Olfactory Responses of Two Coleopteran Species:

The Stored Product Pest *Tribolium castaneum* and The Forest Pest Predator *Dastarcus helophoroides*

DISSERTATION

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Abstract

Olfaction is unavoidable in guiding insect behaviors. Detection of odorant molecules by the principle anatomical structure, the antenna, is thought to result from direct activation of odorant receptors (ORs) by odor molecules. Besides ORs, several other olfactory components are involved in insect olfaction, e.g. odorant binding proteins (OBPs) are highly expressed in insect olfactory tissues, mainly antenna and involved in the first step of odorant reception. Improved knowledge of the olfaction of pest insects or beneficial insects may contribute to identifying new attractants/repellents and support in the development of pest insect control strategies. The overall objective of this study was to identify volatile organic compounds (VOCs) that could modulate the olfactory responses of the stored product pest *Tribolium castaneum* and the forest insect predator *Dastarcus helophoroides*.

The first part of this research work, began with the investigation of the electroantennographic (EAG) responses of the most destructive stored product pest insect *T. castaneum*. Despite the importance of this beetle as "model organism" in population ecology, evolution, and mathematical studies, there is no systemic olfactory information available on this beetle. EAG responses from both sexes of *T. castaneum* to 94 VOCs were recorded and identified more than 90% of tested compounds to elicit antennal responses at high concentration (10^{-1}). Specifically, the volatile compounds undecane, 1-hexen-3-ol, octanal, 2-heptanone, 2-pentanone, hexanoic acid, and ethyl hexanoate were eliciting the strongest EAG responses within the compound group of chemical similarity. Additionally, comparison of vapor pressure with the strength of the EAG amplitudes revealed these compounds were outstanding within a homologous series of compounds. This effort is the first to identify several perceived compounds by both sexes of *T. castaneum* beetles.

RNAi experiments and EAG recordings were performed to investigate the roles of the highly expressed OBPs *TcasOBP9A* and *TcasOBP9B* in the olfactory reception of both sexes of *T. castaneum*. RNAi-treated *dsTcasOBP9A* and *dsTcasOBP9B* beetles were injected into the conjunctivum between fourth and fifth abdominal segments of each *T. castaneum* pupae. It was found that the *dsTcasOBP9A* injected beetles showed highly significant EAG response reduction to 4,8-dimethyldecanal, (*E*)-2-heptenal, 2-hexanone, 6-methyl-5-hepten-2-one, *cis*-3-hexen-1-ol, and β-ionone at most tested dilutions (10^{-1}-10^{-8}). In male beetles, knock-down of *TcasOBP9B* shows less effective and both gene knock-down revealed a similar effect as the *TcasOBP9A* single knock-down. But in female beetles, the *TcasOBP9B* knock-down caused a significant EAG response reduction to all tested VOCs. Similar to the double knock-down situation, which reveal significantly high pronounced response reduction to all tested VOCs at all dilutions. These results provide the first evidence that both *TcasOBP9A* and *TcasOBP9B* play important crucial roles in the perception of diverse VOCs.

A practical application of using OBPs is to develop a portable biosensor device with sensing element for detecting disease marker compounds. The above EAG results and the RNAi knockdown studies enabled me to select two OBPs, *TcasOBP9A* and *TcasOBP9B*, from *T. castaneum* and their respective binding ligand 6-methyl-5-hepten-2-one and 3-octanol. Consequently, for a proof of principle study. These two OBPs were used as sensing element functionalized in a reduced graphene oxide field effect transistor (rGO-FET). Real-time binding affinity measurements by the rGO-FET sensor with selected OBPs to their ligands (*in-vitro*) and antennal responses of *T. castaneum* (*in-vivo*) to the aforementioned ligands revealed to follow the Langmuir model for ligand-OBP interactions. The results demonstrate that *TcasOBP9A* and *TcasOBP9B* are able to bind selected odorants when immobilized on rGO-FET and discriminate between ligands binding within a range of concentrations. These results led to a promising possibility to develop a portable sensor system based on rGO-FET and OBP as a sensing element to detect the compound that acts as an indicator for the toxin, early insect infestation on stored products, food contamination, and food degradation by microbial organisms.

The second part of this research work, the bothriderid predatory beetle *D. helophoroides* was selected for examining its olfactory perception, since this beetle's life cycle is strongly related to several of the most important cerambycid forest tree insect pests. For instance, *Monochamus* spp., *Anoplophora glabripennis, Massicus raddei, Apriona germari, A. swainsoni* and *Batocera horsfieldi* were considered major prey species for *D. helophoroides*. Antennae of both sexes of *D. helophoroides* were tested for EAG and behavioral responses to VOCs emitted from their prey infested trees, prey perceiving host volatiles, and larval frass of many preys. Both sexes of the beetle elicited EAG responses to nonanal, octanal, *cis*-3-hexenol, 3-carene, (R)-(+)-α-pinene, (S)-(-)-α-pinene, (R)-(+)-limonene, and (S)-(-)-limonene. In addition, behavioral tests of *D. helophoroides* showed that both sexes were significantly attracted to nonanal, *cis*-3-hexenol, 3-carene, or (R)-(+)-limonene even at low concentrations but were repelled to high concentrations of *cis*-3-hexenol or (S)-(-)-limonene. These highly attracted compounds might be useful either individually or in mixtures for developing efficient attractants to lure this predatory beetle into forest stands damaged by different cerambycids.

By using all these results together with available molecular information of both coleopterans, it should be possible to develop semiochemicals, RNAi approach, or rGO-FET biosensor based integrated pest management (IPM) strategies for controlling stored product pest like *T. castaneum* and forest insect pests. Furthermore, understanding the regulation of olfactory responses in the model organism *T. castaneum* will enhance our understanding of the olfaction in beetles in general, and in the long term may lead to developing new pest control strategies.

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CONTENTS

Abbreviations

AL	Antennal lobe
ABP II	Antenna binding protein II
APTES	Aminopropyltriethoxysilane
BEF	Beetle entering frequency
BSD	Beetle staying duration
CHS	Chalcone synthase
CSP	Chemosensory protein
cVA	11-*cis* Vaccenyl acetate
DIG	Digoxigenin
DMD	4,8-Dimethyldecanal
DPI	Days post injection
EAG	Electroantennography
EBQ	2-Ethyl-1,4-benzoquinone
FISH	Fluorescence in situ hybridization
GC-EAD	Gas chromatography coupled with electroantennographic detector
GC-SSR	Gas chromatography coupled with single sensillum recorder
GO	Graphene oxide
GOBP	General odorant binding protein
GPCR	G-protein coupled receptor
GR	Gustatory receptor
HSP70	Heat shock protein 70
IPM	Integrated pest management
IR	Ionotropic glutamate-like receptor
LH	Later horn

LoD	Limit of detection
MB	Mushroom body
MBQ	2-Methyl-1,4-benzoquinone
MOP	Mosquito oviposition protein
1-NPN	N-phenyl-1-naphthylamine
OB	Olfactory bulb
OBP	Odorant binding protein
ODE	Odorant degrading enzyme
OR	Odorant receptor
OrCo	Odorant receptor co-receptor
ORN	Odorant receptor neuron
OSN	Odorant sensory neuron
PBS	Phosphate buffer solution
PBSE	1-Pyrenebutanoic acid succinimidyl ester
PTFE	Polytetrafluoroethylene
rGO-FET	Reduced graphene oxide-field effect transistor
RNAi	RNA interference
SB	San Bernardino
SEM	Scanning electron microscopy
SNMP	Sensory neuron membrane protein
SO	Silicone oil
SSR	Single sensillum recorder
swCNT-FET	single walled Carbon nanotube-field effect transistor
TDM	Total distance movement
VOC	Volatile organic compound
VP	Vapor pressure

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CHAPTER 1

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1. General Introduction

Beetles have a significant economic impact on humankind through the destruction of field food crops, stored food products and forests (Schoonhoven et al. 2005; Rees, 2004; Vega and Hofstetter, 2015). On the other hand, some beetles have significant beneficial impact through the predation of vast insect species of economically important crop insect pests and forest insect pests (Lövei and Sunderland, 1996). Both groups of beetles perceives chemical volatiles around them through the chemical senses, olfaction and gustation. In fact, olfaction is an excellent modality for insects to navigate, explore, orient and detect changes in air-borne odorants in the environment to locate its primary needs. Insecticides are frequently used for the protection of food crops, stored food products, and forest trees (Heaps, 2005; Miller et al. 1994). However, the use of broad-spectrum insecticides in food crops, stored conditions and forests has resulted in the eradication of beneficial insects. Additionally, some economically important insects have developed resistance against overused insecticides (Metcalf, 1983). Therefore, there is a need for the development of semiochemicals-based, environmentally friendly and relatively economical pest management strategies for controlling harmful insects, which would presence in different habitats.

1.1. The nature and mechanism of the insect olfaction

Chemosensory neurons present in the morphological structures of animals react with volatile molecules in the surrounding environment. This process leads to a fast detection and recognition of chemicals that are present or any changes in the concentration of chemicals, allowing prompt behavioral responses (Cardé and Willis, 2008). Gustation requires the animal to make direct contact with the source of chemical compounds. Olfaction allows the insect to detect the source volatile compounds from a far distance. For instance, the black jewel beetle, *Melanophila acuminata* De Geer (Coleoptera : Buprestidae) is able to detect smoke odor as far as 50 km to locate forest fires, where burned woods are available for their offspring that can develop only in such a wood (Lindsey, 1943). In fact insects use olfaction to locate their food sources, find mates, offspring, recognize territories, oviposition site and to avoid danger. For these necessities, the olfactory system has to

recognize discrete molecular structures of odorants. In general, odorants may vary in molecular size, functional groups, branching patterns, three-dimensional structures and numerous other physio-chemical features. Therefore, olfaction of insects requires an enormous capacity for detection, discrimination, description and recognition of different odor molecules. The odorant molecules are typically hydrophobic and weigh between 26 and 300 daltons (Mori and Yoshihara, 1995 and reference therein). Volatility of odorant molecule is depend on their size; for instance heavier molecules are able to remain short time in the air compared to lighter molecules, which remains longer and able to reach the olfactory organ (Turin and Yoshii, 2003). Additionally, not all the odorant receptors (ORs) be able to accommodate larger molecules than specific required molecule size (Andersson et al. 2015).

Odorant molecules are first absorbed on the cuticular surface of the sensillum and enter the interior part through the wall pores. The conversion of extracellular chemical signal from odorant molecule to a neuron electrical signal is known as signal transduction (Figure 1-1). In insects, odorants are carried through odorant binding proteins (OBPs) to respective ORs after they interact with the sensillum lymph, while in vertebrate they interact with the nasal mucosa (Pelosi and Maida, 1990; Leal, 2013). The cluster of biochemical processes, which take place between the sensilla wall pores and the dendritic membrane of the sensory neurons, are known as "perireceptor events" (Getchell et al. 1984; Pelosi, 1996). Sensilla harbor odorant receptor neurons (ORNs), whose dendrites are housed in an aqueous fluid termed the sensillum lymph, which forms a hydrophilic barrier for the hydrophobic airborne stimuli (Leal, 2003). After reaching the sensillum lymph, the odorant molecule may interact with different classes of soluble proteins such as odorant binding proteins (OBPs) (Pelosi, 1994, 1996; Pelosi et al. 2006; Leal, 2013), chemosensory proteins (CSPs) (Angeli et al. 1999; Nagnan-Le Meillour et al. 2000; Pelosi et al. 2005), for binding. Then odorant-OBP complex binds to a specific odorant receptor (ORs) for activation of an ion channel gate. The odor coding process starts in ORs that are presence in ORNs, which translate the odor information into a new pattern, pass on to the antennal lobe (AL) for further processing and finally send it to the higher brain centers, which elicit behavioral actions (Figure 1-1) (Leal, 2013). Most ORs bind a range of odorant molecules, depending on the physical and chemical properties of the molecules, the concentration and the ligand affinity leads to varying ORNs and glomeruli activation (Malnic et al. 1999; Wang et al. 2003). The processed information send to the primary olfactory center, the

antennal lobe which correspondingly is passed on to the olfactory bulb (OB) and subsequently send to the higher brain centers, the mushroom bodies (MB) and the lateral horn (LH) for memory formation and organization of behavior (Strausfeld et al. 1998; Heisenberg, 2003; Leal, 2013).

1.2. Components of the insect olfactory system

1.2.1. Odorant binding proteins

So far two classes of soluble proteins have been identified in the sensillum lymph of chemosensilla, namely OBPs and CSPs (Pelosi, 1994, 1996, 1998; Nagnan-Le Meillour et al. 2000; Calvello et al. 2003; Calvello et al. 2005; Pelosi et al. 2005, 2006). Odorant binding proteins (size 10 to 30 kDa) are involved in the first step of odorant reception where they bind, solubilize and deliver the odorant molecules to the respective ORs in the insect antenna (Figure 1-1) (Leal, 2003). At the beginning of 1980s, the first insect OBP was discovered in the giant moth *Antheraea polyphemus* Cramer (Lepidoptera: Saturniidae) by using the tritium labeled specific pheromone (*E*, *Z*)-6, 11-hexadecadienyl acetate as a probe (Vogt and Riddiford, 1981). The OBPs expressed specifically in male antennae that bind to the female released sex pheromones are called pheromone binding proteins (PBPs) and other antenna-specific OBPs that are expressed in both sexes are called general odorant binding proteins (GOBPs) (Leal, 2003).

Odorant binding proteins are not synthesized by the odorant receptor neuron (ORNs) but produced in accessory cells related with them and secreted into the sensillum lymph surrounding the outer dendritic segment (Figure 1-1) (Leal, 2003, Brito et al. 2016). The process of N-terminal signal peptide encoding in OBPs primary sequence leads to the transport to their final destination. This means that N-terminal signal peptides are key responsible for synthesizing target proteins (OBP) and their transport to the secretory pathway (Scaloni et al. 1999; Calvello et al. 2003). Each OBP has six highly conserved cysteine residues forming three interlocking disulfide bonds between 6 α –helices, which provide a high stability to these proteins. CquiOBP1 was the first OBP isolated from the antennae of *Culex quinquefasciatus* Say (Diptera: Culicidae) by the native gel electrophoresis method and additionally cloned from cDNA to obtain full-length sequence (Ishida

et al. 2002). It was identified in a subset of trichoid sensilla including one type responding to

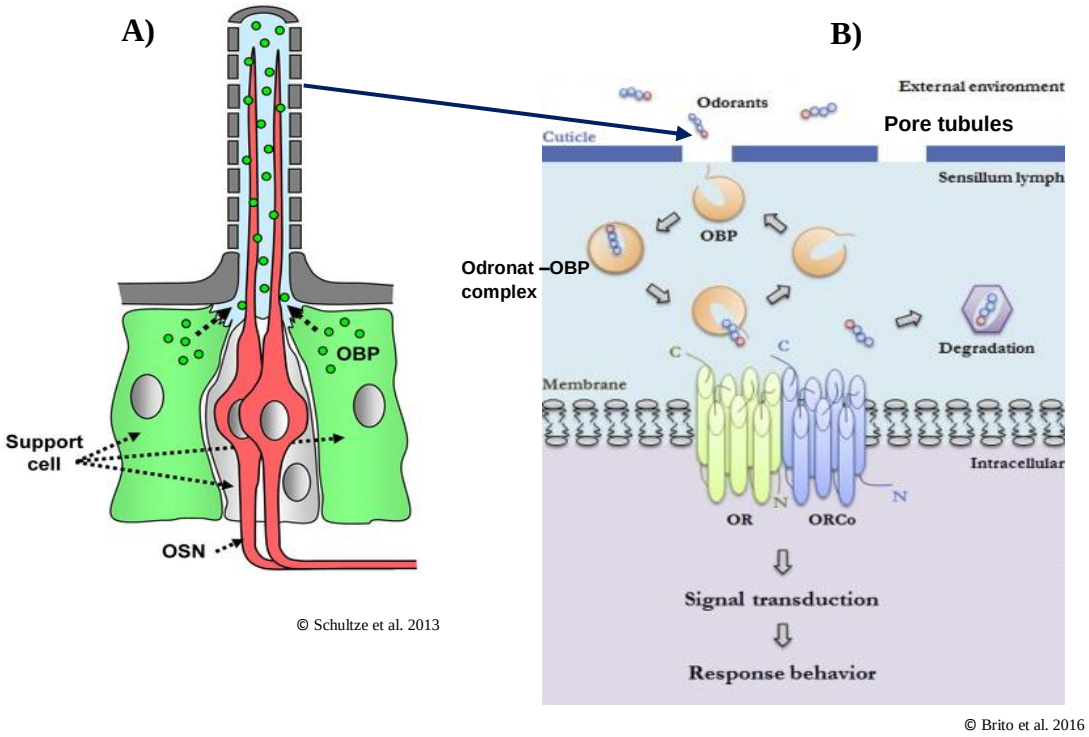

Figure 1-1. Graphical representation of the odorant perception process in insects. **A**) Graphical view of a trichoid sensillum hair: two olfactory sensory neurons (OSNs, red) project their dendrites into the sensillum lymph (blue). The cell bodies of the OSNs are surrounded by three support cells, two of which express "classic" odorant binding proteins (OBPs, dark green) and secret them into the sensillum lymph (after Schultze et al. 2013); **B**) Schematic view of the mechanism of insect olfaction: odorant reach the sensillar lymph through pore tubules and bind to odorant-binding proteins (OBPs). The odorant-OBP complex is transported through the sensillar lymph and activates receptors (OR) while bound odorant receptor co-receptor (OrCo) or it releases the ligand that directly activates receptors (OR) and that leads further processing for signal transduction and response behavior (after Brito et al. 2016)

mosquito oviposition pheromone (MOP) but not in basiconic sensilla on the maxillary palp and in the grooved peg sensilla of *Cx. quinquefasciatus* (Leal et al. 2008). Later research work led to the find and cloning of OBPs from other mosquito species such as AgamOBP1 from *Anopheles gambiae* Giles (Diptera: Culicidae) (Biessmann et al. 2002; Leal et al. 2008) and AaegOBP1 from *Aedes aegypti* L. (Diptera: Culicidae) (Ishida et al. 2004; Leite et al. 2009). These two proteins from different mosquito species were crystallized as a dimer in an asymmetric unit and their structural character is studied. This kind of study allowed using the full sequenced genome of several insect

species to the identification of large multigenic families of "classic -OBPs", which is structurally similar to other group of insect OBPs. To date, 33 classic OBPs from *A. gambiae*, 34 classic OBPs from *A. aegypti*, 55 classic OBPs from *Cx. quinquefasciatus*, and 20 classic OBPs from *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) have been identified (Zhou et al. 2008; Pelletier and Leal, 2009; Dippel et al. 2014).

The olfactory functional roles of insect odorant binding proteins have been studied by using an OBP from *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) called LUSH. It is evidenced that the deletion of LUSH gene suppresses electrophysiological and behavioral response to the male pheromone 11-*cis*-vaccenyl acetate (cVA) (Xu et al. 2005). Another study showed that octanoic acid and hexanoic acid originating from the host plant *Morinda citrifolia* L. (Gentianales: Rubiaceae), act as oviposition attractants for *Drosophila sechellia* Tsacas & Baechli (Diptera: Drosophilidae) but as repellents for *D. melanogaster*. This result was obtained by deleting OBP57d and OBP57e genes in *D. melanogaster* which lead to eliminate the avoidance behavior, while reinserting the orthologous genes of *D. sechiella* into *D. melanogaster* results in attraction to these fatty acids (Matsuo et al. 2007). Similarly, RNAi mediated gene silencing of protein AgamOBP1 of mosquito *A. gambiae,* do not recognize the oviposition attractant indole and without this protein the mosquito antennal receptor cells do not elicit electroantennography (EAG) response to this compound (Biessmann et al. 2010). The "empty neuron" system of *D. melanogaster* mutants was used with the silk moth *Bombyx mori* L. (Lepidoptera: Bombycidae) pheromone binding protein (BmorPBP1) and pheromone receptor BmorOR1 to investigate the response of sex pheromone bombykol. Both BmorPBP1 and BmorOR1 carrying flies showed significantly higher electrophysiological responses than flies having BmorOR1 only (Syed et al. 2006).

1.2.2. Chemosensory proteins

CSPs are members of another family of small sized (10-15kDa) soluble proteins in insect's olfactory system. The first chemosensory proteins were purified from the chemosensory organs antennae, tarsi and labrum of the desert locust *Schistocerca gregaria* Forsskål (Orthoptera: Acrididae) (Angeli et al. 1999). Several CSP members are highly expressed in the chemosensory sensillum lymph and exhibit binding activity towards odorant molecules or pheromones (Pelosi et al. 2005). However, not all of CSPs are expressed in chemoreception organs and this protein do not share their sequence similarity with insect OBPs and has only 4 conserved cysteins. The dynamic

role of CSPs in insect chemical communication clearly indicates that these polypeptides are involved as a second class of OBPs (Nagnan-Le Meillour et al. 2000; Calvello et al. 2005; Xu et al. 2009). A recent study identified a specific chemosensory protein Bdor-CSP2 from *Bactrocera dorsalis* Hendel (Diptera: Tephritidae) to be highly involved in the reception of certain host volatile compounds (Yi et al. 2014). Using RNA interference technique to silence the Bdor-CSP2 protein showed lower electrophysiological responses to (3*Z*)-hex-3-en-1-ol, *trans*-2-hexenal, 6-methylhept-5-en-2-one, and 3-methylbutyl acetate from dsBdor CSP2 treated flies compared to untreated control flies (Yi et al. 2014).

1.2.3. Odorant Receptors

The function of odorant receptors is to recognize biologically meaningful chemical ligands and shape responses of olfactory sensory neurons (OSNs) to regulate different behaviors (Figure 1-1). The first odorant receptor gene was discovered in the rat by Linda Buck and Richard Axel in 1991, who won the Nobel Prize for this discovery in 2004 (Buck and Axel, 1991). In invertebrate olfactory system: odorant receptor neurons (ORNs), odorant receptor (OR) genes, and G-protein coupled receptors (GPCRs; a large family of receptors ~ 60 multigenes), is responsible for the first step of olfactory signal transduction. The first OR genes were identified in *D. melanogaster* in 1999 and they belong to a distinct gene family encoding heteromeric ligand-gated ion channels comprised of a variable sensing component including an obligatory co-receptor called Orco (Neuhaus et al. 2005; Nakagawa et al. 2005; Lundin et al. 2007; Smart et al. 2008; Sato et al. 2008). A completed *D. melanogaster* genome sequence led to initial identification of members of the OR gene family and found 60 OR genes that encode for 62 OR proteins analyzed by alternative splicing techniques (Clyne et al. 1999; Vosshall et al. 1999; Robertson et al. 2003). Later, a family of 79 OR genes in the *A. gambiae* genome, 126 OR genes in the *A. aegypti* genome, 158 putative OR genes in the *Cx. quinquefasciatus g*enome, around 170 ORs in the honey bee *Apis mellifera* L. (Hymenoptera: Apidae) and a substantially greater number of 341 OR genes in *T. castaneum* were identified (Hill et al. 2002; Robertson and Wanner, 2006; Bohbot et al. 2007; Engsontia et al. 2008; Pelletier et al. 2010).

To functionally characterize odorant receptors several methods have been implemented. OR43a from *D. melanogaster* was the first insect odorant receptors functionally characterized. An experiment demonstrated that overexpression of OR43b in the insect antenna, or heterologous

expression in *Xenopus laevis* Daudin (Anura: Pipidae) oocytes (Stortkuhl and Kettler, 2001) led to identification of the ligands: benzaldehyde, cyclohexanone, cyclohexanol, and benzyl alcohol. Another sophisticated method called "empty neuron" system developed in *Drosophila* successfully paved the path in an *in vivo* expression system to study odorant receptors from other insects. For example, the deletion of OR22a and OR22b receptor genes present in the ab3A antennal neuron could not detect any odor molecules in the empty neuron test. Two OR genes from *A. gambiae*, namely AgamOR1 and AgamOR2 were expressed and studied by using this system: AgamOR1 was able to detect 1-methylphenol while AgamOR2 could detect 4-methylphenol from human sweat (Hallem et al. 2004). The co-expression pattern of odorant receptors with Orco (Odorant receptors co-receptors) has been well documented in the silk moth *B. mori*. BmOR1 is co-expressed with BmOR2 (Orco) to detect their sex pheromone bombykol, likely the combination of BmOR3-BmOR2 elicits a response to bombykal in vivo (Nakagawa et al. 2005). A recent study from *D. melanogaster* found the highly conserved olfactory circuit receptor Or55a specifically detects the toxic compound geosmin produced by harmful microbes (Stensmyr et al. 2012).

1.3. Insect olfactory components are molecular targets for pest control

In insects, olfactory components such as OBPs, ORs, ODEs, and CSPs contribute to the sensitivity and selectivity of the insect olfactory response. Hence these olfactory components are the potential molecular target for the development of new environmentally-friendly insecticides (Venthur and Zhou, 2018). The OBP, One among the insect olfactory component plays a significant role in the transportation of odor ligand from insect sensillum lymph to respective OR. Hence, specific insect OBP can be utilized by molecular tool like fluorescence competitive binding assay for wide screening of volatile organic compounds to find out high affinity odor ligand (Xu et al. 2010; Qiao et al. 2011; Deng et al. 2013; Wang et al. 2013). In fluorescence competitive binding assays, N-phenyl-1-naphthylamine (1-NPN) was used as a reporter ligand to monitor fluorescence intensity when add OBPs, thus help to find high affinity ligand, based on low fluorescence intensity (Wang et al. 2013). For example, the CquiOBP1 of mosquito *Cx. quinquefasciatus* was used as a molecular target *in vitro* binding assays to identify high binding affinity odor ligand as trimethylamine (TMA), nonanal and skatole. These compounds role in *Cx. quinquefasciatus* olfaction has been confirmed by using gas chromatography-electroantennographic detection (GC-EAD) and along with field bioassays (Leal et al. 2008). Similarly, ligand binding properties of HoblOBP3 and HoblOBP4 of

scarab beetle *Holotrichia oblita* Faldermann (Coleoptera: Melolonthidae) in 42 ligands were measured in competitive binding assays found that HoblOBP4 show high affinity to 1-hexenol, (*E*)-2-hexenal, butyl hexanoate, hexyl hexanoate and cinnamaldehyde, while HoblOBP3 show more specific binding affinity to α-ionone and β-ionone (Wang et al. 2013). Likewise, the functional role of OBP7 of the parasitoid wasp *Sclerodermus* sp. (Hymenoptera: Bethylidae) (SspOBP7) was used to screen 19 chemicals, to find out behaviorally active compounds (Yi et al. 2018). In this study, the authors identified only 6 compounds (terpinolene, (+)-α-longipinene, (-)-limonene, trans-2-hexen-1-ol, cis-2-penten-1-ol, decanal) which bind to SspOBP7 in a fluorescence quenching binding assays. Subsequent behavioral experiments confirmed significant preference for 2 compounds: (+)-α-longipinene and terpinolene that had a good binding affinity with SspOBP7 (Yi et al. 2018). This kind of study is called the reverse chemical ecology approach, and helps to understand the insect olfactory mechanisms and can lead to the discovery of active semiochemicals that could be used to manipulate insect behaviors for pest management (Leal, 2017). Similarly, the discovery of other molecular component like ORs have more sensitive targets for such reverse chemical ecology (Wang et al. 2016). For instance, an OR36 from mosquito *Cx. quinquefasciatus* (CquiOR36) was functionally characterized through heterologous expression in *X. laevis* oocytes and the response to 230 odorants was tested (Choo et al. 2018). The results indicate that CquiOR36 is highly sensitive to acetaldehyde and this result was further confirmed by electroantennogram recordings from antennae of fruit flies engineered to carry CquiOR36. In recent decades, RNA interference (RNAi) technique has been used to silence the expression of olfactory protein genes in the antenna of insects to influence the selectivity and sensitivity of host volatile compounds.

1.3.1. RNA interference as a molecular tool

RNA interference (RNAi) is an effective molecular tool used to study the function of genes in insects and plants. The first RNAi (posttranscriptional gene silencing) experiment was demonstrated in petunia plants to silence the expression of chalcone synthase (CHS) gene by introduction of a CHS transgene (Napoli et al. 1990).

It has also been used in different fields of research, such as developmental biology, cellular biology, evolutionary biology and functional genomics as an efficient molecular technique to gain loss of function phenotypes (Bucher et al. 2002; Tomoyasu and Denell, 2004; Grunwald et al. 2013). Initial attempt made in nematode *Caenorhabditis elegans* Maupas Rhabditida: Rhabditidae)

discovered that the RNAi effect could be seen throughout the organism regardless of the dsRNA injected site (Fire et al. 1998). The fruit fly *D. melanogaster* is one of the best known genetic models and the first RNAi experiment on this insect was demonstrated by Hammond et al. (2000). The authors report that loss of gene functions can be created in cultured *Drosophila* S2 cells by introduction with specific double-stranded RNAs. The systemic RNAi can be used to analyze the olfactory functional role of OBPs in insect olfaction in many different group of insects: including mosquitoes *A. gambiae*, *A. aegypti*, *Cx. Pipiens* L. (Diptera: Culicidae) , the moths *B. mori*, *Manduca sexta* L. (Lepidoptera: Sphingidae), the honey bee *A. mellifera*, the ladybird beetles *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae), the scarab beetle *Protaetia brevitarsis* Lewis (Coleoptera: Scarabaeidae), the leaf beetle *Diabrotica virgifera* LeConte (Coleoptera: Chrysomelidae) and the stored product pest *T. castaneum* (Bucher et al. 2002; Blandin et al. 2002; Zhu et al. 2003; Amdam et al. 2003; Tabunoki et al. 2004; Tomoyasu and Denell, 2004; Niimi et al. 2005; Baum et al. 2007; Eleftherianos et al. 2007; Sim and Denlinger, 2008; Kim et al. 2008). Most of the RNAi studies in insect olfaction is performed by injection of dsRNA directly into the cell rather than feeding bioassays. For example, *T. castaneum* beetles were injected with TcOr1 dsRNA at their pupal stage showed no significant antennal response to their aggregation pheromone 4,8-dimethyldecanal (DMD), supporting that TcOr1 plays a significant role in pheromone reception (Engsontia et al. 2008). Likewise, the EAG responses of dsAlinOBP4-injected *Adelphocoris lineolatus* Goeze (Hemiptera: Miridae) to butyl butanoate, 1-hexyl butyrate, (*E*)-2-hexenyl butyrate and hexyl hexanoate were significantly decreased (Zhang et al. 2017). However, delivery of dsRNA is also possible through feeding, suggesting a feasible insect pest control technique (Singh et al. 2013). Thus enhance the RNAi technique will be more useful in the field of integrated pest management approaches, either to defect the insect olfaction system with respect to deviate from the food sources or make them unresponsive to the habitat volatile compounds.

1.4. Coleoptera

The insect order Coleoptera is the most diverse insect group on earth and beetles are belong to this largest order of insects representing approximately 40% of all known insect species. The over 380,000 described Coleopterans, exhibit extraordinary morphological and ecological diversity and play important roles in terrestrial and freshwater ecosystems (Crowson, 1981; Ślipiński et al. 2011; Gressitt, 2018). Coleopteran insects attract attention for many different reasons, including their

economic importance as herbivores, grain feeders, predators, fungivores, and carnivores. About 75% of beetle species are plant feeders in both larval and adult stages. Most of them damage on economically important crop plants and stored food grain products (Gilliott, 1995). The majority of plant feeders belong to the beetle families Curculionidae, Chrysomelidae, Cerambycidae, Buprestidae, and Tenebrionidae, whereas predators are often found amongst Coccinellidae, Carabidae, Staphylinidae, Cleridae, and Bothrideridae.

1.5. Stored product insect pests

For the future use of humankind, primary products of cereal grains and its secondary products are often stored in a man-made environment, to prevent qualitative and quantitative deteriorations as a results from biotic and abiotic factors. This kind of food grain storage practice have been adopted and followed more than 6,000 years ago when humans began storing food grains in small pits, mud houses, and wooden enclosures to avoid food famine (Pimentel, 1991; Reed, 1992; Levinson and Levinson, 1994). Subsequently then different storing methods have been implemented depending on the food grain products. These stored food products are used personally and are supplied to the consumer market all over the year. Biotic factors including insects, mites, fungi and vertebrate pests like rodents probably cause 5-10% of commodity losses worldwide, with this number likely to be higher in tropical regions (Mondal and Port, 1994; Siddiqi, 2007). There are 26 families of stored product insect pests identified worldwide and original habitats of most of these insects include the underside of tree bark, seeds, leaf litter, fungi, mold, carrion and nests (Good, 1936; Rees, 2004). These insects became cosmopolitan due to increased commerce and were spread throughout the world by different modes of trading (Good, 1936).

Pests of food grain and its by-product are either primary pests, insects that can damage whole seeds, or secondary pests, insects that can only feed on damaged grains and seeds. Insects feeding on fungal growth on food grain is usually a supplemental type of feeding, providing insects with nutrients that are absent from the infested commodity. However, some species complete their life cycle on mold and cannot survive on grain alone. Storage pests reduce the marketability and value of the bulk grain by lowering the food quality directly through larval and adult feeding, or indirectly by contamination from larval frass, exuviae, adult body parts, and compounds secreted by insects (Mason, 2003). Contamination of processed and value-added food products through

insect byproducts such as frass, defensive secretions, etc. may cause allergic reactions, unpleasant odor and off-coloration of food (Scott, 1991; Olsen et al. 2001).

Based on their feeding habit, stored product insects are classified as either internal feeders - feeding inside of the grain, or external feeders - feeding on the surface of the grain, on broken kernels, and on processed products such as flour. Internal feeders include: rice weevils *Sitophilus oryzae* L., maize weevil *S. zeamais* Motschulsky, granary weevil *S. granarius* L. (Coleoptera: Curculionidae), stored-product bostrichid *Callosobruchus phaseoli* Gyllenhal, *C. analis* F., *C. maculatus* F., *C. chinensis* L. (Coleoptera: Bruchidae), the Angoumois grain moth, *Sitotroga cereallela* Oliv. (Lepidoptera: Geleichiidae), dried bean beetle *Acanthoscelides obtectus* Say (Coleoptera: Chrysomelidae), groundnut borer *Caryedon serratus* Oliv. (Coleoptera: Chrysomelidae), and rice moth *Corcyra cephalonica* Stainton (Lepidoptera: Pyralidae). External food grain feeders include the majority of storage pests, such as the flour beetles *T. castaneum* Herbst , *T. confusam* Jacquelin du Val (Coleoptera: Tenebrionidae), indian meal moth *Plodia interpunctella* Hübner (Lepidoptera: Pyralidae), saw-toothed grain beetle *Oryzaephilus surinamensis* L. (Coleoptera: Silvanidae), warehouse beetle *Trogoderma granarium* Everts (Coleoptera: Dermestidae), cigarette beetle *Lasioderma serricorne* F. (Coleopter: Ptinidae) and long headed flour beetle *Latheticus oryzae* Waterhouse (Coleoptera: Tenebrionidae) (Hagstrum and Subramanyam, 2006).

1.5.1. Control of stored product insect pests

The control or management of storage pests is relatively difficult compare to the control of crop pests, because chemical residues need to be avoided in or on food stuffs in order to get food authorization certificate. There are numerous chemical and physical methods of storage pests control strategies available. Chemical methods are the primary method of insect control and are relatively fast-acting, have broad spectrums of activity and are economically feasible. For example, methyl bromide is an important fumigant and commonly used to control insects in storage conditions. Because of its chemical properties as active ozone-depleter, using methyl bromide was banned in most countries around the world in the late 1980s. Finding new alternative fumigants for methyl bromide is difficult because of the cost involved in registration (Bell et al. 1996). Present days, there are only two fumigants such as phosphine and sulfuryl fluoride available on the market and considered viable methyl bromide alternatives for stored product pest management.

A study reported that only 50% of *T. castaneum* adults die within 72 hours of exposure to phosphine gas (at 28°C) but adding 20% CO_2 leads to 100% mortality within the same time frame (El-Lakwah et al. 1989). The fumigation of sulfuryl fluoride causes 100% mortality for larval, pupal and adult stages of *T. castaneum* but noticed 99.3% survival of *T. castaneum* eggs (Tsai et. al. 2006). In contrast, methyl bromide fumigation kills 100 % of egg, larval and adult stages, with over 99.6% of pupae dying as well (Tsai et. al. 2006). Another group of insecticide organophosphates (OP), for instance pirimiphos-methyl was introduced to the market in the 1960s (Snelson, 1987). It acts quickly on a variety of pest insects, also on mites (Wilkin and Hope, 1973), and is effective against many strains of *T. castaneum* that are resistant to both lindane and malathion (Pieterse et al. 1972).

Use of non-chemical methods such as biological control, physical control and bio-pesticides are also widely adopted against storage pest. For example, in a physical method insects are exposed to heat conditions, it showed that young *T. castaneum* larvae and pupae are more tolerant of heat than adults, late instars and eggs (Mahroof et al. 2003; Mahroof et al. 2005). This heat tolerance occurs due to presence of heat shock proteins, which helps to protect an insect's biological system against negative effects of excess heat (Chapman, 1998). A recent study revealed that the heat shock protein 70 (HSP70) levels increased in young *T. castaneum* larvae upon exposure to 40°C while eggs have reduced levels and other stages showed no differences (Mahroof et al. 2005). Likewise, different insect species show great differences in susceptibility to cold. For instance, *T. castaneum* and *T. confusum* are the most susceptible to cold while *P. interpunctella* is a relatively cold-tolerant species among storage pests (Fields, 1992). However, chemical and non-chemical methods of control against storage pest are sometimes economically not feasible and additionally enhance insecticide resistance strains in different group of insects. Therefore, we need an effective control or management strategies as eco-friendly, to protect stored food products for several years from vertebrate and invertebrate pests.

1.5.2. Stored product pest and model organism *T. castaneum* (Herbst)

The information of insect olfaction has prominent value in designing strategies for monitoring, managing, and control harmful insect species in stored food products. In this study, the olfactory response of red flour beetle *Tribolium castaneum* Herbst, 1797 (Coleoptera: Tenebrionidae) to a

broad spectrum of volatile compounds and their functional role in olfaction have been investigated. Results from this study would give details about the chemical perception of *T. castaneum* and that could be useful in developing semiochemical-based IPM strategies for controlling stored product pests. Furthermore, understanding the olfactory responses in the model organism like *T. castaneum* will enhance our idea of the olfaction in beetles in general, and additional amount of research may lead to the development of new pest control strategies for other coleopteran pests.

Adult red flour beetle *T. castaneum* is reddish-brown in color and 3 mm in size. Female beetles are able to lays up to 18 eggs daily into the feeding substrate, usually whitish and of oval shape. Average fecundity is 1200 eggs per female and it varies depending on the feeding substrate in different environment. Eggs hatch within 3 to 5 days. The larvae are fairly active but generally hide within food and avoid light (Cotton, 1963). The larvae pupate freely after 4 molts in the substrate. To complete life cycle from egg to adult, beetles required about 93 days at 22°C. Though adult beetles possesses fully developed wings, the beetles do not fly at temperatures below 25°C. However, sometimes beetles show a tendency to fly when temperature increases in the storage conditions but there is no information about perfect flying condition requirements. As a stored product pest, the beetles do not survive at low temperatures, habitually they are protected against quick cooling or heating by insulation of their feeding substrate. The beetles' required temperature for development is ranged between 22-40°C with an optimum at 32-37°C and the relative humidity ranged 10% - 95% with an optimum at 70 -90% (White et al. 1995; Reichmuth et al. 2007).

T. castaneum have been reported as stored product pest from grain and other cereal products, beans, cotton seed, shelled nuts, dried fruits, dried vegetables, drugs, chocolates and milk powder (Rees, 2004). There are some studies suggesting that the ancestral *Tribolium* habitats may have included decomposing wood and extant species have been found under bark feeding on decaying organic matter, fungi and bacteria (Good, 1936 and reference therein; Levinson and Levinson, 1985). It is believed that several thousand years ago there was a great famine and drought in the natural habitats of this beetle, which forced them to change their habitat into dried environment where in ancient times food products were stored (Levinson and Levinson, 1985; Levinson and Levinson, 1994). Among the tenebrionids, *T. castaneum* is an important and very destructive pest of stored products, since it is feeding on an extensive number of food products. Infested flour or food products turn pink-colored and pick up a specific unpleasant odor and bitter taste resulting

from the quinone secretion of the beetles (Alexander and Barton, 1943). These defensive compounds are highly volatile and have communication properties which lead to quality changes of products, consequently cause higher losses than direct feeding (Villaverde et al. 2007).

The beetles have defensive glands which secret quinone compounds such as 2-methyl-1,4-benzoquinone (MBQ), 2-ethyl-1,4-benzoquinone (EBQ) and hydroquinone (Howard, 1987). These defensive secretions are act as inhibitors of fungal growth of several microorganisms and also involve in cause of cancer (Yezerski et al. 2005, 2007; Lis et al. 2011). For example, biscuits made of *Tribolium* contaminated flour as well as of 1,4- benzoquinone contaminated flour fed to Swiss albino mice caused approximately 35 % animals to develop hepatocellular carcinoma and lympholeukemia (El-Mofty et al. 1992). Further studies from different researchers found that these quinone compounds are released together with some unsaturated hydrocarbons, the most prominent being 1-pentadecene, which may work as solvent and surfactant for the poisonous quinones (Suzuki et al. 1988; Verheggen et al. 2007). A male-specific aggregation pheromone, 4, 8-dimethyldecanal (DMD), was first identified by Suzuki (1980), which is used in pheromone trapping system and until now only DMD is used to monitor red flour beetles in stored product facilities (Hussain et al. 1994).

1.5.3. Olfactory system of *T. castaneum*

The insect's antenna possesses three primary segments called the scape, pedicel and the flagellum. In *Tribolium*, antennae and maxillary palp are the major olfactory organs (Dippel et al. 2016). The capitate antennal type of Tenebrionid beetles consists of 11 segments and depending on the species, the antenna may broaden gradually distally, or some segments may be enlarged to give a 3-7 segmented antennal club (Roth and Willis, 1951). In case of the *T. castaneum* antenna, the distal three articles are enlarged into a club shaped structure and have morphologically differentiated from other species of the same group (Figure 1-2) (Herbst, 1797; Roth and Willis, 1951). Based on the shape, size and structures, the olfactory sensilla are divided into sensilla basiconica, sensilla trichodea (chemo and mechano function), sensilla coeloconica, sensilla campaniformes, sensilla chaetica and spines or spatulate bristles (Figure 1-3) (Roth and Willis, 1951; Dippel et al. 2016). Under the microscope, wall pores may be observed on the sensillary cuticle of the sensillae through which the airborne odorant molecules can enter into the sensilla to activate the respective receptors (Slifer, 1961).

Figure 1-2. Adult *Tribolium castaneum*

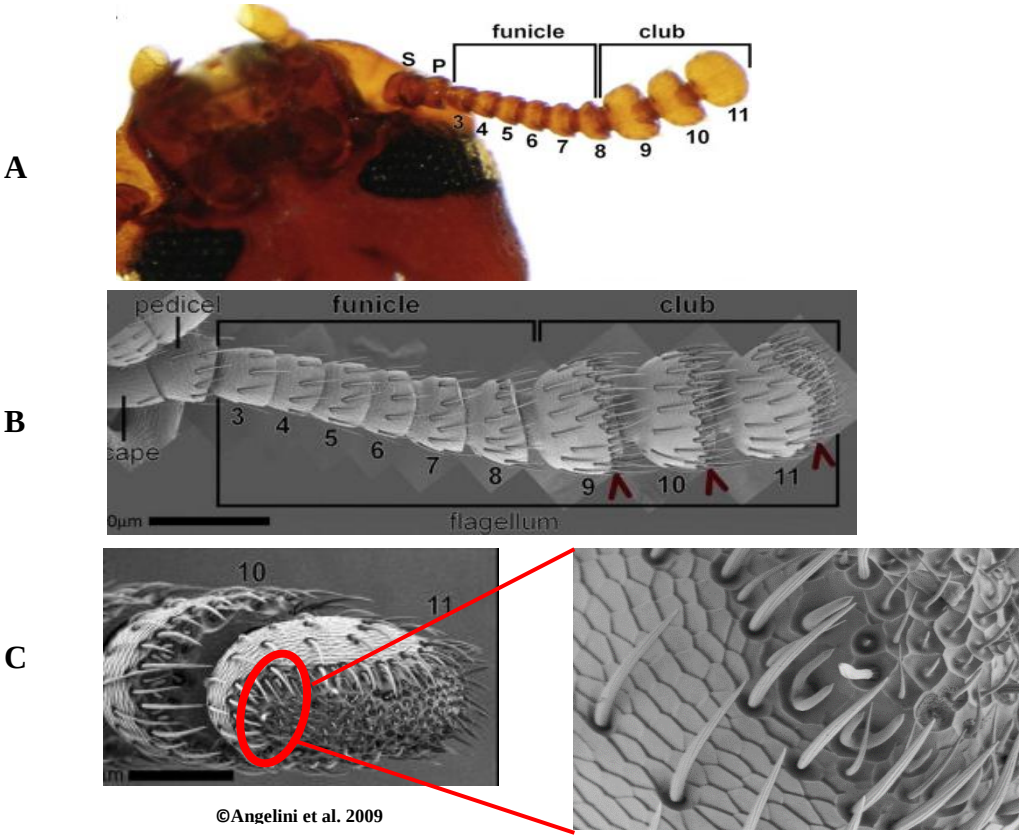

Figure 1-3. Morphology of the adult *Tribolium castaneum* antenna. A) *Tribolium castaneum* antenna; **B**) Scanning electron micrographs (SEM) of the antenna of a *T. castaneum*; **C**) sensilla distribution on the last segments of *T. castaneum* antenna (after Angelini et al. 2009)

Each olfactory sensillum harbors ORNs, whose dendrites are housed in an aqueous fluid called sensillum lymph. The sensillum lymph forms a hydrophilic barrier for the hydrophobic airborne stimuli that reach the ORNs, which translate chemical stimuli into electrical signals, which are send through the antennal lobe to reach the brain. Recent studies reported that there are about 230 olfactory sensilla, housing approximately 600 ORNs in *T. castaneum* antenna, projecting into roughly 70 glomeruli (Dreyer et al. 2010; Dippel et al. 2016). Gene annotation and other molecular analyzed data revealed that 50 OBPs, 20 CSPs, 338 ORs, 207 gustatory receptors (GRs), 23 ionotropic glutamate-like receptors (IRs) and 7 sensory neuron membrane proteins (SNMPs) are housed in the red flour beetle olfaction system (Engsontia et al. 2008; Richards et al. 2008; Nichols and Vogt, 2008; Croset et al. 2010; Vieira and Rozas, 2011; Dippel et al. 2016).

