.

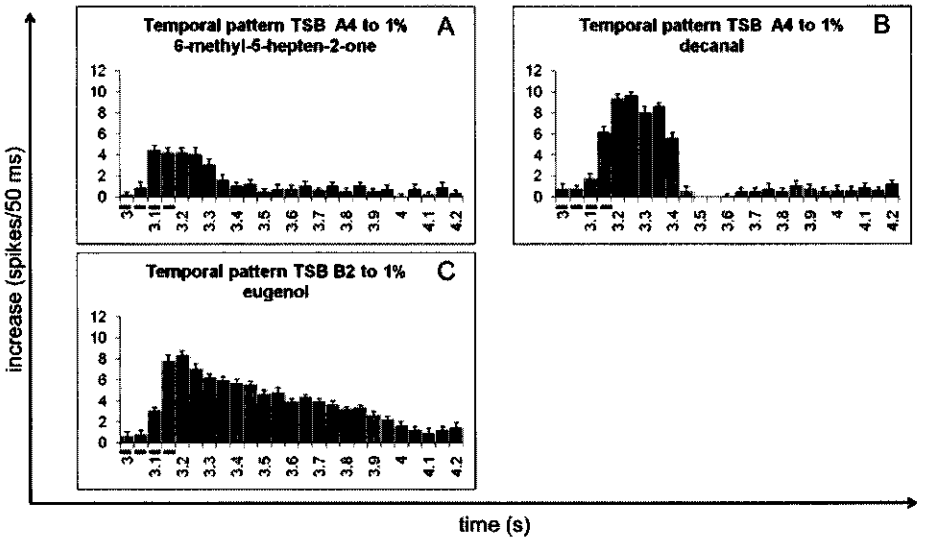

Figure 9: Temporal characteristics of the ORNs associated with TSB in response to three different odorants. Each bar represents an average amount of spikes per 50-ms bin (n=7). The dashed line indicates the 200 ms stimulus that started after 3s in a 10s recording. **A)** temporal characteristics of TSB type A4 in response to 6-methyl-5-hepten-2-one. **B)** temporal characteristics of the response of TSB type A4 to decanal. **C)** temporal characteristics of TSB B2 in response to eugenol.

Tuning width of the different response types

To visualise the specificity of neuron responses, tuning curves were generated for each ORN response type (Fig. 10). A statistical measure of the "peakedness" of the distribution is the kurtosis (K) value. These values were calculated for each response type to allow a quantitative comparison (K-values in Fig. 10). Within TSB the difference seen in the response profiles is further confirmed by different kurtosis values for the different types (Fig. 10). The two weakly responsive response types A3 and B1 have a similar tuning width and kurtosis value (Fig. 10). All other response types have a distinct different tuning curve and thus a different Kurtosis value.

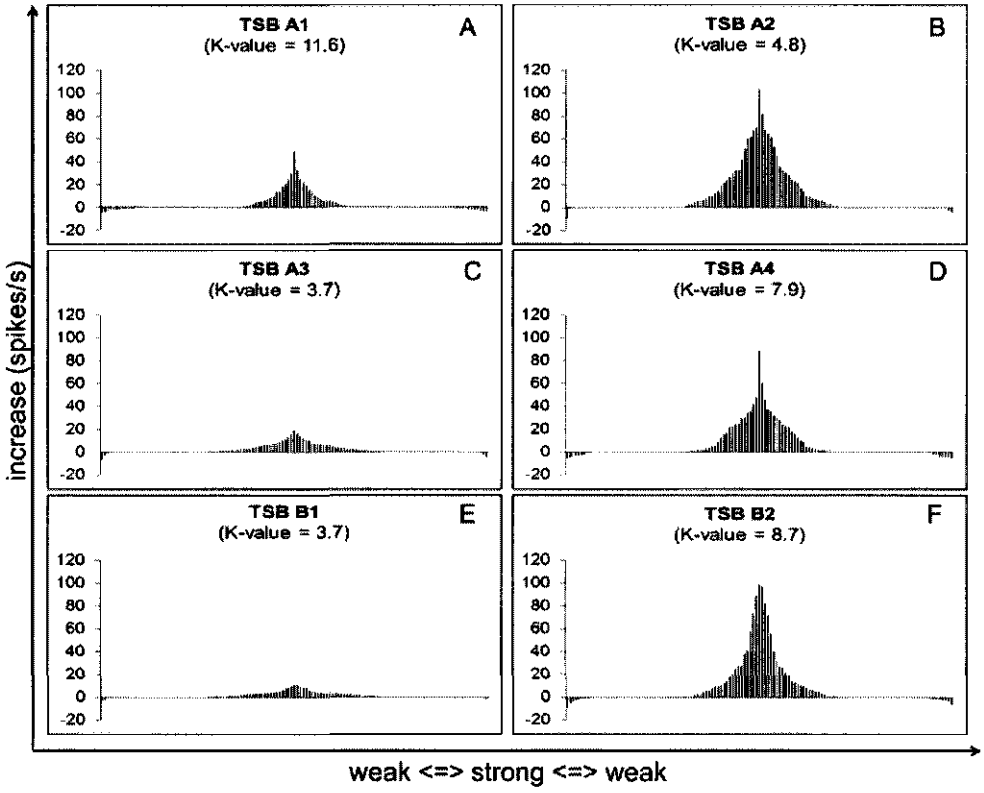

Figure 10A-F: Tuning curves for the response types of trichoid sensilla B. Each figure represents the tuning width of a specific response type to our odorant panel. The 132 odourants are arranged along the X-axis according to the strength of the response they elicit from each response type. The odorants that elicit the strongest responses are placed near the centre of the distribution; those that elicit the weakest responses are placed near the tails. The order of odorants is therefore different for each response type.

The tuning curves of the response types of TSD are less broad because they respond to fewer odours. This explains the larger Kurtosis values. Especially type B2 seemed narrowly tuned, responding strongly to only three odours. The overall tuning widths, given by their Kurtosis values were different but not as distinct as seen in the K-values of the response types of TSB. The Kurtosis values of the three response types for the A neuron of TSD are close together as are the 2 response types for the B neuron of TSD. This could be expected based on their similar response profiles (Fig. 7).

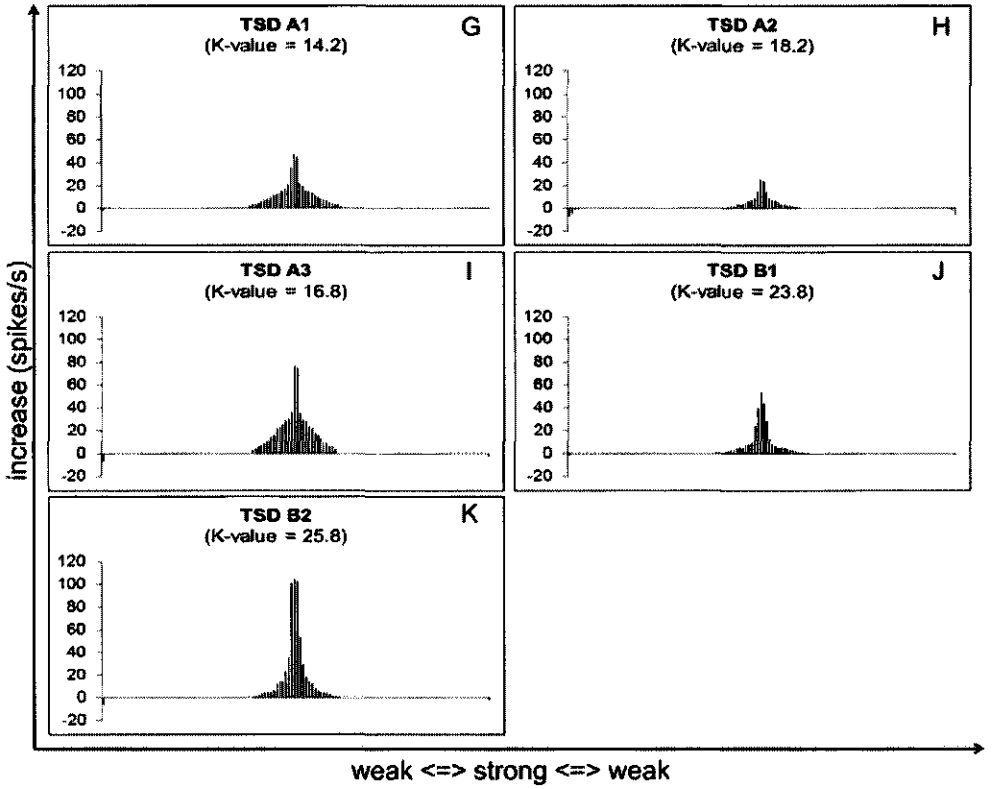

Figure 10G-K: Tuning curves for the response types of trichoid sensilla D. Each figure represents the tuning width of a specific response type to our odorant panel. The 132 odourants are arranged along the X-axis according to the strength of the response they elicit from each response type. The odorants that elicit the strongest responses are placed near the centre of the distribution; those that elicit the weakest responses are placed near the tails. The order of odorants is therefore different for each response type.

Comparison of *in vivo* data with DmHES characterisation of AgORs

The projection to latent structures (PLS) analysis, explaining the *in vivo* characterised trichoid sensilla B data by the DmHES characterised AgOR data, extracted 3 significant principal components. Figure 11 shows the first 2 principal components which explained 44.1% of the variation in the data ($R^2X_{cum} = 0.541$, $R^2Y_{cum} = 0.453$, $Q^2_{cum} = 0.297$).

The regression coefficients were used to describe the relationship between the TSB ORNs and the AgORs (Fig. 12). The AgOR with the highest coefficient is most related to that ORN. As seen in Fig. 12 the regression coefficients were not high. Comparison of the profiles of the TSB ORNs with the AgORs revealed that lower coefficients showed less comparable odour profiles, while the matches with higher coefficients seemed more similar in odour profile (Fig.13).

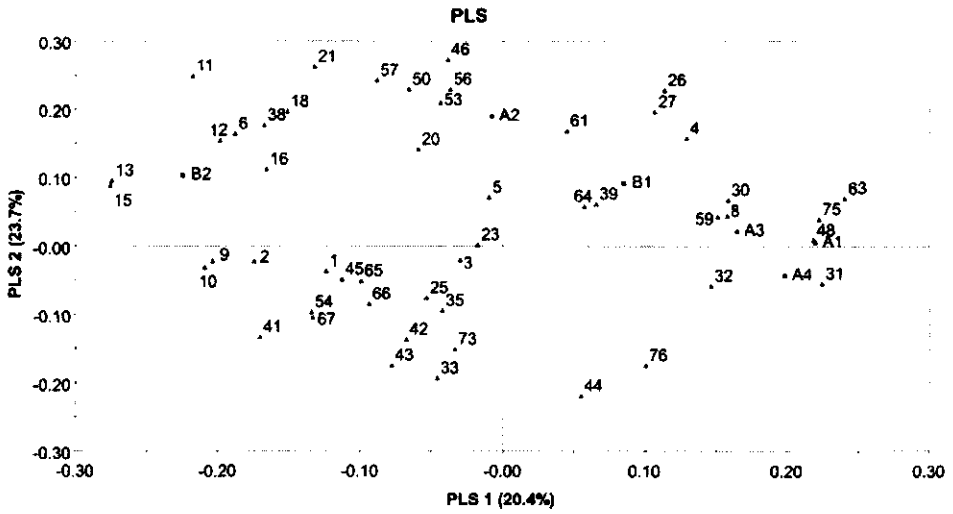

Figure 11: A multivariate analysis on the sensitivities of the six identified olfactory receptor neurons (ORNs) of TSB and all 50 DmHES characterised *Anopheles gambiae* olfactory receptors (AgORs). The loading plot of the first two components of the projection to latent structures (PLS) obtained by plotting the ORNs (Y) against the AgORs (X).

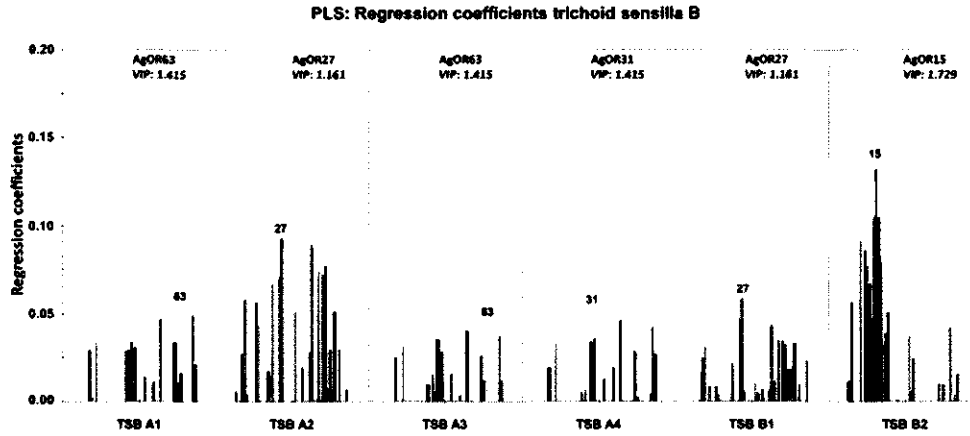

Figure 12: The regression coefficient overview plot of the olfactory receptor neurons (ORNs) of trichoid sensilla B (TSB). On the X-axis for each ORN of TSB are the 50 *Anopheles gambiae* olfactory receptors (AgORs). On the Y-axis the regression coefficients of the AgORs for that specific ORN are plotted. The coefficients represent the degree of correspondence between the response profiles of that AgOR and the specific ORN. The AgOR with the highest coefficient is given together with their variable importance to the projection (VIP).

The PLS on the data for trichoid sensilla D extracted 2 significant principal components (Fig. 14) that explained 28.3% of the variation in the data (R^2X_{cum} = 0.283, R^2Y_{cum} = = 0.483, Q^2_{cum} = 0.0818).

Figure 15 shows the regression coefficients overview plot for ORNs of trichoid sensilla D. The three A-neurons show very similar regression coefficient plots indicating that they are highly correlated. The two B-neurons are also strongly correlated. Clearly AgOR11 (VIP: 2.374) and AgOR6 (VIP: 2.498) are the most related to, respectively, the A-neurons associated with TSD and the B-neurons associated with TSD.

Comparison of the profiles of the *in vivo* response types with the DmHES AgORs reveal similar odour profiles for the B-neurons and less similar profiles for the A-neurons (Fig.16).

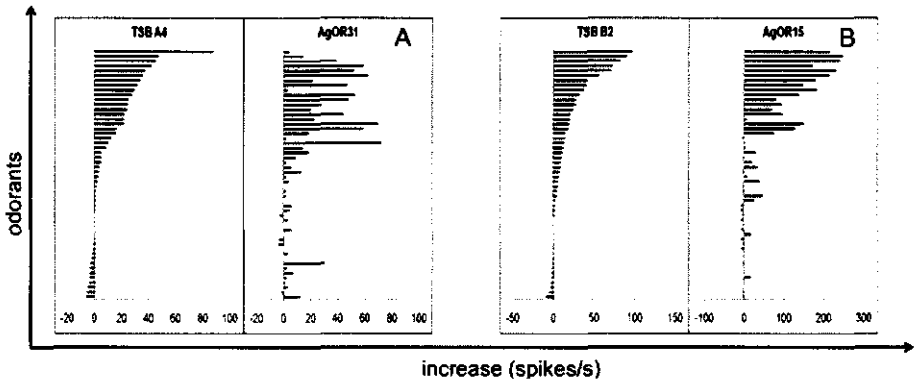

Figure 13: Profiles of related ORNs of trichoid sensilla B and AgORs. The profiles were obtained by sorting the responses of the ORN from strong to weak responses and subsequent sorting of the matched AgOR according to the same ranking of stimulatory effectiveness of the odours. **A)** profiles of TSB A4 and AgOR31. **B)** profiles of TSB B2 and AgOR15.

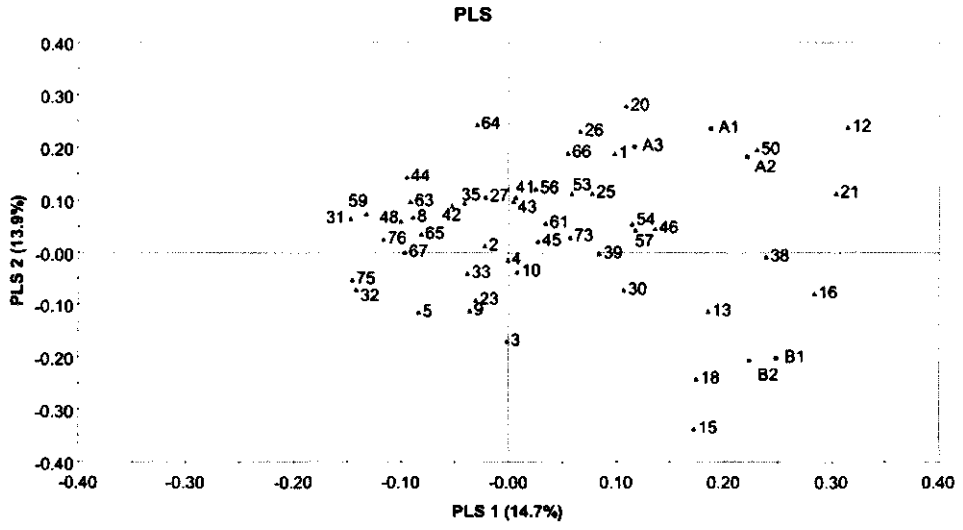

Figure 14: Multivariate analysis of the response profiles of the identified olfactory receptor neurons (ORNs) of trichoid sensilla D (TSD) and all 50 DmHES characterised *Anopheles gambiae* olfactory receptors (AgOR). The loading plot of the first two components of the projection to latent structures (PLS) obtained by plotting the ORNs (Y) against the AgORs (X).

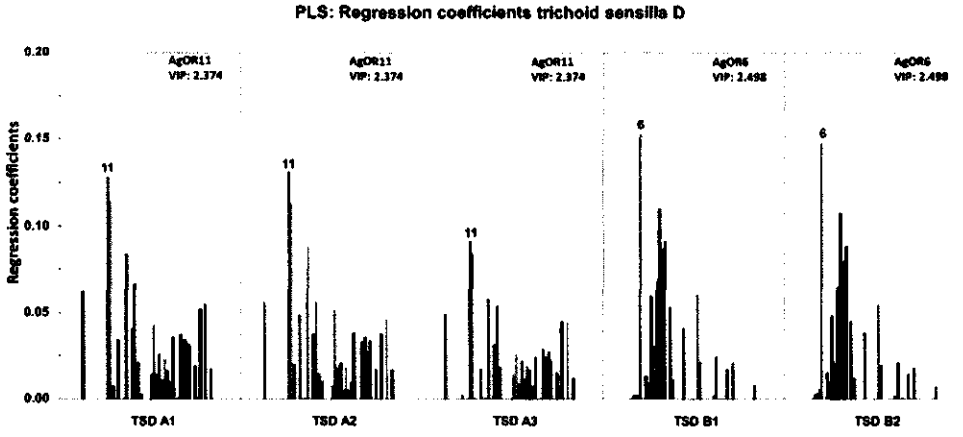

Figure 15: The regression coefficient overview plot of the olfactory receptor neurons (ORNs) of trichoid sensilla D (TSD). On the X-axis for each ORN are the 50 *Anopheles gambiae* olfactory receptors. On the Y-axis are the regression coefficients for the relation between AgOR and that specific ORN. The coefficients represent the degree of correspondence between the response profiles of the AgOR and the specific ORN. The AgOR with the highest coefficient is given together with their variable importance to the projection (VIP).

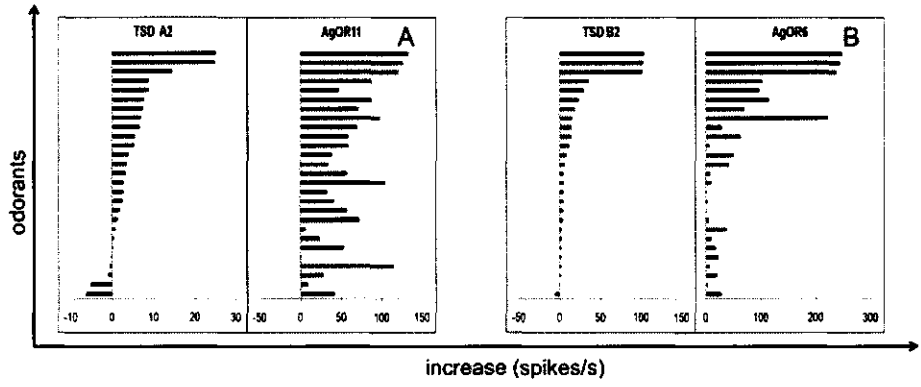

Figure 16: Response profiles of ORNs of trichoid sensilla D and of AgORs putatively expressed therein. The profiles were obtained by sorting the responses of the *in vivo* characterised ORN response profiles from strong to weak responses and subsequent sorting of the matched AgOR according to the same ranking of odours. **A)** profiles of TSD A2 and AgOR11. **B)** profiles of TSB B2 and AgOR6.

Discussion

Responses of the ORNs associated with TSA

None of the odours in the panel were capable of activating the ORNs associated with the trichoid sensilla type A. Similar findings were also reported for the longest sharp-tipped sensilla in *Ae. aegypti* (Ghaninia *et al.* 2007b) and *Cx. quinquefasciatus* (Hill *et al.* 2009). This indicates that this sensillum type might be highly specialistic and that possible ligands were just not present in the odour panel used for this study. Specialistic channels are thought to respond primarily to biologically essential odours (Wilson and Mainen 2006). Further research should focus on using different biologically relevant odours from various sources because finding the ligand of such a specialistic ORN might lead to an odour possibly capable of disrupting/redirecting the mosquito's behaviour.

Response characteristics of the ORNs associated with TSB and TSD

From the 132 odours in the panel tested at a 1% concentration, 71 compounds elicited responses from ORNs present in TSB and 35 odours elicited responses from TSD. Almost all of the responses recorded were excitatory, only few were inhibitory. The low incidence of inhibition-type responses may have been due to the evaluation criteria we used. We tested with a 200 ms stimulus and analysed spike frequency during 500 ms after the stimulus. It is very likely that inhibition will only take place during the stimulus (200 ms) but at a low spontaneous firing rate, with a maximum of 16.9 spikes/s, on average only 3-4 spikes are measured as spontaneous activity during this 200 ms. As this is lower than the standard deviation of the mean spontaneous activity (lowest being 4.1 spikes/s) our rules of using the standard deviation for indicating the boundaries of responses are not suited for detection of inhibition. One way to better detect inhibition at low spontaneous firing frequencies would be to prolong the stimulus duration and equalize the stimulus and analysis duration.

Most of the excitatory responses measured for TSD ORNs rarely exceeded 50 spikes/s, which is fairly low compared with the highest responses (160 spikes/s) measured in other trichoid sensilla of *An. gambiae* (Qiu *et al.* 2006). Response strengths of TSB ORNs were slightly higher than those of ORNs in TSD but still not as high as compared to responses from TSE and TSC ORNs (Qiu *et al.* 2006). Interestingly, responses from AgORs when expressed in a heterologous system seem to be much higher. Carey *et al.* (2010) recorded responses from AgORs via the empty neuron system that very often elicited response intensities exceeding 100 spikes/s sometimes even exceeding 250 spikes/s. This means that a comparison between both systems should rely more on ratio's between responses from several good ligands than on the absolute intensity of the response.

Clustering of the ORNs associated with TSB and TSD

The effort to classify ORNs into specific response types by using cluster analysis was challenging. It was difficult to measure the complete panel of 132 compounds on one single sensillum. To record the responses to all 132 compounds in the panel, while leaving at least a minute time between subsequent stimulations, means that the ORN had to stay in good condition for over three hours. This rarely occurred, resulting in incomplete measurement series that are not suitable for a hierarchical cluster analysis. Based on our present experiences, an ideal number of odours would be around 75 to 80 odours.

Apart from the cluster analysis, the profiles of the three response types for the A neurons of TSD could not be visually distinguished (Fig. 7A). It seemed as if the three response types had similar response spectra but response intensities differed. Thus, finding specific response types for TSD during additional measurements is not feasible. Because of a more qualitative difference, the various TSB response types were easier to identify. Using a diagnostic panel of six compounds, identification of response type can be achieved, making it a more feasible target for further research.

Response spectra of ORNs associated with TSB and TSD

Among the 71 odorants that elicited responses in TSB, 38 have been shown to be emitted by humans (Appendix 2: Bernier *et al.* 1999, Bernier *et al.* 2000, Penn *et al.* 2007, Gallagher *et al.* 2008). Of the 35 compounds eliciting responses in TSD, 15 were reported as being present in human effluents (Appendix 2). The response spectra of ORNs from both types of trichoid sensilla are very different although overlap has been recorded (Figs. 3 and 7).

Twenty-eight of all 132 odorants in our panel elicited responses from both TSB and TSD and of these 28, a total of 13 have been reported as being emitted by humans (Appendix 2). Five of these 13 human odorants are produced by bacteria growing on human feet (Verhulst *et al.* 2009), the preferred body part for feeding by *An. gambiae* (de Jong and Knols 1995a). Moreover, two of the 13 human odorants, namely acetophenone and benzaldehyde, were the best ligands of several response types. These two compounds have been tested for behavioural responses using olfactometers; acetophenone was found to be attractive and benzaldehyde repellent (G. Bukovinszkine Kiss, unpubl. data).

Both trichoid sensilla types in this study showed a clear difference in response profiles between the A-neurons and B-neurons. Similar findings were reported for the two trichoid sensilla previously characterised for *An. gambiae* (TSE and TSC) (Qiu *et al.* 2006). Exceptions are the TSB housing ORNs belonging to response types A3 and B1, because they show no clear responses to any of the compounds tested. All other ORNs co-compartmentalised in the same sensilla can have some overlap in their response spectra but for the most part they are different. This pairing increases the width of the response profile of that single sensillum.

All response types identified were responding to multiple odours. In general, the response types of TSB represented more odours to which higher average responses were recorded than those identified for TSD but all response types that were excited by our panel showed responses to more than 10 different chemicals. A single odour may evoke responses from multiple ORNs. Benzaldehyde, for example, elicits responses from all five response types of TSD as well as from neurons A2 and B2 in TSB. These findings are in line with the model of combinatorial coding: it is the pattern of activated receptors that is used for identification of odour blends, not the response of a single receptor. (Qiu *et al.* 2006, Ghaninia *et al.* 2007b, Hill *et al.* 2009, Carey *et al.* 2010). Although responses were observed towards several human-derived odorants, like 3-methyl-1-butanol and benzaldehyde, none of the response types identified responded to amines or short-chain carboxylic acids that have already been shown to be involved in host-seeking behaviour of *An. gambiae* (Knols *et al.* 1997a, Smallegange *et al.* 2005b, Okumu 2008, Okumu *et al.* 2009, Smallegange *et al.* 2009). These acids did elicit responses from trichoid sensilla C and E and grooved peg sensilla in *An. gambiae* (Qiu *et al.* 2006) and from all trichoid sensilla response types identified in *Aedes aegypti* and *Culex quinquefasciatus* (Ghaninia *et al.* 2007b, Hill *et al.* 2009). Though many good ligands of the response types described here are odorants described as emanating from human skin, many of these odorants are also found in plant and/or oviposition-site emanations. Accordingly, they may also be used to induce sugar feeding or egg laying behaviour.

Nonetheless all of these strong ligands represent possible candidates for behavioural disruption; some for disruption of host seeking, like the human-derived odours, others for the disruption of the search for a sugar meal or a suitable oviposition place. It is not inconceivable that only a few compounds already account for many of the different searching behaviours. It might be that it is the combination and the specific ratios of a few compounds that influence all behaviours. It is known that a physical change like a blood meal alters the sensitivity of ORNs (Qiu *et al.* 2006, Siju *et al.* 2010). Further experiments should provide more insight into the behavioural effects of the ligands presented in this study.

Two response types were characterised by an absence of strong responses (> 50 spikes/s, which is half of the maximum increase observed) to any of the odours from our panel. It is possible that they are very narrowly tuned ORNs containing specific ORs tuned to ligands that were absent from our panel. As mentioned above such ORNs have been postulated to act as specialised channels that respond only to biologically crucial odours (Wilson and Mainen 2006). Similar spontaneously active but non-responsive ORNs have been found in the trichoid sensilla of *Ae. aegypti* (sbtII1B and sst2A) and *Cx. quinquefasciatus* (sst 2A). All of these ORNs might be narrowly tuned to specific compounds that were not in the panels used in this study. Even though our panel contained 80 human odours in our panel, Bernier *et al.* (2000) identified 346 compounds from human hand emanations. This leaves 266 other odours that might be used for host-seeking mosquitoes to find a human host. However, these ORNs that do not respond to the odorants tested, might also be tuned to plant odours, oviposition-site related odours or mating stimuli that have not been included in the compound panels used for the

characterisation. If these ORNs truly respond only to one or two compounds, these odorants will make excellent candidates for behavioural testing. However, it is very tedious work to test hundreds to perhaps thousands of compounds using single sensillum recording electrophysiology. With the recent progress in knowledge on insect olfactory mechanisms (Su *et al.* 2009, Silbering and Benton 2010), it might be possible in the future to provide a better way to select a panel of chemicals based on their molecular structure and the binding properties of the corresponding receptors.

Even though the trichoid sensilla of three mosquito species, *An. gambiae*, *Ae. aegypti* and *Cx. quinquefasciatus*, have been partly characterised, it is difficult to compare the response types and profiles found in these three species with each other even with similar odorant panels. Each study used different equipment and methods, but the most important factor is probably that different concentrations of odorants have been used. This study as well as the previous study done on *An. gambiae* (Qiu *et al.* 2006), has used a 1% concentration as standard concentration for the characterisation of the different ORNs. However, the characterisation studies done on both *Ae. aegypti* (Ghaninia *et al.* 2007b) and *Cx. quinquefasciatus* (Hill *et al.* 2009) used 10% concentrations. A 10-fold higher concentration likely has a large impact on both the number of ORNs that will respond to a compound as well as the strength of the response. It is a common finding that higher concentrations of odorants elicit activity from greater numbers of ORNs (de Bruyne *et al.* 1999, de Bruyne *et al.* 2001, Wang *et al.* 2003, Su *et al.* 2009). Comparison of the response types of *An. gambiae* found in this study combined with the response profiles reported by Qiu *et al.* (2006) with the types found in *Ae. aegypti* and *Cx. quinquefasciatus* reveals a clear overlap in overall odour space between the three species, eleven of the 13 odours used in all three panels elicit responses from all three species. But each species has its own unique response types with different odours as best ligands.

Comparison of response types with AgORs expressed in the DmHES

Lu *et al.* (2007) characterised the response profiles of ORNs in the palpal capitate peg sensilla and showed that AgOR8 and AgOR28 were localised in two of the associated ORNs. The response profiles of the endogenous ORNs were found similar to that of the AgORs functionally expressed in the oocytes of *Xenopus*. Carey *et al.* (2010) demonstrated that, when expressed in the empty neuron system, the response profile of AgOR8 still resembled that of the endogenous ORN. Based on these findings, we expected that we could match results from functional studies on AgORs in heterologous systems with those from SSR studies on other endogenous ORNs. To date, however, none of the other AgORs have been localised and therefore it is still not known in which sensillum or sensilla each AgOR is expressed. By matching response profiles obtained from *in vivo* electrophysiology and electrophysiology from AgORs expressed in a heterologous system we may have an indication of the sensilla in which some AgORs are expressed, which could then be verified by localisation studies done in a similar way as done by Lu *et al.* (2007). Matching the two datasets is for various reasons

(dissimilar panels, different methods, overall higher responses in empty neuron system, etc.) quite challenging. By comparison of the odour profiles of our response types with those of the 50 functional AgORs expressed in the empty neuron system of *D. melanogaster* (Carey *et al.* 2010), we could match some of our ORN response patterns with the AgORs expressed in the heterogeneous system but not all (Figs. 13 and 16).

It is to be expected that the highest similarity between both systems is found in the responses to their best ligands, as is the case for the capitate peg sensilla (Lu *et al.* 2007). Therefore, the responses towards the best ligands of each of the response types described in this study should have a larger weight in the comparison. According to our knowledge it is not yet possible to assign such weights in a PLS. Considering the response types for the TSB sensillum, two out of the six response types (i.e. the response types A3 and B1 for TSB) do not show responses higher than 25 spikes/s. In the AgOR characterisation all AgORs showed some responses (either inhibitory or excitatory) (Carey *et al.* 2010), but 17 AgORs showed only some inhibitory responses, among them is AgOR63 which is matched with TSB A1 and TSB A3.

TSB response type A1 shows *in vivo* the strongest increase when stimulated with linalool oxide. There are two AgORs that respond strongly to linalool oxide: AgOR20 and AgOR50. However, the profiles of these AgORs do not match with that of TSB A1. Next to linalool oxide, isoamyl acetate, 2-ethylhexanol, 1-octen-3-ol and 6-methyl-2-hepten-1-one are good ligands. None of the AgORs tested by Carey *et al.* (2010) respond strongly to more than two of these compounds. This explains why TSB A1 is matched with an AgOR like AgOR63, which does not show strong responses to any of these compounds. If there are no strong responses the PLS will match weaker responses, leading to matches that are suboptimal. Thus, based on the PLS combined with our match of the best ligands, we are unable to select a good candidate AgOR for this ORN.

The best ligand for TSB response type A2 is isoamyl acetate followed by ethyl butyrate, 4-methylcyclohexanol, 3-methyl-1-butanol and 1-hexanol. TSB A2 was most closely related to AgOR27 according to the PLS analysis. Again this is because the odours in the lower part of response profile (with the weaker responses) match well between these two types but that is less relevant for our search. The second best match was AgOR46 and this AgOR responds strongly to most of the better ligands of TSB A2 except for the best one; isoamyl acetate. AgOR46 does respond to isoamyl acetate but not very strongly. However, isoamyl acetate only elicits weak to intermediate responses among the complete set of 50 AgORs. At the lower part of the response profile, where the odours with weaker responses are, the match with AgOR46 is weaker. Even though *in vivo* isoamyl acetate always gives the strongest responses and the weaker responses do not match very well, AgOR46 still represents the best candidate for TSB A2.

For TSB response type A4, the best ligands are the aldehydes decanal, octanal and nonanal, and the compounds 1-octen-3-ol, ethyl hexanoate and phenethyl acetate. None of the AgORs seem to show excitatory responses to decanal, octanal or nonanal and only intermediate to low responses were recorded in response to ethyl hexanoate and phenethyl acetate. The PLS

put AgOR31 forward as the best matching AgOR. But again the match was mainly for the odours eliciting the weaker responses. Thus AgOR31 does not present a good match. And since the best ligands for this OR do not elicit strong responses from other AgORs, no good candidate can be put forward.

Considering the TSB response type B2, some of the best and characteristic ligands are acetophenone, 2-acetylthiophene, 2-acetylpyridine, benzaldehyde, methyl-2-methyl benzoate and 2-methyl phenol. The one AgOR that responds strongly to all these compounds is AgOR15. However, this AgOR does not show strong responses towards alcohols while several alcohols do excite the ORN *in vivo*. Yet, AgOR15 was also rendered by the PLS analysis as the best match for this ORN. Thus AgOR15 represents a good candidate for TSB A4.

The PLS analysis matched all three A neurons of TSD with AgOR11, with AgOR12 as a good second receptor. The best ligands for all three A neurons of the TSD sensillum are the same; 4-methylcyclohexanol, 2-etyltoluene, methylcyclohexanol, 4,5-dimethylthiazole and benzaldehyde. These two AgORs, AgOR11 and AgOR12, differ mostly in response strength, and are the only AgORs responding to these ligands. The regression coefficient overview plot also showed all three A-neurons of TSD were strongly correlated. Together with the rather similar response profiles as seen in Fig. 7A-C, this provides further indication that only one AgOR is expressed in the A-neurons associated with trichoid sensilla type D and AgOR11 could be this AgOR; AgOR12 is a good second possibility.

Just like the A-neurons of TSD, the B-neurons of TSD also seemed strongly correlated, having similar odour profiles (Fig.7 D-E) and thus similar regression coefficient plots (Fig. 15). The PLS analysis matched both of them with AgOR6. When plotted next to each other their profiles are quite alike (Fig. 16B). Both ORNs responded strongly to acetophenone, 2-acetylthiophene, 2-acetylpyridine, benzaldehyde, methyl benzoate. Only three of the AgORs showed strong responses towards these compounds, i.e. AgOR6, AgOR15 and AgOR18. We argue that AgOR15 might not be present in ORNs associated with these TSD sensilla because AgOR15 also showed strong responses to several phenols and other compounds that did not excite the B-neuron of TSD. The responses of these 5 ligands together with responses to several phenols exhibited by AgOR15 match very closely to the response profile of TSB B2 as mentioned above. Since AgOR6 clearly represented both B-neurons better according to the PLS analysis, AgOR6 appears to be the best candidate for TSD B1.

Four of our 11 response types do not match the AgOR profiles reported by Carey *et al.* (2010). Two of these show weak or no excitatory responses (types A3 and B1 of TSB). The two other response types show some clear and in our system strong (>50 spikes/s) excitatory responses. The inability to match these profiles of the *in vivo* response types with the AgOR odour profiles assessed in heterologous expression platforms can have several reasons. *Anopheles gambiae* and *D. melanogaster* have some overlap in odour space but a large part has been shown to be substantially different between both species (Carey *et al.* 2010). One difference between the two systems could be that the odorant binding proteins (OBPs) in the perireceptor space are

different. Even though bioinformatics showed some high similarity between *Anopheles* OBP-encoding genes and *Drosophila* genes, they also indicated several genes with little similarity thus encoding for different OBPs (Li *et al.* 2005). In the empty neuron system the AgOR is expressed in the basiconic sensilla of *D. melanogaster*. This sensillum type is a single-walled structure containing pores just like trichoid sensilla but their shape is quite different. In fact the basiconic sensilla resemble the capitate peg sensilla found on the maxillary palp of *An. gambiae* most. It is therefore possible that the perireceptor spaces differ substantially between the *Drosophila* basiconic sensilla and the native mosquito trichoid sensilla. This would explain the lack of responses towards straight-chain aldehydes and as such the lack of a match for response type TSB A4. However, this cannot explain the lack of similarity between TSB A1 and any of the AgORs since there are AgORs that do respond very strongly to linalool oxide.

Another possible explanation for the incomplete match between response profiles of single AgOR and ORN *in vivo* electrophysiology is that multiple AgORs might be expressed in the same ORN. The paradigm was that each neuron only expresses a single OR, co-expressed together with members of the *Drosophila* Or83b co-receptor family (AgOR7 for *An. gambiae*) to form an odour-gated ion channel (Sato *et al.* 2008). However, Goldman et al. (2005) found two functional OR genes encoding for functional ORs expressed in the same ORN innervating sensilla on the maxillary palp of *D. melanogaster*. Similar results were obtained for two AgGRs (*Anopheles gambiae* Gustatory Receptors), which are thought to have a similar structure as AgORs (also containing an inverted 7-transmembrane topology). Lu *et al.* (2007) proved that expression of multiple AgGRs occurred in the A neuron of the capitate peg sensilla of *An. gambiae*. Unfortunately, no study has yet mapped specific AgORs to ORNs associated with antennal sensilla. Such a study would provide answers to several questions e.g. how many AgORs are being expressed per neuron.

Another conceivable explanation can be found in the recent findings of an additional olfactory receptor family, the IR's (Ionotropic Receptors) (Benton *et al.* 2009, Touhara 2009). These ionotropic gated channels have been shown to convey an olfactory function in *D. melanogaster*. Liu *et al.* (2010) have demonstrated that in *An. gambiae* larvae these IRs form a second olfactory signal-transduction pathway different from the normal AgOR pathway. The AgIR they used responded to an amine, butylamine. It may, therefore, be possible that the response profiles that we have found were not due to AgORs but to AgIRs or maybe to both. Both of these pathways could be present in the same ORN although recent findings in *D. melanogaster* argue against this showing that these IRs only were present in ORNs associated with coeloconic sensilla and were not expressed together with olfactory or gustatory receptors (Benton *et al.* 2009).

Conclusion

This study has shown that ORNs associated with trichoid sensilla type A do not respond to any of the 132 odours tested while ORNs associated with trichoid sensilla type B and D respond to many different odours from ten distinct chemical groups. Characterisation of the ORNs associated with TSB and TSD identified six response types for trichoid sensilla B and five response types for trichoid sensilla D. The ORN responses to the large odorant panel were distinct although some overlap was recorded. Of the 80 human-derived compounds tested, 44 elicited responses in one of the two TS types and 13 odorants of human origin gave responses in both types of TS. Two of these odorants of human origin represented the best ligands of one or more response types. The profiles of seven of the 11 identified response types were significantly correlated with profiles of single AgORs as identified via the *Drosophila melanogaster* heterologous expression system.

Acknowledgements

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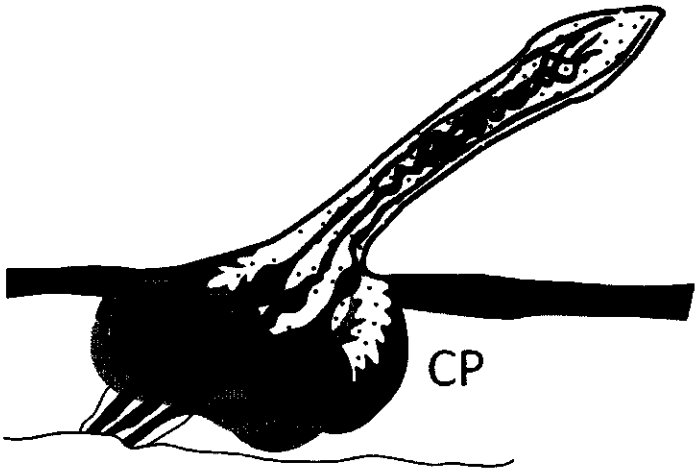
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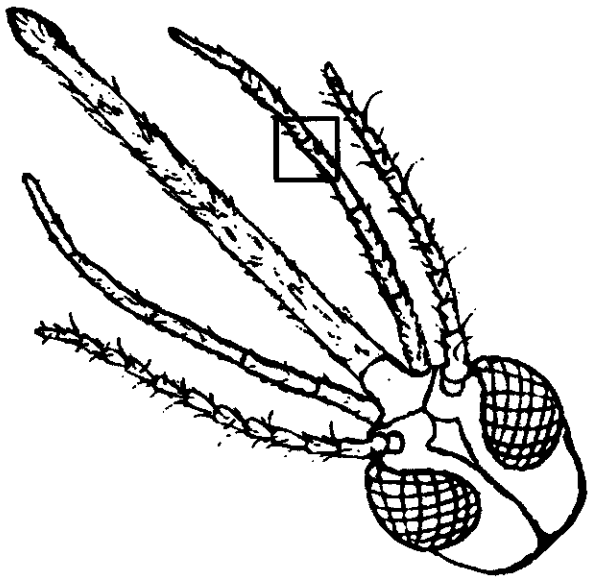

Erratum

Dear reader,

Unfortunately, some errors have occurred during the printing process and it was too late to correct them before the final print of my dissertation. The corrections have been outlined below.
My sincere apologies for any inconvenience.

Kind regards,
Remco A. Suer

Correction 1:
The illustrations on page 74 should be placed on page 92 and *vice versa*.

Correction 2:
The title and abstract of Chapter 3 (page 75) should read:

Chapter 3

Comparing heterologous expression system responses with *in vivo* responses from maxillary palp olfactory sensilla of *Anopheles gambiae s.s.* to binary odour mixtures

Remco A. Suer, Yu Tong Qiu and Joop J.A. van Loon

The African malaria mosquito *Anopheles gambiae* Giles *sensu stricto* is predominantly guided by odours when seeking for human hosts. The odours are detected by the receptors of olfactory neurons associated with different types of olfactory sensilla on the antennae or maxillary palps. The response profile of one of these receptors, AgOR8, has been established through *in vivo* electrophysiology on *An. gambiae* females and this profile when assayed in two heterologous systems matched consistently. These heterologous systems, therefore, have the potential to function as a high-throughput screening assay of potentially behaviourally relevant olfactory receptor complexes. A pilot study using the *Drosophila melanogaster* heterologous expression system (DmHES) reported inhibitory actions of four compounds on the responses of AgOR8 to 1-octen-3-ol, its best ligand and a known mosquito attractant. The current study examined whether electrophysiological responses to binary mixtures containing 1-octen-3-ol recorded from the ORN expressing this AgOR8 in the capitate peg sensilla on the maxillary palp corresponded with the responses obtained from the DmHES expressing AgOR8. Differences were found between *in vivo* and heterologous electrophysiological activity of AgOR8 in response to binary mixtures. These differences were dependent on the concentration of 1-octen-3-ol. These findings restrict the extrapolation of results from the search for compounds that inhibit electrophysiological activity to strong ligands of single, identified ORs as expressed in the DmHES.

Chapter 3

The influence of physiological state on the responsiveness of olfactory receptor neurons on the maxillary palps of *Anopheles gambiae* to odorants produced by human skin bacteria

Remco A. Suer, Yu Tong Qiu and Joop J.A. van Loon

Host-seeking and oviposition site selection behaviour of African malaria mosquitoes are mainly mediated by odours. After a blood meal, required for egg development, the female mosquito stops host seeking behaviour and gradually starts seeking an oviposition site. The host seeking behaviour returns after females have laid their eggs. Olfactory receptor neurons (ORNs) associated with antennal grooved peg and trichoid sensilla of both *Anopheles* and *Aedes* mosquitoes have been shown to become less responsive to host-derived compounds like ammonia and lactic acid after having taken a blood meal. Responsiveness of trichoid sensilla in both species increased after a blood meal when stimulated with oviposition site-related odorants like indole. Capitate peg sensilla located on the maxillary palps of mosquitoes contain three ORNs, one of which detects CO_2. As this is the only ORN-type capable of CO_2 detection, the maxillary palp plays an important role in host seeking behaviour. Despite this clear role the effect of physiological state on the responses of capitate peg ORNs has not been elucidated. This study describes the responses of the three capitate peg ORNs to 11 odorants produced by human feet microbiota. Ten of these compounds elicited responses from one or more of the capitate peg ORNs. One ORN has been found that consistently showed lower responses after a blood meal to most of these bacterial volatiles. For the first time in mosquito literature an increased sensitivity to host seeking compounds is reported in mosquitoes that have oviposited compared to non-blood fed mosquitoes of similar age. Five odorants of the 11 tested in this study completely suppressed the response to CO_2. This is the first record documenting that host-derived compounds inhibit the CO_2.receptor neuron in *Anopheles gambiae*.

