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Functional characterisation of olfactory receptor neurons in the cabbage moth, *Mamestra brassicae* L. (Lepidoptera, Noctuidae).

Gas chromatography linked to single cell recordings and mass spectrometry.

Doctoral Thesis

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Papers included in the thesis

This thesis is based on the following papers that will be referred to by these Roman numerals:

I. Ulland S., Ian E., Borg-Karlson A.-K. and Mustaparta H. (2007) Plant Volatiles Activating Specific Olfactory Receptor Neurons of the Cabbage Moth *Mamestra brassicae* L. (Lepidoptera, Noctuidae). Submitted, Chemical Senses

II. Ulland S., Ian E., Borg-Karlson A.-K. and Mustaparta H. (2006) Discrimination between Enantiomers of Linalool by Olfactory Receptor Neurons in the Cabbage Moth *Mamestra brassicae* (L.). Chemical Senses 31: 325-334

III. Ulland S., Ian E., Mozuraitis R., Borg-Karlson A.-K., Meadow R. and Mustaparta H. (2007) Methyl salicylate, identified as primary odorant of a specific receptor neuron type, inhibits oviposition by the moth *Mamestra brassicae* L. (Lepidoptera, Noctuidae). Chemical Senses, under revision.

Introduction

Most of us are well aware of the many odours surrounding us in nature and enjoy the pleasant smell of flowers or culinary herbs. In evolutionary context the sense of smell is considered as the oldest of our senses and is involved in the search for and intake of food, in reproductive and social behaviour, interspecific communication etc. Most people may not be aware of the important role of olfaction in animals like the insects. Whereas the perception of a flower fragrance in humans may be limited to “the plant smells good”, herbivore insects are able to smell not only the species but also the physiological state of the plant, being indispensable for their survival. In contrast to visual and audio stimuli that are defined by one feature, the wavelength, olfactory stimuli are characterized by several interactive features of the molecule. Thus, screening volatiles on sensory cells in order to find the most effective odorant among the enormous number in nature does not seem to be a promising approach. One of the challenges in mechanistic olfactory research is to identify the biologically relevant odorants detected by the receptor neurons (RNs) in the various model organisms. Even though the gross anatomy of the olfactory system in vertebrates and insects appear very different, like the vertebrate noses and insect sensilla, striking similarities exists as concerns some basic structures and functional principles. For instance, the sensory cells of the olfactory epithelium in the vertebrate nose are bipolar neurons with cilia embedded in mucus and an axon projecting in one of the glomeruli of the olfactory bulb, the primary olfactory centre in the brain. This corresponds to the bipolar RNs in insect olfactory sensilla, having dendrite branches embedded in the receptor lymph and an axon projecting in glomeruli of the antennal lobe, the insect primary olfactory centre. Because the insect olfactory system, including the RNs on the antennae as well as the brain, is relatively easily accessible for electrophysiological studies, insects are suitable model organisms for studying olfactory mechanisms underlying behavioural responses.

Plant odorants

Plants release numerous volatiles that are important in interactions with other organisms, like herbivorous vertebrates and insects, predators and parasites of insects, microorganims and even neighbouring plants. These volatiles are products of biosynthetic pathways, constituting the secondary metabolism of the plants, which is considered to be maintained for defence or advantageous interspecific interactions. The primary metabolism is essential for plant growth and development and the products are termed "primary metabolites" (Hartmann, 1996). Some compounds may act both as a primary and a secondary metabolite. The secondary compounds were first described as by-products of the plant primary metabolism (Sachs, 1873) and considered as metabolic wastes and de-toxification products (Haslam, 1986; Luckner, 1990). Fraenkel (1959) suggested a defensive role of these secondary compounds against herbivory. Later, Ehrlich and Raven (1964) proposed that the plant produced secondary compounds were evolved in a co-evolutionary arms race of plant-defences and herbivore responses. Today, plant produced secondary compounds are considered to be an essential part of the plant's biochemical equipment to cope with factors such as herbivore and pathogen attack (Paré and Tumlinson, 1999; Gouinguené and Turlings, 2002).

Plants are known to emit more than 1000 different volatile compounds (Dudareva *et al.*, 2004). Some of them may act directly on the attacking organisms, for instance deterring oviposition by lepidopteran herbivores (De Moraes *et al.*, 2001; Kessler and Baldwin, 2001). Others may act indirectly, by attracting natural enemies of the herbivores (Turlings *et al.*, 1990). Plant defence can be either "constitutive" or "induced"; constitutive defences meaning that the compounds are continuously produced, stored in specialized structures, and released upon attack. Induced defences on the other hand is triggered by herbivore or pathogen attack (Paré and Tumlinson, 1999; Gouinguené and Turlings, 2002). The advantage of induced defence is a low physiological cost, i.e. production of volatiles occurring only during the

attack (Gershenzon, 1994). Absence of an attacking organism does not necessarily mean that the compounds are not produced, but rather that the production is low and then increases upon attack.

At least four biosynthetic pathways have been implicated in the production of insect-induced plant volatiles (Paré and Tumlinson, 1999). Isopropenoid precursors, such as isopentenyl pyrophosphate (IPP), are derived from the mevalonate and alternative IPP pathways. These precursors serve as substrates for sesquiterpenes, monoterpenes and derived homo-terpenes. “Green leaf volatiles” are derived from the fatty acid/lipoxygenase pathway, mediated by oxidation of membrane-derived fatty acids. Aromatic compounds, for example indole and methyl salicylate (MeS), are products of the shikimic acid/tryptophan pathway (Dudareva *et al.*, 2004; De Moraes *et al.*, 2004). Interestingly, recent studies have demonstrated that plants can emit specific volatile blends that differ depending on the attacking species, even closely related herbivore species (De Moraes *et al.*, 1998; Arimura *et al.*, 2004). Diurnal changes of emitted volatiles have also been shown in tobacco plants (*Nicotiana tabacum*), releasing some compounds exclusively at night, which repels nocturnal female moths (*Heliothis virescens*) (De Moraes *et al.*, 2001). Induction and release of volatile compounds can also be triggered by abiotic factors, such as UV radiation, ozone and temperature (Johnson *et al.*, 1999; Pichersky and Gershenzon, 2002; De Moraes *et al.*, 2004). Altogether, the vast amounts of relevant and non-relevant volatile components released by plants constitute a major challenge for insect species in their search for a suitable host plant. The challenge is met by the use of an extremely sensitive and specialised olfactory system.

Anatomy of the insect olfactory system

Olfactory sensilla

The olfactory organs (sensilla) of insects are usually located on the antennae. Many sensilla have an outer hair formed cuticular structure and contain one or more RNs surrounded by three accessory cells (Schneider, 1964; Schneider and Steinbrecht, 1968). Inside the hair is a lumen filled with receptor lymph that surrounds the dendrites of the olfactory RNs. Numerous pores perforating the hair wall allow air-borne molecules to enter the hair lumen of the sensilla and interact with the membrane receptor proteins of the dendrites (Steinbrecht, 1999). According to external as well as internal anatomy, the olfactory sensilla are classified into different types, like *sensilla trichodea* and *sensilla basiconica* (Ernst, 1969; Keil, 1984; Steinbrecht, 1992; Keil *et al.*, 2001). Typical differences between these two sensilla types are relatively long hairs and thick cuticular wall with fever pores and dendrite branches of *s. trichodea* as compared to the short hairs and extensively perforated thin walls of *s. basiconica* with large numbers of dendrite branches in the lumen. In lepidoteran species, single sensilla of the two types contain 1-5 RNs.

In lepidoptera, sexually dimorphic antennae appear in many species, like in the silk moths where the males have featherlike antennae with sex specific *s. trichodea* (Steinbrecht, 1999). In heliothine moths, the male specific *s. trichodea* type I mediates pheromone information whereas *s. trichodea* type II and *s. basiconica* are present in both sexes mediating plant odour information (Almaas and Mustaparta, 1990; Almaas and Mustaparta, 1991; Koh *et al.*, 1995; Færavaag, 1999). In addition, *s. trichodea* type II is involved in pheromone detection in the males (Almaas and Mustaparta, 1991). Like in heliothine moths, the antenna of *M. brassicae* is filiform, segmented and tapered. The 10 mm long antenna consists of 72 segments, each showing similar organisation by the dorsal side containing two rows of scales, and the ventral side covered with olfactory sensillae. Renou and Lucas (1994) describe two

groups of *s. trichodea*, the lateral and the medial. The lateral sensilla, only present in males, have relatively long hairs (60-193 μm) that are hooked and settled in four to five parallel rows on each segment. The medial hairs (35-55 μm) present in both females and males are distributed on the whole surface (Figure 1). The total number of olfactory hairs is estimated to approximately 10 000 on each antenna, about 82 % located on the proximal 42 segments (Renou and Lucas, 1994). In general, the terminology of olfactory sensilla has been inconsistent among different species of lepidoptera. For instance the lateral hairs are in *Mamestra configurata* termed as *s. trichodea* type III, whereas the medial hairs are termed *s. trichodea* type I and II (Liu and Liu, 1984). In the study of *M. brassicae* by Renou and Lucas (1994), the focus has been on the pheromone sensitive trichoid sensilla in males whereas sensilla responsible for plant odours reception have not been studied.

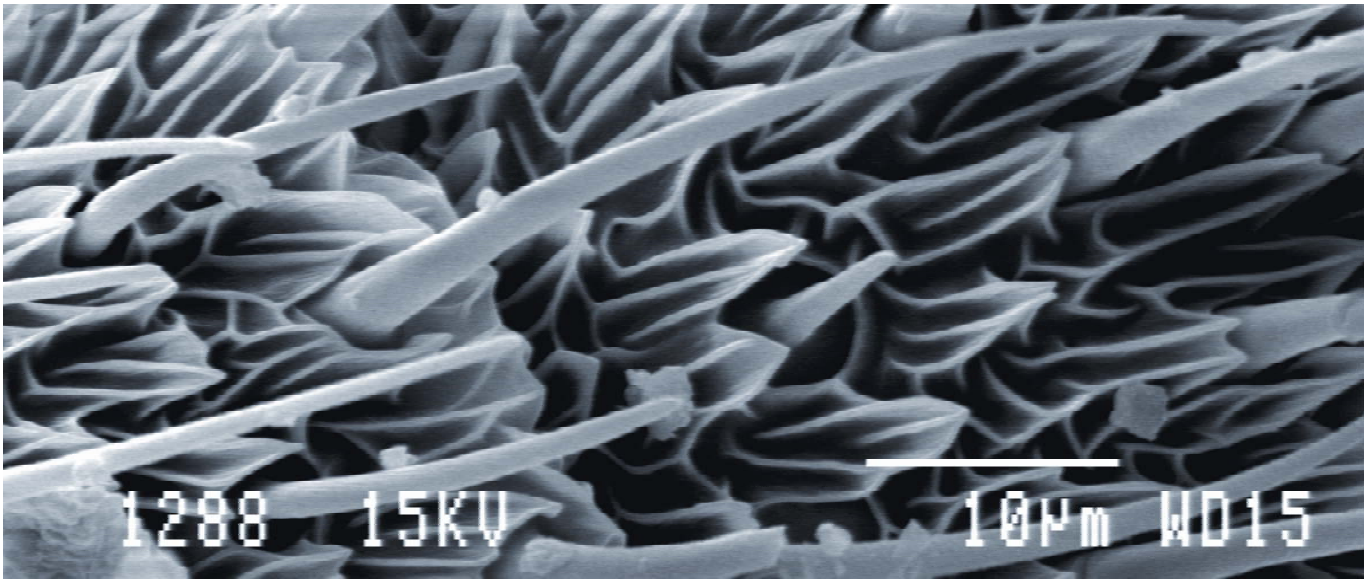

Figure 1. Olfactory sensory hairs located on the antennae of female *M. brassicae*.

Central olfactory pathways

The axons of the primary RNs form the antennal nerve that project directly in the antennal lobe, the primary olfactory centre of the insect brain (Homberg *et al.*, 1989; Berg *et al.*, 1998). The bilateral deutocerebrum is divided into two distinct regions; the antennal lobe (AL) and

the antennal mechanosensory and motor centre (AMMC) in lepidoptera. Whereas the AL receives input from the olfactory RNs, the AMMC receive information from the antennal mechanosensory neurons. In the AL, the olfactory information is transmitted from the RNs to the central neurons in synapses that are condensed in particular neuropil areas, the glomeruli (Boeckh and Tolbert, 1993). Two major morphological types of AL neurons, the local interneurons and the projection neurons, innervating the glomeruli, receive and process the olfactory information. The local interneurons, with extensive branches in almost all glomeruli, are important for interglomerular inhibition, enhancing the contrast between activated and non-activated glomeruli (Sachse and Galizia, 2002). They may also play an important role in creating synchronisation of firing of the projection neurons, as shown particularly in *Locusta migratoria* (Laurent and Naraghi, 1994). The projection neurons innervating single or several glomeruli, have axons that follow one of three major tracts to higher olfactory neuropil areas of protocerebrum (Homberg *et al.*, 1988; Flanagan and Mercer, 1989; Christensen *et al.*, 1993; Rø *et al.*, 2007). These tracts, named the inner-, outer- and the middle-antenno cerebral tract in lepidoptera, have been recognised in all insect species studied (Homberg *et al.*, 1988; Rø *et al.*, 2007; Schachtner *et al.*, 2005) The two major olfactory protocerebral areas are the mushroom bodies and the lateral horn. Whereas the mushrom bodies are attributed an important role in olfactory memory and learning, the lateral horn is a premotoric area considered important for a fast but coarse odour analysis (Menzel, 2001; Heisenberg, 2003; Strausfeld, 1976). Mushroom body output neurons make connections to the lateral protocerebrum (Rybak and Menzel, 1998; Mauelshagen, 1993). The lateral protocerebrum and accessory lobes are considered as the prominent output areas of the brain, from which descending fibres projects to motor neurons in the suboesophageal ganglia (SOG) and the thoracic ganglia.

Peripheral events

The odorants entering the hair lumen through the pores are lipophilic and must pass through the aquatic sensillum lymph before interacting with the receptor proteins. The mechanism behind the "transport" of the odorants are not fully understood and a matter of an ongoing discussion. Small proteins, the odorant binding proteins (OBPs), are believed to have the function of transporting olfactory molecules through the receptor lymph (Vogt and Riddiford, 1981; Prestwich *et al.*, 1995; Steinbrecht, 1998). The presence of these proteins is demonstrated in several insect species (Vogt *et al.*, 1999; Nagnan-Le-Meillour *et al.*, 2004). A vide variety of hypotheses have suggested other functions of the OBPs (Rützler and Zwiebel, 2005; Leal, 2001; Kaissling, 1998; Leal *et al.*, 2005). These hypotheses include removal and inactivation of the odorants, concentration of odorants in the receptor lymph by acting as filters to screen out subsets of odorants, and finally transportation of the odorants through the lymph to the olfactory receptors, possibly acting as co-initiators of the signal transduction process or releasing the odorants at the charged membrane. A recent study has shown that the pheromone binding protein in heliothine moths sharpen the tuning of the RN to the primary odorant (pheromone component) (Große-Wilde *et al.*, 2007). Activation of the receptor proteins by binding of the ligand (odorant molecule) leads to an intracellular G-protein mediated cascade reaction, which produces the second messenger inositol 1,4,5-triphosphate (IP_3) or adenosine 3', 5'-monophosphate(cAMP) (Breer *et al.*, 1990; Paysan and Breer, 2001; Krieger and Breer, 1999). In insects, the IP_3 pathway is considered as the main excitatory pathway. The release of the second messengers opens ion channels, which in turn trigger the action potential that is mediated to the insect brain.

The recognition of an odorant is based on the interactions with specific receptor proteins. A breakthrough in olfactory research was the identification of the large family of genes coding for olfactory receptors in rat (Buck and Axel, 1991), the work granted with the

Nobel price in 2004. These G-protein coupled receptors are characterised by seven transmembrane domains showing sequence conservation as well as sequence diversity at specific domains, and are exclusively expressed in the olfactory tissue. Afterwards, many studies have revealed putative genes encoding olfactory receptor proteins in vertebrates and invertebrates (Mombaerts, 1999; Mombaerts, 2004a; Keller and Vosshall, 2003; Breer, 2003; Rützler and Zwiebel, 2005; Clyne *et al.*, 1999). In a recent study of *Drosophila melanogaster*, functional evidence for 62 odorant receptor genes were demonstrated (Hallem *et al.*, 2004). An important principle shown in vertebrates as well as in insects by molecular biological studies is that each RN expresses only one type of receptor proteins (Ressler *et al.*, 1993; Vassar *et al.*, 1993; Vosshall *et al.*, 1999; Hallem *et al.*, 2004; Clyne *et al.*, 1999). This implies in general that each organism has subsets of olfactory RNs, each subset with one type of receptor proteins. One exception from this principle is later shown in a study of *D. melanogaster*, where the presence of two receptor protein types in the same RN of the maxillar palp was found (Goldman *et al.*, 2005). Another interesting principle demonstrated in vertebrates as well as insects, is that all RNs of one type converge into one or two specific glomeruli of the primary olfactory centres (Ressler *et al.*, 1994; Strotmann *et al.*, 1994; Gao *et al.*, 2000; Vosshall *et al.*, 2000; Treolar *et al.*, 2002; Mombaerts, 2004b). This principle has been termed “the molecular logic of smell” (Axel, 1995).

Electrophysiological recordings

Since the earliest electrophysiological recordings of olfactory responses from insect antennae (Schneider, 1955; Schneider, 1957), two in principal different methods have been employed to study sensitivity to different odorants in various species. Recordings of electroantennograms (EAGs), being a summated receptor potential, has been extensively used by many research groups in searching for relevant odorants, pheromones and plant volatiles

that are detected by the various species. Recordings from single RNs were first performed with glass-capillary (Schneider, 1957) and then with tungsten microelectrodes (Boeckh, 1962) placed into the base of the sensillum, the latter being the common technique used in recordings from sensilla with short hairs. Refinement of the technique has resulted in stable recordings that can last over several days, as exemplified in the present studies. Tip-recordings from cut olfactory sensilla is another technique to obtain activity of single RNs, particularly suitable for sensilla with long hairs (Kaissling, 1974), and have extensively been used in studies of pheromone detection. Obviously, the importance of recording from single units was to resolve how odour information is received by single olfactory RNs, e.g. whether each RN is specialised for single odorants or respond to a broad range of compounds. However, these studies faced two major problems. All naturally produced odorants exist in complex blends of volatiles and commercially available compounds are rarely 100% pure. Therefore, screening such compounds may be misleading, i.e. the response might be to the impurity of compounds of unknown identity. In addition, stimulation with high concentrations of non-relevant odorants may elicit unspecific responses of an olfactory RN. The method of linking gas chromatography with electrophysiological recordings from single cells (GC-SCR) resolves these problems. The GC-separated volatiles in a naturally produced blend are directly tested on single neurons, showing which odorants that influence the activity and which ones have no effect. This method was first carried out in studies of pheromone detection (Wadhams, 1982), and was later introduced for studying plant odour detection (Tømmerås and Mustaparta, 1989; Blight *et al.*, 1995; Wibe and Mustaparta, 1996; Wibe *et al.*, 1996; Wibe *et al.*, 1997). An important refinement of the method was introduced by Røstelien *et al.* (2000), installing two GC-columns in parallel. Because of the different separation properties of the two columns, the identification of the active GC-peak became more reliable. Another important refinement was the introduction of one chiral column to test the effect of pure

enantiomers (Stranden *et al.*, 2003a; Bichão *et al.*, 2005b; Ulland *et al.*, 2006). Because commercially optically pure compounds hardly exist, chiral GC-separation is the only way to test the effect of optically pure enantiomers.

In the early studies on insect olfaction, the RNs were classified into two principal different classes. “Odour specialists” were RNs specialised for one compound being a pheromone, in contrast to “odour generalists” responding to many different odorants, being plant odours (Lacher, 1967; Schneider, 1992). Another important feature was whether different RNs showed “overlapping reaction spectra” (now termed molecular receptive ranges, MRRs) (Vareschi, 1971). For a long time the different tuning of pheromone RNs as specialists and plant odour RNs as generalists was the common view, as discussed by Masson and Mustaparta (1990). Studies, particularly carried out during the past decade have shown RNs that are narrowly tuned and very sensitive to a single plant odorant, i.e. similar to the pheromone “odour specialists”. These studies include both the use of direct stimulation (Hansson *et al.*, 1999; Larsson *et al.*, 2001; Jönsson and Anderson, 1999; Shields and Hildebrand, 2001) and GC-SCR (Wibe and Mustaparta, 1996; Stranden *et al.*, 2003b; Røstelien *et al.*, 2000b; Røstelien *et al.*, 2005; Stensmyr *et al.*, 2001; Stensmyr *et al.*, 2003; Barata *et al.*, 2002; Bichão *et al.*, 2003; Bichão *et al.*, 2005a). Particularly the latter studies using the GC-SCR method gives precise information about the RN specificity by ruling out the numerous non-active compounds present in blends produced by the plants (headspace volatiles and essential oils).

The view on RNs as specialists and generalists implied two principles on how the olfactory information is mediated from the periphery to the CNS. The “labelled-line system” means that the information about one odorant is mediated by one type of RN (“odour specialist”), in contrast to the “across-fibre pattern” mechanism implying that many RN types (“odour generalists” with overlapping MRRs) mediate the information about one compound.

Whether the olfactory information is mediated via labelled lines or across-fibre patterns is still a matter of debate or interpretations.

Mamestra brassicae L. (Lepidoptera: Noctuidae)

In the studies of the present thesis, we have used the cabbage moth *Mamestra brassicae* L. to study how plant odour information is encoded in the RNs. This moth is highly polyphageous with larvae that can survive on more than 70 plant species from 22 plant families. The species overwinters as prepupae in the soil close to its host plant. From mid-June to August the adults emerge and start searching for a host. The female moth oviposit up to several hundred eggs under the leafs of the plant. Although being a polyphageous species, *M. brassicae* caterpillars prefer a host plant of the genus *Brassica* (CAB International, 2005; Skou, 1991). Feeding by the caterpillars causes severe damage on the plants, mostly due to chewing and fouling rather than the amount of plant tissue eaten (CAB International, 2005). In Norway, *M. brassicae* produces one brood each year, while in warmer climates they may produce two-three broods in the same period. This species can be a notorious pest on monocultures, and pesticides have traditionally been used in pest control. The undesirable side-effects of pesticides have led to the current focus in research on other methods of protecting plants from insect pests. Whereas studies of microbial control agents against *M. brassicae* larvae have demonstrated higher mortality rate at the presence of pathogenic fungi (Klingen and Meadow, 2002), other studies have focused on behaviourally modifying olfactory cues (Rojas, 1999; Renou, 1991; Renou and Lucas, 1994). The latter includes insect produced compounds, pheromones and interspecific signals, the effect of which has been studied by recordings from single RNs. Previous to the studies carried out in the present thesis, stimulatory effect of plant odorants had only been recorded as EAGs in *M. brassicae*. In the present studies, the method of GC-SCR has been employed to precisely identify biologically relevant plant odorants detected by

M. brassicae and determine the specificity of the RNs that are involved in detecting host as well as non-host plants. Based on this information the behavioural significance of the odorants can be carried out, which is performed for one odorant in this thesis.

Aims of the thesis

The aims of the thesis were:

1. To identify naturally produced plant volatiles that are detected by RNs in the moth *M. brassicae* by the use GC-SCR.

2. To characterise and classify plant odour RNs into functional types based on their responses to different odorants, i.e. their MRRs, including primary and secondary odorants.

3. To determine the sensitivity and specificity of the characterised plant odorant RNs.

4. To use a behaviourally bioassay for showing the biological relevance of one primary odorant in *M. brassicae*.

Survey of the individual papers

Paper I

The aim of the study described in paper I was to employ two column GC-SCR followed by GC-MS and retesting authentic compounds, in order to identify biologically relevant plant odorants in *M. brassicae* and functionally characterise the responsive RNs. The latter includes classification of the RNs according to their MRRs, as well as primary and secondary odorants. Volatiles from host and non-host plants were collected using a headspace technique. This means aeration of plants and plant materials from which volatiles were trapped in adsorbents, and then washed out by a solvent. Samples of these mixtures of volatiles from various host plants, as well as of essential oils and of commercially available compounds were screened by the use of GC-SCR. This in order to identify which compounds that influenced the activity of single RNs as well as compounds with no effect. Stable recordings were obtained from 53 RNs that were classified into 21 RN types. These RN types responded to compounds of the following chemical groups: terpenoids, aromatics, aliphatics and thiocyanates. All RNs showed a narrow tuning and responded selectively to one or a few structurally related volatiles (e.g. *E,E*-α-farnesene, TMTT, citronellol, *o*-methylanisole, *p*-methylanisole). Between the different RN types no or minimum overlap of the MRRs appeared. Only three pairs of the RN types showed overlap of a single compound; *racemic* linalool eliciting responses in two RN types (8 and 10), (3*Z*)-hexenylacetate in two others (RN type 11 and 13), and *p*-methylanisole in another pair (RN type 15 and 16). Whereas the tuning of some RNs had not been shown before, others had the same primary odorant as RNs identified in other insects, heliothine moths and weevil species. For instance, three RN types, co-located within a sensillum, showed the same response characteristics and tuning as three co-located RNs described in two polyphagous and an oligophagous species of heliothine moths living on different host plants than *M. brassicae*. The presence of similar RN types across different

insect species implies a conservation or reappearance of the RN types, which in this case included independence of the evolution of oligophagy and polyphagy. The 21 RN types so far identified in *M. brassicae* represent only a part of the olfactory RN types present in this species. Considering the number of glomeruli (67±1) in the antennal lobe of *M. brassicae* (Rospars, 1983) and the fact that RNs of each type project in only one or two specific glomeruli, we expect a number in the range of 30-60 RN types in this species.

All compounds identified as odorants of the *M. brassicae* RNs in these studies are known constituents of plant species. Whereas a few of the odorants, such as the thiocyanates, are specific for plants of the genus *Brassica*, others are common in many plant families. Several of the activating compounds are known as induced volatiles being important in the defence mechanism of plants against herbivores. The results suggest that the moths of *M. brassicae* uses a combination of compounds that are plant specific as well as generally present in many plants, for locating a suitable host for nectar feeding and oviposition.

Paper II

In paper II, the RN type tuned to (*R*)-(-)-linalool were characterised based on the same methods as in paper I, using GC-SCR, but here employing a chiral GC-column for separating enantiomers, in parallel with a polar column. Among the recorded RNs in *M. brassicae*, the linalool responsive RNs appearing in 12 experiments, constitute the most abundant RN type. Responses by these RNs to linalool were obtained by testing samples of both host and non-host plants as well as synthetic *racemic* linalool. Optimal separation of *racemic* linalool in the chiral column demonstrated the enantioselectivity of these RNs, responding strongest to (*R*)-(-)-linalool being ten times more effective as (*S*)-(+)-linalool. This was verified by parallel dose-response curves obtained by direct stimulation with enantiomeric samples, showing a

shift of the (*R*)-(-)-linalool curve one log unit to the left of the (*S*)-(+)-linalool curve. The 10-fold difference in response strength to the enantiomers showed that the chiral centre at carbon 3 in the linalool molecule is important for the odorant-receptor interaction. Complete overlap of the temporal response pattern elicited by the two enantiomers, (*S*)-(+)-linalool at 10-fold higher concentration than (*R*)-(-)-linalool, suggested that the two enantiomers are perceived by *M. brassicae* as the same odour quality but with different intensity. The narrow tuning of the linalool RNs was further demonstrated by their weak responses to a few structurally related compounds, the enantiomers of tetrahydrolinalool and dihydrolinalool, and no responses to the other numerous plant released volatiles.

Paper III

In this study employing GC-SCR, a particular type of specifically responding RNs appeared. Screening the separated headspace volatiles from *Arabidopsis thaliana*, elicited response to a single compound, identified as MeS. Confirmation of the response to this compound was shown by testing a standard containing several compounds including MeS and methyl benzoate (MB) in about the same concentration. The marked strong response to MeS confirmed the identification and showed its role as primary odorant in these RNs. The weak response to the structurally similar MB and two unidentified compounds was the only other responses obtained in these RNs. The two aromatic compounds MeS and MB, methylated by the same enzyme from salicylic acid and benzoic acid, occur together in several plant species. The responses by the same RN type to the two compounds indicate that they are perceived as the same odour quality but with different intensity in *M. brassicae*. In two, recordings the MeS neuron was co-located with another RN type responding to six green leaf volatiles: 1-hexanol, (3*Z*)-hexen-1-ol, (2*E*)-hexen-1-ol, (3*Z*)-hexenyl acetate, (2*Z*)-hexen-1-ol and an unidentified compound. The two neurons showed marked different sensitivity, the latter being

much less sensitive. Whereas the specific RNs detected minor amounts of MeS present in some intact plants, the compound was not found by gas chromatography linked to mass spectrometry in any of the intact plants investigated. However, in an induction experiment, MeS was shown to be emitted after herbivore attack. The behavioural significance of MeS was studied in outdoor test arenas with *Brassica napus* and artificial plants. These behavioural experiments indicated that mated *M. brassicae* females avoid plants carrying dispensers emitting MeS. Thus, MeS may mediate a message to *M. brassicae* females that a potential host plant is already occupied by herbivore larvae of own or of a sympatric species.

Discussion

The receptor neurons

The results included in this thesis (paper I-III) contribute to the growing data on how plant odour information is handled by the RNs in herbivorous insects in general, as well as in *M. brassicae* in particular. The narrow tuning of the presented RNs of *M. brassicae*, including classification into distinct functional types according to one primary odorant and a few secondary odorants of related structures, is in agreement with results obtained in heliothine moths as well as weevil species investigated in the same lab, by the use of the same test protocols (Røstelien *et al.*, 2000a; Røstelien *et al.*, 2000b; Stranden *et al.*, 2003b; Røstelien *et al.*, 2005; Stranden *et al.*, 2002; Stranden *et al.*, 2003a; Mustaparta and Stranden, 2005; Bichão *et al.*, 2003; Bichão *et al.*, 2005a; Bichão *et al.*, 2005b). Also other studies using GC-SCR have resulted in narrowly tuned plant odour RNs in herbivorous insects (Stensmyr *et al.*, 2001; Stensmyr *et al.*, 2003; Barata *et al.*, 2002). These data are in contrast to the broadly tuning found for vertebrate olfactory RNs (Buck, 2000). Possibly this difference is related to the evolution of odour detecting RNs in organisms using the odour information in different contexts. The intimate relationships between herbivorous insects and host plants may require a high degree of specialisation and discrimination ability of plant odours mediating different messages. In the search for a suitable host plant, we can imagine that the odour cues act over a relatively long distance in these insect species. Thus, RNs of high sensitivity and specificity may allow these insect species over some distances to sharply discriminate between plant species as well as between plant individuals of different conditions.

The attention to olfactory RN tuning is obviously important for understanding the mechanisms of odour discrimination. Thus, from the earliest studies of single olfactory RNs in insects, screening of numerous volatiles was performed in order to determine their MRRs. As compared with the specific responses of pheromone RNs (“odour specialists”), RN

responding to plant odours ("odour generalists") showed unspecific and weak responses to many compounds. However, whereas the pheromone compounds were known, the relevant plant odorants were not. This means that most of the studies might have suffered from lacking the biologically relevant plant odorants among the test compounds, as well as impurities present in the commercially available compounds that were tested. Testing high concentrations of irrelevant compounds, may elicit weak responses as well as responses to impurities of unknown identity, giving a false impression of the RN tuning. Therefore, employing GC-SCR for studying the functional properties of olfactory RNs in insects have been valuable and have resulted in a different picture, showing narrowly tuned plant odour RNs in herbivorous moths,weevils and other insect species (Røstelien *et al.*, 2000a; Røstelien *et al.*, 2000b; Stranden *et al.*, 2003b; Røstelien *et al.*, 2005; Stranden *et al.*, 2002; Stranden *et al.*, 2003a; Mustaparta and Stranden, 2005; Bichão *et al.*, 2003; Bichão *et al.*, 2005a; Bichão *et al.*, 2005b; Stensmyr *et al.*, 2001; Stensmyr *et al.*, 2003; Barata *et al.*, 2002; Wibe *et al.*, 1997), including *M. brassicae* (papers I-III). The results from these studies, have also demonstrated a high sensitivity of plant odour RNs, detecting concentrations down to picograms. In fact, as shown in paper II some RNs have shown high responses to elution of the primary odorant at a concentration level below the detection limit of the FID detector.

Recognition of chiral odorants is another topic of RN tuning that has a long-standing interest in olfaction as well as in other research areas where chiral recognition by receptor proteins and enzymes has been well documented. Humans can perceive some enantiomers as different qualities and/or having different intensity (Friedman and Miller, 1971; Ohloff, 1994; Brenna *et al.*, 2003). The importance of chiral recognition has been well documented in insect pheromone communication, where optical configurations of pheromone components are critical and contribute to population differences within species as well as to isolation between species (Tumlinson *et al.*, 1977; Birch *et al.*, 1980; Lanier *et al.*, 1980). Enantioselective RNs

responsible for the detection of pheromone enantiomers were first described in bark beetles (Mustaparta *et al.*, 1980) and for a lepidopteran species, *Lymantria dispar* (Hansen *et al.*, 1983), and later for other species (Wojtasek *et al.*, 1998; Plettner *et al.*, 2000; Nikonov and Leal, 2002) by testing synthetic samples of enantiomers. These samples contained a small amount of the opposite enantiomer, and the question whether the opposite enantiomer might have caused the weak responses, remained to be answered. This question was later resolved for plant odorants by employing chiral GC-SCR in studying enantioselectivity of plant odour RNs in heliothine moths (Stranden *et al.*, 2002). Separation of the enantiomers of the plant produced volatile germacrene D, showed 10 times higher effect of the (-) – than of the (+)-configuration. The second type of RNs tested for enantioselectivity via a chiral GC-column was RNs tuned to linalool, recorded in *M. brassicae* (paper II) and in the strawberry weevil (Bichão *et al.*, 2005a). Whereas the strawberry weevil had two types of RNs, one tuned to (*S*)-(+)-linalool and the other to (*R*)-(-)-linalool, the linalool RN in *M. brassicae* showed the same tuning to (*R*)-(-)-linalool. Like for the germacrene D neurons, all linalool RNs, both in the weevil and the moth, responded to both enantiomers, one configuration being 10 times more effective than the other. As shown in paper II for the RNs of *M. brassicae*, ten times higher concentration of (*S*)-(+)-linalool elicited the same response as (*R*)-(-)-linalool, and showed identical temporal response patterns. This implies that *M. brassicae* would not discriminate well between the odour quality of the two enantiomers, but perceive (*R*)-(-)-linalool with higher intensity unless the secondary response to linalool by RN type 8 play a significant role in the perception of linalool. It is interesting that this is similar to the perception of linalool enantiomers in humans, perceiving them as the same quality, but (*R*)-(-)-linalool as more intensive than (*S*)-(+)-linalool.

