Olfaction in the Spruce Bark Beetle, *Ips typographus*

–Receptor, Neuron and Habitat

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Cover: *Ips typographus* on spruce needles
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Abstract

The bark beetle *Ips typographus* regularly kills spruce trees in the Palearctic. Spruces are colonized by means of attraction to an aggregation pheromone. Attraction is modulated by anti-attractive volatiles (NHV) from non-host plants. In this thesis, olfaction in *I. typographus* was studied. At the molecular level, putative odorant receptors (ORs) were identified. When compared with OR sequences of *Tribolium castaneum*, a drastic extension of bark beetle OR function was indicated. The ORs are situated in the membrane of olfactory receptor neurons (ORNs). By recording odor responses from these neurons, 17 ORN classes that strongly responded to pheromone, host, and non-host compounds, were characterized. Surprisingly, almost 25 % of these responded to anti-attractive NHV. The ORN for the essential pheromone compound *cis*-verbenol (cV), was in some sensilla co-localized with an ORN for the host plant compound 1,8-cineole (Ci). Ci inhibited pheromone attraction in the field. In addition, while the ORN for Ci responded, the response in the co-localized cV ORN was inhibited, indicating interactions between ORNs.

In electrophysiological studies of olfaction, odor stimuli are often based on a known amount of compound put on a filter paper, but the amount in the vapor phase (the actual stimulus) is unknown. Using a photoionization detector, vapor amounts of compounds released from stimulus cartridges were measured. A large variation between compounds, solvents, and successive stimulations were recorded. This indicated that stimulus doses often must be corrected for differences in volatility, and that consistent test protocols are required. In nature, insects orient in semiochemically diverse habitats with intermixing odor plumes. We studied the attraction to separated pheromone components, and pheromone and anti-attractant sources in the bark beetle and in the moth *Spodoptera littoralis*. While the beetle responded to spacing distances of a few decimeters, the moth responded to only a few centimeters. This may reflect different processing mechanisms in the sex pheromone system of the moth as compared to the aggregation pheromone system of the beetle. In addition, a long-distance (at least 2 m) effect of NHV was found, indicating a potential for these anti-attractants in forest protection against the beetle.

Keywords: *Ips typographus*, odorant receptor, olfactory receptor neuron, electrophysiology, co-localization, photoionization detector, odor spacing

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Sammanfattning

Granbarkborren. *Foto G. Birgersson.*

Insekter använder sig av doftsinnet för att finna värdväxter och partners. I denna avhandling studerades doftsinnet hos den åttatandade granbarkborren, *Ips typographus*. Granbarkborren är en allvarlig skadeinsekt på gran i Europa och delar av Asien. Attacker på träd samordnas med hjälp av ett aggregationsferomon (*cis*-verbenol och 2-metyl-3-buten-2-ol) som avges av hanar när de funnit ett lämpligt värdträd att kolonisera. Attraktionen till feromonet minskas av dofter från bredbladiga icke-värdväxter (s.k. non-host volatiles, NHV).

På gen-nivå identifierades kandidater för de receptorer på antennen som binder doftmolekyler. Proteinsekvenserna för dessa doftreceptorer jämfördes med de doftreceptorer som tidigare har identifierats hos mjölbaggen, *Tribolium castaneum*. Analysen visade att ungefär hälften av de 40 receptorer som vi fann hos barkborren avskiljdes tillsammans i en grupp specifik för barkborren. Möjligen är dessa receptorer anpassade för detektion av doftämnen som är speciellt viktiga för barkborrar. Exempel på sådana dofter kan vara ämnen från granar eller feromonsubstanser som används i kommunikationen mellan olika barkborre-arter.

Doftreceptorerna sitter i cellmembranen på doftceller i insektens antenn. När en doftmolekyl binds till en receptor, skickas en nervsignal från cellen till doftcentra i hjärnan. Varje cell har oftast enbart en typ av receptor och varje receptor är oftast specifik för ett eller ett fåtal doftämnen. Genom att stimulera insektens antenn med olika doftämnen och samtidigt mäta nervsvaret från cellen elektrofysiologiskt, kan man karakterisera doftresponsen hos de olika doftcellerna. Vi testade drygt 30 dofter på ett hundratal celler och kunde identifiera 17 klasser av starkt svarande doftceller hos barkborren. Nästan en fjärdedel av dessa svarade specifikt på de dofter från icke-värdväxter som minskar feromonattraktionen. Att en insekt använder en så stor del av sitt doftsinne till detektion av dofter den undviker har än så länge inte upptäckts hos någon annan insekt. De övriga klasserna av doftceller svarade specifikt på olika barkborre-feromoner eller gransubstanser.

Barkborrens antenn med dofthår.

Doftcellerna hos insekter finns i speciella sensiller (dofthår) på antennen och varje sensill innehåller oftast två till fyra olika celler. Oftast är grupperingen av celler mycket strikt och insekter placerar normalt feromonceller i specifika sensiller och celler för växtsubstanser i andra sensiller. Granbarkborren verkar bryta detta mönster eftersom doftcellen för den essentiella feromonkomponenten *cis*-verbenol, i vissa sensiller, förekom tillsammans med doftcellen för gransubstansen 1,8-cineol. Intressant var att när cellen för cineol

svarade med ett kraftigt nervsvar, minskades samtidigt nervsvaret till låga feromondoser i den intilliggande feromoncellen. Detta tyder på att det sker en viss grad av integrering av doftsignaler redan ute på antennivå. Vidare visade sig cineol finnas i speciellt stora mängder i granar som var massivt attackerade av granbarkborrar. I ett fältförsök fann vi att cineol starkt minskade attraktionen till feromonfällor. Möjligen representerar detta ämne en överkoloniserad gran eller en gran med kraftigt kemiskt försvar.

I elektrofysiologiska doftstudier baseras doserna oftast på en känd mängd doftämne applicerad på ett filterpapper. Filterpappret placeras i t.ex. en Pasteurpipett, och när insekten stimuleras blåses luften med doften i från pipetten över insektens antenn. Eftersom olika ämnen har olika flyktighet och därmed avdunstar med olika hastighet, kan antalet molekyler i gasfasen vara väldigt varierande beroende på vilket ämne man testar. Om samma doftpipett används flera gånger kan dessutom antalet molekyler variera mellan olika stimuleringar. Avsaknaden av kunskap om hur mycket doft man egentligen stimulerar insekten med kan leda till felaktiga slutsatser om ämnesspecificitet och känslighet. Vi använde en s.k. fotojoniseringsdetektor för att mäta avgivning av 26 ämnen från doftpipetter och fann en enorm skillnad mellan olika ämnen. Vid upprepade stimuleringar med samma pipett uppmättes, för de mest flyktiga ämnena, en mycket snabb reduktion i antalet luftburna molekyler. Dessutom observerades en stor skillnad mellan ämnen utspädda i olika lösningsmedel; koncentrationen av ämnen lösta i pentan minskade klart snabbare än den för ämnen i paraffinolja. Genom att sammankoppla mätningarna med nervsvar från insektens doftceller, fann vi att inkonsekvent hantering av doftpipetter kan leda till felaktig klassificering av doftceller.

Tidigare studier har visat att angrepp av skadeinsekter verkar vara mindre vanliga i habitat med hög biodiversitet. En del av förklaringen kan vara att habitat med hög biodiversitet även har en hög doftdiversitet, vilket resulterar i att dofter från attraktiva källor blandas med dofter från andra källor.

För att undersöka hur insekter påverkas i miljöer med överlappande doftplymer, studerade vi hur attraktionen till feromon hos barkborren och det egyptiska bomullsflyet (*Spodoptera littoralis*) påverkades av fysisk separering av doftkällor. Vi testade attraktionen både till separerade feromonkomponenter och till separerade källor av feromon och anti-attrahent (NHV för barkborren, feromonantagonist för bomullsflyet). Hos båda arterna ledde ökat avstånd mellan feromonkomponenterna till minskad fångst i feromonfällan. Ökat avstånd mellan feromon och anti-attrahent ledde till ökad fångst. Bomullsflyet var dock mycket känsligare än barkborren för separering mellan doftkällor; bomullsflyet påverkades av separering av enbart ett fåtal centimeter, medan barkborren reagerade på separering av flera decimeter. Detta antyder att doftsignaler integreras på olika sätt hos de två arterna. I ett ytterligare experiment med barkborren där vi använde ett fåtal NHV-källor eller tusentals små NHV-doftflingor, fann vi indikationer på att NHV kan ha effekt över ett par meters avstånd från feromonet. Detta tyder på att syntetiska eller naturliga NHV-källor kan användas i skogen som skydd mot barkborreangrepp.

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 Martin N. Andersson, Jonas M. Bengtsson, Ewald Grosse-Wilde, Marcus C. Stensmyr, Ylva Hillbur, Fredrik Schlyter, Bill S. Hansson. Olfactory receptor genes of the spruce bark beetle, *Ips typographus* (manuscript).

II Martin N. Andersson, Mattias C. Larsson, Fredrik Schlyter (2009). Specificity and redundancy in the olfactory system of the bark beetle *Ips typographus*: Single-cell responses to ecologically relevant odors. *Journal of Insect Physiology* 55, 556-567.

III Martin N. Andersson, Mattias C. Larsson, Miroslav Blaženec, Rastislav Jakuš, Qing-He Zhang, Fredrik Schlyter (2010). Peripheral modulation of pheromone response by inhibitory host compound in a beetle. *Journal of Experimental Biology* 213, 3332-3339.

IV Martin N. Andersson, Fredrik Schlyter, Sharon Rose Hill, and Teun Dekker. What reaches the olfactory receptor? -Factors affecting vapor concentration and interpretation of chemosensory studies (manuscript).

V Martin N. Andersson, Muhammad Binyameen, Medhat M. Sadek, Fredrik Schlyter. Attraction modulated by spacing of pheromone components and anti-attractants in a bark beetle and a moth (submitted).

Abbreviations

AL	Antennal lobe
Ci	1,8-Cineole
cV	*cis*-Verbenol
GC	Gas chromatograph
GLV	Green leaf volatile
MB	2-Methyl-3-buten-2-ol
OR	Odorant receptor
ORN	Olfactory receptor neuron
NHV	Non-host volatiles
PID	Photo ionization detector
SSR	Single sensillum recordings

1 Objectives

The objective was to investigate molecular, physiological, and ecological aspects of olfaction in the spruce bark beetle *Ips typographus* (L.). The specific goals were to identify the odorant receptors and to characterize peripheral odor coding mechanisms and odor response profiles of the olfactory receptor neurons. In addition, to learn more about insect orientation in semiochemically diverse habitats, the response to separated attractant and anti-attractant sources was studied in the bark beetle and compared to the response of a moth.