Introduction

Anopheles gambiae is one of the major malaria vectors in sub-Saharan Africa. The high vectorial capacity of this mosquito species is mainly due to the high degrees of anthropophily, endophagy and endophily of female *An. gambiae* mosquitoes and its susceptibility to infection by *Plasmodium falciparum,* causing the most fatal form of malaria. Female *An. gambiae* use olfactory cues to seek mating partners, blood hosts, sugar sources and breeding sites (Takken and Knols 1999, Zwiebel and Takken 2004).

Natural odour sources consist of mixtures of volatile compounds. Human odours are complex mixtures of volatile organic compounds of more than 350 substances that have been identified from human skin emanations alone (Bernier *et al.* 1999, Bernier *et al.* 2000, Curran *et al.* 2005, Penn *et al.* 2007, Gallagher *et al.* 2008).

Behavioural studies have demonstrated that several of these compounds act as mosquito attractants (Knols *et al.* 1997b, Qiu *et al.* 2004, Smallegange *et al.* 2005b, Okumu 2008, Smallegange *et al.* 2009). Synthetic mixtures have been produced using ammonia, lactic acid and several carboxylic acids that catch *Anopheles* mosquito numbers close to or even exceeding those caught with humans as odour source (Smallegange *et al.* 2009, Okumu *et al.* 2010). A recent study showed that cultured skin microflora taken from feet of attractive humans produced several compounds that attracted *An. gambiae* including several carboxylic acids, aldehydes and alcohols (Verhulst *et al.* 2009).

Creating and optimising a multi-component mixture which can be used for attracting and trapping mosquitoes is clearly possible but generates lots of challenges (Okumu *et al.* 2009, Smallegange *et al.* 2009, Verhulst *et al.* 2009, Okumu *et al.* 2010). An alternative way of behavioural manipulation through olfaction would be to use repellent volatiles or to mask attractants (Logan *et al.* 2008). For blood-sucking insects the most widely applied repellent is DEET (*N,N*-diethyl-*m*-toluamide). Until 2008 it was hypothesised that DEET inhibited or interfered with the responses of olfactory neurons to attractive chemical signals (Davis and Sokolove 1976, Davis 1985). Ditzen *et al.* (2008) indeed showed that DEET inhibited the response to 1-octen-3-ol, a compound known to attract several mosquito species (Takken and Kline 1989, Takken and Knols 1999). The odorant receptor complex consisting of the co-receptors AgOR7 and AgOR8 found in the capitate peg sensilla on the maxillary palps of female *Anopheles gambiae* responds to 1-octen-3-ol with an exceptionally low threshold (Lu et al. 2007). Ditzen *et al.* (2008) concluded that DEET masks host odours by inhibition of olfactory receptors that need the OR83b co-receptor (the *Drosophila* orthologue of AgOR7) to form a functional odorant receptor complex (Ditzen *et al.* 2008). However, Syed and Leal (2008) could not find such inhibition caused by DEET in the maxillary palp of *Culex* mosquitoes, which contain a 1-octen-3-ol responsive neuron in the capitate peg sensilla (Syed and Leal 2007). These authors showed that DEET had a fixative effect when applied in the same odour cartridge as 1-octen-3-ol but not when presented separately from 1-octen-3-ol. They also showed that

specific ORNs on the antenna of *Culex quinquefasciatus* were capable of direct detection of DEET (Syed and Leal 2008).

So far, of the 79 putative olfactory receptors (AgOR) of *An. gambiae (Hill et al. 2002)*, 52 have been functionally expressed and characterised in several heterologous systems (Carey *et al.* 2010, Wang *et al.* 2010). One possible application of such heterologous olfactory systems is to function as a high-throughput screening assay of potentially behaviourally relevant olfactory receptor complexes. This might lead to the identification of new attractants or repellents with which mosquitoes can be manipulated.

The response profile to ligands of AgOR8 as established in 2 heterologous expression platforms, the *Drosophila melanogaster* heterologous expression system (DmHES) and the *Xenopus laevis* oocyte assay, was similar to the profile for the ORN expressing AgOR8 in the maxillary palp of *An. gambiae* females in response to single compounds (Lu *et al.* 2007, Carey *et al.* 2010, Wang *et al.* 2010). All response profiles obtained from *in vivo* electrophysiology on olfactory receptor neurons of different mosquito species (Qiu *et al.* 2006, Ghaninia *et al.* 2007b, Lu *et al.* 2007, Syed and Leal 2007, Hill *et al.* 2009) were created by single-compound studies. Even though all natural odour sources produce mixtures of odours, mixtures are still to be tested on mosquito ORNs. The effect of combining two odour stimuli on the response of a single ORN still needs to be elucidated.

A pilot study using the DmHES revealed four compounds causing inhibition of the response of AgOR8 to 1-octen-3-ol (J.R Carlson, pers. comm.). In the present study, it was examined whether *in vivo* electrophysiology generated similar inhibitory responses towards binary mixtures as found for the DmHES to validate that the latter system could be used in the search for inhibitors of the responses mediated by identified AgORs. Such a comparison can at present only be achieved by studying one of the ORNs innervating the capitate peg sensilla of the maxillary palps, since until now these are the only neurons from which it is known which AgOR is expressed (Lu *et al.* 2007). The influence of concentration differences on inhibition was also examined and two odour stimulation methods were compared to examine whether odour retention or a fixative effect occurred.

Material and methods

Drosophila melanogaster heterologous expression system (DmHES)

All data on *Drosophila melanogaster* Meigen were collected in the laboratory of Dr. J.R. Carlson (Yale University, USA) by assistants Molly Dillon and Eliza Kelley-Swift and were kindly provided by Dr. J.R. Carlson for comparative purposes.

Cloning and expression of AgORs

Cloning of AgOR cDNAs was done according to standard procedures and the construction of ab3A mutant flies and Or22a-GAL4 was achieved as described previously (Dobritsa *et al.* 2003, Hallem *et al.* 2004, Carey *et al.* 2010).

Electrophysiology

Extracellular single-unit electrophysiology was performed as described previously (Dobritsa *et al.* 2003, Hallem *et al.* 2004, Carey *et al.* 2010). Five odorants were used each of the highest purity available (Sigma-Aldrich); 1-octen-3-ol, isobutyl propionate, 3-heptanone, 4-heptanone and 2-methylhexanoic acid. All odorants were diluted in paraffin oil (Fluka) to a 1% concentration (w/w).

The stimulation method for the single compounds was the same as described in Carey *et al.* (2010). For the testing of binary mixtures they used the two-filter-disk method, which is the same as for the single compounds except for the placement of two filter paper disks in the same Pasteur pipette instead of a single filter paper disk. Placement of filter paper disks in the pipette was done according to Figure 1.

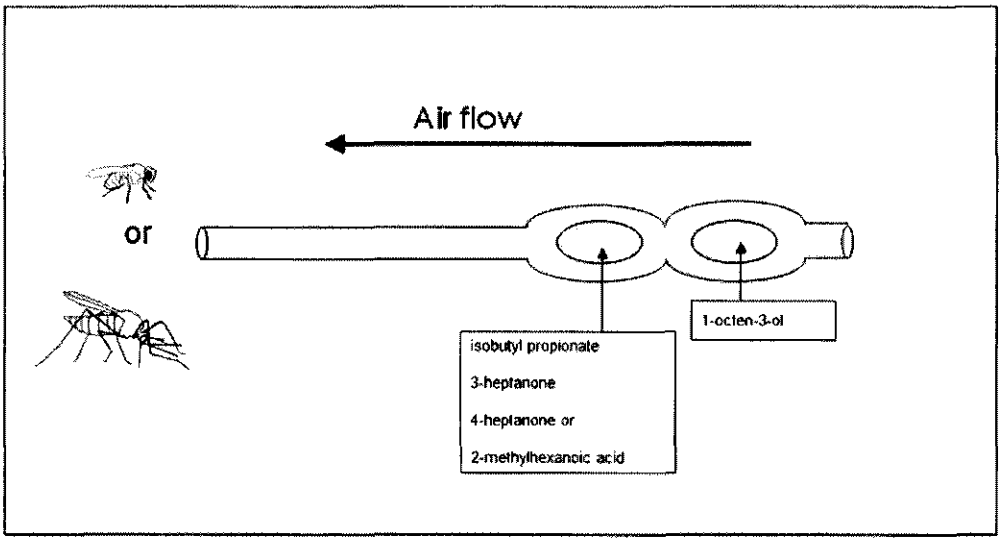

Figure 1. The two-filter disk method encompasses placing two filter paper disks in the same Pasteur pipette in line. The disks were placed in the same sequence in each case, ensuring that the air first passed over the disk with 1-octen-3-ol (the excitatory compound) and then over the other disks containing one of the putatively inhibitory compounds.

In vivo experiments with *Anopheles gambiae*

Mosquitoes

Since 1988 a colony of *Anopheles gambiae* Giles *sensu stricto* (hereafter *An. gambiae*) has been cultured at Wageningen University, The Netherlands. This colony originated from Suakoko, Liberia and was made available to us by Prof. M. Coluzzi. Standard insectary conditions of 27 ± 1°C, 80 ± 5% RH, adjusted 12:12 day-night rhythm, with light phase starting at 12:00 am were used. Adults were kept in 30×30×30 cm^3 gauzed cages with access to a 6% (w/v) glucose solution on filter paper. Female mosquitoes were fed human blood from an arm twice a week. Eggs were laid on damp filter paper that was placed in plastic cups with tap water. Larvae were fed daily with Tetramin® baby fish food (Melle, Germany). Pupae were collected daily and allowed to emerge in the aforementioned cages.

For experiments, non-blood-fed females of 6-8 d old were used. Responsive mosquitoes were collected by selection of females that responded to the odour of the hand of the experimenter held next to the cage. Females were collected by means of an aspirator and placed individually in small 5-ml-pipette points covered by 1x1 mm mesh gauze.

In vivo *electrophysiology*

The preparation and single sensillum recording procedures were exactly the same as described in Chapter 2. Recordings were made from the B-neuron of the capitate peg sensilla on the maxillary palps. Previous molecular identification studies showed that AgOR8 was expressed in this B-neuron (Lu *et al.* 2007).

Stimuli

Five different odorants were tested; 1-octen-3-ol, isobutyl propionate, 3-heptanone, 4-heptanone and 2-methylhexanoic acid. All five were of the highest purity grade (≥96%) commercially available (Fluka, Sigma-Aldrich). All odorants were diluted in paraffin oil (Merck, Germany) which was used as the control. Of each solution, 10 µl was applied to a strip of filter paper (0.5x1.5 cm) inserted into a Pasteur pipette. These odour cartridges were closed with a 1ml pipette-point and Parafilm® and kept at -25 °C. Each odour cartridge was used up to a maximum of 10 times for delivering the stimuli, after which it was replaced by a freshly prepared cartridge.

Three different concentrations of 1-octen-3-ol were used: 0.01%, 0.001%, and 0.0001% dilutions. The other four compounds were tested mostly at 1% dilutions and once at a 10% dilution.

Two-filter disk method

The odour cartridges were made according to the method described above (Figure 1). Vapours of the odorants were delivered at 0.5 l/min to the preparation via injection into a constant, charcoal-filtered and humidified airstream of 0.5 l/min. This was done by a stimulus controller that also supplied a compensation airstream simultaneously into the main air stream to keep the total airflow constant. The odorants were injected for a duration of 200 ms.

Two-airstream method

A stimulation method was designed to enable simultaneous application of two stimuli through separate channels. In this experiment one Pasteur pipette containing 1-octen-3-ol was used together with a different Pasteur pipette containing one of the putatively inhibitory compounds (Dr. J.R. Carlson, unpubl. results). A stimulus controller separated the flow over two channels by adding a "Y" splitter, with one line connected to the 1-octen-3-ol cartridge and the other connected to the test compound cartridge. Both channels delivered odorants at 0.5 l/min into the mainstream of 0.5 l/min at the same distance from the preparation. The odorants were injected for a duration of 200 ms.

Analysis

Analysis of electrophysiological recordings

Single sensillum recordings were automatically filtered to increase signal-to-noise ratio using Autospike software (filter settings: 20 - 1694 Hz) and analysed in combination with Automacrorecorder (ReadmeSoft, http://www.readmesoft.com) software. Though all recordings showed the activity of a maximum of three neurons, we only focussed on the B-neuron, which has the second largest spike amplitude and expresses AgOR8 (Lu *et al.* 2007).

Recordings of 10 sec. were taken to analyse the responses towards the binary mixture. For each measurement the effect of each odour was quantified by the change in action potential firing frequency, which was calculated as the number of action potentials during 500 ms after the onset of the stimulus minus the average of firing frequency during four intervals of 500 ms preceding the onset of the stimulus. The time it took for the injected air volume to pass down the glass tube to reach the antenna was calculated. This delay represented the travelling time for an odour stimulus to reach the antenna. Thus, for our analysis, the counting started at the time point determined by adding this delay to the onset of stimulus delivery.

Statistical analysis

Statistical analysis of both datasets was done using Genstat software (Genstat 14, VSN International Ltd, UK). Treatment differences (test compound, concentration or stimulation

method) were analysed using a Generalized Linear Model with Poisson distribution with a log link function. When the variance between mosquitoes was significant, the P-value for the effect of the treatment was corrected for in the model. If the overall model yielded a significant treatment effect, two-sided t-probabilities were calculated to test pairwise differences between the means. Effects were considered to be significant at $P < 0.05$.

Results

Responses to binary mixtures in both the DmHES system and the *An. gambiae in vivo* using the two-filter disk method

In the *Drosophila* empty neuron system significant treatment effects were found among the odorants when mixed with 1% 1-octen-3-ol (GLM, P = 0.005; Fig. 2A). The 1% concentrations of 3- and 4-heptanone and 2-methylhexanoic acid significantly inhibited the response to 1% 1-octen-3-ol (GLM, P = 0.002). Isobutyl propionate did not result in significant inhibition, possibly due to a low number of replications, but a tendency toward inhibition was seen (GLM, P = 0.089).

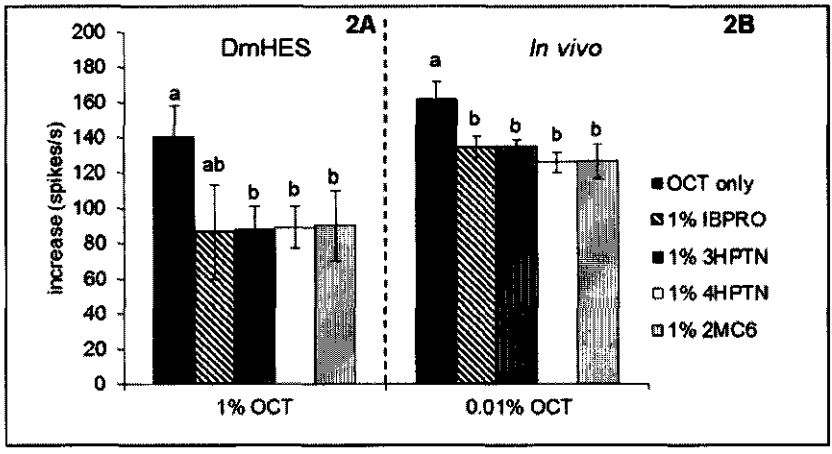

Figure 2: Comparison of the *Drosophila* melanogaster heterologous expression system (DmHES) with *An. gambiae in vivo* electrophysiology. The y-axis displays the neuron response in spikes/s increase relative to spontaneous activity. **2A)** Responses to binary mixtures of 1% 1-octen-3-ol (OCT) in combination with one of four test odours (IBPRO: isobutyl propionate, 3HPTN: 3-heptanone, 4HPTN: 4-heptanone and 2MC6: 2-methylhexanoic acid) using the two-filter disk method obtained from AgOR8 in the empty neuron system (n=6, except for isobutyl propionate; n=3). **2B)** Responses obtained *in vivo* from the B-neuron of the capitate peg sensilla when exposed to the binary mixtures of 0.01% 1-octen-3-ol and the four putative inhibitors (n=10). Error bars represent standard error of the mean. Unrelated letters indicate significant differences between concentrations per bin (GLM, $P < 0.05$).

In vivo data obtained via the two-filter disk method shows similar results (Fig. 2B) as those of the DmHES, although the concentration of 1-octen-3-ol was much lower in the *in vivo* experiments than in the empty neuron experiments (see Material and methods). A treatment effect was found (GLM, $P < 0.001$) and *in vivo* all four compounds tested significantly reduced the response to 0.01% 1-octen-3-ol ($P \leq 0.001$).

Overall, the increases in spike frequencies upon stimulation with the binary mixtures are significantly larger for the *in vivo* preparation than the increases obtained via the DmHES, despite the 100-times lower concentration of 1-octen-3-ol (GLM, $P < 0.001$).

Concentration effect of the best ligand

Significant differences were observed among the four test compounds in combination with 0.0001% 1-octen-3-ol (GLM, $P < 0.001$; Fig. 3). Adding 3-heptanone resulted in a significant increase in response compared to 1-octen-3-ol and the other three mixtures (GLM, $P < 0.001$), the responses to which did not significantly differ from the response to 1-octen-3-ol alone. No significant differences were found among the four binary mixtures and 0.001% 1-octen-3-ol (GLM, $P = 0.242$).

Overall, the responses recorded for the test odorants in combination with 0.01% 1-octen-3-ol were significantly higher than with 0.001% 1-octen-3-ol (GLM, $P < 0.001$), which elicited higher responses than 0.0001% 1-octen-3-ol (GLM, $P < 0.001$).

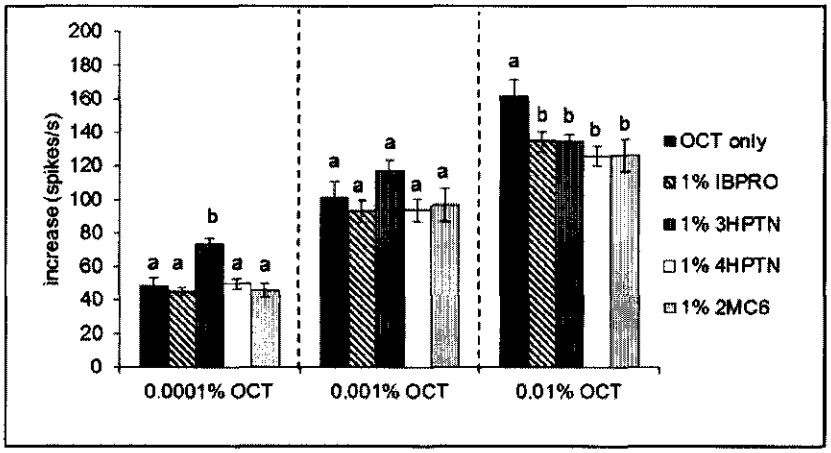

Figure 3: Concentration effects observed *in vivo* for the capitate peg B-neuron responses of *An. gambiae* using the two-filter disk method. On the x-axis the concentration of 1-octen-3-ol (OCT) used together with each of the four compounds is presented (IBPRO: isobutyl propionate, 3HPTN: 3-heptanone, 4HPTN: 4-heptanone and 2MC6: 2-methylhexanoic acid). On the y-axis the increase in firing frequency of the B-neuron of the maxillary capitate peg sensilla is given in spikes/s increase relative to spontaneous activity (n=10, error bars represent standard error of the mean). Data for 0.01% 1-octen-3-ol are reproduced from Figure 2 to allow direct comparison. Unrelated letters indicate significant differences between concentrations per bin (GLM, $P < 0.05$).

Responses of *An. gambiae* to binary mixtures *in vivo* using the two-airstream method

With the two-airstream method, treatment differences were observed among the odorants when mixed with 0.0001% 1-octen-3-ol (GLM, $P < 0.001$; Fig. 4). The mixture of 0.0001% 1-octen-3-ol and 1% 3-heptanone elicited a significantly higher response compared with 1-octen-3-ol alone (GLM, $P < 0.001$; Fig. 4). The other three mixtures tested elicited responses similar to those found to 1-octen-3-ol alone.

A significant treatment effect was found when test odorants were simultaneously applied with 0.001% 1-octen-3-ol (GLM, $P = 0.004$). In the middle section of Figure 4 it is shown that adding either isobutyl propionate or 4-heptanone to 1-octen-3-ol significantly reduced the response compared with 1-octen-3-ol alone (GLM, both $P = 0.001$). The response to 3-heptanone was significantly higher than the response to isobutyl propionate ($P = 0.043$) and 4-heptanone (GLM, $P = 0.027$). The mixtures of 1-octen-3-ol and 2-methylhexanoic acid ($P = 0.057$) or 3-heptanone ($P = 0.197$) elicited responses of similar intensities as 1-octen-3-ol alone.

Significant inhibition of the B-neuron response by each of the four compounds was found by mixing these with 0.01% 1-octen-3-ol (GLM, $P \leq 0.001$; Fig. 4).

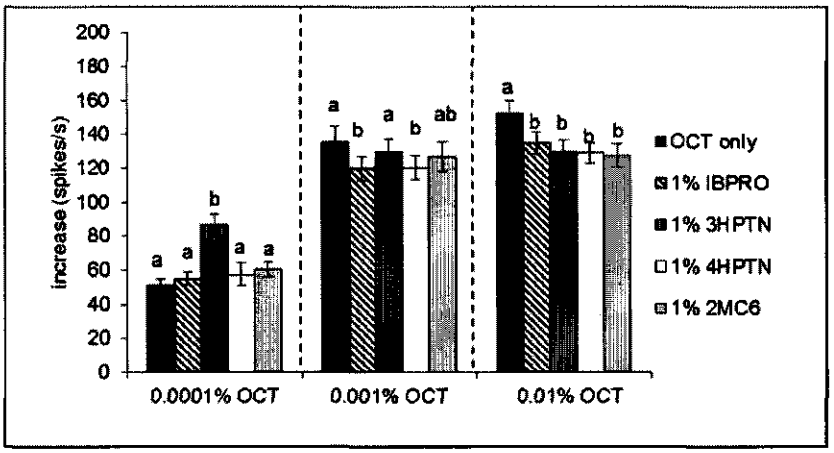

Figure 4: Concentration effects observed *in vivo* for the capitate peg B-neuron responses of *An. gambiae* using the two-airstream method. On the x-axis the concentration of 1-octen-3-ol is plotted, on the y-axis the response intensity of the B-neuron in the capitate peg sensilla in spikes/s increase relative to spontaneous activity to binary mixtures of 1-octen-3-ol plus one of the four odorants indicated (IBPRO: isobutyl propionate, 3HPTN: 3-heptanone, 4HPTN: 4-heptanone and 2MC6: 2-methylhexanoic acid) (n=10, error bars represent standard error of the mean). Unrelated letters indicate significant differences between concentrations per bin (GLM, $P < 0.05$).

Using the two-airstream method, the increases in firing frequencies of the highest and middle concentration (0.01% and 0.001%) of 1-octen-3-ol were not significantly different from each other (GLM, $P = 0.083$; Fig. 4), while using in the two-filter disk method the middle concentration produced significantly lower responses than the highest concentration of 1-octen-3-ol (GLM, $P < 0.001$; Fig. 3).

All the responses recorded when applying the two-airstream method at the concentration of 0.001% 1-octen-3-ol were significantly higher than the responses measured through the two-filter disk method (GLM, P <0.001), while at the highest and lowest concentration of 1-octen-3-ol no significant differences were seen between both methods.

A clear treatment effect was observed when 10% of each of the test compounds was applied simultaneously with 0.0001% 1-octen-3-ol (GLM, P < 0.001; Fig. 5). The responses to 0. 0001% 1-octen-3-ol mixed with either 3-heptanone, 4-heptanone or isobutyl propionate were significantly higher than the responses to 1-octen-3-ol alone (GLM, respectively P < 0.001, P < 0.001 and P = 0.019). The response to the mixture of 0.0001% 1-octen-3-ol with 10% 2-methylhexanoic acid was not significantly different from the response to 1-octen-3-ol alone (GLM, P = 0.467; Fig. 5).

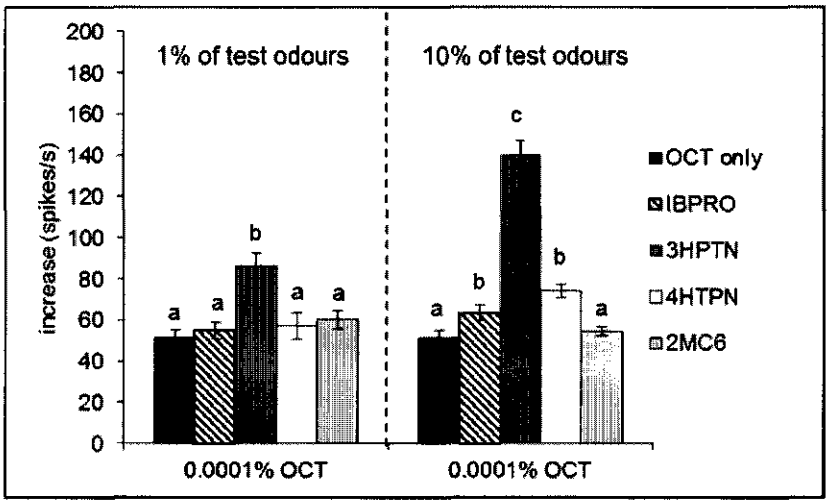

Figure 5: Concentration effects on *An. gambiae in vivo* responses using the two-airstream method. On the x-axis the concentration of 1-octen-3-ol (OCT) used together with each of the four compounds is presented (IBPRO: isobutyl propionate, 3HPTN: 3-heptanone, 4HPTN: 4-heptanone and 2MC6: 2-methylhexanoic acid). On the y-axis response intensity of the B-neuron in the capitate peg sensilla in spikes/s increase relative to spontaneous activity (n=10, error bars represent standard error of the mean). Data for 0.0001% 1-octen-3-ol in combination with 1% of the test compound is reproduced from Figure 4 to allow direct comparison. Unrelated letters indicate significant differences between concentrations per bin (GLM, P < 0.05).

The responses to the mixture of 0.0001% 1-octen-3-ol with 1% of each of the four test compounds were significantly different from the responses to the mixture with 10% of the four test compounds (GLM, P < 0.001). At 10% concentration the increase of both 3-heptanone and 4-heptanone was significantly greater than at 1% of these odorants (GLM, P < 0.001 and P = 0.050). The responses to isobutyl propionate and 2-methylhexanoic acid in combination with 1-octen-3-ol were similar for the 1% and 10% concentrations (GLM, respectively P = 0.148 and P = 0.279).

Responses to single compounds in both the DmHES and the *An. gambiae in vivo* using the two-filter disk method

Of the three odorants tested in the DmHES, 3- and 4-heptanone elicited significant excitation responses (GLM: $P < 0.001$), whereas 2-methylhexanoic acid was inactive ($P = 0.729$; Fig. 6).

In vivo, significant excitation responses were found to the application of the single compounds isobutyl propionate, 3-heptanone and 4-heptanone at a 1% concentration (GLM, $P < 0.001$). The response to 2-methylhexanoic acid did not significantly differ from spontaneous activity (GLM: $P = 0.960$; Fig. 6).

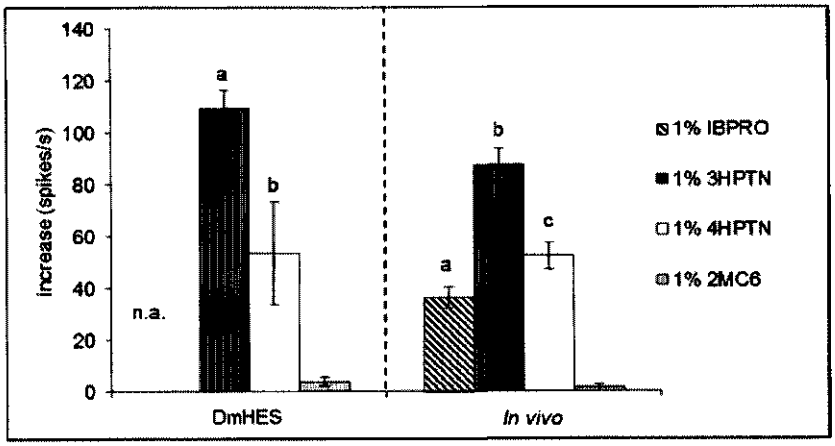

Figure 6: Response intensities elicited by the single test odorants (IBPRO: isobutyl propionate, 3HPTN: 3-heptanone, 4HPTN: 4-heptanone and 2MC6: 2-methylhexanoic acid) in both assay systems, indicated along the x-axis. The response to isobutyl propionate was not available (n.a.) for the Drosophila melanogaster heterologous expression system. The y-axis represents the response intensity in spikes/s increase relative to spontaneous activity. Isobutyl propionate was not tested in the empty neuron system. (n=10, error bars represent standard error of the mean). Unrelated letters indicate significant differences between concentrations per bin (GLM, $P < 0.05$). No letters mean no significant differences from spontaneous activity.

Discussion

This study examined whether electrophysiological responses to binary mixtures recorded *in vivo* from the B-neuron in the capitate peg sensilla on the maxillary palp of female *An. gambiae* that expresses the AgOR8 receptor protein endogenously, corresponded with the responses obtained from the DmHES expressing AgOR8 heterologously.

Different sensitivities between *in vivo* and DmHES

The accurate recording of responses of ORNs in capitate peg sensilla to 1% of 1-octen-3-ol *in vivo* posed technical problems. The B-neuron responding to 1-octen-3-ol was so sensitive that the neuron started firing even when the odour cartridge was only approaching the airstream. If a 1%-stimulus was presented, the spike amplitude showed pinching, seen as a very strong excitation response during which the amplitude of the action potentials diminishes into the background noise level making it practically impossible to reliably count action potentials. In addition, delivery of the 1%-dose of 1-octen-3-ol often silenced the neuron. To prevent pinching and silencing, a concentration of 0.01% was used as highest concentration of 1-octen-3-ol applied in the *in vivo* experiments. This concentration elicited similar increases in spike frequency as the 1% 1-octen-3-ol in the empty neuron system, around 150 spikes/s, and was the highest 10-fold concentration step that produced spikes that, even though some pinching occurred, could be counted reliably. The responses to the four test compounds, when tested singular, elicited similar increases in spike frequencies from the AgOR8-expressing DmHES as from the endogenous neuron. Thus, the *in vivo* assay was considerably more sensitive than the DmHES. In previous electrophysiological studies it seemed that, overall, the increases in spike frequencies measured *in vivo* were actually lower than the frequencies recorded from single AgORs expressed in the heterologous system (Qiu *et al.* 2006, Carey *et al.* 2010, Chapter 2). It appears that some odorants elicit much stronger responses in one system than in the other, while others elicit comparable responses in the same two systems. One possible explanation for this phenomenon might be the lack of mosquito-specific odorant binding proteins (OBPs) in the heterologous empty neuron system. A crucial function ascribed to OBPs is to act as carriers for odour molecules from the sensillum wall pores to the receptor neuron membrane (Pelosi 2006).

Comparison of inhibitory effects between DmHES and *in vivo* assays

If the concentration of 1-octen-3-ol used, when testing the binary mixtures in the heterologous assay (1%), was diluted 100-fold to yield a concentration (0.01% 1-octen-3-ol) that elicited a similar response intensity *in vivo* in the endogenous neuron, the significant inhibitory effects observed in the heterologous system could be replicated *in vivo*. All four compounds, isobutyl propionate, 3- and 4-heptanone and 2-methylhexanoic acid, tested at 1%, significantly inhibited the response to 1-octen-3-ol.

Concentrations

These findings suggest that the concentration of the strongly excitatory odour is decisive for the effect of the additional odorants. It was found that reducing the concentration of 1-octen-3-ol by 10-fold, leading to 30% reduction in response intensity, produced completely different results. The inhibitory effects found for the test odorants when added to 0.01% 1-octen-3-ol disappeared when added to a 10-fold lower concentration of 1-octen-3-ol. When the concentration of 1-octen-3-ol was again diluted 10-fold to 0.0001%, resulting in a drop of 50% in response strength, addition of the previously inhibitory compound, 3-heptanone, now elicited an excitation response greater than that elicited by 0.0001% 1-octen-3-ol alone. The response of 0.0001% 1-octen-3-ol mixed with 1% 3-heptanone produced an increase in firing frequency of the B-neuron similar to that of 1% 3-heptanone alone. This resembles the hypoadditivity interaction described by Duchamp-Viret *et al.* (2003) in which the response to the mixture is equal to but not higher than the response to the best ligand of the two. It seems that the response pattern of a binary mixture can go from suppression to hypoadditivity by lowering the concentration of the stronger ligand (Duchamp-Viret *et al.* 2003). To date, lower concentrations of 1-octen-3-ol have not been tested in the heterologous system. It would be interesting to see if the responses to the binary mixtures varied in a similar way in both systems. Nonetheless, it is unexpected that the inhibitory effect of the weaker ligand in a binary mixture compound turns into a hypo-additive effect at a lower concentration of the best ligand.

Stimulus delivery methods

Syed and Leal (2008) discovered that a fixative effect could occur when odours are presented in the same pipette as was the case in the two-filter disk method we applied. They showed that DEET lowered the response to 1-octen-3-ol in the maxillary palp capitate peg sensilla of *Culex quinquefasciatus* Say when using a two-filter disk method. However, by splitting the airstream before it reached the filter papers and joining the two airstreams together after the air has passed both separated filter papers, this inhibition was no longer observed (Syed and Leal 2008). To test for a fixative effect of 1-octen-3-ol with a candidate stimulus we used a similar two-airstream method and compared it with our two-filter disk method.

At both the lowest and highest concentration of 1-octen-3-ol tested (0.01% and 0.0001%), the two-airstream method showed similar results as the two-filter disk method. However, at the intermediate concentration of 0.001% 1-octen-3-ol, the two methods showed different response patterns for the different treatments. With the two-filter disk method no significant differences were found between 0.001% 1-octen-3-ol and the 1% dosages of the four different odorants. In contrast, using the two-airstream method, both isobutyl propionate and 4-heptanone inhibited responses at 0.001% of 1-octen-3-ol.

Another difference was that all the responses recorded through the two-airstream method were higher than the two-filter disk method at 0.001% 1-octen-3-ol, while at both other 1-octen-3-ol concentrations no differences were seen between both methods. The higher

response intensity recorded through the two-airstream method at the intermediate concentration was similar to that found in response to the highest concentration.

As we aimed to keep the concentration of each odour stimulant similar in both systems, the airflow reaching the palp was higher in the two-airstream method than in the two-filter disk method. This resulted in a difference in flow rate between the two methods, which may have resulted in higher responses to mixtures containing 0.001% 1-octen-3-ol when using the two-airstream method as compared to the two-filter disk method.
We conclude that not the stimulus delivery method, either allowing or preventing sorptive interactions before reaching the preparation, but differences in concentration of the stimulus caused the largest differences in responses to the additional compounds between the two methods.

Further steps need to be taken to unravel whether these differences are truly concentration-based, whether the delivery method used has an effect or whether it is a combination of both. These steps may entail equalizing the stimulation methods to ensure that exactly similar amounts of odour molecules reach the preparation. To quantify concentration effects more accurately, smaller concentration steps should be used. In addition, more combinations of the concentration of both the best ligand and the putative inhibitory compound should be tested to obtain a better quantitative understanding of the physico-chemical interactions that take place.

Responses to the single additional compounds

The fact that, at a reduced concentration of 1-octen-3-ol, the inhibitory effects of the four compounds disappeared, combined with the fact that at the lowest concentration we even measured a stronger excitation than with 1-octen-3-ol alone, was not consistent with the possibility of blocking the AgOR8 membrane-bound receptor that would then result in a reduced receptor potential. Instead, it led us to hypothesise that the test odorants acted as excitatory ligands and competed with 1-octen-3-ol for the same receptor. To test this hypothesis, the four additional odorants (isobutyl propionate, 3- and 4-heptanone and 2-methylhexanoic acid) were tested singly on the capitate peg sensilla *in vivo* and three of them (3- and 4-heptanone and 2-methylhexanoic acid) on the AgOR8 receptor expressed in the DmHES (Dr. J.R. Carlson, unpubl. data). Indeed, three of the four putative inhibitors elicited excitatory responses from AgOR8. Only 2-methylhexanoic acid elicited no responses from AgOR8. The responses towards 2-methylhexanoic acid *in vivo* and in the heterologous system were not significantly different from the spontaneous activity generated by the neuron or from the response to the solvent paraffin oil (data not shown).

The inhibitory effect on the response to 1-octen-3-ol seen for isobutyl propionate, 3- and 4-heptanone could be attributed to competitive interactions between two agonists differing in their affinity or activation effectiveness of the same main receptor site. It is clear that 1-octen-3-ol elicits much stronger responses because even at a concentration 100 times lower, 1-octen-3-ol elicits a higher response of the olfactory neuron than the other compounds.

If an odorant that elicits a strong response from a particular receptor is combined with an additional odorant that can bind to the same receptor but that elicits a weaker response, the response towards the better ligand will be lower because it cannot bind all available receptors and will lead to an apparent inhibitory effect (Rospars *et al.* 2008).

At the intermediate concentration of 1-octen-3-ol (0.001%), addition of the two weakest ligands, isobutyl propionate and 4-heptanone, still inhibited the response whereas 3-heptanone did not inhibit the response anymore. This might be caused by differences in binding properties of the different odorants, e.g. in the time they occupy the receptor.

At the lowest concentration of 1-octen-3-ol (0.0001%), the mixture with 3-heptanone elicited a response equal to the response to 1% 3-heptanone alone. This is consistent with the hypo-additivity mechanism, just as seen at the highest concentration of 1-octen-3-ol, only now at this low concentration of 1-octen-3-ol, 3-heptanone occupies most receptor sites. The fact that the response of 0.0001% 1-octen-3-ol, when applied singularly, elicited responses lower than those to 1% 3-heptanone supports the role of hypo-additivity as an explanation.

The other odorants elicited similar responses as the lowest concentration of 1-octen-3-ol. This could also be explained by hypo-additivity as upon increasing the concentration of the four test compounds to 10%, it was seen that both mixtures of 10% 3-heptanone or 10% 4-heptanone with 0.0001% 1-octen-3-ol elicited higher responses than the 1% mixtures. As expected, the mixture of 10% 4-heptanone elicited higher responses than 0.0001% 1-octen-3-ol alone.

Rospars *et al.* (2008) showed that if the dose-response relations of the single components of a binary mixture are known, this allows for the correct prediction of around half of the mixture interactions, including most hypo-additivity and suppression type interactions. The other half was attributed to non-competitive interactions like the ability of an odorant to increase or decrease the affinity or efficacy of another odorant (Rospars *et al.* 2008). This would fit the responses that occurred to 2-methylhexanoic acid. Even though 2-methylhexanoic acid does not elicit responses from AgOR8 or the B-neuron of the capitate peg sensilla, it does lower the response towards 0.01% 1-octen-3-ol when tested together in either method. A competition for the same receptors does not seem to explain this experimental data.

Conclusion

Differences were observed between *in vivo* and DmHES electrophysiological activity on olfactory sensilla of the maxillary palpi of *An. gambiae* in response to binary mixtures and showed that these differences strongly depend on the concentration of the best ligand tested, 1-octen-3-ol. These findings put constraints on the possibility to extrapolate from the DmHES in the search for compounds that inhibit electrophysiological activity to strong ligands of single, identified ORs expressed in the *Drosophila melanogaster* heterologous expression system.

Acknowledgements

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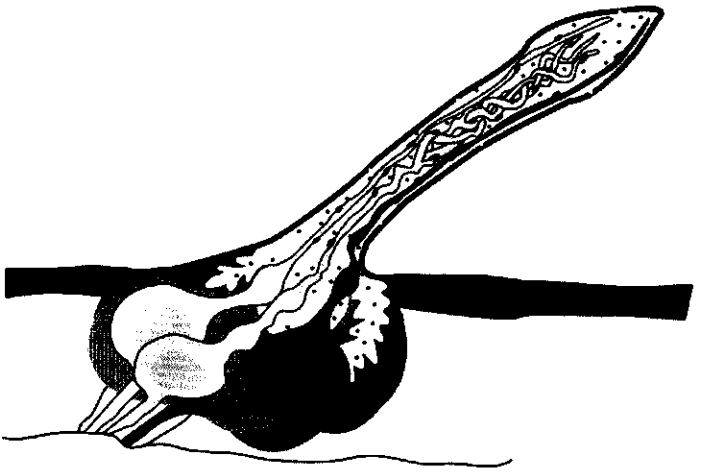
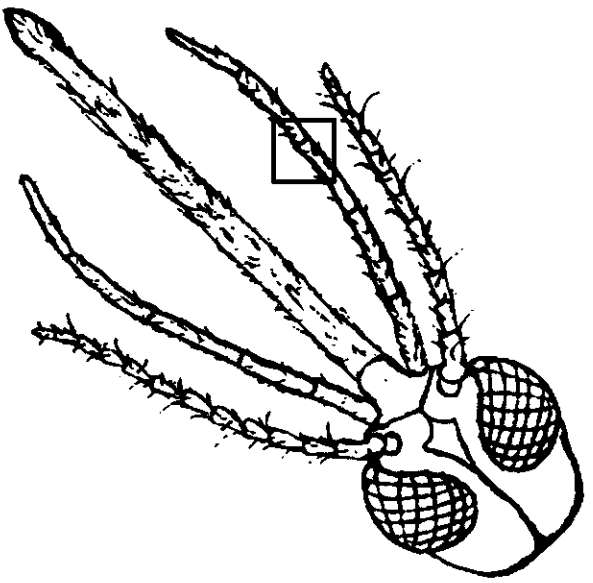




Chapter 4

Physiological state of *Anopheles gambiae* influences the responsiveness of palpal olfactory receptor neurons to human microbiota-derived odorants

Remco A. Suer and Joop J.A. van Loon

Host-seeking and oviposition-site selection behaviour of African malaria mosquitoes are mainly mediated by odours. After a blood meal, required for egg development, the female mosquito stops host-seeking behaviour and gradually starts to seek for an oviposition site. The host-seeking behaviour returns after females have laid their eggs. Olfactory receptor neurons (ORNs) associated with antennal grooved peg and trichoid sensilla of both *Anopheles* and *Aedes* mosquitoes have been shown to become less responsive to host-derived compounds like ammonia and lactic acid after a blood meal. Responsiveness of trichoid sensilla in both species increased after a blood meal when stimulated with oviposition site-related odorants like indole. Many mosquito species utilise CO_2 to orient to their hosts. Capitate peg sensilla located on the maxillary palps of mosquitoes contain three ORNs, one of which detects CO_2. Because this is the only ORN-type capable of CO_2 detection, the maxillary palp plays an important role in host-seeking behaviour. Despite this clear role in odour-mediated behaviour of mosquitoes, the effect of physiological state on the responses of capitate peg ORNs has not been elucidated. This study describes the responses of the three capitate peg ORNs of the malaria mosquito *Anopheles gambiae* to 11 odorants produced by human foot microbiota. Ten of these compounds elicited responses from one or more of the capitate peg ORNs. One ORN exhibited a consistently lower response after a blood meal to most of these bacterial volatiles. For the first time in the mosquito literature an increased sensitivity to host-seeking compounds is reported in mosquitoes that had laid eggs compared to non-blood fed mosquitoes of similar age. Furthermore five odorants of the 11 tested in this study completely suppressed the response to CO_2. This is the first record documenting that host-derived compounds inhibit the CO_2-receptor neuron in *An. gambiae*.

Introduction

Host seeking and reproductive behaviours of malaria mosquitoes are mainly mediated by odours (Takken 1991, Takken and Knols 1999, Takken *et al.* 2001). Depending on physiological state, different odours are used as orientation cues by female mosquitoes. To develop their eggs, female mosquitoes need to take a blood meal. After blood meal ingestion, the mosquito stops host seeking and after a period of digestion and oocyte maturation, it starts to seek an oviposition site (Takken *et al.* 2001). The yellow fever mosquito *Aedes aegypti* L. stopped responding to human odours after having had a blood meal (Klowden and Lea 1978). *Ae. aegypti* females that had taken a blood meal but retained their eggs did not respond to host cues until 24 h after oviposition (Klowden and Lea 1979). Takken *et al.* (2001) found similar results for *Anopheles gambiae* Giles *s.s.*; the first 24 h after a blood meal *An. gambiae* females no longer responded to human sweat odours. Over the next 48 h they gradually restored their response to host cues until 72 h later when they reached the same level of responsiveness.

The behavioural changes in responsiveness to odour cues following a blood meal are associated with changes in sensitivity in the antennal olfactory system. Several electrophysiological studies found that antennal olfactory receptor neuron (ORN) responsiveness to host cues was reduced after a blood meal. Davis (1984) showed that the responsiveness of antennal grooved peg sensilla to lactic acid, a compound eliciting host-seeking in *Ae. aegypti*, is suppressed after a blood meal and that after oviposition the neuronal responses returned to pre-blood meal levels (Davis 1984). Electrophysiological recordings from individual antennal trichoid sensilla of *Ae. aegypti* revealed that most sensilla did not show changes in responsiveness after a blood meal, only neurons housed within four of the five functional types of short blunt-tipped II trichoid sensilla showed increases in response intensity 24 h post blood meal to indole and phenolic compounds (Siju *et al.* 2010). The effect was still visible 72 h post-blood meal, although less pronounced. Though putative host-seeking related odours were present in their test panel, no decreases in response intensity were observed following a blood meal (Siju *et al.* 2010).

