# Olfactory coding and olfactory learning of plant odours in heliothine moths.

**An anatomical, physiological and behavioural study of three related species (*Heliothis virescens*, *Helicoverpa armigera* and *Helicoverpa assulta*).**

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## PAPERS INCLUDED IN THE THESIS

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

I. Skiri, HT, Berg, BG and Mustaparta, H. 2004. Digital 3D atlases of the antennal lobes of the moths *Helicoverpa armigera* (male and female) and *Helicoverpa assulta* (female) based on optical sections acquired by confocal laser scanning microscopy. Submitted to the Journal of Comparative Neurology.

II. Skiri, HT, Galizia, CG and Mustaparta, H. 2004. Representation of primary plant odorants in the antennal lobe of the moth *Heliothis virescens* using calcium imaging. Chem. Senses, 29: 253-267

III. Skiri, HT, Stranden, M, Sandoz, JC, Menzel, R and Mustaparta, H. 2004. Associative learning of the plant odorants *racemic* linalool, β-ocimene and β-myrcene in the moth *Heliothis virescens*. Submitted to the Journal of Experimental Biology.

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# ABBREVIATIONS

AL – antennal lobe

Z11-16:Al - cis-11-hexadecenal

Z9-16:Al - cis-9-hexadecenal

Z9-14:Al - cis-9-tetradecenal

GOBP – general odorant-binding protein

MGC – macroglomerular complex

OBP – odorant-binding protein

PBP - pheromone-binding protein

PER – proboscis extension response

RN – receptor neurone

SOG – suboesophageal ganglion

VUM - ventral unpaired median

# INTRODUCTION

## The olfactory system

The chemical senses, present in all groups of animals even in single cell organisms, are considered as the oldest of our senses. They play a major role in selection of food and in avoiding ingestion of toxins. In addition to be an essential part of what is perceived as the "taste of food" (flavour), olfaction is also important in mating and reproduction, social organisation, predator – prey interaction, as well as in finding suitable places for offsprings, like egglaying in insects. The odours given off from all the sources are complex mixtures of volatiles. Thus, the challenge for the organism is to detect a wide range of molecules that mediates the information of interest and to discriminate these from other, not relevant odorants in the environment. To meet these challenges the various organisms have evolved olfactory systems with special features, many of which are shared across phyla. Similar mechanisms are particularly demonstrated in the first two stages of the olfactory pathway, the receptor neurones (RNs) and the first relay station, the primary olfactory centre, which is the antennal lobe (AL) in insects and the olfactory bulb in vertebrates.

In organisms of all phyla the sensory cells are small bipolar neurones with thin cilia or dendrites containing the receptor proteins, and one thin unmyelinated axon projecting in the primary olfactory centre. Here they make synapses with the central neurones in the spherical structures termed glomeruli. Both in the glomeruli of vertebrates and invertebrates a network is formed by the primary axons, local interneurones and projection neurones, where the first processing of olfactory information takes place. Excitatory synapses between RNs and AL projection neurones, as well as inhibitory and excitatory synapses between local interneurones and projection neurones, are believed to cause excitation within a glomerulus, and lateral inhibition as well as disinhibition between glomeruli in vertebrates and insects (Smith and Shepherd, 1999).

Other similarities are found in the organisation of the olfactory system in two separate pathways, one main system dedicated to food or general odours and another

accessory system dedicated to pheromones. In mammals, the olfactory epithelium of the main olfactory system is located in the upper nasal cavity, from where the primary axons project into the main olfactory bulb. The sensory epithelium of the accessory olfactory system, the vomeronasal organ, is located ventrally in the nasal cavity of many mammals (Døving and Trotier, 1998). It is also present in amphibians and some reptiles. Projections from these RNs are in a separate accessory olfactory bulb, which is found dorsally of the main olfactory bulb in mammals (Wysocki and Meredith, 1987). Also in herbivorous Lepidopterans there are separations between the olfactory pathways for information about insect produced signals and food or host plant odours. The RNs sensitive to the two kinds of odorants are located in different sensilla types. In the male AL the two kinds of odour information is kept separate by the macroglomerular complex (MGC) dedicated to the pheromones and the inter-specific signals, and the numerous ordinary glomeruli to plant odours (Mustaparta, 2002).

Learning and memory play an important role in olfaction. In humans it is claimed that most odours are not inborn, but are easily learned (perhaps except odours of decaying food) (Köster, 2002). This means that we can learn to like or dislike almost all odours. We know the expression that "no memory is as strong as the memory about a bad meal," which refers to flavour aversion learning. The importance of learning odours of a good meal is also experienced in our everyday life. In insects, olfactory learning is well documented in field as well as in laboratory studies. The most extensive studies have been performed on the honeybee. Since von Frisch first demonstrated that honeybees can learn colour in connection with feeding, appetitive learning of odours has also been well documented in this species (rewieved by Menzel, 2001). This thesis is focusing on sensory mechanisms involved in olfactory coding and the use of associative learning to find out how well heliothine moths can learn and discriminate selected odorants of biological relevance.

## Insect antenna and sensilla

In contrast to the inverted olfactory epithelium in vertebrates, the insect olfactory sensilla are situated on the antennae, being exposed to the ambient air. The antenna, showing strikingly different shapes in various insects (Schneider and Steinbrecht,

1968), are filiform in heliothine moths, consisting of two proximal segments (scape and pedicel) and one long flagellum with 80 to 81 annuli (Almaas and Mustaparta, 1990). Only the side frontally oriented during flight, bears the numerous sensilla with different sensory modalities for chemo-, mechano-, temperature- and humidity-sensation (Almaas and Mustaparta, 1991; Jørgensen, 2003; Kvello, 2003; Lassa, 2004). Like in many other species, the olfactory sensilla in heliothine moths are classified in several morphological types *sensilla trichodea*, *sensilla basiconica*, *sensilla auricillia* and *sensilla coeloconica* (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). Different functions are ascribed to these sensilla in various insects, like detection of pheromones by *s. trichodea* and detection of plant odours by *s. basiconica*. In heliothine moths two types of *s. trichodea* are present, the type 1 being male specific, detecting the female produced pheromones and the type 2, present in both sexes, probably detecting pheromone and plant odours in males and only plant odours in females (Almaas and Mustaparta, 1991).