The tuning of olfactory RNs implies how the information about an odorant is mediated to the brain, e.g. whether it follows the “labelled-line” principle or the “across-fiber pattern

mechanisms". Each of the 21 RN types described in paper 1-3 showed a narrow tuning and minimal overlap of the MRR. Only three pairs of RN types showed overlap of the MRR by sharing one compound: *racemic* linalool (RNs 8 and 10), (3*Z*)-hexenylacetate (RNs 11 and 13) and *p*-methylanisole (RNs 15 and 16). This means that the information about at least these three odorants is mediated at high concentrations by an "across-fiber pattern mechanisms" in this species. Since all the other RN types showed no overlap of their MRR, it seems that the information about most of the identified odorants is mediated via the principle of labelled-lines in *M. brassicae*. However, the 21 RN types described in the present studies do not represent all populations of RNs in *M. brassicae*. Future experiments will show whether a similar picture appear for the remaining RN types to be identified. In general, it is likely that both principles are used by the olfactory system in an organisms, e.g. that odorants of particular importance are mediated via labelled lines. Whereas the studies of the moth species in our lab have in general shown minimal overlap of MRR between different RN types, more overlap is shown in the species of weevils, but only between RN types tuned to odorants of the same chemical groups (Wibe *et al.*, 1998; Bichão *et al.*, 2003; Bichão *et al.*, 2005a; Mustaparta and Stranden, 2005). This is logical, since related molecules have a higher chance to interact with the binding sites of the same receptor protein. Tuning of olfactory RNs and MRR has been discussed in studies of the fruit fly *D. melanogaster* as well as in vertebrates, where broadly tuning and overlapping receptive ranges seems to be the general principle (De Bruyne *et al.*, 2001; Buck, 2000). However, in all these studies the tests are performed with pre-selected compounds, which possibly may be the reason for the impression of broadly tuning. As also expressed by De Bruyne *et al.* (2001), some of the RNs in the fruit fly might appear with higher sensitivity and narrower tuning when tested with other odorants. It remains to be seen whether the tuning of the RNs in these organisms give a different impression when tested for other odorants that might be the most relevant. Another question is whether broadly

tuning of some RNs is due to expression of more than one type of receptor proteins, since co-expression of two types in one RN has recently been shown in the *D. melanogaster* (Goldman *et al*., 2005).

Comparison of olfactory RN specificity between closely and distantly related species is interesting in an evolutionary context, i.e. whether the specificity is conserved or changed during the speciation of the insects. In our laboratory, the use of the same test protocols allows us to compare RN types across species of moths and weevils. The presence of RN types tuned to the same primary odorant has been shown in closely as well as in distantly related species living on different host plants. However, the specificity of RNs tuned to the same primary odorant, may have different secondary odorants. Interestingly, closely related species of heliothine moths appear to have RNs of the same type, i.e. with the same primary and secondary odorants, suggesting a functional conservation of the receptor proteins. In contrast, RNs tuned to the same primary odorant in distantly related species may show secondary responses to different odorants, suggesting that they have either arised independently or arised from the same receptor protein and then adapted different through evolution. For instance, the primary odorants of RN type 1 (*E,E*-α-farnesene), 3 (*E*-β-ocimene), 4 (TMTT), 8 ((+)-*E*-verbenol) and 13 (GLV) in *M. brassicae* are also primary odorants of RN types identified in heliothine moths. Other interesting examples are the RNs of *M. brassicae* tuned to the sesquiterpenes *E,E*-α-farnesene (RN type 1), *E*-β-ocimene (RN type 3) and TMTT (RN type 4), RN types that has also been identified in three heliothine species (*Heliothis virescens*, *Helicoverpa armigera* and *H. assulta*). These RN types show in addition similarities between the species as concerns the relative response strengths to the different isomers (*E,E*-α-, *E*-β- and *Z,E*-α-) by RN type 1, secondary responses to β-myrcene by the *E,*-β-ocimene RN and no response to other compounds by the TMTT RN. Further, the primary odorants of RN type 10 ((*R*)-(-)-linalool), 11 ((3*Z*)-hexenylacetate), 15 (*p*-

methylanisole) and 19 (MeS) are the same as the primary odorants of RNs identified in *A. rubi* (Bichão *et al.*, 2005b). In addition, a RN tuned to the terpenoid fenchone (RN type 9) is also described in the weevil *Pissodes notatus* (Bichão *et al.*, 2003). The presence of similar RN types across different insect species implies a strong conservation or reapperance of the RN types, independent of the evolution of oligophagy and polyphagy. On the other hand, primary odorants of some RNs in a species may function as secondary odorants of RNs in other species. For instance, the primary odorants of RN type 6 (eucalyptol), 12 ((2*E*)-hexenal), 14 (3-octanone) and 16 (*o*-methylanisole) of *M. brassicae* (Paper I) are in *Anthonomous rubi* described as secondary odorants of RNs tuned to plant odorants (Bichão *et al.*, 2005a).

Consistency of co-localization of particular RN types in a sensillum, as shown in *M. brassicae* (paper I-III) has also been reported in other species including heliothine moths and the weevil *A. rubi* (Røstelien *et al.*, 2005; Bichão *et al.*, 2005b). The co-located RNs, often belonging to different chemical groups, show no overlap of the MMR. One example in *M. brassicae* is the co-located RNs tuned to MeS (aromatic) neuron and the green leaf volatiles (aliphatic). Another interesting example of co-localization is the RN types tuned to *E*,*E*-α-farnesene, *E*-β-ocimene and TMTT in *M .brassicae* and heliothine moths as they consistently appeared in the same recording. The fourth co-located RN in these recordings was different in *M. brassicae* and the heliothine moths; in *M. brassicae* tuned to *E*,*E*-α-farnesol (RN type 2) and to geraniol in the three heliothine moths. Co-localization of RNs have not only been shown in heliothine moths and *M. brassicae* (Røstelien *et al.*, 2000a; Røstelien *et al.*, 2000b; Stranden *et al.*, 2003a; Stranden *et al.*, 2003b), but also in several other insect species such as the cabbage seed weevil, the fruit chafer and the fruit fly (Blight *et al.*, 1995; De Bruyne *et al.*, 2001; Stensmyr *et al.*, 2001; Stensmyr *et al.*, 2003). The functional significance of co-located RNs is not known. A possible role is that co-located RNs enables high-resolution

spatio-temporal information to be aquired by the CNS as discussed by (Bruce *et al.*, 2005)). It is argued that if a system should be able to determine whether there is complete spatial and temporal coincidence in the arrival of two odorants, the integrating system must sample the air at the same point in space and time. In fact, it has been reported that males of *Helicoverpa zea* can distinguish pulses of a pheromone from those of a behavioural antagonist when the sources were separated by 1mm (thus arriving the antennae with approximately 0.001 s time difference) (Baker *et al.*, 1998). In male moths of lepidoptera, co-localization of RNs tuned to a pheromone component with a RN tuned to an antagonist is common (Baker *et al.*, 1998; Berg *et al.*, 2005), supporting the idea that co-localization of RNs play a role for simultaneously detection of two important signals, mediating opposite behavioural effects.

Chemical ecology

Interestingly, some of the compounds detected by olfactory RNs of *M. brassicae* are known to be induced in many plants. If a plant emits one or several volatiles as a response to herbivore attack or other stress factors, it could mediate important messages to the insect approaching the plant. Evidence for host plant avoidance as a result of induced compounds by herbivory, have been demonstrated for the moth *H. virescens* (De Moraes *et al.*, 2001). Some studies have reported parasitoid attraction (*Cardiochiles nigriceps, Phytoseiulus persimili and Cotesia rubecula*) as well as herbivore attraction (*Maladera matrida, Propillia japonica and Oreina cacaliae*) towards volatiles induced by both conspecifics and heterospecifics (De Moraes *et al.*, 1998; Shimoda and Dicke, 2000; Shimoda and Dicke, 2000; van Poecke *et al.*, 2003; Harari *et al.*, 1994; Loughrin *et al.*, 1996; Kalberer *et al.*, 2001). In paper III, we showed that *M. brassicae* females avoided plants carrying dispensers emitting MeS. Furthermore, an experiment showed that a host plant of *M. brassicae*, *B. oleracea* var. *capitata*, emit MeS upon herbivory by *Pieris napi* caterpillars. We would also expect these

plants to emit MeS when damaged by *M. brassicae* larvae. According to some studies, the volatile blends emitted by brussel sprouts as a response to herbivory by different insect species shows only minor qualitatively differences (Blaakmeer *et al.*, 1994; Geervliet *et al.*, 1997). Emission of MeS may in fact give the following important message to *M. brassicae* females when searching for a host plant: the plant is occupied by another insect and are thus less suitable as a food source for the offspring.

Another interesting aspect of olfactory RNs and plant chemistry is how the MRRs correlate with the products of the biosynthetic pathways in host plants. One example is the RNs tuned primarily to MeS and also responding to MB in *M. brassicae* (paper III) and *A. rubi* (Bichão *et al.*, 2005b). The synthesis of both MeS and the MB is catalyzed by methyltransferases, whereby a methyl group is transferred from the donor molecule *S*-adenosine-*L*-methionine to the carboxyl group of salicylic acid (SA) or benzoic acid (BA), respectively (Chen *et al.*, 2003). In a study of snapdragon, it was shown that a methyltranferase catalyzes the production of MeS along with a small amount of MB (Negre *et al.*, 2002). Obviously, compounds co-produced in the biosynthesis and co-released by the plant may give the same message to the insects. Thus, it is remarkable that RNs evolved for the major constituent like MeS in the biosynthetic pathway respond secondarily to the co-produced compounds like MB, since the insect does not need to discriminate between them. In general, a receptor protein does not need to be more specific than necessary.

Behavioural significance of the plant odorants

Whereas considerable knowledge has recently been acquired about encoding of plant odour information in insect RNs, including for which odorants they have evolved, the behavioural significance of the identified odorants is largely unknown. Relevant questions concerns the importance of single odorants or odour blends in host plant recognition, which

has led to proposal of two major hypotheses. One hypothesize that odour recognition of a host plant relies on specific volatiles in that particular host, termed “token stimuli”, which are not present in unrelated plant species (Fraenkel, 1959). This implies that an insect species, like the moth *M. brassicae* might use one or a few RN types to detect the host specific odours of *Brassica* plants. The other hypothesis proposes that host recognition is based on detecting blends of volatiles generally produced in many plant species, implying that the ratio of concentrations of the many volatiles constitutes the odour cue of a host. Most studies give evidence for the latter hypothesis, expressing that the use of ubiquitous plant volatiles should be seen as the most prevalent mechanism mediating host-plant recognition (Bruce *et al.*, 2005). Considering the primary odorants identified for the 21 RN types in the present studies, most of them belong to volatiles present in species of many different plant families, except for the isothiocyanates that are typical for the cabbage plants. Thus, it seems that *M. brassicae* is equipped with RNs mainly detecting odorants produced by many plant species, suggesting that recognition of host plants is mainly based on the odour blend or ratio of the volatiles released by a plant.

In principle, there are two approaches to study and identify important plant volatiles used by herbivore insects: a top-down approach and a bottom-up approach. The former means to start with behavioural responses to the full boquet of volatiles released by a plant, and then narrow down the blend by separating fractions that contain the important compound. This is a logical way to proceed in identifying the relevant plant odour, and has been used in studies of attractants to parasitiods of herbivore insects (D'Alessandro and Turlings, 2005). However, the results of these studies showed that the important compounds were present in minor amounts and could not be identified by GC-MS. The present study using GC-SCR can be considered as the bottom-up approach starting with detection of the plant volatiles, leaving the behavioural studies to the final stage. The studies included in this thesis, have primarily paid

attention to the identification of relevant plant odorants and classification of RN types, whereas the behavioural effect has only been evaluated for one primary odorant, MeS. Previous studies of *M. brassicae* females (Rojas, 1999) have shown behavioural responses to some of the odorants described in paper I-III. For instance, (2*E*)-hexanal, (3*Z*)-hexenyl acetate and 1.8 cineol (= eucalyptol in paper I) induced upwind flight and landing. In contrast, linalool and MeS did not induce upwind flight and landing. The fact that only five compounds has been evaluated for the behavioural effect, underline the need for further experiments focusing on the behavioural relevance of the 16 remaining compounds known to activate the RNs of *M. brassicae*. Behavioural experiments should focus on not only evaluation of single compounds, but also the effect of odorant blends. In fact, behavioural studies of insects have shown that blends of volatiles are important for oviposition behaviour and attraction, as shown for the moths *Papilio polyxenes* and *M. brassicae* (Baur *et al.*, 1993; Rojas, 1999). On the other hand, some single compounds may be important for some decisions, such as MeS (paper III) and isothiocyanates (paper I), whereas volatile blends govern other behavioural responses. Based on the results in the present thesis, we may assume that both the principle of "token stimuli" and odorant blends could play a role in host plant recognition by *M. brassicae*.

The top-down approaches and the bottom-up approaches has specific advantages. Both approaches have underlined the importance of minor constituents of the plant-released blend of volatiles. Since minor compounds can be identified using GC-SCR, by testing other mixtures that contain larger quantities of the compounds, it might be interesting in future experiments to combine the two approaches.

Concluding remarks and future prospects

The work of this thesis has contributed to the understanding of olfactory mechanisms involved in the interaction between insects and plants. The results obtained show that *M. brassica* detect plant volatiles by narrowly tuned RNs. The 21 RN types identified, respond to one or a few structurally similar odorants, and enantioselectivity has been demonstrated for one RN type. Many of the compounds detected by the RNs of *M. brassicae* are known to be induced in several plant species, here shown in *B. oleracea*. The induced compounds are thought to play an important role in the plants defence system against herbivores. The behavioural tests in outdoor arenas demonstrated that one of the primary odorants, MeS, repelled or inhibited oviposition by *M. brassicae* females. Further experiments should be carried out in order to find out whether MeS can be used in applied pest management against *M. brassicae*. The other primary odorants detected by the RNs form a test panel of odorants that should be tested in future behavioural experiments.

It would be interesting to compare the olfactory RNs of *M. brassicae* with RNs of other insects surviving on *Brassica* species. A good candidate for such studies is the diamondback moth, *Plutella xylostella*, which cause extensive damage on crucifer crops. Comparison of olfactory RN specificity may point out whether herbivore insect species using *Brassica* have evolved RNs for detecting the same odorants, particularly odorants specifically produced in *Brassica* species.

References

Almaas, TJ and Mustaparta, H (1990) Pheromone reception in tobacco budworm moth, *Heliothis virescens. J Chem Ecol* **16;** 1331-1347.

Almaas, TJ and Mustaparta, H (1991) *Heliothis virescens*: response characteristics of receptor neurons in sensilla trichodea type 1 and type 2. *J Chem Ecol* **17;** 953-972.

Arimura, G-I, Huber, D and Bohlmann, J (2004) Forest tent caterpillars (*Malacossoma disstria*) induce local and systemic diurnal emissions of terpenoid volatiles in hybrid poplar (*Populus trichocarpa* x *deltoides*): cDNA cloning, functional characterization, and patterns of gene expression of (-)-germacrene d synthase, *PtdTPS1*. *The Plant Journal* **37;** 603-616.

Axel, R (1995) The molecular logic of smell. *Sci Am* **273;** 154-159.

Baker, TC, Fadamiro, HY and Cossé, AA (1998) Moth uses fine tuning for odour resolution. *Nature* **393;** 530.

Barata, EN, Mustaparta, H, Pickett, JA, Wadhams, LJ and Araújo, J (2002) Encoding of host and non-host plant odours by receptor neurones in the eucalyptus woodborer, *Phoracantha semipunctata* (Coleoptera: Cerambycidae). *J Comp Physiol A* **188;** 121-133.

Baur, R, Feeny, P and Städler, E (1993) Oviposition stimulants for the black swallowtail butterfly: identification of electrophysiologically active compounds in carrot volatiles. *J Chem Ecol* **19;** 919-937.

Berg, BG, Almaas, TJ, Bjaalie, JG and Mustaparta, H (1998) The macroglomerular complex of the antennal lobe in the tobacco budworm moth *Heliothis virescens:* specified subdivision in four compartments according to information about biologically significant compounds. *J Comp Physiol A* **183;** 669-682.

Berg, BG, Almaas, TJ, Bjaalie, JG and Mustaparta, H (2005) Projections of male specific receptor neurons in the antennal lobe of the Oriental tobacco budworm moth, *Helicoverpa assulta*. A unique glomerular organisation among related species. *J Comp Neurol* **486;** 209-220.

Bichão, H, Borg-Karlson, A-K, Araújo, J and Mustaparta, H (2003) Identification of plant odours activating receptor neurones in the weevil *Pissodes notatus* F. (Coleoptera, Curculionidae). *J Comp Physiol A* **189;** 203-212.

Bichão, H, Borg-Karlson, A-K, Wibe, A, Araújo, J and Mustaparta, H (2005a) Molecular receptive ranges of olfactory receptor neurones responding selectively to terpenoids, aliphatic green leaf volatiles and aromatic compounds, in the strawberry blossom weevil *Anthonomus rubi*. *Chemoecology* **15;** 211-226.

Bichão, H, Borg-Karlson, A-K, Araújo, J and Mustaparta, H (2005b) Five Types of Olfactory Receptor Neurons in the Strawberry Blossom Weevil Anthonomus rubi: Selective Responses to Inducible Host-plant Volatiles. *Chem Senses* **30;** 153-170.

Birch, MC, Light, DM, Wood, DL, Browne, LE, Silverstein, RM, Bergot, BJ, Ohloff, G, West, JR and Young, JC (1980) Pheromonal attraction and allomonal interruption of *Ips pini* in California by the two enantiomers of ipsdienol. *J Chem Ecol* **6;** 703-717.

Blaakmeer, A, Geervliet, JBF, Van Loon, JJA, Posthumus, MA, Van Beek, TA and De Groot, Ae (1994) Comparative headspace analysis of cabbage plants damaged by two species of *Pieris* caterpillars: consequenses for in-flight host location by *Cotesia* parasitoids. *Entomol Exp Appl* **73;** 175-182.

Blight, MM, Pickett, JA, Wadhams, LJ and Woodcock, CM (1995) Antennal perception of oilseed rape, *Brassica napus* (Brassicaceae), volatiles by the cabbage seed weevil *Ceutorhynchus assimilis* (Coleoptera, Curculionidae). *J Chem Ecol* **21;** 1649-1664.

Boeckh, J (1962) Electrophysiologische Untersuchungen an einzelnen Geruchsrezeptoren auf den Antennen des Totengräbers (*Necrophorus*, Coleoptera). *Z Vgl Physiol* **46;** 212-248.

Boeckh, J and Tolbert, LP (1993) Synaptic organization and development of the antennal lobe in insects. *Microsc Res Tech* **24;** 260-280.

Breer, H (2003) Olfactory receptors: molecular basis for recognition Ind discrimination of odors. *Anal Bioanal Chem* **377;** 427-433.

Breer, H, Krieger, J and Raming, K (1990) A novel class of binding proteins in the antennae of the silk moth *Antherea pernyi*. *Insect Biochem* **20;** 735-740.

Brenna, E, Fuganti, C and Serra, S (2003) Enantioselective perception of chiral odorants. *Tetrahedron: Assymetr* **14;** 1-42.

Bruce, TJA, Wadhams, LJ and Woodcock, CM (2005) Insect host location: a volatile situation. *Trends Plant Sci* **10;** 269-274.

Buck, L and Axel, R (1991) A novel multigene family may encode odorant receptors: a molecular basis for odour recognition. *Cell* **65;** 175-187.

Buck, LB (2000) The molecular architecture of odor and pheromone sensing in mammals. *Cell* **100;** 611-618.

CAB International (2005) *Mamestra brassicae*. In: Crop Protection Compendium. CAB International, Wallingford.

Chen, F, D'Auria, JC, Tholl, D, Ross, JR, Gershenzon, J, Noel, JP and Pichersky, E (2003) An *Arabidopsis thaliana* gene for methylsalicylate biosynthesis, identified by a biochemical genomics approach, has a role in defense. *The Plant Journal* **36;** 577-588.

Christensen, TA, Waldrop, BR, Harrow, ID and Hildebrand, JG (1993) Local interneurons and information processing in the olfactory glomeruli of the moth *Manduca sexta*. *J Comp Physiol A* **173;** 385-399.

Clyne, PJ, Warr, CG, Freeman, MR, Lessing, D, Kim, J and Carlson, JR (1999) A novel family of divergent seven-transmembrane proteins: Candidate odorant receptors in *Drosophila*. *Neuron* **22;** 327-338.

D'Alessandro, M and Turlings, TCJ (2005) In Situ Modification of Herbivore-Induced Plant Odours: A Novel Approach to Study the Attractiveness of Volatile Organic Compounds to Parasitic Wasps. *Chem Senses* **30;** 739-753.

De Bruyne, M, Foster, K and Carlson, JR (2001) Odor coding in the drosophila antenna. *Neuron* **30;** 537-552.

De Moraes, CM, Lewis, WJ, Paré, PW, Alborn, HT and Tumlinson, JH (1998) Herbivore-infested plants selectively attract parasitoids. *Nature* **393;** 570-573.

De Moraes, CM, Mescher, MC and Tumlinson, JH (2001) Caterpillar-induced nocturnal plant volatiles repel conspecific females. *Nature* **410;** 577-580.

De Moraes, CM, Schultz, JC, Mescher, MC and Tumlinson, JH (2004) Induced plant signaling and its implications for environement sensing. *Journal of Toxicology and Environement Health A* **67;** 819-834.

Dudareva, N, Pirchersky, E and Gershenzon, J (2004) Biochemistry of Plant Volatiles. *Plant Physiol* **135;** 1893-1902.

Ehrlich, PR and Raven, PH (1964) Butterflies and plants: a study in coevolution. *Evolution* **18;** 586-608.

Ernst, KD (1969) Die Feinstruktur von Riechsensillen auf der Antenne des Aaskäfers *Necrophorus* (Coleoptera). *Z Zellforsch* **94;** 72-102.

Færavaag, AC (1999) En transmisjonselektronmikroskopisk- og immuncytokjemisk studie av sensilletyper på antennen hos to arter nattfly, *Heliothis virescens* og *Helicoverpa zea* (Lepidoptera: Noctuidae). *Master thesis, Norwegian University of Science and Technology, Trondheim, Norway*.

Flanagan, D and Mercer, AR (1989) An atlas and 3-D reconstruction of the antennal lobes in the worker honey bee, *Apis mellifera* L. (Hymenoptera: Apidae). *Int J Insect Morphol Embryol* **18;** 145-159.

Fraenkel, GS (1959) The raison d`être of secondary plant substances. *Science* **129;** 1466-1470.

Friedman, L and Miller, JG (1971) Odor incongruity and chirality. *Science* **172;** 1044-1046.

Gao, Q, Yuan, B and Chess, A (2000) Convergent projections of *Drosophila* olfactory neurons to specific glomeruli in the antennal lobe. *Nature Neurosci* **3;** 780-785.

Geervliet, JBF, Posthumus, MA, Vet, LEM and Dicke, M (1997) Comparative analysis of headspace volatiles from different caterpillar-infested or uninfested food plants of *Pieris* species. *J Chem Ecol* **23;** 2935-2954.

Gershenzon, J (1994) Metabolic costs of terpenoid accumulation in higher plants. *J Chem Ecol* **20;** 1281-1328.

Goldman, AL, Van der Goes van Naters, W, Lessing, D, Warr, CG and Carlson, JR (2005) Coexpression of two functional odor receptors in one neuron. *Neuron* **45;** 661-666.

Gouinguené, S and Turlings, TCJ (2002) The effects of abiotic factors on induced volatile emissions in corn plants. *Plant Physiol* **129;** 1296-1307.

Große-Wilde, E, Gohl, T, Bouché, E, Breer, H and Krieger, J (2007) Candidate pheromone receptors provide the basis for the response of distinct antennal neurons to pheromonal compounds. *European J Neurosci* **25;** 2364-2373.

Hallem, EA, Ho, MG and Carlson, JR (2004) The Molecular Basis of Odor Coding in the Drosophila Antenna. *Cell* **117;** 965-979.

Hansen, K, Schneider, D and Boppré, M (1983) Chiral pheromone and reproductive isolation between the gypsy- and nun moth. *Naturwissenschaften* **70;** 466-467.

Hansson, BS, Larsson, MC and Leal, WS (1999) Green leaf volatile-detecting olfactory receptor neurones display very high sensitivity and specificity in a scarab beetle. *Phys Ent* **24;** 121-126.

Harari, AR, Benyakir, D and Rosen, D (1994) Mechanism of aggregation behaviour in Maladera-Matrida Argaman (Coleoptera, Scarabeidae). *J Chem Ecol* **20;** 361-371.

Hartmann, T (1996) Diversity and variability of plant secondary metabolism: a mechanistic view. *Entomol Exp Appl* **80;** 177-188.

Haslam, E (1986) Secondary metabolism - facts and fiction. *Natural Product Reports* **3;** 217-249.

Heisenberg, M (2003) Mushroom body memoir: from maps to models. *Nature Rev Neurosci* **4;** 266-275.

Homberg, U, Christensen, TA and Hildebrand, JG (1989) Structure and function of the deutocerebrum in insects. *Ann Rev Entomol* **34;** 477-501.

Homberg, U, Montague, RA and Hildebrand, JG (1988) Anatomy of antenno-cerebral pathways in the brain of the sphinx moth *Manduca sexta. Cell Tissue Res.* **254;** 255-281.

Johnson, CB, Kirby, J, Naxakis, G and Pearson S. (1999) Substantial UV-B-mediated induction of essential oils in sweet basil (Ocimum basilicum L.). *Phytochemistry* **51;** 507-510.

Jönsson, M and Anderson, P (1999) Electrophysiological response to herbivore-induced host plant volatiles in the moth *Spodoptera littoralis*. *Physiol Entomol* **24;** 377-385.

Kaissling, K-E (1998) Olfactory transduction in moths: II. Extracellular transport, deactivation and degradation of stimulus molecules. *Series on Biophysics and Biocybernetics* **5;** 113-137.

Kaissling,K.E. (1974) *Sensory transduction in insect olfactory receptors.* In Jaenicke,L. (ed.), Biochemistry of sensory functions. Springer-Verlag, Berlin, pp. 243-273.

Kalberer, NM, Turlings, TCJ and Rahier, M (2001) Attraction of a leaf beetle (Oreina cacaliae) to damaged host plants. *J Chem Ecol* **27;** 647-661.

Keil, TA (1984) Reconstruction and morphometry of silkmoth olfactory hairs: A comparative study of sensilla trichodea on the antennae of male *Antherea polyphemus* and *Antheraea pernyi* (Insecta, Lepidoptera). *Zoomorphology* **104;** 147-156.

Keil, TA, Hartlieb, E, Bock, C and Steinbrecht, RA (2001) Die Mikrowelt der Insektantennasen. *Naturwiss Rdsch* **54;** 519-525.

Keller, A and Vosshall, LB (2003) Decoding olfaction in *Drosophila*. *Curr Opin Neurobiol* **13;** 103-110.

Kessler, A and Baldwin, IT (2001) Defensive Function of Herbivore-Induced Plant Volatile Emissions in Nature. *Science* **291;** 2141-2144.

Klingen, I and Meadow, RAT (2002) Mortality of *Delia floralis, Galleria mellonella* and *Mamestra brassicae* treated with insect pathogenic hyphomycetous fungi. *J Appl Ent* **126;** 231-237.

Koh, YH, Park, KC and Boo, KS (1995) Antennal sensilla in adult *Helicoverpa assulta* (Lepidoptera: Noctuidae): morphology, distribution, and ultrastructure. *Annu Entomol Soc Am* **88;** 519-530.

Krieger, J and Breer, H (1999) Olfactory reception in invertebrates. *Science* **286;** 720-723.

Lacher, V (1967) Elektrophysiologische untersuchungen an einzelnen geruchsrezeptoren auf den antennen weiblicher moskitos. (*Aëdes aegypti* L). *J Insect Physiol* **13;** 1461-1470.

Lanier, GN, Claesson, A, Stewart, T, Piston, JJ and Silverstein, RM (1980) *Ips pini*: the basis for interpopulational differences in pheromone biology. *J Chem Ecol* **6;** 677-687.

Larsson, MC, Leal, WS and Hansson, BS (2001) Olfactory receptor neurons detecting plant odours and male volatiles in *Anomala cuprea* beetles (Coleoptera: Scarabaeidae). *J Insect Physiol* **47;** 1065-1076.

Laurent, G and Naraghi, M (1994) Odorant-induced oscillations in the mushroom bodies of the locust. *Neurosci* **14;** 2993-3004.

Leal, WS (2001) Molecules and macromolecules involved in chemical communication of scarab beetles. *Pure Appl Chem* **73;** 613-616.

Leal, WS, Chen, AN, Ishida, Y, Chiang, VP, Erickson, ML, Morgan, TI and Tsuruda, JM (2005) Kinetics and molecular properties of pheromone binding and release. *PNAS* **102;** 5386-5391.

Liu, HJ and Liu, TP (1984) Sensilla on the antennal flagellum of bertha armyworm moth, *Mamestra configurata* Walker (Lepidoptera: Noctuidae): A scanning elektron microscope study. *Ann Entomol Soc Amer* **77;** 236-245.

Loughrin, JH, Potter, DA, HamiltonKemp, TR and Byers, ME (1996) Role of feeding-induced plant volatiles in aggregative behavior of the Japanese beetle (Coleoptera: Scarabaeidae). *Environ Entomol* **25;** 1188-1191.

Luckner,M. (1990) Secondary metabolites in microorganisms, plants, and animals. G. Fischer, Jena.

Masson, C and Mustaparta, H (1990) Chemical information processing in the olfactory system of insects. Part 1. Periphery. (H. Mustaparta). *Physiol Rev* **70;** 199-245.

Mauelshagen, J (1993) Neural correlates of olfactory learning paradigms in an identified neuron in the honeybee brain. *J Neurophysiol* **69;** 609-625.

Menzel, R (2001) Searching for the memory trace in a mini-brain, the honeybee. *Learn Mem* **8;** 53-62.

Mombaerts, P (1999) Seven-transmembrane proteins as odorant and chemosensory receptors. *Science* **286;** 707-711.

Mombaerts, P (2004a) Genes and ligands for odorant, vomeronasal and taste receptors. *Nature Rev Neurosci* **5;** 263-278.

Mombaerts, P (2004b) Odorant receptor gene choice in olfactory sensory neurons: the one receptor-one neuron hypothesis revisited. *Curr Opin Neurobiol* **14;** 31-36.

Mustaparta, H, Angst, ME and Lanier, GN (1980) Receptor discrimination of enantiomers of the aggregation pheromone ipsdienol, in two species of *Ips. J Chem Ecol* **6;** 689-701.

Mustaparta, H and Stranden, M (2005) Olfaction and learning in moths and weevils living on angiosperm and gymnosperm hosts. *Recent Adv Phytochem* **39;** 269-292.

Nagnan-Le-Meillour, P, Francois, M-C and Jacquin-Joly, E (2004) Identification and molecular cloning of putative odorant-binding proteins from the american palm weevil, Rhynchophorus palmarum L. *J Chem Ecol* **30;** 1213-1223.

Negre, F, Kolosova, N, Knoll, J, Kish, CM and Dudareva, N (2002) Novel *S*-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase, an enzyme responsible for biosynthesis of methyl salicylate and methyl benzoate, is not involved in floral scent production in snapdragon flowers. *Arch Biochem Biophys* **406;** 261-270.

Nikonov, AA and Leal, WS (2002) Peripheral coding of a sex pheromone and a behavioural antagonist in the Japanese beetle, Popillia japonica. *J Chem Ecol* **28;** 1075-1089.

Ohloff,G**.** (1994) Scent and Fragrances. The fascination of odors and their chemical perspectives. Springer Verlag, Berlin Heidelberg.

Paré, PW and Tumlinson, JH (1999) Plant volatiles as a defense against insect herbivores. *Plant Physiol* **121;** 325-331.

Paysan, J and Breer, H (2001) Molecular physiology of odor detection: current views. *Pflügers Arch-Eur J Physiol* **441;** 579-586.

Pichersky, E and Gershenzon, J (2002) The formation and function of plant volatiles: perfumes for pollinator attraction and defense. *Current Opinion in Plant Biology* **5;** 237-243.

Plettner, E, Lazar, J, Prestwich, EG and Prestwich, GD (2000) Discrimination of pheromone enantiomers by two pheromone binding proteins from the gypsy moth *Lymantria dispar*. *Biochemistry* **39;** 8953-8962.

Prestwich, GD, Du, G and LaForest, S (1995) How is Pheromone Specificity Encoded in Proteins? *Chem. Senses* **20;** 461-469.

Renou, M (1991) Sex pheromone reception in the moth, *Mamestra thalassina*. Characterization and distribution of two types of olfactory hairs. *J Insect Physiol* **37;** 617-626.

Renou, M and Lucas, P (1994) Sex Pheromone Reception on *Mamestra brassicae* L. (Lepidoptera): Responses of Olfactory Receptor Neurones to Minor Components of the Pheromone Blend. *J Insect Physiol* **40;** 75-85.

Ressler, KJ, Sullivan, SL and Buck, LB (1993) A zonal organization of odorant receptor gene expression in the olfactory epithelum. *Cell* **73;** 597-609.

Ressler, KJ, Sullivan, SL and Buck, LB (1994) Information coding in the olfactory system: Evidence for a stereotyped and highly organized epitope map in the olfactory bulb. *Cell* **79;** 1245-1255.

Rø, H, Müller, D and Mustaparta, H (2007) Anatomical Organization of Antennal Lobe Projection Neuronsin the Moth *Heliothis virescens* . *J Comp Neurol* **500;** 658-675.

Rojas, JC (1999) Electrophysiological and behavioural responses of the cabbage moth to plant volatiles. *J Chem Ecol* **25, no. 8;** 1867-1883.

Rospars, JP (1983) Invariance and sex-specific variations of the glomerular organization in the antennal lobes of a moth, *Mamestra brassicae* and a butterfly *Pieris brassicae* . *J Comp Neurol* **220;** 80-96.

Røstelien, T, Borg-Karlson, A-K, Fäldt, J, Jacobsson, U and Mustaparta, H (2000a) The plant sesquiterpene germacrene D specifically activates a major type of antennal receptor neuron of the tobacco budworm moth *Heliothis virescens*. *Chem Senses* **25;** 141-148.

Røstelien, T, Borg-Karlson, A-K and Mustaparta, H (2000b) Selective receptor neurone responses to *E*-β-ocimene, β-myrcene, *E,E*-α-farnesene and *homo*-farnesene in the moth *Heliothis virescens*, identified by gas chromatography linked to electrophysiology. *J Comp Physiol A* **186;** 833-847.

Røstelien, T, Stranden, M, Borg-Karlson, A-K and Mustaparta, H (2005) Olfactory receptor neurones in two heliothine moth species responding selectively to aliphatic green leaf volatiles, aromatics, monoterpenes and sesquiterpenes of plant origin. *Chem Senses* **30;** 443-461.

Rützler, M and Zwiebel, LJ (2005) Molecular biology of insect olfaction:recent progress and conceptual models. *J Comp Physiol A* **191;** 777-790.

Rybak, J and Menzel, R (1998) Integrative properties of the pe1 neuron, a unique mushroom body output neuron. *Learning & Memory* **5;** 133-145.

Sachs,J. (1873) Lehrbuch der Botanik. W. Engelmann, Leipzig.

Sachse, S and Galizia, CG (2002) Role of inhibition for temporal and spatial odor representation in olfactory output neurons: A calcium imaging study. *J Neurophysiol* **87;** 1106-1117.

Schachtner, J, Schmidt, M and Homberg, U (2005) Organization and evolutionary trends of primary olfactory brain centers in Tetraconata (Crustacea + Hexapoda). *Arthropod Struct Dev* **34;** 257-299.