Females of *An. gambiae* also show changes in olfactory sensitivity depending on the physiological state they are in. Electroantennogram (EAG) responses of *An. gambiae* to incubated human sweat and indole were significantly reduced after a blood meal and returned to normal levels after oviposition (Takken *et al.* 2001). Qiu *et al.* (2006) showed that grooved pegs of *An. gambiae* exhibited lower responses to ammonia, a known attractant, after a blood meal. The intensity of the response of the short sharp-tipped trichoid sensilla to several phenols also decreased after a blood meal. This is consistent with the down-regulation of the gene coding for *Anopheles gambiae* odorant receptor "AgOR1" 12 h after a blood meal (Fox *et al.* 2001). When expressed in the heterologous *Drosophila melanogaster* heterologous expression system (DmHES), this receptor conveys responses to 4-methylphenol, a compound found in human sweat (Hallem *et al.* 2004). Qiu *et al.* (2006) also reported higher responses to indole and several acids by antennal trichoid sensilla after a blood meal.

Most mosquito species utilize CO_2 to orient to animal hosts. In mosquitoes, electrophysiological responses to CO_2 have thus far been described only for one neuron associated with the capitate peg sensilla on the maxillary palps (Kellogg 1970, McIver and Siemicki 1975, Lu *et al.* 2007, Syed and Leal 2007). The capitate peg sensilla also exhibited a remarkable sensitivity for 1-octen-3-ol, another odour that attracts several mosquito species (Lu *et al.* 2007, Syed and Leal 2007) and other insects like tsetse flies (Hall *et al.* 1984). An extensive study on the capitate peg sensilla in *An. gambiae* revealed that the same receptors were always associated with the three ORNs associated with the capitate peg sensilla (Lu *et al.* 2007). The A-neuron responsive to CO_2 contained three *Anopheles gambiae* gustatory receptors (AgGRs), 22, 23 and 24, while both B- and C-neurons expressed a heterodimer formed by AgOR7 with either AgOR8 for the B-neuron and AgOR28 for the C-neuron. All three ORNs associated with the capitate peg sensilla responded to a wide variety of odours and though overlap was noted, three distinct response types were found. Lu *et al.* (2007) showed that next to its high sensitivity for CO_2, the A-neuron exhibited responses to a limited number of other odorants. The B-neuron, that responded strongly to 1-octen-3-ol, was also narrowly tuned to only a few other odours. The C-neuron, however, was reported to act as a more broadly tuned receptor, responding to many acetyl-containing compounds and thiazole derivatives. It was suggested that, since the best ligands of AgOR28 were not involved in host seeking, the C-neuron was not directly involved in the host-seeking behaviour of *An. gambiae*. Instead a function in the detection of nectar sources or oviposition cues was proposed (Lu *et al.* 2007). A similar study in *Culex quinquefasciatus* Say revealed similar highly sensitive responses of the A-neuron and B-neuron to CO_2 and 1-octen-3-ol, however, the tuning widths of these ORNs were completely different from the three capitate peg ORNs in *An. gambiae* (Syed and Leal 2007). In *Cx. quinquefasciatus* the A-neuron of the capitate peg sensilla was solely stimulated by CO_2 and none of the more than 70 other tested odorants could evoke responses from the A-neuron or the C-neuron of the capitate peg sensilla. The B-neuron in the *Cx. quinquefasciatus* capitate peg was more broadly tuned and responded mainly to green leaf volatiles and floral compounds. A more elaborate role for the maxillary palps was proposed in the detection of plant and nectar sources (Syed and Leal 2007).

A recent study by Verhulst *et al.* (2009) revealed 10 volatile compounds that are strongly involved in host-seeking behaviour of *An. gambiae*. These 10 compounds were found in the headspace of bacterial samples that had been taken from human feet and were subsequently grown on agar plates. The synthetic blend composed of these 10 compounds was attractive to female mosquitoes in olfactometer assays. One other compound was found in their study that elicited opposite results (N.O. Verhulst, pers. comm.) and this compound also proved to be a repellent for female *An. gambiae* (R.C. Smallegange, unpubl. data). This compound was therefore included in the test panel.

These 11 bacterial compounds identified by Verhulst *et al.* (2009) were tested for their effectiveness in eliciting activity in the capitate peg sensilla. This study shows a role for all three ORNs of the maxillary palp capitate peg sensilla of *An. gambiae* in the detection of these human skin-derived compounds. Changes in intensity of the responses to these bacterial volatiles following ingestion of a blood meal as well as after oviposition are described and the function of these changes is discussed. Some of these odorants also inhibited the response to CO_2.

Materials and Methods

Insect rearing

A colony of *Anopheles gambiae* Giles *sensu stricto* (hereafter *An. gambiae*), originating from Suakoko, Liberia (courtesy Prof. M. Coluzzi), has been cultured at Wageningen University, The Netherlands, since 1988. The mosquito colony was kept under standard insectary conditions as described in Chapter 2.

For experiments on mosquito females that had not taken a blood meal, henceforth abbreviated as pre-BM, 7-day-old females were used that had not been offered a blood meal and had had *ad libitum* access to a 6% glucose solution (as a proxy for nectar). Females were collected the day before the experiment by means of an aspirator and placed in a small cage with access to water via damp cotton wool placed on top of the cage. Females that were attracted to a hand, held next to the rearing cage were selected to ensure the collection of behaviourally responsive mosquitoes.

To minimise possible effects of age differences on electrophysiological responsiveness, for the post-blood meal (post-BM) experiments 6-day-old females were given the opportunity to feed on a human arm for 10 min and were tested the following day (21 – 24 h post-BM). Only females that had taken a full blood meal, as judged by the appearance of diuretic droplets, were used for experiments and all others were removed from the cage. Access to a 6% glucose solution was given till the day before the experiments, when it was removed and replaced by damp cotton wool placed on top of the cage. For experiments on females that had oviposited, henceforth indicated as post-OP, 4-day-old female mosquitoes were selected and treated in the same way as for the post-BM experiments. After the feeding procedure, 2 small cups of water having damp filter paper on top were positioned in the cage to provide the 6 females in the cage the opportunity to oviposit. The standard procedure to check whether a female has laid her eggs is to check the ovaries under a microscope. Because the preparation method for electrophysiology made it very difficult to keep the mosquito intact after removal from the setup, which is necessary to check by microscopy, the females were given ample time to oviposit and oviposition was judged by egg production on the damp filter papers as only 6 females were present per cage. Both pre-BM and post-BM females were tested at the age of 7

days. For the post-OP experiments females were tested 92 - 96 h after a blood meal, at 8 days of age.

A second series of experiments investigated the potential of five compounds to inhibit the response of the A-neuron to CO_2. These experiments were done with 7-day-old non-blood fed mosquitoes treated in a similar way as the pre- BM mosquitoes described above.

Electrophysiology

Preparation

The preparation for the electrophysiological recording was made as described in Chapter 2. Recordings were made from all three ORNs innervating the capitate peg sensilla on segments 4 and 5 of the maxillary palp. By convention the neuron with the larger amplitude has been called the A-neuron, the one with intermediary amplitude the B-neuron and the one with the smallest amplitude the C-neuron. The A-neuron is also called the CO_2 neuron because it is the only neuron thus far described as capable of CO_2 detection.

Single sensillum recording (SSR)

Two tungsten electrodes were used to contact the mosquito preparation. These electrodes were electrolytically sharpened by repeated dipping in a saturated KNO_2 solution at a voltage of 4 - 9 V until a tip diameter of 1-2 µm was reached. The indifferent electrode was placed in the eye of the mosquito using a mechanical micromanipulator and grounded. A motorised micromanipulator (MX7500L, Siskiyou, Grants Pass, Oregon, USA) was used to position the recording electrode at the basis of a capitate peg sensillum and to pierce the cuticle of the sensillum until action potentials were registered. The analogue signals were fed to an impedance converting preamplifier and digitized by an analogue–digital conversion interface (IDAC4, Syntech, Kirchzarten, Germany) at a sample rate of 10667/s. The digital signals were recorded and visualised using Autospike 3.9 software (Syntech).

Chemical stimuli and stimulus delivery

A panel of 11 chemical compounds identified as volatile products of human skin bacteria (Verhulst *et al.* 2009) was used for these experiments. The compounds were 2- and 3-methylbutanoic acid, 2- and 3-methylbutanal, 2- and 3-methyl-1-butanol, 2,3-butanedione, 1-butanol and 3-hydroxy-2-butanone and 2-phenylethanol and benzaldehyde. All chemicals were of the highest purity commercially available (from 95% to >99% pure; Appendix 2). Five dilutions (0.001%, 0.01%, 0.1%, 1% and 10% w/w in paraffin oil) were made of each compound to establish dose-response relationships.

Of each solution, 10 µl was applied to a strip of filter paper (0.5 x 1.5 cm) inserted into a Pasteur pipette serving as an odour cartridge and were closed with a 1 ml disposable pipette-point and Parafilm® and kept at -25 °C. These cartridges were used for a maximum of 10 stimulations. A constant, charcoal-filtered and humidified airstream of 0.5 l/min was delivered to the antenna via a glass tube. The vapour of test compounds in odour cartridges was injected into the airstream at 0.5 l/min using a stimulus controller (CS-55, Syntech). A synchronised compensation airstream generated by the stimulus controller was injected into the main airstream in order to keep the total airflow constant. Stimulus duration was set at 200 ms.

During the dose-response experiments five of the 11 eleven tested compounds (2,3-butanedione, 2-methyl and 3-methylbutanoic acid and 2-metyl and 3-methylbutanal) showed that they could suppress the activity of the A-neuron of the maxillary palp that also responds to CO_2 (Fig. 1). To further investigate this potential inhibition of the CO_2 response by these five odorants, the recording setup was slightly modified. The stimulus duration was extended to 1 s to allow a better differentiation between true inhibition effects and suspected mechanosensory inhibition of the A-neuron. The main airstream, stimulation method and odour cartridges were identical but now a constant airstream of 15 ml/min containing 4% CO_2 was added, resulting in an increase of +/- 1165 ppm CO_2. Stimulation with the odours was done after a 2 s period giving the neuron the chance to adapt to the CO_2 concentration, which was judged based on the occurrence of a stable increased firing frequency.

Quantification of electrophysiological responses

Single sensillum recordings were automatically filtered to increase signal-to-noise ratio using Autospike software (filter settings 20 Hz - 1694 Hz) in combination with Automacrorecorder (ReadmeSoft) software. All recordings from the capitate peg sensilla showed activities from three neurons. Action potentials from different neurons could be distinguished by spike amplitude, shape and by identification of doublet or triplet wave forms present in a recording. For the dose-response relationships the intensity of response was quantified by the change in action potential firing frequency, calculated as the number of action potentials during the 500 ms after the onset of the stimulus minus the average firing frequency during four intervals of 500 ms preceding the onset of the stimulus. The resulting count was doubled to obtain the number of spikes per second. Because the solvent paraffin oil elicited excitatory responses (significant excitation compared with spontaneous activity) from the A-neuron, this response to paraffin oil was subtracted from the responses of the A-neuron to the bacterial compounds. A significant increase in firing frequency is described as excitation and a decrease as inhibition. The time it took for the injected stimulus to travel down the glass tube to reach the antenna was added to the onset of stimulus time. The calculated delay matched closely with the observed delay between stimulus onset and response onset.

For the experiments investigating inhibition of the CO_2 neuron, the response to the stimulus was analysed in a different way. To represent the time course of inhibitory effects better, the change in response resulting from stimulation by a second compound was measured

as the number of action potentials during small bins of 500 ms, starting 1 s before odour stimulation, for 10 s in total (20 bins). The first second (first 2 bins) represents the elevated stable frequency caused by addition of CO_2.

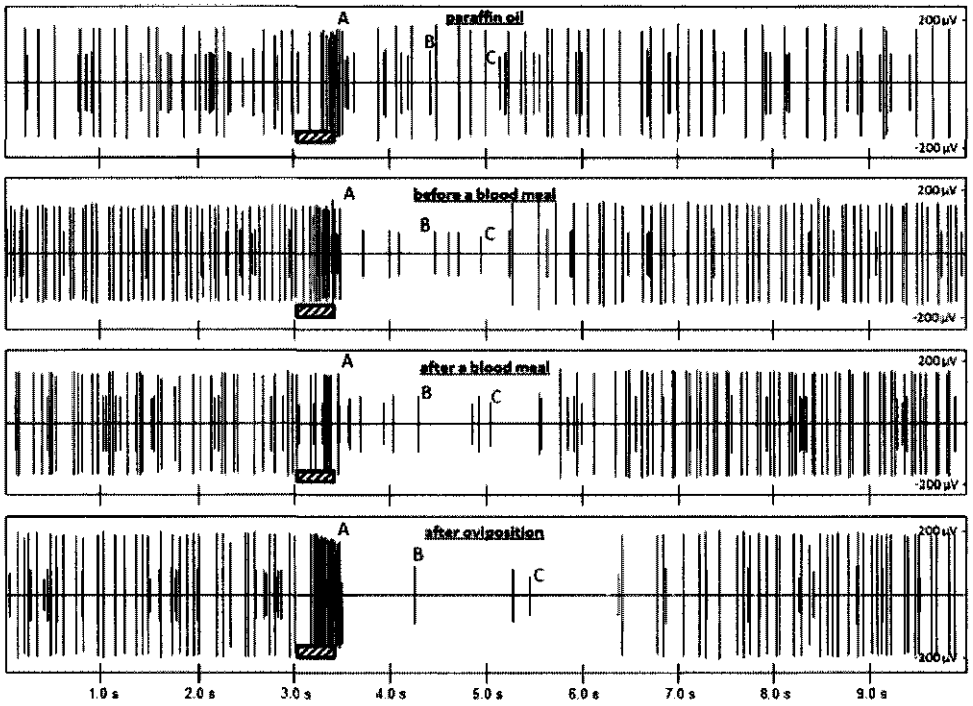


Figure 1: Example of recordings of the responses of the three ORNs innervating the maxillary palp capitate peg sensilla to 1% 3-methylbutanal in the three different physiological states. Vertical lines indicate the action potentials. Hatched bar represents the 200 ms stimulus.

Statistical analysis

Statistical analysis of the electrophysiological data was done using Genstat software (Genstat 14, VSN International Ltd, UK). The overall effect of physiological state of the mosquito was assessed using a Generalized Linear Model with Poisson distribution with a log function. To determine whether the electrophysiological responses in all three physiological stages, i.e. pre-BM, post-BM and post-OP, were significantly different from each other; two-sided t-probabilities were calculated to test pairwise differences between means. Effects were considered to be significant at $P < 0.05$. Data collected on one mosquito were excluded from the post-OP experiment because this mosquito had completely different responses than all other tested mosquitoes. Removal of this specimen from the setup succeeded in keeping it intact allowing dissection. The dissection confirmed that this individual had retained her eggs. The responses displayed by this female were clearly different from both post-BM mosquitoes and post-OP mosquitoes.

For the analysis of the effects on the CO_2 neuron five out of 20 bins have been chosen for statistical analysis. These are bins 3 and 4, coinciding with the 1 s stimulus that was given; bin 5, directly following the stimulus; bin 12: the bin approximately halfway the 10 s recording period and bin 20: the last bin during which the response intensity was quantified. Statistical analysis of these bins was done using Genstat software (Genstat 14, VSN International Ltd, UK). The overall effect of concentration on the response of the A-neuron was calculated per bin using a Generalized Linear Model with Poisson distribution with a log function. To determine which concentration differed significantly from the response to the control (paraffin oil); two-sided t-probabilities were calculated to test pairwise differences between means. Effects were considered to be significant at $P < 0.05$.

Results

Ten out of the 11 odorants tested elicited significant responses from at least one of the three capitate peg olfactory receptor neurons (ORNs); only 3-hydroxy-2-butanone did not cause activation of these neurons. All three ORNs showed changes in response intensity that were dependent on the physiological state of the mosquito for multiple bacterial volatile compounds.

Effect of physiological state

4C-Acids

Physiological state influenced the responsiveness of the capitate peg A-neuron (henceforth referred to as A-neuron) to both aliphatic carboxylic acids having a four-carbon chain (4C), 2-methylbutanoic acid and 3-methylbutanoic acid. The activity of the capitate peg B- and C-neurons (henceforth referred to as B-neuron and C-neuron respectively) were not affected. Significant effects of physiological state were only found when the A-neuron was exposed to 10% of either 2-methylbutanoic acid or 3-methylbutanoic acid (Fig. 2). At this concentration of both acids, the A-neuron of post-OP mosquitoes showed a significantly lower response than the A-neuron in pre-BM mosquitoes.

2-phenylethanol

No differences in response intensity of the A-neuron to stimulation with 2-phenylethanol were found between mosquitoes in different physiological states (Fig. 2). The B-neuron in post-BM mosquitoes displayed significantly lower response intensities to 10% 2-phenylethanol than did the B-neuron in pre-BM mosquitoes. The C-neuron in post-OP mosquitoes showed significantly higher responses to 1% of 2-phenylethanol compared to pre-BM and post-BM mosquitoes. However, at 10% the responsiveness to 2-phenylethanol was similar for mosquitoes in all different states (Fig. 2).

4C-Alcohols

Of all bacterial compounds tested, the alcohols elicited the highest responses from the three capitate peg neurons. The three 4C-alcohols evoked excitatory responses in all three ORNs and the intensity depended on the physiological state of the mosquito (Fig. 2).

The A-neuron showed higher responses to 1% 2-methyl-1-butanol in post-OP mosquitoes compared to pre-BM mosquitoes. There was no difference at 10%. No significant changes in responsiveness were found for the B-neuron in response to 2-methyl-1-butanol. The C-neuron in post-OP mosquitoes responded stronger to 1% and 10% 2-methyl-1-butanol compared to the C-neuron in pre-BM and post-BM mosquitoes (Fig. 2).

The response of the A-neuron to 1% 3-methyl-1-butanol was significantly lower in post-BM mosquitoes than in pre-BM mosquitoes. This difference was not significant at 10%. The response of the B-neuron to 1% 3-methyl-1-butanol was also significantly lower in post-BM mosquitoes compared to pre-BM and post-OP mosquitoes. No significant changes in responsiveness were found for the C-neuron upon stimulation with 3-methyl-1-butanol (Fig. 2).

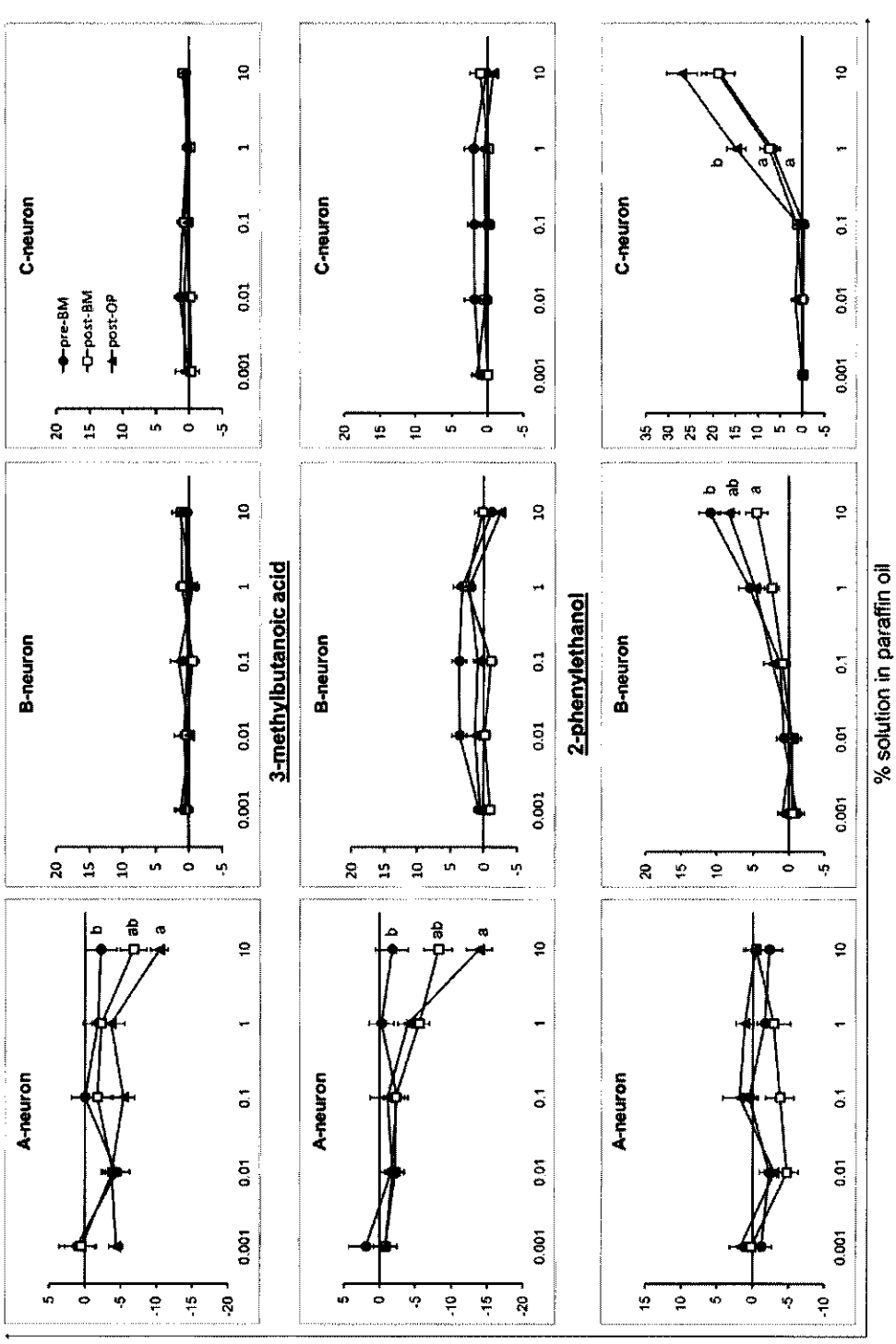
Neither the A nor the C-neuron showed significant differences among mosquitoes with different physiological states, when stimulated with 1-butanol. The B-neuron, however, responded significantly weaker to 10% 1-butanol in post-BM mosquitoes compared to pre-BM (Fig. 2).

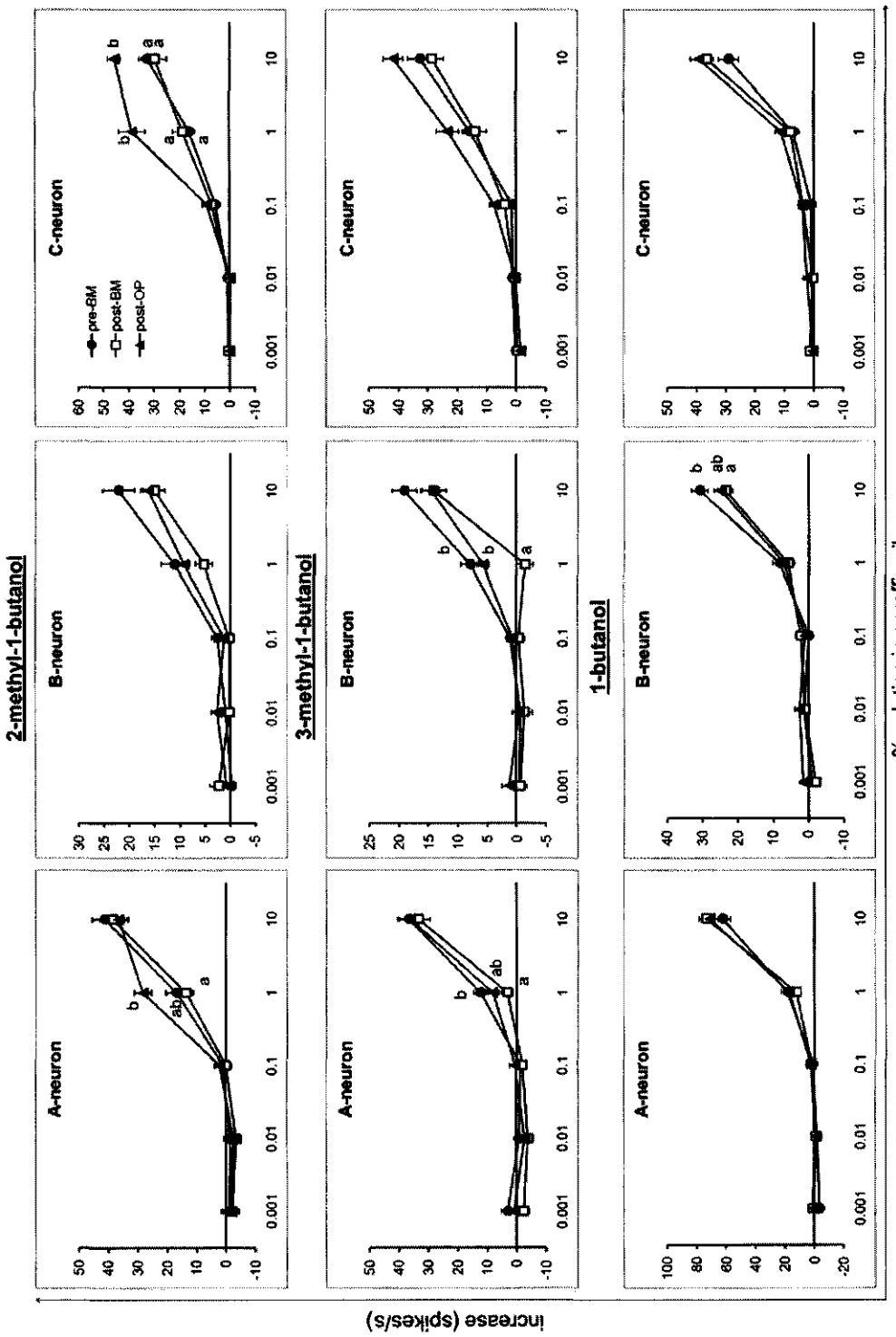
4C-Aldehydes

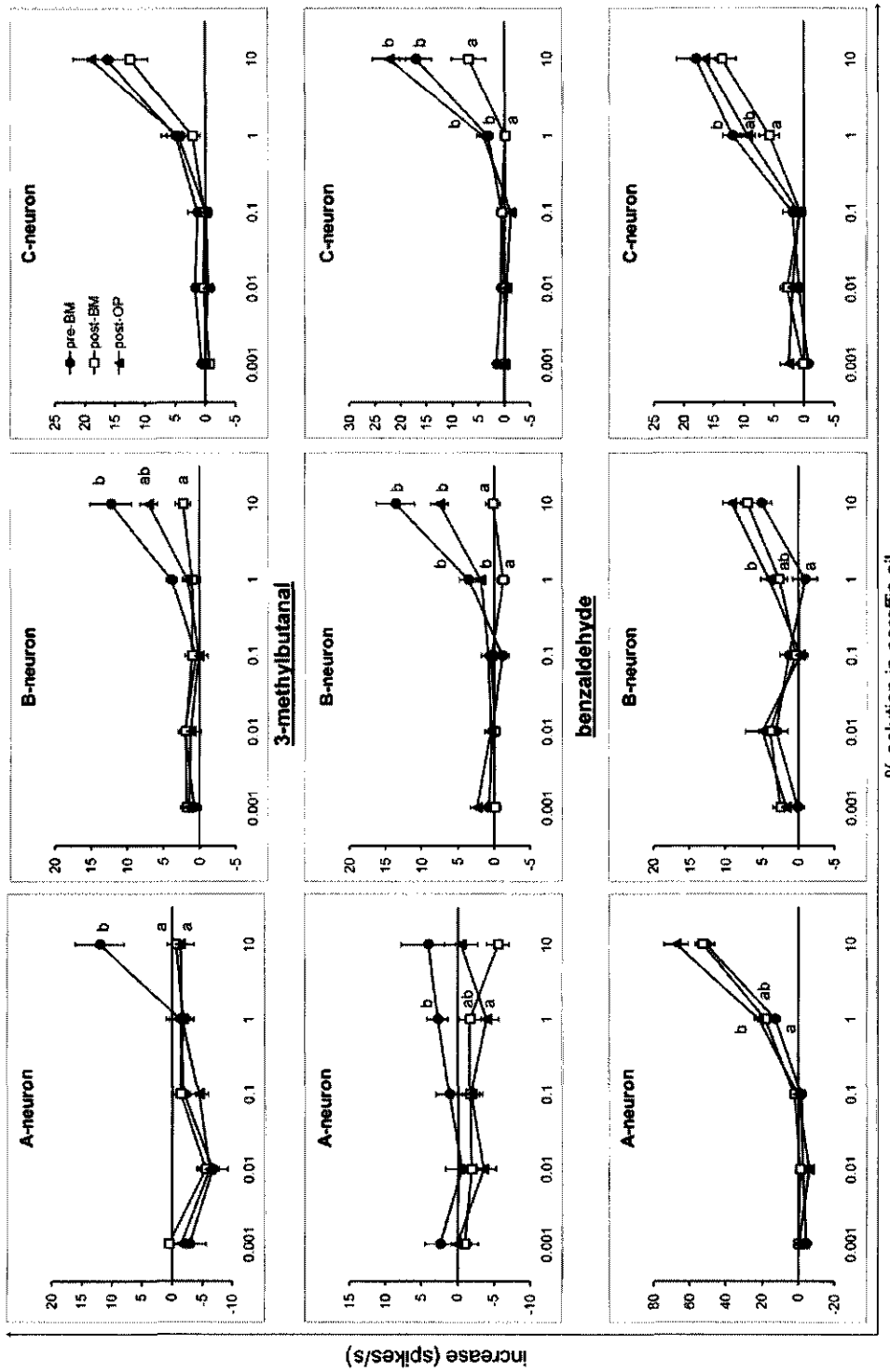
The response of the A-neuron to 10% 2-methylbutanal was significantly lower in post-BM and post-OP mosquitoes than in pre-BM mosquitoes (Fig. 2).
The response of the B-neuron to 10% 2-methylbutanal in post-BM mosquitoes was significantly lower than the response of the B-neuron in pre-BM mosquitoes. No significant difference between mosquitoes in different physiological states was seen in the response of the C-neuron upon stimulation with 2-methylbutanal (Fig. 2).

The response of the A-neuron to 1% 3-methyl-1-butanal was significantly lower in post-OP mosquitoes compared to pre-BM mosquitoes (Figs. 1 and 2). At 10% this difference was not significant anymore. The responses of both the B- and C-neuron to 1% and 10% 3-methyl-1-butanal were significantly lower in post-BM mosquitoes compared to both pre-BM and post-OP mosquitoes (Fig. 2).

All three ORNs showed changes in response intensity that depended on the physiological state of the mosquito when stimulated with 1% benzaldehyde. The response of both the A- and B-neuron to 1% benzaldehyde was significantly higher in post-OP mosquitoes than in pre-BM mosquitoes. The C-neuron, however, showed an opposite pattern, its response


2-methylbutanoic acid
3-methylbutanoic acid
2-phenylethanol
A-neuron
B-neuron
C-neuron
pre-BM
post-BM
post-OP
a
ab
b
increase (spikes/s)
% solution in paraffin oil


2-methyl-1-butanol
3-methyl-1-butanol
1-butanol
A-neuron
B-neuron
C-neuron
pre-BM
post-BM
post-OP
increase (spikes/s)
% solution in paraffin oil


2-methylbutanal
3-methylbutanal
benzaldehyde
A-neuron
B-neuron
C-neuron
pre-BM
post-BM
post-OP
increase (spikes/s)
% solution in paraffin oil

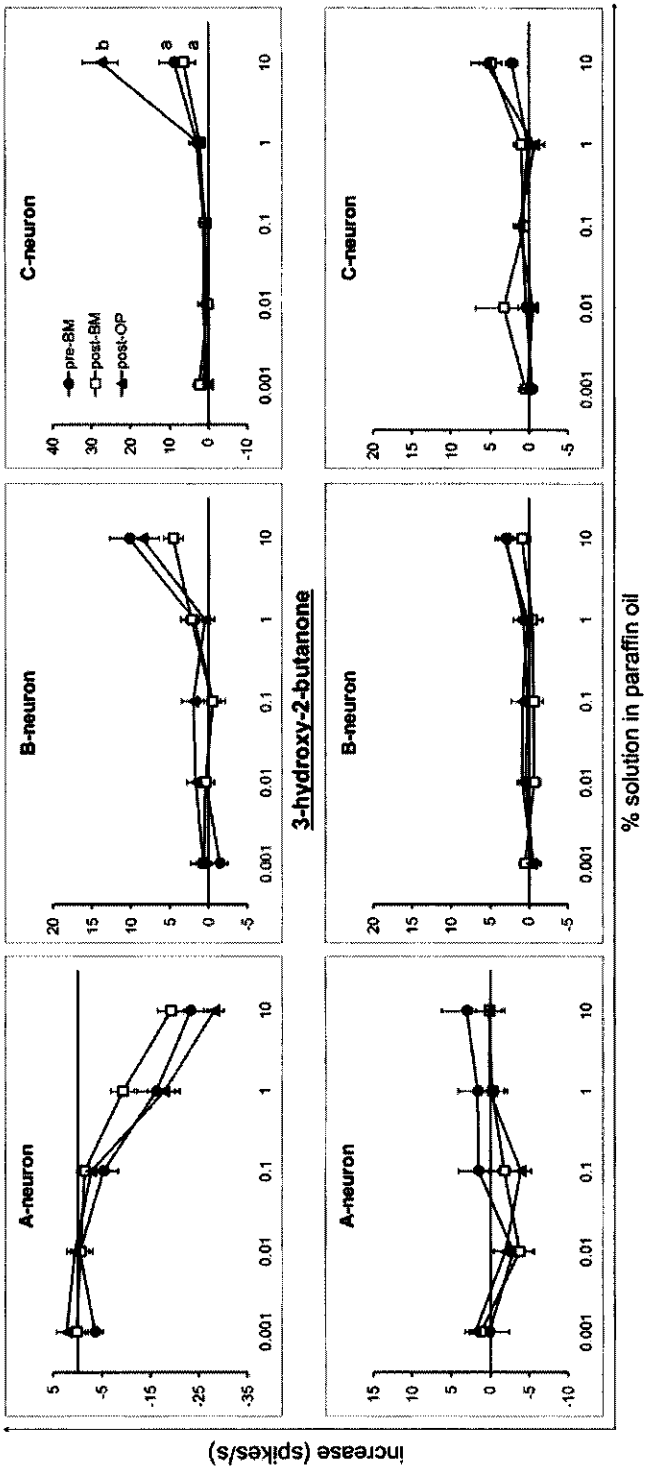


Figure 2: Dose-response curves for 11 odorants for three physiological states; before a blood meal (pre-BM), after a blood meal (post-BM) and after oviposition (post-OP). X-axis shows the percentage dilution (w/w) in paraffin oil. The Y-axis represents the increase in spikes/s compared to the spontaneous activity of the neuron. N=10, error bars represent standard error of the mean. Means with different letters are significantly different (GLM, $P < 0.05$); no letters mean no significant differences among mosquitoes from the different treatments.

to 1% benzaldehyde was significantly higher in pre-BM mosquitoes than in post-BM mosquitoes (Fig. 2).

4C-Ketones

Physiological state of the mosquitoes did not influence the responses of the A- and B-neurons to 2,3-butanedione (Fig. 2). Only the response of the C-neuron to 10% 2,3-butanedione was differed among mosquitoes with different physiological states. The response of the C-neuron in mosquitoes post-OP to 10% 2,3-butanedione was significantly higher than the responses of the C-neuron in pre-BM and post-BM mosquitoes.

Inhibition of the responses of the A-neuron to CO_2

During the experiments with mosquitoes in different physiological state, a short stimulation of 200 ms of five compounds in a 10% concentration was capable of complete inhibition of the spontaneous activity of the A-neuron, some even for more than one second (five times the stimulus; Fig. 3). The A-neuron is the only neuron that responds to CO_2, a known mosquito attractant. For this reason these five odorants were examined for their capacity to suppress the excitation of the A-neuron by CO_2. These five bacterial volatile organic compounds were 2,3-butanedione, 2- and 3-methylbutanoic acid and 2- and 3-methylbutanal (Fig. 2, A-neuron). Because benzaldehyde was rated as a potentially behavioural repellent this compound was also added to the panel of five compounds.

During continuous stimulation with CO_2, a stimulation of 1 s with the solvent paraffin oil alone elicited an initial increase in response for the duration of +/- 0.5 s after which the response decreased again to a level lower than before the paraffin stimulus but still higher than the spontaneous activity level without CO_2 (GLM, $P < 0.001$; Fig. 4).

At the 0.001%, 0.01% and 0.1% doses, all six odorants affected the CO_2 response of the A-neuron with temporal characteristics similar to that of stimulation with paraffin oil (GLM, $P > 0.1$; Fig. 4A). In order to keep the graphical representations concise, these three concentrations were omitted from Figures 4B-4G.

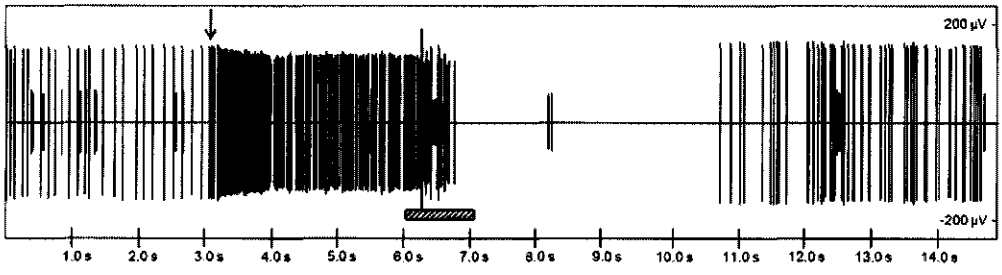


Figure 3: Example of the inhibition of the CO_2 neuron caused by delivery of 10% 3-methylbutanal. Vertical lines indicate action potentials. The arrow shows the moment at which +/- 1165 ppm CO_2 was added, the hatched bar represents the 1 s stimulus of 10% 3-methylbutanal.

4C acids

A significant treatment effect (stimulation with either paraffin oil or 1% or 10% of an odorant) on the response intensity of the A-neuron after stimulation with CO_2 was found for 2-methylbutanoic acid for bins 4, 5, 12 and 20. None of the five 500 ms bins analysed (bins 3, 4, 5, 12 and 20) showed significant differences between paraffin oil and 1% 2-methylbutanoic acid, although for bin 5 a trend was seen (GLM, P = 0.058; Fig. 3A). Stimulation with 10% 2-methylbutanoic acid significantly reduced the response to CO_2 compared to paraffin oil for bins 4, 5, 12 and 20 (Fig. 4A).

3-Methylbutanoic acid elicited similar responses from the A-neuron as 2-methylbutanoic acid (Fig. 4B). A significant treatment effect was found for 3-methylbutanoic acid for bins 4, 5, 12 and 20. For bin 5, stimulation with 1% 3-methylbutanoic acid significantly reduced the response to CO_2 compared to stimulation with paraffin oil. Stimulation with 10% 3-methylbutanoic acid significantly reduced the response to CO_2 for bins 4, 5, 12 and 20 (Fig. 4B).

4C aldehydes

The response intensity of the A-neuron was significantly affected by 2-methylbutanal for bins 4, 5 and 12. For bin 5, stimulation with 1% 2-methylbutanal significantly reduced the response to CO_2 compared to paraffin oil (Fig. 4C). When applied at 10%, 2-methylbutanal reduced A-neuron responses for bins 4, 5 and 12 (Fig. 4C).

A significant treatment effect was found for 3-methylbutanal for bins 4, 5 and 12. Stimulation with 10% 3-methylbutanal significantly reduced the response to CO_2 for bin 4, 5 and bin 12 (Figs. 3 and 4 D).

A significant treatment effect was found for benzaldehyde for bins 3, 4 and 12. Stimulation with 10% benzaldehyde induced a significant increase in the response of the A-neuron for bins 3, 4 and 12 (Fig. 4F).

2,3-butanedione

A significant treatment effect was found for 2,3-butanedione for bins 3, 4 and 12. Stimulation with 10% 2,3-butanedione significantly reduced the response to CO_2 for bin 3 and bin 4. For bin 12 the response to delivery of 10% 2,3-butanedione was significantly higher compared to the response after stimulation with paraffin oil (Fig. 4E).

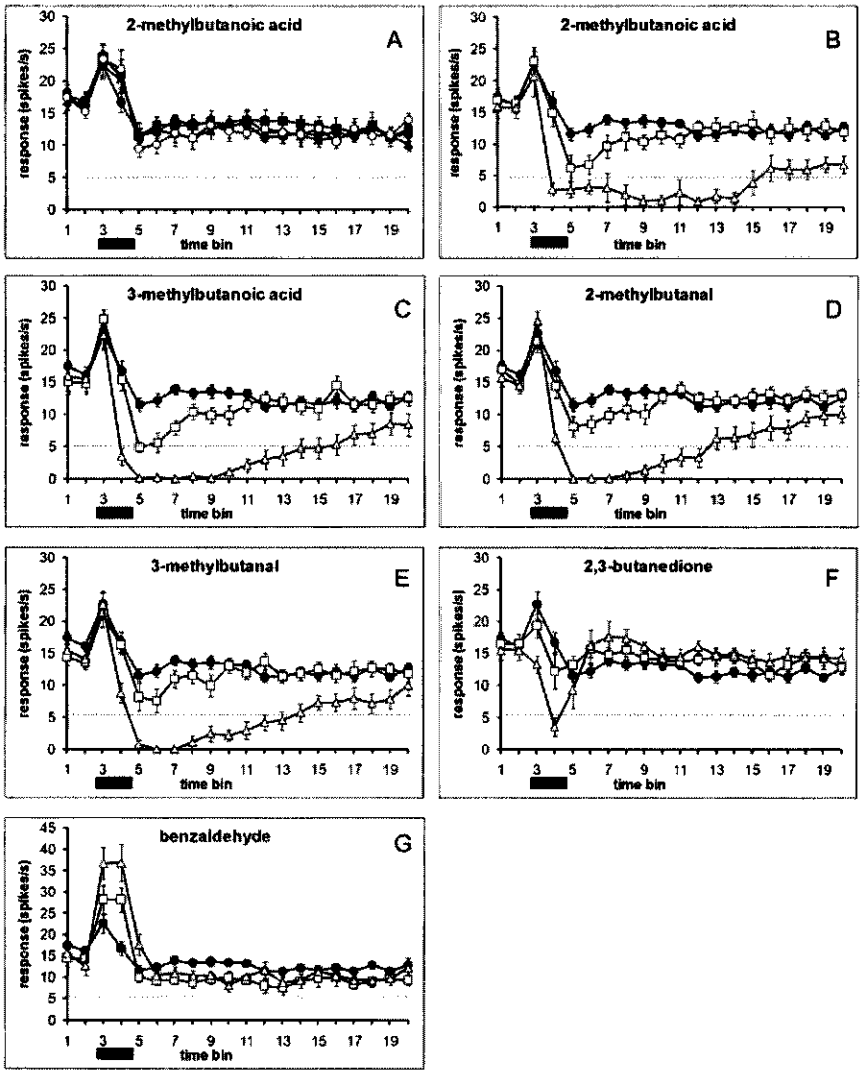


Figure 4: Temporal characteristics of the electrophysiological activity of the A-neuron associated with the capitate peg sensilla in response to the highest two concentrations of six different odorants during continuous stimulation with *ca.* 1165 ppm CO_2. X-axis: 500 ms time bins. Y-axis: the response of the A-neuron in spikes/s. 3A: Temporal characteristics for the three lowest concentrations of 2-methylbutanoic acid. 3B-3G: Temporal characteristics of the two highest concentrations for the different odours. Closed circles: paraffin oil; closed squares: 0.001%; closed triangles: 0.01%; open circles: 0.1%; open squares: 1% concentration of the tested odorant; open triangles: 10% concentration of the tested odorant. The horizontal black bar at the bottom of each graph represents the 1 s stimulus and the dashed line represents the spontaneous activity level of the A-neuron. N=10 and error bars represent standard error of the mean. Significance was tested at 5 bins: bins 3, 4, 5, 12 and 20. Means with different letters are significantly different (GLM, $P < 0.05$); no letters mean no significant differences.

Discussion

This study describes the influence of the physiological state of female *An. gambiae* mosquitoes on the sensitivities of the three palpal capitate peg ORNs to 11 putative host-seeking compounds. The responses of the ORNs to 10 of these 11 odorants were shown to change after ingestion of a blood meal and/or after oviposition. Furthermore, five of the 11 volatile compounds produced by human skin bacteria were shown to inhibit the response of the A-neuron to CO_2.

General trends in the changing response intensities between physiological states

The only compound that did not evoke responses from any of the three ORNs was 3-hydroxy-2-butanone. In contrast, with an increase of +/- 65 spikes/s, 1-butanol represents one of the best ligands for the A-neuron reported thus far (Lu *et al.* 2007). For both the B- and C-neuron, however, the maximum responses to 10% concentrations of respectively 1-butanol (+/- 30 spikes/s) and 2-methyl-1-butanol (+/- 40 spikes/s) represent not even half of the increase caused by their best ligands at a 1% concentration (B-neuron: 1-octen-3-ol and C-neuron: 2,4,5-trimethylthiazole; Lu *et al.* 2007).

In all three ORNs closely related compounds (e.g. the two acids, the two alcohols and the two aldehydes) evoked similar responses and similar changes in response intensity going from one physiological state to another. Nine out of the 11 odorants evoked responses from the A-neuron while both the B- and C-neuron responded to eight of the 11 tested odorants.

During the experiments in which the effects of different physiological states were examined, the response of the A neuron showed some interesting responses. A short stimulation of 200 ms with the highest concentration (10%) of five bacterial compounds (2- and 3-methylbutanoic acid, 2- and 3-methylbutanl and 2,3-butanedione) completely inhibited the spontaneous activity of the A-neuron, for durations sometimes exceeding five times the stimulus duration. Because the responses to 2- and 3-methylbutanal still showed an excitatory response after being corrected for the increase caused by the solvent (paraffin oil), it was deduced that these two compounds first caused an excitatory response of the A-neuron after which they inhibited the response of the A-neuron. Based on these inhibitory effects it was decided to examine whether these five compounds would inhibit the response to CO_2 itself or only lower the spontaneous activity of the A-neuron (see further below).

Another intriguing trend is seen when comparing the responses of the B-neuron of pre-BM mosquitoes with those of post-BM mosquitoes. For almost all odours that evoked responses from the B-neuron, the response post-BM is lower than that pre-BM. For five out of the eight compounds that evoked responses this is significant at one or two of the highest concentrations tested. Two out of the three remaining odours follow the same trend but with ten replicates the recorded differences are not significant. Only benzaldehyde did not evoke a lower response in post-BM mosquitoes compared to pre-BM ones.

