Maria Helena de Carvalho Fernandes Bichão

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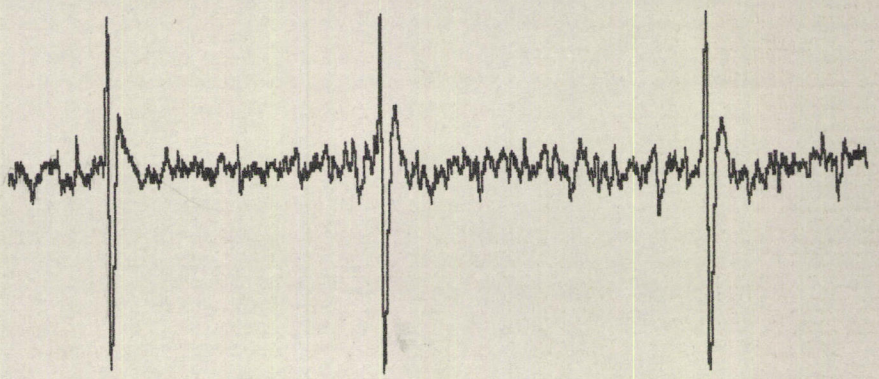
A comparative study, using electrophysiological recordings linked to gas chromatography and mass spectrometry



Orientador: Professor Doutor Jorge Araújo

"Esta tese não inclui as críticas e sugestões feitas pelo júri"
"The thesis does not include changes according to suggestions made by the jury"

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A comparative study, using electrophysiological recordings linked to gas chromatography and mass spectrometry

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Papers included in the thesis

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

I. **Bichão, H.**, Borg-Karlson, A.-K., Araújo, J., Wibe, A., 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

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II. **Bichão, H.**, Borg-Karlson, A.-K., Stranden, M., Araújo, J., and Mustaparta, H. Olfactory receptor neurones in *Pissodes notatus* respond selectively to plant produced *o*-methylanisole and limonene enantiomers. (Manuscript)

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

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IV. **Bichão, H.**, Borg-Karlson, A.-K., Araújo, J., Wibe, A., and Mustaparta H. (2005) Molecular receptive ranges of olfactory receptor neurones responding selectively to terpenoids, aliphatic green leaf volatiles and aromatics, in the strawberry blossom weevil *Anthonomus rubi.* Chemoecology, 15(4): 211-226

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Introduction

Each herbivore in the course of its evolution has became associated with either a particular plant species (monophagous), or a more or less limited group of plants (oligophagous and polyphagous, respectively) (Dethier, 1982; Schoonhoven *et al.*, 1998). The maintenance of these host specializations requires the ability to find and recognize specific plant species, which in natural habitats grow in mixed and complex plant communities. The process of host selection is mediated by the integration in the central nervous system of various sensory inputs, including olfactory, gustatory, visual and tactile cues originating from the plant (Bernays and Chapman, 1994). Several features of the plants are perceived from a distance as well as from close range, like odour, colour and shape, while others, e.g. texture of leaves and taste are only perceived after contact (Miller and Strickler, 1984; Visser, 1986). Phytophagous insects are believed to have had a major role in the evolution of plants by selecting for diverse physical and chemical defences (Ehrlich and Raven, 1964; Kessler and Baldwin, 2001; Marquis, 2004). The latter include the many volatile compounds that constitute the scent of plants.

Odours produced by plants consist of complex blends, comprising a wide scope of constituents that vary in time, space, and between individual sources (Schoonhoven *et al.*, 1998). Finding food or oviposition sites therefore requires the recognition of an odour blend that comprises several dimensions. Traditionally, two opposing theories have been discussed. The early work of Fraenkel (1959) describes the "token stimulus" model according to which recognition of a host relies on highly specific compounds that are not found in unsuitable plant species. Whereas evidence in support of this view is limited (e.g. Guerin *et al.*, 1983; Blight *et al.*, 1995), a considerable amount of data from studies of insect physiology and plant chemistry support the alternative theory that postulates ratio-dependent odour recognition, i.e. odour specificity of a plant is achieved by a precise ratio among constituents that are wide spread in the plant world (reviewed by Bruce *et al.*, 2005). Different insect species may use different strategies or various combinations of the two strategies for recognition of the odour of an acceptable host plant.

Odours are detected in most organisms, including insects, by olfactory receptor neurones (RNs) that bridge the external world and the central nervous system (CNS). In animals, the RNs are small bipolar nerve cells with a thin dendrite containing the receptor proteins and an unmyelinated axon conveying nerve impulses to the CNS. Odour recognition occurs when a particular constellation of compounds matches a model or representation in the

insect's neural world and a specific behaviour is triggered. The information received by sensory cells is integrated in the higher centres of the brain. How neural representations of odour blends are brought about is a challenging topic of many ongoing studies of insects and vertebrates.

The nature of plant odours

Plants release hundreds of volatiles relevant to their interaction with herbivorous insects. These compounds are produced by various biosynthetic pathways that constitute the secondary metabolism of plants. Whereas the products of primary metabolism are essential for plant growth and development, secondary compounds are not (Hartmann, 1996). The secondary compounds were previously considered to be waste products until Stahl (1888) and Fraenkel (1959) suggested a defensive function against herbivory. Later Ehrlich and Raven (1964) proposed that plant secondary chemistry is primarily the result of a co-evolutionary "arms race" between plant defences and herbivore responses, setting the research agenda for decades to come. They suggested a model of stepwise chemical interactions between plants and insects, and proposed that antagonist chemical interactions are primary factors responsible for the adaptive radiation of both plants and insects (Mitchell-Olds *et al.*, 1998).

Today, plant secondary compounds are viewed as an essential part of the plant's biochemical equipment to cope with the environment, including attack by herbivores and pathogens (Hartmann, 1996; Schoonhoven *et al.*, 1998; Harborne, 1999). For a compound to act as a defence against herbivory it must be either repellent or toxic to herbivores or reduce their fitness by others means. For example, plants can reduce herbivory by producing chemical compounds that repel potential herbivores (direct defence), or that attract parasitic and predatory arthropods that will attack the herbivore, and thereby protect the plant (indirect defence) (Kessler and Baldwin, 2001). Plant defence compounds can be 'constitutive' or 'induced'. Constitutive defences are produced all the time, stored in specialised structures and released as a reaction to attack. This is the common strategy of long-lived plants, e.g. conifer trees (Priemé *et al.*, 2000). In contrast, induced defences are those whose production is triggered by insect or pathogen attack (Paré and Tumlinson, 1999; Gouinguené and Turlings, 2002). Inducible compounds may provide effective defence against attack by herbivores while minimizing the physiological costs of production when herbivory is absent (Gershenzon, 1994). However, induced defences do not necessarily mean the production of novel compounds. Many compounds are produced constitutively in small amount and are induced in greater amounts due to stress factors. Generally, plant induced compounds are assumed to

attract predators or parasitoids or to deter herbivores (Ninkovic *et al.*, 2001; Turlings and Wäckers, 2004; Dicke and Van Loon, 2000; De Moraes *et al.*, 2001; Kessler and Baldwin, 2001). However, the same compounds may in addition attract herbivores and thereby incur ecological costs (Bolter *et al.*, 1997; Dicke and Vet, 1999; Horiuchi *et al.*, 2003; Heil, 2004).

Plants attacked by herbivores experience both tissue damage and loss of leaf area which leads to local and systemic induction of volatile production (Dicke and Van Loon, 2000). Volatile compounds released from herbivore infested plants include monoterpenes and sesquiterpenes from the mevalonate and the alternative methylerythritol pathways, "green leaf volatiles" from the fatty acid/lipoxygenase pathway and aromatic metabolites, such as indole and methyl salicylate from the shikimic acid/tryptophan pathway (Dudareva *et al.*, 2004; Paré and Tumlinson, 1996; De Moraes *et al.*, 2004). One of the most interesting findings in this expanding area of research is that the profiles of compounds systemically induced during herbivory vary according to the herbivorous species, both in quality and quantity (De Moraes *et al.*, 1998; Arimura *et al.*, 2004). It has been shown that factors in the saliva of caterpillars induce changes in the production of volatile compounds, which both attract natural enemies (Alborn *et al.*, 1997; Mattiacci *et al.*, 1995; Turlings *et al.*, 1990; Turlings *et al.*, 1995; Paré *et al.*, 2005) and deter oviposition by moths (Kessler and Baldwin, 2001; De Moraes *et al.*, 2001). Furthermore, the induced production is higher at night, when the moths are flying (De Moraes *et al.*, 2001). The induction is caused by activation of a series of genes that up-regulate the activity of specific enzymes, leading to the synthesis of the defence molecules (Halitschke and Baldwin, 2003; Frey *et al.*, 2004).

The composition and quantities of volatiles emitted by plants are also affected by abiotic environmental factors like availability of nutrients, exposure to UV radiation, temperature and ozone (Pichersky and Gershenzon, 2002; De Moraes *et al.*, 2004). The profile of emitted volatiles also shows diurnal (Kolosova *et al.*, 2001) and seasonal variation, superimposed on variation due to individual differences and the phenological state of the plant. This tremendous complexity and variability includes the relevant signals for the herbivore insect in search of a host plant. The challenge for the herbivore insect is being able to distinguish between the relevant chemistry and the background noise. This task is accomplished with the olfactory system.

Detecting odours

Anatomy of the olfactory pathway

The numerous sensilla located in the antennae of insects constitute an efficient filter that sieves the air for odour molecules. Although, the antennae are multimodal sensory structures (olfactory, gustatory, thermo, hygro- and mechanoreceptive sensilla), olfactory sensilla far outnumber the sensilla serving other modalities in almost all insect species (Steinbrecht, 1999). The olfactory sensillum types have different shapes e.g. short and stout hairs called *sensilla basiconica* and long thin hairs denominated *sensilla thricodea.* In general one insect species possesses several types of sensilla. Each sensillum houses one or more RNs, which have the cell body located below the base of the hair and dendrites extending to the sensillum lumen. The lumen is filled with sensillum lymph which isolates only a few RNs in a specific chemical environment. The olfactory receptor proteins are located in the dendrites. The axons of the RNs form the antennal nerves which project directly into distinct areas of the deuterocerebrum, the antenna lobes (ALs). In the ALs, RNs synapse abundantly with AL neurones forming morphological and functional units called glomeruli (Boeckh and Tolbert, 1993; Christensen *et al.*, 1995). Thus, each glomerulus is a condensed region of neuropil where RNs with similar functional properties converge (Vosshall, 2001). In the glomeruli, two major types of neurones receive and integrate information from the antennal sensory neurones. The local interneurones with arborisations in many glomeruli, interconnect the glomeruli within the AL, whereas the projection neurones, the majority branching in only one or a few glomeruli, convey information to higher order centres (Homberg *et al.*, 1988; Flanagan and Mercer, 1989; Christensen *et al.*, 1993; Rø *et al.* unpublished results). These centres are the mushroom bodies, involved in olfactory learning and memory formation, and the lateral horn, a pre-motoric area (Menzel, 1999; Heisenberg, 2003). In weevils the olfactory sensilla of both sexes are in general restricted to the distal part of the antennae, the antennal club. The sensilla distribution occurs in bands described for *Hylobius abietis* by Mustaparta (1973), a pattern that is similar in other weevil species (Dickens, 1990; Saïd *et al.*, 2003; Bichão unpublished results) (Figure 1). Otherwise, the anatomy of the olfactory pathway is not studied in this diverse group of insects.

Perireceptor events and transduction of olfactory stimuli

Olfactory receptor neurones transduce information carried by the odour molecules into electrical signals transmitted within the nervous system. The lipophilic odour molecules

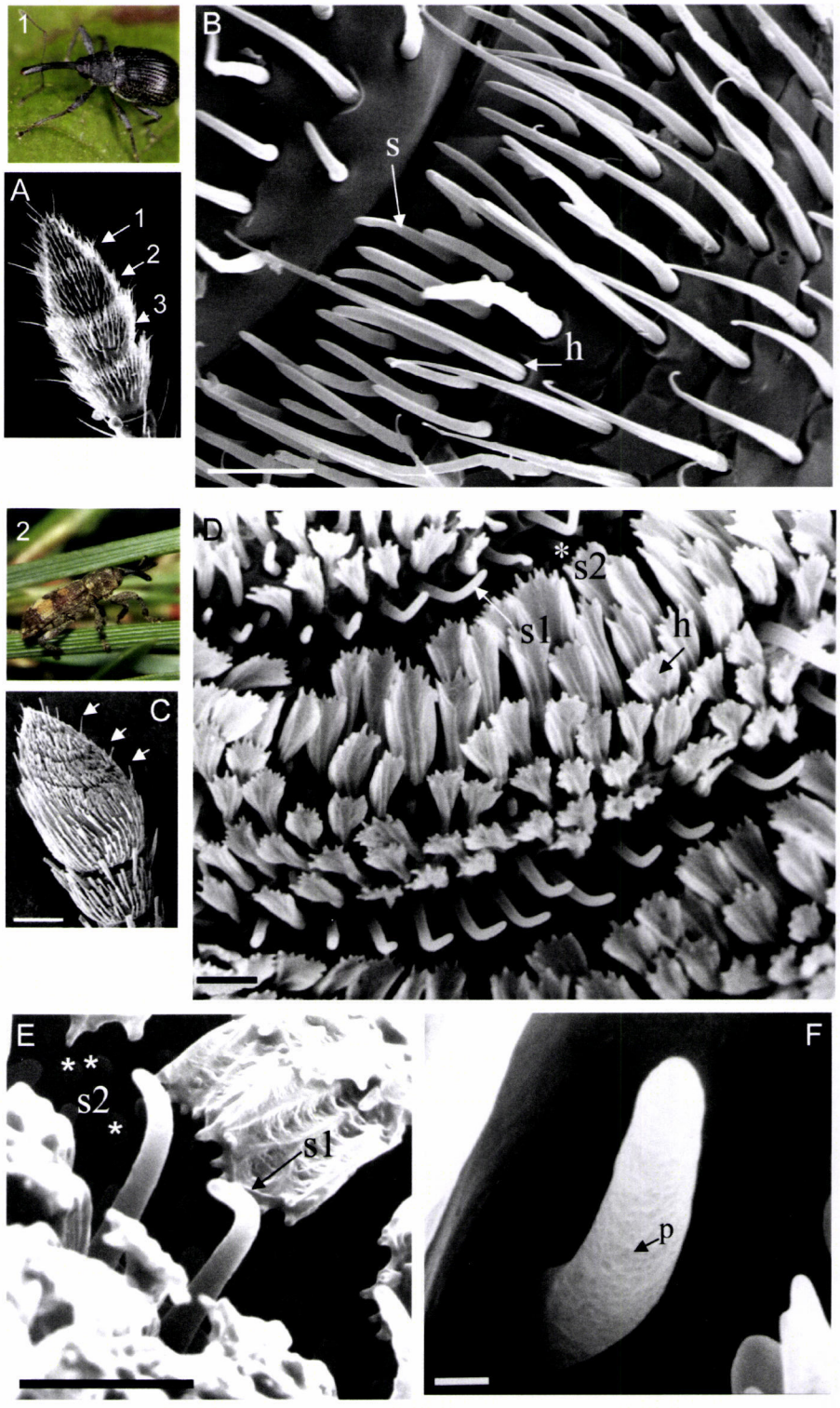
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Figure 1. Scanning electron micrographs of the antenna of *Anthonomus rubi* (A-B) and *Pissodes notatus* (C-F) (scale bars 10 µm). Low magnification side view of the antenna of *A. rubi*. Arrows indicate the three constriction bands where olfactory sensilla are located. B. Detailed view of band 2 showing the olfactory sensilla (s) partially covered by protection hairs with pointed tips and longitudinal furrows (h). C. Low magnification side view of the antenna of *P. notatus* showing the three constriction bands where olfactory sensilla are located. B. Detailed view of constriction bands 1 and 2 showing long and curved sensilla (s1) and short and stout sensilla (s2), partially covered by the antenna's dense scales (h). E. Detail of constriction band 2, showing two long sensilla (s1) and the numerous short sensilla (s2). H. Close-up of one *sensillum*, showing the pored wall. (Colour inserts: 1. *A. rubi* ©Ingrid Altmann; 2 *P. notatus* ©Frank Koehler, with permission) [(A-B) Bichão, NTNU, Trondheim; (C-F) Bichão, CEMUP, Porto)

penetrate the *sensillum* through the pores in the cuticle and must travel across the aqueous sensillum lymph to come in contact with the dendrites of the RNs. The mechanisms by which the odour molecules are transported through the receptor lymph are not fully understood. A widely accepted view is that small odorant binding proteins (OBPs) bind the hydrophobic odorant molecules and thereby facilitate transport across the aqueous moiety (Prestwich *et al.*, 1995; Steinbrecht, 1998). Putative OBPs have been identified in several insect taxa including weevils (Vogt *et al.*, 1999; Nagnan-Le-Meillour *et al.*, 2004). Whether the OBPs, in addition to their transport function, take part in the binding of the odorant to the receptor, are involved in the inactivation of the odorant-receptor complex or release the odorant close to the dendrite membrane, is subject of an ongoing discussion (Kaissling, 1998; Rützler and Zwiebel, 2005). Since OBPs show selective binding to some odorants, they may serve as a first filter preventing many chemicals from reaching the dendrites of the RNs. However, the recognition of olfactory stimuli begins with the interaction of odour molecules with odorant receptor proteins in the dendritic membrane, which determine the specificity of the RNs.

The identity of olfactory receptors was first unravelled in the rat by Buck and Axel (1991), in a milestone paper that granted them the Nobel Prize in physiology and medicine in 2004. A large and extremely divergent gene family, expressed exclusively in the olfactory tissue, encodes heterometric G-protein-coupled receptors with seven transmembrane domains. Since then, putative genes for receptor proteins have been identified in many species of vertebrates and invertebrates (Mombaerts, 1999; Mombaerts, 2004a; Keller and Vosshall, 2003; Rützler and Zwiebel, 2005). The first putative insect olfactory receptor proteins were identified in the fruit fly *Drosophila melanogaster* (Vosshall *et al.*, 1999; Clyne *et al.*, 1999b), and candidate receptor proteins have also been identified in moth and mosquitoes (Krieger *et al.*, 2002; Fox *et al.*, 2001, 2002; Hill *et al.*, 2002). A recent study has provided functional evidence for 62 receptor genes in *D. melanogaster* (Hallem *et al.*, 2004). The same work elegantly demonstrates that the expressed receptor genes confer the functional characteristic to the RN i.e., selectivity and sensitivity, temporal characteristics and mode of the response. Other studies conducted over the past decade in vertebrates and invertebrates indicate that one type of olfactory receptor gene is expressed in a given subset or type of RNs (Ressler *et al.*, 1993; Vassar *et al.*, 1993; Vosshall *et al.*, 1999; Clyne *et al.*, 1999a; Hallem *et al.*, 2004). Additionally, it has been shown that all RNs of one type converge into one or two specific glomeruli in the primary olfactory centre (the antenna lobe in insects and the olfactory bulb in vertebrates) (Ressler *et al.*, 1994; Strotmann *et al.*, 1994; Vosshall *et al.*, 2000; Gao *et al.*, 2000; Dulac and Axel, 1995; Treolar *et al.*, 2002; Keller and Vosshall, 2003; Mombaerts,

2004b). This principle has been termed "the molecular logic of smell" (Axel, 1995). In a recent study Goldman *et al.* (2005) demonstrated that two odorant receptor genes are co-expressed in the same RN of the maxillary palp of *D. melanogaster* and together account for the odorant response profile of the RN. This exception suggests that there might still be more to unravel about expression of receptor protein types in single neurones (see also Mombaerts, 2004b).

Upon binding to an appropriate ligand, olfactory receptor proteins transmit the signal from the extracellular to the intracellular face of the RN membrane by altering the conductance. The intramolecular mechanisms underlying this signal transformation are unknown, but most likely involve ligand induced structural changes of the receptor protein (Krieger and Breer, 2003). The activation of a receptor protein is indicated to trigger intracellular cascade reactions, resulting in the opening of ion channels. Evidence for two intracellular signal pathways operating in RNs have been obtained in an increasing number of animals; one mediated by the second messenger 3,5-cyclic monophosphate (cAMP) and the other by inositol 1,4,5-triphosphate (IP3). In insects, experimental evidence from diverse methods indicates that suitable odorants trigger the formation of IP3 and diacylglycerol (DAG) by activation of the effector enzyme phospholipase C (PLC) (Breer *et al.*, 1990; Stengl, 1994; Laue *et al.*, 1997). More recently, cAMP has also been indicated as a second messenger in insects, first in the cockroach and later in the fruit fly (Krieger *et al.*, 1999; Krieger and Breer, 1999; Gomez-Diaz *et al.*, 2004).

Encoding of information in receptor neurones

Ever since the pioneering works of Schneider (1957), olfactory receptor neurones have been accessible for investigations using electrophysiological methods. The first targets of such investigations were the pheromone detecting neurones in male moths. Such studies revealed that these neurones have a remarkable sensitivity and specificity. Using radio-labelled pheromone it was shown that a single pheromone molecule is sufficient to elicit a nerve impulse (Kaissling, 1986). Further, structure activity investigations demonstrated that recognition of pheromones was highly specific. In the early studies, the terms 'specialist' RN and 'generalist' RN were coined, with the former exemplified by pheromone detectors and the latter by plant odour detectors (Schneider, 1964; Vareschi, 1971). Later, evidence for plant odour RNs being as sensitive and selective as pheromone RNs came from studies using direct stimulation (Dickens, 1990; Hansson *et al.*, 1999; Jönsson and Anderson, 1999; Shields and Hildebrand, 2001) and particularly from studies employing gas- chromatography linked to

electrophysiological recordings from single RNs (GC-SCR) (reviewed by Mustaparta, 2002, 2005). Based on the specificity of studied RNs, two principles have been discussed describing how information is mediated to the CNS. According to the 'labelled line' principle, information about one odorant is mediated to the brain by only on type of RN, whereas information following an 'across fibre pattern' mechanism is mediated to the brain by several RN types with overlapping molecular receptive ranges and different sensitivities (Gesteland *et al.*, 1965; Shepherd, 1984; Mustaparta, 2002). The mediation of pheromone information from the periphery to the antennal lobe of insects seems to follow the principle of labelled lines, while information about food and host odours is commonly believed to be mediated by the more complex across fibre pattern mechanism (reviewed by Lemon and Getz, 1999), despite the fact that narrowly tuned RNs are common in insects. Although both principles are sometimes described as mutually exclusive, they more likely represent extremes of a continuum (Smith and Getz, 1994).

The methods for studying olfactory detection

One of the greatest challenges when studying olfaction is to identify the stimulus. In contrast to the well defined stimuli spectrum of other senses, e.g. the wavelength of light for vision, chemical stimuli do not constitute a known continuum/spectrum. Thus, identification of odorants for insect RNs meets the problem of testing on each RN all the relevant volatiles. The blend of volatiles released from the host plant is likely to contain the most relevant cues about sites suitable for feeding and egg-laying. However, volatiles from non-hosts may also play an important role by providing cues about unsuitable sites (Hardie *et al.*, 1994; Byers *et al.*, 2004). Many of the odorants may be common to host and non-host plants. In order to test the neurones with as many potential odorants as possible, it is important to collect volatiles from many host and non-host plant species using aeration (head-space) methods (Silverstein and Rodin, 1966; Agelopoulos and Pickett, 1998). Collection from intact plants secures ecological relevance, and from cut plant materials the large quantities of compounds necessary for chemical identification.

Finding the biologically active constituents in the mixtures from plants requires the use of techniques of electrophysiological recordings from insect olfactory receptors. These studies must use physiologically/ecologically meaningful concentrations, otherwise they can give misleading results. Indeed, many early studies used stimulus concentrations higher then would occur in nature, leading to the suggestion that plant volatile RNs were generalists i.e.

responding to a broad range of compounds. However, recordings from single neurones using physiologically relevant stimulus concentrations, have demonstrated that host-plant recognition is mediated by highly specific molecular detection. A rough overview of odorants eliciting electrical responses across the olfactory RNs can be obtaind by recordings of electro-antennogram (EAG). However, the most precise way to identify plant produced compounds detected by herbivorous insects, is the method of gas chromatography linked to electrophysiological recordings from single olfactory receptor neurones (GC-SCR) (Wadhams, 1982; Löfstedt *et al.*, 1982; Tømmerås and Mustaparta, 1989). With this method, the constituents of the blends are separated in the column of the gas chromatograph (GC) and tested directly on each neurone. The use of two parallel GC-columns allows each neurone to be tested with the same mixture separated in different ways, namely enatiomeric separation (Røstelien *et al.*, 2000a; Stranden *et al.*, 2002). The next step is the determination of the identity of the active compounds by gas chromatography linked to mass spectrometry (GC-MS). Finally, retesting the same RN types with authentic or synthetic samples confirms their identification. The results obtained with these methods not only provide knowledge about biologically relevant odorants and in which plant species they are produced, but also about the range of odorants the individual RNs respond to, termed the molecular receptive range (MRR).

The weevils

Substantial knowledge of olfactory mechanism in insects stems mainly from the study of species of moths, cockroaches, the honey bee and the fruit fly. This thesis has contributed to elucidate the mechanisms of olfactory detection in another major group of herbivores, the weevils. Weevils are an interesting group to contrast with moth due to their biology, i.e. insects that have a fairly long life and which often form aggregations on the host plant facilitating mating. Reproduction is generally preceded by a feeding period and by the production of aggregation pheromones. In many species of weevils and other beetles, behavioural studies have demonstrated synergism between plant compounds and pheromones in the aggregation phase (e.g. Dickens, 1990; Giblin-Davis *et al.*, 1994; Sant`ana and Dickens, 1998; Phillips and Lanier, 1986; Rochat *et al.*, 2000). Furthermore, like in bark beetles that also form aggregations, many of the weevil pheromones are derivates of host plant secondary compounds that are only produced while on the host plant (Booth and Lanier, 1974; Booth *et al.*, 1983; Chang *et al.*, 1989; Innocenzi *et al.*, 2001).

The weevil family (Curculionidae), is one of the largest families of the Coleoptera with around 60 000 species grouped in 6000 genera (Thompson, 1992; Kuschel, 1995). With its sister group, the Chrysomelidae, they constitute a radiation of phytophagous insects, rivalled in species diversity only by the Lepidoptera (Farrell, 1998a,b). The amazing radiation of this group is thought to have happened contemporary with the radiation of the Angiosperms by multiple shifts in the mode of life. Their origin is referred to a detritivorous or fungivorous ancestor living in the Triassic (248-260 million years ago). From this ancestor, the ancestral lineages of the modern weevils evolved as herbivores in close associations with cycads and gymnosperms, the main groups of plants covering the earth surface at the time (Anderson, 1995; Marvaldi *et al.*, 2002). The ancestral weevil lineage had endophagous larvae, which fed inside coniferous tissues of branches and trunks (Marvaldi *et al.*, 2002). Presently, the numerous weevil species use every plant part and nearly every plant taxon (Anderson, 1995). The majority of species are oligophagous or even monophagous, although exceptions of polyphagous species also exist (e.g. the vine weevil, *Otiorhynchus sulcatus*). Despite its diversity and number of species, the family Curculionidae constitutes a monophyletic group (Marvaldi *et al.*, 2002). Interestingly, lineages often show strong conservatism in the evolution of host use, which means that host associations generally reflect taxonomic relatedness (Marvaldi *et al.*, 2002).

Weevils compete fiercely with humans for food resources. The family Curculionidae includes some of the most important pest insects in agriculture, forestry and stored products. Examples are *Anthonomus grandis* on cotton; *Cylas formicarius* on sweet potato; *Sitona* spp. on beans and peas; *Rhynchophorus* spp. on palm trees, *Hylobius* spp. and *Pissodes* spp. on Pinaceae; *Sitophilus spp.* on corn, rice and wheat (cf. Hoffmann *et al.*, 1963; Hofmann and Meyer, 1995; Nottingham *et al.*, 1989; Nordlander, 1990; Alauzet, 1984; Walgenbach *et al.*, 1987). The damage is generally caused by the activity of both larvae and adults. Historically, searching for pheromones has been the preferred way to find potent attractants for pest insects. However, more than 20 pheromones have been chemically identified in weevils, and after decades of development, only relatively few have found successful application for control (Bartelt, 1999). It has been suggested that the reason for the disappointing results might be the poor understanding of which plant compounds interact with the pheromone attraction. Knowledge has been accumulating describing the means by which weevil species find their host plant, aimed at designing efficient methods to protect crops. Various species of weevils have been shown to be attracted to blends of volatiles of their host plants (see Bichão, 1998, and references therein). However fewer studies have investigated the mechanisms of

olfactory detection in these insects. Examples are studies of the cabbage seed weevil, *Ceutorhynchus assimilis* (Blight *et al.*, 1995), the pine weevil *Hylobius abietis* (Wibe and Mustaparta, 1996; Wibe *et al.*, 1997) and of the cotton boll weevil, *Anthonomus grandis* (Dickens, 1990).

Pissodes and Anthonomus weevils

Two oligophagous weevil species were chosen as model organisms for the present study, the pine weevil, *Pissodes notatus* F. (Coleoptera, Curculionidae) feeding on conifers (Pinaceae), and the strawberry blossom weevil, *Anthonomus rubi* Herbst, (1795) (Coleoptera, Curculionidae), feeding on angiosperms (Rosaceae) (Popov, 1996b) (Appendix I). They represent an ancestral host association (D. Langor, personal communication) and a more recent one, respectively. Both species are important pest insects in their distribution areas. *P. notatus* is widely distributed in Eurasia, and part of Macaronesia, where it is considered a secondary pest in young plantations of *Pinus pinaster* Aiton (De Viedma, 1961; Alauzet, 1984; Ferreira and Ferreira, 1989; Cobos and Robredo, 1982). The eggs are laid under the bark and the larvae feed on the phloem of the trunk, interrupting the sap circulation and eventually causing the death of the tree. Traditional methods of forest protection use "bait logs" to attract ovipositing females, suggesting that host odours are involved in orientation towards the trees (Plata-Negrache and Prendes-Ayala, 1979). However, which volatiles are involved is not known. The other model organism, *A. rubi,* distributed throughout Western and Central Europe, is an important pest of early season strawberries (Cross and Easterbrook, 1998; Popov, 1996a; Aasen, 2001). After laying an egg in an unopened flower bud, the female partially severs the flower stalk with her rostrum (Jary, 1932; Alford, 1984). The flower bud withers and often falls from the plant. The larvae develop within the damaged bud, pupate and the adults emerge in the summer. These adults are in reproductive diapause and feed for a few weeks before migrating to over wintering sites. Each female may sever large numbers of flower buds leading to severe losses. *A. rubi* is oligophagous, feeding and reproducing mainly on strawberry (*Fragaria* × *ananassa* Duchesne) and raspberry (*Rubus idaeus* L.) (Popov, 1996a,b). A male-produced aggregation pheromone of *A. rubi* has been characterized, composed of grandlure I, grandlure II and lavandulol (Innocenzi *et al.*, 2001). However, so far field studies show that it cannot be used alone for pest control, only for monitoring attack (Cross *et al.*, 2005a,b). However, the same studies indicate that the plant compound germacrene D may enhance the attractiveness of the pheromone blend, suggesting

a synergistic effect of plant compounds. Knowing which plant odorants the weevils detect seems to be essential to advance the management of these important pest species.

The investigations in this study aimed at finding out which plant volatiles are detected by RNs of *P. notatus* and *A. rubi.* Electrophysiological recordings from single olfactory receptor neurones were performed during stimulation with plant volatiles separated by gas chromatography. Chemical identification of the active components by GC mass spectrometry followed. Similar test protocols were used for both species in order to be able to compare RN specificities. In this way were identified naturally occurring plant odorants that might be relevant to host finding. The results add to the knowledge of how the olfactory system functions in weevils, and establish a solid basis for the selection of compounds for behavioural studies. In addition, the results may contribute to elucidate evolutionary aspects of olfactory detection.

Aims of the thesis

This thesis focused on the detection and encoding of plant odour information by the olfactory receptor neurones of weevils, using as model organisms the oligophagous weevil species *Pissodes notatus* and *Anthonomus rubi*. These weevils use as hosts Gymnospermeae and Angisopermeae plants, respectively.

The aims of the thesis were:

- To identify host and non host plant produced compounds detected by single olfactory receptor neurones (RN) in the two species
- Characterise the plant odour RNs by their molecular receptive ranges, specificity and sensitivity and elucidate whether the RNs can be classified into distinct functional types.
- Compare RN types across the two weevil species living on different hosts
- Investigate whether the odorants are constitutive in the host plant or induced by insect feeding
- Select relevant compounds for behavioural studies.

Survey of the individual papers

Paper I

In the search for compounds that contribute to the perception of odour quality in the weevil *Pissodes notatus*, single RNs in the antennae were screened for sensitivity to naturally produced plant volatiles by the use of GC-SCR. The active compounds were chemically identified using GC-MS. Volatiles collected from the headspace of the host-plant *Pinus pinaster* and from the sympatric non-hosts, *Pinus pinea* and *Eucalyptus globullus*, were used as test mixtures. These mixtures were tested on most neurones. Headspace from *Picea abies* (sawdust) was also used in order to compare the results with those of a previous study of the weevil *Hylobius abietis* (Wibe and Mustaparta, 1996). Altogether 60 plant odour RNs were classified into 12 distinct types. These responded to 25 of the numerous compounds released by host and non-host plants. All the RNs showed high selectivity responding best to one or two compounds (primary odorants) and weaker to a few chemically related compounds (secondary odorants). The two most abundant RN types responded to α-pinene, β-pinene, and

3-carene (n=17), and to isopinocamphone and pinocamphone (n=17). Other neurone types were tuned to limonene (n=9), β-phellandrene (n=3), and fenchone (n=4). Responses to the sesquiterpene β-caryophyllene (n=1) and to ethanol (n=4) were also recorded. Except for two pairs (types 1 and 2 and types 5 and 6), the RN types did not show overlap of the molecular receptive range. In addition, indication of enantioselectivity of the RNs responding to fenchone appeared when these were tested with standard samples of the enantiomers. Further investigation of enantioselectivity was not preformed in this study. The odorants identified for *P. notatus* were present in the host *P. pinaster* as well as in non-hosts, supporting the principle that herbivorous insects detect the ratio of generaly occuring compounds specific of the host, rather than specific odorants. Many RNs did not respond to any of the test compounds, suggesting that some relevant odorants were lacking in the test mixtures. The results in this study show that plant odour information in *P. notatus* is mediated by a relatively large number of selective RN types. The different types respond specifically to one or a few structurally related compounds. Major as well as minor constituents of the volatile blends seem to be used for host and non-host detection, mainly monoterpenes (85% of the RNs detect bicyclic or monocyclic monoterpenes), but also sesquiterpenes and ethanol. Only a few RNs seem to have receptor specificity similar to that of the RNs of the weevil *H. abietis*.

Paper II

In this paper, nine RN types were described in *P. notatus*, four of which for the first time. The RN type specified for the aromatic compound *o*-methylanisole, present in minute amounts in hosts and non-host materials, constitutes the fifth most abundant RN type (n=5) in the species and had not been found in the previous study. The secondary odorants identified for this neurone type were the related compounds *m*-methylanisole, xylene and *p*-methylanisole. Three new receptors tuned to sesquiterpene hydrocarbons appeared, but the specific odorants were present in trace amounts hindering their definitive chemical identification. The molecular receptive ranges of RN types first described in paper I (types 1, 3, 4 and 12, *cf.* Table I) were confirmed, and some were further elucidated (types 4 and 12, *cf.* table I). The primary response to limonene of RN type 4, (Paper I) and the secondary response to β-myrcene were confirmed. The enantioselectivity was tested using the chiral column, which showed that this RN type responded to both enantiomers but was ca.10 times more sensitive to (-)-limonene. This was confirmed by dose response curves. Two types of RNs responding to ethanol were recorded in this study, one showing strong excitatory responses and another showing an inhibitory response. The high selectivity of the RN type excited by

ethanol was demonstrated by the lack of response to the numerous mixtures and chemical standards tested. Dose response investigations showed that these RNs were 100 000 times more sensitive to the primary odorant ethanol than to the secondary odorant methanol. The very high sensitivity of the RNs excited by ethanol, as well as the existence of one type inhibited by this compound, indicates an important role of ethanol in the chemical ecology of the species.

The results, taken together with those in paper I, indicate that the majority of RNs in *P. notatus* are tuned to bicyclic and monocyclic monoterpenes, both in total number of RNs and number of RN types (6 types). Other groups of naturally produced plant compounds are also detected and may be important for host finding. These are sesquiterpenes (5 RN types), aliphatic alcohols (1 RN type) and aromatics (1 RN type). Particularly interesting are the results showing the sensitivity to ethanol since this compound is emitted in great amounts by stressed or wounded pine trees.

Paper III

In this study, single receptor neurones in the antenna of the oligophagous strawberry blossom weevil *Anthonomus rubi* were screened for sensitivity to naturally produced plant compounds by the use of GC-SCR followed by GC-MS. The compounds detected by five types of RNs in the strawberry weevil are presented. The RNs were narrowly tuned and could be classified in distinct types according to one primary odorant. The selectivity was particularly evident in the most commonly found RN type, which responds to (-)-germacrene D, the (+) - enantiomer having a 100-fold lower effect. The primary odorants of the 5 RN types were (-)-germacrene D, (-)-β-caryophyllene, methyl salicylate, *E*-β-ocimene and *E*-DMNT [(3*E*)-4,8-Dimethyl-1,3,7-nonatriene]. Investigation of the composition of the blend emitted by the flowers before and after weevil attack was made by the collection method Solid Phase Micro Extraction (SPME). The primary odorants and also many of the secondary odorants of *A. rubi* RNs were present in the intact strawberry plant. However, weevil feeding induced their release in higher quantities. It is interesting to note that the two more common RN types are tuned to germacrene D (n=10) and E-*β*-ocimene (n=8), which are also the compounds dominating the induced blend.

The protocols used were designed to allow the comparison with RN types described in other species. Whereas striking similarities were found for the primary odorants, among weevils and moths, the secondary odorants differed, often considerably. For example, both *A. rubi* and the heliothine moths have enantioselective RNs tuned to (-)-germacrene D, with

similar sensitivity, but different secondary odorants. In contrast, no similarities were to be found between the RNs of this weevil and those of *P. notatus*, with the exception of the RNs tuned to the sesquiterpene β- caryophyllene (*cf.* Table I).

The five functional types of RNs in the antennal sensilla of *A. rubi* presented in this paper were narrowly tuned to odorants, which are common secondary metabolites present in many host and non-host plants. These odorants are inducible, suggesting that *A. rubi* may use plant-produced compounds as information about the presence of conspecifics on the host plant. In addition, the results show that RNs tuned to the same compounds exist in unrelated species.

Paper IV

This paper presents 17 RN types identified in *A. rubi*. These increase to 22 the total number of RN types identified in the species (added to the 5 types characterized in paper III), two of which are RNs tuned to pheromone components. The active compounds were terpenoids, aromatics and aliphatic esters, alcohols and aldehydes, some of which are induced in the plant by feeding activity of the weevils (Paper III). The neurones were characterised by a strong response to one or two primary odorants and weaker responses to a few others. With one exception, the molecular receptive range of each neurone type was within one chemical group. Enantiomers of linalool separated in a chiral column activated two RN types with different enantioselectivity. Inhibition by linalool of another RN type, excited by α-pinene, indicated an additional mechanism for coding the information about this compound. Altogether, detection of 54 compounds by olfactory receptor neurones is shown, of which 40 have been chemically identified in this study. Thus *A. rubi* has the ability to detect a large number of odorants that may be used in host selection behaviour. Data presented indicate that the GC-EAG method was not as sensitive as GC-SCR in identifying the complete scope of compounds detected by *A. rubi*.

This study has increased the knowledge about how the plant odour information is encoded in the RNs of *A. rubi*. Excitation was the main response mode of these RNs but inhibition was also recorded. Altogether the results of papers III and IV show that *A. rubi* uses a large number of odorants produced by different biosynthetic pathways to obtain clues about potential food sources, reproduction sites and mates. All the active plant compounds occur in many plant species, and most of the primary and secondary odorants can be classified as induced compounds, produced by the host plant in response to biotic or mechanical damage.