2 Introduction

2.1 Insect olfaction

Insects use their sense of smell to execute behaviors that are crucial for survival and reproduction, such as finding a food source or a mate. Plants and animals send out a multitude of volatile compounds, but in general, only a subset of these are picked up by the insect and used as olfactory cues. Behaviorally, the insect may respond to the signal by attraction towards its source, such as a food item or a sex partner (Andersson *et al.*, 2009), or alternatively by avoidance of other odors, for instance those of non-host plants (Zhang & Schlyter, 2004). Insects may differentiate hosts from non-hosts by detection of specific compounds (Zhang & Schlyter, 2003), but since many compounds are shared among plants and animals, specific combinations and ratios of compounds are often of crucial importance as well (Bruce *et al.*, 2005).

Odor molecules are transported downwind from their source of release, and air turbulence creates an odor plume with a complex structure (Murlis *et al.*, 2000; Vickers, 2000; Cardé & Willis, 2008). Odor molecules in the plume are picked up by odorant receptors (ORs), located mainly in the insect antennae and maxillary palps. Specifically, the receptors are present in the cell membrane of olfactory receptor neuron (ORN) dendrites that, in turn, are housed within olfactory sensilla (Vosshall & Stocker, 2007). The ORs are encoded by a large and diverse family of odorant receptor genes (de Bruyne & Baker, 2008), and each ORN is generally thought to express only one member from this family. When an odor molecule binds to an OR, the corresponding neuron sends a neuronal signal to the primary olfactory center of the brain, the antennal lobe (AL), where integration of odor input takes place (Silbering *et al.*, 2008). Typically, the signal that is generated by an ORN is an increase in the firing frequency of action

potentials (excitation), but some odorants may instead cause a decrease in firing activity (inhibition). ORNs can be divided into classes based on their odor response profiles. Often, ORNs are fairly specific and activated by only one or a few compounds, but some ORNs appear to have a broader tuning. In addition, each compound often activates more than one type of ORN, and thus, the odor input is thought to be constructed as a combinatorial code (Hallem & Carlson, 2006).

The insect ORs show no sequence homology with the receptors of vertebrates. They are also different in that they form heterodimers with a conserved and broadly expressed co-receptor, and have an inverted membrane topology (Benton *et al.*, 2006). In addition, the number of receptors and olfactory neurons is much lower in insects compared to vertebrates. Despite these differences, the insect olfactory system is similar to the vertebrate counterpart in terms of physiology, organization, and development (Hildebrand & Shepherd, 1997; Strausfeld & Hildebrand, 1999; Kaupp, 2010). Thus, the insights gained from insect olfactory research contribute to our understanding of the olfactory sense in animals in general. In fact, the lower complexity of their olfactory sense and the shorter generation time of insects make them suitable models for olfactory research.

2.2 Target species

The European spruce bark beetle, *Ips typographus* L. (Coleoptera, Curculionidae, Scolytinae), is the species of focus in this thesis. To improve our understanding of the molecular aspects of insect olfaction and peripheral encoding of the odor environment, OR gene identification and electrophysiological single sensillum recordings (SSR) from ORNs were performed. In a comparative field trapping study on *I. typographus* and the Egyptian cotton leaf worm, *Spodoptera littoralis* (Lepidoptera: Noctuidae), the behavioral response to physical separation of odor stimuli was investigated. Field tests also included studies of potential long-distance anti-attractive effects of non-host volatiles as a basis for potential use in forest protection against *I. typographus*.

2.3 Host selection of *I. typographus*

The male is the initial host seeking, or "pioneering ", sex of *I. typographus*. Once a male has located a suitable spruce tree to colonize, it releases an aggregation pheromone, a mixture of (4*S*)-*cis*-verbenol and 2-methyl-3-buten-2-ol (Schlyter *et al.*, 1987), which attracts individuals of both sexes. After mating, females lay eggs under the bark. Hatched larvae feed under the bark and then pupate. Emerged adults either leave the tree in search for a new host where a second generation is initiated or they overwinter in the soil or in the tree where they were born. The parent generation typically leaves the first tree and establishes a sister brood on a different tree. Depending on temperature, *I. typographus* goes through one or two (possibly three) generations per year (Wermelinger, 2004, and references therein).

Although the olfactory mediated host location behavior of the spruce bark beetle has been studied for decades, it is still not known how the pioneering males locate a suitable host tree, as no primary attraction (in the absence of pheromone) to spruce volatiles has been demonstrated. However, spruce volatiles may modulate the pheromone response or possibly attract beetles to a suitable habitat (Saint-Germain *et al.*, 2008). It is also possible that pioneering beetles land randomly on trees and assess host quality upon contact (Byers, 1996). However, apart from the few pioneering males, the aggregation pheromone attracts the majority of individuals to the host.

The attraction to the pheromone is modulated by other pheromone compounds that appear in later attack phases. Verbenone and ipsenol are two such compounds that are believed to regulate bark beetle density on attacked trees (Schlyter *et al.*, 1989). In addition, volatiles that are particularly abundant in non-host angiosperm plants (so called non-host volatiles, NHV), such as green leaf volatiles (GLVs) (Zhang *et al.*, 1999) and compounds from the bark, such as C8-alcohols and *trans*-conophthorin (Zhang *et al.*, 2000; 2002), have inhibitory effects on pheromone attraction. Combining these compounds with the anti-aggregation pheromone component verbenone, produces a strong synergistic effect and a very potent anti-attractant blend (Zhang & Schlyter, 2003). Possibly, the individual constituents in the synergistic blend represent different levels in the host selection sequence. The GLVs that are common to broad leaved plants may represent a signal of a non-host dominated habitat (Schlyter & Birgersson, 1999). More specific plant volatiles, such as *trans*-conophthorin may indicate non-hosts at the tree species level, whereas the anti-attractive pheromone components may signal unsuitability of individual spruce trees (Zhang & Schlyter, 2004).

2.4 Ecological relevance and pest status

Some bark beetle species are serious tree killers, destroying forests of great economic value. Currently, the large-scale outbreak of the mountain pine beetle, *Dendroctonus ponderosae*, in North America has resulted in the loss of hundreds of millions m^3 timber and turned the forests into major sources of carbon release (Kurz *et al.*, 2008). In Europe and parts of Asia (Stauffer *et al.*, 1999; Byers, 2004), the spruce bark beetle, *I. typographus*, is considered the most destructive bark beetle of coniferous forests (Økland & Bjørnstad, 2003; 2008). However, at low population densities, *I. typographus* primarily colonizes dying or newly dead Norway spruces [*Picea abies* (L.) Karst.] and starts the decomposition of bark and wood. When large amounts of breeding material is created, for instance after a severe windfall, the beetle multiplies rapidly (Bouget & Duelli, 2004; Wermelinger, 2004) and population densities increase enormously. When the fallen trees are depleted or too old, *I. typographus* attacks healthy, standing trees.

The large number of beetles that synchronously attacks a single tree makes it possible for them to overcome the defense of the tree (e.g. resin) that normally protects against single attacking individuals (Wood, 1982). Eventually, the tree dies as a result of bark beetle feeding in the phloem, as well as the bark beetle-associated pathogenic fungi (e.g. *Ophistoma* and *Ceratocystis* species) that may dry the tissue and induce vascular plugging (Paine *et al.*, 1997). Old trees and trees that are exposed to direct sunlight or drought stress are particularly susceptible to attack (Wermelinger, 2004).

2.5 Management

The spruce bark beetle is managed with the primary aim to minimize attacks on healthy spruce trees. Methods used for this purpose are sanitation felling of infested trees, clearing of wind throws, and pheromone traps for mass trapping (Wermelinger, 2004). Infested trees should be removed from the forest after the next generation of adults emerges. Pheromone traps can be used to reduce population levels, but also to stop an infestation front and to prevent further attacks on living trees (Jakuš, 1998).

The anti-attractive blend of the synergistic NHV and verbenone may also have potential for control of *I. typographus*. For instance, dispensers releasing this blend put on standing trees seem to reduce the probability of *I. typographus* attack (Jakuš *et al.*, 2003; Schiebe *et al.*, 2010). To increase the efficacy of semiochemical based control, the NHV mixture could be used in a 'push-pull' strategy in combination with pheromone traps (Zhang & Schlyter, 2004).

3 Odorant receptors of *I. typographus* (Paper I)

3.1 Insect odorant receptors

The odorant receptors (ORs) are key players in the olfactory system of insects. The binding of an odorant to an OR triggers the cellular machinery of the receptor neuron, which eventually gives rise to a neuronal signal (Sato *et al.*, 2008; Wicher *et al.*, 2008). This signal may, in turn, result in a behavioral response. As olfaction is of crucial importance to the fitness of insects, the OR repertoire is likely adapted to the ecological needs of the insect. Indeed, the low level of sequence homology among insect ORs is thought to reflect a rapid evolution, at least partly, due to adaptation to different environments (Nei *et al.*, 2008).

Sequences of OR genes have been identified mostly through bioinformatic screening of insect genomes. Presumably complete sets of putative OR genes exist for more than 10 *Drosophila* species (Robertson *et al.*, 2003), three mosquitoes (Hill *et al.*, 2002; Bohbot *et al.*, 2007; Arensburger *et al.*, 2010), the honeybee (Robertson & Wanner, 2006), the silkworm moth (Wanner *et al.*, 2007), and the flour beetle, *Tribolium castaneum* (Engsontia *et al.*, 2008). *T. castaneum* is the only coleopteran with identified OR genes, and it stands out among other insects by having a very large number of OR genes (341). However, many of these are pseudogenes or not expressed in the head (Engsontia *et al.*, 2008). *T. castaneum* is a pest of stored products and closely associated with human populations. Thus, for a better understanding of the molecular biology of insect olfaction in the context of natural ecosystems, it would be rewarding to identify OR genes of a natural coleopteran specialist, such as *I. typographus* for which the chemical ecology has been thoroughly investigated. Since the genome of *I. typographus* is not sequenced we used another approach to identify its ORs.

