MOLECULAR AND CELLULAR STUDIES OF MOSQUITO ODORANT RECEPTORS AND OLFACTORY-DRIVEN LARVAL BEHAVIOR

By

Yuanfeng Xia

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Biological Sciences

December, 2008

Nashville, Tennessee

Approved

Professor Clint E. Carter

Professor James G. Parton

Professor Ronald Emeson

Professor Bruce Appel

Professor Laurence J Zwiebel

To My Parents:

Guangjin Xia and Delan Liu

ACKNOWLEDGEMENTS

This work could never have been possible without the guidance, help and support of my mentor, my fellow lab members, my family and the great people at Vanderbilt University. I thank Dr. Laurence J. Zwiebel, my research advisor, who is a great scientist and mentor, who teaches everything needed to be successful in both science and life, and who understands me as a young kid pursuing his dream on a foreign land. I thank my other committee members, Dr. Clint E. Carter, Dr. Bruce Appel, Dr. Ronald Emeson, Dr. James G. Parton. Special thanks to Miss Daniela Buscariollo, who helped me with larval behavioral assays, one of the most important components of this work. This work could not have been done timely without her help. Many thanks to Mr R.Jason. Pitts, who developed SEM technique in the laboratory and showed me the whole procedure. I also want to thank Dr Tan Lu and Dr Hyung-Wook Kwon for their invaluable advice on my *in situ* hybridization experiments. I want to thank the past and present members of the Zwiebel lab, including Pingxi Xu, Michael Rutzler, Jonathan Bohbot, Patricia L. Russell, Hans-Willi Honegger, William B. Walker, David Rinker, Zhen Li, Lujuan Sun, Patrick Martin, Patrick L. Jones, Melissa Zhu, Brittany Guy and Taha Jan. It is them who make the lab a great place to work and stay.

Thank you to the Department of Biological Sciences at Vanderbilt University, which provided me with a graduate student fellowship and an extremely friendly environment during my early graduate career and continuous support throughout my whole graduate school experience.

To all my friends, Thank you. My life-long friends Yudong Wang, Yanmin Zhu, Baolian Ji, who shared the my best high school time with me and helped me to take care of my parents when I was struggling to pursue my dream in a foreign country. My college friends Feng Gao and Zushi Huang, who have never turned their backs on me when I am in need of help. My graduate school friends, Mingyan Li, Qiang Sun, Guoxiang Ruan, Feng Li, Yelin Chen and so many others, have been here with me throughout. I will remember all the joy you have shared with me.

Lastly, I want to thank my parents: Guangjin Xia and Delan Liu, who have loved me unconditionally for the last 29 years, who always have the greatest faith in me, who will give me the best support whenever and wherever I need, who give me the best advice on both life and work, who create the best family I can ever imagine to be born into. I love you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

7TM.....Seven Trans-Membrane

AgOr…….*An. gambiae* odorant receptor

AL…….Antennal lobe

AP……..Alkaline phosphatase

CODEHOP…….COnsensus-DEgenerate Hybrid Oligonucleotide Primer

Cp……Capitate peg

cRNA……...Complementary RNA

DEET……*N*,*N*-diethyl-3-methylbenzamide

DO.....Dorsal Organ

FISH……..Fluorescent *in situ* hybridization

GPCR……G protein-coupled receptor

IRS…….Indoor residual spraying

LAL……..Larval Antennal Lobe

OBP…….odorant binding protein

OR……..Odorant receptor

ORN…….Olfactory receptor neuron

PI……..Performance index

PN.....Projection Neuron

RT…….. Reverse-transcriptase polymerase chain reaction

SEM…….Scanning electron microscopy

Sensory neuron membrane proteins.....SNMPs

TO.....Terminal Organ

VO.....Ventral Organ

WNV……..…….....West Nile Virus

CHAPTER I

INTRODUCTION

The ability to detect a wide range of sensory cues is essential for the survival and vectorial capacity of mosquitoes (Takken and Knols, 1999). Of these sensory stimuli, chemosensory inputs, especially olfactory cues, are crucial in food detection, mating, predator avoidance and other behaviors. Furthermore, olfaction is of great importance in the host-seeking behaviors of several vector mosquitoes that are responsible for the transmittance of malaria, West Nile Virus (WNV) and dengue virus (Zwiebel and Takken, 2004). A deep understanding of the olfactory principles underlying host preference will likely provide novel approaches that target these critical mosquito behaviors to reduce the spread of mosquito-borne diseases. This dissertation focuses on two distinct but ultimately related research investigations: the molecular/cellular analysis of olfactory-driven behavior in malaria vector mosquito *Anopheles gambiae* larvae and characterization of an odorant receptor (OR) from West Nile Virus (WNV) vector mosquito *Culex quinquefasciatus*.

Mosquito-Borne Diseases and Vector Mosquitoes

Human malaria remains the most important mosquito-borne disease in the world WHO, 2007）(Figure 1). According to the latest world Malaria Report by the World Health Organization and the United Nations Children's Fund, there are between 350 and 500 million clinical cases per year world wide, causing more than 1 million deaths annually. In addition to the morbidity and mortality it engenders, malaria is an enormous burden to

the developing countries in Africa, where social and economical stresses originating from malaria are amplified due to the factors such as drug and insecticide resistance, social and environmental changes and population surges (WHO, 2007).

Human malaria is transmitted by female mosquitoes of the genus *Anopheles* (Figure 1). Approximately 422 species of *Anopheles* exist in the world, among which *An. gambiae sensu stricto* is the most effective vector of malaria (Takken and Knols, 1999). *An. gambiae* is extremely anthropophilic, taking its blood meals almost exclusively from humans which factors greatly in its vectorial capacity, an overall assessment of its ability to transmit human malaria (Macdonald, 1957). The selection of blood meal hosts is a complex and not precisely understood process that reflects both the physiological status and sensory inputs. In the latter context, chemosensory cues principally in the form of olfactory signals, together with heat, humility and visual factors, are the most dominant sensory inputs during the host-seeking behaviors in *An. gambiae* and other vector mosquitoes (Takken and Knols, 1999).

Another significant mosquito borne disease is Dengue/Dengue Hemorrhagic Fever which is caused by one of four closely related, but antigenically distinct, virus serotypes (DEN-1, DEN-2, DEN-3, and DEN-4), of the genus *Flavivirus* (Gubler, 1987). Infection with one of these serotypes provides immunity to only that serotype for life, so persons living in a dengue-endemic area can have more than one dengue infection during their lifetime. Dengue fever is the primary disease of tropical and sub tropical areas, and the four different dengue serotypes are maintained in a cycle that involves humans and the *Aedes* mosquito (Yap et al., 1994). However, *Aedes aegypti*, a domestic, day-biting mosquito that prefers to feed on humans, is the most common *Aedes* species. Infections

Disease	Causive agent	Vector Mosquito	Annual cases	Annual Deaths	Countries Affected
Dengue Fever	Dengue Virus	*Aedes*	50-100 million	200,000	>100
Yellow Fever	Yellow Fever Virus	*Aedes*	200,000	30,000	>42
West Nile Virus	West Nile Virus	*Culex*	varies on yearly basis	varies	Africa, Australia Europe, Middle east Asia, and North America
Japanese encephalitis	Japanese encephalitis Virus	*Culex*	50,000	10,000	>10
Malaria	*Plasmodium*	*Anopheles*	500 million	>1 million	>105

Figure 1. Important Mosquito-Borne Diseases

produce a spectrum of clinical illness ranging from a nonspecific viral syndrome to severe and fatal hemorrhagic disease. Important risk factors for dengue fever include the strain of the infecting virus, as well as the age, and especially the prior dengue infection history of the patient (Hayes and Gubler, 2006).

Yellow fever is a mosquito borne arboviral disease that has caused large epidemics in Africa and Americas. It can be recognized from historic texts stretching back 400 years (Barrett and Higgs, 2007). The disease is caused by the yellow fever virus, which also belongs to the *flavivirus* group. In Africa there are two distinct genetic types (called topotypes) associated with East and West Africa. South America has two different types, but since 1974 only one has been identified as the cause of disease outbreaks. Infection causes a wide spectrum of diseases, from mild symptoms to severe illness and death. The "yellow" in the name is explained by the jaundice that affects some patients. Although an effective vaccine has been available for 60 years (Monath, 2005), the number of people infected over the last two decades has increased and yellow fever is now a serious public health issue again (Tomori, 2004).

While considerably less of a public health threat as compared to malaria, and the arboviral maladies mentioned above, WNV has been spreading across North America since it was first recognized in New York City during 1999. Indeed, in 2007, at least 3630 cases of WNV human infections were reported resulting in 124 deaths (Center for Disease Control, 2008). In addition, WNV poses a significant threat to birds as well as other economically important domestic livestock animals such as cattle and horses (Figure 1). The principal mosquito vector of WNV in the United States is *C. quinquefasciatus*, often noted as the major domestic mosquito in many urban areas,

particularly characterized for its propensity for indoor biting (Center for Disease Control). In laboratory studies, *C*. *quinquefasciatus* has also been shown to carry Murray Valley encephalitis virus (Kay et al., 1984). In addition to these important diseases, several other mosquito-borne infectious diseases such as St. Louis Encephalitis still affect many parts of the world including the United States.

Current Malaria Control Strategies

An important component of malaria control strategy focuses on improved access to medical services (Killeen et al., 2003). The availability of healthcare services for diagnosis and treatment is crucial to reducing the mortality risk associated with exposure to *An. gambiae* mosquitoes, but has little effects towards limiting the incidence of clinical malaria in areas of high transmission because it deals only with malaria infections after they occur and has minimal impact on the infectiousness of the human reservoir(Killeen et al., 2000; Lengeler, 2004). Large-scale prophylaxis has played a significant role in some notably successful control program, but may also have limited impacts and even quite dangerous consequences such as the emergence of drug resistance(Raymond et al., 1991). Even intensive infection control with active detection, drug treatment, and follow up cannot eliminate endemic malaria from most parts of sub-Saharan Africa unless rapid re-infection can be prevented by effective vector control, which has been focusing on the control of adult mosquito populations utilizing insecticide spray and pyrethroid-treated bednets (Collins and Paskewitz, 1995).

Domestic insecticide control strategies can substantially lower both morbidity and mortality and remain the most commonly favored approaches for malaria

prevention(Curtis et al., 1996). Bednets have gained great success in the control of malaria in sub-Saharan Africa where high transmission levels result in extremely stable malaria prevalence, incidence and clinical burden(Curtis et al., 1996). Insecticide-treated bednets protect local populations by diverting *An. gambiae* mosquitoes to search for a blood meal elsewhere or by killing those that attempt to feed. Therefore treated nets can prevent malaria in even unprotected individuals by suppressing vector population. However, the results of individual studies often differ and although some trials with *An. gambiae* have demonstrated substantial reductions of its survival rate, others have found little or no effects on the *An. gambiae* population as a whole (Curtis et al., 1996). Moreover with the wide application of insecticide, mosquitoes are developing defense strategies to encounter the effects of the adult insecticide. In as early as 1970s, evidence has been shown that *An. gambiae* mosquitoes started developing resistance towards DDT(Haridi, 1970). *Culex* mosquitoes overproduce nonspecific esterases to resist organophosphate insecticides (Raymond et al., 1991).

Given that the most commonly favored malaria control strategies for protection against adult mosquitoes have clear limitations, it is worth considering other options, not as alternatives but rather as possible additions to current program(Killeen et al., 2000; Marsh and Snow, 1999; Shiff, 2002). The development of novel approaches targeting malaria control, such as transmission-blocking vaccines and genetically modified mosquitoes, are being vigorously advocated and pursued (Ito et al., 2002; Richie and Saul, 2002). These approaches are unlikely to see practical applications for several years or even longer and their likelihood of success has been questioned (Boete and Koella, 2002; Enserink, 2002). In this context, it may be useful to reconsider the simple

larval control methods that enabled the most historically successful attempts to control malaria transmission by *An. gambiae*, before such approaches were abandoned in favor of modern synthetic adultcides (Killeen et al., 2002a).

The Role of Olfaction in Mosquito Host Seeking Behaviors

Human malaria transmission requires the dynamic interplay among three targets -humans, plasmodium parasites pathogens and the mosquito vectors. Effectively, a plasmodium infected mosquito must take a blood meal from a human host, at which point plasmodium sporozoites are transferred to the human host. This malaria transmission cycle; of plasmodium from an infected mosquito to a non-infected human, and from an infected human to a non-infected mosquito, results in a vast reservoir of disease potential in regions endogenous to both suitable *Anopheline* mosquito vectors and plasmodium parasites. Further compounding the prevalence of malaria transmission is the strong preference for human hosts (White, 1974) inherent to the *An. gambiae* mosquito. This unique feature, known as anthropophily, has been thoroughly shown to be mediated by the mosquitoes' sense of smell (Takken, 1991) (Figure 2). Moreover, additional *An. gambiae* characteristics such as its preference for resting indoors (endophily) strongly increases the vectorial capacity (Takken and Knols, 1999).

Numerous electrophysiological and behavioral studies, both in the laboratory and in the field, have been performed to improve our understanding of this particular characteristic. Early studies have shown that host preference in *An. gambiae* was strongly mediated by olfactory cues (Takken, 1991). Furthermore, anthropophily as opposed to zoophily (preference for other animals, such as cattle) was also observed

(Coluzzi et al., 1975; Pates et al., 2001).

The olfactory nature of the attraction towards human hosts was first confirmed in laboratory studies demonstrating that the mosquitoes were responsive to isolated human volatiles in the absence of a human subject (Costantini et al., 1993; Mboera and Takken, 1997). Ever since then, it has been shown that *An. gambiae* mosquitoes are attracted to emanations from human sweat (Braks and Takken, 1997) ,human skin (Takken and Knols, 1999) particularly, from the foot and ankle region, but surprisingly, not from human breath (de Jong and Knols, 1995). Interestingly, the aliphatic fatty acids that constitute the most important components underlying mosquitoes' attraction to human skin emanations (Knols et al., 1997), are actually metabolic byproducts of resident microflora (Nicolaides, 1974). Other human odors implicated in the attraction behavior of *An. gambiae* include ammonia (NH3), lactic acid (Braks et al., 2001; Dekker et al., 2002), and even a synergistic blend of NH3, lactic acid, and carboxylic acids (Smallegange et al., 2005). Finally, while it has been established that many mosquitoes, including *An. gambiae*, respond to carbon dioxide (CO_2) both behaviorally (Dekker et al., 2002; Dekker and Takken, 1998; Knols et al., 1994) as well as electrophysiologically (Lu et al., 2007a), its role in mediating human host seeking behavior of *An. gambiae* is less than certain. While CO_2 cannot be the strict and sole determinant of anthropophily for *An. gambiae,* as it is a prevalent exhalent of all vertebrates (Zwiebel and Takken, 2004), it is clear that it plays an important role in the host seeking behaviors of many mosquitoes (Takken and Kline, 1989). As described above, external determinants of olfactory-driven host seeking behaviors have been extensively investigated.

Recent advances in molecular biology, especially the completion of *An. gambiae*

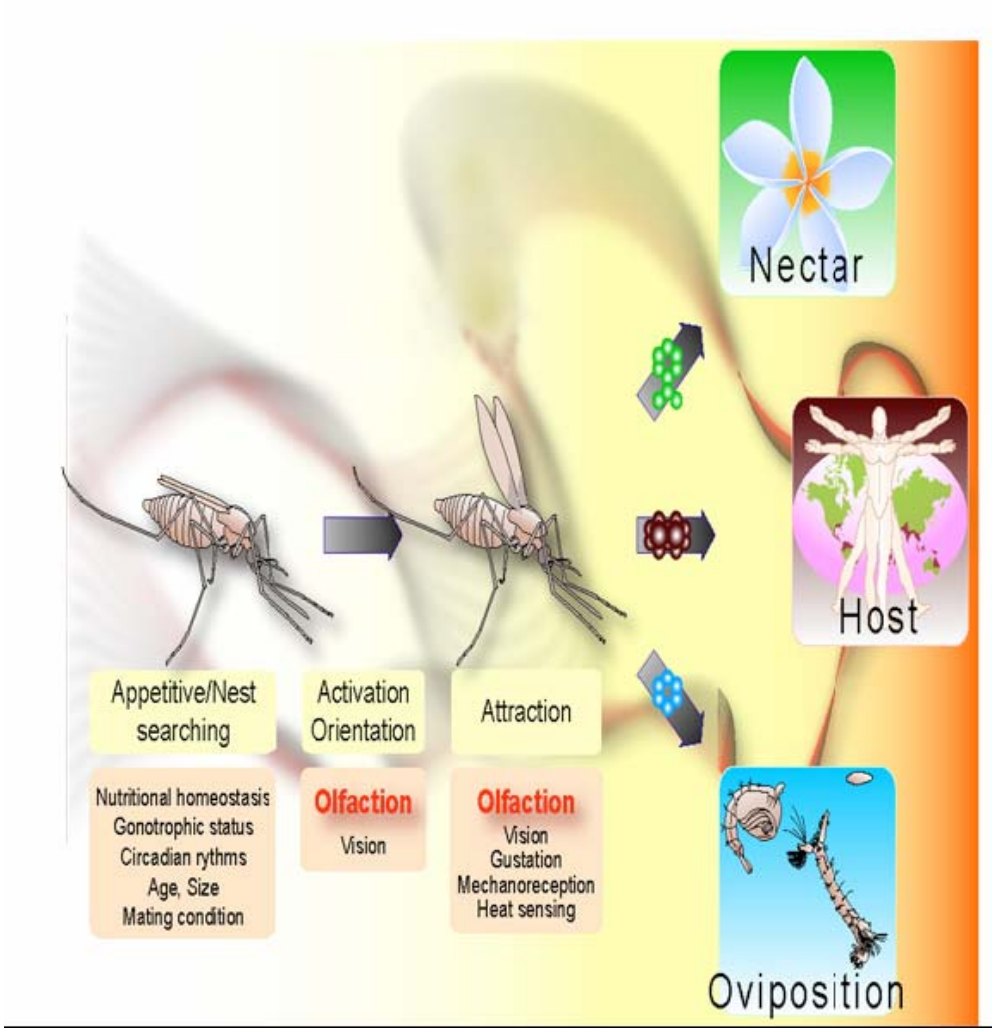


Figure 2. Host seeking behavior is mediated by olfaction.
In *An. gambiae,* a variety of behaviors are influenced by a variety of factors. Importantly, the mosquitoes' sense of smell plays a prominent role in host-seeking behavior. Specifically, *An. gambiae* displays a strong preference for taking blood meals from humans. These facts have dramatic consequences leading to the spread of malaria.Illustration by Dr. Jonathan Bohbot.

genome (Holt et al., 2002) and subsequent identification of 79 putative odorant receptors (ORs) (Hill et al., 2002), have facilitated a greater scrutiny of the molecular mechanisms mediating olfaction at the level of the odorant receptor neuron (ORN). While significant efforts have been made to uncover the neuronal connectivity of *Anopheline* ORNs to higher olfactory processing centers (Ghaninia et al., 2007), it is at the level of the ORN where the primary interface between the mosquito olfactory systemand the environment lies. Thus an integration of knowledge concerning the external olfactory cues with internal olfactory processing mechanisms is necessary for a complete understanding of the most prominent features responsible for olfactory-driven behaviors that may be targeted in the design of novel malaria control strategies (see below).

Insect Olfactory Systems and Signaling Cascades

Insect olfactory systems may be viewed as consisting of both peripheral and central elements which are assumed to be conserved across a range species (Vosshall, 2000). In *Drosophila*, the peripheral olfactory system traditionally is viewed as consisting of the antennae and maxillary palps, both of which exist in pairs extending from the head of the insect. Each antenna contains ~1200 ORNs, whereas each maxillary palp contains ~120 ORNs. Both structures are covered with hair-like structures called sensilla, which can be subdivided into basiconic, coeloconic and trichoid.sub-classes each of which contains the dendrites of up to four ORNs. The antenna contains all three types of olfactory sensilla, whereas the maxillary palp of *An. gambiae* contains only basiconic sensilla otherwise known as the capitate peg (Figure 3). The respective contributions of the antenna and maxillary palp to chemosensory-mediated behaviors are not yet clear.

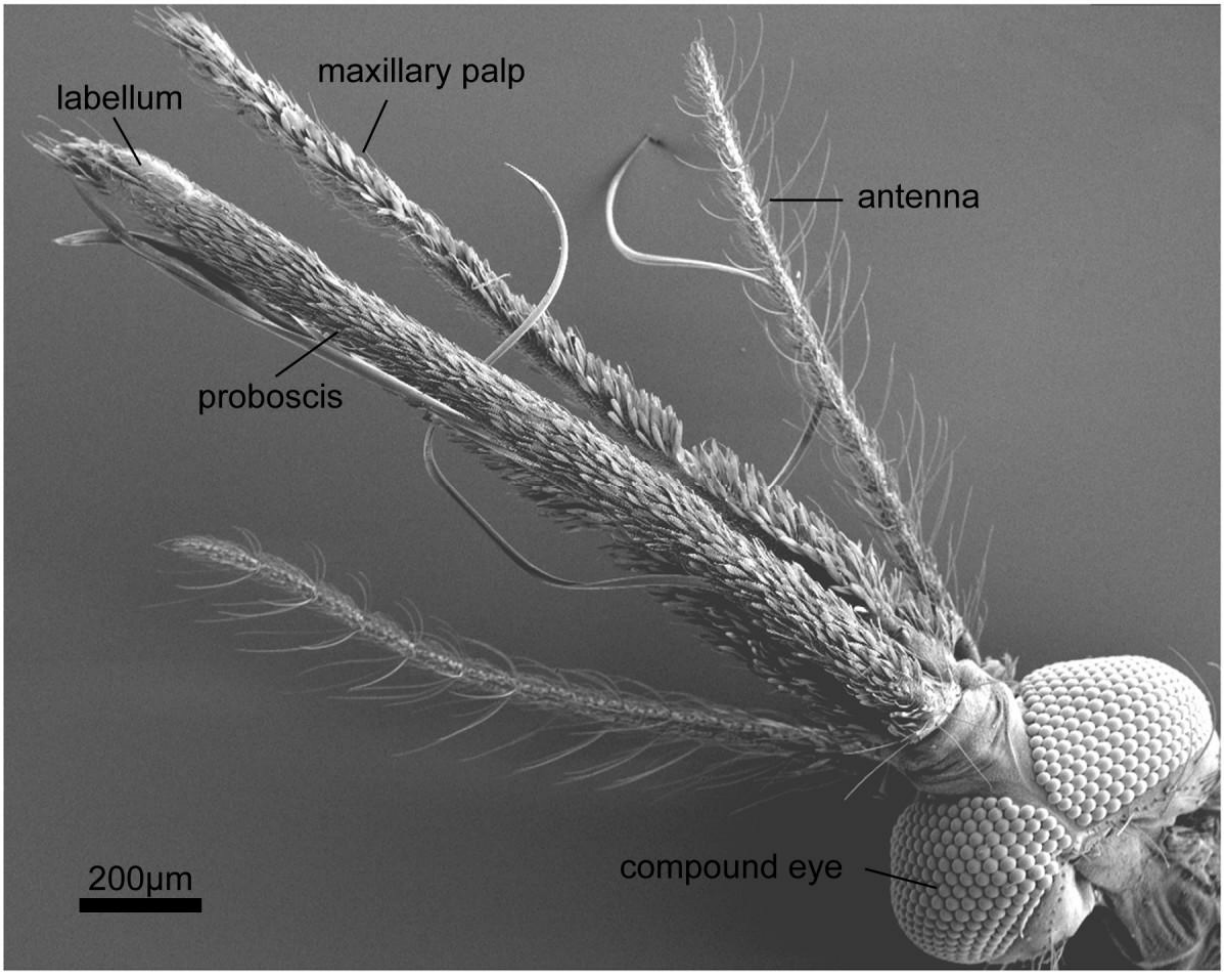


Figure 3. Female Head (Ventral View). Scanning electron micrograph showing the sensory appendages of an adult female *An. gambiae* s.s. Eyes, antennae, and maxillary palps occur in pairs, although the second palp is hidden below the proboscis in this micrograph. The proboscis is a single appendage that encloses the blood-feeding stylets, which appear as ribbon-like tentacles here. At the distal end of the proboscis is the labellum, or labellar lobes (Pitts and Zwiebel, 2006).

Recent studies map the *Drosophila* pheromone receptor to the basiconic sensilla (Kurtovic et al., 2007) and maxillary palps are responsible for CO_2 perception in both *Drosophila* and *An. gambiae*(Jones et al., 2007; Kwon et al., 2007; Lu et al., 2007a).

Olfactory signal transduction begins when an odorant molecule activates a cognate receptor protein within each ORN that is housed with chemosensory sensilla (Figure 4). In this context ORN dendrites reside in an aqueous environment, known as the sensilla lymph. Odorant stimuli are believed to diffuse through the pores on the cuticle that forms the external surface of the sensilla and come into contact this lymph and subsequently with the ORN dendrites (Kreher et al., 2005). Insect ORs are expressed on the dendritic membrane where odorants are believed to interact directly or with the help of OBPs (Figure 4) to generate ORN action potentials. In mammals, the core components of the olfactory signal transduction cascade have been identified. In insects, however, a comprehensive model for olfactory signal transduction remains largely missing.

The first component of this signaling pathway is a soluble class of proteins believed to function upstream of the receptor, so called OBPs (Pevsner et al., 1988; Vogt et al., 1999) . OBPs exist in both mammalian and insect olfactory systems. They were believed to have odorant binding properties. Mammalian and insect OBP families comprise distinct gene families (Scaloni et al., 2001; Xu et al., 2003). With the help of OBPs or alone, the odor ligand will bind to the ORs expressed on the ORN, where the transduction of chemical information into electrical impulses happens. A conformational change in the receptor upon the binding of its ligand recruits and activates an olfactory specific Gs variant known as Golf (Jones and Reed, 1989). The activation of G-proteins

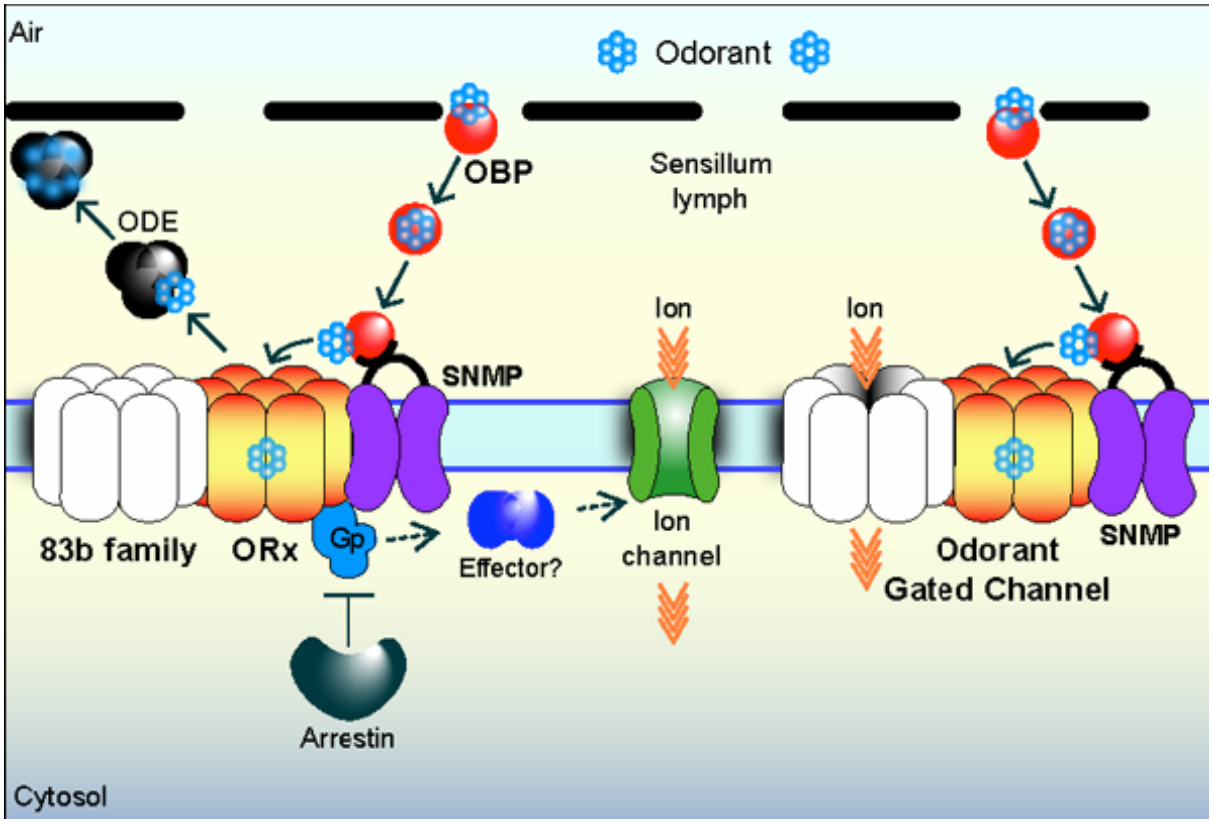


Figure 4. Proposed models of insect olfactory signal transduction. Two models have been proposed. Under both models, odors are presumed to be delivered to the OR through interactions with odorant binding proteins (OBPs). On the left, OR activation is coupled to G protein signaling cascades. On the right, OR activation is directly coupled to the relevant ion channel. Odorant degrading enzymes (ODEs) have been implicated in clearance of active odorants from the sensilla lymph. Sensory neuron membrane proteins (SNMPs) are membrane proteins of unknown function. Illustration by Dr. Jonathan Bohbot.

will trigger the function of an adenylyl cyclase, which leads to a rise in cyclic AMP (cAMP) and consequently the opening of cyclic nucleotide-activated, nonselective cation channels (Dhallan et al., 1990; Nakamura and Gold, 1987). The influx of cations through these channels depolarizes the cell membrane of the OSN, ultimately resulting in an increase in the frequency of action potentials that were transferred along the axons to the glomeruli, globose structures located in the outer part of the olfactory bulb (Mombaerts et al., 1996). The identification of specialized isoforms of Golf, adenylyl cyclase type III and the cyclic nucleotide-activated channel in the olfactory cilia suggests the importance of this pathway. Moreover, gene knockout studies support that the cAMP cascade is dominant in transmitting odorant signals in the olfactory neurons (Brunet et al., 1996), whereas the role of an inositol-1,4,5-triphosphate (IP_3)-mediated pathway remains unclear in vertebrates.

