3. Olfactory physiology of blood-feeding vector mosquitoes

Yu Tong Qiu and Joop J.A. van Loon

Abstract

The olfactory organs of blood-feeding mosquitoes are the antennae, maxillary palps and proboscis. On each of these organs, several morphologically distinct types of multiporous sensilla are present that typically house two to four olfactory receptor neurons (ORNs). Electrophysiological studies of ORN responsiveness have allowed the identification of several functional ORN-classes within each sensillum type. Functional ORN-types are distinguished based on their unique spectrum of responses to a set of volatile organic compounds (VOCs). Each neuron expresses one or a few membrane-bound olfactory receptor (OR) proteins that function as the molecular determinant of the response specificity of the ORN. Genomic analysis has identified 79 and 131 candidate OR-genes in the malaria mosquito *Anopheles gambiae* and the yellow fever mosquito *Aedes aegypti*, respectively. In the sensillum lymph surrounding the dendrites, water-soluble odorant-binding proteins (OBPs) fulfil roles in transport and inactivation of VOCs. In *Anopheles gambiae* 57 OBP-genes have been found and in *Ae. aegypti* this number is 66. The ORNs have been shown to encode odour quality, i.e. molecular structure, odour concentration as well as temporal changes in concentration and spatial distribution. Responses of ORNs belong to two major types: some odour stimuli elicit excitation whereas other odours cause inhibition relative to the pre-stimulus activity. Both response modes are controlled by the same OR. Both generalist ORNs and specialist ORNs are found. The responses of ORNs to odorants are concentration-dependent above a threshold concentration, and both absolute concentration and the change in concentration can be encoded. The ensemble of mosquito ORNs responds to odours emitted by hosts, plants and oviposition sites, which together harbour a substantial diversity of molecular structures. Yet, the number of VOCs tested on the full array of ORN functional types of any mosquito species is still far from exhaustive and rarely exceeds 100 VOCs. A substantial concerted effort of molecular biologists, electrophysiologists and ethologists lies ahead to further unravel the mosquito olfactory system. The information thus obtained is essential for the development of behavioural disruption methods that will contribute to the control of mosquito vector populations.

Keywords: sensillum, neuron coding, odour, receptor

Introduction

Blood feeding mosquitoes are disagreeable to humans not only due to their annoying biting habit but especially because many species transmit fatal human diseases such as malaria, yellow fever, dengue and West Nile virus. Olfaction is essential in the life of a mosquito to ensure survival and successful reproduction. Mosquitoes largely rely on the sense of smell to find a mate, nectar, blood and oviposition sites. Understanding the mechanisms of mosquito olfaction and odour coding is crucial to develop measures based on behavioural disruption to control mosquitoes. Because mosquito species that act as vectors of infectious diseases belong predominantly to the genera *Anopheles, Aedes* and *Culex*, we restrict ourselves mainly to these three mosquito genera in this chapter.

Location and morphology of olfactory organs in adult mosquitoes

The main olfactory organs of insects are the antenna and mouthparts (Figure 1), which carry specialised cuticular extensions, the so-called sensilla that house olfactory receptor neurons (ORNs). The majority of olfactory sensilla of adult mosquitoes are situated on the antennae, yet a smaller number is located on the maxillary palps and the proboscis. Female mosquitoes possess
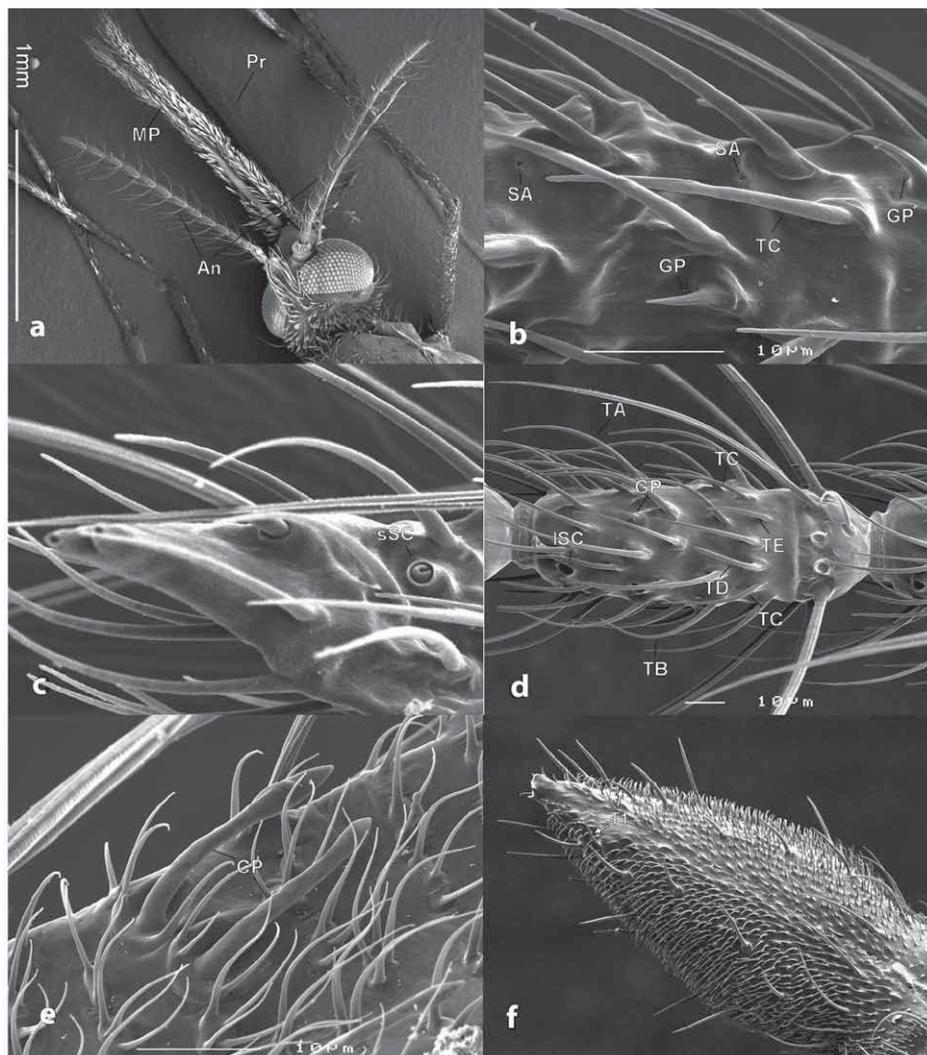

Figure 1. Scanning electron micrograph of olfactory organs of female Anopheles gambiae. *(a) The head showing An: antenna; MP: maxillary palp; Pr: proboscis. (b) Sensilla on the antennae. SA: sensillum ampullacea; GP: grooved peg sensillum; TC: sensillum trichodea subtype C. (c) The 13th segment of the antenna. sSC: small sensillum coeloconicum. (d) One segment of the antenna. lSC: large sensillum coeloconicum; TA, TB, TC, TD or TE: sensillum trichodea subtype A, B, C, D or E. (e) Capitate peg sensilla (CP) on the maxillary palps. (f) The tip of the proboscis showing the olfactory sensilla T1 (SEM photos provided by Hans Smid).*

a much larger number of olfactory sensilla than their male counterparts (McIver 1982, Table 1). Highly specialised in detecting the wing beat sounds from conspecific female mosquitoes (Cator *et al.* 2009), the antennae of male mosquitoes are inhabited by fibrillae on most of the antennal segments, only the proximal and the two most distal segments are populated with olfactory sensilla (Ismail 1962).