Schneider, D (1955) Micro-Electroden registrieren die electrischen Impulse einselner Sinnesnerevenzellen der Schmetterlingsantenne. *Industrie-Electronik (Hamburg)* **3;** 3-7.

Schneider, D (1957) Electrophysiological investigation on the antennal receptors of the moth during chemical and mechanical stimulation. *Experientia* **13;** 89-91.

Schneider, D (1964) Insect antennae. *Ann Rev Entomol* **9;** 103-122.

Schneider, D (1992) 100 years of pheromone research. An essay on lepidoptera. *Naturwissenschaften* **79;** 241-250.

Schneider, D and Steinbrecht, RA (1968) Checklist of insect olfactory sensilla. *Symp Zool Soc Lond* **23;** 279-297.

Shields, VDC and Hildebrand, JG (2001) Responses of a population of antennal olfactory receptor cells in the female moth *Manduca sexta* to plant-associated volatile organic compounds. *J Comp Physiol A* **186;** 1135-1151.

Shimoda, T and Dicke, M (2000) Attraction of a predator to chemical information related to nonprey: when can it be adaptive? *Behav Ecol* **11;** 606-613.

Skou,P**.** (1991) Nordens Ugler. Håndbog over de i Danmark, Norge, Sverige, Finland og Island forekommende arter af Herminiidae og Noctuidae (Lepidoptera). Apollo Books, Stenstrup.

Steinbrecht, RA (1992) Experimental morphology of insect olfaction: Tracer studies, x-ray microanalysis, autoradiography and immunocytochemistry with silkmoth antennae. *Microsc Res Tech* **22;** 336-350.

Steinbrecht, RA (1998) Odorant-binding proteins: Expression and function. *Ann NY Acad Sci* **855;** 323-332.

Steinbrecht,RA (1999) *Olfactory receptors.* In Egudii,E. and Tominaga,Y. (eds.), Atlas of Arthropod Sensory Receptor- Dynamic Morphology in Relation to Function. In prep. Springer, Tokyo.

Stensmyr, MC, Giordano, E, Ballio, A, Angioy, AM and Hansson, BS (2003) Novel natural ligands for *Drosophila* olfactory receptor neurones. *J Exp Biol* **206;** 715-724.

Stensmyr, MC, Larsson, MC, Bice, S and Hansson, BS (2001) Detection of fruit- and flower-emitted volatiles by olfactory receptor neurons in the polyphagous fruit chafer *Pachnoda marginata* (Coleoptera: Cetoniinae). *J Comp Physiol A* **187;** 509-519.

Stranden, M, Borg-Karlson, A-K and Mustaparta, H (2002) Receptor neuron discrimination of the germacrene D enantiomers in the moth *Helicoverpa armigera*. *Chem Senses* **27;** 143-152.

Stranden, M, Liblikas, I, König, WA, Almaas, TJ, Borg-Karlson, A-K and Mustaparta, H (2003a) (-)-Germacrene D receptor neurones in three species of heliothine moths: structure-activity relationships. *J Comp Physiol A* **189;** 563-577.

Stranden, M, Røstelien, T, Liblikas, I, Almaas, TJ, Borg-Karlson, A-K and Mustaparta, H (2003b) Receptor neurones in three heliothine moths responding to floral and inducible plant volatiles. *Chemoecol* **13;** 143-154.

Strausfeld,N.J. (1976) Atlas of an Insect Brain. Springer-Verlag, New York.

Strotmann, J, Wanner, I, Helfrich, T, Beck, A, Meinken, C, Kubick, S and Breer, H (1994) Olfactory neurones expressing distinct odorant receptor subtypes are spatially segregated in the nasal neuroepithelium. *Cell Tissue Res.* **276;** 429-438.

Tømmerås, BÅ and Mustaparta, H (1989) Single cell responses to pheromones, host and non-host volatiles in the ambrosia beetle *Typodendron lineatum*. *Entomol Exp Appl* **52;** 141-148.

Treolar, HB, Feinstein, P, Mombaerts, P and Greer, CA (2002) Specificity of glomerular targeting by olfactory sensory axons. *J Neurosci* **22;** 2469-2477.

Tumlinson, JH, Glein, MG, Doolittle, RE, Ladd, TL and Proveaux, AT (1977) Identification of the female Japanese beetle sex pheromone: Inhibition of male response by an enantiomer. *Science* **197;** 789-792.

Turlings, TCJ, Tumlinson, JH and Lewis, WJ (1990) Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science* **250;** 1251-1253.

Ulland, S, Ian, E, Borg-Karlson, A-K and Mustaparta, H (2006) Discrimination between Enantiomers of Linalool by Olfactory Receptor Neurons in the Cabbage Moth *Mamestra brassicae* (L.). *Chem Senses* **31;** 325-334.

van Poecke, RMP, Roosjen, M, Pumarino, L and Dicke, M (2003) Attraction of the specialist parasitoid *Cotesia rubecula* to *Arabidopsis thaliana* infested by host or non-host herbivore species. *Entomol Exp Appl* **107;** 229-236.

Vareschi, E (1971) Duftunterscheidung bei der Honigbiene - Einzelzell-Ableitungen und Verhaltensreaktionen. *Z Vergl Physiol* **75;** 143-173.

Vassar, R, Ngai, J and Axel, R (1993) Spatial segregation of odorant receptor expression in the mammalian olfactory epithelum. *Cell* **74;** 309-318.

Vogt, RG, Callahan, FE, Rogers, ME and Dickens, JC (1999) Odorant binding protein diversity and distribution among the insect orders, as indicated by LAP, an OBP-related protein of the true bug *Lygus lineolaris* (Hemiptera, Heteroptera). *Chem Senses* **24;** 481-495.

Vogt, RG and Riddiford, LM (1981) Pheromone binding and inactivation by moth antennae. *Nature* **293;** 161-163.

Vosshall, LB, Amrein, H, Morozov, PS, Rzhetsky, A and Axel, R (1999) A Spatial Map of Olfactory Receptor Expression in the *Drosophila* Antenna. *Cell* **96;** 725-736.

Vosshall, LB, Wong, AM and Axel, R (2000) An olfactory sensory map in the fly brain. *Cell* **102;** 147-159.

Wadhams, LJ (1982) Coupled gas chromatography - Single cell recording: a new Technique for use in the analysis of insect pheromones. *Z Naturforsch* **37c;** 947-952.

Wibe, A, Borg-Karlson, A-K, Norin, T and Mustaparta, H (1996) Identification of plant volatiles activating the same receptor neurons in the pine weevil, *Hylobius abietis. Entomol Exp Appl* **80;** 39-42.

Wibe, A, Borg-Karlson, A-K, Norin, T and Mustaparta, H (1997) Identification of plant volatiles activating single receptor neurons in the pine weevil (*Hylobius abietis*). *J Comp Physiol A* **180;** 585-595.

Wibe, A, Borg-Karlson, A-K, Norin, T and Mustaparta, H (1998) Enantiomeric composition of monoterpene hydrocarbons in some conifers and receptor neuron discrimination of alpha-pinene and limonene enantiomers in the pine weevil, *Hylobius abietis. J Chem Ecol* **24;** 273-287.

Wibe, A and Mustaparta, H (1996) Encoding of plant odours by receptor neurons in the pine weevil (*Hylobius abietis*) studied by linked gas chromatography-electrophysiology. *J Comp Physiol A* **179;** 331-344.

Wojtasek, H, Hansson, BS and Leal, WS (1998) Attracted or repelled? - A matter of two neurons, one pheromone binding protein and a chiral center. *Biochem and Biophys Research Commun* **250;** 217-222.

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Individual papers

Paper I

Plant Volatiles Activating Specific Olfactory Receptor Neurons of the Cabbage Moth *Mamestra brassicae* L. (Lepidoptera, Noctuidae)

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Abstract

Herbivore insects are suitable model organisms for studying how plant odour information is encoded in olfactory receptor neurons. By the use of gas chromatography linked to electrophysiological recordings form single receptor neurons, screening for sensitivity to naturally produced plant odorants is possible in order to determine the molecular receptive ranges of the neurons. Using this method, we have in this study of the cabbage moth, *Mamestra brassicae*, classified 21 types of olfactory receptor neurons according to their responses to odorants present in the host plant Brassicae, in the related species Arabidopsis as well as in essential oils of other plants like Ylang-ylang. Most of the receptor neurons were tuned to one or a few structurally similar compounds, showing minimal overlap of their molecular receptive ranges. Whereas some receptor neurons showed a novel tuning, others were tuned to the same compounds as neurons in other species. Three receptor neurons located within the same sensilla, showed the same response characteristics and tuning as three

co-located receptor neurons described in other species of moths, the heliothines living on different host plants. The presence of similar RN types across different insect species implies a strong conservation or reapperance of the RN types, independent of the evolution of oligophagy and polyphagy.

Introduction

In the search by an insect for a proper mate or a suitable host plant, their olfactory system meets the challenge of recognizing relevant information about volatiles released by conspecifics or sympatric species as well as by plants. Whereas much knowledge has been acquired concerning pheromone detection in many insect species, much less data about relevant plant odorants has appeared. In recent years, the use of gas chromatography linked to electrophysiological recordings from single receptor neurons (GC-SCR) has resulted in reliable information about relevant plant odorants in some species of moths, weevils and other beetles (Bichão *et al.*, 2005a; Røstelien *et al.*, 2005; Stranden *et al.*, 2003; Larsson *et al.*, 2001). Plants are known to emit more than 1000 volatile compounds (Dudareva *et al.*, 2004), and the insects must be able to discriminate among these volatiles in order to chose a suitable host. The important objective in this context is to identify the relevant odorants utilized by the insects in their orientation toward a host. A fruitful approach in resolving the problem seems to be identification of the odorants among the numerous volatiles that are detected by single receptor neurons (RNs) and then test the behavioural significance of these odorants.

Another aspect of the results from studies using GC-SCR is the data on the specificity of olfactory RNs for relevant odorants, knowledge of interest in olfaction in general. Since each olfactory RN typically expresses only one type of receptor proteins as shown in insects and vertebrates (Krieger *et al.*, 2002; Mombaerts, 2004; Störtkuhl and Kettler, 2001; Wetzel *et al.*, 2001; Keller and Vosshall, 2003; Hallem and Carlson, 2004; Clyne *et al.*, 1999), we can assume that the specificity of a RN reflect the specificity of the particular receptor protein type expressed in the neuron. By screening via the GC all volatiles present in the blends of various plants on single neurons, the molecular receptive ranges of olfactory RNs have been identified in several herbivorous species. According to this, the neurons have appeared as

distinct functional types like in heliothine moths. In general, each type seems to be specified for one odorant, called primary odorant, and respond weaker to a few structurally similar odorants. Thus, the molecular receptive range (MRR) of each type is narrow, and in heliothine moths, a minimal overlap is found between the neuron types. Comparison of the functional types of olfactory RNs within closely and distantly related species may shed light upon the evolution of the specificity of olfactory receptor proteins. This study allowed comparison of the olfactory RNs with another polyphagous species (*Mamestra brassicae*) preferring different hosts than the heliothines living on plants of sunflower, cotton, corn etc.

M. brassicae is a polyphagous insect species that survives on many species of plants. However, this insect is often choosing host plants of the genus *Brassica* (CAB International, 2005; McKinlay, 1992). The female moths with a large numbers of antennal sensillae for plant odorants, uses odour cues to locate the host plants where she may lay clusters of 50-300 eggs under the leaves. Feeding by the caterpillars causes severe damage on the plants in monocultures, and the species is an economically important pest in agriculture. Research aiming at identifying behaviourally modifying odorants and understanding the olfactory mechanisms involved in host plant location in this species may contribute to integrated pest management. Previous studies of olfaction in this species concern pheromone detection, whereas studies have just started on how plant odour information is encoded in the receptor neurons.

In the present study of olfaction in *M. brassicae*, we have used GC-SCR to identify relevant plant odorants detected by single receptor neurons. Volatiles from the host plants *Brassica napus* L. and *B. oleracea* (Italica), as well as ecotypes of the related species *Arabidopsis thaliana* have been collected and tested. Additionally, volatiles of other plants used in studies

of heliothine moths have been tested. We here present 53 olfactory RNs classified into 21 RN types. The RNs showed a narrow tuning and minimal overlap in the MRR's.

Material and method

Insect material

M. brassicae pupae were supplied by The Norwegian Crop Research Institute, Ås, Norway. The sexed pupae were stored in separate containers placed in climate chambers (22 °C, 14L: 10 D photoperiod, onset of dark cycle at 10 a.m.). After eclosion, the adult insects were kept in cylindrical containers with access to water containing sucrose (5%). The age of adult insects used in the experiments ranged from 2-14 days (showing no differences in responses depending on age). Both sexes were used in the experiments.

Chemicals and headspace samples

Volatiles were collected from several plant species by the use of a headspace technique (Byrne *et al.*, 1975; Pham-Delegue *et al.*, 1989; Røstelien *et al.*, 2000). The plants were placed in a closed oven bag (Look®) through which purified air was sucked (less than 40 ml\min) and led into glass tubes containing the adsorbents (Tenax TA and Porapak Q, 1:1). The air was purified in a filter of activated charcoal before the intake to the bag, and airation was carried out for 24 or 48 hours. The trapped volatiles were eluted by filling the glass tube with the solvent (hexane and ethyl acetate, ratio 1:1) and leading it drop by drop into different vials that were stored in a freezer. The collected volatiles of the plant materials, essential oils and synthetic compounds were used to stimulate the RNs of the insects. Table 1 gives an overview of the materials used for stimulations. The concentrations of all synthetic compounds tested alone as well as those constituting the standards were 1 µg/µl.

Direct stimulation via cartridges

Direct stimulation via glass cartridges was used for screening the receptor neuron sensitivity

to the various samples of headspace and other mixtures, before the tests were performed via the GC. Five µl of each test sample was applied to a filter paper placed inside a cartridge letting the solvent evaporate before use. The RN was exposed for the test sample by puffing air (4 ml/0.5s) through the cartridge and over the insect antenna. Direct stimulation via glass cartridges was also used for determining dose-response curves. In these tests, 100 µl of a test sample (decadic steps) was applied to a filter paper and kept above a flow of N_2 gas for evaporating the solvent. After the evaporation, the filter paper was inserted into the cartridge. The tests were performed from concentrations below the detection limit of the RNs and successively with increased concentrations. Between the stimulations, the antenna was exposed to a continuous flow (500 ml/min) of purified air. The interstimulus interval varied from one minute at low concentrations to 7 minutes at high concentrations.

Gas-chromatography linked to single cell recordings, GC-SCR

The insects were mounted in a Plexiglas holder and the head and antennae were stabilized with tape and wax as described by Røstelien *et al.* 2000. Electrophysiological recordings from single RNs were made by the use of electrolytically sharpened tungsten micro electrodes. The recording electrode was placed into the base of an olfactory sensillum and the reference electrode into the base of the antenna. When activity was obtained, the neurons were initially screened for sensitivity for various test mixtures. When responding to a sample, 0.5-1 µl of the solution was injected into the column of the gas chromatograph (GC). The column was equipped with a splitter at the end, leading half of the effluent to the flame ionization detector (FID) and the other half into a constant airflow (500 ml/min) blowing over the insect antenna. This made it possible, together with the simultaneously single cell recording, to determine which compounds in the mixture elicited the responses. The electrophysiological recording and gas chromatogram was recorded in the software EAD (Syntech, Netherlands). Separation

in the polar column was performed with two different programs, the first and most frequently used program started at the initial temperature 80°C with an increase rate of 6°C/min to 180°C, and a further increase rate of 15°C/min to 220°C. The second program was used to achieve better separation of the compounds in some of the samples: performed from the initial temperature 50°C isothermal for 2 min followed by a 3°C/min increase to 180°C, and a final increase of 15°C/min to 220°C. The FID temperature was set to 230°C for all programs. The GC was equipped with a cold on column injector.

Spike analysis and cell classification

Electrophysiological recordings from RNs were stored and analyzed in the software program Spike 2 (Cambridge Electronic Design Limited, Cambridge, Great Britain). Separations of the cell types in one recording were based on differences in spike amplitudes and waveforms. The RNs were classified according to which odorant elicited the strongest response (primary odorant) as well those having weaker effects (secondary odorants).

Results

The results presented in this paper are based on recordings from 53 olfactory RNs in *M. brassicae* females and males, responding to plant odours. No differences were noticed between RNs obtained in females and males. Most RNs (n=51) were investigated using GC-SCR. The recordings lasted from 30-40 minutes up to 3 days, allowing stimulation of each olfactory RN 1-52 times via the GC. This included tests with numerous plant volatiles present in the headspace samples of plants, essential oils and synthetic standards. Thus, the long-lasting cell contacts enabled repeated tests also of the same samples to verify which compounds elicited responses. All responses were recorded as increased firing rate that followed the concentration profile of the GC peak. The 53 RNs were classified into 21 distinct types (Table 2) according to the compound that elicited the responses, termed the molecular receptive ranges (MRR). In general, there was little overlap between the MRR of the 21 cell types. Only three pairs of the RN types showed overlap of a single compound; *racemic* linalool (RNs 8 and 10), (3Z)-hexenylacetate (RNs 11 and 13 and *p*-methylanisole (RNs 15 and 16). Below the RN are presented according to the group of compounds to which they responded.

Terpenoides

RN type 1: E,E-α-Farnesene

Screening by cartridges with the essential oil of ylang-ylang and standard 8 elicited responses in four RNs, classified as type one. Figure 1A shows a chromatogram of a synthesized mixture of synthetic farnesenes (standard 8) and the simultaneously recorded responses of the RNs. One marked strong response was elicited by *E,E*-α-farnesene, whereas the two structurally similar compounds *E*-β-farnesene and *Z,E*-α-farnesene elicited only weak responses. Spike analysis of the responses to the three compounds confirmed that they

originated from the same RN. Three of the four RNs were recorded together with another RN type displaying different spike amplitude and waveforms. These neurons classified as RN type 2, 3 and 4 respectively, are presented below.

RN type 2: E,E-Farnesol

One RN, recorded together with RN type 1 responded to a terpenoid eluted later than E,E-α-farnesene when stimulated with ylang-ylang oil. GC-MS analyses indicated the active compound to be *E,E*-farnesol. However, the RN was not retested with synthetic *E,E*-farnesol, and further verification with authentic sample need to be carried out. This RN was classified as type 2. Spike analysis of the recording showed that the responses to *E,E*-α-farnesene and *E,E*-farnesol originated from two neurons with different spike amplitudes and waveforms.

RN type 3: E-β-Ocimene

Two RNs were classified as type 3. Both RNs were recorded together with RN type 1, also including type 2 in one recording. They responded strongest to the terpenoid *E*-β-ocimene and weaker by the structurally similar compound β-myrcene. Both compounds were present in the headspace sample of induced cotton. Recordings of RN type 3 were obtained from two neurons.

RN type 4: 4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT)

Two experiments showed a RN that responded when stimulated with the cartridge containing the headspace sample of induced cotton. These RNs classified as type 4 appeared together with RN type 1, 2 and 3 and responded during elution of the terpenoid TMTT when stimulated with induced cotton via the GC. Responses to other compounds were not obtained. Figure 1B shows the responses of RN type 1 and RN type 4 to the GC-eluted compounds,

E,E-α-farnesene and TMTT, respectively. Analysis of spike waveforms and amplitudes showed that the responses to TMTT and *E,E*-α-farnesene originated from two different RNs. In spite of the structural similarity of these compounds, they did not show a secondary effect on the other RNs.

RN type 5: Citronellol

A RN type was activated when stimulated with cartridges containing the essential oils of clove bud, clove and cinnamon. Injection of these essential oils in the GC showed that a compound with the retentiontime of citronellol (Figure 2A) activated the RN. Furthermore, injection of synthetic citronellol verified that this RN type responded to citronellol. Altogether, three RNs responded to the terpenoid citronellol and these RNs were classified as RN type 5. Responses to citronellol were demonstrated eight times when stimulating via the GC-SCR when stimulating with essential oils and synthetic compounds. No responses were obtained to the other plant compounds present in the material tested via the GC (Table 2). Unfortunately, headspace samples were not tested on this RN type.

RN type 6: Eucalyptol

One RN responded to the essential oils of peppermint, ylang-ylang and cinnamon during direct stimulations with cartridges. Injection of peppermint essential oil in the GC followed by GC-MS analysis showed that the RN was activated by eucalyptol. Further tests by injection of ylang-ylang and cinnamon essential oils confirmed the response to eucalyptol. High concentration of eucalyptol in peppermint oil elicited a long lasting response, as compared to the weak responses to the low concentration present in the essential oils of cinnamon and ylang-ylang.

RN type 7: γ-Terpinene

One RN was excited when stimulated with cartridges containing the essential oil of peppermint and cinnamon. Injection of peppermint essential oil in the GC-column elicited three responses, the strongest to γ-terpinene and weaker responses to β-myrcene and β-phellandrene. In addition, this RN responded weakly to α-phellandrene when stimulated with cinnamon essential oil and standard 1.

RN type 8: (+)-trans-Verbenol

One RN responded when stimulated with a cartridge containing the standard 1. Stimulation via the GC showed that the response was to (+)-*trans*-verbenol. In addition, weak responses were obtained to racemic linalool, methylbenzoate and an unknown compound (trace amount) present in the standard 1. This RN was located together with a RN tuned to linalool (type 11). Separation of activity originating from the two cell types was based on analysis of spike waveform and amplitude.

RN type 9: Fenchone

The RN classified as type 9, responded to stimulation with a cartridge containing the essential oil of fennel. Screening with the other essential oils, headspace samples and synthetic standards did not activate this RN type. Stimulation via the GC showed that the RN responded to the eluent of a compound present in high amounts in the fennel oil (Figure 2B). This peak was later identified as fenchone using GC-MS. This RN type appeared in two recordings, both showing weak responses to the high concentrations of fenchone in the fennel oil suggesting that fenchone is not the primary odorant for this RN type.

Aliphatics

RN type 11: (3Z)-Hexenyl acetate

Responses were obtained when stimulating a RN type with cartridges containing the essential oil of ylang-ylang. In addition, the RN responded to the stimulation with standard 2. Injection of ylang-ylang oil and standard 2 in the GC resulted in responses to the compound identified as (3*Z*)-hexenyl acetate. Two RNs responded best to the aliphatic (3*Z*)-hexenyl acetate and were classified as RN type 11. This RN type was stimulated fifteen times via the GC, showing secondary responses to hexyl propanoate and (2*E*)-hexenyl acetate present in standard 6 and 7 (Table 1), respectively. RN type 11 showed a narrow tuning by responding only to three compounds out of more than 40 structurally similar odorants (aliphatics) that were tested, present in standards 2, 5, 6 and 7 (Table 1). Figure 3 shows responses of one RN when tested for three standards that contained the three compounds.

RN type 12: (2E)-Hexenal

A RN responded when stimulated with cartridges containing headspace sample of *A. thaliana* CV, standard 2 and standard 7. Injection of the standards in the GC showed that the RN type responded to (2*E*)-hexenal, while a second compound (trace amount) present in the headspace sample of *A. thaliana* also activated the RN. The RN was stimulated eight times via the GC, showing a narrow tuning by exclusively responding to two of the numerous odorants present in the test mixtures (headspace sample of *A. thaliana*, standard 2, 5, 6 and 7). The second compound activating the RN type was present in a headspace sample of *A. thaliana*, but only in trace amount that did not allow identification by GC-MS. Figure 4 shows response by the RN to (2*E*)-hexenal when stimulated with standard 2 via the GC. The response frequency increased with increasing concentration of the GC-peak, then rapidly decayed and stopped

firing for 10 s after the GC-peak (Figure 4A). This unusually long lasting silence was repeatedly obtained in this RN after stimulation with (2*E*)-hexenal via the GC.

RN type 14: 3-Octanone

Two RNs classified as type 6 were activated by the aliphatic 3-octanone (Figure 4B). These RNs appeared in the same recordings as another RN type responding to linalool (type 10), previously presented in Ulland and others 2006. The RN showed no responses to the numerous other compounds present in the headspace samples, standards or to the synthetic test compounds (Table 2). Separation of the spikes originating from RN type 14 and the linalool type was based on analysis of spike waveforms and amplitudes.

Aromatics

RN type 15: p-Methylanisole

A RN type co-located with RN type 10 responded to stimulation with cartridges containing essential oil of ylang-ylang. Stimulation via the GC showed that the RN type responded to the aromatic *p*-methylanisole present in the ylang-ylang oil. Later, the responses to *p*-methylanisole were verified by stimulation with synthetic *p*-methylanisole. Stimulation with the isomers *o*-, and *m*-methylanisole via the GC did not activate the RN. These RNs were detected two times and was classified as RN type 15. The RNs responded relatively weak, only showing a response of 20 spikes/s when stimulated with approximately 0.5 µg of the compound via the GC.

RN type 16: o-Methylanisol

Stimulation with cartridges containing essential oil of ylang-ylang and a blend of synthetic *o*-, *p*- and *m*-methylanisole activated two RNs. One of these RNs was activated by *o*-

methylanisole and *ρ*-methylanisole, while the other RN was only activated by *o*-methylanisole. In all cases, the RNs responded with a weak response (10-20 spikes/s) to 0.05-0.5 µg of the activating compound when stimulated via the GC. Based on the recordings obtained so far, we classify these RN as type 16. Due to the few recordings obtained in this RN type, it is difficult to determine which of the two compounds that activate this RN type strongest.

RN type 17: Anethole

Two RNs responded when stimulated with cartridges containing essential oils of clove bud, cinnamon and peppermint. Injection of the essential oils of fennel and clove bud in the GC showed that the neurons were activated by the aromatics anethole (Figure 5A) and eugenol (Figure 5B), with the strongest response to anethole. However, the responses were elicited by high concentrations of both compounds, somewhat stronger response to anethole present in largest amounts.

RN type 18: Indole

In three experiments, a RN responded to the aromatic indole when tested by direct stimulation (stimulus load 0.1 µg).When injecting standard 4 containing indole in the GC-column, responses to indole were only obtained for one neuron. The RN showed no responses to the other compounds present in standard 4. This RN type was classified as type 18.

Iso- and thiocyanates

RN type 20: Butyl Thiocyanate

A single neuron was recorded that responded to the compound butyl thiocyanate and this neuron was classified as RN type 20. However, only a weak response was obtained to the

relative high amount of the GC-eluted compound. This RN type did not show responses to other compounds tested present in standard 10.

RN type 21: Phenyl Isothiocyanate

When screening a cartridge containing standard 10, a response of a RN was recorded. Injection of the standard in the GC showed that the RN responded to the compound phenyl isothiocyanate. This RN type was classified as RN type 21. The response was reproduced three times by injection of this standard in the GC. The specificity of the neuron was demonstrated by no responses to the other six compounds present in standard 10 or to other compounds present in the test material.

RN type 10: (R)-(-)-Linalool, RN type 13: GLV, RN type 19: Methylsalicylate.

These three RN types have previously been described in detail by (Ulland *et al.*, 2006) (RN type 10) and Ulland *et al*. (man. in prep.) (RN type 13 and 19). These RNs are included in Table 2, in order to give an overview of the RN types detected in *M. brassicae*.

Discussion

Together with two previously described RN types, the present results, functionally describing 19 types of olfactory RNs in *M. brassicae*, give information about which plant odorants are biologically relevant and how narrowly the 21 types of RNs are tuned to these odorants. By repeated responses of a RN to the same odorant when stimulating via the GC with headspace samples, essential oils and synthetic compounds, the high reproducibility of the results was demonstrated. Thus, the responses of RNs only appearing once (RN type 1, 7-10, 13, 20 and 21) can be considered reliable and indicate that these RNs belong to a particular type. Their rarely appearance may reflect a small number of this population of RNs on the antennae. Other RNs, appearing up to 12 times and showing the same molecular receptive ranges as well as ranking of the odorants according to stimulatory effects, have been classified as one type. The consistency of the MRR within one type is in accordance with the findings in molecular biological studies in vertebrates and insects, that one type of receptor protein is expressed in each olfactory RN (Krieger *et al.*, 2002; Mombaerts, 2004; Störtkuhl and Kettler, 2001; Wetzel *et al.*, 2001; Keller and Vosshall, 2003; Hallem and Carlson, 2004; Clyne *et al.*, 1999).

By testing via the GC headspace samples of host plants like *B. napus* and *B. oleracea*, as well as of non-host plants, the aim was to identify among hundreds of plant produced compounds as many as possible of the relevant odorants and RN types in *M. brassicae*. However, the number of 21 RN types so far found is much less than might be expected. Considering the number of glomeruli (67±1) in the antennal lobe of this species (Rospars, 1983) and the fact that RNs of each type project in only one or two specific glomeruli, one might assume a number in the range of 30-60 RN types in this species. Thus, in future experiments we may find more plant odour RN types along this line. The question is why the number yet obtained

was so low. In addition to a limited number of recordings, the plant samples may have been lacking relevant odorants. We have recorded more than 70 RNs on the antennae of *M. brassicae* that did not show responses to any of the odorants in the plant material tested. Either the lack of responses by these neurons might be due to the low concentrations of the various volatiles in the headspace samples from *Brassica* spp. and *A. thaliana* ecotypes, or that the relevant odorants are not present in intact or mechanical cut materials of these host plants. For instance, comparison of whole plants and macerated tissue has shown that many compounds found in whole plants are missing in macerated tissue, and *vice versa* (Tollsten and Bergström, 1988). In retrospect, we see that it would have been important to test samples from plants with feeding larvae and flowers for obtaining induced compounds, a topic for future experiments. Nevertheless, 21 RN types have been obtained, 19 types in this study. The RNs showed a narrow tuning to the activating odorants, by responding strongly to one primary odorant (RN type 1-21) and weaker to a limited number of secondary odorants (RN type 1, 3, 7-8, 10-11, 13, 16-17 and 19). These results on narrow tuning to one primary odorant are similar to results obtained in heliothine moths and some weevils investigated with the same method of GC-SCR as used in the present study (Røstelien *et al.*, 2005; Bichão *et al.*, 2005b; Wibe *et al.*, 1997). Some of the RNs described in the present study showed a low sensitivity to the odorant identified from essential oils and synthetic compounds. Although we have tested the RNs for numerous odorants present in the headspace samples, essential oils and synthetic standards, it is possible that the primary odorants for these neurons were lacking, meaning that the response was to a secondary odorant.

Among the 21 RN types classified in *M. brassicae*, some showed similar specificity as RNs previously obtained in other species. Other RNs were unique by not being previously characterized by GC-SCR. Particularly interesting are the latter RN types tuned to odorants

that are typical for the host plant genus *Brassica*, like thiocyanates. Surprisingly, only two RNs (classified as type 20 and 21) responded to butyl thiocyanate and phenyl isothiocyanate, respectively. They may belong to a small population of plant odour RNs on the antennae. In an earlier study, Rojas *et al.* (1999a) showed relative weak EAG responses to isothiocyanates in *M. brassicae*, either indicating responses of a small population of RNs or that these compounds might not be the relevant isothiocyanate. The role of a small population of RNs may not be unimportant, but rather give a significant message about the specific host plant, decisive for oviposition. Other RN types obtained for the first time in this study by the use of GC-SCR include RN tuned to compounds that are more common in many plant species, like *E*,*E*-farnesol (RN type 2), eucalyptol (RN type 6), γ-terpinene (RN type 7) and 3-octanone (RN type 14).

In our laboratory, the use of the same test protocols allows us to compare RN types across species of moths and weevils. Not only can we compare the cell types based on their primary odorant, but also on basis of their secondary odorants. For instance, eucalyptol (RN type 6), both (*E*2)-hexanal(RN type 12) and 3-octanone (RN type 14), and finally *o*-methylanisole (RN type 16) are in *Anthonomous rubi* described as secondary odorants of RNs tuned to α-pinene, (3*Z*)-hexenylacetate and (2*E*)-hexenylacetate, and *p*-methylanisole, respectively (Bichão *et al.*, 2005a). The primary odorants of RN type 1, 3, 4, 8 and 13 also function as primary odorants of RNs identified in heliothine moths. Further, the primary odorants of RN type 10, 11, 15 and 19 are the same as the primary odorants of RNs identified in *A. rubi*. In addition, a RN tuned to the terpenoid fenchone (RN type 9) is also described in the weevil *Pissodes notatus* F. (Bichão *et al.*, 2003). Interestingly, RNs tuned to the sesquiterpene *E*,*E*-α-farnesene, *E*-β-ocimene and TMTT are found in three heliothine species (*Heliothis virescens*, *Helicoverpa armigera* and *H. assulta*) and *M. brassicae*. Also the same response strength to

the different isomers (*E,E*-α-, *E*-β- and *Z,E*-α-) of farnesene were similar in the heliothine moths as well as in *M. brassicae*. Weak responses to β-myrcene by the *E*,-β-ocimene RN was also similar in the three heliothine moths and *M. brassicae*, whereas TMTT did not show responses to other compounds. Another interesting aspect of these RN types in the four moth species was the colocalization in the same sensillum as they appeared in the same recording. The fourth colocated RN *E,E*-α-farnesol was different in *M. brassicae* and the three heliothine moth species having a fourth neuron tuned to geraniol responding weaker to citronellol (primary odorant for RN type 5). The presence of similar RN types across different insect species implies a strong conservation or reappearance of the RN types, independent of the evolution of oligophagy and polyphagy. The three specific RN types within the same sensilla in both *M. brassicae* and three heliothine moths give support to the hypothesis that RNs are a result of a conservation.

In addition to provide knowledge about the peripheral olfactory mechanisms, the present results also indicate which odorants might be behaviourally important in *M. brassicae*. In principle all primary odorants should be tested on the behaviour, which is a challenging task considering possible attractive or repulsive effects as well as ratio specificity. As a beginning, single primary odorants might first be tested for attractive, repulsive or synergistic effects, which has been made for methyl salicylate (Ulland et al., under revision Chemical Senses). The responses of these field experiments showed that methyl salicylate inhibited oviposition of mated *M. brassicae* females when added to dispensers on natural as well as artificial plants. Thus, methyl salicylate should not be added to mixtures with other compounds for testing attracting effects. Other behavioural studies of *M. brassicae* have been carried out in wind tunnel experiments (Rojas, 1999a). Here compounds that elicited EAG-responses as well as host plants (undamaged and damaged) were tested for attraction (Rojas *et al.*, 2000; Rojas,

1999b). Interestingly, mated *M. brassicae* females oriented toward (3*Z*)-hexenylacetate, α-terpinene, 1,8-cineole (named eucalyptus in the present paper), chrysanthenone, camphor and (2*E*)-hexanal, which include three primary odorants (RN type 6, 11 and 12) identified in the present paper. Altogether, these behavioural studies are a good beginning for future tests with blends of the primary odorants. In principle, the aim is to identify the mixtures of odorants that have the best attractive effects as well as repellents that inhibit oviposition.

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References

Bichão, H, Borg-Karlson, A-K, Araújo, J and Mustaparta, H 2003. Identification of plant odours activating receptor neurones in the weevil *Pissodes notatus* F. (Coleoptera, Curculionidae). J. Comp. Physiol. A 189**:** 203-212.

Bichão, H, Borg-Karlson, A-K, Wibe, A, Araújo, J and Mustaparta, H 2005a. Molecular receptive ranges of olfactory receptor neurones responding selectively to terpenoids, aliphatic green leaf volatiles and aromatic compounds, in the strawberry blossom weevil *Anthonomus rubi*. Chemoecology 15**:** 211-226.

