Olfaction in the malaria mosquito *Anopheles gambiae*

electrophysiology and identification of kairomones

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Stellingen

1. Inhibitie van de spontane vuurfrequentie van olfactorische neuronen in respons op bepaalde geuren wordt, door onvolledige kennis van perifere olfactorische coderings mechanismen, ten onrechte als een artefact beschouwd.

Olfaction in mosquito-host interactions. Wiley, Chichester (Ciba Foundation Symposium 200). General discussion V. p. 282

2. Het is niet aannemelijk dat bij haematofage muggen alleen de grooved peg sensilla betrokken zouden zijn bij de detectie van gastheergeuren.

Pappenberger B. et al., (1996) Responses of antennal olfactory receptors in the yellow fever mosquito Aedes aegypti to human body odours. In: *Olfaction in mosquito-host interactions. Wiley, Chichester (Ciba Foundation Symposium 200) p. 254-262*
Dit proefschrift

3. Geurvallen zullen alleen dan een positieve bijdrage leveren aan het terugdringen van het aantal malaria gevallen, wanneer ze gecombineerd ingezet worden met additionele antimalaria middelen.

4. Het uitblijven van een bruikbaar middel tegen malaria na tientallen jaren van onderzoek mag geen reden zijn voor verminderde financiering. De toenemende resistentie van *Plasmodium* tegen malaria profylaxis pleit eerder voor het tegenovergestelde.

Butler D. (1997) Briefing malaria. Nature 386: 535-541

5. Het gebruik van vomeroferines in parfums dient, gezien mogelijke consequenties voor de gezondheid, allereerst onderworpen te worden aan uitgebreid wetenschappelijk onderzoek.

Berliner D.L. et al., (1996) The functionality of the human vomeronasal organ (VNO): evidence for steroid receptors. Journal of steroid biochemistry and molecular biology 58: 259-265
Cutler W.B. et al., (1998) Pheromonal influences on sociosexual behavior in men. Archives of sexual behavior 27: 1-13
Sobel N. et al., (1999) Blind smell: brain activation induced by an undetected air-borne chemical. Brain 122: 209-217

6. Het patenteren van menselijke genen dient niet het algemeen maatschappelijk belang.

Reichhardt T. (1998) Patent on gene fragment sends researchers a mixed message. Nature 396 p. 499

7. De notatie van referenties bij publicaties in wetenschappelijke tijdschriften dient op logische wijze gestandaardiseerd te worden. De huidige sterk uiteenlopende notaties zijn inefficiënt en leiden tot een verlaging van de arbeidsproduktiviteit en een verhoging van de bloeddruk.

8. Ten onrechte wordt door een onderzoeker-in-opleiding meer onderwijs gegeven dan genoten.

9. Het volgen van de stroom van tegenstrijdige voedingsadviezen is een dagtaak voor die mensen die een gezond leven proberen na te streven.

10. Tussen 0 en 1 ligt een wereld van nuances.

Stellingen behorend bij het proefschrift:
Olfaction in the malaria mosquito *Anopheles gambiae*
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Jocelijn Meijerink
Wageningen, 26 oktober 1999

Voor mijn ouders

Voor Marcel

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Chapter 1

General introduction

Anopheles gambiae, a prominent vector of malaria

One-third of the world population lives in areas where malaria is a daily threat. Of all cases of malaria 90% occur in sub-Saharan Africa, while 6-7% of the cases take place in India, Brazil, Sri Lanka, Vietnam, Colombia and the Solomon Islands. Reports from Africa mention that people can suffer more than 300 mosquito bites per person per night (Charlwood et al., 1995). With every bite there is a considerable risk of infection with malaria parasites. Each year between 1.5 and 2.7 million people die of malaria, while another 300-500 million people carry the disease and become seriously ill (WHO, 1997).

Female mosquitoes hunting for blood act as the vectors of malaria parasites. A bloodmeal provides the mosquito with proteins necessary for the development of her eggs. During bloodfeeding malaria parasites enter the bloodstream of the human host. In the liver cells, they multiply and after one or two weeks the cells burst and the spores, now called merozoites, invade the red blood cells. This is when the host is starting to get fever. The merozoites multiply and continue invading new bloodcells. If the patient has no immunity or can not be treated, the infection remains or can return at certain intervals and the patient may die. Some of the merozoites form sexual stages, the gametocytes, which stay in the blood. When the host is bitten by a female mosquito, the gametocytes are transmitted. Within the vector, the female gametocytes are fertilized and develop into an ookinete, which traverses the gut wall and than develops as an oocyst on the wall of the foregut. The oocyst will burst after a few weeks and an enormous number of sporozoites will move to the mosquitoes' salivary gland. As soon as the female mosquito takes a new bloodmeal, the sporozoites are transmitted to the host and the whole cycle will start again (Gilles et al., 1993).

Malaria is caused by protozoan parasites of the genus *Plasmodium*. Four types of malaria exist, *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae*, the first being the most dangerous form, accounting for 90% of all malaria cases in Africa.

The mosquito vector of malaria belongs to the *Anopheles gambiae sensu lato* complex which consists of at least seven sibling species (White, 1974; Hunt et al., 1998). *Anopheles gambiae* s.s. is highly anthropophilic and the main vector of human malaria in Africa. *Anopheles arabiensis* is an opportunistic feeder exhibiting varying degrees of anthropophily or zoophily, while *Anopheles quadriannulatus* (species A and B) are zoophilic and not disease vectors. The three remaining species of the complex, *An. bwambae* and the salt-water siblings *An. melas* and *An. merus* are important local vectors of malaria transmission.

Despite decades of research and the use of different vector control strategies, to date the parasites nor the vector are under control. There is an urgent need for the development of novel strategies and improval of existing strategies in the battle against malaria.

Host-seeking behaviour of mosquitoes

It is generally assumed that mosquitoes locate and identify their host on the basis of chemical and physical cues (Takken and Knols, 1999). To date, however, few studies have revealed the identity of the cues that guide a mosquito to its host. Lactic acid, a main constituent of human sweat, was the first component reported to act as an attractant for the yellow fever mosquito *Aedes aegypti* (Acree et al., 1968). As an individual compound, lactic acid is only a weak attractant, but it acts synergistically when combined with carbon dioxide. In addition, human skin wash extracts tested in a windtunnel bioassay lose almost all of their activity after the removal of lactic acid, implicating that the latter component also acts synergistically with other, as yet unidentified components (Geier et al., 1996).

Analysis of cattle odour led to the identification of 1-octen-3-ol, which has since been shown to serve as a powerful attractant for certain species of tsetse flies in the field (Hall et al., 1984; Vale and Hall, 1985). Subsequent studies on mosquitoes have revealed that 1-octen-3-ol affects the host-seeking behaviour of these haematophagous insects as well (Takken and Kline, 1989; Kline et al., 1990a,b, 1991a,b). Traps baited with 1-octen-3-ol have resulted in catches of only a few mosquito species, but in combination with

CO_2 an increase in the collections of especially *Aedes*, *Anopheles*, *Psorophora*, *Coquillettidia* and *Mansonia* species has been observed (Kline, 1994).

Although the role of CO_2 in the host-seeking behaviour of *Anopheles* and other mosquito species is still not fully understood, studies to date indicate a minor role for CO_2 in the attraction of female *An. gambiae s.s.* Field studies conducted in Tanzania showed that only 9% of *An. gambiae* and *An. arabiensis* normally caught in a tent baited with human odour were caught in a tent into which CO_2 was pumped (Mboera et al., 1997). Additional field studies showed that the role of CO_2 depends on the degree of zoophily. The zoophilic *An. quadriannulatus* was equally attracted to a calf and CO_2 (at rates equivalent to that released by a calf), whereas only 20% of the opportunistic *An.. arabiensis* was caught with CO_2 (man equivalent) when compared with a man (Dekker and Takken, 1998).

For *An. gambiae s.s.*, cues involved in the location and selection of a suitable host have been implied to be mainly olfactory (Takken and Knols, 1999). Biting site experiments revealed that *An. gambiae s.s.* has a preference for the lower parts of the human body, the foot and ankles, whilst *An. atroparvus* strongly preferred the head and shoulder region. Washing of the feet and ankles led to a change in biting site preference for *An. gambiae s.s.* The same was observed for *An. atroparvus* after removing the exhaled air (de Jong and Knols, 1995). However, in contrast, Dekker et al. (1998) found that *An. gambiae* females still had a preference for the feet and ankles after washing and suggest that convection currents rather than foot odours lead the mosquito to the lower parts of the human body. Nevertheless, the fact that different species exhibit distinct biting site preferences indicates that they use different cues for close-range attraction.

The striking similarity between foot odour and certain cheeses, at least to the human nose, has led to the assumption that both might release similar components, some of which may act as kairomones. Indeed, testing of Limburger cheese revealed that female *An. gambiae* is attracted to this non-human source of volatiles in a windtunnel bioassay (Knols and De Jong, 1996). Analysis of Limburger cheese identified a range of carboxylic acids. An acid extract of this cheese as well as a synthetic mixture comprising similar acids was also attractive for *An. gambiae* in a windtunnel bioassay (Knols et al., 1997). A broad range of carboxylic acids has been shown to be present on the human skin (Nicolaides, 1974). These acids are major products of microbial activity, suggesting that the kairomones for female *An. gambiae* are of microbial origin.

Human skin glands and their emanations

Distribution of distinct glands on the human skin

The human skin contains three types of glands, the apocrine gland, the eccrine gland and the sebaceous gland. The apocrine gland is mainly restricted to the axilla, pubic area, areola, nipple, anogenital area, edge of the eyelids and the external ear canal and becomes active during adolescence. The eccrine gland is responsible for thermoregulation, is activated by heat stimulation or vigorous exercise and mainly excretes water (99%), urea and lactate; it is widely distributed over the entire body surface. The sebaceous gland has an irregular distribution pattern and has a major secretory area on the forehead. Human skin, however, is exceptionally rich in sebaceous glands and the sebum, the oily liquid secretion product of this gland, covers the entire body surface as a result of normal muscular activity (Albone, 1984).

Secretion products of skin glands and their contribution to human odour

Probably the most important source of the wide range of lipids on the human skin are the sebaceous glands. In the ducts of the sebaceous glands and on the skin surface, triacyl glycerols are hydrolysed by micro-organisms to form free fatty acids, mono- and diacyl glycerols and free glycerol. The triacyl glycerols and their products are only found in the skin surface lipid of human beings, and were not encountered in those of other animals. The fatty acid chains of human skin lipids comprise an uniqueness which is manifested in the number and kind of carbon skeletal types, the extremely wide range of chain lengths and the unusual pattern of unsaturation (Nicolaides, 1974). In addition to the large amount of glycerides present in the human sebum, it also contains sterol esters and wax esters, of which wax esters are common skin lipids in most species of mammals studied. Another major compound, not normally encountered on the skin surface of other species, is the unsaturated C_{30} hydrocarbon, squalene (Nicolaides, 1974, Albone, 1984).

The dominance of the eccrine sweat gland is a characteristically human feature. It has been reported that humans are the only living bipedal mammal with both a naked skin and a totally eccrine-dependent cooling system (Folk and Semken, 1991). Although the main excretion product of this gland is water, it also excretes urea and lactate, of which the latter is produced by the clear cells of the gland (Sato, 1977; Ament et al., 1997). The origin of ammonia in sweat is still unclear. Although it has been suggested that the ammonia content in sweat is mediated by diffusion from the plasma it can also be

produced by the gland cell itself (Ament et al., 1997). However, urease activity of skin bacteria probably also contributes to ammonia production (Ballows, 1991).

The formerly reported studies have focussed on the composition of the liquid sweat. However, the volatile constituents released by the human skin might provide valuable information about the 'headspace' which surrounds a human individual. Analysis of the headspace of human individuals has been performed by Sastry et al. (1980) and Ellin et al. (1974).

Insect olfactory sensilla

Morphology of olfactory sensilla

Detection of odorous molecules in insects is mediated by the olfactory receptor cells innervating different types of antennal sensilla. The sensilla contain pores through which the airborne odorants can enter the sensillum cavity and interact with the receptor cell membrane (Slifer, 1954, 1960; Steinbrecht, 1997). The sensory dendrites of the olfactory cells are bathing in a characteristic protecting fluid, the sensillum lymph, which forms a hydrophilic barrier for the hydrophobic airborne stimuli (Breer et al., 1990).

Two fundamentally distinct categories of olfactory sensilla can be distinguished in insects, the single-walled and double-walled wall-pore sensilla (Fig.1) The single-walled sensilla have cuticular pores which widen into a pore kettle and further extend into several pore tubules. The pore tubules elongate into the lumen where they protrude into the sensillum lymph (Steinbrecht, 1997). Examples of single-walled wall-pore sensilla include pheromone-sensitive sensilla trichodea of the moth and the sensilla trichodea of mosquitoes. In contrast to these single-walled sensilla, double-walled sensilla do not exhibit pore tubules. The lumen where the dendrites of the double-walled sensilla are located are connected to the environment by spoke channels. The cuticular wall of the double walled sensilla consist of several cuticular fingers, which can remain separate or merge, leaving only the radial spoke channels open (Steinbrecht, 1997). Double-walled sensilla are usually smaller than single-walled sensilla and are often located in a pit, like the large sensillum coeloconica in anopheline mosquitoes. The grooved-peg sensillum is another example of a double-walled wall-pore sensillum.

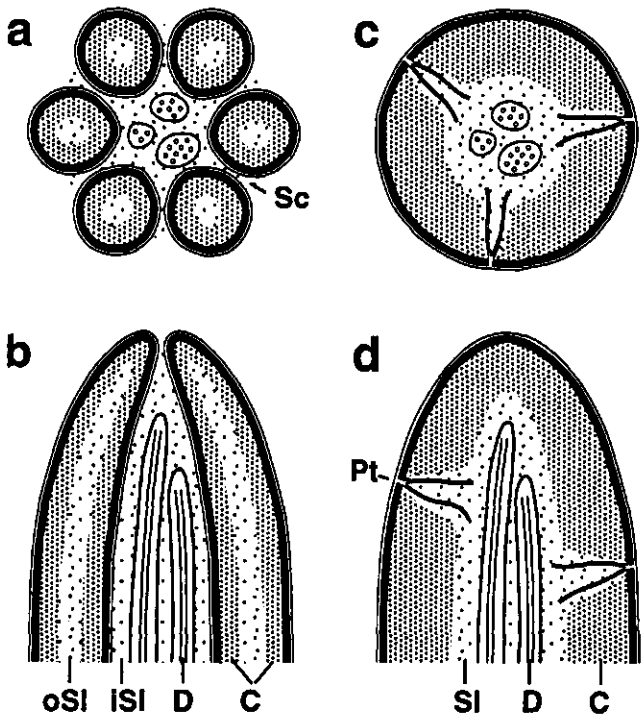

Fig 1. Topological concept of double-walled versus single-walled insect olfactory sensilla (a, c, cross-sections; b, d, longitudinal sections). The sensory dendrites of the double-walled wall-pore sensilla are located outside the cuticular fingers, and surrounded by several cuticular extensions (a, b), while the sensory dendrites of the single-walled wall-pore sensilla are located inside the culicular extension (c, d). Sc = spoke channel, D = dendrite, C = cuticle, Sl = sensillum lymph, oSl = the outer sensillum lymph, iSl = inner sensillum lymph, Pt = pore-tubule systems (after Steinbrecht, 1997).

Chemoreception of odours in insects

Localisation and function of odour binding proteins

The first odour binding protein (OBP) identified for insects was the pheromone odour binding protein (PBP) of the giant silkmoth *Antheraea polyphemus* (Vogt and Riddiford, 1981). This protein specifically binds to the sex pheromone components of the conspecific female moth and is associated with the receptor lymph of pheromone-sensitive sensilla. A pheromone-specific esterase has also been identified in the lymph. It was thought that the PBP was involved in inactivation and the esterase involved in the subsequent degradation of the female sex pheromone. Further studies were involved in

the cloning and sequencing of odour binding proteins of several moth species (Györgyi et al., 1988; Raming et al., 1989; Krieger et al., 1993). In addition to the PBP's, two other subfamilies, called the general odorant binding proteins (GOBPs), were identified (Breer et al., 1990; Vogt et al., 1991a, b).

In several moth species, the PBP has been found to be localised exclusively in the long pheromone-sensitive trichoid hairs of the male moth antennae. In contrast, the GOBP was found in the long trichoid hairs of the female moth antennae and in the basiconica sensilla of both the female and male moth. GOBPs were localised in those sensilla housing sensitive receptor neurons for plant or other 'general' odours. This indicates that the expression of GOBP's reflects different, non-pheromonal specificity of the olfactory receptor neurons involved (Steinbrecht et al, 1995). Although the physiological function of the odour binding proteins is still not known, the above findings have led to a number of hypotheses. OBPs are thought to be involved in the transport of odorants to the receptor membrane (Vogt, 1987) or in the inactivation of odorants after interacting with the receptor site (Vogt and Riddiford, 1981; Kaissling, 1987). Another possibility is that they are involved in both processes (Van den Berg and Ziegelberger, 1991; Ziegelberger, 1995). In addition, the increasing numbers of identified OBPs within the same species suggests that odour binding proteins might be involved in odour molecule recognition as well (Breer et al., 1990; Vogt et al., 1991a, b). Distribution patterns showing a correlation between the functional specificity of olfactory sensilla and the localisation of PBP versus OBP support this suggestion (Steinbrecht et al., 1995).

To date, odour binding proteins have only been identified within the single-wall sensilla and not in the double-walled wall-pore sensilla (Steinbrecht et al., 1995; Steinbrecht, 1997). Steinbrecht (1997) postulated that the pore tubules in single-walled sensilla might facilitate the binding of odours to odour binding proteins along the entire length of the pore tubules, thereby enlarging the contact surface and speeding up the process of odour transport. Double-walled sensilla probably use a different mechanism for the transport of odours, as no odour binding proteins have been discovered in double-walled sensilla. Recently, pores, pore kettles and pore tubules have been identified within the sensilla trichodea of *Aedes aegypti*, indicating that the stimulus conducting structures in single-walled sensilla of mosquitoes are similar to those found in other insects (Muir and Cribb, 1994).

Mosquito sensory physiology

Overview of single cell recordings performed on mosquitoes

Most of the sensory physiology studies undertaken to explore olfactory receptor neuron responses in mosquitoes have focussed on *Aedes aegypti*. For this species, neurons responsive to lactic aid, the only attractant for *Ae. aegypti* identified thus far, were located within the grooved peg sensillum (Davis and Sokolove, 1976). Two types of lactic acid-sensitive neurons were found, lactic acid-excited and lactic acid-inhibited neurons. After a bloodmeal the sensitivity of the lactic acid-excited neuron was found to decrease (Davis, 1984a). The responsible factor for this decrease in sensitivity appeared to be a haemolymph-borne factor, released by the fat body (Klowden et al., 1987; Davis et al., 1987). Further studies in different mosquito species have focussed on the sensitivity of the lactic acid-excited neuron in relation to the physiological state of the mosquito (Bowen et al., 1988; Bowen, 1990; Bowen et al., 1994).

Receptor neurons responsive to carbon dioxide have been found within the sensilla basiconica on the palpi in several mosquito species (Kellogg, 1970; Grant et al., 1995). In the attraction of *Ae. aegypti*, carbon dioxide is known to act synergistically with lactic acid (Acree et al., 1968). Another neuron within the sensilla basiconica was reported to be highly sensitive to 1-octen-3-ol (Grant and O'Connell, 1996). However, the sensitivity of the third neuron innervating this sensillum type is still not known.

Recordings of the neurons innervating the small sensilla coeloconica revealed that these are temperature receptors (Davis and Sokolove, 1975). Responses of grooved peg neurons to water vapour has been reported (Kellogg, 1970), but the relevance of these responses has been questioned (Davis and Sokolove, 1976).

Oviposition-site related and plant-produced volatiles have been reported to elicit responses in the receptor neurons of the sensilla trichodea in *Anopheles stephensi*, *Aedes* and *Culex* species (Bentley et al., 1982; Davis, 1976, 1977; Bowen, 1992). These reports (Bowen, 1991) as well as unpublished results of the authors are reviewed in Davis and Bowen (1994).Table 1 presents a summary of the responses of the antennal and palpal receptor neurons of different mosquito species to host-related, plant-related and oviposition-site related cues.

Table 1. Overview of single sensillum and single cell recordings performed on different mosquito species, chronologically listed

Mosquito species	Stimulus	Response [a]	Sensillum	References
Aedes aegypti	carboxylic acids [1]	+	A1 [1] sensilla	Lacher, 1967
	oils [1]	-	trichodea	
	carboxylic acids [2]	+/-	A2 /A2-m [2] * sensilla trichodea	
Aedes aegypti	water vapour [1]	+	grooved-peg [1]	Kellogg,
	CO_2 [2]	+	basiconica (palpi) [2]	1970
Aedes aegypti	temperature >	+	small sensilla	Davis and
	temperature <	+	coeloconica	Sokolove, 1975
Aedes aegypti	lactic acid	+/-	grooved-peg	Davis and
	water vapour	+		Sokolove,
	repellents	-		1976
	lactic acid related	+/-		
	oviposition-site related	+/-		
	miscellaneous	+/-		
Aedes aegypti	oviposition-site related	+	sensilla trichodea	Davis, 1976
	host-related	+/-	type II	
	repellents	+/-		
	plant-related	+/-		
	miscellaneous	+		
Aedes aegypti (male)	lactic acid	+/-	grooved-peg &	Davis, 1977
	oviposition-site related	+/-	sensilla trichodea	
	host-related	+/-	type II	
	repellents	+/-		
	plant-related	+/-		
	miscellaneous	+/-		
Aedes aegypti	*p*-cresol	+	sensilla trichodea	Bentley et al.,
Aedes triseriatus	4-methylcyclohexanol	+/-	A2, short blunt &	1982
Culex tarsalis			short pointed	
Anopheles stephensi				

Table 1 (continued)

Aedes aegypti	lactic acid [b]	+/-	grooved-peg	Davis, 1984a Davis, 1984b Klowden et al., 1987 Davis et al., 1987
Aedes aegypti	lactic acid-related stimuli	+	grooved-peg	Davis, 1988
Culex pipiens	lactic acid [b]	+	grooved-peg	Bowen et al., 1988; Bowen, 1990
	ethyl proprionate [b]	+	sensilla trichodea A2	
Culex pipiens	terpenes	+	sensilla trichodea A2	Bowen, 1992
	green plant volatiles	+		
	fatty acid esters	+		
	4-methylcyclohexanol	+		
Aedes atropalpus	lactic acid [b]	+/-	grooved-peg	Bowen et al., 1994
Aedes aegypti *Aedes epactius* *Aedes atropalpus* *Culex pipiens*	lactic acid butyric acid	+/- +	short and long length grooved-peg sensilla	Bowen, 1995
Aedes aegypti *Aedes taeniorhynchus* *Anopheles stephensi* *Culiseta melanura* *Culex quinquefasciatus*	CO_2 1-octen-3-ol	+ +	sensilla basiconia (palpi)	Grant et al., 1995; Grant and O'Connell, 1996
Aedes aegypti	Human skin wash		grooved-peg	Pappenberger et al., 1996
	extracts, fractions	+		
	carboxylic acids	+		
	lactic acid	+		
	pentylamine	+		

[a] + represents excitation and - inhibition

[b] studies in which the sensitivity of the olfactory receptor neuron was investigated in relation to the physiological state of the mosquito

[1, 2] Numbers depict sensilla responding to stimuli with a corresponding number.

* The morphological types of A2 sensilla trichodea are reclassified in Davis (1974)

Neural encoding of odours in insects

Peripheral encoding

Two categories of olfactory receptor neuron cells have often been distinguished with respect to odour discrimination, the specialist and the generalist receptor cells. Specialists cells are sensitive to only one key component. Although they can respond to chemically related components, they often show clear-cut differences in sensitivity to compounds within that group. Dose-response characteristics of the individual specialists to key components are similar. Examples of specialists include the pheromone sensitive receptor neurons. Generalists on the other hand, are broadly tuned to a wide spectrum of odours that are not necessarily chemically related. Individual receptor cells display different, partially overlapping response spectra. The distinction between these two cell categories is however, not absolute and intermediate cells exist, exhibiting high sensitivity to a key component but also responses to other components (Kaissling, 1987). These cells are called specialised generalists and it was suggested by Boeckh (1980) that most olfactory receptor cells might exhibit properties between the two extremes of generalist and specialist cells.

Two clear concepts of neural coding of odour quality exist. If the excitation of a specialist cell evokes a specific behavioural response, this cell encodes information according to the 'labeled line' concept. When receptor cells respond to components with overlapping response spectra and each component evokes a characteristic response profile, this is called an 'across fibre pattern'. These concepts are not mutually exclusive, as there are examples of systems exhibiting both concepts (Boeckh, 1980; Kaissling, 1987).

Processing of odour information in the central nervous system: the role of olfactory glomeruli

Information about odour molecules, detected at the olfactory receptor cell, is conveyed along the cell's axon leading to the primary olfactory centre in the brain, the antennal lobe. At the entrance of the antennal lobe axons intermingle and are regrouped, after which they converge on a module called glomerulus. Each axon projects to only one glomerulus and it is thought that the glomeruli function to sort the input of odour information. Primary afferent axons of several olfactory receptor cells converge on one

glomerulus, where they make contact with neurites of olfactory lobe neurons, mainly local interneurons. Local interneurons interact synaptically with other olfactory lobe neurons and connect with different glomeruli. Local interneurons also interact with projection neurons, which extend axons to one or more higher-order olfactory foci in the protocerebrum (Hildebrand, 1996).

Recently, the projection of the antennal and maxillary palp afferents were studied in *Aedes aegypti* (Anton, 1996; Distler and Boeckh, 1997). It was found that afferents of the antennal flagellum project into all glomeruli of the ipsilateral antennal lobe, with exception of an defined glomerulus, which is innervated by the maxillary nerve (Distler and Boeckh, 1997). In the antennal lobe of *Aedes aegypti*, more than 35 distinguishable glomeruli were described, which are innervated by slightly more than 2000 chemosensory neurons from the antenna and maxillary palps (Bausenwein and Nick, 1998). In addition it was reported that males have fewer glomeruli than females (Bausenwein and Nick, 1999).

The most extensively studied glomerulus complex of the antennal lobe in insects is the sexually dimorphic macroglomerular complex of the male moth *Manduca sexta*. The macroglomerular complex comprises at least two distinct glomeruli, the toroid and the cumulus to which the axons of the highly specialised pheromone receptor cells project. Axons of the olfactory cell specific for pheromone component A project to the toroid, while the axons of the olfactory cell specific for pheromone B project to the cumulus (Hansson et al., 1991; Christensen et al., 1995; Hildebrand and Shepherd, 1997).

In contrast to the specialist pheromone-tuned glomeruli, glomeruli receiving projections of generalist olfactory receptor cells are thought to process information according to the "complex labeled line" (Shepherd, 1992; Hildebrand, 1996). These glomeruli receive projections from olfactory receptor cells with the same odour spectra, but they also receive projections of olfactory receptor cells with different, but overlapping response spectra. Some glomeruli are only receiving input from very narrowly tuned olfactory receptor cells, like the pheromone sensitive cells, while others receive projections from different olfactory receptor cells with overlapping response spectra.

Context of the present research project

This study was the first within a research program entitled: 'Odour guided host finding by haematophagous mosquitoes', which consisted of three PhD projects. The study described within this thesis has focussed on the identification and neural encoding of host-odours used in the host-seeking behaviour of *An. gambiae s.s.* In the second project the peripheral coding in three sibling species with different host-preferences, the anthropophilic *An. gambiae s.s.*, the opportunistic *An. arabiensis* and the zoophilic *An. quadriannulatus* was compared. This study was conducted by I.V.F. Van den Broek at the Department of Animal Physiology, University of Groningen. The third project consisted of a behavioural study on host-attractants for female *An. gambiae*, performed by M.A.H. Braks at the Laboratory of Entomology, Wageningen University.

Outline of the thesis

The first goal of this PhD project was to reveal the neural encoding of host odours used by females of the mosquito *Anopheles gambiae* Giles *s.s.* to locate a suitable host. In particular, the receptor neurons innervating the grooved-peg sensilla and the sensilla trichodea were subject of this study. A second goal was the identification of semiochemicals involved in the host-seeking behaviour of *An. gambiae*. This was accomplished by chemical analysis of the headspace of human sweat samples followed by screening for olfactory activity by means of electroantennogram recording.

To reveal the neural encoding of host odours, we first made scanning electron microscopic photographs (SEM) of the different types of sensilla on the antennae of female *An. gambiae*. The distribution of the grooved-peg sensilla, large sensilla coeloconica, small sensilla coeloconica, different subtypes of sensilla trichodea, sensilla ampullacea and the sensilla chaetica were described and the function of these sensilla with respect to the neural encoding was discussed **(chapter 2)**.

Next, we investigated the peripheral sensory responses of antennal olfactory cells of female *An. gambiae* by means of electroantennography. Therefore the technical aspects of the EAG recording technique were more closely examined. Furthermore, dose-response profiles were established for the carboxylic acids 3-methylbutanoic, pentanoic,

hexanoic, heptanoic, octanoic, nonanoic, decanoic, dodecanoic, tetradecanoic and hexadecanoic acid **(chapter 3)**.

The following step was to perform single sensillum recordings on antennal receptor neurons innervating one or more subtypes of the sensilla trichodea population in response to short chain carboxylic acids and 1-octen-3-ol. Carboxylic acids evoked inhibitory or excitatory responses of the olfactory receptor cells, while 1-octen-3-ol evoked excitation. We focussed on the carboxylic acid-inhibited cell type and its temporal pattern of response to different doses of the components. In order to allow a comparison of the stimulatory effectiveness at equivalent doses, corrections were made for differences in volatility between the different compounds **(chapter 4)**.

Identification of potential kairomones for host-seeking *An. gambiae* was the next phase of research. Fresh and incubated human sweat was tested on the behavioural- and on the EAG level. Comparison of the compounds present in the headspace samples of freshly collected sweat with that of incubated sweat and subsequent electroantennographic studies led to the identification of several compounds as olfactory stimulants **(chapter 5)**.

Finally, we document the presence of olfactory receptor neurons responsive to incubated sweat and sweat-borne components on the antennae of female *An. gambiae*. Single cell recordings revealed functionally different subpopulations of receptor neurons within the grooved-peg sensilla and the sensilla trichodea. The dose-response profiles of the responsive olfactory receptor neurons were determined **(chapter 6)**.

The thesis is concluded with a general discussion, in which the results are summarized and the neural coding of the sweat-borne components is discussed **(chapter 7)**. Results are placed in a broader perspective and are discussed with respect to putative neural coding mechanisms on the level of the central nervous system. The peripheral coding of potential kairomones is viewed in the context of olfactory guided host-seeking behaviour of female *An. gambiae*.

References

Acree F Jr, Turner RB, Gouck HK, Beroza M and Smith N (1968) L-Lactic acid: a mosquito attractant isolated from humans. *Science* 161: 1346-1347

Albone ES (1984) Mammalian semiochemistry: the investigation of chemical signals between mammals. Wiley, Chichester, 360 pp.

Ament W, Huizenga JR, Mook GA, Gips CH and Verkerke GJ (1997) Lactate and ammonia concentration in blood and sweat during incremental cycle ergometer exercise. *Int. J. Sports Med.* 18: 35-39

Anton S (1996) Central olfactory pathways in mosquitoes and other insects. In: *Olfaction in mosquito-host interactions.* Wiley, Chichester (Ciba Foundation Symposium 200) pp. 184-192

Ballows A (ed.) (1991) Manual of Clinical Microbiology. Washington, American Society of Microbiology, 5th edition.

Bausenwein B and Nick P (1998) Threedimensional reconstruction of the antennal lobe in the mosquito *Aedes aegypti.* In: *Proceedings of the 26th Goettingen Neurobiology Conference* (eds N Elsner, R Wehner).Thieme, Stuttgart, p. 386

Bausenwein B and Nick P (1999) Reconstruction of the female and male antennal lobes in the mosquito *Aedes aegypti.* In: *Goettingen Neurobiology Report 1999* (eds N. Elsner, U.Eysel) Thieme, Stuttgart, p. 355

Bentley MD, McDaniel IN and Davis EE (1982) Studies of 4-methylcyclohexanol: an *Aedes triseriatus* (Diptera: Culicidae) oviposition attractant. *J. Med. Entomol.* 19: 589-592

Boeckh J (1980) Neural basis of coding of chemosensory quality at the receptor cell level. In: *Olfaction and taste VII*, ed. H. van der Starre, pp. 113-122. IRL London

Bowen MF (1995) Sensilla basiconica (grooved pegs) on the antennae of female mosquitoes: electrophysiology and morphology. *Entomol. Exp. Appl.* 77: 233-238

Bowen MF (1990) Post-diapause sensory responsiveness in *Culex pipiens. J. Insect Physiol.* 36: 923-929

Bowen MF (1991) The sensory physiology of host-seeking behavior in mosquitoes. *Annu. Rev. Entomol.* 36: 139-158

Bowen MF (1992) Terpene-sensitive receptors in female *Culex pipiens* mosquitoes: electrophysiology and behaviour. *J. Insect Physiol.* 38: 759-764

Bowen MF, Davis EE and Haggart DA (1988) A behavioural and sensory analysis of host-seeking behaviour in the diapausing mosquito *Culex pipiens. J. Insect Physiol.* 34: 805-813

Bowen MF, Davis EE, Romo J and Haggart D (1994) Lactic acid sensitive receptors in the autogenous mosquito *Aedes atropalpus. J. Insect Physiol.* 40: 611-615

Breer H, Krieger J and Raming K (1990) A novel class of binding proteins in the antennae of the silk moth *Antheraea pernyi*. *Insect Biochem.* 20: 735-740

Charlwood JD, Smith T, Kihonda J, Heiz B, Billingsley PF and Takken W (1995) Density independent feeding success of malaria vectors (Diptera: Culicidae) in Tanzania. *Bull. Entomol. Res.* 85: 29-35

Christensen TA, Harrow ID, Cuzzocrea C, Randolph PW and Hildebrand JG (1995) Distinct projections of two populations of olfactory receptor axons in the antennal lobe of the sphinx moth *Manduca sexta*. *Chem. Senses* 20: 313-323

Davis EE (1974) Identification of antennal chemoreceptors of the mosquito, *Aedes aegypti*: a correction. *Experientia* 30: 1282-1283

Davis EE (1976) A receptor sensitive to oviposition site attractants on the antennae of the mosquito, *Aedes aegypti*. *J. Insect Physiol.* 22: 1371-1376

Davis EE (1977) Response of the antennal receptors of the male *Aedes aegypti* mosquito. *J. Insect Physiol.* 23: 613-617

Davis EE (1984a) Regulation of sensitivity in the peripheral chemoreceptor systems for host-seeking behaviour by a haemolymph-borne factor in *Aedes aegypti*. *J. Insect Physiol.* 30: 179-183.

Davis EE (1984b) Development of lactic-acid-sensitivity and host-seeking behavior in newly emerged female *Aedes aegypti* mosquitoes. *J. Insect Physiol.* 30: 211-215

Davis EE (1988) Structure-response relationship of the lactic acid-excited neurons in the antennal grooved-peg sensilla of the mosquito *Aedes aegypti*. *J. Insect Physiol.* 34: 443-449.

