

Functional characterisation of olfactory receptor  
neurone types in heliothine moths

*Identification of molecular receptive ranges by the use of single  
cell recordings linked to gas chromatography and mass  
spectrometry*

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## Papers included in the theses

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

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

II. Røstelien T, Borg-Karlson, A.-K. and Mustaparta, H. (2000) Selective receptor neuron responses to *E*- $\beta$ -ocimene,  $\beta$ -myrcene, *E,E*- $\alpha$ -farnesene and homo-farnesene in the moth *Heliothis virescens*, identified by gas chromatography linked to electrophysiology. *J Comparative Physiology A*: 186: 833-847

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

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

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## Introduction

The sense of smell is crucial for most animal species. It is critical for food-finding, reproductive behaviour, predator-prey relationship, kin and mother-infant recognition, homing behaviour and nest finding. The importance of the olfactory systems is reflected in the proportion of the genome that is devoted to the olfactory receptor proteins, e.g. comprising 3-5% in human and mouse (Young and Trask, 2002; Zhang and Firestein, 2002). For a long time the human sense of smell was considered as the most enigmatic of our senses. An intriguing question was; what mechanism could explain our ability of recognizing and remembering more than 10 000 distinct odorants (Buck, 2004). Buck and Axel (1991) performed a breakthrough by the discovery of the large family of olfactory genes in the rat. Buck and Axel were in 2004 honoured with the Nobel Prize in physiology and medicine for this study and the following series of pioneering work on the subject. The knowledge about the olfactory genes is obviously important for studies of the function of the olfactory receptor neurons (RNs), both in solving the transduction mechanisms and the specificity of the RNs. In the search for which odorants the olfactory receptors are evolved, insects are suitable model organisms. Herbivore species are particularly interesting, since many of them share the same plant species and their survival depends on olfactory cues in locating their host for feeding and reproduction (mating and oviposition).

### *The insect olfactory system; anatomy of the olfactory pathway*

The numerous olfactory organs in insects, the sensilla, are mainly located on the antenna (Schneider and Steinbrecht, 1968). The lepidopteran antenna consists of two proximal segments, the scape and pedicel, and the long flagellum. In heliothine moths, the flagellum consists of ~ 80 annuli that carry numerous sensilla mediating information about different modalities, including chemo-, mecano- temperature-, and humidity sensation (Almaas and Mustaparta, 1990, 1991; Jørgensen, 2003; Kvello, 2003; Lassa, 2004). Like in other Lepidopterans, the olfactory sensilla of the moth antenna outnumber by far those of other modalities. The general classification into various morphological types, like *sensilla trichodea*, *s. basiconica*, *s. auricillia* and *s.*

*coeloconica* also applies to olfactory sensilla of heliothine moths (Jefferson et al., 1970; Steinbrecht, 1973; Hallberg, 1981; Keil and Steinbrecht, 1984; Almaas and Mustaparta, 1990; Almaas et al., 1991; Koh et al., 1995; Færavaag, 1999). Extensive studies have been carried out on the structure of *s. trichodea* and *s. basiconica*, involved in pheromone and plant odour detection, respectively (review Steinbrecht, 1997). The cuticle wall of these sensilla is perforated by pores allowing the air-borne volatiles to enter the lumen, which is filled with receptor lymph surrounding the dendrites of the RNs. The membrane spanned receptor proteins are located in the dendrite of these bipolar sensory neurons.

The axons of the antennal RNs form the antennal nerve and project directly to the deutocerebrum, the first relay station of the antennal sensory pathway (Homberg et al., 1989). The bilateral deutocerebrum consist of two distinct regions called the antennal lobe (AL) and the antennal mechanosensory and motor centre (AMMC, also called the dorsal lobe in other species). The olfactory RNs send their axons into the AL, whereas the AMMC receives axons from the mechanosensory neurons (Homberg et al., 1989). Synapses between the RNs and antennal lobe neurons are located in numerous glomerular structures of the antennal lobe. These structures are functional units and represent a physical basis for mapping odour qualities. In herbivorous Lepidopterans, many studies have shown a separation of the glomeruli involved in the two systems of pathways mediating pheromone information and plant odour information. In species of Heliiothinae, the three male specific glomeruli constitute the macroglomerular complex (MGC) dedicated to the pheromone information, and 60-62 ordinary glomeruli dedicated to plant odour information (review Mustaparta, 2002). In the AL two major morphological types of neurons receive and process the olfactory information from the antennal sensory neurons. The local interneurons with arborisation in many glomeruli mediate information within the antennal lobe, whereas projection neurons branching in one or a few glomeruli have an axon conveying information out of the AL to higher order neurons in the protocerebrum. These are located in two areas, the mushroom bodies, shown to be important in learning and memory of odours (review Menzel, 1999), and the lateral horn which is a pre-motoric area (Strausfeld, 1976). In moths, including heliothine, the axons of the projection neurons follow three major tracts from

the AL to the protocerebrum, the inner-, the outer- and the middle antenno-cerebral tract (Homberg et al., 1988; Rø et al., 2003).

### *Peripheral events*

The binding of and interaction between the odorant and the receptor proteins leads to an intracellular cascade reaction (the transduction events), which results in opening of ion channels and depolarisation of the membrane. Lancet and Pace (1987) was the first to identify G-proteins in the olfactory epithelium of vertebrates, suggesting that activation of this protein by the odorant-receptor interaction is the first step of the cascade. In insects, the presence of G-proteins in olfactory neurons was demonstrated by Breer and co-authors (1988). The cascade leading to production of IP<sub>3</sub> (inositol 1,4,5-trisphosphate) as second messenger is, in insects, considered to be the major excitatory pathway, opening the cation channels (Breer et al., 1990; Wegener et al., 1993; Stengl, 1994). In vertebrates, cAMP (adenosine 3,5-monophosphate) is the second messenger for excitation (Nakamura and Gold, 1987; Breer et al., 1990; Breer, 2003b). cAMP has also been indicated as a possible second messenger in insects (Krieger et al., 1999). The odorants reach the receptors via odorant-binding proteins (OBP) present in the receptor lymph. Two major groups of binding proteins are classified in insects, the general odorant binding proteins (GOBPs) and the pheromone specific proteins (PBPs), each consisting of several sub types (Steinbrecht et al., 1992; Laue et al., 1994; Zhang et al., 2001). OBPs are assumed to function as transporters of the air born (hydrophobic) volatiles that have to pass through the liquid receptor lymph in order to reach the receptor proteins. Since OBPs show selective binding to some odorants they may also serve as a filter, protecting the receptors from being exposed to all kinds of volatile compounds. It has been questioned whether OBPs also contribute in odorant-receptor binding and in inactivation of the odorant-receptor complex (Prestwich et al., 1995; Steinbrecht, 1998; Kaissling, 1998; Mohl et al., 2002; Pophof, 2004). Another hypothesis is that the OBPs release the odorants close to the dendrite membrane due to conformational changes caused by the charged membrane (Wojtasek and Leal, 1999). Since the OBPs are present in the chemosensory systems of terrestrial vertebrates and insects, it is suggested that these proteins may be a molecular adaptation to terrestrial life (Breer, 2003a).