Olfactory sensilla are hair- or peg-shaped and have a multiporous wall structure. A typical insect olfactory sensillum is composed of two or more bipolar olfactory receptor neurons (ORNs), although sensilla containing one neuron have also been described (McIver 1982), three auxiliary cells and the surrounding glia, epidermis and cuticle (Figure 2). The surface of the insect cuticle is hydrophobic, a property reducing water evaporation and increasing water repellency. Odour molecules, many of which are non-polar, first have to penetrate through the sensillum wall before reaching the ORNs. There are two types of sensillum walls: single-walled olfactory sensilla contain pore channels through which the non-polar components are excreted to the epicuticle during sensillum formation (Figure 2). Pore channels that remain open are later used for the transportation of the odour molecules into the sensillum lymph (Steinbrecht 1997). In contrast, a double-walled olfactory sensillum is composed of hollow cuticular finger-like structures, which are fused to each other and form at the fusion points, spoke-channels. It is likely that odour molecules get into the sensillum lumen of double-walled sensilla via these channels (Steinbrecht 1997).

Antennal sensilla

The antennae of adult mosquitoes bear five types of sensilla: chaetica, coeloconica, ampullacea, trichodea, and grooved pegs (Figure 1). The latter type is also referred to as sensilla basiconica in some references, which may be confused with the single-walled basiconic sensilla; therefore here we only use the name grooved peg. Sensilla chaetica are mechanosensilla with a thick wall and a socket at their base. Anophelines are the only mosquitoes in the family of Culicidae that have a large type of coeloconic sensilla. Although both large and small sensilla coeloconica have a 'peg in pit' structure, they differ from each other both morphologically and functionally. The small

Table 1. Numbers of olfactory sensilla of mosquito species located on the antennae and maxillary palps (McIver 1982).

| Species	Palpi		Antenna					
			Trichodea		Grooved peg		Large coeloconica	
	♀	♂	♀	♂	♀	♂	♀	♂
Anopheles gambiae	67	14	629	227	84	20	33	14
Anopheles maculipennis	65	13	618	300	62	27	28	12
Anopheles stephensi	98	9	573	194	107	28	32	8
Aedes aegypti	29	17	532	175	105	36	0	0
Culex pipiens	78	15	901	239	265	95	0	0
Culex territans	39	15	537	216	275	174	0	0
Mansonia africana	68	21	623	147	208	36	0	0
Mansonia uniformis	75	17	571	164	212	39	0	0
Wyomia smithii	34	20	218	189	36	30	0	0
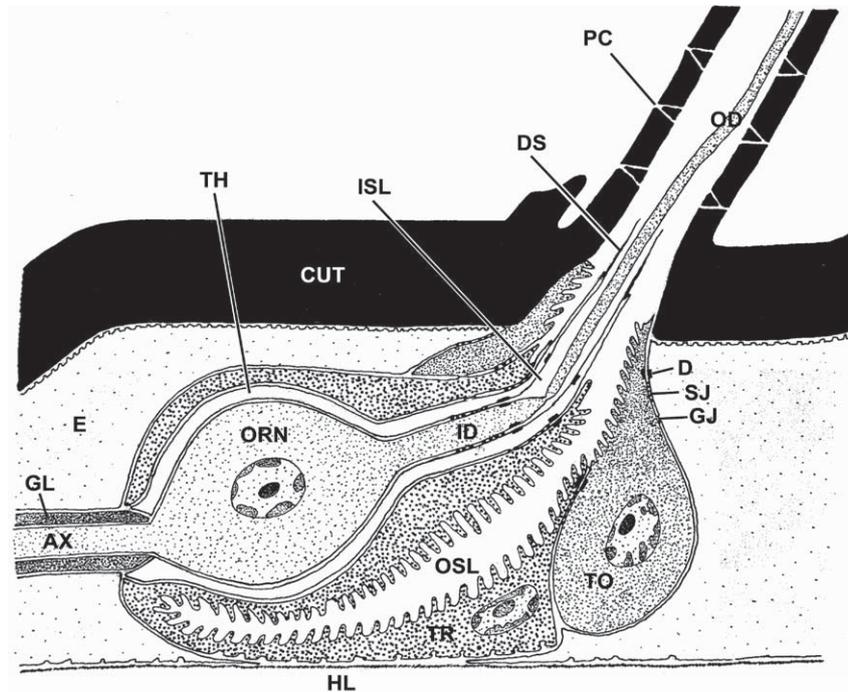
Figure 2. Schematic drawing of the structure of typical olfactory sensilla. AX: axon; CUT: cuticle; D: desmosomes; DS: dendrite sheath; E: epidermis; GJ: gap junctions; GL: glia; HL: haemolymph; ID: inner dendritic segment; ISL: inner sensillum lymph space; OD: outer dendritic segment = ciliary dendrite; ORN: olfactory receptor neuron; OSL: outer sensillum lymph space; PC: pore channel; SJ: septate junction; TH: thecogen cell; TO: tormogen cell; TR: trichogen cell (from Qiu 2005 after Keil and Steinbrecht 1987).

coeloconica contain a single-walled peg, with possibly one or a few pores at the tip (Clements 1999, McIver 1982). A pair of small coeloconic sensilla is located at the tip of mosquito antennae, occasionally these sensilla are found on other antennal segments (McIver 1982, Pitts and Zwiebel 2006). Two of the three neurons innervating these small sensilla coeloconica were found to be sensitive to convection heat, whereas none of the three neurons are olfactory (Davis and Sokolove 1975, Gingl *et al.* 2005, Wang *et al.* 2009). Sensilla ampullacea are thought to function as convection heat detectors because they have a structure similar to that of small sensilla coeloconica (McIver 1982). Only sensilla trichodea, grooved peg sensilla and large coeloconica are innervated by olfactory neurons.

