Chemosensation in the Common Bed Bug, Cimex lectularius

by

Feng Liu

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Approved by

Nannan Liu, Chair, Professor of Entomology and Plant Pathology
Zhanjiang Liu, Professor of Fisheries, Aquaculture and Aquatic Sciences
Arthur Appel, Professor of Entomology and Plant Pathology
Derrick Mathias, Assistant Professor of Entomology and Plant Pathology

Abstract

As an ectoparasite on human beings and other animals, the common bed bug, *Cimex lectulatius*, has been resurgent worldwide and is of concern by both governments and general public for their biting nuisance and potential risk of disease transmission. The host-seeking or risk-avoiding behavior of bed bugs is mediated by their olfaction system in detecting attractive odorants or chemical repellents. Although the constituents of human emanations and chemical insect repellents have been thoroughly elucidated, we know little about which constituents are sensed and how they are recognized by the bed bug's olfactory system, such as olfactory receptor neurons (ORNs) or odorant receptors (ORs). In order to reveal the mechanisms involved in the chemoreception of bed bugs, the current study investigated the responses from olfactory receptor neurons and odorant receptor to both human odorants and chemical insect repellents, especially DEET.

Different types of olfactory sensilla were found to produce distinctive response profiles to human odorants and chemical insect repellents. Particularly, D types of olfactory sensilla possess the widest spectrum in detecting these odorants; E sensilla respond to very few odorants with low firing frequencies and C sensilla were only found to be sensitive to amines and several heterocyclics. Among all different chemical categories of stimuli, bed bug showed bias in detecting the aldehydes /ketones and alcohols and terpene-derived repellents but not the carboxylic acids and non-terpen-derived repellents. In addition, the dosages of

odorants were shown to be an important factor in determining the firing frequency and temporal dynamics of neuronal response in bed bugs.

To further decipher the molecular basis of neuronal responses to these odorants, bed bug ORs expressed in the *Xenopus* oocyte were challenged by odorants from both human being and chemical insect repellents. Each odorant receptor displayed variant tuning curves in response to these odorants and each odorant was encoded by multiple odorant receptors. Aldehydes/ketones, alcohols, heterocyclics and terpenes/terpenoides were more likely to activate bed bug ORs than carboxylic acids, which was consistent with the neuronal response bias. Moreover, dosage and chemical structure of odorants were two critical factors in influencing the responses of ORs.

DEET, one of the most successful synthetic insect repellents, showed very significant repulsive effects to the common bed bug. Dual roles of DEET (activating effect and interfering effect) on the olfactory responses of bed bugs were revealed in this study. ORNs housed in two types of olfactory sensilla (Dα and Dβ sensilla) and at least three bed bug ORs (OR20, OR36 and OR37) were identified to be activated directly by DEET. Meanwhile, it was also proven that DEET could block or mask the neuronal responses of bed bugs to certain odorants by interfering with the function of specific odorant receptors in response to certain odorants. Interestingly, those three DEET-sensitive odorant receptors were found to be even more sensitive to the terpenes/terpenoids, which were originally isolated from plants. Behavior bioassays further indicated that these terpenes/terpenoids were much more effective in repelling bed bugs compared to DEET, suggesting that DEET targeted on the bed bug receptors that were naturally sensitive to terpenes/terpenoids. Thus our finding provided a novel mechanism for DEET's function on insect olfactory systems.

Taken together, my study presented a global picture about the chemoreception of the bed bug to both human odorants and chemical insect repellents, which gave insight to mechanisms of odorant recognition in the bed bug peripheral olfactory system. Particularly, we clarified both the activating and interfering effect of DEET in repelling the common bed bug, which should benefit the development of new repellents in bed bug control.

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List of Abbreviations

AgOR	*Anopheles gambia*e odorant receptor
cDNA	complementary deoxyribose nucleic acid
CDS	coding deoxyribose nucleic acid sequence
ClOR	*Cimex lectularius* odorant receptor
cRNA	complementary ribonucleic acid
DDT	dichloro-diphenyl-trichloroethane
DEET	N,N-Diethyl-meta-toluamide
DMSO	dimethyl sulfoxide
DNA	deoxyribose nucleic acid
EAG	electroantennographic
EC_{50}	half maximal effective concentration
GC-MS	gas chromatography–mass spectrometry
GR	gustatory receptor
HBV	Hepatitis B virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
iGluRs	ionotropic glutamate receptors
IR	ionotropic receptor
JHA	juvenile hormone analogs
LST	long sharp trichoid sensillum
ORN	olfactory receptor neuron
OR	odorant receptor

ORCO	odorant receptor co-receptor
OBP	odorant binding protein
PCA	principal component analysis
PCR	polymerase chain reaction
PG	grooved peg sensillum
P450	cytochrome P450 monooxygenase
SBT	short blunted trichoid sensillum
RT-PCR	reverse transcription polymerase chain reaction
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
RU	response units
SSR	single sensillum recording
SEM	scanning electron microscopy
TEM	transmission electron microscopy
VUAA1	2-(4-ethyl-5-(pyridin-3-yl)-4H-1,2,4-triazol-3-ylthio)-N-(4-ethylphenyl)acetamide

Chapter 1: Literature Review

1.1 General biology of the common bed bug, *Cimex lectularius*

1.1.1 Morphology

The common bed bug, *Cimex lectularius*, (Cimicidae: Hemiptera) possesses three pairs of legs, two dark compound eyes, a short, flat head and no wings. The length of whole body is around 5 to 7 mm with light- to red-brown color. Before taking blood meals, the bed bug body is flat while after taking blood meals, the whole body can be greatly enlarged and become globular.

The common bed bug is among ~7 species that feed on humans and is distributed worldwide. The common bed bug adapts well to human environments and typically lives in temperate climates. There are also other related pests that resemble bed bugs in habits and appearance, including the tropical bed bug (*Cimex hemipterus*) (Boase 2001), bat bug (*Cimex adjunctus* or *Leptocimex boueti*) (Heukelbach et al, 2009), Mexican chicken bug (*Haematosiphon inodora*), barn swallow bug (*Oeciacus vicarius*) (http://extension.colostate.edu/docs/pubs/insect/05574.pdf). Among these pests, the tropical bed bug closely resembles the common bed bug even though they are more likely to be found in tropical or warmer regions including southern states of the USA and Mexico. Morphologically, the major difference between the common bed bug and tropical bed bug is in the shape of the pronotum. The front of the pronotum of the tropical bed bug is moderately excavated, while it is deeply excavated for the common bed bug. Although both species are able to mate between males and females, their offspring tend to be sterile. The differences between the common bed bug and bat bug lie in that a bat bug has a fringe of hairs on its pronotum longer than or equal to the width of its eye, while that of the bed bug is shorter than the width of the eye.

1.1.2 Pathophysiology

Mouthparts of the common bed bug are modified for piercing skin and sucking blood from damaged tissue or directly inserting their tip into a capillary (Elston and Stockwell, 2000; Burnett et al., 1986). Anticoagulants and other active substances are typically found in the biting sites of bed bugs, where they withdraw blood and liquefied epidermal tissues. The clinical reaction to bed bug bites substantially varies between individuals, depending on people's immunocompetence and the degree of previous exposure. Bed bug bites may not be felt initially, but hours later intensive itching may occur. Clinical symptoms include wheal-and-flare response, infiltrated papules, vesicles, or blisters (Sansom et al., 1992; Alexander, 1994).

Besides the biting nuisance, secondary bacterial infections may occur after repeating scratching and excoriation, such as impetigo, ecthyma, cellulitis and lymphangitis (Burnett et al., 1986). Another very important concern is about the potential ability of bed bugs to transmit pathogenic microorganisms that cause disease. Studies indicate that bed bugs are capable of hosting pathogens. For example, bed bug samples collected from northern Transvaal, South Africa, Senegal, Egypt, Ivory Coast, and China were found to be hepatitis B surface antigen positive (Will et al., 1977; Jupp et al., 1978; Hu et al., 1984; El-masry and Kotkat, 1990; Brotman et al., 1973). Bed bugs also tested positive for hepatitis B surface antigen for at least 7.5 weeks but virus replication did not occur after feeding (Ogston et al., 1979; Jupp and McElligott, 1979). Some other studies using PCR (polymerase chain reaction) and Southern hybridization also found that bed bugs were positive for hepatitis B virus (HBV) DNA up to 35 days or 6 weeks after feeding on infected blood from separated studies of Blow et al. (2001) and Silverman et al (2001). Despite their capacity in hosting these pathogens, no study has ever demonstrated bed bug's ability for their transmission (Goddard and deShazo 2009). A previous study aiming to investigate the bed bug's ability to transmit

HBV failed in chimpanzees (Jupp et al., 1991). In that experiment, no chimpanzee got infected after being fed by HBV-infected bed bugs. But when the same HBV sources were injected into the chimpanzees, HBV infection was found to be quickly followed (Jupp et al., 1991). However, a very recent study conducted by research groups of University of Pennsylvania found that bed bugs can efficiently and bi-directionally transmit *Trypanosoma cruzi*, the organism responsible for American trypanosomiasis also known as Chagas' disease (Salazar et al., 2014), to the host mice in the laboratory environment. The investigators found that most bed bugs fed on experimentally infected mice acquired parasites and a majority of previously uninfected mice became infected after cohabitation with exposed bed bugs. Moreover, *T. cruzi* was transmitted to mice after directly exposing to the feces of infected bed bugs, which suggested that the common bed bug may be a competent vector of *T. cruzi* and would post a risk for the transmission of Chagas disease.

1.1.3 Feeding biology

Compared to other haemophagous arthropods, like mosquitoes and ticks (Lehane 1955; Blagburn and Dryden, 2009), bed bugs have a relatively narrow choice of hosts (Reinhardt and Siva-Jothy, 2007), even though bed bugs can also parasitize multiple animals besides human beings, such as the bat, cat, cow, dog, guina pig, hare, mouse, rat, monke, rabbit, duck, goose, hen, pigeon, sparrow, starling and swallow (Rivnay, 1930). Nevertheless, as an urban pest, *C. lectularius* most commonly feeds on human beings while surviving well on other blood sources, like rabbit and chicken blood (Romero et al., 2007; Liu et al., 2014). Generally, the bed bug prefers taking blood meals at night on sleeping hosts even though daytime feeding is also frequently conducted in the laboratory. After full engorgement, bed bugs will move to harborages or shelters for digesting the blood meal.

In order to reach blood sources, random searching with low efficiency is very common phenomenon of bed bugs observed by researchers (Kemper 1936), even though sometimes

the human hosts were found to be undetected in rooms for weeks (Kemper 1929; Marx 1955). Previous study also reported that bed bugs could find hosts as far away as 1.5 m (Marx 1955). In this host seeking process, heat, host kairomones and carbon dioxide are all considered to be very important cues used by bed bugs to locaate human hosts (Weeks et al., 2010). Studies showed that bed bugs could differentiate temperature differences within 1-2 degrees centigrade by their temperature sensors on the antennae (Sioli 1937). Kairomones, for instance, dried human sweat, sebaceous gland material and dried ear secretions, were found to influence their reaction before host contact, which could elicit proboscis extension (Sioli 1937). Some host compounds, like butyric acid, xylol, naphthalene, kerosene, ethanol, and ammonia, however, showed repulsive effects to the bed bugs (Aboul-Nasr and Erakey 1968; Rivnay 1932). A recent study revealed that the initial feeding process of bed bugs was stimulated by blood constituents (Romero and Schal 2013). In this study, they used a membrane-based feeding system to identify chemicals that stimulated acceptance and engorgement responses in various life stages of the common bed bug. They found that adenosine triphosphate was the most effective phagostimulant in adults and nymphs, resulting in >70% of bed bugs fully engorging. ATP was more stimulatory than ADP, which was more effective than AMP. The feeding assays with physiological levels of other blood constituents such as D-glucose, albumin, globulin, cholesterol and mixtures of vitamins and amino acids showed no engorgement stimulation, which suggested that adenine nucleotides are the most important feeding stimulants in bed bugs. This study will contribute toward the development of artificial diets for bed bug rearing and for the development of alternative methods to eliminate bed bug infestations.

1.1.4 Chemical ecology

Semio-chemicals, like host emanations, repulsive volatiles and pheromones (e.g. sex, alarm and aggregation pheromones) play key roles in host seeking, mating, and risk avoidance of

bed bugs. So far, no sex pheromones have been identified in the common bed bug. Previous observation found that male bed bugs will move to any moving bed bug-sized objects, no matter male, female or nymph. Moreover, male bed bugs were found to readily mount and attempt to inseminate even washed dead female bed bugs (Rivnay, 1933), which suggested that it may be unnecessary for females to generate sex pheromone to achieve successful mating. Moreover, sex pheromone production was considered to be costly. The strategy (no sex pheromone production) applied by bed bugs is likely energy-saving and fitness-benefiting.

The alarm pheromone has been isolated using GC-MS analysis by extracting chemicals from the scent glands (Levinson et al., 1974). Two chemicals, (E)-2-octanal and (E)-2-hexanal, were identified as the major components of the bed bug alarm pheromone, which were further confirmed in a behavior test (Benoit et al., 2009; Ryne, 2009). When in a state of alarm, bed bugs released the alarm pheromone from their scent glands to alert conspecifics, which resulted in a fast dispersal from the site of production (Levinson and Bar, 1971). Another previous study also indicated that the behavior threshold for (E)-2-octanal and (E)-2-hexanal is 6×10^{15} and 9×10^{14} molecules/ml, respectively (Levinson et al., 1974). The application of alarm pheromone in controlling bed bugs has been conducted in the lab (Benoit et al., 2009). In this study, when (*E*)-2-hexenal and (*E*)-2-octenal were applied with Dri-die (silica aerogel), water loss increased three folds for the bed bug, which reduced the survival time from 4 days to 1 day. On the other hand, when the alarm pheromone was added into the diatomaceous earth, the effectiveness of diatomaceous earth also showed an increase (Benoit et al., 2009).

For bed bug nymphs, they emit certain nymph-specific alarm pheromone, which contains four chemicals, (*E*)-2-hexenal, (*E*)-2-octenal 4-oxo-(*E*)-2-hexenal and 4-oxo-(*E*)-2-octenal, from their dorsal abdominal glands (Harraca et al., 2010). Harraca et al. (2010) revealed that

nymph-specific alarm pheromone was very important in intraspecific communication of bed bugs. Their experimental results indicated that bed bug nymphs would have the same chance of mating with sperm transfer as females if they failed to release the nymph-specific alarm pheromone. It had been reported that the aldehydes and 4-oxo-(*E*)-2-hexenal were detected by ORNs housed in D and C sensilla. The behavioral experiments showed that application of 4-oxo-(*E*)-2-hexenal or the two aldehydes at a nymph-emitted ratio, could decrease mating frequency of male/female pair during mounting initiation. The mating rate of male/female pair will be comparable to that of a male/nymph pair. These findings suggested that alarm pheromones can reduce the risk of nymphal mating by male bed bugs (Harraca et al., 2010).

C. lectularius were observed aggregating within refugia and returning to harborages after blood feeding (Kemper, 1936), which was considered to be the effect of a specific 'nest odor' (Marx, 1955). The "nest odor" was also called "assembling scent", which was proven to be perceived only by contact chemoreception (Siljander et al., 2007). In dual-choice laboratory experiments conducted by Siljander et al. (2007), they found that juveniles preferred juvenile-exposed paper discs to control discs while males and females preferred male-exposed paper discs to control discs. In addition, none of the juveniles, males, or females liked female-exposed discs more than the control discs. It was suggested that male- and juvenile-specific contact pheromones which shared the same aggregation phenomenon of conspecifics, may have opposite functions of marking safe shelters for juvenile development and growth or adult mate encounters (Siljander et al. 2007). A continuous study from Siljander et al. (2008) tried to identify the major components in airborne aggregation pheromone, which was thought to play the same role as the contact aggregation pheromone in triggering aggregation behaviors in the common bed bug. By analyzing chemical components of the volatiles from experimental *C. lectularius* harborages with liquid chromatography, and bioassaying in dual-choice, still-air olfactometer experiments, 10 compounds (nonanal,

decanal, (E)-2-hexenal, (E)-2-octenal, (2E, 4E)-octadienal, benzaldehyde, (+)- and (−)-limonene, sulcatone, benzyl alcohol) were shown to be essential components of the *C. lectularius* airborne aggregation pheromone (Siljander, 2008).

However, a very recent study by Gries et al. (2015) revealed different chemical components of bed bug aggregation pheromone from the exuviae and feces, which are both present in natural bed bug shelters and have been associated with arrestment behavior. They reported that the bed bug aggregation pheromone comprises five volatile components (dimethyl disulfide, dimethyl trisulfide, (E)-2- hexenal, (E)-2-octenal, 2-hexanone), which attract bed bugs to safe shelters, and one less-volatile component (histamine), which causes their arrestment upon contact. Behavior bioassays indicated that a blend of all six components is very effective in attacting bed bugs into traps.

Although (E)-2- hexenal and (E)-2-octenal were shared in both research result from Siljander et al. (2008) and Gries et al. (2015), the other chemical components are quite different in either studies. This different may lie in the different sampling methods and different stages of bed bugs they collected. However, further study on the neural responses of bed bug olfactory sensilla to these chemicals in both pheromone panels would testify which are the most effective components in bed bug aggregation pheromone.

Another study by Olson et al. (2009) tried to investigate the factors that influence the detectionof bed bugs to the aggregation pheromone in the refugeium They used multiple choice assay to determine differences in aggregation behavior among two strains (established and recently derived strains), multiple life stages (adults and nymphs of different instars), and levels of starvation. They found that there were no differences between established and recently derived strains, or among adults and nymphs of different instars in level of aggregation. The aggregation intensity did decrease after feeding, but preference for

previously stained disks remained very high. They also found that the removal of the pedicel significantly reduced aggregation compared to intact bugs while removal of proboscis or the distal antennal segments didn't show any significant effect on the levels of aggregation. This finding suggested that some key sensory structures on the pedicel may play an essential role in the detection of aggregation odors from the strained disks or refugium. To identify these special aggregation-related sensory structures, a further study conducted by Olson et al. (2014) used Scanning electron microscopy (SEM) to get a full map of sensilla on the bed bug pedicel. They found that serrated hairs were distributed throughout the pedicel which was predicted to be involved in mechanic sensing; but smooth hairs, which were newly described, were present mainly on the distal half. Additionally, a patch of grooved pegs, smooth pegs and immersed cones was shown on the posterior end of the pedicel, which was only found in adults; and they observed cuticular pores in both types of peg sensillum using transmission electron microscopy (TEM), which indicated that the pegs possess olfactory function in their neurons. The smooth hairs were predicted to be similar to gustatory sensilla previously described in *Cimex hemipterus* F. Thus, Olson et al. (2014) concluded that the existence of both olfactory and gustatory sensilla on the pedicel gave strong evidence that those sensilla may play a key role in the sensory basis of off-host aggregation behavior in the common bed bug.

1.1.5 Integrated management

The common bed bug is an indoor pest that is very difficult to control. Just like some other urban insects, physical and chemical managements are the major ways to manage bed bug infestations. For the physical control method, heat steaming is one of the common ways used to remove bed bugs indoors, especiallyplaces infested with a large population of bed bugs. Although this method is very effective in killing large numbers of bed bugs quickly, there are disadvantages, including that the process is time consuming and has high cost. Moreover,

sometimes the eggs cannot be killed thoroughly, which greatly weakens the control efficiency since another bed bug infestaion will come back eventually.

The chemical management of bed bugs includes both chemical repellents or traps and insecticides. Basically, either chemical repellents or traps are olfaction-based management strategies. Some chemical repellents which were originally used for mosquito control were also applied for bed bugs. Different chemicals displayed very different repulsive effects for bed bugs. Previous studies indicated that DEET showed a high level of repellency for bed bugs with more than 94% of the bugs being repelled by DEET for 9 hours at a dose of 10% (Wang et al., 2013). The mechanisms involved in the function of DEET have been extensively investigated in *Drosophila* and mosquitoes. Two types of mechanisms have been suggested in the studies of these insects. The first is that DEET can interfere with insect olfactory sensory neurons or odorant receptors to block the odor recognition (Ditzen et al., 2008; Pellegrino et al., 2011). Another mechanistic hypothesis is that DEET repels insects by activating the olfactory neurons which results in aversion behavior (Syed and Leal, 2008; Xu et al., 2014).

For the first type of mechanism, Ditzen et al. (2008) found that DEET blocked olfactory neuronal responses to attractive odors in both *Anopheles gambiae* and *Drosophila melanogaster*. DEET also showed inhibition to the behavioral attraction to food odors in *Drosophila*, which required the highly conserved olfactory co-receptor OR83b or ORCO. Studies by Pellegrino and co-workers (2011) further proved that DEET functioned as a modulator of the insect odorant receptor complex (OR and ORCO). This modulation effect may either potentiate or inhibit odor-evoked activity and inhibit the suppression of spontaneous activity. The effect depended on the specific odorant receptor and the concentration and identity of the odor ligand.

For the second mechanism, odorant receptors have been identified in the study of mosquito. Xu et al., (2014) demonstrated that DEET and three other typical chemical repellents (picaridin, insect repellent 3535, and p-menthan-3, 8-diol) activated the odorant receptor CquiOR136 in the southern house mosquito, *Cx. quinquefasciatus*. They revealed that the reduction of CquiOR136 transcript level led to a significant decrease in electroantennographic (EAG) responses to DEET and a complete lack of repellency. Thus, they concluded that direct activation of an odorant receptoris necessary for DEET reception in *Culex* mosquitoes.

Taken together, these studies uncovered modes of action for DEET in repelling fruit flies and mosquitoes. Regarding the wide spectrum and high efficiency of DEET in repelling insects, we proposed that probably both indirect and direct mechanisms co-exist in the function of DEET. Further studies in testing both mechanisms in the same insect species would provide a straight-forward answer to this question.

In contrast to the well-studied mechanisms involved in the repelling effect of DEET in mosquitoes and fruit flies, very few studies have examined the role of DEET in bed bugs. Previously, Liu and co-workers (2013) reported that the olfactory neurons housed in the bed bug olfactory sensillum showed no responses to DEET at the dose of 100-fold dilution (v/v). A previous study by Kumar et al. (1995) showed a disturbing effect of DEET on the sensory ability of the tropical bed bug (*C. hemipterus*) to rabbit attractants. For the mechanisms involved in the function of DEET on bed bugs, further studies on the interaction between bed bug odorant receptors and DEET will be required.

Compared to chemical repellents, insecticides are much more effective and faster in the management of bed bug, especially in urban areas (Hwang et al., 2005). Pyrethroids are the major group of insecticides used in bed bug control (Moore and Miller, 2006). A laboratory

investigation of the toxicity of pyrethroids to both a laboratory strain and field strains of bed bugs indicated that the field strains exposed to deltamethrin had much longer LT_{50} (lethal time with 50% of death in the population) value compared with a laboratory strain, which suggested that the field strain was less susceptible to deltamethrin compared to the laboratory strain (Moor and Miller 2006). Another study also showed that bed bug populations collected from human dwellings in Kentucky and Ohio possessed extremely high levels of resistance to two pyrethroid insecticides, deltamethrin and λ-cyhalothrin, relative to a susceptible colony (Romero et al., 2007). All these studies suggested that although pyrethroids were effective for killing the laboratory strains of bed bugs, significant resistance in the field had developed.

With the extensive use of insecticides for bed bug control in different regions across the U.S., the mechanism involved in the insecticides, especially pyrethroids, resistance has always been an interesting question for researchers. The mechanisms involved in pyrethroid resistance mainly included (1) increased metabolic detoxification by P450s, glutathione transferases, and esterases (Feyereisen 2005), and (2) decreased target-site sensitivity of voltage-gated sodium channels (Dong, 2007). The voltage-gated sodium channel is the primary target of pyrethroids and DDT, which is very important for the generation and propagation of action potentials in neurons (Goldin, 2003; ffrench-Constant et al., 2004). Mutations of sodium channels lead to pyrethroid resistance by reducing the binding affinity of pyrethroids to the sodium channel. Yoon et al. (2008) reported two point mutations (V419L and L925I) identified from a highly deltamethrin-resistant population of bed bugs, both of which were considered as the major factors that resulted in deltamethrin resistance in bed bugs. Further investigation on the distribution of these two mutations in 110 bed bug populations collected in the United States showed that 88% of the bed bug populations showed target-site mutation, suggesting the deltamethrin resistance for the sake of target-site insensitivity was widespread among bed bug populations across the United States (Zhu et al.,

2010). Another study also indicated that the bed bug evolved a unique resistance strategy, namely reduced cuticle penetration for pyrethroids (Zhu et al., 2013). In their study, Zhu and colleagues identified 14 molecular markers associated with pyrethroid resistance through transcriptome analysis and they found that most of the resistance-associated genes involved in diverse mechanisms were expressed in the epidermal layer of the integument, which could protect the toxin from reaching the target sites on neurons. Therefore, multiple mechanisms may underlie insecticide resistance in bed bugs, and a comprehensive strategy must be applied for resistance management of this pest.

In addition to using insecticides in bed bug control, juvenile hormone analogs (JHAs), which are relatively harmless to non-arthropods and show effectiveness against cockroach, mosquito and some stored-product pests, were also tested on the bed bug in the laboratory. According to the study of Goodman et al. (2013), two JHA products (Gentrol® and Precor®) were tested for efficacy against various bed bug stages as direct spray or as a dry residue. The results showed that while at 1× and 2× the label rate, Precor® had no significant effect on the development or fecundity of bed bugs and at 2× the label rate, confinement to residues of Gentrol® had no significant effect, but residues at 3× and 10× the label rate caused a reduction in fecundity and impaired development of bed bugs. They also found that field strains were more susceptible to the reproductive effects of Gentrol® than a long-maintained laboratory strain. Therefore, JHA products can act as good alternatives for traditional insecticides in bed bug control based on their inherent safety and special mode of action, although the commercial formulation of these JHA products need to be relabeled more accurately.

1.2 Olfactory physiology of bed bugs

1.2.1 Insect olfactory system

Olfaction plays a vital role for most insects in their host seeking, mate locating and risk

avoiding behaviors. Like the nose of human beings or animals, insects have evolved an elaborate olfactory system involving the antennae. There are usually olfactory sensilla of variant shapes distributed on the antennae. Volatile semiochemicals can disperse into olfactory sensilla through pores and are then bound and transported by odorant binding proteins (OBPs) in the sensillum haemolymph for delivery to olfactory receptors (ORs) on dendrites of olfactory receptor neurons (ORNs), which are located underneath the olfactory sensilla (Leal 2013). Besides the antennae, insects also use maxillary palps or labial palps to detect odorants. For example, mosquitoes use both antennae and maxillary palps to detect volatiles in their environment (Syed and Leal, 2007, 2009; Ghaninia et al., 2007). Fruit flies were found to utilize both antennae and labelum to respond to the odorants of both attractants and repellants (Hallem and Carlson, 2006; Zhang et al, 2013).

1.2.1.1 Odorant binding protein

The odorant-binding proteins (OBPs) are responsible for the transportation of semiochemicals across the haemolymph to odorant receptors on the neuron membrane. In *Culex* mosquitoes, it was found that knocking down one specific OBP gene (CquiOBP1) with the RNA interference (RNAi) technique, resulted in reducedsensitivity of mosquitoes to oviposition attractants (MOP, skatole and indole) in an EAG experiment (Pelletier et al., 2010). In the malaria mosquito, *Anopheles gambiae*, Biessmann et al. (2010) also found that when one OBP, AgamOBP1, was silenced, the olfactory receptor cells showed no response to indole in the absence of this OBP. Both studies provide direct evidence for the vital role of OBPs in odor detection.

Previous studies also showed that OBPs were involved in the selectivity of odorant receptors in response to stimuli, particularly in pheromone recognition. For example, a study carried out by Forstner et al. (2009), found that one silk moth receptor, ApolOR1, responded to all

three types of pheromones at nanomolar concentrations when the compounds were solubilized in dimethyl sulfoxide (DMSO). However, in presence of the subtype ApolPBP2, ApolOR1 responded to picomolar concentrations of the pheromone (*E, Z*)-6, 11-hexadecadienal. For another study conducted by Grosse-wilde et al. (2006), the authors found that pheromone receptors of *Bombyx mori*, BmorOR1 responds to both bombykol and bombykol dissolved in O. However, when one OBP was applied to solubilize these pheromone compounds instead of DMSO, BmorOR1 showed a response to bombykol but not to bombykal. Therefore, OBPs not only transport the hydrophobic odorants, but are probably also involve in the odorants selectivity of ORs.

However, successful studies using the *Xenopus* oocyte to express odorant receptor genes and generate proper responses when odorants are delivered without OBP's involvement caused uncertaintly about the exact role of OBPs. Examining the protocol of oocyte expression experiments indicated that DMSO, which was typically used for dissolving and transferring hydrophobic odorants to the active sites of odorant receptor complex, was probably playing similar role of OBPs in cellular environment.

1.2.1.2 Odorant receptors and co-receptor

Odorant receptors, which are the primary target of odorants in the environment, have shown to be responsible for odorant recognition on the olfactory neurons housed in the olfactory sensilla. Semiochemicals transported from the OBP bind to specific odorant receptors and render open the ion channels comprised by the odorant receptor (OR) and co-receptor. The insect odorant receptor co-receptor was first identified from *D. melanogaster* (Larsson et al., 2004), namely DmelOr83b. Since then, orthologues of DmelOr83b have been identified from many other insects like silk moths (Krieger et al., 2002) and mosquitoes (Hill et al., 2002) which were named OR2 and OR7, respectively. Because DmelOr83b, OR2, and OR7 are

highly conserved in both amino acid sequences and biological functions (Nakagawa et al., 2012), a unified nomenclature was assigned for all of them, namely odorant receptor co-receptor (ORCO) (Vosshall and Hansson, 2011). For example, DmelORCO is a synonym of fruitfly OR83b and BmorORCO is the same name as silkmoth BmorOR2. Recent studies indicated that ORCOs were involved in the delivery of ORs to ORN dendrites and formed heteromeric complexes with other ORs (Neuhaus et al., 2005), therefore enhancing odorant responses without changing ligand specificity when co-expressed with ORs (Benton et al., 2006). Interestingly, another study also found that ORCO was not only involved in chemosensory-dependent behaviors, but also found to be localized to the flagella of *A. gambiae* spermatozoa (Pitts et al. 2014). Pitts et al. (2014) explored the possibility that AgORs mediate responses of spermatozoa to endogenous signaling molecules in the malaria vector, *An. gambiae* and the yellow fever mosquito, *Aedes aegypti*. When ORCO-specific agonists, antagonists, and other odorant ligands were applied in the treatments, robust activation on flagella beating was observed in an ORCO-dependent process. Moreover, ORCO has been found in testes, which is pretty common across distinct insect taxa (Pitts et al., 2014).

Studies on insect olfactory physiology have mostly focused on the model insects *Drosophila*, silk moth and mosquitoes. In *Drosophila*, the odorant receptors have been mapped to each neuron located in common or separate olfactory sensilla. Hallem et al. (2006) investigated the odor coding profiles of each receptor with a chemical panel of over 100 odorants and found that individual receptors range along a contimuum from narrowly tuned to broadly tuned. They also pointed out that the broadly tuned receptors were most sensitive to structurally similar odorants and observed widely spreading inhibitory responses to the odorants among fruit fly receptors. In addition, the temporal dynamics of the receptor repertoire were characterized which provided a rich representation of odor quality, quantity, and duration.

Moreover, they constructed a multidimensional “odor space” based on the responses of each individual receptor and found that the positions of odors depend on their chemical class, concentration, and molecular complexity, which as they stated, provided a basis for predicting behavioral responses to odors.

Similarly, Wang et al. (2010) carried out a systematic functional analysis across the *An. gambiae* odorant receptor (AgOR) repertoire. Thirty-seven odorant receptors of *A. gambiae* were expressed in the *Xenopus* oocytes and the current responses of each odorant receptor to specific odorants were recorded with a two-electrode voltage clamp. The results showed that each AgOR generated a distinct odor-response profile with very diverse tuning breadth. Several AgORs were identified to respond robustly to a range of human emanations that may play important roles in the anopheline host seeking process. AgOR responses were analyzed further by constructing a multi-dimensional odor space which displayed the Euclidean distance between odorants related to both chemical class and concentration.

Almost at the same time, another study conducted by Carey et al. (2010) functionally characterize the *An. gambiae* odorant receptor (AgOR) repertoire by expressing the mosquito ORs in the “empty neuron” of *Drosophila* by way of transgenic techniques and then using single sensillun recording to decode the odorant reception of each OR in the malaria mosquito. They found that mosquito receptors expressed in *Drosophila* “empty” neurons respond strongly to components of human odors which may act in the process of human recognition. In agreement with Wang et al (2010) found within the *Xenopus* expression system, some of the odorant receptors were narrowly tuned, and some salient odorants elicited strong responses from only one or a few receptors. When comparing responses of *An. gambiae* receptors with those of *D. melanogaster*, they found that odorants are differentially encoded by these two species in accordance with their ecological needs, like feeding preference.

1.2.1.3 Ionotropic receptors

Ionotropic glutamate receptors (iGluRs) constitute a novel family of chemosensory receptors, which mediate neuronal communication at synapses throughout vertebrate and invertebrate nervous systems. Ionotropic receptors (IRs) are iGluR-related genes, which are unlike the well described kainate, AMPA, and NMDA classes of iGluRs in that IRs (1) lack their characteristic glutamate interacting residues but have divergent ligand-binding domains and (2) accumulate in sensory dendrites but not at synapses (Benton et al., 2009). A phylogenetic study revealed that IR/iGluR related proteins were conserved across bacteria, plants, and animals, which suggested an evolutionarily ancient mechanism for detecting chemical stimuli through this receptor family (Benton et al., 2009). The first family of IR genes was identified from *D. melanogaster*, including 61 predicted genes and 2 pseudogenes (Benton et al., 2009). In *Drosophila*, each coeloconic olfactory sensory neuron appears to express combinations of several IRs from a repertoire of antennal IR genes, which do not express either insect odorant receptors (ORs) or gustatory receptors (GRs) (Benton et al., 2009). Studies showed that IRs were responsible for responding to organic acids, amines and alcohols in coeloconic OSNs (Benton et al., 2009; Ai et al., 2010). Among all the IRs described in *Drosophila*, IR8a and IR25a were highly conserved across different species and considered to function as co-receptors with other IRs in mediating the olfactory responses to semiochemicals, which resembled the role of ORCO in basiconic and trichoid OSNs (Croset et al., 2010).

1.2.2 Bed bug olfactory system

1.2.2.1 Bed bug antennae

For the common bed bug, olfactory sensilla were only found to be located on the antennae (Steinbrecht and Muller, 1976; Singh et al., 1996). Both antennectomy and electrophysiology studies have been conducted to reveal the function of bed bug antennae involved in olfaction

(Aboul-Nasr and Erakey 1967a, b, 1968; Levinson et al., 1974; Olson et al., 2009; Parashar et al., 2003). Each antenna has four segments, a scapus, a pedicellus and a two-segmented flagellum. The terminal flagellum segment has high sensillum diversity, including olfactory sensilla and bristle-like sensilla. The scapus is covered in bristlelike sensilla, with contact chemoreceptive or mechanoreceptive function (Levinson et al., 1974). Two olfactory regions have been identified on the internal and external edge of the terminal flagellum segment, respectively (Levinson et al., 1974; Harraca et al., 2010). Levinson et al. (1974) pointed out that when the terminal flagellum segment was deprived from the antennae, both male and female bed bugs failed to respond to the alarm pheromone and their assembling scent (aggregation pheromone). However, Olson et al. (2014) indicated that removal of flagellomeres did not affect aggregation, but removal of the whole pedicel or its distal half significantly reduced aggregation and they further identified olfactory sensilla that were related to off-host aggregation on the distal half of the pedicel.

1.2.2.2 Bed bug olfactory sensilla

The first study of the bed bug olfactory sensilla was conducted by Levinson and co-workers in 1974. They systematically described seven types of sensilla on the terminal flagellum segment, with four of them have a porous cuticle, a prerequisite for olfactory function, and were known as types C, D, E1 and E2. The number and distribution of these sensilla were found to be relatively constant and similar in both sexual forms, but differed slightly in the nymph. Another study using both scanning and transmission electron microscopy gave more detailed insight into the fine structure of these sensilla (Steinbrecht and Muller, 1976). Type D and E sensilla possess a simple wall and pores with pore tubules, while type C sensilla have a complex wall structure and spoke channels. Another type of sensillum, the immersed cones of type F has not been described previously. Different types of sensilla displayed varieties in diameter and length of dendrites. Type F sensilla were found to have a peculiar

dendrite innervation, with two dendrites wrapped closely together by a third flat dendrite. In the type D sensilla, dendrites were observed to be grouped in three to eight bundles by multiple sheaths.

While most of the studies focused on olfactory sensilla in the terminal segment of the flagellum, five types of sensilla were recently identified from the pedicel, with two of them (C, D) predicted to have olfactory function and one of them (E3) proposed to have gustatory function (Olson et al., 2014). The existence of both olfactory and gustatory sensilla on the distal half of the pedicel was suggested to be related to the off-host aggregation behavior of bed bugs.

1.2.2.3 Olfactory responses to semiochemical

Electrophysiological technique was first used by Levinson et al (1974) to test the potential response olfactory sensilla to two major components of bed bug alarm pheromone, namely trans-oct-2-en-1-al or trans-hex-2-en-1-al. E sensilla were considered to be responsible for sensing the alarm pheromone (Levinson et al. 1974; Steinbrecht and Muller, 1976). The results showed that minimal concentration of trans-hex-2-en-1-al evoking a receptor potential of E sensilla was about 2×10^{10} molecules per ml air. E sensilla were found to respond also to hexan-1-al, but not to pentan-1-al, butan-1-al, trans-hex-2-ene, and trans-oct-2-ene. They concluded that at least six carbon atoms of chain length and a terminal carbonyl group were chemical-structure prerequisites for optimal odorant activity, and the presence of a Δ^2-double bond seemed to be non-essential for the generation of potential activity from alarm pheromone.

Studies conducted by Harraca et al (2010) reconfirmed the distribution of the 44 olfactory sensilla and identified 3 different sensillum types located at the distal tip of *C. lectularius* antennae by external morphology mapping, which was largely consistent with the previous

report from Levinson et al. (1974). With an electrophysiological characterization of the neuronal responses of each specific sensillum to a panel of relevant odorants, Harraca et al. (2010) further classified the six smooth peg sensilla of the bed bug into three distinct functional classes, namely Dα, Dβ, Dγ. They also found that all nine grooved peg sensilla responded specifically in a dose-dependent manner to ammonia, whereas dimethyl trisulfide, sulcatone, α-pinene, indole, and ethyl butyrate evoked dose-dependent responses within the six smooth peg sensilla. The potential responses of two types of hair-like sensilla (E1 and E2) to the chemical panel were also investigated in this study. E1 sensilla were found to display regular spontaneous activities, while E2 sensilla displayed irregular spontaneous activities in the recording. However, neither type showed any responses to the chemical panel used in the recording. Two major components of alarm pheromone, (*E*)-2-hexenal, (*E*)-2-octenal, were exclusively observed to evoke dose-dependent responses on the six smooth peg sensilla (Dα, Dβ, Dγ). However, neither of them elicited any firing responses on E1 and E2 senailla, which was opposite with the finding from Levinson et al. (1974). A possible explanation given by Harraca et al. (2010) was that other olfactory neurons in adjacent sensilla may contribute to the recorded reception potential, which was probably from the D sensillum.

Another study conducted by Harraca et al. (2012) investigated the potential odorant cues from human bodies implicated in the attraction of bed bugs. In this study, they used aeration extracts from human volunteers to assess the role of olfaction in host searching by bed bugs. Only five compounds were clearly detected by the ORNs housed in the D sensilla of bed bugs. In the still-air arena behavior test, these chemicals showed a dose-dependent repulsive effect. In addition, a higher propensity of local search behavior was found to be associated with human odors containing a lower ratio of sulcatone to C_7–C_{10} aldehydes. They concluded that human odor alone had a weak influence on the behavior of *C. lectularius* and proposed that human kairomones may have a significant impact on bed bug behavior in combination

with heat and carbon dioxide, which at that time were the only known attractive vertebrate cues used by bed bugs for host seeking.