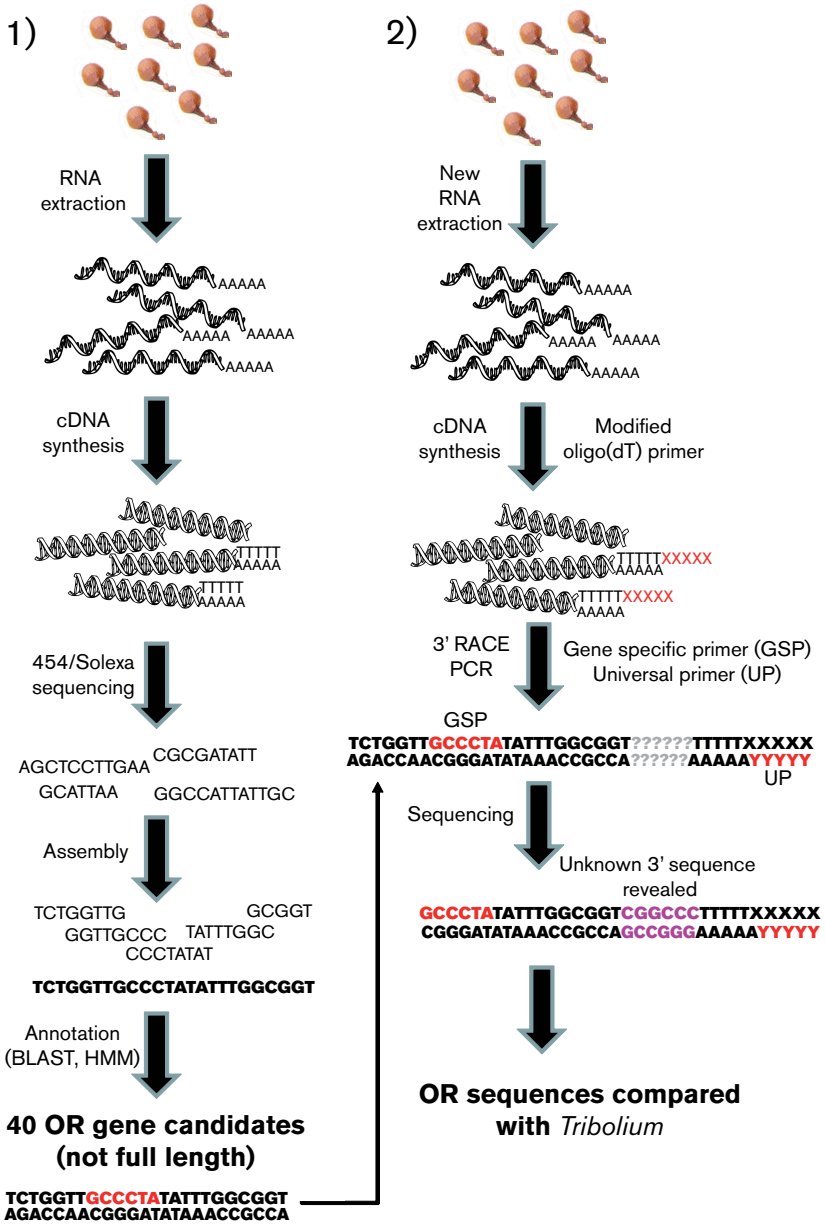

Figure 1. Schematic overview of the steps used for identification 1) and elongation 2) of the putative odorant receptor (OR) genes of *I. typographus*. 1) RNA extracted from antennae was used to construct a normalized cDNA library, which was subjected to 454- and Solexa sequencing. The sequencing produced short reads that were assembled and annotated to identify candidate OR gene fragments. From this sequence information, gene specific primers (GSPs) were designed. 2) The GSPs were used in combination with a universal primer in RACE-PCR to extend each candidate gene fragment in 3'-prime direction.

3.2 Molecular methods in short

The molecular methods are summarized in Fig. 1.

3.2.1 RNA extraction, sequencing and annotation

Since OR genes are transcribed and present as messenger RNA (mRNA) mainly in the antennae, we extracted antennal RNA from ca 200 adult beetles and constructed a normalized cDNA library (Evrogen, Russia). Normalization is done to lower the frequency of highly expressed genes, such as housekeeping genes involved in e.g. the general metabolism. Thus, genes with low expression levels, such as OR genes, will then proportionally be more common (Bogdanova *et al.*, 2008). The cDNA was then sequenced using massively parallel sequencing techniques (454 GS/FLX and Solexa). Sequenced reads were assembled and subsequently annotated using the BLAST algorithm and HMM-based (Hidden Markov Model) searches of the PANTHER database.

3.2.2 Fragment elongation and tissue specific expression

The assembled reads were too short to constitute full length OR genes, so they had to be elongated. This was accomplished by the SMARTer RACE (Rapid Amplification of cDNA Ends) cDNA Amplification Kit (Clontech). For fragments that were going to be elongated in downstream (3'-prime) direction, a special oligo(dT) primer that includes a binding site for a universal primer was used in first strand cDNA synthesis. In the subsequent PCR step, the universal primer was used in combination with a primer specific for each gene of interest. PCR products were visualized on agarose gels, the DNA extracted from the gel, cloned, and then sequenced (Eurofins MWG Operon, Ebersberg, Germany).

Elongated OR genes were tested for tissue specific expression with reverse transcriptase PCR using cDNA from antennae, legs, and abdomens from adult beetles.

3.2.3 Sequence similarity dendrogram

Amino acid sequences of OR candidates were used for multiple sequence alignment with the ORs of the flour beetle, *T. castaneum*. Based on this alignment, a maximum likelihood dendrogram was constructed.

3.3 Putative ORs of *Ips* in relation to ORs of *Tribolium*

Bioinformatic analyses revealed 40 strong OR candidates in the antennal transcriptome of *I. typographus*. RACE-PCR verified 3'-prime ends of eight of these (Paper I). Five of the candidates were specifically expressed in the antennae (not detected in legs or abdomen), which indicated an olfactory function. Expression patterns of remaining candidates have yet to be determined. The number of candidate ORs in *I. typographus* is lower than the number found in the genome of *T. castaneum* (341) (Engsontia *et al.*, 2008). This might appear somewhat counterintuitive as the flour beetle has adopted a comparatively more simplistic lifestyle than the bark beetle, the latter responding behaviorally to a variety of compounds from different ecological sources (Schlyter & Birgersson, 1999; Zhang & Schlyter, 2004). However, many of the genes in *T. castaneum* are present as pseudogenes, and others are not expressed in the adult head. In addition, it is unknown how many of the ca 100 ORs that are expressed in the head are specifically expressed in the olfactory appendages, and the large number of pseudogenes might indicate that the species is in an evolutionary process of losing superfluous receptors (Engsontia *et al.*, 2008).

Amino acid sequences of the 40 strong OR candidates were used for comparison with ORs of *T. castaneum* (Fig. 2). The analyses reveled that almost half of the receptors of *Ips* were grouped together with receptors of *Tribolium*. As the two species belong to distantly related families (Hunt *et al.*, 2007), this could indicate some degree of conservation of ancestral functional patterns among ORs in Coleoptera. One might speculate that these ORs may be involved in the detection of odorant classes important for Coleoptera in general.

Interestingly, more than half of the putative ORs of *Ips* formed an *Ips* dominated subgroup together with two ORs of *Tribolium*. ORs in this group were only distantly related to the all the other ORs. This indicates a drastic extension of OR function in *Ips,* not commonly found in other insects (but see Robertson & Wanner, 2006). Possibly, these *Ips* ORs are involved in the detection of conifer-related odorants or various con- and heterospecific bark beetle pheromone compounds. Indeed, several classes of ORNs that detect such compounds have been identified (Paper II). However, the function of any OR is impossible to infer from a position in a dendrogram, but needs to be determined in a heterologous cell system. To date, comprehensive functional studies of insect ORs have been performed only on the fruit fly and the malaria mosquito, using the 'empty neuron' system of *Drosophila melanogaster* or *Xenopus* oocytes (Hallem & Carlson, 2006; Carey *et al.*, 2010; Wang *et al.*, 2010).

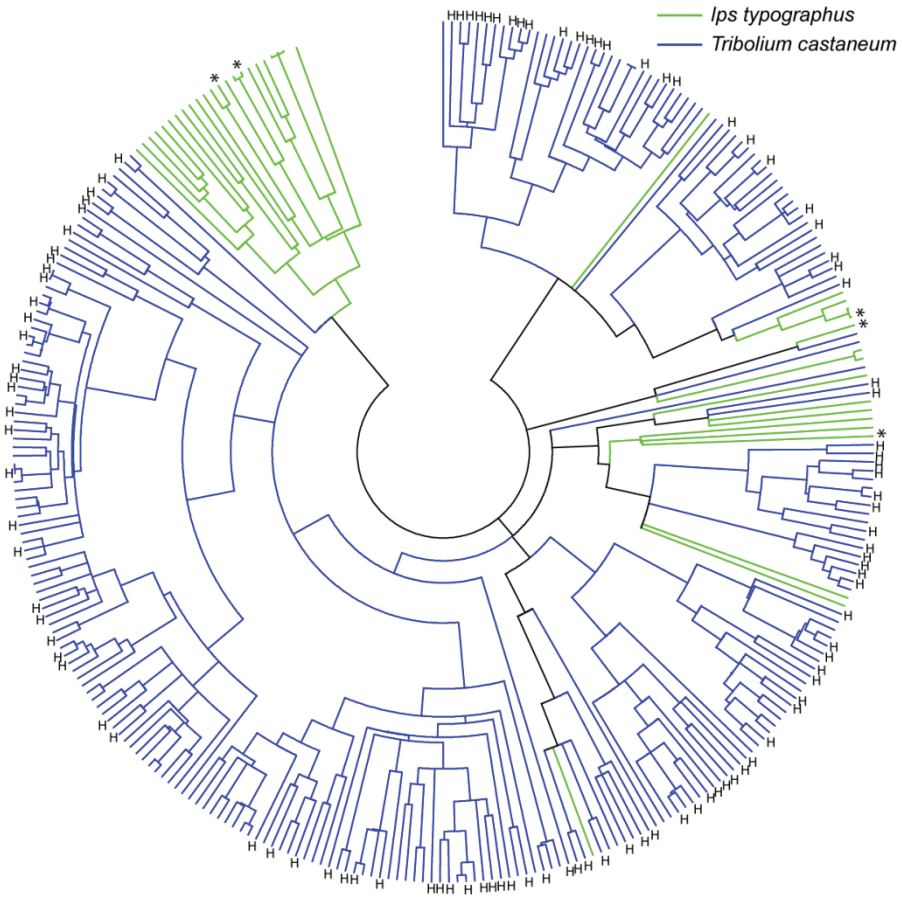

Figure 2. Maximum likelihood dendrogram based on multiple alignment of odorant receptor (OR) amino acid sequences of *Ips typographus* and *Tribolium castaneum*. **H** indicates ORs expressed in the head of adult *T. castaneum*. Asterisk (*) indicates *I. typographus* ORs with confirmed antennal specific expression.