The C-neuron appeared to have a higher sensitivity to several odours in post-OP mosquitoes compared to pre-BM and post-BM mosquitoes. This neuron showed higher responses to seven of the eight compounds that were previously shown to elicit responses from the B- and C-neuron (see above), in post-OP mosquitoes compared to pre-BM and post-BM mosquitoes. For four of the seven odorants that elicited higher responses, this difference was significant with one or both of the two highest concentrations used (1% or 10%). As observed with the B-neuron, benzaldehyde did not show this trend. The other three remaining odorants did not evoke responses at all from the B-neuron.

All 10 bacterial compounds were present in higher quantities in the headspace of blood agar plates inoculated with human foot bacteria compared to blood agar alone (Verhulst *et al.* 2009). However, benzaldehyde was found at lower levels in the attractive bacterial sample plates compared to the samples from blood agar alone. This opposite effect, combined with an apparent repellency of benzaldehyde (R.C. Smallegange, pers. comm.), led us to include this compound in our experiment. Interestingly, benzaldehyde elicited exactly opposite effects compared to most other bacterial compounds. Where stimulation of the A-neuron with most odours resulted in the highest responses in pre-BM mosquitoes (when responses were noted) compared to post-BM and post-OP mosquitoes, stimulation of the A-neuron with benzaldehyde caused the highest responses in post-OP mosquitoes. The B-neuron showed mostly suppression of the responses to the bacterial compounds in post-BM mosquitoes compared to pre-BM mosquitoes while the response of the B-neuron to benzaldehyde was higher in post-BM mosquitoes than in pre-BM. The same applies to the responses of the C-neuron. All bacterial compounds elicited the highest responses of the C-neuron in post-OP mosquitoes except benzaldehyde. Thus benzaldehyde elicits opposite responses from *An. gambiae* both at the level of behaviour (repellence *vs.* attraction in response to the blend of the 10 other bacterial compounds) as well as at the electrophysiological level in ORNs on the maxillary palps.

Inhibition by microbiota-derived compounds of the CO_2 responses of the A-neuron

The five compounds that caused suppression of the spontaneous activity of the A-neuron in the absence of CO_2, were, all five, also capable of lowering the responses of the CO_2 neuron in pre-BM mosquitoes when excited with ca. 1165 ppm extra CO_2. At 10%, the highest concentration tested, complete inhibition of the CO_2 response could be achieved that lasted considerably longer than the initial odour stimulus duration by four of the five odorants, i.e. 2- and 3-methylbutanal along with 2- and 3-methylbutanoic acid. The fifth compound, 2,3-butanedione, also inhibited the CO_2 response but it had a more acute effect, which lasted only for the duration of the stimulus. Nonetheless stimulation with 2,3-butanedione even inhibited the initial excitatory response of paraffin oil on top of the increased response to CO_2.

It seemed that the inhibitory effects of these five compounds on the responses of the A-neuron were even stronger after oviposition when the effects of physiological state on the responses were investigated. This suggests that, after oviposition, the A-neuron becomes more sensitive to these odours. During the experiments the suppression of the spontaneous activity

lasted even longer in post-OP mosquitoes compared to pre-BM mosquitoes (example trace in Fig. 1). If this stronger suppression of activity of the CO_2 neuron would also occur in the presence of higher levels of CO_2, as exhaled by human hosts, remains to be tested.

It has only recently been discovered that the CO_2 neuron on the maxillary palp of *Drosophila melanogaster* Meigen can be inhibited by odorants (Turner and Ray 2009). Both behaviourally and electrophysiologically, stimulation with 2,3-butanedione could directly suppress the olfactory-based avoidance behaviour to CO_2, which is emitted by stressed flies. Stimulation of the flies with 10% 2,3-butanedione could effectively stop the responses to CO_2 of the heterodimeric receptor encoded by Gr21a and Gr63a that detects CO_2. Orthologues of these two receptors are present in the A-neuron of the capitate peg sensilla of *An. gambiae* mosquitoes representing the only ORN capable of CO_2 detection (Kwon *et al.* 2007, Lu *et al.* 2007). Turner and Ray (2009) also tested 2,3-butanedione on the CO_2 neuron of *Cx. quinquefasciatus* females. Whereas 2,3-butanedione did not inhibit the CO_2 response in *Cx. quinquefasciatus*, another compound active on the CO_2 neuron of *D. melanogaster*, 1-butanal, strongly reduced the responses from the CO_2 neuron in the capitate peg sensilla of *Cx. quinquefasciatus*. Though we did not test 1-butanal directly, 2- and 3-methylbutanal elicited similar responses in *An. gambiae* as 1-butanal in *Cx. quinquefasciatus*.

A recent study by Ghaninia *et al.* (2007a) revealed that six dorso-medial (DM8-13) glomeruli in the antennal lobe (AL) of female *An. gambiae* received input from the maxillary palp ORN. Carbon dioxide has been shown innervate neurons projecting to this area. Surprisingly they found that three of these glomeruli (DM10-12) also received input from antennal ORNs. Earlier studies stated that antennal afferents and palpal afferents never converged to the same part of the AL (Anton *et al.* 2003). This is interesting because nine out of the ten bacterial compounds also elicited responses from antennal sensilla (Chapter 2, Qiu *et al.* 2006). The two acids evoked responses from both grooved peg and coeloconic sensilla. So did the two aldehydes and these also elicited responses from several types of trichoid sensilla. The alcohols solely evoked responses from the trichoid sensilla and 2,3-butanedione only from the coeloconic sensilla. This means that all five compounds that suppressed the CO_2 response of the maxillary palp capitate peg ORN, evoke responses from the coeloconic sensilla. The four compounds, i.e. the two acids and two aldehydes, that elicited longer inhibitory periods in the A-neuron, also evoked responses from the grooved pegs. Both types of these olfactory sensilla, grooved pegs and coeloconic sensilla, show responses to ammonia (Qiu *et al.*, 2006; Y.T. Qiu, pers. comm.), a known attractant for *An. gambiae* (Smallegange *et al.* 2005b), and the grooved pegs also to lactic acid, a synergist to ammonia. It might be that these five inhibitory odours reduce the responses to CO_2 that reach the AL, while exciting neurons innervating antennal grooved pegs and coeloconic sensilla, in addition to the other two capitate peg ORNs that send their signals to the same glomeruli.

Roles of maxillary palp ORNs in host seeking

Lower responses to host-seeking compounds after a blood meal, like we found for the capitate peg B-neuron, have previously been reported for ORNs associated with antennal grooved peg sensilla. After a blood meal grooved peg ORNs of *An. gambiae* were less sensitive to ammonia compared to pre blood meal (Qiu *et al.* 2006) and the grooved pegs of *Ae. aegypti* showed decreased responses to lactic acid after a blood meal (Davis 1984). Qiu *et al.* (2006) also reported lower responses of ORNs to ammonia and several phenols in the short sharp-tipped trichoid sensilla on the antennae of *An. gambiae*. A recent study on the effect of a blood meal on the antennal trichoid sensilla of *Ae. aegypti*, using a panel of odorants involved in both host-seeking and oviposition, reported no decreases in responses though such decreases were expected for compounds involved in host-seeking (Siju *et al.* 2010). These authors also reported no changes in the responses of the short sharp-tipped trichoid sensilla of *Ae. aegypti*, contrary to the changed sensitivity observed in short sharp-tipped trichoid sensilla type E of *An. gambiae* (Qiu *et al.* 2006), as well as no changes in the responses of the long sharp-tipped and the short blunt-tipped type I trichoid sensilla (Siju *et al.* 2010). Interestingly, both studies (Qiu *et al.* 2006, Siju *et al.* 2010) found increased responses to indole, a compound known to be released by oviposition sites (Millar *et al.* 1992, Lindh *et al.* 2008a, Lindh *et al.* 2008b). The increased responses to indole found in both studies originated from different types of trichoid sensilla. Qiu *et al.* (2006) showed increased responses to indole from the short sharp-tipped trichoid sensilla E of *An. gambiae* while Siju *et al.* (2010) reported increased responses from the short blunt-tipped type II of *Ae. aegypti*. Siju *et al.* (2010) also reported increased responses to the same phenols that evoked decreased responses in *An. gambiae* after a blood meal. Because phenols have been found both in human emanations and in the odours from oviposition sites (Bentley and Day 1989, Millar *et al.* 1992) and because opposite effects were found in both studies in different types of sensilla, it is plausible that *An. gambiae* uses these phenols as host cues while *Ae. aegypti* uses these phenols to direct them to a suitable oviposition site. It could be that both species have evolved different responses to the same compounds. Though both studies investigated the same physiological state, post-BM, the time frame of both studies was quite different (2 – 24 h in *An. gambiae* or 24 h and 72 h in *Ae. aegypti*). Thus these differences between both studies could also be caused by differences between the different time frames within the post-BM time period.

In our study, increased responses after a blood meal were not observed with any of the odorants. This is in line with the assumption that these compounds represent host cues. Some of the compounds tested in this study (3-methylbutanoic acid, 3-methyl-1-butanol and 2-phenylethanol) were also shown to be emitted from bacterial samples isolated from oviposition sites that were attractive to gravid *An. gambiae* females (Lindh *et al.* 2008b). Therefore, these may just as well have represented oviposition cues.

The decreases in B-neuron responses observed after ingestion of a blood meal confirmed a role for the maxillary palps in host seeking. A recent study suggested that the maxillary palp might have different behavioural roles in different mosquito species (Majeed *et*

al. 2010). These authors showed that the maxillary palps are involved in the landing response of *Ae. aegypti* mosquitoes on a human hand but not in the landing response of *Cx. quinquefasciatus*. Palpectomised *Ae. aegypti* females had a significantly reduced landing response compared to non-palpectomised mosquitoes. Majeed *et al.* (2010) also showed that palpectomised *Cx. quinquefasciatus* females, as reported before (Omer and Gillies 1971), still showed landing responses not significantly different from non-palpectomised females (Majeed *et al.* 2010).

In an earlier study on capitate peg ORNs it was hypothesised that AgOR28, the receptor protein expressed by the C-neuron, would not be involved in host-seeking behaviour but might mediate responses to nectar sources or oviposition-site-related cues (Lu *et al.* 2007). Although we cannot rule out a role in localisation of nectar sources or oviposition sites, we provide evidence that the C-neuron could be involved in host location. Increased responses to several compounds were observed in post-OP mosquitoes and not in post-BM mosquitoes, so only at the time when the mosquito is expected to search for blood. It has been shown that after oviposition the host-seeking behaviour of several mosquito species returned to the pre-BM level (Klowden and Lea 1979, Davis 1984, Rowland 1989, Takken *et al.* 2001). These studies were confined to a period of 72 h since a blood meal was taken. Neither a behavioural nor an electrophysiological study has investigated host-seeking behaviour later than 72 h after a blood meal. We show here that 92 – 96 h after a blood meal the C-neuron in mosquitoes that had oviposited becomes even more sensitive to host-seeking compounds compared to mosquitoes of similar age that never had a blood meal. It would be interesting and relevant to investigate whether mosquitoes become better capable of localising humans after they have completed a complete gonadotrophic cycle. If such effects are present it could be exploited by equipping odour baited traps with odours that elicit higher electrophysiological responses after oviposition. Because mosquitoes need to take a blood meal to become infected with the malaria parasite one would be able to target that part of the mosquito population that can actually transmit malaria, while keeping the chance for resistance by behavioural adaptation very low.

The inhibition of responses to CO_2 by compounds involved in host seeking of *An. gambiae* is also an intriguing finding. It is well known that mosquito species use CO_2 as a long range host cue (Gillies and Wilkes 1968, Takken and Kline 1989, Kline *et al.* 1991b, Kline *et al.* 1991a, Takken 1991, Takken and Knols 1999). However, because *An. gambiae* prefers to bite human feet (de Jong and Knols 1995a, 1996), at one point they need to decide to stop following the CO_2 plume coming from the mouth of a human and direct themselves to their feet. The combined activity of the excitatory responses from both B- and C-neurons together with the inhibitory action on the CO_2 neuron caused by odorants produced by human foot bacteria might play a role in diverting from the CO_2 plume to the preferred biting site.

It would therefore be very interesting to see if these five compounds can alter the behavioural response to an attractive blend combined with CO_2. The first behavioural pilot experiments using a no-choice olfactometer bioassay on two of the five compounds capable of inhibiting the CO_2 response electrophysiologically show promising results (G. Bukovinszkine-Kiss,

pers. comm.). Female *An. gambiae* were attracted to a standard attractive blend containing ammonia, lactic acid and tetradecanoic acid. The addition of CO_2 to this blend increased its attractiveness. Addition of either of both tested bacterial compounds (at 1% concentration) to this attractive blend with CO_2 resulted in suppression of the increase normally seen when CO_2 is added. However the addition of these bacterial volatiles also increased the attractiveness of the standard blend. These are the first indications that, at close-range, human-microbiota-derived are capable of suppression the CO_2 response mosquitoes use to locate humans. Compounds that block the response to CO_2 might represent a new group of odorants capable of disrupting mosquito host-seeking behaviour.

Conclusion

The present study shows that all three ORNs of capitate peg sensilla on the maxillary palps of *An. gambiae* are responding to host-derived cues. Ten odorants emanating from the microbiota on human feet were shown to evoke electrophysiological response from the three ORNs associated with the capitate peg sensilla of the maxillary palp. One neuron was found to express consistently lower responses after a blood meal to most of these bacterially-produced odorants. Five of these compounds caused a significant inhibition of the CO_2-sensitive neuron, A-neuron, associated with the palpal capitate peg sensilla. Furthermore we report an increased sensitivity to host-derived compounds in mosquitoes after oviposition compared to the baseline level of olfactory-induced responses established for non-blood fed mosquitoes. The results suggest that the three neurons studied may mediate host-seeking and oviposition behaviour and are very promising for odour-based strategies against the African malaria mosquito, *Anopheles gambiae* Giles *sensu stricto*.

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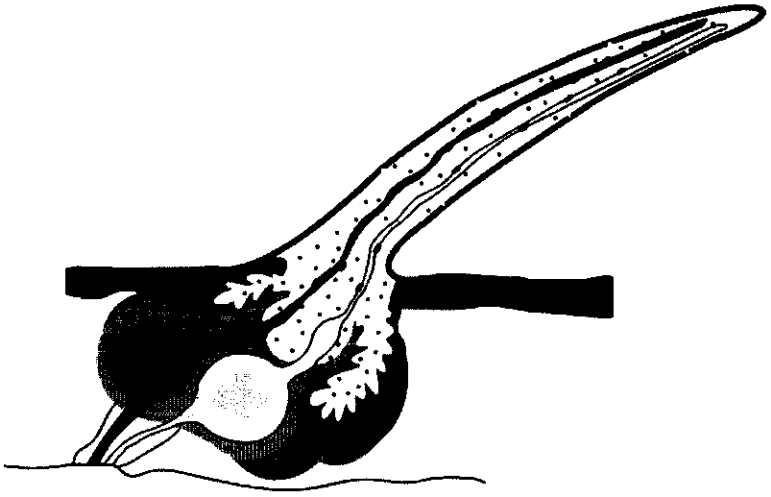

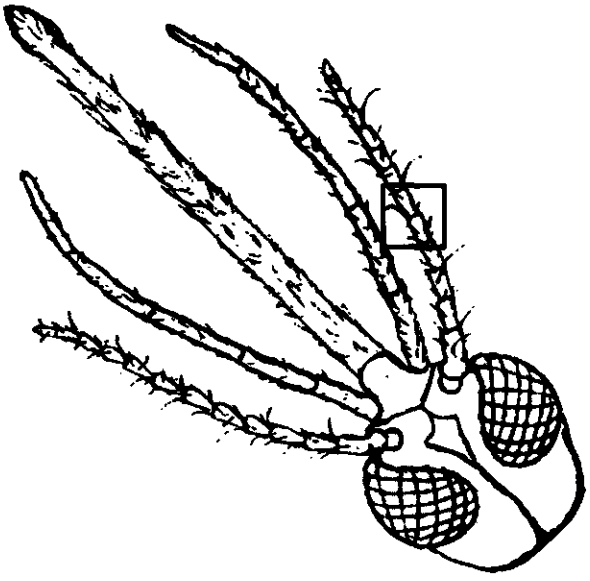

Chapter 5

Behavioural consequences of extensively sustained olfactory receptor neuron responses

Remco A. Suer, Yu Tong Qiu and Joop J.A. van Loon

The African malaria mosquito *Anopheles gambiae* Giles *sensu stricto* localises its blood hosts primarily through olfactory cues. Several studies have shown that human-derived volatile organic compounds elicit electrophysiological responses from the olfactory receptor neurons (ORNs) associated with several types of sensilla present on the antennae and maxillary palps of female mosquitoes. The temporal properties of these ORN responses have been hypothesised to mediate information on odour characteristics. Various temporal ORN activity patterns have been reported but whether these affect behavioural responses has not been addressed. In the current study we describe a novel temporal phenomenon of an unusually prolonged excitatory response of a single ORN associated with the short sharp-tipped trichoid sensillum type E of the antenna of female *An. gambiae*, towards two human-derived odours, i.e. linalool oxide and 2-phenylethanol. A single odour stimulus of two seconds with either compound elicited prolonged excitatory activity from this ORN for more than 10 minutes. We subsequently linked these extensively sustained responses (ESRs) to behavioural changes by using a novel bioassay for recording mosquito behaviour in detail. A pre-treatment of two seconds with these ESR-evoking odours resulted in a clear difference in time allocation to flight behaviour and time spent on behaviours recorded on the odour source in the subsequent five minutes. In contrast, pre-treatment to benzaldehyde, a compound structurally similar to 2-phenylethanol but not evoking ESRs, did not result in these behavioural changes. This is the first report that documents a direct link between temporal characteristics of electrophysiological activity of an insect ORN and ensuing changes in elements of host-seeking behaviour. These findings highlight the importance of temporal features of chemosensory activity in modulating behaviour.

Introduction

Mosquitoes primarily use olfactory cues to find sugar meals, mates, blood hosts and oviposition sites (Takken 1991, Takken and Knols 1999, Zwiebel and Takken 2004). Behavioural evidence suggests that mosquitoes use a blend of odours rather than any single compound during their search for hosts and oviposition sites (Cork and Park 1996, Blackwell and Johnson 2000, Smallegange *et al.* 2005b, Okumu *et al.* 2010, Smallegange *et al.* 2010). Mosquito olfaction has, therefore, received increased interest in the last decade in order to obtain insight in olfactory coding mechanisms.

At the periphery, the olfactory system already shows a great variety of coding possibilities with many different kinds of receptor neurons, associated with different types of sensilla that are distributed on different olfactory organs. In the malaria mosquito *Anopheles gambiae s.s.*, 79 putative olfactory receptors (AgORs) have been discovered (Hill *et al.* 2002). In *Drosophila melanogaster,* 57 ORs were identified (Vosshall *et al.* 1999, Vosshall *et al.* 2000) and similar studies on *Aedes aegypti* revealed 134 putative ORs (Bohbot *et al.* 2007). Recently, a new class of olfactory receptors, ionotropic receptors (IRs), has been described (Benton *et al.* 2009). Morphological and electrophysiological studies have shown that ORs are expressed in different types of sensilla on different appendages (Shanbhag *et al.* 1999, 2000, Pitts *et al.* 2004, Kwon *et al.* 2006, Qiu *et al.* 2006, Ghaninia *et al.* 2007b, Lu *et al.* 2007, Hill *et al.* 2009). These olfactory receptors can have different odour molecules as ligands and one odour can be a ligand of multiple receptors (Vosshall *et al.* 2000, Qiu *et al.* 2006, Ghaninia *et al.* 2007b, Syed and Leal 2007, Hill *et al.* 2009, Carey *et al.* 2010). Multiple ORs, each with a different odour tuning profile, can be expressed in the neurons associated with one sensillum and some examples exist that one neuron can express two ORs (Goldman *et al.* 2005, Lu *et al.* 2007).

Temporal characteristics of olfactory neuron activity provide the peripheral nervous system with an extra dimension in odour coding (Yao *et al.* 2005, Carey *et al.* 2010). Until very recently it was assumed that peripheral neurons only evoke firing patterns that show little temporal variation and that the complex temporal patterns measured in the antennal lobe (AL) of various insects originated from the projection neurons and local neurons in the AL (Wehr and Laurent 1999, Bazhenov *et al.* 2001a, Bazhenov *et al.* 2001b, Laurent 2002). However, recently it was shown that the temporal characteristics of ORN activity underlie the temporal characteristics of neural coding of odours measured in the AL (Raman *et al.* 2010). Recordings from locust antennae revealed that information on identity, concentration and duration of odours was encoded in the varying temporal properties of the odour-elicited firing patterns of the peripheral ORNs.

Varying temporal properties of ORN responses have been noted in several electrophysiological studies in which it was suggested that these properties might provide valuable information on odour characteristics to insects (Bowen *et al.* 1994, Qiu *et al.* 2004, Hallem *et al.* 2006, Olsson

et al. 2006, Qiu *et al.* 2006, Syed 2006, Ghaninia *et al.* 2007b, Lu *et al.* 2007, Syed and Leal 2007, Hill *et al.* 2009, Raman *et al.* 2010). Most excitatory responses of ORNs are either phasic, tonic or phasic-tonic responses. A single odour can evoke different temporal responses depending on the receptor it activates. Phasic responses have been thought to inform insects about odour concentrations and rapid changes in concentrations encountered when crossing the filamentous odour plume (Almaas *et al.* 1991, den Otter and van der Goes van Naters 1992, de Bruyne *et al.* 2001, Olsson *et al.* 2006). Tonic responses have been proposed as signals to sustain upwind flight when loosing contact with an odour plume (Almaas *et al.* 1991, den Otter and van der Goes van Naters 1992, de Bruyne *et al.* 2001). Next to phasic and tonic response types, several other temporal phenomena have been reported. Some electrophysiological studies reported an intermediate type named phasic-tonic response (Meijerink 1999, Qiu *et al.* 2006, Ghaninia *et al.* 2007b, Hill *et al.* 2009). Other authors made a further separation into 'moderate phasic' and/or 'moderate tonic' responses (Olsson *et al.* 2006). Two studies also reported the existence of a post-stimulus inhibition (Qiu *et al.* 2006, Hill *et al.* 2009), which might provide an extra identifying property of the particular odour to the central nervous system.

Another phenomenon that has been reported for several ORs is a prolonged tonic response of a receptor to an odour. The duration of these responses greatly exceeds that of the stimulus, mostly by a few seconds (Olsson *et al.* 2006, Ghaninia *et al.* 2007b, Hill *et al.* 2009, Carey *et al.* 2010, Raman *et al.* 2010) but more than one minute has also been reported (den Otter and van der Goes van Naters 1992). Until now the behavioural effect of the various temporal ORN activity patterns has not been addressed.

During the continuation of the characterisation of trichoid sensilla type E of *An. gambiae*, it was found that one previously identified response type TSE I, reacted to linalool oxide and 2-phenylethanol with unusually prolonged excitatory responses far exceeding a minute. Such a temporal pattern has not been encountered before during the characterisation of olfactory sensilla in *A. gambiae*. Though these two compounds elicit responses from various ORNs (Chapter 2 and Y.T. Qiu, unpubl. data), such a prolonged excitatory response was only recorded from this neuron type. Multiple odorants elicit responses from this ORN. However, only these two odorants, linalool oxide and 2-phenylethanol, that have previously been found in human emanations (Bernier *et al.* 2000, Meijerink *et al.* 2000, Curran *et al.* 2007, Penn *et al.* 2007, Gallagher *et al.* 2008), elicit such a prolonged excitatory responses from this ORN.

The present study combines a detailed electrophysiological and behavioural analysis to investigate the effect of these extensively prolonged excitatory responses of a single olfactory receptor of trichoid sensillum type E (TSE) of female *Anopheles gambiae s.s.* to two human-derived odours, i.e. linalool oxide and 2-phenylethanol.

Materials and Methods

Insect rearing

A colony of *Anopheles gambiae* Giles *sensu stricto* (hereafter termed *An. gambiae*), originating from Suakoko, Liberia (courtesy Prof. M. Coluzzi), has been cultured at Wageningen University, The Netherlands, since 1988. The mosquito colony was kept at standard insectary conditions of 27 ± 1°C, 80 ± 5% RH and at an adjusted 12:12 day-night rhythm, with artificial sunrise and sunset. Adults were kept at 30×30×30 cm^3 gauzed cages with access to a 6% (w/v) glucose solution on filter paper. Female mosquitoes received blood meals from a human arm twice a week. Eggs were laid on damp filter paper that was placed in plastic cups with tap water. Larvae were fed daily with Tetramin® baby fish food (Melle, Germany). Pupae were collected daily and allowed to emerge in the aforementioned cages.

For experiments 6-8 d old females that had not had a blood meal were used. Females that were attracted to a hand held next to the cage were selected to ensure the collection of responsive mosquitoes. Females were collected by means of an aspirator and placed in a small 5 ml pipette-tip covered by 1x1 mm mesh gauze until the experiment.

Electrophysiology

The preparation and single sensillum recording procedures were the same as described in Chapter 2. For this study recordings were made from trichoid sensilla type E (TSE), which are the shortest (16.9±1.4 µm) trichoid sensilla with a sharp tip (Boo 1980, Qiu *et al*, 2006). TSE normally contains two ORNs. The presence of a third ORN was sometimes suspected but could not be proven with certainty. Normally these ORNs are distinguished based on their spike shape and amplitude and spike doublet. The consensus is that the ORN with the largest spike amplitude is called the A-neuron, the smaller one the B-neuron. However the amplitudes of the two olfactory receptor neurons (ORNs) associated with TSE are very similar. So similar that depending on the mosquito recorded from one could assign either A or B to this ORN. Therefore we refrain from giving the label A-neuron or B-neuron to this ORN innervating the TSE. It was however always clear that there was only one neuron that responded to all three stimulants used.

Stimulus and stimulus delivery

Three different odorants were used as stimuli: linalool oxide, 2-phenylethanol and benzaldehyde. Linalool oxide and 2-phenylethanol were chosen because they were the only ones from a panel of 132 compounds (see Chapter 2) that elicited these unusually prolonged excitatory responses. Benzaldehyde was chosen because it elicits excitatory responses from the same neuron but never an prolonged excitatory responses. The highest purity grades of the three compounds commercially available were used (Appendix 2). All odorants were diluted in

paraffin oil and five different concentrations (w/w) of each compound were used: 0.001%, 0.01%, 0.1%, 1% and 10%. Of each solution, 10 µl was applied to a strip of filter paper (0.5 x 1.5 cm) inserted into a Pasteur pipette. These odour cartridges were closed with a 1 ml pipette-tip wrapped with Parafilm® and kept at -25°C. Each cartridge was used for a maximum of 10 experimental mosquitoes.

A constant, charcoal filtered and humidified airstream of 0.5 l/min was delivered to the mosquito antenna via a glass tube. The vapour of test compounds in odour cartridges was injected into the airstream at 0.5 l/min by using a stimulus controller (CS-55, Syntech, Kirchzarten, Germany). A synchronised compensation airstream generated by the stimulus controller was injected into the main airstream in order to keep the total airflow constant. Stimulus duration was 200 ms for the dose-response relationships. In order to study the recovery time of the extensively sustained response (hereafter ESR) back to the spontaneous firing rate, recordings of 30 min were made after a 2 s stimulus of a 1% solution of the odorants.

Analysis

Single sensillum recordings were automatically filtered to increase signal-to-noise ratio using Autospike software (filter settings: 20 - 1694 Hz) and analysed in combination with Automacrorecorder software (ReadmeSoft, http://www.readmesoft.com). Though all recordings showed the activity of maximally two neurons, we only focussed on the neuron that exhibited the ESR. For each recording the effect of each odour was quantified by the change in action potential firing frequency, which was calculated as the number of action potentials during the 500 ms after stimulus onset minus the average of the firing frequency during four intervals of 500 ms preceding the onset of the stimulus. To determine when the firing frequency returned to spontaneous activity level, 500 ms intervals were analysed after every 150 s and compared to the spontaneous activity. The time it took for the injected air volume to pass down the glass tube to reach the antenna was calculated, and counting started at the time point determined by adding this delay to the onset of stimulus delivery.

Behaviour

Bioassay

Behavioural experiments were conducted in a newly developed bioassay setup (Fig. 1). This bioassay consisted of a transparent polymethylmethacrylate (PMMA) cylinder of 49 cm long with an inner diameter of 14.5 cm covered at the ends with metal gauze (mesh size 1x1 mm). At one end of the cylinder a moistened and heated (26±2 °C) airstream was fed into the cylinder via a glass funnel. The funnel also contained a small PMMA cylinder (inner diameter 7.5 cm) that was placed in the middle of the funnel through iron wiring and this small cylinder was pressed against the gauze on that side. At the other end of the cylinder a funnel connected to the laboratory vacuum system was placed to exhaust odours released into the cylinder. As an

attractive odour source a black nylon sock worn for 1 day by a human volunteer was placed in the small cylinder touching the gauze only at the inner circle. The sock was expected to stimulate the mosquito to fly upwind (Fig. 1), because it was previously shown that human odour collected on socks is highly attractive to *An. gambiae* (Pates *et al.* 2001). Mosquitoes were tested from 9:00 till 12:00 am during the last hours of the dark period. The actual test was performed in the dark under red light.

Stimulus and stimulus delivery

Prior to the behavioural experiment, a pre-treatment with the three above-mentioned odours (see stimulus delivery) and paraffin oil was conducted. As a control, paraffin oil, the solvent for the three compounds, was used. Benzaldehyde represented a positive control as an odorant that elicits a response from the same neuron as linalool oxide and 2-phenylethanol but does not elicit an extensively sustained response.

Odour cartridges with 1% concentration of the three odorants were made using the same method as described above for electrophysiology. Odour cartridges were randomly labelled by an independent person in order to keep the observer uninformed as to which odours were being tested.

The same odorant delivery method as used for the electrophysiological study was used to expose the mosquitoes to the three test odours while the mosquitoes were in the 5 ml pipette tips. A constant, charcoal-filtered and humidified airstream of 0.5 l/min was delivered to the mosquito via a glass tube. Vapour of the test compounds in the odour cartridges was injected into the airstream at 0.5 l/min using a stimulus controller (CS-55, Syntech, Kirchzarten, Germany). A compensation airstream applied by the stimulus controller simultaneously was also injected into the main airstream in order to keep the total airflow constant. Stimulus duration was set at 2 s during which the antennae of the mosquito are exposed to the odour.

Experimental protocol

Immediately after the odour exposure the gauze was removed and the pipette-tip was placed at the release hole of the behavioural bioassay setup. To let the mosquito enter the cylinder from the pipette-tip a gentle tap against the tip caused the mosquito to fly and to enter the bioassay cylinder. From the moment of entering the bioassay, the behaviour of the mosquito was scored using a PSION handheld computer equipped with the observational software package The Observer (Noldus Information Technology, Wageningen, The Netherlands). An ethogram consisting of 18 behavioural elements (Table 1) was used to describe mosquito behaviour for 5 min. The behaviours of a single mosquito were continuously recorded during the observation of 5 minutes (Focal sampling, continuous recording). Both frequency as well as duration of behavioural elements was recorded. After 5 minutes of observation the mosquito was removed from the behavioural setup. A maximum number of 15 mosquitoes was studied per pre-treatment.

Statistical Analysis

Statistical analysis of both electrophysiological and behavioural data was done using Genstat software (Genstat 14, VSN International Ltd, UK). For determination of the plateau value, the three highest concentrations were compared using a Generalized Linear Model with a Poisson distribution and a logarithmic link function. If a concentration effect was observed, two-sided t-probabilities were calculated to test pairwise differences between the means. Effects were considered to be significant at $P < 0.05$.

For behavioural data, observational data were uploaded from The Observer to Excel (Microsoft Office) and corrected for the time a mosquito was scored 'out of sight'. 'Out of sight' was recorded in less than 10% of the experimental runs and in each of those runs on average for less than 15% of the total observation time. This time was subtracted from the total observation time. Durations of behaviours were converted to percentage of total observation time, corrected for 'out of sight'. Differences between the four pre-treatments, three odorants and paraffin oil, in frequencies of behavioural elements were tested using a Generalized Linear Model (Poisson distribution, log function). Pre-treatment and day were modelled as factors. If a day effect for a behavioural element was found (GLM, $P < 0.05$) this was implemented in the model. If either day and pre-treatment or pre-treatment alone (when no day effect was present for the particular element) were significant (GLM, $P < 0.05$), two-sided t-probabilities were calculated to test pairwise differences between the means. Effects were considered to be significant at $P < 0.05$.

The analysis of the duration of behavioural elements was done similarly as described above for frequencies using a GLM but the Distribution was changed to a binomial distribution with a logit link function in the GLM.

Table 1: Ethogram of female *Anopheles gambiae* behaviour. This table presents all behavioural elements in the ethogram and their definitions. Every observed combination of location and behavioural element was treated as a separate behavioural element and scored accordingly. Figure 1 specifies the locations for all behaviours.

Behavioural element	**Definition**	**Location**			
Landing (l)	Touching a specific location with all legs after a flight or first time touching a different location with all legs during walking from location to location	Inner circle (li)	Outer circle (lo)	Downwind circle (ld)	Cylinder (lc)
Resting (r)	Staying immobile for more than 5 s	Inner circle (ri)	Outer circle (ro)	Downwind circle (rd)	Cylinder (rc)
Walking (w)	Covering a distance of more than 1.5 cm per 2 s	Inner circle (wi)	Outer circle (wo)	Downwind circle (wd)	Cylinder (wc)
Proboscis contact (p)	Bringing proboscis in contact with and piercing the gauze, directing maxillary palps upward while moving abdomen up and down, head directed downwards	Inner circle (pi)	Outer circle (po)	Downwind circle (pd)	
Flying (fl)	Moving while airborne				
Cleaning legs (cl)	Resting on a surface and preening the legs against each other				
Out of sight	Scored when location of mosquito could not be determined				

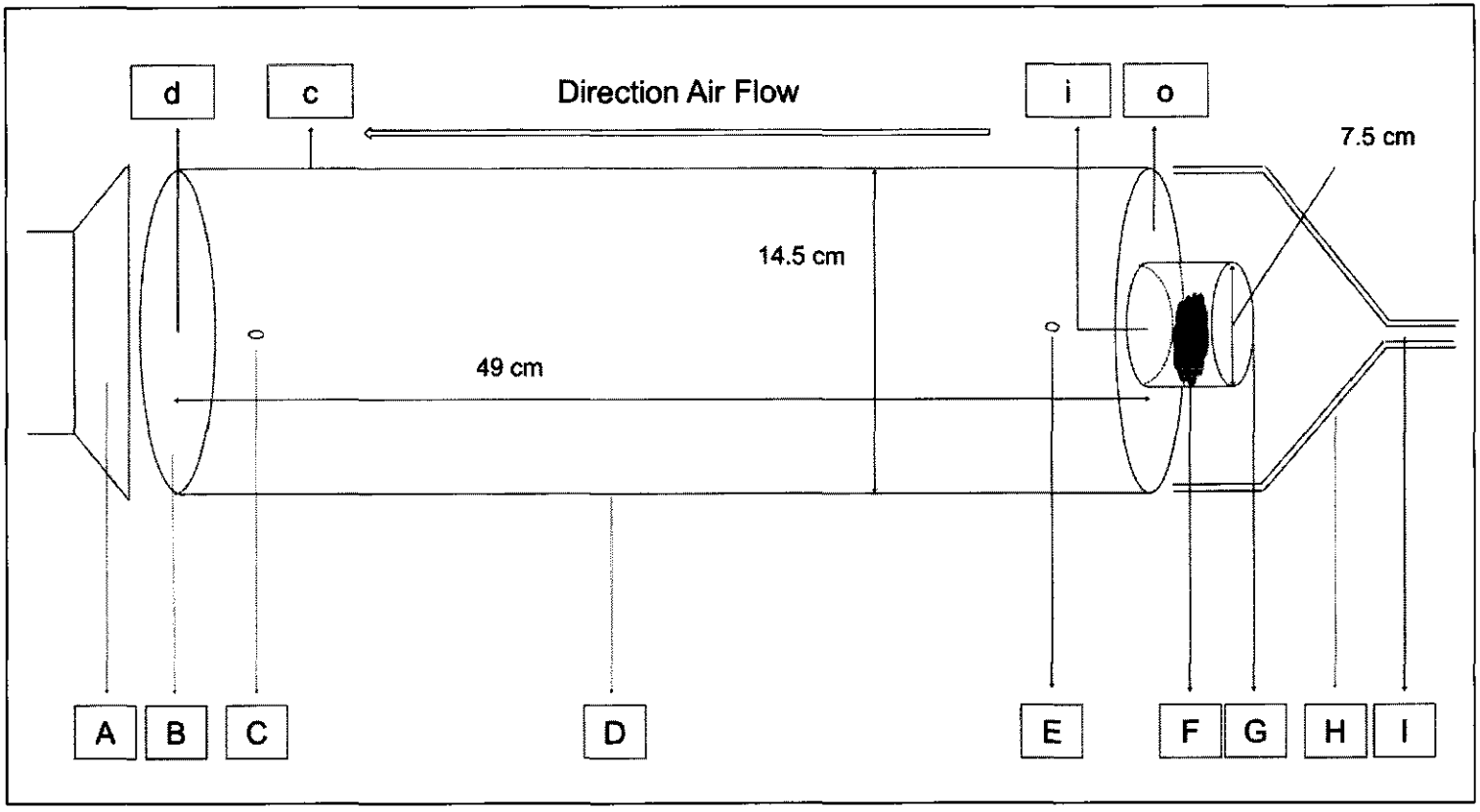

Figure 1: Schematic representation of behavioural bioassay setup that was used to record the behaviour of mosquitoes after a pre-treatment. The lower case letters depict the four locations at which behavioural elements have been scored. d: circle at the downwind side, c: cylinder, i: inner circle on the upwind side and o: outer circle on the upwind side. The capital letters show the different components of the setup. A: Vacuum funnel suction apparatus, B: Metal gauze covered side of the cylinder, same for the other side of the cylinder, C: small opening for releasing mosquitoes, D: transparent PMMA cylinder, E: small exit hole for retrieving the mosquito after the experiment, F: a worn sock as attractive odour source, G: small PMMA cylinder for holding the odour source, H: Funnel and I: tube delivering the moistened and heated

Results

Electrophysiology

Dose-response relations

One of the olfactory receptor neurons innervating the trichoid sensilla type E (TSE) responded to linalool oxide and 2-phenylethanol by strong excitation that lasted far longer than the stimulus duration (Figs. 2 and 3). In contrast, compared to the responses of the other 2 odorants, the response of this olfactory receptor neuron (ORN) to benzaldehyde was several times shorter (Fig. 3). This ORN exhibited a higher sensitivity to linalool oxide and 2-phenylethanol than to benzaldehyde (Fig. 2). At 0.001% the firing rates in response to both 2-phenylethanol and linalool oxide are significantly higher than the spontaneous activity (t-test: P = 0.029 and P = 0.037, respectively) but not for benzaldehyde (P = 0.108). At a 10-fold higher concentration, i.e. 0.01%, benzaldehyde also elicited a significant increase in firing rate compared to the spontaneous firing level (t-test: P < 0.001).

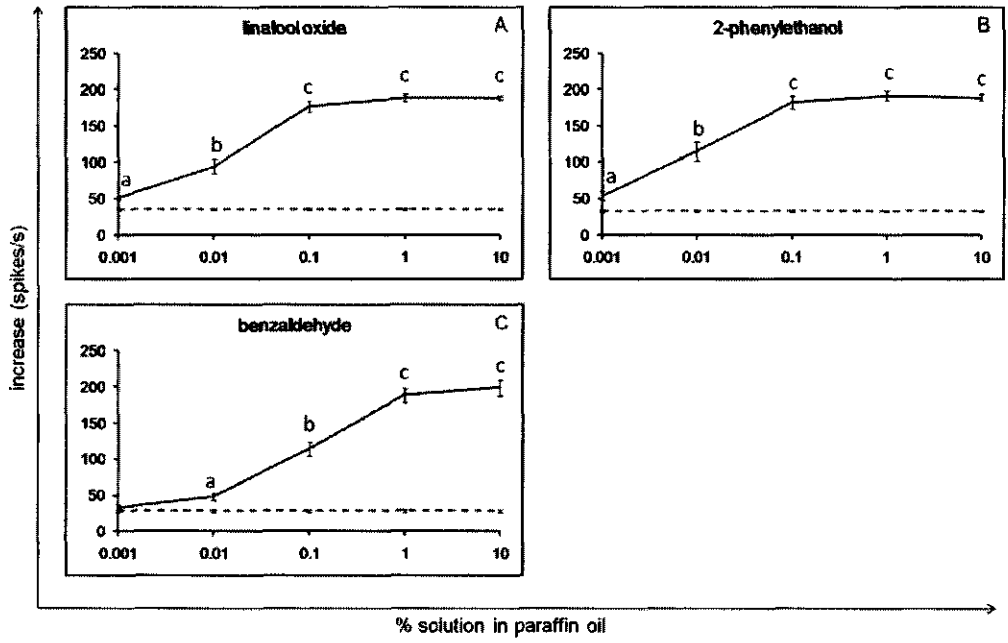

Figure 2: Dose-response relations for the response of a trichoid sensillum E (TSE) ORN to three odorants. X–axis: concentration of the odorant in paraffin oil. Y-axis: increase in firing frequency in spikes/s in relation to the spontaneous activity level. The dashed line signifies the spontaneous activity level of that neuron. **A)** dose-response relation for linalool oxide (n=5); **B)** dose-response relation for 2-phenylethanol (n=5) and **C)** dose-response relation for benzaldehyde (n=7, error bars represent standard error of the mean). Different letters represent significantly different means (P < 0.05). No letters mean no significant differences. The lowest concentration of 0.001% is tested against the spontaneous activity level.

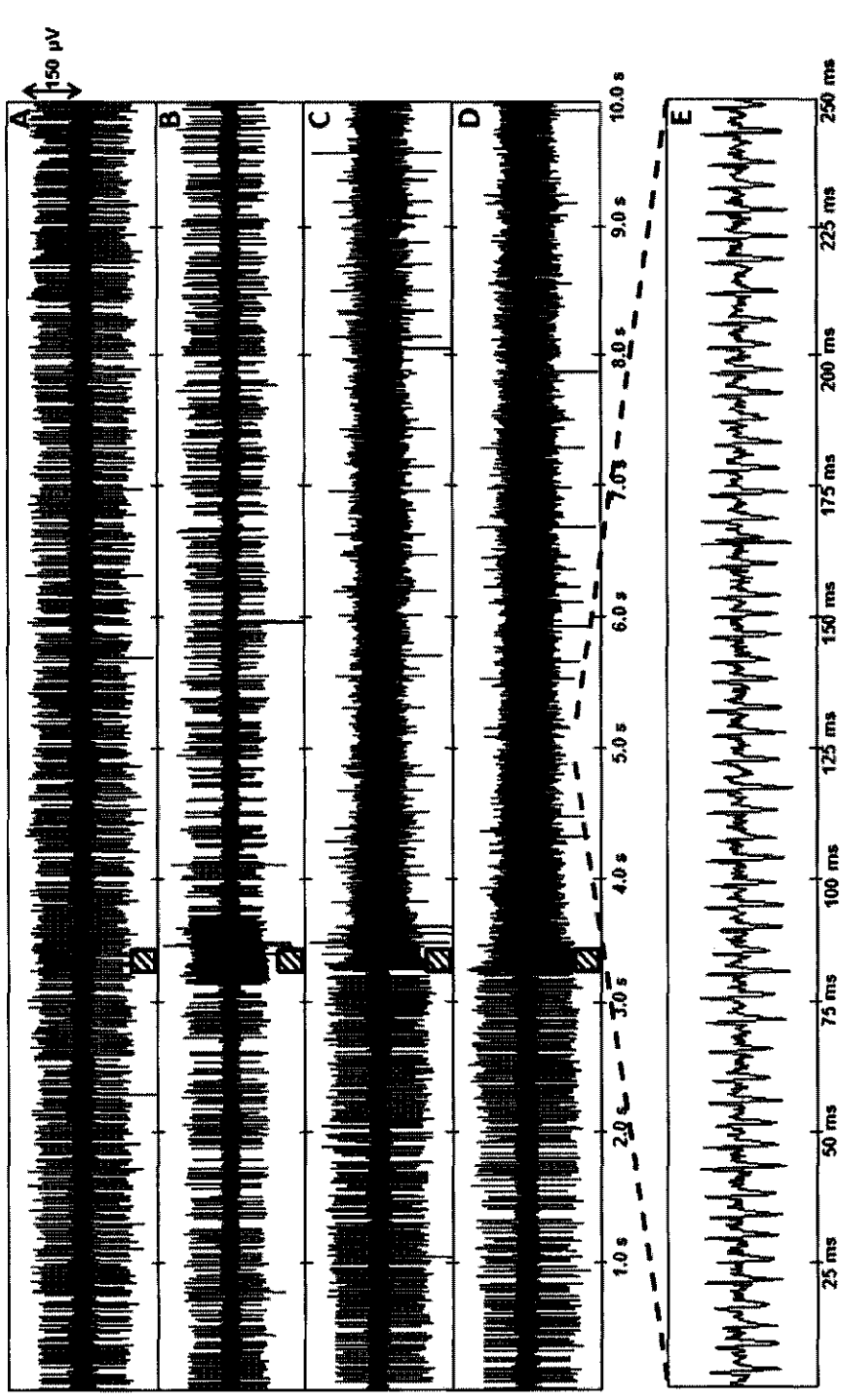

Figure 3: Representative exemplary recordings of 10 s of electrophysiological activity in a trichoid sensillum type E during the dose-response relation measurements. The horizontally striped bars indicate the stimulus of 200 ms. **A)** response to the solvent paraffin oil. **B)** response to a 1% solution of benzaldehyde. **C)** response to 1% linalool oxide. **D)** response to 1% 2-phenylethanol. **E)** Higher time resolution (500 ms in total) for the response to 1% 2-phenylethanol.