1.2.2.4 Bed bug odorant binding protein, odorant receptor and co-receptor

With advances in DNA sequencing techniques and decreases in sequencing costs, bed bug olfactory genes have been elucidated from both transcriptomic and genomic data. For example, Hansen et al (2014) sequenced the bed bug antennae specific transcriptiome and found at least 12 putative odorant binding proteins and 50 putative bed bug odorant receptor genes showing partial coding length. The cDNA sequence of the bed bug ORCO gene was also determined after cloning and sequencing (Hansen et al., 2014). The phylogenetic tree based on amino acid sequences of insect ORCOs indicated that the bed bug shared a close relationship with another blood-feeding hemipteran, *Rhodnius prolixus.* Hansen et al., (2014) also found that the bed bug Orco was widely expressed in different parts of the bed bug including the antennae and sperm. With the treatment of an ORCO agonist, VUAA1, the pheromone-induced aggregation behavior was changed corresponding to specific dosages of VUAA1. In addition, the effect of VUAA1 on the motility of bed bug sperm was tested in their study. The results indicated that VUAA1 inactivated bed bug sperm activity, which suggested a role of ORCO in the bed bug's sperm motility.

Soon after the previous transcriptomic work, the whole genome sequence of the common bed bug was published. According to the annotation of coding DNA sequence (CDS), 11 OBP genes and 49 odorant receptors plus the co-receptor were identified from 14,220 genes (Benoit et al., 2016). Compared to the amount of odorant receptor genes from other phytophagous hemipterans, like pea aphid and stink bug, the OR genes from bed bugs are considered to be reduced, which may result from this species' obligate blood-feeding nature. Actually, another obligate blood-feeding insect, the tsetse fly, has a similar number of OR

genes to bed bugs, possibly because both of them possess moderately complex chemical ecology. However, both bed bugs and tsetse flies havemuch broader host ranges compared to the human body louse (*Pediculus humanus humanus*), which solely feeds on human blood. Interestingly, the number of ORs from the human body louse has been found to be much lower than that of bed bugs, which suggests a correlation between the complexity of chemical ecology and number of OR genes for specific insects.

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Chapter 2: Research Goal and Specific Objectives

2.1 The goal of research and objectives

To fill the gap in our knowledge of bed bug olfactory physiology involved in the host seeking and risk aversion, the long term goal of my research is revealing the cellular and molecular mechanisms of human odorant and chemical repellent reception in the common bed bug, which will provide valuable information for the development of new reagents (attractants and repellents) for bed bug control. To achieve our long-term goal, the following objectives will be addressed in this study: 1) Characterization of bed bug antennal olfactory responses to chemical insect repellents; 2) Deciphering the cellular mechanism involved in human odorant reception of bed bugs; 3) Decoding the molecular mechanism involved in human odorant reception of bed bugs; 4) investigating the cellular and molecular mechanisms involved in the repelling effect of DEET to the common bed bug.

2.1.1 Objective 1: Characterization of bed bug antennal olfactory responses to the chemical insect repellents

Insecticides, particularly the pyrethroid chemicals, have been considered the most efficient method of bed bug control (Yoon et al., 2008). However, insecticide resistance has developed in bed bugs after their consistent exposure to insecticides (Romero et al., 2007; Zhu et al., 2013). Chemical insect repellents, very important alternatives, provide a beneficial complementary strategy for bed bug control (Gillij et al., 2008; Jaenson et al., 2005). Previous studies have isolated many mosquito-targeted chemical repellents, mostly terpenes or terpenoids from plants, which are strongly volatile compared with some synthetic mosquito chemical repellents, such as, DEET, picaridin, insect repellent 3535, and p-menthan-3, 8-diol. Both botanic and synthetic chemical repellents were found to not only repel mosquitoes but also other arthropod species, like fruit flies, house flies, ticks and kissing bugs. Considering

the wide spectrum of efficacy of these chemical repellents among insects, investigating the antennal olfactory responses of bed bugs to chemical insect repellents will provide meaningful insights into bed bug-specific olfactory physiology, as well as general features conserved among insects, and will benefit the development of bed bug repellents.

In this study, we will use 54 commercially available chemical insect repellents, both botanic and synthetic, to stimulate different types of olfactory sensilla on the bed bug antennae. We hypothesized that different types of sensilla will present different response profiles to chemical insect repellents and the firing frequency and temporal dynamics of responses from olfactory receptor neurons (ORNs) housed in the sensilla will be chemical specific and dose-dependent. In addition, olfactory preferences may exist in the detection process of ORNs to these chemical repellents with different molecular structures.

2.1.2 Objective 2: Deciphering the cellular mechanism involved in the human odorant reception of bed bugs

Human odorants are considered to be very important cues in host location of blood-feeding insects, like mosquitoes, bed bugs and kissing bugs (Syned and Leal, 2009). Previous studies that analyzed the chemical components of human-skin emanations revealed up to 400 chemical compounds (Bernier et al., 1999). Some of them have proven to be used by some blood-feeding insects in searching for their preferred hosts. For example, 1-octen-3-ol, L-lactic acid and C3-C5 carboxylic acids are known to attract blood-feeding insects such as mosquitoes, biting midges,and tsetse flies (reviewed in Lehane, 2005). However, both behavioral and electrophysiological studies of the common bed bug did not show similar attraction of these chemicals (Anderson et al., 2009; Wang et al., 2009; Harraca et al., 2010). Therefore, bed bugs may use a different spectrum of human odors in searching for blood sources.

However, no matter what kind of chemical spectrum is utilized by bed bugs, they sense these chemical stimuli with the same olfactory system. They perceive different human odors via different types of olfactory sensilla, which house ORNs inside. Therefore, in this study, we will characterize neuronal responses of bed bug olfactory sensilla to different human odorants in multiple chemical categories. Based on these neuronal responses, we can decipher the coding mechanism of bed bug ORNs to a variety of human odorants, which will further benefit our understanding of bed bug olfactory physiology and development of new reagents, including novel attactants and repellents that may be exploited for bed bug control.

2.1.3 Objective 3: Decoding the molecular mechanism involved in human-odorant reception of bed bugs

In the process of insect chemoreception, chemical stimuli diffuse through pores on the surface of olfactory sensilla and dissolve in the hemolymph. These chemical stimuli are quickly bound by odorant binding proteins dispersed in the hemolymph. Then the odorant binding proteins will transport odorants to specific sites on odorant receptors, which are located in the neuronal membrane (Leal 2013). The odorant receptors and one functionally conserved odorant receptor co-receptor form cation channels, which are triggered to open with the binding of odorants. Action potentials are produced when the neurons are depolarized. Therefore, the odorant receptors are the major factor in determining the neuronal response to chemical stimuli. Moreover, studies have indicated that insects use different combinations of odorant receptors in recognizing different odorants (Wang et al., 2010; Carey et al., 2010). Thus, the odorant receptors are the decisive factors in the coding of odorant identity and intensity in the environment.

Although we still lack the annotation of bed bug odorant receptors from the whole genome, the bed bug odorant receptors and co-receptor genes have been partially identified from RNAseq

data of bed bug anntennae (Hansson et al., 2014). Hansson et al. (2014) identified a total of 50 putative odorant receptors from the bed bug antennae transcriptome, 30 of which possess full cDNA sequences. Therefore, in this study, we are going to characterize the function of these odorant receptors in recognition of human odorants using the *Xenopus* expression system, which may reveal the molecular mechanisms involved in odorant coding of bed bugs.

2.1.3 Objective 4: Investigating the cellular and molecular mechanisms involved in the repelling effect of DEET to the common bed bug

DEET, one of the most effective chemical chemical insect repellents in the world, played and is still playing a crucial role in the management of some blood-feeding pests and vectors of disease agents, like mosquitoes, kissing bugs, bed bugs and ticks (Brown and Hebert, 1997). Since DEET is so efficient in repelling certain insects and ticks, it is extensively marketed to the general public for protection against biting arthropods and the pathogens they may transmit. For the management of bed bugs, DEET has also been considered a good candidate. With regard to the high effectiveness of DEET in repelling msoquitoes, bed bugs and other insects, the mechanisms involved in DEET's function have been of interest to researchers. Currently, two mechanisms have been proposed from studies of Drosophila and mosquitoes. The first is that DEET may interfere with olfactory sensilla to block odor recognition (Dizen et al., 2008; Perigno et al., 2011). The second is that DEET repels insects by activating olfactory neurons, resulting in avoidance behavior (Syed and Leal 2008). For bed bugs, our previous study indicated that all types of olfactory sensilla on the bed bug antennae showed no responses to DEET at the dose of 1:100 v/v (Liu et al., 2014). However, studies of the Culex mosquito revealed that specific sensilla on the mosquito antennae only responded to high doses of DEET (Syed and Leal 2008; Liu et al., 2013). Thus we cannot rule out the possibility that bed bugs may also respond to high doses of DEET. To test this hypothesis and also to investigate whether other mechanisms are involved in the repelling effect of DEET to

bed bugs, we characterized the neural responses of olfactory sensilla to high doses of DEET and detected the possible interfering effect of DEET on the responses of bed bugs to human odorants. We also revealed the interaction of DEET and bed bug odorant receptors at the molecular level.

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Chapter 3: Characterization of Antennal Olfactory Responses to Chemical Insect Repellents in the Common Bed Bug, *Cimex lectularius*

3.1 Abstract

Populations of the common bed bug *Cimex lectularius* (Hemiptera; Cimicidae), a temporary ectoparasite on both humans and animals, have surged in many developed countries. Similar to other haematophagous arthropods, *C. lectularius* relies on its olfactory system to detect semiochemicals in the environment, including both attractants and repellents. To elucidate the olfactory responses of the common bed bug to commonly used chemical chemical insect repellents, particularly haematophagous repellents, we investigated the neuronal responses of individual olfactory sensilla in *C. lectularius'* antennae to 52 chemical chemical insect repellents, both synthetic and botanic. Different types of sensilla displayed highly distinctive response profiles. While C sensilla did not respond to any of the chemical insect repellents, Dγ sensilla proved to be the most sensitive in response to terpene-derived chemical insect repellents. Different chemical repellents elicited neuronal responses with differing temporal characteristics and the responses of the olfactory sensilla to the chemical insect repellents were dose-dependent, with an olfactory response to the terpene-derived chemical repellent but not to the non-terpene-derived chemical repellents. Overall, this study furnishes a comprehensive map of the olfactory response of bed bugs to commonly used chemical insect repellents, providing useful information for those developing new agents (attractants or repellents) for bed bug control.

3.2 Introduction

As a temporary ectoparasite, the development, reproduction and survival of the common bed bug *Cimex lectularius* L. (Hemiptera: Cimicidae) relies on the blood of hosts, including both humans and animals (Bartonicka and Gaisler 2007; Thomas et al. 2004). Although no virus

has yet been reported to be transmitted by *C. lectularius*, the biting nuisance generated by an infestation seriously affects hosts both physically and psychologically (Anderson and Leffler 2008). The introduction and widespread use of chemical insecticides, especially DDT, resulted in the gradual disappearance of bed bugs as a public concern by the end of the 1950s and it was eradicated completely in some developed countries (Romero et al. 2007). However, the reduced use or even prohibition of some insecticides, along with a relaxation in the monitoring and management of bed bugs, the increasing level of international travel, and the development of resistance to many insecticides, have led to a resurgence in bed bug populations worldwide, causing serious problems for public health (Romero et al. 2007; Doggett et al. 2004, 2012; Ter Poorten and Prose 2005; Yoon et al. 2008; Wang et al. 2013; Haynes and Potter 2013). Chemical repellents are now playing a role as an effective alternative for the control of insect pests to overcome the increasing problem of insect control, particularly the development of insecticide resistance (Mumcuoglu et al. 1996; Jones et al. 1996; Jaenson et al. 2005; Gillij et al. 2008). Botanical repellents, especially some terpene-derived chemicals, which are both effective and eco-friendly for insect control, have been used extensively to interrupt the host seeking process of blood-feeding insects or ticks (Peterson and Coats 2001; Bissinger and Roe 2010; Carroll et al. 2007).

Previous studies have indicated that insects detect both attractant and repellent semiochemicals through olfactory receptor neurons (ORNs) housed in the olfactory organs (Hallem et al. 2006; Wang et al. 2009; Hill et al. 2009; Anderson et al. 2009; Leal 2013). For example, *Culex quinquefasciatus, Aedes aegypti* and *Anopheles gambiae* all exhibit an olfactory neuronal response to a wild range of terpene-derived botanical repellents or human emanations (Qiu et al. 2006; Ghaninia et al. 2007; Syed and Leal 2008; Hill et al. 2009; Liu et al. 2013). In particular, *Cx. quinquefasciatus* shows a neuronal response to DEET, which is the most widely-used insect topical repellent in the world (Syed and Leal 2008; Liu et al.

2013). Kissing bugs, *Triatoma infestans*, also respond to odors from their vertebrate hosts (Guerenstein and Guerin 2001). Similar to mosquitoes and other blood-feeding insects, *C. lectularius* detect semiochemicals through ORNs in the olfactory sensilla on their antennae (Levinson et al. 1974; Harraca et al. 2010, 2012). The antennal sensilla in the bed bug are reported to consist of three types of olfactory sensilla, namely type C (grooved peg sensilla), type D (smooth peg sensilla), and type E (hair-like sensilla), with multiple ORNs presented in each sensillum (Levinson et al. 1974). The type D sensillum was later further characterized into Dα, Dβ, Dγ and the type E sensillum was categorized into E1 and E2, each with distinctly different response profiles to a chemical panel (Harraca et al. 2010).

Building on the work of Harraca et al. (2010), who tested the olfactory responses of different types of olfactory sensilla in the common bed bug to nearly thirty chemicals including 5 chemical repellents, this study conducts, for the first time, a systematic study characterizing the electrophysiological responses of olfactory sensilla in the common bed bug to 52 chemicals reported as repellents for different insects or ticks (citations in Table 3.1). Differential response profiles were found for each type of olfactory sensillum, accompanied by dose-dependent responses and different temporal characteristics. In particular, strong responses to terpene-derived chemical repellents were observed. This study provides important knowledge about the olfactory physiology of the common bed bug that will be particularly useful for those engaged in the early screening of new chemical agents (repellents or attractants) for bed bug control.

3.3 Materials and Methods

3.3.1 Insects

The *C. lectularius* colony utilized in this study originated from Ft. Dix, New Jersey, USA (Bartley and Harlan 1974). It is susceptible to pyrethroid insecticides (Romero et al. 2007). Bed bugs were fed with rabbit blood once every week in the lab. Blood was purchased from Hema

Resource and Supply Company (Aurora, OR). All common bed bugs were reared at 25±2°C under a photoperiod of 12:12 (L: D)

3.3.2 Scanning electron microscopy

Scanning Electron microscopy (SEM) experiments were conducted as described by Das et al. (2011). Individual female or male adult bed bugs were decapitated and the antennae excised from the antennal socket under a stereomicroscope (National Microscope, model Direct Current 3-420, Meiji, Japan). The antennae were submerged in tetrachloromethane (CCl_4) at room temperature overnight, after which they were transferred into a PCR tube (200 µl) containing CCl_4 and boiled for 20s, which was repeated 4 times with the CCl_4 renewed each time. The antennae were then dehydrated in a graded ethanol series of 30, 50, 70, 80, 95 and 99.9%, in each concentration for 1 h, and then in absolute alcohol for 15-20 min. The dehydration process was followed by critical point drying (EMX 850). Finally, the specimens were mounted onto aluminum stubs using carbon-coated double-sided sticky tape and sputter coated with gold (EMX 550X auto sputter coater). Samples were examined with an EVO 50 SEM (Carl Zeiss, Jena, Germany), and micrographs were taken of the antennae, flagellum antennomeres (flagellomeres), and sensilla.

3.3.3 Single sensillum recording

Adult bed bugs were randomly selected at least five days after blood feeding. The bed bugs (male or female) were anaesthetized (2-3 min on ice) and mounted on a microscope slide (76×26 mm) between 2 pieces of double-sided tape. Using double-sided tape, the antennae were fixed to a cover slip resting on a small ball of dental wax to facilitate manipulation. The cover slip was placed at a suitable angle to the bed bug head. Once mounted, the bed bug was placed under a LEICA Z6 APO microscope in such a way as to ensure that the antennae were visible at high magnification (×720). Two tungsten microelectrodes were sharpened in 10% KNO_2 at 2-10V to a ~1 µm tip diameter. The reference electrode, which was connected to the

ground, was inserted into the abdomen of the bed bug and the other electrode, which was connected to a preamplifier (10×, Syntech, Kirchzarten, Germany), was inserted into the shaft of the sensillum to complete the electrical circuit and to extracellularly record the olfactory receptor neuron potentials (Den Otter et al. 1980). Controlled manipulation of the electrodes was performed using two micromanipulators (Leica, Germany). The preamplifier was connected to an analog to digital signal converter (IDAC, Syntech, Germany), which in turn was connected to a computer for signal recording and visualization. Signals were recorded for ten seconds, starting one second before stimulation. As a high number of ORNs are co-located in each sensillum type, we were unable to distinguish individual ORN classes based on the shape and amplitude of the action potential response curve. Consequently, the total number of action potential spikes was counted off-line for a 500 ms period before and after stimulation. The number of action potential events after stimulation were calculated by subtracting the number of action potentials before stimulation and then multiplying by 2 to obtain the firing rate change in a single sensillum in spikes per second.

3.3.4 Stimulation and Stimuli

Forty eight chemical repellents from 6 chemical groups (carboxylic acids, esters, aldehydes, alcohols, terpenes, and terpenoids) and four additional chemical repellents that could not be classified in any of the above groups were used in this study (Table 3.1). Each of the chemical repellents was diluted in dimethyl sulfoxide (DMSO), which was confirmed as showing no stimulation in the single sensillum recording, to a stock solution with a concentration of 1:10 v/v. Subsequent decadic dilutions were made from each of the stock solutions. Ten microliters of each dilution was dispersed on a filter paper (3×10 mm) which was then inserted into a Pasteur pipette to create a stimulus cartridge. A filter paper wetted with the solvent alone served as the control. A constant airflow across the antennae was maintained at 1.2 l/min throughout the experiment. Purified and humidified air was delivered to the preparation

through a glass tube (10-mm inner diameter). The glass tube was perforated by a small hole, slightly larger than the tip of the Pasteur pipette, 10 cm away from the end of the tube. Stimulation was achieved by inserting the tip of the stimulus cartridge into this hole in the glass tube. A stimulus controller (Syntech, Germany) diverted a portion of the air stream (0.5 l/min) to flow through the stimulus cartridge for 500 ms to deliver the stimulus to the sensilla. The distance between the end of the glass tube and the antennae was ≤1 cm. At least ten replicates for each recording and each of the different chemicals were conducted on different individuals. The values of the spikes were obtained by averaging all the recordings for the response of each sensillum to each of the chemical repellents. Those sensilla that failed to show a response of ≥ 15 spikes/s were considered to be non-responders (de Bruyne et al. 2001).

3.4 Results

3.4.1 The response profiles of different types of sensilla to chemical repellents

To investigate the roles played by ORNs in bed bug sensilla in response to chemical insect repellents, 52 chemicals previously identified in behavioral studies with other insects (both synthetic and botanic in origin; citations in Table 3.1) were tested using single sensillum recording, at a dose of 1:100 v/v for each chemical. Consistent with the findings reported by previous researchers (Levinson et al. 1974; Harraca et al. 2010), C, Dα, Dβ, Dγ, E1 and E2 sensilla were identified on the antennae of both male and female *C. lectularius*, which share the same sensillum pattern on the antennae, using Scanning Electron Microscopy (SEM) (Fig. 3.1). All sensillum types were tested in this study for a response to each of the 52 chemical repellents by recording their firing frequencies. The results reveal that the C sensilla showed no response to any of the 52 chemical insect repellents in the study, suggesting that these sensilla may not be involved in the bed bug's response to the repellents tested.

The outcome was very different for D sensilla, where Dα, Dβ and Dγ all exhibited neuronal responses to a number of chemicals at concentrations of 1:100 v/v, especially those in the terpene and terpenoid categories (Fig. 3.2). The Dα sensilla showed neuronal responses (≥ 50 spikes/s) to terpenes, including β-caryophyllene (64 spikes/s), α-terpinene (53 spike/s) and terpenoids, (-)-caryophyllene oxide (82 spikes/s), methyl acetate (83 spikes/s), (+)-menthone (103 spikes/s), (-)-menthone (108 spike/s), (-)-α-thujone (169 spikes/s), camphor (169 spikes/s) and eucalyptol (240 spikes/s). For the Dβ sensilla, although none of the neuronal responses elicited in response to any terpene chemical exhibited a firing rate of more than 50 spikes/s, larger responses were seen for certain terpenoid chemicals, including citronellal (50 spikes/s), (-)-caryophyllene oxide (71 spikes/s), methyl acetate (54 spikes/s), (+)-menthone (77 spikes/s), (-)-menthone (71 spike/s), (-)-α-thujone (116 spikes/s), camphor (141 spikes/s) and eucalyptol (186 spikes/s). In particular, the Dβ sensilla responded to linoleic acid with a firing rate of 70 spikes/s, and to oleic acid with a firing rate of 32 spikes/s, both of which are thought to be components of the so-called “death stench” released by gregarious insects such as cockroaches, ants, termites, beetles, and bees (Rollo et al. 1995; Julian and Cahan 1999; Masterman et al. 2001; Ayasse and Paxton 2002; Worden and Parker 2005). The Dγ sensilla showed excitatory responses to all the terpene chemicals, with firing rates ranging from 64 spikes/s for terpinolene to 263 spikes/s for (-)-β-pinene. The Dγ sensilla also presented excitatory responses to many more chemicals in the terpenoid group than either the Dα or Dβ sensilla, including linalyl acetate (53 spikes/s), (+)-menthone (64 spikes/s), (-)-menthone (70 spike/s), geraniol (100 spikes/s), (-)-α-thujone (107 spikes/s), camphor (133 spikes/s), citral (182 spikes/s) and eucalyptol (227 spikes/s). The Dγ sensilla also showed slightly lower responses to linoleic acid and oleic acid, with firing rates of 48 and 35 spikes/s, respectively.

Since all olfactory sensilla have multiple neurons, especially the D types of sensillum with 8-20 neurons, we were unable to differentiate between neurons housed in the same sensillum

in response to chemical repellents based only on the shape or amplitude of their action potentials. However, we did observe differences in the responses of the same sensillum to chemical repellents, with some chemicals eliciting strong action potential spikes, while the spikes produced for others were much smaller. For instance, both (-)-α-pinene and citral produced a strong neural response (>1 mv) in the Dγ sensillum, while stimulation with geraniol and linoleic resulted in small amplitude (<1 mv) action potential spikes (Fig. 3.3). These results suggest that different neurons in the same sensillum responded to different chemical repellents.

Two types of E sensilla, E1 and E2, were identified on the bed bug antennae, with the E1 sensilla located close to the Dγ sensilla and the E2 sensilla distributed closer to the tip of the antennae (Fig. 3.1). We found that the E1 sensilla showed weak excitatory responses to several terpene chemicals, including terpilonene (52 ± 4.3 spikes/s), α-terpinene (25 ± 8.2 spikes/s), R-(+)-limonene (25 ± 3.4 spikes/s), S-(-)-limonene (28 ± 7.3spikes/s), and mycrene (27 ± 12 spikes/s), while E2 sensilla exhibited no excitatory responses to any of the chemical insect repellents (Fig. 3.4). This suggests that E sensilla may indeed be involved in the bed bugs' response to semiochemicals in the environment; no excitatory responses have previously been reported for either E1 or E2 sensilla (Levinson et al. 1974; Harraca et al. 2010).

3.4.2 Temporal dynamics of neuronal responses to chemical insect repellents

There is increasing evidence to suggest that the temporal structures of olfactory responses play a critical role in the odor coding of insects (Laurent et al. 2001; Hallem and Carlson 2006; Qiu et al. 2006; Ghaninia et al. 2007). In this study, we investigated the temporal structure of the primary chemical presentation by examining the firing frequencies of olfactory sensilla as a function of time. Firing frequencies were quantified over the course of

a 2 s period beginning at the onset of chemical stimulation. Responses were plotted onto a bar graph for every 100 ms interval. The results show that the temporal characteristics of ORNs in the olfactory sensilla are indeed stimulus specific. For instance, (-)-α-thujone (10 μg/μl) elicited a relatively phasic response, with short latency on the ORNs of the Dα and Dβ sensilla and almost no latency on the ORNs of the Dγ sensillum, which showed a sharp decrease in the firing rate once the stimulation ceased (Fig. 3.5). However, eucalyptol (10 μg/μl) elicited a tonic response, with a long latency on the ORNs of the Dα, Dβ and Dγ sensilla. In particular, the ORNs of the Dγ sensillum generated a prolonged response throughout the whole 2 s time interval, which was also observed in the response to α-(+)-pinene, α-(-)-pinene, β-(+)-pinene and β-(-)-pinene. In contrast with (-)-α-thujone and eucalyptol, camphor (10 μg/μl) elicited firing responses with a short latency on the ORNs of Dα, Dβ and Dγ sensilla after stimulation (Fig. 3.5). These differences in the temporal dynamics of the neuronal responses of the ORNs contribute to the initial representation of different chemical repellents, which will impact the subsequent representation in the bed bug's nervous system and, in turn, affect behavioral reactions (Laurent et al. 2001).

3.4.3 Dose dependent responses of olfactory sensilla to chemical insect repellents

Since the Dγ sensilla exhibited excitatory responses to a greater number of chemical repellents than any of the other types of sensilla in our test, a dose-dependent response study was performed on the Dγ sensilla to investigate whether the doses of chemical insect repellents play a role in the electrophysiological responses of the bed bugs. For this part of the study we chose a range of chemicals, most of which elicited strong responses in Dγ sensilla, and utilized a series of sequential 10-fold dilutions of each. In general, our results reveal that the bed bug Dγ sensilla exhibit a dose-dependent response to all the chemical repellents tested. Specifically, the α-pinene elicited a much stronger response to the of $1{:}10^3$ dilution (v/v) compared with α-terpinene and 1S-(+)-3-carene at the same concentration, even

though they share comparable firing rates at a dilution of 1:10 (v/v) (Fig. 3.6A). Both eucalyptol and camphor elicited neuronal responses that followed a dose-dependent pattern, but the eucalyptol was found to be much more efficient than camphor in eliciting excitatory responses on the ORNs of the Dγ sensillum, especially at low concentration levels (like $1:10^2$ v/v, Fig. 3.6B). Additionally, although β-caryophyllene generated only relatively mild and dose-dependent responses on the olfactory neurons of Dγ sensillum, these were still much more efficient than for its oxidized form, (-)-caryophyllene oxide (Fig. 3.6C). The Dγ sensillum was also observed to show dose-dependent responses to (-)-α-pinene, (+)-β-pinene, R-(+)-limonene and (-)-menthone (Fig. 3.7) and their corresponding stereoisomers. Here, Dγ sensilla showed quite similar responses to different stereoisomers of these chemicals, with no statistically significant difference at the dose range tested (Fig. 3.8), suggesting the capacity of the olfactory sensilla in the bed bug to detect different chemical structures but not to differentiate between isomers of the same chemical.

3.4.4 Olfactory response tendency of *C. lectularius* to chemical insect repellents

To investigate whether common bed bugs have any special tendency when detecting chemical insect repellents, we categorized the chemical repellents into three groups according to their chemical structures: terpene-derived repellents that originate from terpene $(C_5H_8)_2$; terpenoid repellents, which include the terpene esters, terpene alcohols, and terpene aldehydes; and non-terpene-derived repellents, including non-terpene derived carboxylic acids, alcohols, esters, and aldehydes. In total, there were 12 terpene repellents, 28 terpenoid repellents and 12 non-terpene-derived repellents (Table 3.1). The percentages of repellents in each group that elicited an excitatory response (≥ 50 spikes/s) in any bed bug sensillum were analyzed. The results indicate that almost 100% of the terpene repellents and 40% of the terpenoids elicited an excitatory response (≥50 spikes/s) from the olfactory sensilla of the common bed bugs, but only 8% of the non-terpene derived repellents did so (Fig. 3.9). This particular

tendency in the olfactory receptor neurons indicates that the common bed bugs are clearly more sensitive to terpene-derived chemicals (terpenes and terpenoids) than to non-terpene-derived repellents, suggesting that terpene-derived chemicals may be more likely to be detected or perceived by the bed bugs than non-terpene-derived chemicals, which is very important in developing new repellents or attractants for use in bed bug control.

3.5 Discussion

When examining the antennae of *C. lectularius* in the SEM, six different types of olfactory sensilla were observed, namely C, Dα, Dβ, Dγ, E1 and E2, which is consistent with the findings of previous research (Levinson et al. 1974; Harraca et al. 2010). A comparison of the olfactory sensilla of bed bugs with those of three mosquitoes, *Cx. quinquefasciatus*, *An. gambiae*, and *Ae. aegypti*, revealed morphological conservation of the olfactory sensilla in all of these blood-feeding insects. For example, the C sensilla of the bed bugs are close to the mosquito Grooved Peg sensilla (PG), while the D sensilla appear similar to the mosquito Short Blunted Trichoid sensilla (SBT), and the E sensilla are comparable to the mosquito Long Sharp Trichoid sensilla (LST). Their functional similarity in response to semiochemicals further supports the conservation of olfactory sensilla in these blood-feeding insects. For example, single sensillum recording results indicate that both the D sensilla (Dα, Dβ, Dγ) of the bed bug and the SBT sensilla of *Cx. quinquefasciatus* showed extremely strong responses to two terpenoid repellents, eucalyptol and camphor (Liu et al. 2013). In addition, both the bed bug Dγ sensilla and the *Cx. quinquefasciatus* SBT sensilla showed extremely strong responses to a number of terpene repellents, including α-terpinene, terpinolene, myrcene, α-pinene, (-)-α-pinene, (+)-α-pinene, (+)-β-pinene and (-)-β-pinene (Liu et al. 2013). The C sensilla of the bed bug and the PG sensilla of *Cx. quinquefasciatus* and *An. gambiae* were very sensitive to the human odorant ammonia (Qiu et al. 2006; Syed and Leal 2009; Harraca et al. 2010), and both the E sensilla of bed bugs and the LST sensilla

of *Cx. quinquefasciatus* and *Ae.aegypti* showed no or very weak response to all the semiochemicals tested (Ghaninia et al. 2007; Syed and Leal 2009; Hill et al. 2009).

The results of the single sensillum recording clearly demonstrate thatbed bugs showed particularly strong and consistent responses to terpenes and terpenoids compared to the non-terpene derived insect repellents. This is consistent with a previous study in the mosquito *Cx. quinquefasciatus*, which also revealed strong responses to terpene and terpenoid chemical repellents, the major components in the essential oils often used in mosquito control (Liu et al. 2013). A transgenic study of the odorant receptors on the olfactory neuron of *An. gambiae* mosquitoes indicated that citronellal, a major terpenoid component in Citronella Oil, strongly stimulates specific odorant receptors (Carey et al. 2010), implying that the specific odorant receptors on the olfactory neurons of mosquitoes are responsible for their responses to terpenes and terpenoids. Olfactory receptors in *An. gambiae* have also shown their ability to distinguish specific aromatic semiochemicals found in human sweat (Hallem et al. 2004; Carey et al. 2010; Wang et al. 2010). Since both bed bugs and mosquitoes are blood-feeding insects, similar feeding behaviors and olfactory sensilla types may suggest similar mechanisms for the neuronal response of both to chemicals in the environment. Further studies focusing on the interaction between specific odorant receptors and environmental chemicals in bed bugs could therefore yield interesting results.

DEET, one of the earliest synthetic chemical repellents, first marketed in 1956, is one of the most successful chemical repellents against medically important insects or ticks (Brown and Hebert 1997; Sudakin and Trevathan 2003). Although the mode of action of DEET has yet to be definitively determined (Dickens and Bohbot 2013), DEET is thought to interfere with the olfactory system to block host odor recognition (Ditzen et al. 2008; Pellegrino et al. 2011) or to repel insects by activating olfactory neurons that trigger avoidance behavior (Syed and Leal 2008; Liu et al. 2013; Kain et al. 2013). Previous studies on the interaction between Cx.

quinquefasciatus mosquitoes and DEET have indicated that there are special sensilla on the mosquito antennae that detect DEET at high doses in a direct way (Syed and Leal 2008; Liu et al. 2013). Olfactory neurons on the antennae of the Drosophila melanogaster have also been shown to be very sensitive to DEET (Syed et al. 2011, Kain et al. 2013). In contrast to the relatively well studied DEET repellency in mosquitoes and fruit flies, very little research on DEET repellency for the bed bug has been conducted, although Kumar et al. (1995) reported a disturbing effect of DEET on the sensory ability of the tropical bed bug (C. hemipterus) to human-odorant attractants. Previous studies on mosquitoes have also shown that DEET functions as an odorant antagonist to block the odorant-evoked currents mediated by odorant receptors (Bohbot et al. 2011; Bohbot and Dickens 2012), suggesting that DEET may have a disturbing effect for insects in response to human-odor attractants. Although our current study failed to reveal any direct electrophysiological response of the bed bug to DEET, further work is needed to probe the interaction between DEET and human-odorant attractants in the bed bug.

3.6 References

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Table 3.1 Chemical insect repellents

Chemical category	Specific chemicals	Purity (%)	CAS number	Company	Insects (tested examples)	References
Carboxylic acids	Linoleic acid	≥99%	60-33-3	Sigma	*Periplaneta americana*	[1]
	Oleic acid	≥99%	112-80-1	Sigma	*Periplaneta americana*	[1]
	Palmitic acid	≥99%	57-10-3	Sigma	*Periplaneta americana*	[1]
	Stearic acid	≥98.5%	57-11-4	Sigma	*Periplaneta americana*	[1]
Esters	Dimethyl phthalate	≥99%	131-11-3	SAFC	*Aedes aegypti*	[2]
	Dibutyl phthalate	99%	84-74-2	Aldrich	*Aedes aegypti*	[2]
Aldehyde	trans-Cinnamaldehyde	99%	14371-10-9	Aldrich	*Aedes aegypti*	[3]
Alcohols	Isoamyl alcohol	≥98%	123-51-3	SAFC	*Gryllus domesticus*	[4]
	Cinnamyl alcohol	98%	104-54-1	Aldrich	*Aedes aegypti*	[3,5, 6]
Terpenes	(*R*)-(+)-Limonene	97%	5989-27-5	Sigma	*Aedes aegypti*	[8, 9,10]
	(*S*)-(−)-Limonene	96%	5989-54-8	Aldrich	*Aedes aegypti*	[8, 9, 11]
	α-terpinene	≥95%	99-86-5	Aldrich	*Aedes aegypti*, *Culex pipiens*	[10, 12]
	Myrcene	≥95%	123-35-3	Fluka	*Aedes aegypti*	[9, 10]
	Terpenolene	≥90%	586-62-9	SAFC	*Aedes aegypti*	[8, 11, 15]
	α-Pinene	98%	80-54-8	Aldrich	*Culex pipiens*	[5, 8, 13]
	(+)-α-Pinene	≥99%	7785-80-8	Aldrich	*Culex pipiens*	[13]
	(−)-α-Pinene	≥98%	7785-26-4	SAFC	*Culex pipiens*	[13]
	(-)-β-Pinene	≥99%	18172-67-3	Aldrich	*Tribolium castaneum* *Aedes aegypti*	[11, 14]
	(+)-β-Pinene	≥95%	19902-08-0	Fluka	*Tribolium*	[11, 14]

					castaneum	
					Aedes aegypti	
	S-(+)-3-carene	99%	498-15-7	Aldrich	*Aedes aegypti*	[8, 15, 16]
	β-caryophyllene	≥80%	87-44-5	SAFC	*Aedes aegypti*	[5, 11, 16]
Terpenoids	Phytol	≥97%	7541-49-3	SAFC	*Anopheles gambiae*	[7]
	Citronellic acid	98%	502-47-6	Aldrich	*Aedes aegypti*	[17]
	Citral	≥96%	5392-40-5	Aldrich	*Aedes albopictus*	[18]
	Eugenol	99%	97-53-0	Aldrich	*Anopheles gambiae*	[5, 19, 20, 21]
	Geranyl acetate	98%	105-87-3	Aldrich	*Aedes aegypti*	[9]
	(*S*)-(−)-Perillaldehyde	≥92%	18031-40-8	SAFC	*Anopheles gambiae*	[7, 15]
	(S)-(-)-Perillyl alcohol	96%	18457-55-1	Aldrich	*Anopheles gambiae*	[7, 15]
	(-)-Menthone	90%	14073-97-3	Aldrich	*Culex pipiens,*	[5, 13]
					Aedes aegypti	
	(+)-Menthone	≥98.5%	3391-87-5	Fluka	*Culex pipiens,*	[5, 13]
					Aedes aegypti	
	Thymol	≥99.5%	89-83-8	Sigma	*Culex pipiens*	[12, 15, 21, 22]
	α-terpineol	≥96%	10482-56-1	SAFC	*Culex pipiens Tribolium castaneum*	[5, 8,13, 23]
	(+)-terpinen-4-ol	≥95%	2438-10-0	Fluka	*Aedes aegypti*	[10]
	Citronellal	≥85%	106-23-0	SAFC	*Culex quinquefasciatus*	[19]
	D-neomethol	≥99%	2216-52-6	SAFC	*Aedes aegypti*	[2]
	Menthol	99%	89-78-1	Aldrich	*Aedes aegypti*	[2, 21]
	Geranyl Acetone	≥97%	689-67-8	Aldrich	*Aedes aegypti*	[8]
	Menthyl acetate	97%	89-48-5	Aldrich	*Aedes aegypti*	[2]

	Linalyl acetate	≥97%	115-95-7	SAFC	*Tribolium castaneum*	[24]
	(-)-Linalool	≥95%	126-91-0	Aldrich	*Culex pipiens* *Aedes aegypti*	[5, 10, 12, 22]
	Linalool	97%	78-70-6	Aldrich	*Aedes albopictus* *Culex nigripalpus*	[16,25]
	Geraniol	98%	106-24-1	Aldrich	*Aedes aegypti*	[5, 9]
	Citronellol	≥95%	106-22-9	SAFC	*Culex quinquefasciatus*	[5, 9, 19]
	Carvacrol	≥98%	499-75-2	Aldrich	*Culex pipiens*	[13, 15, 19]
	(*S*)-*cis*-Verbenol	95%	18881-04-4	Aldrich	*Anopheles gambiae*	[7, 15]
	Camphor	≥99%	76-22-2	SAFC	*Aedes aegypti*	[10, 15, 19]
	(-)-α-thujone	96%	546-80-5	Aldrich	*Aedes aegypti*	[16]
	Eucalyptol	≥95%	470-82-6	Fluka	*Culex pipiens,* *Aedes aegypti*	[5, 10, 13, 21, 23]
	(-)-caryophyllene oxide	≥95%	1139-30-6	SAFC	*Anopheles gambiae*	[7, 15]
Others	DEET	97%	134-62-3	Aldrich	*Aedes aegypti*	[3, 26, 27]
	Permethrin	99%	52645-53-1	Sigma	*Aedes aegypti*	[24]
	Naphthalene	99%	91-20-3	Aldrich	*Cephus cinctus*	[28]

All stimuli were tested at a dose of 1:100 v/v, with the exception of dimethyl sulfoxide (DMSO), which was used as the solvent: 100% DMSO was used as the control. Numbers refer to published behavioral studies in which that compound was shown to exhibit repellency to those insects: [1] Rollo et al. (1995); [2] Ansari et al. (2000); [3] Chang et al. (2006); [4] Ufkes and Grams (2007); [5] Tunón et al. (2006); [6] Roadhouse (1953); [7] Omolo et al. (2004) [8] Thorsell et al. (1998); [9] Oyedele et al. (2002); [10] Hwang et al. (1985); [11] Jaenson et al. (2006); [12] Choi et al. (2002); [13] Traboulsi et al. (2002); [14] García et al. (2005); [15] Nerio et al. (2010); [16] Gillij et al. (2008); [17] Essam Abdel et al. (2006); [18] Hao et al. (2008); [19] Ansari et al. (2005); [20] Chogo and Crank (1981); [21] Isman (2006); [22] Tripathi et al. (2000); [23] Klocke et al. (1987); [24] Wirtz et al. (1981); [25]Barnard and Xue (2004); [26] Syed and Leal (2008); [27] Ditzen et al. (2008); [28] Daisy et al. (2002).

