Olfactory Mechanisms of Host Selection in Phytophagous Insects

Behavior, Neuron, and Receptor

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Cover: Egyptian cotton leafworm moth *Spodoptera littoralis* and
Eurasian bark beetle *Ips typographus*
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Olfactory Mechanisms of Host Selection in Phytophagous Insects: -Behavior, Neuron, and Receptor

Abstract

The most challenging tasks for phytophagous insects are the location and selection of mates, food sources, and oviposition sites, all crucial for survival and reproduction. To perform these tasks insects rely largely on their sense of smell (olfaction). I address how the insect olfactory system discriminates between components of complex odor mixtures, modulating behavior and fitness. I have studied modulation of attraction in the moth *Spodoptera littoralis* and the bark beetle *Ips typographus* by separation of pheromone (Ph) and anti-attractants, and of Ph components alone. An antagonist reduced male moth attraction towards the female sex Ph, and a blend of non-host volatiles (NHV) reduced attraction of both sexes of *I. typographus* towards their Ph, insect catches decreased with decreasing odor-source distance. Conversely, increasing distance between Ph components decreased attraction in both insect species. However, moths were more sensitive to small-scale spacing. Reproductive behaviors as well as fecundity and longevity of *S. littoralis* moths were negatively affected in the presence of volatiles from leaves of non-host plants, *Picea abies* or *Adhatoda vasica*. The presence of non-host plants strongly modulated male moths' behavior, reducing their attraction towards the Ph source in flight assays. Gas chromatography-electroantennographic detection (GC-EAD) by female *S. littoralis* antennae with headspace volatile collections from *P. abies* and *A. vasica* revealed eight active compounds, with seven new actives. Single sensillum recordings (SSR) created a functional-morphological map of 49 olfactory sensory neuron (OSN) functional types in six morphological sensillum types in female *S. littoralis*. Proximally located OSNs showed a higher sensitivity, shorter latency, and displayed more phasic responses than distally located OSNs of the same class. GC-SSRs with volatiles from a larval host, cotton plants, and the adult nectar source, lilac flowers, revealed 38 active compounds for female OSNs, including 12 new actives. The odor response specificities of four olfactory receptor (OR) genes of *S. littoralis* were deorphanized by expression in the *Empty Neuron System* (ENS) of *Drosophila melanogaster* using SSR and GC-SSR (GC-SSR-ENS). Two of the ORs responded specifically to single odorants, while the other two responded similarly to the same 9 compounds, but dose-response experiments with new compounds, identified by GC-SSR, revealed specific odor-response profiles.

Keywords: Spodoptera littoralis, *Ips typographus*, olfaction, olfactory sensory neuron, olfactory receptor, single sensillum recordings, plant volatiles, non-host volatiles

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Dedication

To beloved Prophet Muhammad (PBUH)
To my family, especially my Mother (late) and my Father-in-law (late)

In the name of ALLAH, the Most Beneficent, the Most Merciful.

Say: He is ALLAH, the Monoreal! Allah (the Monoreal) is Eternal. The Monoreal does not take or give birth. And there is none comparable to the Monoreal (Al Quran).

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Andersson MN, **Binyameen M**, Sadek MM, Schlyter F (2011). Attraction Modulated by Spacing of Pheromone Components and Anti-attractants in a Bark Beetle and a Moth. *Journal of Chemical Ecology* 37(8), 899-911.

II **Binyameen M**, Hussain A, Yousefi F, Birgersson G, Schlyter F. Modulation of the Reproductive Behaviors by Non-host Plant Volatiles in the Polyphagous Egyptian Cotton Leafworm, *Spodoptera littoralis*. *Submitted.*

III **Binyameen M**, Anderson P, Ignell R, Seada MA, Hansson BS, Schlyter F (2012). Spatial Organization of Antennal Olfactory Sensory Neurons in the Female *Spodoptera littoralis* Moth: Differences in Sensitivity and Temporal Characteristics. *Chemical Senses* 37(7), 613-629.

IV **Binyameen M**, Hansson BS, Birgersson G, Schlyter F. Identification of New Semiochemicals for *Spodoptera littoralis* from Induced Green Plants and Flowers using GC-SSR and GC-MS. *Manuscript.*

V **Binyameen M**, Walker WB III, Montagné N, Chertemps T, Jacquin-Joly E, Schlyter F, Anderson P, Hansson BS, Larsson MC. Deorphanization of Olfactory Receptors Tuned to Host Volatile by Heterologous Expression in *Drosophila Empty Neuron System*, SSR, and GC-SSR-ENS in a Moth. *Manuscript.*

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Abbreviations

AL	Antennal lobe
EAG	Electroantennography
ENS	Empty neuron system
FID	Flame ionization detector
GC	Gas chromatography
GC-EAD	Gas chromatography-electroantennographic detection
GC-MS	Gas chromatography-mass spectrometer
GC-SSR	Gas chromatography-single sensillum recording
GLV	Green leaf volatiles
HIPV	Herbivore induced plant volatiles
LH	Lateral horn
LN	Local neuron
MB	Mushroom body
MGC	Macro glomerular complex
NHV	Non-host volatiles
OBP	Olfactory binding proteins
ODE	Odor degrading enzymes
OR	Olfactory receptor
OSN	Olfactory sensory neuron
PN	Projection neuron
PR	Pheromone receptor
SEM	Scanning electron microscopy
SDH	Semiochemical diversity hypothesis
SNMP	Sensory neuron membrane proteins
SSR	Single sensillum recordings
VOC	Volatile organic compounds

1 Introduction

1.1 Olfaction and host plant selection in phytophagous insects

In nature, insects live in an olfactory landscape of diverse semiochemicals. When selecting host plants, insects may use a variety of senses, such as the sense of smell (olfaction), taste, vision, and touch. All senses are important, but olfaction often is the most important in searching for mates and hosts (Hildebrand & Shepherd, 1997; Bernays & Chapman, 1994).

Olfaction is critical to execute innate behaviors that are crucial for survival and reproduction in phytophagous (plant-feeding) insects, such as recognition of mates, location of food sources, and selection of suitable host plants for oviposition (Bernays & Chapman, 1994; Jaenike, 1990; Visser, 1986). These behaviors could be modulated by the chemical cues released from their sexual partners, host plants or the non-hosts. Pheromones (Ph) are the chemical cues used for intraspecific communication, while kairomones (plant volatiles) are the predominant cues in the host-seeking behaviors in phytophagous insects (Bruce & Pickett, 2011; Schoonhoven *et al.*, 2005; Bernays & Chapman, 1994; Renwick, 1989). Phytophagous insects may have the ability to discriminate between host and non-hosts and between hosts of different quality (Bruce & Pickett, 2011; Gripenberg *et al.*, 2010; Zhang & Schlyter, 2004; Renwick, 1989). Plant volatiles also have been shown to increase male moth attraction towards a female releasing sex Ph while calling for mating (Landolt & Phillips, 1997; Light *et al.*, 1993). How do insects recognize such a large diversity of chemical cues? Numerous electrophysiological and molecular studies provide evidence that insects have a sophisticated olfactory system equipped with many olfactory receptor (OR) proteins (Clyne *et al.*, 1999; Gao & Chess, 1999; Vosshall *et al.*, 1999), which are expressed on the dendritic membranes of olfactory sensory neurons (OSN) housed in olfactory sensilla (Figure 1). The recognition of a host plant is believed to be based on either specific ORs/OSNs

that detect specific odorants released from a specific plant, or combinations of ORs/OSNs that together detect specific ratios of general odorants in a blend (Bruce *et al.*, 2005). Functional deorphanization of ORs in electrophysiological studies have shown that ORs represent the molecular basis for the specificity of the OSNs (Hallem & Carlson, 2006; Hallem *et al.*, 2004).

Modulation and recognition of olfactory signals have been studied in several insect species, including *S. littoralis*, both at behavioral and neuronal levels. The physiological state of an insect plays a vital role in modulating its behaviors. For example, mating modulates behavioral preferences of *S. littoralis* moths; 3 h after mating a female switches its preference from floral (nectar source) to green plants (an oviposition substrate). The floral preference is restored after 24 h (Saveer *et al.*, 2012). Likewise, 3 h after mating, male attraction to female sex Ph and green plants is reduced but there is little effect on attraction to a nectar source (flowers) (Kromann, 2012). Similar modulation in physiological response sensitivity to Ph is also observed both at the periphery (studied by EAG & SSR) and in the antennal lobe (by optical imaging) in the male *S. littoralis* after mating (Kromann, 2012). This modulation in responsiveness could be due to a change in the levels of biogenic amines, as dopamine enhanced the sensitivity of Ph OSNs in *S. littoralis* males 3 h after dopamine injection (Binyameen *et al.*, 2013a). Dopamine injected males also located the Ph source faster compared to untreated males. Other biogenic amines may also contribute in modulating the insect behaviors of different types, e.g. octopamine and serotonin modulate responsiveness to foraging-related stimuli in honeybee *Apis mellifera* (Barron *et al.*, 2002; Erber *et al.*, 1993). Blood-feeding modulates physiological responses in female *Aedes aegypti* mosquitoes by increasing sensitivity of OSNs to indole and phenolic compounds after 24 and until 72 h post-blood feeding (Siju *et al.*, 2010). Based on these observations, one can hypothesize that these different time scales of changes in *Spodoptera* moths and *Aedes* mosquitoes may have ecological and evolutionary relevance in driving host-seeking behavior of females to oviposition sites since *Spodoptera* females start laying eggs few hours after mating and can mate again after 24 h (Saveer, 2012), while *Aedes* females take at least 48-72 h after a blood-meal to start laying eggs and also behaviorally do not respond to oviposition cues at least 24 h post-blood feeding (Klowden, 1995; Davis, 1984). Experience-dependent modulation in behavioral responsiveness to olfactory cues has been shown both in larvae and adults of *S. littoralis* (Anderson *et al.*, 2003; Carlsson *et al.*, 1999). This modulation could be due to evolutionary changes in the olfactory system, as a linkage has been found between host plant use in females and their offspring (Gripenberg *et al.*, 2010). Male and female *S. littoralis* also show host plant

preference hierarchies, indicating that reproductive decisions in both sexes potentially could influence the evolution of host plant range (Thömning *et al*. unpublished; Larsson *et al*. unpublished).

Many different plant-feeding insect species have OSNs/ORs tuned to components of commonly occurring green leaf volatile (GLV) alcohols and aldehydes that are major constituents of green plants (Andersson *et al.*, 2009; Bengtsson *et al.*, 2009; Ulland *et al.*, 2008; Røstelien *et al.*, 2005). Likewise, OSNs detecting floral compounds have been found in several insect species that may represent their common adult feeding ecology, as in most species, adults feed on floral nectars (Bruce *et al.*, 2005; Meagher, 2002; Heath *et al.*, 1992). Herbivore-induced plant volatiles (HIPV) are important signals for an ovipositing female moth to judge the quality of the host plant before laying eggs which is crucial for the survival and development of her offspring (Renwick, 1989). HIPVs have been shown to modulate insect behaviors either acting directly, for instance, by deterring oviposition by a lepidopteran female (Zakir, 2012; Jönsson & Anderson, 1999), or indirectly, by attracting natural enemies of the herbivores (Turlings & Wäckers, 2004; Turlings *et al.*, 1995). OSNs detecting HIPVs, necessary for selecting suitable host plants, have been found in various herbivore species including *S. littoralis* (Binyameen *et al.*, 2012; Bichão *et al.*, 2005a; Stranden *et al.*, 2003).

Volatiles released from non-host plants are also important cues that may be used by insects to avoid non-host or less preferred plants, and to select a right habitat and further select a suitable host (Zhang & Schlyter, 2004). Furthermore, non-host volatiles (NHV) modulate behaviors of bark beetles and moths by reducing their attraction toward pheromones or host kairomones (Schiebe, 2012; Andersson *et al.*, 2011; Jactel *et al.*, 2011; Schiebe *et al.*, 2011). Andersson *et al*. (2009) demonstrated that in the bark beetle *I. typographus* ca. 25% of the responding OSNs were dedicated to the detection of NHV. An inhibitory host compound, 1,8-cineol, modulates the response of *Ips typographus* both at behavioral and neuronal levels by decreasing beetle attraction towards their Ph with decreasing spacing between Ph and 1,8-cineol odor sources (Binyameen *et al.*, 2013b) and by inhibiting the activity of co-localized Ph OSNs when tested as binary mixtures with Ph (Andersson *et al.*, 2010).

