Characterizing the Responses of Antennal Olfactory Sensilla of Bed Bugs, *Cimex lectularius*, to Components of Aggregation Pheromone

by

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Abstract

Responding to urgent call for detecting and monitoring tools of bed bugs, rapid resurgence of which has been noticed by many developed countries recently, aggregation pheromones have been considered as a promising baits for monitor all developmental stages of bed bugs. This study addressed questions concerning how the signals of aggregation pheromones were encoded by olfactory sensilla of the bed bug, *Cimex lectularius*. From broadly tuned spectra of D sensilla with high sensitivity to aggregation pheromone, to narrowly tuned C sensilla, the overall encoding map of sensilla for aggregation pheromone revealed strong responses of odorant-sensillum combinations without showing sexual dimorphism. Majority of sensilla that were well developed in nymph bed bugs generally showed similar response patterns to our odorants panel, except for early instar nymph stages (1^{st} and 2^{nd}), which exhibited significant lower neuronal activity for S-(-)-limonene and R-(+)-limonene compared to 5^{th} nymph and adult stages. We finally tested the olfactory response patterns of the most abundant sensilla, hair-like sensilla, with stimuli from different components of an aggregation pheromone as well as human odorants. Our results revealed 4 distinct functional classes of hair-like sensilla.

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List of Abbreviations

DDT	Dichlorodiphenyltrichloroethane
OBP	Olfactory Binding Protein
OR	Olfactory Receptor
ORN	Olfactory Receptor Neuron
SSR	Single Sensillum Recording

Chapter 1. Literature review and Research goals and objectives

Morphology and biology of the common bed bug

Bed bugs, *C. lectularius,* are members of *Cimicidae* family that is ectoparasite of warm-blooded animals, including dogs, birds, rodents and humans (Usinger 1966). Two regular species that have been found to regularly feed on humans: 1) the common bed bug, *C. lectularius,* which is widely distributed in the temperate and sub-tropical regions of the world, and 2) the tropical bed bug, *C. hemipterus*, which is distributed throughout tropical and sub-tropical regions (Doggett et al. 2012). Adult bedbugs are typically Cimicidae, being wingless, dorsal-ventrally flattened, brownish insects about 4-7mm in length. They are rather oval shape with the short rudimentary wings, or hemelytra, the broad head, and well-developed eyes that are folded beneath the head and thorax (Usinger 1966). Nymphs resemble the adults in shape, but are smaller and lighter in color. Newly hatched nymphs are translucent and smaller than a pinhead (1 mm). The eggs are very small, whitish, and very difficult to see on most surfaces without magnification.

The bed bug is hemimatabolous insects, which will go through five instars to become an adult. Sex of the bed bug can be distinguished from the tip of abdomen; males have a pointed tip whereas females have a rounded abdomen. Bed bug molts at each developmental stage after consuming a blood meal by sheding their skins through ecdysis and discarding their outer exoskeleton. Female adults need to take a blood meal in order to lay eggs. Eggs hatch in about a week at the normal room temperature (27-28 ^{o}C). Each instar lasts approximately a week.

Therefore, under favorable condition, a complete life cycle can be completed in as little as two months. Lower temperatures or fewer blood meals will prolong the development time (Doggett et al. 2012). An adult female can produce about 500 eggs in her entire life. Egg production will stop if lacking of blood meal. Bed bugs are very resilient; both nymphs and adults can persist for months at extreme temperature as low as 55 F or even less without feeding.

Bed bug resurgence and pest management

Bed bugs have been associated with human for more than 3500 years, with the evidence of fossilized bed bugs found in Egyptian tombs (Eva Panagiotakopulu and C. Buckland 1999). Experts suggest that the bed bugs are most likely began to parasitize humans when bat, bugs and human three lived together in caves, then moved out with human to village and city (Usinger 1966). Bed bugs were abundant in human dwelling until 1950s, in which bed bugs were almost eradicated in urban environment due to the use of broad-spectrum synthetic insecticides, most notably dichlorodiphenyltrichloroethane (DDT), and along with greater public awareness (Potter 2011). However, bed bugs have being making a remarkably rapid resurgence in many developed countries in the recent twenty years. All states in the U.S. have shown bed bug infestation based on the online survey conducted by the National Pest Management Association (NPMA) and the University of Kentucky (Potter et al., 2010). Various hypotheses have been proposed for the phenomena of the suddenly resurgence of the bed bugs, including increased international travels, increased second-hand furniture exchange, lack of vigilance of public, poor management practice, and the increase of resistance (Potter et al., 2008).

It has been a challenge to eradicate bed bugs even for well-trained pest control professionals, largely because their cryptic habits which congregate in cracks and crevices. There

are two different managements have been mostly used, i.e., non-chemical methods and chemical controls. Non-chemical methods, such as steam, heat, cold, can achieve high quality eradication when combined with pesticides (Wang and Wen 2011). A survey (Gangloff-Kaufmann et al. 2006) revealed that vacuuming, sticking traps, encasing mattress, steaming, and heating have been the most frequently used non-chemical methods in the control of bed bug. The chemical control of using insecticides has been the most widely tools in bed bug management.

Nowadays, two classes of insecticides commonly used to control bed bugs are pyrethroids and neonicotinoids. Unfortunately, intensive use of pyrethroids has induced high level of resistance in bed bugs (Romero et al., 2007; Zhu et al., 2010). Multiple mechanisms are contributing to the resistance, including target-site mutation, P450 metabolic detoxification, and cuticular proteins that can impede pesticide penetration (Zhu et al. 2010, 2012, Mamidala et al. 2012). Two point mutations on voltage-sensitive sodium channel α-subunit genes, V419L and L925I, are assumed to be involved in the resistance to deltamethrin, a common pyrethroid insecticide on market (Yoon et al. 2008). Subsequently, scientists from worldwide reported these mutations occurred at high rates in the field population (Dang et al., 2015; Durand et al., 2012; Koganemaru & Miller, 2013; Palenchar et al., 2015), indicating the widespread distribution of deltamethrin resistance in bed bug populations (Zhu et al. 2010). A recent research even evaluated and confirmed the presence of cross resistance in field populations to combination of pyrethroid and neonicotinoid products (Gordon et al., 2014).

Due to the restriction on pesticides used indoors, there is low availability of developing a new insecticide as effective DDT. Alternative tools, such as repellent and pheromone related products, have been frequently used in the bed bug control. Detection infestation at the early stage is the key of success in bed bug control, for both eliminating at vulnerable stage and

monitoring the success of treatment. The online survey of pest management professionals (PMPs) suggests visual inspection continues to be the most common method in bed bug detection in 2015 (Potter et al., 2015). Some other alternative methods have also come up in the recent few years, such as odor detection canines and acoustic sensors, but they are either expensive or impractical. Recent researches are seeking a reliable and affordable device to attract bed bugs based on various cues.

Study of aggregation behavior and identification of aggregation pheromone can be very helpful in bed bug monitoring or control. Aggregation pheromone can be a promising tool for bed bug management given the fact that it can be detected at a long distance with a low concentration by insects and its strong attractant effect without being influenced by physiological status. The major attractive components of aggregation pheromone have been identified. When test the formulation in infested apartments, 26 out 27 pheromone-baited traps captured at least one bed bug each (Gries et al. 2015). Subsequently, a trap with this pheromone was developed and has been marketed.

Aggregation behavior of bed bugs

After each blood-meal, *C. lectularius* returns to shelter and aggregates in cluster with both sexes and all developmental stages of conspecific bugs. Bed bugs find little cracks and crevice in human-dwellings as well as roosts of chickens and tree-holes of bats as their shelters. During daytime, bed bugs are usually found in a quiescent state in dark shelters, contacting with fecal matters, egg shells, and exuviae accumulates while digestion takes place (Usinger 1966). Although the mechanisms of aggregation are various from species to species, it has been proposed that ecological benefits are shared among non-social arthropods species, including 1)

indicate food resource 2) facilitate sexual interaction, and 3) protect from natural enemy and severe environmental condition (Wertheim et al., 2005). The hypothesis in Cimicidae has been tested under different conditions. For example, dumped in still-air microhabitats like cracks and crevices can protect bed bugs, especially for early instar nymphs, from quick desiccation, thus increase resistance to dehydration (Benoit et al., 2007). And another research shows nymphs in aggregation grew faster than in solitary (Saenz et al., 2014).

Many efforts have been made that may influence off-host aggregation behavior of bed bugs. Bioassays indicated that physiological factors, including life stages, sexes, starve status, and mating status, do not effect attractant responses of tested bed bugs to intraspecific bug-exposed paper (Olson et al., 2009; Weeks et al., 2012). While extrinsic factors, like circadian rhythms, tactile stimuli, and colors, showed significant effects on arrest of bed bugs according to behavioral tests (Levinson et al., 1971; Reis et al., 2011; Singh et al., 2015). Although experiments showed that bed bugs that had successfully fed tend to aggregate in shelters compared those that had not successfully fed, all bed bugs started to return to harborage to aggregate 2 h prior to the photo phase (Reis and Miller 2011).

Use of aggregation pheromone has been found in many orders of insects (Wertheim et al. 2005). Pheromones are characterized by the behavior that they elicited, aggregation pheromone, by definition, can be produced by either sex or any stage and leads to the formation of conspecific groups of mixed sex and stages (Wyatt, 2003). Early study suggested that bed bugs detect "nest odor" related with scent gland secretion at distance of 75 cm and return to harborage (Marx, 1955). Subsequently, experiments showed that both sexes of adults *C. lectularius* tend to aggregate and rest on filter paper preciously impregnated by the body scent of conspecific adults of *C. lectularius* (Levinson and Bar Ilan 1971). However, volatiles analyzed

from scent gland secretions of bed bugs were failed to show attraction on bed bugs (Schildknecht et al., 1964; Collins, 1968; Levinson et al., 1971). In contrast, functional tests of main components of the volatiles, hex-2-en-1-al and oct-2-en-1-al, elicited a rapid disperse response of tested bed bugs, leading to an investigation of alarming pheromone (Levinson and Bar Ilan 1971, Levinson, Levinson, and Maschwitz 1974).

Until recently, the composition of the bed bug aggregation pheromone was finally revealed. Siljander et al. (2008) were able to find juvenile-specific contact pheromone exist among *C. lectularius.* From their results, juveniles, but not females or male, preferred resting on juvenile-exposed paper discs. Both males and females showed preference on male-exposed paper discs. Neither of juvenile, males, and females preferred female-exposed paper discs to control discs (Siljander et al., 2007). They further verified the hypothesis that both contact and volatiles pheromone contribute aggregation of bed bugs. Ten volatile, including nonanal, decanal, (E)-2-hexanal, (E)-2-octenal, (2E, 4E)-octadienal, benzaldehyde, (+)- and (-)-limonene, sulcatone, benzyl alcohol, extracted from headspace of experimental harborage of *C. lectularius* were proven to be essential for attractant. Although the synthetic blend of 10 essential chemicals combines with juvenile contact pheromone was significantly preferred in lab condition, the combination failed as a field trap. The group's most recent publication reported a field-effective blend of aggregation pheromone components extracted from exuviae and feces of bed bug, which exist in every natural shelter of *C. lectularius.* Use of five reported volatile components (dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS), (E)-2-hexanal, (E)-2-octanal, and 2-hexanone) can attract both juveniles and adults *C. lectularius* into trap, and another less-volatile component, histamine, could lead arrestment of attracted bed bugs (Gries et al. 2015). The identification of aggregation pheromone components is important in the bed bug control and

monitoring. However, the mechanism involved in response of bed bugs to aggregation pheromone components is unexplored.

Peripheral olfactory system of the common bed bug

Opposite to human, insects heavily rely on olfactory system for communication. As we know, the entire olfactory process of insect includes 1) reception of pheromone and other semiochemicals by specialized peripheral olfactory organs called olfactory sensilla, usually located on antenna and maxillary palps; 2) transduction of signals from axons of olfactory receptor neurons (ORNs) to antennal lobe, where numerous glomeruli process various chemical signals; 3) integration of different inputs in the lateral horn and mushroom body with other sensory modalities; 4) finally convert into behavior (Leal 2014). The selectivity and sensitivity of olfaction in part rely on the perception of periphery olfactory system. Olfactory sensilla which house one to several ORNs are basic organs for odor detection, majority of them are located on insect antenna, some exceptions can be found on maxillary or labial palps (de Bruyne and Baker 2008). Little pores on the wall of sensillum were assumed to be prerequisite for olfactory function as odorant molecules can have access to get through the sensilla wall and dissolve in the lymph (Steinbrecht et al., 1976). Olfactory receptors (ORs) embedded on the membrane of dendrites of ORNs are bathed in a lumen filled with mucus-like sensillum nymph (de Bruyne and Baker 2008). Accessary cells not only play an important role in providing extracellular milieu to segregate ORs from outside harmful chemicals, but also produce some useful enzymes and proteins. One class of protein called Olfactory Binding Proteins (OBPs) or Pheromone Binding Proteins (PBPs) isolated from sensillum nymph before ORs were identified (Bruyne et al., 2001). These proteins are abundant in the sensillum lymph which act as “ships” to transport odorant molecules to ORs. Recent genome sequence of bed bugs show that there are 48 genes encoding

49 olfactory receptors, an intermediate level of ORs is in coordinate with the relative simple chemical ecology (Benoit et al. 2016). Also being an obligate blood feeder mosquito, *Aedes aegypti* has 100 ORs in consistent with the fact that the mosquito has broader host range and longer host seeking distance (de Bruyne and Baker 2008).

Nymph hemimetabolous insects add more sensilla as each time they molt, but similar between males and females. Adults have most abundant sensilla as well as ORNs that nymphs do not have (de Bruyne and Baker 2008). Adult bed bugs failed to respond to alarm and aggregation pheromone when deprived distal segment of antenna (Levinson et al., 1974). Structure analysis of the distal flagellum revealed 44 sensilla with pores on the cuticle wall distributed on two opposite olfactory region of the distal segment of antenna. They were classified into three different olfactory sensilla type with different structure characteristics and various ORNs housed in them, consisting of 6 grooved peg sensilla (C sensilla), 9 smooth peg sensilla (D sensilla), 29 hair sensilla (E sensilla) (Levinson et al., 1974). Recent study by Olson et al. (Olson et al., 2009) also suggested that a patch of sensilla located on distal half of pedicel is responsible for bed bug, *C. lectularius,* aggregation behavior. Further structure analysis showed similarity with gustatory sensilla of *C. hemipterus* (Olson et al., 2014). However, no evidence of physiological olfactory response was approved in these sensilla. It is most likely that the sensilla are related to tactile cues detection in aggregation behavior.

A previous electrophysiological study of olfactory system have tested single sensillum response to hundreds of different chemicals like human odorant (Liu and Liu 2015), chemical repellent (Liu et al., 2014), and other compounds related to chemical ecology of bed bugs (Harraca et al., 2009). Although each chemical elicited different responses in different sensilla, these results pointed to same trend. Converging evidence of electrophysiological results and

structure analysis suggest that sensilla have most abundant neurons and pores showed strongest response and widest spectrum. D sensilla are most sensitive type of sensilla in response to various chemical stimuli, especially to aldehydes and ketones. Harraca et al. (2009) further identified three functional groups of D sensilla (Dα, Dβ, Dγ) according to their different olfactory response patterns to 31 chemical compounds. C sensilla are narrowly tuned to few class of chemicals, like amines. There are two types of E sensilla, E1 and E2, with slightly structure difference, the former of which have even cuticle walls whereas the latter have uneven walls. E type of sensilla, which house only 1-3 neurons, reported to show no significant response to majority of tested chemicals. Except E2 type of sensilla that show some strong responses to certain long-chain chemicals in human odorants (Liu and Liu 2015).

Research goals and objectives

The goal of this study is to understand the cellular mechanism of interaction between bed bug olfactory system and aggregation pheromone, trying to provide useful information for alternative pest management of bed bugs. Our hypothesis in this study is that different components of aggregation pheromone are perceived by different olfactory receptor neurons housed in sensilla.

For the first objective in this thesis addresses the question of whether there is sexual dimorphism between male and female in response to aggregation pheromone. I characterize the responses of olfactory sensilla of adult bed bug, *Cimex lectularius,* to components of aggregation pheromone. In addition, I also test dose-dependent response on chemical-sensillum combination that shows strong response at screening stage.

For the second objective, I test the difference of olfactory responses at each nymphal stage. From this study, we can find out not only the change of sensilla number, but also the fluctuation of activities of receptor neurons housed in each olfactory sensillum at different developmental stages.

For the third objective, I use a panel of chemical components that close related to chemical ecology of bed bugs to test if there are different functional types within a certain important type of olfactory sensilla.

Chapter 2：Characterizing the olfactory response of adult bed bugs to components of aggregation pheromone

Introduction

The common bed bug, *Cimex lectularius*, is a temporary ectoparasite of warm-blood animals. It's an obligate blood feeder, which shows preference to human (Aboul-Nasr A.E., 1967). The unprecedented resurgence of bed bugs population in human habitats was reported in the early of 21st century (Potter et al. 2008). Restriction on pesticides used indoors resulted in the application mainly with pyrethroids, to which the bed bugs have shown resistance (Romero et al. 2007, Zhu et al. 2010). Without developing new effective product or alternative methods, rapid infestation of bed bug population could pose a risk to human health.