*“The logic of the sense of smell”*

Olfactory receptor genes first identified in the rat by Buck and Axel (1991) is one of the largest known mammalian gene families, in rats and mouse comprising nearly 1000 genes expressed exclusively in the olfactory tissue. In the many molecular biologically studies that followed, the general finding was that the olfactory information is handled by a large and species-specific number of receptor proteins (Buck and Axel, 1991; reviews Mombaerts, 1999, 2004, Keller and Vosshall, 2003, Breer, 2003a). Studies conducted over the past decade have shown that one type of olfactory receptor gene is expressed in a given subset of RNs (Ressler et al., 1993; Vassar et al., 1993; Clyne et al., 1999; Vosshall et al., 1999; Hallem et al., 2004). Candidate receptor proteins have also been identified in *Heliothis virescens*, showing expression of only one type in each neuron (Krieger et al., 2002, 2004). The olfactory receptor proteins show low homology across phyla. Only one subtype sharing a high degree of sequence identity in several species is co-expressed with other receptor proteins (Clyne et al., 1999; Fox et al., 2002; Krieger et al., 2003; Breer, 2003a). It is assumed that this particular protein has a role other than odorant recognition (Breer, 2003a). Furthermore, molecular biological studies of both vertebrates and invertebrates have shown that each subsets of RNs, expressing the same type of receptor proteins, projects in one or two specific glomeruli of the primary olfactory centres (the antennal lobe in insects and the olfactory bulb in vertebrates) (Axel, 1995; Treloar et al., 2002; Keller and Vosshall, 2003; Mombaerts, 2004). This principle, called “the logic of the sense of smell” suggests a certain relationship between the number of RN types and the number of glomeruli in the primary olfactory centres (Axel, 1995). In insects, this principle has been demonstrated in *Drosophila*, showing that each subset of RNs projects exclusively in one (or sometimes two) homologous glomeruli in each antennal lobe (Gao et al., 2000; Vosshall et al., 2000; Keller and Vosshall, 2003).

Numerous electrophysiological studies have been performed with the aim to functionally classify olfactory RNs (among others, Sicard and Holley, 1984; Ma and Shepherd, 2000; Duchamp-Viret et al., 1999; reviews Shepherd, 1984, Masson and Mustaparta, 1990, Hildebrand and Shepherd, 1997, Todd and Baker, 1999, Mustaparta, 2002, Korsching, 2002). These studies of both vertebrates and invertebrates have shown a large variation of the molecular receptive ranges, from RNs being narrowly tuned and



falling into distinct types, to broadly tuned neurons, often with individually different molecular receptive ranges. The pheromone olfactory receptors in heliothine moth are particular well studied. Since the first identifications of the female produced sexual pheromones in these species, listed in Arn et al. (1992), these biologically important odorants and other interesting chemical analogues have been available for detailed studies of the RN specificity (Masson and Mustaparta, 1990, Mustaparta 1997). Electrophysiological studies have demonstrated a functional classification of three or four RN-types tuned to the insect produced signals in each species. These RNs are characterised by a narrow tuning to one compound and considerably weaker responses to a few chemical analogues. Furthermore, functional tracing of single RNs have demonstrated that the axon terminals of each RN type project in one of the three or four glomeruli of the male MGC (Hansson et al., 1995; Berg, 1998; Berg et al., 1998). These findings have also been supported by optical recordings using  $\text{Ca}^{2+}$  imaging (Galizia et al., 2000). Thus, the results from studies of pheromone receptors in heliothine moth correlate well with the principle of one subset of RNs projecting in one glomerulus.

#### *Chemical aspects of insect-plant interaction*

Plants produce hundreds of compounds that are important in their interaction with insects and other organisms. These compounds are termed secondary metabolites, whereas those essential for growth and development of the plant are called the primary metabolites (Hartmann, 1996). Traditionally, secondary plant metabolites like the volatile compounds emitted from flowers and leaves were looked upon as by-products with no relevance. Ehrlich and Raven (1964) were among the first to suggest that plant produced secondary metabolites are evolved in a co-evolutionary arms race of plant defences and herbivore responses. The plants produce and release volatiles, e.g. for attracting pollinators. Pollinators might as well be herbivorous using these signals for host location (Harborne, 1993). After pollination plants are able to turn off the advertisement to pollinators by gene down regulation, which makes the plant less exposed to herbivory (Tollsten and Bergström, 1989; Dudareva and Pichersky, 2000). Plants may obtain a competitive advantage by producing other specific and reliable chemical signals that repel putative herbivores (direct defence) or attract natural

enemies of the herbivores (indirect defence) (e.g. Bernays and Chapman, 1994; Schoonhoven et al., 1998). Particularly the flowers and seeds, the reproductive parts of a plant, important for the plant fitness, need to be defended against herbivory. The heliothine larvae, mainly feeding on the growing and reproductive parts of the plants, are hazardous to the host plants (Fitt 1989). As defence, the plants produce and accumulate toxins that are damaging to the insects. As a reciprocal response, the insects may detoxify or excrete the toxins. Generalist feeders, which are exposed to a wide spectrum of toxic compounds produced by the plant defence systems, have a well-developed detoxification system, exemplified by the high activity of the MFO-enzyme system (mixed function oxidises) (Brattsten, 1983). Being toxic also to the plant themselves, these compounds are often produced as pro-toxins and are constitutively accumulated in special organs like vacuoles and glandular trichomes (Hartmann, 1985).

Plants are continuously interacting with their surroundings. The profile of volatiles varies during exposure of many biotic and abiotic factors, like nutrition access (e.g. nitrogen deficits), microbial infestation, exposure to UV light and ozone, high temperatures or auto-oxidation by the surrounding air (Janssens et al., 1992; Pichersky and Gershenzon, 2002; De Moraes et al., 2004). The profile of emitted volatiles also shows diurnal and seasonal variations (Hedin, 1976; Dudareva et al., 1999; Kolosova et al., 2001). All these factors might influence the signals exploited by herbivores in their host location. This tremendous complexity and variability of plant volatiles is very challenging in the investigation of biologically significant odorants used by insects and other organisms.

