Odor Perception in three Coleoptera: Molecule, Receptor & Neuron

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Doctoral Thesis
Swedish University of Agricultural Sciences
Alnarp 2010

Acta Universitatis Agriculturae Sueciae
2010:14

Cover: *Pachnoda interrupta* feeding on sorghum
(photo: Jonas M. Bengtsson)

ISSN 1652-6880
ISBN 978-91-576-7491-3

Print: SLU Repro, Alnarp 2010

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Abstract

In this thesis, the sense of olfaction and its links to behavior was studied in the cetoniid chafers *Pachnoda interrupta* and *P. marginata*, and the bark beetle *Ips typographus*. *P. interrupta* is a pest on sorghum in Ethiopia, and *I. typographus* is a spruce pest in the palearctic, and an aim was to uncover new control methods. In *I. typographus*, the olfactory receptors (ORs) that determine response specificity in olfactory receptor neurons (ORNs) were investigated. Phylogenetic analysis showed that *I. typographus* and *Tribolium castaneum* ORs form a Coleoptera-specific subgroup. At the next level of the olfactory system, ORN response to food odorants was compared between *P. interrupta* and *P. marginata*. Both are opportunistic polyphages on fruits and flowers, but are present in disparate habitats. *P. interrupta* is found in savannah, and *P. marginata* in tropical Africa. The two species showed a high degree of overlap in their ORN arrays, indicating that a similar sensory strategy for food search is viable in both habitats. Field trapping with compounds eliciting strong ORN response identified a powerful attractant for *P. interrupta*, 2,3-butane diol. In a *P. interrupta* study, coupled gas chromatographic-electroantennographic detection identified antennally active compounds from sorghum, *Sorghum bicolor*, and another food host, *Abutilon figarianum*. Field tests indicated that single compounds (e.g. methyl salicylate) were more important than mixtures in attraction to these two hosts. Field studies of mating and aggregation in *P. interrupta* showed that unmated females were attractive to males, and to both sexes when combined with food. Female-specific compounds were identified by mass spectrometric comparison of male and female extracts. Field tests of these established that phenylacetaldehyde was highly attractive to both sexes, implying that it is part of the *P. interrupta* pheromone. These findings create novel possibilities for control. Phenylacetaldehyde and attractive food compounds could be used for mass trapping of *P. interrupta*. The identified *I. typographus* ORs make a search for pheromone and repellent receptors possible. Compounds that hyper-excite these receptors could be used for mass trapping or disruption of host search.

Keywords: Pachnoda interrupta, *Pachnoda marginata*, *Ips typographus*, olfaction, electrophysiology, olfactory receptor neurons, olfactory receptor proteins, pheromone, control, mass trapping, monitoring

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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 Jonas M. Bengtsson, Martin N. Andersson, Ewald Grosse-Wilde, Marcus C. Stensmyr, Ylva Hillbur, Fredrik Schlyter, Bill S. Hansson. A Transcriptome Shortcut for Identification of Olfactory Receptor Genes in the Spruce Bark Beetle, *Ips typographus* (manuscript).

II Jonas M. Bengtsson, Hamida Khbaish, Yitbarek Wolde-Hawariat, Andreas Reinecke, Merid Negash, Emiru Seyoum, Bill S. Hansson, Ylva Hillbur, Mattias C. Larsson. Conserved Response to Food Compounds in Olfactory Receptor Neurons of two Scarab Beetles, *Pachnoda interrupta* and *P. marginata* (manuscript).

III Jonas M. Bengtsson, Yitbarek Wolde-Hawariat, Hamida Khbaish, Merid Negash, Bekele Jembere, Emiru Seyoum, Bill S. Hansson, Mattias C. Larsson, Ylva Hillbur (2009). Field Attractants for *Pachnoda interrupta* Selected by Means of GC-EAD and Single Sensillum Screening. *Journal of Chemical Ecology* 35(9), 1063-1076.

IV Jonas M. Bengtsson, Satya Prabhakar Chinta, Yitbarek Wolde-Hawariat, Merid Negash, Emiru Seyoum, Bill S. Hansson, Fredrik Schlyter, Stefan Schulz, Ylva Hillbur. Pheromone-based Mating and Aggregation in the Sorghum Chafer, *Pachnoda interrupta* (submitted).

Abbreviations

GC-EAD	Gas chromatograph-coupled electroantennographic detection
GC-MS	Gas chromatograph-coupled mass spectrometry
OR	Olfactory receptor
ORN	Olfactory receptor neuron
SSR	Single sensillum recording

1 Objectives

The objective was to study the perception of odors at the peripheral level in three Coleoptera: the cetoniid chafers *Pachnoda interrupta* and *P. marginata*, and the spruce bark beetle, *Ips typographus*. The goal was an in-depth understanding of the olfactory function of the antenna, and its response to volatile compounds from hosts and conspecifics, but also to couple this to behavior, and in the longer perspective, to practical control measures for *P. interrupta* and *I. typographus*, both of which are pest insects.

2 Introduction

Olfaction is a sense vital to insects, detecting volatiles relevant to mating, food search, and oviposition (Hartlieb & Anderson, 1999). Out of the varied compounds emitted by odor sources, insects often only detect a handful (Schoonhoven *et al.*, 2005). However, there is a high level of overlap: particular compounds are often emitted by multiple sources, e.g. both plants suitable as food and plants unsuitable as food (Visser, 1986). In some cases, insects have been shown to detect and avoid compounds unique to non-hosts, or to be attracted to compounds unique to hosts (Zhang & Schlyter, 2004; Blight *et al.*, 1995; Nottingham *et al.*, 1991). However, in cases where unique compounds are not found, hosts and non-hosts may differ in the ratio of shared compounds that they emit, and insects have been shown to use this for host choice (Pickett *et al.*, 1998; Bernays & Chapman, 1994). Insects also use volatile compounds for communication, and may emit particular compounds to e.g. attract mates: sex pheromones. In the classical case, an unmated female releases one or more compounds that attract conspecific males (Karlson & Butenandt, 1959). Related species may use similar compounds but attain a higher degree of species specificity in pheromone communication by differing in ratio of compounds emitted, chirality of compounds, or by including one or more unique compounds (Linn & Roelofs, 1995; Löfstedt & van der Pers, 1985). Pheromones are used for a multitude of messages apart from mate attraction, e.g. as an alarm in bees (Boch *et al.*, 1962), and are also involved in aggregation behavior in several species, notably bark beetles (Wood, 1982). The information content of a pheromone may depend on context, and pheromones emitted to attract mates may also attract consexuals in search of resources (Raffa, 2001; Schlyter & Birgersson, 1999), or natural enemies such as predators or parasites (Wertheim *et al.*, 2003).

Mirroring the large role of volatile stimuli in their life history, most insects have a well-developed sense of olfaction. The main olfactory organ is the antenna, but the maxillary and labial palps also play a role (Kwon *et al.*, 2006; de Bruyne *et al.*, 1999; Stange, 1992). On the surface of these organs, morphological structures called sensilla house olfactory receptor neurons (ORNs). The ORNs are the primary units of olfaction, and can generally be divided into distinct classes based on their response spectra. On their surface, ORNs carry proteins that bind odorants, mostly olfactory receptor (OR) proteins (Clyne *et al.*, 1999; Vosshall *et al.*, 1999). The expression of OR proteins has been shown to be necessary and sufficient for odor detection in insects (Hallem *et al.*, 2004). These seven-transmembrane proteins have been found to have an inverted topology compared to olfactory receptor proteins in vertebrates, and have the N-terminus of the protein inside the membrane (Benton *et al.*, 2006). In insect species investigated so far, OR proteins have been found together with an ortholog of the *Drosophila melanogaster* OR83b, and both proteins are necessary for normal olfactory function. ORs are the receptors found in most ORNs, but a subset have other types of receptors, such as ionotropic receptors (IRs) or gustatory receptors (GRs) (Benton *et al.*, 2009; Kwon *et al.*, 2007). GRs are generally associated with the perception of tastants, but phylogenetically, ORs form a subset of GRs. In effect, the GRs thus form a paraphyletic group, named in accordance with function.