4 Olfactory receptor neurons of *I. typographus* (Papers II-III)

Due to its status as a serious pest on an economically important plant, an extensive knowledge regarding the behavioral responses to several attractive and anti-attractive compounds has been established (Schlyter & Birgersson, 1999; Zhang & Schlyter, 2004). However, apart from the olfactory receptor neurons (ORNs) detecting mainly different pheromone compounds (Mustaparta *et al.*, 1984; Tømmerås *et al.*, 1984; Tømmerås, 1985), little was known about the peripheral detection and encoding of odorants in this species.

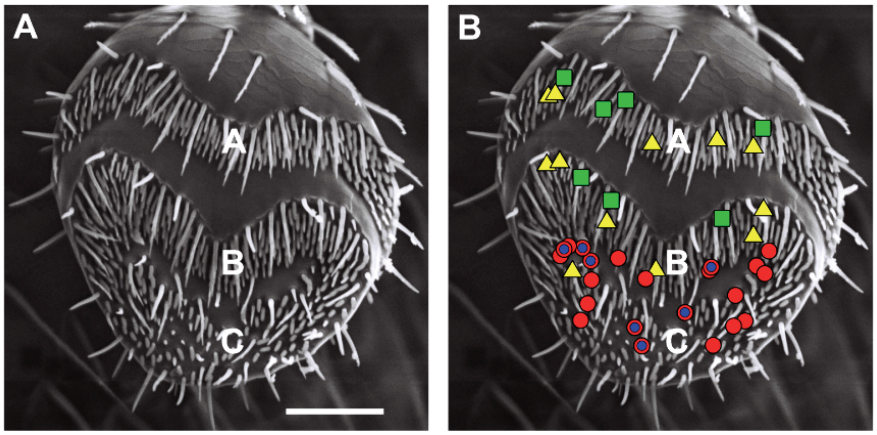

Figure 3. A) Olfactory sensilla are present in three areas (A, B, and C) on the antennal club. B) Spatial distribution patterns of four classes of olfactory receptor neurons (ORNs). ORNs responding to green leaf volatile alcohols (non-host) = green squares, myrcene (host) = yellow triangles, *cis*-verbenol (pheromone) = red circles, 1,8-cineole (host) = blue small circles. Scale bar = 50 μm.

4.1 Single sensillum recordings

Single sensillum recordings (SSR) is an electrophysiological technique. By inserting a tungsten microelectrode into an olfactory sensillum, action potentials (spikes) from the ORNs inside the sensillum can be recorded. During recordings, the insect preparation is continuously exposed to a controlled airstream. When the insect is stimulated with an odorant, a brief odor-pulse is delivered through the airstream and onto the antenna. If the contacted ORNs detect the odorant, the typical response is an increase in action potential firing. In general, ORNs seem to be fairly specific, being sensitive to only one or a few structurally related compounds. In addition to synthetic compounds, natural odor extracts can also be tested through GC-coupled SSR.

4.2 Olfactory receptor neuron classes

Olfactory sensilla of the spruce bark beetle are present in three areas (or bands) on the antenna (Fig. 3A). 150 olfactory sensilla were screened for responses to an odor panel comprised of synthetic pheromone, host and non-host compounds (Paper II). Strong excitatory responses were obtained from 106 ORN; 45 responding specifically to various bark beetle pheromone compounds, 37 to host compounds, and 24 to anti-attractive non-host volatiles (NHV) (Fig. 4). Based on response spectra, the 106 ORNs could be grouped in 17 different classes (Fig. 5). Additionally, 26 neurons (divided into 12 ORN classes) responded only weakly to any test odorant, indicating that the most potent compounds for these ORNs were lacking. 10 of the strongly responding ORN classes were subjected to dose-response trials that indicated that most ORNs are highly specific and sensitive (Fig. 6A-C).

The ORNs were not randomly distributed on the antenna. Instead, ORNs from a particular class were generally found either in both the A and B bands of sensilla, or exclusively in the distal area C (Fig. 3B). This distribution pattern seems to correspond to the distribution of the two morphological types of single-walled sensilla previously identified (Hallberg, 1982).

It is common in insects that the pheromone ORNs are numerous on the antenna, and the most common ORN type is often tuned to the major component (Ljungberg *et al.*, 1993; Baker *et al.*, 2004). In *I. typographus*, the most recurrent ORN class was tuned to *cis*-verbenol. In contrast, there were only few cells (Fig. 5) specific for 2-methyl-3-buten-2-ol (MB), which is produced, and behaviorally active, in much larger quantities (Birgersson *et*

al., 1984; Schlyter *et al.*, 1987). This suggests that the pattern might be reversed in the bark beetle. However, the MB cells were found in a restricted area on the antenna, i.e. on the borderline between the B and C areas, which could have resulted in this cell type being underrepresented among the sampled sensilla. Alternatively, as MB is highly volatile, the low number of cells could be the result of the compound being lost from the stimulus cartridge upon stimulation. Indeed, photoionization detector measurements showed that the headspace concentration of MB drops dramatically upon stimulation. However, the insect ORN still responded vigorously despite the low concentration (Paper IV), rendering this explanation unlikely.

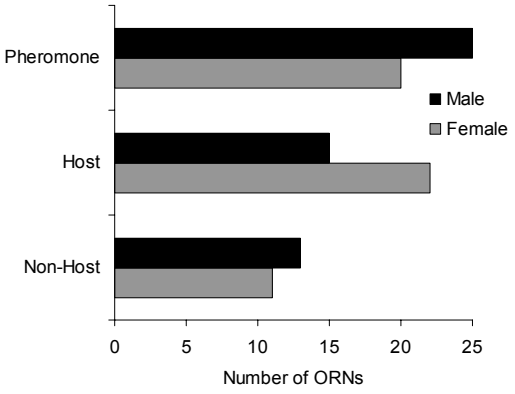

Figure 4. Frequencies of olfactory receptor neurons (ORNs) responding to compounds of different ecological origins.

Given that host kairomones, apart from α-pinene (Erbilgin *et al.*, 2007), have not been demonstrated to be especially important for host attraction, it was a bit surprising to find that several ORN classes were highly selective for conifer-related monoterpenes. Possibly, these compounds may serve as habitat-scale attractants (Saint-Germain *et al.*, 2008), or as modulators of pheromone attraction (Paper III). In addition, it was striking that almost 25 % of the strongly responding ORNs were specifically tuned to NHV. This indicates that insects may devote a lot of olfactory capacity to the detection of compounds that they avoid. Similar results have not been found in any other insect studied so far.

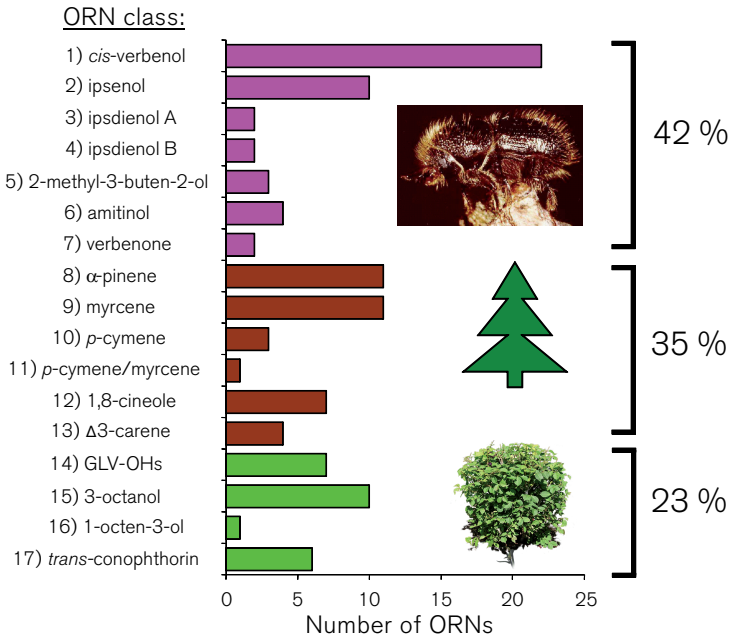

Figure 5. Number of olfactory receptor neurons (ORNs) of the 17 strongly responding classes. ORN classes are labeled according to which compound(s) elicited the strongest response. Pink = bark beetle pheromone compounds, Brown = conifer compounds, Green = non-host volatiles. GLV-OHs = green leaf volatile alcohols.

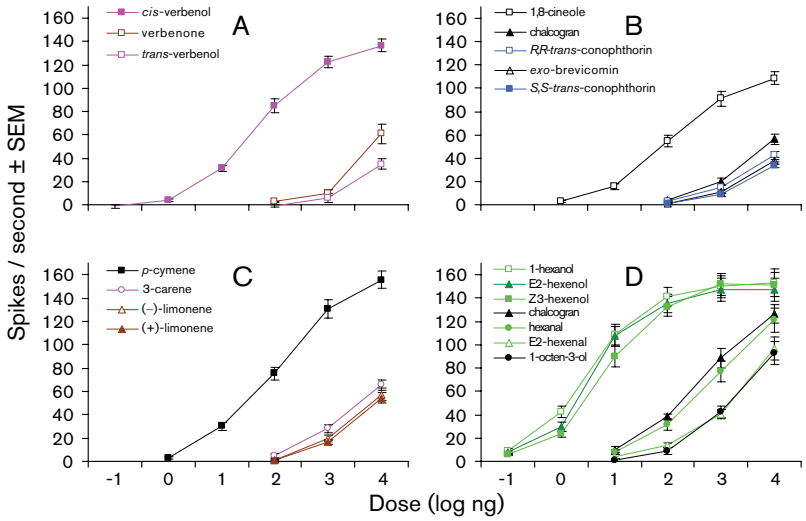

Figure 6. Dose-response curves from four receptor neuron classes, demonstrating specific primary responses to A) the pheromone component *cis*-verbenol, the spruce compounds B) 1,8-cineole and C) *p*-cymene. D) Indiscriminate response to the three green leaf volatiles 1-hexanol, *E*2-hexenol, and *Z*3-hexenol.

4.3 Olfactory receptor neuron responses and behavior

The single sensillum recordings indicated that behavioral responses to several compounds could likely be explained by the responses of the ORNs.