The outer structures of many olfactory sensilla, like *s. trichodea* and *s. basiconica* are hair formed with the cuticle walls perforated by numerous pores, which allow the hydrophobic odour molecules to enter the sensillum (Steinbrecht, 1997). The olfactory sensilla have one to several RNs with dendrites penetrating the hair lumen, which is filled with receptor lymph. The RNs are surrounded by three auxiliary cells, the thecogen, thormogen and trichogen cells (Schneider and Steinbrecht, 1968; Keil and Steinbrecht, 1984). In *Heliothis virescens*, *Helicoverpa assulta* and *Helicoverpa zea* most *s. trichodea* contains 2 to 4 dendrites (Koh *et al.*, 1995; Færavaag, 1999). This number corresponds to the number of units showing activity in electrophysiological recordings from these sensilla (Almaas *et al.*, 1991; Almaas and Mustaparta, 1991; Hansson *et al.*, 1995; Berg *et al.*, 1998; Skiri, 1999; Røstelien *et al.*, 2000a, b; Stranden *et al.*, 2003a, b; Berg *et al.*, 2004).

## Perireceptor events and transduction

The sensillum lymph in insect olfactory sensilla and the mucus of the olfactory epithelium in vertebrates contain odorant-binding proteins (OBPs) that seem to be

important both as a filter to protect the RNs from irrelevant molecules, and as a transporter of the odour molecules to the receptor proteins located in the membrane of the dendrites and the cilia of the RNs (Breer *et al.*, 1990b; Krieger *et al.*, 1993; Prestwich *et al.*, 1995; Garibotti *et al.*, 1997; Steinbrecht, 1998; Pes *et al.*, 1998; Vogt *et al.*, 1999). Particularly interesting in insects is that specific types of OBPs are found in different sensilla types. In noctuid moths pheromone-binding proteins (PBPs) are mainly present in the *s. trichodea* and the general odorant-binding proteins (GOBPs) in *s. basiconica* (Zhang *et al.*, 2001). OBPs have also been identified in other insect species including *Drosophila melanogaster* (Shanbhag *et al.*, 2001; Graham and Davies, 2002). Recent studies have shown that PBPs in moths can bind to different molecules, but only the appropriate ligand induces structural changes in the proteins (Mohl *et al.*, 2002; Bette *et al.*, 2002; Maida *et al.*, 2003). Changes of conformation of the PBP has also been shown with changing pH (Wojtasek and Leal, 1999; Damberger *et al.*, 2000; Horst *et al.*, 2001). It is suggested that unfolding of the PBP structure is triggered by contact with the charged membranes, which might be a mechanism for ligand release upon interaction with the surface of the dendrites (Horst *et al.*, 2001). Another study on the moths *Antheraea polyphemus* and *Bombyx mori* have indicated that PBPs together with the pheromone component seem to contribute to the excitation of the RNs (Pophof, 2004).

After interaction between the odour molecule (or in complex with the OBP) and the receptor protein, an intracellular cascade reaction (the transduction event) takes place, leading to opening of cation channels and depolarisation of the cell membrane. Both in vertebrates and invertebrates the transduction event is mediated by G-proteins (Breer *et al.*, 1988; Jones and Reed, 1989; Laue *et al.*, 1997). However, different intracellular pathways occur in the two groups, in insects via the second messenger inositol 1, 4, 5-trisphospate ($IP_3$) (Breer *et al.*, 1990a) and in vertebrates via the adenosine 3, 5-monophosphate (cAMP) (Nakamura and Gold, 1987). It has also been discussed whether the cAMP pathway may contribute in the transduction elicited by some odorants in insects, because of the presence of a cAMP sensitive ion channel in the antenna (Krieger *et al.*, 1999).

## Olfactory receptor neurones and receptor proteins

The RNs are functionally classified according to which odorants they respond to. In contrast to RNs in vertebrates and in some insect species (De Bruyne *et al.*, 2001), which are found to respond to a wide range of molecules (broadly tuned) (Smith and Shepherd, 1999), the RNs in most studies of Lepidopterans and beetles show a narrow tuning (reviewed by Mustaparta, 2002). After the first study of the silkmoth *B. mori*, demonstrating RNs specialised for the pheromone bombycol, narrowly tuned RN-types for pheromone components have been shown in many insect species, particularly in Lepidopterans (Kaissling and Priesner, 1970; Almaas and Mustaparta, 1990; Almaas *et al.*, 1991; Hansson *et al.*, 1995; Berg and Mustaparta, 1995; Berg *et al.*, 1998; Vickers *et al.*, 1998; Cossè *et al.*, 1998; Skiri, 1999; Larsson *et al.*, 2002; Vickers and Christensen, 2003; Berg *et al.*, 2004). In more recent studies of many species, RN-types responding to plant odorants have been classified in detail by the use of electrophysiology combined with gas chromatography (Blight *et al.*, 1995; Wibe and Mustaparta, 1996; Wibe *et al.*, 1996; Røstelien *et al.*, 2000a, b; Stensmyr *et al.*, 2001; Barata *et al.*, 2002; Stranden *et al.*, 2002; Bichão *et al.*, 2003; Stensmyr *et al.*, 2003; Stranden *et al.*, 2003a, b). These studies have shown a narrow tuning also of plant odour RNs, each type characterised by a major response to one or a few odorants (primary odorants), and much weaker responses to a few other (secondary odorants) having similar chemical structures.

The classification of distinct RN-types is in accordance with the results from molecular biological studies showing that each sensory cell express only one receptor subtype, as shown in insects (*D. melanogaster*) and vertebrates (Ressler *et al.*, 1993; Vassar *et al.*, 1993; Clyne *et al.*, 1999; Vosshall *et al.*, 1999). Also in *H. virescens* the candidate receptor proteins identified so far show expression of one type in each neurone (Krieger *et al.*, 2002). In general the receptor proteins in *H. virescens* have shown low homology with those in the fruitfly *D. melanogaster* and the mosquito *Anopheles gambiae*, with exception of one subtype (the Drosophila Dor83b, the Anopheles AgamGPRor7 and the moth HR2), that share a high degree of sequence identity in several species (Clyne *et al.*, 1999; Vosshall *et al.*, 1999, 2000; Fox *et al.*, 2001; Krieger *et al.*, 2002; Hill *et al.*, 2002; Krieger *et al.*, 2003). Unlike the other

subtypes these proteins are expressed in a large number of antennal neurones and are co-localised with the others, which has indicated a different function in insect olfaction. The olfactory receptor proteins belong to a large family of G-protein linked receptors, the metabotrophic receptor types (7-transmembrane receptors) (Buck and Axel, 1991). Since the first identification and characterisation of these receptor genes, large numbers of olfactory receptor proteins have been identified in many organisms.

