Olfaction in Mosquitoes

-Neuroanatomy and Electrophysiology of the Olfactory System

Majid Ghaninia

Faculty of Landscape Planning, Horticulture and Agricultural Science
Department of Plant Protection Biology
Alnarp

Doctoral thesis
Swedish University of Agricultural Sciences
Alnarp 2007

Acta Universitatis Agriculturae Sueciae
2007: 93

ISSN 1652-6880
ISBN 978-91-576-7392-3

Tryck: SLU Service/Repro, Alnarp 2007

Abstract

Ghaninia, M. 2007. Olfaction in Mosquitoes: Neuroanatomy and Electrophysiology of the Olfactory System. Doctoral dissertation.
ISSN 1652-6880, ISBN 978-91-576-7392-3

Female mosquitoes are vectors of diseases, affecting both livestock and humans. The host-seeking and identification behaviors of mosquitoes are mediated mainly by olfactory cues. The peripheral olfactory organs of mosquitoes which perceive olfactory cues are the antennae and maxillary palps. These appendages bear numerous hair shaped structures, sensilla, in which olfactory receptor neurons (ORNs) are housed. The ORNs detect and discriminate various odorant molecules and send information regarding odor quality, quantity and spatio-temporal patterns to the central olfactory system in the brain for further analysis.

The first goal of this study was to investigate the neuroanatomy of the mosquito central olfactory system. Using different staining techniques, the neuronal architecture of the deutocerebrum as well as 3D reconstructions of antennal lobe (AL) glomeruli were depicted for both sexes of the Afrcian malaria mosquito, *Anopheles gambiae* and the yellow fever mosquito, *Aedes aegypti*.

To study how mosquitoes detect olfactory cues, single sensillum recordings (SSRs) were performed, which allowed me to investigate electrophysiological properties of individual ORNs housed in four morphological types of the most abundant olfactory sensilla, s. trichodea. I was able to identify 11 functional types which their ORNs displayed distinct responses to a set of compounds. As part of this study, axons of functionally defined ORNs were traced by neurobiotin to indicate which glomeruli they targeted. This resulted in a functional map of AL glomeruli. The map indicated that different functional types of ORNs converged onto different spatially fixed glomeruli.

My next step was to identify novel biologically active compounds for the ORNs using gas chromatography coupled SSRs (GC-SSRs). Headspace odors from different human body parts, *i.e.* armpit, feet and trunk regions as well as from a plant used as a mosquito repellent (*Nepeta faassenii*) were collected, extracted and eventually injected onto the GC-column. I found that some of the extract components elicited responses in previously defined ORNs as well as in ORNs of the intermediate sensilla. Some of the compounds, which were subsequently identified by using GC-mass spectrometry (GC-MS) were heptanal, octanal, nonanal and decanal.

Keywords: mosquito, olfactory system, antennal lobe, olfactory receptor neurons, anatomy, physiology

Author's address: Majid Ghaninia, Department of Plant Protection Biology, Division of Chemical Ecology, SLU, SE-230 53 Alnarp, Sweden. majid.ghaninia@vv.slu.se

To my son

Shantia

Good thoughts, good words and good deeds should be the frontispiece of your life

Contents

Appendix

Papers I-IV

The present thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Ignell, R., Dekker, T., Ghaninia, M. & Hansson, B.S. 2005. Neuronal architecture of the mosquito deutocerebrum. *Journal of Comparative Neurology* 493, 207-240.

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

III. Ghaninia, M., Ignell, R. & Hansson B.S. 2007. Functional classification and central nervous projections of olfactory receptor neurons housed in antennal trichoid sensilla of female yellow fever mosquitoes, *Aedes aegypti. European Journal of Neuroscience* doi:10.1111/j.1460-9568.2007.05786.x.

IV. Ghaninia, M., Larsson, M., Hansson, B.S. & Ignell, R. 2007. Identification of natural novel ligands for ORNs of female yellow fever mosquitoes, *Aedes aegypti*, using gas chromatography coupled single sensillum recordings. Submitted.

Objectives

The objectives of this study were 1) to investigate the neuroanatomy of the mosquito central olfactory system, 2) to study how mosquitoes detect olfactory cues and 3) to identify novel biologically active compounds for the olfactory receptor neurons of the mosquitoes.

Introduction

For living organisms, the ability to perceive diverse sensory inputs is a major determinant for increasing survival and fitness. Where there is no photo-, thermo-, mechano- and/or chemoreception, activities that maintain population dynamics such as food seeking, escape from natural enemies and mating are reduced to random occurrences; learning and memorizing experiences would also be totally impaired by the lack of sensory input. Amongst the different sensory modalities, chemoreception is the oldest sense, having been established already during the rise of the prokaryotes (bacteria). Since this time, chemoreception has diversified and specialized tremendously through the evolution of the eukaryotes. The detection of airborne chemicals and chemicals in solution has transformed into what we now think of as olfaction (smell) and gustation (taste) (Blomquist & Vogt, 2003; Doty, 2003).

In insects, the chemical senses (odorant and contact chemosensation) play vital roles throughout development as well as in the establishment and maintenance of various physiological states and in ecological interactions. Olfaction is the primary sense exploited by insects for analyzing the environment. For this reason and several others, the investigation of insect olfaction has drawn considerable attention. The olfactory system of insects shows a striking resemblance to other invertebrates as well as vertebrates in structure, function and development (Hildebrand & Shepherd, 1997). Compared to vertebrates, the insect olfactory system consists of a relatively simple, but sensitive, neuronal network (reviewed by Boeckh & Tolbert, 1993; Menzel & Müller, 1996; Stocker, 2001). In addition, insects display a rich repertoire of behaviors that are instigated by olfactory input. Olfactory cues are used both for interspecific communication (*e.g.* food location) and intraspecific communication (in finding nesting sites, mates and other conspecifics) (reviewed by Hartlieb & Anderson, 1999). Research on the insect olfactory system is needed to establish possibilities to ameliorate the difficulties that arise from insects due to their, to humans, destructive nature as agricultural pests and disease vectors, and the beneficial functions that insects have both for ecosystems (*e.g.* as natural enemies) and for humans (*e.g.* as pollinators and honey producers) (reviewed by Karg & Suckling, 1999). Therefore, insects have become an excellent model for the study of the olfactory system.

Mosquitoes used in this study and their socio-economical importance

'Mosquito' is a Spanish word meaning 'little fly'. Mosquitoes belong to the order Diptera (*i.e.* bearing two wings), known as True Flies (Figure 1). They are found throughout the world except in places that are permanently frozen. There are about 3 500 species of mosquitoes found, where members belonging to two subfamilies, Anophilinae and Culicinae, are the most well known. Both sexes of mosquitoes use plant nectar as their principal food source. However, most females take a vertebrate blood meal (Figure 1) to complete oogenesis (anautogenous mosquitoes) with specialized mouthparts, *i.e.* long piercing-sucking proboscis, while males are obligatory nectar feeders with mouthparts that are not suitable for piercing skin (Clement, 1999; Reiter, 2001).

Figure 1. Female *Aedes aegypti* taking a blood meal from a human host. (Photo by R. Ignell).

Besides being an annoyance to humans and livestock, mosquitoes are efficient vectors of disease agents such as malaria, dengue, yellow fever, lymphatic filariasis, West Nile virus and many other encephalitis-causing agents (Gubler, 1989; Monath, 1989; Snow *et al.,* 2005; see also http://www.who.int). Of these diseases, malaria is the most perilous one, transmitted by members of the genus *Anopheles*. The most effective carriers of malaria are members of the *Anopheles gambiae* species complex. This complex comprises six species including *An. gambiae*, *An. arabiensis*, *An. bwambae*, *An. melas*, *An. merus* and *An. quadriannulatus* (Hunt, Coetzee & Fettene, 1998; Coetzee, Craig & Le Sueur, 2000). The two first of these species are highly efficient vectors of malaria

parasites (*Plasmodium spp.*). *Anopheles gambiae* is the most important agent of human malaria transmission in Afrotropical regions due to several factors, including their high anthropophily, endophagy, endophily, susceptibility to parasite infection and, to some extent, their high survival rate (reviewed by Takken & Knols, 1999). Nearly 49% of the world's population (3.2 billion people) are living in areas at risk of malaria, most of which are in subtropical and tropical regions. Each year ~500 million medical cases of malaria are recorded (an estimated 42 million Disability Adjusted Life Years, DALYs), of which 1-3 million cases result in death, some within an hour after infection. Unfortunately most victims are seen amongst children, pregnant women, and other persons with low capability to fight off the disease (WHO, malaria unit, 2005).

Aedes aegypti is another mosquito species that prefers blood meals from humans (Scott *et al.,* 1993; Harrington, Edman & Scott, 2001). *Aedes aegypti* is the major carrier of yellow fever. It is estimated that 200 000 persons are at risk of yellow fever yearly (616 000 DALYs), from which 30 000 die. Yellow fever epidemics can affect 20% of the exposed population and case-fatality rates may exceed 50% (http://www.who.int). *Aedes aegypti* is also responsible for transmitting another perilous viral disease, dengue fever. There is an estimated 500 000 DALYs lost to the four serotypes of dengue fever (http://www.who.int). In the year 2002, nearly 19 000 persons died from the disease (http://www.who.int/heli/risks/vectors). Over the past two decades the number of yellow and dengue fever infections has risen so that more countries are reporting cases of the diseases (http://www.who.int). Climate factors such as higher global temperatures or global warming play a role in enhancing numbers of people infected and areas at risk. Therefore, we might be expecting more fatality reports in the near future (Reiter, 2001; http://www.who.int)

Attempts made for chemical control and eradication of mosquitoes and mosquito-borne diseases have failed, primarily due to the build up of resistance among both the mosquitoes and the disease agents, *e.g. Plasmodium spp.*, and negative environmental impacts of the mosquitocides such as DDT (WHO, malaria unit, 2005). Application of biological agents (*e.g.* dragonfly, predatory mosquitoes, *Gambusia* fish), insect growth regulators (IGRs) and environmental management have their own limitations and deficiencies as they are either not effective or are badly tested. Using drugs to treat and cure patients of vector borne diseases is now a matter of controversy because they are expensive and sometimes have undesirable side effects. In addition to this, the parasites show resistance to the drugs after frequent usage. Progress in developing suitable vaccines is being made slowly (reviewed by Takken, 2002). Therefore, using an 'integrated' approach to mosquito control that includes mosquito biology, ecology, genetics and behavior is needed as a strategy to minimize vector contact with people (Takken & Knols, 1999).

Due to the socio-economical impact of mosquitoes there have been extensive efforts to identify the mechanisms that regulate the attraction of mosquitoes to their hosts. Mosquito behavior is mediated by various stimuli such as visual, auditory, humidity, thermal, mechanical and chemical cues. Today, comprehensive behavioral studies have indicated that the most crucial cues regulating host-

seeking and identification behaviors are olfactory volatiles emitted from the host (Bowen, 1991; Takken, 1991). These volatiles are subsequently analyzed by the mosquitoes' relatively simple, but sensitive, olfactory system. Therefore, it is expected that an improved understanding of the olfactory system of mosquitoes may help in developing control methods that interfere with mosquito host-seeking behavior.

Importance of olfactory cues in mosquito behavior

Recent studies have shown that the success of mosquito foraging for blood, nectar, and oviposition (Figure 2) are highly dependent on olfactory cues (Braks, Anderson & Knols, 1999; Takken & Knols, 1999; Nighorn & Hildebrand, 2002). Much of the current literature concerning mosquito olfaction focuses on the attractiveness of human sweat or skin emanations to mosquitoes (*e.g.* Smith, Smith & Gouck, 1970; Geier, Bosch & Boeckh, 1999a, 1999b; Bernier *et al.,* 2003; Qiu *et al.,* 2004). Compounds emitted from hosts like carboxylic fatty acids, lactic acid, ammonia, octenol and carbon dioxide are now well known as mosquito attractants (*e.g.* Kline *et al.,* 1990; Braks & Takken, 1999; Dekker *et al.,* 2002; Dekker, Geier & Cardé, 2005).

