Olfactory Responses and Odor Coding of *Culex quinquefasciatus* to Human Odorants

by

Zi Ye

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Approved by

Nannan Liu, Chair, Professor of Entomology and Plant Pathology
Joseph W. Kleopper, Professor of Entomology and Plant Pathology
Nathaniel B. Hardy, Assistant Professor of Entomology and Plant Pathology

Abstract

The southern house mosquito, *Culex quinquefasciatus* (Diptera: Culicidae), transmits a number of potentially fatal diseases, including West Nile virus (WNV). From 1999 to 2013, WNV caused 39,557 cases of human infection and 1,668 deaths in the United States alone, and the lack of a vaccine for WNV makes mosquito control central to reducing the spread of this and other serious diseases. Mosquitoes recognize their hosts by detecting chemical cues via the olfactory receptor neurons (ORN) in their sensilla, most of which are located on the insects' antennae. *Cx. quinquefasciatus'* antennal sensilla are functionally separated based on morphological distinctions and are highly sensitive to plant-derived repellents. However, a comprehensive study of the response of *Cx. quinquefasciatus* to human odorants is still lacking. In this research, single sensillum recording was conducted to investigate *Cx. quinquefasciatus'* neuronal responses to more than one hundred human odorants selected from eleven chemical categories. Five morphological types of antennal sensilla were identified, namely short sharp-tipped (SST), long sharp-tipped (LST), short blunt-tipped type I (SBTI), short blunt-tipped type II (SBTII) and grooved peg (GP). The results of the single sensillum recordings revealed that *Cx. quinquefasciatus* only responded to a very limited number of the human odorants selected. Different types of sensilla presented distinctive response profiles to the human odorants tested and the responses were dose-dependent. In particular, SST, SBTI and SBTII responded to more than one category of human odorants, while GP and LST were narrowly tuned to amines and methyl nonanoate, respectively. Temporal dynamics analysis demonstrated that the temporal

structure is based on the chemical structure. A behavioral study was also carried out that utilized a hand-in-cage bioassay to characterize the odorant modulating behaviors of *Cx. quinquefasciatus,* confirming that heptanal indeed modulates opposite mosquito behaviors and that this behavior is concentration-dependent.

The antennal lobe is the first center that processes olfactory information among insects. Olfactory receptor neurons aggregate in the antennal lobe and construct spherical units called glomeruli and the spatial separation of these glomeruli forms the basis of insect odor-coding. Anterograde fluorescent staining and confocal microscopy were also performed to examine the structure of *Cx. quinquefasciatus'* antennal lobe and the spatial relationship between glomeruli in more detail. Taken together, our study sheds new light on the olfactory physiology and response of *Cx. quinquefasciatus* to human odorants and provides meaningful information to support the development of new reagents for mosquito control.

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ACh	Acetylcholine
AChE	Acetylcholinesterase
cDNA	Complementary DNA
cRNA	Complementary RNA
CHIKV	Chikungunya virus
DDT	Dichlorodiphenyltrichloroethane
DEET	Diethyltoluamide
DENV	Dengue virus
DMSO	Dimethyl sulfoxide
EAG	Electroantennogram
GM	Genetic modification
GP	Grooved peg
LST	Long sharp-tipped
OR	Olfactory receptor
OBP	Odorant-binding protein
ORN	Olfactory receptor neuron
Orco	Olfactory receptor co-receptor
SBTI	Short blunt-tipped type I
SBTII	Short blunt-tipped type I

SLEV	St. Louis encephalitis virus
SNMP	Sensory neuron membrane protein
SSR	Single sensillum recording
SST	Short sharp-tipped
SST-C	Short sharp-tipped curved
VUAA1	2-(4-ethyl-5-(pyridin-3-yl)-4H-1,2,4-triazol-3-ylthio)-N-(4-ethylphenyl)acetamide
WNV	West Nile virus
YFV	Yellow Fever virus

Chapter 1: Literature Review

1.1 Mosquitoes as disease vectors

Culicidae, more commonly known simply as "mosquitoes", is a large and important family of Diptera. More than 3,000 species have been recognized, divided into 13 subfamilies, 44 genera and 145 subgenera. Mosquitoes are vectors for numerous types of diseases because many feed on human blood at some point in their life cycle (Harbach, 2007; Triplehorn and Johnson, 2005). For example, Chikungunya virus (CHIKV) is spread by *Aedes* mosquitoes and the resulting Chikungunya fever induces febrile syndrome and joint pain. Previously epidemic only in Asia and Africa, it has now become a worldwide disease (Dupont-Rouzeyrol et al., 2012; Powers and Logue, 2007). Among the various *Aedes* mosquitoes, the yellow fever mosquito, *Aedes aegypti* (Diptera: Culicidae), primarily transmits Yellow Fever virus (YFV) and Dengue virus (DENV) (van den Hurk et al., 2012), while the malaria mosquito, *Anopheles gambiae* (Diptera: Culicidae), is the primary vector of malaria, causing up to 1.2 million deaths worldwide each year and posing a special threat in Africa, where it is responsible for 90% of childhood morbidity (Boissière et al., 2012; Nayyar et al., 2012). Because of the non-specific symptoms caused by malaria, it is hard to differentiate it from other diseases and thus difficult to diagnose in many cases (Beare et al., 2006).

In the United States, the southern house mosquito, *Culex quinquefasciatus* (Diptera: Culicidae), is responsible for transmitting West Nile virus (WNV), lymphatic filariasis and St. Louis encephalitis virus (SLEV) (Hill et al., 2009; Luby et al., 1969). The parasitic disease

lymphatic filariasis has largely been controlled in America. From 1964 to 2009, an average of 102 cases were reported each year, with the largest outbreak being in 1975 (CDC, 2014). WNV poses a greater threat, however: in 2013, 2,374 cases were reported in 48 states, including 114 deaths. Most of the people infected with WNV have no symptoms, but 1% of the people infected by WNV will develop neurologic illnesses and 10% of those will die (CDC, 2014). *Cx. quinquefasciatus* is spreading across the globe and is now found in both North and South America (Diaz-Badillo et al., 2011). In the United States, *Cx. quinquefasciatus* is most commonly found along a latitude of 36°N and is thus particularly prevalent in AL, MS, AR, TX, GA, FL, and CA (Barr, 1957). Currently, there is no human vaccine against either WNV or SLEV, so mosquito control remains the first line of defense in efforts to restrict their transmission (CDC, 2014; Kramer et al., 2008).

1.2 Life cycle of *Culex quinquefasciatus*

Cx. quinquefasciatus eggs are take the form of rafts that float on the surface of still or stagnant water. This egg stage usually lasts for only one day in tropical areas, with temperature affecting the incubation time. The incubation time for male and female offspring is the same. Those mosquitoes that feed on birds usually lay more eggs than those that feed on humans and mating between different strains may result in offspring death at the embryo stage (Subra, 1981).

The mosquito larvae that hatch from the eggs into the water are called *wigglers* (ACMA, 2013). Each larva sheds its skin four times, referred to as the four instar stages. When a 4th instar larva molts, it develops into a pupa (Gerberg, 1994). The time required for the larva stage ranges from six to eight days, with temperature being one of the determining factors and the male larval stage being shorter than for the females. Another factor at this stage is nutrition, which is why

Cx. quinquefasciatus spawns preferentially in habitats with polluted water containing organisms to provide nutrition for the larvae (Subra, 1981).

Mosquito pupae are called *tumblers*. Still water-borne, the female pupae are generally larger than the males. Pupae are active without feeding during this stage and need to frequently breathe at the surface of the water. When disturbed, they dive deeper into the water using their abdomens (ACMA, 2013; Papathanos et al., 2009; Subra, 1981).

After 1-4 days of the pupa stage, adults emerge from the pupae. Protandry among mosquitoes means that the males emerge earlier than the females to maximize mating opportunities. Females benefit from protandry as it decreases the duration of the pre-reproductive period and increases the time needed to develop into pupae to maximize fecundity. Sunset and the period between 20:00 to 21:00 are peak times for pupae eclosion. In regions where the temperature is more mutable than it is in the tropics, more females emerge than males when the temperature drops (Bradshaw et al., 1997; Kleckner et al., 1995; Subra, 1981). *Cx. quinquefasciatus* adults are dark scaled with metal reflection, their scutal scales are long and sparse and their subcosta intersects the costa before the bifurcation of Radius 2 + Radius 3 (Harbach, 1988). Adults usually fly a short distance away from the larval breeding place, and after 36 hours females will be fertilized before their first blood meals, although cold weather may lengthen the time between emergence and fertilization. Both males and females feed on sugar sources for survival, but females must ingest a blood meal for egg development after mating. The majority of females feed at night. Once the females digest all the blood, eggs form and will be laid on the surface of polluted water. There is an interim period between the oviposition and the next blood meal. *Cx. quinquefasciatus'* feeding preference varies somewhat in different regions,

with anthropophily and ornithophily being the two major types of feeding preference (Foster, 1995; Subra, 1981).

1.3 West Nile virus transmission

More than 60 mosquito species have been found to carry WNV (CDC, 2014). *Cx. quinquefasciatus* is the primary vector of West Nile virus in the southern United States because it feeds both on birds and human hosts. The mosquitoes' preferred habitats, with abundant pollution, correspond to those near humans, especially where human waste is present (Hill et al., 2009). The transmission of WNV by vectors involving birds is called an enzootic cycle. WNV maintains and increases its population in birds that overwinter in warmer southern regions, and can thus be acquired by mosquitoes that feed on those infected birds once the temperature starts to rise in the spring. The vertical transmission of WNV from adult *Cx. quinquefasciatus* to its eggs has also been reported as a mechanism for WNV overwintering. Humans can then be infected with WNV as a result of being bitten by an infected mosquito. This transmission from birds to human is called the epidemic cycle (Savage et al., 2007; Goddard et al., 2003). Fortunately, WNV cannot be transmitted between humans by mosquitoes due to the relatively low virus level (CDC, 2014). *Cx. quinquefasciatus* is also the principal vector of SLEV in the central, southern and eastern United States (CDC, 2014; Pesko and Mores, 2009). SLEV has a similar transmission cycle to that of WNV, infecting humans through mosquitoes that carry SLEV that originated among the local bird population (Richards et al., 2009). Sharing hosts and vectors heightens the possibility of interactions between WNV and SLEV and it has been suggested that one virus infection may induce some immunity of hosts to the other (Pesko and Mores, 2009).

1.4 Mosquito control

Chemical control, biological control and agricultural control have all been reported as effective ways to control *Cx. quinquefasiatus* populations. Recently, novel strategies such as genetic modification and new types of mosquito repellents have succeeded in protecting humans from mosquito bites while incurring much lower ecological risk.

1.4.1 Chemical control

Organochlorine chemicals have been used as insecticides against mosquitoes since the 1940s. The classic example of this is the use of DDT both during and after the Second World War (Subra, 1981). Organochlorines prevent sodium channels on the axon from polarizing after depolarization so the insect's muscles continue to contract and convulse after a single stimulation (Coats, 1990). However, these insecticides began to lose their effectiveness in the 1950s as the target insects began to develop resistance to organochlorine chemicals and these were gradually substituted by organophosphorus chemicals.

Organophosphorus compounds, such as chlorpyrifos, fenthion and diazinon, all of which have shown high effectiveness against mosquitoes in some areas, are used as larvicides and are applied to bodies of water where larvae are concentrated (Subra, 1981). Organophosphorus compounds function by occupying the active site of acetylcholinesterase (AChE), which degrades the neurotransmitter acetylcholine (ACh). The resulting high concentration of ACh constantly stimulates the ACh receptors and cause continuous excitation of the muscles, leading to convulsions (Fukuto, 1990). However, their effectiveness in different areas is inconsistent. Larvicides are designed to be applied in water, but the abuse of larvicides pollutes water and also adversely affects non-targets (Vahitha et al., 2002; Subra, 1981).

An alternative class of insecticides, the carbamates, target adult insects. Their high adulticidal activity extends insecticide categories in an attempt to counter the development of resistance (Georghiou et al., 1966). Carbamates share the same toxic mechanism as that of organophosphorus compounds, but unfortunately *Cx. quinquefasciatus* have already shown resistance to certain kinds of organophosphorus compounds and carbamates by moderating AChE active sites and developing metabolic detoxification (Liu et al., 2005; Subra, 1981).

Nowadays, pyrethroid insecticides synthesized based on the structure of naturally extracted pyrethrum are widely used to control mosquitoes indoors. The mechanism here is to block the regular function of sodium channels on the axon and inhibit the transmission of neuronal signals. However, the problem of the development of resistance to this new class of insecticides has already emerged in *Cx. quinquefasciatus* and in several other sensitive species (Barbosa et al., 2010; Liu et al., 2006; Coats, 1990).

1.4.2 Biological and agricultural control

Biological control methods include botanical insecticides, microbial insecticides, entomopathogenic fungi and predators (Shaalan et al., 2005; Scholte et al., 2004; Subra, 1981). As far back as the first century AD, powdered chrysanthemum was being used as an insecticide in China. Botanical materials were commonly used by human populations plagued by insects around the world before the discovery of synthetic insecticides (Shaalan et al., 2005). Plant extractions are naturally and environmentally safe, being both highly biodegradable and effective. Botanical insecticides have abundant resources from plants and pose a low risk to non-target humans and animals, with different types of plant materials being used to deal with different life stages of mosquitoes. However, there is no evidence that all natural extracts are environmentally safer than synthetic chemicals, and the potential for the development of

resistance is still uncertain (Kovendan et al., 2011; Shaalan et al., 2005). Entomopathogenic bacteria such as *Bacillus thuringiensis* have been increasingly used as a larvicide that causes a high mortality percentage for larvae while posing no risk to human health (Luxananil et al., 2001; Liu et al., 1996).

The so-called mosquitofish, or *Gambusia*, has been widely introduced around the world as a control agent due to its preference for consuming mosquito larvae and pupae and has been shown to be highly effective, adaptive and prolific (Chandra et al., 2008; Krumholz, 1948). However, mosquitofish have also been dubbed plague minnows since they are aggressive predators of other aquatic organisms, including both insects and the eggs of other fish. The growing pupularity of mosquitofish has thus raised concerns regarding their ecological impact on other native species (Chandra et al., 2008; Pyke, 2008).