## The antennal lobe

Evidence for the early idea proposed by Lord Adrian (1951), that glomeruli might be functional units involved in coding of odour quality is particularly given through molecular biological studies in *D. melanogaster* and in vertebrates, showing that RNs expressing the same type of receptor proteins project in one or two glomeruli (Ressler *et al.*, 1994; Vassar *et al.*, 1994; Mombaerts *et al.*, 1996; Vosshall *et al.*, 2000; Gao *et al.*, 2000). Also a number of functional tracing experiments and optical recordings in insects and vertebrates have indicated that each glomerulus is involved in processing information about a particular odour quality. For instance, by optical imaging studies and functional tracing of pheromone RNs and projection neurones in the AL of Lepidopteran species, each MGC glomerulus is shown to be specified for one compound of the pheromones or the insect produced signals (Christensen and Hildebrand, 1987; Hansson *et al.*, 1991; Christensen *et al.*, 1991, 1995; Hansson *et al.*, 1995; Ochieng *et al.*, 1995; Berg *et al.*, 1998; Vickers *et al.*, 1998; Galizia *et al.*, 2000; Carlsson *et al.*, 2002; Vickers and Christensen, 2003; Berg *et al.*, 2004). The tracing studies in *H. virescens* males have shown a correspondence between the input to and the output from the glomeruli in the MGC, in contrast to a mismatch found in *Agrotis segetum* (Hansson *et al.*, 1994; Anton and Hansson, 1999). Correspondence between input and output has also been found in ordinary glomeruli in the honeybee *Apis mellifera* by optical imaging measuring simultaneously from RNs and projection neurones (Sachse and Galizia, 2002). Interesting studies are now being performed with a genetically encoded fluorescence probe, in the fruitfly *D. melanogaster*, allowing optical measurements of projection neurones (Fiala *et al.*, 2002) or RNs (Pelz *et al.*, 2003) of common genetic identity. Intracellular recordings combined with stainings,

have been carried out in a number of species, with the intention to show the functional organisation and output of the ordinary glomeruli (Anton and Hansson, 1994, 1995; Roche King *et al.*, 2000; Sadek *et al.*, 2002; Masante-Roca *et al.*, 2002; Galizia and Kimmerle, 2004; Reisenman *et al.*, 2004; Rø *et al.* in progress). Further evidence for functional organisation of glomeruli are given by the conserved activity pattern across individuals, as well as between the right and left ALs, as shown in calcium imaging studies of the honeybee (Galizia *et al.*, 1998).

The information given to the projection neurones is not only direct, excitatory input from RNs, but also information given via local interneurones, both in insects and vertebrates (Waldrop *et al.*, 1987; Boeckh and Tolbert, 1993; Christensen *et al.*, 1993; Sun *et al.*, 1997). The local interneurones in insects are characterised by dendritic arborisations in several or many glomeruli. These neurones usually have an inhibitory function, most of them being GABAergic in moths and cockroach and GABAergic and histaminergic in the honeybee (Witthöft, 1967; Hoskins *et al.*, 1986; Waldrop *et al.*, 1987; Distler, 1990; Sachse, 2002). Having extensive arborisations in the AL, they are believed to provide contrast enhancement between glomeruli (Sachse and Galizia, 2002; Lei *et al.*, 2002). In *Manduca sexta* they are shown to sharpen the pulsed responses of the projection neurones by providing inhibition between odour pulses (Christensen *et al.*, 2000; Lei *et al.*, 2002; Christensen *et al.*, 2003). A third important function of the local interneurones is to synchronise the activity of populations of projection neurones, as shown by multi-electrode recordings in the pheromone system of the moth *M. sexta* (Lei *et al.*, 2002) and the plant odour system of the cockroach *P. americana* and the honeybee *A. mellifera* (reviewed by Stopfer *et al.*, 1999). In contrast to the studies in *M. sexta* the synchronisation in the two other species was found to be oscillatory and was proposed to contribute to odour discrimination. In accordance with this, blocking of GABAergic local interneurones made the honeybee lose the ability to distinguish between similar odours (Stopfer *et al.*, 1997). It is possible that the different findings in moths vs. honeybees and locusts are due to different coding principles in these species, or to differences in the pheromone vs. plant odour detecting system.

## The mushroom body and the lateral protocerebrum.

In insects the projection neurones conduct the olfactory information to two higher centres in protocerebrum, the mushroom body shown to be important in learning and memory, and the lateral horn, a pre-motoric area. The projection neurones follow three major tracts from the AL to the protocerebrum, both in the honeybee (reviewed by Menzel and Müller, 1996) and the moth (Homberg *et al.*, 1988). In the moth these tracts are named the inner-, the outer- and the middle antenno-cerebral tract. The inner tract projects first in the calyces of the mushroom body and then in the lateral protocerebrum, the outer tract first in the lateral protocerebrum and then in the calyces, whereas the middle tract projects only to the lateral protocerebrum. The calyces, formed by the input regions of the numerous Kenyon cells (Mobbs, 1982) receive the odour information from a much smaller number of AL projection neurones. Each projection neurone give off five collaterals that projects in a large part of the lip region of the calyses, i.e. transmitting information to a large number of Kenyon cells (Müller *et al.*, 2002; Rø *et al.* in progress). The axon terminals show a large number of bouton-like swellings indicating the presynaptic sites in the calyces. Several neurone types have been shown to connect the mushroom body and the lateral protocerebrum. In honeybees, extrinsic cells from the mushroom body are shown to have arborisations in the median and lateral protocerebrum (Mauelshagen, 1993). GABAergic inhibitory interneurones mediating information from the lateral horn to the Kenyon cells of the mushroom body are described in locusts (Perez-Orive *et al.*, 2002). In addition several GABA-immunoreactive feedback neurones have been found in the mushroom bodies of the honeybee, connecting the output and the input region of the Kenyon cells (Grünewald, 1999). Such inhibitory feedback loops are thought to be important for learning dependent plasticity either by regulation of Kenyon cell activity or by controlling input activity to the mushroom bodies.

## The taste pathway

Insects have taste, or contact-chemo sensilla, on many appendages, particularly on the antennae, proboscis and other mouthparts as well as on the feet. Little has been known about the projections of the taste RNs, only that they project in the suboesophageal

ganglion (SOG), which is the primary taste centre. One study in the honeybee has shown that the RNs on the proboscis project in close contact with premotor and motorneurones involved in the proboscis extension response (PER) (Rehder, 1989). In resent studies of the moth *H. virescens* projections of taste RNs on the antennae and on the proboscis have been traced and found to terminate in defined areas of the fronto-dorsal SOG (Jørgensen, 2003; Kvello, 2003). These tracing studies are important both for resolving the central sensory pathways of taste information as well as for identifying the neuronal connections to the olfactory pathways, which are involved in appetitive learning. In the honeybee connections between the SOG and the olfactory pathway have been described. The subesophageal-calycal tract connecting the SOG and the calyces of the mushroom body contain neurones innervating input areas of the mushroom body that does not overlap with the area innervated by the olfactory projection neurones (Schröter and Menzel, 2003). These neurones are thought to provide the mushroom body with sensory information from the proboscis. Of particular interest for olfactory learning is the identification of the ventral unpaired neurone ($VUM_{mx1}$) with cell soma and dendrites in the SOG and extensive arborisations in the AL, the lateral protocerebrum and the calyces of the mushroom body in the honeybee (Hammer, 1993).