Altogether, a large number of attractive and non-attractive volatiles released by plants and their combinations constitute a major challenge for herbivore insects to navigate towards their host plants in a complex olfactory landscape. This challenge is met by the use of an extremely sensitive and specialized olfactory system described below!

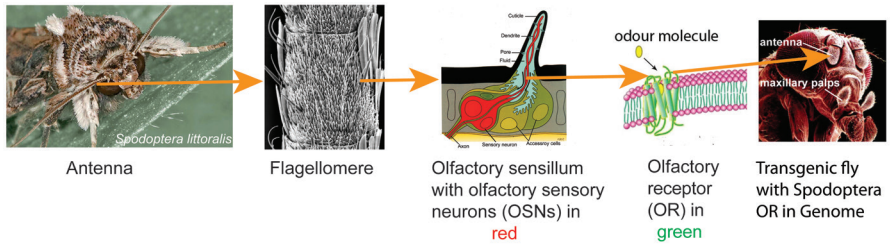


Figure 1. Insect peripheral olfactory system and heterologous expression of olfactory receptors (OR). In the figure, a *Spodoptera littoralis* moth sitting on a cotton leaf. *Spodoptera* adult antenna is comprised of 65-70 flagellomeres covered with olfactory hairs, the sensilla (Paper III). Each sensillum is innervated by two or more olfactory sensory neurons (OSN), which express specific ORs that interact with odorants. For *in vitro* deorphanization of *Spodoptera* ORs, we have used the Empty Neuron System (ENS) of *Drosophila melanogaster* (Paper V), where *S. littoralis* OR genes were cloned and transformed into *D. melanogaster* embryos to produce transgenic flies with *Spodoptera* OR in their genome. Utilizing genetic tools, *S. littoralis* ORs were expressed by the "A-neuron" in the ab3 sensillum of *D. melanogaster*. These transgenic flies were used for electrophysiological recordings to deorphanize *Spodoptera* receptors. Olfactory sensillum drawing is courtesy of Prof. Dr. R. A. Steinbrecht, Max Planck Institute, Seewiesen, Germany.

1.2 The insect olfactory system

The primary olfactory organs in insects are the antennae (Figure 1). Insect antennae vary in shape and size depending on species and their needs, but an antenna can generally be divided into 3 parts: scape (basal segment attached with head capsule), pedicel (a segment attached to scape), and a flagellum that is comprised of a few to many flagellomeres (Keil, 1999; Steinbrecht, 1996). The olfactory sensilla are located mainly on the insect antennae (Figure 1). However, few are present also on the maxillary and/or labial palps (McIver, 1971). The number of olfactory sensilla on an antenna may vary from only a few to more than a 100,000. For example, females of pea aphid *Acyrthosiphon pisum* have 28 sensilla on their antenna, while the male moth of *Manduca sexta* has up to 42,000 trichoid sensilla for the detection of Ph (Keil, 1989) and 75,000 sensilla for the detection of plant compounds (Rospars & Hildebrand, 2000). *S. littoralis* females have approximately 7,000 olfactory sensilla on their antenna (Binyameen *et al.*, 2012). In contrast to adults, the larvae have only few sensilla. For example, 3 olfactory sensilla are present on the Lepidopteran larval antenna (Hansson, 1995).

The olfactory sensilla are classified into different types, such as trichoid, basiconic, coeloconic, auricilic and grooved peg sensilla (Binyameen *et al.*, 2012; Shields & Hildebrand, 2001; Hallberg *et al.*, 1994). The antennae of female *S. littoralis* contain six sensillum types including Ph detecting the long trichoid (Binyameen *et al.*, 2012). However, only few long trichoids are present in females as compared to males (Ljungberg *et al.*, 1993). The number

of OSNs in a sensillum in most insects is 2-3, but in some insects it is up to 200 (Galizia & Rössler, 2010).

Odor molecules enter the sensillum through cuticular pores on the surface, and olfactory-binding proteins (OBP) in the sensillum lymph carry these molecules to the dendritic sensory membrane of OSNs (Figure 1) (Leal, 2012; Sachse & Krieger, 2011; Vogt, 2003). The cell bodies of the OSNs are surrounded by three auxillary cells: thecogen, tormogen, and trichogen, which are involved in the formation of the sensillum during ontogeny, the synthesis of OBPs, and maintaining the ionic composition of the sensillar lymph (Hansson, 1995; Schneider, 1964). In addition, other protein types also have been found in the sensillum lymph e.g. sensory neuron membrane proteins (SNMPs) and odor degrading enzymes (ODEs) (Vogt, 2003), having different functions. For instance, ODEs are considered to be involved in removal and inactivation of the odorants (Leal, 2012). The OR proteins expressed in the dendritic membrane of OSNs are key elements in the molecular recognition and discrimination of odorants (Touhara & Vosshall, 2009). OSNs expressing ORs are thought to be activated by general odorants whereas Ph receptors (PR) are activated by Ph components. The axons of Ph OSNs (in the male moth) project to sexually dimorphic compartment, the macro glomerular complex (MGC) in the AL, while axons of OSNs responding to general odorants project to ordinary glomeruli (Touhara & Vosshall, 2009; Todd & Baker, 1999). The OR proteins interact with the relevant odorants and convert the chemical signals into electrical responses in the OSNs (Leal, 2012; Touhara & Vosshall, 2009). The OSNs project their axons in the AL (Homberg *et al.*, 1989).

The AL consists of the glomeruli, where OSN axons are synaptically interconnected to the projection neurons (PN) and a network of local neurons (LN) (Boeckh & Tolbert, 2005; Homberg *et al.*, 1988). It has been demonstrated that axons of OSNs expressing the same OR converge onto single glomerulus (Galizia & Rössler, 2010; Vosshall *et al.*, 2000). For example, in female *S. littoralis* more than 14,000 OSN axons converge onto about 60 glomeruli (Sadek *et al.*, 2002). In *M. sexta* the degree of convergence is even greater. Over 150,000 axons from cells that are sensitive to plant odors converge onto about 60 glomeruli (Rospars & Hildebrand, 2000). Both inhibitory and excitatory LNs (Huang *et al.*, 2010; Wilson & Laurent, 2005) as well as PNs have been characterized in *D. melanogaster* (Knaden *et al.*, 2012). LNs process and transform incoming olfactory information from the antennal OSNs. The PNs convey this information to higher brain centers, the mushroom body (MB) and the lateral horn (LH) of the protocerebrum (Galizia & Rössler, 2010), where odor signals are translated in the form of specific behaviors.

2 Objectives

The selection of suitable host plants for feeding and oviposition is crucial but a complicated process for phytophagous insect species living in a complex olfactory landscape. The overall objective of this thesis was to elucidate the olfactory mechanisms used by phytophagous insects to select their hosts. In particular, the focus was to provide data on mechanisms and components for modulation of olfaction at different time scales, ranging from short time behavioral changes (ms) to evolution (Myr). I describe the ecological, physiological, and molecular bases of insect olfaction that contribute to our understanding of olfactory mechanisms and their role in host plant selection in moth *Spodoptera littoralis,* a polyphagous pest. These data should provide a basic ground level of knowledge and tools for future behavioral, physiological, and molecular studies to better understand the mechanisms of insect olfaction.
In this thesis, I try to elucidate:

- Modulation of attraction and reproduction by non-host volatiles
- Functional-morphology of OSNs and chemistry of kairomones
- Molecular basis of odor coding

3 Materials and methods

3.1 Behavioral bioassays

3.1.1 Trapping experiments and measurement of odor plumes

Field bioassays involving the capture of insects are the ultimate solution to test the activity of attractants (pheromones and host plant volatiles) or anti-attractants (repellents or anti-attractant non-host volatiles). We studied the attraction of bark beetle *I. typographus* to aggregation Ph in the presence of a NHV blend at different distances from the Ph dispenser and to the separated single Ph components (*cis*-verbenol and 2-methyl-3-buten-2-ol) both vertically (using 19 funnel Lindgren traps, Figure 2A) and horizontally (using 5 funnel wind-vane traps, Figure 2B). For *S. littoralis* we tested the response to horizontally separated (Figure 2C) sex Ph components (major component *Z*9-*E*11-tetradecadienyl acetate [Z9E11-14:Ac], minor component *Z*9-*E*12-tetradecadienyl acetate [Z9E12-14:Ac]). We also tested the response to horizontally separated Ph and an antagonist (*Z*9-tetradecenyl acetate [Z9-14:Ac]). To further investigate the inhibitory effect of NHV on the beetle attraction, eight NHV dispensers were positioned in a ring (with 1, 2, or 3 m radius) around a central Ph trap (Figure 2D) or NHV flakes distributed on the ground around with 2 m radius releasing the same amount as the 8 NHV dispensers (Paper I). The behavioral observations were complemented by measurements of plume structure and overlap in the field using a photo ionization detector (PID) and soap bubble generators (Paper I).

3.1.2 Calling, mating, and oviposition bioassays

Freshly emerged female moths singly, or female and male moths in pairs were kept inside transparent plastic jars (Figure 2E) or in Petri dishes with

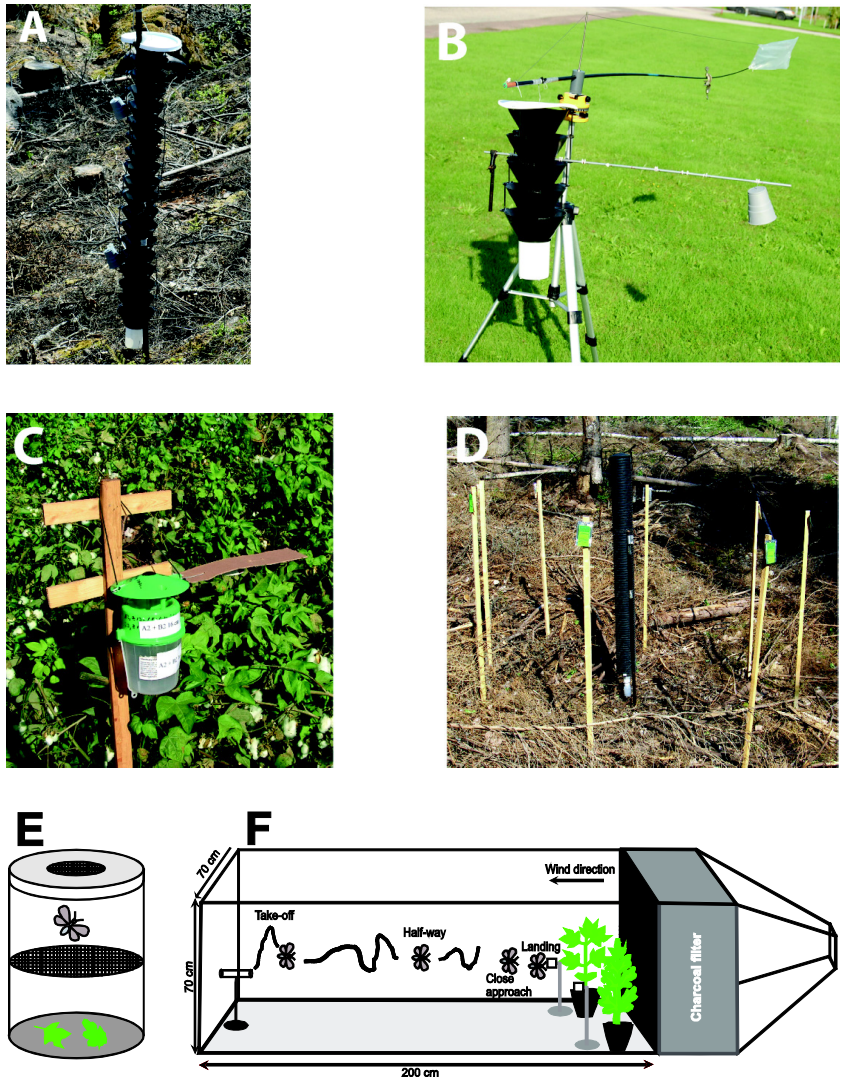


Figure 2. **A)** Lindgren funnel traps (19-funnel size) were used in the vertical spacing tests with the beetle. Dispensers were positioned under grey cups. **B)** A Lindgren trap (5-funnel size) was attached to a wind vane in the horizontal spacing tests with the beetle to ensure constant distance between plumes. **C)** Trap type used in spacing tests with *Spodoptera*. Cardboard protected the dispensers from sunlight. **D)** Pipe trap surrounded by eight non-host volatile dispensers (at 1 m distance) in the beetle anti-attractant background tests. **E)** A 250 ml jar with perforated lid and having plant leaves at the bottom and a metallic net placed 3-4 cm above the leaves to restrict insects to the upper-half of the jar to avoid any physical contact with the leaves. **F)** Schematic drawing of a wind tunnel (Paper II). In dual-choice bioassays, a host (cotton) plant and a non-host (spruce or *Av*) plant were placed upwind in the tunnel, 20 cm apart from each other. One female equivalent pheromone (1FE) blend loaded on a filter paper was used as a source of attraction for the male moth in front of each plant. Males were released downwind from a glass tube.

perforated lids. These containers were empty (negative control) or contained leaves of host and/or non-host plants depending on the treatment. A fine meshed metallic net was placed 3-4 cm above the leaves to restrict insects to the upper half of the jars (Figure 2E) or Petri dishes, thereby preventing direct contact with the leaves. The jars were enclosed by ventilated plastic containers to avoid contamination among different treatments.