1.6. Forest insect pests

Forests are complex ecosystems providing shelter to a diversity of insect groups such as herbivores, fungivores and carnivores. Among them, herbivorous insects are causing extensive damage to important tree species and consequently lead to loss of several billions of dollars each year. These herbivorous insects include many insect species from Lepidoptera, Coleoptera and Hemiptera. Besides other order of insects, Coleopteran insects are considered as major contributors to economic losses. For instance, the Cerambycids *Monochamus alternatus* Hope 1843, *Anoplophora glabripennis* Motschulsky 1853, *Massicus raddei* Blessig and Solsky 1872, *Apriona germari* Hope 1831, *A. swainsoni* (Hope,1840), and *Batocera horsfieldi* (Hope, 1839) (Coleoptera: Cerambycidae), are devastating the hardwood forests in many parts of the world (Mamiya and Enda, 1972; Gao and Li, 2001; Wei et al. 2009) . Control of these highly dangerous pests in terms of chemical or physical methods is almost not feasible due to larger area of attack and outbreak habit. Therefore it is essential to find different strategies to control these problematic pest beetles. For example through a biological control agent like the predatory beetle *D. helophoroides*.

1.6.1. Coleopteran predator *D. helophoroides* for forest insect pests

The second part of this dissertation, the antennal and behavioral responses of a forest insects predator *Dastarcus helophoroides* Fairmaire, 1881 (Coleoptera: Bothrideridae) to their prey related volatile organic compounds have been assessed. Results of this study would provide the information of olfactory cues that would mediates interaction between predator *D. helophoroides* and their prey

cerambycid species. Scuh a antennally perceive volatiles of this predatory beetles could be useful in the development of the IPM strategy, especially to manipulate beetles behaviour and pull them into the forest stands damaged by different species of cerambycids.

The bothriderid beetle *D. helophoroides* is the natural enemy of many economically important insect forest pests, mostly from the family Cerambycidae. This predator mainly occurs in most provinces of China, some parts of Japan (Tadahisa, 2003), and in Korea (Qin and Gao, 1988; Wang et al. 1996; Lim et al. 2012). Their predatory niche includes *M. alternatus*, *A. glabripennis*, *M. raddei*, *A. germari*, *A. swainsoni*, and *B. horsfieldi* (Inoue, 1991; Ogura et al. 1999; Wei, 2007; Wei et al. 2009). Both larvae and adults are preying on the larvae, pupae, and young adults of numerous Cerambycids. However, the larvae feed intensively on immatures of the aforementioned pest beetles. Adult beetles, during most of their lifetime stay under the bark crevice or in the holes of trees, for attacking newly emerge Cerambycids. Nevertheless, there is no report demonstrating predation behavior of adult beetles under field conditions. After mating, adult females lay their eggs twice a year on the outer surface of the bark close to prey entrance holes, prey frass-extrusion holes and around the tunnel walls of prey larvae (Qin and Gao, 1988). Following hatching, the larvae enter into the host entrance hole and actively forage on the immature stage of Cerambycid beetles (Figure 1-4). Research evidence demonstrated that one late instar larva of Asian longhorned beetle *A. glabripennis* or deep mountain longhorn beetle *M. raddei* can support a complete development of 10-35 *D. helophoroides* larvae (Qin and Gao, 1988; Gao et al. 2003). Even so the biology of *D. helophoroides* is known, information about volatile semiochemicals that are used by this beetle to find their prey is still lacking.

Insect predators play a crucial role in agricultural, horticultural, forest ecosystems and are considered important bio-control agents for managing pest insect species. The effectiveness of a predator is based on the availability of the prey species, guiding odor cues, and how long it preying on one insect patch (Charnov, 1976). Predators search and find host prey with the help of visual cues or chemical cues released by its prey or the hosts of its prey. The most reliable cues for the predators would originate primarily from the prey themselves and secondarily from prey damaged host plants (Jeffries and Lawton, 1984; Sullivan et al. 2000; Chuche et al. 2006). Some predatory insects orient towards plant semiochemicals released at short or long distance upon feeding by

herbivores (Moraes et al. 1998; Turlings and Ton, 2006). These volatile compounds, which may include terpenoids, phenolics, ketones and alkaloids, may serve as a part of the plant defense mechanisms by attracting natural enemies during pest attacks. For instance, trapping studies in the forest field demonstrate that the clerid predatory beetle *Thanasimus dubius* is significantly attracted to the traps contain α-pinene, plant-based kairomone for their prey *Dendroctonus frontalis* (Staeben et al. 2015). Similarly, in laboratory olfatormeter tests, the same predatory beetles *T. dubius* responded to frontalin, ipsdienol, (pheromone components of bark beetles) and α -pinene (kairomone for several bark beetle species) in a dose dependent manner (Mizell et al. 1984).

1.6.2. Olfactory system of *D. helophoroides*

The *D. helophoroides* possess no sexual dimorphism in terms of their antennal morphology like sensilla classification or the abundance and distribution of sensilla (Ren et al. 2012). The antennae of Bothrideridae beetles consist of 9 to 11 segments and are usually club-shaped at the tips (Slipiński et al. 2010). However, the predatory beetle *D. helophoroides* antenna, the distal three articles end in an abrupt, rounded knob and form a capitate shaped structure and are morphologically differentiated from other species of the same group. The antennal sensilla of both sexes of *D. helophoroides* are similar and divided into eight different types, based on their morphological shape and structures, (Ren et al. 2012). Among eight types of sensilla, three types are sensilla chaetica, two types are sensilla trichodea, two types are sensilla basiconica and a Bohm'm bristles (Figure 1-5) (Ren et al. 2012). In a scanning electron microscopy (SEM) study on sensilla chaetica no dentritic branches were found (Ren et al. 2012) and concluding that these type of sensilla in *D. helophoroides* might have protective function to other chaemosensilla as in the pine weevil *Hylobius abietis* L.(Coleoptera: Curculionidae), plum curculio *Conotrachelus nenuphar* Herbst (Coleoptera: Curculionidae) (Mustaparta, 1973; Alm and Hall, 1986).

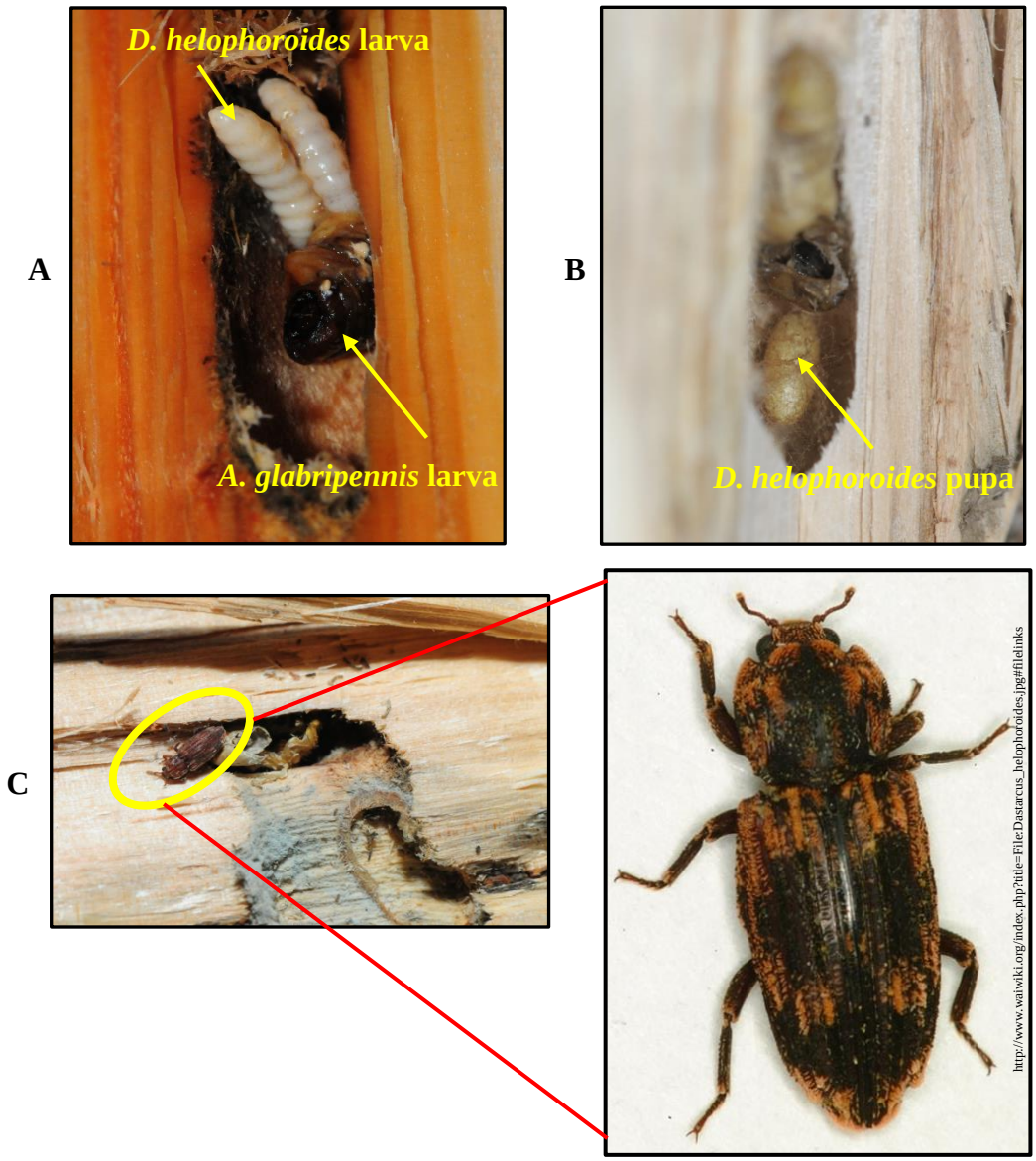

Figure 1-4. Predation of *Dastarcus helophoroides* .**A**) Larvae of *Dastarcus helophoroides* feeding on larva of *Anoplophora glabripennis;* **B**) Pupal cocoon of *D. helophoroides* in the larval tunnel of *A. glabripennis*; **C**) Newly emerged adult *D. helophoroides* from the cocoon that stays in the tunnel of *A. glabripennis* larva (with courtesy of Dr. Lili Li Ren)

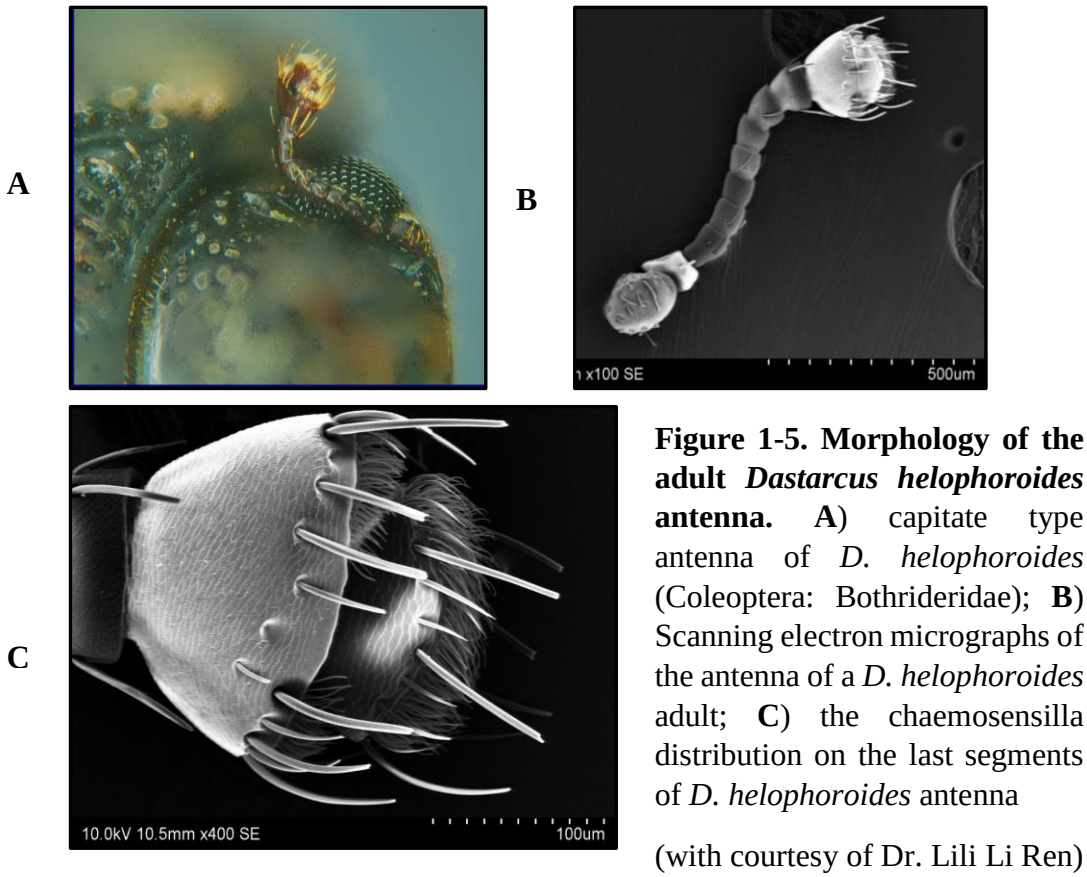

Figure 1-5. Morphology of the adult *Dastarcus helophoroides* antenna. **A**) capitate type antenna of *D. helophoroides* (Coleoptera: Bothrideridae); **B**) Scanning electron micrographs of the antenna of a *D. helophoroides* adult; **C**) the chaemosensilla distribution on the last segments of *D. helophoroides* antenna

(with courtesy of Dr. Lili Li Ren)

A recent molecular study from Wang et al. (2014) reported that the *D. helophoroides* possesses 52 OBPs, 19 CSPs,10 ORs, 8 IRs, 2 gustatory receptors (GRs), and 5 sensory neuron membrane proteins (SNMPs), suggesting a differentiated role in olfaction.

1.7. Electrophysiological recording of insects

A commonly used instrument in chemical ecology field is electroantennogram (EAG), which helps to measure the volatile perception of insect antenna by generating an electrical signal. It produces a bulk measure of the responses of the electrical depolarisations of many olfactory receptor neurons, when the insect antenna is exposed to an adequate stimulus. This equipment was developed based on the innovation discovered by the German biologist Dietrich Schneider (1957). He recorded small voltage fluctuations between tip and base of a male silk moth, *B. mori* antenna during stimulation with pheromones. Schneider has used freshly excised antenna and stimulated it with an air puff containing the sex pheromone "bombykol". The response to stimuli is mainly associated with odorant receptor cells present in the sensilla of insect antenna (Mayer et al. 1984). Another model as a portable EAG recording system was first described by Koch (1990) and used to measure the

pheromone concentration in the field (Sauer et al. 1990; 1992). Recent findings reported that the response of stimulus produced from receptor cells and the change in the EAG potential are interdependent measures of the stimulus strength (Mayer 2001; Thakeow et al. 2008; Drilling and Dettner, 2009). The EAG recording with *Sulcacis affinis* Gyllenhal (Coleoptera: Ciidae) to 1-octen-3-ol showed that significant perceptional differences can exist between male and female insects (Drilling and Dettner, 2009).

There are two kind of antennal preparation known for EAG recording. The first one is that the antenna is excised from the insect and reference and recording electrodes are attached to the base and the tip of the antenna respectively (Thakeow et al. 2008). The second method is by inserting the reference electrode anywhere in an immobilized insect and the recording wire to the tip of the antenna (Park and Hardie, 1998; Park et al. 2002). The latter method has the advantage that the insect antenna remains alive for a longer time period than the excised antenna.

1.8. Objectives of this study

The main purpose of this PhD dissertation was to investigate the olfactory response of stored product pest *T. castaneum* and a forest insect predator *D. helophoroides*. To achieve this results the following specific objectives were carried out:

1. Olfactory detection of food grains VOCs by the stored product insect *T. castaneum* (Chapter 2).

2. Functional characterization of odorant binding proteins *TcasOBP9A* and *TcasOBP9B* from *T. castaneum* through RNAi method (Chapter 3).

3. Assessment of the *T. castaneum* OBPs *TcasOBP9A* and *TcasOBP9B* as sensing elements in an rGO-FET based electronic biosensor for the detection of disease markers (Chapter 4).

4. Olfactory detection and behavioral responses of *D. helophoroides* to prey related VOCs (Chapter 5).

Objectives achievement in detail: Despite the described importance of both above mentioned coleopterans on humankind, the exact information of the olfactory function of these beetles has not been elucidated yet. At the first part of this dissertation, I wanted to explore the olfactory function

of the *T. castaneum* to different VOCs from various stored food grains and also VOCs related to other storage pests, with special regards to influencing carbon chain lengths and vapor pressure of VOCs. The high expression of the OBPs *TcasOBP9A* and *TcasOBP9B* in the antennal tissue was confirmed by transcriptome analysis (Dippel et al. 2014). To functionally describe these proteins, I wanted to use the RNAi-treatment to interfere with the olfactory function of these OBPs genes. As a precondition for subsequent analysis, I aimed to determine the time window of the RNAi-mediated knock-down through the EAG recordings. In order to find *T. castaneum* specific odorants that need a given OBP for their detection, I wanted to perform loss of gene function studies through RNAi with EAG recordings.

To analyze the practical application of *TcasOBPs* in the modern 'bioelectronics nose', I wanted to develop an rGO-FET based portable biosensor device with OBP as a sensing element. Subsequently, the functionality of the biosensor device (*in-vitro*) was to be tested with the different concentrations of marker compounds and compared with *in-vivo* data.

At the second part of this dissertation, encouraged by the high degree of prey related odor cues plays an important role on predator olfactory system, I aimed to analyze the antennal responses of the *D. helophoroides* to its prey related VOCs. Subsequently, in order to confirm the chemotaxis of the perceived compound, I needed to perform the behavioral experiments with *D. helophoroides*.

To conclude, the olfactory analyses of the stored product insect pest *T. castaneum* and the forest pest predator *D. helophoroides* were performed to reveal possible perceived VOCs that play a crucial role for reaching their primary needs. In addition to these results with a number of advanced molecular techniques (RNAi), available molecular information (Dippel et al. 2014; Wang et al. 2014), completion of the whole genome sequence (Richards et al. 2008) will provide the potential future to develop semiochemicals, RNAi approach or rGO-FET biosensor based IPM strategy for controlling stored product pests and forest insect pests.

1.9. References

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CHAPTER 2

Electroantennographic Responses of Red Flour Beetle *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) to Volatile Organic Compounds

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Balakrishnan K : Developed original idea**,** improved and established EAG setup and performed all EAG experiments, analyzed the data, wrote original draft and corresponding author.

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2.1. Abstract

The red flour beetle *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) is the most encountered and destructive stored product pest of cereal grains and seeds. Electroantennogram responses were recorded from both sexes of *T. castaneum* to 94 selected volatile organic compounds including alkanes, alkenes, alcohols, aldehydes, ketones, carboxylic acids, esters, terpenoids and aromatic compounds. Overall, female and male *T. castaneum* exhibited similar EAG responses. Compounds eliciting the strongest EAG responses within their groups were undecane, 1-hexen-3-ol, octanal, 2-heptanone, hexanoic acid and ethyl hexanoate. Comparison of vapor pressure and EAG responses within homologous series of compounds revealed responses to undecane, octadecane, octanal, nonanal, 2-heptanone, hexanoic acid and octanoic acid as outstanding. Such are best candidates for evaluation in behavioral studies to unravel their potential for application in integrated pest management (IPM) strategies of *T. castaneum*.

Keywords: Electroantennography, host and non-host volatiles, *Tribolium castaneum*

2.2. Introduction

Arthropod pests cause considerable losses of about 10% p.a. of worldwide grain production ranging between 8% and 25% in developed countries and 70% and 75% in developing countries (Mason 2003). For example, monetary losses in the United States are estimated to amount to $3 billion p.a. (Siddiqi 2007). Among beetles, *Sitophilus granarius* Linnaeus, *S. oryzae* Linnaeus, *S. zeamais* Motschulsky (Curculionidae), *Callosobruchus maculatus* Fabricius (Chrysomelidae), *Oryzaephilus surinamensis* Linnaeus (Silvanidae), *Tribolium castaneum* Herbst and *T. confusum* Jacquelin Du Val (Tenebrionidae) are the most important storage insect pests. The red flour beetle *T. castaneum* feeds mainly on damaged or ground or milled or processed cereal grains, spices, nuts and seeds (Rees 2004). Additional losses are due to the secretion of malodorant compounds leading to discoloration of food grains and deterioration of the pleasant smell of food grain products. Beetle feeding activity also promotes the growth of fungi, which can either support beetle growth or be noxious by producing mycotoxins (Holighaus and Rohlfs 2016). Quinones act as a defensive secretion from *T. castaneum* like in most tenebrionids (Tschinkel 1975). They are also antimicrobials that might favour beetle survival by inhibition of mycotoxin producing fungi (Yezerski et al. 2007). In general, results from different approaches, including molecular physiology of *T. castaneum* olfaction, suggest a wide perceptional range to different volatiles acting as allomones, pheromones and kairomones (Trematerra et al. 2000; Jonfia-Essien et al. 2007; Engsontia et al. 2008; Richards et al. 2008; Arthur et al. 2011; Campbell 2012; Dippel et al. 2014).

The objective of our study was to explore the peripheral olfactory responses of *T. castaneum* to 94 volatile organic compounds. These compounds were selected based on present literature about electroantennography (EAG) activity, perception and behavioral responses of *Tribolium* species, closely related tenebrionids, and of other storage insect pests from the order Coleoptera (Table 2-1). We used these volatile compounds to record antennal detection of both sexes using EAG (Schneider 1962). EAG has been used widely for screening of volatiles and selecting efficiently compounds for further behavioral tests (Schütz et al. 1999; Weissbecker et al. 2004; Thakeow et al. 2008; Beck et al. 2012). *T. castaneum* is still the only beetle among model insects whose genome is fully annotated (Richards et al. 2008). In particular with such support, this study might pave the way for novel research about biological functions of compounds detected by *T. castaneum* and their respective olfactory components in the olfaction process, such as odorant binding proteins and

odorant receptor proteins, to finally optimize sustainable control strategies not only for *T. castaneum.*

2.3. Materials and Methods

2.3.1. Red Flour Beetle

Stock cultures of San Bernardino strain of *T. castaneum* were reared in transparent plastic boxes at 30°C, 40% relative humidity under a photoregime of light 12 h : dark 12 h. Adults were provided with 95% organic whole wheat flour, added with 5% brewer's yeast by weight basis. The beetles were separated into female and male as pupae, based on their genital organs, and 20 beetles each were maintained in boxes.

2.3.2. Volatile Compounds

A total of 94 volatile organic compounds were chosen based on an extensive literature search to represent a broad screening of potentially ecologically relevant odors to stored product insects and include chemical groups such as alkanes, alkenes, alcohols, aldehydes, ketones, carboxylic acids, esters, terpenoids and aromatic compounds (Table 2-2). All compounds were obtained from commercial suppliers (Table 2-2).

2.3.3. Antennal Preparation and EAG Recording

We developed an improved procedure for *T. castaneum* preparation for EAG. The adult beetles were starved for 24 h. For antennal preparation, a beetle was put into a plastic micropipette (200 µl) tip. We used slight air pressure to wedge the beetle into the tip, with the head turned forward. Then, the tip was cut at the small end, directly in front of the beetle and a second time approximately 2 mm behind the beetle. With the insertion of plasticine material into the back of the tip, the beetle was prevented from moving and forced to protrude the antenna out of the plastic tip along with part of the thorax, where the reference electrode would later be inserted. The beetle holding plastic tip was placed under a stereo microscope (MZ16, Leica, Wetzlar, Germany) and fixed with plasticine to the edge of a plastic slide that provides a platform to fix the antenna and apply the electrodes. Due to the toughness of the cuticle, a small hole was made in the thorax region of the beetle with an electrolytically sharpened tungsten wire. This hole was used to insert a reference glass electrode containing haemolymph Ringer solution (Kaissling and Thorson 1980), in contact with an Ag/AgCl wire. To hold the antennal segment in place, we used a sharpened tungsten wire as supporting

material. Then, we made a puncture at the last antennal segment with a sharpened tungsten wire using a micromanipulator (Joystick Manipulator, MN-151, Narishige, Tokyo, Japan). Without delay, a recording glass electrode (GB150F-8P, 0.86 9 1.50 9 80 mm with filament, Science Products GmbH, Hofheim, Germany) filled with haemolymph Ringer solution and in contact with an Ag/AgCl wire was inserted. Glass capillary electrodes were drawn from borosilicate glass by an electrode puller (Puller, Model No. PP-830, Narishige group, Tokyo, Japan). The recorded EAG signal was amplified with a preamplifier (Universal AC/DC probe, Syntech, Kirchzarten, Germany), which was connected to an EAG amplifier. The total amplification factor was 1009, and a data acquisition controller (Syntech, IDAC-4) processed and digitized the amplified signals. With the customized EAG program (Syntech, version 2.7, EAG 2000), the resulting EAG amplitude (negative potential) was computed as the difference between the baseline level and the maximum amplitude reached during odor stimulation.

2.3.4. Odor Presentation

Selected chemical compounds were diluted in silicone oil M 200 (*Carl Roth* GmbH + Co. KG, Germany) and concentrations in logarithmic steps 10^{-1}, 10^{-2} and 10^{-3} (w/w) were prepared. A glass Pasteur pipette (Pasteur capillary pipette 150 mm, Brand, Wertheim, Germany), containing a folded piece of filter paper (approx. 7 9 40 mm, Whatman No. 1), was impregnated with 20 µl of diluted stimulus. The prepared stimulus apparatus was used to deliver a one second puff of odor to the antenna. An air stimulus controller (CS-55, Syntech) was used, allowing non-intermittent application of a continuous flow (18 l/h) of charcoal- filtered and humidified air through the tube, placed at 1 cm distance from the antenna. The stimulus carrying Pasteur pipette was inserted into the rear end of the tube (18 cm from the hole to the outlet of the tube), and the stimulus puff was triggered by the automated controller. Pure silicone oil was used as a negative control. The aggregation pheromone 4,8-dimethyldecanal (DMD) at 10^{-3} (w/w) dilution in silicone oil was used as a positive control to monitor for changes in EAG detection. At each series of test compounds, the positive and negative control were applied again. Intervals between dilution steps and different compound series were 1 and 2 min, respectively. Throughout the EAG recording, ascending doses of different stimuli were applied within one compound series to minimize the effect of olfactory adaptation. Responses of three antennae from different adult beetles of each sex were recorded with every compound. Three separate runs of each dilution series of odor stimuli were measured with each antenna to compensate for fluctuations of antennal sensitivity with time.

Table 2-1. Effects of volatile compounds on Tenebrionidae insects and other grain feeding Coleopterans

(Experiments: Electroantennography- **E**; Behavior- **B**; Toxicity- **T;** ×- No; ✓- yes)

Insect species	Volatile compounds	Odor source	Insect response			Reference
			E	**B**	**T**	
Red flour beetle *Tribolium castaneum* Herbst (Coleoptera (**Col**):Tenebrionidae (**Tene**))	2-hydroxy-4-methoxy propiophenone	insect	×	×	✓	Howard et al. 1986
	valeraldehyde, maltol, vanillin	synthetic compounds	×	✓	×	Phillips et al. 1993
	acetophenone, phenyl ethanol, ethyl butyrate, 3-methyl butyraldehyde, butyl methacrylate	dry cocoa beans	×	✓	×	Jonfia-Essien et al. 2007
Confused flour beetle *T. confusum* Jacquelin Du Val (Col:Tene)	benzoquinones, ethyl-1,4-benzoquinone, methyl- 1,4-benzoquinone, 1-tetradecene, 1-pentadecene, 1,6-pentadecadiene, 1-hexadecene, 1,7-hexadecadiene, methyl-hydroquinone, 1-heptadecene, 1,8-heptadecadiene, 1,8,11-heptadecatriene, ethyl-hydroquinone	insect	✓	✓	×	Verheggen et al. 2007
	palmitic acid, stearic acid, oleic acid	wheat germ	×	✓	×	Tamaki et al. 1971a
	1-palmito-2,3-diolein, 2-linoleo-1,3-dipalmitin, 1-palmito-2-linoleo-3-olein	wheat germ	×	✓	×	Tamaki et al. 1971b
Desert Tenebrionid *Parastizopus armaticeps* Peringuey (Col:Tene)	3-methyl phenol, ethyl-1,4-benzoquinone, ethyl-11-eicosenoate, Isopropyl-11-eicosenoate, propyl-11-eicosenoate, ethyl-13-docosenoate, methyl-heptacosane, n-nonacosane, methyl-nonacosane, dimethyl-nonacosane, methyl-hentriacontane, dimethyl-hentriacontane,	insects synthetic compounds	×	✓	×	Geiselhardt et al. 2008

	dimethyl-hentriacontane, methyl-tritriacontane, dimethyl-tritriacontane					
Epitracus sallaei Champion (Col:Tene)	3-carene, α-pinene, limonene, ocimene, myrcene, terpinolene	mango flowers syntheic compounds	×	✓	×	Cruz-López et al. 2001
Lesser mealworm beetle *Alphitobius diaperinus* Panzer (Col:Tene)	(R)-(+)-limonene, (*E*)-β-ocimene, 2-nonanone, (S)-(+)-linalool, (R)-(+)-daucene	insect synthetic compounds	×	✓	×	Bartelt et al. 2009
subtribe Stizopina Opatrini (Col:Tene)	α-pinene, camphene, sabinene, β-pinene, *p*-cymene, α-terpinene, α-phellandrene, limonene, 1,4-benzoquinone, methyl-1,4-benzoquinone, ethyl-1,4- benzoquinone, isopropyl-1,4- benzoquinone, propyl-1,4-benzoquinone, phenol, 3-methyl-phenol, 3-ethyl-phenol	insect	×	✓	×	Geiselhardt et al. 2009
Fungivorous beetle *Bolitophagus reticulatus* Linnaeus (Col:Tene)	1-octen-3-ol , 3-octanone, 3-octanol	wood fungi synthetic compounds	✓	✓	×	Holighaus et al. 2014
Insect species from stored product pest mainly in Coleoptera						
Lesser grain borer *Rhyzopertha dominica* Fabricius (Col: Bostrichidae)	(S)-(+)-1-methylbutyl (*E*)-2-methyl-2-pentenoate, (*E*)-2,4-dimethyl-2-pentenoate,	insect synthetic compounds	×	✓	×	Williams et al. 1981
Saw toothed grain beetle *Oryzaephilus surinamensis* Linnaeus (Col:Silvanidae)	1-octen-3-ol (R,S-enantiomer) , 3-octanone, nonanal	infested oat synthetic compounds	✓	✓	×	White and Chambers, 1989
Granary weevil, *Sitophillus granarius* Linnaeus (Col:Curculionidae (**Cur**))	1-butanol,1-pentanol, 1-hexanol, 3-methyl-1-butanol, butanal, pentanal, hexanal, heptanal, (*E*)-2-hexenal, (*E*,*E*)-2,4-heptadienal, (*E*,*E*)-2,4-nonadienal, (*E*,*E*)-2,4-decadienal, 2-pentanone, 2-	synthetic compounds	×	✓	×	Germinara et al. 2008

	hexanone, 2-heptanone, 2,3-butanedione, maltol, furfural, phenylacetaldehyde, vanillin					
	2-pentanone, 2-hexanone, 2-heptanone, 2,3-butanedione	synthetic compounds	×	✓	×	Germinara et al. 2012
Foreign grain beetles *Ahasverus advena* Waltl (Col:Cucujidae)	1-octen-3-ol, (*Z*)-2-octenol, 1-nonanol, and (*Z*)-3-nonenol, hexanal, nonanal	infested oats	×	✓	×	Pierce et al. 1991
Larger grain borer *Prostephanus truncatus* Horn (Col:Bostrichidae) and *Sitophilus zeamais* Motschulsky (Col:Cur)	nonanal, decanal, hexanoic acid, nonanoic acid, vanillin , 1-hydroxyheptan-2-one	fresh cassava and maize	✓	✓	×	Pike et al. 1994
Pulse beetle, *Callosobruchus maculatus* Fabricius (Col:Chrysomelidae)	1-pentanol, 1-octen-3-ol, (*E*)-2-octenal, nonanal, 3-carene	dried green peas synthetic compounds	×	✓	×	Ndomo-Moualeu et al. 2015

Table 2-1. References numbers

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Table 2-2. Selected synthetic volatile compounds used as olfactory stimuli in the electroantennography experiments tested with red flour beetle *T. castaneum* antennae.

No.	Chemicals	CAS No.	VP (mbar) *	Purity %	Source
1.	undecane	1120-214	0.565	99	Acros
2.	decane	124-185	2.123	99	Acros
3.	dodecane	112-403	0.199	99	Acros
4.	octadecane	593-45-3	< 0.001	99	Acros
5.	tetradecane	629-594	0.016	99	Acros
6.	hexadecane	544-76-3	0.002	99	Acros
7.	docosane	629-97-0	< 0.001	99.5	Fluka
8.	1,7-hexadecadiene	125110-62-5	0.007	≥ 90	Fluka
9.	1-pentadecene	13360-61-7	0.007	99.5	Fluka
10.	1-tetradecene	1120-36-1	0.022	97	Fluka
11.	1-heptadecene	6765-39-5	0.001	99	Aldrich
12.	1-hexadecene	629-73-2	0.004	92	Aldrich
13.	1-hexen-3-ol	4798-44-1	6.518	98	Aldrich
14.	2-octanol	4128-31-8	0.369	99	Chemos
15.	3-methyl-2-pentanol	565-606	4.300	99	Aldrich
16.	2-hexanol	626-93-7	4.326	99	Aldrich
17.	(±) octan-3-ol	589-98-0	0.727	97	Merck
18.	1-pentanol	71-41-0	3.120	99	Aldrich
19.	(R)-(-)-1-octen-3-ol	3687-48-7	1.447	99	Acros
20.	2-methyl-1-butanol	137-326	4.151	99	Aldrich
21.	(*Z*)-3-hexen-1-ol	928-96-1	1.959	98	SAFC
22.	(S)-(+)-1-octen-3-ol	24587-53-9	0.707	99	Acros
23.	(S)-(+)-3-octanol	22658-92-0	0.682	97	Aldrich
24.	(+)-2-pentanol	6032-29-7	8.360	99	Acros
25.	1-decanol	112-30-1	0.013	98	Aesar
26.	1-hexanol	111-27-3	1.682	99	Aldrich
27.	(±) 1-octen-3-ol	3391-86-4	1.447	98	Merck
28.	2-ethyl-1-hexanol	104-76-7	0.323	99	Merck
29.	1-nonanol	143-08-8	0.038	98	Aldrich
30.	1-octanol	111-87-5	0.141	98	Aldrich
31.	octanal	124-13-0	3.274	99	Aldrich
32.	4,8- dimethyldecanal	75983-36-7	0.067	99	TRÉCÉ
33.	(*E*)-2-hexenal	6728-26-3	5.484	99	Acros
34.	hexanal	66-25-1	14.081	98	Aldrich
35.	nonanal	124-19-6	0.866	95	Aldrich
36.	(*E*)-2-heptenal	18829-55-5	3.720	97	Aldrich
37.	(*E*)-2-octenal	2548-87-0	1.622	≥ 95	Aldrich
38.	pentanal	110-62-3	44.417	97	Fluka
39.	benzaldehyde	100-527	1.729	98	Fluka
40.	citral	5392-40-5	0.095	98	Merck
41.	2-heptanone	110-43-0	5.316	99	Aldrich
42.	2-pentanone	107-87-9	52.344	99	Chemos

43.	3-octen-2-one	1669-44-9	1.195	97	Chemos
44.	3-octanone	106-68-3	3.149	96	VWR
45.	2- hexanone	591-78-6	9.992	98	Aldrich
46.	6-methyl-5-hepten-2-one	110-93-0	1.701	98	ABCR
47.	acetophenone	98-86-2	0.528	99	Aldrich
48.	2-nonanone	821-55-6	0.453	≥ 99	Aldrich
49.	3-methyl-2-pentanone	565-617	25.920	99	Aldrich
50.	β-ionone	14901-07-6	0.023	96	ABCR
51.	**2H-4M propiophenone	6270-44-6	UN	> 98	Specs
52.	hexanoic acid	142-62-1	0.071	98	Merck
53.	pentanoic acid	109-52-4	0.428	99	Aldrich
54.	octanoic acid	124-07-2	0.005	99	Merck
55.	stearic acid	57-11-4	< 0.001	98.5	Aldrich
56.	palmitic acid	57-10-3	< 0.001	99.5	Fluka
57.	oleic acid	112-80-1	< 0.001	99	Aldrich
58.	acetic acid	64-19-7	11.248	99	Fluka
59.	ethyl hexanoate	123-66-0	1.718	99	Chemos
60.	hexyl benzoate	6789-88-4	0.004	98	Aesar
61.	2-phenylethyl benzoate	94-47-3	UN	98	Acros
62.	phenyl benzoate	93-99-2	0.004	99	Aesar
63.	butyl benzoate	136-60-7	0.051	99	Aldrich
64.	eucalyptol	470-82-6	2.597	99	Aldrich
65.	sabinene	3387-415	9.805	75	ABCR
66.	(-)-β-pinene	18172-673	4.783	98	Aldrich
67.	(±)-camphor	76-22-2	0.462	95	Fluka
68.	α-terpinene	99-86-5	2.217	85	Aldrich
69.	β-myrcene	123-35-3	2.794	95	Fluka
70.	(-)-α-copaene	3856-25-5	0.051	> 90	Fluka
71.	β-caryophyllene	8744-5	0.017	98.5	Aldrich
72.	α-phellandrene	99-83-2	2.424	≥ 85	Aldrich
73.	(S)-(-)-α-pinene	7785-26-4	7.925	99	Fluka
74.	(+)-cuparene	16982-00-6	0.051	99	Fluka
75.	(+)-camphene	5794-03-6	5.768	> 90	Aldrich
76.	(S)-(-)-limonene	5989-54-8	1.975	96	Aldrich
77.	(*E*)-ocimene	3779-61-1	2.132	> 96	Chemos
78.	linalool	78-706	0.226	97	Acros
79.	*p*-cymene	99-87-6	2.099	97	Aldrich
80.	(R)-(+)-limonene	5989-27-5	3.113	97	Aldrich
81.	(R)-(+)-α-pinene	7785-70-8	7.748	99	Aldrich
82.	3-carene	13466-78-9	2.480	90	ABCR
83.	methyl-1,4-benzoquinone	553-97-9	0.933	98	Fluka
84.	phenol	108-95-2	0.394	99	Aldrich
85.	1,4-benzoquinone	106-51-4	0.261	98	Aldrich
86.	ethyl-hydroquinone	2349-704	UN	97	ABCR
87.	vanillin	121-33-5	< 0.001	99	Merck
88.	methyl-hydroquinone	95-71-6	< 0.001	99	Aldrich

89.	3-ethyl phenol	620-17-7	0.074	95	ABCR
90.	*m*-cresol	108-39-4	0.018	99	ABCR
91.	2-n-pentylfuran	3777-69-3	0.250	98	ABCR
92.	maltol	118-71-8	UN	99	Aldrich
93.	naphthalene	91-20-3	0.103	99	Merck
94.	indole	120-72-9	0.016	99	Fluka

* Vapor pressure calculated at 25°C and based on the book: Yaws CL, 2007. The Yaws Handbook of Vapor Pressure-Antoine Coefficient. Gulf Publishing Company, Houston, Texas.
** 2-hydroxy-4-methoxy-propiophenone
UN-unknown

For comparison of the antennal response to different compounds, firstly we subtracted the EAG response to silicone oil from the EAG responses to the following dilution series. Secondly, we normalized the subtracted values to the respective EAG response to silicone oil. Thirdly, we calculated the mean value of the separate runs of each compound with the same antenna. Fourthly, means of these mean results were calculated and this value with the three different antennae of each sex and the standard error were displayed. This procedure was applied to account for two different sources of errors as temporal variation and inter individual variation of EAG response by the beetle antenna. However, the silicone oil response showed a considerable standard deviation (35.2% to the mean EAG response; n = 282; mean = 0.17 mV; SD = 0.0598 mV). EAG responses up to 35% could be mere statistical fluctuation of silicone oil response. Consequently, we regarded only responses bigger than 50% as 'measurable EAG response'.

2.4. Results

2.4.1. EAG Detection

The EAG amplitudes in our whole-body beetle preparations were much more stable than in isolated antennal preparations as utilized for *T. confusum* (Verheggen et al. 2007). Thus, for *T. castaneum*, improved antennal cuticle penetration for the recording electrode combined with the use of a whole-body immobilization enabled us to get reliable EAG recordings for >5 h. Antennae of *T. castaneum* respond to distinct volatile organic compounds from various chemical groups. The normalized EAG responses of female and male *T. castaneum* to 94 volatile compounds at three concentrations are shown in Figure 2-1. Of these 94 tested compounds, we found that at higher concentration (10^{-1}),

79 and 85 compounds elicited measurable EAG responses from females and males, respectively. In case of intermediate concentration (10^{-2}), 74 compounds elicited measurable EAG responses from females and 72 from males. However, at lower concentration (10^{-3}) of all tested compounds, only 25 compounds elicited measurable EAG responses from females and 30 compounds from males. Both sexes show the strongest antennal response amplitude to undecane, 1-hexen-3-ol, octanal, 2-heptanone, hexanoic acid and ethyl hexanoate at all tested concentrations within their chemical groups (Figure 2-1). Male and female beetles did not show any significant sex-specific differences between their EAG responses to the same compound at the same concentration (n = 3; Wilcoxon–Mann–Whitney U-test $P > 0.05$ in all compounds). However, this result might be due to the low number of biological replications. For instance, we found a significant difference between both sexes of the beetles in response to 4,8-DMD at 10^{-3} concentration with higher biological replications (n = 282; Student's t-test $P = 0.0356$). Thus, we decided to present the normalized mean value ± SE of EAG responses of female and male beetles separately (Figure 2-1).

2.4.2. Comparison of EAG Responses and Vapor Pressure in Homologous Series of Stimulus Compounds

Looking at the EAG response of *T. castaneum* antennae to aliphatic hydrocarbons with different functional groups, a general trend becomes visible: EAG responses to the stimuli generated by the same dilutions in silicone oil are decreasing with chain length of the molecules. This trend becomes plausible considering that vapor pressure is declining within a homologous series with increasing number of carbon atoms that is molecular weight. Thus, fewer molecules are available for detection at the same silicone oil dilution and higher molecular weight. Also, as the dilution was made as w/w, the solution already contains less molecules for the heavier substances (Figure 2-2a). If receptors of the antenna are only counting molecules of a compound class, the EAG response will decline with carbon chain length (Figure 2-2b) (vapor pressure rule). In this case, the presence of molecules of one compound class is perceived without differentiating their size. If the receptors of the antenna are more specific at differentiating between molecules of too small, fitting, and too big chain length, the EAG response throughout a homologous series will show deviations from the 'vapor pressure rule' favouring specific compounds with comparably higher EAG responses (Figure 2-2c). Deviations from this rule can hint at compound-specific increased sensitivity of the olfactory receptor to important stimuli of outstanding ecological importance.