Many studies have been performed on tritrophic interactions, i.e. between plants, herbivores and herbivorous predators or parasitoids. Particularly interesting are the findings showing increased production and release of volatiles during caterpillar attack (Turlings and Benrey, 1998; Paré and Tumlinson, 1999; Dicke and Van Loon, 2000; Schmelz et al., 2003). Furthermore, profiles of compounds systemically induced during herbivory show species specificity, as regards quality and quantity, which is also shown for attack by heliothine species (Mori et al., 2001; Röse and Tumlinson, 2004; De Moraes and Mescher, 2004). Thus, the volatiles released by plants in response to insect feeding are directly associated with the feeding herbivore species. This induction is caused by activation of a series of genes that up-regulate the specific defence in plants

(Halitschke and Baldwin, 2003; Frey et al., 2004). The larval oral secretion contains several factors (e.g. volicitin) that induce plant defence responses (Alborn et al., 1997; Mori et al., 2001; Spiteller et al., 2001). Various toxins, like the tannins and gossypol, present in high amounts in flower buds of cotton (*Gossypium hirsutum*) are enzymatically ignited or induced in response to caterpillar feeding (Bezemer et al., 2004). These toxins have a negative effect on the development and survival of several cotton pest insects (Sharma and Agarwal, 1982; Stipanovic et al., 1990; Hedin et al., 1991). In *Nicotiana* species, the content of nicotine increases after herbivory or mechanical damage (Euler and Baldwin, 1996). These toxic plant metabolites are deterrents (inhibit feeding) to several pest insects and protect plants against predation (Bernays and Chapman, 1994).

The complex blends of volatiles produced by a plant can be trapped by various methods of headspace collection, distillation or extraction (review Silverstein and Rodin, 1966). More plant constituents present in nature are identified continuously as more sensitive analytical methods are employed. Gas chromatography, which separates different molecules, linked to or followed by mass spectrometry is a common method used for identifying volatile compounds in plants. These compounds belong to many different chemical groups, like short chain alcohols, aldehydes, ketones and esters, aromatic compounds (like benzenoids), mono- and sesquiterpenes (reviews Gibbs, 1974, Smith, 1976, Knudsen et al., 1993, Bernays and Chapman, 1994, McDonough et al., 1994, Ohloff, 1994, Schoonhoven et al., 1998). A few compounds are mainly found in restricted plant taxa, e.g. the isothiocyanates in Brassicacea (reviews Kjær, 1976, Fahey et al., 2001). Others, commonly occurring, are “green leaf volatiles” (mainly six-carbon alcohols, aldehydes and esters) that are products of the lipid metabolism catalysed by the enzyme lipoxygenase present in green leaves (Hatanaka, 1993; Rosahl, 1996; Croft et al., 1993; Heiden et al., 2003). Some compounds like the terpenoids (linalool, geraniol, limonene, myrcene, *E*- $\beta$ -ocimene, farnesene, nerolidol and caryophyllene, among others), are common constituents of flowers, but are also present in vegetative tissues, where they serve as defence compounds (Knudsen et al., 1993).

### *The heliothine moths*

The subfamily Heliothinae (Insecta; Lepidoptera; Noctuidae) constitute a large group of herbivore insects, of which the three important agricultural pest species *Heliothis virescens*, *Helicoverpa armigera* and *Helicoverpa assulta* were chosen for the present studies. *H. armigera* and *H. virescens* are both generalist feeders (polyphagous) exploiting a wide range of plant species across different families (e.g. *Leguminosae*, *Solanaceae*, *Malvaceae* and *Compositae*) (Fitt 1989; Matthews 1991). Many host plants exploited by one or both species are economically important agricultural crops, like cotton, sunflower, tobacco, maize, chickpeas and sorghums (Zalucki et al. 1986; Fitt, 1989; Firempong and Zalucki, 1990). *H. assulta* is considered oligophagous, exploiting a more narrow range of plant species, mainly within the family *Solanaceae* (Hill, 1983; Matthews, 1991). The two genera *Helicoverpa* and *Heliothis* are considered monophyletic, i.e. having a common origin (Matthews 1999). For millions of years, the American tobacco budworm moth *H. virescens* has been geographically separated from the closely related *H. armigera*, living at the Eurasian, African and Australian continents. The Oriental tobacco budworm *H. assulta*, partly sympatric with *H. armigera*, is distributed in Asia and Australia. The species, living at different continents, have been separated for a long time, and presumably exploited different host plant species, at least prior to the introduction of crop hosts they have in common. This might have led to evolutionary changes of the olfactory system.

The introduction of non-selective insecticides to control pest species, disrupted in many cases the natural balance of herbivore and predator/parasite populations (Bottrell and Adkinsson, 1977). Some insect species, like *H. virescens*, became new major pests because of their remarkable capability to quickly evolve resistance to the insecticides, which threatens the success of pest control (Fitt, 1989). The increasing awareness concerning the ecological consequences of the wide-spread use of insecticides enforces the search for ecologically viable alternative methods in pest management programs. Increased knowledge about the sensory receptor system of these species, their behaviour and ecology, may help minimize the level of crop damage as well as the amounts of insecticides used. This is being tested by combining mating disruption by pheromones and precise timing of low level exposures of insecticides. In

another attraction-kill strategy, the idea is to use plant odorants to attract the females to a gluing material with insecticides.

### **Aims of the thesis**

When the study of this thesis was initiated, hardly any work had been carried out on how plant odour information was encoded by the olfactory RNs in heliothine moths. The method of gas chromatography linked to single cell recordings (GC-SCR) was employed and improved for identifying naturally occurring plant odorants that are detected by single RNs and can be considered as biologically relevant. Three species of the subfamily Heliothinae were included in this work, the two polyphagous *H. virescens* and *H. armigera* and the oligophagous *H. assulta*. The American *H. virescens* is geographically separated from the other two species. *H. armigera* and *H. assulta* are partly sympatric in Asia and Australia.

The aims of the thesis elucidated in Papers I-IV were as follows:

1. To identify plant produced volatiles detected by antennal RNs in the three species of the subfamily Heliothinae.
2. To elucidate whether the single RNs can be classified into distinct types according to their specificity.
3. To characterise the plant odour RN types by their molecular receptive ranges, sensitivity and specificity.
4. To compare the specificity of plant odour RN types across the three related species of Heliothinae, with the aim to reveal any differences in the peripheral olfactory system that may have evolved through evolution.