Table I. Survey of the olfactory receptor neurone types identified in the weevil species *Pissodes notatus* and *Anthonomus rubi* ([a] paper I; [b] paper II; [c] paper III; [d] paper IV)

Primary odorant	*Pissodes notatus*	*Anthonomus rubi*
Monoterpenes		
α-Pinene	Type 1 [a],VIII [b]	Type 3 [d], 4 [d]
Limonene	Type 4 [a],II [b]	
(-)-Limonene	Type II [b]	
E-β-Ocimene		Type IV [c]
E-DMNT		Type V [c]
β-Phellandrene	Type 5 [a]	
Oxygenated monoterpenes		
Fenchone	Type 2 [a]	
Isopinocamphone	Type 3 [a],IX [b]	
(-)-Linalool		Type 1 [d]
(+)-Linalool		Type 2 [d]
E-Grandisol		Type 17 [d]
Lavandulol		Type 16 [d]
unidentified	Types 8 , 9 [a]	Type 12 [d],13 [d]
Sesquiterpenes		
β-Caryophyllene	Type 7 [a]	Type II [c]
(-)-Germacrene D		Type I [c]
unidentified	Types 10,11 [a] V-VII [b]	
Aromatic compounds		
Eugenol		Type 10 [d]
o-Methylanisole	Type I [b]	
p-Methylanisole		Type 9 [d]
Methyl salicylate		Type III [c]
Aliphatic compounds		
Ethanol	Type 12 [a]III,IV [b]	
1-Octen-3-ol		Type 5 [d]
(3*Z*)-Hexenyl acetate (2*Z*)-Hexenyl acetate		Type 6 [d]
(2*E*)-Hexen-1-ol		Type 7 [d]
Dodecanal		Type 8 [d]
unidentified		Type/group 11 [d]

◊ incompletly characterized types 14 [d] and 15 [d] are not represented, since the chemical group could not be identified with certainty by the GC-MS work.

Discussion

Insect olfactory receptor neurones (RNs) have been accessible for investigations using electrophysiological methods since the pioneering works of Schneider (1957) and Boeckh (1962), who recorded RN responses to pheromones in the silk moth, and to host odours in the beetle *Necrophorus*. Whereas pheromones of numerous insect species have been identified, much less is known about the ligands for which plant RNs are evolved. The interest for this topic has become accentuated with the molecular identification of olfactory receptor proteins. To identify the ligands that activate these protein receptors, has been one of the great challenges in olfactory research. Since each RN expresses only one type of receptor protein (with few exceptions), its responses reflect the specificity of the receptor proteins. The method linking gas chromatography to electrophysiological recordings from single RNs (GC-SCR) has provided reliable information about the specificity of the plant odour RNs in several insect species (Blight *et al.*, 1995; Wibe *et al.*, 1997; Røstelien *et al.*, 2000a,b, 2005; Stranden *et al.*, 2002, 2003a,b; Stensmyr *et al.*, 2001; Barata *et al.*, 2002). The present study has contributed to this field with knowledge about functional properties of RNs of the pine weevil *Pissodes notatus* and the strawberry blossom weevil *Anthonomus rubi*.

Which plant compounds are detected?

In the search for food or oviposition sites, an herbivorous insect could assess the identity of a plant by using one or a few compounds present only in the specific host plants as a direct clue of plant identity. Alternatively, it could detect a wider spectrum of general plant compounds, and identify the specific combination emitted by the adequate host plant (Visser, 1986); Bruce *et al.*, 2005). The weevils investigated in this study, detect a wide range of volatiles present both as major and minor constituents of the headspace of plants. Altogether the data show that *P. notatus* and *A. rubi* detect 35 and 78 compounds, respectively, of which 20 and 63 have been chemically identified. The odorants are monoterpenes, sesquiterpenes, aromatic compounds, aliphatic alcohols, aldehydes and esters, many of which are produced by the hosts of both species. For example, both weevils can detect α-pinene and β-caryophyllene with specialized RNs (Table I, papers I and III). Obviously, not all volatiles common to the two host plants are detected by both weevils. Two good examples are (-)-germacrene D and *E*-β-ocimene, which are emitted by both pine and strawberry, but are found to be detected only by *A. rubi* RN (Paper III). The compounds identified as odorants in this

thesis are widespread in the plant world. Thus, in general the single compounds do not seem to provide a basis for host recognition, which supports the hypothesis that insects use a blend of many compounds in a certain ratio to recognize a host plant. This does not exclude that some compounds may advertise the presence of a host plant within a more restricted group of plants. For instance *P. notatus* detects the widespread sesquiterpene β-caryophyllene, which is characteristic of the preferred host *Pinus pinaster* within the pines (Borg-Karlson, personal communication).

The development of analytical methods available for studying plant volatile emissions has revealed that many plants release the same compounds. Even plants belonging to distantly related groups such as gymnosperms and angiosperms have enzymes producing the same compounds. Some of these enzymes may have changed little since the separation of the two groups, while others seem to have undergone convergent evolution for the production of the same compounds, as revealed by phylogenetic distances among them (Martin *et al.*, 2004). Examples of the latter are limonene, myrcene and linalool synthases found in the two goups of plants (Bohlmann *et al.*, 1998; Martin *et al.*, 2004). Still, considerable differences exist between the emitted blends of plants, reflecting the characteristics of the various enzymes of the secondary metabolims and the influence of environmental factors. The result is that each plant species has a chemical signature. As a consequence, insects exploiting different hosts are likely to detect different ensembles of volatiles with more or less common compounds.

How are compounds detected

Narrowly tuned RNs

The results on functional types of RNs in the pine weevil *P. notatus* and the strawberry blossom weevil *A. rubi* elucidate peripheral mechanisms involved in the detection of plant odour information in weevils. The RNs were narrowly tuned, responding consistently and selectively to one primary odorant (eliciting the strongest response) and a few secondary odorants (eliciting weaker responses) (Papers I to IV). The uniform response pattern within one RN type correlates well with the current information from molecular biology studies showing that subsets of olfactory RNs in general express one type of receptor protein in insects and vertebrates (Störtkuhl and Kettler, 2001; Wetzel *et al.*, 2001; Keller and Vosshall, 2003; Hallem *et al.*, 2004). The narrow tuning of RN is further demonstrated by the drop of stimulatory effect as a consequence of small differences in the molecular structure of the primary odorant. One example is the (-)-germacrene D RN type in *A. rubi*, in which the

direction of the isopropyl group relative to the ten carbon ring explains a one hundred fold difference in activity of the (-)- and the (+)-enantiomer (Paper III, Table I). Also the RNs responding to *o*-methylanisole in *P. notatus*, had a 100-fold weaker response when the relative positions of the methyl and methoxy groups were changed (Paper II). Molecular features of importance in receptor-ligand interactions revealed by the structure activity studies of the RNs of weevils were chirality, carbon chain length, electron dense parts and flexibility of the molecules, which are reflected by enantiomers, number of C-atoms, double bonds and open vs. cyclic structures. These features found in the olfactory system seem to be universal among receptor-ligand interactions. (Kafka, 1974; Priesner, 1977, 1979; Bengtsson *et al.*, 1990; Ohloff, 1986, 1994; Masson and Mustaparta, 1990; Leal, 2001; Laska *et al.*, 1999, 2004 among others).

Overlap of the molecular receptive range (MRR) of RNs has traditionally been discussed in connection with the principles governing the transmission of information about one odorant to the brain (i.e. 'labelled lines' and 'across-fibre' patterns) (Mustaparta , 2002). In *A. rubi*, and *P. notatus* was observed both non-overlap as well as some overlap of the MRR of the narrowly tuned RNs. This indicates that, depending on the odorants, both principles co-exist in weevils, in accordance with previous studies of RNs in weevils and other beetles (e.g. Wibe *et al.*, 1998; Barata *et al.*, 2002). The results indicate that in these weevils, odour quality is partly coded in a combinatorial manner. Combinatorial codes for odours have been discussed in insects, e.g. the honey bee, based on studies using calcium imaging to measure glomerular activity in the antennal lobe (Galizia *et al.*, 2000). The non-overlapping MRRs indicate in addition a labelled-line mechanism for odour discrimination. However, identification of more RNs may reveal higher or lower degrees of overlap of the MRR, in these insects. In contrast to the highly selective RNs obtained by GC-SCR, RNs showing broader tuning and more overlap of the MRRs have been found in studies using pre-selected compounds as test samples. Examples are RNs of the fruit fly *D. melanogaster* and of vertebrates (De Bruyne *et al.*, 2001; Buck, 2000). Some of the RNs in these organisms might appear with higher sensitivity and narrower tuning when tested with other odorants (De Bruyne *et al.*, 2001). On the other hand, it is possible that in vertebrates and *D. melanogaster* the receptor proteins are actually broadly tuned. Alternativelly, some RNs may express more than one receptor protein as recently shown for one RN type in *D. melanogaster* (Goldman *et al.*, 2005).

Inhibition – a peripheral coding mechanism?

In many electrophysiological studies of insect RNs using GC-SCR, mostly excitatory responses have been obtained (e.g. Paper I and II, Wibe and Mustaparta, 1996; Røstelien *et al.*, 2005; Barata *et al.,* 2002). However, inhibition has been reported in various studies using direct stimulation, including recent studies of the fruit fly *D. melanogaster* (Boeckh, 1967; Ma and Visser, 1978; Kaissling *et al.*, 1989; De Bruyne *et al.*, 2001; Shields and Hildebrand, 2001; Saïd *et al.*, 2003; Hallem *et al.*, 2004). In these works the RNs are in general inhibited by one odorant and activated by another. In the present study of *A. rubi*, one RN type was inhibited by *racemic* linalool and 1,2-dihydrolinalool, and excited by α-pinene (Paper IV). The results from *A. rubi* are of particular interest since inhibition by remarkably low concentrations of 100% pure compounds was demonstrated. The fundamental implication of the existence of two modes of response in the same RN is that this may allow the RN to function as an integrating unit, as proposed e.g. for the lobster (Ache, 1994; Boekhoff *et al.*, 1994). Boeck (1967) proposed that a mechanism for the existence of two modes of response in the same RN would include separate binding sites of the same receptor protein. The markedly different molecular structures of the excitatory/inhibitory odorant pair described in *A. rubi* (α-pinene/linalool), as well as other insects, support this view. In a recent study of *D. melanogaster*, a model is proposed based on a steady state of the membrane receptors which can be disturbed by ligands in either direction of the equilibrium (Hallem *et al.*, 2004). The existence of excitatory and inhibitory detection by the same receptor may not be consistent with a single transduction pathway in insect olfactory RNs. It is possible that insects, like the lobster, have two transduction pathways, one mediating each of the modes of response. Recent studies indicate that pathways involving messengers other than the IP_3, may be present also in insects (Krieger and Breer, 2003; Gomez-Diaz *et al.*, 2004), and in vertebrates multiple transduction pathways have also been suggested (Vogler and Schild, 1999).

Understanding peripheral integration of odorants requires stimulation of the RNs with mixtures of the activating and inhibiting compounds in variable ratios. Studies using relatively high concentrations of stimuli, have reported higher thresholds for inhibition than for excitation in insect RNs (Boeckh, 1967). Inhibitory odour input can serve to increase the diversity of the neuronal patterns for odour discrimination, (Ache, 1994; Malnic *et al.*, 1999). In the study of *D. melanogaster* inhibition was proposed to enhance the contrast of activity in the glomeruli, by one compound inhibiting a population of RNs and activating another, thereby contributing to signal recognition (De Bruyne *et al.*, 2001). This may also be the case in *A. rubi* having two types of RNs excited by linalool and another type inhibited by the same

molecule. The existence of inhibitory responses may also explain why EAG studies often give different results when compared with SCRs (Paper IV, see also Wibe, 2004; Blight *et al.*, 1995; Jönsson and Anderson, 1999).

Co-location: A basis for spatial-temporal discrimination?

A conspicuous feature of the olfactory system in insects is the co-location of functional RN types (Papers I-IV; Blight *et al.*, 1995; De Bruyne *et al.*, 2001; Stensmyr *et al.*, 2003; Røstelien *et al.*, 2000a,b, 2005; Stranden, *et al.*, 2003a,b among others). The RNs appear in co-located stereotyped groups within each species. It has also been demonstrated that these groups can be similar across closely related species of heliothine moths. The same four functional RN types are collocated in *Heliothis virescens, Helicoverpa armigera* and *Helicoverpa assulta* (Røstelien *et al.*, 2000b; Stranden *et al.*, 2003b). In the weevils, RN types appear in pairs which may show overlap of MRR (e.g. in *A. rubi*, paper III, IV) but often the primary odorants activating each RN belong to different chemical groups and overlap is not observed (Papers I to IV). The functional significance of co-localization is not known. A suggested function of increased spatio-temporal resolution in detecting arrival of odorants comes from studies of the pheromone system (Bruce *et al.*, 2005). Baker *et al.*, (1998), have shown in behavioural studies that males of the moth *Helicoverpa zea* are attracted when the pheromone blend is pulsed from a point source. Pulsing an antagonist interspecific signal from the same point source, reduces the attraction. However, if the same antagonist signal is pulsed from a source 1 mm apart from the pheromone source (i.e arriving at the antenna with ca. 0,001-0,003 s time difference), the effect is significantly less pronounced. Interestingly, in the male moths, RNs detecting one of the attractive pheromone components are often co-located with a RN specifically tuned to a behavioural antagonist (Baker *et al.*, 1998; Berg *et al.*, 2005). The authors propose that the co-location of RNs is the morphological basis for the moths' ability to discriminate odour plumes in which the attractive and antagonist signals are released from one source, from those in which the odorants are released from different sources (Baker *et al.*, 1998). Thus it is thought that the spatio-temporal resolution exhibited by the moth can only be achieved by sampling the air at the same point in space and time i.e. by co-located RNs.

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Comparison of RN specificity

Comparative studies of RN specificity across species can give interesting information about conservation of, or changes in functional properties of RNs throughout evolution. Detailed comparisons of RN specificity among the species of weevils in this study and moths have been enabled by the use of similar test protocols (Røstelien *et al.* 2000a,b, 2005; Stranden *et al.,* 2002, 2003a,b; Ulland *et al.*, 2006, reviewed by Mustaparta and Stranden, 2005). The use of the GC-SCR method has provided information with the degree of detail required by comparisons among species. The method gives information about the compounds that activate the RNs and also rules out all the components in the various mixtures that do not elicit responses, giving a very precise MRR. It is important to note that these studies were not specifically designed to investigate evolutionary relationships. However, the relatively large numbers of RNs classified in weevils and moths reveal striking similarities across distantly related species that inspire some considerations (Table II).

One could hypothesise that RNs which show similar MRR across taxa are likely to have been conserved through evolutionary time. For instance, the (-)-linalool and *E*-β-ocimene RNs in *A. rubi* and the heliothine moths have the same primary odorant and ranking of the secondary odorants, with only slight differences in width of the MRR (Paper IV, Table II). Within the two weevils species investigated, the (-)-β-caryophyllene and α-pinene RN types are other example of possibly conserved types (Table II). Conversely, RNs with a common primary odorant but with different secondary odorants may have undergone convergent evolution. Examples are the RNs tuned to (-)-germacrene D found in weevils and heliothine moths (Paper III, Table II). These have the same sensitivity and the same discrimination of germacrene-D enantiomers, but differ in the other secondary odorants. Whereas *A. rubi* RNs respond secondarily to caryophyllene and bourbonene, RNs in the moths respond to copaenes and ylangenes (Paper III, Stranden *et al.,* 2003a). These receptor neurones may have evolved as adaptations to a convergent evolution of the host plants biosyntheses (Martin *et al.*, 2004). Alternatively, the secondary odorants might not have been subject to selection pressure. To shed more light on the topic of evolution of RN specificity, one needs to know the molecular structures of receptor proteins in the different insect species. So far, sequence diversity within and across species (fruit fly, mosquito and moth), has been found in insect olfactory receptor genes (Breer, 2003), as well as cases of remarkable sequence conservation (Hill *et al.*, 2002; Krieger *et al.*, 2003).

Table II. Molecular receptive range of selected receptor neurone types identified in species of weevils (*Pissodes notatus and Anthonomus rubi*) (papers III-IV) and moths (*Heliothis virescens, Helicoverpa armigera, Helicoverpa assulta and Mamestra brassica*)[1] using GC-SCR and similar test protocols. Primary odorants indicated in bold. (* enantiomers were not tested; The order of elution of enantiomers of dihydrolinalool is not known: [a] indicates the enantiomers eluting first; [b] the enantiomer eluting second, cf. Paper IV)

Receptor neurone types	Weevils		Moths			
	P. notatus	*A. rubi*	*H. virescens*	*H. armigera*	*H. assulta*	*M. brassica*
(-)-Germacrene D		**(-)-Germacrene D** (+)-Germacrene D (-)-β-Caryophyllene α-Humulene β-Bourbonene 3-Pentanone 3-Hexanone	**(-)-Germacrene D** (+)-Germacrene D (-)-α-Ylangene (+)-β-Ylangene (+)-α-Copaene β-Copaene	**(-)-Germacrene D** (+)-Germacrene D (-)-α-Ylangene (+)-β-Ylangene (+)-α-Copaene β-Copaene	**(-)-Germacrene D** (+)-Germacrene D (-)-α-Ylangene (+)-β-Ylangene (+)-α-Copaene β-Copaene	
(*S*)-(+)-Linalool		**(*S*)-(+)-Linalool** (*R*)-(-)-Linalool (+)-Dihydrolinalool [b] (-)-Dihydrolinalool [a]	**(*S*)-(+)-Linalool** (*R*)-(-)-Linalool Dihydrolinalool*	**(*S*)-(+)-Linalool** (*R*)-(-)-Linalool Dihydrolinalool*		
(*R*)-(-)-Linalool		**(*R*)-(-)-Linalool** (*S*)-(+)-Linalool Dihydrolinalool [a] Dihydrolinalool [b]				**(*R*)-(-)-Linalool** (*S*)-(+)-Linalool Dihydrolinalool [a] Dihydrolinalool [b]
***E*-β-Ocimene**		***E*-β-Ocimene** β-Myrcene *Z*-β-Ocimene *E*-DMNT Citronellol Geraniol Geranial Neral Limonene γ-Terpinene β-Phellandrene	***E*-β-Ocimene** β-Myrcene *Z*-β-Ocimene *E*-DMNT	***E*-β-Ocimene** β-Myrcene *Z*-β-Ocimene *E*-DMNT	***E*-β-Ocimene** β-Myrcene *Z*-β-Ocimene *E*-DMNT	
(-)-β-Caryophyllene	**(-)-β-Caryophyllene** α-Humulene	**(-)-β-Caryophyllene** α-Humulene				
α-Pinene	**α-Pinene** β-pinene (+)-3-carene	**α-Pinene** β-pinene				

[1] (Røstelien *et al.*, 2000b, 2005; Stranden *et al.*, 2002a,b, 2003; Ulland *et al.*, 2006)

Chirality is an important feature of volatiles from angiosperms and gymnosperms (Norin, 1996) and plant odour RNs of insects often show enantioselectivity (Papers II-IV; Wibe *et al.*, 1998; Stranden *et al.* 2002). As a rule, when enantioselectivity of RNs has been demonstrated, each species has only one RN type, tuned to one of the enantiomers and responding weaker to the other (cf. Table II and III). This implies that the enantiomers may not be dricriminated by the insect, and both convey the same message. The RNs tuned to (-)-germacrene D follow this rule in *A. rubi* and moths (Table II). In contrast, in *A. rubi*, separate RNs tuned to (-)- and to (+)-linalool were identified (Paper IV). This means that (-)-linalool and (+)-linalool may carry different messages to the insect. Interestingly, this correlates well with the presence of the different enantiomers in plants. Whereas (-)-germacrene D is often the dominating enantiomer occurring in higher plants (Lorimer and Weavers, 1987; Bülow and König, 2000; Mozuraitis *et al.*, 2002), the ratio of (+)- and (-)-linalool varies within and among the different plant species (Borg-Karlson *et al.*, 1996; Casabianca *et al.*, 1998; Borg-Karlson, unpublished results).

Chemical ecology aspects

Comparing species living in angiosperms and gymnosperms

Insects exploiting different hosts, like Angiospermeae and Gymnospermeae, are likely to detect different ensembles of compounds, corresponding to an adaptation to differences in emissions. As a comparison, table III presents a survey of RN types tuned to the same primary odorants found in *A. rubi* and *P. notatus*, as well as other species of Coleoptera and Lepidoptera, previously studied. A tendency is apparent suggesting that the detection of sesquiterpenes is more prominent in species living on flowering plants (e.g. 72% of the RNs in Heliothine moths are tuned to sesquiterpenes), whereas many RNs seems to be dedicated to the detection of bicyclic monoterpenes in the species living on conifers (e.g. 72 % in Pissodes and 85% in *Hylobius abietis* (Røstelien *et al.*, 2005; Wibe *et al.*, 1997). Thus a general correlation seems to appear among the primary odorants of RNs and the principal emissions of the two groups of plants. Of course exceptions exist, e.g. of angioperms extremely rich in bycicilc monoterpene emission. Interestingly, *A. rubi* appears in an intermediate position with 30% of the RNs tuned to sesquiterpenes and 32% to monoterpenes. However, the majority of these monoterpene RNs belong to the type tuned to *E*-β-ocimene, which is also typically induced by herbivory in leaves and flowers of angiosperms (Paper III; Borg-Karlson *et al.*, 1994; Dudareva and Pichersky, 2000; De Moraes *et al.*, 2001; Arimura *et al.*, 2004). The RNs

of *A. rubi* may reveal the ancestral host association of the weevils (conifers) modified by the adaptation to an angiosperm host (strawberry) (Tables II and III). Detection of C6 alcohols and esters (commonly designated "green leaf odours") seems also to be more common in species living in angiosperms. Whereas some species living on conifers detect ethanol (Table III), some may also detect green leaf volatiles as indicated by EAG studies of bark beetles (Huber *et al.*, 2000). This correlates well with the emission of plants subjected to mechanical damages. Flowering plants emit green leaf odours after damage of the tissue (Bernays and Chapman, 1994), while pines also emit ethanol after damage (Sjödin *et al.*, 1989). Another aspect emerging from the comparisons is that species living on angiosperms detect compounds that are commonly induced defences in may plants (e.g. *E*-β-ocimene, *E*- DMNT, linalool and methyl salicylate), while conifer living species detect primarily constitutive defences of conifers (i.e. bicyclic monoterpenes). It is important to note that these comparisons include few species and reflects only a part of the total population of RNs of each species.

The role of induced compounds

Perhaps one of the most interesting findings in this thesis is that the majority of the RNs in *A. rubi* are tuned to odorants that are induced in the strawberry plant by feeding activity of conspecifics, e.g. (-)-germacrene D, *E*-β-ocimene, *E*-DMNT and methyl salicilate (Papers III and IV). Whether these compounds are attractive for *A. rubi,* or the weevil uses them to avoid infested plants, is not known. Since *A. rubi* mates on the host plant, after a feeding period, one can speculate that these induced compounds may guide weevils to plants infested by conspecifics, facilitating aggregation and mate encounters. While many studies have focused on tri-trophic interactions, others have investigated the effects of induced compounds on the herbivores. Mainly from studies with Lepidoptera, it is assumed that compounds induced by feeding mark occupied resources and thus repel the herbivores or limit egg-layng, minimizing competition (Kessler and Baldwin, 2001). Avoidance behaviour in response to induced compounds has been recorded e.g. for the beet armyworm caterpillars (*Spodoptera exigua*) and for ovipositing females of the moth *Heliothis virescens* (Turlings and Tumlinson, 1992; De Moraes *et al.*, 2001). In contrast, scarabaeid and leaf beetles (*Maladera matrida*, *Propillia japonica* and *Oreina cacaliae*) were attracted to host plants infested with conspecific and heterospecific herbivores (Harari *et al.*, 1994; Loughrin *et al.*, 1996; Kalberer *et al.*, 2001). Both strategies can confer adaptive advantages depending on the

Table III. Survey of RN types tuned to the primary odorants of the weevils *Pissodes notatus* and *Anthonomus rubi*. [(●) RN types characterised by GC-SCR. (+) RN characterised by direct stimulation. (-) compound tested, no RNs found. (?) unclear whether RNs exist. (open cell) compound not tested or not mentioned; (*) separate test of enantiomers not done. [1] species living on conifers. [2] species living on angiosperms]

		Coleoptera												Lepidoptera								
Primary odorants		*Trypodendron lineatum*[1]	*Dendroctonus pseudotsugae*[1]	*Hylobius abietis*[1]	***Pissodes notatus***[1]	***Anthonomus rubi***[2]	*Anthonomus grandis*[2]	*Ceuthorhynchus assimilis*[2]	*Rhynchophorus palmatum*[2]	*Leptinotarsa decemlineata*[2]	*Pachnoda marginata*[2]	*Phyllopertа diversa*[2]	*Phoracharita semipunctata*[2]	*Mamestra brassica*[2]	*Heliothis virescens*[2]	*Helicoverpa armigera*[2]	*Helicoverpa assulta*[2]	*Spodoptera littoralis*[2]	*Manduca sexta*[2]	*Bombyx mori*[2]	*Trichoplusia ni*[2]	*Antheraea pernyi*[2]
Monoterpenoids	α-Pinene	+	●	●	●	●	-			-			●	-	-	-		-	-			
	Limonene *		●	●	●	-	-				-			-	-	-		-	-			
	(-)-Limonene			●	●	-			+					-	-	-						
	β-Phellandrene			-	●	-								-	-	-						
	Fenchone			●	●	-								●	-	-						
	Isopinocamphone			-	●	-									-	-						
	E-β-Ocimene			-	-	●	+			+			●	●	●	●	●		-			
	E- DMNT				-	●									-	-						
	Linalool*			●	-	●	+	●		-	●		●	●	●	●		+	+	+		+
	(-)-Linalool			-	-	●								●	-	-						
	(+)-Linalool			-	-	●									●	●					+	
	E-Grandisol					●																
	Lavandulol					●																
Sesquiterpenoids	β-Caryophyllene			-	●	●	+			+	-			○	-	-		+	-			
	(-)-Germacrene D				-	●								○	●	●	●					
Aromatics	Eugenol				-	●				-	●	+		○	-	-		+	-			
	Methyl salicylate				-	●		●		+	●			●	-	-						
	o-Methylanisole			●	●	●								?	-	-						
	p-Methylanisole				-	●								●	-	-						
Aliphatics	Ethanol	+		●	●	-			+													
	1-0cten-3-ol				-	●		●														
	(3Z)- Hexenyl acetate				-	●		●		-	●	+		●	●	●		+				
	(2*E*)- Hexen-1-ol				-	●	+			+	●	-	●	●					+			
	Dodecanal				-	●																
References		1	2	3	4	5	6	7#	8	9	10#	11	12	13	14	15	16	17	18	19	20	21

biology of the herbivore. It has also been shown in species of weevils and mites that the role of the induced compounds is concentration dependent (Heil, 2004; Horiuchi *et al.*, 2003). These organisms prefer weakly induced plants to intact plants. However, heavily induced plants are not preferred. A low concentration of the induced blend may signal an acceptable plant populated by mates and be attractive, while high concentrations of the induced blend indicate an overcrowded resource.

Biosynthesis and insect detection – do they coincide?

As the knowledge about the biosynthetic pathways, genes and enzymes involved in the production of secondary metabolites in plants is growing, it is interesting to observe the coincidence of the selectivity of insect RNs with the products of biosynthetic pathways. In a recent study, the synthase involved in the production of *E*-β-ocimene in *Arabidopsis thaliana* has been shown to produce mainly *E*-β-ocimene (90%), with *Z*-β-ocimene and β-myrcene as by-products (Fäldt *et al.*, 2003). This metabolic pattern coincides with the primary odorant and the two next best compounds activating the *E*-β-ocimene neurones in *A. rubi* (Paper III) and the heliothine moths (Stranden *et al.*, 2003a). Strawberry flower emissions after weevil feeding activity showed a similar profile, where *E*-β-ocimene was the most abundant volatile followed by *Z*-β-ocimene and a small amount of myrcene (Paper III). Another study, describes a carboxyl methyl transferase in snapdragon flowers that catalyzes the production of methyl salicylate (main product), and also of methyl benzoate in smaller amount (Negre *et al.*, 2002). This also correlates with the sensitivity of RNs in *A. rubi* (Paper III) and in the moth *Mamestra brassica* (Ulland, personal communication). Another interesting observation is that rearrangement products derived from the main product of a pathway also activate RNs specified for the main product. For example, β-bourbonene, which is a product of rearrangement of germacrene D by UV radiation (susceptible of occurring in living plants), activated the RN types specified for (-)-germacrene D in this study (Paper III, see also

Table III. (cont.) 1. (Tømmerås and Mustaparta, 1989); 2. (Dickens *et al.*, 1984); 3. (Wibe *et al.*, 1997; Houtari *et al.*, 2003); 4. Papers I and II; 5. Papers III and IV; 6. (Dickens, 1990); 7. (Blight *et al.*, 1995); 8. (Saïd *et al.*, 2003) *et al.*, 2002; 9. (Ma and Visser, 1978); 10. (Stensmyr *et al.*, 2001); 11. (Nikonov *et al.*, 2001; Hansson *et al.*, 1999); 12. (Barata *et al.*, 2002); 13. (Ulland *et al.*, 2006), Ulland personal communication; 14, 15, 16. (Stranden *et al.*, 2002), (Stranden *et al.*, 2003b), (Røstelien *et al.*, 2000a), (Røstelien *et al.*, 2000b), (Røstelien *et al.*, 2005); 17. (Jönsson and Anderson, 1999), (Anderson *et al.*, 1995); 18. (Shields and Hildebrand, 2001); 19. (Kaissling *et al.*, 1989); 20.(Todd and Baker T.C., 1993) # GC-SCR studies in which tested compounds are not listed comprehensively.

Stranden *et al.*, 2003a; Røstelien *et al.* 2005). This may mean that the RNs of insects are optimized to detect main products of particular biosynthetic pathways, and that the by-products and rearrangement products reinforce the response to the primary odorant.

Implications for behavioural studies

After knowing for which odorants the receptor neurones are tuned, studies of the behavioural significance of single odorants and mixtures are needed. Given the complexity of the stimuli involved, selection of compounds is a crucial aspect of behavioural investigation. A general assumption is that both RN sensitivity and the total number of RNs tuned to a particular odorant are important for the distance over which the odorant is detected. In general, a large number of RNs with high sensitivity is thought to mediate long range attraction, while rare RNs with lower sensitivity may be recruited at closer range and be involved in host selection in the vicinity of the plant. According to this principle, α-pinene, isopinocamphone and *S*-(-)-limonene are potential long range behaviour modulators for *P. notatus* (Papers I and II). Interestingly, the air above the canopy of *P. pinaster* is dominated by α-pinene and limonene, together with β-pinene and 3-carene (Riba, 1991). In addition, detecting ethanol may allow *P. notatus* to select weakened trees (Sjödin *et al.*, 1989), which are favourable host individuals. The high sensitivity and selectivity of the RNs responding to *o*-methylanisole (Paper II), is interesting, especially in light of the recent findings that this compound is a deterrent of the pine weevil *H. abietis* (Borg-Karlson *et al.*, 2006). The behavioural responses to the electrophysiologically active compounds in *P. notatus,* is the object of ongoing studies. Preliminary results from bioassays indicate that ethanol may synergise α-pinene in the attraction of *P. notatus*, similar to what was found in studies of other beetles exploiting stressed trees (Nordlander, 1987; Tilles *et al.*, 1986; Tømmerås and Mustaparta, 1989). For *A. rubi*, (-)-germacrene D and *E*-β-ocimene may play a significant role over long distance, together with methyl salicylate, β-caryophyllene and *E*-DMNT (Paper III). Recent findings indicate that (-)-germacrene D enhances the attraction of *A. rubi* to pheromone traps in the field (Cross *et al.*, 2005a,b). Linalool is another important odorant for behavioural studies given the fact that three RN types with different sensitivities where found, which indicates a particularly fine tuning for this compound (Paper IV).

In addition to the innate responses to odorants, changed behaviour due to learning induced by previous experience must also be considered. Important results on odorant learning and memory come from studies with the honeybee, the fruit fly and more recently

moths (e.g. Carew, 2000; Davis, 2004; Skiri *et al.*, 2005). These results are based on conditioning experiments using various behavioural paradigms (e.g. the proboscis extension reflex or PER). In weevils, the possibility of learning is only starting to be investigated. The effect of larval or earlier adult experience on the preference for odours of adults of the granary weevil *Sitophilus granarius* and of the pine weevil *H. abietis* has been shown using multiple choice bioassays (Rietdorf and Steidle, 2002; Roten *et al.*, unpublished results). *A. rubi* and *P. notatus*, like weevils in general, are relatively long-lived insect species whose life cycle alternates between a reproductively active phase on the host plant, and a dormant period living in overwintering sites. This may make weevil species particularly interesting models for studies of learning and memory.

Concluding remarks and future prospects

The studies included in this thesis have provided information about which plant odorants the weevils *Pissodes notatus* and *Anthonomus rubi* detect. The results establish a solid basis for the selection of compounds to be tested in behavioural studies, contributing significantly to the progress toward alternative modes of pest management. This information is used in ongoing behavioural studies in the laboratory and in the field. The detailed knowledge of ligands activating each RN type is also a crucial contribution to ongoing studies of quantitative structure-activity relationships (QSAR), where modelling is used to determine which part of the molecules interact with the binding sites of the olfactory receptor proteins.

The results concerning *A. rubi* represent, to my knowledge, the only model in which both the selectivity of RNs and the induced emission of the host plant are known. They constitute the basis for a very interesting study of the coincidence of emission from the plant and the detection by the insect.

The results add to the growing knowledge about functional types of RNs also in a comparative perspective, i.e., in relation to RNs of closer and more distantly related insect species that have adapted to different host plants. Major differences between the particular species considered, using gymnosperms and angiosperms hosts, appeared e.g. in the number of neurons tuned to monoterpenes and sesquiterpenes, respectively. Also striking similarities were found regarding tuning of RNs among weevils that live on angiosperms and moths. Future studies e.g. of moths living in conifers and of other weevil species living on angiosperms will shed more light on the interesting topic of the evolution of specificity of RNs. The study of varied model species may allow us to unify principles while avoiding the pitfall of simplifying natures' ways.

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Appendix I: Taxonomic position of the insects

Pissodes notatus (Fabricius) 1787
Common name:
Ord. Coleoptera
SubOrd. Polyphaga
*SuperFam.*Curculionoidea
Fam. Curculionidae Latreille, 1802
SubFam. Molytinae Schoenherr, 1823
Tribe Pissodini Gistel 1856
Genus Pissodes Germar, 1817

Anthonomus rubi (Herbst), 1795
Common name:
Ord. Coleoptera
SubOrd. Polyphaga
*SuperFam.*Curculionoidea
Fam. Curculionidae Latreille, 1802
SubFam. Curculioninae
Tribe Anthonomini
Genus Anthonomus Germar, 1817

Abstract

Each herbivore in the course of its evolution became associated with particular plants. The maintenance of host specializations requires the ability to find and recognize plants, which in natural habitats often grow in mixed and complex vegetations. The process of host selection is mediated by integration in the central nervous system of various sensory inputs, including olfactory cues originating in the plant. Odours from plants are complex blends, comprising a wide scope of constituents, which vary in time, space, and between individual sources. Odours are in most organisms, including insects, detected by olfactory receptor neurones (RNs). The detection of olfactory stimuli begins with the interaction of odour molecules with odorant receptor proteins, which determine the specificity of the RNs [i.e. its molecular receptive range (MRR)]. Substantial knowledge about olfactory mechanisms in insects stems mainly from studies of species of moths, cockroaches, the honey bee and the fruit fly. This thesis has contributed to elucidate the mechanisms of olfactory detection in another major group of herbivores, the weevils (Curculionidae).

The family Curculionidae includes some of the most important pest insects in agriculture, forestry and stored products. Historically, searching for pheromones has been the preferred way to find potent attractants for pest insects. However, more than 20 pheromones have been chemically identified in weevils, and after decades of development, only relatively few have found successful application for control. It has been suggested that the reason for the disappointing results might be the poor understanding of which plant compounds interact with the pheromone attraction. Two oligophagous weevil species were chosen as model organisms for the present study, the pine weevil, *Pissodes notatus* F. (Coleoptera, Curculionidae) feeding on pines (Pinaceae), and the strawberry blossom weevil, *Anthonomus rubi* Herbst, (1795) (Coleoptera, Curculionidae), feeding on strawberry (Rosaceae). Both species are economically important pests in their areas of distribution.

The work in this thesis focuses on how plant odour information is detected and encoded by the RNs of weevils. The aims were: (1) To identify host and non host plant produced compounds detected by single olfactory receptor neurones (RN) in the two species; (2) To characterise the plant odour RNs by their molecular receptive ranges, specificity and sensitivity, and elucidate whether the single RNs can be classified into distinct functional types; (3) Compare RN types across the two weevil species living on different hosts; (4)

Investigate whether the odorants are constitutive in the host plant or induced by insect feeding; (5) Select the relevant compounds for behavioural studies.

Electrophysiological recordings from single RNs were performed during stimulation with plant volatiles separated by gas chromatography (GC-SCR), followed by chemical identification of the active components by GC mass spectrometry (GC-MS). Collection of volatiles from headspace of host and non-host plants was done by entrainment techniques, using adsorbents and by Solid phase micro extraction (SPME).

The thesis is organized in two parts. The first part includes the introduction to the topics and the methods, a presentation of the aims of the investigation, and a survey of the results followed by a general discussion with concluding remarks. The second part presents the original publications that form the basis of the thesis.

The weevils investigated in this study, detect a wide range of plant volatiles present both as major and minor constituents of the headspace of plants. The odorants are monoterpenes, sesquiterpenes, aromatic compounds, and also aliphatic alcohols, aldehydes and esters, many of which are produced by the hosts of both species. The compounds identified as odorants in this thesis are ubiquitous in the plant world. Thus, in general the single compounds do not seem to provide a basis for host recognition. These findings support the hypothesis that insects use a blend of many compounds in a certain ratio to recognize a host plant. The RNs were narrowly tuned, responding consistently and selectively to one primary odorant (eliciting the strongest response) and a few secondary odorants (eliciting weaker responses), and could be classified in types. Both in *A. rubi*, and in *P. notatus*, functional types of RNs may appear in stereotyped pairs, as found in studies of other weevils, moths and other insects. Both in *A. rubi* and in *P. notatus,* was observed some overlap of the MRR of the narrowly tuned RNs, showing that information about one odorant is conveyed to the brain by more than one RN type. Thus the results indicate that the odour quality is partly coded in a combinatorial manner. The majority of the responses elicited in the RNs were excitatory. However, one RN type in *A. rubi* showed excitatory responses to α-pinene and an inhibitory response to linalool. This exception raises the interesting question of whether integration of olfactory stimuli starts in the periphery.

The importance of chirality for bioactivity is well known. The use of GC-columns that separate enantiomers has allowed demonstrating that RNs tuned to plant odorants can also show enantioselectivity. In *A. rubi*, separate RNs tuned to (-)- and to (+)-linalool exist. This means that each of the two enantiomers may carry different messages to the insect. In contrast, only one type of RN tuned to (-)-germacrene D was identified. Enatioselective RNs

were also found in *P. notatus*, selectively responding to (-)-limonene. Other molecular features of importance in receptor-ligand interactions revealed by the structure activity studies of the RNs were carbon chain length, electron dense parts and flexibility of the molecules, which are reflected by enantiomers, number of C-atoms, double bonds and open vs. cyclic structures.

Detailed comparisons of RN specificity among different species of weevils, other beetles and moths have been enabled by the use of similar protocols in the investigations. Few similar RN types were found among the two weevil species. Interestingly, striking similarities were found regarding tuning of RNs among *A. rubi* and moths. Interesting aspects for further research in a comparative approach are, e.g. the large numbers of RNs specified for bicyclic monoterpenes found in species living in conifers versus large numbers of RNs for sesquiterpenes in species associated with flowering plants. The majority of RNs of *A. rubi* are tuned to compounds emitted in greater amounts by strawberry subjected to feeding by the weevils. Interestingly, the emissions are dominated by *E*-β-ocimene and germacrene D, which are the primary odorants of the two most abundant RN types of the weevil. On the other hand, *P. notatus* detects many of the compounds of the constitutive defences of the host, as well as ethanol, a sign of stress in the host tree. Whether the electrophysiologically active compounds are attractants or repellents for the weevils is not known.

The thesis has provided information about which plant odorants the weevils *Pissodes notatus* and *Anthonomus rubi* detect. The results establish a solid basis for the selection of compounds to be tested in behavioural studies, contributing significantly to the progress toward alternative modes of pest management. This information is used in ongoing behavioural studies in the laboratory and in the field. The detailed knowledge of ligands activating each RN type is also a crucial contribution to ongoing studies of quantitative structure-activity relationships (QSAR) where modelling is used to find out which part of the molecules interact with the binding sites of the olfactory receptor proteins. The results also add to the growing knowledge about functional types of RNs in a comparative perspective. Future studies may shed more light on the interesting topic of the evolution of specificity of RNs.