## Olfactory learning

The knowledge about learning in insects comes particularly from studies of the honeybee. This species learns colour and odour of the flower, and can after experience choose the flower with the best reward (rewieved by Menzel, 2001). Olfactory learning of host plant odours is also demonstrated in other insect species, like phytophagous Lepidopteran. In one heliothine species, *Helicoverpa armigera*, field experiments have demonstrated that the moths prefer nectar foraging and oviposition on flowering host species on which they have previous experienced (Cunningham *et al.*, 1998a, b, 1999). Furthermore, wind tunnel experiments have shown that this moth preferred odours previously paired with a sucrose reward (Cunningham *et al.*, 2004). The mechanisms for olfactory learning in connection with nectar feeding are particularly well studied in the honeybee, which has become a model for studying appetitive learning in other insect species (Bitterman *et al.*, 1983; Menzel and Müller, 1996; Menzel, 2001). In these

classical conditioning experiments, stimulation with sucrose to the taste sensilla, which elicit the proboscis extension, represent the unconditioned stimulus (US). When the US is paired with the neutral odour stimulus (the conditioned stimulus, CS), the honeybee will develop a conditioned PER to the odour. The connection between the CS and the US pathway is mediated by the octopaminergic VUMmx1 neurone (Hammer, 1993; Hammer and Menzel, 1998). This neurone was shown to respond to sucrose stimulation applied to the antennae and proboscis, and depolarisation of this neurone could substitute sucrose stimulation as the reinforcer (US) in conditioning experiments.

Olfactory learning and memory involves modulation of the responses to odour stimulation. Comparisons of odour responses before and after olfactory conditioning have indeed shown changed responses in the AL and the mushroom body (Mauelshagen, 1993; Faber *et al.*, 1999; Faber and Menzel, 2001; Sandoz *et al.*, 2003). The importance of the mushroom bodies in acquisition, storage and retrieval of olfactory memory has been demonstrated both in the fruitfly and the honeybee (reviewed by Menzel, 1999; Menzel, 2001; Heisenberg, 2003). The fruitfly is a particularly interesting model because of the numerous mutants showing deficiencies in olfactory learning. Four of the well studied mutants (dunce, amnesiac, rutabaga and DCO) have defected genes coding for enzymes in the cAMP pathway, which is shown to be involved in learning and memory in other organisms (Heisenberg, 2003). In addition, the learning ability is affected in mutants lacking parts of the mushroom bodies (Pascual and Préat, 2001). This correlates with results found by chemical ablation of the mushroom bodies (De Belle and Heisenberg, 1994). However, it is shown that the honeybee can still perform elementary olfactory discrimination even with partial mushroom body ablation, indicating that the remaining part of the mushroom bodies are sufficient or that the mushroom bodies are not necessary in this kind of simple learning tasks (Malun *et al.*, 2002). In both insect species, the honeybee and the fruitfly, output from the mushroom body seems not necessary for acquisition, but is needed for retention of olfactory memories (Dubnau *et al.*, 2001; Lozano *et al.*, 2001; McGuire *et al.*, 2001).

## Heliothine moths

The three heliothine moths included in the study of this thesis were *H. virescens* (Fabricius), *H. armigera* (Hübner) and *H. assulta* (Guenée). They are all members of the subfamily Heliothinae (Insecta; Lepidoptera; Noctuida) which consist of more than 80 species, distributed in all five continents (Todd, 1978; Matthews, 1999). Many of these species are polyphagous, like *H. armigera* and *H. virescens* that are found on a wide variety of host plant species belonging to several families [e.g. *H. armigera* is found on more than 120 cultivated or wild host plants of 39 families (Reed and Pawar, 1982; Zalucki *et al.*, 1986)]. The variety of host plants chosen by these species are reflected by the many names given to them, e.g. *H. armigera* is named "corn ear worm," "tomato grub," "tobacco budworm" and "cotton bollworm" (Zalucki *et al.*, 1986). As indicated by the names, these insects live on economically important plants and are therefore considered to be major pests in many parts of the world. *H. armigera* is widespread in South Europe, North Africa, Asia, Australia and the Eastern Pacific, the Oriental tobacco budworm moth *H. assulta* is found in Asia and Australia and the tobacco budworm moth *H. virescens* is found on the American continent. *H. assulta* is considered oligophagous with a more narrow host plant range, mainly feeding on plant species of Solanacea.

The pest status of these heliothine moths is the reason for the extensive studies of the pheromone communication and identification of the sex pheromones, which have been made in 8 species (reviewed by Arn *et al.*, 1992). Although several compounds are identified as constituents of the pheromone blends, only two compounds are necessary and sufficient in each species, for male attraction and sexual behaviour (Vetter and Baker, 1983). The major pheromone component is cis-11-hexadecenal (Z11-16:Al) in all the species studied, except for *H. assulta*, using cis-9-hexadecenal (Z9-16:Al). The presence of the second principal pheromone compound in a certain ratio with the major component, provides the species specificity of the pheromone blend (Vetter and Baker, 1984). Another interesting aspect is the inter-specific effect of the second component, which is shown to interrupt the attraction of sympatric males (Shaver *et al.*, 1982; Vetter and Baker, 1983; Kehat and Dunkelblom, 1990; Boo *et al.*, 1995). The pheromones of the various heliothine species, as well as chemical analogues, have been

available for studying the specificity of the pheromone RNs in males. These studies have shown in each species a functional classification of three to four RN-types (reviewed by Mustaparta, 2002). The neurones are characterised by a narrowly tuning to one compound and much weaker responses to a few analogues. Similarities of the RN specificity across species have also been shown, for instance for the RN-type tuned to Z11-16:Al. Also interesting differences of the RN-specificity have been found for interspecific signals. These RNs responding to insect produced signals, have been used in combined electrophysiological and staining experiments to study the functional organisation of the MGC as mentioned above.