3.1.3 Wind tunnel flight bioassay

A wind tunnel bioassay system is usually used to observe the upwind flight response for mate finding or host-seeking in insects. In the wind tunnel, insects are presented with the Ph of their sex partners or host and/or non-host plants or plant odorants. The wind tunnel bioassay system has some advantages over field bioassays involving capture of insects. For example, temperature, humidity, wind velocity, and odor plume conditions can be reproduced, and the experiments may not face problems of daily variation in results common to field experiments (Elkinton & Carde, 1984). Another key advantage of wind tunnels over field tests is that experiments can be performed throughout the year. Wind tunnel assays were used to test the inhibitory effect of non-host plants on *S. littoralis* male moths' attraction towards the female sex Ph (Figure 2F).

3.2 Electrophysiological recordings (GC-EAD & GC-SSR)

Screening of complex volatile blends in order to identify the biologically relevant odorants is one of the biggest challenges in olfactory research. Luckily, the insect olfactory system, especially the peripheral nervous system, is an excellent model for electrophysiological studies. Insects' ability to smell is often analysed by electrophysiological recordings from the whole antenna (EAG) or individual olfactory sensilla (SSR) (Figure 3).

Since the first electrophysiological recordings from insect antennae (Schneider, 1957), two different techniques have been used to study sensitivity and selectivity to different odorants in insect, and to identify biologically active odorants. The most common and extensively used techniques for identification of bio-active compounds (e.g. pheromones and plant odorants), are electroantennogram (EAG) and gas chromatograph-electroantennographic detection (GC-EAD) (Saveer, 2012; Zakir, 2012; Fraser *et al.*, 2003; Park *et al.*, 2002; Pearson & Schal, 1999; Anderson *et al.*, 1993). An antenna is placed between two electrodes, either while it is still attached to the insect, or immediately after its removal (Figure 3). The potential difference between the two electrodes is recorded when a puff of air carrying an odor is blown over

the antennal surface and changes the potential. The change in potential is a measure of the summed receptor potentials of all the nerve cells in the antenna that respond to the odor, as the EAG amplitude is proportional to the number of sensilla present (White, 1991).

Recordings from individual neurons are called single-cell recordings (SCR) or single sensillum recordings (SSR) (Figure 3). SSR is a more reliable and precise method than EAG. Recordings from single OSNs were first performed with glass-capillary electrodes (Schneider, 1957) and later with tungsten electrodes (Boeckh *et al.*, 1965). Recordings are done by inserting a reference electrode either in the eye or in the abdomen and recording electrode in the base of a sensillum or sensillum cavity (depending on the sensillum type) to establish a contact with the OSNs in the sensillum lymph. Action potentials of the OSNs are amplified through an interface amplifier. Change in potential (spike frequency) is recorded upon stimulation with an odorant.

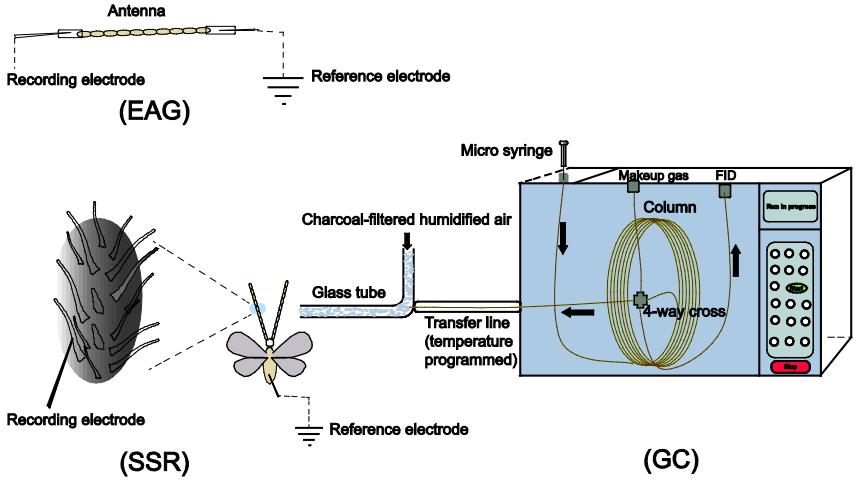


Figure 3. Schematic drawing of the gas chromatography-coupled single sensillum recording (GC-SSR) or electroantennogram (EAG) techniques in moths. Headspace volatile extracts are injected into the GC using a micro syringe onto a capillary column situated in an oven. As the oven temperature increases, the components of the extract are separated, travel through the column and reach a split point (4-way cross). Makeup gas balances the gas flows and half of the effluent from the column goes to a flame ionization detector (FID) as in a conventional GC. The other half leaves the column and passes through a transfer line to a glass tube where a continuous charcoal-filtered humidified air blows the separated components of the extract over the moth antenna. For SSRs, one tungsten wire serving as reference electrode is placed into the abdomen tip and an electrolytically sharpened electrode connected to pre-amplifier is injected at the base of a single sensillum. For EAG, an antenna is placed between two electrodes, either while it is still attached to the insect, or immediately after its removal. The potential difference between the two electrodes is recorded when stimulated with an odor stimulus.

Functional characterization of OSNs/ORs by SSR is usually done *in vivo* or more exactly *in vitro* (by expressing ORs in a heterologous expression system, Figure 1). The main aim of SSRs is to know how odor information is coded by single OSNs, e.g. whether each OSN is specialized for single odorants or respond to a broad range of compounds. However, naturally produced odorants exist in complex blends; therefore, screening for bio-active compounds in such natural mixtures of unknown composition is not possible with SSR alone. The method of combining GC with electrophysiological recordings from single neurons (GC-SSR) resolves this issue. The GC-separated volatiles in a blend are directly tested on OSNs. Thus, studies using the GC-SSR technique give more precise information about the OSN specificity or odor-response spectra. This method was first carried out in studies of Ph detection (Wadhams, 1982), and was later also used for studying plant odor detection (Ulland *et al.*, 2008; Røstelien *et al.*, 2005; Wibe, 2004; Stranden *et al.*, 2003; Røstelien *et al.*, 2000a).

In EAG and SSR, synthetic standards or biological extracts are delivered to the antenna by a delivery system, while in GC-EAD and GC-SSR the activity of a biological extract or the synthetic standard is determined by injecting a small amount, usually 1-2 µL, of them into a GC, where a 4-way cross installed at the end of the GC-column led half of the effluent into the charcoal-filtered and humidified air stream flushing over the insect antenna through a glass tube and the other half to the flame ionization detector (FID). Thus, the activity of an OSN and the gas chromatogram of the components separated in the GC-column are recorded simultaneously (Figure 3). If an active compound is found in an extract, its identity is then revealed using GC-MS (described below).

I have used EAG and GC-EAD (Paper II) for the identification of active compounds from non-host plants. I have also used SSR (Papers III-V) and GC-SSR (Papers IV & V) to identify more putative odorants for *Spodoptera* OSNs/ORs from several host and non-host plants.

3.3 Chemical identification of compounds (GC-MS analyses)

The chemical identification of bio-active peaks in our GC-EAD and GC-SSR studies was done by combining GC and mass spectrometer (GC-MS). Headspace extract samples were injected into the GC-MS by means of an auto sampler. The identity of active compounds was determined according to a standard protocol (Anonymous, 2008; Birgersson oral communication) by looking into their mass spectra and calculating (Kovat's retention indices), in comparison with references from mass spectral libraries (NIST, Wiley, and

Alnarp11). Synthetic standards were then used in GC-MS and GC-EAD/GC-SSR to confirm the chemical identity and biological activity of active peaks, respectively.

3.4 *Drosophila* Empty Neuron System (ENS)

The fruit fly *Drosophila melanogaster* provides an excellent opportunity to deorphanize individual ORs *in vitro*. In *Drosophila*, a basiconic sensillum type, "ab3", houses 2 OSNs named ab3A and ab3B. In wild type *D. melanogaster*, the A-neuron expresses two ORs, OR22a and OR22b, while mutant *D. melanogaster* lack OR22a/b. This is due to the Delta-Halo chromosome that carries a deletion spanning the locus of the *D. melanogaster* OR22a/b genes. For the expression of the SlitOR in the empty neuron, ab3A (Hallem *et al.*, 2004; Dobritsa *et al.*, 2003), male flies of the genotype Delta-Halo/Cyo; SlitORx are mate paired with female flies of the genotype: Delta-Halo/Cyo; OR22a-Gal4. This system utilizes the Gal4-UAS gene expression system (Brand & Perrimon, 1993), such that, in progeny flies with the genotype, Delta-Halo/Delta-Halo; OR22a-Gal4/UAS-SlitORx, the promoter for the DmOR22a gene drives expression of the yeast Gal4 transcription factor in the ab3A neuron, whereafter Gal4 binding to the UAS elements drives expression of the downstream transgene, SlitORx. As these flies are homozygous for the Delta-Halo deletion, the ab3A neuron lacks its endogenous receptor, DmOR22a, thus, all odorant induced neuronal activity is attributed to the transgenic SlitOR.

3.5 Study organisms

3.5.1 *Spodoptera littoralis* (Lepidoptera: Noctuidae)

In the studies of the present thesis, we have used the Egyptian cotton leafworm moth *Spodoptera littoralis* (Boisduval) to study how plant odor information is encoded by the OSNs. Field-collected pupae of *S. littoralis* were imported from Egypt to establish a culture. Larvae were reared on a semi-synthetic diet and all stages of the insect were kept at 25 ± 1 °C, 60-70% RH and 16:8 L:D photoperiods. *S. littoralis* is distributed throughout the warm-temperate and subtropical regions in the Mediterranean countries of Africa, Southern Europe, and the Middle East (Staneva, 2009; Brown & Dewhurst, 1975). It is a serious pest on a variety of

crops, such as cotton, soybean, maize, cowpea, and vegetables (Salama *et al.*, 1971). This moth is highly polyphagous; larvae can survive on more than 80 plant species from 40 different plant families (Brown & Dewhurst, 1975). The female moth oviposits up to several hundred eggs on the underside of the leaves of the plant. Larvae hatched from the eggs start feeding on the leaves that causes severe damage to the plants. The undesirable side-effects of insecticide to control insect pests have led to the current focus in research on alternative plant protection methods. Several studies have focused on behaviorally modifying olfactory cues, however, the focus has been to understand the Ph communication system or to study plant odorants processing in the antennal lobe (Guerrieri *et al.*, 2012; Carlsson *et al.*, 2007; Carlsson *et al.*, 2002; Sadek *et al.*, 2002; Anton & Hansson, 1995; Ochieng *et al.*, 1995; Campion *et al.*, 1980). At the peripheral level, only a few studies have been done with EAG or GC-EAD (Saveer, 2012; Zakir, 2012; Jönsson & Anderson, 1999), and SSR (Anderson *et al.*, 1995). I studied the ecological relationship of this species with host and non-host plants in behavioral bioassays and by EAG and GC-EAD experiments. Furthermore, I characterized a functional-morphological map from the peripheral olfactory system and studied the molecular basis of insect olfaction by employing SSR and GC-SSR both *in vivo* and *in vitro* recordings from the antennal OSNs/ORs, and identified biologically relevant plant odorants detected by *S. littoralis* from several host plants as well as from non-host plants.