Bichão, H, Borg-Karlson, A-K, Araújo, J and Mustaparta, H 2005b. Five Types of Olfactory Receptor Neurons in the Strawberry Blossom Weevil Anthonomus rubi: Selective Responses to Inducible Host-plant Volatiles. Chem. Senses 30**:** 153-170.

Byrne, KJ, Gore, WE, Pearce, GT and Silverstein, RM 1975. Porapak-Q collection of airborne organic compounds serving as models for insect pheromones. J. Chem. Ecol. 1**:** 1-7.

CAB International (2005) *Mamestra brassicae.* In: Crop Protection Compendium. CAB International, Wallingford.

Clyne, PJ, Warr, CG, Freeman, MR, Lessing, D, Kim, J and Carlson, JR 1999. A novel family of divergent seven-transmembrane proteins: Candidate odorant receptors in *Drosophila*. Neuron 22**:** 327-338.

Dudareva, N, Pirchersky, E and Gershenzon, J 2004. Biochemistry of Plant Volatiles. Plant Physiol 135**:** 1893-1902.

Hallem, EA and Carlson, JR 2004. The odor coding system of Drosophila. Trends Genetics 20**:** 453-459.

Keller, A and Vosshall, LB 2003. Decoding olfaction in *Drosophila*. Curr Opin Neurobiol 13**:** 103-110.

Krieger, J, Raming, K, Dewer, YME, Bette, S, Conzelmann, S and Breer, H 2002. A divergent gene family encoding candidate olfactory receptors of the moth *Heliothis virescens*. Eur. J. Neurosci. 16**:** 619-628.

Larsson, MC, Leal, WS and Hansson, BS 2001. Olfactory receptor neurons detecting plant odours and male volatiles in *Anomala cuprea* beetles (Coleoptera: Scarabaeidae). J Insect Physiol 47**:** 1065-1076.

McKinlay,R.G. (1992) Vegetable Crop Species. McMillan Press.

Mombaerts, P 2004. Odorant receptor gene choice in olfactory sensory neurons: the one receptor-one neuron hypothesis revisited. Curr Opin Neurobiol 14**:** 31-36.

Pham-Delegue, MH, Etievant, P, Guichard, E and Masson, C 1989. Sunflower volatiles involved in honeybee discrimination among genotypes and flowering stages. J. Chem. Ecol. 15**:** 329-343.

Rojas, JC 1999a. Electrophysiological and behavioural responses of the cabbage moth to plant volatiles. J. Chem. Ecol. 25, no. 8**:** 1867-1883.

Rojas, JC 1999b. Influence of Host Plant Damage on the Host-Finding Behavior of *Mamestra brassicae* (Lepidoptera: Noctuidae). Environ Entomol 28**:** 588-593.

Rojas, JC, Wyatt, TD and Birch, MC 2000. Flight and Oviposition Behaviour Toward Different Host Plant Species in the Cabbage Moth, *Mamestra brassicae* (L.)(Lepidoptera: Noctuidae). Journal of Insect Behaviour 13**:** 247-254.

Rospars, JP 1983. Invariance and sex-specific variations of the glomerular organization in the antennal lobes of a moth, *Mamestra brassicae* and a butterfly *Pieris brassicae* . J. Comp. Neurol. 220**:** 80-96.

Røstelien, T, Borg-Karlson, A-K, Fäldt, J, Jacobsson, U and Mustaparta, H 2000. The plant sesquiterpene germacrene D specifically activates a major type of antennal receptor neuron of the tobacco budworm moth *Heliothis virescens*. Chem. Senses 25**:** 141-148.

Røstelien, T, Stranden, M, Borg-Karlson, A-K and Mustaparta, H 2005. Olfactory receptor neurones in two heliothine moth species responding selectively to aliphatic green leaf volatiles, aromatics, monoterpenes and sesquiterpenes of plant origin. Chem Senses 30**:** 443-461.

Störtkuhl, KF and Kettler, R 2001. Functional analysis of an olfactory receptor in *Drosophila melanogaster*. PNAS 98**:** 9381-9385.

Stranden, M, Røstelien, T, Liblikas, I, Almaas, TJ, Borg-Karlson, A-K and Mustaparta, H 2003. Receptor neurones in three heliothine moths responding to floral and inducible plant volatiles. Chemoecology 13**:** 143-154.

Tollsten, L and Bergström, G 1988. Headspace volatiles of whole plants and macerated plant parts of *Brassicae* and *Sinapsis*. Phytochemistry 27**:** 2073-2077.

Ulland, S, Ian, E, Borg-Karlson, A-K and Mustaparta, H 2006. Discrimination between Enantiomers of Linalool by Olfactory Receptor Neurons in the Cabbage Moth *Mamestra brassicae* (L.). Chemical Senses 31**:** 325-334.

Wetzel, CH, Behrendt, H-J, Gisselmann, G, Störtkuhl, KF, Hovemann, B and Hatt, H 2001. Functional expression and characterization of a *Drosophila* odorant receptor in a heterologous cell system. PNAS 98**:** 9377-9380.

Wibe, A, Borg-Karlson, A-K, Norin, T and Mustaparta, H 1997. Identification of plant volatiles activating single receptor neurons in the pine weevil (*Hylobius abietis*). J. Comp. Physiol. A 180**:** 585-595.

Figure 1 Gas chromatogram of a sample (upper trace) and the simultaneously recording of a RN (trace below) **A** Injection of a mixture of farnesenes and the RN of type 1 responding to three compounds: *E*-β-farnesene (secondary odorant), *Z,E*-α-farnesene (secondary odorant) and *E,E,*-α-farnesene (primary odorant). The structural similarity of the farnesene isomers is shown to the right. **B** Injection of a headspace sample of induced cotton (*G. herbaceum* L.) and the RN of type 4 responding to the compound TMTT. The co-located RN of type 1 responded weakly to *E,E*-α-farnesene present in the sample (trace amount). (inset) Separation of RN types by analysis of spike amplitude and waveform.

Figure 2 Gas chromatograms (upper trace) and simultaneously recorded activity of a RN (trace below). **A** Injection of essential oil of cinnamon and the response of a RN type 5 at the retentiontime of citronellol. **B** Injection of an essential oil of fennel and the response of a RN of type 9 to the eluent of fenchone.

Figure 3 Gas chromatograms of synthetic standard 2, 6 and 7 (upper trace) and simultaneously recording of activity from a single RN (trace below) of type 12. The RN responded at the retentiontimes of three different compounds: (3*Z*)-hexenyl acetate (primary odorant), (2*E*)-hexenylacetate and hexyl propanoate (secondary odorants). The structurally properties of the three compounds is shown (right, below).

Figure 4 Gas chromatogram of a sample (upper trace) and the simultaneously recording of a RN (trace below) **A** Injection of standard 2 and the response of RN type 12 to (*E*2)-hexenal. The RN showed a increased firing rate after stimulation with (*E*2)-hexenal and a rapid decay at stimulus (GC-peak) offset followed by a silent period. (right) A trace of the recorded

activity showing the response characteristics of the RN. **B** The RN of type 14 responded to the stimulation with 3-octanone.

Figure 5 Gas chromatograms of essential oils (upper trace) and simultaneously recorded activity of a RN (trace below) of type 17. **A** The RN responded to the eluent of anethole (primary odorant) present in the essential oil of fennel. **B** Injection of clove bud essential oil and the response of the RN type 17 to the eluent of eugenol (secondary odorant).

Table 1 An overview of the chemical standards, single compounds, headspace samples and essential oils used to stimulate the RNs via the GC

Synthetic standards

Standard 1	**Standard 2**	**Standard 3**	**Standard 4**	**Standard 5**
Camphor[a]	1-Heptanol[f]	*racemic* Citronellol	Benzylalcohol[j]	2,3-Butan-diol[k]
Isoborneol[b]	(2*E*)-Hexenal[g]	Dihydromyrcene	Benzylcyanide[j]	Butyl-caprylate[k]
racemic Linalool[c]	1-Hexanol[h]	Dihydromyrcenol	(+)-3-Carene[j]	i-Butyl-hexanoate[k]
Methyl benzoate[d]	(3*Z*)-Hexen-1-ol[i]	Geraniol	Citral [Geranial and Neral 1:1][j]	Butyl-pentanoate[k]
Methyl salicylate[e]	(2*E*)-Hexen-1-ol[h]	Myrcene	Indole[j]	Ethyl-2-methyl-propanoate[k] Hexyl-butyrate[k]
(*Z*)- and (*E*)-β-Ocimene (70%)/ Limonene (25%)[c]	(2*Z*)-Hexen-1-ol[h]	Myrcenol		Hexyl-formate[k]
(+)-(*E*)-Verbenole[b]	(3*Z*)-Hexenylacetate[g]	(*Z*)- and (*E*)-β-Ocimene		(±)-2-Methyl-1-butanol[k] Methyl-methyl-butyrate[k]
	1-Octanol[l]	2,6-Dimethyl-2,7-octadiene		Propyl-butyrate[k]
	3-Octanone[b]	3,7-Dimethyl-1-octanol		i-Propyl-butyrate[k]
		3,7-Dimethyl-1,6-octanol		Pentyl-hexanoate[k]

Standard 6	**Standard 7**	**Standard 8**	**Standard 9**	**Standard 10**
Butyl-propionate[k]	Butyl-butyrate[k]	*E,E*-β-Farnesene[k]	(-)-Verbenone[i]	Allyl isotiocyanate[k]
Ethyl-butyrate[k]	Butyl-hexanoate[k]	Farnesenes[k]	(-)-(*E*)-Verbenole[i]	3-Butenyl isotiocyanate[k]
Ethyl-2,4,deco-dienoates[k]	Ethyl-2-methyl-butyrate[k]	Farnesol[b]	(+)-(*Z*)-Verbenole[i]	Butyl tiocyanate[k]
Hexyl-hexanoate[k]	(2*E*)-Hexenal[k]	mix α-Farnesol[b]		3-Me-3-buten tiocyanate[k]
Hexyl-2-methyl-butyrate[k]	(2*E*)-Hexenyl-acetate[k]			Phenetyl isotiocyanate[k]
Hexyl-propanoate[k]	(3*Z*)-Hexenyl-hexanoate[k]			Phenetyl tiocyanate[k]
3-Methyl-1-butanol[k]	3-Methyl-butyl-hexanoate[k]			Phenyl isotiocyanate[k]
2-Methyl-1-butyl-butyrate[k]	2-Methyl-hexyl-butanoate[k]			
2-Methyl-1-butyl-hexanoate[k]	Sec-propyl-butanoate[k]			
2-Methyl-butyl-propanoate[k]				
Pentyl-butyrate[k]				

Synthetic compounds, headspace samples and essential oils

Terpenoids	**Aromatics**	**Ester**	**Headspace samples**	**Essential oils**
Camphor[a]	Ethyl benzoate[d]	Linalyl acetate[h]	*Arabidopsis thaliana* (Cape Verdi)[m]	Basil (*Ocimum basilicum* L.)[o]
racemic Citronellol	Eugenol[h]		*A. thaliana* (Columbia)[m]	Bergamot (*Citrus bergamia*)[o]
Dihydrolinalool[b]	*m*-Methylanisole[h]		*A. thaliana* (Landsberger)[m]	Clove bud (*Syzygium aromaticum* L.)[o]
Geraniol	*o*-Methylanisole[h]		*A. thaliana* (Wassilewskija)m	Clove (*Syzygium aromaticum* L.)[o]
3-Octanone[b]	*p*-Methylanisole[h]		*Brassica napus* L.[m]	Cinnamon (*Cinnamomum zeylanicum* L.)[o]
racemic 1-Octen-3-ol[h]	Methyl jasmonate[d]		*B. oleracea* (Italica)[m]	Fennel (*Foeniculum vulgare* Mill.)[o]
(*S*)-(+)-1-Octen-3-ol[l]			Cotton induced *Gossypium herbaceum L.*[n]	Lemon (*Citrus medica* L.)[o]
(*S*)-(+)-Linalool[b]				Lilac (*Syringa vulgaris* L.)[b]
racemic Linalool[c]				Ylang-ylang (*Cananga odorata* Hook)[g]
racemic Tetrahydrolinalool[h]				

[a]Kebo; [b]A.-K. Borg-Karlson(KTH); [c]Fluka; [d]Lancaster; [e]Merck; [f]Jansen-Chemica; [g]Dragoco RGB; [h]Aldrich;iSigma;[j]J. Anderson;(KTH); [k]I. Liblikas(KTH); [l]J. Fäldt (KTH); [m]S. Ulland (NTNU); [n]S. Gouinguené and T. Turlings (UniNE); [o]NMD (Norsk Medisinal Depot)

Table 2 The RN types identified in *M. brassicae* and the activating compounds tested via the GC

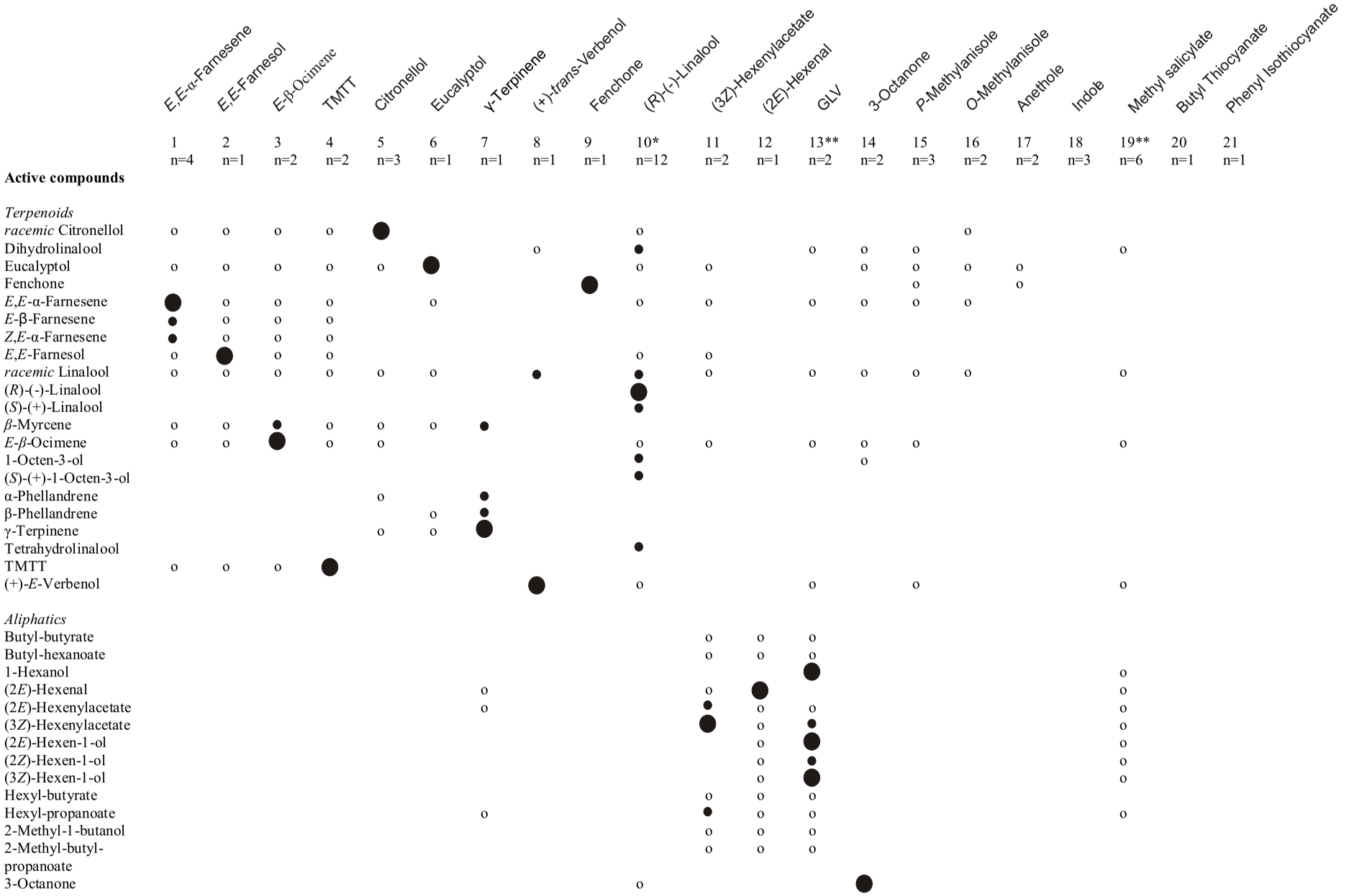


Table 2 continued

Aromatics																	
Anethole							o						●				
Eugenol				o									•				
Indole														●			
o-Methylanisol							o	o	o		o	●			o		
p-Methylanisol	o	o	o		o		o	o	o	o	●	●			o		
Methyl benzoate	o					•		o	o	o	o				•		
Methyl salicylate	o				o	o		o	o	o	o	o			●		
Tiocyanates																	
Butyl Tiocyanate																●	
Phenyl Isotiocyanate																	●

● primary response

• secondary response

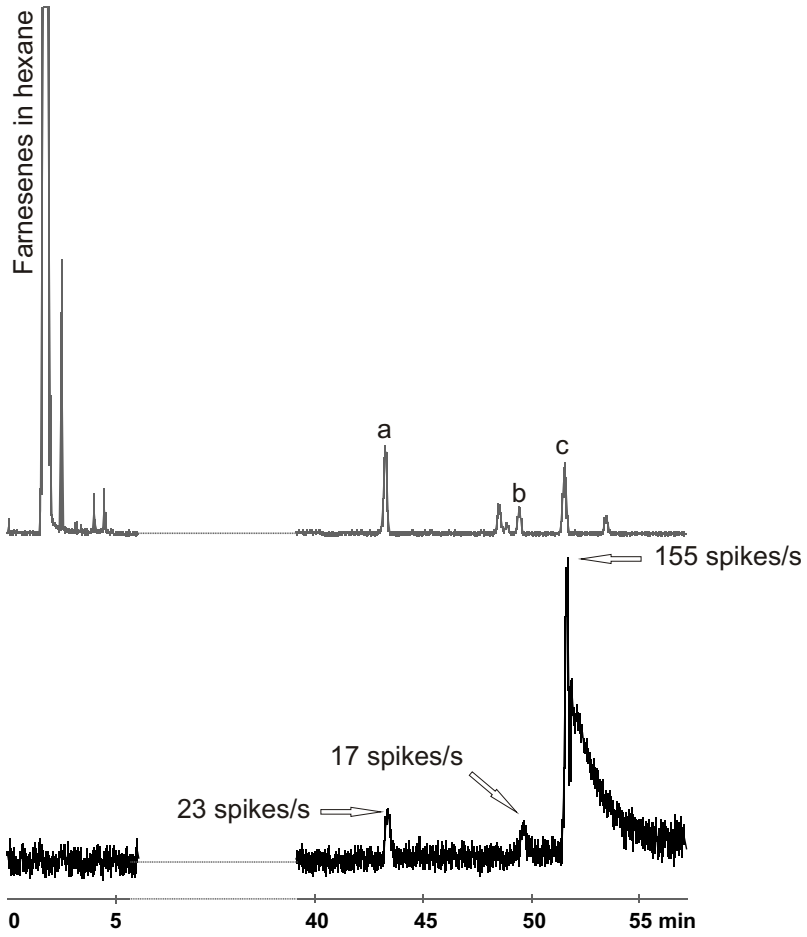
o no response

*previously described by Ulland *et al.* 2006
*Ulland *et al.,* under revision Chemical Senses

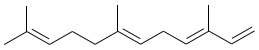
Figure 1

A

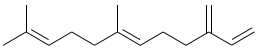
RN type 1, *E,E*-α-Farnesene



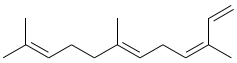
a: *E*,β-Farnesene



b: *Z,E*-α-Farnesene



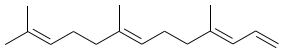
c: *E*,*E*-α-Farnesene



B

RN type 4, *E,E*-TMTT

d: *E*,*E*-TMTT



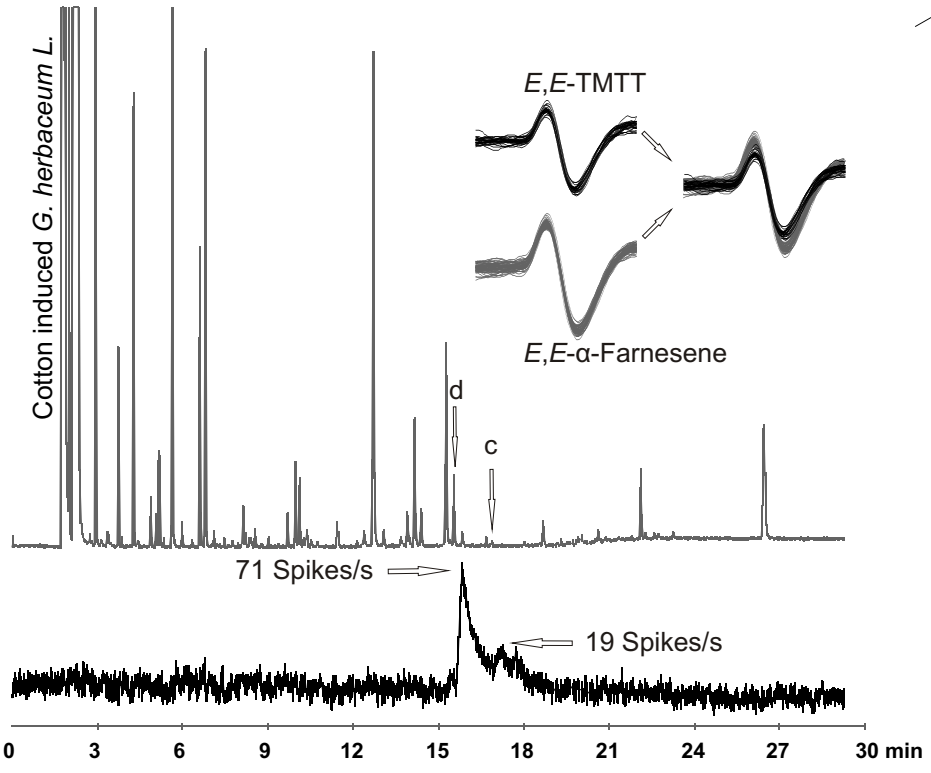
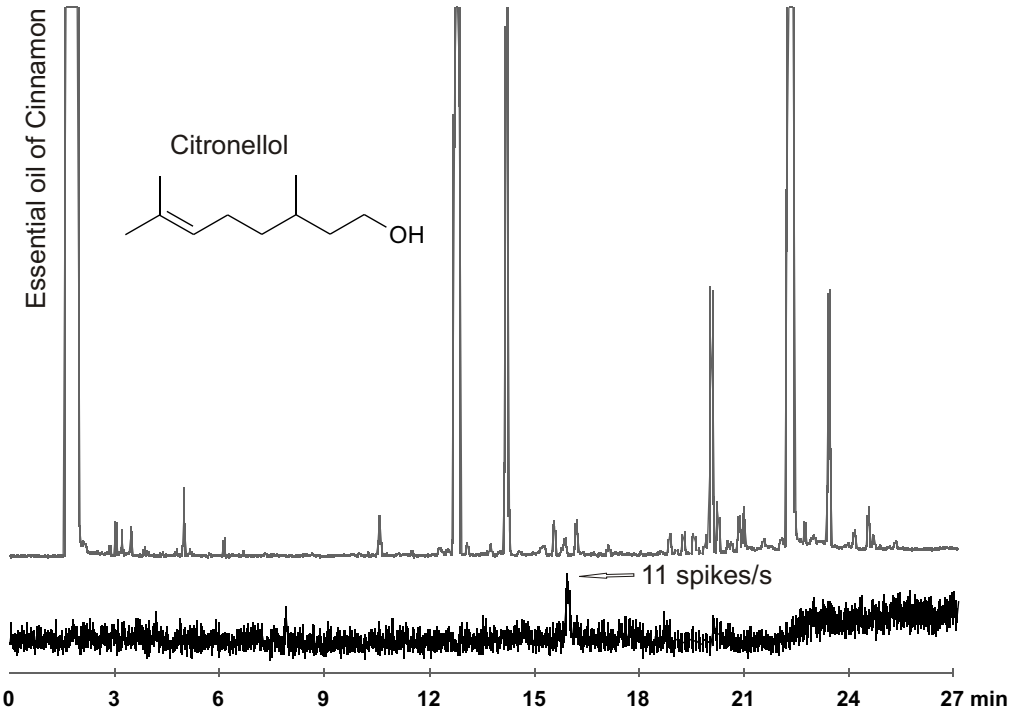


Figure 2

A

RN type 5, Citronellol



B

RN type 10, Fenchone

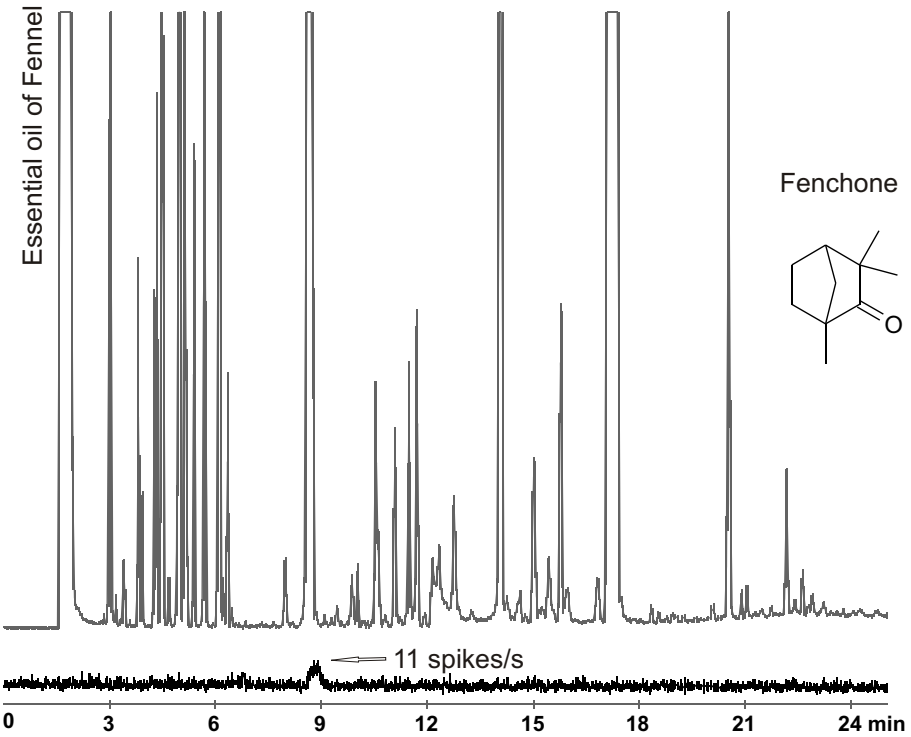
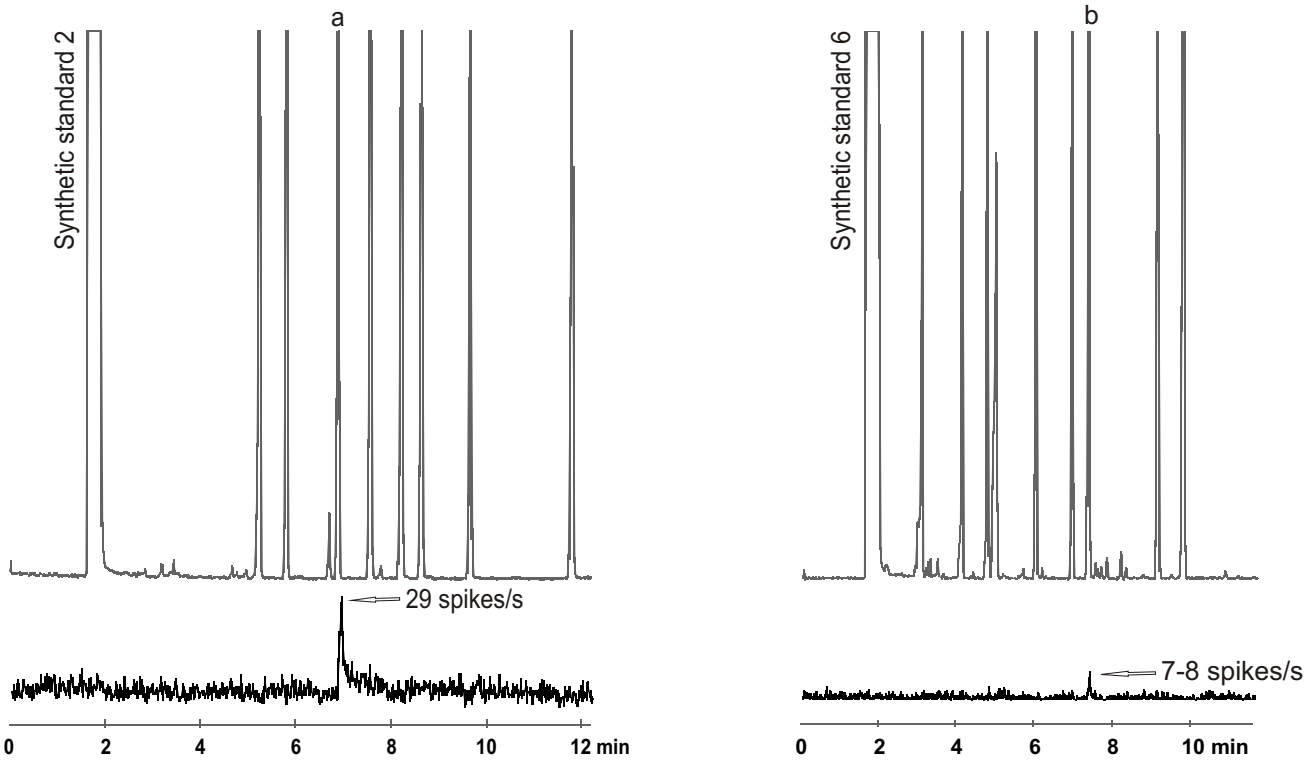
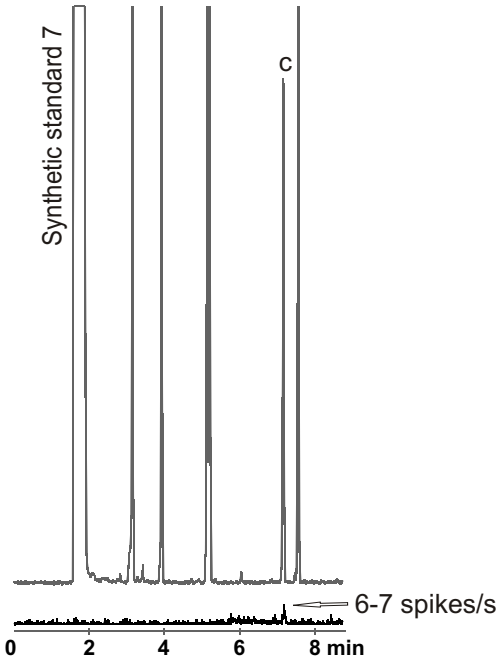


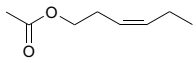
Figure 3

RN type 11, (3*Z*)-Hexenylacetate

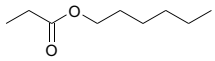




a: (3*Z*)-Hexenylacetate



b: Hexyl propanoate



c: (2*E*)-Hexenylacetate

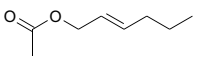
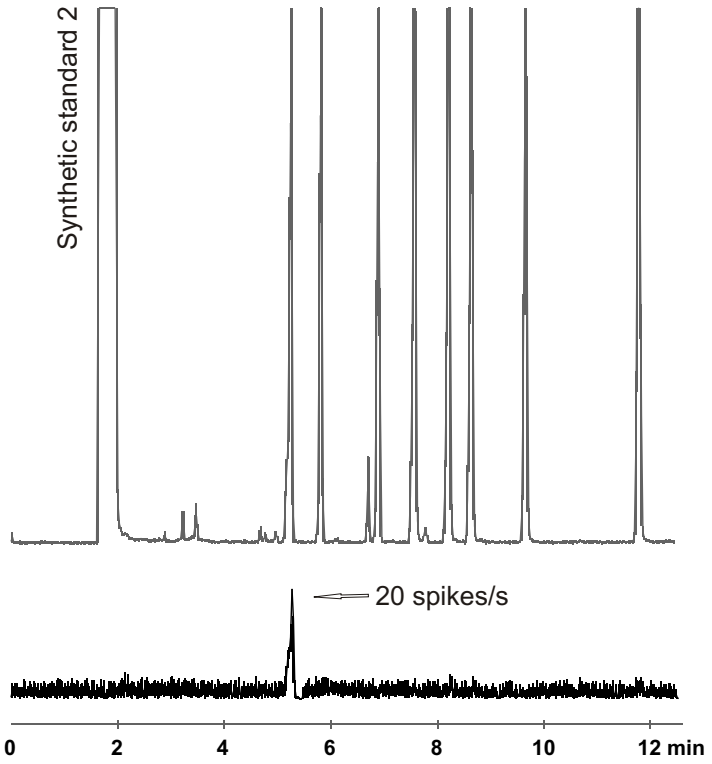
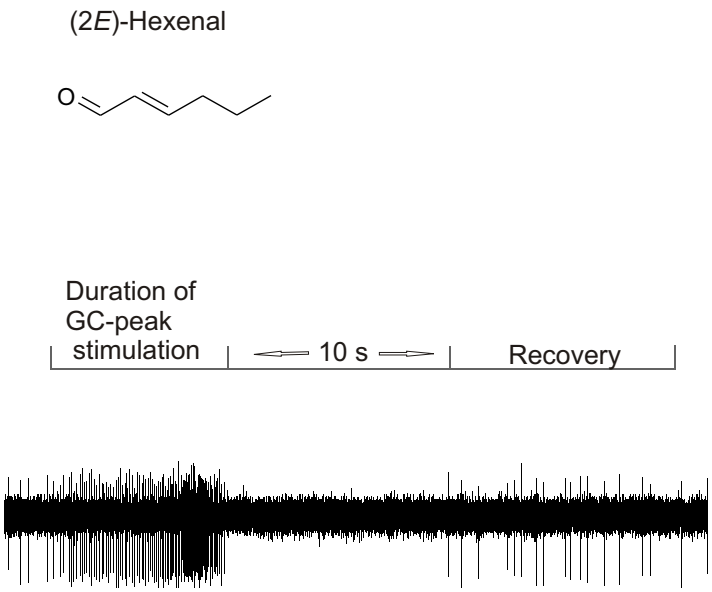


Figure 4

A

RN type 12, (2*E*)-Hexenal





B

RN type 14, 3-Octanone

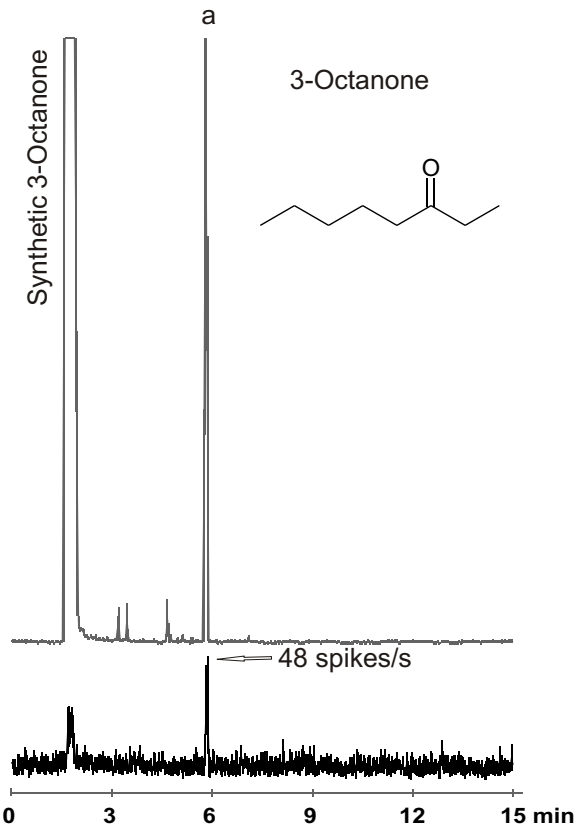
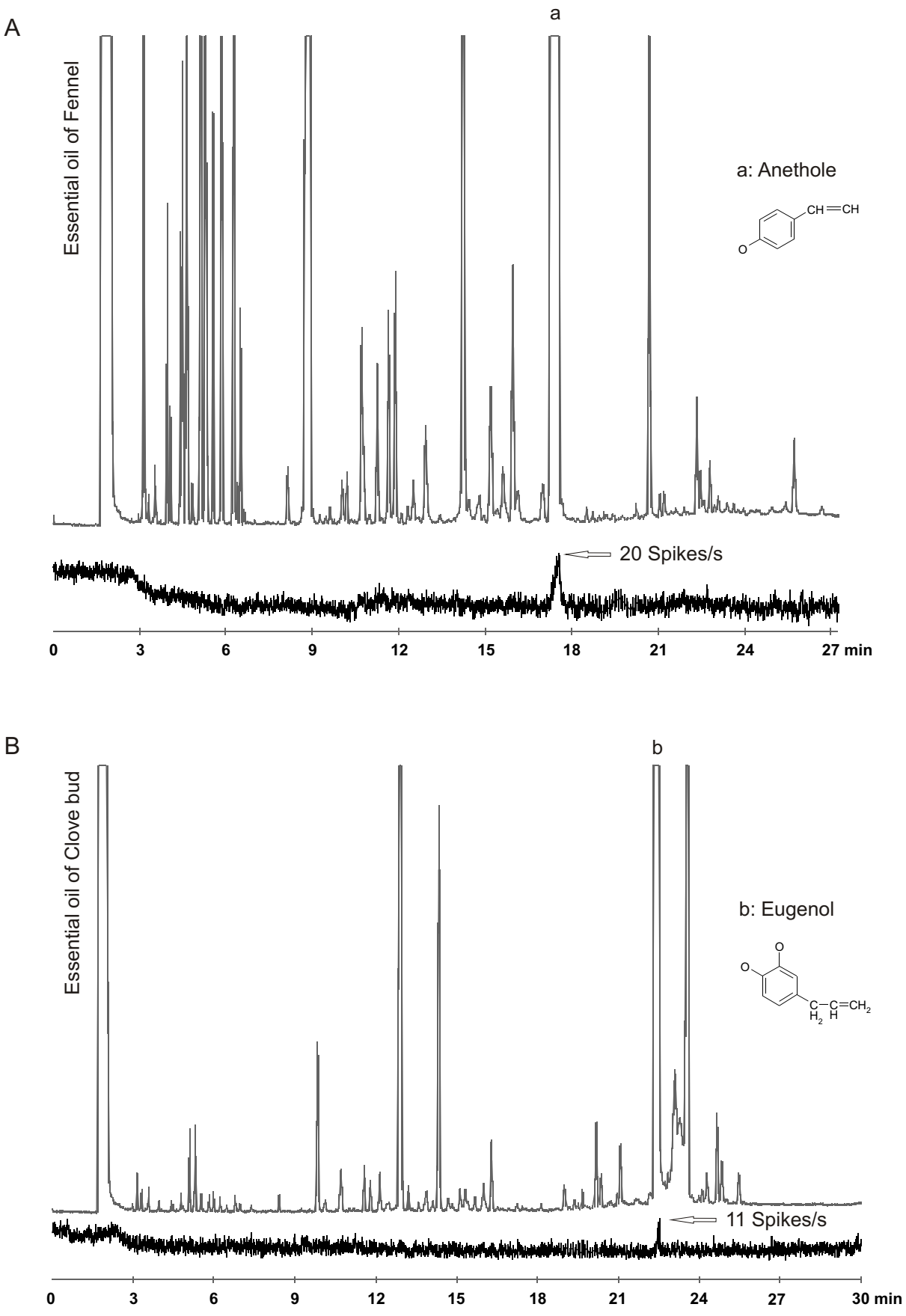


Figure 5

RN type 17, Anethole



Paper II

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Discrimination between Enantiomers of Linalool by Olfactory Receptor Neurons in the Cabbage Moth *Mamestra brassicae* (L.)