Host seeking behavior

Analysis of human skin emanations by gas chromatography coupled mass spectrometry (GC-MS) have indicated that approximately 300-400 compounds are candidate attractants/repellents for mosquitoes (Bernier *et al.,* 2000); among them are around 200 carboxylic acid compounds, some of which have been shown to be behaviorally and/or electrophysiologically active for mosquitoes (reviewed by Cork, 1996). Wind tunnel experiments revealed that mosquitoes are attracted to a mixture of fatty acids with varying chain length (C4 to C18; Knols *et al.,* 1997). In *Ae. aegypti*, a strong attraction was found to short-chained fatty acids (C1-C3) followed by medium (C5-C8) and long chained (C13-C18) ones, indicating that olfactory receptors are able to differentiate the chain length of fatty acids (Bosch, Geier & Boeckh, 2000). L-lactic acid is one of the carboxylic acid components of human skin residue also found in expired breath (Bowen, 1991; Bernier *et al.,* 2002). Although its role in host location has been proven (Dekker *et al.,* 2002), L-lactic acid is considered as a non- or poor-attractive stimulus when used singly in behavioral experiments (Smith, Smith & Gouck, 1970; Bernier *et al.,* 2003). Bernier and coworkers (2003), however, added lactic acid to other human-produced volatiles and found that L-lactic acid acts as a synergist for mosquito-host attraction. Likewise, combining L-lactic acid with ammonia (Geier, Bosch & Boeckh, 1999a) and with fatty acids (Bosch, Geier & Boeckh, 2000) add support to the theory that L-lactic acid acts synergistically with other host volatiles as an attractant.

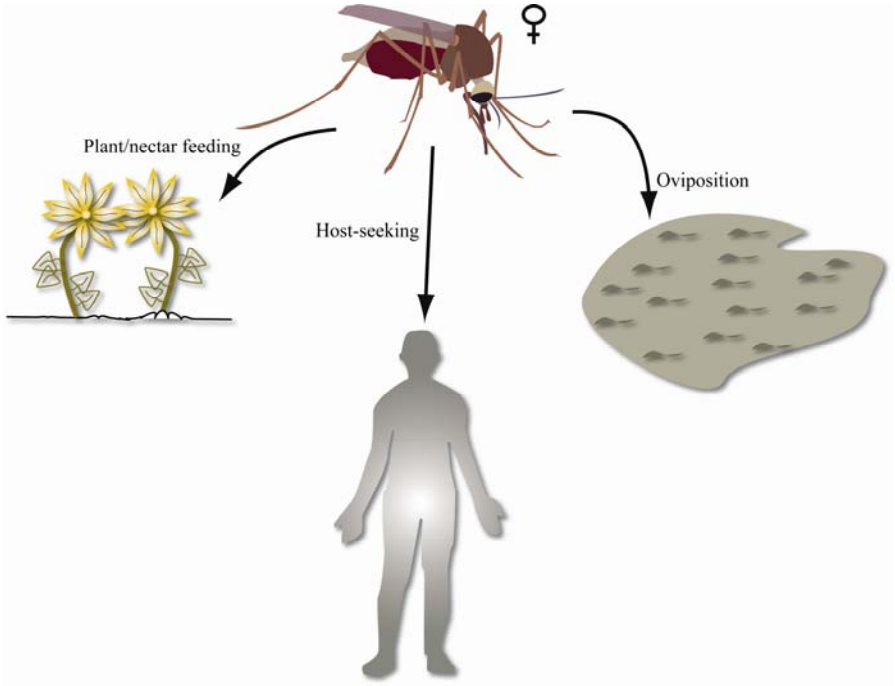

Figure 2. Mosquito behaviors are mediated by olfactory cues. Modified after (Takken & Knols, 1999).

Another important cue, used mainly by zoophilic or generalist mosquitoes, is carbon dioxide (CO_2). From the existing evidence it is known that CO_2 is involved in activation of mosquitoes in host seeking flight and in attraction towards their host (Gillies, 1980). Carbon dioxide is a component of breath exhaled from vertebrates and is also transpired from the skin of the human host. Although, the amount of CO_2 emitted from host skin is quite low compared to exhaled CO_2, the mosquito however takes advantage of skin emitted CO_2 by using the compound as a short-distance olfactory cue while landing on the host (Gillies, 1980; Grant *et al.,* 1995). Experimental evidence of mosquito behavior has been collected by assessing the number of mosquitoes caught in a trap in the presence of CO_2 (reviewed by Cork, 1996). In electrophysiological recordings, mosquitoes were able to detect changes down to 0.01% of the CO_2 concentration (Kellogg, 1970). Apparently not all mosquito species use CO_2 as an olfactory cue for locating their host since it has been demonstrated that CO_2 is more exploited by *Culex* spp than *Anopheles* spp. This finding is supported by the fact that there is no significant effect of human breath on the behavior of *An. gambiae* in response to CO_2 (de Jong & Knols, 1995). One explanation to this is that CO_2 is a general non-specific compound released by mammals. Thereby, CO_2 is believed to only alert *An. gambiae* to the presence of a potential host and, volatiles emitted from feet and ankles from their human hosts are believed to guide the host-seeking behavior of

this anthropophilic species (de Jong & Knols, 1995). One of the most striking effects of CO_2 is its synergistic effects, either acting to modify or augment the response to other stimuli. When particular odors that produce no effect by themselves, *e.g.* L-lactic acid, are combined with CO_2 their degree of attractiveness is markedly increased as measured by the increased rate of take off, flight activity, landing and probing (Gillies, 1980; Eiras & Jepson, 1991). It has been hypothesized that the number and distribution of the CO_2 sensitive sensilla of mosquitoes are arranged in order to enhance the encounter rate of CO_2 with the sensilla: that is to say that the sensilla are arranged on the distal-ventral side of the maxillary palp (McIver, 1971; Braverman & Hulley, 1979). The presence of CO_2 sensitive receptors in basiconic sensilla of non-blood-feeding male mosquitoes has led researchers to hypothesize that males may use CO_2 to locate nectar hosts, *i.e.* plant-emitted CO_2 (another source for CO_2) and/or they might exploit human host associated CO_2 to locate mates, while females are targeting the vertebrate host seeking a blood meal (Grant *et al.,* 1995).

Sugar feeding

Almost all mosquitoes require sugar resources, which are derived from flowers and extrafloral nectaries of their host plants (Figure 2). Female and male mosquitoes feed on nectar to increase their metabolic rate and to reserve more energy for taking flight prior to host seeking (Takken & Knols, 1999). In addition, females need plant carbohydrates to develop eggs and to increase fecundity (Nayar & Sauerman, 1975). It has been argued that females can obtain sufficient nutrients from the blood meal alone (Takken & Knols, 1999). In *Ae. aegypti* it has been shown that female mosquitoes fed on human blood had a greater growth rate than conspecifics fed on human blood supplemented with sucrose (Scott *et al.,* 1997). Depending on age and size, mosquitoes differ in sugar feeding behavior. Small and newly emerged females tend to feed on sugar resources more preferentially than do larger females, probably due to an energy deficiency, *i.e.* lower lipid and glycogen contents (Takken & Knols, 1999).

Orientation and attraction of mosquitoes to their host plant has been shown to be mediated by volatiles given off from the plant (Figure 2). Mono- and bicyclic monoterpenes, such as thujone, are major components of the floral odors. Also, certain green leaf volatiles, such as hexanal, 1-hexanol, and hexenol act as attractants for mosquitoes (reviewed by Takken & Knols, 1999).

Oviposition behavior

Most female mosquitoes require a vertebrate blood meal in order to complete their reproductive cycle and, as such, are effective and efficient disease transmitting agents. Facing a great crowd of mosquitoes repeatedly laying new generations in a confined area such as standing water in an old tire, a tree hole, a pond and a lake shore might raise a question for many of us: what drives mosquitoes to lay their eggs in these areas? How is a specific oviposition site selected by mosquitoes? For species with aquatic larvae, *e.g.* mosquitoes, the selection of an appropriate

oviposition site has a large impact on the survival of the larvae and consequently in the successful production of a new generation (reviewed by Zahiri, 1997; Mokany & Shine, 2003). It is believed that many mosquito species are discriminant in their oviposition site selection as they appear to exploit signals that originate from those sites specifically (reviewed by Zahiri, 1997). Oviposition site selection appears to be species-specific (reviewed by Zahiri, 1997). Mosquitoes appear to track the chemical cues emanating from conspecific larvae (known as oviposition pheromones) and/or bacterial and fungal volatile metabolites released from potential oviposition sites. Females tend to lay their eggs where conspecific larvae have been present previously (Bentley, McDaniel & Davis, 1982; Zahiri, 1997; Takken & Knols, 1999). For instance, attraction of gravid *Culex molestus* to water full of conspecific larvae has been attributed to volatiles produced by the bacteria, *Pseudomonas vesiculari* (Dhileepan, 1997). Other chemicals such as phenol, 4-methylphenol, 4-ethylphenol (produced by bacteria isolated from decaying woods or plants, Bentley *et al.,* 1979; Millar, Chaney & Mulla, 1992), erythro-6-acetoxy-5-hexadecanilide (an oviposition pheromone released by egg rafts residing on natural water, Millar *et al.,* 1994), 3-methylindole (isolated from Bermuda grass infusion, Bentley, McDaniel & Davis, 1982) and 4-methylcyclohexanol (Bentley, McDaniel & Davis, 1982) have also evoked oviposition attraction in different mosquito species.

To conclude, it is worth mentioning that there also are several important physical (abiotic) factors affecting female mosquitoes' ability to discriminate amongst potential oviposition sites such as: temperature, light, water depth, and turbidity of the water bodies (O'Gower, 1958; Zahiri, 1997; cited in Mokany & Shine, 2003). Moreover, there appear to be additional biotic factors involved in mosquito oviposition behavior. For example, presence of interspecific competitors (*e.g.* tadpoles) can inhibit mosquitoes from ovipositing, whereas it was shown that mosquitoes are attracted to the oviposition site where larvae of their own species are maturing (Mokany & Shine, 2003; Blaustein & Kotler, 1993).

The peripheral olfactory system of mosquitoes

In insects, the principle peripheral olfactory organs consist of paired head appendages, the antennae, located on either side of the head capsule. In addition to this, many insects bear additional olfactory organs located on the mouthparts, *e.g.* the labial palps in Lepidoptera and the maxillary palps in Diptera possess a number of olfactory sensilla (see below) (Keil, 1999). Each antenna of anophiline and culicine mosquitoes consists of 13 flagellar segments connected to a round pedicel, containing Johnston's Organ (JO), that is attached to the head (Figure 3A) by a ring-shaped scape to which the antennal associated-muscles are connected (McIver, 1982; Pitts & Zwiebel, 2006). The JO is a sensory apparatus that functions as a hearing organ for mosquitoes and consists of thousands of radially arranged mechanoreceptors (7500 in females and 15000 in males) known as scolopidia (Clements, 1999).

The antennae of mosquitoes are sexually dimorphic. In males, all types of olfactory sensilla (see below) are restricted to the distal-most two segments, while in females they are relatively uniformly distributed on all flagellar segments (Figure 3) (Ismail 1964; McIver, 1982; Pitts & Zwiebel, 2006). Antennae of female mosquitoes are approximately 1.5 mm in length with segments ranging from 90 μm to 160 μm each. The males' antennae are longer, approximately 2.2 mm, with the last two terminal segments slightly enlarged (approximately 380 μm and 200 μm) (van den Broek, 2000).