3.5.2 *Ips typographus* (Coleoptera: Curculionidae, Scolytinae)

The Eurasian bark beetle (*Ips typographus* L.) is a serious pest on trees of Norway spruce, *Picea abies* (L.) (Wermelinger, 2004). Field trapping with separated aggregation Ph components, and Ph and a blend of NHV for *Ips* were done in a comparative field trapping of male moth *S. littoralis* (Paper I).

3.5.3 *Gossypium hirsutum* (Malvales: Malvaceae)

The cotton (*Gossypium hirsutum* L.) plant is a shrub native to tropical and subtropical regions around the world, including the Americas, Africa, and India (Brubaker *et al.*, 1999). Cotton plants used in experiments were grown individually in pots in a growth chamber at 25 ± 2 °C and 65 ± 5% RH.

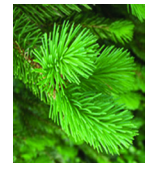
3.5.4 *Syringa vulgaris* (Lamiales: Oleaceae)

Lilac (*Syringa vulgaris* L.) is a deciduous shrub native to the Balkan Peninsula in southeastern Europe (Tutin *et al.*, 1976). Lilac is a common ornamental plant in gardens and parks, because of its attractive, sweet-smelling flowers. Lilac flowers used in headspace volatile collection were collected from Alnarpsgården, Alnarp, Sweden.

3.5.5 *Adhatoda vasica* (Lamiales: Acanthaceae)

Malabar Nut (*Adhatoda vasica* L.) trees grow wild in abundance in Egypt, Sri Lanka, Nepal, India, and Pakistan (Claeson *et al.*, 2000). The synonyms of *Adhatoda vasica* are *Justicia adhatoda* L. and *A. zeylanica* Medic. (Claeson *et al.*, 2000). Twigs of *A. vasica* were imported from Egypt and re-grown in plastic pots in a greenhouse for 12 months prior to use in the experiments.

3.5.6 *Picea abies* (Pinales:Pinaceae)



Norway spruce (*Picea abies* L.) is a species of spruce native to Europe. It is also commonly referred to as the European Spruce. Three to -four year old commercially grown spruce seedlings and 3-4 year old spruce trees grown on an experimental land at SLU, Alnarp were used in different experiments.

4 Results

4.1 Modulation by non-host volatiles/anti-attractants

Responses to separated pheromone and pheromone/anti-attractants (Paper I).

In nature, plumes from attractive and anti-attractive odor sources most likely mix together and may negatively affect the localization of attractive sources, such as host plants or pheromones (Jactel *et al.*, 2011; Schiebe *et al.*, 2011; Jactel & Brockerhoff, 2007; Zhang & Schlyter, 2003). How do the odor plumes released from different attractant and anti-attractant odor sources affect the attraction behavior of two different insect species, the moth *S. littoralis* and the bark beetle *I. typographus*, living in different habitats?

To test the "semiochemical diversity hypothesis" (SDH; Zhang & Schlyter, 2003), we studied the attraction of *I. typographus* to Ph in the presence of a NHV blend at different distances from the Ph dispenser as well as to separated single Ph components. For *S. littoralis* we tested the response to separated sex Ph components to separated Ph and an antagonist. To further investigate the inhibitory effect of NHV on the beetle attraction, eight NHV dispensers were positioned in a ring (with 1, 2, or 3 m radius) around a central Ph trap (Paper I) or NHV flakes distributed on the ground around with 2 m radius releasing the same amount as eight NHV dispensers. The behavioral observations were complemented by measurements of plume structure and overlap in the field using a photo ionization detector (PID) and soap bubble generators (Paper I).

In both species, increased spacing between Ph and anti-attractants increased trap catch (Figure 4), whereas increased spacing between Ph components had the opposite effect (Paper I). However, the two species differed at least an order of magnitude with respect to the spacing distances: beetles responded to separation of a few decimeters while the moths responded to distances of just a few centimeters (Figure 4). Such fine tuning of odor resolution in moths has been reported previously (Fadamiro *et al.*, 1999; Baker *et al.*, 1998). This

difference in odor resolution between the beetle and the moth may reflect the size of the odor plumes from their natural odor sources they orient to. A male moth orients towards a single calling female to mate, while male and female bark beetles may orient to many calling males on a large tree trunk for mating, feeding, and oviposition. The moth Ph system is highly specialized both at peripheral and central levels, as OSNs for Ph components are housed in specific sensilla (Binyameen *et al.*, 2012; Ljungberg *et al.*, 1993), and the processing of Ph signals occurs in the MGC in the AL (Ochieng *et al.*, 1995). In contrast, in the bark beetle, the OSN for a Ph component, *cis*-verbenol, is co-localized with an OSN for the plant compound 1,8-cineole (Andersson *et al.*, 2010; Andersson *et al.*, 2009) and there is no evidence that an MGC exists. Interestingly, in each species, the spacing distances affecting behavior were the same between the Ph component spacing and the Ph/anti-attractant spacing experiments (Paper I).

The bark beetle Ph/NHV spacing experiments showed clear anti-attractive effects of NHV. In the vertical spacing, long distance effects of NHV were found, as at 112 cm spacing significantly fewer beetles were caught than the Ph alone (Figure 4A). In the horizontal spacing, NHV reduced beetles attraction up to 80 cm (Figure 4B).

In the Ph/antagonist experiment with *S. littoralis*, the antagonist Z9-14:Ac inhibited attraction only at the highest dose tested (Figure 4C). In fact, the lowest dose had a synergistic effect, indicating that a low amount of this compound is part of the sex Ph blend (Campion *et al.*, 1980).

In the Ph component spacing in *Ips*, the beetles capture was the same at 0 and 16 cm spacing and reduced significantly at 24 cm and onwards in both horizontal and vertical spacing. However, at long distances of 112 cm in vertical and 80 cm in horizontal spacings, the capture was still higher than for the single components (Paper I).

In the Ph component spacing in *Spodoptera*, the position of the major component (in the trap or moved outward) affected trap capture (Paper I). With the major component in the trap, more males were captured at the 16 cm spacing than at the 8 cm spacing. In fact, the catch in traps with 16 cm spacing was very similar to the catch in traps with the major component alone (Paper I). However, when the minor component was in the trap and major component moved outwards, the catch in traps with 16 cm was similar to the catch in traps with the minor component alone (Paper I). Thus, it seems that the moths oriented to the 'best' alternative at the 16 cm spacing distance, but not at 8 cm spacing. Similar observations have previously been reported (Linn & Gaston, 1981).

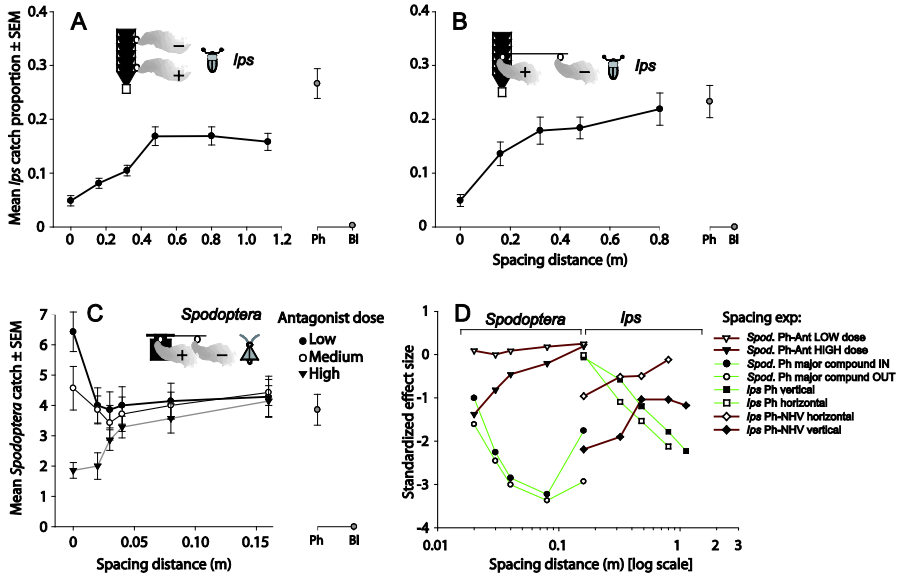


Figure 4. Response of *Ips typographus* to **A)** vertical and **B)** horizontal spacing between the aggregation pheromone and a non-host volatile blend. **C)** Response of male *Spodoptera littoralis* to horizontal spacing between the two-component sex Ph and three doses of a Ph antagonist (Z9-14:Ac). Only the high dose antagonized Ph attraction. The lowest dose enhanced Ph attraction at 0 cm spacing. Right panels in graphs show responses to control treatments: Ph = pheromone only, Bl = blank. **D)** Effect sizes for the various spacing distances in the *I. typographus* and *S. littoralis* spacing experiments (Hedges' unbiased g). The effect size provides a measure of a biological treatment effect, by scaling the difference between the treatment and control means, with the pooled standard deviation for those means. Effect sizes further from zero than 0.8 are regarded as strong effects. In all experiments, the Ph bait alone (zero distance between components) was the control. The 0 cm spacing distance in experiments involving anti-attractants is omitted for clarity.

Effect sizes comparison showed that spacing of odor sources had strong effect on both species, but similar effects were obtained at very different spacing distances (Figure 4D). This comparison highlights the superior sensitivity to small-scale spacing of the moth and the potential long-distance effect of NHV on the beetle.

With the eight NHV sources, bark beetle attraction was significantly reduced both at the 1 m and 2 m spacing distances (Paper I). Similar to the eight point sources, the NHV flakes also reduced Ph attraction. These distances are in accordance with the "active inhibitory range" of NHV of at least 2 m, estimated previously (Zhang & Schlyter, 2003).

Plume visualization with soap bubbles indicated that at 16 cm spacing, overlap started close to the trap (< 20 cm), whereas at 0.5-1 m spacing between

sources, plumes overlapped 1-3 m downwind from the source (Paper I). In addition, plume parameters measured by the PID vary greatly close to the odor source (Paper I), which is similar to results of previous studies (Thistle *et al.*, 2004; Murlis *et al.*, 2000; Murlis & Jones, 1981).

Modulation of reproductive behaviors by non-host plant volatiles (Paper II)

Odor source spacing field experiments (Paper I) showed that localization of attractive sources, such as host plants and pheromones, are negatively affected by the presence of odors from non-host or anti-attractive sources. How do the volatiles released from the non-host plants affect host selection and reproduction in phytophagous insects? In the present study, we studied how non-host plant volatiles affect reproductive behaviors and fitness in *S. littoralis.* We also identified ligands from non-host plants volatiles that potentially could be used in future pest management strategies. Calling, mating, and oviposition behaviors as well as fitness of newly emerged *S. littoralis* moths were studied in the presence of volatiles from leaves of a host plant, *Gossypium hirsutum* (cotton) and two non-host plants, *Adhatoda vasica* (*Av*) or *Picea abies* (spruce) either alone or in host/non-host combinations.

To determine the effect of NHV on the reproduction and sexual performance of *S. littoralis* during the entire reproductive age, we extended the work by Sadek and Anderson, (2007), using similar experimental conditions, except that we restricted the insects from having direct-contact with the leaves, i.e. exposed to volatiles only. Combinations of host and non-host plant leaves were also observed.

Females exposed to cotton volatiles started calling earlier than the females exposed to NHV or a combination of host and NHV, and the blank control (Paper II). The period of calling in females (that were kept alone) was longer than in females kept with males having the opportunity to mate (Paper II). Likewise, moth pairs exposed to cotton volatiles started mating earlier than the ones exposed to NHV or combinations of host and NHV (Paper II). However, the mating duration in the moth pairs exposed to cotton volatiles was not different than in the treatments with non-hosts or blank control (Paper II). In a recent study of *S. littoralis* by Zakir (2012), it was demonstrated that volatiles from cotton damaged by conspecific larvae did not affect the mating duration, but delayed the onset of calling as well as mating. This was presumably due to the inhibitory effects of herbivore-induced volatiles from damaged cotton and NHV may have similar effects. These results are similar to those of Sadek and Anderson, (2007).