Resumo

A maioria dos insectos fitófagos são especialistas, i.e. monófagos ou oligófagos. A conservação das especializações alimentares dos insectos, requer a capacidade de seleccionar a planta adequada, de entre as muitas existentes na natureza. O metabolismo secundário das plantas dá origem a numerosos compostos, muitos dos quais são voláteis e cuja principal função parece ser a de conferir à planta resistência aos factores do meio, incluindo o ataque por insectos fitófagos. As espécies fitófagas, por sua vez, adaptaram-se a estas barreiras químicas, por vezes utilizando-as em benefício próprio. Disto é exemplo a capacidade de utilização de compostos voláteis da planta hospedeira como sinais de orientação para a encontrar. O processo de selecção da planta hospedeira é mediado pela integração, a nível do sistema nervoso central, de estímulos sensoriais de diversa natureza. De entre estes, destacam-se os estímulos olfactivos, com origem nas plantas hospedeira e não hospedeiras. A especialização ao nível da detecção e integração olfactiva desempenha um papel fundamental na estabilização das associações insecto-planta.

Em todos os animais, os estímulos odoríficos são detectados por neurónios receptores olfactivos (RNs). Nos insectos, os RNs encontram-se localizados sobretudo nas sensillas olfactivas das antenas. O reconhecimento de um estímulo olfactivo começa com a interação das moléculas odoríficas com as proteínas receptoras que residem na membrana das dendrites dos RNs. Estas proteinas, determinam a especificidade RNs, isto é, a gama de compostos a que o mesmo responde.

Uma das questões fundamentais colocadas à investigação das interacções insecto-planta e dos mecanismos olfactivos, reside na definição de estímulos e identificação de odores com significado biológico. Este tipo de questão tem vindo a ser elucidada particularmente em insectos que possuem um sistema olfactivo facilmente acessível à análise electrofisiológica. Trabalhos que combinam estudos electrofisiológicos com análise química têem permitido identificar moléculas para cuja recepção os insectos adquiriram sensores especializados ao longo da evolução,.

Os resultados apresentados nesta tese contribuem para o conhecimento sobre os mecanismos da detecção olfactiva num dos grupos de herbívoros mais diversificados, os gorgulhos (Curculionidae). Algumas das mais importantes e destrutivas pragas agrícolas e florestais pertencem a esta família. Historicamente, a procura de feromonas tem sido a via

preferida para encontrar atraentes eficazes no controlo de pragas. No entanto, já foram identificadas mais de 20 feromonas de curculionídeos e, após décadas do desenvolvimento experimental, relativamente poucas foram bem sucedidas no controle de pragas agrícolas. Uma das razões avançadas para estes resultados decepcionantes é o défice de conhecimento sobre os compostos da planta envolvidos no processo de identificação do hospedeiro, e de como estes interagem com as feromonas. Duas espécies de curculionídeos oligófagos foram escolhidas como modelo para este estudo: o gorgulho pequeno do pinheiro *Pissodes notatus* F. (Coleoptera, Curculionidae) cujo hospedeiro preferencial é o pinheiro bravo, *Pinus pinaster* (Pinaceae), e o gorgulho da flor do morangueiro, *Anthonomus rubi* Herbst (Coleoptera, Curculionidae). Ambas as espécies constituem pragas economicamente importantes nas suas áreas de distribuição. Embora sendo este um estudo de investigação fundamental, os resultados obtidos são projectáveis no plano da aplicação, nomeadamente em protecção integrada de povoamentos de pinheiro bravo e da cultura do morangeiro.

O objectivo geral foi o de investigar os mecanismos de detecção e codificação de odores das plantas pelos RNs *de P. notatus* e *A . rubi*, e assim contribuir para o conhecimento de quais os compostos importantes para a localização do hospedeiro nestas espécies. Definiram-se os seguintes objectivos específicos: (1) Determinar a gama de compostos emanados por plantas hospedeiras e não hospedeiras, detectados por RNs de *P.notatus* e *A. rubi*; (2) Caracterizar a especificidade e sensibilidade dos neurónios receptores olfactivos, agrupando-os em classes de acordo com os diferentes compostos constituintes do odor das plantas hospedeiras e não hospedeiras, que detectam; (3) Investigar a indução da produção de compostos voláteis na planta hospedeira; (4) Pesquisar aspectos particulares do olfacto em curculionídeos, através da abordagem comparativa entre espécies; (5) Seleccionar compostos para estudos comportamentais

Para a prossecução destes objectivos foram utilizadas, principalmente, técnicas de registo extracelular de potenciais de acção de neurónios receptores olfactivos, quando estimulados com compostos voláteis produzidos pelas plantas. Os registos electrofisiológicos foram executados durante a estimulação com os voláteis da planta, separados por cromatografia em fase gasosa (GC-SCR). Para a identificação química dos componentes das misturas que geraram resposta electrofisiológica, utilizou-se a técnica de cromatrografia em fase gasosa acoplada a espectrometria de massa (GC-MS). Para obtenção das misturas de voláteis utilizadas nos testes electrofisiológicos, os voláteis emanados por diferentes espécies de plantas hospedeiras e não hospedeiras foram adsovidos em filtros (Porapak Q e Tenax) utilizando técnicas de "headspace", seguidas por desorpção por solvente (hexano e/ou

etilacetato). A recolha de amostras para a investigação da indução de compostos defensivos foi efectuada por "micro extração em fase sólida" (SPME).

A tese está organizada em duas partes: a primeira parte inclui uma introdução geral que apresenta uma revisão da literatura sobre diferentes tópicos relevantes para a contextualização do trabalho desenvolvido. Segue-se o enunciado dos objectivos do trabalho experimental e a sinopse dos resultados experimentais obtidos em cada uma das publicações originais que formam a base desta tese. A discussão geral dos resultados, que procura posicionar os mesmos resultados no contexto actual do conhecimento em mecanismos olfactivos e ecologia química, é seguida pelas considerações finais. São ainda indicadas algumas das questões mais pertinentes para trabalhos futuros. A segunda parte apresenta as publicações originais que formam a base da tese.

Os curculionídeos investigados neste estudo detectam uma gama relativamente vasta de voláteis das plantas. Os compostos detectados são monoterpenos e sesquiterpenos, compostos aromáticos, e também álcoois, aldeídos e ésters alifáticos, muitos dos quais são produzidos pelos hospedeiros de ambas as espécies. Os compostos detectados são comuns no mundo vegetal, fazendo parte do elenco de voláteis emanados por muitas plantas. Assim, parecem não fornecer uma base para o reconhecimento da planta hospedeira em função dos compostos específicos desta. Estes resultados apoiam a hipótese segundo a qual os insectos usam um conjunto de muitos compostos comuns no mundo vegetal, em determinadas proporções, para distinguir as plantas hospedeiras das não hospedeiras. Cada RN respondeu consistente e selectivamente a um espectro estreito de compostos com semelhanças na estrutura molecular. Foi possível agrupar os RNs caracterizados em cada espécie, em classes funcionais, de acordo com o odor primário (i.e. que gera a resposta de maior intensidade) e os odores secundários (que dão origem a respostas mais fracas). Em muitos registos, apareceu actividade de dois neurónios. Isto significa que dois neurónios estão localizados na mesma sensila. Nestes casos, os pares de classes funcionais de RNs são constantes, tal como acontece em muitos outros insectos, sugerindo uma importância funcional da co-localização. Foi observada um certo grau de sobreposição do espectro de resposta das diversas classes de RNs. Isto significa que, de um modo geral, a informação sobre um determinado composto é transmitida ao sistema nervoso central através da actividade combinada de diversos neurónios com sensibilidades diferentes, ou seja segundo um 'across fibre pattern'. Assim os resultados indicam que a qualidade do odor do hospedeiro é codificada em parte, de forma combinatória. Em contraste, alguns voláteis são detectados por apenas uma classe de RN, sendo portanto a informação acerca da sua presença, transmitida por linhas dedicadas ('labeled-lines').

A maioria das respostas dos RNs foram excitatórias, quer dizer traduzidas por um aumento da frequência de potenciais de acção. No entanto, um dos RNs de *A. rubi* respondeu com aumento de frequência dos potenciais de acção ao monoterpeno α-pineno e com um silenciamento total (resposta inibitória), ao linalool. Esta excepção levanta uma questão interessante sobre a hipótese de a integração de estimulos olfactivos poder ocorrer na periferia.

A importância da quiralidade para a bioactividade é bem conhecida, sabendo-se por exemplo que numerosas feromonas são compostos quirais dos quais apenas um dos enantiómeros é activo. Neste estudo, o uso de colunas cromatográficas que separam enantiómeros permitiu demonstrar que RNs adaptados para a recepção dos odores das plants são enantiómero-selectivos. Em *A. rubi*, existem RNs específicos para a detecção de (-)- e de (+)-linalool. Isto significa que cada um dos dois enantiómeros poderá encerrar mensagens diferentes para o insecto. Por outro lado, todos os RNs identificados respoderam preferencialmente a (-)-germacreno D, não se tendo encontrado RNs especialistas para (+)-germacreno. RNs enatioselectivos foram também identificados em *P. notatus*, estes respondendo seletivamente a (-)-limoneno. A enatioselectividade sublinha a especificidade do RNs dedicados à detecção de compostos das plantas. Outras características moleculares com importância nas interacções entre proteína receptora olfactiva e o ligante que a activa, reveladas pelos estudos de estrutura/actividade, foram o comprimento do esqueleto de carbono, as zonas de maior densidade de electrões e flexibilidade das moléculas, que se traduzem em número dos átomos de carbono, ligações duplas e estruturas cíclicas ou não cíclicas.

O uso de protocolos similares nas investigações permitiu a comparação detalhada da especificidade dos RN entre as duas espécie de curculionídeos e com outras espécies de coleópteros e lepidópteros. *P. notatus* e *A rubi* possuem apenas duas classes de RNs com especificidade semelhante. Por outro lado, encontraram-se semelhanças de especificidade entre RNs de *A. rubi* e de borboletas nocturnas da família Heliothinae. Outros aspectos relevantes que emergem das comparações são, por exemplo, um grande número RNs específicos para a detecção de monoterpenos bicíclicos encontrados nas espécies que vivem em coníferas, *versus* um grande número RNs para sesquiterpenos nas espécies associadas com angiospérmicas.

Um dos aspectos mais interessantes dos resultados, é o facto de que a maioria de RNs de *A. rubi* detectam selectivamente os compostos cuja emissão em maior quantidade é induzida nas flores de morangueiro pela actividade alimantar deste curculionídeo. Mais ainda,

a emissão induzida é dominada por *E*-β-ocimeno e por germacreno D, que são os odores primários dos dois tipos de RNs mais abundantes. O gorgulho do pinheiro, por seu lado, detecta maioritáriamente compostos incluídos nas defesas constitutivas do pinheiro, bem como o ethanol, que nas coníferas sinaliza o stress da árvore hospedeira. Estudos comportamentais são necessários para determinar se os compostos electrofisiologicamente activos são atraentes ou repelentes para cada um dos curculionídeos.

Os resultados apresentados nesta tese fornecem informação sobre os odores detectados por *P. notatus* e *A. rubi*. Os resultados estabelecem uma base sólida para a selecção de compostos a ser testados em estudos comportamentais, contribuindo significativamente para o progresso do conhecimento aplicável ao controle biológico destas duas espécies. Esta informação está a ser usada em estudos comportamentais em laboratório e no campo. O conhecimento detalhado dos ligantes que activam cada tipo de RN é também uma contribuição crucial para os estudos quantitativos de actividade/estrutura (QSAR), nos quais se procura modelar o sítio activo das proteínas receptoras olfactivas a partir do conhecimento das moléculas que com ele interagem. Os resultados contribuem também para o conhecimento dos mecanismos olfactivos em curculionídeos e em insectos em geral, numa perspectiva comparativa. Verificaram-se diferenças entre as espécies de curculionídeos estudadas, que tem como hospedeiros gimnospérmicas e angiospérmicas. As semelhanças evidenciadas entre *A rubi* e as borboletas da família Heliothinae são muito interessantes e constituem um primeiro contributo para a compreensão da evolução da especificidade dos neurónios receptores olfactivos dos insectos herbívoros.

Paper I

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ORIGINAL PAPER

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Identification of plant odours activating receptor neurones in the weevil *Pissodes notatus* F. (Coleoptera, Curculionidae)

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Abstract Plants release complex mixtures of volatiles important in the interaction with insects and other organisms. In the search for compounds that contribute to the perception of odour quality in the weevil *Pissodes notatus*, single olfactory receptor neurones on the antennae were screened for sensitivity to naturally produced plant volatiles by the use of gas chromatography linked to single cell recordings. We here present 60 olfactory neurones responding to 25 of the numerous compounds released by host and non-host plants. All the neurones show high selectivity and are classified into 12 distinct types. The two most abundant types respond to α-pinene, β-pinene, and 3-carene ($n=17$), and to isopinocamphone and pinocamphone ($n=17$), respectively. Other neurone types respond to limonene ($n=9$), β-phellandrene ($n=3$), and fenchone ($n=4$). Responses to β-caryophyllene ($n=1$) and to ethanol ($n=4$) are also shown. Except for two pairs, the neurone types do not show overlap of the molecular receptive range. The active compounds are present in the host, *Pinus pinaster*, as well as in non-hosts, supporting the idea that plant odour quality is mediated by the ratio of the compounds rather than specific odorants.

Keywords Gas chromatography · Insect receptor neurones · Plant odours · Single cell recordings · Terpenes

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Abbreviations FID flame ionisation detector · GC gas chromatography · ORN olfactory receptor neurone · ppr peak response range · SCR single cell recording

Introduction

A large part of the olfactory system in herbivorous insects is evolved for detecting and processing plant odour information, reflecting the importance of olfactory cues in host location (Schoonhoven et al. 1998; Mustaparta 2002). The importance of olfaction has been demonstrated for some species of *Pissodes* for which host plant odours act as attractants, both alone and synergistically together with aggregation pheromones (Booth et al. 1983; Phillips and Lanier 1986; Brattli et al. 1998). Whether the weevil *Pissodes notatus* F. (Coleoptera, Curculionidae) uses odours for host location has not been clarified (cf. Chararas 1979; Alauzet 1984). However, traditional methods of forest protection, based on "bait logs", suggest that host odours are involved in orientation towards susceptible trees also in *P. notatus*.

P. notatus feeds and reproduces mainly on conifers of the genus *Pinus* (Pinaceae) and is considered a pest in young plantations of *Pinus pinaster* Aiton (Cobos and Robredo 1982; Alauzet 1984; Bogdanova 1986; Ferreira and Ferreira 1989). The adult weevil lays the eggs under the bark. The damage is caused by the larvae feeding on the phloem of the trunk, interrupting the sap circulation and eventually causing death of the tree. *P. notatus* is widely distributed in Europe, and also found in Siberia, North Africa, Madeira, and the Canary Islands (De Viedma 1961; Alauzet 1984; Ferreira and Ferreira 1989).

The antennae of *P. notatus* respond to several of the components present in the blend of the host plant, *P. pinaster*, as shown in preliminary studies using gas chromatography linked to electroantennogram recording (H. Bichão, unpublished observations). A more precise method to identify naturally produced plant

compounds detected by insect receptor neurones is gas chromatography linked to electrophysiological recordings from single receptor cells (GC-SCR). This technique was first used for the identification of insect pheromones (Wadhams 1982). GC-SCR in combination with GC linked to mass-spectrometry (GC-MS) has been successfully used to identify host plant odorants that are detected by several species of beetles and moths (Blight et al. 1995; Wibe and Mustaparta 1996; Wibe et al. 1997, 1998; Bichão et al. 1997; Røstelien et al. 1997, 2000a, 2000b; Barata et al. 2002; Stranden et al. 2002). In the present study, GC-SCR and GC-MS have been used to identify compounds produced by *P. pinaster* that influence the activity of single receptor neurones in *P. notatus*. Furthermore, we characterized the receptor neurone types according to the odorants that elicited responses.

Materials and methods

Insects

Adult *P. notatus* were obtained from a colony annually infused with feral insects maintained at the Entomology Laboratory in Évora University, Portugal. Upon emergence, adults were fed with pine (*P. pinaster*) branches and kept under long-day conditions (14:10 h) at suitable temperature (22–25°C). Before experiments, all male and female insects used in this study were starved for at least 24 h.

Collection of naturally produced plant volatiles

Volatile compounds released by host and non-host plants were collected using entrainment techniques from the headspace of *P. pinaster*, *Pinus pinea* L. (Pinaceae) and the non-host *Eucalyptus globulus* Labill. (Myrtaceae). The plant materials were collected in June from the forest areas where *P. notatus* occurs (Alentejo region, Portugal). Approximately 400 g of bark, branches, leaves and fruits (weight proportion 2:2:1:1) from three individual trees were cut, mixed and placed in a sealed glass vial (3 l). The volatiles emitted were trapped by blowing N_2 (for 24 h at 25–30°C) through the glass vial into two parallel glass tubes (6.6 cm×0.5 cm i.d.) packed with the adsorbent (150 mg Porapak Q, 80–100 mesh). The average flow measured at the outlet of the tubes was 50–60 ml min^{-1} (i.e. total air volume exchange in the vial, every 50–60 min). Before use the Porapak Q was rinsed with dichloromethane and *n*-hexane and activated overnight in a heating chamber at 180°C while perfused with N_2. The adsorbed plant volatiles were eluted from the Porapak Q with *n*-hexane (pa Merck) and the resulting solutions were used as test samples in the experiments. Entrainment samples of Norway spruce sawdust, *Picea abies* (L.) H. Karst, were also included in this study (see Wibe and Mustaparta 1996 for details of sampling procedure).

Gas chromatography linked to single cell recording (GC-SCR)

The insect was fixed to a Plexiglas cube formed to fit the animal and immobilized by Parafilm and wax. The left antenna was exposed and fastened to the wax layer. Nerve impulses from single olfactory receptor neurones on the antenna club were recorded using tungsten microelectrodes sharpened to a tip less than 0.3 μm (Mustaparta et al. 1979). The electrode tip was inserted at the base of the sensillae that were located at high densities in three different bands on the antennal club.

GC-SCR recordings were obtained from male and female *P. notatus* stimulated with host and non-host mixtures via the gas chromatograph. A sample (ca. 0.8–1 μl) of the hexane solution containing the plant volatiles was injected into the column (J&W DBwax; 30 m; 0.25 mm i.d.; 0.25 μm film thickness) through an on-column injector. Helium served as the carrier gas. The oven was programmed to start at 50°C, where it was held isothermal for 2 min, with an increase of 6°C min^{-1} to 200°C, where it was held isothermal for 15 min. A spilt at the end of the GC column led half of the effluent to the GC detector (flame ionisation detector, FID) and half to a clean airflow (300 ml min^{-1}) that blew across the insect antenna. This resulted in simultaneously recorded gas chromatograms and single cell responses to the separated compounds (Wadhams 1982; Wibe and Mustaparta 1996).

Each receptor neurone was first screened for sensitivity to the different entrainment samples and vapour of fresh plant materials. These tests were performed by blowing air puffs through glass cartridges containing either a filter paper with the test sample or fresh plant material. Neurones responding to any of these stimuli were classified as plant odour receptors and further examined by stimulation via gas chromatography. Retesting some of the neurones with synthetic or authentic reference samples of the compounds, via the GC, confirmed the identification of the active components. The nerve impulse frequency was measured by a window discriminator and displayed as the number of spikes/time unit. Spike activity was recorded on an analogue tape recorder in parallel with the computer program Electro Antenno Detection (version 2.3, Syntech NL, Hilversum, The Netherlands) and analysed with the program AutoSpike-32 (Syntech NL).

Chemical analysis

The chemical identifications were focused mainly on the plant compounds that activate the receptor neurones. The analyses were carried out by GC-MS, using the same type of GC-column described previously for GC-SCR (i.e. J&W DBwax; 30 m; 0.25 mm i.d.; 0.25 μm film thickness). A mass-spectrometer Finningan SSQ 7000 instrument connected to a Varian 3400 gas chromatograph was used for the analysis. The GC was equipped with a split/splitless injector (splitless mode 30 s; injector temperature 200°C; carrier gas He). The MS ion source temperature was 150°C and mass spectra were recorded at 70 eV, 30-400 mu. The temperature program was adjusted to fit the separation obtained in the GC-SCR experiments (40°C, 4°C min^{-1} to 200°C, 26 min at 200 °C).

Results

The results are based on recordings from 60 olfactory receptor neurones (ORNs) from 37 individuals of *Pissodes notatus* (24 females and 13 males). All recordings were obtained from olfactory sensilla located in the constriction bands on the antennal club, most of them in the second band. Care was taken to obtain recordings from single neurones. However, in some experiments recordings were made simultaneously from two neurones, each appearing with different spike amplitudes. An example is presented in Fig. 1 showing responses by neurone A to three compounds and neurone B to two later eluted constituents. The recordings from a single neurone lasted from 40 min to 12 h, which allowed several plant mixtures to be tested via the GC. Whereas 31 neurones were tested once or twice with the host mixture, the others (29) were also tested with the non-host mixtures. The spontaneous activity varied from 0.1 to 17 spikes s^{-1}. The strongest responses ranged from 18 to 59 spikes s^{-1} and the

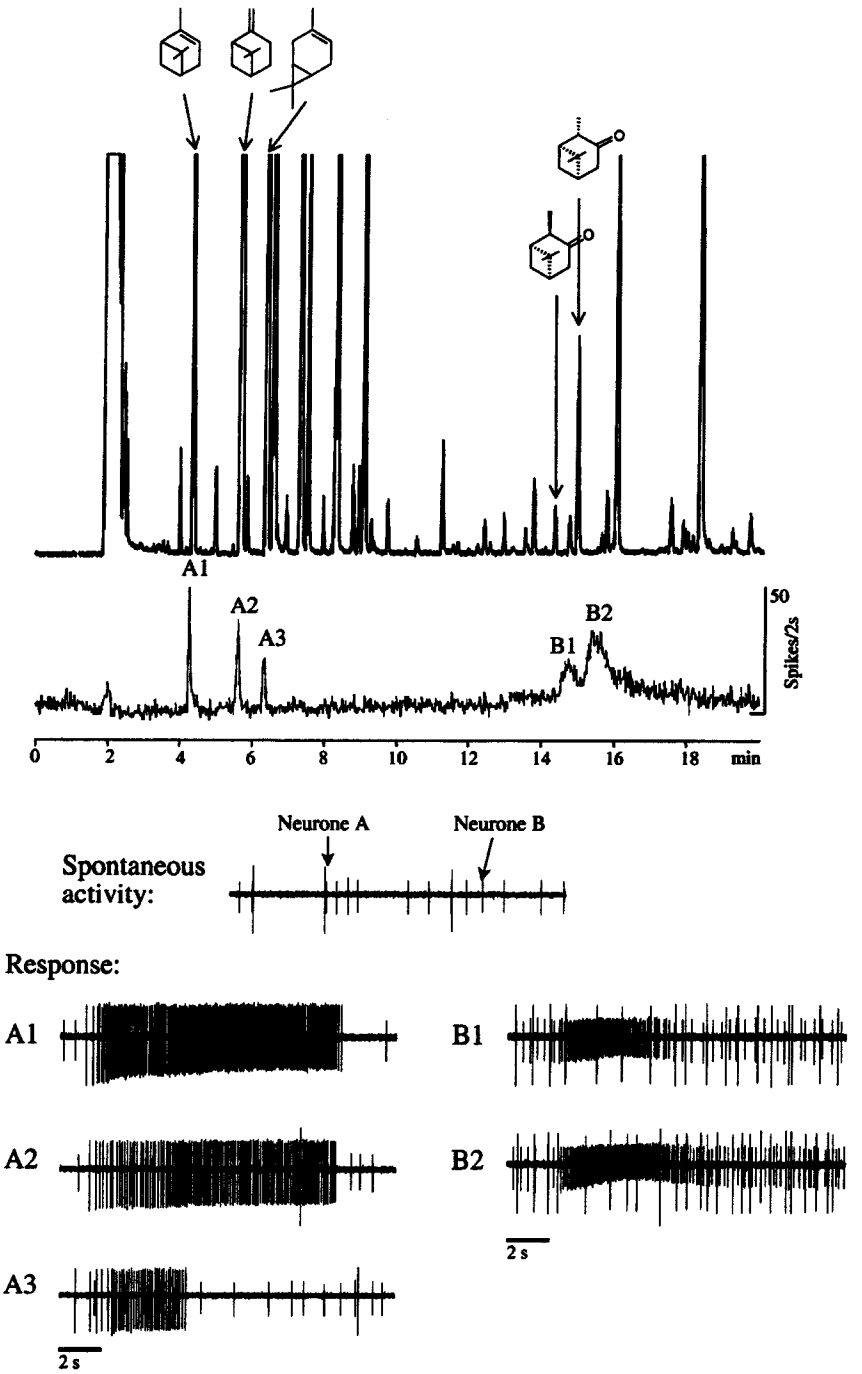

Fig. 1 Gas chromatogram of volatiles collected from *Pinus pinaster* and simultaneously recorded activity of two olfactory receptor neurones, *A* (type1) and *B* (type3), during stimulation with the compounds eluted from the column of the gas chromatograph. The different spike amplitudes of the two neurones clearly showed the specialization of neurone A to α-pinene (*A1*), β-pinene (*A2*) and 3-carene (*A3*), and neurone B to pinocamphone (*B1*) and isopinocamphone (*B2*)

medium and weak secondary responses ranged from 13 to 32 spikes s^{-1} and from 11 to 24 spikes s^{-1}, respectively. The neurones responded consistently at the same retention time when tested for the same or different plant mixtures containing the active components. Responses of the receptor neurones to plant odours were in all cases recorded as excitation, i.e. increased spike firing activity, which in general followed the concentration profile expressed by the GC-peak of the stimulating compound (Fig. 2). In some cases the response outlasted the GC-peak. This was observed often for responses to components eluted in the latter part of the gas chromatogram.

All neurones were tested with the entrainment sample of *P. pinaster* (host) which contained more than 90 components detected by GC. Twenty-two of the compounds elicited responses in one or more of the 60 receptor neurones and 13 of these active constituents were present at a concentration that could be identified by GC-MS. In addition, two other active compounds

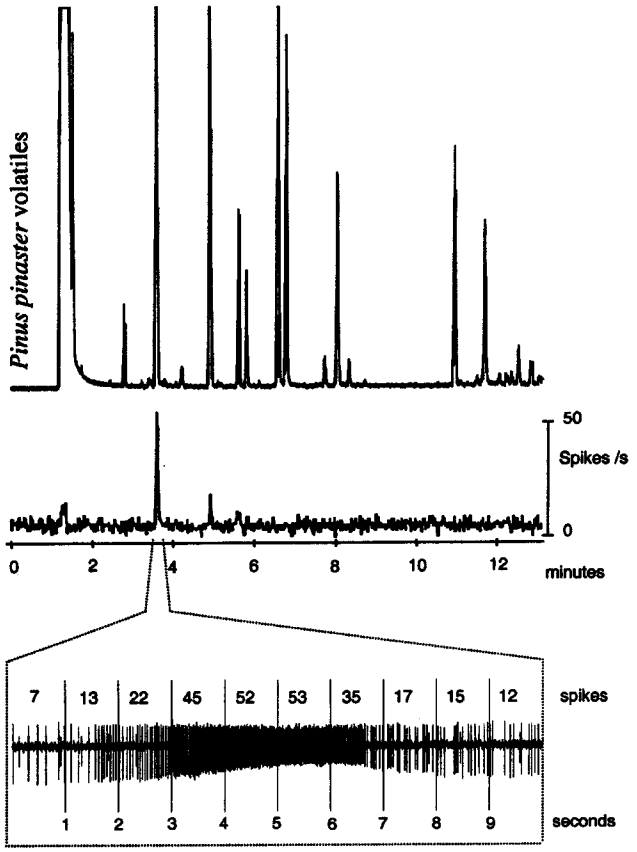

Fig. 2 The response of one receptor neurone, recorded as increased firing rate (number of spikes/second) (*below*), which followed the concentration profile of the active GC peak (*above*)

were identified in the entrainment sample of the non-hosts *E. globulus* and *P. abies*. All olfactory receptor neurones selectively responded to only 1, 2, 3 or 4 components in the host plant mixture. Based on these responses the neurones could be classified into 12 types. The molecular receptive ranges show no overlap, except for the neurone types 1 and 2, and 5 and 6 (Fig. 3). Out of the 12 neurone types, six (types 1, 2, 3, 4, 5 and 11) were found in both sexes, whereas the other six types were recorded in females only. The numbering of the neurone types was made according to the functional categories of the active compounds as shown in Fig. 3 (type 12 is not presented in the figure, since the response of this neurone only appeared during direct stimulation). The active compounds identified were bicyclic, monocyclic, and acyclic monoterpenes, and sesquiterpene hydrocarbons.

Neurones responding best to bicyclic monoterpenes

The most abundant receptor neurones responded to bicyclic monoterpenes and were classified into three types.

Type 1

Seventeen neurones showed the strongest response to α-pinene [peak response range (prr): 18–52 spikes s^{-1}] and had weaker responses to β-pinene (prr: 13–32 spikes s^{-1}) and 3-carene (prr: 11–24 spikes s^{-1}), all being major components in the host mixture (Figs. 1 and 3). No other components of the host and non-host volatiles elicited responses in these neurones. Two of these cells were retested with α-pinene and β-pinene and one cell with 3-carene, confirming the identity of the active components. In 10 out of the 17 recordings obtained from this neurone type activity of a second neurone appeared simultaneously (type 3, see below) (Fig. 1).

Type 2

Four neurones responded strongly to fenchone (prr: 37–59 spikes s^{-1}), a minor constituent in the pine extracts, and much weaker to camphene (30 spikes s^{-1}) present in larger amounts (Fig. 4A, B). In some recordings a weak response to β-pinene and 3-carene also appeared. Two of the neurones tested for the non-host volatiles of *E. globulus* showed a strong response to the major component 1,8-cineole (Fig. 4C). Tests on one of these neurones with authentic standards of fenchone and camphene indicated that the two compounds were the active constituents. Further tests with (+)- and (−)-fenchone showed slightly stronger response to the (−)- than to the (+)-enantiomer (50 and 43 spikes s^{-1}, respectively) (Fig. 5).

Type 3

Seventeen neurones responded strongest to isopinocamphone (prr: 25–34 spikes s^{-1}) and weaker to the smaller amount of the stereoisomer pinocamphone (prr: 16–22 spikes s^{-1}) when tested with the host volatiles of *P. pinaster*. One of these neurones responded by excitation when retested with authentic isopinocamphone. Two other neurones further tested with spruce sawdust volatiles responded to camphor and isopinocamphone in this sample. Retesting with authentic camphor elicited excitation of these neurones. Considering the amounts of the three compounds in the test mixtures, isopinocamphone seems to have the strongest stimulatory effect. All the responses by type 3 neurones showed a slow decay outlasting the GC peak, as exemplified by neurone B in Fig. 1.

Neurones responding to monocyclic and acyclic monoterpenes

Type 4

Nine neurones had their strongest responses to the monocyclic monoterpene limonene (prr: 30–35 spikes s^{-1}) and weaker responses to the acyclic β-myrcene (prr: 15–21 spikes s^{-1}), both major components in the

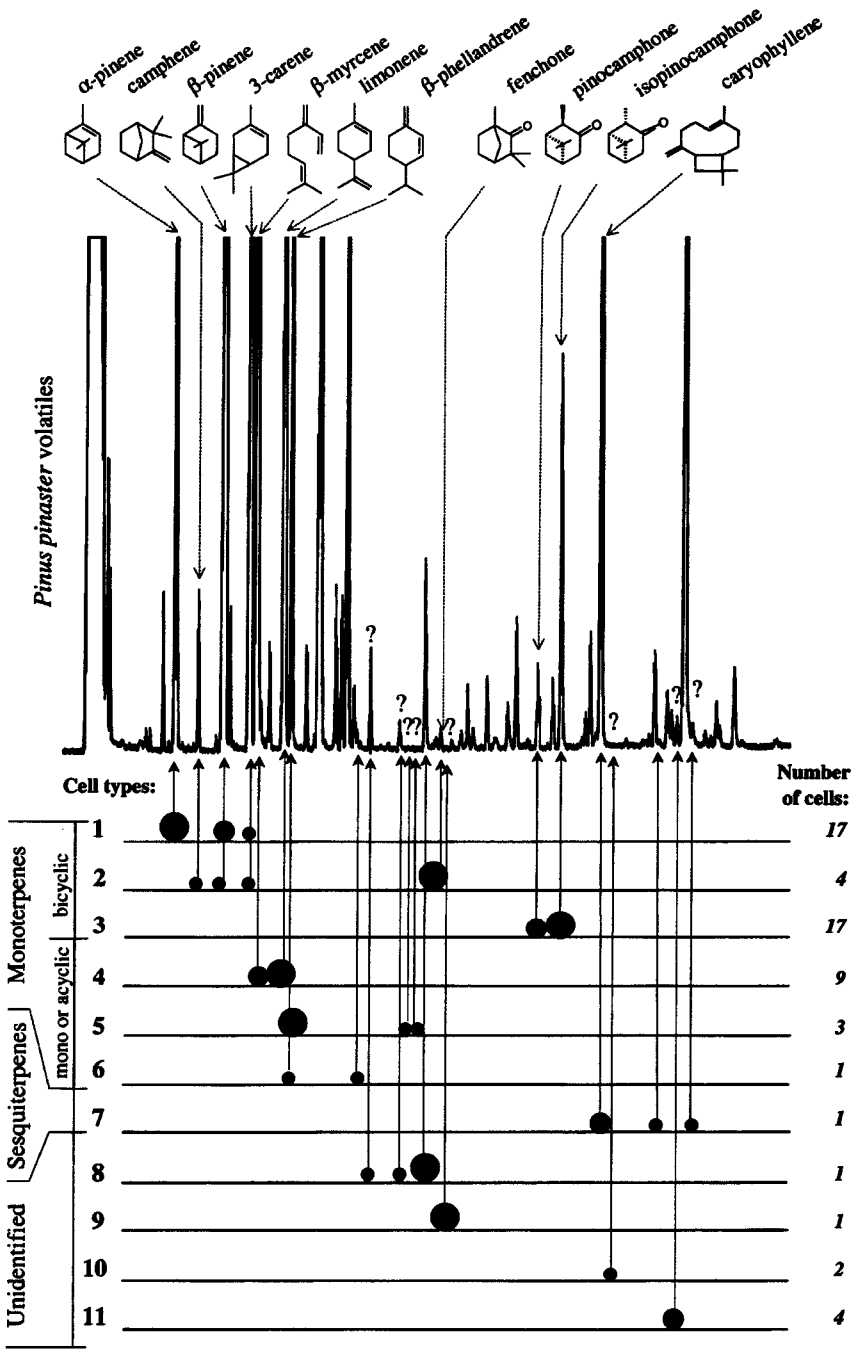

Fig. 3 Overview of the olfactory receptor neurone (ORN) types identified in this study. Classification was done based on the responses of ORNs to the GC-separated compounds. *Top*: gas chromatogram of *P. pinaster* volatiles trapped by headspace of chopped bark, leaves and cones, separated by a DB wax column. *Below*: The selective responses of the classified receptor neurone types (1–11) to 1, 2, 3 or 4 of the eluted compounds of the *P. pinaster* mixture. Within each cell type, the relative response strength is expressed by the size of the circle (three categories: strongest, medium and weak responses, respectively, range 18–59; 13–32; and 11–24 spikes s^{-1}) as indicated for selected neurone types described in the text. The number of neurones assigned to each type is indicated on the right side. (NB: type 12 is not represented since it was not identified using the same method, see Fig. 6)

host plant mixtures. Retesting of one neurone with authentic limonene elicited excitation. Two other neurones tested with the non-host volatiles of spruce sawdust responded to limonene but not to the large amount of β-myrcene.

Type 5

Three neurones had their strongest responses to β-phellandrene (prr: 45–50 spikes s^{-1}) when tested with the headspace sample of the host *P. pinaster*, containing a large amount of this compound. In addition, one neurone responded to two unidentified constituents (retention times 10.13 min and 10.18 min) that were scarcely detected by the GC. Two responses appeared at the corresponding retention times when the neurone was stimulated with the *P. abies* sawdust mixture. Although the amount of the two compounds was at the detection limit of the GC, the antennal responses were stronger than to the large amount of β-phellandrene. A fourth response appeared to a component previously identified by Wibe et al. (1997) as β-cyclocitral (2,6,6-trimethyl-1-

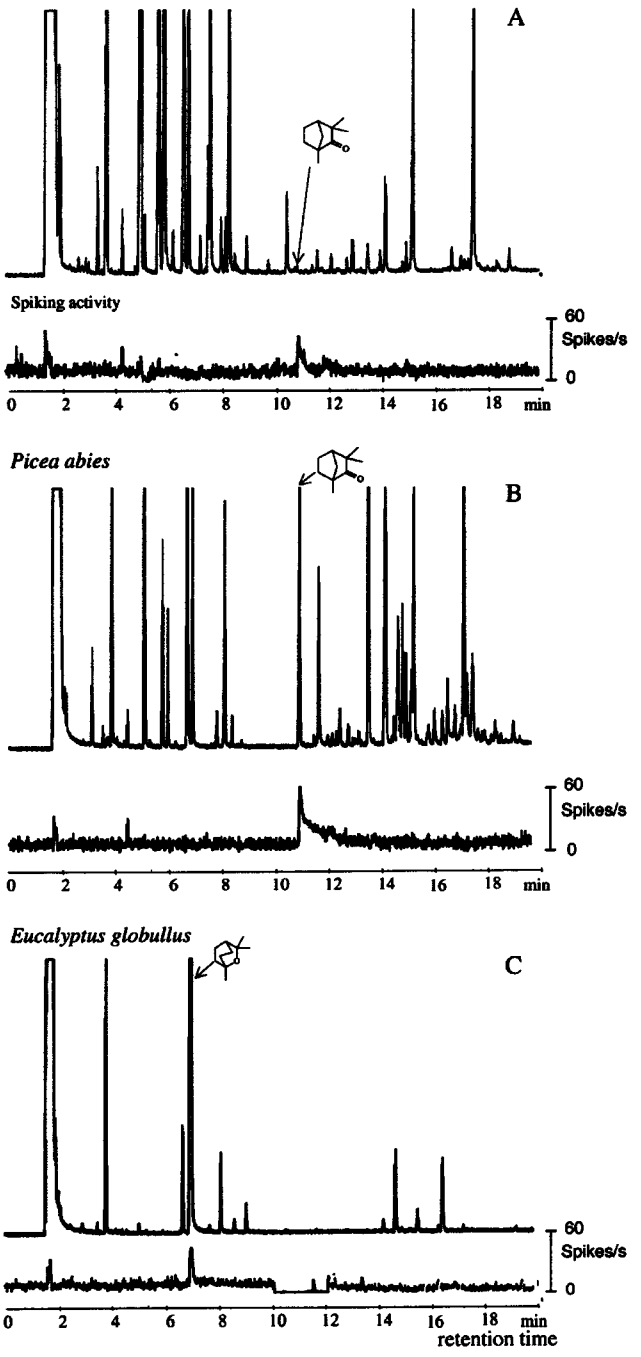

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Fig. 4A–C Recordings from a single neurone during elution of volatiles collected from materials of *P. pinaster* (**A**), *Picea abies* (**B**) and *Eucalyptus globulus* (**C**). Strongest response to fenchone and secondary to camphene appeared during the elution of *P. pinaster* and *P. abies* volatiles. Response to the high concentrations of 1,8-cineol appeared during the elution of volatiles collected from *E. globulus* (in **C**, between 10 min and 12 min a dramatic drop in spontaneous activity of the neurone was observed)

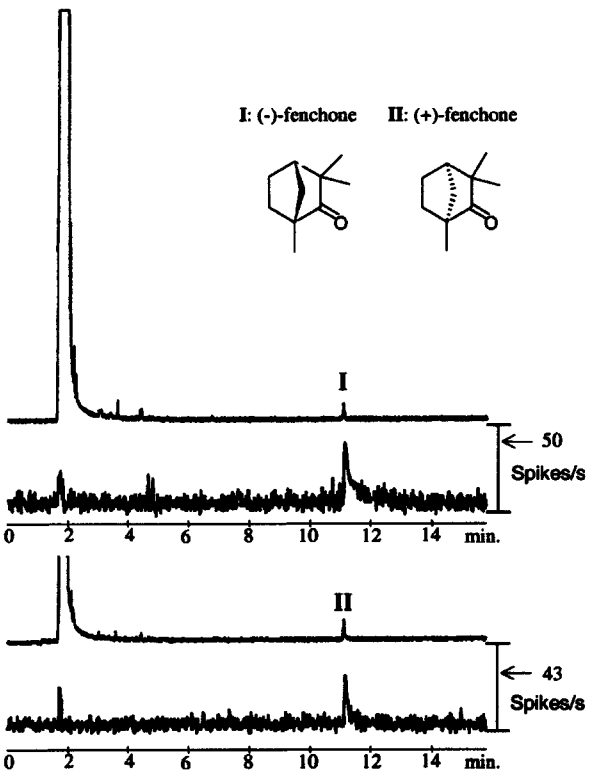

Fig. 5 Recordings from the neurone shown in Fig. 4 shows the responses to both enantiomers of fenchone (standard samples), during elution from the GC column with non-chiral phase

cyclohexen-1-carboxy-aldehyde) by comparing its mass-spectrum with MS library data.