Bed bugs are nocturnal feeders; they leave their harborage to seek blood meals when their hosts are at sleep or minimum activity. During daytime, bed bugs congregate at harborages like conceives or cracks near their hosts and stay in a quiescent state for digestion and reproduction until next host seeking behavior. Previous studies assumed that *C. lectularius* produced an 'assembling scent' that attract conspecific bugs into shelters and maintained by tactile stimuli (Levinson and Bar Ilan 1971, Levinson et al., 1974). Very recent research has confirmed and identified the most effective components of aggregation pheromone; Five volatile components attract bed bugs into shelters and one of less-volatile component arrests bed bug at trap (Gries et al. 2015).

Insects rely heavily on olfactory system to seek their hosts, food resources or others. The sensitivity and selectivity of olfactory system usually determines their living-or-death. Odorants were detected by olfactory receptor housed in the peripheral olfactory organs, then

extracellular action potential was generated and electrical signal was transmitted into nervous system and finally convert into behavior response. With the evidence of antenna amputation, morphology observation, and electrophysiology response of olfactory sensilla, some reports suggested olfactory region of bed bugs located on the terminal segment of flagellum of antenna, on which 44 olfactory sensilla, were distributed sparsely. Bed bug failed to show proper response to alarming pheromone and aggregation pheromone when the olfactory region was amputated (Levinson et al. 1974, Harraca et al. 2009, Liu and Liu 2015). In contrast to previous reports, Olson et al. (2009) found that bed bugs loss the ability of arrestment on stained disks when deprived distal part of pedicel but not flagellum, suggesting sensilla on pedicel play an essential role in arrestment of bed bugs. Further structure analysis of sensilla revealed prospective olfactory sensilla and gustatory sensilla coexist on the pedicel (Olson et al. 2014), but no evidence of olfactory response was shown.

Odorant receptors (Ors) are seven transmembrane-domain proteins encoded by large gene families. *C. lectularius* has 48 genes encoding 49 Ors. Extensive researches on olfactory system of model insect, *Drosophila,* showed that individual members of Ors were expressed in a subset of olfactory receptor neurons (ORNs), which are compartmentalized into sensilla (Hildebrand and Shepherd, 1997; De Bruyne et al., 2001; Elissa A. Hallem, 2005). Three morphological type of sensilla were first described by Levinson et al. (1974) and Steinbrecht (1976) in *C. lectularius*, including grooved peg sensilla (C sensilla) housing 4-5 receptor neurons, smooth peg sensilla (D sensilla) housing 8-12 receptor neurons, and hair sensilla (E sensilla) housing 1-3 receptor neurons. Characterizing the electrophysiological responses of olfactory organs to aggregation pheromone will not only improve our understanding of neuronal basis of aggregation behavior, but can also provide useful information for screening most effective components that elicit strong responses at a low dosage.

Materials and Methods

Insects

The *C. lectularius* colony utilized in the 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, USA). Bed bugs were reared at 25±2 ºC under a photoperiod of 12:12 (L: D). Experiments were conducted using only males and females one week after blood feeding, regardless mating status.

Preparation of bed bugs and stimuli solutions

Single Sensillum Recording method was used in this experiment following the protocol introduced by Feng et al. (2016). The bed bug was fixed on a plastic coverslip with double-side tape and the legs were removed with a fine scissor to localize the body. The antennae of both sides were gently replaced by a pin to stick them on the tape. The coverslip with bed bug was set against on a magnetic stand with a small ball of wax, adjusted the angle to 70~90 degree to facilitate manipulation for recording electrodes. Then the magnetic stand (NARISHIGE, Japan) was placed under a stereo microscope (LEICA Z6 APO, Germany), once the sensillum was found under high magnification (720×), the magnetic stand was turned on to stabilize the position.

Solution of 10% KNO_2 (w/v) was prepared in a 20 ml bottle. Two tungsten microelectrodes were sharpened in KNO_2 solution at 5 V by repeatedly dipping the tungsten electrodes in and out the solution. To be able to puncture the cuticle of bed bug olfactory sensillum (diameter 1.1-2.2μm), Tip of recording electrode was sharpened to reach 0.2-0.5μm in diameter. The tungsten wire was roughly sharpened at first by dipping deep (10 mm)

and slow (1 dips/sec), then the tip was delicately sharpened by dipping shallowly (around 1 mm) and quickly (2 dips/sec). The tip of reference electrode was sharpened to 1 μm in diameter, which was good enough to puncture the body cuticle of bed bugs. Recording electrodes was connected to the preamplifier (10×, Syntech, Kirchzaeten, Germany) which is connected with the signal acquisition controller (IDAC, Syntech, Germany) then to a computer; reference electrode was fixed on a magnetic stand (NARISHIGE, Japan) which was grounded to the earth through a metal clamp.

Chemical stimuli used in this experiment were volatile components of *C. lectularius* aggregation pheromone reported by Silgander et al. (2008) and Gries et al. (2015), including histamine, dimethyl trisulfide, dimethyl disulfide, (2E,4E)-octadienal, (E)-2-octenal, (E)-2-hexanal, sulcatone, decanal, nonanal, benzaldehyde, (+)-limonene, (–)-limonene, benzyl alcohol. All chemicals were bought from authentic companies (Table 1.1) with purity higher than 94%. Each chemical stimulus was diluted in dimethyl sulfoxide (DMSO) to 1:10 v/v as a stock solution. A series of dosage was diluted from 1:10 to $1:10^5$ v/v. Response to $1:10^2$ v/v dilution was tested at the initial screening stage.

Single sensillum recording

When the preparation was done, reference electrode was inserted into the body of bed bugs and fixed with magnetic stands. Recording electrode was inserted to the shaft of a certain olfactory sensillum using a micromanipulator (Leica, Germany). At the same time, extracellular action potentials produced by neuron cells housed in one sensillum were conducted to the amplifier, the voltage and temporal dynamics of the action potentials were detected and each action potential was represented by a specific spike on AutoSpike32 (software). 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 action potential

response curve. Consequently, the total number of action potential spikes of whole sensillum was counted off-line. Once the signal was getting stable, 10 μL of dilution was deposited on a filter paper strip (3×10 mm), then inserted the paper strip into a glass Pasteur pipette. Waiting 2-5 min until the stimulus was completely vaporized in the glass pipette. To deliver the stimulus, a constant humidified air flow (1.2 L/min) was provided by a glass above in front of the antenna, by connecting the Pasteur pipette with the stimulus controller (Syntech, Kirchzaeten, Germany), 500 ms stimuli (0.5 L/min) was puffed into the glass tube as depressing the footswitch. Each chemical stimulus was changed after 2 to 5 times injections. The responses of sensilla were counted on AutoSpike32, two 500 ms periods of action potential spikes were counted, before and after stimulation respectively, the response of single sensillum spontaneous activity were calculated in the below expression:

Response (spikes/s)=(spikes after stimulation-spikes before stimulation) × 2

Responses of olfactory sensilla

The number and distribution of sensilla on olfactory region were shown to be constant between individuals and even between sexes. To denote the relative location of each sensillum, a map of distribution of sensilla (Figure 1.1) was made combining literature review and observation under microscope. There were total 44 olfactory sensilla on the distal part of each antenna: 6 D type sensilla, 9 C type sensilla, and 29 E type sensilla. Although sensilla fell into same type, each single sensillum responses was detected to see if there were different functional classes within one type of sensilla. The only exception were D type of sensilla that has been classified into three functional classes (Dα, Dβ, Dγ) according to their response to a odorant panel using SSR. First experiment was screening the overall responses of olfactory sensilla to tested chemical stimuli. Each type of sensillum was stimulated by a

panel of chemical compounds with 1:10 v/v dilution at the initial screening stage. Pure DMSO was used as controls. Each single sensillum had at least 3 replicates.

Second experiment was designed to test dose-dependent responses of chemical-sensillum combinations that showed strong (>50% maximum response) responses at 1:100 v/v dilutions, each combinations was treated with a series of dilutions increased from $1:10^5$ to 1:10v/v dilution. Dose-response curves of each sensilla type were generated with nonlinear regression (curve fit).

Results

Response spectra of sensilla and sexual dimorphism of olfactory responses

Except six D sensilla that were classified into 3 functional group by Harraca et al. (2009), rest of the olfactory sensilla can only be identified by their morphological traits. To interpret the role of each sensilla in perceiving aggregation pheromone, we tested our 13 components of aggregation pheromone (Table 1) on more than 36 sensilla which yield 88% of total olfactory sensilla on O1 and O2 of both males and female using SSR (Fig. 2.1C), with more than 3 replicates on each single sensillum, covering both three morphological types of sensilla (grooved peg, smooth peg, hair sensilla). The total number (mixed sexes) of recording for each sensillum type was 794 for D sensilla, 311 for the C sensilla, 1536 for the E sensilla. The tuning curves of D and C sensilla were consistent with previous reports of other electrophysiological studies on *C. lectularius,* of which D sensilla showed wide-tuned response spectrum to a large odorant panel (Fig. 2.2), and C sensilla were narrowly tuned to a few odorant families (Fig. 2.3), amines for example (Harraca et al. 2009, Liu and Liu 2015). With the consistancy of sensilla location on the antenna, we were able to label and test each

individual of E sensilla, which generally showed weak response (<50 spikes/s) to tested odorants (Fig. 2.5).

To investigate if there are differences in olfactory responses to aggregation pheromone between males and females, C and D sensilla, which house most abundant ORNs, were choosen to test response difference (Fig. 2.2; Fig. 2.3), having each sensillum been tested at least 6 times for odors panels with different bed bugs. Ninteen odorants-sensillum combinations that elicited strong response (≥100 spiks/s) on either sexes were chosen for statistical analysis (table 2.2). Unpaired-tested method were applied on 19 combinations of males and females and our results showed there was no significant difference, with p value less than 0.05, in olfactory responses between males and females. Thus, for the rest of this thesis, males were mainly used as models for adults.

Responses of D and C sensilla

The foregoing analysis has revealed a set of 5 physiological types of sensilla responsible for the detecting odor composition of aggregation pheromone. Specifically, 44.5 % of 65 entries (13 odors × 5 sensilla) produced a response (Fig. 2.6B). It is difficult to distiguish the activity of each specific neuron in D sensilla by the amplitude of action potential, largely because each of D sensilla housed too many ORNs, therefore, we analyzed the whole response of entire sensillum. Five out of six aldehyde components in aggregation pheromone elicited strong responses in at least one of D sensilla, especially saturated aldehydes like decanal and nonanal evoked very high increase of firng rates of receptor neurons in both three functional types of D (Fig. 2.2). Similar in structure, C-9 aldehyde nonanal elicited twice higher response on average than C-10 aldehyde decanal did in Dα and Dβ. Dγ had broadest response spectrum, showing strong or very strong response to 5 chemical families, including aldehydes [(E)-2-octenal (85 ± 10 spikes/s), decanal (141 ± 15

spikes/s), nonanal (181 ± 13 spikes/s)], ketone [sulcatone (156 ± 11 spikes/s)], limonenes [S-(-)-limonene (125 ± 11 spikes/s), R-(+)-limonene (120 ± 11 spikes/s)]. Three odors, (2E, 4E)-octadienal, (E)-2-hexenal, and nonanal evoked highest increasing firind rates in Dα with 189 ± 16 spikes/s, 196 ± 17 spikes/s, 190 ± 10 spikes/s, respectively.

In this study, we were able to detect two C sensilla (relative locations see Fig. 2.1), C1 and C2. Both C1 and C2 sensilla showed excitatory response to histamine with increaing of firing rates of 54 ± 7 spikes/s and 99 ± 10 spikes/s, respectively. It was worthy noting that C1 was highly excited by a specific aldehyde, (E)-2-hexanal, with increasing firng rate of 152 ± 18 spikes/s. Unlike D sensilla that housed too many ORNs, we can recognize two distinguishable neuronal activities sensilla with different amplitudes of spikes (Fig. 2.3B) when stimulated with (E)-2-hexanal and histamine in C1. This revealed that C1 has a specific ORN that response to (E)-2-hexanal which C2 was absent of. Due to the restriction of the location of other C sensilla, these two sensilla, C1 and C2, were used to represent grooved peg sensilla type. In general, the results show some common traits that C sensilla were narrowly tuned to a few components of aggregation pheromone and both showed excitatory response to histamine.

In general, ORNs housed in C and D type of sensilla showed highest activity in response to aggregation pheromone and different ORNs combinations were housed within one morphological type of sensilla. When combined their response spectra (Fig. 2.4), we found each component of aggregation pheromone elicited strong repsonse in at least one type of sensilla, and each sensillum showed its highest firing rate to each of odorants. For instance, R-(+) and S-(-)-limonene were specifically active strong neuronal response in Dγ, where as histamine could only be percieved by C sensilla.

Response of E sensilla

E sensilla were divided into E1 and E2 based on their anatomical structure of sensilla wall, that E1 had a even wall whereas E2 has an uneven wall from the cross-section slide of the sensilla (Levinson et al. 1974). Unfortunately, we were unable to tell them apart by keen eye during experiement. Moreover, structure similarity can not guarantee consistency of olfactory function. Thus, in this research, we regarded each hair sensillum as an individual and labeled them with specific number based on their order of locations (Fig. 2.1C). Each of 27 E sensilla was evaluated with their own responses to a panel of 13 components of aggregation pheromone.

Considering E sensilla houses 1-3 ORNs showed very sparse spontaneous frequency (Fig 2.7B) under natural condition with the average of fring frequency was 8 ± 5 spike/s, we took response of combinant that exceeded 16 spikes/s as excitatory response, which was 75% of maximal repsonse and happened to approximate the limit of 2-fold of firing frequency of typical neurons bfore stimulation. The same cretia applied as inhibitory response, by inhibiting 75% of maximal inhibitory response (Fig. 2.6A).

Nearly 92% of chemical-sensillum combinants showed neither excitatory nor inhibitory responses across 483 entries. There were 4.89% and 3.15% of combinants showed excitory and inhibitory responses, respectively (Fig. 2.6A). Within excitatory responses of 11 sensilla, E1 (n=2), E6 (n=3), E7 (n=7), E12 (n=6), E14 (n=3), E20(n=4), E22 (n=2), and E23 (n=3) showed extictatory response to either or both decanal and noanal. E1 and E12 also excited by sulcatone. Three sensilla, E5 (n=5), E18(n=5), E21 (n=3), showed inhibitory responses to panel of 13 odorants, strongest inhibitory effects in these sensilla were mostly triggerred by sulcatone (Fig. 2.5).

E12 and E5 were representatives of two stereotypes of E sensilla that showed responses (Fig. 2.7A). They were both narrowly tuned to one or two odorants in response to

components of aggregation pheromone. When stimulated with same sulcatone in dilution of 10^{-2}, E1 and E12 showed reversed effects (Fig. 2.7B).

Dose-dependent response analyses

Both quality and quantity of odorant are essential in encoding information of chemical ecology (De Bruyne et al. 2001). The odorant solutions used in screening tests were at 10^{-2} dilution, assuming all applied pheromone fully evaporated, the concentration of odorant molecules that reached bed bug antenna was 2×10^{-4} μg/μL. It is unlikely that bed bug will encounter this high concentration in natural environment. Indeed, sensilla responded to odorants at low concentration were detected within 12 dose-response curves, and all of the sensilla showed dose-dependent patterns to tested chemicals (Fig 2.8A-F).

Ten out of 13 odorants evoked strong response in D sensilla with exciting firing rate of spikes higher than 100 spikes/s. For the odorants that elicited strong responses in more than one type of sensilla, the only sensilla that showed strongest firing rate were chosen to perform dose-response of the odorant. The lowest response threshold was observed in Dγ sensilla in response to sulcatone (42 ± 14 spikes/s; n=5) at 10^{-5} dilution, followed by threshold of (E)-2-hexenal in Dα sensilla at 10^{-4} dilution (42 ± 27 spikes/s; n=3), those responses of sensilla, which showed low threshold tended to saturate at high dosage (Fig 2.8A and C). In Dβ sensilla, response curve of dimethyl trisulfide was higher than (E)-2-octenal and responded at relatively low concentration (Fig. 2.8 B). Higher response thresholds were shown in dose-response curves for (2E, 4E)-octadienal, (E)-2hexenal in Dα sensilla, (E)-2-octenal in Dβ sensilla, and R-(+)-limonene and dimethyl disulfide in Dγ sensilla all began to show responses when odorant molecules reached antenna at 10^{-3} dilution or higher (Figure. 2.8A-C).

C2 responded to histamine at lower threshold than C1, with response of 82 ± 4 spikes/s and 28 ± 8 spikes/s, respectively, to histamine at dilution of $1:10^3$ v/v (Fig 2.8C and D). However, the highest firing rate of overall responses of C sensilla was observed in C1 when stimulated with 10^{-1} dilution of histamine (174 ± 40 spikes/s). Dose-response curves of (E)-2-hexanal and histamine in C1 were almost overlapped, indicating similar binding specificity of both chemicals to olfactory receptors (Fig. 2.8D).

To test the sensitivity of inhibitory effect in E sensilla, sulcatone was chosen to perform dose-response tests on one of the inhibitory sensilla. Spontaneous firing frequency gradually decreased as concentration of odorant increased exponentially (Fig 2. 8F), especially when concentration increase from 10^{-3} to 10^{-2} dilution.