Pair longevity was significantly decreased either in the absence of cotton or in the presence of *Av*, and spruce leaves (Figure 5A). A likely repellence by NHV could have resulted in sustained locomotor activity, causing insect resource depletion and mortality as was hypothesized by Gabel and Thiéry, (1994). The longevity of insects was also decreased in the control treatment as compared to cotton; therefore, an increase in female mortality could also be due to host deprivation resulting in abnormal, forced or prolonged egg retention and not only due to non-host plant volatiles (Nylin *et al.*, 2000; Gabel & Thiéry, 1994). Similar to our results, Zakir (2012) found that longevity of *S. littoralis* females was significantly shorter in the presence of damaged cotton as compared to the females exposed to undamaged cotton. According to these observations, the presence of a suitable host may have positive effects on insect longevity and hence on fitness as compared to an unavailability of host or presence of unsuitable host as well as non-host plants. However, Sadek and Anderson, (2007) reported that the presence of *Av* leaves did not have any effect on average longevity of both sexes of *S. littoralis* moths.

Fecundity (egg production) was also significantly reduced in moths exposed to a combination of cotton and spruce volatiles (Figure 5B). In oviposition bioassays of *S. littoralis*, it was shown that females lay more eggs on undamaged cotton plants as compared to cotton plants damaged by conspecific larvae (Zakir, 2012; Anderson & Alborn, 1999). Females of *Plutella xylostella* have also shown aversion in oviposition behavior to the odors of pea, a non-host plant (Zhang *et al*., 2007). A decrease in the oviposition either on the non-host plants or on the hosts located in the vicinity of non-host plants may be used as survival strategy that guides female moths to avoid plants that are poor sources of food for their progeny or not in the right habitat (Zhang & Schlyter, 2004).

Furthermore, the effect of NHV on the attraction of 2-3 days old unmated male moths towards the Ph source was studied in a wind tunnel by using cotton, *Av*, and spruce plants in no-choice and dual-choice assays. This was done by placing a filter paper loaded with one female equivalent Ph (1FE) blend in front of a plant in the wind tunnel.

In the no-choice assay, more males arrived at close approach and landed on the Ph source when the host plant, cotton, was offered in the background as compared to the non-hosts (Figure 5C). In the dual-choice assay, more males located the Ph source when cotton-*Av* combination was presented in the wind tunnel (Figure 5D). However, more males landed on the Ph source in front of the host plant as compared to the Ph source in front of non-hosts (Figure 5E-F). Similarly, the attraction of spruce seed moth *Cydia strobilellea* to female sex Ph was inhibited by NHV (Bedard *et al.*, 2002). This study provides evidence

that NHV modulate the reproductive behaviors in *S. littoralis*. It also confirms that NHV may have negative effects on the fitness measures of *S. littoralis* as well as in reducing male attraction to the female-produced sex Ph.

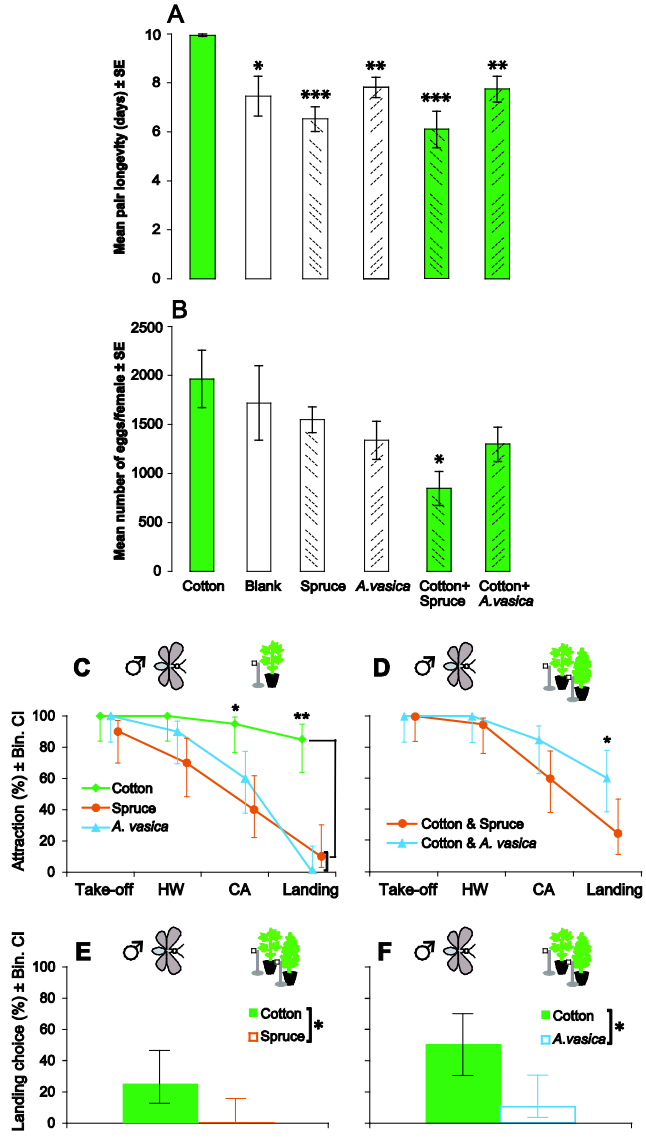


Figure 5. Reduction of fitness parameters and inhibition of male moth attraction to pheromone by non-host plants (Paper II). **A)** Mean pair longevity of *Spodoptera littoralis* recorded over 10 consecutive days after emergence. **B)** Mean number of eggs laid by *S. littoralis* females recorded over 10 consecutive days. **C-F)** Attraction of unmated males towards 1 female equivalent pheromone (1FE) synthetic blend in the wind tunnel and having host or non-host plants in the

background. **C)** No-choice assay where 1FE blend was presented in the middle-front of a host plant (cotton) or a non-host plant (spruce or *Av*). Four sequential behavioral steps were observed (take-off, half way [HW], close approach [CA], and landing). **D)** Dual-choice assay where the Ph blend was presented in front of a host and a non-host, 20 cm apart. **E & F)** Dual-choice landing assay.

GC-EAD by female *S. littoralis* revealed five antennal-active compounds in headspace collections from spruce and three compounds in *Av*, which were subsequently identified through GC-MS (Figure 6), indicating that *S. littoralis* antennae have OSNs for the detection of volatiles from non-host plants. The biological activity of synthetic standards of the identified compounds was further confirmed through GC-EAD and EAG dose-response tests (Paper II). The antennal active compounds identified from spruce headspace extract were mainly monoterpene hydrocarbons. These monoterpenes could be repellent or toxic for *S. littoralis* and might have inhibited reproductive behaviors and reduced fitness. For example, females of diamondback moth showed oviposition aversion in presence of *para*-cymene (Wang *et al.*, 2008), a compound we also found in spruce.

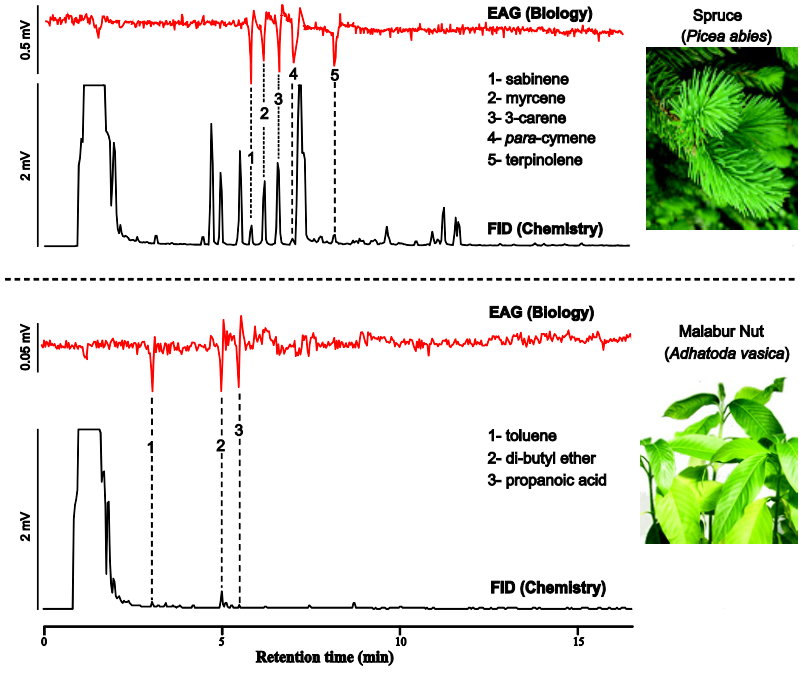


Figure 6. Averaged GC-EAD signals from 2-3 days old, virgin *S. littoralis* female antennae to headspace samples of Norway spruce (n = 3) and *Av* (n = 5). Volatile compounds eluting from an HP-5 coated capillary column and eliciting antennal responses are named accordingly after GC-MS analyses.

4.2 Electrophysiology of OSNs and chemical analyses of kairomones

Characterization of antennal olfactory sensory neurons (Paper III)

In moths, like other insects, volatile cues are detected by OSNs enclosed in antennal sensilla (Shields & Hildebrand, 2001; Hallberg *et al.*, 1994; Hallberg, 1981). In this paper, we studied the morphology and functional physiology of antennal olfactory sensilla in female *S. littoralis* by scanning electron microscopy (SEM) and single sensillum recordings (SSR), respectively.

SEM analyses revealed 6 different morphological sensillum types: Long trichoid (LT), short trichoid (ST), basiconic (BC), coeloconic (CC), auricilic (AC), and grooved peg (GP) sensilla (Figure 7). All of these morphological sensillum types are similar to the antennal olfactory sensilla that have been characterized in other moth species (Shields & Hildebrand, 2001; Hallberg *et al.*, 1994; Hallberg, 1981; Flower & Helson, 1974).

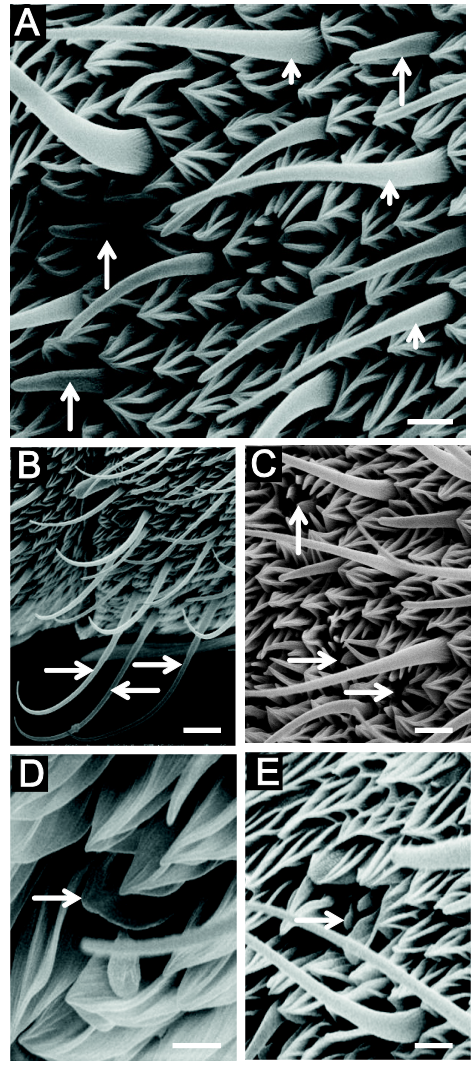


Figure 7. Six morphological types of antennal olfactory sensilla in female *Spodoptera littoralis*. **A)** Short trichoid (ST) (short arrows), a new type not earlier distinguished from the basiconic (BC) sensilla (long arrows). **B)** Long-trichoid (LT) sensilla (arrows) are present at the lateral surfaces. **C)** Coeloconic (CC) sensilla (arrows). **D)** Auricilic (AC) sensilla (arrow). **E)** Grooved peg (GP) sensilla (arrow). Bars represent a scale of 5 µm, except in (D) where the bar represents a scale of 2 µm in the SEM micrographs.

SSRs were obtained from antennal OSNs housed in sensilla located at the base and at the tip of the antenna of female *S. littoralis* using

a panel of 35 odor stimuli (Binyameen *et al.*, 2012). Recordings were made from two antennal segments (15th flagellomeres from the **P**roximal and **D**istal ends) of the antenna of 65-70 segments.