Sensilla trichodea

The most abundant type of antennal sensillum of mosquitoes are sensilla trichodea (Figure 1b, 1c), which are single-walled olfactory sensilla normally containing dendrites of two ORNs, occasionally sensilla trichodea innervated by a single neuron can be found on the most distal antennal flagella (Boo 1980a, McIver 1982) (Figure 3a, 3c). The average number of sensilla trichodea in a species ranges from 200 to 1000 (Table 1, based on McIver (1982). Mosquito antennae carry several different shapes of sensilla trichodea, each of which has distinct functional characteristics. Culicines have
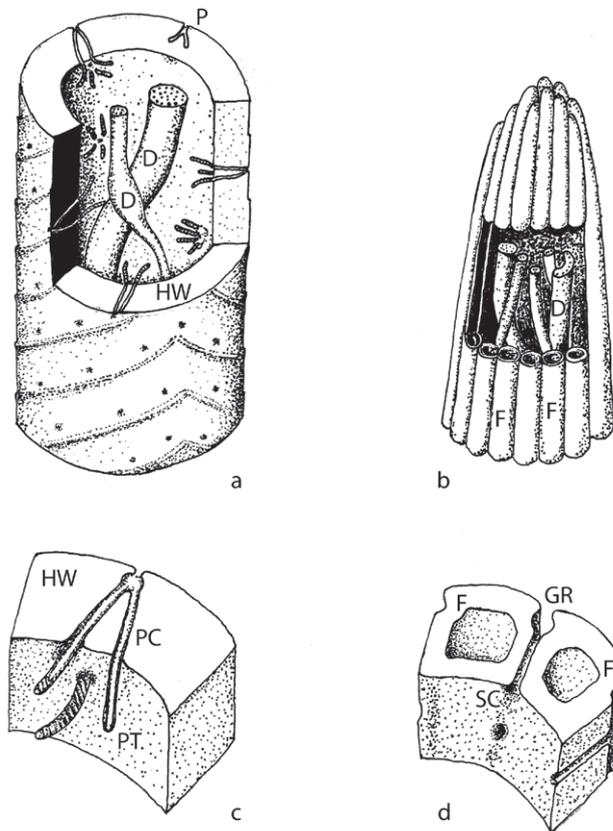
Figure 3 Schematic diagrams of single- and double-wall structure of olfactory sensilla. (a) Section through a sensillum trichodeum (single-walled) and pore tubules. HW: hair wall; D: dendrite; P: pores. (b) Coeloconic sensillum (double-walled), opened to show dendrites therein. The cuticular fingers (F) are hollow and partly fused. (c) Detail of wall of sensillum trichodeum. PC: pore channel; PT: pore tubules. (d) Detail of the double wall of a coeloconic sensillum. SC: spoke channels; F: fingers; GR: groove (from Qiu 2005 after Keil 1999).

basically four types of sensilla trichodea: T1, T2, T3 and T4, classified according to their length, wall thickness and whether the tip is sharp or blunt (reviewed by McIver 1982). This classification does not fit well for anophelines. Transmission microscope studies by Boo (1980a,b) identified five types of sensilla trichodea, designated A – E, on the antennae of *Anopheles stephensi* Liston according to hair length, shape, diameter, wall thickness, neuron branching and pore channel density.

Van den Broek and Den Otter (1999) compared the sensitivities of ORNs in four anopheline species with host preferences ranging from zoophilic to anthropophilic and found differences in the number and sensitivity of ORNs in sensilla trichodea responding to carboxylic acids and 1-octen-3-ol.

Grooved pegs

Grooved peg sensilla are double-walled sensilla containing 2 to 5 neurons one of which is assumed to be a hygroreceptor (Figure 3b, 3d). In *Aedes* and *Culex* species, grooved peg sensilla show great variation in peg length and it was suggested that two types exist and that the groove length correlated with the sensitivity of innervating neurons to lactic acid (Bowen 1995). However, large overlap occurs between length distributions of pegs with different lactic acid sensitivity.

Large coeloconic sensilla

The large coeloconic sensilla are only found on the antennae of anopheline mosquitoes (Table 1, McIver 1982). Double-walled pegs are contained in sunken pits. The number of large coeloconic sensilla on each antenna of female mosquitoes range from 28 for *An. maculipennis* Meigen to 50 for *An. ziemanni* Grünberg and these are innervated by four or five neurons, the function of which has not been studied.

Maxillary palps and proboscis

On the maxillary palps the only sensilla innervated by ORNs are the capitate peg sensilla, which in female anophelines are located on the ventral side of palp segments 2-4 and in male anophelines on segment 4 (McIver and Siemicki 1975). In both sexes of *Culex* and *Aedes* mosquitoes capitate pegs are located on segment 4 (reviewed by McIver 1982). Three neurons are co-compartmentalised in each capitate peg sensillum. The dendrite of one neuron forms numerous lamellae (reviewed by McIver 1982).

At the tip of the labellar lobe there are three types of trichoid sensilla, two of which (T1 and T2) are located externally and one (T3) internally (McIver 1982, Pappas and Larsen 1976). These sensilla were thought to contain mechanical and gustatory neurons, however, a recent study revealed that T2 sensilla contain olfactory receptor proteins and have an olfactory function (Kwon *et al.* 2006).

Olfactory transduction

Transduction is the process by which quality (molecular structure) and quantity (concentration) of odorants are converted to the neural code contained in the frequency and temporal patterns of action potentials (see Chapter 4, this volume). The nature of this process is considered to be common to all insects, although most information is derived from studies on Lepidoptera and the fruit fly, *Drosophila melanogaster* Meigen (Rutzler and Zwiebel 2005). The sensillum lymph of olfactory sensilla contains water soluble proteins, called odorant binding proteins (OBPs) that bind odour molecules and transport them to receptor molecules on the dendritic membrane (Klein 1987, Vogt and Riddiford 1981). The possible functions of OBPs include: selective binding with odour molecules; binding with irrelevant or harmful compounds to reduce their chance of coming into contact with the dendritic membrane (Park *et al.* 2000); selective transport of odour molecules to specific receptor molecules on the dendritic membrane and selective inactivation of odour molecules (Steinbrecht 1998). Based on the presence of six conserved cystein residues and a conserved spacing between the cysteins, 57 putative OBP-genes in *An. gambiae* Giles and 66 in *Aedes aegypti* (L.) have been identified (Xu *et al.* 2003, Zhou *et al.* 2008).