Since the growth of larvae largely relies on organic nutrition from polluted water, an alternative biological approach is to reduce the amount of suitable breeding habitat for *Cx. quinquefasciatus* by removing or covering water containers and continually renewing pond water as a form of mosquito control (Subra, 1981).

A novel strategy to control mosquitoes called genetic modification (GM) is drawing more and more attention and the Brazilian government has begun the first field test of GM mosquitoes (PRI, 2014). Using genetic modification technology, it is possible to insert modified genes into the mosquito genome causing mortality of offspring and reducing the capacity to carry malaria (Aldridge, 2008; Catteruccia et al., 2003). However, the application of genetic modified mosquitoes has still a long way to go due to technological, ethical and cultural limits (Lavery et al., 2008; Benedict, 2003). Several problems have been reported, such as allergies triggered by

female GM mosquito bites, and there is also a risk that other species will overtake GM species (PRI, 2014).

In addition to the control of mosquito populations, personal protection is widely used to break the transmission cycle of mosquito-borne viruses. Mosquito repellents such as DEET offer effective and durable ways to prevent the prevalence of mosquito-borne diseases (Liu et al., 2013; Maia et al., 2011; Fradin et al., 2002). Instead of killing mosquitoes directly, DEET works on the mosquito olfactory system to interfere with mosquitoes' recognition and interactions with hosts (Syed and Leal, 2008).

1.5 The mosquito olfactory system

Insects rely on their olfactory senses when mating, feeding and ovipositing. For mosquitoes, olfaction is central to both host seeking and oviposition site seeking. Mosquitoes detect and analyze chemical information in the environment and mediate their behavior accordingly (Guidobaldi et al., 2014; Zwiebel and Takken, 2004). Ammonia, carbon dioxide and the aldehyde nonanal have been demonstrated to serve as human attractants for mosquitoes (McMeniman et al., 2014; Guidobaldi et al., 2014; Syed and Leal, 2009). The concentration of odors plays an essential role in mosquito behaviors; different concentration levels of the same chemical have been shown to stimulate opposite behaviors in mosquitoes (Logan et al., 2010).

1.5.1 Olfactory receptor neurons

Odorants from human, mammals, plants and oviposition sites are detected by olfactory receptor neurons (ORNs) that are housed in mosquito sensilla on olfactory organs, like antennae, maxillary palps and proboscises (Hill et al., 2009; Syed and Leal, 2009; Hallem and Carlson, 2006; Hallem et al., 2006). Other than the ORNs, parts of the gustatory, thermosensory and

mechanosensory neurons are also located in these olfactory organs (Hallem et al., 2006). Different olfactory organs present different sensitivities to chemicals. Carbon dioxide is mainly detected by maxillary palps in mosquitoes, which has been demonstrated using the single sensillum recording technique (Syed and Leal, 2007; Lu et al., 2007). Chemicals like lactic acid, ammonia and indole are primarily detected by antennae (Anton et al., 2003). ORNs are not only able to detect the quality and quantity of odorants, but also the temporal dynamics of neuronal activities (Qiu et al., 2006). The structure of sensilla is highly conserved across species, with a typical sensillum consisting of a hair-like structure containing dendrites of at least one ORN and sensillum lymph flowing between these dendrites and the sensillum cuticular wall. Multiple pores located on the sensillum cuticular wall provide passage for odor molecules (Hallem et al., 2006; Pelosi and Maida, 1995). Each sensillum is also supported by three accessory cells providing lymph, ions and proteins (Guidobaldi et al., 2014).

Each antenna of *Cx. quinquefasciatus* has thirteen flagellomeres. There are five morphological types of antennal sensilla on *Cx. quinquefasciatus*: coeloconica sensilla, chaetica sensilla, ampullaceal sensilla, grooved pegs and trichodea sensilla. Only grooved pegs (GPs) and trichodea sensilla accommodate ORNs. Meanwhile, five morphological subtypes of trichodea sensilla have been found: short sharp-tipped (SST), long sharp-tipped (LST), two types of short blunt-tipped (SBTI and SBTII) and short sharp-tipped curved (SST-C) (Hill et al., 2009; Xia and Zwiebel, 2006).

1.5.2 The inhibition of olfactory receptor neuron

Commonly, each trichodea sensillum or grooved peg of *Cx. quinquefasciatus* houses one or two ORNs, whereas some grooved pegs have been reported to contain three ORNs (Liu et al., 2013; Syed and Leal, 2009; Ghaninia et al., 2008). ORNs are compartmented in sensilla with

stereotypic patterns in fruit flies, mosquitoes and moths to construct distinct functional types of sensilla. The combinations of ORNs within sensilla contribute to lateral inhibition between neighboring ORNs and modulate insect behavior when several neighboring ORNs are excited at the same time (Su et al., 2013; Su et al., 2012).

1.5.3 Olfactory receptors

Odorants interact with olfactory receptors (ORs) that are located on the membranes of ORN dendrites. Olfactory receptors thought to be G-protein coupled receptors have been verified to be heteromeric ligand-gated non-selective cation channels containing at least one odorant-binding OR subunit and at least one Or83b-like family subunit as olfactory receptor co-receptors (Orco). (Leal, 2013; Pelletier et al., 2010a; Benton et al., 2009; Sato et al., 2008). OR subunits are seven-transmembrane-domain proteins with N termini intracellular and C termini extracellular (Benton et al., 2009; Hallem and Carlson, 2006). It has been suggested that OR subunits are in charge of olfactory selectivity and sensitivity since the replacement and mutation of OR subunits changes the preference and sensitivity, respectively, of ORs toward odorants (Nichols et al., 2011). Or83b-like subunits are highly conserved across insects and are similar to OR subunits, with intracellular N termini and extracellular C termini. CquiOR7 found on *Cx. quinquefasciatus* has been shown to function as an olfactory co-receptor due to its high similarity with other Or83b family members. It is widely accepted that Orco is not able to bind and detect odorants alone, although as an exception, a chemical named VUAA1 has been found to elicit responses from Orco alone. In an OR/Orco complex, a complete olfactory receptor functional structure takes the form of an ion pore. The other functions of Orco are to increase the insect's responsiveness to odorants without changing ligand specificity and to make up heteromeric complexes with other

ORs (Leal, 2013; Jones et al., 2011; Nichols et al., 2011; Benton et al., 2009; Xia and Zwiebel, 2006).

ORs are able to convert chemical signals into electrical signals. Action potential can be produced by the opening of ion channel followed by the influx of cations activated by certain chemicals, which accomplishes the transduction of chemical information into an electrical signal (Sato et al., 2008). The olfactory periphery systems in moths and flies use different mechanisms for sensing odorants, with the ORs of moths being more specifically tuned to chemicals compared to flies. Thus, moths select odorants using ORs, whereas flies detect large numbers of odorants using ORs and select specific odorants within a combining odorant signal (Leal, 2013).

ORs amino acid sequence comparative analysis among three mosquito species, *A. aegypti* (AaegOR2; AaegOR7; AaegOR8; AaegOR10), *A. gambiae* (AgamOR2; AgamOR7; AgamOR8; AgamOR10) and *Cx. quinquefasciatus* (CquiOR2; CquiOR7; CquiOR8; CquiOR10), has revealed that the four ORs in *Cx. quinquefasciatus* are highly conserved between the three species because CquiOR2, CquiOR7, CquiOR8 and CquiOR10 exhibit a high similarity to AaegOR2/AgamOR2, AaegOR7/AgamOR7, AaegOR8/AgamOR8 and AaegOR10/AgamOR10, respectively. However, in general most ORs amino acid sequences share few identical fragments and it has been suggested that they display a high level of divergence (Pelletier et al., 2010a; Xia and Zwiebel, 2006).

1.5.4 Olfaction-related proteins

When odorants reach the cuticular wall, odorant-binding proteins (OBPs) are normally involved in receiving and transporting odorants crossing through the sensillum lymph to the ORs. OBPs have a characteristic pattern of six cysteine residues and an N-terminal signal peptide sequence. The C-terminus covers the binding cavity fixed by two hydrogen bonds of the C-terminus

carboxylate oxygen to Tyr-54 and His-23 (or Arg-23), respectively. There are two hypotheses concerning the delivery of odorants by OBPs. One is that odorants individually stimulate ORs in moths and mosquitoes, while the other posits that odorant-OBP complexes trigger the action of ORs in *Drosophila melanogaster* (Diptera: Drosophilidae). The membrane of the dendrite is negatively charged and therefore the protons are attracted to it and accumulate near the membrane surface, which leads to a low PH level near the membrane. At low PH levels, the hydrogen bonds between OBPs and odorants are unstable, which triggers the release of odor molecules. The low PH theory suggests that odorants are more likely to stimulate ORs by themselves, but it is still unclear whether the odorant-OBP complexes influence the selectivity of ORs. *D. melanogaster* has a unique type of OBP named LUSH and it has been suggested that Odorant-LUSH complexes activate ORs because the activation of ORs by LUSH alone and by mutations that mimic Z11-18OAc pheromone-bound LUSH have been found in *D. melanogaster* T1 sensilla, whereas Z11-18OAc receptors have been shown to be activated by Z11-18OAc alone. CquiOBP1 is the first OBP of *Cx. quinquefasciatus* that has been demonstrated to affect the delivery of certain odorants from the cuticular wall to the ORNs. (Leal, 2013; Pelletier and Leal, 2011; Pelletier et al., 2010b; Syed and Leal, 2009; Laughlin et al., 2008; Zwiebel and Takken, 2004).

Within the insect olfactory system, both the detection and inactivation of odorants are essential for navigation. Insects inactivate odorants to qualify their direction of travel: if insects can constantly detect the odorants after inactivation, this means that they are on the right path towards their target. The mechanism involved in odorant inactivation is still unresolved. Although odorant-degrading enzymes (ODEs) have successfully been isolated that are believed

to be related to odorant degradation, an alternative hypothesis suggests that odorants are inactivated by so-called molecular traps (Leal, 2013).

Other proteins might also play a role in insects' olfaction. Sensory neuron membrane proteins (SNMPs), which are located on the surface of the dendrite and encoded by the CD36 gene family, have been reported to function as pheromone detectors in *D. melanogaster* and moths, whereas their function in mosquitoes is unknown (Pelletier and Leal, 2011; Benton et al., 2009). "Plus-C" odorant-binding proteins ("plus-C" OBPs), which are included in the same family as OBPs although with at least three more conserved cysteine residues than typically found in OBPs, are highly abundant in mosquito antennae, which has cited as evidence for its function in mosquito olfaction (Pelletier and Leal, 2011).

1.5.5 Central nervous system

ORNs project their axons into the antennal lobes, the first center processing olfactory information. Typically, each ORN only expresses one type of OR. The ORNs expressing the same OR converge together in the antennal lobes and form a unit called a glomerulus. Each glomerulus links onto projection neurons, which then transmit neuronal signal into higher-level brain regions. Different odorants are able to activate different combination patterns of glomeruli, and the identities of odorants are encoded by these patterns. The number of glomeruli within antennal lobes varies from species to species. Antennal lobe fluorescent staining and confocal microscopy techniques have been used to demonstrate the 3D structure of antennal lobes (Suh et al., 2014; Carey and Carlson, 2011; Hallem et al., 2006; Anton et al., 2003; Hansson and Anton, 2000). The antennal lobe 3D structures of *A. aegypti* and *A. gambiae* have been constructed and annotated. *A. aegypti* presented 50 and 49 glomeruli in females and males, respectively, while *A.*

gambiae showed 60 and 61 glomeruli in females and males (Ghaninia et al., 2007a; Ignell et al., 2005).

1.6 Electrophysiological techniques

Electrophysiological techniques have been commonly used to investigate insect olfactory responses to stimuli (Olsson and Hansson, 2013; Pellegrino et al., 2010; van der Pers and Minks, 1997; Kaissling, 1995). Combining electrophysiological and antennal lobe fluorescent staining makes it possible to investigate the affiliation between each sensillum and its cognate glomerulus (Ghaninia et al., 2007b).

1.6.1 Electroantennogram (EAG)

As mentioned earlier, the antenna is one of the most essential mosquito olfactory organs since it accommodates the great majority of the olfactory receptor neurons (Hill et al., 2009). I na electroantennogram (EAG), the total electrical potential difference between the distal segments and proximal segments of the antennae are measured after being stimulated by individual chemicals, and it has been suggested that the amplitude of the detected potential is positively related to the antennal length (Olsson and Hansson, 2013). The EAG technique has been widely used to investigate odorants collected from gas-chromatography devices (van der Pers and Minks, 1997; Kaissling, 1995). However, the lack of fine resolution available with EAG makes it suitable only for detecting the responses and not for quantifying them (Olsson and Hansson, 2013; van der Pers and Minks, 1997). Therefore, another technique, single sensillum recording (SSR), has been developed to deliver higher resolution and consistency.

1.6.2 Single sensillum recording (SSR)

Sensilla are electrically separate from each other, suggesting that it is possible to detect the electrical potential within a single sensillum (Kaissling, 1995). Using the SSR technique, a reference electrode and a recording electrode are applied to detect the action potential frequency change in each sensillum after stimulation. Rather than being applied directly to the neurons, SSR actually reads the sum of the action potential from the sensillum lymph. Each action potential waveform is referred to as a "spike" and action potentials are quantified by counting the change in the spike number after stimulation. Spikes emitted by different neurons can be distinguished by their amplitude (Olsson and Hansson, 2013; Pellegrino et al., 2010).

1.6.3 *Xenopus* oocyte system

The African clawed frog *Xenopus laevis* (Anura: Pipidae), is the most commonly used model for investigating the function of ion channels (Lin-Moshier and Marchant, 2013; Papke and Smith-Maxwell, 2009; Dascal, 1987). *Xenopus* oocytes are germ cells in female frogs. In the 1960s and 1970s, *Xenopus* oocyte researchers focused primarily on the process of oocyte growth and the mechanism governing cell division (Dascal, 1987). Gurdon and colleagues successfully expressed exogenous rabbit 9S mRNA in *Xenopus* oocytes (Gurdon et al., 1971), after which Kusano and colleagues defined the response of receptors on the *Xenopus* oocyte membrane to neurotransmitters using electrophysiological methods (Kusano et al., 1977). In the 1980s, several studies demonstrated the expression of receptor genes on the membrane of *Xenopus* oocytes by injecting exogenous RNAs. In 1982, Miledi and colleagues first identified the expression of foreign ion channels in *Xenopus* oocytes and since then *Xenopus* oocytes have developed into one of the first heterologous expression systems (Papke and Smith-Maxwell, 2009; Bianchi et al., 2006; Dascal, 1987).