Investigations of the mechanics of the sense of olfaction have furthered our understanding of how this sense is used throughout the life-cycle of insects (Hartlieb & Anderson, 1999), but have also enabled new control methods for pest insects (Foster & Harris, 1997). For example, the identification of pheromones, and, in some cases, kairomones (signals benefiting receiver but not sender, e.g. plant compounds attractive to herbivores) have enabled mass trapping of pest insects (Cork *et al.*, 2005; Alpizar *et al.*, 2002; Oehlschlager *et al.*, 2002). Another application is mating disruption, where habitat-wide release of sex pheromones has been used to interfere with mate-finding, leading to fewer matings and hence reduction of damage due to lower numbers of larvae (Ioriatti *et al.*, 2008; Witzgall *et al.*, 2007; Louis *et al.*, 1997). Identified pheromones and plant compounds have also been used to devise highly attractive baits that have been used in trapping for monitoring and prediction of pest insects (Liebhold & Tobin, 2008; Grégoire & Evans, 2004).

2.1 Model species in this study

To further our understanding of the reception of odors at the peripheral level, an investigation of OR genes in *I. typographus*, as well as an in-depth comparative SSR study on *P. interrupta* and *P. marginata*, and a study on detection of host volatiles in *P. interrupta* have been undertaken. The use of pheromone communication in mating and aggregation by *P. interrupta* has also been investigated. These studies have uncovered novel possibilities for control of the two pest species *P. interrupta* and *I. typographus*.

2.1.1 *Pachnoda interrupta*: an Ethiopian pest on sorghum

The sorghum chafer, *P. interrupta* Olivier (Coleoptera: Scarabaeidae: Cetoniinae) is present in the Sahel ecoregion of Africa, in dry savannah habitats. In the early 1990s, *P. interrupta* emerged as a serious pest on *Sorghum bicolor* (L.) Moench (Poaceae) in parts of Ethiopia (Hiwot, 2000). In severely infested areas, up to 70% crop loss was reported (Yitbarek & Hiwot, 2000). The beetle is highly polyphagous, feeding on several types of fruits and flowers (Grunshaw, 1992; Clark & Crowe, 1978; Schmutterer, 1969). *P. interrupta* is univoltine, and spends much of the year aestivating underground as an adult. During the mating season, which generally lasts around two weeks and occurs just before the onset of the rainy period in June-July, males and females emerge and mate. Larvae develop in the topsoil and feed on leaf litter, and the new adults eclose in September-October. Before going into aestivation during the dry perdiod, until the mating season the next year, the new adults feed intensively. This feeding period coincides with sorghum having seeds in the milky stage, and this is the season when the beetles cause damage to the crop. Apart from sorghum, *P. interrupta* has also been recorded as a pest on pearl millet (Sastawa & Lale, 2000).

2.1.2 *Pachnoda marginata*: a non-pest African chafer

The fruit chafer *P. marginata* (Coleoptera: Scarabaeidae: Cetoniinae) is found further south than *P. interrupta*, in habitats with higher rainfall and humidity in tropical parts of equatorial Africa (Rigout, 1989). Like the sorghum chafer, it is highly polyphagous and feeds on a number of fruits and flowers

(Schmutterer, 1969). However, it has not been reported as a pest on agricultural crops. Unlike *P. interrupta*, it is active throughout the year and reproduces continuously.

2.1.3 *Ips typographus*: a palearctic pest on spruce

The spruce bark beetle, *I. typographus* L. (Coleoptera: Curculionidae: Scolytiinae), is an important pest on spruce throughout Europe and northern Asia (Schlyter & Witzell, 2009; Grégoire & Evans, 2004). Larvae and adults feed on xylem of live and dead spruce, and have 2-3 generations during the summer, depending on temperature. The use of pheromone communication enables synchronous attack on single trees by hundreds of beetles. The joint effort lets the beetles overcome the active defenses of the tree, e.g. resin, which would easily overpower single beetles (Wood, 1982).

3 Olfactory receptor proteins in the bark beetle *I. typographus*

To further elucidate the mechanics of olfactory reception in the spruce bark beetle, *I. typographus*, a project to identify genes for olfactory receptor (OR) proteins in this species was started. Previous work has established a strong foundation of knowledge regarding the chemical ecology of this species, with both pheromones and kairomones (repellent and attractive) identified (Erbilgin *et al.*, 2007; Zhang & Schlyter, 2003; Zhang *et al.*, 1999), as well as a good understanding of the sensory physiology at the ORN level (Andersson *et al.*, 2009b). The structure and function of OR genes has been extensively studied especially in *Drosophila melanogaster*, but the only previous study on OR genes in Coleoptera is on the red flour beetle, *Tribolium castaneum*, where putative OR genes have been identified from the genome (Engsontia *et al.*, 2007). Compared to other species, *T. castaneum* has a high number of OR genes, and also has an uncommonly high proportion of genes which have lost their ability to code proteins (pseudogenes). These atypical features make it questionable whether *T. castaneum* is a typical Coleoptera with respect to olfactory receptors.

3.1 A transcriptome shortcut to OR gene identification

Identification of OR genes in insects have generally relied on bioinformatic studies of a genome, where search algorithms have identified genes similar to known OR genes. The lack of sequenced genomes for the vast majority of insect species severely restricts the possibilities for OR gene identification using this method. However, other venues for identifying OR genes exist. As the antenna is the major organ of olfaction, OR genes will be expressed here, and will be present as mRNA. Extractions of RNA from *I. typographus* antennae were performed, and from this a normalized cDNA library was

constructed. By normalization, the frequency of common genes is lowered, e.g. housekeeping genes that are highly active throughout the insect, and rare genes such as OR genes will thus be comparatively more common (Bogdanova *et al.*, 2008). The normalized cDNA library was sequenced and putative OR genes were identified by bioinformatic searches using the tBLASTx and HMM algorithms with the NCBI and PANTHER databases. From a total of 140 candidate sequences, the 39 strongest candidates were selected for further bioinformatics analysis. Their predicted transmembrane domains were highly similar to those of confirmed olfactory receptor proteins in *D. melanogaster* (Fig. 1). Insect ORs are characteristic seven transmembrane proteins with an inverted topography compared to vertebrate ORs, with the N terminus of the protein inside the cell membrane (Benton *et al.*, 2006).

3.2 Phylogeny & function

To evaluate the phylogeny of the putative *I. typographus* ORs, they were compared to *D. melanogaster* and *T. castaneum* ORs. In this analysis, a multiple sequence alignment using the MUSCLE 4.0 software (Edgar, 2004a; Edgar, 2004b) was used. The *I. typographus* ORs were more closely related to OR genes in *T. castaneum* than those of *D. melanogaster*, as is shown by a dendrogram based on the analysis (Fig. 2). The majority of the putative *I. typographus* ORs group together with a subset of the *T. castaneum* ORs, with only a few found in subgroups with both *D. melanogaster* and *T. castaneum* ORs. This could indicate the presence of Coleoptera-specific functional similarity in ORs, even though *I. typographus* and *T. castaneum* are part of two distantly related families (Hunt *et al.*, 2007). Previous studies have established a host of behaviorally active compounds, both pheromones and kairomones, detected by the olfactory sense of *I. typographus* (Zhang *et al.*, 2000; Zhang *et al.*, 1999). Apart from an identified aggregation pheromone (Kim *et al.*, 2005; Faustini *et al.*, 1981), such information is scant for *T. castaneum*. It is thus difficult to speculate regarding the exact nature of the overlap between the species. However, as the most commonly expressed ORs are more likely to be found in the transcriptome, the shared functionality should be of the more common parts of the olfactory system.