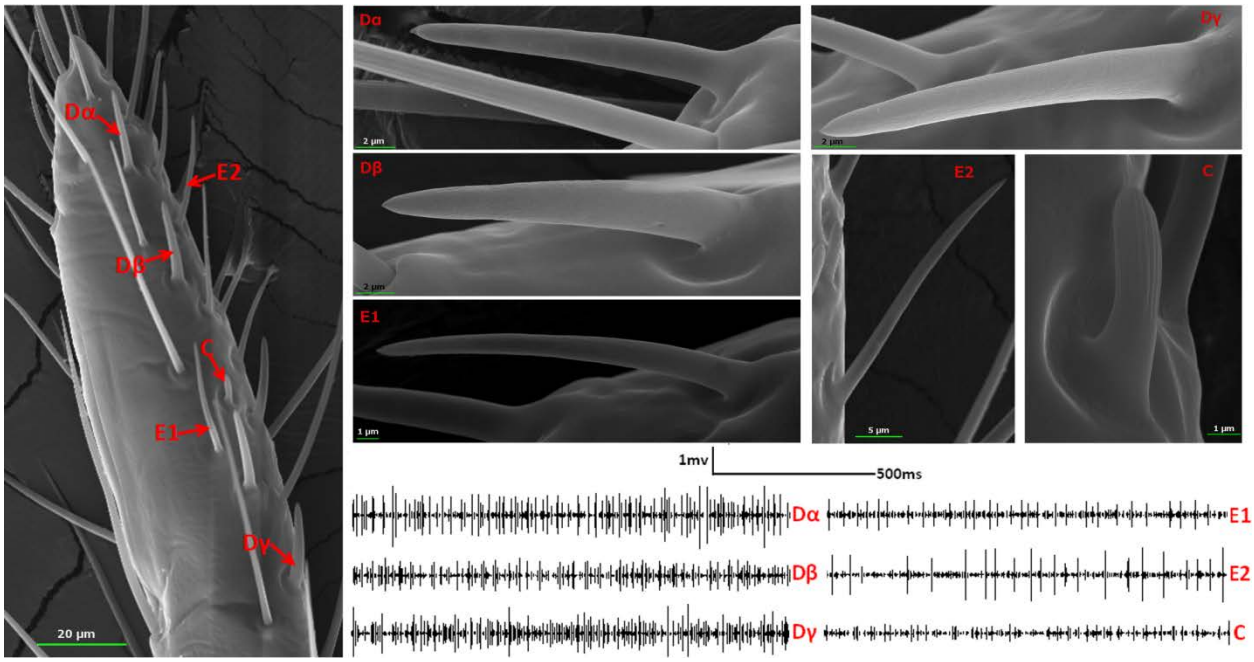

Figure 3.1 SEM photos of bed bug's terminal antennal flagellum. Morphological types of olfactory sensillum and their corresponding spontaneous neuronal activities are shown.

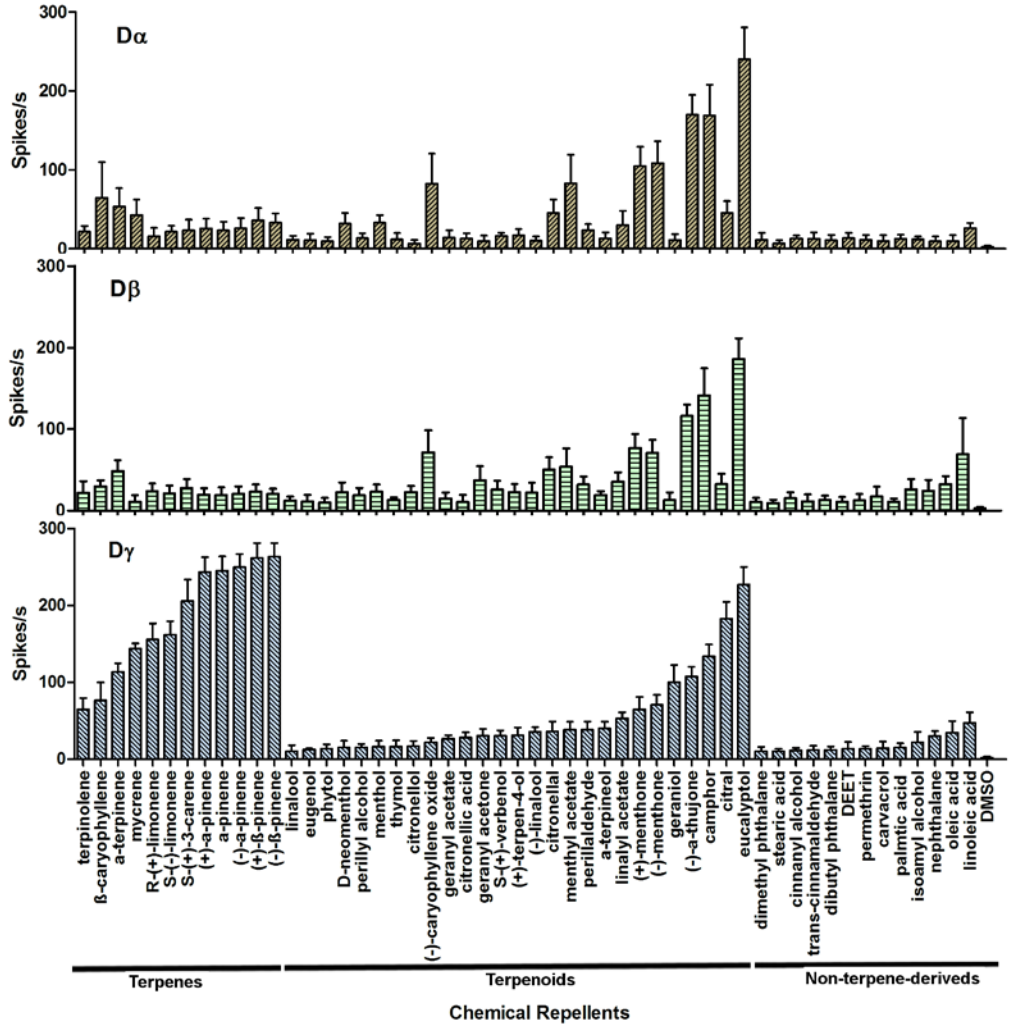

Figure 3.2 Response profiles of D type antennal sensilla to chemical insect repellents. Distinctive response profiles (spikes/s) of Dα, Dβ and Dγ sensilla to different chemical groups of chemical insect repellents were tested through single sensillum recording, with at least ten repeats for each chemical, at a concentration of 1:100 v/v. The solvent, DMSO, produced no stimulation in any of the sensillum types.

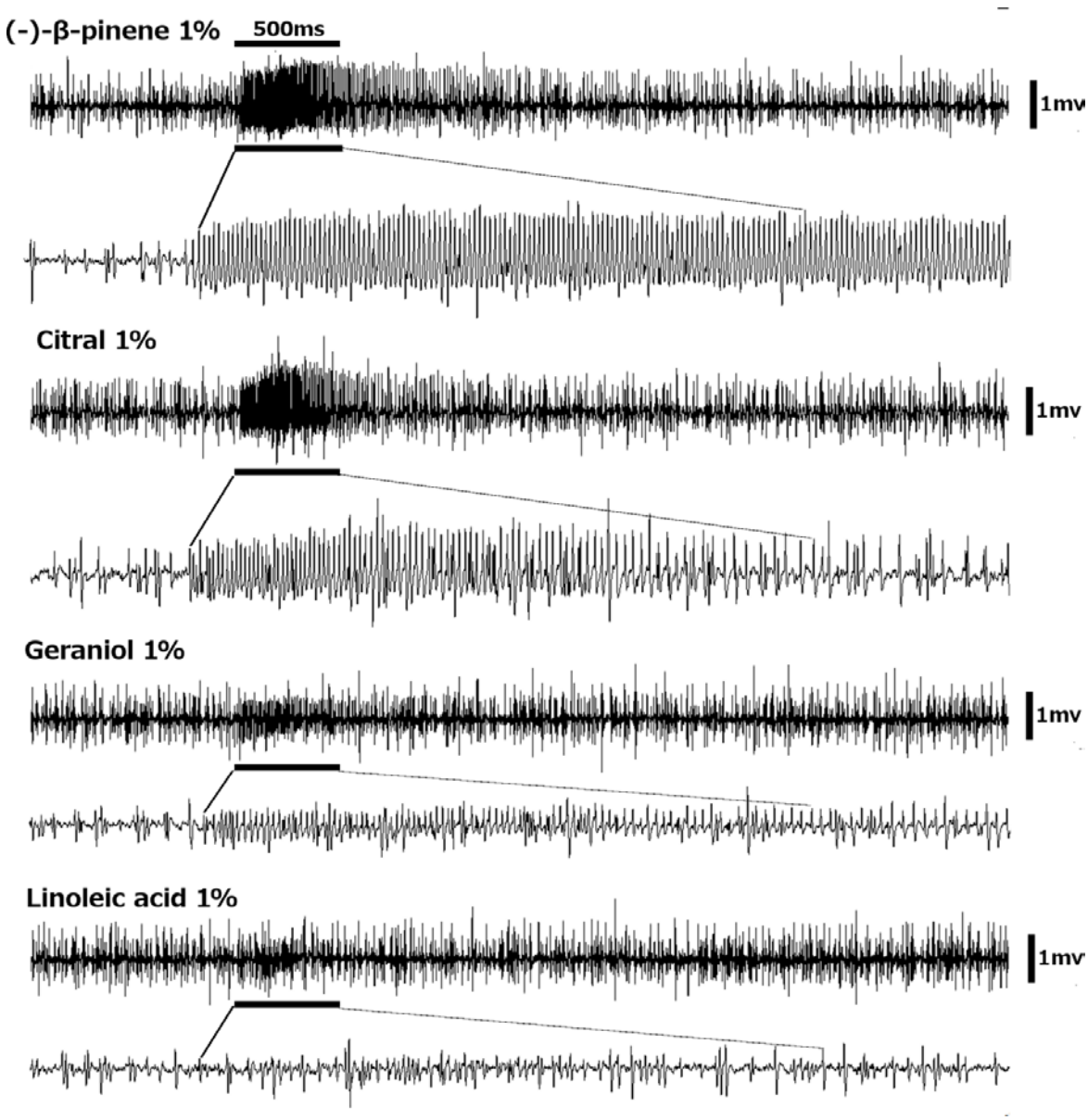

Figure 3.3 Different response patterns of the ORNs in the olfactory sensillum to the chemical insect repellents. Both the (-)-α-pinene and citral stimulated ORNs with large amplitudes on the Dγ sensillum, while geraniol and linoleic acid elicited firing response on the ORNs with small amplitudes in the Dγ sensillum. The enlarged signal shows the action potentials elicited over the 500 ms interval of the stimulation.

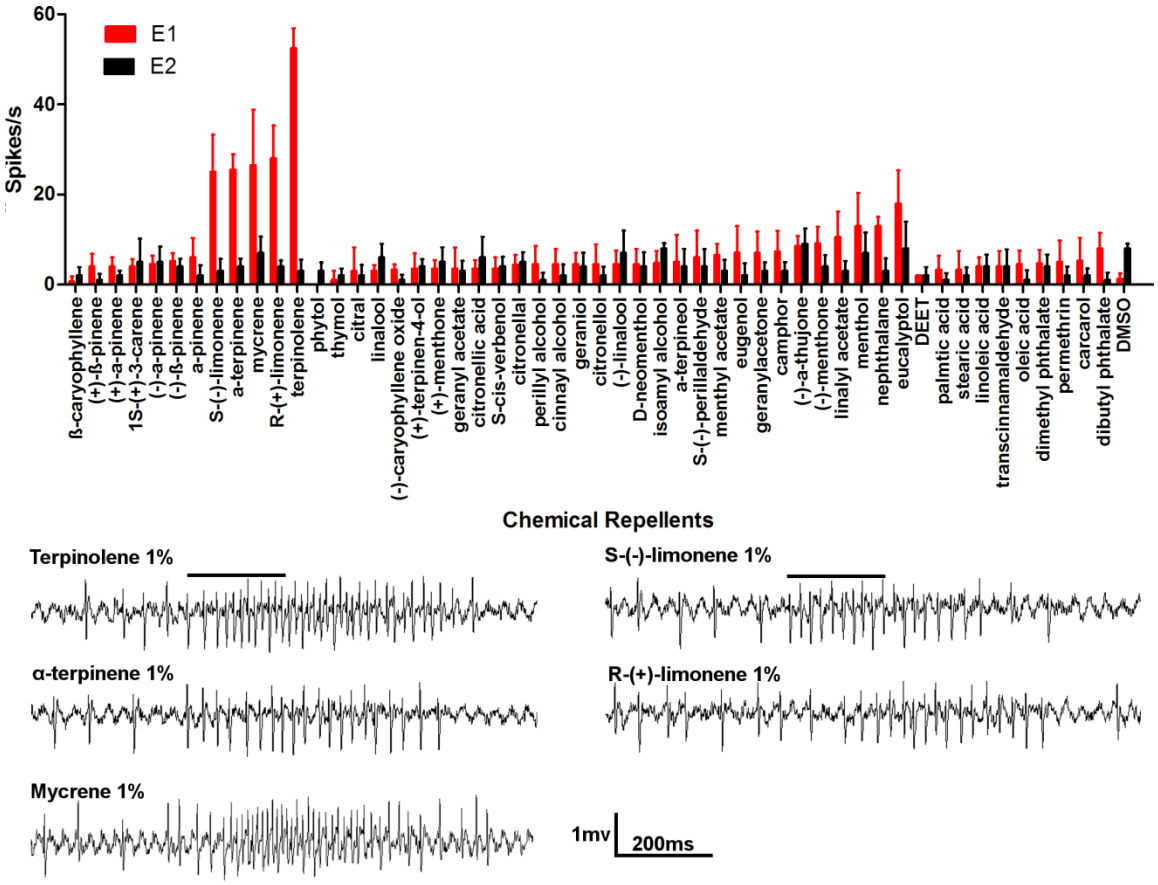

Figure 3.4 The electrophysiological responses of the E1 and E2 sensilla to the chemical insect repellents. Neither of these sensilla showed a response to most of the chemical insect repellents at the test concentration of 1:100 v/v, although the E1 sensilla did exhibit an excitatory response to several terpene chemicals, including S-(-)-limonene, α-terpinene, myrcene, R-(+)-limonene and terpilonene.

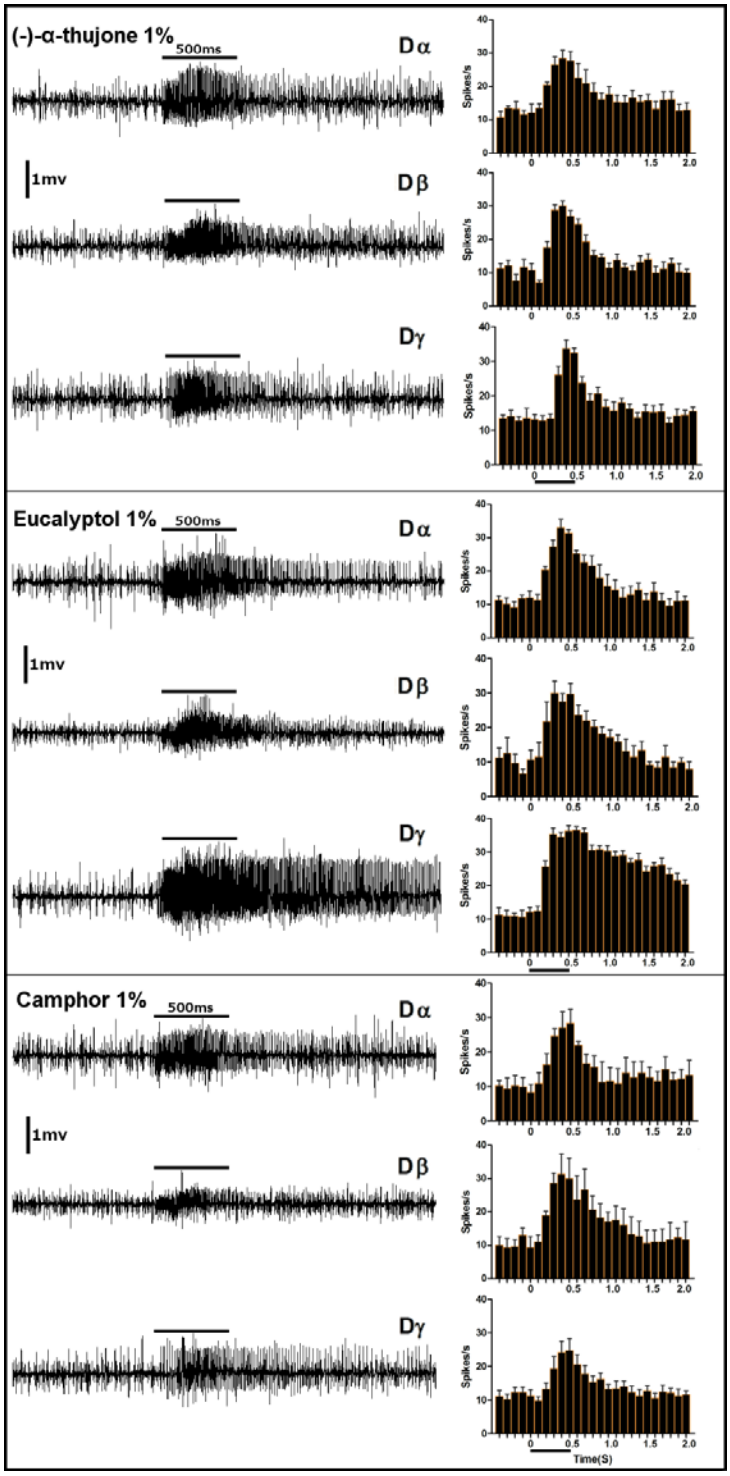

Figure 3.5 Temporal dynamic of response of Dα, Dβ, Dγ sensilla. All three chemical insect repellents were tested at a dose of 1:100 v/v. On the left are the representative firing responses of the ORNs in the Dα, Dβ, Dγ sensilla to (-)-α-thujone, eucalyptol and camphor; the right of the figure shows a histogram representing the number of spikes recorded during each 100 ms sampling period. Horizontal bars indicate the duration of the stimulation (500 ms). Error bars represent the standard error for the mean of the ten readings.

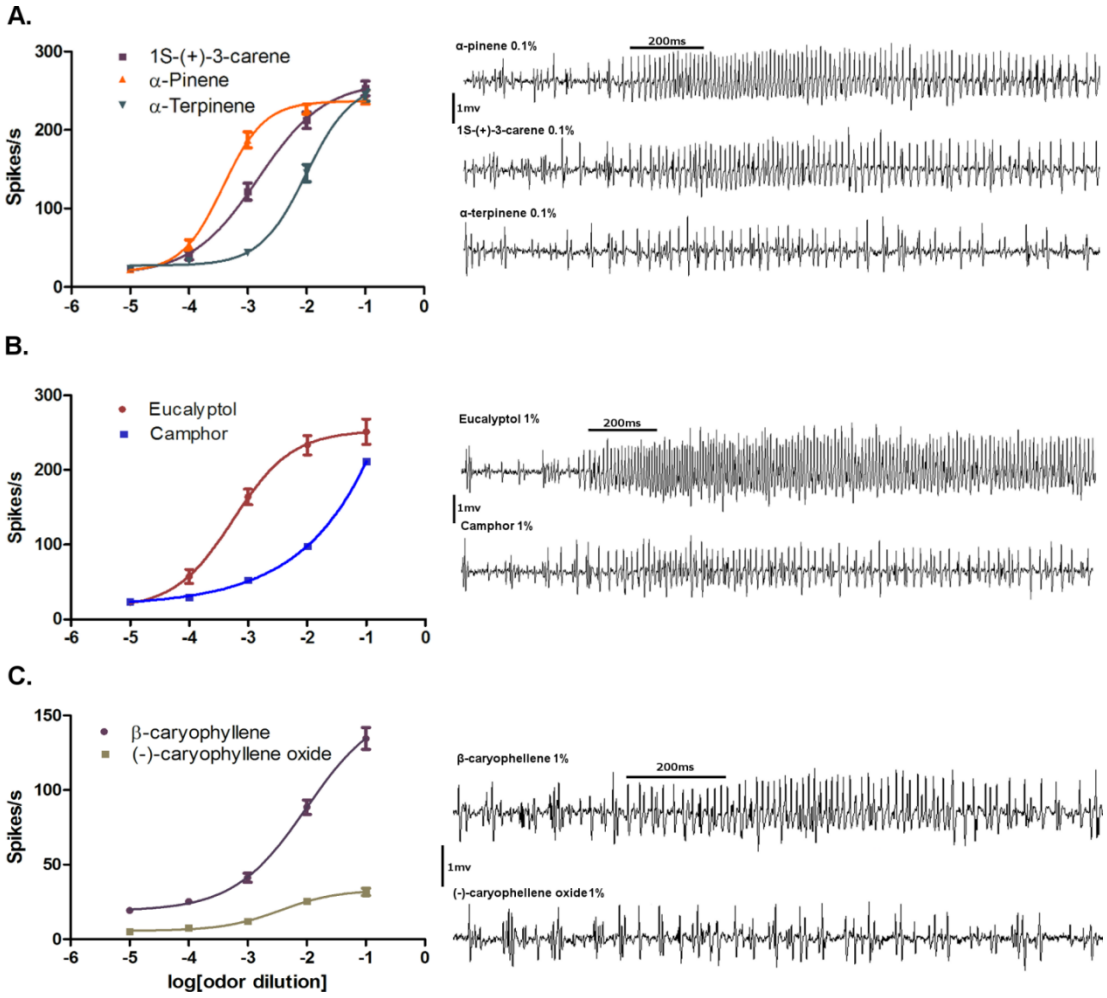

Figure 3.6 Dose-dependent responses of Dγ sensilla to chemical insect repellents. The dose-dependent response curve is presented as a mean value ± SEM. The X axis describes the logarithm dilution series from 1:10 to $1:10^6$ v/v. (A): Dose response curve of three terpene chemicals and their representative firing signal at a dose of $1:10^3$ (0.1%) v/v; (B): Dose response curve of two terpenoid chemicals and their representative firing signal at a dose of $1:10^2$ (1%) v/v; and (C): Dose response curve of β-caryophyllene and (-)-caryophyllene oxide and their representative firing signal at a dose of $1:10^2$ (1%) v/v.

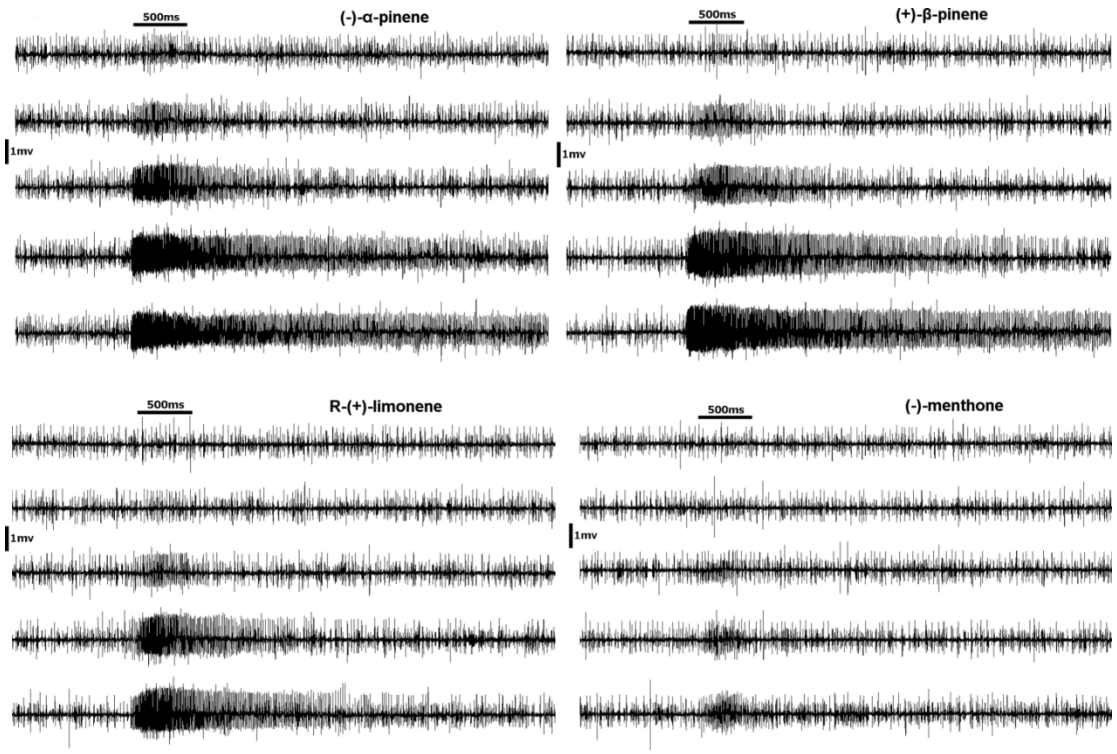

Figure 3.7 Representative excitatory dose-dependent responses of Dγ sensillum to (-)-α-pinene, (+)-β-pinene, R-(+)-limonene and (-)-menthone. As the doses increase from $1{:}10^{6}$ to 1:10 v/v (from top to bottom) for all four chemicals, the firing frequencies of the ORNs in the Dγ sensillum also increased.

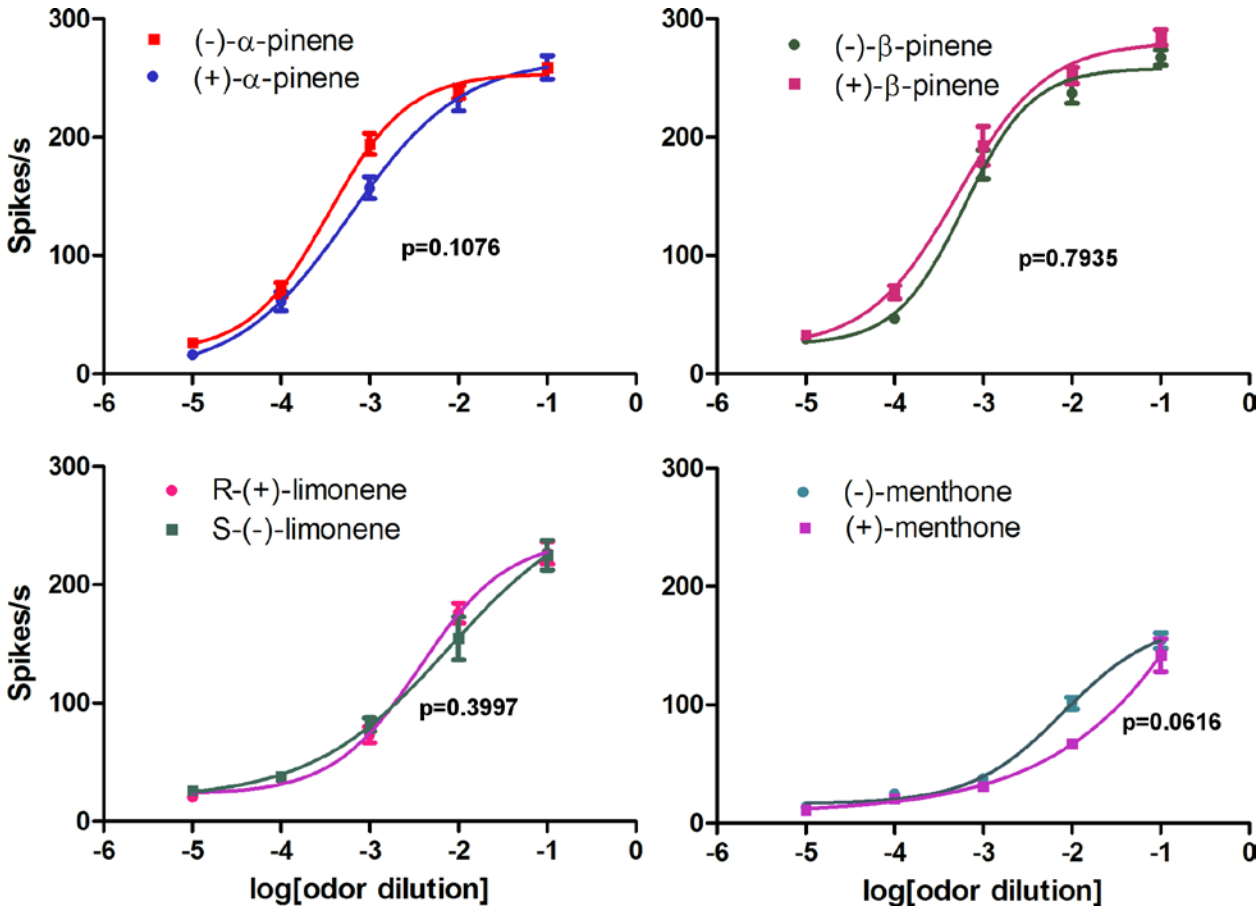

Figure 3.8 Responses of the Dγ sensilla to different stereoisomers. The dose-dependent response curve is presented as a mean value ± SEM. The X axis describes the logarithm dilution series from 1:10 to $1:10^6$ v/v. *F*-tests with Bonferroni correction were conducted to compare the dose-response curve for each pair of stereoisomers of α-pinene, β-pinene, limonene and menthone (n = 8-10). $p < 0.05$ was considered to be a significant difference.

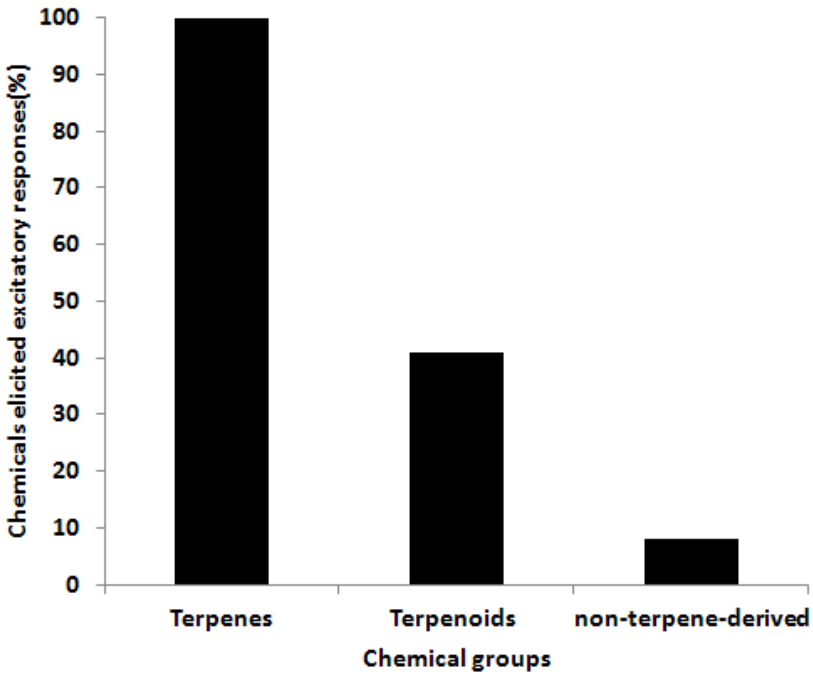

Figure 3.9 Percentage of excitatory responses (≥50 spikes/s) to different chemical groups. Chemical repellents were categorized into three groups according to their chemical structures: terpene repellents, terpenoid repellents, and non-terpene-derived repellents.

Chapter 4: Characterization of Antennal Olfactory Responses to Human Odorants in the Common Bed Bug, *Cimex lectularius*

4.1 Abstract

The common bed bug *Cimex lectularius* is a temporary ectoparasite on humans and is currently resurgent in many developed countries. The ability of bed bugs to detect human odorants in the environment is critical for their host-seeking behavior. This study deciphered the chemical basis of host detection by investigating the neuronal response of olfactory sensilla to 103 human odorants using single sensillum recording and characterized the electro-physiological responses of bed bug odorant receptors to human odorants with the *Xenopus* expression system. The results showed that the D type of olfactory sensilla play a dominant role in detecting the human odorants tested. Various human odorants elicited different neuronal responses with different firing frequencies and temporal dynamics. Particularly, aldehydes and alcohols are the most effective stimuli in triggering strong responses while none of the carboxylic acids showed strong stimulation. Taken together, the findings of this study not only provide exciting new insights into the human odorant detection of bed bugs, but also offer valuable information for developing new reagents (attractants or repellents) for bed bug control.

4.2 Introduction

The common bed bug, *Cimex lectularius,* is a temporary hematophagous ectoparasite on human beings and animals (Rivnay, 1930; Thomas et al., 2004), with all its developmental stages and both sexual forms relying on blood for nutrition and reproduction. Although virus transmission has been rarely reported for *C. lectularius*, the biting nuisance and potential for secondary infections create both physical and psychological disturbances in human hosts (Anderson and Leffler, 2008). The introduction of effective chemical insecticides removed

the common bed bug as a subject of public concern for many years as populations were controlled and almost eradicated in some industrialized countries (Anderson and Leffler, 2008). However, in the early 21st century the common bed bug was reported to be resurgent, causing serious problems for public health (Doggett et al., 2004; Ter Poorten and Prose, 2005; Romero et al., 2007; Wang et al., 2013). The resurgence of the common bed bug led to a search for new sustainable methods to monitor and control this human ectoparasite. Because of increased insecticide resistance of bed bugs, traps baited with attractive cues represent a promising complementary method for bed bug control. As in other blood-feeding insects such as mosquitoes, human odorants possess great potential as attractants for bed bugs. Indeed, previous studies of behavioral responses to human volatiles have revealed that human sweat alone has a significant attraction for all stages and both sexes of bed bugs (Levin 1975), and other studies have indicated that odors from animal skin emanations are also attractive to bed bugs (Rivnay 1932; Aboul-Nasr and Erakey, 1968).

The olfaction system of bed bugs plays an important role in their host-seeking process. Olfactory receptor neurons housed in olfactory sensilla on bed bug antennae are responsible for detecting human odors (Harraca et al., 2012). Odorant receptors on the neuron membrane bind to human odors, resulting in the depolarization of the neuron membrane and the production of action potentials (Hallem et al., 2006; Grant and Dickens, 2011; Leal 2013; Guidobaldi et al., 2014). Three types of olfactory sensilla (C, D, E sensillum) on the bed bug antennae have been morphologically identified by Levinson et al. (1974). More recently, functional studies have further categorized the D type of sensillum into three subtypes, Dα, Dβ, and Dγ, based on their distinctive response profiles to the chemicals in single sensillum recording (SSR) (Harraca et al., 2010; Liu et al., 2014).

Despite the promising application in the bed bug control, only a few human odorants have been tested on bed bugs using single sensillum recording or in behavior assays (Harraca et

al., 2012). In an effort to characterize the interaction between the bed bug olfactory sensilla and human odorants and decipher the molecular basis of odorant detection by the bed bug olfactory system, we conducted a systematic characterization of the neural responses of bed bug olfactory sensilla to 103 commercially available human odorants using single sensillum recording.

4.3 Materials and Methods

4.3.1 Insects, scanning electron microscopy, and single sensillum recording

The *C. lectularius* colony was a gift from Dr. Haynes (University of Kentucky, Lexington, KY). For single sensillum recordings, adult bed bugs were used throughout. Bed bugs were reared at 25±2°C under a photoperiod of 12:12 (L: D). Scanning Electron microscopy (SEM) and single sensillum recording (SSR) experiments were conducted as described by Liu et al. (2014). Briefly, the bed bugs (male or female) were anaesthetized (2-3 min on ice) and mounted on a microscope slide (76×26 mm) between 2 pieces of double-sided tape. The antennae were fixed by double-sided tape to a cover slip resting on a small ball of dental wax to facilitate manipulation. The cover slip was placed at an appropriate angle to the bed bug head. Once mounted, the bed bug was placed under a LEICA Z6 APO microscope and the antennae examined at high magnification (×720). Two tungsten microelectrodes were each sharpened in 10% KNO_2 at 2-10V to a ~1μm tip diameter; the reference electrode, connected to ground, was inserted into the abdomen of the bed bug and the other electrode, connected to a preamplifier (10×, Syntech, Kirchzarten, Netherlands), was inserted into the shaft of olfactory sensillum to complete the electrical circuit in order to extracellularly record the olfactory receptor neuron (ORN) potentials (Den Otter et al., 1980). Controlled manipulation of the electrodes was performed using 2 micromanipulators (Leica, Germany). The preamplifier was connected to an analog to digital signal converter (IDAC, Syntech, Netherlands) and then to a computer for signal recording and visualization. Signals were recorded for 10 s starting 1 s before

stimulation at a sampling rate of 96,000/s, and the action potentials were counted off-line for 500 ms before and after stimulation. Changes in spike rates during the 500 ms pre-stimulation period were subtracted from the activity recorded during the 500 ms stimulation period and the difference converted to the conventional scale of spikes/s.

4.3.2 Stimulation and stimuli

Based on Bernier and colleagues' (2000) GC-MS study on emanations from human skin, 103 commercially available human odorants from 11 chemical groups (carboxylic acids, esters, aldehydes, alcohols, ketones, aliphatics/aromatics, halides, heterocyclics, amines, sulfides and ureas) were used in the study (Table 4.1). Each of the human odorants was diluted in dimethyl sulfoxide (DMSO) to a stock solution with a concentration of 1:10 v/v. Subsequently, serial 10-fold dilutions were made from the stock solution for each of the chemicals. Ten microliters of each dilution were dispersed on a filter paper (10×10 mm) that was then inserted in a Pasteur pipette to create each stimulus cartridge. A pipette containing solvent alone served as the control. A constant airflow across the antenna was maintained at 20 ml/s throughout the experiment. Purified and humidified air was delivered to the preparation through a glass tube (10 mm inner diameter). The glass tube was perforated by a small hole, slightly larger than the tip of the Pasteur pipette, 10 cm away from the end of the tube. Stimulation was achieved by inserting the tip of the stimulus cartridge into the hole of the glass tube. A stimulus controller (Syntech, Germany) diverted a portion of the air stream (0.5 l/min) to flow through the stimulus cartridge for 500 ms, thus delivering the stimulus to the sensilla. The distance between the end of the glass tube and the antennae was $\leq$ 1 cm. All the human odorants were tested on each type of antennal sensillum at least 6 times each and the value of spikes/s obtained by averaging all the recordings for each sensillum to each odorant. Those sensilla that failed to show a response of firing rate of 15 spikes/s, were considered to be non-responders (de Bruyne et al., 2001).

4.3.3 Data analysis

Hierarchical cluster analysis and principle component analysis (PCA) for the odorant space were performed using PASW 18.0 (IBM, NY). Euclidean distance and between-group linkage classification methods were used for the hierarchical cluster analysis (Hallem and Carlson, 2006; Ling et al., 2014). PCA was conducted using the correlation matrix. A value of $P \leq 0.05$ was considered statistically significant.

4.4 Results

4.4.1 Response, tendency and tuning curve of olfactory sensilla to human odorants

We tested the neural responses of each type of olfactory sensilla to 103 chemicals from 11 chemical groups utilizing single sensillum recording (Fig. 4.1A) and found that different sensilla (Fig. 4.1B) displayed markedly distinct characteristic neuronal responses to various human odors. In general, each type of sensilla exhibited its highest exciting response to a different odorant, with the exception of Dα and Dγ, which showed the strongest response to the same human odor, namely nonanal (Fig. 4.1C).

In total, 624 odorant-sensillum combinations, with each chemical being tested for all six types of sensilla, were recorded with at least six replicates on different individuals. Of these combinations, 88.8% (554) of the odorant-sensillum combinations yielded little if any response (<50 spikes/s); 6.1% (38) resulted in responses of ≥ 50, ≤ 100 spikes/s; 2.2% (14) produced a strong response of ≥ 100, ≤ 150 spikes/s; 1.3% (8) resulted in very strong responses of ≥ 150, ≤ 200 spikes/s; and 1.6% (10) generated extremely strong responses of ≥ 200 spikes/s (Fig. 4.2A). This result indicates that strong or even mild neuronal responses to human odorants are uncommon for bed bugs.

To investigate whether the common bed bug had special tendencies or biases towards detecting particular types of human odorants, we compared proportions of the major chemical

groups (carboxylic acid, aldehydes, alcohols, aromatic, and heterocylics) that elicited excitatory responses (≥ 50 spikes/s) in the bed bugs and found that sensilla responded to 82% of the aldehydes, 50% of the alcohols, 40% of the aromatics, and 30% of the heterocyclics (Fig. 4.2B). Interestingly, although carboxylic acids make up the largest chemical group of the human odorants tested, 21 of the 103 chemicals, none of the bed bugs' olfactory antennal sensilla had an excitatory response of ≥ 50 spikes/s to any carboxylic acid (Fig. 4.2B). This distinctive differentiation of neuronal responses in the common bed bugs to human odorants suggests that certain chemical groups (such as the aldehydes) may play a key role in the host-seeking process of bed bugs.