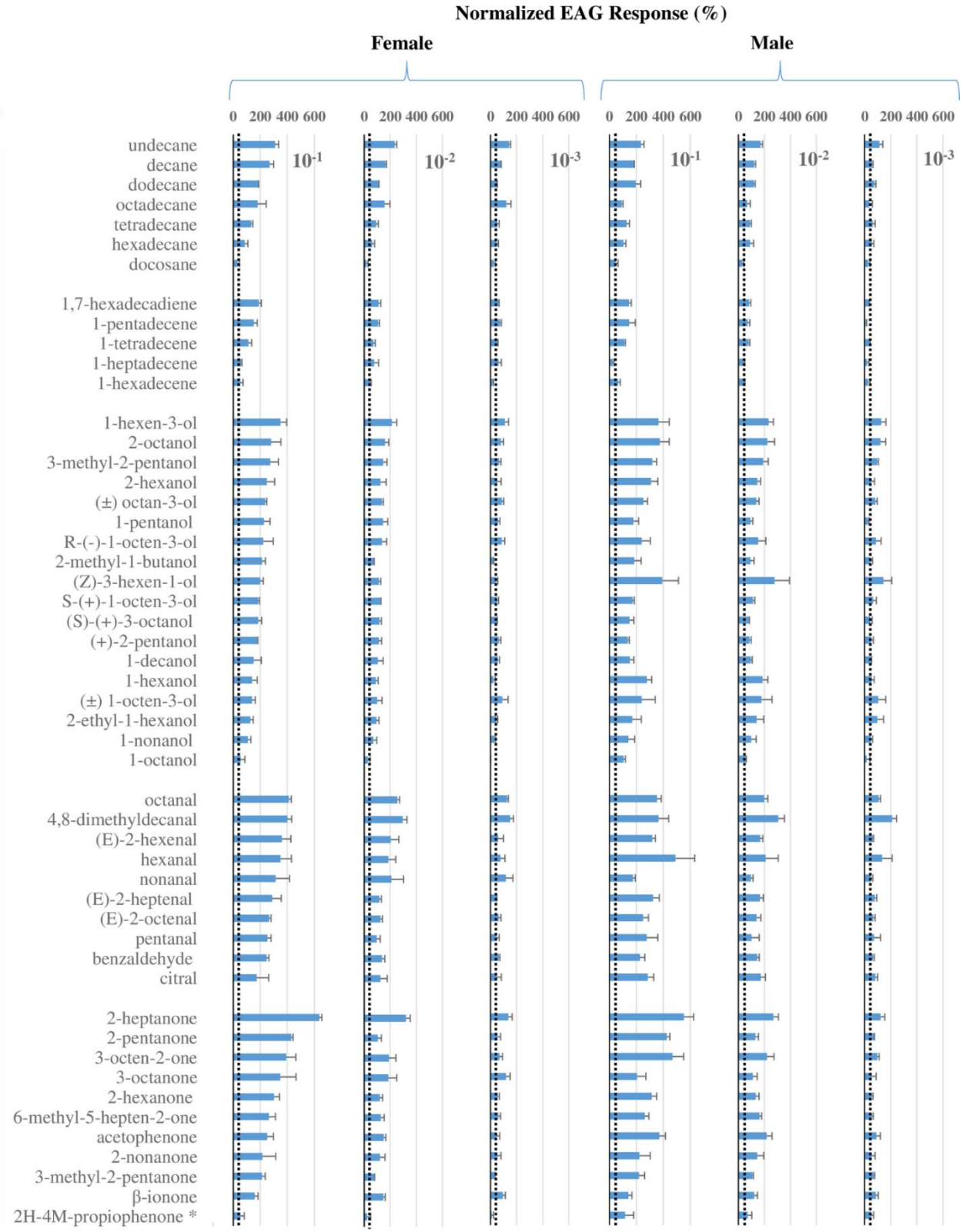


Figure.1 continued

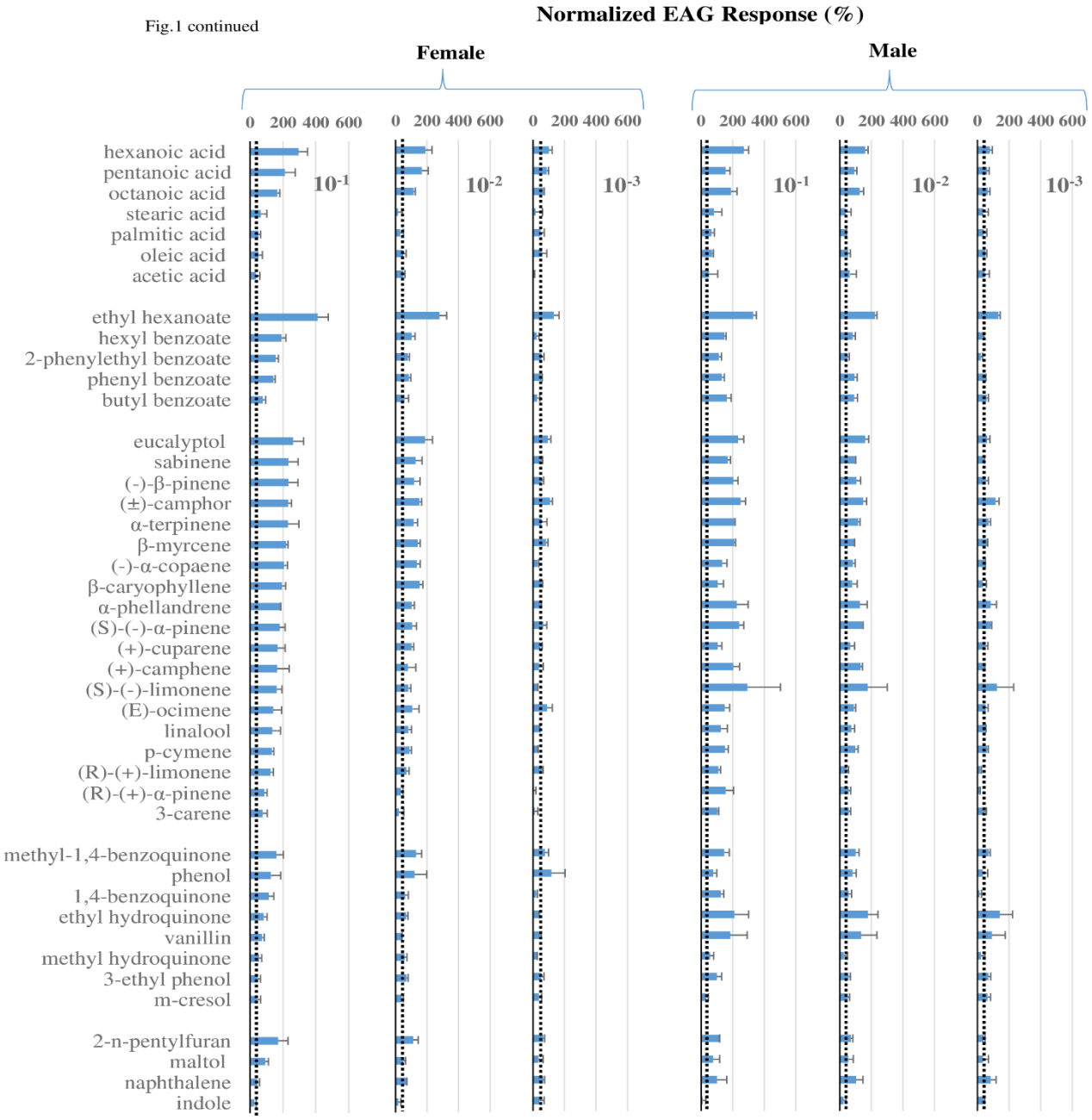


Figure 2-1. ***Tribolium castaneum*** **antennal detection profile of 94 selected volatile organic compounds**. Horizontal columns show EAG responses of both female and male antennae of *T. castaneum*. The compounds are arranged in chemical groups displayed along vertical direction. Within the groups they are arranged according to the EAG response elicited at 10^{-1} dilution in silicone oil. Thus, the odors within each group that elicit the strongest EAG responses at 10^{-1} in females are placed at the top; those that elicit the weakest EAG responses are placed at the bottom. The error bars represent the standard error of the mean (n = 3). EAG response of silicone oil was used as a negative control and to normalize EAG responses (100 %) elicited by other chemicals. The dotted vertical lines represent the limit of what is regarded as a "measurable EAG response". * 2-hydroxy-4-methoxy-propiophenone.

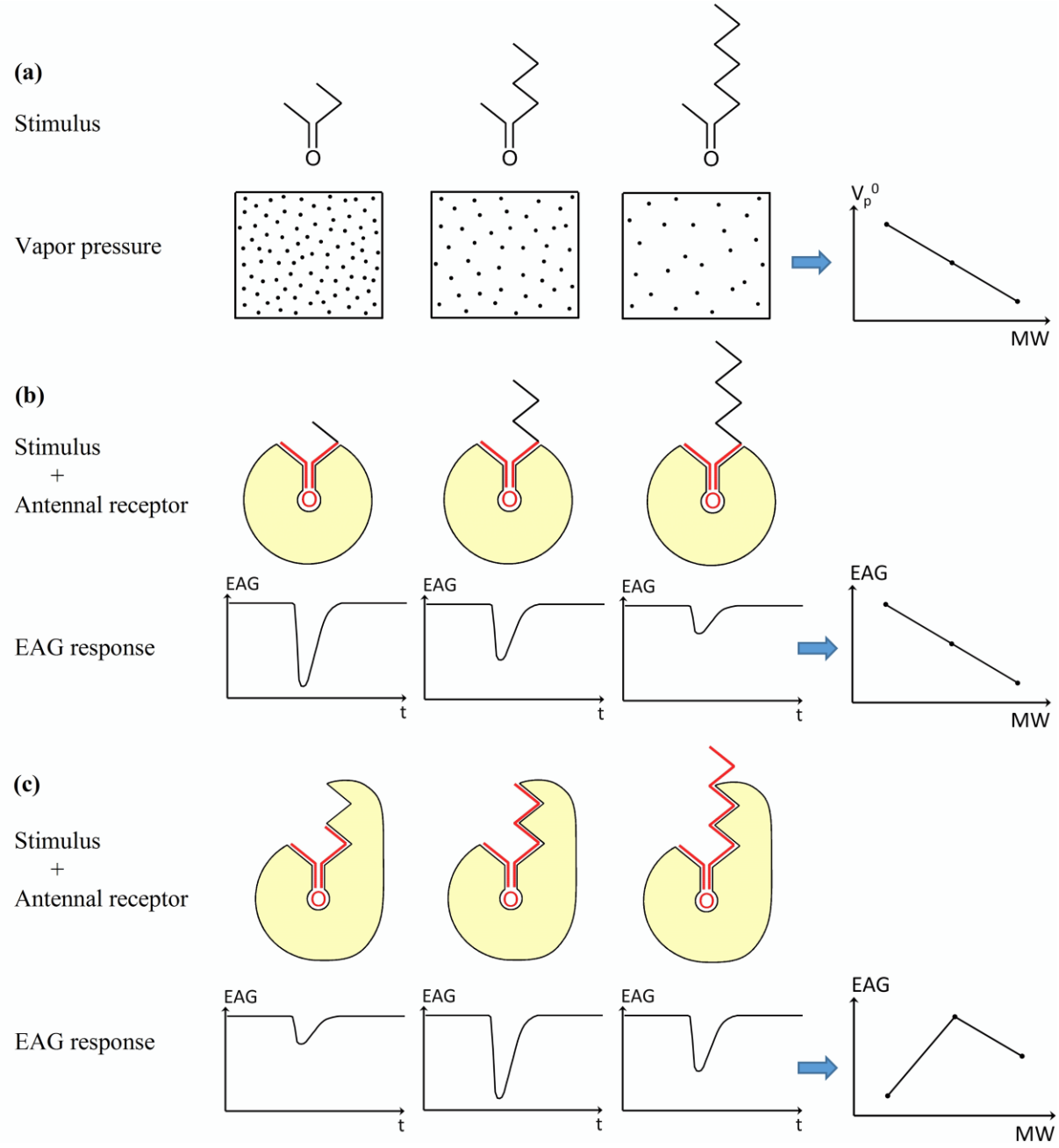


Figure 2-2. Diagram depicting the "vapor pressure rule" relating the EAG response to a series of homologous stimulus compounds. a) Schematic diagram of the 2-ketones with different carbon chain lengths resulting in different vapor pressures; **b**) Schematic diagram of binding of these 2-ketones to a receptor recognizing the 2-ketone groups without differentiating carbon chain lengths resulting in a monotonous decrease of EAG response towards stimuli of an homologous series of increasing molecular weight (vapor pressure rule); **c**) Schematic diagram of binding of these 2-ketones to a receptor recognizing the 2-ketone groups with differentiating carbon chain lengths resulting in a deviation from the "vapor pressure rule".

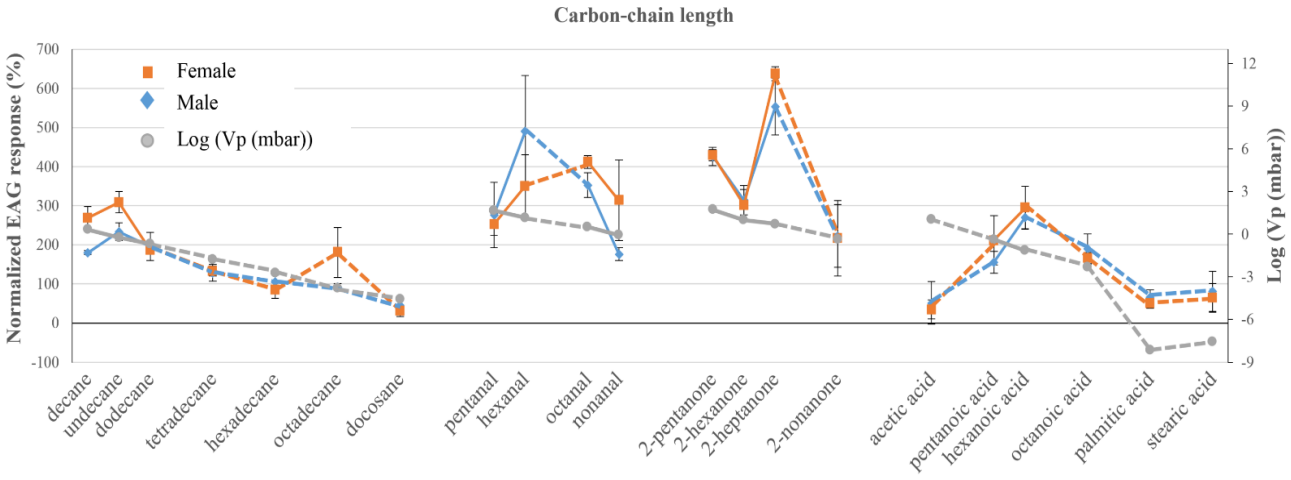


Figure 2-3. Comparison of expected EAG responses according to the vapor pressure rule Vs measured EAG response. Correlation of EAG responses of male and female beetles to 10^{-1} dilution in silicone oil with the vapor pressure (Vp) of the diluted volatile organic compounds. Homologous series of alkanes, aldehydes, ketones and carboxylic acids (n = 3).

Comparing vapor pressure and EAG responses of *T. castaneum* antennae at 10^{-1} concentration to alkane homologous between C_{10} and C_{22} atoms, only two compounds show deviation from the drawn up rule (Figure 2-3): undecane (C_{11}) and octadecane (C_{18}). Both compounds elicit higher female EAG responses than expected according to their vapor pressure. This is visualized in Figure 2-3, following the descending line drawn across the neighbouring alkanes. The other five alkanes follow the vapor pressure rule quite closely. Analogously, EAG responses to the selected aldehydes, 2-ketones and carboxylic acids do not follow the vapor pressure rule consistently throughout. In aldehydes and in carboxylic acids, C_6 and C_8 compounds show strong deviation, respectively, whereas in ketones C_7 compounds stand out.

2.5. Discussion

The present study revealed measurable peripheral olfactory responses (EAG) of both sexes of *T. castaneum* antennae to the majority of the tested host and non-host volatile compounds, indicating that a wide chemical range of volatiles related to different ecological aspects are detected by the beetle. A broad overlap between sexes is not unexpected as both live on the same resources

characterized by volatile cues to locate the feeding substrate and to meet there for mating (Li et al. 1992; Germinara et al. 2007, 2009; Ukeh et al. 2010). Thus, these compounds are potential semiochemicals mediating interactions between organisms enabling olfactory discrimination of host and non-host substrates as well as conspecific beetles. Based on these findings, we speculate that many of the compounds detected by the beetles might also be active in behavioral assays, as many of the selected compounds were previously tested in behavioral studies of other tenebrionids and stored insect pests of Coleopterans (Table 2-1). However, at lower concentrations, we observed decreasing numbers of volatiles detected by the beetles (females 79/74/25 and males 85/72/30 compounds at dilution $10^{-1}/10^{-2}/10^{-3}$, respectively) and only a dozen of volatiles that did not show measurable EAG responses by both sexes at any concentration.

Alkanes used in our study ranged between n-C_{10} and n-C_{22}, with undecane as outstanding. Alkanes of even longer chain length are commonly present in plant and seed surface waxes, playing a role as significant attractants for plant-feeding insects (Udayagiri and Mason 1997; Sarkar et al. 2013). Straight chain alcohols and aldehydes are products of lipid oxidation, caused by food degradation such as rancidification. Pentanoic, hexanoic and octanoic acid are products of further oxidation of these aldehydes (Xiao et al. 2014). In general, the concentrations of aldehydes and carboxylic acids increase with progressing degradation of lipids in grains (Xiao et al. 2014). Thus, low concentrations of these compounds indicate low lipid degradation levels and high food quality for stored product insect pests. Short-chain fatty acids (C_6-C_8) exhibited strong repellent behavior to storage insect pests including *T. castaneum* in contrast to fatty acids with long carbon chain (C_{16}, C_{18}, $C_{18:1}$, $C_{18:2}$) (Cohen et al. 1974). In our study, short-carbon-chain fatty acids (C_5, C_6, C_8) elicited EAG responses highest for hexanoic acid – remarkably it also has the strongest behavioral effect, among the repellent fatty acids in the study of Cohen et al. (1974). This demonstrates that comparative EAG screening approaches in some cases can point to compounds that also have outstanding potential to be behaviorally active, while this is, however, not to be expected. Lipid oxidation results in aldehyde formation not only due to oxidation: the high EAG amplitude of *T. castaneum* to (*E*)-2- hexenal, hexanal, nonanal and (*E*)-2-heptenal – enzymatically produced 'green leaf aldehydes' – together with strong toxic effects of (*E*)-2-hexenal to storage insect pests including *T. castaneum* (Hubert et al. 2008) suggests that a discriminative evaluation of these aldehydes is adaptive for assessing food quality. We therefore suggest that future behavioral tests should

examine single compounds at different concentrations or mixtures that are not only combined due to chemical similarity but also take metabolic origins into account (Wenda-Piesik et al. 2016).

Similar as aldehydes, aliphatic ketones are emitted by various cereal grains (Zhou et al. 1999 and reference therein) and many of them are known to possess antibacterial activity against foodborne bacteria (Bowles and Miller 1993). Additionally, they are also known as repellents to stored product insects (Germinara et al. 2012; Piesik and Wenda-Piesik 2015; Wenda-Piesik et al. 2016) and probably mediate host information for palatability and oviposition. Common fungal semiochemicals such as 2-octanol, octan-3-ol and 3-octanone (Thakeow et al. 2008; Holighaus et al. 2014) showed pronounced EAG responses by female *T. castaneum*. These compounds might be involved in the host choice process as fungal- infested grain seem to be attractive to *Tribolium* (Phillips et al. 1993), while other fungi compete with beetles for the same resources signaling severe toxicity (Holighaus and Rohlfs 2016 and reference therein). EAG active terpenoids, whether of plant or fungal origin, such as eucalyptol, sabinene, (-)-β-pinene, (±)-camphor, (R)-(+) and (S)-(-) α-pinene, β-caryophyllene, (*E*)-ocimene and α-terpinene may contribute to non-host avoidance, as these compounds are causing toxic effects to *T. castaneum* and *S. oryzae* (Lee et al. 2001; Stefanazzi et al. 2011; Chaubey 2012). Referring to *T. castaneum* ecology beside host choice, we demonstrated that methyl-1,4- benzoquinone and 1,4-benzoquinone as major part of its defensive secretion are EAG active, suggesting that they might confer the presence and abundance of conspecifics.

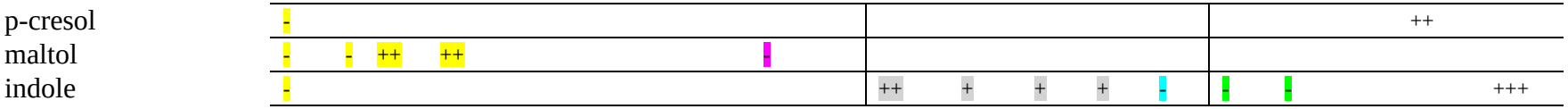
We demonstrated that the EAG responses of *T. castaneum* antennae to stimuli from alkanes, aldehydes, 2-ketones and carboxylic acids followed a rule linking the EAG response linearly to the logarithm of the vapor pressure of each compound. However, we observed deviations from this rule and suggest that these deviations hint at a specialization of the olfactory system to these compounds. The fact that carbon chain lengths of the deviating molecules are varying (C_{11} and C_{18} for alkanes, C_6 for aldehydes, C_7 for 2- ketones and C_8 for carboxylic acids) suggests that it is likely the outcome of evolutionary pressure rendering the sensitive detection of some compounds more useful than others. Sensitivity to carbon chain length and functional groups of insects feeding on similar food sources as *T. castaneum* might have similar underlying evolutionary pressure regarding the sensitive detection of feeding and oviposition substrate-related compounds (Andersson et al. 2015).

Table 2-3. Olfactory response of different group of insect species and their natural enemies to some of our tested volatile organic compounds

(Electroantennography-**E**; Behavior response-**B**; Single sensillum recording-**SSR** ;+++ - high response; ++ - intermediate response; + - low response; - - no response)

Chemicals *	**Insect groups ****																									
	Grain feeders & their parasitoid														Foliage feeders & parasitoid									Fruit feeder			Blood feeder	
	1 E	B	**2** B		**3** B		**4** E	**5** B		**6** E		B	**7** E	B	**8** E		**9** E		**10** E		**11** E		**12** E		**13** E	SSR	B	**14** E	SSR
Alcohols																													
3-octanol	++							++																	++				
1-pentanol	++				+++								+								+++				+++		+		
cis-3-hexenol	+														+++		+++		+++		++		+++		+++		+++		+
2-ethyl-1-hexanol	+																												+
(+)-2-pentanol	+																								+++		+		
1-hexanol	+				++								+								+++				++		+++		
(R,S)-1-octen-3-ol	+						-							-							+				+		-	+++	++
1-nonanol	+							+++							++		++		+++				++		-		++		
1-decanol	+														++		+		+++				+		-				
1-octanol	-							+							+++		+++		+				++		+		+++		-		++
Aldehydes																													
(*E*)-2-hexenal	+++				+++								++								-						++		-		
(*E*)-2-heptenal	+++									++		++	++																
octanal	+++									++		++ +	++														++			-	
(*E*)-2-octenal	+++												+																
nonanal	+++						-			+				++																+++	
hexanal	+++				+++					++ +		++ +	++								+		+++				+++		++		-	
pentanal	++		+	+++	++								+														+++		+		
benzaldehyde	++													++							-						+++		-		
Ketones																													
2-hexanone	+++				+++								++																

2-heptanone	+++			++				+							+++	+		
6-methyl-5-hepten-2-one	+++														+++	-		+++
2-pentanone	+++			+++			+								++	++		
3-octanone	+++				+	+		+										+
acetophenone	++														++	-		+++
2-nonanone	+							+										+
β-ionone	+											-						
Acids																		
hexanoic acid	+++						+		++	+++	++		+	+	++	++	++	
pentanoic acid	++						+								+++	+	+++	-
octanoic acid	+														+	+	-	-
acetic acid	+													++		++	++	-
Esters																		
ethylhexanoate	+++														+++	+		
Terpenoids																		
α-terpinene	++											-						
(-)-β-pinene	++															+		
β-myrcene	++											-		-		+		
sabinene	++											-						
linalool	+											-	+++		++	-		
β -ocimene	+								+++		+++		++	++				
copaene	+													-				
D-limonene	+														+	-		
(R)-(+)-limonene	+								+	-	++		++	-				
(S)-(-)-limonene	+												+					
α-pinene	+								+	+	+		-	-	-			
(E)-β caryophyllene	+								+	+	-		+	+		+		
p-cymene	+															+		
(R)-(+)-α-pinene	-															+		
3-carene	+													-	++	+		
Phenolics																		
vanillin	-	-	++	+			-											



*- The odors are arranged within each group based on descending order of EAG amplitude value of female *T. castaneum* antenna at 10^{-1} dilution in silicone oil

**- different insect groups as follows

Insect No.	Stored product insects	References
1	Red flour beetle- *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae)	Phillips et al. 1993- (**B**); (**EAG**)-our own data
2	Rice weevil -*Sitophilus oryzae* Linnaeus (Coleoptera: Curculionidae)	Phillips et al. 1993- (**B**)
3	Granary weevil -*Sitophilus granarius* Linnaeus (Coleoptera: Curculionidae)	Germinara et al. 2008- (**B**)
4	Saw toothed grain beetle -*Oryzaephilus surinamensis* Linnaeus (Coleoptera: Silvanidae)	White et al. 1989- (**EAG**) & (**B**)
5	Cowpea seed beetle- *Callosobruchus maculatus* Fabricius (Coleoptera:Chrysomelidae)	Adhikary et al. 2015- (**B**)
6	Maize weevil- *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae)	Ukeh et al. 2012- (**EAG**) & (**B**)
	Parasitoid of stored product insects	
7	Parasitic wasp - *Theocolax elegans*- Westwood (Hymenoptera: Pteromalidae)	Germinara et al. 2009- (**EAG**)
	Leaf feeding insects	
8	Tobacco budworm -*Heliothis virescens*- Fabricius (Lepidoptera: Noctuidae)	Park et al. 2002- (**EAG**)
9	Corn earworm- *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae)	Park et al. 2002- (**EAG**)
10	European corn borer - *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae)	Park et al. 2002- (**EAG**)
11	Cabbage seed weevil - *Ceutorhynchus assimilis* Paykull (Coleoptera: Curculionidae)	Blight et al. 1995- (**EAG**)
	Parasitoid of leaf feeding insects	
12	Braconid wasp - *Microplitis croceipes* (Hymenoptera: Braconidae)	Park et al. 2001-(**EAG**); Chen and Fadamiro 2007 - (**EAG**), Park et al. 2002- (**EAG**)
	Fruit feeder	
13	Fruit fly -*Drosophila melanogaster* (Diptera: Drosophilidae)	Zhu et al. 2003- (**EAG**) & (**B**); Park et al. 2002 - (**EAG**), Clyne et al. 1997 -(**SSR**), Hallem et al. 2004 - (**SSR**), Hallem and Carlson 2006 - (**SSR**)
	Blood feeder	

14	Malaria mosquito- *Anopheles gambiae* (Diptera: Culicidae)	Costantini et al. 2001- (**EAG**) & (**B**); Wang et al. 2010- (**SSR**), Carey et al. 2010 -(**SSR**)

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Table 2-4. Olfactory response of different groups of insect species and their natural enemies to selected groups of volatile organic compounds. Bold numbers represent the number of volatile compounds showing at least intermediate olfactory response (++) either in behavior test or electroantennography (EAG) or single sensillum recording (SSR) by different insects of the same group. Numbers in parenthesis indicate carbon chain length of the volatile compounds taken into account. (See details in Table 2-3.) *

Chemical/insect group	Grain feeder	Grain feeder parasitoid	Foliage feeder	Foliage feeder parasitoid	Fruit feeder	Blood feeder
Alcohols	**4** (C_5, C_6, C_8, C_9)		**6** (C_5, C_6, C_8-C_{10})	**3** (C_6, C_8, C_9)	**7** (C_5-C_9)	**2** (C_8)
Aldehydes	**8** (C_5-C_9)	**6** (C_6-C_9)		**1** (C_6)	**5** (C_5-C_8)	**1** (C_9)
Ketones	**6** (C_5-C_8)	**1** (C_6)			**4** (C_5, C_7, C_8)	**2** (C_8)
Acids	**2** (C_5, C_6, C_8)		**1** (C_6)		**3** (C_2, C5, C_6)	**3** (C_2, C_5, C_6)
Esters	**1** (C_8)					
Terpenoids	**5** (C_{10})		**2** (C_{10})	**3** (C_{10})	**3** (C_{10})	
Phenolics	**1** (C_6 , C_8)					**1** (C_7)

*- **Grain feeder**: *Tribolium castaneum, Sitophilus oryzae, Sitophilus granarius, Oryzaephilus surinamensis, Callosobruchus maculatus*; **Grain feeder parasitoid**-*Theocolax elegans*; **Foliage feeder**- *Heliothis virescens, Helicoverpa zea, Ostrinia nubilalis, Ceutorhynchus assimilis*; **Foliage feeder parasitoid**- *Microplitis croceipes*; **Fruit feeder**- *Drosophila melanogaster*; **Blood feeder**- *Anopheles gambiae*

Insects feeding on similar food sources might have similar underlying evolutionary pressure regarding the sensitive detection of feeding and oviposition substrate related compounds. If this is the case, similar peaks of detectivity regarding chain length and functional group are to be expected in antennae of storage pests feeding on grains like *T. castaneum*. Moreover, predators and parasitoids foraging on grain- feeding or plant feeding insects are to be expected to share at least a part of these peaks of detectivity, marking out a habitat likely to

be colonised by their prey. However, electrophysiological data are sparse on these insects (Table 2-3). A compilation of stimulus compounds examined by different insects is displayed, including some foliage feeding insects, antagonists of foliage feeding insects and insects utilising other food sources in Table 2-4. Despite the sparseness of data, the pattern becomes visible that the detectivity for C_6- C_9 aldehydes is increased in grain feeders and their natural enemy *T. elegans* as the detectivity of C_6 2-ketones. Whereas C_8 to C_9 primary alcohols seem to play a major role in foliage-feeding insects and their parasitoid *Microplitis croceipes* Cresson (Hymenoptera: Braconidae).

2.6. Conclusions

In conclusion, the results of this study represent an initial attempt to elucidate EAG sensitivity of the antennae of both sexes of *T. castaneum*. Most of the tested compounds elicited measurable EAG responses for which *T. castaneum* olfactory system houses about 338 odorant receptors, 50 odorant binding proteins and 20 chemosensory proteins, suggesting a broad spectrum of perceived volatiles (Engsontia et al. 2008; Vieira and Rozas 2011). Our results show that some compounds, namely undecane, 1-hexen-3-ol, 2-octanol, 3-methyl-2-pentanol, octan-3-ol, 1-octen-3-ol, octanal, hexanal, nonanal, 2-heptanone, 3-octen-2- one, 3-octanone, β-ionone, hexanoic acid, ethyl hexanoate and (±)-camphor, are more sensitively detected by *T. castaneum* antenna within chemical groups. We recommend to preferably use these individual compounds for behavior tests, as only pentanal, vanillin, maltol, α-pinene, β-caryophyllene and 4,8-dimethyldecanal have been examined till now (Phillips et al. 1993; Campbell 2012; Chaubey 2012). In particular, different combinations and mixtures are promising for developing efficient attractant or repellent formulations as demonstrated for *T. confusum* recently (Wenda-Piesik et al. 2016). This might help to set up a semiochemical-based monitoring and control of *T. castaneum* in future. Besides that, we established an improved long-lasting EAG recording technique for *T. castaneum*. This will benefit future research by the combination of electrophysiology with molecular techniques, to unravel the olfactory system of this beetle.

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CHAPTER 3

Mutual exclusive expression of closely related odorant binding proteins *TcasOBP9A* and *TcasOBP9B* in the antenna of the red flour beetle *Tribolium castaneum*

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Author contributions:

Balakrishnan K: I performed all the EAG experiments, analysed them and prepared the EAG figures (fig s1; fig 1&2).

SD, SS, and **EAW** conceived and initiated the project. **AM**, **KB**, and **SD** performed the experiments. **AM**, **KB**, and **SD** analyzed the data and created the figures. **SD** and **GO** performed the phylogenetic analysis. **EAW** wrote the first draft of the manuscript with contributions from **AM**, **KB**, and **SD**. All authors critically revised and approved of the manuscript**.**

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3.1. Abstract

Olfaction is crucial for insects to find food sources, shelter, and mates. One of the initial steps in olfaction is facilitated by odorant binding proteins (OBPs) that translocate hydrophobic odorants through the aqueous olfactory sensilla lymph to present them to the odorant receptors on the olfactory sensory neurons. The *Tribolium castaneum* (Coleoptera, Tenebrionidae) OBPs encoded by the gene pair *TcasOBP9A* and *TcasOBP9B* represent the closest homologs to the well-studied *Drosophila* homolog Lush (OBP76a), which mediates pheromone reception. By electroantennographic analysis, we can show that these two OBPs are not pheromone-specific but rather enhance the detection of a broad spectrum of organic volatiles. Both OBPs are expressed in the antenna but in a mutual exclusive way, despite their homology and gene pair character by chromosomal location. Phylogenetic analysis reveals, that this gene pair arose at the base of the Cucujiformia, which dates the gene duplication event to about 200 Mio years ago. Therefore, this gene pair is not the result of a recent gene duplication event and the high sequence conservation is potentially the result of a common function in spite of their expression in different sensilla.

Keywords: beetle, odorant binding protein (OBP), olfaction, *Tribolium castaneum*

3.2. Introduction

Insects rely heavily on odorous stimuli to find food or hosts or to recognize partners. Odorant reception occurs in chemosensory sensilla and is mediated by odorant binding proteins (OBPs), odorant receptors (ORs), sensory neuron membrane proteins, ionotropic glutamate-like receptors, gustatory receptors, and odorant degrading enzymes (Leal, 2013). The antennae carry the highest density of olfactory sensilla, which represent hair-like structures that house the dendrites of the odorant receptor neurons and are filled with aqueous lymph. This lymph contains OBPs that are secreted by non-neuronal auxiliary cells (Vogt and Riddiford, 1981; Steinbrecht, 1998). OBPs are globular, rather small (10 to 30 kDa), water-soluble proteins (reviewed in Zhou, 2010) with a hydrophobic pocket for binding ligands (Sandler et al. 2000). Systematic OBP knockdowns in *Drosophila melanogaster* indicate their necessity for correct olfactory behavioral responses and suggest a combinatorial OBP-dependent odorant recognition (Swarup et al. 2011). Moreover, *D. melanogaster* mutants for the OBP Lush (OBP76a; Xu et al. 2005), as well as allelic variation of different OBPs in *D. melanogaster* (Arya et al. 2010) and of an OBP in the fire ant *Solenopsis invicta* (Krieger et al. 2005), as well as several RNAi based experiments in mosquitoes (Biessmann et al. 2010; Pelletier et al. 2010) showed that OBPs are essential for the correct reception of different semiochemicals in these insects. Functional experiments conducted with moth pheromone receptors in heterologous expression systems (Forstner, 2009; Grosse-Wilde et al. 2006, 2007) or *in vivo* using the *D. melanogaster* "empty neuron system" (Hallem et al. 2004; Syed et al. 2006) revealed that the presence of the corresponding OBP (pheromone binding protein, PBP) increases the sensitivity to the pheromone by 2 to 3 orders of magnitude (reviewed in Leal, 2013). Therefore, hydrophobic semiochemicals are believed to first interact with OBPs, which shuttle them through the aqueous sensillar lymph, to finally reach and activate ORs (Leal, 2013). However, OBPs have also been reported to be involved in inactivation of the odor response by removing odorants from the sensillar lymph and the receptors (Vogt and Riddiford, 1981; Ziegelberger, 1995), which then actually enables reactivation of the respective OR (Kaissling, 1986). More recently, Larter et al. (2016) could show in *D. melanogaster* that the deletion of the abundant OBP28a does not cause a reduction of the olfactory response but rather an increase. This indicates a buffering role of this OBP and thus present a potential molecular mechanism of gain control.

Comparative expression data suggest that within the classic OBPs, especially the antenna binding proteins II (ABPII) subgroup has a specific role in olfaction, since all members of *Tribolium*, *Drosophila*, and *Anopheles* are highly expressed and enriched in the antennae

(Dippel et al. 2014). Moreover, this group contains some of the most prominent OBPs such as *Anopheles gambiae Agam*OBP4 that forms cooperative heteromers with other OBPs (Qiao et al. 2011) and *Agam*OBP1 that is co-expressed with other ABPIIs (*Agam*OBP3, *Agam*OBP4, *Agam*OBP19) (Schultze et al. 2013) and mediates indole detection to find blood meals (Biessmann et al. 2010), as well as *D. melanogaster* Lush involved in pheromone detection in trichoid sensilla (Gomez-Diaz et al. 2013; Xu et al. 2005). Lush is at present the most thoroughly investigated OBP and has been demonstrated to bind to the *Drosophila* pheromone 11-*cis*-vaccenyl acetate (Kruse et al. 2003; Laughlin et al. 2008) as well as to other insect pheromones (Katti et al. 2013), short-chain alcohols (Bucci et al. 2006; Thode et al. 2008), and phthalates (Zhou et al. 2004).

The red flour beetle *Tribolium castaneum* (Herbst, Coleoptera, Tenebrionidae) is a major pest of stored products (Sokoloff, 1966) and represents the currently the best coleopteran model organism (Brown et al. 2009) with a number of excellent genetic tools: environmental RNA interference (RNAi) (Bucher et al. 2002; Tomoyasu and Denell, 2004), forward genetics-based insertional mutagenesis (Trauner et al., 2008), transgene-based mis-expression systems (Schinko et al. 2010, 2012), as well as a fully annotated genome sequence (Richards et al. 2008; Kim et al. 2010). These tools dedicate *Tribolium* to investigate findings from *Drosophila* for their generality in insects. In this study, we focused on the functional and expression analysis of *TcasOBP9A* and *TcasOBP9B* which are the two *T. castaneum* OBPs most closely related to the well-studied *D. melanogaster* OBP Lush.

3.3. Material and methods

3.3.1. Red flour beetle *Tribolium* rearing

Tribolium castaneum strain San Bernardino was reared on organic whole wheat flour supplemented with 5% brewers yeast powder in aired transparent plastic boxes at 30°C and 40% relative humidity under a photo regime of 12h light and 12h dark.

3.3.2. Cloning of *TcasOBP9A* and *TcasOBP9B*

To amplify open reading frames (ORFs) of *T. castaneum* OBP9A and OBP9B, PCR using Advantage2Taq polymerase (Clonetech, Mountain View, CA, USA) was performed on total RNA, which was prepared from antennae by using the SMART cDNA synthesis kit (Clonetech, Mountain View, CA, USA) and applying gene specific primers (Table 3-1). These PCR products were cloned into the PCRII vector (Invitrogen, Life Technologies GmbH, Darmstadt, Germany) and confirmed by Sanger sequencing (Macrogen, Seoul, Republic of Korea).

Table 3-1. The primers used for dsRNA synthesis

Forward primer	OBP	Reverse primer
aaaccATGGCTGCGATGTCTG AGGC	*TcasOBP9A EcoRIrev*	TTTGAATTCTCAGGGGAGAAAGTAC TTTTCAGGATTG
aaaccATGGCGATGAGTGAAG CCC	*TcasOBP9B EcoRIrev*	TTTGAATTCTTACGGTAAGAAGTATT TCTCGGGATTATCC

3.3.3. RNA interference

To generate templates for bidirectional in-vitro dsRNA synthesis, a T7 forward and a reverse primer containing a T7 promoter sequence overhang were used in a PCR reaction. The dsRNA was then synthesized by using the T7 Megascript Kit (Ambion, Life Technologies GmbH, Darmstadt, Germany) with the respective PCR fragment as template and following the manufactures protocol. After purification, the resulting RNA was denatured by boiling about 20 minutes and annealing through cooling down to room temperature over 3 hours. The formation of dsRNA was confirmed by gel electrophoresis and the concentration was adjusted to 3 µg/µl with injection buffer (1.4 mM NaCl, 0.07 mM Na2HPO4, 0.03 mM KH2PO4, 4 mM KCL, and 10 % phenol red), using a NanoDrop photometer. To see the combinatorial effects of *TcasOBP9A* and *TcasOBP9B*, the dsRNA concentration was adjusted to 1.5 µg/µl for each OBP, which together made a final dsRNA concentration of 3 µg/µl. The mock dsRNA was made from a PCRII vector containing a 427 bp fragment of dsRed (kindly provided by G. Bucher).

For the injection of dsRNA, the last pupal stage was mounted with double sided adhesive tape on an object slide. Approximately 0.5 µl of the respective dsRNA solution was injected into the conjunctivum between the fourth and fifth abdominal segments of each *T. castaneum* pupae, using a glass capillary coupled to a FemtoJet express (Eppendorf, Hamburg, Germany). The injected pupae were placed in flour filled Petri dishes and stored in an incubator at 30°C until adult beetles emerged, which were then aged before their use for EAG experiments.

3.3.4. Electroantennography

To perform an electroantennography (EAG) recording, we selected the antennally active compounds, 4,8-dimethyldecanal (4,8-DMD), (*E*)-2-heptenal, *cis*-3-hexen-1-ol, β-ionone, 6-

methyl-5-hepten-2-one and 2-hexanone (Balakrishnan et al. 2017). These volatile compounds were selected based on measurable EAG response values, their structural dissimilarities (Figure 3-1), and their biological importance to flour beetles. The selected compounds were all ≥ 96 % pure. The compounds (*E*)-2-heptenal and 2-hexanone from Sigma-Aldrich and β-ionone, 6-methyl-5-hepten-2-one from ABCR (Karlsruhe, Germany) and *cis*-3-hexen-1-ol from SAFC (Germany) were purchased. A well-known *T. castaneum* aggregation pheromone 4,8-DMD was kindly provided by TRÉCÉ Inc. (insect monitoring systems and pheromone, Adair, Oklahoma).

EAG (Syntech, Hilversum, The Netherlands) was used to record the antennal responses of dsRed and dsRNAi-OBP injected beetles to selected volatile compounds. A detailed procedure for EAG recording and odor presentation is described in Balakrishnan et al. (2017). For EAG recording, 10-15 days post injection (DPI) beetles were used for antennal preparation. Selected volatile compounds were diluted in silicone oil M 200 (Carl Roth GmbH + Co. KG, Germany) and dilutions in logarithmic steps 10^{-1} - 10^{-8} (w/w) were prepared. The prepared stimulus apparatus was used to deliver a one-second puff of odor to the antenna. DsRed-injected beetles were used as a negative control. The aggregation pheromone 4,8- DMD at 10^{-3} dilution (w/w) was used as a positive control to monitor for changes in the EAG detection. Intervals between dilution steps and different compound series were one and two minutes respectively. Responses of twelve antennae from different adult beetles of both sexes were recorded for all compounds at each dilution. Three separate runs of each dilution series of odor stimuli were measured with each antenna to compensate for fluctuations of antennal sensitivity with time. Antennal EAG responses in mV were recorded and a dose- response curve was plotted. The recorded EAG signals were amplified, filtered, digitized and analyzed with an EAG program (Syntech, version 2.7, EAG 2000). Significant differences between RNAi-treated and dsRed control beetles were analyzed using Wilcoxon-Mann-Whitney U test. The level of significance was set at $P < 0.05$ (Supplementary Material: Statistical analysis).

3.3.5. Antennal *in situ* hybridization

Synthesis of digoxigenin (DIG) or biotin-labelled RNA probes was conducted using the respective "RNA Labeling Kit (SP6/T7)" (Roche Diagnostics Deutschland GmbH, Mannheim, Germany) with the following modified protocol: 2 µg of linearized recombinant pCRII vector carrying the gene of interest served as template. To this template, 2 µl buffer, 2 µl respective RNA labeling mix and 2 µl SP6 or T7 RNA Polymerase was added. SP6 or T7 RNA

Polymerase was used according to the orientation of the insert and whether a sense or antisense probe was desired to be synthesized. For synthesis of an antisense probe the polymerase that starts at the 5´ side towards the 3´end of the antisense strain was used. Finally, autoclaved MilliQ water was added to a final volume of 20 µl. After an incubation time of 3 h, 1 µl DNaseI (Roche Diagnostics Deutschland GmbH, Mannheim, Deutschland) was added to digest the DNA template. After adding 2.5 µl lithium chloride (5 M) and 75 µl 100 % ethanol, the RNA probe was precipitated via incubation at -80°C for 30 min and subsequent centrifugation at 4°C for 30 min. The obtained RNA probe pellet was washed with 70 % ethanol followed by a second centrifugation step. The dried pellet was dissolved in 50 µl water. By incubation in carbonate buffer (80 mM NaHCO3, 120 mM Na2CO3, pH 10.2) all RNA probes were fragmented to an average length of about 200 bp following the protocol of Angerer and Angerer (1992). Fragmented probes were stored at -20°C in a buffer containing 50 % formamide, 10 % dextran sulfate, 0,2 µg/µl yeast tRNA, 0,2 µg/µl sonicated salmon sperm DNA and 2 x SSC.

Fluorescence in situ hybridization (FISH) on *T. castaneum* antennae was performed as described for *A. gambiae* antennae (Schultze et al. 2013) with several modifications: Unless otherwise noted, all steps were performed in 1.5 ml Eppendorf tubes at room temperature and all used buffers contained 0,03 % Triton X-100 to avoid sticking of the antennae. Beetles were anesthetized on ice before the antennae were severed with forceps. Collected antennae were transferred into ice cold fixation solution (4 % paraformaldehyde in 0.1 M NaCO3, pH 9.5, 0.03 % Triton X-100) and incubated for 24 h at 4°C. The fixated tissue was then put into a silicon form to become embedded in tissue freezing medium ("Tissue-Tek® O.C.T. Compound", Science Services GmbH, München, Germany). To freeze the embedded sample, it was kept at -20°C for 10 min. When completely frozen, the cryotome "Cryostat CM 1950" (Leica, Nussloch, Germany) was used to bisect the antennae by cutting them to slices of a thickness of 50 µm. Subsequently, the frozen slices were collected in a cold Eppendorf tube. 1000 µl PBS were added to melt the tissue freezing medium. After removal of the melted freezing medium, the antennae were washed for 1 min in PBS, followed by a 10 min washing step in 0.2 M HCl and 1 min incubation in PBS + 1 % Triton X-100. Afterwards, the antennae were kept in the hybridization solution (50 % formamide, 5x SSC, 1x denhardt's reagent, 50 µg/ml yeast RNA, 1 % Tween 20, 0.1 % Chaps, 5 mM EDTA, pH 8.0) for 1 to 10 days at 4°C. Prehybridization was achieved by heating the antenna to 55°C for 5 h before adding the desired probes. Probes were diluted 1:50 or 1:100 (depending on the expression level of the transcript that had to be labelled) in hybridization solution (hyb). To ensure that the probe is single

stranded the probe/hyb solution was heated to 65°C for 5 min. Subsequently, the probe/hyb solution was chilled on ice for 10 min before it was added to the antennae. After probe incubation for 3 days at 55°C, the antennae were washed four times for 15 min each in 0,1x SSC at 60°C. In order to block unspecific binding sites for the subsequent antibody incubation the antennae were treated with "Blocking reagent" (Roche Diagnostics Deutschland GmbH, Mannheim, Germany) for 5 h at 4°C. For the detection of DIG-labelled probes, Fab fragments of anti-digoxigenin-AP antibodies (Roche Diagnostics Deutschland GmbH, Mannheim, Germany) were used, diluted 1:500 in blocking reagent. To detect biotin-labelled probes, a Streptavidin-HRP conjugate (PerkinElmer, Rodgau, Germany) was added to the blocking reagent. In case of a desired nuclear staining of the samples, DAPI was added in a dilution of 1: 1000. After 3 days of incubation at 4°C, the antennae were washed 5 x 10 min with TBS + 0.05 % Tween 20. To visualize transcripts labelled with DIG-probes, the "HNPP Fluorescent Detection Set" (Roche Diagnostics Deutschland GmbH, Mannheim, Germany) was used. The incubation time was 1 h to 3 h at room temperature, depending on the applied probe. Biotin-labelled probes were visualized by using the "TSA™, Fluorescein System" or the "TSA™ Plus Fluorescein System" (PerkinElmer, Rodgau, Germany) after washing the respective samples three times for 5 min each in TBS. The incubation time was 3 h at room temperature. Finally, antennae were washed three times for 5 min each in TBS and transferred to PBS before they were embedded in "Mowiol® 4-88" (Sigma-Aldrich Chemie GmbH, München, Germany). The embedded samples were stored at -20°C and analyzed via microscopy.

3.3.6. Microscopy and image processing

Embedded *T. castaneum* antennae that had been subjected to FISH were analyzed with a "Zeiss LSM780 laser scanning microscope" (Carl Zeiss Microscopy GmbH, Jena, Germany) using a 405 nm, 488 nm, and a 561 nm laser. Confocal image stacks were taken from single antennal segments. These stacks were projected to a single picture. If desired, several of these were arranged to show coherent antennal stretches using "PowerPoint" (Microsoft, Unterschleißheim, Germany).