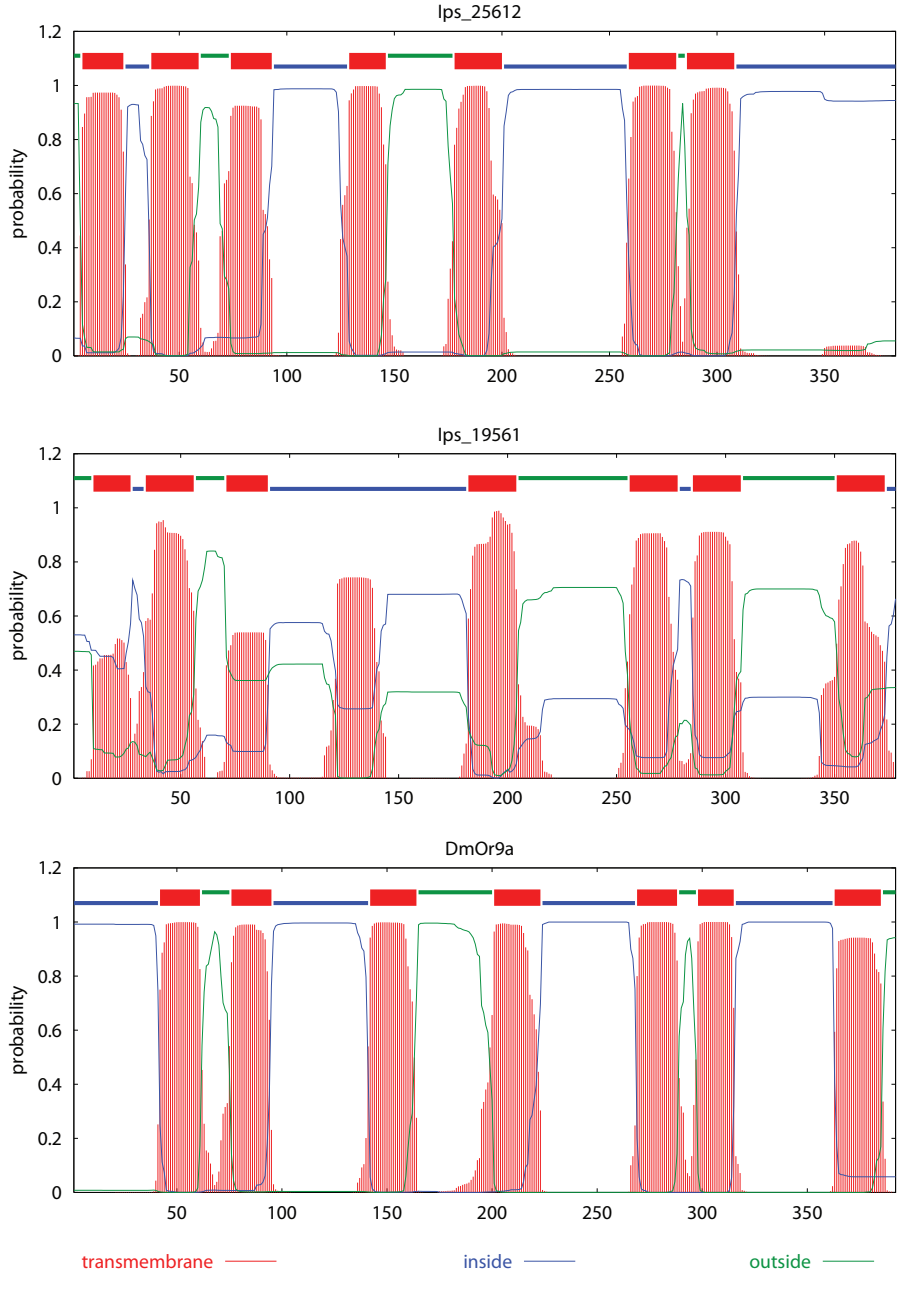

Fig. 1. Predicted transmembrane domains for two putative *I. typographus* OR proteins, compared to those of a previously identified OR protein from *D. melanogaster*.

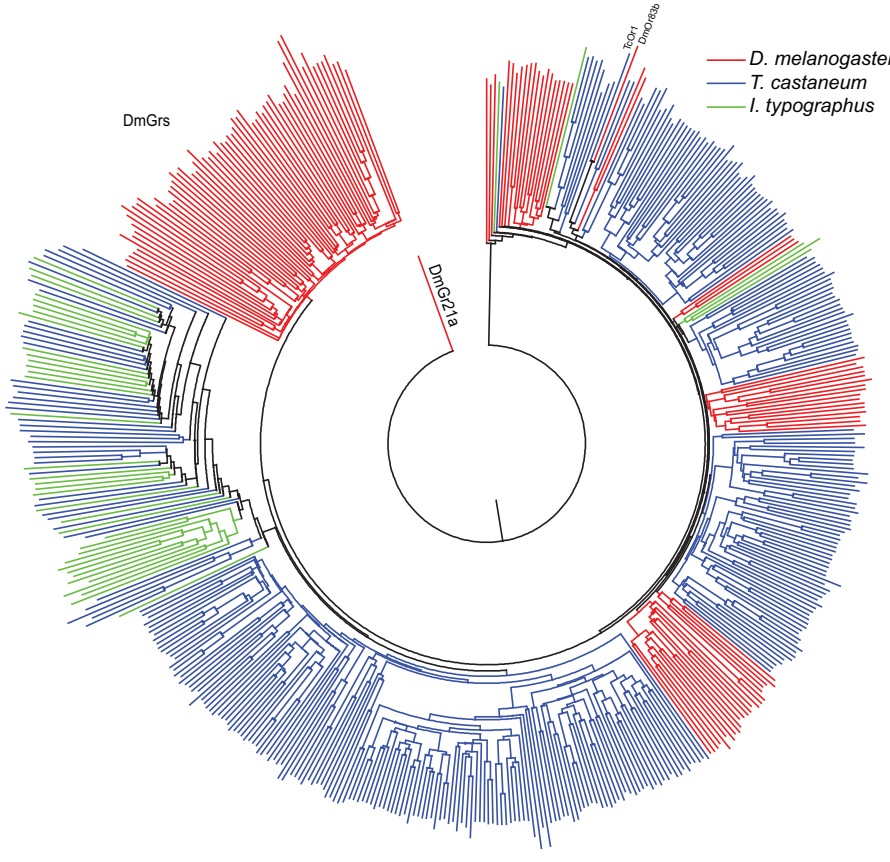

Fig. 2. Dendrogram based on sequence alignment of putative OR and GR genes in *I. typographus*, *D. melanogaster*, and *T. castaneum*.

4 Olfaction in food search in two related polyphagous beetles

In food search, insects use host-specific compounds, as well as specific combinations or ratios of compounds common to hosts and non-hosts (Visser, 1986; Fraenkel, 1959). Polyphagous herbivores face a situation where multiple compounds, combinations and ratios may be found in hosts as well as non-hosts. The mechanisms of host-search in polyphagous insects were investigated by comparing the ORN array in two polyphagous beetles, *P. interrupta* and *P. marginata*, that occur in different habitats (see 2.1.1 and 2.1.2); different food plants are thus available for the two species. In this study, a total of 85 compounds were used. Most of these were related to fruits and flowers, but there were also compounds common to vegetative parts of plants, as well as fermentation and microbial degradation of organic matter, since fermenting fruit is a part of the diet of both species (for a full list of compounds, see Table 1 in paper II; Carasek & Pawliszyn, 2006; Knudsen *et al.*, 2006; Ibáñes *et al.*, 1998; Chatonnet *et al.*, 1992).

The ORNs are located inside the sensilla and form the basic neural unit of odor detection. Recordings from single sensilla (SSR) can thus be used to investigate ORN response to stimulation with volatile compounds. There are multiple morphological types of sensilla, such as hair-like, peg-like and plate-like (Ryan, 2002). *P. interrupta* and *P. marginata* showed a high level of morphological similarity (Fig. 3). The surface of the lamella could be divided into a heterogeneous and a smooth area (Fig. 3A, B). A majority of sensilla were of the placodea (plate) morphological type, with a minority of sensilla of the smooth peg or coeloconic morphologies, found in the heterogeneous area (Fig. 3C, D). The sensilla placodea could be divided into smooth (adjoined to the substrate) and grooved (with a small groove along their edge; Fig. 3E, F).

