

**Reception of Human Odorants and Their Chemical Antagonists in the Yellow Fever Mosquito,  
*Aedes aegypti***

by

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## Abstract

The yellow fever mosquito *Aedes aegypti* (Linnaeus) (Diptera: Culicidae) is a vector of several tropical diseases, including yellow fever, dengue fever, Zika fever, and chikungunya. Mosquitoes use multiple cues such as CO<sub>2</sub>, skin odor, and body heat for host seeking, identifying their hosts on the basis of their specific skin odorant profile. Around 340 compounds have been isolated from human skin emanations, but which of these compounds can be detected by *Ae. aegypti* and which contribute to host seeking remain largely unknown. Unfortunately, *Ae. aegypti* has developed resistance to DEET, the most common active ingredient in insect repellents; DEET-based insect repellents are also probably more toxic to humans than the non-DEET-based products now being registered. Hence, new chemical compounds are needed to protect humans from *Ae. aegypti* mosquitoes.

In this study, 103 commercially available human odorants were examined in the antennal olfactory sensilla of female *Ae. aegypti* using single sensillum recording (SSR). Results showed that *Ae. aegypti* only responded to certain human odorants, including aldehydes, alcohols, aliphatics/aromatics, ketones, amines, and heterocyclics. Carboxylic acids on the panel did not elicit any responses in any of the sensilla types, but the SBTII, GP, and SST sensilla responded to most of the odorants detected by *Ae. aegypti*, with different types of sensilla exhibiting different selectivity and sensitivity. Both olfactory receptor neurons 'A' and 'B' in the trichoid sensilla contributed to the human odor sensation, and aldehydes were more likely to be discriminated by *Ae. aegypti* than aliphatics/aromatics. The sensillar responses of both non-blood fed and blood-fed female mosquitoes to the human odorants detected by *Ae. aegypti* were then investigated at 24-36, 48-60, and 72-84 hours post blood meal. Results indicated that *Ae. aegypti* with a blood meal showed compromised sensitivity to certain aldehydes, alcohols, aliphatics/aromatics, ketones, and amines at one or multiple time points, suggesting that these odorants may be important for *Ae. aegypti* host seeking.

Next, the responses of antennal olfactory sensilla of female *Ae. aegypti* to 48 plant-derived chemical compounds were studied. Results demonstrated that different types of sensillum exhibited different response patterns to the chemical compounds tested. The SST2 sensillum evoked inhibitory responses to several compounds, including eucalyptol, citronellal, and  $\alpha$ -terpinene. When eucalyptol was applied together with other odorants (i.e. 4,5-dimethylthiazole, cyclohexanone or 2-methyl-2-thiazoline) in the SST2 sensilla, the excitatory responses elicited by the odorants were reduced dramatically with increased dose of eucalyptol from 1  $\mu$ g to 10  $\mu$ g. These results indicated that eucalyptol might be used as confusant to protect humans from mosquitoes.

Furthermore, the responses of *Ae. aegypti* odorant receptors to the salient human odorants were characterized using *Xenopus* oocyte expression system and two-electrode voltage clamp. The oocytes expressing AaOR13, AaOR15, or AaOR55 together with the co-receptor AaOrco yielded strong to moderate responses to the human odorants tested but very weak responses to the chemical compounds that elicited inhibitory responses in SST2 sensilla. When a human odorant (benzaldehyde, p-cresol, or sulcatone) and a chemical compound ( $\alpha$ -terpinene or citronellal) were delivered simultaneously to the oocytes that expressed AaOR13, AaOR15, or AaOR55 together with AaOrco, the current oocyte response to an odorant + a chemical compound was significantly weaker than that to an odorant alone. The antagonistic effects of  $\alpha$ -terpinene and citronellal on the reception of human odorants suggested that these chemicals may be good alternates to protect humans from *Ae. aegypti*, though the potency needs to be verified by future behavioral studies.

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## List of Abbreviations

ORN: Olfactory receptor neuron; also referred to as olfactory sensory neuron (OSN)

OR: Odorant receptor

OBP: Odorant-binding protein

SSR: Single sensillum recording

LST: Long sharp tipped

SST: Short sharp tipped

SBTI: Short blunt tipped

SBTII: Short blunt tipped II

GP: Grooved peg

pbm: post blood meal

TEVC: Two-electrode voltage-clamp

## Chapter 1: Literature Review

### 1.1 The yellow fever mosquito

#### 1.1.1 Description and life cycle

The yellow fever mosquito *Aedes aegypti* (Linnaeus) (Diptera: Culicidae), which probably originates in sub-Saharan Africa, has been domesticated in the United States for centuries (Nelson 1986; Powell and Tabachnick 2013). It is a small to medium-sized mosquito, usually 4 to 7 mm. Adult *Ae. aegypti* mosquitoes are very likely to be identified as another mosquito species such as the Asian tiger mosquito *Ae. albopictus*, which was introduced in US in 1985 (Sprenger and Wuthiranyagool 1986). The main difference between the two species is that *Ae. aegypti* adults have white scales on the dorsal side of the thorax that look like a violin (or lyre), whereas a straight white band is formed on the top of the thorax of adult *Ae. albopictus* (Nelson 1986; Hawley 1988). There is white basal band on each tarsal segment of the hind legs of *Ae. aegypti*; the first two tarsal segments of the fore- and mid-legs also possess white bands (Nelson 1986). Female *Ae. aegypti* can be easily distinguished from males because of their relatively larger body sizes and less plumose antennae (Nelson 1986).

The yellow fever mosquito is a holometabolous insect, which means it has an egg, larva, pupa, and adult stage (Nelson 1986). Although adult male yellow fever mosquitoes feed solely on the nectar of plants, females show a strong preference for humans for blood meals, as this provides the proteins needed for egg development (Christophers 1960; McBride et al. 2014). After a blood meal, female *Ae. aegypti* can produce on average 100 to 200 eggs per batch, with each female laying eggs up to five times as long as she has access to blood meals (Christophers 1960; Nelson 1986). Eggs are usually laid on the surface of stagnant water in domestic water-holding containers (such as tree holes and uncovered man-made

containers) or at edges between the water surface and containers (Christophers 1960; Scott et al. 2000). The egg stage is one of the two potentially diapause life cycle stages of *Aedes* (Denlinger and Armbruster 2014). During the diapause period, eggs are environmentally programmed to not respond to a water stimulus, with diapause termination being induced by changes in the photoperiod and increasing temperatures (Lacour et al. 2015). The larvae have four instar stages and usually feed on algae, bacteria, and other microscopic organisms in the aquatic environment where they hatch (Christophers 1960; Nelson 1986). Male larvae develop faster than females, thus they usually enter the pupa stage earlier (Foster and Walker 2002). Pupae are mobile and can respond to environmental stimuli including light, temperature, and mechanical shock (Holmes 1911; Folger 1946; Omardeen 1957). Like male larvae, male adults generally emerge earlier in the growing season than females (Lounibos and Escher 2008).

### **1.1.2 Distribution and the human diseases vectored**

The yellow fever mosquito was originally found in Africa and was introduced into the New World on early trading ships (Powell and Tabachnick 2013). The ancestral form is a zoophilic treehole subspecies of *Ae. aegypti*, *Ae. aegypti formosus*, that still exists in forests and vegetated ecotones in sub-Saharan Africa where non-human animals provide blood meals (Lounibos 1981; Brown et al. 2014). Nowadays, *Ae. aegypti* mosquitoes are widely distributed in both tropical and subtropical regions, including the Americas, Europe/Africa, and Asia/Oceania (Kraemer et al. 2015). This global expansion of mosquito vectors is thought to be associated with the growth of trade between continents and international travel (Tatem et al. 2006). By using four nuclear genes, *apoLp-2* paralogue (Apolipoprotein 2), *SDR* (Short-chain dehydrogenase/reductase), *CYP9J2* (Cytochrome P450), and *DVRF1* (dengue virus restriction factor), in conjunction with more than 1,500 single nucleotide polymorphism markers, genetic analysis of samples collected from 21 localities representing 13 countries has confirmed a single subspeciation event resulted in the pantropic domestic form of *Ae. aegypti* and its subsequent spread around the world (Brown et al. 2014). In the United States, 183 counties in 26 states and the District of Columbia have reported the

occurrence of *Ae. aegypti* between 1995 and 2016 (Hahn et al. 2016). However, from 1,443 to 2,209 U.S. counties may be environmentally suitable for *Ae. aegypti* mosquitoes based on the results of a predictive model using county-level records, historical records, and suitable climate variables (Johnson et al. 2017).

Yellow fever mosquitoes are vectors of many globally important arboviruses, including yellow fever virus, dengue virus (DENV), chikungunya virus (CHIKV), and Zika virus (ZIKV) (Marchette et al. 1969; Jentes et al. 2011; Simmons et al. 2012; Leparc-Goffart et al. 2014). Yellow fever is an acute infectious disease that can be transmitted by *Aedes* spp mosquitoes including *Ae. aegypti* (Jentes et al. 2011; Shearer et al. 2018). According to an official report from the WHO, there were 84,000-170,000 severe cases of yellow fever infection and 29,000-60,000 deaths worldwide in 2013 alone, with most of the infection cases reported in Africa and Central and South America (WHO 2013). The WHO strongly recommends vaccination against yellow fever in as many as 236 countries or regions (WHO 2010), but there may be an even greater number of potential infection risk zones worldwide, as predicted by an analysis of 1155 geographical records from 47 countries across the Americas and Africa of yellow fever infection in humans from 1970 to 2016 (Shearer et al. 2018).

DENV is primarily transmitted by *Ae. aegypti* in tropical and subtropical latitudes; the mosquito *Ae. albopictus*, which has spread dramatically in recent years, has become the secondary vector of DENV (Simmons et al. 2012). There are an estimated 50 million infection cases of DENV per year spread across more than 100 countries or areas in the world and the potential risk zones may soon extend to temperate zones in North America, Australia, Europe, and Japan (Simmons et al. 2012). CHIKV can also be transmitted by both *Ae. aegypti* and *Ae. albopictus* mosquitoes (WHO 2016a). Since 2005, over 1.9 million human infection cases have been identified in the Indian subcontinent, Africa, and Asia. As yet, there is no licensed vaccine or specific treatment available for this disease (Leparc-Goffart et al. 2014; WHO 2016a). Zika is mainly transmitted by *Ae. aegypti*, although many other mosquito species in the genus *Aedes* such as *Ae. africanus*, *Ae. apicoargenteus*, and *Ae. furcifer* can also transmit it (Hayes 2009; Ayres 2016). ZIKV was first isolated from a monkey caged in the Ziika Forest of Uganda in 1947, with the first human case being identified in 1952 (Dick 1952; Cohen 2016). Since 2007, multiple outbreaks of

ZIKV have been reported in Africa, Asia, the Americas, and the Pacific, causing thousands of infections in humans (WHO 2018). In a pregnant woman, infection with ZIKV may cause serious damage to her unborn child, resulting in microcephaly and other serious brain anomalies (Rasmussen et al. 2016).

## **1.2 Mosquito control — current challenges and a brighter future**

### **1.2.1 Chemical and biological methods for larva management**

Adult mosquito populations may be controlled by treating the breeding sites of larvae with biological or chemical agents (Walker 2002), but these methods are only feasible when the breeding site larvae population is relatively small or breeding sites are restricted to man-made containers. Larvicides developed against mosquito larvae include: (1) petroleum-based oils, (2) Paris green, (3) dichlorodiphenyltrichloroethane (DDT, organochloride, which is effective but with high residual activity), (4) temephos (organophosphate, toxic to birds and fish), (5) pyrethrum and pyrethroids (toxic to aquatic nontarget organisms), (6) s-methoprene (an analogue of juvenile hormone (JH), toxic to fish), and (7) diflubenzuron (chitin-synthesis inhibitor, toxic to mammals and crustaceans) (Rose 2001; Walker 2002; Seccacini et al. 2008; van den Berg 2009; Buteler and Stadler 2011). Most of these chemicals/products are no longer used for mosquito larvae control because of their serious side effects on the environment (Walker 2002; van den Berg 2009; Liu 2015). Alternative compounds with mosquitocidal potential, such as botanical phytochemicals, are now being developed for mosquito management (Shaalán et al. 2005; Ghosh et al. 2012). These botanical derivatives are biodegradable and exhibit broad-spectrum target-specific activities against different mosquito species (Benelli 2015). Larvivorous fish, especially those in the family of *Cyprinodontidae*, have also been used to control mosquito larvae in a number of places around the world (Walker 2002; Howard et al. 2007; Chandra et al. 2008). These mosquitofish are released periodically into man-made water-holding structures such as rice fields, irrigation channels, lakes in urban areas to eat the larvae of *Anopheles*, *Aedes*, and *Culex* mosquitoes, and this approach has proven to be fairly effective (Walker 2002; Howard et al. 2007; Chandra et al. 2008).

Bacteria in the genus *Bacillus* may be another good choice for mosquito larvae control. Different serotypes of *B. thuringiensis israelensis* (Bti) and *B. sphaericus* (Bs) with higher toxicity have been isolated and shown to be effective against the larvae of *Aedes* and many other mosquito species (Priest 1992; Porter et al. 1993; Lacey 2007). By releasing Crystal (Cry) and Cytolytic (Cyt) proteins, Bti kills a variety of insects including those in the orders of Lepidoptera, Coleoptera, Diptera, and other invertebrates such as nematodes. These toxic proteins are ingested by susceptible larvae, dissolving their midgut epithelial cells and causing the death of the targets (Bravo et al. 2007). Although lepidopteran insects of both field and laboratory-selected colonies have developed resistance to Bti (Ferré and Van Rie 2002), no such cases have been documented in mosquitoes treated with either commercial Bti or environmental Bti (Ferré and Van Rie 2002; Bravo et al. 2007; Paris et al. 2011). This has been ascribed to the presence of the Cyt1Aa toxin in the Bti crystal inclusions (Georghiou and Wirth 1997; Berry et al. 2002; Ferré and Van Rie 2002). The Cyt1Aa protein synergizes the toxicity of Bti Cry proteins by enhancing the interaction between the insect brush border membrane vesicles and Cry proteins (Georghiou and Wirth 1997; Pérez et al. 2005). Though targeting similar sites, the Bs toxin has a different mode of action, causing severe damage to midgut cells by changing their electron density and destroying the mitochondrial matrix and endoplasmic reticula inside cells (Charles et al. 1996). As yet, Bs has only been used against mosquito larvae, including both *Anopheles* and *Culex* species, but because of intensive application, larval resistance to Bs toxins has been observed in both laboratory-selected and field populations of *Cx. pipiens* (Charles et al. 1996).

### **1.2.2 Chemical control of adult mosquitoes and insecticide resistance**

Currently, four classes of insecticides, namely organochlorines (DDT and methoxychlor), organophosphates (OP), carbamates, and pyrethroids are used for mosquito control (Becker et al. 2003; Zaim and Jambulingam 2007; Kelly-Hope et al. 2008). Most of the insecticides in these four groups are synthetic organics and are toxic to adult mosquitoes, although OP and pyrethroids also show larvicidal

activity (Becker et al. 2003). Organochlorines are currently used only in the endemic areas of some African countries; carbamates are used in Africa, Americas, and the Eastern Mediterranean region; and OP and pyrethroids are available around the world (Zaim and Jambulingam 2007). As to the types of application, organochlorines and carbamates are limited to indoor residual spraying (IRS) (Walker 2002; Zaim and Jambulingam 2007) while OP can be used for both IRS and space spraying (Zaim and Jambulingam 2007). Pyrethroids can be used for IRS, space spraying, and the treatment of mosquito nets, playing a vital role in a mosquito prevention program based on the use of long-lasting insecticidal nets (LLIN) that has been highly effective against mosquito-borne human diseases (Zaim and Jambulingam 2007; Kelly-Hope et al. 2008).

The worldwide use of insecticides for mosquito control continues to increase dramatically each year, specifically organochlorines (DDT), OP (chlorpyrifos, fenitrothion, and malathion), carbamates (bendiocarb), and pyrethroids (deltamethrin and permethrin) (Zaim and Jambulingam 2007). However, the intensive application of these pesticides has caused the widespread development of resistance in a number of different species of mosquito. For example, pyrethroids are the only pesticides approved for the LLIN program, but there are increasing reports of pyrethroid resistance developing in malaria vectors in western and southern Africa (Kelly-Hope et al. 2008). Resistance to organochlorines (DDT) and/or OP has also been documented in *Aedes*, *Anopheles*, and *Culex* mosquitoes (Walker 2002; Becker et al. 2003). In particular, cross resistance or multiple-resistance has arisen in insects treated with two or more types of pesticide (Becker et al. 2003; Davies et al. 2007; Liu 2015), and resistance to these insecticides has substantially compromised their efficacy against human disease vectors.

Two of the mechanisms thought to be responsible for the development of resistance to commonly used pesticides in insects have been well studied, namely metabolic resistance and target-site resistance. Metabolic resistance generally results from the overexpression of enzymes that are capable of detoxifying xenobiotics (e.g. insecticides) or mutations within these genes that cause amino acid substitutions and subsequent alter the affinity of the enzymes to pesticides (Hemingway and Ranson 2000). Three major families of enzymes are known to be involved in insect metabolic resistance: esterases, glutathione S-

transferases (GSTs), and cytochrome P450 monooxygenases (P450s) (Hemingway and Ranson 2000; Liu 2015). In contrast, target-site resistance occurs due to point mutations or structural modifications in the genes/proteins that play important roles in organisms (Hemingway and Ranson 2000; Liu 2015). These salient proteins include sodium channels, acetylcholinesterase (AChE), and  $\gamma$ -aminobutyric acid (GABA) receptors, which are targeted by DDT and pyrethroids, OP and carbamates, and cyclodienes and phenyl pyrazoles, respectively (Liu 2015).

### **1.2.3 Mosquito traps baited with attractants**

To reduce the amount of insecticide applied, an alternative option for adult mosquito control is to use traps baited with attractants. Luring pests toward an attractive source constitutes the pull strategy in push-pull strategies developed for integrated pest management (Figure 1.1A; Cook et al. 2007). The attractants used for mosquito traps include blacklight (UV light), CO<sub>2</sub>, and volatile chemicals (host odor or oviposition attractants). A limited number of studies on this topic have indicated that traps baited solely with light were indeed attractive for a few mosquito species such as *An. gambiae* and *An. Melas*, although the attraction rates of light traps placed at different locations varied greatly (Lines et al. 1991; Overgaard et al. 2012). Traps baited with both CO<sub>2</sub> and light were attractive to more mosquito species, however, including *Aedes* spp., *Anopheles* spp., *Culex* spp., *Ochlerotatus* spp., *Coquillettidia perturbans*, and *Psorophora ferox* (Magbity et al. 2002; Williams and Gingrich 2007; Hoel et al. 2009; Lühken et al. 2014), suggesting the important role of CO<sub>2</sub> in attracting mosquitoes. The attraction efficiency of a trap baited with CO<sub>2</sub> has been shown to be related to the release rate of CO<sub>2</sub>. Reisen et al. (2000) found that traps baited with CO<sub>2</sub> with a release rate of 0.5~1.5 liters/min lured significantly more female *Cx. tarsalis* than those with a release rate of 0.4~0.5 liters/min.

In addition to light and CO<sub>2</sub>, volatile semiochemicals are often included in mosquito traps. Octenol (or 1-octen-3-ol), which is one of the chemicals used as an attractant, has been isolated from human skin emanations (Bernier et al. 2000). When used alone, octenol is not attractive to most mosquito

species (Kline et al. 1990; Becker et al. 1995; Burkett et al. 2001), but traps baited with CO<sub>2</sub> and octenol lured more *An. atropos*, *An. crucians*, *Ae. taeniorhynchus*, *Ae. vigilax*, and *Culicoides furens* compared to CO<sub>2</sub> alone (Kline et al. 1990; Essen et al. 1994). Interestingly, octenol has also been shown to have an inhibitory effect on the attractiveness of CO<sub>2</sub> for certain *Aedes* and *Culex* spp. For example, fewer *Ae. polynesiensis*, *Cx. pipiens*, *Cx. quinquefasciatus*, and *Wyeomyia mitchellii* were collected using traps baited with CO<sub>2</sub> and octenol than traps using CO<sub>2</sub> alone (Kline et al. 1990; Burkett et al. 2001; Russell 2004).

Like octenol, lactic acid, another chemical commonly utilized in mosquito traps, showed no attractiveness to most mosquito species when used alone (Kline et al. 1990; Bernier et al. 2002; Smallegange et al. 2005). However, more *An. atropos*, *An. gambiae*, *Ae. taeniorhynchus*, *Cu. furens*, and *Wy. mitchellii* were lured to CO<sub>2</sub> traps supplemented with lactic acid than those with CO<sub>2</sub> only (Kline et al. 1990; Dekker et al. 2002). Lactic acid is also known to enhance the attractiveness of ammonia to the malaria mosquito *An. gambiae* (Smallegange et al. 2005), although ammonia alone does attract these mosquitoes (Braks et al. 2001; Smallegange et al. 2005). Other semiochemicals used as mosquito attractants include certain aldehydes and ketones (Bernier et al. 2002; Syed and Leal 2009). Recently, a variety of synthetic blends containing multiple odorant attractants have been developed. Impressively, the attraction efficiency of these blends for specific mosquito species has been shown to be comparable to that of human volunteers, suggesting the significant potential of these synthetic blends for adult mosquito control (Okumu et al. 2010; Mukabana et al. 2012).

Chemicals that mediate mosquito oviposition site preferences are another attractant option. Ponnusamy et al. (2008) isolated three bacteria-associated semiochemicals (nonanoic acid, tetradecanoic acid, and tetradecanoic acid methyl ester) from bamboo leaf or white oak leaf infusions and found that a synthetic blend of all three compounds (each at a certain concentration) was highly effective at inducing egg laying in female *Ae. aegypti*. Acetaldehyde that was accidentally isolated from commercially purchased ethanol, a common odorant solvent, has also been found to be an oviposition attractant: an oviposition bioassay indicated that gravid female *Cx. quinquefasciatus* showed a strong preference for

trays baited with acetaldehyde for egg laying (Choo et al. 2018). Other volatile compounds identified as mosquito oviposition attractants include certain aromatics, heterocyclics, terpenes, terpenoids, alcohols and esters (Himeidan et al. 2013).

#### **1.2.4 Sterile insect-based techniques**

Sterile insect techniques (SIT) are a species-specific and environmentally benign method of pest control. In this context, “sterile” refers to (1) chemical- or irradiation-induced sterility, (2) *Wolbachia*-induced cytoplasmic (sperm-egg) incompatibility (CI), or (3) engineered repressible dominant lethal mutations (Alphey et al. 2010). Large numbers of sterile individuals, either males or females, are released to mate with wild mosquitoes to produce non-fertilized eggs or eggs expressing sex-specific lethal genes, leading to the suppression or elimination of the population of a target insect in the field (Benedict and Robinson 2003; Alphey 2014). Since 1960s, SIT using sterilization techniques such as chemosterilization, gamma irradiation, translocation and other chromosomal rearrangements has been used against *Aedes*, *Anopheles*, and *Culex* mosquitoes but only a few cases have been documented as successful (Benedict and Robinson 2003). This may be partially due to the detrimental effect of irritation on the mating competitiveness of irritated males (Harris et al. 2011).

Another SIT method, the incompatible insect technique (IIT), has also been developed for insect pest control. IIT uses *Wolbachia*-infected males that compete with wild males to mate with uninfected-females or females lacking the same *Wolbachia* types, which induces cytoplasmic incompatibility (CI) and embryonic lethality (Werren et al. 2008). *Wolbachia*-induced CI was first introduced in the 1960s to suppress the population of *Culex pipiens fatigans* (Lees et al. 2015). In recent years, there has been remarkable progress regarding to the use of IIT for population suppression in *Cx. p. quinquefasciatus* and *Ae. albopictus* under both laboratory and semi-field conditions (Lees et al. 2015). However, the accidental release of *Wolbachia*-infected females (which does occur to a limited extent due to the methods currently used for sex separation) can have a devastating effect on a population suppression program for a target

insect (Calvitti et al. 2015) because the infected females mate with uninfected males to produce fertile eggs and all these fertilized eggs will hatch into females (the so-called “male killing” effect of *Wolbachia* infection) (Werren et al. 2008).

An alternative approach, namely release of insects carrying a dominant lethal genetic system (RIDL), has therefore been developed to solve the sex separation problem for SIT-based insect management (Alphey et al. 2010; Alphey 2014). Using a genetics-based approach, a strain of the target insect that carries a conditional, dominant, and sex-specific lethal gene can be constructed (Alphey 2002). For example, in a female-lethal version of RIDL, males and females are homozygous for a female-specific dominant lethal genetic construct (i.e. the tetracycline-repressible transcriptional activator protein (tTa) (Thomas et al. 2000). Expression of tTa is repressed in the presence of antibiotic tetracycline under laboratory conditions. After the insects are released from the laboratory/factory, tetracycline is no longer accessible to them and all the engineered females will die because of the activation of tTa, thus driving the expression of effector genes (Alphey 2002). Engineered males can then only mate with wild females, whose F1 progeny receive a dominant female-specific lethal gene. After several generations, the natural population of target organism is therefore suppressed (Thomas et al. 2000; Alphey 2014). Using an RIDL system, the population of *Ae. aegypti* mosquitoes has been successively suppressed under both laboratory and field conditions, suggesting the feasibility of this system for controlling other insects (Phuc et al. 2007; de Valdez et al. 2011; Harris et al. 2011).

### **1.2.5 Cas9-based gene drive**

A number of different gene drive systems have been developed for insect pest control (Champer et al. 2016). The most promising of these is the RNA-guided CRISPR—Cas9 (clustered regularly interspaced short palindromic repeats— CRISPR-associated 9) endonuclease system (Mali et al. 2013). Here, the single guide RNA (sgRNA), which is a fusion form of a CRISPR RNA (crRNA) and a transactivating crRNA (tracrRNA), guides the Cas9 endonuclease to recognize and cleave specific DNA sequences

within the genome of a target insect (Jinek et al. 2012). Using a Cas9-based gene drive system, an advantageous (e.g. pathogen-resistant gene) or disadvantageous (e.g. lethal/sterile or maleness gene) trait can be linked to a gene drive or disrupted by the gene drive. Either trait spreads quickly through the population of the target insect species through the drive system (Adelman and Tu 2016; Champer et al. 2016).

Recently, a polycistronic cluster of anti-ZIKV synthetic microRNAs (miRNAs) has been engineered into a laboratory population of *Ae. aegypti* using a Cas9-based gene drive. The engineered *Ae. aegypti* mosquitoes showed significant resistance to ZIKV infection, dissemination, and transmission (Buchman et al. 2019). Another study utilizing a Cas9-based gene drive demonstrated that disruption of three reproduction-related genes using the CRISPR-Cas9 technique to confer sterility in female *An. gambiae* mosquitoes. The recessive female-sterility phenotype was efficiently spread in their G<sub>2</sub> to G<sub>5</sub> progeny, with transmission rates ranging from 91.4 to 99.6% (Hammond et al. 2016). The first mosquito male-determining factor (M factor) *Nix* was characterized in *Ae. aegypti* (Hall et al. 2015). Theoretically, the *Nix* gene can be engineered into a laboratory population of *Ae. aegypti* through a Cas9-based gene drive system, producing all male progeny with the M factor or lethal/sterile female progeny (Adelman and Tu 2016). The Akbari lab has used a novel CRISPR-based technology, i.e. precision guided SIT (pgSIT), to engineer a system in *Drosophila* that expressed double gRNA (dgRNA) *dgRNA*<sup>*βTub, Sxl*</sup> (*βTub*, *βTubulin 85D*, disruption of male fertility; *Sxl*, *sex lethal*, killing females) (Kandul et al. 2019). Using this system, a population of 100% sterile males was created under laboratory conditions. In addition, according to mathematical modeling predictions pgSIT can induce greater population suppression of local *Ae. aegypti* compared to the currently used suppression technologies, including IIT, RIDL, and female-specific RIDL (fsRIDL) (Kandul et al. 2019). Taken together, the Cas9-based gene drive is an emerging, efficient tool for the control of mosquitoes and mosquito-vector-borne diseases.

### **1.3 Insect olfaction and olfactory system**

#### **1.3.1 Olfactory organs and olfactory sensilla**

Their antennae are the primary olfactory organ of adult insects (Figure 1.2A; Hansson and Stensmyr 2011). Insects detect volatile chemical information on food, mating partners, or oviposition sites mainly through the olfactory sensilla located on antennae (Figure 1.2B; Vosshall and Stocker 2007; Joseph and Carlson 2015). Trichodea (hairs), basiconica (grooved pegs), and coeloconica (pitted pegs) are three major types of antennal olfactory sensilla that have been fully characterized in insects (Pitts and Zwiebel 2006; Joseph and Carlson 2015). Based on their morphological traits, four trichodea and one basiconica olfactory sensilla have been identified on the antennae of mosquitoes, namely long sharp tipped (LST), short sharp tipped (SST), short blunt tipped I (SBTI), and short blunt tipped II (SBTII) trichodea, and grooved peg (GP) basiconica (Figure 1.2B; McIver 1978, 1982). It has been suggested that female mosquitoes possess significantly more olfactory sensilla per antenna than males, which may reflect their different ecological needs in the adult stage (McIver 1982). Unlike gustatory sensilla, which are uniporous (a single pore at the tip of sensilla) and usually found on the mouthparts of insects, antennal trichodea olfactory sensilla are multi-porous (with a larger number of pores through the sensillum wall), which facilitates more efficient communication with odorants (Zacharuk 1980; Joseph and Carlson 2015). The maxillary palp is another important olfactory organ in insects (Vosshall and Stocker 2007; Joseph and Carlson 2015); capitate pegs on mosquito maxillary palps have been shown to be responsible for the detection of both carbon dioxide (CO<sub>2</sub>) and human odor (McIver 1982; Tauxe et al. 2013). Insect larvae can also sense chemo-stimuli through the dorsal organ on their head surface, though the larva's olfactory system is simpler than the adult system (Vosshall and Stocker 2007).

#### **1.3.2 Odorant-binding proteins (OBPs) and odorant-degrading enzymes**

After reaching the sensillar lymph through the pores on the sensillum wall, odorants (which are usually hydrophobic) will bind to odorant-binding proteins (OBPs) (Leal 2013). OBPs can facilitate the

transportation of hydrophobic odorants across the aqueous environment of the sensillar lymph (Pelosi 1996). So far, two models have been proposed to explain the mode of action of OBPs (Leal 2013). The first of these suggests that the odorant itself (after release from the OBPs) can activate an odorant receptor (OR). Evidence found in moths and mosquitoes strongly supports this model. However, the second model proposes that it is actually the OBP/odorant complex that activates an OR, which has been confirmed in the fruit fly. For the first model, it is believed that the release of an odorant ligand from OBPs is closely related to the low pH at the surface of the dendrites on which ORs are expressed (Leal 2013); OBPs exhibit strong affinity to ligands at the high or near-neutral pH found in sensillar lymph but only weak affinity at the low pH close to the surface of dendrites (Wojtasek and Leal 1999; Horst et al. 2001). Regardless of how an OR is activated, there is increasing evidence to suggest that OBPs may play important roles in the selectivity of an insect olfactory system. For example, although HvirOR13 (a pheromone receptor of the tobacco budworm *Heliothis virescens*) expressed in HEK 293 cells responded broadly to four pheromone constituents (Z11-16Ald, Z11-16OAc, Z9-14Ald, and Z9-16OAc), when the same ligands were preincubated with HvirPBP2 (a pheromone-binding protein in *H. virescens*), HvirOR13 displayed enhanced selectivity to Z11-16Ald (Große-Wilde et al. 2007). Similar selectivity resulting from OBPs/PBPs has been observed in the silkworm *Antheraea polyphemus* and the mosquito *Cx. quinquefasciatus* (Forstner et al. 2009; Pelletier et al. 2010).

Inactivation of the odorant signal can avoid continuous stimulation toward olfactory sensory neurons (OSNs). Several different antennal specific enzymes (including esterases, aldehyde oxidase (AOX), and GSTs) are known to be involved in the degradation of odorants in insects. For example, ApolPDE, the first esterase identified in the antennae of male *A. polyphemus*, hydrolyzes the main component of the sex pheromone E6Z11-16OAc (Vogt and Riddiford 1981; Leal 2013). The aldehyde oxidase MsexAOX has been found in both male and female antennae of another moth species, *Manduca sexta* (Rybczynski et al. 1989). MsexAOX is capable of oxidizing bombykal, one of the two components of the sex pheromone in *M. sexta* (Rybczynski et al. 1989, 1990). In *M. sexta*, another enzyme GST-msolf1, which is restricted to the trichoid sensilla of male and female antennae, has exhibited degrading

activity against an aldehyde odorant (trans-2-hexenal) (Rogers et al. 1999). Other enzymes, including epoxide hydrolases, aldehyde dehydrogenases, and P450s, are assumed to be associated with the degradation of odorants in insects, but their precise roles in odorant signal termination have not yet been confirmed (Leal 2013).

### **1.3.3 Olfactory sensory neurons (OSNs) and chemosensory receptors**

Binding of an odorant to a chemosensory receptor will fire or inhibit specific OSNs housed in olfactory sensilla (Ghaninia et al. 2007; Chen et al. 2018). In mosquitoes, there are 600 to 800 trichoidea sensilla (each housing 2 OSNs; Figure 1.2C) and around 100 grooved peg sensilla (each housing 2 to 5 OSNs) on each antenna (McIver 1982). Similarly, there are 200 to 800 trichoidea sensilla (each housing 5 to 15 OSNs) and 40 to 130 basiconica sensilla (each housing 5 to 6 OSNs) on each antenna of kissing bugs (Wigglesworth and Gillett 1934; Catalá and Schofield 1994). However, in the common bed bug *Cimex lectularius*, there are only six D type sensilla (each housing 8 to 19 OSNs), nine C type sensilla (each housing 4 to 5), and 34 E type sensilla (each housing 1 to 3 OSNs) per antenna (Levinson et al. 1974). This huge difference in the total number of OSNs between mosquitoes/kissing bugs and bed bugs may have evolved due to the totally different habitats occupied by the two groups of insects. Mosquitoes and kissing bugs live in open outdoor environments, a world composed of a huge amount of volatile chemicals, while bed bugs prefer closed indoor conditions, which have relatively fewer classes of odorants.

As mentioned above, chemosensory receptors expressed on the membrane of OSNs will interact with the odorants transported by OBPs. A single group of chemoreceptors, the ORs, are responsible for detecting most of the odorants encountered by insects, especially the odor of food, hosts, and mating partners (Nakagawa et al. 2005; Hallem and Carlson 2006; Carey et al. 2010; Liu et al. 2017). Genome studies have identified 117 ORs in *Ae. aegypti*, 76 ORs in *An. gambiae*, 162 ORs in *Cx. quinquefasciatus*, and 93 ORs in *R. prolixus*, whereas there are only 49 ORs in *C. lectularius* (Arensburger et al. 2010;

Benoit et al. 2016; Hill et al. 2002; Mesquita et al. 2015; Matthews et al. 2018). Ionotropic receptors (IR) are another group of chemoreceptors associated with odor reception. In the fruit fly, IRs have been shown to be capable of sensing acids and amines (Ai et al. 2010; Benton et al. 2009; Min et al. 2013). The latest reference genomes have annotated 135 IRs in *Ae. aegypti* and 110 IRs in *An. gambiae* (Matthews et al. 2018), but only 33 and 30 IRs have been identified in the kissing bug *Rhodnius prolixus* and the common bed bug *C. lectularius*, respectively (Mesquita et al. 2015; Benoit et al. 2016). Mosquitoes and kissing bugs have more ORs and/or IRs than bed bugs, which may reflect the different ecological needs of both groups of insects. As noted earlier, mosquitoes can sense CO<sub>2</sub> through their maxillary palps (McIver 1982). Recent studies show that the reception of CO<sub>2</sub> is associated with gustatory receptors (GRs), which are expressed on the membrane of OSNs in capitata pegs (Turner et al. 2011; Tauxe et al. 2013). Unlike ORs, which are restricted to antennae, GRs and IRs are expressed in various locations on the insect's body, including its labellum, wings, legs, and antennae (Joseph and Carlson 2015). Specific GRs have also been mapped in the brain, gut and reproductive systems of insects (Joseph and Carlson 2015).

Two models have been proposed to explain the molecular mechanism of odor reception in insects. In the first, the odorant receptor co-receptor (*orco*) itself forms ionotropic cation channels that are activated by odorants or cyclic nucleotides (Wicher et al. 2008; Missbach et al. 2014). The wingless *Zygentoma silverfish* *Thermobia domestica*, which expresses only *orco*, can respond as many classes of chemicals as the winged Pterygota insects, which express *orco*/ORs (Missbach et al. 2014). Also, *Drosophila orco* (Or83b) expressed in human embryonic kidney (HEK293) cells can be activated by the second messenger cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP) (Wicher et al. 2008). In the second, ORs are co-expressed with the *orco* to form heteromeric ligand-gated ion channels, which is commonly seen in most insect species (Sato et al. 2008; Leal 2013; Missbach et al. 2014). The first *orco*, found to be coexpressed with insect ORs, was *D. melanogaster* Or83b (Larsson et al. 2004). Its ortholog *BmorOR2*, from the silk moth *Bombyx mori*, must be coexpressed with another conventional OR (*BmOR1*) to detect the sex pheromone bombykol, while the complex *BmorOR2/BmOR3* was found to be activated by the oxidized form of the sex pheromone bombykal (Nakagawa et al. 2005).

Evidence from evolutionary studies suggests that insect orco first evolved in the wingless *Zygentoma* silverfish and the complex orco/ORs evolved subsequently in the winged Pterygota insects; ORs evolved with orco that was present before the appearance of ORs (Missbach et al. 2014). Specifically, when expressed independently, the orco of many Pterygota insect species (including *An. gambiae*, *D. melanogaster*, the tobacco budworm *Heliothis virescens*, the Indian jumping ant *Harpegnathos saltator*, and the parasitic fig wasp *Apocrypta bakeri*) can be activated by the agonist VUAA1, suggesting the conserved role of orco across *Zygentoma* and Pterygota insects (Jones et al. 2011; Butterwick et al. 2018).