1.6.3.1 The characteristics of *Xenopus* oocytes

Immature oocytes grow in female frogs until fully-developed. Mature oocytes are orbs about 1.2mm in diameter. Four layers from outer to inner constitute the outer surface of each oocyte: a layer of epithelial cells, the theca layer, a layer of follicular cells and the vitelline membrane, which is a noncellular fibrous layer. The follicular cells are electrically coupled to each other and the oocyte by gap junction that provide passages for small molecules, such as ions (Bianchi et al., 2006; Lampe and Lau, 2004; Dascal, 1987). In most studies, the follicular layer is removed by collagenase to minimize interference and make electrode piercing easier (Bianchi et al., 2006; Goldin, 2006). Each oocyte appears half dark and half pale because of its unbalanced melanin pigment granule concentration. The dark half is known as the animal hemisphere and the pale half the vegetal hemisphere. Cytoskeleton contractile structures concentrate in the animal hemisphere, while most of the RNA is in the vegetal hemisphere (Dascal, 1987).

1.6.3.2 The gene expression in *Xenopus* oocytes

cRNA clones that are transcribed from cDNA in vitro are usually injected into oocytes as exogenous genes. The process of transcription requires a RNA polymerase promoter site T3 or T7. An available cRNA has untranslatable regions at both 5’ and 3’ ends known as the cap and ploy (A) tail, respectively. *Xenopus* β-globin 5’ and 3’ end untranslatable regions can make cRNA more stable and easily translated. The shorter 5’ end untranslatable resign can also boost the expression efficiency (Bianchi et al., 2006; Goldin, 2006).

Several biosynthesis processes are performed after the injection of endogenous cRNA. cRNA interacts with other oocyte components to form polysomes and start the protein biosynthesis process. Initiation, elongation and termination signals will be detected during the biosynthesis. Nascent proteins will be post-translationally processed, including any protein

structure changes and functional modifications. The process requires non-species specific cellular components (Soreq et al., 1985).

1.6.3.3 The advantages of using a *Xenopus* oocyte system

There are several advantages related to using *Xenopus* oocytes as a system to express and investigate exogenous genes. Frogs are easily reared in purified water at the proper temperature (around 19°C; any fluctuations in temperature will lower oocyte activity) and salt concentration. Each frog produces more than 20,000 oocytes that can be extracted by simple surgery, making it possible to select batches of oocytes from a single frog, although special care is necessary during the interval between surgeries. *Xenopus* oocytes are large in size and thus convenient for manual microinjection. Different genes can be injected simultaneously, which is important for mosquito olfactory receptor expression since both OR subunits and Orco are required for olfactory receptor function. In addition, compared with the transfection of mammalian cells, each subunit ratio can be predicted in *Xenopus* oocytes through the specific cRNA ratio used in the microinjection. An oocyte expresses an extremely large number of exogenous receptors compared to endogenous receptors, which minimizes any interference from endogenous receptors (Lin-Moshier and Marchant, 2013; Papke and Smith-Maxwell, 2009; Bianchi et al., 2006; Goldin, 2006; Dascal, 1987).

1.6.3.4 The disadvantages of using a *Xenopus* oocyte system

The major disadvantage is that the *Xenopus* oocyte system is not optimal as it is not able to express every channel or receptor (Goldin, 2006). The strict temperature control entailed might also impede the mutation process in mammalian channels, which requires a higher temperature. The presence of endogenous proteins might lead to the formation of heteromultimers with

exogenous proteins. For instance, in one study the KCNE1 gene was expressed in *Xenopus* oocytes and functioned as an IKs K+ channel, which mistakenly led the researchers to conclude that the KCNE1 protein had independently formed an IKs K+ channel. In fact, the IKs K+ channel was actually formed by endogenous KCNQ1 protein and KCNE1 exogenous protein (Bianchi et al., 2006). Furthermore, *Xenopus* oocytes are not native cells for exogenous genes, which suggests there might be functional differences between oocyte-expressed and native cell-expressed genes (Goldin, 2006).

1.6.3.5 Recent research

Hitherto, 177 ORs have been identified from the *Cx. quinquefasciatus* genome (Leal et al., 2013). Of these, 10 ORs were characterized in the *Xenopus* oocyte system, namely OR1, OR21, OR37, OR44, OR73, OR95b, OR99, OR121, OR136 and OR161 (Xu et al., 2014; Zhu et al., 2013; Xu et al., 2013; Leal et al., 2013; Pelletier et al., 2010a; Hughes et al., 2010). These studies showed distinct response profiles for each OR. OR136 was recently shown to be characterized in response to the repellent DEET and methyl jasmonate (Xu et al., 2014), while OR37 and OR99 have been shown to be narrowly tuned to 4-methylphenol and 4-ethylphenol, both of which are oviposition attractants for *Cx. quinquefasciatus* (Zhu et al., 2013). OR121 responded to the oviposition attractants indole and phenolic compunds (Pelletier et al., 2010a) and OR21 showed a strong preference for skatole (Hughes et al., 2010). OR95b was narrowly tuned to citronellal and ethyl 2-phenylacteate, both of which displayed repellency in *Cx. quinquefasciatus* (Leal et al., 2013). OR1, OR44 and OR73 responded to 1-octen-3-ol, plant-derived terpenoids and phenolic compounds, respectively, while none of the chemicals used were able to stimulate OR161 (Xu et al., 2013).

1.7 Bioasssy of mosquito behaviors

Bioassay experiments are used to investigate how inorganic substances affect living organisms, with different bioassays being used to test mosquito behavioral responses to attractants and repellents. Bioassay results are considered convincing evidence when explaining mosquito behavior (Syed and Leal, 2008).

In order to investigate ovipositing attraction, sticky-screen bioassays and ovitraps are used in laboratory and field experiments, respectively (Ponnusamy et al., 2010). Sticky-screen bioassays mimic mosquito ovipositing sites, with attractants and water added to the treatment and control sites, respectively. Each site is then equipped with a sticky screen to trap female mosquitoes arriving to deposit eggs and the attraction is quantified by counting the number of mosquitoes stuck to the screen (Ponnusamy et al., 2010; Trexler et al., 2003; Trexler et al., 1998). EVS mosquito traps baited with CO_2 and attractants are used in field experiments designed to test host-seeking attractants (Syed and Leal, 2009). However, the efficiency of field experiments depends heavily on the weather, temperature and local mosquito population.

A classic bioassay for mosquito repellents is the surface-landing bioassay. Here human volunteers offer their arms as a mosquito landing surface, with repellents spread on one arm but not on the other. In a given unit of time, the number of mosquitoes landing on each arm is then counted and compared (Logan et al., 2010; Douglas et al., 2005; Fradin and Day, 2002). An odorant-free surface landing bioassay has also been introduced to determine whether repellents stimulate mosquitoes in their own right or simply mask other human attractants. Here, instead of using human skin, Dudley tubes heated with circulating water maintained at 38℃ serve as the landing area (Syed and Leal, 2008).

1.8 Reference

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Chapter 2: Research Goals and Specific Objectives

2.1 The goal of the research and objectives

To develop a comprehensive theory of mosquito olfaction and a novel strategy against mosquito-borne diseases, the long term goal of my research is to investigate the chemical ecology and neurophysiology of mosquito interacting with human beings. To achieve my long goal, 3 objectives have been conducted: 1) Investigation of neuronal response profiles of *Cx. quinquefasciatus* to human odorants; 2) Characterization of dose-dependent patterns and temporal dynamics; and 3) Investigation of the antennal lobe three-dimensional structure of *Cx. quinquefasciatus*.

2.1.1 Objective 1: Investigation of neuronal response profiles of *Cx. quinquefasciatus* to human odorants

In my study, *Cx. quinquefasciatus* was lab-reared at 25±2°C supplied with 10% sucrose solution and an L: D cycle of 12:12 hours (Liu et al., 2011). Single sensillum recording was used to investigate the neuronal responses of *Cx. quinquefasciatus* to more than one hundred human odorants at the concentration of 10µg/µL selected from eleven chemical categories. The experiments were performed on five morphological types of sensilla including SST, SBTI, SBTII, LST and GP. Each treatment was recorded from one second before the stimulation to ten second after. The magnitude of responses was quantified by action potential waveform, known as spike, number changes averaged with at least three repetitions.

To further investigate mosquito odor coding characteristics, hierarchical cluster analysis was performed to calculate and analyze the relationship of responses between odorants. Odorants clustering together was considered to be similarly encoded by mosquito antennae.
I hypothesized that different morphological types of sensilla would display distinct response profiles, and *Cx. quinquefasciatus* encoded odorants largely based on the chemical structure.

2.1.2 Objective 2: Characterization of dose-dependent patterns and temporal dynamics
The chemical information is largely encoded by insect antennae in the aspects of quality, quantity and temporal dynamics (F. Guidobaldi et al., 2014; A. F. Carey and J. R. Carlson, 2011; C. Y. Su et al., 2011; S. R. Hill et al., 2009). To address the dose-dependent patterns and temporal dynamics of *Cx. quinquefasciatus* neuronal responses, single sensillum recording was performed with eleven odorants eliciting strong responses on SBTI and SBTII sensilla. Odorants will be used at a concentration series of 0.001μg/μL, 0.01μg/μL, 0.1μg/μL, 1μg/μL and 10μg/μL to investigate the dose-dependent patterns. Temporal dynamics was recorded in the form of response frequency from one second before the stimulation to four seconds after.

Several odorants stimulated opposite behaviors of mosquitoes at different concentration (J. G. Logan et al., 2010; S. N. Puri et al., 2006; H. D. Douglas et al., 2005). Behavior bioassays were conducted to investigate how odorants modulate *Cx. quinquefasciatus* behaviors, which were compared with former behavior study at different concentration to verify the multi-function of odorants to mosquito behaviors. I hypothesized that the neuronal responses followed dose-dependent curves, and the concentration of odorants played an essential role in modulating mosquito behaviors.

2.1.3 Objective 3: Investigation of the antennal lobe three-dimensional structure of *Cx. quinquefasciatus*

The structure of antennal lobes and the spatial relationship between glomeruli are highly involved in the odor coding and can help us understand the mechanisms of odorant identification and recognition (M. Ghaninia et al., 2007a; R. I. Wilson and Z. F. Mainen, 2006; R. Ignell et al., 2005). The Combination of electrophysiological and antennal lobe fluorescent staining makes it possible to investigate the affiliation between each sensillum and its cognate glomerulus (M. Ghaninia et al., 2007b). To demonstrate the three dimensional structure of *Cx. quinquefasciatus* antennal lobes, anterograde antennal lobe staining and confocal microscopy were conducted. Neurobiotin was used to trace through mosquito antennal nerves to antennal lobes. Mosquito brains were dissected out and incubated in fluorescent dye to mark the structure of antennal lobes. Confocal microscopy was performed to take picture stacks of the antennal lobes from the top layer to the bottom. Picture stacks were introduced into computer program to construct the three dimensional structure.

2.2 Significance of research

The neuronal responses of *Cx. quinquefasciatus* to human odorants are indispensable for a better understanding of mosquito host recognition and West Nile virus transmission. The demonstration of *Cx. quinquefasciatus* antennal lobe structure provides an essential insight into the mosquito central nervous system. This research will shed a new light on disease vector neurophysiology, and contribute to the development of novel mosquito repellents and attractants.

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Chapter 3: Neuronal Response Profiles of *Cx. quinquefasciatus* to Human Odorants

3.1 Abstract

Mosquito control is essential to protect humans from mosquito-borne diseases. The host recognition between mosquitoes and humans is achieved by the mosquito olfactory system. Antennal sensilla, which house olfactory receptor neurons (ORNs), are responsible for detecting chemical cues from hosts. To deepen our understanding of the mechanisms involved in the host seeking behavior of mosquitoes, we conducted an electrophysiological study to investigate the response profile of each type of antennal sensilla to human odorants using single sensillum recording. In this study, more than one hundred human odorants have been applied as stimuli to the 5 morphological types of sensilla. Different types of sensilla present distinctive response profiles to the human odorants tested. In particular, SST, SBTI and SBTII sensilla responded to more than one category of human odorants, while GP and LST were narrowly tuned to amines and methyl nonanoate, respectively. Hierarchical cluster analysis revealed that odorants sharing similar chemical structures are encoded similarly by *Cx. quinquefasciatus*.

3.2 Introduction

Mosquitoes act as disease vectors due to their blood-feeding behavior (Harbach, 2007; Triplehorn and Johnson, 2005). The southern house mosquito, *Culex quinquefasciatus*, is the primary vector transmitting fatal diseases in the U.S., including both West Nile virus (WNV) and Saint Louis encephalitis virus (SLEV). The first case of WNV was reported in New York in

1999, and as of 2013 had caused 39,557 cases of human infection and 1,668 deaths, affecting every state in the U.S. except for Alaska and Hawaii. WNV maintains its population in birds and can be transmitted to humans by *Culex* mosquitoes. As yet there is no vaccine against WNV. Since mosquitoes play a key role in WNV transmission to humans, mosquito management remains our best defense as a way to control the spread of this disease (CDC, 2014; Savage et al., 2007).

Mosquitoes rely on their olfactory system for long-range host seeking (Logan et al., 2008; Zwiebel and Takken, 2004). Odorants from hosts are detected by olfactory receptor neurons (ORNs) housed in hair-like sensilla on a mosquito's olfactory organs. Its antenna are the mosquito's most essential olfactory organs since they accommodate the great majority of its olfactory neurons (Hill et al., 2009; Syed and Leal, 2009; Lu et al., 2007; Hallem et al., 2006). Olfactory receptors (ORs), ligand-gated channels embedded across the ORN membrane, are responsible for converting the information carried by chemical cues to electrical signals. The signal transduction alters the active potential frequency on the ORN membrane, which can be detected by electrophysiological approaches as an indicator of the neuronal response magnitude. Six morphological types of antennal sensilla (SST, SBTI, SBTII, LST, SST-C, GP) have been identified on *Cx. quinquefasciatus*, each of which show distinct neuronal response profiles to chemical cues (Guidobaldi et al., 2014; Suh et al., 2014; Liu et al, 2013; Pellegrino et al., 2010; Hill et al., 2009; Qiu et al., 2006). An earlier study focused on the neuronal responses of *Cx. quinquefasciatus* to plant-derived compounds, but a comprehensive investigation of *Culex* mosquito responses to the human odor profile is still lacking (Liu et al, 2013; Hill et al., 2009).