Recordings showed OSNs with selective responses to plant odors and female sex Ph. The 196 OSNs responding to a panel of 35 stimuli were housed in 32 functional sensillum types: 27 in BC, 3 in LT, 2 in CC, and 3 in AC sensilla (Paper III). The OSNs in BC, CC, and AC sensilla responded to plant odorants, whereas OSNs in LT sensilla were dedicated to detection of the female sex Ph components. OSNs specificity to plant stimuli ranged from highly specific to broadly tuned, which coincides with earlier findings in several moths and other insect species, where both "specialist" and "generalist" OSNs responding to plant volatiles have been characterized (Andersson *et al.*, 2009; De Bruyne & Baker, 2008; Ulland *et al.*, 2008; Ignell & Hansson, 2005). The underlying reason for this has been proposed to be that insects experience complex odor diversity, and hence, the discrimination of host plants may requires the combination of both generalist and specialist OSNs (Ignell & Hansson, 2005; Malnic *et al.*, 1999; Hansson, 1995).

Several studies on moths and other phytophagous insects, reviewed by Bruce *et al.* (2005) and De Bruyne and Baker, (2008), have reported that OSN responses to many odorants are shared across species, irrespective of oligophagy and/or polyphagy, suggesting that the discrimination between odorants may take place at higher levels in the olfactory system. One may also speculate that the presence of functionally similar OSNs or ORs in different species of insects (Ulland, 2007) are due to their common adult feeding ecology, as in most insect species adults feed on floral nectars, irrespective of the evolution of female preferences and larval ecology for reproduction. For example OSN (BC2A) responding to two compounds (Paper III), where phenyl acetaldehyde (PAA) is a common flower produced volatile, thus representing a nectar (adult food) source, whereas (*Z*)3-hexenyl acetate is a common GLV and thus representing plant material like cotton, which is larval food. We also found some OSNs housed in BC and AC sensilla that were broadly tuned to GLVs and other general plant odorants, which demonstrate that female *S. littoralis* moths also have generalist receptors to find plants providing both nectars (food) and oviposition sites.

OSNs of the 2 locations differed in temporal characteristics: OSNs on proximal (P) flagellomere had shorter latency and displayed more phasic responses, whereas those on distal (D) flagellomere had more tonic responses, especially at low stimulus concentrations (Figure 8A). This may convey different information to the central nervous system (CNS) regarding temporal

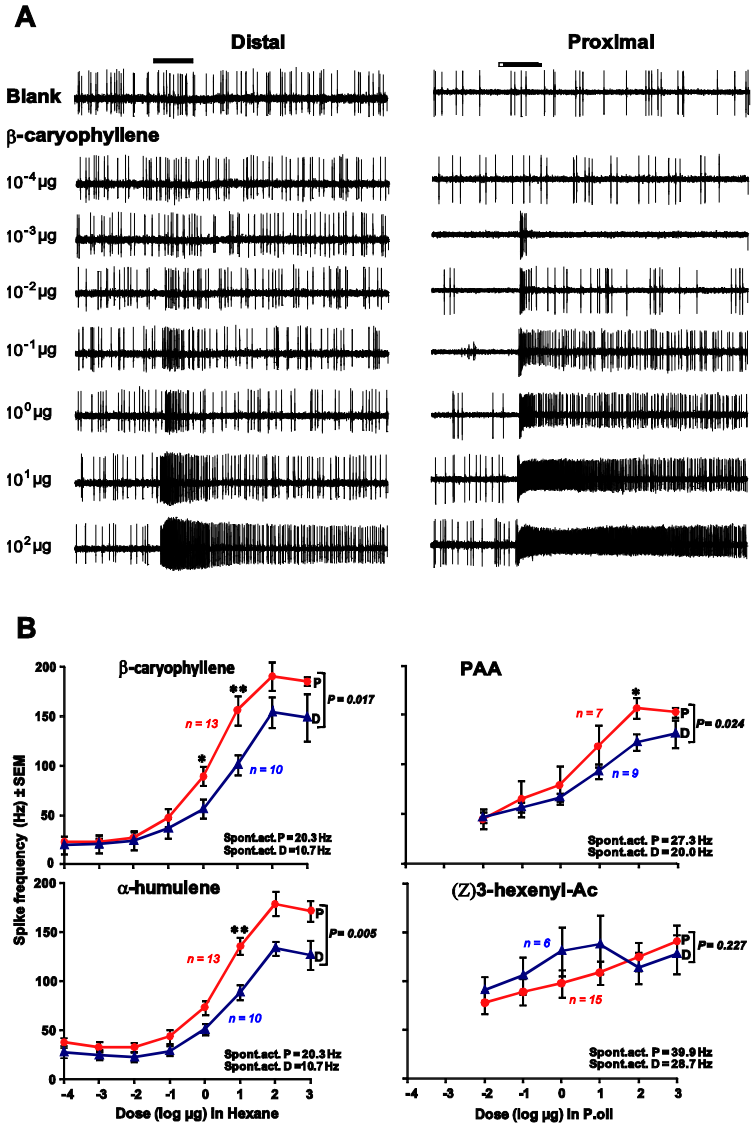


Figure 8. Temporal responses and the sensitivity vary in the same OSN type between the tip and base of the antenna. **A)** Dose response of an OSN (BC3A) housed in BC sensilla on 15th antennal segments from tip and base to different doses (100 pg–100 µg on filter paper) of a sesquiterpene, β-caryophyllene. Horizontal scale bars indicate stimulation of 0.5 s, whereas vertical represents 5 mV. **B)** Sensitivity is compared among functional classes of OSNs (housed in BC sensilla) from a proximal (P) and a distal segment (D) of the antenna. BC3A OSN responds to both β-caryophyllene and α-humulene. An OSN (BC1A) responding to phenyl acetaldehyde. The BC2A cell responding to (*Z*)3-hexenyl acetate. *n* = number of replicates, and ‘‘Spont. Act.’’ denotes spontaneous spike activity ± SEM.

aspects of stimulus occurrence (Raman *et al.*, 2010; Almaas & Mustaparta, 1990). A spatial variation in sensitivity was also observed: OSNs present on the P segment were more sensitive than those on the D (Figure 8B). One may speculate that if the spatial variation in sensitivity is represented in the CNS, it may help in coping with signals of vastly different magnitude. Alternatively, it is possible that paired lower sensitivity sensilla assemblage at antenna tips helps in close proximity, allowing orientation to point sources in clines of high concentrations.

Kairomones for Spodoptera littoralis from green plants and flowers (Paper IV)

Identification of plant volatiles that play a pivotal role in host selection by phytophagous insects is essential for neurophysiological studies as well as for ecological and plant protection strategies (Del Socorro *et al.*, 2010; Gregg *et al.*, 2010; Heath *et al.*, 1992; Hedin *et al.*, 1979). The aim of this study was to employ the GC-SSR technique using plant headspace volatiles followed by GC-MS, in order to identify biologically relevant plant odorants in *S. littoralis*, and functionally characterize the OSNs.

We have analysed airborne volatiles from the host plant, cotton (*Gossypium hirsutum*), damaged by conspecific larvae as well as from flowers of lilac (*Syringa vulgaris*) by GC-SSRs from antennal olfactory sensilla of female *S. littoralis*. Volatiles from larval-damaged cotton plants and lilac flowers were collected using a headspace sampling technique. Aeration of plants were trapped on adsorbents (Super Q), and then washed out by a solvent, n-hexane.

Initially, the single OSNs were screened for sensitivity to 45 synthetic single stimuli (Paper IV), and to pipettes loaded with damaged cotton and lilac volatile extracts. If a neuron responded to any synthetic or to an extract sample, we tested the individual constituents separated in the GC linked to SSR setup. Recordings were obtained from 96 individual sensilla that were classified into 20 previously identified OSN classes (Paper III) and 14 novel classes of OSNs including one new class found in the predominant short trichoid sensillum type (Paper IV). We also found some new ligands for some of the 20 OSNs re-characterized in this study (Paper IV). The GC-SSRs revealed, in total, 39 active peaks in the volatile blends of larval-damaged cotton plants and of lilac flowers (Table 1), 38 of which were subsequently identified through GC-MS and 1 peak which still remains unknown (Figure 9). One of the active identified peaks, (*E*,*E*)-cosmene, was identified based on its mass spectra matching >90% with NIST and Wiley libraries, but due to unavailability of synthetic standard we could not confirm this identification (question marked in Figure 9). Of the active plant compounds identified, 9 from damaged cotton and 11 from lilac were new for female *S. littoralis* compared to earlier GC-

EAD studies (Saveer *et al.*, 2012; Zakir, 2012; Jönsson & Anderson, 1999) (Table 1).

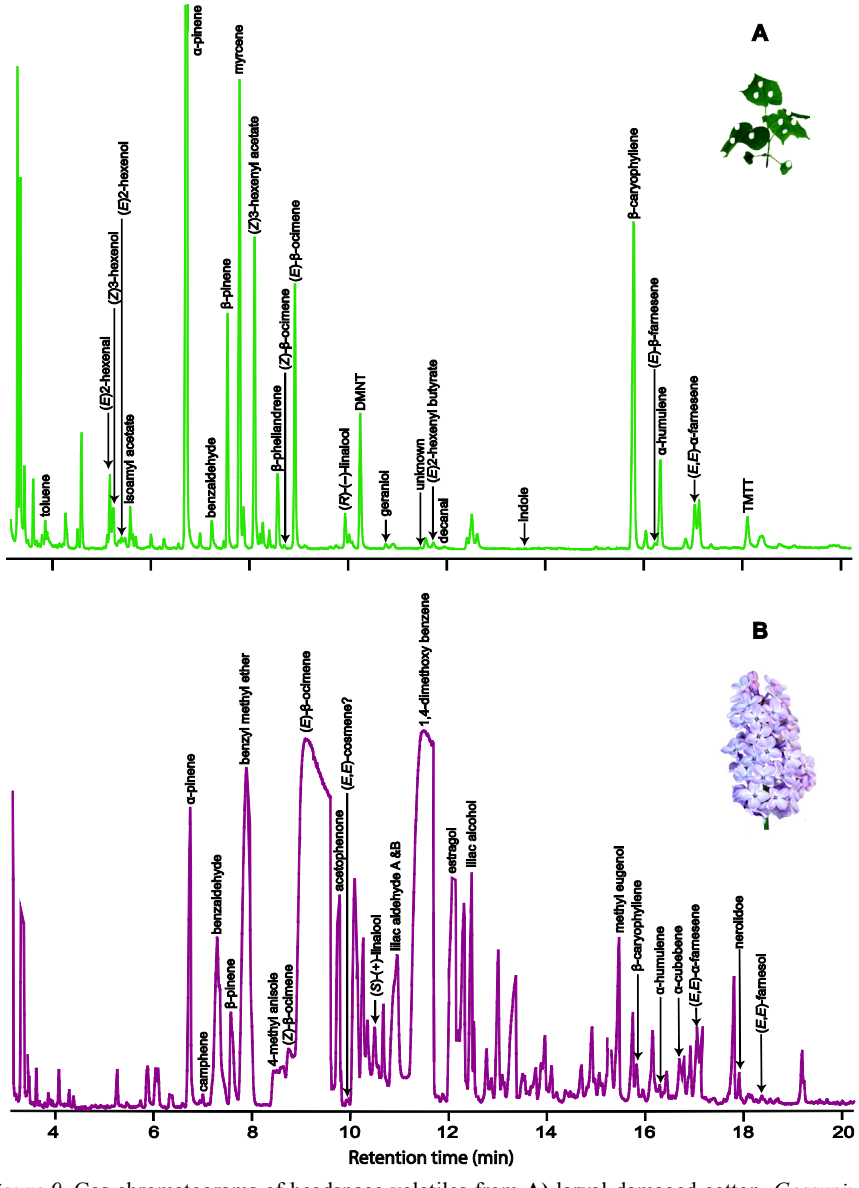


Figure 9. Gas chromatograms of headspace volatiles from **A)** larval-damaged cotton, *Gossypium hirsutum* plants and **B)** flowers of lilac, *Syringa vulgaris*. GC peaks with electrophysiological activity are given names (chemical identity). Standard protocol for identification was followed; comparison of mass spectra with NIST, Wiley, and Alnarp11 MS libraries to give candidate compounds and subsequent injection of candidate synthetic standards on GC-MS and GC-SSR.