## 1.4 Mosquito host-seeking behavior

### 1.4.1 CO<sub>2</sub>

Mosquitoes use multiple cues for host-seeking navigation, including exhaled CO<sub>2</sub>, the odor of skin emanations, and the visual, heat, and humidity signatures of hosts. At as far as 10 m away from their hosts, mosquitoes may be attracted by the CO<sub>2</sub> from host exhaled breath (Cardé 2015). A field study carried out by Gillies and Wilkes (1972) demonstrated that different mosquito species responded to CO<sub>2</sub> at different distances. Mosquitoes *An. ziemanni* Grünb., *Aedes* spp., and *M. africana* (Theo.) were capable of responding to the presence of CO<sub>2</sub> from a distance of 15 m, whereas *Cx. thalassius* Theo. and *Cx. univittatus* Theo. exhibited a response at 7.5 m (Gillies and Wilkes 1972). Subsequent field studies revealed that traps baited with CO<sub>2</sub> alone were effective in luring various mosquito species, including *Ae. taeniorhynchus*, *An. atropos*, *An. crucians*, *Wy. ritchellii*, and *Culex* spp. (Kline et al. 1990; Syed and Leal 2009). Interestingly, the addition of CO<sub>2</sub> substantially boosted the attraction rates of lactic acid, octenol, and nonanal (three components of human odor) to these mosquito species in the field (Kline et al. 1990; Syed and Leal 2009).

Under laboratory conditions, CO<sub>2</sub> also enhances the sensitivity of mosquitoes to the odor of human skin. Dekker et al. (2005) found that the attraction rate of 20% skin odor plus 4% CO<sub>2</sub> for female *Ae. aegypti* was comparable to that of 100% skin odor. More recent studies from Vosshall lab showed that

in a two-port olfactometer assay, human odor plus 10% CO<sub>2</sub> attracted significantly more female *Ae. aegypti* mosquitoes than human odor alone (DeGennaro et al. 2013; McMeniman et al. 2014). CO<sub>2</sub> has also demonstrated a synergistic effect on the attraction efficiency of lactic acid, though lactic acid alone did not displayed any attraction for female *Ae. aegypti* mosquitoes (Bernier et al. 2003; McMeniman et al. 2014). In addition to boosting the attraction efficiency of human odor, CO<sub>2</sub> also plays an important role in the attraction of heat for female mosquitoes. McMeniman et al. (2014) found that 37 °C alone did not trigger any heat-seeking or blood-feeding activities in female *Ae. aegypti* mosquitoes, whereas the same heat condition in the presence of CO<sub>2</sub> attracted female mosquitoes for blood-feeding.

As mentioned previously, CO<sub>2</sub> is sensed through the capitae peg sensilla on mosquito maxillary palps (McIver 1972, 1982; Grant et al. 1995). Kellogg (1970) found that only one of the three OSNs housed in the capitae peg sensilla was responsible for the detection of CO<sub>2</sub>; the other two OSNs in capitae pegs responded to various odorants including n-heptane, acetone, amyl acetate, and 1-octen-3-ol (McIver 1972; Grant et al. 1995). A more recent study demonstrated that the neurons in capitae peg sensilla produce large amplitude spikes in response to CO<sub>2</sub> and have thus been designated as ‘cpA’ (Tauxe et al. 2013). In addition to their response to CO<sub>2</sub>, the cpA neuron in *Ae. aegypti* and *An. gambiae* were also activated by a variety of components of skin odor, insect repellents, and other odorants that have been implicated in mosquito behavior (Lu et al. 2007; Tauxe et al. 2013). The results of many studies have demonstrated that GRs are involved in the detection of CO<sub>2</sub>. The first two GRs (*DmelGr21a* and *DmelGr63a*) that mediate CO<sub>2</sub> sensation were identified in the fruit fly *D. melanogaster*; these two receptors had to function together in order to detect CO<sub>2</sub> (Jones et al. 2007; Kwon et al. 2007). The orthologs of the CO<sub>2</sub>-responding receptors were subsequently cloned and functionally characterized in mosquitoes *An. gambiae* (*AgGr23*, *AgGr22*, and *AgGr24*) and *Ae. aegypti* (*AaGr1* and *AaGr3*) (Lu et al. 2007; Erdelyan et al. 2011; McMeniman et al. 2014). Specifically, the *Gr3<sup>ECFP4</sup>* mutant of *Ae. aegypti* showed neither an electrophysiological nor behavioral response to stimulation by CO<sub>2</sub> or a combination of CO<sub>2</sub> and 37 °C. The host-seeking activities triggered by lactic acid with CO<sub>2</sub> or human odor with CO<sub>2</sub> were also compromised in the mutant line (McMeniman et al. 2014).

### 1.4.2 Skin odor

Different mosquitoes may show divergent preference for humans versus non-human animals when seeking a blood meal. Previous studies found that *Ae. aegypti* was attracted by both human arms and chicken, whereas *Culex* spp. only responded strongly to the odor of chicken, specifically aldehyde compounds (Allan et al. 2006; Syed and Leal 2009), while *Cx. tarsalis* was also attracted to amphibians such as frogs, toads, and lizards (McIver 1968). Even within the same species, mosquitoes collected from different environmental conditions may exhibit distinct preferences for human versus non-human animal odors. McBride et al. (2014) reported that *Ae. aegypti* mosquitoes collected from indoor sites (the ‘domestic’ form) preferred humans to guinea-pigs for their blood meal, while the opposite was true for the outdoor collections (the ‘forest’ form), which were attracted by the odor of guinea-pigs rather than humans. The disparate preferences exhibited by different *Ae. aegypti* colonies for humans vs. non-human animals may be driven by the differential expression of specific ORs. Compared to the forest form, *AaOr4* was highly expressed in the domestic form of *Ae. aegypti*; this receptor responded strongly to sulcatone, a predominant ketone component emitted from humans rather than other warm-blooded animals (McBride et al. 2014).

In addition to the odorants discussed above, many other compounds comprise human odor. An analysis of human skin emanations via gas chromatography/mass spectrometry (GC/MS) identified more than 340 volatile chemicals, including carboxylic acids, aldehydes, alcohols, aliphatics/aromatics, amines, ketones, sulfides, and heterocyclics, among others (Bernier et al. 2000). Interestingly, some human subjects are more attractive to mosquitoes than others, perhaps due to the increased relative abundance of specific compounds. Bernier et al. (2002) found that certain carboxylic acids, ketones, alcohols, and amines were substantially or slightly increased in more attractive human hosts compared to less attractive subjects, though not all chemicals by themselves were attractive to *Ae. aegypti* mosquitoes.

Bernier et al. (2002) found that by themselves, ketones were only slightly attractive to *Ae. aegypti* mosquitoes; when tested individually, carboxylic acids and aliphatics/aromatics did not attract *Ae. aegypti*. However, a binary blend of lactic acid and certain ketones (acetone and butanone) or sulfides (dimethyl sulfide, dimethyl disulfide, and carbon disulfide) attracted *Ae. aegypti* with high efficiency (Bernier et al. 2003; Bernier et al. 2015). Interestingly, a synthetic blend containing aqueous ammonia, CO<sub>2</sub>, lactic acid and seven additional carboxylic acids (each added at their respective optimum concentrations) was optimized and found to be more efficient in attracting *Anopheles*, *Culex* and *Mansonia* mosquitoes than a human volunteer at long range distances in semi-field experiments (Okumu et al. 2010). The synthetic blend also exhibited less but comparable attractiveness to *An. gambiae*, *Culex* spp., and *Mansonia* spp. at short range distances compared to a human volunteer (Okumu et al. 2010). As noted earlier, the presence of CO<sub>2</sub> often increases the sensitivity of mosquitoes to human odor. McMeniman et al. (2014) also found that human odor at a temperature of 37 °C elicited significantly higher levels of blood feeding than the same odor at ambient temperature (26 °C), suggesting the synergistic effect of body heat for the attraction of mosquitoes driven by human odor.

As discussed previously, ORs and IRs are the groups of chemoreceptors responsible for odor reception in insects. Most of the odorants that are important for mosquitoes, especially host odor, are sensed by ORs, whereas acids and amines are detected through IRs (Nakagawa et al. 2005; Hallem and Carlson 2006; Benton et al. 2009; Ai et al. 2010; Carey et al. 2010; Min et al. 2013; Liu et al. 2017). Different ORs may exhibit different tuning breadths (response spectra) to the same odor panel. Some ORs that responded to a great variety of odorants are referred to as generalists, e.g. AgOr57, which was activated by 53 out of the 110 odorants tested (Carey et al. 2010). However, others that are narrowly tuned to one or several compounds are regarded as specialists, e.g. AgOr5 which only responded to 2,3-butanedione (ketone), thiazole (heterocyclic), and 1-butanol (alcohol) (Carey et al. 2010). Interestingly, different chemicals may also display different tuning curves when tested against the same set of ORs. For example, both 3-methylindole (aromatic) and geranyl acetate (terpene) can activate/inhibit only three of the 50 AgOrs examined, while isoamyl acetate (ester) is capable of activating/inhibiting 19 of them

(Carey et al. 2010). So far, genome studies have identified just 76 ORs in *An. gambiae*, hence odor reception in this mosquito species is far from fully decoded. The function of ORs in *Ae. aegypti* and IRs in all mosquito species also remains to be investigated.

### 1.4.3 Visual, heat, and humidity signatures

In addition to CO<sub>2</sub> and skin odor, mosquitoes also use other cues such as body heat, humidity, and visual signatures to navigate toward their hosts. As mentioned above, body heat (37 °C temperature) had a significant synergistic effect on the attraction of mosquitoes driven by host skin odor (McMeniman et al. 2014). However, body heat itself only slightly elicited host-seeking activity (10%~20%) in *Ae. aegypti* and *An. gambiae* mosquitoes (Spitzen et al. 2013; McMeniman et al. 2014; Corfas and Vosshall 2015). Interestingly, the weak attraction triggered by the 37 °C stimuli only happened when mosquitoes were extremely close (within 30 cm) to their hosts (Spitzen et al. 2013; McMeniman et al. 2014). Further investigation revealed that the attraction of mosquitoes to body heat stimuli was tuned by a cation channel TRPA1. The mutant line (*TRPA1*<sup>-/-</sup>) of *Ae. aegypti* did not lose attraction to moderate-temperature stimuli (30~45 °C), rather they became more tolerant to high-temperature stimuli (50~60 °C) compared to wild type individuals (Corfas and Vosshall 2015).

More recently, visual cues have been shown to be involved in the host-seeking activity of mosquitoes. Liu and Vosshall (2019) found that *Ae. aegypti* mosquitoes were more likely to be attracted to dark visual contrasts (e.g. a white background with a 2 cm black circle ‘dot’ at its center) than to no visual contrast (e.g. a white background only) at ambient temperature. The attraction induced by dark visual contrast was not influenced by host-like temperature stimuli or by the host-seeking status of mosquitoes, whether before a blood meal, 48 hrs post-meal, or 96 hrs post-meal (Liu and Vosshall 2019). Calcium imaging also indicated that the lobula, a region in the mosquito’s optic lobe, exhibited a strong response to dark visual contrast in *Ae. aegypti* (Vinauger et al. 2019). Other cues such as humidity may

also contribute to mosquito host-seeking activity, but their precise role remains unclear (Olanga et al. 2010).

## 1.5 Insect repellents and chemical inhibitors of human odor

### 1.5.1 *N,N*-Diethyl-*meta*-toluamide (DEET)

In addition to pull strategies that use attractants to lure and kill mosquitoes, push strategies that protect humans from mosquitoes by applying chemical repellents are also utilized (Figure 1.1A). These chemical compounds repel mosquitoes by acting on their olfactory and/or gustatory systems, thus triggering avoidance behavior in the mosquitoes. *N,N*-Diethyl-*meta*-toluamide (DEET), which is currently the most widely used ingredient in commercially available insect repellents, was discovered and developed by Samuel I. Gertler and his colleagues at the U.S. Department of Agriculture (Gertler 1946; Fradin 1998; Fradin and Day 2002). DEET shows broad-spectrum repellent activity against many biting arthropod species, including mosquitoes, fleas, and ticks (Fradin 1998).

DEET provides protection for humans against biting insects through multiple modes of action. Recent studies have indicated that insects were capable of smelling and tasting DEET, and thus avoided it. *Xenopus laevis* oocytes expressing *An. gambiae* GPROR8 + GPROR7 (the mosquito ortholog of the *Drosophila* OR83b co-receptor) or *Cx. quinquefasciatus* CquiOR136 + CquiOrco evoked particularly strong responses to DEET (Ditzen et al. 2008; Xu et al. 2014). Behavioral studies have also indicated that unlike wild populations, which showed little interest in DEET-treated skin, female *Ae. aegypti orco* mutants were attracted by a human arm treated with 10% DEET, suggesting that the *orco* mutants were no longer sensitive to volatile DEET (DeGennaro et al. 2013). On the other hand, *Drosophila* detected DEET directly via gustatory receptor neurons (GRNs) that expressed *Gr32a*, *Gr33a*, and *Gr66a* (Lee et al. 2010). Three GRNs responding to DEET have also been identified on the labella of female *Ae. aegypti* mosquitoes (Sanford et al. 2013) and DeGennaro et al. (2013) found that female *Ae. aegypti orco* mutants were repelled by DEET-treated human skin within 60 msec of contact, indicating a role for GRs in

repellency in mosquitoes. These results suggest a dual mode of action for DEET that repels mosquitoes via both olfactory and gustatory systems. A third mode of action of DEET may also be involved in protecting humans from biting insects, however. Ditzen et al. (2008) first demonstrated that DEET inhibited the neuronal response of the maxillary palp cp sensilla of *An. gambiae* to a human odorant 1-octen-3-ol (a mosquito attractant). However, Syed and Leal (2008) reported in the same year that DEET did not inhibit the antennal olfactory response of *Culex* mosquitoes to 1-octen-3-ol but instead appeared to have a fixative effect on the release of the odorant, thus decreasing the spike frequency that it elicited. In terms of the toxicity of DEET, more than three decades of studies in mice and rats has revealed no potential in humans for teratogenicity and oncogenicity (Fradin 1998). However, long-term, excessive, or inappropriate use of DEET or the application of highly concentrated DEET may cause death in highly sensitive individuals, especially in children younger than 6 years of age (Fradin 1998). DEET is also more toxic to humans than other insect repellents that have been registered recently (Tavares et al. 2018). A population of *Ae. aegypti* that is resistant to DEET has been successfully selected under laboratory conditions (Stanczyk et al. 2010), which means that alternative chemical compounds are needed to protect humans from biting insects. A number of alternative insect repellents or plant-derived repellents, such as Skin-So-Soft, soybean oil, IR3535, icaridin (picaridin), DEPA, PMD, and methyl jasmonate have been developed for human protection (Fradin 1998; Fradin and Day 2002; Xu et al. 2014; Tavares et al. 2018). However, the results of protection assays have revealed that, except for picaridin, none of the currently available non-DEET repellents provides protection for humans with a duration that is comparable to those achieved by DEET-based insect repellents (Fradin and Day 2002; Tavares et al. 2018). This suggests that new chemical repellents with a different mode of action are required to protect humans from biting insects.

### **1.5.2 Inhibitors/confusants for human odors**

A third strategy that protects humans from mosquitoes is to mask the reception of human odor using chemical inhibitors or confusants, thus interrupting the host-seeking activity of female mosquitoes (Figure 1.1A). For example, an ORN evokes an excitatory response to a human odorant A but a simultaneous inhibitory response to a chemical compound B, so when a mosquito detects odorant A and chemical compound B at the same time, the firing frequency (or spike number) induced by odorant A in the olfactory organ of the mosquito may be reduced due to the inhibitory response induced in response to chemical compound B (Figure 1.1B). ORNs that evoke inhibitory responses to certain odorants have been reported in several mosquito species, including *Ae. aegypti*, *An. gambiae*, and *Cx. quinquefasciatus* (Qiu et al. 2006; Ghaninia et al. 2007; Chen et al. 2018; Xu et al. 2018). A recent study found that the excitatory response induced by an odorant cyclohexanone in the SST2 sensillum of *Cx. quinquefasciatus* was blocked by the chemical inhibitors eucalyptol or fenchone, and the same phenomenon was observed in the *Ae. aegypti* mosquito (Xu et al. 2018). Behavioral assays have indicated that the protection efficacy of the insect repellent methyl salicylate decreased significantly when it was applied in conjunction with the chemical inhibitor eucalyptol (Xu et al. 2018). This indicates that developing chemical inhibitors for human odor may be a good alternative choice to protect humans from mosquitoes and other biting insects.

### **1.6 Techniques for ORN/OR functional studies**

Multiple electrophysiological techniques have been used to investigate the function of insect OSNs and ORs. Unlike electroantennography (EAG), which assesses the receptive range of the whole insect antenna, single sensillum recording (SSR) characterizes the response spectrum of a single olfactory sensillum to chemical stimuli (Olsson and Hansson 2013). In order to identify the chemical compounds of ecological significance in insects, EAG and SSR are usually performed in conjunction with gas chromatography (Olsson and Hansson 2013). For decades, SSR has been used to identify the chemical compounds that may be important for insect host-seeking (or selection), mating, oviposition, warning of

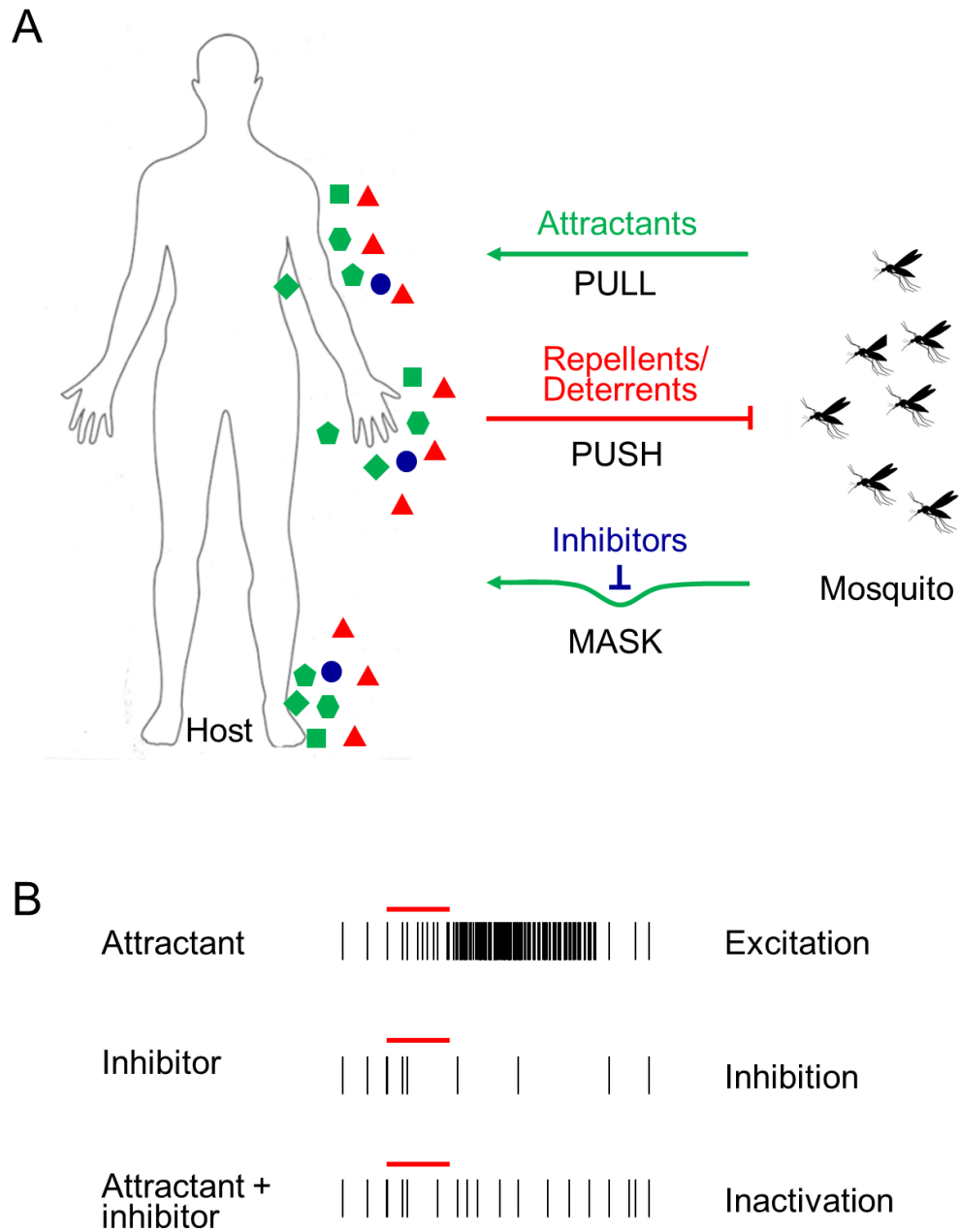
natural enemies, and so on (Den Otter 1977; Van Der Pers and Den Otter 1978; Den Otter et al. 1980; Visser 1986; Kurtovic et al. 2007; Leal et al. 2008; Zhang et al. 2017). In particular, SSR has been utilized to characterize the responses of single olfactory sensilla to botanical chemicals and host odorants, serving as a useful tool for screening new chemical repellents or attractants for a target insect species (Liu et al. 2013, 2014; Liu and Liu 2015; Ye et al. 2016; Chen et al. 2018).

As mentioned earlier, the trichoid sensilla on mosquito antennae have been classified into four different types according to their different morphological traits (McIver 1978, 1982). SSR studies have demonstrated that different types of trichoid sensilla exhibit different response spectra to the same set of odorant stimuli (Qiu et al. 2006; Ghaninia et al. 2007; Ye et al. 2016; Chen et al. 2018). In some cases, more than two functional subtypes were identified for a single morphological type of trichoid sensillum based on the different response profiles (Qiu et al. 2006; Ghaninia et al. 2007; Ye et al. 2016; Chen et al. 2018). These functional subtypes respond differently to the same set of odorants, possibly due to the expression of different ORs on the membrane of ORNs housed in each subtype (Vosshall and Stocker 2007; Leal 2013).

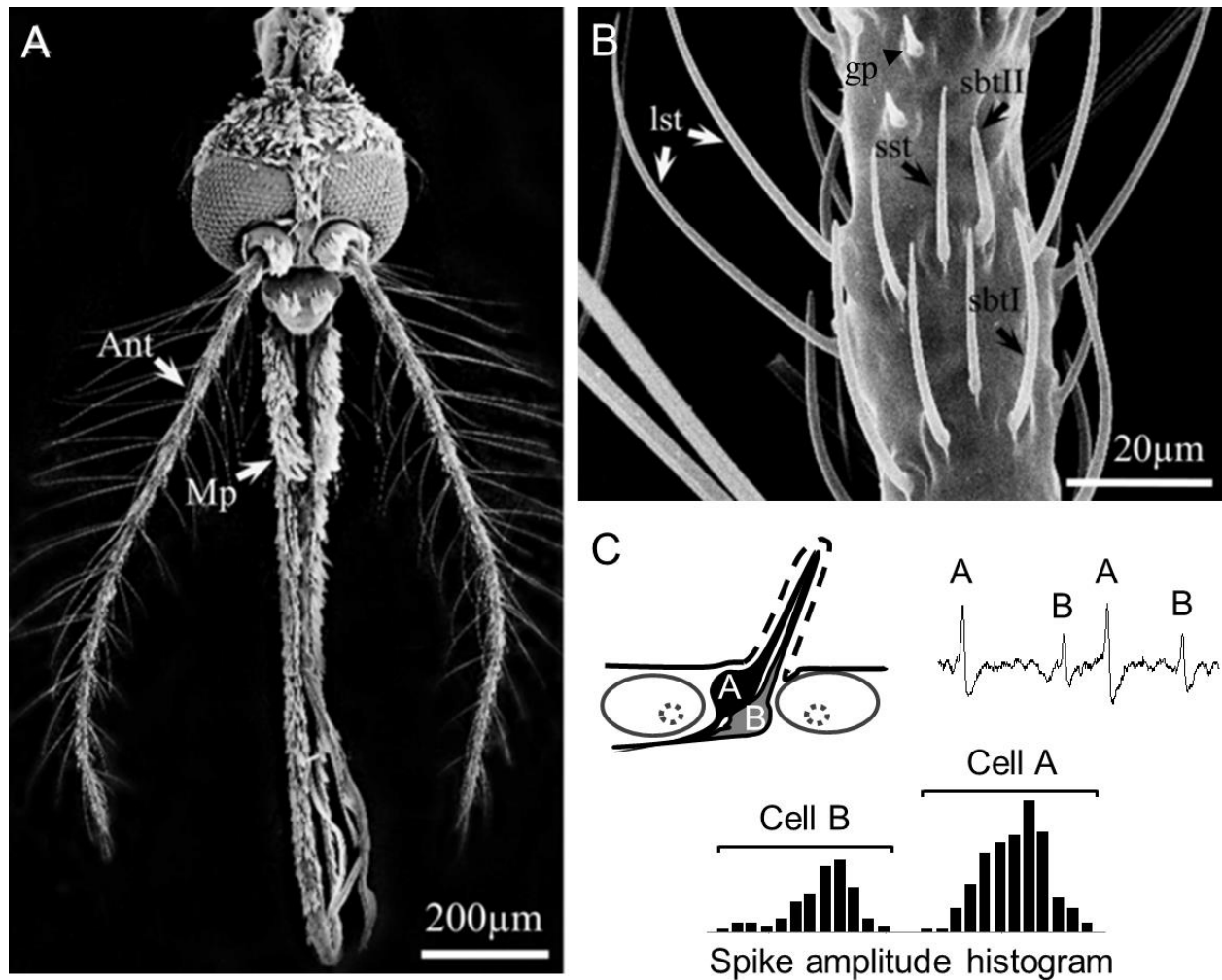
SSR is only capable of characterizing the response of ORNs in individual sensilla to chemical stimuli and cannot identify the response of individual ORs expressed on the membrane of these ORNs. Individual insect ORs are therefore usually expressed in *Xenopus laevis* frog oocytes, human embryonic kidney (HEK293) cells, or *Drosophila* empty neuron systems in order to examine their functions (Wicher et al. 2008; Carey et al. 2010; Wang et al. 2010; Liu et al. 2017). Individual insect ORs are expressed together with their highly conserved co-receptors on the membrane of a *Xenopus* oocyte or HEK293 cell, which forms heteromeric ligand-gated cation-non-selective ion channels (Sato et al. 2008; Wicher et al. 2008). These ligand-gated channels open when they bind to certain odorant ligands, triggering an extracellular  $\text{Ca}^{2+}$  influx. The odor-evoked whole-cell current is therefore obtained via an outside-out patch-clamp (or two-electrode voltage-clamp) (Sato et al. 2008; Wicher et al. 2008).

In addition to the *in vitro* expression systems, individual insect ORs are also expressed in *Drosophila* empty neuron systems, an *in vivo* expression system, for functional studies (Hallem and

Carlson 2006; Syed et al. 2006; Carey et al. 2010; Slone et al. 2017). The empty neuron system is a mutant antennal neuron that lacks any *Drosophila* endogenous ORs (Dobritsa et al. 2003). After the expression of insect ORs in the empty neuron system, odorant responses will be assayed electrophysiologically using SSR (Dobritsa et al. 2003; Hallem and Carlson 2006; Carey et al. 2010; Slone et al. 2017). Other methods that are used to characterize the function of insect ORs/orco include zinc-finger nuclease, transcription activator-like effector nuclease, and CRISPR/Cas9 (DeGennaro et al. 2013; Koutroumpa et al. 2016; Li et al. 2016; Yang et al. 2016).



**Figure 1.1 The push-pull-mask strategies.** (A) Push-pull-mask strategies used for insect control. Insects such as mosquitoes can be attracted by host odor (green dots) (PULL) or repelled by repellents/deterrents (red dots) (PUSH); their attraction to host odor can also be masked by chemical inhibitors (blue dots) (MASK). (B) Excitatory and inhibitory activities of ORNs induced by attractants and chemical inhibitors, respectively. Attractant-induced ORN excitation can be masked by chemical inhibitors, which subsequently causes the inactivation of an ORN.



**Figure 1.2 The olfactory system of a female mosquito.** (A) Antennae (Ant) and maxillary palps (Mp) of a female *Ae. aegypti* mosquito (Ghaninia et al. 2007). (B) Antennal olfactory sensilla (Ghaninia et al. 2007). (C) An antennal trichoid sensillum housing two ORNs, neuron ‘A’ and ‘B’. Right: a single-sensillum recording of the spontaneous activities of both neurons. Action potentials from ORNs ‘A’ and ‘B’ can be distinguished by their amplitudes, with the larger amplitude corresponding to neuron ‘A’ and the smaller amplitude to neuron ‘B’.

## Chapter 2: Research Goals and Specific Objectives

### 2.1 The goals and objectives of this research

More than 300 chemicals have been isolated from human skin emanations through GC/MS (Bernier et al. 2000), but it is largely unknown which of these odorants can be detected by *Ae. aegypti* and which may contribute to the host-seeking activity of *Ae. aegypti* remain largely unknown. A recent study demonstrated that *Ae. aegypti* develop resistance to DEET, the most common active ingredient in commercially available insect repellents, after successive generations of selection under laboratory conditions (Stanczyk et al. 2010). Unfortunately, among the currently available non-DEET repellents, only icaridin has been shown to provide protection for humans with a duration comparable to that of DEET-based insect repellents (Fradin and Day 2002; Tavares et al. 2018). This suggests that new chemical compounds with a different mode of action are needed to protect humans from biting insects. The long-term goal of my research is thus to characterize human odor reception in *Ae. aegypti* and identify new chemical compounds that can protect humans from *Ae. aegypti*. To achieve this long-term goal, four specific objectives will be addressed in this project: 1) identify the human odorants detected by *Ae. aegypti*; 2) examine the effect of a blood meal on the sensitivity of *Ae. aegypti* to these human odorants; 3) screen chemical compounds that inhibit the reception of the salient human odorants in *Ae. aegypti*; and 4) functionally characterize the odorant receptors of *Ae. aegypti* that respond to the human odorants and their chemical antagonists.

#### 2.1.1 Objective 1: Identify the human odorants detected by *Ae. aegypti*

A study by DeGennaro and colleagues found that female *Ae. aegypti* showed a strong preference for humans when seeking a blood meal compared to other warm-blooded animals such as guinea pigs

(DeGennaro et al. 2013). Since both humans and guinea pigs can produce CO<sub>2</sub> and have a similar body temperature, female *Ae. aegypti* must use skin odor for host discrimination. So far, more than 300 compounds have been isolated from human skin emanations (Bernier et al. 2000), but which of these volatile compounds contribute to host discrimination (or host-seeking) in *Ae. aegypti* remains unknown. Thus, in objective 1, the responses of five morphological types of antennal olfactory sensilla (i.e. long sharp tipped (LST), short sharp tipped (SST), short blunt tipped I (SBTI), short blunt tipped II (SBTII), and grooved peg (GP) (McIver 1978; Ghaninia et al. 2007)) of an exclusively sucrose-fed female *Ae. aegypti* were examined against 103 human odorants using SSR. All of these human odorants are commercially available and cover a wide range of different chemical classes, including carboxylic acids, aldehydes, alcohols, aliphatics/aromatics, esters, ketones, amines, sulfides, ureas, halides, and heterocyclics.

### **2.1.2 Objective 2: Examine the effect of a blood meal on the sensitivity of *Ae. aegypti* to these human odorants**

A previous study indicated that, before a blood meal, female *Ae. aegypti* mosquitoes were highly attracted by human odor, while after the blood meal, e.g. at 24-72 hours post blood meal (pbm), blood-fed mosquitoes showed decreased or even zero interest in human odor (Liesch et al. 2013). I thus hypothesize that blood-fed *Ae. aegypti* will evoke reduced olfactory responses to certain human odorants compared to non-blood-fed mosquitoes. These human odorants may be involved in the host-seeking activity of *Ae. aegypti*. Once the human odorants sensed by *Ae. aegypti* had been identified in Objective 1, the effect of a blood meal on the sensitivity of *Ae. aegypti* to these human odorants was investigated at three different time points, i.e. 24-36 hrs, 48-60 hrs, and 72-84 hrs pbm in order to pinpoint the specific human odorants that elicit reduced responses in blood-fed *Ae. aegypti* mosquitoes.

### **2.1.3 Objective 3: Screen chemical compounds that inhibit the reception of the salient human odorants in *Ae. aegypti***

As discussed in Section 1.5.1, DEET protects humans from biting insects through three modes of action, namely repelling insects by acting on their olfactory and gustatory systems and showing a fixative effect on the release of odorants from human skin (Ditzen et al. 2008; Syed and Leal 2008; DeGennaro et al. 2013; Sanford et al. 2013; Xu et al. 2014). However, a population of *Ae. aegypti* showing resistance to DEET has been selected under laboratory conditions, and very few of the currently available non-DEET repellents can provide protection for humans comparable to that achieved by DEET-based insect repellents (Fradin and Day 2002; Stanczyk et al. 2010; Tavares et al. 2018). Alternative compounds with different modes of action are thus urgently needed for human protection against mosquitoes, including *Ae. aegypti*. In addition to DEET, there are a large number of chemical compounds that have been isolated from different plant species that exhibit strong to moderate repellent activity to many biting bugs, including mosquitoes (Liu et al. 2013; Tavares et al. 2018). However, as yet the neuronal activities in very few of these plant-derived chemical compounds have been studied in the antennal olfactory sensilla of *Ae. aegypti*. In Objective 3, 48 plant-derived chemical compounds were examined against solely sucrose-fed *Ae. aegypti* mosquitoes. The same antennal olfactory sensilla that evoke excitatory and inhibitory responses to different odorants have been identified in multiple insect species (Qiu et al. 2006; Ghaninia et al. 2007; Xu et al. 2018). I hypothesized that when applied together, the chemical compounds eliciting inhibitory responses in one olfactory sensillum would block/mask the reception of human odorants, eliciting excitatory responses in the same sensillum of *Ae. aegypti* mosquitoes. Therefore, the chemical compounds eliciting inhibitory responses were applied along with the human odorants eliciting excitatory responses to test their antagonistic effects on the reception of these odorants in *Ae. aegypti*.

#### **2.1.4 Objective 4: Functionally characterize the odorant receptors of *Ae. aegypti* responding to the human odorants and their chemical antagonists**

There are several *An. gambiae* ORs that have been examined against some of the human odorants tested in our study and have shown responses to these human odorants (Carey et al. 2010). Through running BLAST for the amino acid sequences of these AgORs against the genome data of *Ae. aegypti* in the National Center for Biotechnology Information (NCBI, <https://www.ncbi.nlm.nih.gov/>), certain candidates of AaORs that respond to the human odorants from objective 2 and the chemical compounds from objective 3 will be identified. Using acidic guanidine thiocyanate-phenol-chloroform method, the total RNA of the antennae of four- to six-day-old *Ae. aegypti* mosquitoes will be extracted (Chomczynski and Sacchi 2006). Besides, using *Xenopus laevis* oocyte expression system to express the AaORs and two-electrode voltage-clamp (TEVC) technique, the odor-evoked whole-cell current will be detected (Liu et al. 2017). The antagonistic effect of the chemical compounds identified in objective 3 on the reception of the human odorants identified in objective 2 will be examined in the *Xenopus* oocytes, which express specific AaORs together with AaOrco that respond to these chemical compounds and human odorants.

## **2.2 The significance of research**

The odorants that elicit reduced olfactory responses in blood-fed mosquitoes may be involved in the host-seeking activity of *Ae. aegypti* and thus can be developed as chemical attractants for *Ae. aegypti* mosquitoes. Besides, the chemical compounds that exhibit antagonistic effect on the reception of certain salient human odorants in *Ae. aegypti* can be good alternates of DEET to protect humans from mosquitoes. Finally, identification of the AaORs responding to certain important human odorants may contribute to screening antagonists for these receptors in the future studies.

### **Chapter 3: Human Odor Coding in the Yellow Fever Mosquito, *Aedes aegypti***

#### **3.1 Abstract**

Insects use their olfactory systems to obtain chemical information on mating partners, oviposition sites and food. The yellow fever mosquito *Aedes aegypti*, an important vector of human infectious diseases, shows strong preference for human blood meals. This study investigated the chemical basis of host detection by characterizing the neuronal responses of antennal olfactory sensilla of female *Ae. aegypti* to 103 compounds from human skin emanations. Results showed that *Ae. aegypti* only responded to certain human odorants, including aldehydes, alcohols, aliphatics/aromatics, ketones, amines and heterocyclics. The SBTII, GP, and three functional subtypes of SST sensilla responded to most of the odorants tested and different types of sensilla exhibited different selectivity and sensitivity. Both olfactory receptor neurons (ORNs) ‘A’ and ‘B’ in the trichoid sensilla, either activated or inhibited, were involved in the human odor coding process. In addition, chemical class and concentration had effect on the information representation of an odor in insects. Aldehydes were more likely discriminated by *Ae. aegypti* than aliphatics/aromatics probably due to the more diverse response patterns elicited by compounds from the former group. These results enhance our understanding about the human odor reception in *Ae. aegypti* and may contribute to the development of chemical attractants for mosquito management.

Chen, Z., Liu, F., & Liu, N. (2019). Human odour coding in the yellow fever mosquito, *Aedes aegypti*.

*Submitted.*

### 3.2 Introduction

Insects rely to a large extent on olfactory cues to locate mating partners, oviposition sites, or hosts for food (Nakagawa et al. 2005; Ponnusamy et al. 2008; McBride et al. 2014). In particular, the mosquito *Aedes aegypti* (Linnaeus), a vector of many important human diseases including yellow fever, dengue fever and Zika fever, has been found to show strong preference for human hosts over other warm-blooded animals for blood meals (DeGennaro et al. 2013; McBride et al. 2014). At present, insecticides such as pyrethroids are the first choice for strategies to control mosquitoes, but the development of resistance to these chemicals has largely compromised their efficacy for mosquito management (Liu 2015). Alternative approaches based on the use of attractants and other lure-baited traps are therefore now widely used to control mosquito vectors (Kline 2006; Okumu et al. 2010).

Using GC/MS, a previous study isolated around 300 chemicals from the human skin emanations (Bernier et al. 2000). Many compounds, including amines, carboxylic acids, ketones, and sulfides, have been shown to be effective in attracting mosquitoes, either on their own or to enhance the attractiveness of other chemicals, based on the results of behavioural assays (Smallegange and Takken 2010). However, few of these compounds has been examined in *Ae. aegypti* for their olfactory neuronal activities.