Human skin emits about four hundred types of volatile compounds (Dormont et al., 2013; Bernier et al., 2000). People with different ethnicities exhibit different odor compositions, but

their dominant emanations, namely 6-methyl-5-hepten-2-one, nonanal, decanal and geranylacetone, are identical (Dormont et al., 2013; Syed and Leal, 2009). The microorganisms living on human skin are thought to be the major contributors to odor profile differences among individuals, and the volatiles emitted from microbes are heavily involved in blood-sucking insect host recognition (Dormont et al., 2013).

In the current study, single sensillum recording was used to address the mechanisms involved in *Culex* mosquito neuronal responses to human odorants. More than one hundred human odorants selected from eleven categories were used as stimulants on five morphological types of antennal sensilla.

3.3 Materials and methods

3.3.1 Southern house mosquitoes

Southern house mosquitoes, *Cx. quinquefasciatus*, were collected from Huntsville, Alabama, USA. The mosquitoes were reared at 25±2°C and an L: D cycle of 12:12 hours, and supplied with a 10% sucrose solution (Liu et al., 2011).

3.3.2 Single sensillum recording

Female mosquitoes 4 days after emerging were anaesthetized on ice and mounted on a microscope slide (76×26mm). The antennae were fixed using double-sided tape to a cover slip resting on a small ball of dental wax to facilitate manipulation and the cover slip was placed at an appropriate angle to the mosquito head. Once mounted, the specimen was placed under a Leica Z6 APO microscope and the antennae were viewed at high magnification (720×). Two tungsten microelectrodes were sharpened in 10% KNO_2 at 2-10V to a ≈1μm tip diameter. The reference electrode, which was connected to ground, was inserted into the compound eye of the mosquito

and the other was connected to the preamplifier (10×, Syntech, Kirchzarten, Germany) and inserted into the shaft of the trichoid sensillum to complete the electrical circuit to extracellularly record ORN potentials. Controlled manipulation of the electrodes was performed using two micromanipulators (Leica, Germany). The preamplifier was connected to an analog to digital signal converter (IDAC-4, Syntech, Germany), which in turn was connected to a computer for signal recording and visualization. The activity of co-located ORNs in each sensillum was assessed based on the differences in spike amplitude. Neuron cells with larger spike amplitude were indicated as cell A, while cells with smaller spike amplitude were called cell B. Signals were recorded for 10s starting 1s before stimulation, and the action potentials were counted off-line over a 500ms period before and after stimulation. Spike rates observed during the 500ms stimulation were subtracted from the spontaneous activities observed in the preceding 500ms and counts recorded in units of spikes/s (Liu et al., 2013).

3.3.3 Stimulation and stimuli

One hundred and three human odorants from eleven groups (carboxylic acids, aldehydes, alcohols, aliphatics/aromatics, esters, ketones, amines, sulfides, ureas, halides, heterocyclics) were used in the study (**Table 1**). Each human odorant was diluted in dimethyl sulfoxide (DMSO), except for ammonia which was diluted in ddH_2O, to a concentration of 100µg/µL as a stock solution and 10µg/µL as the initial stimulating concentration. For each dilution, a 10µL portion was dispersed onto a filter paper (3×10mm) which was then inserted into a Pasteur pipette to create the stimulus cartridge for that repellent dilution. A sample containing the pure solvent alone served as the control. The airflow across the antennae was maintained at a constant 20mL/s throughout the experiment. Purified and humidified air was delivered to the preparation through a glass tube (10mm inner diameter) perforated by a small hole 10cm away from the end

of the tube into which the tip of the Pasteur pipette could be inserted. The stimulus was delivered to the sensilla by inserting the tip of the stimulus cartridge into this hole and diverting a portion of the air stream (0.5L/min) to flow through the stimulus cartridge for 500ms using a stimulus controller (Syntech, Germany). The distance between the end of the glass tube and the antennae was 61cm. All chemicals were tested on each type of antennal sensillum at least 10 times (Liu et al., 2013). Since different functional types were observed within the several morphological types of sensilla, the most common functional types with at least 3 repeats were selected as results. The number of spikes/s was obtained by averaging the results of each sensillum/chemical combination. Responses less than fifteen spikes/s were considered as no response. The excitatory response of the sensilla was categorized as <20%, 20%-50%, 50%-80% and >80% of the largest firing frequency in the recording.

3.3.4 Hierarchical cluster analysis

Hierarchical cluster analysis of the response profile was conducted by R 3.1.1 based on Euclidean distances and Ward's classification (R Foundation for Statistical Computing, Vienna, Austria).

3.4 Results

3.4.1 Neuronal response profile of *Cx. quinquefasciatus* to human odorants

Five morphological types of sensilla with olfactory function were identified under an optical microscope, namely short sharp-tipped (SST), long sharp-tipped (LST), short blunt-tipped type I (SBTI), short blunt-tipped type II (SBTII) and grooved pegs (GP). Two types of neuron cells (A and B) with distinct spike amplitudes were found in SBTI and SBTII. SST sensilla provided two

different response profiles to the chemicals tested, defined as functional types SST 1 and SST 2. The strongest response was found for SBTII B to octanal (155 spikes/s).

In total, 824 odorant-sensillum combinations were tested (**Figure 1**). 108 out of the 824 combinations showed excitatory responses (≥15 spikes/s). Aldehydes presented the most extensive excitatory responses, with 100% of the aldehydes activating the neurons on at least one type of sensilla. Most of the active stimulations were elicited by aldehydes (33 out of 108), followed by ketones (17 out of 108), heterocyclics (15 out of 108), and aliphatics/aromatics (14 out of 108). Carboxylic acid was the second biggest chemical group among the stimuli tested, with 21 compounds. However, only trans-2,3-dimethylacrylic acid (26±8 spikes/s on SST 1), octanoic acid (43±11 spikes/s on SBTII A) and n-nonanoic acid (25±1 spikes/s on SBTII A; 51±23 spikes/s on SBTII B) elicited excitatory responses. In terms of the sensilla, SBTII was found to be the most sensitive, accounting for 54 out of the 108 responding stimulations (21 out of 108 on SBTII A; 33 out of 108 on SBTII B), followed by SST 1 (23 out of 108).

SST 1 showed highly active responses to aldehydes (113±20 spikes/s to hexanal; 113±16 spikes/s to decanal; 112±12 spikes/s to heptanal), ketones (104±7 spikes/s to 2-pentanone) and heterocyclic compounds (106±14 to 2,6-dimethylpyrazine; 112±7 spikes/s to 2-picoline; 116±11 spikes/s to pyrazine). SST 2 exhibited a low sensitivity to decanal (11±4 spikes/s), heptanal (24±8 spikes/s) and pyrazine (3±3 spikes/s) but did respond to skatole (86±13 spikes/s) and 2-methylbutanal (41±18 spikes/s), to which SST 1 showed no response.

2-decanone (38±3 spikes/s) elicited the highest tonic excitation on SBTI A. SBTI A responded slightly to 6-methyl-5-hepten-2-one (26±5 spikes/s) and methyl nonanoate (27±7 spikes/s). SBTI B showed higher excitatory responses to aldehyde compounds than SBTI A, including octanal (60±7 spikes/s), nonanal (65±10 spikes/s) and decanal (53±16 spikes/s).

Two neuron cells A and B of SBTII showed remarkably distinct response patterns to different classes of human odorants. SBTII A showed specifically strong excitation to aromatics and heterocyclics such as toluene (125±24 spikes/s), styrene (153±5 spikes/s), benzene (115±37 spikes/s), ethylbenzene (118±21spikes/s), xylene (115±21 spikes/s) and indole (152±27 spikes/s). For the most part, the strongest responses of SBTII B were to the aldehydes. Decanal (143±16 spikes/s), nonanal (113±12 spikes/s), heptanal (123±27 spikes/s), propanal (96±15 spikes/s) and octanal (155±12 spikes/s) all elicited extremely active responses on SBTII. SBTII B also responded to 2-hexanone (60±14 spikes/s) and skatole (75±4 spikes/s), which elicited no response in SBTII A. It was difficult to differentiate between the firing frequency amplitude for the A and B cells to benzaldehyde at 10µg/µL, since both responded to benzaldehyde and the amplitude of cell B was irritated to the same level as cell A. Given that the response ratio for A to B at high chemical concentration (10µg/µL) is likely to be consistent with that at lower concentrations (1µg/µL and 0.1µg/µL), at which the spike amplitude of cell B was not irritated, benzaldehyde was assumed to elicit responses of 60±5 spikes/s and 100±8 spikes/s for cells A and B, respectively.

According to earlier studies (Hill et al., 2009; Syed and Leal, 2009), LST exhibited no response to most of the tested chemicals. However, we, for the first time, found LST responded to methyl nonanoate (63±2 spikes/s). GP mildly responded to all the amines tested, including propylamine (36±6 spikes/s), butylamine (37±9 spikes/s) and ammonia (31±8 spikes/s), but yielded no response to any other chemicals.

It is worth mentioning that 1-methylpiperazine (-11±6 spikes/s to GP), 2-decanone (-15±4 spikes/s to SBTII A) and 2-decanol (-13±11 spikes/s to SBTII A) inhibited the firing frequency

after stimulating. This neuronal inhibition is critical for the behavior of *Drosophila*, but its biological significance in the mosquito remains unclear (Su et al., 2011).

3.4.2 Hierarchical cluster analysis

To visualize the odor coding pattern of *Cx. quinquefasciatus*, hierarchical cluster analysis was applied based on the Euclidean distance of the responses (**Figure 2**). The analysis of the response profile showed odorants sharing similar chemical structures clustering together, revealing that chemicals with similar structures are often identically encoded by mosquitoes. Even if some chemicals were not in the same chemical categories, they may still be clustered together according to their structural similarity, especially chemicals with benzene rings.

3.5 Discussion

For this study, single sensillum recording (SSR) was applied to six types of sensilla on *Cx. quinquefasciatus*. 103 types of human odor compounds were used as stimuli. SST, SBTI A, SBTI B, SBTII A and SBTII B responded to more than one category of chemicals, while LST and GP were narrowly tuned to methyl nonanoate and amines, respectively.

Recent research has revealed that multiple cues from hosts, that is, heat, CO_2 and chemical cues, interact to direct mosquito feeding behavior (McMeniman et al., 2014). By using electrophysiological methods, human odorants have been shown to play an essential role in mosquito-involved disease transmission. The individual components of human sweat are of particular concern due to its function in attracting mosquitoes (Hill et al., 2009; Qiu et al., 2006; Hallem et al., 2004; Braks et al., 2001; Meijerink et al., 2001; Cork and Park, 1996). Indole, 1-octen-3-ol and 6-methyl-5-hepten-2-one, all of which are components of human sweat, all elicited neuronal responses in our experiments (McBride et al., 2014; Ghaninia et al., 2007; Cork

and Park, 1996). As an ornithophilic mosquito, *Cx. quinquefasciatus* primarily feeds on birds (Cooperband et al., 2008). Nonanal, an emanation of both human and birds, has been demonstrated as the reason why *Cx. quinquefasciatus* readily switches between birds and human hosts. This host switch enables West Nile virus to maintain its population in birds and then be transmitted by mosquitoes from infected birds to humans (Syed and Leal, 2009; Savage et al., 2007). The results from electroantennograms (EAG) and SSR revealed that nonanal elicits antennal excitation on *Cx. quinquefasciatus*, supporting the results obtained in the field using mosquito traps baited with nonanal, which clearly demonstrated the attraction of nonanal to *Cx. quinquefasciatus* (Syed and Leal, 2009; Puri et al., 2006). Meanwhile, other human-derived aldehydes such as hexanal, heptanal, octanal and decanal have also been detected in pigeons and chickens (Syed and Leal, 2009; Bernier et al., 2008; Bernier et al., 2000). Our data showed those aldehydes all elicited highly active firing excitations on both SST 1 and SBTII B. This suggests the likelihood that these aldehydes are also involved in the *Cx. quinquefasciatus* host switch. Behavioral research has demonstrated that L-lactic acid, one of the strongest human skin emanations, is not attractive to *Cx. quinquefasciatus* alone but functions synergistically with CO_2 as an attractant (Allan et al., 2010; Geier et al., 1999). In this study, L-lactic acid failed to elicit any neuronal response.

Inconsistent results have been found between EAG- and SSR-based studies. According to our data, hexanoic acid, undecanoic acid, and tridecanoic acid failed to elicit firing excitations on any types of sensilla, but all showed strong responses in former EAG experiments (Puri et al., 2006). The reason for this inconsistency might be the relatively low concentrations used in the present study and our focus on trichoids and grooved pegs. Previous researchers have reported

that coeloconica sensilla, which are also present on antennae, mainly detect acids in *Drosophila* (Suh et al., 2014; Hill et al., 2009).

How do mosquitoes identify and distinguish different chemical cues? Normally for insects, each olfactory receptor neuron (ORN) only expresses one type of olfactory receptor (OR). Even though an antenna accommodates thousands of ORNs, the ORNs expressing the same type of OR aggregate together to form a spherical unit called a glomerulus in the antennal lobe. Thus, a chemical stimulus will activate certain glomerulus combinations and the identity of certain chemicals will thus be partly encoded by this combination (Carey and Carlson, 2011; Hill et al., 2009; Hallem and Carlson, 2006). To deeply understand the odor coding of mosquitoes, it is necessary to associate the response profiles from sensilla to OR and, ultimately, to the higher level central nervous system. Currently, researchers have demonstrated several OR response profiles for *Cx. quinquefasciatus* (Xu et al., 2014; Xu et al., 2013; Leal et al., 2013; Zhu et al., 2013; Hughes et al., 2010; Pelletier et al., 2010). In these studies, OR1 was shown to respond to benzaldehyde, 1-octen-3-ol and 6-methyl-5-hepten-2-one, which closely matches the response profiles for SST 1 and SBTII A obtained in this study.