3.3.7. Phylogenetic analysis and interspecies comparison

After subtraction of the signal peptide (SignalP4.1; Petersen et al. 2011) the sequences were aligned using MAFFT (v7.040b; Katoh et al., 2005) as described in (Viera and Rozas, 2011) and the tree was constructed using RAxML (version 7.8.6; Stamatakis, 2006) with WAG substitution model and GAMMA correction. The phylogenetic tree was visualized by iTOL

(Letunic and Bork, 2007) and descriptions were added using inkscape (Harrington et al. last visited Feb. 13th, 2019).

3.4. Results and Discussion

3.4.1. *TcasOBP9A* and *TcasOBP9B* enhance detection of a broad spectrum of volatiles

In order to identify whether *TcasOBP9A* and *TcasOBP9B* facilitate the detection of specific odorants, we employed EAG to examine the antennal response of dsRNA-mediated RNAi-treated beetles to selected volatile compounds (with dsRed as mock control). Our initial EAG screening of 94 compounds (Balakrishnan et al. 2017) identified six volatile organic compounds (VOCs) to be readily detected by *T. castaneum*: 4,8-DMD, (*E*)-2-heptenal, *cis*-3-hexen-1-ol, β-ionone, 6-methyl-5-hepten-2-one and 2-hexanone. To identify the time window of the RNAi-mediated knock-down, we used *dsTcasOBP9A*-injected female beetles from three different time points after injection for EAG recording: 10-15 DPI, 20-25 DPI and 30-35 DPI. This preliminary work (Supplementary Figure 3-1) revealed that the dsRNA injected beetles showed highly significant EAG response reduction at 10-15 DPI to (*E*)-2-heptenal, 2-hexanone, 4,8-DMD, β-ionone, 6-methyl-5-hepten-2-one at all tested dilutions (10^{-1}-10^{-6}). This effect was reduced at 20-25 DPI and gone at 30-35 DPI (Supplementary Figure 3-1) assumingly because of the high transcription rate of OBPs in the auxiliary cells, which titers out the RNAi effect. Therefore, we used 10-15 DPI beetles for further experiments, in which we silenced *TcasOBP9A*, *TcasOBP9B*, and both genes by dsRNA-mediated RNAi. In order to highlight the dose-dependent reduction of EAG-responses after RNAi-treatment to the selected compounds, we used a wide dilution range 10^{-1}-10^{-8} ($\log_{10}$ dilution in silicone oil).

Unexpectedly, the EAG response to all these chemically very diverse odorants, was significantly reduced already when *TcasOBP9A* was knocked-down by RNAi (Figures 3-1 and 3-2). In males, knock-down of *TcasOBP9B* was less effective and the double knock-down revealed a similar outcome as the *TcasOBP9A* single knock-down (Figure 3-1). In females also the *TcasOBP9B* knock-down caused a significant reduction to all tested VOCs (Figure 3-2). Therefore, these highly abundant OBPs (Dippel et al. 2014) do not seem to provide any specificity to the olfactory process, but they enhance the detection of diverse VOCs in an unspecific manner.

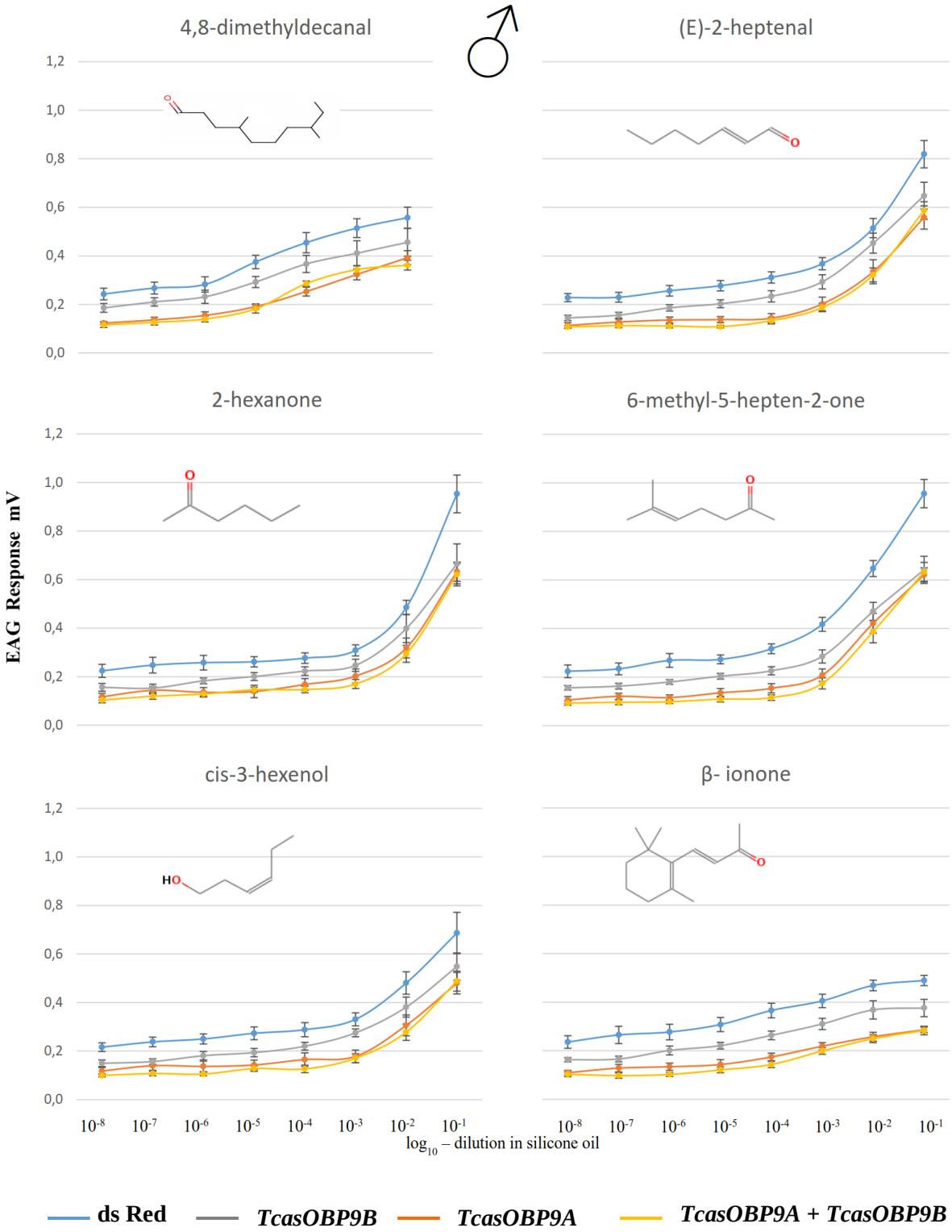

Figure 3-1. Electroantennographic (EAG) responses of dsRNA-treated and dsRed control male *T. castaneum* beetle to six chemicals. EAG responses of male beetles injected with dsRed, dsRNA-*TcasOBP9A*, *TcasOBP9B* and *TcasOBP9A* + *TcasOBP9B* to the olfactory stimulants 4,8-dimethyldecanal, (*E*)-2-heptenal, 2-hexanone, 6-methyl-5-hepten-2-one, *cis*-3-hexen-1-ol and β-ionone. The antennal response of beetles to eight $\log_{10}$ dilutions (10^{-1}-10^{-8}) of aforementioned compounds were recorded, except 4,8-dimethyldecanal (10^{-2} -10^{-8}). Error bars indicate standard error of the mean (SEM). n=6.

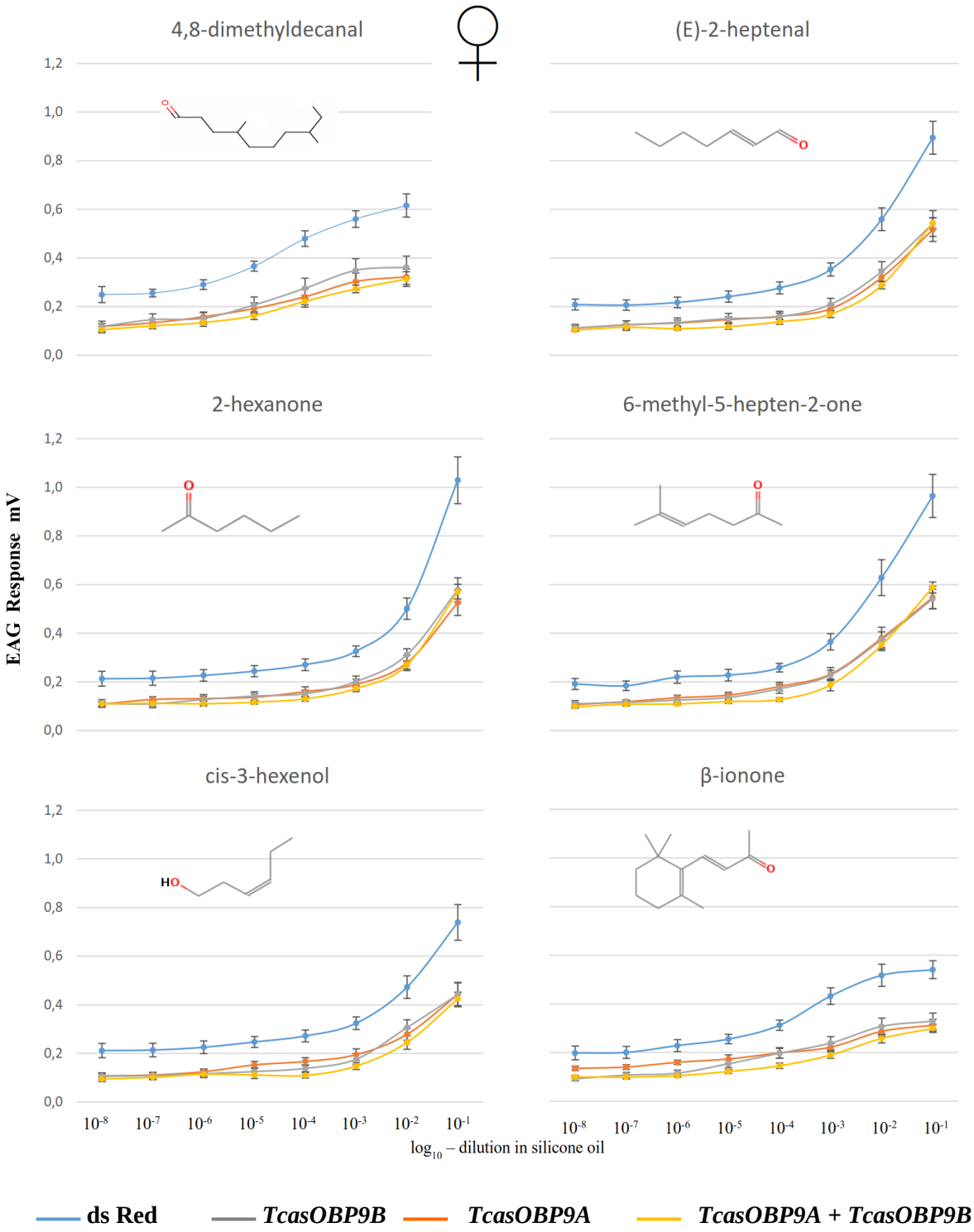

Figure 3-2. Electroantennographic (EAG) responses of dsRNA-treated and dsRed control female *T. castaneum* beetle to six chemicals. EAG responses of female beetles injected with dsRed, dsRNA-*TcasOBP9A*, *TcasOBP9B* and *TcasOBP9A* + *TcasOBP9B* to the olfactory stimulants 4,8-dimethyldecanal, (*E*)-2-heptenal, 2-hexanone, 6-methyl-5-hepten-2-one, *cis*-3-hexen-1-ol and β-ionone. The antennal response of beetles to eight $\log_{10}$ dilutions (10^{-1}-10^{-8}) of aforementioned compounds were recorded, except 4,8-dimethyldecanal (10^{-2} -10^{-8}). Error bars indicate standard error of the mean (SEM). n=6.

3.4.2. Mutual exclusive antennal expression of *TcasOBP9A* and *TcasOBP9B*

Based on quantitative transcriptomics data, *TcasOBP9A* and *TcasOBP9B* belong to the most abundantly expressed OBPs in the *T. castaneum* antenna of both sexes (Dippel et al. 2014). However, these data do not allow to answer the question, whether these OBPs are expressed in all or only a subset of olfactory sensilla. Therefore, we performed FISH experiments to identify the exact pattern of antennal expression for *TcasOBP9A* and *TcasOBP9B* (Figure 3-3). Whereas *TcasOBP9A* is only expressed in the terminal 11th segment of the antenna (Figure 3-3A), *TcasOBP9B* is expressed in all three olfactory responsive club segments (9th -11th segments) (Figure 3-3B). Double FISH with *TcasOBP9B* and *TcasOrco* as a marker for olfactory sensory neurons (Dippel et al. 2016) revealed the distally adjacent location of the auxiliary cells to the olfactory sensory neuron somata (Figure 3-3C) and thereby confirmed the arrangement already described by Roth and Willis (1951). In contrast to other insects, both the auxiliary cells as well as the olfactory sensory neuron somata are located not directly beneath the sensilla cuticle but rather more proximally within the club segments.

Double FISH with *TcasOBP9A* and *TcasOBP9B* indicates that there is no overlap of expression (Figure 3-3D, 3E, 3F). While *TcasOBP9A* is expressed more centrally in the terminal segment, *TcasOBP9B* is expressed radially in all three olfactory club segments. This expression pattern could correlate with the distribution of trichoid sensilla centrally in the terminal segment and basiconic sensilla radially in segments 9-11 (Dippel at al. 2016). Therefore, it is likely that these two genes are mutually exclusively expressed in different types of sensilla. This is somewhat surprising, as the two genes resemble a gene pair directly neighboring each other on the ninth chromosome with only a 1.5 kb intergenic region between them (Dippel et al. 2014).

3.4.3. Phylogeny of *TcasOBP9A* and *TcasOBP9B*

By comparing OBPs only across different insect orders (Dippel et al. 2014), a detailed picture of gene duplication events cannot be provided. Therefore, we carried out a phylogenetic analysis for specifically Lush-related OBPs across a variety of insect orders but also including a large set of coleopteran species (Figure 3-4). This analysis shows, that specific *TcasOBP9A* and *TcasOBP9B* orthologs can be identified across Cucujiform beetles, which indicates that the gene duplication event must date to about 200 Mio years ago (Zhang et al. 2018). Thus, despite the close chromosomal localization and the best homology within *T. castaneum*, this is not a gene pair derived from a recent gene duplication. There was much time for those genes to gain independent expression. In this respect, it is interesting to note that *TcasOBP9A* expression is restricted to the head, whereas *TcasOBP9B* is also significantly expressed in the

legs (Dippel et al. 2014), which resembles the expression of the respective orthologues in the sweet potato weevil *Cylas formicarius*, *CforOBP11* and *CforOBP4* (Bin et al. 2017).

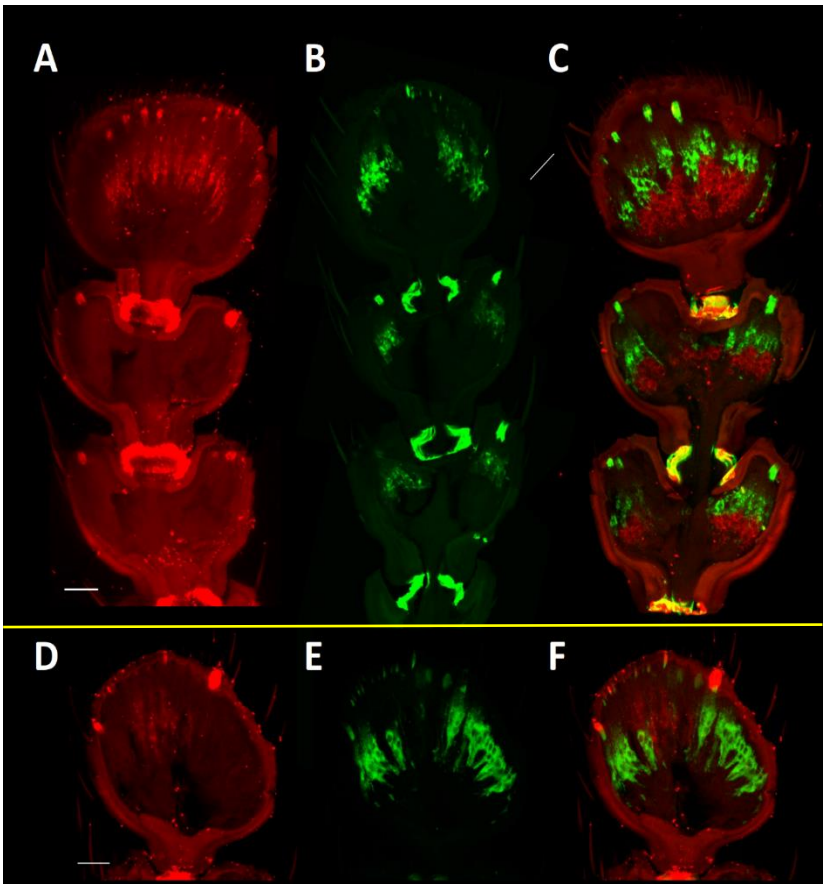

Figure 3-3. Expression of *TcasOBP9A* and *TcasOBP9B* in *T. castaneum* antennae. Depicted are projected image stacks of a stretch of segments 9 – 11 (A-C), which represent the olfactory responsive club segments (Dippel at al. 2016), or the terminal segment 11 (D-F) after single or double FISH. (**A**) Digoxigenin-labelled probe targeting *TcasOBP9A* transcripts visualized by the HNPP/FastRed detection system for digoxigenin-labelled probes (red fluorescence). (**B**) Biotin-labelled probe targeting *TcasOBP9B* transcripts visualized by the TSA detection system for biotin-labelled probes (green fluorescence). (**C**) Double FISH with a digoxigenin-labelled probe targeting *TcasOrco* transcripts (Dippel et al., 2016) and a biotin-labelled probe targeting *TcasOBP9B* transcripts. Hybridized transcripts were visualized by the HNPP/FastRed detection system for digoxigenin-labelled probes (red fluorescence) followed by the TSA detection system for biotin-labelled probes (green fluorescence). Overlay of both fluorescence channels is shown. (**D**-**F**) Double FISH with a digoxigenin-labelled probe targeting *TcasOBP9A* transcripts and a biotin-labelled probe targeting *TcasOBP9B* transcripts. Hybridized transcripts were visualized by the HNPP/FastRed detection system for digoxigenin-labelled probes (red fluorescence, D) followed by the TSA detection system for biotin-labelled probes (green fluorescence, E). The separate fluorescence channels (A and B) and the overlay of both fluorescence channels are shown in (F). Please note that the strong fluorescence between single segments and at the segments distal cuticle where the olfactory sensilla rise is non-specific. Scale bar is 20 µm.

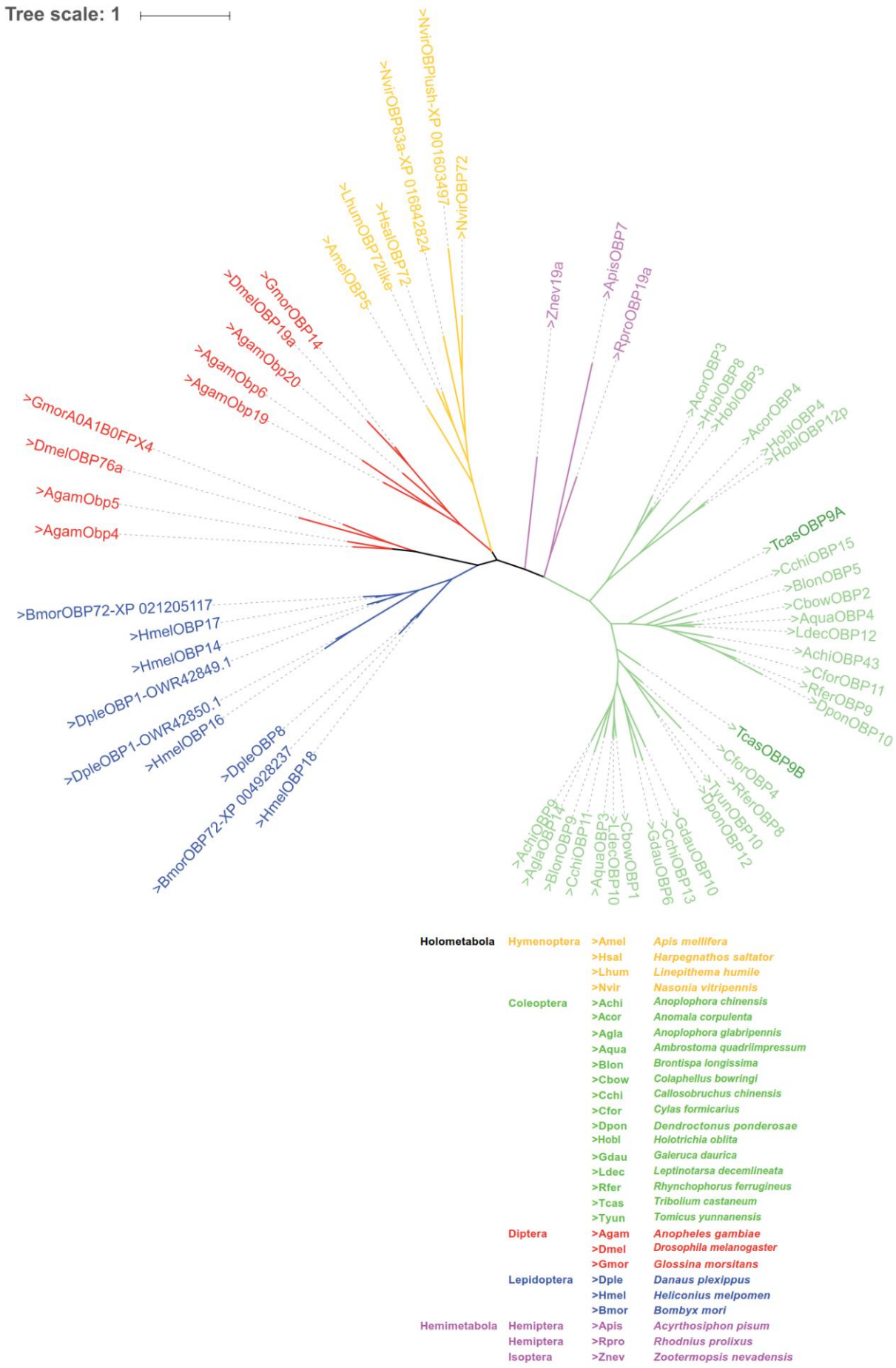

Figure 3-4. Phylogenetic tree of *TcasOBP9A* and *TcasOBP9B* homologs.

However, since the *TcasOBP9A* and *TcasOBP9B* gene pair is not the result of a recent gene duplication and their expression has become mutual exclusive, the reason for their still close sequence homology must lie in a common function. For some Lush orthologs (Figure 3-4), it has been shown that they can form functional heterodimers that bind odorants better than single OBPs: e.g. *AgamOBP4* (Andronopoulou et al. 2006; Qiao et al. 2011) and the homologue *HoblOBP4* of the scarab beetle *Holotrichia oblita* (Wang et al. 2013). Both *AgamOBP4* and *HoblOBP4* can bind to a wide variety of VOCs (Qiao et al. 2011; Wang et al. 2013) and *AgamOBP4* has been shown to be abundantly expressed in many sensilla and to be co-expressed with other OBPs in specific sensilla (Schultze et al. 2013). Such a function as a kind of Co-OBP would also be consistent for *TcasOBP9A* and *TcasOBP9B* with their abundant expression and the enhancement of detection of very diverse odorants. Future analysis of the detailed expression of more *T. castaneum* OBPs (Dippel et al. 2014) will reveal potential co-expression with *TcasOBP9A* or *TcasOBP9B* and thus identify candidates for the study of heterodimer formation in this beetle.

3.5. Acknowledgements

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3.7. Supplementary material: Supplementary Figure 3-1

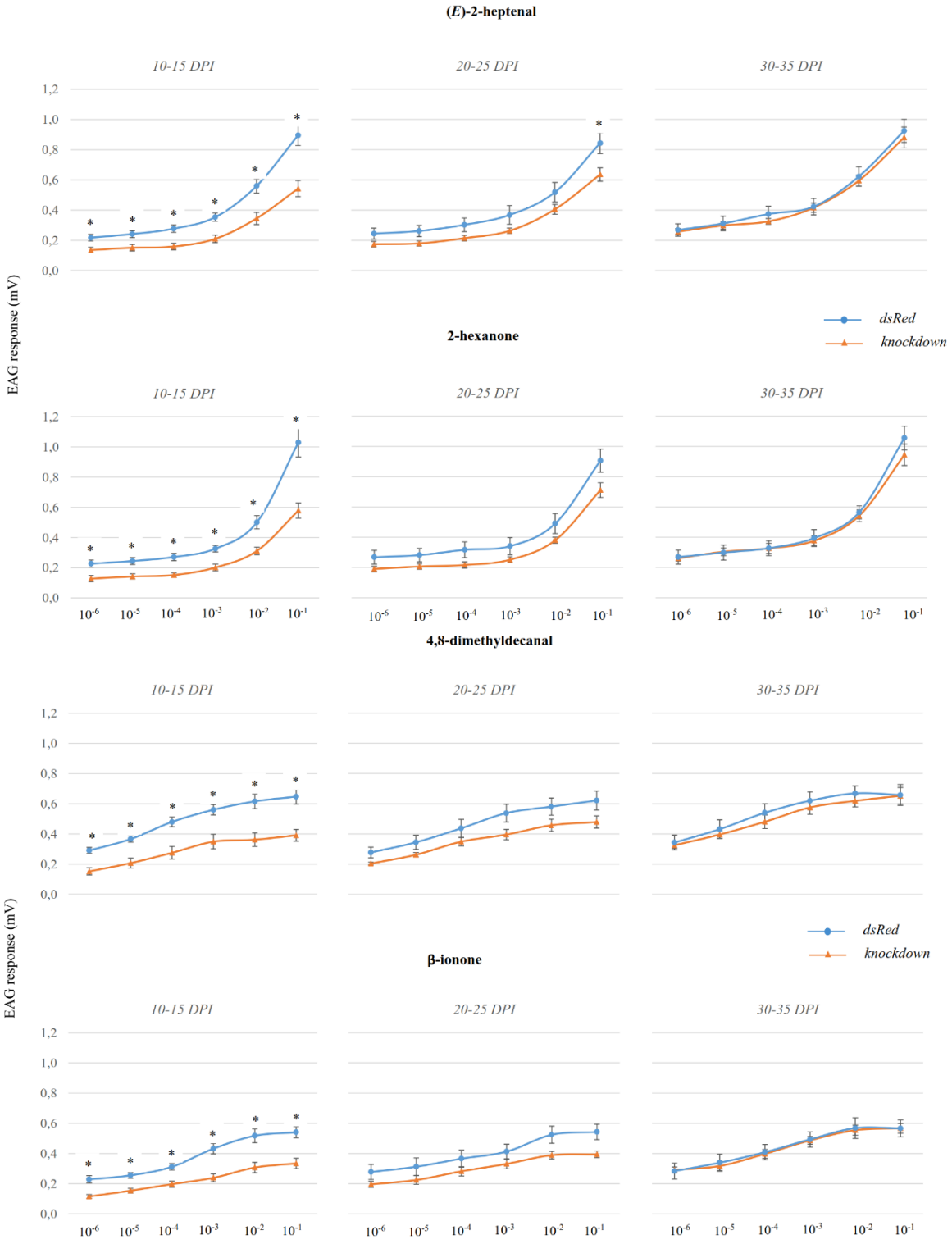
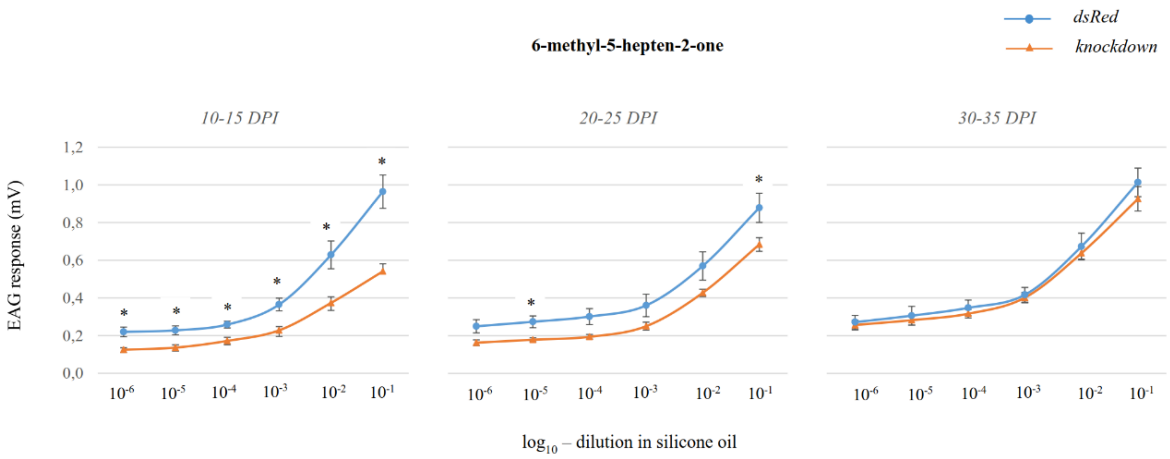


Figure continued…..



Supplementary Figure 3-1.

EAG responses of female *T. castaneum* beetles injected with dsRed and dsRNA-*TcasOBP9A* to the olfactory stimulants (*E*)-2-heptenal, 2-hexanone, 4,8-dimethyldecanal, β-ionone, 6-methyl-5-hepten-2-one and measured at three different days post injections (DPI) periods. The antennal response of beetles to six log_{10} dilutions (10^{-1} -10^{-6}) of aforementioned compounds were recorded. Error bars indicate standard error of the mean (SEM). *- indicate significant EAG response reduction due to *TcasOBP9A* knockdown. (Wilcoxon-Mann-Whitney U test $P < 0.05$, n=6).

Supplementary Table 3-1A. Statistical Analysis

Wilcoxon Mann Whitney U Test (P ≤ 0.05)

Male beetle (N=6)								
***TcasOBP9A* Vs ds Red**								
Chemicals	**10^{-8}**	**10^{-7}**	**10^{-6}**	**10^{-5}**	**10^{-4}**	**10^{-3}**	**10^{-2}**	**10^{-1}**
4,8-dimethyldecanal	0.0051	0.0051	0.0131	0.0051	0.0051	0.0083	0.0203	n.r
(*E*)-2-heptenal	0.0051	0.0051	0.0051	0.0051	0.0051	0.0083	0.0657	0.0051
2-hexanone	0.0131	0.0308	0.0203	0.0203	0.0308	0.0308	0.0131	0.0051
6-methyl-5-hepten-2-one	0.0083	0.0131	0.0051	0.0051	0.0051	0.0051	0.0051	0.0051
cis-3-hexenol	0.0051	0.0203	0.0131	0.0131	0.0203	0.0083	0.0308	0.0657
β-ionone	0.0051	0.0131	0.0131	0.0051	0.0051	0.0051	0.0051	0.0051
***TcasOBP9B* Vs ds Red**								
4,8-dimethyldecanal	0.1096	0.1285	0.2301	0.6576	0.1738	0.1738	0.1738	n.r
(*E*)-2-heptenal	0.0051	0.0131	0.0455	0.0308	0.0455	0.0455	0.0929	0.0308
2-hexanone	0.0308	0.0308	0.0657	0.0455	0.1285	0.1285	0.0657	0.0455
6-methyl-5-hepten-2-one	0.0929	0.0657	0.0455	0.0203	0.0131	0.0203	0.0308	0.0203
cis-3-hexenol	0.0203	0.0083	0.0455	0.0455	0.0657	0.0929	0.0929	0.1285
β-ionone	0.0929	0.0455	0.1285	0.0657	0.0308	0.0455	0.0657	0.0657
***TcasOBP9A* + *TcasOBP9B* Vs ds Red**								
4,8-dimethyldecanal	0.0051	0.0051	0.0051	0.0051	0.0051	0.0083	0.0051	n.r
(*E*)-2-heptenal	0.0051	0.0051	0.0051	0.0051	0.0051	0.0051	0.0051	0.0083
2-hexanone	0.0051	0.0083	0.0051	0.0051	0.0051	0.0051	0.0051	0.0131
6-methyl-5-hepten-2-one	0.0051	0.0051	0.0051	0.0051	0.0051	0.0051	0.0051	0.0051
cis-3-hexenol	0.0051	0.0051	0.0051	0.0051	0.0051	0.0051	0.0131	0.0929
β-ionone	0.0051	0.0051	0.0051	0.0051	0.0051	0.0051	0.0051	0.0051

Highly significant - <0.000-0.009

Significant - <0.010-<0.05

No significant – >0.05

Supplementary Table 3-1B. Statistical Analysis

Wilcoxon Mann Whitney U Test (P ≤ 0.05)

Female beetles (N=6)								
***TcasOBP9A* Vs ds Red**								
Chemicals	**10^{-8}**	**10^{-7}**	**10^{-6}**	**10^{-5}**	**10^{-4}**	**10^{-3}**	**10^{-2}**	**10^{-1}**
4,8-dimethyldecanal	0.0051	0.0051	0.0051	0.0051	0.0051	0.0051	0.0051	n.r
(*E*)-2-heptenal	0.0083	0.0203	0.0131	0.0051	0.0083	0.0051	0.0131	0.0083
2-hexanone	0.0083	0.0083	0.0083	0.0051	0.0203	0.0051	0.0051	0.0051
6-methyl-5-hepten-2-one	0.0203	0.0203	0.0164	0.0131	0.0308	0.0308	0.0308	0.0083
cis-3-hexenol	0.0051	0.0051	0.0051	0.0051	0.0051	0.0131	0.0051	0.0131
β-ionone	0.0929	0.0308	0.0083	0.0203	0.0131	0.0051	0.0051	0.0051
***TcasOBP9B* Vs ds Red**								
4,8-dimethyldecanal	0.0203	0.0203	0.0083	0.0164	0.0203	0.0308	0.0083	n.r
(*E*)-2-heptenal	0.0083	0.0203	0.0203	0.0203	0.0131	0.0083	0.0203	0.0083
2-hexanone	0.0203	0.0203	0.0203	0.0131	0.0051	0.0131	0.0083	0.0083
6-methyl-5-hepten-2-one	0.0308	0.0203	0.0131	0.0131	0.0203	0.0203	0.0131	0.0083
cis-3-hexenol	0.0051	0.0131	0.0083	0.0051	0.0051	0.0051	0.0657	0.0203
β-ionone	0.0051	0.0083	0.0051	0.0131	0.0131	0.0051	0.0131	0.0131
***TcasOBP9A* + *TcasOBP9B* Vs ds Red**								
4,8-dimethyldecanal	0.0051	0.0051	0.0051	0.0051	0.0051	0.0051	0.0051	n.r
(*E*)-2-heptenal	0.0051	0.0131	0.0051	0.0051	0.0051	0.0051	0.0051	0.0083
2-hexanone	0.0308	0.0131	0.0051	0.0051	0.0051	0.0051	0.0051	0.0051
6-methyl-5-hepten-2-one	0.0083	0.0131	0.0051	0.0051	0.0051	0.0083	0.0131	0.0051
cis-3-hexenol	0.0083	0.0131	0.0051	0.0051	0.0051	0.0051	0.0051	0.0051
β-ionone	0.0051	0.0051	0.0051	0.0051	0.0051	0.0051	0.0051	0.0051

Highly significant - <0.000-0.009
Significant - <0.010-<0.05
No significant – >0.05
Not Recording – n.r

Supplementary Table 3-1C. Statistical Analysis

Wilcoxon Mann Whitney U Test ($P \leq 0.05$)

Male beetles (N=6)								
***TcasOBP9A* Vs ds Red**								
Chemicals	**10^{-8}**	**10^{-7}**	**10^{-6}**	**10^{-5}**	**10^{-4}**	**10^{-3}**	**10^{-2}**	**10^{-1}**
4,8-dimethyldecanal	**	**	*	**	**	**	*	n.r
(*E*)-2-heptenal	**	**	**	**	**	**	n.s	**
2-hexanone	*	*	*	*	*	*	*	**
6-methyl-5-hepten-2-one	**	*	**	**	**	**	**	**
cis-3-hexenol	**	*	*	*	*	**	*	n.s
β-ionone	**	*	*	**	**	**	**	**
***TcasOBP9B* Vs ds Red**								
4,8-dimethyldecanal	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.r
(*E*)-2-heptenal	**	*	n.s	*	n.s	n.s	n.s	*
2-hexanone	*	*	n.s	n.s	n.s	n.s	n.s	n.s
6-methyl-5-hepten-2-one	n.s	n.s	n.s	*	*	*	*	*
cis-3-hexenol	*	**	n.s	n.s	n.s	n.s	n.s	n.s
β-ionone	n.s	n.s	n.s	n.s	*	n.s	n.s	n.s
***TcasOBP9A* + *TcasOBP9B* Vs ds Red**								
4,8-dimethyldecanal	**	**	**	**	**	**	**	n.r
(*E*)-2-heptenal	**	**	**	**	**	**	**	**
2-hexanone	**	**	**	**	**	**	**	*
6-methyl-5-hepten-2-one	**	**	**	**	**	**	**	**
cis-3-hexenol	**	**	**	**	**	**	*	n.s
β-ionone	**	**	**	**	**	**	**	**

Highly significant - <0.000-0.009 - **
Significant - <0.010-<0.05- *
No significant – >0.05- n.s
Not Recording- n.r

Supplementary Table 3-1D. Statistical Analysis

Wilcoxon Mann Whitney U Test (P ≤ 0.05)

Female beetle (N=6)								
***TcasOBP9A* Vs ds Red**								
Chemicals	**10^{-8}**	**10^{-7}**	**10^{-6}**	**10^{-5}**	**10^{-4}**	**10^{-3}**	**10^{-2}**	**10^{-1}**
4,8-dimethyldecanal	**	**	**	**	**	**	**	n.r
(*E*)-2-heptenal	**	*	*	**	**	**	*	**
2-hexanone	**	**	**	**	*	**	**	**
6-methyl-5-hepten-2-one	*	*	*	*	*	*	*	**
cis-3-hexenol	**	**	**	**	**	*	**	*
β-ionone	n.s	*	**	*	*	**	**	**
***TcasOBP9B* Vs ds Red**								
4,8-dimethyldecanal	*	*	**	*	*	*	**	n.r
(*E*)-2-heptenal	*	*	*	*	*	**	*	**
2-hexanone	*	*	*	*	**	*	**	**
6-methyl-5-hepten-2-one	*	*	*	*	*	*	*	**
cis-3-hexenol	**	*	**	**	**	**	n.s	*
β-ionone	**	**	**	*	*	**	*	*
***TcasOBP9A* + *TcasOBP9B* Vs ds Red**								
4,8-dimethyldecanal	**	**	**	**	**	**	**	n.r
(*E*)-2-heptenal	**	*	**	**	**	**	**	**
2-hexanone	*	*	**	**	**	**	**	**
6-methyl-5-hepten-2-one	**	*	**	**	**	**	*	**
cis-3-hexenol	**	*	**	**	**	**	**	**
β-ionone	**	**	**	**	**	**	**	**

Highly significant - <0.000-<0.009- **
Significant - >0.010-<0.05- *
No significant – >0.05- n.s
Not Recording – n.r

CHAPTER 4

Monitoring crop disease markers by odorant binding proteins of *Tribolium castaneum* – Electroantennogram versus reduced graphene-oxide based electronic biosensor

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Status: "in preparation for re-submission to the journal PNAS"

Contribution to this paper

Balakrishnan K: I performed the EAG experiments, analysed them and prepared the EAG graphical data, reviewing and editing the manuscript

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4.1. Abstract

Early stage detection of crop disease is highly desirable for economical, ecological and food safety reasons. In this study, the method of choice is a sensor based detection of low concentrations of volatile disease markers. Inspired by insect olfaction, the latest generation of biosensors relies on the binding of these markers to odorant binding proteins, functionally coupled to a technical transducer.

Along these lines, we recently established a reduced graphene-oxide field effect transistor, functionalized by an odorant binding protein, for real-time detection of unlabeled volatiles. Here we report on the use of this high-throughput analytical tool, capable of detecting small, low affinity ligands in a reversible, concentration dependent manner, for screening the insect odorant binding proteins for its interactions with ligands of wheat and maize disease markers. The OBPs *TcasOBP9A* and *TcasOBP9B* from the red flour beetle *Tribolium castaneum* were identified as capture molecules for the analytes 6-methyl-5-hepten-2-one, and 3-octanol. This is the key for a bioelectro-interfacial nano-sensor, towards future agricultural applications.

Deciphering the odorant's capability i) to interact with odorant binding proteins and ii) to activate the odorant receptor (OR) for insect's olfactory response of the antenna was investigated with two biosensors, *in-vitro* and *in-vivo*. The *in-vitro* method is an OBP-functionalized rGO-FET based sensor which monitors the odorant interaction with the odorant binding protein. An *in-vivo* orthogonal method was able to identify the odorant´s capability to activate the complex natural olfactory system to get insect's olfactory response of the antenna.

Keywords: Graphene-oxide field effect transistor, Odorant binding proteins, Electroantennogram, Electronic biosensor

4.2. Introduction

The red flour beetle *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) is an economically important pest for stored products, infesting granaries, flour storage and food grain processing units all over the world causing damages of billions of dollars every year (APHLIS, 2011; Cao et al. 2002). The beetle is conspicuous of attacking grains that are slightly damaged and show early symptoms of disease (Ahmad et al. 2012). Grains in living plants showing severe disease symptoms repel the beetles by plant defence compounds and grains in stored conditions that deteriorated by fungi, producing mycotoxins act as an infochemicals to the beetles in order to avoid intoxication (Dunkel, 1988). Thus, olfaction of the *Tribolium* beetle might provide binding proteins for marker compounds related to early stages of plant diseases in important crops like wheat and maize. Such a marker compound can help to early detection of symptoms of plant disease in the field and also detection of early symptoms on stored products, which is prone to insect attack. Reducing crop diseases in the field and in the stored products are important strategies to fight against poverty and hunger in the world. These are key steps to avoid food famine and develop toward a sustainable food production and safe food supply throughout the year (www.un.org/millenniumgoals).

The whole genome of *T. castaneum* is annotated and provides an excellent starting position for screening the insect‘s proteome for identifying proteins that bind marker compounds of interest (Richards et al. 2008; Li et al. 2013). The molecular structure and the amino acid sequence of the highly homologous odorant binding proteins (OBPs) *TcasOBP9A* and *TcasOBP9B* are given in Figure 4-1 from *T. castaneum*. These two OBPs were selected, because of high expression levels in the antennae, suggested an important role in odorant reception of this beetle (olfaction) (Li et al. 2013; Dippel et al. 2014).

As a proof of principle, two C-8 marker compounds 6-methyl-5-hepten-2-one and 3-octanol with different chemical structure and different biochemical pathways were selected from a list of 94 compounds known to be detected by *T. castaneum* (Balakrishnan et al. 2017). The compound 6-methyl-5-hepten-2-one, a branched unsaturated C-8 ketone being a degradation product of terpenes and thus indicating breakdown of plant defenses. Similarly, the compound 3-octanol, a linear saturated secondary C-8 alcohol being a degradation product of lipids and indicating the activity of fungi (Holighaus et al. 2014) and thus potential presence

of mycotoxins (Sherif, 2016). The RNA-interference knocking down of both above mentioned proteins rendered a massive loss of sensitivity of *T. castaneum* antennae to one of selected C-8 marker compounds (6-methyl-5-hepten-2-one) (Montino, Balakrishnan et al., in preparation), related to early stages of plant diseases in important crops like wheat and maize.

To this end, we introduce a technical sensing platform for real-time and high-throughput, quantitative analysis of protein-ligand interactions based on reduced graphene oxide-field effect transistor (rGO-FET) sensing (Larisika et al. 2015). In this study, we report the detection of selected odorant molecules with reduced graphene-oxide based electronic biosensor functionalized with selected *TcasOBP*, as *in-vitro*. Additionally, as the antennal response of *T. castaneum* to selected compounds is considered the final step of the natural olfactory system, results from the artificial sensor rGO-FET were partially compared with antennal response of *T. castaneum* to tested volatile compounds, as *in-vivo*.

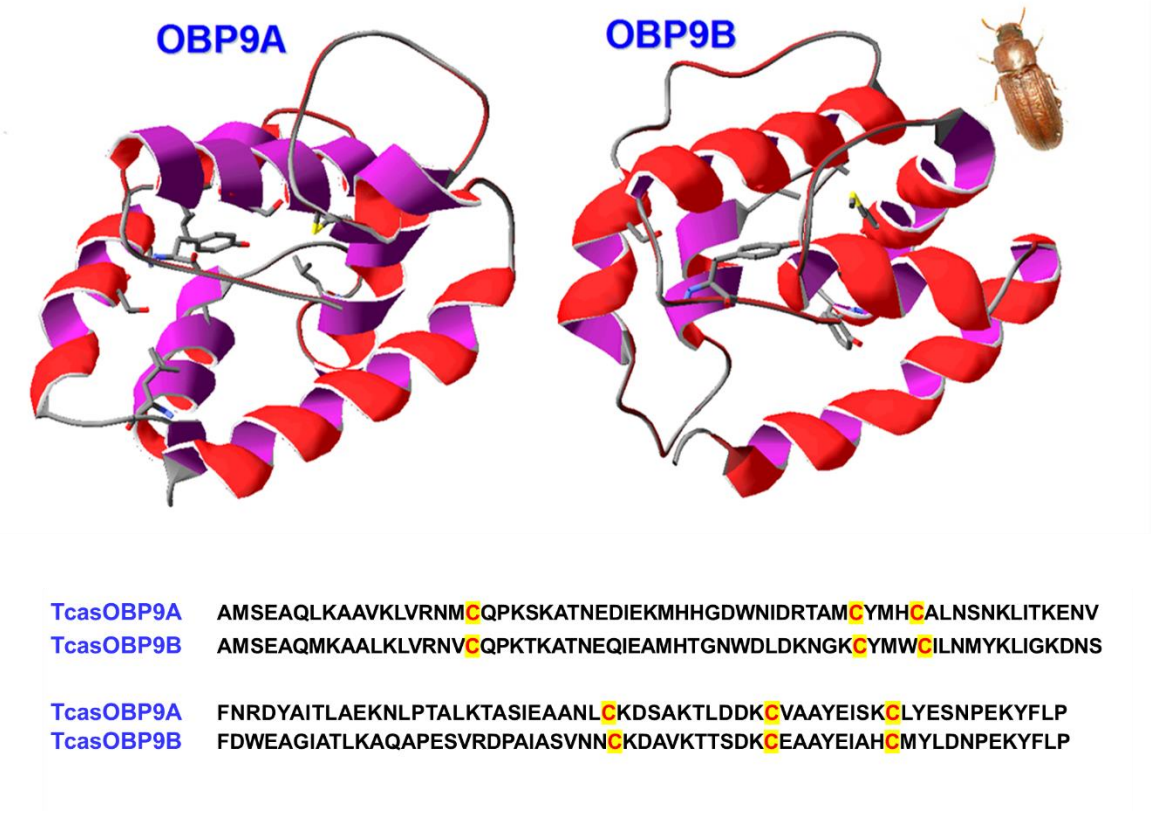


Figure 4-1. Odorant binding proteins of *Tribolium castaneum*. Differences of the amino acid sequence and the resulting tertiary structure of the two odorant binding proteins, *TcasOBP9A* and *TcasOBP9B*, respectively, from the red flour beetle, *T. castaneum*.