Several volatiles from non-host plants were previously shown to inhibit pheromone attraction of the spruce bark beetle (Zhang *et al.*, 1999; 2000; 2002). The three GLVs 1-hexanol, *E*2-hexenol, and *Z*3-hexenol all reduced pheromone attraction to a similar extent. However, combining the three did not produce a stronger inhibition of attraction, a phenomenon defined as redundancy (Zhang & Schlyter, 2003). Interestingly, the only ORN that was sensitive to any of these volatiles had a more or less identical sensitivity to all three of them (Fig. 6D). Thus, it appears as if the bark beetle cannot differentiate between the compounds at the physiological level, which could explain their behavioral redundancy. In contrast, the compounds verbenone and *trans*-conophthorin that synergize the inhibition are detected by different ORNs. Interestingly, the pheromone component, chalcogran, of the sympatric *Pityogenes chalcographus*, was primarily detected by the same neuron as *trans*-conophthorin. Chalcogran also inhibits pheromone attraction of *I. typographus* (Byers, 1993).

Most insects house their ORNs for pheromone compounds in sensilla that are distinct from the ones that detect plant compounds (Hansson *et al.*, 1999; Larsson *et al.*, 2001). However, in *I. typographus* we found that the ORN for the aggregation pheromone component *cis*-verbenol (cV), in some sensilla, is co-localized with an ORN that responds to the plant compound 1,8-cineole (Ci) (Fig. 3B). This lack of segregation between ORNs detecting pheromones and plant volatiles may suggest that host finding in bark beetles is an integrated process that involves both pheromones and plant volatiles. Interestingly, when the ORN for Ci responded, the co-localized cV cell was inhibited. In addition, Ci was found to be particularly abundant in heavily attacked spruce trees and the compound strongly reduced pheromone attraction (88 % reduction in trap catch) in the field (Paper III). Possibly, Ci is a signal of an unsuitable (crowded) host or a well-defended tree.

4.4 Peripheral modulation of pheromone response

Co-localization of insect ORNs is thought to increase the spatiotemporal resolution of odor stimuli (Fadamiro *et al.*, 1999) and ratio detection of ecologically relevant odor mixtures (Bruce *et al.*, 2005). In addition, the presence of two or more neurons in the same sensillum may provide opportunities for signal modulation in the periphery.

In *I. typographus*, not all cV neurons were co-localized with the neuron for Ci. These cV neurons were instead found together with another ORN type that did not respond to any test odorant. The Ci seemed to inhibit the cV cell only when the two neurons were co-localized, which implied that the inhibition might be due to interactions between ORNs. To test this hypothesis, both types of cV sensilla, (with or without the Ci cell; Fig. 7A), were tested for responses to binary cV/Ci mixtures.

Indeed, it was found that not only the spontaneous activity, but also the ORN response to the lowest cV dose (1 ng) was inhibited by simultaneous stimulation with high doses of Ci (1-10 μg). This inhibition occurred only in sensilla that also contained the Ci cell (Fig. 7B-C). In addition, the response to the higher cV dose (10 ng), was more strongly inhibited when the Ci cell was co-localized. Thus, it seems plausible that the two ORNs interact, possibly by means of passive electrical interactions (Vermeulen & Rospars, 2004). However, if or to which extent the behavioral inhibition can be explained by the inhibition of the pheromone ORN remains unknown, as the excitatory input from the two ORNs also provides the means for central integration.

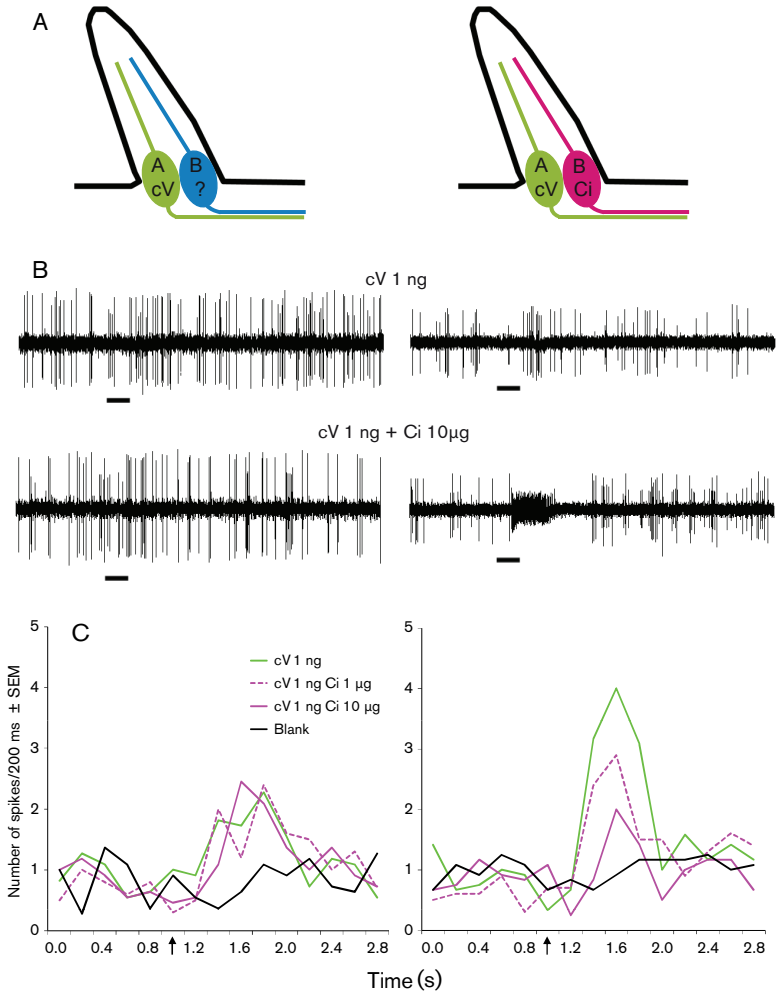

Figure 7. A) Schematic drawing of the two types of sensilla containing the A cell (large amplitude spikes) for *cis*-verbenol (cV), accompanied either by a non-responsive (*left column*) B cell (small amplitude), or a B cell for 1,8-cineole (Ci) (*right column*). B) Responses of both sensillum types to 1 ng cV (*upper traces*), and a binary mixture of 1 ng cV and 10 µg Ci (*lower traces*). Note the inhibition of the cV response during the response to Ci in the B cell. Black horizontal bars indicate the 0.5 s stimulation period. C) Detailed response curves to cV and binary cV:Ci mixtures showing a Ci dose-dependent inhibition of the cV response only in sensilla that also contain the Ci cell (N = 10-12). Arrows indicate the onset of the 0.5 s stimulation period.

5 Measurements of airborne odor amounts (Paper IV)

5.1 What reaches the antenna?

In many electrophysiological studies on insect olfaction, odor stimuli are prepared based on a known amount (e.g. in nano- or microgram) of compound applied to a piece of filter paper. The filter paper is then often placed inside an odor cartridge, such as a Pasteur pipette. Upon stimulation, the headspace in the cartridge is blown over the insect preparation. However, depending on compound, solvent, and how many times the cartridge has been used etc., the quantity of molecules reaching the insect can be highly variable. This may confound conclusions regarding, for instance, physiological response thresholds and specificities of ORNs (Cometto-Muñiz *et al.*, 2003; Tsukatani *et al.*, 2003).

A photoionization detector (PID) was used to measure airborne odor quantities released from Pasteur pipette stimulus cartridges in an odor delivery system commonly deployed in electrophysiological recordings (Paper IV). A PID uses a UV light source to ionize molecules. The charge produced by the ions is measured by a sensor and is proportional to the concentration of molecules. As the measured concentration of a compound is dependent on the ionization probability of the detected molecule, absolute concentrations for different compounds are not comparable. Therefore, in the present study the measured concentration of each compound was converted to molar amounts by means of a GC-FID (flame ionization detector) calibration approach (Paper IV).

5.2 Variation in headspace amounts

In electrophysiological recordings, stimulus pipettes are often loaded just once and then used for many consecutive stimulations, sometimes during a full day of experiments. To mimic a very productive day of recordings, we measured how the airborne amount of 26 compounds (C_2-C_{10}) was depleted (reduced) during 50 successive stimulations (10 min between stimulations) with the same stimulus pipette. Paraffin oil and pentane were used as solvents.

A huge variation among compounds dissolved in paraffin oil (100 μg dose on the filter paper) was observed (Fig. 8A). For the most volatile compounds (i.e. ethanol, 1-butanol, and MB) ca 80 % of the headspace was lost at the first puff, and airborne odor amount was below detection threshold already after a few stimulations. On the other side of the spectrum, pipettes loaded with heavier and less polar compounds such as linalool and 1-octanol showed a more constant release throughout the test protocol. Pipettes containing lower doses of compounds on the filter paper tended to be depleted at a proportionally higher rate (Fig. 8B).

All compounds dissolved in pentane demonstrated much higher rates of depletion as compared to their depletion from paraffin oil (Fig. 8C). The type of solvent also affected the order of depletion rates among compounds; some compounds with relatively low depletion rates in paraffin (e.g. monoterpene hydrocarbons and methyl octanoate) were released very quickly when dissolved in pentane.

Keeping stimulus cartridges containing the more volatile compounds (e.g. MB and Z3-hexenol) at room temperature for more than a few hours significantly reduced headspace amounts. The loss of headspace was also significant, albeit less extreme and appeared after longer storage for the heavier compounds (i.e. methyl salicylate and 3-octanol). Except for MB, pipettes stored at −20 °C retained their headspace amount for at least 2 days.