By building a hierarchical clustering tree based on the response profile, three major groups were found to be differently encoded: ketones, aldehydes and aromatics (**Figure 2**). Interestingly, benzaldehyde and indole were also categorized among the aromatics. The benzene ring in both is likely to be the reason why benzaldehyde and indole fall into the same group as the aromatics, suggesting that the odor coding of *Cx. quinquefasciatus* is in fact based on chemical structures.

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Table 1. Human odorants within 11 categories used as stimuli in this study.

Chemicals	CAS	Company	Purity	Chemicals	CAS	Company	Purity
Carboxylic acids				**Aromatics and Aliphatics**			
acetic acid	64-19-7	Sigma	99	hexane	110-54-3	Sigma	95
propionic acid	79-09-4	Fisher	99.5	n-heptane	142-82-5	Sigma	99
hexanoic acid	142-62-1	Sigma	99.5	n-octane	111-65-9	Sigma	98
heptanoic acid	111-14-8	Sigma	96	n-nonane	111-84-2	Fisher	100
octanoic acid	124-07-2	Sigma	98	n-decane	124-18-5	Fisher	99
n-nonanoic acid	112-05-0	Sigma	97	2,4-dimethyl hexane	589-43-5	Fidher	99
decanoic acid	334-48-5	Sigma	98	n-pentadecane	629-62-9	Acros Organics	99
lauric acid	143-07-7	Sigma	99	hexadecane	544-76-3	Acros Organics	99
n-tridecanoic acid	638-53-9	Sigma	98	n-heptadecane	629-78-7	Sigma	99
myristic acid	544-63-8	Sigma	98	n-octadecane	593-45-3	Sigma	99
n-pentadecanoic acid	1002-84-2	Sigma	99	benzene	71-43-2	Sigma	99.8
heptadecanoic acid	506-12-7	Sigma	98	ethylbenzene	100-41-4	Sigma	99
acrylic acid	79-10-7	Acros Organics	99	propylbenzene	103-65-1	Sigma	98
undecanoic acid	112-37-8	Sigma	98	styrene	100-42-5	Sigma	99
benzoic acid	65-85-0	Sigma	99.5	squalene	111-02-4	Sigma	98
adepic acid	124-04-9	Acros Organics	99	toluene	108-88-3	Sigma	99.8
pimelic acid	111-16-0	Acros Organics	98	xylene	106-42-3	Sigma	99.5
4-hydroxybenzoic acid	99-96-7	Acros Organics	99	2-pentene	109-68-2	Aldrich	99
L-(+)-lactic acid	79-33-4	Sigma	98	trans-2-octene	13389-42-9	Aldrich	97
DL-3-methylvaleric acid	105-43-1	Acros Organics	97	trans-3-octene	14919-01-8	Aldrich	98
trans-2,3-dimethylacrylic acid	80-59-1	Acros Organics	98	trans-4-octane	14850-23-8	Aldrich	98
				1-hexadecene	629-73-2	Aldrich	99
Aldehydes				1-tetradecene	1120-36-1	Aldrich	97
propanal	123-38-6	Sigma	97	**Esters**			
butanal	123-72-8	Sigma	99	methyl tridecanoate	1731-88-0	Acros Organics	97
pentanal	110-62-3	Sigma	97	methyl nonanoate	1731-84-6	Acros Organics	95
hexanal	66-25-1	Sigma	98	**Ketones**			
heptenal	111-71-7	Sigma	92	2-butanone	78-93-3	Sigma	99.7
octanal	124-13-0	Sigma	99	2-pentanone	107-87-9	Fisher	99
nonanal	124-19-6	Aldrich	95	2-hexanone	591-78-6	Fluka	96
decanal	112-31-2	Sigma	98	2-decanone	693-54-9	Aldrich	98
Isobutanal	78-84-2	Sigma	99	3-pentanone	96-22-0	Fisher	99
2-methylbutanal	96-17-3	Sigma	90	sulcatone	110-93-0	Sigma	98
benzaldehyde	100-52-7	Sigma	99	**Halides**			
Alcohols				1-chloroheptane	629-06-1	Aldrich	99
p-cresol	106-44-5	Acros Organics	99	lauryl chloride	112-52-7	Acros Organics	99
4-methylphenol	123-07-9	Acros Organics	97	1-chlorotetradecane	2425-54-9	Acros Organics	98
1-hexen-3-ol	4798-44-1	Sigma	98	1-chlorohexadecane	4860-03-1	Aldrich	95
cis-2-hexen-1-ol	928-94-9	Aldrich	95	1-chlorohexane	544-10-5	Fisher	95

trans-2-hexen-1-ol	928-95-0	Acros Organics	96	benzyl chloride	100-44-7	Sigma	99
trans-2-octen-1-ol	18409-17-1	Acros Organics	98	**Amines**			
2-decanol	1120-06-5	Sigma	98	propylamine	107-10-8	Aldrich	99
phenelethyl alcohol	60-12-8	Sigma	99	butylamine	109-73-9	Aldrich	99.5
glycerol	56-81-5	Sigma	99	ammonia	7664-41-7	Aldrich	100
phenol	108-95-2	Sigma	99	**Sulfides**			
1-tetradecanol	112-72-1	Sigma	97	carbon disulfide	75-15-0	Fisher	99.9
2-hexadecanol	14852-31-4	Sigma	99	methyl disulfide	624-92-0	Sigma	99
1-octen-3-ol	3391-86-4	Aldrich	99	**Ureas**			
Heterocyclics				methyl urea	598-50-5	Sigma	97
N-piperidineethanol	3040-44-6	Acros Organics	99	thiourea	62-56-6	Sigma	99
1-methylpiperazine	109-01-3	Acros Organics	99.5	urea	57-13-6	Sigma	99
2-methylfuran	534-22-5	Acros Organics	99				
thiazolidine	504-78-9	Acros Organics	98	DMSO	67-68-5	Sigma	100
3-methylindole	83-34-1	Acros Organics	98				
3-aminopyridine	462-08-8	Acros Organics	99				
4-aminopyridine	504-24-5	Acros Organics	98				
pyridine	110-86-1	Acros Organics	100				
2,6-dimethylpyrazine	108-50-9	Acros Organics	96				
coumarin	91-64-5	Acros Organics	99				
4-piperidinemethanamine	7144-05-0	Acros Organics	97				
2-picoline	109-06-8	Acros Organics	98				
indole	120-72-9	Aldrich	99				

*All stimuli were diluted to 10µg/µL. DMSO was tested as control with 100% purity. Benzene, ethylbenzene, styrene, toluene, xylene, indole, hexanal, heptanal, octanal, nonanal and decanal were used in dose-dependent tests and diluted to a series of 0.001µg/µL, 0.01µg/µL, 0.1µg/µL, 1µg/µL and 10µg/µL.

	SST 1	SST 2	SBTI A	SBTI B	SBTII A	SBTII B	LST	PG
Carboxylic Acids								
acetic acid	○	○	○	○	○	○	○	○
myristic acid	○	○	○	○	○	○	○	○
hexanoic acid	○	○	○	○	○	○	○	○
heptanoic acid	○	○	○	○	○	○	○	○
propionic acid	○	○	○	○	○	○	○	○
heptadecanoic acid	○	○	○	○	○	○	○	○
L-(+)-lactic acid	○	○	○	○	○	○	○	○
n-pentadecanoic acid	○	○	○	○	○	○	○	○
benzoic acid	○	○	○	○	○	○	○	○
trans-2,3-dimethylacrylic acid	•	○	○	○	○	○	○	○
DL-3-methylvaleric acid	○	○	○	○	○	○	○	○
octanoic acid	○	○	○	○	●	○	○	○
decanoic acid	○	○	○	○	○	○	○	○
undecanoic acid	○	○	○	○	○	○	○	○
lauric acid	○	○	○	○	○	○	○	○
n-tridecanoic acid	○	○	○	○	○	○	○	○
adepic acid	○	○	○	○	○	○	○	○
pimelic acid	○	○	○	○	○	○	○	○
4-hydroxybenzoic acid	○	○	○	○	○	○	○	○
acrylic acid	○	○	○	○	○	○	○	○
n-nonanoic acid	○	○	○	○	•	●	○	○
Aldehydes								
hexanal	●	●	○	○	○	●	○	○
propanal	●	○	○	○	○	●	○	○
decanal	●	○	•	●	○	●	○	○
nonanal	●	○	•	●	○	●	○	○
benzaldehyde	•	○	○	○	●	●	○	○
heptenal	●	•	○	•	○	●	○	○
pentanal	●	●	○	○	○	●	○	○
octanal	●	•	•	●	○	●	○	○
butanal	○	○	○	○	○	•	○	○
isobutanal	○	○	○	○	○	•	○	○
2-methylbutanal	○	●	○	○	●	•	○	○
Alcohols								
2-decanol	○	○	○	○	–	•	○	○
phenol	○	○	○	○	○	○	○	○
trans-2-hexen-1-ol	○	○	○	○	○	●	○	○
1-tetradecanol	○	○	○	○	○	○	○	○
2-hexadecanol	○	○	○	○	○	○	○	○
cis-2-hexen-1-ol	○	○	○	○	○	●	○	○
glycerol	○	○	○	○	○	•	○	○
1-hexen-3-ol	●	●	○	○	●	○	○	○

	SST 1	SST 2	SBTI		SBTII		LST	PG
			A	B	A	B		
1-octen-3-ol	●	●	○	○	●	•	○	○
trans-2-octen-1-ol	○	○	○	○	○	●	○	○
4-ethyl phenol	○	○	○	○	○	○	○	○
p-cresol	○	○	○	○	○	○	○	○
phenelethyl alcohol	○	○	○	○	○	•	○	○
Aliphatics/Aromatics								
n-heptadecane	○	○	○	○	○	○	○	○
toluene	○	○	○	○	●	○	○	○
1-hexadecene	○	○	○	○	○	○	○	○
1-tetradecene	•	○	○	○	○	○	○	○
n-nonane	○	○	○	○	○	○	○	○
benzene	○	○	○	○	●	○	○	○
squalene	○	○	○	○	○	○	○	○
propylbenzene	•	○	•	○	●	○	○	○
n-pentadecane	○	○	○	○	○	○	○	○
hexadecane	○	○	○	○	○	○	○	○
trans-2-octene	○	○	○	○	○	○	○	○
n-octadecane	○	○	○	○	○	○	○	○
styrene	○	○	○	○	●	○	○	○
n-decane	○	○	○	○	○	○	○	○
xylene	○	○	○	○	●	○	○	○
ethylbenzene	○	○	○	○	●	○	○	○
2,4-dimethyl hexane	○	○	○	○	○	○	○	○
trans-4-octene	○	○	○	○	○	●	○	○
trans-3-octene	○	○	○	○	○	•	○	○
n-octane	○	○	○	○	○	•	○	○
2-pentene	○	○	○	○	○	•	○	○
hexane	○	○	○	○	○	•	○	○
n-heptane anhyd	○	○	○	○	○	○	○	○
Esters								
methyl nonanoate	●	○	•	●	○	●	●	○
methyl tridecanoate	○	○	○	○	○	○	○	○
Ketones								
2-hexanone	●	●	○	○	○	●	○	○
2-pentanone	●	●	○	○	○	○	○	○
3-pentanone	●	●	○	○	●	•	○	○
2-decanone	●	○	●	•	–	•	○	○
2-butanone	○	○	○	○	○	○	○	○
6-methyl-5-hepten-2-one	●	○	•	•	●	○	○	○
Amines								
propylamine	○	○	○	○	○	○	○	●
butylamine	○	○	○	○	○	○	○	●
ammonia	○	○	○	○	○	○	○	●

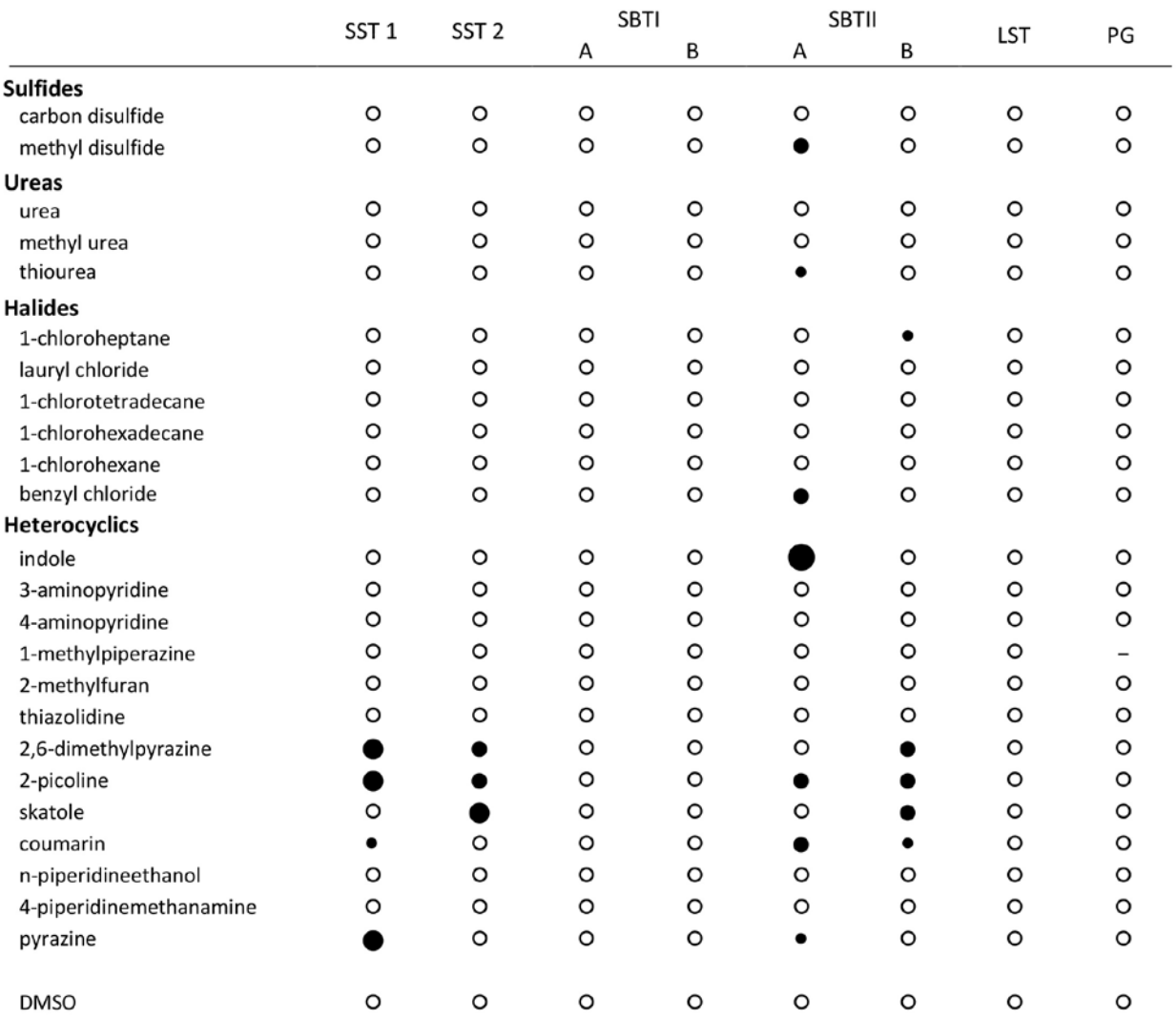

Figure 1. Response profiles of SST, SBTI, SBTII, LST and GP to 103 types of human odorants. Responses less than 15 spikes were considered as no response (○). Black circles indicate less than 20% (●), 20-50% (●), 50-80% (●), and more than 80% (●) responding spikes of the highest response which is 155 spikes/s.