Antennal sensilla of mosquitoes

The antennal surface of mosquitoes, like in most other insects, is covered by many cuticular hair-shaped structures called sensilla (Figure 3). A sensillum is the smallest functional sensory structure of the insect sensory system (Figures 3B, 4A) (Keil, 1999). All mosquito olfactory sensilla display the same basic morphology (Figure 4A) (McIver 1973; Boo, 1980b; Bowen, 1995). Briefly, they contain 1 to 5 bipolar olfactory receptor neurons (ORNs). The dendrites of the ORNs extend into the sensillum lymph cavity formed by auxiliary cells (Figure 4A). Cell bodies of the ORNs are situated directly beneath the base of the sensillum and their axons project into the antennal lobes of the brain via the antennal nerve (Figure 4A). Other components of the sensillum are the auxiliary, or sheath, cells including the thecogen, tormogen and trichogen cells (Figure 4A). Sheath cells are involved in sensillum formation during ontogeny, as well as in the regulation of the ionic composition of the sensillum lymph (Keil, 1999). Considering that trichogen and tormogen cells have secretory duties, it is assumed that they are responsible for the secretion of odorant binding proteins (OPBs; see below; Steinbrecht, 1998). The conventional cuticular surface of a sensillum is perforated allowing the odor molecules to enter the sensillum lymph (Figure 4A) (Boo, 1980b; McIver, 1982; Keil, 1999). Based on the wall structure of the sensilla two types of olfactory sensilla have been described, single-walled and double-walled sensilla. In single-walled sensilla, the pores are connected to the lymph by pore channels. The innermost surface of the channels is covered by lipid layers allowing the transportation of the predominately lipophilic odor molecules into the lymph. In doubled-wall sensilla such channels do not exist. Rather, the cuticular structure of the sensilla is composed of intermittently invaginated surface, rendering them closely arranged finger-like looking. The channels made between the fused finger-like structures of the sensilla are called 'spoke channels'. It is assumed that odor molecules might pass through these channels in order to enter the lymph (Keil, 1999).

Sensilla are responsible for detecting olfactory cues. It is estimated that 90% and 83% of the antennal sensory neurons (which fall into trichoid and grooved peg sensilla; see below) in female and male mosquitoes, respectively, have olfactory function (McIver, 1978; McIver, 1982). In addition, mechano-, hygro-, and thermo-receptive neurons are housed within the sensillar array (McIver, 1982). The antennal sensilla are differentiated into several types based on gross morphological characteristics, neuronal patterns of innervation and neuronal function. Five morphological types of sensilla: sensilla chaetica, s. ampullacea, s.

coeloconica, s. trichodea and grooved peg sensilla, are found on the anopheline (*e.g. An. gambiae*) and culicine (*e.g. Ae. aegypti*) antenna (McIver, 1982; see also Pitts & Zwiebel, 2006). The three first sensillum types are innervated by mechano-, thermo- or hygro-receptor cells (McIver, 1982). Sensilla trichodea and grooved peg sensilla, which constitute ninety percent of the total antennal sensillum population, are the only antennal olfactory sensilla of the mosquitoes (McIver, 1978, 1982). These sensilla house 2 or 3 olfactory receptor neurons (ORNs) (McIver, 1974, 1978), and have been shown to respond to behaviorally active compounds (Lacher, 1971; Davis, 1976; Davis & Sokolove, 1976; Bentley, McDaniel & Davis, 1982; Davis & Bowen, 1994). Each antenna of female *Ae. aegypti* and *An. gambiae* possess approximately 900-1000 sensilla from which roughly 2000 receptor cells originate and extend axons to the brain (McIver, 1982; Pitts & Zwiebel, 2006).

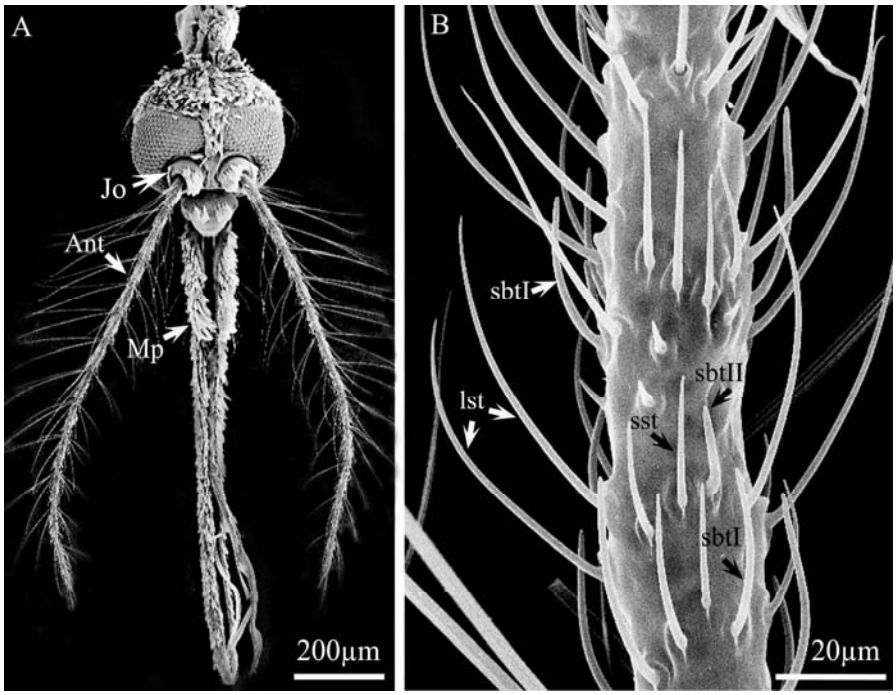

Figure 3. (A) Scanning electron micrographs of the olfactory organs, the antennae (Ant) and maxillary palps (Mp), and (B) an individual segment of the antenna of a female *Aedes aegypti* showing short sharp-tipped (sst), short blunt-tipped I (sbtI), short blunt-tipped II (sbtII) and long sharp-tipped (lst) s. trichodea. Jo: Johnston's organ (Ghaninia, Ignell & Hansson, 2007).

Trichoid sensilla of mosquitoes

Sensilla trichodea (Figure 4B) comprise two thirds of all sensilla and is thus the most abundant type of sensory structure on the antennal flagella of mosquitoes (Ismail, 1964; Pitts & Zwiebel, 2006). The number of these sensilla is estimated to be 650 and 800 for each antenna of female *An. gambiae* and *Ae. aegypti*,

respectively (McIver, 1982; Pitts & Zwiebel, 2006). These sensilla are present on all female flagellar segments, and on the two terminal segments in male mosquitoes (Boo, 1980a,b). In female mosquitoes, however, s. trichodea are sparse on the first segment. The highest density of these sensilla can be found on segments 4 to 13 (Van den Broek, 2000).

Based on length, shape and wall thickness, four distinct morphological subtypes of s. trichodea can easily be distinguished in *Ae. aegypti* under a light microscope: short sharp-tipped (sst), long sharp-tipped (lst), short blunt-tipped type I (sbt I) and short blunt-tipped type II (sbt II) (Figure 4B). There are, however, numerous intermediate sensillum types that do not fit into these four classes (Davis & Rebert, 1972). Ultrastructural evidence has revealed that all trichoid subtypes are innervated by 2 neurons except for the short sharp-tipped sensilla which possess only one receptor neuron (McIver, 1978).

Functionally, s. trichodea are olfactory chemosensory sensilla. Sensilla trichodea are the principal olfactory sensory organs where peripheral sensitivity to host odors has been investigated (Van den Broek, 2000), probably due to the relative ease of recording electrophysiologically from them. The long, sharp tipped s. trichodea (also called A1 setae) have been suggested to mediate information concerning attractive odors (such as fatty acids from a human hand) and has also been shown to be sensitive to some essential oils (Lacher, 1967). Fatty acids have been shown to excite neurons of long, sharp tipped sensilla, whereas essential oils, usually associated with plant and floral odors inhibit them (Lacher, 1971). Furthermore, short blunt-tipped type I (A2 setae) sensilla have been shown to be sensitive to the commercial repellent, DEET (reviewed by McIver, 1982). The short blunt tipped II sensilla are reported to be sensitive to plant related defense compounds like α-pinene and α-thujone (Davis, 1976, 1977; Bowen, 1992).

Peripheral events in odor reception

Transportation of an odorant molecule through the sensillum lymph

Odorant molecules released from a source such as a human host are diluted in the environment. The insect olfactory system is only able to detect these odors after they have passed through the porous cuticle and ultimately reached the odor receptors (Figure 4B). Olfactory cues are predominantly non-polar volatiles, which poses an interesting question as to how these are transported through the aqueous sensillum lymph to the olfactory receptors on the membrane of the ORNs. In 1981, Vogt and Riddiford discovered the expression of high concentrations (15 mg/antenna) of small water-soluble proteinaceous molecules (~ 14 kDa; 120 to 150 amino acids) in the antennal sensillum lymph of the silk moth, *Antheraea polyphemus*. These proteins were demonstrated to be able to bind to odorant molecules and were therefore named odorant binding proteins, OBPs (Vogt & Riddiford, 1981). The OBPs are synthesized by the auxiliary cells and secreted

into the aqueous sensillar lymph (Blomquist & Vogt, 2003). In 1985, a mechanism by which odorant molecules are transported to the neuronal receptor proteins by such OBPs was described (Vogt & Riddiford, 1985). According to this theory, once entering the sensillum lymph, odor molecules are primarily recognized and bound to OBPs, which subsequently transport the volatiles to the receptor proteins on the ORNs. Not only are odorant binding proteins responsible for the discrimination and transportation of odor molecules through the lymph, but they might also be involved in the interaction of odorant molecules with the receptor proteins and, potentially, the degradation in order to vacate the receptor for another arriving molecule (reviewed by Stengl *et al.,* 1999). Odorant binding proteins have been postulated to be either specific, binding to pheromone molecules (*i.e.* pheromone binding proteins; PBPs) or generalists, binding to olfactory molecules present in *e.g.* food or oviposition substrates (*i.e.* general odorant binding proteins; GOBPs) (reviewed by Stengl *et al.,* 1999). The roles of OBPs are still under debate.

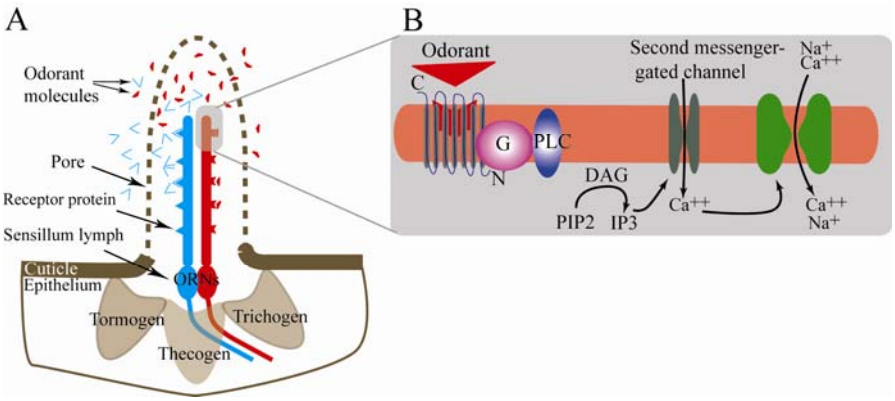

Figure 4. (A) Schematic overview of an insect olfactory sensillum comprising a porous cuticular shaft, olfactory receptor neurons (ORNs), three auxiliary cell types including trichogen, tormogen and thecogen cells that together make up the sensillum lymph cavity where the lymph is floating (see reviews in Hansson, 1999). Once an odorant molecule passes through the pore it will be transferred by odorant binding proteins (OBPs, see the text) dissolved in the lymph to the dendrite where olfactory receptor proteins are expressed (B). Once an odorant molecule binds to the odorant receptor, generation of phospholipase C (PLC) begins. The PLC converts phosphatidylinositol 4,5-biphosphate (PIP2) to the second messengers IP_3 (inositol 1,4,5 triphosphate) and diacylglycerol (DAG). IP3 influences the opening or closing of membrane ion channels (Ca^{++} and Na^{+}) leading to the depolarization or hyperpolarization of the dendrite. Modified after (Buck & Axel, 1991).