In insects, several downstream components of OR signaling pathways have been indicated as functioning in *Drosophila* olfactory signal transduction cascade. These include genes encoding G protein (Kalidas and Smith, 2002), phospholipase C (Riesgo-Escovar et al., 1995), phosphatidylinositol transfer protein (Riesgo-Escovar et al., 1994), cAMP phosphodiesterase (Gomez-Diaz et al., 2004), cyclic nucleotide (Baumann et al., 1994) and voltage-gated ion channels (Dubin et al., 1998). While the function of these downstream elements remains largely unknown, they match the overall paradigm of G-proteins-coupled-receptor (GPCR)-mediated signal transduction in vertebrate systems. Moreover, these data suggest the presence of multiple signaling pathways that activate AC and PLC cascades. Indeed, dual signaling pathways have been observed in ORNs of the lobster (Hatt and Ache, 1994) and other animals(Ache

and Zhainazarov, 1995). However, as previously mentioned, there are emerging evidence that insect olfactory signal transduction may not embrace canonical GPCR signaling (Benton et al., 2006). Thus a comprehensive model for olfactory signal transduction in *D. melanogaster* and other insects remains undefined (Figure 4).

Information processing pathways downstream of ORNs are of central importance in defining odor coding. Here ORN axons to connect to the AL (Figure 5) of the central nervous system (CNS) where axons forms bundles of fibers called glomeruli (Stocker, 1994). The functional organization of insect AL is remarkably similar to that of the olfactory bulb in vertebrates (Hildebrand and Shepherd, 1997). The AL glomeruli are innervated by PN dendrites that are linked to the mushroom body (MB) and lateral horn of the protocerebrum which represent the higher CNS processing centers (Figure 5).

Odorant Receptors

Following the first odorant receptors cloned from rat olfactory epithelium in 1991, numerous studies towards understanding the structure and function of ORs were done in mammals. Initial speculation was based upon observations that odorant stimulation of the olfactory epithelium led to measurable cellular increase of cyclic adenosine monophosphate (cAMP) (Pace et al., 1985) catalyzed by adenylyl cyclase (AC) enzymes that are coupled to G-protein signal transduction pathways. Current evidence supports the model that mamalian ORs belong to the GPCR superfamily and are seven-trans-membrane (7TM) proteins (Gaillard et al., 2004)

Since then, candidate 7TM GPCR ORs have been identified and characterized in

numerous mammalian species (Mombaerts, 1999). With the completion of mouse and rat genome, 1200-1400 putative ORs have been classified in these model vertebrate systems (Ache and Young, 2005). In humans, more than 800 putative ORs have been identified in the genome alone, but over half of these turn to be pseudogenes, based upon the presence of premature stop codons and other deleterious mutations within the coding sequence (Niimura and Nei, 2003), compared to a much lower prevalence of pseudogenes in rat and mouse, which is about 20-25 percent only (Ache and Young, 2005).

Similar RT-PCR approaches utilized in cloning of the mammalian ORs were initially unsuccessful in insects. In what has proven to be the hallmark for the cloning of all insect ORs, the completion of the *Drosophila* genome project provided a breakthrough in that it facilitated a bioinformatics-based approach for OR identification. Here, a novel computer algorithm that searched for diagnostic features of the GPCR superfamily, including hydropathy, polarity and weighted amino acid composition of the predicted protein successfully identified multiple putative OR genes from fly genome (Kim et al., 2000). These ORs form a highly divergent family of genes, displaying between 10% and 75% identity and bearing no significant homology to any other GPCR family (Clyne et al., 1999b; Vosshall et al., 1999). Subsequent efforts using similar approaches have been employed to identify ORs from other insect species (see below).

Compared to mammalians, Insects have far fewer putative ORs. Only 62 candidate DmORs were identified in *D. melanogaster* (Clyne et al., 1999b; Gao and Chess, 1999; Robertson et al., 2003; Vosshall et al., 1999). Similarly, there are 79 OR genes in *An. gambiae* (Hill et al., 2002), 131 in A. aegypti(Bohbot et al., 2007), 170 in *Apis. Mellifera*

(Robertson and Wanner, 2006), ~ 48 in silkworm *Bombyx mori* and ~341 in *Triboulium castaneum* (Abdel-Latief, 2007). In contrast to mammalian ORs, as well as those from the nematode, *C. elegans,* which have approximately 25% pseudogenes, (Robertson, 1998), there are few if any known pseudogenes in the OR families of insects (Robertson et al., 2003). Another distinct characteristic of insect ORs is the presence of alternative splicing, which is absent in mammalian ORs (Mombaerts, 1999; Robertson et al., 2003).

While precise protein structures for insect ORs have not been determined, there is little doubt that they form functional heterodimers and are the central component in odor detection (Hague et al., 2004). Insect ORNs generally express a highly divergent "conventional" odorant binding OR together with a highly conserved "non-conventional" OR belonging to the OR83b subfamily of proteins (Hallem et al., 2004b; Kreher et al., 2005; Kwon et al., 2006; Lu et al., 2007b; Xia et al., 2008). While the biochemical processes inducing the activation of downstream molecular targets are not clearly defined, recent studies suggest that insect ORs exhibit either ionotropic or alternatively both iono and metabotropic properties. On one hand, the kinetics of OR response observed in heterologous expression systems is consistent with the behavior of ligand-gated ion channels that do not require the involvement of G-proteins (Sato et al., 2008). Interestingly, another study indicates an additional metabotropic response requiring the activation of G-protein-mediated secondary-messenger pathways that is somewhat delayed relative to the initial ionotropic response (Wicher et al., 2008). Although at present, no definite answers can be given to this question, the incorporation of G-protein dependent pathways that presumably require the synthesis of secondary messengers is also consistent with numerous reports linking insect ORs to GPCR

signaling pathways (Kalidas and Smith, 2002; Woodard et al., 1992; Yang et al., 1996).

Mosquito Odorant Receptors

As mentioned above, the completion of *An. gambiae* genome has provided an opportunity to study mosquito olfaction at the molecular level of ORs (Fox et al., 2001; Fox et al., 2002; Hill et al., 2002). *An. gambiae* has a family of 79 *AgOR* genes, which are dispersed throughout the three chromosomes (Hill et al., 2002). Fluorescence *in situ* hybridization(FISH) has mapped a subset of ORs to a spatially conserved population of ORNs in adult olfactory and even gustatory appendages (Kwon et al., 2006; Lu et al., 2007a). Phylogenic studies revealed AgORs share low level of conservation with *Dm*ORs (Hill et al., 2002). There is a large subfamily of 27 AgORs with no close *D. melanogaster* relatives (Hill et al., 2002).

Multiple AgORs have been functionally characterized in *Xenopus oocytes* (Lu et al., 2007a; Xia et al., 2008), HEK293 cell lines and the *Drosophila* “empty neuron” system (Hallem et al., 2004a). When expressed in these heterologous systems, AgOR proteins confer responses to a subset of odors tested (Lu et al., 2007a; Xia et al., 2008). Similar to those observed for *DmORs*, responses of the *AgORs* to odors are pervasive. Importantly, the responses of several *AgORs* in heterologous systems are consistent with those of the endogenous ORNs as observed *in vivo* using single sensillum recordings (Kwon et al., 2006; Lu et al., 2007a), suggesting ORs as the molecular basis for odor perception at the ORN level.

In a similar manner, a total of 131 candidate *AaOR*-encoding genes were identified and characterized in the genome of *Ae. aegypti* (Bohbot et al., 2007). Of these, 100

AaOr genes encode putative, complete functional proteins, 10 are incompletely annotated genes, and 21 are pseudogenes. Phylogenetic analysis reveals several gene species-specific expansions in *Ae. aegypti* and *An. gambiae*. Overall, the *AaOr* family is significantly expanded relative to that of *An. gambiae*. Interestingly, there exists a large set of 40 *AaOr*s with no closely related *An. gambiae* homologues. The expansion and diversification of Ors from these two mosquito species may reflect a rapid evolution of the insect chemosensory receptors (Robertson et al., 2003). The OR diversity between these two mosquitoes indicates that many species-specific evolution in sensory perception has happened in the 140–200 million years since the *Anopheline/Culicine* divergence (Krzywinski and Besansky, 2003; Krzywinski et al., 2001)

*AaOr*s are expressed during both the larval and adult stages. An RT-PCR survey determined the spatial expression profiles of these *AaOr*s. Eighty-three of the 123 surveyed *AaOr*s (70%, excluding the previously characterized *AaOr*7) are expressed in the antennae although transcripts from only three *AaOr*s can be detected in the maxillary palps of *Ae. aegypti*. Consistent with the data from *An. gambiae*, a subset of 12 *AaOr*s (excluding *AaOr*7) are also expressed in the proboscis of *Ae. aegypti*. Moreover 23 *AaOr*s were found in the larval antenna, of which fifteen turn out to be larval specific as their transcripts were not detected in adult. Most larval *AaOr* genes from monophyletic clusters and/or are linked on genome.

Lastly, the characterization of the first OR from WNV vector *C. quinquefasciatus* that is detailed in chapter V of this thesis marks an initial (and at present the only) step in understanding the olfactory capacity in this common southern mosquito. With the

imminent completion of *C. quinquefasciatus* genome project, more and more CqOrs are likely to be identified and characterized, providing invaluable information for effective control towards this WNV vector in the US and worldwide.

ORs, ORNs and Olfactory-Driven Behavior in Insects

The first evidence that directly correlates functional OR expression in insect ORNs with olfactory-related behavior comes from *Drosophila*. DmOR83b mutant flies, which suffer a dramatic loss of odor-evoked potentials to a broad range of general odors at the level of the whole antenna and single ORN, become non-responsive to odors which normally elicit strong behavioral responses in wild-type animals, a phenotype that can be rescued with the restoration of DmOR83b expression (Larsson et al., 2004). As DmOR83b is broadly expressed, the deletion of this gene inevitably affects the normal function of multiple classes of ORNs, which ultimately causes the loss of olfactory-related behavior in flies.

Recent studies with *Drosophila* pheromone perception revealed that a single class of ORNs is sufficient to mediate the behavioral response to sex pheromone 11-*cis*-vaccenyl acetate (cVA) (Kurtovic et al., 2007). Mutant flies lack of DmOR67d, whose expression is restricted to a single class of ORNs housed in the T1 type sensilla on *Drosophila* antennae, present inappropriate courtship behaviors, Indicating that odor identity can be encoded by the activation of a subset of ORNs that express a single OR. Moreover, further studies suggest a single functional neuron can provide sufficient information to permit chemotaxis behavior towards odorant stimuli (Fishilevich et al., 2005). Even with only one functional OSN expressing one individual OR42a, *Drosophila*

larvae can still respond to 22 odorants out of a 53 odor panel. These results suggest that a complex behavior can be mediated by a single OSN expressing an individual OR, which constitutes the molecular basis for olfactory-driven behavior in insects.

Relevance of This Work

The investigation of the olfactory system of *An. gambiae,* coupled with existing knowledge of behaviorally relevant human odorants known to mediate host seeking behavior will foster the development of better mosquito attractants and repellents. These products, when coupled with numerous other strategies that are being considered to combat malaria, may dramatically reduce the immense human suffering and financial burden that currently is shouldered by developing countries and regions which are suffering from this deadly disease.

CHAPTER II

AN INTRODUCTION TO LARVAL STAGE MOSQUITOES

An. gambiae Larvae as Important Target for Vector Control

Sir Ronald Ross, who was awarded Nobel Prize in 1902 for his discovery of the life cycle of malaria parasite plasmodium thus uncovering the role of *An. gambiae* as its vector, observed “The most vulnerable point in the history of gnats is when they are larvae; they can be destroyed wholesale.”

An. gambiae mosquitoes have four distinct developmental stages: egg, larva, pupa, and adult. Immature stages of *An. gambiae* mosquitoes require water to complete their life cycle. Larvae will emerge from eggs within 2-3 days after oviposition when environmental conditions are ideal. The normal development of larvae can takes from 4 to 14 days depending on the temperature of the water. The larvae will go through four developmental stages called instars. First instars are barely noticeable to the human eyes while the fourth instar larvae can be approximately 1/2 inch (12.7mm) long under normal nutritional levels. In natural environment, A*n. gambiae* larvae inhabit small water bodies that are often numerous, scattered, sunlit, temporary, and close to human dwellings.

As the larvae are exclusively aquatic, their distribution is determined by the locations of potential larval habitats. Generally, pre-adult *Anopheline* mosquitoes prefer slow-moving or still water in which they can stay close to the surface with their breathing orifices open to the air. Furthermore, unlike some other mosquito species, *Anopheles*

mosquitoes require relatively clean water for development (Fillinger et al., 2004). Therefore before any larval control intervention program can be implemented, the majority of the vector larvae's productive breeding sites must be located. This poses a significant impediment in areas where many breeding sites are inaccessible or ephemeral. Consequently larval control is generally very promising in urban areas, given the high probability of locating the potential breeding site(s)(Keiser et al., 2005) (Caldas de Castro et al., 2004). Indeed, modeling of the combined impacts of insecticide-treated bednets and larval control predicted that a 50% reduction in vector emergence from breeding sites could contribute to an overall 15–25-fold reduction in entomological inoculation rate, even in highly endemic areas (Killeen et al., 2000). It is generally agreed that aggressive larval control was a critical component in eradicating epidemic malaria from Brazil in the early 20th century (Soper and Wilson, 1943) and can therefore provide a more effective supplement to current adult control strategies, or even an alternative in areas where insecticide-treated bednets are not available.

One of most important components of larval control is the use of chemical larvicides to eliminate or reduce the larval population by direct killing. Indeed, chemical larviciding was widely employed prior to the commercialization of DDT, particularly for control of malaria in urban and peri-urban areas (Gratz, 1999). In addition, it has been widely practiced to control nuisance-biting mosquitoes, particularly in the USA (Florida Coordinating Council on Mosquito Control, 1998). Because they normally have very low human toxicity and short environmental persistence, certain larvicides such as temephos are applied to drinking water sources for vector control in some countries (United States Environmental Protection Agency, 2002; 2003).

Not surprisingly, a range of chemical larvicides has been developed and used successfully against malaria mosquitoes. Petroleum oils were applied over 100 years ago to asphyxiate larvae of malaria vectors and other mosquitoes (Gratz, 1999). The poison Paris Green (copper acetoarsenite) was employed against *Anopheles* larvae extensively until the 1940s, by application as a fine powder that floated on the water surface where it was eaten by *Anopheles* larvae (Killeen et al., 2002a). Systematic use of Paris Green over approximately 54 000 km^2 of apparently ideal habitat in north-east Brazil during the 1930s contributed to elimination of *An. gambiae.* from this region where it had been accidentally introduced (Killeen et al., 2002a). A very effective organophosphate-based larvicidal formulation, temephos, exhibits very low mammalian toxicity (FCCMC, 1998) and has been used routinely for malaria vector control in several countries including India, Mauritius and Oman (Gopaul, 1995; Kumar et al., 1994; Parvez et al., 1985).

However, the use of chemical larvicides is limited by issues such as toxicity and resistance. For example, although inexpensive and highly effective, the use of Paris Green is no longer recommended, due to the risks posed by its high toxicity towards humans (Coosemans and Carnevale, 1995). In addition, some synthetic pyrethroids are very effective but are problematic as larvicides due to their collateral toxicity to aquatic non-target organisms (W.H.O., 2006). Larval resistance to some of the more widely applied larvicides such as temephos is also a growing problem(Coosemans and Carnevale, 1995; Majori et al., 1986). The efficacy of chemical larvicide relies on several factors including the formulation, water quality, and the susceptibility of the targeted larvae(Walker and Lynch, 2007). The available larvicides focus on high efficiency of

killing and low toxicity towards other organism. Interestingly a lot of efforts have been put into developing novel repellent and attractant in adult mosquitoes, but little similar studies have been done in larvae. Coupled with certain larval attractants, the larvicide may have a greater chance to kill their larvae targets. Larval repellent may also help to eliminate potential larval habitats. These approaches require further study regarding the behavior of An. gambiae larvae, especially olfactory -driven behavior.

The Drosophila Larval Olfactory System as a Model

The cephalic chemosensory apparatus of the Drosophila larva includes 3 external sense organs, dorsal organ (DO), terminal organ (TO), and ventral organ (VO), as well as 3 internal, pharyngeal organs (Gendre et al., 2004; Python and Stocker, 2002; Singh, 1997). Each of them consists of several sensilla, a sensillum comprising one to several sensory neurons and 3 accessory cells, all housed below a common cuticular structure or terminal pore. The major olfactory organ of *Drosophila* larva is the DO. It is composed of the central "dome" and 6 peripheral sensilla. The dome, whose wall is perforated by thousands of pore tubules, is innervated by the profuse dendritic arbors of 21ORNs.

Recent studies have demonstrated that the logic of *Or* gene expression in the larval olfactory system, despite its simplicity, is surprisingly similar to the adult and mammalian design (Fishilevich et al., 2005; Kreher et al., 2005; Larsson et al., 2004). In total, 25 *Or* genes are shown to be expressed in the DO both by RNA *in situ* hybridization and by *Or-Gal4* trans-gene expression (Fishilevich et al., 2005; Kreher et al., 2005). Each of the 21 larval ORNs expresses the non-conventional receptor gene *Or83b*ng with OR83b,

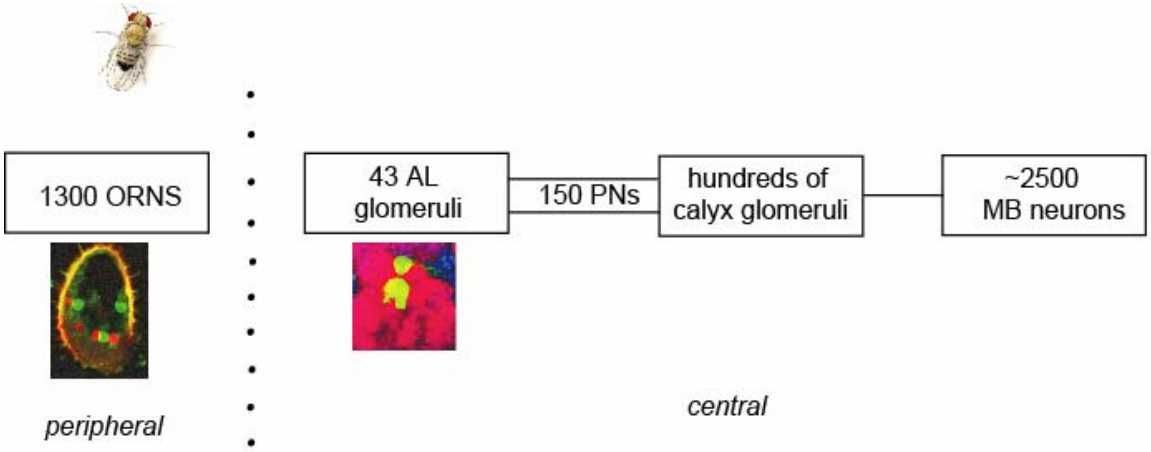


Figure 5. Wiring diagram of the adult olfactory system.
There are ~1300 ORNs in adult *Drosophila* olfactory system. These ORNs project their axons to 43 glomeruli in the antennae lobe (AL). The 43 AL glomeruli are connected to hundreds of calyx glomeruli by about 150 projection neurons (PN). There are an estimated 2500 neurons in the higher processing center: mushroom body (MB).

(Larsson et al., 2004*)*. While the majority of the neurons express one conventional OR and at least two ORNs were shown to co-express two conventional ORs besides OR83b (Fishilevich et al., 2005). Of the 25 *Or* genes, 13 appear to be larval specific (Fishilevich et al., 2005; Kreher et al., 2005). Based on the discrepancy between the number of Ors and the number of ORNs, a few more cases of triple OR expression may be expected, although an exhaustive study has not, as yet, been reported.

The odor response spectra of 11 larval ORs were studied by recording electrophysiological responses to a panel of 29 odorants in empty neuron system (Kreher et al., 2005). The response spectra of these ORs are very diverse and odorants that elicit strong responses can activate multiple receptors. Most of the recorded responses are excitatory, but some receptors are strongly inhibited by one compound and excited by another. Response dynamics and odor sensitivities vary largely among different receptors. Direct electrophysiological recordings from dorsal organ confirmed that the dome could respond to multiple odorants, which can activate at least one Or in empty neuron recordings.

Drosophila larvae can manifest chemotaxis behavior towards a variety of odorants stimuli (Larsson et al., 2004). OR83b mutant larvae lost their behavior response towards all behaviorally active compounds, indicating the importance of olfactory system in larval chemotaxis behavior (Larsson et al., 2004). Moreover the ablation of single OSN reveals the existence of a functional redundancy in the larval olfactory system: A given OR is only necessary for chemotaxis to a relatively small subset of odorants tested. Larvae with only a single functional neuron can chemo-tax robustly toward a number of odor stimuli (Fishilevich et al., 2005). Combinatorial coding offered by the entire ensemble of

ORs is not strictly necessary for *Drosophila* larvae to perceive and respond behaviorally toward an odor. However, adding to a single-neuron animal a second functional OSN, which by itself is not sufficient to mediate chemotaxis, produces enhanced behavioral responses to a subset of odors (Fishilevich et al., 2005). At a behavioral level, a single OSN is sufficient to detect the presence of an olfactory stimulus and that the combinatorial activation of different ORs participates in the formation of whole olfactory perception process.

A recent study found, to some degree, several aspects of *Drosophila* larval behavior could be explained and even predicted from the activities of the ORs they express (Kreher et al., 2008). The overall strength of an attractive chemotaxis response has a linear relationship with the total magnitude of the input from OR level. Even when subject to an integrated olfactory input from two odorants (odor masking), the larvae would manifest a response that could be described to a large extent by the response profile of the ORs. Interestingly, the odor masking appeared more sensitive to the identity of the responding receptors (the different odorants the larvae could respond to) than to the magnitudes of their responses (the number of spikes in single sensillum recordings) (Kreher et al., 2008).

Mosquito Larval Behavior

One notable behavior presented by almost all mosquito larvae is aggregation. In behavioral terms, aggregation is a congregation of animals that doesn't depend upon social attraction. Studies have shown that aggregated distribution of larvae within their natural habitats exists in a number of mosquito species, including *An. gambiae, An.*

arabiensis and Ae. aegypti. Despite its widespread occurrence the fundamental biology underlying larval aggregation remains largely unknown. One study indicates the high aggregation of *Culex* larvae may result from oviposition behaviors of female adults, although whether this is involves olfactory cues is unknown (Pickett and Woodcock, 1996) Another brief report of aggregation of *Aedes stimulans* larvae in a woodland pool suggested that water temperature and light intensity may play an important role.(Fernald and Burger, 1980) This is consistent from our own observation and experiments in *Ae. aegypti* larvae (Xia and Zwiebel, unpublished observation). Another interesting observation from *C. quinquefasciatus* larvae revealed that larval aggregation is more intensive when the larvae were both crowded and deprived of food (Workman and Walton, 2003).

In nature, mosquito larvae are vulnerable to many predators and accordingly develop avoidance behaviors. In this respect, *C. pipiens* larvae exhibit avoidance behavior in response to a variety of cues, and react to potential predators by reducing their movement and concentrating at the edge of the container(Kasap, 1980; Kasap, 1981). In one experiment, *Culex* larvae responded significantly to water that had contained both predator and larval prey, indicating the persistence of chemical cues that act on the chemosensory pathways of the mosquito larvae(Thangam and Kathiresan, 1996). In addition to avoidance behavior, larvae that are at water surface respond to certain stimuli with escape behaviors, often called alarm responses. Usually this involves diving, at a more rapid rate than is typically undertaken in feeding dives. Lastly, mechanical and optical stimuli are believed to trigger escape response, although some evidence may suggest the involvement of olfactory cues(Clements, 1993).

Mosquito larvae manifest a number of behavioral responses towards different kinds of stimuli such as light, food and color etc (Merritt et al., 1992). Responses towards food sources, often referred to as feeding behavior, are believed to be largely driven by olfactory chemosensory stimuli (Merritt et al., 1992) and have been studied in many mosquito species. *Aedes vexans* larvae, when placed in a dish with eight incompletely separated compartments, congregated in the four compartments that contained pellets of fishmeal or wheat flour (Aly, 1985). In addition, *C. quinquefasciatus* larvae became concentrated, to a modest degree, in regions of water containing casein hydrolysate or the amino acids phenylalanine, aspartic acid and proline (John T. Barber, 1983). It has been established that mosquito larvae accumulate in regions where there is food as the result of orthokinetic responses to soluble constituents diffusing from the food with the involvement of their olfactory systems (Merritt et al., 1992).

Larval Contributions to the Adult Olfactory System

In insects, it is generally believed that larval sensilla are derived from embryonic structures and are normally lost during metamorphosis after which they are replaced by pupal and adult stage sensilla originating from imaginal discs (Levine et al., 1995). Interestingly, studies have revealed that a subset of larval visual sensory neurons persist and are integrated into adult visual pathways (Malpel et al., 2002). Even the metamorphosis of central neuronal circuits, as indicated by a recent larval brain map (Pereanu and Hartenstein, 2006), involves essentially the integration of a group of secondary neuronal lineages into an embryonically derived tract system.

During metamorphosis, DO ganglia move progressively backward from its

peripheral site. The larval ORNs thereby become increasingly proximal to the antennal imaginal disc, which is the origin of adult ORNs. Adult ORN afferents join and extend through the larval antennal nerve (Tissot et al., 1997) and reach the brain by 16–20 hr. after pupa formation (Jhaveri et al., 2000). The adult AL derives from a brain region distinct from the larval AL(LAL) which is derived when larval ORN terminals become gradually pruned (Jefferis et al., 2004). Many larval olfactory interneurons become integrated in the adult system. Local GABAergic interneurons are present both in the LAL and adult AL of several species, but whether they are identical at both stages is not clear (Homberg and Hildebrand, 1994; Python and Stocker, 2002). It is obvious that larval PNs which extend from the AL to higher brain centers in the *Drosophila* CNS survive through metamorphosis (Stocker et al., 1997). In the adult AL, at least 15 glomeruli are innervated by embryonically derived PNs. These glomeruli are morphologically distinct from those innervated by larva-derived PNs. Moreover, the embryonic-born PNs in the adult appear to be the same cells as the mature larval PNs (Marin et al., 2005). Until 12 hr after pupa formation, their dendrites in the LAL are gradually pruned and ultimately disappear, together with LAL neurophil as a whole. At the same time, new dendritic arbors grow from the main PN process at a novel site, dorsal and posterior to the LAL. This secondary area then develops into the adult AL (Jefferis et al., 2004; Marin et al., 2005).