In recent studies using gas chromatography linked to single cell recordings, 18 functional types of plant odour RNs have been identified in heliothine moths (Røstelien *et al.*, 2000a, b; Stranden *et al.*, 2002, 2003a, b; Røstelien *et al.* in progress; Ulland *et al.* in progress). Like the pheromone RNs, these neurones are also characterised by a major response to one or two compounds (primary odorants) and weaker responses to compounds of related molecular structures (secondary odorants). One interesting type, frequently occurring in *H. virescens*, *H. armigera* and *H. assulta*, is the (-)-germacrene D RN-type. The similar structure-activity relationship of these neurones has indicated that all of them belong to the same functional type in the three species (Røstelien *et al.*, 2000a; Stranden *et al.*, 2002, 2003a). The behavioural importance of the sesquiterpene (-)-germacrene D is demonstrated as increased attraction and oviposition by mated *H. virescens* females (Mozuraitis *et al.*, 2002). The primary odorants for some of the other RN-types are the *E*,*E*-α-farnesene, *E*-β-ocimene, geraniol and (S)-(+)-linalool. Also these RNs show similarities across the heliothine species. Due to the knowledge about the specificity, particularly of the plant odour RNs, the three closely related heliothine species are especially interesting model organisms for studying the functional organisation of the glomeruli in the AL as well as studying olfactory learning.

# AIMS OF THE THESIS

The aims of the thesis were:

I. To elucidate anatomical and functional organisation of the glomeruli in the AL of heliothine moths.

II. To demonstrate the ability of heliothine moths to learn and to discriminate biological relevant plant odorants, and correlate the behavioural responses to odour induced activity in the glomeruli and the RNs.

# SURVEY OF THE INDIVIDUAL PAPERS

The aims given above are elucidated by:

- Making digital 3D atlases of the glomeruli in the ALs of *H. armigera* males and females and *H. assulta* females, as a complement to the previously made atlases of *H. assulta* males and *H. virescens* males and females.
- Measuring optically the activity in AL glomeruli of one heliothine species, *H. virescens*, during stimulation with identified primary and secondary odorants of different RN-types.
- Carrying out olfactory conditioning experiments to demonstrate the ability of the moth to learn and to discriminate three selected odorants with known RN-specificity, which were also tested in the optical imaging experiments.

## Paper I

Three dimensional reconstructions of the AL glomeruli of *H. armigera* males and females and *H. assulta* females were made by the use of synaptic antibody staining combined with confocal laser scanning microscopy. The digital 3D reconstructions were made in the AMIRA software. Atlases of four AL preparations within each sex of *H. armigera* and two AL preparations of *H. assulta* females were made separately. Comparison between the atlases allowed identification of glomeruli across individuals.

Corresponding glomeruli were given the same number and colour. There was a large consistency in glomerular size and position across individuals. In *H. armigera* 64 glomeruli were identified in females and males. In males these numbers included 3 glomeruli constituting the MGC, located at the entrance of the antennal nerve, well separated from the assembly of the ordinary glomeruli. The three glomeruli were named the cumulus, the anterior dorso-medial glomerulus and the posterior dorso-medial glomerulus. In females, the numbers included two glomeruli located at the entrance of the antennal nerve, named the central large female glomerulus and the medial large female glomerulus. In *H. assulta* 61 glomeruli were found, including the two female specific glomeruli. In both species, two cell clusters densely packed with clearly visible somata, were seen. They were named the lateral cell cluster and the medial cell cluster. Together with the previous study (Berg *et al.*, 2002) we now have 3D atlases of the glomeruli in both sexes of three heliothine moths (*H. virescens*, *H. armigera* and *H. assulta*). Comparison across species showed some pronounced glomeruli that seemed to be homologous by having similar size and position in all species. These atlases provide a necessary tool in studies of the functional organisation of the glomeruli in the three species.

## Paper II

The study of paper II was based on the findings of narrowly tuned RNs in heliothine mots, each responding mainly to one or two odorants (primary odorants) and weaker to a few odorants (secondary odorants) of similar chemical structures (reviewed by Mustaparta, 2002). In addition, a calcium imaging study of the AL of *H. virescens* confirmed and extended the results on the functional organisation of the MGC (Galizia *et al.*, 2000), previously shown by functional tracing of RNs and projection neurones (Christensen *et al.*, 1995; Hansson *et al.*, 1995; Berg *et al.*, 1998; Vickers *et al.*, 1998). A few selected plant odorants included as stimulants in the study by Galizia et al. indicated odour specific activity patterns in the ordinary glomeruli of the AL. This encouraged a more extensive optical imaging study of the plant induced activity in the AL of this species, resulting in paper II.

In principle the insect preparation was made according to the previous study by Galizia et al. (2000), by opening the head capsule, exposing the brain, and staining with calcium green 2 AM for one hour. This assured uptake of the dye before the extracellular dye was washed away. During the optical imaging experiments we recorded the spatio-temporal activity pattern of the glomeruli in the anterior AL during odour stimulation. Single plant odorants, most of which were known to activate specific RN-types, two essential oils containing several of the selected odorants, as well as the principal pheromone component were tested. Different from the previous study was the staining with a second dye, to make the glomerular structures visible in epifluorescent light, after the optical imaging recordings. In this way the activated foci could be ascribed to glomeruli in the AL. Attempts were made to identify the activated glomeruli according to the available 3D atlas (Berg *et al.*, 2002). However, this was in general difficult for the ordinary glomeruli, whereas the MGC and two large glomeruli (labial pit organ glomerulus and the neighbouring glomerulus) could be easily identified. In spite of this an across individual comparison could be performed, showing eight glomeruli with corresponding plant odour responses and positions in the AL within each sex. Each odorant was found to activate one or a few glomeruli. Like in previous studies, the major pheromone component elicited activity exclusively in the large MGC glomerulus, the cumulus, whereas single plant odorants and the two essential oils elicited activity in the ordinary glomeruli only. Specific activation in one or a few glomeruli was found, e.g. for linalool, β-ocimene/ β-myrcene (activating the same RN-type) and germacrene D, which each specifically activated glomeruli that were not activated by any of the other odorants. These results could be expected based on the knowledge about the RN specificity (Røstelien *et al.*, 2000a, b; Stranden *et al.*, 2002, 2003a, b; Røstelien *et al.* in progress) and the convergence of the same RN-type in one or two glomeruli (Vosshall *et al.*, 2000; Gao *et al.*, 2000). Surprisingly, one to three glomeruli in the two sexes were activated by more than one odorant. Thus the monoterpenes, linalool and β-ocimene/ β-myrcene, activated 1 or 2 glomeruli and the sesquiterpenes, germacrene D and α-farnesene, another glomerulus. Stimulation with the two essential oils containing some of the single test odorants, elicited activity that covered the glomeruli activated by the single constituents. The dose-response study

showed that increased odour concentration led to stronger activation in the AL. This was shown both as increased response strength in the odorant-specific glomeruli as well as by recruitment of less sensitive glomeruli. This corresponds to the results obtained in the honeybee (Sachse and Galizia, 2003).