Insects mainly use their antennal olfactory sensilla for odor sensation (Joseph and Carlson, 2015; Matthews et al., 2016). Inside each olfactory sensillum, two or more olfactory receptor neurons (ORNs) are housed, on which membrane specific odorant receptors (ORs) and the common OR coreceptor (Orco) are expressed (McIver 1978; Ghaninia et al. 2007; DeGennaro et al. 2013; Leal 2013). Binding of odorants to specific ORs fires their host ORNs, thus inducing action potentials in these ORNs; the action potentials are finally sent to the brain of insects and trigger behavioral responses such as attraction or avoidance (Takken 1991; Gao et al. 2000; Vosshall et al. 2000; Zwiebel and Takken 2004; Ditzen et al. 2008; Syed and Leal 2008). Animals encode the identity of an odor in terms of its chemical structure and the concentration they encounter (Hallem and Carlson 2006; Carey et al. 2010), and this information can be visualized through the temporal dynamic of the ORN response evoked by the odor (Friedrich and Laurent 2001; Laurent et al. 2001; Hallem and Carlson 2006; Carey et al. 2010).

In this study, the neuronal responses of antennal olfactory sensilla of female *Ae. aegypti* to 103 commercially available human odor-related compounds were examined. Also, the roles of olfactory receptor neurons (ORNs) ‘A’ and ‘B’ of trichoid sensilla in odor coding for the peripheral sensory system of *Ae. aegypti* mosquitoes were explored. The temporal dynamics of the neuronal responses elicited by structurally related compounds and how the spatial relationships between these compounds were represented in odor space were studied as well.

### **3.3 Materials and Methods**

#### **3.3.1 Insects**

*Ae. aegypti* mosquitoes (Orlando strain, obtained from Dr. James Becnel, USDA, ARS, Mosquito and Fly Research Unit) were maintained at  $25 \pm 2^\circ\text{C}$  and a photoperiod of 12: 12 (L:D) h (lights on 8 am). Females and males were reared together after eclosion and supplied with unlimited 10% sucrose solution throughout. Four- to six-day-old only sucrose-fed female mosquitoes were used in all the experiments in this study. Adult females were fed blood samples from horses (Large Animal Teaching Hospital, College of Veterinary Medicine, Auburn University) for stock maintenance.

#### **3.3.2 Single sensillum electrophysiology**

Extracellular single sensillum recording (SSR) was carried out as previously described (Liu et al. 2013). Briefly, 4- to 6-day-old female mosquitoes were used after being anaesthetized on ice (1-2 min) and fixed with a 200  $\mu\text{L}$  pipet tip. Mosquitoes were fixed by dental wax and a cover slip ( $22 \times 22$  mm) with double-sided tape. The reference tungsten electrode, connected to ground, was inserted into one eye of the tested mosquito and the recording electrode, which was connected to a preamplifier (Universal AC/DC Probe Gain 10 $\times$ , Syntech), was inserted into the shaft of the test sensillum under a microscope (LEICA Z6 APO) using a micromanipulator (Leica, Cat #: 115378). The signal acquired by the preamplifier was digitized using an IDAC 4 (Syntech). Action potentials (i.e. spikes) evoked by a stimulus or control puff

were recorded for 10 s, beginning 1 s before the stimulation. Action potentials were counted off-line for 500 ms before and after the stimulation. Firing rates recorded during the 500 ms post-stimulation period were subtracted from the spontaneous activities observed during the 500 ms pre-stimulation period and the outcome was multiplied by two to convert it to the conventional scale of spikes per second. Excitatory responses were recognized if the firing rate exceeded the response to the diluent control by 15 spikes/s and the inhibitory responses were identified as those where the firing rate diminished by 10 spikes/s or more (Ghaninia et al. 2007). The ORN evoking action potential with the larger amplitude was designated cell 'A', while the one yielding the action potential with the smaller amplitude was designated cell 'B' (Ghaninia et al. 2007).

### **3.3.3 Stimulation and stimuli**

One hundred and three commercially available human odorants, identified through GC-MS analysis on skin emanations (Bernier et al. 2000), were used in the study (Table S3.1). These odorants fell into 11 chemical classes, namely carboxylic acids, aldehydes, alcohols, aliphatics/aromatics, esters, ketones, amines, sulfides, ureas, halides, and heterocyclics. Except for ammonia, which was diluted in ddH<sub>2</sub>O, the other odorants used as stimuli were freshly prepared every two weeks in dimethyl sulfoxide (DMSO) at the desired concentrations. For the dose-dependent assays, chemical stimuli were delivered to the sensillum under test from low to high dose and the delivery was performed as previously described (Liu et al. 2013). In brief, 10  $\mu$ L of each stimulant solution was dispersed onto a piece of filter paper (3  $\times$  45 mm), which was then inserted into a glass Pasteur pipette and allowed to stand for 30 sec to create a stimulus cartridge. Ten microliters of 100% DMSO was used as the control stimulus. The preparation was bathed in a purified and humidified air stream (20 mL/s) flowing from a Stimulus Controller CS-55 (Syntech), to which the stimulus pulse at a rate of 0.5 L/min for 500 ms was added. For each odorant, each recording was from a separate sensillum. No more than three sensilla were tested per insect.

### 3.3.4 Data analysis

Hierarchical cluster analysis using Euclidean distance and Ward's method, heatmap construction, and principal component analysis (PCA) for odor space were performed with PAST 3.20 (University of Oslo). Tuning breadth curves and dose-dependent curves were fitted using GraphPad Prism 7.0. Statistical analysis was conducted by IBM SPSS Statistics (version 20, <https://www.ibm.com/us-en/marketplace/spss-statistics>). Error bars indicate s.e.m. unless otherwise noted.

## 3.4 Results

### 3.4.1 Responses of antennal olfactory sensilla to human odorants in *Ae. aegypti*

Five morphological types of olfactory sensilla have been previously identified on the antennae of *Ae. aegypti* mosquitoes, namely long sharp tipped (LST), short sharp tipped (SST), short blunt tipped I (SBTI), short blunt tipped II (SBTII), and grooved peg (GP) (McIver 1978; Ghaninia et al. 2007). In this study, we examined the neuronal response of each type of olfactory sensilla of female *Ae. aegypti* mosquitoes against 103 human odorants from 11 chemical classes that have been isolated from skin emanations (Bernier et al. 2000). Hierarchical cluster analysis on the responses of these five morphological types of sensilla to the 103 human odorants revealed seven physiological clusters (Figure S3.1). In particular, three functional subtypes of SST sensilla (55% of SST1, 14% of SST2, and 31% of SST3) were identified and defined according to their distinctive neuronal response profiles to the odorants on the panel (Figure 3.1A and 3.1B). Sensilla SST1 and SST2 were reported in a previous study (Chen et al. 2018).

In total, 721 odorant-sensillum combinations were tested for this study. Of these, 89 elicited significant excitatory ( $\geq 15$  spikes/s) or inhibitory ( $\leq -10$  spikes/s) responses (Figure 3.1A, Table S3.2). Specifically, 23 out of the 89 combinations yielded significant responses in the SBTII sensilla, followed by 19 in the SST3 sensilla, 17 in the SST2 sensilla, 16 in the SST1 sensilla, 9 in the SBTI sensilla, and 5 in the GP sensilla. The LST sensilla were not activated or inhibited by any of the human odorants

included in the panel. All the aldehydes (11/11), ketones (6/6), and amines (3/3) elicited either excitatory or inhibitory responses in one or more types of sensilla (Figure 3.1A, Table S3.2). The aldehyde-sensillum combination contributed to most of the odorant-sensillum combinations (37/89) that induced significant responses. However, only 7 out of 13 heterocyclics, 5 out of 13 alcohols, and 7 out of 23 aliphatics/aromatics activated one or more types of olfactory sensilla. None of the carboxylic acids, esters, sulfides, ureas, and halides tested elicited excitatory or inhibitory responses in any of the olfactory sensilla types. These findings suggest that female *Ae. aegypti* mosquitoes display bias toward detecting specific groups of human odorants such as aldehydes, ketones, and amines and these odorants may be involved in their host-seeking activities.

Different types of olfactory sensilla also exhibited differential preference in sensing human odorants. The tuning curves show that the GP sensilla were very selective, being narrowly tuned to five compounds (3 amines and 2 heterocyclics, with a  $k$  value of 44; Figure 3.1A and 3.1C), and were thus defined as specialists. The SST3 and SBTII sensilla responded to more odorants, however, being tuned to 19 and 23 chemicals, respectively (7 aldehydes, 2 alcohols, 2 aliphatics/aromatics, 5 ketones, and 3 heterocyclics in the SST3 sensilla; 11 aldehydes, 2 alcohols, 6 aliphatics/aromatics, 2 ketones, 1 amine, and 1 heterocyclic in the SBTII sensilla; both with a  $k$  value of 7; Figure 3.1A and 3.1C), and were thus regarded as generalists. Moreover, different types of sensilla gave their strongest responses to different odorants. For instance, the GP, SST2, and SBTI sensilla were tuned to ammonia (amine), 2-picoline (heterocyclic), and xylene (aliphatic/aromatic), respectively, while the SST1, SST3, and SBTII sensilla responded strongly to trans-2-octen-1-ol (alcohol), 2-hexanone (ketone), and octanal (aldehyde), respectively (Figure 3.1C). In addition, although the SST1, SST3, and GP sensilla evoked only excitatory responses to the chemicals tested, the SST2 and SBTII sensilla evoked both excitatory and inhibitory responses (Figure 3.1A and 3.1B). Taken together, the differential sensation preferences of different types of olfactory sensilla, possibly due to the expression of different ORs in each type of sensilla, reveal the capacity of female *Ae. aegypti* mosquitoes to encode a wide range of odorants from diverse chemical groups in their environment.

### 3.4.2 Integration of human odorant information via ORNs ‘A’ and ‘B’

Two ORNs were classified in the antennal trichoid sensilla of *Ae. aegypti* mosquitoes, which is consistent with the findings of a previous study (Ghaninia et al. 2007). Based on the histogram of spike amplitudes, the ORN with larger spike amplitudes was named as ‘A’ neuron, while the one with smaller spike amplitudes was assigned to ‘B’ neuron (Figure 3.2A). The odorants that elicited significant excitatory ( $\geq 15$  spikes/s) or inhibitory ( $\leq -10$  spikes/s) responses in each type of sensilla were chosen for the analysis of the response intensity of the ‘A’ and ‘B’ neurons. The results showed that the ‘A’ neuron alone was responsible for detecting all the aldehydes and aliphatics/aromatics that elicited excitatory responses in the SBTI sensilla (Figure 3.2B), but both the ‘A’ and ‘B’ neurons in the SBTII sensilla were involved in sensing human odorants. The ‘A’ neuron in the SBTII sensilla was activated or inhibited by aldehydes, whereas the ‘B’ neuron was inhibited by aliphatics/aromatics (Figure 3.2C). Interestingly, in the SBTII sensilla two aldehydes (nonanal and heptanal) activated the neuron ‘A’ but inhibited its neighbouring neuron ‘B’ (Figure 3.2C). Although similar patterns were observed for toluene and benzene, in this case the outcomes were reversed, with the two aliphatic/aromatic compounds activating neuron ‘B’ but inhibiting its neighbouring neuron ‘A’ (Figure 3.2C). Two alcohols (trans-2-octen-1-ol and cis-2-hexen-1-ol) and one heterocyclic compound (indole) also activated neuron ‘A’ in the SBTII sensilla, while the amine (butylamine) inhibited neuron ‘B’.

ORNs in all three functional types of SST sensilla showed distinctive patterns of activation or inhibition when exposed to the same panel of human odorants. Specifically, only neuron ‘A’ in the SST1 sensilla was triggered by aldehydes, alcohols, and ketones (Figure 3.2D). In sensilla SST2, however, the situation was more complicated. For example, although neuron ‘A’ in the SST2 sensilla was inhibited by several aldehydes (hexanal, nonanal, heptanal, and octanal) and all ketones except for 2-butanone, it was activated by two alcohols (cis-2-hexen-1-ol and p-cresol), one aliphatic/aromatic (benzene), two heterocyclics (2-picoline and pyrazine), and several other aldehydes (benzaldehyde, butanal, isobutanal,

and 2-methylbutanal) (Figure 3.2D). In the same sensilla, neuron 'B' was fired by benzaldehyde and 2, 6-dimethylpyrazine (Figure 3.2D). Finally, compared to those in the SST1 and SST2 sensilla, neuron 'A' in the SST3 sensilla evoked more intense excitatory responses to ketones and heterocyclics (Figure 3.2D). The neuron 'B' in the SST3 sensilla was activated by the aldehyde compound benzaldehyde (Figure 3.2D). All these findings suggest that both ORNs 'A' and 'B' in the antennal trichoid sensilla of *Ae. aegypti*, whether activated or inhibited, are necessary for the detection of human odorants.

### 3.4.3 Sensation of human odorants is dose-dependent

To determine whether ORNs that responded strongly to certain odorants also responded with high sensitivity, we examined their responses to serial doses of compounds that elicited strong responses ( $\geq 50$  spikes/s or  $\leq -10$  spikes/s) at a  $10^{-2}$  dilution. Three aldehyde compounds (hexanal, nonanal, and octanal) elicited dose-dependent excitatory responses in the SST1 sensilla but inhibitory responses in the SST2 sensilla (Figure 3.3A). In the SST3 sensilla, ketones including 2-hexanone, 2-pentanone, and 3-pentanone induced stronger excitatory responses when their concentrations increased (Figure 3.3B). 2-hexanone also produced dose-dependent responses in the SST2 sensilla but in an inhibitory manner (Figure 3.3B). The SST2 sensilla showed a particularly high sensitivity to a heterocyclic compound (2-picoline), with a firing rate of 47 spikes/s at a  $10^{-4}$  dilution (Figure 3.3C).

Next, the sensitivities of the SBTII sensilla against aldehydes and aliphatics/aromatics and the GP sensilla against amines were tested. The SBTII sensilla were found to be very sensitive to nonanal, with a firing rate of 54 spikes/s at a  $10^{-5}$  dilution, followed by octanal (24 spikes/s) and heptanal (16 spikes/s) (Figure 3.3D). Butanal, however, produced dose-dependent inhibitory responses in the same sensilla. Three aliphatic/aromatic compounds (toluene, benzene, and styrene) induced excitatory responses in the SBTII sensilla, with the response increasing slowly with the concentration (Figure 3.3E). One amine compound (butylamine) elicited inhibitory responses in the SBTII sensilla, but these responses did not follow a dose-dependent pattern (Figure 3.3E). The GP sensilla showed high sensitivity to ammonia, with

the response increasing sharply with the concentration (Figure 3.3F). A further two amines also elicited excitatory responses in the GP sensilla, although the sensitivity of the GP sensilla to these two compounds was lower than their sensitivity to ammonia (Figure 3.3F). These results suggest that *Ae. aegypti* shows different sensitivities to different human odorants, which perhaps reflects the ecological significance of certain compounds in mosquito host-seeking activities.

#### **3.4.4 Temporal dynamics of olfactory sensillum responses to human odorants**

Temporal dynamics may explain how an odorant is represented in the peripheral sensory systems of insects (Laurent et al. 2001; Carey et al. 2010). To investigate the temporal dynamics of the *Ae. aegypti* ORN responses to human odorants, strong responses induced by a 500-ms pulse of air through the compound cartridge were plotted to construct a set of temporal firing activities. Aliphatics/aromatics (including toluene, benzene and styrene) induced typical phasic responses in the SBTII sensilla, with the highest firing rates occurring 500 ms post-stimulus followed by rapid declines during the next 1.5-sec of the analysis period (Figure 3.4A). When the same sensilla were challenged with certain aldehydes (octanal, nonanal, hexanal, decanal, and heptanal), typical tonic excitatory responses were observed and these responses were sustained throughout the 4-sec observation period (Figure 3.4A). Other aldehydes elicited inhibitory responses in the same ORNs, but the temporal structures of these responses were different. In the SBTII sensilla, propanal induced significant phasic inhibitory responses, while other aldehydes (pentanal, butanal, isobutanal, and 2-methylbutanal) produced prolonged/tonic responses (Figure 3.4A). Ketones also elicited more phasic responses in the SST3 sensilla (Figure 3.4B), while three amine compounds (ammonia, propylamine, and butylamine) induced more tonic responses in the GP sensilla (Figure 3.4C). Finally, the concentration of an odorant was found to influence the temporal structure of the response it elicited. For example, at a  $10^{-2}$  dilution, toluene elicited a typical phasic response in the SBTII sensilla, but at lower concentrations it induced more tonic responses in the same sensilla (Figure 3.4D). These results suggest that compounds from different chemical groups (and in some

cases, those from the same group) may be represented differently in the peripheral sensory system of insects and this may be influenced by the concentration of an odorant that insects encounter.

### **3.4.5 A primary presentation of odor space among the olfactory sensilla**

To discover whether compounds from the same chemical group elicit similar ORN response patterns, the spatial relationships among the 39 human odorants yielding at least one response with a firing rate of  $\geq 15$  or  $\leq 10$  spikes/s at a  $10^{-2}$  dilution in any type of sensilla were examined in the odor space. The seven-dimensional (7D) odor space was constructed based on the primary responses of ORNs to the 39 compounds across seven types of sensilla. To quantify the relationships, the Euclidean distances between all possible pairs (a total of 741 pairs) of the 39 compounds were compared. Skatole and trans-3-octene (8 spikes/s), skatole and 1-hexen-3-ol (9 spikes/s), and 1-hexen-3-ol and trans-3-octene (9 spikes/s) were the three closest pairs, followed by 2-pentanone and 3-pentanone (12 spikes/s) and n-piperidineethanol and 4-piperidinemethanamine (12 spikes/s). Of these, the compounds in the last two pairs were structurally related. Octanal and 2-picoline (264 spikes/s) and nonanal and 2-picoline (260 spikes/s) were the two pairs whose members were farthest apart in the odor space. The members of three additional pairs, octanal and isobutanal (256 spikes/s), octanal and pentanal (251 spikes/s), and nonanal and isobutanal (249 spikes/s) were also separated by large Euclidean distances, even though all the compounds involved came from the same chemical class.

A hierarchical cluster analysis was then performed to visualize the spatial relationships among the 39 human odorants in the odor space. Though in no case was there a cluster which contained all members from the same chemical group, in general compounds with similar structures were tightly clustered together, such as 2-pentanone and 3-pentanone, propanal and butanal, and propylamine and butylamine (Figure 3.5A and 3.5B). To represent the original 7D odor space in 3D space, principal component analysis (PCA) was carried out. In the 3D odor space, compounds from the aldehyde group were more widely dispersed from each other than those from the aliphatic/aromatic group (with a mean inter-odorant

distance of  $0.388 \pm 0.023$  for aldehydes and  $0.205 \pm 0.028$  for aliphatics/aromatics;  $P < 0.001$ , two-tailed  $t$ -test), suggesting that compounds from the aldehyde group may elicit more variable response patterns across the seven different types of sensilla, while those from the aliphatic/aromatic group may induce more similar response patterns.

### 3.5 Discussion

In a previous study, LST2, one of the two functional subtypes of LST sensilla in *Ae. aegypti*, was found to respond to two botanic terpenoid compounds (myrcene and terpinolene) (Chen et al. 2018). However, the LST sensilla were not activated or inhibited by any of the human odorants on the panel used for this study. This may be because the LST sensilla of *Ae. aegypti* are narrowly tuned to specific compounds, including myrcene and terpinolene. The GP sensilla of *Ae. aegypti* responded to amine compounds, as reported previously on the same type of sensilla in other mosquito species (Qiu et al. 2006; Ye et al. 2016).

Insect ORs are responsible for the sensation of most odorants. However, IRs, another group of chemoreceptors responsible for odor sensation, and GRs, the group of receptors responsible for taste sensation, are thought to be involved in the detection of amines (Min et al. 2013; Hussain et al. 2016; MacWilliam et al. 2018). As with ORs, IRs have been shown to be expressed in the antennae of *Anopheles gambiae* mosquitoes, though the exact sensillar localization of these IRs remains to be established (Pitts et al. 2017). However, GRs have been mapped in various locations in insects, including their antennae (Vosshall and Stocker 2007; Liman et al. 2014; Joseph and Carlson 2015). Carboxylic acids included in the panel did not induce responses in any types of antennal olfactory sensilla in *Ae. aegypti*, which is consistent with the findings reported in the southern house mosquito *Culex quinquefasciatus* (Ye et al. 2016). Similarly, the *Drosophila* ORNs expressing *An. gambiae* ORs in the empty neuron system were not triggered by acids (Carey et al. 2010). Previous studies indicated that IRs expressed in the ORNs of coeloconic sensillum were responsible for acid sensation in *Drosophila*

(Benton et al. 2009; Ai et al. 2010; Silbering et al. 2011). The reception of acids in mosquitoes needs to be examined in more detail.

How an odorant is encoded in the peripheral sensory system of insects remains largely unknown. In *Ae. aegypti*, two aldehydes (nonanal and heptanal) activated the 'A' neuron of SBTII sensilla but inhibited its neighbouring 'B' neuron; whereas two aliphatic/aromatic compounds (toluene and benzene) activated the 'B' neuron of SBTII sensilla but inhibiting its neighbouring 'A' neuron. Interestingly, Su et al. (2012) reported similar lateral neuronal inhibition in fruit flies and mosquitoes. In *Drosophila*, the ab3A neuron activated by methyl hexanoate was inhibited when its neighbouring ab3B neuron was activated by 2-heptanone or a pulse of light. In *Anopheles*, the cpA neuron activated by CO<sub>2</sub> was also inhibited when its neighbouring cpB neuron was activated by 1-octen-3-ol. Unlike the lateral inhibition of ORNs found in *Ae. aegypti*, where it is caused by the same compound, the inhibition observed in both *Drosophila* and *Anopheles* results from a different stimulus. Moreover, we found that certain aldehydes and ketones (such as nonanal and 2-hexanone) activated the 'A' neuron of the SST1 and/or SST3 sensilla in *Ae. aegypti* while at the same time inhibited the 'A' neuron of the SST2 sensilla. These results suggest that coding of an odor in insects may require multiple ORNs from the same or different olfactory sensilla. Precisely how different ORNs process chemical signals at the peripheral sensory centre and how electrical signals are modified at higher olfactory processing centres awaits further investigation.

**Table S3.1** Human odorants tested against the antennal olfactory sensilla in *Ae. aegypti*

Chemicals*	CAS number	Company	Purity (%)	Behavioural activity	SSR activity	
					Antenna ORN	maxillary palp ORN
<b>Carboxylic acids</b>						
acetic acid	64-19-7	Sigma	99	[1-3]	[4-7]	
myristic acid	544-63-8	Sigma	98	[2, 8-9]		
hexanoic acid	142-62-1	Sigma	99.5	[2, 9]	[5-6, 10-11]	[12]
heptanoic acid	111-14-8	Sigma	96	[2-3]		[12]
propionic acid	79-09-4	Fisher	99.5	[1-3]	[4-6, 10]	
heptadecanoic acid	506-12-7	Sigma	98	[2]		
L-(+)-lactic acid	79-33-4	Sigma	98	[1-2, 9, 13-17]	[4, 10]	
n-pentadecanoic acid	1002-84-2	Sigma	99	[2]		
benzoic acid	65-85-0	Sigma	99.5	[2]		
trans-2,3-dimethylacrylic acid	80-59-1	Acros Organics	98			
DL-3-methylvaleric acid	105-43-1	Acros Organics	97			
octanoic acid	124-07-2	Sigma	98	[2, 9]	[5-6]	[12]
decanoic acid	334-48-5	Sigma	98	[2, 8]		
undecanoic acid	112-37-8	Sigma	98	[2]		
lauric acid	143-07-7	Sigma	99	[2, 8]		
n-tridecanoic acid	638-53-9	Sigma	98	[2]		
adipic acid	124-04-9	Acros Organics	99			
pimelic acid	111-16-0	Acros Organics	98			
4-hydroxybenzoic acid	99-96-7	Acros Organics	99			
acrylic acid	79-10-7	Acros Organics	99			
n-nonanoic acid	112-05-0	Sigma	97	[2-3, 8]		
<b>Aldehydes</b>						
hexanal	66-25-1	Sigma	98	[2, 9]		
propanal	123-38-6	Sigma	97			
decanal	112-31-2	Sigma	98	[2]	[18]	
nonanal	124-19-6	Aldrich	95	[2]	[18]	
benzaldehyde	100-52-7	Sigma	99			[12]
heptanal	111-71-7	Sigma	92	[2, 9]	[18]	
pentanal	110-62-3	Sigma	97			
octanal	124-13-0	Sigma	99	[2]	[18]	[12]
butanal	123-72-8	Sigma	99			
isobutanal	78-84-2	Sigma	99			
2-methylbutanal	96-17-3	Sigma	90			[12]
<b>Alcohols</b>						
2-decanol	1120-06-5	Sigma	98			
phenol	108-95-2	Sigma	99		[6]	

trans-2-hexen-1-ol	928-95-0	Acros Organics	96			
1-tetradecanol	112-72-1	Sigma	97			
2-hexadecanol	14852-31-4	Sigma	99			
cis-2-hexen-1-ol	928-94-9	Aldrich	95			
glycerol	56-81-5	Sigma	99	[2]		
1-hexen-3-ol	4798-44-1	Sigma	98	[9]		
1-octen-3-ol	3391-86-4	Aldrich	99	[3, 9, 13, 15, 17, 19]	[7]	[11, 16, 20]
trans-2-octen-1-ol	18409-17-1	Acros Organics	98			
4-ethyl phenol	123-07-9	Acros Organics	97		[6]	
p-cresol	106-44-5	Acros Organics	99			
pheneethyl alcohol	60-12-8	Sigma	99			
<b>Aliphatics/Aromatics</b>						
n-heptadecane	629-78-7	Sigma	99			
toluene	108-88-3	Sigma	99.8	[2]		
1-hexadecene	629-73-2	Aldrich	99			
1-tetradecene	1120-36-1	Aldrich	97			
n-nonane	111-84-2	Fisher	100			
benzene	71-43-2	Sigma	99.8	[2]		
squalene	111-02-4	Sigma	98	[2]		
propylbenzene	103-65-1	Sigma	98			
n-pentadecane	629-62-9	Acros Organics	99			
hexadecane	544-76-3	Acros Organics	99			
trans-2-octene	13389-42-9	Aldrich	97	[2]		
n-octadecane	593-45-3	Sigma	99			
styrene	100-42-5	Sigma	99	[2]		
n-decane	124-18-5	Fisher	99			
xylene	106-42-3	Sigma	99.5			
ethylbenzene	100-41-4	Sigma	99			
2,4-dimethylhexane	589-43-5	Fidher	99			
trans-4-octene	14850-23-8	Aldrich	98			
trans-3-octene	14919-01-8	Aldrich	98			
n-octane	111-65-9	Sigma	98			
2-pentene	109-68-2	Aldrich	99			
hexane	110-54-3	Sigma	95		[7]	[20]
heptane anhydrous	142-82-5	Sigma	99	[2]		
<b>Esters</b>						
methyl nonanoate	1731-84-6	Acros Organics	95			
methyl tridecanoate	1731-88-0	Acros Organics	97			

<b>Ketones</b>						
2-hexanone	591-78-6	Fluka	96			
2-pentanone	107-87-9	Fisher	99	[2]		
3-pentanone	96-22-0	Fisher	99	[2]		
2-decanone	693-54-9	Aldrich	98	[2]		
2-butanone	78-93-3	Sigma	99.7	[2]		[12]
sulcatone	110-93-0	Sigma	98	[2-3]	[21]	
<b>Amines</b>						
propylamine	107-10-8	Aldrich	99			
butylamine	109-73-9	Aldrich	99.5			
ammonia	1336-21-6	Aldrich	28	[2, 9, 22]		
<b>Sulfides</b>						
carbon disulfide	75-15-0	Fisher	99.9	[2]		
methyl disulfide	624-92-0	Sigma	99	[2]		
<b>Ureas</b>						
urea	57-13-6	Sigma	99			
methylurea	598-50-5	Sigma	97			
thiourea	62-56-6	Sigma	99			
<b>Halides</b>						
1-chloroheptane	629-06-1	Aldrich	99			
lauryl chloride	112-52-7	Acros Organics	99			
1-chlorotetradecane	2425-54-9	Acros Organics	98			
1-chlorohexadecane	4860-03-1	Aldrich	95			
1-chlorohexane	544-10-5	Fisher	95			
benzyl chloride	100-44-7	Sigma	99			
<b>Heterocyclics</b>						
indole	120-72-9	Aldrich	99	[2, 9]	[5-7, 11]	[12]
3-aminopyridine	462-08-8	Acros Organics	99			
4-aminopyridine	504-24-5	Acros Organics	98	[2]		
1-methylpiperazine	109-01-3	Acros Organics	99.5			
2-methylfuran	534-22-5	Acros Organics	99			
thiazolidine	504-78-9	Acros Organics	98			
2,6-dimethylpyrazine	108-50-9	Acros Organics	96			
2-picoline	109-06-8	Acros Organics	98			
skatole	83-34-1	Acros Organics	98	[9]		
coumarin	91-64-5	Acros Organics	99			
n-piperidineethanol	3040-44-6	Acros Organics	99			
4-peridinemethanamine	7144-05-0	Acros Organics	97			

pyrazine	290-37-9	Acros Organics	100	[12]
DMSO	67-68-5	Sigma	100	

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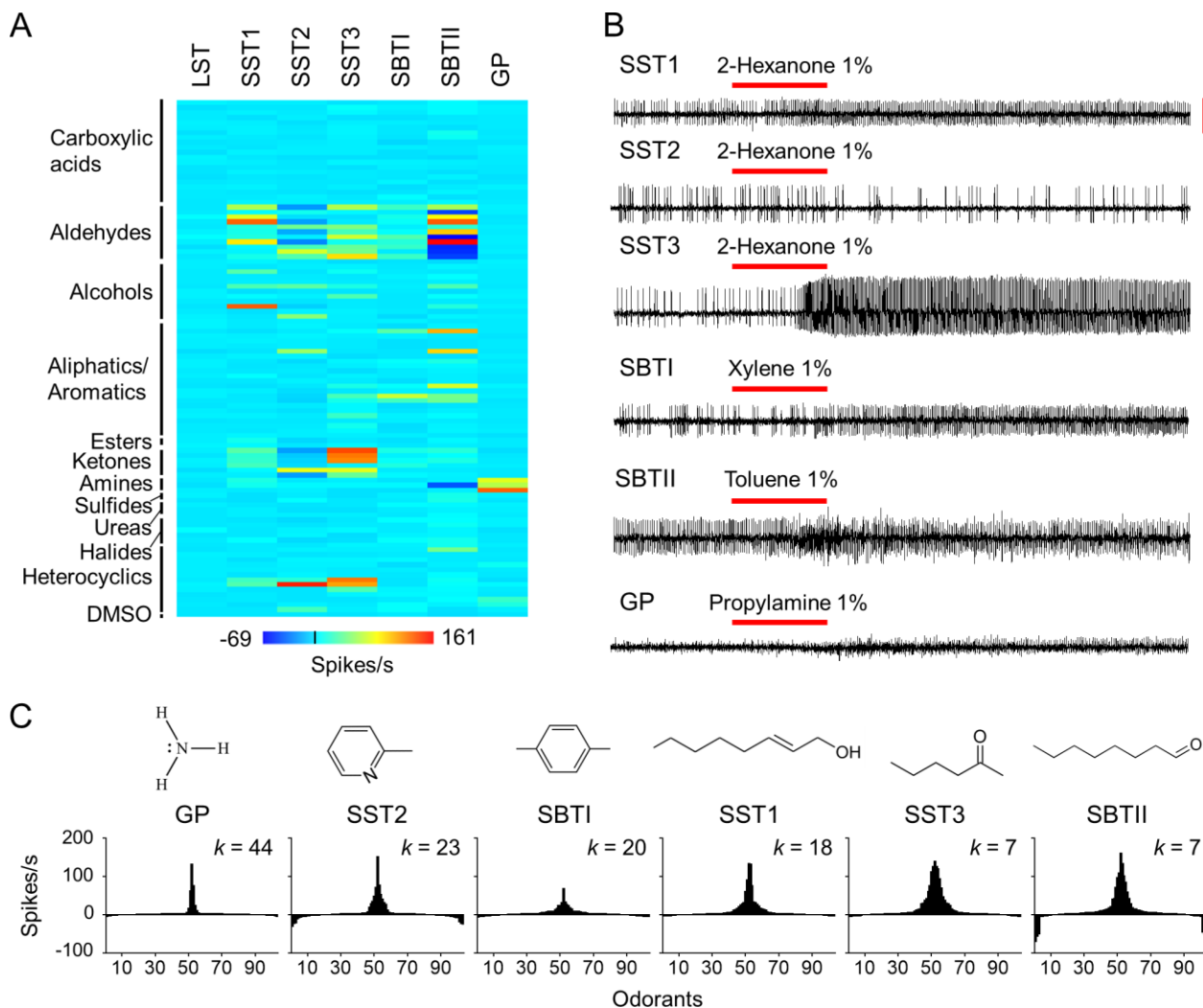
\*Human odorants were selected according to Bernier et al. (2002). All odorants were tested at a dilution of  $10^{-2}$ , except for nonanal, heptanal, and octanal in the SBTII sensilla, which were tested at dilutions of  $10^{-4}$ ,  $10^{-3}$ , and  $10^{-3}$ , respectively, while ammonia was tested in the GP sensilla at a dilution of  $0.5 \times 10^{-3}$ . Aldehydes (nonanal, heptanal and octanal) and amine (ammonia) at a dilution of  $10^{-2}$  elicited such strong responses in the SBTII and GP sensilla that the responses were immediately inhibited upon delivery. Dimethyl sulfoxide (DMSO) was used to dilute all compounds except for ammonia, which was diluted in ddH<sub>2</sub>O. Numbers refer to previous behavioural studies or SSR studies on antennal/maxillary palp sensilla in *Ae. aegypti*: [1] Takken 1991; [2] Smallegange and Takken 2010; [3] Saratha and Mathew 2016; [4] Davis and Sokolove 1976; [5] Ghaninia et al. 2007; [6] Siju et al. 2010; [7] Stanczyk et al. 2010; [8] Ponnusamy et al. 2008; [9] Mathew et al. 2013; [10] Pappenberger et al. 1996; [11] DeGennaro et al. 2013; [12] Tauxe et al. 2013; [13] Canyon et al. 1997; [14] Williams et al. 2006; [15] Cook et al. 2011; [16] McMeniman et al. 2014; [17] Vinauger et al. 2014; [18] Ghaninia et al. 2008; [19] Majeed et al. 2016; [20] Grant and Dickens 2011; [21] McBride et al. 2014; [22] Bosch et al. 2000.