## Survey of the individual papers

### *Paper I*

The study of paper I was the first carried out in the heliothine moth, with the aim to identify plant odorants by the use of gas chromatography linked to electrophysiological recordings from single receptor cells (GC–SCR). Volatiles released by a large number of host and non-host plants, intact as well as cut materials, were collected by headspace techniques, i.e. by trapping organic molecules from the air surrounding the plants. The volatile constituents were led through a tube containing an adsorbent and were subsequently eluted with a solvent. These headspace mixtures were then used as test samples on the RNs. The gas chromatograph was installed with two columns in parallel, each linked to the electrophysiological setup by a split at the outlet. In this way, half of the effluent is led to the GC detector and the other half out of the oven and into an air stream blowing over the insect antenna. This made it possible to test each single neuron with the compounds separated via two columns with different properties. The results were obtained as simultaneous recordings of gas chromatograms and neuron activity with responses to the active compounds. A large number of RNs were tested for numerous mixtures of plants volatiles. One particular type of neuron frequently appeared in nearly 80% of the recordings from *H. virescens* females. The neurons responded with high sensitivity and selectivity to one compound present in several hosts as well as non-host materials. The active compound was identified as a sesquiterpene hydrocarbon by the use of linked gas chromatography–mass spectrometry (GC-MS). Isolation of the compound from a sesquiterpene fraction of cubebe oil provided enough material for identification by NMR (nuclear magnetic resonance). The identification of the compounds as germacrene D was verified by retesting the purified compound via the gas chromatograph, which showed a significant response to germacrene D. All RNs responding to germacrene D showed a weak response to another sesquiterpene hydrocarbon. However, due to the lack of reference material, this compound could not be identified. Thirteen sesquiterpenes structurally related to germacrene D were found to have no effect. The germacrene D neurons presented in this paper was the first example of a narrowly tuned plant odour receptor type in a polyphagous moth species shown by

the use of GC-SCR, where hundreds of naturally occurring plant volatiles were screened on each neuron.

### *Paper II*

In Paper II, the specificity of three other RN types in *H. virescens* were identified on the basis of results obtained with the methods of GC-SCR and GC-MS. The method with two parallel columns was described in detail in this paper. The advantage by testing the same neurons with the same sample sequentially via a polar and a non-polar column was demonstrated. The various samples collected from host as well as non-host (intact and cut) materials were used in the studies of all four papers included in this thesis. The headspace techniques used for collecting the volatiles was described in paper II. In this study activity of three RNs occurred in the same recordings and these neurons were assumed to be co-located in one sensillum. Occasionally, one or two of them occurred alone in the recordings, or all three occurred together with a fourth neuron for which the compounds were not identified. By screening the neuron for sensitivity to a large number of plant samples containing hundreds of volatiles, all three neurons were found to have a high sensitivity and selectivity for one odorant (primary odorant) by showing weaker responses to a few other compounds with related structures (secondary odorants). On the basis of the mass spectra of the GC-MS analyses, the primary and secondary odorants were identified for neuron type 1 as *E*- $\beta$ -ocimene,  $\beta$ -myrcene, *Z*- $\beta$ -ocimene and DMNT (4,8-dimethyl-1,3,7-nonatriene, named *homo*-myrcene in Paper II), for neuron type 2 as *E,E*- $\alpha$ -farnesene and *E*- $\beta$ -farnesene, and for neuron type 3 as TMTT (4,8,12-trimethyl-1,3,7,11-tridecatetraene, named *homo*-farnesene in Paper II). The responses by neuron type 1 to *E*- and *Z*- $\beta$ -ocimene and  $\beta$ -myrcene was verified by retesting reference samples. Several of the other primary and secondary odorants were retested in the work of Paper III.

### *Paper III*

The study of paper III, using the same method with two parallel columns linked to electrophysiological recordings from single RNs, showed recordings from females of the three heliothine moths *H. virescens*, *H. armigera* and *H. assulta*. Based on 135

tests by GC-SCR from 52 RNs in the three species, the four co-located RNs reported in paper II were functionally described and compared in the three related moth species. From the study of paper II, the primary and most of the secondary compounds were known for three of the RNs in *H. virescens*. In paper III, the primary and secondary odorants of the fourth type were identified in *H. virescens*, and the same four RN types were demonstrated in the other two heliothine species. Additional data on the molecular receptive ranges of the former identified RN types were also provided. Thus the primary (underlined) and secondary odorants for the four neuron types in the three related moths were described as follows: For RN type I, *E*- $\beta$ -ocimene,  $\beta$ -myrcene, *Z*- $\beta$ -ocimene, DMNT and dihydromyrcene, for RN type II *E,E*- $\alpha$ -, *Z,E*- $\alpha$ - and *E*- $\beta$ -farnesene, for RN type III, TMTT, and for RN type IV geraniol, citronellol, (*S*)-(+)- and (*R*)-(-)-linalool, in addition to one unidentified compound.

All neurons of the four types were narrowly tuned, by only responding to these odorants out of hundreds naturally occurring plant volatiles tested. Each RN type of the three species showed similar ranking of primary and secondary compounds according to the response strength, indicating a functional similarity. In addition, all four RN types occurred together in the same recordings of the three species, indicating a similar co-location in the sensilla. These similarities indicate a common evolutionary line of these RNs in the heliothine moths.

Paper III also provides an attempt to trace the axons of the four co-located plant odour RNs into the antennal lobe of the insect brain. The fluorescent dye was applied to the base of the sensillum from which the recordings were made. In one successful staining of *H. assulta* four selectively stained axons in the antennae and four axon terminals in the antennal lobe were obtained. Three of them were located in different areas close to the entrance of the antennal nerve, and the fourth was located in the ventro-medial part of the lobe.

#### *Paper IV*

Using the same methods of GC-SCR (with two parallel GC-columns) and GC-MS, results obtained in the study of paper IV contribute with identification and classification of fourteen out of totally nineteen RN types recorded in the polyphagous heliothine



species *H. virescens* and *H. armigera*. This paper also provides an overview of the plant odour RN types identified so far in the three heliothine species (*H. virescens*, *H. armigera* and *H. assulta*). Altogether these results demonstrate that the olfactory RNs in heliothine species can be classified into distinct types, which correlate well with the principle of one receptor protein type expressed in each neuron of *H. virescens* females (Krieger et al. 2002). The RN types were functionally identified according to the compound eliciting the strongest response (the primary odorant) of which the most frequently recorded type of neurons in this study showed enantioselective responses to the acyclic monoterpene (+)-linalool. The primary odorant for the other RN types were (3Z)-hexenyl acetate, (+)-3-carene, *E*-pinocarveol, *E*-verbenol, vinylbenzaldehyde, 2-phenylethanol, methyl benzoate,  $\alpha$ -caryophyllene and caryophyllene oxid.

Five of the RN types were found in the two species *H. virescens* and *H. armigera*. These types, like the five previously reported RN types (Paper I-III, Strandén et al. 2002, 2003), showed similarities that were noteworthy across the heliothine moths. Not only in the molecular receptive ranges and relative response strengths of primary and secondary compounds, but also the co-locations of RN types corresponded. This indicates that genes coding for important plant odorant receptors in the monophyletic heliothine species studied are conserved through evolution.

All compounds identified were known to be general constituents in several plant materials, e.g. floral compounds, oxidation products of the common monoterpenes  $\alpha$ - and  $\beta$ -pinene, and aliphatic green leaf volatiles. Many of them are known as inducible, e.g. by caterpillar attack. Putative biological functions of the various odorants were discussed, either as attractants for nectar feeding or oviposition stimulants vs. repellents.

Table 1 Survey of the RNs identified in the three heliothine species *H. virescens*<sup>1</sup>, *H. armigera*<sup>2</sup> and *H. assulta*<sup>3</sup> (for details see Table 2, Paper IV) (\* Refers to publications by Strandén et al. 2002; 2003).