The tuning curves of the neuronal responses revealed the preference for each type of olfactory sensillum in detecting semiochemicals in the environment. Among the six different types of olfactory sensilla on the bed bug antennae, the tuning curve ranged from extremely narrow (in the C sensillum with a K value of 13) to very broad (in the Dβ sensillum with a K value of 5.1), displaying a continuous pattern (Fig. 4.2C). The narrowly tuned C sensillum responded to only a few chemicals, mostly amines with very high firing frequencies, while the most broadly tuned Dβ sensillum responded strongly to human odorants with very diverse chemical structures (Fig. 4.3). This difference among the tuning curves for different types of olfactory sensilla indicates their potential capacity in detecting odorants from human hosts. Particularly, based on their broad tuning curve to human odorants in this study and also supported by the findings in previous studies (Harraca et al., 2010; Liu et al., 2014), we conclude that the D type olfactory sensilla, particularly Dβ and Dγ, play the dominant role for the bed bug to detect chemical stimuli in the environment, including human odors.

4.4.2 Olfactory responses of D sensilla to human odorants

As noted above, D sensilla (Dα, Dβ and Dγ) play the most important role in detecting the major chemical groups (aldehydes, alcohols, heterocyclics, and aromatics) in human

odorants, far outpacing the other types of olfactory sensilla. Specifically, Dα sensilla responded to 55%, 15% and 15% of the aldehydes, alcohols, and heterocyclics, respectively, with a firing frequency of ≥ 50 spikes/s; Dβ sensilla responded to 82%, 38% and 30% of the aldehydes, alcohols, and aromatics, respectively, with a firing frequency of ≥ 50 spikes/s; and Dγ sensilla responded to 64%, 26% and 15% of the aldehydes, aromatics, and heterocyclics, respectively, with a firing frequency of ≥ 50 spikes/s.

Interestingly, the D sensilla also showed strong responses to a few chemicals in the minor chemical groups (ketones, halides, etc.) in human odorants. For example, the Dα sensilla reacted to one of the halides (1-chlorohexane) with a neuronal response of 136 ± 13.59 spikes/s (Fig. 4.3, Table 4.2). The Dβ sensilla also showed strong excitatory responses to two halides (1-chloroheptane and 1-chlorohexane) with firing rates of 146 ± 12.59 and 131 ± 3.53 spikes/s, respectively (Fig. 4.3, Table 4.2). Moreover, both the Dβ and Dγ sensilla were very sensitive to several ketones. Dβ sensilla showed strong responses to 2-pentanone, 2-hexanone, 2-decanone and 3-pentanone, with firing rates of 102 ± 4.8, 138 ± 9.12, 100 ± 5.2, 122 ± 4.8 spikes/s, respectively, while the Dγ sensilla showed strong responses to 2-hexanone and sulcatone, with firing rates of 111 ± 6.1 and 226 ± 7.36 spikes/s, respectively (Fig. 4.3, Table 4.2).

4.4.3 Olfactory responses of C, E1 and E2 sensilla to the human odorants

The grooved peg C sensilla (nine on each antenna) each house 4-5 sensory neurons, and these were found to exhibit much lower sensitivities to most of the human odorants tested than the smooth peg D sensilla. C sensilla revealed no systematic response to several of the major chemical groups in human odorants, including carboxylic acids, aldehydes, alcohols and aromatics. However, the grooved peg C sensilla did exhibit systematic sensitivity to amines, including ammonia, propylamine, and butylamine, with firing frequencies of 200 ± 6.97, 195 ± 15.93, and 144 ± 12.06 spikes/s, respectively (Table 4.2). Two additional heterocyclics,

1-methylpiperazine and thiazolidine, were also found to be strong stimuli for the C sensilla, with firing rates of 176 ± 41 and 130 ± 36 spikes/s, respectively (Table 4.2).

The hair-like E sensilla are the most abundant sensilla on bed bug antennae, although they house far fewer sensory neurons (1-3 sensory neurons) and pores on the sensilla cuticle compared to the D and C sensilla. In this study, two types of E sensilla, E1 and E2, exhibited very different neuronal signals. The E1 sensilla did not respond to any of the human odorants except for weak responses to two chemicals, octanal and methyl tridecanoate, with firing frequencies of 30 ± 4.45 and 23 ± 2.93 spikes/s, respectively (Table 4.2). However, the E2 sensilla showed much greater activity in response to the long-chain chemicals in human odorants. Marked excitatory responses were observed in the E2 sensilla in response to several human odorants, three of which, N-pentadecanoic acid, 1-tetradecene, and 1-chlorododecane, elicited responses with firing frequencies of 49 ± 4.4, 60 ± 5.63, 59 ± 9.49 spikes/s, respectively (Table 4.2). The E2 sensilla also show weaker responses to another five human odorants, namely hexadecane, 1-hexadecene, methyl tridecanoate, 1-chlorotetradecane, and 1-chlorohexadecane, with neuronal responses of 35 ± 3.5, 42 ± 5.29, 31 ± 2.93, 39 ± 4.6, 20 ± 1.38 spikes/s, respectively (Table 4.2). Since all the chemicals that generated responses from the E2 sensilla possess more than ten carbons in their molecular backbone, it seems likely that the E2 sensilla on the common bed bug antennae are responsible for detecting the long-chain chemicals in human odorants.

4.4.4 Dose dependent responses of olfactory sensilla to human odorants

To investigate the effect of chemical dosage on the neuronal responses of olfactory sensilla to human odorants, the responses of Dα, Dβ, Dγ and C sensilla to different doses were tested. Human odorants that had previously shown strong stimulations at a 10-fold dilution (v/v) were chosen for this dose-response study. Basically, all different types of olfactory sensilla

tested showed a dose-dependent response to the human odorants. One particularly interesting result was the comparison of two alcohols, trans-2-hexen-1-ol and cis-2-hexen-1-ol, with Dα sensilla, where the results showed that as the doses increased from $1{:}10^5$ to 1:10 v/v, the neuronal response of Dα sensilla to both chemicals increased accordingly, rising from ≤ 20 spikes/s to ≥ 200 spikes/s (Fig. 4.4A). The very similar dose-dependent curves may result from their similar chemical structures.

A number of ketones (2-pentanone, 2-butanone, 2-hexanone, 2-decanone and 3-pentanone) and halides (1-chlorohexane and 1-chloroheptane) were also chosen for the dose-response test for the Dβ sensilla, which displayed the highest firing frequency to these human odorants at the original dose of 1:10 v/v. Here, the lowest dose-response curve was observed in 2-butanone and the highest in 2-hexanone (Fig. 4.4B). The 2-pentanone/3-pentanone and 1-chlorohexane/1-chloroheptane pairs showed quite similar dose-dependent stimulation for the Dβ sensilla at different doses (Fig. 4.4B/C), which make sense based on their similarities in chemical structure.

For the Dγ sensilla, aromatics (ethylbenzene, propylbenzene, and methylbenzene (toluene)) and aldehydes (from propanal to decanal) were chosen for dose-response tests. All these human odorants showed their strongest stimulation on the Dγ sensilla compared to other types of sensilla at the original dose of 1:10 v/v. For the three aromatic human odorants, the Dγ sensilla showed statistically significantly stronger responses to ethylbenzene and propylbenzene compared with methylbenzene (F test, $P < 0.0001$) (Fig. 4.4D). For the aldehydes, hexanal, heptanal and octanal generated the strongest stimulations with the threshold of responses at least one-log dose lower than nonanal, two-log doses lower than decanal and pentanal and three-log doses lower than propional and butanal (Fig. 4.4E).

The two human odorants that showed the strongest stimulation on the grooved peg C sensilla, propylamine and butylamine, were chosen to conduct the dose-response test for the C sensilla. The C sensilla displayed quite similar responses to both amines, with no statistically significant differences in the responses at doses of 10^5, 10^4, 10^3 and 10-fold dilutions (v/v) (*t* test, $P > 0.05$). However, at the 10^2-fold dilution (v/v) doses, the firing frequency of C sensilla to butylamine (223 ± 20 spikes/s) was significantly higher than that for propylamine (94 ± 20 spikes/s) (*t* test, $P < 0.001$) (Fig. 4.4F). Taken together, these results indicated that the specific dosage of human odors is very important in triggering the olfactory neural responses of bed bugs to their hosts.

4.4.5 Temporal dynamics of olfactory sensilla in response to human odorants

Besides the firing frequency, the temporal structure of an olfactory neural response is considered to be another important factor involved in the odor coding process (Laurent et al., 2001; Hallem, and Carlson, 2006; Qiu et al., 2006; Ghaninia et al., 2007). To investigate the temporal structure of these neural responses in the bed bug, we examined the firing frequencies of olfactory sensilla over a 2 s period beginning at the onset of chemical stimulation. Responses were plotted onto a continuous line graph at 100 ms intervals. The results show that the temporal characteristics of ORNs in the olfactory sensilla are indeed both stimulus and dose specific. For instance, the temporal structure of the Dγ sensillum's response to aldehydes at a dose of 1:100 v/v varied considerably (Fig. 4.5A). Propional, butanal and decanal were more likely to elicit a phasic neuronal response, while pentanal, hexanal, heptanal, octanal and nonanal instead tended to generate a tonic neuronal response, with the firing rates remaining at a high level (≥ 30 spikes/100 ms) throughout the 2s time period. Sulcatone was the only ketone that presented a tonic response; all the others (2-hexanone, 2-pentanone, and 2-decanone) displayed more phasic responses from the olfactory neurons (Fig. 4.5A). Aromatic chemicals generally elicited phasic neuronal

responses at a dose of 100-fold dilution (v/v), with no typically tonic responses observed (Fig. 4.5A). The cluster analysis based on the temporal structures of these neural responses further distinguished human odors with the same categories. For example, the aldehydes (C3-C10) were evidently separated into two groups according to their differences in neural temporal structure (Fig. 4.5A).The same is true for ketones, among which sucaltone was obviously discriminated from the aliphatic ketones, perhaps resulting from their differences in molecular structure (Fig. 4.5A). For the aromatics, the response to ethylbenzene varied compared to those of other aromatics with a relatively short cluster distance (Fig. 4.5A). In conclusion, the wide variations in the temporal structures of neural responses may influence further odorant recognition for bed bugs.

Furthermore, the temporal dynamics of neuronal responses were also significantly influenced by the odor dosages or intensity. Low doses of human odors appeared to generate more phasic-neuronal responses, while high doses were more likely to elicit tonic responses from the olfactory neurons. For some human odors, like hexanal, nonanal and sulcatone, the firing processes were prolonged greatly as the doses increased from 10^5-fold to 10^2-fold dilution (v/v) and the temporal dynamics shifted from predominantly phasic to become more tonic (Fig. 4.5B), which was also the case for several other stimuli, including heptanal, octanal, 1-chloroheptane and 1-chlorohexane (Data not shown).

4.4.6 Primary presentations of odorant space among the olfactory sensilla

Our results clearly showed that human odors elicit vairant patterns of response combinations from different bed bug olfactory sensilla. To investigate the ability of bed bugs to differentiate between human odors in different categories, we examined the primarily spatial relationships among odorants in an odorant space created by the responses of each olfactory sensillum to each of the odorants tested. In this six-dimensional odorant space, Euclidean

distances in spikes/s between all possible pairs of the 103 tested human odorants were used to evaluate the spatial differences involved in the process of bed bug olfaction.

Of the 5,356 pairs of human odorants tested, five of the top 10 closest pairs, which showed smallest Euclidean distance, structurally and chemically fell into the same categories (Table 4.3). The top 10 odorant pairs that were farthest apart in odorant space were found to all share one member: nonanal (Table 4.3). Although all the C5-C10 aldehydes (pentanal, hexanal, heptanal, octanal, nonanal and decanal) were very far away (≥ 100 spikes/s) from almost all the other chemicals in different categories, especially the amine odorants (with a Euclidean distance ≥ 200 spikes/s), nonanal was consistently farthest out. The three amine odorants were also a long distance (≥ 100 spikes/s) from almost all the other chemical categories, especially the aldehyde odorants, apart from two exceptions: the aromatics thiazolidine and 1-methylpiperazine. These results suggest that both aldehydes (C5-C10) and amines are very important but mutually distinctive chemical components in human odorants for chemoreception in bed bugs.

To visualize the relationships among odorants in this space, a hierarchical cluster analysis was performed on the odorants based on the responses of each olfactory sensillum. We found that odorants in the same chemical group often, though not always, clustered together (Fig. 4.6A). Particularly, certain structurally similar molecules were observed to be tightly clustered, for example, cis-2-hexen-1-ol and trans-2-hexen-1-ol; 2-pentanone, 3-pentanone, 2-hexanone and 2-decanone; hexanal, heptanal and nonanal (Fig. 4.6B).

As another way of analyzing the relationships among odors, principle component analysis (PCA) was used to represent the six-dimensional odor space in a three-dimensional odor space. As in the hierarchical cluster analysis, odorants of aldehydes (green dots) or amines (pink dots) were more likely to cluster together (Fig. 4.6C). Acids were the most dispersive

chemical groups in this odor space and some intermingling was observed in the odor space among odors of different classes (Fig. 4.6C). These results indicate that chemical class is one of the critical factors involved in determining the pattern of activation among olfactory sensilla on bed bug antennae.

4.5 Discussion

Bed bugs rely heavily on blood from their host, either human or animal, for survival and development, and the neural responses of bed bug antennae to human odorants provide the primary messages that enable them to identify a potential blood source. Previous studies have tended to emphasize the importance of heat and carbon dioxide in attracting bed bugs, and studies that have focused on the role of human odorants in the process of host seeking have been very limited (Harraca et al., 2012). This study provides a systematic description of the neural responses of the olfactory antennal sensilla of bed bugs to 103 human odorants, and elucidates the different response profiles of the olfactory sensilla to various human odorants. Our results revealed that bed bugs exhibited neural responses to at least 42 human odorants with firing rates higher than 50 spikes/s, which suggests that at least at the olfactory sensillum level, bed bugs are sensitive to a number of human odorants, which included several aliphatic aldehydes(C7-C10) and one ketone (sulcatone) that have been used in a behavioral assay that showed greater attraction to the bed bug at low concentrations but repellency at high concentrations (Harraca et al., 2012).

Traps that combine CO_2, heat and chemical lures have been tested in the lab, but the results revealed no significant additive effects of the chemical lure on the number of bed bugs captured compared to traps consisting of CO_2 and heat alone (Wang et al., 2009; Anderson et al., 2009). The major components of these chemical lures were carboxylic acids, which are known attractants for blood-feeding insects such as mosquitoes, biting midges, kissing bugs and tsetse flies (Lehane 2005). However, in our study we found that bed bugs showed no

neural responses to any of the carboxylic acids tested. Therefore, our finding may partially explain why no additive effects were observed in the bed bug catches when carboxylic acids were added to the traps.

In this study, we found that the amines tested in our study were exclusively recognized by the neurons housed in the grooved-peg C type olfactory sensilla, while aldehydes are most likely to activate the olfactory neurons housed in the D type of olfactory sensilla of bed bugs. This result is consistent with the findings reported in previous studies on mosquitoes (*Culex quinquefasciatus*) and kissing bug (*Triatoma infestans*), where the grooved-peg sensilla also showed very strong responses to the amine chemicals (Syed and Leal, 2009; Diehl et al., 2003). Our results also revealed that the amine chemicals exhibited the most significant difference with the aldehydes in odor space. This huge difference in the chemoreception may result from the distinctive expression of two different types of olfactory receptors, odorants receptors (ORs) and ionotropic receptors (IRs), in the olfactory neurons of D type sensilla and C type sensilla, respectively. Ionotropic receptors were uncovered as a new family of insect chemoreceptors recently, which were proved to be responsible for the recognition of polar molecules, like the amines and acids (Spletter and Luo, 2009; Abuin et al., 2011; Missbach et al., 2014). Interestingly, IRs have been widely reported in the coeloconic sensillum in *Drosophila melanogaster* (Spletter and Luo, 2009; Abuin et al., 2011), and in bed bugs grooved peg C sensilla shared similar cuticle and pore structure with the coeloconic sensillum of other insects (Levinson et al., 1974). Given that amines are exclusively detected by the C sensilla, we propose that IRs may be expressed in the neurons housed in C type olfactory sensilla.

4.6 References

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Table 4.1 Human odorants list

Chemicals*	CAS	Company	Purity	Behavior Activity	SSR Activity	Reference
Carboxylic acids						
acetic acid	64-19-7	Sigma	99%			
propionic acid	79-09-4	Fisher	99.5	+		[1, 2]
hexanoic acid	142-62-1	Sigma	99.5		+	[3]
heptanoic acid	111-14-8	Sigma	96			
octanoic acid	124-07-2	Sigma	98			
N-nonanoic acid	112-05-0	Sigma	97%			
decanoic acid	334-48-5	Sigma	98			
lauric acid	143-07-7	Sigma	99			
N-tridecanoic acid	638-53-9	Sigma	98			
myristic acid	544-63-8	Sigma	98			
N-pentadecanoic acid	1002-84-2	Sigma	99			
heptadecanoic acid	506-12-7	Sigma	98			
acrylic acid	79-10-7	Acros Organics	99			
undecanoic acid	112-37-8	Sigma	98			
benzoic acid	65-85-0	Sigma	99.5			
adepic acid	124-04-9	Acros Organics	99			
pimelic acid	111-16-0	Acros Organics	98			
4-hydroxybenzoic acid	99-96-7	Acros Organics	99			
L-(+)-lactic acid	79-33-4	Sigma	98	+	+	[1, 2, 3]
DL-3-methylvaleric acid	105-43-1	Acros Organics	97			

trans-2,3-dimethylacrylic acid	80-59-1	Acros Organics	98			
Aldehydes						
propanal	123-38-6	Sigma	97			
butanal	123-72-8	Sigma	99			
pentanal	110-62-3	Sigma	97			
hexanal	66-25-1	Sigma	98			
heptenal	111-71-7	Sigma	92	+	+	[4]
octanal	124-13-0	Sigma	99	+	+	[4]
nonanal	124-19-6	Aldrich	95	+	+	[4, 5]
decanal	112-31-2	Sigma	98	+	+	[4, 5]
isobutanal	78-84-2	Sigma	99			
2-methylbutanal	96-17-3	Sigma	90			
benzaldehyde	100-52-7	Sigma	99	+	+	[3, 5]
Alcohols						
p-cresol	106-44-5	Acros Organics	99			
4-methylphenol	123-07-9	Acros Organics	97		+	[3]
1-hexen-3-ol	4798-44-1	Sigma	98			
cis-2-hexen-1-ol	928-94-9	Aldrich	95			
trans-2-hexen-1-ol	928-95-0	Acros Organics	96			
trans-2-octen-1-ol	18409-17-1	Acros Organics	98			
2-decanol	1120-06-5	Sigma	98			
phenelethyl alcohol	60-12-8	Sigma	99			
glycerol	56-81-5	Sigma	99			

phenol	108-95-2	Sigma	99		+	[3]
1-tetradecanol	112-72-1	Sigma	97			
2-hexadecanol	14852-31-4	Sigma	99			
1-octen-3-ol	3391-86-4	Aldrich	99	+	+	[1, 2, 3]
Aromatics/Aliphatics						
hexane						
N-heptane	142-82-5	Sigma	99			
n-octane	111-65-9	Sigma	98			
N-nonane	111-84-2	Fisher	100			
n-decane	124-18-5	Fisher	99			
2,4-dimethyl hexane	589-43-5	Fidher	99			
N-pentadecane	629-62-9	Acros Organics	99			
hexadecane	544-76-3	Acros Organics	99			
n-heptadecane	629-78-7	Sigma	99			
n-octadecane	593-45-3	Sigma	99			
benzene	71-43-2	Sigma	99.8			
ethylbenzene	100-41-4	Sigma	99			
propylbenzene	103-65-1	Sigma	98			
styrene	100-42-5	Sigma	99			
squalene	111-02-4	Sigma	98			
toluene	108-88-3	Sigma	99.8			
xylene	106-42-3	Sigma	99.5			
2-pentene	109-68-2	Aldrich	99			
trans-2-octene	13389-42-9	Aldrich	97			
trans-3-octene	14919-01-8	Aldrich	98			

trans-4-octane	14850-23-8	Aldrich	98			
1-hexadecene	629-73-2	Aldrich	99			
1-tetradecene	1120-36-1	Aldrich	97			
		Esters				
methyl tridecanoate	1731-88-0	Acros Organics	97			
methyl nonanoate	1731-84-6	Acros Organics	95			
		Ketones				
2-butanone	78-93-3	Sigma	99.7		+	[3]
2-pentanone	107-87-9	Fisher	99			
2-hexanone	591-78-6	Fluka	96			
2-decanone	693-54-9	Aldrich	98			
3-pentanone	96-22-0	Fisher	99			
sulcatone	110-93-0	Sigma	98	+	+	[3, 4, 5]
		Halides				
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			
		Amines				
propylamine	107-10-8	Aldrich	99			
butylamine	109-73-9	Aldrich	99.5			
ammonia	7664-41-7	Aldrich	100		+	[3]

		Sulfides	
carbon disulfide	75-15-0	Fisher	99.9
methyl disulfide	624-92-0	Sigma	99
		Ureas	
methyl urea	598-50-5	Sigma	97
thiourea	62-56-6	Sigma	99
urea	57-13-6	Sigma	99
		Heterocyclics	
N-piperidineethanol	3040-44-6	Acros Organics	99
1-methylpiperazine	109-01-3	Acros Organics	99.5
2-methylfuran	534-22-5	Acros Organics	99
thiazolidine	504-78-9	Acros Organics	98
3-methylindole	83-34-1	Acros Organics	98
3-aminopyridine	462-08-8	Acros Organics	99
4-aminopyridine	504-24-5	Acros Organics	98
pyridine	110-86-1	Acros Organics	100
2,6-dimethylpyrazine	108-50-9	Acros Organics	96
coumarin	91-64-5	Acros Organics	99
4-piperidinemethanamine	7144-05-0	Acros Organics	97
2-picoline	109-06-8	Acros	98

		Organics			
indole	120-72-9	Aldrich	99	+	[3]
DMSO	67-68-5	Sigma	100		

*Human odorants were selected referring to the study of Bernier et al., 2000. All the human odorants were tested at the dose of 1:100 v/v, with the exception of dimethyl sulfoxide (DMSO), which was used as the solvent and 100% of DMSO was used as the control. Numbers refer to published behavioral studies or SSR studies of specific human odorant on the bed bugs: [1] Anderson et al. (2009); [2] Wang et al. (2009); [3] Harraca et al. (2010); [4] Harraca et al. (2012); [5] Siljander et al. (2008).

Table 4.2 Firing rates of responses of different olfactory sensilla to human odorants (n=6~10, Mean ± SEM)

Odorants	Dα±SEM	Dβ±SEM	Dγ±SEM	C±SEM	E1±SEM	E2±SEM
acetic acid	16.67±2.31	35.33±7.17	15.00±2.30	17.33±2.01	1.50±1.37	4.33±0.98
propionic acid	20.67±1.67	36.00±1.39	23.33±4.49	-1.33±2.57	-0.50±0.77	13.20±1.98
hexanoic acid	19.75±5.58	30.86±4.18	29.67±6.62	7.00±2.46	1.60±0.51	4.40±1.07
heptanoic acid	10.57±1.89	48.29±2.71	14.33±3.63	15.33±2.13	4.00±0.80	4.33±1.47
octanoic acid	10.80±2.62	31.20±4.10	24.50±2.55	4.40±1.54	5.33±0.62	15.60±2.07
N-nonanoic acid	9.14±1.44	22.00±3.91	22.67±3.73	19.20±5.70	3.33±0.80	5.00±1.00
decanoic acid	9.14±1.76	17.00±1.40	16.00±4.27	11.67±1.20	3.50±1.35	7.25±1.50
lauric acid	8.00±2.06	15.50±2.75	8.67±2.67	6.40±1.79	2.57±0.78	4.22±0.83
N-tridecanoic acid	10.29±2.19	21.75±1.33	15.20±2.82	7.60±0.83	3.56±0.91	6.25±1.13
myristic acid	14.22±2.79	48.00±4.96	9.56±2.11	16.40±2.79	2.00±0.53	8.67±3.46
N-pentadecanoic acid	8.86±1.99	18.29±2.16	23.09±3.16	12.40±2.43	2.00±0.40	49.00±4.40
heptadecanoic acid	6.00±0.80	10.00±1.39	21.33±4.19	18.00±1.60	1.20±0.44	8.00±1.60
acrylic acid	15.00±1.60	22.44±1.52	11.20±1.38	7.20±1.98	2.57±0.19	8.80±1.12
undecanoic acid	14.57±2.38	16.86±3.04	13.20±2.40	12.80±2.50	3.33±1.07	7.00±1.00
benzoic acid	9.14±1.67	15.50±1.45	18.91±4.32	8.33±1.24	2.50±0.80	4.75±1.38
adepic acid	12.67±2.49	22.75±3.98	12.67±2.79	10.80±2.18	5.00±0.93	5.25±1.43
pimelic acid	6.33±1.47	22.29±1.93	14.22±2.25	5.60±0.51	3.33±0.53	8.57±1.24
4-hydroxybenzoic acid	11.75±1.15	15.00±2.40	16.89±3.08	12.80±4.54	3.00±1.07	7.40±1.52
L-(+)-lactic acid	25.56±3.36	32.25±2.38	24.29±2.87	6.40±2.49	2.29±0.39	2.33±2.23
DL-3-methylvaleric acid	5.33±1.67	14.00±2.26	9.43±2.30	24.67±1.22	3.50±0.77	3.60±1.15
trans-2,3-dimethylacrylic acid	7.33±0.92	32.00±1.13	15.25±2.96	20.00±1.39	-1.50±1.65	7.20±1.54
propanal	7.50±0.40	40.67±3.33	36.67±2.80	29.33±4.82	7.50±1.51	2.33±0.44
butanal	35.50±3.02	51.00±7.32	43.33±3.30	24.67±6.99	4.00±0.57	8.57±1.63
pentanal	140.86±12.9	183.00±8.67	167.11±8.24	14.00±2.82	2.75±1.10	6.86±1.45

hexanal	220.22±13.6	180.00±14.20	186.25±5.50	22.00±4.38	6.67±1.42	6.67±1.65
heptenal	218.50±8.04	214.33±13.09	208.80±14.51	33.71±5.07	12.40±0.88	5.67±1.20
octanal	135.00±9.18	200.00±4.62	161.71±9.95	17.33±2.01	29.50±4.45	10.00±1.33
nonanal	248.50±13.9	212.67±10.18	223.43±9.97	21.00±3.60	16.40±2.29	4.67±1.60
decanal	74.50±3.6	85.33±2.44	107.67±8.94	8.67±1.85	13.20±3.38	5.00±1.33
Isobutanal	14.00±1.73	30.00±4.00	32.29±4.63	13.33±3.23	4.00±1.13	9.67±1.73
2-methylbutanal	38.20±4.38	69.00±6.4	63.00±4.80	13.00±1.03	2.86±1.08	8.50±2.45
benzaldehyde	19.80±3.9	55.25±2.83	41.11±5.83	22.00±4.20	5.33±0.95	4.25±1.45
p-cresol	6.86±1.53	19.71±2.61	10.25±2.33	7.67±1.24	3.60±1.41	6.67±1.01
4-methylphenol	13.67±2.00	17.71±1.67	12.75±2.10	1.67±0.67	3.00±0.67	9.25±1.90
1-hexen-3-ol	38.80±3.36	71.50±4.20	46.44±4.56	8.00±2.58	5.14±0.85	11.78±2.31
cis-2-hexen-1-ol	153.56±11.0	62.22±2.92	28.57±4.15	5.60±1.04	2.29±0.85	9.14±1.59
trans-2-hexen-1-ol	113.33±16.1	49.78±3.40	21.25±4.01	10.00±2.95	7.20±1.54	5.00±2.00
trans-2-octen-1-ol	8.86±2.95	52.00±7.12	20.29±2.97	16.80±4.82	4.80±0.90	8.80±1.40
2-decanol	21.50±3.19	40.50±3.44	31.00±5.18	6.67±2.07	3.20±0.70	5.60±1.00
phenelethyl alcohol	26.00±3.55	51.25±4.46	44.00±2.92	9.60±3.01	7.60±1.73	6.40±1.00
glycerol	13.33±2.36	73.00±4.46	41.33±9.50	4.40±1.64	11.00±3.80	4.80±1.10
phenol	9.00±1.39	8.57±2.64	10.67±2.13	21.43±4.18	-1.50±1.77	5.00±1.87
1-tetradecanol	7.75±1.13	12.50±2.35	13.40±2.59	7.33±1.16	6.00±1.07	14.40±2.62
2-hexadecanol	10.25±1.70	17.80±2.32	21.50±4.40	8.40±2.07	3.50±1.00	14.33±4.68
1-octen-3-ol	36.00±4.93	59.67±5.48	45.00±3.80	31.43±6.29	8.00±1.60	7.33±0.62
hexane	24.50±3.22	14.00±2.26	30.67±4.40	15.50±2.86	2.50±0.40	11.00±1.47
N-heptane	14.00±2.21	33.43±5.42	21.25±5.17	15.33±4.22	3.60±1.09	7.20±0.38
n-octane	22.89±2.27	53.25±2.30	31.25±3.70	15.60±1.54	4.33±1.20	7.67±1.38
N-nonane	12.86±4.33	25.14±3.66	20.00±4.42	22.67±5.39	3.50±0.60	5.50±1.24
n-decane	14.20±2.64	33.50±4.05	21.14±2.81	4.00±1.52	2.40±0.51	8.86±2.30
2,4-dimethyl hexane	15.60±2.75	34.25±3.68	56.00±6.40	15.33±2.61	2.33±1.02	4.33±1.92
N-pentadecane	7.00±1.40	17.71±1.73	15.50±3.00	15.60±3.71	2.67±0.89	7.80±1.20

hexadecane	8.33±2.58	30.00±2.67	14.67±2.37	5.20±1.54	-3.60±0.58	34.89±3.50
n-heptadecane	16.22±2.94	35.00±3.50	25.00±4.50	5.67±1.55	5.33±0.89	12.33±3.18
n-octadecane	13.33±2.25	29.71±3.98	19.50±5.20	6.00±1.13	2.29±0.42	8.00±2.99
benzene	15.33±4.89	42.00±3.39	32.33±7.13	24.67±3.61	3.00±1.03	5.00±1.07
ethylbenzene	22.67±2.51	55.00±6.31	68.86±5.64	16.00±3.27	-6.00±1.20	8.00±1.80
propylbenzene	25.67±2.34	40.25±5.60	84.00±1.90	18.00±5.94	7.60±2.43	7.50±2.60
stylene	21.00±2.12	72.00±5.25	81.43±7.98	18.00±5.60	6.80±1.84	6.33±1.73
squalene	15.00±1.66	14.50±3.49	19.14±2.73	8.33±2.84	-8.80±1.02	6.33±1.40
toluene	21.67±2.55	62.57±7.24	65.14±6.21	12.00±2.95	5.20±1.02	6.00±2.49
xylene	18.00±4.45	50.67±1.22	64.86±7.23	23.33±1.22	13.50±4.50	6.29±1.96
2-pentene	15.00±3.28	32.00±4.20	29.71±3.33	2.00±0.80	3.60±0.83	6.67±1.20
trans-2-octene	9.33±1.8	41.33±5.80	35.20±5.72	5.00±0.84	2.67±0.89	5.71±0.64
trans-3-octene	35.33±2.96	71.60±6.40	34.67±4.33	4.57±1.51	3.71±0.39	6.57±1.00
trans-4-octene	30.75±2.30	69.78±6.12	29.56±3.04	14.00±3.43	5.00±1.47	5.50±2.08
1-hexadecene	10.22±1.70	43.78±3.50	12.57±2.78	5.67±1.38	2.00±0.46	41.67±5.29
1-tetradecene	14.73±2.16	38.33±5.51	13.67±2.53	13.00±2.62	4.29±1.31	60.00±5.63
methyl tridecanoate	22.67±5.79	24.67±2.31	28.67±5.08	18.00±0.80	22.50±4.54	31.33±2.93
methyl nonanoate	19.67±3.01	34.25±6.53	29.50±3.14	8.00±2.09	6.00±2.24	10.00±2.13
2-butanone	12.29±3.21	59.71±7.80	10.75±3.28	23.33±5.58	6.00±1.60	3.00±1.00
2-pentanone	36.00±4.45	102.00±4.80	59.00±5.40	29.33±4.89	7.00±3.55	4.00±1.33
2-hexanone	50.00±4.07	138.00±9.12	110.75±6.10	7.14±1.95	9.25±1.18	3.50±1.26
2-decanone	30.73±4.73	100.00±5.20	86.75±4.78	9.60±2.29	9.71±1.01	11.14±2.39
3-pentanone	46.18±5.17	122.67±4.80	59.00±4.64	6.67±1.87	5.67±1.20	14.67±2.41
sulcatone	24.67±5.76	40.00±7.16	226.33±7.36	12.86±2.65	5.67±1.20	3.33±1.10
1-chloroheptane	61.33±4.50	131.43±3.53	76.22±2.94	12.33±4.39	8.67±1.60	4.25±0.79
1-chlorododecane	18.80±2.78	33.71±4.24	21.43±4.38	5.20±0.72	6.33±0.76	57.33±9.49
lauryl chloride	10.57±1.51	17.75±3.64	10.50±2.21	-9.20±2.09	14.00±2.00	58.80±6.00
1-chlorotetradecane	12.33±1.63	25.75±3.81	17.71±2.71	17.33±2.61	1.60±0.51	39.20±4.60

1-chlorohexadecane	17.40±2.80	39.00±3.07	21.75±2.75	23.00±4.10	3.14±0.62	20.33±1.38
1-chlorohexane	136.00±13.6	146.25±12.59	41.33±5.74	23.20±5.44	-7.20±2.05	14.50±0.40
benzyl chloride	8.86±2.57	21.50±5.22	37.14±4.88	6.80±2.01	2.80±0.83	13.60±1.90
propylamine	3.50±1.20	28.00±3.32	22.00±3.20	194.57±15.93	4.00±1.73	5.33±0.98
butylamine	5.00±1.03	14.67±1.73	18.00±2.49	144.00±12.06	6.00±1.96	-0.33±0.67
ammonia	8.50±0.40	34.00±3.39	48.00±6.14	200.00±6.97	1.00±2.96	2.57±0.78
carbon disulfide	6.57±1.51	28.67±4.70	9.56±1.31	-7.67±1.63	5.60±1.47	9.60±1.60
methyl disulfide	6.00±2.09	94.00±18.35	7.67±1.38	5.67±1.38	4.50±1.40	7.67±1.40
methyl urea	6.29±1.04	10.00±1.33	11.56±2.61	10.80±1.22	3.14±0.98	5.20±1.38
thiourea	6.00±0.69	10.25±1.53	16.22±3.65	5.20±0.90	2.50±0.80	10.57±0.95
urea	13.14±1.67	23.75±2.90	14.50±2.35	6.00±1.28	2.33±0.44	6.89±1.64
N-piperidineethanol	7.71±2.14	18.57±3.05	14.50±3.44	30.67±5.78	3.50±1.40	5.50±1.60
1-methylpiperazine	16.29±4.65	10.00±2.73	12.25±3.51	176.80±16.34	-4.50±1.00	2.80±0.53
2-methylfuran	11.67±0.79	47.56±5.13	19.71±2.33	9.33±2.38	3.71±1.04	8.40±1.24
thiazolidine	5.33±0.79	21.71±2.33	18.57±3.51	129.60±14.30	5.60±1.41	4.00±1.07
3-methylindole	6.00±1.60	14.86±2.35	17.33±2.96	9.60±2.37	3.33±0.89	6.89±2.00
3-aminopyridine	11.14±2.12	21.78±2.23	11.33±2.31	4.80±1.34	4.00±0.53	11.25±2.58
4-aminopyridine	10.33±1.73	21.75±1.30	10.75±1.58	11.20±2.62	4.00±0.27	9.40±1.52
pyridine	7.14±1.31	25.14±2.22	23.25±1.78	8.40±1.15	2.25±0.73	8.40±1.60
2,6-dimethylpyrazine	18.00±1.60	74.00±6.72	56.00±5.69	10.40±1.41	4.86±0.72	7.40±2.27
coumarin	5.14±1.89	19.14±1.53	20.00±2.60	1.00±0.40	3.75±1.30	9.20±1.28
4-piperidinemethanamine	4.67±0.46	5.00±0.57	18.86±3.54	21.33±7.85	3.00±2.12	7.60±2.30
2-picoline	6.00±1.79	39.75±5.17	41.43±4.10	18.00±5.08	7.67±1.73	4.00±0.71
indole	12.00±1.60	51.67±5.93	70.57±5.52	28.57±3.54	5.33±1.20	6.29±0.85
DMSO	1.64±0.48	2.89±0.49	2.44±0.55	2.86±0.43	1.20±0.38	1.00±0.44

Table 4.3 Euclidean Distance (ED) of the top ten closest and farthest odorant pairs in the odor space of bed bugs.

Closest odorant pairs	ED (Spikes/s)	Farthest odorant pairs	ED (Spikes/s)
Decanoic acid/4-hydroxybenzoic acid	3.6	Nonanal/ Propylamine	406.2
Urea/Tridecanoic acid	4.1	Nonanal/ Methylpyrazine	405.3
Methylindole/ Benzoic acid	4.4	Nonanal/ Butylamine	394.9
Decanoic acid/ Methylindole	4.5	Nonanal/ Ammonia	390.6
Urea /Acrylic acid	4.6	Nonanal/ Thiozilidine	386.6
Pentadecane/ Decanoic acid	4.6	Nonanal/methyl urea	380.7
Decane/ Octadecane	4.7	Nonanal/ Perperidinemethamine	380.3
Undecanoic acid/4-hydroxybenzoic acid	5.0	Nonanal/ Phenol	380.3
Pimelic acid/ Tridecanoic acid	5.1	Nonanal/ Methylvaleric acid	380.2
Adipic acid/ Tridecanoic acid	5.1	Nonanal/ Lauric acid	378.5

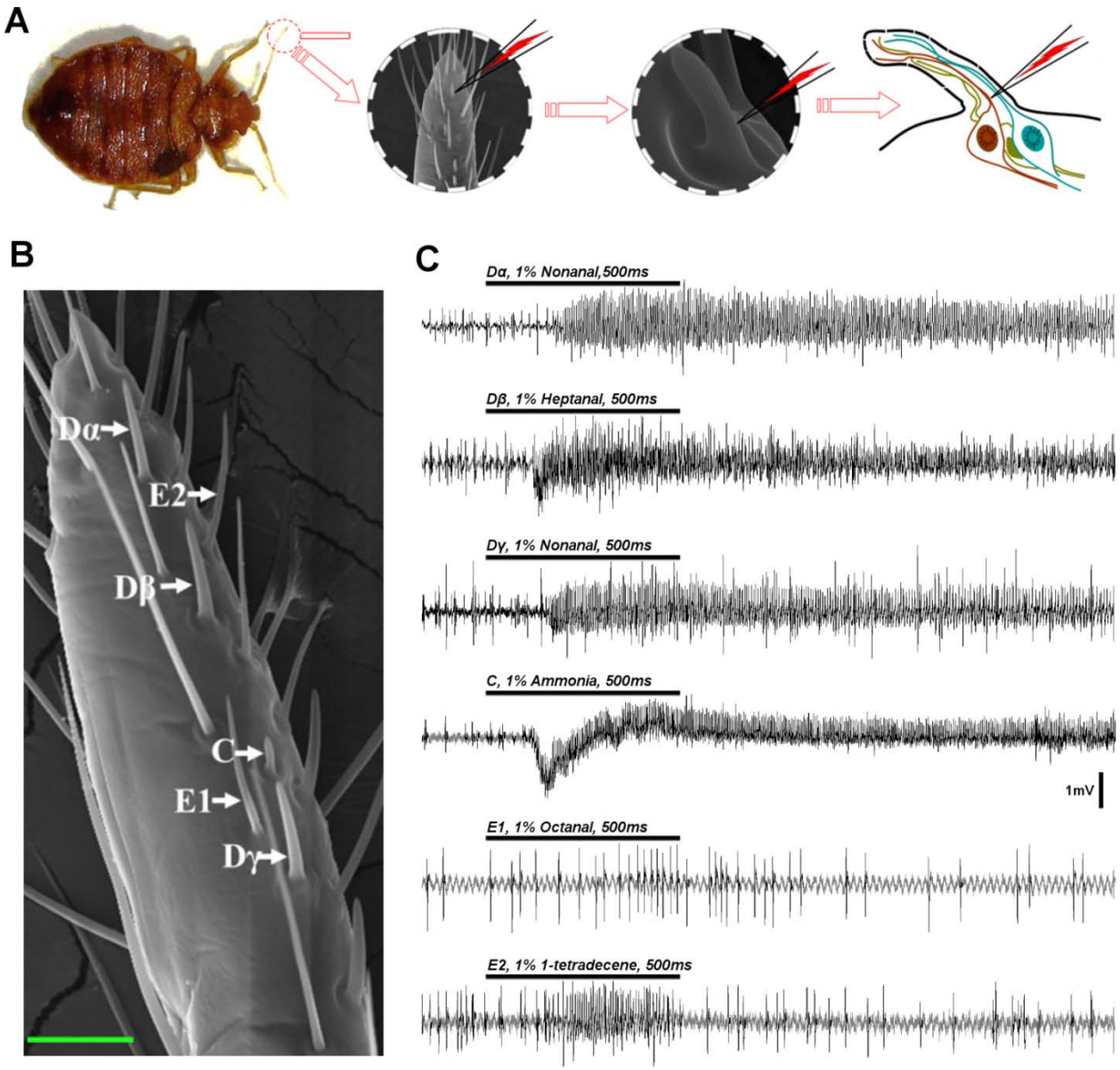

Figure 4.1 Single sensillum recording on different types of olfactory sensilla in the common bed bug, *C. lectularius*. A) Schematic image of single sensillum recording in the olfactory sensilla on bed bug antennae. **B)** SEM photo (modified from Liu et al., 2014) showing the different types of olfactory sensilla on bed bug antennae. The scale bar indicates 20 µM. **C)** The highest neural responses for each type of olfactory sensillum to different human odorants.