**Table S3.2** Responses of different types of antennal olfactory sensilla to the human odorants tested (mean  $\pm$  s.e.m., spikes/s)

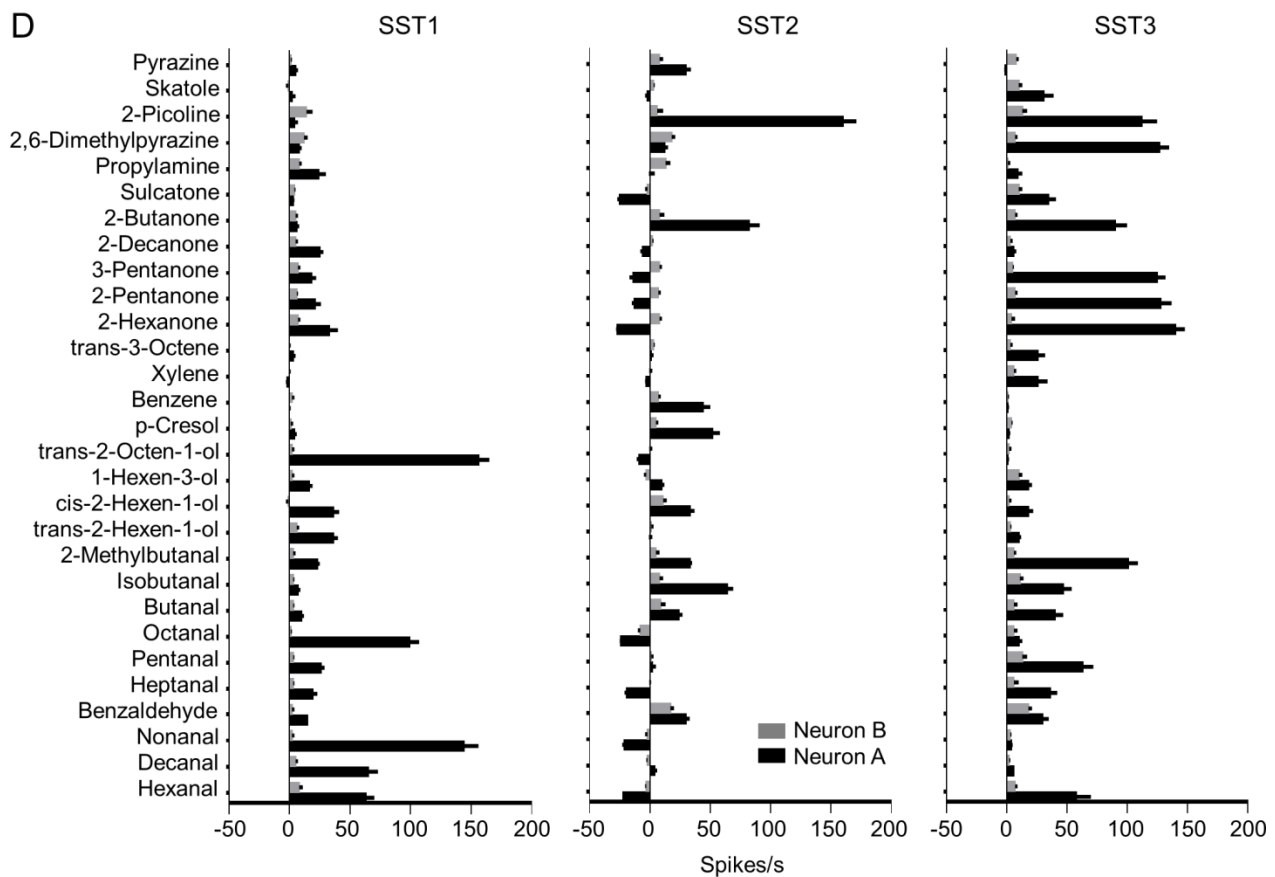
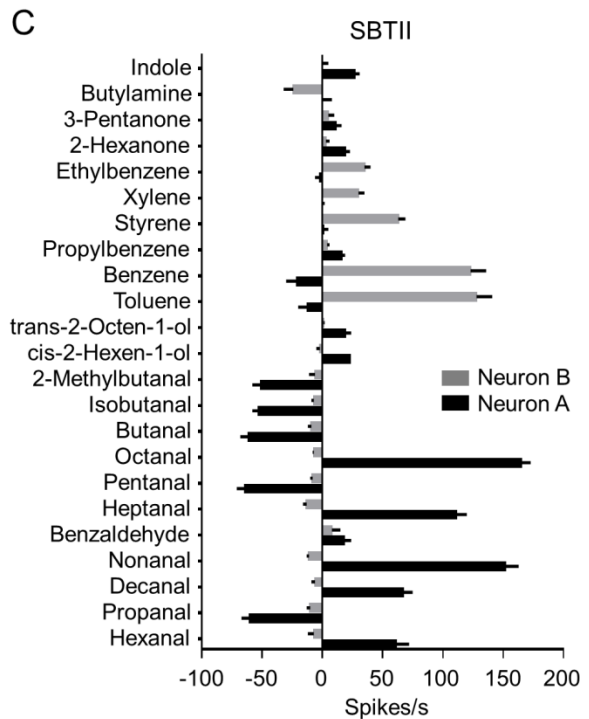
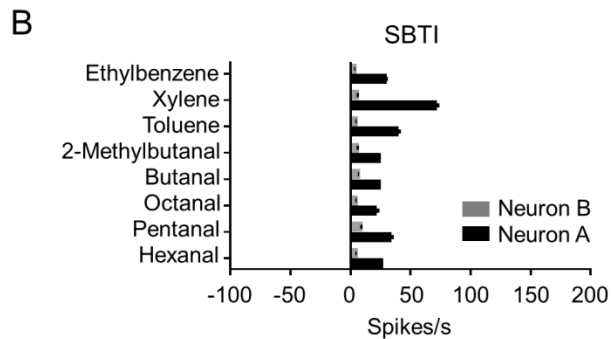
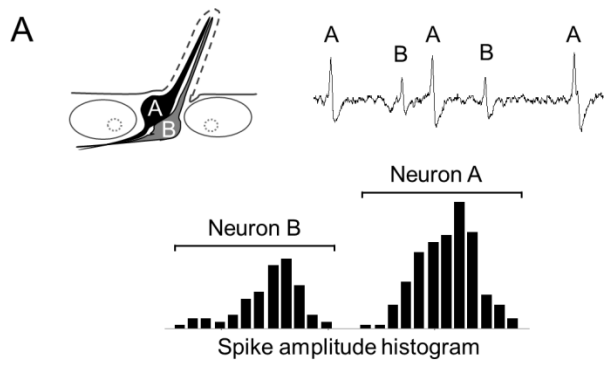
<b>Chemicals\ Sensilla</b>	<b>LST</b>	<b>SST1</b>	<b>SST2</b>	<b>SST3</b>	<b>SBTI</b>	<b>SBTII</b>	<b>GP</b>
<b>Carboxylic acids</b>							
acetic acid	4 $\pm$ 2	2 $\pm$ 1	2 $\pm$ 1	-1 $\pm$ 2	1 $\pm$ 4	6 $\pm$ 5	1 $\pm$ 2
myristic acid	-2 $\pm$ 2	0 $\pm$ 1	0 $\pm$ 1	8 $\pm$ 3	3 $\pm$ 3	6 $\pm$ 2	3 $\pm$ 2
hexanoic acid	1 $\pm$ 2	3 $\pm$ 2	1 $\pm$ 2	5 $\pm$ 4	1 $\pm$ 2	6 $\pm$ 2	2 $\pm$ 2
heptanoic acid	1 $\pm$ 1	-2 $\pm$ 1	2 $\pm$ 1	-2 $\pm$ 3	-2 $\pm$ 2	0 $\pm$ 3	1 $\pm$ 2
propionic acid	3 $\pm$ 2	2 $\pm$ 2	-2 $\pm$ 2	2 $\pm$ 5	2 $\pm$ 4	1 $\pm$ 4	1 $\pm$ 2
heptadecanoic acid	2 $\pm$ 2	3 $\pm$ 1	0 $\pm$ 2	4 $\pm$ 3	0 $\pm$ 2	-2 $\pm$ 3	1 $\pm$ 2
L-(+)-lactic acid	-2 $\pm$ 2	3 $\pm$ 1	1 $\pm$ 2	4 $\pm$ 3	0 $\pm$ 3	12 $\pm$ 6	0 $\pm$ 2
n-pentadecanoic acid	2 $\pm$ 1	1 $\pm$ 1	-1 $\pm$ 1	3 $\pm$ 3	-1 $\pm$ 2	10 $\pm$ 3	1 $\pm$ 2
benzoic acid	-1 $\pm$ 2	0 $\pm$ 2	1 $\pm$ 2	2 $\pm$ 2	-5 $\pm$ 1	-1 $\pm$ 3	-1 $\pm$ 2
trans-2,3-dimethylacrylic acid	1 $\pm$ 2	-1 $\pm$ 2	2 $\pm$ 1	2 $\pm$ 4	2 $\pm$ 2	0 $\pm$ 2	3 $\pm$ 2
DL-3-methylvaleric acid	-2 $\pm$ 2	3 $\pm$ 2	2 $\pm$ 2	2 $\pm$ 2	-2 $\pm$ 2	0 $\pm$ 3	2 $\pm$ 1
octanoic acid	4 $\pm$ 2	0 $\pm$ 2	-2 $\pm$ 2	-2 $\pm$ 3	-2 $\pm$ 2	-4 $\pm$ 3	1 $\pm$ 2
decanoic acid	3 $\pm$ 2	2 $\pm$ 2	0 $\pm$ 2	4 $\pm$ 2	3 $\pm$ 3	2 $\pm$ 3	1 $\pm$ 1
undecanoic acid	-1 $\pm$ 2	0 $\pm$ 2	0 $\pm$ 2	-2 $\pm$ 3	2 $\pm$ 3	1 $\pm$ 2	0 $\pm$ 2
lauric acid	-1 $\pm$ 2	3 $\pm$ 1	2 $\pm$ 2	1 $\pm$ 3	0 $\pm$ 3	-2 $\pm$ 3	0 $\pm$ 2
n-tridecanoic acid	-2 $\pm$ 2	0 $\pm$ 1	-1 $\pm$ 1	1 $\pm$ 3	0 $\pm$ 1	3 $\pm$ 3	-2 $\pm$ 2
adipic acid	0 $\pm$ 2	1 $\pm$ 2	0 $\pm$ 2	0 $\pm$ 2	4 $\pm$ 3	-2 $\pm$ 3	-1 $\pm$ 1
pimelic acid	2 $\pm$ 2	3 $\pm$ 1	0 $\pm$ 1	2 $\pm$ 3	-3 $\pm$ 1	1 $\pm$ 4	3 $\pm$ 2
4-hydroxybenzoic acid	1 $\pm$ 2	0 $\pm$ 1	-1 $\pm$ 2	-2 $\pm$ 2	-1 $\pm$ 1	4 $\pm$ 3	0 $\pm$ 1
acrylic acid	1 $\pm$ 2	1 $\pm$ 1	3 $\pm$ 2	0 $\pm$ 3	7 $\pm$ 3	-2 $\pm$ 2	0 $\pm$ 1
n-nonanoic acid	0 $\pm$ 1	4 $\pm$ 2	2 $\pm$ 2	2 $\pm$ 2	2 $\pm$ 2	5 $\pm$ 2	1 $\pm$ 2
<b>Aldehydes</b>							
hexanal	0 $\pm$ 3	60 $\pm$ 3	-24 $\pm$ 2	60 $\pm$ 6	24 $\pm$ 2	58 $\pm$ 4	3 $\pm$ 2
propanal	1 $\pm$ 3	6 $\pm$ 1	12 $\pm$ 2	5 $\pm$ 3	2 $\pm$ 2	-49 $\pm$ 4	2 $\pm$ 2
decanal	7 $\pm$ 3	75 $\pm$ 4	-2 $\pm$ 2	-2 $\pm$ 3	13 $\pm$ 9	85 $\pm$ 6	-1 $\pm$ 1
nonanal	1 $\pm$ 2	131 $\pm$ 6	-21 $\pm$ 1	1 $\pm$ 2	9 $\pm$ 5	133 $\pm$ 12	0 $\pm$ 1
benzaldehyde	0 $\pm$ 2	16 $\pm$ 1	40 $\pm$ 2	46 $\pm$ 5	2 $\pm$ 3	21 $\pm$ 6	2 $\pm$ 2
heptanal	3 $\pm$ 2	13 $\pm$ 2	-18 $\pm$ 3	28 $\pm$ 7	8 $\pm$ 4	101 $\pm$ 11	1 $\pm$ 2
pentanal	7 $\pm$ 3	16 $\pm$ 2	2 $\pm$ 2	69 $\pm$ 7	31 $\pm$ 3	-69 $\pm$ 5	2 $\pm$ 2
octanal	2 $\pm$ 2	90 $\pm$ 4	-29 $\pm$ 2	12 $\pm$ 4	18 $\pm$ 2	161 $\pm$ 6	2 $\pm$ 2
butanal	-3 $\pm$ 2	5 $\pm$ 2	28 $\pm$ 2	38 $\pm$ 4	23 $\pm$ 2	-59 $\pm$ 5	1 $\pm$ 2
isobutanal	3 $\pm$ 3	4 $\pm$ 1	71 $\pm$ 4	42 $\pm$ 5	1 $\pm$ 2	-56 $\pm$ 5	2 $\pm$ 2
2-methylbutanal	0 $\pm$ 2	13 $\pm$ 3	32 $\pm$ 3	93 $\pm$ 8	24 $\pm$ 2	-50 $\pm$ 6	-1 $\pm$ 2
<b>Alcohols</b>							
2-decanol	2 $\pm$ 2	4 $\pm$ 2	1 $\pm$ 2	-3 $\pm$ 1	-1 $\pm$ 2	-4 $\pm$ 2	3 $\pm$ 2
phenol	2 $\pm$ 2	0 $\pm$ 1	2 $\pm$ 4	3 $\pm$ 3	0 $\pm$ 2	4 $\pm$ 3	2 $\pm$ 2
trans-2-hexen-1-ol	1 $\pm$ 2	32 $\pm$ 2	-1 $\pm$ 2	10 $\pm$ 2	1 $\pm$ 2	0 $\pm$ 2	1 $\pm$ 2
1-tetradecanol	1 $\pm$ 2	1 $\pm$ 1	1 $\pm$ 1	0 $\pm$ 2	3 $\pm$ 1	7 $\pm$ 3	1 $\pm$ 2
2-hexadecanol	1 $\pm$ 2	2 $\pm$ 1	2 $\pm$ 2	1 $\pm$ 2	1 $\pm$ 2	2 $\pm$ 3	1 $\pm$ 2
cis-2-hexen-1-ol	0 $\pm$ 1	29 $\pm$ 2	34 $\pm$ 3	15 $\pm$ 5	2 $\pm$ 3	29 $\pm$ 3	0 $\pm$ 1
glycerol	3 $\pm$ 2	-2 $\pm$ 1	0 $\pm$ 2	-1 $\pm$ 2	1 $\pm$ 2	-3 $\pm$ 2	2 $\pm$ 2
1-hexen-3-ol	3 $\pm$ 2	6 $\pm$ 2	3 $\pm$ 2	30 $\pm$ 3	0 $\pm$ 1	8 $\pm$ 5	3 $\pm$ 2
1-octen-3-ol	1 $\pm$ 1	0 $\pm$ 2	-1 $\pm$ 4	1 $\pm$ 4	0 $\pm$ 3	1 $\pm$ 2	1 $\pm$ 1
trans-2-octen-1-ol	-1 $\pm$ 2	133 $\pm$ 6	-7 $\pm$ 2	1 $\pm$ 2	0 $\pm$ 2	17 $\pm$ 4	3 $\pm$ 3
4-ethyl phenol	1 $\pm$ 1	-2 $\pm$ 2	3 $\pm$ 2	3 $\pm$ 1	1 $\pm$ 1	-2 $\pm$ 2	0 $\pm$ 2
p-cresol	3 $\pm$ 2	0 $\pm$ 2	48 $\pm$ 3	2 $\pm$ 2	-4 $\pm$ 1	4 $\pm$ 2	2 $\pm$ 3

phenelethyl alcohol	4±2	1±1	3±2	2±2	3±3	0±3	1±1
<b>Aliphatics/Aromatics</b>							
n-heptadecane	4±2	1±2	4±2	1±2	-3±3	3±2	1±2
toluene	0±2	-2±1	1±3	8±4	33±3	108±4	1±2
1-hexadecene	3±2	2±1	4±1	2±2	3±2	1±3	-1±2
1-tetradecene	2±2	3±1	2±2	1±1	-2±1	-1±2	1±2
n-nonane	2±2	1±1	2±1	1±2	-2±1	-1±3	1±2
benzene	-3±2	-1±2	53±3	0±3	-1±2	97±6	0±2
squalene	1±1	3±2	3±2	-3±3	-1±2	-2±1	2±2
propylbenzene	3±2	0±2	-4±2	4±2	8±3	15±4	0±2
n-pentadecane	3±2	-1±1	0±2	2±2	5±3	5±3	2±2
hexadecane	1±2	0±2	1±2	-3±2	1±3	3±2	1±1
trans-2-octene	2±2	7±3	-1±3	3±3	1±2	2±3	-1±2
n-octadecane	0±1	0±2	3±2	1±2	-3±1	2±2	-1±1
styrene	-1±1	-2±2	0±2	14±5	8±5	71±3	4±2
n-decane	3±2	2±2	-3±1	0±2	3±2	2±2	1±2
xylene	3±3	0±2	-5±1	28±9	68±4	42±3	2±2
ethylbenzene	0±3	0±2	-6±1	13±5	26±2	40±2	-1±2
2,4-dimethylhexane	-1±2	2±1	1±1	5±2	-3±2	-1±2	0±2
trans-4-octene	2±2	0±2	-2±2	5±4	-1±3	2±2	1±1
trans-3-octene	3±2	3±2	1±2	23±7	3±3	6±3	1±2
n-octane	-1±2	0±2	0±2	1±2	2±3	1±1	0±1
2-pentene	1±2	1±2	1±1	12±4	-3±2	3±3	1±2
hexane	1±2	0±1	2±1	5±4	-2±2	4±2	2±2
heptane anhydrous	2±2	4±2	2±1	2±2	2±3	0±2	-2±1
<b>Esters</b>							
methyl nonanoate	2±2	12±3	-5±3	0±4	-2±4	5±5	1±2
methyl tridecanoate	4±2	5±3	0±2	4±5	0±2	2±2	1±2
<b>Ketones</b>							
2-hexanone	2±4	33±5	-22±2	139±5	14±2	18±4	-3±1
2-pentanone	-1±2	20±3	-9±2	125±8	3±3	7±2	0±1
3-pentanone	3±4	22±3	-6±2	120±8	9±4	15±4	1±2
2-decanone	2±4	27±4	-4±2	3±3	16±13	3±2	2±1
2-butanone	-3±2	5±2	77±3	75±7	1±3	1±3	0±1
sulcatone	2±2	2±2	-22±2	38±4	5±2	3±1	0±2
<b>Amines</b>							
propylamine	0±2	15±4	0±3	2±2	-1±3	2±2	75±4
butylamine	-2±2	12±4	5±3	5±4	5±4	-44±3	62±4
ammonia	2±2	1±1	2±2	-2±2	-2±2	2±2	132±6
<b>Sulfides</b>							
carbon disulfide	3±2	-2±1	1±1	-2±3	2±2	13±4	2±2
methyl disulfide	-1±2	-2±2	7±3	0±2	1±3	14±3	-1±2
<b>Ureas</b>							
urea	2±2	0±1	-1±2	0±2	6±6	5±2	3±3
methylurea	0±1	2±2	-2±2	-2±3	1±3	3±2	2±2
thiourea	0±2	2±1	2±2	2±2	0±2	6±3	1±2
<b>Halides</b>							
1-chloroheptane	0±2	-1±2	-4±3	0±2	7±5	7±4	3±2
lauryl chloride	0±2	1±1	-1±2	1±2	1±2	10±3	3±2
1-chlorotetradecane	7±2	0±2	3±1	5±2	1±3	4±1	-2±2

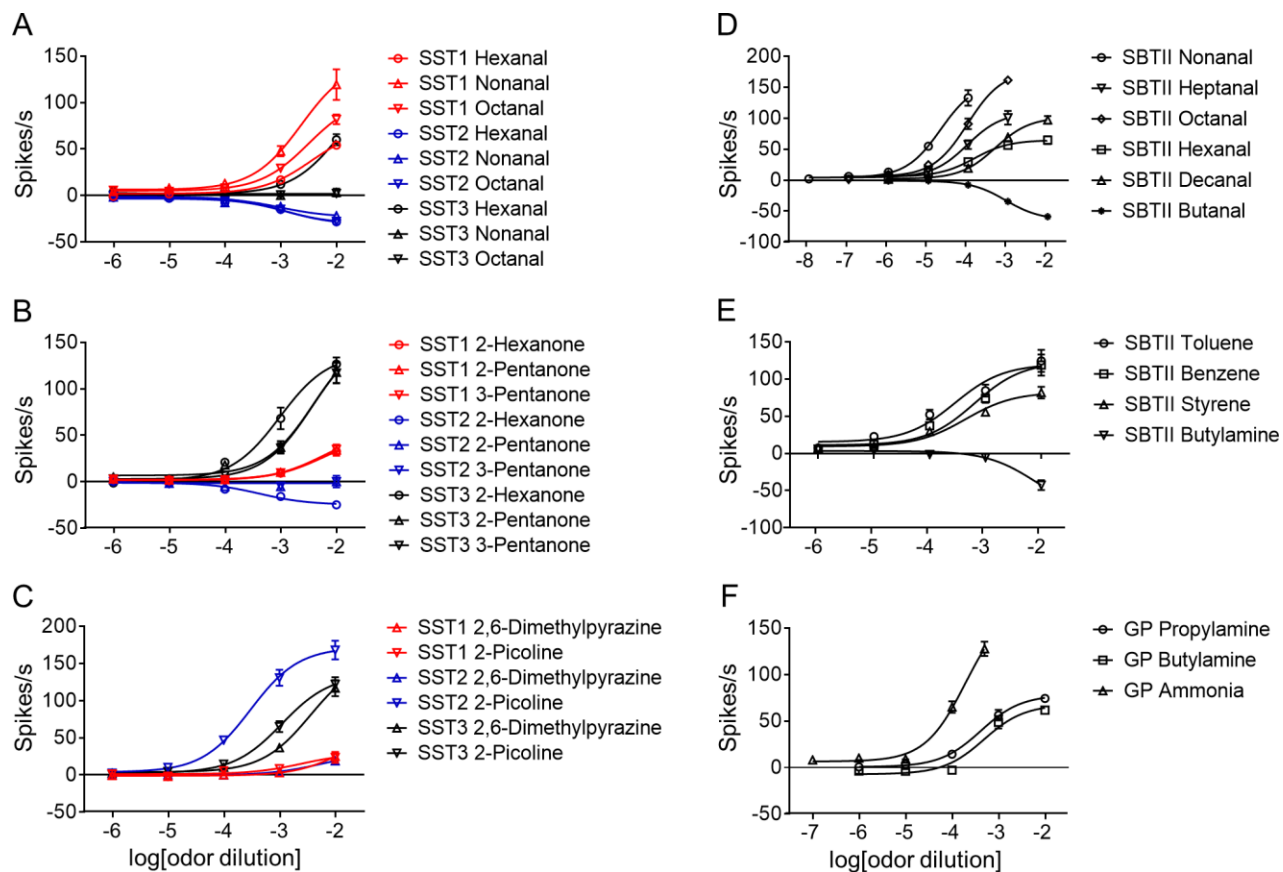
1-chlorohexadecane	-1±1	0±1	4±2	-3±3	3±2	4±3	0±2
1-chlorohexane	1±2	7±4	-4±1	-2±2	8±3	12±4	0±2
benzyl chloride	0±2	1±1	0±2	4±1	3±2	12±3	2±2
<b>Heterocyclics</b>							
indole	-1±2	1±2	3±2	2±3	2±3	42±3	0±2
3-aminopyridine	3±3	2±2	-1±2	0±2	1±2	1±3	1±2
4-aminopyridine	2±2	-2±1	4±2	4±2	5±2	1±3	0±2
1-methylpiperazine	2±2	1±1	3±2	3±2	-3±1	2±3	10±3
2-methylfuran	2±2	2±1	2±2	1±2	2±2	0±2	1±2
thiazolidine	2±1	-1±2	4±2	1±3	0±2	6±2	0±2
2,6-dimethylpyrazine	1±2	25±6	19±2	125±5	-1±2	10±4	-3±2
2-picoline	4±5	31±6	151±9	112±7	-1±1	8±3	5±3
skatole	3±2	-1±3	-1±1	30±5	4±3	6±2	1±2
coumarin	-1±2	1±1	-1±2	4±2	5±3	5±2	0±2
n-piperidineethanol	0±1	1±2	-1±2	-1±3	1±3	7±2	23±4
4-peridinemethanamine	1±2	-2±2	3±2	1±2	8±7	3±1	16±5
pyrazine	0±2	0±2	27±3	2±2	8±5	-2±2	2±2
DMSO	0±1	0±1	0±1	-1±1	1±3	-3±2	-1±1



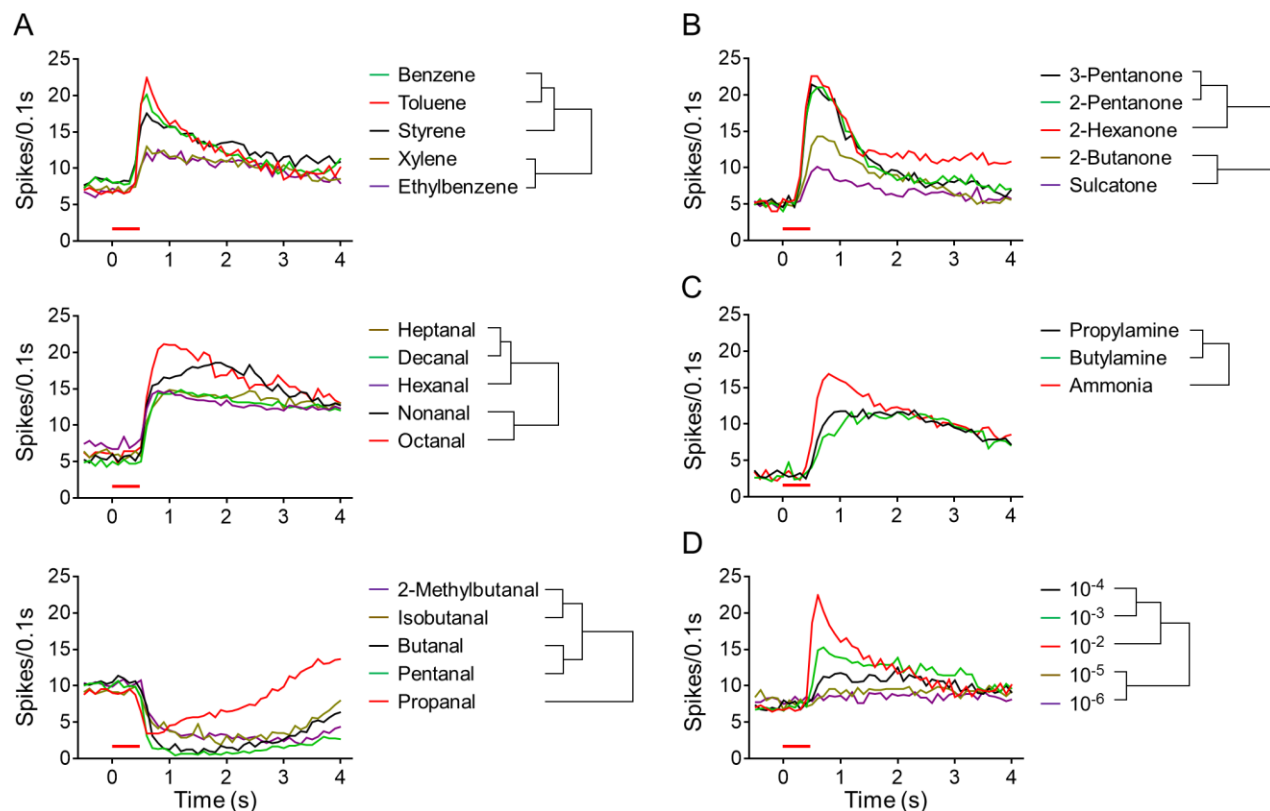
**Figure 3.1 Functional characterizations of antennal olfactory sensilla in *Ae. aegypti*.** (A) Heat map of the neuronal responses of different types of sensilla to 103 human odorants. Compounds and numerical values are presented in Table S3.2,  $n = 7-73$ . For compounds that elicited significant excitatory responses ( $\geq 15$  spikes/s) or inhibitory responses ( $\leq -10$  spikes/s),  $n = 9-73$ . All odorants were tested at a dilution of  $10^{-2}$ , except that nonanal, heptanal, and octanal were examined against SBTII sensilla at dilutions of  $10^{-4}$ ,  $10^{-3}$ , and  $10^{-3}$ , respectively, while ammonia was tested against GP sensilla at a dilution of  $0.5 \times 10^{-3}$ . (B) Extracellular recording of sensillum neuronal response to a 500-ms pulse (horizontal red bar) of air through the odorant cartridge. Vertical red bar indicates 4 mV. (C) Tuning breadths of olfactory sensilla to human odorants. The 103 odorants are arranged along the  $x$ -axis according to the strength of the response they elicited in each type of sensilla. The odorants that elicited the strongest responses are placed near the centre of the distribution, while those that elicited the weakest responses are placed near the edges. The order of odorants is therefore different for each type of sensilla. The kurtosis value,  $k$ , indicates the ‘peakedness’ of each plot. Chemical structures of the odorants eliciting the strongest responses in each type of sensilla (ammonia (GP), 2-picoline (SST2), xylene (SBTI), trans-2-octen-1-ol (SST1), 2-hexanone (SST3), and octanal (SBTII)) are shown above each plot.



**Figure 3.2 Comparison of response intensities for ORNs 'A' and 'B'.** (A) A trichoid sensillum that houses two ORNs, 'A' and 'B'. Right: a single-sensillum recording of the spontaneous activities of both neurons. Action potentials from both ORNs can be distinguished by their amplitude, with the larger amplitude corresponding to Neuron 'A' and the smaller amplitude to Neuron 'B'. Distribution of spike amplitudes of the two neurons is shown below. Response intensities for neuron 'A' (black bar) and neuron 'B' (grey bar) from sensilla SBTI (B), SBTII (C), and SST (D) are plotted. Only compounds that elicited significant responses ( $\geq 15$  spikes/s or  $\leq -10$  spikes/s) were used to analyse the response intensity for neurons 'A' or 'B'.  $n = 7-13$ . Error bars indicate s.e.m.

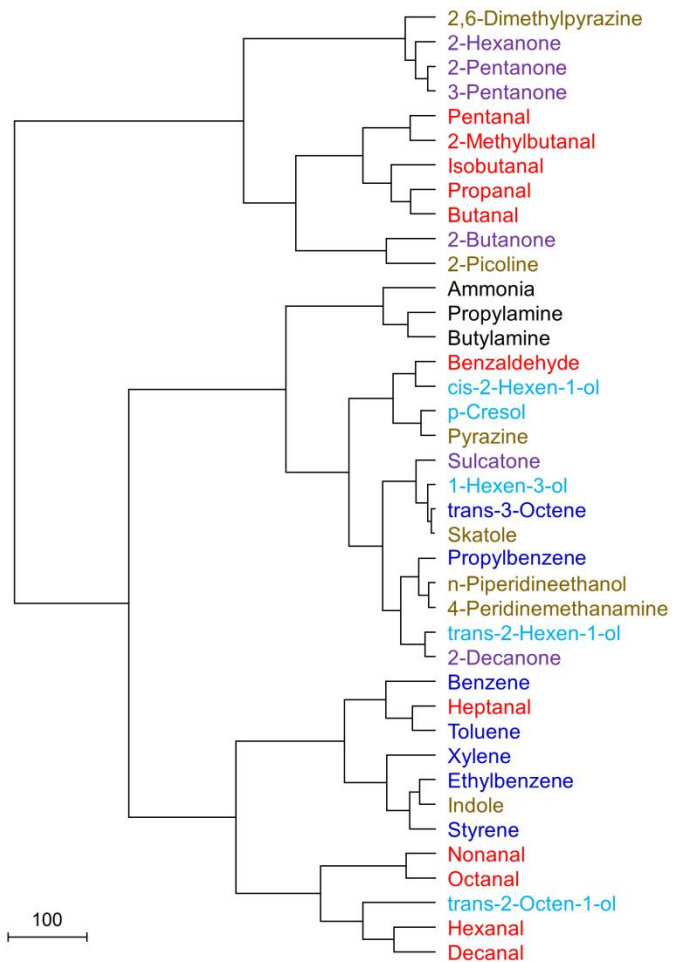


**Figure 3.3 Dose-dependent responses of olfactory sensilla to human odorants.** (A) Aldehydes, (B) ketones, and (C) heterocyclics tested in SST1 (red line), SST2 (blue line), and SST3 (black line) sensilla at serial  $10^{-2}$ - $10^{-6}$  dilutions. (D) Aldehydes tested in SBTII sensilla. Nonanal tested at serial  $10^{-4}$ - $10^{-8}$  dilutions; heptanal and octanal at serial  $10^{-3}$ - $10^{-7}$  dilutions; hexanal, decanal, and butanal at serial  $10^{-2}$ - $10^{-6}$  dilutions. (E) Three aliphatics/aromatics and one amine tested in SBTII sensilla at serial  $10^{-2}$ - $10^{-6}$  dilutions. (F) Amines tested in GP sensilla. Propylamine and butylamine tested at serial  $10^{-2}$ - $10^{-6}$  dilutions; ammonia tested at serial  $0.5 \times 10^{-3}$ - $10^{-7}$  dilutions.  $n = 5-7$ . Error bars indicate s.e.m.

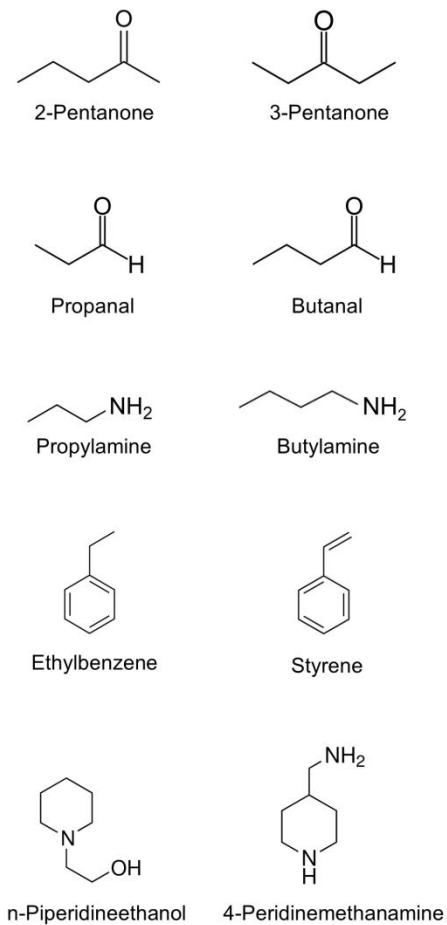


**Figure 3.4 Temporal structures of neuronal responses to human odorants.** Traces indicate the mean values of spikes ( $n = 7-9$ , error bars are not shown) recorded during each 100-ms sampling period, with the corresponding relationships within each group revealed by hierarchical cluster analysis. **(A)** SBTII sensilla responding to aliphatics/aromatics (upper) and aldehydes (middle and lower). All odorants were tested at a dilution of  $10^{-2}$ , except that nonanal, heptanal, and octanal were examined at dilutions of  $10^{-4}$ ,  $10^{-3}$ , and  $10^{-3}$ , respectively. **(B)** SST3 sensilla responding to ketones at a dilution of  $10^{-2}$ . **(C)** GP sensilla in response to amines. All chemicals were tested at a dilution of  $10^{-2}$ , except for ammonia which was examined at a dilution of  $0.5 \times 10^{-3}$ . **(D)** Toluene was examined in the SBTII sensilla at serial  $10^{-2}$ - $10^{-6}$  dilutions. The horizontal red bar above the  $x$ -axis indicates a 500-ms pulse of stimulation.

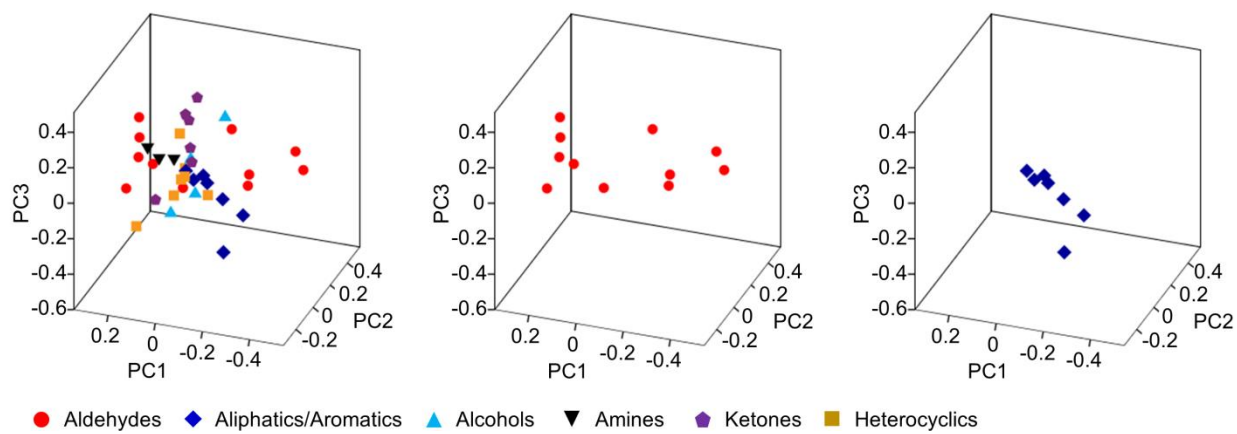
A



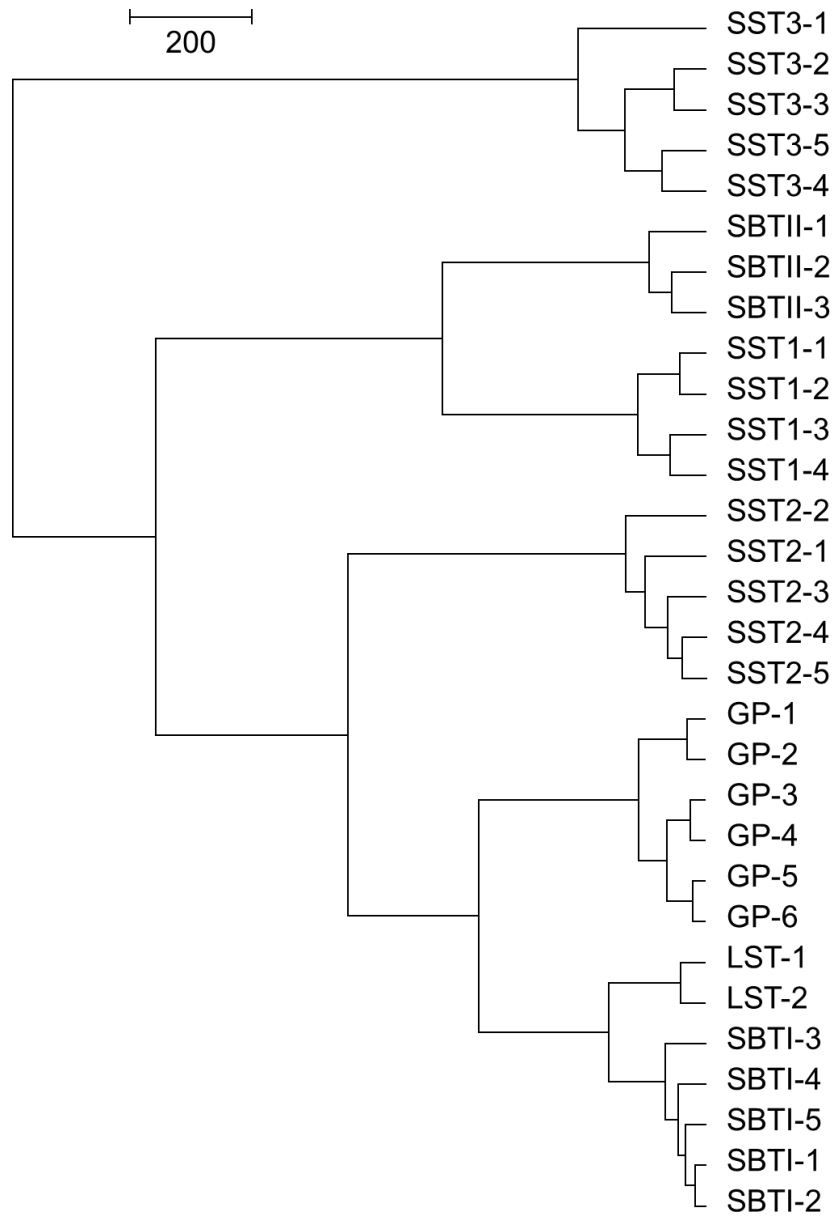
B



C



**Figure 3.5 A primary presentation of odor space.** (A) Hierarchical cluster analysis for 39 human odorants based on the Euclidean distances between odorants. These odorants elicited at least one response  $\geq 15$  spikes/s or  $\leq -10$  spikes/s at a  $10^{-2}$  dilution in any of the types of olfactory sensilla. Odorants are colour coded by chemical class. (B) Odorant pairs with close chemical structures are clustered together in the hierarchical cluster analysis. (C) Odor spaces as visualized by PCA for all 39 odorants (left), aldehydes alone (middle), and aliphatics/aromatics alone (right). By performing PCA for the neuronal responses of the seven types of olfactory sensilla to the 39 odorants, the original seven-dimensional odor space was represented in a three-dimensional space using the first three principal components. This three-dimensional representation accounts for 83.22% of the variance in the original seven-dimensional data set. Odorants are colour coded by chemical class, as shown below.



**Figure S3.1 Hierarchical cluster analysis of the responses of five morphological types of sensilla.** Seven branch clusters suggest there are seven physiological types of sensilla, namely LST, SST1, SST2, SST3, SBTI, SBTII, and GP. Three to seven replicates were performed for each type of sensilla, as indicated by the numbers (1-6).