On the plasma membrane of the olfactory neuron dendrite one to three ligand-binding olfactory receptor (OR) genes are co-expressed with a conservative co-receptor gene, the Or83b family

in *Drosophila* and AgOR7 in *An. gambiae*. Both OR protein and the co-receptor protein have a seven-transmembrane domain, but unlike the vertebrate G-protein-coupled receptors (GPCRs) their amino termini locate intracellularly. From the genome of *An. gambiae*, 276 G-protein-coupled receptor homologous genes were identified and 79 of these are considered as putative OR-genes (Hill *et al.* 2002). Recently, 131 putative odorant receptor genes have been identified from the genome sequence of *Ae. aegypti* (Bohbot *et al.* 2007; Chapter 2, this Volume). In *An. gambiae* conventional AgORs are co-expressed in ORNs with a highly conserved OR gene, AgOR7, and form a heterodimer (Pitts *et al.* 2004). Recent studies suggest that insect ORs respond to odour ligands along two distinct pathways (Sato *et al.* 2008, Wicher *et al.* 2008). Firstly, insect OR heterodimers function as a ligand-gated ion channel. Secondly, the OR heterodimers also follow a metabotropic pathway by forming a non-selective cation channel activated by cyclic nucleotides and odorant-sensing units. When the depolarisation of the membrane resulting from the respective ion flows reaches a threshold, action potentials are generated at the point where the axon exits the perikaryon and travel along the axon, which projects to the antennal lobe in the brain (see Chapter 4, this volume).

Peripheral odour coding

Properties of odour coding

By using the single sensillum recording (SSR) method, the action potentials of ORNs can be recorded *in situ*; their specificity and sensitivity in response to odour stimuli can be studied by quantifying the frequency of action potentials over defined post-stimulus time intervals. ORNs have been shown to encode odour quality, concentration as well as temporal changes in odour concentration and spatial distribution (De Bruyne *et al.* 2001, Heinbockel and Kaissling 1996, Mustaparta 2002).

ORNs respond selectively to odour compounds with certain structural features such as chain length, electron cloud distribution, the position of double bonds and functional groups of the odour molecule (Boeckh and Ernst 1983, Liliefors *et al.* 1985, 1987, Shields and Hildebrand 2001). The specificity range or odour tuning width of an ORN is largely determined by the olfactory receptor that is expressed therein. An elegant method to study the function of an single OR-gene is to delete the endogenous OR gene expressed in a specified neuron in the *D. melanogaster* antenna and to replace it by a single exogenous OR gene (Dobritsa *et al.* 2003, Hallem *et al.* 2004a). Not only the differences but also the overlap of the response spectra between different ORNs is important information for the central nervous system to discriminate the quality of an odour compound (De Bruyne *et al.* 2001, Den Otter and Van der Goes van Naters 1993, Sass 1978). These ORN properties allow several degrees of freedom for odour coding. Based on the response spectra of ORNs to a panel of odorant stimuli, ORNs and the sensilla containing them can be classified into different functional groups (De Bruyne *et al.* 2001, Ghaninia *et al.* 2007, Qiu *et al.* 2006).

ORN responses belong to two major types: some odour stimuli elicit excitation whereas other odours cause inhibition relative to the pre-stimulus activity (De Bruyne *et al.* 2001, Ghaninia *et al.* 2007, Qiu *et al.* 2006, Shields and Hildebrand 2001). This process is controlled by the same OR and can be explained by a simple model (Hallem *et al.* 2004b). Without odour stimulus, ORs are present as two forms in homeostasis: active and inactive. Once an OR binds with a ligand that elicits an excitation response, the active form is stabilised and the firing frequency is increased; in case an OR binds with a ligand that elicits an inhibition-type response, the inactive form is stabilised and the firing frequency is decreased.

The responses of ORNs to odorants are concentration-dependent above a threshold concentration, and both absolute concentration and the change in concentration can be detected, provided that the quality of the odorant is coded independently (Todd *et al.* 1992). It seems that pheromone-sensitive-ORNs in lepidopterans are only sensitive to the concentration change (Kaissling 1998). Carbon dioxide-sensitive receptor neurons of some insect species can detect both absolute and relative concentrations (Bogner 1992, Bogner *et al.* 1986, Den Otter and Van der Goes van Naters 1992, Grant *et al.* 1995). More ORs are activated when exposed to higher concentrations of odours, which might be a mechanism for insects to detect the absolute odour concentrations (Hallem *et al.* 2004a).

In most cases two or more ORNs are co-compartmentalised in one sensillum providing the nervous system with one more dimension for odour coding (Todd and Baker 1999). Such an arrangement might be particularly important in the detection of odorant mixtures. The fact that sex pheromone-sensitive receptor neurons in moths, which respond behaviourally to a species-specific ratio of often two major components, are commonly housed in the same sensillum, implies a function of co-compartmentalisation in discrimination of ratios between components of odour blends (Baker *et al.* 1988, Cossé *et al.* 1995, Hansson *et al.* 1987, O'Connell 1975).

Temporal characteristics of ORN responses to odours are another feature of odour coding, which is especially important to enable odour discrimination while flying or walking upwind (Baker 1985, Kennedy *et al.* 1981, Kramer 1992). A response is called phasic if the frequency of firing action potentials decreases abruptly shortly after the onset of the excitation response. A tonic response is characterised by an increase in firing frequency that outlasts the duration of stimulation. The temporal response characteristics of an ORN to a certain stimulus seem largely independent of dosage (De Bruyne *et al.* 2001, Qiu *et al.* 2006).

Natural odours utilised by mosquitoes

Flower- and honeydew-associated odours

To meet their energetic needs, both female and male mosquitoes of all physiological stages need carbohydrates, obtained mainly from floral and extra-floral nectar and honeydew (Foster 1995 2008). Flower extracts and synthetic plant odours were shown to attract mosquitoes (Foster 2008, Foster and Hancock 1994, Hancock and Foster 1993, Healy and Jepson 1988, Jepson and Healy 1988, Mauer *et al.* 1999). Information on the compounds in floral fragrances attractive to mosquitoes is scarce (Jhumur *et al.* 2008). Floral fragrances are composed of a rich diversity of compounds among which terpenes, phenols, aldehydes, fatty acid derivatives and benzenoids are most likely attractants of mosquitoes (Foster 1995, Jhumur *et al.* 2008)

Human odours

Female mosquitoes are guided to their blood hosts by the physical and chemical cues emanating from these hosts. Heat, moisture and visual cues from blood hosts are perceived by searching female mosquitoes at a close range. Gillies and Wilkes (1968) reported that carbon dioxide could attract mosquitoes at distances of 18-36 m and natural odours from a calf at distances of 54-73 m.