All three dose-response curves reached the same plateau value of approximately 200 spikes/s (GLM; $P = 0.581$). For both linalool oxide and 2-phenylethanol this plateau is reached at 0.1%. There was no significant difference in response intensity between the three highest concentrations for both linalool oxide and 2-phenylethanol (GLM; $P = 0.273$ and $P = 0.598$ respectively). For benzaldehyde, significant differences in the responses to the 0.1%, 1% and 10% concentrations of this compound were recorded (GLM; $P < 0.001$). The response of TSE to 0.1% benzaldehyde was significantly lower than the response to 1% (GLM; $P < 0.001$) whereas the responses to 1% and 10% did not differ from each other (GLM; $P < 0.506$).

Recovery time

Recovery times were measured after a 2 s-stimulation of a 1% solution of the three compounds. Clear differences in recovery time were found between the two odorants causing ESRs (linalool oxide and 2-phenylethanol) and benzaldehyde (Fig. 4).

Both linalool oxide and 2-phenylethanol elicited an elevated firing level compared to the spontaneous activity 600 s after stimulus onset (t-test: $P = 0.033$ and $P = 0.024$ respectively). The response to 2-phenylethanol was still sustained 20 minutes after stimulus onset (t-test: $P = 0.048$).

Benzaldehyde caused strong responses lasting longer than the 2 s-stimulus duration. However, 1 s after the end of the stimulus, the firing frequency had returned to the spontaneous activity level (t-test: $P = 0.135$) (Fig. 4B). It also seemed that a form of adaptation takes place when stimulating with benzaldehyde; 1 s after the stimulus onset, which was half way the stimulus duration, the firing rate was significantly lower than at the onset (t-test: $P = 0.006$). The TSE-ORN did not show adaptation during the stimulation with both linalool oxide and 2-phenylethanol (t-test: $P < 0.001$).

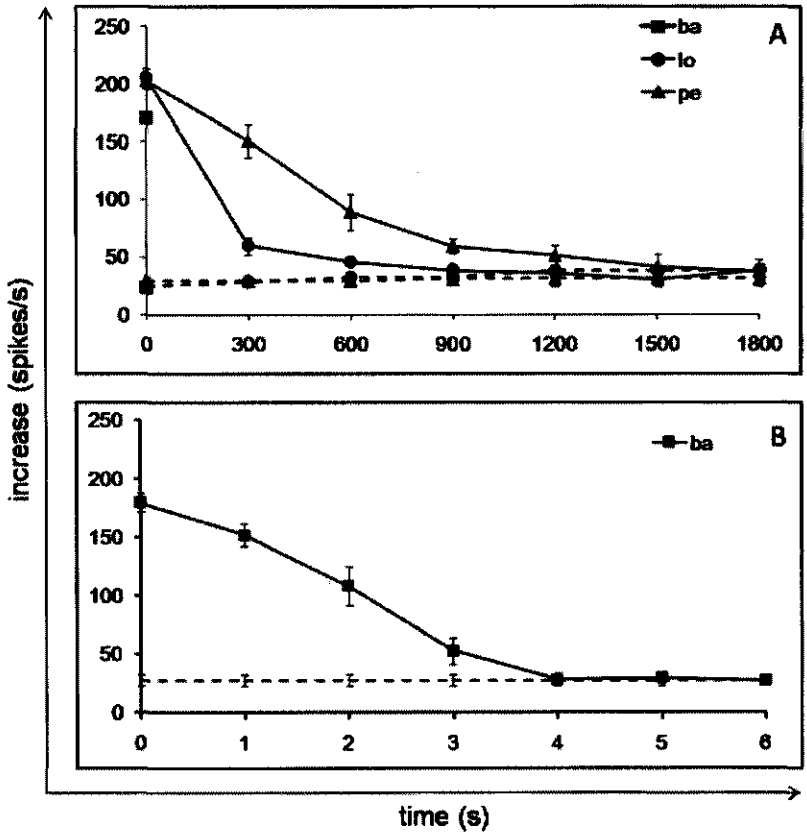

Figure 4: Recovery time for the ORN innervating the trichoid sensilla type E to return to spontaneous activity levels after a 2 s stimulus. X-axis: time (s) since the start of stimulus application. Y-axis: increase in firing frequency in spikes/s in relation to the spontaneous activity level. Continuous lines: response to 1% concentration of the stimuli; dashed lines: spontaneous activity levels; these values overlap to a large extent. **A)** recovery time for stimulation with 1% for all three odorants; ba: benzaldehyde, pe: 2-phenylethanol, lo: linalool oxide. **B)** higher time resolution for the response towards 1% benzaldehyde. Error bars represent standard error of the mean, n=5 for all data points.

Behaviour

Latency of behavioural elements

None of the latencies of the behaviours on the inner and outer circle, defined as the time from the start of the observation until that behaviour was first recorded, differed significantly as a result of pre-treatment with any of the three different compounds (GLM, $P > 0.1$).

Frequency of behavioural elements

Per odour pre-treatment the number of times a specific behaviour occurred was recorded. Only 'flying' (Fig. 5C) and 'resting on the inner circle' (Fig. 5B) differed significantly among the four pre-treatments (GLM, $P = 0.021$ and $P = 0.035$ respectively). For all other behaviours no significant effects of the pre-treatment were found.

The frequency of 'flying' for benzaldehyde-treated mosquitoes was not significantly different from that of control mosquitoes (pre-treated with solvent only; paraffin oil); whereas the frequency of 'flying' for linalool oxide and 2-phenylethanol-treated mosquitoes differed significantly from mosquitoes that had experienced solvent only (GLM, $P = 0.023$ and $P = 0.009$ respectively; Fig. 5C).

The frequency of 'resting on the inner circle' for mosquitoes that had experienced a pre-treatment with linalool oxide was significantly lower than for the mosquitoes that had experienced solvent only (GLM, $P = 0.011$; Fig. 5B). No differences among the other pre-treatments were found for the frequency of this behaviour (Fig. 5B).

Duration

Several behaviours were also compared for the average proportion of time a female mosquito spent on that specific behaviour over the total observation time. This comparison showed clear differences in the time spent on 'proboscis contact on the inner circle' between mosquitoes pre-treated with linalool oxide or 2-phenylethanol and mosquitoes that had experienced benzaldehyde or solvent only (GLM, $P < 0.001$; Fig. 6B). Mosquitoes that experienced a pre-treatment with linalool oxide and 2-phenylethanol spent significantly more time on 'proboscis contact' than the solvent only-treated mosquitoes (GLM, $P < 0.001$ and $P = 0.001$ respectively). No significant difference was found between the mosquitoes receiving the solvent only pre-treatment or the pre-treatment with benzaldehyde (Fig. 6B). All mosquitoes spent more time (20-50% of the observation time) on 'proboscis contact on the inner circle' and 'flying' (Figs. 6B and 6C). Less than 15% of the time was spent on any of the other behaviours (Fig. 6).

Similar differences between ESR eliciting compounds and the other pre-treatments were found when comparing the time spent on 'walking on the inner circle' (GLM, $P = 0.002$). Mosquitoes pre-treated with linalool oxide or 2-phenylethanol spent significantly less time 'walking on the inner circle' than the mosquitoes that had experienced solvent only (GLM, P =

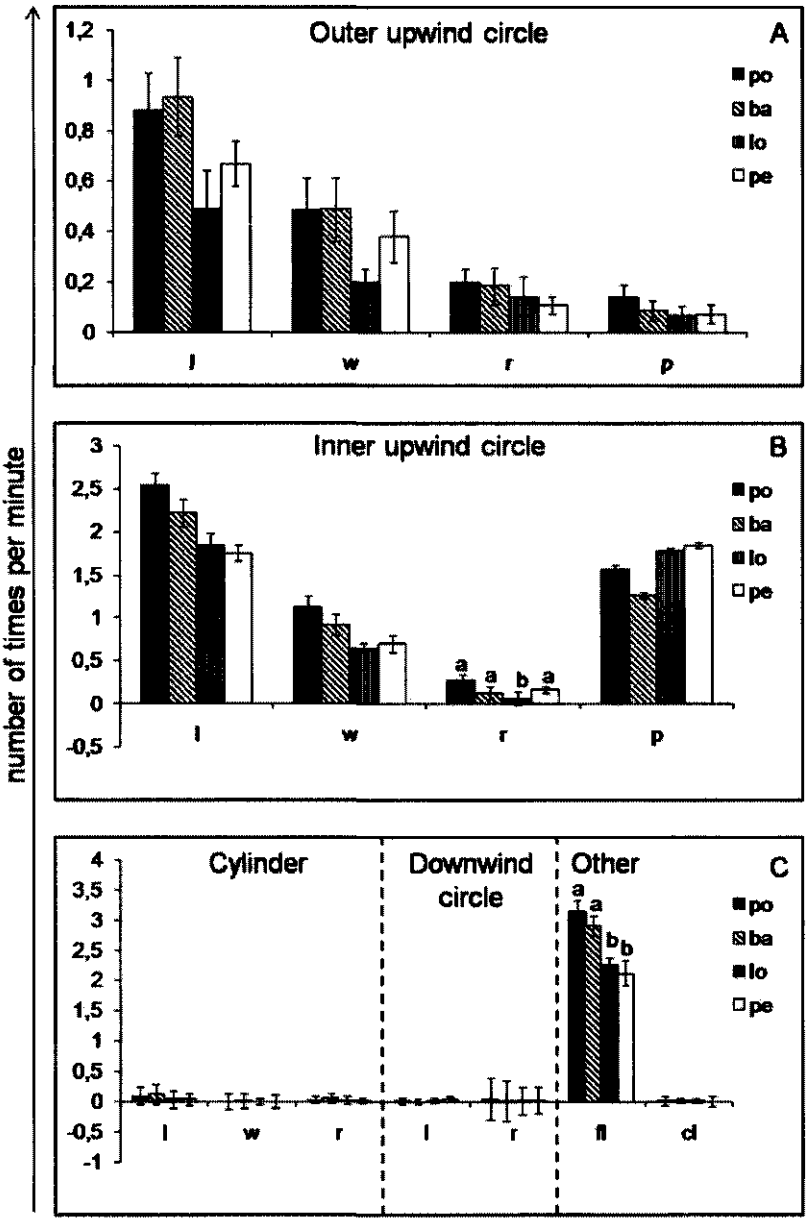

Figure 5: The frequency of behavioural elements for each pre-treated group of mosquitoes. X-axis: the different behavioural elements that were recorded; l: landing, w, walking, r: resting, p: proboscis contact, fl: flying and cl: cleaning legs. Y-axis: number of times a behavioural element was scored per minute of observation. Pre-treatments; po: paraffin oil, ba: benzaldehyde, pe: 2-phenylethanol, lo: linalool oxide. **A)** number of times behavioural elements occurred on the outer circle. **B)** number of times behavioural elements occurred on the inner circle. **C)** number of times all other recorded behavioural elements occurred on either the cylinder or downwind circle except for flying and cleaning legs. Error bars represent standard error of the mean; n=16. Means with different letters are significantly different (GLM, $P < 0.05$); no letters mean no significant differences among mosquitoes from the different pre-treatments.

0.001 and P = 0.007; Fig. 6B). The difference between benzaldehyde-treated mosquitoes and mosquitoes that had experienced solvent only was close to significance (GLM, P = 0.055; Fig. 6B). A significant pre-treatment effect was found for time spent 'flying' (GLM, P = 0.029; Fig. 6C). The proportion of time spent 'flying' was not significantly different between odour pre-treatments and the solvent control. However, benzaldehyde-treated mosquitoes spent significantly more time 'flying' than mosquitoes that were pre-treated with linalool oxide or 2-phenylethanol (GLM, P = 0.016 and 0.007 respectively).

Behavioural sequences

We only analysed sequence differences for behavioural elements occurring at the inner circle, close to the odour source and focussed on the elements following directly after a specific behavioural element that took place on the inner circle.

After 'landing on the inner circle', the behavioural elements 'walking', 'resting' and 'proboscis contact on the inner circle' or 'flying' were observed. Figure 7 shows that significant alterations in behavioural sequence between mosquitoes exposed to different pre-treatments were seen for 'walking on the inner circle' (GLM, P < 0.001), 'proboscis contact' (GLM, P < 0.001) and 'flying' (GLM, P < 0.001).

For solvent only and benzaldehyde-treated mosquitoes, 'landing on the inner circle' was significantly more often followed by 'walking on the inner circle' and 'flying' compared with mosquitoes pre - treated with linalool oxide and 2-phenylethanol (GLM, P = 0.005 and P = 0.002 respectively). 'Landing on the inner circle' was more often followed by 'flying' for mosquitoes that experienced solvent only or benzaldehyde than for linalool oxide and 2-phenylethanol treated mosquitoes (GLM, P = 0.003 and P = 0.001). Mosquitoes pre-treated with linalool oxide or 2-phenylethanol exhibited significantly more often 'proboscis contact on the inner circle' directly after ' landing on the inner circle' than did benzaldehyde-treated mosquitoes and mosquitoes that had experienced solvent only (GLM, P < 0.001 and P < 0.001 respectively; Fig. 7). Behavioural sequences following 'proboscis contact on the inner circle' did not differ between the four pre-treatments (GLM, P > 0.1; Fig. 8).

The different pre-treatments resulted in different frequencies of subsequently observed behavioural elements following 'walking on the inner circle' (Fig. 9). There was no significant difference in the number of times differently pre-treated mosquitoes walked from the inner circle to the outer circle (landing on the outer circle). No difference was found for the propensity to initiate flight after having walked on the inner circle.

However, the exposure to different pre-treatments resulted in differences in the frequency of occurrence of 'resting on the inner circle' after 'walking on the inner circle' (GLM, P = 0.007). Only for mosquitoes pre-treated with benzaldehyde 'walking on the inner circle' was significantly more followed by 'resting on the inner circle' (GLM, P = 0.002; Fig. 9).

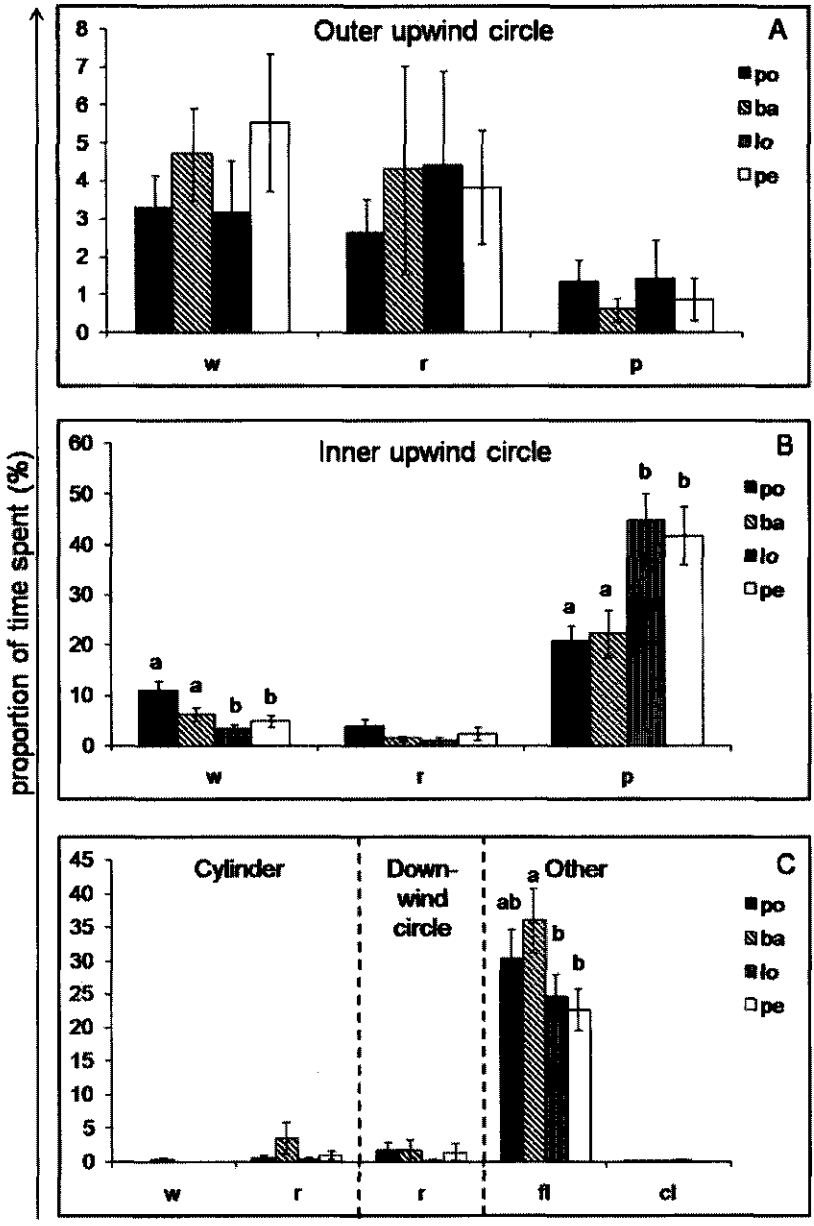

Figure 6: Duration spent on behavioural elements for each pre-treated group of mosquitoes. X-axis: the different behavioural elements that were recorded; l: landing, w, walking, r: resting, p: proboscis contact, fl: flying and cl: cleaning legs. Y-axis: the proportion of time spent on each behavioural element during the 5 min observation. Pre-treatments; po: paraffin oil, ba: benzaldehyde, pe: 2-phenylethanol, lo: linalool oxide. **A)** proportion of time spent on behavioural elements that occurred on the outer circle. **B)** proportion of time spent on behavioural elements that occurred on the inner circle. **C)** proportion of time spent on all other behavioural elements that occurred on the either the cylinder or downwind circle except for flying and cleaning legs. Error bars represent standard error of the mean (n=16). Means with different letters are significantly different (GLM, $P < 0.05$); no letters mean no significant differences among mosquitoes from the different pre-treatments.

A difference close to significance was found for the frequency that mosquitoes showed 'proboscis contact on the inner circle' after 'walking on the inner circle' (GLM, P = 0.057; Fig. 9). The 2-phenylethanol-treated mosquitoes exhibited significantly more 'proboscis contacts on the inner circle' after 'walking on the inner circle' (GLM, P = 0.014). A close to significant difference was found between mosquitoes that had experienced solvent only and linalool oxide-treated mosquitoes (GLM, P = 0.079) for 'proboscis contact on the inner circle' after 'walking on the inner circle' (Fig. 9).

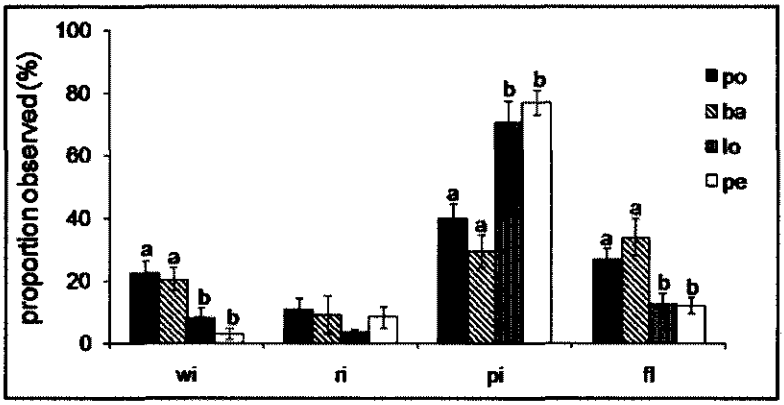

Figure 7: The proportion of times that landing on the inner circle (li) was followed by a specific behavioural element for each pre-treated group of mosquitoes. X-axis: the behavioural elements that followed landing on the inner circle; wi (walking on the inner circle), ri (resting on the inner circle), pi (proboscis contact on the inner circle) or fl (flying). Y-axis: proportion of times li was followed by any behaviour. Pre-treatments; po: paraffin oil, ba: benzaldehyde, pe: 2-phenylethanol, lo: linalool oxide. Error bars represent standard error of the mean (n=16). Means with different letters are significantly different (GLM, P < 0.05); no letters mean no significant differences among mosquitoes from the different pre-treatments.

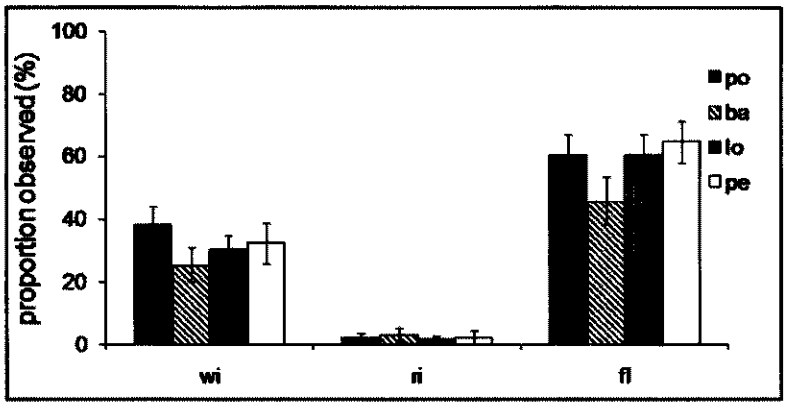

Figure 8: The proportion of times proboscis contact on the inner circle (pi) was followed by a specific behavioural element for each pre-treated group of mosquitoes. X-axis: the behavioural elements that followed proboscis contact on the inner circle; wi (walking on the inner circle), ri (resting on the inner circle), or fl (flying). Y-axis: proportion of times pi was followed by any behaviour. Pre-treatments; po: paraffin oil, ba: benzaldehyde, pe: 2-phenylethanol, lo: linalool oxide. Error bars represent standard error of the mean (n=16). Means with different letters are significantly different (GLM, $P < 0.05$); no letters mean no significant differences among mosquitoes from the different pre-treatments.

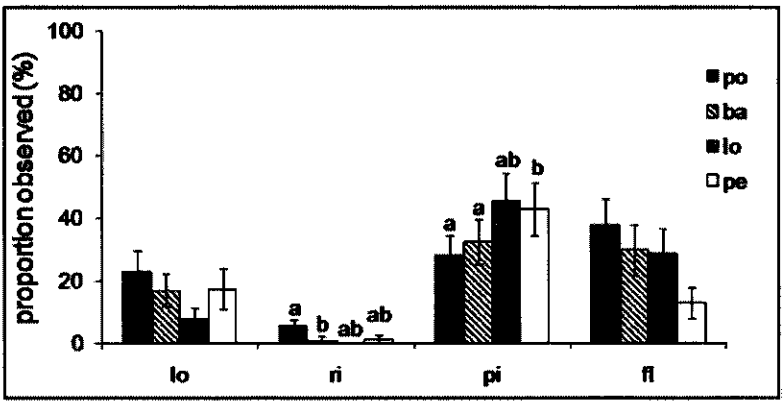

Figure 9: The proportion of times walking on the inner circle (wi) was followed by a specific behavioural element for each pre-treated group of mosquitoes. X-axis: the behavioural elements that followed walking on the inner circle; lo (landing on the outer circle), ri (resting on the inner circle), pi (proboscis contact on the inner circle) or fl (flying). Y-axis: proportion of times wi was followed by any behaviour. Pre-treatments; po: paraffin oil, ba: benzaldehyde, pe: 2-phenylethanol, lo: linalool oxide. Error bars represent standard error of the mean (n=16). Means with different letters are significantly different (GLM, $P < 0.05$); no letters mean no significant differences among mosquitoes from the different pre-treatments.

Discussion

This study reports on an unusually prolonged tonic response of an olfactory receptor neuron (ORN) innervating trichoid sensilla type E (TSE) of *An. gambiae* as elicited by two compounds. After a short (2 s) stimulation with either of two odour compounds a prolonged excitatory response with a duration of more than 10 min. was observed. We have named these responses 'extensively sustained responses', abbreviated as ESR.

Stimuli-exceeding tonic responses have been reported in the literature, but the duration rarely exceeded one minute and these responses have not been subject of detailed investigations (den Otter and van der Goes van Naters 1992, Olsson *et al.* 2006, Ghaninia *et al.* 2007b, Hill *et al.* 2009, Carey *et al.* 2010, Raman *et al.* 2010).

Out of the more than 90 odorants that have been tested on this ORN, only two compounds, i.e. linalool oxide and 2-phenylethanol, elicited an ESR. Only one out of the more than 36 ORN response types that have been characterised thus far (Qiu *et al.* 2006, Lu *et al.* 2007, Chapter 2) showed this unusual temporal response type. This same ORN is activated by 46 of the 96 odorants (Qiu et al. 2006), such as benzaldehyde and shows different temporal characteristics, both phasic and tonic to various odorants, but in no other case an ESR has been observed. Both linalool oxide and 2-phenylethanol elicit responses from various ORNs in other types of sensilla (Chapter 2 and Chapter 3) but they only evoke an ESR from this ORN. Recently Carey *et al.* (2010) reported one out of 50 AgORs that mediated prolonged excitatory responses in the *Drosophila melanogaster* heterologous expression system (DmHES). This receptor, AgOR20, produced a prolonged response after stimulation with linalool oxide (Carey *et al.* 2010). The fact that the ORN measured from is the only neuron thus far exhibiting ESRs, and AgOR 20 is the only olfactory receptor that produced a prolonged response to the same stimulus, linalool oxide, makes it plausible that AgOR20 is the olfactory receptor mediating the ESR in TSE1. A similar prolonged response in the DmHES has not been reported yet for 2-phenylethanol. If 2-phenylethanol would elicit similar responses form AgOR20 as observed for linalool oxide, this would increase the probability that AgOR20 is expressed in the ORN of TSE1, although *in-situ* hybridisation is still required to prove this.

Our electrophysiological results underline the importance of temporal aspects of ORN responses and potential concentration differences. Without consideration of these two aspects all three odorants tested in this study elicited similar increases in responses at higher concentrations and reached the same plateau value. This value might represent the maximum possible response intensity for this neuron. It is, however, possible that we are measuring at the limit of the software we used. At higher response intensities the software is not capable of visualising the response on top of the noise level due to the shrinking of the spike amplitude. Using a dose-response relation study we showed that the threshold for detection of benzaldehyde occurred at a dose 10-fold higher than that of both linalool oxide and 2-phenylethanol. The electrophysiological responses to the latter two compounds could only be distinguished by their temporal differences as they were characterised by similar threshold and plateau values.

A new behavioural observation setup is presented here that enabled us to analyse the latency, frequency, duration and sequences of 18 predefined behavioural elements. We were able to show that a pre-treatment of 2 s with either of the two odorants eliciting ESR (linalool oxide and 2-phenylethanol) resulted in several differential behavioural changes towards the odour of a worn sock in the 5 min. observation period immediately following exposure to these odorants. This is a remarkable finding that has not been reported before in the insect olfactory literature as far as we are aware.

After pre-exposure to linalool oxide or 2-phenylethanol, the frequency of flights within 5 min was reduced but the durations of these flights were longer. The largest difference between mosquitoes treated with solvent only or benzaldehyde and mosquitoes pre-treated with either of the two compounds triggering ESRs was found in the time spent on behaviours on the inner circle, *i.e.* the area closest to the attractive complex odour source emitted by a worn sock (Fig. 1). After pre-treatment with linalool oxide or 2-phenylethanol mosquitoes spent 50% more time on 'proboscis contact' and less time on 'walking' on the odour source than mosquitoes pre-treated with the solvent only or benzaldehyde. It seems that by a brief exposure to the ESR-evoking compounds, the mosquito is arrested on the landing spot and spends more time on *'proboscis contact'* and less time on *'walking'* or *'flying'*.

The sequential data further supported the notion that the mosquitoes treated with ESR-evoking compounds were spending more time on the odour source. Mosquitoes pre-treated with linalool oxide or 2-phenylethanol more often started 'proboscis contact on the inner circle' immediately after 'landing on the inner circle' than solvent only or benzaldehyde-treated mosquitoes, which more often first walked around or flew to a different spot. When linalool oxide or 2-phenylethanol treated mosquitoes did walk on the odour source (inner circle), they were more likely to follow this up by 'proboscis contact' than mosquitoes pre-treated with solvent only or benzaldehyde.

It appears that a short encounter with ESR-evoking odours can influence the decision of staying (arrestment) or leaving a possible feeding site. Examples of odorants causing arrestment have been recorded in several studies on parasitoids. Based on the amount of kairomones released from the combination of hosts and their plant food and from nearby patches, parasitoids adapt their time allocation (Waage 1979, van Alphen *et al.* 2003). Arrestment could be caused by direct effects of these specific odours. In our study, however, because the detection of the ESR-evoking odours and the actually observed behaviours were separated in time, it could be that the ESR-evoking odours increase the sensitivity towards odours in our attractive odour source causing prolonged proboscis contact, which leads to more time spent on a possible feeding site. Such sensitization where an odour increased the sensitivity towards a second odour has also been observed in other insect species (Kelling *et al.* 2002, Said 2005, Schroder and Hilker 2008), although, during most of these cases, the sensitization was observed during a continuous presence of a "background" odour. A form of sensitization, possibly similar to that occurring in the current study, would be the increased sensitivity of *Aedes aegypti* (L.) to skin odours by CO_2

(Dekker *et al.* 2005). Dekker *et al.* (2005) showed that encounters with CO_2 filaments increased the sensitivity towards the skin odours located upwind of the CO_2 release point by at least a fivefold. The effects of a single, short exposure to an ESR-evoking odorant, as described in this study have not been reported in the literature so far for any other organism, and may be interpreted as a form of sensitisation.

Even though both odours that elicited ESR are human-derived compounds, we do not interpret the behavioural effects that we observed as evidence for attraction. Attraction would probably have resulted in smaller latencies for behaviours on the odour source (inner circle), thus smaller time periods between the release in the flight chamber and the first time a behavioural event was observed, leading to a more rapid localisation of the odour source. This was not the case for any of the observed behaviours.

The findings presented here generate new questions, the answers to which can enhance our understanding of the olfactory system and may bring us a step closer to odour-mediated mosquito control strategies. Does continuous stimulation with ESR-evoking compounds, the actual situation occurring during foraging, elicit similar behavioural changes? Identification of the threshold dose at which the behavioural changes still occur is also necessary? This is especially important for a possible applied aim. It would also be interesting to investigate the possible involvement of the ESR in learning (McCall and Eaton 2001): would a previous encounter with the ESR-evoking odours, when rewarded with a blood meal, enhance the behavioural effects observed in this study? Using the 3D-tracking system for mosquito flight that is available in our laboratory (Spitzen *et al.* 2008), flight parameters can be quantified in detail, possibly yielding identification of previously unknown effects of human-derived odours on mosquito behaviour.

Another question relevant for a possible application of these effects is what constitutes the simplest possible blend that, when combined with these two odours, still elicits this enhanced 'proboscis contact' behaviour? Behavioural experiments have shown that a synthetic blend of ammonia, lactic acid and carboxylic acids can significantly attract *Anopheles* mosquitoes (Smallegange *et al.* 2005b, Smallegange *et al.* 2009, Okumu *et al.* 2010). But when given a choice, mosquitoes still preferred a sock (similar to the odour source in the present study) over this basic blend (Smallegange *et al.* 2010). The replacement of the human sock by a simple blend while still maintaining the effects as seen by these ESR-evoking compounds could lead to improved mosquito attract-and-kill strategies. Insecticide-treated surfaces, for example, or surfaces treated with entomopathogenic fungi could benefit from addition of these odorants. If mosquitoes can be kept on a specific surface for a longer period of time exposing more of the body parts to this surface by proboscis contact, the exposure times and pick-up rates of fungal spores or insecticide will go up, leading to higher infection and killing rates.

Conclusion

The present study has revealed the existence of an ORN in *Anopheles gambiae* females that exhibits an extensively sustained electrophysiological response, defined as an excitation response lasting for more than 10 minutes, to two human-derived odorants, i.e. linalool oxide and 2-phenylethanol. These electrophysiological responses have been linked to behavioural effects, using a new bioassay. We have shown that pre-treatment of a few seconds to ESR-evoking odorants resulted in behavioural changes of mosquitoes searching for a blood meal that lasted up to five minutes. This cause-effect relationship between temporal ORN responses and changes in host-seeking behaviour is novel in the insect neurobiology literature.

Acknowledgements

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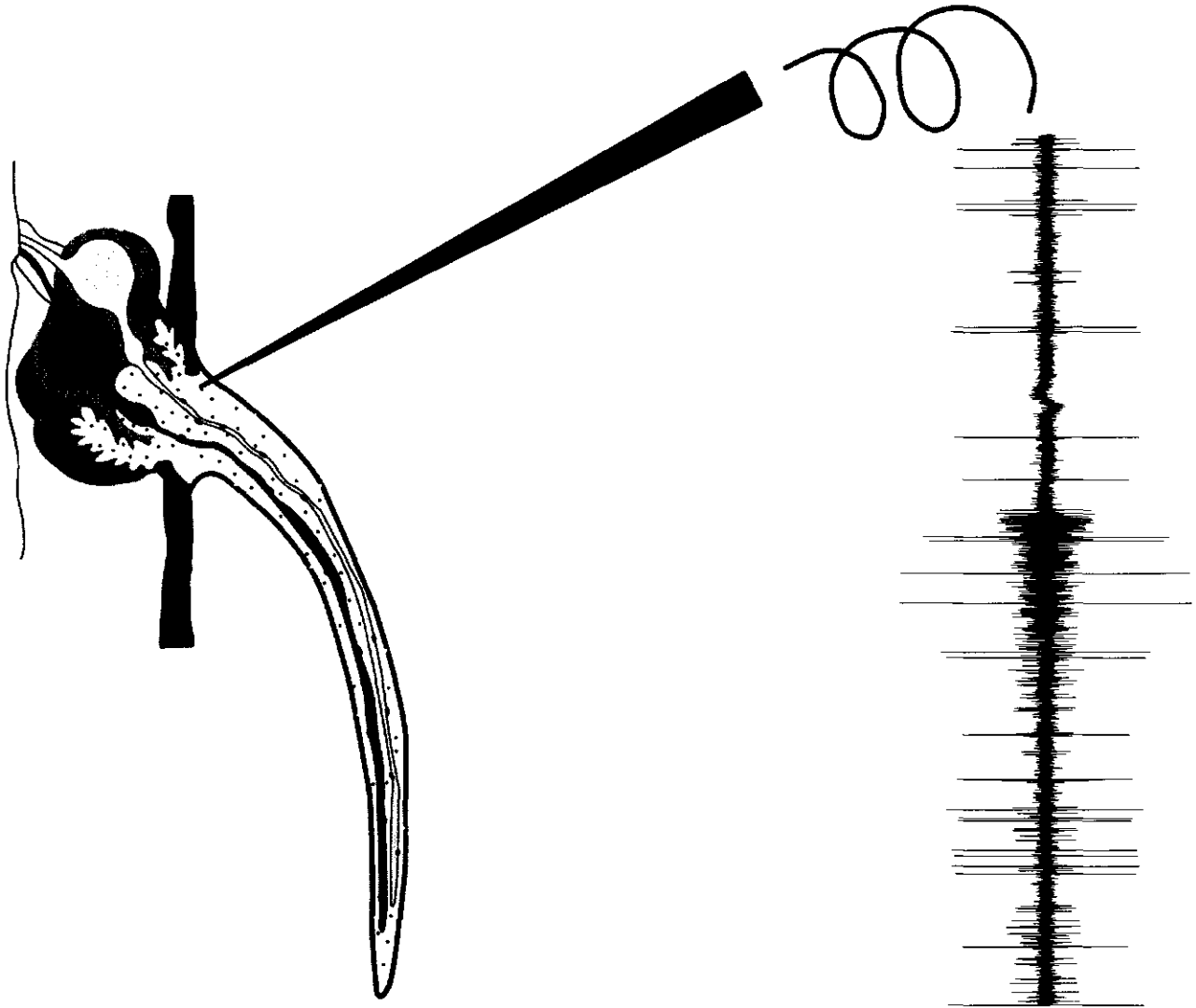

Chapter 6

Summarising discussion

Remco A. Suer

The work described in this thesis expands our knowledge on the functioning of the peripheral olfactory system of *Anopheles gambiae* Giles *s.s.* in response to various chemical components including volatiles found in human emanations and from mosquito oviposition sites.

The main experimental findings have been discussed at length at the end of each chapter. The most important results are therefore briefly summarised in this chapter according to the objectives articulated in **Chapter 1**. In addition, future perspectives based on the work described in this thesis are presented and discussed here.

Objective 1: Classify and characterise the response spectra of the ORNs innervating the three trichoid sensilla types A, B and D

The characterisation of the response spectra of olfactory receptor neurons (ORNs) associated with various types of sensilla has been performed for several mosquito species (Appendix 1). Such characterisations indicate not only which odours are detected by the mosquito but also which odours might be more important for olfactory-based behaviour like host seeking and oviposition-site selection, as it may be expected that odours that cause the strongest response of an ORN, thus the best ligands of the olfactory receptors of that ORN, will have a more pronounced role in these olfactory-based behaviours than less responsive ones. This response can be excitatory or inhibitory, both of which can cause attraction or repellency.

The A-, B-, and D-subtypes of the trichoid sensilla (TS) present on the antenna of *An. gambiae* have been characterised using a panel of 132 odours, 80 of which are also found in human emanations (**Chapter 2**). These results have been added to the previous results on the C- and E-subtypes of trichoid sensilla and the grooved pegs of *An. gambiae* summarised in Appendix 1 (Table 1). A start was made with the characterisation of the response spectra of TSA, the longest sharp-tipped trichoid sensilla; however, it became clear that none of the odours in the panel were capable of activating the TSA-ORNs. Because similar findings were reported for the longest sharp-tipped sensilla in *Aedes aegypti* (Ghaninia *et al.* 2007b) and *Culex quinquefasciatus* (Hill *et al.* 2009), the focus was shifted towards the other two trichoid sensilla subtypes, TSB and TSD. In total 71 odorants elicited responses from TSB (**Chapter 2**). Of these 71 odorants, 38 have been shown to be emitted by humans. TSD-ORNs responded to 35 odours, 15 of which being reported as present in human effluents (**Chapter 2**). Based on differences in the response spectra of the two ORNs associated with various morphologically similar trichoid sensilla, six different response types were identified for TSB; four for the A-neuron of TSB and two for the B-neuron. Five response types were identified for TSD; three for the A-neuron and two for the B-neuron. Two of the TSB-response types, A3 and B1, showed weak or no responses to the odours in our panel. The other four can be seen as generalistic response types, responding to multiple odours. These four response types of TSB had partly overlapping response spectra but were different enough to allow identification by the use of a diagnostic panel of six odorants. The response types of TSD were quite different from those of TSB. Though still responsive to multiple odours, TSD-response types were activated by a much lower number of odours. The differentiation between the response types of TSD was also not very

Table 1: Summary of all characterisation data of olfactory sensilla of *An. gambiae*. Light grey presents the data obtained by this thesis, dark grey is missing data. n.d.) not done; P) possibly.

Mosquito species	Organ	Sensilla	Subtype	Functional - response types	Olfactory receptor
Anopheles gambiae	antenna	trichodea	A (longest sharp-tipped)	TA	n.d.
			B (after A longest sharp-tipped with widest diameter)	TB A1	n.d.
				TB A2	P) AgOR46
				TB A3	P) AgOR83
				TB A4	n.d.
				TB B1	P) AgOR83
				TB B2	P) AgOR15
			C (medium-length sharp-tipped; intermediate in all characteristics)	TC1	
				TC2	
				TC3	
				TC4	
			D (short blunt-tipped)	TD A1	P) AgOR11 or AgOR12
				TD A2	P) AgOR11 or AgOR12
				TD A3	P) AgOR11 or AgOR12
				TD B1	P) AgOR6
				TD B2	P) AgOR6
			E (short sharp-tipped)	TE1	
				TE2	
		grooved peg	GP1		
			GP2		
			GP3		
			GP4		
			GP5		
	maxillary palp	capitate peg		A-neuron	AgGR 22;23;24
				B-neuron	AgOR7 + AgOR8
				C-neuron	AgOR7 + AgOR28
	proboscis	T2		A-neuron	AgOR7 + AgOR6; 53; 12; 18; 3; 15; 19; 28; 55; 62; 70
				B-neuron	

The "-" sign indicates that none of the compounds tested elicited this response type. References can be found in the reference list of Appendix 1.

Compounds eliciting		Ref.
Excitation	Inhibition	
-	-	Chapt.2
henkel 100; linalool oxide; 2-ethyl-1-hexanol; isoamyl acetate; 6-methyl-5-hepten-2-one; 1-octen-3-ol; octanal; 2-isobutylthiazole; 3-octanone; amyl acetate; (S)-(+)-carvone; ethyl hexanoate; (R)-(−)-carvone	-	Chapt.2
isoamyl acetate; (-)-α-thujone; 4-methylcyclohexanol; 2-isobutylthiazole; henkel 100; 3-methyl-1-butanol; 1-hexanol; benzaldehyde; ethyl butyrate; 3-methyl-2-cyclohexen-1-ol; (R)-(−)-carvone; 1-hexen-3-ol; 2-methyl-1-butanol; (S)-(+)-carvone; 2-acetylpyridine; 2-acetylthiophene; acetophenone; 4,5 dimethyl thiazole; amyl acetate; 4-methylthiazole; cis-3-hexen-1-ol; 6-methyl-5-hepten-2-one; ethylene glycol butyl ether; ethyl hexanoate; pyridine; 2-ethyl-1-hexanol; linalool oxide; methyl salicylate; 2-furaldehyde	2-methylbutanal	Chapt.2
decanal; 1-hepten-3-ol; hexanal; 1-hexanol	thiazole	Chapt.2
decanal; henkel 100; octanal; 1-octen-3-ol; ethyl hexanoate; phenethyl acetate; 2-nonanone; dimethyl adipate; 1-hexanol; 3-octanone; 2-decanone; (S)-(+)-carvone; 2-nonenal trans; amyl acetate; methyl octanoate; 6-methyl-5-hepten-2-one; 1-hepten-3-ol; (R)-(−)-carvone; cis-3-hexen-1-ol; isoamyl acetate; nonanal; methyl caprylate; methyl nonanoate; geranyl acetone; 2-octene trans; 2-ethyl-1-hexanol	-	Chapt.2
1-hepten-3-ol		Chapt.2
eugenol; benzaldehyde; acetophenone; 4,5-dimethyl thiazole; 2,4,5-trimethylthiazole; 2-acetylthiophene; henkel 100; 2-acetylpyridine; 2-methylphenol; 2-acetylthiazole; methyl-2-methyl benzoate; methyl benzoate; 2-furaldehyde; 2-ethylphenol; 4-methylthiazole; pyridine; 4-methylphenol; 3-methylindole; 3-methyl-2-cyclohexen-1-ol; 3-methylphenol; methyl salicylate; 4-methylcyclohexanol; phenol; cyclohexanone; 4-ethylphenol; indole	(S)-(+)-carvone; dimethyl adipate	Chapt.2
ammonia; phenol; 4-methylphenol; 4-ethylphenol	-	9;10;11;12
geranyl acetone	-	9;10;11;12
indole; phenol; 2-methylphenol; 4-methylphenol; 4-ethylphenol	-	9;10;11;12
ammonia ; 4-ethylphenol	-	9;10;11;12
4-methylcyclohexanol; 2-ethyl toluene; 3-methyl-2-cyclohexen-1-ol; benzaldehyde; 4,5-dimethyl thiazole; 2-methyl-1-butanol	-	Chapt.2
4-methylcyclohexanol; 2-ethyl toluene	-	Chapt.2
3-methyl-2-cyclohexen-1-ol; 2-ethyl toluene; (+)-fenchone; (−)-fenchone; 4,5-dimethyl thiazole; 4-methylcyclohexanol; benzaldehyde; styrene; 1-hepten-3-ol; 3-methylindole; cyclohexanone; acetophenone; 2-ethylphenol	-	Chapt.2
acetophenone; 2-acetylpyridine; 2-acetylthiophene; henkel 100; 2-acetylthiazole; benzaldehyde; 3-methyl-2-cyclohexen-1-ol	-	Chapt.2
2-acetylpyridine; acetophenone; 2-acetylthiophene; henkel 100; methyl benzoate; 2,4,5-trimethylthiazole; benzaldehyde; 3-methyl-2-cyclohexen-1-ol; 2-acetylthiazole; cyclohexanone; 4-methylthiazole; ethylene glycol butyl ether; 4-methylcyclohexanol	1-hepten-3-ol	Chapt.2
indole; phenol; 2-methylphenol; 4-methylphenol; 4-ethylphenol; 1-hexen-3-ol; 5-7C acids; 3-methyl-1-butanol; ammonia ;2-phenoxyethanol	3-4C acids	9;10;11;12
4-methylphenol; 4-ethylphenol; geranyl acetone; 2-nonanone; 1-hexen-3-ol; 1-hepten-3-ol; 1-octen-3-ol; 7-octenoic acid; 3-methyl-2-hexenoic acid; 3C; 3-methylbutanoic acid; 6C; 9C; 10C; 12C acids; 2-oxobutanoic acid; 2-oxopentanoic acid; ammonia	-	9;10;11;12
ammonia; 1-butylamine; 1-pentylamine	-	9
ammonia; 1-butylamine; 1-pentylamine; 2-oxobutanoic acid; 2-oxopentanoic acid	-	9
ammonia; 1-butylamine; 2-oxobutanoic acid; lactic acid	-	9
ammonia; 1-butylamine; 2-oxobutanoic acid; lactic acid	3-6C and 9C acids ; 1-hepten-3-ol	9
ammonia; 1-butylamine; 1-pentylamine; 4-6C acids	-	9
CO2; thiazole; cyclohexanone; 4-methyl thiazole; 2-methyl-1-butanol; 3-methyl-1-butanol; 1-butanol; 2-methylbutanal; benzaldehyde	indole; 3-methyl indole; 3-octanone; 1-hepten-3-ol; L-(+)-lactic acid; heptanal; 2-iso-butyl thiazole; 2,3-butanedione; 2-methylbutanal; 3-methylbutanal; 2-methylbutanoic acid; 3-methylbutanoic acid	13, Chapt. 4
1-octen-3-ol; 1-hepten-3-ol; 3-octanone; 1-hexen-3-ol; henkel 100; ethyl hexanoate; amyl acetate; cis-3-hexen-1-ol; 6-methyl-5-hepten-2-one; 2-phenylethanol, 2-methyl-1-butanol; 3-methyl-1-butanol; 1-butanol; 2-methylbutanal; 3-methylbutanal; benzaldehyde; 2,3-butanedione	4-methylcyclohexanol; 2,4,5-trimethyl thiazole; 2-ethylphenol; 3-methyl-2-cyclohexen-1-ol; 2-acetylthiophene; cyclohexanone; 2-acetyl thiazole; DEET; 4-methylthiazole; methyl-2-methyl benzoate; acetylpyridine; acetophenone; 4,5-dimethyl thiazole; methyl benzoate; ethyl propionate; linalool oxide; R-(-)-&S-(+)- carvone	13, Chapt. 4
2,4,5-trimethylthiazole; acetophenone; 2-acetylthiophene; 3-metyl-2-cyclohexen-1-ol; 4-methylcyclohexanol; 4,5-dimethylthiazole; 2-acetylthiazole; 6-methyl-5-hepten-2-one; cyclohexanone; linalool oxide; 2-iso-butylthiazole; methyl-2-methylbenzoate; cis-3-hexen-1-ol; 1-hepten-3-ol; methyl benzoate; 1-hexen-3-ol; 4-methylthiazole; (-)-fenchone; hexanal; ethyl hexanoate; S-(+)-carvone; (+)-fenchone; amyl acetate; 2-phenylethanol, 2-methyl-1-butanol; 3-methyl-1-butanol; 1-butanol; 2-methylbutanal; 3-methylbutanal; benzaldehyde; 2,3-butanedione	-	13, Chapt. 4
oxovaleric acid; butyl amine; acetothiophene; acetic acid; oxobutylic acid; acetylpyridine; acetylthiazole	-	14
acetylthiophene; acetylthiazole; acetylpyridine; acetophenone; oxobutylic acid; oxovaleric acid; butylamine	-	14

clear. All three response types for the TSD-A-neuron comprised a similar spectrum of odours that elicited responses; they only seemed to differ in the strength of the response. The latter finding also applied to the B-neuron of TSD.