## Chapter 4: Effects of a Blood Meal on the Sensitivity of *Ae. aegypti* to Human Odor

### 4.1 Abstract

The results of chapter 3 identified that only 39 of the 103 human odorants tested were sensed by female *Ae. aegypti* mosquitoes; however, which of the odorants detected by *Ae. aegypti* get involved in their host-seeking activities remains unknown. This study examined the effect of a blood meal on the sensitivity of female *Ae. aegypti* to the 39 human odorants. The results showed that the SST1 sensilla of blood-fed *Ae. aegypti* showed decreased sensitivity to four aldehydes at 24-60 hours post blood meal (pbm) and three alcohols at 24-84 hours pbm compared to that of non-blood fed mosquitoes. The SST2 sensilla of a blood-fed mosquito also evoked reduced responses, in either excitatory or inhibitory manner, to certain aldehydes, ketones, and alcohols. However, the SST3 sensilla of blood-fed mosquitoes indicated enhanced sensitivity to four aldehydes, one ketone, and one heterocyclic. The SBTI and SBTII sensilla in *Ae. aegypti* with a blood meal demonstrated compromised sensitivity to certain aldehydes and aliphatics/aromatics as well. The GP sensilla only evoked reduced response to propylamine at 48-60 hours pbm in blood-fed *Ae. aegypti*. The odorants eliciting diminished responses in blood-fed mosquitoes may be important in *Ae. aegypti* host-seeking activity and thus can be developed as chemical attractants for mosquito management.

Chen, Z., Liu, F., & Liu, N. (2019). Human odour coding in the yellow fever mosquito, *Aedes aegypti*.

*Submitted.*

## 4.2 Introduction

Like many other mosquito species, female *Ae. aegypti* also uses multiple cues for host-seeking activities, including CO<sub>2</sub>, skin odor, host body heat and humidity, and so on (Cardé 2015). A recent study indicated that female *Ae. aegypti* mosquitoes showed strong preference for human hosts, instead of guinea pigs, to get blood meals (DeGennaro et al. 2013). Considering that both guinea pigs and humans release CO<sub>2</sub> and have similar body heat temperature, female *Ae. aegypti* should use skin odor for host discrimination.

Another study demonstrated that more than 300 chemicals were isolated from the human skin emanations (Bernier et al. 2000). However, it is still not clear which of these human odorants can be detected by *Ae. aegypti* and which of them may contribute to the host-seeking activity of *Ae. aegypti*.

Female mosquitoes after a blood meal have shown reduced attraction to human hosts (Takken et al., 2001; Liesch et al. 2013; Matthews et al. 2016). It has been suggested that decreased neuronal responses to certain human odorants may account for this diminished host-seeking activity (Qiu et al. 2006). The major olfactory organs of insects are their antennae, in which olfactory sensory neurons (OSNs) are located (Vosshall and Stocker 2007; Hansson and Stensmyr 2011; Joseph and Carlson 2015). Odorant receptors (ORs), ionotropic receptors (IRs), and in some cases gustatory receptors (GRs) together with their highly conserved co-receptors form heteromeric ligand-gated ion channels on the membrane of OSNs in the antennae, thus contributing to the selectivity of odorant ligands in insects (Nakagawa et al. 2005; Vosshall and Stocker 2007; Sato et al. 2008; Joseph and Carlson 2015; Pitts et al. 2017). A blood meal has been shown to cause the down-regulation of specific olfactory receptor genes in hematophagous insects, which may explain the decreased neuronal responses to certain human odorants in blood-fed individuals (Fox et al. 2001; Rinker et al. 2013; Latorre-Estivalis et al. 2016; Matthews et al. 2016; Chen et al. 2017).

In chapter 3, 103 commercially available human odorants were tested in female *Ae. aegypti* and only 39 of them were sensed by these mosquitoes. Although the 39 human odorants elicited either excitatory or inhibitory responses in the antennal olfactory sensilla of *Ae. aegypti*, it does not mean that all of them may be involved in the host-seeking activity of *Ae. aegypti* mosquitoes. Blood-fed female *Ae.*

*aegypti* mosquitoes have shown decreased or even zero interest to the human odor at 24-72 hours post blood meal (pbm), but the strong interest returns at 96 hours pbm (Liesch et al. 2013). This suggests that the attraction (or olfactory response) of blood-fed *Ae. aegypti* mosquitoes to certain human odorants may decrease at 24-72 hours pbm, and these odorants may be important in the host-seeking activity of *Ae. aegypti* mosquitoes. In this study, both blood-fed (BF) and non-blood-fed (NBF) *Ae. aegypti* were tested against the 39 human odorants at three different time points (i.e. 24-36, 48-60 and 72-84 hours post blood meal (pbm)), in order to see if there will be any difference of antennal olfactory sensillar responses between BF and NBF mosquitoes.

### **4.3 Materials and Methods**

#### **4.3.1 Insects**

*Ae. aegypti* mosquitoes (Orlando strain, obtained from Dr. James Becnel, USDA, ARS, Mosquito and Fly Research Unit) were maintained at  $25 \pm 2^\circ\text{C}$  and a photoperiod of 12: 12 (L:D) h (lights on 8 am). Females and males were reared together after eclosion and supplied with unlimited 10% sucrose solution throughout. To examine the effect of a blood meal on the neuronal responses of antennal olfactory sensilla to human odorants, one group of female mosquitoes was fed once with the arm of a volunteer at the third day morning (8 am) after eclosion, while the other group was kept non-blood-fed and used as the control. Both blood-fed and non-blood-fed mosquitoes were thereafter supplied with unlimited 10% sucrose solution. The responses of the olfactory sensilla in both BF and NBF mosquitoes were tested against odorants at 24-36, 48-60, and 72-84 hours pbm.

#### **4.3.2 Single sensillum electrophysiology**

Extracellular single sensillum recording (SSR) was carried out as described in section 3.3.2.

### 4.3.3 Stimulation and stimuli

According to the results of chapter 3, 39 out of the 103 human odorants were detected by the antennal olfactory sensilla in *Ae. aegypti* (Table S4.1). The effect of a blood meal on the sensitivity of *Ae. aegypti* to these odorants were thus examined. Except for ammonia, which was diluted in ddH<sub>2</sub>O, the other odorants used as stimuli were freshly prepared every two weeks in dimethyl sulfoxide (DMSO) at the desired concentrations. All stimulations were applied as described in section 3.3.3. For each odorant, each recording was from a separate sensillum. No more than three sensilla were tested per insect.

### 4.3.4 Data analysis

Statistical analysis was conducted by IBM SPSS Statistics (version 20, <https://www.ibm.com/us-en/marketplace/spss-statistics>). Error bars indicate s.e.m. unless otherwise noted.

## 4.4 Results

### 4.4.1 Effect of blood feeding on the sensitivity of SST sensilla

To identify the human odorants that may be involved in the host-seeking activity of *Ae. aegypti*, the antennal ORNs responses of blood-fed and non-blood-fed mosquitoes to the 39 odorants sensed by *Ae. aegypti* were compared. The results showed that a blood meal had different effect on the sensitivity of different subtypes of SST sensilla in *Ae. aegypti* mosquitoes. The SST1 sensilla in blood-fed mosquitoes evoked significantly weaker responses to several aldehydes (hexanal, decanal, and octanal) at 24-36 and 48-60 hours pbm compared to those in non-blood-fed individuals (Figure 4.1). The SST1 sensilla of blood-fed mosquitoes also evoked reduced responses to another aldehyde compound (nonanal) and three alcohols, with the inhibitory effect from the blood meal lasting for up to 84 hours (Figure 4.1). The SST2 sensilla of blood-fed *Ae. aegypti* also indicated decreased sensitivity to certain aldehydes, two ketones and one alcohol odorant when compared to those in non-blood-fed mosquitoes. For example, the SST2 sensilla of blood-fed mosquito evoked diminished inhibitory responses to three aldehydes (hexanal,

nonanal, and octanal) and two ketones (2-hexanone and sulcatone) while reduced excitatory response to benzaldehyde and p-cresol (Figure 4.2). Interestingly, the SST2 sensilla of blood-fed mosquitoes evoked a stronger response to butanal than those of non-blood-fed individuals at 48-60 and 72-84 hours pbm as well (Figure 4.2). Besides, in contrast to the reduced responses observed in the SST1 and SST2 sensilla of blood-fed mosquitoes, the SST3 sensilla of blood-fed mosquitoes evoked increased responses to four aldehydes (hexanal, pentanal, butanal, and isobutanal) at 48-60 hours pbm, one ketone (3-pentanone) and one heterocyclic (2-picoline) odorant at 24-36 hours pbm (Figure 4.3).

#### **4.4.2 Effect of blood feeding on the sensitivity of SBTI, SBTII, and GP sensilla**

The neuronal responses of the SBTI, SBTII, and GP sensilla to certain human odorants were also affected in *Ae. aegypti* mosquitoes after a blood meal. In comparison with non-blood-fed individuals, blood-fed mosquitoes evoked decreased responses to four aldehydes (hexanal, pentanal, butanal, and 2-methylbutanal) and three aliphatics/aromatics (toluene, xylene, and ethylbenzene) through their SBTI sensilla, though the response to octanal increased (Figure 4.4). The SBTII sensilla in blood-fed mosquitoes also showed compromised responses to specific aldehydes (nonanal, heptanal, and octanal) and two aliphatics/aromatics (toluene and styrene) at 24-36 or 48-60 (in some cases 72-84) hours pbm (Figure 4.5). However, the response of the GP sensilla in blood-fed mosquitoes to propylamine only reduced at 48-60 hours pbm (Figure 4.5). Taken together, these results suggest that certain aldehydes, alcohols, aliphatics/aromatics, ketones, and amines, all of which elicited decreased responses in blood-fed mosquitoes, may be involved in the host-seeking response of female *Ae. aegypti* mosquitoes.

#### **4.5 Discussion**

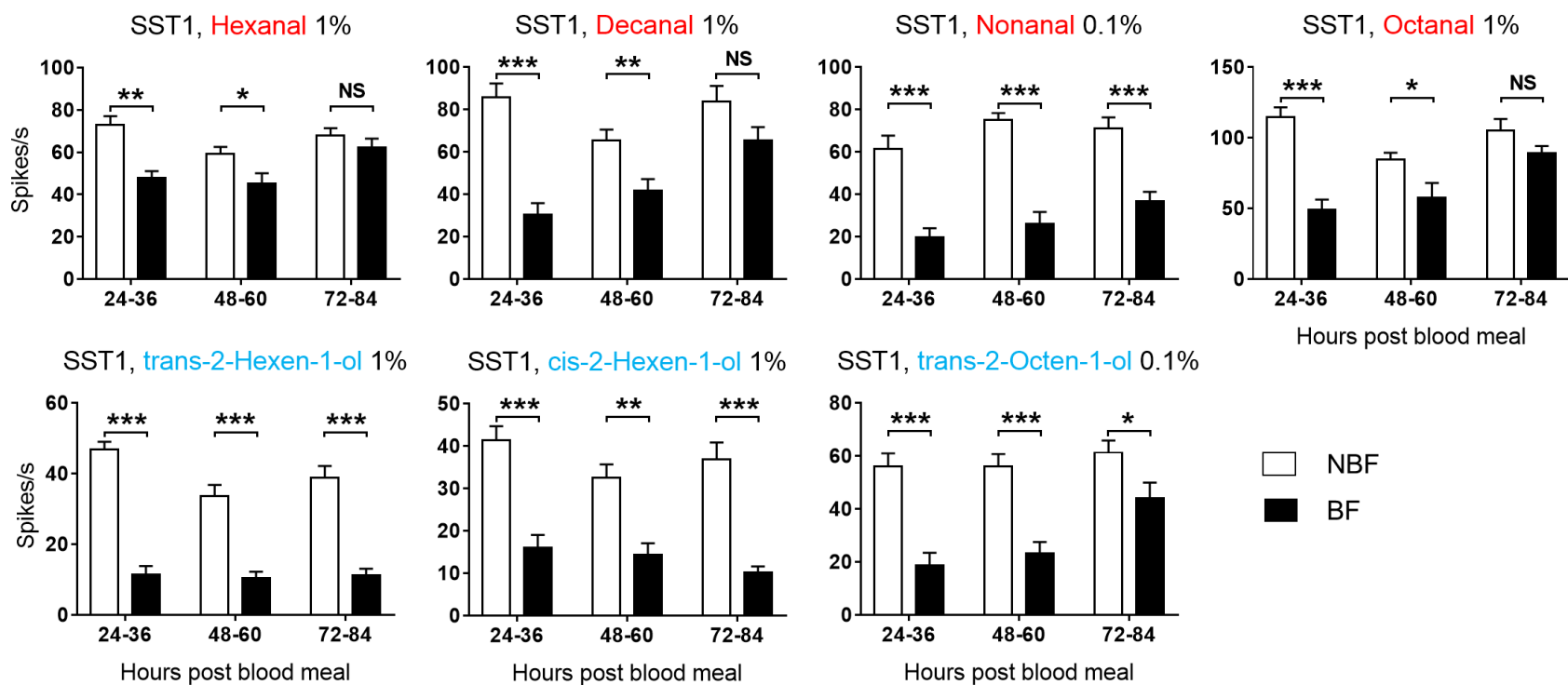
Previous studies have revealed the inhibitory effect of a blood meal on the host-seeking responses of female mosquitoes (Takken et al. 2001; Liesch et al. 2013; Matthews et al. 2016). In this study, blood-fed female *Ae. aegypti* showed decreased ORN responses to certain human odorants, including aldehydes,

alcohols, aliphatics/aromatics, ketones, and amines on the panel. These compounds may thus be important in their host-seeking activities. Ammonia has been found to be effective in attracting *An. gambiae* females at low doses and has also been reported to enhance the attractiveness of lactic acid to both *Aedes* and *Anopheles* mosquitoes (Smallegange et al. 2005; Williams et al. 2006). Specific ketones were also slightly attractive to female *Ae. aegypti* (Bernier et al. 2002). Interestingly, McBride and colleagues found that the preference of *Ae. aegypti* for humans was associated with a high expression level of *Or4* and its high sensitivity to sulcatone (a ketone odorant from human skin emanation), although sulcatone perfumed with Guinea-pig odor was not preferred over the odor of Guinea-pig alone by human-preferring mosquitoes (McBride et al. 2014). Alcohols and aliphatics/aromatics were not attractive to *Ae. aegypti* on their own (Bernier et al. 2002). Aldehydes demonstrated little attractiveness or even repellence (at high concentrations) to female *Ae. aegypti* mosquitoes (Bernier et al. 2002; Douglas et al. 2005; Logan et al. 2008). This suggests that a mixture composed of multiple human odorants (each at an optimized concentration) may be useful as an attractant for female mosquitoes.

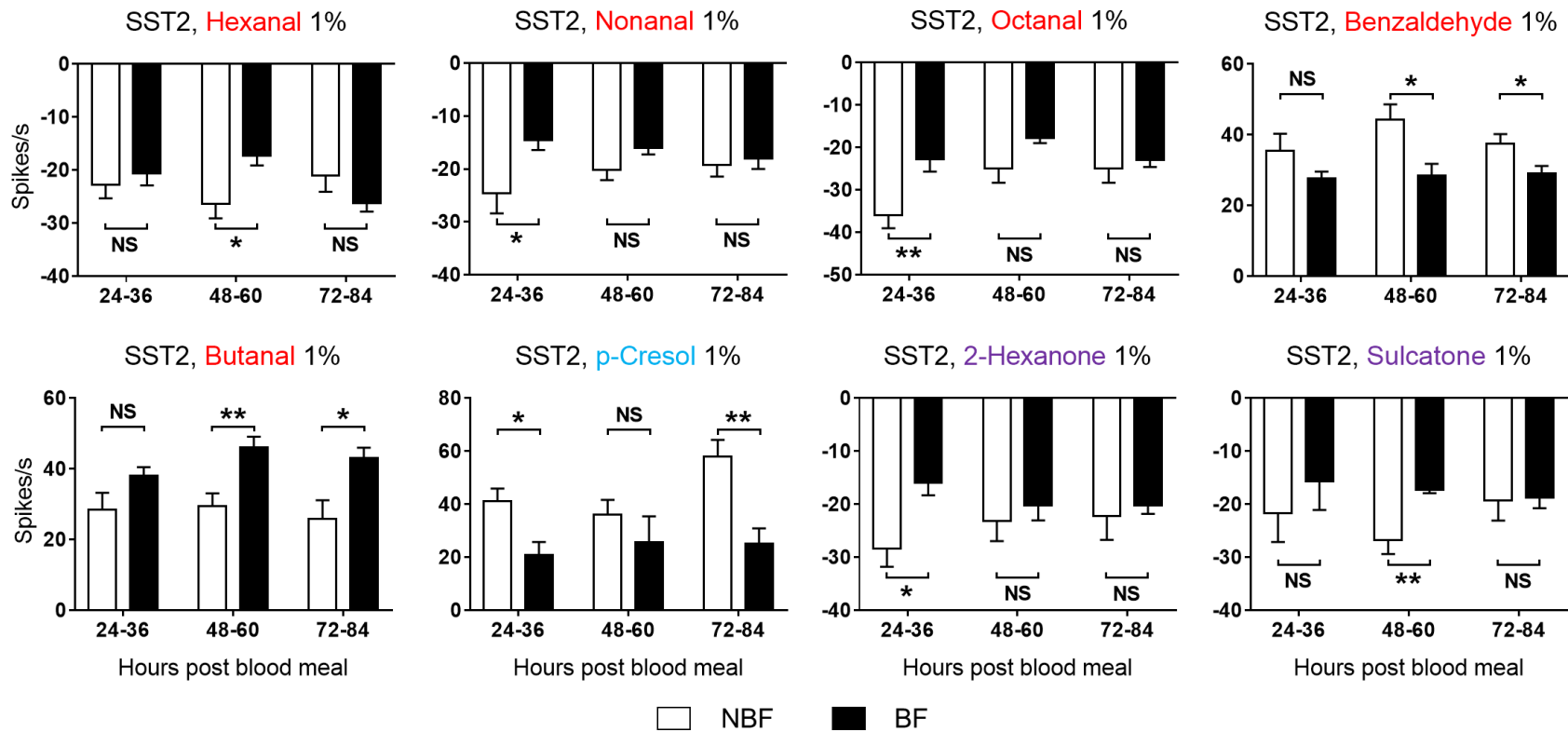
The compromised responses to human odorants may be due to the down-regulation of certain olfactory receptor genes in blood-fed insects (Fox et al. 2001; Rinker et al. 2013; Latorre-Estivalis et al. 2016; Matthews et al. 2016; Chen et al. 2017). However, regardless of the overall decreased response levels, blood-fed female *Ae. aegypti* also evoked increased responses to four aldehydes, one ketone and one heterocyclic through their SST3 sensilla. The enhanced responses to these human odorants may result from the overexpression of specific olfactory receptor genes (Rinker et al. 2013; Matthews et al. 2016; Chen et al. 2017), which may also respond to chemicals related to oviposition site cues. Blood-fed mosquitoes have been found to show increased ORN sensitivity to oviposition site cues, including heterocyclics (indoles), alcohols (phenols), ketones and carboxylic acids (Qiu et al. 2006; Siju et al. 2010). Taken together, a blood meal may exert a dual effect on the expression of olfactory receptor genes in insects, which finally modulates their ORN responses to cues from both hosts and oviposition sites.

**Table S4.1** Human odorants used to test the effect of a blood meal on the sensitivity of *Ae. aegypti*

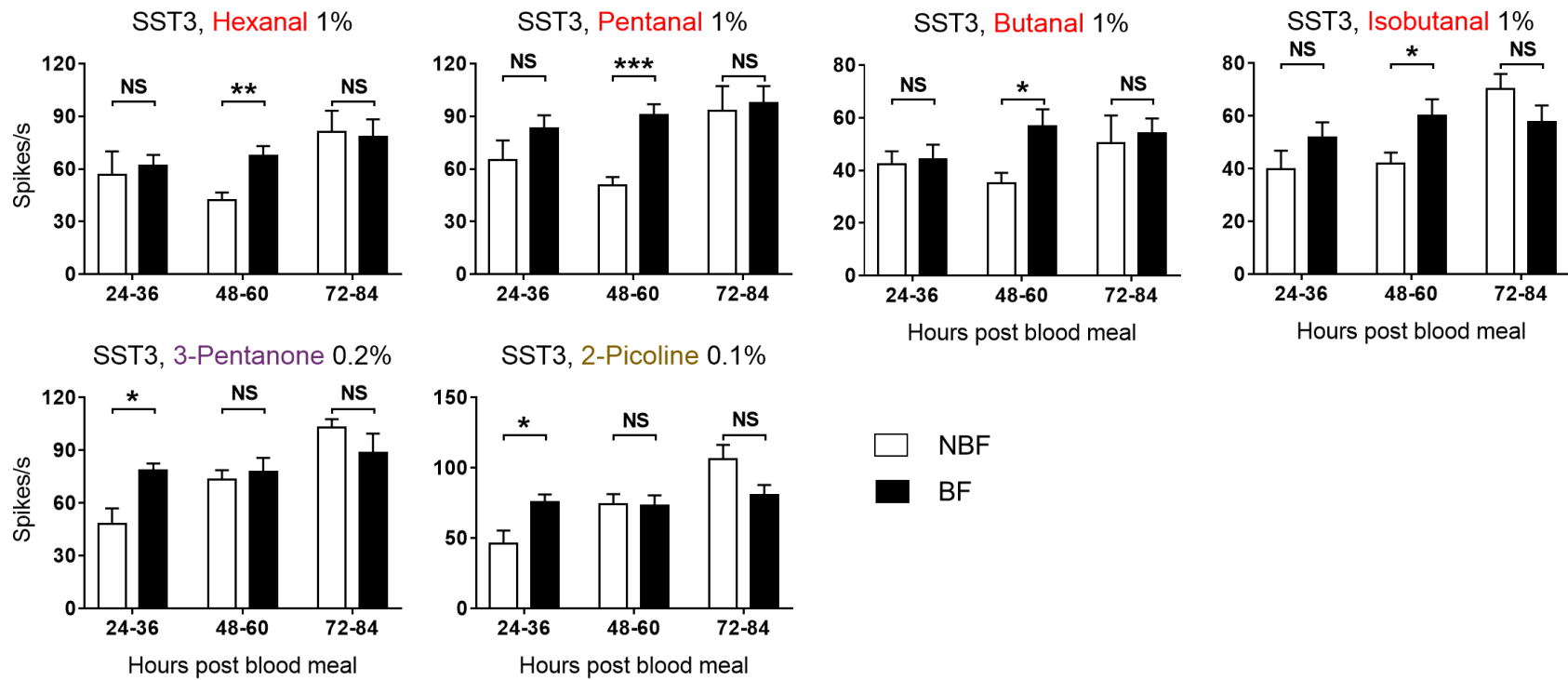
<b>Chemicals</b>	<b>CAS number</b>	<b>Company</b>	<b>Purity (%)</b>
<b>Aldehydes</b>			
hexanal	66-25-1	Sigma	98
propanal	123-38-6	Sigma	97
decanal	112-31-2	Sigma	98
nonanal	124-19-6	Aldrich	95
benzaldehyde	100-52-7	Sigma	99
heptanal	111-71-7	Sigma	92
pentanal	110-62-3	Sigma	97
octanal	124-13-0	Sigma	99
butanal	123-72-8	Sigma	99
isobutanal	78-84-2	Sigma	99
2-methylbutanal	96-17-3	Sigma	90
<b>Alcohols</b>			
trans-2-hexen-1-ol	928-95-0	Acros Organics	96
cis-2-hexen-1-ol	928-94-9	Aldrich	95
1-hexen-3-ol	4798-44-1	Sigma	98
trans-2-octen-1-ol	18409-17-1	Acros Organics	98
p-cresol	106-44-5	Acros Organics	99
<b>Aliphatics/Aromatics</b>			
toluene	108-88-3	Sigma	99.8
benzene	71-43-2	Sigma	99.8
propylbenzene	103-65-1	Sigma	98
styrene	100-42-5	Sigma	99
xylene	106-42-3	Sigma	99.5
ethylbenzene	100-41-4	Sigma	99
trans-3-octene	14919-01-8	Aldrich	98
<b>Ketones</b>			
2-hexanone	591-78-6	Fluka	96
2-pentanone	107-87-9	Fisher	99
3-pentanone	96-22-0	Fisher	99
2-decanone	693-54-9	Aldrich	98
2-butanone	78-93-3	Sigma	99.7
sulcatone	110-93-0	Sigma	98
<b>Amines</b>			
propylamine	107-10-8	Aldrich	99
butylamine	109-73-9	Aldrich	99.5
ammonia	1336-21-6	Aldrich	28
<b>Heterocyclics</b>			
indole	120-72-9	Aldrich	99
2,6-dimethylpyrazine	108-50-9	Acros Organics	96
2-picoline	109-06-8	Acros Organics	98
skatole	83-34-1	Acros Organics	98
n-piperidineethanol	3040-44-6	Acros Organics	99
4-peridinemethanamine	7144-05-0	Acros Organics	97
pyrazine	290-37-9	Acros Organics	100
<b>DMSO</b>	67-68-5	Sigma	100



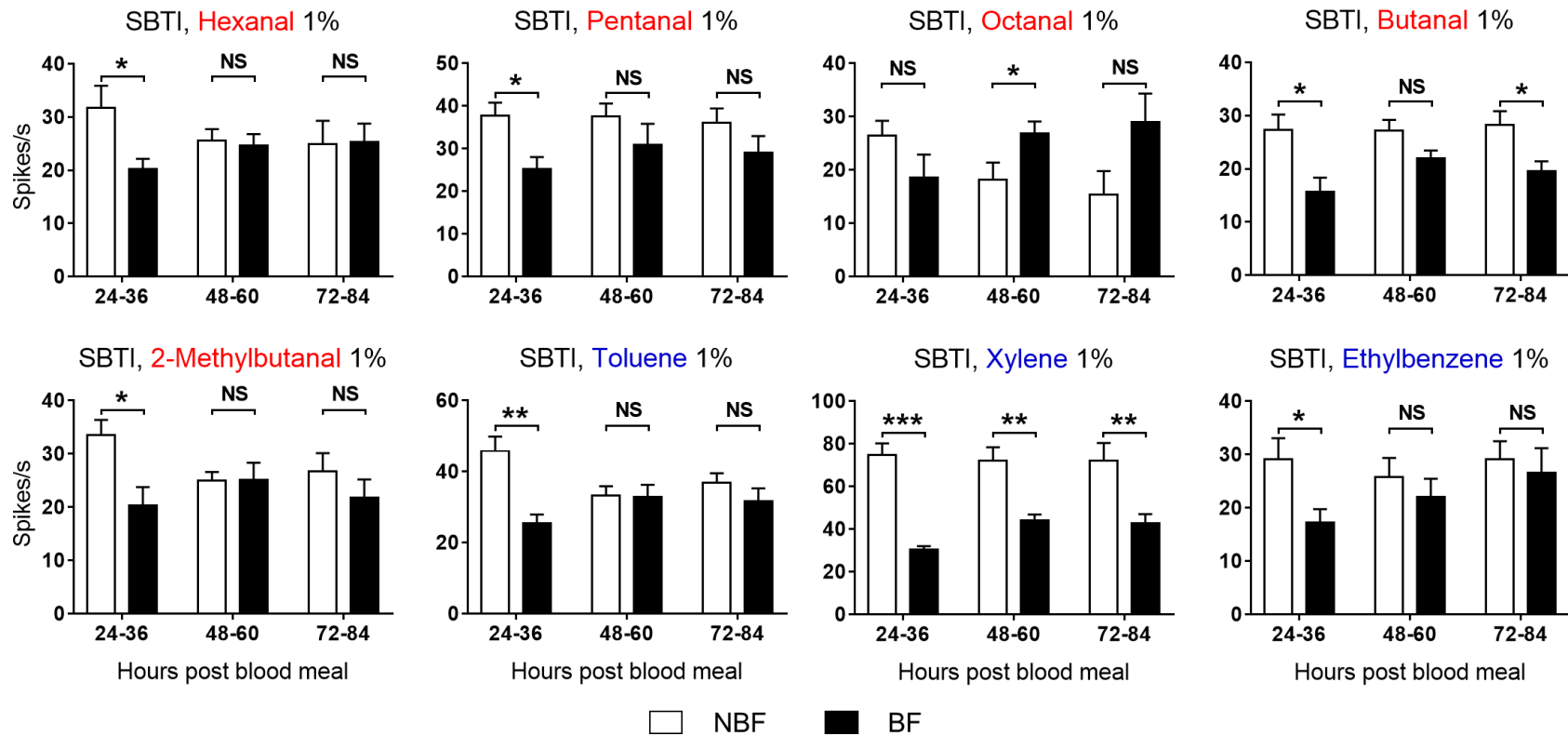
**Figure 4.1 Effect of blood feeding on the sensitivity of SST1 sensilla to human odorants.** Responses of the SST1 sensilla in BF (black bar) and NBF (white bar) female mosquitoes were compared at 24-36, 48-60, and 72-84 h pbm. Odorants are color coded by chemical class: aldehydes, red; alcohols, light blue.  $n = 8-21$ . An asterisk above the bar indicates: \*\*\*,  $P < 0.001$ ; \*\*,  $0.001 < P < 0.01$ ; \*,  $0.01 < P < 0.05$ ; NS, not significant, according to two-tailed  $t$ -test. Error bars indicate s.e.m.



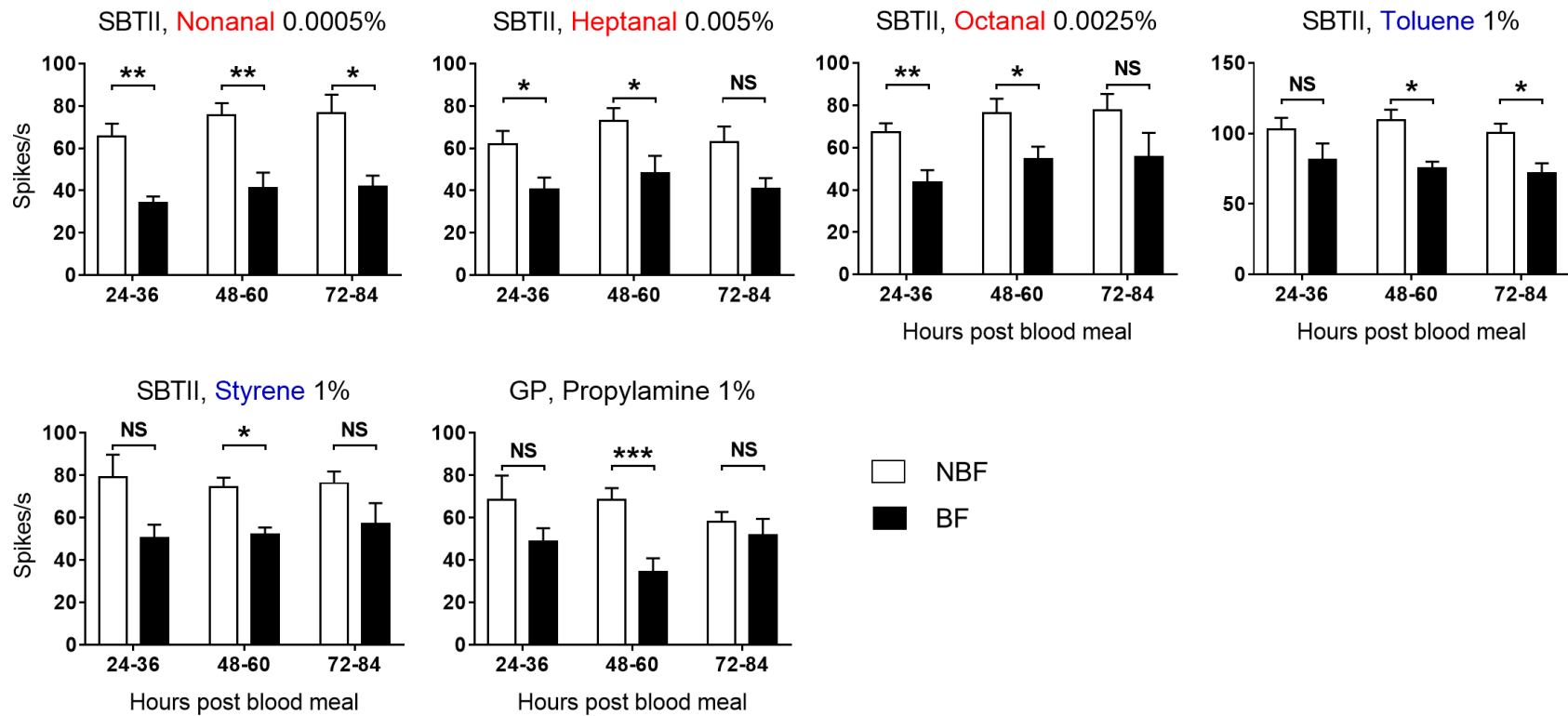
**Figure 4.2 Effect of blood feeding on the sensitivity of SST2 sensilla to human odorants.** Responses of the SST2 sensilla in BF (black bar) and NBF (white bar) female mosquitoes were compared at 24-36, 48-60, and 72-84 h pbm. Odorants are color coded by chemical class: aldehydes, red; alcohols, light blue; ketones, purple.  $n = 6-14$ . An asterisk above the bar indicates: \*\*\*,  $P < 0.001$ ; \*\*,  $0.001 < P < 0.01$ ; \*,  $0.01 < P < 0.05$ ; NS, not significant, according to two-tailed  $t$ -test. Error bars indicate s.e.m.



**Figure 4.3 Effect of blood feeding on the sensitivity of SST3 sensilla to human odorants.** Responses of the SST3 sensilla in BF (black bar) and NBF (white bar) female mosquitoes were compared at 24-36, 48-60, and 72-84 h pbm. Odorants are color coded by chemical class: aldehydes, red; ketones, purple; heterocyclics, gold.  $n = 6-10$ . An asterisk above the bar indicates: \*\*\*,  $P < 0.001$ ; \*\*,  $0.001 < P < 0.01$ ; \*,  $0.01 < P < 0.05$ ; NS, not significant, according to two-tailed  $t$ -test. Error bars indicate s.e.m.



**Figure 4.4 Effect of blood feeding on the sensitivity of SBTI sensilla to human odorants.** Responses of the SBTI sensilla in BF (black bar) and NBF (white bar) female mosquitoes were compared at 24-36, 48-60, and 72-84 h pbm. Odorants are color coded by chemical class: aldehydes, red; aliphatics/aromatics, blue.  $n = 7-10$ . An asterisk above the bar indicates: \*\*\*,  $P < 0.001$ ; \*\*,  $0.001 < P < 0.01$ ; \*,  $0.01 < P < 0.05$ ; NS, not significant, according to two-tailed  $t$ -test. Error bars indicate s.e.m.



**Figure 4.5 Effect of blood feeding on the sensitivity of SBTII and GP sensilla to human odorants.** Responses of the SBTII and GP sensilla in BF (black bar) and NBF (white bar) female mosquitoes were compared at 24-36, 48-60, and 72-84 h pbm. Odorants are color coded by chemical class: aldehydes, red; aliphatics/aromatics, blue; amines, black. SBTII,  $n = 7-22$ ; GP,  $n = 11-20$ . An asterisk above the bar indicates: \*\*\*,  $P < 0.001$ ; \*\*,  $0.001 < P < 0.01$ ; \*,  $0.01 < P < 0.05$ ; NS, not significant, according to two-tailed  $t$ -test. Error bars indicate s.e.m.

## **Chapter 5: Antennal Neuronal Responses of Insect Chemical Repellents and Their Antagonistic Effect on Odor Reception in *Ae. aegypti***

### **5.1 Abstract**

The yellow fever mosquito, *Aedes aegypti*, is a vector of many human diseases such as yellow fever, dengue fever, and Zika. As insecticide resistance has been widely reported, chemical repellents have been adopted as alternative options for mosquito and mosquito-borne disease control. This study characterized the responses of olfactory receptor neurons (ORNs) in different types of antennal olfactory sensilla in *Ae. aegypti* to 48 chemicals that exhibited repellent activity in various insect species. Both excitatory and inhibitory responses were observed from ORNs in response to these chemicals and differential tuning properties were also observed among ORNs. Remarkable excitatory responses were recorded from the ORNs in sensilla SST1, SST2, SBTI, SBTII, and LST2, while inhibitory activities were detected from a neuron in sensillum SST2 in response to several terpene/terpenoid compounds. Simultaneous application of eucalyptol and three odorants in the SST2 sensilla dramatically reduced the excitatory responses of the odorants. Moreover, the temporal dynamics of neuronal responses were found to be compound-specific and concentration dependent. Hierarchical cluster analysis and principal component analysis of the response to each compound across ORNs in seven types of olfactory sensilla in *Ae. aegypti* revealed that odor reception depended not only on chemical class but also specific chemical structure. Results of this study give new insights into the sensory physiology of *Aedes* mosquitoes to the chemical repellents and should contribute to the development of new repellent reagents for human protection.

Chen, Z., Liu, F., & Liu, N. (2018). Neuronal responses of antennal olfactory sensilla to insect chemical repellents in the yellow fever mosquito, *Aedes aegypti*. *Journal of chemical ecology*. 44(12), 1120-1126.

Xu, P., Choo, Y.M., Chen, Z., Zeng, F., Tan, K., Chen, T.Y., Cornel, A.J., Liu, N., & Leal, W.S. (2019). Odorant inhibition in mosquito olfaction. *iScience*, accepted.

## 5.2 Introduction

The mosquito *Aedes aegypti* (Linnaeus) acts as a vector for many important human infectious diseases, including yellow fever, dengue fever and Zika (Gubler 2004; Marchette et al. 1969; Monath 2001), with female *Ae. aegypti* preferring human hosts to other warm-blooded animals for blood meals (DeGennaro et al. 2013; McBride et al. 2014). The development of resistance to insecticides such as pyrethroids in mosquitoes has been widely reported, compromising their efficacy for mosquito management (Liu 2015). A wide range of insect repellents, that are either natural products (e.g. terpenes/terpenoids) or synthesized (e.g. DEET (*N, N*-diethyl-*meta*-toluamide)) have been developed to provide alternative protection for humans against mosquitoes and other biting arthropod species (Campbell et al. 2011; Fradin and Day 2002; Gertler 1946; Isman 2006; Trongtokit et al. 2005). However, few of these compounds have been examined for the neuronal responses in antennal olfactory sensilla of *Ae. aegypti*.

Insects sense odors through morphologically distinctive olfactory sensilla located on their peripheral olfactory organs such as antennae (Joseph and Carlson 2015; Matthews et al. 2016). Housed inside each type of olfactory sensilla are certain combinations of olfactory receptor neurons (ORNs). On the membrane of ORNs specific olfactory receptors (ORs), which interact with odorant molecules, and the highly conserved co-receptor (Orco) are expressed together forming heteromeric ligand-gated ion channels (Nakagawa et al. 2005; Sato et al. 2008). Using this system insects obtain information about an odor based on its chemical properties and the concentration at which it is represented (Carey et al. 2010; Hallem and Carlson 2006; Liu and Liu 2015). This information can be represented through the temporal dynamics of primary neuronal responses (Hallem and Carlson 2006; Laurent et al. 1996; Lei et al. 2004; Liu and Liu 2015; Stopfer et al. 1997).