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Abstract

Plants emit complex blends of volatiles, including chiral compounds that might be detected by vertebrates and invertebrates. Insects are ideal model organisms for studying the underlying receptor neuron mechanisms involved in olfactory discrimination of enantiomers. In the present study, we have employed two-column gas chromatography linked to recordings from single olfactory receptor neurons of *Mamestra brassicae*, in which separation of volatiles in a polar and a chiral column was performed. We here present the response properties of olfactory receptor neurons tuned to linalool. The narrow tuning of these receptor neurons was demonstrated by their strong responses to (*R*)-(−)-linalool, the weaker responses to the (+)-enantiomer as well as a few structurally related compounds, and no responses to the other numerous plant released volatiles. The enantioselectivity was verified by parallel dose-response curves, that of (*R*)-(−)-linalool shifted 1 log unit to the left of the (*S*)-(+)-linalool curve. A complete overlap of the temporal response pattern was found when comparing the responses of the same strength. Analysis of the spike amplitude and waveform indicated that the responses to the two enantiomers originated from the same neuron.

Key words: enantiomeric discrimination, GC–SCR, *Mamestra brassicae*, olfactory receptor neurons, (*R*)-(−)-linalool

Introduction

Chiral recognition by receptors and enzymes is well demonstrated in biochemical, pharmaceutical, and chemosensory research. Many chiral odorants are perceived by humans as different qualities and/or having different intensities (Friedman and Miller, 1971; Leitereg *et al.*, 1971; Pike *et al.*, 1988; Ohloff, 1994; Brenna *et al.*, 2003), like (*R*)-(−)-linalool which smells slightly different from (*S*)-(+)-linalool, and has a much stronger intensity (Christoph and Drawert, 1985). In contrast, the odor of other enantiomers may not be distinguished by humans (Ohloff, 1994). The importance of enantiomers in olfaction is well demonstrated in insects where optical configurations of pheromone components may be critical and contribute to population differences within species as well as to isolation between species (Tumlinson *et al.*, 1977; Birch *et al.*, 1980; Lanier *et al.*, 1980; Hagaman and Cardè, 1984; Wallner *et al.*, 1984). The presence of different olfactory receptor neurons (RNs) for these enantiomers have been demonstrated by extracellular recordings in several insect species (Mustaparta *et al.*, 1980; Hansen *et al.*, 1983; Wojtasek *et al.*, 1998; Plettner *et al.*, 2000; Nikonov and Leal, 2002).

Studies of herbivorous insects have also provided interesting data on RN detection of chiral compounds emitted by plants. Most interesting are the results obtained by the use of gas chromatography (GC), employing chiral columns linked to electrophysiological recordings from single olfactory RNs [gas chromatography–single cell recording (GC–SCR)] (Stranden *et al.*, 2002, 2003a,b; Bichão *et al.*, 2005b). This is because chiral GC is a means of providing optically pure compounds, which is a challenge when testing the effects of enantiomers. In three related heliothine moths, the sesquiterpene germacrene D, consisting of two enantiomers, activates a major group of RNs (Røstelien *et al.*, 2000a; Stranden *et al.*, 2003a). Stimulation with compounds eluting from a chiral column have shown that all the RNs responding to this compound in heliothine moths were tuned to (−)-germacrene D that has a 10-fold stronger effect than the (+)-enantiomer. A few other compounds of related structures had a weak effect. In the strawberry blossom weevil *Anthonomus rubi*, RNs tuned to germacrene D were found to have a similar enantioselectivity but differed in respect to other secondary odorants (Bichão *et al.*, 2005a). RNs tuned to linalool have been

found or indicated in several insect species, where linalool may act as a host plant attractant or repellent (Hori, 1998) or as a pheromone, or synergist to the pheromone (Ochieng *et al.*, 2002; Borg-Karlson *et al.*, 2003). In addition, other RN types tuned to plant odorants with a chiral center have been reported in various insect species. However, these RNs have not been tested with 100% pure enantiomers separated via chiral GC columns. We here present results obtained using GC–SCR with two different columns installed in parallel to stimulate enantioselective RNs tuned to linalool in the herbivorous moth species, *Mamestra brassicae*.

Materials and methods

Insects

Mamestra brassicae pupae were supplied by The Norwegian Crop Research Institute, Ås, Norway. The sexed pupae were stored in separate containers placed in climate chambers (22°C, 14:10h light:dark photoperiod, onset of dark cycle at 10:00 AM.). After eclosion, the adult insects were kept in cylindrical containers with access to water containing sucrose (5%). The age of adult insects used in the experiments ranged from 2 to 14 days. Both sexes were used in the experiments.

Chemicals and headspace samples

Volatiles were collected from several plant species by using a headspace technique (Byrne *et al.*, 1975; Pham-Delegue *et al.*, 1989; Wibe and Mustaparta, 1996; Røstelien *et al.*, 2000b). The plants were placed in a closed oven bag through which purified air was sucked and led into glass tubes containing the adsorbents (Tenax TA and Porapak Q, 1:1). The air was purified by a filter of activated charcoal before the intake to the bag, and the collection was carried out for 24 or 48 h. The trapped volatiles were eluted with hexane and ethyl acetate (1:1) and stored in vials kept in a freezer. The plant materials used for collecting volatiles were *Brassica oleracea*, *Brassica napus*, and four ecotypes of *Arabidopsis thaliana*. Table 1 gives an overview of the headspace samples, essential oils, standard mixtures, and single compounds used to stimulate the RNs via the GC.

Direct stimulation via cartridges

Direct stimulation via glass cartridges was used for screening of RN sensitivity to the various samples of headspace and other mixtures. Five microliters of each test sample was applied to a filter paper placed inside the cartridge letting the solvent evaporate before use. The RN was exposed for the test sample by puffing air (8 ml/s) through the cartridge and over the antenna. Direct stimulation via glass cartridges was also used for determining dose-response curves. In these tests, 100 µl of each dilution of linalool enantiomers (decadic steps) was applied to a filter paper, and the solvent was evaporated by N_2 flow before inserting the filter paper into the glass cartridge. This resulted in test tubes containing the following amounts of linalool: 0.01 ng, 0.1 ng, 1 ng, 0.01 µg, 0.1 µg, and 1 µg. In dose-response experiments, using direct stimulation, the tests started with concentrations below the detection limit of the RN and continued toward higher concentrations. Between the stimulations, the antenna was exposed to a continuous flow (500 ml/min) of purified air. The interstimulus interval varied from 30 s at low concentrations to 3 min at high concentrations.

GC linked to single-cell recordings, GC–SCR

The insects were mounted in a Plexiglas holder, and the head and antennae were stabilized with tape and wax as described by Røstelien *et al.* (2000b). Electrophysiological recordings from single RNs were made by the use of electrolytically sharpened tungsten microelectrodes; the recording electrode placed into the base of a sensillum and the reference electrode into the base of the antenna. The neurons were initially screened for responses to mixtures of plant odors and single compounds via cartridges. When responding, 0.5–1 µl of the solution was injected into the column of the GC. The column was equipped with a splitter at the end, leading half of the effluent to the flame ionization detector (FID) and the other half into a constant airflow (500 ml/min) blowing over the insect antenna (Røstelien *et al.*, 2000b). This made it possible, together with the simultaneous single-cell recording, to determine which compounds in the mixture elicited the responses. The spike rate and the gas chromatogram were recorded using EAD software (Syntech, Netherlands), while the spikes were recorded and stored using Spike2 software (Cambridge Electronic Design Limited, Cambridge, Great Britain). The GC was equipped with two columns of different separation properties, installed in parallel (Stranden *et al.*, 2002). In the present experiments, we used a DBwax [25 m, inner diameter (i.d.) 0.25 mm, film thickness 0.25 µm, J&W Scientific, Agilent Technologies, Palo Alto, CA] and a chiral column [25 m, i.d. 0.25 mm, octakis (6-O-methyl-2,3-di-O-pentyl)-γ-cyclodextrin (80% in OV 1701), König *et al.*, 1990]. Separation in the polar column was performed with two different programs, the first and most frequently used program starting at the initial temperature 80°C with an increase rate of 6°C/min to 180°C and a further increase rate of 15°C/min to 220°C. The second program was used to achieve better separation of the compounds in some of the headspace samples: performed from the initial temperature 50°C isothermal for 2 min followed by a 3°C/min increase to 180°C and a final increase of 15°C/min to 220°C. In the chiral column, enantiomeric separation of linalool, dihydrolinalool, and tetrahydrolinalool was optimal at the isothermal temperature 80°C. The FID temperature was set to 230°C for all programs. The GC was equipped with a cold on–column injector.

Spike analysis and cell classification

The spikes from RNs were analyzed using the Spike2 software. Separation of the cell types in one recording was based on differences in spike amplitudes and waveforms. The RNs

Table 1 Overview of the samples tested on each linalool RN via the two-GC columns

	RN no. I	RN no. II	RN no. III	RN no. IV	RN no. V	RN no. VI	RN no. VII	RN no. VIII	RN no. IX	RN no. X	RN no. XI	RN no. XII
Test samples												
Racemic linalool[a]	12	2	1	1	—	1	—	—	2	1	—	—
(*S*)-(+)-linalool[b]	7	1	—	—	—	—	—	1	1	—	1	1
Headspace/essential oils												
Arabidopsis thaliana Cvi[c]	2	—	—	—	—	—	—	1	—	—	—	—
Arabidopsis thaliana Col[c]	2	—	—	—	—	—	—	1	—	—	—	—
Arabidopsis thaliana L[c]	3	—	—	—	—	—	—	—	—	—	—	—
Arabidopsis thaliana Was[c]	—	—	—	—	—	—	—	1	—	—	—	—
Brassica napus[c]	2	—	—	—	—	—	—	—	—	—	—	—
Brassica oleracea[c]	2	—	—	—	—	—	—	—	—	—	—	—
Cinnamon essential oil[d]	—	—	—	—	—	—	—	—	—	—	1	—
Lemon essential oil[d]	1	—	—	—	—	—	—	1	—	—	—	—
Ylang ylang essential oil[e]	—	—	—	1	1	1	1	—	1	1	1	—
Synthetic compounds												
Camphor[b]	—	1	—	—	—	—	—	—	—	—	—	—
Racemic citronellol	1	—	—	—	—	—	—	—	—	—	—	—
Racemic dihydrolinalool[b]	6	—	—	—	—	—	—	—	—	—	—	1
Linalyl acetate[e]	1	—	—	—	—	—	—	—	—	—	—	—
3-Octanone[b]	1	—	—	—	—	—	—	—	2	—	1	—
Racemic 1-octen-3-ol[e]	1	—	—	—	—	—	—	—	2	—	1	—
p-, *m*-, *o*-Methylanisol[f]	—	—	—	1	—	—	—	—	—	—	—	—
Racemic tetrahydrolinalool[e]	5	—	—	—	—	—	—	—	—	—	—	1
Standard 1	2	—	1	—	—	—	—	2	—	—	1	1
3,7-Dimethyl-1-Octanol												
3,7-Dimethyl-1,6-Octanol												
2,6-Dimethyl-2,7-Octadiene												
Racemic citronellol												
Dihydromyrcene												
Myrcenol												
Dihydromyrcenol												
Geraniol												
Myrcene												
(*Z*)- and (*E*)-β-ocimene (1:1)												
Standard 2	—	2	—	—	—	—	—	1	—	—	—	—
(*Z*)- and (*E*)-β-ocimene (70 %)/												
Limonene (25%)[a]												
Camphor												
Racemic linalool[a]												

Table 1 Continued

	RN no. I	RN no. II	RN no. III	RN no. IV	RN no. V	RN no. VI	RN no. VII	RN no. VIII	RN no. IX	RN no. X	RN no. XI	RN no. XII
Methyl benzoate[g]												
Isoborneol[b]												
(+)-(*E*)-Verbenol[b]												
Methyl salicylate[g]												
Standard 3	1	—	—	—	—	—	—	—	—	—	—	—
(+)-3-Carene												
Citral [Geranial and Neral (1:1)]												
Benzylalcohol												
Benzylcyanide												
Indole												

Numbers indicate how many times the stimulation was performed on the individual RNs.
[a]Fluka, Buchs, Switzerland.
[b]Borg-Karlson, Royal Institute of Technology RIT, Stockholm, Sweden.
[c]Ulland, Norwegian University of Science and Technology,Trondheim, Norway.
[d]Norsk Medisinal Depot (NMD), Oslo, Norway.
[e]Aldrich, Steinheim, Germany.
[f]Lancaster, Lancashire, UK.
[g]Merck, Darmstadt, Germany.

were classified according to which odorant elicited the strongest response (primary odorant) as well as those having weaker effects (secondary odorants).

Results

General response characteristics

Excitation and temporal response pattern

Out of 43 olfactory RNs recorded in *M. brassicae* females and males, 12 neurons responded best to the plant volatile linalool. The 12 RNs were obtained in six females and six males. Altogether, the RNs were tested 89 times via the GC (GC = 89), out of which 49 tests were performed on the same neuron (Table 1, RN no. I). In 30 out of the 49 tests, this neuron responded strongly to linalool present in the various headspace samples of host and nonhost plants as well as to synthetic linalool. In addition, the other RNs tested repeatedly with samples containing linalool demonstrated reproducibility of the responses, which appeared as increased firing rate upon stimulation with linalool (Figure 1A). Three other compounds elicited weaker responses in the most sensitive neurons. No inhibition was observed. When stimulating via the GC, the firing rate of the RNs followed the concentration profile of the active GC peak, regaining the spontaneous activity within half a minute or a few minutes. Responses to high concentrations elicited responses that far outlasted the GC peak. Most of the 12 RNs showed responses also to the solvents hexane and ethyl acetate. Stimulation directly with linalool via cartridges, giving a fast onset and more constant concentration profile, showed a phasic-tonic response pattern. After stimulation with high concentrations of linalool, the RNs showed adaptation which might last for several minutes. Most recordings presented from the GC–SCR indicated activity of two or three RNs responding to different compounds. The responses of the RNs were distinguished based on spike analysis in the Spike2 software. Similar amplitudes and waveforms of the spikes were ascribed to the same RN.

Concentration dependency

Ten out of the twelve RNs responded to linalool at low concentrations. The threshold concentration for the most sensitive neurons was approximately 0.5 ng of *racemic* linalool when tested via the polar GC column. Dose-dependent excitation was shown by stimulation with decadic increase of concentrations over 5 log units. As shown in Figure 1B, the most sensitive linalool RN displayed an approximately linear increase of the firing frequency over the three highest concentrations (5 ng–0.5 μg), reaching a maximum firing rate of more than 200 spikes/s. The temporal response pattern of the same RN when stimulated with the three highest concentrations is shown in Figure 1C. The firing rate of the neuron increased from the onset of the stimulus, that is, when the GC peak appeared, reaching a maximum after 300–400 ms at the peak of the concentration profile. During the high firing frequency of the strongest response, the spike amplitude decreased below the noise level making spike counting

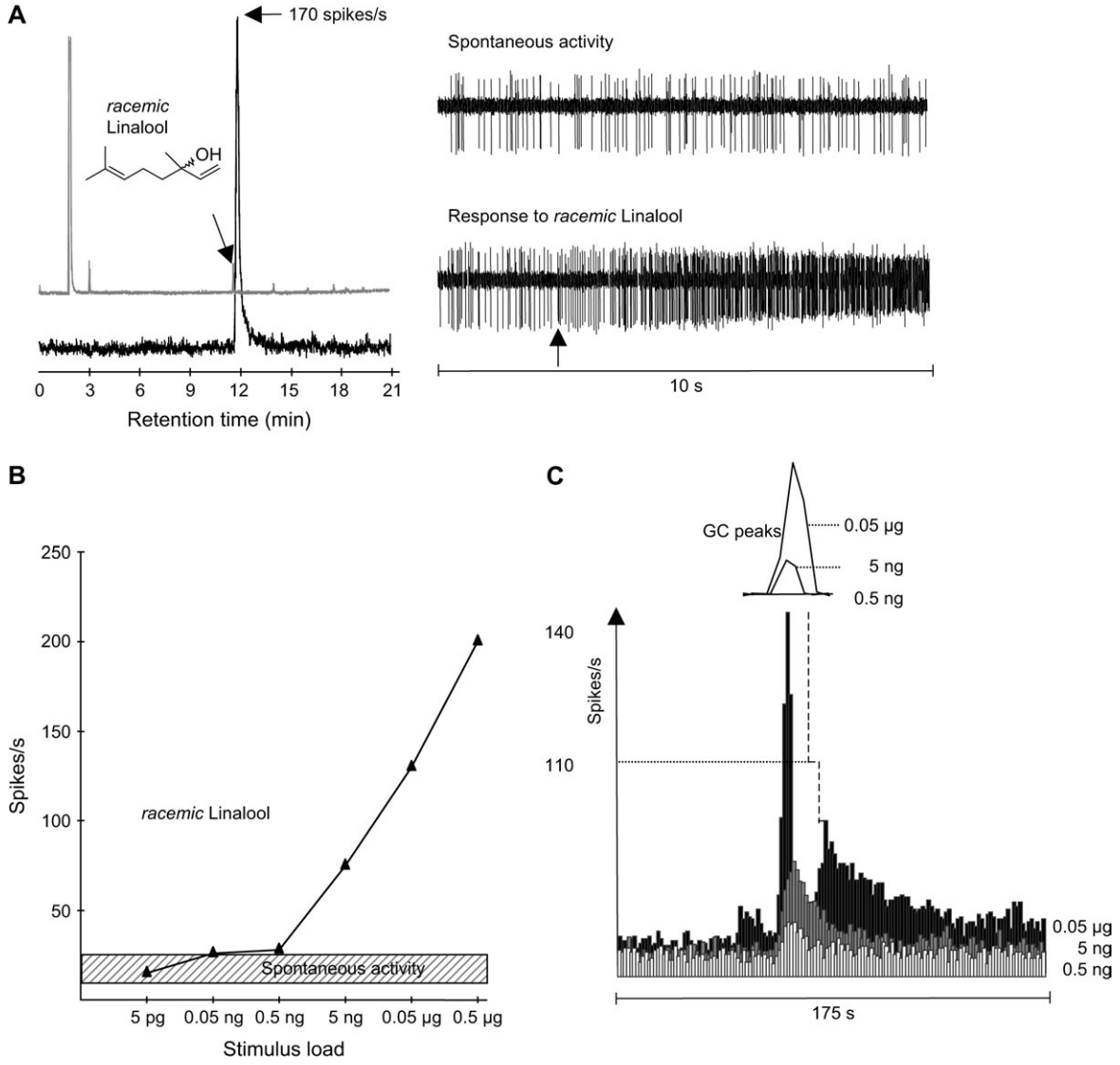

Figure 1 Response characteristics of a single RN to stimulations with *racemic* linalool via the polar GC column. **(A)** Gas chromatogram of standard 1 (GC trace above) and simultaneously recorded activity of the RN (trace below), showing response to linalool eluted at the retention time 11.29 min (left). Traces (10 s) of the spontaneous activity and the response (right). Arrow indicates the start of the stimulation. **(B)** Dose-response curve indicating threshold concentration at 0.5 ng and an approximately linear increase up to 0.5 µg. **(C)** Temporal patterns of the responses to decadic increase of the concentration (0.5 ng–0.05 µg). The stimulus period and relative concentrations of the GC peak is indicated above. Maximum firing rate appeared at about the same time after onset of the stimulus. The decay of the strongest response was particularly prolonged. The sudden drop appearing at the maximum firing rate was caused by the decrease of spike amplitudes, disabling spike counting in the Spike2 software. The dashed line indicates the real decay of the response.

difficult. This was the cause of the apparently rapid drop in spike frequency in Figure 1C. Nevertheless, recovery of spike amplitude showed that the actual spike frequency recovered fairly quickly toward baseline levels, that is, to about 50% of maximum within 5 s.

The molecular receptive range and enantioselectivity

Stimulation with compounds eluting from the polar column

The molecular receptive range was described by stimulating the neurons with headspace samples, essential oils, and synthetic compounds via the polar GC column. Figure 2A shows the gas chromatogram of the headspace sample of the host plant *B. oleracea* var. *italica* and the simultaneously recorded activity of a single RN. The response appeared at the retention time of linalool (11.29 min), present in trace amount below the detection limit of the FID. In addition, weak responses to two other compounds appeared at the retention times 12.47 and 14.07 min. The trace amount did not allow identification of these two compounds by GC–mass spectrometry. Responses during the elution of the large amounts of the solvents (hexane and ethyl acetate) were

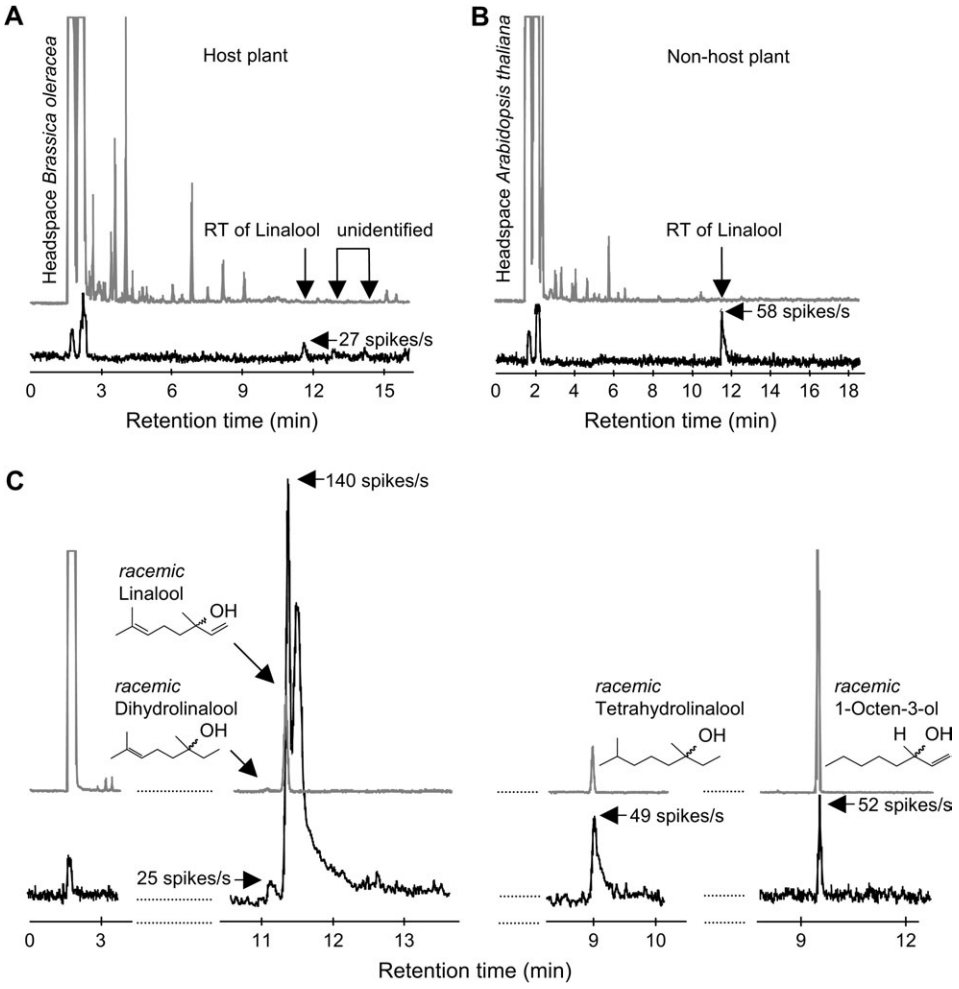

Figure 2 Gas chromatograms of headspace samples of the host *Brassica oleracea* **(A)** and the nonhost *Arabidopsis thaliana* Cvi **(B)** separated in the polar GC column (GC trace above). Below, simultaneously recorded activity of a single RN stimulated with the separated components, showing responses at the retention time (RT) 11.29 min of linalool (trace amount). Two unidentified compounds present in *B. oleracea* elicited weak responses (retention times 12.47 and 14.07 min). Responses to the high concentrations of the solvents (hexane and ethyl acetate) also appeared. **(C)** Responses of the same RN (traces below) to stimulation via the polar GC column (GC trace above) with synthetic material of *racemic* linalool, *racemic* tetrahydrolinalool, and *racemic* 1-octen-3-ol.

also obtained in this type of neuron of high sensitivity. When stimulating with the headspace sample of *A. thaliana*, the most sensitive neurons responded during the elution of the two solvents and at the retention time (11.29 min) of linalool (Figure 2B). Also in this sample, the amount of linalool was below the detection limit of the FID. Altogether, six headspace samples of *Brassica* spp. and *A. thaliana* and two essential oils were tested on this neuron, all eliciting responses at the retention time of linalool. *Racemic* linalool injected in the polar column elicited one strong response to the linalool peak. Also, a weaker response to dihydrolinalool appeared before the elution of linalool. In addition, weak responses were obtained to two structurally related compounds, tetrahydrolinalool and 1-octen-3-ol (Figure 2C). The selectivity for linalool was further demonstrated by no responses to the numerous other compounds present in the standards and headspace samples tested.

Stimulation with compounds eluting from the chiral column

Eight of the twelve RNs (RN no. I, II, V, VIII–XII) were tested with compounds eluted from the chiral column. All of them showed enantioselectivity by responding stronger to (*R*)-(−)-linalool than to (*S*)-(+)-linalool. Approximately a 10-fold stronger effect of the (−)-enantiomer than of (*S*)-(+)-linalool was found for all eight neurons. This is illustrated for RN no. I in Figure 3A where the concentrations 0.05 μg of (*R*)-(−)-linalool and 0.5 μg of (*S*)-(+)-linalool elicited the same firing frequencies. Analysis of the spikes of the two responses showed similar amplitudes and waveforms, indicating that they originated from the same neuron. The 10-fold difference in stimulatory effect was also shown by the dose-response curves obtained by direct stimulation, the (*R*)-(−)-linalool curve shifted 1 log unit to the left of the (*S*)-(+)-linalool curve (Figure 3B). Enantioselectivity was also found for the responses to dihydrolinalool and

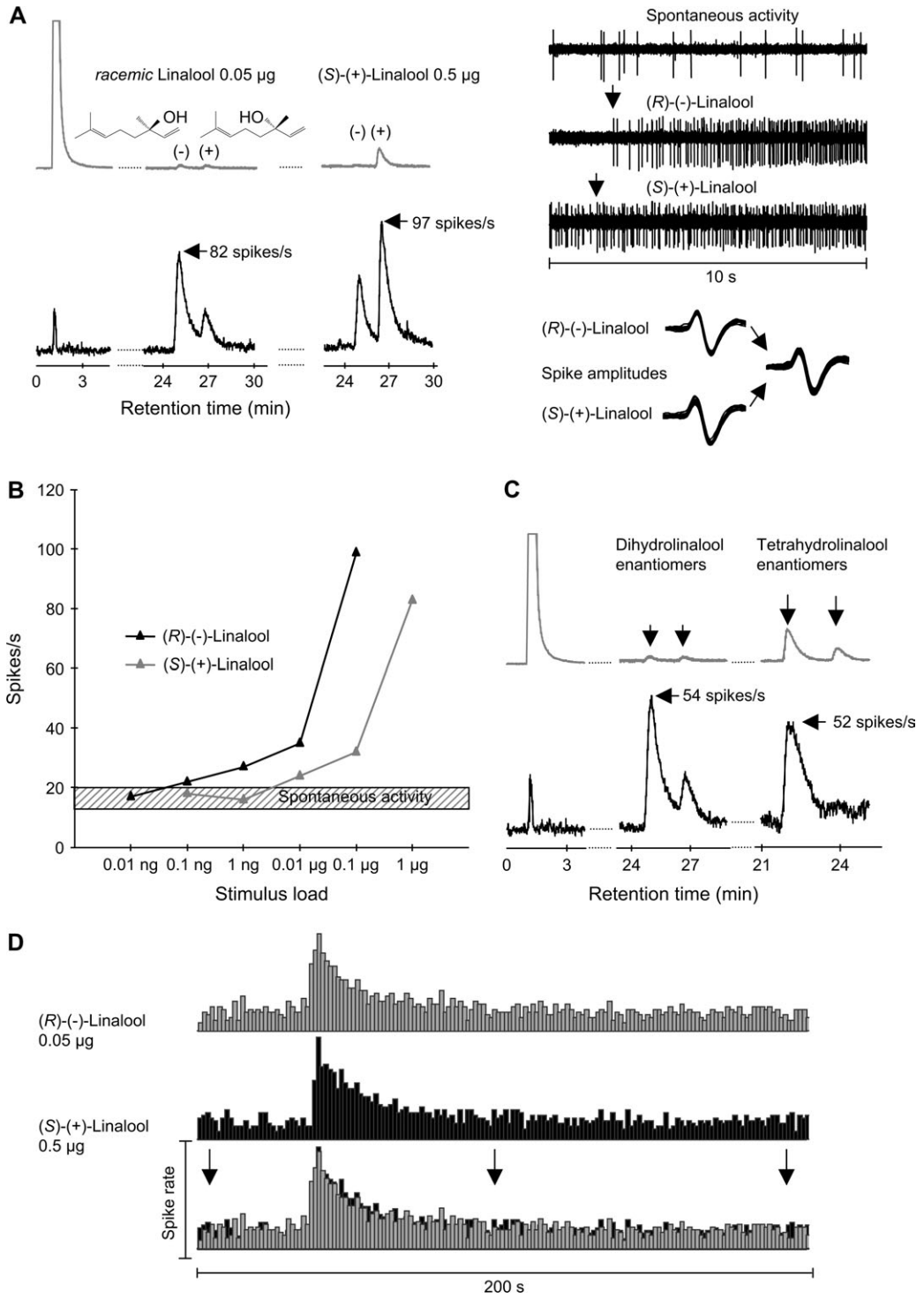

Figure 3 **(A)** Responses of a single RN (traces below) to enantiomers of linalool during elution from a chiral GC column (GC traces above) (left). The responses elicited by solutions with different enantiomeric ratios show about 10 times higher effect of (*R*)-(−)-linalool than (*S*)-(+)-linalool. Traces of the spontaneous activity and the responses to the enantiomers (right). Arrows indicates the start of the stimulations. Overlapping spike waveforms and amplitudes indicated that the responses originated from the same RN. **(B)** Dose-response curves for linalool enantiomers obtained by direct stimulation with two samples, one containing 96% (*S*)-(+)-linalool and the other 97% (*R*)-(−)-linalool, verified a 10-fold stronger effect of (*R*)-(−)-linalool. **(C)** Responses of the RN (traces below) to synthetic *racemic* dihydrolinalool and *racemic* tetrahydrolinalool eluted from the chiral GC column (GC trace above) shows a marked stronger response to one of the enantiomers (the sequences of the enantiomers are unknown). **(D)** A complete overlap of the temporal patterns of the responses to 0.05 µg (*R*)-(−)-linalool and to 0.5 µg (*S*)-(+)-linalool is shown.

tetrahydrolinalool by two RNs (RN no. I and XII) stimulated with the compounds eluted from the chiral GC column (Figure 3C). However, the elution sequence of these enantiomers in the chiral column is not known. Comparison of the responses to 0.05 μg (*R*)-(−)-linalool and to 0.5 μg (*S*)-(+)-linalool showed complete overlap of the temporal response pattern (Figure 3D).