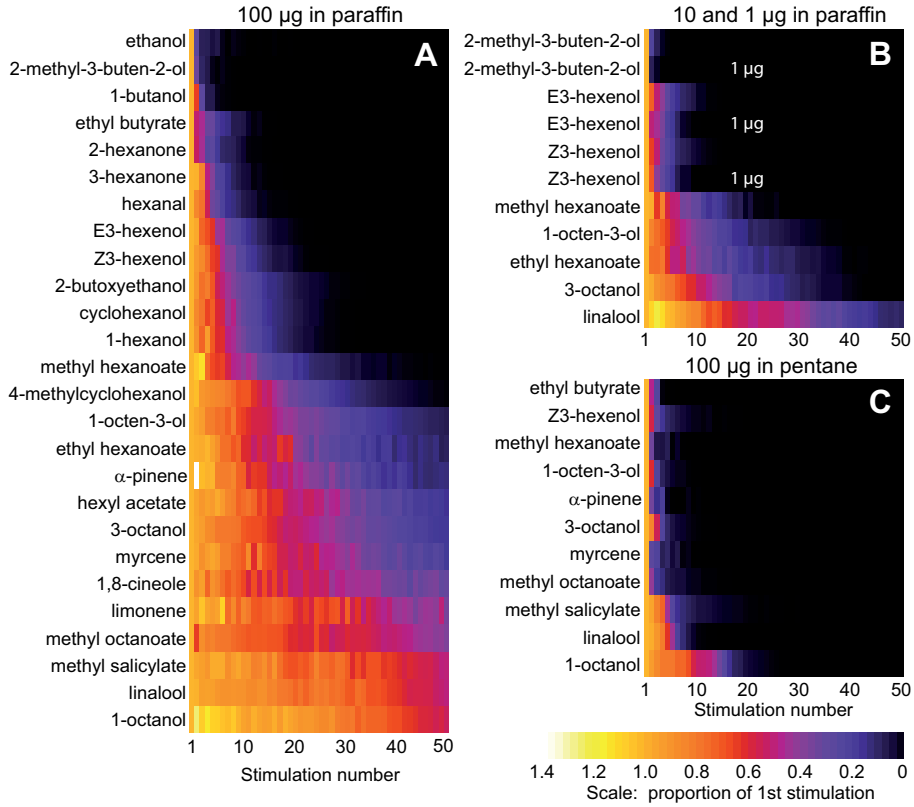

Figure 8. Depletion of compounds in paraffin oil at a A) 100 µg dose on the filter paper ($N = 4$) and B) two lower doses, 10 µg and 1 µg (indicated in plot; $N = 3$-4), represented in heat plots. C) Depletion of compounds dissolved in pentane at the 100 µg dose ($N = 4$). For all compounds, setting the average initial response of the detector to 1.0 normalized the responses and other responses are expressed as a proportion of this value.

5.3 Implications for single sensillum recordings

The large variation observed between compounds, solvents, and successive stimulations, could easily bias electrophysiological responses in insects. During the initial stimulations, the more volatile compounds were released in clearly larger amounts than the less volatile ones. However, after a few stimulations, the airborne amount of less volatile compounds was larger. By using fresh (not used) and 'old' (used 10 times) pipettes, the response of the 3-octanol ORN of *I. typographus* (Paper II) to two C_8-alcohols (3-octanol and 1-octen-3-ol) and two C_6-alcohols (Z3-hexenol and 1-hexanol) was analyzed. The responses of the ORN to fresh stimulus cartridges were clearly different from the response to the 'old' pipettes (Fig. 9). Especially the response to the C_6-alcohols was clearly lower when old pipettes were

used. In fact, the different response of the ORN actually implied that recordings were made from two ORN classes with different response specificity.

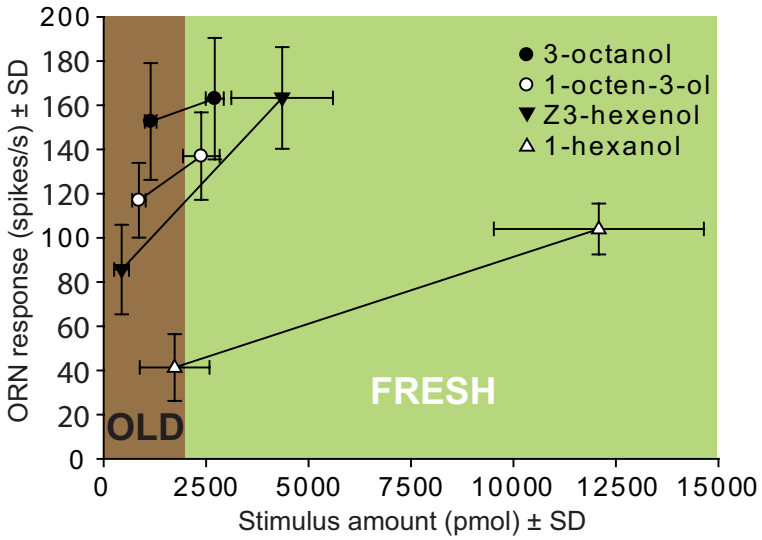

Figure 9. The response of the 3-octanol olfactory receptor neuron (ORN) ($N = 6$) to airborne amounts of four compounds from old (i.e. puffed 10 times before; *left*) and fresh (*right*) stimulus pipettes. Responses elicited by the same compound are connected with a line.

By alternately stimulating the PID and the insect ORNs with a single stimulus pipette, the sensitivity of ORNs to key and non-key ligands was compared to the sensitivity of the PID. In this test, the highly specific MB ORN of *I. typographus,* as well as the short sharp trichoid 3A (sst 3A) ORN of the mosquito *Culex quinquefasciatus* were tested. The sst 3A neuron responds best to 1-octen-3-ol, and secondarily to 2-butoxyethanol and 4-methylcyclohexanol (Hill *et al.*, 2009). For the two key ligands, MB and 1-octen-3-ol, the response of the corresponding ORNs remained relatively strong despite large reductions in airborne concentration. In addition, the change in response to non-linear reductions in airborne amounts was linear in both types of ORNs (Fig. 10A-B). However, the reduction in ORN response to the secondary odorants in the mosquito more closely followed the response curve of the PID (Fig. 10C-D).

Our novel dose-response procedure provides far more data points and requires fewer stimulus pipettes than the traditional method that is based on log-step compound doses applied on different filter papers. In addition, as the new method is based on airborne amounts, it also provides more accurate information about ligand specificity and sensitivity.

5.4 Recommendations

The PID measurements suggest that there might be a need to measure airborne stimulus amounts in olfactory research. Although it may not be important or practical to do this for all compounds in every study, it may sometimes be absolutely necessary in order to draw the correct conclusions (Ditzen *et al.*, 2008; Syed & Leal, 2008). In addition, it is recommended to: (i) compensate for differences in vapor amounts if possible, (ii) handle stimulus cartridges consistently, (iii) use volatile stimuli only for a few stimulations, (iv) store pipettes at low temperature and not for too long, and (v) use the same solvent, preferably paraffin oil if possible.

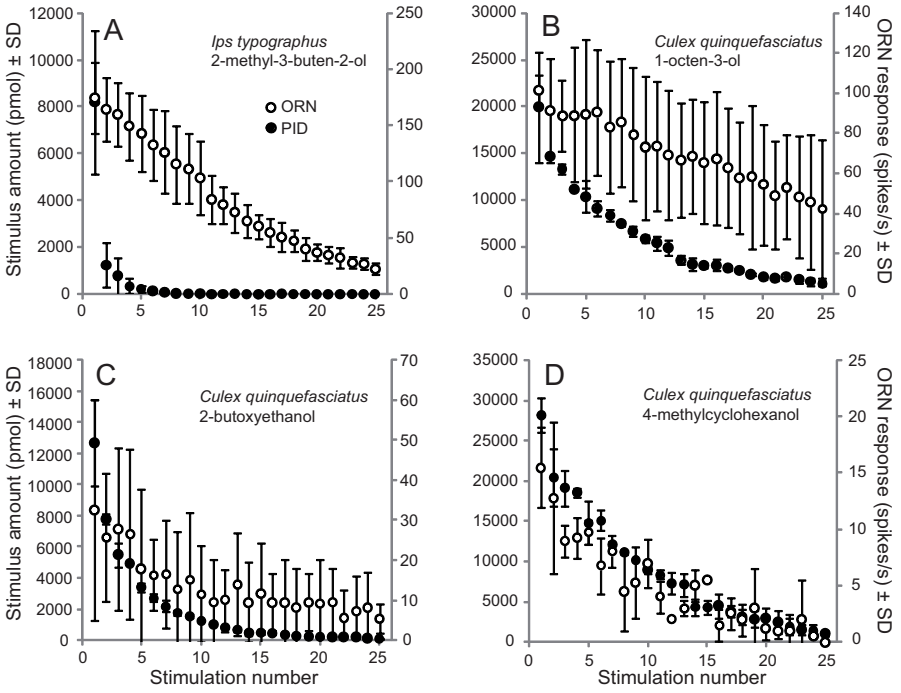

Figure 10. Response of the insect olfactory receptor neurons (ORNs) and the photo ionization detector (PID) to successive stimulations with A) 2-methyl-3-buten-2-ol in *Ips typographus* ($N = 4$); and B) 1-octen-3-ol, C) 2-butoxyethanol, and D) 4-methylcyclohexanol in *Culex quinquefasciatus* short sharp trichoid 3A neuron ($N = 2$).

6 Behavioral responses to separated odor sources (Paper V)

6.1 Detection and behavior in odor-diverse habitats

The insect response to an attractant may be displayed as an upwind flight towards the odor source. In contrast, the detection of a repulsive compound may lead to abortion of upwind flight and avoidance behavior. In environments with a high "semiochemical diversity" (Zhang & Schlyter, 2003) where odor plumes from different sources likely intermix, localization of attractive sources, such as host plants, may be seriously hampered by the presence of odors from non-hosts (Jactel & Brockerhoff, 2007; Jactel *et al.*, 2010). Thus, it may be possible to reduce the risk of bark beetle attacks by making the environment more semiochemically diverse. Homogenous mixing of odor plumes from different sources is, however, contradicted by the partitioning of plumes into "odor packages" (or filaments) that are interspersed with pockets of "clean air" (Vickers, 2000; Cardé & Willis, 2008). The filamentous nature of plumes is thought to facilitate plume discrimination by insects.

6.2 The comparative approach

It was known from previous studies that placing a NHV mixture inside a pheromone trap greatly reduces trap catch of *I. typographus* (Zhang & Schlyter, 2003). To test the "semiochemical diversity hypothesis", pheromone attraction in the presence of NHV at different distances from the pheromone dispenser, was investigated (Paper V). To further study responses to separated baits, the response to separated pheromone components (*cis*-verbenol and 2-methyl-3-buten-2-ol) was also tested (in the absence of NHV). As beetles colonize both standing and fallen trees, odor source spacing was done both vertically (Fig. 11A) and horizontally

(Fig. 11B). In addition, the response of the beetle was compared to the response of the Egyptian cotton leaf worm, *Spodoptera littoralis* (Lepidoptera; Noctuidae). *S. littoralis* is distributed throughout the warm-temperate and subtropical regions in the Old world (Brown & Dewhurst, 1975) and is a pest on a variety of crops, such as cotton, soybean, and vegetables (Salama *et al.*, 1971). For this species we tested the response to horizontally separated (Fig. 11C) sex pheromone components (major compound *Z*9-*E*11-tetradecadienyl acetate [Z9E11-14Ac], minor compound *Z*9-*E*12-tetradecadienyl acetate [Z9E12-14Ac]), in addition to horizontally separated pheromone and behavioral antagonist (*Z*9-tetradecenyl acetate [Z9-14Ac]) sources (Kehat *et al.*, 1976; Campion *et al.*, 1980; Haines, 1983).