## Paper III

Studies of olfactory learning have shown that the insects' preference for host plants can be modulated by odour experience in combination with a food reward, either at the host plant site or in controlled laboratory experiments. In heliothine moths, the responses of olfactory RNs have been very well characterised and represent an interesting model for evaluating possible changes in odour responses through learning. The present study represents an effort to describe olfactory learning mechanisms in *H. virescens*, before an extensive search for learning-induced changes in this species can begin. By the use of the PER, classical conditioning experiments were performed on *H. virescens* to study the ability to learn three selected odorants identified as biologically relevant in this moth (Røstelien *et al.*, 2000b; Stranden *et al.*, 2003b; Røstelien *et al.* in progress). Two of the odorants activate the same RN-type, β-ocimene stronger than β-myrcene, whereas *racemic* linalool activates two other RN-types, one as a primary odorant and the other as a secondary odorant. Acquisition curves showed that the moth could learn all three odorants, and they could keep the memory of a learned odour CS up to 24 hours. However, the maximum percentage of moths showing PER was lower than in the honeybee and more learning trials were needed to induce memory. One set of experiments was performed to determine the inter-stimulus interval (ISI) giving the optimal learning conditions. The best learning performance was obtained for ISIs of one to three seconds, which is in accordance with previous results obtained in honeybee and moths (Menzel and Müller, 1996; Fan *et al.*, 1997). In another set of experiments, the effect of odour concentration was studied. For all odorants, an increased concentration improved learning performance. However, a much lower concentration threshold for learning was found for *racemic* linalool than for the two other odorants. This seemed to be due to a higher sensitivity to linalool, as demonstrated by electroantennogram recordings in this study and by optical imaging in paper II. The ability of the moths to

discriminate between the three odorants was evaluated in differential conditioning tests with odour concentrations eliciting the same percentage of conditioned responses. The best discrimination was found with β-myrcene rewarded and *racemic* linalool unrewarded. The opposite combination gave lower discrimination, indicating a higher salience for β-myrcene than for linalool. The moths could also discriminate between β-ocimene and *racemic* linalool, and between β-ocimene and β-myrcene, but no difference in salience was found between these odorants. The discrimination between β-ocimene and β-myrcene was surprising, since they activate the same RN-type. However, these two odorants were more easily confused than the other odour pairs, as shown in the generalisation tests.

# DISCUSSION

## Anatomy and functional organisation of the antennal lobe

The present study on the functional organisation and odour discrimination in heliothine moths is based on the knowledge about RN specificity for primary and secondary odorants. This is an advantage since screening of chemicals for possible sensitivity and relevance is difficult. This particularly applies to the plant odours, being produced in large numbers, as hundreds of different compounds. In addition, interaction between intra-molecular features makes it difficult to screen for a continuum of important characteristics to find the most efficient stimulants. In the search for relevant odorants, the use of gas chromatography linked to single cell recordings, has ensured that in principle most volatiles emitted by a plant are tested on each single RN. In addition the method ensures the purity of the odorants. The resulted classification of distinct RN-types with narrowly tuned and similar dose-response relationship has shown the biological significance of the identified odorants. Having the knowledge about the primary and secondary odorants the purpose of paper I and II was to find out how these odour qualities might be represented in the AL. These studies have in principle given two kinds of information; a comparison of the anatomical organisation of the glomeruli, both the male specific MGC and the ordinary glomeruli, and the odour quality representation in the glomeruli.

The results of the two papers have extended the knowledge about the MGC. With the completion of the 3D atlases of the glomeruli in three closely related heliothine moths, *H. armigera*, *H. assulta* and *H. virescens*, the anatomical organisation of the MGC can be compared in more detail across species. The confocal laser scanning microscopy method, non-invasive to the heliothis brain, have given accurate measures of the glomeruli, making the comparison more reliable. For instanse the volumes of the cumulus in the three species, shows a volume range of 221000 to 299000 $\mu m^3$ in the four *H. armigera* preparations, a volume of 304200 $\mu m^3$ in *H. assulta* (preparation 1) and 481200 $\mu m^3$ in *H. virescens* (preparation 1). Interestingly, the number of three glomeruli forming the MGC of *H. armigera* is the same as in the two other previously

studied species of the genus *Helicoverpa* (*H. assulta* and *H. zea*), whereas two species of the genus *Heliothis* (*H. virescens* and *Heliothis subflexa*) have four glomeruli (Hansson *et al.*, 1995; Berg *et al.*, 1998; Vickers *et al.*, 1998; Skiri, 1999; Vickers and Christensen, 2003; Berg *et al.*, 2004). The anatomical division of these glomeruli becomes more interesting when comparing the function. The previous studies using functional tracing and optical imaging have shown that the cumulus in all species receives information about the major pheromone component (Christensen *et al.*, 1995; Hansson *et al.*, 1995; Berg *et al.*, 1998; Skiri, 1999; Galizia *et al.*, 2000; Berg *et al.*, 2004). One dorso-medial compartment receives information about Z9-14:Al in all species except *H. subflexa*, independent of whether this compound act as the second principal pheromone component or an inter specific signal [Indirect evidence for this principle is shown in *H. assulta* where the two RN-types tuned to the major pheromone component and to Z9-14:Al are co-localised (Berg *et al.*, 2004)]. The third and fourth glomeruli show more species variation when it comes to odour specificity. Particularly interesting is the comparison between *H. armigera* and *H. zea*. The two species, geographically separated on different continents, are believed to be paraphyletic. Attempts of cross mating between these two species gave fertile eggs and viable offspring (reviewed by Matthews, 1999). The relatedness of these two species are also reflected in the same anatomical and functional organisation of all the glomeruli of the MGC; the cumulus, anterior dorso-medial and posterior dorso-medial compartments (Almaas *et al.*, 1991; Vickers *et al.*, 1998; Skiri, 1999). The only difference found is the response to Z11-16:Ac in *H. zea*, but not in *H. armigera*, the component being an inter-specific signal produced by the American sympatric species *H. subflexa*. The knowledge about the MGC in several heliothine species is interesting, since species-specific differences are reflected in a different anatomical and functional organisation of the MGC, suggesting that the MGC has been exposed to evolutionary changes.