4.3. Experimental procedures

4.3.1. Preparation of the FET sensors and their electrical characterization

4.3.1.1. Preparation of large graphene oxide (GO) sheets

Graphene oxide (GO) was prepared by using a modified Hummers/Offeman method (Hummers and Offeman, 1958). In a typical experiment flake graphite (1 g, 125–250 μm) (Su et al. 2009) and sodium nitrate (1 g) were mixed with concentrated sulfuric acid (30 ml, 98 %). The suspension was mixed in an overhead shaker for 1 h at 25 °C (RT). Subsequently, potassium permanganate (3 g) was interspersed over a period of 3 h and the reaction was kept shaking for 12 h at RT. Thereafter, the mixture was slowly poured into ice-cooled deionized water (30 ml) and hydrogen peroxide (30 %) was then added until the suspension turned golden. After centrifugation, it was dialyzed until the conductivity of the washing water was below 2 μS/cm. The low ionic strength is essential to achieve delamination of GO by osmotic swelling.

In a second approach, we replaced the last steps by skimming the GO from the water-air interface, diluting it directly in 0.5 l distilled H_2O, and stirring this solution at a low velocity (250 rpm) for 2-3 days in order to obtain better exfoliation results.

4.3.1.2. Preparation of rGO-FETs

The employed protocol followed a procedure summarized before (Larisika et al. 2015). Briefly, silicon substrates with a 300 nm oxide layer were chosen as basal layer for the FETs. The SiO_2 substrates were cleaned with a standard RCA cleaning procedure. The substrates were then submerged in a 2 % aminopropyltriethoxysilane (APTES) solution in ethanol for 1 h; APTES forming a self-assembled monolayer used to increase the adsorption of graphene oxide sheets. After rinsing with ethanol, the substrates were heated to 120 °C for two hours and afterwards cooled to room temperature. The graphene oxide flakes were applied onto the APTES-modified Si-Wafer via drop casting of the supernatant of the graphene-oxide solution. The devices were then treated in hermetically sealed glass petri dishes with hydrazine at 70 °C overnight to accomplish the graphene-oxide reduction. Flake distribution was first checked with an optical microscope and selected devices then characterized using scanning electron microscope (SEM) (Figure 4-2).

4.3.1.3. Characterization of the electrical performance of the devices

Electrodes were applied consisting of gold (60-100 nm) with an adhesive layer of chromium (2-3 nm) using a standard evaporation process with a shadow mask. A chip-holder was designed for this process, ensuring the central positioning of the electrodes as well as reducing electrode geometry glazing because of unwanted shadow offset during the evaporation process. Success of the used reduction technique via hydrazine was probed as described before (Larisika et al. 2012). In order to assure a sufficient conductivity of used graphene layers, all devices displaying higher conductivity than ~800 Ohms were dismissed. A silver-silver chloride reference electrode (Flex ref, World Precision Instruments) was used to operate the FET device in a liquid gate configuration with a constant gate bias, V_G = -0.6 V (cf. Figure 4-3), and a constant source-drain bias, V_{SD} = 0.05 V.

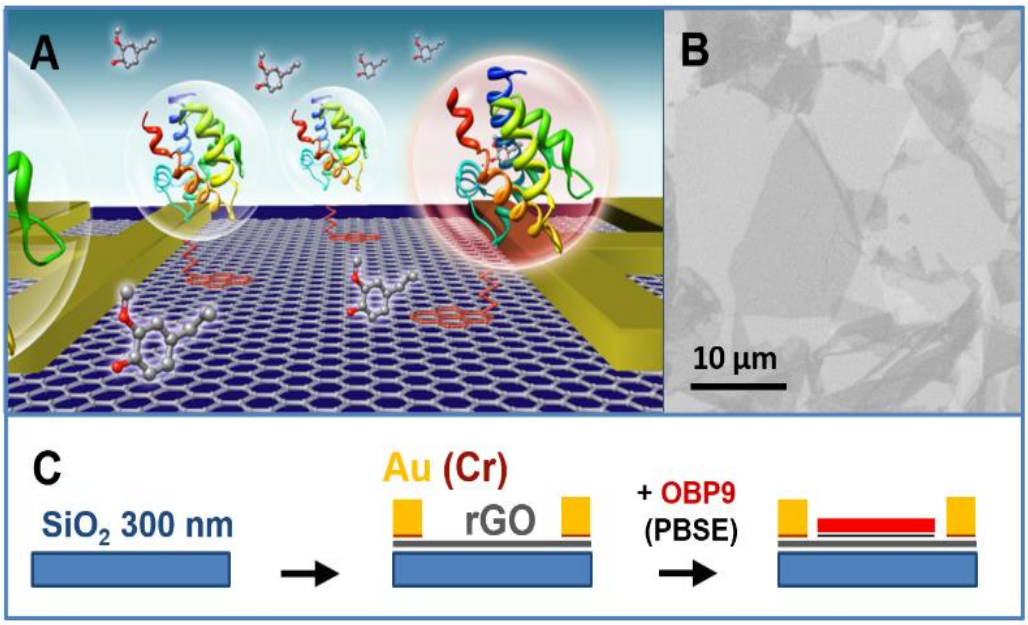


Figure 4-2. Schematic illustration of the individual fabrication steps of the rGO-FET biosensor device. (**A**) Artist's view of the graphene-based transistor with immobilized odorant binding proteins used for odorant binding studies; (**B**) light microscopic image of the GO flakes on the chip substrate (before reduction to rGO and before being coated by the electrodes; (**C**) schematic illustration of the individual fabrication steps of the graphene biosensor device: onto a SiO_2 coated Si substrate, GO flakes from a dispersion were assembled and reduced to rGO; next, source and drain Au electrodes were evaporated (with a thin layer of Cr as an adhesion promotor) and coated (by self-assembly) by a linker, PBSE, and finally functionalized by the attachment of odorant binding proteins.

4.3.1.4. Odorant sensing with OBP-functionalized rGO-FETs

For the detection of different odorants, the rGO-based biosensors were functionalized with odorant binding proteins *TcasOBP9A* or *TcasOBP9B*, respectively, from *T. castaneum*. For the attachment of the recombinantly synthesized protein to the sensing area, the graphene surface

was chemically modified by a bi-functional linker, 1-pyrenebutanoic acid succinimidyl ester (PBSE) (Figure 4-2). On one end, the linker firmly attaches to the graphene surface through π-π interactions with a pyrene group and on the other end covalently reacts with the amino groups of the protein to form an amide bond. Therefore, 20 µl of a 5 µM PBSE solution in THF was placed onto the rGO-FET channel, followed by 10 µl of a 10 µM OBP solution in PBS buffer (10 mM; pH 8.0) which was deposited onto the detection area and incubated for 2 h at 4 °C.

The general procedure of the sensing experiment started with continuously flushing the detection area with pure buffer (1 mM PBS, pH = 8.0), until a stable baseline of the source-drain current, I_{SD}, was established (Reiner-Rozman et al. 2015).

As we will show, the odorant binding protein-based biosensor is able to discriminate between different odorants and responds in a quantitative way to different concentrations of a given ligand binding to its receptor, the OBP, immobilized on the sensor surface. The fundamental concept for sensing of different odorant concentrations by a field-effect-transistor is based on the observation that the biosensor's response, i.e., the source-drain-current, I_{SD}, being a function of the applied gate voltage, V_G, depends on the presence of ligands bound to the receptor proteins immobilized on the gate. This is demonstrated in Figure 4-3: the characteristic V-shaped current change upon tuning the gate voltage, V_G, from -0.75 V to +0.75 V shows an appreciable dependence on the concentration of the ligand in solution. This controls the surface coverage by binding of the ligand to the OBP. The set-up is therefore very well suited for ligand sensing. Given this dependence of I_{SD} on the ligand concentration a gate voltage of V_G = -0.6 V was chosen in the following experiments.

4.3.1.5. Preparation of odorant solutions for FET measurements

Odorant solutions of 6-methyl-5-hepten-2-one and 3-octanol (chemical structures of the employed ligands are given in Figure 4-4) were prepared as 1 mM stock solutions in phosphate buffer (1 mM PBS, pH = 8.0) and further diluted (from 0.3 10^{-3} M to 6 10^{-3} M) by successive dilutions in PBS buffer. All solutions were freshly prepared on the day of the experiment.

4.3.2. Electroantennographic (EAG) measurements

4.3.2.1. Insects

Stock cultures of San Bernardino (SB) strain of *T. castaneum* were reared at 30 ºC, 40 % relative humidity, and 12 h photophase. Adults were provided with 95 % organic whole wheat flour seeded with 5 % (w/w) brewer's yeast.

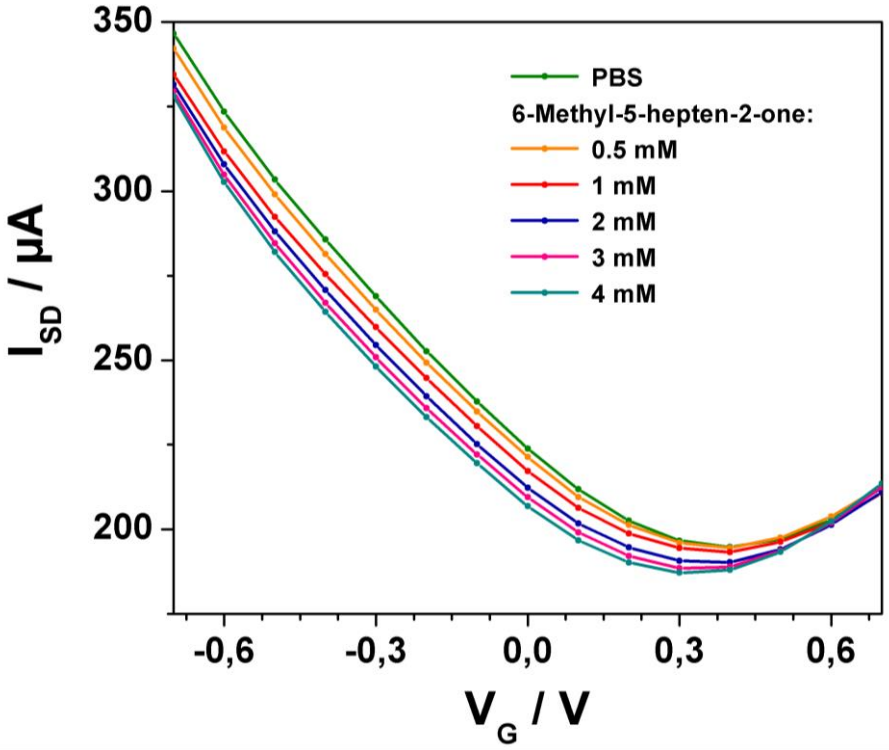
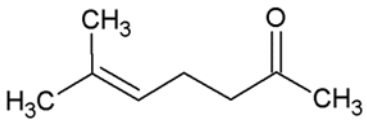


Figure 4-3. Gate voltage dependent rGO FET biosensor response: source-drain-current ISD-vs-gate voltage (VG) of a rGO FET sensor, functionalized by *TcasOBP9B*, at different concentrations of 6-methyl-5-heptene-2-one.

a) 6-methyl-5-hepten-2-one



b) 3-octanol

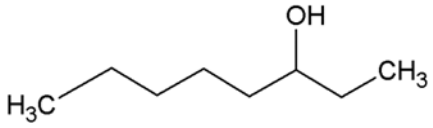


Figure 4-4. Chemical structure of the ligands investigated by the *in-vivo* sensor, EAG, and by the *in-vitro* rGO-FET sensor.

4.3.2.2. Stimulus compounds

Selected compounds 6-methyl-5-hepten-2-one and 3-octanol were diluted in silicone oil M 200 (Carl Roth GmbH + Co. KG, Germany) and concentrations of 10^{-1}, 0.3 10^{-1}, 10^{-2}, 0.3 10^{-2}, 10^{-3}, and 0.3 10^{-3} (w/w) were prepared. A glass Pasteur pipette (Pasteur capillary pipette, 150 mm Wu Mainz, Deutschland), containing a folded piece of filter paper (approx. 7×40 mm, Whatman No.1) was impregnated with 20 µl of diluted stimulus compound. The stimulus delivery system (CS-55, Syntech, Hilversum, Netherlands) was used to deliver an one second-puff of odour to the antenna allowing non-intermittent stimulus application at a continuous flow (3 l/min) of charcoal-filtered and humidified air through the tube, placed at 1 cm distance from the antenna. The stimulus carrying Pasteur pipette was inserted into the rear end of the tube (18 cm from the hole to the outlet of the tube), and the stimulus flow was triggered by the automated controller. The pheromone 4,8-dimethyldecanal (DMD) at 10^{-3} (w/w) concentration was applied as positive control and silicone oil was applied as a blank control to monitor possible changes in EAG sensitivity. Intervals between series and stimuli were 1 and 2 minutes respectively. Throughout the EAG recording, ascending doses of different stimuli were applied in order to minimize the effect of olfactory adaptation. Responses from ten antennae of different adult beetles of both sexes were recorded with every compound.

4.3.2.3. EAG recording

The beetle was put into a plastic micropipette tip. We used slight air pressure to wedge the beetle into the tip, with the head turned forward protruding the antenna out of the plastic tip along with part of the thorax that would later carry the reference electrode. Due to toughness of the cuticle, a small hole was made at the thorax region of the beetle with an electrolytically sharpened tungsten wire. This hole was used to insert a reference glass electrode containing Ringer solution, in contact with an Ag/AgCl wire. After making a puncture at the last antennal

segment with a sharpened tungsten wire by using a micromanipulator (Joystick Manipulator, MN-151, Narishige-Japan), a recording glass electrode (GB150F-8P, 0.86×1.50×80 mm with filament, Science products GmbH, Hofheim-Deutschland) filled with Ringer solution and in contact with a Ag/AgCl wire was immediately inserted. Glass capillary electrodes were drawn from borosilicate glass by electrode puller (Puller, Model No. PP-830, Narishige group, Japan). The recorded EAG signal was amplified with a pre-amplifier (Universal AC/DC probe, Syntech, Netherlands), which was connected to an EAG amplifier (x10). A data acquisition controller (IDAC-4, Syntech) processed and digitized the amplified signals (Figure 4-5B). With the customized EAG program (Version 2.7, EAG 2000, Syntech), the resulting EAG amplitude was computed as the difference between the baseline level and the maximum amplitude reached during odour stimulation.

4.4. Results and Discussions

4.4.1. In vitro measurements with the rGO FET sensors

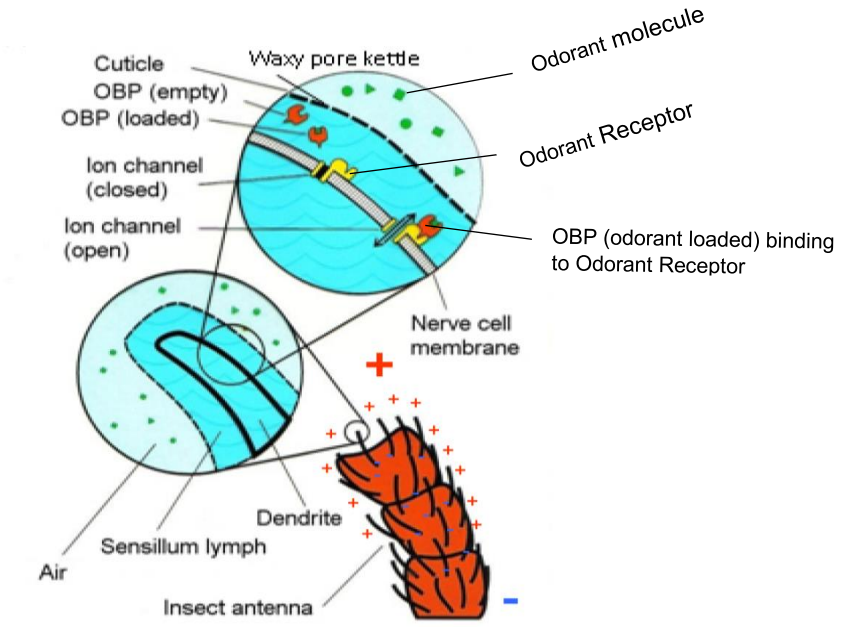
A first series of odorant sensing experiments was performed with a transducer architecture schematically depicted in Figure 4-6A: the rGO gate surface was modified with the linker system PBSE used to immobilize the odorant binding protein *TcasOBP9A*. After the baseline was established, the change in the source-drain current, ΔISD, was monitored *on-line* in real time while the odorant, 6-methyl-5-hepten-2-one, concentration in the analyte solution was stepwise increased from c_0 = 500 μM to 6 mM. This global analysis is shown in Figure 4-6 B. The following rinsing step with pure buffer re-set the system to the pristine baseline level, demonstrating the full reversibility of the affinity reaction between the surface immobilized receptor and its ligand binding from solution. This is an important pre-requisite for the applicability of the Langmuir model for the quantitative analysis of the binding data, allowing for the evaluation of kinetic rate constants, k_{on} and k_{off}, respectively, as well as for the titration of the equilibrium surface coverages, allowing for the determination of affinity constants, K_A.

An important aspect that we address next before going into a detailed analysis of the binding data is the question of non-specific adsorption of ligand molecules to the sensor surface. To this end we assembled FET devices with only the linker layer, i.e., without the final preparation step of immobilizing an odorant binding protein (Figure 4-7A). Upon rinsing various high concentration solutions of the studied ligand systems through the attached flow

cell a small, almost negligible response of the device could be observed (Figure 4-7B). The recorded current changes were all below the 5-10 % level of the response of the sensor in the presence of odorant proteins binding the ligands specifically.

Figure 4-8 summarizes a complete set of data of the global analysis with sensors functionalized with *TcasOBP9B*, exposed to analyte solutions of different concentrations of 6-methyl-5-hepten-2-one. Figure 4-8A gives the kinetic response of the FETs upon increasing the analyte concentration (from the pure buffer baseline level) in a stepwise fashion from c_0 = 500 µM to 4 mM (green arrows with concentrations as indicated), followed by a pure buffer rinse (blue arrow). The black dots are the experimental data; the red curves are fits assuming single exponential time dependence.

A



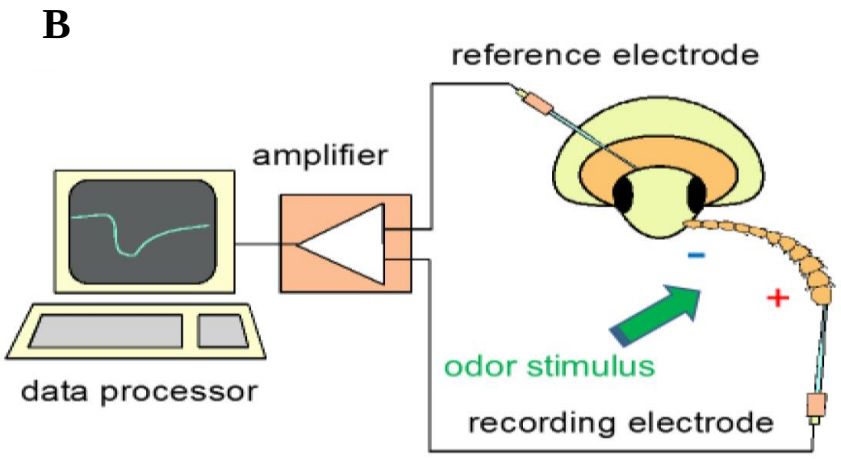
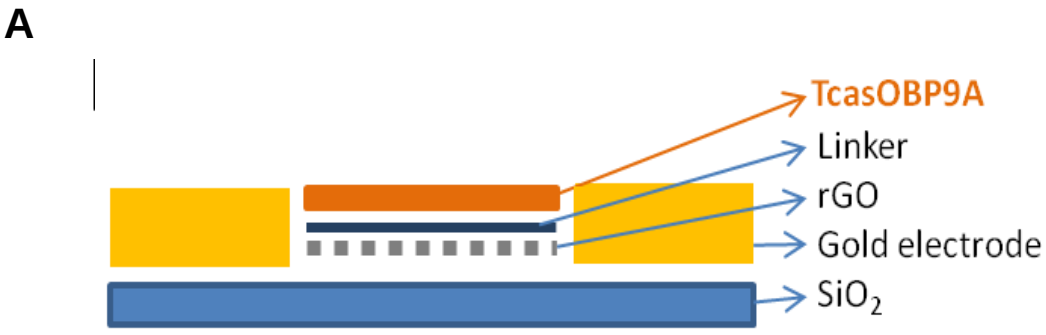
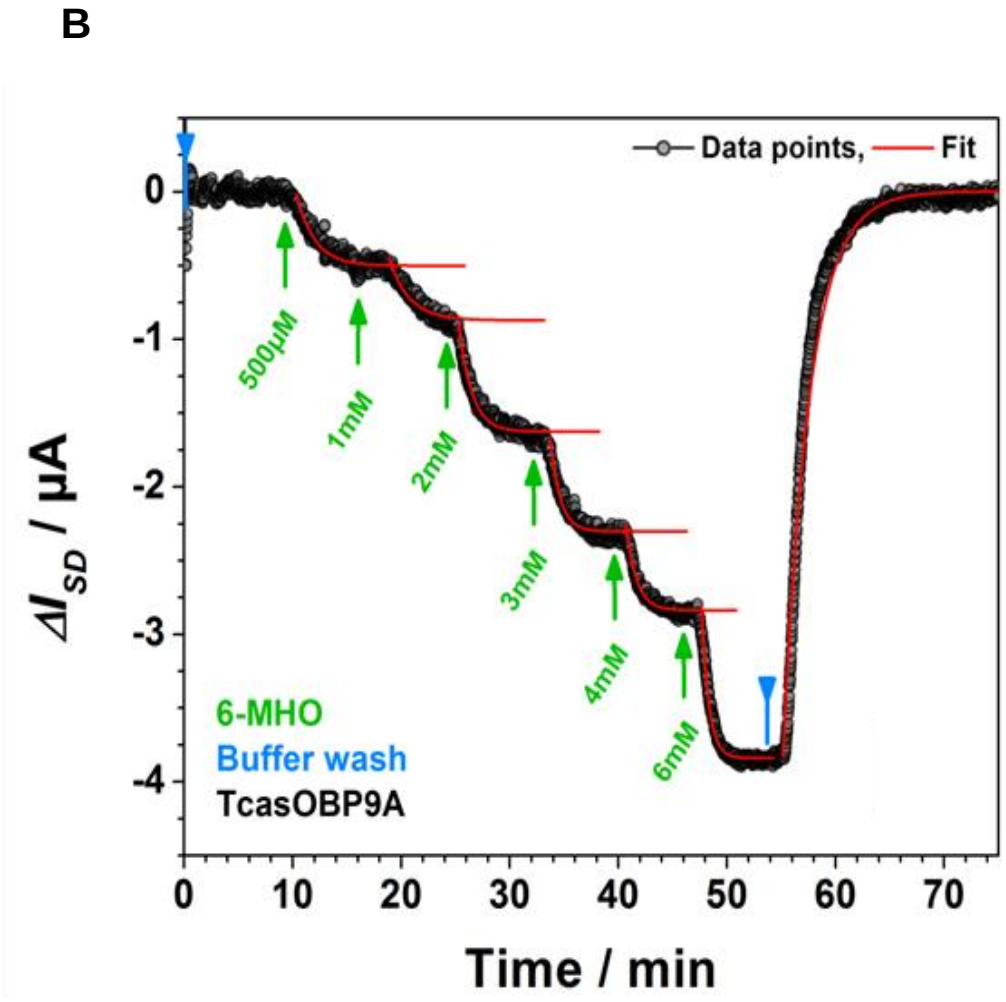


Figure 4-5. Schematic view of the mechanism of odorant binding and activation of odorant receptor and of the EAG recording setup. (**A**) Artist's view of an insect antenna containing dissolved odorant binding proteins in the sensillum lymph, binding to odorants and shuttling them to the membrane-bound odorant receptors giving raise to a depolarization of the stimulated nerve cells which can be recorded macroscopically as a temporary dipole of the antenna; (**B**) Schematic of an electroantennographic (EAG) set-up measuring the temporary dipole of the insect antenna generated upon stimulation with an odorant stimulus.





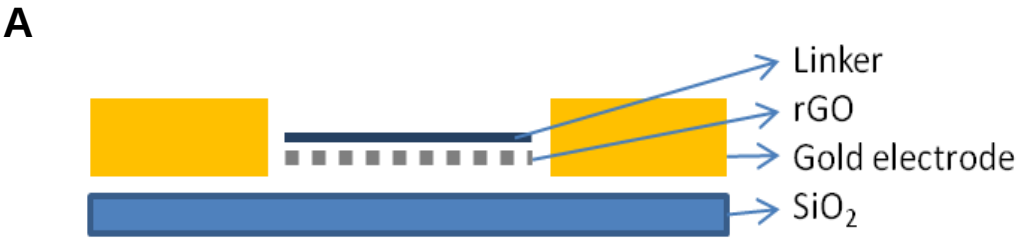


Figure 4-6. Schematic illustration of the rGO-FET biosensor device, functionalized with OBP. (**A**) Schematic illustration of the biosensor device with the rGO gate, a linker layer, PBSE, for the chemical coupling of the receptor, and the odorant binding protein, TcasOBP9A. (**B**) Real-time monitoring of the current change, ΔI_{SD}-vs-time, of a TcasOBP9A-functionalized biosensor to different concentrations of 6-methyl-5-hepten-2-one (6-MHO) (global analysis).

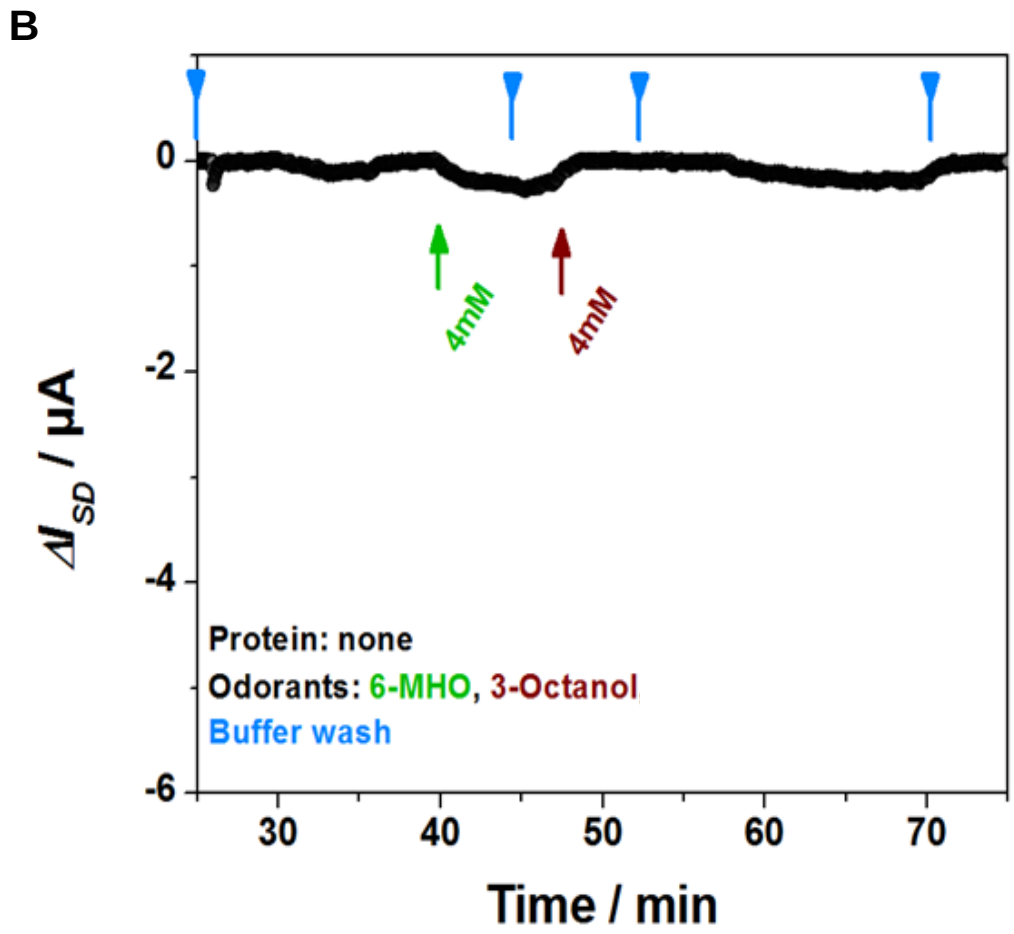


Figure 4-7. Schematic illustration of the rGO-FET biosensor device, functionalized without OBP (**A**) Schematic illustration of the biosensor device with the rGO gate, a linker layer, PBSE, for the chemical coupling of the receptor, and without protein functionalization. (**B**) Real-time monitoring of the current change, control measurement without protein, ΔI_{SD}-vs-time response to 4 mM solutions of 6-methyl-5-hepten-2-one (6-MHO) and 3-octanol, respectively, as indicated by the different colors.

If one plots all the measured (fit) k-values as a function of the bulk concentration as it is done in Figure 4-8B, one finds a linear increase of the rate constant as it is predicted by the Langmuir model:

$$k = k_{on}\, c_0 + k_{off}$$

From the straight line through the data points in Figure 4-8B, one obtains k_{on} = 1.3 $M^{-1}s^{-1}$ for the binding of 6-methyl-5-hepten-2-one to *TcasOBP9B* from the beetle *T. castaneum*. The intersection of the fit line with the ordinate, i.e., k for $c_0 = 0$, yields k_{off} = 0.004 s^{-1}.

According to the Langmuir model the relation between the rate constants and the affinity constant is given according to

$$K_A = k_{on} / k_{off}$$

which results then in $K_A = 3.2 \times 10^2\ M^{-1}$ or $K_d = 3.0$ mM.

Alternatively, one can analyze the surface coverages obtained at equilibrium with any (new) bulk concentration. Such a titration data set can be also derived from the global analysis presented in Figure 4-8A by plotting the level of the source-drain current after changing the concentration of the bulk odorant solution. The result is shown in Figure 4-8C for 3 different sets of measurements. The fit to the data gives a dissociation constant of $K_d = 2.5$ mM, in excellent agreement with the value from the pure kinetic analysis. This supports the approach of using the Langmuir model for a quantitative evaluation of the binding data recorded by rGO FETs.

Table 4-1 summarizes the obtained results for the two employed odorant binding proteins, *TcasOBP9A* and *TcasOBP9B*, respectively, for the two employed ligands. For comparison, we add the values for another protein, *AmelOPB14* from the honey bee *Apis mellifera* L. (Hymenoptera: Apidae), for a series of different odorants (ligands) as indicated (Kotlowski et al. 2018). For this system, the dissociation constants K_d (as well as the k_{on} values) differ by almost three orders of magnitude from a strong ligand like homovanillic acid to a weak ligand, like geraniol. This range of K_d- and k_{on}- values demonstrates that the sensor employed also for the *TcasOBP9A* and *TcasOBP9B* measurements is well able to differentiate between ligands with different binding strengths and kinetics. As expected, the sequence wise highly similar *TcasOBP9A* and *TcasOBP9B* show very similar K_d to the two C-8 marker compounds.

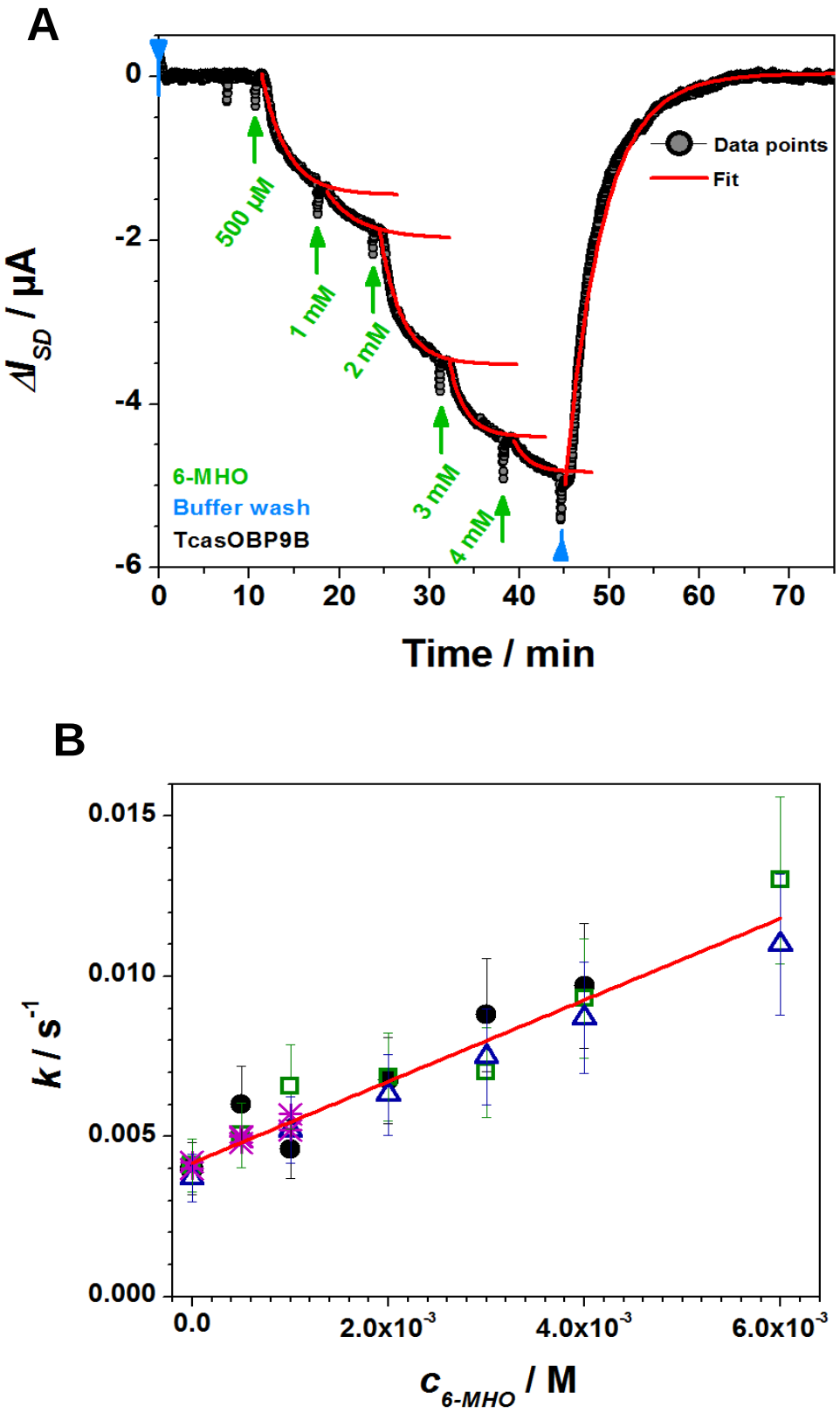


Figure 4-8 continued…..

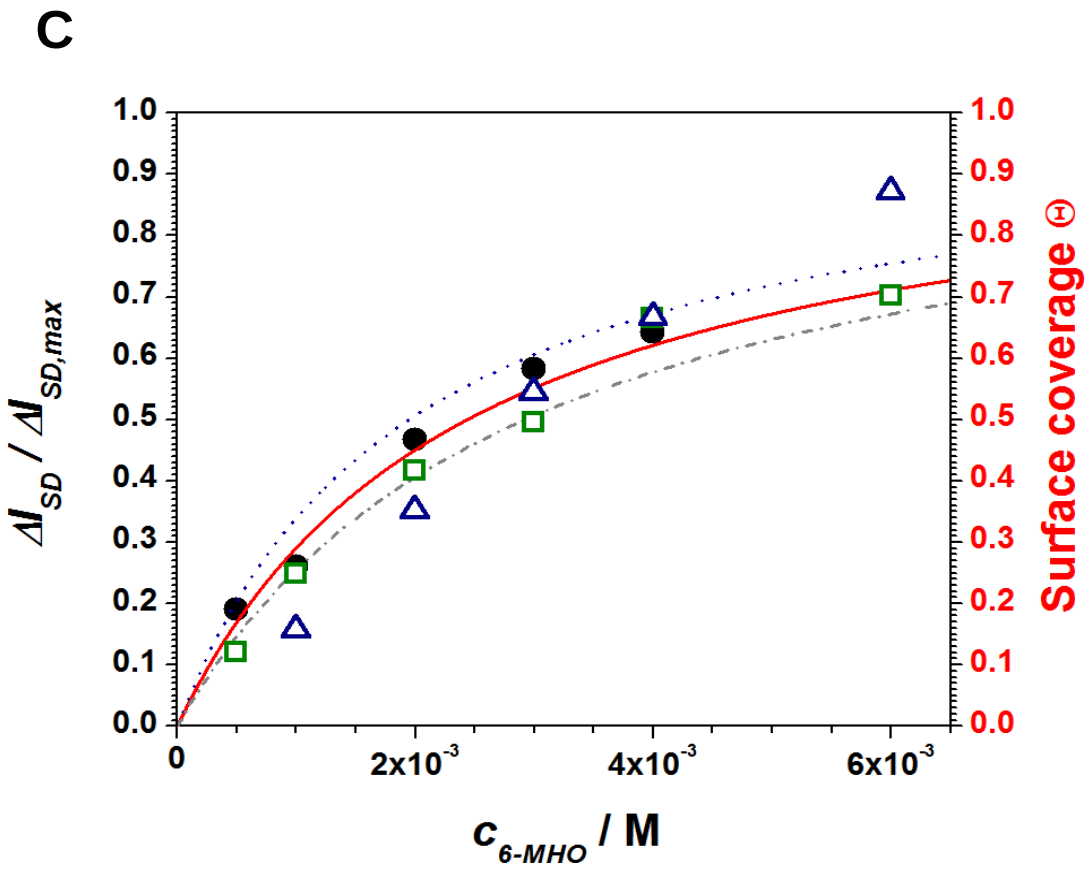


Figure 4-8. Affinity data and kinetic analysis of 6-methyl-5-hepten-2-one (6 MHO) binding to the protein *TcasOBP9B*. (**A**) Real-time response of a *TcasOBP9B*-functionalized biosensor to the binding of 6-methyl-5-hepten-2-one: the current decreases with the increase of the bulk concentration of 6-methyl-5-hepten-2-one (from c_0 = 500 µM to 6 mM) and gradually saturates. Blue arrows indicate runs with pure buffer, while green arrows indicate experiments with 6-methyl-5-hepten-2-one solutions. Red curves indicate the fitting of the raw data by kinetic simulations of the association and dissociation processes based on the Langmuir model. (**B**) Analysis of the reaction rate constants, k, obtained from the fitted data of (**A**) as a function of the 6-methyl-5-hepten-2-one concentration; different symbols were taken from 3 individual repeats. Linear regression gives the kinetic constants k_{off} = 0.004 s^{-1}, k_{on} = 1.3 M^{-1} s^{-1}, and the dissociation constant K_d= 3.0 x 10^{-3} M. (**C**) Langmuir adsorption isotherm, obtained for 3 individual repeats; the red fit curve gives K_d = 2.5 x 10^{-3} M.

Table 4-1. Individual dissociation constants K_d and reaction rate constants, k_{on} and k_{off}, respectively, of odorants binding to different OBPs, as indicated.

Protein (Insects)	Ligand	Dissociation constant K_d/μM	k_{on}/ $M^{-1}s^{-1}$	k_{off}/ s^{-1}
TcasOBP9A	6-methyl-5-hepten-2-one	3000	3	0.008
(*T. castaneum*)	3-octanol	2000	3	0.006
TcasOBP9B	6-methyl-5-hepten-2-one	4000	1	0.004
(*T. castaneum*)	3-octanol	3000	2	0.006
AmelOBP14	homovanillic acid	4	1130	0.008
(*Apis mellifera*)	methyl vanillate	20	235	0.010
	eugenol	40	170	0.006
	citral	800	9	0.003
	methyl eugenol	1400	6	0.006
	geraniol	3300	3	0.003
	6-methyl-5-hepten-2-one	3500	5	0.008

Particularly in view of the following comparison with affinity measurements by whole antenna recordings we focus on two more aspects of these odorant binding studies with an artificial device, i.e., the repeatability of the kinetic association and dissociation measurements, as well as the accuracy and limit of detection (LoD) for the recording of any change of the source-drain current upon exposing the sensor to a very low bulk concentration of the analyte solution, way below the half saturation concentration $c_{1/2}$ (= K_d).

The results of the repeatability test with a FET device that was functionalized by *TcasOBP9B* upon repeated exposure to a low (c_0=500μM) and a moderate (c_0=1mM) concentration of the analyte 6-methyl-5-hepten-2-one, alternating with pure buffer solutions, is given in Figure 4-9A. The full red curves represent single exponential fits to the data and result in kinetic rate constants that are summarized in Table 4-2. As one can see, the repeatability is quite satisfactory. The typical time constants for both, the association and the dissociation process, are in the few minutes regime.

The second important aspect concerns the accuracy of the rate constant determination and the LoD, Figure 4-9B gives an example for a measurement with a sensor that was functionalized with *TcasOBP9A*, and was exposed – after equilibration with pure buffer – to a 300 μM solution of 3-octanol.

Table 4-2. Kinetic rate constants, k_{on} and k_{off}, respectively, as well as the dissociation constant, K_d, as obtained from the fits to the repeatability experiments presented in Figure 4-8A.

6-methyl-5-hepten-2-one / mM	k / s^{-1}	k_{on} / s^{-1} M^{-1}	K_{off} / s^{-1}	K_d / M
0.5	0.0050	2	0.0040	2×10^{-3}
0.5	0.0048	1.6	0.0040	3×10^{-3}
1	0.0057	1.5	0.0042	3×10^{-3}
1	0.0052	1	0.0042	4×10^{-3}

Note the excellent signal-to-noise of the recorded current change! The full red curve is a single exponential fit to the experimental data and confirms further the excellent quality of the fit with an acceptable accuracy as far as the rate constant determination is concerned. The dotted light blue and the dash-dotted light green curves correspond to rate constants that are 20 % higher or lower than the best value, respectively.

Further added in Figure 4-9B is a simulated binding curve in grey that represents a theoretical response of the sensor upon the addition of a fictive 300 μM solution of 3-octanol. One can see that the signal would give a curve that could be clearly discriminated from the baseline given as a dashed grey line in Figure 4-9B, and represents an estimate for the LoD of this sensor configuration.

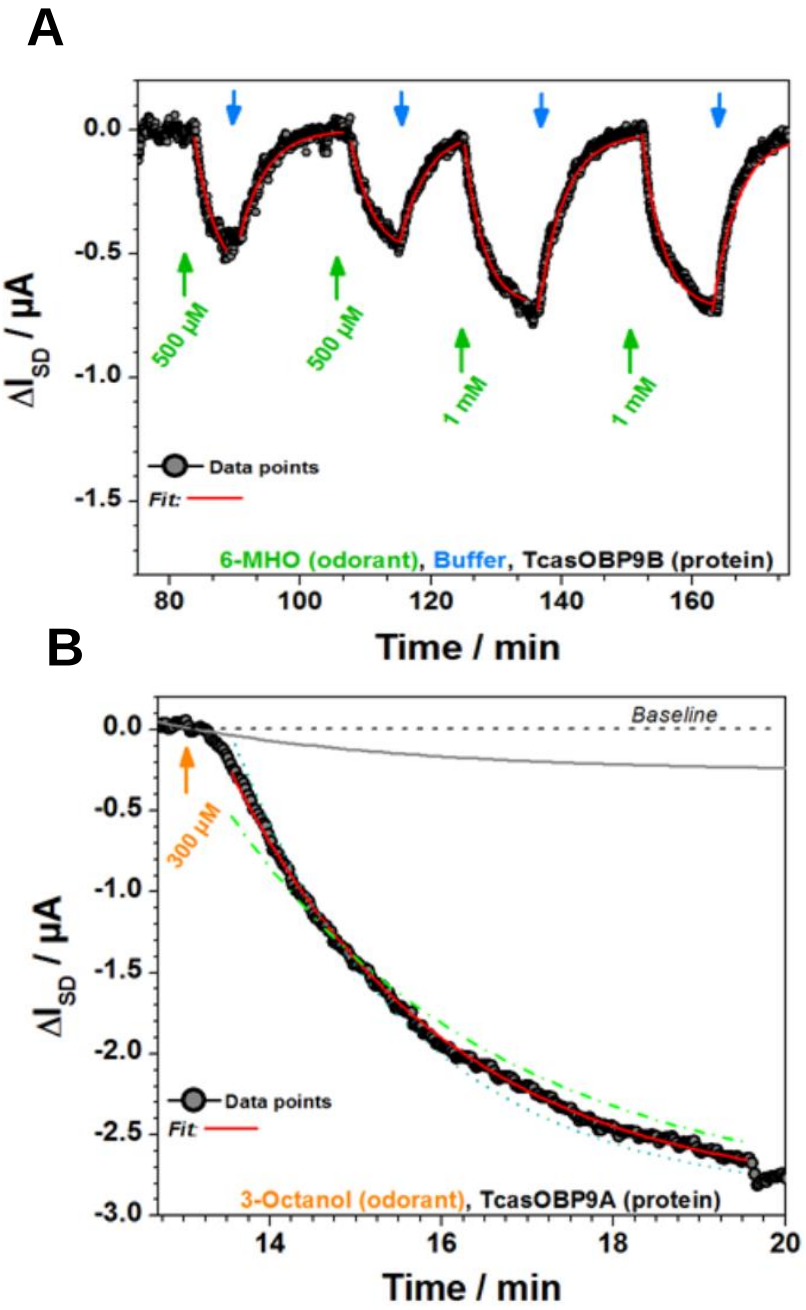


Figure 4-9. Kinetic response of rGO-FET biosensor device functionalized with *TcasOBP*. **A**) Repeatability test for the kinetic association and dissociation response of an *TcasOBP9B* functionalized FET sensor upon exposure to low and moderate concentrations of 6-methyl-5-hepten-2-one (6 MHO) solutions red curve is a (single-exponential) fit to the experimental data points with a rate constant k= and to pure buffer, respectively; **B**) kinetic response of the sensor, functionalized with *TcasOBP9A*, to a change of the analyte solution from pure buffer to a 300 μM solution of 3-octanol. The full 0.0243 s^{-1}, with the dashed light blue curve being a lower (k=0.0194 s^{-1}) and the dash-dotted light blue curve being an upper bound of the rate constant (k=0.02916 s^{-1}).