CHAPTER III

THE MOLECULAR AND CELLULAR BASIS OF OLFACTORY-DRIVEN BEHAVIOR IN *AN. GAMBIAE* LARVAE

Preface

The information presented in this chapter was published in the *Proceedings of the National Academy of Sciences* (105: 6433-6438). My contribution to this body of work include all the identification and cloning of the larval *AgOrs*, all *in situ* hybridizations as well as most of the behavioral studies. Guirong Wang performed the *Xenopus oocytes* recordings. Whole mount antibody staining of AgOR7 was performed by R. Jason Pitts. Daniela Buscariollo finished SEM and part of the behavior assays.

Introduction

Human malaria is transmitted by several species of *Anopheles* mosquitoes, most notably *An. gambiae* which is the principal afrotropical vector for this disease. (Adams et al.). Current anti-malaria strategies largely focus on domestic protection against adult mosquitoes and improved access to effective diagnosis and treatment(Goodman et al., 2001; Greenwood and Mutabingwa, 2002; Nabarro and Tayler, 1998). Insofar as vector control is concerned this concept is favored because of the early success of dichlorodiphenyltrichloroethane (DDT) as an insecticide in 1950s as well as the successful and increasingly widespread use of pyreithroid-treated bednets in the last decade(Garrett-Jones and Grab, 1964; Killeen et al., 2002a). However, the effects of resource limitations as well as increasing levels of insecticide resistance among

mosquito vectors (Killeen et al., 2002a; Killeen et al., 2002b), has made the effective suppression of malaria via intensive use of these methods more and more difficult in many parts of the world(Curtis, 1998; Schellenberg et al., 2001; Winch et al., 1997). In the face of these growing limitations related to vector control strategies directed against adult mosquitoes, improving or developing novel approaches targeting the aquatic mosquito larvae represents a potentially significant strategic augmentation. Indeed, there are several examples of the successful control and in some cases elimination of *Anopheles* vectors accomplished by specifically targeting mosquito larvae and/or reducing the amount of suitable habitats for immature *Anopheles* mosquitoes around human dwellings (Killeen et al., 2002a; Killeen et al., 2002b). For example, when the accidental introduction of *An. gambiae* to Brazil resulted in a catastrophic malaria epidemic in 1938, a vector control campaign focusing on the eradication of mosquito larvae from the periphery of the infested area towards the center, the 1938-1940 campaign gained unprecedented success. This resulted in the halting the invasion of *An. gambiae* and its eradication in less than two years (Barber, 1940; Killeen et al., 2002a). Similar success in the removal of *An. gambiae* vectors was also achieved by employing the same strategy in Egypt in 1945 (Killeen et al., 2002a). However, despite being one of the oldest, and arguably the most historically successful strategies for the prevention of human malaria larval control paradoxically is sparsel implemented in Africa and other disease endemic regions (Fillinger et al., 2004; Killeen et al., 2002a)**.**

While numerous studies support the hypothesis that olfactory signals play an essential role in the host-seeking and other essential behaviors that contribute to the vectorial capacity of female adult mosquitoes(Takken and Knols, 1999) and rapid progress has

been achieved towards a better understanding of the adult *An. gambiae* olfactory system(Fox et al., 2001; Hallem et al., 2004a; Kwon et al., 2006; Merrill et al., 2003; Pitts et al., 2004), little is known about olfactory processes and the relevant behaviors of pre-adult stage malaria vector mosquitoes. In addition to its potential importance for disease control, the simplicity of the insect larval olfactory system makes it an excellent model to study olfactory-related behaviors. Indeed, recent work in the arbovirus vector mosquito *Aedes aegypti* (*A. aegypti*) has revealed the larval expression of 24 OR genes, 15 of which are larval specific (Bohbot et al., 2007) while elegant work using the *Drosophila* insect model has identified and characterized the role of 25 ORs expressed in 21 larval ORNs in each of the two dorsal organs, which constitute the olfactory apparatus of the fly larva(Couto et al., 2005; Fishilevich et al., 2005; Kreher et al., 2005). In this study we have designed and utilized a novel olfactory-based assay to characterize robust and concentration-dependent behavioral responses in *An. gambiae* larvae to a range of synthetic and natural chemosensory stimuli. We have focused on the role of the larval olfactory system in this process as ablation of the larval antennae effectively eliminates these behavioral responses. In order to further establish an underlying basis for these diverse behaviors we have used molecular approaches to identify a subset of *AgOr* genes as larval, and in some cases larval specific, ORs (L-AgORs). Consistent with expectations, L-AgOR transcripts have been mapped by fluorescence in situ hybridization (FISH) to a distinctive population of ORNs located

within the larval antennae. Functional analyses of L-AgORs was carried out using heterologous expression and voltage clamp studies in *Xenopus oocytes* which validate their roles as bona fide OR proteins and demonstrate that L-AgORs encompass both broadly and narrowly tuned receptors. Taken together, these studies expand our understanding of pre-adult life stages by shedding light on the molecular basis of olfactory-based behavior in *An. gambiae* larvae, thereby providing alternatives to adult-based mosquito control strategies.

Results

Ultrastructure of the *An. gambiae* Larval Antenna

In *An. gambiae*, the main larval olfactory organ is the antenna, which manifests a number of apical structures as well as integumental sensilla (Figure 6A). One of the apical structures is an elongated cone found at the antennal tip that is innervated by 12 bipolar neurons all of which express high levels of *AgOr7* (Pitts et al., 2004). A single *An. gambiae* larval antenna consists of two basal segments, the scape and pedicel, and a cylindrical flagellum, the antennal shaft, which has freedom to move laterally.

A total of six micro-appendages comprising five structural types are found in a stereotypic array at the terminal region of the antennae, all are directed anterad antennal

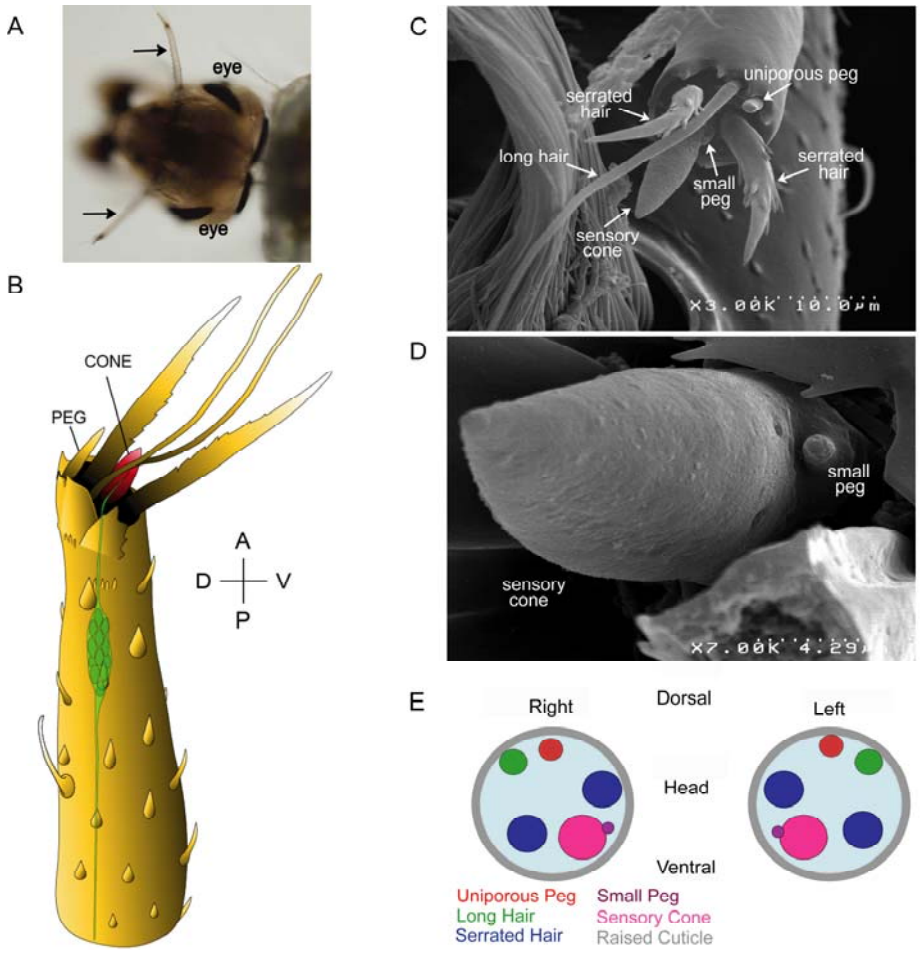


Figure 6. Ultrastructure of the *An.gambiae* larval antenna.

(*A*) Bright-field image of a larval head; the arrow indicates the antenna structure.

(*B*) Schematic representation of the external morphology of the fourth instar larval antennae.

(*C*) SEM image of the tip of a larval antenna, showing the multiple hair structures.

(*D*) SEM image of the sensory cone and small peg structures.

(*E*) Schematic diagram representing the microappendage distribution pattern at the tip of larval antennae.

tip (Figure 6) and all correspond to sensory structures that have been characterized from the in *A. aegypti* larvae (Zacharuk et al., 1971). Of these, two types are believed to be involved in chemosensation: the sensory cone and the uniporous peg. The sensory cone is conically shaped and comes to an aporous point with a finely ridged external texture. The cone consists of the dendrites of the olfactory neurons and is considered to be chemically permeable (Zacharuk et al., 1971). In *A. aegypti* L4 the cone has been classified as an olfactory organ with a thin and aporous cuticle that is permeable to chemicals such as crystal-violet stain yet as a result of its aquatic environment lacks a pore tubule system common to other insect olfactory sensilla(Zacharuk et al., 1971). The semi-transparent and unpigmented cuticle of the sensory cone of the *An. gambiae* larva appears to be similarly thin in bright-field imaging (data not shown). The uniporous peg is the most dorsal terminal structure/sensillum of the *An. gambiae* larvae and is aligned with the single hair located along the antennal shaft. The peg's base seems to be continuous with the terminal membrane of the antennae, and is halfway encircled by the raised cuticle.

Besides the sensory cone and the uniporous peg, there are three other types of non-chemosensory hair structures: serrated hairs, long hair and single hair. Two serrated hairs of approximately equal length and diameter are found at the terminal region of the antennal shaft (Figure 6 B, C), with one located ventrally to the cone and distally to the head, and the other located dorsally to the cone and proximally to the head. The base of each serrated hair is continuous with the terminal membrane of the antenna and lacks a

socket, but retains limited motility and is able to project in different directions. The long hair is the longest terminal sensillum of the *An. gambiae* larvae (Figure 6 B, C). It is found adjacent to the uniporous peg, away from the head. The long hair is highly flexible and can project in a wide variety of directions. The cuticular wall is apparently aporous. The long hair emerges from a pitted base surrounded by cuticle that is continuous with the raised cuticle originating from the antennal shaft (Figure 6 B, C). A socketed hair, termed single hair, is generally situated 1/4 of the length of the antennal shaft distally on the medial aspect and is directed anteromedially (Figure 6 B, C). In the course of this study twenty larvae were examined by bright-field microscopy. In all cases, the single hair was present on the dorsal side of the shaft, where it aligned with the uniporous peg sensillum located apically on the antenna. The most dorsal sensillum is the uniporous peg (Figure 6 C, D). Another organ, termed long hair, is situated to its outer side and away from the head (Figure 6 C, D). One of the two serrated hairs is situated dorsal to the long hair, and the other is found across from the first and on the side of the uniporous peg closer to the head (Figure 6 C, D). The sensory cone is located between the two serrated hairs (Figure 6 C, D) and a small peg invariably emerges from the base of its dorsal surface (Figure 6 C, D). While all six sensilla are situated at the periphery of the terminal region, their distance from the raised cuticle varies slightly per individual and as a result of the precise larval developmental stage which are typically denoted as L1-4 instars. The peg and long hair are consistently found adjacent to the raised cuticle while the two serrated hairs and sensory cone are generally located closer to the center of the

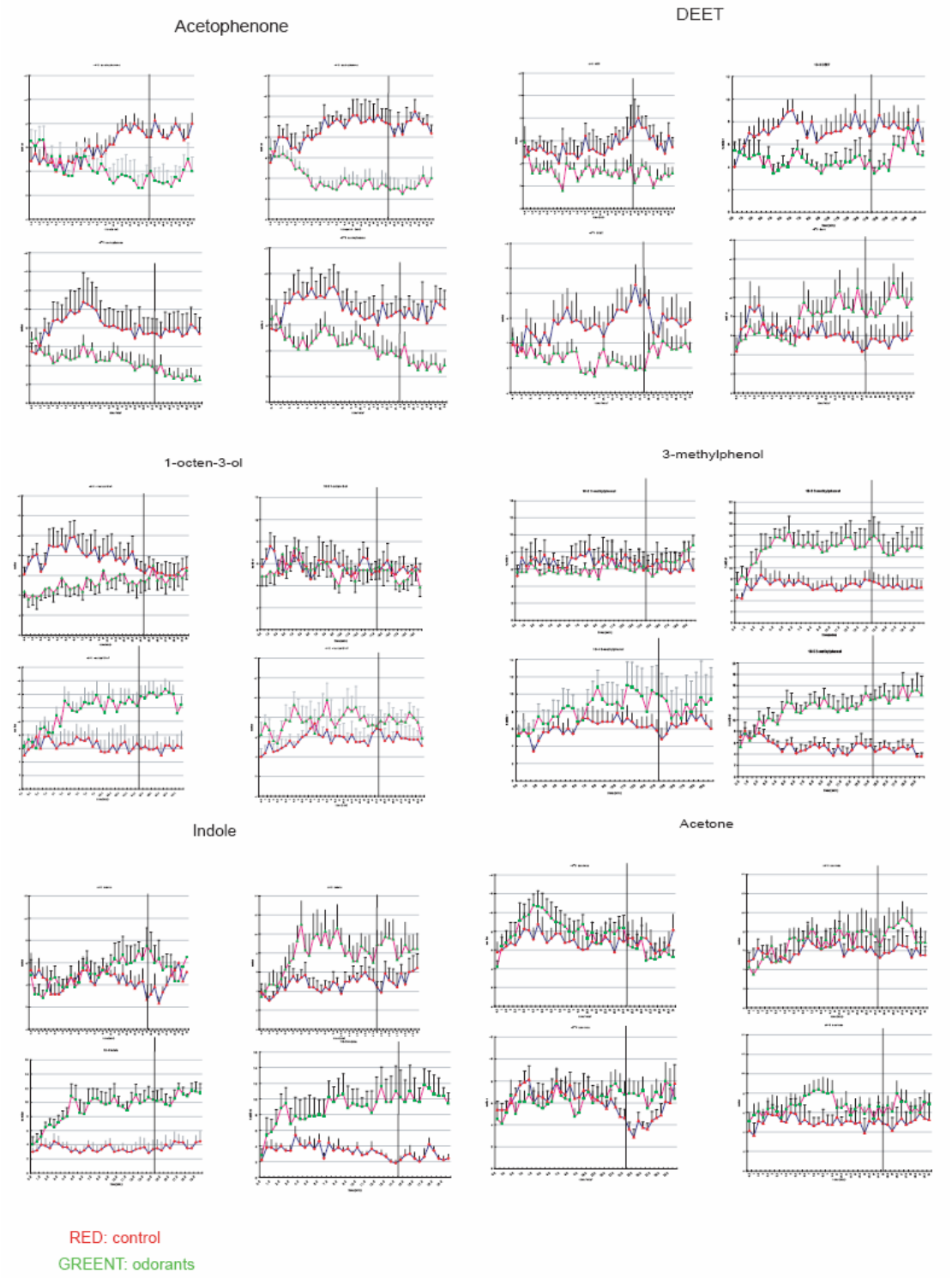
antennal tip in L1 but are situated closer to the periphery in L4, probably as a result of growth.

An. gambiae Larva to Respond to Synthetic and Natural Odorants

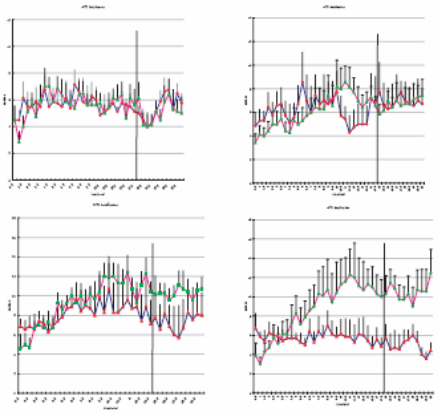
The logical output of the larval olfactory system is to generate distinct behavior responses. To understand the relationship between olfactory inputs and behavioral outputs in *An. gambiae* larvae, we established a sensitive olfaction-based behavior assay that tracks the ability of *An. gambiae* larvae to respond to a range of synthetic and natural odorant stimuli (Figure 7, 8; Table 1). In this assay, the distribution of 100 2nd and 3rd instar aquatic-stage larvae is monitored to a range of odorant stimuli as well as appropriate negative controls throughout a twenty-two minute time course (Figure 7).

To access the real-time distribution of *An. gambiae* larvae under the influence of the odorant stimuli, we counted the number of larvae present in both odorant zone and control zone throughout all time-points in our twenty-two minute assay (Figure 7). If the distribution of the larvae is equal across the surface of the dish, ~20 larvae are expected in the control zone. The obvious discrepancy between the actual and expected number of larvae in the control zone may originate from the aggregation behavior by *An. gambiae* larvae (see Chapter II), they prefer gathering around the edge of the container in the laboratory. A slightly increase in the number of larvae (8-9) found in the control zone was observed from compounds like acetophenone and DEET

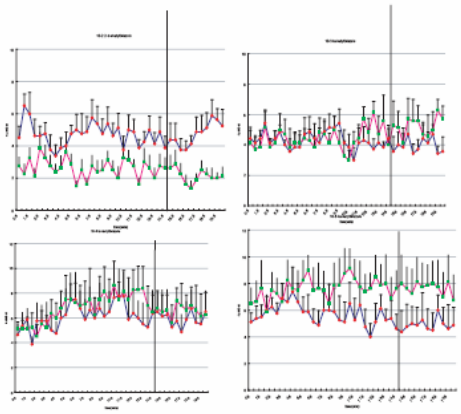
Figure 7. The real-time distribution of *An. gambiae* larvae exposed to different odorants. The red series indicate the distribution of the larvae in control zone, while the green shows the number of larvae in the odorant zone. The vertical line indicates 15 minute time-point. (continued)


Acetophenone
DEET
1-octen-3-ol
3-methylphenol
Indole
Acetone
RED: control
GREENT: odorants

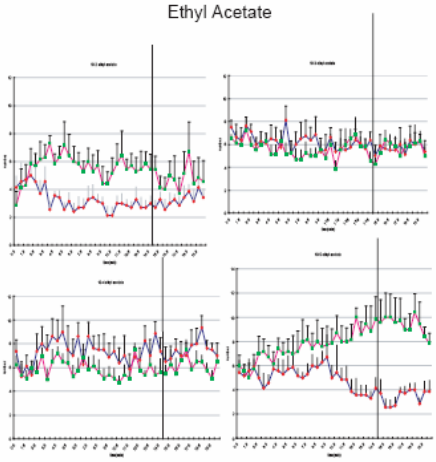
n-Butylamine



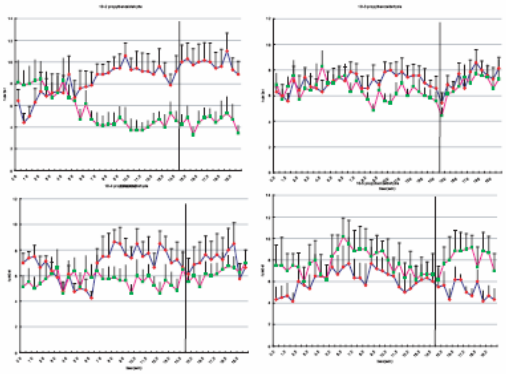
2-isobutylthiazole



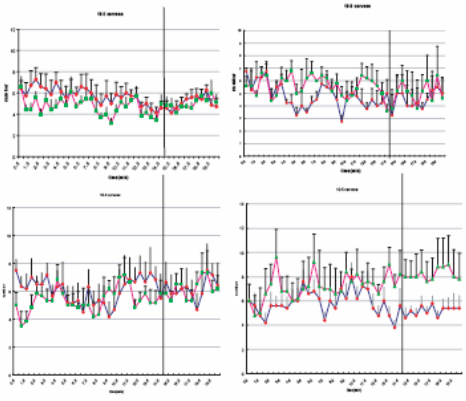
Ethyl Acetate



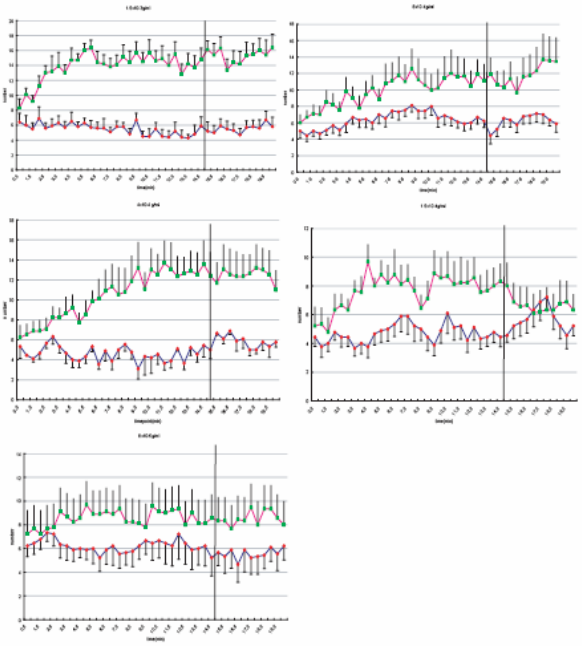
propylbenzaldehyde



Carvone



Yeast



RED: control

GREENT: odorants

(Figure 7), which elicit avoidance behavior. A consistent phenomenon observed from repulsive odorants or some odorants at higher concentrations was the reduced number of larvae in the odorant zone (Figure 7). An average of only 2-3 larvae were found in the odorant zone. For odorants such as, 4-methylcyclohexanol, fish food, 1-octen-3ol, 2-methylphenol, 3-methlyphenol,4-methylphenol, yeast, around 23 larvae gathered in the odorant zone, compared to about 6 in the control area. No difference in the number of larvae found in odorant zone and control zone was observed from odorants like acetone, carvone and butylamine etc (Figure 7).

Although most of the odorants we used in our assays spread slowly in the water, *An. gambiae* larvae present a relatively fast response towards behaviorally-active compounds, both repellent and attractants. We start seeing a different distribution of larvae exposed to 10-5 2-methylphenol at 2.5 minute time-point. In majority of the cases, a noticeable response can be observed within 5 minutes upon the application of a behaviorally-active odorant and, once elicited, the response can last throughout the whole assay.

The real-time distribution of the larvae provides us unique and invaluable information regarding the effects of individual odorant upon *An. gambiae* larvae, while we found it very difficult to compare the response profile of different odorants or even the same odorant at different concentrations. To address this concern, we used a widely employed value named performance Index (PI) in *Drosophila* behavioral assays.

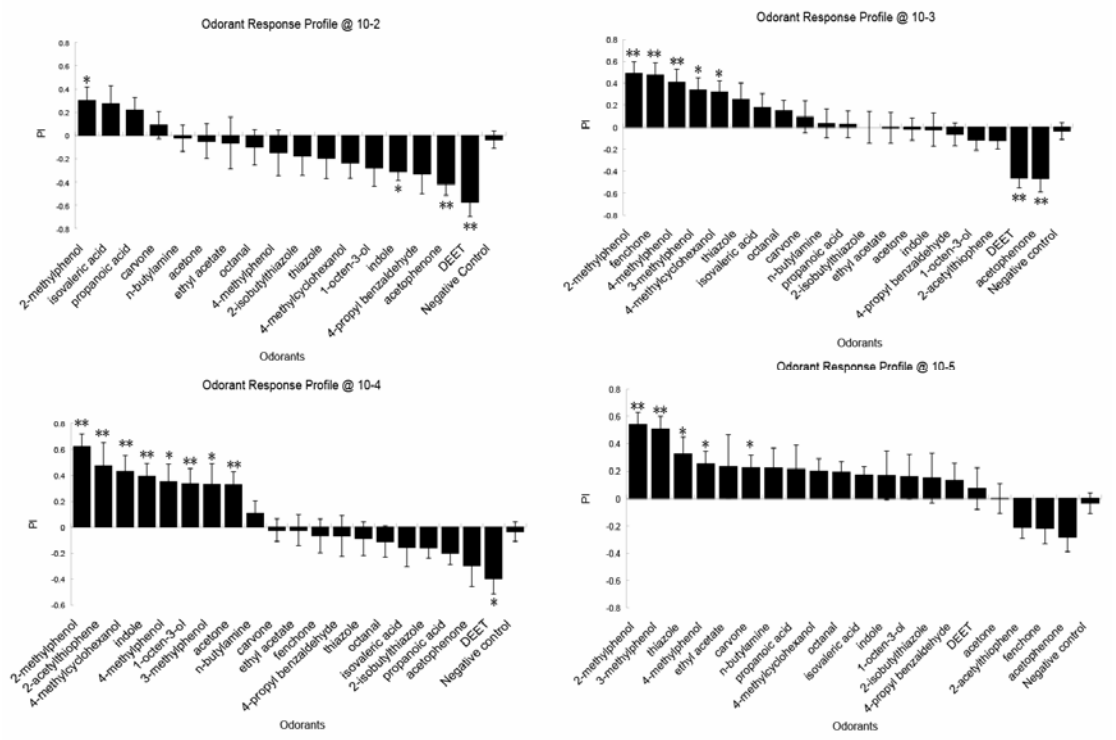


Figure 8 An overview of odorant response profile of different odorants at four different concentrations the PI of the negative control was derived from assays performed with odorant-less stock placed in both odorant zone and control zone; error bar stands for S.E.M; two-tailed unpaired student T-test was performed between PI of specific odorant at specific concentration versus PI of the negative control: ** p< 0.01; * p<0.05; n=8

Performance index was calculated as:

PI=($\#_{odorant}$-$\#_{control}$)/($\#_{odorant}$+$\#_{control}$). A performance index at 15min is calculated to represent the response characteristics of each odorant with +1 indicative of full attraction while –1 represents complete repulsion. Overall, *An. gambiae* larvae display significant responses to just below 35% (11 of 33) of the odorants tested across four different concentrations (Figure 8, 9)

When exposed to higher concentrations of the odorants, *An. gambiae* larvae normally displayed an avoidance behavior (Figure 8). As the concentration lowers, more and more odorants start generating positive PIs (Figure 8). Interestingly, most of the odorants which elicited behavioral responses were aromatics and all of the cresols tested, 2-methylphenol (o-cresol) 3-methylphenol (m-cresol) and 4-methylphenol (p-cresol) strongly attracted *An. gambiae* larvae at dilutions as low as 10^{-5} (Figure 9 C-E). At the other end of the behavioral spectrum, acetophenone, which is used to create almond and cherry scents, was extremely repulsive to *An. gambiae* larvae at similarly high dilutions (Figure 9F). Indole, another aromatic compound, induces different responses in a concentration dependant manner. Here, *An. gambiae* larvae were strongly attracted to a 10^{-4} dilution of indole, while they largely avoided higher (10^{-2}) concentration (Figure 9G). The mono-unsaturated eight-carbon alcohol 1-octen-3-ol is a well-established odorant cue for adult mosquitoes that has been isolated from human