The present results on the ordinary glomeruli (paper I and II) are related to several aspects in olfaction: 1) the number of glomeruli within a species in relation to the number of RN-types, 2) the number and homology of glomeruli across related species and 3) the representation in the glomeruli of odour qualities, particularly of primary and secondary odorants. Based on the findings from molecular biological studies that each RN-type project in one or two glomeruli as shown in some vertebrates

and insects (Ressler *et al.*, 1994; Vassar *et al.*, 1994; Mombaerts *et al.*, 1996; Vosshall *et al.*, 2000; Gao *et al.*, 2000), one expects that the number of glomeruli might indicate the number of RN-types present. The 64 ordinary glomeruli in female *H. armigera*, including the labial pit organ glomerulus (probably receiving information about $CO_2$) and two female specific glomeruli that might be involved in intra-specific information, leave 61 glomeruli for plant odour information. With a ratio of 2 glomeruli for each RN-type, at least 30 RN-types should be present in these moths. So far, 18 different RN-types have been identified in the heliothine species (Røstelien *et al.*, 2000a, b; Stranden *et al.*, 2002, 2003a, b; Ulland *et al.* in progress; Røstelien *et al*. in progress). Considering the large number of olfactory sensilla on the antenna [17 000 in females and 12 000 in males of *H. virescens* (Almaas and Mustaparta, 1990)], most of which contain one-four RNs (Færavaag, 1999), one can expect more RN-types to be identified in future experiments. Also taking into account the large number of host plant species used by these insects, many odorants might not yet have been tested on the RNs, which might have caused the missing identification of some RN-types.

The atlases presented in paper I, together with the previous atlases (Berg *et al.*, 2002), have revealed a relative constant number of ordinary glomeruli also across the heliothine species. In addition, some of them seem to be homologous, as indicated by position, size and in some cases by similar function, like the already discussed MGC in males, the large labial pit organ glomerulus shown to receive input from the labial pit organ in *H. virescens* and *M. sexta* (Kent *et al.*, 1986; Kvello, 2003; Almaas, personal communication), and the two female specific glomeruli, which may either receive plant odour information as shown in *M. sexta* (Roche King *et al.*, 2000; Reisenman *et al.*, 2004) or be involved in intra-specific signal information about male produced compounds (Teal and Tumlinson, 1989). Some other glomeruli also show similarities in size and position. An interesting question is whether the functional organisation is similar across these species. This question remains unanswered until more species have been investigated according to glomerular specificity.

In the studies of the functional organisation of the glomeruli, one important goal is to resolve the codes for the odour qualities in the AL. For this purpose optical imaging is a suitable method since the activity in a large number of glomeruli can be measured simultaneously, as done in the honeybee (Galizia *et al.*, 1999; Sachse *et al.*,

1999). With this method and the knowledge about the RN specificity for identified odorants, the study of paper II was found particularly interesting, since the activity elicited by each of the primary odorants make one able to predict which RN-type is mediating the information. Furthermore, since the RN-types classified have shown only minimal overlap in molecular receptive ranges, one question was whether this might be reflected in glomerular activity in a single or a few glomeruli when stimulating with each odorant. When this study started, only a few primary and secondary odorants had been identified that were available in large enough quantities to be included, like (-)-germacrene D, *E*,*E*-α-farnesene, *E*-β-ocimene, *E*-β-myrcene (secondary odorant to the *E*-β-ocimene RN-type) and (*S*)-(+)-linalool. Based on the knowledge about RN-types and the converging projection of RNs in the AL we expected a specific non-overlapping response pattern for each of the primary odorants. Indeed we found glomeruli that each responded to one of the odorants, e.g. one glomerulus in females responded specifically to germacrene D, one or two glomeruli in males responded only to β-ocimene and β-myrcene and another responded only to linalool. Surprisingly we also found a few glomeruli that seemed to respond to several of the odorants. One glomerulus in females responded to both of the sesquiterpenes germacrene D and α-farnesene, while one or two glomeruli (males and females, respectively) responded to both monoterpenes β-ocimene and linalool. In the honeybee, optical imaging studies have shown that each odour activates a few glomeruli and that each glomerulus can be activated by several odorants. These results indicate that each odour is coded by an across-glomeruli pattern code (reviewed by Galizia and Menzel, 2000). So far this overlapping response pattern is only found for a few of the glomeruli in the moth AL. It might be that these glomeruli are involved in coding of other properties than odour quality. However, far more odorants need to be tested in order to know if the odour quality is coded by an across-glomeruli pattern also in the moth. In addition, optical imaging studies of the projection neurones in the heliothine moths should be made, in order to investigate whether the output from the AL is contrast enhanced in the same way as in the honeybee (Sachse and Galizia, 2002).

Paper II included a study of the effects of mixtures on the activity pattern in the AL that are interesting for revealing inter-glomerular interactions like inhibition. In this

study essential oils were used as mixtures. They were chosen because they contained large numbers of odorants and were primarily used to test the sensitivity of the insect AL in the start of each experiment. The two mixtures contained several of the single components tested and the expectation was therefore that the AL responses of the mixtures should cover the response areas activated by the single constituents. This was also what we found in the results of the calcium imaging experiments. However, no interaction in the form of inter-glomerular inhibition could be observed, which might have been masked by the different quantities of the constituents. In future experiments compounds with defined amounts should be used in the mixtures, in order to compare with the responses to the single compounds, and search for across-glomerular inhibitions. This mechanism is interesting in contrast enhancement in olfaction, and is found in imaging experiments on the honeybee (Joerges *et al.*, 1997; Sachse, 2002; Sachse and Galizia, 2003).

Optical imaging has been demonstrated as a useful technique to study the activation of glomeruli in a large part of the AL. However, the studies are challenging in respect to the long preparation where the insect brain is exposed for almost two hours before the measurement starts. This is the reason for the relative short time of carrying out experiments in each preparation (up to two hours). As a complement to optical recordings, it is therefore advantageous to carry out electrophysiological studies. Intracellular recordings in the AL combined with staining are very challenging in these small insects, but have the advantage of specific measurements from single neurones and of a precise anatomical tracing of the dendrites in the glomeruli where the information is received. Correlation between the present optical measurements and intracellular recordings has been shown in one case for a linalool specific glomerulus in males (Rø *et al.* in progress). We hope to obtain more results for comparing calcium responses (mainly representing the input to the glomeruli) with electrophysiological responses of projection neurones (representing the output of the glomeruli). Additional primary odorants activating new RN-types have been identified (Røstelien *et al*., in progress), which awaits further studies.