Temporal dynamics of olfactory sensilla

Duration of response to an odorant is also an important parameter in encoding environmental signal (De Bruyne et al. 2001, Hallem and Carlson 2004). To investigate the temporal dynamics of ORNs to certain odorant, means of firing frequency of 100 ms intervals were successively calculated from the beginning of stimulation to 2.2 s afterwards. Responses of ORNs can be abruptly ended or prolonged depending on the identity and concentration of the stimulus. In this study, most of odorants from various families elicited phasic neuronal responses at 10 μg/μL, including (2E, 4E)-octadienal and nonanal in Dα, (E)-2-octenal and dimethyl trisulfide in Dβ, and R-(+)-limonene and S-(-)-limonene in Dλ. By contrast, (E)-2-hexenal and sulcatone evoked tonic neuronal responses in Dα and Dλ which continued high firing rates after 0.5 s (Fig. 2.9A-2.9C), indicating that higher concentration tended to prolong high firing rates. Dynamic structure of C1 sensilla exhibited tonic activity as concentration increased to 100 μg/μL, whereas it showed phasic response when stimulated as odorant in 10μg/μL (Fig. 2.10A).

Temporal dynamics structure also provided a comprehensive map of how different concentrations of odorants were encoded. For example, in dose-response curve of C2 sensilla to histamine (Fig. 2.8F), the degrees of firing frequency of C2, which responded to histamine at 10μg/μL and 100 μg/μL, were shown no significant difference. However, by looking at the temporal dynamic graphs (Fig. 2.1C), it was apparent that two modes of response structure were presented, in which extreme high concentration (100 μg/μL) induced a quiescence period after stimulation and high concentration at 10 μg/μL evoked tonic neuronal response, followed by relative phasic neuronal response at 1 μg/μL (Fig. 2.10B).

Discussion

Our results confirmed that olfactory sensilla on the distal part of antenna of bed bugs played an essential role in perceiving volatile components of aggregation pheromone, with no significant difference observed between genders. The response spectra and sensitivities to different compositions determined the encoding process. We found 12 components elicited olfactory response (>60 spikes/s) in at least one type of sensilla, with no significant response to benzyl alcohol observed in any type of sensilla, and each sensillum showed strong response (>100 spikes/s) to a subset of tested odorants. Our results indicated that D and C sensilla had a complimentary encoding map of response to aggregation pheromone, specifically, D sensilla tuned to most aldehydes in aggregation pheromone, moreover, Dγ sensilla were also sensitive to (+), (-)-limonene and sulcatone. Our results are very similar as reported by Gries et al. (2015), in which C sensilla were specifically responsible for histamine and was proposed to be related to arrestment behavior of bed bugs (Gries et al. 2015). The similar results were consistent with previous electrophysiological studies on bed bugs antenna, which has been concluded that C and D sensilla played critical roles in

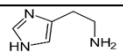
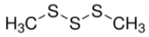
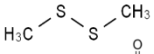
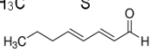
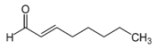
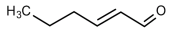
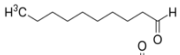
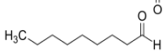
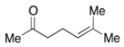
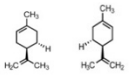
olfaction related behavior like alarming dispersal as well as host seeking (Harraca et al., 2009, Liu et al., 2014, Liu and Liu, 2015). Our study has, for the first time, systematically recorded the inhibitory effects showed in three E sensilla, especially to sulcatone. It has been proposed that inhibitory effect may serve as a way to reduce noise for particular important signals, which was coincident with the fact that sulcatone excited E12 and inhibited E5 (Hallem and Carlson, 2006). Trying to classify E sensilla into functional groups based on their responses to odors related to bed bug chemical ecology, a side project was attached in appendix I. Thus, we can deduct the conclusion that ORNs housed in different sensilla have to work together to encode the comprehensive map of a certain environmental signals.

Both excitatory and inhibitory responses showed dose-dependent responses. The right ratio and concentration of odorants are important in triggering appropriate behaviors of bed bugs. In fact, some components of aggregation pheromone were even overlapped with human odorant as well as insect repellents. For examples, decanal, nonanal and sulcatone are major components of human emanation (Liu and Liu, 2015); S-(-) and R-(+)-limonene act as insect repellents in some market products (Liu et al., 2013); (E)-2-hexanal and (E)-2-octenal are the most abundant components of bed bug alarming pheromone (Levinson et al., 1974). Our results provided solid evidence to explain the mechanism of behavior switches from aggregation to dispersal as bed bugs encountered same odorant at increasing concentration. Took (E)-2-hexenal as an example, Dα exhibited excitatory activity at 10^{-4} dilution (42 ± 27 spikes/s) and showed stronger neuronal response as dosage increased 100-fold at 10^{-2} dilution (117 ± 41 spikes/s), which were correspond with the concentration for effective arrestment behavior [1.7×10^{-3} μg/ml; (Gries et al., 2015)] and threshold of alarming behavior [1.3-2.4 μg/ml; (Siljander et al., 2008)]. Although it is unlikely that bed bugs would encounter pure odorants in nature environment, the sensitivity for each component still provided valuable information for produce effective attractants. For instance, (E)-2hexanal and sulcatone can be

detected at very low concentration, which may indicate smaller usage of chemical can achieve attractive effect. However, an effective mixer might elicit stronger response at a much lower concentration than what the experiment used.

Temporal dynamics of ORNs not only add a degree of freedom for peripheral olfactory system encoding odorant information, but also play an important role in successfully locate the source, especially critical for insects who required long-distance source seeking, like mosquitos and *drosophila* (De Bruyne et al. 2001; Ye et al. 2016). Due to the close parasite-host association with humans, bed bugs have relative simple chemical ecology as their active diameter are often limited within 1.5 m (Weeks et al. 2011), resulting in a reduced number of olfactory sensilla as well as ORNs. Temporal dynamics of ORNs can represent the kinetic structure of binding affinity of odorant to receptors. As it is well established, different receptors expressed in neuron membranes were coexisted in sensillum, certain type of sensilla was responsible for certain chemical family. Our results suggested that odorants evoked fast increase of firing rates in Dα sensilla might indicate high binding affinities to one or more olfactory receptor housed in this sensilla type; whereas some other odorants that evoked slow increase slope might indicate relative weak binding affinities to receptors in the sensilla, such as dimethyl trisulfide in Dβ sensilla. We also found that same odorant can elicit immediate high response in one sensilla, and showed gradual increase response in another sensilla when stimulated with same concentration of the chemicals. Finding of Dα and Dγ that exhibited prolonged responses to most of the aldehydes and of neuronal responses in Dβ that terminated abruptly might indicate Dα and Dγ were responsible for encoding long-lasting “memory” of some components aggregation pheromone, whereas Dβ responded to spontaneous signals.

Table 2.1. Chemicals used as components of aggregatin pheromone of *Cimex lectularius*

Compound Type	Chemicals	Purity [a] (%)	Structure	Company	CAS-number	References [b]
Amine	Histamine	≥ 97%		SIGMA	51-45-6	2
Sulfide	Dimethyl trisulfide	≥ 98%		SIGMA-ALDRICH	3658-80-8	2
	Dimethyl disulfide	99%		SIGMA-ALDRICH	624-92-0	2
Aldehyde	(2E,4E)-Octadienal	95%		SIGMA-ALDRICH	5577-44-6	1
	(E)-2-octenal	≥ 94%		SIGMA-ALDRICH	2548-87-0	1,2
	(E)-2-hexenal	99%		ACROS	6728-26-3	1,2
	Decanal	95%		ACROS	112-31-2	1
	Nonanal	95%		ALDRICH	124-19-6	1
	Benzaldehyde	98%		ALFA AESAR	100-52-7	1
Alcohol	Benzyl alcohol	≥ 98%		flasher science education	100-51-6	1
Ketone	Sulcatone	≥ 98%		SIGMA	110-93-0	1
Limonene	S-(-)-limonene	96%		SIGMA	5989-54-8	1
	R-(+)-limonene	97%		SIGMA	5989-27-5	1

[a] Purity of chemical compounds were higher that 94%, chemicals were dissolved in dimethyl sulfoxide (DMSO) to dilution of 1:10 (v/v for liquid or w/v for solid) as stock dilution.

[b] Numbers indicates published reference which identified specific compound as component of aggregation pheromone; 1) Siljander et al. (2009); 2) Gries et al. (2015).

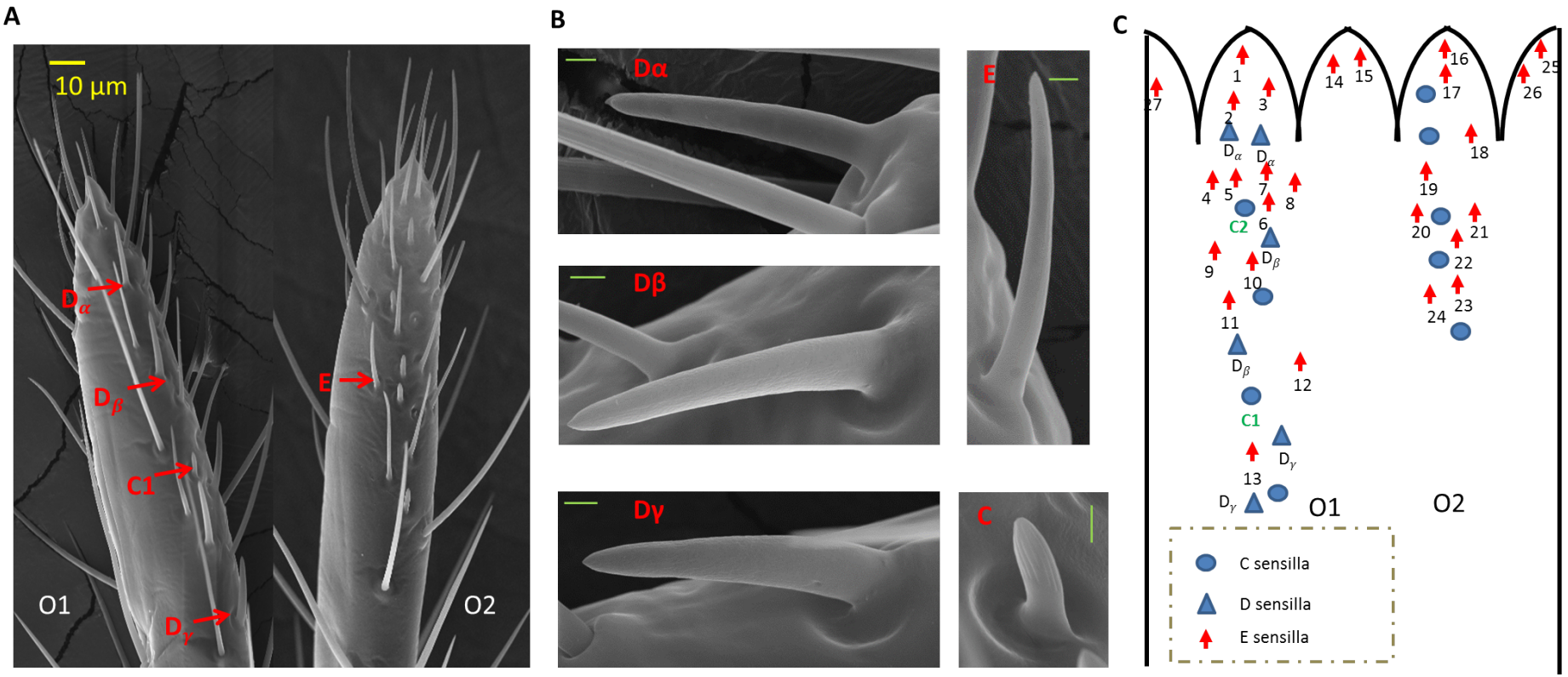


Figure 2.1. Distribution of olfactory sensilla on surface of distal antennal part of adult *Cimex lectularius*. (A) Scanning electron micrograph (Photo took by Liu. F) showing the two opposite olfactory regions (O1 and O2) of distal part of antenna of a male bed bug, different type of olfactory sensilla scattering on these regions. Scale bars indicates 10µm; (B) SEM of different type of sensilla, scale bar indicates 2µm (C) Schematic map of distribution of olfactory sensilla on O1 and O2 are based on observation under microscope and maps proposed by Steinbrecht and Müller (1976) and Harraca et al. (2009), including 6 smooth peg sensilla (D sensilla), 9 grooved peg sensilla (D sensilla), and 27 hair sensilla (E sensilla). Each sensillum was labeled by a specific symbol or number.

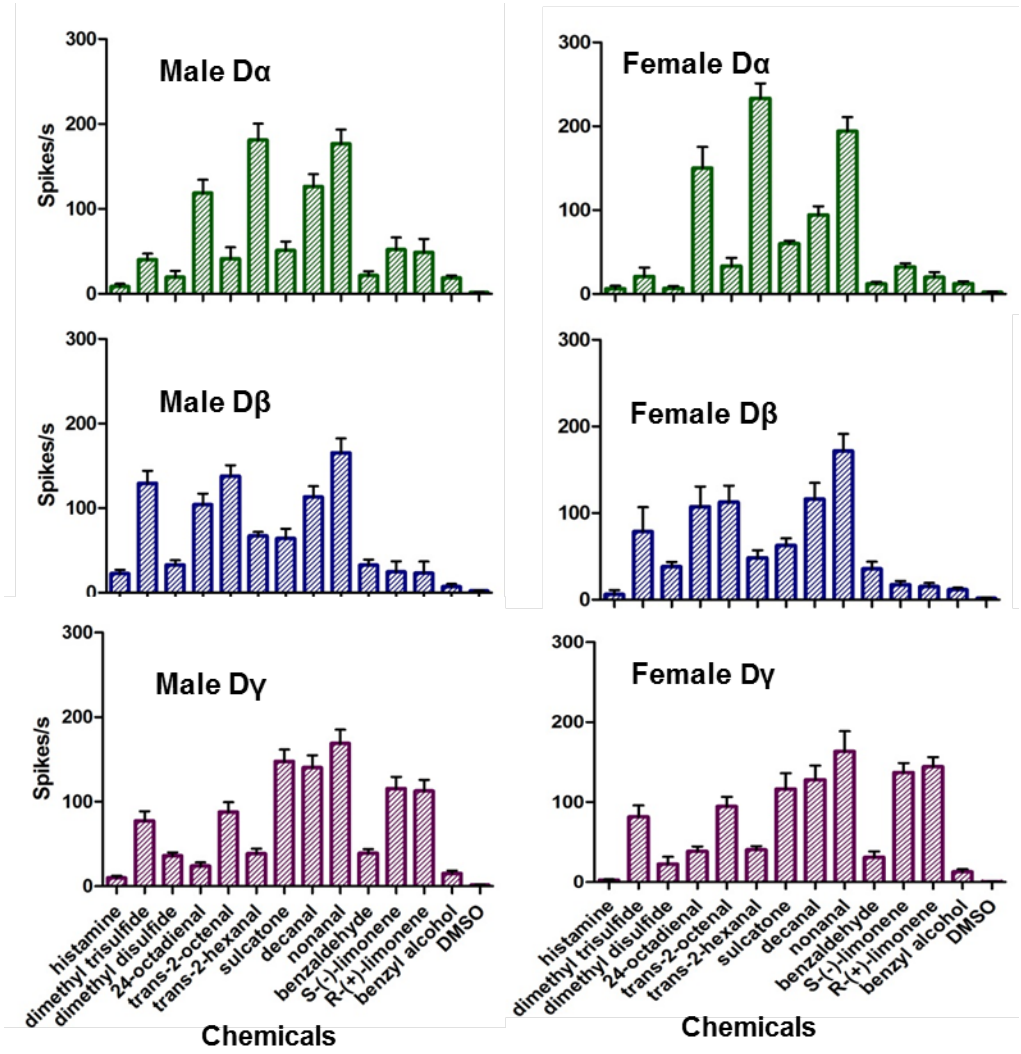
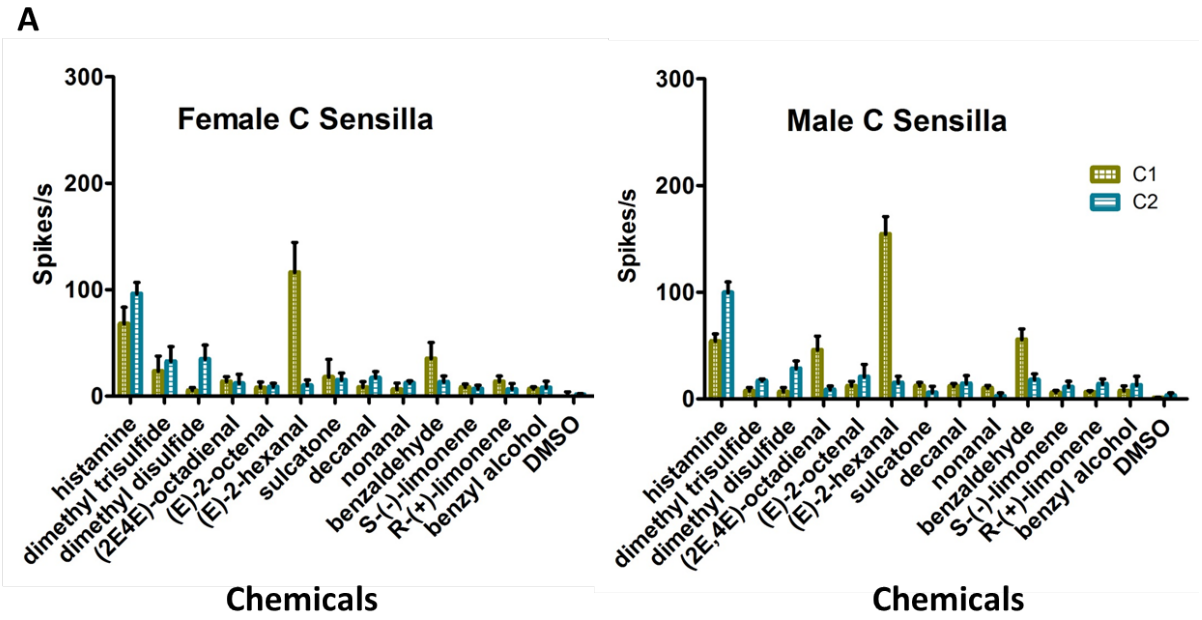


Figure 2.2. Olfactory response patterns of smooth peg sensilla (D sensilla) of adult bed bugs to components of aggregation pheromone. Each column represents response (mean ± SEM, n≥6) of certain type of olfactory sensilla to specific components at 1:100 v/v dilutions. Pure DMSO was used as control. Different each color indicates one functional type of D sensilla. **(**A) Olfactory responses of males to 13 components of aggregation pheromone; (B) Responses of females.