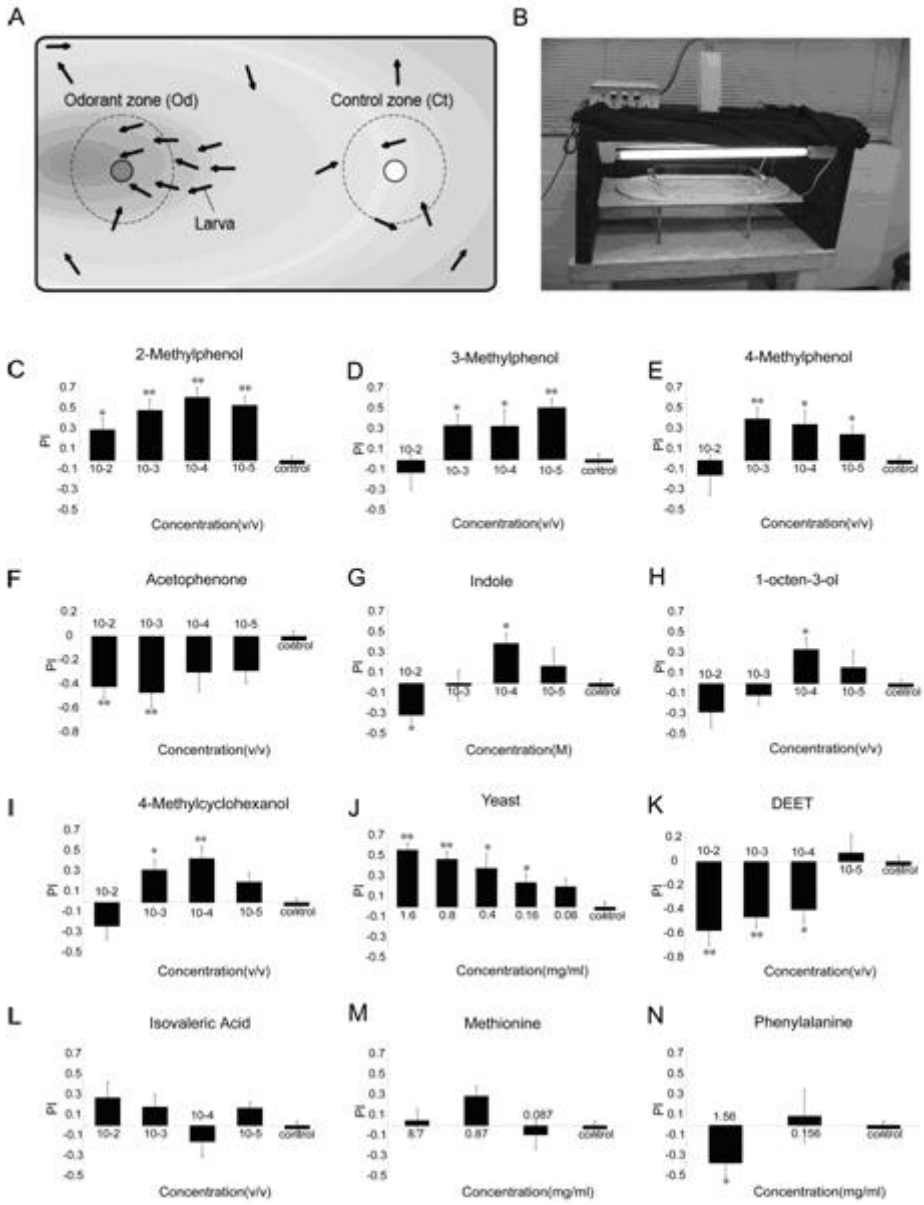


Figure 9. The larvae behavior assay set-up and response profile of different odorants at different concentrations

A, A schematic diagram showing the setup of the behavior assay

B, A picture of the actual setup showing the container, the light source and the camera

C-N, The response profile of 2-methylphenol, 3-methylphenol, 4-methylphenol, acetophenone, indole, 1-octen-3-ol, 4-methylcyclohexanol, yeast, DEET, isovaleric acid, methionine, phenylalanine ;the PI of the control was derived from assays performed with odorant-less stock placed in both odorant zone and control zone; error bar stands for S.E.M; two-tailed unpaired student T-test was performed between PI of specific odorant at specific concentration versus PI of the control: ** $p < 0.01$; * $p<0.05$; $n \geq 8$

and cattle odors (Cork and Park, 1996; Hall et al., 1984; Kline et al., 1991; Mboera et al., 2000; Takken et al., 1997) was attractive at a only a single (10^{-4}) dilution with an average PI value of +0.34 (Figure 9 H). Several acids, including isovaleric acid, which has been shown to act as a strong attractant for adult *An.gambiae* (Costantini et al., 2001), did not evoke statistically significant behavioral effects in larvae across the four different dilutions tested (Figure 9 L).

In order to better assess responses to potential larval food sources, yeast extract as well as two amino acids methionine and phenylalanine which have been shown to attract *Culex quinquefasciatus* larvae (John T. Barber, 1983) were employed in our behavioral paradigm. In these studies, *An. gambiae* larvae showed robust attraction to yeast across a range of concentrations (Figure 9J) while, apart from some avoidance behavior against 1.56mg/ml phenylalanine, *An. gambiae* larvae didn't manifest any preference towards these tow amino acids (Figure 9 M, N). Not surprisingly the widely used insect repellent DEET (N, N diethyl-*m*-toluamide) which is believed to act as a neurotoxin (Curtis et al., 1987; McIver, 1981; Osimitz and Grothaus, 1995) consistently evokes dose-dependent and highly significant repellency at dilutions ≥ 10^{-4} (Figure 9K).

To provide additional evidence that the behavioral responses we observed are indeed mediated by the larval olfactory system, an antennal ablation study was carried out. Here both antennae were carefully removed, and the larvae were allowed to recover under normal conditions for 1 day before behavioral analyses. Moreover, to control for

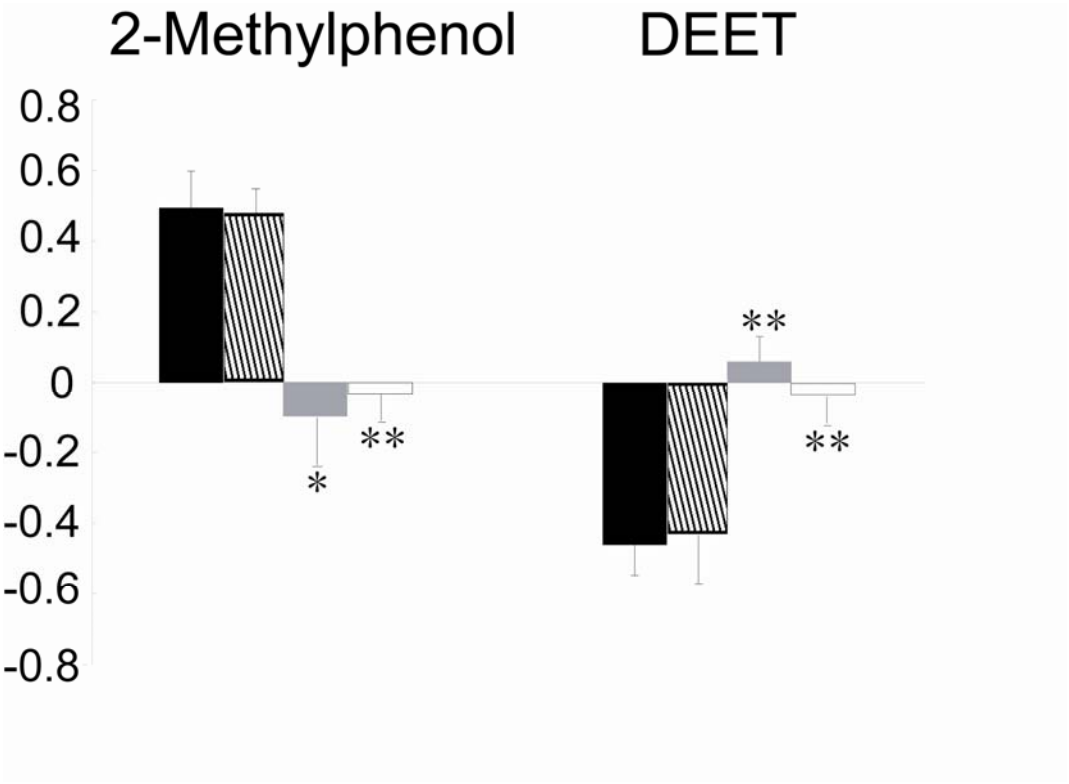


Figure 10. Ablation of the larval antenna reduces olfactory responses. Behavioral responses for unablated larvae (black bars,*n*=8); sham/maxilla ablations (cross-hatched bars, *n*=3); antennal ablations (gray bars, *n*=3) and no odorant/unablated control larvae (open bars, *n*=8). Both 2-methylphenol and DEET were used at 10-3 dilutions. Error bar indicates SEM. Two-tailed unpaired student's *t* tests were performed: ******, *P* =0.01; *****, *P* =0.05 relative to unablated larvae.

potential artifactual effects of surgical injury, sham ablations of the larval maxilla were also undertaken. Importantly, with regard to overall mobility (distance traveled in 30 min.) ablated larvae were indistinguishable from un-ablated controls (data not shown), indicating an absence of any general locomotor defect. Behavioral responses to two compounds that normally manifest strong but opposite reactions (2-methylphenol and DEET) were then examined. In each instance, ablation of the larval antennae resulted in a dramatic loss of odorant-driven behavioral responses (Figure. 10). Of note, the PIs of larvae that had undergone maxilla ablations were statistically indistinguishable from those of un-ablated animals, providing strong correlative data linking olfactory input via the larval antenna to odor-driven behavioral output.

OR Expression in Larval Olfactory Sensory Neurons

At a molecular level, a set of putative ORNs have previously been identified on larval antenna on the basis of the expression of the non-conventional AgOR7 co-receptor (Pitts et al., 2004). To determine the precise number of AgOR7^{+} ORNs, whole-mount labeling of the larval antenna with the same antibody was carried out. A detailed examination of multiple (n > 10) preparations revealed that 12 ORNs were labeled with the AgOR7 antibody, and the dendrites of these neurons were observed to project into the sensory cone (Figure. 11A).

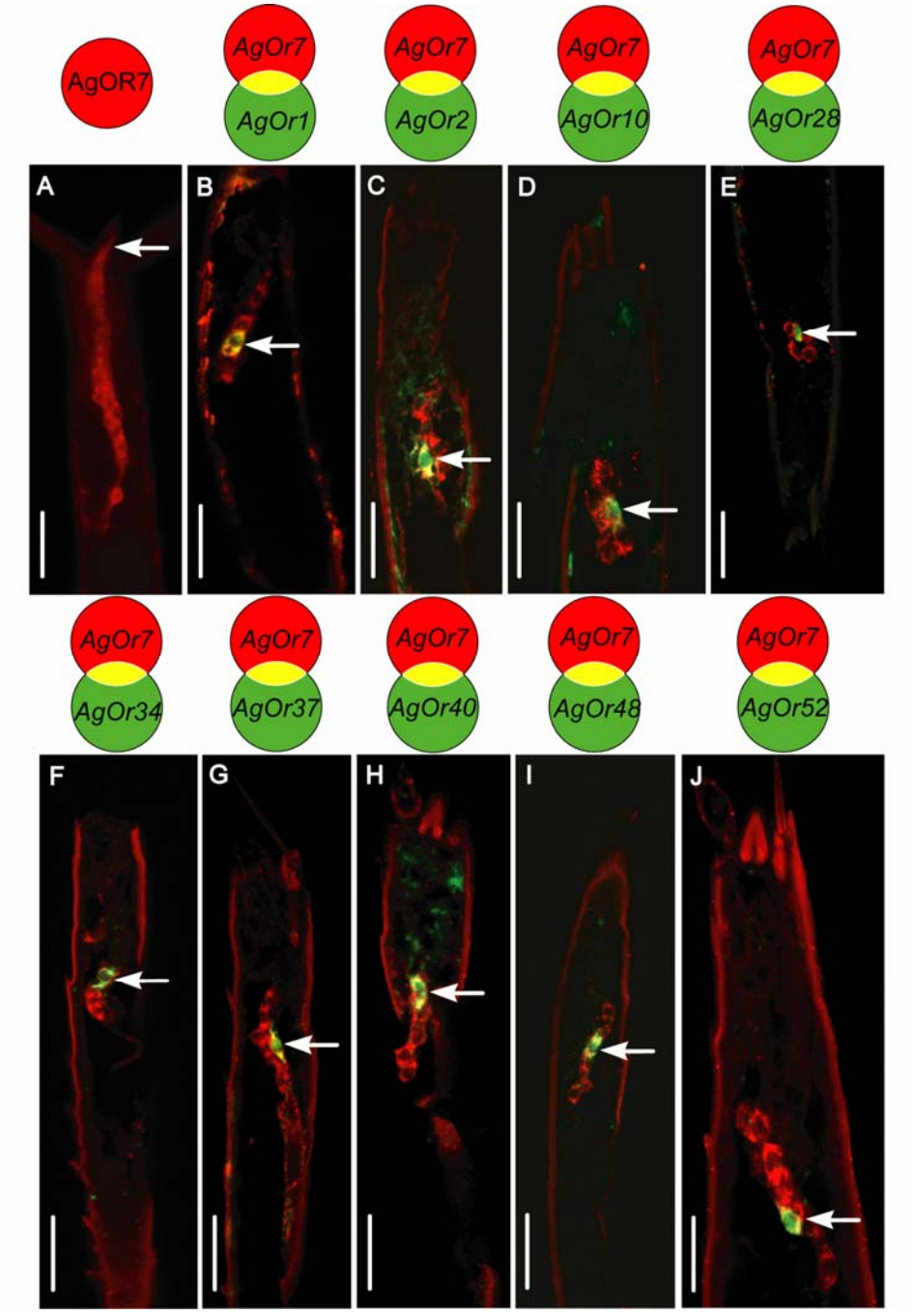


Figure 11. Expression of AgOr genes in the larval antenna

(A) whole-mount staining of *An. gambiae* larval antennae with AgOR7 antibody. The arrow indicates the dendrites projecting into the sensory cone

(B) (B-J) AgOr FISH on 8um section results revealed that each individual conventional AgOr is solely co-expressed with AgOr7 in a single larva OSN. Arrow indicate the individual neuron (yellow) with AgOr7 (red) and one conventional AgOr(green) co-expressed. (Scale bar, 25um)

Two different approaches were then taken to characterize any conventional *AgOr*s that were presumed to be co-expressed along with *AgOR*7 in the larval ORNs. Initially, an RT-PCR based screen employing intron-spanning primers from all 79 *AgOrs* reactions was carried out with L4 larval antennae cDNA as template. For each individual *AgOr*, three independent PCR trials were performed along with appropriate controls. In this manner, transcripts derived from 12 conventional *AgOr* were consistently amplified from larval cDNA preparations. These products were subsequently cloned and confirmed by sequencing. Of these, *AgOr*1, *AgOr*2, *AgOr*6, *AgOr*10, *AgOr*28, *AgOr*34, *AgOr*48 and *AgOr*49 have also been detected in the olfactory appendages (antennae, maxillary palps & proboscis) from adult *An. gambiae* mosquitoes (Fox et al., 2001; Hill et al., 2002; Kwon et al., 2006; Pitts et al., 2004). Four L-*AgOr*s, *AgOr*37, *AgOr*40, *AgOr*52 and *AgOr*58, are determined to be exclusively expressed in *An. gambiae* larvae, as no amplification was observed in similar experiments carried out with adult olfactory appendages (L.J.Z., unpublished observation).

Based on the concordant number of L-*AgOr*s and the larval ORNs, we then asked whether each conventional L-*AgOr* is co-expressed with the non-conventional *AgOr7* in a single larval ORN. To test this hypothesis as well as extend our investigation, FISH was employed, in which digoxygenin-labeled anti-sense riboprobes of the each conventional L-*AgOr*s was co-hybridized with a fluorescein-labeled *AgOr7* probe on 8μm paraffin sections of larval antennae. In these studies, 9 of the 12 conventional *AgOrs*

including *AgOr1, AgOr2, AgOr10, AgOr28, AgOr34, AgOr37, AgOr40, AgOr48* and *AgOr52* were detected while *AgOr6, AgOr49* and *AgOr58* failed to generate consistent FISH signals (Figure 11 B-J). The lack of FISH signals is consistent with the relatively weak amplification of these *AgOr*s in semi-quantitative RT-PCR studies, suggesting they may be expressed at very low levels in the larval antennae. Furthermore, an exhaustive examination of multiple sections for individual and pairs of FISH-detectable L-*AgOrs* (n>5), supports the hypothesis that each conventional L-*AgOr* detected by FISH is expressed together with *AgOr7* in distinct and indeed, stereotypic larval ORNs (data not shown). For example, mixed probes of *AgOr34* and *AgOr37* always label 2 distinct neurons, suggesting no co expression of these 2 *AgOr*s in the same neuron (n=4, data not shown). Interestingly, in *Drosophila*, similar expression profile was observed in larval olfactory systems with minor exceptions (Fishilevich et al., 2005; Kreher et al., 2005).

Odor Response Spectra of *An.gambiae* Larval ORs

Having demonstrated that a subset of *AgOrs* are expressed in larval ORNs, we used heterologous expression in *Xenopus oocytes* to examine whether these genes are functional and likely to facilitate larval olfaction. This system has been used to characterize numerous insect odorant and pheromone receptors (Krieger et al., 2004; Lu et al., 2007a; Nakagawa et al.,

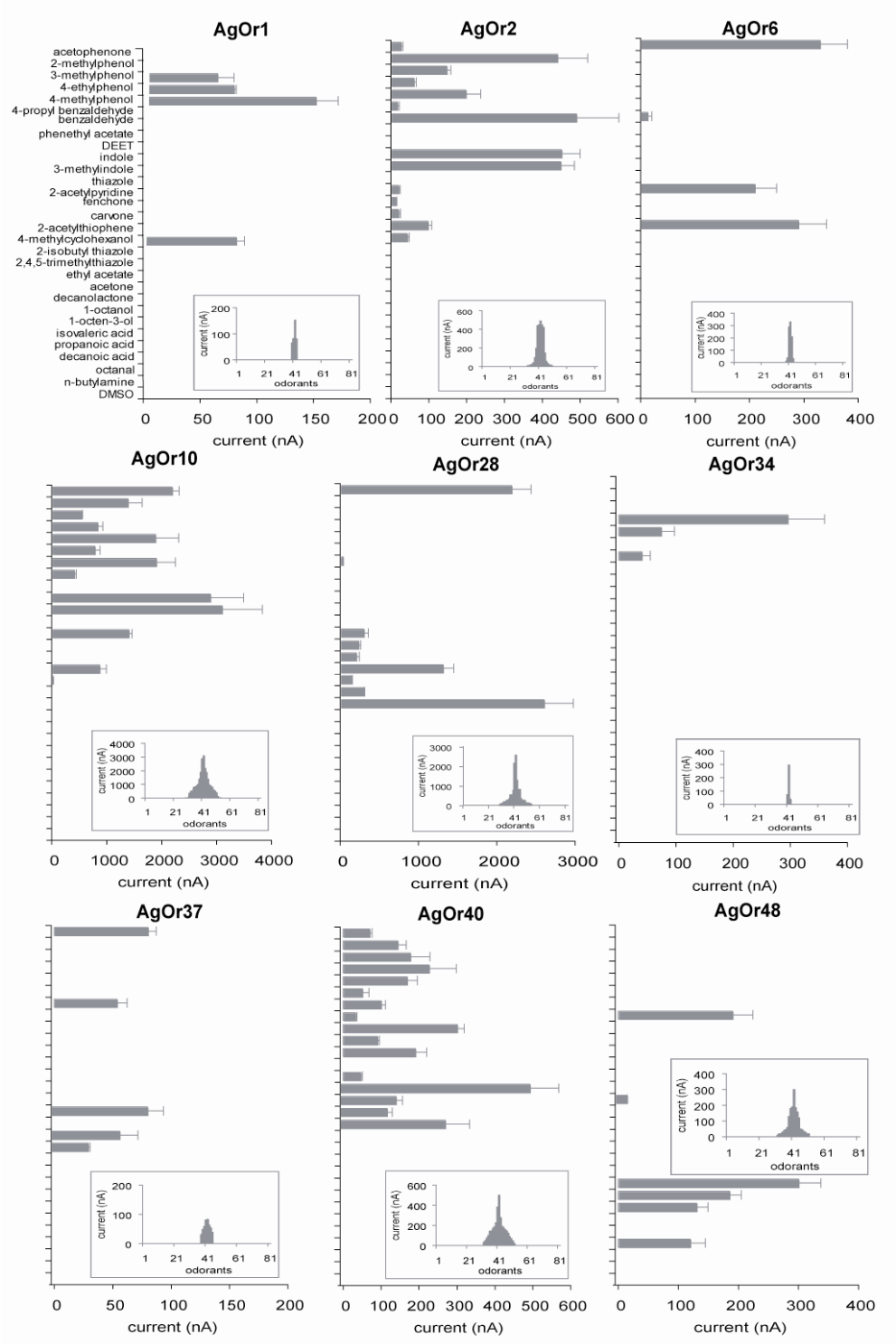


Figure 12. Odor response spectra of larval AgORs.

Response is measured as induced currents, expressed in nA. Error bars indicate the SEM (*n* = 5–8). The corresponding tuning curve for a given receptor is placed in the *Insets*. The 82 odorants are displayed along the *x* axis, with those eliciting the strongest responses being placed near the center, and those eliciting the weakest responses placed near the edges.

2005; Sakurai et al., 2004; Wetzel et al., 2001). The test panel of 29 core chemical stimuli used for larval behavioral studies was augmented by an additional 53 compounds or odorant mixtures to enhance odorant representation across a range of chemical classes. In these functional analyses, nine larval-expressed *AgOrs* (*AgOR1, AgOr2, AgOR6, AgOR10, AgOR28, AgOR34, AgOR37, AgOR40* and *AgOR48*) facilitated responses to at least two odorants in the test panel (Figure.12, 13), whereas three (*AgOR49, AgOR52* and *AgOR58*) failed to generate any detectable odor-induced currents in *oocytes* (data not shown).

Not surprisingly, the response spectrum of each individual *AgOR* varies. For example, *AgOR1* and *AgOR34* each responded to a very narrow set of odorants, whereas *AgOR10* and *AgOR40* manifested much broader spectra (Figure. 11, 13). Furthermore, the absolute response amplitude also differed significantly between different *AgOrs*. For example, the indole response current of *AgOR10* was as high as ~3000nA, and 4-methylphenol, the strongest activator of *AgOR34*, generated a ~75-nA current (Figure. 12). It is not possible at this point to distinguish whether these effects reflect either differential expression or odorant-binding affinities between individual *AgOrs* in this system. Importantly, several of the compounds that elicit larval behavioral responses were also able to activate multiple *AgOrs* (Figure. 12). Of these, 4-methylphenol, which evokes strong responses in behavioral assays, also

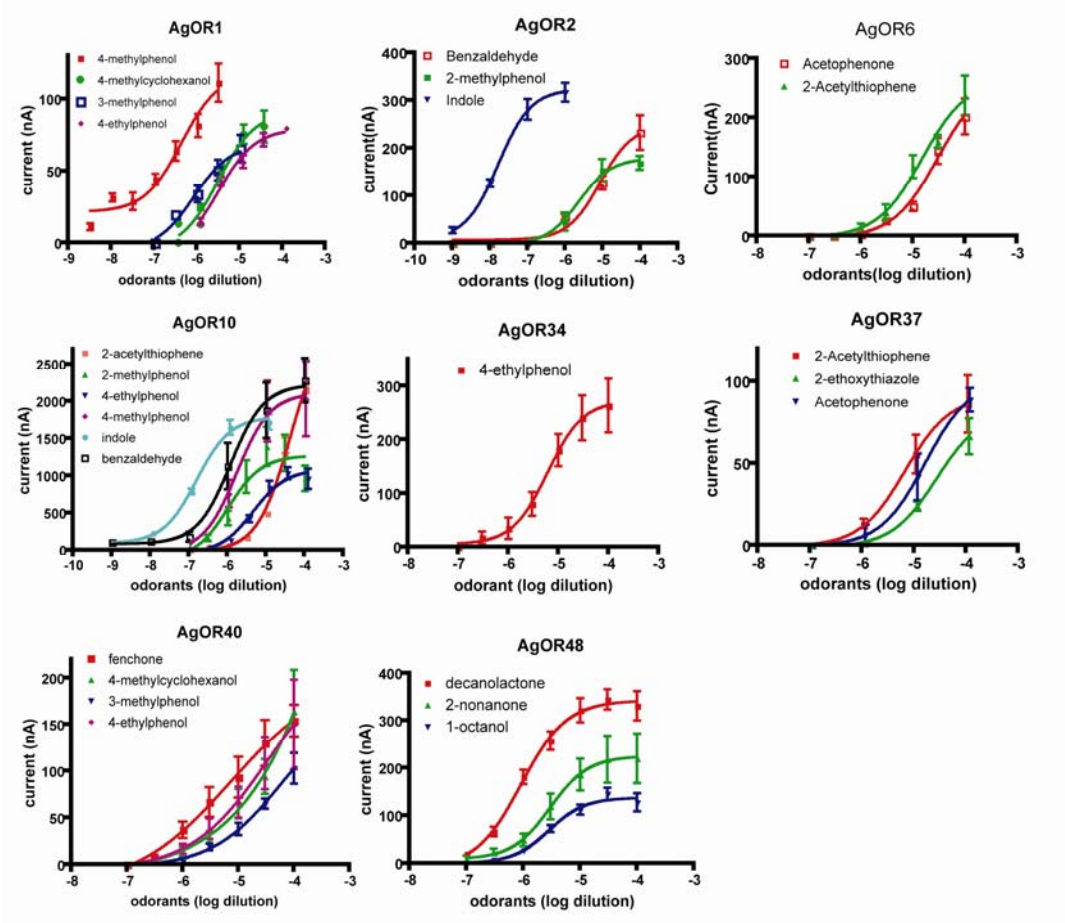


Figure 13. Dose-response curves of Larval AgORs to their most effective ligands. Each point represents the means (SEM) of *n* = 5–6 independent oocytes. EC50 values for each AgOR/odorant are as follows: AgOR1: 4-methylphenol (4.45 x10^{-7}); 4-methylcyclohexanol (3.17 x10^{-6}); 3-methyl phenol (8.54 x10^{-7}); 4-ethylphenol (3.20x10^{-6}). AgOR2: benzaldehyde (8.58x10^{-6}); 2-methylphenol (2.30x10^{-6}); indole (1.66x10^{-8}). AgOR6: acetophenone (3.02x10^{-5}); 2-acetylthiophene (1.51x10^{-5}). AgOR10: 2-acetylthiophene (4.36x10^{-5}); 2-methylphenol (1.05x10^{-6}); 4-ethylphenol (4.61x10^{-6}); 4-methylphenol (1.75x10^{-6}); indole (1.58x10^{-7}); benzaldehyde (1.20x10^{-6}). AgOR34: 4-ethylphenol (6.07x10^{-6}). AgOR37: 2-acetylthiophene (6.67x10^{-6}); 2-ethoxythiazole (2.7x10^{-5}); acetophenone (1.64x10^{-5}). AgOR40: fenchone (4.89x10^{-6}); 4-methylcyclohexanol (3.00x10^{-5}); 3-methylphenol (2.60x10^{-5}); 4-ethylphenol (1.57x10^{-5}). AgOR48: decanolactone (9.13x10^{-7}); 2-nonanoone (2.95x10^{-6}); 1-octanol (2.78x10^{-6}).

produced robust currents in *oocytes* expressing *AgOR1, AgOr2, AgOR10, AgOR34* and *AgOR40*. In a similar context, *AgOR6, AgOR10, AgOR28, AgOR37* and *AgOR40* all responded to acetophenone (Figure. 12, 13), which evoked measurable avoidance behaviors in *An.gambiae* larvae even at source dilutions as low as 10^{-5} (Figure. 9). Over and above these observations, we note that most larval *AgOrs* elicited strong responses to specific odorant groups when expressed in *Xenopus oocytes*. Each of the nine *in situ*-hybridization-positive larval *AgOrs* responded to at least two of the aromatics tested, while *AgOR1, AgOR6, AgOR10, AgOR28, AgOR37* and *AgOR40* responded to a number of heterocyclic compounds. Interestingly, *AgOR48* was the only larval *AgOR* that responded to acid, alcohols, and ketones (Figure 12). Of the four larval-specific *AgOrs*, two (*AgOR37* and *AgOR40*) manifested a distinct odorant response spectra; *AgOR40*, however, was more of a generalist that characteristically evoked large currents and was the only larval *AgOR* that responded to DEET. *AgOR37* appeared to be narrowly tuned to five odorants with smaller currents. Dose Response data for eight larval *AgOrs* (Figure 13) and AgOR28 (Lu et al., 2007a) revealed EC_{50} values ranging from $1.66X10^{-8}$ (AgOR2 and indole) to $1.51X10^{-5}$ (AgOR6 and 2-acetylthiophene). The other two larvae-specific genes, *AgOR52* and *AgOR58*, showed no response to any odorants tested, suggesting that they may be tuned to a different group of odorants not included in the test panel. *AgOR49*, which is also expressed in adult olfactory appendages, similarly failed to yield any odorant response, suggesting it may be tuned to undefined yet biologically significant odorants. Alternatively, the absence of oocyte responses in

these instances may result from a lack of threshold levels of functional *AgOR* expression.