Among the best ligands of all these response types were several odorants also detected in human emanations like linalool oxide, acetophenone and benzaldehyde. The latter two, when tested behaviourally in a dual-choice olfactometer, evoked changes in host-seeking behaviour from female mosquitoes; acetophenone evoked increased attraction of an attractive blend at the highest dose tested while benzaldehyde decreased the attraction of this attractive blend at the lowest dose tested (G. Bukovinszkine Kiss, unpubl. data).

By comparing the response spectra of the identified response types with the response spectra obtained via individual AgORs expressed in the *Drosophila melanogaster* heterologous expression system (DmHES), potential AgOR candidates were identified for the various response types of both TSB and TSD (Table 1). Definitive proof that the selected AgORs are indeed expressed by these ORNs should be obtained through *in situ* hybridisation studies.

Objective 2: Evaluate if AgORs expressed in the DmHES can be used in the search for inhibitors of AgOR responses

Several human-derived compounds were shown to elicit strong responses from a number of ORNs of *An. gambiae* (**Chapter 2** and **Chapter 4**). If the mosquito uses these compounds to locate a human, inhibitors of the ORN responding to these odours that block the receptor or otherwise nullify the responses to such compounds, could be used as repellents or masking odours. Two studies reported the characterisation of individual AgORs by use of heterologous expression systems (Carey *et al.* 2010, Wang *et al.* 2010). These heterologous expression systems have been put forward as potential high-throughput screening (HTS) tools for possible attractants or repellents. The only comparison made between *in vivo* ORNs and their heterologously expressed AgORs was done for the AgORs expressed by capitate peg ORNs on the maxillary palps. It was shown that the response spectra obtained by *in vivo* electrophysiology matched the response spectra obtained by the identified capitate peg AgORs when heterologously expressed. A pilot study on one of these identified AgORs, AgOR8, when expressed in the DmHES, found four inhibitors of the best ligand of AgOR8; 1-octen-3-ol, a known mosquito attractant. These four inhibitors were tested *in vivo* to evaluate if the results obtained by expression in the DmHES could be reproduced *in vivo* (**Chapter 3**). Differences between *in vivo* and DmHES electrophysiological activity in response to binary mixtures were observed. The concentration of 1-octen-3-ol used in the heterologous system (1%) could not be used for *in vivo* measurements because this concentration completely silenced the ORN. The concentration of 1-octen-3-ol had to be lowered 100-fold to generate comparable response intensities between both systems. When the concentration of 1-octen-3-ol in the binary blend was reduced even further, the potential inhibitors even enhanced the responses to 1-octen-3-ol. To exclude possible fixative effects reported by Syed and Leal (2008) for DEET, different stimulation methods were used; however,

both methods generated mostly similar results. The main influential factor was the concentration of 1-octen-3-ol (the best ligand for this ORN) and not the method used. Further experiments revealed that the potential inhibitors when tested singularly elicited responses from the same ORN as responsive to 1-octen-3-ol. The inhibition observed at higher concentrations of 1-octen-3-ol is probably caused by competition of two ligands for the same receptor. The differences observed at different concentrations stress the importance of testing multiple concentrations especially in binary mixtures. As concentration has such large effect and the responses to similar concentrations can be so different between *in vivo* and DmHES some caution should be exerted when extrapolating data obtained in the heterologous expression system to *in vivo* responses. Additional caution against DmHES to *in vivo* extrapolation comes from (1) the lack of correspondence between *in vivo* response types and DmHES AgORs ligand spectra (**Chapter 2**) and (2) the fact that not every odour that elicits responses *in vivo* does so in the heterologous system (Carey *et al.* 2010, Chapter 2, Wang *et al.* 2010). To increase the credibility of the heterologous expression system, it is essential that complete dose-response relations of binary blends are compared with *in vivo* data. It is very likely that OBPs are playing a major role in the differences observed between *in vivo* and DmHES. In the *Xenopus* oocyte expression system OBPs are completely absent and it is known that there is little homology between OBPs of different species (Zhou 2008), thus the DmHES contains different OBPs than present in the olfactory sensilla of *An. gambiae*. As different morphological types of olfactory sensilla have different response spectra, it might be that different morphological types of sensilla also contain different types of OBPs. Another possible explanation for the differences observed between *in vivo* and heterologous electrophysiological results could be the newly identified IR olfactory pathway (Benton *et al.* 2009). A recent study identified this new family of chemoreceptor genes in *An. gambiae* and revealed that these IRs form a different olfactory pathway that responds to odours the AgOR pathway does not respond to, like butylamine (Liu *et al.* 2010). It is probable that these AgIRs will also respond to various carboxylic acids and ammonia, which are odours that also do not elicit responses from AgOR characterised via the DmHES.

Objective 3: Investigate the role of the maxillary palp capitate peg ORNs in host seeking

and

Objective 4: Examine the possible effect a transition of one physiological state to another might have on the responsiveness of these capitate peg ORNs

The maxillary palps of mosquitoes play an important role in host-seeking behaviour. Palpectomised *Ae. aegypti* females had a significantly reduced landing response compared to non-palpectomised mosquitoes (Majeed *et al.* 2010). Most, if not all, mosquito species also utilise CO_2 as a long range activator and guidance towards their hosts (Gillies and Wilkes 1968, Gillies 1980, Takken and Kline 1989, Takken 1991, Spitzen 2008). The only ORN capable of CO_2 detection is found associated with the capitate peg sensilla on the maxillary palp. These

capitate peg sensilla contain two additional ORNs next to this CO_2-sensitive neuron, one of which has a remarkable sensitivity to 1-octen-3-ol, which attracts several mosquito species and other insects like tsetse flies (Hall *et al.* 1984, Lu *et al.* 2007, Syed and Leal 2007).

Verhulst *et al.* (2009) discovered that 10 volatiles produced by bacterial samples taken from human feet affect host-seeking behaviour. In this study, benzaldehyde was added to this list of ten compounds because of reported repellency together with a reduced presence in volatiles emanating from more attractive bacterial samples. These 11 compounds were used in the study described in **Chapter 4** to investigate the role of the maxillary palps in host-seeking behaviour. Ten of the eleven odours elicited electrophysiological responses of at least one of the capitate peg ORNs showing that all three ORNs are involved in the detection of host-derived cues. The only odour that did not elicit any responses from the capitate peg ORN was 3-hydroxy-2-butanone. To date this compound has never elicited responses from any of the olfactory sensilla currently tested in *An. gambiae* but it does enhance the attractiveness of an attractive basic blend when tested in a behavioural setup (R.C. Smallegange, pers. comm.). The only sensillum type of *An. gambiae* that has not been characterised to date is the coeloconic sensillum (Table 1). It would be interesting to check if ORNs innervating the coeloconic sensilla are responsive to this compound. The characterisation of the coeloconic sensilla is currently being finished within our lab (Y.T. Qiu, unpubl. data).

As host-seeking behaviour stops during 48 h after a female mosquito has taken a blood meal and it gradually returns after 72 h or more (Takken *et al.* 2001), changes in the responses of ORNs could be indicative of involvement in either host seeking or oviposition-site selection depending on the nature of the change in sensitivity. Such changes in responsiveness have been shown for several antennal olfactory sensilla and further evidence has been provided by up-and down regulation of genes involved in olfaction after the ingestion of a blood meal (Qiu *et al.* 2006, Siju *et al.* 2010). The changes in responsiveness between different physiological states (pre-blood meal, post-blood meal and post-oviposition) in response to the 11 bacterial volatiles provided further evidence of the involvement of the maxillary palps in host seeking (**Chapter 4**). One of the capitate peg ORNs of post-blood meal mosquitoes consistently expressed lower responses to most of the bacterially produced odorants compared to pre-blood meal mosquitoes. The lower sensitivity of this ORN post-blood meal correlates very well with the 48 h period of inhibition of host-seeking behaviour post blood meal. The third capitate peg ORN was found to have an increased sensitivity to several of the odours emanating from the microbiota of the human feet after oviposition compared to the responses measured for pre-blood meal mosquitoes. This could indicate that after a complete gonotrophic cycle a mosquito's olfactory system is more sensitive for detecting and responding to human-derived volatiles.

Benzaldehyde has repeatedly been shown to elicit responses from many antennal ORNs (**Chapter 2**) and behavioural experiments in an olfactometer showed that addition of this compound to an attractive basic blend (consisting of ammonia, lactic acid and tetradecanoic acid) decreased the attractiveness of the blend to female *An. gambiae* (G. Bukovinszkine Kiss, pers. comm.). In the analysis of Verhulst *et al.* (2009) it seemed that this component was less

present in the more attractive bacterial samples they analysed (N.O. Verhulst, pers. comm.). The three capitate peg ORNs responded differently in the different physiological states to stimulation with benzaldehyde compared to any of the other bacterial volatiles. Where the A-neuron showed the strongest responses to most odours in pre-blood meal mosquitoes, benzaldehyde excited the A-neuron most in post-oviposition mosquitoes. Similar results were seen for the B-neuron. For most odours, these palpal responses of post-blood meal mosquitoes were lower compared to pre-blood meal mosquitoes. However, the B-neuron of post-blood meal mosquitoes responded strongest to benzaldehyde compared to pre-blood meal or post-oviposition mosquitoes. For the C-neuron, post-oviposition mosquitoes showed the highest responses for most odours, except for benzaldehyde. These results suggest that benzaldehyde is an interesting candidate for behavioural manipulation of *An. gambiae*, possibly as a spatial repellent.

Five of these bacterial odours (2- and 3-methylbutanoic acid, 2- and 3-methylbutanal and 2,3-butanedione) also suppressed the spontaneous activity of the ORN that responds to CO_2 in every physiological state assessed (i.e. pre blood meal, post blood meal, post oviposition). Even in the presence of levels of CO_2 higher than 1100 ppm these five compounds are capable of completely suppressing the activity of the CO_2 neuron. At a 10% solution the duration of the suppression by four of these five inhibitors lasted for several times longer than the stimulus duration. Preliminary behavioural experiments with some of these odorants have shown that even at a 1% concentration these odours significantly altered the behavioural response to CO_2 in both no-choice and dual-choice bioassays (G. Bukovinszkine Kiss, pers. comm.). This discovery is very important. It has long been known that mosquitoes use CO_2 to locate a host from afar (Gillies and Wilkes 1968, Takken and Kline 1989, Kline *et al.* 1991b, Kline *et al.* 1991a, Takken 1991, Takken and Knols 1999). At close range other odours become increasingly more important for host selection. *Anopheles gambiae* prefers to bite the lower body parts of humans, more particularly the feet (de Jong and Knols 1995a, 1996). This means that at a certain moment in their approach to the host they have to divert from the exhaled CO_2 plume coming from the mouth of a host toward the feet. This thesis shows that volatiles produced by microbiota on the feet of humans induce responses from the olfactory sensilla present on the maxillary palps while at the same time inhibiting the responses to elevated levels of CO_2. Some of these bacterial volatiles also elicit responses from antennal ORNs. It was already known that the synthetic blend of the 10 compounds mentioned above was capable of attracting female *An. gambiae* in the absence of CO_2 (Verhulst *et al.* 2009). Behavioural experiments were undertaken to test each of the 10 volatiles separately in a behavioural setup in combination with an already attractive blend. In the laboratory in the absence of CO_2 several of these volatiles increased the attractiveness of the basic blend. In a semi-field setup in Kenya, when tested singularly with the same basic blend and CO_2, two of the five inhibitors significantly increased the trap entry responses of *An. gambiae* (Verhulst 2010). These results combined with the behavioural experiments performed in the laboratory showing both inhibition of the CO_2 response by these same inhibitors at a distance of 160 cm (roughly the distance between

the feet and the mouth) in a laboratory bioassay and attraction by some of the other bacterial volatiles, suggest that the specific blend of odours coming from the human feet is responsible for the divergence from the CO_2 plume coming from the mouth of a human host towards the odour plume coming from the feet.

A further intriguing finding is the fact that the sensitivity towards these bacterial odours is higher immediately after oviposition compared to pre-blood meal (**Chapter 4**). This also seemed to be the case for the responses of the A-neuron to the four strongest CO_2 inhibitors. After oviposition the persistence of the complete inhibition of the spontaneous activity of the A neuron, in the absence of elevated levels of CO_2, appeared to last longer than when tested pre-blood meal. It should be further investigated if this stronger suppression is also present at elevated CO_2 levels and if this is a permanent change or if it only occurs over a short time span, after which the responses might gradually return to pre-blood meal levels.

A detailed description of a rare temporal phenomenon and its behavioural consequences

An intriguing temporal phenomenon was encountered during the characterisation study reported in **Chapter 2**. Though not one of the original objectives, studying the temporal aspects of neural responses is of great importance because it provides the peripheral nervous system with an extra dimension in odour coding (Yao *et al.* 2005, Carey *et al.* 2010) (**Chapter 1**). Recently, evidence was presented indicating that temporal properties of the odour-elicited firing patterns of the peripheral ORNs are involved in encoding the information on identity, concentration and stimulus duration of odours (Raman *et al.* 2010). During characterisation studies, different temporal properties of responses were noted. In particular, one ORN in TSE of *An. gambiae* showed an extremely intense response with a unique temporal pattern to one of the bacterial volatiles (2-phenyl ethanol; **Chapter 4**) as well as another human-derived compound that represents the best ligand of one of the previously identified response types of TSB (linalool oxide; **Chapter 2**). A 200-ms stimulation with either of the two compounds evoked a response that lasted several or even many minutes. Because of this unusual excitation pattern, we decided to devote a separate study to this novel temporal feature displayed by these two ORN-response types (**Chapter 5**).

It was hypothesised that such strong, long-lasting neural signals might cause behavioural changes. Because the odours eliciting such responses were present in human emanations, changes in host-seeking behaviour were expected. It was shown that a 2 s stimulus evoked a response for more than 10 min. for linalool oxide and for even more than 20 min. for 2-phenylethanol (**Chapter 5**). These long-lasting responses were named 'extensively sustained responses' (ESRs). By use of a newly designed bioassay that accommodated more detailed observation of mosquito behaviour, these electrophysiologically obtained ESRs were linked to changes in behaviour. A pre-exposure of 2 s using an identical stimulation method and concentration as practised in the SSR-electrophysiology, resulted in various behavioural

changes to the odour of a worn sock in the 5 min observation period immediately following exposure to these odorants. One of the most interesting behavioural changes observed was that the mosquito showed increased arrestment on the landing spot (closest to the odour source) and spends more time on extending the proboscis and less time walking or flying. Mosquitoes pre-exposed to either ESR-evoking compound were also shown to more often follow up landing and walking on the odour source with proboscis extensions on the odour source compared to control mosquitoes that were pre-exposed to the solvent only. This is the first time such a direct link between temporal characteristics of electrophysiological activity of an insect ORN and the resulting changes in elements of host-seeking behaviour have been described. The arrestment behaviour combined with a higher time allocation to proboscis extension might offer some possibilities for future use in behavioural manipulation. For further investigation, the next logical experimental step would be to investigate if these effects are also observed under continuous stimulation with these ESR-eliciting compounds. The study presented in **Chapter 5** has shown that temporal properties are definitely important and should be examined in more detail in future research.

Future perspectives

Odour cartridge concentrations: linking electrophysiology to behaviour

The concentrations in the odour cartridges used for characterisation studies on olfactory sensilla have varied between studies and mosquito species (Qiu *et al.* 2006, Ghaninia *et al.* 2007b, Hill *et al.* 2009). Though all used a similar odour cartridge made out of a Pasteur pipette with a small (+/- 0.5 x 1.5 cm) piece of filter paper with 10 µl of odour, some used 10% for a specific odour on the filter paper while other used a 1% concentration. Based on the characterisation experiments for TSB and TSD, the use of a 1% concentration instead of 10% is preferred. A 1% concentration still generated a number of responses that were absent at 0.1% but also gave the opportunity to investigate the characteristics of the response to a higher concentration when determining the dose-response relations (**Chapter 2**). The factor concentration has always been a point of debate. It has been shown that higher concentrations elicit activity from more olfactory receptors than is recorded with lower concentrations (de Bruyne *et al.* 1999, de Bruyne *et al.* 2001, Wang *et al.* 2003, Su *et al.* 2009). A 10-fold higher concentration will have a large impact on both the proportion of ORNs that will respond to a compound as well as the strength of the response. Most relevant in the context of our study is correspondence between concentrations tested electrophysiologically and concentrations actually encountered by the mosquitoes during the host-seeking sequence. To my knowledge no study has focussed on the concentrations of behaviourally relevant compounds that mosquitoes encounter in the wild while host seeking. These concentrations will likely span a wide range because mosquitoes use olfactory cues to locate a host from afar, to navigate to it and to land on it. It is possible that different odours are used for the different stages of host

seeking; however it might just as well be that different receptors are used for the different concentration ranges of the same odour. It has been shown that odours are perceived by various response types or AgORs and that they differ in the strength of their responses to the same odour (Qiu *et al.* 2006, Lu *et al.* 2007, Carey *et al.* 2010). Behavioural experiments conducted in our laboratory have shown that the concentration range we are using for electrophysiology elicits behavioural responses in the mosquitoes (Chapter 4, Chapter 5, Smallegange *et al.* 2005b, Smallegange *et al.* 2009, Verhulst *et al.* 2009) and that sometimes these responses change with different concentrations. For these reasons the use of 10 µl of a 1% dilution of an odour compound is a good starting point for an electrophysiological characterisation study. As a necessary follow-up for an electrophysiological study, however, a dose-response study on compounds that are active at the 1%-dose supplies indispensable information.

Please note that many other factors are also of great importance. The airstream used for passage through the odour cartridge is, with 0.5l/min, the same for all studies. But the background airstream varies from 0.5l/min to 1l/min, the distance between injection of the odour in the background airstream and the prepared mosquito is different, the distance between the antenna of the mosquito and end of the glass tube leading the airstream over the preparation is different and so is the diameter of the glass tube. All of these are factors influencing the end concentration that is delivered at the antenna of the mosquito. For better comparison between various characterisation studies it would be preferred to agree on a standardized method of stimulation. Because we are all using similar equipment it should be possible.

Characterisation of response spectra of trichoid sensilla A and the coeloconic sensilla

The wall structure of the trichoid sensillum type A indicates an olfactory function (Boo 1980, McIver 1982). The fact that not even one of the 132 odours evoked a response in this sensillum type indicates that this type is probably highly specialistic. By *in situ* hybridization it should be possible to identify which olfactory receptors (ORs) are expressed in the associated TSA-ORNs. The DmHES or other high-throughput heterologous expression systems can then be used to rapidly screen for potential ligands. Specialistic channels are thought to only respond to biologically essential odours (Wilson and Mainen 2006). Therefore, finding the ligand of such specialistic ORNs might lead to the identification of an odour capable of disrupting/redirecting the mosquito's behaviour.

As can be deduced from Table 1, except for TSA, there is only one really interesting olfactory sensillum type left to characterise: the antennal coeloconic sensilla. This olfactory sensillum type has a similar structure as the grooved pegs, the only type of sensilla so far that has ORNs activated by ammonia, several human-derived amines and several carboxylic acids shown to be attractive to mosquitoes (Smallegange *et al.* 2005a, Qiu *et al.* 2006, Smallegange *et al.* 2010).

None of the AgORs when expressed in the heterologous expression systems could convey responses to ammonia or amines (Carey *et al.* 2010, Wang *et al.* 2010). As mentioned above, a novel family of olfactory receptors has only recently been discovered: the ionotropic receptors (IRs). In *D. melanogaster* these are not co-expressed together with traditional ORs and these IRs are found in the *D. melanogaster* equivalent of the mosquito grooved pegs, confusingly called coeloconic sensilla. Recently Liu *et al.* (2010) identified a family of these IRs in *An. gambiae*. By RNAi-mediated gene silencing of the traditional AgOR pathway via the non-conventional co-receptor AgOR7, Liu *et al.* (2010) revealed the involvement of IRs in the detection of butylamine which did not activate any of the AgORs. This indicates that IRs could be involved in host detection and that these IRs are probably expressed in the grooved pegs of mosquitoes. Because coeloconic sensilla have a very similar double walled structure, these coeloconic sensilla might also contain IRs and might therefore be of great interest for further studies on odours that can be deployed in behavioural manipulation of mosquitoes. Experiments done in our laboratory on these coeloconic sensilla have revealed that these sensilla are indeed capable of detecting ammonia, amines and carboxylic acids (Y.T. Qiu, unpubl. data).

In-situ hybridisation studies to identify AgORs associated with trichoid sensilla B and D

An interesting follow-up on the characterisation study would be an *in situ*-hybridisation study of AgORs expressed in TSB- and TSD-ORNs. At present the only *in situ*-hybridisation studies conducted in *An. gambiae* are for the palpal capitate peg sensilla (Lu *et al.* 2007) and for the T2-chemosensilla of the proboscis (Kwon *et al.* 2006). Only for the first, the capitate peg AgORs, did the authors compare the response spectra of heterologously expressed AgORs with the actual ORNs expressing these AgORs *in vivo*. *In situ*-hybridisation on the TSB- and TSD-AgORs and the subsequent comparison of the response spectra of the heterologously expressed AgORs and the response spectra of the AgOR *in vivo* provides much stronger evidence for the value that a heterologous expression system can have in identification of good ligands for identified AgORs. These results could provide a better understanding of why certain response types found in **Chapter 2** could not be matched with known AgORs.

Further investigation of the effect of physiological state on olfactory responses

Building upon the knowledge that physiological state of the female mosquito can influence the sensitivity of the ORNs (**Chapter 4**), it would be worthwhile to investigate if the increased sensitivity observed in females that completed a full gonadotrophic cycle is permanent or transient. If the sensitivity changes are permanent it would indicate that physiological plasticity occurs after having taken a blood meal that provides the female with sufficient nutrients for egg development. Fundamentally, this leads to a very interesting new research area of phenotypic plasticity of insect behaviour. What causes this increased sensitivity? Are more or different AgORs activated or expressed? What happens after a 'poor' blood meal that did not contain enough or had fewer nutrients for egg development? From a more practical and

applied point of view it would be interesting to know if an association takes place between nutrient-rich blood and the odour pattern of the host. What if a female *An. gambiae* takes its first blood meal from a cow? Do the receptors still show a greater sensitivity to human odours or rather to cow-related odours? With the knowledge that *An. arabiensis*, the more zoophilic member of the *An. gambiae* complex, has a greater preference for livestock than humans, it would be of interest to see to what extent the responses between both species are the same. Can a single first blood meal alter or influence its host specificity?

More importantly, if the sensitivity changes are permanent, this may provide the opportunity to develop a blend of odours that specifically targets that part of the mosquito population that already had a blood meal and thus has the potential to transmit diseases. Odours that elicit stronger responses from specific ORNs after oviposition compared to the same ORNs in non-bloodfed mosquitoes can be used to develop an attractive blend of odours that targets the disease-transmitting part of the mosquito population. Similar reasoning also holds for odours that were down-regulated directly after the blood meal, because host seeking is reduced in this period. As previously mentioned, in the light of these findings it would also be exciting to investigate if the prolonged suppression of the CO_2 neuron also occurs at elevated levels of CO_2.

Further investigation of the potential of CO_2 inhibitors

The inhibition of responses on both electrophysiological as well as behavioural levels represents a clear embodiment of the overall goal, 'the discovery and utilisation of infochemicals to disrupt host-seeking behaviour of the malaria mosquito *Anopheles gambiae*', of the Grand Challenge in Global Health project #121 that constituted the framework for this thesis. With odours that inhibit the CO_2 response it may be possible to alter host seeking, but some caution should be exercised in assigning a repellent action to inhibitors of CO_2-sensitive neurons. The work presented in this thesis clearly shows that odours that block the CO_2 receptor can at the same time elicit responses from multiple ORNs on both antenna and maxillary palps (**Chapter 2** and **Chapter 4**). The behavioural experiments carried out so far underline these results. A prerequisite for using CO_2-neuron inhibitors is that they are also tested for their effect in the absence of CO_2. There might be compounds that only block the CO_2 receptor without evoking responses from other ORNs. Still, in the line of reasoning that host-location by *An. gambiae* uses other odours than CO_2 at a closer range (Spitzen 2008), CO_2-neuron inhibitors cannot be used as repellents but as masking odours or as blockers of the CO_2-based long range searching behaviour.

A potential use for odours like these five bacterial compounds that by CO_2-neuron inhibition divert the attention of the mosquito towards other odours for host location, is in a barrier-trapping system. By placing odour-release systems around houses or villages with directly behind it a trapping system with an attractive blend, like the blend described by Okumu *et al.* (2010), the CO_2 released by the people in the huts act as a CO_2 source. By placing a barrier of these CO_2 inhibitors, one can divert the mosquitoes away from the CO_2 emanating from the

huts to the odours released from the traps placed just behind the barrier. Important at this stage is to investigate the effect of the synthetic blend of bacterial volatiles tested by Verhulst *et al.* (2009) in the presence of CO_2 and to test whether the concentration of the inhibitors can be lowered if tested all together.

Testing the best ligands for their effect on oviposition behaviour

Though the main objective of this project was the use of odours for disruption of host-seeking behaviour, several of the odours identified in **Chapter 2** as well as some of the bacterial volatiles used in **Chapter 4**, have also been described as potentially involved in the selection of oviposition sites. If these odours do not seem to affect host seeking much they should be further investigated for possible effects on the selection of oviposition sites. Compounds like 4-methylcyclohexanol, several phenols, 3-methylbutanoic acid and 2-phenylethanol have been described as emanating from oviposition sites via grass infusions or bacterial volatiles (Bentley 1981, Bentley *et al.* 1982, Millar *et al.* 1992, Lindh *et al.* 2008b). At the moment, since the discovery of the IR pathway and the lack of responses to known human-derived mosquito attractants like the carboxylic acids and ammonia, it seems as if the trichoid sensilla may be less important for host-seeking behaviour. Nonetheless, it may be that these odours that elicit strong responses from the trichoid sensilla are involved in different odour-based behaviours of the mosquito like nectar feeding, oviposition-site selection or mating behaviour. Each of these stages in the mosquito's life cycle provides a potential target for odour-based disruption of mosquito behaviour in order to reduce malaria transmission.

Study the effect *Plasmodium* infection has on the olfactory responses to humans

An infection with the *Plasmodium* parasite alters the behaviour of the mosquito. Mosquitoes infected with sporozoites, the transmittable form of the parasite, were shown to take larger blood meals than uninfected mosquitoes. A higher proportion of sporozoite-infected mosquitoes took blood meals from different persons on the same night (Koella and Packer 1996, Koella *et al.* 1998). More recently Ferguson and Read (2004) showed that *Plasmodium chabaudi*-infected *An. stephensi* were more drawn to already infected mice. This suggests that a *Plasmodium* infection not only alters the host odour, it also suggests that infected mosquitoes might use other or additional odours to locate hosts compared to uninfected mosquitoes. To study this in more detail it is possible to check if the ORN-response types identified thus far will change when mosquitoes become infected with *Plasmodium*. If differences are observed this would provide a starting point for further characterisation using human-derived odours. Possibly, stronger or weaker responses might be indicative of odours involved in the additional attraction of infected mosquitoes. A tailored volatile blend based on this knowledge can be more effective in targeting the infected part of the mosquito population.

Conclusion

Malaria still remains an enormous problem for many tropical countries. In 2010 the malaria report of the WHO revealed that malaria still caused the deaths of over 781.000 persons, 85% of them children (WHO 2010). The African malaria mosquito *An. gambiae* Giles *sensu stricto* is one of the major malaria vectors in sub-Saharan Africa capable of transmitting *P. falciparum*, the parasite that accounts for the majority of all deaths that occurred. Females of *An. gambiae* predominantly use odours to locate their sugar meals, human blood hosts and oviposition sites. The results presented in this thesis increase our knowledge on the peripheral olfactory system of the malaria mosquito *An. gambiae* and identifies several candidate odours for olfactory manipulation of host-seeking behaviour. It provides a firm basis for further electrophysiological research and at the same time offers additional suggestions for behavioural research. Especially the changing sensitivity of palpal ORNs with changing physiological state and its possible effect on inhibition of the CO_2-sensitive neuron are interesting targets for further behavioural research. All of these findings are promising for future odour-based control and/or killing strategies for malaria mosquitoes, which can present a valuable asset for the integrated vector-control measures in the fight against malaria.

References

Allan, S. A., and D. L. Kline. 1995. Evaluation of organic infusions and synthetic compounds mediating oviposition in *Aedes albopictus* and *Aedes aegypti* (Diptera: Culicidae). Journal of Chemical Ecology 21: 1847-1860.

Almaas, T. J., T. A. Christensen, and H. Mustaparta. 1991. Chemical communication in heliothine moths. Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology 169: 249-258.

Anton, S., J. J. A. van Loon, J. Meijerink, H. M. Smid, W. Takken, and J. P. Rospars. 2003. Central projections of olfactory receptor neurons from single antennal and palpal sensilla in mosquitoes. Arthropod Structure & Development 32: 319-327.

Arensburger, P., K. Megy, R. M. Waterhouse, J. Abrudan, P. Amedeo, B. Antelo, L. Bartholomay, S. Bidwell, E. Caler, F. Camara, *et al.* 2010. Sequencing of *Culex quinquefasciatus* establishes a platform for mosquito comparative genomics. Science 330: 86-88.

Bazhenov, M., M. Stopfer, M. Rabinovich, H. D. I. Abarbanel, T. J. Sejnowski, and G. Laurent. 2001a. Model of cellular and network mechanisms for odor-evoked temporal patterning in the locust antennal lobe. Neuron 30: 569-581.

Bazhenov, M., M. Stopfer, M. Rabinovich, R. Huerta, H. D. I. Abarbanel, T. J. Sejnowski, and G. Laurent. 2001b. Model of transient oscillatory synchronization in the locust antennal lobe. Neuron 30: 553-567.

Beehler, J. W., J. G. Millar, and M. S. Mulla. 1994. Field evaluation of synthetic compounds mediating oviposition in *Culex* mosquitoes (Diptera: Culicidae). Journal of Chemical Ecology 20: 281-291.

Bentley, M. D. 1981. Oviposition attractants and stimulants of *Aedes triseriatus* (Say) (Diptera: Culicidae). Environmental Entomology 10: 186-189.

Bentley, M. D., and J. F. Day. 1989. Chemical ecology and behavioral aspects of mosquito oviposition. Annual Review of Entomology 34: 401-421.

Bentley, M. D., I. N. McDaniel, and E. E. Davis. 1982. Studies of 4-methylcyclohexanol: An *Aedes triseriatus* (Diptera: Culicidae) oviposition attractant. Journal of Medical Entomology 19: 589-592.

Bentley, M. D., I. N. McDaniel, M. Yatagai, H. P. Lee, and R. Maynard. 1979. Para-cresol: Oviposition attractant of *Aedes triseriatus* (Diptera: Culicidae). Environmental Entomology 8: 206-209.

Benton, R. 2006. Molecular biology of insect odorant receptors. Journal of Neurogenetics 20: 83-84.

Benton, R., K. S. Vannice, C. Gomez-Diaz, and L. B. Vosshall. 2009. Variant ionotropic glutamate receptors as chemosensory receptors in *Drosophila*. Cell 136: 149-162.

Bernier, U. R., M. M. Booth, and R. A. Yost. 1999. Analysis of human skin emanations by gas chromatography/mass spectrometry. 1.Thermal desorption of attractants for the yellow fever mosquito (*Aedes aegypti*) from handled glass beads. Analytical Chemistry 71: 1-7.

Bernier, U. R., D. L. Kline, D. R. Barnard, C. E. Schreck, and R. A. Yost. 2000. Analysis of human skin emanations by gas chromatography/mass spectrometry. 2. Identification of volatile compounds that are candidate attractants for yellow fever mosquito (*Aedes aegypti*). Analytical Chemistry 72: 747-756.

Bernier, U. R., D. L. Kline, C. E. Schreck, R. A. Yost, and D. R. Barnard. 2002. Chemical analysis of human skin emanations: Comparison of volatiles from humans that differ in attraction of *Aedes aegypti* (Diptera: Culicidae). Journal of the American Mosquito Control Association 18: 186-195.

Bieber, S. L., and D. V. Smith. 1986. Multivariate analysis of sensory data: a comparison of methods. Chemical Senses 11: 19-47.

Biessmann, H., E. Andronopoulou, M. R. Biessmann, V. Douris, S. D. Dimitratos, E. Eliopoulos, P. M. Guerin, K. Iatrou, R. W. Justice, T. Kröber, *et al.* 2010. The *Anopheles gambiae* odorant binding protein 1 (AgamOBP1) mediates indole recognition in the antennae of female mosquitoes. PLoS ONE 5: e9471.

Blackwell, A., and S. N. Johnson. 2000. Electrophysiological investigation of larval water and potential oviposition chemoattractants for *Anopheles gambiae sensu stricto*. Annals of Tropical Medicine and Parasitology 94: 389-398.

Bohbot, J., R. J. Pitts, H. W. Kwon, M. Rutzler, H. M. Robertson, and L. J. Zwiebel. 2007. Molecular characterization of the *Aedes aegypti* odorant receptor gene family. Insect Molecular Biology 16: 525 - 537.

Boo, K. S. 1980. Fine structure of the antennal sensory hairs in female *Anopheles stephensi*. Zeitschrift für Parasitenkunde 61: 161-171.

Boo, K. S., and S. B. McIver. 1975. Fine-structure of sunken thick-walled pegs (sensilla ampullacea and coeloconica) on antennae of mosquitos. Canadian Journal of Zoology 53: 262.

Boo, K. S., and S. B. McIver. 1976. Fine structure of surface and sunken grooved pegs on the antenna of female *Anopheles stephensi* (Diptera: Culicidae). Canadian Journal of Zoology 54: 235-244.

Bowen, M. 1995. Sensilla basiconica (grooved pegs) on the antennae of female mosquitoes: Electrophysiology and morphology. Entomologia Experimentalis et Applicata 77: 233-238.

Bowen, M. F., E. E. Davis, J. Romo, and D. Haggart. 1994. Lactic-acid sensitive receptors in the autogenous mosquito *Aedes atropalpus*. Journal of Insect Physiology 40: 611-615.

Braks, M. A. H., and W. Takken. 1999. Incubated human sweat but not fresh sweat attracts the malaria mosquito *Anopheles gambiae sensu stricto*. Journal of Chemical Ecology 25: 663-672.

Braks, M. A. H., R. A. Anderson, and B. G. J. Knols. 1999. Infochemicals in mosquito host selection: Human skin microflora and *Plasmodium* parasites. Parasitology Today 15: 409-413.

Braks, M. A. H., J. Meijerink, and W. Takken. 2001. The response of the malaria mosquito, *Anopheles gambiae*, to two components of human sweat, ammonia and L-lactic acid, in an olfactometer. Physiological Entomology 26: 142-148.

Carey, A. F., G. Wang, C. Y. Su, L. J. Zwiebel, and J. R. Carlson. 2010. Odorant reception in the malaria mosquito *Anopheles gambiae*. Nature 464: 66-71.

Cator, L. J., K. R. Ng'Habi, R. R. Hoy, and L. C. Harrington. 2010. Sizing up a mate: Variation in production and response to acoustic signals in *Anopheles gambiae*. Behavioral Ecology 21: 1033-1039.

Clements, A. N. [ed.] 1999. The Biology of Mosquitoes: Sensory Reception and Behaviour. CABI, Wallingford, UK.

Clyne, P. J., C. G. Warr, M. R. Freeman, D. Lessing, J. H. Kim, and J. R. Carlson. 1999. A novel family of divergent seven-transmembrane proteins: Candidate odorant receptors in *Drosophila*. Neuron 22: 327-338.

Coetzee, M. 2004. Distribution of the African malaria vectors of the *Anopheles gambiae* complex. American Journal of Tropical Medicine and Hygiene 70: 103 - 104.

Coetzee, M., M. Craig, and D. Le Sueur. 2000. Distribution of African malaria mosquitoes belonging to the Anopheles gambiae complex. Parasitology Today 16: 74 - 77.

Coluzzi, M., A. Sabatini, A. Della Torre, M. A. Di Deco, and V. Petrarca. 2002. A polytene chromosome analysis of the *Anopheles gambiae* species complex. Science 298: 1415-1418.

Coluzzi, M. M. 1979. Chromosomal differentiation and adaptation to human environment in the *Anopheles gambiae* complex. Transactions of the Royal Society of Tropical Medicine and Hygiene 73: 483.

Cork, A., and K. C. Park. 1996. Identification of electrophysiologically-active compounds for the malaria mosquito, *Anopheles gambiae*, in human sweat extracts. Medical and Veterinary Entomology 10: 269-276.

Couto, A., M. Alenius, and B. J. Dickson. 2005. Molecular, anatomical, and functional organization of the *Drosophila* olfactory system. Current Biology 15: 1535-1547.

Curran, A. M., S. I. Rabin, P. A. Prada, and K. G. Furton. 2005. Comparison of the volatile organic compounds present in human odor using SPME-GC/MS. Journal of Chemical Ecology 31: 1607-1619.

Curran, A. M., C. F. Ramirez, A. A. Schoon, and K. G. Furton. 2007. The frequency of occurrence and discriminatory power of compounds found in human scent across a population determined by SPME-GC/MS. Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences 846: 86-97.

Curtis, C. F. 1996. Options for the control of malaria vectors. Pesticide Outlook: 20-24.

Davis, E. E. 1976. Receptor sensitive to oviposition site attractants on antennae of mosquito, *Aedes aegypti*. Journal of Insect Physiology 22: 1371-1376.

Davis, E. E. 1984. Regulation of sensitivity in the peripheral chemoreceptor systems for host-seeking behaviour by a haemolymph-borne factor in *Aedes aegypti*. Journal of Insect Physiology 30: 179-183.

Davis, E. E. 1985. Insect repellents: Concepts of their mode of action relative to potential sensory mechanisms in mosquitoes (Diptera: Culicidae). Journal of Medical Entomology 22: 237-243.

Davis, E. E. 1988. Structure response relationship of the lactic acid-excited neurons in the antennal grooved-peg sensilla of the mosquito *Aedes aegypti*. Journal of Insect Physiology 34: 443-449.

Davis, E. E., and P. G. Sokolove. 1976. Lactic acid-sensitive receptors on antennae of mosquito, *Aedes aegypti*. Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology 105: 43-54.

Davis, E. E., and M. F. Bowen. 1994. Sensory physiological basis for attraction in mosquitos. Journal of the American Mosquito Control Association 10: 316-325.

de Bruyne, M., P. J. Clyne, and J. R. Carlson. 1999. Odor coding in a model olfactory organ: The *Drosophila* maxillary palp. Journal of Neuroscience 19: 4520-4532.

de Bruyne, M., K. Foster, and J. R. Carlson. 2001. Odor coding in the *Drosophila* antenna. Neuron 30: 537-552.

de Jong, R., and B. G. J. Knols. 1995a. Selection of biting sites on man by two malaria mosquito species. Cellular and Molecular Life Sciences 51: 80-84.

de Jong, R., and B. G. J. Knols. 1995b. Olfactory responses of host-seeking *Anopheles gambiae s.s.* Giles (Diptera: Culicidae). Acta Tropica 59: 333-335.

de Jong, R., and B. G. J. Knols. 1996. Selection of biting sites by mosquitoes. Olfaction in mosquito host interactions 200: 89-103.

Dekker, T., M. Geier, and R. T. Carde. 2005. Carbon dioxide instantly sensitizes female yellow fever mosquitoes to human skin odours. Journal of Experimental Biology 208: 2963-2972.

den Otter, C. J., and W. M. van der Goes van Naters. 1992. Single cell recordings from tsetse (*Glossina m.morsitans*) antennae reveal olfactory, mechano- and cold receptors. Physiological Entomology 17: 33-42.

Ditzen, M., M. Pellegrino, and L. Vosshall. 2008. Insect odorant receptors are molecular targets of the insect repellent DEET. Science 319: 1838 - 1842.

Dobritsa, A. A., W. van der Goes van Naters, C. G. Warr, R. A. Steinbrecht, and J. R. Carlson. 2003. Integrating the molecular and cellular basis of odor coding in the *Drosophila* antenna. Neuron 37: 827-41.

Du, Y. J., and J. G. Millar. 1999. Electroantennogram and oviposition bioassay responses of *Culex quinquefasciatus* and *Culex tarsalis* (Diptera: Culicidae) to chemicals in odors from Bermuda grass infusions. Journal of Medical Entomology 36: 158-166.

Duchamp-Viret, P., A. Duchamp, and M. A. Chaput. 2003. Single olfactory sensory neurons simultaneously integrate the components of an odour mixture. European Journal of Neuroscience 18: 2690-2696.

Eriksson, L., E. Johansson, N. Kettaneh-Wold, J. Trygg, C. Wikström, and S. Wold [eds.]. 1996. Multi- and Megavariate Data Analysis: Part 1 Basic Principles and Applications. Umetrics AB.

Ferguson, H., and A. Read. 2004. Mosquito appetite for blood is stimulated by *Plasmodium chabaudi* infections in themselves and their vertebrate hosts. Malaria Journal 3: 12.

Foster, W. A. 1995. Mosquito sugar feeding and reproductive energetics. Annual Review of Entomology 40: 443-474.

Foster, W. O., and W. Takken. 2004. Nectar-related vs. human-related volatiles: Behavioural response and choice by female and male *Anopheles gambiae* (Diptera: Culicidae) between emergence and first feeding. Bulletin of Entomological Research 94: 145-157.

Fox, A., R. Pitts, H. Robertson, J. Carlson, and L. Zwiebel. 2001. Candidate odorant receptors from the malaria vector mosquito *Anopheles gambiae* and evidence of down-regulation in response to blood feeding. Proceedings of the National Academy of Sciences of the United States of America 98: 14693 - 14697.

Gallagher, M., C. J. Wysocki, J. J. Leyden, A. I. Spielman, X. Sun, and G. Preti. 2008. Analyses of volatile organic compounds from human skin. British Journal of Dermatology 159: 780-791.

Gao, Q., and A. Chess. 1999. Identification of candidate *Drosophila* olfactory receptors from genomic DNA sequence. Genomics 60: 31-39.

Geier, M., H. Sass, and J. Boeckh. 1996. A search for components in human body odour that attract females of *Aedes aegypti*. CIBA Foundation Symposia: 132-148.

Ghaninia, M., B. S. Hansson, and R. Ignell. 2007a. The antennal lobe of the African malaria mosquito, *Anopheles gambiae* - innervation and three-dimensional reconstruction. Arthropod Structure & Development 36: 23-39.

Ghaninia, M., R. Ignell, and B. S. Hansson. 2007b. Functional classification and central nervous projections of olfactory receptor neurons housed in antennal trichoid sensilla of female yellow fever mosquitoes, *Aedes aegypti*. European Journal of Neuroscience 26: 1611-1623.

Gibson, G. 1995. A behavioural test of the sensitivity of a nocturnal mosquito, *Anopheles gambiae*, to dim white, red and infra-red light. Physiological Entomology 20: 224-228.

Gillies, M. T. 1980. The role of carbon dioxide in host-finding by mosquitoes (Diptera: Culicidae): A review. Bulletin of Entomological Research 70: 525-532.

Gillies, M. T., and T. J. Wilkes. 1968. A comparison of the range of attraction of animal baits and of carbon dioxide for some West African mosquitoes. Bulletin of Entomological Research 59: 441-456.

Gillies, M. T., and M. Coetzee. 1987. A supplement to the Anophelinae of Africa South of the Sahara. The South African Institute for Medical Research, Johannesburg.

Goldman, A. L., W. V. van Naters, D. Lessing, C. G. Warr, and J. R. Carlson. 2005. Coexpression of two functional odor receptors in one neuron. Neuron 45: 661-666.

Grant, A. J., B. E. Wigton, and J. G. Aghajanian. 1995. Electrophysiological responses of receptor neurons in mosquito maxillary palp sensilla to carbon dioxide. Journal of

Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology 177: 389-396.

Greenwood. 2005. Malaria. The Lancet 365: 1487.

Hall, D. R., P. S. Beevor, A. Cork, B. F. Nesbitt, and G. A. Vale. 1984. 1-octen-3-ol: A potent olfactory stimulant and attractant for tsetse isolated from cattle odours. Insect Science and its Application 5: 335-339.

Hallem, E. A., A. Dahanukar, and J. R. Carlson. 2006. Insect odor and taste receptors. Annual Review of Entomology 51: 113-135.

Hallem, E. A., A. N. Fox, L. J. Zwiebel, and J. R. Carlson. 2004. Olfaction: Mosquito receptor for human-sweat odorant. Nature 427: 212-213.