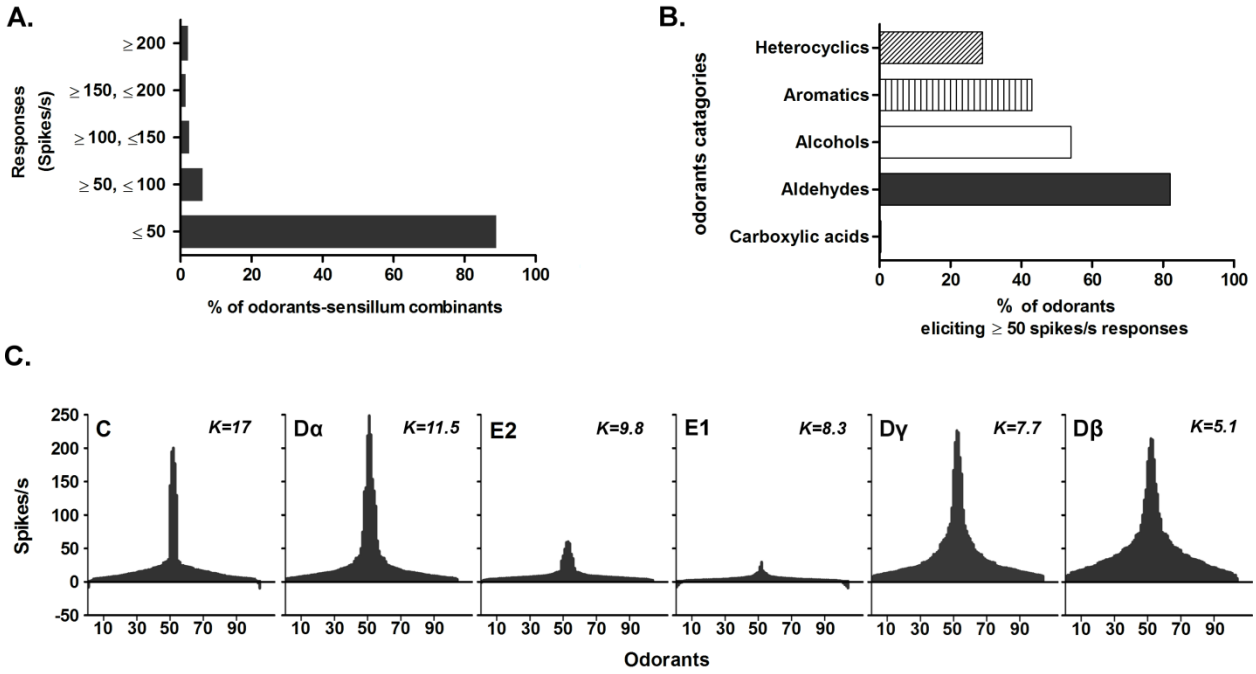

Figure 4.2 Summary of the responses of olfactory sensilla in bed bugs to human odorants. A) Distribution of firing frequencies for different strengths of responses to different odorant/sensillum combinations; **B**) Response biases to different odorant categories with firing frequencies greater than 50 spikes/s. Sensilla that failed to show a response ≥ 15 spikes/s were considered non-responders. The excitatory response of 50 spikes/s was selected as the criterion which represents 20% of the largest firing frequency recorded (248.5 spikes/s, for nonanal in Dα sensilla). **C**) Tuning curves of olfactory sensilla for human odorants. The 103 odorants are distributed along the x axis according to the strengths of the responses they elicited from each sensillum. The odors that elicited the strongest responses are near the center of the distribution; those that elicited the weakest responses are near the edges. The order of the odorants therefore differs among sensilla. Negative values indicate inhibitory responses.The kurtosis value, K value, as a statistical measure of 'peakedness', is shown on the right side for each plot.

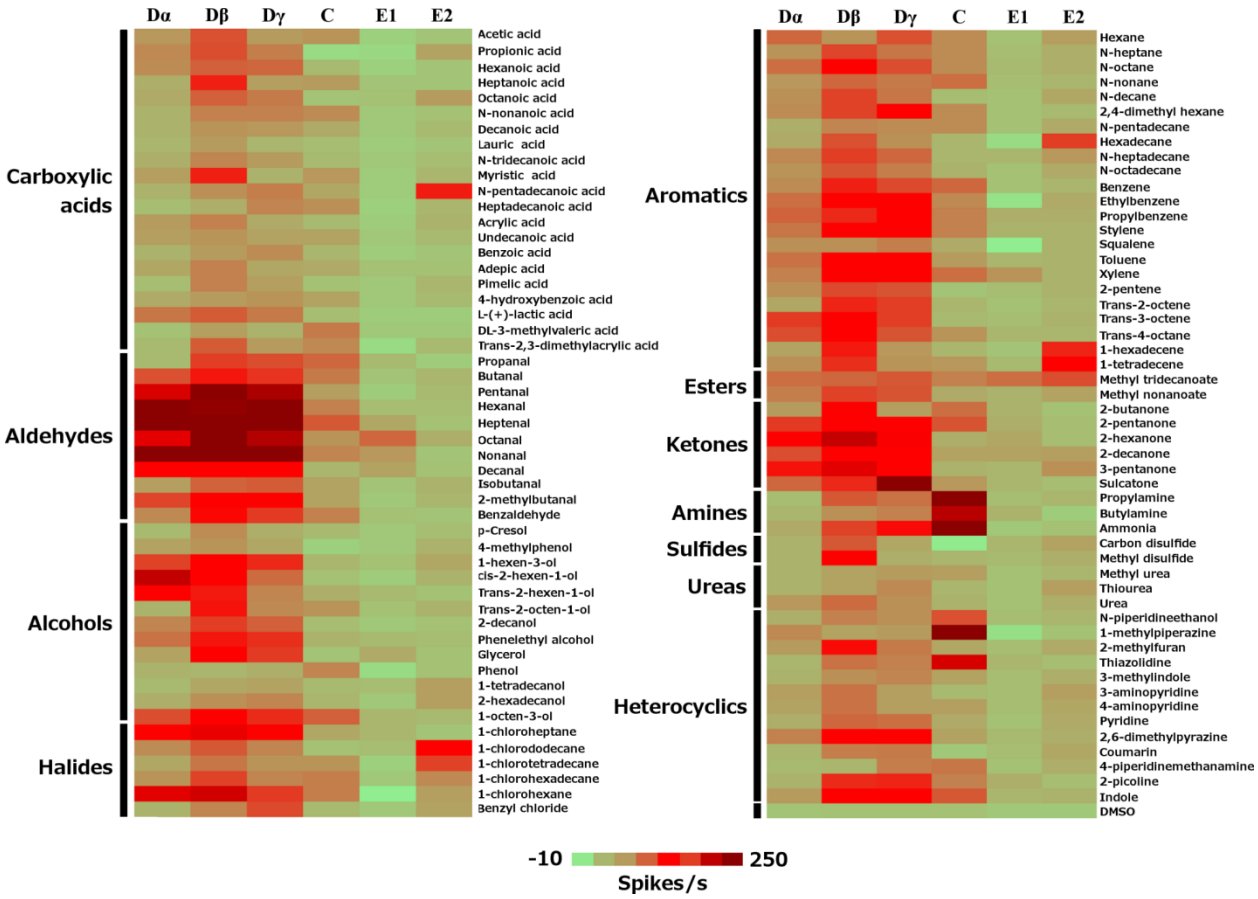

Figure 4.3 Heatmap presentations of the responses of olfactory sensilla to human odorants. Distinctive response profiles (spikes/s) of Dα, Dβ, Dγ, C, E1 and E2 sensilla to different chemical groups of human odorants were tested through single sensillum recording, with at least six replicates for each odorant on different individual sensilla at a dose of 1:100 v/v. The solvent, DMSO, produced no stimulation in any of the sensilla types.

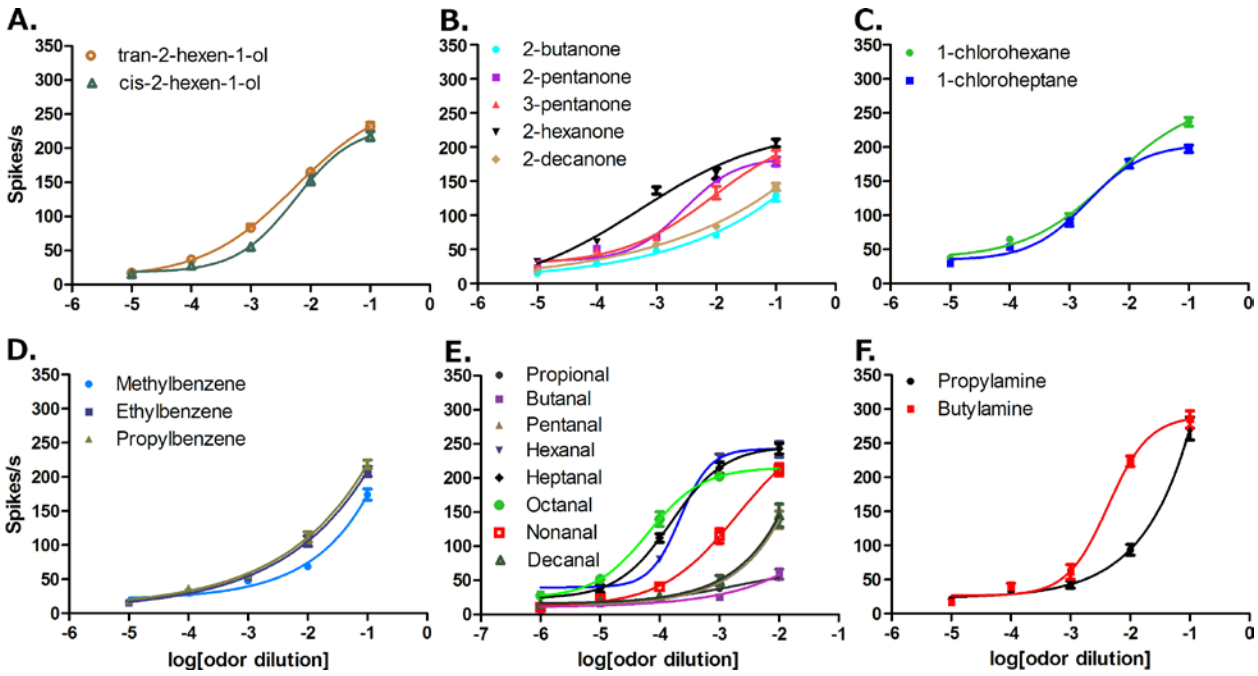

Figure 4.4 Dose-dependent responses of bed bug olfactory sensilla to human odorants. The dose-dependent response curve is presented as a mean value ± SEM, n ≥ 6. **A)** Dose-dependent response of Dα sensilla to two stereoisomers of 2-hexen-1-ol; **B)** Dose-dependent response of Dβ sensilla to ketones; **C)** Dose-dependent response of Dβ sensilla to halides; **D)** Dose-dependent response of Dγ sensilla to aromatics; **E)** Dose-dependent responses of Dγ sensilla to aldehydes; and **F)** Dose-dependent response of grooved peg C sensilla to two amines, propylamine and butylamine. The X axis describes the logarithm dilution series from 1:10 to $1:10^5$ v/v in A, B, C D and F, and from $1:10^2$ to $1:10^6$ v/v in E.

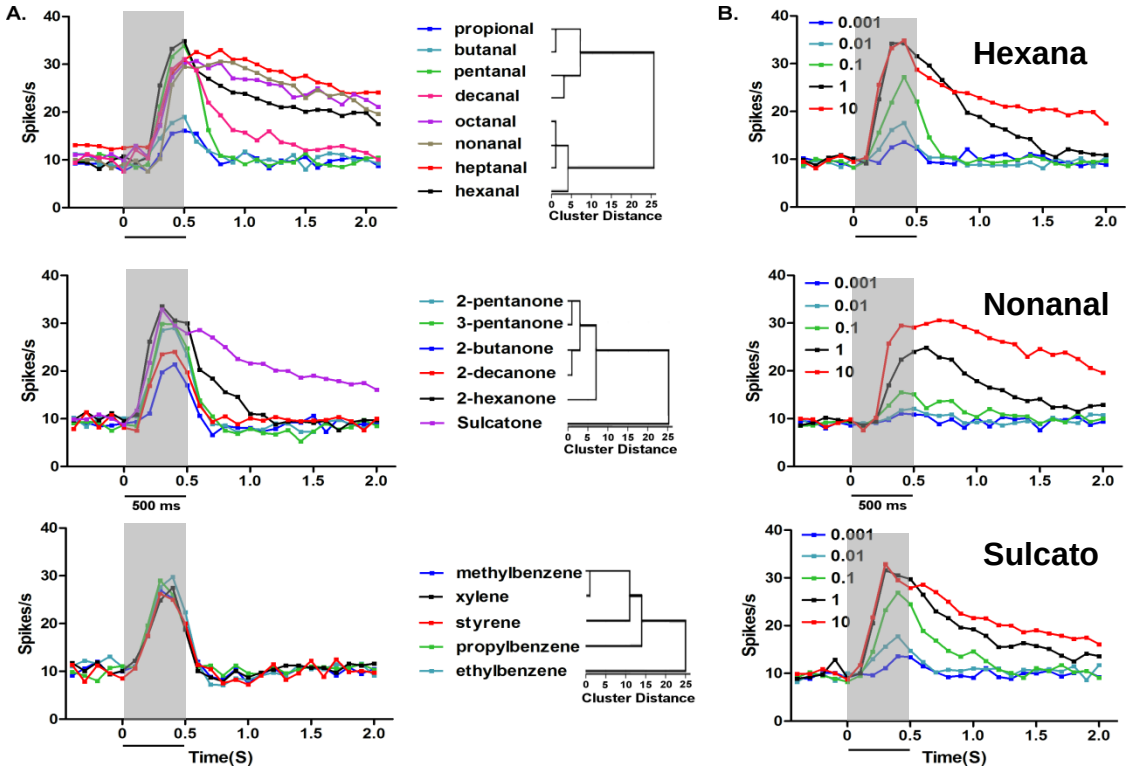

Figure 4.5 Temporal dynamics of olfactory sensilla in response to human odorants. A) Temporal structures of neuronal responses of Dα sensilla in response to aldehyde, ketone and aromatic odorants at a dose of 1:100 v/v. The left side of the figure shows a trace representing the mean value of spikes (n=8, error bars are not shown) recorded during each 100 ms sampling period. The right side of the figure shows the hierarchical cluster analysis for the odorants, with the corresponding categories based on the action potential number in each single 100ms sampling period. **B)** Temporal structures of dose-dependent responses of Dα sensilla in response to hexanal, nonanal and sucaltone at doses ranging from $1{:}10^2$ to $1{:}10^6$ v/v. Horizontal bars indicate the duration of the stimulation (500 ms).

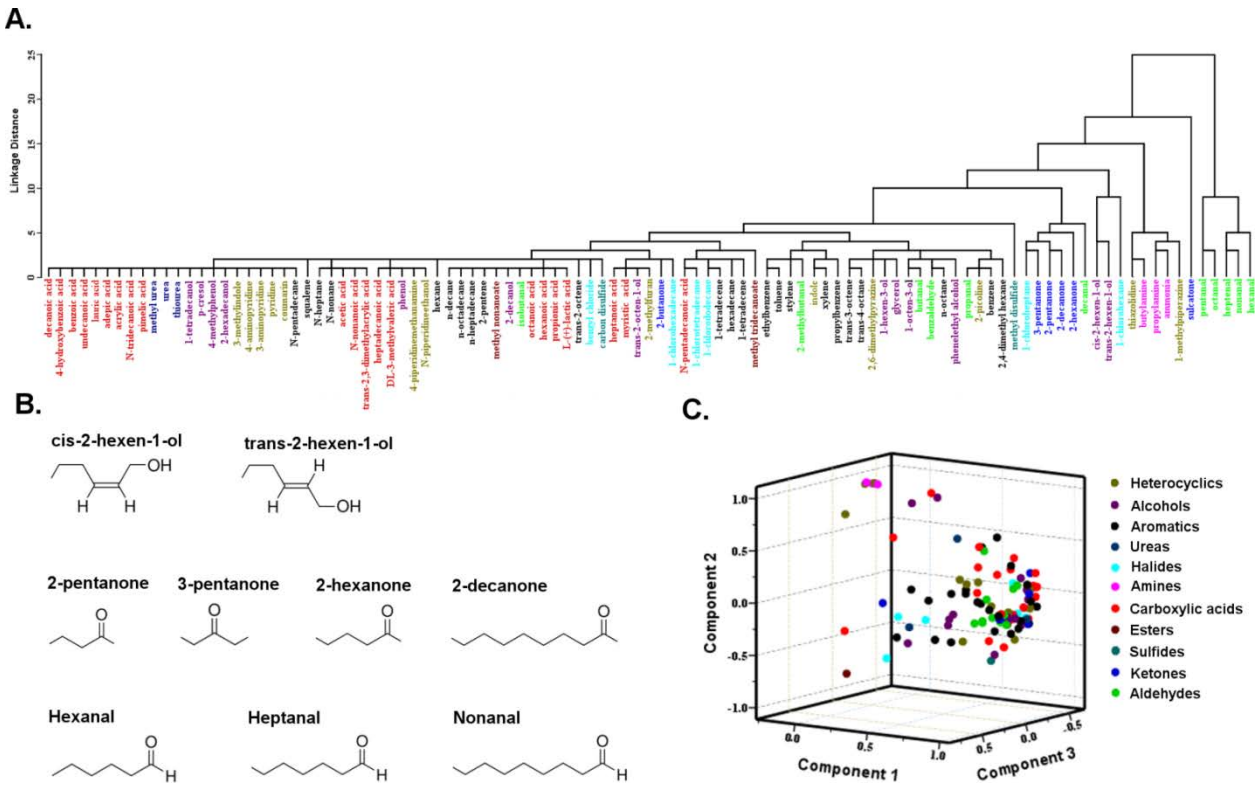

Figure 4.6 Primary presentations of odorant space among the olfactory sensilla. A) Hierarchical cluster analysis for human odorants based on the Euclidean distances between them. Odorants are color coded by chemical class. **B)** Typical odorants with close chemical structure are clustered together in the Hierarchical cluster analysis. **C)** Relationships among human odorants of the indicated chemical classes at a dose of $1{:}10^2$ v/v revealed by PCA. Odorants are color coded by chemical class as in Fig. 6A. In PCA, vectors quantifying the responses of the 6 antennal sensilla to each tested odor are projected onto a three-dimensional region. Each axis represents the normalized neuronal responses of the olfactory sensilla in a new coordinate system determined by PCA. This three-dimensional representation captures 87.67% of the variation in the original 6-dimensional data set.

Chapter 5: Molecular Basis of Chemoreception in the Common Bed Bug

5.1 Abstract

As one of the most notorious ectoparasites, bed bugs rely heavily on human or animal blood sources for survival, mating and reproduction. Chemoreception mediated by the odorant receptors on the membrane of olfactory sensory neurons, plays a vital role in the process of host seeking and risk aversion of bed bugs. To understand the molecular basis of chemoreption in the common bed bug, we investigated the responses of odorant receptors to a large spectrum of semiochemicals, including human odorants and plant-released volatiles. We found that strong responses were sparse and aldehydes/ketones were the most efficiency stimuli, while carboxylic acids and aliphatics/aromatics were comparatively less effective in eliciting the responses from the bed bug odorant receptors. Odorant reception of bed bug follow a combinatory model with each odorant receptors responded to single/multiple odorants or individual odorant was recognized by single/multiple receptors presenting a continuing tuning breaths. Both the odorant identity and dosages played important roles in determining the strength of responses. The odor space constructed based on the responses from all the tested odorant receptors revealed that odorants within the same chemical groups were widely dispersed while odorants from different groups intermingles together, suggesting the complexity of odorant encoding in the bed bug ORs. This study provides a comprehensive picture of olfactory coding mechanisms of bed bugs that ultimately benefit the design and development of novel olfactory-based strategies for reducing the biting nuisance and disease transmission from bed bugs.

5.2 Introduction

Chemoreception is critical for insects in locating the hosts, finding mate, identifying oviposition site and avoiding predators in their environment. The common bed bug, *Cimex*

lectularius, as a resurgent parasite for human being or animals, has showed a strong ability to detect a large panel of stimuli from human odorants or plant-released volatiles which have been used as chemical repellents for mosquitoes or other hematophagous arthropods (Liu et al., 2014, 2015; Harraca et al., 2012). The olfactory receptor neurons (ORNs) housed in the olfactory sensilla on the bed bug antennae showed extremely sensitive to several chemical classes, such as aldehydes/ketones and amines, in the human odors or some terpene-derived stimuli extracted from the plants (Liu and Liu, 2015).

In the olfactory receptor neurons, olfactory receptors on the neuron membrane are responsible for detecting the chemical stimuli in the surrounding. Odorant receptors (OR) as the most extensively investigated clade of olfactory genes, have played a fundamental role in the chemoreception of various insect species. Odorants with biological meanings for insects will be specifically recognized by the ORs in the neuron membrane and trigger the firing process in the ORNs (Hallem et al., 2006; Carey et al., 2011; Leal 2013), which provides the primary olfactory information for further odor identification in the central nervous system.

Previous studies on the bed bug olfactory system indicated that bed bugs possess a degenerative olfactory system with much fewer olfactory sensilla and fewer ORs compared to other insect species (Benoit et al., 2016). Indeed, there are only about 44 sensillum (29 E sensillum/6 D sensillum/9 C sensillum) on the second flagellum (Harraca et al., 2010) and a few olfactory sensillum (2 E sensillum/2 D sensillum/6 C sensillum) on the first flagellum of bed bug antennae (Olson et al., 2014). The transcriptome analysis for the bed bug antennae only identified 16 ORs with significant expression (Hansen et al., 2014). The recently published bed bug genome sequence revealed about 47 ORs of bed bugs (Benoit et al., 2016). Considering that bed bugs, as one of the wingless insects, always live closing to their hosts and aggregate together, their olfactory system may not be required to handling very complex chemical environment as other insects often do, like the yellow fever mosquitoe (*Aedes*

aegypti) and honeybees (*Apis mellifera*), which own 131 and 170 ORs, respectively (Bohbot et al., 2007; Robertson and Wanner, 2006).

Even though the olfactory neuronal responses of bed bugs to human odorants or some insect chemical repellents have been extensively characterized, very few research has been conducted to decipher the molecular basis of the chemoreception in the common bed bugs. Previous study in our group initiated the investigation on the function of two bed bug ORs (OR5 and OR9b, previously named as OR1 and OR2, respectively) in response to 42 odorants from human odorants and very limited information of molecular basis of chemoreception had been revealed (Liu and Liu, 2015). Therefore, to gain a better understanding the function of bed bug ORs and deorpherize the molecular basis of chemoreception in the common bed bugs, in this study, we successfully characterized the function of 15 bed bugs ORs in response to a much bigger chemical panel, 148 odorants from both human odorants and botanical chemical stimuli, which provided a much more informative and general picture about the sensory ecology of bed bugs.

5.3 Materials and Methods

5.3.1 Insects

The *C. lectularius* colony utilized in this study originated from Ft. Dix, New Jersey, USA. It is susceptible to pyrethroid insecticides (Romero et al. 2007). The bed bugs were fed with rabbit blood once every week in the laboratory. Blood was purchased from Hema Resource and Supply Company (Aurora, OR). All bed bugs were reared at 25±2°C under a photoperiod of 12:12 (L: D)

5.3.2 RT-PCR, cDNA cloning and cRNA synthesis

Adult bed bugs were cold anesthetized with ice. Olfactory tissues (antennae) were hand dissected and stored in dry ice for RNA extraction. Total RNA was extracted from adult olfactory appendages using the acidic guanidine thiocyanate-phenolchloroform method (Liu

and Scott. 1997) and used for oligo (dT)-primed cDNA synthesis with SuperScript III reverse transcriptase (Invitrogen) for the generation of templates for subsequent PCR reactions using full-length primers with specific restriction enzyme cutting site added (Table S1). The purified PCR products were cloned into pT7Ts vector (a gift from Dr. Wang in Institute of Plant Protection, CAAS), with a Kozak sequence added behind the cutting site in the forward primer. The constructed vectors were linearized and cRNAs synthesized from the linearized vectors with mMESSAGE mMACHINE T7 (Ambion, Carlsbad, CA).

5.3.3 *Xenopus* oocyte expression system and two-electrode voltage-clamp

Mature healthy oocytes (stage V–VII) (Nasco, Salida, CA) were treated with collagenase I (GIBCO, Carlsbad, CA) in washing buffer (96 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$, and 5 mM HEPES [pH = 7.6]) for about 1 h at room temperature. After being cultured overnight at 18°C, oocytes were microinjected with 5 ng cRNAs of both ORs and ORCO. After injection, oocytes were incubated for 4–7 days at 18°C in 1X Ringer's solution (96 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$, 0.8 mM $CaCl_2$, and 5 mM HEPES [pH = 7.6]) supplemented with 5% dialyzed horse serum, 50 mg/ml tetracycline, 100 mg/ml streptomycin and 550 mg/ml sodium pyruvate. Whole-cell currents were recorded from the injected *Xenopus* oocytes with a two-electrode voltage clamp. Odorants-induced currents were recorded with an OC-725C oocyte clamp (Warner Instruments, Hamden, CT) at a holding potential of −80 mV. Odorants (~100% purity) were dissolved in DMSO at a 1:10 ratio to make stock solutions and then the stock solution was further diluted with 1× Ringer's solution to the desired concentrations (Wang et al., 2010). Data acquisition and analysis were carried out with Digidata 1440A and pCLAMP 10.2 software (Axon Instruments Inc., CA). Dose-response data were analyzed by GraphPad Prism 5.0 (GraphPad Software Inc, CA).

5.3.4 Statistical analysis

Principle component analysis and hierarchical cluster analysis were performed using PASW Statistic 18 (IBM, NY).

5.4 Result

5.4.1 Evolutionary stability of bed bug OR family

Based on the genomic data from Benoit and his colleagues (2016), we did the phylogenetic analysis for ORs of two blood-sucking Hemipterans, bed bug (C. lectularius) and kissing bug (*Rhodnius proxilus*), and one plant-feeding Hemipteran, stink bug (*Halyomorpha halys*). Forty seven predicted ORs were used to build the phylogenetic tree with 72 ORs from kissing bug (www.vectorbase.org) and 133 ORs from stink bug (http://www.ncbi.nlm.nih.gov/). According to the phylogenetic tree, Orco genes from all three organisms are clustered together due to their highly conserved amino acid sequence (Fig. 5.1). Specific OR gene expansion was observed in both kissing bug and stink bug when two branches of stink bug ORs rarely presented any relative ORs from either bed bugs or kissing bugs and one branch of kissing bug ORs showed no putative orthologs from bed bugs and stink bugs. However, we found that no bed bug-specific OR gene expansion was demonstrated in the phylogenetic tree with most of the bed bug ORs clearly clustering with certain ORs from either kissing bugs or stink bugs, which suggested a slowly evolving rate of bed bug OR gene family. The relative conservativeness of the OR gene family also suggested a comparatively stable chemosensory ecology in bed bugs, which may result from their obligate blood-feeding requirement, narrow host spectrum and relatively simple habitat (always close to the hosts).

5.4.2 Response profiles of bed bug ORs to odorant stimuli

The *Xenopus* oocyte expression system has been successfully used in characterizing the function of ORs from multiple insect species (Bohbot and Dickens, 2009; Wang et al., 2010; Xu et al., 2014). In our study, 15 of 21 of the ORs tested gave rise to specific odorant-induced response profiles when co-expressed with ORCO in *Xenopus* oocytes. The remaining six ORs

gave no responses to any odor panel component. Overall, 2220 odorant-receptor combinations were tested individually in the oocyte voltage-clamp system with functional interactions that displayed significant variation in the absolute amplitude of OR current responses.

To facilitate a comparison between all OR-odorant pairs, responses were normalized by defining the maximal odorant response for each receptor as 100 response units (RU). In this light, strong current responses were really sparse (Fig. 5.2). Only 3.96% of OR-odorant pairs displaying responses falling into the range of ≥ 20, ≤ 40 RU of the maximal responses; 1.71% of OR-odorant pairs displayed responses in ≥ 40, ≤ 60 RU; 0.59% of OR–odorant pairs displayed responses in ≥ 60, ≤ 80 RU; only 0.95% of OR–odorant pairs displayed responses higher than 80 RU (Fig. 5.3A).

This normalization allowed us to assess bed bug OR responses among different chemical groups. We found the average frequency of strong responses (>20 RU) evoked by odorants in different chemical groups are quite variant. Specifically, aldehydes/ketones are the most efficient group that elicited strong responses (>20 RU) from 2.6 ORs per odorant. The alcohols, terpenes/terpenoids and heterocyclics also triggered strong responses (>20 RU) on at least 1 ORs per odorant, compared with that in aliphatics/aromatics (0.38 OR/odorant) and carboxylic acid (0.17 OR/odorant) (Fig. 5.3B).

Previously, almost the same panel of odorants had been used to test the neuronal responses of bed bugs by the single sensillum recording (SSR) system (Liu et al., 2014; Liu and Liu, 2015), which enables us to make a comparison between the sensory spectrums of olfactory receptor neurons (ORNs) and ORs. No surprisingly, most of the odorants (45 out of 67) eliciting active ORNs responses (≥ 50 spikes/s or 20% of the maximal responses) were also very effective in activating the ORs (Fig. 5.3C). If we looked into the major odorant groups tested in both experimental system, variances were showed in the receptive spectrum within these

odorant groups (Fig. 5. 3D). For example, all the aldehydes active in ORNs (SSR system) were also effective in ORs (oocyte expression system), while only half of odorants active in ORNs demonstrated effectiveness in activating ORs. Considering that we only characterized the response spectrum of 15 ORs (about one third of the total ORs), it was very promising that most of the odorants active in SSR system will be covered by the oocyte expression system. However, to the opposite, certain odorants active in ORs were not perceived by the ORNs, which may lie in the disadvantage of the oocyte expression system that only naked ORs were tested with no involvements of other factors, such as odorant binding protein, which may play a selective role in delivering the odorants to ORs on the neuron membrane. Therefore, it makes sense for ORs possessing a larger response spectrum than ORNs.

5.4.3 Tuning breadth of bed bug OR repertoire

In order to compare the specific response spectrum of individual bed bug OR to the odorants, OR tuning curves were generated (Fig. 5.4) referring to the study of Wang et al. (2010). Several ORs (e.g. OR15, 17, 9b, 37) appeared to be specialists, with each OR responding strongly to very few odorants. For example, OR15 displayed particular sensitivity to β-caryophyllene, OR17 was strongly response to coumarin and OR37 was found to be very sensitive to citral (Fig. 5.4). At the other end of the spectrum, OR1, OR20, OR19, OR36 were more likely to be classified as generalists as they showed responses to multiple odorants within several chemical groups. For example, OR36 was found to respond strongly to about 30 structurally diverse odorants, including aldehydes, ketones, aliphatics/aromatics, terpenes/terpenoids, and alcohols (Fig. 5.4). All these tuning curves from 15 bed bugs ORs clearly demonstrated that the receptive range of bed bug ORs followed a continuing pattern and varies smoothly from very narrow to broadly tuned, which was consistent with previous findings in fruit fly (*Drosophila melanogaster*) (Hallem and Carlson, 2006) and malaria mosquito (*An. gambia*) (Carey et al., 2010; Wang et al., 2010).

Interestingly, some narrowly tuned ORs showed extremely strong response to compounds that are biologically important for bed bugs. For example, both OR9b and OR21 responded strongly to decanal (Fig. 5.4), which is a very important component of bed bug aggregation pheromone linked to bed bug's aggregation behavior (Siljander et al. 2008; Gries et al. 2015). Furthermore, OR37 is narrowly tuned with citral (Fig. 5.4), which showed very strong excito-repellency for bed bugs (unpublished data).

5.4.4 Odor coding and odorant identity

When we looked into the response profiles of bed bug ORs to different odorants, it is evident that odorant identity showed great impact on the responses of individual OR, especially among some structurally similar odorants. For instance, OR15 is exclusively sensitive to β-caryophyllene, but showing very weak response to (-)-caryophyllene oxide (Fig. 5.2). Another very significant example is OR36, which presented remarkable response to trans-3-octene and trans-4-octene but very weak responses to trans-2-octene, which suggested that the double bond position in the molecule is vital for the activating efficiency for the OR36 (Fig. 5.2).

The bed bug ORs not only showed strict requirements for the chemical structures, but also for the stereotypes of the isomers of the same chemical. For example, (+)-menthone was found to evoke remarkable current response (242 nA) on OR46 while (-)-menthone only activated minor current response (25 nA) (Table S2). Similarly, (+)-β-pinene (315 nA) elicited much stronger response on OR20 comapred to (-)-β-pinene (55 nA). These results further suggested the superior capacity for bed bug ORs to discriminate odorants with subtle variances in their chemical structure.

As different odorants were recognized or encoded by different ORs of bed bug, it would be interesting to compare the receptor spectrum response to different odorants. In order to better

present our results, we generated the “odorant tuning curves” to represent the odorant-activated receptors with differential responses, which is the reciprocal of receptor tuning curves and considered as a complementary analysis approach in identifying receptors and odorants that are important for innate insect behavioral responses (Carey et al., 2010). Tuning curves of 32 odorants, which showed very effective in activating single or multiple ORs were selectively presented according to their tuning breadth (Fig. 5). Similar to the receptor tuning curve, some odorants were found to be narrowly recognized by very few ORs while some other odorants were broadly recognized by multiple ORs. For instance, trans-3-octene and trans-4-octene were only found to be recognized by OR36; citral was also solely encoded by OR37. Both trans-3-octene and trans-4-octene elicited strong neuronal responses in SSR (Liu and Liu, 2015) and were found to be components of human emanation (Bernier et al., 2000), which may hint their possible role involved in the host location of bed bugs. In addition, citral existed as the “narrowly tuned” odorant recognized by “narrowed tuned” receptor, OR37. It also turned out to be a very efficient repellent for bed bugs.

5.4.5 Dose-dependent response of ORs to odorant stimuli

Numerous studies have indicated that dosage is a very critical factor in determining the responses of ORs to odorants (Carey et al., 2010; Wang et al., 2010; Xu et al., 2014; Pelletier et al., 2010). In this study, we also found that the responses of ORs were dramatically influenced by the dosages of odorants. It is very obvious that low dosages of odorants only elicited very weak responses from ORs while high doses of odorants ($1{:}10^3$ or $1{:}10^4$ v/v) activated a large number of ORs (Fig. 5.6A). To further compare the sensitivity of ORs to the odorant stimuli, EC_{50} value of odorants for different ORs were calculated (Fig. 5.6B). Based on the dose-response curves of ORs to different odorants, we found that certain ORs could only respond to certain odorants at high doses. For example, OR17 and OR11 only displayed strong responses to coumarin and 2-decanone at the dose of $1{:}10^4$ v/v, respectively. However,

some other ORs appeared to be extremely sensitive to odorants with a low dose. For example, OR36 can be activated by (+)-menthone and (-)-menthone with an EC_{50} of $9.67x10^{-8}$ and $1.64x10^{-7}$ v/v, respectively and OR37 can be activated by citral and (+)-menthone with EC_{50} of $3.32x10^{-8}$ and $1.93x10^{-7}$ v/v, respectively (Fig. 5.6). As all these EC_{50}s are in the range of nanomolar, they are probably the cognate ligand for these ORs (Bohbot and Pitts, 2015).

5.4.6 Odor presentation based on the response of odorant receptors

As indicated in this study, odorants are usually recognized combinatorically by multiple ORs, which is consistent with the scenario found in *Drosophila* (Hallem and Carlson, 2006), mosquito (Carey et al., 2010; Wang et al., 2010) and mammalian (Malnic et al., 1999) ORs. To examine the relationship between the chemical nature of odorant stimuli and OR responses, we constructed a multidimensional odor space to display the non-normalized current responses of these 15 functional ORs in our *Xenopus* oocyte expression system. Euclidean distances (in nanoamperes, nA) between all responsive pairs of ORs and odorants were mapped in the odor space.

The odor space built in this study only represents a subset of all possible OR-odorant response combinations, and thus compromises only part of the bed bug's overall chemosensory inputs. However, we presumed that odorants clustered together with small Euclidean distance within this odor space, generally share significant chemical characteristics and are difficult for bed bug to differentiate. To visualize the relationships among odorants in this space, a hierarchical cluster analysis was performed on the odorants based on the Euclidean distance within each odorant pair (Fig. 5.7A). We found that odorants in the same chemical group often, though not always, clustered together. Moreover, an inspection of these clusters revealed many examples of structurally similar molecules that are tightly clustered, such as several of the aliphatic aldehydes (propional, butanal, pentanal, hexanal) and aromatics (toluene, ethylbenzene, propylbenzene), which only possess minor variance in their carbon train (Fig. 5.7B).

As another way of analyzing the relationships among odorants, principle component analysis (PCA) was used to represent the 15-dimensional odor space in a three-dimensional odor space which caught about 55% of the original variances (Fig. 5.7C). As showed in the 3D odor space, odorants from each chemical groups were more likely to disperse in the whole odor space even though limited clustering were observed for small number of odorants. On the other hand, odorants from different chemical groups appeared to intermingle together with no significant separation, suggesting that chemical class is a critical factor but not the only factor that involves in the odorant encoding process of bed bug odorant receptors.

5.5 Discussion

Semiochemicals, such as human odorants and chemical repellents play a critical role in the host seeking and risk aversion process of bed bugs. Our previous work (Liu et al., 2014; Liu and Liu, 2015) have extensively descripted the olfactory neuronal response of bed bugs to human odorants and some potential chemical repellents. However, studies focused on the molecular basis of bed bugs' chemoreception were very limited, even though some preliminary endeavor has been exerted (Hansen et al., 2014; Liu and Liu, 2015). Therefore, this study provided the first general picture about the molecular basis of bed bugs' chemoreception by investigating the responses of 15 odorant receptors to a large panel of odorants stimuli from both human emanation and plant volatiles.

When comparing the olfactory neuronal responses of olfactory sensilla to the same panel of odorants stimuli with the current responses from the odorant receptor tested in this study, it is clear that about 67 % of the odorants elicited strong neuronal responses are likely to evoke strong current responses on the odorant receptor, which confirmed that ORs are the primary target of odorants on the neuronal membrane and activated ORs are responsible for neuronal firing. As more bed bug ORs will be functionally characterized, we posited that certain ORs will be identified for most of these odorants which are effective in triggering neuron firing.