Discussion

This work follows previous molecular studies (Pitts et al., 2004) and is consistent with numerous field and laboratory-based work in mosquitoes(Merritt et al., 1992) as well as more recent studies using *Drosophila* (Fishilevich et al., 2005; Louis et al., 2008) . That said, it is important to appreciate the pre-adult fruit flies and mosquitoes reside in totally different environment. *An.gambiae* larvae inhabit small water bodies that are often numerous, scattered, sunlit, turbid, temporary, and lack of consistent foot resources (Merritt et al., 1992), whereas *Drosophila* larvae typically exist in a terrestrial environment containing a high concentration of food. Not surprisingly, although both systems display a robust odor-coding capacity, each species has a distinct larval odor-response spectrum. We used a simple mobility assay to identify olfactory-based responses to an odorant panel spanning multiple chemical groups and biological contexts, the majority (~60%) of which failed to elicit any significant behavioral response. Although detailed time-lapse studies are required to precisely define the nature of odorant induced behavioral response profile of *An.gambiae* larvae we have identified, these data nevertheless provide unequivocal initial evidence of a repertoire of larval

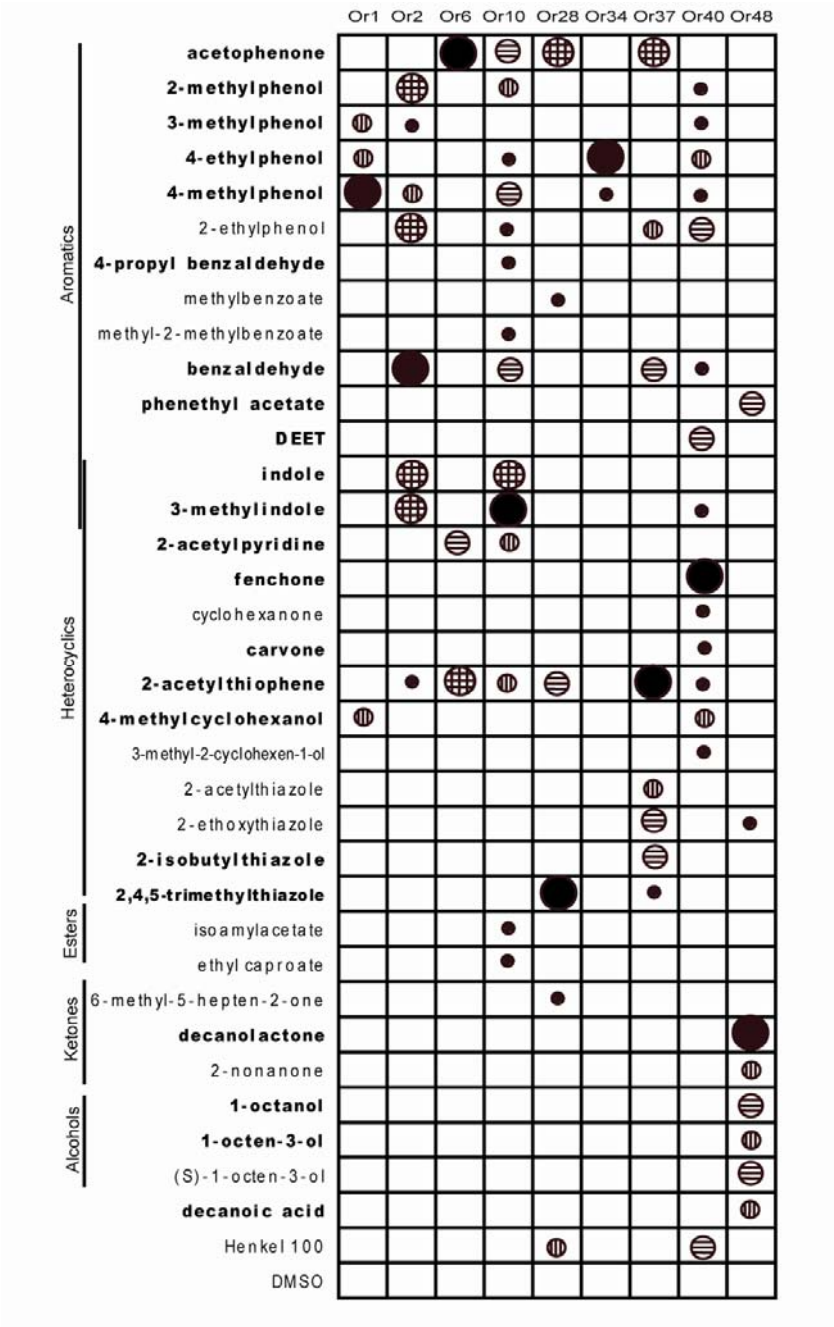


Figure 14. Combinatorial coding of odors in *An.gambiae* larvae.

Filled circles represent the maximal response for each AgOR. Checkered circles represent 80–99% of the maximal response of given AgOR. Horizontally striped circles represent 60–79% of the maximal response of given AgOR. Vertically striped circles represent 40–59% of the maximal response of given AgOR. Crosshatched circles represent 20–39% of the maximal response of given AgOR. Odorants are classified into different categories according to their functional groups (aromatics, heterocyclics, esters, ketones, alcohols, and acids). The odorants highlighted in bold were also evaluated in behavioral assays.

olfactory-based behaviors.

Interestingly, aromatics comprise most of the 10 odorants that are shown to be associated with significant larval responses. Of these, the positive PIs manifested by several cresol derivatives, such as 2-methylphenol, 3-methylphenol, 4-methylphenol, 4-methylcyclohexanol, and indole, over a range of concentrations, are consistent with the effects of attractants. These compounds are products of organic decay, which constitute a major food source for mosquito larvae (Thiery et al., 1991). Of these, indole, 3-methylphenol, and 4-methylcyclohexanol have also been shown to evoke strong electrophysiological activity from the antennae of female adult *An.gambiae* (Blackwell and Johnson, 2000). This interesting parallel between the adult and larval olfactory systems is consistent with the co-expression of several *AgOrs* in systems as well as with the suggestion that compounds that foster larval development might also act as potential oviposition attractants for adults.

Two other compounds, acetophenone and DEET, provoked negative PIs that are consistent with potentially repulsive behaviors when tested against *An.gambiae* larvae. Acetophenone has been shown to be attractive to *D. melanogaster* larvae (Fishilevich et al., 2005); DEET, however, is the major commercial insect repellent, although to date this has been used exclusively to target adults. 1-Octen-3-ol, which is present in the body odor of several vertebrates and is an attractant for many insect species including *Anopheles* mosquitoes (Ramoni et al., 2001), evoked positive PIs from *An.gambiae*

larvae, albeit at a single, relatively high dilution (10^{-4}). Although it is difficult to parse the potential biological significance of such a narrowly tuned behavioral response, it is possible that 1-octen-3-ol is normally found within the context of other compounds where it plays a synergistic role.

We examined the role of the larval antennae to define the cellular basis for these responses. Indeed, specific ablation of the larval antennae in *An.gambiae* dramatically compromised these responses, thereby validating their olfactory basis. However, larvae subjected to sham maxilla ablations and un-ablated controls both maintained normal response parameters (Figure 10). Consistent with our previous studies, immunohistochemistry localized *AgOR7* to define 12 putative ORN cells within the larval antennae. At the same time, a molecular survey of the larval antennae defined an identical number of conventional *AgOrs* that, together with *AgOr*7, are likely to be responsible for the olfactory specificity in *An.gambiae* larvae. Of these, the expression of four *AgOrs* was specifically restricted to the larval olfactory system. This is a significant overall reduction relative to the 23 ORs that are detected in larval stage of both *D. melanogaster* (Kreher et al., 2005) and *Ae. aegypti* (Bohbot et al., 2007), of which 10 or 15, respectively, are larval specific. Of these, apart from AgOr7, eight larval *AgOrs* have homologs in *Ae. aegypti*, and yet share similarity to any *Drosophila* Ors (Hill et al., 2002). This high degree of OR conservation suggests that, although the odor space of *Ae. aegypti* larvae remains undefined, it is likely to share significant characteristics with that of *An.gambiae*, in which the overall reduction in the size of the olfactory system may

reflect unique features of the larval ecology relative to other mosquito species.

Our *In situ* hybridization studies support the idea that each larval ORN stereotypically co-expresses a single conventional AgOr together with *AgOr*7. Although we cannot rule out that co-expression of conventional *AgOrs* ever occurs, in contrast to what has been observed for *D. melanogaster* (Fishilevich et al., 2005; Kreher et al., 2005), this hypothesis is buttressed by several observations. These include the presence of an identical number of conventional *AgOrs* and *AgOr7*$^{+}$ ORNs as well as the absence of a single instance (over numerous double-labeling experiments) in which the transcripts for any conventional *AgOr* were detected either together with another *AgOr* or, alternatively, in more than one individual larval ORN.

Of the 82 odorants tested against nine larval *AgOrs*, 35 evoked a response at least from *oocytes* expressing a single conventional AgOR. Although the inherently limited odorant panel precludes any assumption this represents the complete response spectrum, it is nevertheless sufficiently broad to suggest these data provide a comprehensive survey. Although several *AgOrs—AgOR2, AgOR10, AgOR28* and *AgOR40*—manifested relatively broad tuning responses and *AgOr1, AgOr6 and AgOr34* exhibited a more narrowly tuned response, there was an overall bias towards compounds with aromatic and heterocyclic functional groups. Indeed, of the nine *AgOrs* that function in *Xenopus oocytes*, only AgOr48, which is tuned to a modest number of odorant ligands displayed any significant responses outside the aromatic/heterocyclic range. Moreover, in keeping with the paradigms established in

vertebrate (Malnic et al., 1999) and in both larval- (Kreher et al., 2005) and adult-stage (Hallem et al., 2004b) *Drosophila* systems, several odorants elicited responses from multiple *AgOrs* (Figure 12). In a similar manner, this suggests that a functional redundancy insofar as *AgOR* tuning and that a combinatorial odorant coding mechanism acts to encode odorant information in *An.gambiae* larvae. The most striking element of this analysis was the relatively large number of odorants that activated a very narrow range of the larval AgOR repertoire. Indeed, just over half of the 35 active odorants in this survey elicited responses from a single larval AgOR; if one considers odorants that activate up to two larval *AgOrs*, this rises to > 65%. This is consistent with similar observation for combinatorial odor coding in the larval olfactory system of *Drosophila* (Kreher et al., 2005) and may reflect an implicit restriction in how the response spectrum is maintained in a significantly more restricted neuronal system.

The link between peripheral olfactory sensitivity and larval behavioral output is obvious, although not straightforward. In this context, we can easily rationalize the effects of odorant stimuli, such as acetophenone and 2, 3 or 4-methylphenol, which stimulated multiple *AgOrs* and also evoked robust larval behaviors. Similarly, we never observed a behaviorally active stimulus that failed to activate at least one functionally characterized larval AgOR. One of the most striking examples is the behavioral and AgOR40-expressing oocyte response to the adult insect repellent DEET (Figures 7, 9 , 13 and 14), which, based on the larval specific expression of AgOr40, may indicate the presence of additional DEET-sensitive *AgOrs* in the adult. An alternative suggestion is

that DEET may not act as a true behavioral repellent but rather by inhibiting the attraction to another compound, perhaps through antagonistic mechanisms at the molecular level.

Several compounds such as 2-acetlythiophene, benzaldehyde, and 3-methyl indole, strongly activated multiple *AgOrs* in *Xenopus* oocyte recordings, yet did not evoke significant behavioral responses from *An.gambiae* larvae within the context of our assays. This may result from an inherent limitation of our behavioral paradigm or it might be that sensory input at a molecular level is necessary but may not always be sufficient to generate behavioral responses. This is not unique to mosquitoes, as ethyl acetate activates three Ors expressed in *Drosophila* larvae (Hallem et al., 2004b; Kreher et al., 2005), despite the fact that wild-type *Drosophila* larvae are largely indifferent to ethyl acetate in a chemotaxis assay (Fishilevich et al., 2005).

Aqueous larval habitats inherently represent a confined and therefore more easily targeted venue for mosquito control strategies. In addition to addressing the underlying basis for olfactory coding in insects, these studies shed light on the mechanistic elements of the larval olfactory system in *Anopheles* that may facilitate the development of novel approaches targeting larval feeding and other behaviors to potentially enhance the effectiveness of current vector control strategies.

Methods

Mosquito Rearing

An. gambiae sensu stricto, originated from Suakoko, Liberia, was reared as described (Fox et al., 2001). For stock propagation, 4- to 5-days-old female mosquitoes were blood-fed for 30–45 min on anesthetized mice following the guidelines set by Vanderbilt Institutional Animal Care and Use Committee.

Scanning Electron Microscopy

Larva heads were fixed in 4% paraformaldehyde, 0.1% Triton X-100 in PBS followed by dehydration in ethanol series from 50–100% (10% increments) and ethanol:hexamethyldisilazane (HMDS) at (v/v) 75:25, 50:50, 25:75 and 0:100. The heads were then dried in a fume hood, mounted onto aluminum pins with colloidal silver paint and sputter-coated for 30 s with gold–palladium. The images were captured by using Hitachi S-4200 scanning electron microscope with Quartz PCI image acquisition software version 6.0 (Quartz Imaging Corp. Vancouver, BC).

***In situ* Hybridization**

Heads were hand-dissected from *An. gambiae* third-instar larvae, embedded in paraffin and sectioned at 8μm on a sliding microtome (HM340E; Microm). *In situ* hybridization was performed as described with modification(Kwon et al., 2006).

Anti-sense DIG and FITC riboprobes were transcribed from *AgOr* templates derived from cDNA synthesized from larvae antennae total RNA. Two-color *in situ* hybridization was performed with digoxigenin- and fluorescein-labeled riboprobes, detected first with Fast Red Tablets (digoxigenin; Roche) and then with TSA-plus Tyramide Amplification System (fluorescein; Perkin Elmer). Anti-Fluorescein-AP and anti-Digoxigenin-POD were diluted 1:500 (Roche).

Receptor Expression in *Xenopus Oocytes* and Two-Electrode Voltage Clamp Electrophysiological Recording

Full-length coding sequences of *AgOr*1, *AgOr*2, *AgOr*6, *AgOr7*, *AgOr10, AgOr28, AgOr34, AgOr37, AgOr40, AgOr48, AgOr52* and *AgOr58* were PCR amplified from female *An. gambiae* maxillary palp cDNA. *AgOr7* coding sequence was cloned into pT7TS (a gift from G. Lepperdinger with permission of D. Melton); coding sequences of *AgOr*1, *AgOr*2, *AgOr*6, *AgOr10, AgOr28, AgOr34, AgOr37, AgOr40, AgOr48, AgOr52* and *AgOr58* were first cloned into pENTR/D-TOPO (Invitrogen) and then sub-cloned into pSP64DV by means of the Gateway LR reaction. The pSP64DV vector was a Gateway-compatible destination vector converted from pSP64T-Oligo (a gift from A. George) using the Gateway Vector Conversion System (Invitrogen). cRNAs were synthesized from linearized vectors using mMESSAGE mMACHINE or mMESSAGE mMACHINE T7 Ultra (Ambion). Human $G\alpha_{15}$ RNA was transcribed from pSGEM-$G\alpha_{15}$ (a gift from H. Hatt) (Wetzel et al., 2001).

Mature healthy *oocytes* (stage V-VII) were treated with 2 mg/ml collagenase S-1 in washing buffer (96 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$ and 5 mM HEPES, pH 7.6) for 1-2 h at room temperature. *Oocytes* were later micro-injected with *AgOr*1, *AgOr*2, *AgOr*6 , *AgOr10, AgOr28, AgOr34, AgOr37, AgOr40, AgOr48, AgOr52* and *AgOr58*, 27.6 ng *AgOr7* cRNA and 0.276 ng $G\alpha_{15}$ cRNA. After injection, *oocytes* were incubated for 3-5 days at 18°C in 1XRinger's solution (96 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$, 0.8 mM $CaCl_2$ and 5 mM HEPES, pH 7.6), supplemented with 5% dialyzed horse serum, 50 μg/ml tetracycline, 100 μg/ml streptomycin and 550 μg/ml sodium pyruvate.

Whole cell currents were recorded from the injected *Xenopus oocytes* with a two-electrode voltage clamp. Odorants were dissolved in DMSO at a 1:10 ratio to make stock solutions. Prior to recording, stock solutions were diluted in 1XRinger's solution to the indicated concentrations before being applied to *Xenopus oocytes* in an RC-3Z oocyte recording chamber (Warner Instruments). Odorant-induced currents were recorded using an OC-725C oocyte clamp (Warner Instruments) at a holding potential of –80 mV. Data acquisition and analysis were carried out using Digidata 1322A and pCLAMP software (Axon Instruments).

Larval Behavior Assay and Data Analysis

100 *A. gambiae* 2nd or 3rd instar larva were picked and washed carefully to eliminate any food particles. Washed larva were kept in 27 ^{O}C distilled water and starved for 2 hrs.

Odorant stocks were made by dissolving specific amount of the odorants in pre-heated 2% NuSieve GTG low melting temperature agarose (Cambrex Bio Science). The assay was performed in a 38.1 x25.4 x 5.08 cm Pyrex baking dish containing 500mls 27^0C distilled water. A test zone and control zone were determined and outlined. The larva were released in the center of the dish and allowed to swim freely for 1 hr. The odorant/control stock was inserted into a mesh ring, which was used to prevent direct contact between larva and odorant stock, and then placed in the center of the zone area accordingly. Real-time pictures were taken every 30 seconds with a CCD camera controlled by Scion Image 1.63. The assay lasted for 21 minutes.

The number of larva in both test and control zone was counted throughout all time-point.. Performance index at 15 min was calculated as:

$PI=(\#_{odorant}-\#_{control})/(\#_{odorant}+\#_{control})$

Table 1. Natural or synthetic odorants used in *Xenopus* oocyte recordings and behavioral assays

Group	Odorant	Chemical abstract service no. (CAS)
Aromatics	Benzaldehyde	100-52-7
	2-Methylphenol	95-48-7
	3-Methylphenol	108-39-4
	4-Ethylphenol	123-07-9
	4-Methylphenol	106-44-5
	3-Methylindole	83-34-1
	Acetophenone	98-86-2
	DEET (*N*,*N*-diethyl-m-toluamide)	134-62-3
	Indole	120-72-9
Hyterocyclics	2-Isobutyl thiazole	18640-74-9
	2,4,5-Trimethylthiazole	13623-11-5
	2-Acetylpiridine	1122-62-9
	2-Actothiophene	88-15-3
	4-Methylcyclohexanol	589-91-3
	Carvone	2244-16-8
	Fenchone	4695-62-9
	Thiazole	288-47-1
Acids	Decanoic acid	143-07-7
	Isovaleric acid	503-74-2
	Propanoic acid	1979-9–4
Esters	Phenetyl acetate	103-45-7
	Ethyl acetate	141-78-6
Alcohols	1-Octanol	111-87-5
	1-Octen-3-ol	3391-86-4
Ketones	Decanolactone	706-14-9
	Acetone	67-64-1
Amine	*N*-butylamine	109-73-9
aldehyde	Octanal	124-13-0
Amino acids	**Methionine**	
	Phenylalanine	
Larvae nutrition	**Yeast**	
	Fish food	

Odorants highlighted in bold were only tested in behavioral assays.

CHAPTER IV

INTRODUCTION TO THE WEST NILE VIRUS AND ITS VECTOR MOSQUITO *CULEX QUINQUEFASCIATUS*

West Nile Virus

WNV is a mosquito-borne flavivirus originally isolated in 1937 from the blood of a febrile woman in the West Nile province of Uganda (Smithburn KC 1940). The virus is maintained through a bird–mosquito transmission cycle. The virus is widely distributed and has been associated with asymptomatic infections and sporadic disease outbreaks in humans and horses in Africa, Europe, Asia and Australia (Hayes and Gubler, 2006). In 1999, WNV underwent a dramatic expansion of its geographic range, and was reported for the first time in the western hemisphere during an outbreak of human and equine encephalitis in New York City (Nash et al., 2001). The outbreak was accompanied by extensive and unprecedented avian mortality. Since then, WNV has dispersed across the Western Hemisphere and is now found throughout the USA, Canada, Mexico and the Caribbean, and parts of Central and South America (Hayes and Gubler, 2006). Moreover, after 1994, outbreaks have occurred with a higher incidence of severe human disease, particularly affecting the nervous system (Hayes and Gubler, 2006).

Since 1999, at least 19,500 cases of WNV infection have been reported in the USA alone, of which 8,606 (44%) caused neuroinvasive disease with 771 fatalities (3·9% of all) (Kramer et al., 2007). Normally people develop symptoms between 3 and 14 days after bitten by the infected mosquito (Mackenzie et al., 2004). Paradoxically, approximately 80 percent of people who are infected with WNV do not show any symptoms at all (Jeha et al., 2003). That said, up to 20 percent of the people who become infected have symptoms such as fever, headache, and body aches, nausea, vomiting, and sometimes swollen lymph glands or a skin rash on the chest, stomach and back. Symptoms can last for as short as a few days, though some people may become sick for several weeks (Jeha et al., 2003). Only about one in 150 people infected with WNV will develop severe illness. The symptoms can include high fever, headache, neck stiffness, stupor, disorientation, coma, tremors, convulsions, muscle weakness, vision loss, numbness and paralysis (Jeha et al., 2003). The symptoms may last several weeks, and neurological effects may be permanent.

WNV Vector Mosquito *C. quinquefasciatus*

Often known as the common southern mosquito, the adult *C. quinquefasciatus* is less than 3/16 inch long. The distinguishing features of *Culex* mosquitoes are: crossed veins on narrow wings, blunt abdomen, short

maxillary palps, and no pre-spiracular or post-spiracular setae (CDC 2007). Larvae are recognized by their long breathing tubes and whip-like tail movements when disturbed (CDC 2007).

C. quinquefasciatus can be found in many areas of the world and studies indicate that a considerable regional diversity exists regarding host species chosen for blood meals (Zinser et al., 2004). Some reports suggest that in North America *C. quinquefasciatus* feed predominantly on birds and less than 1% of the time on humans (Reisen et al., 1990), although other studies find no strong evidence supporting this theory. Indeed, a recent report from California found *C. quinquefasciatus* feeds approximately equally on mammals and birds (Zinser et al., 2004). Analysis of blood-meal sources from *C. quinquefasciatus* in two urban sites and one country site in Louisiana suggests that the *Culex* mosquitoes are opportunistic feeders that feed readily on humans or birds (Niebylski and Meek, 1992). Mosquitoes from a site adjacent to a dog kennel had >96% dog blood meals and typical residential areas yielded 65–70% dog, 9–15% human, and 6–30% bird blood (Niebylski and Meek, 1992; Zinser et al., 2004).

Adult *C. quinquefasciatus* mosquitoes are attracted to a number of natural and synthetic odorants. In an odorant-baited trap study, significantly more *C. quinquefasciatus* responded to foot odors collected on nylon stockings than to clean nylon ones (Mboera et al., 2000). It was also found that outdoors, a carbon dioxide baited trap collected over 12 times more *C.*

quinquefasciatus adults than an un-baited one (Mboera et al., 2000). Later studies have shown that *C. quinquefasciatus* mosquitoes are attracted to the filtrates of some bacteria found from their breeding sites (Poonam et al., 2002), which may contain the potential oviposition pheromone. Given its strong avian host preference, it is not surprising that odors from fresh chicken feces in water elicited upwind flight of host-seeking female *C. quinquefasciatus* mosquitoes in a dual-choice olfactometer (Cooperband et al., 2008).

Insecticide Resistance in *Culex* mosquitoes

In the last forty years, the wide application of adult insecticides against disease vectors has caused the development of insecticide resistance in many mosquito species (McCarroll et al., 2000). This has greatly increased the cost of current mosquito control events and is the focus of extensive studies, particularly in *C. quinquefasciatus* (Labbe et al., 2007).

C. quinquefasciatus uses one predominant resistance mechanism that occurs in more than 80% of insecticide-resistant *Culex* worldwide (Hemingway and Karunaratne, 1998) which originated in one population and accumulated through evolutionary adaptation before spreading rapidly (Labbe et al., 2007). This resistance depends on the stable germline amplification of two esterase enzymes and an aldehyde oxidase (Hemingway et al., 2000); the esterases are attributed to closely linked loci denoted as A and B according to substrate preference (Hemingway et al., 2000; Raymond et al., 1991), and the

over-production of B-type esterases is due to gene amplification (Peiris and Hemingway, 1996). The esterases found in resistant mosquitoes are expressed at very high levels in the gut and salivary glands, in some cases where up to 80 copies of this esterases B amplicon can be found per cell (McCarroll et al., 2000).

The increasing concern regarding insecticide resistance strongly argues for the development of new insecticides as well as novel control strategies. In this light, the ability to modulate vector behavior provides a potentially important opportunity to reduce disease transmission by targeting vectorial capacity. Inasmuch as olfaction is of great importance in nearly all mosquito behaviors (Takken and Knols, 1999), a deeper understanding of olfactory system of *C. quinquefasciatus* may very well provide valuable information for effective control against the WNV vector mosquito. Our characterization of the first odorant receptor from *C. quinquefaciatus* detailed in the next chapter of this thesis marks an initial but nevertheless important step towards this ultimate goal.

CHAPTER V

IDENTIFICATION AND CHARACTERIZATION OF AN OR FROM THE WEST NILE VIRUS MOSQUITO, *CULEX QUINQUEFASCIATUS*

Preface

The information presented in this chapter was published in the *Insect Biochemistry and Molecular Biology* (36(3):169-76). The author's contribution to this body of work includes all the research covered in this paper.

Introduction

West Nile Virus (WNV) has been spreading across North America since it was first recognized in New York City during 1999. By 2003, at least 9862 cases of WNV human infections were reported resulting in 264 deaths. In addition, WNV poses a significant threat to birds as well as economically important domestic animals such as cattle and horses. It has been established that WNV transmission is driven by the requirement for a vertebrate blood meal by female mosquitoes in order to complete their gonotrophic reproductive cycle. Olfactory signals provide important sensory inputs that a female mosquito uses to locate and attack a blood meal host (Takken and Knols, 1999) and, accordingly, shapes the negative impact of these and many other insects of economic and medical importance (Zwiebel and Takken, 2004). Therefore, a deeper understanding of the mosquito olfaction system may facilitate the

development of methods that can interfere with the interaction of insect vectors with their host organisms.