Hansson, B. S. 1999. Insect olfaction. Springer, Berlin.

Hay, S. I., C. A. Guerra, A. J. Tatem, A. M. Noor, and R. W. Snow. 2004. The global distribution and population at risk of malaria: Past, present, and future. The Lancet Infectious Diseases 4: 327-336.

Healy, T. P., and M. J. W. Copland. 2000. Human sweat and 2-oxopentanoic acid elicit a landing response from *Anopheles gambiae*. Medical and Veterinary Entomology 14: 195-200.

Hill, C. A., A. N. Fox, R. J. Pitts, L. B. Kent, P. L. Tan, M. A. Chrystal, A. F. Cravchik, H. Collins, H. M. Robertson, and L. J. Zwiebel. 2002. G-protein-coupled receptors in *Anopheles gambiae*. Science 298: 176 - 178.

Hill, S. R., B. S. Hansson, and R. Ignell. 2009. Characterization of antennal trichoid sensilla from female Southern house mosquito, *Culex quinquefasciatus* Say. Chemical Senses 34: 231-252.

Holt, R. A., G. M. Subramanian, A. Halpern, G. G. Sutton, R. Charlab, D. R. Nusskern, P. Wincker, A. G. Clark, J. M. C. Ribeiro, R. Wides, *et al.* 2002. The genome sequence of the malaria mosquito *Anopheles gambiae*. Science 298: 129-149.

Hunt, R. H., M. Coetzee, and M. Fettene. 1998. The *Anopheles gambiae* complex: a new species from Ethiopia. Transactions of the Royal Society of Tropical Medicine and Hygiene 92: 231-5.

Ismail, I. A. 1964. Comparative study of sense organs in the antennae of culicine and anopheline female mosquitoes. Acta Tropica 21: 155-68.

Jones, W. D., P. Cayirlioglu, I. Grunwald Kadow, and L. B. Vosshall. 2007. Two chemosensory receptors together mediate carbon dioxide detection in *Drosophila*. Nature 445: 86-90.

Jones, W. D., T. A. T. Nguyen, B. Kloss, K. J. Lee, and L. B. Vosshall. 2005. Functional conservation of an insect odorant receptor gene across 250 million years of evolution. Current Biology 15: 119-121.

Kelling, F. J., F. Ialenti, and C. J. d. Otter. 2002. Background odour induces adaptation and sensitization of olfactory receptors in the antennae of houseflies. Medical and Veterinary Entomology 16: 161-169.

Kellogg, F. E. 1970. Water vapour and carbon dioxide receptors in *Aedes aegypti*. Journal of Insect Physiology 16: 99-108.

Kent, L. B. 2008. The Gr family of candidate gustatory and olfactory receptors in the yellow-fever mosquito *Aedes aegypti*. Chemical Senses 33: 79-93.

Kliewer, J. W., T. Miura, R. C. Husbands, and C. H. Hurst. 1966. Sex Pheromones and mating behavior of *Culiseta inornata* (Diptera: Culicidae). Annals of the Entomological Society of America 59: 530-532.

Kline, D. L., J. R. Wood, and J. A. Cornell. 1991a. Interactive effects of 1-octen-3-ol and carbon dioxide on mosquito (Diptera: Culicidae) surveillance and control. Journal of Medical Entomology 28: 254-258.

Kline, D. L., D. A. Dame, and M. V. Meisch. 1991b. Evaluation of 1-octen-3-ol and carbon dioxide as attractants for mosquitoes associated with irrigated rice fields in Arkansas. Journal of the American Mosquito Control Association 7: 165-169.

Klowden, M. J. 1987. Role of the fat-body in the regulation of host-seeking behavior in the mosquito, *Aedes aegypti*. Journal of Insect Physiology 33: 643-646.

Klowden, M. J., and A. O. Lea. 1978. Blood meal size as a factor affecting continued host-seeking by *Aedes aegypti* (L). American Journal of Tropical Medicine and Hygiene 27: 827-831.

Klowden, M. J., and A. O. Lea. 1979. Humoral inhibition of host-seeking in *Aedes aegypti* during oocyte maturation. Journal of Insect Physiology 25: 231-235.

Knols, B. G., R. de Jong, and W. Takken. 1995a. Differential attractiveness of isolated humans to mosquitoes in Tanzania. Transactions of the Royal Society of Tropical Medicine and Hygiene 89: 604 - 606.

Knols, B. G. J., W. Takken, D. Charlwood, and R. d. Jong. 1995b. Species-specific attraction of *Anopheles* mosquitoes (Diptera: Culicidae) to different humans in South Tanzania, pp. 201-206, Proceedings of Experimental and Applied Entomology, Amsterdam.

Knols, B. G. J., W. Takken, A. Cork, and R. DeJong. 1997a. Odour-mediated, host-seeking behaviour of *Anopheles* mosquitoes: A new approach. Annals of Tropical Medicine and Parasitology 91: S117-S118.

Knols, B. G. J., J. J. A. Van Loon, A. Cork, R. D. Robinson, W. Adam, J. Meijerink, R. De Jong, and W. Takken. 1997b. Behavioural and electrophysiological responses of the female malaria mosquito *Anopheles gambiae* (Diptera: Culicidae) to Limburger cheese volatiles. Bulletin of Entomological Research 87: 151-159.

Koella, J., and M. Packer. 1996. Malaria parasites enhance blood-feeding of their naturally infected vector *Anopheles punctulatus*. Parasitology 113: 105 - 109.

Koella, J. C., F. L. Sorensen, and R. A. Anderson. 1998. The malaria parasite, *Plasmodium falciparum*, increases the frequency of multiple feeding of its mosquito vector, *Anopheles gambiae*. Proceedings of the Royal Society B: Biological Sciences 265: 763 - 768.

Kwon, H., T. Lu, M. Rutzler, and L. Zwiebel. 2006. Olfactory responses in a gustatory organ of the malaria vector mosquito *Anopheles gambiae*. Proceedings of the National Academy of Sciences of the United States of America 103: 13526 - 13531.

Kwon, J. Y., A. Dahanukar, L. A. Weiss, and J. R. Carlson. 2007. The molecular basis of CO_2 reception in *Drosophila*. Proceedings of the National Academy of Sciences of the United States of America 104: 3574-3578.

Laarman, J. J. 1955. The host-seeking behaviour of the malaria mosquito *Anopheles maculipennis atroparvus*. PhD dissertation, University of Leiden 144.

Lacher, V. 1967. Elektrophysiologische untersuchungen an einzelnen geruchsrezeptoren auf den antennen weiblicher moskitos (*Aedes aegypti* L.). Journal of Insect Physiology 13: 1461-1464, IN3, 1465-1470.

Laurent, G. 2002. Olfactory network dynamics and the coding of multidimensional signals. Nature Reviews Neuroscience 3: 884-895.

Li, Z. X., J. A. Pickett, L. M. Field, and J. J. Zhou. 2005. Identification and expression of odorant-binding proteins of the malaria-carrying mosquitoes *Anopheles gambiae* and *Anopheles arabiensis*. Archives of Insect Biochemistry and Physiology 58: 175-189.

Lindh, J. M., A. K. Borg-Karlson, and I. Faye. 2008a. Transstadial and horizontal transfer of bacteria within a colony of *Anopheles gambiae* (Diptera: Culicidae) and oviposition response to bacteria-containing water. Acta Tropica 107: 242-250.

Lindh, J. M., A. KÃ¤nnaste, B. G. J. Knols, I. Faye, and A. K. Borg-Karlson. 2008b. Oviposition responses of *Anopheles gambiae s.s.* (Diptera: Culicidae) and identification of volatiles from bacteria-containing solutions. Journal of Medical Entomology 45: 1039-1049.

Linley, J. R. 1989. Laboratory tests of the effects of p-cresol and 4-methylcyclohexanol on oviposition by three species of *Toxorhynchites* mosquitoes. Medical and Veterinary Entomology 3: 347-352.

Liu, C., R. J. Pitts, J. D. Bohbot, P. L. Jones, G. Wang, and L. J. Zwiebel. 2010. Distinct olfactory signaling mechanisms in the malaria vector mosquito *Anopheles gambiae*. PLoS Biology 8: e1000467.

Logan, J., M. Birkett, S. Clark, S. Powers, N. Seal, L. Wadhams, A. Mordue, and J. Pickett. 2008. Identification of human-derived volatile chemicals that interfere with attraction of *Aedes aegypti* mosquitoes. Journal of Chemical Ecology 34: 308-322.

Lu, T., Y. T. Qiu, G. Wang, J. Y. Kwon, M. Rutzler, H.-W. Kwon, R. J. Pitts, J. J. A. van Loon, W. Takken, J. R. Carlson, *et al.* 2007. Odor coding in the maxillary palp of the malaria vector mosquito *Anopheles gambiae*. Current Biology 17: 1533-1544.

Majeed, S., S. R. Hill, J. Cook, J. G. Logan, G. Birgersson, and R. Ignell. 2010. Differential responses to two human kairomonal cues in *Aedes aegypti* and *Culex quinquefasciatus* mosquitoes., International Society of Chemical Ecology, Tours, France.

Marchand, R. P. 1984. Field observations on swarming and mating in *Anopheles gambiae* mosquitoes in Tanzania. Netherlands Journal of Zoology 34: 367-387.

Mboera, L. E. G. 2000. Comparison of carbon dioxide-baited trapping systems for sampling outdoor mosquito populations in Tanzania. Medical and Veterinary Entomology 14: 257-263.

McCall, P. J., and G. Eaton. 2001. Olfactory memory in the mosquito *Culex quinquefasciatus*. Medical and Veterinary Entomology 15: 197-203.

McIver, S. 1978. Structure of sensilla trichodea of female *Aedes aegypti* with comments on innervation of antennal sensilla. Journal of Insect Physiology 24: 383-390.

McIver, S., and R. Siemicki. 1975. Palpal sensilla of selected anopheline mosquitoes. Journal of Parasitology 61: 535-538.

McIver, S. B. 1968. Host preferences and discrimination by the mosquitoes *Aedes aegypti* and *Culex tarsalis* (Diptera: Culicidae). Journal of Medical Entomology 5: 422-428.

McIver, S. B. 1972. Fine structure of pegs on the palps of female culicine mosquitoes. Canadian Journal of Zoology 50: 571-576.

McIver, S. B. 1974. Fine structure of antennal grooved pegs of mosquito, *Aedes aegypti*. Cell and Tissue Research 153: 327-337.

McIver, S. B. 1982. Sensilla of mosquitoes (Diptera: Culicidae). Journal of Medical Entomology 19: 489-535.

Meijerink, J. 1999. Olfaction in the malaria mosquito *Anopheles gambiae*: Electrophysiology and identification of kairomones, Entomology. Wageningen University, Wageningen.

Meijerink, J., and J. J. A. van Loon. 1999. Sensitivities of antennal olfactory neurons of the malaria mosquito, *Anopheles gambiae*, to carboxylic acids. Journal of Insect Physiology 45: 365-373.

Meijerink, J., M. A. H. Braks, and J. J. A. van Loon. 2001. Olfactory receptors on the antennae of the malaria mosquito *Anopheles gambiae* are sensitive to ammonia and other sweat-borne components. Journal of Insect Physiology 47: 455-464.

Meijerink, J., M. A. H. Braks, A. A. Brack, W. Adam, T. Dekker, M. A. Posthumus, T. A. Van Beek, and J. J. A. van Loon. 2000. Identification of olfactory stimulants for *Anopheles gambiae* from human sweat samples. Journal of Chemical Ecology 26: 1367-1382.

Melo, A. C. A., M. Rützler, R. J. Pitts, and L. J. Zwiebel. 2004. Identification of a chemosensory receptor from the yellow fever mosquito, *Aedes aegypti*, that is highly conserved and expressed in olfactory and gustatory organs. Chemical Senses 29: 403-410.

Millar, J. G., J. D. Chaney, and M. S. Mulla. 1992. Identification of oviposition attractants for *Culex quinquefasciatus* from fermented Bermuda grass infusions. Journal of the American Mosquito Control Association 8: 11-17.

Muir, L. E., and B. W. Cribb. 1994. *Aedes aegypti*: Sensilla trichodea and stimulus-conducting structures. Journal of Insect Physiology 40: 1017-1023.

Nene, V., J. R. Wortman, D. Lawson, B. Haas, C. Kodira, Z. Tu, B. Loftus, Z. Xi, K. Megy, M. Grabherr, *et al.* 2007. Genome sequence of *Aedes aegypti*, a major arbovirus vector. Science 316: 1718-1723.

Neuhaus, E. M., G. Gisselmann, W. Zhang, R. Dooley, K. Stortkuhl, and H. Hatt. 2005. Odorant receptor heterodimerization in the olfactory system of *Drosophila melanogaster*. Nature Neuroscience 8: 15-17.

Nijhout, H. F., and G. B. Graig. 1971. Reproductive isolation in *Stegomyia* mosquitoes III: Evidence for a sexual pheromone. Entomologia Experimentalis et Applicata 14: 399-412.

Okumu, F., E. Titus, E. Mbeyela, G. Killeen, and S. Moore. 2009. Limitation of using synthetic human odours to test mosquito repellents. Malaria Journal 8: 150.

Okumu, F. O. 2008. Medium range olfactory responses of the malaria vector *Anopheles gambiae s.s* to synthetic odor blends. University of Nairobi, Nairobi.

Okumu, F. O., G. F. Killeen, S. Ogoma, L. Biswaro, R. C. Smallegange, E. Mbeyela, E. Titus, C. Munk, H. Ngonyani, W. Takken, *et al.* 2010. Development and field evaluation of a synthetic mosquito lure that is more attractive than humans. PLoS ONE 5: e8951.

Olanga, E., M. Okal, P. Mbadi, E. Kokwaro, and W. Mukabana. 2010. Attraction of *Anopheles gambiae* to odour baits augmented with heat and moisture. Malaria Journal 9: 6.

Olsson, S. B., C. E. Linn, and W. L. Roelofs. 2006. The chemosensory basis for behavioral divergence involved in sympatric host shifts II: Olfactory receptor neuron sensitivity and temporal firing pattern to individual key host volatiles. Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology 192: 289-300.

Pappas, L. G., and J. R. Larsen. 1976. Gustatory hairs on the mosquito, *Culiseta inornata*. Journal of Experimental Zoology 196: 351-360.

Pates, H. V., W. Takken, K. Stuke, and C. F. Curtis. 2001. Differential behaviour of *Anopheles gambiae sensu stricto* (Diptera: Culicidae) to human and cow odours in the laboratory. Bulletin of Entomological Research 91: 289-296.

Pelletier, J., A. Guidolin, Z. Syed, A. Cornel, and W. Leal. 2010. Knockdown of a mosquito odorant-binding protein involved in the sensitive detection of oviposition attractants. Journal of Chemical Ecology 36: 245-248.

Pelosi, P. P. 2006. Soluble proteins in insect chemical communication. Cellular and Molecular Life Sciences 63: 1658-1676.

Penn, D. J., E. Oberzaucher, K. Grammer, G. Fischer, H. A. Soini, D. Wiesler, M. V. Novotny, S. J. Dixon, Y. Xu, and R. G. Brereton. 2007. Individual and gender fingerprints in human body odour. Journal of the Royal Society Interface 4: 331-340.

Pennetier, C., B. Warren, K. R. Dabiré, I. J. Russell, and G. Gibson. 2010. "Singing on the wing" as a mechanism for species recognition in the malaria mosquito *Anopheles gambiae*. Current Biology 20: 131-136.

Pitts, R. J., and L. J. Zwiebel. 2006. Antennal sensilla of two female anopheline sibling species with differing host ranges. Malaria Journal 5:26.

Pitts, R. J., A. N. Fox, and L. J. Zwiebel. 2004. A highly conserved candidate chemoreceptor expressed in both olfactory and gustatory tissues in the malaria vector *Anopheles gambiae*. Proceedings of the National Academy of Sciences of the United States of America 101: 5058-5063.

Polerstock, A. R. 2002. Mating alters the cuticular hydrocarbons of female *Anopheles gambiae sensu stricto* and *Aedes aegypti* (Diptera: Culicidae). Journal of Medical Entomology 39: 545-552.

Qiu, Y. T., J. J. A. van Loon, W. Takken, J. Meijerink, and H. M. Smid. 2006. Olfactory coding in antennal neurons of the malaria mosquito, *Anopheles gambiae*. Chemical Senses 31: 845-863.

Qiu, Y. T., R. C. Smallegange, S. Hoppe, J. J. A. van Loon, E.-J. Bakker, and W. Takken. 2004. Behavioural and physiological responses of the malaria vector mosquito *Anopheles gambiae* to human skin emanations. Medical and Veterinary Entomology 18.

Raman, B., J. Joseph, J. Tang, and M. Stopfer. 2010. Temporally diverse firing patterns in olfactory receptor neurons underlie spatiotemporal neural codes for odors. Journal of Neuroscience 30: 1994-2006.

Rejmankova, E. 2005. Volatile substances from larval habitats mediate species-specific oviposition in *Anopheles* mosquitoes. Journal of Medical Entomology 42: 95-103.

Rospars, J.-P., P. Lansky, M. Chaput, and P. Duchamp-Viret. 2008. Competitive and Noncompetitive Odorant Interactions in the Early Neural Coding of Odorant Mixtures. Journal of Neuroscience 28: 2659-2666.

Rowland, M. 1989. Changes in the circadian flight activity of the mosquito *Anopheles stephensi* associated with insemination, blood-feeding, oviposition and nocturnal light intensity. Physiological Entomology 14: 77-84.

Said, I. 2005. Interactions between acetoin, a plant volatile, and pheromone in *Rhynchophorus palmarum*: Behavioral and olfactory neuron responses. Journal of Chemical Ecology 31: 1789-1805.

Sato, K., M. Pellegrino, T. Nakagawa, L. B. Vosshall, and K. Touhara. 2008. Insect olfactory receptors are heteromeric ligand-gated ion channels. Nature 452: 1002-1006.

Schroder, R., and M. Hilker. 2008. The relevance of background odor in resource location by insects: A behavioral approach. BioScience 58: 308-316.

Scott, J. A., W. G. Brogdon, and F. H. Collins. 1993. Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain-reaction. American Journal of Tropical Medicine and Hygiene 49: 520-529.

Service, M. W. [ed.] 1993. Mosquitoes (Culicidae). Chapman & Hall, London.

Shanbhag, S. R., B. Muller, and R. A. Steinbrecht. 1999. Atlas of olfactory organs of *Drosophila melanogaster* - 1. Types, external organization, innervation and distribution of olfactory sensilla. International Journal of Insect Morphology & Embryology 28: 377-397.

Shanbhag, S. R., B. Muller, and R. A. Steinbrecht. 2000. Atlas of olfactory organs of *Drosophila melanogaster* 2. Internal organization and cellular architecture of olfactory sensilla. Arthropod Structure & Development 29: 211-229.

Siju, K. P., S. R. Hill, B. S. Hansson, and R. Ignell. 2010. Influence of blood meal on the responsiveness of olfactory receptor neurons in antennal sensilla trichodea of the yellow fever mosquito, *Aedes aegypti*. Journal of Insect Physiology 56: 659-665.

Silbering, A. F., and R. Benton. 2010. Ionotropic and metabotropic mechanisms in chemoreception: Chance or design? EMBO Reports 11: 173-179.

Smallegange, R. C., Y. T. Qiu, J. J. A. van Loon, and W. Takken. 2005a. Synergism between ammonia, lactic acid and carboxylic acids as kairomones in the host-seeking behaviour of the malaria mosquito *Anopheles gambiae sensu stricto* (Diptera: Culicidae). Chemical Senses 30: 145 - 152.

Smallegange, R. C., Y. T. Qiu, G. Bukovinszkiné-Kiss, J. J. A. Van Loon, and W. Takken. 2009. The effect of aliphatic carboxylic acids on olfaction-based host-seeking of the malaria mosquito *Anopheles gambiae sensu stricto*. Journal of Chemical Ecology 35: 933-943.

Smallegange, R. C., B. G. J. Knols, and W. Takken. 2010. Effectiveness of synthetic versus natural human volatiles as attractants for *Anopheles gambiae* (Diptera: Culicidae) *sensu stricto*. Journal of Medical Entomology 47: 338-344.

Smallegange, R. C., Y. T. Qiu, J. J. A. van Loon, and W. Takken. 2005b. Synergism between ammonia, lactic acid and carboxylic acids as kairomones in the host-seeking behaviour of the malaria mosquito *Anopheles gambiae sensu stricto* (Diptera: Culicidae). Chemical Senses 30: 145-152.

Smith, C. N., N. Smith, H. K. Gouck, D. E. Weidhaas, I. H. Gilbert, M. S. Mayer, B. J. Smittle, and A. Hofbauer. 1970. L-lactic acid as a factor in the attraction of *Aedes aegypti* (Diptera: Culicidae) to human hosts. Annals of the Entomological Society of America 63: 760-770.

Snow, R. D. L. 2005. The global distribution of clinical episodes of *Plasmodium falciparum* malaria. Nature 434: 214-217.

Spitzen, J. 2008. Effect of human odours and positioning of CO_2 release point on trap catches of the malaria mosquito *Anopheles gambiae sensu stricto* in an olfactometer. Physiological Entomology 33: 116-122.

Spitzen, J., C. W. Spoor, S. Kranenbarg, J. Beeuwkes, F. Grieco, L. P. J. J. Noldus, J. L. van Leeuwen, and W. Takken. 2008. Track3D: Visualization and flight track analysis of *Anopheles gambiae s.s.* mosquitoes. Proceedings of Measuring Behavior 2008.

Steinbrecht, R. A. 1997. Pore structures in insect olfactory sensilla: A review of data and concepts. International Journal of Insect Morphology & Embryology 26: 229-245.

Su, C. Y., K. Menuz, and J. R. Carlson. 2009. Olfactory Perception: Receptors, Cells, and Circuits. Cell 139: 45-59.

Sumba, L. A., K. Okoth, A. L. Deng, J. Githure, B. G. Knols, J. C. Beier, and A. Hassanali. 2004. Daily oviposition patterns of the African malaria mosquito *Anopheles gambiae* Giles (Diptera: Culicidae) on different types of aqueous substrates. Journal of Circadian Rhythms 2: 6.

Sutcliffe, J. F. 1994. Sensory bases of attractancy: morphology of mosquito olfactory sensilla - A review. Journal of the American Mosquito Control Association 10: 309-315.

Syed, Z., and W. S. Leal. 2007. Maxillary palps are broad spectrum odorant detectors in *Culex quinquefasciatus*. Chemical Senses 32: 727-738.

Syed, Z., and W. Leal. 2008. Mosquitoes smell and avoid the insect repellent DEET. Proceedings of the National Academy of Sciences of the United States of America 105: 13598 - 13603.

Syed, Z. A. D. 2006. Pheromone reception in fruit flies expressing a moth's odorant receptor. Proceedings of the National Academy of Sciences of the United States of America 103: 16538-16543.

Takken, W. 1991. The role of olfaction in host-seeking of mosquitoes: A review. Insect Science and its Application 12: 287-295.

Takken, W., and D. L. Kline. 1989. Carbon dioxide and 1-octen-3-ol as mosquito attractants. Journal of the American Mosquito Control Association 5: 311-316.

Takken, W., and B. G. J. Knols. 1999. Odor-mediated behavior of Afrotropical malaria mosquitoes. Annual Review of Entomology 44: 131 - 57.

Takken, W., B. G. J. Knols, and H. Otten. 1997. Interactions between physical and olfactory cues in the host-seeking behaviour of mosquitoes: The role of relative humidity. Annals of Tropical Medicine and Parasitology 91.

Takken, W., M. J. Klowden, and G. M. Chambers. 1998. Effect of body size on host seeking and blood meal utilization in *Anopheles gambiae sensu stricto* (Diptera: Culicidae): the disadvantage of being small. Journal of Medical Entomology 35: 639-645.

Takken, W., J. J. A. van Loon, and W. Adam. 2001. Inhibition of host-seeking response and olfactory responsiveness in *Anopheles gambiae* following blood feeding. Journal of Insect Physiology 47: 303 - 310.

Touhara, K. 2009. Insect olfactory receptor complex functions as a ligand-gated ionotropic channel, pp. 177-180. *In* T. E. Finger [ed.].

Tripet, F. 2004. The "wingbeat hypothesis" of reproductive isolation between members of the *Anopheles gambiae* complex (Diptera: Culicidae) does not fly. Journal of Medical Entomology 41: 375-384.

Turner, S. L., and A. Ray. 2009. Modification of CO_2 avoidance behaviour in *Drosophila* by inhibitory odorants. Nature 461: 277-281.

Vale, G. A., and D. R. Hall. 1985. The role of 1-octen-3-ol, acetone and carbon dioxide in the attraction of tsetse flies, *Glossina spp.* (Diptera: Glossinidae), to ox odour. Bulletin of Entomological Research 75: 209-218.

van Alphen, J. J. M., C. Bernstein, and G. Driessen. 2003. Information acquisition and time allocation in insect parasitoids. Trends in Ecology & Evolution 18: 81-87.

van den Broek, I. V. F., and C. J. den Otter. 1999. Olfactory sensitivities of mosquitoes with different host preferences (*Anopheles gambiae s.s., An. arabiensis, An. quadriannulatus, An. m. atroparvus*) to synthetic host odours. Journal of Insect Physiology 45: 1001-1010.

van den Broek, I. V. F., and C. J. den Otter. 2000. Odour sensitivity of antennal olfactory cells underlying grooved pegs of *Anopheles gambiae s.s.* and *An. quadriannulatus*. Entomologia Experimentalis et Applicata 96: 167-175.

Verhulst, N. O. 2010. The role of skin microbiota in the attractiveness of humans to the malaria mosquito *Anopheles gambiae* Giles, pp. 236, Entomology. Wageningen University, Wageningen.

Verhulst, N. O., H. Beijleveld, B. G. Knols, W. Takken, G. Schraa, H. J. Bouwmeester, and R. C. Smallegange. 2009. Cultured skin microbiota attracts malaria mosquitoes. Malaria Journal 8.

Vosshall, L. B., A. M. Wong, and R. Axel. 2000. An olfactory sensory map in the fly brain. Cell 102: 147-159.

Vosshall, L. B., H. Amrein, P. S. Morozov, A. Rzhetsky, and R. Axel. 1999. A spatial map of olfactory receptor expression in the *Drosophila* antenna. Cell 96: 725-736.

Waage, J. K. 1979. Foraging for patchily-distributed hosts by the parasitoid, *Nemeritis canescens*. Journal of Animal Ecology 48: 353-371.

Wang, G., A. F. Carey, J. R. Carlson, and L. J. Zwiebel. 2010. Molecular basis of odor coding in the malaria vector mosquito *Anopheles gambiae*. Proceedings of the National Academy of Sciences of the United States of America 107: 4418-4423.

Wang, J. W., A. M. Wong, J. Flores, L. B. Vosshall, and R. Axel. 2003. Two-photon calcium imaging reveals an odor-evoked map of activity in the fly brain. Cell 112: 271-282.

Wehr, M., and G. Laurent. 1999. Relationship between afferent and central temporal patterns in the locust olfactory system. Journal of Neuroscience 19: 381-390.

Wetzel, C. H., H.-J. Behrendt, G. Gisselmann, K. F. Störtkuhl, B. Hovemann, and H. Hatt. 2001. Functional expression and characterization of a *Drosophila* odorant receptor in a heterologous cell system. Proceedings of the National Academy of Sciences of the United States of America 98: 9377-9380.

White, G. B. 1974. *Anopheles gambiae* complex and disease transmission in Africa. Transactions of the Royal Society of Tropical Medicine and Hygiene 68: 278-302.

WHO. 2009. World Malaria Report 2009. World Health Organization.

WHO. 2010. World Malaria Report 2010. World Health Organization.

Wicher, D., R. Schafer, R. Bauernfeind, M. C. Stensmyr, R. Heller, S. H. Heinemann, and B. S. Hansson. 2008. *Drosophila* odorant receptors are both ligand-gated and cyclic-nucleotide-activated cation channels. Nature 452: 1007-1011.

Wilson, R. I., and Z. F. Mainen. 2006. Early events in olfactory processing. Annual Review of Neuroscience 29: 163-201.

Xia, Y., and L. J. Zwiebel. 2006. Identification and characterization of an odorant receptor from the West Nile Virus mosquito, *Culex quinquefasciatus*. Insect Biochemistry and Molecular Biology 36: 169-176.

Xu, P. X., R. Atkinson, D. N. Jones, and D. P. Smith. 2005. *Drosophila* OBP LUSH is required for activity of pheromone-sensitive neurons. Neuron 45: 193 - 200.

Yao, C. A., R. Ignell, and J. R. Carlson. 2005. Chemosensory coding by neurons in the coeloconic sensilla of the *Drosophila* antenna. Journal of Neuroscience 25: 8359-8367.

Yuval, B. 2006. Mating system of blood-feeding flies. Annual Review of Entomology 51: 413-440.

Zeng, X. N., J. J. Leyden, A. I. Spielman, and G. Preti. 1996. Analysis of characteristic human female axillary odors: Qualitative comparison to males. Journal of Chemical Ecology 22: 237-257.

Zeng, X. n., J. J. Leyden, H. J. Lawley, K. Sawano, I. Nohara, and G. Preti. 1991. Analysis of characteristic odors from human male axillae. Journal of Chemical Ecology 17: 1469-1492.

Zhou, J. J. 2008. Identification of odorant-binding proteins of the yellow fever mosquito *Aedes aegypti*: Genome annotation and comparative analyses. Insect Molecular Biology 17: 147-163.

Zwiebel, L., and W. Takken. 2004. Olfactory regulation of mosquito-host interactions. Insect Biochemistry and Molecular Biology 34: 645 - 652.

Appendix 1

Summary of all characterisation data of the ORNs associated with the olfactory sensilla of *Aedes aegypti*, *Culex quinquefasciatus* and *Anopheles gambiae*. Light grey presents the data that will be obtained by this thesis, dark grey is missing data (n.d.: not done; P: possibly). The "-" sign indicates that none of the compounds tested excited this response type.

References for Appendix 1

1. **Ghaninia, M., R. Ignell and B. S. Hansson. 2007.** Functional classification and central nervous projections of olfactory receptor neurons housed in antennal trichoid sensilla of female yellow fever mosquitoes, *Aedes aegypti*. European Journal of Neuroscience 26: 1611-1623.
2. **Siju, K. P., S. R. Hill, B. S. Hansson and R. Ignell. 2010.** Influence of blood meal on the responsiveness of olfactory receptor neurons in antennal sensilla trichodea of the yellow fever mosquito, *Aedes aegypti*. Journal of Insect Physiology 56: 659-665.
3. **Davis, E. E. 1976.** Receptor sensitive to oviposition site attractants on antennae of mosquito, *Aedes aegypti*. Journal of Insect Physiology 22: 1371-1376.
4. **Davis, E. E. and P. G. Sokolove. 1976.** Lactic acid-sensitive receptors on antennae of mosquito, *Aedes aegypti*. Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology 105: 43-54.
5. **Grant, A. J., B. E. Wigton and J. G. Aghajanian. 1995.** Electrophysiological responses of receptor neurons in mosquito maxillary palp sensilla to carbon dioxide. Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology 177: 389-396.
6. **Hill, S. R., B. S. Hansson and R. Ignell. 2009.** Characterization of antennal trichoid sensilla from female Southern house mosquito, *Culex quinquefasciatus* Say. Chemical Senses 34: 231-252.
7. **Syed, Z. and W. S. Leal. 2009.** Acute olfactory response of Culex mosquitoes to a human- and bird-derived attractant. Proceedings of the National Academy of Sciences of the United States of America 106: 18803-18808.
8. **Syed, Z. and W. S. Leal. 2007.** Maxillary palps are broad spectrum odorant detectors in *Culex quinquefasciatus*. Chemical Senses 32: 727-738.
9. **Qiu, Y. T., J. J. A. van Loon, W. Takken, J. Meijerink and H. M. Smid. 2006.** Olfactory coding in antennal neurons of the malaria mosquito, *Anopheles gambiae*. Chemical Senses 31: 845-863.
10. **Meijerink, J. and J. J. A. van Loon. 1999.** Sensitivities of antennal olfactory neurons of the malaria mosquito, *Anopheles gambiae*, to carboxylic acids. Journal of Insect Physiology 45: 365-373.
11. **Meijerink, J., M. A. H. Braks and J. J. A. van Loon. 2001.** Olfactory receptors on the antennae of the malaria mosquito *Anopheles gambiae* are sensitive to ammonia and other sweat-borne components. Journal of Insect Physiology 47: 455-464.
12. **van den Broek, I. V. F. and C. J. den Otter. 1999.** Olfactory sensitivities of mosquitoes with different host preferences (*Anopheles gambiae s.s., An. arabiensis, An. quadriannulatus, An. m. atroparvus*) to synthetic host odours. Journal of Insect Physiology 45: 1001-1010.
13. **Lu, T., Y. T. Qiu, G. Wang, J. Y. Kwon, M. Rutzler, H.-W. Kwon, R. J. Pitts, J. J. A. van Loon, W. Takken, J. R. Carlson, *et al.* 2007.** Odor coding in the maxillary palp of the malaria vector mosquito *Anopheles gambiae*. Current Biology 17: 1533-1544.
14. **Kwon, H., T. Lu, M. Rutzler and L. Zwiebel. 2006.** Olfactory responses in a gustatory organ of the malaria vector mosquito *Anopheles gambiae*. Proceedings of the National Academy of Sciences of the United States of America 103: 13526 - 13531.

Mosquito species	Organ	Sensilla	Subtype	Functional types	Olfactory receptor	Compounds eliciting		Ref.
						Excitation	Inhibition	
Aedes aegypti	antenna	trichoid	short sharp-tipped	sst1		4-methylcyclohexanol; 2-butoxyethanol; indole; ethyl lactate	2-3C acids	1
				sst2		2-butoxyethanol; indole	-	1
				sst3		4-methylcyclohexanol; 2-butoxy ethanol; α-thujone; ethyl butarate; ethyl propionate	3C; 6C acids	1
				sst4		4-methylcyclohexanol; 2-butoxyethanol; ethyl-L-lactate	α-thujone; acetic acid, propionic acid; ethyl propionate; methyl butyrate; ethyl butyrate	1
			long sharp-tipped	lst		-	-	1
			blunt-tipped I (long)	sbt I 1		4-methylcyclohexanol; 2-butoxy ethanol; α-thujone; α-pinene; acetic acid; ethyl lactate; methylbutyrate,ethylbutyrate	α-thujone	1
				sbt I 2		ethyl butyrate; ethyl-L-lactate; ethyl propionate; 2-butoxyethanol	-	1
				sbt I 3		2-butoxyethanol; 4-methylcyclohexanol	-	2
			blunt-tipped II (short)	sbt II 1		esters of 2-4C acids; 1-octen-3-ol; 2-butoxyethanol; 4-methylphenol; α-pinene	2-3C acids	3
				sbt II 2		-	2-3C acids; indole	1
				sbt II 3		indole	propionic acid	1
				sbt II 4		hexanoic acid; octanoic acid; α-thujone; acetic acid	ethyl-D-lactate; 2-butoxyethanol	1
				sbt II 5		indole; α-thujone; hexanoic acid	propionic acid, indole, 2-butoxyethanol	1
						acetic acid; propionic acid; hexanoic acid; phenol; 3-methylphenol; 4-methylphenol; 4-ethylphenol; indole; 3-methylindole; 2-undecanone	-	2
		grooved peg	lactic acid excited			lactic acid; acetic acid; propionic acid; fluoro-lactate; ammonia	DEET; fluoro-lactate	4
			lactic acid non-responding			pyruvic acid; ammonia	acetic acid; propionic acid; pyruvic acid; ethyl lactate; 2-butoxyethanol	4
			lactic acid inhibited			*ammonia; water; pyruvic acid; acetic acid*	lactic acid; DEET; fluoro-lactate; *pyruvic acid;acetic acid; propionic acid*	4
	maxillary palp	capitate peg		A-neuron	AaGR1;2,3			
				B-neuron	AaOR7 + AaOR8			
				C-neuron	AaOR7 + AaOR49			
Culex quinquefasciatus	antenna	trichodea	short sharp-tipped	sst1		2-butoxyethanol; 4-methylcyclohexanol; ethylene glycol; *4-methylphenol; 4-ethylphenol;* 3-*methylindole;* α-pinene; verbanone; menthone; eugenol; isopropyl acetate; (-)-ethyl lactate;	C2-C6; 1-octen-3-ol;2-butoxyethanol; phenol; 3-methylphenol; 3-methylindole; α-pinene; verbanone; limonene; menthone; thymol; citral; nonanal; dimethyl trisulfide; linalool	6
				sst2		1-octen-3-ol; 2-butoxyethanol; 4-methylcyclohexanol; (-)-α-thujone, citral; linalool	2-butoxyethanol	6
				sst3		lactic acid; C7; 1-octen-3-ol; 2-butoxyethanol; 4-methylcyclohexanol; benzyl alcohol; ethylene glycol; geranyl acetone; 2-undecanone; (-)-α-thujone; verbanone; limonene; eugenol; citral; methyl butyrate; ethyl lactate; nonanal; methyl propyl disulfide; linalool	-	6
				unknown		linalool; eucalyptol; α- & β-thujone; skatole; geranyl acetone; 1-octen-3-ol; ethylhexanoate, indole, 2-*methylphenol, 6-methyl-5-hepten-2-one; 5-methyl-2-hexanone; 2-heptanone; citral; nerol;* geraniol; 4-methylcyclohexanol; heptalactone	-	7
			long sharp-tipped			-	-	6,7
			blunt-tipped I (long)	sbtI1		5C-8C acids; 1-octen-3-ol; 2-butoxyethanol; 4-methylcyclohexanol; benzyl alcohol; 3-methylphenol; 4-methylphenol; 4-ethylphenol; citral; nonanal;	3-4C acid; indole; linalool	6
				sbtI2		6C-7C acid; 1-octen-3-ol; 2-butoxyethanol; 4-methylcyclohexanol; 3-methylphenol; 4-methylphenol; 4-ethylphenol; 3-methylindole; (-)-α-thujone; verbanone; menthone; eugenol; citral; ethyl lactate; nonanal	-	6
				sbtI3		1-octen-3-ol; 2-butoxyethanol; indole; 3-methylindole; menthone; linalool	3-methylphenol; (-)-α-thujone; verbanone; eugenol; thymol;	6
				sbtI4		1-octen-3-ol; indole; 3-methylindole; citral; linalool	-	6
				sbtI5		1-octen-3-ol; 4-methylcyclohexanol; indole; 3-methylindole; ethyl butyrate; isopropyl acetate; linalool	-	6
				sbtI6		2C acid; 1-octen-3-ol; 2-butoxyethanol; ethylene glycol; phenol; 3-methylphenol; 4-methylphenol; 4-ethylphenol; indole; 3-methylindole; 2-tridecanone; 2-undecanone; (-)-α-thujone; α-pinene; limonene; menthone; eugenol; citral; methyl butyrate; ethyl butyrate; isopropyl acetate; ethyl lactate; linalool	-	6
				sbtI7		2-butoxyethanol; 4-methyl phenol; naphthalene	-	6
				sbtI8		4-methylcyclohexanol; (-)-α-thujone;	-	6
				sbtI9		1-octen-3-ol; 4-methylcyclohexanol; nonanal	-	6
			blunt-tipped II (short)	sbtII1		2-8C acids; 1-octen-3-ol; 2-buthoxyethanol; 4-methylcyclohexanol;benzyl alcohol; phenol; 3-methylphenol; 4-methylphenol; 4-ethylphenol; indole; 3-methylindole; 2-undecanone; (-)-α-thujone; citral; nonanal; linalool; naphthalene	7C acid; 3-methylphenol; isopropyl acetate	6
				sbtII2		2C acid; 7C acid; 2-butoxyethanol; 3-methylphenol; indole; citral; (-)-ethyl lactate; nonanal; naphthalene	1-octen-3-ol; 4-etylphenol; 2-undecanone;	6
				sbtII3		2-8c acids; 1-octen-3-ol; 2-buthoxyethanol; 4-methylphenol; 4-ethylphenol; indole; 3-methylindole; 2-undecanone;(-)-α-thujone; menthone; citral; nonanal; naphthalene	1-octen-3-ol; 3-methylphenol; 4-ethylphenol; 2-undecanone	6

Mosquito species	Organ	Sensilla	Subtype	Functional types	Olfactory receptor	Compounds eliciting		Ref.
						Excitation	Inhibition	
Culex quinquefasciatus	antenna	trichodea	blunt-tipped II (short)	sbtII4		4C-8C acids; 2-butoxyethanol; 4-methylphenol; 4-ethylphenol; indole; 3-methylindole; citral; (-)-ethyl lactate; nonanal; naphthalene	indole; (-)-ethyl lactate	6
				sbtII5		lactic acid; 2C-8C acids; 16C acid; 1-octen-3-ol; 2-butoxyetanol; 4-methylcyclohexanol; 3-methylphenol; 4-ethylphenol; indole; 2-undecanone; menthone; citral; ethyl propinoate; nonanal; linalool; naphthalene	3C-4C acid; linalool	6
			blunt-tipped I or II	unknown		nonanal; indole; 2-methylphenol; heptanal; octanal; phenol; (*E*)-2-hexanal; 3 & 6-methylindole; 7-octenoic acid	-	7
		grooved peg		unknown		ammonia; trimethylamine; propylamine; butylamine; pentanoic acid; hexanoic acid; lactic acid; cadaverine; putrescine	-	7
	maxillary palp	Capitate peg		A-neuron		CO2	-	8
				B-neuron		1-octen-3-ol; butanol; pentanol; hexanol; heptanol; octanol; 3-octanol; 1,5-octadiene-3-ol; pentanal; hexanal; heptanal; octanal; ethyl-3-hydroxybutyrate; ethyl-3-hydroxyhexanoate; ethyl lactate; 1-hexanal; 1-hexanol; 1-hexen-3-ol; (*E*) & (*Z*)-2- & 3-hexen-1-ol; b-myrcene; racemic linalool; α-pinene; β-caryophylene; 6-methyl-5-hepten-2-one; α & β-thujone	-	8
				C-neuron		-	-	8
Anopheles gambiae	antenna	trichodea			n.d.	n.d.	n.d.	
					n.d.	n.d.	n.d.	
			C (medium-length sharp-tipped; intermediate)	TC1		ammonia; phenol; 4-methylphenol; 4-ethylphenol	-	9;10;11;12
				TC2		geranyl acetone	-	9;10;11;12
				TC3		indole; phenol; 2-methylphenol; 4-methylphenol; 4-ethylphenol	-	9;10;11;12
				TC4		ammonia ; 4-ethylphenol	-	9;10;11;12
					n.d.	n.d.	n.d.	
			E (short sharp-tipped)	TE1		indole; phenol; 2-methylphenol; 4-methylphenol; 4-ethylphenol; 1-hexen-3-ol; 5-7C acids; 3-methyl-1-butanol; ammonia ;2-phenoxyethanol	3-4C acids	9;10;11;12
				TE2		4-methylphenol; 4-ethylphenol; geranyl acetone; 2-nonanone; 1-hexen-3-ol; 1-hepten-3-ol; 1-octen-3-ol; 7-octenoic acid; 3-methyl-2-hexenoic acid; 3C; 3-methylbutanoic acid; 6C; 9C; 10C; 12C acids; 2-oxobutanoic acid; 2-oxopentanoic acid; ammonia	-	9;10;11;12
		grooved peg	GP1			ammonia; 1-butylamine; 1-pentylamine	-	9
			GP2			ammonia; 1-butylamine; 1-pentylamine; 2-oxobutanoic acid; 2-oxopentanoic acid	-	9
			GP3			ammonia; 1-butylamine; 2-oxobutanoic acid; lactic acid	-	9
			GP4			ammonia; 1-butylamine; 2-oxobutanoic acid; lactic acid	3-6C and 9C acids ; 1-hepten-3-ol	9
			GP5			ammonia; 1-butylamine; 1-pentylamine; 4-5C acids	-	9
	maxillary palp	capitate peg		A-neuron	AgGR 22;23;24	CO2; thiazole; cyclohexanone; 4-methyl thiazole	indole; 3-methyl indole; 3-octanone; 1-hepten-3-ol; L-(+)-lactic acid; heptanal; 2-iso-butyl thiazole	13
				B-neuron	AgOR7 + AgOR8	1-octen-3-ol; 1-hepten-3-ol; 3-octanone; 1-hexen-3-ol; henkel 100; ethyl hexanoate; amyl acetate; cis-3-hexen-1-ol; 6-methyl-5-hepten-2-one	4-methyl cyclohexanol; 2,4,5 trimethyl thiazole; 2-ethylphenol; 3-methyl-2-cyclohexen-1-ol; 2-acetylthiophene; cyclohexanone; 2-acetyl thiazole; DEET; 4-methylthiazole; methyl-2-methyl benzoate; acetylpyridine; acetophenone; 4,5 dimethyl thiazole; methyl benzoate; ethyl propionate; linalool oxide; R-(-)- & S-(+)- carvone	13
				C-neuron	AgOR7 + AgOR28	2,4,5-trimethylthiazole; acetophenone; 2-acetylthiophene; 3-methyl-2-cyclohexen-1-ol; 4-methylcyclohexanol; 4,5-dimethylthiazole; 2-acetylthiazole; 6-methyl-5-hepten-2-one; cyclohexanone; linalool oxide; 2-iso-butylthiazole; methyl-2-methylbenzoate; cis-3-hexen-1-ol; 1-hepten-3-ol; methyl benzoate; 1-hexen-3-ol; 4-methylthiazole; (-)-fenchone; hexanal; ethyl hexanoate; S-(+)-carvone; (+)-fenchone; amyl acetate	-	13
	proboscis	T2		A-neuron	AgOR7 + AgOR6; 53; 12; 18; 3; 15; 19; 28; 55; 62; 70	oxovaleric acid; butyl amine; acetothiophene; acetic acid; oxobutylic acid; acetylpyridine; acetylthiazole	-	14
				B-neuron		acetylthiophene; acetylthiazole; acetylpyridine; acetophenone; oxobutylic acid; oxovaleric acid; butylamine	-	14

Appendix 2

Summary of all chemical compounds used in this thesis. The name of the compounds as referred to in this project is shown in column A, along with the used purity (2nd column), CAS number (3rd column) and the company that supplied the compound. The 4th column refers to articles showing the compound as being present in human emanations and the 5th column refers to literature showing behavioural activity from mosquitoes caused by this compound.