Molecular cloning and identification of OBPs have been reported from several insect species belonging to Lepidoptera, Coleoptera, Hymenoptera, Hemiptera and Diptera (Blomquist & Vogt, 2003). In *Drosophila,* 38 isolated OBPs genes have been reported (Graham & Davies, 2002). In *An. gambiae,* 32 OBP candidates have been isolated and reported to be strongly expressed in olfactory tissues of female antennae, indicating that OBPs most likely are involved in regulating mosquito behavior (Zhengxi *et al.,* 2004). Investigation of species-specificity of the OBPs in mosquitoes has revealed differential expression between *Anopheles gambiae* and *Anopheles arabiensis,* in which the latter species showed relatively higher

expression intensity of OBPs. Considering that *An. arabiensis* is not as anthropophilic as *An. gambiae* (varying from anthropophilic to zoophilic), it is plausible that *An. arabiensis* should require more OBPs expressed in its olfactory auxiliary cells than *An. gambiae* in order to support host selection preference (Zhengxi *et al.,* 2004).

Olfactory receptor proteins

Olfactory receptor proteins (ORs) (Figure 4B) were first discovered in the rat (Buck and Axel, 1991), a discovery which led to the 2004 Nobel Prize in medicine and physiology to Linda Buck and Richard Axel. Some years later, the first reports on the discovery of potential OR genes in insects (*i.e. Drosophila*) were announced (Clyne *et al.,* 1999; Gao & Chess, 1999; Vosshall *et al.,* 1999). Today, ORs of other insect species, including *An. gambiae* (Fox *et al.,* 2001), *Heliothis virescens* (Krieger *et al.,* 2002), *Bombyx mori* (Sakuari *et al.,* 2004), *Apis mellifera* (Robertson & Wanner, 2006) and *Ae. aegypti* (Bohbot *et al.,* 2007) have been identified.

In mammals, odor molecules bind to seven-transmembrane G-protein coupled receptors (GPCRs) that are located in the plasma membrane of ORN dendrites (Buck & Axel, 1991). In insects, it is hypothesized that the receptor protein is reversed, or inverted, in the plasma membrane as compared to ORs of mammals, so that its C-terminal is outside and N-terminal is located inside the membrane (Figure 4B) (Benton *et al.,* 2006).

The ORs are encoded by a remarkably diverse gene superfamily. In the rat, for example, 500-1000 gene families have been proposed to encode the ORs whereas in fish this number is about 30-100 (Buck & Axel, 1991; Ngai *et al.,* 1993) suggesting that a more limited range of odors are detected by fish ORNs. The genes reside as clusters on the chromosomes. With some exceptions, it is now known that in both insects and vertebrates each functional type of ORN expresses only one type of unique functional olfactory receptor gene (therefore only one receptor protein) (Clyne *et al.,* 1999; Vosshall *et al.,* 1999; Dobrista *et al.,* 2003; Elmore *et al*., 2003). A different strategy is found in the nematode *Caenorhabditis elegans* in which 1000 olfactory receptor genes are expressed in nearly 32 ORNs (Bargmann & Kaplan, 1998).

In insect ORNs, an additional, highly conserved receptor gene, *e.g. Or83b* in *Drosophila* and *Heliothis*, and *Or7* in *An. gambiae*, is co-expressed with the conventional receptor gene (Larsson *et al.,* 2004; Benton *et al.,* 2006), a phenomenon that has not yet been reported in mammals. Although the common receptor protein may not directly function in ligand binding (Larsson *et al.,* 2004), it appears to assist the unique receptor proteins to bind ligands properly and acting as co-receptor. Moreover, the silencing of *Or83b* leads to the localization of the receptor proteins to the ORN cell bodies rather than their dendrites (Dahanukar, Hallem & Carlson, 2005; Benton *et al.,* 2006). Therefore, it is hypothesized that the Or83b protein might be involved in the transportation and localization of canonical receptor proteins from the endoplasmic reticulum, where they are synthesized to the dendrites of ORNs, where they are expressed (Benton *et al.,*

2006). Nevertheless, there seem to be exceptions to the 'one OR type to one ORN' hypothesis as some ORNs truly co-express two receptor proteins in insects. This phenomenon has been very well documented in *Drosophila* (Fishilevich & Vosshall, 2005; Goldman *et al.,* 2005) and could be hypothesized in mosquitoes as well. For example in *An. gambiae* and *Ae. aegypti* seventy nine and one hundred and thirty one OR genes have been identified respectively (Hill *et al.,* 2002; Bohbot *et al.,* 2007). However, there are 50-60 glomeruli located in each antennal lobe, each of which is supposedly targeted by ORNs expressing a single receptor gene. In this situation, either the remaining OR genes must be pseudogenes or some ORNs must express more than one type of OR. Multiple gene expression has recently been demonstrated in *Drosophila* (Goldman *et al.,* 2005). On the basis of the existing data, it is tempting to speculate that the 'hypothesis' one receptor-one neuron (-one glomerulus) is being shifted to 'multi receptor- one neuron-one glomerulus'. Perhaps multireceptor expression in ORNs will provide an additional way for coding of odor information in the peripheral olfactory system (Goldman *et al.,* 2005). Under such a circumstance an ORN response is expanded to a wider range of odorous compounds. An ORN expressing multiple receptors might be simultaneously responding to *e.g.* food and pheromone sources, though, only one glomerulus is targeted. In contrast, in the case of single receptor-expressing ORNs, at least two glomeruli are needed for processing of odors released from two different sources. Therefore, behavioral responses of the insect may be different between these two different odor processing pathways. It has been suggested that multiple receptor expression of the ORNs is a way to facilitate signal integration (Goldman *et al.,* 2005).

Olfactory transduction mechanism

The olfactory transduction process is mediated by transductory proteins, *i.e.* OBPs, ORs, arrestins (for shutting off the transduction pathway), G-proteins (we currently do not know whether insect ORs are associated with G-proteins) and enzymes (Figure 4B) (reviewed by Rutzler & Zwiebel, 2005; Benton *et al.,* 2006). Signal transduction amplifies the input signal and can cause changes in receptor potential, resulting in excitation or inhibition of the ORNs (Hildebrand & Shepherd, 1997; Zwiebel & Takken, 2004). Excitation or inhibition of the ORNs in response to odorous compounds is one of the criteria that the odor discrimination phenomenon is based upon (Hallem, Dahanukar & Carlson, 2006).

The G-protein coupled receptor hypothesis of olfactory transduction is a historically accepted model. In this model, the GPCRs represent key parts of the sensory pathways (Hildebrand & Shepherd, 1997; Zwiebel & Takken, 2004). In general, binding of odor ligands to olfactory receptors leads to the activation of its associated G-protein, which subsequently triggers a signal transduction cascade (Figure 4B) (Hildebrand & Shepherd, 1997). Both in insects and vertebrates the activation of GPCRs leads to generation of phospholipase C and eventually the production of second messengers, IP_3 (the major pathway in insects) and/or cAMP (Figure 4B) (Hildebrand & Shepherd, 1997). The activation of the second messenger pathways results in the opening or closing of membrane ion channels leading to depolarization or hyperpolarization of the dendrite membrane (Figure

4B). The receptor potential travels along the dendrite to a spike initiation site where, if the local membrane potential reaches/surpasses a threshold potential, action potentials will be generated. The action potentials travel along the ORN axon to the antennal lobe(s), conveying information regarding quality, quantity and spatio-temporal patterns of an odorant (Hansson, 1995; Hildebrand & Shepherd, 1997; Field, Pickett & Wadhams, 2000; Nighorn & Hildebrand, 2002).

Olfactory coding

Insects are able to discriminate thousands of odorants in their environment (Hildebrand & Shepherd, 1997). Although discrimination and integration of odors and odor blends is a function of the insect central nervous system (CNS), peripheral ORNs play a principal and basic role in detecting, identifying and discriminating odor molecules, thus providing the required information to the CNS. To unravel odor coding in insects it is, therefore, essential to identify individual ORNs through the classification of their activation by specific odors (Malnic *et al.,* 1999). Since specific ORNs innervate distinct parts of the antennal lobe, the peripheral odor code is transformed in the glomeruli from where the olfactory message is conveyed to higher brain centers for assimilation, which ultimately leads to a behavioral response if the appropriate code is given.

Two odor coding schemes have been hypothesized at the peripheral level. The first theory suggests that a ligand only activates a particular type of receptor cell and from there the information is transferred directly to the antennal lobe and thence to higher brain centers without pooling or modulating the input signals. This odor coding scheme is named labeled-line, in which one odor molecule activates one glomerulus (reviewed by Hansson & Christenson, 1999). A well known example of this is the coding of pheromone components in male moths by ORNs projecting to the macroglomerular complex. Several examples of labeled-line coding systems can also be found in mosquitoes. For example, CO_2 sensitive receptor neurons are exclusively housed in sensilla basiconica of the maxillary palps and apparently project to a single glomerulus in the brain (Kellogg, 1970; Distler & Boeck, 1997; Anton *et al.,* 2003). Lactic acid-sensitive neurons as well as temperature sensitive neurons in grooved peg and coeloconica sensilla, respectively could be other examples of the labeled-line coding system in mosquitoes (Davis & Sokolove, 1975, 1976).

The other canonical odor coding system is across fiber patterning. According to this system, the ORNs respond more broadly to a range of compounds. In other words, more than one functional type of ORN is involved in detecting a certain odor and therefore more than one glomerulus is activated. Thereby, discrimination between two odors is facilitated at the central level (reviewed by Hansson & Christenson, 1999). Examples of this type of odor coding are the ORNs sensitive to plant or oviposition related compounds that can be found in mosquitoes and other insects (*e.g.* Davis, 1976; Dethier, 1976).

Neither of the canonical hypotheses alone can describe odor coding in insects. Rather, a novel way of odor coding has been hypothesized (Malnic *et al.,* 1999). According to this new coding system, distinct ORNs express given receptor

proteins that are broadly tuned to a set of compounds with differential affinities (selectivity). Characteristics of odorant molecules such as shape, charge distribution and hydrophobicity of functional groups may affect the affinity (reviewed by Ignell & Hansson, 2005). Thereby, the ORNs generate a differential response profile to various compounds. Additionally, an odorant can activate several ORNs with differential affinities (Hallem & Carlson, 2006). This phenomenon was first described by Malnic *et al*. (1999) and termed as combinatorial coding of odorants. Optical imaging techniques can demonstrate this phenomenon. The application of a single odorant to an antenna activates a subset of glomeruli in a fixed region surrounded by less activated glomeruli, and as the odor concentration increases the number of involved glomeruli increases (reviewed by Galizia & Menzel, 2001). The dose-dependent response suggests that the identity of a compound in the AL is not only established by the contribution of various functionally different glomeruli receiving input from various ORNs but also by a combination of information from targeted glomeruli being read by higher brain centers (Ng, 2002).

Central elements of olfactory processing

The central olfactory system of insects has attracted less attention compared to what is known about *e.g.* the central visual system. This could partially be due to the complexity of the insect olfactory system, *i.e.* its neuronal connectivity and neuropilar substructures (Homberg, Montague & Hildebrand, 1988). On the other hand, it is generally believed that olfaction has a broader effect on insect behavior such as host-seeking, mate finding, egg laying *etc.* than any other sensory modality. Therefore, studying the anatomical aspects of insect olfactory processing centers in conjunction with studies of physiological mechanisms involved in olfaction might lead us to a better understanding of behaviors central to insect life.