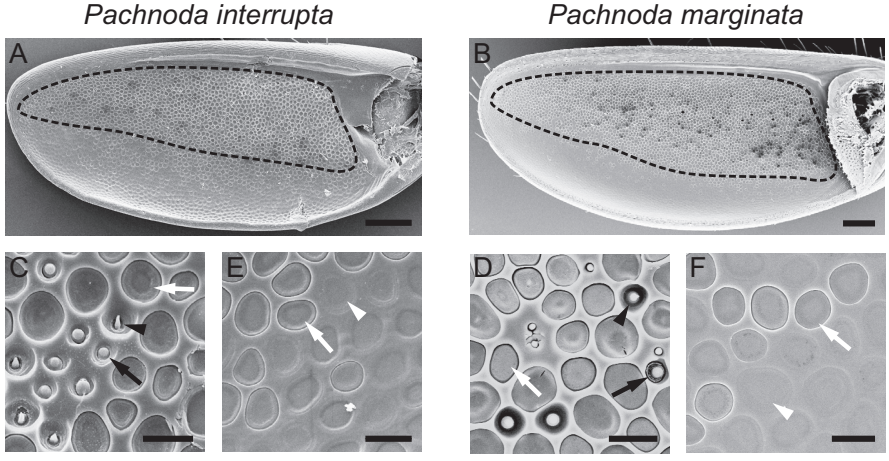

Fig. 3. Scanning electron micrographs of *P. interrupta* and *P. marginata* antennae. A, B surface of lamella, with the heterogeneous area encircled by a dashed line. C, D closeup of heterogeneous area, showing smooth peg (black arrow), coeloconic (black arrowhead) and grooved placodea (white arrow). E, F closeup of border between heterogeneous and smooth areas, showing grooved placodea (white arrow) and smooth placodea (white arrowhead).

Our single sensillum recordings stem from sensilla of the two placodea morphologies, as we only got intermittent contacts from coeloconic and smooth peg sensilla. We recorded from a total of 875 sensilla in both species, and encountered 422 responding ORNs. Sensilla generally contained two neurons, but most responding neurons (88%) were encountered together with a non-responding neuron. There were few persistent patterns of combination when two responding ORNs were encountered together, but on several occasions, neurons responding to a GLV were combined with a neuron responding to an aromatic or ester compound (see e.g. Fig. 4).

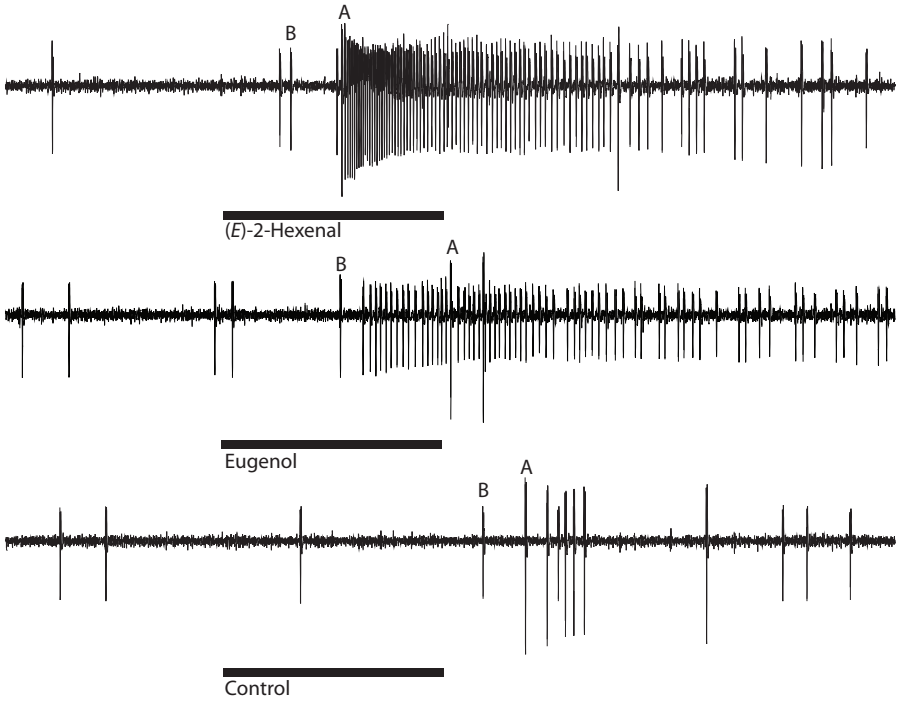

Fig. 4. The two ORNs shown above are present in a single sensillum, and could be distinguished based on their amplitude. The neuron with the larger amplitude (denoted "A") responded to (*E*)-2-hexenal, while the neuron with the smaller amplitude ("B") responded to eugenol. The horizontal bar denotes the stimulation period (0.5 sec).

4.1 Detection of similar food-related compounds

To classify ORNs encountered in single sensillum recordings from both species according to response, cluster analysis was employed (using average linkage and Euclidean distance). To a large extent, the olfactory arrays of *P. interrupta* and *P. marginata* overlapped, and cluster analysis shows that ORNs from both species group together (supplement A of paper II, see also Fig. 5). A large proportion of ORN types in both species detect esters and aromatic compounds typical for fruit and flowers, e.g. eugenol, methyl octanoate and benzaldehyde. However, several of the ORN classes that were most common in both species detect green leaf volatiles (GLVs), e.g. (*E*)-2-hexenal, (*E*)-2-hexenol, (*Z*)-3-hexenol, and (*E*)-3-hexenol, compounds generally associated with vegetative parts of plants. As GLVs are widely present in plants, these compounds could be involved in host search, where

particular combinations or ratios could be more common in hosts or non-hosts. Most ORN classes had narrow response spectra, and were excited by single compounds or small groups of structurally and functionally related compounds. A notable exception is one of the most common classes, which responded to such diverse compounds as butyl butyrate, 3-octanol and sulcatone (Fig. 6).

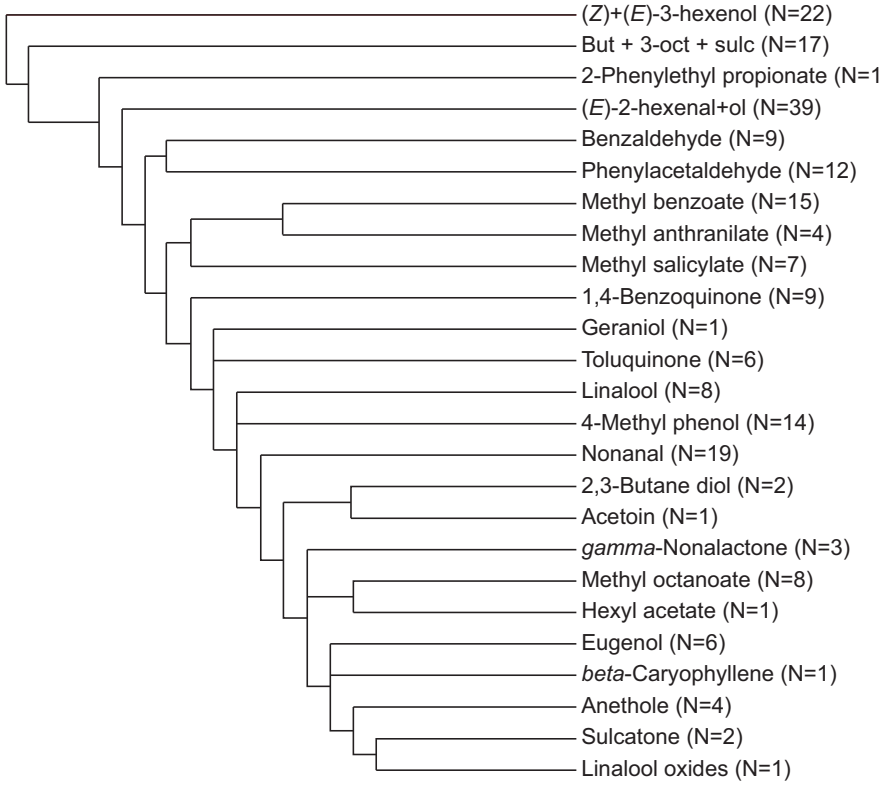

Fig. 5. Condensed dendrogram showing olfactory receptor neuron classes in *P. interrupta* and *P. marginata*.