Type 6

One neurone that responded weakly to β-phellandrene differed from type 5 neurones by responding to another minor component of the host mixture, not chemically identified.

Neurones responding best to sesquiterpenes

Type 7

Only one neurone was found that responded to the sesquiterpene β-caryophyllene (26 spikes s^{-1}), a major component in the headspace sample of the host. Re-testing with authentic β-caryophyllene elicited excitation. Two weaker responses, one to α-caryophyllene (α-humulene) and the other to an unidentified compound, also appeared. This neurone was co-localized with a second neurone that did not respond to any of the compounds tested.

Neurones responding to unidentified components

Four neurone types were classified for which the active compounds were not identified. One neurone (type 8)

was activated during the elution of three distinct GC peaks (retention times 8.67, 9.36 and 10.17 min) in the host sample. The strongest response appeared at 10.17 min. These three compounds were not detected by the GC-MS and could not be identified. Another neurone (type 9) responded strongly to the compound tentatively identified as β-cyclocitral (Wibe et al. 1997) and weaker to fenchone when tested with the *P. abies* sawdust sample. Stimulation with *P. pinaster* entrainment sample elicited one response at the retention time corresponding to β-cyclocitral. However, the amount of the active component was below the detection limit for the GC-MS. One neurone (type 10) responded to one component in the *P. pinaster* mixture (retention time 20.58 min), possibly an "anethole type" as suggested by GC-MS. Four neurones (type 11) were found in an early phase of the study, which responded to a compound eluting at 19.17 min, (using 4 min at 50°C initial temperature instead of that described previously for the rest of the experiments) just before germacrene D.

Some neurones in *P. notatus* responded to the vapour of fresh host material directly blown over the insect antenna. However, no response appeared to stimulation with the entrainment samples of host and non-hosts. Four of these neurones (type 12) showed a strong response when stimulated with ethanol via a glass cartridge (Fig. 6). These responses were characterized by a large increase in the firing rate with decreasing spike amplitudes followed by recovery of the amplitudes and a sudden stop of firing before the end of the stimulation period. After a short period the spike activity re-appeared.

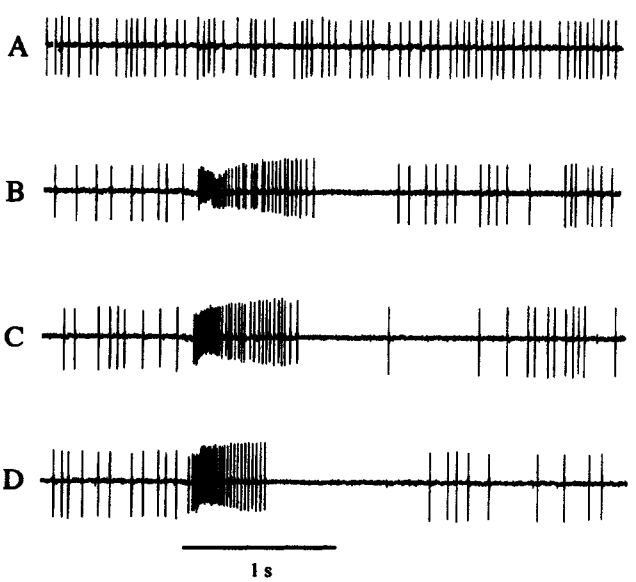

Fig. 6 Spike activity of one receptor neurone during direct stimulation with ethanol. *Trace A*: no change of the activity during stimulation with purified air. *Traces B, C, D*: responses to three consecutive stimulations with 1 μl pure ethanol on filter paper (same cartridge). Inter-stimulation period 30 s; stimulus period (1 s) indicated below

Discussion

The results obtained in the present study using GC linked to single cell recording (GC-SCR) have shown that single olfactory receptor neurones (ORNs) of the forest weevil, *P. notatus*, detect one or a few of the numerous volatiles released by host and non-host plants, suggesting that these compounds are biologically relevant for the weevil. The selective and strong responses to minute amounts of compounds present in the plant mixtures indicate that ORNs tuned to these plant odours in *P. notatus* might be as selective and sensitive to their key stimuli as pheromone receptor neurones present in other species (Dickens 1990; Mustaparta 2002).

Altogether the 60 neurones recorded showed responses to 25 of the components present in the various plant materials. Most of the identified active constituents were monoterpenes (12 out of 16), activating the largest number of neurones. This demonstrates the importance of this group of compounds for the weevil. However, the significant response by one neurone to β-caryophyllene, one of the most distinctive constituents of *P. pinaster*, suggests that sesquiterpenes may also be relevant to these weevils. Furthermore, mass-spectra indicate that among the unidentified active components other sesquiterpenes and oxygenated terpenes were present.

The strong and selective responses of the ORNs facilitated the classification of neurones into distinct types showing specialized rather than broadly tuned ORNs. The consistent responses to the same components when testing one neurone with the same or different plant materials demonstrated the reproducibility of the recorded molecular receptive range of the ORNs. Furthermore, the appearance of the same neurone type in up to 17 recordings (types 1 and 3) indicates the significance of these ORN types. The proportion of ORNs mediating information about α-pinene, pinocamphone as well as limonene further indicates that these plant components are particularly important. The marked best response to one compound, appearing in most units (Fig. 3), gives the impression that the ORNs are specialized for that compound and respond weaker to two or three others. For example, α-pinene seems to have the best effect on neurone type 1, which responds markedly weaker to β-pinene and 3-carene, all major compounds in the host. For other types it is particularly important to consider the amount of the compounds present in the mixtures. For instance, neurone type 3 showed the strongest response to isopinocamphone and a weaker response to the smaller amount of pinocamphone. In this case, it was not possible to determine which of the two compounds is the most efficient. Similarly, the neurone type 5 showed the strongest response to the β-phellandrene peak and weaker responses to two minor unidentified constituents scarcely detected by the GC. The significant response by these neurones to the minimal amount of the unidenti-

fied components indicates that the minor components and not β-phellandene are the most relevant odorants. In future experiments, dose-response curves could determine the relative stimulatory effect of the active compounds. The present classification of 12 types, of which some were represented by a single neurone, suggests that more ORN types might appear with a larger number of recordings.

In those cases where two or three compounds were identified activating the same neurone, they showed similarities in their molecular structures. For example, the type 1 neurones responded to bicyclic monoterpenes, best to α-pinene and second to β-pinene, type 2 to fenchone and camphene, type 3 to isopinocamphone and pinocamphone, and type 4 responded best to the monocyclic monoterpene limonene, and second to the acyclic monoterpene β-myrcene. The structural similarities between these components appear in Fig. 3. The chiral configuration of the active compounds was not studied in detail. However, in the case of fenchone, one neurone was tested separately with two enantiomeric standards, showing a slightly higher response to (−)-fenchone. Since these tests were made using a non-chiral GC-column, we do not know whether both enantiomers activate the neurone. Many of the active monoterpenes are chiral compounds produced by separate enzymes, resulting in different enantiomeric ratios in the various species, individuals, and parts of the plants (Almquist et al. 2000; Sjödin et al. 2000). Thus, separation with chiral GC columns (Stranden et al. 2002) will be important in future experiments with *P. notatus*.

Interestingly, the molecular receptive range of the 12 types of neurones classified here showed little overlap. This indicates that information about one compound is conveyed to the brain mainly by one type of ORN. Only two pairs of neurone types (type 1 and 2 and type 5 and 6) showed slight overlaps. More neurones with overlapping molecular receptive ranges might appear in a larger data set. A similar overlap in the molecular receptive range for neurones responding to chemicals of the same groups, e.g. bicyclic monoterpenes, was also demonstrated in the related pine weevil *Hylobius abietis* (Coleoptera, Curculionidae) (Wibe and Mustaparta 1996) as well as in the cerambycid beetle *Phoracantha semipunctata* (Coleoptera, Cerambycidae) (Barata 1997; Barata et al. 2002). One may speculate whether the overlapping of molecular receptive range is biologically significant. At long distances the concentration of the secondary odorants might be too low. However, when the insect is on the host, boring into the bark, it is obviously exposed to high concentrations of the compounds. Thus, odorants having a secondary effect on an ORN may play an important role at close ranges.

The classification of distinct neurone types raises the question of whether the specificity of neurones is similar in closely and distantly related species. In a study of the related *H. abietis* living on conifers, 30 types of neurones have been classified (Wibe and Mustaparta 1996; Wibe et al. 1997). Four of the types in *P. notatus* correspond to neurones in *H. abietis* by having their greatest response to the same compounds; α-pinene (type 1), fenchone (type 2), pinocamphone/camphor (type 3) and limonene (type 4). The next question is how similar the neurone types are with respect to secondary active compounds. In the case of the α-pinene neurone, one type in *H. abietis*, responding second best to β-pinene seems to be similar to the type 1 in *P. notatus*. Another interesting similarity appears when comparing the recordings from the co-located type 1 and type 3 neurones in *P. notatus* (Fig. 1) with the recordings in *H. abietis*. In fact tests with both species, with the same entrainment sample (*P. abies* sawdust), showed similar response patterns, i.e. both showed strongest responses to α-pinene and camphor. In *H. abietis* the responses have been interpreted as originating from one neurone. However, it is possible that in this species the responses were in fact originating from two neurones (A. Wibe, personal communication). The other pairs of neurone types seem to differ between the two species with respect to their secondary responses. The fenchone type responded secondarily to camphene (Fig. 5) in *P. notatus* and to a different (unidentified) compound in *H. abietis*. The neurones responding best to limonene in *P. notatus* did not respond to any other component in the *P. abies* mixture, whereas in *H. abietis* the limonene neurones had secondary responses to several other compounds. However, comparison of weak responses can be misleading considering the variation of sensitivity of neurones and changes (e.g. oxidation) of the test mixture that might have occurred over time. When comparing *P. notatus* with the more distantly related *P. semipunctata,* living in a non-conifer host, *Eucalyptus globulus*, two neurone types corresponded with *P. notatus*. The α-pinene type differed in the secondary responses, whereas the limonene type in both species also responded to myrcene. These results suggest that the two related weevil species may have similar ORNs for some of the plant compounds. However, many ORNs are functionally different, as described above, accounting for the use of species-specific odour blends for the location of host plants.

The active compounds identified in this study were present in entrainment mixtures from host and non-host plants. None of the ORNs found in *P. notatus* were specific for the detection of compounds only present in non-host plants. Moreover, the identified active compounds are widely distributed, mainly present in the resin of conifers, but in different proportions both within and between tissues and species (Sjödin et al. 1996, 2000). This indicates that this weevil discriminates a host from a non-host plant by the ratio of the active compounds released by the individuals rather than specific sets of compounds present in the host or a non-host species. This has been suggested also for other beetles such as *H. abietis* (Wibe et al. 1997) and *P. semipunctata*, although in the latter species neurones specifically tuned to non-host compounds were found (Barata et al. 2002).

The adult *P. notatus* weevil uses its host for different purposes; i.e. feeding on the soft part of the bark for maturation and laying eggs in the trunk. Moreover, *P. notatus* is able to develop and reproduce in different species of *Pinus*. Thus, the oligophagous weevil is challenged by different odour blends produced under different conditions by the same as well as different plant species. This may account for the many different types of ORNs evolved. It is interesting that *P. notatus*, which is attracted to weakened trees, has selective ORNs for detecting ethanol, a compound produced in higher amounts by stressed conifers. Ethanol has been shown to play an important role for many beetles in the interaction with the host plant (Byers et al. 2000, Nordlander 1990).

In conclusion, the results show that plant odour information in *P. notatus* is mediated by a relatively large number of selective ORN types. The different types are specifically tuned to one or a few structurally related compounds, only a few having a slight overlap of response spectra. Major as well as minor constituents of plant volatile blends seem to be used for host and non-host detection, mainly including monoterpenes (bicyclic and monocyclic), but also sesquiterpenes and ethanol. Only a few ORNs seem to have receptor specificity similar to that of the ORNs of the related weevil *H. abietis*, indicating that the two related species use different odour blends for locating their hosts.

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Paper II

Olfactory receptor neurones in *Pissodes notatus* respond selectively to plant produced ethanol, *o*-methylanisole and limonene enantiomers

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Running head: RN types in the weevil *Pissodes notatus*

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Taxa: *Pissodes notatus,* Coleoptera, Curculionidae

Abstract

Plants release complex mixtures of volatile compounds that are important in the interaction with insects and other organisms. In the present study 18 selective olfactory receptor neurones on the antenna of the weevil *Pissodes notatus* were functionally characterized by the use of electrophysiological recordings from single cells linked to gas chromatography. A wide range of naturally plant produced volatiles were tested on the neurones separated by both a polar and a chiral column installed in parallel in the gas chromatograph. The olfactory receptor neurones were classified into 9 types according to the odorant eliciting the strongest response, termed primary odorant. One receptor neurone type was specified for *o*-methylanisole, one for *S*-(-)-limonene, two for ethanol, and three for chemically unidentified sesquiterpenes. The two remaining types responded to monoterpenes.

Introduction

The weevil *Pissodes notatus* is an oligophagous species with wide distribution in Eurasia, constituting a pest in plantations of maritime pine (*Pinus pinaster,* Aiton) in southern Europe and the Mediterranean area (Cobos and Robredo, 1982; Alauzet, 1984; Ferreira and Ferreira, 1989). The weevils use weakened species of conifer trees of the genus *Pinus* as host plants (Alauzet, 1984). The adults aggregate on suitable hosts and mate. The females lay eggs under the bark and the larvae develop feeding on the phloem of the trunk. The feeding activity of the larvae interrupts the circulation of sap in the phloem leading to the death of the tree. Traditional methods used to control this pest insects are based on trap logs laid on the ground (Plata-Negrache and Prendes-Ayala, 1979), indicating that *P. notatus* uses olfaction to find the host. In a previous study of plant olfactory receptor neurones (RNs) in *P. notatus* (Bichão *et al.* 2003), we have presented 12 olfactory RN types, identified by using gas chromatography linked to single cell recordings (GC-SCR) and gas chromatography linked to mass-spectrometry (GC-MS). The majority of these RNs were specified for monoterpenes. Only one type responded to a sesquiterpene, another to ethanol, and the odorants activating four types could not be chemically identified.

In the present study, a wider range of plant mixtures was used as test stimuli compared to the previous study. In addition, a more advanced method of GC-SCR was employed, in which two GC-columns, one polar and one chiral, aided the separation of the constituents in the various samples (Røstelien *et al.*, 2000a; Stranden

et al., 2002). The 18 single RNs recorded on the antenna of *P. notatus* presented here were tuned to monoterpenes, sesquiterpenes, ethanol and aromatic compounds, and were classified into 9 types. We present one RN type tuned to aromatic compounds, three to sesquiterpenes and one to ethanol, which have not been previously reported. Information regarding enantioselectivity and molecular receptive range of four previously described RN types is also included.

Material and methods

Insects:

Adult P. notatus were obtained from a colony kept under long-day conditions (14:10 hours) at suitable temperature (22-25 C), and fed with logs, twigs and leaves of Pinus sylvestris. All insects were starved for at least 24 hours, before experiments. The colony was started with insects collected from P. pinaster in the area of Toulouse, France (kindly provided by Dr. Alain Rocques).

Plant volatiles:

The plant compounds used to stimulate the RNs were constituents in headspace mixtures and essential oils of host (*P. pinaster*) and non-host plants (*Pinus pinea, Eucalyptus globulus*, among others, conf. Tab. II). Reference samples of the relevant authentic and synthetic compounds were also used to confirm identifications.

Volatile compounds released by host and non-host plants were collected using dynamic headspace entrainment techniques. For collection details reference is made to Bichão *et al.,* (2003). Briefly, the volatiles emitted by plant materials were trapped by blowing nitrogen (during 24 hours at 25-30 C) through a glass vial into two parallel glass tubes (6.6 cm x 0.5 cm i.d.) packed with the adsorbent (150 mg Porapak Q, 80-100 mesh). The average flow measured at the outlet of the tubes was 50-60 ml/min (i.e. total air volume exchange in the vial, every 50-60 min). The adsorbed plant volatiles were eluted from the Porapak Q with n-hexane (pa Merck) and the resulting solutions were used as test samples in the experiments.

Linked gas chromatography single cell recordings (GC-SCR):

The insect preparation and the electrophysiological recordings of nerve impulses from single olfactory RNs on the antennae were carried out as described previously by Bichão *et al.* (2003). Contact with the neurone was made with a tungsten microelectrode inserted at the base of the *sensillum*. Each neurone was tested for

sensitivity to plant volatiles by direct stimulation with different mixtures, i.e. by blowing air through a cartridge containing a small sample of each solution on filter paper. If a cell responded, its selectivity was tested by injection of the various mixtures into the GC-column. A spilt at the end of the GC-column led half of the effluent to the GC-detector and half to the clean airflow that blew over the insect antenna, resulting in recording of responses to the separated compounds, simultaneously with recording the gas chromatogram of the volatile mixture. The nerve impulse frequency was measured by a spike integrator and displayed as the number of spikes/time unit with the computer program Electro Antenno Detection (version 2.3, Syntech NL, Hilversum, The Netherlands). In parallel, spike activity was recorded and analysed with the Software Spike 2 (Cambridge Electronic Design Limited, Cambridge, Great Britain). Two GC-column types were used in parallel: a polar DBwax column (J&W, 30 m; 0.25 mm i.d.; 0.25 m film thickness), and one chiral column [octakis-(6-methyl-2,3-di-O-pentyl)-γ-cyclodextrin (80% in OV1701, 25m, 0.25 mm i.d.; 0.25 m film thickness) (König *et al.*, 1990). Separations in the non-chiral column were performed from an initial temperature of 50 C (alternatively from 80 C), where it was held isothermal for 2 min, with a subsequent increase of 6 C/min (alternatively from 80 C) to 180 C, and a further increase rate of 15 C/min to 220 C. The enantiomers of limonene were separated in the chiral column by an initial temperature of 55 C for 15 min, followed by an increase of 1 C/min to 75 C.

Chemical analysis:

The active compounds were identified by GC-MS, using a Finningan SSQ 7000 MS instrument connected to a Varian 3400 GC, using the same column type as for the GC-SCRs. The MS ion source temperature was 150 °C at 70 eV, and the mass range was 30-400 mu.

Results

The results are based on recordings from 18 single RNs on the antenna of *P. notatus* that responded to naturally plant produced compounds. The responses appeared consistently at the same retention time in repeated tests with the same and different samples that contained the active compounds. All RNs responded strongest to one compound (primary odorant), and weaker to a few compounds of similar structure (secondary odorants). The RNs could therefore be classified in 9 types according to the primary odorant. These include five RN types that have not been recorded previously and 4 types that have been reported in a previous study (Bichão *et al.* 2003) (Tab. I). For two of the latter types more detailed information about the molecular receptive range is presented here. The primary odorants of the RNs were aromatic compounds (*o*-methylanisole), sesquiterpenes (unidentified), monoterpenes [S-(-)-limonene, α-pinene, and isopinocamphone], and ethanol. For clarity, the RN types presented are numbered with consecutive roman numerals. For previously described types, the corresponding number (arabic numerals) attributed by Bichão *et al.*, (2003), is added in parentheses.

Type I: RNs tuned to o-*methylanisole*

Five RNs responded with high selectivity and sensitivity to the aromatic compound *o*-methylanisole (Fig. 1). Their primary odorant was found in minute amounts in the headspace of cut materials of the host *P. pinaster* (Fig. 1A) and the non-host *Helianthus annus* (Fig. 1B). Numerous headspace samples and essential oils of various plants were also tested, including hosts and non-hosts, as well as various synthetic or isolated compounds (Tab. II). Weak responses to the structural isomer *m*-methylanisole appeared whereas the *p*-methylanisole did not elicit responses (Fig. 1C). Stimulation with xylene (a simple aromatic ring), elicited a weak response similar in strength to the response to *m*-methylanisole. The dose-response curves in Fig. 1D show that a 100 times higher concentrations of the two secondary odorants are needed to elicit the same response as *o*-methylanisole. Many other aromatic plant compounds tested on these RNs did not elicit any responses Tab. II; Fig. 1E).

Type II (4): RN responding to S-(-) limonene

One RN type (n=1) responded strongest to limonene and weaker to β-myrcene when stimulated with the host headspace samples, similarly to previously described RNs (Bichão *et al.,* 2003). No other constituents elicited responses. The RN was further tested with reference samples of *S*-(-)- and *R*-(+)-limonene via the chiral column. A

clearly stronger response to *S*-(-)-limonene then to the *R*-(+)-enantiomer was recorded (Fig. 2A). Dose-response curves obtained by direct stimulation showed that *S*-(-)-limonene was about 100 times more effective than *R*-(+)-limonene (Fig. 2B).

Types III (12) and IV: RNs responding to ethanol

Two RN types responded to ethanol, type III (n=2) showing excitatory responses while the mode of response of type IV (n=2) was inhibition (Fig. 3). All four neurones were tested for sensitivity to numerous samples of plant volatiles and showed no response to any of the numerous constituents. The only responses were elicited by ethanol. When stimulated with different doses of ethanol via the GC-column, type III RNs showed dose-dependent excitatory responses (Fig. 3A). Several other saturated alcohols of different chain length were tested. The saturated alcohols with longer chain length, 1-hexanol, 1-octanol and 1-nonanol, did not elicit responses, whereas a weak response was elicited by methanol. Dose-response curves determined by direct stimulation showed a 100 000 fold lower effect of methanol compared to ethanol (Fig. 3B) underlining the selectivity of these neurones to ethanol. The other ethanol type, (RN type IV, n=2) responded consistently by inhibition to high concentrations of ethanol (Fig. 4). None of the test compound elicited excitatory responses in these RNs.

Types V, VI, VII: RN types responding to sequiterpenes

Three RN types which responded to sesquiterpene hydrocarbons have not previously been described for this species. RNs of type V (n=2) responded to three of the compounds present in the essential oil of ylang-ylang, which were tentatively identified by mass spectrum comparisons as bicyclic sesquiterpene hydrocarbons. Type VI (n=1) RNs responded to another compound also present in ylang-ylang essential oil, which was tentatively identified as an elemene isomer. The type VII RN (n=1) responded to a compound eluting just before humulene, present both in the ylang-ylang essential oil and in the host headspace. None of these could be positively identified due to co-elution with other compounds and the small amounts present in the samples.

Types VIII (1) and IX (3): RNs tuned to bicyclic monoterpenes

Among the recorded RNs were two types, one showing best response to α-pinene (Type VII; n=2) and the other one to isopinocamphone (type IX; n=2). These RN types showed secondary responses to β-pinene and 3-carene and to pinocamphone,

which correlates with the previously characterized RNs responding to α-pinene and isopinocamphone, respectively.

Discussion

The results presented here add to the knowledge on functional characteristics of olfactory RNs of the weevil *P. notatus,* partly by showing additional types of RNs and partly by adding more detailed information about previously reported RN types (Bichão *et al.*, 2003). The data are interesting for the understanding of olfactory mechanisms in this species and for comparing RN specificity with other species. Like plant RNs described in previous studies of *P. notatus* and other insect species, the RNs were narrowly tuned, showing best response to one compound (primary odorant) and weaker responses to a few structurally related compounds (secondary odorants). Most responses were excitatory, but inhibitory responses to ethanol also appeared in one RN type. Based on the selective responses, the RNs could be classified in types showing a characteristic molecular receptive range and ranking of sensitivity to the active odorants in accordance with what has been shown in previous GC-SCR studies for RNs of other weevils and beetles, as well as moths (Blight *et al.*, 1995; Wibe and Mustaparta, 1996; Røstelien *et al.,* 2000a,b, 2005; Stensmyr *et al.,* 2001, 2003; Barata *et al.,* 2002; Stranden *et al.,* 2002, 2003a,b; Bichão *et al.,* 2003, 2005a,b; Ulland *et al.,* 2006). In this study, the selectivity of the RNs is particularly well illustrated by the RN types tuned to ethanol and to *o*-methylanisole. These responded to only two or three compounds, respectively, out of the hundreds present in the more than 30 mixtures of volatiles tested (Tab. I, Fig. 1E).

One of the greatest challenges when studying olfaction is to identify the stimulus. In contrast to the well defined stimuli spectrum of other senses, e.g. the wavelength of light for vision, volatile stimuli range does not constitute a known continuum/spectrum. In addition, it is impossible to stimulate each RN with all known volatile compounds. This problem was exemplified by the present study in which including more plant samples as stimuli revealed RN types that were not found in previous studies of *P. notatus* (Bichão *et al.,* 2003). Due to the huge variability displayed by plant volatile production, even within the same species, using several samples of the host is important. For example the aromatic flower compound *o*-methylanisole, identified as a primary odorant in the present study (Fig. 1A), was present in minute amount in only one out of the three host headspace mixtures used.

This compound was also present in non-host mixtures, e.g. sunflower (Fig. 1B). This RN type adds the aromatic compounds, to the scope of chemical groups of compounds detected by *P. notatus* (i.e. monoterpenes, sesquiterpenes and ethanol, Bichão *et al.,* 2003). Three other RN types tuned to sesquiterpene hydrocarbons were identified due to the presence of the primary odorant in the essential oil of ylang-ylang. Only one was present in the host headspace. These examples show how important it is to stimulate with as many compounds as possible when studying the response spectra of olfactory RNs. The common method of stimulating with only a restricted selection of commercial compounds or a few host blends is likely to restrict the results obtained, failing to identify all olfactory RN types.

An interesting question in olfactory research is which molecular features of the odorant are important in the interaction with the receptor proteins at the surface of the dendrites. Chirality, chain length and functional groups are features that have been suggested to be important in insects (Priesner, 1979; Schneider *et al.*, 1977; Bengtsson *et al.*, 1990; Masson and Mustaparta, 1990; Leal, 2001; Stranden *et al.*, 2002; Bichão *et al.*, 2005a, b; Ulland *et al.,* 2006, among others). Results concerning the *S*-(-)-limonene RN type show that the direction of the isopropenyl group is important for activating the RN: the stimulatory effect of the *R*-(+)-limonene was about 100 fold weaker as compared to the (-)-enantiomer (Fig. 2, A and B). Similar enatioselective RNs responding strongest to the *S*-(-)-limonene have also been described in the large pine weevil *Hylobius abietis* (Wibe *et al.*, 1998). In the case of the RN type tuned to *o*-methylanisole, the relative position of the methyl and the ester group is an important feature, as shown by minimal and no effect of the structural isomers *m*-methylanisole and *p*-methylanisole, respectively (Fig. 1C, D).

From an evolutionary and ecological point of view, it is interesting to compare the specificity of RNs of closely and distantly related species, and species that use the same or different host plants. These comparisons are only possible if experimental protocols are similar. Several of the RN types described in *P. notatus* show similarities to RN types described in other insect species. Recordings from a RN sensitive to *o*-methylanisole have been obtained from *H. abietis* (Wibe, personal communication). Interestingly, *o*-methylanisole is found in the faeces of *H. abietis* and is most probably a lignin degradation product (Borg-Karlson *et al.*, 2006), Interestingly, this compound act as antifeedants for the pine weevil, which also feeds and reproduces in conifers. In the strawberry weevil *Anthonomus rubi*, adapted to

different host plants, only RNs tuned to *p*-methylanisole were found showing secondary responses to *o*- and *m*-methylanisole (Bichão *et al.,* 2005b). Other similarities between the conifer feeding *P. notatus* and *H. abietis* are that both have neurones tuned to the monoterpenes α-pinene, *S*-(-)-limonene, and to ethanol (Wibe *et al.*, 1998; Bichão *et al.*, 2003; Huotari *et al.*, 2003; Stranden., personal communication). Only a few electrophysiological studies describe RNs tuned to ethanol (Tømmerås and Mustaparta, 1989; Bichão *et al.*, 2003; Saïd *et al.*, 2003), despite the fact that the behavioural effect of ethanol has been demonstrated in various insect species including *H. abietis* (e.g. Hunt and Raffa, 1989; Brattli *et al.*, 1998, Tilles *et al.*, 1986, Nordlander *et al.*, 1986). In *P. notatus,* RNs responding to ethanol by excitation have been found in the previous and the present studies. In addition, a RN type inhibited by ethanol has appeared in this study (Fig. 4), suggesting the importance of this odorant for the weevil.

The results in this study, taken together with the results from Bichão *et al.,* (2003), indicate that the majority of RNs in *P. notatus* are tuned to bicyclic and monocyclic monoterpenes, both in total number of RNs (72%, n=78) and number of types (6 out of 17). Other groups of naturally occurring compounds are also detected, and may be important in the interaction with the plant [(5 RN types responded to sesquiterpenes (11,8% of the total), 2 types to alcohols (8%) and 1 type to aromatic compounds (6.6%)]. In analogy with the pheromone system, a general assumption is that both RN sensitivity and the total number of RNs tuned to a particular odorant are important for the distance over which the odorant is detected. In general, numerous RNs with high sensitivity are thought to mediate long range attraction while rarer RNs, with low sensitivity, may be recruited at closer range and be involved in the selection of the host. According to this principle, α-pinene, isopinocamphone and *S*-(-)-limonene are potential long range behaviour modulators for *P. notatus*. Interestingly, the air above the canopy of *P. pinaster* is dominated by α-pinene and limonene (Riba, 1991), together with β-pinene and 3-carene. In addition, detecting ethanol may allow *P. notatus* to smell weakened trees, which are favourable host individuals. The synergistic attractive effect of monoterpenes and ethanol has been reported for other beetle species associated with conifers (Tilles *et al.*, 1986), and is suggested in *P. notatus* in preliminary laboratory behavioural tests (Bichão, unpublished). The high sensitivity of the RNs responding to *o*-methylanisole, indicate an ecological relevance of this odorant. Finally, sesquiterpenes may be more important at closer range.

However behavioural studies are needed to understand the biological significance of the identified odorants for *P. notatus*.

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Figure legends

Figure 1. Molecular receptive range of a receptor neurone of *Pissodes notatus* tuned to *o*-methylanisole, defined by responses to host and non host volatiles (A-B) and structure activity studies (C-E).A. Gas chromatogram of the headspace volatiles of pine (*Pinus pinaster*), *above*, and simultaneously recorded activity in a single receptor neurone during stimulation with the separated constituents *below*. The strong and selective response was elicited by *o*-methylanisole. The bond-line structure of the active constituent is shown. B. Gas chromatograms of the headspace of the non host-plant sunflower (*Helianthus annus*), *above,* and simultaneously recorded activity of another receptor neurone of the same type, showing the selective response to minute amounts of the primary odorant, *below*. C. Gas chromatograms of reference samples of *o*-, *m*- and *p*-methylanisole, *above*, and simultaneously recorded activity of a single receptor neurone tuned to *o*-methylanisole, *below*. Bond-line structures of the active compounds are shown. (The arrow head indicates a response to an minute amount of *o*-methylanisole in the sample). D. Dose-response curves for the same receptor neurone as in A obtained by stimulation with the three odorants via the polar GC-column, showing a 100-fold loss in activity by moving the methyl group from position 2 (*o*-methylanisole) to position 3 (*m*-methylanisole). The simple ring xylene had a similar stimulatory effect as *m*-methylanisole. E. Bond-line representation of the structures of tested non-active compounds. (1) Highest dose tested: 100ng/µl; (*) tested only by direct stimulation.

Figure 2. A. Gas chromatograms of reference samples of *S*-(-)- *(left)* and *R*-(+)-limonene *(right)* separated in the chiral GC-column, *above*. The simultaneously recorded activity of a single neurone in *Pissodes notatus*, shows responses to both enantiomers, but a stronger response to the (-)-limonene, *below*. B. Dose-response curves of a receptor neurone tuned to (-)- and (+)-limonene showing a 100-fold higher sensitivity to the (-)- enantiomer, obtained by direct stimulation with decadic dilutions of reference samples of the enantiomers. The points show the mean of two responses elicited by repeated stimulation with the same cartridge. The shaded area marks the spontaneous activity.

Figure 3. A. Gas chromatograms of two dilutions of ethanol separated in the GC-column, *above* and the simultaneously recorded activity of a single receptor neurone in *Pissodes notatus*, *below*. B. Dose-response curves of a receptor neurone excited by ethanol, obtained by direct stimulation with decadic dilutions of ethanol and methanol. The points show the mean of two responses elicited by repeated stimulation with the same cartridge. The shaded area marks the spontaneous activity.

Figure 4. Inhibitory responses of single receptor neurones when stimulated directly with ethanol (saturated). Inhibition of spiking activity is visible after stimulations with ethanol compared to no response when stimulating with control (air) (Black bars: stimulus duration).

Table I. Overview of the olfactory receptor neurone types recorded in this study, classified according to the primary and secondary odorants. The number of olfactory receptor neurones (n) of each type is indicated. For the types previously described is indicated the corresponding type number (square parentheses) in Bichão *et al.* (2003) and the number of neurones recorded in the previous study (round parentheses).

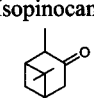
RNs	Primary odorant	Secondary odorants
Type I n=5	*o*-Methylanisole 	*m*-Methylanisole Xylene
Type II [4] n=1 (9)	*S*-(-)-Limonene 	*R*-(+)-Limonene β-Myrcene
Type III [12] n=2 (4)	Ethanol 	Methanol
Type IV n=2	Ethanol (inhibition) 	
Type V n=2	*Bicyclic sesquiterpene hydrocarbon*	*Sesquiterpene hydrocarbon* *Sesquiterpene hydrocarbon*
Type VI n=1	*Elemene isomer*	
Type VII n=1	*Unidentified sesquiterpene*	*Unidentified sesquiterpene*
Type VIII [1] n=2 (17)	α-Pinene 	β-Pinene 3-Carene
Type IX [3] n=2 (17)	Isopinocamphone 	Pinocamphone

Table II. Mixtures and single compounds tested on the RN types responding to *o*-methylanisole and ethanol, identified in *Pissodes notatus*. Direct stimulation was done by blowing air through a cartridge containing a sample of the test mixture on filter paper. (++++ strong response; ++ medium response; + weak response; ± equivalent to solvent response; ø no response; - compound not tested)

	RN types			
	o-Methylanisole		*Ethanol*	
Mixtures	*Direct*	*Via GC*	*Direct*	*Via GC*
Pinus pinaster *(Headspace)*	+++	+++	ø	-
Cananga odorata (essential oil) Dragoco	++	+	ø	-
Helianthus annuus (headspace) Stranden	+++	++	ø	-
Juniperus virginiana *Stranden*	ø	-	ø	-
Pinus sylvestris (essential oil) NMD	ø	-	ø	-
Turpentine Borg-Karlson	ø	-	ø	-
Cedar tree oil	ø	-	ø	-
Orange peal (headspace) Stranden	ø	-	ø	-
Cupressus sempervirens	ø	-	ø	-
Eucalyptus globulus *(headspace)*	ø	-	ø	-
Clove bud (essential oil) NMD	ø	-	ø	-
Cinnamon (essential oil) NMD	ø	-	ø	-
Basil (essential oil) NMD	ø	-	ø	-
Bergamot (essential oil) NMD	ø	-	ø	-
Lemon (essential oil) NMD	ø	-	ø	-
Clove (essential oil) NMD	ø	-	ø	-
Syringa (essential oil) Borg-Karlson	ø	-	ø	-
Fennikel (essential oil) NMD	ø	-	ø	-
Peppermint (essential oil) NMD	ø	-	ø	-
Arabidopsis thaliana (headspace of 4 ecotypes)	ø	-	ø	-
Brassica napus (head space)	±	ø	ø	-
Apple esters 1	ø	-	ø	-
Apple esters 2	ø	-	ø	-
Apple esters 3	ø	-	ø	-
St1 (various sesquiterpenes)	ø	-	ø	-
St2	ø	-	ø	-
St2a (misture of farnesenes)	ø	-	ø	-
St3a	ø	-	ø	-
St4 [(+)-3-carene, (+/-) pinene[a]]	ø	-	ø	-
Isothiocyanates	ø	-	-	-
div. Verbenones	ø	-	-	-

Table II (continued)

	RN types			
	o-Methylanisole		*Ethanol*	
Single compounds	*Direct*	*Via GC*	*Direct*	*Via GC*
Anthole	ø	-	-	-
Benzylalcohol	ø	ø	ø	-
Benzylcyanide	ø	-	-	-
β-Cyclocitral	ø	-	-	-
Ethanol	±	-	++++	++++
Ethyl benzoate	±	ø	-	-
Eugenol	ø	-	-	-
(-)-Fenchone	ø	-	-	-
(+)-Fenchone	ø	-	-	-
Grandisol	ø	-	-	-
Hexane	±	-	+	+
Indole	±	-	-	-
Linalool	ø	-	-	-
Methanol	-	-	+	+
p-Methoxyanethole	ø	-	-	-
p-Methylanisole	ø	ø	-	-
m-Methylanisole	++	++	-	-
o-Methylanisole	++++	++++	-	-
Methyl benzoate	±	ø	-	-
Methyl jasmonate	±	ø	-	-
Methyl salicylate	ø	-	-	-
Myrcene	ø	-	-	-
Pinocarveol	ø	-	-	-
1-Octen-3-ol	ø	-	ø	-
β-Ocimene	ø	-	-	-
1-Octanol	ø	-	-	-
Xylene	++	++	-	-

Apple esters 1: (Ethyl-2-methyl-propanoate, Methyl-methyl-butyrate, Propyl-butyrate, i-butyl-butyrate, (±)-2-Methyl-1-butanol, Hexyl-formate, Butyl-pentanoate, i-Butyl-hexanoate, Hexyl-butyrate, Pentyl-hexanoate, Butyl-caprylate, 2,3-Butandiol); **Apple esters 2** (Ethyl-butyrate, Butyl-propionate, 2-Methyl-butyl-propanoate, 3-Methyl-1-butanol, 2-Methyl-1-butyl-butyrate, Pentyl-butyrate, Hexyl-propanoate, Hexyl-2-methyl-butyrate, 2-Methyl-1-butyl-hexanoate, Hexyl-hexanoate, Ethyl-2,4,deco-dienoates) **Apple esters 3** (Ethyl-2-methyl-butyrate, Sec-propyl-butanoate, Butyl-butyrate, (2*E*)-Hexenal, (2*E*)-Hexenyl-acetate, Butyl-hexanoate, 2-Methyl-hexyl-butanoate, 3-Methyl-butyl-hexanoate, (3Z)-Hexenyl-hexanoate); **St1** [-cubebene 69%, (-)- -cubebene, -copaene, -elemene, cadinener, div. muurolener, amorphen, -muurolene, δ-cadinene, -muurolen, + -cadinene]; **St2** [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)]; **St2a** (misture of farnesenes)(Farnesol, Mix -farnesol (from Ilme), Farnesenes in hexane, E,E,- -Farnesene; **St3a** [Tetrahydrolinalool (99%), Linalool F 21-25[c], Linalool F 13-15 [c], Linalool F 10 [c], Racemic linalool, Linalyl 97% acetate, **Isothiocyanates** (phenyl isotiocyanate, butyl tiocyanate, allyl isotiocyanate, 3-me-3-buten tiocyanate, phenetyl isotiocyanate, phenetyl tiocyanate, 3-butenyl isotiocyanate1-octen-3-ol (98%), 3-octanone]

Figure 1.