Primary olfactory center (the antennal lobe)

The antennal lobes (ALs) of insects, which are equivalent to the vertebrate olfactory bulbs, are two protruding spheroid neuropils located on either side of the oesophagus (Figure 5) (Anton & Homberg, 1999). Within the AL are many globular-shaped structures called glomeruli, the sites of the ORN axons' first synaptic contacts (Homberg, Christensen & Hildebrand, 1989). Anatomical characteristics of the glomeruli have been extensively investigated in several species belonging to Lepidoptera (Rospars, 1983; Rospars & Hildebrand, 2000; Sadek *et al.,* 2002), Hymenoptera (Galizia *et al.,* 1999; Smid *et al.,* 2003) and Diptera (Laissue, *et al.,* 1999; Ignell *et al.,* 2005; Ghaninia, Hansson & Ignell, 2007). The antennal lobes are the primary olfactory processing centers of an insect. The glomeruli receiving input from antennae, maxillary palps and labium or labial palps are not only functionally distinct but are also spatially fixed

forming a functional map within the lobes (Vosshall, Wong & Axel, 2000; Galizia & Menzel, 2001).

The glomeruli are usually arranged in one or two layers around a central fiber core consisting of central interneuron processes, and glomeruli are generally separated from each other by a glial sheath (Copenhaver, 1993; Anton & Homberg, 1999; Hansson & Anton, 2000; see also Ignell *et al.,* 2005). The number, shape, size and other characteristics of glomeruli are species- as well as sex-specific (reviewed by Anton & Homberg, 1999). In some insects, like locusts, the AL is composed of multilayered microglomeruli (Anton & Hansson, 1996). The number of glomeruli in the AL varies from 43 in *Drosophila melanogaster* (Diptera) to over 1000 in locusts (Orthoptera) and wasps (Hymenoptera) (Laissue *et al.,* 1999; Rospars, 1988). Between the two ALs of an insect, glomeruli are bilaterally symmetrically located so that it is possible to identify the homologous glomeruli of the two ALs (Rospars, 1983). The glomerular array can be sexually different. The best understood cases of sexually dimorphic glomeruli are the macroglomerular complex (MGC) of male moths and the macroglomerulus of male cockroaches that lack a female counterpart (reviewed by Anton & Homberg, 1999). These neuropils, which are believed to be derived from 'ordinary' glomeruli (shared glomeruli in both sexes of the same species), are located close to the junction with the antennal nerve and have been found to receive input exclusively from pheromone-sensitive neurons (Hansson *et al.,* 1992; Anton & Homberg, 1999).

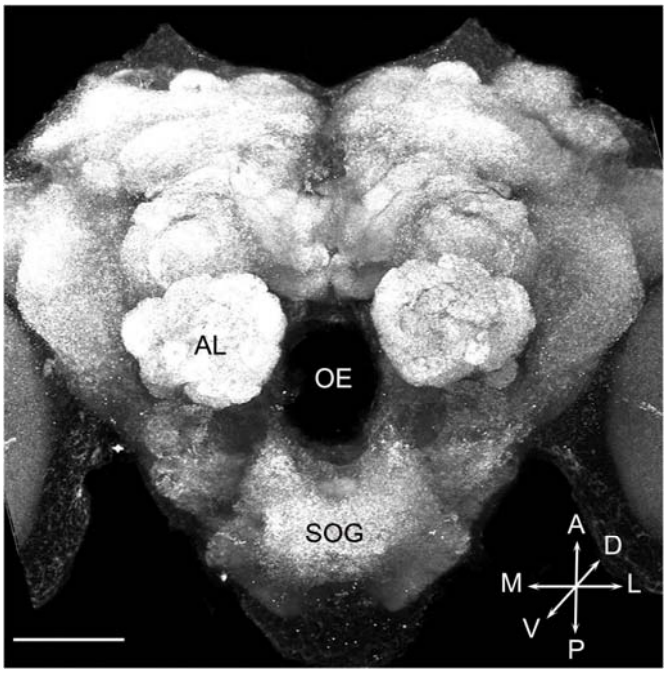

Figure 5. The antennal lobe (AL) of a female *Anopheles gambiae* mosquito. OE: oesophagus; SOG: suboesophageal ganglion; A: anterior; P: posterior; L: lateral; M: medial; D; dorsal; V: ventral. Scale bar= 50µm (Ghaninia, Hansson & Ignell, 2007).

Cellular components of the antennal lobe

At the glomerular level four distinct classes of neurons can be distinguished, the input neurons (ORNs), and three classes of central neurons including local interneurons (LNs), projection neurons (PNs) and centrifugal neurons. The ORNs have their cell bodies in the olfactory organs and dispatch axons through the antennal nerve bundle to the AL targeting the glomeruli (Figure 6) (reviewed by Anton & Homberg, 1999). The tracing of the projection patterns of afferent neurons within the AL glomeruli have been described in mosquitoes (Anton & Rospars, 2004; Anton *et al.,* 2003; Ignell *et al.,* 2005; Ghaninia, Hansson & Ignell, 2007) as well as several other insects (Boeckh *et al.,* 1984; Homberg, Montague & Hildebrand, 1988). In most insect species, ORNs arising from one antenna only innervate the ipsilateral AL. In *D. melanogaster*, however, most of the antennal ORNs (approximately 80%) arborize bilaterally (Stocker, 2001). It was hypothesized that the bilateral arborization found in *Drosophila* may be a representative trait of the dipterans, however in mosquitoes it has been found that ORN input from the antennae is restricted to the ipsilateral AL (Anton *et al.,* 2003; Ignell *et al.,* 2005). Individual ORNs not sharing a similar function (*i.e.* odor selectivity) consistently converge on different distinct glomeruli and do not physically overlap (Hansson *et al.,* 1992; Anton *et al.,* 2003; Dobritsa *et al.,* 2003). In moths, pheromone-specific neurons of the male project to a sexually dimorphic neuropil in the AL, the MGC. Olfactory receptor neurons specific to different components of a pheromone blend, target different glomeruli of the MGC (Hansson *et al.,* 1992). The ORNs terminating in the glomeruli make synapses with central neurons, local interneurons and projection neurons (Figure 6) (reviewed by Anton & Homberg, 1999).

Local interneurons (LNs) lack axons and do not send terminals out of the AL. Therefore, LNs are exclusively restricted to the AL, innervating and interconnecting most (and in some cases all) glomeruli (Figure 6). The presence of LNs has been reported in all insect orders studied so far (Hildebrand and Shepherd, 1997). Cell bodies of the LNs are located as clusters at the periphery of the ALs. These clusters can be found in three major regions of the AL, the anterior, medial and lateral AL (Ignell *et al.,* 2005). Based on their arborization pattern three types of LNs have been classified: multiglomerular LNs, with dendritic arborizations evenly distributed within the glomeruli; multiglomerular LNs, with dendritic arborizations unevenly distributed within the glomeruli; and finally oligoglomerular LNs, innervating only a very limited number of glomeruli (Hansson & Anton, 2000). In mosquitoes, the LNs belonging to the first category were found to be the great majority innervating the glomeruli (Ignell *et al.,* 2005).

Projection neurons (PNs) have their cell bodies in the periphery of the AL (Anton & Homberg, 1999). The dendritic arborizations of the PNs are both uni- and multiglomerular, and the axons leave the AL through various fiber tracts. The tracts are formed through congregation of PN axons connecting the AL to other parts of the brain such as the protocerebrum and the calyces of the mushroom bodies (Figure 6) (Hansson & Anton, 2000). Overall, five major tracts containing PN axons can be found in the majority of insect species studied so far; the inner antenno-cerebral tract (IACT), the outer antenno-cerebral tract (OACT), the

middle antenno-cerebral tract (MACT), the dorsal antenno-cerebral tract (DACT) and the dorso-medial antenno-cerebral tract (DMACT) all connect the antennal lobe to other regions of the brain (Figure 6) (Hansson & Anton, 2000).

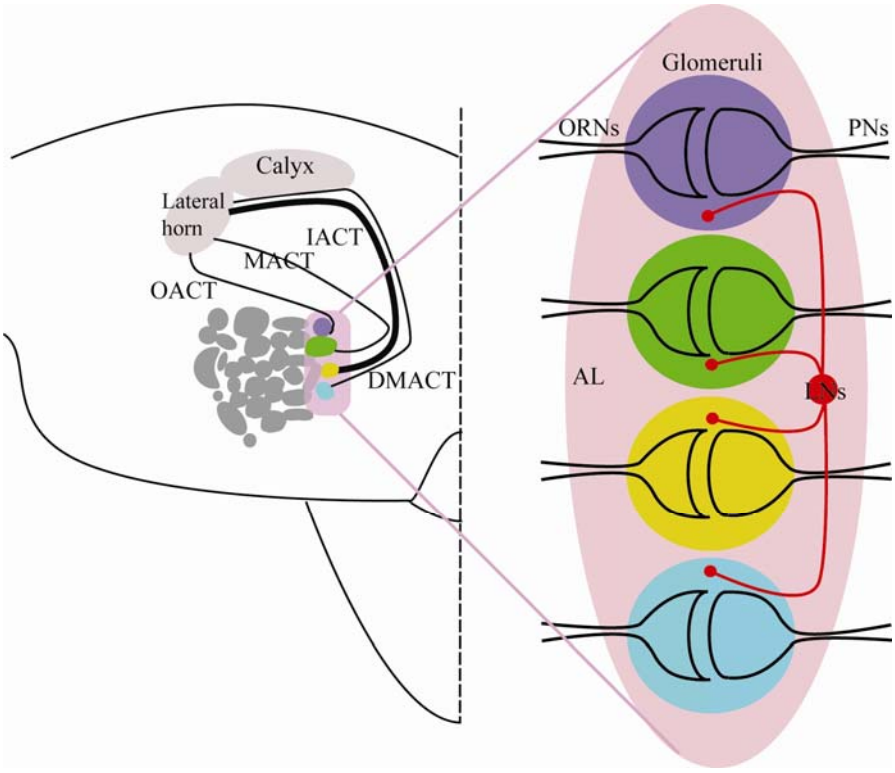

Figure 6. Cellular components of the insect antennal lobe (AL). In a glomerulus the olfactory receptor neurons (ORNs) synapse with the central neurons, *i.e.* projection neurons (PNs) and local interneurons (LNs). The LNs are restricted within the AL, whereas axons of PNs extend to the higher brain centers (*e.g.* calyx and lateral horn) via several tracts including the inner antenno-cerebral tract (IACT), outer antenno-cerebral tract (OACT), middle antenno-cerebral tract (MACT), and dorso-medial antenno-cerebral tract (DMACT).

Centrifugal neurons in mosquitoes appear to share many characteristics with those in other insect species, including moths, bees and locusts (Ignell *et al.*, 2005; Hansson & Anton, 2000). There is only single type of serotonin-immunoreactive centrifugal neurons found in each AL of mosquitoes. The neuron has its cell body in the dorso-lateral AL, extending the primary neurite to the IACT from where the neurite bifurcates and sends a recurrent fiber back into the AL and arborizes the AL glomeruli. (Siju *et al.*, in preparation). The function of this type of neurons is poorly understood but it is assumed that they are involved in the modulation of AL activity (reviewed by Anton & Homberg, 1999).

Electrophysiological techniques (SSR, GC-SSR)

Different electrophysiological techniques have been employed to study the nervous system of insects since the late 1950s. In 1957, Schneider for the first time announced that it was possible to measure the electrophysiological responses from the antennae of an insect, *Bombyx mori*, using an electroantennogram (EAG). The EAG technique has since been developed for several insect species of various insect orders (reviewed by Millar & Haynes, 1998). Despite its usefulness, the method is limited in its use. For instance, the sensitivity of the technique is low, a shortcoming often observed in insects whose antennae have *e.g.* a limited number of sensilla (reviewed by Millar & Haynes, 1998). To overcome this problem, Boeckh (1962) developed a new technique, the so-called single sensillum recordings (SSRs), which can measure responses of single ORNs (Figure 7) (reviewed by Millar & Haynes, 1998). Single sensillum recordings allow for the analyses of specific types of sensilla and for the determination of the mechanisms by which insects code for different odors.