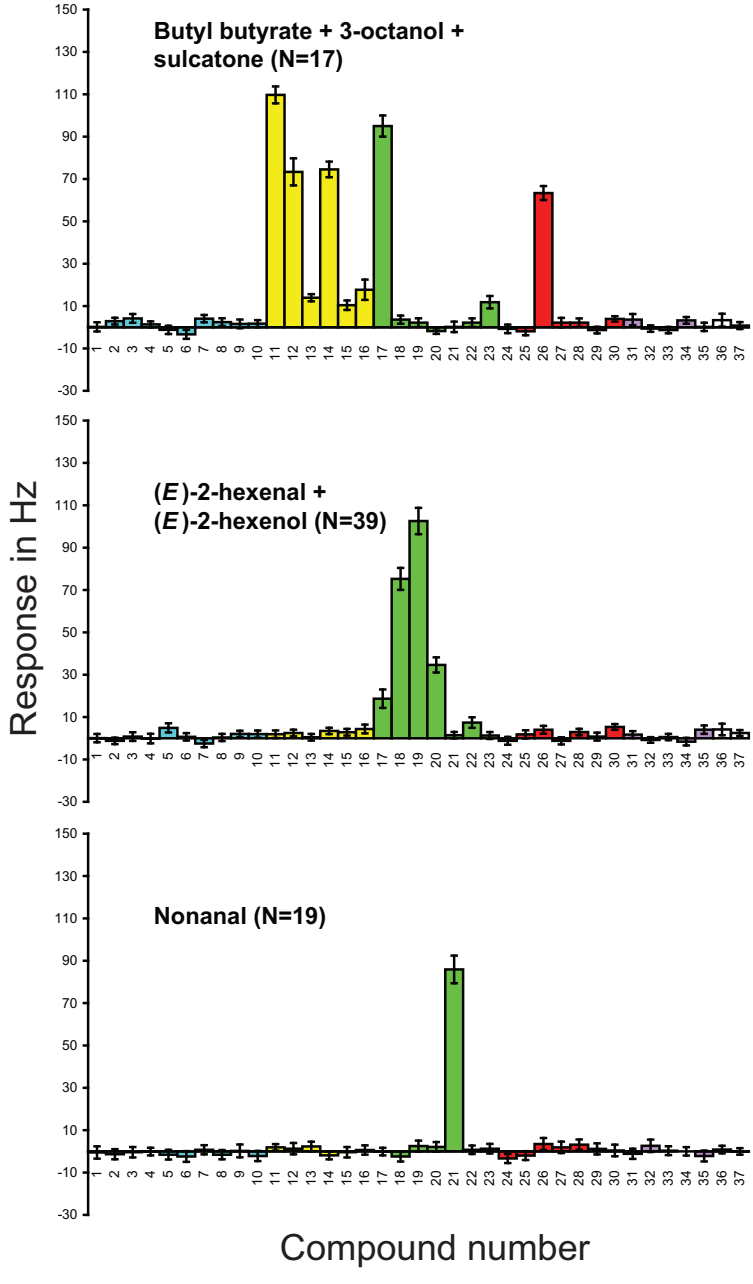

Fig. 6. Average responses to ligands of three ORN classes. The class responding to butyl butyrate had the widest response spectrum encountered, while two other classes (responding to (*E*)-2-hexenal and nonanal, respectively) had more narrow response spectra, similar to those of most ORN classes encountered (for legend, see paper II, Fig. 6C).

4.2 Shifts in response spectra of ORNs

In most cases, ORN classes had identical response spectra in both species, but in some cases, we found indications of shifts in response. In one example of this, ORNs responding strongly to methyl benzoate and fairly strongly to methyl anthranilate were quite common in *P. marginata*, but were not found in *P. interrupta* (Fig. 7). An ORN type responding strongly to methyl anthranilate, but only weakly to methyl benzoate, was however found in *P. interrupta*, but not *P. marginata*. This also correlates with behavior: while both methyl benzoate and methyl anthranilate were attractive to *P. marginata*, only the latter was attractive to *P. interrupta*.

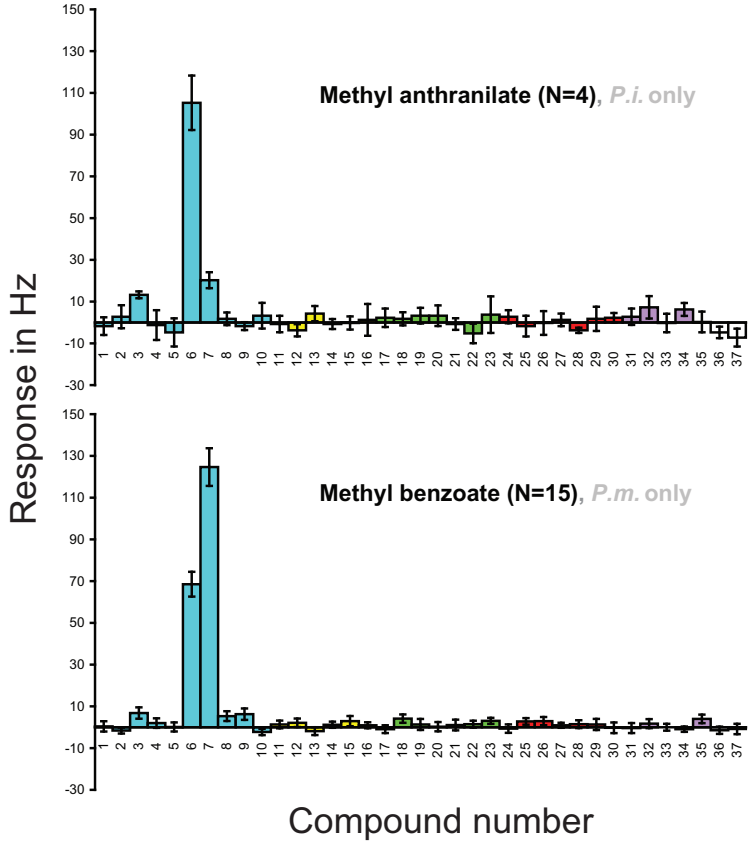

Fig. 7. Average responses to ligands of two olfactory receptor neuron classes unique to either species: methyl anthranilate (only found in *P. interrupta*) and methyl benzoate (only found in *P. marginata*). Stimulus 6, methyl anthranilate; 7, methyl benzoate (for full legend, see paper II, Fig. 6C).

4.3 Different frequencies of ORN classes

Most ORN types were present in similar numbers in both species, but some classes were more common in one species, and the two classes responding to *gamma*-nonalactone and methyl benzoate both appeared to be unique to *P. marginata*. As single compounds appear to be strong attractants for both species, as well as related scarabs (Bengtsson *et al.*, 2009; Wolde-Hawariat *et al.*, 2007; Larsson *et al.*, 2003; Donaldson *et al.*, 1990), it is possible that these ORN classes represent adaptations of *P. marginata* to hosts unique to its habitat.

4.4 Implications of peripheral detection for the olfactory code

Research on vertebrates indicates that multiple ORN classes respond to single compounds (see e.g. the review by Nei *et al.*, 2008), but this scenario does not seem to hold up in insect systems, where compounds generally activate single ORN classes when physiologically relevant doses are used (Andersson *et al.*, 2009b; Hallem & Carlson, 2006; Hansson *et al.*, 1999). ORN classes identified in *P. interrupta* and *P. marginata* show high specificity, and respond to single compounds or narrow groups of functionally and structurally similar compounds (Fig. 6, 7, see also Fig. 6A-C in paper II). Only a minority of active compounds (6%) activated more than a single ORN class. Results from *P. interrupta*, *P. marginata*, as well as other species (ibid) imply that compound identity is determined at the periphery, rather than by integration at higher levels. The number of different compounds a particular insect can detect would thus be roughly equivalent to the number of OR genes expressed in ORNs. However, while response spectra are generally non-overlapping, some ORNs detect more than one compound (Fig. 6, 7, Fig. 6A-C in paper II, see also Hallem & Carlson, 2006). Results from host-search studies imply that higher-level integration is used to evaluate blends of different compounds and specific ratios of compounds (Bruce *et al.*, 2005). Most herbivores seem to distinguish hosts from non-hosts by either unique blends or unique ratios between compounds. Single compounds that are reliable indicators for a host ("token stimuli") appear to be the exception rather than the rule. A lack of token stimuli would make higher-level integration for host identification, rather than for identifying single compounds, adaptive.

5 Selective perception: GC-EAD on host volatiles in *P. interrupta*

Plants release a multitude of volatiles compounds, but only a few of them are detected by insect herbivores and used in host search (Schoonhoven *et al.*, 2005). Entrapment of airborne compounds and subsequent fractionation by gas chromatography, where the insect antenna is used as a an additional detector (GC-EAD), has been used to identify active compounds (Arn *et al.*, 1975). Single sensilla are sometimes employed as detectors (GC-SSR), but more commonly the whole antenna is used (GC-EAD). The former is more sensitive, but the latter has the advantage that it allows screening for any compound detected by the whole antenna, meaning that fewer trials are generally needed (Wibe, 2004).