In this study, the responses of ORNs in the antennal olfactory sensilla of *Ae. aegypti* to 48 compounds were characterized. These compounds showed repellent activity in insects including *Ae. aegypti* in behavioral studies (Ansari et al. 2000; Liu et al. 2013, 2014; Ufkes and Grams 2007). The dose dependent activity of the compounds that elicited strong responses and the temporal dynamics of responses evoked by structurally related compounds among the ORNs in different types of sensilla were studied as well. In addition, the spatial relationships in an odor space among 25 compounds that elicited at least one significant response in the ORNs of any type of sensilla were investigated. Finally, the antagonistic effect of eucalyptol on the responses of three odorants in the SST2 sensilla was examined.

### **5.3 Materials and Methods**

#### **5.3.1 Insects**

*Aedes aegypti* mosquitoes (Orlando strain obtained from Dr. James Becnel, USDA, ARS, Mosquito and Fly Research Unit) were maintained at  $25 \pm 2$  °C under a photoperiod of 12: 12 (L:D) h. Eggs were hatched in deoxygenated deionized water. Larvae were cultured in deionized water and fed yeast (Fleischmann's Rapid Rise Instant Yeast) as needed. Adult female and male mosquitoes were reared together after eclosion with 10% sucrose solution. Four- to five-day-old only sucrose-fed female mosquitoes were used in all the experiments in this study. Adult females were fed blood samples from horses (Large Animal Teaching Hospital, College of Veterinary Medicine, Auburn University) for stock maintenance.

#### **5.3.2 Single sensillum recording**

Extracellular single sensillum recording (SSR) was carried out as described in section 3.3.2.

### 5.3.3 Stimulation and stimuli

The responses to 48 compounds were tested for this study, with 45 compounds covering six chemical classes (terpenes, terpenoids, carboxylic acids, esters, aldehyde, and alcohols) plus DEET, permethrin, and naphthalene (Table S5.1). These compounds, which exhibit repellent activity against various insect species including *Ae. aegypti*, and three other odorants were applied as stimuli to examine the neuronal responses of different types of antennal olfactory sensilla in *Ae. aegypti* using SSR (Table S5.1; Liu et al. 2013, 2014). All stimulations were applied as described in section 3.3.3. For each compound, each recording was from a separate sensillum, with no more than three sensilla tested per insect.

### 5.3.4 Data analysis

The heatmap construction and the hierarchical cluster analysis based on Euclidean distance and Ward's method were performed using PAST 3.20 (University of Oslo). Principal component analysis (PCA) was conducted using IBM SPSS Statistics (version 20, <https://www.ibm.com/us-en/marketplace/spss-statistics>). Error bars represent SEM unless otherwise noted.

## 5.4 Results

### 5.4.1 Distinctive response profiles of ORNs to chemical repellents

Previous studies on the antenna of *Ae. aegypti* revealed five morphological types of olfactory sensillum, including long sharp tipped (LST), short sharp tipped (SST), short blunt tipped I (SBTI), short blunt tipped II (SBTII), and grooved peg (GP) sensilla (Ghaninia et al. 2007; McIver 1978). In this study, the ORNs in each type of sensilla were tested against 48 compounds. Two functional subtypes of sensilla were identified for both LST (77.3% of LST1 and 22.7% of LST2) and SST (77.4% of SST1 and 22.6% of SST2) according to their different response profiles to the compound panel (Figure 5.1a). Also, two neurons with different amplitudes (neuron 'A', with larger amplitudes, and neuron 'B', with smaller amplitudes) were found in all four types of trichoid sensilla (Figure 5.1b and c).

Single sensillum recording generated a data matrix of 336 compound-sensillum combinations (Figure 5.1a; Table S5.2). ORNs in sensilla LST2, SST1, SST2, SBTI and SBTII displayed distinctive response profiles to the panel of compounds (Figure 5.1a). Specifically, neuron ‘A’ in SST1 sensilla showed excitatory activity to a large number of the compounds tested, with particularly strong responses to three terpenoids including camphor ( $147 \pm 5$  spikes/s), eucalyptol ( $141 \pm 6$  spikes/s), and  $(-)\text{-}\alpha\text{-thujone}$  ( $99 \pm 7$  spikes/s) (Figure 5.1c). Neuron ‘A’ in SST2 sensilla evoked inhibitory responses ( $>50\%$  of firing rate loss) to almost all of these compounds with the exception of isoamyl alcohol, which elicited a strong excitatory response with a firing frequency of  $159 \pm 11$  spikes/s (Figure 5.1c). In addition, ORNs in SBTII sensilla showed an overall broader response profile than those in SBTI (Figure 5.1a and c). The neuron ‘A’ in sensilla SBTI and SBTII was responsible for the response to all compounds, except for menthyl acetate and naphthalene (Figure 5.1c). Menthyl acetate and naphthalene elicited a response from the ‘B’ neuron in SBTI and SBTII sensilla, respectively (Figure 5.1c). The ORN in sensillum LST2 evoked significant excitatory responses to two terpene compounds (myrcene and terpinolene) (Figure 5.1c). ORNs in sensilla LST1 and GP did not evoke any responses to the panel of compounds tested.

#### **5.4.2 Excitatory and inhibitory responses across concentrations**

To determine whether ORNs housed in the antennal olfactory sensilla are able to sense odorants at lower concentrations, their responses to serial doses of compounds that elicited strong excitatory responses ( $\geq 50$  spikes/s) at a  $10^{-2}$  dilution were tested. All responses followed a dose-dependent pattern with the firing rate positively correlated with concentration of the odorant. Naphthalene and oleic acid elicited excitatory responses ( $\geq 15$  spikes/s, dark grey bar) in the ORN of SBTII at a  $10^{-4}$  dilution (Figure 5.2a). The terpenoid,  $(S)\text{-}(-)\text{-perillaldehyde}$  appeared to be more effective in activating the SBTII ORNs, which elicited significant responses at a  $10^{-5}$  dilution (Figure 5.2a). However, three terpenoids (camphor, eucalyptol, and  $(-)\text{-}\alpha\text{-thujone}$ ) and one terpene ( $\alpha\text{-terpinene}$ ) triggered significant excitatory responses in the SST1 ORNs at lower concentrations, while inhibitory responses were induced by the same

compounds in SST2 ORNs (Figure 5.2b). Isoamyl alcohol elicited dose-dependent excitatory responses in the ORNs of both SST1 and SST2 sensilla, with greater potency in SST2 ORNs (Figure 5.2b).

### 5.4.3 Temporal dynamics of ORN repertoire

To explore the temporal dynamics of the responses from the *Ae. aegypti* ORN repertoire, significant responses elicited by a 500-ms pulse of air through the compound cartridge were plotted to construct a set of temporal firing activity covering a 2-sec analysis window for the phasic response and a 4-sec window for the tonic response. Several terpenes/terpenoids (camphor, eucalyptol, (-)- $\alpha$ -thujone, (-)-menthone, and  $\alpha$ -terpinene) induced typical phasic responses in SST1 ORNs, with the highest firing rates occurring 500 ms post-stimulus followed by rapid declines during the subsequent 1.5-sec analysis period (Figure 5.1b and 5.3a). However, when tested against the ORNs of SST2 sensilla the same terpenes/terpenoids triggered prolonged inhibitory responses (Figure 5.1b and 5.3a). Two terpenoids, (S)-(-)-perillaldehyde and citronellal elicited typical tonic/super-sustained excitatory responses in the ORNs of SBTII sensilla, with the strong responses continuing throughout the 4-sec observation period (Figure 5.1b and 5.3a). Interestingly, although camphor, eucalyptol, (-)- $\alpha$ -thujone, (-)-menthone, and (S)-(-)-perillaldehyde are structurally related, as shown in Figure 5.2a and b, the temporal dynamics of the neuronal responses induced by these compounds in SST1 and SBTII ORNs were different.

Insects in a natural habitat typically experience a dynamic olfactory environment in which odors are encountered at varying concentrations. Thus, the temporal firing activity of neuronal responses across serial dilutions were examined as well. For example, eucalyptol elicited different temporal firing activities at two concentrations, with a more phasic response at high concentration (e.g.  $10^{-2}$  dilution) compared with a more tonic response at lower concentrations (e.g.  $10^{-3}$  dilution) (Figure 5.3b). However, eucalyptol and (S)-(-)-perillaldehyde at either a  $10^{-2}$  or  $10^{-3}$  dilution elicited prolonged responses in the ORNs of SST2 (inhibitory response) and SBTII (excitatory response) sensilla, respectively (Figure 5.3b).

#### 5.4.4 A presentation of odor space

To further ask which compounds elicit similar patterns of ORN activation and which elicit very different patterns in *Ae. aegypti*, the spatial relationships among 25 compounds that elicited at least one response  $\geq 15$  spikes/s in the ORNs of any type of sensilla were examined in an odor space, which was constructed based on the responses of ORNs in seven types of antennal sensilla. To quantify relationships among compounds in the odor space, the Euclidean distances between all possible pairs of the 25 tested compounds were measured and compared. Of the 300 pairs, the five pairs whose members were closest were  $\alpha$ -pinene and d-neomethol (8 spikes/s),  $\alpha$ -pinene and (+)-3-carene (10 spikes/s), (+)-terpinen-4-ol and d-neomethol (10 spikes/s),  $\alpha$ -pinene and (+)-terpinen-4-ol (11 spikes/s), and (+)-terpinen-4-ol and linalyl acetate (11 spikes/s). The compound pairs that were farthest apart in odor space were isoamyl alcohol and eucalyptol (187 spikes/s), isoamyl alcohol and camphor (179 spikes/s), and isoamyl alcohol and (-)- $\alpha$ -thujone (172 spikes/s).

To visualize relationships among compounds in the odor space, we carried out a hierarchical cluster analysis (Figure 5.4a). Results demonstrated that compounds from the same chemical class tended to cluster together, though in no case was there a cluster that included all members of a class (Figure 5.4a). Specifically, compounds with similar structures were tightly clustered together, such as eucalyptol, (-)- $\alpha$ -thujone and camphor (Figure 5.2b and 5.4a).

By performing principal component analysis, the seven-dimensional odor space was represented in a three dimensional space (Figure 5.4b). Consistent with the hierarchical cluster analysis, compounds of the same class, such as terpenes (blue dots) and terpenoids (red dots), tended to cluster together but were mutually highly separated (Figure 5.4b). Despite the fact that compounds from the same class were clustered, an intermingling among one terpene ( $\alpha$ -terpinene) and five terpenoids ((-)-menthone, (S)-cis-verbenol, eucalyptol, (-)- $\alpha$ -thujone and camphor) formed in the odor space (dashed oval) (Figure 5.4b). These results suggest that the chemical class is one but not the only feature that determines the response pattern of ORNs in *Ae. aegypti*.

#### 5.4.5 Antagonistic effect of eucalyptol on odorant reception in SST2 sensilla

Since the chemical compound eucalyptol elicited inhibitory response in the SST2 sensillum of *Ae. aegypti*, whether it would reduce the excitatory response induced by other odorants from the same sensillum when two odorants were applied simultaneously needs to be answered. A few other odorants was examined in the SST2 sensilla of *Ae. aegypti* and it was found that 4,5-dimethylthiazole (5  $\mu\text{g}$ ), cyclohexanone (0.5  $\mu\text{g}$ ), and 2-methyl-2-thiazoline (5  $\mu\text{g}$ ) elicited excitatory responses in the ORN 'A' of SST2 sensilla with a firing rate of  $68 \pm 7$ ,  $76 \pm 2$ , and  $60 \pm 6$  spikes/s, respectively. Next, the odorant 4,5-dimethylthiazole (5  $\mu\text{g}$ ) and the chemical compound eucalyptol (1  $\mu\text{g}$ , 5  $\mu\text{g}$ , or 10  $\mu\text{g}$ ) were delivered to the SST2 sensilla simultaneously. Results showed that the response of ORN 'A' in SST2 sensilla to 4,5-dimethylthiazole decreased dramatically when the dose of eucalyptol increased (Figure 5.5a); about 91% of the response induced by 4,5-dimethylthiazole was inhibited by eucalyptol at its highest dose (Figure 5.5b). Also, the chemical compound eucalyptol showed dose-dependent antagonistic effect on the responses of another two odorants, cyclohexanone and 2-methyl-2-thiazoline, in the SST2 sensilla of *Ae. aegypti*, which caused 59% and 115% loss of the response, respectively, at its highest dose (Figure 5.6).

### 5.5 Discussion

This study characterized the responses of ORNs in the antennal olfactory sensilla of *Ae. aegypti* to 48 compounds that exhibit repellent activity in various insect species. *Aedes aegypti* ORNs in different types of sensilla demonstrated distinct response profiles to the panel of compounds. In contrast to the remarkable responses evoked in the ORNs in sensilla LST2, SST1, SST2, SBTI and SBTII, ORNs in sensilla LST1 and GP did not respond to any of the 48 compounds tested in *Ae. aegypti*. ORNs in LST1 may be narrowly tuned to certain compounds that are not included in our stimulus spectrum, as found for ORNs in LST sensilla in other mosquito species (Ghaninia et al. 2007; Hill et al. 2009; Qiu et al. 2006). ORNs in GP sensilla of the malaria mosquito *Anopheles gambiae* are tuned to amines and acids (Qiu et al. 2006). ORNs in SST1 sensilla evoked excitatory responses to seven terpene/terpenoids tested whereas

those in SST2 sensilla showed inhibitory activity to these compounds. Odor-induced inhibition in peripheral olfactory neurons has been reported in various insect species (De Bruyne et al. 2001; Qiu et al. 2006; Ghaninia et al. 2007; Hill et al. 2009; Liu et al. 2017). Cao et al. (2017) found that odor-evoked ORN inhibition independently drove attraction or avoidance behavior in *Drosophila*, as did odor-evoked ORN excitation. Odor-evoked excitation and inhibition in peripheral ORNs may expand the odor-coding capacity in insects (Cao et al. 2017), a property that requires further examination. In addition, we found that the chemical compound eucalyptol that elicited inhibitory response in the SST2 sensilla of *Ae. aegypti* was capable of reducing the excitatory responses induced by three odorants in the same sensilla, indicating the great potential of eucalyptol to be used as a confusant to protect humans from mosquitoes.

The terpenoid repellent (S)-(-)-perillaldehyde was effective in firing the ORNs of *Ae. aegypti* SBTII sensilla at a  $10^{-5}$  dilution. ORNs of other insects have also exhibited high sensitivity to odors at a relatively low dose. For example, fruit flies can detect the complex odors of an apple, a banana, or a strawberry at a  $10^{-4}$  dilution (Hallem and Carlson 2006). Minute quantities of bombykol, the sex pheromone released by female silk moths *Bombyx mori* (von Butenandt et al. 1959), also trigger significant electrical responses in the antenna of male silk moths (Boeckh et al. 1965). Being capable of sensing odors at low concentrations is critical for animals to locate food, mates, oviposition sites and potential enemies.

Compounds of the same class, such as camphor and (S)-(-)-perillaldehyde, induced different temporal dynamics of activity in *Ae. aegypti* ORNs. Studies in other insects have also demonstrated that temporal dynamics are involved in the information coding of structurally similar odors (Laurent et al. 1996; Stopfer et al. 1997; Lei et al. 2004; Hallem and Carlson 2006). In addition, the concentration of an odor that is encountered by insects also influences the shape of dynamic structure. Eucalyptol induced a phasic response in *Ae. aegypti* SST1 ORNs at a  $10^{-2}$  dilution, whereas a more prolonged response was observed at a  $10^{-3}$  dilution, consistent with findings from *Drosophila* by Hallem and Carlson (2006). In addition, the spatiotemporal structure of an odor plume is determined by the duration of an odor pulse, too. Hallem and Carlson (2006) revealed that more prolonged odor pulses elicited more prolonged

responses, which perhaps indicates the ability to encode information about the duration of an odor stimulus and this ability may be essential for insects to navigate toward/away from an odor source.

Terpene or terpenoid compounds tended to cluster together in the hierarchical cluster analysis but were widely distributed in the odor space, suggesting that chemical class serves as an important factor in the odor-coding process in *Ae. aegypti*. However, there was an intermingling formed among one terpene and five terpenoids, which tightly clustered together in the odor space. These structurally similar compounds may be more difficult for *Ae. aegypti* to distinguish than compounds that map far apart in the space, as found in other insects (Hallem and Carlson 2006; Carey et al. 2010; Liu and Liu 2015). Briefly, odor reception, especially odorant mixture reception in insects, is a complex process and the mechanism involved remains to be established.

**Table S5.1** Chemicals characterized in the ORNs of antennal olfactory sensilla in *Aedes aegypti*

<b>Chemical*</b>	<b>Purity (%)</b>	<b>CAS number</b>	<b>Company</b>
<i>Carboxylic acids</i>			
Oleic acid	≥99	112-80-1	Sigma
Palmitic acid	≥99	57-10-3	Sigma
<i>Esters</i>			
Dimethyl phthalate	≥99	131-11-3	SAFC
Dibutyl phthalate	99	84-74-2	Aldrich
<i>Aldehyde</i>			
trans-Cinnamaldehyde	99	14371-10-9	Aldrich
<i>Alcohols</i>			
Isoamyl alcohol	≥98	123-51-3	SAFC
Cinnamyl alcohol	98	104-54-1	Aldrich
<i>Terpenes</i>			
(R)-(+)-Limonene	97	5989-27-5	Sigma
(S)-(-)-Limonene	96	5989-54-8	Aldrich
α-Terpinene	≥95	99-86-5	Aldrich
Myrcene	≥95	123-35-3	Fluka
α-Pinene	98	80-56-8	Aldrich
(+)-α-Pinene	≥99	7785-80-8	Aldrich
(-)-α-Pinene	≥98	7785-26-4	SAFC
(-)-β-Pinene	≥99	18172-67-3	Aldrich
(+)-β-Pinene	≥95	19902-08-0	Fluka
(+)-3-Carene	99	498-15-7	Aldrich
Terpinolene	≥90	586-62-9	SAFC
β-Caryophyllene	≥80	87-44-5	SAFC
<i>Terpenoids</i>			
Phytol	≥97	7541-49-3	SAFC
(-)-Caryophyllene oxide	≥95	1139-30-6	SAFC
Citronellic acid	98	502-47-6	Aldrich
Eugenol	99	97-53-0	Aldrich
(S)-(-)-Perillaldehyde	≥92	18031-40-8	SAFC
(S)-(-)-Perillyl alcohol	96	18457-55-1	Aldrich
(-)-Menthone	90	14073-97-3	Aldrich
Thymol	≥99.5	89-83-8	Sigma
α-Terpineol	≥96	10482-56-1	SAFC
(+)-Terpinen-4-ol	≥95	2438-10-0	Fluka
Citronellal	≥85	106-23-0	SAFC
D-Neomenthol	≥99	2216-52-6	SAFC
Menthol	99	89-78-1	Aldrich
Geranyl acetone	≥97	689-67-8	Aldrich
Menthyl acetate	97	89-48-5	Aldrich
Linalyl acetate	≥97	115-95-7	SAFC
(-)-Linalool	≥95	126-91-0	Aldrich
Geraniol	98	106-24-1	Aldrich
Citronellol	≥95	106-22-9	SAFC
Carvacrol	≥98	499-75-2	Aldrich
(S)-cis-Verbenol	95	18881-04-4	Aldrich
Camphor	≥99	76-22-2	SAFC

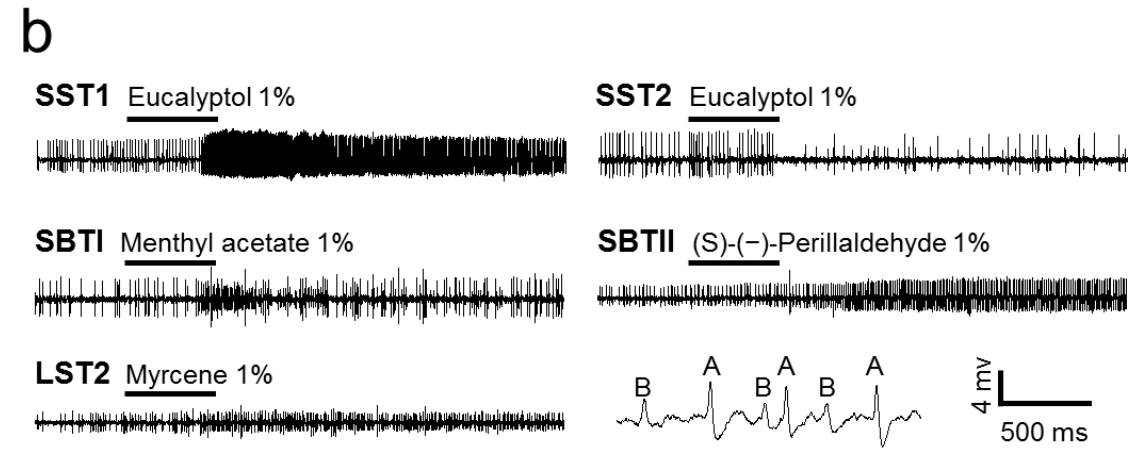
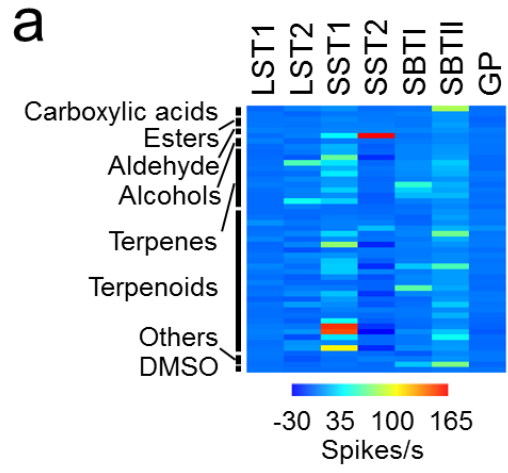
Eucalyptol	≥95	470-82-6	Fluka
Citral	≥96	5392-40-5	Aldrich
Linalool	97	78-70-6	Aldrich
(-)- $\alpha$ -Thujone	96	546-80-5	Aldrich
<i>Others</i>			
DEET	97	134-62-3	Aldrich
Permethrin	99	52645-53-1	Sigma
Naphthalene	99	91-20-3	Aldrich

\*Chemicals that indicated repellent activity in previous studies with references described in Liu et al. (2013, 2014).

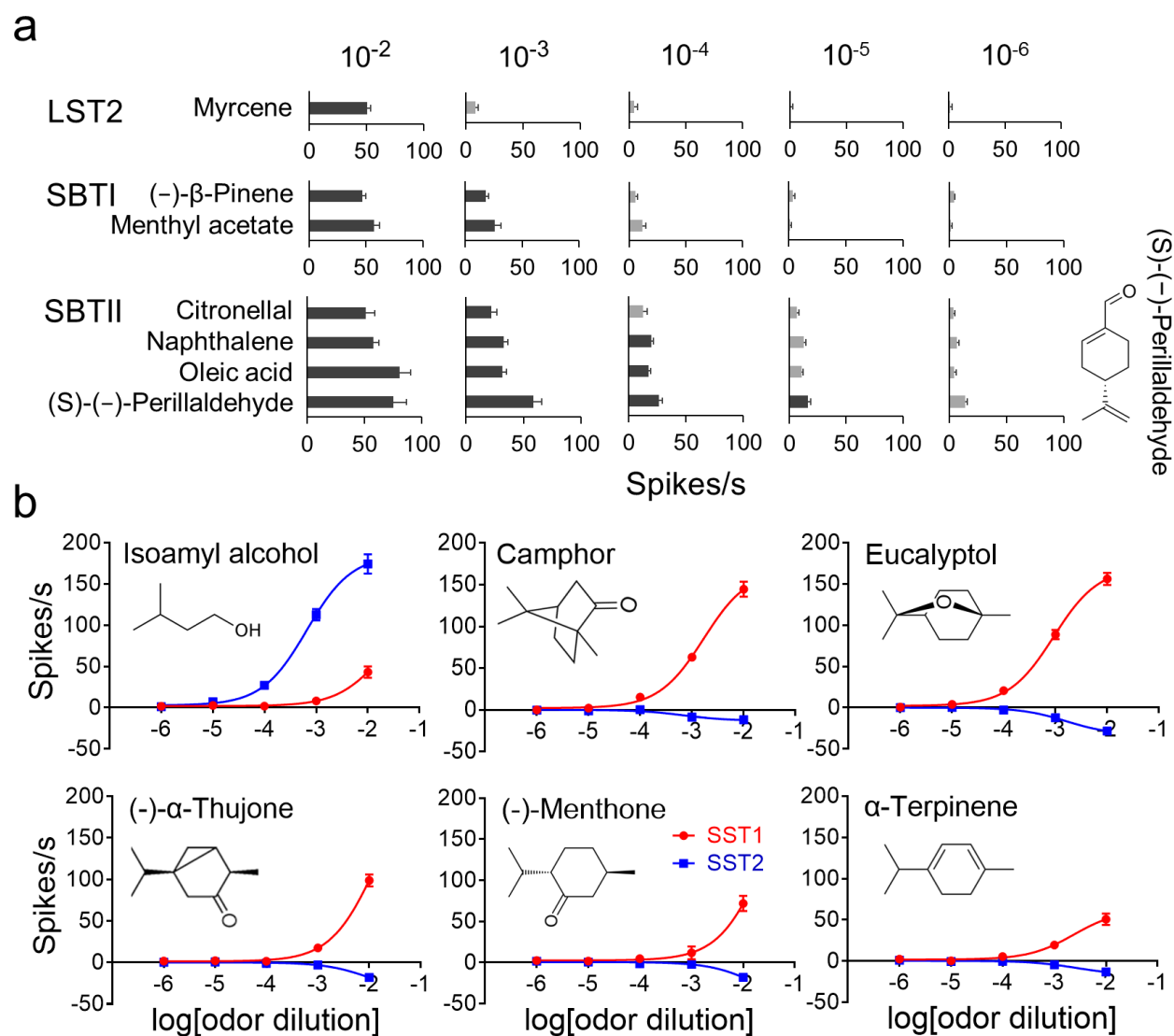
**Table S5.2** Response profiles (mean  $\pm$  SEM, spikes/s) of antennal olfactory neurons to insect chemical repellents in *Ae. aegypti*

Chemical\ Sensillum	LST1	LST2	SST1	SST2	SBTI	SBTII	GP
<i>Carboxylic acids</i>							
Oleic acid	-2 $\pm$ 1	4 $\pm$ 3	8 $\pm$ 3	0 $\pm$ 2	1 $\pm$ 2	76 $\pm$ 5	0 $\pm$ 2
Palmitic acid	1 $\pm$ 2	-2 $\pm$ 1	2 $\pm$ 1	2 $\pm$ 2	4 $\pm$ 2	10 $\pm$ 2	0 $\pm$ 2
<i>Esters</i>							
Dimethyl phthalate	1 $\pm$ 2	2 $\pm$ 5	6 $\pm$ 2	3 $\pm$ 2	2 $\pm$ 2	9 $\pm$ 2	-3 $\pm$ 2
Dibutyl phthalate	2 $\pm$ 1	1 $\pm$ 6	4 $\pm$ 1	4 $\pm$ 3	2 $\pm$ 2	12 $\pm$ 2	-1 $\pm$ 2
<i>Aldehyde</i>							
trans-Cinnamaldehyde	4 $\pm$ 3	3 $\pm$ 3	5 $\pm$ 2	5 $\pm$ 3	5 $\pm$ 2	9 $\pm$ 2	3 $\pm$ 3
<i>Alcohols</i>							
Isoamyl alcohol	0 $\pm$ 1	4 $\pm$ 4	34 $\pm$ 4	159 $\pm$ 11	1 $\pm$ 2	5 $\pm$ 3	1 $\pm$ 1
Cinnamyl alcohol	4 $\pm$ 2	-2 $\pm$ 2	3 $\pm$ 1	-1 $\pm$ 2	3 $\pm$ 2	6 $\pm$ 3	2 $\pm$ 2
<i>Terpenes</i>							
(R)-(+)-Limonene	-1 $\pm$ 2	4 $\pm$ 6	17 $\pm$ 3	-4 $\pm$ 2	5 $\pm$ 2	9 $\pm$ 2	1 $\pm$ 2
(S)-(-)-Limonene	-2 $\pm$ 2	2 $\pm$ 3	15 $\pm$ 3	-3 $\pm$ 1	5 $\pm$ 2	10 $\pm$ 2	1 $\pm$ 2
$\alpha$ -Terpinene	-1 $\pm$ 2	-5 $\pm$ 2	59 $\pm$ 5	-16 $\pm$ 3	5 $\pm$ 2	12 $\pm$ 2	-1 $\pm$ 2
Myrcene	0 $\pm$ 2	53 $\pm$ 5	29 $\pm$ 5	-2 $\pm$ 2	8 $\pm$ 4	22 $\pm$ 2	-1 $\pm$ 2
$\alpha$ -Pinene	1 $\pm$ 2	5 $\pm$ 6	17 $\pm$ 3	1 $\pm$ 2	8 $\pm$ 3	18 $\pm$ 3	-1 $\pm$ 2
(+)- $\alpha$ -Pinene	-1 $\pm$ 2	0 $\pm$ 9	30 $\pm$ 6	0 $\pm$ 2	7 $\pm$ 4	16 $\pm$ 2	3 $\pm$ 3
(-)- $\alpha$ -Pinene	1 $\pm$ 2	6 $\pm$ 6	10 $\pm$ 3	-2 $\pm$ 1	5 $\pm$ 2	9 $\pm$ 2	1 $\pm$ 2
(-)- $\beta$ -Pinene	3 $\pm$ 2	2 $\pm$ 5	11 $\pm$ 2	-1 $\pm$ 2	46 $\pm$ 2	16 $\pm$ 2	-1 $\pm$ 2
(+)- $\beta$ -Pinene	0 $\pm$ 2	4 $\pm$ 7	23 $\pm$ 3	-1 $\pm$ 5	24 $\pm$ 3	13 $\pm$ 3	2 $\pm$ 3
(+)-3-Carene	-1 $\pm$ 2	4 $\pm$ 3	8 $\pm$ 2	-5 $\pm$ 5	18 $\pm$ 4	18 $\pm$ 4	0 $\pm$ 2
Terpinolene	-1 $\pm$ 2	38 $\pm$ 5	24 $\pm$ 3	-4 $\pm$ 3	4 $\pm$ 3	8 $\pm$ 3	1 $\pm$ 2
$\beta$ -Caryophyllene	-1 $\pm$ 2	4 $\pm$ 5	0 $\pm$ 1	-2 $\pm$ 2	0 $\pm$ 2	6 $\pm$ 2	-1 $\pm$ 3
<i>Terpenoids</i>							
Phytol	-1 $\pm$ 2	-1 $\pm$ 2	0 $\pm$ 2	-1 $\pm$ 2	1 $\pm$ 3	9 $\pm$ 3	3 $\pm$ 2
(-)-Caryophyllene oxide	1 $\pm$ 2	-2 $\pm$ 3	3 $\pm$ 3	1 $\pm$ 2	-3 $\pm$ 2	12 $\pm$ 2	3 $\pm$ 1
Citronellic acid	8 $\pm$ 2	2 $\pm$ 4	4 $\pm$ 2	4 $\pm$ 2	-2 $\pm$ 2	8 $\pm$ 3	2 $\pm$ 2
Eugenol	0 $\pm$ 3	-1 $\pm$ 5	4 $\pm$ 1	19 $\pm$ 10	4 $\pm$ 2	17 $\pm$ 3	8 $\pm$ 2
(S)-(-)-Perillaldehyde	1 $\pm$ 2	5 $\pm$ 5	25 $\pm$ 3	0 $\pm$ 3	5 $\pm$ 2	62 $\pm$ 6	2 $\pm$ 3
(S)-(-)-Perillyl alcohol	5 $\pm$ 2	-3 $\pm$ 7	8 $\pm$ 2	1 $\pm$ 1	2 $\pm$ 2	15 $\pm$ 3	3 $\pm$ 2
(-)-Menthone	-2 $\pm$ 2	5 $\pm$ 4	70 $\pm$ 9	-18 $\pm$ 2	4 $\pm$ 2	9 $\pm$ 2	3 $\pm$ 2
Thymol	-1 $\pm$ 1	-7 $\pm$ 3	0 $\pm$ 1	2 $\pm$ 4	3 $\pm$ 2	4 $\pm$ 3	0 $\pm$ 2
$\alpha$ -Terpineol	1 $\pm$ 2	7 $\pm$ 4	9 $\pm$ 2	1 $\pm$ 3	3 $\pm$ 2	7 $\pm$ 2	3 $\pm$ 2
(+)-Terpinen-4-ol	0 $\pm$ 2	4 $\pm$ 3	21 $\pm$ 3	-4 $\pm$ 2	4 $\pm$ 2	7 $\pm$ 2	1 $\pm$ 2
Citronellal	5 $\pm$ 2	0 $\pm$ 2	23 $\pm$ 3	-16 $\pm$ 3	21 $\pm$ 3	52 $\pm$ 4	2 $\pm$ 2
D-Neomenthol	2 $\pm$ 3	4 $\pm$ 4	22 $\pm$ 3	5 $\pm$ 2	3 $\pm$ 2	11 $\pm$ 3	2 $\pm$ 3
Menthol	0 $\pm$ 2	-4 $\pm$ 3	7 $\pm$ 2	-7 $\pm$ 4	6 $\pm$ 3	11 $\pm$ 3	-1 $\pm$ 2
Geranyl acetone	4 $\pm$ 2	-2 $\pm$ 5	-2 $\pm$ 2	-3 $\pm$ 2	5 $\pm$ 3	11 $\pm$ 2	0 $\pm$ 2
Menthyl acetate	0 $\pm$ 2	3 $\pm$ 5	2 $\pm$ 1	-3 $\pm$ 4	57 $\pm$ 4	14 $\pm$ 3	-1 $\pm$ 2
Linalyl acetate	1 $\pm$ 2	7 $\pm$ 2	20 $\pm$ 3	-14 $\pm$ 5	6 $\pm$ 3	7 $\pm$ 3	2 $\pm$ 2
(-)-Linalool	-3 $\pm$ 2	-4 $\pm$ 1	11 $\pm$ 2	-3 $\pm$ 1	-2 $\pm$ 2	10 $\pm$ 5	3 $\pm$ 2
Geraniol	1 $\pm$ 2	11 $\pm$ 6	7 $\pm$ 2	-6 $\pm$ 1	0 $\pm$ 2	22 $\pm$ 4	1 $\pm$ 2
Citronellol	0 $\pm$ 2	-3 $\pm$ 3	4 $\pm$ 2	3 $\pm$ 3	2 $\pm$ 2	7 $\pm$ 2	2 $\pm$ 2
Carvacrol	0 $\pm$ 2	0 $\pm$ 3	1 $\pm$ 1	-1 $\pm$ 2	2 $\pm$ 2	16 $\pm$ 2	-1 $\pm$ 2
(S)-cis-Verbenol	1 $\pm$ 2	-4 $\pm$ 6	39 $\pm$ 4	-4 $\pm$ 5	0 $\pm$ 2	5 $\pm$ 2	-2 $\pm$ 3
Camphor	3 $\pm$ 2	2 $\pm$ 4	147 $\pm$ 5	-14 $\pm$ 2	-1 $\pm$ 2	7 $\pm$ 3	-3 $\pm$ 2

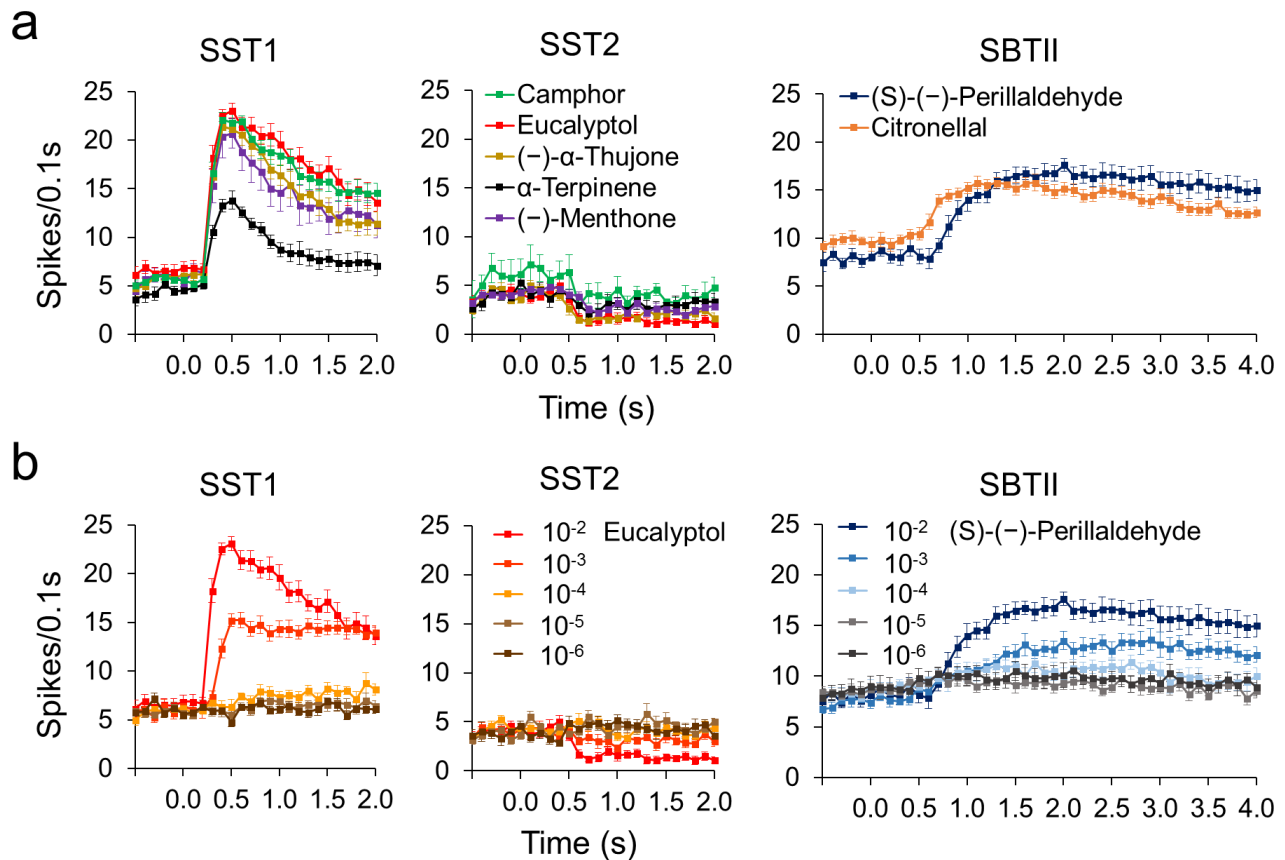
Eucalyptol	4 ± 3	9 ± 7	141 ± 6	-27 ± 2	7 ± 2	21 ± 3	-5 ± 2
Citral	3 ± 2	-8 ± 3	29 ± 4	-2 ± 3	2 ± 2	38 ± 4	-1 ± 2
Linalool	0 ± 2	8 ± 6	6 ± 2	-2 ± 3	2 ± 2	10 ± 2	-2 ± 2
(-)- $\alpha$ -Thujone	1 ± 2	10 ± 7	99 ± 7	-17 ± 3	7 ± 2	11 ± 3	-1 ± 2
<i>Others</i>							
DEET	2 ± 2	-3 ± 5	2 ± 1	-1 ± 3	-5 ± 3	6 ± 2	2 ± 3
Permethrin	1 ± 2	-6 ± 6	0 ± 1	4 ± 3	3 ± 2	9 ± 2	2 ± 2
Naphthalene	0 ± 2	-2 ± 2	1 ± 1	-1 ± 2	21 ± 4	65 ± 4	-2 ± 2
DMSO	1 ± 1	0 ± 0	0 ± 0	0 ± 1	1 ± 1	3 ± 1	1 ± 1



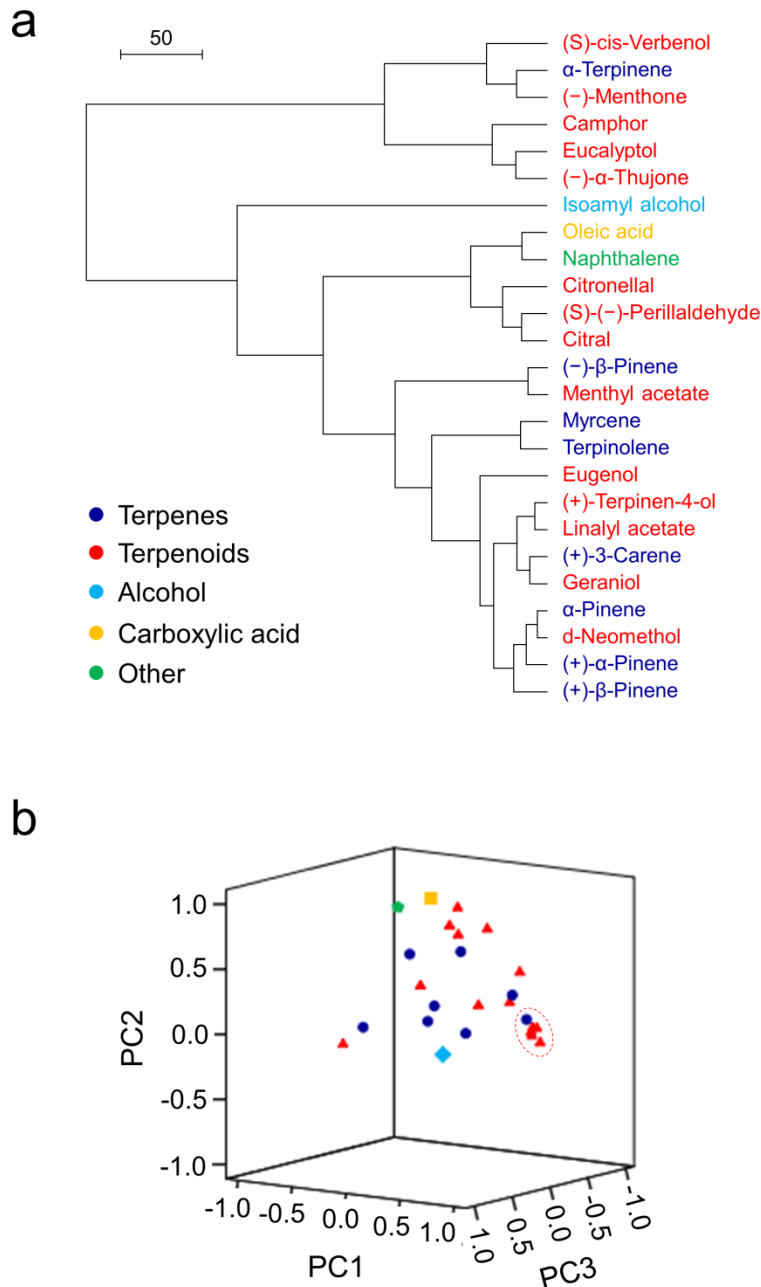
**Figure 5.1 Responses of ORNs in antennal sensilla of *Ae. aegypti* to insect repellents.** (a) Heat map of the responses of ORNs in different types of sensilla to 48 compounds. Compounds and numerical values are presented in Table S5.2,  $n = 5-35$ . For compounds that elicited significant excitatory responses ( $\geq 15$  spikes/s) or inhibitory responses ( $\leq -10$  spikes/s),  $n = 10-35$ . All repellents were tested at a  $10^{-2}$  dilution. (b) Extracellular recordings of ORNs to a 500-ms pulse of air through the repellent cartridge. Action potentials from both ORNs housed in the sensillum can be distinguished by amplitude, with the larger amplitude corresponding to the neuron 'A' and the smaller amplitude to the neuron 'B'. (c) Response intensity of neuron 'A' (black bar) and neuron 'B' (grey bar) in different types of sensilla. Only compounds that elicited significant responses ( $\geq 15$  spikes/s or  $\leq -10$  spikes/s) were used to analyze the response intensity for neuron 'A' or 'B'. Error bars indicate SEM.