Primary odorant	INDIVIDUAL PAPERS				
	I	*	II	III	IV
(-)-Germacrene D	1	2,3			
<i>E</i> - $\beta$ -Ocimene			Type 1 <sup>1</sup>	Type I <sup>1,2,3</sup>	
<i>E,E</i> - $\alpha$ -Farnesene			Type 2 <sup>1</sup>	Type II <sup>1,2,3</sup>	
<i>E</i> -TMTT (4,8,12-trimethyl-1,3,7,11-tridecatetraene)			Type 3 <sup>1</sup>	Type III <sup>1,2,3</sup>	
Geraniol			Type 4 <sup>1</sup> (unidentified)	Type IV <sup>1,2,3</sup>	
(+)-Linalool					Type 1 <sup>1,2</sup>
(+)-3-Carene					Type 2 <sup>1,2</sup>
<i>E</i> -Pinocarveol					Type 3 <sup>1</sup>
<i>E</i> -Verbenol/verbenone					Type 4 <sup>1(2)</sup>
$\alpha$ -Caryophyllene					Type 5 <sup>1,2</sup>
Caryophyllene oxid					Type 6 <sup>1</sup>
Cadinane-type					Type 7 <sup>1</sup>
Vinylbenzaldehyde					Type 8 <sup>1(2)</sup>
Methyl benzoate					Type 9 <sup>2</sup>
2-Phenylethanol					Type 10 <sup>1</sup>
(3 <i>Z</i> )-Hexenol/ (3 <i>Z</i> )-Hexenyl acetate					Type/group 11 <sup>1</sup>
Unidentified					Types 12-14 <sup>1,2</sup>

## Discussion

Through the history of olfactory research, a central question has been which compounds activate the single RNs and can be considered as biologically relevant odorants in the various species. Even today, with the knowledge about the genes coding for olfactory receptor proteins, this question is still unresolved in most vertebrate and invertebrate species for food and plant odour information. When this study started, there was virtually no knowledge on how plant odour information is encoded in the RNs of heliothine moths. Many electrophysiological studies on insect olfactory RNs had been made by direct stimulation with synthetic compounds (review Masson and Mustaparta, 1990; Dickens, 1990). Also in parallel with the present study results on RN tuning obtained by direct stimulation with selected odorants were made (Anderson et al., 1995; Jönsson and Anderson, 1999; De Bruyne et al., 1999; De Bruyne et al., 2001; Shields and Hildebrand, 2001, among others). Altogether, the various studies have reported broadly tuned RNs as well as RNs responding specifically to one or a few compounds. However, tests with selected compounds are restrictive in it self, leaving the question open whether other compounds not tested might in fact be the biologically relevant odorants for the neuron. The method of GC-SCRs was used to test a single neuron for a large number of compounds, for instance sampled from the host plants by headspace collections. The method of GC-SCR, first used in studies of the pheromone RNs (Wadhams, 1982; Löfstedt et al., 1982), were later employed for examining plant odour RNs (Tømmerås and Mustaparta, 1989; Wibe and Mustaparta, 1996; Blight et al., 1995; Stensmyr et al., 2001, 2003; Barata et al., 2002). The improved GC-SCR method with two parallel columns, used in the studies of this thesis, allowed each neuron to be tested for the same mixture via two columns with different properties. This was an important upgrading since the active odorants often were found among the minor constituents, sometimes having overlapping retention times with other components. Thus, the shift of retention times in the polar- and the non-polar columns was important for identifying the active constituents in the GC-MS analyses. After the successful use of the GC-SCR method, a further important step was made by exchanging one of the two columns with a column exhibiting chiral separation properties (Stranden et al., 2002). This was made to study the effect of pure enantiomers on the RNs, as shown in Paper III. The results presented in the papers I-IV, as well as results of other studies in our laboratory, have

demonstrated the importance of using this method of GC-SCR with two parallel columns for identifying biologically relevant plant odorants in insects (Stranden et al. 2002, 2003; Bichão et al., 2003; Bichão et al. in Press; Ulland et al., 2003).

### *Comparative aspects*

Comparative studies of the RNs in related species are important, since the results can provide information about conservation or changes of the functional properties through evolution. The results also become strengthened when the same RN types appear in more than one species. The plant odour receptor system in heliothines is particularly interesting, since these species are considered as a monophyletic insect group, i.e. having a common origin (Matthews, 1999). The two species *H. virescens* and *H. armigera*, living on different continents, have been geographically separated for a long time and presumably exploited different host plant species, at least prior to the introduction of crop hosts they have in common. This suggests that changes between the species in sensitivity to plant odours might have evolved. However, the present studies have shown similarities of RN specificity that is noteworthy. All RN types found in more than one species showed remarkable similarities in molecular receptive ranges as well as in the relative sensitivity to the primary and secondary odorants as demonstrated by the dose-response relationships (Paper III, IV, Stranden et al. 2003). The germacrene D RN type first classified in *H. virescens* (Paper I) and later in the other species (Stranden et al. 2002, 2003), showed striking similarities across the species, both concerning the response properties and as the most frequently occurring type. For all of them (-)-germacrene D had 10 times stronger stimulatory effect than the (+)-enantiomer, and the other compounds like (-)- $\alpha$ -ylangene elicited weaker responses (Stranden et al. 2002; 2003). Also the other RN types identified in two or three species were similar (Papers III-IV). Another interesting feature was the co-location of the same RN types in the three species, shown for the four types presented in paper II and III. Similarities of the olfactory system within and between the heliothine species have also been found in the antennal lobe, by the invariance of number, size, form and position of the ordinary glomeruli (Berg et al., 2002; Skiri HT, Berg BG and Mustaparta H, submitted). Altogether, these studies suggest that both peripheral and some central

features of the olfactory system are conserved in the three species of Heliothinae studied.

It is hypothesised that the species of the subfamily Heliothinae are evolved in a close relationship to agricultural host plants. Thus, the RNs might have been challenged by similar volatiles from the cultivated plants, which may have influenced RN specialisation during evolution. The question of which mechanisms make the heliothine species choose different host plants remains to be answered. Possibly this rely on species-specific olfactory RNs not yet identified or on differences in the central processing of odour information, if not, solely based on the contact chemoreception. In addition to the innate responses, the olfactory system has the capacity of plasticity as shown in experiments on olfactory learning and memory (e.g. reviews Menzel, 2001, Davis, 2004). It is hypothesised that previous experience might induce changes in the host preferences and thereby influence host-selection behaviour in heliothine moth, which could increase the utilization of abundant plants, like in monocultures (Firempong and Zalucki, 1991; Schoonhoven et al., 1998; Cunningham et al., 1999; West and Cunningham, 2002; Jallow et al., 2004). In studies combining appetitive olfactory learning and dual-choice wind tunnel tests, Cunningham et al. (2004) showed that *H. armigera* females trained on a certain odour preferred plants that were enhanced with the particular odour. However, olfactory learning does not seem to be the only mechanism influencing the different host plant choices. Laboratory experiments carried out with virgin females have shown that *H. armigera* and *H. assulta* choose different plants when given equal options (Wang et al., 2004).