As *P. interrupta* is a pest on sorghum, a study was conducted to determine how it uses olfaction to locate this host. A polyphagous herbivore like *P. interrupta* has to detect several hosts. In other insects, this has often been accomplished by detecting compounds found in several hosts and non-hosts, and evaluating blends and ratios (Bernays & Chapman, 1994). GC-EAD (Fig. 8) was thus employed to search for active compounds in the volatile blend emitted by *Sorghum bicolor* (L.) Moench (Poaceae). For comparison, GC-EAD was also used with volatiles from another plant that is highly attractive to *P. interrupta*, *Abutilon figarianum* Webb (Malvaceae).

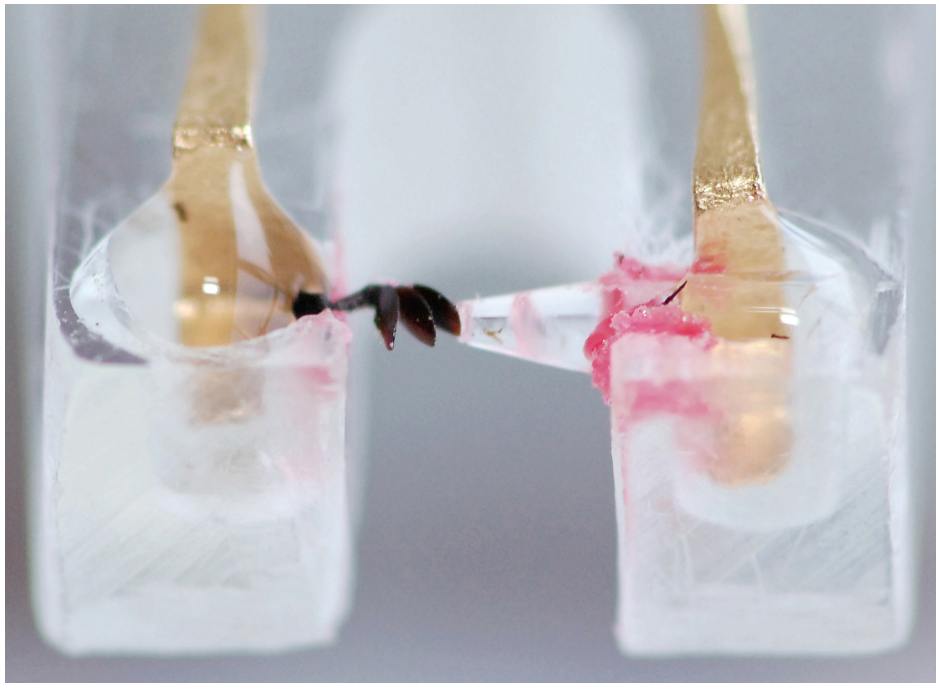

Fig. 8. Mounting of *P. interrupta* antenna for GC-EAD.

5.1 Many compounds released by plants – few detected by insects

By use of GC-EAD on headspace collections, active compounds detected by *P. interrupta* in the volatile blend released by the two hosts were identified by gas chromatograph-coupled mass spectrometry (GC-MS). In sorghum, (*Z*)-3-hexenol, 1-octanol, 1-octen-3-ol, and tridecane were found, and in abutilon (*Z*)-3-hexenol, methyl anthranilate, methyl salicylate, and tetradecane (Fig. 9).

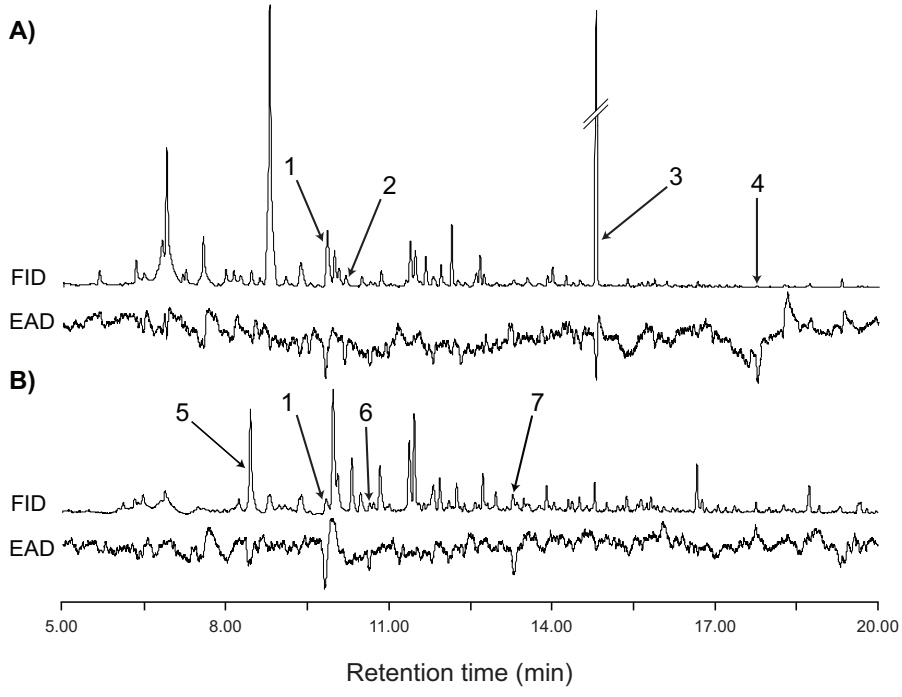

Fig. 9. GC-EAD of host volatiles from A) abutilon and B) sorghum using antennas from *P. interrupta* males. Results from females were similar (not shown). The upper trace in each figure represents the GC-FID (flame ionization detector), and the lower trace the EAD. The active compounds in abutilon were identified as 1) (*Z*)-3-hexenol, 2) tetradecane, 3) methyl salicylate, and 4) methyl anthranilate, and in sorghum 5) tridecane, 1) (*Z*)-3-hexenol, 6) 1-octen-3-ol, and 7) 1-octanol.

We performed trapping tests in the field of sorghum and abutilon blends, where these compounds were present in ratios mimicking those found in headspace collections (Fig. 10). The blends attracted *P. interrupta*, but similar numbers of beetles were caught by traps baited with the most attractive single components (methyl anthranilate, methyl salicylate, tridecane and 1-octanol), and some compounds seemed redundant (especially (*Z*)-3-hexenol and tetradecane).

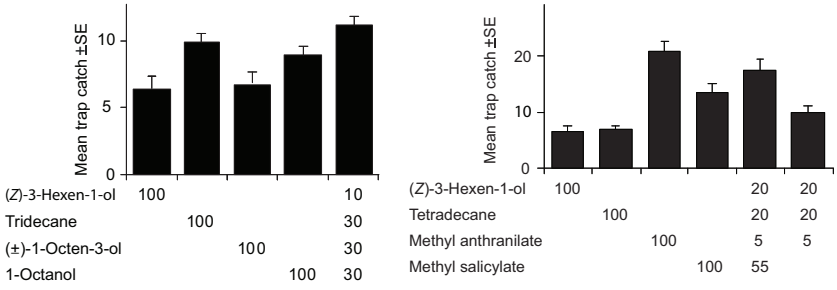

Fig. 10. Field trapping results for single compounds and host mimic blend for sorghum and abutilon.

5.2 Compound blends, ratios, and host search

Single compounds are clearly potent attractants for *P. interrupta*. Single compounds or simple blends of 2-3 compounds have been observed to be highly attractive also to other scarab species (Potter & Held, 2002; Donaldson *et al.*, 1990). For species feeding of fruits and flowers, one possibility is that they overhear stereotyped signals used by the plants to attract symbionts for pollination and fruit (seed) dispersal. As it is adaptive for plants to provide these symbionts with clear and unambiguous signals, it may be difficult for them to avoid that herbivores utilize these signals for host location.

6 Olfaction, aggregation, and mating in *P. interrupta*

During the mating season, which lasts for approximately two weeks and occurs just before the rainy season in June-July, *P. interrupta* have been observed to form aggregations on host plants (Fig. 11). In other insects, aggregations are sometimes mediated by visual stimuli at the landscape level, or volatile stimuli induced in hosts, but pheromones are also commonly involved (Wertheim *et al.*, 2005). We thus performed field experiments to determine if a pheromone was involved in the mating or aggregation behavior of *P. interrupta*.