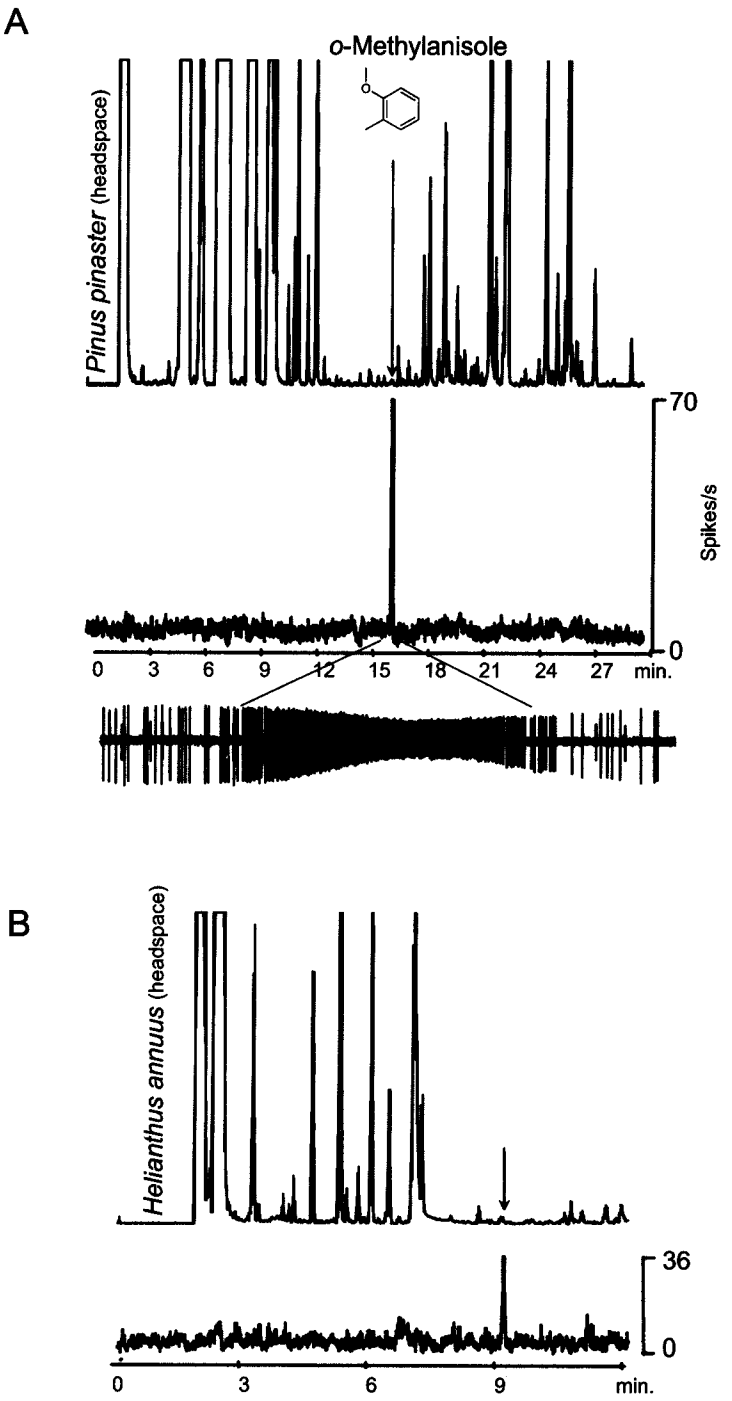
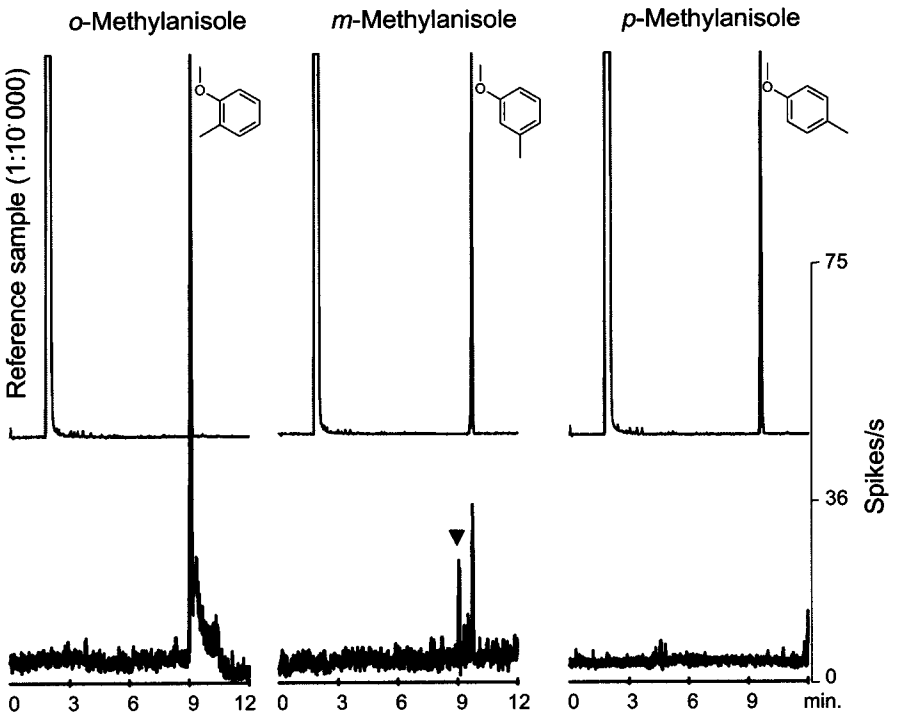
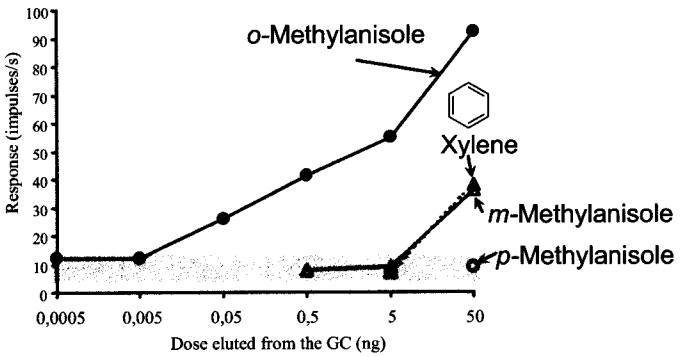

A
o-Methylanisole
Pinus pinaster (headspace)
70
Spikes/s
0
0 3 6 9 12 15 18 21 24 27 min.
B
Helianthus annuus (headspace)
36
0
0 3 6 9 min.

Figure 1.

C



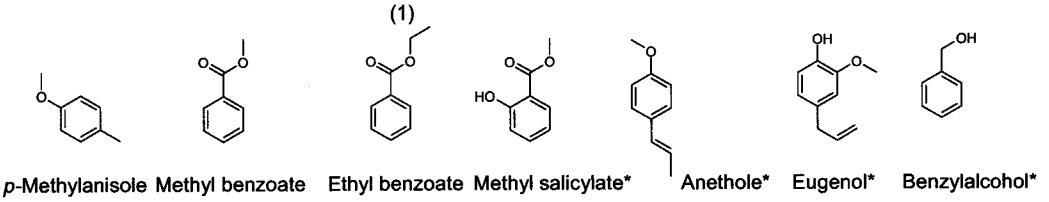
D



E

Inactive structures

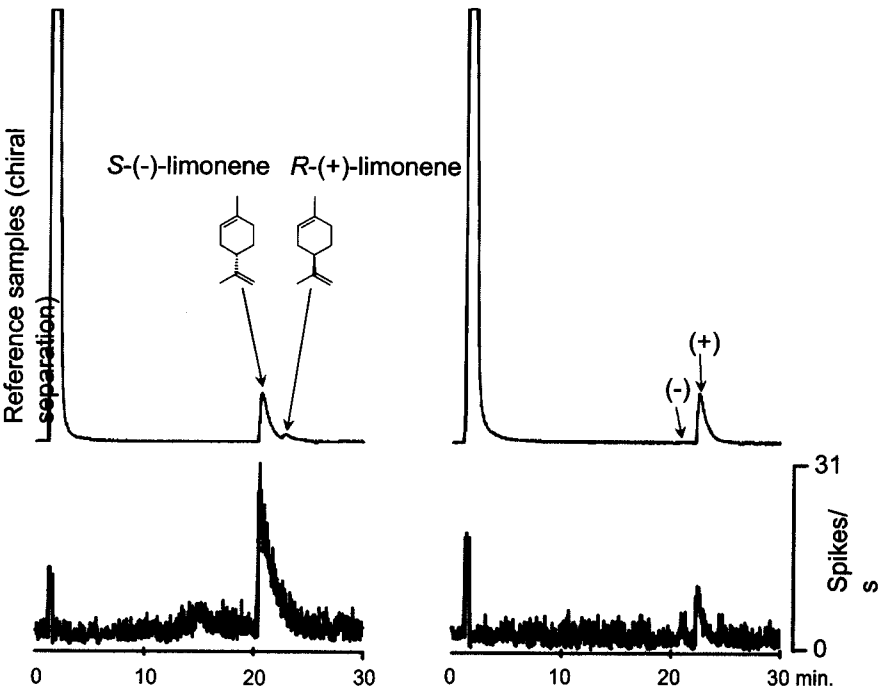
(1)



p-Methylanisole Methyl benzoate Ethyl benzoate Methyl salicylate* Anethole* Eugenol* Benzylalcohol*

Figure 2.

A


Reference samples (chiral separation)
S-(-)-limonene
R-(+)-limonene
(+)
(-)
31
Spikes/ s
0
0
10
20
30
0
10
20
30 min.

B

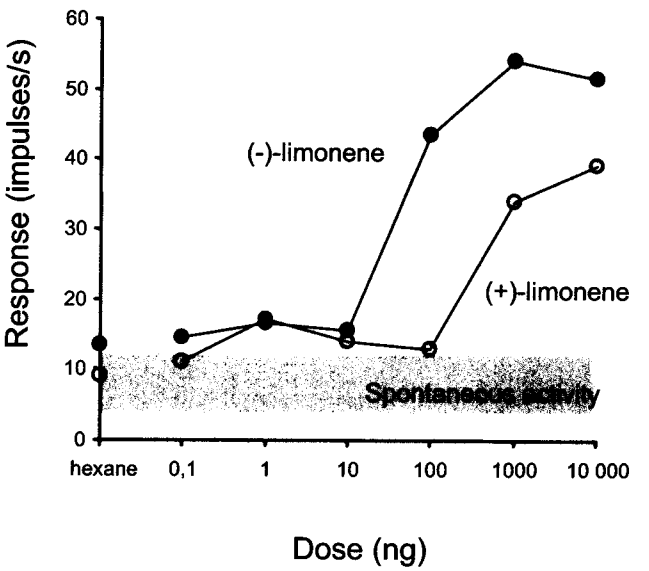

Response (impulses/s)
60
50
40
30
20
10
0
(-)-limonene
(+)-limonene
Spontaneous activity
hexane
0,1
1
10
100
1000
10 000
Dose (ng)

Figure 3.

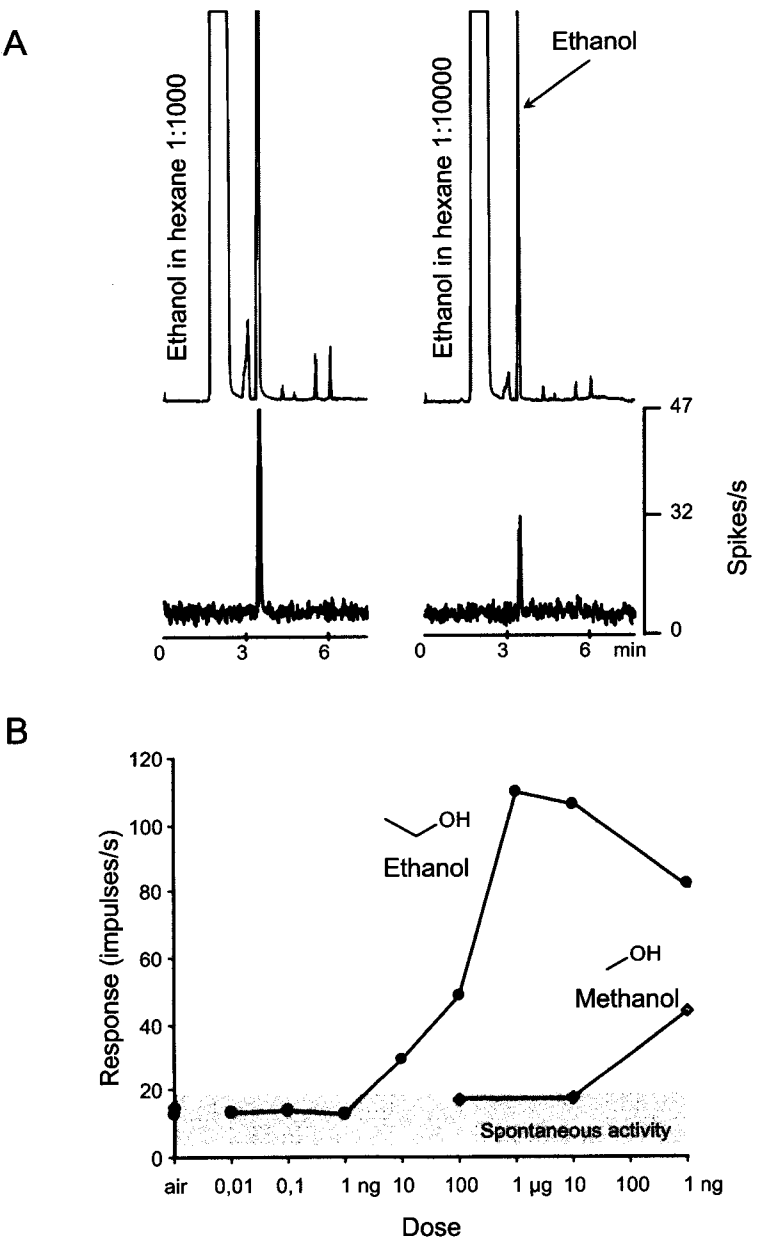
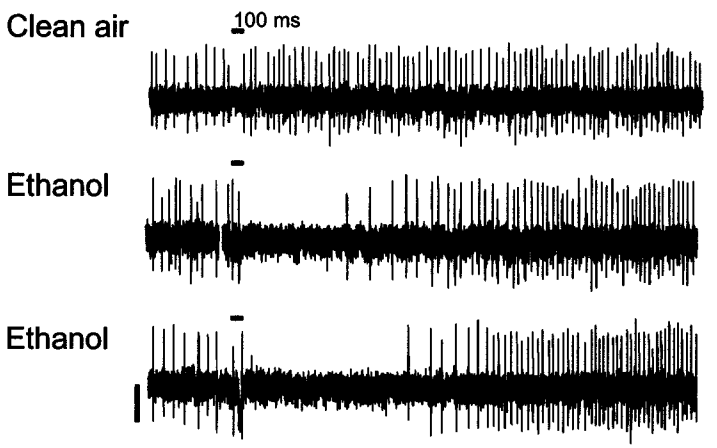

A
Ethanol in hexane 1:1000
Ethanol in hexane 1:10000
Ethanol
47
32
0
Spikes/s
0
3
6
0
3
6
min
B
Response (impulses/s)
120
100
80
60
40
20
0
OH
Ethanol
OH
Methanol
Spontaneous activity
air
0,01
0,1
1 ng
10
100
1 µg
10
100
1 ng
Dose

Figure 4.



Paper III

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Five Types of Olfactory Receptor Neurons in the Strawberry Blossom Weevil *Anthonomus rubi*: Selective Responses to Inducible Host-plant Volatiles

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Abstract

Plants release hundreds of volatiles that are important in the interaction with herbivorous animals, but which odorants are detected by which species? In this study, single receptor neurons on the antenna of the oligophagous strawberry blossom weevil *Anthonomus rubi* were screened for sensitivity to naturally produced plant compounds by the use of gas chromatography linked to electrophysiological recordings from single cells. The narrow tuning of the neurons was demonstrated by responses solely to a few structurally related sesquiterpenes, aromatics or monoterpene hydrocarbons out of hundreds of plant constituents tested. We present five olfactory receptor neuron types, identified according to one primary odorant i.e. the compound to which the neurons are most sensitive. These odorants, (−)-germacrene D, (−)-β-caryophyllene, methyl salicylate, *E*-β-ocimene and (3*E*)-4,8-dimethyl-1,3,7-nonatriene, present in the intact strawberry plant, are induced in higher amounts by weevil feeding. This suggests that these compounds can provide information about the presence of conspecifics. We used protocols especially designed to allow comparison with previously investigated species. Striking similarities, but also differences, are demonstrated between receptor neuron specificity in the strawberry weevil and moths.

Key words: GC-MS, GC-SCR, insect olfaction, plant odours, terpenes, aromatics

Introduction

A large part of the olfactory system in herbivorous insects, including weevils, is evolved for the detection and processing of plant odour information, reflecting the importance of olfactory cues in host location for feeding and oviposition (Schoonhoven *et al.*, 1998; Mustaparta, 2002). Plants produce a large variety of secondary metabolites, many of which are in common, while some are restricted to certain plant groups. The blend of compounds released by a plant does not only vary with species and strain, but can also be influenced by abiotic or biotic factors like herbivore feeding (Paré and Tumlinson, 1999). While mechanical injuries increase the quantity of compounds released from the wounded site, feeding by insects often induces systemic production of new compounds and/or different relative amounts of the compounds. Species-specific compounds present in the oral secretions of herbivore larvae can induce these changes (Mattiacci *et al.*, 1995; Alborn *et al.*, 1997). Among the compounds shown to be systemically induced by insect feeding activity are the sesquiterpenes germacrene D and β-caryophyllene, the monoterpene *E*-β-ocimene, the *homo*-monoterpene *E*-DMNT (*E*-4,8-dimethyl-1,3,7-nonatriene) and the aromatic compound methyl salicylate (Innocenzi *et al.*, 2001; Arimura *et al.*, 2004; Colazza *et al.*, 2004; reviewed by Paré and Tumlinson, 1999; Dicke and Van Loon, 2000). It has been shown that compounds released after herbivory appear to protect plants by repelling or deterring herbivores, or by attracting the enemies of herbivores (De Moraes *et al.*, 2001; Pichersky and Gershenzon, 2002; reviewed by Turlings and Wäckers, 2004). In addition to providing information about competitors or food quality, inducible plant compounds may be reliable cues that advertise the presence of conspecifics and thereby promote aggregation of individuals. Thus these compounds can be important synergists to aggregation pheromones (Rochat *et al.*, 2000; Yang *et al.*, 2004).

The strawberry blossom weevil, *Anthonomus rubi* (Herbst) 1795, is an oligophagous species that feeds and reproduces

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on plants of the family Rosacea (Popov, 1996), mainly strawberry (*Fragaria* × *ananassa* Duchesne) and raspberry (*Rubus idaeus* L.). Adult weevils migrate early in the spring from overwintering shelters to the strawberry fields, where they feed on the foliage and start to mate at the onset of bud formation. After laying a single egg in an unopened bud, the female partially severs the bud pedicel. The larvae develop and pupate inside the withered bud, and emerge in late summer. This species constitutes a serious pest in strawberry throughout Europe, in some cases responsible for damages of up to 80% of the berry yield (Popov, 1996; Cross and Easterbrook, 1998). The role of olfaction for host location in *A. rubi* has not been completely clarified. The pheromone system has been investigated by Innocenzi *et al.* (2001), who showed that three components of a male-produced aggregation pheromone blend attract weevils to baited traps, but the role of host plant odours has not been examined.

An important question in insect–plant interactions is which of the numerous volatiles released by plants are detected by the insects and might constitute biologically relevant odorants for host location. In a preliminary study using gas chromatography linked to electroantennogram recordings (GC-EAG), the antennae of *A. rubi* were found to respond to several components present in the blend of the host plant (unpublished data). A more accurate method of identifying relevant odorants is recordings from single olfactory receptor neurons (RNs) linked to gas chromatography. With this method, each olfactory receptor neuron can be screened with numerous separated compounds of naturally produced blends, and repeatedly tested for confirming the responses. This allows determination of the molecular receptive range of the RNs. Gas chromatography linked to single cell recording (GC-SCR), in combination with GC linked to mass spectrometry (GC-MS), has been successfully used to identify host plant odours that are detected by several species of beetles and moths (Blight *et al.*, 1995; Wibe *et al.*, 1997, 1998; Røstelien *et al.*, 2000a,b; Stensmyr *et al.*, 2001; Barata *et al.*, 2002; Stranden *et al.*, 2002, 2003a,b; Bichão *et al.*, 2003). In the present study we identified the compounds that are detected by five types of RNs in the strawberry weevil and investigated structure–activity relationships. The protocols used also allowed the comparison with RN types described in other species. The primary odorants of the RNs were monoterpenes, sesquiterpenes and aromatic compounds that were emitted in greater amounts from flowers of strawberry subjected to weevil feeding.

Materials and methods

Insects

Adult *A. rubi* were collected from unsprayed strawberry fields (Dragvoll, Trondheim, Norway) during the summer seasons (mid-May to August) of 2002 and 2003. Prior to recordings, the insects were kept in the laboratory under natural scotophase at room temperature (22–25°C) for a maximum of 1 week. Fresh food material, consisting of young and mature leaves, flowers and flower buds of strawberry, was provided every third day. All insects used in this study were starved for at least 12 h before the experiments. The weevils were sexed after the experiments using the method described by Innocenzi *et al.* (2002).

Plants and collection of plant volatiles

In this study the host plant materials used were strawberry (*F.* × *ananassa*, Duschene, variety Korona) and raspberry (*R. idaeus* L.) from fields and green areas in Trondheim, Norway. Two different methods were used to collect volatiles from the plants, solid-phase micro-extraction (SPME) and dynamic headspace entrainment using the sorption material Porapak Q (80/100 mesh, Supelco).

Volatiles emitted by strawberry flowers, both intact and infested by overwintered weevils, were collected by SPME, using a 65 μm polydimethylsiloxane-divinylbenzene fibre (Supelco™) (Zhang and Pawliszyn, 1993; Borg-Karlson and Mozuraitis, 1996). The volatiles were collected from three individual plants before and after adding the beetles. The flowers were enclosed separately in small glass vials, of ~10 ml, the fibre was placed at a distance of 5 mm above the flower and the odours were sampled during 24 h. The samples obtained were analysed using the equipment and the GC-MS parameters specified below. These samples were used to characterize emissions from intact and infested flowers.

For the GC-SCR experiments, volatile compounds released by host plant materials were collected from freshly cut material of strawberry and raspberry (foliage and buds) using dynamic headspace techniques. The plant material was confined in an entrainment chamber (1500 ml) and a carrier gas (N_2 flow measured at the outlet: 50–60 ml/min) was blown through the chamber. The volatile compounds released by the plant sample were carried by the gas through two parallel glass tubes (6.6 × 0.5 cm i.d.) containing a solid trap, Porapak Q (80/100 mesh, Supelco), where the compounds were adsorbed and pre-concentrated. The adsorbed volatiles were eluted [with *n*-hexane (>99%), ethyl acetate (absolute) or a mixture of both (1:1)] and used as test samples in the experiments. Before use, the Porapak Q was rinsed with dichloromethane and *n*-hexane, and activated overnight in a heating chamber at 180°C while perfused with N_2. These samples were used to test the RNs for sensitivity to host odours.

Other test samples

In addition to the host headspace mixtures, the neurons were tested with volatile constituents in extracts, headspace samples and essential oils of several plant materials (Table 1). For details of extraction of other sample mixtures used in this study, reference is made to Bichão *et al.* (2003), Stranden *et al.* (2003a) and Wibe and Mustaparta (1996). Standard samples

Table 1 Samples used to test single olfactory receptor neurons (RNs) in *A. rubi* via the gas chromatograph

Test samples	RN types				
	I	II	III	IV	V
Headspace					
Chrysanthemum sp.[a]	1	1	1	–	–
Maritime pine (*Pinus pinaster*), Aiton	–	3	3	2	–
Orange (*Citrus* sp.)[b]	1	–	–	1	1
Raspberry (*Rubus idaeus*)	2	2	2	2	2
Strawberry (*Fragaria* × *ananassa* var. Korona)	15	3	7	5	4
Spruce sawdust (*Picea abies* L.)[c]	1	–	–	–	–
Wild briar (*Rosa dumalis* Bechst.)[b]	1	–	–	–	–
Essential oils and fractions					
Cubebe pepper (*Piper cubeba* L.) containing (–)-β-caryophyllene and (–)-germacrene D[d]	11	2	1	–	–
Cinnamon oil (*Cinnamomum sp.*)[NMD]	–	1	1	–	–
Clove bud oil (*Syzygium aromaticum* L.)[NMD]	–	–	–	2	–
Lavender oil (*Lavandula angustifolia* Mill.)[e]	–	–	–	4	1
Lemon oil (*Citrus medica*, L)[NMD]	–	–	–	2	2
Orange oil (fractions 23–30) (*Citrus* sp.)[f]	4	–	–	–	–
Orange oil (fraction 60) (*Citrus* sp.)[f]	4	–	–	–	–
Orange oil (fractions 115–117) (*Citrus* sp.)[f]	4	–	–	–	–
Germacrene D, thermal degradation products (230°C)[f]	1	–	–	–	–
Germacrene D, UV degradation products (1 h)[f]	4	–	–	–	–
Germacrene D, UV degradation products (2 h)[f]	1	–	–	–	–
(+)-Germacrene D fraction [90% (+) and 10%(–)][g]	21	–	–	–	–
(–)-Germacrene D fraction [90%(–) and 10% (+)][g]	1	–	–	–	–
Ylang-ylang (*Cananga odorata*, Hook)[h]	12	5	4	3	–
Ylang-ylang (*Cananga odorata*, Hook)[i]	11	–	–	–	–

Numbers indicate the total of times each sample was tested on each olfactory receptor neuron (RN) type (I–IV). Sources: [a]Ulland, Norwegian University of Science and Technology, Trondheim, Norway; [b]Stranden *et al.* (2003); [c]Wibe and Mustaparta (1996); [d]Stranden *et al.* (2002); [e]Rolhoff, Norwegian University of Science and Technology, Trondheim, Norway; [f]Liblikas, Estonian Agriculture University, Tartu, Estonia; KIT, Stockholm, Sweeden; [g]König, University of Hamburg, Germany; [h]Dragoco; [i]Firmenich; [NMD]norsk medisinaldepot AS.

of chemical compounds (synthetic and authentic) were used to re-test the neurons and confirm identification of the active compounds. The source and purity of the chemical standards included in the experiments are presented in Table 2.

Reference samples of (–)- and (+)-germacrene D enantiomers, containing 90% of the main enantiomer and 10% of the opposite enantiomer [optical purity 79% *ee.* for the (+)-sample and 76% *ee.* for the (–)-sample; for details see Stranden *et al.*, 2002], were kindly provided by Prof. Dr W.A. König (University of Hamburg, Germany). The samples containing acid catalysed, photochemical and thermal induced rearrangement products of germacrene D were prepared by Dr Ilme Liblikas (Estonian Agriculture University, Tartu, Estonia and KTH, Stockholm, Sweden) according to the procedures described by Bülow and König (2000).

Gas chromatography linked to single cell recordings

Each weevil was fixed and immobilized in dental wax in a Plexiglas holder made to fit the insect. The antennae were exposed on a layer of wax and secured with tungsten hooks. Nerve impulses from single RNs on the antenna club were recorded using tungsten microelectrodes sharpened to a tip <0.3 μm (Mustaparta *et al.*, 1979). The tip of the recording electrode was inserted at the base of the sensilla, which are located in three bands on the antennal club. The reference electrode was inserted ventrally in the head. Most recordings were obtained from the two distal bands where the density of sensilla is higher.

Each RN was first screened for sensitivity to volatiles in the different test samples and in fresh plant materials. These tests were performed by puffing filtered air through glass cartridges containing either a filter paper with the test sample or fresh plant material. Neurons responding to any of these stimuli were classified as plant odour RNs and further examined by stimulation via the gas chromatograph (GC). A sample of 0.8–1 μl of the solution with the plant volatiles was injected into the GC-column through a cold on-column injector. Helium (99.9%) served as the carrier gas. A split at the end of the GC-column led half of the effluent to the GC-detector (Flame Ionization Detector, FID) and half to a clean airflow (300 ml/min) that blew over the insect antenna. This resulted in simultaneously recorded gas chromatograms and single cell responses to the separated compounds (Wadhams, 1982; Wibe and Mustaparta, 1996). The nerve impulse signal was amplified (×1800) and a spike integrator (Syntech, NL, Hilversum, The Netherlands) measured frequency. Spike activity was recorded in parallel with the computer programs Electro Antenno Detection (version 2.3, Syntech NL,) and Spike 2 (Cambridge Electronic Design Limited, Cambridge, UK).

The GC was equipped with two parallel columns allowing testing the neurons for the same mixture, with two different separation patterns. The types of columns used in this study were: one polar (J&W DBwax; 30 m; 0.25 mm i.d.; 0.25 μm film thickness) and one chiral [heptakis-(6-O-*t*-butyldimethylsilyl-2, 3-di-O-methyl)-β-cyclodextrin (50% in OV1701, 25m, i.d. 0.25mm)] (Schmidt *et al.*, 1998 König *et al.*, 1999;). The separation of the mixtures in the polar column used as a reference was obtained using the following temperature program: initial temperature of 80°C held isothermal for 2 min, then a

Table 2 Chemical standards and reference samples used to test single olfactory receptor neurons (RNs) in *A. rubi* via the gas chromatograph

Chemical standards (purity)	I	II	III	IV	V
Monoterpene hydrocarbons					
β-Myrcene[a]	–	–	–	11	–
Z- and *E*-β-Ocimene[b]	–	–	–	11	–
Limonene[c]	–	–	–	5	1
3,7-Dimethyl-1,6-octadiene[a]	–	–	–	5	1
Oxygenated monoterpenes					
Neral	–	–	–	3	3
Geranial	–	–	–	3	3
Myrtenal	–	–	–	1	1
Camphor[d] (98%)	1	1	3	–	–
Citronellol [32%(–) and 68% (+)][a]	–	–	–	5	1
3,7-Dimethyl-1-octanol	–	–	–	5	1
Geraniol	–	–	–	5	1
Racemic linalool[b] (97%)	1	1	3	5	1
Myrcenol[a]	–	–	–	5	1
(+)-*E*-Verbenol	1	1	3	–	–
iso-Borneol	2	1	3	1	1
Sesquiterpene hydrocarbons					
α-Bulnesene[e] (84%)	1	–	–	–	–
Cadinenes (from Scots pine)[a]	3	–	–	–	–
δ-Cadinene mix (from conifers)[a]	–	–	–	–	–
(–)-β-Caryophyllene[b] (99%)	1	12	11	–	–
α-Copaene (mainly (–)-enantiomer)[a]	3	–	–	–	–
(–)-α-Cubebene[a]	3	–	–	–	–
β-Cubebene[a]	3	–	–	–	–
(+)-Cyclosativene[b] (>99%)	1	–	–	–	–
β-Elemene[a]	3	–	–	–	–
(*E,E*)-α-Farnesene[a]	–	–	–	11	–
α-Guajene[e] (94.3%)	1	–	–	–	–
α-Humulene = α-Caryophyllene[e] (>95%)	1	–	–	–	–
(+)-Longicyclene[b] (97%)	1	–	–	–	–
(+)-Longifolene[b] (>99%)	1	–	–	–	–
(+)-Longipinene[b] (>99%)	1	–	–	–	–
α-Muurolene[e] (95,8%)	1	–	–	–	–
γ-Muurolene[e] (54,5%)	1	–	–	–	–
Muurolenes (distillation fraction from Scots pine)[a]	3	–	–	–	–
γ-Muurolene[a]	3	–	–	–	–
α-Muurolene[a]	3	–	–		–
Aromatic esters					
Methyl salicylate[f] (99%)	5	1	7	–	–
Ethyl benzoate[g] (99%)	5	1	7	–	–
Methyl benzoate[g] (99%)	5	1	7	–	–
Aliphatic compounds					
(2*E*)-Hexenal[f]	–	–	–	1	–
Butyl butyrate[h]	–	–	–	1	–
Butyl hexanoate[h]	–	–	–	1	–
Ethyl-2-methyl butyrate[h]	–	–	–	1	–
(2*E*)-Hexenyl acetate[h]	–	–	–	1	–
(3*Z*)-Hexenyl acetate[h]				1	
(3*Z*)-Hexenyl hexanoate[h]	–	–	–	1	–
2-Methylhexyl butanoate[h]	–	–	–	1	–
3-Methylbutyl hexanoate[h]	–	–	–	1	–
sec-Propyl butanoate[h]	–	–	–	1	–
Methyl jasmonate[g]	4	–	4	–	–

Numbers indicate the total of times each sample was tested on each olfactory receptor neuron (RN) type (I–V). Sources: [a]Borg-Karlson, Royal Institute of Technology RIT, Stockholm, Sweden; [b]Fluka; [c]Barata, University of Evora, Evora, Portugal; [d]Kebo; [e]Hartlieb and Remboldt (1996); [f]Merck; [g]Lancaster; [h]Liblikas, Estonian Agriculture University, Tartu, Estonia.

temperature increase rate of 6°C/min to 180°C, followed by a further increase rate of 15°C/min to 220°C. In several experiments the temperature program was altered in order to obtain optimal separation in certain areas. For better separation of the monoterpenes, the initial temperature was lowered to 50°C, and for complex mixtures like the orange headspace, the initial increase rate used was lowered to 2°C/min. Separations with the chiral column were performed by holding the temperature constant at 125°C.

For each cell type, protocols of stimulation sequences were established for the investigation of structure activity relationships. These were also designed to compare the functionally identified neuron types with those of other weevils and moths (Wibe *et al.*, 1997; Røstelien *et al.*, 2000b; Bichão *et al.*, 2003; Stranden *et al.*, 2003a,b).

Analysis of the recordings was carried out using the Spike 2 program. In this program construction of templates is based on the recorded data, and sorting of spikes is made by template matching. All counts were checked manually. In the experiments showing responses to several of the components by more than one RN, the selectivity of each neuron was resolved on the basis of different spike amplitudes and waveforms. Spontaneous activity rate of each neuron (expressed by minimum and maximum) was determined by random counts during 1 s intervals.

Chemical analysis

The chemical analyses were carried out by GC-MS, using the same type of GC-columns described previously for GC-SCR. Mass spectra were obtained with a Finningan SSQ 7000 spectrometer connected to a Varian 3400 gas chromatograph. The GC was equipped with a split/splitless injector (splitless mode 30 s; injector temperature 200°C; carrier gas helium). The MS source was kept at 150°C, and mass spectra were obtained at 70 eV, with a mass range 30–300 mass units. Temperature programs were adjusted to reproduce the separation obtained in the GC-SCR experiments.

Results

In this study we present the results from five olfactory receptor neuron (RN) types (Figure 1) that respond specifically to induced compounds identified in the volatiles of infested flowers. The volatiles emitted by the healthy uninfested flowers were minute amounts of benzaldehyde, germacrene D and methyl salicylate (Figure 2). After 24 h of infestation the emission of flower volatiles had increased and was dominated by *E*-β-ocimene, germacrene D, (*E,E*)-α-farnesene, and methyl salicylate. Other sesquiterpenes such as α-muurolene, δ-cadinene, β-caryophyllene and β-bourbonene were also present together with linalool and benzylalcohol. After only 2 h of infestation large amounts of terpenes were produced. The volatile profile was consistent among plants.

The results are based on recordings from single RNs located in sensilla within the two distal bands of the antennal club of male and female *A. rubi*, representing the data of 175 GC-SCR experiments. Recordings from the single neurons, lasting for periods of 40 min to several hours, allowed up to 23 GC-SCR experiments to be performed from each neuron. The responses of the neurons appeared consistently at the same retention time when stimulated with the same or different mixtures containing the active components. In all experiments reported here responses were recorded as excitation, i.e. increased spike firing activity, which in general followed the concentration profile expressed by the GC-peak of the active components. In some cases the neurons showed a slow decay of the response,

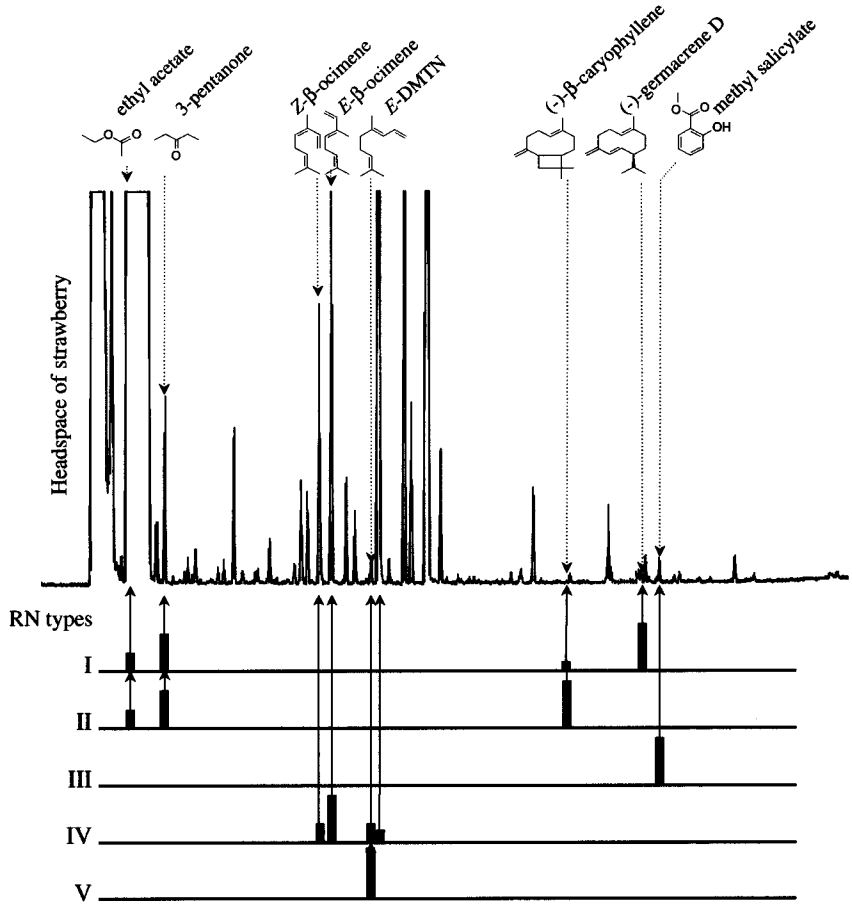
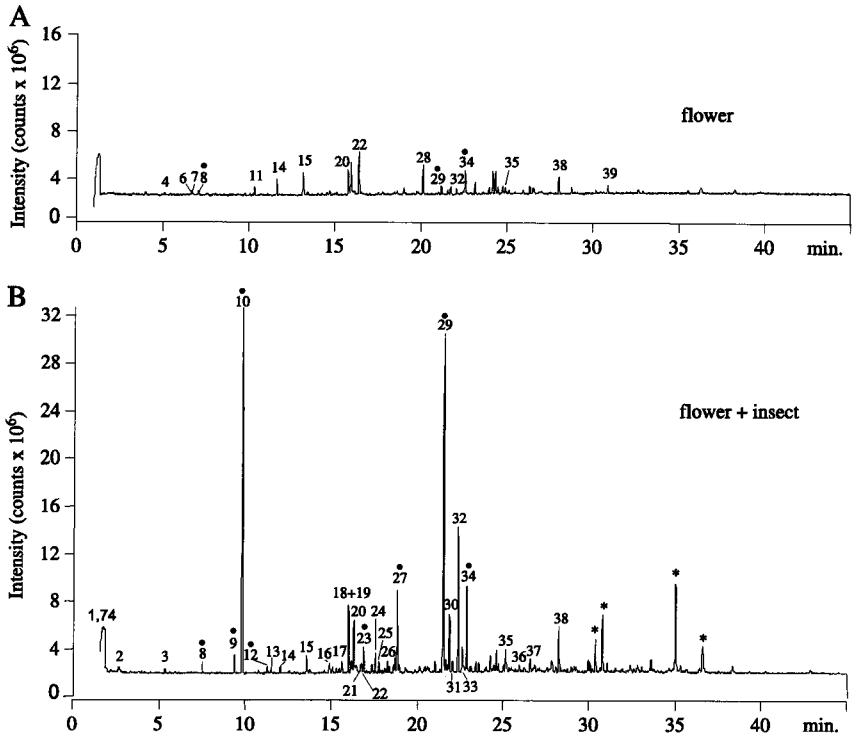


Figure 1 Overview of the responses of the five olfactory receptor neuron (RN) types identified in *A. rubi*. Top: gas chromatogram of the headspace volatiles from cut materials of strawberry (*F.* × *ananassa* var. Korona). Structures of the active components are shown. Bottom: vertical bars indicate the selective responses of the RN types (I–V). Within each RN type bar height indicates the relative response strength.