Davis EE and Bowen MF (1994) Sensory physiological basis for attraction in mosquitoes. *J. Am. Mosq. Control Assoc.* 10: 316-325

Davis EE, Haggart DA and Bowen MF (1987) Receptors mediating host-seeking behaviour in mosquitoes and their regulation by endogenous hormones. *Insect Sci. Applic.* 8: 637-641

Davis EE and Sokolove PG (1975) Temperature responses of antennal receptors of the mosquito *Aedes aegypti*. *J. Comp. Physiol.* 96: 223-236

Davis EE and Sokolove PG (1976) Lactic acid-sensitive receptors on the antennae of the mosquito, *Aedes aegypti*. *J. Comp. Physiol.* 105: 43-54.

De Jong R and Knols BGJ (1995) Selection of biting sites on man by two malaria mosquito species. *Experientia* 51: 80-84

Dekker T and Takken W (1998) Differential responses of mosquito sibling species *Anopheles arabiensis* and *An. quadriannulatus* to carbon dioxide, a man or a calf. *Med. Vet. Entomol.* 12: 136-140

Dekker T and Takken W, Knols BGJ, Bouman E, Van Der Laak S, De Bever S and Huisman PWT (1998) Selection of biting sites on a human host by *Anopheles gambiae sensu stricto, An. arabiensis* and *An. quadriannulatus*. *Entomol. Exp. appl.* 87: 295-300

Distler PG and Boeckh J (1997) Central projections of the maxillary and antennal nerves in the mosquito *Aedes aegypti*. *J. Exp. Biol.* 200: 1873-1879

Ellin RI, Farrand RL, Oberst FW, Crouse CL, Billups NB, Koon WS, Musselman NP, and Sidell FR (1974) An apparatus for the detection and quantitation of volatile human effluents. *J. of Chrom.* 100: 137-152

Folk GE Jr and Semken HA Jr (1991) The evolution of sweat glands. *Int. J. Biometeorol* 35: 180-186

Geier M, Sass H and Boeckh J (1996) A search for components in human body odour that attract females of *Aedes aegypti*. In: *Olfaction in mosquito-host interactions*. Wiley, Chichester (Ciba Foundation Symposium 200) pp. 132-144

Gilles HM, Warrell DA and Bruce-Chwatt (1993) *Bruce-Chwatt's essential malariology*. 3rd Edition. Edward Arnold, London. 340 pp.

Grant AJ and O'Connell RJ (1996) Electrophysiological responses from receptor neurons in mosquito maxillary palp sensilla. In: *Olfaction in mosquito-host interactions*. Wiley, Chichester (Ciba Foundation Symposium 200) pp. 233-248

Grant AJ, Wigton BE, Aghajanian JG and O'Connell RJ (1995) Electrophysiological responses of receptor neurons in mosquito maxillary palp sensilla to carbon dioxide. *J. Comp. Physiol. A* 177: 389-396

Györgyi TK, Roby-Shemkovitz AJ and Lerner MR (1988) Characterization and cDNA cloning of the pheromone-binding protein from the tobacco hornworm, *Manduca sexta*: A tissue-specific developmentally regulated protein. *Proc. Natl. Acad. Sci. USA* 85: 9851-9855

Hall DR, Beevor PS, Cork A, Nesbitt BF and Vale GA (1984) 1-Octen-3-ol: a potent olfactory stimulant and attractant for tsetse isolated from cattle odours. *Insect Sci. Applic.* 5: 335-339

Hansson BS, Christensen TA and Hildebrand JG (1991) Functionally distinct subdivisions of the macroglomerular complex in the antennal lobes of the sphinx moth *Manduca sexta*. *J. Comp. Neurol.* 312: 264-278

Hildebrand JG (1996) Olfactory control of behavior in moths: central processing of odor information and the functional significance of olfactory glomeruli. *J. Comp. Physiol. A* 178: 5-19

Hildebrand JG and Shepherd GM (1997) Mechanisms of olfactory discrimination: converging evidence for common principles across phyla. *Annu. Rev. Neurosci.* 20: 595-631

Hunt RH, Coetzee M and Fettene M (1998) The *Anopheles gambiae* complex: a new species from Ethiopia. *Trans. R. Soc. Trop. Med. Hyg.* 92: 231-235

Kaissling K–E, (1987) R. H. Wright Lectures on Insect Olfaction. Simon Fraser University, Burnaby, Canada

Kellogg FE (1970) Water vapour and carbon dioxide receptors in *Aedes aegypti. J. Insect Physiol.* 16: 99-108

Kline DL (1994) Olfactory attractants for mosquito surveillance and control: 1-octen-3-ol. *J. Am. Mosq. Control Assoc.* 10: 280-287

Kline DL, Dame DA and Meisch MV (1991a) Evaluation of 1-octen-3-ol and carbon dioxide as attractants for mosquitoes associated with irrigated rice fields in Arkansas. *J. Am. Mosq. Control Assoc.* 7: 165-169

Kline DL, Takken W, Wood JR and Carlson DA (1990a) Field studies on the potential of butanone, carbon dioxide, honey extract, 1-octen-3-ol, L-lactic acid and phenols as attractants for mosquitoes. *Med. Vet. Entomol.* 4: 383-391

Kline DL, Wood JR and Cornell JA (1991b) Interactive effects of 1-octen-3-ol and carbon dioxide on mosquito (Diptera: Culicidae) surveillance and control. *J. Med. Entomol.* 28: 254-258

Kline DL, Wood JR and Morris CD (1990b) Evaluation of 1-octen-3-ol as an attractant for *Coquillettidia perturbans*, *Mansonia* spp. and *Culex* spp. associated with phosphate mining operations. *J. Am. Mosq. Control Assoc.* 6: 605-611

Klowden MJ, Davis EE and Bowen MF (1987) Role of the fat body in the regulation of host-seeking behaviour in the mosquito, *Aedes aegypti. J. Insect Physiol.* 33: 643-646

Knols BGJ and De Jong (1996) Limburger cheese as an attractant for the malaria mosquito *Anopheles gambiae s.s. Parasitol. Today* 12: 159-160

Knols BGJ, van Loon JJA, Cork A, Robinson RD, Adam W, Meijerink J, De Jong R and Takken W (1997). Behavioral and electrophysiological responses of the female malaria mosquito *Anopheles gambiae* (Diptera: Culicidae) to Limburger cheese volatiles. *Bull. Entomol. Res.* 87:151-159

Krieger J, Gänβle H, Raming K and Breer H (1993) Odorant binding proteins of *Heliothis virescens*. *Insect Biochem. Molec. Biol.* 23: 449-456

Lacher V (1967) Elektrophysiologische Untersuchungen an einzelnen Geruchsrezeptoren auf den Antennen weiblicher Moskitos (*Aedes aegypti* L.). *J. Insect Physiol.* 13: 1461-1470.

Mboera, LEG, Knols BGJ, Takken W and della Torre A (1997) The response of *Anopheles gambiae s.l.* and *An. funestus* (Diptera: Culicidae) to tents baited with human odour or carbon dioxide in south-east Tanzania. *Bull. Entomol. Res.* 87: 173-178

Muir LE and Cribb BW (1994) *Aedes aegypti*: sensilla trichodea and stimulus-conducting structures. *J. Insect Physiol.* 40: 1017-1023

Nicolaides N (1974) Skin Lipids: Their Biochemical Uniqueness. *Science* 186: 19-26

Pappenberger B, Geier M and Boeckh J (1996) Responses of the antennal olfactory receptors in the yellow fever mosquito *Aedes aegypti* to human body odours. In: *Olfaction in mosquito-host interactions.* Wiley, Chichester (Ciba Foundation Symposium 200) pp. 254-266.

Raming K, Krieger J and Breer H (1989) Molecular cloning of an insect pheromone-binding protein. *FEBS Lett.* 256: 2215-2218

Sastry SD, Buck KT, Janák J, Dressler M and Preti G (1980) Volatiles emitted by humans. In: *Biochemical Applications of Mass Spectrometry* (G.R. Waller & O.C. Dermer Eds.) John Wiley & Sons Ltd., New York. pp. 1086-1123.

Sato K (1977) The physiology, pharmacology, and biochemistry of the eccrine sweat gland. *Rev. Physiol. Biochem. Pharmacol.* 79: 51-131

Shepherd GM (1992) Modules for molecules. *Nature* 358: 457-458

Slifer EH (1954) The permeability of the sensory pegs on the antennae of the grasshopper (Orthoptera, Acrididae) *Biol. Bull. (Woods Hole)* 106: 122-128

Slifer EH (1960) A rapid and sensitive method for identifying permeable areas in the body wall of insects. *Entomol. News* 71: 179-182

Steinbrecht RA (1997) Pore structures in insect olfactory sensilla: a review of data and concepts. *Int. J. Insect Morphol. Embryol.* 26: 229-245

Steinbrecht RA, Laue M and Ziegelberger G (1995) Immunolocalization of pheromone-binding protein and general odorant-binding protein in olfactory sensilla of the silk moths *Antheraea* and *Bombyx*. *Cell Tissue Res.* 282: 203-217

Takken W and Kline DL (1989) Carbon dioxide and 1-octen-3-ol as mosquito attractants. *J. Am. Mosq. Control Assoc.* 5: 311-316

Takken W and Knols BGJ (1999) Odor-mediated behavior of Afrotropical malaria mosquitoes. *Annu. Rev. Entomol.* 44: 131-157

Vale GA and Hall DR (1985) The role of octenol, acetone and carbon dioxide in the attraction of tsetse flies, *Glossina* spp. (Diptera: Glossinidae) to ox odour. *Bull. Entomol. Res.* 75: 209-217

Van den Berg MJ and Ziegelberger G (1991) On the function of the pheromone-binding protein in the olfactory hairs of *Antheraea polyphemus*. *J. Insect Physiol.* 37: 79-85

Vogt RG (1987) Molecular basis of pheromone reception: its influence on behavior. In: *Pheromone Biochemistry*, ed. GD Prestwich, GJ Blomquist, pp. 385-431. New York: Academic

Vogt RG, Prestwich GD and Lerner MR (1991a) Odorant-binding-protein subfamilies associate with distinct classes of olfactory receptor neurons in insects. *J. Neurobiol.* 22: 74-84

Vogt RG and Riddiford LM (1981) Pheromone binding and inactivation by moth antennae. *Nature* 293: 161-163

Vogt RG, Rybczynski R and Lerner MR (1991b) Molecular cloning and sequencing of general odorant-binding proteins GOBP1 and GOBP2 from the tobacco hawk moth *Manduca sexta*: comparison with other insect OBPs and their signal peptides. *J. Neurosci.* 11: 2972-2984

White GB (1974) *Anopheles gambiae* complex and disease transmission in Africa. *Trans. R. Soc. Trop. Med. Hyg.* 68: 278-301

World Health Organisation (1997) World malaria situation in 1994. Part I. Population at risk. Weekly Epidemiological Record. 72 (36): 269-74

Ziegelberger G (1995) Redox-shift of the pheromone-binding protein in the silkmoth *Antheraea polyphemus*. *Eur J. Biochem.* 232: 706-711

Chapter 2

Sensillar structures on the antenna of the female mosquito, *Anopheles gambiae*

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Abstract

The antennal segments of a female anopheline mosquito possess six distinct types of sensilla. Here we show scanning electron microscopic (SEM) photographs of the large sensilla coeloconica, small sensilla coeloconica, grooved peg sensilla, sensilla trichodea, sensilla ampullacea and the sensilla chaetica. Observation of the various subtypes of sensilla trichodea revealed two subtypes which resemble subtype D and E as classified for *An. stephensi*. The distribution of the various sensillar types on the antennal segments is discussed in the context of the electrophysiological studies in the following chapters.

Introduction

The antenna of the mosquito *Anopheles gambiae* Giles *sensu stricto* bears six different types of sensilla: large sensilla coeloconia, small sensilla coeloconica, grooved peg sensilla, sensilla trichodea, sensilla ampullacea and sensilla chaetica (McIver, 1982; Ismail, 1964). The female antenna possesses sensilla on all thirteen segments contrasting the male antenna which exclusively bears sensilla on the two top segments. Nevertheless, all distinct types of sensilla are represented in the male also.

The large sensilla coeloconica are restricted within the mosquito species to the anophelines and although to date no electrophysiological studies are reported on *An. gambiae s.s.* revealing the function of this type of sensillum, studies on other insect species, like the desert locust *Schistocerca gregaria, Drosophila melanogaster* and *Bombyx mori* imply that neurons innervating this sensillum type are sensitive to odours (Ochieng and Hansson, 1999; Clyne et al., 1997; Pophof, 1997). For *An. gambiae* electrophysiological recordings (this thesis chapter 4 and 6; Meijerink and Van Loon, 1999; Meijerink et al., submitted; Van den Broek and Den Otter, 1999) from neurons innervating the grooved peg sensilla and sensilla trichodea have shown that these receptor neurons are responsive to odours. Also for *Aedes aegypti* several studies have reported responses of olfactory neurons innervating these two types of sensilla (Pappenberger et al., 1996; Bowen, 1995; Davis, 1988; Lacher, 1967). Neurons innervating the grooved peg sensilla of *Aedes aegypti* are sensitive to lactic acid, a stimulus known to be involved in host seeking behaviour of this mosquito species (Davis and Sokolove, 1976).

Although it was assumed that the grooved peg sensillum of *Ae. aegypti* contained an apical pore (McIver, 1982) through which odour molecules could enter the sensillum, later studies showed that the pores were restricted to the area of the grooves and that this sensillum did not contain an apical pore like contact-chemoreceptor sensilla (Cribb and Jones, 1995). For the same mosquito species stimulus-conducting structures consisting of pores, pore kettles and pore tubules have been demonstrated for the sensilla trichodea. This indicates that the general mechanism of odour molecule entrance and transport, finally leading to the interaction with molecular receptors in the dendritic membrane can be extended to mosquitoes (Muir and Cribb, 1994).

Single cell recordings from *Ae. aegypti*, revealed that receptor neurons within the small sensilla coeloconica are responsive to heat (Davis and Sokolove, 1975). One of the receptor neurons innervating this sensillum responded by an increase in the spontaneous spike frequency in response to an increase in temperature while a second neuron showed a increase in spike frequency in response to a decrease in temperature. Based on the morphological similarity of the inner structure of the small sensilla coeloconica and the sensilla ampullacea, receptor neurons innervating the latter sensilla are thought to be thermo-sensitive as well (McIver, 1982). The sensilla chaetica or bristles are considered to function as mechanosensilla.

Although the morphology of the various sensilla on the antennae of mosquitoes species like *Ae. aegypti, An. stephensi* etc. has been extensively studied by McIver (1982) and others (Boo, 1980a, b) hardly any morphological data exist on *An. gambiae*. This study shows the first scanning electron microscopic photographs of the various types of sensilla and reveals the distribution of the different types of sensilla on the different segments on the female antennae of *An. gambiae*.

Material and Methods

Insects

Anopheles gambiae s.s. originated from Suakoko, Liberia (courtesy of Prof. M. Coluzzi, Rome). Mosquitoes were reared at 27 °C, 80% R.H. and 12:12 L:D photo /scotophase. Adults were kept in gauze cages (30 x 30 x 30 cm). They had access to a 6% glucose solution and were given the opportunity to feed on a human arm twice a week. Eggs were laid on wet filter paper and transferred to water trays. Larvae were fed Tetramin® fish food.

Scanning electron microscopy

Two different fixation procedures were used to prepare antennae for viewing. For the tetrachloromethane fixation females were cooled in a freezer (- 5 °C) for ± 1.5 min. Heads were cut off and fixed in pure tetrachloromethane by boiling the preparations 4 times during 30-60 sec, after which the heads were air-dried (Cuperus, 1986). Heads were embedded in silver, coated with gold-palladium for 2 min and subsequently examined in a Philips 535 M scanning electron microsope.

For the acetone fixation heads were fixated in acetone for 2-5 days (Hardie et al., 1994). After critical-point drying, heads were coated with gold-palladium for 2 min and viewed with a Philips 535 M scanning electron microsope.

Results and Discussion

Fig. 1A-F show the scanning electron microscopic photographs of the six distinct types of sensilla present on a female antenna. An overview of one of the thirteen segments of the antenna is shown in Fig. 1G.

Large sensilla coeloconica were only present on segment 1 to 9 which is in line with studies performed by Ismail (1964). Grooved peg sensilla were observed on segment 5 to 13. The number per segment increases gradually towards the apex of the antennae. Ismail (1964) mentioned the presence of two grooved peg sensilla on segment 4, however, our SEM photographs never revealed any grooved pegs on this segment.

For *An. stephensi* five different subtypes of sensilla trichodea, namely A, B, C, D, and E, have been described (Boo, 1980a). For *An. gambiae* most subtypes of sensilla trichodea are not easily distinguished. However, on segment 3 to 11, but more frequently on the basal segments, a subtype sensillum trichodeum was observed with a very pronounced appearance (Fig 2A, B). It resembles subtype D in *An. stephensi* by having a round tip. Also the diameter of the sensillum, like in *An. stephensi*, hardly changes along its entire length. We have not made any recordings from this subtype sensillum trichodeum. Similar subtypes in other mosquito species, however, have been found to be sensitive to oviposition site-related odours (Davis and Bowen, 1994). Fig. 3 shows a SEM photograph of the shortest subtype sensillum trichodeum. In several cases receptor neurons innervating this sensillum subtype responded by excitation to indole, 3-methyl-1-

butanol and occasionally to 6-methyl-5-hepten-2-one. Receptor neurons were most sensitive to indole (chapter 6). Neurons innervating a similar subtype were found to be inhibited by short chain carboxylic acids (chapter 4). This subtype sensillum trichodeum is most comparable to subtype E in *An. stephensi*. Possible other subtypes within the remaining subtypes of sensilla trichodea were hard to distinguish as they exhibit a gradual transition in both form as well as sensillum length. Nevertheless, sensilla trichodea subtypes housing receptor neurons responsive to either geranyl acetone or indole and to 3-methyl-1-butanol, 6-methyl-5-hepten-2-one (chapter 6) and short chain carboxylic acids (chapter 4) displayed an intermediate length and therefore mostly resemble subtype C of *An. stephensi*. In general, the total number of sensilla trichodea per segment increases towards the top of the antenna.

Being the smallest among the six types of sensilla, the sensilla ampullacea were only occasionally observed on segment 1, 2, 3, and 6. Moreover, small sensilla coeloconica were found at the top of segment 13. The sensilla chaetica occur in whorls at the base of segments 2-13. Fig. 4 shows a SEM photograph of the first segment of a female antenna exhibiting numerous microtrichia and scales. Segment 2 and 3 possess fewer of these structures and they are totally absent on segments 4-13. According to McIver (1982), microtrichia are not innervated.

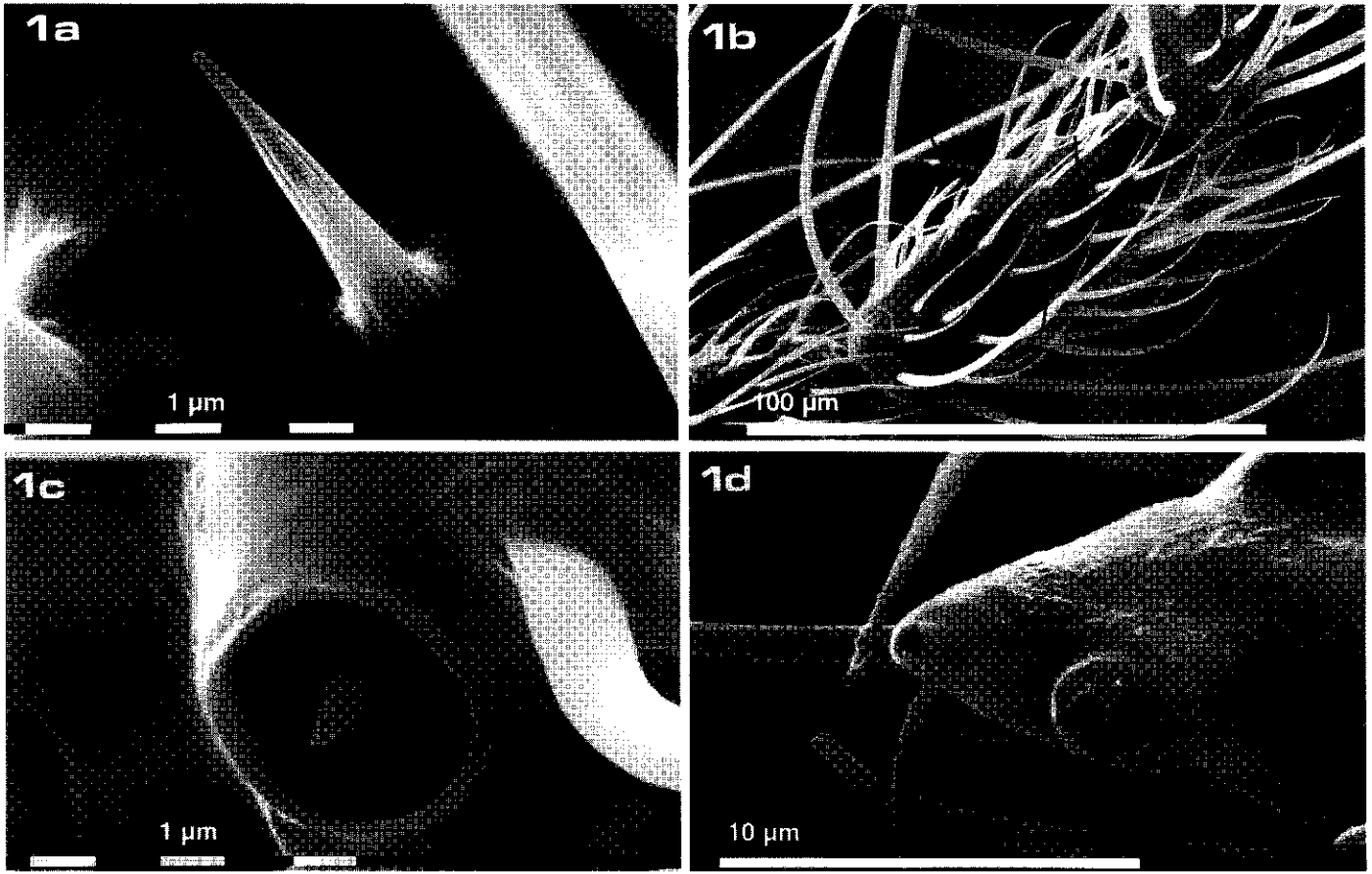

Fig. 1 Scanning electron microscopic photographs showing the distinct types of sensilla of a female *An. gambiae* antenna (A) grooved peg sensillum, scale bar 1 µm; (B) various subtypes sensilla trichodea (see arrows), scale bar 100 µm; (C) large sensillum coeloconica, scale bar 1 µm; (D) small sensilla coeloconica, scale bar 10 µm.

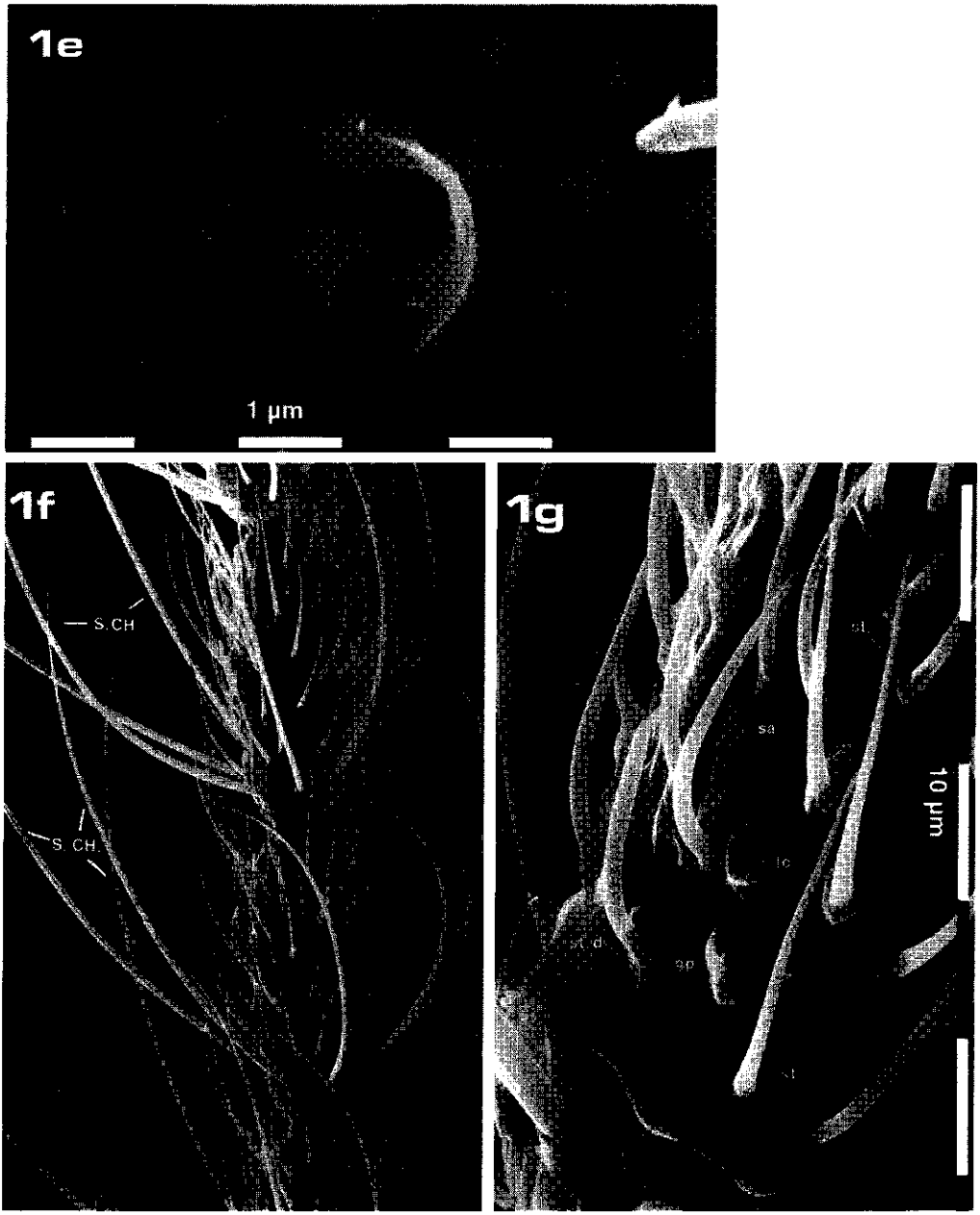

Fig. 1 (continued) Scanning electron microscopic photographs showing the distinct types of sensilla of a female *An. gambiae* antenna. (E) sensillum ampullacea, scale bar 1 µm and (F) sensilla chaetica, indicated by s. ch., x 482 (G) SEM photograph showing a part of the sixth segment of a female antenna. Letters point to grooved peg sensillum (gp), large sensillum coeloconica (lc), sensillum ampullacea (sa) and sensilla trichodea (st.) and sensillum trichodeum subtype D (st. d), scale bar, 10 µm.

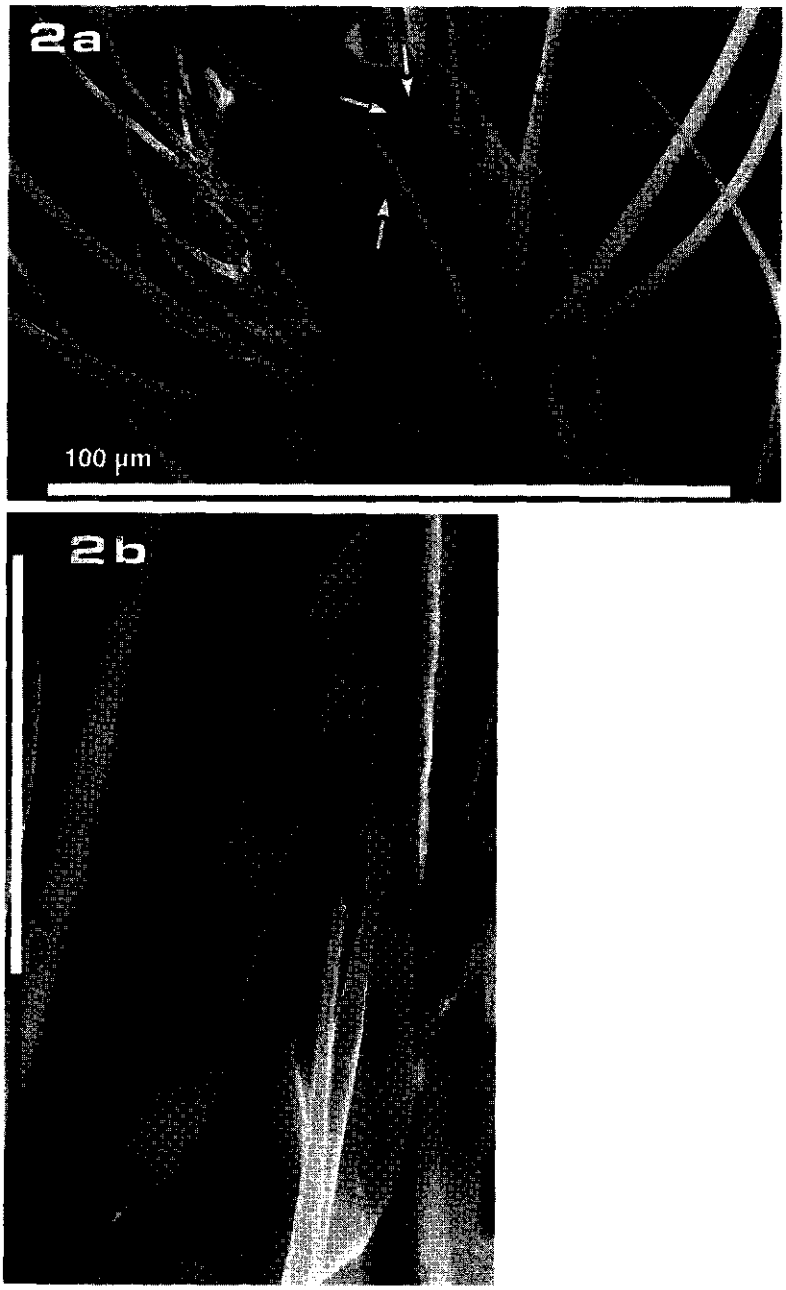

Fig. 2A Scanning electron microscopic photographs of a subtype sensillum trichodeum (see arrow) located at the fourth segment of a female antenna. According to the classification made for *An. stephensi* it resembles most subtype D. Scale bar, 100 µm.
2B Detailed picture of above described subtype sensillum trichodeum. Scale bar, 10 µm

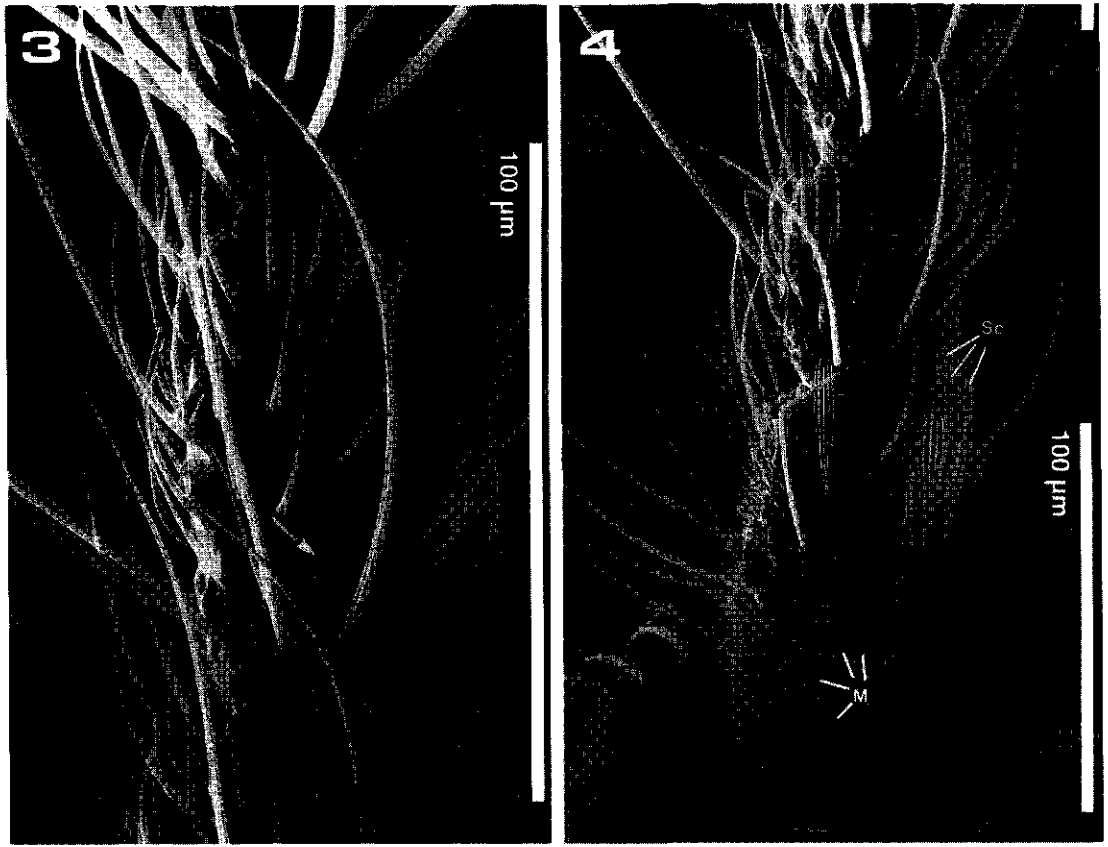

Fig. 3. Scanning electron microscopic photograph showing the shortest subtype sensilla trichodea situated on the fifth segment of a female antenna. Neurons innervating some of these subtypes were sensitive to indole (chapter 6). According to the classification made for *An. stephensi*, these sensilla resemble subtype E. Scale bar, 100 µm.

Fig. 4. Scanning electron microscopic photograph of the first segment of a female antenna exhibiting numerous microtrichia (m) and several scales (Sc). Scale bar, 100 µm.