To resolve the questions about the mechanisms underlying host plant selection, more studies are required. We know from studies of pheromone receptions in heliothine moths that RNs with similar specificity mediate different behavioural response in different species. According to this, the first step is to find out whether the identified plant odorants elicit similar or different behavioural responses in the females of the three species. So far, the primary odorant (-)-Germacrene D activating functionally similar RNs in all three species (Paper I, Strandén et al. 2002; 2003), has been shown to mediate attraction both of *H. virescens* and *H. armigera* females (Mozuraitis et al., 2002; Gregg, personal communication). The aliphatic leaf odorant (3Z)-hexenyl acetate, which is the primary odorant of RN type 11 in *H. virescens* (Paper IV), is one of the

compounds released by tobacco plants at night when infested with *H. virescens* larvae. Thus, it might be involved in host repellence of *H. virescens*, preventing egg-laying on plants already occupied by conspecific caterpillars (De Moraes et al. 2001). The presence of (3Z)-hexenyl acetate responding RNs in *H. armigera* is indicated by electroantennographic responses (Burguiere et al., 2001). In this species, (3Z)-hexenyl acetate mediate attraction to unmated *H. armigera* females when presented as a single compound in wind tunnel experiments (Gregg and Del Socorro, 2002). The opposite behavioural response observed in the two studies might either be due to species-specific differences of responses by different neurones to (3Z)-hexenyl acetate. Alternatively, mated and unmated females may respond differently. Possibly the repellence shown in *H. virescens* may in fact have been caused by other compounds. An interesting comparison of neuron specificity and behavioural responses across species has been made for the taste system of heliothine caterpillars. The deterrent sensitive neurons of the taste sensilla (*sensilla styloconica*) of two heliothine caterpillars (*Heliothis subflexa* and *H. virescens*) showed no differences, neither in firing rate nor in adaptation to the taste stimuli tested (Bernays and Chapman, 2000). However, the behavioural threshold for rejection of toxic plant compounds (selected deterrents) during feeding was found to be lower in caterpillars of the monophagous *H. subflexa* than in those of *H. virescens* (Bernays et al., 2000). This implies a loss through evolution in the polyphagous *H. virescens* larvae to detect the compounds, after overcoming the toxicity. These results led to the conclusion that the different feeding behaviour of the two species were caused by different coding in the CNS rather than by differences in the peripheral sensory system. A similar principle may apply to the olfactory system.

When comparing RNs specificity in related species, one question is whether the same types are also present in unrelated species, which may point to a convergent evolution of the receptor proteins. RNs specialized for the same primary odorants as those found in heliothine species are also found in distantly related species. For instance RNs detecting *E*- $\beta$ -ocimene has been shown in *Spodoptera* moths as well as in the two weevils *Anthonomus grandis* (cotton weevil) and *Anthonomus rubi* (strawberry weevil) (Dickens, 1990; Jönsson and Anderson, 1999; Stensmyr et al., 2001; Bichão et al., in press). In some cases it is difficult to make a complete comparison of RN specificity because different test protocols have been used. In the studies of the strawberry weevil

*A. rubi* (Coleoptera: Curculionidae) and the lepidopteran moth *Mamestra brassica* similar test protocols as for the heliothine species were used (Bichão et al., in press; Ulland et al., 2003). Both similarities and differences of the RNs molecular receptive ranges appeared across these species. RNs tuned to linalool that were identified in all species (except *H. assulta*) responded secondarily to dihydrolinalool (Ulland et al. 2004). In contrast, other RNs tuned to the same primary odorants showed differences in sensitivity to the secondary odorants. The (-)-germacrene D RN type in the weevil (*A. rubi*) showed response to  $\beta$ -caryophyllene and no response to  $\alpha$ -ylangene, whereas the opposite was the case for the (-)-germacrene D RNs of the heliothine moths (Stranden et al., 2002, 2003; Bichão et al., in press). The differences in molecular receptive ranges observed for some of the RN types in the distantly related species may reflect an independent evolution of the RN specificity during the adaptation to similar compounds of different host plants, or to chance mutation of common ancestral genes.

#### *Coding of odour quality*

The basis for recognition and discrimination of odour qualities in animals is the presence of RNs with different specificities for the odorants. In contrast to the visual system, operating with only three types of cones as bases for colour vision, the olfactory system is equipped with a much larger number of receptor protein types (Buck and Axel 1991). Each type is expressed in subsets of RNs, which projects to one or two glomeruli in the primary olfactory centre (reviewes Axel, 1995, Breer, 2003b). This implies a certain ratio between the number of glomeruli and the number of sensory neurons. Whereas important processing of the visual information occurs in the retina, the olfactory information in vertebrates and insects is directly conducted by the RN axons to the primary olfactory centre of the brain. Certain principles of information processing typical in vision, like convergence on higher orders of neurons and lateral inhibition, is also important in the olfactory system, particularly studied in the primary olfactory centre (the AL of insects and the olfactory bulb of vertebrates). In insects, a large number of olfactory RNs converge on a smaller number of AL neurons (with a ratio of  $\sim 1000:1$ ). Local AL inter-neurons provide lateral inhibition between glomeruli, which seems to be important in enhancing the contrast between active and inactive glomeruli

(Smith and Shepherd, 1999). Thus, activation of specific glomeruli by an odorant represents the code of the odour quality. This is particularly studied by optical recordings in honeybees, heliothine moths and the fruit fly (Galizia et al., 1999; Galizia et al., 2000; Galizia and Kimmerle, 2004; Skiri et al., 2004; Hallem and Carlson, 2004b). For interpreting results from this kind of studies, knowledge about the RN specificity is important and is in the present study provided for several RN types in heliothine moths.