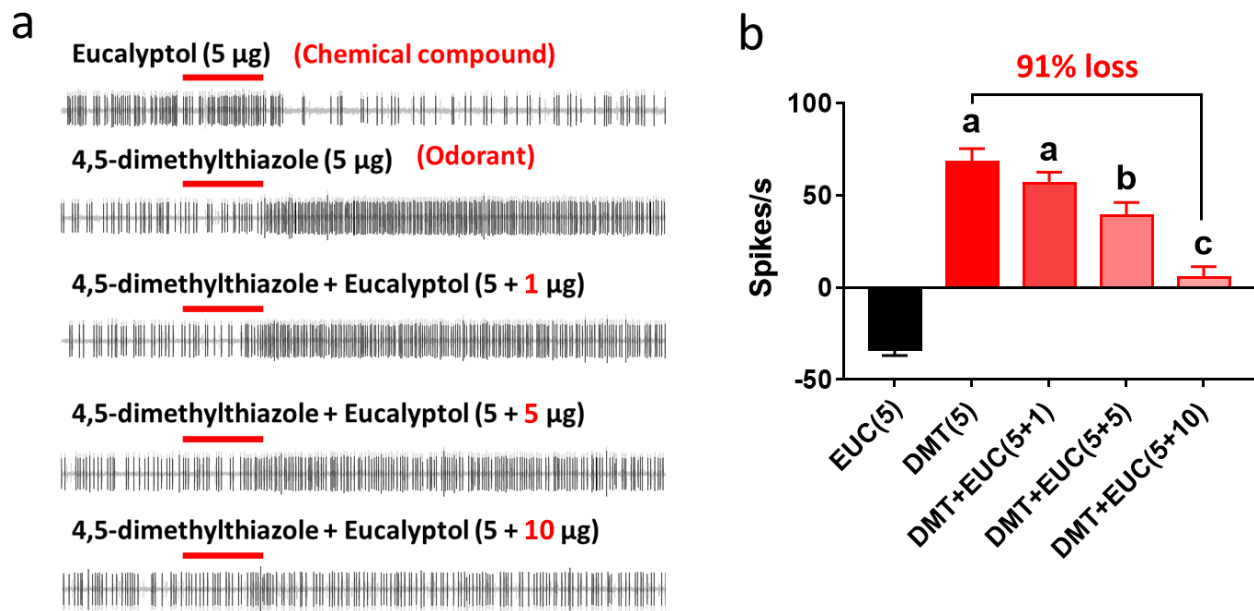
**Figure 5.2 Dose-dependent responses of ORNs in olfactory sensilla to insect repellents.** Only compounds that induced significant responses ( $\geq 50$  spikes/s) at a  $10^{-2}$  dilution were tested at lower concentrations. **(a)** Responses of ORNs in the LST2, SBTI, and SBTII sensilla to insect repellents at serial  $10^{-2}$ - $10^{-6}$  dilutions.  $n = 5$ -11. Dark grey bar, response with a firing rate of  $\geq 15$  spikes/s. Error bars indicate SEM. **(b)** Responses of ORNs in SST1 (red line) and SST2 (blue line) sensilla to compounds at serial  $10^{-2}$ - $10^{-6}$  dilutions.  $n = 8$ -11. Error bars indicate SEM.



**Figure 5.3 Temporal dynamics of ORNs in olfactory sensilla to insect repellents.** (a) Temporal firing activities constructed for the primary responses of ORNs to compounds at a  $10^{-2}$  dilution. ORNs in SST1 (left) and SST2 (middle) sensilla tested against Camphor, Eucalyptol, (-)- $\alpha$ -Thujone, (-)-Menthone, and  $\alpha$ -Terpinene over a 2-s observation period; ORNs in SBTII (right) sensilla against (S)-(-)-Perillaldehyde and Citronellal over a 4-s period.  $n = 5-16$ . Error bars indicate SEM. (b) Temporal firing activities constructed for the responses of ORNs to compounds at serial  $10^{-2}$ - $10^{-6}$  dilutions. ORNs in the SST1 (left) and SST2 (middle) sensilla tested against Eucalyptol over a 2-s period; ORNs in SBTII (right) sensilla against (S)-(-)-Perillaldehyde over a 4-s period.  $n = 6-13$ . Error bars indicate SEM.

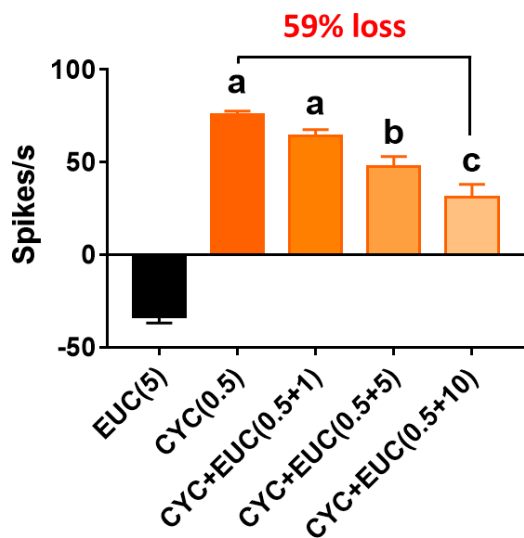


**Figure 5.4 A presentation of odor space.** (a) Hierarchical cluster analysis for 25 compounds that elicited at least one response  $\geq 15$  spikes/s at a  $10^{-2}$  dilution in the ORNs of any types of sensilla based on the Euclidean distances between compounds. Compounds are color coded by chemical class. (b) An odor space as visualized by PCA for 25 compounds. These compounds elicited at least one response  $\geq 15$  spikes/s at a  $10^{-2}$  dilution in the ORNs of any types of sensilla. By performing PCA for the ORN responses in seven types of sensilla to the 25 compounds, the original seven-dimensional odor space was represented in a three-dimensional space using the first three components extracted. This three-dimensional representation accounts for 84% of variance in the original seven-dimensional data set. Compounds are color coded by chemical class as shown in (a).

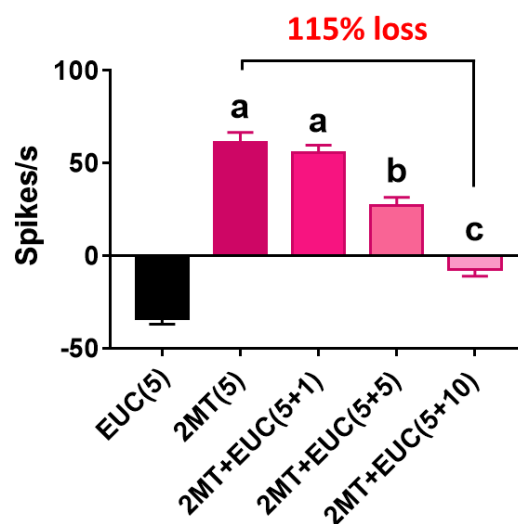


**Figure 5.5 Antagonistic effect of eucalyptol on 4,5-dimethylthiazole reception in *Ae. aegypti*.** (a) Extracellular recordings of the ORN ‘A’ in SST2 sensilla to a 500-ms pulse (as indicated by a horizontal red bar) of air through the odorant cartridge. (b) Bars depicting the responses shown in (a). EUC, eucalyptol; DMT, 4,5-dimethylthiazole.  $n = 6$ . Error bars indicate SEM. Bars with different lower letters are considered statistically different at  $\alpha = 0.05$  level, according to Tukey’s test.

**a** Effect of eucalyptol (EUC) on cyclohexanone (CYC) response



**b** Effect of eucalyptol (EUC) on 2-methyl-2-thiazoline (2MT) response



**Figure 5.6 Antagonistic effect of eucalyptol on the reception of cyclohexanone and 2-methyl-2-thiazoline in *Ae. aegypti*.** Responses of the ORN ‘A’ in SST2 sensilla to (a) cyclohexanone and (b) 2-methyl-2-thiazoline are shown. EUC, eucalyptol; CYC, cyclohexanone; 2MT, 2-methyl-2-thiazoline.  $n = 6$ . Error bars indicate SEM. Bars with different lower letters are considered statistically different at  $\alpha = 0.05$  level, according to Tukey’s test.

## Chapter 6: Characterization of the *Ae. aegypti* Odorant Receptors Responding to Human Odorants and Their Chemical Antagonists

### 6.1 Abstract

The study reported in Chapter 5 revealed that several chemical compounds reduced the responses of three non-human odorants in the SST2 sensilla of *Ae. aegypti*. However, whether these compounds show similar antagonistic effect on the responses of human odorants in *Ae. aegypti* remains unknown. In this study, a *Xenopus* oocyte expression system was used to examine the antagonistic effect of seven chemical compounds on the reception of human odorants that may be important for *Ae. aegypti* host-seeking activity. Three odorant receptors, i.e. AaOR13, AaOR15 and AaOR55, were identified from *Ae. aegypti* that responded to the human odorants and chemical compounds tested. All chemical compounds indicated antagonistic effects on the reception of the human odorant benzaldehyde through one or multiple AaORs. Specifically,  $\alpha$ -terpinene and citronellal demonstrated an overall stronger antagonistic effect on benzaldehyde reception via AaOR55 compared to the other chemical compounds;  $\alpha$ -Terpinene and citronellal also indicated dose-dependent inhibition on the responses of a further two human odorants, p-cresol and sulcatone, in AaOR55. Taken together, these results suggest  $\alpha$ -terpinene and citronellal may be good alternatives to DEET for protecting humans from *Ae. aegypti* mosquitoes.

Chen, Z., Liu, F., & Liu, N. (2019). Plant-derived chemical antagonists for human odor reception in the yellow fever mosquito, *Aedes aegypti*. *In preparation*.

### 6.2 Introduction

The yellow fever mosquito, *Aedes aegypti*, is the principal vector of a number of serious tropical diseases, including yellow fever, dengue fever, chikungunya and Zika fever (Marchette et al. 1969; Jentes et al.

2011; Simmons et al. 2012; Leparc-Goffart et al. 2014). However, although insecticides such as organochlorines, organophosphates, carbamates, and pyrethroids are widely used for mosquito control (Becker et al. 2003; Zaim and Jambulingam 2007; Kelly-Hope et al. 2008), repeated application of these pesticides has caused widespread resistance to develop in multiple mosquito species, thus substantially compromising their efficacy against disease vectors (Walker 2002; Becker et al. 2003; Kelly-Hope et al. 2008; Liu 2015). Botanical essential oils or synthesized compound-based insect repellents provide alternative protection for humans against mosquitoes (Fradin and Day 2002; Campbell et al. 2011), but the mode of action for most insect repellents remains largely unknown.

DEET (*N, N*-diethyl-metatoluamide), the most common ingredient in insect repellents, has been intensively studied. Mosquitoes' olfactory systems express specific ORs and the highly conserved co-receptor Orco, enabling them to smell DEET and thus avoid humans utilizing it as a repellent (Ditzen et al. 2008; Syed and Leal 2008; Stanczyk et al. 2010; Xu et al. 2014). Female *Ae. aegypti orco* mutants have been observed to be repelled by a DEET-treated human arm within 60msec of contact, suggesting the role of gustatory receptors in its repellency action (Lee et al. 2010; DeGennaro et al. 2013). A recent study indicated that the gustatory receptor neurons responsible for DEET detection are located on the legs of *Ae. aegypti* mosquitoes (Dennis et al. 2019). A third mode of action for DEET may be its fixative effect on the release of host odor, thus effectively hiding the otherwise attractive odor from the mosquitoes (Ditzen et al. 2008; Syed and Leal 2008; Afify et al. 2019). Unfortunately, DEET-based insect repellents may be more toxic to humans than other non-DEET-based products registered recently, and none of the currently used non-DEET-based products provides protection for humans with a duration comparable to that of DEET-based insect repellents (Fradin 1998; Fradin and Day 2002; Tavares et al. 2018). A population of *Ae. aegypti* showing resistance to DEET has been successively selected under laboratory conditions (Stanczyk et al. 2010), which means that new chemical compounds with a different mode of action are needed to protect humans from biting insects including mosquitoes.

In a previous study, several chemical compounds that elicited inhibitory responses in the SST2 sensilla of *Ae. aegypti* were found to be capable of reducing the excitatory responses of certain non-

human odorants in the same sensilla (Xu et al. 2018). Another study discovered additional chemical compounds that elicited inhibitory responses in the SST2 sensilla, which should show a similar antagonistic effect on odorant reception in *Ae. aegypti* (Chen et al. 2019). In this study, we examined the antagonistic effect of all these chemical compounds on the reception of human odorants that are thought to be involved in the host-seeking activity of *Ae. aegypti* mosquitoes. Seven odorant receptors were cloned from the antennae of *Ae. aegypti*, which were predicted by blasting the amino acid sequences of *Anopheles gambiae* ORs responding to few compounds on our list (Carey et al. 2010). We found that three of the cloned odorant receptors (ORs), i.e. AaOR13, AaOR15, and AaOR55, responded to the human odorants and chemical compounds tested. Two additional chemical compounds,  $\alpha$ -terpinene and citronellal, were found to show an overall stronger antagonistic effect on the reception of a human odorant, benzaldehyde, among the three AaORs, and  $\alpha$ -Terpinene and citronellal also demonstrated a dose-dependent antagonistic effect on the reception of a further two human odorants, p-cresol and sulcatone, through AaOR55.

## **6.3 Materials and Methods**

### **6.3.1 Insects**

The *Aedes aegypti* (Orlando strain) mosquitoes used in this study were obtained from the laboratory of Dr. James Becnel at the USDA/ARS Mosquito and Fly Research Unit. The colony was maintained at  $25 \pm 2^\circ\text{C}$ ,  $75 \pm 5\%$  relative humidity, and under a photoperiod of 12: 12 (L:D) h. Larvae were cultured in deionized water and fed yeast (Fleischmann's Rapid Rise Instant Yeast) as needed. Female and male mosquitoes were reared in the same cage after eclosion and supplied with unlimited 10% sucrose solution. Adult mosquitoes were fed cow blood samples supplied by the Large Animal Teaching Hospital at Auburn University for stock maintenance.

### 6.3.2 OR cloning and cRNA synthesis

Total RNA samples were extracted from around 800 four-to-six day-old *Ae. aegypti* antennae using the acidic guanidine thiocyanate-phenolchloroform method (Chomczynski and Sacchi 2006). The total RNA was subsequently digested with TURBO DNA-free kit (Invitrogen) to remove DNase,  $Mg^{2+}$  and  $Ca^{2+}$ . Antennal cDNA was synthesized from 1.5  $\mu$ g of DNA-free RNA using the Oligo d(T)<sub>20</sub>-primed SuperScript IV First-Strand Synthesis System (Invitrogen). The full-length coding sequences of *Ae. aegypti* OR genes were cloned by PCR using gene-specific primers containing restriction endonuclease sites and Kozak consensus sequence (Table S6.1). PCR products, purified with a QIAquick gel extraction kit (Qiagen), were digested by restriction enzymes and cloned into pT7Ts vector (a gift from Dr. Guirong Wang at the Institute of Plant Protection, CAAS, China). After the transformation using One Shot TOP10 Chemically Competent *E. coli* (Invitrogen) and the culture in Terrific Broth medium, plasmids were extracted using E.Z.N.A. Plasmid DNA Mini kit (Omega). All recombinant plasmids were sequenced (GENEWIZ, NJ) and confirmed through VectorBase database (<https://www.vectorbase.org/>). Linearized plasmids were used for synthesizing cRNAs with a mMMESSAGE mMACHINE T7 kit (Ambion, Carlsbad, CA).

### 6.3.3 *Xenopus* oocyte expression system and two-electrode voltage-clamp

Mature healthy oocytes (stage V-VII) were obtained by performing aseptic surgery on African clawed frogs, *Xenopus laevis* (Nasco, Salida, CA). The Care and Use of Laboratory Animals were approved and monitored by Auburn University's Institutional Animal Care and Use Committee (approved protocol # 2016-2987). Before microinjection, *Xenopus* oocytes were digested using collagenase I (GIBCO, Carlsbad, CA) in washing buffer (96 mM NaCl, 2 mM KCl, 5 mM  $MgCl_2 \cdot 6H_2O$ , 5 mM HEPES sodium salt, and 10  $\mu$ g/mL gentamycin, pH = 7.6) for 40-60 min at ambient temperature. Digested oocytes were then kept in modified Barth's saline (88 mM NaCl, 1 mM KCl, 0.33 mM  $Ca(NO_3)_2 \cdot 4H_2O$ , 0.41 mM  $CaCl_2 \cdot 2H_2O$ , 0.82 mM  $MgSO_4 \cdot 7H_2O$ , 2.4 mM  $NaHCO_3$ , and 10 mM HEPES sodium salt, pH = 7.6)

supplemented with 10  $\mu\text{g}/\text{mL}$  of gentamycin and 10  $\mu\text{g}/\text{mL}$  of streptomycin at 18°C overnight. Each oocyte was microinjected with 20 nL of the premixed cRNA of OR and Orco (both at a concentration of 250 ng/ $\mu\text{L}$ ). Injected oocytes were subsequently kept in modified Barth's saline at 18°C for 4-7 days. Whole-cell currents were recorded from *Xenopus* oocytes with a two-electrode voltage-clamp (TEVC) as previously described (Liu et al. 2017). Briefly, stock solutions of odorants were prepared in DMSO at a concentration of 1:10 v/v and then diluted to desired concentrations using 1 $\times$  Ringer's solution (91 mM NaCl, 2 mM KCl, 1.8 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 2 mM  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 5 mM HEPES sodium salt, pH = 7.6). Odorant-induced whole-cell currents at a holding potential of -80 mV were recorded from oocytes bathed in perfusion 1 $\times$  Ringer's solution and amplified with an OC-725C amplifier (Warner Instruments, Hamden, CT). Data acquisition and analysis were performed using Digidata 1440A and pCLAMP 10.2 software (Axon Instruments Inc., CA). Human odorants and chemical compounds on the panel included: benzaldehyde, butanal, isobutanal, 2-methylbutanal, cis-2-hexen-1-ol, p-cresol, benzene, 2-butanone, sulcatone, 2-picoline, pyrazine,  $\alpha$ -terpinene, (-)-menthone, citronellal, linalyl acetate, camphor, eucalyptol, and (-)- $\alpha$ -thujone.

## 6.4 Results

### 6.4.1 AaORs responding to human odorants and chemical compounds

To identify the chemoreceptors that respond to human odorants, seven AaORs and the coreceptor AaOrco were cloned from the antennae of *Ae. aegypti* using gene-specific primers (Table S6.1). These AaORs were examined against the human odorants most likely to be involved in the host-seeking activity of *Ae. aegypti* and the chemical compounds known to be capable of moderating the responses of several non-human odorants in both *Ae. aegypti* and *Culex quinquefasciatus* mosquitoes (Xu et al. 2018; Chen et al. 2019). When expressed together with AaOrco, three of the cloned AaORs, i.e. AaOR13, AaOR15, and AaOR55, responded strongly to certain human odorants and chemical compounds tested (Figure 6.1; Table S6.2). Specifically, AaOR13 and AaOR15 showed strong responses to two human odorants, cis-2-

hexen-1-ol and p-cresol, while AaOR15 and AaOR55 demonstrated robust responses to another human odorant benzaldehyde (Figure 6.1; Table S6.2). Chemical compounds generally elicited weak to moderate responses in all three AaORs (Figure 6.1; Table S6.2). *Xenopus* oocytes expressing AaOR21, AaOR26, AaOR54, or AaOR71 indicated weak or no responses to the odorants or compounds on the panel (Table S6.2). Raw oocytes were not activated by any human odorants or chemical compounds tested in the study (Table S6.2).

To determine the role of AaOrco in *Ae. aegypti* odor reception, *Xenopus* oocytes injected with only AaOR55, only AaOrco, or both AaOR55 and AaOrco were examined against the human odorant (benzaldehyde) and two chemical compounds ( $\alpha$ -terpinene and citronellal). Consistent with raw cells, oocytes expressing only AaOR55 or AaOrco responded to neither the two chemical compounds at a concentration of  $10^{-4}$  v/v nor to the human odorant at serial concentrations (Figure 6.2; Table S6.3). However, oocytes expressing both AaOR55 and AaOrco showed weak inward responses to the two chemical compounds and dose-dependent responses to the human odorant benzaldehyde (Figure 6.2; Table S6.3). This suggests that AaORs need to be coexpressed with the coreceptor AaOrco to perform their functions.

In addition, two different response patterns were identified from the oocytes expressing different AaORs. When challenged by the human odorant p-cresol, oocytes expressing AaOR55+AaOrco or AaOR15+AaOrco evoked typical phasic responses, which recovered to the resting current level very rapidly after the stimulation (Figure 6.3). However, oocytes expressing AaOR13+AaOrco gave prolonged, tonic responses to the same odorant p-cresol and another human odorant cis-2-hexen-1-ol (Figure 6.3). These two distinct response patterns probably result from the different binding affinity of human odorants to different AaORs.

#### 6.4.2 $\alpha$ -Terpinene and citronellal masked benzaldehyde reception

Since the human odorant benzaldehyde elicited moderate to strong responses in AaOR13, AaOR15, and AaOR55 (Figure 6.1) and had been implicated in the host-seeking activity of *Ae. aegypti* mosquitoes (Chen et al. 2019), whether the chemical compounds eliciting inhibitory responses in SST2 sensilla showed an antagonistic effect on the reception of benzaldehyde in *Ae. aegypti* remains to be established (Chen et al. 2018). Although eliciting very weak or almost no responses, all seven chemical compounds at a concentration of  $10^{-4}$  v/v demonstrated a significant antagonistic effect on the reception of benzaldehyde in one or multiple AaORs (Figure 6.1 and 6.4). Specifically,  $\alpha$ -terpinene and citronellal exhibited overall stronger inhibition on benzaldehyde reception in all three AaORs than any of the other chemical compounds tested (Figure 6.4). All chemical compounds yielded an averaged inhibition rate of 62% on the response of benzaldehyde in AaOR55, higher than the 47% seen in AaOR13 and the 30% in AaOR15.

Moreover, the dose-dependent antagonistic effects of  $\alpha$ -terpinene and citronellal on benzaldehyde reception in AaOR55 were examined. Results indicated that  $\alpha$ -terpinene showed an apparent dose-dependent antagonistic effect on the reception of benzaldehyde in AaOR55 (Figure 6.5).  $\alpha$ -Terpinene at a concentration of  $10^{-5}$  v/v inhibited about 33% of the response induced by benzaldehyde at a concentration of  $10^{-4}$  v/v; however, at a concentration of  $10^{-4}$  v/v it blocked 82% of the response from benzaldehyde (Figure 6.5). Another chemical compound, citronellal, also indicated a similar pattern of antagonistic effect on benzaldehyde reception in AaOR55 (Figure 6.6). Citronellal at a concentration of  $10^{-5}$  v/v masked around 32% of the response of benzaldehyde, while at a concentration of  $10^{-4}$  v/v it reduced 68% of the response (Figure 6.6). Benzaldehyde applied alone before and after the combination stimulation elicited consistent responses in AaOR55, suggesting that the inhibition of the benzaldehyde response was a result of the antagonistic effect of chemical compounds rather than oocyte signal reduction.

### 6.4.3 $\alpha$ -Terpinene and citronellal blocked the reception of other human odorants

As there are many more human odorants that may be involved in the host-seeking activity of *Ae. aegypti* (Chen et al. 2019), the antagonistic effect of  $\alpha$ -terpinene and citronellal on the reception of an additional two human odorants, p-cresol and sulcatone, in AaOR55 were examined as well. Results showed that both  $\alpha$ -terpinene and citronellal exerted a dose-dependent antagonistic effect on the response of p-cresol in AaOR55 (Figure 6.7), while  $\alpha$ -Terpinene, at a concentration of either  $0.5 \times 10^{-4}$  v/v or  $10^{-4}$  v/v, dramatically reduced the response of p-cresol in AaOR55 (Figure 6.7). Citronellal at the same concentrations also significantly inhibited the response of p-cresol, though to a lesser extent (Figure 6.7). The two chemical compounds demonstrated similar patterns of inhibition on the reception of another human odorant, sulcatone, in AaOR55 and  $\alpha$ -Terpinene at all concentrations, i.e.  $10^{-5}$  v/v,  $0.5 \times 10^{-4}$  v/v, and  $10^{-4}$  v/v, significantly reduced the reception of sulcatone (Figure 6.8). Compared to  $\alpha$ -terpinene, citronellal was less effective and only markedly masked sulcatone reception at concentrations of  $0.5 \times 10^{-4}$  v/v or  $10^{-4}$  v/v (Figure 6.8).  $\alpha$ -terpinene at a concentration of  $10^{-4}$  v/v reduced the response of p-cresol by 71% and the response of sulcatone by 64%, while citronellal at the same concentration only blocked 50% and 46% of the responses of the two odorants, respectively. This suggests that the chemical compound  $\alpha$ -terpinene may be a better candidate to mask human odor from *Ae. aegypti* mosquitoes.

## 6.5 Discussion

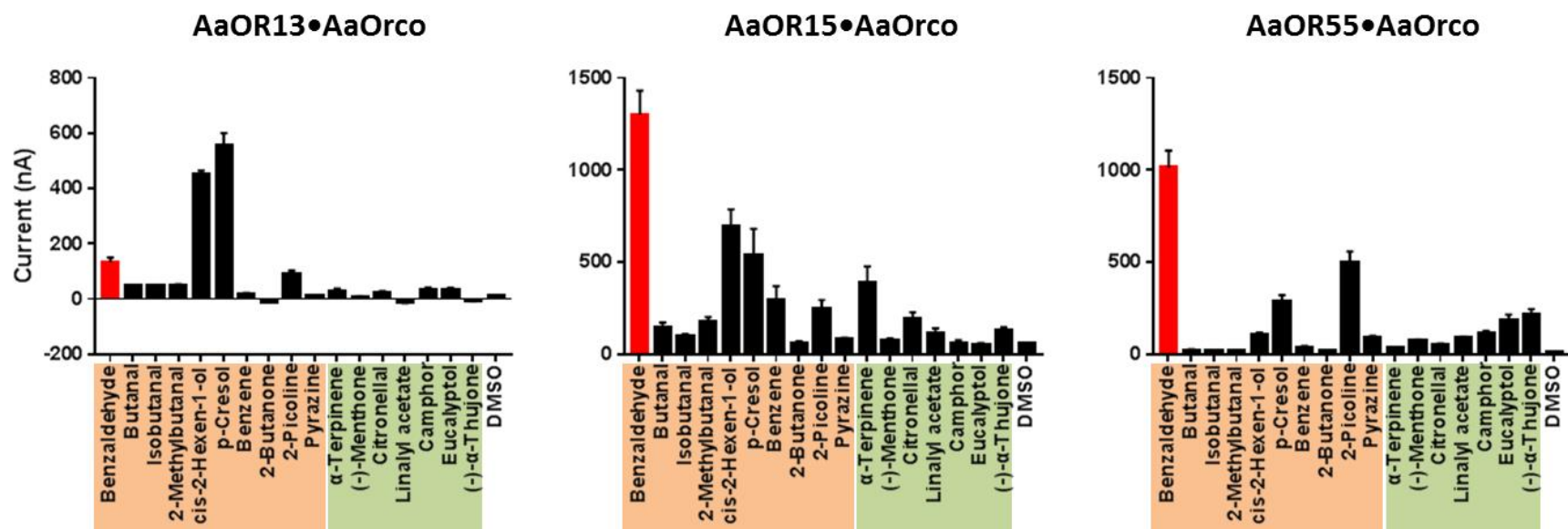
Female *Ae. aegypti* mosquitoes have shown a strong preference for humans when seeking blood meals compared to guinea pigs and this strong preference was found to be associated with a high expression level of AaOR4, an odorant receptor showing high sensitivity to the human odorant sulcatone (McBride et al. 2014). Liesch et al. (2013) discovered that before a blood meal, female *Ae. aegypti* were highly attracted to human odor, but at 24-72 hours post blood meal (pbm) blood-fed mosquitoes showed decreased or even zero interest in human odor. A previous study indicated that at 24-84 hours pbm female *Ae. aegypti* exhibited reduced sensitivity to certain aldehydes, alcohols, aliphatics/aromatics, ketones, and

amines, suggesting the important role of these human odorants in their host-seeking activity (Chen et al. 2019). In this study, three ORs, AaOR13, AaOR15, and AaOR55, were identified from *Ae. aegypti*, that were responsible for the mosquitoes' ability to sense these salient human odorants. Specifically, AaOR15 and AaOR55 evoked strong responses to benzaldehyde, but only moderate responses to sulcatone.

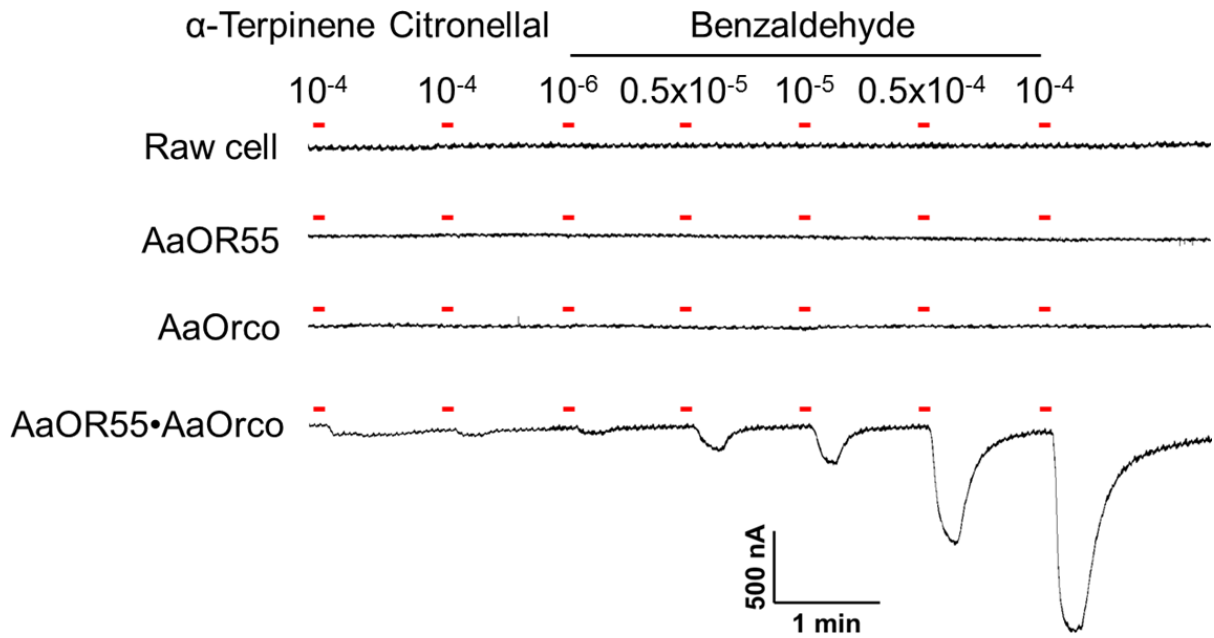
Many chemical compounds extracted from plants have shown strong to moderate repellent activities to biting insects, including mosquitoes (Liu et al. 2013), although their modes of action have yet to be established. In a previous study, we identified several chemical compounds that elicited inhibitory responses in the antennal SST2 sensilla of female *Ae. aegypti* (Chen et al. 2018). Some of these chemical compounds were able to moderate the reception of specific non-human odorants in both *Ae. aegypti* and *Cx. quinquefasciatus* mosquitoes (Xu et al. 2018). In the current study, the antagonistic effect of these chemical compounds on the reception of salient human odorants in *Xenopus* oocytes expressing AaORs and AaOrco were examined.  $\alpha$ -terpinene and citronellal were capable of reducing the responses of three human odorants (benzaldehyde, p-cresol and sulcatone) in AaOR55, unlike the modes of action for DEET (Syed and Leal 2008; Lee et al. 2010; DeGennaro et al. 2013).  $\alpha$ -terpinene also exhibited repellent activity to *Aedes* and *Culex* mosquitoes that was comparable to that of DEET for the first 60 min (Park et al. 2005; Gu et al. 2009). Citronellal has also been used to as a repellent against *Ae. aegypti* mosquitoes, though with a relatively low efficacy (Fradin and Day 2002; Specos et al. 2010). Given that *Ae. aegypti* has developed resistance to DEET, and DEET-based insect repellents are thought to be more toxic to humans than the new non-DEET-based products registered recently,  $\alpha$ -terpinene and citronellal may be good alternatives to protect humans from *Ae. aegypti* mosquitoes (Stanczyk et al. 2010; Tavares et al. 2018).