The behavioral observations were complemented by measurements of plume structure and overlap in the field using a photo ionization detector (PID) and soap bubble machines (Fig. 11E-F).

6.3 Beetle vs. moth

6.3.1 Different scales

In both species, increased spacing between pheromone and anti-attractants led to increased trap catch (Fig. 12), whereas increased spacing between pheromone components had the opposite effect (Fig. 13). However, the two species differed greatly with respect to the spacing distances that affected their behavior. While the beetle responded to separation of a few decimeters, the moth responded to distances of just a few centimeters. Such high resolution of moths has been observed also previously (e.g. Rothschild, 1974; Witzgall & Priesner, 1991; Liu & Haynes, 1993; Fadamiro *et al.*, 1999). In each species, the spacing distances affecting behavior did not differ between the pheromone component spacing and the pheromone/anti-attractant spacing experiments.

6.3.2 Plume structure and overlap

Measurements of plume structure and overlap were only possible to obtain for the spacings used in the bark beetle tests. Plume visualization with soap bubbles indicated that at ca 0.5-1 m spacing between sources (distances affecting the response of the beetle), plumes typically overlapped ca 1-3 m from the trap. In contrast, at 16 cm spacing, overlap started close to the trap (< 2 dm). Still, moths were able to resolve much smaller spacing distances. In addition, plume parameters measured by the PID were very different close to the trap as compared to 2 m downwind. At 2 m distance, few odor bursts were detected and they contained much lower odor concentration as

compared to 1-2 dm downwind. Thus, the amount of odor insects encounter while orienting in plumes seems to vary greatly depending on distance to the source. The changes in plume parameters were similar to results of previous studies (Murlis & Jones, 1981; Murlis *et al.*, 2000; Thistle *et al.*, 2004).

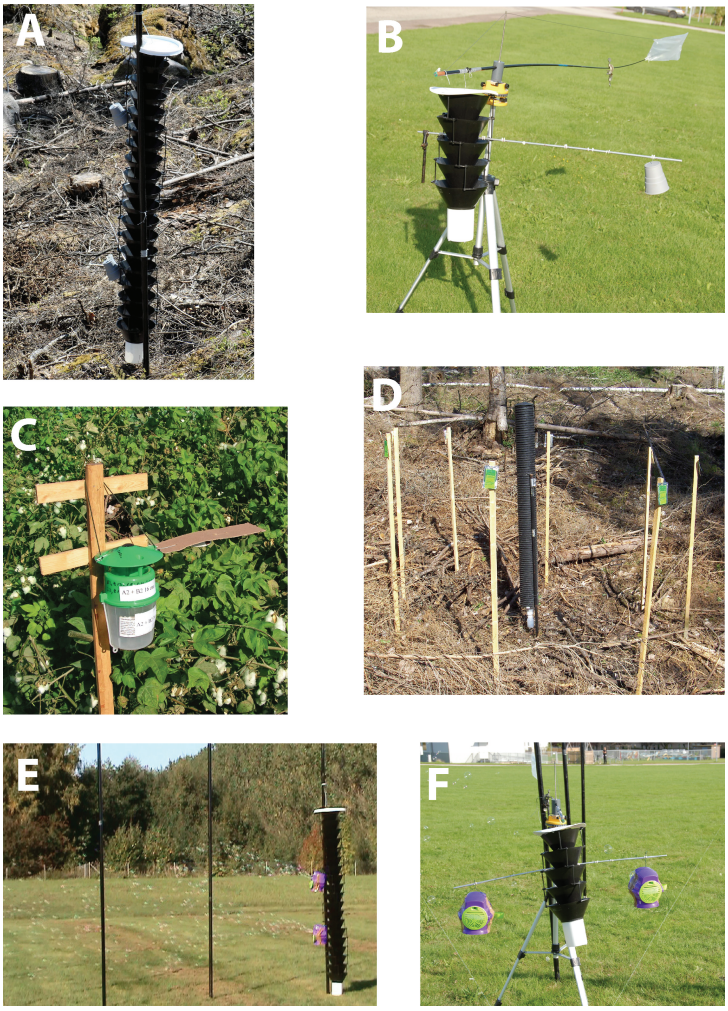

Figure 11. A) Lindgren funnel traps (19-funnel size) were used in the vertical spacing tests with the beetle. Dispensers positioned under grey cups. B) A Lindgren trap (5-funnel size) was attached to a wind vane in the horizontal spacing tests with the beetle to ensure constant distance between plumes. C) Trap type used in spacing tests with *Spodoptera*. Cardboard protected dispenser from sunlight. D) Pipe trap surrounded by eight non-host volatile dispensers (at 1 m distance) in the beetle anti-attractant background tests. E) Soap bubble visualization of vertical plume overlap at the 48 cm spacing distance. Distance between black poles = 1 m. F) Horizontal bubble plume overlap at the 80 cm spacing distance.

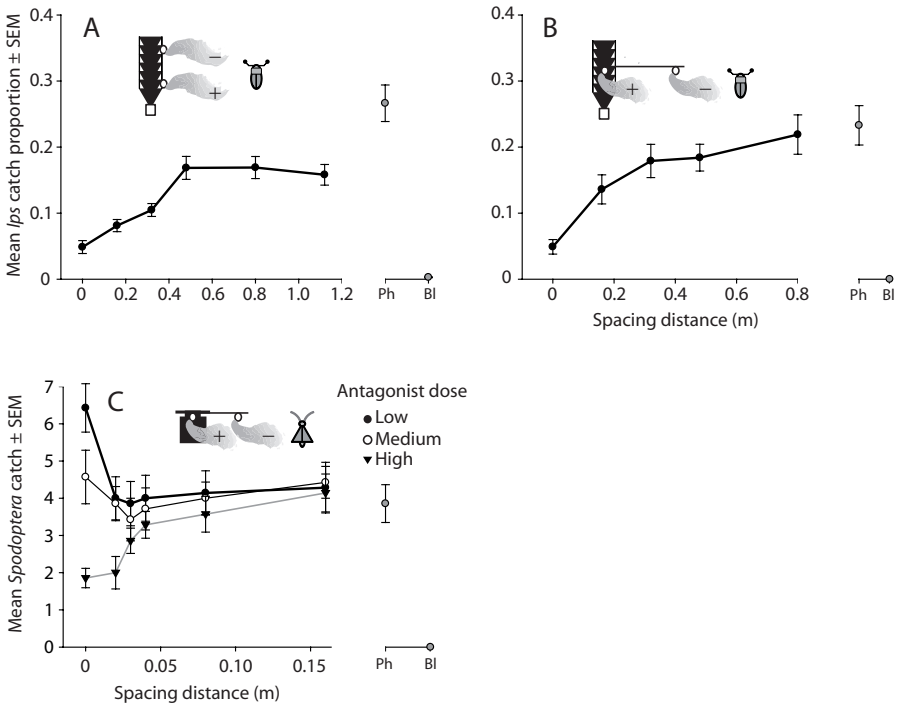

Figure 12. Response of *Ips typographus* to A) vertical ($N = 16$) and B) horizontal ($N = 14$) spacing between the two-component aggregation pheromone and an anti-attractive non-host volatile blend. C) Response of male *Spodoptera littoralis* to horizontal spacing between the two-component sex pheromone and three doses (Low, Medium, High: 2.5, 25, 250 μg, respectively) of a pheromone antagonist (Z9-14Ac; $N = 7$). Only the high dose antagonized pheromone attraction. The lowest dose enhanced pheromone attraction at 0 cm spacing. Right panels in graphs show responses to control treatments: Ph = pheromone only, Bl = blank.

6.3.3 Sex vs. aggregation

The difference in resolution between the beetle and the moth may reflect the size of the natural odor sources (and plumes) they orient to. While a male moth orients towards a single calling female, male and female bark beetles may orient to large tree trunks with hundreds of calling males.

The moth sex pheromone system is highly specialized. A male moth flies towards a female that releases her pheromone to get mated, whereas the bark beetle aggregation pheromone is a signal of mates, food, and oviposition sites. The specialization of the pheromone subsystem in moths is seen both peripherally and centrally. The ORNs for pheromone compounds are housed in specific sensilla, different from the ones that

detect plant odors (Ljungberg *et al.*, 1993), and the processing of pheromone signals occurs in a specialized compartment (the macroglomerular complex, MGC) of the AL (Ochieng *et al.*, 1995). In contrast, the bark beetle groups the *cis*-verbenol pheromone ORN together in the same sensilla as an ORN for the plant compound 1,8-cineole (Papers II-III). In addition, preliminary observations indicate no apparent MGC in the AL.

Coincidence detection of pheromone components and antagonists seems to be absolutely crucial to elicit proper behavioral responses in moths (Fadamiro *et al.*, 1999). The lower sensitivity of the beetle to spatial separation of odor sources might indicate that coincidence detection is of less importance for bark beetles.

6.3.4 Effects of position and dose on *S. littoralis*

In the pheromone/anti-attractant experiment with *Spodoptera*, the antagonist Z9-14Ac inhibited attraction only at the highest dose tested (Fig. 12C). In fact, at the lowest dose it actually increased trap catch, indicating that a low amount of the compound is part of the sex pheromone blend.

In the pheromone component spacing experiment with *Spodoptera*, the position of the major component (inside trap or moved outward) affected trap capture (Fig. 13B-C). With the major component in the center of the trap, more males were attracted to the 16 cm spacing distance than to the 8 cm spacing distance. This was not the case when the minor component was positioned inside the trap (major component moved outwards). In fact, the catch in traps with 16 cm between components and the major component in the center was very similar to the catch in traps with the major component alone. Thus, it seems possible that the moths oriented to the 'best' alternative at the 16 cm spacing distance, but at 8 cm spacing they did not. Similar observations have been made also before (Linn & Gaston, 1981; Lux *et al.*, 1994).