References

Boo KS (1980a) Fine structure of the antennal sensory hairs in female *Anopheles stephensi*. *Z. Parasitenkd.* 61: 161-171

Boo KS (1980b) Antennal sensory receptors of the male mosquito, *Anopheles stephensi*. *Z. Parasitenkd.* 61: 249-264

Bowen MF (1995) Sensilla basiconica (grooved pegs) on the antennae of female mosquitoes: electrophysiology and morphology. *Entomol. Exp. Appl.* 77: 233-238

Clyne P, Grant A, O'Connell R and Carlson JR (1997) Odorant response of individual sensilla on the *Drosophila* antenna. *Invertebr. Neurosc.* 3: 127-135

Cribb BW and Jones MK (1995) Reappraisal of the pore channel system in the grooved pegs of *Aedes aegypti*. *Tissue Cell* 27: 47-53

Cuperus PL (1986) A comparative electron microscopical study on antennae of small ermine moths (Lepidoptera: Yponomeutidae). *PhD thesis Rijksuniversiteit Groningen,* Groningen, The Netherlands, p. 97

Davis EE (1988) Structure-response relationship of the lactic acid-excited neurons in the antennal grooved-peg sensilla of the mosquito *Aedes aegypti*. *J. Insect Physiol.* 34: 443-449

Davis EE and Bowen MF (1994) Sensory physiological basis for attraction in mosquitoes. *J. Am. Mosq. Control Assoc.* 10: 316-325

Davis EE and Sokolove PG (1975) Temperature responses of antennal receptors of the mosquito, *Aedes aegypti*. *J. Comp. Physiol.* 96: 223-236

Davis EE and Sokolove PG (1976) Lactic acid-sensitive receptors on the antennae of the mosquito, *Aedes aegypti*. *J. Comp. Physiol.* 105: 43-54

Hardie J, Visser JH and Piron PGM (1994) Perception of volatiles associated with sex and food by different adult forms of the black bean aphid, *Aphis fabae*. *Physiol. Entomol.* 19: 278-284

Ismail IAH (1964) Comparative study of sense organs in the antennae of Culicine and Anopheline female mosquitoes. *Acta Trop.* XXI: 155-168

Lacher V (1967) Elektrophysiologische Untersuchungen an einzelnen Geruchsrezeptoren auf den Antennen weiblicher Moskitos (*Aedes aegypti* L.). *J. Insect Physiol.* 13: 1461-1470

McIver SB (1982) Sensilla of mosquitoes (Diptera: Culicidae). *J. Med. Entomol.* 19: 489-535

Meijerink J and Van Loon JJA (1999) Sensitivities of antennal olfactory neurons of the malaria mosquito, *Anopheles gambiae*, to carboxylic acids. *J. Insect Physiol.* 45: 365-373

Meijerink J, Braks MAH and Van Loon JJA. Olfactory receptors on the antennae of the malaria mosquito *Anopheles gambiae* are sensitive to ammonia and other sweat-borne components. Submitted to *J. Comp. Physiol. A*

Muir LE and Cribb BW (1994) *Aedes aegypti*: sensilla trichodea and stimulus-conducting structures. *J. Insect Physiol.* 40: 1017-1023

Ochieng AA and Hansson BS (1999) Responses of olfactory receptor neurones to behaviourally important odours in gregarious and solitarious desert locust, *Schistocerca gregaria. Physiol. Entomol.* 24: 28-36

Pappenberger B, Geier M and Boeckh J (1996) Responses of the antennal olfactory receptors in the yellow fever mosquito *Aedes aegypti* to human body odours. In: *Olfaction in mosquito-host interactions*. Wiley, Chichester (Ciba Foundation Symposium 200) pp. 254-266

Pophof B (1997) Olfactory responses recorded from sensilla coeloconica of the silkmoth *Bombyx mori. Physiol. Entomol.* 22: 239-248

Van den Broek IVF and Den Otter CJ (1999) Olfactory sensitivities of mosquitoes with different host preferences (*Anopheles gambiae s.s., An. arabiensis, An, quadriannulatus, An. m. atroparvus*) to synthetic host odours. *J. Insect Physiol.* 45: 1001-1010

Chapter 3

Electroantennogram responses of female *An. gambiae* to carboxylic acids

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With: Anouk Brack

Part of this chapter has been published as: Knols B.G.J., Van Loon J.J.A., Cork A., Robinson R.D., Adam W., Meijerink J., De Jong R., and Takken W. (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.

Abstract

The electrical potential difference measured over a cotton thread exposed to carboxylic acids, 1-octen-3-ol and fresh and incubated sweat was compared for glass- versus tungsten electrodes. Potential differences varying between –1.28 and –3.21 mV were measured when applying 1% propionic acid, butyric acid and hexanoic acid. These components did not generate a potential difference when glass electrodes were used. Neither did 1-octen-3-ol, fresh sweat, incubated sweat, ether or water generate a potential difference when using glass- or tungsten electrodes. These results indicate that electroantennographic (EAG) studies of carboxylic acids performed with tungsten electrodes might give artefactual results. EAG studies performed with glass electrodes in response to the carboxylic acids 3-methylbutanoic, pentanoic, hexanoic, heptanoic, octanoic acid, nonanoic acid, decanoic acid, dodecanoic, tetradecanoic and hexadecanoic acid revealed that hexanoic acid evoked the highest response. Highest EAG amplitudes were elicited by acids with a chain length of C_5-C_8, while weaker though significant responses were obtained with the less volatile acids C_9 to C_{14}. Only hexadecanoic acid (C_{16}) did not elicit a detectable response. The headspace of Limburger cheese rich in carboxylic acids and a synthetic mixture comprising 12 carboxylic acids elicited EAG responses as well.

Introduction

The female mosquito *Anopheles gambiae* Giles *s.s.* (Diptera: Culicidae), Africa's most important vector of malaria, is highly anthropophilic (White, 1974). Although field studies concerning the host-seeking behaviour of *An. gambiae* are limited, it is generally assumed that odours play an important role during the process of host-seeking and host location of its human host (Takken, 1991; Takken and Knols, 1999). Odours are perceived by the olfactory receptor neurons innervating distinct antennal or palpal sensilla (McIver, 1982).

The first synthetic mixture shown to attract female *An. gambiae* in a windtunnel bioassay comprised the carboxylic acid isomers iso-C_4 and iso-C_5 and a number of aliphatic

carboxylic acids with carbon chain lengths of C_2-C_6, C_8, C_{10}, C_{12}, C_{14}, C_{16} (Knols et al., 1997). Carboxylic acids are present on the human skin, either esterified or free, where they display an enormous diversity both in the extremely wide range of chain lengths as well as in the unusual patterns of saturation. Free fatty acids together with glycerol, mono- and diacylglycerides are formed during the hydrolysis of triacylglycerides, an excretion product of the sebaceous gland which makes up 60% of the lipid content of the human sebum. Triacylglycerides are hydrolysed by lipases, mainly originating from the skin microflora (Nicolaides, 1974).

Also Limburger cheese, reminiscent to human foot odour to the human nose, contains a high number of aliphatic carboxylic acids. The latter are formed by microbial activity, and the odour of this cheese has been shown to be attractive for female *An. gambiae* mosquitoes in a behavioural bioassay (De Jong and Knols, 1995; Knols and De Jong, 1996).

For *Aedes aegypti*, the only mosquito species for which olfactory receptors have been characterized to some extent, carboxylic acid sensitive receptors cells have been found within distinct sensilla. For instance, sensitive neurons for propionic acid, butyric acid and iso-butyric acid were found in the grooved peg sensillum of *Aedes aegypti* (Davis, 1988). Likewise, for the same species, pentanoic acid and hexanoic acid evoked responses of grooved-peg associated neurons (Pappenberger et al., 1996). In *Aedes epactius,* however, grooved peg-associated neurons responding to butyric acid were found to be distinct from lactic acid sensitive neurons (Bowen, 1995). Different subtypes of sensilla trichodea were reported to respond by excitation or inhibition to aliphatic carboxylic acids. Inhibition was elicited by acids with short carbon chains, while acids with higher carbon chains evoked excitation of receptor neurons innervating the same sensillum subtype (Lacher, 1967).

This study was a first initiative to investigate the peripheral sensory responses of antennal olfactory cells of female *An. gambiae* by means of electroantennography (EAG). Firstly technical aspects of the EAG recording technique were more closely examined. To investigate whether electrodes potentials, irrespective of the electroantennographic potentials obtained upon stimulation, might interfere with the biological signal, experiments were performed by replacing the female head preparations by a cotton thread. The properties of both tungsten electrodes as well as glass electrodes were tested. Stimulants were: propanoic acid, butanoic acid, hexanoic acid, 1-octen-3-ol, fresh sweat, incubated sweat, ether and water. Furthermore, to quantify antennal olfactory sensitivity to carboxylic acids, electroantennographic studies were conducted with *An. gambiae* excised head preparations. Dose-response profiles were established for the carboxylic acids 3-

methylbutanoic, pentanoic, hexanoic, heptanoic, octanoic acid, nonanoic acid, dodecanoic, tetradecanoic and hexadecanoic acid.

Material and methods

Mosquitoes

The *An. gambiae* strain used originated from Suakoko, Liberia (courtesy of Prof. M. Coluzzi, Rome) and was maintained under standard laboratory conditions (27 ± 1°C, 80 ± 5% rh and 12 h scotophase). Adults were kept in 30 cm square gauze covered cages and fed on 6% glucose solution. Females were given the opportunity to feed from a human arm twice weekly for 10 min. Eggs were laid on wet filter paper, hatched in water trays and larvae were fed on Tetramin® fishfood. Pupae were collected daily from the trays and adults allowed to emerge in cages. Experimental females were 4-8 days old and had not received a bloodmeal. Females which exhibited an alighting response to a hand of the experimenter were selected from adult cages and used for electroantennography.

Electrophysiology

Electrode potentials. A cotton thread was soaked in insect Ringer (9 g/l NaCL, 2 g/l KCl, 1.34 g/l $CaCl_2.2H_2O$ and 5 g/l PVP in distilled water) and connected to tungsten electrodes or glass electrodes. Tungsten electrodes (tungsten wire of 0.2 mm diameter, Drijfhout, The Netherlands) were electrolytically sharpened (5 min at 10 V and then 15 min at 3 V in a 8% solution of KNO_2 in H_2O). Glass capillaries were filled with insect Ringer and AgCl-coated Ag-wires inserted. Signals were amplified 10x and directly imported via an IDAC interface box and A/D converter (Syntech, Hilversum, The Netherlands) into an Intel® Pentium-based personal computer. Recordings were analyzed by means of EAG software version 2.6 (Syntech, Hilversum, The Netherlands).

A moistened, charcoal filtered, continuous airstream (14.5 ml/ sec) was led through a glass tube (i.d. 1 cm) ending 0.5 cm from the preparation holder. Stimulus puffs (volume 1.6 ml) lasted for 0.5 sec and were injected into the airstream, at a location 10 cm from the outlet of the tube, by using a stimulus controller (Syntech, Hilversum, The Netherlands).

EAGs from excised head preparations. Excised mosquito head preparations removed from cooled (*c.* 90 s at -5° C) females were used for electrophysiological recording. The excised head was mounted between two glass electrodes filled with 0.1 M KCl. AgCl-coated Ag-wires were inserted into a glass capillary, placed in a holder, and connected to a home-made DC amplifier (amplification 100x). The recording electrode was slid over the tip of the antenna, from which the terminal segment had first been removed. The indifferent electrode was inserted through the foramen magnum of the head. The potential differences between the electrodes were visualised on a paper chart recorder set to a full scale sensitivity of 2 mV. EAG-amplitudes were measured manually to an accuracy of $\pm$ 10 μV. A standard off-line method of stimulus delivery was used (Van Loon et al., 1992). An airstream (33 ml/sec), saturated with water vapour, blew continuously over the preparation.

Odours tested

Chemical stimuli. All chemicals used were purchased from Merck, Aldrich or Sigma and were more than 99% pure, except for heptanoic acid (> 98%) and 1-octen-3-ol (> 97%).

Electrode potentials. Propanoic acid, butanoic acid, hexanoic acid and 1-octen-3-ol were dissolved in ether. Stimuli were prepared by applying 25 μl of the sweat or a dissolved compound on a 2.4 cm^2 filterpaper (Schleicher & Schuell, Dassel, Germany). The solution on the filterpaper was allowed to evaporate for 10 sec, which in case of diethyl ether resulted in the removal of the solvent. Sweat samples still contained water after 10 sec. Filterpapers were placed in a 150 mm glass Pasteur pipette that was sealed with parafilm at both ends.

Fresh sweat was collected from a female volunteer (age 28) after physical exercise in a warm (30 °C) and humid room (70% rh). Incubated sweat was obtained after incubation for 42-52 hours under aerobic conditions at 37 °C .

Excised head preparations. A) Limburger cheese headspace - In a preliminary set of experiments, headspace odour of Limburger cheese was used to stimulate the preparation. A known amount of cheese (50 - 100 mg) was placed in a 30 ml glass bottle, which was then capped with a aluminium screwcap incorporating a rubber septum. After 30 min, 1 ml aliquots of head space odour were withdrawn from the bottle with a syringe. This volume was injected manually, in a *c.* 1 s pulse, into the continuous airstream over the preparation. As a control, an injection of 1 ml of air drawn from a similar but empty bottle was applied.

B) Chemicals tested - Aliphatic carboxylic acids were volatilized from an aliquot of paraffin oil (25 µl) that was put on a filter paper strip (30 x 3 mm) and inserted into a glass Pasteur pipette. Odours were allowed to volatilize in the pipette for *c.* 30 min and than injected into the continuous airstream by inserting the tip of the pipette through a small hole in the side of the glass tube (distance of the hole from preparation *c.* 10 cm). Doses are expressed as % in paraffin oil (v/v; e.g.1% means 225 µg of compound). Fatty acids tested were: 3-methylbutanoic, pentanoic, hexanoic, heptanoic, octanoic, nonanoic, decanoic, dodecanoic, tetradecanoic and hexadecanoic acid. Each compound was tested in a series of five doses in a random order, and no more than three chemicals were tested on a given mosquito head preparation. This process was repeated for 5-8 individuals per test series. A synthetic mixture of the acids in the ratio in which they occur in the headspace of the acid extract as given in table 1 in Knols et al. (1997) was also tested. The maximum EAG response was calculated for each compound/dose and expressed relative to the response elicited by the solvent (paraffin oil). The solvent response was measured at regular intervals during the testing sequence. In some experiments, 2-ethyl hexanoic acid (previously shown to be electrophysiologically active) was used as a standard. Differences between response amplitudes of test odours and controls as well as between different doses of the same chemical were analysed using Mann-Whitney *U* tests.

Results

Electrode potentials

Table 1 shows the potential values measured between two glass- respectively tungsten electrodes connected by a cotton thread. Propanoic acid, butanoic acid and hexanoic acid generated a potential difference at a concentration of 1%, when using tungsten electrodes. Electrode potentials were ranging between –1.28 and -3.21 mV. Lower concentrations of butanoic acid never generated any potential change. Propanoic acid, butanoic acid and hexanoic acid generated no electrode potentials when glass electrodes were used. 1-Octen-3-ol, fresh sweat, incubated sweat, ether and water never generated any electrode potentials regardless of the type of electrodes used.

Table 1. Potential difference over a cotton thread as measured by respectively glass- or tungsten electrodes.

stimulus	glass electrodes			tungsten electrodes		
	potential difference (mV)	SEM	n	potential difference (mV)	SEM	n
1% propanoic acid	0.06	0.05	8	-3.21	1.12	5
0.01% butanoic acid	0.00	0.00	4	0.06	0.06	2
0.1% butanoic acid	0.08	0.08	5	0.17	0.17	3
1% butanoic acid	0.08	0.06	7	-2.00	1.11	6
1% hexanoic acid	0.00	0.00	6	-1.28	0.80	5
1% 1-octen-3-ol	0.03	0.03	5	0.07	0.04	5
10% 1-octen-3-ol	0.00	0.00	6	0.08	0.08	4
fresh sweat	0.00	0.00	6	0.00	0.00	3
incubated sweat	0.01	0.01	5	-0.07	0.14	5
Ether	0.20	0.10	8	0.04	0.03	6
Water	0.00	0.00	5	0.28	0.28	4

EAGs from excised head preparations

A well-defined and reproducible EAG response was observed when mosquitoes were stimulated with Limburger cheese volatiles. The mean response amplitude was 300 μV (SEM 40 μV; n=8). No measurable response was seen upon injection of clean air from the control bottle. EAG response amplitudes from the synthetic acid mixture (at doses ≥ 0.1%) differed significantly from the solvent stimulus, and were positively correlated with the dose (Fig. 1).

Figure 2 shows that dose-dependent EAG-responses were observed for 3-methylbutanoic, pentanoic, hexanoic, heptanoic, and octanoic acid, with a threshold of between 0.001 and 0.1 % (i.e. *c.* 0.23 and 22.5 μg of pure compound) with the stimulus delivery method used. The mean absolute response to paraffin oil was 140 μV (n=8; data from one experimental series). Significant but lower amplitude EAG responses were elicited by nonanoic acid (0.01 and 1%), decanoic and dodecanoic acid (at the 0.1% and higher doses), and these increased significantly with dose. Tetradecanoic acid elicited a significant EAG response, but only at the 10% level, and no significant responses were recorded upon exposure of the head preparation to doses of up to 10% hexadecanoic acid.

Discussion

A potential difference measured over a cotton thread was observed when applying 1% propanoic, butanoic and hexanoic acid. This potential difference was only obtained with tungsten electrodes while no potential changes were observed when using glass electrodes. No electrode potential was generated by lower concentrations of butanoic acid. Of the three carboxylic acids tested the measured potential changes seemed to be correlated with the pKa values of the acids.

Using tungsten electrodes, extreme EAG values (-9.04 mV) have been reported to be elicited by formic acid, but also ethanoic, propionic, butanoic, pentanoic and hexanoic acid

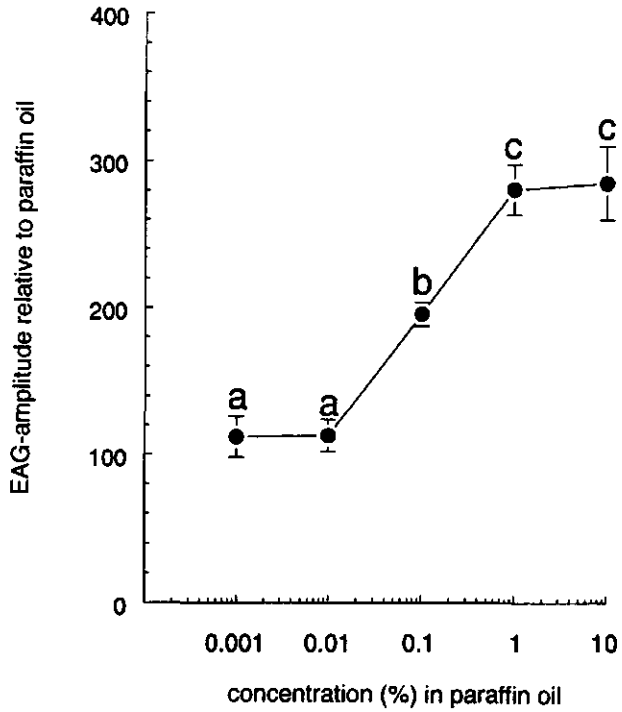

Fig. 1. EAG responses (± SEM) relative to the response to paraffin oil (average set at 100%) of *Anopheles gambiae* to a mixture of aliphatic carboxylic acids (composition see material and methods) at different doses. Doses marked with 'a' depict responses similar to paraffin oil. Doses without letters in common are significantly different from each other at $P < 0.05$.

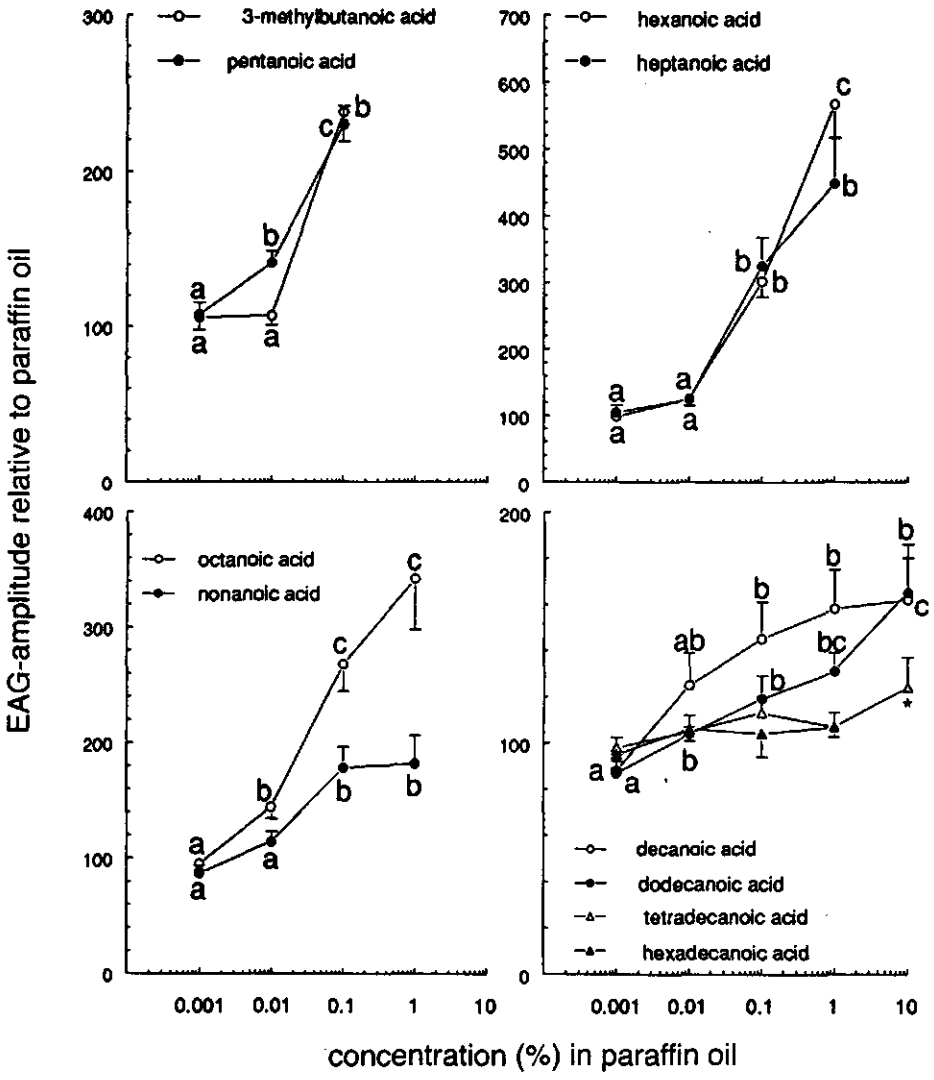

Fig. 2. EAG responses (with one-sided SEM) relative to the response to paraffin oil (average set at 100%) of *Anopheles gambiae* to individual aliphatic carboxylic acids at different doses. Doses marked with 'a' depict responses similar to paraffin oil. Doses without letters in common are significantly different from each other at $P < 0.05$. Tetradecanoic acid gave only a significant response at a dose of 10% (see asterisk). Hexadecanoic acid did not elicit any significant responses. Notes: Y-axes have different scales. Letters shown only refer to dose-response analyses within a compound.

evoked EAG values between –1.7 and –4.55 mV (Cork and Park, 1996). With the use of glass electrodes, we never obtained EAG responses of female *An. gambiae* antennae exceeding –1 mV (chapter 5). The largest EAG response elicited by 1% hexanoic acid as measured with glass electrodes was -0.84 mV. Nevertheless, it should be noted that Cork and Park (1996) used higher doses. This might have elicited higher EAG responses. No electrode potentials were generated in our experimental set-up by other stimuli like 1-octen-3-ol, fresh sweat or incubated sweat. Although sweat contains a wide range of carboxylic acids, the total dose of acids present in the sweat probably did not exceed doses generating electrode potentials. These results strongly indicate that caution should be taken when conducting electroantennographic experiments in response to carboxylic acids or other acidic compounds by means of tungsten electrodes.

Responses to carboxylic acids

EAG responses obtained upon stimulation with the carboxylic acids iC_5, C_5-C_{10}, C_{12}, C_{14} and C_{16} showed that hexanoic acid offered at a 1% concentration evoked the highest response. Also, heptanoic acid and octanoic acid elicited high EAGs at this concentration. EAG responses to the more volatile acids 3-methylbutanoic and pentanoic acid were lower, suggesting that there are more antennal receptor neurons sensitive to hexanoic acid. However, as the EAG is considered to be a summed potential of (a part of) the sensory cells on the antennae, the lower EAG responses evoked by the short chain carboxylic acids might be caused by inhibitory responses. Inhibitory responses of neurons innervating (a) subtype(s) of the sensilla trichodea population were elicited by the short chain carboxylic acids C_2, C_3, C_4, iC_4 and iC_5 but not by higher chain acids (C_5-C_{16}). Excitation of a different cell type, however, was evoked by short chain carboxylic acids as well as by pentanoic and hexanoic acid (chapter 4).

Thresholds for the acids with a carbon chain ranging between iC_5-C_8 were between 0.001%-0.1%. Higher chain carboxylic acids, like C_9, C_{10}, C_{12}, and C_{14} evoked small responses and displayed higher thresholds, while no significant responses were observed for C_{16}. Acids with a carbon chain length of C_9 and higher showed a decreasing EAG amplitude with increasing chain lengths. This might be explained by the fact that an increasing chain length of these components corresponds with a decreasing volatility. Additionally, the solvent (paraffin oil) may have influenced the dose arriving at the antenna, because it has the properties of a slow release medium.

Analyses of the composition of human sweat samples revealed ethanoic and hexadecanoic acid as the most abundant carboxylic acids (Cork and Park, 1996).

Hexanoic acid, evoking the highest EAG response during our measurements, was present in a much lower amount. Hexadecanoic acid has a lower volatility compared to shorter chain length acids and therefore fewer molecules are expected to reach the antennal olfactory receptors of the mosquito. Analyses of the composition of the headspace of human sweat or a human subject might reveal additional information about the content of the individual acids in the air.

Our results show that the antenna of *An. gambiae* displays olfactory sensitivity to a number of carboxylic acids. This together with the fact that a synthetic mixture comprising aliphatic carboxylic acids has been shown to attract female *An. gambiae* in a windtunnel bioassay implies that carboxylic acids might play a role during the host seeking and host locating process of *An. gambiae*. Further behavioural studies have to be conducted to elucidate the function of these components. Experiments with synthetic mixtures comprising both individual carboxylic acids as well as ammonia, which has recently been shown to attract female *An. gambiae* in a windtunnel bioassay (Braks et al., submitted; this thesis chapter 6) might yield more information about the identity of the attractive carboxylic acids.

References

Bowen MF (1995) Sensilla basiconica (grooved pegs) on the antennae of the female mosquitoes: electrophysiology and morphology. *Entomol. Exp. Appl.* 77: 233-238.

Braks MAH, Meijerink J and Takken W. The role of human sweat components, ammonia and L-lactic acid, in the behavior of the anthropophilic malaria mosquito, *Anopheles gambiae* (Culicidae; Diptera). Submitted to *J. Comp Physiol.*

Cork A and Park KC (1996) Identification of electrophysiologically-active compounds for the malaria mosquito, *Anopheles gambiae*, in human sweat extracts. *Med. Vet. Entomol.* 10: 269-276.

Davis EE (1988) Structure-response relationship of the lactic acid-excited neurons in the antennal grooved-peg sensilla of the mosquito *Aedes aegypti*. *J. Insect Physiol.* 34: 443-449.

De Jong R and Knols BGJ (1995) Olfactory responses of host-seeking *Anopheles gambiae* s.s. Giles (Diptera: Culicidae). *Acta Trop.* 59: 333-335.

Knols BGJ and De Jong R (1996) Limburger cheese as an attractant for the malaria

mosquito *Anopheles gambiae s.s. Parasitol. Today* 12: 159-161.

Knols BGJ, Van Loon JJA, Cork A, Robinson RD, Adam W, Meijerink J, De Jong R and Takken W (1997) Behavioural and electrophysiological responses of the female malaria mosquito *Anopheles gambiae* (Diptera: Culicidae) to Limburger cheese volatiles. *Bull. Entomol. Res.* 87: 151-159.

Lacher V (1967) Elektrophysiologische Untersuchungen an einzelnen Geruchsrezeptoren auf den Antennen weiblicher Moskitos (*Aedes aegypti l.*). *J. Insect Physiol.* 13: 1461-1470

McIver SB (1982) Sensilla of mosquitoes (Diptera: Culicidae). *J. Med. Entomol.* 19: 489-535.

Nicolaides N (1974) Skin Lipids: Their Biochemical Uniqueness. *Science* 186:19-26

Pappenberger B, Geier M and Boeckh J (1996) Responses of the antennal olfactory receptors in the yellow fever mosquito *Aedes aegypti* to human body odours. In: *Olfaction in mosquito-host interactions.* Wiley, Chichester (Ciba Foundation Symposium 200) pp. 254-266.

Takken W (1991) The role of olfaction in host-seeking of mosquitoes: a review. *Insect Sci. Applic.* 12: 287-295.

Takken W and Knols BGJ (1999) Odor-mediated behavior of Afrotropical malaria mosquitoes *Annu. Rev. Entomol.* 44: 131-157.

Van Loon JJA, Frentz WH and Van Eeuwijk FA (1992) Electroantennogram responses to plant volatiles in two species of *Pieris* butterflies. *Entomol. Exp. Appl.* 62: 253-260.

White GB (1974) *Anopheles gambiae* complex and disease transmission in Africa. *Trans. R. Soc. Trop. Med. Hyg.* 68: 278-301.

Chapter 4

Sensitivities of antennal olfactory neurons of the malaria mosquito, *Anopheles gambiae*, to carboxylic acids

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Abstract

Single sensillum recordings on the antennae of female *Anopheles gambiae s.s.* mosquitoes revealed neurons sensitive to aliphatic carboxylic acids within (a) subtype(s) sensilla trichodea. The aliphatic acids, acetic acid, propionic acid, butyric acid, iso-butyric acid and iso-valeric acid evoked an inhibition reaction in one of the cell-types recorded from. A different cell-type was excited in response to the former aliphatic acids, but showed a broader range of sensitivity, as acids with a longer carbon chain length, like caproic acid, elicited excitations as well. In addition, occasionally 1-octen-3-ol elicited an excitation reaction. This article focuses on the carboxylic acid-inhibited cell type and its temporal pattern of response to different doses of the compounds. Furthermore, in order to compare the stimulatory effectiveness of the compounds on a per molecule basis, corrections were made for differences in volatility by determining the absolute number of molecules in the stimulus puff.

Introduction

Female haematophagous mosquitoes of the species *Anopheles gambiae* Giles *s.s.* (Diptera: Culicidae) are highly anthropophilic and the transmission of *Plasmodium* parasites while bloodfeeding makes them one of the most important vectors of human malaria (White, 1974). During the process of host-seeking behaviour female mosquitoes are guided by physical and chemical emanations of the host. In the vicinity of the host body heat and moisture are thought to play an important role, while long-distance attraction seems to be mainly mediated by olfactory cues (Gillies, 1980; Takken, 1991). Behavioural studies on *An. gambiae* indicate a minor role for carbon dioxide during the process of host seeking and host location when compared to opportunistic and zoophilic species and human breath compounds other than carbon dioxide and acetone have never shown any attractancy (De Jong and Knols, 1995a; Costantini et al., 1996; Takken et al., 1997). Biting site experiments showed that female *An. gambiae* have a preference for the lower parts of the body, namely the foot and ankles, but interpretation of these

experiments seems difficult. Conflicting results have been obtained after washing the foot, indicating that either convection currents along the body or/and specific foot odours might lead the mosquito to the lower parts of the body (De Jong and Knols, 1995b; Dekker et al., 1998). However, as *An. gambiae* is a highly anthropophilic mosquito it is generally thought that human specific odours released by the skin might serve as kairomones (Knols and Meijerink, 1997).

The first group of compounds which appeared to be attractive for *An. gambiae* in a windtunnel bioassay consisted of a synthetic mixture of aliphatic carboxylic acids (Knols et al., 1997). Carboxylic acids have been identified in human sweat as well as in Limburger cheese from which they are thought to be released as a result of the microbial activity of related bacterial strains (Cork and Park, 1996; Nicolaides, 1974).

Sensitivities at the peripheral level to carboxylic acids have been reported for the yellow fever mosquito *Aedes aegypti.* Sensory cells associated with the grooved peg sensillum, known for its sensitivity to lactic acid, seem to respond to the short chain carboxylic acids, butyric, iso-butyric and propionic acid as well (Davis, 1988). Responses of the grooved peg-associated receptor cells in *Ae. aegypti* were also elicited by valeric and caproic acid (Pappenberger et al., 1996). In *Aedes epactius,* however, butyric acid sensitive cells associated with the grooved peg sensillum were found to be distinct from the cells responding to lactic acid (Bowen, 1995). In addition to the grooved peg associated receptor neurons, other sensillum types on the antennae of *Ae. aegypti* were reported to house carboxylic acid sensitive cells. Cells associated with A1 type trichodea as well as cells associated with a subtype A2 sensillum trichodeum responded by excitation when exposed to aliphatic acids, in contrast to cells underlying a different subtype A2 sensillum trichodeum. These cells were inhibited in response to aliphatic acids with a short carbon chain length and excited by longer chain acids (Lacher, 1967).

Electroantennogram responses to carboxylic acids have recently been shown for *An. gambiae s.s.*, however to date no reports are available at the single cell level (Cork and Park,1996; Knols et al., 1997). Here we document the responses of olfactory receptor cells, innervating (a) subtype(s) of the sensilla trichodea population, to aliphatic carboxylic acids. Two types of carboxylic acid-sensitive neurons were found within this sensilla. The reactions of carboxylic acid-inhibited neurons responding by a decrease in spontaneous spike frequency as well as the responses of carboxylic acid-excited cells will be described within this paper. Furthermore, to allow a comparison of the stimulatory effectiveness at equivalent doses, corrections were made for differences in volatility between the different compounds.

Material and methods

Insects.

Anopheles gambiae sensu stricto originated from Suakoko, Liberia (courtesy of Prof. M. Coluzzi, Rome). Mosquitoes were reared at 27 °C, 80% rh. and 12:12 L:D photoperiod. Adults were held in gauze cages (30 x 30 x 30 cm). They had access to a 6% glucose solution and were given the opportunity to feed on a human arm twice a week. Eggs were laid on wet filter papers and transferred to water trays. Larvae were fed Tetramin® fishfood.

Chemical stimuli

All chemicals used were purchased from Merck, Aldrich or Sigma and were more than 99% pure, except for heptanoic acid (>98%) and 1-octen-3-ol (>97%). Stimulants were dissolved in diethyl ether, after which 25 µl was applied to a 2.4 cm^2 filterpaper (Schleicher & Schuell, Dassel, Germany). The solution on the filterpaper was allowed to evaporate for 10 sec, resulting in the removal of the solvent. Filterpapers were placed in a glass Pasteur pipette that was sealed with Parafilm® at both ends. Mixture C_5-C_{16} contained the carboxylic acids with the following chain length: C_5, C_6, C_7, C_8, C_9, C_{10}, C_{12}, C_{14}, C_{16}. Mixture C_8-C_{16} contained: C_8, C_9, C_{10}, C_{12}, C_{14}, C_{16}. Acids were pooled in a ratio 1:1.

Electrophysiology

Preparation. Five to eight days old non-bloodfed female mosquitoes were lured by a human hand, cooled in a freezer (- 5 °C) for ± 1.5 min, and mounted on a small perspex holder (Lacher, 1971). Wings were glued to the holder surface with Perfax glue (Henkel, The Netherlands), legs were removed, and the antennae were carefully fastened to double-sided sticky tape. The preparation was viewed with an Olympus CK2 inverted microscope at 600x magnification.