Discussion

Discrimination between odors is based on the presence of a large number of olfactory receptor protein types, each expressed in subsets of sensory neurons, as shown in various vertebrates and insects (Buck and Axel, 1991; Clyne *et al.*, 1999; Krieger and Breer, 1999; Mombaerts, 1999; Vosshall, 2001). The convergence of axons belonging to one subtype in one or two specific glomeruli in the primary olfactory center, the olfactory bulb in vertebrates, and antennal lobe in insects further elucidate the principle called "the molecular logic of smell" (Axel, 1995). Depending on the tuning of the receptors, the information about one compound may be mediated by one or several types of RNs resulting in activation of one or more glomeruli. Among the olfactory RN types recorded in *M. brassicae*, the neurons tuned to linalool constituted the largest group. They all showed similar functional characteristics, indicating that *M. brassicae* detect linalool by one type of RNs that has a narrow tuning to (*R*)-(−)-linalool. This RN type is 10 times more sensitive to (*R*)-(−)-linalool than to (*S*)-(+)-linalool and show the same temporal response pattern to the two enantiomers. It suggests that *M. brassicae* perceive the odors of these enantiomers as similar quality but with different intensity. Although RNs tuned to (*S*)-(+)-linalool have not been found in this species, we can not exclude the possibility that they might be present among the large number of olfactory RNs responding to plant odors, for example, indicated by the numerous ordinary glomeruli (67 ± 1) in the antennal lobe of this species (Rospars, 1983). It is also possible that the two enantiomers of linalool have differential effects on RNs tuned to other compounds, resulting in different across-glomerular stimulation patterns and behavioral discrimination. In another insect species, the strawberry blossom weevil *A. rubi*, two types of linalool RNs have been described, one tuned to the (*S*)-(+)-linalool and the other to the (*R*)-(−)-linalool, both showing considerably lower sensitivity to the opposite enantiomer (Bichão *et al.*, 2005b). So far, the results obtained in these two species indicate that the strawberry blossom weevil may easily discriminate between the two enantiomers, whereas *M. brassicae* may perceive these odors as being of the same or similar quality. How the information about the two enantiomers is represented by specific glomerular activation is not known in these species. However, in another species of moths *Manduca sexta*, two specific glomeruli have been identified that receive enantioselective information about linalool (Reisenman *et al.*, 2004). According to these results, *M. sexta* certainly possesses at least two populations of RNs that discriminate linalool enantiomers. In fact, another study of linalool responding RNs of *M. sexta* showed individual variations of the molecular receptive ranges when directly stimulated with numerous selected compounds (Shields and Hildebrand, 2001). It would be interesting to test these neurons by GC-SCR and with the same protocol as in *M. brassicae*, for comparison of specificity and enantioselectivity.

Linalool is a typical floral constituent produced in a wide range of plants. It is synthesized via a condensation of dimethyl allyl pyrophosphate and isopenthyl pyrophosphate to geranyl diphosphate (GPP) and converted to linalool in a reaction catalyzed by linalool synthase, which is enantioselective (Dudareva *et al.*, 1996; Cseke *et al.*, 1998). Both enantiomers of linalool are present in many plant species, but in a few species only one of them is present (Borg-Karlson *et al.*, 1996; Casabianca *et al.*, 1998). Each inflorescence can produce its own specific composition of the enantiomers (A.-K. Borg-Karlson, unpublished data). It is not known whether *M. brassicae* moths uses a narrow or a broad range of flowering plants for nectar feeding, but their larvae are particularly associated with plants of the genus *Brassica* (Skou, 1991). The enantiomeric ratio of linalool released by these host plants has previously not been reported and was not investigated in the present study by separating headspace samples in the chiral column. However, in *A. thaliana* (Columbia ecotype) of the same family, Brassicaceae, a larger amount of (*R*)-(−)-linalool than (*S*)-(+)-linalool is emitted (ratio 2:1) (Chen *et al.*, 2003). Furthermore, a gene coding for a protein involved in catalyzing GPP to pure (*S*)-(+)-linalool has been identified, which implies that there is also a particular gene coding for the (−)-enantiomer. Emission of linalool in *A. thaliana* (Columbia ecotype) is shown exclusively from the flowering parts, and the release is continuous with no indications of induction by stress (J. Gershenzon, personal communication). If Brassicaceae plants have a similar distribution of linalool enantiomers, we may assume that *M. brassicae* uses (*R*)-(−)-linalool as a cue in the nectar feeding and not in the oviposition. In fact, in wind tunnel experiments, mated *M. brassicae* females did not show upwind flight to stimulation with linalool (Rojas, 1999).

Important in demonstrating enantioselectivity of RNs is to obtain an optimal separation in the chiral GC column. This is in order to make the sensory neurone able to recover enough from adaptation to the first eluted enantiomer before stimulated with the second one, which was the case in the present study. The RN responded to (*S*)-(+)-linalool although it eluted after the stronger response to (*R*)-(−)-linalool. The 10-fold difference of the stimulatory effect of the (−)- and (+)-enantiomers also demonstrated by direct stimulation corresponds to what is found in other enantioselective plant odor RNs in insects when stimulated via the chiral GC column (Stranden *et al.*, 2002, 2003a; Bichão *et al.*,

2005a). The molecular properties of enantiomers are interesting in considering odor-receptor interactions. The present results show that the chiral center at carbon 3 in the linalool molecule is important in the interaction with the receptor of *M. brassicae*. The molecule with hydroxyl group in *R* configuration at carbon 3 in combination with the allylic double bond elicits the highest response, followed by the *S* configuration. A similar ratio of the stimulatory effect can be assumed for the corresponding configurations of the weaker stimulants dihydrolinalool and tetrahydrolinalool, for which the sequence of elution is not known. When stimulating with the smaller molecule, 1-octen-3-ol, lacking both of the methyl groups at carbon 3 and 7, the response is even more reduced. Speculations about the binding between the receptor protein and the linalool enantiomers are difficult without knowing the amino acid sequence of the binding area of the receptor protein.

Because the linalool enantiomers are common in many plant species, it would be interesting to know the enantioselectivity of the receptors in insects of different genera and families as well as in vertebrates. Based on this, it might be possible to figure out why the receptors for detecting these enantiomers have evolved similar or different specificities across species and why some species have one and others have two types of enantioselective linalool receptors.

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References

Axel, R. (1995) *The molecular logic of smell*. Sci. Am., 273, 154–159.

Bichão, H., Borg-Karlson, A.-K., Araújo, J. and **Mustaparta, H.** (2005a) *Five types of olfactory receptor neurons in the strawberry blossom weevil* Anthonomus rubi: *selective responses to inducible host-plant volatiles*. Chem. Senses, 30, 153–170.

Bichão, H., Borg-Karlson, A.-K., Wibe, A., Araújo, J. and **Mustaparta, H.** (2005b) *Molecular receptive ranges of olfactory receptor neurones responding selectively to terpenoids, aliphatic green leaf volatiles and aromatic compounds, in the strawberry blossom weevil* Anthonomus rubi. Chemoecology, 15, 211–226.

Birch, M.C., Light, D.M., Wood, D.L., Browne, L.E., Silverstein, R.M., Bergot, B.J., Ohloff, G., West, J.R. and **Young, J.C.** (1980) *Pheromonal attraction and allomonal interruption of* Ips pini *in California by the two enantiomers of ipsdienol*. J. Chem. Ecol., 6, 703–717.

Borg-Karlson, A.-K., Tengö, J., Valterová, I., Unelius, C.R., Taghizadeh, T., Tolasch, T. and **Francke, W.** (2003) *(S)-(+)-Linalool, a mate attractant pheromone component in the bee* Colletes cunicularius. J. Chem. Ecol., 29, 1–14.

Borg-Karlson, A.-K., Unelius, C.R., Valterova, I. and **Nilsson, L.A.** (1996) *Floral fragrance chemistry in the early flowering shrub* Daphne mezereum. Phytochemistry, 41, 1477–1483.

Brenna, E., Fuganti, C. and **Serra, S.** (2003) *Enantioselective perception of chiral odorants*. Tetrahedron: Assymetr., 14, 1–42.

Buck, L. and **Axel, R.** (1991) *A novel multigene family may encode odorant receptors: a molecular basis for odour recognition*. Cell, 65, 175–187.

Byrne, K.J., Gore, W.E., Pearce, G.T. and **Silverstein, R.M.** (1975) *Porapak-Q collection of airborne organic compounds serving as models for insect pheromones*. J. Chem. Ecol., 1, 1–7.

Casabianca, H., Graff, J.B., Faugier, V., Fleig, F. and **Grenier, C.** (1998) *Enantiomeric distribution studies of linalool and linalyl acetate*. J. High Resolut. Chromatogr., 21, 107–112.

Chen, F., Tholl, D., D'Auria, J.C., Farooq, A., Pichersky, E. and **Gershenzon, J.** (2003) *Biosynthesis and emission of terpenoid volatiles from* Arabidopsis *flowers*. Plant Cell, 15, 1–14.

Christoph, N. and **Drawert, F.** (1985) *Olfactory thresholds of odour stimuli determined by gas chromatographic sniffing technique; structure-activity relationships*. In Berger, R.G., Nitz, S., Schreier, P., Eichhorn, H. and Marzling-Hangenham (eds), Proceedings of the International Conference on Topics in Flavor Research. Freising-Weihenstephan.

Clyne, P.J., Warr, C.G., Freeman, M.R., Lessing, D., Kim, J. and **Carlson, J.R.** (1999) *A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in* Drosophila. Neuron, 22, 327–338.

Cseke, L., Dudareva, N. and **Pichersky, E.** (1998) *Structure and evolution of linalool synthase*. Mol. Biol. Evol., 15, 1491–1498.

Dudareva, N., Cseke, L., Blanc, V.M. and **Pichersky, E.** (1996) *Evolution of floral scent in Clarkia: novel patterns of S-linalool synthase gene expression in the* C. breweri *flower*. Plant Cell, 8, 1137–1148.

Friedman, L. and **Miller, J.G.** (1971) *Odour incongruity and chirality*. Science, 172, 1044–1046.

Hagaman, T.E. and **Cardè, R.T.** (1984) *Effect of pheromone concentrations on organization of preflight behaviours of the male gypsy moth,* Lymantria dispar *(L.)*. J. Chem. Ecol., 10, 17–23.

Hansen, K., Schneider, D. and **Boppré, M.** (1983) *Chiral pheromone and reproductive isolation between the gypsy- and nun moth*. Naturwissenschaften, 70, 466–467.

Hori, M. (1998) *Repellency of rosemary oil against* Myzus persicae *in a laboratory and in a screenhouse*. J. Chem. Ecol., 24, 1425–1432.

König, W.A., Icheln, D., Runge, T., Pforr, I. and **Krebs, A.** (1990) *Cyclodextrins as chiral stationary phases in capillary gas chromatography, part VII: cyclodextrins with an inverse substitution pattern—synthesis and enantioselectivity*. J. High Resolut. Chromatogr., 13, 702–707.

Krieger, J. and **Breer, H.** (1999) *Olfactory reception in invertebrates*. Science, 286, 720–723.

Lanier, G.N., Classon, A., Stewart, T., Piston, J.J. and **Silverstein, R.M.** (1980) Ips pini*: the basis for interpopulational differences in pheromone biology*. J. Chem. Ecol., 6, 677–687.

Leitereg, T.J., Guadagni, D.G., Harris, J., Mon, T.R. and **Teranishi, R.** (1971) *Chemical and sensory data supporting the difference between the odours of the enantiomeric carvones*. J. Agric. Food Chem., 19, 785.

Mombaerts, P. (1999) *Molecular biology of odorant receptors in vertebrates*. Annu. Rev. Neurosci., 22, 487–509.

Mustaparta, H., Angst, M.E. and **Lanier, G.N.** (1980) *Receptor discrimination of enantiomers of the aggregation pheromone ipsdienol, in two species of* Ips. J. Chem. Ecol., 6, 689–701.

Nikonov, A.A. and **Leal, W.S.** (2002) *Peripheral coding of sex pheromone and a behavioural antagonist in the Japanese beetle,* Popillia japonica. J. Chem. Ecol., 28, 1075–1089.

Ochieng, S.A., Park, K.C. and **Baker, T.C.** (2002) *Host plant volatiles synergize responses of sex pheromone-specific olfactory receptor neurones in male* Helicoverpa zea. J. Comp. Physiol. A, 188, 325–333.

Ohloff, G. (1994) *Scent and fragrances. The fascination of odours and their chemical perspectives*. Springer Verlag, Heidelberg, Berlin.

Pham-Delegue, M.H., Etievant, P., Guichard, E. and **Masson, C.** (1989) *Sunflower volatiles involved in honeybee discrimination among genotypes and flowering stages*. J. Chem. Ecol., 15, 329–343.

Pike, L.M., Enns, M.P. and **Hornung, D.E.** (1988) *Quality and intensity of carvone enantiomers when tested separately and in mixtures*. Chem. Senses, 13, 307–309.

Plettner, E., Lazar, J., Prestwich, E.G. and **Prestwich, G.D.** (2000) *Discrimination of pheromone enantiomers by two pheromone binding proteins from the gypsy moth* Lymantria dispar. Biochemistry, 39, 8953–8962.

Reisenman, C.E., Christensen, T.A., Francke, W. and **Hildebrand, J.G.** (2004) *Enantioselectivity of projection neurons innervating identified olfactory glomeruli*. J. Neurosci., 24, 2602–2611.

Rojas, J.C. (1999) *Electrophysiological and behavioural responses of the cabbage moth to plant volatiles*. J. Chem. Ecol., 25, 1867–1883.

Rospars, J.P. (1983) *Invariance and sex-specific variations of the glomerular organization in the antennal lobes of a moth,* Mamestra brassicae *and a butterfly* Pieris brassicae. J. Comp. Neurol., 220, 80–96.

Røstelien, T., Borg-Karlson, A.-K., Fäldt, J., Jacobsson, U. and **Mustaparta, H.** (2000a) *The plant sesquiterpene germacrene D specifically activates a major type of antennal receptor neuron of the tobacco budworm moth* Heliothis virescens. Chem. Senses, 25, 141–148.

Røstelien, T., Borg-Karlson, A.-K. and **Mustaparta, H.** (2000b) *Selective receptor neurone responses to E-β-ocimene, β-myrcene, E,E-α-farnesene and homo-farnesene in the moth* Heliothis virescens*, identified by gas chromatography linked to electrophysiology*. J. Comp. Physiol. A, 186, 833–847.

Shields, V.D.C. and **Hildebrand, J.G.** (2001) *Responses of a population of antennal olfactory receptor cells in the female moth Manduca sexta to plant-associated volatile organic compounds*. J. Comp. Physiol. A, 186, 1135–1151.

Skou, P. (1991) *Nordens Ugler. Håndbog over de i Danmark, Norge, Sverige, Finland og Island forekommende arter af Herminiidae og Noctuidae (Lepidoptera)*. Apollo Books, Stenstrup.

Stranden, M., Borg-Karlson, A.-K. and **Mustaparta, H.** (2002) *Receptor neuron discrimination of the germacrene D enantiomers in the moth* Helicoverpa armigera. Chem. Senses, 27, 143–152.

Stranden, M., Liblikas, I., König, W.A., Almaas, T.J., Borg-Karlson, A.-K. and **Mustaparta, H.** (2003a) *(−)-Germacrene D receptor neurones in three species of heliothine moths: structure-activity relationships*. J. Comp. Physiol. A, 189, 563–577.

Stranden, M., Røstelien, T., Liblikas, I., Almaas, T.J., Borg-Karlson, A.-K. and **Mustaparta, H.** (2003b) *Receptor neurones in three heliothine moths responding to floral and inducible plant volatiles*. Chemoecology, 13, 143–154.

Tumlinson, J.H., Glein, M.G., Doolittle, R.E., Ladd, T.L. and **Proveaux, A.T.** (1977) *Identification of the female Japanese beetle sex pheromone: inhibition of male response by an enantiomer*. Science, 197, 789–792.

Vosshall, L.B. (2001) *The molecular logic of olfaction in* Drosophila. Chem. Senses, 26, 207–213.

Wallner, W.E., Cardé, R.T., Xu-Chonghua, Weselhof, R.M., Xilin, S., Jingjun, Y. and **Schaefer, P.W.** (1984) *Gypsy moth (*Lymantria dispar *L.) attraction to disparlure enantiomers and the olefin precursor in the people's republic of China*. J. Chem. Ecol., 10, 753–757.

Wibe, A. and **Mustaparta, H.** (1996) *Encoding of plant odours by receptor neurons in the pine weevil* (Hylobius abietis) *studied by linked gas chromatography-electrophysiology*. J. Comp. Physiol. A, 179, 331–344.

Wojtasek, H., Hansson, B.S. and **Leal, W.S.** (1998) *Attracted or repelled?—a matter of two neurons, one pheromone binding protein and a chiral center*. Biochem. Biophys. Res. Commun., 250, 217–222.

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Paper III

Methyl salicylate, identified as primary odorant of a specific receptor neuron type, inhibits oviposition by the moth *Mamestra brassicae* L. (Lepidoptera, Noctuidae).

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Abstract

The cabbage moth, *Mamestra brassicae* L. (Lepidoptera, Noctuidae), is a polyphageous species that is often choosing plants of *Brassica* as hosts for oviposition. In the search for biologically relevant odorants used by these moths, gas chromatography linked to electrophysiological recordings from single receptor neurons has been employed, resulting in classification of distinct types of neurons. This study presents specific olfactory receptor neurons responding to methyl salicylate as primary odorant, and showing a weak response to methyl benzoate; the two aromatic compounds occurring together in several plant species. In two cases the neuron was co-located with another receptor neuron type responding to six green-leaf volatiles: 1-hexanol, (3*Z*)-hexen-1-ol, (2*E*)-hexen-1-ol, (3*Z*)-hexenyl acetate, (2*Z*)-hexen-1-ol and an unidentified compound. Whereas the specific receptor neurons detected the minor amounts of methyl salicylate in some plants, the compound was not found by gas chromatography linked to mass spectrometry in intact plants, but it was found after herbivore attack. The behavioural effect of methyl salicylate was studied in outdoor test arenas with *Brassica napus* and artificial plants. These behavioural experiments indicated that mated *Mamestra brassicae* females avoid plants with dispensers emitting methyl salicylate. As it is induced by caterpillar feeding, this compound may mediate a message to mated *Mamestra brassicae* females that the plant is already occupied.

Introduction

Olfactory receptor neurons (RNs) in herbivore organisms may have evolved to detect volatiles that are specifically produced by certain species of host plants or that are widely present in many families of plants. In several species of moths, weevils and beetles, distinct types of olfactory RNs have been found using gas chromatography linked to electrophysiological recordings from single RNs (Røstelien *et al.*, 2000; Stranden *et al.*, 2003a; Stranden *et al.*, 2003b; Røstelien *et al.*, 2005; Bichão *et al.*, 2005a; Bichão *et al.*, 2005b; Ulland *et al.*, 2006). The results have demonstrated RNs of polyphagous as well as oligophagous insects, which respond to compounds present in a wide range of plant species. However, each of the neuron types has appeared with a relatively narrow molecular receptive range, by strongly responding to one primary odorant and weaker to a few structurally similar compounds, termed secondary odorants (Røstelien *et al.*, 2000). In the related heliothine moths, the same functional types of RNs have been found in the polyphagous moths, using a broad range of plants in monocultures (cotton, corn, tomato, maize, etc.) and in oligophagous species, preferring a more narrow range of host plant within the family Solanaceae. In this context, one may ask whether other moth species, like *Mamestra brassicae*, preferring different plant species as hosts, may have evolved olfactory RNs detecting different odorants used in their orientation toward suitable host plants.

Plant defence can be either “constitutive” or “induced”; constitutive defences meaning that the compounds are continuously produced, stored in specialized structures, and released upon attack. Induced defences on the other hand can be triggered by herbivore or pathogen attack (Paré and Tumlinson, 1999; Gouinguené and Turlings, 2002). Induction and release of volatile compounds can also be triggered by abiotic factors, such as UV radiation, ozone and temperature (Johnson *et al.,* 1999; Pichersky and Gershenzon, 2002; De Moraes *et al.*, 2004). An example of a well known compound to be induced is the aromatic compound methylsalicylate (MeS), which is shown to be induced upon attack by herbivores in several

plant species (Van den Boom *et al.*, 2004; Chen *et al.*, 2003; van Poecke *et al.*, 2001; Bichão *et al.*, 2005b). It is also shown that plants can emit specific blends of volatiles that differ depending on the attacking species, even closely related species as shown for *Heliothis virescens* and *Helicoverpa armigera* (De Moraes *et al.*, 1998). Altogether, insects in search for a suitable host plant needs to unravel the vast amount of non-relevant and relevant components released by plants. The challenge is met by the use of a highly sensitive and specialised olfactory system.

The cabbage moth, *M. brassicae*, is widespread around the world, and is common in the southern parts of Norway. The species overwinters as prepupae in the soil close to its host plant. From mid-June to August the adults emerge and after mating the females start searching for a host plant. *M. brassicae* is polyphagous and survives on many species of plants, and the caterpillars are often associated with host plants of the genus *Brassica* (CAB International, 2005; Skou, 1991). Feeding by the caterpillars causes severe damage on the plants, mostly due to chewing and fouling rather than the amount of plant tissue eaten (CAB International, 2005). Plant production in agriculture has been dependent on insecticides for over half a century. The undesirable side effects of many of these insecticides have led to the current focus on research on other methods of protecting plants from insect pests. Thus, identification of biologically relevant odorants provides key compounds to be included in integrated control of herbivorous pest insects. As with other herbivorous moths, the important olfactory cues used by *M. brassicae* include pheromones and plant odours. Whereas the limited number of compounds produced by female moths makes the pheromone blend relatively simple to resolve, it is much more complicated to identify the broad range of numerous plant-produced volatiles that are detected by the olfactory RNs. In *M. brassicae*, specific tuning of single RNs to the female-produced sex pheromones are well described (Renou and Lucas, 1994). Studies on the specificity of the plant odour RNs, including tuning to induced compounds, have just started by using gas chromatography linked to single cell recordings (GC-SCR). In a previous

study, we presented one RN type in *M. brassicae* tuned to linalool (Ulland *et al.*, 2006). In the present study we ask whether *M. brassicae* has other RN types specifically tuned to plant odorants, systemically produced or induced by herbivory. We here present two types of plant odour RNs, one tuned to methylsalicylate (MeS) and the other to green leaf volatiles (GLV). The behavioural effect of MeS was tested in a field bioassay. The results indicated that MeS has an inhibitory effect on the oviposition of mated *M. brassicae* females.

Materials and methods

Electrophysiological experiments

Insect material

M. brassicae pupae were from our cultures at The Norwegian Institute for Agricultural and Environmental Research, Ås, Norway. The sexed pupae were stored in separate containers placed in climate chambers (22 C, 14:10 light:dark regime, onset of dark cycle at 10 a.m.). After eclosion, the adult insects were kept in cylindrical containers (approximately 1350 cm^3) with access to water containing sucrose (5%). The age of adult insects used in the experiments ranged from 3-15 days. Both sexes were used in the experiments.

Headspace samples and synthetic compounds

Volatiles were collected from several plant species using a headspace technique (Byrne *et al.*, 1975; Pham-Delegue *et al.*, 1989; Røstelien *et al.*, 2000). The plants were placed in a closed oven bag (Look®) through which purified air (flow below 40 ml/min) was passed into glass tubes containing the adsorbents Tenax TA and Porapak Q (1:1). The air was purified by a filter of activated charcoal before the intake to the bag, and the collection was carried out for 24 or 48 hours. The trapped volatiles were eluted by filling the glass tube with the solvent (hexane and ethyl acetate, ratio 1:1) and leading it drop by drop into different vials that were stored in a freezer. The plant materials used for collecting volatiles were *Brassica oleracea (L.), Brassica napus (L.)* as well as the related species *Arabidopsis thaliana*, a potential host of *M. brassicae*. With the known genom and pathways of biosynthesis, *A. thaliana* was considered as an interesting species as concerns relevant volatiles. Synthetic material of MeS (99%) and other compounds was also used to stimulate the RNs. Table I gives an overview of the plant material from which volatiles were collected as well as the tested synthetic

compounds, standards and essential oils. The concentrations of all synthetic compounds tested alone as well as those constituting the standards were 1 µg/µl.

Direct stimulation via cartridges

Direct stimulation via glass cartridges was used for screening the RNs for sensitivity to the various headspace samples of plants, essential oils and synthetic mixtures. A five-µl dilution of each sample (1 µg/µl) was applied to a filter paper placed inside the cartridge, letting the solvent evaporate before use. The RN was exposed for the test sample by puffing air (8 ml/s) through the cartridge and over the antenna. Direct stimulation via glass cartridges was also used for determining dose-response curves. In these tests, 100 µl of each MeS solution diluted in decadic steps was applied to a filter paper. The solvent was evaporated by N_2 flow before inserting the filter paper into the glass cartridge. In dose-response experiments, the tests were performed from low to high concentrations (range 0.12 ng to 1.2 µg on each filter paper). Between stimulations, the antenna was exposed to a continuous flow (500 ml/min) of purified air. The inter-stimulus interval varied from one minute at low concentrations to seven minutes at high concentrations.

Gas chromatography linked to single cell recordings, GC-SCR

The insects were mounted in a Plexiglas holder and the head and antennae were stabilized with tape and wax as described by Røstelien *et al.* 2000. Electrophysiological recordings from single RNs were made by the use of electrolytically sharpened tungsten microelectrodes; the recording electrode placed into the base of an olfactory sensillum and the reference electrode into the base of the antenna. The neurons were initially screened for responses to the mixtures of plant odours and single compounds via cartridges. Each sample consisted of 0.5-1 µl of the solution, which was injected into the column of the gas chromatograph (GC). The column was equipped with a splitter at the end, leading half of the effluent to the flame ionization detector

(FID) and the other half into a constant airflow (500 ml/min) blowing over the insect antenna (Røstelien *et al.*, 2000). This made it possible, together with the simultaneous single cell recording, to determine which compounds in the mixture elicited the responses. The spike rate and the gas chromatogram were recorded using EAD software (Syntech, Netherlands), while the spikes were recorded and stored using Spike2 software (Cambridge Electronic Design Limited, Cambridge, Great Britain). The GC was installed with a polar column [DBwax, 25 m, inner diameter (i.d.) 0.25 mm, film thickness 0.25 μm, J&W Scientific]. Separation in the polar column was performed with two different programs, the first and most frequently used program started at an initial temperature of 80°C with an increase in rate of 6°C/min to 180°C, and a further increase in rate of 15°C/min to 220°C. The second program, used to achieve better separation of the compounds in some of the samples, was performed from the initial temperature of 50°C isothermal for 2 min followed by a 3°C/min increase to 180°C, and a final increase of 15°C/min to 220°C. The FID temperature was set to 230°C for all programs. The GC was equipped with a cold on-column injector.

Spike analysis and cell classification

The spikes from RNs were analysed using the software Spike2. Separation of the cell types in one recording was based on differences in spike amplitudes and waveforms. The RNs were classified according to which odorant elicited the strongest response (primary odorant) as well as those having weaker effects (secondary odorants).

Identification of MeS in headspace of cabbage plants.

The Solid Phase Micro Extraction (SPME) technique (Pawliszyn, 1997) was used to collect volatiles released from individual potted cabbage plants (*B. oleracea* var. capitata L.), bearing 6 – 7 leaves. The collection lasted for 24 hours and it was carried out under laboratory conditions at 20:4 h light:dark regime. Two 400W metal halide lamps (Philips HPI-T Plus)

were used as the light source. The temperature was kept at 26 ± 2 °C during the photophase and 24 ± 2 °C during the scotophase. The aboveground part of the plant was placed into a glass jar. At the bottom, the jar was closed by folding-plates covered with aluminium foil to prevent soil odours from entering the jar. Before the collection periods, the routine conditioning of the SPME fibre (100 mm polydimethylsiloxane) was done at 225 °C for about 10 min in a GC injector. The tip of the syringe with the cleaned fibre was then placed in the jar through the inlet hole, which was covered by aluminium foil. Volatiles were collected from five individual healthy plants and five caterpillar-wounded plants (*B. oleracea var. capitata* L.). Three *Pieris napi (L.)* caterpillars of about 8 mm body length were allowed to feed on each plant for 48 hours, and afterwards they were removed from the plant just before sampling. Volatiles were analyzed by means of a Varian 3400 gas chromatograph (GC), connected to a Finnigan SSQ 7000 mass spectrometer (MS). A SPB-1 and DB-Wax silica capillary column (30 m, i.d. 0.25 mm, film thickness 0.25 µm, J & W Scientific) was used with a temperature program of 40 °C (1 min), increased by 4 °C/min to 200 °C, then by 10 °C/min up to 230 °C and thereafter held isothermally at 230 °C for 6 min. The split/splitless injector temperature was 225 °C and the splitless period lasted for 60 s. Helium was used as the carrier gas, with an inlet pressure of 70 kPa. Electron ionization mass spectra were determined at 70 eV with an ion source at 150 °C. MeS was identified by comparison of mass spectral data and GC-retention times of volatiles with the corresponding data of a synthetic standard.

Behavioural experiments

The behavioural experiments were carried out during three separate periods (June 2004, August 2004 and June 2005) at Ås, Akershus, in southern Norway. All experiments took place in outdoor test arenas, consisting of aluminium-framed cages (2x2x2m) covered with plastic mesh (Figure 1). Experiments were performed in three arenas simultaneously. Real

plants of *B. napus* var. *napobrassica* (*L.*) Wilhelmsburger and artificial plants were used separately. The artificial plants, made from paper (four leaves) were placed over a black pot. The test odorants, diluted in hexane (0.15 mg/µl), were applied on a small piece of towel inserted into a 6 mm piece of rubber tube [outer diameter (o.d.) 0.75 mm, i.d. 0.30 mm]. To each of these "test dispensers", 3 µl of the odour solution was added and the hexane was allowed to evaporate before starting the experiment. The release rate of the test odorants (50-100 ng/hour) was measured by collecting a headspace sample of the "test dispenser" by the use of SPME. In all experiments, we used eight plants in each arena. Four of the plants were supplied with a test dispenser while the remaining four served as control plants with empty dispensers. The eight plants were placed in a circle on the floor of the arena in a randomized order; e.g. in one arena, every second plant was a control and in the second and third arena two test and two control plants were placed side by side. Prior to the main experiment we carried out pilot experiments testing four different compounds, including MeS, in each arena. Individual plants were supplied with a test dispenser containing one of the compounds. In the main experiments, we only used test dispensers containing MeS. Before, as well as during the experiments, female and male *M. brassicae* were allowed to mate and drink sucrose water. We used up to 20 insects in each arena, more than half of them being females. Each experiment started shortly before dusk and ended the following morning.

Data analysis

The number of eggs was counted at the end of each experiment. Only eggs oviposited on the plants were included in the data analysis. The number of eggs on the test plants and the control plants was analysed using the Wilcoxon matched pair test.

Results

Electrophysiology

RN responding to MeS

Out of the 43 olfactory RNs classified according to the compounds influencing their activity, six responded to the aromatic MeS. These neurons were tested in total 13 times via the GC (GC = 13) with various headspace samples of plants (Figure 2A) as well as synthetic MeS present in standard 1 (Figure 2B). No differences were noticed between RNs obtained in females and males. The recordings demonstrated high reproducibility of the responses, which appeared as increased firing rate. We did not observe any indications of inhibition, by reduced firing rate or stop of firing as responses to an eluted compound. The selectivity of the RNs appeared by a strong response to MeS (primary odorant) in headspace samples as well as in the standard and a weak response to the aromatic methyl benzoate (MB) (secondary odorant) in the standard. The amounts of MeS and MB in all headspace samples were below the detection limit by the FID. Two other, unidentified compounds (retention times 10.51 min and 15.01 min) present in *A. thaliana*, one of them also present in *B. oleracea* (retention time 15.01 min), activated the RN presented in Figure 2A. However, they had a lower effect than MeS that elicited a response of 25 spikes/s at the trace amount, below the FID detection limit. In comparison the larger amounts of the unidentified compounds appearing in the gas chromatogram elicited a weaker response of ~20 spikes/s, suggesting a role as secondary odorants. Another MeS RN tested for the headspace sample of *A. thaliana* did not show responses to these compounds, probably because of a general lower sensitivity of this RN. Like the other MeS RNs, this RN also responded to the larger amount of MB present in the standard. Although the two other secondary odorants present in *A. thaliana* and *B. oleracea* could be detected by the FID, the amounts were too small to identify their mass spectra. The increased firing rate as a response to the GC eluted MeS followed the concentration profile of

the GC peak, regaining the spontaneous activity within one or a few minutes after the peak. Spike analysis of the responses to MeS and MB showed similar amplitudes and waveforms (Figure 2B), indicating that they originated from the same RN. Dose-dependency was shown by direct stimulation with decadic increase of MeS concentrations over 6 log units (Figure 2C). The threshold concentration for the most sensitive RN was in the range of 1.2 ng-12 ng.

Responses originating from co-located RNs

In recordings from two of the six MeS RNs, an additional RN with large spike amplitudes responded to green leaf volatiles (GLV) present in standard 2 (Figure 3A). Three of the activating compounds, 1-hexanol, (3*Z*)-hexen-1-ol and (2*E*)-hexen-1-ol, elicited relative strong responses in this neuron, whereas three others, (3*Z*)-hexenyl acetate, (2*Z*)-hexen-1-ol and an unidentified compound were less potent. Co-injection of standard 1 and standard 2 containing nearly equal amounts of MeS, MB and the GLV (Figure 3B), showed a much stronger response by the MeS RN (100 spikes/s) than the GLV RN (30 spikes/s). A clear difference between the spike amplitudes and waveforms of the two RNs appeared as shown by the spike analysis in Figure 3B. Thus, the response to the GLVs originated from a different RN, co-located with the MeS RN.

Induction of Methyl salicylate in cabbage plants.

The SPME analyses of headspace samples from intact *B. oleracea* showed no detectable amount of MeS in the GC-MS. Because caterpillars of *M. brassicae* were not available at the time of these experiments, the sympatric *P. napi* was used. After the feeding by *P. napi* caterpillars on the plants for 48 h, MeS could be identified in samples of all five test plants. The amount produced was found to be 0.9 ± 0.4 ng/day (Figure 4).

Behavioural experiments.