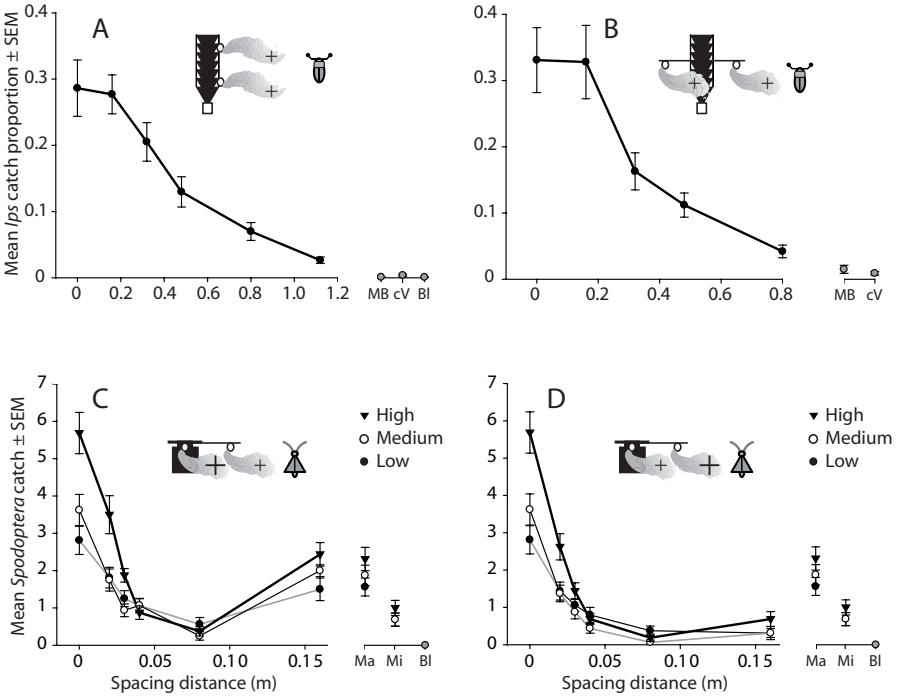

Figure 13. Response of *Ips typographus* to A) vertical, and B) horizontal spacing between pheromone components *cis*-verbenol (cV) and 2-methyl-3-buten-2-ol (MB) ($N = 14$). Response of male *Spodoptera littoralis* to horizontal spacing between three doses (Low, Medium, High: 5, 50, 500 μg, respectively, based on major compound) of the pheromone compounds Z911-14Ac (major component) and Z9E12-14Ac (minor component), with major C) or minor D) component in the center of the trap ($N = 16$). Right panels in graphs show responses to control treatments: Ma = major component, Mi = minor component, Bl = blank.

6.3.5 Long-distance effects of NHV on *I. typographus*

The bark beetle pheromone/NHV spacing experiments indicated clear anti-attractive effects of NHV despite the fact that they were positioned several decimeters away from the pheromone bait. Especially in the vertical test, indications of long-distance effects of NHV were found since the 112 cm spacing treatment caught significantly fewer beetles than the pheromone alone positive control (Fig. 12A).

To further investigate potential effects of NHV at longer distances, pheromone attraction was studied in the presence of a synthetic background of NHV (Paper V). In the first test, eight NHV point sources (2 doses) were positioned in a ring (with either 1, 2, or 3 m radius) around a central pheromone trap (Fig. 11D). In a second test, instead of using eight point sources, we distributed ca 6000 small (ca 3 x 3 mm) NHV impregnated

flakes (releasing the same amount of compounds as eight dispensers) on the ground around a pheromone trap, covering an area with 2 m radius.

With the eight NHV sources, bark beetle attraction was significantly reduced both at the 1 m and 2 m spacing distance (Fig. 14). At the 3 m distance, there was still a tendency for reduced attraction. These distances are in accordance with the "active inhibitory range" of NHV of at least 2 m that was estimated previously (Zhang & Schlyter, 2003). Similar to the eight point sources, the NHV flakes also reduced pheromone attraction.

The pheromone dose in these experiments was comparable to an ongoing mass attack, which is a very strong signal. Thus, it is quite striking that volatiles from non-host plants can inhibit attraction when they are released a few meters away from the pheromone source. This indicates that avoiding not only non-host species, but also non-host habitats, would likely improve bark beetle fitness.

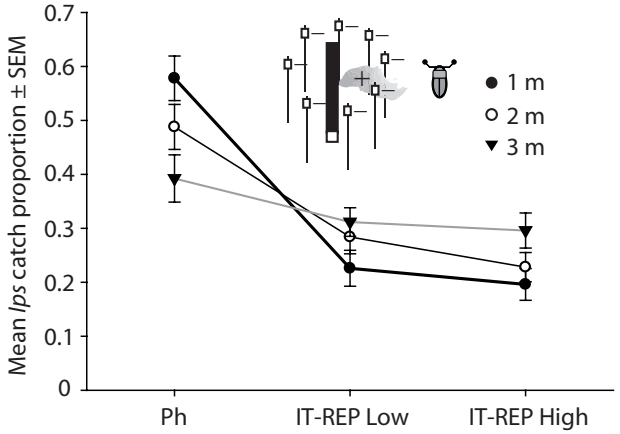

Figure 14. Reduction in *Ips typographus* trap catch by the presence of eight non-host volatile sources (Low dose = 1 dispenser/position, High dose = 3 dispensers/position) at different distances (1, 2, and 3 m) from a central pheromone trap (N = 8-10). Ph = pheromone control treatment, IT-REP = semi-commercial non-host volatile dispenser (Fytofarm Ltd. Slovakia).

7 Applied aspects

A meta-analysis showed that conifer pest insect infestations typically are less common in diversified habitats (Jactel & Brockerhoff, 2007), which in part may be due to the presence of inhibitory NHV. The indication that NHV, from a distance of at least 2 m, can reduce attraction to "the smell of a mass attack" suggests a potential for NHV in forest protection. However, pheromone attraction was not shut down (Fig. 14) so it may be more likely that instead of counteracting ongoing mass attacks, synthetic or natural NHV sources may reduce the risk of spruces being attacked in the first place. Indeed, spruce trees were previously successfully protected by NHV dispensers attached to every second tree, demonstrating a protective effect of ca 2 m (Jakuš *et al.*, 2003). In another recent study, groups of ten standing trees were all protected by 20 NHV dispensers, and bark beetle attacks were diverted to trees >15 m away (Schiebe *et al.*, 2010).

The identification of the bark beetle odorant receptors (Paper I), paves the way for the development of potential novel management strategies in the future. By expressing the ORs in heterologous cell systems, the receptors for the pheromone components and the anti-attractive NHV can be identified. By means of high throughput screening systems, it might then be possible to identify ligands that pharmacologically block the pheromone receptors or hyper-stimulate (Triballeau *et al.*, 2008) receptors for non-host volatiles. If such compounds are identified, they might be dispensed in the forest to disrupt bark beetle pheromone communication and host tree localization.

8 Conclusions and future directions

In this thesis, 40 strong odorant receptor (OR) candidates of *I. typographus* were identified (Paper I). Comparing their sequences with *T. castaneum* ORs indicated an extension of bark beetle OR function. Possibly, the receptors in the bark beetle-specific group detect compounds that are especially relevant for bark beetle ecology. Such compounds could be the spruce-related mono- or sesquiterpenes, or the pheromone compounds from various bark beetle species that *I. typographus* responds to. Indeed, the existence of several olfactory receptor neuron (ORN) classes that are selectively tuned to monoterpenes and bark beetle pheromone compounds suggests that this scenario is not unlikely. However, this speculation needs to be validated experimentally by expressing the ORs in a heterologous cell system and then by screening them for odor responses. De-orphanization of the *Ips* ORs is facilitated by our knowledge of the array of compounds that elicit physiological responses in the ORNs (Paper II).

It was striking that almost one fourth of the strongly responding ORNs were tuned to anti-attractant compounds from non-host plants. This finding likely reflects the ecological and evolutionary significance of NHV. Additionally, the multitude of ORN classes that responded to compounds from conifers implied that detection of host kairomone is important as well. Behaviorally, the spruce compound 1,8-cineole antagonized pheromone attraction in the field (Paper III). There were several ORNs that responded only weakly, or not at all, to any test odorant. By including additional compounds in SSR, such as e.g. conifer-related sesquiterpenes, or by testing natural odor extracts using GC-coupled SSR, several additional odor ligands for these ORNs would likely be identified.

Unlike most other insects, *I. typographus* has sensilla that house pheromone ORNs together with ORNs for plant compounds. In addition, it was found that the response of the ORN for the plant compound 1,8-

cineole inhibited the response of the co-localized pheromone neuron to low doses of pheromone (Paper III). Possibly, the peripheral modulation of pheromone response by the neighboring cell for 1,8-cineole may explain parts of the inhibition of pheromone attraction in the field.

One hypothesis why insect co-localize specific ORNs in the same sensilla is that it allows for improved spatiotemporal resolution of odor stimuli (Fadamiro *et al.*, 1999). To test this hypothesis, a recently initiated experiment will compare the response of *I. typographus* to spacing between pheromone and 1,8-cineole, with the response to spacing between pheromone and verbenone, the latter compound being detected by an ORN that is never co-localized with a pheromone neuron. Predictably, the beetle should be more 'sensitive' to small-scale spacing of the 1,8-cineole as compared to the verbenone. Unfortunately, the collected number of replicates is yet too small for any conclusion to be drawn.

The photoionization detector (PID) measurements of amounts released from stimulus cartridges revealed a very large variation between compounds, solvents, and, in some instances, also successive stimulations with the same cartridge (Paper IV). These results question the traditional way of preparing odor stimuli that is based on the same weight of compounds put on the filter paper. The results also stress the need of consistent handling of stimulus pipettes. Theoretically it should be possible, after measuring vapor amounts of systematically chosen compounds, to produce an algorithm/database that allows for calculation of a corrected dose-on-filter paper to give the same concentration of airborne molecules for different compounds.

The odor source spacing experiments revealed that the spruce bark beetle and the moth *S. littoralis* respond to spacing distances that differ by approximately one order of magnitude (Paper V). The superior resolution of the moth may reflect a principal difference between sex and aggregation pheromone systems. In addition, the difference between species may imply that different central processing mechanisms are operating. To investigate whether the high resolution of the moth is restricted to the sex pheromone system, it would be very interesting to investigate the response to spacing between the pheromone and a plant-derived behavioral antagonist. Unfortunately, no anti-attractive plant volatiles are yet identified for this polyphagous moth.

As the PID does not distinguish between different molecules, it could not be used to measure plume overlap in the field. However, with a portable single sensillum recording device (Van der Pers & Minks, 1993), the sensillum that contains the ORNs for *cis*-verbenol and 1,8-cineole could be used as a biological detector for detailed measurements of plume filament

overlap in the field. Measurements from this sensillum would be highly useful to study if filaments from overlapping plumes are detected coincidently or not. It would also provide some indirect clue if beetles temporally integrate filaments from different plumes to a larger degree than the moth, which could explain the difference in response to spacing in the two species.

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