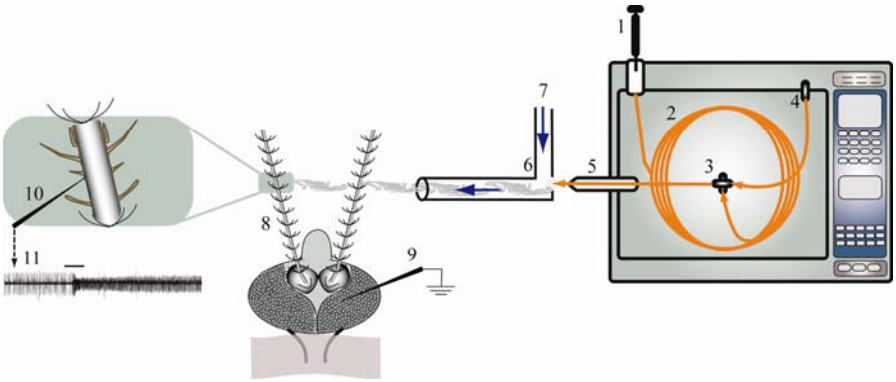

Figure 7. The GC-SSR technique in mosquitoes. At the GC part, headspace extracts are injected by a microsyringe (1) onto a GC column (2). The column is situated in an oven. As the oven temperature increases the components of the extract are separated and travel down through the column and reach a split (3) from where half of the effluent goes to a flame ionization detector (FID) (4). The other half leaves the column and passes through a transfer line (5) to a glass tube (6) where a continuous humidified/purified airflow (7) blows the separated components of the extract over the mosquito antenna (8). At the SSR part, two tungsten electrodes, a ground and a reference (9 and 10), are placed into the eye and at the base of a single sensillum, respectively. Action potentials of the ORNs housed in a sensillum and their responses to the odor components are recorded (11).

In SSRs, two sharpened tungsten electrodes are used: a ground electrode is in contact with the haemolymph while the recording electrode is inserted near the base or in the shaft of a single sensillum (Figure 7). Voltage differences generated between the electrodes, when amplified, can be viewed on an oscilloscope when placed within the circuit (reviewed by Millar & Haynes, 1988; see also de Bruyne, Foster & Carlson, 2001). The pioneers of electrophysiological studies describing the functional characteristics of ORNs were Lacher, 1967; Schneider &

Steinbrecht, 1968; and Kellogg, 1970. Davis & Sokolove (1976) used SSRs on flagellar sensilla and found that receptor cells housed in grooved peg sensilla responded to lactic acid. Using the SSR technique, receptor neurons responding to CO_2 were found in sensilla basiconica of the maxillary palps (Kellogg, 1970; Grant *et al.,* 1995). During the last decade, several experiments leading to functional characterization of individual sensilla have been carried out in sugar fed mosquitoes of the *Anopheles* species as well as in *Aedes aegypti* (Meijerink & van Loon, 1999; Van den Broek & den Otter, 1999; Meijerink, Braks & van Loon, 2001; Qiu *et al.,* 2006; Ghaninia, Ignell & Hansson, 2007). However, these studies are still in their infancy and additional analysis is required to obtain detailed information about the functional characteristics of the peripheral olfactory system.

The introduction of gas chromatography (GC) coupled SSRs, first used in moths and aphids (Arn, Städler & Rauscher, 1975; Wadhams, 1982; Dawson *et al.,* 1987) (Figure 7) allowed for the first detection of active components within a complex blend of compounds. This technique has later been used to identify novel ligands of ORNs in a large number of insect species. At the GC part an extract likely to contain odorants detected by the ORNs is injected onto the GC-column. The column is located in an oven where it is possible to regulate the column temperature. As the temperature of the column is increased the components of the extract are separated while traveling down the column and exit the GC set-up (Figure 7). The separated components of the extracts encounter the single sensillum from which a stable electrical contact is established (Figure 7). Responses of the ORNs housed in a single sensillum to the extract components are recorded (Figure 7). The chemical identity of the response eliciting component(s) can be further identified using mass spectrometry (MS) (*e.g.* Stensmyr *et al.,* 2003).

Summary of results

Neuro-anatomy of the mosquito deutocerebrum (Papers I, II)

In mosquitoes, the structure and, to some extent, function of the antennal and maxillary palp sensilla are well studied (Kellogg, 1970; McIver, 1971, 1974, 1978; Boo, 1980a,b; Grant *et al.,* 1995; Meijerink & van Loon, 1999; Van den Broek & den Otter, 1999; Meijerink, Braks & van Loon, 2001; Pitts & Zwiebel, 2006; Qiu *et al.,* 2006). However, structural studies of the central olfactory system has, for many years, remained scarce, mainly due to technical problems coupled with the physical nature of the mosquito brain (Christophers, 1960, Childress & McIver 1984; Anton *et al.,* 2003; Anton & Rospars, 2004). As part of my doctoral thesis project I investigated the neuroanatomical organization of the deutocerebrum in both sexes of *Ae. aegypti* and *An. gambiae* using several staining methods, including monoclonal antibody stainings, anterograde neurobiotin backfills, reduced silver staining and Golgi impregnation.

General description

The mosquito deutocerebrum is, like in other insects, divided into two neuropils, the antennal lobe (AL) and the antennal mechanosensory and motor center (AMMC). The neuroarchitecture of the AMMC is described below. In both species, the ALs protrude ventrally on either side of the oesophagus (Figure 5). A subset of the ALs glomeruli are surrounded by a thin glial sheath. The glomeruli are arranged in one to two layers around a central fiber core. The AL and its glomeruli is a specialized site for receiving input from antennal, palpal and labial olfactory sensory neurons. In order to understand how the glomerular array within the AL is organized I made 3-dimensional maps of the AL glomeruli of both sexes of the two mosquito species. Using the above-mentioned staining methods the structures were visualized. In the case of AL reconstructions, confocal image slices of the ALs were loaded onto a computer equipped with the AMIRA software. Here, individual glomeruli were manually demarcated and subsequently three-dimensionally reconstructed. The glomeruli were then compared and matched between specimens (n=5) and given specific names. Glomerulus delineation and comparison were started with 'Class 1' glomeruli whose position, shape, size and ease of demarcation were more or less constant. Based on the relative position to Class 1 glomeruli, 'Class 2' glomeruli, lacking one of the above-mentioned criteria, were identified. 'Class 3' glomeruli were more poorly demarcated and varied in more than one criterion.

Antennal Lobe

In the ALs of *Ae. Aegypti*, 49 male glomeruli and 50 female glomeruli were identified. Male and female *An. gambiae* possessed 61 and 60 AL glomeruli, respectively. In general, male glomeruli were more difficult to delineate than those of females as more class 2 and 3 glomeruli were found in males (Table 1 in papers I and II). Male AL glomeruli are more tightly positioned than the female counterpart, making it more difficult to differentiate the glomerular border line. This difficulty may be related to a lower number of afferent fibers innervating male AL glomeruli compared to female mosquitoes (~ 2000/500 and ~1500/500 in female/male *Ae. aegypti* and *An. gambiae*, respectively; McIver, 1982). Additionally, technical constraints, such as subtle differences in the strength of antibody staining, may be involved in difficulties in delineation of glomeruli. In order to confirm that the drawn neuropils were true glomeruli, I compared the overview immunohistochemical preparations with anterograde filled preparations. The ORNs projecting to the AL by way of three possible routes, namely the antennae, maxillary palps and labium were backfilled. Neuropils targeted by specific neurons were considered as individual glomeruli. In general the two staining methods coincided. Numerically, male and female glomeruli in both species were approximately the same, although glomeruli correspondence between male and female of these two species is difficult to ascertain due to intersexual variability in the distribution of the glomeruli. However, some glomeruli were found in similar geographical positions, *e.g.* AV1/AV1, AV2/AV3, VM3/VM3 in female and male *An. gambiae* respectively (Figures 2-5 paper II) and AM1-AM2,

AD1-AD2, PM1-PM2 in both sexes of *Ae. aegypti* (Figures 3-6 paper I) suggesting that these are sexually isomorphic.

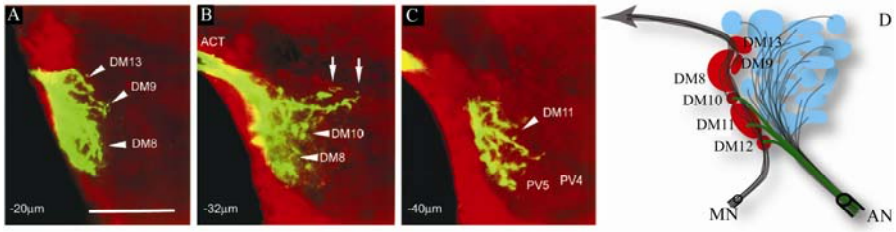

Figure 8. (A-D) In female *Anopheles gambiae*, maxillary palp associated neurons (MN) innervate 6 AL glomeruli, DM8-DM13, and project via the antenno-commissural tract to corresponding glomeruli in the contralateral AL (ACT in B and arrow in D). Three of these glomeruli, DM10-DM12, also receive input from flagellar ORNs (D). Arrows in B indicate termination of the neurons in the vicinity of, or in additional DM glomeruli in the AL. AN: antennal nerve; Scale bar= 25µm.

Anterograde stainings of the antennae, maxillary palps and labium revealed that the AL glomeruli of mosquitoes are organotopically organized so that they are consistently innervated by the neurons housed in the sensilla of these appendages. In *Ae. aegypti*, all glomeruli of both sexes, except three (MD1, MD2 and MD3), receive input from the ipsilateral antennal flagellar nerve. MD1-3 receive ipsilateral innervation from afferents projecting through the antenno-suboesophageal tract (AST). Moreover, afferent input from the labial palps also targets the MD3 glomerulus. In *An. gambiae*, flagellar ORNs innervate the great majority of the ipsilateral AL glomeruli, 49 in females and 43 in males. Five dorso-medially located glomeruli, *i.e.* DM5, DM7, DM8, DM11 and DM12 in male (Figure 7 paper II), and six dorso-medially located glomeruli, *i.e.* DM8-DM13 in female *An. gambiae* (Figure 8) received afferent input originating from the maxillary palps. In contrast to *Ae. aegypti*, maxillary palp afferents in *An. gambiae* innervated both ipsilateral glomeruli and the corresponding glomeruli in the contralateral AL. Interestingly, three female-associated maxillary palp glomeruli, *i.e.* DM10, DM11 and DM12, received input from the flagellar nerve (Figure 8D). I did not observe any flagellar and palpal nerve overlapping input areas in male *An. gambiae*. In addition, I repeatedly observed innervation from the labium into a single glomerulus, DM7, in male *An. gambiae*, and MD3 in both sexes of *Ae. aegypti* (Figure 9 paper II).

Besides afferents innervating the ALs, the AL has its own interneurons (Figure 9). I observed amacrine LNs whose the cell bodies were located in a lateral cell cluster while their primary neurites in the AL innervated most of the glomeruli homogenously. In both sexes and both species, I identified projection neurons (PNs) exiting the AL and ascending into to higher brain centers (*e.g.* lateral horn of the protocerebrum, calyces of the mushroom bodies). The unilateral PNs showed uni- or multi-glomerular arborizations and sent their axons through four distinct tracts, including the inner (IACT), dorsal (DACT), medial (MACT) and dorsomedial (DMACT) antennocerebral tracts. I also observed bilateral PNs that

interconnected the ALs. In both sexes and species, two commissural tracts, one superior (SCT) and one inferior (ICT), contained PNs that innervates most of the AL glomeruli. These neurons' cell bodies were located in a medial cell cluster. In *An. gambiae*, however, an additional commissural tract named the antennal commissural tract (ACT), primarily containing palpal ORNs, innervated the contralateral AL in which a bilateral PN interconnecting the palpal associated glomeruli was found (Figure 9).