Human odour is composed of a complex of volatiles released from human skin and exhaled in human breath. GC-MS analysis of human skin emanations collected on glass beads revealed 346 compound peaks (Bernier *et al.* 2000). More than 100 compounds were identified from exhaled

human breath (Krotoszynski *et al.* 1977). Ellin *et al.* (1974) analysed the composition of total human body effluvia and identified 135 out of more than 300 compounds detected.

Human skin secretes higher amounts of lactic acid compared to other vertebrates, which compound proved to be a mosquito attractant (Dekker *et al.* 2002, Smith *et al.* 1970). Lactic acid is attractive by itself for *Ae. aegypti* (Smith *et al.* 1970) but not for *An. gambiae,* whereas a synergistic effect was found between lactic acid, ammonia and a mixture of aliphatic carboxylic acids (Smallegange *et al.* 2002). Braks and Takken (1999) reported that incubated human sweat was attractive to *An. gambiae* and that most of this effect could be ascribed to the emission of ammonia produced by microbial activity in sweat. When freshly produced sweat was incubated, the abundance of other components, such as indole, 1-dodecanol, 6-methyl-5-hepten-2-one and geranyl acetone, changed. Incubated sweat was found to be highly attractive whereas the attraction to fresh sweat was weak (Meijerink *et al.* 2000).

Female mosquitoes were strongly attracted to nylon stockings worn by a human (Pates *et al.* 2001). GC-MS analysis of the extracts of Limburger cheese, which resembles, to a human nose, the smell of unwashed human feet, revealed the major components to be carboxylic acids (Knols *et al.* 1997). Forty compounds were identified from a diethyl-ether extract of human sweat, the major components being aliphatic carboxylic acids (Healy and Copland 2000). Oxocarboxylic acids were found in human blood and urine (Chalmers and Lawson 1982). Six oxocarboxylic acids were reported to stimulate landing responses of *An. gambiae* (Healy *et al.* 2002). Chemical analysis revealed that the typical human axillary odour is composed of C_6 to C_{11} straight-chain, branched and unsaturated acids (Zeng *et al.* 1991, 1996).

Oviposition-site related odours

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

Mosquito breeding sites produce semiochemicals, mainly of microbial origin, that stimulate mosquito oviposition (Beehler *et al.* 1994a, Hasselschwert and Rockett 1988, Hazard *et al.* 1967). Infusions from decaying wood were found attractive to gravid *Ae. triseriatus* (Say) and an active component was identified as *p*-cresol (4-methylphenol) (Bentley *et al.* 1979, 1981). Grass infusions were shown to contain oviposition stimuli for *Culex* mosquitoes, the attractive compounds include, among others, 3-methylindole, 4-methylphenol and indole (Du and Millar 1999, Millar *et al.* 1992, Mboera *et al.* 2000, Lindh *et al.* 2008). The behavioural responses of mosquitoes to olfactory cues is reviewed in detail in the laboratory (Chapter 7), and in the (semi-)field (Chapter 8) elsewhere in this Volume.

Response spectra of mosquito ORNs

The response characteristics of ORNs are determined by the ORs expressed therein (Hallem *et al.* 2004b). Normally ORNs in morphologically different sensilla have different response spectra indicating that they express different ORs. Moreover, sensilla that appear morphologically similar may house ORNs with different response spectra.

ORNs can be classified according to their spectrum of response specificity to a panel of odour stimulants. The selection of the odour panel has a direct bearing on the classification. An ideal odour panel should contain the best stimulant of each ORN under investigation. Unfortunately, which stimulants are the best for the ORNs is unknown beforehand and in fact it is the central research question that first needs to be answered. In most studies, odours that are known to elicit behavioural activities and odours that typically occur in volatile blends emitted by nectar, hosts or oviposition sites are included in the test panel. However, these odours are not necessarily the best stimulants for the ORNs. For example, the most potent stimulant of the C neuron innervating capitate peg sensilla on the maxillary palps of female *An. gambiae* is 2,4,5-trimethylthiazole which is a flavour compound and has not previously been identified as a mosquito infochemical (Lu *et al.* 2007).

Anopheline and culicine mosquitoes show similarities as well as differences in the subtypes of olfactory sensilla and the function of ORNs. We summarised the results of several electro-physiological studies on different morphological subtypes of sensilla trichodea and grooved peg sensilla in both mosquito families in an attempt to make a comparison (Table 2). For examples,

Table 2. Function of ORNs in sensilla trichodea and grooved peg sensilla in the mosquito Subfamilies Culicinae and Anophelinae.

Mosquito subfamily	Sensilla	Subtype	Functional types
Culicinae	trichodea	short sharp-tipped	
			sst1
			sst2
			sst3
			sst4
		long sharp-tipped	
		blunt I (long)	
			sbtI1
			sbtI2
		blunt II (short)	
			sbtII1
			sbtII2
	grooved peg	lactic acid excited	
		lactic acid non-responding	
		lactic acid inhibited	

ORNs sensitive to ammonia and lactic acid are found in grooved peg sensilla in both families; indole-sensitive ORNs are found in short sharp-tipped sensilla trichodea; four functional types have been found in the short sharp-tipped ST in *Ae. aegypti*, whereas two were found in a similar morphological type of ST in *An. gambiae*. This comparison is necessarily limited at this time because (1) not all sensillar sub-types have been studied; (2) in anophelines three additional morphological ST-subtypes are present; (3) disparate odour panels have been used in different studies.

Sensilla trichodea (ST)

Among the five subtypes of ST (A-E) of anophelines only the medium-length sharp-tipped subtype C and short sharp-tipped subtype E have been well studied for their function. By testing the response of olfactory sensilla to a panel of 44 compounds that are components of human odour, plant volatile blends or derived from oviposition sites, Qiu *et al.* (2006) identified four functional types among ST subtype C and two functional types among subtype E in female *An. gambiae*. 'Generalist' ORNs that are tuned to a broad range of odours were found in ST subtype E, whereas 'moderate specialist' ORNs that are tuned to a narrow range of odours were found