4.4.2. EAG

In order to demonstrate the insect's olfactory response to 6-methyl-5-hepten-2-one we measured the antenna's odorant reception *in vivo* using electroantennography of the antenna of *T. castaneum.*

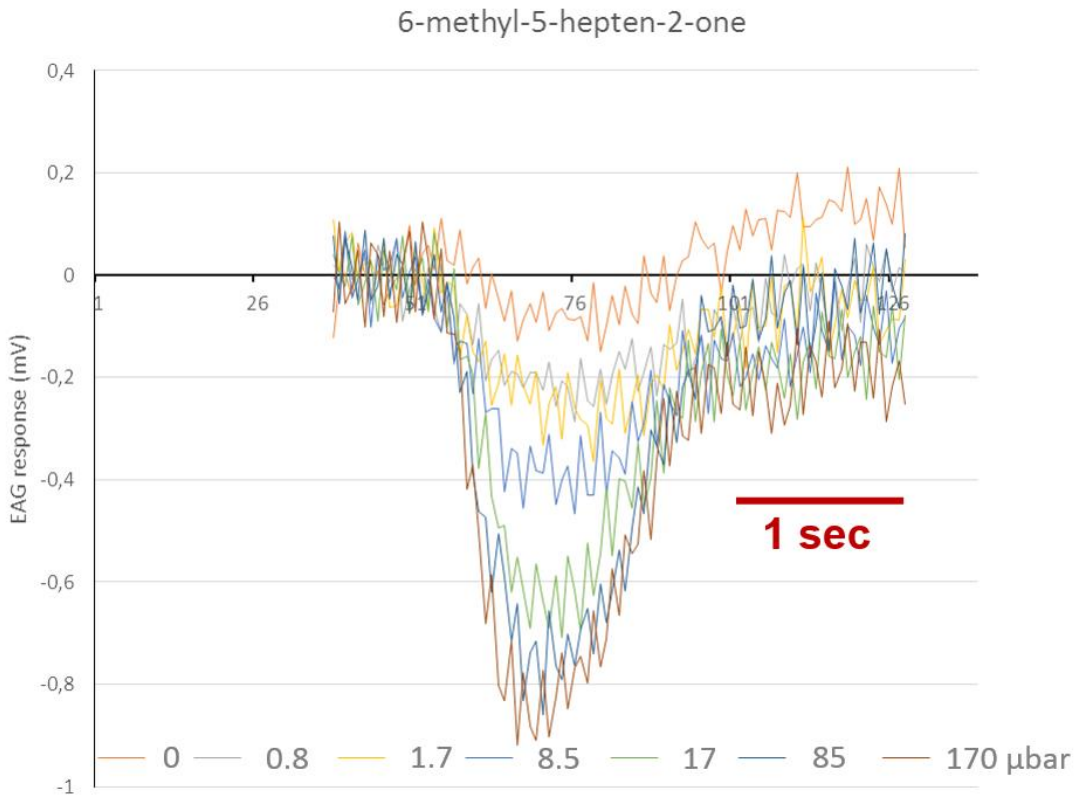


Figure 4-10. Overlay of real-time responses by a *T. castaneum* antenna to the stimulation by 6-methyl-5-hepten-2-one. The electroantennographic potential increases with the increase of the concentration of 6-methyl-5-hepten-2-one in air (from 0.8 µbar to 170 µbar partial pressure) and gradually saturates. Response times are below 1 s and recovery times are in the range of 2-3 seconds (26 units on the time axis are one second).

The response of the antenna follows a similar kinetics for both compounds with a dose-dependent response time to 90 of 1000 to 300 ms and a dose-independent recovery rate. A series of kinetic runs for 6-methyl-5-hepten-2-one are summarized in Figure 4-10. Blank measurements with silicone oil (SO) show a small and reproducible response by mechanoreceptors, whereas positive control measurements with the pheromone 4,8-

dimethyldecanal (DMD) gave the expected high response (data not shown). The detected signal represents the potential of multiple responding neurons within the antenna to the odorant. Our results show the relevance of the investigated odorants for the insect as a potential marker for damaged grains and diseased plants.

In an attempt to quantify the kinetic EAG response of the whole beetle and to generate a set of parameters that would allow for a quantitative comparison with the FET sensor data we (tried to) analyze the individual response curves at the various odorant concentration in air. Three examples of the time-dependent EAG data, taken at partial pressures (concentrations) of the odorant 6-methyl-5-hepten-2-one that cover a concentration range of 2 orders of magnitude are given in Figure 4-11A-C. Despite the somewhat noisy signal traces, we tried to fit the curves with single exponentials (inspired by what a Langmuir type binding behavior would suggest). We should point out that, of course, the red curves are not real fits, rather they give a qualitative impression of how a fit could look like.

The apparent kinetic rate constants thus obtained are given in Figure 4-12, as obtained for 6-methyl-5-hepten-2-one from the data of Figure 4-11, covering the full partial pressure range of the experiments with the stimulating odorant from 0.8 μbar to 170 μbar. The kinetic rate constants are called "apparent" because they do not necessarily relate only to the OBPs tested in the rGO-FET-arrangement, but reflect the activity of the whole OBP-pool of the insect antenna. Kinetic rate constants of both different approaches being similar suggest that the OBPs contributing the major part of the effect are examined. Three major findings should be pointed out: i), the EAG response at each partial pressure of the odorant is fully reversible: the signal increases upon exposure of the antenna to the odorant in the carrier gas, but returns completely back to the base-line level after the spiked pulse is over. This is a central requirement for the applicability of the Langmuir model to analyze the data; ii), despite the noise in the time-traces one can see that the association process, i.e., the time-dependent generation of the action potentials in the antenna, becomes faster at higher concentrations of the odorant in the air stream. This is already very obvious in the original data displayed in Figures 4-10 and 4-11, respectively. This again is in agreement with the expectation from a Langmuir picture of the process;

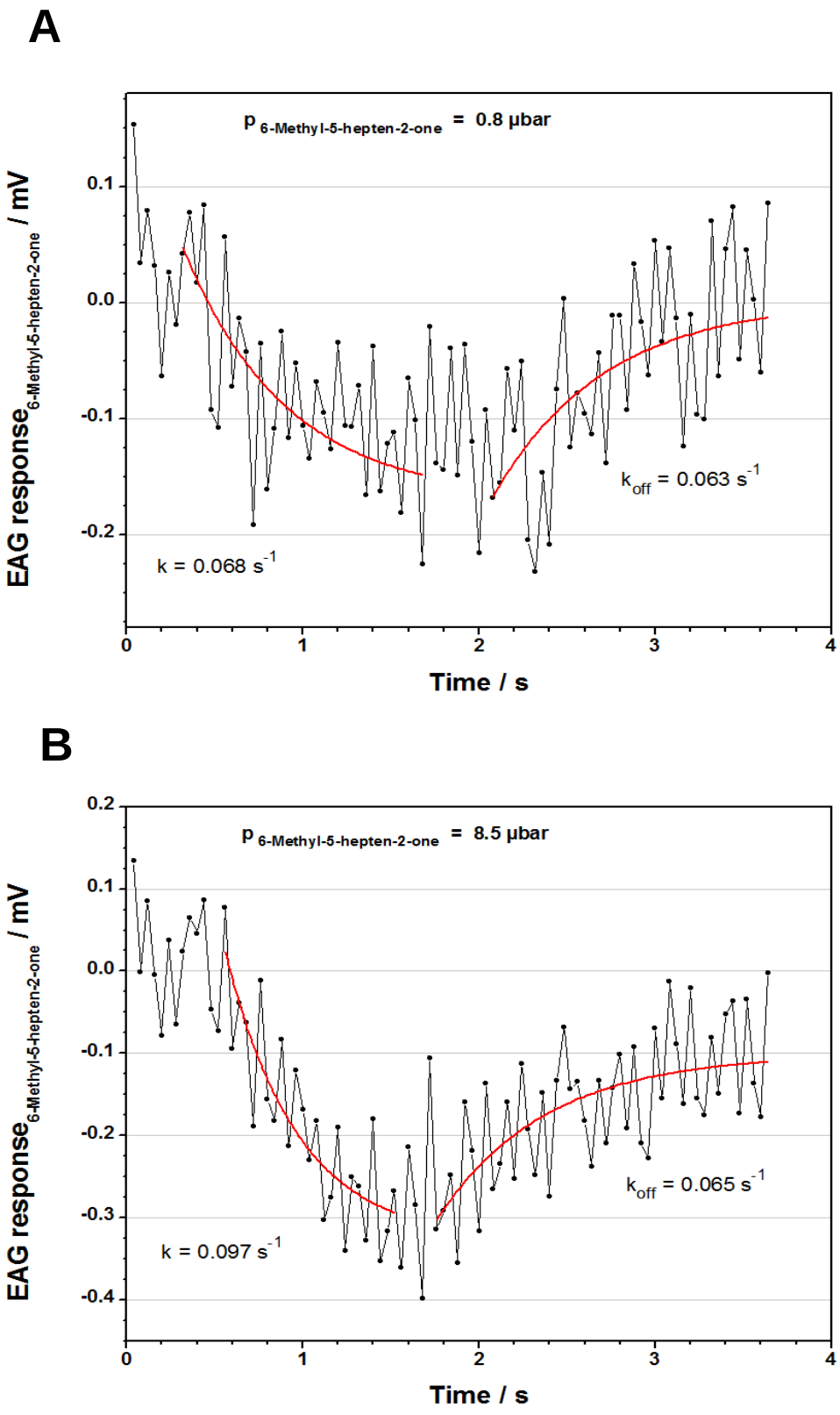


Figure 4-11 continued…..

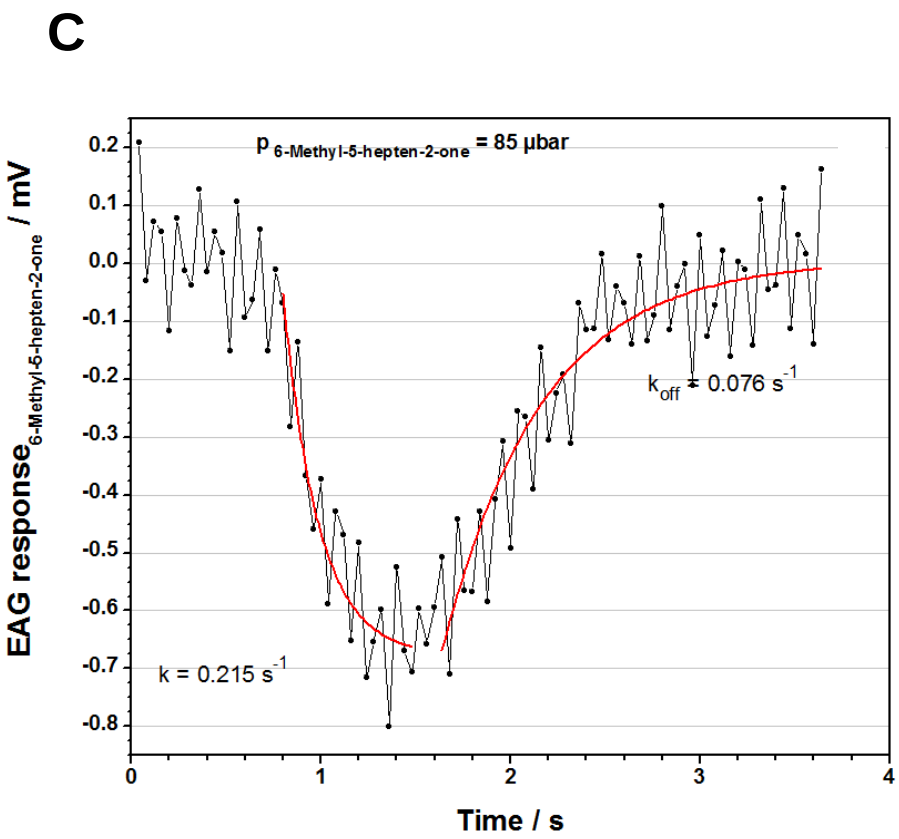


Figure 4-11. Examples for fitting of the raw data of electroantennographic recordings (after subtraction of silicone oil background signal) at different partial pressures (A: P=0.8 µbar; B: P=8.5 µbar; C: P=85 µbar) of 6-methyl-5-hepten-2-one in order to determine apparent rate constants of association and dissociation (cf. Figure 4-12).

iii) furthermore, independent of the level of excitation in the antenna at different stimuli concentrations, the dissociation process is concentration independent. Again, this is very clearly seen already in the original time traces (cf. Figure 4-10 and 4-11) and is also in full agreement with the expectations of the Langmuir model.

As in the case of a Langmuir adsorption isotherm analysis, one can obtain from the apparent kinetic rate constants in Figure 4-12 the association and the dissociation rate constants, respectively, for this fictive binding event of the ligand 6-methyl-5-hepten-2-one to the putative odorant binding protein: we obtain from the straight fit lines $k^*_{off} = 0.08\ s^{-1}$, and $k^*_{on} = 0.001\ \mu bar^{-1}\ s^{-1}$. The apparent dissociation constant thus obtained from the kinetic measurements amounts to $K^*_d = 80\ \mu bar$.

The second Langmuir-inspired protocol for the analysis of the EAG response data refers to the "equilibrium levels" reached for each new odorant concentration spiked to the carrier

gas (keeping in mind that this process of neural signal generation is basically a non-equilibrium process). As best seen in the overlay of the real-time responses in Figure 4-10, the new "equilibrium level" of the EAG data increases with increasing partial pressure until it reaches saturation at higher concentrations.

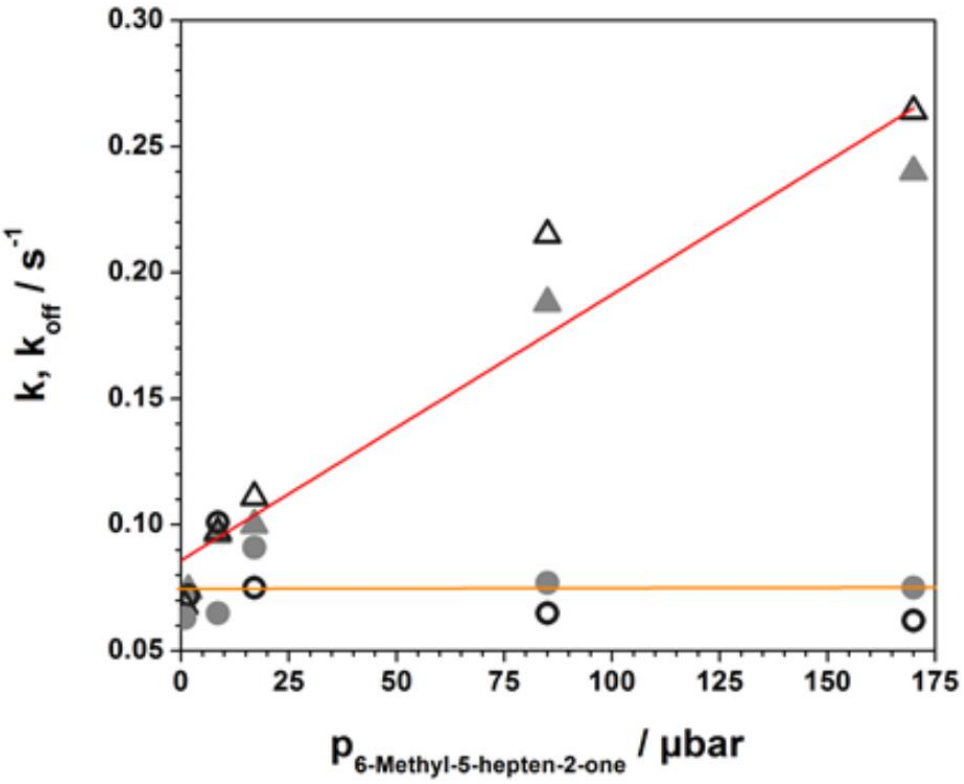


Figure 4-12. Analysis of the electroantennographically determined apparent reaction rate constants k* as a function of the 6-methyl-5-hepten-2-one -concentration. Linear regression yields the apparent kinetic constants k^*_{off} = 0.08 s^{-1}, k^*_{on} = 0.001 µbar^{-1} s^{-1}, and the apparent dissociation constant K^*_d= 80 µbar, assuming the OBP binding being the limiting step in the generation of EAG responses.

The corresponding "equilibrium" EAG response values for 6-methyl-5-hepten-2-one are all plotted linearly as a function of the log of the partial pressure in Figure 4-13. The full red curve is a fit to the data based on the Langmuir model, assuming a half-saturation partial pressure of $p^*_{1/2}$ = 6 µbar. This is considerably different from the value obtained from the kinetic analysis, i.e., $K^*_d = p^*_{1/2}$ = 80 µbar (added for comparison in Figure 4-13 as the grey dashed curve); however, given the approximation and the uncertainly as to whether the Langmuir model is a valid approach at all to quantify the data from the whole antenna

recordings this difference of about one order of magnitude is rather encouraging for our intent to take these values and compare them with the sensor data.

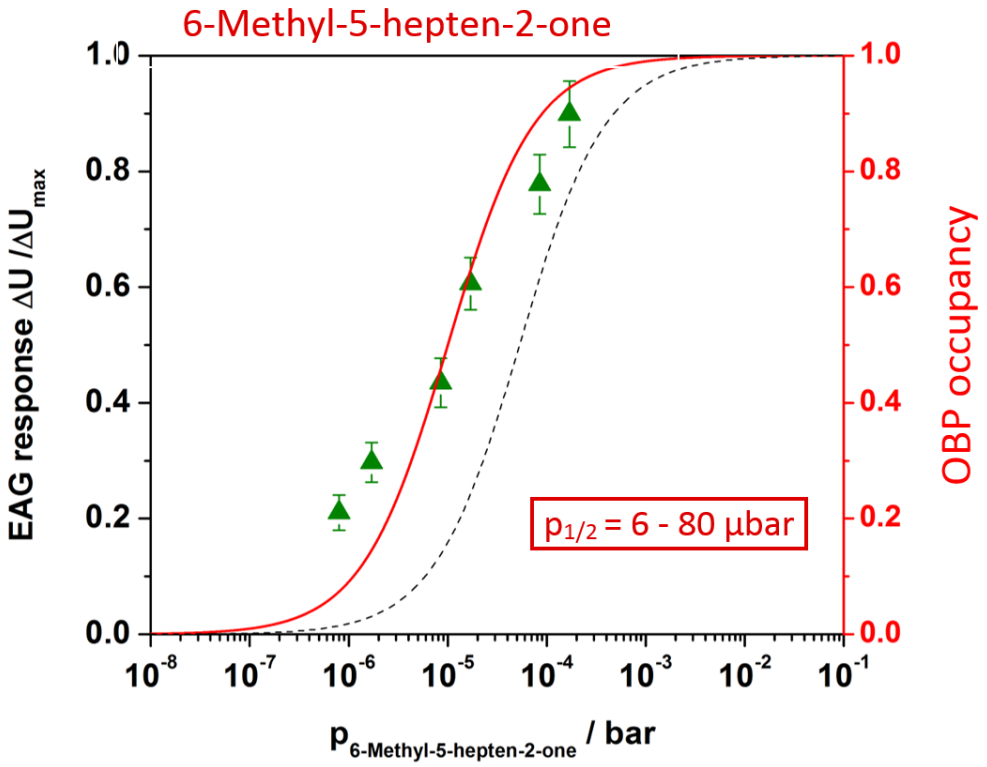


Figure 4-13. In-vivo antennal dose-response relation to OBP occupancy. EAG measurements of the responses to a dilution series of 6-methyl-5-heptene-2-one in air: the EAG response increases with the odorant concentration. Error bars show standard deviations from 10 repeats.

4.4.3. Comparison between *in-vitro* and *in-vivo* studies

The comparison of an artificial nanosensor-system based on a rGO field-effect transistor with a natural insect antenna has to be interpreted with much care. The natural system is stimulated by an odor stimulus in air consists of odorants reach the sensillar lymph through a hydrophobic chitin cuticle containing waxy pore kettles. Entered odorants is selectively binding and carrying by the soluble odorant-binding proteins (OBP), forming an odorant-OBP complex and transport to a specific membrane bound odorant receptors for processing the signal by opening of ion channels in the membrane of the sensory neuron (Figure 4-5A) (*in-vivo*).

In contrast, the electronic nanosensor-system is stimulated by an odorant molecules dissolved in water that delivered in a micro-flow cell, consists of OBPs immobilized to a

graphene gate of a FET for further electric conduction process by opening the gate channel (*in-vitro*). However, this artificial system is partially demonstrate about the natural system of insect olfaction and ideal for comparison of olfactory information process up to OBP level. Additionally, a more detailed comparison might only be useful if the OBP utilized for the experiment is one with a major contribution to the sum signal of the whole insect antenna (EAG response).

For a first step of a more detailed comparison, the stimulus quantity has to be aligned. Henry's law provides a relation between the concentration of a compound in an aqueous solution, ca, and its vapor pressure, p:

$$c_a = H^{cp} \cdot p$$

As an example, we compare the situation for 6-methyl-5-hepten-2-one for which Henry's law constant, is known:

$$H^{cp}=0.14 \text{ Mol m}^{-3} \text{ Pa}^{-1}$$

With the measured range of $p^*_{1/2}$ = 6-80 μbar we obtain an equivalent 6-methyl-5-hepten-2-one concentration in the sensillum lymph for which half of the EAG-response is measured of $c_{1/2}$ = 0.09 - 1.2 mM. Using H_{cp} = 0.31 Mol m^{-3} Pa^{-1} of 3-octanol and the measured range of $p^*_{1/2}$ = 0.7-7 μbar we obtain an equivalent 3-octanol concentration in the sensillum lymph for which half of the EAG-response is measured of $c_{1/2}$ = 0.02 – 0.2 mM.

Our lowest concentration of 3-octanol in the flow cell was 300 μM which makes sense given the K_d= 3 mM, but it could have been much lower (certainly measurements are possible with 30 μM, corresponding to a partial pressure of 1 μbar, cf. simulations displayed in Figure 4-9B).

Hence, the dilutions measured by natural and artificial olfaction systems are in a similar range of magnitude and these two techniques have shown comparable sensitivity to the tested stimuli, though artificial system partially mimic the natural system. However, the natural system shows a much quicker response yielding times at the milli-seconds level and recovery times at the seconds level in comparison to minutes-level in the artificial system. This hints at the fact that both binding and removal of the ligand and exhibiting olfactory response in the

natural systems are significantly quicker due to involvement of several olfactory components. For instance, comparing the binding constants of the OBPs in both experiments shows one to two orders of magnitudes higher values for k_{on} and for k_{off} in the EAG measurements.

The artificial system might display a delayed response because of immobilization effects delaying the approach of odorants to the tested OBPs. However, the same immobilizing strategy was used for *OBP14* of *A. mellifera* yielding significantly higher k_{on} for selected ligands, rendering immobilization effects might be one of explanation for the different response times of the artificial system unlikely (Larisika et al. 2015). Unfortunately, there are no data about rate constants for all the components of the natural *T. castaneum* system available. Thus, we can speculate that the fact of the k_{on} of the natural system being much quicker, might be due to additional phase-transfer catalytic process (22) employed during the delivery of the hydrophobic odorants from the external lipid layer through waxy pore kettles to the sensillum lymph (Sharma et al. 2015). Because of the low water solubility of many semiochemicals, transfer odorants from sensillum lymph to respective odorant receptor by OBPs may be a rate limiting step in the artificial system. The fact that both, the artificial and the natural system display similar K_d, but showing equally increased kinetic constants for association and dissociation in the natural system is highly suggesting the involvement of a catalytic process, which accelerates both, back- and forth-reaction.

4.5 Conclusion

We have demonstrated the detection of specific odorant molecules using an artificial olfactory biosensor based on reduced graphene oxide-field effect transistor (rGO-FET) functionalized with odorant binidng protein *TcasOBP9A* and *TcasOBP9B* from *T. castaneum* beetle as sensing elements. These sensor devices respond to different concentration of 3-octanol and 6-methyl-5-hepten-2-one, even at a low concentration in real-time affinity assays. Based on this results, in comparision to portable biosensor available in the market, our new rGO-FET biosensor improved detection limit and sensitivity of odor molecules and can be used for directly measuring the affinities of interested ligand with binding protein. The obtained results from artificial sensor device is compared with an EAG response of the whole antenna of a living beetle by using fitting data based on the Langmuir model. This study represents a first step towards the development of olfactory sensor device closely mimicking the complex natural olfactory system of an insect antenna.

4.6 Suggestion remarks

Manuscript of this chapter has been submitted to the journal and among authors, the peer-review process is under debating. Because certain weakness of this manuscript has not yet solved, I would like to address those issues with the following remarks:

I. The artificial sensor system is assembled based on the olfaction process depicted in Figure 4-5A and this model has been suggested and proved by Smith (2007). The author shown that while binding LUSH (pheromone binding protein of *Drosophila*) to 11-*cis*-vaccenyl acetate (cVA) form a unique OBP-odor complex (undergo conformational changes) that together functions as the ligand for the activation of respective odorant receptors to open ion channels (Smith, 2007; Laughlin et al. 2008). However, some years later, this step of the insect olfaction process has been published as completely not possible and proven that there is no conformational property for cVA/LUSH complex (Gomez-Diaz et al. 2013). Based on this evidence, our arguments stands into question, mainly in comparison between *in-vivo* and *in-vitro* studies. We assume that odorant-OBP complex activate the OR (as in Figure 4-5A) and expect that our artificial sensor device function is similar. In fact, the function of our artificial sensor device is based only on the process of OBP level of the natural olfactory system and not on the process of other olfactory components level like odorant receptor (OR). This clearly indicates that we misinterpret and compare the rGO FET sensor data to OR level of natural olfactory system, instead of comparing at the OBP level.

II. Comparing the affinity measurements by an artificial device of OBPs to tested odorants with the EAG response of the whole antenna of a living beetle underestimates the complex process of the natural olfactory system. Primarily, in the natural olfactory system, the odorant molecule binds to an OBP that transports it to an OR and co-receptor (Orco) team on the surface of the olfactory receptor neuron (ORN). Thus then leads to the neuron firing an action potential down the axon. Besides, the insect OBPs play different roles as carrier (OBP bind to odorants and carry them to OR), buffer (trap the odorants efficiently at high concentrations), some of them degrade by odorant degrading enzymes (ODEs) before they reach the OR and some odorant-OBP complexes may not reach the OR or may reach the OR but do not activate (Pelosi, 1994; Leal 2013). Therefore, our assumption that high occupancy of OBP in the sensor device correlates directly with the exhibited EAG response by the whole antenna of a living beetle to a particular compound at different concentration (data here with Langmuir model) is not realistic.

4.7. Acknowledgements

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CHAPTER 5

EAG Response and Behavioral Orientation of *Dastarcus helophoroides* (Fairmaire) (Coleoptera: Bothrideridae) to Synthetic Host-Associated Volatiles

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Author contributions:

Balakrishnan K : Improved EAG method for *Dastarcus helophoroides* beetle, performed all EAG recordings, analyzed data, re-wrote the whole manuscript**,** reviewing and editing

Ren LL : Developed idea and performed all behavioral experiments, data analysis, wrote the first draft

Luo YQ : conceptualization, supervision

Schütz S : conceptualization, supervision

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5.1. Abstract

Dastarcus helophoroides Fairmaire (Coleoptera: Bothrideridae) is an effective predatory beetle of larvae and pupae of several cerambycid beetles including *Monochamus alternatus* and *Anoplophora glabripennis*. Electroantennography (EAG) and a dynamic two-choice olfactometer were respectively used to measure the antennal and behavioral responses of both sexes to selected volatile compounds. Female and male *D. helophoroides* exhibited similar EAG and behavioral responses. Significant dose-dependent EAG responses in both sexes were elicited by nonanal, octanal, cis-3-hexenol, 3-carene, (R)-(+)-α-pinene, (S)-(-)-α-pinene, (R)-(+)-limonene and (S)-(-)-limonene. Female and male beetles were repelled at high concentration by cis-3-hexenol and (S)-(-)-limonene, respectively. Both sexes of *D. helophoroides* were significantly attracted to nonanal, cis-3-hexenol, 3-carene and (R)-(+)-limonene even at low concentrations. These compounds might be used either individually or in mixtures for developing biological control methods to attract this predatory beetle into forest stands threatened by cerambycid beetles.

Keywords: natural enemy, long-horned beetles, host plant volatiles, electroantennogram, behavioral bioassay

5.2. Introduction

The bothriderid beetle *Dastarcus helophoroides* Fairmaire, 1881 (Coleoptera: Bothrideridae) (syn. *Pathodermus helophoroides* Fairmaire, 1881; *Dastarcus longulus* Sharp, 1885) is a predator of the larvae and pupae of important cerambycid forest tree pests such as *Monochamus alternatus* Hope 1843, *Anoplophora glabripennis* Motschulsky 1853, *Massicus raddei* Blessig & Solsky 1872, *Apriona germari* Hope 1831, *A. swainsoni* (Hope,1840), and *Batocera horsfieldi* (Hope, 1839), (Wei, 2007; Wei et al. 2009; Inoue, 1991). Some of these long-horned beetles are causing significant economic losses in China and other countries. For example, *A. glabripennis*, native to China and other Asian countries, has been introduced into North America and some European countries, devastating the hardwood forests by vigorously feeding on tree trunks. Additionally, *M. alternatus* is a vector of pine wilt disease by carrying its causative agent, the pine wood nematode *Bursaphelenchus xylophilus*. Therefore, *D. helophoroides* could be an option as a potential biological agent for controlling various cerambycid pest species.

Female *D. helophoroides* deposit egg clusters close to entrance holes, gallery walls and the frass of longicorn beetles larvae (Inoue, 1991; Qin and Gao, 1988). The hatched predatory larvae are active and search for prey, which, once located, are paralyzed before feeding. Predators of plant feeders commonly use semiochemicals to locate their prey at both long and short distances. These semiochemicals might emanate from their prey or from trees attacked by their prey (Weißbecker et al. 2006; Boer et al. 2008; Fournet et al. 2001; Li et al. 2015). Thus, semiochemicals including monoterpenes, sesquiterpenes, and homoterpenes (Boer et al. 2008) can serve as indirect plant defense compounds, attracting specific predators to kill the attacking pest insects. For example, both sexes of *D. helophoroides* were attracted to volatiles emanating from larval tunnels and the frass of two cerambycid beetle species (Wei et al. 2013). To date, only one strong attractant (R)-(+)-limonene has been identified for *D. helophoroides* (Wei et al. 2008). However, nothing is known about the antennal detection of *D. helophoroides* to any previously tested compounds including (R)-(+)-limonene. Additionally, no study has examined the dose-dependent behavioral responses of *D. helophoroides* to any prey related volatile compounds (Wei et al. 2008, 2013).

The antennal sensilla of *D. helophoroides* have been morphologically studied and the typical chemosensitive structures (wall-pores) were found on the different types of sensilla (Ren et al. 2012). Moreover, recent transcriptome analyses identified multiple chemosensory

gene families in *D. helophoroides* that may be involved in olfaction (Zhang et al. 2014; Wang et al. 2014).

The purpose of our study was to explore the antennal and behavioral responses of *D. helophoroides* to prey-related volatile compounds (Table 5-1). A total of nine volatile compounds were chosen for antennal detection and behavior experiments. Among them, 3-carene, (R)-(+)-α-pinene, (S)-(-)-α-pinene, (R)-(+)-limonene, (S)-(-)-limonene, and trans-β-caryophyllene were selected based on volatiles perceived by *Monochamus* spp. (a prey of *D. helophoroides*) from *Pinus* spp. trees **(**Weißbecker et al. 2006; Fan et al. 2007). Additionally, octanal, nonanal, and cis-3-hexenol were selected from among volatiles released by *A. glabripennis* attacked *Populus nigra* (Hu et al. 2009; Scholz and Schütz, 2012). Moreover, the monoterpenes 3-carene, (R)-(+)-α-pinene, (S)-(-)-α-pinene, (R)-(+)-limonene, and (S)-(-)-limonene were reported as actively perceived by *D. helophoroides* from larval frass of *A. glabripennis, A. swainsoni* and *M. raddei* (Wei et al. 2008, 2013). Being associated with different prey species, these selected volatile compounds are potentially biologically important to *D. helophoroides*. We used electroantennography (EAG) (Schneider, 1962) to record antennal detection and a dynamic two-choice ten track olfactometer to analyze the behavioral responses of *D. helophoroides* to these compounds (Paczkowski et al. 2014).

5.3. Materials and methods

5.3.1. Insects

Cocooned *D. helophoroides* pupae were obtained from a laboratory colony reared at the Natural Enemy Research Institute of the Beijing Agriculture College, China. This colony was established with *M. alternatus* larvae and pupae that were collected from the trunks of *Pinus massoniana* in Guangdong Province, China. No specific permissions were required for collecting *M. alternates* at this site because it's a common herbivorous insect in the afforestation pine plantations. The field studies did not involve endangered or protected species. In a controlled atmosphere, the *D. helophoroides* pupae were reared in ventilated plastic boxes at 25 °C, 45% RH, and a photoperiod of light 16 h : dark 8 h. After hatching, the adults were collected, transferred to cages and fed an artificial diet (Qiong et al. 2005). The beetles were then imported into Germany and were maintained in a climate chamber with identical environmental conditions as in China. The age of the beetles used in the EAG and the behavioral experiments was approximately 40 days after hatching. After each experiment,

actively responding beetles were sexed reliably by dissecting the reproductive organs (Tang et al. 2007). Ten antennae from different adult beetles and 70-100 adult beetles of each sex were used in EAG and behavioral experiments respectively.

Table 5-1. Selected synthetic volatile compounds used as olfactory stimuli in the EAG and the behavioral experiments tested with predatory beetle *D. helophoroides*

Chemicals	Purity %	Source	Vapor pressure Vp 25°C (mbar)	Dilutions used in the experiments	
				EAG (in paraffin mg/mg)	Behavior (in silicone oil mg/mg)
octanal	98	Tci	3.27	10^{-1}-10^{-6}	10^{-1}, 10^{-3}, 10^{-5}
nonanal	95	Aldrich	0.71	10^{-1}-10^{-6}	10^{-1}, 10^{-3}, 10^{-5}
cis-3-hexenol	98	Aldrich	1.39	10^{-1}-10^{-6}	10^{-1}, 10^{-3}, 10^{-5}
3-carene	90	Aldrich	2.48	10^{-1}-10^{-6}	10^{-1}, 10^{-3}, 10^{-5}
(R)-(+)-limonene	99	Tci	2.05	10^{-1}-10^{-6}	10^{-1}, 10^{-3}, 10^{-5}
(S)-(-)-limonene	95	Tci	2.05	10^{-1}-10^{-6}	10^{-1}, 10^{-3}
(R)-(+)-α-pinene	95	Tci	4.65	10^{-1}-10^{-6}	10^{-1}, 10^{-3}
(S)-(-)-α-pinene	98	Fluka	4.65	10^{-1}-10^{-6}	10^{-1}, 10^{-3}
trans-β-caryophyllene	98.5	Aldrich	0.017	10^{-1}-10^{-6}	10^{-1}, 10^{-3}

TCI -Shanghai Development Co. Ltd., China; Aldrich- Steinheim, Germany; Fluka- Buchs, Switzerland

5.3.2. Chemical stimuli

The selected compounds octanal, nonanal, cis-3-hexenol, 3-carene, (R)-(+)-limonene, (S)-(-)-limonene, (R)-(+)-α-pinene, (S)-(-)-α-pinene and trans-β-caryophyllene were diluted in paraffin oil (mg/mg) (Uvasol®, Merck, Darmstadt, Germany) and silicone oil M 200 (mg/mg) (Carl Roth GmbH + Co. KG, Germany) for EAG and behavioral experiments respectively. All compounds were obtained from commercial suppliers (Table 5-1).

5.3.3. Electroantennogram and odor delivery

The procedure used to prepare the beetles for the electroantennographic recording is described in (Balakrishnan et al. 2017) and was adapted to the special requirements of *D. helophoroides*. Beetles were starved for 24 h before the experiments. A sharpened tungsten wire prepared by electrolytic etching was used to make a small hole in the thorax region. Later, this hole was used for the insertion of a reference glass electrode filled with Ringer's solution in contact with an Ag/AgCl wire. The beetle was placed under a high magnification compound microscope (Leica MZ16, Leica Microsystems GmbH, Germany). To stabilize the antenna, a sharpened

tungsten wire was used to hold the antennal segment in place. Then, the last antennal segment was punctured with a sharpened tungsten wire using a micromanipulator. This hole was used to insert a recording glass capillary (GB150F-8P, 0.86 × 1.50 × 80 mm with the filament; Science products GmbH, Hofheim-Deutschland) containing Ringer's solution in contact with an Ag/AgCl wire. The EAG responses were detected through a combi-probe (INR-II; Syntech, the Netherlands). The DC potential was recorded (Universal AC/DC probe), processed and analyzed using EAG 2000 software (Syntech, Hilversum, Netherlands).

An air stimulus controller (CS55; Syntech, Hilversum, the Netherlands) was used to deliver purified air and odor. A constant flow (18 l/h) of filtered air was passed over the prepared antenna through the open end of the metal tube positioned close to the antenna. The pulse duration time was 1 s. Each volatile dilution was tested in a 1 min interval. The time interval between each volatile compound was 2 min. Ascending concentrations of each compound (10^{-6} to 10^{-1} in paraffin mg/mg) were applied to avoid olfactory adaptation. A standard green leaf volatile cis-3-hexenol at 10^{-3} (in paraffin oil mg/mg) stimulation was done at the beginning and at the end of each recording to correct for the loss of sensitivity of the antennal preparation. Similarly, a control paraffin oil stimulation was done at the beginning and at the end of each recording to subtract the blank value from the antennal responses. For each compound, EAG responses of ten antennae from different adult beetles of each sex were recorded.

5.3.4. Olfactometer and behavioral test

A custom-built dynamic two-choice olfactometer with 10 parallel tracks was used in the behavior test as described in (Paczkowski et al. 2014). A 40 × 180 × 170 mm olfactometer was constructed from PTFE (Polytetrafluoroethylene). The 10 parallel walking chambers were covered with a glass lid. The non-transparent PTFE-walls between tracks isolated the tested beetles from each other as well as both chemical and visual cues. Each walking chamber was 20 mm deep and 150 mm long (Figure 5-1). The width of each chamber was enlarged to 10 mm to allow *D. helophoroides* to turn around freely inside the chamber. A white LED plate (200 × 200 mm) was used as a light source beneath the walking tracks. At both ends of each walking chamber, 200 µl filter pipette tips (Sarstedt AG&CO, Nümbrecht, Germany) were inserted: 10 filter tips on one end were drenched with 65 µl of volatile compound, and 10 filter tips on the other end were drenched with pure silicone oil control. Silicone oil was used as a

solvent for the behavioral assays, because of the unfavorable viscosity of paraffin oil (used in EAG) tended to obstruct the filter tips of the olfactometer. Filtered and humidified air was fed into both ends of each track at the identical constant flow rate of 0.080 L/min.

A live video tracking system, the Etho vision XT 8.0 (Noldus Information Technology, the Netherlands), was used to recognize and record the behavior of the beetles. Three compartment zones of olfactometer were defined as zone 1, zone 2, and the beetle releasing point as "neutral" zone (Figure 5-1). Ten insects were introduced into the olfactometer at the neutral zone in each trial, with the released beetles unable to contact each other because of the physical separation in the walking arenas. The volatile compound and the silicone oil were placed randomly in the direction of zone 1 or zone 2 in each trial. Each trial began with a 30 s accommodation phase for the beetles and lasted for 15 min. The record was valid as soon as the middle point of a beetle entered zone 1 or zone 2. The olfactometer was cleaned with methanol, and the position of the volatile compound was changed after each trial. To avoid possible influence of asymmetries in illumination on recording data, the olfactometer was turned by 180° after each true trials.

In behavioral tests, initially, we used all compounds at 10^{-1} and 10^{-3} doses. Later, those compounds showing significant differences between control and treatment zones at a dose 10^{-3} were further tested at a dose of 10^{-5}. A control experiment with silicone oil vs. silicone oil was conducted to test the behavior of *D. helophoroides* without stimuli. Fifty replications were conducted for all compounds at selected concentration (10^{-1}, 10^{-3}, 10^{-5}; Table 5-1).

To measure the behavioral responses of *D. helophoroides* beetles responding to both treatment and control zones, we calculated the total distance movement (TDM) of beetles in each trial. Beetles were released in the neutral zone of each track, and the total moving distance for 15 min in any direction in the track (to treatment zone, to control zone, returning to the neutral zone or moving to either of control and treatment zones etc.,) was calculated. Our estimation of TDM assumes that beetles respond behaviorally towards either of the tested zones (control or treatment) in each track, but it does not reveal the kinesis activity of beetles. To evaluate the attraction of beetles to tested stimuli, we calculated the beetle entering frequency (BEF) responses to treatment or control zones within 15 min. Beetles were released in the neutral zone of each track, and scored a single BEF count as the beetle moved into the treatment

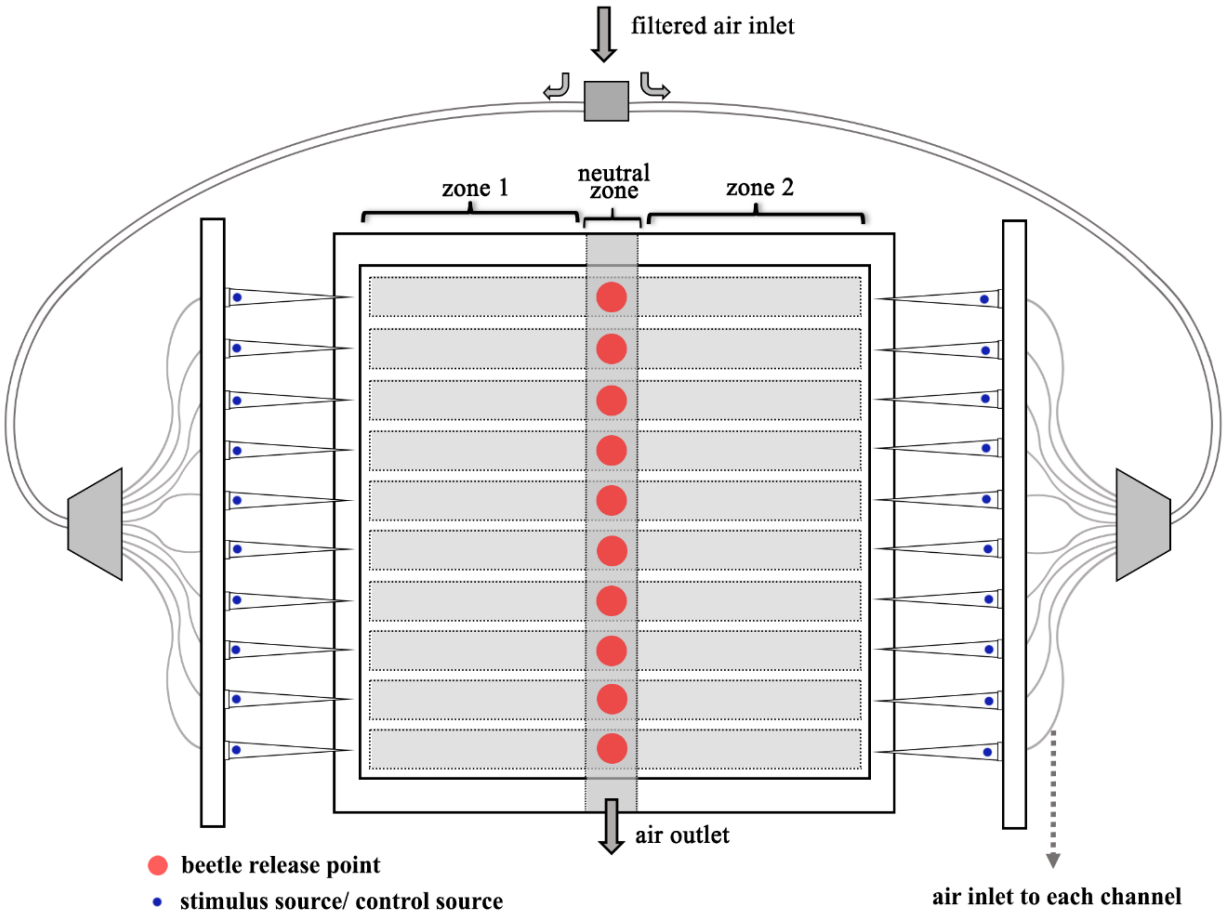

Figure 5-1. Schematic representation of the custom-built dynamic two-choice olfactometer with 10 tracks and showing the walking arena defined as zone 1, 2 and the beetle releasing point as neutral zone.

or control zone (may or may not reach the track end) and return to the neutral zone. As a measure of how beetles show high attraction to chemical stimuli, we calculated the beetle staying duration (BSD) responses at 5, 10, and 15 min intervals in both treatment and control zones. We scored the BSD as beetles that entered the treatment or control zone, and noted how long each beetle stayed in a particular zone across different time durations (5, 10, 15 min). Observations were stopped once the beetle exited of the zone to the releasing point (neutral zone) and resumed when the beetle entered the same zone within the assigned time duration.