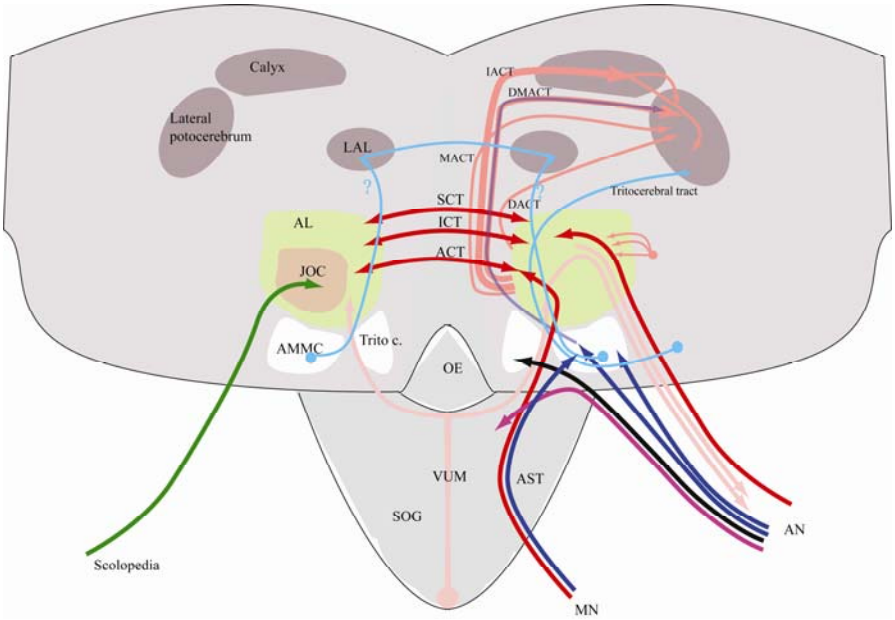

Figure 9. Summary schematic of the neuronal architecture of the mosquito deutocerebrum. ⟵ afferents innervating the antennal lobe (AL); ⟶ efferent antennal lobe neurons; ⟵ mechanosensory flagellar afferents innervating the antennal mechanosensory and motor centre (AMMC); ⟶ afferent originating from scolopidia projecting into the Johnston's organ centre (JOC); ⟵ local interneurons (LNs); ↑ tracts containing unilateral antennal lobe projection neurons; ⟷ tracts containing bilateral projection neurons; ↑ the AMMC projection neurons; ⟵ afferents innervating the tritocerebrum; ? non-defined tract; AN: antennal nerve; AST: antenno-subesophageal tract; SOG: suboesophageal ganglion; VUM: ventral unpaired medial neuron; LAL: lateral accessory lobe; OE: oesophagus; IACT: inner antenno-cerebral tract, MACT: middle antenno-cerebral tract; DACT: dorsal antenno-cerebral tract; DMACT: dorso-medial antenno-cerebral tract.

Two types of AL efferents were also identified. The first type displayed spiny dendritic arborizations in single glomeruli and extended its axons out of the AL into the antennal nerve bundle. It is speculated that these neurons are neuropeptidergic with cell bodies located in the flagellum (Meola et al., 1998, 2000). The second type of efferent neurons was identified as ventral unpaired median (VUM) neurons with cell bodies located in the posterior-apical region of the suboesophageal ganglion (SOG). These neurons sent their primary neurites in the antero-ventral part of the SOG and projected symmetrically into the dorsal region of the AL and extended their axons into the antennal nerves (Figure 9).

The Johnston's organ center

Neurobiotin staining of the flagellar nerve bundle revealed that the bundle divides into two branches prior of entering the AL; one projects to the AL glomeruli and the other one exclusively targets a multi-lobed non-distinct neuropil. Considering that the latter nerve bundle originates from the Johnston's organ, the neuropil was named the Johnston's organ center (JOC). Depending on sex, the neuropil occupies one-forth to one-third of the AL volume. As mentioned before, the JO of mosquitoes consists of numerous radially arranged mechanoreceptors, scolopidia (Clements, 1999). The afferents originating from the Johnston's organ were somatotopically organized and terminated in different regions of the JOC.

Antennal Mechanosensory and Motor Center

The AMMC of mosquitoes receives afferents from the antennae and the maxillary palps. Based on the thickness of the axons, it is assumed that neurons originating from mechanosensory sensilla, *i.e. s. chaetica*, innervate the neuropil. Mechanosensory neurons originating from the antennae medially targeted the AMMC and arborized throughout the neuropil. Another set of antennal neurons specifically targeted the posterior part of the neuropil. Mechanosensory neurons originating from the maxillary palp innervated the postero-dorsal part of the AMMC. A third type of AMMC-associated afferent neurons was found in both species. These neurons targeted the AMMC and projected to the protocerebrum, via the DMACT, where they innervated the superior medial protocerebrum (Figure 9; see also Figure 19A-D paper I). Cell bodies of antennal motor neurons innervating the AMMC were found in three locations; a lateral cluster (LC) close to the AL; a posterior cluster (PC) close to the AMMC; and a cluster in an apical part of the suboesophageal ganglion (SC) (Figure 18A,B paper I). Moreover, two types of AMMC interneurons were found. The first type arborized in the postero-dorsal part of the AMMC and projected to the ventro-lateral protocerebrum via the tritocerebral tract. The second type showed bilateral innervation in the AMMC and projected to the ventro-lateral protocerebrum via a non-defined tract.

Functional classification and central nervous projection of olfactory receptor neurons (Paper III)

Exploring the physiology of the mosquito ORNs in order to understand how odor coding is accomplished is of great importance. In this study, I concentrated on the most abundant antennal olfactory sensilla of female *Ae. aegypti*, *s. trichodea* in order to understand odor coding patterns at peripheral level. These sensilla can be divided into four distinct morphological sub-types that can easily be distinguished under a light microscope. The sensilla are named based on their morphological characteristics: short sharp-tipped (sst), long sharp-tipped (ls), short blunt-tipped I (sbtI) and short blunt-tipped II (sbtII) *s. trichodea* (Figure 3B). There are ~800 of these sensilla whose function, until now, have not been thoroughly investigated (McIver, 1982).

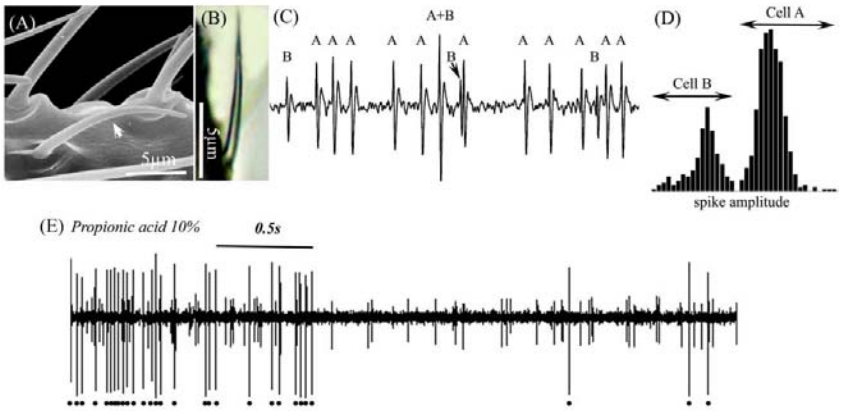

Figure 10. Single sensillum recordings from a 'sst' sensillum trichodeum. Scanning electron and light microscopic micrographs of the sensillum are shown in A and B. Differential spike amplitudes, displayed in C, correspond to the two ORNs, *i.e.* A and B, housed in the sensillum. The spike frequency of the two neurons are occasionally superimposed (A+B). Distribution of spike amplitudes of the two neurons is shown in D. Inhibition of the A neuron in response to propionic acid is shown in E.

Using single sensillum recordings (SSRs), I investigated the function of single ORNs housed in the *Ae. aegypti s. trichodea*. To do so, the antenna was first mounted on a microscope slide bearing double-sided sticky tape. The antennal sensilla were clearly visible under a light microscope with high magnification (750X). One sharpened tungsten microelectrode was positioned in the eye (ground electrode) and another (recording electrode) at the base of the sensillum until a contact was established (Figure 7). The spontaneous activity of individual ORNs, spikes or action potentials, housed in a single sensillum was separated based on shape and amplitude. The spontaneous activity as well as the shape and amplitude of single ORNs are believed to be a function of the physical parameters of a particular neuron, *e.g.* number of dendrites, length and diameter of dendrites *etc.* (Hansson *et al.,* 1994). Spike amplitude classes are believed to be a good representation of the number of ORNs residing in a single sensillum. Conventionally, the ORN with the larger amplitude is named 'A' and the ORN with the smaller amplitude is named 'B'. In some cases the firing of A- and B-neurons coincide, resulting in superimposed spikes (A+B) and thereby a higher amplitude (Figure 10).

In order to functionally classify the ORNs I stimulated them with physiologically relevant odor ligands. The ligands chosen were previously found to elicit either electrophysiological and/or behavioral responses in mosquitoes. A collection of 16 compounds, representing three different classes of compounds, *i.e.* oviposition attractants and human- and plant-related compounds, were diluted and delivered to the animal through a stimulator.

Based on 85 successful recordings, 18 functional classes of ORNs were found. In addition, four ORNs were non-responding. The ORNs were stereotypically

paired in 11 functional classes of sensilla, where each ORN displayed a unique response to the panel of tested compounds. In addition, I observed a consistent signaling mode (excitation and inhibition), and temporal dynamic pattern (phasic and tonic) of single ORN types.

Through molecular/cellular approaches, we know from other insects (and vertebrate) that functional types of ORNs project to spatially distinct glomeruli in the AL (Hansson *et al.,* 1992; Vosshall, Wong & Axel, 2000). In order to investigate how olfactory information is organized in mosquitoes, through anterograde neurobiotin staining of functionally defined ORNs the targed AL glomeruli of female *Ae. aegypti* were located and compared with our previously made 3-dimensional spatial map (*i.e.* paper I). This led us depict a functional map of the antennal lobe of the female *Ae. aegypti* (Figure 11).

Based on the number of successful stainings (10 out of 70 attempts), it was evident that the two ORNs originating from an individual sensilla were targeting single glomeruli in the ipsilateral antennal lobe. Interestingly, in some cases one neuron was more intensely stained than its counterpart. I assume that the differential staining of the neurons was due to differential excitatory response to the stimulus, a phenomenon that was found by Hansson *et al.* (1992).

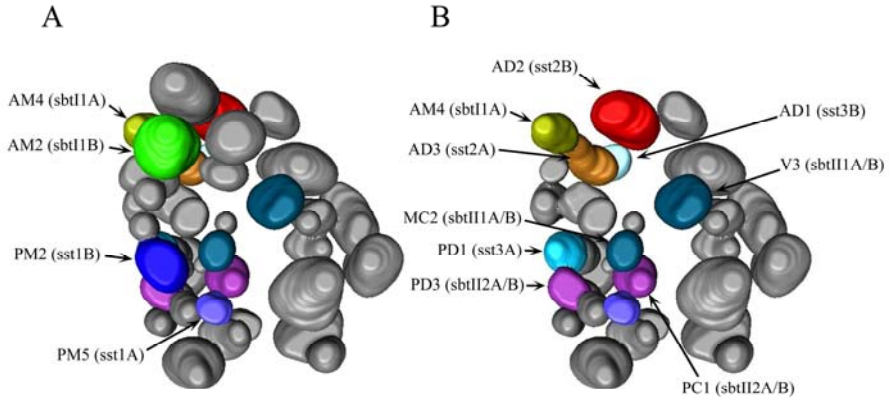

Figure 11. (A, B) A combined 3D reconstruction and functional map of the AL of a female *Aedes aegypti*. Outside the bracket letters indicate the glomerulus name (see Ignell *et al.,* 2005) and inside the bracket letters indicate the functional classes of the ORNs targeting the glomeruli.