Recording technique. During the experiments a sharpened tungsten electrode was used as the indifferent electrode, which was inserted into the thorax or eye of the mosquito. The measuring electrode (tungsten wire of 0.2 mm diameter, Drijfhout, The Netherlands) was electrolytically sharpened (5 min at 10 V and 15 min at 3 V in a 8% solution of KNO_2 in

H_2O). For recording the 'surface-contact' technique was used (Den Otter et al., 1980), in which the electrode is carefully moved around the base of a sensillum till electrophysiological activity is recorded. Signals were amplified 1000x, recorded on a tape recorder (Racal recorder store7DS) or directly imported, via an IDAC interface box and A/D converter (Syntech, Hilversum, The Netherlands), into an Intel Pentium based personal computer. Recordings were analysed by means of Autospike™ software (Syntech, Hilversum, The Netherlands). Spikes were assigned to different cells based on the occurrence of discrete classes in an amplitude histogram.

Stimulus delivery. A moistened, charcoal filtered, continuous airstream (28.7 cm/sec) was led through a glass tube (i.d. 1 cm) ending 0.5 cm from the preparationholder. Stimulus puffs (volume 1.6 ml) lasted for 0.2 sec and were injected into the airstream, at a location 10 cm from the outlet of the tube, by using a stimulus controller (Syntech, Hilversum, The Netherlands).

Dose-response curves were determined, starting with the lowest dose. Not all the stimuli could be tested on each individual, as the signal-noise ratio decreased over time, however butyric acid was tested at all doses on each individual, thereby serving as a standard.

Sensilla. Recordings were made from neurons innervating different subtypes of sensilla trichodea. Based on the classification for *An. stephensi* (Boo, 1980) sensilla trichodea subtypes recorded from mostly resemble subtype C and E. No recordings were made from subtype D, which is mainly distributed on the basal subsegments and is easily recognized by its round tip.

Response criteria. Cells responding to stimuli tested at one dose, namely 2500 μg, were classified as slightly inhibited respectively inhibited when a decrease of 20%-40% or 40% or more of the spontaneous spike frequency was observed in response to a 0.2 sec pulse in a time period of 3 sec following stimulation. For determining the temporal pattern of response, all cells showing a decrease of the spontaneous spike frequency of at least 20% in response to a 0.2 sec pulse of 2500 μg butyric acid in a time period 3 sec upon stimulation were classified as carboxylic acid inhibited cells. Doses of stimuli which did not evoke a difference in spike frequency twice as big as the standard deviation of the spontaneous spike frequency during the first sec upon stimulation were regarded as ineffective. For the dose-response relationships, responses were expressed as the

difference between the mean spontaneous spike activity determined in the 3 sec before stimulation and the mean spike fequency in the 7 sec during and after stimulation starting at the onset of stimulation. Responses were classified as excitation when the maximum spike frequency during 0.1 sec upon stimulation was 150% or more than the spontaneous spike frequency.

Volatility determinations

Volatility measurements were performed on a Carlo Erba Vega 6000 gas-chromatograph fitted with a Grob split/splitless injector (220 °C) and a FID detector (260 °C) and a DB-wax column (60 m x 0.324 mm), with a film thickness of 0.5 μm (J & W Scientific, Folsom, U.S.A.). Determination of volatile concentrations in the headspace was performed isothermally at 200 °C. As carrier gas N_2 was used (15 cm/sec). Glass Pasteur pipettes containing filterpapers loaded with stimuli were prepared in the same way as when used in electrophysiological experiments. Samples of 250 μl each were taken from the air inside the pipette by using a glas syringe (Hamilton, Bonaduz, Switzerland) and injected into the GC. Samples of 1000 μl each were taken for stimulus loads of 25 μg, because the amount of stimulus in 250 μl headspace at this stimulus load was below the detection limit of the GC. Three samples were taken for each compound at each dose. Quantification of the headspace samples was performed by comparing the peak areas with a corresponding standard, which was a known amount of a solution of the same compound in diethylether.

Results

Neurons innervating (a) subtype(s) of the sensilla trichodea population were found to respond to 1-octen-3-ol and to carboxylic acids at a load of 2500 μg with a stimulus duration of 0.2 sec. Fig.1 shows a scanning electron micrograph of one of the segments of a female *An. gambiae* including the sensilla trichodea subtype(s) recorded from. Reactions to the short chain carboxylic acids acetic acid, propionic acid, butyric acid, iso-butyric acid and iso-valeric acid, further referred to as C_2, C_3, C_4, iC_4 and iC_5 were obtained frequently, while only occasionally responses to 1-octen-3-ol and higher chain carboxylic acids like valeric acid (C_5) and caproic acid (C_6) were observed. No responses were found to other sweat borne compounds like dimethyldisulfide, ethylhexenoic acid

and hydrocinnamic acid.

Table 1 shows the response of eleven sensilla out of 9 females to the afore mentioned substances. Due to the short length of the recordings, not all the stimuli could be tested on the same sensillum. However, some differences in reaction spectra seemed very pronounced. The inhibited cell type responded by inhibition to C_2, C_3, C_4, iC_4 and iC_5 and was slightly inhibited by valeric acid. Acids or mixtures of acids with a higher chain length elicited no inhibitory responses. Responses of the carboxylic-acid inhibited cell to 1-octen-3-ol were only occasionally observed. An excitatory response was observed during a simultaneous recording were one cell was inhibited by the tested short chain aliphatic acids while the other cell was excited by the same acids. However, responses of the carboxylic acid excited cell were not only limited to the short chain acids as the cell was also excited by the higher chain carboxylic acids C_5 and C_6. Fig. 2 shows such a simultaneous recording in which both neurons respond by a different type of reaction.

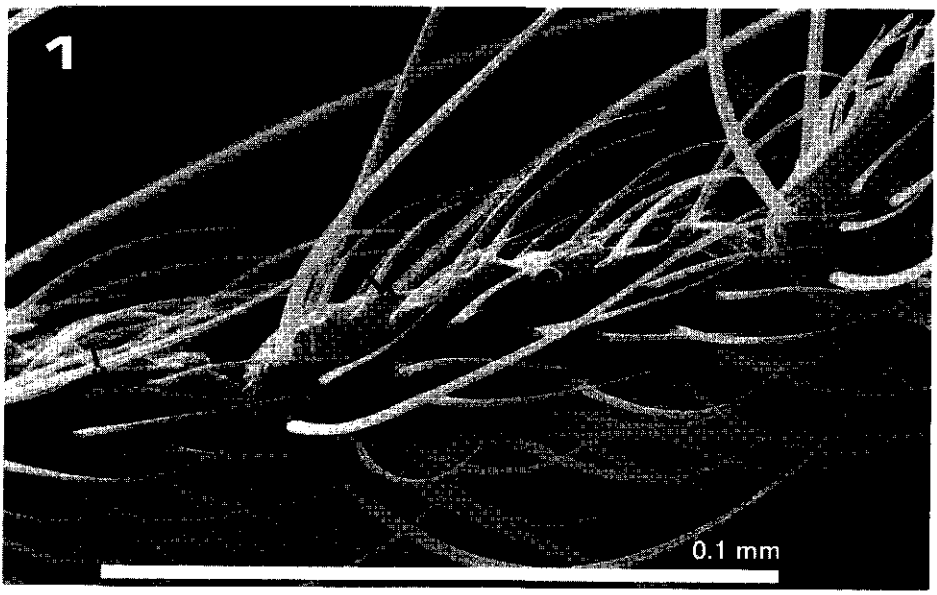

Fig. 1. Scanning electron micrograph of one of the segments of the antennae of a female *An. gambiae*. Arrows show sensilla trichodea which house carboxylic acid sensitive neurons. Scale bar = 0.1 mm

Table 1 Response of 11 sensilla from nine females to various compounds

Compound[a]	Sensillum[b]													
	1a	1b	2	3a	3b	4	5	6a	6b	7	8	9	10	11
acetic acid	O	O								O	O	O	O	–
propionic acid	o	–	O	o	O	O	O	O	●	O	O	O	O	–
butyric acid			O	O	O	O	O	O	●	o	O	O	O	–
iso-butyric acid							O			O				
iso-valeric acid							O			–				
valeric acid			o	–	–			o	●	–	–		–	–
mixture C_5-C_{16}				–	–			o	●					
caproic acid								–	●		–		–	–
heptanoic acid											–			–
mixture C_8-C_{16}											–			–
1-octen-3-ol	–	●				–		–	–	●	–			–

[a] Mixture C_5-C_{16} contains C_5, C_6, C_7, C_8, C_9, C_{10}, C_{12}, C_{14}, C_{16}; mixture C_8-C_{16} contains C_8, C_9, C_{10}, C_{12}, C_{14}, C_{16}.

[b] Numbers represent the different neurons associated with the same subtype(s) sensilla trichodea. a, b indicate two different neurons recorded at the base of the same sensillum. O, inhibition (cells responded with a decrease in spike frequency of 40% or more); o, slightly inhibited (cells responded with a 20-40% decrease in spike frequency), ●, excitation; –, no response; no sign, compound not tested.

Temporal pattern of response for short chain carboxylic acids

Response characteristics of the inhibited neuron were determined by studying not only the strength of the reaction, in response to a range of concentrations, but also the time period during which the spontaneous spike activity was suppressed.

Recordings usually did not last long enough to test the full set of compounds on one sensillum. Therefore each sensillum was always tested with the full range of doses from 2.5 μg to 2500 μg of C_4 and the full range of doses of one of the other compounds (stimulus duration 0.2 sec). In this way C_4 served as a reference compound. The entire set

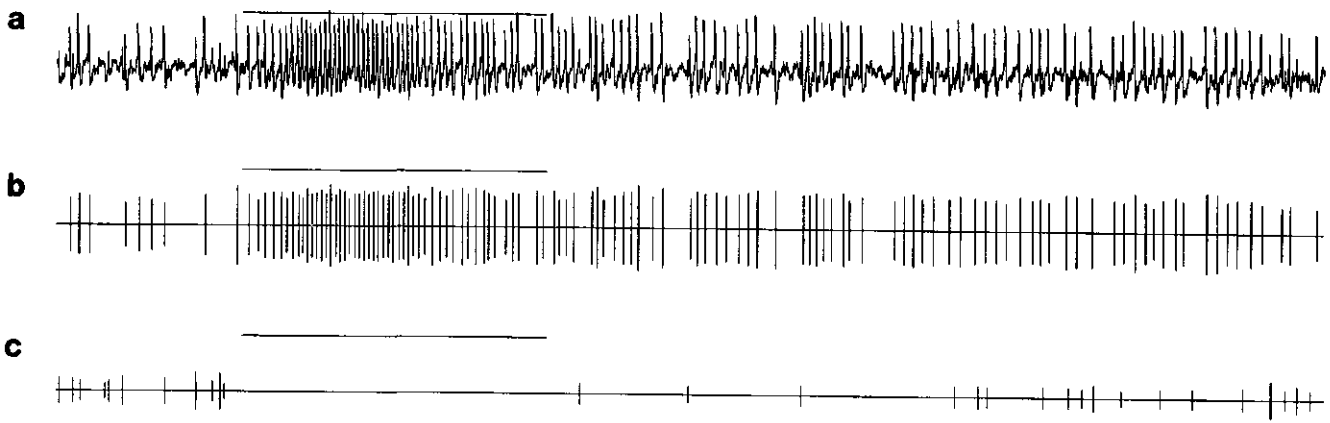

Fig. 2. Single sensillum recording showing the action potential response (A) and the amplitude-based analyses of two different neurons reponding with an increase in spike frequency (B) and a decrease in spike frequency (C) upon stimulation with a dose of 2500 µg propionic acid in diethyl ether loaded on filter paper in a stimulus cartridge. The bars show the duration of the stimulus (500 ms).

of doses of C_4 and one of the other compounds was presented to 26 sensilla of 19 different insects. In most cases the activity of only one of the two neurons, thought to innervate these sensilla trichodea, was registered. In 22 sensilla, one celltype was found which responded by inhibition. In the four other sensilla another celltype was observed which was excited upon stimulation. In one case the excited cell showed, only in response to iC_4, iC_5 and C_5, an inhibition after the excitation. Excitation as well as inhibition was observed to the lower chain carboxylic acids C_2, C_3, C_4, iC_4 and iC_5, however, responses to C_5 and C_6 were only recorded from the celltype that responded by excitation (n=2). Fig. 3 shows the action potential response of a carboxylic acid inhibited neuron to 25 µg, 250 µg and 2500 µg of C_4. The temporal pattern of response of 22 carboxylic acid inhibited cells in response to C_2 (n=6), C_3 (n=5), C_4 (n=22), iC_4 (n=6) and iC_5 (n=6) is shown in Fig. 4 for the doses 25 µg, 250 µg and 2500 µg. No responses were observed to stimulus loads of 2.5 µg. In addition, lower stimulus loads of 250 ng never produced any response. Stimulation with 25 µg C_4 resulted in 9 percent of the cells in a decrease in spike activity, which was bigger as or at least twice as big as the standard deviation. Ten fold and hundred fold higher doses of stimuli gave stronger and especially hundred fold higher doses longer lasting inhibition.

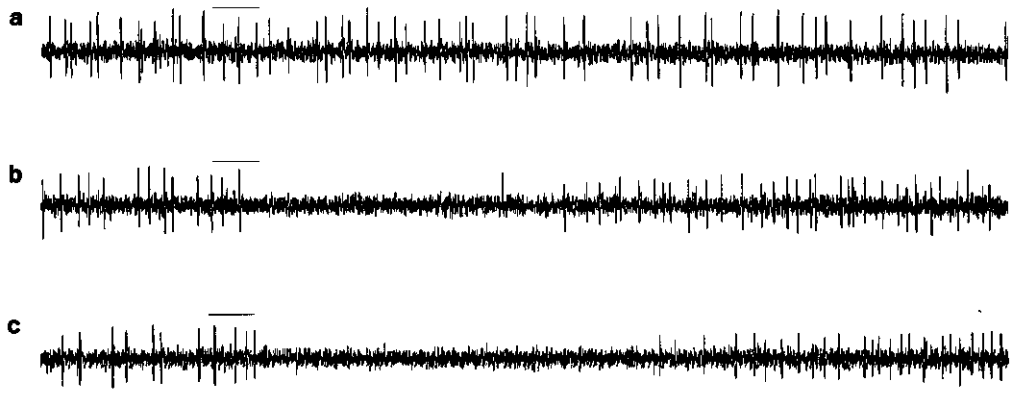

Fig. 3. Action potential response of a carboxylic acid inhibited neuron in response to doses of 25 µg (a), 250 µg (b) and 2500 µg (c) of iso-butyric acid in diethyl ether loaded on filter paper in a stimulus cartridge. The bars show the duration of the stimulus (200 ms).

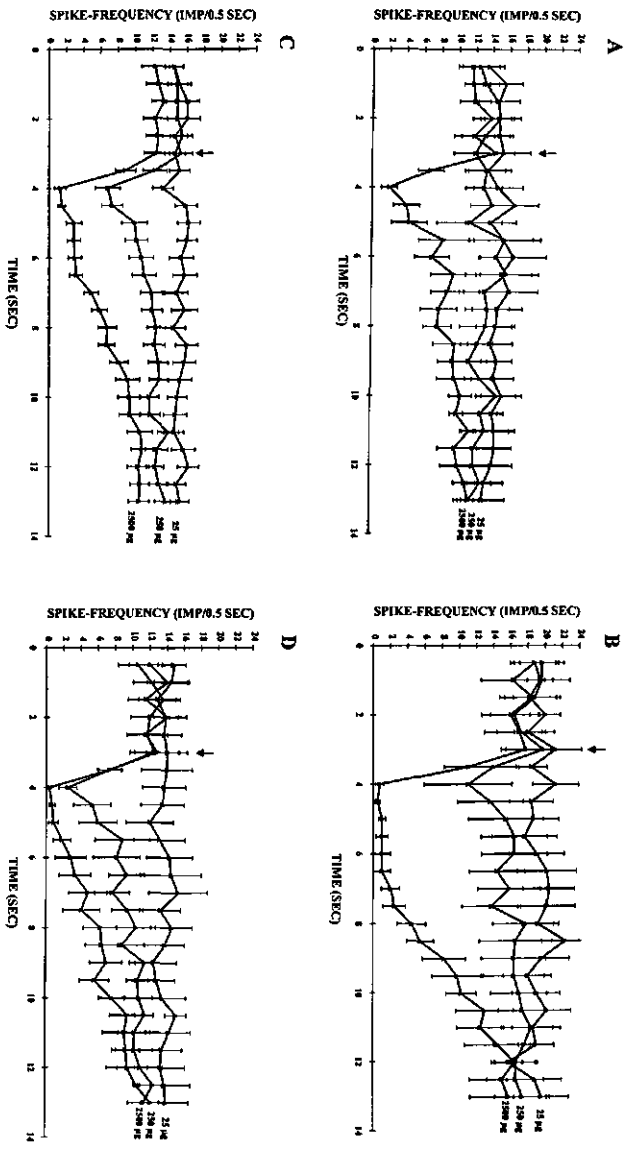

Fig. 4. Temporal pattern of response (mean + SEM) of a carboxylic acid inhibited neuron in response to a 0.2 sec puff led over of doses of 25 µg, 250 µg and 2500 µg of acetic acid (A; n=6), propionic acid (B; n=5), butyric acid (C; n=22), iso-butyric acid (D; n=6) and iso-valeric acid (E; n=6). Arrow indicates moment of stimulation.

Dose-response relationships

In order to assess possible differences in sensitivity of the carboxylic acid inhibited neuron in response to the tested short chain carboxylic acids corrections were made for the differences in volatility by determining the absolute number of molecules in a stimulus puff. This was done by taking vapour samples from the odour inside a glass Pasteur pipet, containing one of the stimuli. Fig. 5 shows the dose-response relationships for the tested carboxylic acids corrected for the differences in volatility. Although the carboxylic acid inhibited cell seemed to be more sensitive to iC_4 at a dose of 250 µg then for instance to C_2 (Mann-Whitney *U* test, P = 0.004), when judged on the basis of stimulus load on filter paper, no differences in sensitivity for the short chain carboxylic acids at any dose could be seen after correction, indicating that this carboxylic acid inhibited neuron is equally sensitive for the short chain carboxylic acids tested (ANCOVA, F = 1.69, df = 1,4; P = 0.156). Carboxylic acid sensitive neurons showed an inhibitory reaction to doses ranging from 53 x 10^{-11} mol/0.2 sec to 13.4 x 10^{-9} mol/0.2 sec with a threshold between 16 x 10^{-11} and 53 x 10^{-11} mol/ 0.2 sec.

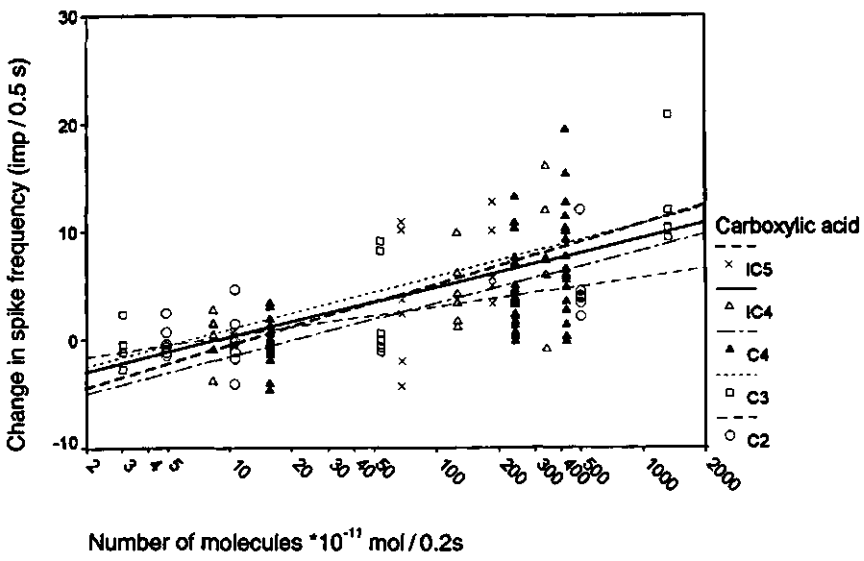

Fig. 5. Dose-response relationships based on regression analysis with number of stimulus molecules released as covariate (ANCOVA) of acetic acid (C_2), propionic acid (C_3), butyric acid (C_4), iso-butyric acid (IC_4) and iso-valeric acid (IC_5). See text for the way in which values for the number of stimulus molecules released were obtained.

Discussion

Response patterns and dose response relationships

Dose-response relationships are commonly based on doses of a certain stimulus in the liquid phase, namely the dose applied to the filterpaper, rather than to the absolute numbers of molecules to which the animal is exposed during stimulation (Bengtsson et al., 1990). When related to the dose applied to the filter paper, dose-response relationships for the carboxylic acid inhibited cell type showed a significant difference in suppression of the spontaneous spike activity between for instance iC_4 and C_2 at a dose of 250 µg. However, when the response of the carboxylic acid inhibited cell was related to the absolute number of molecules in the odour puff, no statistical differences in sensitivity of the carboxylic acid inhibited neuron for the tested compounds could be observed, indicating that the carboxylic acid inhibited cell type is equally sensitive for the carboxylic acids C_2, C_3, C_4, iC_4 and iC_5. These data show the importance of relating dose-response relationships to the actual amount of compound to which an animal is exposed during stimulation instead of neglecting possible differences in volatility between the different compounds tested.

The carboxylic acid inhibited cell type responded upon stimulation by a long lasting decrease in spontaneous spike frequency which could last for several seconds when stimulated with the highest concentration. This was in contrast with the response pattern of the excited cell type, which showed a phasic tonic response with a short burst of maximum activity. Differences in temporal response patterns for different neurons have been described by Moore (1994). Model olfactory receptor cells show different response characteristics with respect to adaptation and the recovery of adaptation. It is thought that by having different response characteristics, olfactory cells can filter the spatially and temporally variable odour signals present in the natural environment.

Relation between sensillum subtype and response type

Here we reported the observation that neurons innervating (a) subtype(s) of the sensilla trichodea population respond to carboxylic aliphatic acids. Although the majority of the observed carboxylic acid sensitive neurons reponded by inhibition, occasionally carboxylic acid excited neurons were found. Observations indicate that the exact position of the tungsten electrode during recording might determine the celltype recorded from. The tungsten electrode was predominantly placed at the same spot, namely at the distal

half of the sensillum base. The carboxylic acid excited cells, however, were usually found when the electrode was placed at a different position. Occasionally recordings were made in which two cells were observed simultaneously. Some of these recordings show that both cells were inhibited; one recording shows one cell which is inhibited while the other one is excited in response to the same compounds. The former observations might indicate that a part of the sensilla trichodea house cells which respond by a similar type of reaction, namely inhibition, while other sensilla house cells showing both reaction types. Observations of different cells innervating the same sensillum but responding by a different type of reaction, namely by inhibition or excitation, to the same stimulus have been documented (Schneider et al., 1964; Davis and Sokolove, 1976; Altner and Loftus, 1985). Other studies on the single cell level with *Necrophorus* beetles showed excitation to carboxylic acids with a carbon chain length of six to nine carbon atoms, while carboxylic acids with a shorter chain length caused inhibitory responses (Boeckh, 1962).

Carboxylic acid sensitive olfactory neurons have been found within sensilla trichodea in *Aedes aegypti* (Lacher, 1967). It was shown that different response patterns were linked to different subtypes sensilla trichodea. In our work no association between reaction spectrum and sensillum subtype within the morphological category studied have been observed. It should be noted, however, that the different sensillum trichodeum subtypes are not easily distinguished in *An. gambiae* (Ismail, 1964; McIver, 1982).

Inhibition

Although inhibitory responses are less common and/or less described then odor-evoked excitation, other studies also reveal a possible role of odor-evoked inhibition in the process of olfactory coding. Odor-evoked inhibition in lobster olfactory receptor neurons has been shown to be mediated by a different second messenger pathway than odor-evoked excitation (Michel and Ache, 1992; Fadool and Ache, 1992). In addition, intracellular measurements of these olfactory neurons show that the propagated output of primary olfactory neurons in response to inhibitory stimuli acts within the natural sampling interval, suggesting that inhibitory odor input is functional in olfactory coding, by increasing the diversity of the neuronal patterning (Michel and Ache, 1994). Although studies to identify a distinct inhibition mediated second messenger pathway in insects were negative (Breer et al., 1990; Ziegelberger et al., 1990) some studies show clear correlations between the behavioural response and an odor or chemical evoked inhibition. Wall-pore single-walled olfactory sensilla of the termite *Schedorhinotermes lamanianus*

house an olfactory neuron that is inhibited in response to natural concentrations of CO_2 that occur in the nest. Inhibition in response to natural concentrations lasts around 2 sec and blocks excitatory responses to other volatiles (Ziesmann, 1996). Inhibitory responses of neurons innervating the grooved peg sensillum have been found for lactic acid in the mosquito species *Aedes aegypti.* Lactic-acid inhibited neurons as well as excited neurons were found within the grooved peg sensillum responding to concentrations of lactic acid equal to concentrations emanating from the human skin. The sensitivity of lactic-acid excited neurons decreased after mosquitoes received a bloodmeal, in contrast to lactic acid inhibited neurons (Davis, 1984; Davis et al., 1987).

We found carboxylic acid inhibited as well as carboxylic acid excited neurons within (a) subtype(s) of the sensilla trichodea population, responding to equal concentrations of carboxylic acids. Whether the combination of those differently responding cells might be a general phenomenon in mosquitoes is not known. However, results indicate that carboxylic acid sensitive neurons are not limited to the subtype(s) of sensilla trichodea reported here as preliminary data showed carboxylic acid sensitive cells within the grooved peg sensillum, comparable to *Aedes aegypti.*

Behaviour

Responses to carboxylic acids are not limited to mosquitoes only, but have been described for a range of insect species as well as for other arthropods. Neurons innervating sensilla basiconica of the carrion beetle *Thanatophilus rugosus* are inhibited by propionic acid (Boeckh, 1967). In addition, short chain carboxylic acid sensitive receptors were also found for the tropical bont tick *Amblyomma variegatum* (Steullet and Guerin, 1994) and inhibitory as well as excitatory responses were obtained to butyric acid in female butterflies of the species *Pieris brassicae* (Den Otter et al., 1980). Apart from responses obtained at the sensory level, carboxylic acids have been shown to act as an attractant as well as a repellent on the behavioural level for different insect species. For mosquitoes, *alpha* substituted aliphatic carboxylic acids with a 2- to 5-carbon chain length were reported to be attractive for *Aedes aegypti* (Carlson et al., 1973), although earlier work with the same mosquito species yielded less clear results (Roessler, 1961; Müller, 1968). Caproic acid was found repellent for the tsetse species *Glossina morsitans* and *G. pallidipes* when used in an odour-baited trap (Vale, 1980), while butyric acid at high concentrations acts as an oviposition attractant for the gravid fruit fly *Dacus tryoni* (Eisemann and Rice, 1992).

Attractants which have been identified under laboratoy conditions might

eventually be used in the field and have its utilization as odour-baited traps to monitor mosquito densities for the study of population dynamics. If the carboxylic acids alone are sufficient for application of an optimal bait or whether a combination with other compounds might be essential to lure *An. gambiae* mosquitoes under field conditions is still under investigation.

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References

Altner H and Loftus R (1985) Ultrastructure and function of insect thermo- and hygroreceptors. *Annu. Rev. Entomol.* 30: 273-295.

Bengtsson M, Liljefors T, Hansson BS, Löfstedt C and Copaja SV (1990) Structure-activity relationships for chain-shortened analogs of (Z)-5-decenyl acetate, a pheromone component of the turnip moth, *Agrotis segetum*. *J. Chem. Ecol.* 16: 667-684.

Boeckh J (1962) Elektrophysiologische Untersuchungen an einzelnen Geruchsrezeptoren auf den Antennen des Totengräbers (*Necrophorus*, Coleoptera). *Z. Vgl. Physiol.* 46: 212-248.

Boeckh J (1967) Inhibition and excitation of single insect olfactory receptors and their role as a primary sensory code. In *Olfaction and Taste*, eds Y. Zotterman, Vol. II, pp. 721-735. Pergamon Press, New York.

Boo KS (1980) Fine Structure of the Antennal Sensory Hairs in Female *Anopheles stephensi*. *Z. Parasitenkd.* 61: 161-171.

Bowen MF (1995) Sensilla basiconica (grooved pegs) on the antennae of the female mosquitoes: electrophysiology and morphology. *Entomol. Exp. Appl.* 77: 233-238.

Breer H, Boekhoff I and Tareilus E (1990) Rapid kinetics of second messenger formation in olfactory transduction. *Nature* 345: 65-68.

Carlson DA, Smith N, Gouck HK and Godwin DR (1973) Yellow fever mosquitoes: Compounds related to lactic acid that attract females. *J. Econ. Entomol.* 66: 329-331.

Cork A and Park KC (1996) Identification of electrophysiologically-active compounds for the malaria mosquito, *Anopheles gambiae*, in human sweat extracts. *Med. Vet. Entomol.* 10: 269-276.

Costantini C, Gibson G, Sagnon N, Della Torre A, Brady J and Coluzzi M (1996) Mosquito responses to carbon dioxide in a West African Sudan savanna village. *Med. Vet. Entomol.* 10: 220-227

Davis EE (1984) Regulation of sensitivity in the peripheral chemoreceptor systems for host-seeking behaviour by a haemolymph-borne factor in *Aedes aegypti. J. Insect Physiol.* 30: 179-183.

Davis EE (1988) Structure-response relationship of the lactic acid-excited neurons in the antennal grooved-peg sensilla of the mosquito *Aedes aegypti. J. Insect Physiol.* 34: 443-449.

Davis EE, Haggart DA and Bowen MF (1987) Receptors mediating host-seeking behaviour in mosquitoes and their regulation by endogenous hormones. *Insect Sci. Applic.* 8: 637-641.

Davis EE and Sokolove PG (1976) Lactic Acid-sensitive Receptors on the Antennae of the Mosquito, *Aedes aegypti. J. Comp. Physiol. A* 105: 43-54.

De Jong R and Knols BGJ (1995a) Olfactory responses of host-seeking *Anopheles gambiae* s.s. Giles (Diptera: Culicidae). *Acta Trop.* 59: 333-335.

De Jong R and Knols BGJ (1995b) Selection of biting sites on man by two malaria mosquito species. *Experientia* 51: 80-84.

Dekker T, Takken W, Knols BGJ, Bouman E, van der Laak S, de Bever A and Huisman PWT (1998) Selection of biting sites on a human host by *Anopheles gambiae sensu stricto*, *An. arabiensis* and *An. quadriannulatus*. *Entomol. Exp. Appl.* 87: 295-300

Den Otter CJ, Behan M and Maes FW (1980) Single cell responses in female *Pieris brassicae* (*Lepidoptera: Pieridae*). *J. Insect Physiol.* 26: 465-472.

Eisemann CH and Rice MJ (1992) Attractants for the gravid Queensland fruit fly *Dacus tryoni. Entomol. Exp. Appl.* 62: 125-130.

Fadool DA and Ache BW (1992) Plasma membrane inositol 1,4,5-trisphosphate-activated

channels mediate signal transduction in lobster olfactory receptor neurons. *Neuron* 9: 907-918.

Gillies MT (1980) The role of carbon dioxide in host-finding by mosquitoes (Diptera: Culicidae): a review. *Bull. Entomol. Res.* 70: 525-532.

Ismail IAH (1964) Comparative Study of Sense Organs in the Antennae of Culicine and Anopheline Female Mosquitoes. *Acta Trop.* 21: 155-168.

Knols BGJ, van Loon JJA, Cork A, Robinson RD, Adam W, Meijerink J, De Jong R and Takken W (1997) Behavioural and electrophysiological responses of the female malaria mosquito *Anopheles gambiae* (Diptera: Culicidae) to Limburger cheese volatiles. *Bull. Entomol. Res.* 87: 151-159.

Knols BGJ and Meijerink J (1997) Odors Influence Mosquito Behavior. *Science Med.* 4: 56-63.

Lacher V (1967) Elektrophysiologische Untersuchungen an einzelnen Geruchsrezeptoren auf den Antennen weiblicher Moskitos (*Aedes aegypti l.*). *J. Insect Physiol.* 13: 1461-1470.

Lacher V (1971) Electrophysiological equipment for measuring the activity of single olfactory nerve cells on the antennae of mosquitoes. *J. Econ. Entomol.* 64: 313-314.

McIver SB (1982) Sensilla of mosquitoes (Diptera: Culicidae). *J. Med. Entomol.* 19: 489-535.

Michel WC and Ache BW (1992) Cyclic nucleotides mediate an odor-evoked potassium conductance in lobster olfactory receptor cells. *J. Neurosci.* 12: 3979-3984.

Michel WC and Ache BW (1994) Odor-evoked inhibition in primary olfactory receptor neurons. *Chem. Senses* 19: 11-24.

Moore PA (1994) A model of the role of adaptation and disadaptation in olfactory receptor neurons: implications for the coding of temporal and intensity patterns in odor signals. *Chem. Senses* 19: 71-86.

Müller W (1968) Die Distanz- und Kontakt-Orientierung der Stechmücken (*Aedes aegypti*) (Wirtsfindung, Stechverhalten und Blutmahlzeit). *Z. Vgl. Physiol.* 58: 241-303.

Nicolaides N (1974) Skin Lipids: Their Biochemical Uniqueness. *Science* 186: 19-26.

Pappenberger B, Geier M and Boeckh J (1996) Responses of the antennal olfactory receptors in the yellow fever mosquito *Aedes aegypti* to human body odours. In: *Olfaction in mosquito-host interactions*. Wiley, Chichester (Ciba Foundation Symposium 200) pp. 254-266.

Roessler HP (1961) Versuche zur geruchlichen Anlockung weiblicher Stechmücken (*Aedes aegypti* L., Culicidae). *Z. Vgl. Physiol.* 44: 184-231.

Schneider D, Lacher V and Kaissling KE (1964) Die Reaktionsweise und das Reaktionsspektrum von Riechzellen bei *Antheraea pernyi* (*Lepidoptera, saturniidae*). *Z. Vgl. Physiol.* 48: 632-662.

Steullet P and Guerin PM (1994) Identification of vertebrate volatiles stimulating olfactory receptors on tarsus I of the tick *Amblyomma variegatum* Fabricius (Ixodidae) II. Receptors outside the Haller's organ capsule. *J. Comp. Physiol. A* 174: 39-47.

Takken W (1991) The role of olfaction in host-seeking of mosquitoes: a review. *Insect Sci. Applic.* 12: 287-295.

Takken W, Dekker T and Wijnholds YG (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). *J. Insect Behav.* 10: 395-407.

Vale GA (1980) Field studies on the responses of tsetse flies (Glossinidae) and other Diptera to carbon dioxide, acetone and other chemicals. *Bull. Entomol. Res.* 70: 563-570.

White GB (1974) *Anopheles gambiae* complex and disease transmission in Africa. *Trans. R. Soc. Trop. Med. Hyg.* 68: 278-301.

Ziegelberger G, van den Berg MJ, Kaissling KE, Klumpp S and Schultz JE (1990) Cyclic GMP levels and guanylate cyclase activity in pheromone-sensitive antennae of the silkmoths *Antheraea polyphemus* and *Bombyx mori*. *J. Neurosci.* 10: 1217-1225.

Ziesmann J (1996) The physiology of an olfactory sensillum of the termite *Schedorhinotermes lamanianus*: carbon dioxide as a modulator of olfactory sensitivity. *J. Comp. Physiol. A* 179: 123-133.