Compounds eliciting		References
Excitation	Inhibition	
ethyl propanoate, 4-methylcyclohexanol		Kuthiala *et al.* 1992
4-methylcyclohexanol; 2-butoxyethanol; indole; ethyl lactate	2-3C acids	Ghaninia *et al.* 2007
2-butoxyethanol; indole		Ghaninia *et al.* 2007
4-methylcyclohexanol; 2-butoxyethanol; α-thujone; ethyl butarate; ethyl propione	3C, 6C acids	Ghaninia *et al.* 2007
4-methylcyclohexanol; 2-butoxyethanol; ethyl-L-lactate	several fatty acids and esters of fatty acids	Ghaninia *et al.* 2007
2-10C acids	volatile oils	Lacher 1967
7-10C acids; volatile oils; eugenol; citronellol	2-5C acids; eugenol; citronellol	Lacher 1967
4-methylcyclohexanol; 2-butoxyethanol; α-thujone; α-pinene; acetic acid; ethyl lactate; methyl-& ethyl butyrate		Ghaninia *et al.* 2007
ethyl butyrate; ethyl-L-lactate; ethyl propionate; 2-butoxyethanol		Ghaninia *et al.* 2007
esters of 2-4C acids; 1-octen-3-ol; 2-butoxyethanol; 4methylphenol; α-pinene	2-3C acids	
	2-3C acids; indole	Ghaninia *et al.* 2007
indole	propionic acid	Ghaninia *et al.* 2007
lactic acid; 2-3C acids; fluoro-lactate; ammonia	DEET; fluoro-lactate	Davis and Sokolove 1976
pyruvic acid; ammonia	2-3C acids; pyruvic acid; ethyl lactate; 2-butoxyethanol	Davis and Sokolove 1976
ammonia; water; pyruvic acid; acetic acid	lactic acid; DEET; fluro-lactate; pyruvic acid; 2-3C acids	Davis and Sokolove 1976

Table 2. Continued.

Mosquito subfamily	Sensilla	Subtype	Functional types
Anophelinae	trichodea	C (medium-length sharp-tipped)	TC1
			TC2
			TC3
			TC4
		E (short sharp-tipped)	
			TE1
			TE2
		C or E	
	grooved peg	GP1	
		GP2	
		GP3	
		GP4	
		GP5	

in subtype C, with one 'specialist' ORN tuned to only geranyl acetone. Phenols were among the most effective stimulants for several neuron types belonging to different functional classes in both subtype C and E. Subtype E, but not subtype C, also houses neurons responding to aliphatic carboxylic acids, oxocarboxylic acids as well as 1-octen-3-ol and its homologs. ORNs responding to 4-methylcyclohexanol and 4-methylphenol were found in short sharp-tipped ST of *An. stephensi* (Bentley *et al.* 1982).

The function of ORNs in ST with various shapes was reported for several species of culicines (Bentley *et al.* 1979, Bowen 1990, Davis 1976, Klowden and Blackmer 1987, Kuthiala *et al.* 1992, Lacher 1967) (Table 2). A recent study by Ghaninia *et al.* (2007) classified 11 functional groups within ST using a panel of 16 compounds from six different chemical groups (Table 2). The short sharp-tipped ST contains at least one neuron that is sensitive to 2-butoxy ethanol, which is a component of plant volatiles and attracts *Culex quinquefasciatus* Say (http: //www.pherobase. net/database/species/species-Culex-.php, accessed 25 August 2009). ORNs in the short sharp-tipped ST respond strongly to indole, 4-methylcyclohexanol and esters of short-chain carboxylic acids; these compounds stimulate oviposition by gravid culicine mosquitoes (Allan and Kline 1995, Beehler *et al.* 1994b, Bentley *et al.* 1982, Perry and Fay 1967), whereas indole is also a major

Compounds eliciting		References
Excitation	Inhibition	
ammonia; phenols		Qiu *et al.* 2006
geranyl acetone		Qiu *et al.* 2006
indole; phenols		Qiu *et al.* 2006
ammonia		Qiu *et al.* 2006
4-methylcyclohexanol; 4-methylphenol		Bentley *et al.* 1982
indole; phenols; 1-hexen-3-ol; 5-7C acids; 3-methyl-1-butanol, ammonia	3-4C acids	Qiu *et al.* 2006
4-methylphenol; 4-ethylphenol; geranyl acetone; 2-nonanone; 1-hexen-3-ol; 1-hepten-3-ol; 1-octen-3-ol; 7-octenoic acid; 3-methyl-2-hexenoic acid; 3C, 3M4C,6C, 9C, 10C, 12C acids; oxo-4-5C acids; ammonia		Qiu *et al.* 2006
geranyl acetone, 3-methyl-1-butanol, sulcatone, ammonia, 2-6C acids; 1-octen-3-ol; 3- & 4-methyl phenol	2-6C acids	Meijerink and van Loon 1999, Meijerink *et al.* 2001, Van den Broek and Den Otter 1999
ammonia; 4-5C amines		Qiu *et al.* 2006
ammonia; 4-5C amines; oxo-4-5C acids		Qiu *et al.* 2006
ammonia; 1-butylamine; 2-oxo-butanoic acid; lactic acid		Qiu *et al.* 2006
ammonia; 1-butylamine; 2-oxo-butanoic acid; lactic acid	3-6C and 9C acids	Qiu *et al.* 2006
ammonia; 4-5C amines; 4-6C acids		Qiu *et al.* 2006

component of aged human sweat (Meijerink *et al.* 2000). Short blunt-tipped ST contain neurons sensitive to esters of short-chain carboxylic acids, 4-methylphenol and alcohols, the former two types of compounds were reported to attract gravid mosquitoes and considered to be oviposition stimulants (Bentley *et al.* 1979, Davis 1976, Klowden and Blackmer 1987). An ORN strongly tuned to the plant volatile, α-thujone, is also found in the short blunt-tipped ST (Ghaninia *et al.* 2007). The long blunt-tipped STs were found responding to C2-C5 carboxylic acids by inhibition, but to C7-C10 by excitation; they showed excitatory responses to terpineol but were inhibited by eugenol and citronellol, the latter three being terpenoids of plant origin (Lacher 1967). Except for some responses to C2-C10 carboxylic acids recorded by Lacher (1967), long sharp-tipped STs were not responsive to the compounds that have been tested in the study by Ghaninia *et al.* (2007).

Grooved peg sensilla

Grooved peg sensilla in *An. gambiae* and *An. quadriannulatus* Theobald were reported to respond to polar compounds such as ammonia, lactic acid, acetone, butylamine as well as to complex natural odour mixtures such as incubated sweat, cow odour and Limburger cheese odour, which

is perceived by the human nose to resemble human foot odour (Meijerink *et al.* 2001, Van den Broek and Den Otter 1999).

Qiu *et al.* (2006) found that all sensilla they recorded from in *An. gambiae* contained at least one neuron that responded to ammonia and 1-butylamine. ORNs in GP sensilla were found responding to oxocarboxylic acids, short-chain carboxylic acids and lactic acid, compounds that are shown to attract mosquitoes or to enhance landing responses (Braks *et al.* 2001, Healy and Copland 2000, Knols *et al.* 1997, Smallegange *et al.* 2005).