Novel electrophysiologically active ligands (Paper IV)

Female *Aedes aegypti* mosquitoes are the primary vectors of dengue and yellow fever (Gubler, 1989; Monath, 1989). It is the host-seeking behaviors, including their high degree of anthropophily and endophily, of these mosquitoes that makes them such efficient vectors. The predominant cues driving the host-seeking behavior are odor volatiles. In a previous study (paper III) I demonstrated that antennal ORNs of female *Ae. aegypti* are divided into functionally distinct classes.

The axons of these ORN classes project and terminate stereotypically into 51 ipsilateral antennal lobe glomeruli. The compounds used had been found in previous studies to be either behaviorally and/or electrophysiologically relevant for *Ae. aegypti* and other mosquito species (*e.g.* Davis, 1977; Bentley, McDaniel & Davis, 1982; Bowen, 1992; Puri *et al.,* 2006). However, it is likely that more relevant key ligands remain to be discovered for some olfactory receptor neurons on the mosquito antenna.

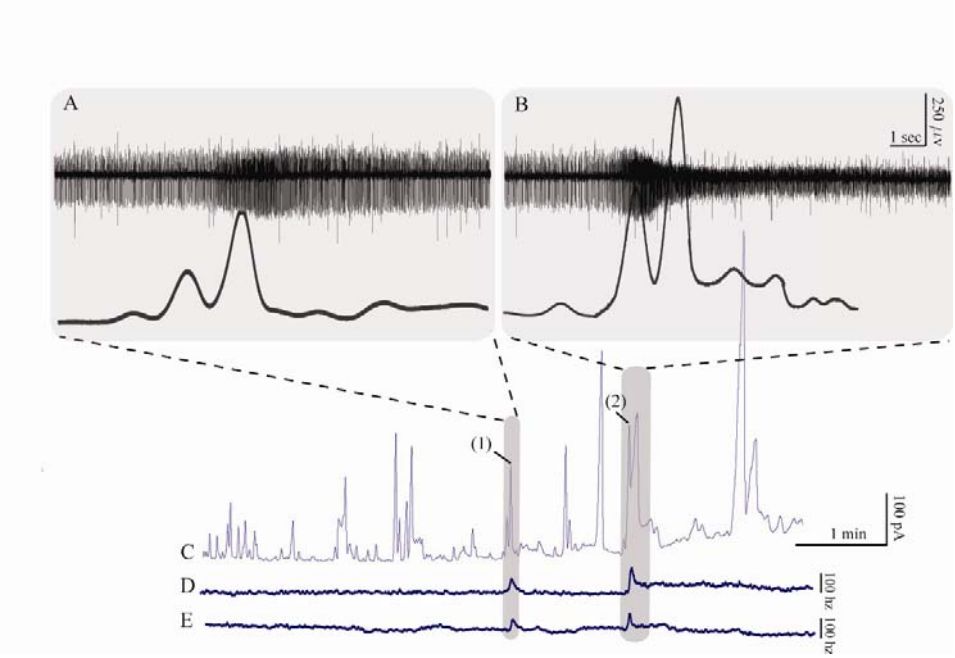

Figure 12. Coupled GC-SSR from short blunt subtype II sensilla trichodea, sbtII2. (A and B) Electrophysiological responses of the 'A' neuron to two FID peaks (1,2) obtained from injection of a sock headspace extract. Mass spectrometry (MS) resulted in the identification of (1) octanal and (2) nonanal. C: GC-trace; D, E: the corresponding single sensillum recordings presented in line form.

Here, gas chromatography-single sensillum recordings (GC-SSR) on antennal s. trichodea and intermediate sensilla (the most abundant sensilla) were performed, for the first time (Figure 7) on female *Ae. aegypti* to elucidate the chemical identity of compounds to which the ORNs respond. The technique has previously been used in several insect specie (*e.g.* Arn, Städler & Rauscher, 1975; Wadhams, 1982; Dawson *et al.,* 1987; Stensmyr *et al.,* 2003; Kristoffersen, 2006). I used headspace collections and solvent washes from biologically relevant sources, such as different human body parts including foot, armpit and trunk regions. For foot odor collection, volunteers were given new socks. For armpit odor collection the volunteers used sterile gauze pads. For torso odor collection I used the night T-shirt and underwear of the volunteers. In all cases the volunteers were asked not to take a bath or use deodorant for 2 days during the experiment. The items were then collected and isolated into separate plastic bags. The bags were connected to

a pump (KNF Neuberger, Stockholm) through which air was blown into the bag, circulated and then sucked out through a filter. Using Porapak Q filters (Supelco) I could trap the odors. Head-space collections in the filters were then washed out with hexane and delivered to single sensilla through the GC. The GC separates the injected extracts into single components while passing through a capillary column. During the separation process, a carrier gas (hydrogen) pushes the extract along the column which temperature is raising (10ºC/min). The extract then gets separated and the single components then pass, based on their boiling point, through the column linked to the SSR setup.

I found that several types of ORNs responded strongly to the extracts injected. By using the GC-MS (GC-mass spectrometry) technique a number of compounds eliciting a response were tentatively identified (Figure 12). The compounds have earlier been reported to be present in human emanations (Curran *et al.,* 2005; Bernier *et al.,* 2000, 2002). Examples are heptanal, octanal, nonanal and decanal. Sensitivity of the ORNs housed in two functional sensillum types was then examined using different concentrations (ranging from 0.001 to 10%) of the synthetic compounds.

One of the environmentally safe strategies in the field control of mosquitoes is using odor-based traps in which different mosquito attractants can be combined for luring them to the trap. Therefore, identification of novel natural ligands may help us to develop traps with a more optimal bait composition.

Conclusion and future directions

With the insight provided in this study into mosquito deutocerebral neuroarchitecture and peripheral neurophysiology, a significant step towards a better understanding of the mosquito olfactory system has been taken. In general, we need to know how the neural connectivity looks in the mosquito central nervous system to establish a firm base to understand olfactory-guided behavior in mosquitoes. I could show the applicability of the AL 3D map in paper III. Taking advantage of a high resolution 3D map in *Ae. aegypti* followed by an attempt to trace functionally defined ORNs has led me to partially define a central functional map. In addition, I was able to show that different morphological types of antennal sensilla are divided into different physiological subtypes, similar to what *e.g.* Qiu *et al.,* 2006 on mosquitoes and de Bruyne, Foster & Carlson, 2001 on flies found. These studies are important for understanding of coding patterns in the olfactory system. Although, my physiological data support the one receptor neuron-one glomerulus hypothesis, I am unable to discard the possibility of a 'multi receptor cell-one glomerulus' system. Such a system could exist in insects in order to improve odor coding, and to allow appropriate behavioral decisions. To increase our understanding of olfactory coding of the classified neurons my fourth project was designed to identify novel ligands for the functionally classified receptor neurons. Identification of novel natural ligands for mosquitoes may help us to develop or optimize bait-based traps, as an environmentally safe method for

mosquito control. Moreover, the compounds identified could be used in a push-pull strategy in mosquito control: pulling them to the trap using attractants and pushing them from the people using repellents.

What I have provided in my thesis has neither been the first attempt nor is it going to be the last one in mosquito neurobiology. Stated another way, there are and will be numerous other issues related to mosquito olfaction that need to be addressed in the future by researchers. Functional mapping of the ALs of other mosquito species, particularly of the *Anopheles* complex (similar to what Kondoh and coworkers, 2003 carried out in *Drosophila* complex) is a desirable project. Comparison between different maps belonging to the *Anopheles* complex will allow us to make interpretation of mosquito evolution, ecology, behavior and sexual dimorphism. For functional classification of the ORNs, one could shift from s. trichodea to other olfactory sensilla like grooved pegs and intermediate ones. The compounds used or identified in this study could be tested in behavioral experiments. In line with the GC-SSR work which could be performed on *Anopheles* complex perhaps other analytical methods like HPLC-MS as well as other odor sampling methods should be carried out.

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Acknowledgements

For me joining such a productive and cheerful lab with its kind members was a great chance. Now, it is my turn to express my deepest gratitude to those who have been directly or indirectly involved in my investigations.

First and foremost, I'd like to express my sincere gratitude to my main supervisor, **Professor Bill Hansson**. Words fail to adequately thank you for you did not spare any effort or time to improve my scientific ability. I am really impressed by your knowledge, wisdom and dignity. Thanks for accepting me in your wonderful lab and giving me such a marvellous opportunity to get acquainted with novel scientific topics. Your spiritual and logistic support will never be forgotten. Without such support, I would have never achieved the success I feel I have today. I know that I cannot repay for your favors. But at least I can promise the best investment of the many lessons I have learned from you.

I wish to express my deepest gratitude to my co-supervisor, **Dr. Rickard Ignell** from whom I have learned a lot. Rickard, thanks for the inspiring guidance, encouragement and valuable criticism during my Ph.D. work. Thanks for being always accessible and caring about my scientific progress. I shall confess that the deadlines that you put for me throughout my study period worked out perfectly.

Mattias
It was a great chance to carry out my last project with you. I'm indebted to you for creating such a gainful, and yet enjoyable, atmosphere for me. Also thanks for patiently answering my questions.

Medhat and Sharon
My sincere thanks are due to you (my respectable teachers) who, without any expectation, spent much of your valuable time to boost my scientific level.

Teun
Your smiley face and the way you made lovely jokes will never go away from my mind. Thanks for the help with equipment.

Rita, Ylva, Marie-luis, Marrie, Elizabeth
I'm deeply grateful to you for the assistance, and for being a family for me while I was away from my original one.

My office-mates
Kattis (still I don't forget the first time when I met you in the office, be successful wherever you are), Nanna, Agnieszka, Jonas (don't forget to close the window during the freezing Swedish winter time), Yitbarek (you have been my first friend when I arrived to Sweden, do you remember the guest house in September/October 2003? All the best with your Ph. D career), Merid, Siju (see below!!), Satoshi, Maryam (of course, you have been everywhere with me!!).

Many thanks to:
Fredrik, Anna-Carin, Peter W., Peter A., Niels, Jocelijn (for showing me how to work with the SSR), Göran (such a great chance to sit down with such an experienced scientist), Anna B., Christian, Holger, Wiltrud, Felipe, David, Johannes, Per M., Sonia, Sussanna, Zsolt, Nerilda, Eraldo, Nelia, Mikaela (your wonderful wedding was unforgettable), Paola, Yang, Martin A., Juliane, Geir, Daniel, Elin, Eraldo, Franscesca, Andreas, Malin, Irene, Isabella, Micke, Marcus Sj., Marcus St., Per N., Martin, Lena, Marco, Simon (for drawing the mosquito displayed in my thesis cover page).

My friends who are striving to get through with their education (Have fun!!)
Martin A., Sophie, Tina, Miriam, Hamida, Siju, Anneli, Lina, Jonas, Ulf.

My family
There is no doubt that reaching the final point of my career was impossible if my adorable wife, Maryam, was not beside me. Thanks Maryam for the provision of an appropriate atmosphere in order for me to more comfortably deal with my educations. Thanks for putting up with all the material deficiencies that I had as a student.

My deepest sense of gratitude is also to my parents, brother and sister who have always been concerned about my living and studying situation in Sweden.

Siju
Friend of my loneliness, to whom I used to tell my personal problems. Thanks for being a sympathetic listener. I will certainly miss you so much. Good luck with your Ph. D. studies.

The ministry of science research and technology of Iran has supported me all along my Ph. D. work and was always ready to solve any technical problems. So, I'm grateful to them.

Thank you all for the creation of an exceptionally educational and exciting chapter in my life.

Good bye!