Chapter 5

Identification of olfactory stimulants for *Anopheles gambiae* from human sweat samples

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Abstract

The behavioural and electro-antennogram (EAG) responses of female *Anopheles gambiae* mosquitoes to pooled samples of freshly collected human sweat and human sweat incubated for 42-52 hours were compared. No behavioural or EAG response was obtained to pooled fresh sweat samples while the incubated pooled sweat samples produced a behavioural as well as an EAG response. GC-MS analysis of the headspace composition of the fresh sweat revealed ethanol (15.1% of the total amount of volatiles trapped), acetic acid (10.9%) and 3-hydroxy-2-butanone (9.5%) as most abundant compounds; a wide range of ethyl esters was present as well. None of the ethyl esters were detected in the headspace collections from incubated sweat while the relative amounts of ethanol, acetic acid and 3-hydroxy-2-butanone were strongly reduced. In the latter collections, indole (27.9%), 1-dodecanol (22.4%) and 3-methyl-1-butanol (10%) were present in high amounts while they were absent or present in only minor amounts in the headspace collections from fresh sweat. Geranyl acetone (6%) and 6-methyl-5-hepten-2-one (1.9%) were relatively abundant in both the fresh and in the incubated headspace samples. EAG responses were observed in response to indole, 6-methyl-5-hepten-2-one and geranyl acetone.

Introduction

Finding a suitable host is crucial for the reproduction of most haematophagous mosquito species, as proteins from the host's blood are essential for the development of the eggs. For the anthropophilic malaria mosquito *Anopheles gambiae* Giles *sensu stricto* (Diptera: Culicidae), the process of seeking and locating a potential host is mediated by chemical and physical cues. Odours emanating from the human body are thought to elicit attraction from longer distances, while physical cues like heat exert their effect in the direct vicinity of the host (Gillies, 1980; Takken, 1991).

Of the few human breath compounds tested, only acetone and carbon dioxide have shown attractiveness for *An. gambiae* (Takken et al., 1997). However, carbon

dioxide has been reported to be of minor importance in the process of host seeking and host location when compared with the more opportunistic and zoophilic species (De Jong and Knols, 1995; Costantini et al., 1996; Mboera et al., 1997).

It is generally assumed that the highly anthropophilic *An. gambiae* mosquito is mainly attracted by volatiles emanating from the human skin (Knols and Meijerink, 1997). The first synthetic blend of odours which attracted *An. gambiae* in a windtunnel bioassay consisted of a mixture of carboxylic acids (Knols et al., 1997). Carboxylic acids are constituents of human skin emanations and are thought to be produced by microbial activity (Nicolaides, 1974). Recently, the first electrophysiological study on the single cell level for *An. gambiae* reported single antennal olfactory neurons sensitive to aliphatic carboxylic acids (Meijerink and Van Loon, 1999).

Behavioural experiments conducted with sweat showed that alkalinization of an attractive sweat sample, which results in a decreased release of acidic compounds, had no effect on the attractiveness of the sweat (Braks et al., 1997). This finding suggests that in addition to carboxylic acids other sweat-borne compounds play a role in the host seeking behaviour of *An. gambiae.* However, when repeating this study with freshly collected sweat from other volunteers, it appeared to be not attractive at all (Braks and Takken, 1999).

In the past, conflicting results have been reported concerning the attraction of *Aedes aegypti* mosquitoes to complex odour mixtures of human origin as well. Although most studies report attraction to arm pit sweat and odours emanating from a hand or arm, inconsistent results have been published about sweat originating from other parts of the body, for instance the chest (Willis, 1947; Parker, 1949; Brown et al., 1952; Thompson and Brown, 1955; Müller, 1968; Price et al., 1979; Eiras and Jepson, 1991; Eiras and Jepson, 1994).

The first indication that the differential attractiveness of sweat samples for *An. gambiae* might be due to differences in chemical composition of the sweat samples came from pH measurements showing that attractive sweat samples had an alkaline pH of 8, in contrast to freshly obtained sweat samples which had an acidic pH around 5.5 (Noble and Somerville, 1974; Braks et al., 1997, Braks and Takken, 1999). Surprisingly, freshly collected sweat from the tested individuals was not attractive for *An. gambiae*, while incubation of the sweat resulted in a strong attraction. Incubation of the sweat was accompanied by a distinct growth of microflora, suggesting that the change in pH as well as the attractiveness of the incubated sweat sample might be the result of chemicals produced by these bacteria (Braks and Takken, 1999).

Our approach was to identify (potential) kairomones for *An. gambiae* which are generally produced by every human host. Therefore, we decided to pool sweat samples and neglect possible differences between individuals.

Here we report the behavioural and antennal olfactory response of female *An. gambiae* mosquitoes to pooled samples of fresh and incubated sweat. Furthermore, GC-MS analyses of the headspace of fresh and incubated sweat were made in order to identify potential kairomones. Comparison of the compounds present in the headspace analyses of freshly collected sweat with that of incubated sweat and subsequent electroantennographic studies led to the identification of several compounds as olfactory stimulants.

Methods and materials

Insects

Anopheles gambiae s.s. originated from Suakoko, Liberia (courtesy of Prof. M. Coluzzi, Rome). Mosquitoes were reared at 27 °C, 80% rh and 12:12 L:D photoperiod. Adults were held in gauze cages (30 x 30 x 30 cm). They had access to a 6% glucose solution and were given the opportunity to feed on a human arm twice a week. Eggs were laid on wet filter paper and transferred to water trays. Larvae were fed Tetramin® fishfood. For the behavioural and EAG studies 5 – 8 days old non-bloodfed female mosquitoes were used.

Sweat collection

Sweat collection from volunteers engaged in physical exercise by cycling on a hometrainer took place in a humidified (70% rh) room at 30 °C. Fourteen Caucasian volunteers, nine males and five females with ages ranging from twenty-one to fifty-two were asked each to collect 3 ml sweat from the forehead. Volunteers had not used soap or any other cosmetic products 24 hours prior to the sweat collection. From each individual 1.5 ml sweat was directly placed in a freezer (-5 °C), while the other 1.5 ml was incubated for 42-52 hours under aerobic conditions at 37 °C and subsequently stored in a freezer. Both sweat samples will further be referred to as 'fresh sweat' and 'incubated sweat' respectively. A pooled sweat sample (pool A) comprised of the individual sweat samples was obtained by pooling ± 1.2 ml sweat from each of the individuals; this was

done both for the fresh and for the incubated sweat. A second sweat sample (pool B) was obtained from three male Caucasian volunteers with ages ranging from thirty-three to fifty-one, each of whom collected 6 ml sweat from the forehead. Equal volumes of their sweat samples were pooled. The pH of all the sweat samples was determined before and after incubation (Indicator Paper, Merck).

Behavioural assay

A dual-port olfactometer, consisting of a perspex flight chamber (1.6 x 0.66 x 0.43 m) (modified after Braks and Takken, 1999) was used to study the attractiveness of sweat samples. Charcoal filtered, humidified (65 ± 5% rh) air with a temperature of 27 ± 0.5 °C was led, via perspex trapping devices, through two ports (diameter 0.04 m, 0.28 m apart) into the flight chamber with a speed of 20 cm/ sec. Light (1 lux) was produced by one incandescent light bulb (75 W) and was filtered and scattered by a piece of yellow cloth hanging 1 m above the flight chamber.

The behavioural responses to fresh and incubated sweat samples of pool A and B were tested. For each test 50 µl sweat was applied on a sand-blasted glass slide (5 x 2 cm) which was placed in the left or right perspex trapping device and was tested to an equivalent amount of distilled water. Mosquitoes were released from a container at the rear end of the flight chamber. During each 15 min-test, 30 mosquitoes had the opportunity to choose between one of the ports. Each stimulus was tested six times against its control. Stimuli were alternated between the right and left port with each test. Behavioural tests were performed during the last four hours of the dark period.

Headspace collection

Sweat samples were placed in a 50 ml glass flask with 3-mm-ID inlet and outlet openings. A nitrogen stream filtered through activated charcoal was led through the flask at a flow of 120 ml/min. The outlet of the flask was connected via a screwed joint to the end of a glass tube (ID 8 mm), which was fitted with screw thread. The glass tube, approximately shaped like a Pasteur pipette, contained 53 mg Tenax in the middle part (ID 3 mm x 56 mm). The nitrogen stream left the tube at the other end (ID 1 mm). Connections were made with 4-mm-ID Teflon tubing.

Headspace collections were made by trapping sweat volatiles during a 6 hour period on the Tenax containing glass tube. Headspaces collected during a shorter period

of time did not evoke a detectable EAG response. Each sweat sample was divided in two portions of 2.7 ml each, after which volatiles from both portions successively were trapped for 3 hours on the same Tenax tube. The second portion was kept in the freezer (- 5 °C) until it was used. In this way the period of bacterial growth within the sweat sample was limited to 3 hours thereby preventing 'aging' of the sweat sample. Volatiles adsorbed on Tenax were eluted with 500 µl pentane/ether (2:1). Most of this eluate was used for EAG recordings. The remaining part (70 µl) was evaporated with nitrogen to 1.5 µl and used for GC-MS analyses (injection mode). The pH of the sweat samples was measured before and after collection.

For GC-MS analyses employing thermal desorption, volatiles from ± 2.7 ml sweat were trapped for 3 hours on 90 mg Tenax-TA packed in a Pyrex glass tube (160 x 6 mm OD) which was connected to the outlet of the flask through 4-mm-ID Teflon tubing. After collection, the adsorption tube was closed with 1/4 inch brass Swagelok caps and kept in a dark, dry place at room temperature prior to analysis.

Electrophysiology

Female mosquitoes were lured by a human hand, caught in an aspirator, cooled in a freezer (- 5 °C) for ± 1.5 min., and mounted on a small perspex holder (Lacher, 1971). Wings were glued with Perfax glue (Henkel, The Netherlands). Legs were removed, and one of the antennae was carefully fastened with the proximal segments to double-sided sticky tape (Sellotape, H.P.N., The Netherlands), after which the tip of the distal segment of the antenna was removed. The other antenna was fastened over the entire length to the double- sided sticky tape to enhance the stability of the preparation. Electroantennograms were recorded with AgCl-coated Ag-electrodes. Glass electrodes were filled with a saline solution containing 9 g NaCl, 2 g KCl, 1.34 g $CaCl_2.2H_2O$ and 5 g PVP per liter H_2O. The tip of the glass electrodes was removed and the recording electrode was placed over the cut end of the antenna. The indifferent electrode was inserted into the eye of the mosquito. A moistened, charcoal filtered, continuous airstream (14.5 ml/ sec) was led through a glass tube (i.d. 1 cm) ending 0.5 cm from the preparation holder. Stimuli were prepared by applying 25 µl of the sweat, its headspace or a dissolved compound on a 2.4 cm^2 filterpaper (Schleicher & Schuell, Dassel, Germany). The solution on the filterpaper was allowed to evaporate for 10 sec, which in case of diethyl ether and pentane/ether (2:1) resulted in the removal of the solvent. Sweat samples still contained water after 10 sec. Filterpapers were placed in a 150 mm glass Pasteur pipette that was sealed with

parafilm at both ends. Stimulus puffs (volume 1.6 ml) lasted for 0.5 sec and were injected into the airstream, at a location 10 cm from the outlet of the tube, by using a stimulus controller (Syntech, Hilversum, The Netherlands).

Signals were amplified 10x and directly imported via an IDAC interface box and A/D converter (Syntech, Hilversum, The Netherlands) into an Intel® Pentium-based personal computer. Recordings were analyzed by means of EAG software version 2.6 (Syntech, Hilversum, The Netherlands).

To normalize the electroantennogram responses, EAG values were corrected for the response to the control, which in case of the sweat samples was the same amount of water. For the headspace samples and the chemicals respectively pentane/ether (2:1) and diethylether were used. Corrected values were expressed as percent response to the standard 1-octen-3-ol, which was a 1% solution (25 µl) in diethylether (v/v). Each preparation was stimulated with the standard to allow comparison of relative effectiveness of a stimulus among individuals. EAG responses did not decrease during the life-span of a preparation, most likely because living mosquitoes were used. Nevertheless, for the EAG experiments performed in response to the identified components, the standard was applied after every two stimulations.

Headspace analysis

Thermodesorption mode. Collected headspace volatiles were released from the absorbents by heating in a Thermodesorption Cold Trap Unit (Chrompack 16200) at 250 °C for 10 min by flushing with a He flow of 10 ml/min and cryofocussed in a cold trap at –90 °C. Flash heating of the trap (220 °C) resulted in an injection of the volatiles on the column (Supelcowax 10, 60 m x 0.25 mm, film thickness 0.25 µm). A temperature program was used starting with 40 °C for 4 min, after which the temperature was raised by 4 °C/min till 270 °C (4 min). The linear velocity of the He gas was 25 cm/ sec at 40 °C. The column was directly coupled to the ion source of the mass spectrometer (Finnigan MAT 95) which operated in the 70 eV EI ionization mode. Scanning was performed from mass 24 to 400 at 0.7 sec/ dec. Thermodesorption was used for pool A headspace samples and individual headspace samples from 2 individuals who had contributed to the pool B samples. Chemical analyses of pool B headspace samples were performed using the injection mode. Identification of compounds was performed by comparing mass spectra with those in the Mass Spectral Databases and by checking retention indices with literature values (where available). Identified compounds in

headspace samples of the freshly collected and incubated sweat were highly comparable for the different sweat sources analyzed. An exception were the ethyl esters, which were only found in the headspace collections of the fresh sweat samples of pool A.

Chemicals

Indole, 1-dodecanol and 6-methyl-5-hepten-2-one were obtained from Sigma. Geranyl acetone was purchased from Fluka and 1-octen-3-ol from Merck. The first four chemicals were more than 99% pure; 1-octen-3-ol was more than 97% pure. All compounds were dissolved in diethyl ether (>99.5% pure). A dose of 25 µl of a 1% and 10% solution of indole (w/v), 1-dodecanol (w/v), geranyl acetone (v/v) and 6-methyl-5-hepten-2-one (v/v) was used.

Results

Behavioural responses to fresh and incubated sweat

The trapping devices baited with incubated sweat samples of both pool A and B, caught a significantly larger number of mosquitoes than the control trap (Table 1). The catches of the two fresh samples were not significantly different from the control. The total number of mosquitoes caught in the stimulus port in six replicates were compared with the control using Chi-squared tests.

EAG recordings from fresh and incubated sweat

Electroantennogram responses to the different sweat samples were corrected for the response to the control and compared. Mean responses to the incubated sweat samples of pool A (- 0.24 mV $\pm$ 0.02; N = 35) as well as of pool B (- 0.21 mV $\pm$ 0.03; N = 11) were significantly larger (pool A: $P < 0.001$; pool B: $P < 0.01$; Wilcoxon's matched pair signed rank test) than those obtained from the fresh sweat samples of pool A (0.00 mV $\pm$ 0.01; N = 35) and B (0.01 mV $\pm$ 0.02; N = 11). No significant differences were observed between the incubated sweat samples from pool A and B ($P = 0.2$; Mann-Whitney U test). The mean pH value for the fresh and incubated sweat from pool A was 5 and 8 respectively and for the fresh and incubated sweat samples from pool B these values were 7 and 9. Mean normalized EAG values corrected for their response to the control and expressed as

percent response to the standard 1-octen-3-ol are shown in Fig.1.

Table 1. Data from the dual-port olfactometer experiments with sweat samples collected from 14 volunteers (Pool A) and three volunteers (Pool B). Shown are the numbers of *Anopheles gambiae s.s.* caught in trapping devices baited with fresh sweat samples against the control (an equivalent amount of distilled water) and incubated sweat samples against the control. The results of the Chi-squared test are indicated.

Sweat	**Pool A**		**Pool B**	
Test	Fresh vs Control	Incubated vs Control	Fresh vs Control	Incubated vs Control
1	5 : 8	11 : 0	1 : 2	7 : 0
2	4 : 5	15 : 4	3 : 3	10 : 2
3	8 : 2	20 : 3	4 : 1	13 : 3
4	0 : 7	8 : 6	1 : 2	3 : 0
5	4 : 3	16 : 1	4 : 4	5 : 1
6	3 : 7	15 : 2	8 : 7	5 : 2
	24 : 32	85 : 16	21 : 19	43 : 8
X^2	ns	*	ns	*

ns: not significant ($P > 0.05$)
* $p < 0.05$,

EAG recordings from fresh and incubated headspace collections

The first 400 µl pentane/ether eluate of a headspace collection from pool B incubated sweat samples gave a stronger response (- 0.16 mV ± - 0.12; N = 5) than the eluate of a headspace collection from pool B fresh sweat samples (0.00 mV ± 0.00; N = 2). The second eluate (700 µl) from the same pool B incubated headspace collections failed to produce an EAG response (- 0.02 mV ± - 0.02; N = 5). Incubated sweat samples used to trap volatiles were tested after collection of the volatiles in order to test the remaining activity of the sweat. Mean EAG-values in response to pool B incubated sweat samples from which during a 3 hour period a headspace collection had been obtained (- 0.23 ± - 0.15; N = 7) showed that the samples retained their activity.

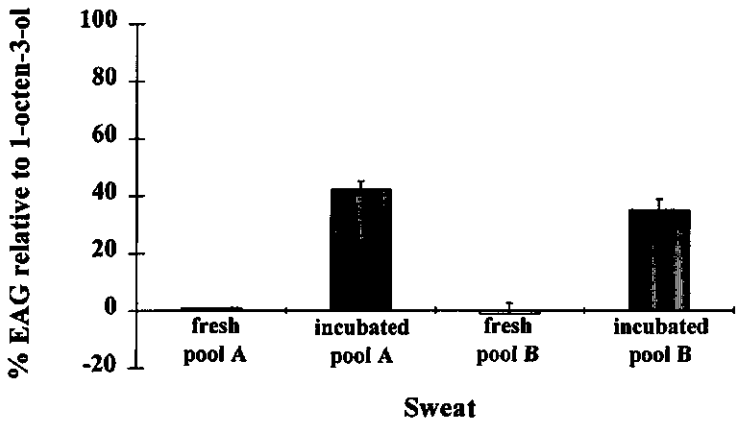

Fig. 1. Normalised mean EAGs expressed as percent response to the standard 1-octen-3-ol (25 µl of 1% solution in diethylether (v/v)) from *An. gambiae* females in response to fresh and 42-52 hours incubated sweat samples from a pooled sample of 14 (pool A) and a pooled sample of 3 (pool B) volunteers respectively. Markers above and below bars represent ± SEM.

Chemical analysis of headspace volatiles

The composition of the headspaces of fresh and incubated sweat and the percentage of the total peak area for the identified volatiles are shown in Table II.

Headspace analysis of the fresh samples revealed the presence (relative amount > 1.75 %) of ethanol, 3-hydroxy-2-butanone, 6-methyl-5-hepten-2-one, nonanal, decanal, geranyl acetone, acetic acid, propanoic acid, 2-ethylhexanoic acid and the esters ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl 4-methyldecanoate, ethyl dodecanoate, ethyl 4-methyldodecanoate, ethyl 9-tetradecenoate and ethyl 9-hexadecenoate. Additional ethyl esters were present in minor or trace amounts. The three most abundant compounds were ethanol (15.1%), acetic acid (10.9%) and 3-hydroxy-2-butanone (9.5%).

The headspace analysis of the incubated pooled sweat samples comprised (relative amount > 1.75%) ethanol, 3-methyl-1-butanol, 6-methyl-5-hepten-2-one, acetic acid, 6-methyl-3,5-heptadien-2-one, 1-nonanol, geranyl acetone, 1-dodecanol, 1-tetradecanol, 1-hexadecanol and indole. The relative amounts of ethanol (3.6%), acetic

acid (3.1%) and 3-hydroxy-2-butanone (1.2%) were strongly reduced when compared with the fresh samples, while propanoic acid, 2-ethylhexanoic acid and the esters were absent in the incubated headspace samples. 6-Methyl-5-hepten-2-one (1.9%) comprised the same relative amount in the fresh as well as the incubated headspace collections, while geranyl acetone (6%) and 6-methyl-3,5-heptadien-2-one (2.5%) were found in higher amounts. Indole, 1-dodecanol and 3-methyl-1-butanol constituted 27.9%, 22.4% and 10% of the total amount of compounds respectively. These were present in only minor or trace amounts in the headspace collections from fresh sweat.

EAG responses to sweat-borne compounds

Volatiles tested in electrophysiological experiments were selected based on their increased relative amounts in the incubated headspace collections when compared to the fresh collections. In addition, abundant compounds (relative amount > 2.5 %) which were present in both the incubated and the fresh headspaces were tested as well. Selected compounds were indole, 1-dodecanol, 3-methyl-1-butanol, geranyl acetone and 6-methyl-3,5-heptadien-2-one. An exception was made for 6-methyl-5-hepten-2-one; in addition to the fresh and incubated headspaces (relative amount respectively 1.8 % and 1.9 %) this volatile was also observed in headspace collections from human feet (data not shown). Therefore it was decided to test this compound as well. Major components in the fresh headspace samples, like ethanol, acetic acid and 3-hydroxy-2-butanone, which showed a large quantitative decrease in the incubated headspace collections were not included in this bioassay. Unfortunately, 6-methyl-3,5-heptadien-2-one was not commercially available. The responses to 3-methyl-1-butanol are discussed in another manuscript (Meijerink et al., submitted).

Fig. 2 shows the mean normalized EAG response expressed as percent response to the standard 1-octen-3-ol of 11 *An. gambiae* females to a dose of 25 µl of a 1% and 10% solution of indole, 1-dodecanol, geranyl acetone and 6-methyl-5-hepten-2-one. Except for 1-dodecanol, EAG-amplitudes in response to these, were all significantly different from the control for both concentrations tested ($P < 0.01$; Wilcoxon's matched-pair signed rank test).

Table 2 GC-MS analyses from headspaces from fresh and incubated sweat samples from 14 volunteers

No.	Compound	Percentage [a]	
		fresh	incubated
1	acetone	0.5	
2	3-ethyl-2,2-dimethyloxirane	0.2	
3	2-propanol	0.5	0.3
4	ethanol	15.1	3.6
5	ethyl butanoate	0.3	
6	ethyl 2-methylbutanoate	0.1	
7	hexanal	0.3	
8	ethyl 3-methylbutanoate	0.4	
9	4-methyl-3-penten-2-one	0.2	
10	1-methoxy-2-propanol	0.3	0.2
11	1-butanol	0.8	0.5
12	unknown m/z 41,55,69(100%),109,123,152M	0.2	
13	heptanal	~0.2	
14	ethyl isohexanoate [t, oi]	0.1	
15	4-methyl-4-penten-2-ol [t]	0.2	0.4
16	pyridine	0.1	0.5
17	2-methyl-1-butanol		~0.4
18	3-methyl-1-butanol		10.0
19	1-methoxy-2-propyl acetate	0.1	
20	ethyl hexanoate	2.4	
21	1-pentanol	0.2	0.4
22	octanal	0.5	
23	3-hydroxy-2-butanone	9.5	1.2
24	ethyl 4-methylhexanoate	0.5	
25	cyclohexanone	0.2	
26	3-methyl-2-buten-1-ol [oi]		0.5
27	ethyl heptanoate	0.8	
28	6-methyl-5-hepten-2-one	1.8	1.9
29	1-hexanol		0.7
30	ethyl 4-methylheptanoate [t, oi]	0.2	
31	2-nonanone		0.2
32	nonanal	2.4	0.6
33	ethyl octanoate	3.4	
34	linalooloxide B		0.6
35	1-heptanol		0.6
36	6-methyl-5-hepten-2-ol		0.6
37	acetic acid	10.9	3.1
38	linalooloxide A		0.4
39	furfural	0.3	
40	ethyl 4-methyloctanoate	0.3	

41	decanal	3.9	0.6
42	3,5,5-trimethyl-1-hexanol [t]		0.8
43	ethyl nonanoate	1.5	
44	1-octanol		1.0
45	propanoic acid	2.2	
46	6-methyl-3,5-heptadiene-2-one [t]	1.0	2.5
47	benzonitrile		0.6
48	butanoic acid	1.0	~0.8
49	ethyl decanoate	2.0	
50	menthol	0.6	
51	1-nonanol		1.8
52	furfuryl alcohol		0.4
53	3-methylbutanoic acid		~0.4
54	ethyl 4-methyldecanoate	1.8	
55	dec-9-enoic acid, ethyl ester [t]	0.9	
56	ester C13.H26.O2	0.4	
57	ester C13.H26.O2	0.7	
58	ethyl undecanoate	0.4	
59	1-decanol		1.7
60	ethyl 4-methylundecanoate	0.9	
61	ethyl isododecanoate	1.2	
62	ethyl dodecanoate	4.2	
63	hexanoic acid	1.0	
64	geranyl acetone	5.0	6.0
65	benzyl alcohol		1.1
66	nicotine		0.9
67	ethyl 4-methyldodecanoate [oi]	1.8	
68	phenylethanol		0.6
69	ethyl isotridecanoate [oi]	1.6	
70	ethyl isotridecanoate [oi]	0.7	
71	2-ethylhexanoic acid	2.0	
72	1-dodecanol	1.3	22.4
73	ethyl isotetradecanoate [oi]	0.8	
74	ethyl tetradecanoate	1.0	
75	unknown m/z 88(100%),102,127,129	1.4	
76	ethyl 9-tetradecenoate	3.1	
77	ethyl pentadecanoate	0.7	
78	1-tetradecanol	0.9	2.0
79	ethyl 9-hexadecenoate	2.0	
80	1-hexadecanol	0.7	1.8
81	indole		27.9

[a] Percentages are calculated by dividing the peak area of a compound by the total area of compounds identified in the headspace of a pooled fresh sweat sample or a pooled incubated sweat sample of 14 volunteers.

Abbreviations: [t] = tentative identification; [oi] = or isomer

Discussion

The behavioural bioassay showed that sweat samples incubated for 2 days were attractive to *An. gambiae* females in contrast to the fresh pooled sweat samples which showed no significant attractiveness. In addition to Braks and Takken (1999) who found that sweat obtained from two out of three individuals became attractive after an incubation period of two days, we found that pooled sweat samples from a group of 14 as well as from a group of 3 volunteers gave a similar degree of attractiveness after incubation. The observed change in pH, the abundant growth of microflora after incubation (Braks et al., in prep.) and the fact that the sweat only elicits a behavioural and olfactory response after incubation, strongly indicates that attractants are produced by microbial activity. Field experiments conducted by Haddow (1942) showed that unwashed children are more attractive to *An. gambiae* than washed children and corroborate indications that the skin microflora might play a role in the production of kairomones.

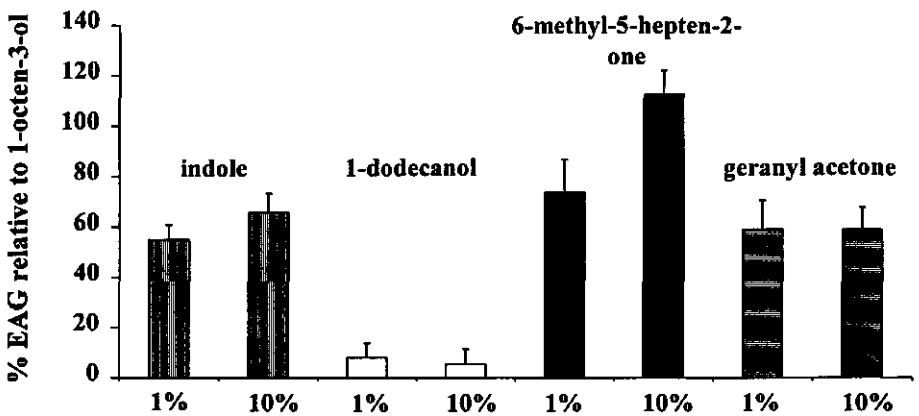

Fig. 2. Normalized mean EAGs expressed as percent response to the standard 1-octen-3-ol (25 µl of 1% solution in diethylether (v/v)) from intact *An. gambiae* females in response to a dose of 25 µl of a 1% and 10% solution in diethylether of the identified sweat-borne compounds: indole (w/v), 1-dodecanol (w/v), 6-methyl-5-hepten-2-one (v/v) and geranyl acetone (v/v). Markers above and below bars represent ± SEM.

During exercise, volunteers collected droplets from the skin of the forehead which most likely comprised a mixture of sebum- and eccrine gland products. The eccrine gland which is involved in thermoregulation is activated by heat stimulation or vigorous excercise thereby excreting mainly water (99%), urea and lactate. The sebaceous gland produces sebum, which is equally distributed over the skin surface during muscular activity; it has a major excretory area on the forehead (Folk and Semken, 1991; Albone, 1984). Sebum excretions comprise a broad range of carboxylic acids (esterified or free), wax esters, sterol esters, and mono-, di- and triacylglycerides (Nicolaides, 1974). Because wax esters, sterol esters, and glycerides consist of long carbon chains they are less volatile and therefore not expected to be detected in headspace collections. Methyl and ethyl esters, however, have a higher vapor pressure than carboxylic acids with comparable carbon chain lengths, explaining why a broad range of esters was detected while only some of the short chain carboxylic acids were present. Ethyl esters were solely found in the headspace collections of the fresh sweat samples of pool A and not in the headspace samples of individuals who had contributed to pool B samples. Fatty acid ethyl esters have recently been mentioned as markers for alcohol intake and can be detected in the serum up to 24 hours after alcohol consumption (Laposata, 1997). Fatty acid ethyl esters are formed by esterification of ethanol with fatty acids. Wine itself contains ethyl esters as well (Ferreira et al., 1995), but orally ingested ethyl esters are degraded in the gastrointestinal tract (Laposata, 1997) and are therefore not expected to be found in the serum. Our volunteers followed no diet or had no consumption restrictions and the broad range of ethyl esters found in the fresh sweat samples might therefore be the result of the consumption of alcohol one day prior to the sweat collections. No ethyl esters were observed in the headspace collections of the incubated sweat which is likely due to the esterase activity of the skin microbes present in the sweat (Holland, 1993).

Regarding the carboxylic acids, acetic acid has been found as one of the most abundant compounds in fresh headspace collections which is consistent with other sweat analyses (Perry et al., 1970; Cork and Park, 1996). Carboxylic acids have recently been studied on the behavioural-, electroantennogram- and single cell level (Knols et al., 1997; Meijerink and Van Loon, 1999). Acetoin or 3-hydroxy-2-butanone, another abundant volatile present in headspace collections of fresh sweat, has been shown to be a constituent of human breath (Preti et al., 1992). Preliminary studies with acetoin, however, did not reveal any EAG activity for *An. gambiae* (unpublished data).

In order to identify volatiles in the way they are perceived by the mosquitoe's olfactory receptors, we tried to reveal the identity and chemical composition of the

volatiles present in the headspace of a behaviourally attractive natural source. A recently published chemical study, performed to elucidate the identity of attractants for the mosquito species *Aedes aegypti*, used glass beads as a medium to collect human hand palm emanations (Bernier et al., 1999). Our approach was to focus on the volatile components produced by both the eccrine and sebaceous glands and to avoid the collection of non-volatile skin components. Therefore it was decided to use sweat as a natural source. Interestingly, behavioural studies with *Ae. aegypti* showed that these mosquitoes are repelled by incubated sweat, while fresh sweat attracts them (Müller, 1968), indicating that *Ae. aegypti* might use different olfactory cues to locate its human host than *An. gambiae*.

No EAG reactions were observed in response to the fresh sweat samples, indicating that the nature or quantity of volatiles emanating from freshly collected sweat is not adequate to produce measurable EAG-responses. However, volatiles within the fresh sweat might act as synergists on the behavioural level with compounds produced during incubation. Therefore, it was decided to test not only the compounds which were solely present in the incubated sweat, but also compounds which were abundant in the incubated as well as the fresh sweat.

The analysis and subsequent comparison of the compounds present in the headspace of the incubated and fresh sweat revealed several interesting components. Indole was solely present in the headspace of the incubated sweat samples and elicited an EAG response for both concentrations tested. Indole has been identified from fermented Bermuda grass infusion and serves within a blend of four other compounds as an oviposition semiochemical for *Culex quinquefasciatus* (Millar et al., 1992). For several other Diptera indole has been identified as a compound with potential kairomonal activity or has been shown to act as an attractant in behavioural or field bioassays (Mulla et al., 1977; Mulla and Ridsdill-Smith, 1986; Cork, 1994; Cossé et al., 1995; Cossé and Baker, 1996). For instance the Australian bush fly *Musca vetustissima*, and the house fly *Musca domestica* are attracted to complex odourant mixtures which comprise indole (Mulla et al., 1977; Mulla and Ridsdill-Smith, 1986; Cossé and Baker, 1996). In this study geranyl acetone, indole and 6-methyl-5-hepten-2-one were identified as olfactory stimulants for *An. gambiae*. All of these components seem to act as semiochemicals for several other insect species (Andersen and Metcalf, 1986; Hammack 1996; Keegans et al., 1993; Micha et al., 1993; Takács et al., 1997).

No EAG response was elicited by 1-dodecanol, although it has been consistently found in all headspace analyses of the incubated sweat samples, and not or in minor

amounts in the headspace analyses of the fresh sweat samples.

This study attempted to reveal the chemical identity of potential attractants for the malaria mosquito *An. gambiae* contained in the complex mixture of human sweat. By means of comparing headspace samples of behaviourally attractive and non-attractive pooled sweat samples, potentially interesting compounds were identified. Whether the identified compounds act as attractants is presently studied in windtunnel- as well as in field bioassays. Furthermore, single cell recordings have been conducted to reveal the sensillar types sensitive to the incubated sweat and to the identified compounds, indole, geranyl acetone and 6-methyl-5-hepten-2-one.

Compounds showing kairomonal activity in a field situation can be applied in studies of the population dynamics and used in mosquito density monitoring programs thereby serving as an alternative for human biting catches. Eventually application of an optimized bait might lead to a reduction in malaria mosquito populations which in combination with other malaria control measures might contribute to malaria control

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References

Albone ES (1984) Mammalian semiochemistry: the investigation of chemical signals between mammals. Wiley, Chichester, 360 pp.

Andersen JF and Metcalf RL (1986) Identification of a volatile attractant for *Diabrotica* and *Acalymma* spp. from blossoms of *Cucurbita maxima* Duchesne. *J. Chem. Ecol.* 12: 687-699.

Bernier UR, Booth MM and Yost RA (1999) Analyses 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. *Anal. Chem.* 71:

1-7.

Braks MAH, Cork A and Takken W (1997) Olfactometer studies on the attraction of *Anopheles gambiae sensu stricto* (Diptera: Culicidae) to human sweat. *Proc. Exp. Appl. Entomol. N.E.V.* Amsterdam 8: 99-104.

Braks MAH and Takken W (1999) Incubated human sweat but not fresh sweat attracts the malaria mosquito *Anopheles gambiae sensu stricto*. *J. Chem. Ecol.* 25: 663-672.

Brown AWA, Sarkaria DS and Thompson RP (1952) Studies of the responses of the female *Aedes* mosquito. Part I.-The search for attractant vapours. *Bull. Entomol. Res.* 42: 105-114.

Cork A (1994) Identification of electrophysiologically-active compounds for New World screwworm, *Cochliomyia hominivorax*, in larval wound fluid. *Med. Vet. Entomol.* 8: 151-159.