Figure 2.3. Olfactory response patterns of grooved peg sensilla (C sensilla) of adult bed bugs to components of aggregation pheromone. A) Each column represents response (mean ± SEM, n≥6) of certain type of olfactory sensilla to specific components at 1:100 v/v dilutions. Pure DMSO was used as control. Two grooved peg sensilla, C1 and C2, located on different places (location see Figure 2.1) were presented by two different colors. B) SSR recording of C1 sensilla of male in response to E)-2-hexenal and histamine at same dosage, showing two different excited neurons response with different amplitudes.



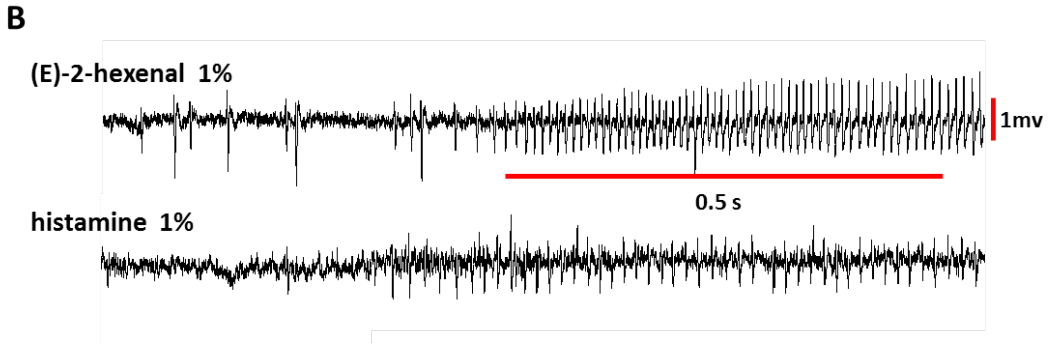


Figure 2.4. Combinatorial coding of aggregation pheromone by C and D sensilla of adult bed bugs. Females have similar coding map with males. The 3-D map showing overall responses of two types of sensilla that houses most abundant ORNs when stimulated with individual odorant at 10^{-2} dilution. Responses of three functional type D sensilla, Dα, Dβ, and Dλ, were labeled with green, purple and blue respectively. Responses of two C sensilla, C1 and C2, were labeled with yellow and red respectively.

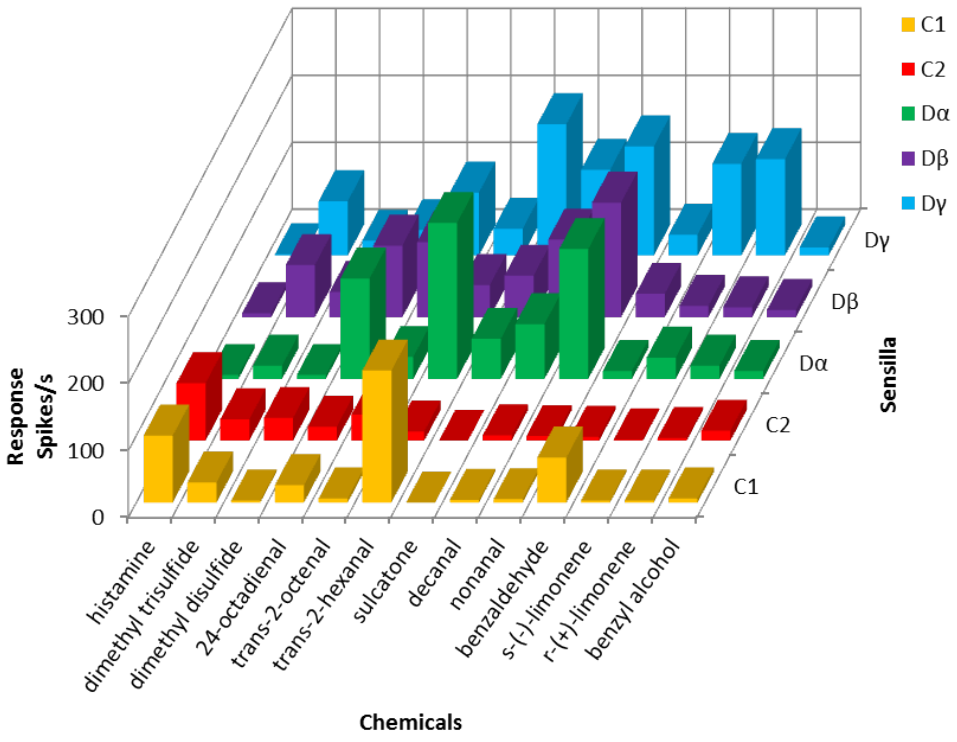


Table 2.2. Results of unpaired t-test of olfactory responses between males and females

	Chemicals [a]	Male Mean ± SEM (Spikes/s)	Female Mean ± SEM (Spikes/s)	Difference between means [b] (Spikes/s)	P value [c]
Dα	(2E,4E)-octadienal	189 ± 15.8 N=6	148 ± 11.4 N=8	40.9 ± 19.5	0.06
	(E)-2-hexenal	196 ± 17.2 N=13	250 ± 10.8 N=6	-54.8 ± 26.7	0.06
	Decanal	127 ± 14.4 N=13	82.7 ± 14.4 N=6	44.3 ± 23.5	0.08
	Nonanal	190 ± 10.7 N=13	194 ± 17.0 N=6	-4.46 ± 21.6	0.84
Dβ	(2E,4E)-octadienal	114 ± 10.2 N=12	131 ± 21.7 N=6	-17.0 ± 20.9	0.43
	(E)-2-octenal	136 ± 12.6 N=12	113 ± 19.0 N=9	23.3 ± 22.0	0.30
	Decanal	118 ± 13.4 N=12	116 ± 18.8 N=7	2.17 ± 22.7	0.92
	Nonanal	178.8 ± 11.05 N=12	171.7 ± 19.82 N=6	7.167 ± 20.88	0.74
	Dimethyl trisulfide	56.7 ± 12.0 N=9	78.5 ± 28.3 N=6	-21.8 ± 25.6	0.41
Dγ	(E)-2-octenal	85.6 ± 10.4 N=14	94.6 ± 12.1 N=10	-9.03 ± 16.0	0.58
	Sulcatone	156 ± 11.6 N=16	145 ± 23.5 N=7	11.3 ± 23.3	0.63
	Decanal	141 ± 15.4 N=14	128 ± 17.7 N=10	13.1 ± 23.6	0.58
	Nonanal	181 ± 13.3 N=14	163 ± 25.3 N=10	17.3 ± 26.4	0.52
	S-(-)-limonene	125 ± 11.1 N=13	137 ± 11.9 N=10	-12.2 ± 16.4	0.46
	R-(+)-limonene	120 ± 10.5 N=13	144 ± 12.0 N=10	-24.7 ± 16.0	0.14
	Dimethyl trisulfide	84.3 ± 9.58 N=12	73.2 ± 8.56 N=12	11.1 ± 12.8	0.40
C1	Histamine	54.3 ± 7.05 N=6	68.0 ± 15.6 N=6	-13.7 ± 15.1	0.39
	(E)-2-hexenal	152 ± 18.3 N=9	116 ± 28.1 N=6	36.1 ± 32.0	0.28
C2	Histamine	99.7 ± 10.0 N=7	96.2 ± 10.7 N=6	3.51 ± 14.9	0.82

[a] chemicals elicited strong response (≥100 spikes/s) to certain type of sensilla of both sexes were chosen to compare the sexual dimorphism. Total 19 combinations were tested.

[b] difference between means were calculated by means of females minus means of males.

[c] P value of unpaired t-test, $P < 0.05$ was considered to show significant difference. $P \geq 0.05$ was considered to have no statistically significant difference.

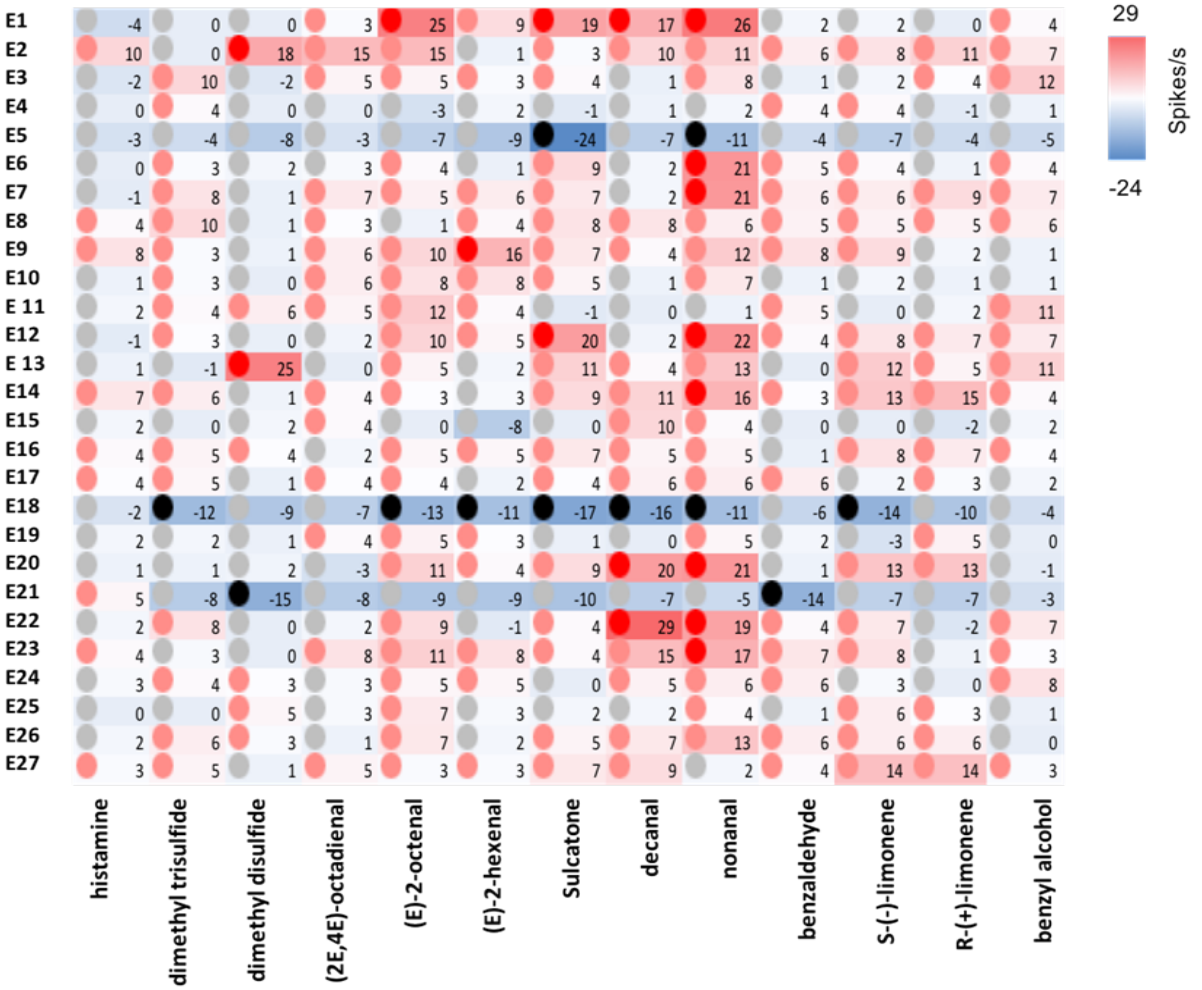


Figure 2.5. Heat map of responses of E sensilla to panel of aggregation pheromone. 1251 odorant-sensillum combinants was recorded in total, with individual sensillum being tested by at least 2 replicates of whole panel of odorants. Cells filled with gradient color from red to blue indicating excitatory and inhibitory response, respectively. Deep red or black dot represented stronger responses of excitatory or inhibitory, respectively. "Red dot" indicates the response exceeded that of spontaneous frequency by n ≥ 16 spike/s; "pale red" dot by 16 > n ≥ 4 spikes/s; "gray dot" by 4> n ≥ -10 spikes/s; "black dot" by -10 > n spikes/s. These frequencies represented 75%. 50% and 25% of 29 spikes/s, the maximal firing frequency of E sensilla across this table. All odorants were diluted 1: 100 v/v in DMSO.

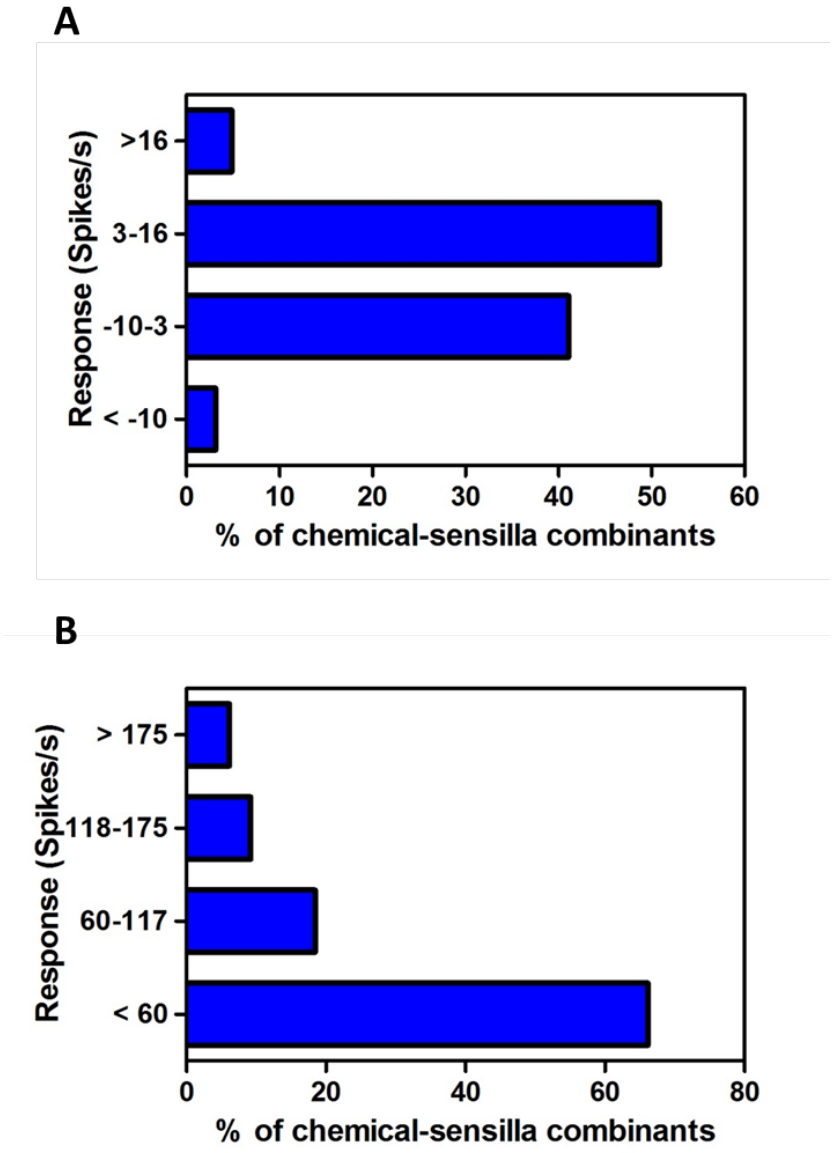
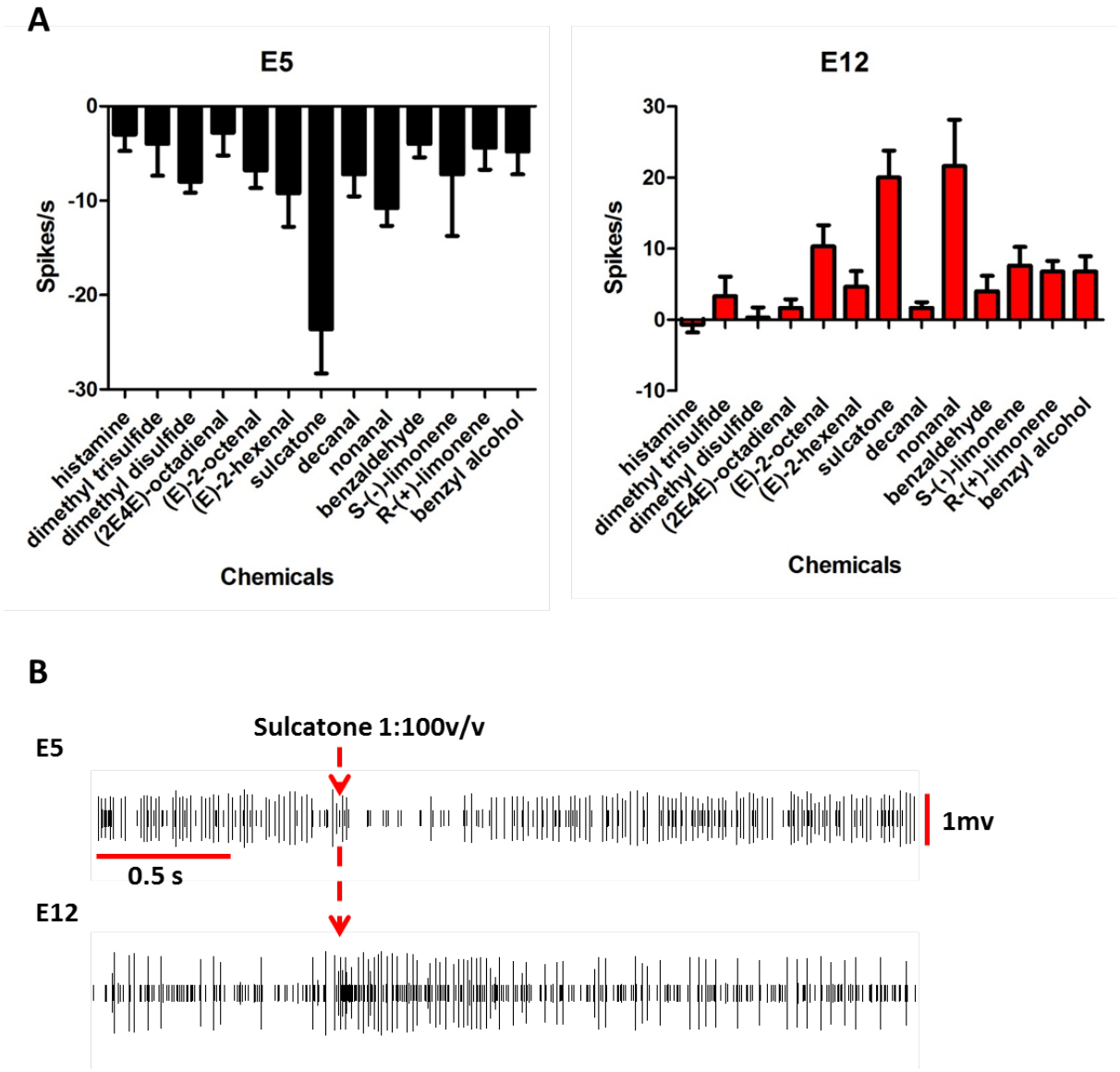