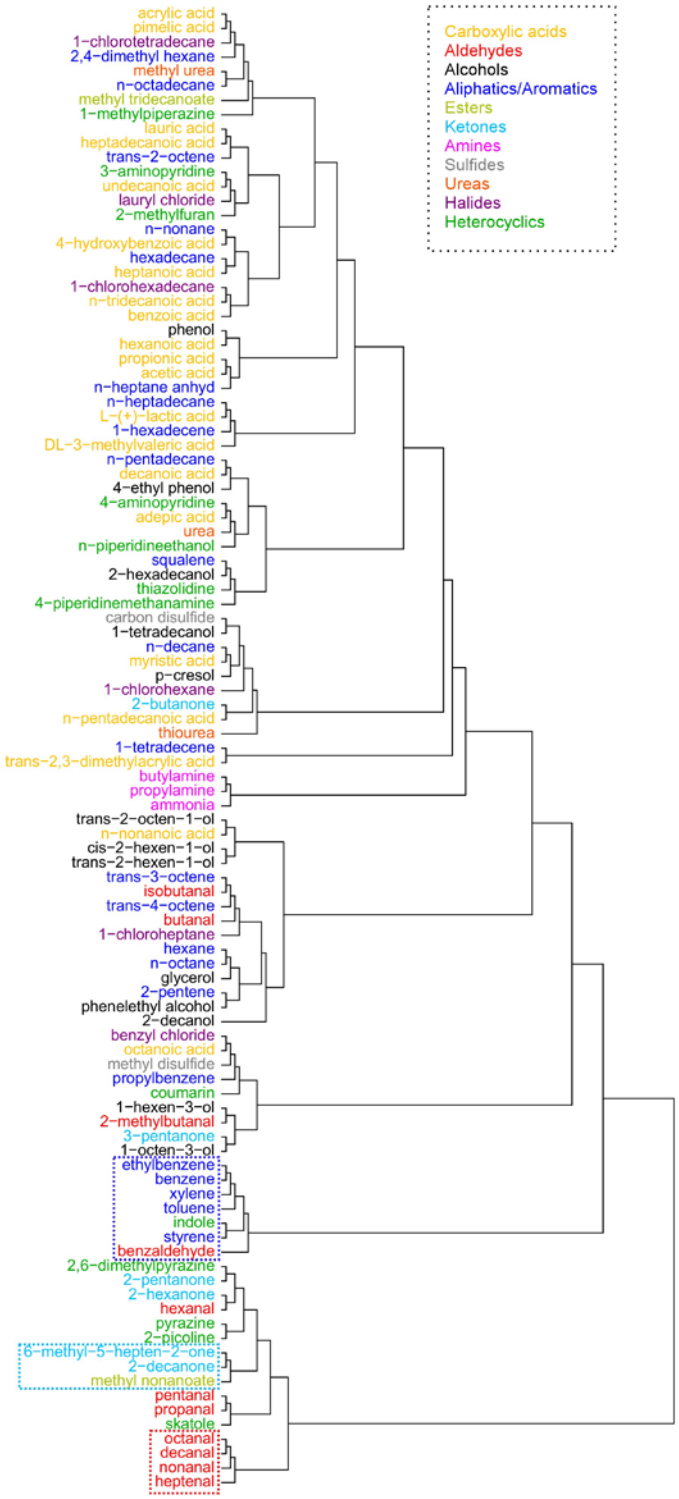

Figure 2. Hierarchical cluster analysis based on Euclidean distance between chemical responses. Chemicals within the same categories were marked with the same color. Clusters mainly consisted by aldehydes, aromatics and ketones were outlined.

Chapter 4: Dose-dependent Patterns and Temporal Dynamics

4.1 Abstract

In insect antennae, chemical information is largely encoded in terms of quality (response profiles), quantity (dose-dependent patterns) and temporal dynamics. Researchers have emphasized the importance of temporal dynamics for chemical discrimination by showing that engineered insects with a single ORN are still able to distinguish odorants and modulate their behavior accordingly. To more deeply understand the mechanisms involved in mosquito odor coding, we conducted an electrophysiological study to investigate the dose-dependent patterns and temporal dynamics of SBTI and SBTII antennal sensilla to eleven human odorants previously found to elicit strong neuronal responses using single sensillum recording. The results obtained showed that all the neuronal responses followed a dose-dependent curve. Styrene and indole, which share similar structures based on benzene ring substituent groups, presented different temporal dynamics from the other aromatics, which suggests that temporal dynamics may be encoded based on chemical structures.

A behavioral study showed that nonanal, decanal and octanal elicited opposite behaviors at different concentrations. To generalize our understanding of odorant-mediated behaviors, we conducted a hand-in-cage bioassay using heptanal, which elicited strong mosquito neuronal responses. These results confirm the earlier hypothesis that heptanal, like other aldehydes, stimulates opposite mosquito behavior at different concentrations

4.2 Introduction

Mosquitoes act as disease vectors due to their blood-feeding behavior (Harbach, 2007; Triplehorn and Johnson, 2005). The southern house mosquito, *Culex quinquefasciatus*, is the primary vector transmitting fatal diseases in the U.S., particularly West Nile virus (WNV) and Saint Louis encephalitis virus (SLEV). The first case of WNV was reported in New York, 1999 and by 2013 the disease had caused 39,557 cases of human infection and 1,668 deaths across the U.S., affecting every state aprt from Alaska and Hawaii. WNV maintains its population in birds and is then transmitted to humans by *Culex* mosquitoes. Hitherto, no vaccine has been found with activity against WNV. Since the mosquito plays a key role in WNV transmission, mosquito management is thus the most effective way to control the disease's prevalence (CDC, 2014; Savage et al., 2007).

Mosquitoes rely on their olfactory system for long-range host seeking (Logan et al., 2008; Zwiebel and Takken, 2004). Odorants from hosts are detected by olfactory receptor neurons (ORNs) housed in hair-like sensilla on the mosquito's olfactory organs. The antennae are the most essential of the mosquito's olfactory organs since they accommodate the majority of the olfactory neurons (Hill et al., 2009; Syed and Leal, 2009; Lu et al., 2007; Hallem et al., 2006). Olfactory receptors (ORs), which are ligand-gated channels embedded across the ORN membrane, are responsible for transforming the information carried by chemical cues into neuronal electrical signals. This signal transduction alters the active potential frequency on the ORN membrane, which can then be detected by electrophysiological approaches as an indicator of neuronal response magnitude. Six morphological types of antennal sensilla (SST, SBTI, SBTII, LST, SST-C, GP) were identified on *Cx. quinquefasciatus* and each has been shown to

exhibit distinct neuronal response profiles to chemical cues (Guidobaldi et al., 2014; Suh et al., 2014; Liu et al, 2013; Pellegrino et al., 2010; Hill et al., 2009; Qiu et al., 2006).

The chemical information is largely encoded by insect antennae in terms of its quality (response profiles), quantity (dose-dependent patterns) and temporal dynamics (Guidobaldi et al., 2014; Carey and Carlson, 2011; Su et al., 2011; Hill et al., 2009). The chemical quality and quantity are encoded based on the identity and number of activated ORs. Temporal dynamic coding largely relies on the properties of the ORs. Researchers have emphasized the importance of temporal dynamics for chemical discrimination, reporting that engineered insects with a single ORN are still able to distinguish odorants and modulate their behavior accordingly (Su et al., 2011; Su et al., 2009; Hallem and Carlson, 2006).

A number of human odorants have been identified as mosquito attractants by behavioral studies, including ketones, carboxylic acids and aldehydes (McBride et al., 2014; Syed and Leal, 2009; Puri et al., 2006; Douglas et al., 2005). Octanal, decanal and nonanal all display attraction at low concentration for *Cx. quinquefasciatus* but repellency at relatively high concentrations (Logan et al., 2010); although heptanal has been shown to be attractive to *Cx. quinquefasciatus* at 0.02μg/μL, its repellency at higher concentrations has not yet been demonstrated (Puri et al., 2006).

In this study, single sensillum recording was conducted to investigate the dose-dependent patterns and temporal dynamics of neuronal responses in *Cx. quinquefasciatus*. To generalize this to enhance our understanding of odorant-mediated behaviors, we also conducted a bioassay using heptanal, which is known to elicit a strong mosquito neuronal response.

4.3 Materials and methods

4.3.1 Southern house mosquitoes

Southern house mosquitoes, *Cx. quinquefasciatus*, were collected from Huntsville, Alabama, USA. Mosquitoes were reared at 25±2°C under a L: D cycle of 12:12 hours and supplied with a 10% sucrose solution (Liu et al., 2011).

4.3.2 Single sensillum recording

Female mosquitoes 4 days after emerging were anaesthetized on ice and mounted on a microscope slide (76×26mm). The antennae were fixed using double-sided tape to a cover slip resting on a small ball of dental wax to facilitate manipulation and the cover slip was placed at an appropriate angle to the mosquito head. Once mounted, the specimen was placed under a Leica Z6 APO microscope and the antennae were viewed at high magnification (720×). Two tungsten microelectrodes were sharpened in 10% KNO_2 at 2-10V to a ≈1μm tip diameter. The reference electrode, which was connected to ground, was inserted into the compound eye of the mosquito and the other was connected to the preamplifier (10×, Syntech, Kirchzarten, Germany) and inserted into the shaft of the trichoid sensillum to complete the electrical circuit to extracellularly record ORN potentials. Controlled manipulation of the electrodes was performed using two micromanipulators (Leica, Germany). The preamplifier was connected to an analog to digital signal converter (IDAC-4, Syntech, Germany), which in turn was connected to a computer for signal recording and visualization. The activity of co-located ORNs in each sensillum was assessed based on the differences in spike amplitude. Neuron cells with larger spike amplitudes were indicated as cell A, while cells with smaller spike amplitudes were called cell B. Signals were recorded for 10s starting 1s before stimulation, and the action potentials were counted off-line over a 500ms period before and after stimulation. Spike rates observed during the 500ms stimulation were subtracted from the spontaneous activities observed in the preceding 500ms and counts recorded in units of spikes/s (Liu et al., 2013).

4.3.3 Stimulation and stimuli

Eleven odorants eliciting strong neuronal responses on SBTI and SBTII sensilla were used in this study (Hexanal, heptanal, octanal, nonanal, decanal, benzene, ethylbenzene, xylene, toluene, styrene and indole) (**Table 1**). Each human odorant was diluted in dimethyl sulfoxide (DMSO) to a concentration of 100μg/μL as a stock solution and a concentration series of 0.001μg/μL, 0.01μg/μL, 0.1μg/μL, 1μg/μL, 10μg/μL used for the stimulation. For each dilution, a 10μL portion was dispersed onto a filter paper (3×10mm) that was then inserted into a Pasteur pipette to create the stimulus cartridge for that repellent dilution. A sample containing the pure solvent alone served as the control. The airflow across the antennae was maintained at a constant 20mL/s throughout the experiment. Purified and humidified air was delivered to the preparation through a glass tube (10mm inner diameter) perforated by a small hole 10cm away from the end of the tube into which the tip of the Pasteur pipette could be inserted. The stimulus was delivered to the sensilla by inserting the tip of the stimulus cartridge into this hole and diverting a portion of the air stream (0.5L/min) to flow through the stimulus cartridge for 500ms using a stimulus controller (Syntech, Germany). The distance between the end of the glass tube and the antennae was 61cm. All chemicals were tested on each type of antennal sensillum at least 3 times (Liu et al., 2013). The number of spikes/s was obtained by averaging the results of each sensillum/chemical combination. The temporal dynamics were recorded in the form of the response frequency from one second before the stimulation to four seconds afterwards.

4.3.4 Hand-in-cage bioassay

A modified hand-in-cage bioassay (Logan et al., 2010; Douglas et al., 2005) was used to investigate the behavioral functions of heptanal at 10μg/μL. The trials were performed in collapsible cages (20cm ×20cm ×20cm; Bioquip). 25 1-3-week-old female adult mosquitoes

were added into each cage and starved for 24 hours. The mosquitoes had never taken a blood meal or encountered the human odorants tested. Two male student volunteers were recruited from Auburn University. Volunteers washed their hands and wrists with unscented hand soup before the trials. Paper circles (9cm diameter) were cut out from chromatography paper (Whatman) and loaded with 50μL heptanal as the treatment; DMSO was used as the control. In each trial, one paper circle was attached to and fully covered the back of the fist with a rubber band. Chemicals were uniformly dispersed along the edge of the paper circles. The trial began with the fist and its attached control paper circle being inserted into the cage and brought to rest on the bottom of the cage. The number of mosquitoes landing on the hand was recorded for consecutive 15s-periods for 3mins. The fist was rolled over to count the mosquitoes beneath, and the mosquitoes were then gently shaken off every 15s. The treatment was conducted with another hand bring inserted into the same cage immediately after the control trial was over. Each cage was only tested once. The repellency was quantified by comparing the total number of mosquitoes landing on the controls with those on landing on the treatments during the 3mins.