The first investigation utilizing cryo-electron microscopy (EM) to determine the structure of insect OR co-receptor (Orco) has been reported recently for the parasitic fig wasp *Apocrypta bakeri* (Butterwick et al. 2018). Insect olfactory receptors are heteromeric ligand-gated ion channels, which are composed of specific ORs and the highly conserved Orco. Identification of the cryo-EM structure for an insect OR•Orco heterotetramer could be a useful approach for investigating the binding sites of the ion

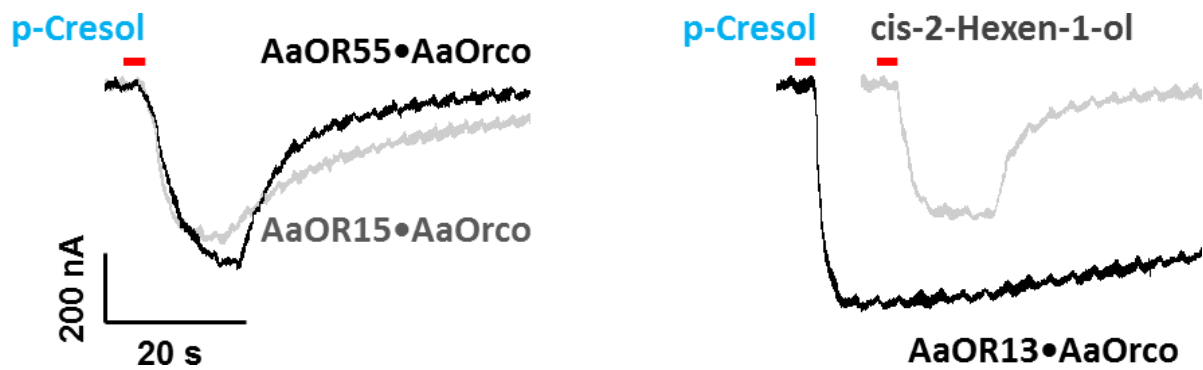
channel and thus its interactions with potential chemical ligands. Based on the cryo-EM structures for OR•Orco heterotetramers, the mode of action of the chemical compounds that exhibit an antagonistic effect on human odor reception in *Ae. aegypti* could also shed new light on the mechanisms involved.



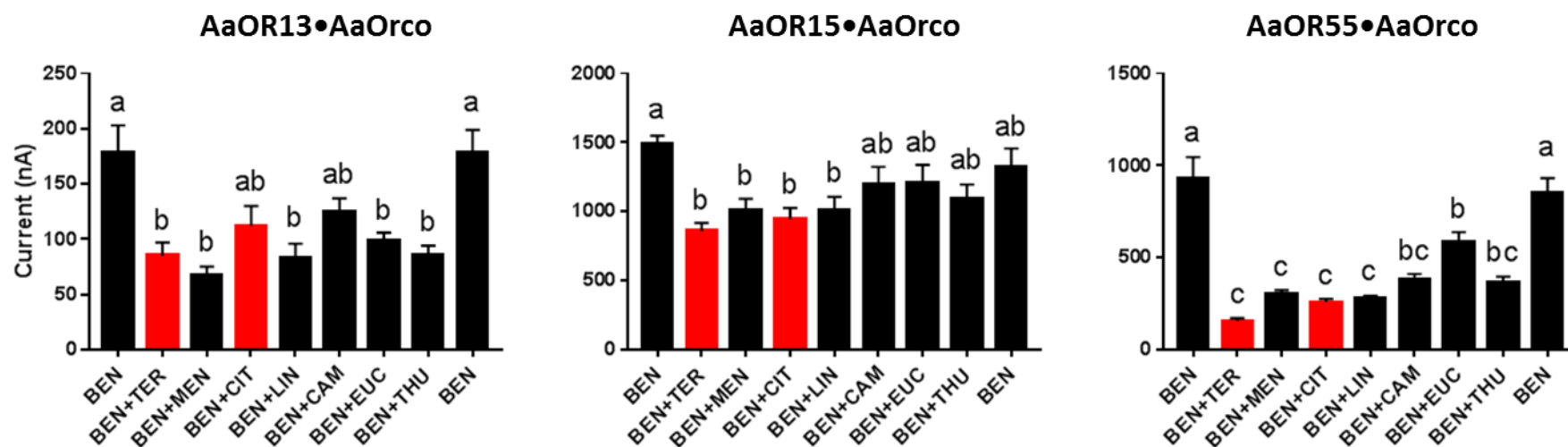
**Figure 6.1 Responses of AaORs to human odorants and chemical compounds.** *Left*, AaOR13•AaOrco,  $n = 8-14$ ; *middle*, AaOR15•AaOrco,  $n = 8-14$ ; *right*, AaOR55•AaOrco,  $n = 6-12$ . Red color, human odorants; green color, chemical compounds. All odorants were tested at a concentration of  $10^{-4}$  v/v. Error bars indicate SEM.



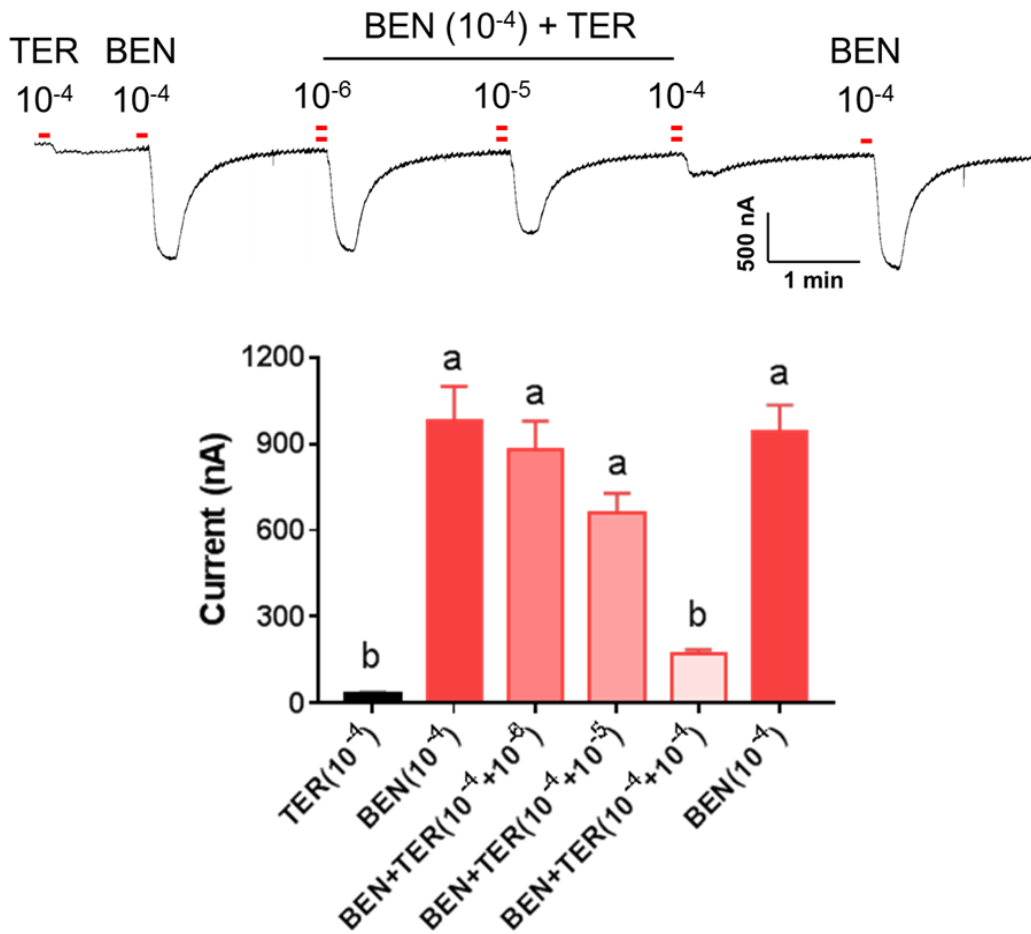
**Figure 6.2** *Xenopus* Oocytes expressing both AaORs and AaOrco responded to human odorants. Chemical compounds  $\alpha$ -terpinene and citronellal were tested at a concentration of  $10^{-4}$  v/v; the human odorant benzaldehyde was tested at serial concentrations as indicated.  $n = 3$ .



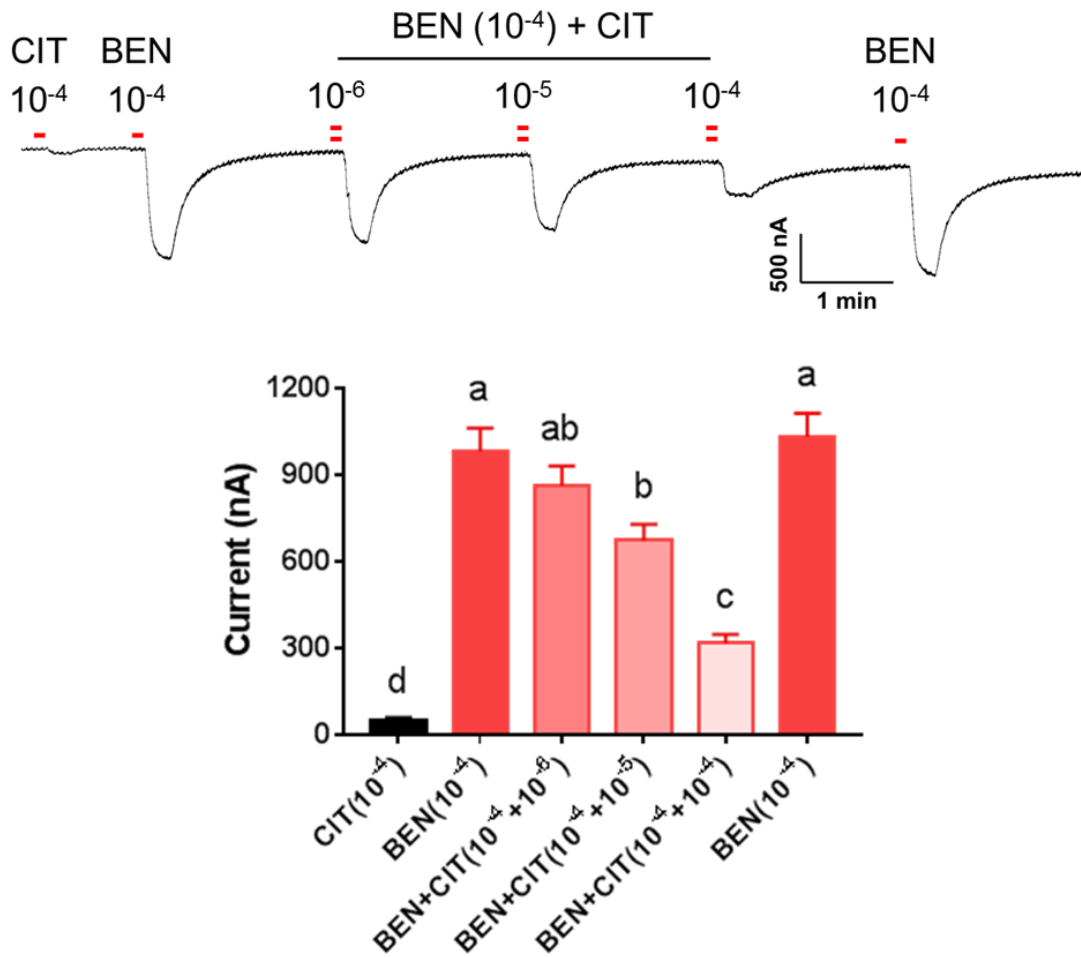
**Figure 6.3** Different response patterns of AaORs to human odorants. *Left*, responses of AaOR55•AaOrco and AaOR15•AaOrco to p-cresol; *right*, responses of AaOR13•AaOrco to p-cresol and cis-2-hexen-1-ol.



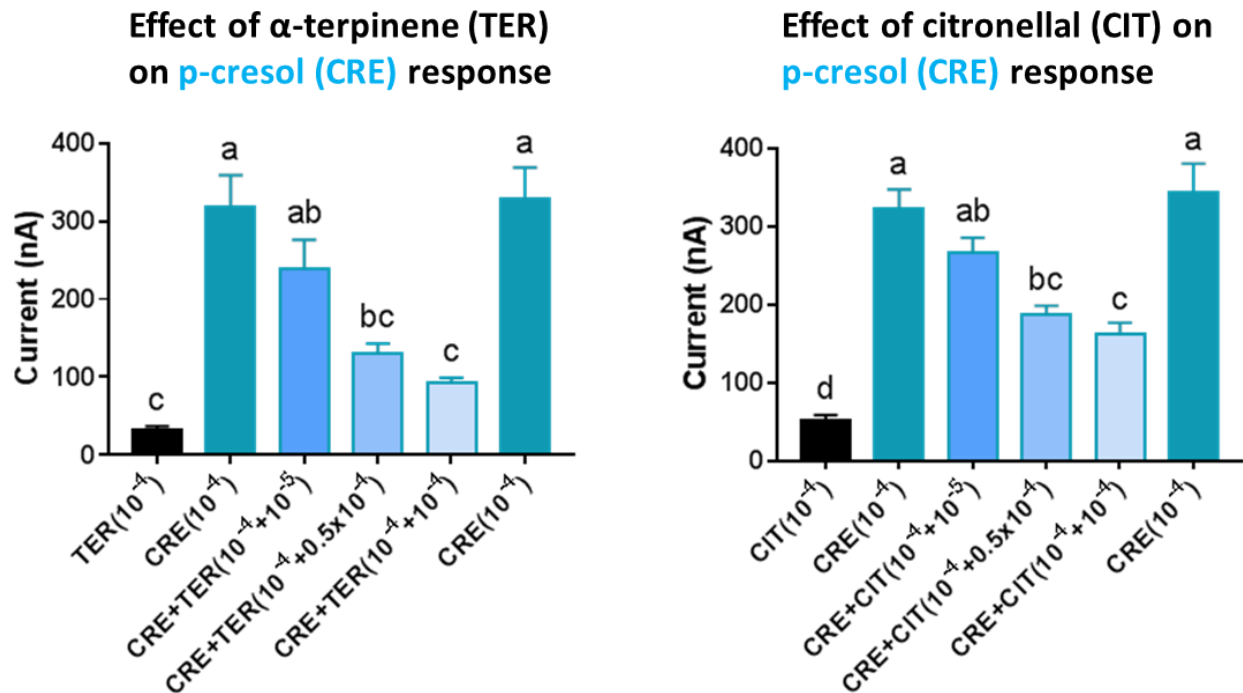
**Figure 6.4 Antagonistic effects of chemical compounds on benzaldehyde reception.** All chemical compounds and the human odorant benzaldehyde were examined at a concentration of  $10^{-4}$  v/v. *Left*, AaOR13•AaOrco,  $n = 6$ ; *middle*, AaOR15•AaOrco,  $n = 6$ ; *right*, AaOR55•AaOrco,  $n = 6$ . BEN, benzaldehyde; TER,  $\alpha$ -terpinene; MEN, (-)-menthone; CIT, citronellal; LIN, linalyl acetate; CAM, camphor; EUC, eucalyptol; THU, (-)- $\alpha$ -thujone. Error bars indicate SEM. Bars with different lower letters are considered statistically different at  $\alpha = 0.05$  level, according to Tukey's test.



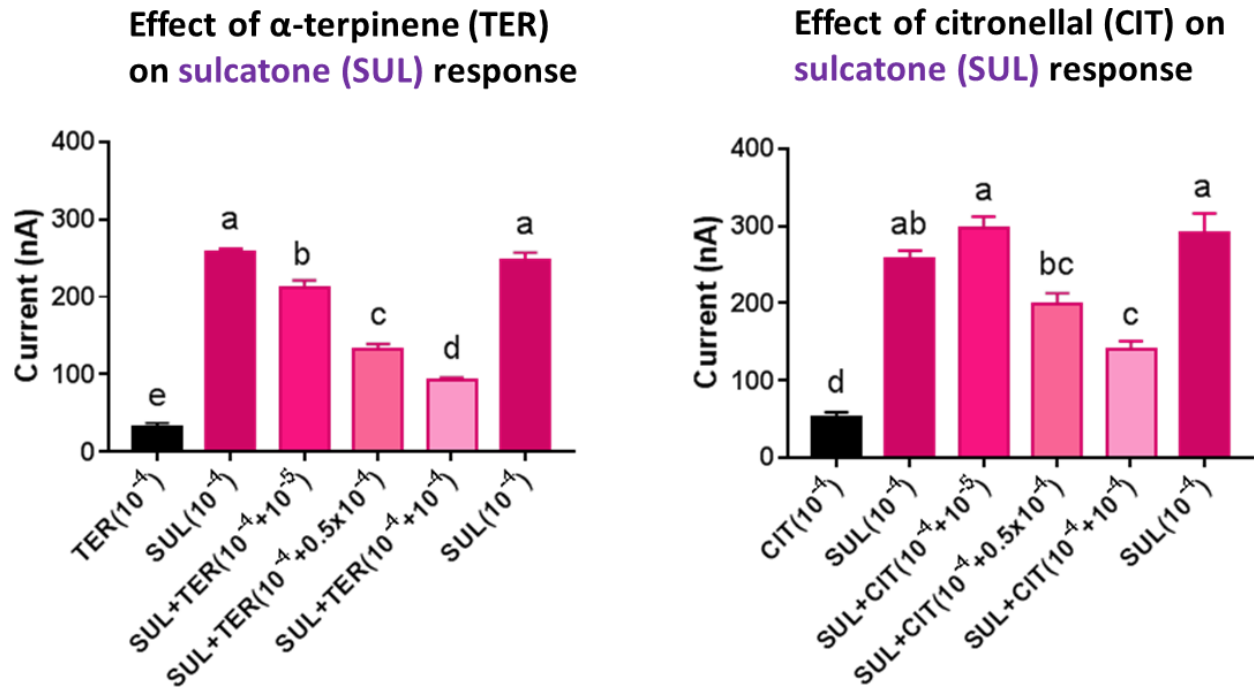
**Figure 6.5 Dose-dependent antagonistic effect of  $\alpha$ -terpinene on benzaldehyde reception in AaOR55•AaOrco.** BEN, benzaldehyde; TER,  $\alpha$ -terpinene.  $n = 6$ . Error bars indicate SEM. Bars with different lower letters are considered statistically different at  $\alpha = 0.05$  level, according to Tukey's test.



**Figure 6.6 Dose-dependent antagonistic effect of citronellal on benzaldehyde reception in AaOR55·AaOrco.** BEN, benzaldehyde; CIT, citronellal.  $n = 6$ . Error bars indicate SEM. Bars with different lower letters are considered statistically different at  $\alpha = 0.05$  level, according to Tukey's test.



**Figure 6.7 Antagonistic effects of  $\alpha$ -terpinene and citronellal on p-cresol reception in *AaOR55*•*AaOrco*.** *Left*, effect of  $\alpha$ -terpinene (TER) on p-cresol (CRE) reception,  $n = 6$ ; *right*, effect of citronellal (CIT) on p-cresol (CRE) reception,  $n = 6$ . Error bars indicate SEM. Bars with different lower letters are considered statistically different at  $\alpha = 0.05$  level, according to Tukey's test.



**Figure 6.8 Antagonistic effects of  $\alpha$ -terpinene and citronellal on sulcatone reception in AaOR55•AaOrco.** *Left*, effect of  $\alpha$ -terpinene (TER) on sulcatone (SUL) reception,  $n = 6$ ; *right*, effect of citronellal (CIT) on sulcatone (SUL) reception,  $n = 6$ . Error bars indicate SEM. Bars with different lower letters are considered statistically different at  $\alpha = 0.05$  level, according to Tukey's test.

**Table S6.1** Primers used for cloning *Ae. aegypti* OR genes

Gene	Forward primer*	Reverse primer*
Orco	ccggctagc <span style="color: green;">gccacc</span> ATGAACGTCCAACCGACA	ctaggc <span style="color: red;">ggccgc</span> TTATTTCAACTGCACCAACA
OR13	ccggctagc <span style="color: green;">gccacc</span> ATGTGGCAGCCGTTGAGA	ctaggc <span style="color: red;">ggccgc</span> CTAGAATCTATTCTTCAGAA
OR15	ccggctagc <span style="color: green;">gccacc</span> ATGAAGTACTTTGAGCTG	ctaggc <span style="color: red;">ggccgc</span> TTACAACCTGATCCTTTAGTA
OR21	ccggctagc <span style="color: green;">gccacc</span> ATGGATCTTATAGAAACG	ctaggc <span style="color: red;">ggccgc</span> TTACTCAGTAAATTGAATCA
OR26	ccggctagc <span style="color: green;">gccacc</span> ATGGTATTATTTTCCCGC	ctaggc <span style="color: red;">ggccgc</span> TTAATGACCTTGGTCTCGAT
OR54	ccggctagc <span style="color: green;">gccacc</span> ATGAATACTAATTCACAA	ctaggc <span style="color: red;">ggccgc</span> TCACTTATCTCCATAGAATT
OR55	ccggctagc <span style="color: green;">gccacc</span> ATGCAGCAAAAACCTCCG	ctaggc <span style="color: red;">ggccgc</span> CTATTCTCCGTAGAACTGCT
OR71	ccggctagc <span style="color: green;">gccacc</span> ATGGAACTGTCCTACCAT	ctaggc <span style="color: red;">ggccgc</span> TCAAAGCCGGTCCAGAACAT

\*Purple color, protective nucleotides; red color, restriction endonuclease site; green color, Kozak sequence; black color, gene-specific primer sequence

**Table S6.2** Current response profiles (nA, mean  $\pm$  SEM) of *Xenopus* oocytes expressing AaORs and AaOrco to odorants

Odorant*	OR							
	raw cell (n = 5)	13 (n = 8-14)	15 (n = 8-14)	21 (n = 3)	26 (n = 3)	54 (n = 3)	55 (n = 6-12)	71 (n = 3)
Benzaldehyde	2 $\pm$ 4	134 $\pm$ 16	1301 $\pm$ 129	13 $\pm$ 7	13 $\pm$ 7	17 $\pm$ 9	1013 $\pm$ 92	30 $\pm$ 6
Butanal	8 $\pm$ 2	45 $\pm$ 3	145 $\pm$ 27	17 $\pm$ 3	10 $\pm$ 0	23 $\pm$ 3	22 $\pm$ 5	30 $\pm$ 6
Isobutanal	10 $\pm$ 4	46 $\pm$ 5	94 $\pm$ 15	23 $\pm$ 3	0 $\pm$ 6	20 $\pm$ 6	15 $\pm$ 3	27 $\pm$ 9
2-Methylbutanal	14 $\pm$ 2	48 $\pm$ 5	175 $\pm$ 27	13 $\pm$ 3	3 $\pm$ 9	13 $\pm$ 7	20 $\pm$ 4	30 $\pm$ 12
cis-2-Hexen-1-ol	14 $\pm$ 2	448 $\pm$ 17	693 $\pm$ 94	20 $\pm$ 6	10 $\pm$ 0	13 $\pm$ 3	103 $\pm$ 14	27 $\pm$ 12
p-Cresol	4 $\pm$ 4	555 $\pm$ 45	534 $\pm$ 146	10 $\pm$ 6	3 $\pm$ 7	17 $\pm$ 7	285 $\pm$ 36	13 $\pm$ 3
Benzene	12 $\pm$ 2	19 $\pm$ 3	291 $\pm$ 80	17 $\pm$ 9	-7 $\pm$ 3	23 $\pm$ 7	38 $\pm$ 7	17 $\pm$ 9
2-Butanone	4 $\pm$ 6	-8 $\pm$ 3	59 $\pm$ 11	27 $\pm$ 7	3 $\pm$ 7	7 $\pm$ 3	15 $\pm$ 3	17 $\pm$ 9
2-Picoline	6 $\pm$ 2	89 $\pm$ 14	246 $\pm$ 48	13 $\pm$ 3	3 $\pm$ 3	17 $\pm$ 7	500 $\pm$ 58	27 $\pm$ 7
Pyrazine	10 $\pm$ 0	10 $\pm$ 3	79 $\pm$ 11	23 $\pm$ 3	10 $\pm$ 0	20 $\pm$ 6	90 $\pm$ 11	27 $\pm$ 3
$\alpha$ -Terpinene	16 $\pm$ 2	26 $\pm$ 12	390 $\pm$ 87	-3 $\pm$ 7	0 $\pm$ 10	10 $\pm$ 17	32 $\pm$ 5	30 $\pm$ 6
(-)-Menthone	6 $\pm$ 2	4 $\pm$ 6	74 $\pm$ 13	13 $\pm$ 3	3 $\pm$ 3	37 $\pm$ 12	72 $\pm$ 9	20 $\pm$ 6
Citronellal	10 $\pm$ 3	24 $\pm$ 5	193 $\pm$ 35	-3 $\pm$ 7	20 $\pm$ 0	13 $\pm$ 3	52 $\pm$ 7	17 $\pm$ 9
Linalyl acetate	16 $\pm$ 4	-10 $\pm$ 5	114 $\pm$ 27	7 $\pm$ 13	0 $\pm$ 6	17 $\pm$ 9	88 $\pm$ 8	27 $\pm$ 3
Camphor	4 $\pm$ 6	33 $\pm$ 8	61 $\pm$ 14	20 $\pm$ 6	13 $\pm$ 3	7 $\pm$ 13	112 $\pm$ 16	43 $\pm$ 19
Eucalyptol	2 $\pm$ 5	31 $\pm$ 9	51 $\pm$ 9	27 $\pm$ 3	0 $\pm$ 6	17 $\pm$ 3	185 $\pm$ 31	30 $\pm$ 6
(-)- $\alpha$ -Thujone	6 $\pm$ 2	-4 $\pm$ 4	130 $\pm$ 17	40 $\pm$ 6	7 $\pm$ 3	30 $\pm$ 6	217 $\pm$ 28	30 $\pm$ 10
DMSO	0 $\pm$ 0	10 $\pm$ 3	55 $\pm$ 2	13 $\pm$ 9	3 $\pm$ 3	3 $\pm$ 13	13 $\pm$ 2	10 $\pm$ 0

\*All odorants were tested at a concentration of  $10^{-4}$  v/v.

**Table S6.3** Responses (nA, mean  $\pm$  SEM) of *Xenopus* oocytes expressing AaOR55, AaOrco, and AaOR55+AaOrco to odorants

Odorant	Concentration (v/v)	Raw cell (n = 3)	OR55 (n = 3)	Orco (n = 3)	OR55+Orco (n = 3)
$\alpha$ -Terpinene	$10^{-4}$	-3 $\pm$ 3	3 $\pm$ 3	3 $\pm$ 3	47 $\pm$ 9
Citronellal	$10^{-4}$	-3 $\pm$ 3	3 $\pm$ 3	0 $\pm$ 6	63 $\pm$ 18
Benzaldehyde	$10^{-6}$	0 $\pm$ 0	-3 $\pm$ 3	7 $\pm$ 3	23 $\pm$ 3
Benzaldehyde	$0.5 \times 10^{-5}$	0 $\pm$ 0	-7 $\pm$ 3	0 $\pm$ 0	143 $\pm$ 9
Benzaldehyde	$10^{-5}$	-3 $\pm$ 3	-3 $\pm$ 3	3 $\pm$ 3	243 $\pm$ 35
Benzaldehyde	$0.5 \times 10^{-4}$	3 $\pm$ 3	0 $\pm$ 0	0 $\pm$ 0	810 $\pm$ 58
Benzaldehyde	$10^{-4}$	7 $\pm$ 3	0 $\pm$ 0	7 $\pm$ 3	1307 $\pm$ 258

## Chapter 7: Research Summary and Future Directions

Female mosquitoes use multiple cues, including CO<sub>2</sub>, skin odor, and body heat, in their host-seeking behavior (Cardé 2015). Female *Ae. aegypti* mosquitoes have recently been found to show a strong preference for humans when seeking blood meals compared to another mammal, in this case guinea pigs (McBride et al. 2014). Considering that both humans and guinea pigs produce CO<sub>2</sub> and have a similar body temperature, female *Ae. aegypti* are likely to use skin odor for host discrimination. A previous study has isolated more than 300 compounds from human skin emanations (Bernier et al. 2000), although which of these compounds can be detected by *Ae. aegypti* and which of them contribute to the host-seeking activity of *Ae. aegypti* remain largely unknown.

In Objective 1, the neuronal responses of *Ae. aegypti* antennal olfactory sensilla to 103 commercially available human odorants were examined using the SSR technique. The olfactory sensilla of female *Ae. aegypti* only showed responses to certain human odorants, including aldehydes, alcohols, aliphatics/aromatics, ketones, amines, and heterocyclics. Carboxylic acids on the panel did not elicit any responses in any of the different types of *Ae. aegypti* antennal olfactory sensilla, which is consistent with the findings reported in the southern house mosquito *Cx. quinquefasciatus* (Ye et al. 2016). Previous studies have demonstrated that IRs expressed in the ORNs of coeloconic sensillum are responsible for acid sensation in *Drosophila* (Benton et al. 2009; Ai et al. 2010; Silbering et al. 2011), but acid sensation in mosquitoes also remains to be examined in more detail.

A previous study by Liesch et al. (2013) discovered that before a blood meal, female *Ae. aegypti* was highly attracted to human odor, but after the blood meal, e.g. at 24-72 hours pbm, blood-fed mosquitoes showed decreased or even zero interest in human odor. I therefore hypothesized that blood-fed female *Ae. aegypti* would evoke reduced olfactory responses to certain human odorants. In Objective 2, the antennal sensillar responses of both non-blood fed and blood-fed female *Ae. aegypti* mosquitoes to

the human odorants identified in Objective 1 were investigated at 24-36, 48-60, and 72-84 hours pbm. Results indicated that after a blood meal, female *Ae. aegypti* showed reduced sensitivity to certain aldehydes, alcohols, aliphatics/aromatics, ketones, and amines at one or multiple time points, which indicates that these human odorants may be involved in the host-seeking activity of *Ae. aegypti*. Okumu et al. (2010) developed a synthetic blend containing optimum levels of aqueous ammonia, CO<sub>2</sub>, lactic acid and seven additional carboxylic acids (each added at their respective optimum concentrations) and found it more attractive to *Anopheles*, *Culex* and *Mansonia* mosquitoes compared to a human volunteer at long range distances in semi-field experiments. This indicates that a blend composed of different human odorants and other olfactory cues should be more efficient in attracting female *Ae. aegypti* than an individual cue or odorant, although the optimum concentration for each ingredient needs to be determined.

DEET is a synthetic compound and also the most common ingredient of commercially available insect repellents. However, a population of *Ae. aegypti* showing resistance to DEET has been successively selected under laboratory conditions (Stanczyk et al. 2010), raising the specter of the widespread development of resistance to one of the most effective weapons in our preventative arsenal. DEET-based insect repellents may also be more toxic to humans than the non-DEET-based products registered recently (Tavares et al. 2018). This suggests that we stand in urgent need of new chemical compounds to protect humans from mosquitoes. Although many botanical compounds have shown strong to moderate repellent activity to biting insects, including mosquitoes, few has yet been functionally studied in the antenna olfactory sensilla of *Ae. aegypti* mosquitoes. In Objective 3, the responses of *Ae. aegypti* antennal olfactory sensilla to 48 plant-derived chemical compounds were studied. Different types of sensilla exhibited different response patterns to the chemical compounds tested. Specifically, the SST2 sensillum evoked inhibitory responses to several compounds, including eucalyptol, citronellal, and  $\alpha$ -terpinene. I then hypothesized that the chemical compounds eliciting inhibitory responses in the SST2 sensillum would reduce the excitatory responses induced by other odorants in the same sensillum. When the chemical compound eucalyptol and other odorants (i.e. 4,5-dimethylthiazole, cyclohexanone or 2-

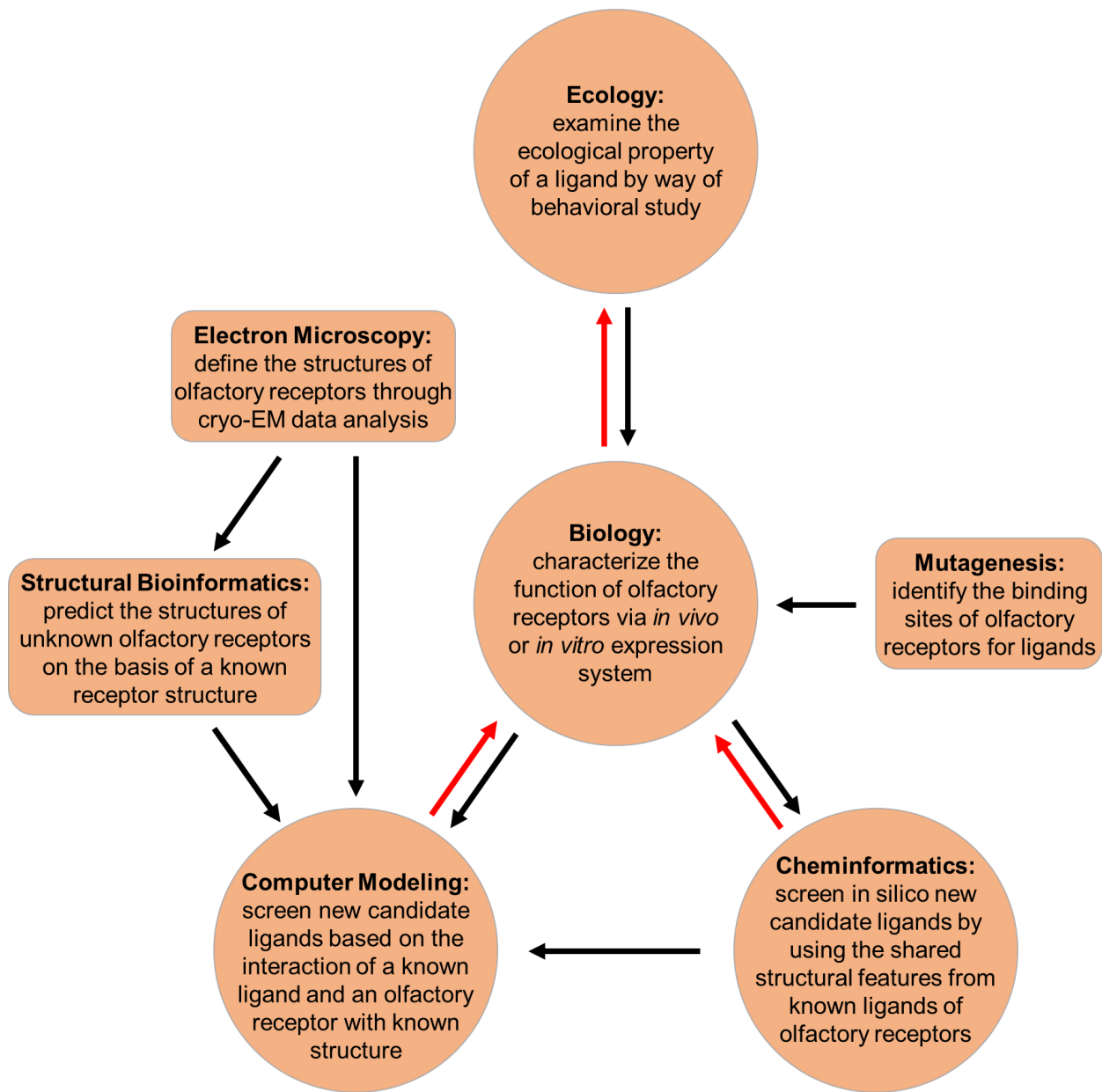
methyl-2-thiazoline) were applied simultaneously in the SST2 sensilla of *Ae. Aegypti* mosquitoes, the excitatory responses of the odorants reduced dramatically with increasing doses of eucalyptol. These results suggest that the chemical compound eucalyptol may be a good candidate for use as a confusant to protect humans from mosquitoes, though the potency needs to be confirmed by future behavioral studies.

McBride et al. (2014) found that the strong preference of female *Ae. aegypti* for human blood meals was associated with a high expression level of AaOR4, an odorant receptor showing high sensitivity to the human odorant sulcatone. In Objectives 1 and 2, human odorants including certain aldehydes, alcohols, aliphatics/aromatics, ketones, and amines were found to be involved in the host-seeking activity of *Ae. aegypti*. A recent genome study identified 117 ORs from *Ae. aegypti* (Matthews et al. 2018). Objective 4 aimed at identifying the *Ae. aegypti* ORs responsible for the sensation of these salient human odorants. Seven ORs candidates from *Ae. aegypti* were identified by running BLAST for the amino acid sequences of *An. gambiae* ORs that evoked responses to some of the compounds on our list. The responses of *Xenopus* oocytes expressing the AaORs together with the coreceptor AaOrco against the human odorants on the panel were examined. Only oocytes injected with AaOR13, AaOR15, or AaOR55 yielded strong to moderate responses to the odorants tested, indicating the important roles of the three AaORs in human odor sensation in *Ae. aegypti*.

Also, the chemical compounds that elicited inhibitory responses in the SST2 sensillum were tested in the oocytes expressing any of the three AaORs, but only very weak or no responses were observed, suggesting that the three AaORs are not target receptors for these chemical compounds. However, when the human odorant benzaldehyde was delivered with the individual chemical compounds (one odorant + one chemical compound each time) to the oocytes expressing AaOR13, AaOR15, or AaOR55 at the same time, the current response of benzaldehyde +  $\alpha$ -terpinene (or citronellal) was significantly weaker than that of the odorant benzaldehyde alone.  $\alpha$ -Terpinene and citronellal also reduced the current responses of another two human odorants, p-cresol and sulcatone, in a dose-dependent manner. These results suggest that the two chemical compounds,  $\alpha$ -terpinene and citronellal, have

antagonistic effects on the reception of human odorants in *Ae. aegypti*, although this needs to be verified in further behavioral studies.

Traditionally, odorants with ecological significance are screened via behavioral studies and their chemoreceptors are then identified using electrophysiological (e.g. SSR) and molecular methodologies (e.g. transgenic fruit flies, *Xenopus laevis* oocyte expression system, and two-electrode voltage-clamp) (Figure 7.1). Despite the progress made in a few model insects (e.g. *D. melanogaster*) during past decades, there are many more insect species with either medical (diseases vectors) or ecological (insect pollinators) importance whose olfactory systems are yet to be characterized. Recently, the first cryo-electron microscopy (EM) structure of insect OR co-receptor (Orco) has been identified from the parasitic fig wasp *Apocrypta bakeri* (Butterwick et al. 2018). However, insect olfactory receptors are heteromeric ligand-gated ion channels, which are composed of both specific ORs and the highly conserved Orco. Future studies that identify the cryo-EM structure of an insect OR•Orco heterotetramer will be beneficial for studies in the following two areas: (1) predicting the EM structures of undefined OR•Orco heterotetramers from the same or different insect species through structural bioinformatics studies; and (2) screening ligands for specific ORs from thousands of candidate compounds in a short time via computer modelling studies on the basis of the well-defined EM structures of their OR•Orco heterotetramers (Figure 7.1). This is an exciting time to be working in this field as future studies integrating the results from multiple disciplines are expected to boost our understanding of insect olfactory systems considerably.



**Figure 7.1** An illustration of interdisciplinary studies on insect olfactory systems.

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