Out of 44 behavioural experiments, 14 resulted in oviposition and are included in the results. In the initial six "pilot experiments" using real plants, we never observed oviposition on the plants having dispensers with MeS, only on the control plants and the plants having dispensers with other odorants. In the experiments designed for the present study, testing plants with MeS dispensers contra control plants, eggs were laid in nine of 38 experiments. One of the nine experiments was terminated because of heavy rain and low temperature. Thus, the presented results are based on eight experiments, five with real plants and three with artificial plants (Figure 5A). We never observed differences in oviposition preference between experiments with real and artificial plants. In six experiments, four with real plants and two with artificial plants, oviposition was exclusively on the control plants. The total number of eggs deposited in each of these experiments was in the range of 52 to 300. In only two of the experiments, a few eggs were deposited on the plants with MeS dispensers; in one of the two experiments, four eggs were placed on these plants, as compared to 150 eggs on the control plants. Statistical analysis of the number of eggs deposited on the control plants versus the test plants showed a preference for the control plants (Wilcoxon matched pair test, $P < 0.02$). The overall percentage of eggs deposited on the test plants contra the control plants are shown in Figure 5B.

Discussion

In principle, there are two approaches to study and identify important plant volatiles used by herbivorous insects: a top down and a bottom up approach. The former starts with the behavioral responses to the full bouquet of volatiles released by a plant, and then narrow down the blend by separating fractions containing the effective compounds (D'Alessandro and Turlings, 2005). The present study can be considered a bottom-up approach, starting with identifying the plant-released volatiles that are detected by the RNs and leaving behavioral studies to the final stage. The two approaches have particular advantages and challenges; the former giving direct behavioral information, but having difficulties in identifying the minor components in the effective fractions. The advantage of the latter approach, using GC-SCR, is that relevant odorants present in minor amounts, even below the detection limit of the GC, can be identified because of the very sensitive RNs. This is indeed shown in the present study for the MeS RN that responded to the odorant in the headspace samples of the host plants at amounts below the detection limit of the FID (Figure 2A). By testing the synthetic standard, the RN showed a strong response to the MeS peak having the same retention time as the effective component of the headspace sample (Figure 2B). Similarly, the structurally related MB, present as a minor component or absent in some headspace samples, was identified as a second compound eliciting a weak response in this neuron type. The two other compounds present in *A. thaliana* and *B. oleracea*, eliciting marked weaker responses than MeS, could not be identified. They were present in too small amounts for GC-MS identification and they were not among available compounds present in the standards. A way to identify these compounds would be to test the RNs for headspace samples containing a larger quantity of them, e.g. samples of other plant species (Røstelien et al 2000).

The results of the present study add to the growing data on narrowly tuned RNs responding to plant odorants in herbivorous insects. Each RN, tested for hundreds of volatiles naturally produced by a plant, responds to only one or to a few compounds of related

chemical structures. In the latter case, one compound has a marked strongest effect, and is termed primary odorant, whereas those of weaker effects are termed secondary odorants. This is typically exemplified by the MeS RN type. The marked higher effect of MeS qualified for the term primary odorant, whereas MB and the two unknown compounds were termed secondary odorants. The other RN type, appearing co-located with the MeS RN in two recordings, responded to six structurally related compounds; three of them, 1-hexanol, (3*Z*)-hexen-1-ol, (3*Z*)-hexenyl acetate, having a marked best effect. These rarely appearing GLV RNs need to be further tested for dose-response properties, in order to precisely determine the specificity. A relevant question is whether one of the compounds has a marked best effect as primary odorant or if all of the three flexible molecules fit into the same receptor pocket with similar receptor affinity. Although responding to six odorants, these RNs are also quite narrowly tuned, considering the numerous compounds in the various mixtures that had no effect. On the basis of the primary odorant and the molecular receptive ranges, the plant odour RNs in *M. brassicae* as well as in all species studied by GC-SCR fall into distinct functional types (Røstelien *et al.*, 2000; Stranden *et al.*, 2003a; Stranden *et al.*, 2003b; Røstelien *et al.*, 2005; Bichão *et al.*, 2005a; Bichão *et al.*, 2005b; Blight *et al.*, 1995; Stensmyr *et al.*, 2001; Barata *et al.*, 2002; Wibe *et al.*, 1997). This correlates well with the general principle that subsets of olfactory RNs express only one type of receptor proteins in insects (Störtkuhl and Kettler, 2001; Wetzel *et al.*, 2001; Keller and Vosshall, 2003; Hallem and Carlson, 2004; Clyne *et al.*, 1999). Thus, the recorded responses from each RN reflect the specificity of one expressed receptor protein type, e.g. specified for MeS.

The results on the narrow tuning of the plant odour RN types *M. brassicae* is in accordance with results obtained in other species using the method of GC-SCR. In the studies of heliothine moths and herbivorous weevils, carried out in the same lab using similar test protocols, has made it possible to compare the RN specificity across the species (Røstelien *et al.*, 2000; Stranden *et al.*, 2003b; Røstelien *et al.*, 2005; Bichão *et al.*, 2005a; Bichão *et al.*,

2005b). For instance, in the strawberry blossom weevil *Anthonomus rubi* one type of RN responding to MeS has been functionally characterized (Bichao *et al*., 2005b). Like in *M. brassicae*, they showed a weak response to MB. However, the RNs in *A. rubi* in addition responded weakly to ethyl benzoate, in contrast to the MeS RNs of *M. brassicae*. Thus, in the adaptation to different host plants these two distantly related insect species have evolved RNs for MeS with slightly different molecular receptive ranges. Olfactory RNs responding to MeS have also been shown in two other species, the cabbage seed weevil, *Ceutorhynchus assimilis* (Blight *et al.*, 1995) and the fruit chafer, *Pachnoda marginata* (Stensmyr *et al.*, 2001), by the use of GC-SCR. However, because of different test protocols, comparison of the RN specificity cannot be done between these species and *M. brassicae* and *A. rubi*. Sensitivity to MeS and some of the GLVs presented in this study have previously been shown in *M. brassicae* by the use of GC-EAGs (Rojas, 1999a). Whereas the tool of EAG recordings is suitable for screening the antennae for general sensitivity to various volatiles, the techniques of GC-SCR give precise information about the specificity of single RNs. Large groupings of RNs responding weakly to secondary odorants may result in relatively strong EAG responses, whereas small groups of RNs specifically responding to a primary odorant may elicit small EAGs. Thus, the contribution of the present results is that *M. brassicae* has evolved specific RNs for detecting MeS as well as GLVs.

Another interesting aspect of olfactory RNs is how the molecular receptive ranges correlate with the products of the biosynthetic pathways in host plants. In recent studies the biosynthesis of MeS and MB have been resolved in several plant species (Dudareva *et al.*, 1998; Chen *et al.*, 2003; Dudareva *et al.*, 2004). Both the MeS and the MB synthesis are catalysed by methyltransferases, whereby a methyl group is transferred from the donor molecule *S*-adenosine-*L*-methionine to the carboxyl group of salicylic acid (SA) or benzoic acid (BA), respectively (Chen *et al.*, 2003). Like in many species, including the snapdragon, *Antirrhinum majus*, it has been shown that a methyltranferase catalyses the production of MeS

along with a small amount of MB (Negre *et al.*, 2002). The large number of plant species emitting MeS with a smaller amount of MB suggests that MeS is the important compound, whereas MB apparently does not play a significant role in insects using these plants as hosts. In fact, no MB was detected by the RNs in our headspace samples. The two compounds MeS and MB, activating the same RN, may be perceived by *M. brassicae* as the same odour quality but with different intensity. In contrast, humans clearly discriminate the two floral compounds that are perceived as two distinctly different odour qualities; MeS containing the hydroxyl group is experienced as a sweet odour, whereas MB is not as pleasant.

The second part of this study concerns the behavioural effect of MeS. The present finding that *M. brassicae* in general avoids ovipositing on plants with MeS dispensers is in accordance with results obtained in a previous behavioural study of *M. brassicae*. By the use of wind-tunnel experiments, Rojas (1999a) found that *M. brassicae* did not show upwind flight to a cotton wool wick loaded with MeS. In our study, we only found a few eggs on plants with MeS dispensers in two of the experiments. In comparison, most experiments showed 50-300 eggs oviposited on the control plants, indicating that the females reject plants with MeS that they are able to detect by the very sensitive and narrowly tuned olfactory RNs on the antennae. MeS is shown to be induced in several plant species during herbivore attack (Van den Boom *et al.*, 2004; Chen *et al.*, 2003; Bichão *et al.*, 2005b). For instance, herbivory by *Pieris rapae* larvae has previously been shown to induce emission of MeS in *A. thaliana* (van Poecke *et al.*, 2001), a potential host plant of *M. brassicae*. In this study, MeS was shown to be induced in the host plant *B. oleracea*. We used caterpillars of *P. napi* but assume a similar induction of MeS caused by *M. brassicae* caterpillars. In any case, it might be important for mated *M. brassicae* females to detect which host individuals that are already occupied. These findings seems contradictory to those of wind tunnels experiments presented by Rojas (1999b). He found that *M. brassicae* females are attracted to and oviposit more eggs on plants (cabbage and tomato) damaged by female locusts (*Schistocerca gregaria*), and

orient more often to cabbage plants damaged by conspecific caterpillars than to undamaged plants. Several reasons may underline the different results. Obviously numerous plant-released volatiles are involved in host plant attraction and avoidance, and damaged plant release larger quantities of volatiles than undamaged plants. Whereas our results were based on comparison of the behavioural effect of only MeS added to artificial or to intact plants, the results of Rojas involves the whole bouquet of systemically or induced plant released volatiles. Thus other induced compounds with strong attractive effect may have been involved in the experiments by Rojas.

In the only experiment terminated because of heavy rain, eggs were found on test plants with MeS dispensers. The reason for terminating this experiment was that the release of volatiles would be low in the cold weather and the motivation of the moths might be to search for shelter under such conditions rather than a suitable oviposition site. In a few other experiments carried out during cold weather and heavy rain resulting in no oviposition, the moths flew to the closest plant apparently for finding shelter. However, the possibility exists that one female actually chose the plant emitting MeS. The existence of individuals, with a different preference (e.g. for plants with MeS), may reflect a built-in flexibility that enables species to cope with changing conditions in the environment as discussed by Schoonhoven *et al.* (2005). In the case of a very polyphagous species like *M. brassicae*, this possibility should not be ignored.

The present study has elucidated the sensory significance and indicated a behavioural effect of MeS showing its specific effect as primary odorant on one RN type, which mediates avoidance or inhibition of oviposition on artificial and intact plants to which MeS is added. Future experiments should follow up concentration dependency with additional behavioural studies. This should include tests with MeS added to undamaged as well as damaged plants. Also, the behavioural significance of the GLV, activating the other RN type in this study, should be tested. One might expect that these compounds have the opposite effect of MeS,

since one of them, (3*Z*)-hexenyl acetate, has previously been shown to induce upwind flight of mated *M. brassicae* females in wind tunnel experiments (Rojas, 1999a). Obviously, many more odorants are involved in host plant localization and selection, and the challenge in future experiments are to find out the behavioural relevance of all plant odorants and their mixtures. For practical application of the results, supplemental studies should be done to determine whether mechanical damage in the absence of insect feeding would induce MeS production. If this is the case, well-timed mechanical damage could help prevent attack by *M. brassicae*.

Acknowledgements

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References

Barata EN, Mustaparta H, Pickett JA, Wadhams LJ and Araújo J. 2002. Encoding of host and non-host plant odours by receptor neurones in the eucalyptus woodborer, *Phoracantha semipunctata* (Coleoptera: Cerambycidae). J Comp Physiol A 188**:** 121-133.

Bichão H, Borg-Karlson A-K, Wibe A, Araújo J and Mustaparta H. 2005a. Molecular receptive ranges of olfactory receptor neurones responding selectively to terpenoids, aliphatic green leaf volatiles and aromatic compounds, in the strawberry blossom weevil *Anthonomus rubi*. Chemoecology 15**:** 211-226.

Bichão H, Borg-Karlson A-K, Araújo J and Mustaparta H. 2005b. Five Types of Olfactory Receptor Neurons in the Strawberry Blossom Weevil Anthonomus rubi: Selective Responses to Inducible Host-plant Volatiles. Chem Senses 30**:** 153-170.

Blight MM, Pickett JA, Wadhams LJ and Woodcock CM. 1995. Antennal perception of oilseed rape, *Brassica napus* (Brassicaceae), volatiles by the cabbage seed weevil *Ceutorhynchus assimilis* (Coleoptera, Curculionidae). J Chem Ecol 21**:** 1649-1664.

Byrne KJ, Gore WE, Pearce GT and Silverstein RM. 1975. Porapak-Q collection of airborne organic compounds serving as models for insect pheromones. J Chem Ecol 1**:** 1-7.

CAB International (2005) *Mamestra brassicae.* In: Crop Protection Compendium. CAB International, Wallingford.

Chen F, D'Auria JC, Tholl D, Ross JR, Gershenzon J, Noel JP and Pichersky E. 2003. An *Arabidopsis thaliana* gene for methylsalicylate biosynthesis, identified by a biochemical genomics approach, has a role in defense. The Plant Journal 36**:** 577-588.

Clyne PJ, Warr CG, Freeman MR, Lessing D, Kim J and Carlson JR. 1999. A novel family of divergent seven-transmembrane proteins: Candidate odorant receptors in *Drosophila*. Neuron 22**:** 327-338.

D'Alessandro M and Turlings TCJ. 2005. In Situ Modification of Herbivore-Induced Plant Odours: A Novel Approach to Study the Attractiveness of Volatile Organic Compounds to Parasitic Wasps. Chem Senses 30**:** 739-753.

De Moraes CM, Lewis WJ, Paré PW, Alborn HT and Tumlinson JH. 1998. Herbivore-infested plants selectively attract parasitoids. Nature 393**:** 570-573.

De Moraes, CM, Schultz JC, Mescher MC and Tumlinson JH. 2004. Induced plant signaling and its implications for environement sensing. J Toxicol Environ Health A 67**:** 819-834.

Dudareva N, Pirchersky E and Gershenzon J. 2004. Biochemistry of Plant Volatiles. Plant Physiol 135**:** 1893-1902.

Dudareva N, Raguso RA, Wang J, Ross JR and Pichersky E. 1998. Floral Scent Production in Clarkia breweri[1]. Plant Physiol 116**:** 599-604.

Gouinguené S and Turlings TCJ. 2002. The effects of abiotic factors on induced volatile emissions in corn plants. Plant Physiol 129**:** 1296-1307.

Hallem EA and Carlson JR. 2004. The odor coding system of Drosophila. Trends Genetics 20**:** 453-459.

Johnson CB, Kirby J, Naxakis G and Pearson S. 1999. Substantial UV-B-mediated induction of essential oils in sweet basil (Ocimum basilicum L.). Phytochemistry 51**:** 507-510.

Keller A and Vosshall LB. 2003. Decoding olfaction in *Drosophila*. Curr Opin Neurobiol 13**:** 103-110.

Negre F, Kolosova N, Knoll J, Kish CM and Dudareva N. 2002. Novel *S*-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase, an enzyme responsible for biosynthesis of methyl salicylate and methyl benzoate, is not involved in floral scent production in snapdragon flowers. Arch Biochem Biophys 406**:** 261-270.

Paré PW and Tumlinson JH. 1999. Plant Volatiles as a Defense against Insect Herbivores. Plant Physiol 121**:** 325-331.

Pawliszyn J. 1997. Solid-Phase Microextraction: Theory and Practice. VCH New York 275.

Pham-Delegue MH, Etievant P, Guichard E and Masson C. 1989. Sunflower volatiles involved in honeybee discrimination among genotypes and flowering stages. J Chem Ecol 15**:** 329-343.

Pichersky E and Gershenzon J. 2002. The formation and function of plant volatiles: perfumes for pollinator attraction and defense. Curr Opin Plant Biol 5**:** 237-243.

Renou M and Lucas P. 1994. Sex Pheromone Reception on *Mamestra brassicae* L. (Lepidoptera): Responses of Olfactory Receptor Neurones to Minor Components of the Pheromone Blend. J Insect Physiol 40**:** 75-85.

Rojas JC. 1999a. Electrophysiological and behavioural responses of the cabbage moth to plant volatiles. J Chem Ecol 25**:** 1867-1883.

Rojas JC. 1999b. Influence of Host Plant Damage on the Host-Finding Behavior of *Mamestra brassicae* (Lepidoptera: Noctuidae). Environ Entomol 28**:** 588-593.

Røstelien T, Borg-Karlson A-K, Fäldt J, Jacobsson U and Mustaparta H. 2000. The plant sesquiterpene germacrene D specifically activates a major type of antennal receptor neuron of the tobacco budworm moth *Heliothis virescens*. Chem Senses 25**:** 141-148.

Røstelien T, Stranden M, Borg-Karlson A-K and Mustaparta H. 2005. Olfactory receptor neurones in two heliothine moth species responding selectively to aliphatic green leaf volatiles, aromatics, monoterpenes and sesquiterpenes of plant origin. Chem Senses 30**:** 443-461.

Schoonhoven L.M., Van Loon J.J.A. and Dicke M. 2005 Insect-Plant Biology. Oxford University Press.

Skou P. 1991 Nordens Ugler. Håndbog over de i Danmark, Norge, Sverige, Finland og Island forekommende arter af Herminiidae og Noctuidae (Lepidoptera). Apollo Books, Stenstrup.

Stensmyr MC, Larsson MC, Bice S and Hansson BS. 2001. Detection of fruit- and flower-emitted volatiles by olfactory receptor neurons in the polyphagous fruit chafer *Pachnoda marginata* (Coleoptera: Cetoniinae). J Comp Physiol A 187**:** 509-519.

Störtkuhl KF and Kettler R. 2001. Functional analysis of an olfactory receptor in *Drosophila melanogaster*. PNAS 98**:** 9381-9385.

Stranden M, Liblikas I, König WA, Almaas TJ, Borg-Karlson A-K and Mustaparta H. 2003a. (-)-Germacrene D receptor neurones in three species of heliothine moths: structure-activity relationships. J Comp Physiol A 189**:** 563-577.

Stranden M, Røstelien T, Liblikas I, Almaas TJ, Borg-Karlson A-K and Mustaparta H. 2003b. Receptor neurones in three heliothine moths responding to floral and inducible plant volatiles. Chemoecol 13**:** 143-154.

Ulland, S, Ian, E, Borg-Karlson, A-K and Mustaparta, H. 2006. Discrimination between Enantiomers of Linalool by Olfactory Receptor Neurons in the Cabbage Moth *Mamestra brassicae* (L.). Chem Senses 31**:** 325-334.

Van den Boom CEM, Van Beek TA, Posthumus MA, De Groot, A and Dicke M. 2004. Qualitative and quantitative variation among volatile profiles induced by *Tetranychus urticae* feeding on plants from various families. J Chem Ecol 30**:** 69-89.

van Poecke RMP, Posthumus MA and Dicke M. 2001. Herbivore-induced volatile production by *Arabidopsis thaliana* leads to attraction of the parasitoid *Cotesia rubecula*: Chemical, behavioural, and gene-expression analysis. J Chem Ecol 27**:** 1911-1928.

Wetzel CH, Behrendt H-J, Gisselmann G, Störtkuhl KF, Hovemann B and Hatt H. 2001. Functional expression and characterization of a *Drosophila* odorant receptor in a heterologous cell system. PNAS 98**:** 9377-9380.

Wibe A, Borg-Karlson A-K, Norin T and Mustaparta H. 1997. Identification of plant volatiles activating single receptor neurons in the pine weevil (*Hylobius abietis*). J Comp Physiol A 180**:** 585-595.

Figure 1 Scheme of the arena used in the behavioural experiments. The plants were placed on the ground, randomly distributed in a circle. The insects were released from the middle of the cage (*i*) prior to scotophase.

Figure 2 A-B Gas chromatogram (upper trace) of samples injected in the polar GC-column and simultaneously recorded activity (lower trace) of a RN. (**A)** The RN responded to two compounds in the sample of *B. oleracea*, one unidentified (15.01 min) and MeS (16.12 min). The trace amount of MeS in both headspace samples was below the detection limit of the FID but still detected by the RNs (left). The RN responded to three compounds in the headspace sample of *A. thaliana sp.*, two unidentified (10.51 min and 15.01 min) and MeS (16.12 min) (right). (**B)** The RN responded to MB (13.13 min) and MeS (16.12 min) present in the synthetic standard 1 (left). Spike traces of spontaneous activity and responses to MB and MeS (right). Spike analysis of the two responses showed overlap of spike shape and amplitude. (**C)** Dose response curve based on stimulation via cartridges containing MeS in decadic concentration steps from (0.12 ng-1.2 µg). Threshold concentration of the RN was in the range of 1.2 ng – 12 ng.

Figure 3 Gas chromatogram (upper trace) of sample injected in the polar GC-column and simultaneously recorded activity (lower trace) of the RNs. (**A)** The RN responded to the eluents of: (3*Z*)-hexenyl acetate, 1-hexanol, (3*Z*)-hexen-1-ol, (2*E*)-hexen-1-ol and (2*Z*)-hexen-1-ol. **(B)** Co-injection of synthetic standard 1 and 2 and the responses of the RNs to the eluents of: 1-hexanol, (3*Z*)-hexen-1-ol, (2*E*)-hexen-1-ol, MB and MeS (right). Spike analysis showed that the first three responses originated from a different RN than the two last responses (left).

Figure 4 Total ion chromatogram records of volatiles obtained from *B. oleracea* plants. (**A)** Intact plant before introduction of caterpillars. (**B)** Plant wounded by three *P. napi* caterpillars. Total ion chromatogram in the range *m/z* 30 - 400; SPB-1 fused silica capillary column; peak size represents about 1 ng of MeS.

Figure 5 (**A)** Histogram of eggs oviposited on plants with MeS and control plants, which resulted from 8 experiments carried out at night. Experiment 1-5, natural plants. Experiment 6-8, artificial plants. (**B)** Percentage of eggs oviposited on the control plants as compared to plants with MeS dispensers, showing a significantly higher number of eggs deposited on the control plants (99.6%, Wilcoxon matched pair test, $P < 0.02$).

Table I Plant material and compounds used to stimulate the MeS and GLV RNs

Chemical standards

Standard 1	**Standard 2**	**Standard 3**	**Standard 4**	**Standard 5**
(*Z*)- and (*E*)-β-Ocimene (70%)/ Limonene (25%)[a]	(2*E*)-Hexenal[f]	Ethyl 2-methylbutyrate[j]	Ethyl butyrate[j]	Ethyl 2-methylpropanoate[j]
Camphor[b]	3-Octanone[d]	*i*-Propyl butanoate[j]	Butyl propionate[j]	Methyl methylbutyrate[j]
racemic Linalool[a]	(3*Z*)-Hexenyl acetate[f]	Butyl butyrate[j]	2-Methylbutyl propanoate[j]	Propyl butyrate[j]
Methyl benzoate[c]	1-Hexanol[g]	(2*E*)-Hexenal[j]	3-Methyl-1-butanol[j]	i-Butyl butyrate[j]
Isoborneol[d]	(3*Z*)-Hexen-1-ol[h]	(2*E*)-Hexenyl acetate[j]	2-Methyl-1-butyl butyrate[j]	(±)-2-Methyl-1-butanol[j]
(+)-*trans*-Verbenol[d]	(2*E*)-Hexen-1-ol[g]	Butyl hexanoate[j]	Pentyl butyrate[j]	Hexyl formate[j]
Methyl salicylate[e]	(2*Z*)-Hexen-1-ol[g]	2-Methylhexyl butanoate[j]	Hexyl propanoate[j]	Butyl pentanoate[j]
	1-Heptanol[i]	3-Methylbutyl hexanoate[j]	Hexyl-2-methyl butyrate[j]	i-Butyl hexanoate[j]
	1-Octanol[h]	(3Z)-Hexenyl hexanoate[j]	2-Methyl-1-butyl hexanoate[j]	Hexyl butyrate[j]
			Hexyl hexanoate[j]	Pentyl hexanoate[j]
			Ethyl 2,4-decadienoates[j]	Butyl octanoate[j]
				2,3-Butan-diol[j]

Essential oils	**Headspace samples**	**Synthetic compounds**
Basil (*Ocimum basilicum* L.)[k]	*Arabidopsis thaliana* sp.[l]	Ethyl benzoate[c]
Lilac (*Syringa vulgaris* L.)[k]	*Brassica napus*[l]	Methyl salicylate[e]
Ylang ylang (*Cananga odorata* Hook)[f]	*Brassica oleracea*[l]	*o*-,*p*-,*m*- Methylanisol[d]
		(-)-Verbenone
		(-)-*trans*-Verbenol

Sources: [a]Fluka; [b]Kebo; [c]Lancaster; [d]Borg-Karlson, KTH, Sweden; [e]Merck; [f]Dragoco; [g]Aldrich; [h]Sigma; [i]Janssen Chimika; [j]Synthesised by Ilme Liblikas; [k]NMD (”Norsk Medisinal Depot”); [l]Headspace sample, Ulland, NTNU, Norway

Figure 1

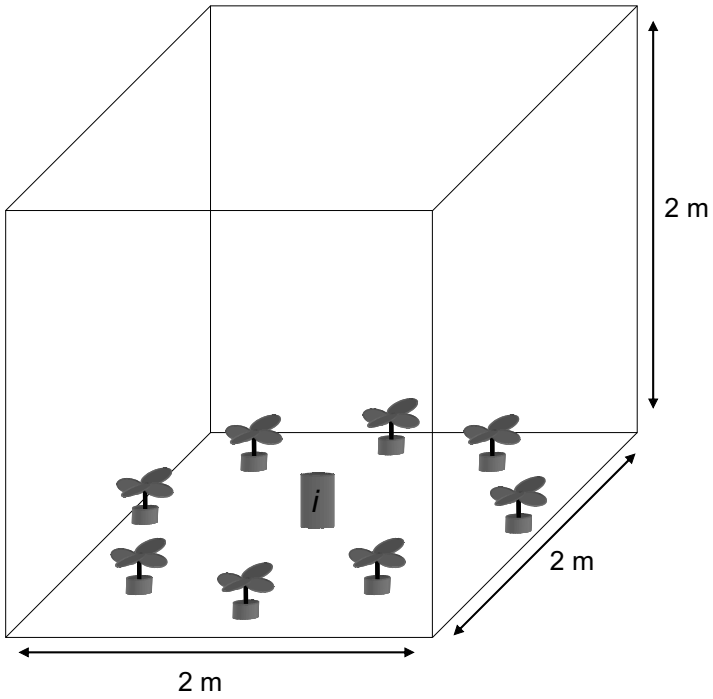


Figure 2

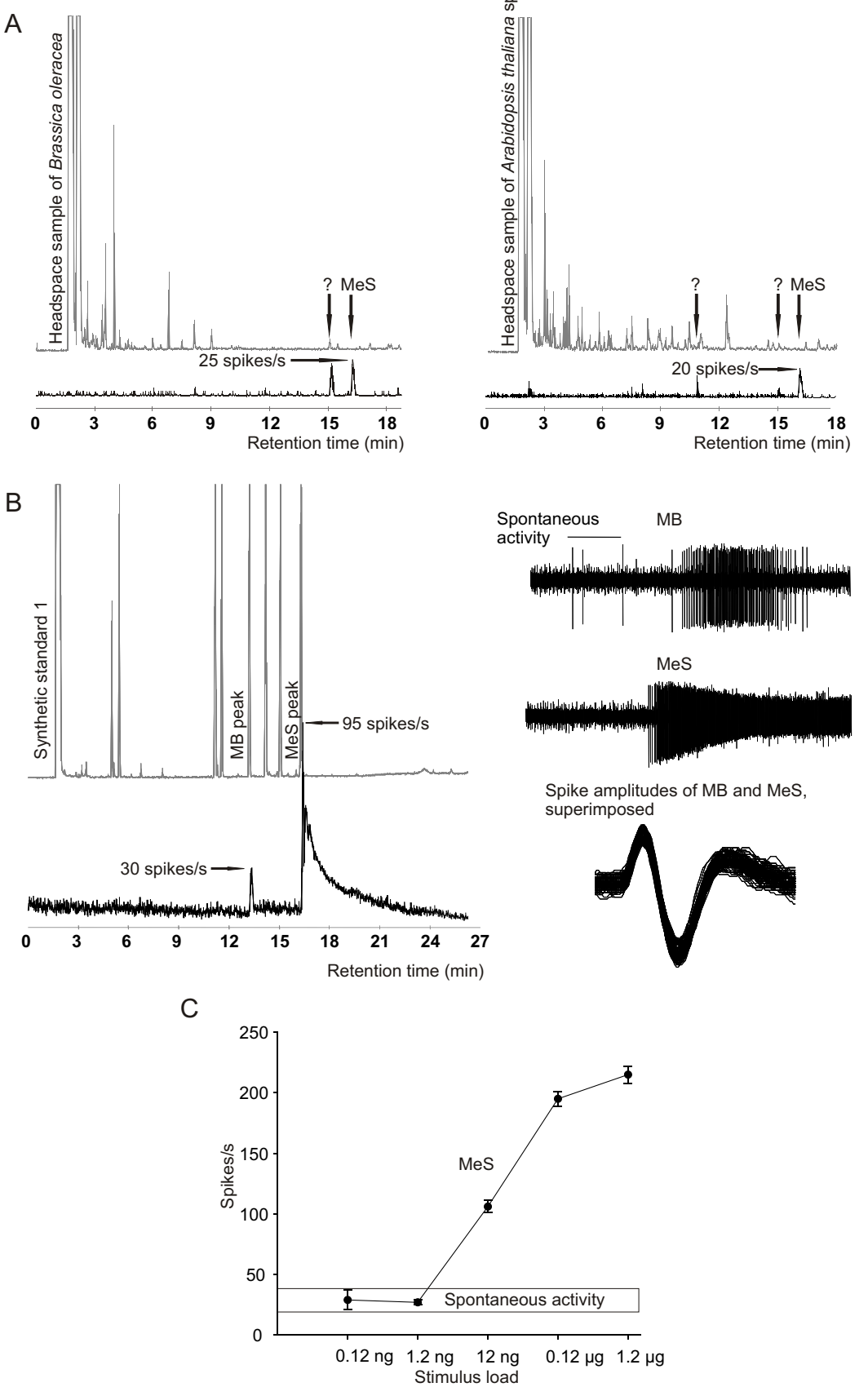

A
Headspace sample of Brassica oleracea
? MeS
25 spikes/s
0 3 6 9 12 15 18
Retention time (min)
Headspace sample of Arabidopsis thaliana sp.
? ? MeS
20 spikes/s
0 3 6 9 12 15 18
Retention time (min)
B
Synthetic standard 1
MB peak
MeS peak
95 spikes/s
30 spikes/s
0 3 6 9 12 15 18 21 24 27
Retention time (min)
Spontaneous activity
MB
MeS
Spike amplitudes of MB and MeS, superimposed
C
Spikes/s
250
200
150
100
50
0
MeS
Spontaneous activity
0.12 ng 1.2 ng 12 ng 0.12 µg 1.2 µg
Stimulus load

Figure 3

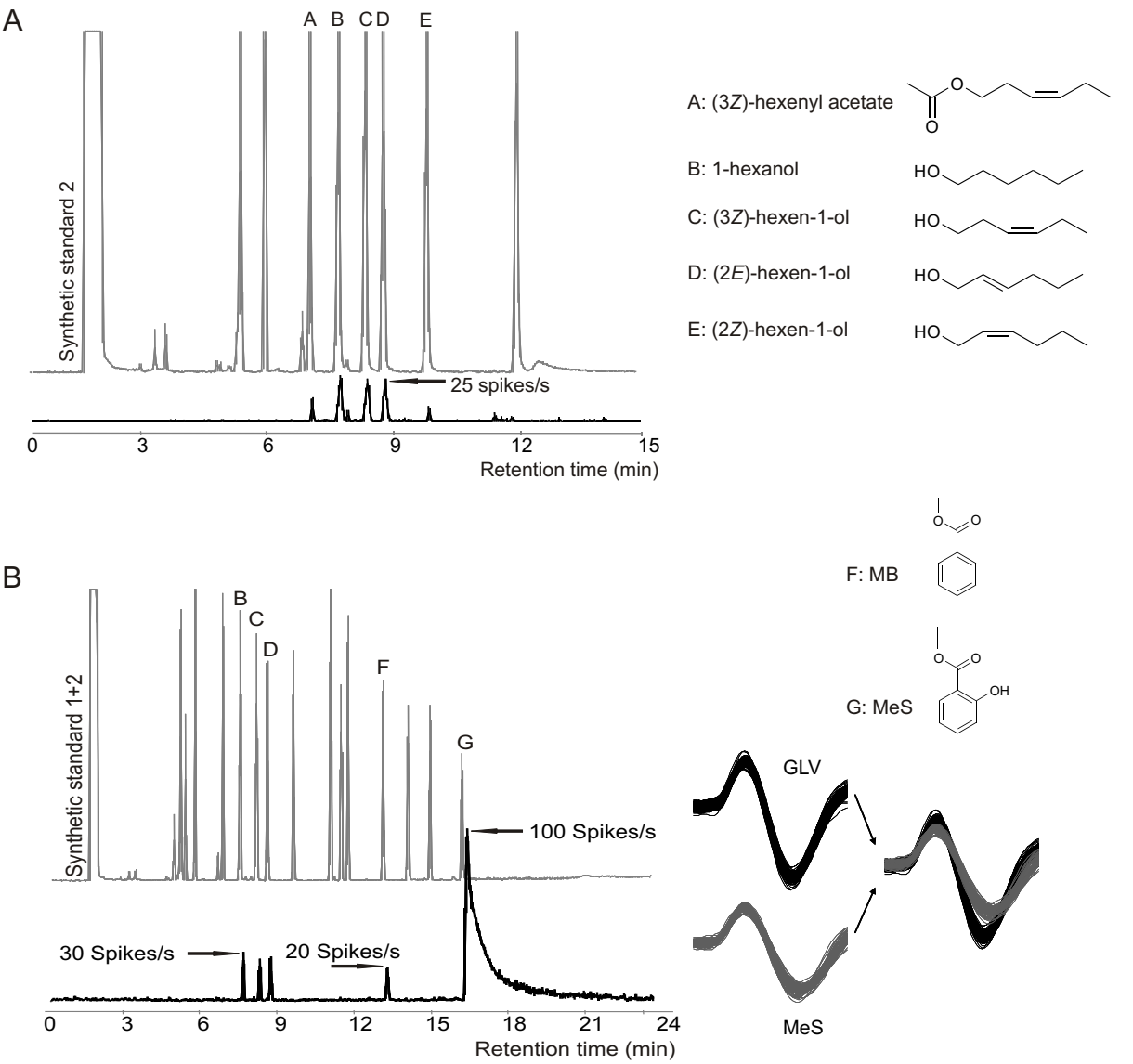

A
A B C D E
Synthetic standard 2
25 spikes/s
0 3 6 9 12 15
Retention time (min)
A: (3Z)-hexenyl acetate
B: 1-hexanol
C: (3Z)-hexen-1-ol
D: (2E)-hexen-1-ol
E: (2Z)-hexen-1-ol
HO
O
B
B C D F G
Synthetic standard 1+2
100 Spikes/s
30 Spikes/s
20 Spikes/s
0 3 6 9 12 15 18 21 24
Retention time (min)
F: MB
G: MeS
OH
GLV
MeS