Here, we report the identification and characterization of CqOr7 that represents the first, albeit non-conventional, candidate member of the OR (OR) family of proteins from *C. quinquefasciatus*. As is the case for other members of this group of non-conventional ORs, CqOr7 is widely expressed in olfactory appendages of both immature and adult stages, and shares great similarity with apparent orthologs from several other insects. These include *An. gambiae* (AgOr7) (Hill et al., 2002); *Aedes aegypti* (*AaOr*7) (Melo et al., 2004); *Drosophila melanogaster* (DOr83b) (Clyne et al., 1999a; Gao and Chess, 1999; Vosshall et al., 1999); *Heliothis virescens* (HvirR2)(Krieger et al., 2003), as well as *Apis mellifera* (AmelR2) (Krieger et al., 2003). The high conservation level across species and the wide expression in chemosensory tissues of *C. quinquefasciatus* suggests that this receptor and its orthologs represent an OR sub-family that may play an important role in the chemosensory signal transduction in this mosquito and other insects. The best-studied member of this non-conventional OR sub-family, *Drosophila* DOr83b has been shown to act as a nearly essential dimerization partner for other, conventional ORs in heterozygous systems (Neuhaus et al., 2005). Furthermore, DOr83b mutant flies manifest abnormal cytoplasmic aggregation of other co-expressed ORs and display dramatically impaired electrophysiological responses to some odorants (Larsson et al., 2004). As such, members of this particular gene

sub-family (which we propose to hereafter designate the OR 83b sub-family) may prove useful as targets for disruption of the insect olfactory signal transduction pathway. Indeed, the study of this unique candidate OR sub-type may lead to novel approaches designed to reduce olfactory sensitivity and, therefore, the vectorial capacity of mosquitoes by disrupting vector/host interactions.

Materials and Methods

Mosquito Rearing

C. quinquefasciatus were reared as-described (Fox et al., 2001). For stock propagation, 4- to 5-days-old female mosquitoes were blood-fed for 30–45 min on anesthetized mice following the guidelines set by Vanderbilt Institutional Animal Care and Use Committee.

Molecular Cloning

Primary amino acid sequences of the following genes were retrieved from GenBank: *Drosophila melanogaster* Or83b (NM079511), *An. gambiae* Or7 (AY363725,AY363726), *Aedes aegypti* Or7 (AY582943). Blocks of sequences were generated using BlockMaker (http:// blocks.fhcrc.org/blocks/make_blocks.html) and oligonucleotide primers for PCR amplification were designed from blocks using CODEHOP algorithm

(http://blocks.fhcrc. org/blocks/codehop.html). Three primers were used in subsequent PCR amplifications:\ Deg 502: CATCGCCCTGGCCAARATGMGNAA; Deg 301 : CGGAGCCGTCGTACCARTGRCA; Deg302 : GGTAGCCGATCACGGTGAAGSCRTANACRTT*:*

PCR templates were prepared from hand-dissected antennae from 1000 female *C. quinquefasciatus* mosquitoes that were used to generated total RNA with RNeasy (Qiagen, Valencia, CA) protocols followed by the preparation of and adaptor-ligated cDNAs using the Marathon cDNA Construction reagents (BD Biosciences Clontech, Palo Alto, CA). PCR reactions were carried out with a 1:10 dilution of antennal cDNAs and CODEHOP primers in a PTC-200 (MJ Research, Waltham, MA) thermal-cycler for 35 cycles with an optimal annealing temperature of 55 1C along with appropriate control reactions. All experimental-specific PCR products were gel-purified using QIAquick gel extraction reagents (Qiagen, Valencia, CA), cloned into the pCRII-TOPO cloning vector (Invitrogen, Carlsbad, CA) and subsequently sequenced in the DNA Core Facility at Vanderbilt University. Full length CqOr7 cDNA were obtained using RACE amplifications in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA) under conditions as-described in Marathon kit manual with Adaptor primer 1 (AP1, Marathon cDNA kit, BD Biosciences Clontech, Palo Alto, CA) and CqOr7 specific RACE primers—RACE primer 1: AAGGTACCGCTTCTCGCAAATCAGGTCA and RACE primer 2:

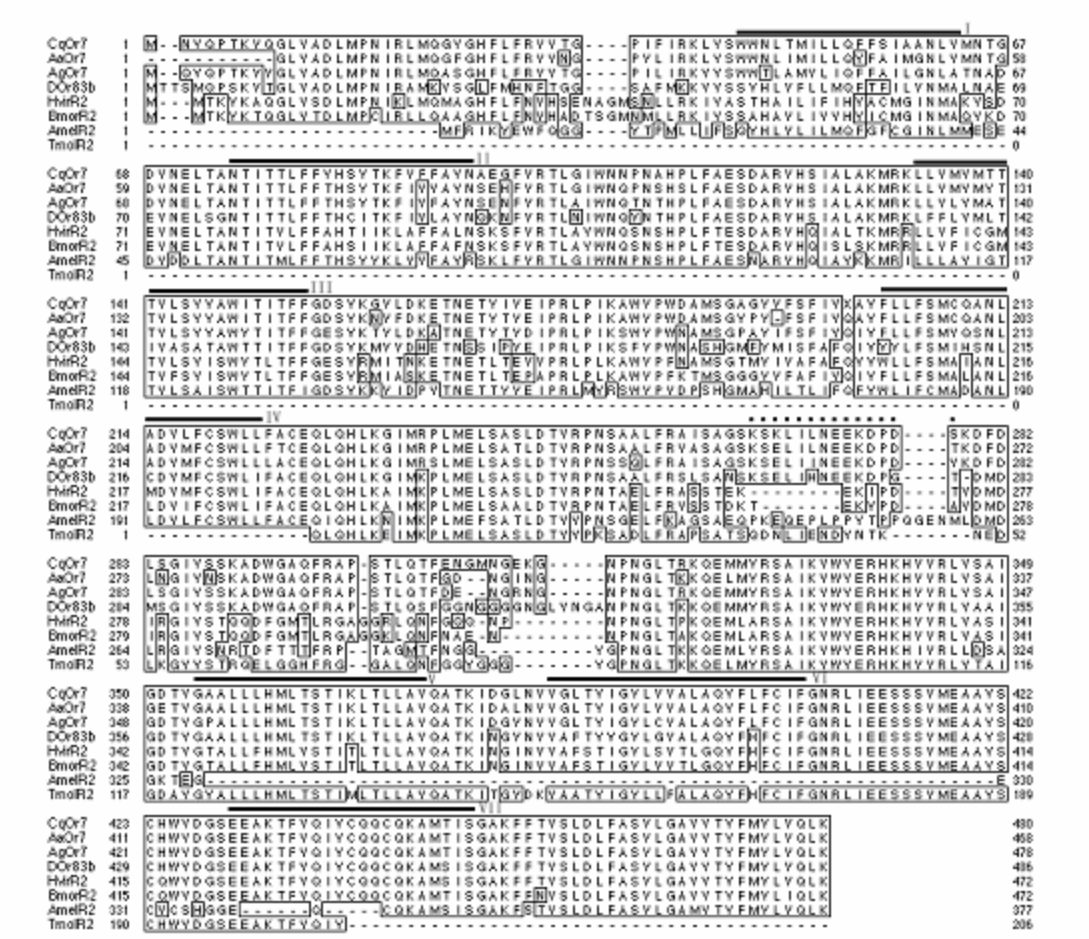


Figure 15. Alignment of CqOr7 ortholog peptides using the single amino acid code. Identical residues are shaded and boxed. Trans-membrane domains I–VII are indicated with black bars. Dotted line indicates peptide used for generating OR7 antiserum. For a list of genes and accession numbers see materials and methods.

CAGGTACCTGTGCACGGTTGCATCGGA. PCR products were further cloned into the pCRII-TOPO cloning vector (Invitrogen, Carlsbad, CA) and sequenced as-described above. The complete CqOr7 nucleotide sequence has been deposited to Genbank where it has been assigned the accession number DQ231246.

RNA Expression

Total RNA was isolated from the following *C. quinquefasciatus* tissues using the RNeasy reagents and protocols (Qiagen, Valencia, CA) as-described above—early instar larvae (2–4 days old), late instar larvae (10–14 days old), pupae, or adult tissues (4–6 days old). Firststrand cDNA synthesis was carried out by using Superscript II reverse transcriptase (Invitrogen Inc., Carlsbad, CA) and 0.5 mg of *C. quinquefasciatus* RNA according to the manufacturer's instructions. In order to control for any genomic DNA contamination, all subsequent PCR reactions were carried out using the following intron-spanning (based on partial genomic sequencing, data not shown) primers—CqOR7 501: CACATGCTGACCTCGACCAT and CqOR7 301: CAGCTGCACCAACTCCATGAA for 30 cycles with an optical temperature of 60 1C. All RT–PCR reactions were replicated at least eight times. Furthermore, the Culex homolog for the ribosomal protein S7 gene (CqRPS7, Genbank accession AF272670) was amplified in tandem as a control for cDNA integrity by using the primers CqRPS7a: CTGGAGATGAACTGGACCT and CqRPS7b:

CTTGTACACCGACGTGAAGG. PCR products were gel-purified, subcloned into the pCRIITOPO cloning vector and sequenced as described above.

Scanning Electron Microscopy

Adult heads were fixed in 4% paraformaldehyde, 0.1% Triton X-100 in PBS followed by dehydration in ethanol series from 50–100% (10% increments) and ethanol:hexamethyldisilazane (HMDS) at (v/v) 75:25, 50:50, 25:75 and 0:100. The heads were then dried in a fume hood, mounted onto aluminum pins with colloidal silver paint and sputtercoated for 30 s with gold–palladium. The images were captured by using Hitachi S-4200 scanning electron microscope with Quartz PCI image acquisition software version 6.0 (Quartz Imaging Corp. Vancouver, BC).

Immunocytochemistry

Immunocytochemistry was performed using a rabbit polyclonal peptide antisera derived against amino acid residues 268–281 of the AgOR7 sequence (Pitts et al., 2004) with the sole modification of incubating the secondary antibody reaction overnight at 4 1C. Confocal images were captured by using LSM 510 META system attached to an Axioplan fluorescence microscope (Zeiss). Other images were captured by using DP70 charge-coupled device camera attached to a BX-60 fluorescent microscope (Olympus Inc., Bethpage, NY).

Results

CqOR7 Transcripts

Degenerate primers were synthesized with the assistance of the CODEHOP web server based on a multiple sequence alignment of 83b family members from *An.gambiae*, *A. aegypti* and *D. melanogaster*. These primers were used to amplify a partial sequence of CqOr7 from a Marathon cDNA library prepared from *C. quinquefasciatus* antennae and confirmed by sequencing. Based on this, additional gene-specific primers were designed and used in RACE reactions to yield both N-terminal and C-terminal CqOr7 sequences. The predicted amino acid sequence of CqOR7 was aligned with those of other 83b sequences from *An.gambiae*, *A. aegypti*, *D. melanogaster*, *H. virescens, A. mellifera, Bombyx mori* and *Tenebrio molitor*. As shown in Figure 15, the eight sequences have >80% similarity and >60% identity. Moreover, the 150 amino acids that comprise the C-terminal region show extremely high conservation at levels that approach >90% identity. Specifically, CqOR7 shares 90% identity and 93% similarity in the terminal 230 amino acids with its most evolutionarily related homolog from *A. aegypti*, *AaOr*7. Compared with other insects' orthologs, CqOr7 shares a higher level of conservation with AgOr7 and *AaOr*7.

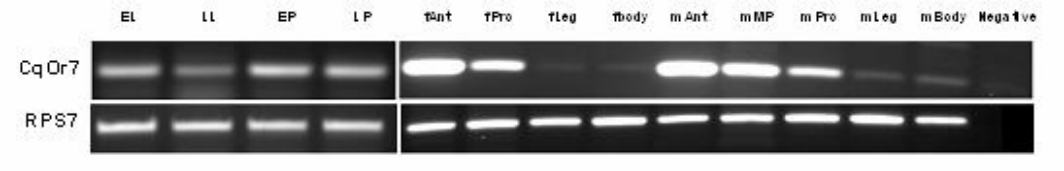


Figure 16. Expression of CqOR7 in pre-adult and adult *C. quinquefasciatus*.

Lane as follows—early larvae (EL), late larvae (LL), early pupae (EP), late pupae (LP), female antennae (fAnt), female proboscis (fPro), female leg (fLeg), female body (fBody), male antennae (mAnt), male maxillary palp (mMP), male proboscis (mPro), male leg (mLeg), male body (mBody), negative control. RPS7 acts as positive control.

RNA Expression

ORs are expressed in sensory neurons in olfactory appendages of insects, including the antennae and maxillary palps (Vosshall, 2000). We examined the expression pattern of CqOr7 transcripts by non-quantitative RT-PCR analyses of tissues isolated from several developmental stages and adult tissues (Figure. 16). In order to control for artifactual amplification from genomic DNA contamination, an intron-spanning primer set that results in diagnostic products from either cDNA (215 bp) or genomic DNA (523 bp) templates was designed. PCR products with an expected size of 215 bp can be observed with cDNA templates from different tissues, and the intensity of the products (relative to a constitutively expressed internal control) suggests variable expression levels in different tissues. For example, in addition to a significant expression throughout larval and pupal pre-adult life stages CqOr7 mRNA is robustly expressed in antennae from both male and female adult mosquitoes. CqOr7 expression is also observed in male and female mouthparts. CqOr7 transcripts are undetectable in these assays from female legs, while RNA derived from male legs display relatively weak CqOr7 expression as compared with antennae, maxillary palps and proboscis. Overall, as expected, CqOr7 expression is observed in the major olfactory tissues of *C. quinquefasciatus* comprising the antennae and maxillary palps, as well as in traditionally non-olfactory appendages such as the proboscis and legs from adults. In developmental studies, CqOr7 is first found in the early larvae stage and can

be readily detectable throughout all developmental stages.

Protein Expression

We used a rabbit polyclonal antisera that was raised against a 14 amino acid polypeptide of the deduced amino acid sequence of AgOR7 (Pitts et al., 2004) to examine the localization of the CqOr7 protein (Figure. 15, dotted line). Although there are two amino acid substitutions in the corresponding peptide sequence of CqOR7, the AgOR7 polyclonal antibody specifically labeled neurons throughout proximal, intermediate and distal segments of the female *C. quinquefasciatus* antenna (Figures. 16(C), (D), and (G)). Through systematic analyses of overlapping immunolabeled sections, each consisting of between five to eight consecutive antennal segments, we were able to observe CqOr7-specific labeling in all 13 flagellar segments in *C. quinquefasciatus*. Since the thicker and shorter proximal segments can be reasonably distinguished from the longer and thinner distal segments, we conclude that CqOr7-specific labeling is present in all flagellar segments. As was the case for both AgOR7 (Pitts et al., 2004) and *AaOr7* (Melo et al., 2004) homologs, CqOr7 labeling was observed within dendrites and cell bodies of sensilla trichodica in *C. quinquefasciatus* (Figure. 17D) where specific labeling can be detected in every sensillum examined. As a positive control, anti-horseradish peroxidase conjugated with FITC (HRP) was used to label neuronal cell bodies and axons (Jan and Jan, 1982);

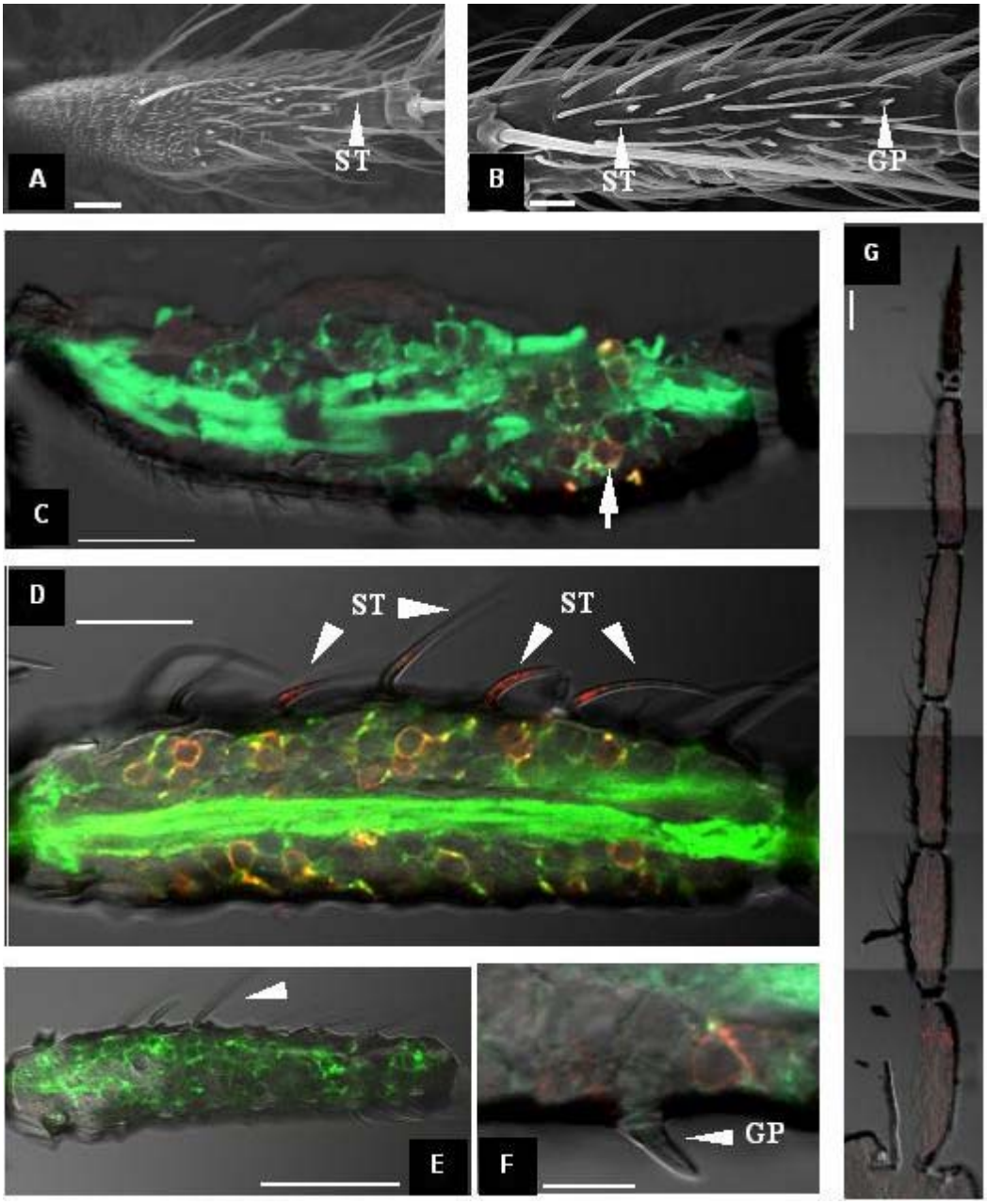


Figure 17. Localization of CqOR7 protein in female *C. quinquefasciatus* antennae.
Red is anti-AgOR7 marked with Cy3-labelled secondary antibody. Green is anti-horseradish peroxidase conjugated to FITC.
(A) SEM of the first antenna segment ST—sensillum trichodica (scale bar is 40 mm).
(B) SEM of the second antenna segment GP—grooved pegs, (scale bar is 40 mm).
(C) CqOr7 labeling of the first antenna segment, arrows shows the labeling of a neuron cell body, (scale bar is 20 mm).
(D) CqOr7 labeling of the second antenna segment, (scale bar is 20 mm).
(E) control reaction using pre-immune serum as primary antibody, arrow shows an unlabeled sensillum trichodica (scale bar is 40 mm).

(Sun and Salvaterra, 1995). In any one plane of section and with the exception of the first (proximal) antennal segment (Figure. 17C), approximately 20–30% of the HRP-positive neurons were labeled with AgOR7 antibody. Although overlapping of HRP:FITC (green) and AgOR7:Cy3 (red) signals were observed in many instances (Figure. 17), there were considerable sections where no obvious overlap was observed on the same cell body, suggesting these two antibodies may label the different sides of the membrane. This phenomenon is consistent with AgOR7 and *AaOr*7 staining (Melo et al., 2004; Pitts et al., 2004). Dendrites from sensilla trichodica were in general strongly labeled with AgOR7 antisera (Figure. 18D), although in these instances HRP labeling was typically not observed, suggesting the lack of the HRP epitope in the dendrites of this type of sensillum. Interestingly, basiconic sensilla from *C. quinquefasciatus* antennae, which are sometimes referred to as grooved pegs, apparently express neither CqOr7 nor HRP epitopes (Figure. 17F) consistent with observations in of both *An.gambiae* and *A. aegypti* mosquitoes (Melo et al., 2004; Pitts et al., 2004).

Interestingly, CqOr7 labeling was restricted to only the distal part of the first antenna segment where clear cell body labeling could be observed (Figure. 14C). This result stands in contrast to *A. aegypti*, where *AaOr*7 was detected throughout the first antennal segment (Melo et al., 2004) and to *An.gambiae*,

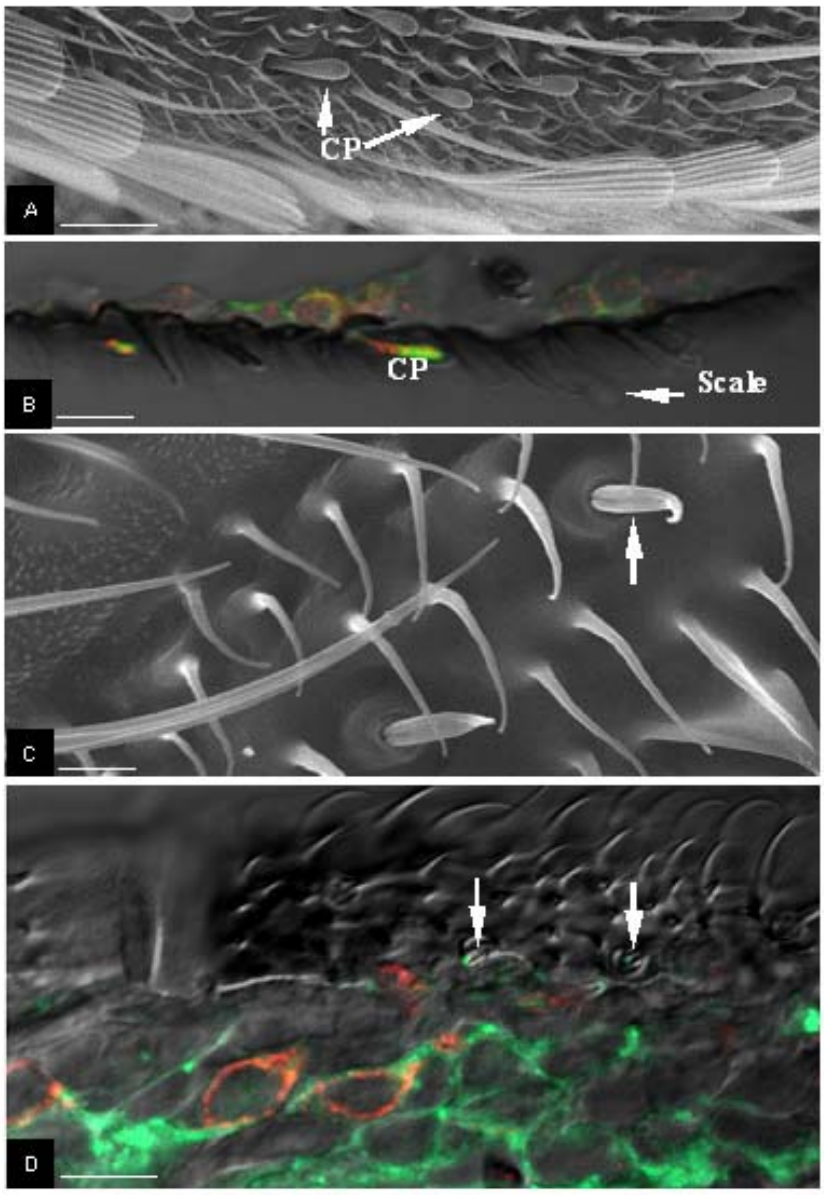


Figure 18. Localization of CqOR7 protein in female *C. quinquefasciatus* maxillary palp and proboscis.

Red is anti-AgOR7 marked with Cy3-labeled secondary antibody. Green is anti-horseradish peroxidase conjugated with FITC.

(A) SEM of a female C. quinquefasciatus maxillary palp CP—capitate pegs (scale bar is 25 mm).

(B) CqOr7 labeling of the capitate pegs (scale bar is 25 mm).

(C) SEM of a female C. quinquefasciatus proboscis region, arrow shows a distinct type of sensillum (scale bar is 5 mm).

(D) CqOr7 is labeled in a distinct type of sensilla shown by arrow (scale bar is 20 mm).

where the entire first antennal segment was devoid of AgOr7 labeling (Pitts et al., 2004). This likely reflects the fact that in *C. quinquefasciatus*, sensilla trichodea are only present in the distal part of the first antennal flagellum (Figure. 16A). Importantly, CqOr7 labeling was never observed within scales, microtrichia or sensilla chaetica (Figure. 17(C) and (D).

Consistent with the localization of CqOr7 mRNA (Figure. 16) immunoreactivity was also observed in the maxillary palps of the female *C. quinquefasciatus* (Figure. 18B). While a total of four types of sensory hair structures are found on *C. quinquefasciatus* maxillary palps—non-innervated incrotrichia and scales as well as mechanosensory sensilla chaetica and thin walled capitate peg sensilla (Figure. 18A). CqOr7 labeling was restricted to the dendrites but, interestingly, not cell bodies of capitate peg sensilla (Figure. 18B). As was the case for antennae, HRP labeling was also observed in capitate pegs on the maxillary palp where it was localized to many dendrites. Some weak background staining, which was present in the pre-immune control (data not shown), was also observed on cell bodies. Once again, mechanosensory, microtrichia and scales remained unlabeled in these preparations (Figure. 18B).

AgOR7 antisera also labeled a distinct subset of neuronal cells from the distal labellum of the proboscis of female *C. quinquefasciatus* (Figure. 18D), which has been characterized as the principal gustatory organ in mosquitoes. This is in agreement with similar data from *An.gambiae* and *A. aegypti* (Melo et

al., 2004; Pitts et al., 2004), but in contrast to the localization of DOr83b whose adult expression is limited to the antennae and maxillary palps (Vosshall et al., 1999). In *C. quinquefasciatus*, clear cell body and dendrite were also labeled (Figure. 16D). When compared with antennae, however, far fewer cells were labeled in proboscis, although the labeling within those cells is very strong. From the SEM studies it appears that only a limited number of chemosensory sensilla are distributed across the labellum of the proboscis (Figure. 18C) and these sensilla are only found on the upper part of the labellum.

Discussion

We have identified and characterized CqOr7, a non-conventional member of the OR family of proteins from the WNV vector mosquito *C. quinquefasciatus*. As expected, CqOr7 shares an extremely high conservation of its primary amino acid sequence with other 83b sub-family members from other insect species(Clyne et al., 1999b; Gao and Chess, 1999; Hill et al., 2002; Krieger et al., 2003; Melo et al., 2004; Vosshall et al., 1999). These genes are apparent orthologs based on both functional (Jones et al., 2005) and sequence conservation. Indeed, there is compelling evidence to suggest that members of this sub-family of non-conventional OR proteins do not themselves bind odorant ligands but rather form heterodimers with co-expressed ‘‘conventional’’ ORs (Neuhaus et al., 2005) and, moreover, these complexes are required for localization of ORs to dendrites (Larsson et al., 2004). As such,

it is not surprising to note the extreme conservation displayed by members of this sub-family of ORs despite the general divergence and species-specific gene expansions that are characteristic of the evolution of other insect Ors (Hill et al., 2002).

CqOr7 is expressed in the main chemosensory organs of the adult mosquito—antennae, maxillary palps, proboscis, legs, whilst expression in other parts of the adult body is largely undetectable. Furthermore, from a developmental standpoint, CqOr7 RNA is detectable in early larvae stages, which is in agreement with the expression pattern of its mosquito orthologs from *An.gambiae* and *A. aegypti* (Melo et al., 2004; Pitts et al., 2004). In *D. melanogaster*, DOr83b is one of 23 ORs found to be expressed in larval stages (Kreher et al., 2005; Vosshall et al., 1999). The expression of CqOr7 in the pre-adult stage suggests a chemosensory role of this gene during the early development of *C. quinquefasciatus*, whereby it is likely to play an important role in larval feeding and other behaviors.