Cork A and Park KC (1996) Identification of electrophysiologically-active compounds for the malaria mosquito, *Anopheles gambiae*, in human sweat extracts. *Med. Vet. Entomol.* 10: 269-276.

Cossé AA and Baker TC (1996) House flies and pig manure volatiles: wind tunnel behavioral studies and electrophysiological evaluations. *J. Agric. Entomol.* 13: 301-317.

Cossé AA, Todd JL, Millar JG, Martínez, LA and Baker TC (1995) Electroantennographic and coupled gas chromatographic-electroantennographic responses of the Mediterranean fruit fly, *Ceratitis capitata*, to male-produced volatiles and mango odor. *J. Chem. Ecol.* 21: 1823-1836.

Costantini C, Gibson G, Sagnon N, Della Torre A, Brady J and Coluzzi M (1996) Mosquito responses to carbon dioxide in a West African Sudan savanna village. *Med. Vet. Entomol.* 10: 220-227.

De Jong R and Knols BGJ (1995) Olfactory responses of host-seeking *Anopheles gambiae* s.s. Giles (Diptera: Culicidae). *Acta Trop.* 59: 333-335.

Eiras AE and Jepson PC (1991) Host location by *Aedes aegypti* (Diptera: Culicidae): a wind tunnel study of chemical cues. *Bull. Entomol. Res.* 81: 151-160.

Eiras AE and Jepson PC (1994) Responses of female *Aedes aegypti* (Diptera: Culicidae) to host odors and convection currents using an olfactometer bioassay. *Bull. Entomol. Res.* 84: 207-211.

Ferreira V, Fernández P, Peña C, Escudero A and Cacho JF (1995) Investigation on the role played by fermentation esters in the aroma of young spanish wines by

multivariate analysis. *J. Sci. Food Agric.* 67: 381-392.

Folk GE Jr. and Semken HA Jr. (1991) The evolution of sweat glands. *Int. J. Biometeorol.* 35: 180-186.

Gillies MT (1980) The role of carbon dioxide in host-finding by mosquitoes (Diptera: Culicidae): a review. *Bull. Entomol. Res.* 70: 525-532.

Haddow AJ (1942) The mosquito fauna and climate of native huts at Kisumu, Kenya. *Bull. Entomol. Res.*33: 91-142.

Hammack L (1996) Corn volatiles as attractants for northern and western corn rootworm beetles (Coleoptera: Chrysomelidae: *Diabrotica* Spp.). *J. Chem. Ecol.* 22: 1237-1253.

Holland KT (1993) Nutrition of cutaneous resident microorganisms. In: *The skin microflora and microbial skin disease.* Noble WC, pp. 33-72.

Keegans SJ, Billen J, Morgan ED and Gökcen OA (1993) Volatile glandular secretions of three species of new world army ants, *Eciton burchelli*, *Labidus coecus*, and *Labidus praedator*. *J. Chem. Ecol.* 19: 2705-2719.

Knols BGJ and Meijerink J (1997) Odors influence mosquito behavior. *Science Med.*. 4: 56-63.

Knols BGJ, Van Loon JJA, Cork A, Robinson RD, Adam W, Meijerink J, de Jong R and Takken W (1997) Behavioral and electrophysiological responses of the female malaria mosquito *Anopheles gambiae* (Diptera: Culicidae) to Limburger cheese volatiles. *Bull. Entomol. Res.* 87: 151-159.

Lacher V (1971) Electrophysiological equipment for measuring the activity of single olfactory nerve cells on the antennae of mosquitoes. *J. Econ. Entomol.* 64: 313-314.

Laposata M (1997) Fatty acid ethyl esters: short-term and long-term serum markers of ethanol intake. *Clin. Chem.* 43: 1527-1534.

Mboera LEG, Knols BGJ, Takken W and della Torre A (1997) The response of Anopheles gambiae s.l. and An. funestus (Diptera: Culicidae) to tents baited with human odour or carbon dioxide in south-east Tanzania. *Bull. Entomol. Res.* 87: 173-178.

Meijerink J, Braks MAH and Van Loon JJA Olfactory receptors on the antennae of the malaria mosquito *Anopheles gambiae* are sensitive to ammonia and other sweat-borne components. Submitted to *J. Comp. Physiol.*

Meijerink J and Van Loon JJA (1999) Sensitivities of antennal olfactory neurons of the malaria mosquito, *Anopheles gambiae*, to carboxylic acids. *J. Insect Physiol.* 45:

365-373.

Micha SG, Stammel J and Höller C (1993) 6-Methyl-5-hepten-2-one, a putative sex and spacing pheromone of the aphid hyperparasitoid, *Alloxysta victrix* (Hymenoptera: Alloxystidae). *Eur. J. Entomol.* 90: 439-442.

Millar JC, Chaney JD and Mulla MS (1992) Identification of oviposition attractants for *Culex quinquefasciatus* from fermented Bermuda grass infusions. *J. Am. Mosq. Control Assoc.* 8: 11-17.

Mulla MS, Hwang Y -S and Axelrod H (1977) Attractants for synanthropic flies: chemical attractants for domestic flies. *J. Econ. Entomol.* 70:644-648.

Mulla MS and Ridsdill-Smith JT (1986) Chemical attractants tested against the Australian bush fly *Musca vetustissima* (Diptera: Muscidae). *J. Chem. Ecol.* 12: 261-270.

Müller W (1968) Die Distanz- und Kontakt-Orientierung der Stechmücken (*Aedes aegypti*) (Wirtsfindung, Stechverhalten und Blutmahlzeit). *Z. Vgl. Physiol.* 58: 241-303.

Nicolaides N (1974) Skin Lipids: Their Biochemical Uniqueness. *Science* 186: 19-26.

Noble WC and Somerville DA (1974) *Microbiology of human skin.* W. B. Saunders Company Ltd. London.

Parker AH (1949) Stimuli involved in the attraction of *Aëdes aegypti* I. to man. *Bull. Entomol. Res.* 39: 387-397.

Perry T L, Hansen S, Diamond S, Bullis B, Mok C and Melancon SB (1970) Volatile fatty acids in normal human physiological fluids. *Clin. Chim. Acta* 29: 369-374.

Preti G, Clark L, Cowart BJ, Feldman RS, Lowry LD, Weber E and Young IM (1992) Non-oral etiologies of oral malodor and altered chemosensation. *J. Periodontol.* 63: 790-796.

Price GD, Smith N and Carlson DA (1979) The attraction of female mosquitoes (*Anopheles quadrimaculatus* Say) to stored human emanations in conjunction with adjusted levels of relative humidity, temperature, and carbon dioxide. *J. Chem. Ecol.* 5: 383-395.

Takács S, Gries G and Gries R (1997) Semiochemical-mediated location of host habitat by *Apanteles carpatus* (say) (Hymenoptera: Braconidae), a parasitoid of clothes moth larvae. *J. Chem. Ecol.* 23: 459-472.

Takken W (1991) The role of olfaction in host-seeking of mosquitoes: a review. *Insect Sci. Applic.* 12: 287-295.

Takken W, Dekker T and Wijnholds YG (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). *J. Insect Behav.* 10: 395-407.

Thompson RP and Brown AWA (1955) The attractiveness of human sweat to mosquitoes and the role of carbon dioxide. *Mosq. News* 15: 80-84.

Willis RE (1947) The olfactory responses of female mosquitoes. *J. Econ. Entomol.* 40: 769-778.

Chapter 6

Sensitivity of olfactory receptor neurons of the malaria mosquito *Anopheles gambiae* to ammonia and other sweat-borne components.

This chapter has been submitted as: Meijerink J, Braks M.A.H and Van Loon J.J.A. Olfactory receptors on the antennae of the malaria mosquito *Anopheles gambiae* are sensitive to ammonia and other sweat-borne components. *Journal of Comparative Physiology.*

Abstract

Electrophysiological studies on female *An. gambiae* s.s. mosquitoes revealed a receptor neuron within a subpopulation of the antennal grooved-peg sensilla sensitive to incubated sweat, that did not respond to fresh sweat. This receptor neuron was sensitive to ammonia as well, a component which attracted female *An. gambiae* in a windtunnel bioassay. Neurons innervating a different subpopulation of grooved-peg sensilla did not show a response to incubated sweat. In the latter sensilla however, one type of neuron responded to water or water containing solutions, while an other receptor neuron was inhibited when stimulated with dry air, ether or ethanol. Neurons innervating sensilla trichodea, a more abundant antennal type of olfactory sensillum, did not respond to fresh nor to incubated sweat. However, receptor neurons within the sensilla trichodea responded with excitation to several sweat-borne components. A subpopulation of the sensilla trichodea was found to be innervated by neurons sensitive to geranyl acetone. A second subpopulation housed receptor neurons sensitive to indole. 3-Methyl-1-butanol and 6-methyl-5-hepten-2-one evoked excitation of receptor neurons within both subpopulations of sensilla trichodea. Neurons were most sensitive to indole and geranyl acetone with a threshold of 0.01% and higher. Thresholds to 3-methyl-1-butanol were around 0.1% while 6-methyl-5-hepten-2-one only elicited responses when stimulated with concentrations of at least 1%. These findings are discussed in the context of host-seeking behaviour.

Introduction

The female mosquito *Anopheles gambiae* Giles *sensu stricto* transmits malaria parasites during bloodfeeding. This nocturnally active mosquito is thought to be guided to its host predominantly by olfactory cues (Takken and Knols, 1999). Odours are perceived by olfactory receptor neurons situated within sensilla on the antennae and palps. For *An. gambiae* six different types of antennal sensilla can be recognized of which the sensilla trichodea are the most abundant (McIver, 1982). The sensilla trichodea can be

morphologically classified in a number of subtypes, which are not easily distinguished in *An. gambiae* (McIver, 1982; Ismail, 1964). In *Aedes aegypti* and related anophelines, olfactory receptor neurons innervating grooved-peg sensilla and sensilla trichodea were shown to be sensitive to odours (Lacher, 1967; Davis, 1988; Davis and Bowen, 1994; Bowen, 1995; Pappenberger et al., 1996). In particular, lactic acid, the only attractant for *Aedes aegypti* known thus far, evokes responses from grooved peg-associated neurons (Davis and Sokolove, 1975; Davis, 1984; Davis, 1988). Receptor neurons sensitive to a human skin wash extract and the extract's behaviourally active chromatographic fractions, have been reported to innervate the grooved peg sensillum (Pappenberger et al., 1996).

The first single sensillum studies for *An. gambiae s.s.* reported neurons associated with (a) subtype(s) sensillum trichodeum which responded to aliphatic carboxylic acids (Meijerink and Van Loon, 1999; Van den Broek and Den Otter, 1999). Aliphatic carboxylic acids are volatile products of skin bacteria and a synthetic blend comprising short chain carboxylic acids was shown to be attractive for *An. gambiae* in a windtunnel bioassay (Knols et al., 1997).

Behavioural studies indicated that other compounds in addition to carboxylic acids might be involved in the odour-mediated host finding of *An. gambiae* (Braks et al., 1997). Sweat freshly collected from the human forehead, used as a natural source of volatiles, was not attractive to *An. gambiae*, while incubation of the sweat at body temperature resulted in an attractive mixture (Braks and Takken, 1999). Incubated sweat evoked electroantennogram responses in contrast to fresh sweat, which did not elicit any response (Meijerink et al., accepted). Components identified through GC-MS analysis of the headspace of the incubated sweat were tested for their sensory activity by electroantennographic recording (Meijerink et al., accepted). Three sweat-borne components, indole, geranyl acetone and 6-methyl-5-hepten-2-one gave significant EAG responses, indicating that they might serve as kairomones. Additional studies revealed that the incubated sweat contains large amounts of ammonia, which attracts female *An. gambiae* in a windtunnel bioassay (Braks et al., submitted).

This report describes the response characteristics of the antennal olfactory neurons of *An. gambiae* to human sweat and identified components from the fresh and incubated sweat. We focussed on the receptor neurons within the grooved-peg sensilla and sensilla trichodea to investigate detection of behaviourally active and potential host-emitted attractants. Therefore the receptor neurons were exposed to the natural sources of the volatiles, fresh and incubated sweat and 7 chemicals identified from these sources.

Materials and methods

Insects

Anopheles gambiae sensu stricto originated from Suakoko, Liberia (courtesy of Prof. M. Coluzzi, Rome). Mosquitoes were reared at 27 °C, 80% rh and 12:12 L:D photoperiod. Adults were held in gauze cages (30 x 30 x 30 cm). They had access to a 6% glucose solution and were given the opportunity to feed on a human arm twice a week. Eggs were laid on wet filter papers and transferred to water trays. Larvae were fed Tetramin® fishfood.

Electrophysiology

Preparation. Five to eight days old non-bloodfed female mosquitoes were lured by a human hand, sucked into an aspirator, cooled in a freezer (- 5 °C) for $\pm$ 1.5 min, and mounted on a small perspex holder (Lacher, 1971). Wings were glued to the holder surface with Perfax glue (Henkel, The Netherlands), legs were removed, and the antennae were carefully fastened to double-sided sticky tape. The preparation was viewed with an Olympus CK2 inverted microscope at 600x magnification.

Recording technique. During the experiments a sharpened tungsten electrode was used as the indifferent electrode, which was inserted into the thorax or eye of the mosquito. The recording electrode (tungsten wire of 0.2 mm diameter, Drijfhout, The Netherlands) was electrolytically sharpened (5 min at 10 V and 15 min at 3 V in a 8% solution of KNO_2 in H_2O). The electrode was carefully moved around the base of a sensillum until electrophysiological activity was recorded (Meijerink and Van Loon, 1999). Signals were amplified 1000x, recorded on a tape recorder (Racal recorder store7DS) or directly imported, via an IDAC interface box and A/D converter (Syntech, Hilversum, The Netherlands), into an Intel Pentium based personal computer.

Stimulus delivery. A moistened, charcoal filtered, continuous airstream (28.7 cm/sec) was led through a glass tube (i.d. 1 cm) ending 0.5 cm from the preparation holder. Stimulus puffs lasted for 0.5 sec and were injected into the airstream, at a location 10 cm from the outlet of the tube, by using a stimulus controller (Syntech, Hilversum, The Netherlands).

Stimuli

Indole, geranyl acetone, 3-methyl-1-butanol, 6-methyl-5-hepten-2-one, 1-dodecanol, adipic acid and ammonia were all identified in the headspace of the fresh and/or incubated sweat or in the incubated sweat liquid (Meijerink et al., accepted; Braks et al., submitted). Butylamine and pentylamine were identified in the headspace analysis of a human individual (Ellin et al., 1974). Indole, 1-dodecanol, adipic acid and 6-methyl-5-hepten-2-one were obtained from Sigma. Geranyl acetone and 3-methyl-1-butanol were purchased from Fluka and ammonia from Merck. Butylamine and pentylamine came from Aldrich. All chemicals used were more than 99% pure, except for ammonia. The latter consisted of a 25% aqueous solution, which was further diluted with distilled water. Pure compounds were dissolved in diethyl ether, except for butylamine, pentylamine and ammonia which were diluted in distilled water. For preparing the stimuli 25 µl of the sweat, dissolved compound, water (control) or diether (control) was applied to a 2.4 cm^2 filterpaper (Schleicher & Schuell, Dassel, Germany). The solution on the filterpaper was allowed to evaporate for 10 s, which in case of diethyl ether resulted in the removal of this solvent. Filterpapers were placed in a glass Pasteur pipette that was sealed with parafilm® at both ends. For the water or water containing stimuli, air inside the Pasteur pipette had a rh of $\pm$ 100%. The moistened continuous airstream had a rh of $\pm$ 73%.

Sweat collection

Sweat was collected from volunteers engaged in physical exercise by cycling on a hometrainer and took place in a humidified (70% rh) room at 30 °C. Fourteen Caucasian volunteers, nine males and five females with ages ranging from twenty-one to fifty-two were each asked to collect 3 ml sweat from the forehead. Volunteers had not used soap or any other cosmetic products 24 hours prior to the sweat collection. From each individual 1.5 ml sweat was directly placed in a freezer (-5 °C), while the other 1.5 ml was incubated for 42-52 hours under aerobic conditions at 37 °C and subsequently stored in a freezer. Both sweat samples will further be referred to as 'fresh sweat' and 'incubated

sweat' respectively. A pooled sweat sample (pool A) comprised of the individual sweat samples was obtained by pooling ± 1.2 ml sweat from each of the fourteen individuals; this was done both for the fresh and for the incubated sweat. A second sweat sample was obtained from a different collection. Fifteen Caucasian volunteers, eleven males and four females collected 3 ml sweat from the forehead under the formerly mentioned conditions. Sweat samples were treated as described above and pooled (pool B), again keeping the fresh and the incubated sweat separately.

The fresh and incubated sweat of pool A were used to stimulate the grooved-peg sensilla during the first set of experiments. During the experiments in which ammonia was applied as a pure compound, grooved-peg neurons were exposed to the fresh and incubated sweat of pool B. Both the incubated sweat of pool A and pool B have been shown to attract female *An. gambiae* mosquitoes in a windtunnel bioassay (Meijerink et al., accepted; Braks et al., submitted).

Sensilla

Grooved-peg sensilla. No morphological distinctions could be observed within the grooved-peg population. Most grooved-peg sensilla are situated on the distal segments, consequently single sensillum recordings were obtained from sensilla located at the distal segments 9-13.

Sensilla trichodea. Recordings were made from neurons innervating different subtypes of sensilla trichodea. Earlier studies with *An. gambiae* showed that neurons innervating this subtype(s) respond to carboxylic acids (Meijerink and Van Loon, 1999). Based on the classification for *An. stephensi* (Boo, 1980) sensilla trichodea subtypes recorded from resembled subtype C and E most. No recordings were made from subtype D, which is mainly distributed on the proximal antennal segments and is easily recognized by its round tip. Recordings were obtained from sensilla situated at the segments 8-13.

Data analyses

Grooved-peg. Separation of the different receptor neurons was accomplished by using Sapid Tools version 16 (Smith et al., 1990). Responses were expressed as the difference between the mean spontaneous spike frequency determined in the 3 sec before stimulation and the spike frequency during the first 300 ms after stimulus onset in

response to a 0.5 sec puff.

Sensilla trichodea. Recordings were analysed by means of Autospike™ software (Syntech, Hilversum, The Netherlands) and further analysed by Sapid Tools version 16 (Smith et al., 1990) in order to distinguish both cells based on their different waveform characteristics and amplitudes. Responses were expressed as the difference between the mean spontaneous spike frequency determined in the 3 sec before stimulation and the spike frequency during the first 300 ms after stimulus onset in response to a 0.5 sec puff. For the single dose experiments the magnitude of excitation was assigned to four sensitivity categories: 75%-100%, 50%-75%, 25%-50% and less than 25% of the maximum excitation observed in one of the two neurons innervating one sensillum, irrespective of the stimulus eliciting this maximal response.

Results

Grooved-peg sensilla

Fresh and incubated sweat. Sixteen grooved-peg sensilla of nine females were exposed to fresh and incubated sweat (pool A). Usually, the spontaneous spike activity of three or four receptor neurons innervating this sensillum could be distinguished. Nine grooved-peg sensilla housed a receptor neuron with a large spike amplitude, which responded with excitation to the incubated sweat. This receptor neuron did not respond to the fresh sweat. The spontaneous spike frequency of the large amplitude receptor neuron varied within the range of 6.7-27.7 impulses/ sec. The change in spike frequency upon stimulation varied between 53-97 impulses/ sec. Neurons innervating seven other grooved-peg sensilla never showed a response to the incubated sweat. In these 7 sensilla, however, the spike frequency of a large amplitude receptor neuron was increased in response to water or water-containing stimuli (100% rh). A receptor neuron within the same sensilla from which a smaller spike amplitude was recorded, was inhibited in response to ether, ethanol or dry air in contrast to the water sensitive receptor neuron which was not or only partly inhibited by the latter stimuli (Fig.1).

Fig 1. Recording of neuronal activity of receptor neurons innervating a grooved-peg sensillum. This sensillum belonged to a subpopulation of grooved-peg sensilla housing receptor neurons which responded to water and dry air. (A) shows excitation of a receptor neuron innervating a grooved peg sensillum in response to water (B) Inhibition of activity recorded from receptor neurons of a grooved peg sensillum in response to ether. The bars show the duration of the stimulus (500 ms)

Ammonia. Electrophysiological recordings of the grooved-peg sensilla revealed that the incubated sweat-sensitive neuron also responded to ammonia. Thirteen sensilla of nine females were exposed to incubated sweat, fresh sweat (pool B) and ammonia. Seven sensilla contained a neuron from which a large amplitude was recorded, which was excited upon stimulation with both incubated sweat and ammonia offered at concentrations of 0.25%-25%. Doses were within the range attracting *An. gambiae* in a windtunnel bioassay. The change in spike frequency of the large amplitude receptor in response to incubated sweat and different doses of ammonia is given in table 1. Cells innervating five different sensilla did not respond to the incubated sweat or ammonia, while one receptor neuron within a different sensillum was excited by a dose of 10% ammonia but not by the incubated sweat. Fig. 2 shows the responses of grooved-peg receptor neurons to incubated sweat and different doses of ammonia. Additionally, preliminary results showed that excitation was evoked by butylamine, while pentylamine mostly evoked inhibition and only occasionally excitation (n=1). Inhibition was also elicited by 3-methyl-1-butanol. Apart from the inhibition observed in response to ether-containing solutions, no responses of the ammonia-sensitive receptor neuron or any other grooved-peg receptor neuron was evoked by geranyl acetone, indole, adipic acid, 1-dodecanol or 6-methyl-5-hepten-2-one.

Table 1. Responses of grooved-peg neurons upon stimulation with different doses of ammonia and a standard dose of incubated sweat (25 µl).

Ammonia dose	**Response** [a]		**n**
	Ammonia	Standard Incubated sweat	
25%	18 ± 1	14 ± 1	3
10%	26 ± 2	17 ± 2	3
2.5% *	18	30	1
0.25%*	11		

[a] Change in spike frequency (mean ± SEM) during the first 300 ms after stimulus onset in response to a 0.5 puff of incubated sweat or ammonia.
n represents the number of grooved-peg sensilla recorded from.* both concentrations of ammonia were tested on the same individual

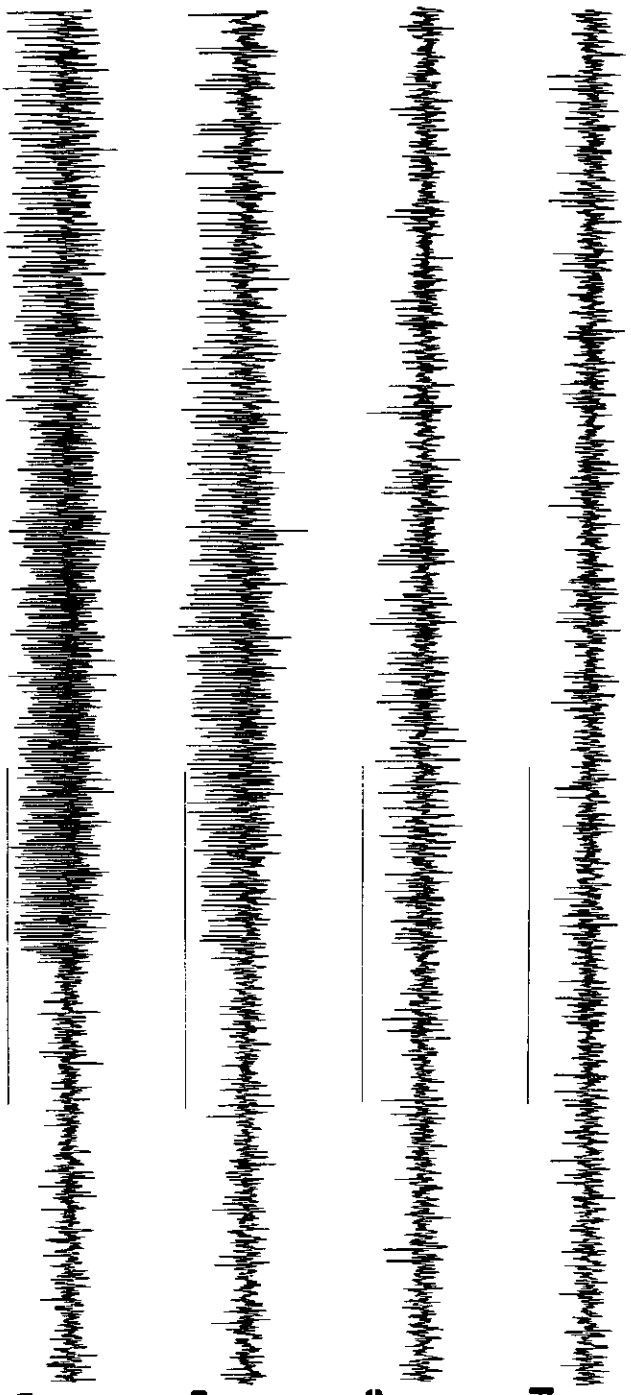

Fig 2. Recording of neuronal activity of receptor neurons innervating a grooved-peg sensillum. This sensillum belonged to a subpopulation of the grooved-peg sensilla housing receptor neurons, which responded to incubated sweat and ammonia. The figure shows excitation of a grooved-peg receptor neuron in response to (A) incubated sweat, (B) 2.5% ammonia and (C) 0.25% ammonia. (D) shows the neuronal activity of the same grooved-peg receptor neuron which is not responding to fresh sweat. The bars show the duration of the stimulus (500 ms).

Sensilla trichodea

Single dose experiments. In order to identify additional putative neurons sensitive to the sweat-borne compounds, single sensillum recordings were made from 21 sensilla trichodea. From these sensilla usually activity from two neurons with overlapping impulse amplitudes was recorded, which could be distinguished after analysis. Sensilla trichodea tested belonged to one or possibly two morphologically different subtypes of the sensilla trichodea population. In an earlier study neurons innervating this subtype(s) sensilla trichodea have been shown to respond to carboxylic acids (Meijerink and Van Loon, 1999).

Table 2 shows the response of olfactory neurons within nine sensilla trichodea in response to the sweat-borne components. These recordings were selected based on the quality of the signal-noise ratio and the length of the recording. Receptor neurons within the other 12 sensilla showed a similar response pattern. All components were tested at a dose of 10%, except for ammonia which was applied at 10% and 0.25%. Two functionally different subpopulations of sensilla trichodea were observed. Neurons innervating one subpopulation responded to geranyl acetone, 6-methyl-5-hepten-2-one and/or 3-methyl-1-butanol, while neurons innervating a different subpopulation of sensilla trichodea responded to indole, 6-methyl-5-hepten-2-one and/or 3-methyl-1-butanol. Occasionally in both sensillum subpopulations a small increase in spike frequency was observed in response to 10% ammonia; in one case incubated sweat elicited a weak excitation. No responses were observed to adipic acid, 1-dodecanol, 0.25% ammonia or fresh sweat.

Dose response. Thirteen sensilla trichodea of twelve females were exposed to geranyl acetone, 6-methyl-5-hepten-2-one, 3-methyl-1-butanol and indole in concentrations ranging from 0.001% to 1%. In most cases the spikes from two neurons were registered.

In six sensilla one receptor neuron was found which responded with excitation to geranyl acetone (Fig 3A). Maximum excitation was elicited by a dose of 0.1 %, while thresholds were observed for doses of 0.01% or occasionally in response to a dose of 0.001 %. Neurons within the same sensillum showed an increase in the spike frequency in response to 3-methyl-1-butanol at doses of 0.1% and higher (Fig 3B); in four out of six cases the geranyl acetone-excited receptor neuron responded to 3-methyl-1-butanol as well, while in the other two cases the other neuron responded. A lower sensitivity was observed to 6-methyl-5-hepten-2-one, which evoked responses at doses of 1% and higher (Fig 3C). In five out of six sensilla receptor neurons were excited when stimulated with

Table 2 Response spectra of neurons innervating sensilla trichodea in response to sweat borne compounds

Component [c]	Sensillum [a]	1		2	3		4		5		6		7		8		9
	Neuron [b]	A	B	A	A	B	A	B	A	B	A	B	A	B	A	B	A
6-Methyl-5-hepten-2-one		+	++	++	+	+++	++	0	+++	0	0	+++	+++	0	0	+++	+++
Geranyl acetone		++	++	+++	++	+	0	+	+++	0	0	0	0	0	0	0	0
Indole		0	0	0	0	0	0	0	0	0	0	+	++	++	+0	++	+++
Adipic acid		0	0	0	0	0	0	0	0	0	0	0	0	0	-	-	-
1-Dodecanol		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-
3-Methyl-1-butanol		0	+++	+++	0	+++	++	0	-	-	+	+0	-	-	-	-	+++
Ammonia 10%		0	0	+	+	0	0	0	-	-	0	0	-	-	0	+0	-
Ammonia 0.25%		0	0	0	0	0	0	0	-	-	0	0	-	-	-	-	0
Incubated sweat		0	0	0	+0	0	0	0	0	0	0	0	0	0	-	-	-
Fresh sweat		0	0	0	0	0	0	0	0	0	0	0	-	-	-	-	-

[a] Numbers represent different sensilla. [b] A, B indicate different neurons recorded at the base of the same sensillum. +: excitation; 0: no response; -: not tested. The magnitude of excitation was assigned to four sensitivity categories (+0: 0-25%; +: 25%-50%; ++: 50%-75%; +++: 75%-100%) The maximum excitation observed during each single sensillum recording was set to 100%. [c] Components were applied at a dose of 10%, except for those components where other doses are mentioned. Fresh and incubated sweat were applied at a dose of 25 µl.

6-methyl-5-hepten-2-one. Four of these receptor neurons also responded to geranyl acetone, while in one sensillum the other receptor neuron responded to geranyl acetone. In two cases both receptor neurons recorded from responded upon stimulation. Generally, it was found that within some sensilla the receptor neuron responding to geranyl acetone was excited upon stimulation with 3-methyl-1-butanol and/or 6-methyl-5-hepten-2-one. In other sensilla the neuron which did not respond to geranyl acetone was excited by 3-methyl-1-butanol and/ or 6-methyl-5-hepten-2-one. Also the response patterns of the two latter components did not always correlate. It should be noted, however, that the spike amplitudes of both receptor neurons were overlapping and it was not always possible to reliably separate both cells. None of the receptor neurons responded to indole. According to the classification made for *An. stephensi*, sensilla recorded from mostly resembled subtype C.

In six other sensilla a receptor neuron was found which was excited upon stimulation with indole (Fig 4A). Sensitivities of the indole-excited cell were in the same order of magnitude as that of the geranyl acetone-excited neuron, with thresholds around 0.01%. Neurons within the same sensillum showed responses to 3-methyl-1-butanol as well (Fig 4B). Owing to the instability of the recordings from sensilla housing indole responsive neurons, the full range of doses of 3-methyl-1-butanol and 6-methyl-5-hepten-2-one was only tested in four of the six sensilla. In three of the four recordings the indole-excited cell responded to 3-methyl-1-butanol. Responses to 6-methyl-5-hepten-2-one were only observed once. Geranyl acetone never evoked an increase in the spontaneous spike frequency of both neurons innervating the indole sensitive sensilla. Indole sensitive neurons were found within subtype C and E, according to the classification for *An. stephensi.*

One sensillum was found of which none of the cells responded to either indole or geranyl acetone. One of the cells was excited upon stimulation with 3-methyl-1-butanol while the other cell was excited in response to 6-methyl-5-hepten-2-one. Thresholds were in the same order of magnitude as observed in both formerly mentioned subpopulations sensilla trichodea.

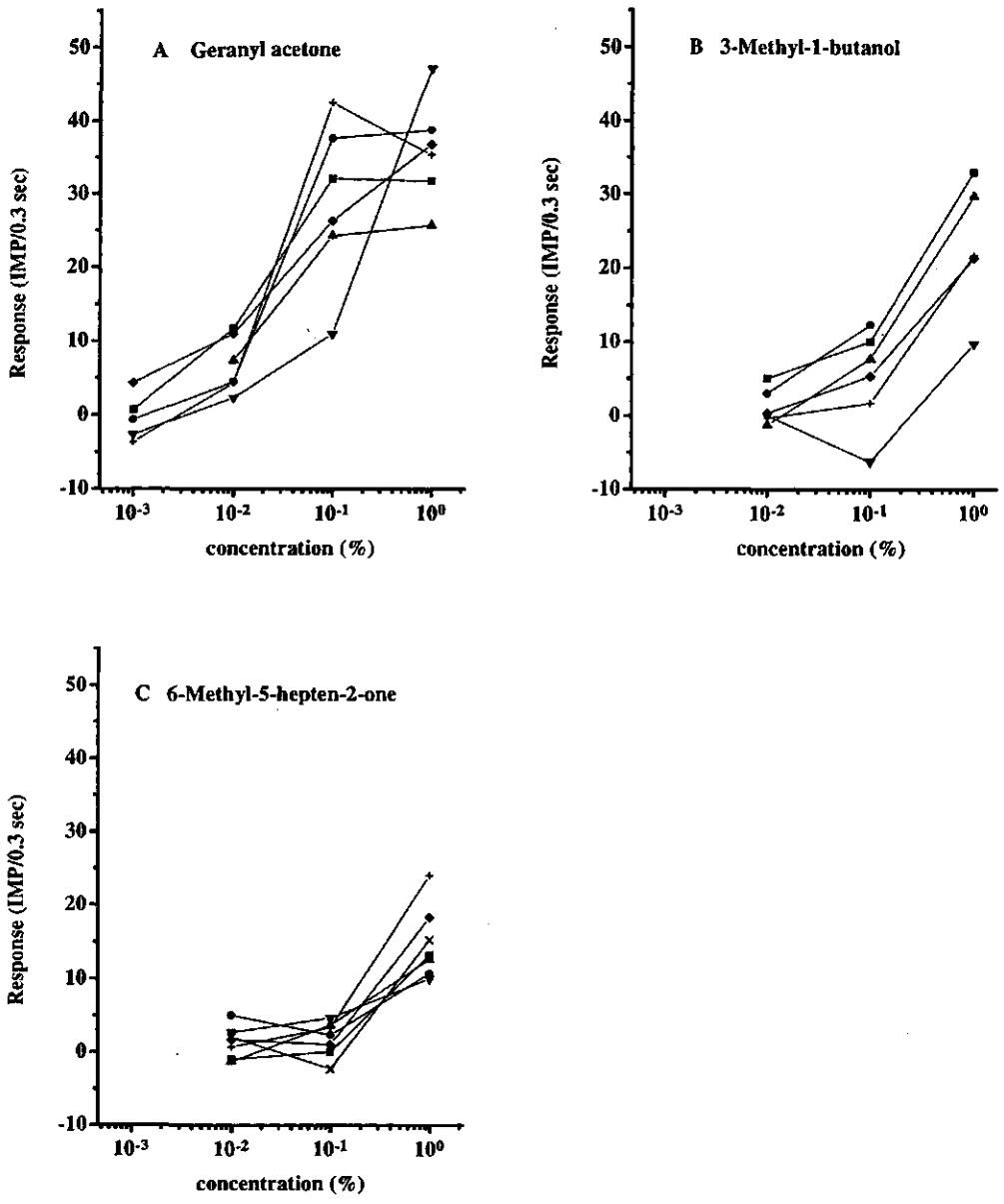

Fig 3. Dose-response relationships for receptor neurons within geranyl acetone-sensitive sensilla trichodea to varying doses of (A) geranyl acetone, (B) 3-methyl-1-butanol and (C) 6-methyl-5-hepten-2-one. Each symbol represents data for an individual neuron. Responses evoked by the three components were not necessarily restricted to the same neuron. One receptor neuron could respond to geranyl acetone, while the other responded to 3-methyl-1-butanol and/or 6-methyl-5-hepten-2-one. Occasionally both neurons reponded to the same component.