Fig. 11. *P. interrupta* adults on Acacia *spp*.

6.1 Mating & aggregation behavior: implications for use of pheromones

Field trapping experiments with live beetles as bait were performed during the mating season. To evaluate the influence of host volatiles, live beetles were also combined with a preferred food, banana. Unmated female beetles were attractive to males, but not to females, while mated females, males or males together with females did not attract either sex (Fig. 12). This indicates the presence of a female-emitted sex pheromone attractive to males. Unmated females combined with banana were highly attractive to both sexes, while other combinations of beetles and banana were not more attractive than banana by itself. The aggregations observed during the mating season could thus be caused by attraction to a combination of host volatiles and unmated females.

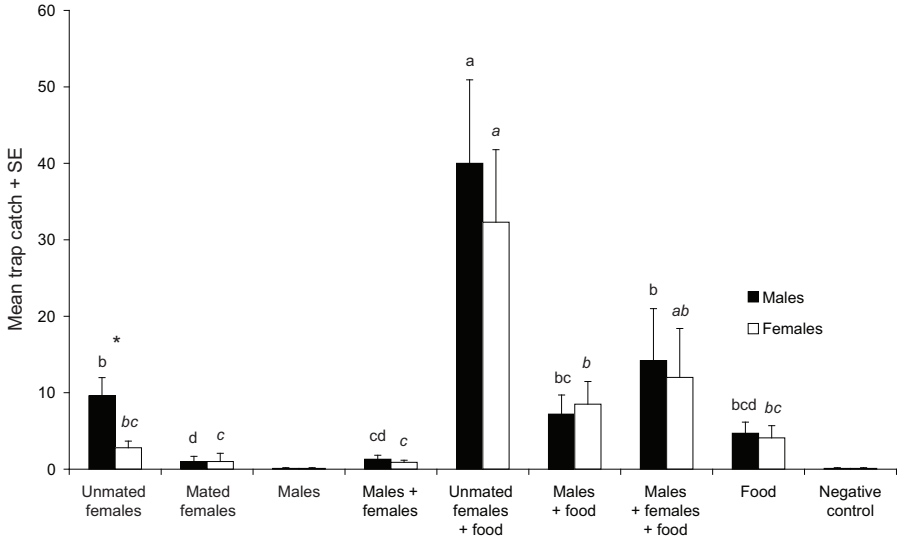

Fig. 12. Average trap catch over five days for different treatments using live beetles combined with a food source, banana. Treatments designated with different letters have a significantly different trap catch at α=0.05 (ANOVA with Tukey's post hoc). Treatments denoted with an asterisk have a significantly different sex ratio to the positive control treatment (not shown here, see Fig. 2 in paper IV).

6.2 Identification of phenylacetaldehyde as a possible pheromone compound

To identify the female-released pheromone, hexane extractions of male and female beetles were performed during the mating season. These extractions were compared by GC-MS to identify female-unique compounds. Nineteen female-unique compounds were tested in the field, singly and in a mixture. Most compounds did not attract more beetles than control (empty) traps, but traps baited with phenylacetaldehyde were strongly attractive to both sexes (Fig. 13). This implies that this female-specific compound is involved in pheromone communication in mating and aggregation in *P. interrupta*.

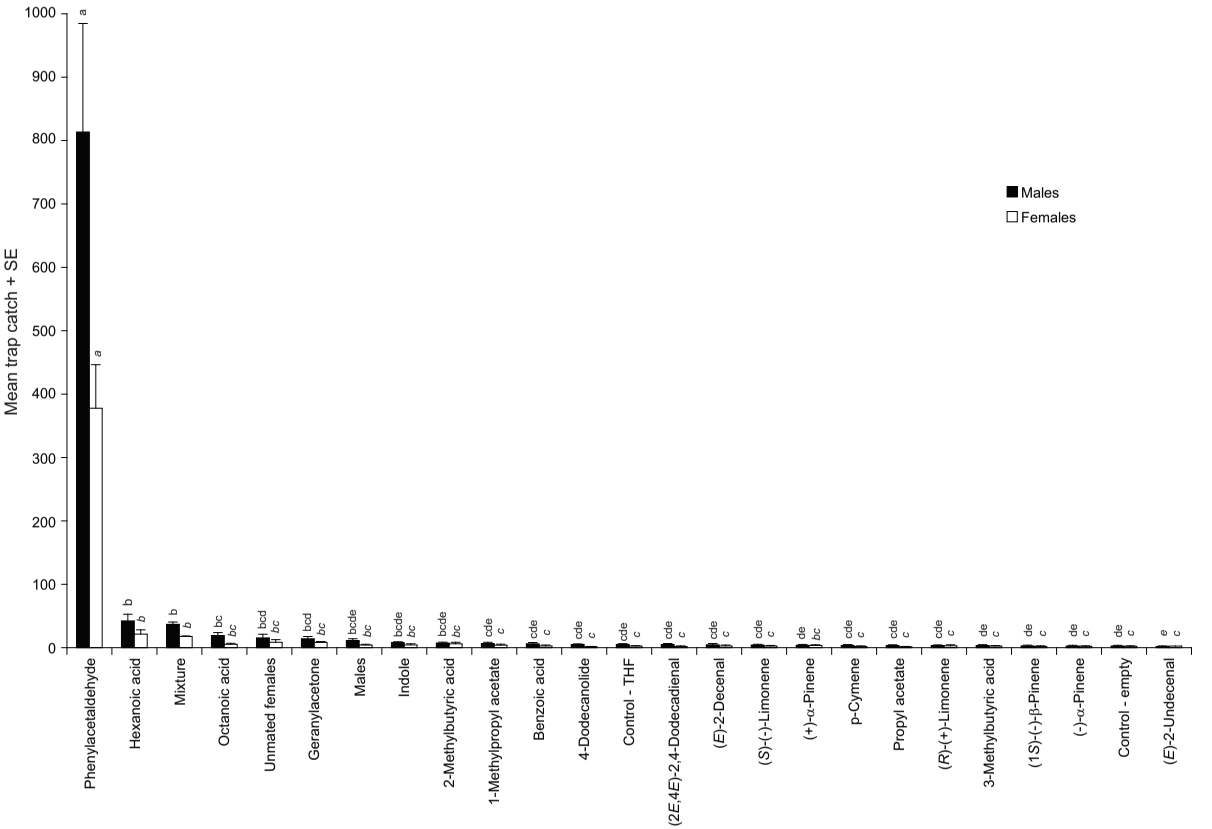

Fig. 13. Average trap catch over three days for female-unique compounds. Treatments designated with different letters have a significantly different trap catch at α=0.05 (ANOVA with Tukey's post hoc).

7 Practical applications for *P. interrupta* and *I. typographus*

The identification of pheromones and kairomones has led to successful control measures in several insect species. Compared to conventional insecticides, control using pheromones and kairomones have several advantages. The doses used are very low, and no residues of lepidopteran pheromones have been fond in crops for which they have been used for control (Tinsworth, 1990). While insects often develop resistance to pesticides, continued use of pheromones generally improves the effect (Weddle *et al.*, 2009; Ioriatti *et al.*, 2008; Varner *et al.*, 2001). Several control methods have been employed, one of which is mating disruption. Here, pheromone dispensers are deployed throughout the area that is treated, and while the exact mechanisms are unclear, this leads to lower mating success in the pest species, and hence less damage to the crop (Jones *et al.*, 2009; Ioriatti *et al.*, 2008; Varner *et al.*, 2001; Louis *et al.*, 1997; Waldner, 1997). Pheromones have also been used in mass trapping, sometimes together with kairomones. The aim of this control method is to trap enough individuals to negatively impact the pest population, and lead to damage decrease. It has been successful in control for a number of pest species, such as the brinjal fruit borer *Leucinodes orgonalis* (Cork *et al.*, 2005; Cork *et al.*, 2003; Cork *et al.*, 2001), the American palm weevil *Rhynchophorus palmarum* (Said *et al.*, 2005; Oehlschlager *et al.*, 2002; Oehlschlager *et al.*, 1992), the coffee white stemborer *Xylotechus quadripes* (Hall *et al.*, 2006), and the northern bark beetle *Ips duplicatus* (Schlyter *et al.*, 2003). Pheromones may also be used for monitoring, either of invasive insect species (Liebhold & Tobin, 2008; El-Sayed *et al.*, 2006) or for species with a short activity period where it can be reached by pesticides (Andersson *et al.*, 2009a; Boddum *et al.*, 2009; Hillbur *et al.*, 2005; Hillbur *et al.*, 1999), or to determine if the species is reaching levels where the damage motivates control efforts (Wall *et al.*, 1987).