However, we still found that certain odorants, which showed no effects on the ORNs but activated ORs in this study. For example, dimethyl phthalate at the dose of $1:10^4$ v/v evoked very strong current response (552 nA) on OR36, while no olfactory sensillum ever showed very strong responses to dimethyl phthalate in the single sensillum recording (Liu and Liu, 2015). Similar phenomenon was also existed for coumarin, which was found to be very effective stimuli on OR17, OR42, and OR47. However, very weak or no neuronal responses were observed from the olfactory sensillum (Liu and Liu, 2015). The reasons for this inconsistence may lie in: 1) bed bugs probably have rare chance to encounter certain chemicals like dimethyl phthalate or coumarin with such high doses in their natural habitat; 2) there is no specific odorant binding protein (OBPs) in the sensillum lymph that are responsible for transporting these odorants to their target ORs. Future studies on the actual dosages of odorants in bed bug's natural environment and characterization the function of bed bugs' OPBs would benefit to the addressment of this puzzle.

In this study, we further demonstrated that aldehydes/ketones are the most promising stimuli released from human bodies that bed bugs are sensitive to, which is very consistent with our finding from the ORNs (Liu and Liu, 2015). Moreover, certain odorants from aldehydes/ketones (such as nonanal, sulcatone), some alcohols (such as 1-octane-3-ol) and heterocyclics (such as skatole) that bed bug ORs were also very sensitive to been reported as active attractants for mosquitoes (Kline 1994; Olagbemiro et al., 2004; Zyed and Leal, 2009; Mcbride et al., 2014). Therefore, these odorants from aldehydes/ketones, alcohols and heterocyclics, are probably very important factor in the host locating process of bed bugs. A finely designed behavior bioassay is needed to further test this hypothesis.

In addition, terpenes and terpenoids were found to be very active in evoking the current responses from the bed bug ORs, too, which also confirmed their high potency in eliciting the firing in the ORNs housed in the olfactory sensilla on the bed bug antennae (Liu et al., 2014).

Previous behavioral studies on both bed bugs and mosquitoes have indicated that plant-released terpenes or terpenoids stimuli were very repulsive for them (Choi et al., 2002; Omolo et al., 2004; Gillij et al., 2008; unpublished bioassay data for bed bugs). Several terpenes or terpenoids (citral, (+)-menthone, geranyl acetate and 1s-(+)-3-carene) even displayed a higher efficiency than DEET, which is one of the most important and successful "all round" synthetic chemical repellent (Osimitz and Grothaus, 1995), in repelling the bed bugs in the two-choice behavior bioassay (unpublished bioassay data for bed bugs). It is obvious that certain bed bugs' ORs, are specifically sensitive to terpenes or terpenoids and initiated the firing in the ORNs, which lead to the aversive behaviors responses. Therefore, terpenes or terpenoids should be very promising candidates in screening new chemical repellents for the bed bug control.

As indicated in this study and also previous finding in the fruit flies and mosquitoes (Hallem and Carlson, 2006; Wang et al., 2010), the dosages of odorants are very critical in triggering the responses from odorant receptors. Some of the ORs can be activated only at high doses while some other ORs are capable to respond to very low doses of odorants. Considering the actual doses of odorants in the natural environment are very low, maybe much lower than what we used in this experiment, we believed that responses elicited by low doses of odorants are, to some extent, more closing to the natural odorant reception in the bed bugs' ORNs.

Moreover, in this study, although the multi-dimensional odor space generated based on the relationships of odorants and ORs' response gave us remarkable information about the ability of bed bugs to discriminate these odorants, it is noticeable that the odor space we defined here only covered a partial range of bed bug's olfaction. As more bed bug ORs are functionally characterized, we should gain a much more complex picture about how these odorants are encoded by the ORs. Even after we can finally clarify the reception spectrum of all these bed bug ORs, we still need to determine how bed bugs respond to 1) a plume (blends) of odorants

rather than a single odorants; 2) quickly changing dosages of odorants in their natural environment rather than the equally defined concentrations in this *Xenopus* oocyte ex vivo expression system. To address these questions, more sophisticated approaches, like the patch clamp recording directly in the antennal lobe or calcium imaging, are necessary to be applied in the investigation.

5.6 References

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Table 5.S1 Primers for cloning the ORs of bed bugs

Gene[&]	Forword primer*	Reverse primer*
OR1	ccgactagtgccaccATGAGTAAAGTAACGATA	ctaggcggccgcTTAATGTTTCTTCGAATCAG
OR5	ccggctagcgccaccATGTGGAAAGTAGCGAGC	ctaggcggccgcTCACAATTTAGAAGACACCG
OR7	ccggctagcgccaccATGCCAGGGAAAAAGGGT	ctaggcggccgcTTAATTTCCTCTTGAAGCGT
OR8	ccggctagcgccaccATGGCCGGTAAGGAAAAG	ctaggcggccgcTTAATTTTGCGATGCGTTGA
OR9b	ccggctagcgccaccATGGGAACTGTAAAAACA	ctaggcggccgcACCCATGAGGGCTTTGAGTA
OR11	ccggctagcgccaccATGGGTAAAGGAGGATCA	ctaggcggccgcTTATCTCGTGGCTTTTAAGG
OR12b	ccggctagcgccaccATGGCTCAGCTCTTCGAC	ctaggcggccgcTTAATTATCGACAGTTCTTA
OR14	ccggctagcgccaccATGCAATCAAAATGTATC	ctaggcggccgcTCAAATAAATAACAATCGCA
OR15	ccggctagcgccaccATGGTCGGCCGAAGGGAT	ctaggcggccgcTCATTGCAGGCTTTGAAGAA
OR16	ccggctagcgccaccATGAACGAAAATTTAAAA	ctaggcggccgcTCAATACATTGATTTAAGCA
OR17	ccggctagcgccaccATGAACGAGAACATGAAG	ctaggcggccgcTCAATTCATCGATTTGAGTA
OR19	ccggctagcgccaccATGACAGAATTGAAGAAA	ctaggcggccgcTTATATATCGAAATGCTGAA
OR20	ccggctagcgccaccATGAAGTTCGGAAGATAT	ctaggcggccgcTCAAAAGTTCATATTTTTGA
OR21	ccggctagcgccaccATGGCGGTGGATGTAAAG	ctaggcggccgcTCATCTCTCATTTAGGAAAA
OR23	ccggctagcgccaccATGGGAAAAGAGAAAAGT	ctaggcggccgcTTAAGACCTCTCCATTGTCC
OR27	ccggctagcgccaccATGACGGCGCTTTCCGGT	ctaggcggccgcCTATAGTTTTTCGCTTGATG
OR36	ccggctagcgccaccATGGCAAGTATTCAGGAC	ctaggcggccgcTTAAGATTTCGTAGCGATAA
OR37	ccggctagcgccaccATGACCTACTGGAAAGAA	ctaggcggccgcCTATGTCTTGATATCAATCG
OR42	ccggctagcgccaccATGGTTTCGGAAGGGATA	ctaggcggccgcTCAGCGGTTCATTTGTTTAA
OR46	ccggctagcgccaccATGGAGTCGACAGAGATT	ctaggcggccgcTCACTTCTCAAGAACGAACT
OR47	ccggctagcgccaccATGGAAGAGGTGATGTCG	ctaggcggccgcTTAATTGAGTTTCTTCAGCA

&: yellow color showed OR genes expressed in *Xenopus* oocytes with no responses to any odorants used in this experiment. *pink color: protective nucleotides; red color: restriction enzyme cutting site; green color: Kozak sequence; black color: gene specific primer sequence

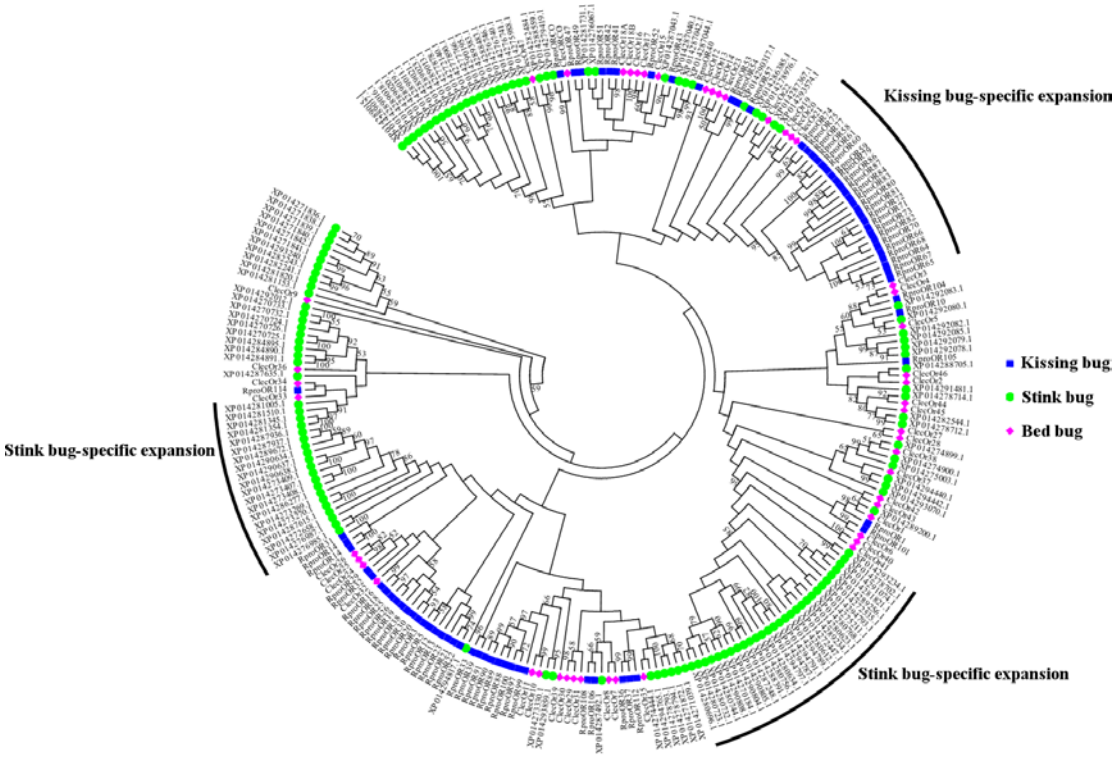

Figure 5.1 Phylogenetic analyses of odorant receptor genes among bed bug, Kissing bug and stink bug. All 47 bed bug ORs (pink color) were retrieved from bed bug genome annotation (www.hasc.org). 76 ORs from kissing bug (Blue color) (*Rhodnius proxilus*) were retrieved from Vectorbase (www.vectorbase.org). 133 ORs from stink bug (green color) (*Halyomorpha halys*) were retrieved from the NCBI (http://www.ncbi.nlm.nih.gov). The tree was constructed with MEGA6 based on a Clustal alignment of the amino acid sequences. Numbers above branches represent the percentage of 1,000 bootstrap replication trees in that branch, with only those above 50% shown.

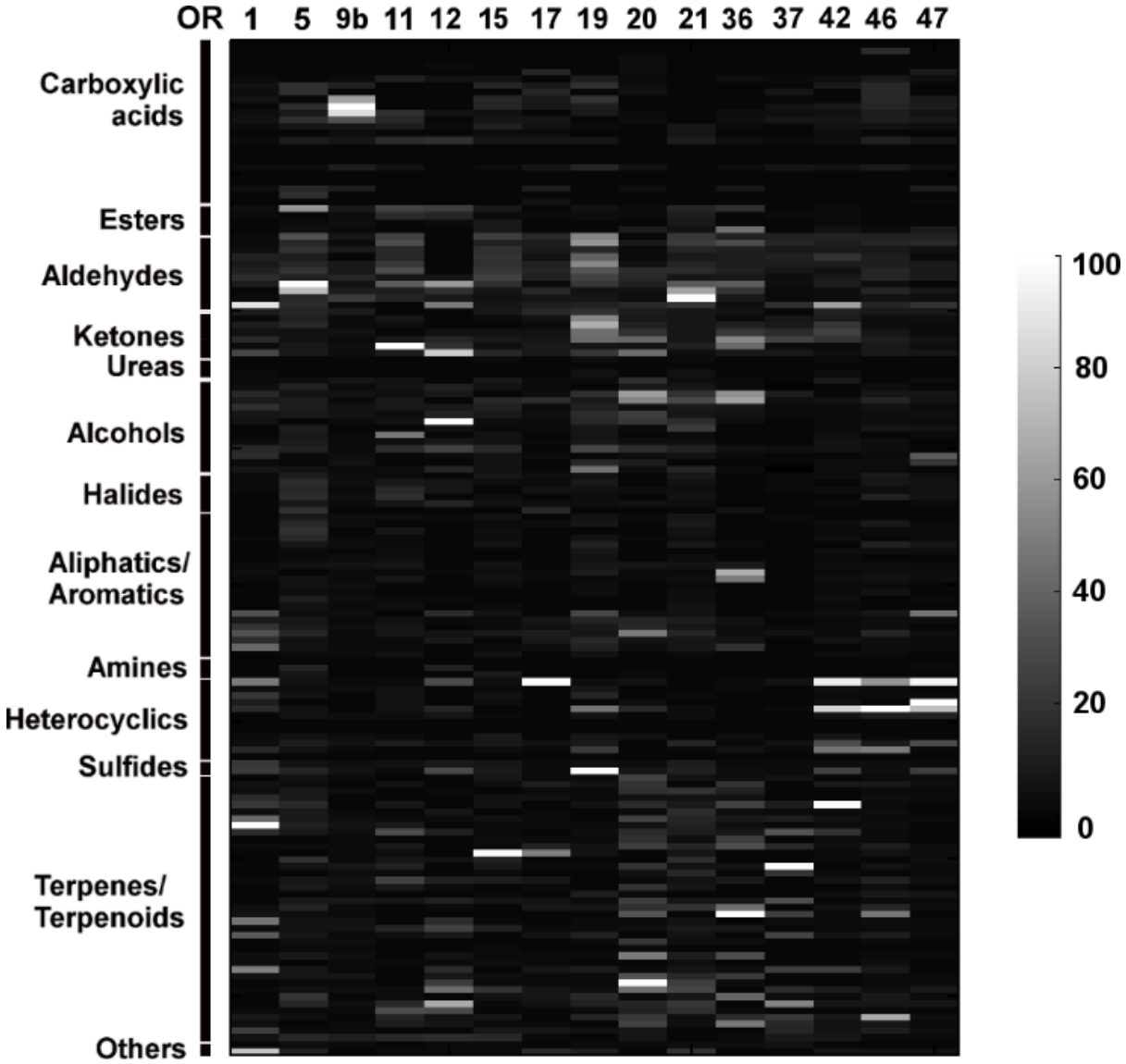

Figure 5.2 Heatmap presentation of the response profiles of bed bug ORs to odorants. Each bed bug OR was co-expressed with the ORCO gene in the *Xenopus* oocytes. Distinctive response profiles (spikes/s) of each OR to different chemical groups of odorants were tested through two-electrode voltage clamp, with at least three independent replicates for each odorant on different individual OR at a dose of 1:1000 v/v. The solvent, 0.1% DMSO Ringer's solution, produced no stimulation in any of the ORs.

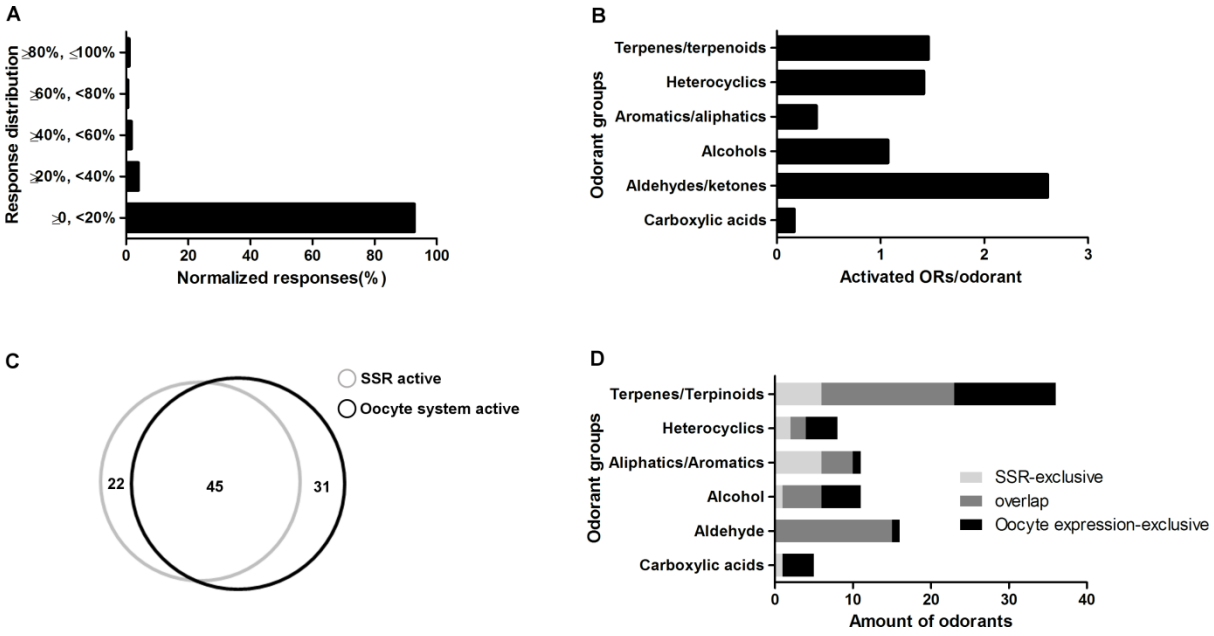

Figure 5.3 Summary of the current responses of bed bug ORs to odorant stimuli. A) Distribution of current responses with different strength evoked from variant odorant/OR combinations. Strong responses (≥20% RU) were sparse among all the odorant-OR combinations. B) Effectiveness of odorants in different chemical groups in eliciting ≥20% response unit (RU). The average number of ORs activated by individual odorant was calculated by dividing the total number of strong responses with total number of odorants within the chemical group. For instance, all 16 aldehydes/ketones elicited 34 strong responses (≥20% RU). Then the average number of ORs activated by aldehydes/ketones would be 2.6, as shown in the bar chart. C) Overlap of the SSR-active odorants and the oocyte expression system active odorants. Odorants that were active in both SSR and oocyte expression system were placed in the overlapped area of the cycles. Un-overlapped areas represented SSR-exclusive (gray cycle) or oocyte expression system (black cycle) exclusive odorants. D) Odorants within major chemical groups of odorants which are active in SSR or oocyte expression system. Light gray color bar means odorants only active in SSR system. Black bar means odorants only active in oocyte expression system. Dark gray bar means odorants active in both systems.

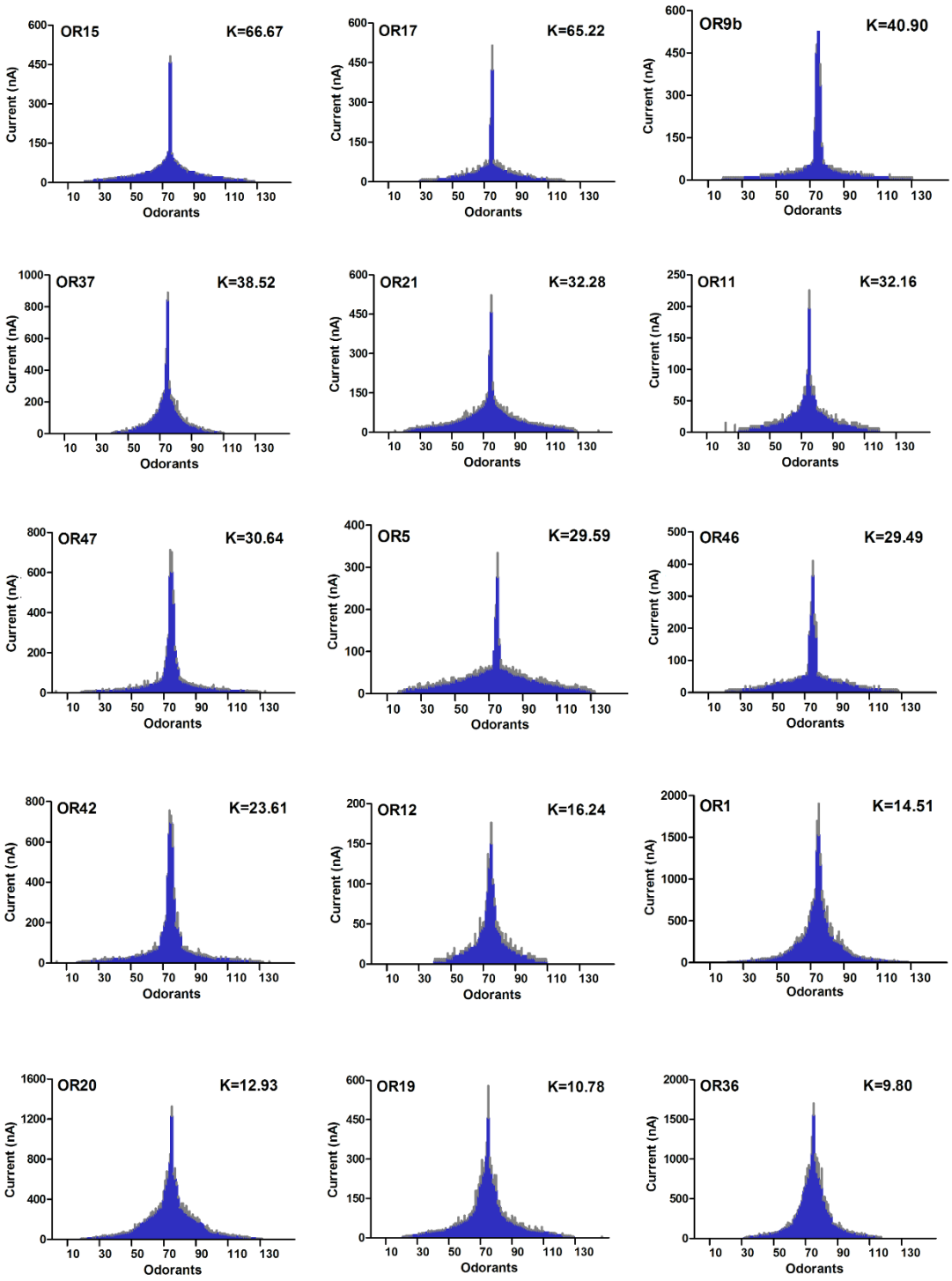

Figure 5.4 Tuning curves of bed bug ORs. nonnormalized bed bug OR (ClOR) responses were presented referring to Carey et al (2010) and Wang et al (2010). The 149 odorants were displayed along the x axis, with those eliciting the strongest response placed near the center, and those eliciting the weaker responses placed near the edges. Therefore, the order of odorants is different for each receptor. The kurtosis value, k, a statistical measure of 'peakedness' was placed along each ORs. The tuning curve of each ClOR was arranged from small to large kuitosis value.

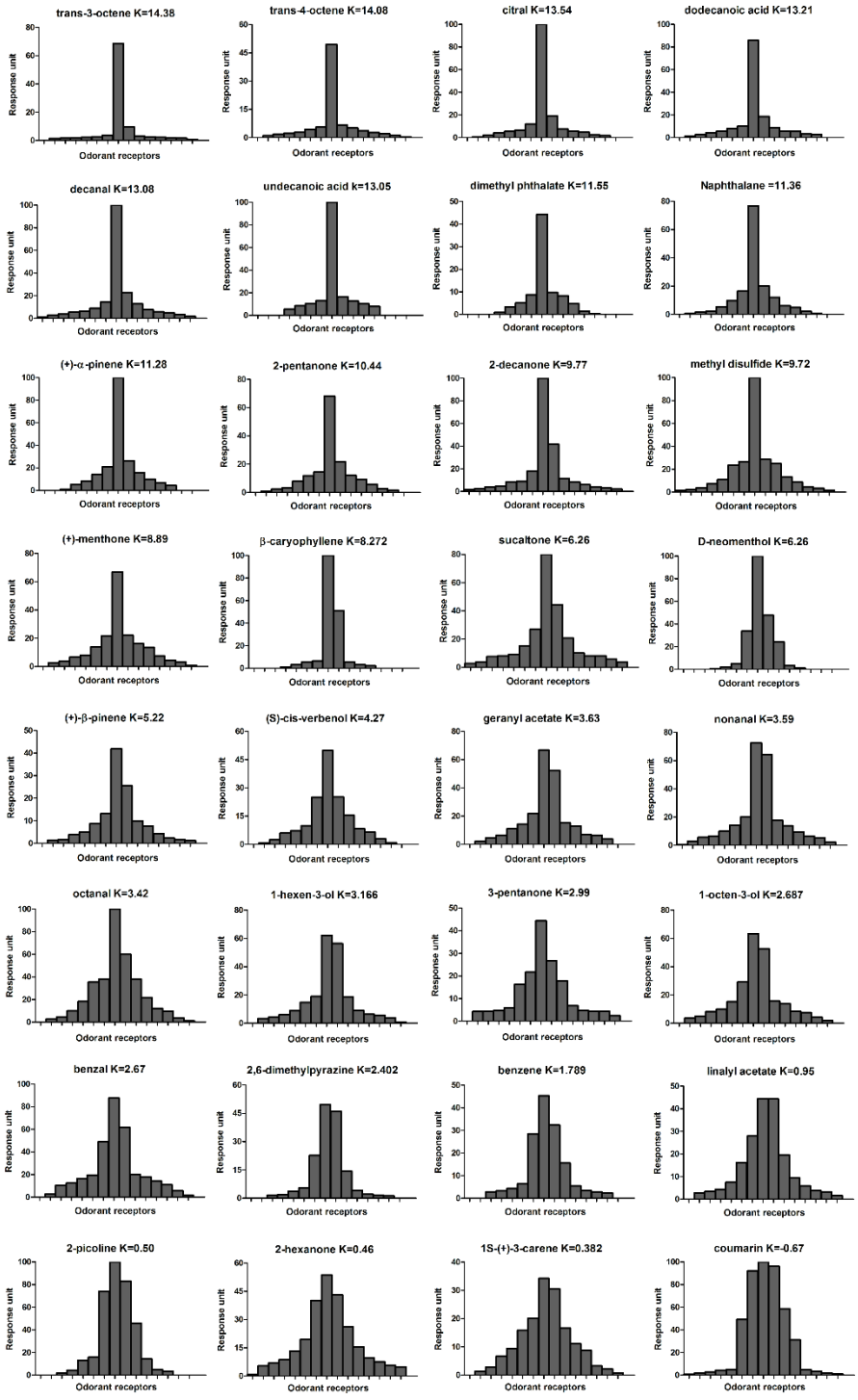

Figure 5.5. Tuning curves of odorants. The normalized responses of the 15 ORs were ordered along the x-axis according to the magnitude of the response they generate for each odorant. The receptor with the strongest response is placed at the center of the distribution; those that have the weakest responses are at the edges. The order of receptors is therefore different for each odorant. The kurtosis value is indicated in each graph. 32 odorants with tuning curves from very narrow to very broad were selectively presented. The tuning curve of each odorants was arranged from small to large kuitosis value.

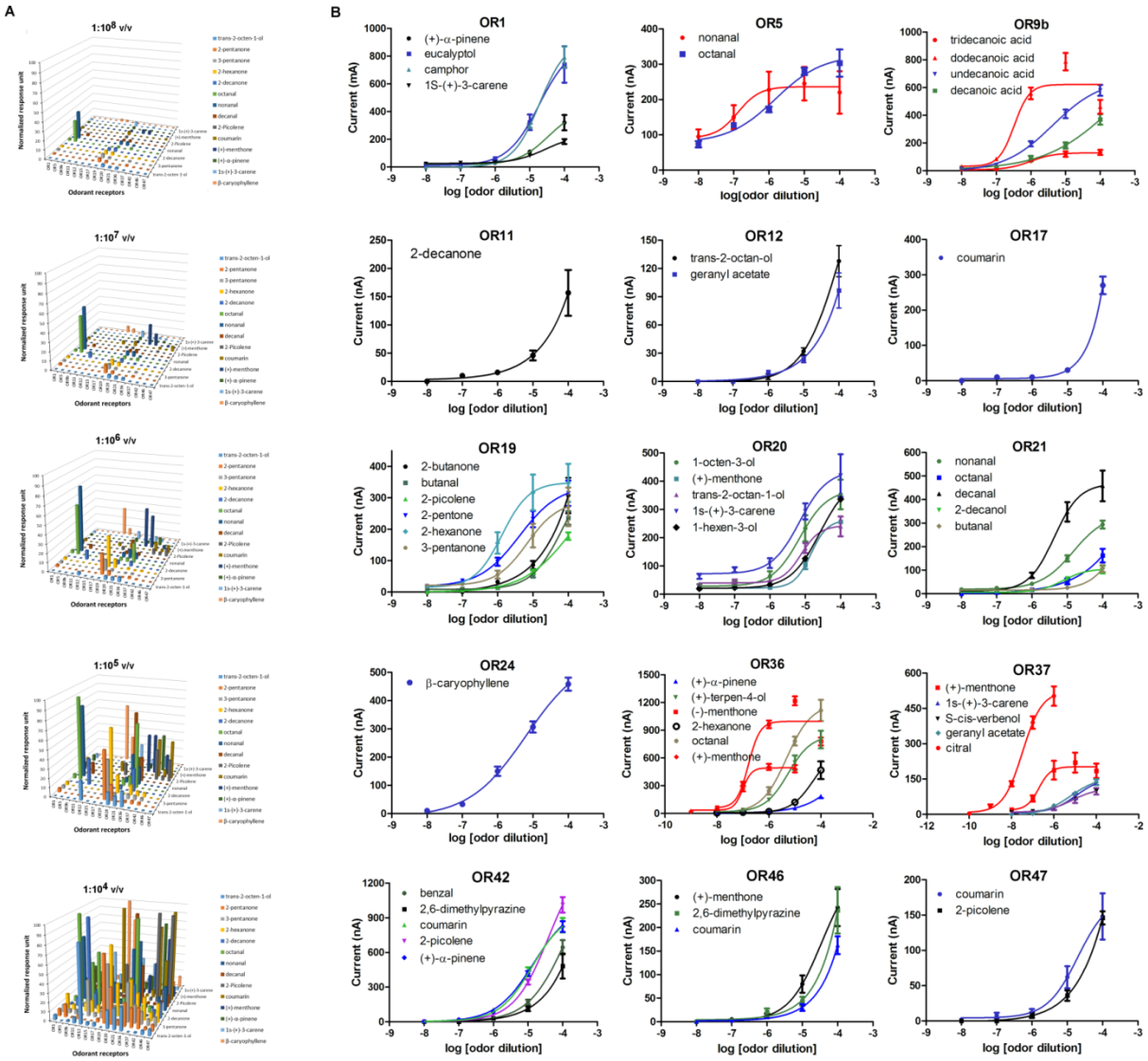

Figure 5.6 Dose response curve of ClORs (written in bold here) and their strongest ligands. A) Normalized responses of 15 ORs to a subset of 14 odorants displayed dose-dependent characteristics. Odorants are listed on the Z-axis sequentially from trans-2-octen-1-ol to β-caryophyllene (n=3-6). B) dose-response curves of ORs to different odorants (means ± SEM, n=3-6). EC_{50} values are expressed as dilutions of pure reagents in Ringer's solution (Wang et al., 2010). Red dose–response curves indicate ligands whose EC_{50} values are in the range of 10^{-7} dilution or below.

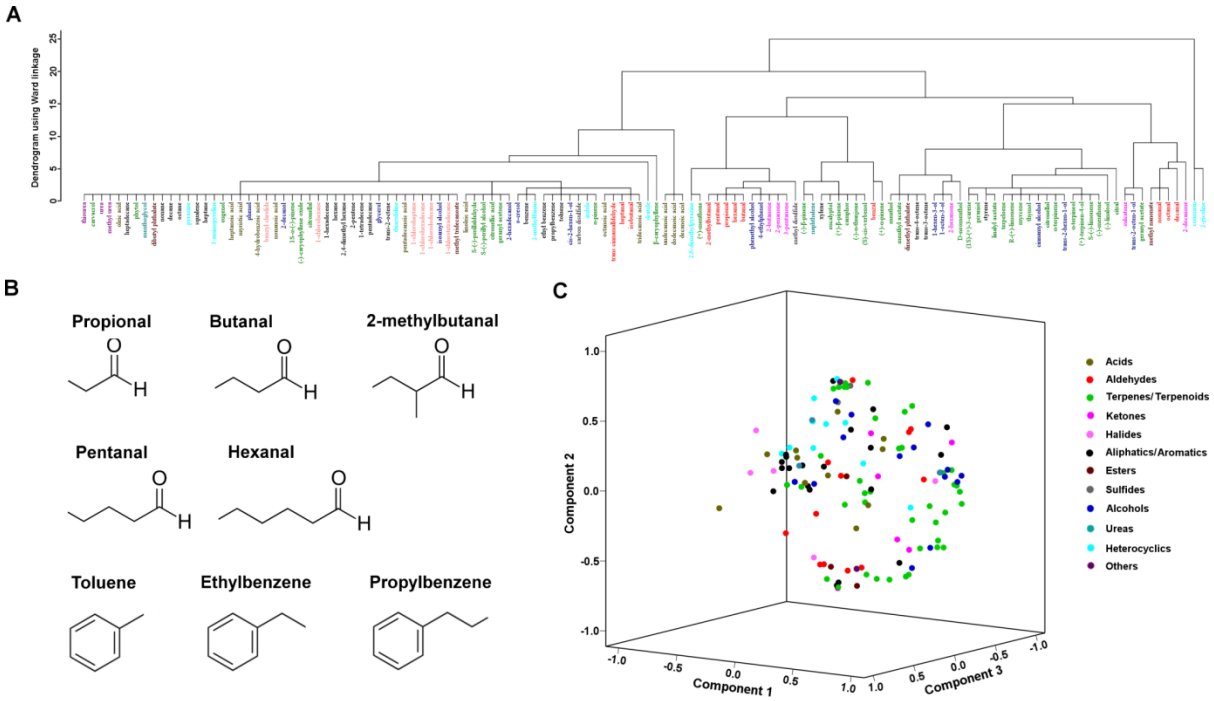

Figure 5.7 Bed bug odor space. (A) Hierarchical cluster analysis for odorants based on the Euclidean distance between odors. Odorants are highlighted in color according to chemical classes. (B) Typical odorants with similar chemical structure are clustered together in the Hierarchical cluster analysis. (C) Relationships among human odorants of the indicated chemical classes at a dose of $1{:}10^4$ v/v revealed by PCA. Odorants were color coded by chemical class as in Fig. 7A. In PCA, vectors quantifying the responses of the 15 odorant receptors to each tested odor are projected onto a three-dimensional region. Each axis represents the normalized current responses of the ORs in a new coordinate system determined by PCA. This three-dimensional representation captures 55% of the variance in the original 15-dimensional data set.

Chapter 6: Dual Role of DEET Involved in the Olfactory Reception of Bed Bugs

6.1 Abstract

As the most extensively used chemical repellent, DEET showed repellency to a wide range of insects, including the common bed bug, *Cimex lectularius*. The neuronal or molecular basis involved in DEET's repelling effect on the mosquitos and fruit flies have been clearly elucidated. However, no or very few work has been engaged into revealing DEET's function on the common bed bug. To get insight into the mechanisms of DEET's repulsive effect to the common bed bug, we characterized the neuronal response of bed bugs to DEET and identified olfactory receptors that are targeted by DEET and demonstrated the interfering effect of DEET on bed bug's responses to human odorants. High doses of DEET were required in activating olfactory receptor neurons in the sensillum of bed bugs. Three DEET-sensitive receptors were also found to be even more sensitive to certain terpenes/terpenoids, which also presented repellency of variant extent to bed bugs, suggesting that DEET may target on the receptors originally responding to terpenes/terpenoids. In addition, DEET showed blocking effect on neuronal responses of bed bugs to specific human odors, which probably results from the inhibitory effect of DEET on the function of odorant receptors in response to human odors. Taken together, our study showed that DEET would function as a stimulus in triggering the avoidance behaviors and also a molecular 'confusant' in the process of odor recognition of bed bugs, which would benefit our understanding in the mechanisms involved in the function of DEET in repelling the common bed bug and provide valuable information for developing new reagents for bed bug control.

6.2 Introduction

As an ectoparasite and obligated blood-feeding insect, bed bugs rely heavily on human and animal blood sources for survival, development and reproduction. Compared to other blood-feeding arthropods (e.g., black fies, mosquitoes, body lice, fleas, and ticks), which are

also served as disease vectors, bed bugs have long been considered to be lack of the capacity of disease transmission (Silverman et al., 2001), until a very recent study indicated that bed bugs may transmit the protozoan, *Trypanosoma cruzi*, which causes the Chagas disease (Salazar et al., 2015). However, the biting nuisance from bed bug infestation still presents a huge stress and disturbance to the hosts both physically and psychologically. To efficiently control this pest, insecticides, especially DDT and pyrethroids (Maryanna et al., 2005; Gangloff-Kaufmann et al., 2006), were extensively used to suppress the bed bug populations worldwide. In some developed countries or regions, bed bugs were considered to be efficiently controlled and out of public concern after 1950s. However, at the end of 1990s, bed bugs showed a resurgent trend in some developed countries or areas (Maryanna et al., 2005; Wang et al., 2013), which is partially result from the abolishment of highly efficient insecticides, and development of insecticide resistance (Yoon et al., 2005; Romero et al., 2007; Zhu et al., 2013).

As one of the most successful synthetic chemical repellents, DEET, played and is still playing a critical role in the insect management. Particularly, DEET displays repellency to a wide range of insect species, such as fruit flies (*Drosophila melanogaster*), mosquitos (*Aedes aegypti*; *Anopheles gambiae*; *Culex quinquefacistus*), kissing bug (*Triatoma rubida*), the common bed bug (*Cimex* lectularius) and tropical bed bug (*Cimex hemipterus*) (Syed et al., 2011; Syed and Leal, 2008; Badolo et al., 2004; Terriquez et al., 2013; Kumar et al., 1995; Wang et al., 2013). Currently, two contrasted mechanisms have been proposed in documents. The first one is that DEET can act as “confusant” in interfering the odorant recognition within the insect olfactory receptor neurons (ORNs) or odorant receptors (ORs) (Ditzen et al., 2008; Pellegrino et al., 2011; Bohbot et al., 2011, 2012) while another one is that DEET acts as “stimulus” in repelling insects by activating the ORNs or ORs which will result in the avoidance behavior (Syed and Leal 2008; Xu et al., 2014). For example, Ditzen et al (2008)

found that DEET could significantly block the neuronal response of *An. gambiae* to one human odorant, 1-octen-3-ol. Another study by Pellegrino and the colleagues (2011) indicated that DEET somehow scrambled the olfactory responses of *D. melanogaster* to odors. Bohbot et al (2011) further revealed that DEET significantly inhibited the function of mosquitos' odorant receptors in response to the odorants. All these work indicated that the interfering effect of DEET on the function of insect olfactory system. At almost the same time, several other studies actually identified either olfactory receptor neurons or olfactory receptors (e.g. OR136b in mosquito *Culex quinquefaciatus*) that were activated by DEET with marked electrophysiological responses (Syed and Leal 2008; Xu et al., 2014), which suggested the activating effect of DEET on the insect olfactory system. In addition, DEET was also found to work as a contact chemical repellent at close range by activating gustatory receptor neurons or gustatory receptors in *Drosophila melanogaster* (Lee et al., 2010).

With all these studies focusing on the fruit fly or mosquitoes, very few studies have been engaged into the role of DEET in repelling the common bed bugs. Moreover, very little endeavor has been added to elucidate if DEET can act as both "confusant" and stimulus in insect olfactory system. Therefore, in this study, we tried to reveal the mechanisms involved in the repulsive effect of DEET to bed bugs by testing the olfactory neuronal responses of bed bugs to DEET, identifying the DEET-activated odorant receptors and also investigating the interfering effect of DEET on the responses of bed bug to human odorants on the level of either ORNs or ORs.

6.3 Materials and Methods

6.3.1 Insects and chemicals

The *C. lectularius* colony originated from Ft. Dix, New Jersey, USA (Bartley and Harlan 1974). It is susceptible to pyrethroid insecticides (Romero et al., 2007). All the common bed bugs were reared at 25±2°C under a photoperiod of 12:12 (L: D). All chemicals were

commercially purchased from the company with high purity. Each of odorants or DEET was diluted in dimethyl sulfoxide (DMSO) to a stock solution at the dose of 1:10 v/v and stored at 4 degree. Subsequently, the decadic dilution (10-fold dilutions) was made from the stock solution.