Similar as for anophelines, grooved peg sensilla in culicine mosquitoes have also been found responding to short-chain carboxylic acids but hardly to plant and oviposition site-associated volatiles (Davis 1988, Davis and Sokolove 1976). However, the responses of the GP sensilla in *Ae. aegypti* to ammonia and lactic acid are strikingly different from the responses recorded from the homologous sensilla in *An. gambiae*. Almost all the GP sensilla in *An. gambiae* contain ORNs that were excited by ammonia, whereas only 8% of those in *Ae. aegypti* responded to ammonia (Davis and Sokolove 1976, Qiu *et al.* 2006). In contrast, the LA-excited neuron population in *Ae. aegypti* was present in a higher proportion of grooved peg sensilla (58.8%) than that in *An. gambiae* (40%) (Qiu *et al.* 2006). The difference in sensitivity of peripheral neurons to ammonia and lactic acid reflects the different roles of these two compounds in the behaviour of these two mosquito species: for *An. gambiae* ammonia alone is attractive to the females, but lactic acid alone has a negligible effect; while for *Ae. aegypti* the relative importance of the two compounds in attraction appears reversed (Steib *et al.* 2001, Geier *et al.* 1999, Smallegange *et al.* 2005).

Labellar sensilla

Because the characteristic co-receptor AgOR7 was found to be expressed in T2 sensilla on the proboscis of *An. gambiae* and *Ae. aegypti*, it has been hypothesised that the proboscis of these mosquitoes also functions as an olfactory organ (Melo *et al.* 2004, Pitts *et al.* 2004). Subsequent studies by Kwon *et al.* (2006) tested the response of ORNs in T2 sensilla on the proboscis of female *An. gambiae* to a range of compounds and found that these neurons were responding to butylamine, acetic acid, oxo-carboxylic acids and ketones with a heterocyclic ring, including acetothiophene, acetylpyridine, acetylthiazole, and acetylphenone. *In situ* hybridisation revealed the co-expression of the conventional AgOR6 localised in sensilla T2.

Capitate peg sensilla

For blood feeding mosquitoes and biting midges (Diptera: Ceratopogonidae), a receptor neuron sensitive to CO_2 was found in sensilla basiconica on the maxillary palps (Grant and Kline 2003, Grant and O'Connell 1996, Grant *et al.* 1995, Kellogg 1970). Because almost all the sensilla containing CO_2 receptors are innervated by one neuron with an expanded lamellar structure, it has been postulated that this is the neuron that is sensitive to CO_2. The capitate peg sensilla of mosquitoes also house a neuron that is highly sensitive to 1-octen-3-ol (Grant and O'Connell 1996).

The function of the three neurons innervating capitate sensilla on the maxillary palps of *An. gambiae* was studied by Lu *et al.* (2007). Activity of three neurons has been identified. A neuron from which the largest spike amplitude was recorded responded to CO_2 over a narrow concentration range. This neuron showed excitatory responses to several heterocyclic compounds and inhibitory responses to several compounds including indole. The B-neuron was extremely sensitive to 1-octen-3-ol, it also responded to several analogues of 1-octen-3-ol, but with lower

sensitivity, and several ketones including 6-methyl-5-hepten-2-one. The C neuron in the capitate sensilla was broadly tuned to heterocyclic compounds, ketones and alcohols. *In situ* hybridisation indicated the coexpression of three AgGRs (AgGR22, 23 and 24) in neuron A and the co-receptor AgOR7 was expressed together with AgOR8 in the B neuron and with AgOR28 in the C neuron. Expression of these AgORs in heterologous systems verified that the response spectra of the individual receptors matched the *in vivo* responses of the three neurons.

Effects of physiological state

The behaviour of a mosquito is determined by the prevalent physiological state. For example, a female mosquito does not take her first blood meal before she reaches a certain age; after a blood meal has been taken, a female mosquito will not respond to host-derived cues for the next 48 to 72 hours until the eggs have matured, in due time she needs to find a suitable oviposition site to deposit the eggs. Does the peripheral olfactory system respond to changes in physiological state? Would mating, age, blood meal, gonotrophic cycle, body size and infection state affect the response of ORNs to odours? Several studies addressed these questions and provided some answers, whereas most of the questions remain unanswered.

Age

Davis (Davis 1984a) reported that the sensitivity of grooved peg sensilla in *Ae. aegypti* increased with increasing post-emergence time, which was in accordance with increased host-seeking activity. By comparing the response of the CO_2 receptor neuron in capitate peg sensilla of *Ae. aegypti*, Grant and O'Connell (2007) found that newly emerged female mosquitoes were much less sensitive to CO_2 than older females, whereas for males no such pronounced difference was found. The CO_2 sensitivity was in accordance with the host-seeking activity.

Blood meal and oviposition

Lactic acid sensitivity was found to be down-regulated after female mosquitoes took a blood meal, suggesting an involvement of the peripheral olfactory system in the modulation of foraging behaviour of *Ae. aegypti* (Davis 1984b). After oviposition, the lactic acid sensitivity returns to the pre-blood-fed level. Davis and Takahashi (1980) found that ORNs in blunt-tipped type II sensilla of gravid females were more sensitive to oviposition site-related compounds, namely methyl- and ethyl-esters of C2-C4 carboxylic acids, than the non-gravid females.

After a blood meal, a new functional type of sensilla trichodea E was found for *An. gambiae*, which was highly sensitive to indole and 3-methyl indole, and sensitive to C5-C9 carboxylic acids as well as 7-octenoic acid, which is a component of human axillary odours (Qiu *et al.* 2006, Zeng *et al.* 1991). Increasing sensitivity to these compounds might facilitate female mosquitoes to locate oviposition sites and hosts in the next gonotrophic cycle more efficiently. The overall sensitivity of ORNs in gravid mosquitoes to ammonia and phenols was lower (Qiu *et al.* 2006), supporting a role in the observed suppression of host-seeking behaviour. Up to 435 gene products are either up- or down-regulated after the ingestion of a blood meal by female *An. gambiae* (Holt *et al.* 2002, Ribeiro 2003). One of the putative OR genes (AgOR1) was strongly down regulated 12 h after blood feeding (Fox *et al.* 2001). When AgOR1 was expressed in a *Drosophila* ORN of which the original OR was deleted ('empty neuron'), it was found specifically responsive to 4-methylphenol (Hallem *et al.* 2004a). The overall decrease of sensitivity to phenols found by Qiu *et al.* (2006) might be caused by the down-regulation of these and similar genes.