• Compounds with electrophysiological activity

Peak #	Compound	Flower	Flower+ insect
1	Acetone		x
2	2-Butenal**		x
3	1-Butene-2-one**		x
4	α-Pinene	x	
5	2-Methyl furane**		x
6	Hexanal	x	
7	3-Carene	x	
8	Myrcene	x	x
9	Z-β-Ocimene		x
10	E-β-Ocimene		x
11	Octanal	x	
12	E-DMNT		x
13	(3Z)-Hexenyl acetate		x
14	Sulcatone	x	x
15	Nonanal	x	x
16	Alloocimene**		x
17	Linalool oxide (furane type)		x
18	2-Pinen-10-ol**		x
19	Myrtenal**		x
20	Decanal	x	x
21	Alcanfor**		x
22	Benzaldehyde	x	x
23	Bourbonene		x
24	Linalool		x
25	Cinerone**		x
26	β-Ylangene		x
27	β-Caryophyllene		x
28	β-Farnesene	x	
29	Germacrene D	x	x
30	α-Muurolene		x
31	Elemene		x
32	(E,E)-α-Farnesene	x	x
33	δ-Cadinene		x
34	Methyl salicylate	x	x
35	Benzylalcohol	x	x
36	2-Phenylethanol		x
37	β-Ionone		x
38	Anisaldehyde	x	x
39	Benzoic acid	x	

* oxygenated sesquiterpenes,

** identified by MS-library reference spectra

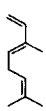
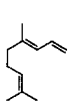
Figure 2 Gas chromatograms of the volatiles released from flowers on potted strawberry plants (*F.* × *ananassa* var. Korona) collected by solid-phase microextraction (SPME). Clear differences are shown both in the composition and in the relative amounts of the various compounds emitted from intact flowers **(A)** and from the flowers with weevils feeding **(B)**. Numbers indicate the identified structures listed below.

which outlasted the GC-peak. All neurons were tested with the sample of the host strawberry (*F.* ×*ananassa*), which contained >90 compounds detected by the GC flame ionization detector. In addition, mixtures of volatiles from many other plants were tested. In all cases the responses to the most active components were confirmed by re-testing with standard mixtures of synthetic or authentic materials. In this investigation, a total of 29 RNs were classified into five types, according to the

compound that elicited the best response (Table 3). The RNs responded selectively to a few compounds (in the range of 3–12), one or two of which had a markedly better effect (primary odorant). All neuron types were found in both males and females. No particular distribution of the different RN types was found on the two antennal bands.

In some recordings two types of RNs appeared together, indicating co-location in the same sensillum. For example, in some recordings of type III RNs, tuned to the aromatic ester methyl salicylate, additional activity of one neuron of type I, or type II, appeared [tuned to the sesquiterpenes (−)-germacrene D and (−)-β-caryophyllene, respectively]

Table 3. Summary of the olfactory receptor neurone (RN) types in *Anthonomus rubi*, classified according to specificity of response to odours. The primary odorant (eliciting best response) and secondary odorants are indicated. Number of neurones identified (n) in a number of GC-SCR experiments (e) is indicated. Total number of times the response to each active compound was observed (r), is given with indication of the stimulation method (GC, gas chromatograph equipped with polar column; Ch, gas chromatograph equipped with chiral column; D, direct stimulation). Numbers in superscript indicate methods used for identification of the compounds (No number means use of comparison of mass spectra and retention times, in polar or chiral column, and re-testing on the neurones with reference samples; [1] Comparison of mass spectra and retention times on a polar column; [2] Comparison of mass spectra)

RN types	Primary odorant		Secondary odorants	
I	**Germacrene D**	r=52 (GC)	(+)-Germacrene D	r=16(Ch)+35(D)
n=10	**(-)-Germacrene D**	r=33(Ch)+40(D)	(-)-β-Caryophyllene	r=33(GC)
e=100			α-Humulene [1]	r=27(GC)
			(-)-β-Bourbonene [2]	r= 6 (GC)
			3-Pentanone [1]	r=16(GC)
			3-Hexanone [1]	r= 5 (GC)
			Ethyl acetate	r=18(GC)
II	**(-)-β-Caryophyllene**	r=28 (GC)	α-Humulene [1]	r=5 (GC)
n=3			3-Pentanone [1]	r=8 (GC)
e=31			3-Hexanone [1]	r=2 (GC)
			Ethyl acetate	r=6 (GC)
III	**Methyl salicylate**	r=13 (GC)	Methyl benzoate	r=8 (GC)
n=5			Ethyl benzoate	r=8 (GC)
e=24				
IV	***E*-β-Ocimene**	r=39 (GC)	*Z*-β-Ocimene	r=22(GC)
n=8			β-Myrcene	r=23(GC)
e=42			Dihydromyrcene	r=4 (GC)
			E-DMNT [1]	r=3 (GC)
			Limonene	r=17(GC)
			Terpinene [1]	r=4 (GC)
			β-Phellandrene	r=5 (GC)
			Geraniol	r=5 (GC)
			Citronellol	r=2 (GC)
			Neral	r=1 (GC)
			Geranial	r=4 (GC)
V	***E*-DMNT [1]**	r=6 (GC)	Neral	r=5 (GC)
n=3			Geranial	r=4 (GC)
e=25			Geraniol	r=6 (GC)
			Citronellol	r=2 (GC)

(Figure 3A). The type IV neurons tuned to the monoterpene *E*-β-ocimene were sometimes recorded simultaneously with type V tuned to the monoterpene *E*-DMNT (Figure 3A). In these recordings of co-localized RNs, the relative size of the spike amplitudes of each type were consistent, i.e. the type II RNs showed larger spikes than type III RN and the type IV RN larger spikes than type V (Figure 3B).

Type I

One neuron type responded best to (−)-germacrene D (n = 10) and to another early eluting compound in the host plant mixture. Examples of GC-SCR recordings from this type of RNs are given in Figure 4. In a tentative identification, the second active component was indicated to be 3-pentanone. Weaker responses to (−)-β-caryophyllene and α-humulene were also recorded when mixtures containing high amounts of these compounds were injected into the GC (Figures 4C and 5A), as well as weak responses to 3-hexanone. When tested with an UV degraded germacrene D sample, a secondary response to the rearrangement product β-bourbonene appeared (structure shown in Figure 5D). An additional response appeared when the neurons were stimulated with ylang-ylang essential oil (Firmenich) and the *Chrysanthemum* headspace (Figure 4C,D). This response was elicited by a sesquiterpene that could not be identified. A sustained response during the elution of the solvent ethyl acetate was also observed in all recordings (Figure 4A).

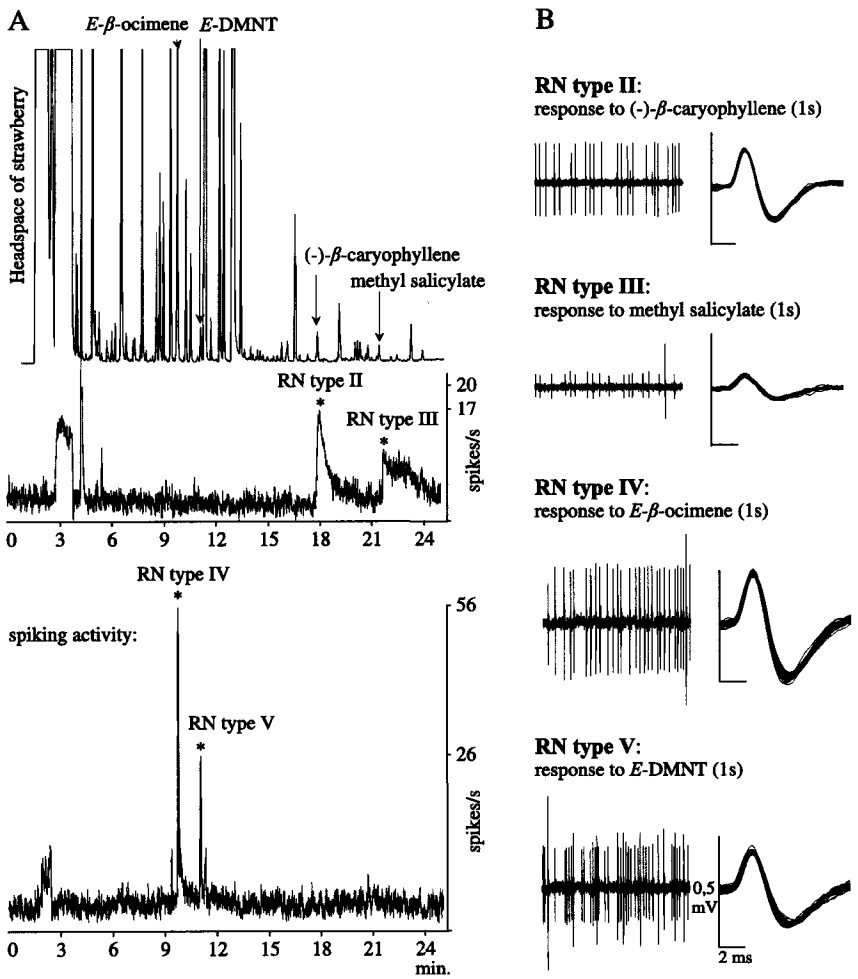


Figure 3 Responses of olfactory receptor neurons (RNs) located in the same sensillum of the weevil *A. rubi*. **(A)** Top: gas chromatogram of the headspace volatiles of strawberry (*F.* × *ananassa* var. Korona) and recording from a RN of type II responding primarily to (−)-β-caryophyllene and a type III RN responding to methyl salicylate (middle). The responses to the early-eluted compounds, ethyl acetate, 3-pentanone and 3-hexanone, originated from the RN type II. The recordings from the two other co-located RNs in the lower trace show responses to *E*-β-ocimene and to *E*-DMNT by a type IV and a type V RN, respectively. **(B)** Spike activity during 1 s after the start of each of the responses (marked with * in A). The differences in spike amplitude and waveform allowed each response to be ascribed to the RN types II–V. The spikes of each response are presented as overlays.

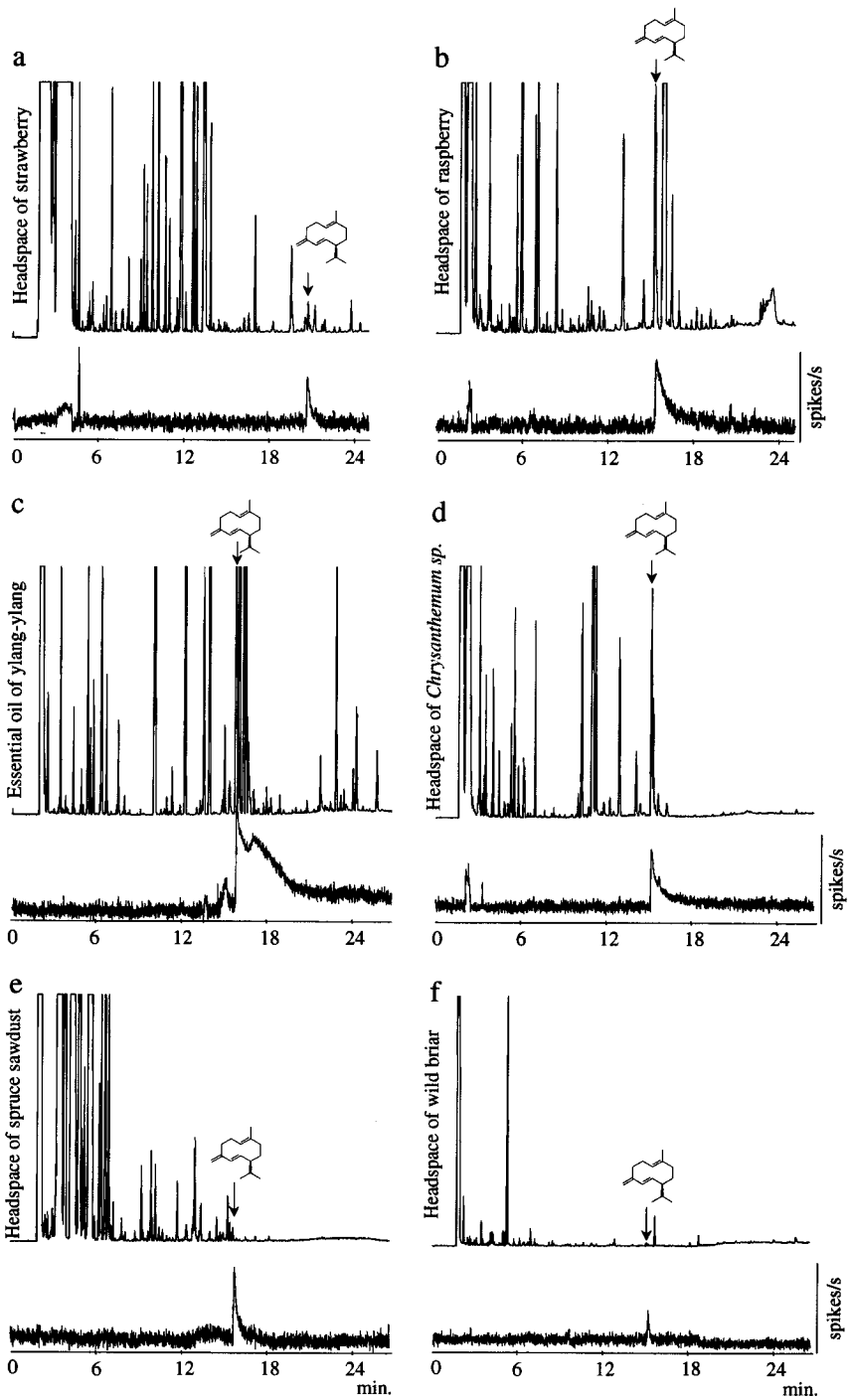


Figure 4 Selective responses of an olfactory receptor neuron (RN) type I in *A. rubi* to germacrene D and related compounds in the headspace of the host plant strawberry (*F.* × *ananassa* var. Korona) **(a)**, raspberry (*R. idaeus*) **(b)** and of four non-host plant materials **(c–d)**, separated by a polar GC column. Simultaneously recorded activity of a type I RN is shown below each chromatogram. Selective responses are shown to the primary odorant germacrene D, both at a high (c) and at a low concentration (f). Weaker responses are visible to secondary odorants in four of the recordings (a–d).

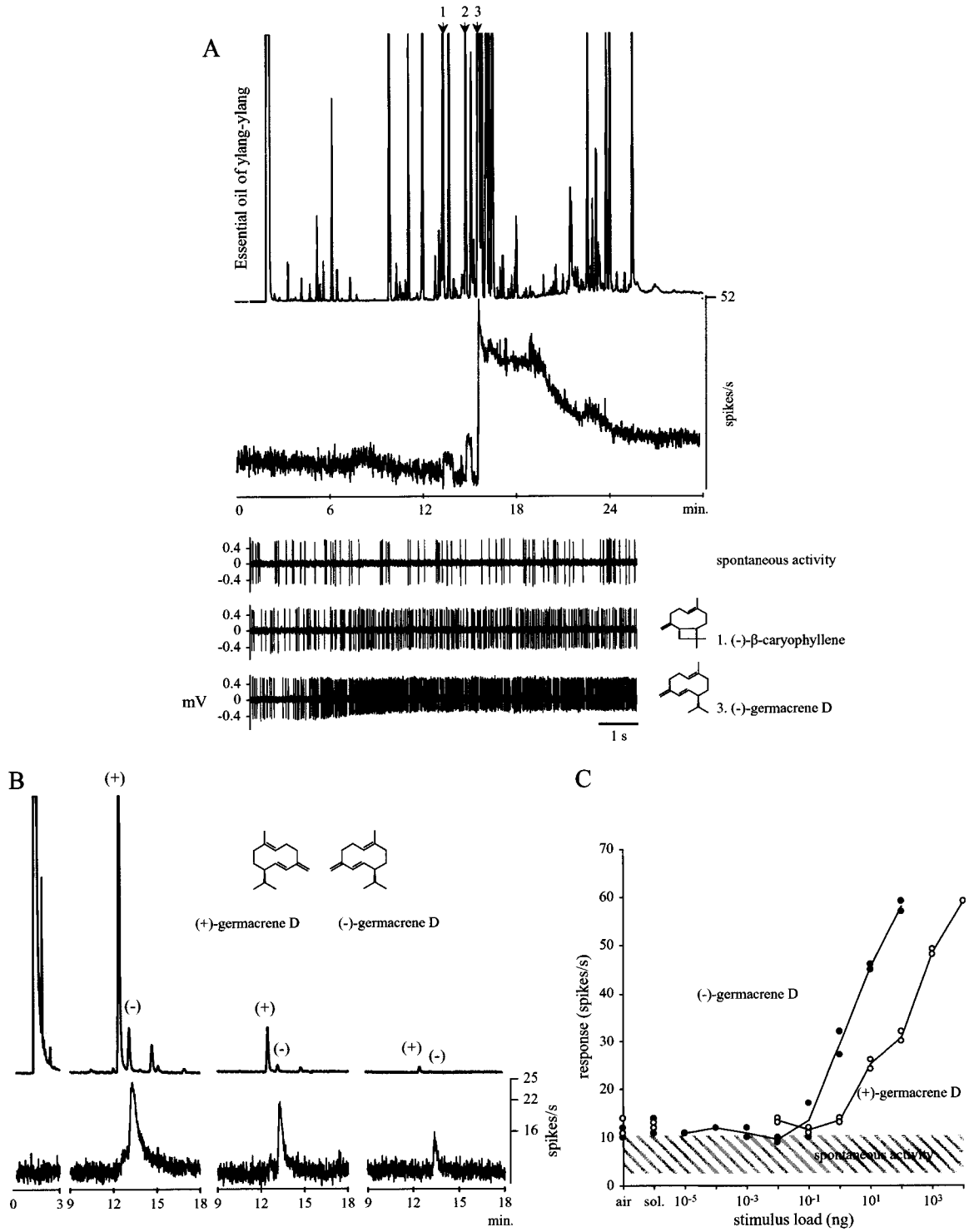


Figure 5 Sensitivity and molecular receptive range of the olfactory receptor neuron (RN) type I in *A. rubi*. **(A)** Gas chromatogram of an essential oil of ylang-ylang separated in the polar column, and simultaneously recorded responses of a type I RN. The primary response are shown to the sesquiterpene (−)-germacrene D (3) and the weak secondary responses to the related compounds (−)-β-caryophyllene (1) and α-humulene (2). **(B)** Decadic dilutions of a reference sample of (+)-germacrene D [containing 90% (+)- and 10% (−)-germacrene D] separated in the chiral column and simultaneously recorded activity of a type I RN, showing stronger response to the (−)-germacrene D than to the (+)-enantiomer (detection limit of the chiral column = 0.5 ng). **(C)** Dose–response curves

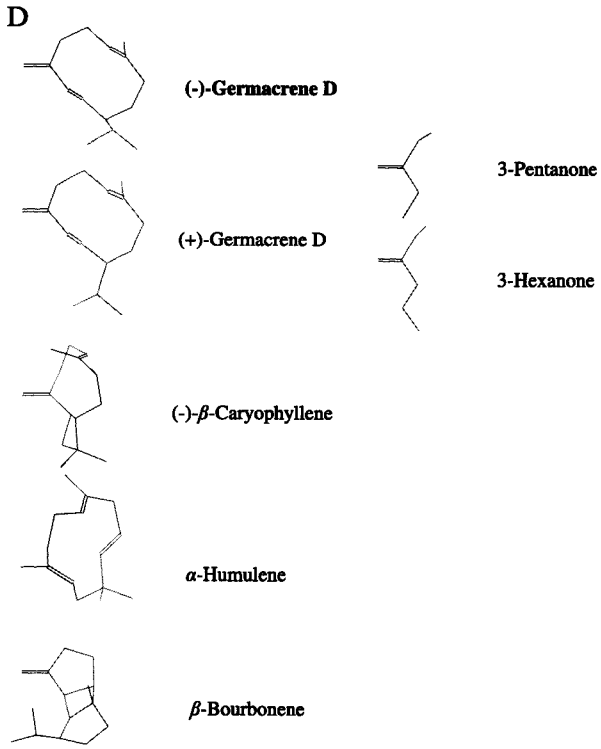


Figure 5 Continued.

We tested the neurons for sensitivity to the (−)- and (+)-germacrene D enantiomers by stimulating with reference samples separated in the chiral GC-column. Dose-dependent responses to both enantiomers were demonstrated for seven of these neurons. This was accomplished by separation in the chiral column of decadic dilutions of the (+)-germacrene D standard sample (Figure 5B). The neurons showed a higher sensitivity to (−)-germacrene D than to the (+)-germacrene D. This was confirmed by the dose–response curves obtained by direct stimulation of the neurons with decade step concentrations of the (+)- and (−)-germacrene D standard samples. Figure 5C presents an example of duplicated dose–response curves obtained for one neuron. The (+)-germacrene D curve is shifted ~1 log unit to the right of the curve for the (−)-enantiomer, indicating at least a 10-fold higher sensitivity for (−)-germacrene D. In one of the recordings activity of one type III RN was recorded simultaneously (details are given below). No overlap of the molecular receptive ranges of the two RN types was found.

Type II

Three neurons were specifically tuned to (−)-β-caryophyllene and responded also to the component identified as 3-pentanone. Secondary responses of these neurons to α-humulene and 3-hexanone were also recorded (Figure 6), as well as a sustained high firing rate during the elution of the solvent ethyl acetate. Dose–response studies performed by injecting decadic dilutions of a reference sample of (−)-β-caryophyllene into the GC-column revealed a high sensitivity of this neuron type, which responded to amounts below the minimum GC-detector threshold [in the range of picograms (data not shown)]. Injection of several mixtures of plant volatiles and standards into the chiral column of the GC showed that none of the samples used contained detectable amounts of (+)-β-caryophyllene [the detection limit of the chiral column is 0.5 ng as calculated by Stranden *et al.* (2002)]. Whether the neurons also respond to the (+)-enantiomer remains to be tested.

Interestingly, the RNs of type I and II, tuned to different sesquiterpenes [(−)-germacrene D and (−)-β-caryophyllene, respectively], showed overlapping molecular receptive ranges for several secondary compounds, as well as response of type I to (−)-β-caryophyllene. The common secondary odorants were α-humulene, 3-pentanone and 3-hexanone, as well as the solvent ethyl acetate (Figure 6 and Table 3). Another similarity was the pronounced phasic pattern of the responses to 3-pentanone and 3-hexanone, as compared with the phasic-tonic, long-lasting responses to the primary odorants in both neuron types.

Type III

The RN type III (n = 5) responded best to methyl salicylate, as shown by separation of the volatiles of the host plant, strawberry (Figure 3A). Three of these neurons appeared in the same recordings as RN type II, whereas the fourth type III neuron appeared together with a type I neuron and the fifth was recorded alone. Secondary responses (for three of the neurons) to methyl benzoate and ethyl benzoate were demostrated by injection of a standard mixture, as shown in Figure 7A (the remaining two neurons were not tested for these compounds). Figure 7B shows dose–response curves obtained by injection of three dilutions of another mixture containing the active compounds. It is worth noting that the spontaneous activity of these neurons was low (0–2 spikes/s) and that the response to the primary odorant showed a tonic, long-lasting firing rate even at relatively low concentrations. The high sensitivity and tonic response

obtained by direct stimulation with samples of (−)- and (+)-germacrene D (both containing 10% of the opposite enantiomer), showing 10–100 times stronger effect of the (−)-germacrene D. The curves show the mean of two responses obtained by repeated stimulation with the same cartridge. **(D)** Structures of the compounds that constitute the molecular receptive range of the RN type I.

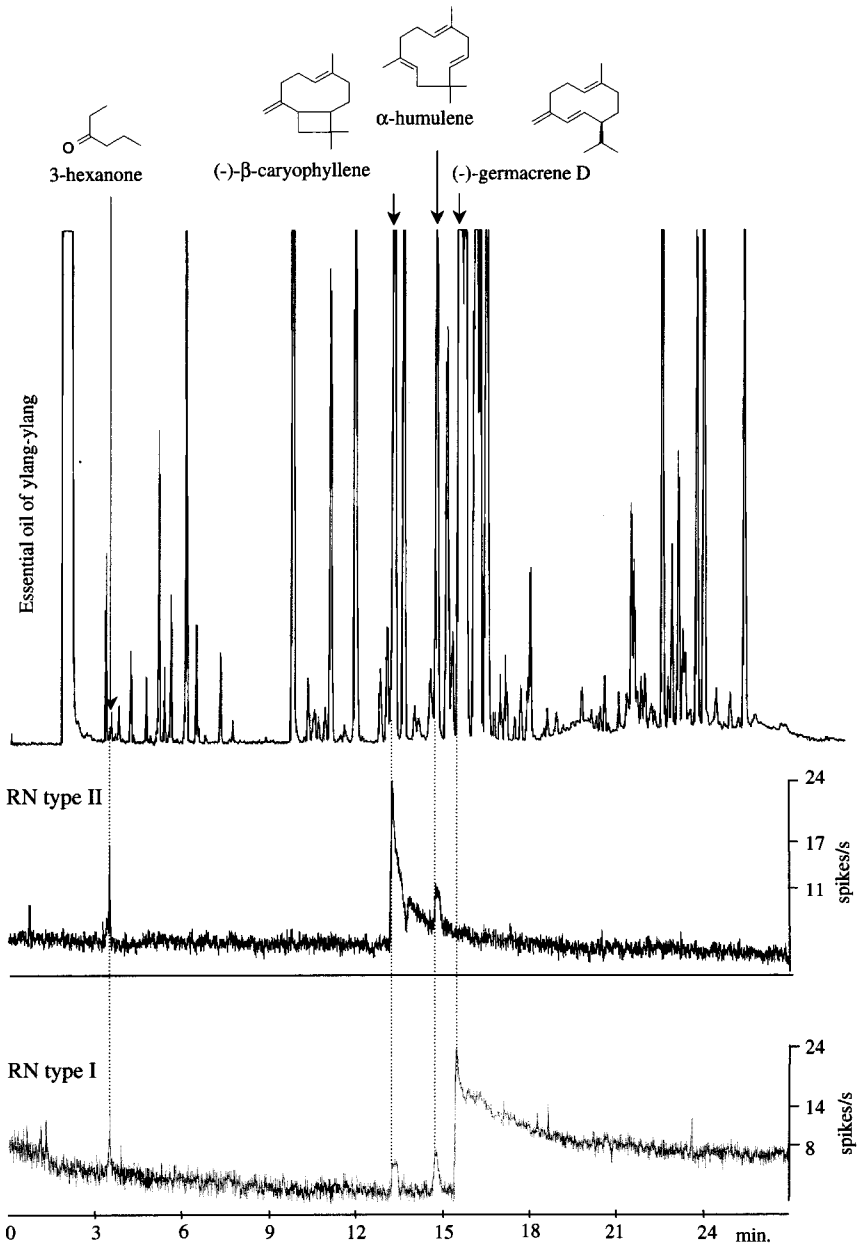


Figure 6 Gas chromatogram of an essential oil of ylang-ylang separated in the polar column and simultaneously recorded activity of a type II olfactory receptor neuron (RN) showing the strongest response to (−)-β-caryophyllene and weaker responses to α-humulene and 3-hexanone. The overlap between the molecular receptive ranges of the RN types I and II is shown by presenting (in grey) a recording from a type I RN (obtained in the same individual).

property of this RN type to the primary odorant was further shown by injecting a small amount of methyl salicylate. A 0.5 ng amount elicited a response that outlasted the GC-peak (65 s), although the highest firing frequency was only 13 spikes/s. In contrast, the responses to methyl benzoate and ethyl benzoate were relatively brief, lasting from 10 to 20 s. In the recording showing activity of only one type III neuron, the firing rate was much higher, i.e. maximum firing rate of the response was 31 spikes/s, and the spontaneous activity varied from 0 to 6 spikes/s.

Type IV

The neurons showing highest sensitivity to the acyclic monoterpene *E*-β-ocimene (n = 8) were classified as type IV. Altogether, 11 compounds elicited secondary responses in these RNs. Examples of GC-SCR recordings from this type

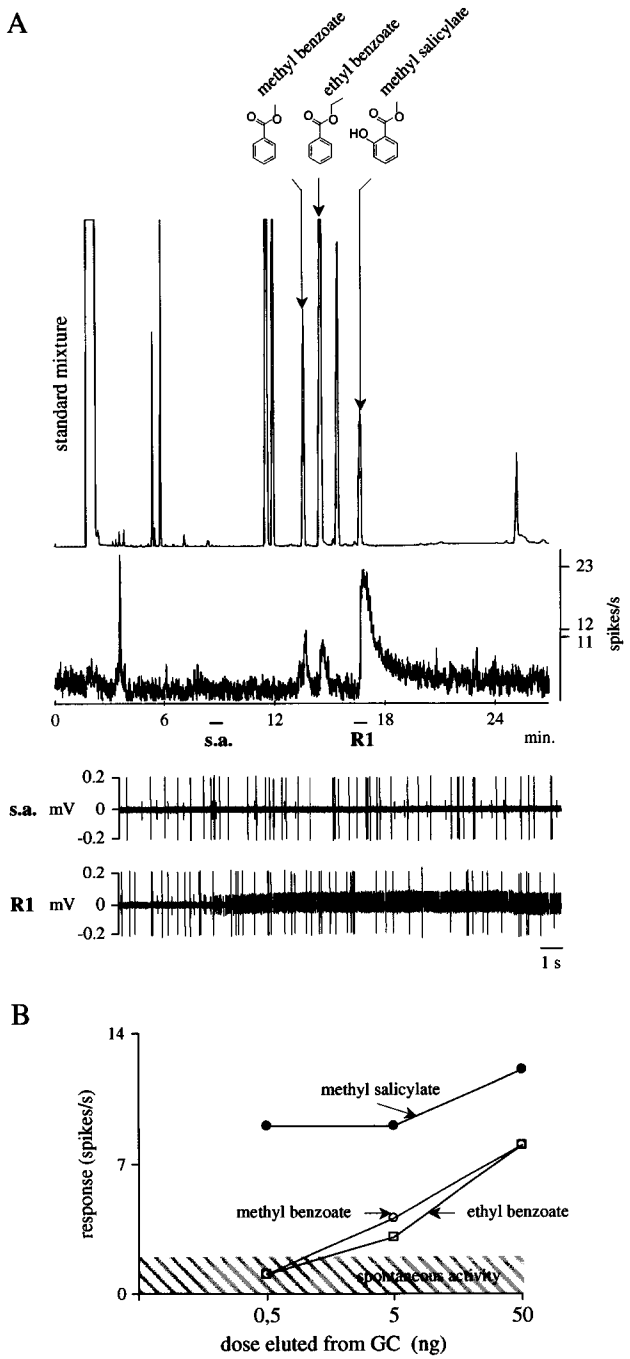


Figure 7 **(A)** Gas chromatogram of a standard mixture separated in the polar column and simultaneous recorded activity of a type III receptor neuron (RN) showing response to three components. Top: the molecular structures are shown of the three active compounds. Bottom: spike activity of the neuron during 20 s (black bars) of spontaneous activity (s.a.) and during the response to methyl salicylate (R1). **(B)** Dose–response curves for the three odorants activating the type III RN, obtained by stimulation via the polar column showing a stronger effect of the methyl salicylate (primary odorant) than of methyl benzoate and ethyl benzoate (secondary odorants).

of neurons are shown in Figure 8, with secondary responses to *Z*-β-ocimene and *E*-DMNT. In addition, dihydromyrcene, citronellol, geraniol, geranial and neral present in some mixtures (lavender and lemon essential oils) elicited weak responses. Responses to the cyclic monoterpenes limonene, γ-terpinene and β-phellandrene appeared when the neurons were stimulated with mixtures containing very large amounts (μg) of these compounds. Verification of the identity of all active compounds on this RN type was performed by stimulating the neurons with standard samples (except γ-terpinene). Dose–response experiments, performed by injecting standard decadic dilutions into the GC-column, indicated the specificity of these neurons by *E*-β-ocimene having a stronger effect than the geometrical isomer *Z*-β-ocimene and β-myrcene (Figure 8B). Three of these neurons appeared together with a RN of type V. Interestingly, the two neuron types had overlapping molecular receptive ranges, i.e. both responded to the odorants *E*-DMNT, citronellol, geraniol, geranial and neral (Figure 9B). Neuron type IV responded weakly to *E*-DMNT, the primary odorant for neuron type V. The synchronous responses of the two neurons to the stereoisomers neral and geranial are shown in Figure 9B.

Type V

The RNs of type V responded strongest to the monoterpene *E*-DMNT ($n = 3$) when tested with the host volatiles (Figure 8A). The compound *E*-DMNT eluted simultaneously with (3*E*)-hexenyl acetate in the strawberry headspace mixture, but re-testing with a (3*E*)-hexenyl acetate standard showed no response. Furthermore, testing the neurons for the alternative host raspberry that contained a clean *E*-DMNT peak confirmed the response. Strong responses to neral and weaker responses to geranial were detected when the neurons were stimulated with lemon essential oil and confirmed by injection of decadic dilutions of reference samples (results for one dilution shown in Figure 9A). Additional weaker responses to geraniol and citronellol were recorded when the neurons were stimulated with lavender essential oil (Figure 9B).

Discussion

The present results on functional types of olfactory receptor neurons (RNs) in the strawberry weevil *A. rubi* elucidate peripheral mechanisms involved in the detection of plant odour information. The results add to the growing knowledge about functional types of RNs in general, and in a comparative perspective, i.e. in relation to RNs of closely (weevils) and distantly (moths) related insect species that have adapted to different host plants. The use of GC-SCR has allowed each single neuron to be tested for hundreds of naturally produced volatiles in various host and non-host plants. The consistent and selective responses to one or a few primary and secondary odorants indicate that these compounds are biologically significant to the strawberry weevil. The narrowly

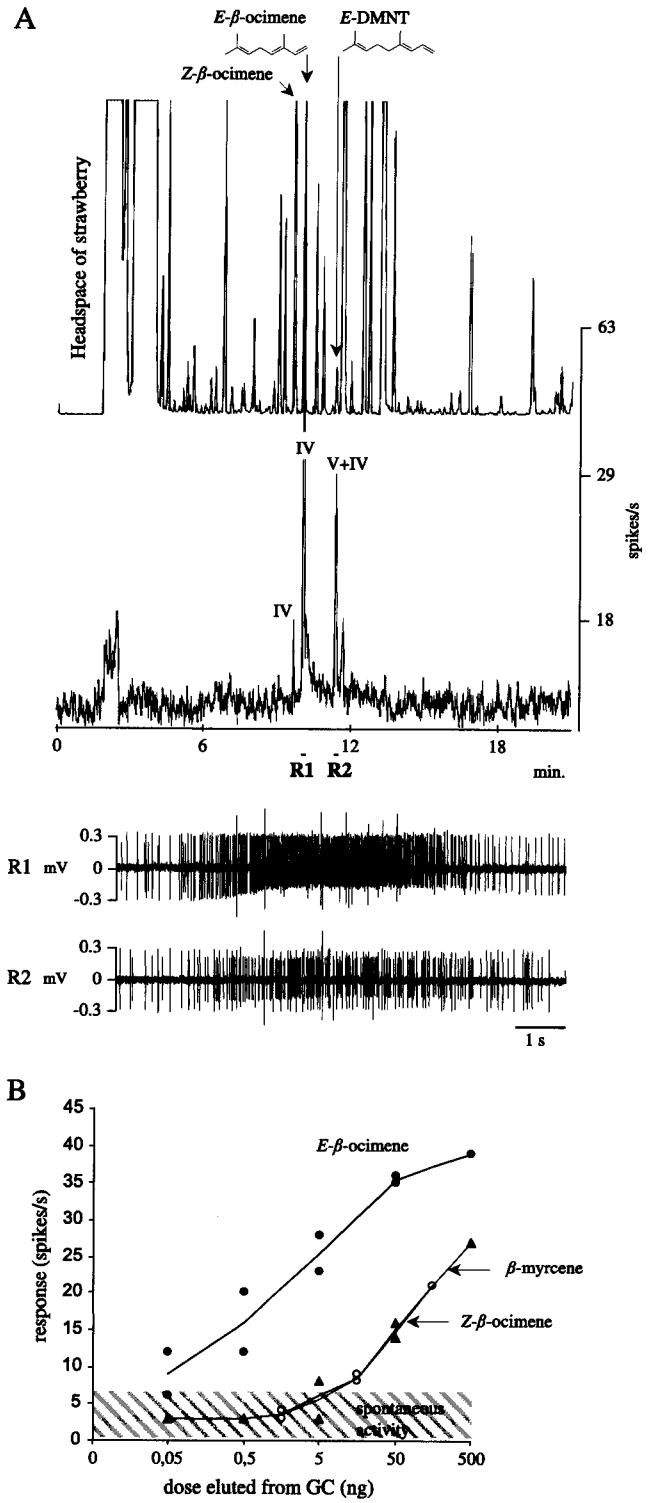


Figure 8 **(A)** Gas chromatogram of the headspace volatiles of strawberry (*F.* × *ananassa* var. Korona) and simultaneously recorded activity of a type IV and a type V olfactory receptor neurons (RN). Molecular structures of the active compounds are presented above. Spike activity elicited by the two most potent odorants, *E*-β-ocimene and *E*-DMNT, is shown during 10 s of the responses (indicated by black bars) R1 and R2. **(B)** Dose–response curves for the RN type IV stimulated via the GC with three odorants, *Z*-β-ocimene, *E*-β-ocimene and β-myrcene, showing 10–100 times stronger effect of the primary odorant, *E*-β-ocimene.

tuned RNs presented here could be classified into five distinct types, the neurons of each type having similar molecular receptive range and similar ranking of the primary and secondary odorants. The uniform response pattern within one neuron type correlates well with the current information from molecular biology studies showing that subsets of olfactory RNs express one type of receptor protein in insects and vertebrates (Störtkuhl and Kettler, 2001; Wetzel *et al.*, 2001; Keller and Vosshall, 2003; Hallem *et al.*, 2004). The primary odorants (eliciting the strongest response) and the majority of the secondary odorants (eliciting weaker responses) were shown to be inducible compounds, i.e. produced in higher amounts by the strawberry inflorescences on which weevils were feeding (Figure 2).

Structurally similar molecules have a higher probability to bind to the same odorant-binding and receptor protein. This explains the molecular similarity among the primary and secondary odorants activating one RN type, and also the pattern of overlap of molecular receptive ranges of different types. The principle that RNs which respond to the same chemical group show some overlap, whereas no overlap is found between RN types that respond to different chemical groups, e.g. monoterpenes versus sesquiterpenes, is shown in the present as well as in previous studies of weevils, other beetles and moth species (Wibe and Mustaparta, 1996; Wibe *et al.*, 1997; Stensmyr *et al.*, 2001; Barata *et al.*, 2002; Bichão *et al.*, 2003). In this study, the monoterpene molecules activating RN type IV and V are similar. For RN type IV, the primary and secondary odorants *E*-β-ocimene and *Z*-β-ocimene are geometrical isomers, and one other secondary odorant, β-myrcene, differs only in the position of one of the double bonds (Figure 9). The weaker effect of two other secondary compounds, dihydromyrcene and *E*-DMNT, is probably due to higher flexibility and the effect of one additional carbon atom, thus allowing less interactions with the receptor compared to the primary odorant. The RN of type V responded strongest to *E*-DMNT, which has a chain one carbon longer than ocimene and myrcene. The aldehyde group in neral, the second most active compound for type V RNs, might have an electrophilic similarity with the methylene group of *E*-DMNT, which may explain the relatively high secondary effect. In addition, the acyclic monoterpenes geranial and geraniol elicited weak responses in both type IV and V, whereas the cyclic monoterpenes activated only the type IV RNs.