The majority of the compounds identified as odorants for *S. littoralis* OSNs in these studies are known constituents of plant species. Whereas a few of the odorants may be specific for damaged cotton plant, others are common in many plant families. Herbivore-induced compounds are important in the defense of plants against herbivores. Detection of herbivore-induced chemicals has previously been shown for *S. littoralis* both at antennal (Zakir, 2012) and neuronal levels (Jönsson & Anderson, 1999). Several physiologically active compounds; e.g. (*E*,*E*)-α-farnesene, (*E*)-β-farnesene, (*E*)-β-ocimene, linalool, indole, DMNT, and TMTT that we found in damaged cotton have been reported earlier in herbivore larvae-damaged cotton plants and are proposed as *de novo* synthesized in response to insect feeding (Zakir, 2012; Rose & Tumlinson, 2005; Paré & Tumlinson, 1997; McCall *et al.*, 1994). For example, OSN5A responded to two compounds, 4,8,12-trimethyl-1,(*E*)3,(*E*)7,11-tridecatetraene (TMTT) present in the damaged cotton headspace and (*E*,*E*)-α-farnesene present in both damaged cotton and lilac headspace (Figure 10). Results in this study also suggest that *S. littoralis* uses a combination of compounds that are plant specific as well as generally present in many plants, for locating a suitable host for nectar feeding and oviposition.

In our previous antennal mapping study (Paper III), we found two functional classes of OSNs, BC11A and BC19B, responding to racemic linalool. In this study, separation of racemic linalool in the GC demonstrated the enantioselectivity of these OSNs. BC11A neuron responded to (*S*)-(+)-linalool (coriandrol), while BC19B responded to (*R*)-(−)-linalool (licareol). Similarly, strawberry weevil had two types of OSNs, one tuned to coriandrol and the other to licareol (Bichão *et al.*, 2005b). Enantioselective responses in OSNs of moths of other species have also been demonstrated. For instance, in *Mamestra brassicae* an OSN responded ten times stronger to coriandrol as compared to licareol (Ulland, 2007). Enantioselectivity to linalool was also presented in *Manduca sexta* both at neuronal (Reisenman *et al.*, 2004) and behavioral levels (Reisenman *et al.*, 2010).

This study contributes to understanding the insect-plant relationships and the development of sustainable plant protection strategies as well as to underlying neural mechanisms of olfactory coding.

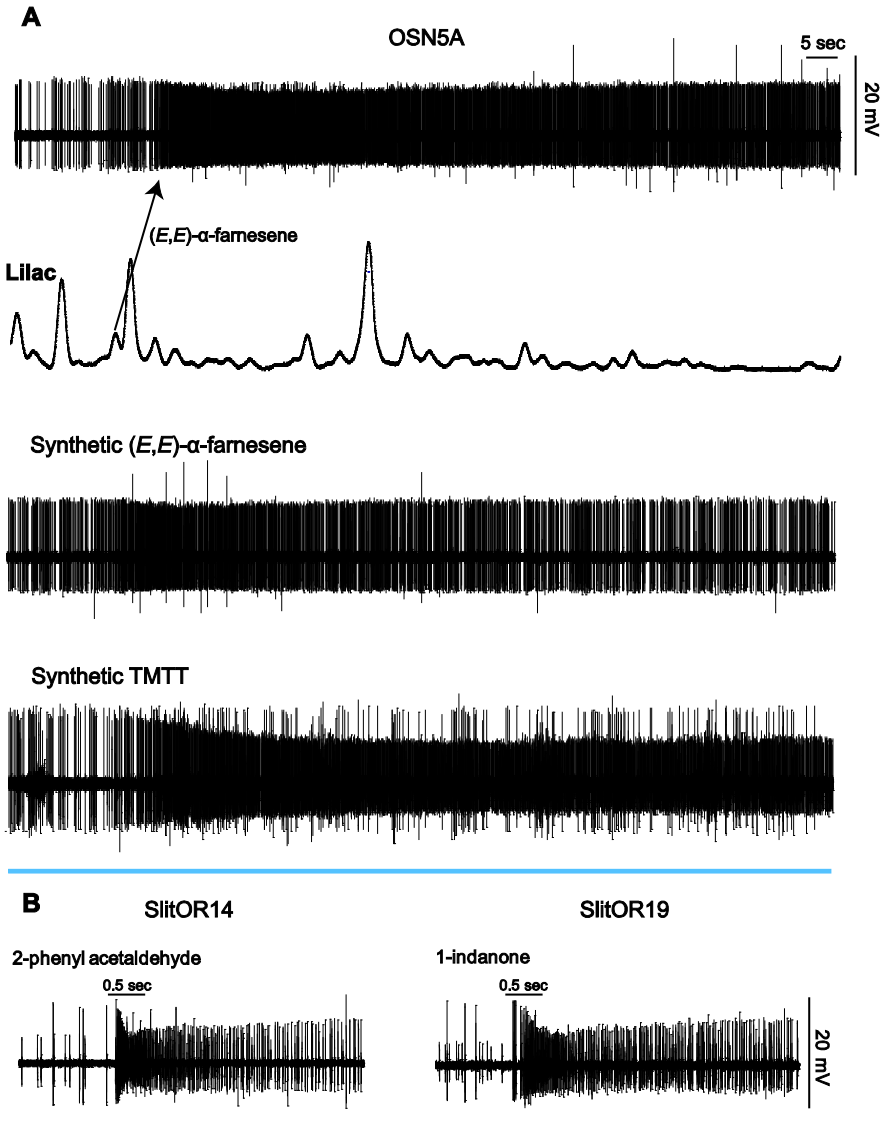


Figure 10. GC-SSR recordings from OSN5A cells and *Drosophila* ENS using damaged cotton and lilac headspace as well as synthetic standard candidates for the active peaks (Paper IV & V). **A)** Responses of OSN5A to (*E*,*E*)-α-farnesene peak present in lilac headspace and to GC-injection of 10 ng synthetic (*E*,*E*)-α-farnesene and 4,8,12-trimethyl-1,(*E*)3,(*E*)7,11-tridecatetraene (TMTT), respectively. Horizontal and vertical scales are the same for all traces. **B)** Single sensillum recordings (SSR) from two narrowly tuned odorant receptors; SlitOR14 and SlitOR19. The SlitOR14 responded with high sensitivity and selectivity to a floral compound, 2-phenyl acetaldehyde. The SlitOR19 responded to an oviposition deterrent compound, 1-indanone, which was previously identified from feces of *S. littoralis* larvae.

4.3 Molecular basis of odor coding in *Spodoptera littoralis*

Deorphanization of olfactory receptor genes (Paper V)

Each OSN generally expresses one particular olfactory receptor (*OR*) gene selected from a large *OR* gene repertoire and a specific coreceptor, ORco (Ray *et al.*, 2007; Benton, 2006; Larsson *et al.*, 2004). These ORs, which are expressed on the dendritic membrane of OSNs, interact with volatile compounds, however, functional characterization (deorphanization) of ORs tuned to compounds other than Ph, has only been done in 3 Dipterans (Carey *et al.*, 2010; Hughes *et al.*, 2010; Pelletier *et al.*, 2010; Wang *et al.*, 2010; Hallem & Carlson, 2006; Hallem *et al.*, 2004), a Hymenopteran (Wanner *et al.*, 2007), a Coleopteran (Mitchell *et al.*, 2012), and only 1 moth (Jordan *et al.*, 2009).

In this study, we have deorphanized four *OR* genes in female *S. littoralis* by determining the receptive range of each OR via a heterologous expression system, *Drosophila* ENS (Empty Neuron System), by means of SSRs. We have also utilized GC analyses of headspace extracts of different host plants, coupled to the SSR (GC-SSR-ENS).

Two (SlitOR14 & SlitOR19) of the ORs deorphanized in this study showed a high degree of specificity by responding to single compounds (Figure 10B). In contrast, the other two ORs (SlitOR24 & SlitOR36) showed identical qualitative odor spectra (Table 2) when tested at one dose with a panel of 53 synthetic stimuli (Paper V). However, when these two broadly tuned ORs were subjected to dose-response tests with synthetic standards including newly identified ligands (Figure 11 A-D), we were able to observe that they are different with respect to their response spectra (Table 2), temporal response patterns (Paper V), and sensitivity to different odors (Figure 11E), which represent quality, temporal characteristics, and intensity of odorants, respectively (Brand & Perrimon, 1993). These observations may lead to a better understanding of the coding capacity of the insect olfactory system and mechanisms involved in host selection.

GC-SSR-ENS revealed eight physiologically active odorants identified by GC-MS from damaged cotton and lilac flowers (Figure 11), which are ecologically relevant odor sources for moths. Earlier studies using GC-SSRs have identified narrowly tuned plant odor OSNs in herbivorous insects (Ulland *et al.*, 2008; Røstelien *et al.*, 2005; Larsson *et al.*, 2003; Stranden *et al.*, 2003; Stranden *et al.*, 2002; Stensmyr *et al.*, 2001). In the present study, there was a great overlap in the receptive range of SlitOR24 and SlitOR36. This overlapping coding strategy may represent a molecular mechanisms used by the olfactory system to discriminate odorants (Leal, 2012). However, no overlap was found between the other two ORs; SlitOR14 and SlitOR19. In the

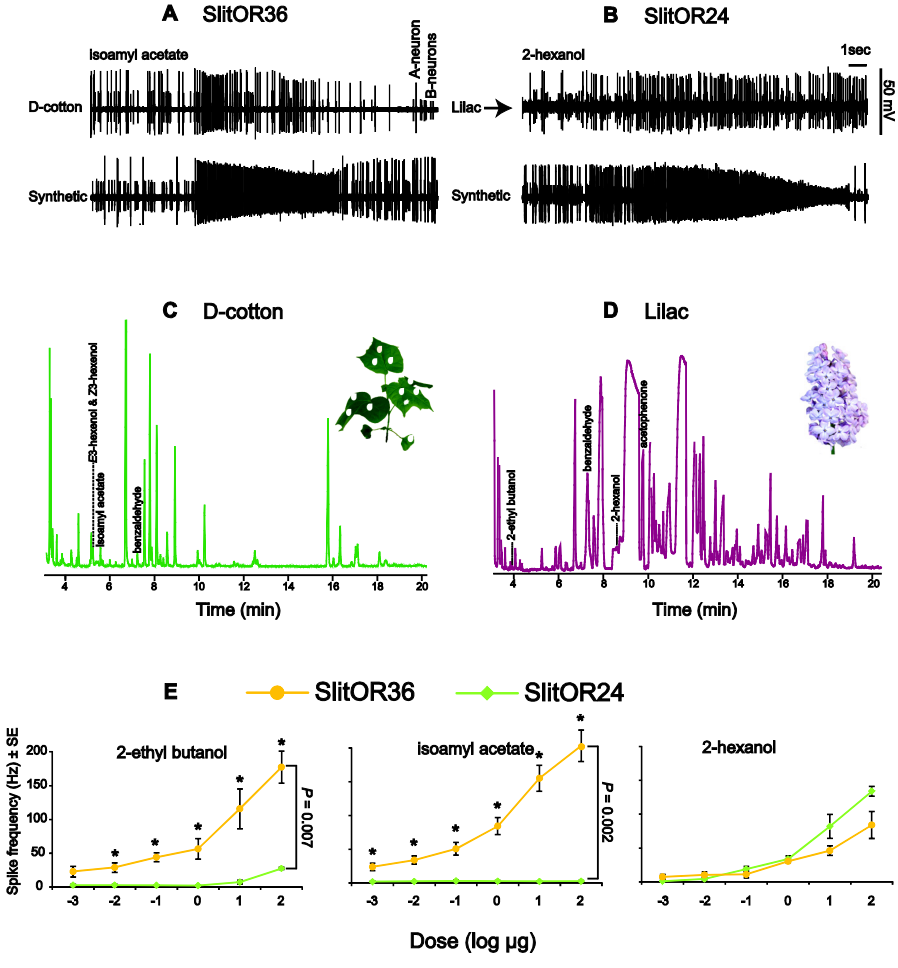


Figure 11. Examples of recordings from SlitORs in *Drosophila* ENS showing responses to headspace volatiles of different plants and their synthetic standards used in GC-SSRs. **A)** GC-SSRs from SlitOR36 responding to isoamyl acetate present in headspace of larvae-damaged cotton and to 100 ng of synthetic isoamyl acetate injected into the GC combined to the SSR setup. **B)** GC-SSR responses obtained from SlitOR24 using lilac extract and the synthetic 2-hexanol identified from lilac flower volatiles. **C-D)** Gas chromatograms from headspace volatile collections of damaged cotton and lilac flowers, respectively. The peaks are given names after their chemical identification and confirmation of biological activity. Neurons were differentiated based on difference in their spike amplitudes, i.e. A (larger spikes) and B (smaller spikes). **E)** Dose-relationship curves from SlitOR24 and SlitOR36 responses to six different concentrations of 3 diagnostic odorants. *P-values* represent the overall (pooled doses) sensitivity difference between SlitOR24 and SlitOR36, when means were compared by independent samples t-test. *) indicates the sensitivity difference between SlitOR24 and SlitOR36 at individual doses of individual compounds, tested by nonparametric independent samples Mann-Whitney U test. The α-level was set at 0.05.

earlier study (Paper III) on female *S. littoralis*, an OSN type (BC1A) responding to only 2-phenyl acetaldehyde was found (Paper III), which is identical in its response to the response of heterologously expressed SlitOR14 (present study). Such narrowly tuned ORs may mediate signals that activate specialized circuits in the brain, resulting in discrete behaviors as SlitOR14 detects a floral compound, 2-phenyl acetaldehyde (Saveer *et al.*, 2012), whereas SlitOR19 detects an oviposition deterrent compound, 1-indanone, found in the larval feces of *S. littoralis* (Jönsson & Anderson, 1999). So, these two receptors may induce attraction and avoidance behaviors, respectively, in *S. littoralis*.