Figure 2.6. Distribution of firing frequency of different chemical-sensillum combinants. A) 483 chemical-sensillum combinants in E type of sensilla were divided into 4 groups by their means of responses (Fig 2.5), 16, 3, and -10 were indicating responses of which elevated firing frequency of maximal excitatory response by 75%, 50% or inhibited firing frequency by 75% of maximal inhibitory response; B) distribution of response 65 chemical-sensillum combinants in D and C sensilla. 175, 118, and 60 were indicating responses of which elevated firing frequency of maximal excitatory response by 75%, 50%, 25%, respectively. 50% and 75% of the maximal firing frequency approximate the one-fold and two-fold of average firing frequency before stimulation.

Figure 2.7. Two stereotypes of olfactory responses of E sensilla to components of aggregation pheromone. A) Olfactory response (mean ± SEM spike/s ; n=5) of E5 and E12 to panel of 13 components of aggregation pheromone. B) Typical SSR recording of E5 and E12 in response to 10^{-2} dilution of sulcatone, showing inhibitory and excitatory response, respectively.



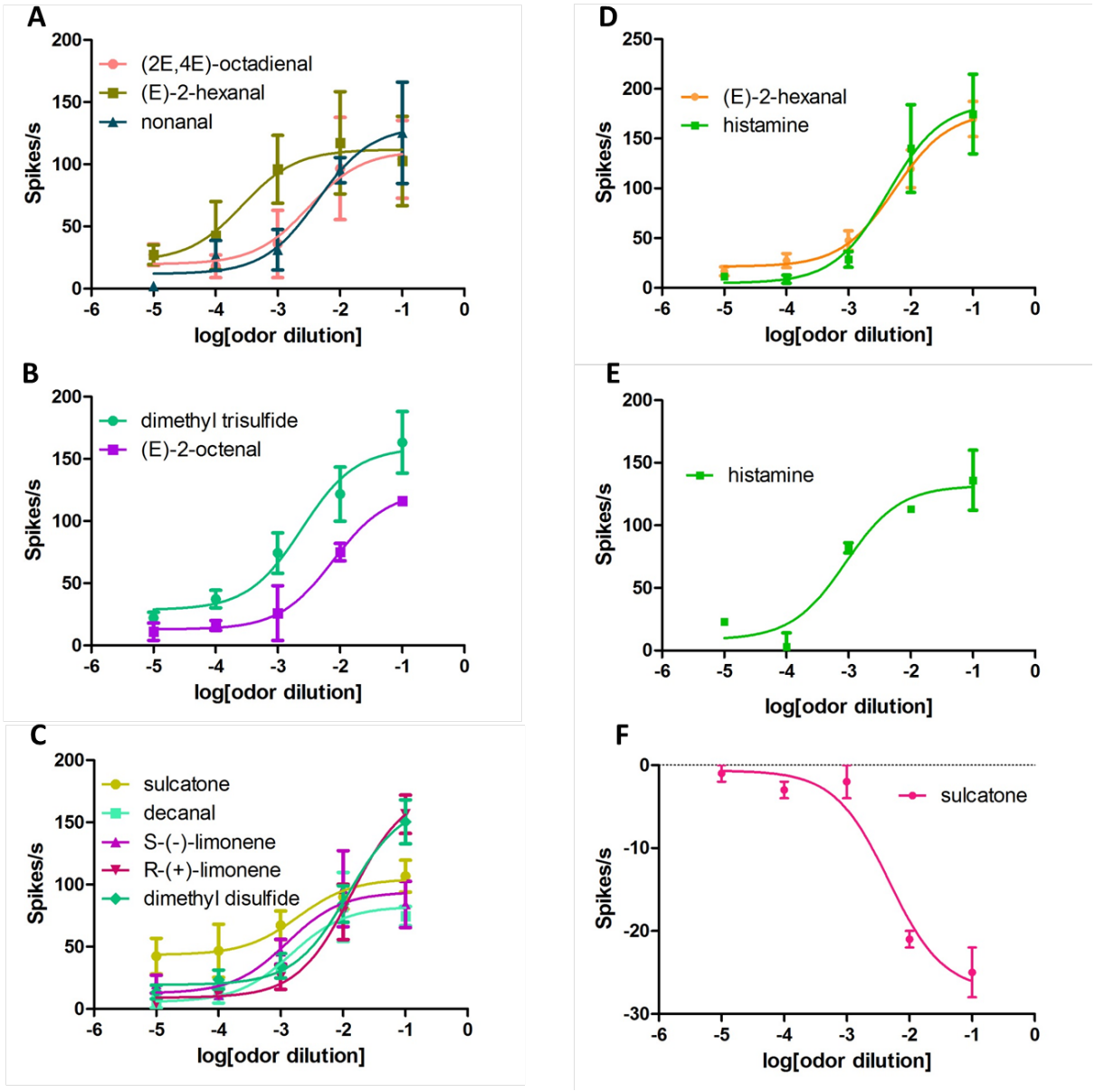
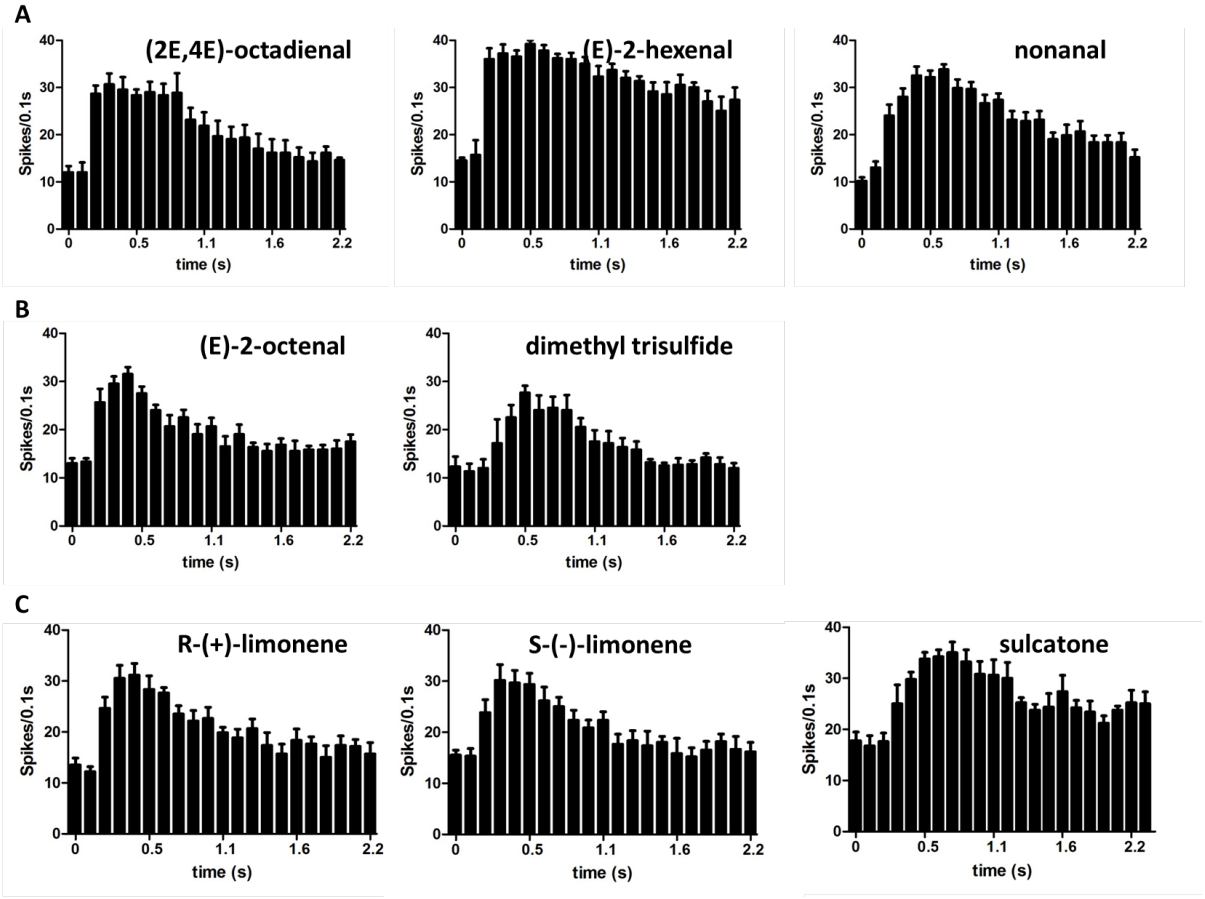


Figure 2.8. Dose-response curves of odorants that elicited strong responses at initial screening stage. X axis showed logarithm of dosage series exponentially increased from 10^{-5} to 10^{-1} v/v. Responses of sensilla were represented by mean ± SEM spike/s; N ≥ 3; nonlinear regression analyses of dose-responses were performed to generate curves. Each graph represented dose-response of each type of sensilla. A) Dα sensilla; B) Dβ sensilla; C) Dγ sensilla; D) C1 sensilla; E) C2 sensilla; F) dose-dependent inhibitory responses of E5.

Figure 2.9. Temporal dynamics of smooth peg sensilla (D sensilla) in response to components of aggregation pheromone. Starting from 0s when a 0.5s stimulation was pumped out of Pasture pipette, spontaneous firing frequency (mean ± SEM spikes/0.1s, n=6) of receptor neurons in 0.01s was consecutively calculated until 2.2s, with all odorants at 1: 100 v/v dilution. The concentration of odorant molecules when reached the antenna was approximate 10^{-4} μg/μL; A) Dα sensilla B) Dβ sensilla C) Dγ sensilla.



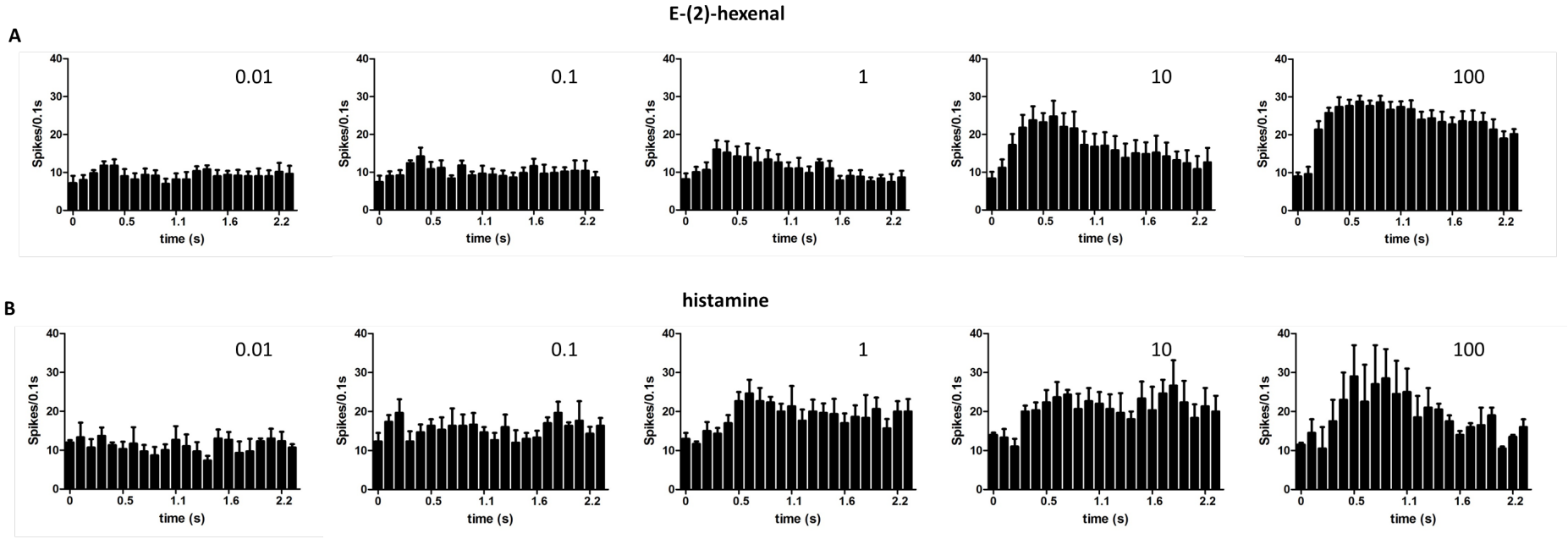


Figure 2.10. Temporal dynamics of grooved peg sensilla (C sensilla) in response to components of aggregation pheromone at different dosage. Dosage series exponentially increased from 0.01- 100 μg/ μL on the filter paper, Starting from 0s when a 0.5 ms stimulation was pumped out of Pasture pipette, spontaneous firing frequency (mean ± SEM spikes/0.1s, n=6) was shown for successive 100 ms interval until 2.2s. Due to the distance from glass tube to antenna, the stimulation to antenna was ended around 520 ms; A) Temporal dynamics of C1 sensilla in response to (E)-2-hexenal; B) temporal dynamics of C2 sensilla in response to histamine.

Chapter 3. Test the difference of olfactory responses between developmental stages of bed bugs, *Cimex lectularius,* to components of aggregation pheromone

Introduction

The common bed bug, *C. lectularius,* is hemimetabolous insect that have 5 nymph instars. After hatching from eggs, each time bed bug nymphs moult into next developmental stage require at least one blood meal (Usinger, 1966). Fed bed bugs return to harborage and aggregate with conspecifics as a result of volatile aggregation pheromone and tactile stimuli. During day time, bed bugs stay quiescently in their harborage digesting the blood meal, mating, and reproducing progeny. Aggregation behavior of bed bugs of both nymphs and adults enable them successfully find the shelter as after blood meals, greatly reducing the risk of being found out by hosts.

Previous behavior tests demonstrated that juvenile bed bugs preferred to aggregate on discs exposed by nymph bed bugs than discs exposed by males or females, which suggested the existence of juvenile specific aggregation pheromone (Siljander et al., 2007). Moreover, Harraca et al. (2010) discovered that 5^{th} instars bed bugs also produce juvenile alarming pheromone to defend males from sexual harassment. Together, these findings suggest different developmental stage of bed bugs may have different response to certain chemical signals. Little is known on this physiological basis of these behaviors.

Chemical communication relies on the chemoreceptor neurons housed in sensilla. The numbers of olfactory sensilla increased each time as the bed bugs move into higher nymphal instars, with no sexual dimorphism observed between male and female (Levinson et al., 1974).

Specifically, numbers of grooved peg sensilla (C sensilla) and hair sensilla (E sensilla) are dramatically reduced in 1st and 2nd instar bed bugs, except for the grooved pegs sensilla (D sensilla). Only 4 C sensilla remain in O2 region and 5 C sensilla in O1 region are dismissed in early nymphal stages. Previous studies mainly focus on late instar nymphs of bed bugs, specifically 4th and 5th nymphs. Still, Siljander et al. (2010) claimed that complete synthetic aggregation pheromone showed attractive effect on bed bugs of all developmental stages regardless their feeding status, physiological states, or sexes. Foregoing study of this thesis has demonstrated that males and females responded to components of aggregation pheromone without showing sexual dimorphism, and also described certain types of sensilla that exhibited strong responses to aggregation pheromone components. It will be worth to investigate whether nymph bed bugs have different coding map in response to aggregation pheromone and how important an odorant was in eliciting olfactory responses during different developmental stages of bed bugs.

Materials and methods

Insects

1-5 nymph instar bed bugs were used in the experiments; 1st instar nymphs were hatching from eggs within one week, 2-5 instar nymphs were moulting from last instar and had not been fed. The *C. lectularius* colony utilized in the 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, USA). Bed bugs were reared at 25±2 ºC under a photoperiod of 12:12 (L: D).