In the case of the RN type I, the most effective molecules are (−)-germacrene D and (+)-germacrene D. The direction of the isopropyl group on the 10-carbon ring might explain the different effect of the two enantiomers. The much lower

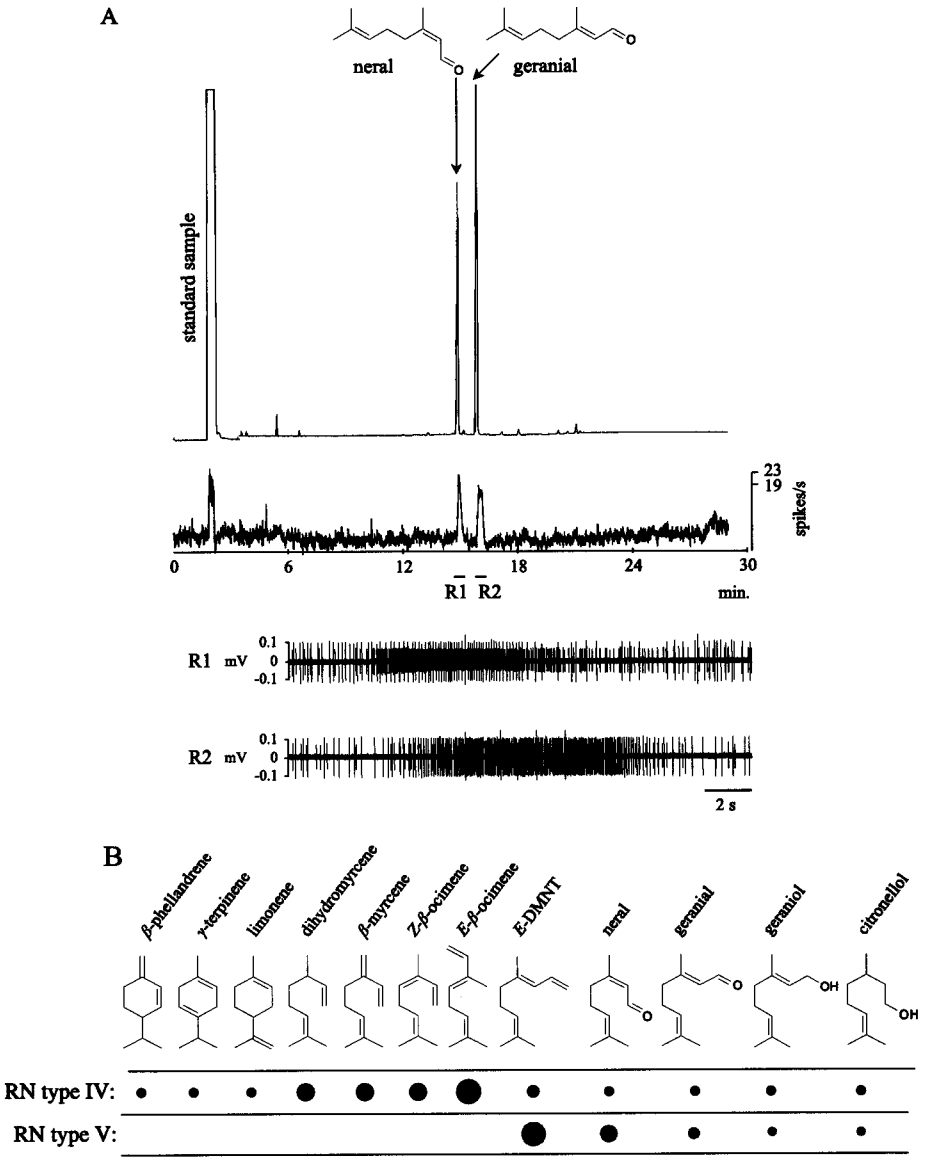


Figure 9 **(A)** Gas chromatogram of a standard mixture of neral and geranial, and simultaneously recorded responses of the two olfactory receptor neuron (RN) types (IV and V) located in the same sensillum. Top: molecular structures of the active compounds are shown. Bottom: spike activity during the 20 s periods R1 and R2 (indicated by black bars), of the type IV (large amplitude spikes) and the type V (small amplitude spikes) RNs, shows responses of both neurons to the two odorants. **(B)** The molecular receptive ranges of the two RN types are shown with indication of the relative response strength within each RN type (size of the circles).

effect of (−)-β-caryophyllene and α-humulene, which are molecules with similar hydrophobicity as germacrene D, can be explained by the different ring systems, and the absence of the isopropyl group (Figure 5D). In the case of the type II RNs tuned to (−)-β-caryophyllene, the receptor seems to be slightly different, so that the germacrene D does not fit well into the receptor. More surprising was the response of these two sesquiterpene RN types to the structurally unrelated molecules 3-pentanone, 3-hexanone and ethyl acetate. However, these are small molecules that might easily fit into the receptor pocket. The similarities in electrophilic properties between the carbonyl group in the ketones and the methylene group in the sesquiterpene molecule may be important features. Possibly the interaction is only with a part of the receptor site, which correlates with the responses to the small molecules showing a short and phasic pattern compared with the long-lasting responses elicited by the sesquiterpenes (cf. Figure 3A). Alternatively, the responses to the small molecules could have originated from a different neuron recorded simultaneously. However, since the spike

amplitude and waveform of the responses were similar, and the responses always occurred in the numerous recordings of these neurons, they seem to originate from the same neuron.

Consistent co-location of functional RN types in one sensillum has been found in this study as well as in previous studies of weevils and moths, and other insects (Blight *et al.*, 1995; Røstelien *et al.*, 2000b; De Bruyne *et al.*, 2001; Stranden *et al.*, 2003b), but the functional importance of co-location is not known. It is interesting to note, however, that in *A. rubi*, the five functional types occurred in three different pairs, two of them showing no overlap of the molecular receptive range between the two elements of the pair: I and III [(−)-germacrene D and methyl salicylate], and II and III [(−)-β-caryophyllene and methyl salicylate], i.e. type III occurred together with both types I and II. In the other neuron pair, IV and V, both RNs were tuned to related terpenes [*E*-β-ocimene and *E*-DMNT] and showed an extensive overlap of the molecular receptive range (cf. Figure 9).

From an evolutionary point of view it is interesting to compare the specificity of RNs responding to plant volatiles in closely and distantly related insect species. In this study we used experimental protocols designed to allow comparison with studies of weevils and heliothine moths carried out in our laboratory. Particularly interesting is the RN type tuned to (−)-germacrene D, which constitutes the largest number of the five RN types in *A. rubi* and is also the most abundant RN in the heliothine moths (Røstelien *et al.*, 2000a; Stranden *et al.*, 2002, 2003a). In both insect groups these RNs show similar enantioselectivity to the dominant enantiomer in higher plants (−)-germacrene D, which has a 10- to 100-fold stronger effect than (+)-germacrene D (Figure 5C). However, the secondary odorants were different. In the weevil *A. rubi*, the neurons responded secondarily to (−)-β-caryophyllene, α-humulene and β-bourbonene, whereas in the heliothine moths the germacrene D RN type responds to ylangenes and copaenes (Stranden *et al.*, 2003a). Thus, the RN type evolved for the detection of (−)-germacrene D in *A. rubi* is different in specificity from the type evolved in the three heliothine moths, indicating that they have evolved independently in the adaptation to their different host plants. An RN type corresponding to type IV tuned to *E*-β-ocimene in *A. rubi* has also been identified in heliothine moths (Røstelien *et al.*, 2000b; Stranden *et al.*, 2003b). In this case, a striking similarity occurred in the secondary responses to *Z*-β-ocimene and β-myrcene in both insect groups. However, in *A. rubi* these RNs responded weakly to several cyclic monoterpenes and oxygenated monoterpenes, which was not the case in the heliothine moths. In two other weevil species, *Pissodes notatus* and *Hylobius abietis*, which live on conifers, no RNs have been found that were tuned primarily to germacrene D or to *E*-β-ocimene (Wibe *et al.*, 1997; Bichão *et al.*, 2003), although the headspaces of the host plants contain large quantities of these compounds. In the cabbage moth *Mamestra brassicae*, one RN type is tuned to methyl salicylate (S. Ulland, personal communication) and responds secondarily to methyl benzoate, similar to the RN type III in *A. rubi*. But again differences emerge when considering the secondary responses, *A. rubi* showing an additional response to ethyl benzoate.

Receptor neurons specified for the same odorants as RN types II and III, are also found in other species of beetles. A RN type with primary odorant β-caryophyllene is found in *P. notatus* (Bichão *et al.*, 2003) and RN types with primary odorant methyl salicylate are found in the weevil *Ceuthoynchus assimilis* (Blight *et al.*, 1995) using brassica plants as hosts, and in the fruit chafer *Pachnoda marginata* (Stensmyr *et al.*, 2001). However, in these cases different protocols were used and therefore the comparison concerning secondary responses is limited. Whereas the mentioned studies are based on GC-SCR, screening with synthetic odorants has also revealed RN types for the same primary odorants in the cotton weevil *Anthonomus grandis*, e.g. RN types tuned to *E*-β-ocimene and to β-caryophyllene (Dickens, 1990). Using yet another method, GC-EAG, Kalinová *et al.* (2000) recorded responses to caryophyllene in the apple blossom weevil *Anthonomus pomorum*. Altogether these comparisons show that species of weevils and moths have evolved RNs tuned to the same primary odorants, but these RNs show differences in the secondary odorants. We do not know the significance of secondary odorants, but one can speculate that these differences reflect independent (convergent) evolution in the adaptation to different host plants. Alternatively, these RNs may have evolved from a common ancestral RN type that has later undergone chance mutations.

In recent years the advances in plant chemistry have shown that the blends of volatiles emitted from plants contain a large number of compounds most of which are common to many plants. The insects are equipped with RNs that detect these odorants. Thus, they probably use for host location a large number of compounds that are common to many plant species. This is in contrast to the idea that the plant odour RNs are specified for a few compounds characteristic of the particular host plants. Another principle, emerging from the results of this and other studies, is that plant odour RN types in insects are specified for compounds emitted in particular conditions by the host plant (Stranden *et al.*, 2003b). In this study, all the primary odorants identified for the five RN types were collected in higher amounts in the headspace of strawberry flowers on which *A. rubi* had been feeding than on intact flowers (cf. Figure 2). Whether these compounds contribute to enhanced or reduced attraction to the host plant is not known. In the case of attraction, the ability to recognize a plant on which conspecifics are feeding could have an adaptive role by favouring aggregation and therefore encounters of potential mates. Since these weevils lay only one egg per flower bud, it is also possible that induced volatiles might be a cue for the females to avoid attacked flower buds.

The five functional types of RNs in the antennal sensilla of *A. rubi* presented here are narrowly tuned and all respond to compounds which are common secondary metabolites present in many host and non-host plants. However, these compounds are inducible, i.e. emitted systemically in altered ratios when the weevils are feeding on the host plants, suggesting that *A. rubi* uses plant-produced compounds as information about the presence of conspecifics on the host plant. In addition, the results show that RNs tuned to the same compounds exist in unrelated species that utilize host plants belonging to different plant groups, suggesting a general importance of these compounds for plant herbivore interactions.

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CHEMOECOLOGY

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*

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Summary. An important question in insect-plant interactions is which of the numerous plant compounds contribute to the perception of odour qualities in herbivorous insects and are likely to be used as cues in host-searching behaviour. In order to identify which plant-produced volatiles the strawberry blossom weevil *Anthonomus rubi* detects, we have used electrophysiological recordings from single olfactory neurones linked to gas chromatography and mass spectrometry. We here present 15 receptor neurone types specialised for naturally produced compounds present in the host and non-host plants and two types for two aggregation pheromone components. The active compounds were terpenoids, aromatic and aliphatic esters, alcohols and aldehydes, some of which are induced by feeding activity of the weevils. The neurones were characterised by a strong response to one or two primary odorants and weaker responses to a few others having similar chemical structure. With one exception, the molecular receptive range of each neurone type was within one chemical group. Enantiomers of linalool separated on a chiral column activated two neurone types with different enantioselectivity. Inhibition by linalool of another neurone type, excited by α-pinene, indicated an additional mechanism for coding the information about this compound. Altogether, detection of 54 compounds by olfactory receptor neurones is shown, of which 40 have been chemically identified in this study. Thus *A. rubi* has the ability to detect a large number of odorants that may be used in host selection behaviour.

Key words. GC-SCR – GC-MS – Primary odorants – Induced plant compounds – Linalool enantiomers
Taxa. Anthonomus rubi – Coleoptera – Curculionidae

Introduction

The olfactory system in insects, as well as other organisms, is exposed to myriads of volatiles in the environment. This raises the question, which compounds are detected by their olfactory receptor neurones (RNs). Herbivorous insects are exposed to plant emitted volatiles that not only vary with the species, but also with individuals and the condition of each plant in response to biotic or abiotic factors (Paré & Tumlinson 1999; Gouinguené & Turlings 2002). In recent years several studies have focused on induction of compounds by insects, *i.e.* compounds systemically released by the plants during larval feeding. Compounds in the saliva of the larvae induce the release of certain volatiles, not only in larger quantities but also in modified composition of the constituents (Alborn *et al.* 1997; Mattiacci *et al.* 1995). The large number of compounds of different chemical groups and the variability of the blends emitted by individual plants has made it complicated to identify the mixtures of odorants used by the various insect species in the location of adequate feeding and oviposition sites. To meet the challenge of detecting the large diversity of molecules, the olfactory system operates with a large number of receptor proteins, each type expressed in subsets of neurones, as shown in vertebrates and in invertebrates, *e.g.* the fruit fly *Drosophila melanogaster* (reviewed by Mombaerts 1999; Hallem *et al.* 2004). Whether each RN responds to a few or to a large number of odour molecules, might limit or extend the discrimination ability of the organism. Obviously, in both cases a large number of odorants can be detected, which makes the identification of odorants for which the RNs have evolved, a challenging task.

In insects, the functional properties of RNs, including the specificity, have been studied by the use of gas chromatography linked to electrophysiological recordings from single cells (GC-SCR) (Wadhams 1982). This method allows testing plant produced odorants, separated by gas chromatography, directly on each RN. The active compounds are chemically identified by further analyses with gas chromatography linked to mass spectrometry (GC-MS). Knowledge of the specificity of single RNs is interesting both for the understanding of the olfactory coding mechanisms as well as for the interpretation of which odorants may be most important and should be tested in behavioural bioassays. The GC-SCR method has been used for studying

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Table I Samples used to test single olfactory receptor neurones in *Anthonomus rubi* via the gas chromatograph

Plant material Source	Sampling method (# samples)	Source
Strawberry (*Fragaria x ananassa* Duchesne)	headspace (2)	
Raspberry (*Rubus idaeus* L.)	headspace (3)	
Maritime pine (*Pinus pinaster* Ait.)	headspace (3)	Bichão *et al.* 2003
Umbrella pine (*Pinus pinea* L.)	headspace	Bichão *et al.* 2003
Eucalyptus (*Eucalyptus globullus* Labyll.)	headspace	Bichão *et al.* 2003
Orange peel (*Citrus* sp.)	headspace (2)	Stranden
Bergamot (*Citrus bergamia* Risso)	essential oil	NMD Norsk Medisinaldepot
Cedar tree (*Juniperus* sp.)	essential oil	Dragoco GRB
Clove (*Syzygium aromaticum* L.)	essential oil	NMD
Clove bud (*Syzygium aromaticum* L.)	essential oil	Aqua oleum
Cinnamon (*Cinnamonum zeylanicum* L.)	essential oil	NMD
Cubebe peper (*Piper cubeba* L.) containing (-)-_-caryophyllene and (-)-germacrene D	essential oil	Stranden *et al.* 2002
Lavender oil (*Lavandula angustifolia* Mill.)	essential oil	Jens Rolhof
Lemon oil (*Citrus medica* L)	essential oil	NMD
Orange (*Citrus* sp.) (fraction 40 % Germacrene D)	essential oil	R.C. Treatt
Ylang-ylang (*Cananga odorata* Hook)	essential oil	Dragoco GRB

plant odour reception in several insects, *e.g.* moths and beetles, providing accurate information about the selectivity of the RNs involved (Blight *et al.* 1995; Wibe *et al.* 1997, 1998; Bichão *et al.* 1997, 2003; Røstelien *et al.* 2000a,b, 2005; Stensmyr *et al.* 2001; Barata *et al.* 2002; Stranden *et al.* 2002, 2003a,b).

The strawberry blossom weevil, *Anthonomus rubi* Herbst, (1795) (Coleoptera, Curculionidae), is an oligophagous species that feeds and reproduces on angiosperms of the Rosaceae family, mainly strawberry (*Fragaria x ananassa* Duchesne) and raspberry (*Rubus idaeus* L.) (Popov 1996a). Adult weevils migrate from overwintering shelters to the strawberry fields early in the spring where they feed on the foliage and start to mate at the onset of bud formation. When flowers open, the weevil feeds also on pollen and petals. After laying a single egg in an unopened bud, the female partially severs the bud pedicel. The larvae develop and pupate inside the withered bud, and emerge in late summer. This weevil constitutes a serious pest on strawberry throughout Europe (Popov 1996b; Cross & Easterbrook 1998). In addition to the basic question about the RN specificity in this weevil, knowledge about plant odours that may influence the behaviour of this pest insect can also be useful for applied purposes.

In the present study we used GC-SCR in order to identify natural plant produced odorants for the RNs of *A. rubi.* We describe and classify 17 RN types, which adding to the five types previously described (Bichão *et al.* 2005), increase the number of identified RN types in *A. rubi* to 22, including two RN types tuned to pheromone components. The RNs are narrowly tuned to a few structurally related compounds and show some overlap of the molecular receptive range. Some of the active odorants are inducible plant compounds *e.g.* linalool, here shown to influence the activity of four different neurone types. Similarities among RNs in *A. rubi* and RNs in other species are discussed.

Materials and methods

Insects

Adult *A. rubi* were collected from an unsprayed strawberry field (Dragvoll, Trondheim, Norway), during the summers (mid May to August) of 2002 and 2003. The main strawberry variety planted was Korona. The insects were kept in the laboratory prior to the recordings for a maximum of one week under ambient photophase and temperature (22-25 °C). Fresh food material, consisting of young and mature leaves, flowers and flower buds, was provided every third day. All males and females used in this study were starved for at least 12 hours before the experiments.

Test samples

The plant volatiles used were constituents of extracts, headspace samples, and essential oils of plant material (Table I), as well as reference samples of compounds (synthetic or authentic, Table II).

Collection of naturally produced plant volatiles

Volatile compounds released by host plant materials were collected from the headspace of strawberry (*Fragaria x ananassa,* var. Korona) and raspberry (*Rubus idaeus*), using entrainment techniques. Several dynamic headspace procedures were tested to select a method yielding as many compounds as possible in detectable amounts and a sufficient volume of sample. The best results were obtained from cut plant materials using N2 as carrier gas. The procedure of extraction was carried out as described in Bichão *et al.* (2003) with minor modifications. In general, N2 was blown through a glass vial (1500 ml) containing cut plant material (leaves and flower buds) and, together with the plant volatiles, passed through two parallel glass tubes (6.6 cm × 0.5 cm i.d.) containing the adsorbent Porapak Q (80/100mesh, Supelco). The average flow measured at the outlet of the tubes was 50-60 ml/min. The sampling period was 24 hours and was carried out at 22-25 °C. The trapped volatiles were eluted with n-hexane (>99 %), ethyl acetate (absolute) or a mix of both (1:1) and used as test samples in the experiments. Before use, the Porapak Q was rinsed and activated

Table II Chemical standards and reference samples used to test single olfactory receptor neurones in *Anthonomus rubi* via the gas chromatograph ([1]components of the mixture designated "green leaf volatiles"; [2]components of the synthesized standard mixture designated "apple esters"; KTH, Royal Institute of Technology, Stockholm, Sweden)

Compounds (purity % GC)	Source
Monoterpene hydrocarbons	
Limonene	Fluka
Z- and *E*-β-Ocimene	Fluka
(+)-α-Pinene (>99 %)	Fluka
(−)-α-Pinene (>99,5 %)	Fluka
Oxygenated monoterpenes	
Camphor (98 %)	Kebo
trans-Grandisol (90 %)	
racemic Linalool (97 %)	Fluka
(*R*)-(−)-Linalool (97 %)	Fluka
(+)-*trans*-Verbenol (83,6 %)	
iso-Borneol	
Aliphatic compounds	
(2*E*)-Hexenal[1,2]	Dragoco GRB
Dodecanal	Avocado
1-Hexanol (98 %)[1]	Aldrich
(2*E*)-Hexen-1-ol[1] (97 %)	Aldrich
(2*Z*)-Hexen-1-ol[1] (95 %)	Aldrich
(3*Z*)-Hexen-1-ol[1] (98 %)	Sigma
1-Heptanol (98 %)[1]	Jansen Chemica
1-Octanol (99 % liquid)[1]	Sigma
racemic 1-Octen-3-ol (97 %)	Aldrich
(*R*)-(−)-1-Octen-3-ol	Fäldt (KTH)
(*S*)-(+)-1-Octen-3-ol	Fäldt (KTH)
3-Octanone[1]	Borg-Karlson (KTH)
Butyl butyrate[2]	Liblikas (KTH)
Ethyl-2-methyl butyrate[2]	Liblikas (KTH)
(2*E*)-Hexenyl acetate[1,2]	Liblikas (KTH)
(3*Z*)-Hexenyl acetate[1]	Dragoco GRB
(3*Z*)-Hexenyl hexanoate[2]	Liblikas (KTH)
2-Methylhexyl butanoate[2]	Liblikas (KTH)
3-Methylbutyl hexanoate[2]	Liblikas (KTH)
sec-Propyl butanoate[2]	Liblikas (KTH)
Methyl jasmonate (98 %)	Lancaster
Aromatic compounds	
Eugenol	
p-Methylanisole (99 %)	Aldrich
m-Methylanisole (99 %)	Aldrich
o-Methylanisole (99 %)	Aldrich
Methyl salicylate (99 %)	Merck
Ethyl benzoate (99 %)	Lancaster
Methyl benzoate (99 %)	Lancaster

by solvent and heat (Bichão *et al.* 2005). The plant materials were collected in May and June from the same field where the insects were collected.

Gas chromatography linked to single cell recording (GC-SCR)

Each insect was fixed to a Plexiglas holder formed to fit the animal and immobilised by dental wax. The antennae were exposed, fastened to the wax layer and secured with tungsten hooks. The tip of a tungsten microelectrode [less than 0.3 µm (Mustaparta *et al.* 1979)] was inserted at the base of olfactory sensilla located in three bands on the antennal club. Nerve impulses from single RNs innervating the sensilla were then recorded. The two distal antennal bands had a denser population of sensilla and, due to the curvature of the antennal club, were more accessible to electrode insertion and exposure to test compounds. During the recordings we could not detect which *sensillum* was penetrated by the electrode because they are partly covered by other hairs.

Each RN was first screened for plant volatile sensitivity by blowing air puffs through glass cartridges containing either filter paper with the test sample or fresh plant material. Neurones responding to any of these stimuli were further examined by stimulation with test samples via the gas chromatograph (GC). For this purpose, a sample (ca. 0.8-1.0 µl) of the hexane solution containing the plant volatiles was injected into the GC-column through an on-column injector. Helium served as the carrier gas. A glass splitter at the end of the GC-column directed half the effluent to the GC-detector (Flame Ionisation Detector, FID) and half to a clean airflow (300 ml/min) that blew across the insect antenna. This resulted in simultaneously recorded gas chromatograms, and single cell responses to the separated compounds (Wadhams 1982; Tømmerås & Mustaparta 1989). The nerve impulse signal was amplified (1800x) and the impulse frequency was measured by a spike integrator and displayed as the number of spikes/time unit. Spike activity was recorded simultaneously with two different software programs: Electro Antenno Detection-EAD (version 2.3, Syntech NL, Hilversum, The Netherlands) and Spike 2 (Cambridge Electronic Design Limited, Cambridge, Great Britain). Analysis of the electrophysiological recordings was done using the Spike 2 program, which constructs spike templates based on the detected nerve impulses. The spikes were compared, sorted and counted using template matching, and the reliability of the counts was verified manually.

The GC was equipped with two columns installed in parallel. The two types of columns used in this study were one polar column (J&W DBwax; 30 m; 0.25 mm i.d.; 0.25 µm film thickness), and one chiral column [octakis-(6-methyl-2,3-di-O-pentyl)-γ-cyclodextrin (80 % in OV1701, 25 m, 0.25 mm i.d.; 0.25 µm film thickness)] (König *et al.* 1990). For separation of the mixtures in the polar column we used mainly a temperature program with an initial temperature of 80 °C, held isothermal for 2 min, with an increase of 6 °C/min to 180, and a further increase rate of 15 °C/min to 220 °C. In some cases this temperature program had to be altered to obtain better separation of specific regions of the chromatogram. For example, the initial temperature was lowered to 50 °C in some experiments and for very complex mixtures (*e.g.* the orange peel headspace) the temperature increase rate was lowered to 2 °C/min. Separations with the chiral column were performed isothermally (80 °C for linalool; 60 and 80 °C for 1-octen-3-ol).

Criteria for classification of functional RN types

The classification of different RN types was based on the following criteria according to previous studies (*e.g.* Wibe and Mustaparta 1996; Wibe *et al.* 1997; Røstelien *et al.* 2000a, b; Barata *et al.* 2002; Bichão *et al.* 2003, 2005; Stranden *et al.* 2002, 2003a, b). First, the recordings of each neurone were highly reproducible *i.e.* when tested repeatedly the neurones showed responses to the same compounds. Non reproducible alterations of the spontaneous activity were not considered as responses to specific compounds. Secondly, a group of neurones was considered one RN type when the neurones composing it showed the same molecular receptive range and ranking of the odorant effectiveness *i.e.* showed best response to the same primary odorant and weaker responses to the same secondary odorants. Each RN type was then designated by the primary odorant and numbering was attributed for simplicity of the writing.

Gas chromatography linked to electroantennogram recordings (GC-EAG)

For the electroantennograms (EAGs) the insects were prepared as described for the GC-SCR. An indifferent glass electrode containing insect Ringer solution was placed in contact with the insect's haemolymph through a hole made at the base of the head. The recording glass electrode containing the same solution was placed in contact with the tip of the antenna and pressed onto it. When the recording baseline was stable

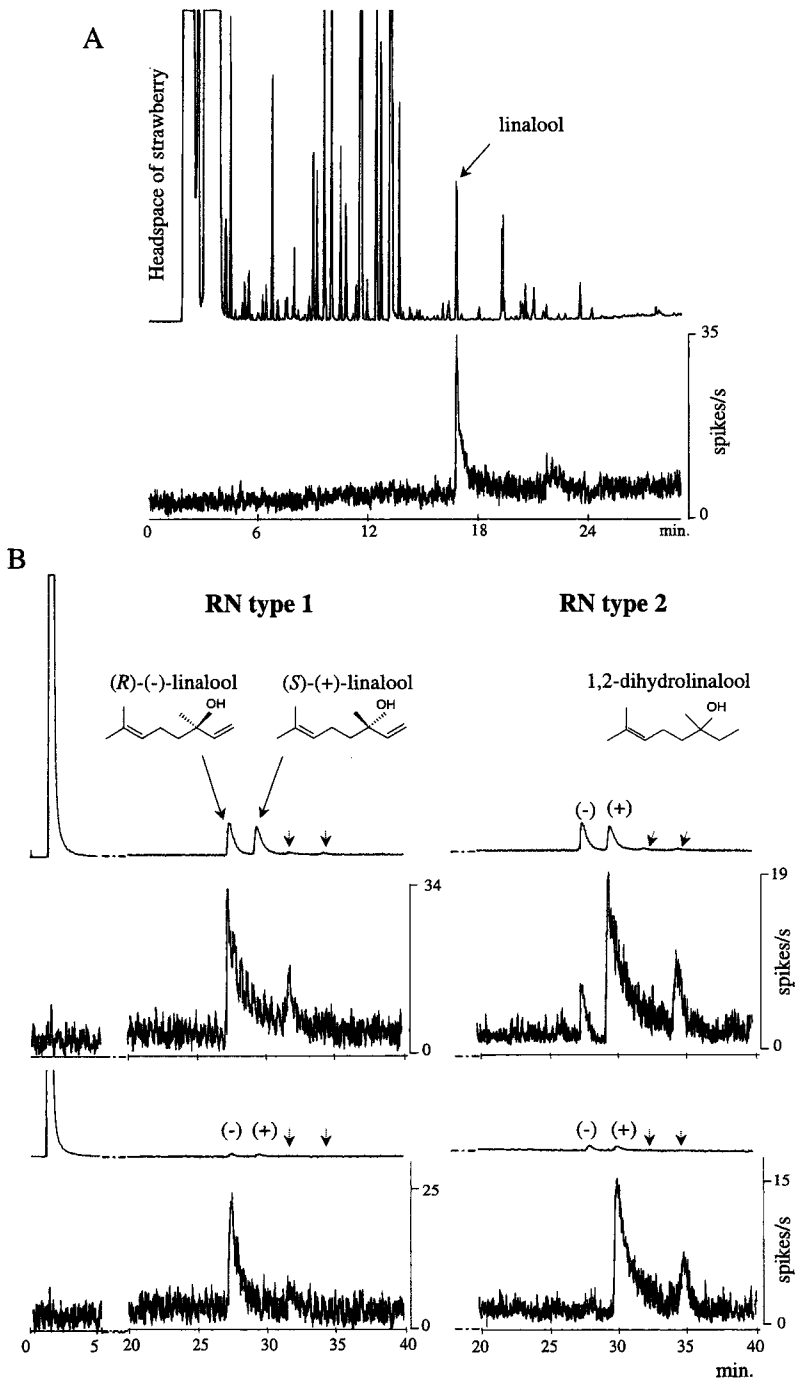


Fig. 1 A) Gas chromatogram of a headspace sample of cut strawberry leaves and flower buds (*Fragaria x ananassa*, var. Korona) (*above*) and simultaneously recorded response to linalool of a type 1 olfactory receptor neurone of the strawberry blossom weevil (*Anthonomus rubi*) (*below*). B) Responses of olfactory receptor neurones of type 1 and 2 to the (–)- and (+)-enantiomers of linalool separated by a chiral GC-column (solid arrows). Type 1 olfactory receptor neurone responded more strongly to (–)-linalool than to (+)-linalool, whereas the type 2 olfactory receptor neurone showed a primary response to (+)-linalool and a secondary response to (–)-linalool. Both neurone types also showed secondary responses to one enantiomer each of 1,2-dihydrolinalool (dashed arrows; elution order of enantiomers not known)

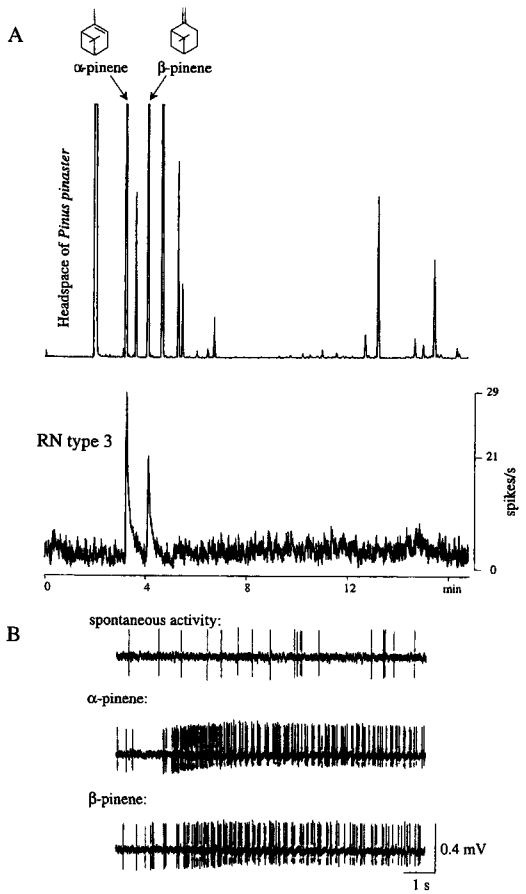


Fig. 2 A) Gas chromatogram of a headspace sample of maritime pine (*Pinus pinaster*) (*above*) and the simultaneously recorded activity of an olfactory receptor neurone of type 3 of *Anthonomus rubi* (*below*). The strongest response to α-pinene and a weaker response to β-pinene are shown. B) Spike activity (10 s each) recorded during spontaneous activity, the response to α-pinene, and during the response to β-pinene

the insects were stimulated directly (via glass cartridges) with several mixtures and those eliciting clear EAGs were selected for injection in the GC.

Gas chromatography linked to mass spectrometry (GC-MS)

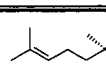
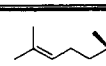
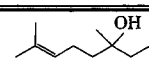
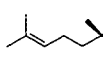
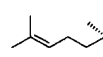
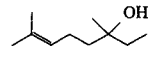
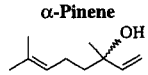
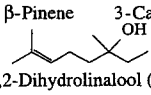
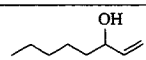
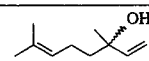
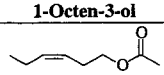
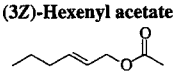
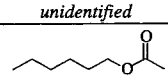
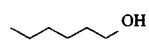
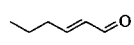
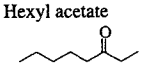
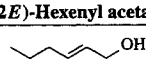
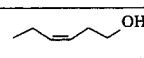
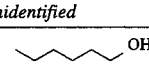
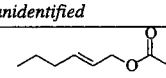
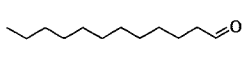
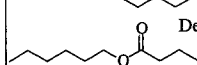
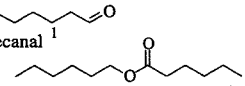
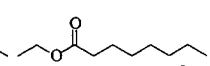
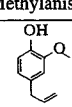
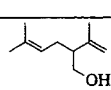
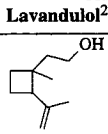
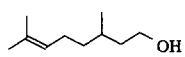
The chemical analyses were carried out by GC-MS, using the same types of GC-columns as described above for GC-SCR [J&W DBwax; 30 m; 0.25 mm i.d.; 0.25 µm film thickness and octakis-(6-methyl-2,3-di-O-pentyl)-γ-cyclodextrin (80 % in OV1701, 25 m, 0.25 mm i.d.; 0.25 µm film thickness)]. A mass-spectrometer Finningan SSQ 7000 instrument was used connected to a Varian 3400 GC. The GC was equipped with a split/splitless injector (splitless mode 30 s; injector temperature 200 °C; carrier gas Helium). The ion source temperature was 150 °C, mass spectra were recorded at 70 eV, and the mass range was 30-300 m/z. Several temperature programs were used to correspond with the separation obtained in the different GC-SCR.

Results

The results are based on recordings from single RNs innervating olfactory sensilla in the two distal bands of the antennal club of male and female *Anthonomus rubi*. Each RN was stimulated with test samples separated by the GC-columns from 1 to 33 times [gc (number of GC runs) = 1-33]. The initial classification was based on the responses to the compounds present in the headspace sample of leaves and flower buds of the host plant strawberry (*Fragaria x ananassa* var. Korona). This sample contained more than 90 constituents detected by the GC-detector (FID). Responses to compounds in the headspace samples and essential oils from other plant species (Table I) contributed to the identification of the molecular receptive range of each RN type. The reproducibility of the results was demonstrated by the consistent responses of each RN at the retention time of the active components present in many test mixtures. Most responses were recorded as excitation with increased firing frequency that followed the concentration profile of the GC-peak, while others had a slow decay that outlasted the GC-peak. Only one RN showed an inhibitory response to two of the tested compounds, in addition to excitatory responses to others. Altogether 30 RNs showed responses to plant odours, whereas 23 RNs did not respond to any of the compounds tested. All the 30 RNs showed selective responses to a few (3-8) compounds, of which one or two (primary odorants) elicited a marked strongest response. The RNs were classified into 17 types according to the primary and secondary odorants. The primary odorant of 11 RN types were chemically identified (Table III). The molecular receptive range of each type was either within the terpenoid, aliphatic or aromatic compounds. Two RN types responded to aggregation pheromone components.

RNs of type 1 and type 2 responding to linalool enantiomers: Four neurones (gc=25) responded selectively to linalool present in the headspace sample of the host (Fig. 1A) and non-host plants, as well as in essential oils and standards. In addition, responses appeared to the small amount of the structurally related compound 1,2-dihydrolinalool that was only present in the synthetic sample of *racemic* linalool. The recordings showed a high sensitivity of the RNs to both linalool and 1,2-dihydrolinalool. The enantioselectivity of these RNs was tested by stimulation via the chiral column that separated the linalool enantiomers. Responses to both enantiomers were obtained but with different relative strengths. One RN, classified as type 1 (gc=14), had its strongest response to (−)-linalool (Fig. 1B). Two RNs, type 2 (gc=11), had their greatest response to (+)-linalool. The enantioselectivity of these three RNs was apparently demonstrated by different responses to the enantiomers of 1,2-dihydrolinalool, which were separated in the chiral column (order of elution of enantiomers not known) (Fig. 1B). The fourth RN was not tested with the separated linalool enantiomers and therefore could not be further classified. The two RNs of type 2 were recorded together with the activity of another RN type (distinguished by spike amplitude) that responded to two compounds in the host plant sample (type 14). These compounds could not be identified, due to the small amounts present in the test samples.

Table III Summary of the olfactory receptor neurone types in *Anthonomus rubi*, classified according to specificity of response to odorants. All responses were excitatory except those marked otherwise. The number of neurones (n) and GC-SCR (gc) is indicated. Numbers in superscript indicate methods used for identification of the compound: No number means comparison of mass spectra, retention times and re-testing the neurones with reference samples;[1] Comparison of mass spectra and retention times;[2] Comparison of mass spectra)

RN type	Primary odorants	Secondary odorants
1 n=1 gc=14	 **(R)-(−)-Linalool**	 (S)-(+)-Linalool  1,2-Dihydrolinalool
2 n=2 gc=11	 **(S)-(+)-Linalool**	 (R)-(−)-Linalool  1,2-Dihydrolinalool
3 n=2 gc=7	 **α-Pinene**	 β-Pinene
4 n=1 gc=11	 **α-Pinene**  *racemic*-**Linalool** (inibition)	 β-Pinene  3-Carene  1,8-Cineole[1]  Camphene  1,2-Dihydrolinalool (inibition)
5 n=2 gc=33	 **1-Octen-3-ol**	? *unidentified*  *racemic*-Linalool
6 n=2 gc=31	 **(3Z)-Hexenyl acetate**  **(2*E*)-Hexenyl acetate**	 Hexyl acetate  1-Hexanol  (2*E*)-Hexenal  3-Octanone ? *unidentified* ? *unidentified*
7 n=2 gc=31	 **(2*E*)-Hexen-1-ol**	 (3Z)-Hexen-1-ol  1-Hexanol  (2*E*)-Hexenyl acetate
8 n=4 gc=14	 **Dodecanal[1]**	 Decanal [1]  Hexyl butyrate[2]  Hexyl hexanoate[2]  Ethyl octanoate[2]
9 n=1 gc=11	 ***p*-Methylanisole**	 *m*-Methylanisole  *o*-Methylanisole
10 n=2 gc=8	? *unidentified*	 Eugenol
11-15	(see text)	
16 n=1 gc=8	 **Lavandulol[2]**	? *unidentified*
17 n=1 gc=2	 **cis-Grandisol[1]**	 Citronellol[1]

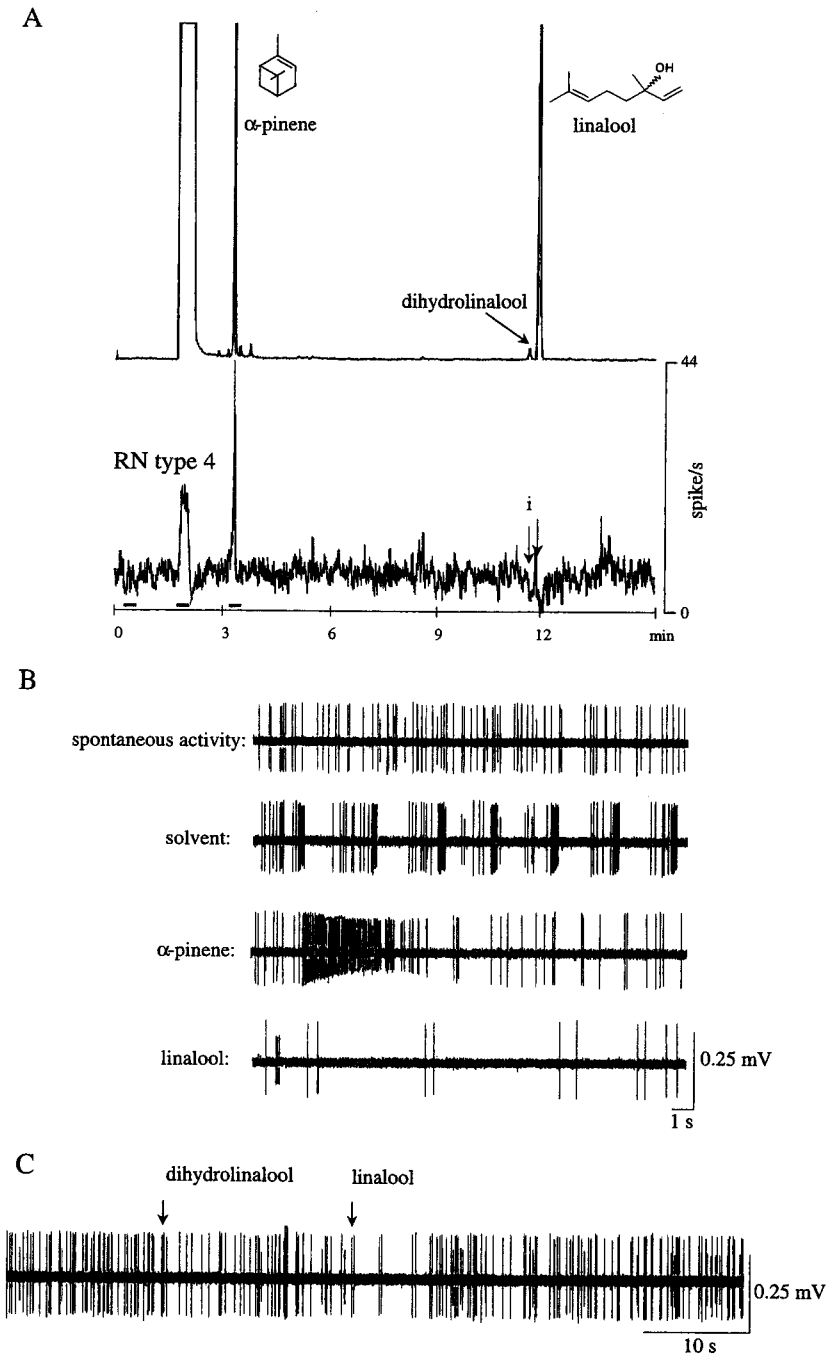


Fig. 3 A) Gas chromatogram of a standard mixture containing (–)-α-pinene,1,2-dihydrolinalool and *racemic* linalool (*above*) and the simultaneously recorded activity of a type 4 olfactory receptor neurone of *Anthonomus rubi* (*below*). An excitatory response to (–)-α-pinene and an inhibitory response (i) to *racemic* linalool were recorded. Increased spike frequency during elution of the solvent (hexane) is also shown. B) Sequences of spike activity (10s each) during the spontaneous activity, the response to hexane, to (–)-α-pinene, and to linalool. C). Spike activity of the same olfactory receptor neurone as in A, during the elution of 1,2-dihydrolinalool and linalool (start of elution indicated by arrows). Reduced spike frequency was recorded in response to both compounds.