The papers I-IV present 19 types of distinctly classified olfactory RNs in heliothine moths. However, a larger number is expected to be present, both indicated by the present electrophysiological data, and by the number of about 60 ordinary glomeruli in the antennal lobe of the three heliothine species studied (Berg et al., 2002; Skiri HT, Berg BG and Mustaparta H, submitted). With a ratio of 1:1 or 1:2 between the number of RNs and the ordinary glomeruli the heliothine antenna is expected to comprise in the range of 30-60 RN types. In future GC-SCR studies, we expect that additional RN types are recorded and classified according to their molecular receptive ranges. From the results of RNs so far obtained, the sharp tuning to one primary odorant and the low overlap of the molecular receptive ranges give the impression that the information about each odorant is mainly mediated by one RN type, similar to the pheromone system in these and other moth species. This correlates well with the expression of one receptor protein type in each RN, as also indicated by the molecular biological study of olfactory gene expression in *H. virescens* (Krieger et al., 2002; 2004). Thus, the plant odour system in the heliothine moths seems to operate according to the principle of “labelled-line” system, at least at low concentrations. However, this principle does not hold true for all the identified primary and secondary odorants. For instance (+)-linalool is the primary odorant for one RN type and a secondary odorant for another type (papers III and IV). A second example of overlap is between secondary odorants of two RN types responding to oxygenated bicyclic monoterpenes (Paper IV). Whether overlapping molecular receptive ranges is an important feature in the coding of plant odour information in heliothine moths remains to be seen in future studies, when the molecular receptive ranges of more RN types are identified. The relatedness of the few molecules out of hundreds tested, which activates the same type of RNs, support the principle that structurally similar molecules have a higher probability to bind to the



same receptor proteins. This also explains the few cases of overlapping molecular receptive ranges within the same chemical group. This has also been shown in other studies of moths and weevils. Typical in all studies is that RNs of different chemical groups show no, or only minimal, overlap (Wibe and Mustaparta, 1996; Wibe et al., 1997; Stensmyr et al., 2001; Barata et al., 2002; Bichão et al., 2003). These results appear different from what is discussed according to results obtained in *Drosophila*, where a larger degree of overlap is found between the olfactory RNs (De Bruyne et al. 1999; 2001; Stensmyr et al. 2003; review Hallem and Carlson, 2004a).

The structure-activity relationships of the various RNs presented in this thesis indicate several molecular features of importance in receptor-ligand interaction. These are chirality, carbon chain length, electron dense parts and the flexibility of the molecules, which are reflected by enantiomers, number of C-atoms, double bonds and open vs. cyclic structures. These features are considered universal among receptor-ligand interactions in the olfactory system (Kafka, 1974; Priesner, 1977, 1979; Schneider et al., 1977; Bengtsson et al., 1990; Ohloff, 1986, 1994; Masson and Mustaparta, 1990, Mustaparta, 2002; Leal, 2001; Wibe et al., 1997, 1998; Borg-Karlson et al., 2003; Bichão et al., 2003; Bichão et al., in press; Laska, 2004, among others).

The results obtained in this thesis have been, and are currently, used in various other studies. This includes the use of optical recordings to study the representation of plant odorant qualities in the antennal lobe (Galizia et al., 2000; Skiri et al., 2004). Specific activity in distinct areas of the AL, mainly covering one or two glomeruli, has been shown for single odorants (Skiri et al., 2004). In addition, attempts have been made to trace the olfactory RN axons in the antenna lobe of heliothine females. These results show some correlation with the results from optical recordings (Paper III and Strandén et al. 2003). Thus, these preliminary results indicate that RNs responding to the same primary odorant project in one or a few glomeruli in the antennal lobe similar to what is found for the pheromone system in heliothine males (Berg et al., 1998; Berg, 1998). This is also in accordance with the current knowledge particularly from molecular studies in vertebrates and insects. For instance in *Drosophila*, the projections in one or two glomeruli are determined for RNs with identified genes coding for receptor proteins and described molecular receptor ranges (Keller and Vosshall, 2003; Hallem and Carlson, 2004a).

Ultimately, determining how odour information is coded in the brain requires linking a specific olfactory input with a behavioural output and correlating this with a measure of synaptic activity in the brain. This can be done in tests showing the ability of animals to discriminate between biologically relevant odorants, for instance by the use of the proboscis extension reflex in nectar feeding insects. In *H. virescens*, this reflex has been used to test some of the identified primary and secondary odorants (Skiri et al., in press), and these kind of studies are continuing for the other odorants identified in the present studies. As expected, Skiri and co-authors showed that *H. virescens* females, in a dose-dependent manner, were able to learn and to discriminate between linalool and both  $\beta$ -ocimene and  $\beta$ -myrcene, identified as primary and secondary odorants in paper III-IV. Surprisingly, the moths also showed the ability to discriminate between  $\beta$ -ocimene and  $\beta$ -myrcene, which in our experiments always activated the same RNs (Papers II, III). These findings, which were supported by results from  $\text{Ca}^{2+}$ -imaging experiments (Skiri et al., 2004) were explained by the possible presence of other RN types not yet identified, which responded to only one of the two odorants. In addition, impurities present in the samples at the relatively high concentrations tested might influence the discrimination. Also further processing of the olfactory information in AL projection neurons as well as higher orders of neurons (in the mushroom bodies and lateral protocerebrum) are important and may account for the discrimination of the two similar odorants (review Davis, 2004). For instance, in one study of the honeybee, synchronisation and temporal coding is suggested to be important in the discrimination of similar odorants (Stopfer et al., 1997).

#### *Coding of odour intensity*

The olfactory system seems to have the capacity to give information about odour intensity over several orders of magnitudes. The mechanisms involved in the coding of intensity can be ascribed to different response strengths of each RN to increased concentrations, to different sensitivity of each RN, as well as to central nervous mechanisms. The present studies present information about plant odour RNs types that is very sensitive to the primary odorants. In addition, the RNs within each type also show some variation in sensitivity. The best example comes from the frequently recorded (-)-germacrene D RNs, of which the most sensitive neurons responded to

concentrations lower than the GC-detection limit, i.e. 1-10 pg (see Paper I) whereas higher concentrations are needed to activate other (-)-germacrene D RN. The dose-response relationship showed that these neurons increase firing rates over 4-5 log units (Paper I, Strandén et al., 2003). Obviously, this intensity range can be transmitted directly to the AL neurons. Further increase of intensity might be provided by recruitment of RNs with lower sensitivity. This mechanism has previously been suggested for detection of the major pheromone compound Z-11-16: AL in heliothine moths, having numerous sensilla along the antenna with RNs showing the same selectivity but different sensitivity to the same compounds. AL projection neurons of these moths respond with different sensitivity to antennal stimulation with the major component. This may be due to direct input from RNs with different sensitivity or to different numbers of RNs converging on each AL neuron. For the plant odour system the data that correlate sensitivity of the RNs to the sensitivity of the projection neurons are scarce. In general, a low sensitivity is observed for the projection neurons responding to antennal stimulation with plant odorants (Roche King et al., 2000; Anton and Hansson, 1995; Greiner et al., 2002; Masante-Roca et al., 2002; Reisenman et al., 2004). This may be due to down regulation of the neurone sensitivity, by modulation of serotonin or octopamin (Kent et al., 1987; Sun et al., 1993; Kloppenburg and Hildebrand, 1995; Mercer et al., 1996). Alternatively, the responses recorded from the projection neurons are not ascribed to stimulation with the primary odorant of the RNs giving the input. Results from optical recordings experiments ( $\text{Ca}^{2+}$ -imaging) in various species, including heliothine, shows that an increasing number of glomeruli were recruited with increasing odour concentrations (Sachse and Galizia, 2003; Carlsson and Hansson, 2003; Skiri et al., 2004). In the honeybee, this increase seems to be due to overlapping molecular receptive ranges of the RNs. 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. The large number of very sensitive RNs responding to germacrene D in *H. virescens* indicates that this compound may play a significant role over a long distance, probably in attraction (Mozuraitis et al., 2002). Recruitment of (-)-germacrene D RNs with lower sensitivity may be activated closer to the odour source, and give additional information about the intensity at short range.