Stimuli

Chemical stimuli used in this experiment were 13 volatile components of *C. lectularius* aggregation pheromone reported by Silgander et al. (2008) and Gries et al. (2015), including histamine, dimethyl trisulfide, dimethyl disulfide, (2E,4E)-octadienal, (E)-2-octenal, (E)-2-hexanal, sulcatone, decanal, nonanal, benzaldehyde, (+)-limonene, (–)-limonene, benzyl alcohol. Each chemical was diluted in dimethyl sulfoxide (DMSO) at 1:100 v/v dilution.

Single sensillum recording

Method details stay the same as described in chapter 2. Only D sensilla were well-developed as adults in both number and shape in nymph bed bugs, while early nymph bed bugs lack of C sensilla in O1 (Figure. 2.1A). C and D sensilla of nymphs, which distributed on the same location of antenna, were chosen to practice stimulation of chemical panel. E sensilla were not taken into consideration primarily because they changed both in numbers and relative locations during development and were impractical to find out their identity in each instar. Moreover, they showed relative weak response in adults. Each type of D sensilla was tested for at least 6 times on different bed bugs. While each type of C sensilla has at least 3 replicates of recordings.

Data analysis

The odorant-sensilla combinants which showed strong responses at 10^{-2} dilution in at least one developmental stage were chosen to perform One-way ANOVA tests, including 16 chemical-sensillum combinations in D sensilla and 2 combinations in C sensilla. If groups showed significant difference in F-test, multiple comparison procedure will be practiced by Duncan test or Tukey's tests, comparing the means of variance between groups.

Results and Discussion

Three types of D sensilla in each instar were tested by 13 chemical panel of aggregation pheromone (Fig. 3.2 A and B). Sixteen chemical-sensillum combinations, which elicited strong neuronal activities within D sensilla, were chosen to perform One-way ANOVE to test difference between instars (Table 2.1), including 4 chemicals in Dα sensilla, 5 chemicals in Dβ sensilla, and 7 chemicals in Dγ sensilla (Fig. 3.4 and 3.5). Twelve out of 16 combinations showed similar strong responses with F-test ($P > 0.05$, Table 3.1). The results indicated that not only did D sensilla look similar in the shape between nymphs and adults, but they also play same important roles in perceiving components of aggregation pheromone. However, we found Dα and Dγ sensilla of nymph bed bugs showed significant weaker responses to saturate aldehydes and limonene components of aggregation pheromone, respectively (Fig. 3.3A and B). While first instar bed bugs showed weakest activity with lowest neuronal responses of 20 ± 1.155, 51.5 ± 9.5, 44 ± 6.2, 48 ± 4.3 spikes/s to decanal, nonanal, S-(-)-limonene, and R-(+)-limonene, respectively, followed by early nymphal stage (2^{nd} instar and 3^{rd} instar), and 4^{th} and 5^{th} instar bed bugs showed similar responses as adults.

Siljander et al. (2007) reported bed bug juvenile-specific aggregation pheromone, and also suggested the pheromone was perceived by contact chemoreception. Through our study, we revealed the 'adult-specific' volatile aggregation pheromone components, S-(-) and R-(+)-limonene, which elicited strong response in adults but not in bed bugs of early nymphal stages (Fig. 3.3 B). It is worth noting that it is male bed bugs that produced largest quantity of limonene (Siljander et al. 2008), and both males and females preferred to aggregate on male-exposed discs

but not females. Taken together, our result provided physiological basis of components that may play a part of role in the preference of aggregation behavior of adults, that is, (+)- and (-)-limonene produced by males may act as attractant for males and females but not for juveniles, it can be further verified by behavior tests.

ORNs responding to same structure of odorants may be expressed at multiple sensilla at different developmental stages. For instance, decanal and nonanal elicited high neuronal activities in three types of D sensilla of all developmental stages, except for Dα sensilla of 1-4th instar (Fig. 3.2 A-F). Here, we provide two hypotheses for this phenomenon: 1) in early nymphal stage, some sensilla assembled incomplete repertoire of ORNs compared with 5th instar nymphs and adults. And the number as well as diversity of ORNs increased as well as each time they molting into the next instar; 2) the sensitivities of ORNs to certain chemical structure were differently regulated between nymphs and adults, this is supported by the response of Dγ sensilla in different instars, which Dγ of bed bugs in all instars did show excitatory responses to limonene (Fig. 3.3B), although adults exhibited higher firing frequency. It can be explained by the fact that the larger the sensillum was growth, the more pores were presented on the cuticle wall of sensilla and the more odorant molecules were likely to be absorbed through the sensillum lymph. Alternatively, the expression level of Odorant Binding Protein (OBPs) may be regulated differently between nymphs and adult, the OBPs were thought to be related to the sensitivity of ORs (Xu et al. 2005).

C1 and C2 sensilla were not so well-developed in 1st-2nd instars and 1st instar, respectively. Therefore, only instars that had well-developed sensilla were taken into account for difference analysis. Although (E)-2-hexenal and histamine elicited relative low spontaneous

firing rates in early nymphal stages, no significant difference was detected between instar groups by One-way ANOVA test. bed bug 3rd and 4th instar showed significant higher responses (198 ± 59 spike/s, n=3; 148 ± 15.77 spikes/s, n=5) to histamine than other developmental stages with F-tests ($p=0.0032 < 0.05$, Table 3.1). From the 3-D map of responses of C and D sensilla to components of aggregation pheromone in each instar and adult, we can find only slight difference between adults and nymphs higher than 2nd instar. For the early stage nymphs, although C1 and C2 were not well-developed, but there are another 4 C sensilla distributed on olfactory regions may play the same role as C1 and C2 does.

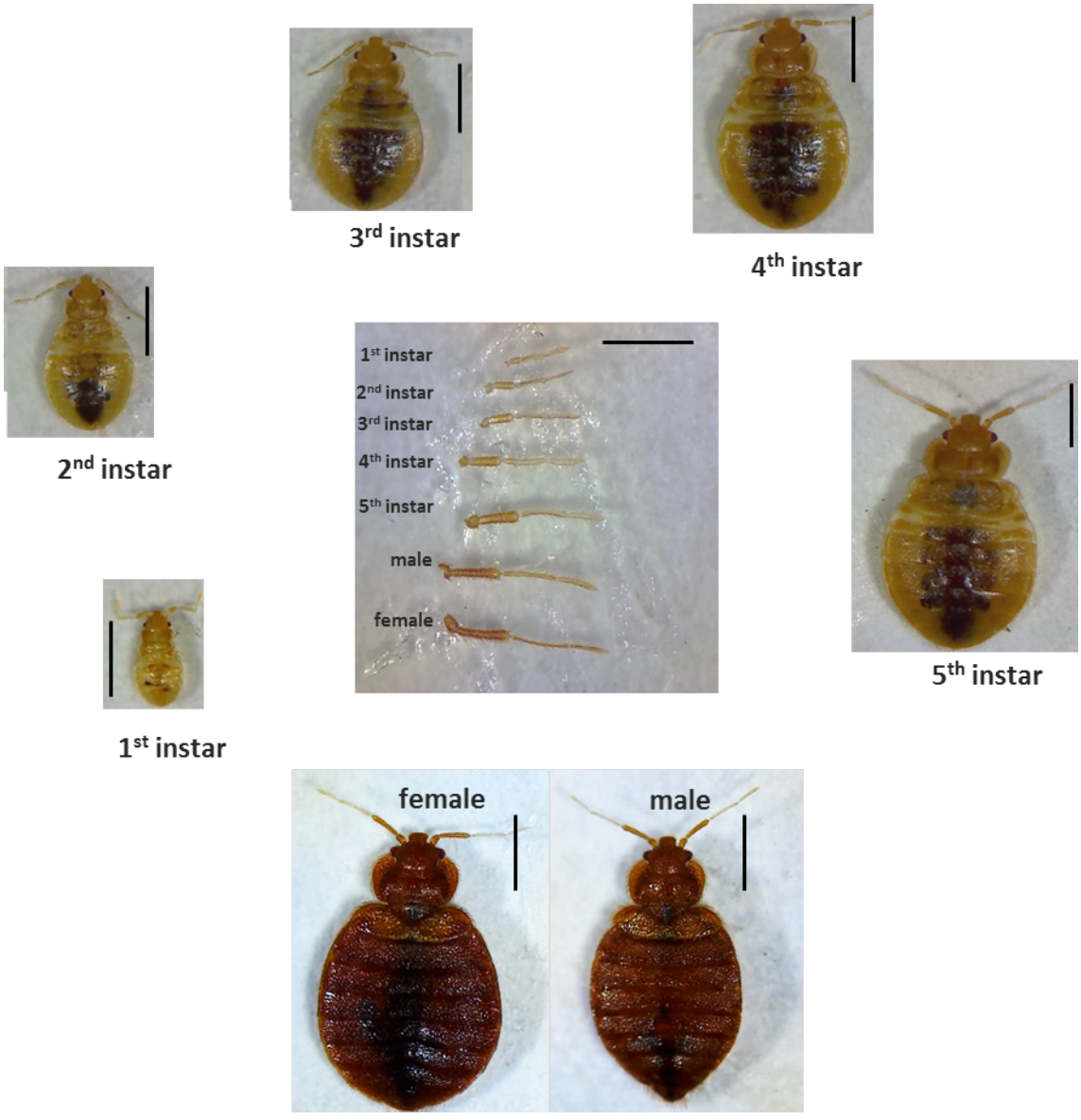


Figure 3.1. Different developmental stages of bed bugs. Scale bars indicate 1mm. bed bugs were generally one week after blood feeding. Dissection of antenna of different instars and sexes showed the relative length of antenna as development of bed bugs.

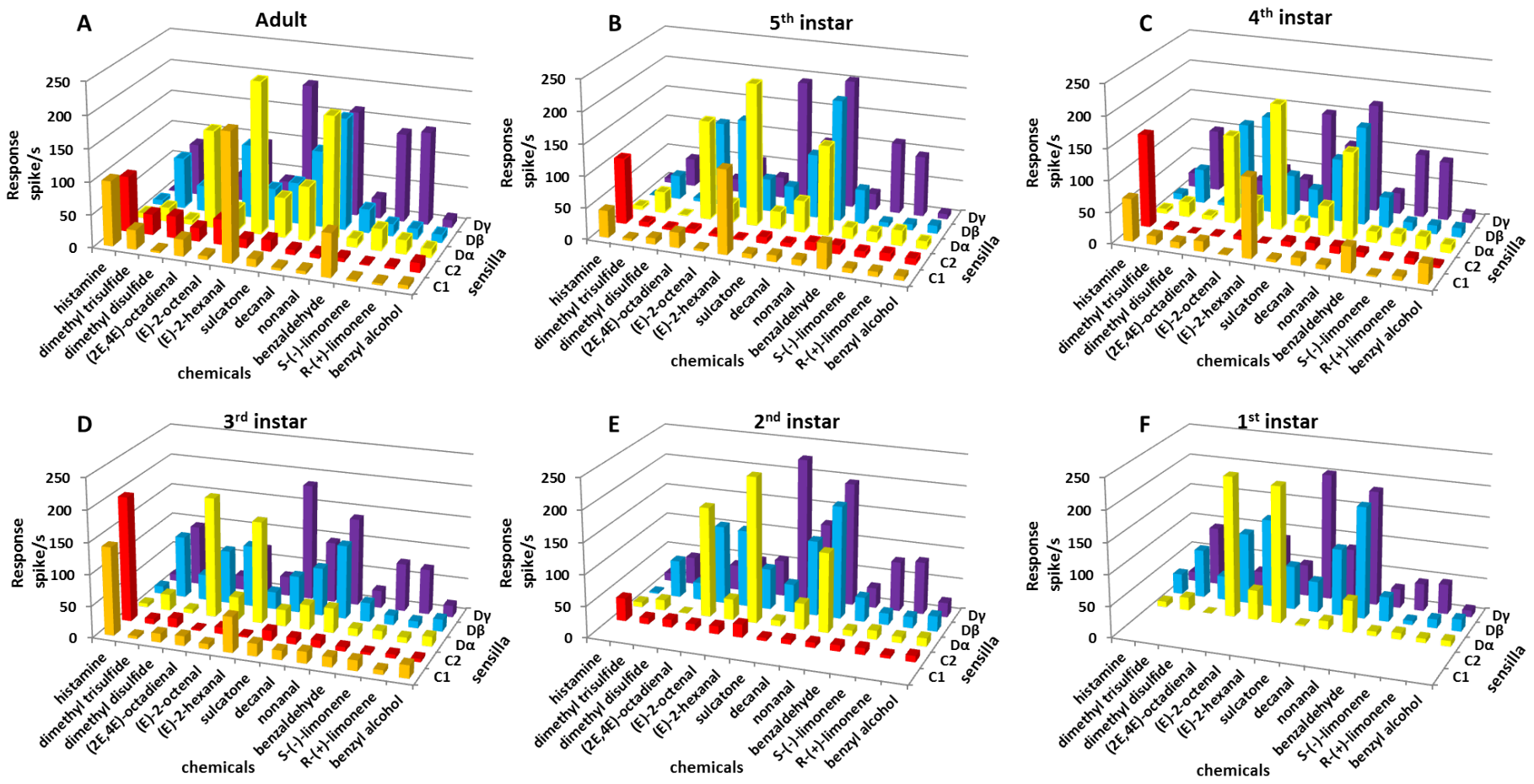


Figure 3.2. 3-D maps of responses of each developmental stages of *Cimex lectularius* to components of aggregation pheromone.

Three functional types of D sensilla and two C sensilla in response to 13 components of aggregation pheromone at 1:100 v/v dilution. With D sensilla have more than 6 replicates and C sensilla have at least 3 replicates. A-F showing encoding map of decreasing instar bed b

Table 3.1. F-tests results of strong chemical-sensillum combination from different developmental stage bed bugs

Sensilla	Chemicals [a]	Mean ± SEM (spikes/s) Number of values (n)						One-way ANOVA	Does means significant different? [b]
		1st instar	2nd instar	3rd instar	4th instar	5th instar	adult	p value	yes/no
Dα	(2E,4E)-octadienal	208 ± 21.39 (3)	175.4 ± 8.92 (7)	190.4 ± 9.108 (5)	148.3 ± 13.15 (6)	176.4 ± 21.41 (5)	165.3 ± 10.6 (14)	0.1983	no
	(E)-2-hexenal	218 ± 18.49 (4)	221.6 ± 23.79 (5)	161.7 ± 14.07 (7)	198.3 ± 16.68 (7)	239 ± 17.19 (6)	212.8 ± 13.45 (19)	0.1269	no
	Decanal	20 ± 1.155 (3)	41.78 ± 6.62 (9)	39.43 ± 9.892 (7)	46.57 ± 7.718 (7)	73.67 ± 25.57 (6)	112.9 ± 11.65 (19)	< 0.0001 ***	yes
	Nonanal	51.5 ± 9.5 (4)	110 ± 25.16 (5)	61.71 ± 15.43 (7)	136.9 ± 14.22 (7)	177.7 ± 27.32 (7)	190.6 ± 8.889 (17)	< 0.0001 ***	yes
Dβ	(2E,4E)-octadienal	113.3 ± 8.257 (6)	116 ± 11.46 (9)	89.82 ± 9.863 (11)	138 ± 14.41 (7)	133.7 ± 18.73 (6)	119.7 ± 9.733 (18)	0.1597	no
	(E)-2-octenal	140.7 ± 4.89 (6)	123.8 ± 9.713 (9)	114.6 ± 1.784 (7)	154.9 ± 16 (7)	143.3 ± 6.401 (6)	133.5 ± 10.32 (19)	0.2671	no
	Decanal	107 ± 9.22 (6)	88.67 ± 16.49 (9)	76.15 ± 13.11 (13)	100.3 ± 14.77 (7)	101.3 ± 23.96 (6)	117.4 ± 10.62 (19)	0.246	no
	Nonanal	180.4 ± 17.03 (5)	130 ± 23.05 (8)	121.4 ± 22.32 (7)	156 ± 25.1 (7)	192 ± 17.51 (6)	172 ± 9.005 (17)	0.0729	no
	Dimethyl trisulfide	99.45 ± 7.237 (11)	79.6 ± 22.66 (5)	99 ± 23.56 (6)	54.86 ± 8.105 (7)	50.4 ± 9.289 (5)	75.4 ± 12.92 (10)	0.0714	no

Table 2.1 continued

Dγ	(E)-2-octenal	85.11 ± 12.88 (9)	68 ± 16.8 (8)	72.31 ± 7.486 (13)	52.67 ± 9.201 (9)	60 ± 10.11 (9)	89.33 ± 7.76 (24)	0.0844	no
	Sulcatone	203.4 ± 17.11 (7)	167.5 ± 24 (8)	185.7 ± 10.49 (12)	149.2 ± 19.61 (10)	193.8 ± 9.272 (10)	152.7 ± 10.51 (23)	0.0608	no
	Decanal	95.75 ± 18.16 (8)	89.5 ± 19.79 (8)	97.56 ± 11.24 (9)	102.2 ± 14.88 (10)	100.8 ± 13.94 (10)	135.7 ± 11.46 (24)	0.1132	no
	Nonanal	184.8 ± 15.62 (8)	138.3 ± 33.55 (8)	140 ± 15.79 (11)	171.8 ± 14.31 (9)	204.4 ± 12.4 (10)	173.5 ± 12.87 (24)	0.1217	no
	S-(-)-limonene	44 ± 6.719 (8)	79.43 ± 7.141 (7)	76.46 ± 15.75 (13)	101.2 ± 8.94 (10)	113 ± 13.02 (10)	130.1 ± 8.051 (23)	< 0.0001 ***	yes
	R-(+)-limonene	48 ± 6.349 (8)	74.5 ± 13.56 (8)	72.29 ± 11.78 (14)	101.2 ± 8.94 (10)	96.4 ± 9.53 (10)	130.4 ± 8.155 (23)	< 0.0001 ***	yes
	Dimethyl trisulfide	93.6 ± 13.78 (5)	67.2 ± 9.222 (5)	96 ± 5.55 (5)	99 ± 14.2 (6)	59.2 ± 8.823 (5)	78.71 ± 6.38 (24)	0.1377	No
C1	(E)-2-hexenal			56.67 ± 5.925 (3)	127.3 ± 26.03 (3)	133 ± 47.95 (4)	160.5 ± 16.35 (11)	0.1053	no
C2	Histamine		79.43 ± 19.8 (7)	198 ± 59 (3)	148.4 ± 15.77 (5)	103.6 ± 13.3 (5)	98.25 ± 7.037 (12)	0.0032 **	yes

[a] chemicals elicited strong response (≥100 spikes/s) to certain type of sensilla of both sexes were chosen to compare the sexual dimorphism. Total 18 combinations were tested.