4.3.5 Statistics

The temporal dynamics and dose-dependent data were analyzed by GraphPad Prism 5.0 (GraphPad Software Inc, CA). The dose-dependent curves were fitted with Graphpad Prism 5.0 using the equation: Y=Bottom+(Top-Bottom)/(1 +10^[LogEC50-X]). Hierarchical cluster analysis of the temporal dynamics data was conducted by R 3.1.1 (R Foundation for Statistical Computing, Vienna, Austria). The results of the hand-in-cage bioassay were analyzed using independent 2-group t-test by R 3.1.1 (R Foundation for Statistical Computing, Vienna, Austria).

4.4 Results

4.4.1 Dose-dependent response analysis

Dose-dependent tests were performed on SBTII A (**Figure 3A**) and SBTII B (**Figure 3B**) with 11 chemicals that were known to elicit strong responses. Responding cells were identified at low concentration (**Figure 3C**). All the chemicals tested showed a clearly dose-dependent pattern, with positive slopes in most cases. Benzene, ethylbenzene, indole, styrene, toluene and xylene all elicited strong excitatory responses on SBTII A at 10µg/µL, while toluene elicited responses from 17±3 to 160±2 spikes/s even at the lowest threshold of 0.01µg/µL. Indole, styrene and benzene exhibited a 1 order of magnitude higher threshold than toluene, while xylene and ethylbenzene displayed the highest thresholds at 1µg/µL. SBTII B responded strongly to hexanal, heptanal, octanal, nonanal and decanal at 10µg/µL. Negative slopes of the dose-dependent curves were recorded for nonanal (152±24 spikes/s at 1µg/µL; 143±25 spikes/s at 10µg/µL) and octanal (144±11 spikes/s at 1µg/µL; 129±12 spikes/s at 10µg/µL).

4.4.2 Temporal dynamics of the olfactory responses

A temporal analysis was conducted based on the dose-dependent data to investigate different temporal patterns at 10µg/µL. In this study, typical phasic/short-truncated neuronal responses were observed from certain aromatic human odors, such as xylene, toluene, ethylbenzene and benzene (**Figure 4A**). Cluster analysis based on the temporal structure of neuronal responses indicated that styrene and indole were more likely to be distinguished from the other aromatics, with a long-lasting tonic pattern with a frequency above 50Hz lasting more than 2 seconds after the onset of stimulation, much longer than that for the other aromatics, which all lasted for less than 1 second (**Figure 4B**). Typical tonic/super-sustained neuronal responses were observed from certain aldehydes from hexanal to decanal (**Figure 4C**). Also, cluster analysis suggested

that mosquitoes are more likely to identify heptanal from other aldehyde human odors (**Figure 4D**).

4.4.3 Hand-in-cage bioassay

Heptanal was used as the chemical cue to investigate *Cx. quinquefasciatus* behavior in this study. As noted above, several aldehydes displayed opposite behavioral function at different concentrations (Logan et al., 2010). In this study, heptanal which had previously been reported to function as a mosquito attractant at relatively low concentrations (Puri et al., 2006), instead showed significant repellent impact ($P<0.05$) at higher concentrations (**Figure 5**).

4.5 Discussion

In this research, three aspects of insect peripheral odor-coding were analyzed: response profiles (quality), dose-dependent patterns (quantity) and temporal dynamics (Guidobaldi et al., 2014; Carey and Carlson, 2011; Su et al., 2011; Hill et al., 2009). By building a hierarchical clustering tree based on the response profile, three major groups were found to be differently encoded: the ketones, the aldehydes and the aromatics (**Figure 2**). Within each major group, the dose-dependent patterns of chemicals were similar but the temporal dynamics of the neuronal responses was different, which suggests that temporal dynamics might be most decisive when encoding chemicals with similar structures. It is worth mentioning that indole and styrene are similarly encoded on all three aspects, which drew our attention to their chemical structure. Indole and styrene share benzene rings with other aromatics, and the presence of a benzene ring might be the reason why indole is in the group that otherwise consists primarily of aromatics. The carbon-carbon double bond in the substituent on the benzene ring might be the key structure

which causes indole and styrene to activate a long-sustained neuronal response that is different from that of other aromatics (**Figure 6**).

Concentration dramatically influences the behavioral function of aldehydes to *Cx. quinquefasciatus*. Although octanal and decanal functioned as mosquito repellents at a high concentration (10%), both significantly lost their repellency and even showed attraction at lower concentrations (0.1% and 0.001%, respectively) (Logan et al., 2010; Douglas et al., 2005), which suggests that aldehydes might help mosquitoes track hosts at moderate doses but actually repels mosquitoes at more intense doses since aldehydes elicit very strong neuronal excitation signals and might thus scare mosquitoes away. Our behavioral bioassay proved the same mechanism was in operation for heptanal as this neuronal "over-excitation" was found in our experiments. Nonanal and octanal optimized their neuronal response with increasing concentration from 0.001 to 1μg/μL, but the response declined when the concentration rose to 10μg/μL.

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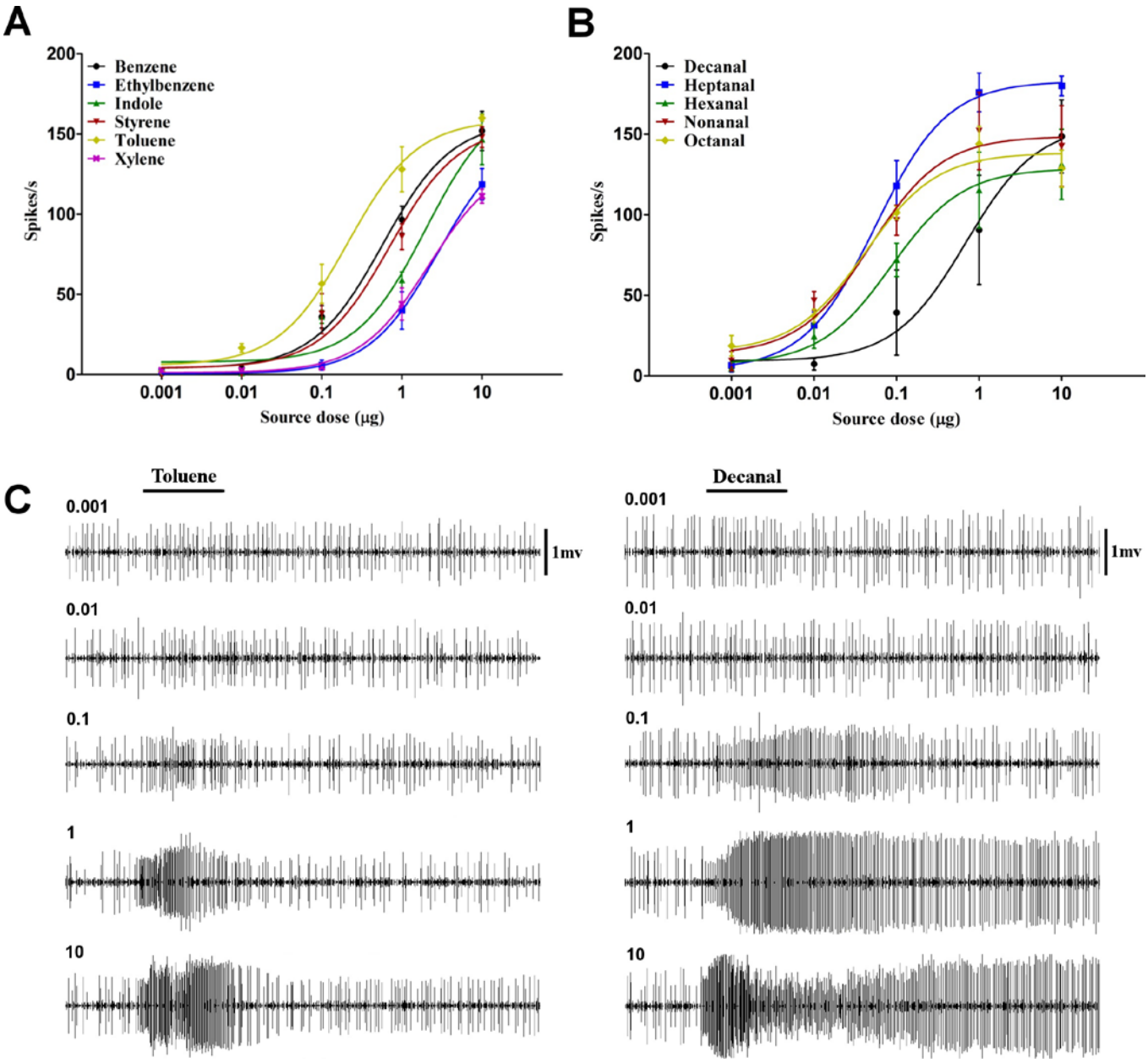

Figure 3. Dose-dependent curves of SBTII A to 5 aromatics and indole (**A**) and SBTII B to 5 aldehydes (**B**); Dose-dependent neuronal responses of SBTII with large amplitude spikes to toluene and small amplitude spikes to decanal (**C**). Horizontal bars beneath the chemical names indicate the duration of stimulation (500ms).

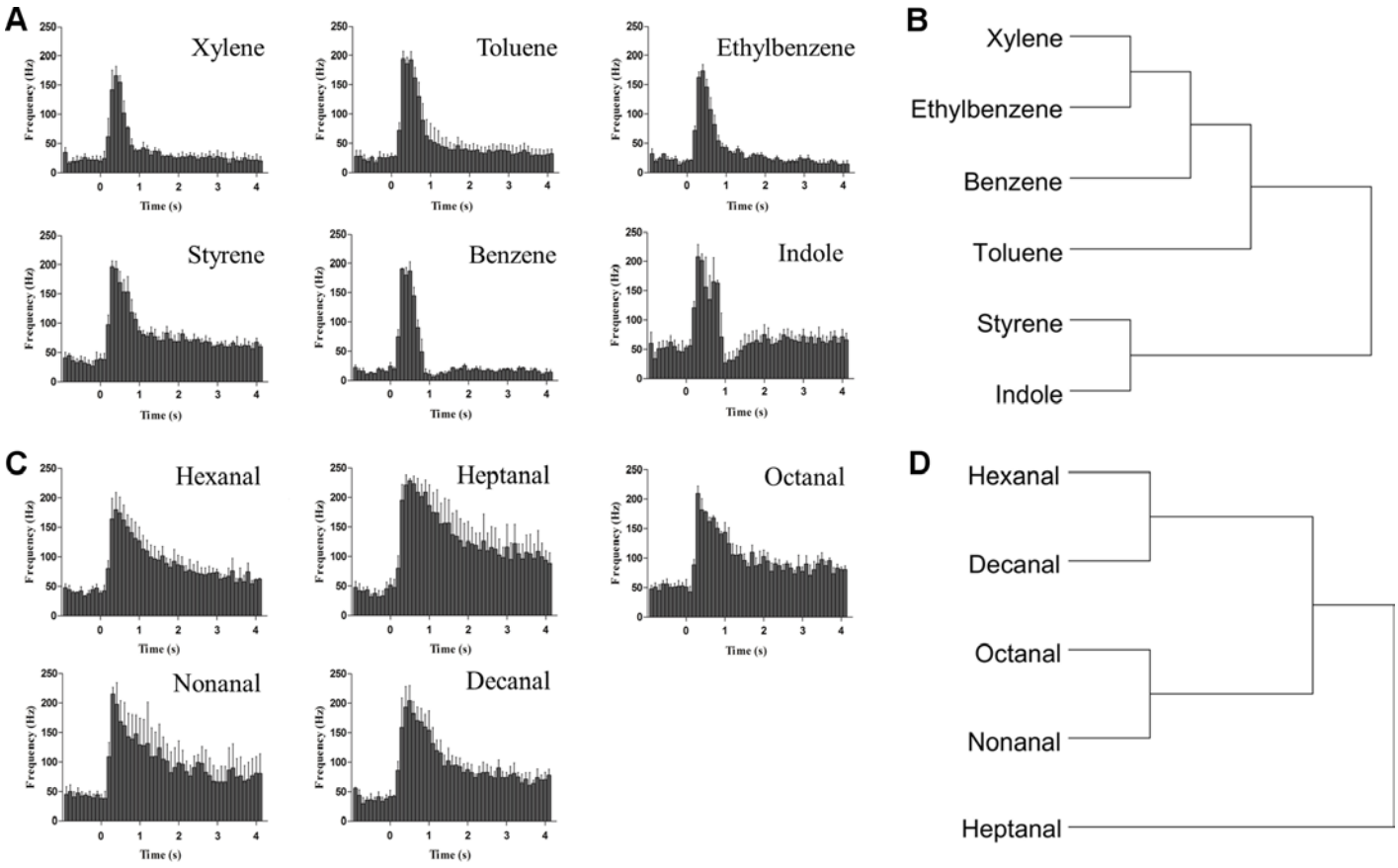

Figure 4. Temporal structure of neuronal responses to different human odorants, (**A**): aromatics and indole; (**C**): aldehydes and the corresponding relationships within the aromatics (**B**) and aldehydes (**D**) using hierarchical cluster analysis based on Euclidean distance.

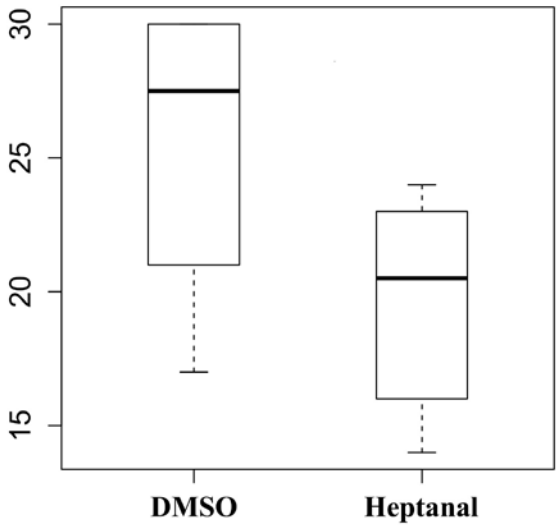

Figure 5. Number of mosquito landing in 3min using DMSO and 10µg/µL heptanal as repellents (n=6, P<0.05).