### *Behavioural implications*

During recent years, attention has been given to induction of compounds emitted by a plant. Both biotic and abiotic factors influence the production and release of volatiles. Interesting biotic factors are volicitin and structural analogue compounds present in caterpillar saliva (Alborn et al., 1997; Mori et al., 2001, 2003). During feeding, these are suggested to induce production and release of certain compounds like (3Z)-hexenyl acetate, (3Z)-hexenol, *E*- $\beta$ -ocimene, linalool, *E*-4,8-dimethyl-1,3,7-nonatriene, *E*- $\beta$ -farnesene, and *E,E*- $\alpha$ -farnesene (Loughrin et al., 1994; McCall et al., 1994; Röse et al., 1996; De Moraes et al., 2001; Heiden et al., 2003; Röse and Tumlinson, 2004). It is also assumed that the induced release of volatiles is partly due to the constant cutting of leaf tissue during feeding. However, both qualitative and quantitative differences of the induced volatile profiles appear by feeding of different moth species, including the heliothines (Mori et al., 2001; Röse and Tumlinson, 2004; De Moraes and Mescher, 2004). This indicates that species specific factors in the saliva influence the induction. Compounds induced by *H. virescens* caterpillars have been suggested to mediate host repellence, preventing mated conspecific females to lay eggs on infested plants (De Moraes et al., 2001). This repellence behaviour might be mediated by several of the RN types identified in this study (Papers II-IV); detecting *E*- $\beta$ -ocimene [secondary odorant *E*-4,8-dimethyl-1,3,7-nonatriene], (3Z)-hexenyl acetate and (3Z)-hexenol, *E,E*- $\alpha$ -farnesene [secondary odorant *E*- $\beta$ -farnesene], (+)- and (-)-linalool, respectively. The enantiomeric ratio of linalool emitted during caterpillar feeding is not reported.

To become an insect repellent signal, the compound produced by the direct defence mechanisms in infested plants might be associated with increased concentrations of toxins or a lower nutrition value, having a negative effect on the development and survival of the offspring (Mori et al., 2001). The fitness advantages to herbivores avoiding oviposition on induced plants are obvious, as these plants are likely to host not only larvae that represent potential competitors for the offsprings, but may also attract natural enemies. Parasitoids and predators of the heliothines have the ability to learn to discriminate between plant emitted volatiles that are induced by different species of caterpillars (Meiners et al., 2002, 2003). Two of the compounds commonly induced by heliothine caterpillars (e.g. *H. virescens*), linalool and  $\beta$ -ocimene, are attractive to various parasites and predators (De Moraes et al., 1998; De Moraes and

Mescher, 2004, Röse and Tumlinson 2004). The two compounds are not induced in the host plant *Physalis angulata* when attacked by caterpillars of the monophagous *Helicoverpa subflexa* (De Moraes and Mescher, 2004). This is explained by the adaptation of *H. subflexa* to monophagy on *Physalis* fruits, lacking linolenic acid. Other plant species contain this acid, which is required both for development and morphogenesis of most insects, and important for the induction of linalool and  $\beta$ -ocimene in plants (De Moraes and Mescher, 2004). In this way, *H. subflexa* caterpillars feeding of *Physalis* fruits exhibit a clear competitive advantage as compared with *H. virescens*, by overcoming the lack of linolenic acid and not inducing attraction to the predators (De Moraes and Mescher 2004).

Plants emit blends of volatiles that vary both qualitatively and quantitatively in different parts of the plant, as well as with age or during the diurnal or seasonal cycles. Herbivore insects might exploit signals specific for these conditions when searching for a host. In nocturnal emission of flowering tobacco plants, it is found a four-fold increase in the amount of aromates like 2-phenylethanol, methyl benzoate and benzaldehyde (Raguso et al., 2003). It was suggested that these floral compounds are produced for attracting night active pollinators and may also serve as cues for the noctuid herbivorous moths in their search for nectar (Raguso et al., 2003). This is for instance shown for 2-phenylethanol tested in a two-choice olfactometer (Gregg and Del Socorro, 2002). Many other compounds identified as primary and secondary odorants for the heliothine species are shown to be attractive to mated or unmated females in various behavioural bioassays. These include linalool, 3-carene, geraniol,  $\alpha$ -caryophyllene,  $\beta$ -caryophyllene and (-)-germacrene D, which were either tested as single compounds or constituents added to blends (Rembold and Tober, 1985; Rembold et al., 1991; Jallow et al., 1999; Bruce and Cork, 2001; Hartlieb and Rembold, 1996; De Moraes et al., 2001; Mozuraitis et al., 2002; Gregg and Del Socorro, 2002; Robert Heath, personal communication). (-)-Germacrene D is particularly interesting because of the numerous RNs found on the antenna of the three heliothine species. This compound, tested in two independent studies, has been indicated to act as an oviposition stimulant and/or attractant for mated *H. virescens*, and as an attractant for virgin *H. armigera* females (Mozuraitis et al., 2002; Peter Gregg, personal communication).

*Concluding remarks and directions for future work*

Striking similarities were found when comparing the RN specificity of the three heliothine species. Furthermore, the RNs were tuned to general plant constituents of which many seems to be related to certain condition of the plant e.g. caterpillar attack. Because these species are able to utilize a broad range of host plants, it is possible that other RN-types might be revealed when including more plant species. More electrophysiological data, in particular on the less studied oligophagous *H. assulta*, is required for detailed comparisons of the RN types. In addition, other species of the subfamily heliothine may be included in future studies. *H. subflexa* is particularly interesting because of its specialisation on *Physalis* fruits. Although our results are complementary to other studies showing behavioural significance of some of the odorants identified, more behavioural studies are required to elucidate the biological role of the various odorants. Interesting objectives are studies concerning the ability of the moths to learn and to discriminate single components and mixtures of the identified odorants.

The results obtained in the present studies have shown 19 types of olfactory RNs of which primary and several secondary odorants are identified for 16 of them (Paper I-IV, Strandén et al. 2002, 2003). These data may be used in future studies of the peripheral olfactory events, including odorant-receptor interactions and transduction mechanisms. This requires molecular biological characterisations of olfactory genes and receptor proteins in heliothine moths, studies that are in progress by Krieger et al. (2002; 2004). The identified odorants are also used in studies of the central processing of olfactory information and olfactory learning, carried out in our laboratory. In addition, the use of plant odorants in integrated control of heliothine moths makes the present results interesting also for applied research.

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**Individual papers**