Table 1. List of physiologically active plant odorants identified from host and non-host plants, in the present GC-EAD and GC-SSR studies on female *S. littoralis*, through GC-MS analyses, and their amounts found in larval damaged cotton, lilac flowers, spruce, and malabar nut plants.

Nr	Compound	Odorants released (ng min^{-1})				Antennal active compounds identified earlier by GC-EAD studies on *S. littoralis*		
		Damaged cotton	Lilac	Spruce	Malabar Nut	Damaged cotton	Undamaged cotton	Lilac
1	**toluene★**	0.23			0.03			
2	**di-butyl ether**				1.85			
3	**propanoic acid**				0.04			
4	(*E*)2-hexenal	0.37				1, 2‡		
5	(*Z*)3-hexenol	0.30				1, 2		
6	**(*E*)2-hexenol**	0.08						
7	**isoamyl acetate**	0.20						
8	**α-pinene**	6.25	11.21					
9	**camphene**		0.72					
10	benzaldehyde	0.22	15.13				3	3
11	β-pinene	1.24	5.83			1		
12	benzyl methyl ether		25.72					3
13	4-methylanisole		2.03					3
14	**sabinene**			0.32				
15	myrcene	2.50		0.68		1, 2	3	
16	**3-carene**			0.76				
17	***para*-cymene**			2.23				
18	**terpinolene**			0.04				
19	(*Z*)3-hexenyl acetate	1.70				1, 2	3	
20	**β-phellandrene**	0.53						
21	(*Z*)-β-ocimene	0.02	5.46					3
22	(*E*)-β-ocimene	1.43	119.37			1, 2		3
23	acetophenone		4.47					3
24	**(*E*,*E*)-cosmene**		0.38					
25	(*S*)-(+)-linalool		4.78					3
26	(*R*)-(−)-linalool	0.22				1, 2	3	
27	DMNT†	0.83				1, 2		
28	lilac aldehyde A		14.61					3
29	lilac aldehyde B		5.55					3
30	**(*E*)2-hexenyl butyrate**	0.07						
31	1,4-dimethoxy benzene		92.31					3
32	decanal	0.04					3	
33	estragol†		10.79					3
34	lilac alcohol		10.47					3
35	indole	0.01				1, 2		
36	**methyl eugenol**		8.32					
37	β-caryophyllene	2.66	1.30			1, 2		
38	**(*E*)-β-farnesene**	0.05						
39	α-humulene	0.73	4.73			2		
40	**α-cubebene**		2.48					
41	(*E*,*E*)-α-farnesene	0.39	3.02			2		
42	TMTT†	0.34				1, 2		
43	nerolidol		2.16			2		
44	**(*E*,*E*)-farnesol**		0.66					
Number of active compounds		23	23	5	3	14	5	12
New compounds found in this thesis		7	6	4	3			

†) DMNT stands for (4,8-dimethyl-1,(*E*)3,7-nonatriene), estragol (1-methoxy-4-(2-propenyl)-benzene), TMTT (4,8,12-trimethyl-1,(*E*)3,(*E*)7,11-tridecatetraene). ‡) 1= Jönsson et al. 1999, 2= Zakir 2012, 3= Saveer *et al*. 2012. ★) Names in bold are compounds not earlier reported as actives for *Spodoptera littoralis*.

Table 2. Response spectra of *Drosophila Empty Neuron System* (ENS) equipped with transgenic *Spodoptera littoralis* receptors (SlitORs) studied by SSR and GC-SSR.

Compound	**Ligands only found by GC-SSR-ENS**	SlitOR14	SlitOR19	SlitOR24	SlitOR36	Odor initial source		
						Synthetic	Damaged cotton	Lilac flower
benzaldehyde				●	•	*	*	*
2-phenyl acetaldehyde		●		●	•	*		
acetophenone				●	•	*		*
(*E*)2-hexenol				●	●	*		
(*E*)3-hexenol	**X**			●	●		*	
(*Z*)3-hexenol				●	●	*	*	
1-hexanol				●	●	*		
2-hexanol	**X**			●	●			*
benzyl alcohol				●	●	*		
2-ethyl butanol	**X**				●			*
isoamyl acetate					●		*	
1-indanone			●			*		
Number of encountered sensilla		8	9	5-7	5-9			

The spike frequency in second (Hz) is used as a measure of response strength, (•) indicate 15-50 Hz, (●) (51-80 Hz), (●) (81-110 Hz) and (●) indicate >110Hz. No inhibitory responses were observed in any OSN/OR. *indicate the initial source of odorants, i.e. compound was either present in the panel of synthetic odors tested and it was also found in headspace extract or it was not present in synthetic panel and found only in headspace extract of respective plant.

5 Conclusions and future prospects

The work of this thesis has contributed to the understanding of olfactory mechanisms involved in the interactions between insects and plants as well as the underlying neural and molecular mechanisms of odor coding used by phytophagous insects to select their hosts.

Papers I-II contribute to behavioral modulation by NHV, hypothesized as "semiochemical biodiversity hypothesis" (Zhang & Schlyter, 2003); an increase in semiochemical diversity seems to lead to decreased herbivore pest attacks or at least make it difficult for insects to locate their host plants. Field experiments by separating odor sources showed that anti-attractants modulate insect behaviors by reducing attraction of male *S. littoralis* moths and both sexes of *I. typographus* beetles towards female-produced sex Ph and aggregation Ph, respectively. This indicates that anti-attractants have the potential to be used in pest management (Schlyter, 2012). It showed that insects were able to differentiate between odor plumes released from different sources and the concept of odor source spacing may have potential to be used in mating disruption for pest control strategies (Byers, 1987). It also indicated that the insect olfactory system is highly capable and sensitive and not only detects odorants from their host plants but also of the non-hosts (Andersson *et al.*, 2009). Reproductive behaviors including oviposition bioassays and the behavioral tests in the wind tunnel demonstrated that non-host plants not only modulate behaviors by inhibiting reproduction activities but also effect insects' fitness negatively. To the best of our knowledge, this is the first study of NHV on a polyphagous moth. Thus, further experiments should be carried out in order to find out whether NHV can be used in pest management of generalists.

Papers III-IV contribute to the growing data on how plant odor information is coded by the OSNs in herbivorous insects. These studies also provide mechanisms for behavior and physiological studies to further dissect modulation and evolution of host plant range in phytophagous insects. We have identified 44 physiologically active compounds in GC-EAD, GC-SSR,

and GC-SSR-ENS studies (Table 1). The results obtained in these studies show that the majority of the OSNs/ORs in *S. littoralis* detecting plant volatiles are narrowly tuned. Of the 49 OSN classes characterized (Paper III-IV), 29 OSNs responded to one or a few structurally related odorants. The presence of both narrowly and broadly tuned OSNs indicates that the olfactory information could be mediated from the periphery to the CNS either through "labeled-line coding", in which the information about one odorant is coded by one type of OSN/OR, or through the "combinatorial coding" through which many OSNs either respond to the same compound or that the same compound can stimulate more than one neuron (Galizia & Rössler, 2010; Malnic *et al.*, 1999; Todd & Baker, 1999). Both coding mechanisms have also been suggested in the AL (Hansson & Christensen, 1999). However, from this thesis and similar other studies that have been done previously or ongoing in our lab, it appears that the "labeled-line" concept is getting more experimental support over time, in particular when stimulating neurons with lower doses.

Our deorphanization study shows a very nice example of specificity at lower doses: SlitOR14 and SlitOR19 responded specifically to single odorants, whereas SlitOR24 and SlitOR36 responded similarly to the same nine compounds when tested only with the synthetic panel doses. However, from our GC-SSR analyses of plant headspace, we found two key ligands for SlitOR36 that did not elicit any response in SlitOR24 (Paper V). This indicates that even apparent broadly tuned OSNs/ORs can display specific responses to some odorants, when tested at low, ecologically relevant doses. This also shows that employing GC-SSR for functional classification of OSNs in insects is very valuable, and it has resulted in a different picture, as OSNs specifically tuned to plant odorants have been reported in herbivorous moths, weevils, and beetles (Andersson *et al.*, 2012; Andersson *et al.*, 2009; Ulland, 2007 and references therein). Some of these studies also show that enantioselectivity is an important feature of OSNs specificity. Thus, OSNs of high sensitivity and specificity may allow insects over some distances to discriminate between plant species to select a right habitat as well as between plants of different quality. This study also gives the tools to work directly on molecular mechanisms involved in modulation of specific receptors as well as allow evolutionary studies of a kind of hitherto only possible in *Drosophila*. Future detailed deorphanization of ORs in this polyphagous moth will show whether or not a similar picture appears for other ORs.

The studies included in this thesis have mainly paid attention to the functional characterization of OSNs/ORs and identification of relevant plant odorants, while the behavioral modulation have mainly been evaluated for non-host plant volatiles. Whereas considerable knowledge is now acquired about

encoding of plant odor information by OSNs/ORs in *S. littoralis*, in all 44 compounds identified in the studies included in this thesis, the behavioral significance of only few of the compounds has been evaluated (Saveer, 2012; Zakir, 2012; Jönsson & Anderson, 1999). This underlines the need for further experiments focusing on the behavioral relevance of these compounds. In addition to common plant odorants, many of the compounds detected by the OSNs of *S. littoralis* are known to be induced compounds in cotton. Emission of induced compounds from cotton may provide an important message to *S. littoralis* females searching for a host plant to oviposit that the plant is under attack by conspecifics and thus not a good food source for the offspring and also may not be safe from natural enemies. Thus, the odorants detected by the OSNs from the test panel of compounds and natural headspace from host as well as non-host plants should be tested in future behavioral experiments. Behavioral experiments should focus on both single compounds as well as on the effect of odorant blends. Currently, the ratio-specific hypothesis is favored by most studies (Bruce *et al.*, 2005), as many behavioral studies have shown that blends of volatiles are important for oviposition behavior and attraction, as shown for the moths (Saveer, 2012; Del Socorro *et al.*, 2010; Gregg *et al.*, 2010; Ulland *et al.*, 2008; Rojas, 1999). On the other hand, some single compounds may be important for some decisions, such as linalool in *Manduca sexta* (Reisenman *et al.*, 2010) and *Mamestra brassica* (Ulland *et al.*, 2006).

Based on the results in the present thesis, we may assume that both single compounds and odorant blends could play a role in host plant recognition by *S. littoralis*. It would be interesting to compare the ORs of *S. littoralis* with ORs of specialist moths, as the presence of similar OR types across different insect species implies a strong conservation or reappearance of the same OSN types, independent of the evolution of oligophagy and polyphagy. A detailed comparison of OSNs specificity may reveal whether herbivore insects have evolved functionally similar ORs for detecting the same odorants by chance or necessity?

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