Figure 4

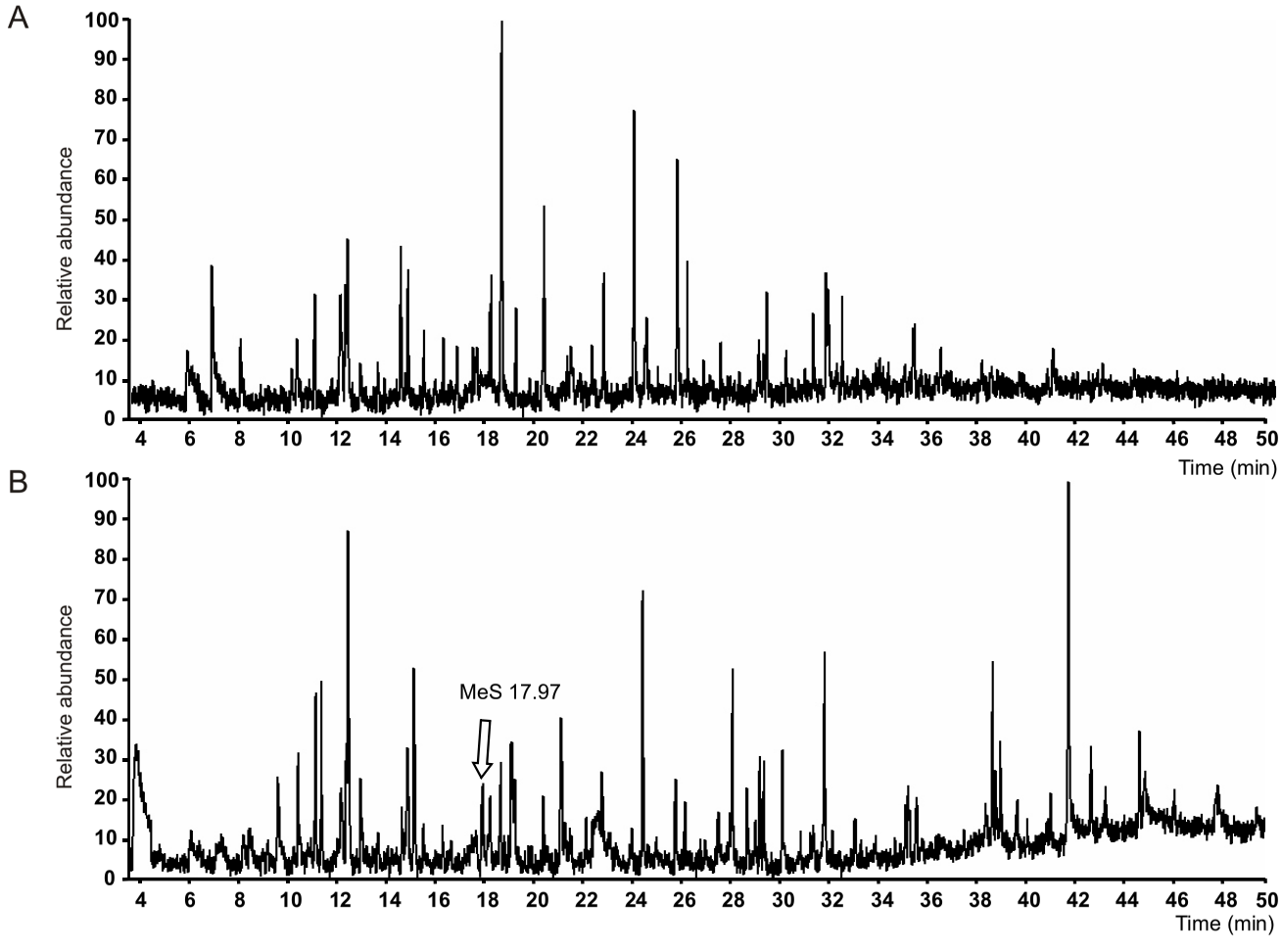
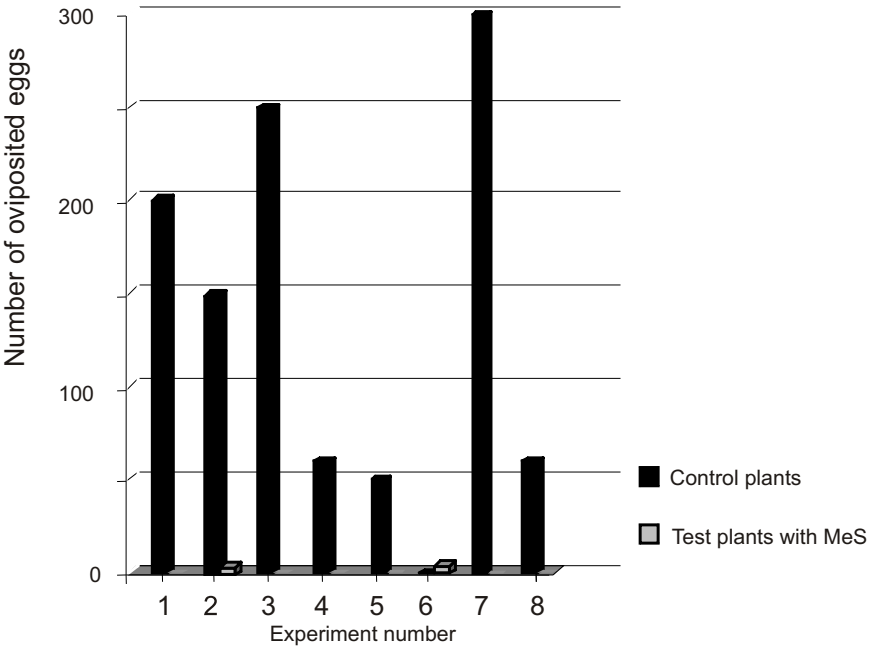
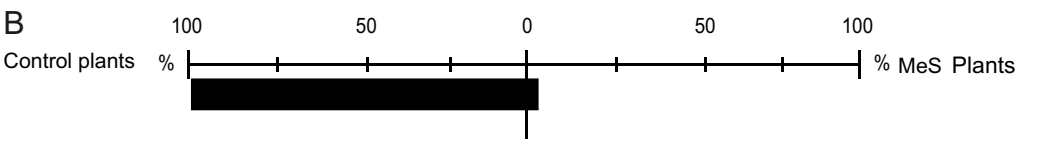

A
Relative abundance
100
90
80
70
60
50
40
30
20
10
0
4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
Time (min)
B
Relative abundance
MeS 17.97
100
90
80
70
60
50
40
30
20
10
0
4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
Time (min)

Figure 5

A


Number of oviposited eggs
300
200
100
0
1
2
3
4
5
6
7
8
Experiment number
Control plants
Test plants with MeS

B


100
50
0
50
100
Control plants
%
% MeS Plants

Doctoral theses in Biology
Norwegian University of Science and Technology
Department of Biology

Year	Name	Degree	Title
1974	Tor-Henning Iversen	Dr. philos Botany	The roles of statholiths, auxin transport, and auxin metabolism in root gravitropism
1978	Tore Slagsvold	Dr. philos. Zoology	Breeding events of birds in relation to spring temperature and environmental phenology.
1978	Egil Sakshaug	Dr.philos Botany	"The influence of environmental factors on the chemical composition of cultivated and natural populations of marine phytoplankton"
1980	Arnfinn Langeland	Dr. philos. Zoology	Interaction between fish and zooplankton populations and their effects on the material utilization in a freshwater lake.
1980	Helge Reinertsen	Dr. philos Botany	The effect of lake fertilization on the dynamics and stability of a limnetic ecosystem with special reference to the phytoplankton
1982	Gunn Mari Olsen	Dr. scient Botany	Gravitropism in roots of *Pisum sativum* and *Arabidopsis thaliana*
1982	Dag Dolmen	Dr. philos. Zoology	Life aspects of two sympartic species of newts (*Triturus, Amphibia*) in Norway, with special emphasis on their ecological niche segregation.
1984	Eivin Røskaft	Dr. philos. Zoology	Sociobiological studies of the rook *Corvus frugilegus.*
1984	Anne Margrethe Cameron	Dr. scient Botany	Effects of alcohol inhalation on levels of circulating testosterone, follicle stimulating hormone and luteinzing hormone in male mature rats
1984	Asbjørn Magne Nilsen	Dr. scient Botany	Alveolar macrophages from expectorates – Biological monitoring of workers exosed to occupational air pollution. An evaluation of the AM-test
1985	Jarle Mork	Dr. philos. Zoology	Biochemical genetic studies in fish.
1985	John Solem	Dr. philos. Zoology	Taxonomy, distribution and ecology of caddisflies (*Trichoptera*) in the Dovrefjell mountains.
1985	Randi E. Reinertsen	Dr. philos. Zoology	Energy strategies in the cold: Metabolic and thermoregulatory adaptations in small northern birds.
1986	Bernt-Erik Sæther	Dr. philos. Zoology	Ecological and evolutionary basis for variation in reproductive traits of some vertebrates: A comparative approach.
1986	Torleif Holthe	Dr. philos. Zoology	Evolution, systematics, nomenclature, and zoogeography in the polychaete orders *Oweniimorpha* and *Terebellomorpha*, with special reference to the Arctic and Scandinavian fauna.
1987	Helene Lampe	Dr. scient. Zoology	The function of bird song in mate attraction and territorial defence, and the importance of song repertoires.
1987	Olav Hogstad	Dr. philos. Zoology	Winter survival strategies of the Willow tit *Parus montanus*.

1987 Jarle Inge Holten	Dr. philos Bothany	Autecological investigations along a coust-inland transect at Nord-Møre, Central Norway
1987 Rita Kumar	Dr. scient Botany	Somaclonal variation in plants regenerated from cell cultures of *Nicotiana sanderae* and *Chrysanthemum morifolium*
1987 Bjørn Åge Tømmerås	Dr. scient. Zoology	Olfaction in bark beetle communities: Interspecific interactions in regulation of colonization density, predator - prey relationship and host attraction.
1988 Hans Christian Pedersen	Dr. philos. Zoology	Reproductive behaviour in willow ptarmigan with special emphasis on territoriality and parental care.
1988 Tor G. Heggberget	Dr. philos. Zoology	Reproduction in Atlantic Salmon (*Salmo salar*): Aspects of spawning, incubation, early life history and population structure.
1988 Marianne V. Nielsen	Dr. scient. Zoology	The effects of selected environmental factors on carbon allocation/growth of larval and juvenile mussels (*Mytilus edulis*).
1988 Ole Kristian Berg	Dr. scient. Zoology	The formation of landlocked Atlantic salmon (*Salmo salar* L.).
1989 John W. Jensen	Dr. philos. Zoology	Crustacean plankton and fish during the first decade of the manmade Nesjø reservoir, with special emphasis on the effects of gill nets and salmonid growth.
1989 Helga J. Vivås	Dr. scient. Zoology	Theoretical models of activity pattern and optimal foraging: Predictions for the Moose *Alces alces*.
1989 Reidar Andersen	Dr. scient. Zoology	Interactions between a generalist herbivore, the moose *Alces alces*, and its winter food resources: a study of behavioural variation.
1989 Kurt Ingar Draget	Dr. scient Botany	Alginate gel media for plant tissue culture,
1990 Bengt Finstad	Dr. scient. Zoology	Osmotic and ionic regulation in Atlantic salmon, rainbow trout and Arctic charr: Effect of temperature, salinity and season.
1990 Hege Johannesen	Dr. scient. Zoology	Respiration and temperature regulation in birds with special emphasis on the oxygen extraction by the lung.
1990 Åse Krøkje	Dr. scient Botany	The mutagenic load from air pollution at two work-places with PAH-exposure measured with Ames Salmonella/microsome test
1990 Arne Johan Jensen	Dr. philos. Zoology	Effects of water temperature on early life history, juvenile growth and prespawning migrations of Atlantic salmion (*Salmo salar*) and brown trout (*Salmo trutta*): A summary of studies in Norwegian streams.
1990 Tor Jørgen Almaas	Dr. scient. Zoology	Pheromone reception in moths: Response characteristics of olfactory receptor neurons to intra- and interspecific chemical cues.
1990 Magne Husby	Dr. scient. Zoology	Breeding strategies in birds: Experiments with the Magpie *Pica pica*.
1991 Tor Kvam	Dr. scient. Zoology	Population biology of the European lynx (*Lynx lynx*) in Norway.
1991 Jan Henning L'Abêe Lund	Dr. philos. Zoology	Reproductive biology in freshwater fish, brown trout *Salmo trutta* and roach *Rutilus rutilus* in particular.
1991 Asbjørn Moen	Dr. philos Botany	The plant cover of the boreal uplands of Central Norway. I. Vegetation ecology of Sølendet nature reserve; haymaking fens and birch woodlands
1991 Else Marie Løbersli	Dr. scient Botany	Soil acidification and metal uptake in plants

1991	Trond Nordtug	Dr. scient. Zoology	Reflctometric studies of photomechanical adaptation in superposition eyes of arthropods.
1991	Thyra Solem	Dr. scient Botany	Age, origin and development of blanket mires in Central Norway
1991	Odd Terje Sandlund	Dr. philos. Zoology	The dynamics of habitat use in the salmonid genera *Coregonus* and *Salvelinus*: Ontogenic niche shifts and polymorphism.
1991	Nina Jonsson	Dr. philos.	Aspects of migration and spawning in salmonids.
1991	Atle Bones	Dr. scient Botany	Compartmentation and molecular properties of thioglucoside glucohydrolase (myrosinase)
1992	Torgrim Breiehagen	Dr. scient. Zoology	Mating behaviour and evolutionary aspects of the breeding system of two bird species: the Temminck's stint and the Pied flycatcher.
1992	Anne Kjersti Bakken	Dr. scient Botany	The influence of photoperiod on nitrate assimilation and nitrogen status in timothy (*Phleum pratense* L.)
1992	Tycho Anker-Nilssen	Dr. scient. Zoology	Food supply as a determinant of reproduction and population development in Norwegian Puffins *Fratercula arctica*
1992	Bjørn Munro Jenssen	Dr. philos. Zoology	Thermoregulation in aquatic birds in air and water: With special emphasis on the effects of crude oil, chemically treated oil and cleaning on the thermal balance of ducks.
1992	Arne Vollan Aarset	Dr. philos. Zoology	The ecophysiology of under-ice fauna: Osmotic regulation, low temperature tolerance and metabolism in polar crustaceans.
1993	Geir Slupphaug	Dr. scient Botany	Regulation and expression of uracil-DNA glycosylase and O^6-methylguanine-DNA methyltransferase in mammalian cells
1993	Tor Fredrik Næsje	Dr. scient. Zoology	Habitat shifts in coregonids.
1993	Yngvar Asbjørn Olsen	Dr. scient. Zoology	Cortisol dynamics in Atlantic salmon, *Salmo salar* L.: Basal and stressor-induced variations in plasma levels ans some secondary effects.
1993	Bård Pedersen	Dr. scient Botany	Theoretical studies of life history evolution in modular and clonal organisms
1993	Ole Petter Thangstad	Dr. scient Botany	Molecular studies of myrosinase in Brassicaceae
1993	Thrine L. M. Heggberget	Dr. scient. Zoology	Reproductive strategy and feeding ecology of the Eurasian otter *Lutra lutra*.
1993	Kjetil Bevanger	Dr. scient. Zoology	Avian interactions with utility structures, a biological approach.
1993	Kåre Haugan	Dr. scient Bothany	Mutations in the replication control gene trfA of the broad host-range plasmid RK2
1994	Peder Fiske	Dr. scient. Zoology	Sexual selection in the lekking great snipe (*Gallinago media*): Male mating success and female behaviour at the lek.
1994	Kjell Inge Reitan	Dr. scient Botany	Nutritional effects of algae in first-feeding of marine fish larvae
1994	Nils Røv	Dr. scient. Zoology	Breeding distribution, population status and regulation of breeding numbers in the northeast-Atlantic Great Cormorant *Phalacrocorax carbo carbo*.
1994	Annette-Susanne Hoepfner	Dr. scient Botany	Tissue culture techniques in propagation and breeding of Red Raspberry (*Rubus idaeus* L.)

1994	Inga Elise Bruteig	Dr. scient Bothany	Distribution, ecology and biomonitoring studies of epiphytic lichens on conifers
1994	Geir Johnsen	Dr. scient Botany	Light harvesting and utilization in marine phytoplankton: Species-specific and photoadaptive responses
1994	Morten Bakken	Dr. scient. Zoology	Infanticidal behaviour and reproductive performance in relation to competition capacity among farmed silver fox vixens, *Vulpes vulpes*.
1994	Arne Moksnes	Dr. philos. Zoology	Host adaptations towards brood parasitism by the Cockoo.
1994	Solveig Bakken	Dr. scient Bothany	Growth and nitrogen status in the moss *Dicranum majus* Sm. as influenced by nitrogen supply
1995	Olav Vadstein	Dr. philos Botany	The role of heterotrophic planktonic bacteria in the cycling of phosphorus in lakes: Phosphorus requirement, competitive ability and food web interactions.
1995	Hanne Christensen	Dr. scient. Zoology	Determinants of Otter *Lutra lutra* distribution in Norway: Effects of harvest, polychlorinated biphenyls (PCBs), human population density and competition with mink *Mustela vision*.
1995	Svein Håkon Lorentsen	Dr. scient. Zoology	Reproductive effort in the Antarctic Petrel *Thalassoica antarctica*; the effect of parental body size and condition.
1995	Chris Jørgen Jensen	Dr. scient. Zoology	The surface electromyographic (EMG) amplitude as an estimate of upper trapezius muscle activity
1995	Martha Kold Bakkevig	Dr. scient. Zoology	The impact of clothing textiles and construction in a clothing system on thermoregulatory responses, sweat accumulation and heat transport.
1995	Vidar Moen	Dr. scient. Zoology	Distribution patterns and adaptations to light in newly introduced populations of *Mysis relicta* and constraints on Cladoceran and Char populations.
1995	Hans Haavardsholm Blom	Dr. philos Bothany	A revision of the *Schistidium apocarpum* complex in Norway and Sweden.
1996	Jorun Skjærmo	Dr. scient Botany	Microbial ecology of early stages of cultivated marine fish; inpact fish-bacterial interactions on growth and survival of larvae.
1996	Ola Ugedal	Dr. scient. Zoology	Radiocesium turnover in freshwater fishes
1996	Ingibjørg Einarsdottir	Dr. scient. Zoology	Production of Atlantic salmon (*Salmo salar*) and Arctic charr (*Salvelinus alpinus*): A study of some physiological and immunological responses to rearing routines.
1996	Christina M. S. Pereira	Dr. scient. Zoology	Glucose metabolism in salmonids: Dietary effects and hormonal regulation.
1996	Jan Fredrik Børseth	Dr. scient. Zoology	The sodium energy gradients in muscle cells of *Mytilus edulis* and the effects of organic xenobiotics.
1996	Gunnar Henriksen	Dr. scient. Zoology	Status of Grey seal *Halichoerus grypus* and Harbour seal *Phoca vitulina* in the Barents sea region.
1997	Gunvor Øie	Dr. scient Bothany	Eevalution of rotifer *Brachionus plicatilis* quality in early first feeding of turbot *Scophtalmus maximus* L. larvae.
1997	Håkon Holien	Dr. scient Botany	Studies of lichens in spurce forest of Central Norway. Diversity, old growth species and the relationship to site and stand parameters.
1997	Ole Reitan	Dr. scient. Zoology	Responses of birds to habitat disturbance due to damming.

1997 Jon Arne Grøttum	Dr. scient. Zoology	Physiological effects of reduced water quality on fish in aquaculture.
1997 Per Gustav Thingstad	Dr. scient. Zoology	Birds as indicators for studying natural and human-induced variations in the environment, with special emphasis on the suitability of the Pied Flycatcher.
1997 Torgeir Nygård	Dr. scient. Zoology	Temporal and spatial trends of pollutants in birds in Norway: Birds of prey and Willow Grouse used as Biomonitors.
1997 Signe Nybø	Dr. scient. Zoology	Impacts of long-range transported air pollution on birds with particular reference to the dipper *Cinclus cinclus* in southern Norway.
1997 Atle Wibe	Dr. scient. Zoology	Identification of conifer volatiles detected by receptor neurons in the pine weevil (*Hylobius abietis*), analysed by gas chromatography linked to electrophysiology and to mass spectrometry.
1997 Rolv Lundheim	Dr. scient. Zoology	Adaptive and incidental biological ice nucleators.
1997 Arild Magne Landa	Dr. scient. Zoology	Wolverines in Scandinavia: ecology, sheep depredation and conservation.
1997 Kåre Magne Nielsen	Dr. scient Botany	An evolution of possible horizontal gene transfer from plants to sail bacteria by studies of natural transformation in *Acinetobacter calcoacetius*.
1997 Jarle Tufto	Dr. scient. Zoology	Gene flow and genetic drift in geographically structured populations: Ecological, population genetic, and statistical models
1997 Trygve Hesthagen	Dr. philos. Zoology	Population responces of Arctic charr (*Salvelinus alpinus* (L.)) and brown trout (*Salmo trutta* L.) to acidification in Norwegian inland waters
1997 Trygve Sigholt	Dr. philos. Zoology	Control of Parr-smolt transformation and seawater tolerance in farmed Atlantic Salmon (*Salmo salar*) Effects of photoperiod, temperature, gradual seawater acclimation, NaCl and betaine in the diet
1997 Jan Østnes	Dr. scient. Zoology	Cold sensation in adult and neonate birds
1998 Seethaledsumy Visvalingam	Dr. scient Botany	Influence of environmental factors on myrosinases and myrosinase-binding proteins.
1998 Thor Harald Ringsby	Dr. scient. Zoology	Variation in space and time: The biology of a House sparrow metapopulation
1998 Erling Johan Solberg	Dr. scient. Zoology	Variation in population dynamics and life history in a Norwegian moose (*Alces alces*) population: consequences of harvesting in a variable environment
1998 Sigurd Mjøen Saastad	Dr. scient Botany	Species delimitation and phylogenetic relationships between the Sphagnum recurvum complex (Bryophyta): genetic variation and phenotypic plasticity.
1998 Bjarte Mortensen	Dr. scient Botany	Metabolism of volatile organic chemicals (VOCs) in a head liver S9 vial equilibration system in vitro.
1998 Gunnar Austrheim	Dr. scient Botany	Plant biodiversity and land use in subalpine grasslands. – A conservtaion biological approach.
1998 Bente Gunnveig Berg	Dr. scient. Zoology	Encoding of pheromone information in two related moth species
1999 Kristian Overskaug	Dr. scient. Zoology	Behavioural and morphological characteristics in Northern Tawny Owls *Strix aluco*: An intra- and interspecific comparative approach

1999	Hans Kristen Stenøien	Dr. scient Bothany	Genetic studies of evolutionary processes in various populations of nonvascular plants (mosses, liverworts and hornworts)
1999	Trond Arnesen	Dr. scient Botany	Vegetation dynamics following trampling and burning in the outlying haylands at Sølendet, Central Norway.
1999	Ingvar Stenberg	Dr. scient. Zoology	Habitat selection, reproduction and survival in the White-backed Woodpecker *Dendrocopos leucotos*
1999	Stein Olle Johansen	Dr. scient Botany	A study of driftwood dispersal to the Nordic Seas by dendrochronology and wood anatomical analysis.
1999	Trina Falck Galloway	Dr. scient. Zoology	Muscle development and growth in early life stages of the Atlantic cod (*Gadus morhua* L.) and Halibut (*Hippoglossus hippoglossus* L.)
1999	Torbjørn Forseth	Dr. scient. Zoology	Bioenergetics in ecological and life history studies of fishes.
1999	Marianne Giæver	Dr. scient. Zoology	Population genetic studies in three gadoid species: blue whiting (*Micromisistius poutassou*), haddock (*Melanogrammus aeglefinus*) and cod (*Gradus morhua*) in the North-East Atlantic
1999	Hans Martin Hanslin	Dr. scient Botany	The impact of environmental conditions of density dependent performance in the boreal forest bryophytes *Dicranum majus*, *Hylocomium splendens*, *Plagiochila asplenigides*, *Ptilium crista-castrensis* and *Rhytidiadelphus lokeus*.
1999	Ingrid Bysveen Mjølnerød	Dr. scient. Zoology	Aspects of population genetics, behaviour and performance of wild and farmed Atlantic salmon (*Salmo salar*) revealed by molecular genetic techniques
1999	Else Berit Skagen	Dr. scient Botany	The early regeneration process in protoplasts from *Brassica napus* hypocotyls cultivated under various g-forces
1999	Stein-Are Sæther	Dr. philos. Zoology	Mate choice, competition for mates, and conflicts of interest in the Lekking Great Snipe
1999	Katrine Wangen Rustad	Dr. scient. Zoology	Modulation of glutamatergic neurotransmission related to cognitive dysfunctions and Alzheimer's disease
1999	Per Terje Smiseth	Dr. scient. Zoology	Social evolution in monogamous families: mate choice and conflicts over parental care in the Bluethroat (*Luscinia s. svecica*)
1999	Gunnbjørn Bremset	Dr. scient. Zoology	Young Atlantic salmon (*Salmo salar* L.) and Brown trout (*Salmo trutta* L.) inhabiting the deep pool habitat, with special reference to their habitat use, habitat preferences and competitive interactions
1999	Frode Ødegaard	Dr. scient. Zoology	Host spesificity as parameter in estimates of arhrophod species richness
1999	Sonja Andersen	Dr. scient Bothany	Expressional and functional analyses of human, secretory phospholipase A2
2000	Ingrid Salvesen, I	Dr. scient Botany	Microbial ecology in early stages of marine fish: Development and evaluation of methods for microbial management in intensive larviculture
2000	Ingar Jostein Øien	Dr. scient. Zoology	The Cuckoo (*Cuculus canorus*) and its host: adaptions and counteradaptions in a coevolutionary arms race
2000	Pavlos Makridis	Dr. scient Botany	Methods for the microbial econtrol of live food used for the rearing of marine fish larvae
2000	Sigbjørn Stokke	Dr. scient. Zoology	Sexual segregation in the African elephant (*Loxodonta africana*)

2000 Odd A. Gulseth	Dr. philos. Zoology	Seawater tolerance, migratory behaviour and growth of Charr, (*Salvelinus alpinus*), with emphasis on the high Arctic Dieset charr on Spitsbergen, Svalbard
2000 Pål A. Olsvik	Dr. scient. Zoology	Biochemical impacts of Cd, Cu and Zn on brown trout (*Salmo trutta*) in two mining-contaminated rivers in Central Norway
2000 Sigurd Einum	Dr. scient. Zoology	Maternal effects in fish: Implications for the evolution of breeding time and egg size
2001 Jan Ove Evjemo	Dr. scient. Zoology	Production and nutritional adaptation of the brine shrimp *Artemia* sp. as live food organism for larvae of marine cold water fish species
2001 Olga Hilmo	Dr. scient Botany	Lichen response to environmental changes in the managed boreal forset systems
2001 Ingebrigt Uglem	Dr. scient. Zoology	Male dimorphism and reproductive biology in corkwing wrasse (*Symphodus melops* L.)
2001 Bård Gunnar Stokke	Dr. scient. Zoology	Coevolutionary adaptations in avian brood parasites and their hosts
2002 Ronny Aanes	Dr. scient	Spatio-temporal dynamics in Svalbard reindeer (*Rangifer tarandus platyrhynchus*)
2002 Mariann Sandsund	Dr. scient. Zoology	Exercise- and cold-induced asthma. Respiratory and thermoregulatory responses
2002 Dag-Inge Øien	Dr. scient Botany	Dynamics of plant communities and populations in boreal vegetation influenced by scything at Sølendet, Central Norway
2002 Frank Rosell	Dr. scient. Zoology	The function of scent marking in beaver (*Castor fiber*)
2002 Janne Østvang	Dr. scient Botany	The Role and Regulation of Phospholipase A_2 in Monocytes During Atherosclerosis Development
2002 Terje Thun	Dr.philos Biology	Dendrochronological constructions of Norwegian conifer chronologies providing dating of historical material
2002 Birgit Hafjeld Borgen	Dr. scient Biology	Functional analysis of plant idioblasts (Myrosin cells) and their role in defense, development and growth
2002 Bård Øyvind Solberg	Dr. scient Biology	Effects of climatic change on the growth of dominating tree species along major environmental gradients
2002 Per Winge	Dr. scient Biology	The evolution of small GTP binding proteins in cellular organisms. Studies of RAC GTPases in *Arabidopsis thaliana* and
2002 Henrik Jensen	Dr. scient Biology	Causes and consequenses of individual variation in fitness-related traits in house sparrows
2003 Jens Rohloff	Dr. philos Biology	Cultivation of herbs and medicinal plants in Norway – Essential oil production and quality control
2003 Åsa Maria O. Espmark Wibe	Dr. scient Biology	Behavioural effects of environmental pollution in threespine stickleback *Gasterosteus aculeatur* L.
2003 Dagmar Hagen	Dr. scient Biology	Assisted recovery of disturbed arctic and alpine vegetation – an integrated approach
2003 Bjørn Dahle	Dr. scient Biology	Reproductive strategies in Scandinavian brown bears
2003 Cyril Lebogang Taolo	Dr. scient Biology	Population ecology, seasonal movement and habitat use of the African buffalo (*Syncerus caffer*) in Chobe National Park, Botswana
2003 Marit Stranden	Dr.scient Biology	Olfactory receptor neurones specified for the same odorants in three related Heliothine species (*Helicoverpa armigera, Helicoverpa assulta* and *Heliothis virescens*)

2003	Kristian Hassel	Dr.scient Biology	Life history characteristics and genetic variation in an expanding species, *Pogonatum dentatum*
2003	David Alexander Rae	Dr.scient Biology	Plant- and invertebrate-community responses to species interaction and microclimatic gradients in alpine and Artic environments
2003	Åsa A Borg	Dr.scient Biology	Sex roles and reproductive behaviour in gobies and guppies: a female perspective
2003	Eldar Åsgard Bendiksen	Dr.scient Biology	Environmental effects on lipid nutrition of farmed Atlantic salmon (*Salmo Salar* L.) parr and smolt
2004	Torkild Bakken	Dr.scient Biology	A revision of Nereidinae (Polychaeta, Nereididae)
2004	Ingar Pareliussen	Dr.scient Biology	Natural and Experimental Tree Establishment in a Fragmented Forest, Ambohitantely Forest Reserve, Madagascar
2004	Tore Brembu	Dr.scient Biology	Genetic, molecular and functional studies of RAC GTPases and the WAVE-like regulatory protein complex in *Arabidopsis thaliana*
2004	Liv S. Nilsen	Dr.scient Biology	Coastal heath vegetation on central Norway; recent past, present state and future possibilities
2004	Hanne T. Skiri	Dr.scient Biology	Olfactory coding and olfactory learning of plant odours in heliothine moths. An anatomical, physiological and behavioural study of three related species (*Heliothis virescens, Helicoverpa armigera* and *Helicoverpa assulta*).
2004	Lene Østby	Dr.scient Biology	Cytochrome P4501A (CYP1A) induction and DNA adducts as biomarkers for organic pollution in the natural environment
2004	Emmanuel J. Gerreta	Dr. philos Biology	The Importance of Water Quality and Quantity in the Tropical Ecosystems, Tanzania
2004	Linda Dalen	Dr.scient Biology	Dynamics of Mountain Birch Treelines in the Scandes Mountain Chain, and Effects of Climate Warming
2004	Lisbeth Mehli	Dr.scient Biology	Polygalacturonase-inhibiting protein (PGIP) in cultivated strawberry (*Fragaria* x *ananassa*): characterisation and induction of the gene following fruit infection by *Botrytis cinerea*
2004	Børge Moe	Dr.scient Biology	Energy-Allocation in Avian Nestlings Facing Short-Term Food Shortage
2005	Matilde Skogen Chauton	Dr.scient Biology	Metabolic profiling and species discrimination from High-Resolution Magic Angle Spinning NMR analysis of whole-cell samples
2005	Sten Karlsson	Dr.scient Biology	Dynamics of Genetic Polymorphisms
2005	Terje Bongard	Dr.scient Biology	Life History strategies, mate choice, and parental investment among Norwegians over a 300-year period
2005	Tonette Røstelien	PhD Biology	Functional characterisation of olfactory receptor neurone types in heliothine moths
2005	Erlend Kristiansen	Dr.scient Biology	Studies on antifreeze proteins
2005	Eugen G. Sørmo	Dr.scient Biology	Organochlorine pollutants in grey seal (*Halichoerus grypus*) pups and their impact on plasma thyrid hormone and vitamin A concentrations.

2005	Christian Westad	Dr.scient Biology	Motor control of the upper trapezius
2005	Lasse Mork Olsen	PhD Biology	Interactions between marine osmo- and phagotrophs in different physicochemical environments
2005	Åslaug Viken	PhD Biology	Implications of mate choice for the management of small populations
2005	Ariaya Hymete Sahle Dingle	PhD Biology	Investigation of the biological activities and chemical constituents of selected *Echinops* spp. growing in Ethiopia
2005	Ander Gravbrøt Finstad	PhD Biology	Salmonid fishes in a changing climate: The winter challenge
2005	Shimane Washington Makabu	PhD Biology	Interactions between woody plants, elephants and other browsers in the Chobe Riverfront, Botswana
2005	Kjartan Østbye	Dr.scient Biology	The European whitefish *Coregonus lavaretus* (L.) species complex: historical contingency and adaptive radiation
2006	Kari Mette Murvoll	PhD Biology	Levels and effects of persistent organic pollutans (POPs) in seabirds Retinoids and α-tocopherol – potential biomakers of POPs in birds?
2006	Ivar Herfindal	Dr.scient Biology	Life history consequences of environmental variation along ecological gradients in northern ungulates
2006	Nils Egil Tokle	Phd Biology	Are the ubiquitous marine copepods limited by food or predation? Experimental and field-based studies with main focus on *Calanus finmarchicus*
2006	Jan Ove Gjershaug	Dr.philos Biology	Taxonomy and conservation status of some booted eagles in south-east Asia
2006	Jon Kristian Skei	Dr.scient Biology	Conservation biology and acidification problems in the breeding habitat of amphibians in Norway
2006	Johanna Järnegren	PhD Biology	Acesta Oophaga and Acesta Excavata – a study of hidden biodiversity
2006	Bjørn Henrik Hansen	PhD Biology	Metal-mediated oxidative stress responses in brown trout (*Salmo trutta*) from mining contaminated rivers in Central Norway
2006	Vidar Grøtan	phD Biology	Temporal and spatial effects of climate fluctuations on population dynamics of vertebrates
2006	Jafari R Kideghesho	phD Biology	Wildlife conservation and local land use conflicts in western Serengeti, Corridor Tanzania
2006	Anna Maria Billing	phD Biology	Reproductive decisions in the sex role reversed pipefish *Syngnathus typhle*: when and how to invest in reproduction
2006	Henrik Pärn	phD Biology	Female ornaments and reproductive biology in the bluethroat
2006	Anders J. Fjellheim	phD Biology	Selection and administration of probiotic bacteria to marine fish larvae
2006	P. Andreas Svensson	phD Biology	Female coloration, egg carotenoids and reproductive success: gobies as a model system
2007	Sindre A. Pedersen	phD Biology	Metal binding proteins and antifreeze proteins in the beetle *Tenebrio molitor* - a study on possible competition for the semi-essential amino acid cysteine

2007	Kasper Hancke	phD Biology	Photosynthetic responses as a function of light and temperature: Field and laboratory studies on marine microalgae
2007	Tomas Holmern	phD Biology	Bushmeat hunting in the western Serengeti: Implications for community-based conservation
2007	Kari Jørgensen	phD Biology	Functional tracing of gustatory receptor neurons in the CNS and chemosensory learning in the moth *Heliothis virescens*