7.1 Mass trapping for control of *P. interrupta*

For *P. interrupta*, where adults are the damaging stage, trapping may be a suitable control method. Mass trapping with combined pheromone and kairomone baits has been efficiently employed to reduce pest populations to levels below those where they are problematic (El-Sayed *et al.*, 2006; Alpizar *et al.*, 2002; Oehlschlager *et al.*, 2002). Our studies have led to the identification of a highly attractive putative pheromone, phenylacetaldehyde (Fig. 13). Field testing of plant compounds selected by GC-EAD (Fig. 10) and SSR (Fig. 14) has also led to the identification of several highly attractive compounds. This opens up for further testing of mass trapping for control of *P. interrupta*.

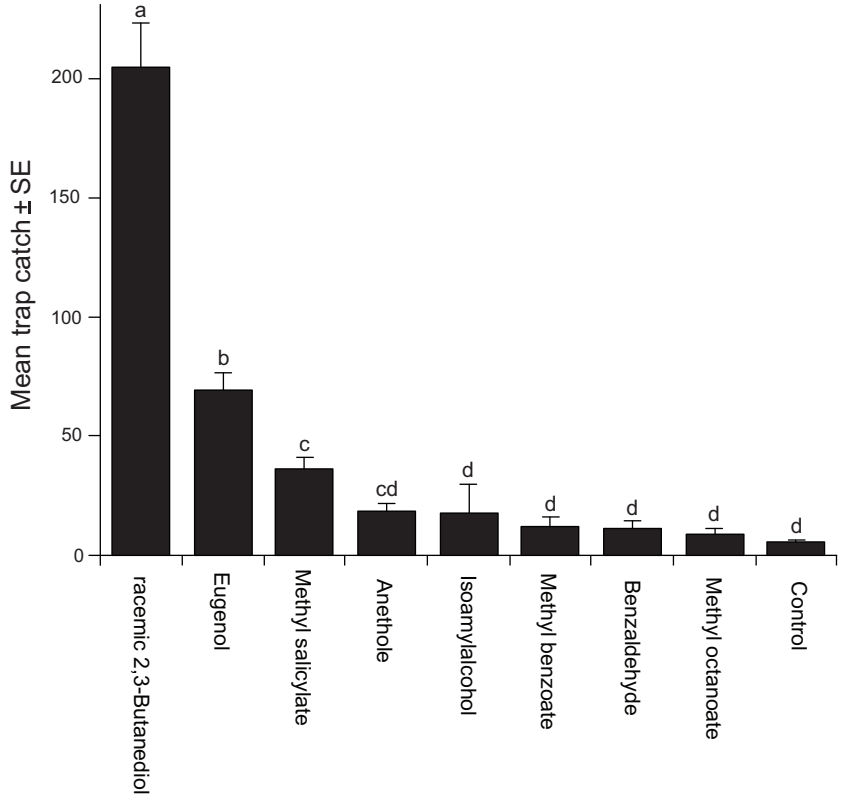

Fig. 14. Average trap catch over five days with food-related compounds selected based on SSR results, identifying a new attractant, 2,3-butane diol, which outperforms the previous best attractants, methyl salicylate and eugenol. Treatments denoted with different letters have a significantly different trap catch at α=0.05 (ANOVA with Tukeys post-hoc).

7.2 Monitoring for prediction of outbreaks for *P. interrupta*

Detection of pest insects by pheromone trapping is an important tool in making decisions for control, either by conventional pesticide treatment or by some form of semiochemical-based method (Grégoire & Evans, 2004). Monitoring of *P. interrupta* during the mating season could give an indication of the population size of the next generation that will appear during the fall, which is the one doing damage to sorghum (Hiwot, 2000). Despite the general kairomone nature of the compounds used as lures, *P. interrupta* were caught with high specificity; the percentage of side catch was low (less than 1%) for compounds such as 2,3-butane diol, eugenol and methyl salicylate (total catch shown in Fig. 14, side catch not shown). This could possibly be due to high population levels of *P. interrupta*, but similar levels of specificity have been achieved at different sites and years, where catch of sorghum chafers have been tenfold less (comparing the same compounds on the same dispensers; data not shown). The trap design (Japanese beetle trap) in combination with the compounds used thus seems to achieve a high level of specificity even at conditions indicating low populations of *P. interrupta*.

7.3 Inhibitors or excitors for receptors in *I. typographus*

The identification of OR genes in *I. typographus* makes it possible to search for compounds that pharmacologically block or hyper-excite these receptors. Compounds that hyper-excite receptors detecting repellents, as well as compounds blocking pheromone receptors and thus make the beetles anosmic to their own pheromone, have novel potential in pest management. Compounds that interfere with pheromone reception could possibly be applied with a methodology similar to that of mating disruption, and could be efficient both towards species using a sex pheromone system, as well as bark beetle species that rely on an aggregation pheromone to succeed in overcoming the active defenses of conifers by simultaneous attack. As of yet, research on repellents for control is scant, but application of material from a non-host (deciduous) tree has been shown to reduce attack frequency on pine by pine processionary moth, *Thaumetopoea pityocampa* (Jactel *et al.*, 2010; Schlyter & Jactel, 2006). Studies on bark beetles indicate that while repellents may not be able to give full protection during high population levels, they lead to damage mitigation (Blazenec *et al.*, 2007; Jakuš *et al.*, 2003).

8 Conclusions

Our studies of olfaction at the peripheral level have shown that there appears to be a Coleoptera-specific OR gene expansion, possibly reflecting an overlap in the sense of olfaction within the Coleoptera. This could indicate that while adaption of olfaction at the peripheral level is useful, often a different higher-level processing of the same compounds can be used as well. The large overlap in ORN classes between *P. interupta* and *P. marginata* suggests that a similar sense of olfaction is efficient for host search in diverse habitats, as long as no drastic shifts in diet are undertaken. Furthermore, understanding the olfactory periphery makes it possible to choose non-redundant compounds for field-testing. By choosing compounds that activate unique ORN types, the set of compounds will activate a large fraction of the ORN array, increasing the likelihood of identifying attractants. Higher-level processing of pheromone and kairomone olfactory stimuli is likely to underlie the aggregation behavior in *P. interrupta*, as very strong attraction of both sexes is observed to the combination of host volatiles and pheromone emitted by unmated females. The identification of pheromone and kairomone compounds opens for new control measures, such as monitoring or mass trapping.

9 Acknowledgements

First of all, I would like to thank my family, without whose support my PhD would never have been possible. Ylva has been a fantastic supervisor, even though we haven't always agreed on everything (especially not regarding the minimum amount of field work necessary). Mattias has been my single cell guru and has also given great feedback on my manuscripts, as has Fredrik, who's also been a great help in thinking up field experiments, and devising statistics to interpret them. Bill has given lots of good, solid advice on science and writing, and Emiru & Bekele have been instrumental in getting the field experiments off the ground. Stefan & Satya did a fantastic job analysing a huge number of beetle extracts for the pheromone identification, and it wouldn't have been possible without them. I've been fortunate to work together with some very dedicated and talented people, and without Yitbarek and Merid I don't think the field experiments would have been possible. In single cell, I've been sharing successes as well as the long, slow haul with Hamida, and Martin, Ewald and Liwen have been adamant in the bark beetle OR gene project. I'd also like to thank the gang at Alnarp - it's been great working together with you! Special thanks go out to the local guys in Rasa and Embuay Bad, Ethiopia, for great work in helping set up the field experiments, and Gitachew for being the best driver a field scientist could wish for. Last but not least, I'd like to thank all my friends for cheering me on, and putting up with numerous e-mails and stories about the hardships of field work in Ethiopia...

This work has mainly been financed by a SIDA/Sarec grant. Additional support has been provided by the IC-E^3 programme at SLU, the Addis Ababa University, and the Max Planck Institute for Chemical Ecology. The field studies have received extra resources from the Lars Hierta foundation and Ångpanneföreningen.

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