A polyclonal antiserum directed against a highly conserved sequence of amino acid was used to localize CqOr7 protein in *C. quinquefasciatus*. While the peptide used to generate antisera is known to be unique to AgOR7 in *An.gambiae*, the absence of data on the *C. quinquefasciatus* proteome prevents similar exclusions. Nevertheless, the fact that AgOR7 peptide antiserum specifically labels olfactory sensilla and neuronal cell bodies in *C. quinquefasciatus* as well as *An.gambiae* (Pitts et al., 2004) and *A. aegypti*

(Melo et al., 2004) strongly supports its utility as a general marker for OR7 family members in mosquitoes. The specificity of the antisera is further supported by a lack of labeling in pre-immune control in *C. quinquefasciatus* and other mosquitoes. This antibody labels CqOR7 in three kinds of tissues—antennae, maxillary palps and proboscis, where signals are restricted to three types of sensilla, of which two have been described to function in the perception of a variety of odorants and carbon dioxide (Bowen, 1996; Grant and O'Connell, 1996), while the third has been implicated in contact chemosensation and mechanosensation (Pappas and Larsen, 1976). Interestingly, mosquito-grooved peg sensilla are specifically not labeled with CqOR7, AgOR7 or *AaOr*7, although grooved pegs of *An.gambiae* and *A. aegypti* have been shown to be sensitive to a variety of odors, some of which are known kairomones for host seeking (Meijerink et al., 2001). Indeed, the absence of OR7 proteins in olfactory responsive mosquito-grooved peg sensillum suggests the presence of another pathway for olfactory signal transduction that is independent of OR7 function. Similarly, while there are significant effects on olfactory signaling in *Drosophila* DOr83b mutants (Larsson et al., 2004) or with RNA interference-mediated silencing of DOr83b (Neuhaus et al., 2005), it is important to note that in *Drosophila* not all olfactory neurons co-express both conventional and non-conventional OR proteins (Clyne et al., 1999b; Vosshall et al., 1999).

CqOr7 mRNA and protein is also found in the proboscis, which is usually

viewed as a contact chemosensory appendage associated with gustation in mosquitoes (Pappas and Larsen, 1976). In *D. melanogaster* no ORs, including the widely expressed DOr83b, have been shown to be expressed in the fruitfly proboscis. While in *An.gambiae* and *A. aegypti*, AgOR7 and *AaOr*7 are both robustly expressed in proboscis (Melo et al., 2004; Pitts et al., 2004) as is the case forHvR2 fromH. virescens (Krieger et al., 2002). Based on the essential role that DOr83b play in the localization and function of co-expressed DOr proteins (Larsson et al., 2004; Neuhaus et al., 2005), the expression of Or83b orthologs in the proboscis across these mosquito species strongly suggests the presence of cryptic olfactory inputs derived from these gustatory organs. Such olfactory responses derived from an appendage that typically comes into extremely close approximation to human skin volatiles, may play a critical role in the penultimate steps of blood-feeding behaviors. Further study of this non-conventional receptor will facilitate our understanding of chemosensation in mosquitoes and, ultimately, may facilitate the development of novel anti-malarial programs that target olfactory-based behaviors of vector mosquitoes.

CHAPTER VI

DISCUSSION/CONCLUSION

Summary

In the course of this thesis work I have worked with members of the Zwiebel laboratory to characterize the molecular and cellular basis of olfactory-driven behavior in malaria mosquito *An.gambiae* larvae. I have also carried out pioneering work in *C. quinquefaciatus* that represents the first OR ever identified and characterized in this important WNV vector mosquito. This work is grounded in the maxim that inasmuch as olfaction plays an important role mediating host-seeking and other olfactory-related behaviors, a deeper understanding of the mosquito olfactory systems in both adult and pre-adult will aid in the development of novel strategies targeting mosquito control and ultimately, disease reduction.

In our behavioral studies, we tested 21 odorants in total. Of these odorants, several displayed strong attractant-like or repellent-like characters. Interestingly, most of these behaviorally active compounds correspond to aromatics. However, it is clear that these studies must be viewed as an initial effort in this regard. In order to develop this question further it will be necessary to expand our current test panel to include a considerably more diverse panel of biologically relevant as well as synthetic compounds that span multiple

chemical classes. While it will always be impossible to encompass the entire spectrum of potential chemical odorants, we would ideally be testing between 100 and 300 compounds in order to achieve a reasonable approximation of the "odor space" of *Anopheline* larvae.

Our molecular studies have revealed that eight of 12 larval *AgOrs* are also expressed in adults. The biological basis of this phenomenon remains unknown as are similar phenomena observed in *Drosophila* (Fishilevich et al., 2005; Kreher et al., 2005). A possible explanation might be both adult and larvae are able to respond to the same or similar odorants source. Indeed, electrophysiological studies have shown that female *An.gambiae* antennae can respond to odorant extracts isolated from larval habitats (Blackwell and Johnson, 2000), suggesting the presence of olfactory cues that can be recognized by both female adults and larvae. One hypothesis is that such compounds could correspond to potential oviposition pheromones that would direct gravid females to lay their eggs in habitats that are well suited for larval growth.

Indeed, the simplicity of larval olfactory system and sensitive behavior assays provide the opportunity to easily identify biological-active compounds which might play important roles in the breeding behavior of female *An. gambiae* mosquitoes. The behaviorally active compounds identified from our larval behavior assays will be examined with female adults behaviorally. These approaches may help to find potential oviposition-attractant that, once masked

or interfered, will reduce the breeding efficiency of the female mosquitoes, and eventually reduce mosquito population.

Our behavior studies have proved that *An.gambiae* larvae are capable of responding to a variety of odorants. In our assays, the larvae gathered around, migrated away from or remained non-responsive to specific odorant source. But the mechanism of this behavioral response remains unknown. In *Drosophila* numerous reports have indicated fly larvae display chemotaxis behavior towards odorant stimuli (Fishilevich et al., 2005; Louis et al., 2008), during which the larvae underwent oriented movement towards odorant source. In mosquitoes, *Aedes aegypt*i larvae migrated away from strong light source. During their movement, they made numerous turns without specific orientation, a phenomenon often defined as kinesis (Muir et al., 1992). Using a small petri-dish, we were able to track the movement of individual larva exposed to a concentration gradient of specific odorant. Our preliminary results suggest the olfactory behavior pattern presented by *An. gambiae* larvae can be defined as kinesis. When exposed to strong attractant, the larva will slow down once entering the odorant gradient, a phenomenon called orthokinesis. The larva displayed a different behavior towards DEET, the most successful and widely marketed insect repellent worldwide. Here *An. gambiae* larvae moved faster and turned frequently, trying to stay as far from the odorant source as possible.

DEET, was recently reported to function by blocking OR/odorant responses by targeting OR83b/Or7 family members within olfactory pathways

(Ditzen et al., 2008). Interestingly, in the larval behavioral assays developed here, *An.gambiae* larvae were apparently directly repelled by DEET alone, a phenomenon not observed in *Drosophila* larvae (Fishilevich et al., 2005) and which is inconsistent with a role as odorant /OR blocker. In contrast, our data suggest that instead of acting to block odorant activation of insect ORs, DEET acts as a direct excito-repellent. Consistent with this hypothesis, in our studies, of the 12 conventional *AgOrs* expressed in *An.gambiae* larvae, AgOr40 responds to DEET across a wide range of concentrations when expressed in *Xenopus oocytes*. Moreover, the ablation of larval antennae completely removed the responses towards DEET, further indicating DEET directly targets specific larval ORs that are expressed on the larval antennae, especially AgOR40.

To examine this hypothesis more fully, it will be necessary to use stable germline transformation with *RNAi* construct or directly inject dsRNA to eliminate AgOr40 transcripts. The larvae will be tested against DEET to determine the knockdown effects. As an alternative, heterologous expression of AgOR40 in *Drosophila* larvae, which are, as mentioned above, lack of response towards DEET, may also help to determine whether AgOR40 is a DEET receptor. The AgOr40 cDNA will be cloned into p-UAS vector for stable transformation and *Drosophila* larvae-specific promoters will be used to drive AgOR40 expression in specific *Drosophila* larval ORN. Using the single larval

Chemicals	Diffusion Coefficient (cm2/sec) in water	Diffusion Coefficient (cm2/sec) in air
Acetophenone	8.66 e-6	0.070
2-methylphenol	9.17 e-6	0.077
DEET	6.24 e-6	0.0558
Indole	9.13 e-6	n/a
1-octen-3-ol	7.32 e-6	0.0659

Table 2. Comparison of the Diffusion Coefficient of several chemical compounds in water and air.
Estimated from EPA On-line Tools for Site Assessment Calculation

behavior assay (Fishilevich et al., 2005), we will be able to determine the response profile to DEET in our AgOR40-expressing *Drosophila* larvae (Fishilevich et al., 2005). This study will server to critically expand our knowledge towards understanding the mechanisms of insect repellent.

An. gambiae **Larvae Manifest a Highly Sensitive Olfactory System**

Our behavior studies have confirmed that *An. gambiae* larvae are able to respond to a variety of odorant sources across a wide range of concentrations. The next question is how sensitive is this larval olfactory system. As mentioned, the olfactory signal transduction relies on the delivery of the odorant molecule to the ORs expressed on the membrane of the ORNs. The majority of chemical odorants isolated from the larval habitats are hydrophobic compounds, which spread slowly in the water. While the lack of a precise understanding of the fluid dynamics of odorant distribution makes it difficult to determine the actual concentration of an odorant at a specific position and point in time, an appreciation of the odorant diffusion coefficients in both water and air provides useful information insofar as evaluating the sensitivity of our behavioral assays.

As shown in the Table 2, a 10,000 fold difference exists between the diffusion coefficients of several behaviorally-active compounds in our behavior assays in water as compared to air. Using 1-octen-3-ol as an example, it should be noted that to elicit strong behavioral responses from terrestrial

Drosophila larvae, a source of 100% 1-octen-3-ol has to be used in behavioral assays (Fishilevich et al., 2005) In *An. gambiae* where larvae are aquatic, similar behavioral responses were observed using as little as a 10^{-4} dilution of 1-octen-3-ol as a source dilution. Therefore if we also take into consideration the 10,000 fold difference in the diffusion coefficient, *An. gambiae* larvae manifest a significantly stronger olfactory response as compared to *Drosophila melanogaster*. This may reflect the requirement for a much more sensitive olfactory system reflective of biology of aquatic mosquito larvae that typically develop in nutrient-poor environments as compared to the terrestrial-based *Drosophila* larvae which develop inside rotting fruit and accordingly have constant access to a virtually unlimited amount of food,

The Bridge Between OR Response Profiles and Larval Behavior

Little is known about the molecular and cellular mechanisms underlying insect attraction and repulsion. Generally, based on whether the responses are directionally related or unrelated to external stimuli, the behavior can be defined as taxis or kinesis respectively. Taxis are movements of the animals that are oriented directly towards or directly away from stimuli. Responses towards chemical stimuli are normally called chemotaxis. On the other hand, kinesis are un-oriented movements made in response to particular stimuli, strength of response being related to intensity of stimulation. There are two types of kinesis. Orthokinesis is a change in linear speed or in frequency of

locomotion; Klinokinesis is a change in the rate or frequency of turning. Numerous studies have described that *Drosophila* larvae manifest chemotaxis behavior towards some chemical stimuli (Fishilevich et al., 2005; Kreher et al., 2005; Louis et al., 2008). In *Culex* mosquitoes, food particles seem to elicit orthokinesis responses from the larvae. Once the larvae entered the area containing food resources, their movements were slowed down (John T. Barber, 1983). In *An. gambiae*, the nature of larval response towards the chemicals is largely unknown.

The problem is of interest partly because of its importance in understanding the principles of odor perception and partly because of its practical implications. Mosquitoes act as nuisances, and far more importantly, transmit diseases such as malaria to hundreds of millions of people each year. Compounds that could repel/attract mosquitoes are therefore in great demand. A novel attractant would potentially augment a good mosquito trap that could reduce adult populations and while new repellents might serve direct mosquitoes away from people and thereby reduce the incidence of human blood meal. Efforts to identify new attractants/repellents that are effective, safe, and cost efficient however, have been severely hampered by the inefficiency of the available behavioral screening methods. Current methods often require the rearing of large number of mosquitoes or entail difficult field studies; such behavioral studies are often complicated by variables that are difficult to control. A correlation between the identification of insect attractant/repellents

and a rapid physiological screen of receptor activity could thus be of great value in the control of mosquitoes and even insect pests.

In our studies, the link between peripheral olfactory sensitivity and larval behavioral output is obvious although not straightforward in some cases. Odorant stimuli such as acetophenone and 2, 3 or 4-methylphenol, which could stimulate multiple AgORs, also evoke robust larval behaviors. Similarly, we never observe a behaviorally active stimulus that fails to activate at least one functionally characterized larval AgOR. This lays a foundation for the identification of future behaviorally active compounds. Those chemicals that can activate multiple AgORs may ultimately have a greater chance to elicit behavior responses from the animals. Such chemicals could therefore be prioritized for the much more labor intensive behavioral tests resulting in a much higher level of throughput in the search for novel repellents and attractants.

Larval ORs in *Ae. aegypti*

An RT-PCR screen with *Ae. aegypti* larval antennal cDNA identified 23 Ors (Bohbot et al., 2007). Interestingly, 15 of these ORs were not detected in the survey conducted using cDNA from adult olfactory appendages, indicating their expression to be larval-specific. This is a big increase in number of larval-specific Ors in *Ae. aegypti* compared to that of *An.gambiae* larvae. A bigger population of ORs would potentially increase the odor-coding capacity

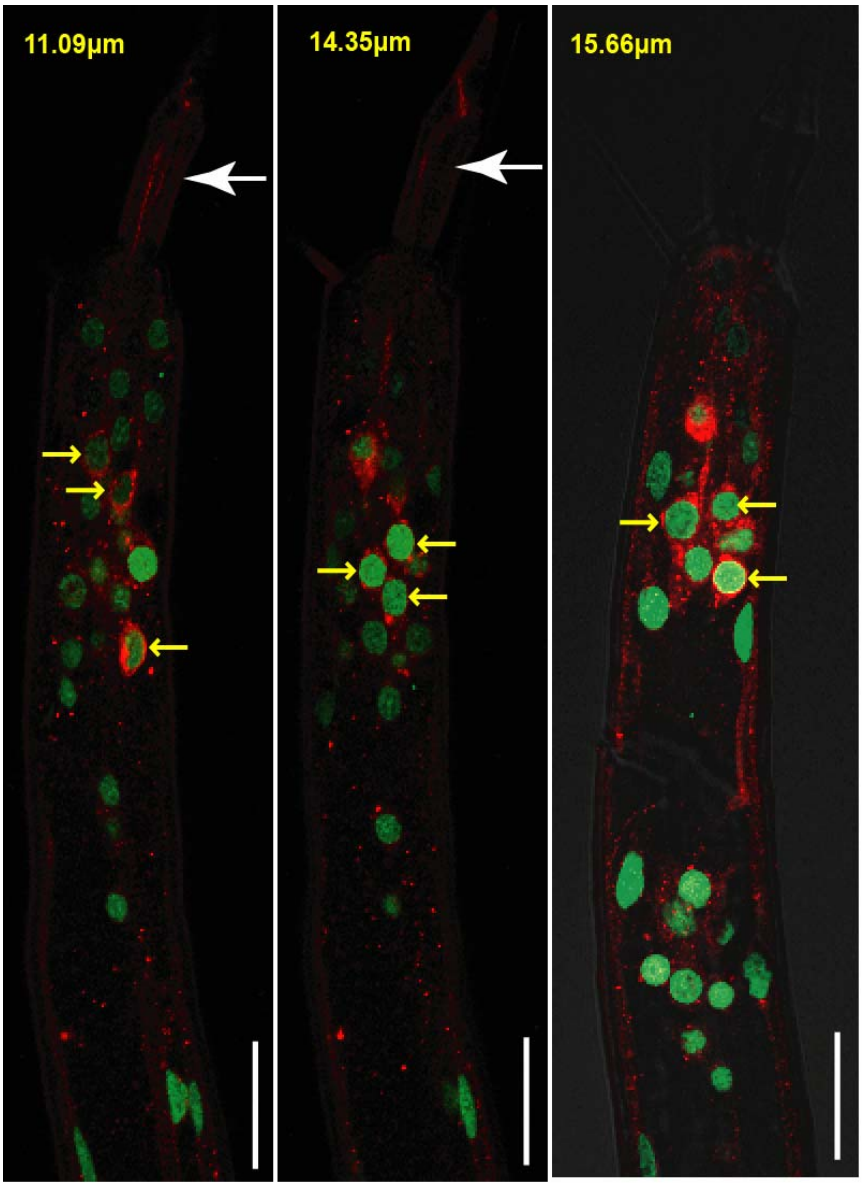


Figure 19. Whole-mount staining of *Ae. aegypti* larval antennae with AgOR7 antibody. Representative confocal-images from a z-stack series were shown, with their relative position indicated by the distance (µm) towards the first image of the z-stack. Multiple distinct neurons were labeled with both *AaOr*7 (red) and nuclei marker Yoyo1 (green), indicated by yellow arrow. *AaOr*7 (red) labeling was also observed in the sensory cone located at the tip of the *Ae. aegypti* larval antenna, indicated by white arrow. Scale bar equals 25µm.

of the organism, for example, the number of insect ORs ranging from 60 to 150 (Vosshall, 2001), compared to more than 1000 in dog and human being (Mombaerts, 1999). A whole mount antibody staining study using AgOR7antibody has identified 21 *AaOr7*$^{+}$ neurons (Figure 19), a number that almost doubles that of *An.gambiae* larvae. The numerical similarity between the number of OR and that of the OSN may indicate that each ORN only expresses one individual OR. Whether this increase in the OR number suggests a more robust olfactory system remains unknown, due to the lack of knowledge in the odorant-induced behavior in *Ae. aegypti* larvae. We will expand our behavior study to *Ae. aegypti*, as our preliminary data suggests the olfactory behavior paradigm works well with *Ae. aegypti* larvae. The ORs identified in RT-PCR studies will be examined for their spatial expression using sensitive fluorescence *in situ* hybridizations. The odor response spectra of these ORs will be determined by *Xenopus* oocytes recordings.

Designing Novel Larval Control Strategies

An. gambiae larvae inhabit small water bodies that are often numerous, scattered, sunlit, turbid, temporary, and close to human dwellings. Thus effective larval control strategy must promote the involvement of local community members, the people who are responsible for the creating, maintaining, or sometimes even using larval habitats. Detection of larval habitats relies mainly on visual inspection of water bodies or sampling the

water with pipette to determine presence or absence of larvae (Killeen et al., 2002a). These activities require a reasonable amount of field experience that is typically absent in the majority of disease endemic community members. .

Obtaining a better understanding of the larval olfactory systems will greatly facilitate the development of new larval-control strategies. *Xenopus oocytes* recordings provide insight as to candidate odorants that may elicit behavioral responses from larvae that can be tested in the sensitive behavior assays developed during the course of my thesis work. Strong attractants and repellants may ultimately prove to be very valuable. For example most insecticides have to be ingested (or otherwise contacted) at a sufficient level to reach particular lethal concentrations in order to be fully effective. In actual field-work, this could translate into high amounts of materials used as well as the frequency of their reapplication. In our studies, 2-methylphenol was shown to strongly attract *Anopheline* larva at very low concentrations (10^{-5} dilutions) for a long period of time (at least 2 hrs in our assays). If certain strong attractants such as these cresols can be used as baits, it could help to increase the larval density proximate to insecticides and thereby greatly enhance their effectiveness. This would also serve to reduce the potential environmental hazards and save considerable costs related to the acquiring and applying insecticide. Moreover potential attractants can also be used to more effectively examine whether specific water bodies have been infected by *An. gambiae* larvae. Indeed, vector surveillance has traditionally relied on

larval sampling protocols (Killeen et al., 2002b) and improvements obtained through this method would increase the accuracy level of the current visual or experimental methods for screening larva habitats.

Candidate repellents also offer the promise of practical utility even though the biological mechanism behind the repellency remains unknown. One possible reason would be the repulsive chemicals may act as a warning cue used by larva to signal the presence of danger. Or they may be toxic to larva at a physiological level. Revealing the fundamental principles underlying this phenomenon will definitely help develop cheaper and more effective insecticide to reduce mosquito larvae population or reduce their survival rate by creating a hostile environment for their development.

REFERENCES

Abdel-Latief, M. (2007). A family of chemoreceptors in Tribolium castaneum (Tenebrionidae: Coleoptera). PLoS ONE *2*, e1319.

Ache, B. W., and Young, J. M. (2005). Olfaction: diverse species, conserved principles. Neuron *48*, 417-430.

Ache, B. W., and Zhainazarov, A. (1995). Dual second-messenger pathways in olfactory transduction. Curr Opin Neurobiol *5*, 461-466.

Adams, M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G., Scherer, S. E., Li, P. W., Hoskins, R. A., Galle, R. F*., et al.* (2000). The genome sequence of Drosophila melanogaster. Science *287*, 2185-2195.

Aly, C. (1985). Feeding rate of larval Aedes vexans stimulated by food substances. J Am Mosq Control Assoc *1*, 506-510.

Barber, M. (1940). The present status [summer 1939]of *Anopheles gambiae* in Brazil. Am J Trop Med *20*, 249-267.

Barrett, A. D., and Higgs, S. (2007). Yellow fever: a disease that has yet to be conquered. Annu Rev Entomol *52*, 209-229.

Benton, R., Sachse, S., Michnick, S. W., and Vosshall, L. B. (2006). Atypical membrane topology and heteromeric function of Drosophila odorant receptors in vivo. PLoS Biol *4*, e20.

Blackwell, A., and Johnson, S. N. (2000). Electrophysiological investigation of larval water and potential oviposition chemo-attractants for Anopheles gambiae s.s. Ann Trop Med Parasitol *94*, 389-398.

Boete, C., and Koella, J. C. (2002). A theoretical approach to predicting the success of genetic manipulation of malaria mosquitoes in malaria control. Malar J *1*, 3.

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

Bowen (1996). Sensory aspects of host location in mosquitoes, In Olfaction in

mosquito-host interactions, G. R. Bock, and G. Cardew, eds. (New York: John Wiley & Sons), pp. 197-211.

Braks, M. A., and Takken, W. (1997). Olfactometer studies on the attraction of *Anopheles gambiae* sensu stricto (Diptera: Culicidae) to human sweat. Proceedings of Experimental and Applied Entomology *8*, 99-104.

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

Brunet, L. J., Gold, G. H., and Ngai, J. (1996). General anosmia caused by a targeted disruption of the mouse olfactory cyclic nucleotide-gated cation channel. Neuron *17*, 681-693.

Caldas de Castro, M., Yamagata, Y., Mtasiwa, D., Tanner, M., Utzinger, J., Keiser, J., and Singer, B. H. (2004). Integrated urban malaria control: a case study in dar es salaam, Tanzania. Am J Trop Med Hyg *71*, 103-117.

Clements, A., N (1993). The Biology of Mosquitoes, Volume 2: Sensory Reception and Behavior (New York: CABI publishing).

Clyne, P. J., Certel, S. J., de Bruyne, M., Zaslavsky, L., Johnson, W. A., and Carlson, J. R. (1999a). The odor specificities of a subset of olfactory receptor neurons are governed by Acj6, a POU-domain transcription factor. Neuron *22*, 339-347.

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

Collins, F. H., and Paskewitz, S. M. (1995). Malaria: current and future prospects for control. Annu Rev Entomol *40*, 195-219.

Coluzzi, M., Di Deco, M., and Petrarca, V. (1975). [Propensity for feeding on blood under laboratory conditions and chromosomal polymorphism in Anopheles stephensi]. Parassitologia *17*, 137-143.

Cooperband, M. F., McElfresh, J. S., Millar, J. G., and Carde, R. T. (2008). Attraction of female Culex quinquefasciatus Say (Diptera: Culicidae) to odors from chicken feces. J Insect Physiol *54*, 1184-1192.

Coosemans, M., and Carnevale, P. (1995). Malaria vector control: a critical review on chemical methods and insecticides. Ann Soc Belg Med Trop *75*,

13-31.

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

Costantini, C., Birkett, M. A., Gibson, G., Ziesmann, J., Sagnon, N. F., Mohammed, H. A., Coluzzi, M., and Pickett, J. A. (2001). Electroantennogram and behavioural responses of the malaria vector Anopheles gambiae to human-specific sweat components. Med Vet Entomol *15*, 259-266.

Costantini, C., Gibson, G., Brady, J., Merzagora, L., and Coluzzi, M. (1993). A new odour-baited trap to collect host-seeking mosquitoes. Parassitologia *35*, 5-9.

Couto, A., Alenius, M., and Dickson, B. J. (2005). Molecular, anatomical, and functional organization of the Drosophila olfactory system. Curr Biol *15*, 1535-1547.

Curtis, C. F. (1998). Insecticide treated mosquito nets. Trop Doct *28*, 63.

Curtis, C. F., Lines, J. D., Ijumba, J., Callaghan, A., Hill, N., and Karimzad, M. A. (1987). The relative efficacy of repellents against mosquito vectors of disease. Med Vet Entomol *1*, 109-119.

Curtis, C. F., Myamba, J., and Wilkes, T. J. (1996). Comparison of different insecticides and fabrics for anti-mosquito bednets and curtains. Med Vet Entomol *10*, 1-11.

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

Dekker, T., Steib, B., Carde, R. T., and Geier, M. (2002). L-lactic acid: a human-signifying host cue for the anthropophilic mosquito Anopheles gambiae. Med Vet Entomol *16*, 91-98.

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

Dhallan, R. S., Yau, K. W., Schrader, K. A., and Reed, R. R. (1990). Primary structure and functional expression of a cyclic nucleotide-activated channel from olfactory neurons. Nature *347*, 184-187.

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

Dubin, A. E., Liles, M. M., and Harris, G. L. (1998). The K+ channel gene ether a go-go is required for the transduction of a subset of odorants in adult Drosophila melanogaster. J Neurosci *18*, 5603-5613.

Enserink, M. (2002). Malaria. Ecologists see flaws in transgenic mosquito. Science *297*, 30-31.

Fernald, G., and Burger, J. (1980). Effect of temperature and light on aggregation behavior of Aedes stimulans larvae (Piptera: Culicidae) in a woodland pool. *J New York Entomol Soc 88*.

Fillinger, U., Sonye, G., Killeen, G. F., Knols, B. G., and Becker, N. (2004). The practical importance of permanent and semipermanent habitats for controlling aquatic stages of Anopheles gambiae sensu lato mosquitoes: operational observations from a rural town in western Kenya. Trop Med Int Health *9*, 1274-1289.

Fishilevich, E., Domingos, A. I., Asahina, K., Naef, F., Vosshall, L. B., and Louis, M. (2005). Chemotaxis behavior mediated by single larval olfactory neurons in Drosophila. Curr Biol *15*, 2086-2096.

Fox, A. N., Pitts, R. J., Robertson, H. M., Carlson, J. R., and Zwiebel, L. J. (2001). Candidate odorant receptors from the malaria vector mosquito Anopheles gambiae and evidence of down-regulation in response to blood feeding. Proc Natl Acad Sci U S A *98*, 14693-14697.

Fox, A. N., Pitts, R. J., and Zwiebel, L. J. (2002). A Cluster of Candidate Odorant Receptors from the Malaria Vector Mosquito, Anopheles gambiae. Chem Senses *27*, 453-459.

Gaillard, I., Rouquier, S., and Giorgi, D. (2004). Olfactory receptors. Cell Mol Life Sci *61*, 456-469.

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

Garrett-Jones, C., and Grab, B. (1964). The Assessment of Insecticidal Impact on the Malaria Mosquito's Vectorial Capacity, from Data on the Proportion of Parous Females. Bull World Health Organ *31*, 71-86.

Gendre, N., Luer, K., Friche, S., Grillenzoni, N., Ramaekers, A., Technau, G. M., and Stocker, R. F. (2004). Integration of complex larval chemosensory organs into the adult nervous system of Drosophila. Development *131*, 83-92.

Ghaninia, M., Hansson, B. S., and Ignell, R. (2007). The antennal lobe of the African malaria mosquito, *Anopheles gambiae* – innervation and three-dimensional reconstruction. Arthropod Struct Dev *36*, 23-39.