Diapause

The lactic acid-sensitive neurons found in active females do not respond in the diapausing females of *Cx. pipiens* Linnaeus, whereas neurons that are sensitive to putative oviposition stimulants are unaffected during diapause (Bowen *et al.* 1988). A subsequent study showed that in female *Cx. pipiens* that had terminated diapause high sensitivity to lactic acid was restored.

Translation of physiology to behaviour

Input-output relations

As discussed above, mosquito ORNs have varied specificity spectra and sensitivity ranges. Across-fibre patterning is the most plausible coding principle operating in the mosquito olfactory system (Ghaninia *et al.* 2007, Qiu *et al.* 2006). What conclusions can we draw from the electrophysiological studies in terms of their behavioural relevance? Can we predict which compound is attractive or repellent to mosquitoes based on our electrophysiological data? Can we say anything at all about the behavioural relevance of a compound or compound mixture after the electrophysiological activity these elicit has been recorded? Does the response intensity of ORNs correlate with behavioural response? Has one compound a higher behavioural impact when more ORNs are tuned to it or when it is tuned to only by few specific neurons? Do excitatory and inhibitory responses have a different behavioural effect? We must establish that we have only preliminary answers to some of these questions. Correlations between ORN specificity and sensitivity to a compound and behavioural significance of this compound have been found in several cases, but a full picture is still out of reach. Several examples are given below.

In all the tested species CO_2 receptors are found in the capitate peg sensilla on the maxillary palps (Grant *et al.* 1997, Grant and O' Connell 1996, Lu *et al.* 2007). As expected, CO_2 is indeed generally attractive to mosquitoes (Dekker *et al.* 2001, Gillies 1980, Mboera and Takken 1997, Qiu *et al.* 2007). It is noteworthy that the CO_2-sensitive neurons respond within a narrow concentration range and the response intensity is not particularly high. An ORN responding to 1-octen-3-ol is co-compartmentalised in the same capitate pegs as the CO_2 receptor neurons. The sensitivity to 1-octen-3-ol is exceptionally high compared to other ligands, and the response intensity is much higher than observed for the CO_2-sensitive neuron. Yet in behavioural tests, 1-octen-3-ol showed much more variable effects to various mosquito species and on its own is rarely attractive to mosquitoes (Kline 1994).

For ammonia, an abundant component of incubated sweat, a better match between electrophysiology and behaviour has been found. Ammonia elicits excitatory responses in all functional types of grooved peg sensilla and some types of sensilla trichodea in *An. gambiae* (Meijerink *et al.* 2001, Qiu *et al.* 2006), which finding nicely correlates with the reliable attraction of female *An. gambiae* to sources of this compound (Braks *et al.* 2001, Smallegange *et al.* 2005). Lactic acid has a similar behavioural effect on female *Ae. aegypti* as ammonia on female *An. gambiae*, and neurons that are sensitive to this compound are found in the majority of the grooved peg sensilla, as mentioned before.

N,N-diethyl-3-methylbenzamide (DEET) is a strong repellent for many species of mosquitoes and several electrophysiological studies attempted to reveal its mode of action. It was found in *Ae. aegypti* that blunt-tipped type I ST contain neurons that can be excited by DEET (Lacher 1967). Other studies suggested that DEET makes female mosquitoes anosmic to lactic acid (Davis

1985, Davis and Sokolove 1976) and oviposition stimulants (Kuthiala *et al.* 1992). A recent study showed that DEET reduced the sensitivity of a neuron expressing AgOR8 responsive to 1-octen-3-ol (Ditzen *et al.* 2008). More recently, however, Syed and Leal (Syed and Leal 2008) showed that the decrease of 1-octen-3-ol activity is due to the adsorbing properties of DEET, as deduced from the observation that when the two compounds were delivered separately the response to 1-octen-3-ol was unaffected. These authors also found DEET sensitive neurons on the short sharp-tipped ST in *Cx. quinquefasciatus*.

Conclusions and outlook

Understanding the chemical ecology of natural odours mediating mosquito behaviours is extremely important. Many natural odours from different sources share the same chemical components. It is the combination of different components and the ratios between the amounts of these components that determine characteristic profiles of natural odours. For example, carboxylic acids, indoles and phenols are found among plant volatiles, human emanations and oviposition site odours alike. From behavioural studies it has become clear that not a single VOC, but a subset of VOCs (the most effective composition of which is as yet unknown) as occurring in the complex compound mixtures that natural sources provide, form an ensemble code that allows the olfactory system to discriminate between a nectar source, a host body or an oviposition site. Components in a mixture may synergise or antagonise the activity of each other already at the peripheral level (Ochieng *et al.* 2002). As an example, it will be of considerable interest to analyse the possible peripheral interactions occurring between ammonia, lactic acid and carboxylic acids when these are offered as different blends of these three components that have been shown to cause behavioural synergism (Smallegange *et al.* 2005). Electrophysiological studies on volatile blends are rare, as a strong bias for single compound stimulations has been noted. Subsequent processing of all olfactory input takes place in the olfactory lobe of the brain to either generate or suppress a behavioural response (Chapter 4, this volume).

The amount of electrophysiological data on ORN-functional types has grown over the past decade but is still modest and indeed far from exhaustive. The large number of sensilla (Table 1) and even more so, ORNs, on mosquito antennae and the absence of regionalised sensillar distributions make it difficult to carry out an exhaustive inventory. It is to be expected that a larger sample size of the total ORNs studied enhances the chance of identifying additional functional classes. Thus, increasing this sample size is still a first and foremost but also tedious task, yet essential to increase the likelihood that the sample attains a size representative for the total ORN population. The availability of cloned OR-genes allows for screening of their ligand-specificity spectra gene by gene using heterologous platforms. This necessitates close collaboration between molecular biologists and electrophysiologists as for verification of the data thus obtained in the donor mosquito species itself, the use of SSR-techniques remains a crucial final step.

Looking at mosquito olfaction from the perspective of the complex chemistry of natural VOC-mixtures released by hosts, oviposition sites and nectar- and honeydew sources, the number of VOCs tested on the full array of ORN functional types of any mosquito species is still far from exhaustive and rarely exceeds 100 VOCs. Increasing the panel of VOCs is likely to reveal new functional ORN-types. Expanding this panel to the 300 or so VOCs that are produced by the human body is one goal, but including non-natural analogs and indeed, synthetic compounds, is bound to aid our insight in ORN specificity and olfactory coding in blood feeding vector mosquitoes. The predictability of olfaction-mediated behavioural responses based on quantified olfactory input is

a real scientific challenge for any sensory physiologist and bears particular societal relevance in case of mosquito species that are such threatening disease vectors.

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