5.3.5. Statistical analyses

To correct the EAG responses in comparison to the paraffin oil control, the mean of the blank responses before and after the measurements were subtracted from that to elicited by the volatile compound. All EAG responses data were then normalized to cis-3-hexenol (10^{-3}) as follows:

$$\frac{A \; - \; (\text{EAG (ctl1)} + \text{EAG (ctl2)}) / 2}{(\text{EAG (std1)} + \text{EAG (std2)}) / 2 - (\text{EAG(ctl1)} + \text{EAG (ctl2)}) / 2}$$

where A is the amplitude (mV) of the EAG response to compound; EAG (ctl1) is the EAG response to control at the beginning of the recording; EAG (ctl2) is the EAG response to control at the end of the recording; EAG (std1) is the EAG response to standard at the beginning of the recording; EAG (std2) is the EAG response to standard at the end of the recording.

First, we compared the normalized EAG-responses to selected compounds between the sexes with two-way ANOVA, followed by multiple comparisons corrected with the Bonferroni test (Prism 5, Graphpad Software). Second, the mean EAG responses relative to the standard at different dilutions of each compound were analyzed by two-way ANOVA, followed by multiple comparisons with Fisher's LSD test (Prism 5, Graphpad Software). Third, we checked for significant differences in EAG response among tested compounds at 10^{-1} and 10^{-2} by Tukey's multiple comparisons test (Prism 5, Graphpad Software).

In the behavioral tests, the time period s *D. helophoroides* stayed (BSD) in each zone (5, 10 and 15 min) were compared using a multiple t-test (Prism 5, Graphpad Software). Additionally, the frequencies of the beetles entering (BEF) each zone during 15 min of testing were compared using a chi-square test (SPSS Statistics 22, IBM). The TDM, BEF and BSD response measurements by *D. helophoroides* in the olfactometer were normalized and compared using one-way ANOVA, followed by Tukey's multiple comparisons test (Prism 5, Graphpad Software).

5.4. Results

5.4.1. EAG responses of *D. helophoroides* to compounds from cerambycid-attacked trees

The normalized EAG responses of female and male *D. helophoroides* to nonanal, octanal, cis-3-hexenol, 3-carene, (R)-(+)-limonene, (S)-(-)-limonene, (R)-(+) - α-pinene, (S)-(-)-α-pinene and trans-β-caryophyllene at six doses (10^{-1} - 10^{-6} diluted in paraffin oil mg/mg) are shown in Figure 5-2. Of these, the compounds octanal, nonanal and (S)-(-)-limonene elicited clear dose-dependent EAG-responses down to 10^{-6} dose in both sexes. On the other hand, the common plant sesquiterpene trans-β-caryophyllene did not elicit the antennal response at lower doses (10^{-3}, 10^{-4} and 10^{-5}) in either sex of the beetles (Figure 5-2). The compound (S)-(-)-α-pinene elicited dose-dependent EAG responses down to 10^{-6} dose in male beetles only. However, female and male beetles did not show any significant sex specific differences between the amplitudes of their EAG responses ($P>0.05$, Tukey's multiple comparisons test) (Figure 5-2; Table 5-2).

When comparing the normalized EAG responses of both sexes of *D. helophoroides* to all nine volatile compounds at the two highest doses (10^{-1} and 10^{-2}) (Table 5-2), the highest EAG response to the aldehydes octanal and nonanal was recorded for female beetles at a dose of 10^{-1} ($P<0.05$, Tukey's multiple comparisons test). In contrast, at a dose of 10^{-2}, both sexes showed significantly higher EAG response amplitudes to octanal and nonanal than to the other tested compounds (Table 5-2) ($P<0.05$, Tukey's multiple comparisons test). The EAG response amplitudes of both sexes to trans-β-caryophyllene at 10^{-1} and 10^{-2} were significantly lower than to all other compounds (Table 5-2) ($P<0.05$, Tukey's multiple comparisons test).

5.4.2. Behavioral responses of *D. helophoroides* to selected volatile compounds

5.4.2.1. Total distance movement (TDM)

We calculated the total distance moved of *D. helophoroides* as a combined behavioral response to different concentrations (10^{-1}, 10^{-3}, 10^{-5} diluted in silicone oil mg/mg) of all compounds and silicone oil control within 15 min (Table 5-3). Comparison of TDM for both sexes to all compounds at different doses relative to the control stimulus showed that the female beetles exposed to trans-β-caryophyllene at 10^{-1} exhibited a significantly increased TDM. Moreover, the TDM of female beetles was significantly reduced when exposed to a 10^{-5} dose of cis-3-hexenol. However, both sexes exposed to 3-carene and (R)-(+)-limonene showed significantly increased TDM at high (10^{-1}) doses compared to low (10^{-5}). Male beetles showed this effect to trans-β-caryophyllene even at a dose of 10^{-1} relative to 10^{-3} dose (Table 5-3).

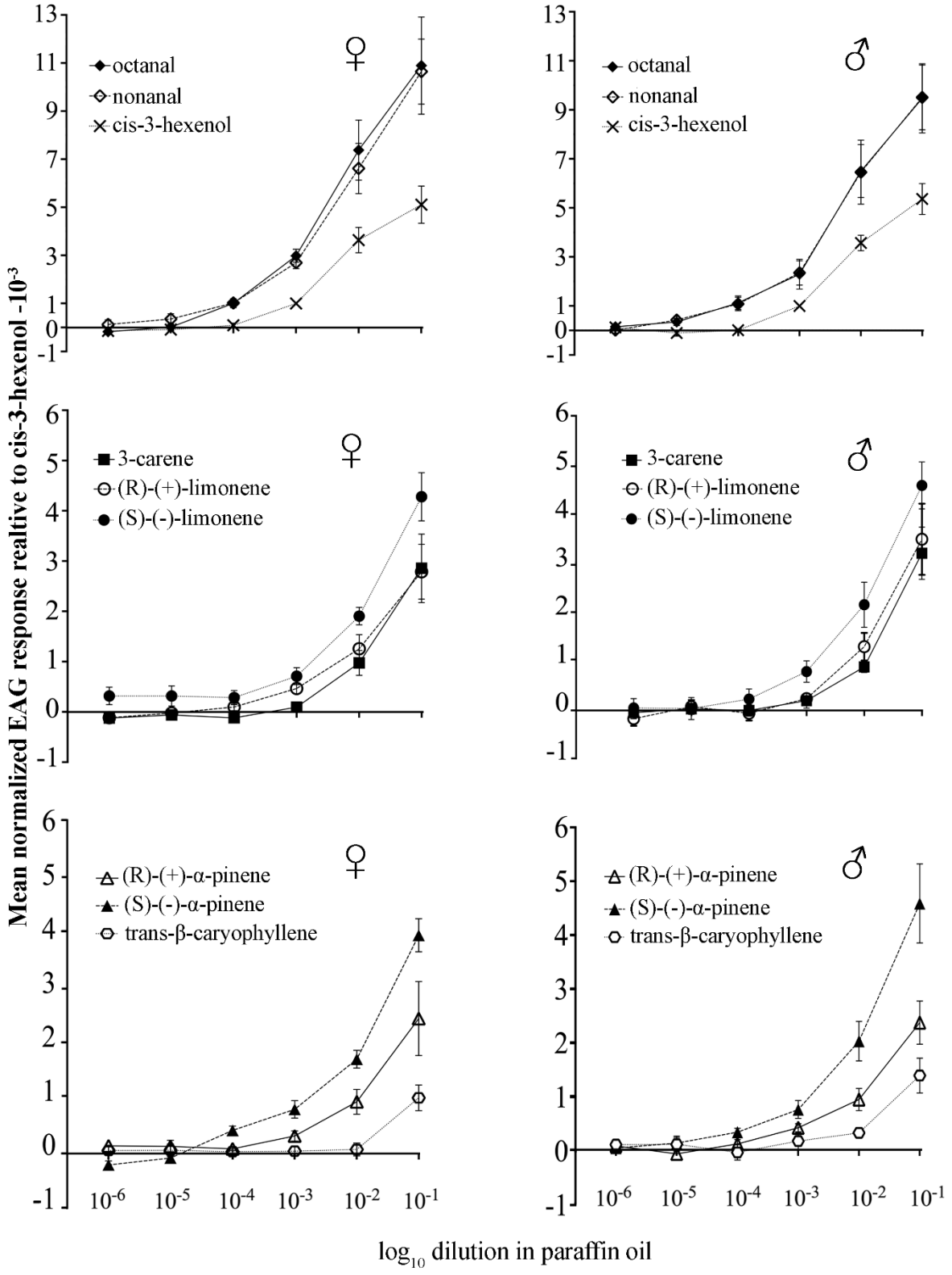

Figure 5-2. Normalized EAG responses of both female and male *D. helophoroides* to selected volatile compounds. The error bars represent the mean standard error (n = 10).

Table 5-2. Normalized mean EAG responses of *D. helophoroides* to different compounds of the same dilution

Normalized mean EAG response to different compounds (diluted in paraffin oil mg/ mg)												
Volatile compounds	**10^{-1}**		**10^{-2}**		**10^{-3}**		**10^{-4}**		**10^{-5}**		**10^{-6}**	
	Female	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**
octanal	**10.89± 2.02 a**	**9.47± 1.41 a**	**7.38± 1.25 a**	**6.50± 1.08 a**	**2.98± 0.27 a**	**2.30± 0.60 a**	1.01± 0.18 a	1.13±0.28 a	0.01± 0.13 a	0.35± 0.15 a	0.18±0.23 a	0.15± 0.20 a
nonanal	**10.65± 1.35 a**	**9.51± 1.32 a**	**6.62± 1.04 a**	**6.46± 1.30 a**	**2.70± 0.25 a**	**2.35± 0.50 a**	1.02± 0.14 a	1.08±0.28 a	0.35± 0.22 a	0.43± 0.16 a	0.12± 0.19 a	0.01± 0.12 a
cis-3-hexenol	5.11± 0.77 b	5.36± 0.63 b	3.63± 0.53 b	3.57± 0.32 b	1.00± 0.00 b	1.00± 0.00 b	0.09± 0.09 a	0.01±0.06 a	0.09± 0.05 a	0.11± 0.12 a	0.14± 0.05 a	0.12± 0.11 a
3-carene	2.86± 0.68 c	3.20± 0.53 c	0.98± 0.25 cd	***0.89± 0.11 d***	0.09± 0.08 b	0.20± 0.15 b	0.12± 0.11 a	0.00±0.12 a	0.06± 0.07 a	0.03± 0.11 a	0.12± 0.05 a	0.04± 0.13 a
(R)-(+)-limonene	2.79± 0.54 c	3.49± 0.73 d	1.26± 0.28 cd	1.30± 0.28 cd	0.46± 0.10 b	0.24± 0.11 b	0.10± 0.07 a	0.06±0.15 a	0.02± 0.04 a	0.08± 0.14 a	0.12± 0.07 a	0.17± 0.15 a
(S)-(-)-limonene	4.28± 0.48 b	4.58± 0.48 d	1.91± 0.17 c	2.15± 0.46 c	0.71± 0.17 b	0.79± 0.22 b	0.29± 0.14 a	0.23±0.20 a	0.32± 0.20 a	0.04± 0.23 a	0.32± 0.17 a	0.05± 0.19 a
(R)-(+)-α-pinene	2.43± 0.67 c	2.37± 0.40 c	0.93± 0.22 cd	0.93± 0.21 cd	0.32± 0.09 b	0.41± 0.08 b	0.08± 0.03 a	0.11±0.08 a	0.13± 0.11 a	0.08± 0.08 a	0.14± 0.07 a	0.07± 0.13 a
(S)-(-)-α-pinene	3.93± 0.30 bc	4.58± 0.74 d	1.70± 0.16 c	2.02± 0.37 cd	0.80± 0.16 b	0.75± 0.17 b	0.42± 0.08 a	0.32±0.08 a	0.08± 0.09 a	0.12± 0.13 a	0.2± 0.07 a	0.03± 0.09 a
trans-β-caryophyllene	***1.00± 0.23 d***	***1.38± 0.32 e***	***0.07± 0.12 d***	***0.31± 0.10 d***	0.04± 0.09 b	0.16± 0.15 b	0.03± 0.08 a	0.05±0.14 a	0.06± 0.11a	0.10± 0.13 a	0.06± 0.06 a	0.09± 0.10 a

Different letters indicate significant differences among different compounds (P<0.05, Tukey's multiple comparisons test at the same dilution level). Values are means (± standard error). (n = 10).

5.4.2.2. Beetle entering frequency (BEF) into the selected zones

To see the effect of volatile compounds on *D. helophoroides* behavior, we calculated the sum of entering frequencies for each treatment- and silicone oil control- zones over a 15 min stimulus period (Figure 5-3). We found that both sexes exposed to 3-carene and (R)-(+)-limonene showed significantly higher ($P < 0.05$, chi-square test) BEF than silicone oil alone at nearly all tested doses (10^{-1}, 10^{-3}, 10^{-5} diluted in silicone oil mg/mg) (Figure 5-3D and 5-3E). Similarly, the compound nonanal at 10^{-3}, 10^{-5} and cis-3-hexenol at a dose of 10^{-5} elicited significantly increased BEF for both sexes into the treatment zone compared to the control zone ($P < 0.05$, chi-square test) (Figure 5-3B and 5-3C). However, only male beetles showed significantly increased BEF into the zones treated with (R)-(+)-α-pinene at 10^{-3} and (S)-(-)-α-pinene at 10^{-1} and 10^{-3} doses (Figure 5-3G and 5-3H) compared to control zones. In contrast, the BEF of male beetles into control zones was significantly higher compared to zones treated with (S)-(-)-limonene and trans-β-caryophyllene at a dose of 10^{-1} (Figure 5-3F and 5-3I). Neither sex of *D. helophoroides* showed any significant differences in BEF when silicone oil was tested *versus* silicone oil ($P > 0.05$, chi-square test) (Figure 5-3J). The overall comparisons of BEFs for both sexes over a 15 min interval to all tested compounds at different doses (10^{-1}, 10^{-3}, 10^{-5} diluted in silicone oil mg/mg) are presented in Table 5-4.

5.4.2.3. Beetle staying duration (BSD) in the selected zones

In order to measure the attractive behavior of *D. helophoroides* to all tested compounds at different doses (10^{-1}, 10^{-3}, 10^{-5} diluted in silicone oil mg/mg), we compared the mean beetle staying durations at 5, 10 and 15 min stimulus periods in treatment- and silicone oil control-zones (Figure 5-4). In both sexes at 5 min, the volatile compounds nonanal, cis-3-hexenol, 3-carene, (R)-(+)-limonene and (S)-(-)-α-pinene elicited significantly increased BSD in treatment zones ($P< 0.05$, t-test) compared to control zones mostly at lower doses (10^{-3}, 10^{-5}) (Figure 5-4B, 5-4C, 5-4D and 5-4E). Similarly, beetles exposed to different dilutions of compounds for 10 and 15 min periods revealed that the BSD in zones treated with nonanal, cis-3-hexenol, and (R)-(+)-limonene were significantly increased ($P< 0.05$, t-test) compared to control zones at a lower dose in both sexes of the beetles (Figure 5-4B, 5-4C and 5-4E). Additionally, higher concentrations (10^{-1}) of (R)-(+)-α-pinene and (S)-(-)-α-pinene elicited significantly increased BSD at 10 min and at 5, 10 and 15 min respectively in treated zones compared to control zones of both sexes (Figure 5-4G and 5-4H). In contrast, female beetles exposed to cis-3-hexenol at a higher dose (10^{-1}) for 10 and 15 min showed significantly higher BSD in the control zones (Figure 5-4C). Neither sex tested in silicone oil *versus* silicone oil showed any significant difference in BSD at any tested period (Figure 5-4J) ($P> 0.05$, t-test). The overall comparisons of BSD for both sexes within 5, 10 and 15 min to all compounds at different doses (10^{-1}, 10^{-3}, 10^{-5} diluted in silicone oil mg/mg) are shown in Table 5-5, 5-6, 5-7.

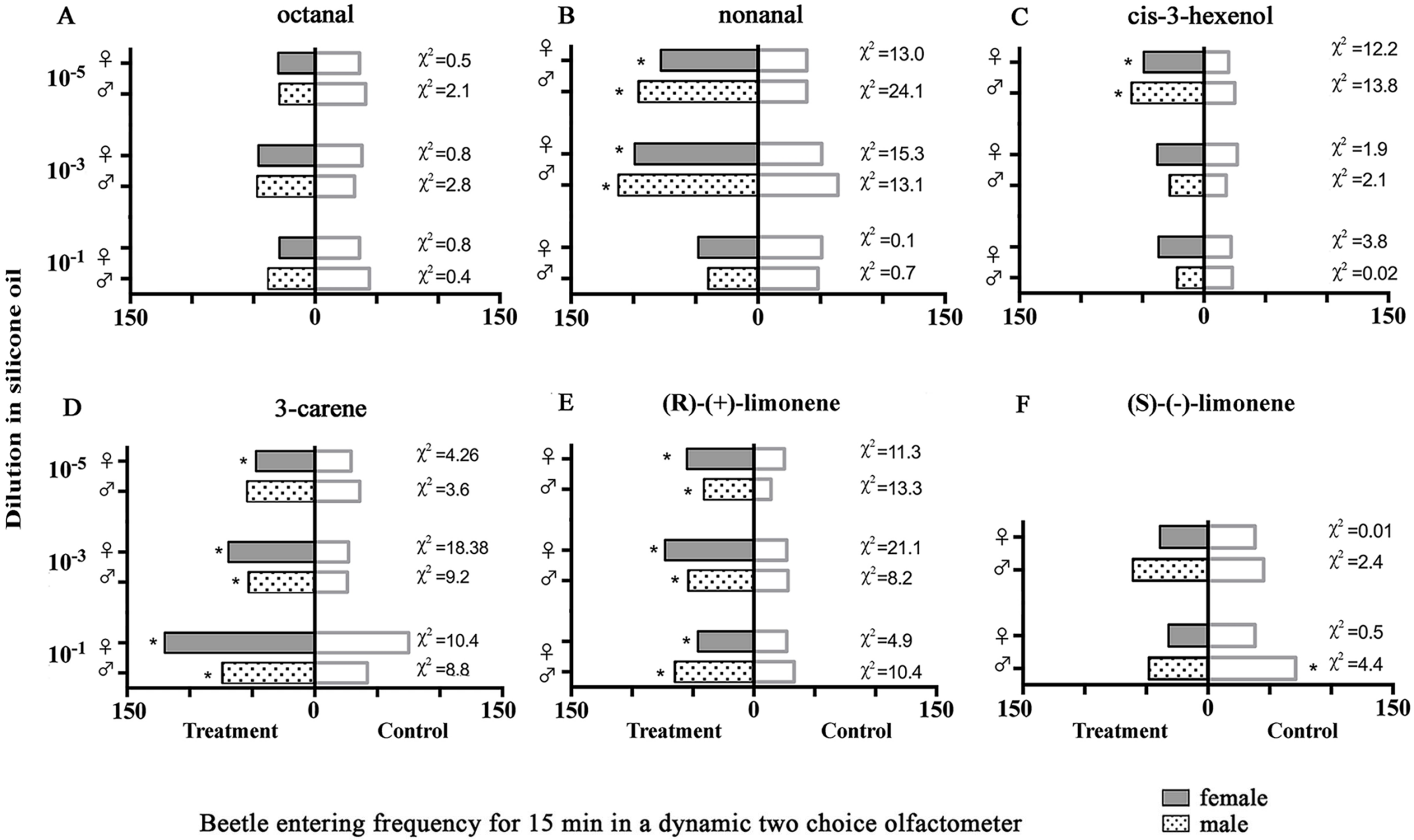

Figure 5-3 continued ……..

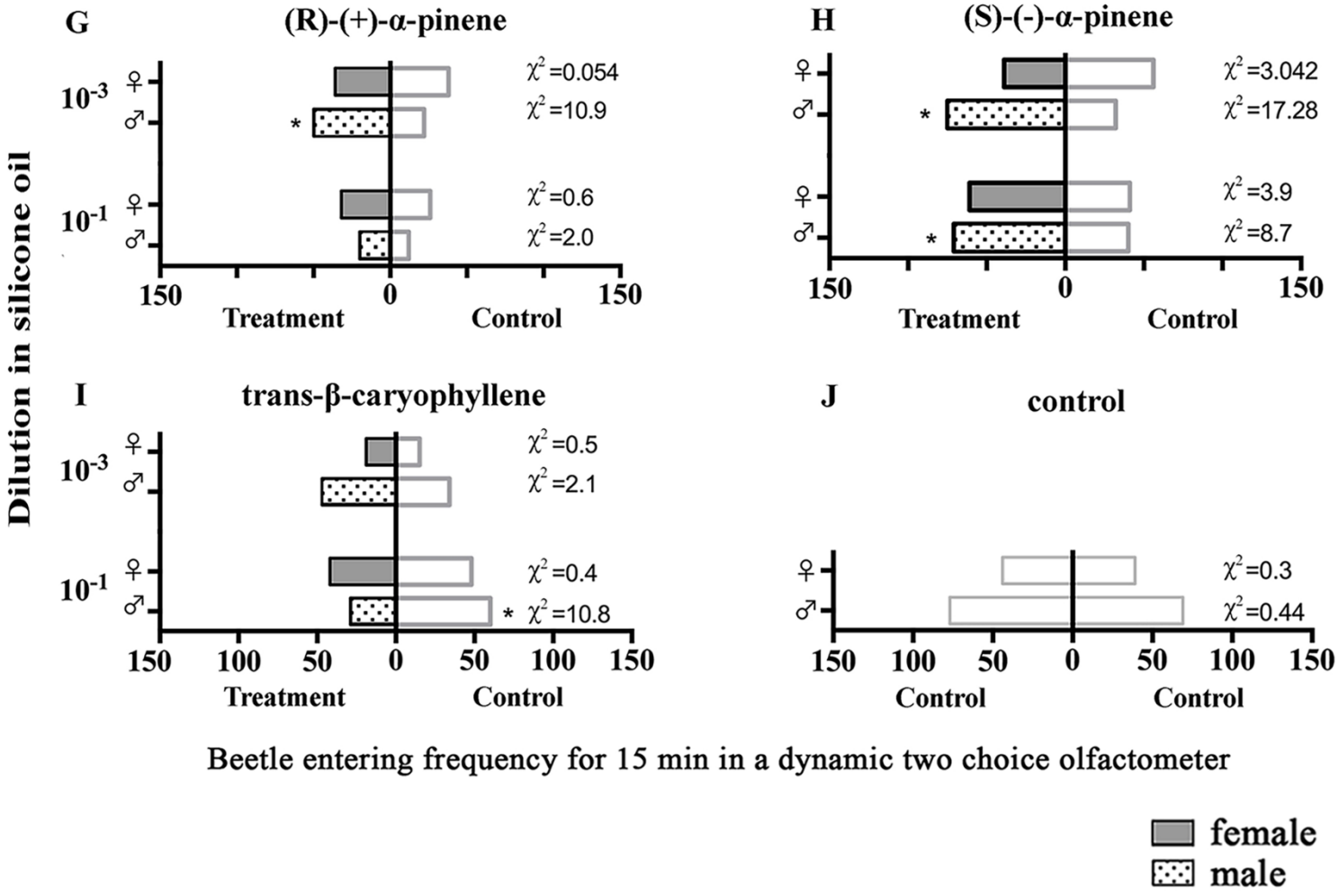

Figure 5-3. Beetle entering frequency (BEF) of *D. helophoroides* to treatment- and silicone oil control- zones within 15 min of exposure to different concentrations of volatile compounds diluted in silicone oil (mg/mg). Left bars indicate entering frequency to treatment zones, right bars indicate entering frequency to control zones. Asterisk indicate significant differences among different treatments ($P < 0.05$, chi-square test, $n = 50$). Control- silicone oil.

Table 5-3. Total distance movement (TDM) in 15 min by *D. helophoroides* exposed to different concentrations (10^{-1}, 10^{-3}, 10^{-5} diluted in silicone oil mg/mg) of volatile compounds

Volatile compounds	Total distance moved in 15 min (cm)					
	Female			Male		
	10^{-1}	10^{-3}	10^{-5}	10^{-1}	10^{-3}	10^{-5}
octanal	20.23±2.52 cd	20.89±2.17 cd	17.74±2.21 cd	18.33±2.37 cd	19.56±1.80 cd	24.89±3.68 bc
nonanal	22.11±2.01 bc	27.24±1.62 b	22.13±2.42 bc	24.53±2.04 bc	26.85±2.03 bc	22.90±3.07 bc
cis-3-hexenol	20.24±2.12 cd	22.13±2.36 bc	***15.73±1.48 d***	16.31±1.26 cd	18.62±1.65 cd	16.62±1.18 cd
3-carene	31.70±2.77 ab	21.31±1.94 bc	18.60±1.22 cd	30.90 ±3.72 ab	21.46±2.18 bc	19.73±1.35 cd
(R)-(+)-limonene	28.71±3.56 ab	27.17±5.37 bc	17.84±1.40 cd	30.73±3.36 ab	23.05±2.41 bc	16.13±1.33 cd
(S)-(-)-limonene	19.86±1.43 cd	21.30±1.74 c	-	20.91±1.27 cd	22.10±1.95 bc	-
(R)-(+)-α-pinene	25.45±1.82 bc	30.65±2.29 ab	-	24.43±2.55 bc	24.29±2.32 bc	-
(S)-(-)-α-pinene	19.76±1.45 cd	22.76±1.93 bc	-	19.23±1.40 cd	22.17±1.66 bc	-
trans-β-caryophyllene	**34.07±5.31 a**	23.93±2.70 bc	-	30.39±4.84 ab	23.71±2.32 bc	-
silicone oil control	*23.13±17.34 bc*			*22.24±18.59 bc*		

Different letters indicate significant difference among different compounds (P<0.05, one-way ANOVA followed by Tukey's multiple comparisons test). Values are means (± standard error). n=50

Table 5-4. Beetle entering frequency (BEF) in 15 min by *D. helophoroides* exposed to different concentrations (10^{-1}, 10^{-3}, 10^{-5} diluted in silicone oil mg/mg) of volatile compounds

Volatile compounds	Beetle entering frequency in 15 min (number of times)					
	Female			Male		
	10^{-1}	10^{-3}	10^{-5}	10^{-1}	10^{-3}	10^{-5}
octanal	0.58±0.14 b	0.92±0.19 b	0.60±0.11 b	0.76±0.20 b	0.94±0.36 b	0.58±0.12 b
nonanal	0.96±0.19 b	**1.98±0.26 a**	**1.56±0.24 a**	0.80±0.12 b	**2.24±0.24 a**	**1.92±0.33 a**
cis-3-hexenol	0.74±0.20 b	0.76±0.19 b	0.98±0.17 b	0.44±0.11 b	0.56±0.14 b	1.18±0.21 b
3-carene	**2.40±0.34 a**	**1.38±0.39 a**	0.94±0.20 b	1.48±0.22 ab	1.08±0.21 b	1.08±0.13 b
(R)-(+)-limonene	0.92±0.15 b	**1.46±0.27 a**	1.10±0.25 b	**1.30±0.23 a**	1.08±0.20 b	0.82±0.14 b
(S)-(-)-limonene	0.64±0.12 b	0.78±0.11 b	-	0.96±0.28 b	**1.22±0.29 a**	-
(R)-(+)-α-pinene	0.64±0.14 b	0.72±0.13 b	-	0.40±0.11 b	1.00±0.29 b	-
(S)-(-)-α-pinene	**1.22±0.20 a**	0.78±0.11 b	-	**1.42±0.31 a**	1.50±0.29 ab	-
trans-β-caryophyllene	0.84±0.17 b	0.38±0.09 b	-	0.58±0.16 b	0.94±0.22 b	-
silicone oil control		*0.78±0.15 b*			***1.38±0.20 a***	

Different letters indicate significant difference among different compounds (P<0.05, one-way ANOVA followed by Tukey's multiple comparisons test). Values are means (± standard error). n=50

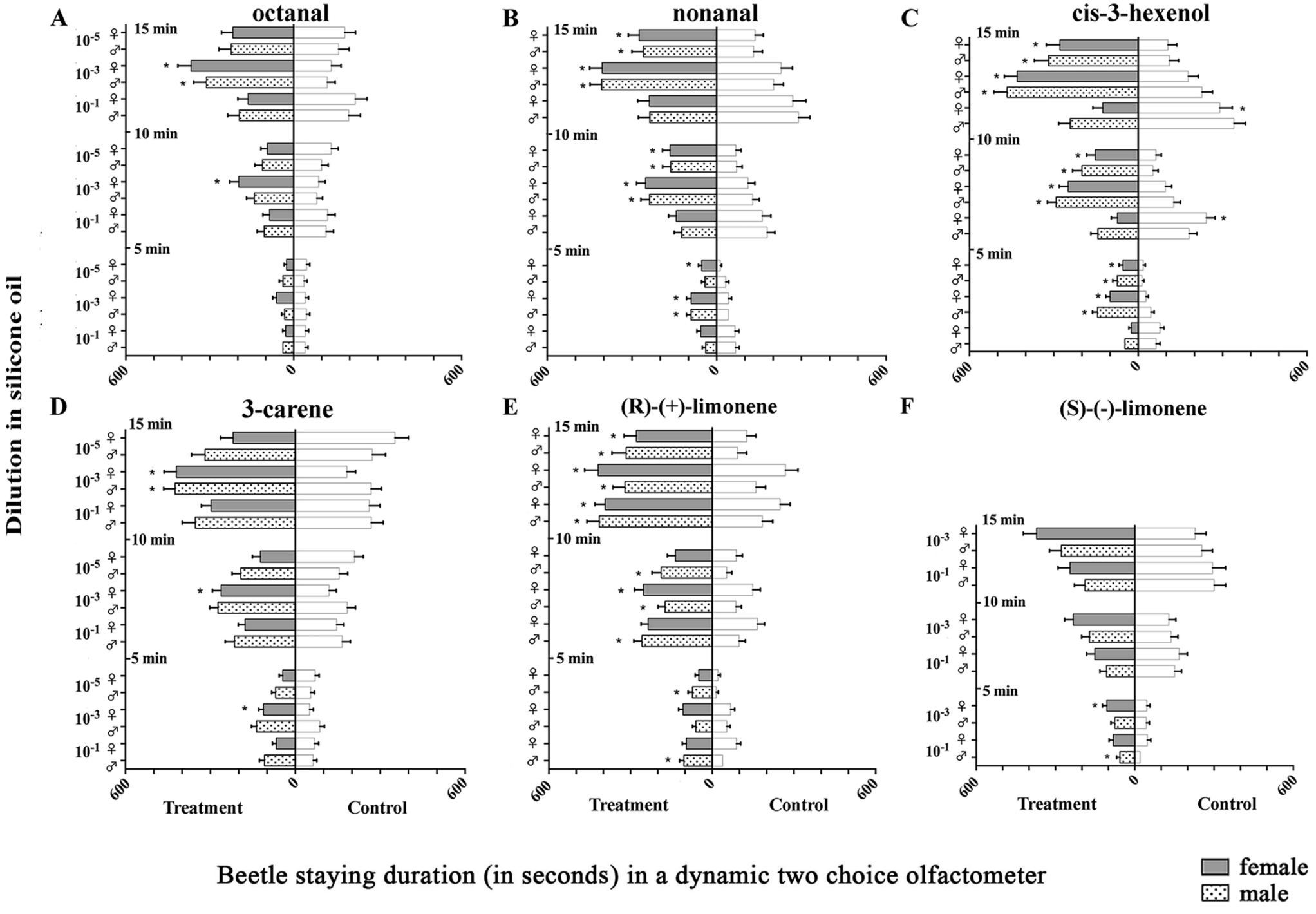

Figure 5-4 continued ……..

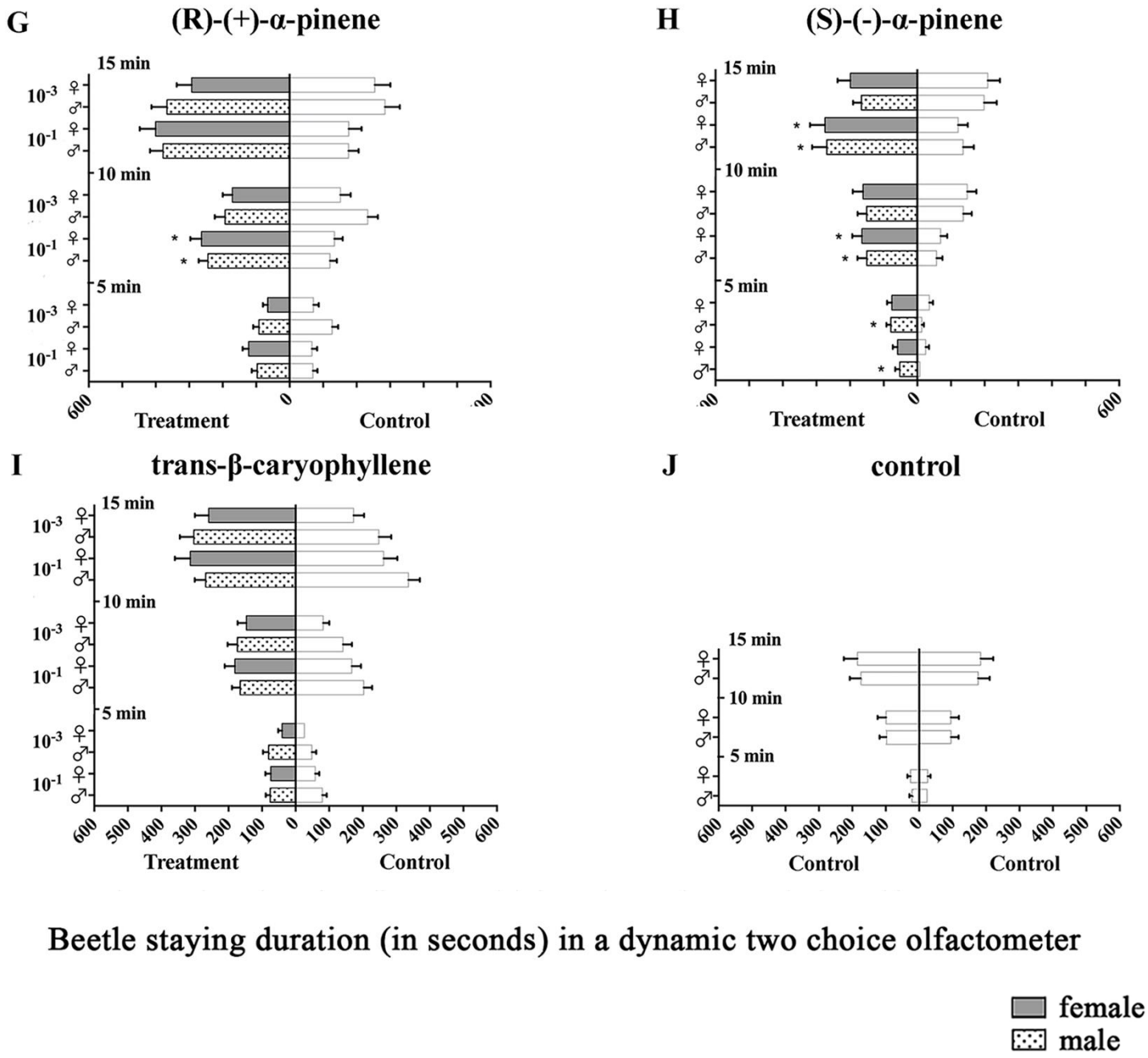

Figure 5-4. Beetle staying duration (BSD) (in seconds) of both sexes of *D. helophoroides* in treatment and silicone oil control zones within 5, 10 and 15 min of exposure to different concentrations (diluted in silicone oil mg/mg) of volatile compounds. Left bars indicate staying duration in treatment zones, right bars indicate staying duration in control zones. Asterisk indicate significant differences among different treatments ($P < 0.05$, multiple t-test). The error bars represent the mean standard error (n = 50). Control- silicone oil.

Table 5-5. Beetle staying duration (BSD) in 5 min by *D. helophoroides* exposed to different concentrations (10^{-1}, 10^{-3}, 10^{-5} diluted in silicone oil mg/mg) of volatile compounds

Volatile compounds	Beetle staying duration in 5 min (in seconds)					
	Female			Male		
	10^{-1}	10^{-3}	10^{-5}	10^{-1}	10^{-3}	10^{-5}
octanal	28.98±10.02 b	61.19±13.31 b	25.57±8.56 b	38.84±11.07 b	32.76±10.08 b	38.84±12.39 b
nonanal	56.80±13.30 b	90.48±15.76 ab	52.84±11.49 b	39.23±11.34 b	90.94±15.55 ab	41.73±11.5 b
cis-3-hexenol	24.64±8.54 b	100.06±15.16 ab	54.18±13.60 b	47.33±10.57 b	**144.81±17.59 a**	74.97±15.5 ab
3-carene	67.21±13.52 ab	112.35±16.91 ab	44.30±12.68 b	109.29±17.56 ab	137.03±17.66 ab	69.55±13.96 ab
(R)-(+)-limonene	94.95±14.67 ab	107.09±16.19 ab	48.98±13.35 b	104.05±15.61 ab	60.13±12.36 b	72.86±15.59 ab
(S)-(-)-limonene	81.31±15.85 ab	105.55±16.34 ab	-	56.30±12.25 b	75.34±14.78 ab	-
(R)-(+)-α-pinene	122.33±17.92 ab	65.73±3.82 b	-	97.26±5.84 ab	91.9±17.07 ab	-
(S)-(-)-α-pinene	59.17±13.48 b	75.64±14.40 ab	-	52.36±13.54 b	78.84±13.02 ab	-
trans-β-caryophyllene	74.11±15.54 ab	40.3±11.57 b	-	75.69±13.43 ab	80.85±16.02 ab	-
silicone oil control	*25.39±8.63 b*			*23.49±8.80 b*		

Different letters indicate significant difference among different compounds (P<0.05, one-way ANOVA followed by Tukey's multiple comparisons test). Values are means (± standard error). n=50

Table 5-6. Beetle staying duration (BSD) in 10 min by *D. helophoroides* exposed to different concentrations (10^{-1}, 10^{-3}, 10^{-5} diluted in silicone oil mg/mg) of volatile compounds

Volatile compounds	Beetle staying duration in 10 min (in seconds)					
	Female			Male		
	10^{-1}	10^{-3}	10^{-5}	10^{-1}	10^{-3}	10^{-5}
octanal	86.51±23.54 b	196.52±31.98 ab	94.42±22.68 b	105.75±24.93 b	140.48±27.77 ab	111.73±27.16 b
nonanal	142.96±27.13 ab	251.69±31.96 ab	164.99±26.65 ab	124.30±25.57 b	238.05±30.15 ab	163.30±27.89 ab
cis-3-hexenol	73.92±21.51 b	249.36±31.23 ab	152.54±30.44 ab	143.62±24.40 ab	**291.57±31.10 a**	200.02±33.17 ab
3-carene	177.41±24.13 ab	262.29±30.23 ab	123.33±28.30 b	214.59±33.05 ab	272.94±29.05 ab	192.67±30.63 ab
(R)-(+)-limonene	235.16±26.08 ab	252.48±32.94 ab	135.09±29.43 ab	257.91±30.23 ab	173.17±25.91 ab	187.39±33.38 ab
(S)-(-)-limonene	151.03±31.11 ab	232.82±32.40 ab	-	107.46±24.38 b	171.59±29.52 ab	-
(R)-(+)-α-pinene	263.40±33.31 ab	171.19±28.80 ab	-	244.06±27.48 ab	192.84±30.93 ab	-
(S)-(-)-α-pinene	164.66±28.52 ab	161.36±30.54 ab	-	150.65±27.27 ab	150.95±26.42 ab	-
trans-β-caryophyllene	181.20±30.36 ab	146.94±25.92 ab	-	165.35±24.10 ab	173.88±28.77 ab	-
silicone oil control	*94.79±24.31 b*			*95.13±22.57 b*		

Different letters indicate significant difference among different compounds (P<0.05, one-way ANOVA followed by Tukey's multiple comparisons test). Values are means (± standard error). n=50

Table 5-7. Beetle staying duration (BSD) in 15 min by *D. helophoroides* exposed to different concentrations (10^{-1}, 10^{-3}, 10^{-5} diluted in silicone oil mg/mg) of volatile compounds

Volatile compounds	Beetle staying duration in 15 min (in seconds)					
	Female			Male		
	10^{-1}	10^{-3}	10^{-5}	10^{-1}	10^{-3}	10^{-5}
octanal	162.63±36.97 c	366.74±47.08 ab	217.5±39.92 bc	194.62±40.85 c	311.90±45.46 bc	223.51±43.52 bc
nonanal	238.76±40.73 bc	405.44±44.34 ab	274.33±38.67 bc	237.24±39.78 bc	407.80±40.60 ab	259.56±40.67 bc
cis-3-hexenol	125.84±35.41 c	430.15±45.51 ab	278.20±48.5 bc	242.12±40.1 bc	**466.14±46.39 a**	318.91±50.40 b
3-carene	297.62±33.9 bc	419.82±43.26 ab	219.82±43.77 bc	353.40±45.97 ab	424.94±39.79 ab	318.63±47.59 b
(R)-(+)-limonene	393.58±37.22 ab	419.28±49.11 ab	278.82±44.86 bc	414.84±37.22 ab	320.65±44.19 b	316.02±52.27 b
(S)-(-)-limonene	244.85±45.83 bc	371.58±50.45 ab	-	188.83±39.44 c	278.06±44.67 bc	-
(R)-(+)-α-pinene	400.34±47.19 ab	292.44±44.74 bc	-	378.27±38.95 ab	366.53±45.9 ab	-
(S)-(-)-α-pinene	274.80±44.06 bc	199.08±37.85 c	-	269.58±42.99 bc	166.55±24.55 c	-
trans-β-caryophyllene	313.99±45.93 b	258.83±41.17 bc	-	268.29±32.4 bc	303.41±41.19 bc	-
silicone oil control	*183.90±37.78 c*			*176.66±34.21 c*		

Different letters indicate significant difference among different compounds (P<0.05, one-way ANOVA followed by Tukey's multiple comparisons test). Values are means (± standard error). n=50

5.5. Discussion

Previous results from our laboratory showed that the antenna of *D. helophoroides* contains different types of sensilla with a porous cuticle, suggesting that these sensilla might be involved in olfaction (Ren et al. 2012). In the present study, the EAG responses of *D. helophoroides* were recorded to explore the antennal detection of their prey-associated volatile compounds (Weißbecker et al. 2006; Fan et al. 2007; Wei et al. 2008, 2013; Hu et al. 2009; Scholz and Schütz, 2012; Li et al. 2016). To the best of our knowledge, this study is the first to investigate both electrophysiological and behavioral responses of *D. helophoroides* to their prey-associated volatile compounds at different dilutions. The aldehydes octanal and nonanal eliciting the highest EAG response amplitudes in both sexes of the beetles suggesting that more olfactory chemoreceptors are involved in the detection of these aldehydes (Kaissling, 1986). However, in our behavioral tests, both sexes showed statistically significant responses only to nonanal. This shows that highly EAG-active compounds do not always necessarily elicit behavioral responses. Nevertheless, mixtures of these aldehydes might elicit significant behavioral responses by *D. helophoroides* in an olfactometer as demonstrated by their prey *A. glabripennis* (Wickham et al. 2012).

Both, female and male antennae showed high EAG responses to cis-3-hexenol. However, different concentrations of this compound have different effects on the behavioral responses of the two sexes. For example, female beetles were significantly repelled when exposed to cis-3-hexenol at a dose of 10^{-1}, whereas at a dose of 10^{-3}, the same compound was attractive. Even an attractive effect was elicited by cis-3-hexenol at a dose of 10^{-5} for both female and male beetles, suggesting a role in finding mating partners and oviposition substrates (Table 5-8). Moreover, this green leaf volatile is released from damaged deciduous trees, eliciting strong EAG and attractive behavioral responses to *B. horsfieldi* and *A. glabripennis*, prey species of *D. helophoroides* (Li et al. 2003; Nehme et al. 2009; Yang et al. 2010). Thus, cis-3-hexenol might serve as a kairomone for deciduous trees by providing a clue to the cerambycid herbivores and serve as a synomone by luring predators of these herbivorous beetles.

The common plant sesquiterpene trans-β-caryophyllene elicited a weak EAG response and a strong repellent behavior in both female and male beetles. This is underlined by significantly increased TDM response of *D. helophoroides* when exposed to trans-β-caryophyllene suggesting that this compound may contribute to non-host avoidance.

Table 5-8. Different behavioral response patterns of *D. helophoroides* to selected volatile compounds at different concentrations (10^{-1}, 10^{-3}, 10^{-5} diluted in silicone oil mg/mg) as determined by the TDM, BEF and BSD assay parameters

Chemicals	Dilution (mg/mg)	TDM	BEF	BSD	Behavior response	
					Female	Male
octanal	10-1	-	-	-	-	-
	10-3	-	-	F+M+	Att.	Att.
	10-5	-	-	-	-	-
nonanal	10-1	-	-	-	-	-
	10-3	-	F+M+	F+M+	H. Att.	H. Att.
	10-5	-	F+M+	F+M+	H. Att.	H. Att.
cis-3-hexenol	10-1	-	-	F-	Rep.	-
	10-3	-	-	F+M+	Att.	Att.
	10-5	F-	F+M+	F+M+	H. Att.	H. Att.
3-carene	10-1	F+M+	F+ M+	-	Att.	Att.
	10-3	-	F+M+	F+M+	H. Att.	H. Att.
	10-5	-	F+	-	Att.	-
(R)-(+)-limonene	10-1	F+M+	F+M+	F+M+	H. Att.	H. Att.
	10-3	-	F+M+	F+M+	H. Att.	H. Att.
	10-5	-	F+M+	F+M+	H. Att.	H. Att.
(S)-(-)-limonene	10-1	-	M-	-	-	Rep.
	10-3	-	-	-	-	-
(R)-(+)-α-pinene	10-1	-	-	F+M+	Att.	Att.
	10-3	F+	M+	-	RM	Att.
(S)-(-)-α-pinene	10-1	-	M+	F+M+	Att.	Att.
	10-3	-	M+	-	-	Att.
trans-β-caryophyllene	10-1	F+M+	M-	-	RM	Rep.
	10-3	-	-	-	-	-

'TDM' Total Distance Movement; 'BEF' Beetle Entering Frequency; 'BSD' Beetle Staying Duration. '-' no significant response; 'F+' significant positive response of female; 'M+' significant positive response of male; 'F-' significant negative response of female; 'M-' significant negative response of male.