References to Appendix 2

1. **Gallagher, M., C. J. Wysocki, J. J. Leyden, A. I. Spielman, X. Sun and G. Preti. 2008.** Analyses of volatile organic compounds from human skin. British Journal of Dermatology 159: 780-791.
2. **Meijerink, J., M. A. H. Braks, A. A. Brack, W. Adam, T. Dekker, M. A. Posthumus, T. A. Van Beek and J. J. A. van Loon. 2000.** Identification of olfactory stimulants for *Anopheles gambiae* from human sweat samples. Journal of Chemical Ecology 26: 1367-1382.
3. **Ellin, R. I., R. L. Farrand, F. W. Oberst, C. L. Crouse, N. B. Billups, W. S. Koon, N. P. Musselman and F. R. Sidell. 1974.** An apparatus for the detected and quantitation of volatile human effluents. Journal of Chromatography A 100: 137-152.
4. **Bernier, U. R., M. M. Booth and R. A. Yost. 1999.** Analysis of human skin emanations by gas chromatography/mass spectrometry. 1.Thermal desorption of attractants for the yellow fever mosquito (*Aedes aegypti*) from handled glass beads. Analytical Chemistry 71: 1-7.
5. **Bernier, U. R., D. L. Kline, D. R. Barnard, C. E. Schreck and R. A. Yost. 2000.** Analysis of human skin emanations by gas chromatography/mass spectrometry. 2. Identification of volatile compounds that are candidate attractants for yellow fever mosquito (*Aedes aegypti*). Analytical Chemistry 72: 747-756.
6. **Lindh, J. M., A. KÃ¤nnaste, B. G. J. Knols, I. Faye and A. K. Borg-Karlson. 2008.** Oviposition responses of *Anopheles gambiae s.s.* (Diptera: Culicidae) and identification of volatiles from bacteria-containing solutions. Journal of Medical Entomology 45: 1039-1049.
7. **Bernier, U. R., D. L. Kline, C. E. Schreck, R. A. Yost and D. R. Barnard. 2002.** Chemical analysis of human skin emanations: comparison of volatiles from humans that differ in attraction of *Aedes aegypti* (Diptera: Culicidae). Journal of the American Mosquito Control Association 18: 186-195.
8. **Cork, A. and K. C. Park. 1996.** Identification of electrophysiologically-active compounds for the malaria mosquito, *Anopheles gambiae*, in human sweat extracts. Medical and Veterinary Entomology 10: 269-276.
9. **Wahl, H. G., A. Hoffmann, D. Luft and H. M. Liebich. 1999.** Analysis of volatile organic compounds in human urine by headspace gas chromatography-mass spectrometry with a multipurpose sampler. Journal of Chromatography A 847: 117-125.
10. **Healy, T. P., M. J. W. Copland, A. Cork, A. Przyborowska and J. M. Halket. 2002.** Landing responses of Anopheles gambiae elicited by oxocarboxylic acids. Medical and Veterinary Entomology 16: 126-132.
11. **Healy, T. P. and M. J. W. Copland. 2000.** Human sweat and 2-oxopentanoic acid elicit a landing response from *Anopheles gambiae*. Medical and Veterinary Entomology 14: 195-200.
12. **Qiu, Y. T. 2005.** Sensory and behavioural responses of the malaria mosquito *Anopheles gambiae* to human odours.
13. **Zeng, X. N., J. J. Leyden, H. J. Lawley, K. Sawano, I. Nohara and G. Preti. 1991.** Analysis of characteristic odors from human male axillae. Journal of Chemical Ecology 17: 1469-1492.
14. **Zeng, X. N., J. J. Leyden, A. I. Spielman and G. Preti. 1996.** Analysis of characteristic human female axillary odors: Qualitative comparison to males. Journal of Chemical Ecology 22: 237-257.

15. **Akutsu, T., K. Sekiguchi, T. Ohmori and K. Sakurada. 2006.** Individual comparisons of the levels of (*E*)-3-methyl-2-hexenoic acid, an axillary odor-related compound, in Japanese. Chemical Senses 31: 557-563.

16. **Kostelc, J. G., G. Preti and P. R. Zelson. 1980.** Salivary volatiles as indicators of periodontitis. Journal of Periodontal Research 15: 185-192.

17. **Millar, J. G., J. D. Chaney and M. S. Mulla. 1992.** Identification of oviposition attractants for *Culex quinquefasciatus* from fermented Bermuda grass infusions. Journal of the American Mosquito Control Association 8: 11-17.

18. **Bentley, M. D., I. N. McDaniel and E. E. Davis. 1982.** Studies of 4-methylcyclohexanol: An *Aedes triseriatus* (Diptera: Culicidae) oviposition attractant. Journal of Medical Entomology 19: 589-592.

19. **Munk, S., P. Munch, L. Stahnke, J. Adler-Nissen and P. Schieberle. 2000.** Primary odorants of laundry soiled with sweat/sebum: Influence of lipase on the odor profile. Journal of Surfactants and Detergents 3: 505-515.

20. **Kostelc, J. G., G. Preti and P. R. Zelson. 1984.** Oral odors in early experimental gingivitis. Journal of Periodontal Research 19: 303-312.

21. **Bentley, M. D., I. N. McDaniel, M. Yatagai, H. P. Lee and R. Maynard. 1979.** Para-cresol: Oviposition attractant of *Aedes triseriatus* (Diptera: Culicidae). Environmental Entomology 8: 206-209.

22. **Bernier, U. R., K. D. Furman, D. L. Kline, S. A. Allan and D. R. Barnard. 2005.** Comparison of contact and spatial repellency of catnip oil and N,N-diethyl-3-methylbenzamide (Deet) against mosquitoes. Journal of Medical Entomology 42: 306-311.

23. **Puri, S. N., M. J. Mendki, D. Sukumaran, K. Ganesan, S. Prakash and K. Sekhar. 2006.** Electroantennogram and Behavioral responses of *Culex quinquefasciatus* (Diptera: Culicidae) females to chemicals found in human skin emanations. Journal of Medical Entomology 43: 207-213.

24. **Curran, A. M., S. I. Rabin, P. A. Prada and K. G. Furton. 2005.** Comparison of the volatile organic compounds present in human odor using SPME-GC/MS. Journal of Chemical Ecology 31: 1607-1619.

25. **Curran, A. M., C. F. Ramirez, A. A. Schoon and K. G. Furton. 2007.** The frequency of occurrence and discriminatory power of compounds found in human scent across a population determined by SPME-GC/MS. Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences 846: 86-97.

26. **Logan, J., M. Birkett, S. Clark, S. Powers, N. Seal, L. Wadhams, A. Mordue and J. Pickett. 2008.** Identification of human-derived volatile chemicals that interfere with attraction of *Aedes aegypti* mosquitoes. Journal of Chemical Ecology 34: 308-322.

27. **Costantini, C., M. A. Birkett, G. Gibson, J. Ziesmann, N. F. Sagnon, H. A. Mohammed, M. Coluzzi and J. A. Pickett. 2001.** Electroantennogram and behavioural responses of the malaria vector *Anopheles gambiae* to human-specific sweat components. Medical and Veterinary Entomology 15: 259-266.

28. **Phillips, M., K. Gleeson, J. M. B. Hughes, J. Greenberg, R. N. Cataneo, L. Baker and W. P. McVay. 1999.** Volatile organic compounds in breath as markers of lung cancer: A cross-sectional study. The Lancet 353: 1930-1933.

29. **Knols, B. G. J., J. J. A. van Loon, A. Cork, R. D. Robinson, W. Adam, J. Meijerink, R. De Jong and W. Takken. 1997.** Behavioural and electrophysiological responses of the female malaria mosquito *Anopheles gambiae* (Diptera: Culicidae) to Limburger cheese volatiles. Bulletin of Entomological Research 87: 151-159.

30. **Smallegange, R. C., Y. T. Qiu, J. J. A. van Loon and W. Takken. 2005.** Synergism between ammonia, lactic acid and carboxylic acids as kairomones in the host-seeking behaviour of the malaria mosquito *Anopheles gambiae sensu stricto* (Diptera: Culicidae). Chemical Senses 30: 145-152.

31. **Takken, W., T. Dekker and Y. G. Wijnholds. 1997.** Odor-mediated flight behavior of *Anopheles gambiae* Giles *sensu stricto* and *An. stephensi* liston in response to CO_2, acetone, and 1-octen-3-ol (Diptera: Culicidae). Journal of Insect Behavior 10: 395-407.

32. **Braks, M. A. H., J. Meijerink and W. Takken. 2001.** The response of the malaria mosquito, *Anopheles gambiae*, to two components of human sweat, ammonia and L-lactic acid, in an olfactometer. Physiological Entomology 26: 142-148.

33. **Czarnowski, D. and J. Gorski. 1991.** Sweat ammonia excretion during submaximal cycling exercise. Journal of Applied Physiology 70: 371-374.
34. **Smallegange, R. C., Y. T. Qiu, G. Bukovinszkine-Kiss, J. J. A. van Loon and W. Takken. 2009.** The effect of aliphatic carboxylic acids on olfaction-based host-seeking of the malaria mosquito *Anopheles gambiae sensu stricto*. Journal of chemical ecology 35: 933-943.
35. **Omolo, M. O., D. Okinyo, I. O. Ndiege, W. Lwande and A. Hassanali. 2004.** Repellency of essential oils of some Kenyan plants against *Anopheles gambiae*. Phytochemistry 65: 2797-2802.
36. **Soini, H. A., K.E. Bruce, I. Klouckova, R. G. Brereton, D. J. Penn and M. V. Novotny. 2006.** In situ surface sampling of biological objects and preconcentration of their volatiles for chromatographic analysis. Analytical Chemistry 78: 7161-7168.
37. **Ghaninia, M., M. Larsson, B. S. Hansson and R. Ignell. 2008.** Natural odor ligands for olfactory receptor neurons of the female mosquito *Aedes aegypti*: Use of gas chromatography-linked single sensillum recordings. Journal of Experimental Biology 211: 3020-3027.
38. **Tangerman, A., M. T. Meuwese Arends and J. H. M. Van Tongeren. 1983.** A new sensitive assay for measuring volatile sulphur compounds in human breath by Tenax trapping and gas chromatography and its application in liver cirrhosis. Clinica Chimica Acta 130: 103-110.
39. **Penn, D. J., E. Oberzaucher, K. Grammer, G. Fischer, H. A. Soini, D. Wiesler, M. V. Novotny, S. J. Dixon, Y. Xu and R. G. Brereton. 2007.** Individual and gender fingerprints in human body odour. Journal of the Royal Society Interface 4: 331-340.
40. **Silva, I. M., A. E. Eiras, D. L. Kline and U. R. Bernier. 2005.** Laboratory evaluation of mosquito traps baited with a synthetic human odor blend to capture *Aedes aegypti*. Journal of the American Mosquito Control Association 21: 229-233.
41. **Qiu, Y. T., R. C. Smallegange, C. J. F. Ter Braak, J. Spitzen, J. J. A. van Loon, M. Jawara, P. Milligan, A. M. Galimard, T. A. Van Beek, B. G. J. Knols, *et al.* 2007.** Attractiveness of MM-X traps baited with human or synthetic odor to mosquitoes (Diptera: Culicidae) in the Gambia. Journal of Medical Entomology 44: 970-983.
42. **Acree Jr, F., R. B. Turner, H. K. Gouck, M. Beroza and N. Smith. 1968.** l-lactic acid: A mosquito attractant isolated from humans. Science 161: 1346-1347.
43. **Braks, M. A. H. and W. Takken. 1999.** Incubated human sweat but not fresh sweat attracts the malaria mosquito *Anopheles gambiae sensu stricto*. Journal of Chemical Ecology 25: 663-672.
44. **Dekker, T., B. Steib, R. T. Cardé and M. Geier. 2002.** L-lactic acid: A human-signifying host cue for the anthropophilic mosquito *Anopheles gambiae*. Medical and Veterinary Entomology 16: 91-98.
45. **Bernier, U. R., D. L. Kline, S. A. Allan and D. R. Barnard. 2007.** Laboratory comparison of *Aedes aegypti* attraction to human odors and to synthetic human odor compounds and blends. Journal of the American Mosquito Control Association 23: 288-293.
46. **Bernier, U. R., D. L. Kline, K. H. Posey, M. M. Booth, R. A. Yost and D. R. Barnard. 2003.** Synergistic attraction of *Aedes aegypti* (L.) to binary blends of L-Lactic acid and Acetone, Dichloromethane, or Dimethyl Disulfide. Journal of Medical Entomology 40: 653-656.
47. **Douglas III, H. D., J. E. Co, T. H. Jones, W. E. Conner and J. F. Day. 2005.** Chemical odorant of colonial seabird repels mosquitoes. Journal of Medical Entomology 42: 647-651.
48. **Allan, S. A., U. R. Bernier and D. L. Kline. 2006.** Attraction of mosquitoes to volatiles associated with blood. Journal of Vector Ecology 31: 71-78.
49. **Geier, M., H. Sass and J. Boeckh. 1996.** A search for components in human body odour that attract females of *Aedes aegypti*. CIBA Foundation Symposia: 132-148.
50. **Geier, M. and J. Boeckh. 1999.** A new Y-tube olfactometer for mosquitoes to measure the attractiveness of host odours. Entomologia Experimentalis et Applicata 92: 9-19.
51. **Geier, M., O. J. Bosch and J. Boeckh. 1999.** Ammonia as an attractive component of host odour for the yellow fever mosquito, *Aedes aegypti*. Chemical Senses 24: 647-653.
52. **Geier, M., O.J. Bosch and J. Boeckh. 1999.** Influence of odour plume structure on upwind flight of mosquitoes towards hosts. Journal of Experimental Biology 202: 1639-1648.

53. **Bosch, O. J., M. Geier and J. Boeckh. 2000.** Contribution of fatty acids to olfactory host finding of female *Aedes aegypti*. Chemical Senses 25: 323-330.

54. **Williams, C. R., R. Bergbauer, M. Geier, D. L. Kline, U. R. Bernier, R. C. Russell and S. A. Ritchie. 2006.** Laboratory and field assessment of some kairomone blends for host-seeking *Aedes aegypti*. Journal of the American Mosquito Control Association 22: 641-647.

55. **Smith, C. N., N. Smith, H. K. Gouck, D. E. Weidhaas, I. H. Gilbert, M. S. Mayer, B. J. Smittle and A. Hofbauer. 1970.** L-lactic acid as a factor in the attraction of *Aedes aegypti* (Diptera: Culicidae) to human hosts. Annals of the Entomological Society of America 63: 760-770.

56. **Haze, S., Y. Gozu, S. Nakamura, Y. Kohno, K. Sawano, H. Ohta and K. Yamazaki. 2001.** 2-Nonenal newly found in human body odor tends to increase with aging. Journal of Investigative Dermatology 116: 520-524.

Compounds	Purity (%)	CAS number	Company	Human emanation	Behavioural activity in mosquitoes
(S)-(+)-carvone	96,0	2244-16-8	Sigma Aldrich		35
(+)-fenchone	≥ 99,5	4695-62-9	Fluka		35
1-butanol	≥ 99,5	71-36-3	Sigma Aldrich	1,2,3,56	
2-ethylphenol	99,0	90-00-6	Sigma Aldrich		
2-methylbutanal	95,0	96-17-3	Sigma Aldrich	4,5	
2-methyl-1-butanol	≥ 98,0	137-32-6	Fluka	2	
2-methylpropanoic acid	99,0	79-31-2	Sigma Aldrich	8	29
(+/-)-2-methylbutanoic acid	98,0	116-53-0	Sigma Aldrich		
2-methylphenol	≥ 99,5	95-48-7	Fluka		
2-pentanone	≥ 99,8	107-87-9	Fluka	5,7	7
cis-3-hexen-1-ol	≥ 95,0	928-96-1	Sigma Aldrich		
3-methylbutanal	≥ 98,0	590-86-3	Fluka	4	
3-methyl-1-butanol	≥ 99,8	123-51-3	Fluka	2,9	4,6,12
3-methylbutanoic acid	99,0	503-74-2	Sigma Aldrich	1,2,8,19	6,29,34,41,48,
3-methylphenol	≥ 98,0	108-39-4	Fluka		
3-pentanone	≥ 99,5	96-22-0	Fluka	5,7	7
4-ethylphenol	99,0	123-07-9	Sigma Aldrich		12,17,41
4-methylcyclohexanol	99,0	589-91-3	Fluka		18
4-methylphenol	≥ 99,0	106-44-5	Sigma Aldrich	1,8,9,16,19,20	17,21
6-aminocaproic acid	≥ 99,0	60-32-2	Sigma Aldrich		
7-octenoic acid	≥ 95,0	18719-24-9	Sigma Aldrich	13,14	12,27,41
cis-9-octadecenoic acid	≥ 95,0	112-80-1	Sigma Aldrich	7,8,11	29
amyl acetate	≥ 99,0	628-63-7	Sigma Aldrich	3	
acetone	≥ 99,5	67-64-1	Merck	1,2,3,9,16,28,	12,31,45,46,54
2-acetylthiophene	≥ 99,8	88-15-3	Fluka		
ammonia	25,0	7664-41-7	Merck	3,32,33	30,32,34,41
2-acetylpyridine	> 99,0	1122-62-9	Sigma Aldrich		
acetophenone	≥ 99,5	98-86-2	Fluka	1	
2-acetylthiazole	99,0	24295-03-2	Sigma Aldrich		
benzaldehyde	≥ 99,0	100-52-7	Fluka	1,5,7,16,24,25,26,28	
1-butylamine	≥ 99,0	109-73-9	Sigma Aldrich	3	
2-phenylethanol	≥ 99,0	60-12-8	Fluka	1,5,19,25,39	6
benzyl acetate	>99,0	140-11-4	Sigma Aldrich		

Compounds	Purity (%)	CAS number	Company	Human emanation	Behavioural activity in mosquitoes
butanone	99,0	78-93-3	Sigma Aldrich	5,7,9	
2,3-butanedione	≥ 99,0	431-03-8	Fluka		
decanoic acid	≥ 99,0	334-48-5	Sigma Aldrich	4,5,7,8,11,13,14,25	29,23
dodecanoic acid	99,0	143-07-7	Sigma Aldrich	1,4,5,7,8,11,24,25	29,23
tridecanoic acid	98,0	638-53-9	Sigma Aldrich	1,5,11	23,53
tetradecanoic acid	≥ 99,0	544-63-8	Sigma Aldrich	1,4,5,8,11,24	3,29,34,41,48,53
pentadecanoic acid	≥ 99,5	1002-84-2	Fluka	1,4,5,7,11,39	23,53
hexadecanoic acid	99,0	57-10-3	Sigma Aldrich	1,4,5,7,8,11,39	23,29,53
heptadecanoic acid	≥ 99,0	506-12-7	Fluka	1,4,5,7,11,39	23,53
octadecanoic acid	≥ 99,0	57-11-4	Sigma Aldrich	4,5,7,8,11	23,29,48,53
acetic acid	≥ 99,0	64-19-7	Sigma Aldrich	1,2,5,8,19,28,56	29
propanoic acid	99,0	79-09-4	Sigma Aldrich	1,2,5,8	12,23,29,34,41,53
butanoic acid	≥ 99,0	107-92-6	Sigma Aldrich	1,2,8,11,19,56	12,29,34,41,48
pentanoic acid	≥ 99,0	109-52-4	Sigma Aldrich	8,19	29,34,53
hexanoic acid	99,0	142-62-1	Sigma Aldrich	1,2,5,8,11,13,14,19	12,23,29,34,41,47,53,54
heptanoic acid	98,0	111-14-8	Sigma Aldrich	5,8,11,13,14,19	12,23,34,48,53
octanoic acid	≥ 99,0	124-07-2	Sigma Aldrich	1,5,8,11,13,14,19,26	23,29,34,53
nonanoic acid	97,0	112-05-0	Sigma Aldrich	5,8,13,14	23,29,53
(R)-(−)-carvone	98,0	6485-40-1	Sigma Aldrich		35
cyclohexanone	≥ 99,5	108-94-1	Fluka	2,3	
dimethyl adipate	≥ 99,0	627-93-0	Sigma Aldrich	24,25	
decanal	99,0	112-31-2	Sigma Aldrich	1,2,4,5,24,25,26,28,36,37,56	26
2-decanone	≥ 99,5	693-54-9	Fluka	5,7,25	
dodecane	≥ 98,0	112-40-3	Fluka	24,25,56	
2-dodecanone	≥ 97,0	6175-49-1	Fluka		
N,N-diethyl-3-methylbenzamide	97,0	134-62-3	Sigma Aldrich		22,35
dimethyl malonate	≥ 99,0	108-59-8	Sigma Aldrich	24,25	
dimethyldisulfide	≥ 95,0	624-92-0	Acros	5,9,16,20,38	40,45,46
1-dodecanol	≥ 99,5	112-53-8	Sigma Aldrich	2,20,36	6
4,5-dimethyl thiazole	≥ 97,0	3581-91-7	Sigma Aldrich		
ethyl butyrate	≥ 99,7	105-54-4	Fluka	2,19	
ethyl formate	≥ 99,5	109-94-4	Fluka		
ethylene glycol butyl ether	≥ 99,8	111-76-2	Fluka		

Compounds	Purity (%)	CAS number	Company	Human emanation	Behavioural activity in mosquitoes
ethyl 3-hydroxybutyrate	≥ 97,0	5405-41-4	Fluka		
2-ethyl-1-hexanol	≥ 98,0	104-76-7	Merck Schuchard	1,3,26,56	
(-)-ethyl L-lactate	≥ 99,0	687-47-8	Fluka	1	
ethyl propanoate	99,0	105-37-3	Sigma Aldrich		
2-ethyl toluene	99,0	611-14-3	Sigma Aldrich		
2-ethoxythiazole	≥ 99,0	15679-19-3	Sigma Aldrich		
eugenol	99,0	97-53-0	Sigma Aldrich	39	35
E-2-hexenal	98,0	6728-26-3	Sigma Aldrich		
ethyl hexanoate	≥ 99,0	123-66-0	Sigma Aldrich	2	
(−)-fenchone	≥ 99,6	7787-20-4	Fluka		35
2-furaldehyde	99,0	98-01-1	Sigma Aldrich	2,3,24,25	
furfuryl alcohol	99,0	98-00-0	Sigma Aldrich	1,2,3,5,24,25	
geranyl acetone	96,0	3796-70-1	Sigma Aldrich	1,2,5,24,25,26,36,37	12,26,41
3-hydroxy-2-butanone	≥ 97,0	513-86-0	Fluka		
heptacosane	≥ 99,5	593-49-7	Fluka		
heptane	99,0	142-82-5	Sigma Aldrich	3,5,7	
heptanal	95,0	111-71-7	Sigma Aldrich	2,3,4,5,7,24,25,37,56	
1-hepten-3-ol	99,0	4938-52-7	Sigma Aldrich	5,7	7
1-hexen-3-ol	≥ 98,0	4798-44-1	Sigma Aldrich	5	
hexanal	98,0	66-25-1	Sigma Aldrich	2,4,19,24,25,26,56	47
1-hexanol	≥ 99,9	111-27-3	Fluka	2,3,56	
isoamyl acetate	≥ 97,0	123-92-2	Sigma Aldrich		
2-isobutylthiazole	99,0	18640-74-9	Sigma Aldrich		
indole	≥ 99,0	120-72-9	Sigma Aldrich	2,5,7,16,20,26	6,12,17
L(+) lactic acid	≥ 70,0	79-33-4	Merck	1,3,4,5,7,10,11,32,33,42,43	30,32,34,41,44,45,48-55
linalool oxide	> 97,0	60047-17-8	Fluka	2	
methyl benzoate	≥ 99,5	93-58-3	Fluka		
3-methyl-2-cyclohexen-1-ol	96,0	21378-21-2	Sigma Aldrich		
methyl caprylate	≥ 99,8	111-11-5	Fluka	24,25	
3-methyl-2-hexenoic acid	≥ 95,0	35205-70-0	Sigma Aldrich	13,14,15	27
6-methyl-5-hepten-2-one	99,0	110-93-0	Sigma Aldrich	1,2,5,7,24,25,26	7,12,26
3-methylindole	99,0	83-34-1	Sigma Aldrich	16	17
methyl-2-methyl benzoate	99,0	89-71-4	Sigma Aldrich		

Compounds	Purity (%)	CAS number	Company	Human emanation	Behavioural activity in mosquitoes
methyl nonanoate	≥ 99,8	1731-84-6	Fluka	5,24,25	
methyl propionoate	99,0	554-12-1	Sigma Aldrich		
methyl salicylate	≥ 99,5	119-36-8	Fluka		
4-methylthiazole	99,0	693-95-8	Sigma Aldrich		
nerylacetone	≥ 98,0	3879-26-3	Fluka		
nonanal	≥ 95,0	124-19-6	Fluka	1,2,4,5,7,24,25,26,28,36,37,56	26
2-nonanone	≥ 99,0	821-55-6	Sigma Aldrich	2	6
2-octene trans	≥ 98,0	13389-42-9	Fluka	3,5,7	
1-octen-3-ol	≥ 98,0	3391-86-4	Fluka	1,5,8,19	31
3-octanone	≥ 98,0	106-68-3	Sigma Aldrich		
2-oxopropanoic acid	≥ 98,0	127-17-3	Fluka	3	
2-oxobutanoic acid	≥ 95,0	600-18-0	Sigma Aldrich		10
2-oxopentanoic acid	≥ 95,0	1821-02-9	Sigma Aldrich		10,11
2-oxohexanoic acid	97,0	13022-85-0	Sigma Aldrich		10
1-pentylamine	99,0	110-58-7	Sigma Aldrich	3	
pentacosane	≥ 99,5	629-99-2	Fluka	5,7	
2-phenoxyethanol	≥ 99,5	122-99-6	Fluka		
phenethyl acetate	≥ 99,0	103-45-7	Sigma Aldrich		
phenol	> 99,5	108-95-2	Sigma Aldrich	1,5,8,9,13,14,16,20,24,25	
2-propylphenol	98,0	644-35-9	Sigma Aldrich		
pyridine	≥ 99,8	110-86-1	Fluka	1,2,5,7,16,20,24,25	
styrene	≥ 99,5	100-42-5	Fluka	3,5,7,16,28	
tetradecane	≥ 99,5	629-59-4	Fluka	5,24,25	
(-)-α-thujone	≥ 96,0	546-80-5	Fluka		35
2-nonenal trans	97,0	18829-56-6	Sigma Aldrich	24,25,26,56	
triacontane	≥ 99,0	638-68-6	Fluka		
2,4,5-trimethylthiazole	98,0	13623-11-5	Sigma Aldrich		
thiazole	99,0	288-47-1	Sigma Aldrich		
delta-undecalactone	> 97,0	24901617	Sigma Aldrich		
urethane	≥ 99,0	51-79-6	Fluka	1	
vinyl acetate	≥ 99,0	108-05-4	Fluka		
Henkel 100					

Samenvatting

Malaria is wereldwijd een van de meest voorkomende parasitaire infectieziekten die de gezondheid van mens en dier bedreigen. Elk jaar sterven er meer dan 780.000 mensen aan de gevolgen van malaria, het merendeel kinderen jonger dan 6 jaar. Malaria wordt veroorzaakt door parasieten van het geslacht *Plasmodium*. Deze parasieten hebben zowel de mug als de mens nodig om hun levenscyclus te volbrengen. De overdracht van deze parasieten wordt veroorzaakt door muggen van het geslacht *Anopheles*. *Anopheles gambiae* Giles *sensu stricto* (verder afgekort als *An. gambiae*) is de meest effectieve vector (overbrenger) van malariaparasieten, doordat deze soort een sterke voorkeur heeft voor mensen als gastheer en steekt en rust binnenshuis. Juist *An. gambiae* verspreidt voornamelijk *Plasmodium falciparum*, de parasiet die de meest dodelijke vorm van malaria veroorzaakt.

In de laatste jaren blijkt de parasiet steeds resistenter te worden tegen geneesmiddelen en onderwijl wordt ook de mug steeds beter bestand tegen de gebruikte insecticiden. Doordat de mug gebruik maakt van geuren tijdens het zoeken van gastheren, nectar en plaatsen voor eileg, kunnen geurstoffen gebruikt worden om het gedrag van de mug te manipuleren. Met de juiste geuren kan de mug worden afgestoten of juist ergens naar toe gelokt worden. Met het oog op de groeiende resistentie tegen de huidige middelen in de strijd tegen malaria, worden strategiën gebaseerd op gedragsmanipulatie door geuren steeds belangrijker. Kennis over het reukvermogen van de malariamug is hiervoor van essentieel belang.

Dit proefschrift is een onderdeel van een groot internationaal project. Dit project had als doel het ontdekken en toepassen van geurstoffen waarmee het gastheerzoekgedrag van de mug gemanipuleerd kan worden. In dit proefschrift ligt de nadruk op de identificatie van voornamelijk menselijke geurstoffen die middels de reukorganen van de mug waargenomen kunnen worden en hun mogelijke rol in het zoeken naar een gastheer.

De reukorganen van de mug zijn 2 antenne's, 2 monddelen (palpen) en een steeksnuit (zie figuur 1). Op elk van deze onderdelen bevinden zich verschillende soorten vaak haarachtige structuren genaamd sensilla (zie figuur 2). Deze sensilla kunnen verschillende functies hebben. Er zijn mechanosensorische sensilla (reageren op mechanische stimuli zoals beweging van de sensilla veroorzaakt door wind), sensilla voor detectie van warmte en vochtigheid en sensilla voor de detectie van vluchtige stoffen. In dit proefschrift zijn alleen de reuk- oftewel olfactorische sensilla bestudeerd. Deze olfactorische sensilla kunnen weer onderverdeeld worden in de sensilla trichodea, sensilla coeloconica en grooved peg sensilla op de antenne (figuur 2a), de capitate peg sensilla op de monddelen (figuur 4a) en de zogenaamde T2-chemosensilla op de steeksnuit (figuur 4b). De sensilla trichodea hebben op hun beurt in de malariamug weer 5 onderverdelingen (genaamd type A tot en met E) op basis van enkele uiterlijke kenmerken zoals de vorm van de punt, stomp of scherp, de lengte en de diameter van

de sensilla. Onder de verschillende typen olfactorische sensilla bevinden zich tussen de 2-5 neuronen (zenuwcellen, ORN in figuur 3) die met dendritische uitlopers het sensillum op de cuticula innerveren. Deze olfactorische neuronen brengen meestal een tweetal moleculaire receptoren voor geurstoffen (OR in figuur 3) tot expressie die geurstoffen kunnen binden en elk van deze receptoren kan meestal binden aan verschillende geurstoffen. In dit proefschrift is gebruik gemaakt van een elektrofysiologische techniek genaamd 'single sensillum recording' (SSR). Deze techniek maakt het mogelijk om de responsen (reacties) van de neuronen op stimulering met verschillende geurstoffen te meten en te visualiseren.

In voorafgaand promotie-onderzoek aan *An. gambiae*, dat gebruik maakte van dezelfde techniek, is het 'responsspectrum', de deelverzameling geuren die een reactie opwekken, van de verschillende neuronen geassocieerd met de grooved pegs, de sensilla trichodea type C en sensilla trichodea type E in kaart gebracht aan de hand van de responsen op een verzameling van 45 geurstoffen.

Het eerste deel van dit proefschrift (**hoofdstuk 2**) beschrijft een systematische SSR-studie van het responsspectrum van de neuronen die 3 typen sensilla trichodea innerveren, typen A, B en D, die tot nog toe niet of nauwelijks bestudeerd zijn. Deze karakterisering van de responsspectra is uitgevoerd aan de hand van een verzameling bestaande uit 132 vluchtige chemische verbindingen waarvan er 80 beschreven zijn als stoffen die vrijkomen van de menselijke huid. Op basis van de reactiesterkte van de verschillende neuronen op de 132 stoffen, konden de neuronen onderverdeeld worden in verschillende responstypen, op basis van overeenkomstige responsspectra. De neuronen geassocieerd met de type B sensilla trichodea, een lang sensillum met een scherpe punt, konden worden onderverdeeld in 6 responstypen; de neuronen van sensilla trichodea type D, een kort sensillum met een stomp uiteinde, konden onderverdeeld worden in 5 verschillende responstypen. Geurstoffen die een sterke reactie veroorzaken, worden verondersteld een belangrijke rol te spelen in oriëntatiegedrag zoals gastheerzoekgedrag. Een aantal van de geuren die de sterkste reacties veroorzaakten in sensilla trichodea type B en D zijn stoffen die vrijkomen van de menselijke huid of uit secreties. Geen enkele van de geteste 132 chemische verbindingen veroorzaakte een reactie van de neuronen geassocieerd met sensilla trichodea type A. Een mogelijke verklaring is dat de geurstoffen waar dit sensillum op reageert niet in onze verzameling aanwezig waren.

De Amerikaanse partners binnen dit project hebben, aan de hand van de complete genoomsequentie van de malariamug, 79 moleculaire olfactorische receptoren geïdentificeerd. Door middel van een heteroloog expressie-systeem (DmHES), met behulp waarvan per neuron een enkele olfactorische receptor van de mug tot expressie gebracht kan worden in een olfactorisch neuron van een bepaald sensillum op de antenne van de fruitvlieg *Drosophila melanogaster* waarvan de oorspronkelijke receptor is uitgeschakeld, werden de responsspectra bepaald van 50 van de 79 olfactorische receptoren. Het is onbekend welke olfactorische receptor in welk olfactorisch neuron van de mug tot expressie komt. De responsspectra van de individuele olfactorische receptoren werden daarom vergeleken met de responsspectra van de 11 geïdentificeerde responstypen van sensilla trichodea type B en D om na te gaan of een

correlatie tussen moleculaire receptor en responstype ontdekt kan worden. Zeven van de 11 responstypen vertoonden een significante correlatie met de responsspectra van enkele olfactorische receptoren. Voor 4 responstypen werd geen correlatie gevonden. Indien er een goede correlatie bestaat tussen de reacties gevonden met behulp van het heterologe expressiesysteem en de electrofysiologische reacties gemeten in de mug zelf, is het heterologe expressie systeem efficiënter voor de zoektocht naar potentiële, afstotende dan wel aantrekkende, geuren, die door het reuksysteem van de malariamug waargenomen kunnen worden.

De olfactorische receptoren AgOR8 en AgOR28 zijn de enige receptoren waarvan bekend is in welke neuronen expressie optreedt en wat hun responsspectra zijn. In **hoofdstuk 3** werd een directe vergelijking gemaakt tussen de elektrofysiologische responsen van AgOR8, tot expressie gebracht in het *Drosophila melanogaster* hereroloog expressie systeem (DmHES), en het neuron in *An. gambiae* (*in vivo*) dat deze receptor bevat (neuron B van de capitate peg sensilla op de maxillaire palp). Deze vergelijking gaf aan dat er verschillen zijn tussen beide systemen in de wijze van reactie op bepaalde stoffen in verschillende concentraties. Concluderend geeft dit aan dat enige voorzichtigheid in acht moet worden genomen bij de extrapolatie van gegevens van het heterologe systeem naar de *in vivo* situatie.

De experimenten beschreven in **hoofdstuk 4** van dit proefschrift hadden tot doel meer informatie te verschaffen over de rol van de monddelen in het gastheerzoekgedrag. Op de monddelen van *An. gambiae* bevindt zich slechts één type olfactorisch sensillum, het capitate peg sensillum. Dit sensillum wordt geïnnerveerd door drie olfactorische neuronen, A, B en C. Neuron A bevat 3 gustatoire receptoren en is het enige type neuron van de malariamug dat reageert op koolstofdioxide (CO_2) een belangrijke lokstof voor de malariamug. Neuron B bevat de al eerder genoemde receptor AgOR8 en reageert zeer gevoelig op 1-octen-3-ol, een geurstof die voor meerdere muggensoorten aantrekkelijk is bevonden. Daarom werd verwacht dat de monddelen een rol spelen in het gastheerzoekgedrag van de mug.

Binnen het overkoepelende project waar dit proefschrift deel van uit maakt, is ontdekt dat huidbacteriën aanwezig op menselijke voeten geurstoffen produceren die aantrekkelijk zijn voor *An. gambiae* vrouwtjes die op zoek zijn naar een gastheer. Een veertiental vluchtige stoffen zijn geïdentificeerd van in cultuur gebrachte bacterie monsters afkomstig van voor de malariamug aantrekkelijke mensen en komen in grotere hoeveelheden daarin voor dan in de bacteriemonsters van minder aantrekkelijke mensen. In **hoofdstuk 4** wordt de rol van de monddelen in gastheerzoekgedrag onderzocht aan de hand van de reactie van de neuronen geassocieerd met de capitate peg sensilla op 10 van deze 14 bacteriële geurstoffen. De responsen van de drie capitate peg neuronen zijn getest en vergeleken in muggen van 3 verschillende fysiologische stadia; muggen die geen bloedmaaltijd hebben gehad, muggen die zich wel al met bloed gevoerd hebben maar nog geen eieren gelegd hebben en muggen die na een bloedmaaltijd ook al eieren gelegd hebben. Negen van deze tien geuren werden gedetecteerd door de drie olfactorische neuronen geassocieerd met de capitate peg sensilla op

de monddelen in muggen die geen bloedmaaltijd gehad hebben. Dit geeft aan dat alle drie de olfactorische neuronen in staat zijn stoffen waar te nemen die aantrekkelijk zijn voor de mug. De sterkte van de reactie veranderde naar gelang de fysiologische toestand waarin de mug zich bevond. Uit eerder onderzoek is al gebleken dat gedurende de eerste 48 uur na een bloedmaaltijd het gastheerzoekgedrag volledig onderdrukt wordt. Neuron B vertoonde lagere responsen voor verschillende bacteriële geurstoffen. Neuron C bleek echter juist in bloedgevoerde muggen die eieren gelegd hadden sterker te reageren op deze van de menselijke huid afkomstige geurstoffen vergeleken met muggen die nooit gevoerd hadden. Dit is het eerste verslag van een verhoogde gevoeligheid na eileg. Dit zou erop kunnen duiden dat muggen nadat ze een keer mensenbloed gehad hebben, nog beter in staat zijn om mensen te localiseren aan de hand van hun geurpatroon. Gedurende deze experimenten bleek tevens dat vijf van deze 10 bacteriële stoffen in staat waren om de respons op CO_2 volledig te kunnen onderdrukken. Deze vijf geurstoffen induceerden ook responsen in zowel de andere olfactorische neuronen geassocieerd met de capitate peg sensilla als in neuronen geassocieerd met antennale olfactorische sensilla. Deze bevindingen suggereren dat geurstoffen geproduceerd door op de voeten levende bacteriën door de mug gebruikt kunnen worden om de mens te localiseren. Dezelfde stoffen zouden gebruikt kunnen worden om het gastheerzoekgedrag van de malariamug te manipuleren.

Een van de 10 bacteriële geurstoffen (2-fenylethanol) veroorzaakte een extreem sterke reactie met een uniek temporeel (tijdsafhankelijk) patroon van een van de twee olfactorische neuronen geassocieerd met de kortste sensilla trichodea met een scherpe punt, type E. Dit werd ook waargenomen voor linalooloxide, een van de stoffen die de sterkste respons gaf van een van de responstypen van sensilla trichodea type B. Vanwege dit unieke patroon hebben deze responsen de naam 'extensively sustained responses' (langdurig aanhoudende responsen) gekregen. Deze responsen zijn gedefinieerd als een excitatie reactie die meer dan 10 minuten voortduurt na een enkele korte stimulans van 200 ms. In **hoofdstuk 5** wordt dit ongewone elektrofysiologische tijdspatroon in detail beschreven en gekoppeld aan geobserveerde gedragsveranderingen die mogelijk gebruikt kunnen worden voor het manipuleren van gastheerzoekgedrag. Het was duidelijk zichtbaar dat een korte blootstelling aan deze 2 geurstoffen, ertoe leidde dat gedurende de 5 minuten die volgden op de stimulans de muggen meer tijd besteden aan gedragingen op de geurbron zoals het contact maken met de steeksnuit en vervolgens prikken van de steeksnuit door het gaas waarachter de geurbron zich bevond. Een dergelijk rechtstreeks verband tussen bepaalde temporele eigenschappen van elektrofysiologische activiteit van een olfactorisch neuron en de daaruit voortvloeiende veranderingen in elementen van gastheerzoekgedrag is niet eerder beschreven voor insecten. Deze bevindingen benadrukken het belang van temporele kenmerken van neurale activiteit die ten grondslag ligt aan de gedragsrespons op geuren.

De resultaten gepresenteerd in dit proefschrift verruimen onze kennis over het functioneren van het perifere olfactorisch systeem van de malariamug *An. gambiae*. Verschillende geurstoffen zijn geïdentificeerd die een sterke electrofysiologische respons veroorzaken in de mug. Dit zijn veelbelovende aanwijzingen dat deze geuren gebruikt kunnen worden voor manipulatie van het gastheerzoekgedrag van muggen. De vergelijking van resultaten bereikt met het heterologe expressie systeem en die gevonden voor de malariamug zelf geeft aan dat er correlaties gevonden worden maar ook dat er een aantal verschillen zijn waardoor niet alle resultaten van het ene systeem automatisch gelden voor het andere systeem. Er is ook bewezen dat de neuronen van de capitate peg sensilla op de maxillaire palp geurstoffen geproduceerd door bacteriën die op de menselijke huid voorkomen kunnen waarnemen, dat hun gevoeligheid verandert met het fysiologisch stadium waarin de mug zich bevindt, dat na eileg een van de drie neuronen nog sterker reageert op menselijke stoffen en dat vijf van deze bacteriële stoffen de respons op CO_2 volledig kunnen onderdrukken. Tevens is aangetoond dat temporele aspecten van belang zijn voor geurcodering.

De in dit proefschrift gepresenteerde nieuwe informatie kan bijdragen aan het ontrafelen van de samenstelling van optimale geurmengsels die bijvoorbeeld gebruikt kunnen worden om veranderingen in de muggenpopulatie op tijd op te merken. Een andere mogelijkheid voor het gebruik van dergelijke mengsels is als onderdeel van bestrijdingsstrategieën gebaseerd op geuren zoals "push-pull" systemen. In deze systemen worden afstotende geuren gebruikt om de muggen te weren uit dorpen (push) en tegelijktijd aan te trekken door middel van aantrekkelijke geurstoffen (pull) geplaatst in een vallensysteem aan de buitenrand van de dorpen. Op deze manier kan de frequentie van de interacties tussen mug en mens worden verlaagd wat zal leiden tot minder muggenbeten en daarmee een kleinere kans op het overbrengen van de malariaparasiet.

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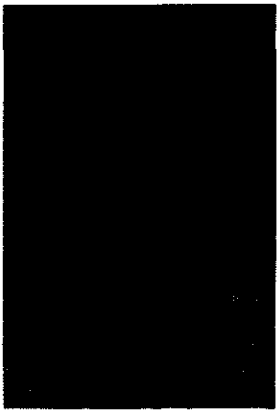
Curriculum Vitae



Remco A. Suer was born on the 7th of June 1982 in Purmerend, The Netherlands. At the age of 12 he moved to Belgium where he finished his secondary school at the Koninklijk Atheneum Keerbergen in 2000.

That same year Remco started studying Biology at the University of Utrecht back in The Netherlands. During his BSc he developed an interest in neurobiology and ethology. During his MSc he chose his internships accordingly. In 2004 he started his first internship at the Department of Functional Neurobiology at the University of Utrecht. The internship entailed gaining a better understanding of the human visual system by studying the temporal properties of centre-surround interaction in human motion perception. The second internship was supervised by the Department Ethology and Welfare of the University of Utrecht. This project focussed on the identification of behaviours that could be used as indicators for stress in captive bottlenose dolphins with the aim of improving the welfare of the animals. After obtaining his MSc in 2006, Remco continued to work for three months at the Ethology and Welfare Department on a project that investigated long-term decision making in rats.

In September 2006 he started his PhD on unravelling the malaria mosquito's sense of smell at the Laboratory of Entomology, Wageningen University, the results of which have been presented in this dissertation.

During the last few months of his PhD research, Remco founded, together with Bart Knols and Marit Farenhorst, his own company In2Care. He will continue his scientific career with a combination of fundamental and applied research by developing, re-engineering and validating products against disease-transmitting insects.

PE&RC PhD Education Certificate

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 32 ECTS (= 22 weeks of activities)

Review of literature (6 ECTS)

- Olfactory physiology of the African malaria mosquito, *Anopheles gambiae* Giles *s.s.*

Writing of project proposal (2.5 ECTS)

- Using dogs to detect transmissible malaria infections in humans; Grand Challenges Explorations round 2 (2009)

Post-graduate courses (5.5 ECTS)

- Insect chemical ecology; SLU Sweden (2007)
- Advanced statistics; PE&RC (2008)

Laboratory training and working visits (3 ECTS)

- PhD Excursion; Rothamsted Research, University of Oxford, London School of Hygiene and Tropical Medicine, University of Southampton (2007)

Deficiency, refresh, brush-up courses (1.5 ECTS)

- Basic statistics (2007)

Competence strengthening / skills courses (3.2 ECTS)

- Project- and time management; WGS (2007)
- Entrepreneurial boot camp; WBS (2009)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.5 ECTS)

- Introduction weekend; PE&RC (2007)
- PE&RC Days (2007, 2008)

Discussion groups / local seminars / other scientific meetings (8.6 ECTS)

- PhD Discussion group Entomology (2006-2010)
- Monthly Seminars at the laboratory of Entomology (2006-2010)
- GCGH Young Investigator Meeting (2007-2010)
- NEV Days (2007-2009)
- Netherlands Annual Ecology Meeting: **NERN** (2008-2009)

International symposia, workshops and conferences (4.2 ECTS)

- European Symposium for Insect Taste and Olfaction: ESITO (2009)
- European Chemoreception Research Organisation: ECRO (2009)

Lecturing / supervision of practical's / tutorials; 8 days (2.4 ECTS)

- Biology and management of plant pathogens, insects and weeds (2008-2009)

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