Gomez-Diaz, C., Martin, F., and Alcorta, E. (2004). The cAMP transduction cascade mediates olfactory reception in Drosophila melanogaster. Behav Genet *34*, 395-406.

Goodman, C. A., Mnzava, A. E., Dlamini, S. S., Sharp, B. L., Mthembu, D. J., and Gumede, J. K. (2001). Comparison of the cost and cost-effectiveness of insecticide-treated bednets and residual house-spraying in KwaZulu-Natal, South Africa. Trop Med Int Health *6*, 280-295.

Gopaul, R. (1995). [Entomological surveillance in Mauritius]. Sante *5*, 401-405.
Grant, A., and O'Connell, R., eds. (1996). Electrophysiological responses from receptor neurons in mosquito maxillary palp sensilla (Wiley, Chichester).

Gratz, N. G. (1999). Emerging and resurging vector-borne diseases. Annu Rev Entomol *44*, 51-75.

Greenwood, B., and Mutabingwa, T. (2002). Malaria in 2002. Nature *415*, 670-672.

Gubler, D. J. (1987). Dengue and dengue hemorrhagic fever in the Americas. P R Health Sci J *6*, 107-111.

Hague, C., Uberti, M. A., Chen, Z., Bush, C. F., Jones, S. V., Ressler, K. J., Hall, R. A., and Minneman, K. P. (2004). Olfactory receptor surface expression is driven by association with the beta2-adrenergic receptor. Proc Natl Acad Sci U S A *101*, 13672-13676.

Hall, D. R., Beevor, P. S., Cork, A., Nesbitt, B. F., and Vale, G. A. (1984). 1-octen-3-ol, a potent olfactory stimulant and attractant for tsetse isolated from cattle odours. Insect Sci Appl *5*, 335-339.

Hallem, E., Fox, A. N., Zwiebel, L. J., and Carlson, J. R. (2004a). A mosquito odorant receptor tuned to a component of human sweat. Nature *427*, 212-213.
Hallem, E., Ho, M. G., and Carlson, J. R. (2004b). The Molecular Basis of Odor Coding in the Drosophila Antenna. Cell *117*, 965-979.

Haridi, A. M. (1970). DDT resistance in the Anopheles gambiae complex. Trans R Soc Trop Med Hyg *64*, 27.

Hatt, H., and Ache, B. W. (1994). Cyclic nucleotide- and inositol phosphate-gated ion channels in lobster olfactory receptor neurons. Proc Natl Acad Sci U S A *91*, 6264-6268.

Hayes, E. B., and Gubler, D. J. (2006). West Nile virus: epidemiology and clinical features of an emerging epidemic in the United States. Annu Rev Med *57*, 181-194.

Hemingway, J., Coleman, M., Paton, M., McCarroll, L., Vaughan, A., and Desilva, D. (2000). Aldehyde oxidase is coamplified with the World's most common Culex mosquito insecticide resistance-associated esterases. Insect Mol Biol *9*, 93-99.

Hemingway, J., and Karunaratne, S. H. (1998). Mosquito carboxylesterases: a review of the molecular biology and biochemistry of a major insecticide resistance mechanism. Med Vet Entomol *12*, 1-12.

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

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

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

Homberg, U., and Hildebrand, J. G. (1994). Postembryonic development of gamma-aminobutyric acid-like immunoreactivity in the brain of the sphinx moth Manduca sexta. J Comp Neurol *339*, 132-149.

Ito, J., Ghosh, A., Moreira, L. A., Wimmer, E. A., and Jacobs-Lorena, M. (2002). Transgenic *Anopheline* mosquitoes impaired in transmission of a malaria parasite. Nature *417*, 452-455.

Jan, L., and Jan, Y. (1982). Antibodies to horseradish peroxidase as specific neuronal markers in Drosophila and in grasshopper embryos. Proceedings of the National Academy of Sciences USA *79*, 2700-2704.

Jefferis, G. S., Vyas, R. M., Berdnik, D., Ramaekers, A., Stocker, R. F., Tanaka, N. K., Ito, K., and Luo, L. (2004). Developmental origin of wiring specificity in the olfactory system of Drosophila. Development *131*, 117-130.

Jeha, L. E., Sila, C. A., Lederman, R. J., Prayson, R. A., Isada, C. M., and Gordon, S. M. (2003). West Nile virus infection: a new acute paralytic illness. Neurology *61*, 55-59.

Jhaveri, D., Sen, A., Reddy, G. V., and Rodrigues, V. (2000). Sense organ identity in the Drosophila antenna is specified by the expression of the proneural gene atonal. Mech Dev *99*, 101-111.

John T. Barber, E. G. E., Burnton C. plaster (1983). Chemotaxis of Culex pipiens quinquefasciatus larvae (diptera: Culicidae) in response to amino acids. JMed Entomol *20*, 641-643.

Jones, D. T., and Reed, R. R. (1989). Golf: An Olfactory Neuron Specific-G Protein Involved in Odorant Signal Transduction. Science *244*, 790-795.

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

Jones, W. D., Nguyen, T. A., Kloss, B., Lee, K. J., and Vosshall, L. B. (2005). Functional conservation of an insect odorant receptor gene across 250 million years of evolution. Curr Biol *15*, R119-121.

Kalidas, S., and Smith, D. P. (2002). Novel genomic cDNA hybrids produce effective RNA interference in adult Drosophila. Neuron *33*, 177-184.

Kasap, M. (1980). Response of mosquitoes to mechanical stimuli. 1. Response of the larvae and pupae of Aedes aegypti, Anopheles stephensi and Culex pipiens (Dipetera: Culicidae) to surface disturbance. Commun Fac Sci Univ Ankara, ser C, Zool *24*, 43-55.

Kasap, M. (1981). Response of mosquitoes to mechanical stimuli. II. An observation on the response of the larvae and pupae of Aedes aegypti, Anopheles stephensi and Culex pipiens (Dipetera: Culicidae) to taps at the edge of an experimental dish. Commun Fac Sci Univ Ankara, ser C, Zool *25*, 25-35.

Kay, B. H., Fanning, I. D., and Carley, J. G. (1984). The vector competence of Australian Culex annulirostris with Murray Valley encephalitis and Kunjin

viruses. Aust J Exp Biol Med Sci *62 (Pt 5)*, 641-650.

Keiser, J., Singer, B. H., and Utzinger, J. (2005). Reducing the burden of malaria in different eco-epidemiological settings with environmental management: a systematic review. Lancet Infect Dis *5*, 695-708.

Killeen, G. F., Fillinger, U., Kiche, I., Gouagna, L. C., and Knols, B. G. (2002a). Eradication of Anopheles gambiae from Brazil: lessons for malaria control in Africa? Lancet Infect Dis *2*, 618-627.

Killeen, G. F., Fillinger, U., and Knols, B. G. (2002b). Advantages of larval control for African malaria vectors: Low mobility and behavioural responsiveness of immature mosquito stages allow high effective coverage. Malar J *1*, 8.

Killeen, G. F., Knols, B. G., and Gu, W. (2003). Taking malaria transmission out of the bottle: implications of mosquito dispersal for vector-control interventions. Lancet Infect Dis *3*, 297-303.

Killeen, G. F., McKenzie, F. E., Foy, B. D., Schieffelin, C., Billingsley, P. F., and Beier, J. C. (2000). The potential impact of integrated malaria transmission control on entomologic inoculation rate in highly endemic areas. Am J Trop Med Hyg *62*, 545-551.

Kim, J., Moriyama, E. N., Warr, C. G., Clyne, P. J., and Carlson, J. R. (2000). Identification of novel multi-transmembrane proteins from genomic databases using quasi-periodic structural properties. Bioinformatics *16*, 767-775.

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

Knols, B. G., de Jong, R., and Takken, W. (1994). Trapping system for testing olfactory responses of the malaria mosquito Anopheles gambiae in a wind tunnel. Med Vet Entomol *8*, 386-388.

Kramer, L. D., Li, J., and Shi, P. Y. (2007). West Nile virus. Lancet Neurol *6*, 171-181.

Kreher, S. A., Kwon, J. Y., and Carlson, J. R. (2005). The molecular basis of odor coding in the Drosophila larva. Neuron *46*, 445-456.

Kreher, S. A., Mathew, D., Kim, J., and Carlson, J. R. (2008). Translation of sensory input into behavioral output via an olfactory system. Neuron *59*,

110-124.

Krieger, J., Grosse-Wilde, E., Gohl, T., Dewer, Y. M., Raming, K., and Breer, H. (2004). Genes encoding candidate pheromone receptors in a moth (Heliothis virescens). Proc Natl Acad Sci U S A *101*, 11845-11850.

Krieger, J., Klink, O., Mohl, C., Raming, K., and Breer, H. (2003). A candidate olfactory receptor subtype highly conserved across different insect orders. J Comp Physiol A Neuroethol Sens Neural Behav Physiol *189*, 519-526.

Krieger, J., Raming, K., Dewer, Y. M., Bette, S., Conzelmann, S., and Breer, H. (2002). A divergent gene family encoding candidate olfactory receptors of the moth Heliothis virescens. Eur J Neurosci *16*, 619-628.

Krzywinski, J., and Besansky, N. J. (2003). Molecular systematics of Anopheles: from subgenera to subpopulations. Annu Rev Entomol *48*, 111-139.

Krzywinski, J., Wilkerson, R. C., and Besansky, N. J. (2001). Toward understanding Anophelinae (Diptera, Culicidae) phylogeny: insights from nuclear single-copy genes and the weight of evidence. Syst Biol *50*, 540-556.
Kumar, A., Sharma, V. P., Sumodan, P. K., Thavaselvam, D., and Kamat, R. H. (1994). Malaria control utilizing Bacillus sphaericus against Anopheles stephensi in Panaji, Goa. J Am Mosq Control Assoc *10*, 534-539.

Kurtovic, A., Widmer, A., and Dickson, B. J. (2007). A single class of olfactory neurons mediates behavioural responses to a Drosophila sex pheromone. Nature *446*, 542-546.

Kwon, H. W., Lu, T., Rutzler, M., and Zwiebel, L. J. (2006). Olfactory responses in a gustatory organ of the malaria vector mosquito Anopheles gambiae. Proc Natl Acad Sci U S A *103*, 13526-13531.

Kwon, J. Y., Dahanukar, A., Weiss, L. A., and Carlson, J. R. (2007). The molecular basis of CO2 reception in Drosophila. Proc Natl Acad Sci U S A *104*, 3574-3578.

Labbe, P., Berticat, C., Berthomieu, A., Unal, S., Bernard, C., Weill, M., and Lenormand, T. (2007). Forty years of erratic insecticide resistance evolution in the mosquito Culex pipiens. PLoS Genet *3*, e205.

Larsson, M. C., Domingos, A. I., Jones, W. D., Chiappe, M. E., Amrein, H., and Vosshall, L. B. (2004). Or83b encodes a broadly expressed odorant receptor essential for Drosophila olfaction. Neuron *43*, 703-714.

Lengeler, C. (2004). Insecticide-treated bed nets and curtains for preventing malaria. Cochrane Database Syst Rev, CD000363.

Levine, R. B., Morton, D. B., and Restifo, L. L. (1995). Remodeling of the insect nervous system. Curr Opin Neurobiol *5*, 28-35.

Louis, M., Huber, T., Benton, R., Sakmar, T. P., and Vosshall, L. B. (2008). Bilateral olfactory sensory input enhances chemotaxis behavior. Nat Neurosci *11*, 187-199.

Lu, T., Qiu, Y. T., Wang, G., Kwon, J. Y., Rutzler, M., Kwon, H. W., Pitts, R. J., van Loon, J. J., Takken, W., Carlson, J. R., and Zwiebel, L. J. (2007a). Odor coding in the maxillary palp of the malaria vector mosquito Anopheles gambiae. Curr Biol *17*, 1533-1544.

Lu, T., Qiu, Y. T., Wang, G., Kwon, J. Y., Rutzler, M., Kwon, H. W., Pitts, R. J., Van Loon, J. J., Takken, W., Carlson, J. R., and Zwiebel, L. J. (2007b). Odor Coding in the Maxillary Palp of the Malaria Vector Mosquito Anopheles gambiae. Curr Biol *17*, 1-12.

Macdonald, G. (1957). The Epidemiology and Control of Malaria (London: Oxford University Press).

Mackenzie, J. S., Gubler, D. J., and Petersen, L. R. (2004). Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. Nat Med *10*, S98-109.

Majori, G., Sabatinelli, G., Villani, F., and Petrarca, V. (1986). Studies on insecticide susceptibility of Anopheles gambiae s.1. and Culex quinquefasciatus in the area of Ouagadougou, Burkina Faso (west Africa). J Am Mosq Control Assoc *2*, 305-309.

Malnic, B., Hirono, J., Sato, T., and Buck, L. B. (1999). Combinatorial receptor codes for odors. Cell *96*, 713-723.

Malpel, S., Klarsfeld, A., and Rouyer, F. (2002). Larval optic nerve and adult extra-retinal photoreceptors sequentially associate with clock neurons during Drosophila brain development. Development *129*, 1443-1453.

Marin, E. C., Watts, R. J., Tanaka, N. K., Ito, K., and Luo, L. (2005). Developmentally programmed remodeling of the Drosophila olfactory circuit. Development *132*, 725-737.

Marsh, K., and Snow, R. W. (1999). Malaria transmission and morbidity.

Parassitologia *41*, 241-246.

Mboera, L. E., Takken, W., and Sambu, E. Z. (2000). The response of Culex quinquefasciatus (Diptera: culicidae) to traps baited with carbon dioxide, 1-octen-3-ol, acetone, butyric acid and human foot odour in Tanzania. Bull Entomol Res *90*, 155-159.

Mboera, L. E. G., and Takken, W. (1997). Carbon dioxide chemotropism in mosquitoes (Diptera: Culicidae) and its potential in vector surveillance and management programmes. Rev Med Vet Entomol *85*, 355-368.

McCarroll, L., Paton, M. G., Karunaratne, S. H., Jayasuryia, H. T., Kalpage, K. S., and Hemingway, J. (2000). Insecticides and mosquito-borne disease. Nature *407*, 961-962.

McIver, S. B. (1981). A model for the mechanism of action of the repellent DEET on Aedes aegypti (Diptera: Culicidae). J Med Entomol *18*, 357-361.

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

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

Merrill, C. E., Pitts, R. J., and Zwiebel, L. J. (2003). Molecular characterization of arrestin family members in the malaria vector mosquito, Anopheles gambiae. Insect Molecular Biology *12*, 641-650.

Merritt, R. W., Dadd, R. H., and Walker, E. D. (1992). Feeding behavior, natural food, and nutritional relationships of larval mosquitoes. Annu Rev Entomol *37*, 349-376.

Mombaerts, P. (1999). Molecular biology of odorant receptors in vertebrates. Annu Rev Neurosci *22*, 487-509.

Mombaerts, P., Wang, F., Dulac, C., Chao, S. K., Nemes, A., Mendelsohn, M., Edmondson, J., and Axel, R. (1996). Visualizing an olfactory sensory map. Cell *87*, 675-686.

Monath, T. P. (2005). Yellow fever vaccine. Expert Rev Vaccines *4*, 553-574.

Muir, L. E., Kay, B. H., and Thorne, M. J. (1992). Aedes aegypti (Diptera: Culicidae) vision: response to stimuli from the optical environment. J Med Entomol *29*, 445-450.

Nabarro, D. N., and Tayler, E. M. (1998). The "roll back malaria" campaign. Science *280*, 2067-2068.

Nakagawa, T., Sakurai, T., Nishioka, T., and Touhara, K. (2005). Insect Sex-Pheromone Signals Mediated by Specific Combinations of Olfactory Receptors. Science.

Nakamura, T., and Gold, G. H. (1987). A cyclic nucleotide-gated conductance in olfactory receptor cilia. Nature *325*, 442-444.

Nash, D., Mostashari, F., Fine, A., Miller, J., O'Leary, D., Murray, K., Huang, A., Rosenberg, A., Greenberg, A., Sherman, M.*, et al.* (2001). The outbreak of West Nile virus infection in the New York City area in 1999. N Engl J Med *344*, 1807-1814.

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

Nicolaides, N. (1974). Skin lipids: their biochemical uniqueness. Science *186*, 19-26.

Niebylski, M. L., and Meek, C. L. (1992). Blood-feeding of Culex mosquitoes in an urban environment. J Am Mosq Control Assoc *8*, 173-177.

Niimura, Y., and Nei, M. (2003). Evolution of olfactory receptor genes in the human genome. Proc Natl Acad Sci U S A *100*, 12235-12240.

Osimitz, T. G., and Grothaus, R. H. (1995). The present safety assessment of deet. J Am Mosq Control Assoc *11*, 274-278.

Pace, U., Hanski, E., Salomon, Y., and Lancet, D. (1985). Odorant-sensitive adenylate cyclase may mediate olfactory reception. Nature *316*, 255-258.

Pappas, L. G., and Larsen, J. R. (1976). Gustatory Hairs on the Mosquito, *Culiseta inornata*. Journal of Experimantal Zoology *196*, 351-360.

Parvez, S. D., Akhtar, K., and Sakai, R. K. (1985). Two new mutations and a linkage map of *Anopheles stephensi*. Journal of Heredity *76*, 205-207.

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

Peiris, H. T., and Hemingway, J. (1996). Effect of fenthion treatment on larval densities of insecticide-resistant Culex quinquefasciatus in an urban area of Sri Lanka. Med Vet Entomol *10*, 283-287.

Pereanu, W., and Hartenstein, V. (2006). Neural lineages of the Drosophila brain: a three-dimensional digital atlas of the pattern of lineage location and projection at the late larval stage. J Neurosci *26*, 5534-5553.

Pevsner, J., Reed, R. R., Feinstein, P. G., and Snyder, S. H. (1988). Molecular cloning of odorant-binding protein: member of a ligand carrier family. Science *241*, 336-339.

Pickett, J. A., and Woodcock, C. M. (1996). The role of mosquito olfaction in oviposition site location and in the avoidance of unsuitable hosts. Ciba Found Symp *200*, 109-119; discussion 119-123, 178-183.

Pitts, R. J., Fox, A. N., and Zwiebel, L. J. (2004). A highly conserved candidate chemoreceptor expressed in both olfactory and gustatory tissues in the malaria vector, Anopheles gambiae. Proc Natl Acad Sci U S A *101*, 5058-5063.

Pitts, R. J., and Zwiebel, L. J. (2006). Antennal sensilla of two female *Anopheline* sibling species with differing host ranges. Malar J *5*, 26.

Poonam, S., Paily, K. P., and Balaraman, K. (2002). Oviposition attractancy of bacterial culture filtrates: response of Culex quinquefasciatus. Mem Inst Oswaldo Cruz *97*, 359-362.

Python, F., and Stocker, R. F. (2002). Adult-like complexity of the larval antennal lobe of D. melanogaster despite markedly low numbers of odorant receptor neurons. J Comp Neurol *445*, 374-387.

Ramoni, R., Vincent, F., Grolli, S., Conti, V., Malosse, C., Boyer, F. D., Nagnan-Le Meillour, P., Spinelli, S., Cambillau, C., and Tegoni, M. (2001). The insect attractant 1-octen-3-ol is the natural ligand of bovine odorant-binding protein. J Biol Chem *276*, 7150-7155.

Raymond, M., Callaghan, A., Fort, P., and Pasteur, N. (1991). Worldwide migration of amplified insecticide resistance genes in mosquitoes. Nature *350*, 151-153.

Reisen, W. K., Meyer, R. P., Tempelis, C. H., and Spoehel, J. J. (1990). Mosquito abundance and bionomics in residential communities in Orange and Los Angeles Counties, California. J Med Entomol *27*, 356-367.

Richie, T. L., and Saul, A. (2002). Progress and challenges for malaria vaccines. Nature *415*, 694-701.

Riesgo-Escovar, J., Raha, D., and Carlson, J. R. (1995). Requirement for a phospholipase C in odor response: overlap between olfaction and vision in Drosophila. Proc Natl Acad Sci U S A *92*, 2864-2868.

Riesgo-Escovar, J. R., Woodard, C., and Carlson, J. R. (1994). Olfactory physiology in the Drosophila maxillary palp requires the visual system gene rdgB. J Comp Physiol [A] *175*, 687-693.

Robertson, H. M. (1998). Two large families of chemoreceptor genes in the nematodes Caenorhabditis elegans and Caenorhabditis briggsae reveal extensive gene duplication, diversification, movement, and intron loss. Genome Res *8*, 449-463.

Robertson, H. M., and Wanner, K. W. (2006). The chemoreceptor superfamily in the honey bee, Apis mellifera: expansion of the odorant, but not gustatory, receptor family. Genome Res *16*, 1395-1403.

Robertson, H. M., Warr, C. G., and Carlson, J. R. (2003). Molecular evolution of the insect chemoreceptor gene superfamily in Drosophila melanogaster. Proc Natl Acad Sci U S A *100 Suppl 2*, 14537-14542.

Sakurai, T., Nakagawa, T., Mitsuno, H., Mori, H., Endo, Y., Tanoue, S., Yasukochi, Y., Touhara, K., and Nishioka, T. (2004). Identification and functional characterization of a sex pheromone receptor in the silkmoth Bombyx mori. Proc Natl Acad Sci U S A *101*, 16653-16658.

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

Scaloni, A., Paolini, S., Brandazza, A., Fantacci, M., Bottiglieri, C., Marchese, S., Navarrini, A., Fini, C., Ferrara, L., and Pelosi, P. (2001). Purification, cloning and characterisation of odorant- and pheromone- binding proteins from pig nasal epithelium. Cell Mol Life Sci *58*, 823-834.

Schellenberg, J. R., Abdulla, S., Nathan, R., Mukasa, O., Marchant, T. J., Kikumbih, N., Mushi, A. K., Mponda, H., Minja, H., Mshinda, H.*, et al.* (2001).

Effect of large-scale social marketing of insecticide-treated nets on child survival in rural Tanzania. Lancet *357*, 1241-1247.

Shiff, C. (2002). Integrated approach to malaria control. Clin Microbiol Rev *15*, 278-293.

Singh, R. N. (1997). Neurobiology of the gustatory systems of Drosophila and some terrestrial insects. Microsc Res Tech *39*, 547-563.

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

Smithburn KC , H. T., Burke AW (1940). A neurotropic virus isolated from the blood of a native of Uganda Am J Trop Med *20*, 471-492.

Soper, F., and Wilson, D. (1943). *Anopheles gambiae* in Brazil (New York: The Rockefeller Foundation).

Stocker, R. F. (1994). The organization of the chemosensory system in *Drosophila melanogaster* : a review. Cell & Tissue Research *275*, 3-26.

Stocker, R. F., Heimbeck, G., Gendre, N., and de Belle, J. S. (1997). Neuroblast ablation in Drosophila P[GAL4] lines reveals origins of olfactory interneurons. J Neurobiol *32*, 443-456.

Sun, B., and Salvaterra, P. M. (1995). Characterization of Nervana, a Drosophila melanogaster Neuron-Specific Glycoprotein Antigen Recognized by Anti-Horseradish Peroxidase Antibodies. J Neurochem *65*, 434-443.

Takken, W. (1991). The Role of Olfaction in Host-Seeking of Mosquitoes: A Review. Insect Science Applications *12*, 287-295.

Takken, W., Dekker, T., and Wijnholds, Y. G. (1997). Odor-mediated flight behavior of Anopheles gambiae Giles Sensu Stricto and A-stephensi Liston in response to CO2, acetone, and 1-octen-3-ol (Diptera: Culicidae). Journal of Insect Behavior *10*, 395-407.

Takken, W., and Kline, D. L. (1989). Carbon dioxide and 1-octen-3-ol as mosquito attractants. J Am Mosq Contr Assoc *5*, 311-315.

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

Thangam, T. S., and Kathiresan, K. (1996). The prey consumption and prey preference of the larvae of the mosquito Culex (Lutzia) raptor on the larvae of Culex quinquefasciatus. Experientia *52*, 380-382.

Thiery, I., Nicolas, L., Rippka, R., and Tandeau de Marsac, N. (1991). Selection of cyanobacteria isolated from mosquito breeding sites as a potential food source for mosquito larvae. Appl Environ Microbiol *57*, 1354-1359.

Tissot, M., Gendre, N., Hawken, A., Stortkuhl, K. F., and Stocker, R. F. (1997). Larval chemosensory projections and invasion of adult afferents in the antennal lobe of Drosophila. J Neurobiol *32*, 281-297.

Tomori, O. (2004). Yellow fever: the recurring plague. Crit Rev Clin Lab Sci *41*, 391-427.

Vogt, R. G., Callahan, F. E., Rogers, M. E., and Dickens, J. C. (1999). Odorant binding protein diversity and distribution among the insect orders, as indicated by LAP, an OBP-related protein of the true bug Lygus lineolaris (Hemiptera, Heteroptera). Chem Senses *24*, 481-495.

Vosshall, L. B. (2000). Olfaction in Drosophila. Curr Opin Neurobiol *10*, 498-503.

Vosshall, L. B. (2001). The molecular logic of olfaction in Drosophila. Chem Senses *26*, 207-213.

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

Walker, K., and Lynch, M. (2007). Contributions of Anopheles larval control to malaria suppression in tropical Africa: review of achievements and potential. Med Vet Entomol *21*, 2-21.

Wetzel, C. H., Behrendt, H., Gisselmann, G., Storkuhl, K. F., Hovemann, B., and Hatt, H. (2001). Functional expression and characterization of a *Drosophila* odorant receptor in a heterologous cell system. PNAS *98*, 9377-9380.

White, G. B. (1974). *Anopheles gambiae* comples and disease transmission in Africa. Transactions of the Royal Society of Tropical Medicine and Hygiene *68*, 278-301.

Wicher, D., Schafer, R., Bauernfeind, R., Stensmyr, M. C., Heller, R.,

Heinemann, S. H., and Hansson, B. S. (2008). Drosophila odorant receptors are both ligand-gated and cyclic-nucleotide-activated cation channels. Nature.

Winch, P. J., Makemba, A. M., Makame, V. R., Mfaume, M. S., Lynch, M. C., Premji, Z., Minjas, J. N., and Shiff, C. J. (1997). Social and cultural factors affecting rates of regular retreatment of mosquito nets with insecticide in Bagamoyo District, Tanzania. Trop Med Int Health *2*, 760-770.

Woodard, C., E, A., and J, C. (1992). The rdgB gene of Drosophila: a link between vision and olfaction. Journal of Neurogenetics *8*, 17-31.

Workman, P. D., and Walton, W. E. (2003). Larval behavior of four Culex (Diptera: Culicidae) associated with treatment wetlands in the southwestern United States. J Vector Ecol *28*, 213-228.

Xia, Y., Wang, G., Buscariollo, D., Pitts, R. J., Wenger, H., and Zwiebel, L. J. (2008). The molecular and cellular basis of olfactory-driven behavior in Anopheles gambiae larvae. Proc Natl Acad Sci U S A *105*, 6433-6438.

Xu, P. X., Zwiebel, L. J., and Smith, D. P. (2003). Identification of a Distinct Family of Genes Encoding Atypical Odorant-Binding Proteins in the Malaria Vector Mosquito, *Anopheles gambiae*. Insect Molecular Biology *12*, 549-560.

Yang, T. T., Cheng, L., and Kain, S. R. (1996). Optimized codon usage and chromophore mutations provide enhanced sensitivity with the green fluorescent protein. Nucleic Acids Res *24*, 4592-4593.

Yap, H. H., Chong, N. L., Foo, A. E., and Lee, C. Y. (1994). Dengue vector control: present status and future prospects. Gaoxiong Yi Xue Ke Xue Za Zhi *10 Suppl*, S102-108.

Zacharuk, R. Y., Yin, L. R.-S., and Blue, S. G. (1971). Fine Structure of the Antenna and its Sensory Cone in the Larvae of *Aedes aegypti* (L.). Journal of Morphology *135*, 273-298.

Zinser, M., Ramberg, F., and Willott, E. (2004). Culex quinquefasciatus (Diptera: Culicidae) as a potential West Nile virus vector in Tucson, Arizona: blood meal analysis indicates feeding on both humans and birds. J Insect Sci *4*, 20.

Zwiebel, L. J., and Takken, W. (2004). Olfactory Regulation of Mosquito-Host Interactions. Insect Biochem Mol Biol *34*, 645-652.