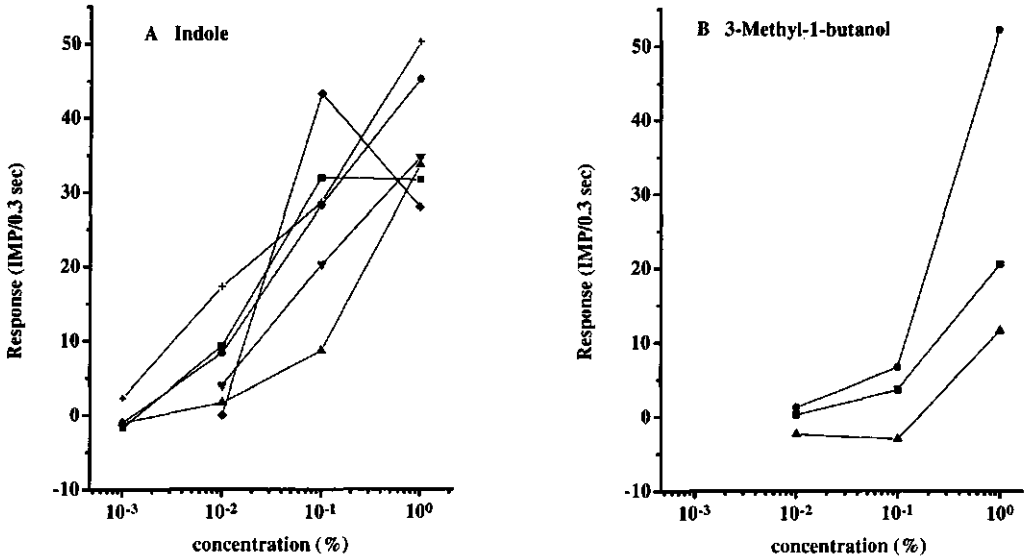

Fig 4. Dose-response relationships for receptor neurons within indole-sensitive sensilla trichodea to varying doses of (A) indole and (B) 3-methyl-1-butanol. Each symbol represents data for an individual neuron. Responses to indole and 3-methyl-1-butanol were not necessarily restricted to the same neuron.

Discussion

Grooved peg sensilla

Our experiments revealed the presence of ammonia-sensitive receptor neurons within a subpopulation of the grooved-peg sensilla on the antenna of *An. gambiae* mosquitoes. This type of receptor neuron responded to ammonia as well as to the incubated sweat from which ammonia is released. Both excitation as well as inhibition were evoked by butyl- and pentylamine. Although this receptor neuron was not as sensitive to these components as to ammonia, headspace collections have been shown to contain butylamine and pentylamine (Ellin et al., 1974). Further studies should reveal whether other components than ammonia also contribute to the strength of the response elicited by the incubated sweat.

For other haematophagous arthropods ammonia-sensitive receptor neurons have been identified in grooved-peg sensilla as well. Nymphs of the blood-sucking bug

Triatoma infestans possess two different types of ammonia-excited receptors located in two different grooved-peg subpopulations; one of these receptors was shown to display thresholds in the same range as observed in the behavioural bioassay (Taneja and Guerin, 1997). Similarly, two types of ammonia-sensitive neurons, with different sensitivities, have been found on the first tarsi of the tick *Rhipicephalus sanguineus* (Haggart and Davis, 1980). Electrophysiological studies on *Amblyomma variegatum* ticks revealed two types of ammonia-sensitive neurons both within the wall-pored sensilla located outside the Haller's organ capsule. A third receptor type located in a different sensillum displayed a weak response to high concentrations of ammonia (Steullet and Guerin, 1994). Similarly, we found receptor neurons within the sensilla trichodea responding in a relatively insensitive way when stimulated with ammonia, while most other sweat-borne components evoked stronger responses. For other mosquito species, responses of grooved-peg receptor neurons to ammonia have only been briefly mentioned for *Aedes aegypti* (Kellogg, 1970; Davis and Bowen, 1994). Grooved-peg receptor neurons in *Aedes aegypti* have been found to respond to water vapour (Kellogg, 1970). We found a large amplitude receptor neuron in a part of the grooved-peg sensilla population sensitive to water or water-containing stimuli. No neurons within this population of grooved-peg sensilla responded to incubated sweat or ammonia. The spike frequency of the large amplitude receptor neuron as well as a different receptor neuron was decreased in response to dry air, or stimuli lacking water like ether or ethanol. During the experiments a moist airstream is continuously blown over the preparation and stimulation with stimuli lacking water vapour therefore results in a temporal decrease of the water vapour pressure. Generally, however, hygro-sensitive sensilla contain 3 receptor neurons, one cold receptor, one moist receptor and one dry receptor. Moist receptors respond to sudden increases in humidity by excitation, while dry receptors responds by excitation to sudden decreases in humidity (Altner and Loftus, 1985). We found a receptor cell that was inhibited when stimulated with dry air. Stimulation with extremely dry air has been reported to inhibit moist air cells of the stick insect, *Carausius morosus* (Tichy and Loftus, 1990). Behavioural experiments conducted in our laboratory revealed that moist air is attractive to *An. gambiae* in a windtunnel bioassay and both ports of the windtunnel always release air with a higher relative humidity in order to obtain a trap-entry response.

Sensilla trichodea

Single sensillum recordings from the sensilla trichodea revealed receptor neurons

sensitive to a number of sweat-borne components. However, hardly any clear responses were evoked by the source of the components, namely the incubated or fresh sweat. Several explanations for this contradiction are possible. First, receptor neurons might respond to incubated sweat with an increase in spike frequency of only a few spikes/per sec. Such a slight response is very hard to detect. The convergence ratio of the receptor neurons innervating the ca. 600 sensilla trichodea on the antennae, of which at least a part is stimulated by sweat-borne components, to the olfactory lobe is different from the convergence ratio of grooved-peg receptor neurons. The input of grooved-peg neurons to the central nervous system is less as there are only a total of 84 sensilla present on the antennae. Secondly, components produced during the 2 days of incubation are most likely of microbial origin. Microbial products are very common in nature and usually widely distributed thereby serving as semiochemicals for different insect species (Millar et al., 1992). Responses observed to the sweat-borne components might be the sensory basis for different behavioural activities such as location of oviposition sites or nectar feeding. Indole, for instance, has been shown to be involved in the oviposition behaviour of *Culex quinquefasciatus* (Millar et al., 1992) and recently this component has been implicated to have a similar role in the oviposition behaviour of *An. gambiae s.s.* (Blackwell, personal communication). Additionally, geranyl acetone, 6-methyl-5-hepten-2-one and 3-methyl-1-butanol seem to act as semiochemicals for several other insect species and originate from sources as diverse as plants, animal excretions, defensive excretions or microbial degradation products (Andersen and Metcalf, 1986; Hammack, 1996; Keegans et al., 1993; Micha et al., 1993; Pierce et al., 1991; Takács et al., 1997).

Thirdly, the incubated sweat might contain additional inhibitory compounds, which suppress the excitatory response evoked by the sweat-borne components tested. Fourth, the amount of (some) odours released by a human skin might be higher than the amount of volatiles emanating from the incubated sweat.

Although the grooved-peg sensillum is considered to be the sensillum type housing receptor neurons sensitive to odours involved in the host-seeking behaviour, we found responses to sweat-borne components within the sensilla trichodea as well. Whether the sensitive receptor neurons respond to odours with a kairomonal function in host-seeking behaviour or whether the odours act as semiochemicals in oviposition- or nectar feeding behaviour has to be resolved in future behavioural studies.

Relationship between morphologically and functionally distinct subpopulations

Two morphologically and functionally distinct classes of grooved-peg sensilla have been distinguished for *Culex pipiens* and a number of *Aedes* species (Bowen, 1995). Short grooved peg sensilla are innervated by neurons responding with excitation to lactic acid. These lactic acid-excited cells, however, are absent in the longer grooved peg sensilla. We revealed the presence of two subpopulations of grooved-peg sensilla. A neuron innervating one of the subpopulations responded to ammonia and incubated sweat, while neurons within another subpopulation responded to water. No morphological differences were found for the grooved peg sensilla in *An. gambiae* during scanning electron microscopic observations. Other studies on *Aedes aegypti* report that grooved-peg neurons are thought to display overlapping response spectra. Human skin wash extracts and different behaviourally active fractions evoke overlapping response spectra and some of the cells display different relative sensitivities to the fractions tested (Pappenberger et al., 1996). For *An. gambiae*, testing of other behaviourally relevant odours might reveal an additional more complex response pattern of the grooved-peg neurons involved.

Neurons innervating a part of the sensilla trichodea population responded to geranyl acetone, indole, 3-methyl-1-butanol and 6-methyl-5-hepten-2-one. The sensilla subtypes recorded from mostly resemble subtype C and E as classified for *An. stephensi* (Boo, 1980). Neurons innervating the same subtypes have been shown to be responsive to carboxylic acids and 1-octen-3-ol (Meijerink and Van Loon, 1999). However, the two functionally distinct subpopulations, the indole-sensitive sensilla and geranyl acetone-sensitive sensilla were partly correlated with the morphologically distinct subtypes C and E. Neurons innervating subtype E, the shortest among the different subtypes sensilla trichodea, were sensitive to indole. No responses of neurons innervating this sensillum subtype were observed for geranyl acetone. Neurons innervating subtype C, however, responded to either indole or geranyl acetone.

Behavioural relevance

This report is the first study on *An. gambiae* describing the responses of receptor neurons associated with different antennal sensilla to a number of sweat-borne components. Ammonia, indole, geranyl acetone, 3-methyl-1-butanol, 6-methyl-5-hepten-2-one, 1-dodecanol, adipic acid are all components identified from (the headspace of) fresh and/or incubated human sweat (Meijerink et al., accepted; Braks et al., submitted). The fact that ammonia has been shown to attract female *An. gambiae* in a windtunnel bioassay (Braks

et al., submitted) together with the fact that ammonia sensitivity was found within the grooved-peg sensillum, implicate that ammonia might be an important kairomone for female *An. gambiae*. Ammonia is emitted from the human skin as a microbial breakdown product of excreted sweat products like urea and nitrogen-rich components (Bergeim and Cornbleet, 1943; Jenkinson et al., 1974). Whether ammonia is attractive under field conditions remains to be tested. In addition windtunnel studies are underway to elucidate the role of geranyl acetone, indole, 3-methyl-1-butanol and 6-methyl-5-hepten-2-one with respect to host seeking behaviour of female *An. gambiae*.

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References

Altner H and Loftus R (1985) Ultrastructure and function of insect thermo- and hygroreceptors. *Annu. Rev. Entomol.* 30:273-295.

Andersen JF and Metcalf RL (1986) Identification of a volatile attractant for *Diabrotica* and *Acalymma* spp. from blossoms of *Cucurbita maxima* Duchesne. *J. Chem. Ecol.* 12: 687-699.

Bergeim O and Cornbleet T (1943) The antibacterial action of the lactic acid and volatile fatty acids of sweat. *Am. J. Med. Sci* 785-792.

Boo KS (1980) Fine structure of the antennal sensory hairs in female *Anopheles stephensi*. *Z. Parasitenkd.* 61: 161-171.

Bowen MF (1995) Sensilla basiconica (grooved pegs) on the antennae of the female mosquitoes: electrophysiology and morphology. *Entomol. Exp. Appl.* 77: 233-238.

Braks MAH, Cork A, and Takken W (1997) Olfactometer studies on the attraction of *Anopheles gambiae sensu stricto* (Diptera: Culicidae) to human sweat. *Proc. Exp. Appl. Entomol.* N.E.V. Amsterdam 8: 99-104.

Braks MAH, and Takken W (1999) Incubated human sweat but not fresh sweat attracts the malaria mosquito *Anopheles gambiae sensu stricto. J. Chem. Ecol.* 25:663-672.

Braks MAH, Meijerink J and Takken W. The role of human sweat components, ammonia and L-lactic acid, in the behavior of the anthropophilic malaria mosquito, *Anopheles gambiae* (Culicidae; Diptera). Submitted to *J. Comp. Physiol. A*

Davis EE and Sokolove PG (1975) Temperature responses of antennal receptors of the mosquito, *Aedes aegypti. J. Comp. Physiol.* 96: 223-236.

Davis EE (1984) Regulation of sensitivity in the peripheral chemoreceptor systems for host-seeking behaviour by a haemolymph-borne factor in *Aedes aegypti. J. Insect Physiol.* 30: 179-183.

Davis EE (1988) Structure-response relationship of the lactic acid-excited neurons in the antennal grooved-peg sensilla of the mosquito *Aedes aegypti. J. Insect Physiol.* 34: 443-449.

Davis EE, and Bowen MF (1994) Sensory physiological basis for attraction in mosquitoes. *J. Am. Mosq. Control Assoc.* 10: 316-325.

Ellin RI, Farrand RL, Oberst FW, Crouse CL, Billups NB, Koon WS, Musselman NP, and Sidell FR (1974) An apparatus for the detection and quantitation of volatile human effluents. *J. Chrom.* 100: 137-152.

Haggart DA and Davis EE (1980) Ammonia-sensitive neurons on the first tarsi of the tick, *Rhipicephalus sanguineus. J. Insect. Physiol.* 26: 517-523.

Hammack L (1996) Corn volatiles as attractants for northern and western corn rootworm beetles (Coleoptera: Chrysomelidae: *Diabrotica* Spp.). *J. Chem. Ecol.* 22:1237-1253.

Ismail IAH (1964) Comparative study of sense organs in the antennae of culicine and anopheline female mosquitoes. *Acta Trop.* 21: 155-168.

Jenkinson DM, Mabon RM, Manson W (1974) The effect of temperature and humidity on the losses of nitrogenous substances from the skin of Ayrshire calves. *Res. Ver. Sci.* 17: 75-80.

Keegans SJ, Billen J, Morgan ED and Gökcen OA (1993). Volatile glandular secretions of three species of new world army ants, *Eciton burchelli, Labidus coecus*, and *Labidus praedator. J. Chem. Ecol.* 19:2705-2719.

Kellogg FE (1970) Water vapour and carbon dioxide receptors in *Aedes aegypti. J. Insect*

Physiol. 16:99-108.

Knols BGJ, Van Loon JJA, Cork A, Robinson RD, Adam W, Meijerink J, de Jong R, and Takken W (1997) Behavioral and electrophysiological responses of the female malaria mosquito *Anopheles gambiae* (Diptera: Culicidae) to Limburger cheese volatiles. *Bull. Entomol. Res.* 87:151-159.

Lacher V (1967) Elektrophysiologische Untersuchungen an einzelnen Geruchsrezeptoren auf den Antennen weiblicher Moskitos (*Aedes aegypti* L.). *J. Insect Physiol.* 13: 1461-1470.

Lacher V (1971) Electrophysiological equipment for measuring the activity of single olfactory nerve cells on the antennae of mosquitoes. *J. Econ. Entomol.* 64: 313-314.

McIver SB (1982) Sensilla of mosquitoes (Diptera: Culicidae). *J. Med. Entomol.* 19: 489-535.

Meijerink J and Van Loon JJA (1999) Sensitivities of antennal olfactory neurons of the malaria mosquito, *Anopheles gambiae*, to carboxylic acids. *J. Insect Physiol.* 45: 365-373.

Meijerink J, Braks MAH, Brack A, Adam W, Dekker T, Posthumus MA, Van Beek TA and Van Loon JJA. Identification of olfactory stimulants for *Anopheles gambiae* from human sweat samples. *J. Chem. Ecol.* (accepted)

Micha SG, Stammel J and Höller C (1993) 6-Methyl-5-hepten-2-one, a putative sex and spacing pheromone of the aphid hyperparasitoid, *Alloxysta victrix* (Hymenoptera: Alloxystidae). *Eur. J. Entomol.* 90:439-442.

Millar JC, Chaney JD and Mulla MS (1992) Identification of oviposition attractants for *Culex quinquefasciatus* from fermented Bermuda grass infusions. *J. Am. Mosq. Control Assoc.* 8:11-17.

Pappenberger B, Geier M and Boeckh J (1996) Responses of the antennal olfactory receptors in the yellow fever mosquito *Aedes aegypti* to human body odours. In: *Olfaction in mosquito-host interactions*. Wiley, Chichester (Ciba Foundation Symposium 200) pp. 254-266.

Pierce AM, Pierce HD, Borden JH, and Oehlschlager AC (1991) Fungal volatiles: semiochemicals for stored-product beetles (Coleoptera: Cucujidae). *J. Chem. Ecol.* 17: 581-597.

Smith JJB, Mitchell BK, Rolseth BM, Whitehead AT and Albert PJ (1990) SAPID Tools: microcomputer programs for analysis of multi-nerve recordings. *Chem. Senses* 15: 253-270.

Steullet P and Guerin PM (1994) Identification of vertebrate volatiles stimulating olfactory receptors on tarsus I of the tick *Amblyomma variegatum* Fabricius (Ixodidae). II. Receptors outside the Haller's organ capsule. *J. Comp. Physiol.* 174: 39-47.

Takács, S., Gries, G., and Gries, R. (1997). Semiochemical-mediated location of host habitat by *Apanteles carpatus* (say) (Hymenoptera: Braconidae), a parasitoid of clothes moth larvae. *J. Chem. Ecol.* 23:459-472.

Takken W and Knols BGJ (1999) Odor-mediated behavior of Afrotropical malaria mosquitoes. *Annu. Rev. Entomol.* 44: 131-157.

Taneja J and Guerin PM (1997) Ammonia attracts the haematophagous bug *Triatoma infestans*: behavioural and neurophysiological data on nymphs. *J. Comp. Physiol.* 181: 21-34.

Tichy H and Loftus R (1990) Response of moist-air receptor on the antenna of the stick insect, *Carausius morosus*, to step changes in temperature. *J. Comp. Physiol.* 166:507-516.

Van den Broek IVF and Den Otter CJ (1999). Olfactory sensitivities of mosquitoes with different host preferences (*Anopheles gambiae s.s., An. arabiensis, An. quadriannulatus, An. m. atroparvus*) to synthetic host odours. *J. Insect Physiol.* 45: 1001-1010.

Chapter 7

General discussion

Studies documented in this thesis were undertaken to identify new potential attractants for host-seeking *An. gambiae* and to reveal the response characteristics and response spectra of olfactory receptor neurons involved in the detection of host-odours. In this chapter results are summarised and the function of the olfactory receptor neurons responsive to host-produced odours is discussed. Based on the observed response spectra of human odour-responsive receptor neurons, hypothetical models for the projection of receptor neuron axons to the glomeruli are given. The results are viewed in the context of olfactory-guided host-seeking behaviour of female *An. gambiae.*

Sensitivity and specificity of different olfactory neurons within distinct sensillar subtypes

The composition of the volatile constituents of the headspace of pooled sweat samples of fresh and incubated sweat was revealed by GC-MS (chapter 5). Components were selected based on their relative and absolute amounts and subsequently submitted to EAG recording. Indole, geranyl acetone and 6-methyl-5-hepten-2-one evoked EAG responses. Indole was solely present in the headspace of the incubated sweat while geranyl acetone and 6-methyl-5-hepten-2-one were present in both the headspace of the fresh and incubated sweat. In order to reveal sensitivity and specificity of the receptor neurons responding to these components, single cell studies were undertaken. In addition, 3-methyl-1-butanol, a component abundantly present in the headspace of the incubated sweat was tested as well. The source of the volatiles, namely the fresh and incubated sweat, as well as ammonia, which is present in large amounts in the incubated sweat were also tested.

Table 1 gives a summary of all single cell recordings undertaken in this study. It summarises the responses of receptor neurons to components which have been implicated to play a role in the host-seeking behaviour of *An. gambiae*, like the carboxylic acids, or to the formerly mentioned identified sweat-borne components. Three additional compounds were tested. Butylamine and pentylamine were identified in volatile collections of humans (Ellin et al., 1974), while 1-octen-3-ol is an attractant for several mosquito species (Kline, 1994).

Table 1. Sensitivity and specificity of the olfactory receptor neurons innervating different sensilla on the female antennae.

Sensillum	Subtype	Effective stimuli	Response [b]	Threshold [c]	n
grooved-peg	I	incubated sweat	+		16
		ammonia	+	< 0.25%	7
		butylamine	+		2
		pentylamine	-/+		2/1
		3-methyl-1-butanol	-		1
grooved-peg	II	water	+		9
		dry air, ether, ethanol	-		9
		butylamine	-		1
		pentylamine	-		1
		3-methyl-1-butanol	-		1
sensilla trichodea	C [a]	geranyl acetone	+	0.01%	11
		3-methyl-1-butanol	+	0.1%	10
		6-methyl-5-hepten-2-one	+	1%	11
		ammonia	+	< 10%	2
sensilla trichodea	E [a], C [a]	indole	+	0.01%	10
		3-methyl-1-butanol	+	0.1%	5
		6-methyl-5-hepten-2-one	+	1%	5
		ammonia	+	< 10%	1
sensilla trichodea	E [a], C [a]	acetic acid	-/+	16-53 x 10^{-11} [d]	11/1
		propionic acid	-/+	16-53 x 10^{-11} [d]	15/4
		butyric acid	-/+	16-53 x 10^{-11} [d]	31/4
		iso-butyric acid	-/+	16-53 x 10^{-11} [d]	8/3
		iso-valeric acid	-/+	16-53 x 10^{-11} [d]	7/1
		valeric acid	-/+		2/3
		caproic acid	+		3
		1-octen-3-ol	+		2

[a] The different subtypes of sensilla trichodea were classified according to the classification for *An. stephensi* (Boo, 1980)

[b] + indicates that the receptor neuron was excited upon stimulation, while - means that the neuron was inhibited, -/+ means that both reaction types were observed.

[c] Thresholds are only given for those components for which thresholds could be determined from a dose-response curve. Threshold given for ammonia is the lowest concentration tested.

n represents the number of sensilla innervated by neurons which showed a response upon stimulation with the relevant component tested. It represents the total number of responding sensilla tested in the one-dose experiments as well as the dose-response curves. Only recordings were included in which the signal-noise ratio and the stability of the recording were judged adequate for analysis.

[d] Response threshold given are only for the carboxylic acid inhibited cell type

Sensilla trichodea

Responses to carboxylic acids

In chapter 4 subtypes of the sensilla trichodea population are documented which respond by inhibition to carboxylic acids. The function, however, of inhibitory responses of receptor neurons in chemosensory coding is still not known. For *Aedes aegypti* a binary coding mode has been implicated for the lactic acid sensitive receptor neurons. Two types of lactic acid-sensitive neurons have been found within the grooved peg sensilla of *Aedes aegypi*; receptor neurons responding by an increase and receptor neurons responding by a decrease of the spontaneous spike frequency upon stimulation with lactic acid. After a bloodmeal the sensitivity of the lactic acid-excited neuron did decrease while the sensitivity of the lactic acid-inhibited neuron did not change (Davis et al., 1987). It has been suggested that the sum of the change in spike frequency of the lactic acid-excited neurons and the lactic acid-inhibited neurons determines the behavioural response of the female mosquito. Thus, if the sensitivity of the lactic acid-excited neuron is decreased after a bloodmeal, the sum of change in spike frequency of the inhibited and excited neuron will be negative. A total negative change in spike frequency will result in an inhibition of host-seeking behaviour. The role of the lactic acid-inhibited neuron is hypothesised to actively prevent a behavioural response to large lactic acid signals in a situation, such as oocyte development, when a response to a host-attractant is inappropriate (Davis et al., 1987).

Analogously, we found two types of carboxylic acid-sensitive neurons. Carboxylic acid-excited neurons, however, seemed to be sensitive to a broader range of components than the carboxylic acid-inhibited neurons, as longer chain carboxylic acids like valeric acid and hexanoic acid elicited responses as well (chapter 4). Furthermore, carboxylic acid-excited neurons were revealed to be more sensitive than the carboxylic acid inhibited-neurons (Van den Broek and Den Otter, 1999), a difference which was not observed for the two types of lactic acid-sensitive cells in *Aedes aegypti*. These observations indicate that the inhibitory response we observed might have a different function than the inhibitory response elicited by lactic acid.

The inhibitory responses we found are similar to carboxylic acid-inhibited responses described for the carrion beetle (Boeckh, 1967). The time course of the carboxylic acid-inhibited neuron is similar to the time course we obtained (chapter 4) and differs from excited cells. It has been suggested that a stimulant might evoke excitation in its own specialist cell type, while it simultaneously inhibits another specialist cell type

sensitive to other components (Boeckh, 1967). We did not simultaneously stimulate carboxylic acid-inhibited cells with both short chain carboxylic acids and the excitatory stimulants geranyl acetone, indole, 3-methyl-1-butanol and 6-methyl-5-hepten-2-one to check whether this also occurs in *An. gambiae*. As the response spectra to these stimulants overlap, it would be interesting to see whether the inhibitory response will overrule the excitatory response. Such a correlation between behavioural response and peripheral encoding was implicated for the termite *Schedorhinotermes lamanianus*. Levels of CO_2 occurring in the termite nest evoked inhibition of a receptor neuron, while excitatory responses elicited by other stimuli were blocked in the presence of CO_2 (Ziesmann, 1996).

Inhibitory responses we observed might also function to block the response of a specialist receptor neuron normally responding by excitation to other stimuli. By blocking the input of other 'concurrent' stimuli, the input to the central nervous system is restricted and the processing of odour information might be in 'favour' of the stimulant which evokes excitation of it's own specialist receptor cell. Whether the central integration of this "binary code", namely inhibition and excitation, leads to repellency or attractancy might differ between insect species or even between different input afferents.

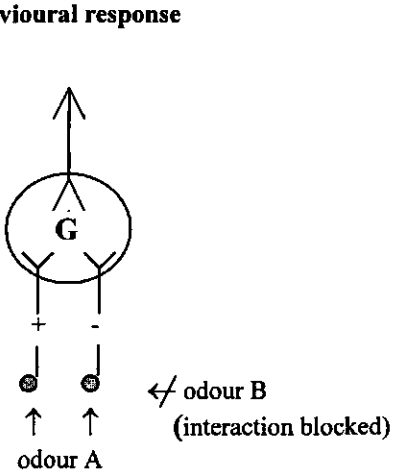


Fig. 1. Hypothetical model of the central integration of the input from an inhibitory (-) and excitatory (+) olfactory receptor neuron response. G represents a glomerulus. ◍ represents an olfactory receptor neuron. ↚ indicates that the receptor neuron response to the indicated odour is blocked.

Fig. 1. shows a hypothetical model of the central integration of the input from an inhibitory and excitatory olfactory receptor neuron response evoked by the same odour A. The excitatory receptor neuron response elicited by odour B is blocked by the inhibitory response evoked by odour A.

Responses to sweat-borne components

Analyses of the headspace composition of the fresh and incubated sweat, followed by EAG recording, revealed that geranyl acetone, indole, 3-methyl-1-butanol and 6-methyl-5-hepten-2-one were detected by the antennal olfactory neurons (chapter 5). Consecutive single cell recordings showed that neurons innervating (a) subtype(s) of the sensilla trichodea population were sensitive to these sweat-borne components. The lowest thresholds were obtained for geranyl acetone and indole. Both components evoked excitation in a different subpopulation of the sensilla trichodea population. Indole has been identified solely in the headspace of the incubated sweat, while geranyl acetone was present in both the fresh and incubated sweat in equal amounts (chapter 5).

Sensilla housing the geranyl acetone sensitive neuron were more frequently found. Although this could indicate that the antennae house more neurons sensitive to geranyl acetone than to indole, it is more likely that the routine of the experimenter when recording and fastening the mosquito's antennae have a big influence on the sensilla types recorded from. There are several examples in which different experimenters found different subpopulations of neurons sensitive to different odours. In *Ae. aegypti* responses of grooved-peg neurons to carboxylic acids were frequently registered, but no responses were obtained to lactic acid. When a different experimenter started, sensitive neurons to lactic acid were immediately found (M. Geier *pers. comm.*) Likewise, different responses like excitation or inhibition were found by different experimenters, or in different time periods, in response to exactly the same odours (Davis, 1996; I.V.F. Van den Broek, *pers. comm.*). In our experiments, carboxylic acids usually evoked inhibition in sensilla trichodea associated receptor neurons (chapter 4). During recording the tungsten electrode was predominantly placed at the base of the socket of the sensillum. During the recordings in which the same stimuli elicited excitation the recording electrode was placed at the top of the socket or almost behind the sensillum, indicating that the exact position of the electrode might determine the neuron recorded from.

3-Methyl-1-butanol and 6-methyl-5-hepten-2-one elicited excitation in both geranyl acetone and indole sensitive subpopulations of sensilla trichodea. In some cases

the same neuron that responded to indole or geranyl acetone was excited, in other cases the other neuron that innervates these sensilla was responding. Thresholds for 3-methyl-1-butanol and 6-methyl-5-hepten-2-one were higher than those for geranyl acetone and indole, however, it should be stressed that no corrections were made for differences in volatility.

Grooved peg-sensillum

For *Aedes aegypti*, the grooved peg-sensillum has been considered to be the type of sensillum housing receptor neurons sensitive to host attractants. Neurons sensitive to lactic acid, the only attractant for host-seeking *Ae. aegypti* chemically identified thus far, are located within the grooved pegs (Davis and Sokolove, 1976; Davis, 1984, 1988). Also, fractions of human skin emanations which are attractive for *Ae. aegypti* in a windtunnel bioassay elicited responses of the grooved peg-neurons (Pappenberger et al., 1996). We found excitation of a "large amplitude" receptor neuron innervating a subpopulation of the grooved peg population in response to ammonia and the incubated sweat (chapter 6). These data indicate that also for *An. gambiae* sensitivity to host attractants is provided by the receptor neurons within the grooved peg sensilla. The fact that ammonia attracted female *An. gambiae* in a windtunnel bioassay (Braks et al., submitted) together with the fact that ammonia sensitivity was found within the grooved-peg sensillum implicate that ammonia might be an important kairomone for female *An. gambiae*. Another subpopulation of the grooved peg population was innervated by a receptor neuron which was excited upon stimulation with water (chapter 6). In order to obtain a trap-entry response of female mosquitoes, from both ports of the windtunnel bioassay, air is released with a higher relative humidity, indicating that moist air is used by *An. gambiae* during the process of host seeking and host location. In addition, preliminary data indicate that lactic acid sensitive receptor neurons are also located within the grooved peg sensillum (Meijerink, unpublished results), similar to the situation in *Ae. aegypti*. Recently, behavioural studies revealed that lactic acid can attract *An. gambiae* mosquitoes (Braks et al., submitted). In contrast to ammonia, however, lactic acid was only attractive when offered at one particular dose. These data taken together strongly indicate that the grooved peg sensilla are innervated by neurons sensitive to host cues used by host seeking *An. gambiae*. The grooved peg sensillum might therefore be the prime focus of further sensory studies into the peripheral encoding of host odours.

Neural encoding of sweat-borne components

The role of sensilla trichodea receptor neurons in the detection of host-odours

Although evidence supporting the hypothesis that grooved-peg neurons encode for host attractants is clearly existing, a role of the sensilla trichodea cannot be excluded. Despite the fact that for *Ae. aegypti* neurons innervating the sensilla trichodea have been mentioned to encode for plant-related and oviposition site-related volatiles (Davis and Bowen, 1994), our data show that neurons innervating the sensilla trichodea are responding to sweat borne components as well (chapter 6). Also other evidence supports the assumption that neurons within the sensilla trichodea might be involved in the detection of host odours. Mosquitoes of the species *Wyeomyia smithii* which were obtained from an obligatorily autogenous population, i.e. a blood meal is not required for egg maturation nor is one taken, bear significantly less sensilla trichodea and grooved pegs than *Wyeomyia aporonoma*. *Wyeomyia aporonoma* is an anautogenous mosquito, i.e. it has an absolute requirement for a blood meal for egg maturation, which feeds on large mammals (McIver and Hudson, 1972; McIver, 1982). The fact that not only the grooved peg sensilla but also the number of sensilla trichodea is greatly reduced, indicates that the latter structures might house host odour encoding neurons as well. Similar to our findings, neurons innervating the sensilla on the surface of tarsus I of the tick *Amblyomma variegatum,* respond to single host odour components, but not to the vertebrate odour mixtures collected on Porapak (Steullet and Guerin, 1994).

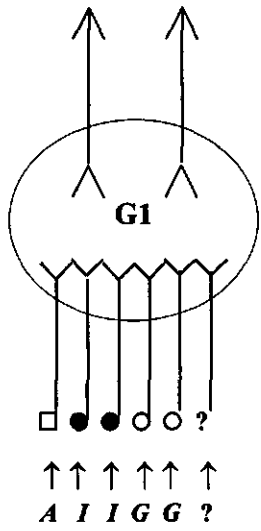
Sensitive neurons for carbon dioxide have been located in the sensilla basiconica on the maxillary palps of several mosquito species (Grant and O'Connell, 1996). Carbon dioxide has been shown to act as an important cue during host-seeking behaviour of many mosquito species (Gillies, 1980). Likewise, neurons responding to 1-octen-3-ol were found innervating the same sensilla basiconica (Grant and O'Connell, 1996). Neurons responding to 1-octen-3-ol have also been found within the sensilla trichodea of *An. gambiae* (chapter 4). A comparative study of the anthropophilic *An. gambiae,* the opportunistic *An. arabiensis* and the zoophilic *An. quadriannulatus* and *An. m. atroparvus,* four *Anopheles* species with different host preferences revealed sensitive neurons within the sensilla trichodea as well (Van den Broek and Den Otter, 1999). In addition, occasionally sensitivity to 1-octen-3-ol was found in the grooved peg neurons of the tested mosquito species *An. gambiae* and *An. quadriannulatus* (Van den Broek and

Den Otter, submitted). 1-Octen-3-ol has been shown to attract several mosquito species in the field (Kline, 1994). These data indicate that sensitive neurons encoding host odours are not only restricted to the grooved peg neurons.