[b] means of responses was tested by One-way ANOVA with $P > 0.05$, was defined to be significant different with one another group.

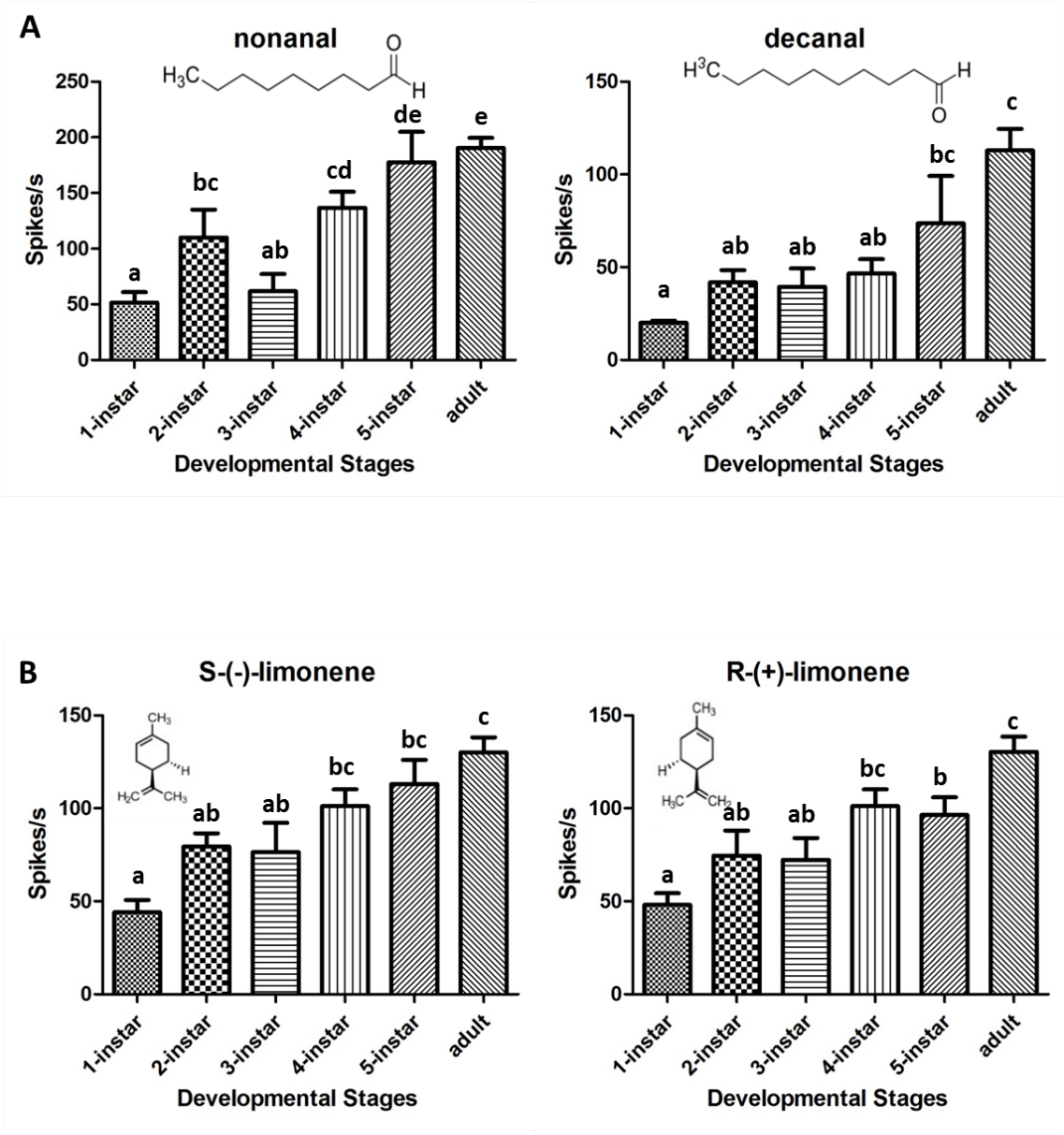
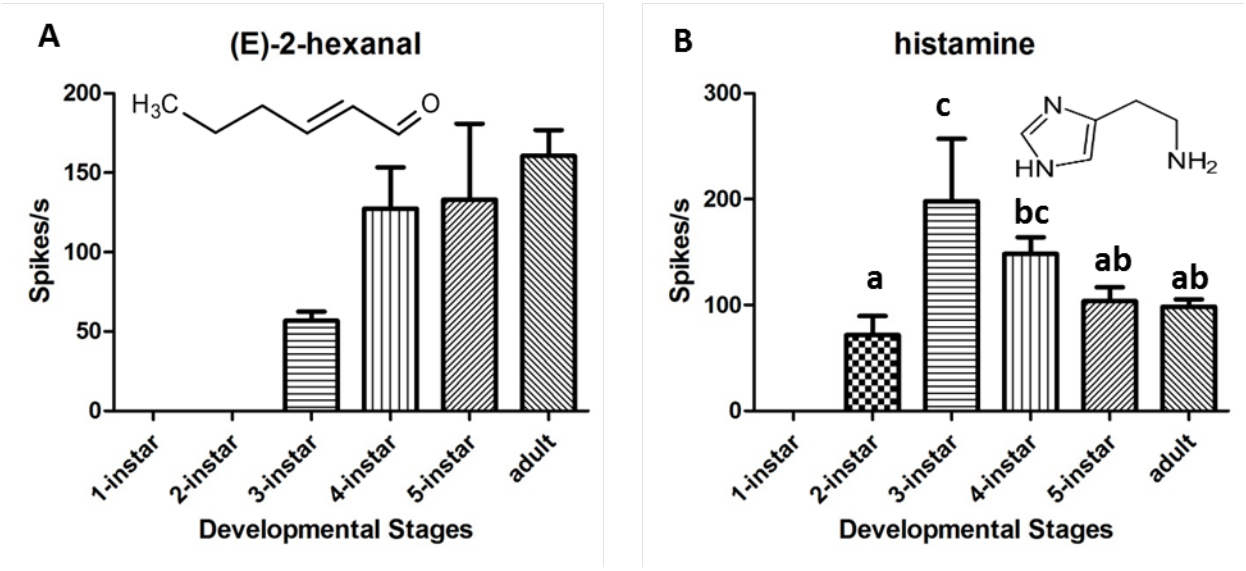


Figure 3.3. Components of aggregation pheromone that elicited significant different responses of D sensilla in different developmental stages. Responses of same chemical-sensillum combination in different instars with F-test, $P < 0.05$ ($n > 6$), followed by multiple comparison by Tukey's test. The columns with at least one same letter were not significant different from each other. A) responses of Dα sensilla to nonanal and decanal; B) responses of

Dγ sensilla to S-(+)-limonene and R-(-)-limonene. Related chemical structures were presented above. Response of adults includes both males and females.

Figure 3.4. Multiple comparisons of responses of C sensilla in different instars. (E)-2-hexenal and histamine were two components that elicited strong neuronal activities in C1 and C2 sensilla ($n \geq 3$), respectively. Significant difference between groups was analyzed by One-way ANOVA test. And groups without data were not taken into analysis. A) responses of C1 sensilla with F-test P=0.1053, C1 was not observed in 1st and 2nd instar bed bugs; B) responses of C2 sensilla to histamine with P=0.0032, 1st instar do not have C2 sensilla, the bars of instar with the same letter were not significant different from one another, analyzed by tukey test ($P < 0.05$).



Chapter 4: Classification of E sensilla into functional groups

Introduction

Hair-like sensilla, also called E sensilla, are the most abundant olfactory sensilla of *Cimex lectularius*, yielding more than 60% of the total 44 olfactory sensilla distributed on the distal part of last flagellum of bed bug antenna. Levinson et al. (1974) characterized two morphological groups of E sensilla, E1 and E2, with even an uneven sensillum wall, respectively. Cross-section of sensilla also suggested E1 sensilla housed 1-3 neurons with 2 pores on the sensillum wall, and E2 sensilla house 2 neurons with less than 2 pores on the sensillum wall (Steinbrecht and Müller, 1976). However, we cannot distinguish E1 and E2 under microscope by keen eyes, in this study, we referred all 27 hair-like sensilla as E sensilla.

Due to the recent resurgence of bed bug infestation, many efforts have been made on the research of olfactory system of bed bugs, revealing the critical function roles of smooth peg sensilla in response to many bed bug behavior related odorants, like human odorant (Liu and Liu, 2015), chemical repellents (Liu et al., 2014), and bed bug alarming pheromone (Harraca et al. 2009). However, little has been done on characterizing the physiological function of E sensilla, general because many E sensilla barely showed any response to previous used chemicals. Except E2 were reported to show some excitatory responses to some long chain human odorants. Yet no report has systematically tested the olfactory response of all E sensilla.

To investigate whether there are different functional groups within E sensilla, we designed this experiment to tests overall olfactory responses of each single E sensillum to 16 chemical odorants using Single Sensillum Recording (SSR) method as described in the previous

chapters. Odorants were chosen from 13 components of bed bug aggregation pheromone and three long-chain human odorants, 1-tetradecane, lauryl chloride, and N-pentadecanoic acid.

Materials and Methods

Insects

The *C. lectularius* colony utilized in the 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, USA). Bed bugs were reared at 25±2 ºC under a photoperiod of 12:12 (L: D). Due to the decreased E sensilla in nymph bed bugs, Experiments were conducted using only males and females one week after blood feeding, regardless mating status.

Stimuli

Sixteen chemicals from components of aggregation pheromone and human odorant were chosen to test response of 27 hair-like sensilla, including histamine, dimethyl trisulfide, dimethyl disulfide, (2E,4E)-octadienal, (E)-2-octenal, (E)-2-hexanal, sulcatone, decanal, nonanal, benzaldehyde, (+)-limonene, (–)-limonene, benzyl alcohol, 1-tetradecane, lauryl chloride, and N-pentadecanoic acid. Chemicals obtained purity more than 94%. Stimulations were diluted in dimethyl sulfoxide to 1: 100 v/v for liquid or 1:100 w/v for solid.

Single sensillum Recording

The details of methodology were as described in Chapter 2, total 27 E sensilla were labeled, although they were similar in morphology, their positions on the olfactory region were

consistent between male and female. Each sensillum was tested by same odorant panel with at least three replicates on different bed bugs.

Analysis data

To classify functional groups based on olfactory responses of ORNs in 27 different sensilla, we performed hierarchical cluster analysis using Ward's classification method and Euclidean distances by SPSS (IBM SPSS Statistics 20). This method has previously been used by similar electrophysiological studies previously (Hallem and Carlson, 2006, Harraca et al., 2009). It quantitated the olfactory responses of each sensillum to 16 odorants by constructing a 16-dimensional sensillum space in which each odorant represented an axis. Sensilla with similar response patterns would show shorter distance, thus will be clustered into same group.

Results and Discussion

E sensilla houses 1-3 neurons, in most of recordings, we observed very neat spikes of firing rates (Fig. 4.2 D), indicating only one neurons housed in the sensillum. Sometimes we could distinguish different neurons by different amplitudes firing rates of spikes (Fig. 4.2B). However, it was usually distinguishable only when stimulated by certain odorants, thus we only analyzed the overall responses of a sensillum.

In our 1505 SSR recordings over 27 E sensilla with panel of 16 odorants, E sensilla generally exhibited sparse spontaneous firing rates as well as weaker neuronal responses than D and C sensilla with highest excitatory response of 32 spikes/s. We found some different patterns of different receptors that were activated by different combinations of odorants. To examine the

relationships between ORNs in different sensilla, we constructed a Dendrogram cluster of sensilla based on overall responses of ORNs in each sensillum to 16 tested odorants (Fig. 4.1B). Four clusters of sensilla group were well separated. Means of responses of odorant-sensillum combinations were arranged in the order of Dendrogram by heat map (Fig. 4.1A). Accordingly, we call the four types of group as EI, EII, EIII, and EIV, sensilla grouped in one type did show similar patterns to tested odorants. Within each group, response patterns of sensilla to odorant panel showed no significant difference (Fig. 4.1A).

First of all, a small group of sensilla consisting of E5, E18 and E21 were separated from others at the beginning of hierarchical tree. EIV type of sensilla exhibited inhibitory effect to 13 components of aggregation pheromone (Fig. 4.1B). This type of sensilla always showed regular and relative high spontaneous firing rates (Fig. 4.2D). In foregoing studies, E sensilla have been characterized as showing inhibitory effect to all tested odorants, revealing that EIV sensilla had specifically inhibitory responses to bed bug aggregation pheromone but not to human odorants. This results can be explained by the hypothesis that inhibitory effect of neuronal activities may help reducing noises for important signal information (Hallem and Carlson, 2006).

Another distinguishable group was EIII which separated from EI and EII at second joint; 7 sensilla fell into EIII type shared one feature of which was sensitive to human odorants. Many features of EIII type of sensilla indicate that they belonged to E2 sensilla, including its irregular spontaneous firing rate (Fig. 4.2C), and E2 sensilla were reported to be responsible for human odorant detection (Liu and Liu, 2015).

Most of sensilla in EII type specifically showed excitatory response to decanal, nonanal and sulcatone at 100-fold dilution. Whereas EI type sensilla showed no significant response to

any of the 16 odorants used in this experiment. There results indicated that EII type of sensilla were responsible for detecting pheromone-related odorants and EI type sensilla had been evolved into other functions. EI type consisting 10 sensilla in total is the largest group in E sensilla, however, the function of these EI type sensilla needs to be explored.

Hierarchical tree has been used as the tool for the groups with greater similarity at closer cluster, with which we can found by the fact that representatives of EI and EII were more similar in response spectra than EIII and EIV (Fig. 4.2A-D). It is worth noting that E19 and E20 were neighbors on the position of antenna (Fig. 4.3), had 2 neurons in it (by looking at two different amplitudes of spikes of recording activity), and showed similar spontaneous firing rates. But they still fell into different functional classes with difference can be distinguished. For example, nonanal was observed to elicit increase of firing rate in short amplitude spikes in E20 but not in E19 (Fig. 4.1A and B).

Combined distribution map of sensilla proposed by Steinbrecht and Müller (1976), we mapped the position of each functional type of E sensilla on the antenna (Fig. 4.3). The relative positions of sensilla were quite constant between individuals of adult. Majority of E sensilla distributed on the two opposite regions (O1 and O2) of distal part of last flagellum of bed bug antenna. Each type of sensilla was distributed evenly on the olfactory regions without divided into specific functional area like in *Drosophila* (De Bruyne et al., 2001). This may be correlated with the morphological and behavioral difference among species. For example, bed bugs have reduced wings with a relative narrow activity diameter, whereas *Drosophila* requires high sensitivity of receptors to follow and locate chemical cues during fast movement.