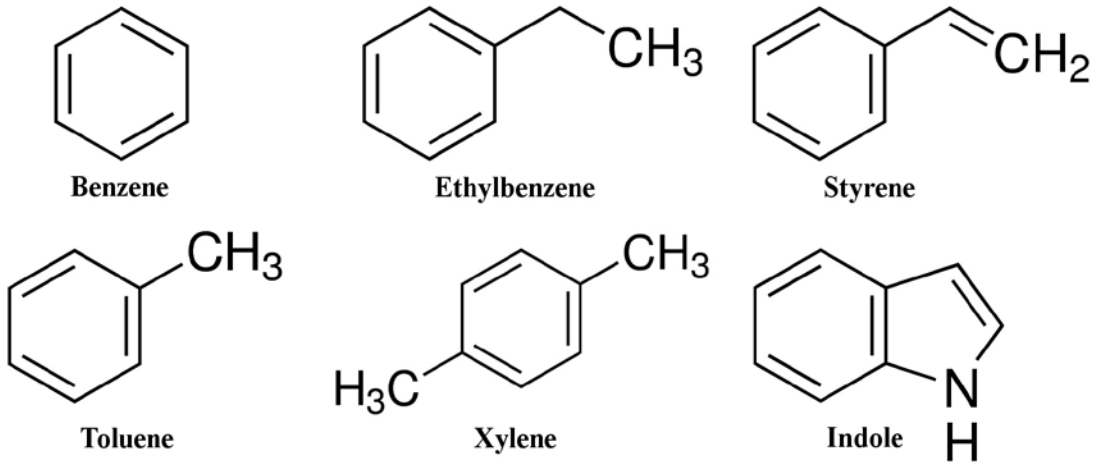

Figure 6. Chemical stuctures of benzene, toluene, ethylbenzene, xylene, styrene and indole.

Chapter 5: Future Studies

5.1 The antennal lobe three-dimensional structure of *Cx. quinquefasciatus*

5.1.1 Introduction

Mosquitoes are major disease vectors due to their blood-feeding behavior (Harbach, 2007; Triplehorn and Johnson, 2005). The southern house mosquito, *Culex quinquefasciatus*, is the primary vector transmitting fatal diseases in the U.S., including both West Nile virus (WNV) and Saint Louis encephalitis virus (SLEV). Since the first case of WNV was reported in New York in 1999, by 2013 WNV had caused 39,557 cases of human infection and 1,668 deaths across the U.S. affecting every state apart from Alaska and Hawaii. WNV maintains its population in birds from whence it is transmitted to humans by *Culex* mosquitoes. As yet, no vaccine has been found with activity against WNV. Since mosquito plays a key role in WNV transmission, mosquito management is currently the most effective way to control the prevalence of this serious disease (CDC, 2014; Savage et al., 2007).

Mosquitoes rely on their olfactory system for long-range host seeking (Logan et al., 2008; Zwiebel and Takken, 2004). Odorants from hosts are detected by olfactory receptor neurons (ORNs) housed in hair-like sensilla on the mosquito's olfactory organs. Its antenna serve as the mosquito's most essential olfactory organ since they contain the majority of its olfactory neurons (Hill et al., 2009; Syed and Leal, 2009; Lu et al., 2007; Hallem et al., 2006). Olfactory receptors (ORs), which are ligand-gated channels embedded across the ORN membrane, are responsible

for transforming the information carried by chemical cues into neuron electrical signal (Guidobaldi et al., 2014; Suh et al., 2014).

ORNs project their axons into the antennal lobes, the primary center processing olfactory information. Typically, each ORN only expresses one type of OR. The ORNs expressing the same OR converge together in the antennal lobes and form a unit which is called a glomerulus. Each glomerulus links to projection neurons that transmit the neuronal signals into higher-level brain regions. Different odorants are thus able to activate different combination patterns of glomeruli, and the identities of the odorants are encoded by these patterns. The number of glomeruli within antennal lobes is determined by the insect species. Antennal lobe fluorescent staining and confocal microscopy techniques have been used to demonstrate the three-dimensional (3D) structure of antennal lobes (Suh et al., 2014; Carey and Carlson, 2011; Hallem et al., 2006; Anton et al., 2003; Hansson and Anton, 2000). The antennal lobe 3D structures of *A. aegypti* and *A. gambiae* have been constructed and annotated by previous researchers in this area. *A. aegypti* presented 50 and 49 glomeruli in female and male mosquitoes, respectively, while *A. gambiae* showed 60 and 61 glomeruli in its females and males (Ghaninia et al., 2007a; Ignell et al., 2005).

The structures of the antennal lobes and the spatial relationship between glomeruli are highly involved in the odor coding and can thus help us to understand the mechanisms of odorant identification and recognition (Ghaninia et al., 2007a; Wilson and Mainen, 2006; Ignell et al., 2005). This combination of electrophysiological and antennal lobe fluorescent staining makes it possible to investigate the affiliation between each sensillum and its cognate glomerulus (Ghaninia et al., 2007b). In this study, anterograde antennal lobe staining and confocal

microscopy were conducted to demonstrate the three dimensional structure of the *Cx. quinquefasciatus* antennal lobes.

5.1.2 Materials and methods

5.1.2.1 Southern house mosquitoes

Southern house mosquitoes, *Cx. quinquefasciatus*, were collected from Huntsville, Alabama, USA. Mosquitoes were reared at 25±2°C under an L: D cycle of 12:12 hours and supplied with 10% sucrose solution (Liu et al., 2011).

5.1.2.2 Anterograde antennal lobe staining

Mosquitoes were mounted on Petri dishes using double-sided tape. The antennae were cut off with two to three segments remaining. Open-end glass capillaries were filled with 2% biotin-dextran (Sigma-Aldrich, St. Louis, MO, USA) diluted in distilled water and placed over the cut end of the antennae. Mosquitoes were then kept in the fridge at 4ºC overnight. The mosquito brains were dissected out in Ringer's solution with fined forceps and fixed in 4% formaldehyde overnight. The brains were then washed four times for ten minutes each in 1% PBS solution and incubated in streptavidin conjugated to Alexa 488 (Molecular Probes, Eugene, OR, USA), after which they were then washed again six times for ten minutes each and mounted in Depex Mounting Medium (Electron Microscopy Sciences, Hatfield, PA, USA) (Das and Fadamiro, 2013; Ghaninia et al., 2007a).

5.1.2.3 Confocal microscopy and three-dimensional reconstruction

The brain samples were examined with a Nikon A1 confocal laser-scanning microscope. The Alexa 488 (conjugated to streptavidin) was excited at a wavelength of 488 nm in order to obtain

digital image stacks from laser scanning. Images from the brain tissues were obtained using 40× oil-immersion objectives at a resolution of 1024×1024 pixels. The optical sections from each antennal lobe were obtained in a stack of 100 images. Complete stacks of images were imported into the three-dimensional analysis software AMIRA 5.3.3 (Visage Imaging, San Diego, CA, USA) (Das and Fadamiro, 2013).

5.1.3 Preliminary Results

The three-dimensional structure of the antennal lobe was constructed out of the confocal microscopy image stacks (**Figure 7**). Each glomerulus was manually segmented. The width of a *Cx. quinquefasciatus* brain is typically around 698μm and the volume of the antennal lobe is around $105.4\times10^3\mu m^3$.

44 glomeruli were identified in the female *Cx. quinquefasciatus* antennal lobe, which suggests that 44 types of ORNs are accommodated in *Cx. quinquefasciatus* antennae. The antennal lobes are innervated by the nerves from antennae, maxillary palps and labia, but the anterograde antennal lobe staining technique was only able to reveal the glomeruli formed by the antennal nerves. Due to the relatively lower number of afferent nerves, anterograde staining suffered a low success rate and hence a low resolution for glomerulus differentiation (Ghaninia et al., 2007a).

Future studies will focus on fluorescent staining of the antennal lobes with antibodies. The monoclonal antibody nc82 has been used in several studies, and has shown the most distinct segmentation compared to other antibodies (Ghaninia et al., 2007a; Ignell et al., 2005; Laissue et al., 1999). Instead of staining the antennal lobes using nervous tracing, nc82 antibody directly binds to antennal lobes and can thus be detected by the secondary antibody conjugated with fluorescent dye. A possible sexual-dimorphism of the antennal lobes is another interesting topic

that could shed new light on our understanding of the mechanisms responsible for the olfactory and behavioral discrepancies between male and female insects, especially for blood-feeding insects due to the divergence in their feeding behavior.

5.2 Characterization of olfactory receptors

An olfactory receptor AeagOR4 controlling the detection of a human-specific odorant, sulcatone, has been identified in the mosquito *A. aegypti*, and this research has also revealed that mosquitoes with high AeagOR4 expression levels preferred feeding on humans to other mammals (McBride et al., 2014). The *Xenopus* oocyte system has brought an exceptional platform for use in characterizing olfactory receptors and olfactory receptors can now be expressed on the membrane of *Xenopus* oocytes. The functional agonism of olfactory receptors will be identified by detecting the cation influx using a voltage clamp. Based on the findings of the research presented in this thesis, *Cx. quinquefasciatus* are highly sensitive to aldehydes, ketones and aromatics. The identification of the molecular targets for these human odorants will promote our understanding of host recognition and the development of new mosquito control strategies.

5.3 Identification of glomerular targets of antennal sensilla

Earlier studies have revealed that insects encode chemicals by activating certain combinations of glomeruli (Ghaninia et al., 2007b). The anterograde single sensillum staining technique will make it possible to trace each type of sensilla and mark the relevant cognate glomeruli, thus bridging the gap between sensilla and glomeruli. This study provides further evidence demonstrating the importance of further investigations of the mosquito's odor-coding mechanisms at the central nervous system level.

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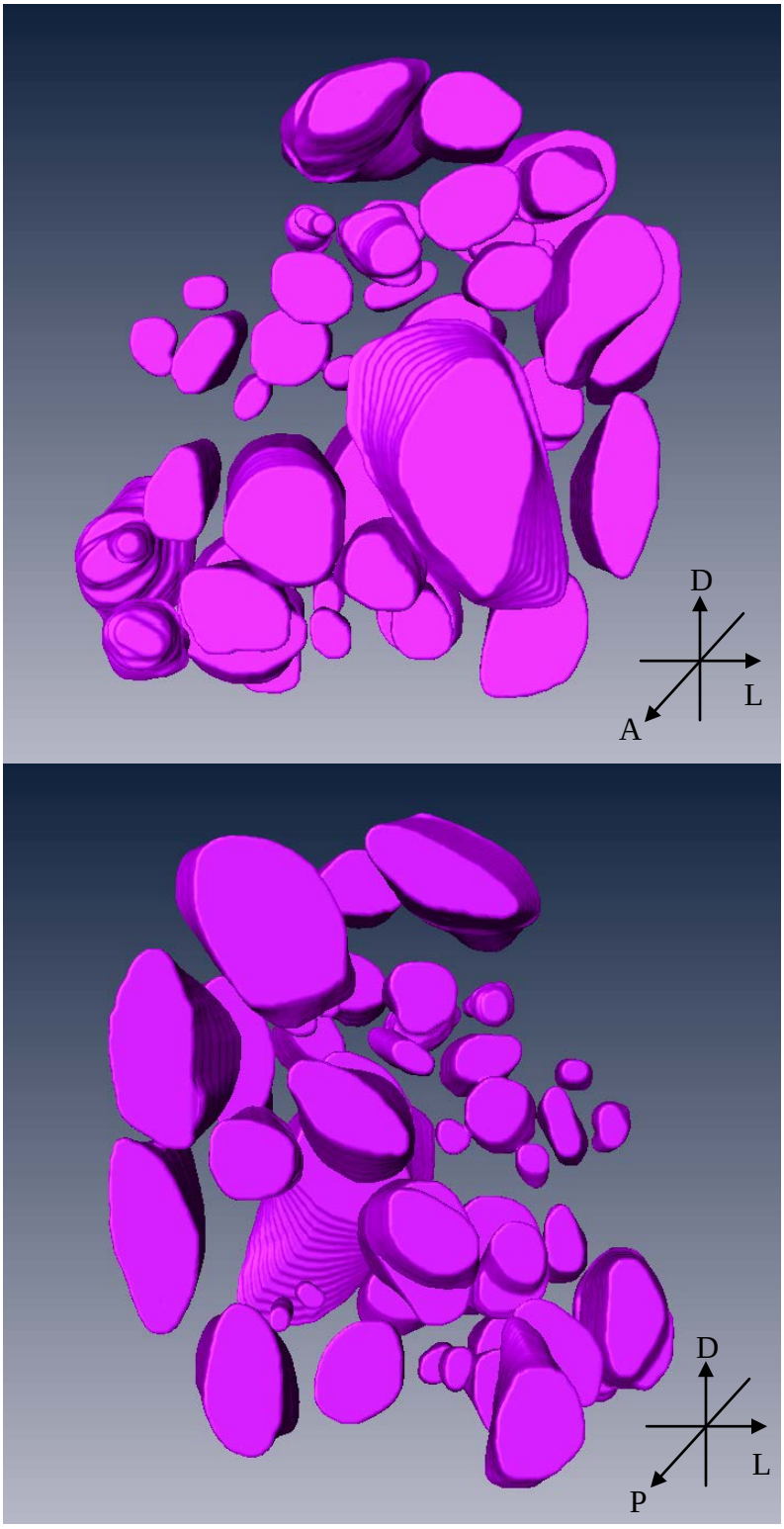

Figure 7. Three-dimensional structure of *Cx. quinquefasciatus* antennal lobe. A: anterior; P: posterior; L: lateral; D: dorsal.

Chapter 6: The Conclusion and Research Summary

The overarching theme of this research has focused on the neuronal responses of the southern house mosquito, *Cx. quinquefasciatus*, to human odorants. Single sensillum recording was conducted to investigate the neuronal responses to more than one hundred human odorants. Based on the response profiles recorded, it seems likely that *Cx. quinquefasciatus* antennae are involved in the odor coding and rely on the functional compartments of the sensilla. Their chemical sharing structures were found to be similarly encoded. The temporal dynamics of the neuronal responses present clear discriminations between chemicals. It has been suggested that the temporal dynamics are a factor in the odorant differentiation of insects (Su et al., 2011). Our research supports this suggestion by showing that the distinct temporal dynamics are based on different chemical structures. A behavioral bioassay was conducted to investigate the function of heptanal and generalize the concept that the concentration of chemicals affects the odorant modulating behavior of *Cx. quinquefasciatus*. The antennal lobe structure of *Cx. quinquefasciatus* was reconstructed by anterograde fluorescent staining and confocal microscopy. Forty four glomeruli were identified.

Reference

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