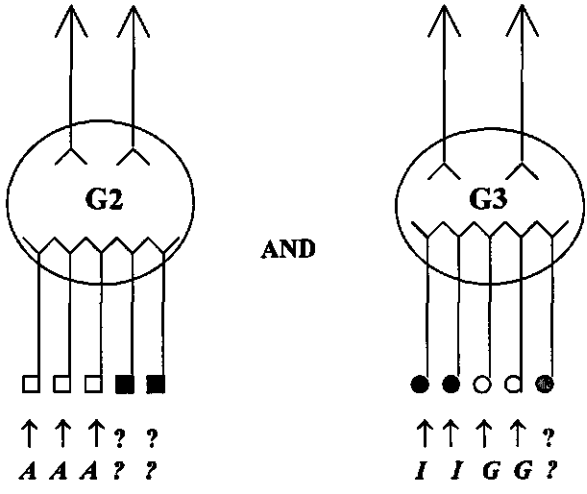
Why the natural source of the volatiles only evokes responses within the grooved peg neurons is still puzzling. One of the answers might be that the convergence factor to the glomeruli within the antennal lobe might be different as there are many more sensilla trichodea than grooved peg sensilla. Studies investigating to which glomeruli sensilla trichodea and grooved peg neurons project might yield more insight revealing the function with respect to the encoding of host odours of the olfactory receptor neurons within these two types of sensilla. Such studies are momentarily conducted for *An. gambiae* (S. Anton, unpubl. results). Two hypothetical models of the projection of the sensilla trichodea and grooved peg sensilla to the olfactory lobe are given in fig. 2. In model A the afferents of grooved peg- and sensilla trichodea receptor neurons project to the same glomerulus. In this case it is assumed that receptor neurons innervating both types of sensilla are involved in the detection of host-odours. Further processing of this input might lead to a behavioural output correlated with host-seeking behaviour. In model B afferents of grooved-peg- and sensilla trichodea receptor neurons project to distinct glumeruli. Only receptor neurons innervating grooved-peg neurons encode for host odours, while receptor neurons innervating the sensilla trichodea encode for different behavioural activities such as location of oviposition sites or nectar feeding.

Another interesting question is whether the grooved peg neurons in *An. gambiae* are inhibited after a bloodmeal, like the lactic acid-excited neuron in *Aedes aegypti*, and if this also holds for the neurons innervating (a part of) the sensilla trichodea population. Studies on *An. gambiae* addressing this subject have recently been performed on the EAG level and seem to indicate that peripheral sensitivity indeed is modified. Nevertheless, behavioural studies in a windtunnel bioassay and/or field studies elucidating the role of the sweat borne components geranyl acetone, indole, 3-methyl-1-butanol and 6-methyl-5-hepten-2-one might reveal a role of the sensilla trichodea neurons with respect to the encoding of host odours.

A Host-seeking behaviour



B Host-seeking behaviour other behaviour (oviposition or nectar feeding)



Across fibre pattern coding

Receptor neurons innervating (a) morphological subtype(s) of the sensilla trichodea population responded to the sweat-borne components geranyl acetone, indole, 3-methyl-1-butanol and 6-methyl-5-hepten-2-one (chapter 6). Two different subpopulations were clearly distinguished with one subpopulation of receptor neurons responding to geranyl acetone, while the other subpopulation was sensitive to indole. Reaction spectra of these two stimuli did not overlap. Thresholds for both receptor neurons were around 0.01 %. For 6-methyl-5-hepten-2-one and 3-methyl-1-butanol the response spectra of the neurons overlapped in such a way that responses were elicited in both subpopulations of sensilla trichodea. 6-Methyl-5-hepten-2-one, however, hardly evoked any response in the indole-sensitive sensilla, while it elicited excitation in almost every geranyl acetone-sensitive sensillum. Thresholds for 6-methyl-5-hepten-2-one and 3-methyl-1-butanol were around 1% and 0.1% respectively.

Also, occasionally receptor neurons were found within both subpopulations responding to high doses of ammonia. These data suggest the presence of a "specialised generalist" receptor cell. A specialised odour generalist has been described as a receptor cell responding sensitively to a key component, but showing different spectra of responses to other components (Kaissling, 1987). The reaction pattern observed indicates across fibre pattern coding, although the non-overlapping reaction patterns elicited by indole and geranyl acetone might fit into the labeled line coding concept. Most olfactory receptor cells are thought to react in between the two extreme response patterns described. At one side there are the highly specialised pheromone receptor neurons reacting according to the labeled line pattern, while on the other side there are the generalist neurons responding to almost every component without displaying any high sensitivity (Boeckh, 1980). Carboxylic acids as well as 1-octen-3-ol have been shown to elicit responses in the same type of sensilla trichodea (chapter 4). It would be interesting to know whether these response patterns would overlap or restrict to the geranyl acetone- or indole-sensitive subpopulations. For instance indole responsive receptor neurons might be excited by 1-octen-3-ol as well, or the other receptor neuron housing within the same

Fig. 2 (p. 120) Two hypothetical models (A and B) showing the projection of olfactory receptor neuron axons of the grooved peg- and sensilla trichodea neurons to the glomeruli. □,■ represent different grooved peg receptor neurons; ○, ◍, ●, represent different sensilla trichodea receptor neurons. *A* represents ammonia; represents indole and *G*, geranyl acetone, ? represents an unknown component or unknown receptor neuron. G1, 2, 3: glomeruli.

sensilla might be sensitive to 1-octen-3-ol. Additional data might provide more information about the specificity of the receptor neurons to the different components and their significance for encoding host-seeking behaviour or other behaviours. In addition, increases of the spike frequency evoked by indole and geranyl acetone did vary, suggesting that the receptor neurons for both components differ in their sensitivity.

For *Aedes aegypti* grooved peg neurons have been mentioned to react according to the across fibre pattern coding in response to behaviourally active fractions of human skin wash extracts (Pappenberger et al., 1996). We found a subpopulation within the grooved peg population exhibiting a large amplitude receptor neuron sensitive to both ammonia and incubated sweat (chapter 6). Also for *Culex pipiens* and several *Aedes* species, including *Ae. aegypti,* the population of the grooved-peg sensilla has been reported to consist out of two functionally and morphologically distinct classes. Short grooved peg sensilla were innervated by neurons excited by lactic acid. These excited grooved peg cells were absent in long grooved peg sensilla (Bowen, 1995). It seems that although overlapping patterns in response to several host odour related components exist, clearly functionally and even morphologically distinguishable classes are present as well. Further studies for *An. gambiae*, including other behaviourally relevant odours, probably reveal responses of neurons showing more complex overlapping patterns of the grooved peg neurons involved. However, testing of individual components might not give a clear picture about the reaction patterns elicited by natural complex sources, because possible peripheral interactions might occur in response to the latter.

Sensory physiology and behaviour

Our electrophysiological studies (this thesis) as well as the behavioural studies conducted on female *An. gambiae* (M.A.H. Braks, thesis) indicate that ammonia is an important kairomone for *An. gambiae*. Single cell recordings of the neurons innervating the grooved peg neurons revealed that one of the active substances within the incubated sweat causing excitation of one of the receptor neurons within the grooved peg sensillum is the highly volatile component ammonia. However, results from the electrophysiological studies as well as from the behavioural data indicate that there are more kairomonal odours present in the sweat which play a role during the host-seeking behaviour of *An. gambiae*. First, the increase in the spike frequency of the grooved peg neurons in response to doses of ammonia similar to the ammonia contents of the incubated sweat, versus the response

upon stimulation with incubated sweat is inconsistent. The incubated sweat was observed to elicit much more intense responses than would be expected from the ammonia content present. Although during stimulation the ammonia release from a complex mixture like the incubated sweat is different from the release of the same contents of ammonia, these results can also indicate that there are more candidates within the incubated sweat evoking excitation of this receptor neuron.

Secondly, behavioural data imply that there are additional non-highly volatile components involved which attract female *An. gambiae* in a windtunnel bioassay. Experiments conducted with panty socks which had been worn by a human volunteer showed that the socks lost a part of their attractivity during the first hour, while the socks still remained some of their attractivity during the following hours (T. Dekker, *pers. comm.*). Ammonia might have caused the attractivity observed during the first hours. However, one or more less volatile components presumably caused the longer-lasting attraction. Also EAG responses elicited by the headspace of incubated sweat were obtained only after longer time periods of sampling (chapter 5), indicating that these EAG responses were evoked by other, less volatile components than ammonia. Geranyl acetone and indole, both components eliciting EAG responses as well as excitatory responses of single receptor neurons, might be likely candidates.

Thirdly, behavioural observations conducted with fresh and incubated sweat showed that fresh sweat contains attractive components as well. While the fresh sweat itself is already more attractive than its control (water), it becomes more attractive after the water content has evaporated (M.A.H. Braks, *pers. comm.*). This attractiveness might be due to lactic acid, but also other components might be retained in the water volume which in the natural situation would be released from the human skin. But also the observed attractiveness of the fresh sweat might be due to components which are present in the fresh as well as in the incubated sweat. Geranyl acetone as well as 6-methyl-5-hepten-2-one are present in both sweat sources (chapter 5) and responsive neurons have been found in a subpopulation of the sensilla trichodea pointing to a potential role in host seeking *An. gambiae*. As the incubated sweat as an odour source is not identical to the human skin it is possible that the incubated sweat has become even more attractive than the natural source. Although it is difficult to rule out the contribution of moist air and temperature, it would yield some important information to test a human hand against the incubated sweat. Likewise, the contribution of ammonia to the attractiveness of the incubated sweat might be tested by adding ammonia to the fresh sweat till it contains doses similar to ammonia contents within the incubated sweat. In this way the release rate

of the ammonia from the fresh sweat probably equals that of the incubated sweat, and it can be seen to what extent the attractiveness of the incubated sweat can be attributed to ammonia. Similarly, the same could be studied for the single cell recordings of the grooved peg neurons. Experiments with equal concentrations of ammonia in the fresh and incubated sweat might reveal whether additional components within the incubated sweat excite the large amplitude receptor neuron innervating the grooved peg.

Also carboxylic acids might be candidates. However, on the behavioural level attraction of a synthetic mixture consisting of carboxylic acids, shown to attract female *An. gambiae* in a windtunnel bioassay, could not be repeated (M.A.H. Braks, thesis). Limburger cheese, on the other hand, continuously attracted *An. gambiae* when tested. This indicates that other components than carboxylic acids have caused (a part of) the attractiveness of Limburger cheese. Ammonia being a volatile constituent of cheeses might be a likely candidate. But also indole has been reported to be present in Limburger cheese (Parliment et al., 1982). Nevertheless, the fact that carboxylic acids evoke receptor neuron responses and have been demonstrated to elicit a behavioural response from female *An. gambiae* indicates that they play a role during the host-seeking behaviour of this mosquito species.

New putative kairomonal odours can be identified by single cell-recording (SCR) coupled to a GC. Gaschromatographic coupled electroantennogram recordings (GC-EAG) conducted at our laboratory were not successful (unpublished results), which was due to the low amplitude of EAG responses obtained from *An. gambiae* antennae. EAG amplitudes never exceeded -1 mV and were usually around - 0.3 mV, indicating that the coupled EAG-GC technique is probably too insensitive for the detection of electrophysiological active odours. GC-SCR, however, might provide a powerful tool for the identification of additional potential attractants.

Until now one component, namely ammonia, was found as attractant for female *An. gambiae* in a windtunnel bioassay. A number of putative attractants like the sweat borne components geranyl acetone, indole, 3-methyl -1-butanol and 6-methyl-5-hepten-2-one might have kairomonal activity as well. Mixtures of these components together with ammonia are interesting to test. Often, kairomones have been shown to act synergistically at the behavioural level. The fact that ammonia is attractive when offered solely and the fact that it is still attractive at very high concentrations implies that it is a very powerful kairomone for female *An. gambiae*. Further field studies might reveal if this odour can attract female *An. gambiae* mosquitoes in their natural environment in Africa.

References

Boeckh J (1967) Inhibition and excitation of single insect olfactory receptors and their role as a primary sensory code. In *Olfaction and Taste*, eds Y. Zotterman, Vol. II, pp. 721-735. Pergamon Press, New York

Boeckh J (1980) Neural basis of coding of chemosensory quality at the receptor cell level. In: *Olfaction and taste VII*, ed. H. van der Starre, pp. 113-122. London: IRL

Boo KS (1980) Fine structure of the antennal sensory hairs in female *Anopheles stephensi*. *Z. Parasitenkd.* 61: 161-171

Bowen MF (1995) Sensilla basiconica (grooved pegs) on the antennae of female mosquitoes: electrophysiology and morphology. *Entomol. Exp. Appl.* 77: 233-238

Braks MAH, Meijerink J and Takken W. The role of human sweat components, ammonia and L-lactic acid, in the behavior of the anthropophilic malaria mosquito, *Anopheles gambiae* (Culicidae; Diptera). Submitted to *J. Comp Physiol. A*

Braks MAH. *Human skin emanations in the host-seeking behaviour of the malaria mosquito* Anopheles gambiae. PhD thesis. Landbouw Universiteit Wageningen, Wageningen, The Netherlands (submitted)

Davis EE (1984) Regulation of sensitivity in the peripheral chemoreceptor systems for host-seeking behaviour by a haemolymph-borne factor in *Aedes aegypti*. *J. Insect Physiol.* 30: 179-183

Davis EE (1988) Structure-response relationship of the lactic acid-excited neurons in the antennal grooved-peg sensilla of the mosquito *Aedes aegypti*. *J. Insect Physiol.* 34: 443-449

Davis EE (1996) In: *Olfaction in mosquito-host interactions*. Wiley, Chichester (Ciba Foundation Symposium 200) pp. 265

Davis EE and Bowen MF (1994) Sensory physiological basis for attraction in mosquitoes. *J. Am. Mosq. Control Assoc.* 10: 316-325

Davis EE, Haggart DA and Bowen MF (1987) Receptors mediating host-seeking behaviour in mosquitoes and their regulation by endogenous hormones. *Insect Sci. Applic.* 8: 637-641

Davis EE and Sokolove PG (1976) Lactic acid-sensitive receptors on the antennae of the mosquito, *Aedes aegypti*. *J. Comp. Physiol.* 105: 43-54

Ellin RI, Farrand RL, Oberst FW, Crouse CL, Billups NB, Koon WS, Musselman NP, and Sidell FR (1974) An apparatus for the detection and quantitation of volatile human effluents. *J. of Chrom.* 100: 137-152

Gillies MT (1980) The role of carbon dioxide in host-finding by mosquitoes (Diptera: Culicidae): a review. *Bull. Entomol. Res.* 70: 525-532

Grant AJ and O'Connell RJ (1996) Electrophysiological responses from receptor neurons in mosquito maxillary palp sensilla. In: *Olfaction in mosquito-host interactions.* Wiley, Chichester (Ciba Foundation Symposium 200) p. 233-248

Kaissling K –E, (1987) R. H. Wright Lectures on Insect Olfaction. Simon Fraser University, Burnaby, Canada

Kline DL (1994) Olfactory attractants for mosquito surveillance and control: 1-octen-3-ol. *J. Am. Mosq. Control Assoc.* 10: 280-287

McIver SB (1982) Sensilla of mosquitoes (Diptera: Culicidae). *J. Med. Entomol.* 19: 489-535.

McIver S and Hudson A (1972) Sensilla on the antennae and palps of selected *Wyeomyia* mosquitoes. *J. Med. Entomol.* 9: 337-345

Pappenberger B, Geier M and Boeckh J (1996) Responses of the antennal olfactory receptors in the yellow fever mosquito *Aedes aegypti* to human body odours. In: *Olfaction in mosquito-host interactions.* Wiley, Chichester (Ciba Foundation Symposium 200) pp. 254-266.

Parliment TJ, Kolor MG and Rizzo DJ (1982) Volatile components of limburger cheese *J. Agric. Food Chem.* 30: 1006-1008

Steullet P and Guerin PM (1994) Identification of vertebrate volatiles stimulating olfactory receptors on tarsus I of the tick *Amblyomma variegatum* Fabricius (Ixodidae). II. Receptors outside the Haller's organ capsule. *J. Comp. Physiol.* 174: 39-47

Van den Broek IVF and Den Otter CJ (1999) Olfactory sensitivities of mosquitoes with different host preferences (*Anopheles gambiae s.s., An. arabiensis, An, quadriannulatus, An. m. atroparvus*) to synthetic host odours. *J. Insect Physiol.* 45: 1001-1010

Van den Broek IVF and Den Otter CJ. Odour sensitivity of antennal olfactory cells underlying grooved pegs of *An. gambiae s.s.* and *An. quadriannulatus*. Submitted to *Entomol. Exp. Appl.*

Ziesmann J (1996) The physiology of an olfactory sensillum of the termite *Schedorhinotermes lamanianus*: carbon dioxide as a modulator of olfactory sensitivity. *J. Comp. Physiol.* 179: 123-133.

Summary

Female mosquitoes of the species *Anopheles gambiae* Giles *sensu stricto* are important vectors of human malaria in Africa. It is generally assumed that they locate their human host by odours. These odours are detected by olfactory receptor neurons situated within cuticular extensions on the antenna. These cuticular extensions, called sensilla, contain numerous pores through which the odours can enter the sensillum and reach the olfactory receptor neuron membrane. Despite the fact that these mosquitoes are so important for the transmission of malaria, hardly any sensory studies have been performed to date. Therefore, the goal of this study was to analyze the response spectra and characteristics of the olfactory receptor neurons encoding human-derived odours in female *An. gambiae*. Another goal of this study was the identification of human odours which guide female *An. gambiae* to its host. This was accomplished by making chemical analyses of the odour profile of human sweat.

Firstly, a scanning electron microscopic (SEM) study was undertaken to identify the different types of sensilla exhibited on the antennae of female *An. gambiae*. Chapter 2 shows SEM photographs of the six different types of antennal sensilla: large and small sensilla coeloconica, grooved peg sensilla, sensilla trichodea, sensilla ampullacea and sensilla chaetica. The distribution of the different sensilla on the thirteen segments is tentatively described. Odours present on the human skin or identified in the headspace of human sweat evoked responses of grooved peg- and sensilla trichodea receptor neurons (chapter 4 and 6). Although the grooved peg sensillum is easily recognized during SEM studies and light microscopic observations, the different subtypes of sensilla trichodea are hard to distinguish in *An. gambiae*. SEM photographs of two different types of sensilla trichodea housing receptor neurons responsive to sweat-borne components are shown in chapter 2.

The antennal olfactory responses of female *An. gambiae* were studied by means of electroantennography (chapter 3). The electroantennogram (EAG) is considered to be the summed activity of all (or a part of the) responsive sensory receptor neurons on the antenna. Initially, the technical aspects of the EAG recording technique were closely examined. It was found that when using tungsten electrodes, artefactual electrode potentials were generated by the carboxylic acids, propionic acid, butyric acid and hexanoic acid. No artefactual electrode potentials were obtained with glass electrodes. A blend of carboxylic acids has been reported to be attractive for female *An. gambiae* mosquitoes. These are present on the human skin where they display an enormous

diversity in chemical structure. To quantify the antennal olfactory sensitivity to carboxylic acids, EAG studies were conducted with glass electrodes. Carboxylic acids with carbon chain lengths of 5-8 (C_5-C_8) elicited high EAG amplitudes, while lower responses were evoked by the less volatile acids, C_9-C_{14}. Hexanoic acid evoked the highest EAG response.

Single sensillum studies were undertaken to reveal antennal olfactory receptor neurons responsive to carboxylic acids (chapter 4). Neurons innervating one or two of the morphologically different subtypes sensilla trichodea were found to respond to the short chain carboxylic acids: acetic acid (C_2), propionic acid (C_3), butyric acid (C_4), iso-butyric acid (iC_4) and iso-valeric acid (iC_5). Usually the receptor neurons responded by inhibition, but receptor neurons were also found responding by excitation to the short chain carboxylic acids. Occasionally receptor neurons were found which responded by excitation to 1-octen-3-ol. Dose-response characteristics were assessed for the carboxylic acid-inhibited cell type. It was demonstrated that the carboxylic acid-inhibited neuron was equally sensitive to the short chain acids tested. This was revealed by making corrections for the differences in volatility of the different short chain acids. It is suggested that in this case an inhibitory response might function to block the response of a specialised cell normally responding by excitation to other stimuli (chapter 7).

Because behavioural studies indicated that in addition to carboxylic acids other components are involved in the host-seeking behaviour of female *An. gambiae*, studies were undertaken to identify new putative attractants (chapter 5). Rather than searching for minor differences in chemical odour profiles between different individuals, we focussed on components which are generally produced by every human host. Freshly collected pooled sweat samples obtained after physical exercise from a group of volunteers neither attracted female *An. gambiae* in a windtunnel bioassay, nor evoked a detectable EAG response. Incubation of the sweat samples, however, resulted in a behaviourally attractive source of volatiles which evoked reproducible EAG responses. Sweat obtained during physical exercise is most likely to originate from the eccrine and sebaceous glands. Several observations strongly indicated that during incubation attractants are produced by microbial activity. Although the fresh sweat did not elicit a behavioural or EAG response, it was possible that it may contain components which acted as synergists at the behavioural level together with components produced during incubation. Headspace analysis of the fresh and incubated sweat revealed that geranyl acetone (5%-6%) and 6-methyl-5-hepten-2-one (1.8%-1.9%) were relatively abundant in both the fresh and incubated headspace samples. Headspace samples of the incubated sweat comprised large

amounts of indole (27.9%), 1-dodecanol (22.4%) and 3-methyl-1-butanol (10%). These components were absent or only present in minor amounts in the headspace samples of the fresh sweat. Indole, geranyl acetone and 6-methyl-5-hepten-2-one evoked an EAG response, while 1-dodecanol did not elicit any response. 3-Methyl-1-butanol was only tested at the single cell level (chapter 6).

In order to reveal olfactory receptor neurons responsive to the identified sweat-borne components, studies were conducted at the single cell level (chapter 6). For other mosquito species, such as *Aedes aegypti*, the grooved peg sensillum is considered to house receptor neurons sensitive to host odours. Indeed, incubated sweat elicited excitation of a receptor neuron innervating a subpopulation of the grooved peg sensilla in *An. gambiae*. The same receptor neuron was excited by ammonia, which was found to be present in large amounts in the incubated sweat. This strongly implies that ammonia is causing (most of) the attractiveness of the incubated sweat.

However, chemically identified components from the headspace of the fresh and incubated sweat (chapter 5) did not elicit responses of grooved peg receptor neurons. They evoked excitation of receptor neurons associated with the sensilla trichodea. Two different subpopulations were found, one was innervated by receptor neurons sensitive to indole, while the other subpopulation housed receptor neurons sensitive to geranyl acetone. 3-Methyl-1-butanol and 6-methyl-5-hepten-2-one elicited responses of receptor neurons associated with both subpopulations. Receptor neurons displayed lower sensitivity to 3-methyl-1-butanol and 6-methyl-5-hepten-2-one. Sensilla trichodea receptor neurons only occasionally responded to the source of the components, the incubated sweat.

It is suggested that not only the grooved peg receptor neurons encode host-odours but also the sensilla trichodea receptor neurons fulfill a function. The incubated sweat may evoke an increase in the spike frequency of only a few spikes per second and responses like these are very hard to detect. Another explanation might be that sensilla trichodea receptor neurons encode other behavioural activities, such as nectar feeding or location of oviposition sites. Identified sweat-borne components are very likely of microbial origin. Microbial products are very common in nature and therefore not restricted to human emanations (chapter 6).

Further studies on the behavioural level might elucidate the role of the identified sweat-borne components for the behaviour of *An. gambiae*. The function of the different olfactory receptor neurons in host-seeking *An. gambiae* is further discussed in chapter 7.

Samenvatting

Vrouwelijke muggen van de soort *Anopheles gambiae* Giles *sensu stricto* zijn belangrijke vectoren van *Plasmodium falciparum*, de belangrijkste verwekker van malaria bij de mens in Afrika. Het wordt over het algemeen aangenomen dat zij hun menselijke gastheer opsporen door middel van geuren. Deze geuren worden gedetecteerd door de geur-gevoelige receptor neuronen die zich bevinden in sensilla, cuticulaire uitstulpingen van de antenne. Deze sensilla bezitten talrijke porieën waardoor de geuren het sensillum kunnen binnenkomen, waar ze vervolgens de membraan van het olfactorisch receptor-neuron bereiken. Ondanks het feit dat *Anopheles gambiae* een zeer belangrijke rol speelt in de transmissie van malaria waren er bij de aanvang van het hier beschreven onderzoek nauwelijks enige sensorische studies aan deze muggensoort uitgevoerd. Het doel van dit proefschrift was het bestuderen van de respons-spectra en -karakteristieken van de geur-gevoelige receptor neuronen, van vrouwelijke muggen van de soort *Anopheles gambiae*, die gevoelig zijn voor menselijke geuren. Een ander doel van deze studie was de identificatie van die menselijke geuren die *An. gambiae* gebruikt voor het vinden van zijn gastheer. Dit werd bewerkstelligd door het analyseren van geur-profielen van menselijk zweet.

Ten eerste werd een scanning elektronen microscopische studie (SEM) ondernomen om de verschillende typen sensilla op de antenne van *An. gambiae* te identificeren. Hoofdstuk 2 toont SEM-foto's van de 6 verschillende typen antennale sensilla: grote en kleine sensilla coeloconica, "grooved peg" sensilla, sensilla trichodea, sensilla ampullacea en de sensilla chaetica. De verdeling van de verschillende sensilla over de dertien segmenten is globaal beschreven. Geuren die voorkomen op de menselijke huid of die geïdentificeerd werden in de headspace van menselijk zweet wekten responsen op in receptor-neuronen geassocieerd met "grooved peg" sensilla en sensilla trichodea (hoofdstuk 4 en 6). Alhoewel het "grooved peg" sensillum makkelijk herkend kan worden gedurende SEM studies en licht-microscopische observaties, zijn de verschillende subtypen sensilla trichodea in *An. gambiae* moeilijk te onderscheiden. Hoofdstuk 2 toont SEM-foto's van twee verschillende typen sensilla trichodea waarvan de receptor neuronen reageren op uit zweet afkomstige componenten.

De antennale geur-responsen van vrouwelijke *An. gambiae* muggen werden bestudeerd d.m.v. electroantennografie (hoofdstuk 3). Het electroantennogram (EAG) wordt beschouwd als de gesommeerde activiteit van alle (of een deel van de) responsieve sensorische neuronen aanwezig op de antenne. In eerste instantie werden de technische

aspecten van de EAG-recording techniek grondiger bestudeerd. Vastgesteld werd dat wanneer gebruikt werd gemaakt van wolfraam-elektroden, artefactuele elektrode potentialen werden gegenereerd door de carboxyl zuren propionzuur, boterzuur en hexaanzuur. Wanneer gebruik werd gemaakt van glas-elektroden werden geen artefactuele elektrode-potentialen verkregen. Uit gedragsstudies is gebleken dat een mengsel van carboxyl-zuren attractief is voor vrouwelijke *An. gambiae* muggen. Carboxyl-zuren zijn aanwezig op de menselijke huid waar ze een enorme variatie in chemische structuur ten toon spreiden. Om de antennale geurgevoeligheden voor carboxyl zuren te kwantificeren werden EAG studies uitgevoerd met glas-elektroden. Carboxyl zuren met koolstof-ketenlengtes van 5-8 (C_5-C_8) veroorzaken hoge EAG-amplitudes, terwijl lagere responsen werden opgewekt door de minder vluchtige zuren (C_9-C_{14}). Hexaanzuur gaf de hoogste EAG-respons.

Single-sensillum studies werden ondernomen om de antennale geur-gevoelige olfactorische neuronen te identificeren die reageren op carboxyl zuren (hoofdstuk 4). Het werd gevonden dat neuronen die een of mogelijk twee morfologisch herkenbare subtypen sensilla trichodea innerveren, reageerden op de kort-ketenige carboxyl zuren: azijnzuur (C_2), propion zuur (C_3), boterzuur (C_4), iso-boter zuur (iC_4), en iso-pentaan zuur (iC_5).

De receptor neuronen reageerden meestal met inhibitie, maar er werden ook receptor neuronen gevonden die in respons op de kort-ketenige carboxyl zuren reageerden met excitatie. Af en toe werden receptor neuronen gevonden die d.m.v. excitatie reageerden op 1-octen-3-ol. Dosis-response karakteristieken werden bepaald voor het carboxyl-zuur geïnhibeerde celtype. Het carboxyl zuur-geïnhibeerd neuron was in gelijke mate gevoelig voor de geteste kort-ketenige zuren. Dit werd uitgewezen door correcties te maken voor de verschillen in vluchtigheid voor de verschillende kort-ketenige zuren. Als hypothese werd geformuleerd dat in dit geval een inhibitie-type respons kan functioneren om de respons van de geinhibeerde cel, die mogelijk gespecialiseerd is voor andere vooralsnog onbekende stimuli en met excitatie op deze stimuli reageert, te blokkeren (hoofdstuk 7).

Naar aanleiding van gedragsstudies, die er op duiden dat naast carboxyl-zuren ook andere componenten een rol spelen in het gastheer-zoekgedrag van vrouwelijke *An. gambiae* muggen, werden studies ondernomen gericht op identificatie van nieuwe potentiële attractantia (hoofdstuk 5). Hierbij is er gericht gezocht naar die componenten die wel uit geïncubeerd zweet maar niet uit vers zweet vrijkwamen. Uit gedragsstudies was gebleken dat vers verzamelde gepoolde zweet samples verkregen na lichamelijke inspanning van een groep vrijwilligers niet aantrekkelijk waren voor *An. gambiae* in een

windtunnel set-up. Het bleek tevens dat deze verse monsters geen meetbare EAG responsen opwekten. Incubatie van de zweet samples resulteerde echter in een attractieve bron van geurstoffen die reproduceerbare EAG responsen opwekte. Zweet verkregen na lichamelijke inspanning is zeer waarschijnlijk afkomstig van de eccriene en sebum-klieren. Verscheidene observaties geven sterke aanwijzingen dat attractantia gedurende incubatie worden geproduceerd door microbiële activiteit. Alhoewel het verse zweet geen gedrags- of EAG respons opwekt, is het mogelijk dat het componenten bevat die op gedragsniveau synergistisch werken met componenten die geproduceerd worden gedurende incubatie. Uit headspace analyses van het verse en geïncubeerde zweet bleek dat geranyl aceton (5%-6% van de totale opbrengst) en 6-methyl-5-hepten-2-one (1.8%-1.9%) relatief veel voorkwamen in zowel de verse als de geïncubeerde headspace monsters. Headspace monsters van het geïncubeerde zweet bevatten grote hoeveelheden indool (27.9%), 1-dodecanol (22.4%) en 3-methyl-1-butanol (10%). Deze componenten waren afwezig of alleen in relatief kleine hoeveelheden aanwezig in de headspace monsters van het verse zweet. Indool, geranyl-aceton en 6-methyl-5-hepten-2-one gaven een EAG respons, terwijl 1-dodecanol geen respons veroorzaakte. 3-Methyl-1-butanol werd alleen getest op single cell niveau (hoofdstuk 6).

Om de geur-gevoelige receptor neuronen te identificeren die reageren op de uit zweet afkomstige geïdentificeerde componenten, werden op single cell-niveau studies ondernomen (hoofdstuk 6). Voor andere muggensoorten, zoals de gele koorts vector *Aedes aegypti*, wordt het "grooved peg" sensillum verondersteld receptor neuronen te bevatten die gevoelig zijn voor gastheer-geuren. Geïncubeerd zweet veroorzaakte inderdaad excitatie van een receptor neuron dat een subpopulatie van de "grooved peg" sensilla in *An. gambiae* innerveert. Ditzelfde receptor neuron werd geëxciteerd door ammonia, dat in grote hoeveelheden voorkomt in het geïncubeerde zweet. Dit is een sterke aanwijzing dat ammonia in belangrijke mate bijdraagt aan de attractiviteit van het geïncubeerde zweet.

Echter, chemisch geïdentificeerde componenten uit de headspace van het verse en geïncubeerde zweet (hoofdstuk 5) veroorzaakten geen responsen in "grooved peg" receptor neuronen. Deze componenten wekten excitatie op in receptor neuronen geassocieerd met sensilla trichodea. Twee verschillende subpopulaties werden gevonden, één subpopulatie werd geïnnerveerd door receptor neuronen gevoelig voor indool, terwijl de andere subpopulatie receptor neuronen bevatten die gevoelig bleken voor geranyl-aceton. 3-Methyl-1-butanol en 6-methyl-5-hepten-2-one veroorzaakten reacties van receptor neuronen geassocieerd met beide subpopulaties. Receptor neuronen vertoonden

een lagere gevoeligheid voor 3-methyl-1-butanol en 6-methyl-5-hepten-2-one. Sensilla trichodea receptor neuronen reageerden alleen in een aantal gevallen op de bron van de componenten, het gïncubeerde zweet.

Als hypothese werd geformuleerd dat niet alleen de "grooved peg" receptor neuronen coderen voor gastheer geuren, maar dat ook de sensilla trichodea in deze een functie vervullen. Het geïncubeerde zweet zou een verhoging van de spike frequentie van slechts enkele spikes per seconde kunnen opwekken; responsen zoals deze zijn zeer moeilijk te detecteren. Een andere verklaring kan zijn dat sensilla trichodea receptor neuronen coderen voor andere gedrags-activiteiten, zoals 'nectar feeding' of het zoeken van locaties voor ovipositie. Uit geïncubeerd zweet afkomstige geïdentificeerde componenten zijn zeer waarschijnlijk van microbiële oorsprong. Microbiële produkten, echter, komen overal voor in de natuur en zijn daarom niet beperkt tot menselijke uitscheidingen (hoofdstuk 6).

Verdere studies op gedrags-niveau zijn nodig om de rol van de uit zweet afkomstige geïdentificeerde componenten m.b.t. het gedrag van *An. gambiae* te kunnen ophelderen. De functie van de verschillende geur-gevoelige receptor neuronen in het gastheer zoekgedrag van *An. gambiae* is verder bediscussieerd in hoofdstuk 7.

Nawoord

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List of Publications

Knols BGJ and Meijerink J (1997) Odors influence mosquito behavior. *Science and Medicine* 4:56-63.

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

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

Meijerink J, Braks MAH, Brack A, Adam W, Dekker T, Posthumus MA,. Van Beek TA and Van Loon JJA. Identification of olfactory stimulants for *Anopheles gambiae* from human sweat samples. *Journal of Chemical Ecology* (accepted)

Meijerink J, Braks MAH and Van Loon JJA. Olfactory receptors on the antennae of the malaria mosquito *Anopheles gambiae* are sensitive to ammonia and other sweat-borne components. *Journal of Comparative Physiology A* (submitted)

Braks MAH, Meijerink J, Takken W. The role of human sweat components, ammonia and L-lactic acid, in the behaviour of the anthropophilic malaria mosquito, *Anopheles gambiae* (Diptera ; Culicidae). *Journal of Comparative Physiology A* (submitted)

Curriculum vitae

Jocelijn Meijerink was born on 31-08-1966 in Sittard, The Netherlands. In 1984 she graduated from Scholengemeenschap Groenewald (VWO-B) in Stein.
From 1984 to 1985 she studied music at the Conservatorium in Maastricht and from 1985 to 1986 she studied architecture at the Technical University Delft.

In 1986 she started the biology curriculum at the University of Utrecht. The specialisations during her MSc ('doctoraal') study were microbiology, performed at the Veterinary faculty at the University of Utrecht and physiology, performed at the Hubrecht Laboratory, Netherlands Institute for Developmental Biology in Utrecht. She graduated in 1992. In 1991-1992 she was employed for 6 months within a research project during the International Microgravity Laboratory I project of the space-shuttle flight the Discovery, Cape Canaveral, Florida, USA. In 1993 she joined a research project on the electrophysiology of zebrafish neural crest cells at the Netherlands Institute for Developmental Biology in Utrecht.

From 1994 to 1999 she conducted a PhD study at the Wageningen Agricultural University, Department of Entomology. This project concerned an electrophysiological study on the olfactory receptor neurons of *Anopheles gambiae*. In 1995 two months were spent at the Natural Resources Institute in Chatham, Great Britain. In 1998 she visited the International Centre for Insect Physiology and Ecology in Nairobi, Kenya for a short research period.