F+/M+ in TDM- robust movement towards tested zones in the same track (does not alone reveal the kinesis activity of the beetles).
F-/M- in TDM- little movement towards any tested zones in the same track (does not alone reveal the kinesis activity of the beetles).
F+/M+ in BEF- Attraction

F-/M- in BEF- Repulsion
F+/M+ in BSD- Attraction
F-/M- in BSD- Repulsion
***Att**.- Attraction, **H. Att**.- High Attraction (**Att.** in both BEF and BSD), **Rep**.- Repulsion, **RM.**- Robust Movement, **LM.**- Little Movement.*

The monoterpenes 3-carene, (R)-(+)-limonene, (S)-(-)-limonene, (R)-(+)-α-pinene and (S)-(-)-α-pinene elicited intermediate EAG responses. No significant sex specific antennal response differences to any of these compounds was observed. Moreover, behavioral responses of both sexes towards these monoterpenes are quite similar. Additionally, there was no sexual dimorphism in EAG and behavioral responses to enantiomers of α-pinene and limonene. This is not unexpected as both sexes live on resources characterized by prey associated volatile cues. Similar results have been demonstrated in the cerambycid *B. horsfieldi* (Yang et al. 2010). In both sexes, we found a strong discrimination between the enantiomers of limonene, both in EAG and behavioral responses of *D. helophoroides*. (S)-(-)-limonene elicited significantly higher EAG responses in both sexes. However, in behavioral tests only (R)-(+)-limonene elicited an attraction response in both sexes. These results are in agreement with a previous report (Wei et al. 2008), which demonstrated that *D. helophoroides* adults were significantly attracted by (R)-(+)-limonene derived from the frass odor of the prey species *M. raddei*. In addition to these findings, we confirmed significant attractive responses of *D. helophoroides* to (R)-(+)-limonene over a broad range of concentrations. Similar enantiomeric discrimination has been observed in diverse forest tree pests like *Xylotrechus rusticus, Dendroctonus sp., Hylobius abietis* (White and Hobson, 1993; Wibe et al. 1998; Yan et al. 2006; Staeben et al. 2015) as well as prey species of *D. helophoroides* including *A. glabripennis* and *M. alternatus* (Fan et al. 2007, 2007a; Liqing et al. 2013). This highlights the importance of enantiomeric discrimination for finding suitable plant and/or prey. Consequently, highly specific enantiomer discrimination of prey associated compounds might sharpen the searching ability of predators like *D. helophoroides* to reach their prey.

The prey-associated plant volatiles played a crucial role in the prey location process of *D. helophoroides*, as demonstrated in another coleopteran predator *Thanasimus dubius* for the forest pest *Dendroctonus frontalis* (Staeben et al. 2015). *D. helophoroides* is an oligophagous predator that preys on at least six forest pest species of cerambycids (Wei et al. 2008, 2009). Thus, adult *D. helophoroides* need to adapt to a broad range of prey-associated chemical cues that are not

necessarily specific to one prey insect. EAG and behaviorally active compounds in this beetle, such as nonanal, cis-3-hexenol, 3-carene, (R)-(+)-limonene, (R)-(+)-α-pinene and (S)-(-)-α-pinene, are common host volatiles or feeding-induced volatiles of several cerambycid beetle species (Wei et al. 2008, 2009; Costello et al. 2008). For example, nonanal, cis-3-hexenol, 3-carene, (R)-(+)-α-pinene and (S)-(-)-α-pinene were previously reported to generate robust EAG responses and elicit behavioral responses to *A. glabripennis* and *B. horsfieldi* (Li et al. 2003; Nehme et al. 2009; Yang et al. 2010; Zhuge et al. 2010; Wickham et al. 2012; Liqing et al. 2013). Similarly, (R)-(+)-α-pinene and 3-carene were also reported generate robust EAG responses in *M. alternatus* (Fan et al. 2007). This suggests that many cerambycid beetles use host plant volatile cues for finding their host and assessing the suitability for feeding or oviposition. Similarly, an olfactory response of *D. helophoroides* to prey associated volatile compounds either from their prey host plant or prey frass (Wei et al. 2008, 2013; Nehme et al. 2009; Zhuge et al. 2010; Liqing et al. 2013) is viewed as an adaptive strategy to locate the prey. This also suggests that *D. helophoroides* is mainly relying on different prey associated volatile cues either in individual or in particular ratios to find prey, as well as for mating and oviposition site selection.

In conclusion, the EAG results of this study represent an initial attempt to demonstrate the electrophysiological sensitivity of both sexes of *D. helophoroides* to prey-associated plant volatiles. In addition to previous behavioral studies (Wei et al. 2008, 2013), we found that the beetles exhibited concentration dependent behavioral responses with different behavioral response affected/triggered. Most of the selected compounds elicited notable EAG and behavioral responses. The beetle *D. helophoroides* has 52 odorant binding proteins (OBPs), 19 chemosensory proteins (CSPs),10 olfactory receptors (ORs), 8 ionotropic receptors (IRs), 2 gustatory receptors (GRs), and 5 sensory neuron membrane proteins (SNMPs) in their antennae (Wang et al. 2014), suggesting a differentiated role in olfaction. Our results can foster future research approaches combining electrophysiological methods with molecular techniques such as RNAi knockdown to understand the olfactory mechanism of this predatory beetle in detail. Our results show that octanal, nonanal, cis-3-hexenol, 3-carene, (S)-(-)-α-pinene and (R)-(+)-limonene are most acutely perceived by *D. helophoroides* antenna. These compounds might be useful either individually or in mixtures for developing efficient attractants to lure this predatory beetle into forest stands damaged by cerambycid herbivores. For instance, the green leaf volatile cis-3-hexenol is

sensitively perceived by *D. helophoroides* but not by its prey *M. alternatus* (Fan et al. 2007). Therefore, cis-3-hexenol might be used as an allochthonous kairomone (Wehnert and Müller, 2012) to attract the *D. helophoroides* beetles into the *M. alternatus* attacked forest stands without attracting more *M. alternatus.* Additionally, the most effective compounds should be tested in various combinations in the field trapping experiments or in biological control sites to see if the compounds increase the attraction of *D. helophoroides* and by extension enhance predation rates.

5.6. Acknowledgements

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CHAPTER 6

6. General Discussion and Outlook

The general purpose of this dissertation is the investigation of olfactory response information of two coleopterans for the development of integrated pest management strategies (IPMs). Olfaction is obligatory in guiding insect behaviors to reach their food habitat, find mating partner, shelter and oviposition substrate. Volatile organic compounds (VOCs) from surroundings are the source of the odor that guide insects to exhibit a different behavioral pattern (Cardé and Willis, 2008). In order to manipulate insect behavior in IPMs, it is necessary to understand the olfactory responses to their host- related volatile compounds (Riolo et al. 2012; Ndomo-Moualeu et al. 2016). In the first part of this dissertation, I started with the investigation of the olfactory response of the stored product pest *Tribolium castaneum* to a broad spectrum of VOCs. Host association of *T. castaneum* is multifaceted and therefore cause difficulty to select a particular group of food compounds for the olfactory analysis. This results in gathering VOCs not only ecologically related to *T. castaneum* but also to their closely related species and other storage pest insects (Chapter 2). In the second part of this dissertation, investigated the olfactory responses of the important forest insect predator *Dastarcus helophoroides* to its prey related VOCs. The understanding of the predator-prey interaction is essential to develop an effective IPM strategy for this beetle as well. It is discussed in Chapter 5, how far the prey related VOCs play a role in *D. helophoroides* olfaction and also what their differential behavior pattern to perceptive VOCs is. Electroantennography (EAG) is capable to identify the antennally perceive compounds at different concentrations, that could be used in traps either individually or in mixtures to manipulate insect behavior as shown in Ndomo-Moualeu et al. (2016) (semiochemical-based IPM strategy; Chapter 2 & 5). It was also possible to demonstrate that using improved EAG technique for stable and reliable antennal response recordings for a series of chemical compounds (Chapter 2 & 5), which it is not the case in other methods (Verheggen et al. 2007). In order to use the valuable olfactory information from the EAG results, further research work examined the functions of odorant reception of two OBPs *TcasOBP9A* and *TcasOBP9B* from *T. castaneum* through an RNAi-mediated gene silencing approach (Chapter 3). This work revealed the involvement of *TcasOBP9A* and *TcasOBP9B* in the

detection of diverse VOCs (Chapter 3). The practical application of such laboratory experiments led to the development of an rGO-FET biosensor for field applications, functionalized with *TcasOBP9A* and *TcasOBP9B* as a sensing element (Chapter 4). This artificial olfactory biosensor has shown that rapid detection of two disease markers is possible and results are promising to compare between *in-vitro* and *in-vivo* responses (electronic olfactory sensor based IPM strategy; Chapter 4).

6.1. Electroantennography and behavioral study reveal the basic information of the insect olfaction

The first chapter of this dissertation provides details about the literature evidence supporting the necessity of findings of antennally perceived VOCs by both coleopterans, *T. casataneum* and *D. helophoroides*. With an EAG, it would be possible to obtain sum-signals of the olfactory receptors of the whole insect antenna, would results in dose-response curve representing primarily the number of olfactory dendrites responding to the stimulus at a given concentration (Schneider, 1957, 1962; Mayer et al. 1984; Park and Hardie, 1998; Thakeow et al. 2008). This might confer some ecological information, assuming that compounds which stimulate many olfactory dendrites have to provide some important chemotaxis information to perceived insects, either moving towards or away from the odor sources.

Olfactory response of stored product pest insect *Tribolium castaneum*

In order to establish effective pest management strategies, one must atleast understand the chemosensory information of the insects. There are several VOCs released from stored grains and food products at various conditions like ripening stages, mechanical damages, during growth of microorganisms and infestations by primary pests. These play a major role in guiding insect behaviors (Dunkel, 1988; Schütz et al. 1999; Ahmad et al. 2012). Those VOCs belong to primary alcohols, terpenoids, carboxylic acids, aldehydes, alkenes, esters, alkanes, ketones, and phenolic compounds. Obviously, not all classes of compounds are releasing at an unambiguous time but depend on different stages and conditions of stored products. Red flour beetle *T. castaneum,* as a secondary pest, exhibit complex food grain associations and has a polyphagous feeding habit (Rees, 2004) (Chapter 1). Consequently, for olfactory analysis, the information of VOCs of many

food items is needed, in which *T. castaneum* inhabiting and additionally need the information about the effects of VOCs on many other stored product pests (see Table 2-1 in Chapter 2). Based on the literature evidence, it would be possible to hypothesize that some infochemicals from other stored product pests might also be a cue for *T. castaneum* to reach the food items, as this beetle is considered secondary pest. However, to narrow the VOC selection for recording the antennal response of *T. castaneum*, I would suggest using the gas chromatography for analyzing volatile compounds of beetles food items, together with electroantennographic detection (GC-EAD), which would be required to simplify the research in an appropriate way, as shown in *Cis boleti* (Thakeow et al. 2008). When I tried to establish stable GC-EAD recordings for *T. castaneum* experienced that reliable results would not be possible with excised antennae, as it has a too short lifetime and since most chemoreceptors are covered by Ringer solution that interfere response to stimuli after contact is made in the antenna holder. Therefore additional effort is required in future work to overcome this issues.

It is surprising that the *T. castaneum* has been used as a model organism for many decades but no olfactory information had been published besides for the pheromone 4,8-dimethyldecanal (Engsontia et al. 2008). This encouraged me to focus on this beetles olfaction to be able to present the first report of peripheral olfactory responses of both female and male *T. castaneum* antennae to 94 putative natural volatile compounds (Chapter 2), reveal which that 90 % of compounds are detected by the antennae of both sexes (Balakrishnan et al. 2017). Both sexes of *T. castaneum* elicited high EAG amplitudes to undecane, 1-hexen-3-ol, octanal, 2-heptanone, 2-pentanone, hexanoic acid, and ethyl hexanoate (Chapter 2). The ecological importance of these perceptive compounds might differ at various needs of the beetles. In the case of alkanes like undecane that present in plant and seed surface waxes, playing a role as significant attractants for plant-feeding insects (Sarkar et al. 2013). On the other hand, octanal and hexanoic acid are considered as products of lipid oxidation and concentrations of these compounds increase with progressing degradation of lipids in food grains, indicates food quality for *T. castaneum* (Xiao et al. 2014). Similarly, the possible role of other perceived compounds by both sexes of *T. castaneum* are discussed in detail in Chapter 2. Additionally, the general importance of carbon chain length, molecule size, and vapor pressure of compounds that are proposed in the ability of insects in odor discrimination and specialization are also discussed. Furthermore, the role of vapor pressure in relating to the EAG response of tested VOCs is considered and it could be shown speculatively

that the binding site of odorant receptors for odorants influence the strength of the EAG response of the *T. castaneum,* as reviewed in Andersson et al. (2015). It has also been possible to demonstrate that the olfactory system of *T. castaneum* might have evolved odor discriminative power to deal with a broad range of compounds and benefit from an informational value on food quality. It was possible to conclude that the host finding behavior of *T. castaneum* is generally processing with the combination of certain compounds, as in nature not one specific host compound acts as a principal compound to identify the habitat, except for sex-specific pheromone compounds (Stengl, 2010). This is true in the majority of phytophagous insects that follow bouquets of volatile compounds released by their host, in particular ratios rather than a single compound are important (Najar-Rodriguez et al. 2010; Bruce and Pickett, 2011; Ndomo-Moualeu et al. 2016).

Olfactory response of the predatory beetle *Dastarcus helophoroides*

Insect biocontrol agents such as predators might utilize the specific volatile cues released from the frass of their prey or plant volatiles released upon feeding by herbivores, as a host finding strategy (Grégoire et al. 1991; Thaler, 1999; Staeben et al. 2015). In Chapter 5 of this dissertation, we investigated both electrophysiological and behavioral responses of *D. helophoroides* to their prey-associated volatile compounds. Both sexes of this beetles elicited promising antennal responses to prey related VOCs: nonanal, octanal, *cis*-3-hexenol, 3-carene, (R)-(+)-α-pinene, (S)-(-)-α-pinene, (R)-(+)-limonene and (S)-(-)-limonene (Ren et al. 2017). The sensitivity of the chemoreceptor neuron of *D. helophoroides* to the tested volatiles are clearly revealed here with different concentrations of the same compound. Two aldehydes, nonanal and octanal, released from *A. glabripennis* attacked *Populas nigra* trees elicited strong EAG response in both sexes. In addition, a behavioral responses to one of the aldehyes (nonanal) indicates the general importance in predator-prey-host association (Scholz and Schütz, 2012; Hu et al. 2009), (Chapter 5). Because of the key function of the host plant releasing volatile compounds for plant feeders in finding their host plant and its palatability (Weißbecker et al. 2006; Fan et al. 2007), the general importance of prey perceiving host plant volatiles is proposed an infochemical for predatory insect like *D. helophoroides*, to encounter their specific prey (Staeben et al. 2015).

In order to identify the kinesis activity of insects, I would prefer the EAG experiments together with behavioral assays, since it is necessary to confirm the behavioral activity of

antennally detected compounds (Holighaus et al. 2014; Thakeow et al. 2008). Differences in animal lifestyles are expected to correspond to the differences in chemosensory perceptions. In holometabolous insects, like all Bothriderids species, adult and larvae display very distinctive lifestyles (Ren et al. 2012). Adult *D. helophoroides* beetles have to fly and orient over considerable distances to find prey, mates and oviposition sites (Li and Wu, 1993) but larvae are less locomotive and live directly on their prey. Therefore short and long-range infochemicals are ideal for adult beetles, performing perfect orientation towards their prey source. However, depending on the adult insect primary needs, the same odor compound may or may not lead to exhibit different activity patterns in the chemosensory pathways (Chapter 5). In some cases, the antennally detected compounds might elicit differential behavioral pattern at the insect brain centers depending on the concentrations, including compounds eliciting low EAG amplitude (Riolo et al. 2012; Holighaus et al. 2014; Ren et al. 2017). *D. helophoroides* is either repelled or attracted to the green leaf alcohol *cis*-3-hexenol, suggesting a discriminative decision role in finding oviposition substrates (Chapter 5). Both female and male beetles were strongly attracted to nonanal, *cis*-3-hexenol, 3-carene, or (R)-(+)-limonene even at low concentrations. But, female and male beetles were repelled to a high concentration of *cis*-3-hexenol and (S)-(-)-limonene, respectively. However, positive behavioral response to the compound that elicited high EAG amplitude is not expected and repetitive behavioral response from a perceived stimulus, either attraction or repulsion, is well interpreted. In addition to providing specific stimulus input, *D. helophoroides* is able to discriminate chemotaxis of the compound even at an enantiomeric level for (R)-(+)-limonene and (S)-(-)-limonene, as shown in Thakeow et al. (2008). These compounds, might, in turn, mediate intraspecific signals among the individuals of the same sex in beetle populations. Therefore, such a compound might presumably be involved in sharpening the decisive ability of *D. helophoroides* towards the aggregation status of conspecifics, to avoid overpopulation on the same prey resources. Overall behavioral activity of the *D. helophoroides* shows increasing compound concentration about the preferred range, generates increased locomotor activity (total distance movement), and results ultimately in beetles moving to the desirable odor source (see table 5-8 in Chapter 5). Therefore, olfaction studies (EAG and behavioral assays) support the knowledge of utilizing the same plant volatile as cues for both predators and prey to reach their prey and host plant, respectively, suggesting possible dual functions of host plant volatile compounds (Fan et al. 2007; Yang et al. 2010; Li et al. 2003; Liqing et al. 2013; Zhuge et al. 2010). However, it should be

tested, whether those perceived compounds by *D. helophoroides* in the lab would give similar results under field conditions, by applying them individually or in mixtures of compounds in a trap.

Previously, several EAG studies have been conducted either in lab conditions (Park et al. 2001; Visser et al. 1996; Germinara et al. 2009, 2016; Chen and Fadamiro, 2007) or in field conditions (Sauer et al. 1992; Karg and Sauer, 1995; Milli et al. 1997; Schütz et al. 1999). However, most of the EAG studies have shown considerable variations in the lifetime of an insect antenna and usually noticed with the lifetime of excised insect antenna do not last > 2 h (Hardie et al. 1994; Visser et al. 1996; Visser and Piron, 1997) and often less than 30 min (Van Giessen et al. 1994). Moreover, attaining a stable and long last EAG recording is a major constrain in many insect species, especially in those having clavate, capitate or club-shaped antenna (Verheggen et al. 2007; Blažytė-Čereškienė et al. 2016). Because, those antenna types have their chemoreceptors mostly on the last antennal segment, where the electrode contact is made and electrolyte solution covers most of the chemoreceptors, which results in no response to a given stimulus.

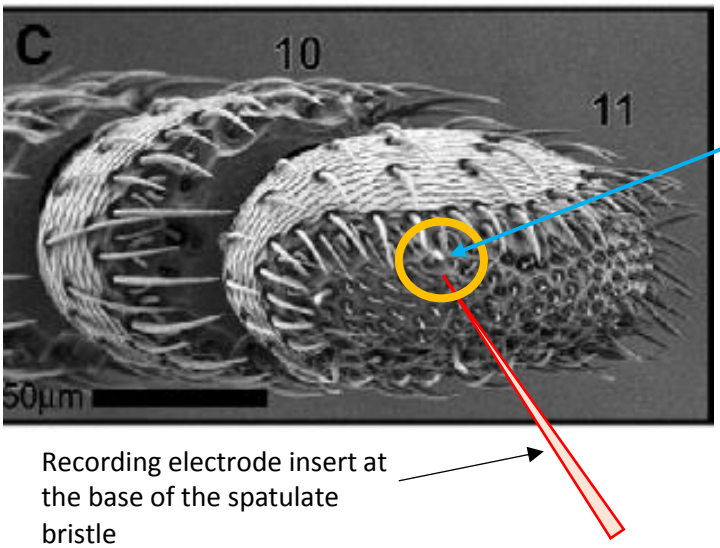

Figure 6-1. Scanning electron micrograph of the distal tip (11th segment) of the *Tribolium castaneum* antenna. Blue arrow indicate the spatulate bristle type of sensilla. The EAG recordings were always performed by inserting the recording electrode at the base of the same spatulate bristle sensilla as marked in round shape (after Angelini et al. 2009)

In this dissertation, I have improved the EAG experimental setup based on the whole body-beetle preparation and this has not only increased the lifetime of the *T. castaneum* antenna (like > 5 h) but also allowed to get the stable and reliable EAG recordings by inserting the recording electrode at the tip of the last antennal segment (details in methodology of Chapter 2). Additionally, to gain consistency in the EAG responses between the individuals of *T. castaneum*

throughout the experiment, I inserted the recording electrode always at the base of a specific spatulate bristle sensillum (Figure 6-1). When preparing the beetle antenna at the same position as shown in Figure 6-1, the above mentioned sensillum is easily identifiable, as it appears bigger in size and slight pinkish in color (Roth and Willis, 1951). It is also possible for other insects to get stable and reliable EAG recordings with this improved setup, as demonstrated here for *D. helophoroides*.

The mechanism of olfactory transduction of odorant stimuli is processed in the antennal lobe and higher brain center of the insects, where behavioral responses are initiated. As a limitation, no behavioral assay was examined in this dissertation for *T. castaneum*. Nevertheless, the EAG response data is enough to identify detectable or undetectable volatile compounds by both coleopteran antennae (Park et al. 2002; Weissbecker et al. 2004; Schütz et al. 1999). Additionally, olfactory response results of both *T. castaneum* and *D. helophoroides* suggest that they are able to discriminate the important infochemicals among detected VOCs through the process of odor coding and recognition as shown in other insects (Hallem and Carlson, 2006; Hansson, and Stensmyr, 2011; Keesey et al. 2015). It was also possible to conclude that this olfactory study and recent molecular studies on both *T. castaneum* and *D. helophoroides* offer the possibility to evaluate the perceived VOCs and their specific binding proteins, that would allow to find out the best option for controlling *T. castaneum* and forest insects ((Bucher et al., 2002; Tomoyasu et al., 2008; Dippel et al. 2014, 2016; Wang et al. 2014; Venthur and Zhou, 2018).

6.2. Role of Odorant Binding Proteins in the insect olfaction

The first step in the development of an RNAi-based IPM strategy is the identification of effective and specific target genes in the targeted pest insects. Such a set of genes is extremely important for certain development aspects of insect like growth, metamorphosis, food digestion, detoxification and VOCs detection (Tomoyasu et al. 2008; Bucher et al. 2002; Tomoyasu and Denell, 2004; Biessmann et al. 2010; Dong et al. 2017). Besides other genes, OBP genes of *T. castaneum* play essential roles in the olfactory responses to several VOCs. Consequently, silencing of these OBP genes would result in altered antennal response to VOCs. *T. castaneum* is a model species for different fields of research (Tomoyasu et al. 2008; Trauner et al. 2009; Bucher et al. 2002; Tomoyasu and Denell, 2004; Grunwald et al. 2013). Stimulated by those studies, which demonstrate the possibilities of RNAi approach on this beetle, performed RNAi-mediated gene

silencing to functionally analyse the two OBPs *TcasOBP9A* and *TcasOBP9B* (highly expressed in the antennae of *T. castaneum*) (Dippel et al. 2014) in this dissertation. This first attempt surprisingly reveals the EAG responses of *dsTcasOBP9A*-treated *T. castaneum* was significantly reduced to all chemically diverse odorants: 4,8-dimethyldecanal , (*E*)-2-heptenal, *cis*-3-hexen-1-ol, β-ionone, 6-methyl-5-hepten-2-one and 2-hexanone (Chapter 3). Specifically, in male beetles, knock-down of *TcasOBP9B* was less effective compare to double knock-down that would result in a similar effect as the *TcasOBP9A* single knock-down. However, in female beetles also the *TcasOBP9B* caused significant EAG response reduction to all tested VOCs at all dilutions (10^{-1}-10^{-8}) (Chapter 3). These results imply that the availability of odor molecules in each dilution would decide the intensity of the EAG amplitude. Nevertheless, further, to confirm the odor-binding function of these highly expressed OBPs, additionally competitive binding assays are required. Preliminary binding assays demonstrated that *TcasOBP9A* are able to bind many VOCs including *cis*-3-hexen-1-ol, and β-ionone (personal data from Dr. Stefan Dippel; Figure 6-2). However, the ligand binding experiments should be conducted for *TcasOBP9A* and *TcasOBP9B* with all above mentioned six compounds, as shown for OBPs in scarab beetle *Holotrichia oblita* (Wang et al. 2013), to find out the affinity level of the OBPs to each ligand which would reveal their contribution in *T. castaneum* olfaction.

Expression analysis of these two OBPs in the antenna of *T. castaneum* shows that they are independently expressed in different sensilla (Chapter 3). Furthermore, *TcasOBP9A* is expressed more centrally in the last segment only, probably in trichoid sensilla but *TcasOBP9B* is expressed radially in all last three antennal segments, probably in basiconic sensilla (Dippel et al. 2016). Despite independent expression, these OBP's roles in beetle olfaction seem to be similar (Chapter 3). However, to gain the particular functional role of *TcasOBP9A* and *TcasOBP9B* in chemokinesis of *T. castaneum* beetles, an RNA interference based loss of olfactory function approach, combined with a behavioral study should be performed, as done for *Helicoverpa armigera* (Dong et al. 2017). Previously, our lab tried to demonstrate the behavioral responses of *T. castaneum* with different types of olfactometers including a dynamic two-choice ten track olfactometer, which, however, reveal inconsistent results due to beetles tend to stop while releasing air stimuli. Therefore, additional effort is required to build an olfactometers, probably without air stream and mimic the original habitat for future research on this beetle.

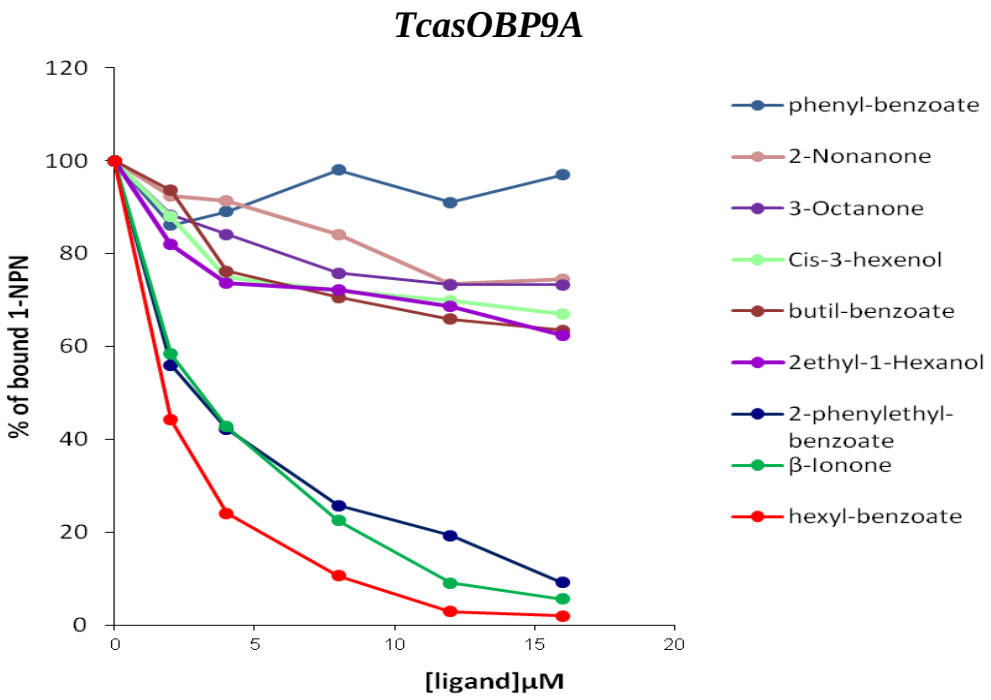

Figure 6-2. Competitive binding curves of selected ligands to recombinant TcasOBP9A of *Tribolium castaneum*. A solution of the protein and 1-NPN in Tris-buffer, both at a concentration of 2 µM, was titrated with 1 mM solution of each potentially competing ligand to final concentrations of 2–20 µM. Fluorescence intensities are reported as percent of the values in the absence of competitor. (This figure is kindly provided by Dr. Stefan Dippel)

6.3. OBPs as a sensing element in rGO- FET based olfactory sensors

In recent decades, portable nanosensor systems are widely used to rapidly detect the marker odorant molecules that would vaporize in insect-infested materials, contaminated food items or diseased items. Previous nanosensor devices are functioning mainly based on human olfactory receptors functionalized with a single-walled carbon nanotube field effect transistors (swCNT-FETs) (Kim et al. 2009). However, very recent years 'bioelectronic noses' have been advancing with OBPs of insects as a sensing element in a graphene-based liquid-gated field effect transistor (Reiner-Rozman et al. 2015, 2016; Larisika et al. 2015; Son et al. 2016; Kotlowski et al. 2018). In Chapter 4 of this dissertation, I present a technical sensing platform for the quantitative analysis of OBP-odor ligand interactions based on the reduced graphene oxide-field effect transistor (rGO-FET) and for the first time we report the comparison between in-*vitro* data from the device and *in-vivo* data from the antennal response of *T. castaneum* to the same tested compounds. The highly expressed OBPs *TcasOBP9A* and *TcasOBP9B* in the antenna of *T. castaneum* were selected and

subjected to the RNA-interference knock-down of both OBPs, which showed massively decreased antennal detectivity to different compounds including 6-methyl-5-hepten-2-one (Chapter 3; Montino et al. in preparation). We, therefore, assembled our rGO-FET sensor device functionalized with these proteins. In the first attempt of such an artificial olfactory system, odor molecule detection of this sensor is monitored by a thin film transistor with a graphene gate by immobilization of OBPs as a bio-mimetic building block (Reiner-Rozman et al. 2016). We aimed through this approach to reduce the complexity of the function of natural insect olfactory system and use these two OBPs as a robust element in the sensor for giving an affinity response to 6-methyl-5-hepten-2-one and 3-octanol. However, the expected results from *in-vivo* (EAG recordings) to 6-methyl-5-hepten-2-one and 3-octanol are potentially less conclusive to compare it with *in-vitro* (rGO-FET device) data and difficult to interpret, since no competitive binding assays were demonstrated for these OBPs to selected compounds. On the other hand, the comparison and interpretation of the affinity data of OBP-Odorant between the *in-vivo* and *in-vitro* systems should be done at the OBP process level and not at the odorant receptor (OR) process level, which brings weakness to these findings. Therefore, to circumvent these issues, I would propose the artificial olfactory system in a simple way with the following two models:

Model I: As OBPs certainly play a major role in detecting and recognizing of the odor molecules with selectivity, sensitivity, and specificity: for this model, preferably select OBPs which show high affinity and sensitivity to the toxic compound, which then could be used as a sensing element in rGO-FET sensor. Figure 6-3 briefly summarizes preparation steps for the Model I device. To detect the highly toxic compound at the intermittent time, OBP-odorants affinity measurements (association) can be recorded when odor molecules reach the surface of the device, where the OBPs are immobilized. After measuring the OBP-odorant affinity, the OBP does not release the bound-odorant molecule, which is not necessary, as we are interested mainly to

measure the detectivity level of the toxic compounds and the presence at an unsafe level would trigger the alarm (Figure 6-3).

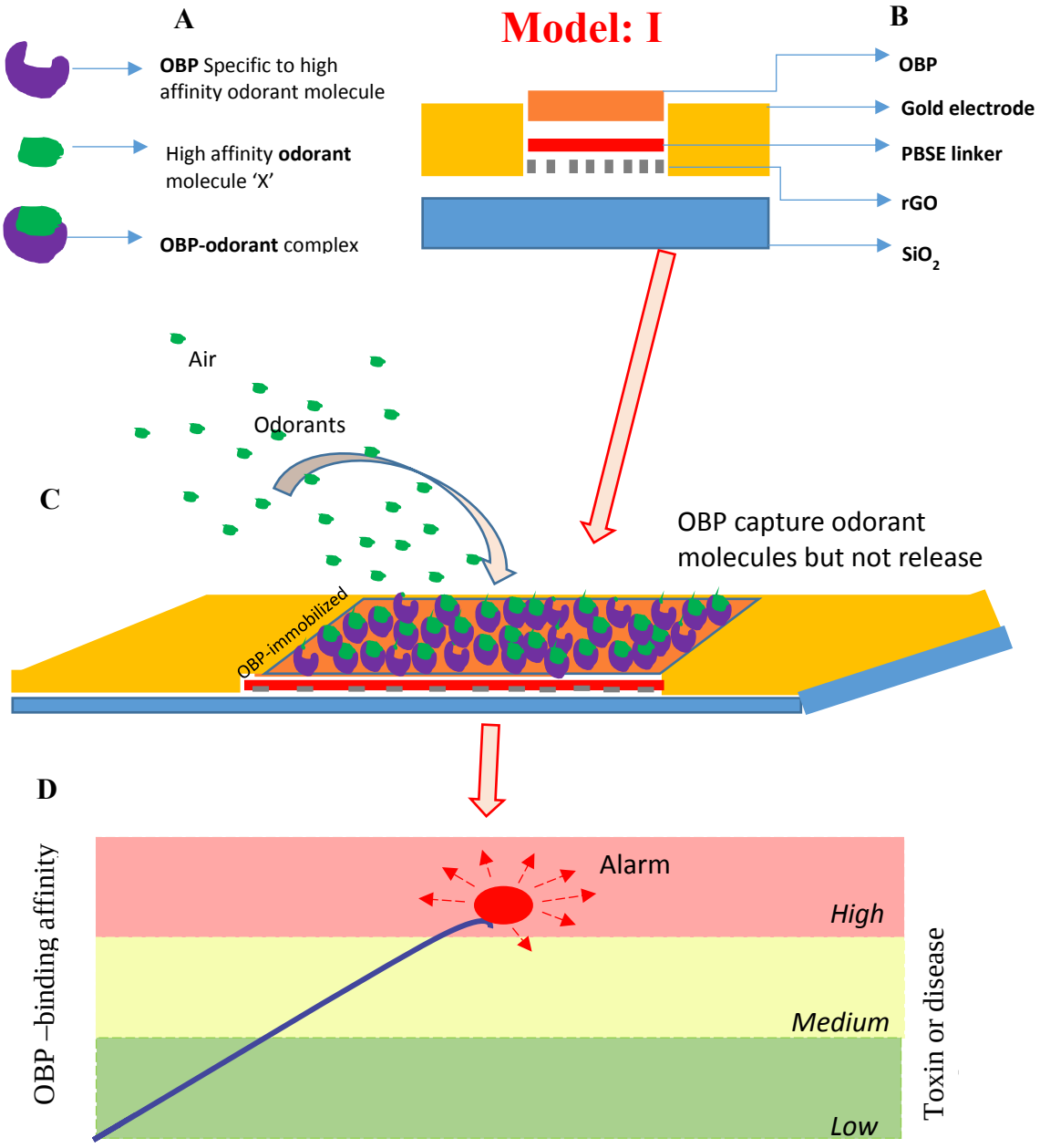

Figure 6-3. Proposed schematic diagram of artificial olfactory device-model I. **A**) Selected odorant binding proteins (OBPs) specifically bind to high-affinity odorant, either toxin or disease marker; **B**) Illustration of the biosensor device with the rGO gate, a linker layer-PBSE for the chemical coupling of the OBP, selected OBP and other material of the device; **C**) Plausible binding mechanisms of odorants from air to OBP (immobilized state in the device), establishing active OBP-odorant forms and odorants once locked in OBP but not release; **D**) Quantifying active OBP-odorant forms (affinity level) of toxin or disease marker at intermittent time, when reach to unsafe level, will trigger the alarm.

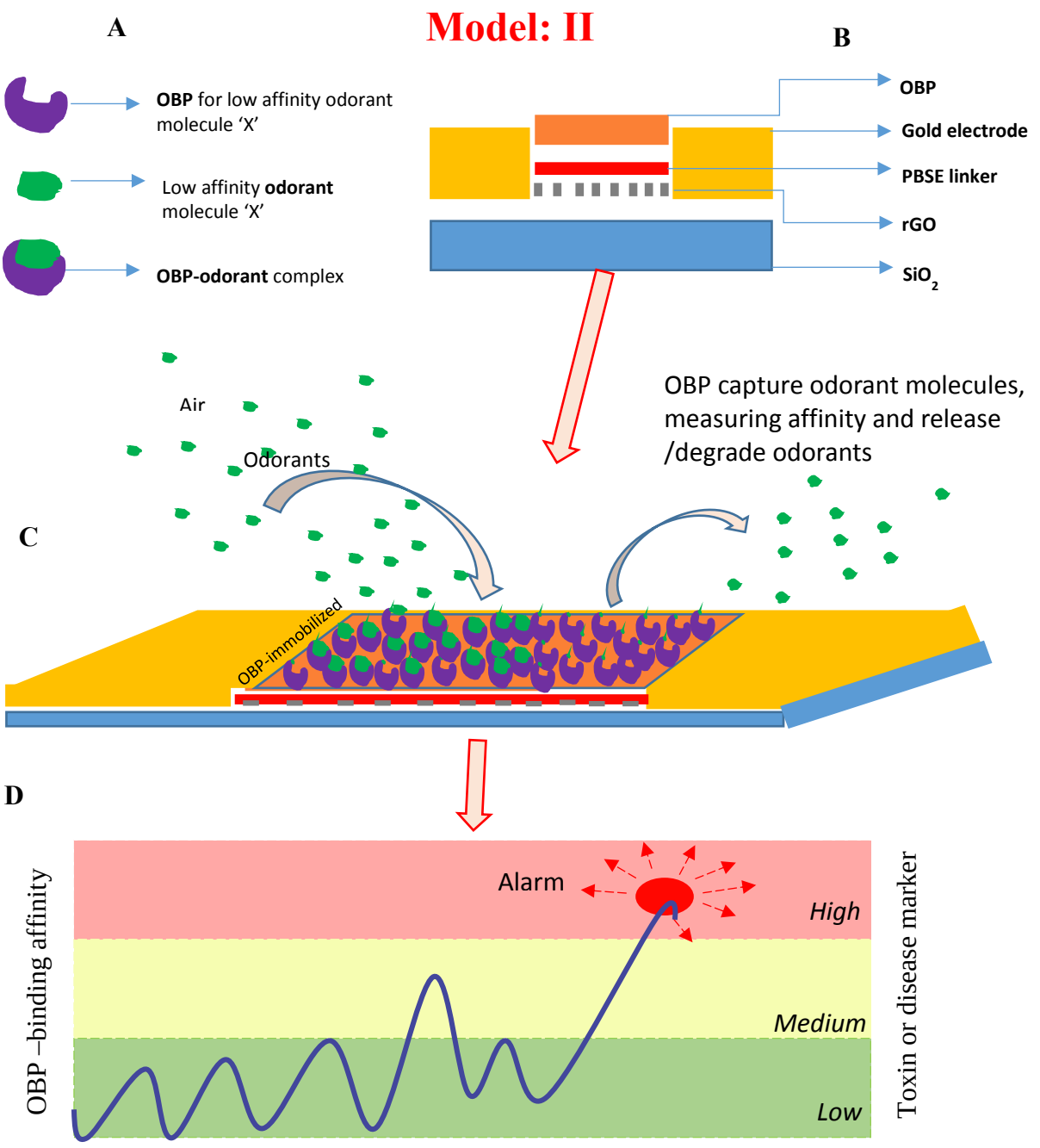

Figure 6-4. Proposed schematic diagram of artificial olfactory device-model II. **A**) Selected odorant binding proteins (OBPs) bind to low-affinity odorants, either toxin or disease marker; **B**) Illustration of the biosensor device with the rGO gate, a linker layer-PBSE for the chemical coupling of the OBP, selected OBP and other material of the device; **C**) Plausible binding mechanisms of odorants from air to OBP (immobilized state in the device), establishing active OBP-odorant forms and once measured affinity level, odorants that locked in OBP either release/degrade; **D**) Quantifying active OBP-odorant forms (affinity level) of toxin or disease marker on-line in real time, when reach to unsafe level, will trigger the alarm.

Model II: In a similar approach as Model I, one can prepare another type of artificial olfactory system to monitor the toxic compound *on-line* in real time. For this model, using low-affinity OBPs could be the best option for binding a toxic compound. Figure 6-4 briefly summarizes preparation steps for the Model II device. With this device model, the OBP-odorants affinity (association) can be measured continuously (accumulation of specific odorant) and after a while, OBPs release bound-odorants or destroy then (disassociation) by means of using any possible methods.

Because the affinity response of OBP-odorant interaction and recovery to originate state needs very long time, thus cannot be directly compared to natural olfactory system, where there are constant replenishment of OBPs and additional interactions with ORs, the SNMPs and ODEs that actively degrade the odorants as described in the review of a recent publication (Pelosi et al. 2018). Additionally, while using OBP as a sensing element in the rGO-FET sensor, data comparison between *in-vitro* and *in-vivo* studies would be inappropriate since OBP is not directly involved in the exhibition of action potentials of the olfactory sensory neurons. Therefore, in contrast to using the OBP in an artificial olfactory device, I would suggest using the OR to detect the specific toxic molecule in the air, as previously demonstrated in a bioelectronics nose functionalized with human OR *hOR2AG1* (Kim et al. 2009). I assume that using OR in the rGO-FET sensor has a number of advantages: (i) the OR gives direct active or inactive information of specific odor molecules, this would be questionable with an OBP as it plays a different role in odorant receptions (Pelosi, 1994); (ii) the OR in a sensor device is more convincing while comparing data between *in-vitro* and *in-vivo*, because odorant-OR active form trigger the action potentials (Leal, 2013); (iii) easy to identify specificity of OR to the specific toxic compound from a bouquet of compounds with gas chromatography-linked single sensillum recording (GC-SSR), as shown in *Drosophila* Or56a that specifically respond to the toxic compound geosmin (Stensmyr et al. 2012).

By using all these aforementioned ideas, it should be possible to develop a portable sensor system based on rGO-FET, which is mimicking the natural olfactory system. Such an artificial olfactory device with not only be useful to detect the toxic compounds but also allow to detect the compound that would be an indicator for early-insect infestations on stored products, food contamination, food degradation, and presence of disease organism. It is necessary now, as insect

pests and diseases are causing extensive food losses both in the field and in stored conditions of important food plants globally and causing several hundred million dollars losses (Oerke, 1994; Mason, 2003).

6.4. Conclusion and future prospectives

As an overall conclusion, the olfactory studies of two coleopteran species would seem legitimate in the development of different components in IPM strategies. The stored product pest *T. castaneum* perceived a broad spectrum of volatile compounds with high sensitivity to C_6-C_9 compounds from alkanes, aldehydes, alcohols, ketones, and esters. Specifically, both sexes of *T. castaneum* exhibited reliable antennal responses to undecane, 1-hexen-3-ol, octanal, 2-heptanone, 2-pentanone, hexanoic acid, and ethyl hexanoate, within compound groups of chemical similarity. It is necessary to unravel the perceptive volatiles of *T. castaneum* as this beetle's used as a model organism for more than six decades without knowledge on its olfactory information processing (see reference in Richards et al. 2008). Furthermore, understanding the regulation of olfactory responses in the model organism like *T. castaneum* will enhance our understanding of the olfaction in beetles in general, and in the long term may lead to the development of new pest control strategies for other coleopterans. The cerambycids predator *D. helophoroides* perceived the volatiles from prey infested trees, prey larval frasses, and prey perceived compound groups. Both sexes of *D. helophoroides* elicited significant antennal responses to nonanal, octanal, *cis*-3-hexenol, 3-carene, (R)-(+)-α-pinene, (S)-(-)-α-pinene, (R)-(+)-limonene and (S)-(-)-limonene. Furthermore, in behavioral experiments, both sexes of *D. helophoroides* are significantly attracted to nonanal, *cis*-3-hexenol, 3-carene or (R)-(+)-limonene even at low concentrations and repelled at high concentration of *cis*-3-hexenol or (S)-(-)-limonene, respectively. For these two coleopterans, an improved EAG method resulted in promising antennal responses to tested VOCs and provides a platform for measuring the antennal response from a tiny insect with a complicated antennal structure, in which chemoreceptors are located only at the last antennal segments.

RNAi-mediated gene silencing of *TcasOBP9A* and *TcasOBP9B* genes showed to interfere with odorant perception of both sexes in *T. castaneum* to 4,8-dimethyldecanal (*E*)-2-heptenal, *cis*-3-hexen-1-ol, β-ionone, 6-methyl-5-hepten-2-one and 2-hexanone. The results showed that these two OBPs are exclusively expressed in different sensilla but involved in a similar olfactory function. Besides, these two aforementioned OBPs are used as a sensing element in rGO-FET

biosensor and are able to reliably detect disease markers such as 6-methyl-5-hepten-2-one and 3-ocatanol at different concentrations.

The experimental work performed in this dissertation resulted in the identification of several very promising perceptive compounds from both coleopteran species, but it is still necessary to evaluate these compounds in the original habitat or in field conditions. Additionally, experiments with single sensillum recording (SSR) should be performed to investigate the olfactory systems of both coleopterans in detail, leading to a possible explanation for which type of sensillum is responsible for what specific compound. A further task could be the development of an odorant receptor (OR)-based rGO-FET biosensor for detecting marker compounds and that would mimic the natural olfactory system more precisely, since ORs are directly involved in the activation and deactivation of on channels. It is well known that insects express ORs with high sensitivity to either beneficial or harmful compounds (Stensmyr et al. 2012) and such ORs could be immobilized in rGO-FET biosensor as done with human ORs (Park et al. 2012).

6.5. References

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Kotlowski, C., **Balakrishnan, K.**, Ramach, U., Rayne, K., Breu, J., Gabriel, M., Kleber, C., Mastrogiacomo, R., Schütz, S., and Knoll, W. Monitoring crop disease markers by odorant binding proteins of *Tribolium castaneum* –Electroantennogram versus reduced graphene oxide based electronic biosensor. 30 Pp

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