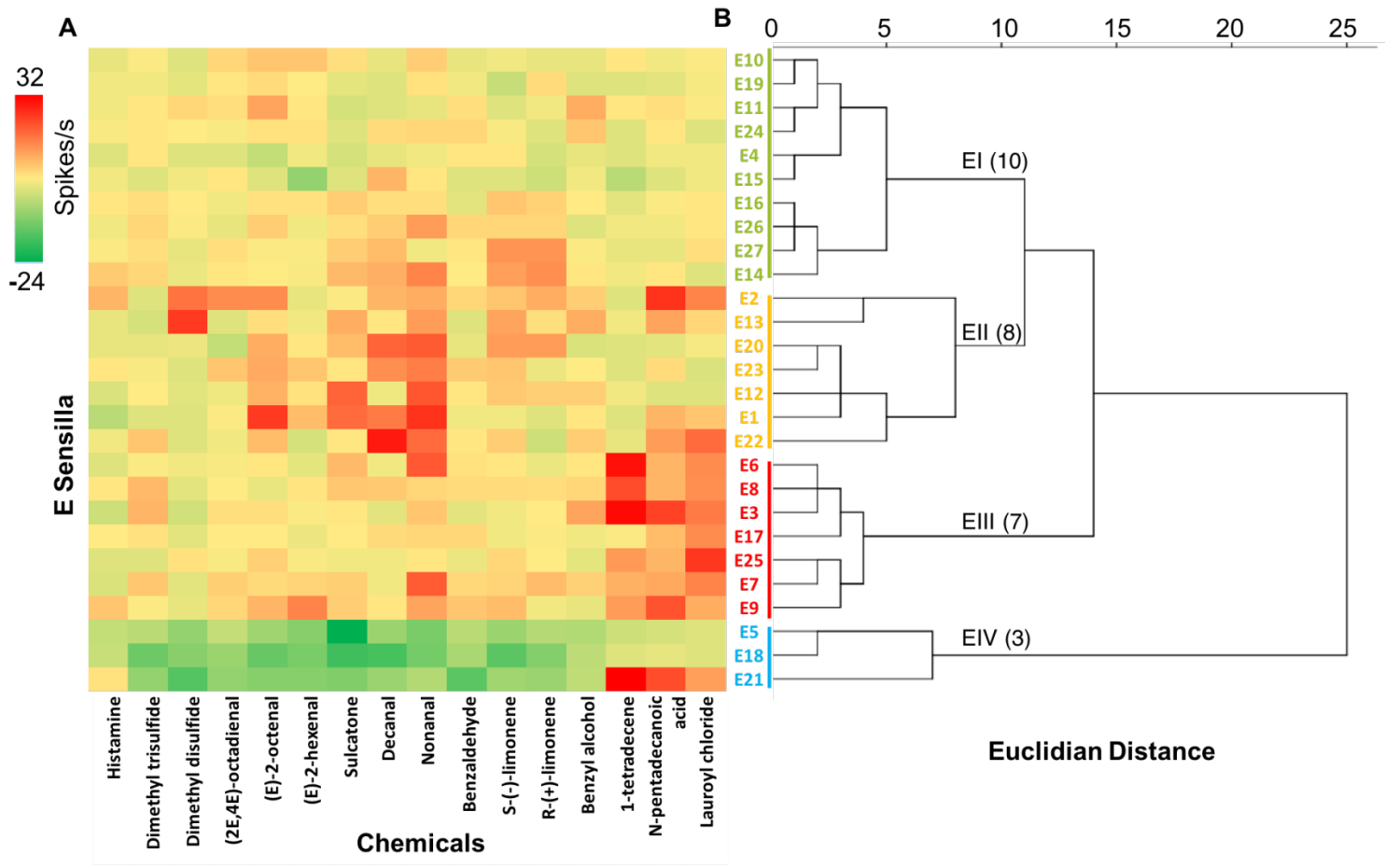
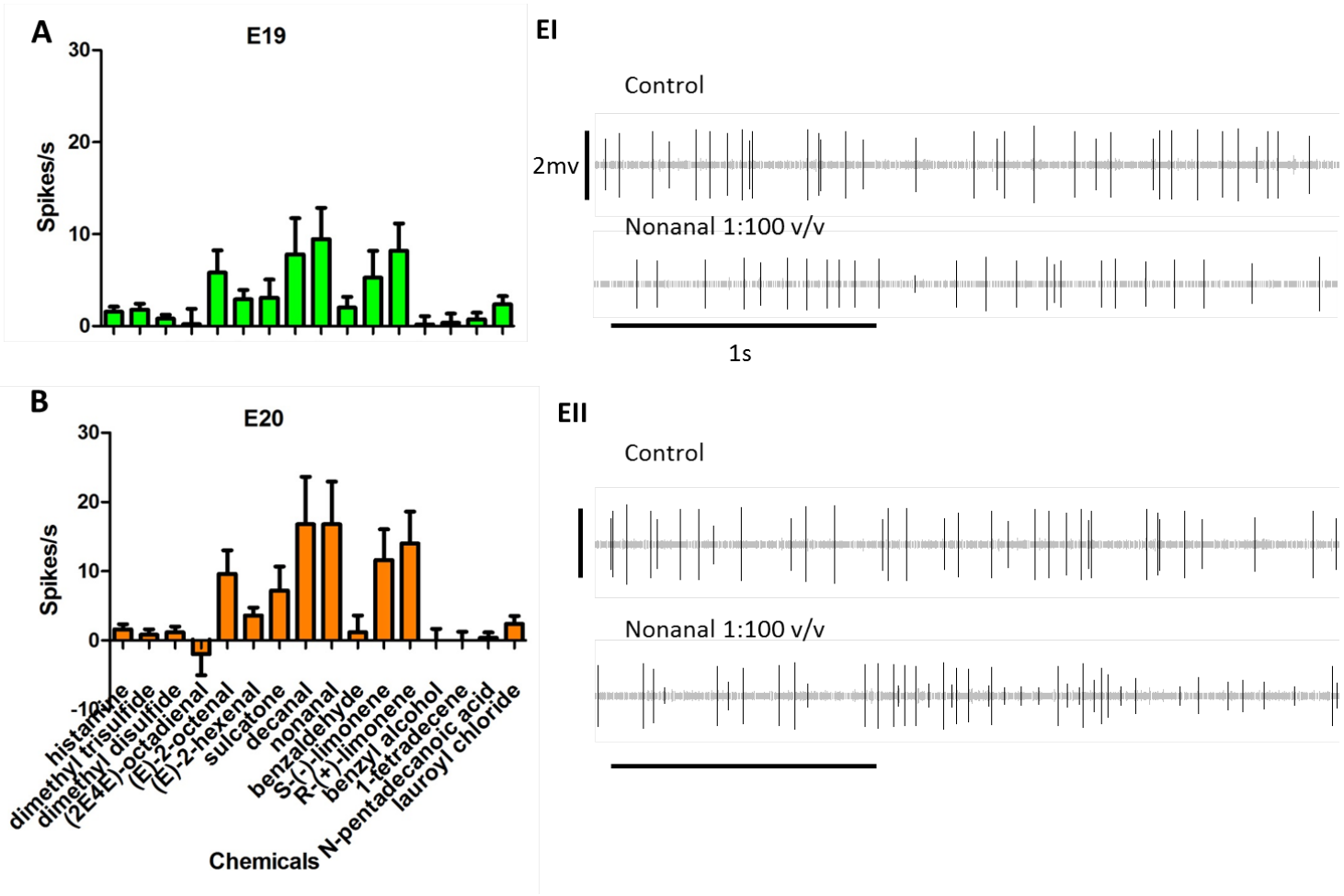


Figure 4.1. Classification of E sensilla based on olfactory responses. A) Heat map of olfactory responses of ORNs house in E sensilla in response to 16 odorant stimulation, each cell with at least 3 replicates. Means of response spikes were represented by gradient color, red indicates excitatory responses and green indicates inhibitory responses. Horizontal axis indicates 16 chemicals at

1:100 v/v dilution, vertical direction showed 27 E sensilla in total. B) Dendrogram cluster of E sensilla based on olfactory responses of each sensillum to same 16 odorant panels using Ward's cluster analysis, horizontal axis indicated Euclidean distance.



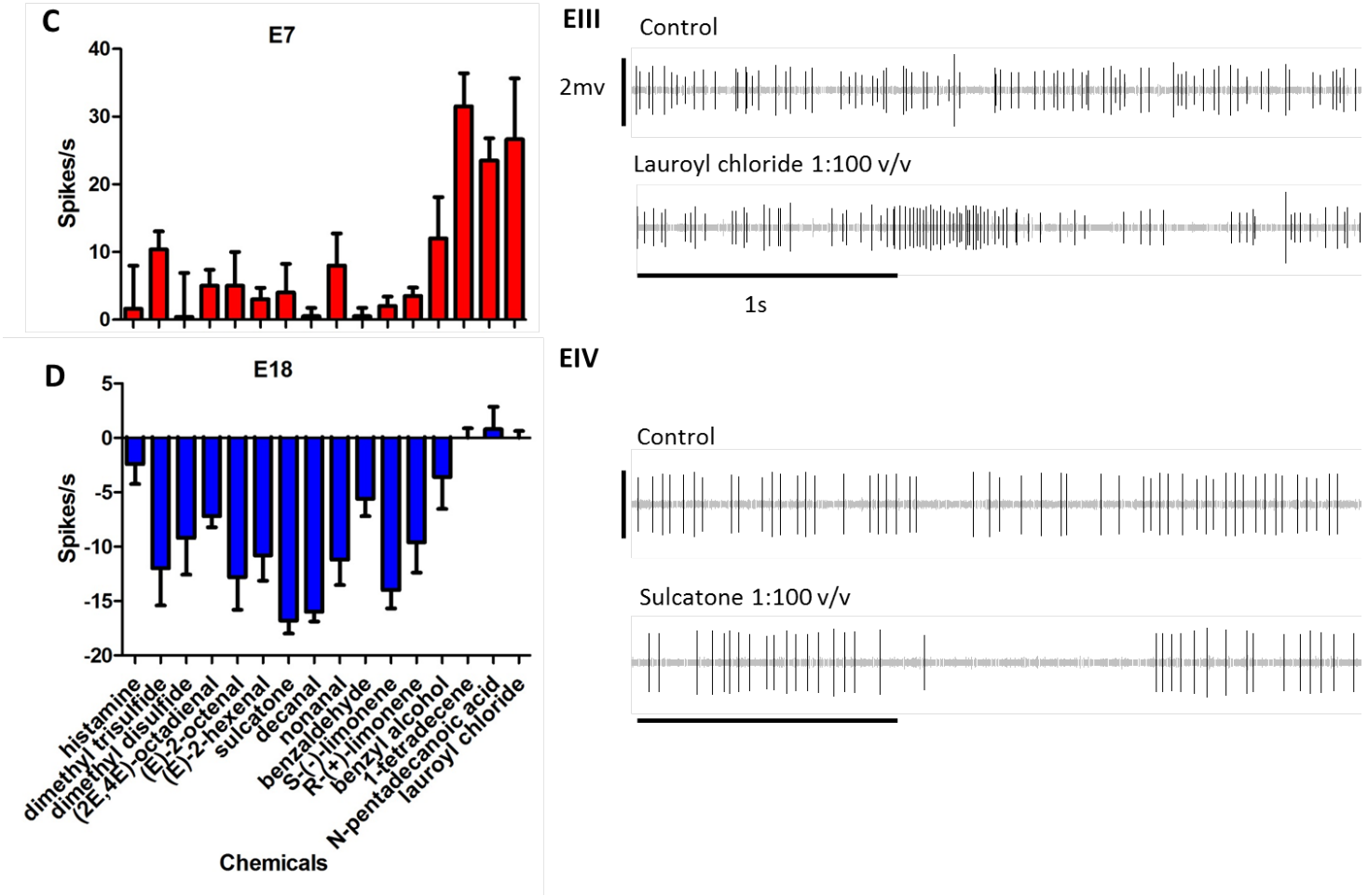


Figure 4.2. Four stereotypes of four functional types of E sensilla. Column graphs on the right showing means of response to 16 odorants at 1:100 v/v dilutions, A) EI type with n=8; B) EII type with n=6; C) EIII type with n=9; D) EIV with n=6. SSR recordings on the left are some representative responses to certain odorant with DMSO as control, except for E19 sensilla which showed overall weak response to chemical panel.

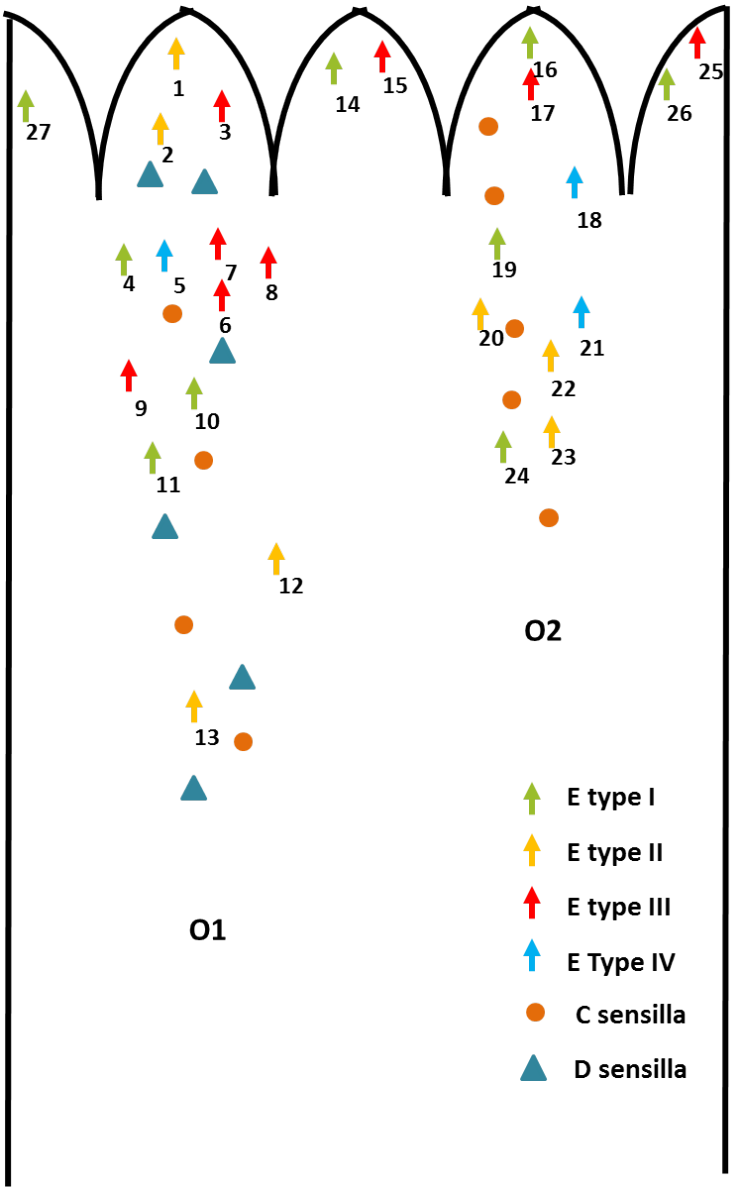


Figure 4.3. Distribution of functional types of E sensilla on distal part of antenna of *Cimex lectularius*. Different colors of arrow marks represented different functional types of E sensilla based on our hierarchical cluster analysis. Relative positions of sensilla were consistent with their real positions on the antenna. C and D sensilla were represented by circle and triangle symbols.

Chapter 5: Summary and Future Directions

In this study, I mainly focus on the electrophysiology of bed bug olfactory sensilla, to decipher the encoding map of olfactory response to components of aggregation pheromone by using SSR.

In Chapter 2, I characterized the response spectra, dose-dependent responses and temporal dynamics of different types of sensilla. 12 out of 13 components of aggregation pheromone elicited excitatory neuronal responses in at least one type of sensilla. Combinatorial responses spectra of D and C sensilla played complimentary roles in perceiving aggregation pheromone. Consistent with previous electrophysiological studies (Harraca et al. 2009, Liu and Liu, 2015), D sensilla generated broad spectrum to tested odorants, showing dose-dependent responses with different sensitivities. Specifically, the highest sensitivities were observed in responses of Dα and Dγ to sulcatone and (E)-2-hexenal, respectively, showing significant neuronal excitatory effect (>50 spikes/s) at dilution of 10^{-4}, While other odorants were detected at 10- or 100-fold dilutions. We also found that higher odor concentration not only increased the firing frequency of neuron cells, but also prolonged response to the odorant. It is worth of noting that C sensilla were narrowly tuned to histamine, which has been thought to be related to arrestment behavior of bed bugs (Gries et al., 2015). Finding out the relation between ORNs in C sensilla and arrestment behavior of bed bugs will be very valuable in understanding the aggregation behavior of bed bugs.

In the chapter 3, we addressed the question concerning whether there are differences of encoding process of olfactory sensilla between nymphs and adults of bed bugs. Previous studies suggested that unknown juvenile- and adult-specific aggregation pheromone may cause the

preference of aggregation of different instar (Siljander et al., 2007). Interestingly, we did find four chemical-sensillum combinations that evoked significant higher response in higher instar bed bugs. Moreover, S-(-)-limonene and R-(+)-limonene were mainly produced in males (Siljander et al., 2008), suggesting their potential roles in constructing adult/sexual-specific aggregation pheromone. Taken together, in my first two chapters, I characterized comprehensive neuronal responses of peripheral olfactory system to aggregation pheromone. Not only did our results reveal sensitivities of ORNs in sensilla to each component, but also provided solid physiological evidence to explain behavior preference of aggregation behavior.

In the chapter 4, we explored different functions of a large sensilla family. For the first time, four functional classes of E sensilla were described based on response patterns of each 27 E sensilla to 16 odorants panel. Sensilla in II, III, and IV functional type showed distinct response patterns than another type, with only minor difference between individuals in same functional type. However, the function of 10 sensilla in Type I remains unknown, with no response showed to tested odorants.

Taken together, I characterized comprehensive neuronal responses of peripheral olfactory system to aggregation pheromone. Not only did our results reveal sensitivities of ORNs in sensilla to each component, but also provided solid physiological evidence to explain behavior preference of aggregation behavior. My effort in building connection between physiology and chemical ecology of bed bugs have shed light on cellular mechanism of behaviors of bed bugs. However, SSR has limited ability in distinguish individual ORN in single sensillum. Future research on investigating the molecular mechanism of neuronal responses to aggregation pheromone will be very interesting. For example, to locate the receptor family, which responses to histamine, can alter the behavior of bed bugs.

Another direction pointed to application of developing effective tools for bed bug monitor and control. To determine the most effective ratio of components, our result have provided clues for chemicals that can be detected at different dosages, although in practical mixed components may elicit stronger response with a much lower concentration. Based on the neuronal response patterns, for instance, (E)-2hexenal, sulcatone, decanal and nonanal showed much strong stimuli for ORNs than other chemicals. Also, followed by behavioral tests, there is great chance in developing attractant that will be specifically effective adult with (+)- and (-)-limonene.

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