6.3.2 Single sensillum recording

Adults of the common bed bug were randomly selected at least five days after blood feeding. The bed bugs (male or female) were anaesthetized (2-3 min on ice) and mounted on a microscope slide (76×26 mm) between 2 pieces of double-sided tape. Using double-sided tape, the antennae were fixed to a cover slip resting on a small ball of dental wax to facilitate manipulation. The cover slip was placed at a proper angle to the bed bug head. Once mounted, the bed bug was placed under a LEICA Z6 APO microscope in such a way as to ensure that the antennae were visible at high magnification (×720). Two tungsten microelectrodes were sharpened in 10% KNO_2 at 5V to a ~1 μm tip diameter. The reference electrode, which was connected to the ground, was inserted into the abdomen of the bed bugs and the other electrode, which was connected to a preamplifier (10×, Syntech, Kirchzarten, Germany), was inserted into the shaft of the sensillum to complete the electrical circuit and extracellularly record the olfactory receptor neuron potentials (Den Otter et al. 1980). Controlled manipulation of the electrodes was performed using two micromanipulators (Leica, Germany). The preamplifier was connected to an analog to digital signal converter (IDAC, Syntech, Germany), which in turn was connected to a computer for signal recording and visualization. Signals were recorded for ten seconds starting one second before stimulation. As a high number of ORNs co-located in each sensillum type, we didn't calculate the firing rate for each ORN within the same sensillum. Instead, the total numbers of action potentials were counted off-line in a 500 ms period before and after stimulation for the whole sensillum. The number of action potentials after stimulation was subtracted from the number

of action potentials before stimulation and multiplied by two in order to quantify the firing rate change in one sensillum in spikes per second.

6.3.3 Stimulation and Stimuli

Ten microliter of diluted human odorants alone, or diluted DEET, or the mixtures of human odors were dispersed on a filter paper (20×3 mm) which was inserted in a Pasteur pipette to create the stimulus cartridges. Solvent or DEET alone served as control. A constant airflow across the antennae was maintained at 20 ml/s throughout the experiment. Humidified air was delivered to the preparation through a glass tube (10-mm inner diameter). The glass tube was perforated by a small hole, slightly larger than the tip of the Pasteur pipette, 10 cm away from the end of the tube. Stimulation was achieved by inserting the tip of the stimulus cartridge into the hole of the glass tube. A stimulus controller (Syntech, Germany) diverted a portion of the air stream (0.5 l/min) to flow through the stimulus cartridge for 0.5 sec, delivering the stimulus to the sensilla. The distance between the end of the glass tube and the antennae was ≤1 cm. At least six replicates for each recording experiment with different stimuli were conducted on different individuals. The values of the spikes were obtained by averaging all the recordings for the response of each sensillum to each chemical. Dose-response data were analyzed by GraphPad Prism 5.0 (GraphPad Software Inc, CA).

6.3.4 *Xenopus* oocyte expression system and two-electrode voltage-clamp

The entire coding region of bed bug odorant receptors and co-receptor (ClORs and ClORCO) was amplified using the primers with a cutting site added. The purified PCR products were cut with *Not*I-HF/*Nhe*I-HF (New England Biolabs, MA) and then cloned into pT7Ts vector (a gift from Dr. Wang), with a Kozak sequence added behind the cutting site in the forward primer. The constructed vectors were linearized with specific restriction enzyme and cRNAs were synthesized from linearized vectors with mMESSAGE mMACHINE T7 (Ambion, Carlsbad, CA). Mature healthy oocytes (stage V–VII) (Nasco, Salida, CA) were harvested from African

Clawed Frog (*Xenopus laevis*) and treated with collagenase I (GIBCO, Carlsbad, CA) in washing buffer (96 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$, and 5 mM HEPES [pH = 7.6]) for about 1 h at room temperature. After being cultured overnight at 18°C, oocytes were microinjected with 5 ng cRNAs of both ClORs and ClORCO. After injection, oocytes were incubated for 4–7 days at 18°C in 1X Ringer's solution (96 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$, 0.8 mM $CaCl_2$, and 5 mM HEPES [pH = 7.6]) supplemented with 5% dialyzed horse serum, 50 mg/ml tetracycline, 100 mg/ml streptomycin and 550 mg/ml sodium pyruvate. Whole-cell currents were recorded from the injected *Xenopus* oocytes with a two-electrode voltage clamp. Odorants-induced currents were recorded with an OC-725C oocyte clamp (Warner Instruments, Hamden, CT) at a holding potential of −80 mV. Odorants and DEET were dissolved in DMSO at a 1:10 ratio to make stock solutions that were diluted in 1× Ringer's solution to the indicated concentrations Data acquisition and analysis were carried out with Digidata 1440A and pCLAMP 10.2 software (Axon Instruments Inc., CA).

6.3.5 Olfactometer bioassay

The olfactometer bioassay was followed the procedure in the study of Gries et al (2015) with minor modification. Bioassays were conducted in dual-choice olfactometers, which consisted of two lateral Petri dishes (with lid) and a central dish (without lid) (3 × 9 cm inner diameter). The central dish was connected with two lateral dishes via a plastic tube (2.5 cm long × 1 cm inner diameter). The dishes in this olfactometer mimic the natural still-air shelters in which bed bugs spend the day. Prior to the start of bioassays, a disc of filter paper (9 cm diameter) was placed into each dish and a strip of filter paper (24 × 0.6 cm) was inserted into the connecting tubing to provide traction for walking bed bugs. In addition, a piece of filter paper was placed into each lateral dish and covered with a piece of cardboard (2.2 × 2.2 cm) as a refuge for bioassay insects. An inverted lid of a 4-ml vial was placed on top of the corrugated cardboard shelter in the randomly assigned treatment dish of the olfactometer. All these olfactometers

were placed in a small room with very good air circulation. Before adding the stimuli or DMSO, the connected tube will be sealed using a small piece of Parafilm membrane (Sigma) and a single male or female adult bed bug was released into the central chamber of 20-60 olfactometers per experiment at the end of the 12-h photophase. Then chemical stimulus formulated in equal amounts in DMSO was pipetted into the lid of experimental treatment while in the control treatment only DMSO was added into the inverted lid. The bed bug in each olfactometer was then allowed to explore the central dish for 1h of darkness. After 1h of darkness, the Parafilm membrane will be removed in the connected tubes, which enables bed bugs to detect odorants in either side of dish. After the 12-h darkness period, the bed bug's position within each olfactometer was recorded. Any insect not found in a lateral chamber was recorded as a nonresponder. Olfactometers were washed with unscented detergent (Beaumont Products, GA, USA), rinsed with distilled water, and dried at room temperature between each bioassay.

6.4 Result

6.4.1 Olfactory responses of bed bugs to high doses of DEET

To reveal the neural responses of olfactory sensillum in the bed bug antennae to DEET, we screened all different types of olfactory sensillum, including Dα, Dβ, Dγ, C, E1 and E2 sensillum using the single sensillum recording. The results showed that no evident responses from different sensillum has been detected at the doses of ≤ 1:100 v/v dilution, which is consensus with our previous finding that no remarkable firing responses have been generated from the stimulation of DEET at the dose of 1:100 v/v dilution. However, we also notified that a slight response were elicited from doses at the 1:10 v/v on the Dα and Dβ sensilla, with the firing rate of 23± 1.8 and 38± 2.7 spikes/s respectively (Fig. 6.1). When the pure DEET with no dilution was used to stimulate the Dα and Dβ sensilla, the firing rate of excitatory response increased to 50±4.8 and 53±3.7 spikes/s, respectively (Fig. 6.1). All these results

indicated that the olfactory neurons on the bed bug antennae can detect high doses of DEET, while showed no sensitivity to low doses of DEET. These results are consistent with studies in the mosquitoes which were also found to be only sensitive to high doses of DEET (≥1:100 v/v or 10 ug/ul) but not low concentrations (Syed and Leal 2008; Liu et al., 2013). Therefore, high doses of DEET are required to activate the ORNs in the bed bug antennae.

6.4.2 DEET activated multiple ORs of bed bugs

Insect odorant receptors (ORs) in the plasma membrane of ORNs are responsible for sensitizing odorants and producing the neuronal firings (action potential). To further identify which bed bug odorant receptor(s) are targeted by DEET, 15 bed bug ORs expressed in the *Xenopus* oocytes were challenged by DEET at the dose of $1{:}10^4$ v/v with the two-electrode voltage clamp. The results showed that at least three of these ORs, OR20, OR36 and OR37 showed remarkable current response (≥100 nA) to DEET (Fig. 6.2A). The responses of these ORs, OR20, OR36 and OR37, to DEET also followed a dose-dependent manner with EC_{50} values of $7.5x10^{-6}$, $7.1x10^{-6}$ and $6.5x10^{-6}$, respectively (Fig. 6.2B).

Very interesting, all these ORs that are activated by DEET were also found to be most sensitive to certain terpenes/terpenoids compared to odorants from other chemical classes (Liu et al. manuscript for reviewing). Particularly, OR37 were found to be exclusively activated by terpenes/terpenoids (Liu et al. manuscript for reviewing). OR20, OR36 and OR37 showed much stronger responses to (-)-linlool, (-)-menthone and citral compared to DEET (Fig. 6.3), of which, (-)-menthone and citral presented EC_{50} values of 43- and 195-fold lower than that of DEET on the OR36 and 37, respectively. As all these terpenes/terpenoids are major components of essential oil or other botanical repellents, which are repulsive to the blood-feeding mosquitoes (summarized in Liu et al., 2014), probably bed bugs will also show aversive response to these terpenes/terpenoids.

6.4.3 Bed bugs are behaviorally aversive to DEET and terpenes/terpenoids

To test behavioral responses of bed bugs to DEET and terpenes/terpenoids, we applied dual-choice olfactometer bioassay as described in the study of Gries et al (2015). Both male and female bed bugs showed to be significantly repelled by pure DEET while no difference in response to 10% of DEET and the control solvent, DMSO, which suggested that only highly doses of DEET were able to elicit the aversive response of bed bugs (Fig. 6.4A and 4B). As for the terpenes and terpenoids, the efficiency of repulsion was quite variant among these chemical stimuli (Fig. 6.5). For instance, eugenol and carvacrol, which couldn't activate any DEET-sensitive ORs, also showed no influence on the behavior choices of bed bugs, even at the doses of 100% (Fig. 5A, B). However, 1% of linalyl acetate, menthyl acetate, (-)-linalool (Fig. 6.5C, D, E), (+)-menthone (Fig. 6.S1A) or 5% of citral, geranyl acetate and 1S-(+)-3-carene (Fig. 6.S1B, C, D) already displayed very strong repulsive effect to bed bugs. Interestingly, all these terpenes/terpenoids they presented high efficiency in activating the DEET-sensitive ORs. Another terpene odorant, (+)-β-pinene, which displayed similar effect as DEET on the receptors, also showed similar repellency to bed bugs only at the dose of 100% (Fig. 6.5F).

Since DEET is the synthetic chemical repellent which does not naturally exist until 1944, it is impossible that certain insect ORs are functionally responsible for detecting DEET. Instead, it is more likely that some existed ORs, which are originally targeted by some unpleasant odorants, are coincidently responding to DEET. Here, in our study, we highly poised that DEET was recognized by the ORs of terpenes/terpenoids, some of which demonstrated even stronger repellency than DEET in the bed bug's behavior responses. However, apparently not every terpene/terpenoid-sensitive OR showed response to DEET. For example, the broadly tuned OR1 showed strong response to nearly 10 terpenes/terpenoids (≥ 100 nA) in previous study (unpublished manuscript by Liu et al) while presenting inhibitory response to DEET

(Fig. 6.2A). Therefore, we hypothesized that terpene/terpenoids-sensitive ORs, but not all of them, are responsible for detecting DEET in the environment, which will lead to the avoidance behavior of bed bugs.

6.4.4 Interfering effect of DEET on the responses of ORNs to human odorants

Although we confirmed that DEET can activate the ORNs of the bed bugs directly, many previous studies from the fruit fly also indicated that DEET might exert an interfering effect on the neural responses to the odorants (Ditzen et al., 2008; Pellegrino et al., 2011). To investigate whether DEET displayed the same function on the olfactory system of the bed bugs, we characterized the neural response of Dγ and C sensillum to the combination of DEET and human odorants and compared them with the responses to solely human odorants. Human odorants from different classes were chosen based on their strong stimulation on the Dγ or C sensillum which was characterized in our previous study (Liu et al., 2015). Very interestingly, we found that the responses of bed bug to human odorants were totally "scrambled" when DEET was added into the stimulation (Fig. 6.6 and Fig. 6.S2-3). For example, Dγ sensillum showed no significant difference in the dose-dependent response to the combinant of DEET and hexanal or DEET and toluene compared to hexanal or toluene alone (Fig. 6.6B and Fig. 6.S2A). However, for some other aldehyde chemicals, like heptanal, octanal, nonanal and decanal, a very significant blocking effect was observed in the neuronal responses to the DEET-added combinants with the dose-dependent curve much lower than that of aldehydes alone (Fig. 6.6C, 6D, 6E, 6F). For the chemical pentanal, the firing frequencies at the dose of $1:10^6$-$1:10^3$ v/v slightly increased when DEET was added into the stimulation (Fig. 6.6A). Nevertheless, the responses to 10^2-fold dilution of pentanal and DEET was inhibited compared with non-DEET-added pentanal (Fig. 6.6A). It is worth to notice that while majority of the strongest blocking effect appeared at the highest dose (1:100 v/v) of human odorants, a small portion of them, were presented at some medium

doses, such as 1: 10^4 v/v of heptanal, 1: 10^3 v/v of octanal (Fig. 6.6C and 6D). It seems that the high doses of heptanal and octanal can overcome the blocking effect of DEET on the ORNs. Although DEET showed an extensively blocking effect on the neural responses of Dγ sensillum to certain chemicals, no interfering effect was observed on the response of C sensillum to two amines tested, propylamine and butylamine (Fig. 6.S3).

Temporal dynamic of neuronal responses is also considered to be very important feature for odorant encoding or recognition. To test whether DEET showed any influence on the temporal characteristic of neural response to human odorants, we compared temporal dynamics of response to the combinants of DEET and odorants with that of solely human odors. We found that the DEET did change the temporal structure of neural response to certain odors and this changing is odor-specific and dose-specific (Fig. 6.7). For instance, DEET showed a huge modification on the temporal structure of responses to nonanal at the doses of 1:1000 v/v by inhibiting the peak firing from the ORNs housed in the Dγ sensillum (Fig. 6.7C), while no effect was displayed on the temporal structure of responses to hexanal at the same doses (Fig. 6.7A). For another human odor, DEET showed a large impact on the response to heptanal at the dose of 1:1000 v/v with the temporal structure converted from more tonic to more phasic (Fig. 6.7B). However, when we increased the dose of heptanal to 1:100 v/v, DEET's influence was diminished and again, it seems that high doses of heptanal can overcome the interference of DEET on the temporal structure of neural response.

6.4.5 Interaction between DEET and bed bug odorant receptor

Previous studies in mosquitoes have proved that DEET can actually work on the ion channel formed by the complex of odorant receptors and co-receptor and disturb the recognition of odorant receptors to their ligands (Bohbot et al., 2011; Bohbot and Dickens, 2012). To investigate if similar mechanism also involved in the repulsive effect of DEET to the bed bug, we tested the current responses of bed bug odorant receptor (OR19) to several

human-odor stimuli (pentanal, butanal, 2-butanone, 2-pentanone, 2-hexanone) with or without DEET added into the perfusion. The result showed that, basically, all these stimuli without mixing with DEET, elicited typical strong responses on the OR19/ORCO. For example, 2-butanone and 2-pentanone triggered remarkable current responses on the OR19/ORCO, even though the responses will decrease slightly when this receptor was challenged repeatedly with high doses of odorants (Fig. 6.8A). However, when DEET was added into either 2-butanone or 2-pentanone solution, the current responses of OR19 to both stimuli were dramatically decreased (Fig. 6.8B). Not only 2-butanone and 2-pentanone, the efficiencies of all the other human-odor stimuli in triggering the current response from OR19/ORCO were also significantly reduced after DEET was included into the perfusion (Fig. 6.8C).

The effect of DEET on the dose-dependent response of OR19/ORCO to 2-butanone and 2-pentanone was also investigated in this study. We found that DEET at the dose of $1{:}10^3$ v/v showed a very clear antagonistic effect to the dose-dependent responses of OR19/ORCO to both 2-butanone and 2-pentanone (Fig. 6.9A/B). The doses of DEET also influenced a lot on the antagonistic effect of DEET. As we increased the dose of DEET, the antagonistic effect also significantly increased. For example, the antagonistic effect of DEET on the dose-dependent response of OR19/ORCO to 2-pentanone and 2-butanone was significantly stronger at the dose of $1{:}10^3$ v/v than $1{:}10^4$ v/v (Fig. 6.9C). .

Therefore, our result clearly indicated the DEET could actually interact with the bed bug odorant receptors and inhibited the current response of specific receptor to the human odors. This antagonistic effect of DEET on the odorant receptor (s) probably conferred to the blocking effect on the ORNs in response to human odors.

6.5 Discussion

In this study, we characterized the neuronal responses of olfactory sensillum on the bed bug antennae to high doses of DEET (≧10%) but not low doses (≦1%) and revealed that DEET activated multiple bed bug odorant receptors which primarily involve in the detection of terpenes or terpenoids. Behavioral bioassay confirmed the repulsive effect of high-dose of DEET while much lower doses of terpenes/terpenoids on the common bed bugs. When we examined the constituents of some commercial insect repellents, particularly the mosquito repellents, they were usually labeled to be containing 10-40% of DEET and minor addition of essential oils which are majorly constituted with terpenes/terpenoids. As we found in this behavioral bioassay, the thresholds for certain terpenes/terpenoids in repelling the bed bugs were much lower than that of DEET. This rose the deduction that the minor portion of terpene/terpenoids in the commercial insect repellents probably contribute dramatically in repelling the insects besides providing the fragrance, even though DEET was typically considered to play the major role in protecting from mosquito biting or other insect annoyance.

In testing the neuronal responses of bed bugs to DEET, we found that although we used very high doses (≥10%) of DEET in the stimulation, only mild responses were observed from both Dα and Dβ sensillum, while very strong responses were recorded when terpenes/ terpenoids were used in stimulating the Dα and Dβ sensillum (Liu et al., 2014). Similarly, the bed bug odorant receptors that showed mild responses to DEET are also coincidently showed much stronger responses to certain terpenes/terpenoids. Apparently, the binding affinity of DEET to these bed bug ORs are much lower than that of terpenes/terpenoids. Very interestingly, Syed and the colleagues also reported that the specific olfactory sensillum on the antennae of *Cx. quinquefaciatus* mosquitoes present much weaker response to DEET compared with several terpenes/terpenoids (Syed and Leal 2008). As DEET is a synthetic odorant which might not

exist in nature before 1950s, it is highly impossible for insects to independently evolve some novel ORs that are specifically targeted on DEET or some novel functions for recognizing DEET among the existing ORs. Based on this study, we strongly proposed that the insect ORs which are originally targeted by terpenes/terpenoids, play a critical role in detecting DEET in their environment. Since terpenes/terpenoids are found to be broadly detected by ORs or ORNs of various insect species (Hallem and Carlson, 2006; Qiu et al., 2006; Ghaninia et al., 2007; Hill et al., 2009; Carey et al., 2010; Wang et al., 2010; Liu et al., 2013, 2014), some of these ORs may naturally possess the capacity in sensing DEET, which also give insight into the question why DEET showed such a broad spectrum in repelling different insect species.

In addition to the activating effect of DEET on the ORNs or odorant receptors, we also revealed that DEET demonstrated interfering effect in the process of human odors sensation for bed bugs. It is interesting to notice that DEET scrambled the odor coding process of ORNs in Dγ sensillum to most human odors, while no significant influence was presented on the C sensillum. Morphologically, Dγ sensilla are close to the short blunted trichoid sensilla with ORNs expressing ORs while C sensilla resembled the *coeloconic sensilla* with ORNs expressing IRs in mosquitoes or other insects (Levinson et al., 1974). ORs have been proved to be responsible for some alcohols, aldehydes, esters and aromatics (Hallem and Carlson, 2006; Carey et al., 2010; Wang et al., 2010), while IRs were responsible for detecting some polar molecules, such as acids and amines (reviewed by Joseph and Carlson, 2015; Mcbride, 2016). Previous study also showed that DEET interfered with the function of mosquito ORs in recognition of 1-octen-3-ol, a human odor (Bohbot and Dickens, 2012). In our study, DEET was also found to block responses of ORNs to human odors which may probably result from the interaction with ORs in ORNs. In addition, no previous studies had elucidated

the interaction between IRs and DEET. According to our finding that DEET showed no effect on the ORNs' response to amines tested, we keep a negative view about DEET's role in interfering with the IRs or inhibiting the function of IRs in response to certain human odors.

As we found in this study, DEET showed interfering effect on the function of the complex of OR/ORCO and changed the binding affinity of chemical legends to the OR/ORCO complex. But we are still lack of the direct proof on which part of this complex, OR or ORCO that DEET is targeting on. Since DEET showed repulsive effects on a wide spectrum of insects, Ditzen et al. (2008) proposed that DEET may act on the ORCO, which is highly conserved among different insects, to inhibit the responses of OR/ORCO complex to their ligands. However, another study by Tsitoura et al. (2015) reported that there was no inhibition of ORCO could be observed even at 10mM DEET by using the lepidopteran insect cell system to express the *Ae. aegypti* ORCO, suggesting that DEET have no influence on the function of ORCO. Moreover, in our study and also some other studies (Xu et al., 2014), multiple ORs were actually found to be activated by DEET. If DEET inhibited the function of ORCO, it would be impossible to observe the electrophysiological responses of these in vitro-expressed ORs to DEET. Therefore, we proposed that DEET was more likely to work on the ORs but not ORCO when interfering with the function of OR/ORCO complex in the insect olfactory system.

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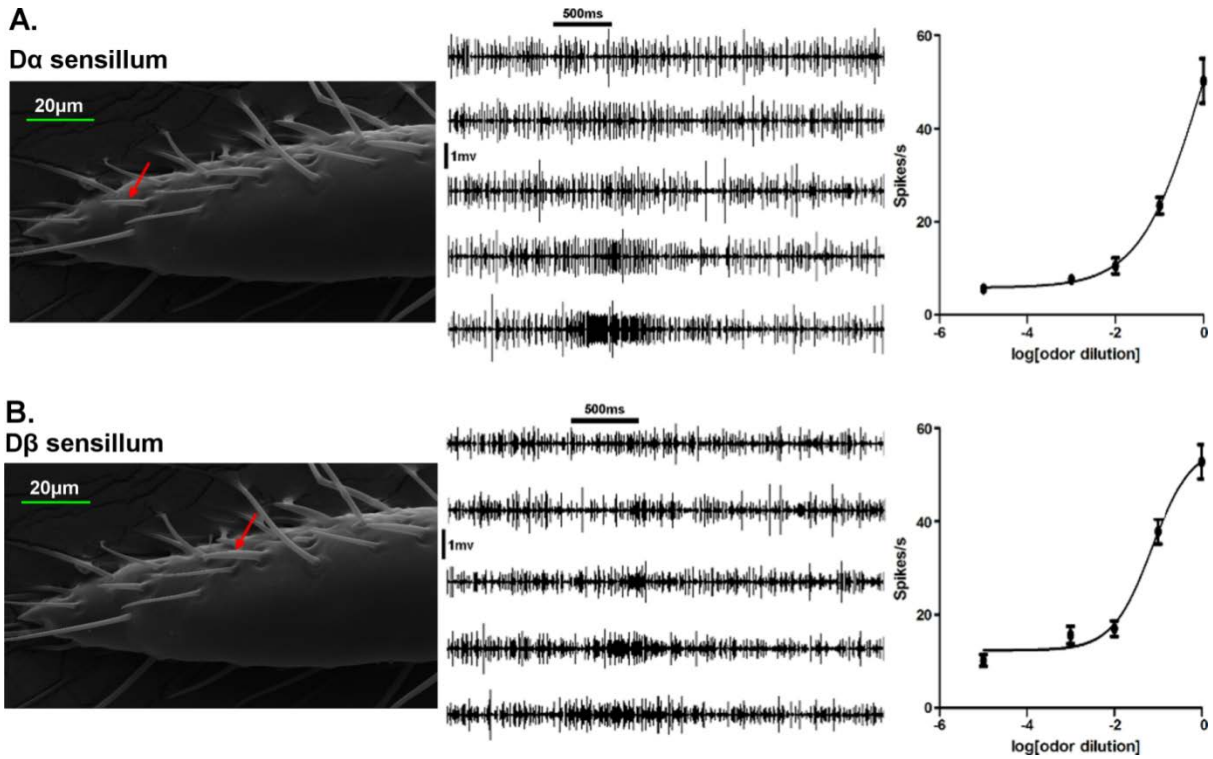

Figure 6.1 Neuronal responses of olfactory sensillum to DEET in the common bed bug. A) Dose-dependent responses of Dα sensillum (red arrow) to DEET. B) Dose-dependent responses of Dβ sensillum (red arrow) to DEET. The representative firing traces of ORNs in responses to different doses of DEET (from top to bottom: $1:10^{8}$-$1:10^{4}$ v/v). Dose-response curve was fit with the Sigmoidal dose-response model with variable slope using the Graphpad Prism 5**.**

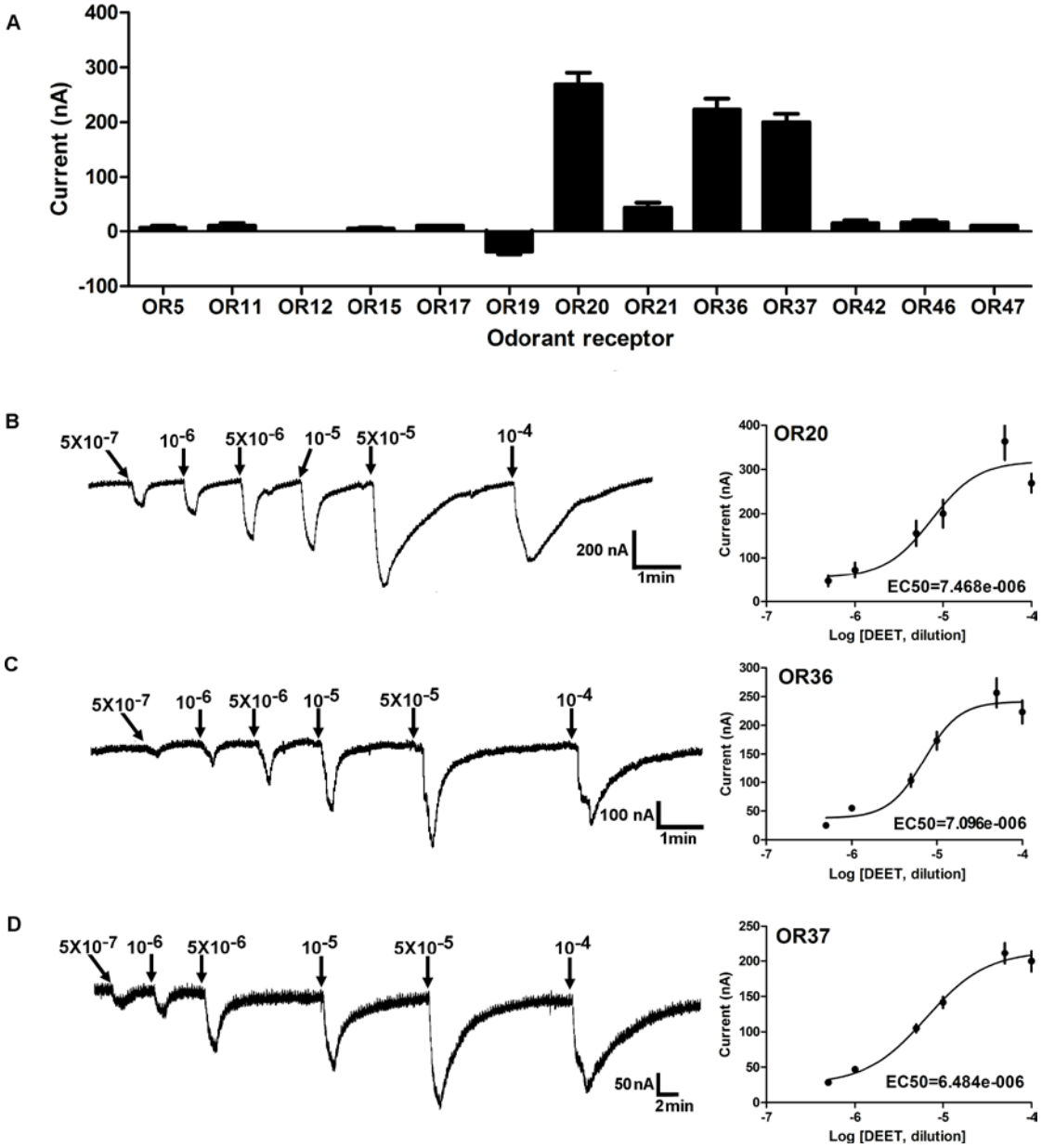

Figure 6.2 Activating effect of DEET on multiple ORs in the common bed bugs. A) Three out of thirteen bed bug ORs showed current responses more than 100 nA to the perfusion of DEET at the dose of $1{:}10^4$ v/v, n=6-10. B) dose-dependent responses of OR20 to DEET from the dose of 1: $5x10^7$ to $1{:}10^4$ v/v. C) dose-dependent responses of OR37 to DEET from the dose of 1: $5x10^7$ to $1{:}10^4$ v/v. The value of the current responses from the complex of OR/ORCO were presented as the M (mean) ± SEM. Dose-response curve was fit with the Sigmoidal dose-response model with variable slope using the Graphpad Prism 5.

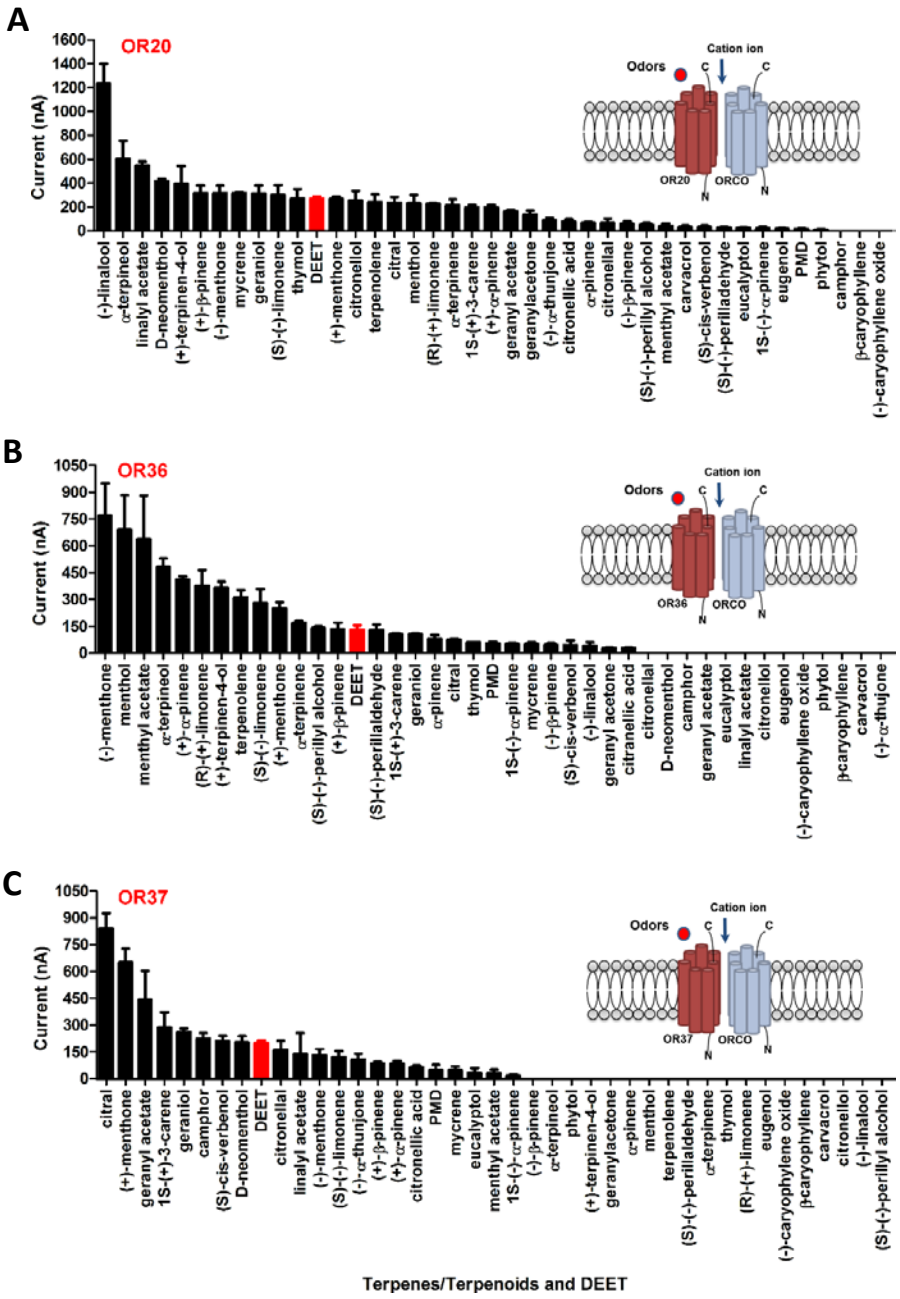

Figure 6.3 The current responses of three DEET-sensitive ORs to terpenes/terpenoids and DEET. A) responses of OR20 to terpenes/terpenoids and DEET; B) responses of OR36 to terpenes/terpenoids and DEET; C) responses of OR37 to terpenes/terpenoids and DEET. The responses to DEET were emphasized with red color. All the data (Mean±SEM) from OR's response to terpenes/terpenoids were retrieved from the study of Liu et al, 2016 (manuscript).

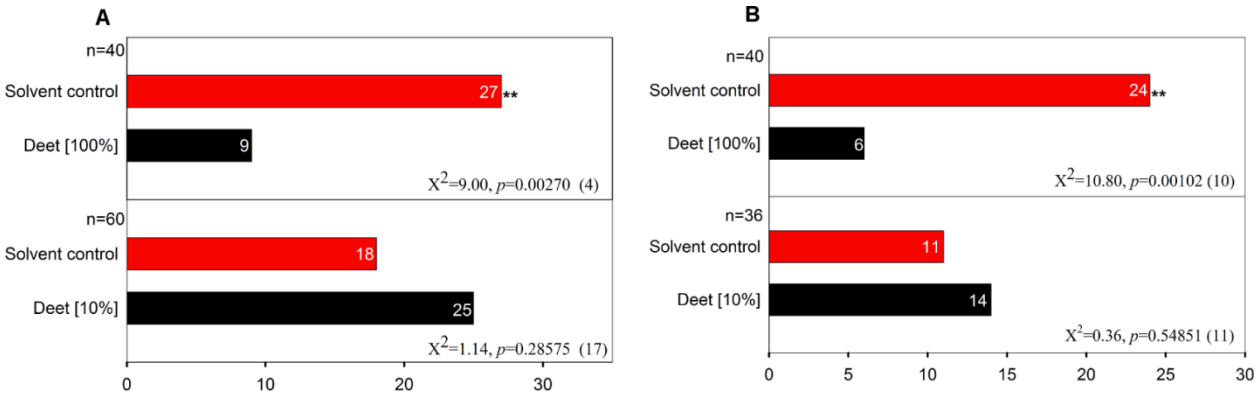

Figure 6.4 Repellency of high doses of DEET to the common bed bug. A) Behavior bioassay of male bed bugs to three doses of DEET (10%, 100%); B) Behavior bioassay of female bed bugs to three doses of DEET (10%, 100%). For each experiment, an asterisk indicates a significant response to DEET; $\chi 2$ test with Yates correction for continuity; *P<0.05; **P<0.01 (Siljander et al., 2008). 50 µl DEET of different doses was applied in each test. The value of n indicated the replicates for the two-choice olfactometer bioassay of single bed bug. DMSO was used as the control solvent for each replicates. Numbers in parentheses indicate the number of bed bugs not responding to DEET.

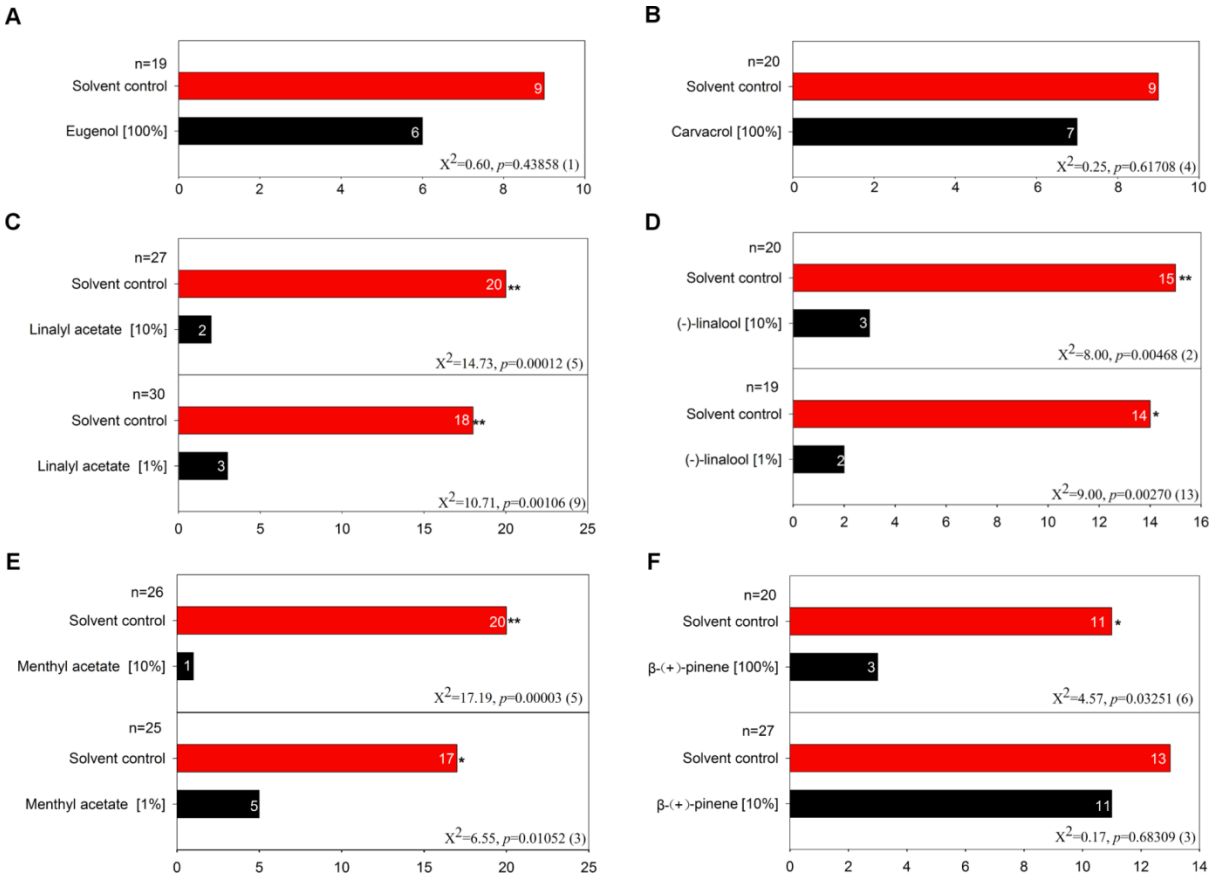

Figure 6.5 Repellency of different doses of four terpenes/terpenoids to the common bed bug. A) olfactometer bioassay of bed bugs to 100% of eugenol; B) olfactometer bioassay of bed bugs to 100% of carvacrol; C) olfactometer bioassay of bed bugs to two doses of linalyl

acetate (1%, 10%); D) olfactometer bioassay of bed bugs to two doses of (-)-linalool (1%, 10%); E) olfactometer bioassay of bed bugs to two doses of menthyl acetate (1%, 10%); F) olfactometer bioassay of bed bugs to two doses of (+)-β-pinene (10%, 100%). For each experiment, an asterisk indicates a significant response to the treatment stimulus; $\chi 2$ test with Yates correction for continuity; *P<0.05; **P<0.01 (Siljander et al., 2008). 50 µl treatment stimulus of different doses was applied in each test. The value of n indicated the replicates for the two-choice olfactometer bioassay of single bed bug. DMSO was used as the control solvent for each replicates. Numbers in parentheses indicate the number of bed bugs not responding to either test stimulus.