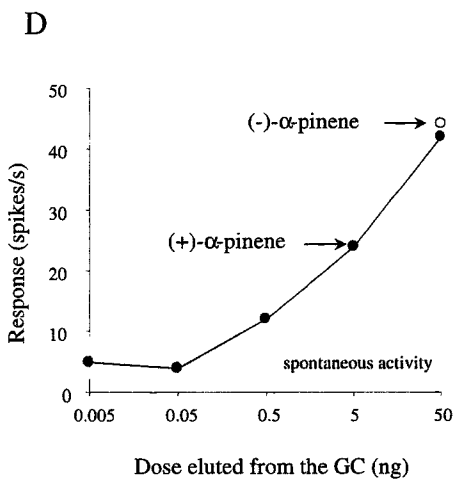


Fig. 3 **D)** Dose-response curve obtained from the same neurone as in A, by stimulation via the GC-column with decadic dilutions of reference samples of (+)-α-pinene and one concentration of (−)-α-pinene

RNs of type 3 and type 4 responding to α-pinene: Recordings were obtained from three RNs strongly activated by α-pinene and weaker by β-pinene. Two of these RNs (classified as type 3, Fig. 2) did not respond to other compounds in the host plant mixture either via the GC (gc=7) or by direct stimulation. The third neurone (type 4) (gc=11) also showed its strongest response to α-pinene and a weaker response to β-pinene. However, minor responses to camphene, 3-carene and 1,8-cineole were also recorded (table III). Interestingly, an inhibitory response to linalool was obtained when the RN was tested with the headspace sample of the host plant (not shown). This was the only neurone that showed inhibitory responses. In Figs. 3A and B the responses of this RN are shown during stimulation with GC-separated α-pinene and linalool. The inhibitory response to linalool was confirmed by re-testing this neurone with synthetic *racemic* linalool, and an additional inhibitory response to the contaminant 1,2-dihydrolinalool was also observed (Figs. 3A and C). Both inhibitory responses outlasted the elution of the GC-peak. Direct stimulation with the two reference samples of the enantiomers of linalool (concentration ca. 0,1 µg/µl) indicated inhibition by both enantiomers. The chiral column did not separate the enantiomers of α-pinene. Therefore, enantioselectivity of the RN was tested by injecting samples of (+)- and (−)-α-pinenes in the non-chiral column. Both enantiomers elicited responses of similar strength at one concentration as shown in Fig. 3D. Only (+)-α-pinene was tested at several concentrations. During the elution of the solvent hexane, this neurone responded by a series of short bursts of spikes followed by silent periods (Fig. 3A).

RNs of type 5 responding to 1-octen-3-ol: Two RNs had their greatest response to 1-octen-3-ol (gc=33). One of these

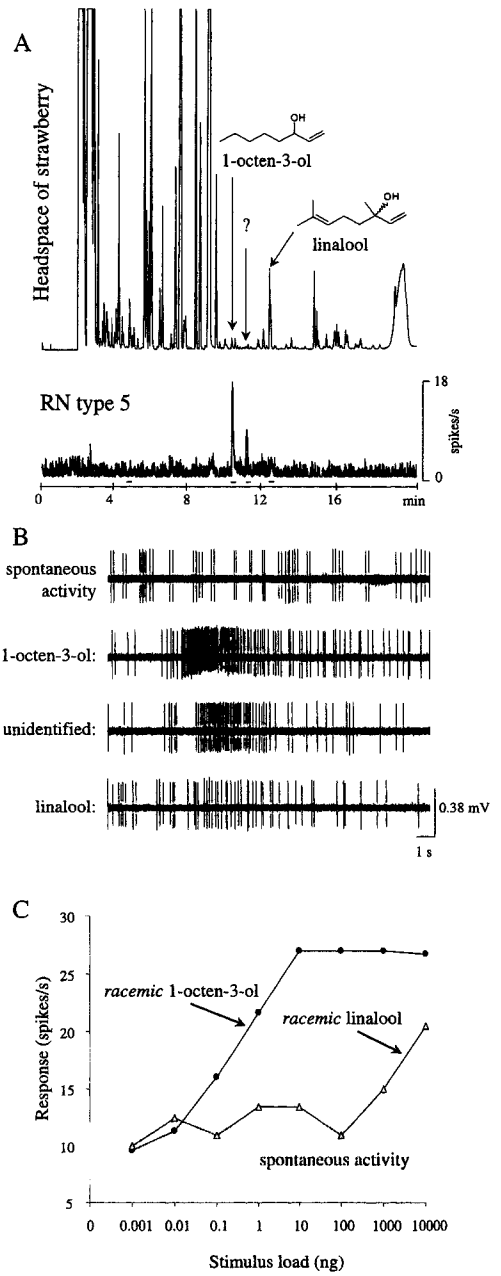


Fig. 4 A) Gas chromatogram of the headspace sample of cut strawberry leaves and flower buds (*Fragaria x ananassa*, var. Korona) (*above*), and simultaneously recorded activity of a type 5 olfactory receptor neurone of *Anthonomus rubi* (*below*), showing its greatest response to 1-octen-3-ol and secondary responses to an unidentified component (marked with "?") and to linalool. B) Spike activity (10 s each) of the neurone during spontaneous activity and of each of the responses [marked by short horizontal bars in the recording shown in A)]. C) Dose-response curves obtained by direct stimulation of the same olfactory receptor neurone with decadic dilutions of *racemic* 1-octen-3-ol and *racemic* linalool (points represent the average value of two stimulations)

neurones, with a particular high sensitivity to the primary odorant, showed in addition weaker responses to an unidentified compound and to linalool, both present in the host plant mixture (Fig. 4A). The chiral column did not separate *S*-(+)- and *R*-(−)-1-octen-3-ol completely, but the strongest response was elicited by *S*-(+)-1-octen-3-ol (not shown). Dose-response curves obtained by direct stimulation with decadic dilutions of *racemic* 1-octen-3-ol and *racemic* linalool showed a shift of four log units in the dose-response curves, the RN being far less sensitive to linalool (Fig. 4). The minimum threshold concentration was 10 pg for 1-octen-3-ol and ~100 ng for linalool. When the RN was stimulated with the enantiomers via the chiral GC column, the responses to the two enantiomers of linalool were similar (not shown). The second neurone of type 5 was 10-100 times less sensitive to the primary odorant and showed no response to any other compounds. The spontaneous activity of this RN was very low or not detected.

RNs of type 6 responding to aliphatic C_6 *esters:* Two neurones (gc=31) responded strongest to (3*Z*)-hexenyl acetate, one of the major constituents of the mixture of the host plant volatiles (cut leaves and flower buds). Weaker responses occurred to the small amount of (2*E*)-hexenyl acetate, to hexyl acetate, to 1-hexanol and to one unidentified component (peak 7 in Fig. 5A) present in the host mixture (Fig. 5A). The structures of the active components in the host-plant mixture are shown in Fig. 5B. Responses to 3-octanone, (2*E*)-hexenal and also to one unidentified compound present in very small amount were recorded when the RNs were tested for the standard mixtures "Green leaf volatiles" and "Apple esters" (cf. table II) (not shown). Dose-response curves, determined for (3*Z*)-hexenyl acetate and (2*E*)-hexenyl acetate by stimulation via the GC-column, showed complete overlap (Fig. 5C). The high sensitivity of the two neurones was demonstrated by the detection of these compounds at 10 and 50 pg, respectively (lowest doses tested). The secondary odorant 3-octanone had a ~100 times weaker effect than (3*Z*)–hexenyl acetate and (2*E*)–hexenyl acetate (Fig. 5C). The responses of these two neurones to (3*Z*)-hexenyl acetate showed the highest firing rate recorded in this species (68 spikes/s when stimulated with a dose of 50 ng via the GC).

RNs of type 7 responding to aliphatic C6 *alcohols:* The two type 7 RNs were recorded simultaneously with the two RNs of type 6 (gc=31) (*i.e.* co-located in the same sensilla). When stimulated with the headspace of cut leaves and flower buds of strawberry, these neurones responded strongest to (2*E*)-hexen-1-ol and showed secondary responses to two other aliphatic C6 alcohols and one C6 ester (Table III, Fig. 5A). Fig. 5D shows the dose-response curve obtained by stimulating one neurone with decadic dilutions of the primary odorant (2*E*)-hexen-1-ol via the GC-column. The two RN types 6 and 7 showed overlap in their molecular receptive ranges regarding their responses to (2*E*)-hexenyl acetate and 1-hexanol (Fig. 5B).

RNs of type 8 responding to aliphatic aldehydes: Type 8 RNs (n=4; gc=14) showed their greatest response to hexyl acetate, one component of the headspace mixture of orange peel, that partly co-eluted with the sesquiterpene germacrene D (Fig. 6A). Secondary responses were recorded to four other compounds in this mixture. Tentative identification, indicated dodecanal (also called lauraldehyde) as the primary odorant and decanal, hexyl butyrate, *n*-hexyl hexanoate, and ethyl octanoate as secondary odorants (Fig. 6B). Verification of the identity of these odorants by testing the neurones with reference samples remains to be done. Response to germacrene D was excluded when there was no response to GC-injection of the essential oil of ylang-ylang containing large amount of this compound. None of the components of the plant odour mixtures tested (Table I) elicited responses of these RNs, either when tested via the GC or when blown directly over the antenna. Decanal and dodecanal are known volatiles of strawberry, which are emitted by both healthy and damaged leaves of strawberry (collection method: SPME. Borg-Karlson, unpublished results), but the strawberry samples used in this study did not contain either in detectable amounts (collection method: adsorption on Porapak Q). This RN type was recorded simultaneously with another RN that did not respond to any of the components tested.

RN of type 9 responding to p-methylanisole: One neurone (gc=11) had its greatest response to the *p*-methylanisole present in the essential oil of ylang-ylang. Re-testing the neurone with a commercially available sample of *p*-methylanisole and the related structures *o*- and *m*-methylanisole (Table III) showed that the neurone responded strongest to *p*-methylanisole, secondarily to *o*-methylanisole, and weaker to *m*-methylanisole. The detection limit for *p*-methylanisole was in the order of the ng and the neurone seemed to be 10 times more sensitive to this compound than the most effective secondary odorant, *o*-methylanisole.

RNs of type 10 responding to eugenol: Two of the RNs (gc=8) showed strong responses to clove bud, clove, and cinnamon essential oils, to eugenol and to (2*E*)-hexenal, when stimulated directly via glass cartridges. However, no responses were obtained when these RNs were stimulated via the GC-column with the same mixtures or standard compounds. A relatively high amount of eugenol (0.5 µg) was necessary to elicit response in these RNs (dose-response curve not shown), suggesting that eugenol might not be the primary odorant for this neurone. Therefore, eugenol was here considered a secondary odorant (Table III), and the primary odorant remains to be identified.

Other RN types

Some recordings showed responses to aliphatic compounds, terpenoids and/or aromatic compounds. These RNs were difficult to characterise either due to difficulties in sorting the spikes, and therefore ascribing responses to individual RNs, or due to small amounts and/or incomplete separation of the active compounds.

In two series of experiments from different insects (n=2, gc=8), identical patterns of response activity were obtained to seven odorants present in the host headspace sample (type/group 11, Fig. 7). Three of the compounds eliciting responses could be tentatively identified [(3*Z*)-hexenyl acetate, 1-heptanol and methyl salicylate]. In addition, responses were

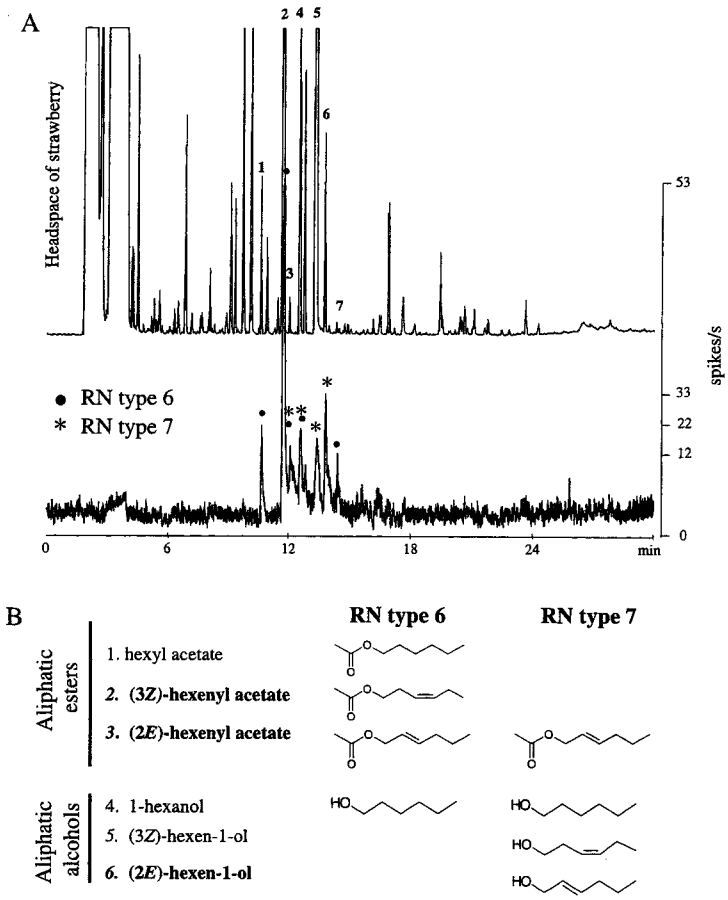


Fig. 5 A) Gas chromatogram of the headspace sample of cut strawberry leaves and flower buds (*Fragaria x ananassa*, var. Korona) (*above*), and simultaneously recorded activity of two olfactory receptor neurones of *Anthonomus rubi* (*below*), showing responses to aliphatic esters (type 6) and aliphatic alcohols (type 7) (Peak 7 in the chromatogram is not chemically identified). B) Molecular structures of the active compounds with primary odorants indicated in bold

recorded to methyl butyl hexanoate, (3*Z*)-hexenyl hexanoate and an unidentified compound (present in small amount), when these RNs were stimulated via the GC-column with a synthetic standard mixture of apple esters (Table II). Spike analysis could not ascertain whether these recordings were produced by more than one neurone.

The RN type/group 12, responded to nerol acetate and geraniol present in the essential oil of lavender (gc=4). These responses were consistent but weak considering the large amount of compounds used during the stimulation of the RNs. It is therefore possible that neither of the two compounds were primary odorants. Weak responses to coumarin in the essential oil of lavender, and to isoeugenol in the essential oil of basil, were also recorded in the same GC-SCRs. Although these responses may originate from another neurone since added amplitudes of simultaneously occurring spikes were visible during the runs, the analysis did not allow ascribing the responses to separate neurones. One neurone (type 13, gc=1) responded to camphor and (+)-*trans*-verbenol. However, the tested concentrations were ca. 10 ng/µl, making it difficult to be certain whether any of these compounds were the primary odorant. Another two types of RNs (types 14 and 15) responded to constituents that could not be identified due to the minute amounts present and/or their co-elution with other constituents. Type 14 (n=3), co-located with type 2 RNs, responded to two components of the headspace mixture of the host plant. RN type 15 (n=1) co-located with the RN type 4, responded to a minor compound in the essential oil of lavender.

RN types responding to aggregation pheromone components

One neurone (type 16; gc=8) responded most strongly to lavandulol, one of the components of aggregation pheromone of this species (Innocenzi *et al.* 2001), present in the essential oil of lavender (Fig. 8). Weak responses were also recorded when stimulating with essential oil of cedar

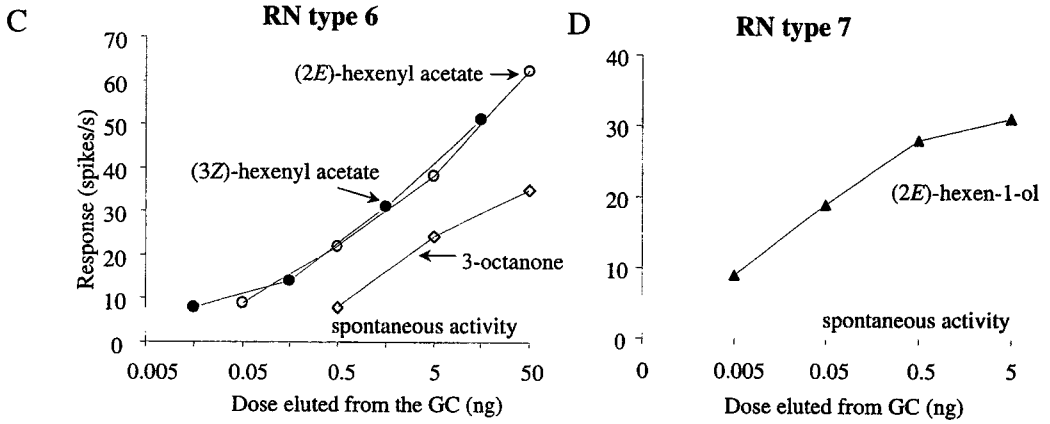


Fig. 5 C) Dose-response curves obtained by stimulating one type 6 receptor neurone via the GC-column with decadic dilutions of three odorants. D) Dose-response curve of a type 7 receptor neurone stimulated with the primary odorant (2*E*)-hexen-1-ol, obtained as in C)

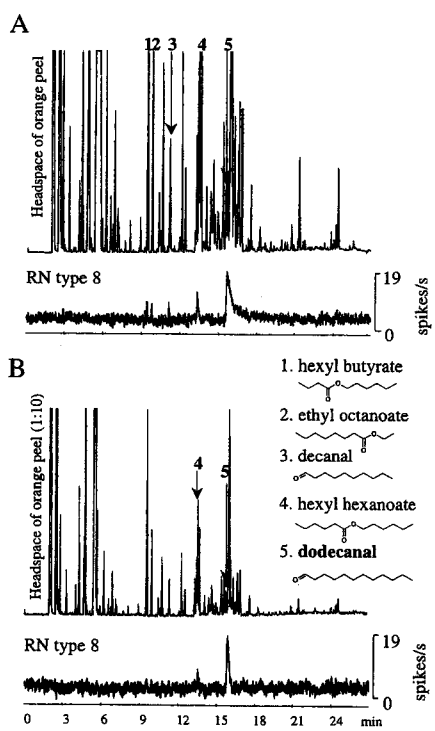


Fig. 6 A) Gas chromatograms of cut orange peel headspace (*above*) and simultaneously recorded activity of one type 8 olfactory receptor neurone of *Anthonomus rubi* (*below*), showing responses to the primary odorant dodecanal (5) and to four other odorants [hexyl butyrate (1); ethyl octanoate (2); decanal (3); *n*-hexyl hexanoate (4)]. B) Recorded activity of the same neurone stimulated with orange peel headspace volatiles diluted 1:10 via the GC-column. The molecular structures of the active odorants are shown (primary odorant in bold)

wood and of ylang-ylang. Due to incomplete GC-separation from other components in the mixtures, the active compounds could not be identified. Activity of another RN appeared in the same recordings, but this RN did not respond to any of the compounds tested.

In another experiment, one RN (type 17) responded strongly to the reference sample of authentic *trans*-grandisol (concentration ca. 0,1 µg/µl) during direct stimulation. This sample was tested on the neurone via the GC. Tentative identification of the components eliciting responses indicates that the aggregation pheromone component *cis*-grandisol (Innocenzi *et al.* 2001), and citronellol present as impurities in the sample, were the active compounds. The main constituent in the sample, *trans*-grandisol did not elicit a response.

GC-EAG recordings

Recordings of EAGs and GC-EAGs were carried out to assess the sensitivity of whole antennae to plant odours in seven males and four females of *A. rubi*. Direct stimulation elicited very clear EAGs (Fig. 9A), while GC-EAG recordings were often unstable and variable (examples shown in Figs. 9B and C) and did not correlate well with one another. One example, shown in Figs. 9A and B, is the case of the essential oil of ylang-ylang, which elicited a strong response by direct stimulation, whereas the GC-EAG showed only very weak responses to the components in the same mixture. Altogether, GC-EAG experiments showed that 11 compounds present in the host plant mixtures elicited consistent EAG-responses by antennae of the weevils (Fig. 9C). Seven compounds present in mixtures other than the host plant, also elicited consistent EAG responses (not shown). The comparison of the responses to host-plant constituents obtained with GC-EAG and GC-SCR is presented in Fig. 9D, showing that 8 components eliciting single cell responses did not elicit consistent EAGs, while 2 that elicited EAGs were not detected by the GC-SCRs.

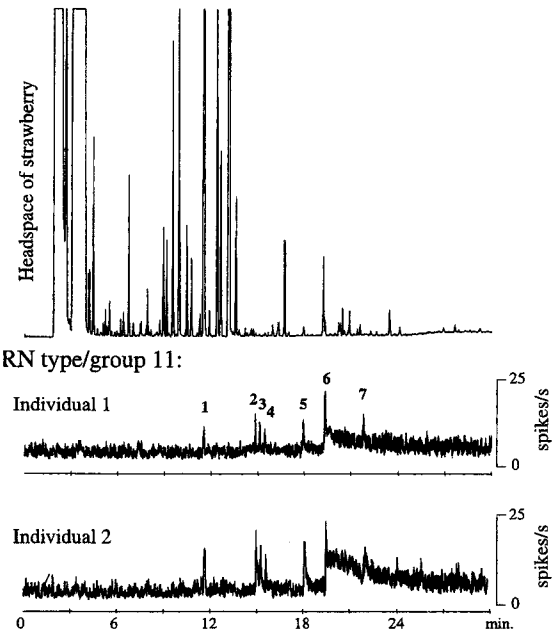


Fig. 7 Gas chromatogram of the headspace sample of cut strawberry leaves and flower buds (*Fragaria x ananassa*, var. Korona) (*above*) and simultaneously recorded activity of neurone type/group 11, of *Anthonomus rubi (middle). Below*: Recording of another neurone of the same type, in another individual, stimulated with the same volatile mixture separated in the GC-column, showing the consistent pattern of responses in recordings from neurones/neurone groups of the same type. Numbers indicate responses to (3*E*)-hexenyl acetate (1), 1-heptanol (2), methyl salicylate (7), and to unidentified compounds (3-6)

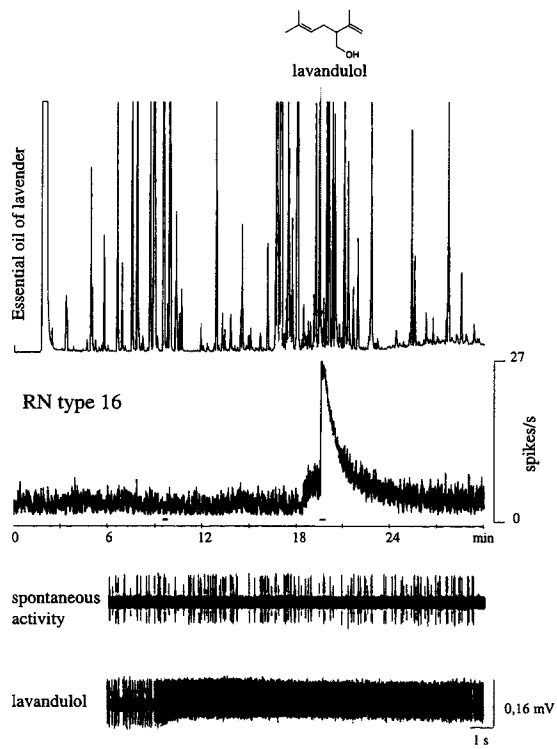


Fig. 8 Gas chromatogram of lavender essential oil (*above*) and simultaneously recorded activity of a neurone type 16 of *Anthonomus rubi* stimulated with the GC-separated volatiles, showing strong response to the aggregation pheromone component lavandulol (*middle). Below:* Spike activity of the same neurone during 20s of spontaneous activity and during the response to lavandulol (indicated by short horizontal bars under the middle trace)

Discussion

During recent years the use of GC-SCR has provided knowledge about the molecular receptive ranges of plant odour RNs in a few species of weevils, other beetles and heliothine moths (Blight *et al.* 1995; Wibe and Mustaparta 1996; Wibe *et al.* 1997; Røstelien *et al.* 2000a, b, 2005; Stensmyr *et al.* 2001; Barata *et al.* 2002; Stranden *et al.* 2002; 2003a, b; Bichão *et al.* 2003, 2005). The present results on the oligophagous weevil *Anthonomus rubi,* whose larvae and adults use strawberry as host for feeding and reproduction, are interesting both to understand olfaction in this species, and to make comparisons with other species. Particularly interesting are the comparisons to the forest weevils *Pissodes notatus* and *Hylobius abietis*, and also the heliothine moths, since similar test protocols were used for all of them. The present study provides the molecular receptive ranges of 17 types of RNs in *A. rubi*. Together with the five types previously reported (Bichão *et al.* 2005), functional properties of 22 RN types have been described. The molecular receptive ranges of these RN types also give the number and identity of natural plant produced volatiles that activate the RNs, *i.e.* 78 compounds, including the 63 chemically identified and the 15 indicated by GC-retention times, in this and the previous study. Like in the forest weevils and the heliothine moths, the RNs of *A. rubi* were narrowly tuned, showing a strong response to one or two compounds (primary odorants) and weaker responses to a few (3-7) structurally related compounds (secondary odorants) out of the hundreds tested. The term secondary odorants is useful in discussing the RN tuning, but does not mean that these odorants are behaviourally unimportant. Since all RNs responded by excitation to all compounds with only two inhibitory responses, the classification of the RNs was defined according to the compounds that elicited excitation.

An interesting feature of the olfactory RNs is the degree of overlap of the molecular receptive ranges, which traditionally has been discussed in connection with the principles by which information about an odorant is transmitted to the brain ("labelled lines" and "across-fibre" patterns, cf. Mustaparta, 2002). The present results show some overlap of the molecular receptive ranges of these narrowly tuned RNs. For instance the bicyclic monoterpene α-pinene activated both RN types 3 and 4, and both enantiomers of

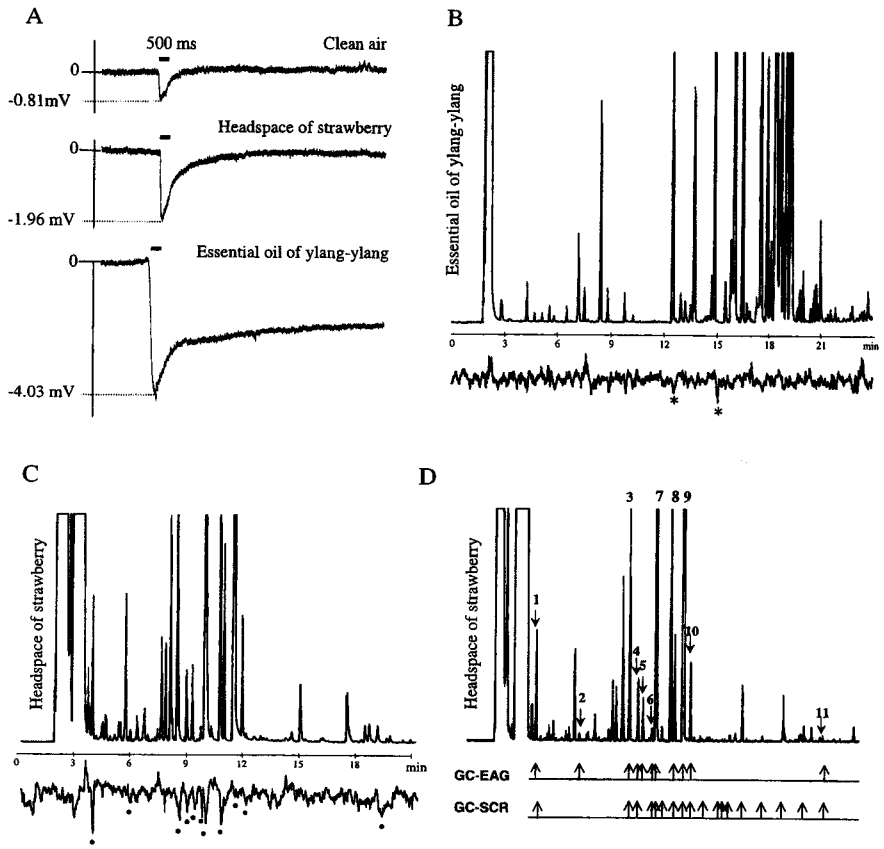


Fig. 9 A) Electroantennograms obtained by direct stimulation of *Anthonomus rubi* antenna with the mixtures of volatiles from the headspace of strawberry leaves and flower buds (*Fragaria x ananassa*, var. Korona) and the essential oil of ylang-ylang (*Cananga odorata*). B) Gas chromatogram of the essential oil of ylang-ylang (*above*), and simultaneously recorded electroantennogram (*below*) showing responses to *p*-methylanisole and linalool indicated by asterisks (n=7). C) Gas chromatogram of the headspace of strawberry (*above*) and simultaneously recorded electroantennogram (*below*) showing 11 responses to different compounds of the host plant volatile mixture (solid circles indicate consistent responses, n=11). D) Gas chromatogram of the headspace of strawberry (*above*). *Below:* Summary of the responses to strawberry plant headspace components identified by the gas chromatography linked to electroantennography (1. 3-pentanone; 2.; 3. *E*-α-ocimene; 4. unidentified; 5. unidentified; 6. (3*E*)-hexenyl acetate + *E*-DMNT [(3*E*)-4,8-dimethyl-1,3,7-nonatriene]; 7. (3*Z*)-hexenyl acetate; 8. 1-hexanol; 9. (3*Z*)-hexen-1-ol; 10. (2*E*)-hexen-1-ol; 11. methyl salicylate) and by gas chromatography linked to single cell recordings, for comparison (this study and Bichão *et al.* 2005)

the chiral alcohol linalool activated the RN types 1, 2, and 5 as well as inhibited type 4 (Figs. 1, 2 and 3). Also RN types 6 and 7, co-located in the same sensillum, showed overlap of responses in the case of the aliphatic compounds 1-hexenol and (2*E*)-hexenyl acetate (Fig. 6). The principle of overlapping molecular receptive ranges within the same compound class has also been shown in the previous studies of the pine weevils and the cerambycid beetle *Phoracantha semipunctata* (Wibe *et al.* 1997; Barata *et al.* 2002; Bichão *et al.* 2003) as well as in heliothine moths (Stranden *et al.* 2003b; Røstelien *et al.* 2005). The degree of overlap found in *A. rubi* was similar to that found in other weevils and beetles, in contrast with the RNs in heliothine moths which only occasionally showed overlap of the molecular receptive ranges (Stranden *et al.* 2003b, Røstelien *et al.* 2005). Only in one case in *A. rubi* did overlap of the molecular receptive range of two RN types occur across chemical groups. This overlap occurred where RN type 5 responded primarily to the aliphatic alcohol (+)-1-octen-3-ol and also weakly to the monoterpene alcohol linalool, the primary odorant of RN types 1 and 2 (Table III). However, the structural similarity of these compounds is striking, suggesting a narrow tuning of the RNs. All identified odorants activating the same RNs in these studies have shown molecular structures with similar features, indicating binding with different affinity to narrowly tuned receptor proteins. In contrast, studies using pre-selected compounds as test samples have shown broader tuning and more overlap, both in the fruit fly *Drosophila melanogaster* and in vertebrates (De Bruyne *et al.* 2001; Buck 2000), suggesting that each receptor protein is less specific. Whether these neurones would appear with a higher sensitivity and narrower tuning when stimulated with

other odorants remains to be seen (De Bruyne *et al.* 2001). However, it is certainly possible that the receptor proteins of insect and vertebrate species possess considerably different selectivity.

The drop in stimulatory effect with small changes of the molecular structure of the primary odorant further demonstrates the narrow tuning of the RNs in the present study. Important features of the molecules that affected the responses were the position of the double bond (*e.g.* α-pinene and β-pinene in RN types 3 and 4, Table III), the presence of double bonds as compared to saturation [(3*Z*)-hexenyl acetate and (2*E*)-hexenyl acetate compared to hexyl acetate in RN type 6 (Fig. 5B)] and chain length (dodecanal and decanal in RN type 8, Fig. 6). Interestingly, certain small changes of the molecular structure did not reduce stimulatory effect, like the *Z*- and *E*-configuration and the position of the double bond [(3*Z*)-hexenyl acetate and (2*E*)-hexenyl acetate (Fig. 5C)] for RN type 6, and saturation of the allylic bond (linalool and 1,2-dihydrolinalool) for RN types 1 and 2 (Fig. 1B). Also surprising was the similar, although weak effect of decanal and hexyl hexanoate on RN type 8 (Figs. 6A and B), which may be explained by the possible conformation of the hexyl hexanoate molecule exposing the carbonyl group for optimal interaction with the binding site in the receptor protein.

The chiral configuration is a particular feature of odour molecules that has been discussed throughout the history of olfactory research (Ohloff 1994). Whereas enantioselective RNs for pheromones have been described in several insect species (Mustaparta *et al.* 1980; Hansen *et al.* 1983; Leal *et al.* 1995; Larsson *et al.* 1999), enantioselectivity of plant odour RNs has been only recently reported in a few cases (Wibe *et al.* 1998; Stranden *et al.* 2002, 2003a, b; Bichão *et al.* 2005, Røstelien *et al.* 2005). Behavioural discrimination of enantiomers has been shown in insects (Tooker *et al.* 2002; Borg-Karlson *et al.* 2003). In the present study, the pure linalool enantiomers separated on a chiral column, were each found to be the primary odorant for one of two RN types (types 1 and 2), whereas the opposite enantiomer had a lower effect (Fig. 1). Enantioselective RN types for linalool have also been demonstrated in species of moths, both *Heliothis virescens* and *Mamestra brassicae* (Røstelien *et al.* 2005, Stig Ulland, personal communication). In *Manduca sexta* two groups of antennal lobe projection neurones have been identified, which showed enantioselective responses to linalool and had arborisations in separate glomeruli (Reisenman *et al.* 2004). Altogether, these results indicate that discrimination of linalool enantiomers may be common in insect species. Possessing separate RN types tuned to each of the enantiomers of linalool, *A. rubi* may have the capacity to discriminate between (+)- and (−)-linalool, which are produced by different enzymes in plants (Raguso & Pichersky 1999). In contrast, the enantiomers of germacrene D also produced by separate enzymes, are detected by only one RN type with strongest response to (−)-germacrene D both in *A. rubi* (Bichão *et al.* 2005) and in the heliothine moths (Stranden *et al.* 2002, 2003a). This suggests that discrimination between linalool enantiomers may be more important for the insect's behaviour than discrimination of germacrene D enantiomers. The presence of the enantioselective RNs for linalool and germacrene D corresponds with the presence of the enantiomers in plants. Whereas (−)-germacrene D is often the dominating enantiomer occurring in higher plants (Lorimer & Weavers 1987; Bülow & König 2000; Stranden *et al.* 2002), the ratio of (+)- and (−)-linalool varies within and among the different plant species (Borg-Karlson *et al.* 1996; Casabianca *et al.* 1998; Borg-Karlson, unpublished results).

In most electrophysiological studies of insect RNs excitatory responses have been obtained. However, inhibition has also been reported and is particularly discussed in the recent studies of the fruit fly *D. melanogaster* (De Bruyne *et al.* 2001; Hallem *et al.* 2004). Both in *D. melanogaster* and the silk moth *Bombyx mori* inhibition is elicited by linalool (Kaissling *et al.* 1989; De Bruyne *et al.* 2001). In the present study, *racemic* linalool and 1,2-dihydrolinalool elicited the only inhibitory responses in a RN type that was excited by α-pinene (Figs. 3A and C). To our knowledge this is the only case of inhibition reported in GC-SCR studies, indicating that inhibition is a less common response mode in insect olfactory RNs. However, it should be taken into consideration that inhibition might only be seen in RNs with a certain spontaneous activity. In the study of *D. melanogaster* inhibition was proposed to provide a mechanism for enhancing signal recognition, by one compound inhibiting a population of RNs and activating another, thereby increasing the contrast of activity in the glomeruli (De Bruyne *et al.* 2001). This may also be the case in *A. rubi* having two types of RNs excited by linalool and another type (RN type 4) inhibited by linalool.

In recent years much attention has been paid to how plant emissions are influenced by herbivores. A general principle seems to be that plants emit greater amounts and different profiles of volatiles after herbivore damage (Paré & Tumlinson 1999; Dudareva *et al.* 2004). In a previous study we have reported that numerous compounds are induced in strawberry flowers during feeding of *A. rubi* (Bichão *et al.* 2005). These include linalool and aliphatic alcohols and aldehydes, in addition to germacrene D, β-caryophyllene, *E*-β-ocimene, *E*-DMNT [(3*E*)-4,8-dimethyl-1,3,7-nonatriene] and methyl salicylate. The present and the previous study show that many of the characterised RNs of *A. rubi* are tuned to these compounds, *e.g.* the RN types tuned to linalool and those tuned to green leaf volatiles (types 1, 2, 4, 6 and 7). The detection by *A. rubi* of plant compounds induced by conspecifics may play a role in aggregation as shown for other insects (Rochat *et al.* 2000; Yang *et al.* 2004). Induced compounds may also be used by *A. rubi* females as cues to avoid flower buds that already contain eggs. Other compounds, like the aromatic compounds that are emitted only during the flowering period (Borg-Karlson, unpublished results) could provide cues describing the phenological state of the plant.

Most of the compounds detected by RNs in *A. rubi* and other insects are common in many species of plants. When comparing the RNs across insect species, the types tuned to C_6 aliphatic alcohols, esters and aldehydes (*i.e.* commonly designated green leaf volatiles) and types tuned to 1-octen-3-ol are found in weevils, others beetles, and moths living on angiosperms (Dickens 1990; Blight *et al.*1995; Hansson *et al.* 1999; Røstelien *et al.* 2005; Barata *et al.* 2002; Stensmyr *et al.* 2001; Ulland, unpublished results) but so far, no RNs tuned to these compounds have been found in the

well studied conifer weevils *P. notatus* and *H. abietis* (Bichão *et al.* 2003; Wibe *et al.* 1997)

As shown in the previous and present study of *A. rubi* and other insect species the method of GC-SCR has the advantage of giving precise, results about the RNs' specificity. Although many experiments have been made, more data are required to identify the molecular receptive range of all olfactory RN types in the antenna. GC-EAG is another method used to identify plant compounds detected by insects (Guerin *et al.* 1983; Baur *et al.* 1993, among others). In order to compare results with the two methods, we also carried out GC-EAG experiments. Except for two, all responses obtained by GC-EAG could be documented by GC-SCR, whereas several active compounds identified by GC-SCR could not be detected by the GC-EAG (Fig. 9). Particularly surprising was that linalool and germacrene D, shown to be important by the GC-SCR, did not elicit clear responses by GC-EAG. EAG, assumed to measure the summated receptor potential, is a coarse method that may not reveal all responses of the RNs. The EAG recordings are particularly sensitive to the time course of the stimulus delivery, which may be one reason why GC-EAG gave limited results. Other studies comparing GC-SCR and GC-EAG have reported similar observations (Jönsson & Anderson 1999; Wibe 2004).

In summary, this study has increased the knowledge about how the plant odour information is encoded in the RNs of *A. rubi*, by functionally characterising 17 RN types in addition to the five previously reported. Excitation was the main response mode of these RNs. Inhibition was only elicited by racemic linalool in one RN type excited by α-pinene. The different excitation elicited by each of the linalool enantiomers in two other RN types, suggests a special coding mechanism for this compound, which in turn must reflect the importance of the compound for the insect. The results show that *A. rubi* uses a large number of odorants produced by different biosynthetic pathways, to obtain clues about potential food sources, reproduction sites and mates. All the active plant compounds occur in many plant species and most of the primary odorants can be classified as induced by biotic or mechanical damage. Whether these compounds are attractive or repellent, and how *A. rubi* uses them for host finding, oviposition behaviour or mating, is not known and is the object in ongoing investigations. Some odorants may be attractive and act in synergy with aggregation pheromones favouring aggregation and mating. It is also possible that certain odorants or blends are cues indicating the phenological state of the plant appropriate for feeding and/or oviposition, or mating flower buds occupied by conspecific eggs inhibiting oviposition behaviour.

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