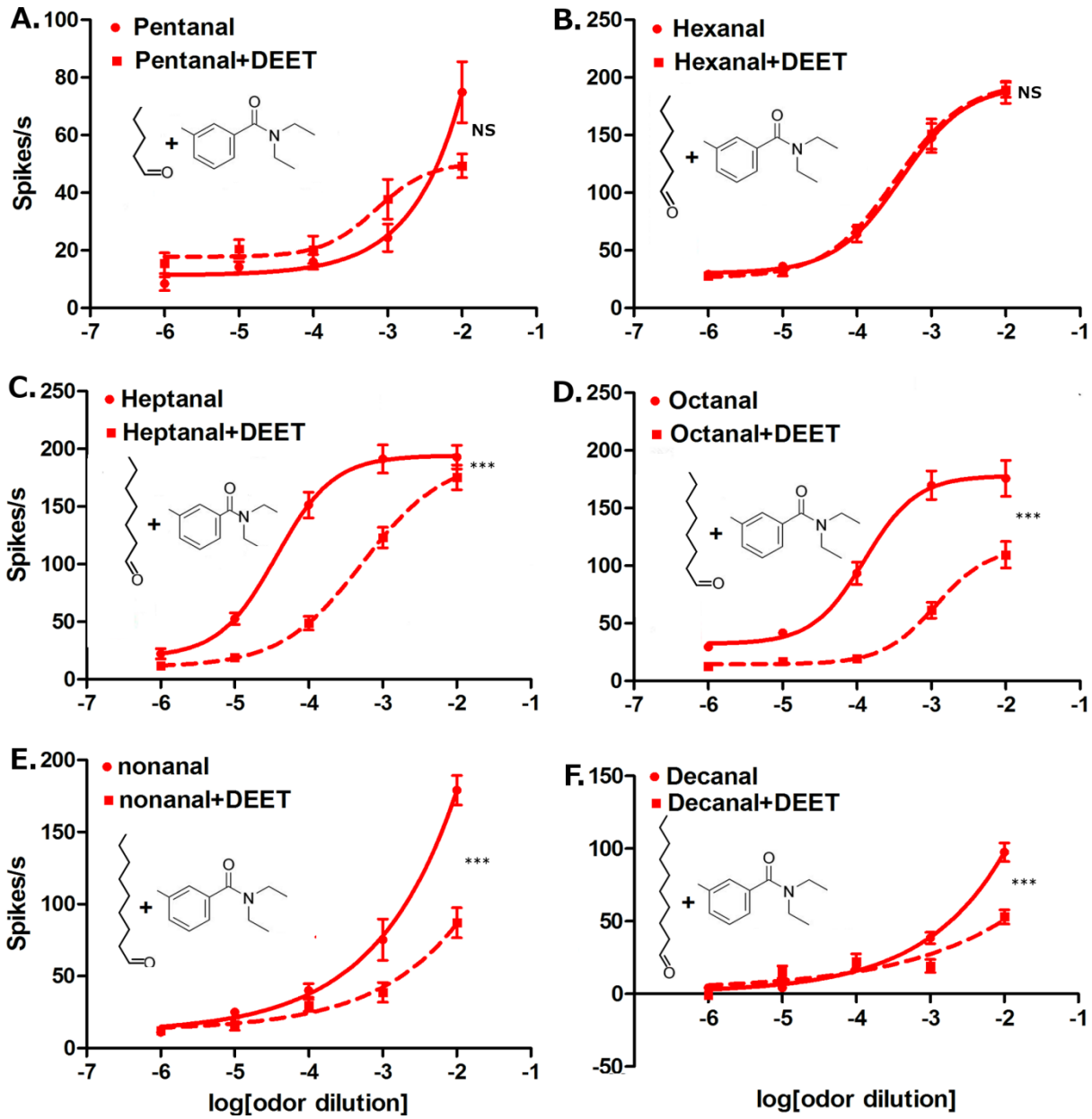

Figure 6.6 Modulation of DEET on the neuronal responses of bed bug Dγ sensilla to aldehyde odorants. A) Dose–response curves of ORNs in Dγ sensilla to pentanal ($1{:}10^2$ v/v) with (solid line) or without DEET (dash line); B) Dose–response curves of ORNs in Dγ sensilla to hexanal ($1{:}10^2$ v/v) with (solid line) or without DEET (dash line); C) Dose–response curves of ORNs in Dγ sensilla to heptanal ($1{:}10^2$ v/v) (solid line) or without DEET

(dash line); D) Dose–response curves of ORNs in Dγ sensilla to octanal ($1{:}10^2$ v/v) (solid line) or without DEET (dash line); E) Dose–response curves of ORNs in Dγ sensilla to nonanal ($1{:}10^2$ v/v) (solid line) or without DEET (dash line); F) Dose–response curves of ORNs in Dγ sensilla to decanal ($1{:}10^2$ v/v) (solid line) or without DEET (dash line). (F-test with Bonferroni correction; mean±SEM., n=6–10; NS, no significance; *P<0.05; **P<0.01; ***P<0.001) . Dose-response curve was fit with the Sigmoidal dose-response model with variable slope using the Graphpad Prism 5.

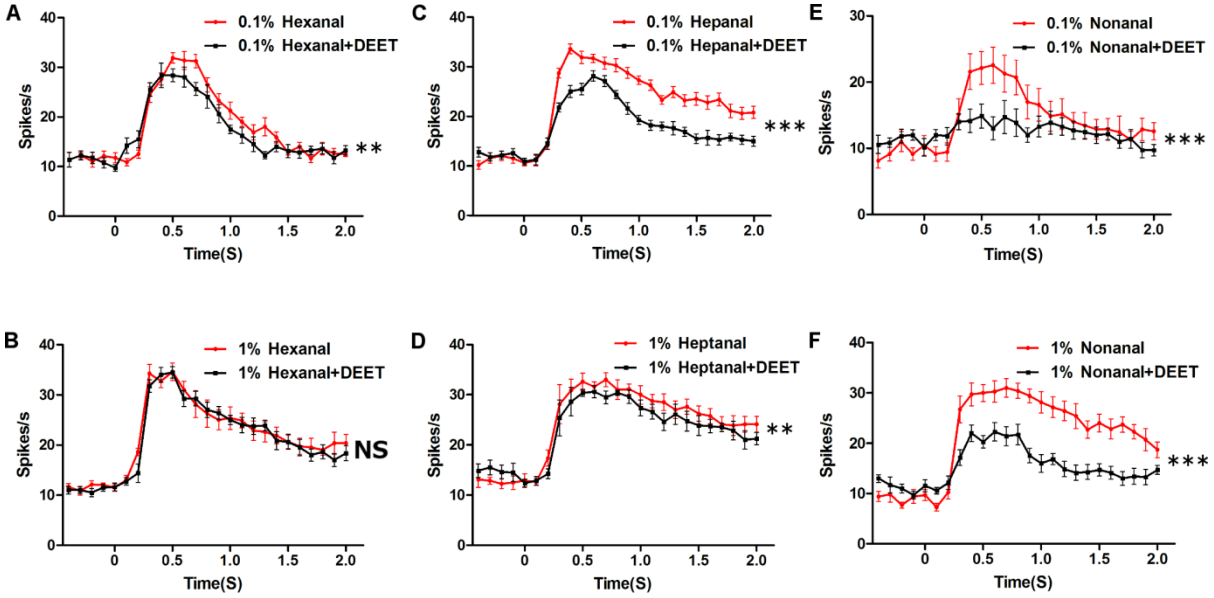

Figure 6.7 Modulation of DEET on the temporal dynamic of responses to odorants. A) Temporal dynamic of responses to hexanal at the dose of $1{:}10^3$ v/v with (black line) or without DEET (red line); B) Temporal dynamic of responses to hexanal at the dose of $1{:}10^2$ v/v with (black line) or without DEET (red line); C) Temporal dynamic of responses to heptanal at the dose of $1{:}10^3$ v/v with (black line) or without DEET (red line); D) Temporal dynamic of responses to heptanal at the dose of $1{:}10^2$ v/v with (black line) or without DEET (red line); E) Temporal dynamic of responses to noanal at the dose of $1{:}10^3$ v/v with (black line) or without DEET (red line); F) Temporal dynamic of responses to nonanal at the dose of $1{:}10^2$ v/v with (black line) or without DEET (red line) (F-test with Bonferroni correction; mean±SEM., n=6–10; NS, no significance; *P<0.05; **P<0.01; ***P<0.001).

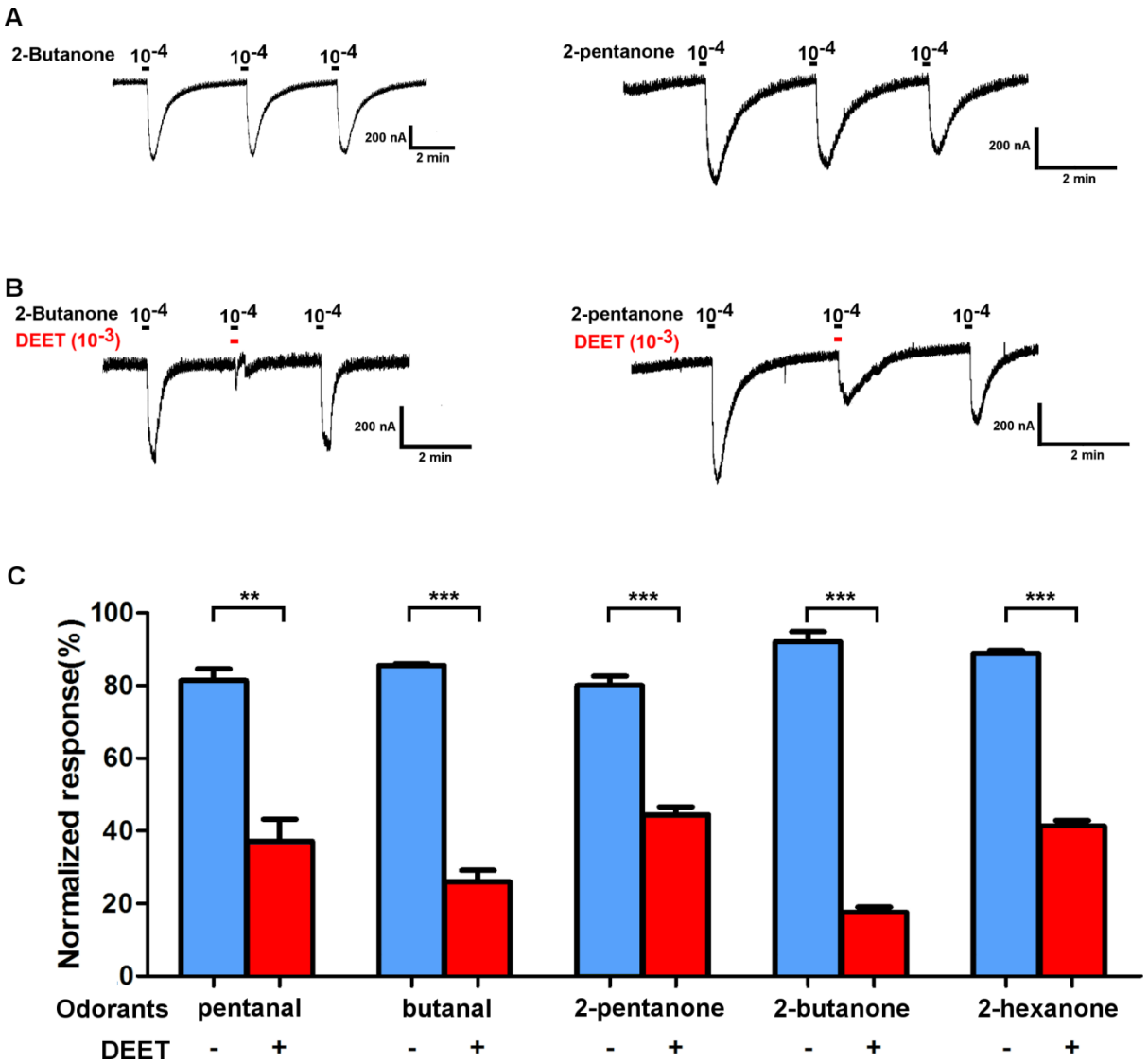

Figure 6.8 Antagonistic effect of DEET on the current responses of OR19/ORCO to odorants. A) 2-butanone and 2-pentanone at the dose of 1:104 v/v elicited macroscopic inward currents in oocytes expressing OR19/ORCO, respectively. During a repetitive stimulation, the agonist-evoked amplitudes will be slightly desensitized. B) Current response evoked by 2-butanone and 2-pentanone (at the dose of $1:10^4$ v/v) was considerably inhibited by DEET ($1:10^3$ v/v). C) DEET significantly antagonized the current responses of OR19/ORCO to the odorants. To enable a fair comparison, responses evoked from the second stimulation with or without DEET will be normalized with the responses evoked from the first stimulation (*t*-test with Bonferroni correction; mean±SEM, n=6; *P<0.05; **P<0.01; ***P<0.001).

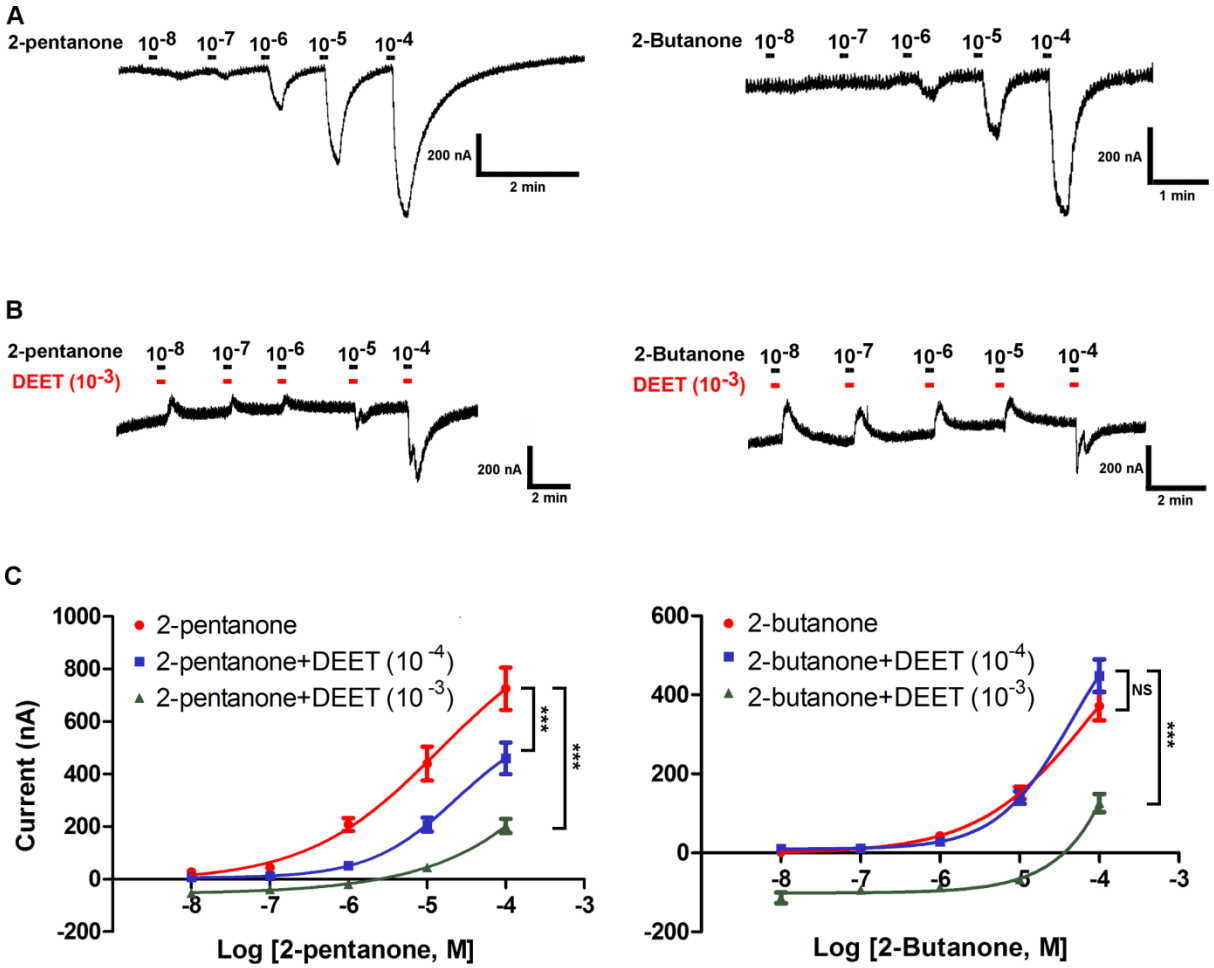

Figure 6.9 Antagonistic effect of DEET on the dose-dependent responses of OR19/ORCO to 2-butanone and 2-pentanone. A) Does-dependent responses of OR19/ORCO to 2-butanone and 2-pentanone without DEET added into the perfusion. B) Does-dependent responses of OR19/ORCO to 2-butanone and 2-pentanone with DEET ($1{:}10^3$ v/v) added into the perfusion. C) Fitted dose-response curve of OR19/ORCO to 2-pentanone and 2-pentanone with (blue line $1{:}10^4$ v/v, green line $1{:}10^3$ v/v) or without DEET (red line) (F-test with Bonferroni correction; mean±SEM., n=6–10; NS, no significance; *P<0.05; **P<0.01; ***P<0.001) . Dose-response curve was fit with the Sigmoidal dose-response model with variable slope using the Graphpad Prism 5.

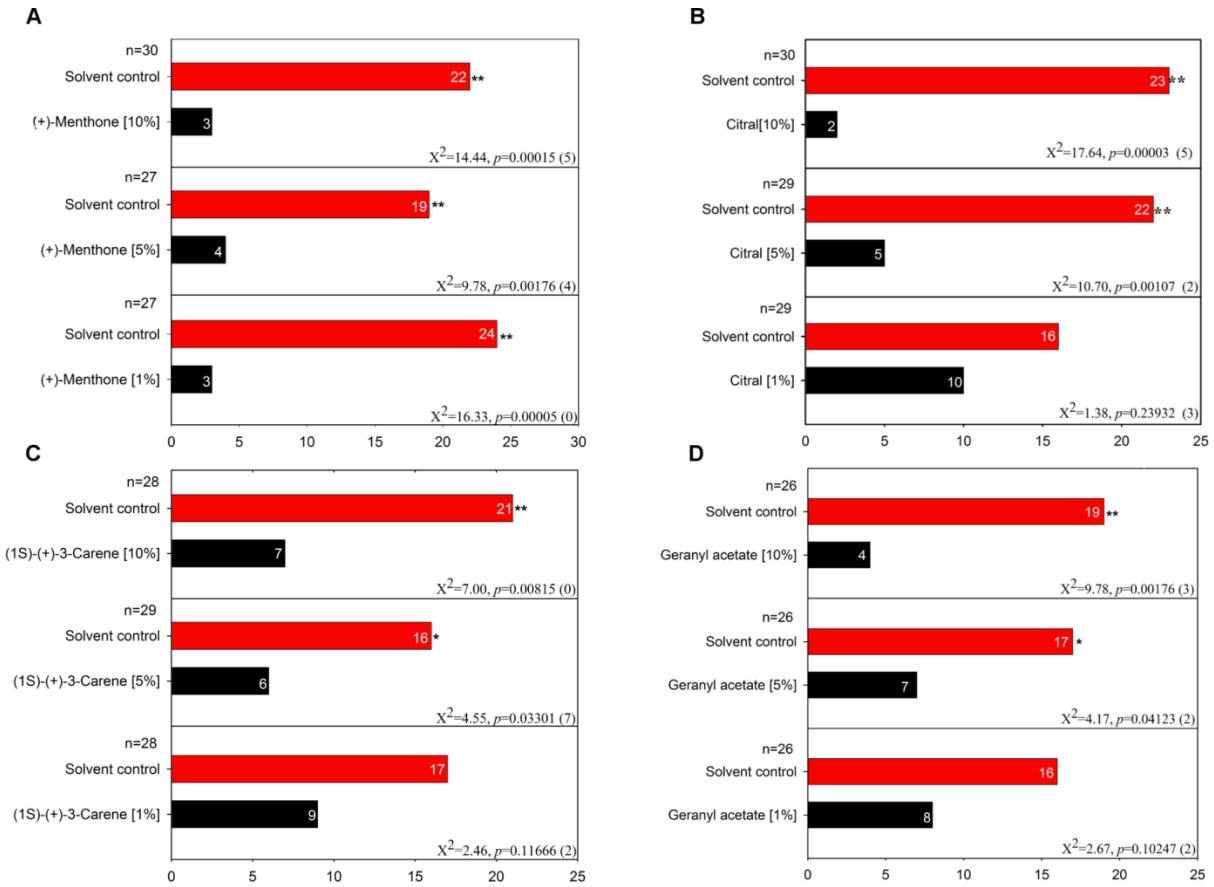

Figure 6.S1 Behavior bioassay of bed bugs in response to terpenes/terpenoids. A) olfactometer bioassay of bed bugs to three doses of (+)-menthone (1%, 5%, 10%); B) olfactometer bioassay of bed bugs to two doses of citral (1%, 5%, 10%); C) olfactometer bioassay of bed bugs to two doses of 1S-(+)-3-carene (1%, 5%, 10%); D) olfactometer bioassay of male bed bugs to three doses of geranyl acetate (1%, 5%, 10%). For each experiment, an asterisk indicates a significant response to the treatment stimulus; $\chi 2$ test with Yates correction for continuity; *P<0.05; **P<0.01 (Siljander et al., 2008). 50 µl treatment stimulus of different doses was applied in each test. The value of n indicated the replicates for the two-choice olfactometer bioassay of single bed bug. DMSO was used as the control solvent for each replicates. Numbers in parentheses indicate the number of bed bugs not responding to either test stimulus.

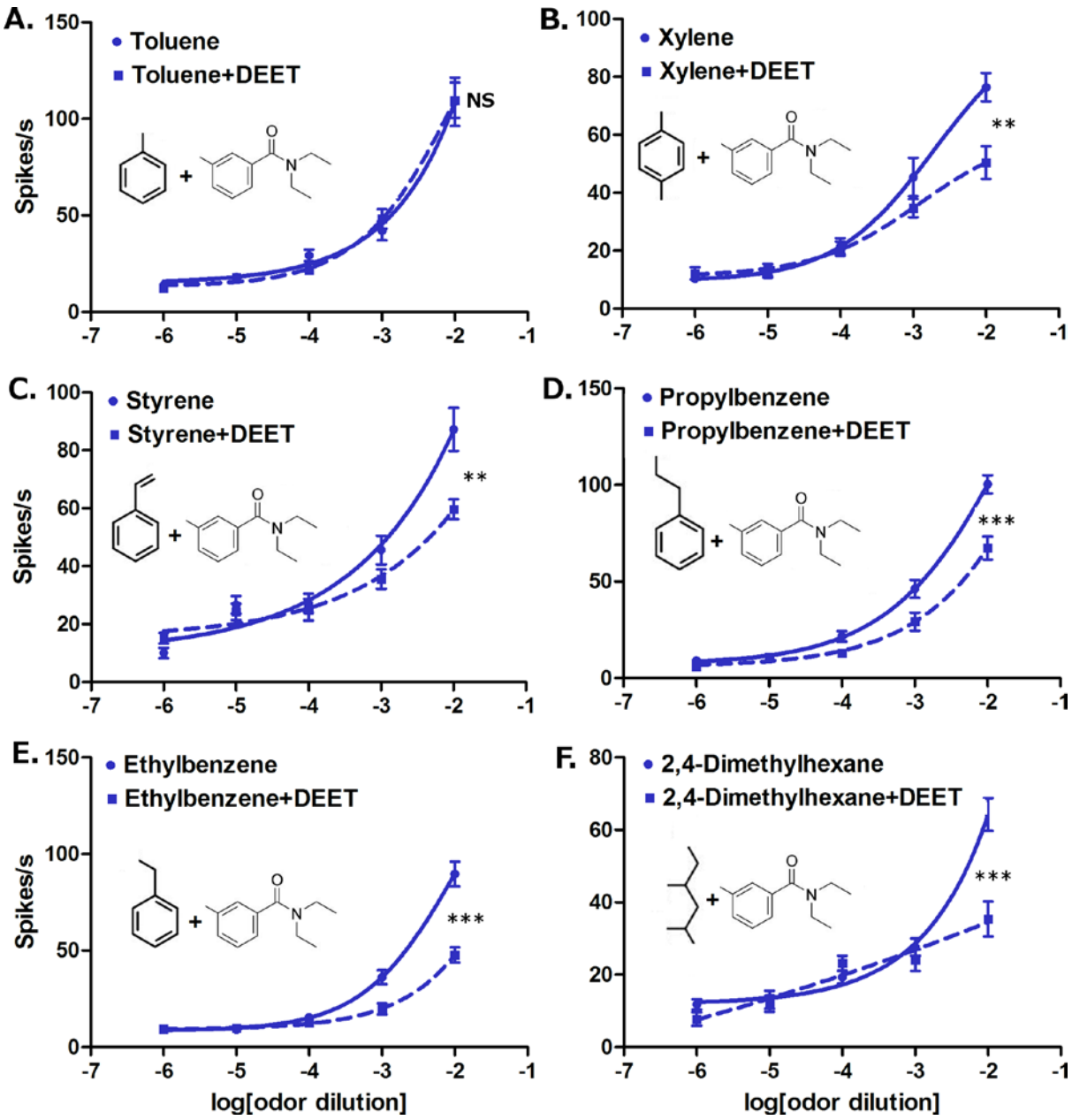

Figure 6.S2 Modulation of DEET on the neuronal responses of bed bug Dγ sensilla to aromatic/aliphatic odorants. A) Dose–response curves of ORNs in Dγ sensilla to toluene (1:10^2 v/v) with (solid line) or without DEET (dash line); B) Dose–response curves of ORNs in Dγ sensilla to xylene (1:10^2 v/v) with (solid line) or without DEET (dash line); C) Dose–response curves of ORNs in Dγ sensilla to styrene (1:10^2 v/v) (solid line) or without DEET (dash line); D) Dose–response curves of ORNs in Dγ sensilla to propylbenzene (1:10^2 v/v) (solid line) or without DEET (dash line); E) Dose–response curves of ORNs in Dγ sensilla to ethylbenzene (1:10^2 v/v) (solid line) or without DEET (dash line); F) Dose–response curves of ORNs in Dγ sensilla to 2,4-dimethylhexane (1:10^2 v/v) (solid line) or without DEET (dash line). (F-test with Bonferroni correction; mean±SEM., n=6–10; NS, no significance; *P<0.05; **P<0.01; ***P<0.001). Dose-response curve was fit with the Sigmoidal dose-response model with variable slope using the Graphpad Prism 5.

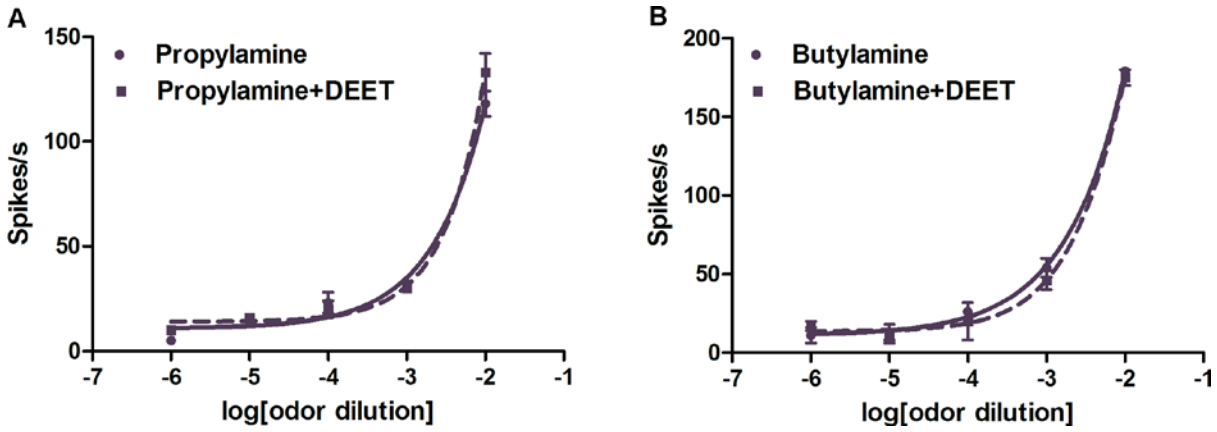

Figure 6.S3 No impact of DEET on the neuronal responses of bed bug Dγ sensilla to amine odorants. A) Dose–response curves of ORNs in C sensilla to propylamine ($1:10^2$ v/v) with (solid line) or without DEET (dash line); B) Dose–response curves of ORNs in C sensilla to butylamine ($1:10^2$ v/v) with (solid line) or without DEET (dash line). (F-test with Bonferroni correction; mean±SEM., n=6–10; NS, no significance; *P<0.05; **P<0.01; ***P<0.001) . Dose-response curve was fit with the Sigmoidal dose-response model with variable slope using the Graphpad Prism 5.

Chapter 7: Research Summary and Future Study

7.1 Research Summary

My research majorly focused on the chemoreception in the common bed bug, *Cimex lectularius*. Specifically, the cellular and molecular mechanisms of olfaction of bed bugs were investigated. Two general categories of odorants were selectively used in this study, including human odorants, which were considered as important cues in the host seeking process of bed bugs, and chemical insect repellents, which have been reported as chemical repellents in other insect species, particularly some blood-sucking insects, like mosquitoes and kissing bugs. Four objectives have been addressed in this study, including 1) Characterizing the olfactory neuronal responses of bed bugs to human odorants; 2) Investigating the olfactory neuronal responses of bed bugs to chemical repellents; 3) Deciphering the molecular mechanisms involved in the odorants reception of bed bugs; 4) Revealing the mechanisms of DEET's repulsive effect to the bed bugs.

For the first objective, we answered the question about how the ORNs of bed bugs respond to the human odorants, excitatory or inhibitory. By using the single sensillum recording technique, we found that different types of olfactory sensilla produced distinctive excitatory response profiles to human odorants. Particularly, we found that D types of olfactory sensilla, including Dα, Dβ, Dγ sensilla, have the widest spectrum in detecting the odorants, E sensilla respond to very few odorants with low firing frequencies and C sensilla were only found to be sensitive to the amines and several heterocyclies. Among all different chemical categories of human odorants, bed bug showed preference in detecting the aldehydes /ketones and alcohols but not the carboxylic acids. In addition, the dosages of human odorants were found to affect significantly on the firing frequency and temporal dynamics of neuronal response in bed bugs.

For the second objective, we answered the question about how the ORNs of bed bugs respond to the chemical insect repellents, excitatory or inhibitory. Using the single sensillum recording method, we found that different olfactory sensilla displayed different excitatory response profiles to the chemical repellents. Similar to the first objective, D types of sensilla showed responses to a wide spectrum of chemical repellents while E types of sensilla presented very weak responses and C sensilla showed no responses to the chemical repellents. Comparatively, the common bed bugs are more likely to sensitize the terpene-derived (terpene and terpenoids) chemical repellents but not the non-terpene-deriveds, which suggested the promising application for these terpen-derived chemical repellents in the management of bed bugs.

For the third objective, we answered the question about the molecular basis of neuronal responses of bed bugs to both human odorants and chemical repellents. In this study, we used the Xenopus oocytes expression system coupled with two electrode voltage clamp to test the function of individual bed bug odorant receptor in responses to both human odorants and chemical repellents. Thirteen odorant receptors were successfully expressed in the frog oocytes and showed significant responses to at least one odorants from either human beings or plant volatiles. We found that each odorant receptor respond to multiple odorants and each odorant was encoded by multiple odorant receptors. Bed bug odorant receptors were more likely to detect the aldehydes/ketones, alcohols, heterocyclics and terpenes/terpenoides but not the carboxylic acid. We also found that the dosages of odorants greatly impacted the responses of odorant receptors. With the increase of the dosages of odorants, the current responses from bed bug odorant receptors would increase. By calculating the EC50 values of certain odorants for specific odorant receptors, the potential cognate ligands were identified in this study. When we used the responses from these thirteen odorant receptors to build a bed bug odor space, we found that certain odorants with similar structures were clustered

together and showed very short Euclidean distance from each other, which suggested that more efforts were required for bed bug ORs to distinguish odorants with similar structures. Meanwhile, we also found that some odorants were widely dispersed in the odor space with a big Euclidean distance, which were probably differentiated easily from each other by the bed bug olfactory system.

For the fourth objective, we answered the question about the cellular and molecular basis of bed bug's responses to DEET. DEET as the most extensively used chemical insect repellents, had also showed repulsive effect to the bed bugs. By combining both the single sensillum recording and *Xenopus* oocytes expression system, we were able to reveal the cellular and molecular basis involved in the aversive responses of bed bugs to DEET. In this study, we identified two types of olfactory sensilla, including Dα and Dβ sensilla, showed excitatory responses to DEET. Then we also found that at least three bed bug odorant receptors were involved in the detection of DEET. Meanwhile, these three odorant receptors were also found to be even more sensitive to the terpenes/terpenoids, which were originally isolated from the plants. Behavior bioassay further indicated that these terpenes/terpenoids were much more effective in repelling bed bugs compared to DEET, which required a very high dose to show a significant repulsive effect on the bed bug behavior responses. Therefore, we raised a hypothesis that DEET probably targeted on the bed bug receptors that were naturally sensitive to terpenes/terpenoids, which was a novel mechanism for DEET's function on insect olfactory system. In addition to the direct activating effect of DEET on the bed bug olfactory receptor neurons or odorant receptors, we also found that DEET could block or mask the neuronal responses of bed bugs to certain odorants by interfering with the function of specific odorant receptors in response to certain odorants. Thus, this study confirmed dual role of DEET (activating effect and interfering effect) on the repulsive responses of bed bugs,

which would provide valuable information in the development of novel reagents in the bed bug control.

7.2 Future studies

Investigating the cellular and molecular mechanisms of chemoreception of bed bugs is very important for understanding the olfactory physiology/chemical ecology and developing efficient management tools or tactics. However, all our work are focusing on the peripheral nervous system of bed bugs without information on how the central nervous system process this peripheral olfactory information input from the olfactory receptor neurons on the bed bug antennae. In addition, although characterized the function of odorant receptors involved in the chemoreception of bed bugs, we have no idea about the function of other two families of olfactory receptors, including the ionotropical receptors and gustatory receptors, which may play a complementary role in the odorant sensation. Moreover, although we know the neuronal responses of bed bugs to these odorants, we cannot determine their role as attractants or repellents. Therefore, further studies focus on the following three aspects of bed bug chemoreception would greatly benefit our understanding on the bed bug sensory physiology and ecology.

7.2.1 Investigating odorants encoding in the antennal lobe of bed bugs

In the peripheral olfactory system, odorants go through the pores on the cuticle of olfactory sensilla and dissolve into the sensillum lymph. The odorants are first band by the odorant binding proteins (OBPs) and delivered to the odorant receptors on the neuron membrane. Once the odorants are released by the OBPs and activate the odorant receptors, the ORNs will be depolarized and produce the action potential. Therefore the firing information of ORNs will be further sent to the insect brain. In most insect brain, antennal lobe is the first processing center for olfactory information from the ORNs. In the antennal lob, there are

glomeruli, which are the clusters of terminals from ORNs and dendrites from the projection neurons from the mushroom body. Terminals from ORNs with the same odorant receptor will project into the same glomerulus and transfer the firing information to the projection neurons. Therefore, the glomeruli in the antennal lobe are actually sophisticated sites for authentic olfactory signal transmission. Different glomeruli are responsible for different firing information transmission. Previous studies suggested that firing information from ORNs will be normalized through lateral enhancing or inhibition by the lateral neurons surrounding the glomeruli (Hong et al., 2013). Therefore, the antennal lobe is the key structure for processing the olfactory information perceived form the outside environment. Previous studies have characterized the architecture of antennal lobe in several Hemipterian insects, like the stink bug and kissing bugs and glomeruli have been morphologically identified and described (Kristoffersen et al., 2008; Barrozo et al., 2009). However, no study have been engaged into the understanding the architecture of antennal lobe s in the brain of bed bugs and the information processing mechanism inside it. Thus, for our future studies, it would be meaningful to characterizing the 3D-structure of bed bug antennal lobe first, then to investigating the mechanisms involved in the odorant encoding process.

In order to reveal the organization of glomeruli in the bed bug antennal lobe, histology and immunocytochemistry experiment will be conducted to prepare the sample of central nervous system for confocal microscopy. The fruitfly-originated antibody will be used in this study to specifically bind with the cell membrane of the antennal lobe as suggested by Kristoffersen et al., (2008). The well-stained sample will be used for scanning under the confocal microscopy layer by layer. After that, we will use the graphic software Amira to re-construct the bed bug antennal lobe. Thus, we build the foundation for further study on the odorant presentation in or among the glomeruli.

To get a clear view about how the odorants are encoded by the glomeruli in the bed bug antennal lobe, a powerful technique is two-photo calcium imaging system, which can *in vivo* monitor the excited neurons in specific glomerulus of distinct locations in the antennal lobe. By loading membrane-permeant fluorescent indicator dyes in large populations of cells, then using two-photon Ca^{2+} recordings to image through the intact brain to detect the excited glomerulus in the antennal lobe, we can pinpoint which glomeruli are responsible for the secondary encoding of the odorants.

7.2.2 Characterizing the function of IRs and GRs of bed bugs

Besides ORs, both ionotropic receptors (IRs) and gustatory receptors (GRs) play fundamental roles in the odorant reception of insects. Previous studies indicated that GRs in mosquitoes are important in detecting the CO2 released from host bodies (Kent et al., 2008). Meanwhile, GRs are involved in the taste reception, particularly for some sugars, bitters and salt (Sato et al., 2011; Ling et al., 2011; Zhang et al., 2013). IRs are kind of ancient olfactory receptors, which are targeted by mostly polar air-borne molecules, like the amines, acids and aldehydes (Croset et al., 2010; Rytz et al., 2013). Interestingly, in our studies on the responses of bed bugs to human odorants, we found that the ORNs or ORs of bed bugs are insensitive to carboxylic acids emanated from the human bodies. The promising explanation is that acids are majorly covered by IRs but not ORs. Most the ORNs we tested in the laboratory were expressed ORs but not IRs. Since IRs and GRs are so important for bed bugs' host-seeking, predator-avoiding, and pheromone-sensing behavior, it would be meaningful to unravel the function of specific IRs and GRs.

Previous studies showed that *Xenopus* oocyte expression system coupled with two electrode voltage-gated recording is powerful technique to elucidate the function of either IRs and GRs in moth or fruit fly (Sato et al., 2011; Abuin et al., 2011). Therefore, for our future study, we

can still use the *Xenopus* oocyte expression system to express the bed bug IRs and GRs. Then two electrode voltage-gated recording can be used to detect the responses of IRs or GRs to different stimuli from either human bodies or plant-released volatiles, which may serve as chemical repellents for bed bugs.

7.2.3 Determining behavior responses of bed bugs to odorants

In our current studies, we characterized the neuronal responses of bed bugs to a large panel of odorants, from both human emanation or plant volatiles. However, not many of them are able to activate strong responses from ORNs or ORs. Although we did behavior tests to some terpenes/terpenoids using the two-choice olfactometer, for most these odorants that bed bugs are extremely sensitive to, we still have no idea about their role in determining the behavior of bed bugs. Therefore, future study focusing on the behavior responses of bed bugs to neuron-active odorants would benefit a lot for our understanding about the function of these stimuli (attractant or repellents) and build the basis for field application in the bed bug management.

As a brilliant way to test the behavior of bed bugs to odorants, the two-choice olfactometer described by Gris and his colleagues (2015) would be also useful in testing the behavior responses of bed bugs to odorants which bed bug showed strong responses to. The two-choice olfactometer was majorly formed by three Petri-dishes with a small tube to connect each other. Filter papers will be placed into each Petri dish in order for bed bug's convenient moving. A single bed bug will be allocated in the middle Petri dish. Odorant with certain dose will be placed in one of the side Petri dish while the control solvent, DMSO, will be added into another side Petri dish. A small piece of cardboard paper will be placed in both side of the Petri dish. In this way, we can observe which side of the Petri dish that bed bug

will creep into. Based on the behavioral choice of bed bugs, we can make the conclusion about the attractiveness or repulsiveness of odorant that is tested.

7.3 References

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