## Coding of odour intensity

The results of the optical imaging experiments have given information about how odour intensity is coded in the AL. We know that RNs increase their firing rate with increasing concentration. We also know that RNs and projection neurones of the same type may have different sensitivity, as shown for the pheromone and plant odour system in heliothine moths (Almaas and Mustaparta, 1991; Christensen *et al.*, 1995; Stranden *et al.*, 2003a, b). If all RNs of one type project into the same (one or two) glomeruli, the activation elicited by the odours should be limited to these glomeruli, which should also show dose-dependency. Another possibility would be that some glomeruli receive input from the most sensitive RNs and others from less sensitive RNs, resulting in recruitment of more glomeruli with increasing odour concentration. In fact, recruitment of less sensitive glomeruli was found in the present study. An increased odour concentration elicited stronger responses in sensitive odour specific glomeruli in addition to weaker responses in a few others. However, the reason for the recruitment of glomeruli is not known, and is not necessarily due to input from less sensitive, odour specific RNs. Many RN-types are not yet identified, and we can not exclude the possibility that there are non-identified RN-types for which the tested odorants act as secondary odorants. Recruitment of glomeruli with increased odour concentration has also been shown in the optical imaging experiments in the honeybees (Sachse and Galizia, 2003), in other moth species (Carlsson and Hansson, 2003; Skiri *et al.*, 2004) and in mice (Fried *et al.*, 2002). In the honeybee the simultaneous measurements of activity in RNs and in a group of projection neurones, showed that the projection neurones only received and conveyed further the information from the strongly activated glomeruli. Sachse and Galizia suggested that these more strongly activated glomeruli dealt with the odour quality, while the additional weakly activated glomeruli dealt with odour quantity information, converged to higher centres by multi-glomerular projection neurones. Since multiglomerular projection neurones were not investigated in that study, this question remains to be answered in future experiments. It is important to notice that the olfactory system in the insects has different mechanisms for detecting odour intensity over a large range, based on both a temporal (increased firing rate in each neurone) and a spatial mechanism (recruitment of less sensitive neurones). Low concentrations are

particularly important for detecting odours released by sources over a large distance and sensation of high intensity seems particularly important when the insect is sitting on the flower, and olfactory conditioning takes place.

## Odour sensitivity

Several studies have shown low sensitivity to plant odorants during single cell recordings in the AL. This was also the case in the calcium imaging study included in paper II, where strong responses to the major pheromone component were elicited at doses of 1 µg on the filterpaper, whereas higher concentration were often necessary for eliciting responses to plant odorants at all. Also in other studies, odour doses up to 10 or 100 µg of the component on the filter paper was needed to obtain responses in local interneurones and projection neurones of the AL (Anton and Hansson, 1995; Roche King *et al.*, 2000; Shields and Hildebrand, 2001; Greiner *et al.*, 2002; Masante-Roca *et al.*, 2002; Reisenman *et al.*, 2004; Rø *et al*., in progress). This concentration level is quite high as compared to the doses of 0.1 to 1 ng, which are enough to elicit responses in RNs as shown in single RN recordings (Røstelien *et al.*, 2000a; Stranden *et al.*, 2003a, b). One would rather expect the opposite sensitivity difference of RNs and AL neurones like in the pheromone system, where the projection neurones in the AL respond to concentrations 100 times lower than the RNs (Almaas *et al.*, 1991; Christensen *et al.*, 1991, 1995; Berg and Mustaparta, 1995; Berg *et al.*, 1995, 1998; Vickers *et al.*, 1998). The differences between the pheromone and plant odour system might partly be due to a larger convergence of the pheromone responding RNs in the few MGC glomeruli as compared to the convergence of the plant odour RNs in about 60 glomeruli. In comparison of optical and electrophysiological recordings it is also possible that optical recordings are less sensitive. Whereas the electrophysiological recordings in heliothine moths have shown RN responses down to concentrations of 0.1 ng, the calcium imaging experiments in this study showed a threshold of 100 ng or often higher, which might be due to some fatigue of the preparations. Interestingly, optical imaging studies with simultaneous recordings from projection neurone signals and compound signals (mainly responses from RNs) in honeybees have shown no difference in sensitivity (Sachse and Galizia, 2003). One general explanation for the relative low

sensitivity of AL projection neurones in the plant odour system is that stimulation has been made with compounds that are not primary odorants. This, together with a possible downregulation of sensitivity due to neuromodulation in the AL might be the reason for the low sensitivity recorded in most species. Neuromodulation of the AL neurones are known from studies of honeybees and moths. Seretonin can function as neurotransmitters, neuromodulators and neurohormones in insects and thereby modulate the sensitivity of neurones (reviewed by Erber *et al.*, 1993). One seretonin-immunoreactive neurone has been identified in each AL of *M. sexta*, which make direct output synaptic contact with local interneurones (Kent *et al.*, 1987; Sun *et al.*, 1993). Intracellular recordings of this moth as well as of cultured AL neurones, have shown that seretonin modulates the responses of local and projection neurones, reducing their excitatory responses at low concentrations and enhancing the responses at high concentrations (Kloppenburg and Hildebrand, 1995; Mercer *et al.*, 1996). Interestingly, high pressure liquid chromatoghrapy studies have shown that the seretonin level in the ALs fluctuate over a 24 hr period, with the highest levels when the moths are most active, indicating influence on the performance of odour–dependent behaviour (Kloppenburg *et al.*, 1999). The presence of one seretonin immunoreactive neurone in each AL, has also been identified in *H. virescens* (Almaas and Homberg, unpublished data), suggesting a similar neuromodulation in this species.

## Olfactory learning and odour discrimination

The ability of an organism to discriminate between odour qualities is first of all based on the presence of different types of RNs. In contrast to colour vision, having only three types of photoreceptors, olfactory discrimination is based on a large number of different RN-types. In common is the expression of one type of sensory proteins in each cell, and in both systems the further processing of information is obviously important for how well the organism discriminate the different qualities of the stimuli. In insect olfaction, information about odour qualities is processed in odorant specific glomeruli where contrast enhancement may play a significant role in the odour discrimination. In addition comes the involvement of the Kenyon cells of the mushroom body, a process important for learning and memory of odour qualities. Although *H. virescens* do not

show the same high learning success as the honeybee (50% compared to 80-90%) (Bitterman *et al.*, 1983), the knowledge about the RN-specificity made this heliothine moth particularly interesting for studies of olfactory learning. In paper III, we therefore used the simple learning paradigm, PER conditioning, to study the ability of the moths to learn and to discriminate the three odorants, β-ocimene and β-myrcene activating the same RN-type and *racemic* linalool two other types. What also makes the discrimination tests in this study interesting is that the concentrations were adjusted so that the different odours were tested with concentrations giving the same degree of learning success. This eliminated a concentration effect on learning, leaving only subjective similarities and salience as the causes for distinction between odour qualities. In this study the expectation was a higher discrimination success with the odour pairs activating different RN-types, than with the two odours activating the same type. Surprisingly we found that the moths had the ability to discriminate between all three odorants tested, including β-ocimene and β-myrcene at the adjusted concentrations. This indicates the existence of olfactory neurones mediating information about one and not the other odorant. This has not yet been found in the previous electrophysiological studies of the RNs. However, the calcium imaging experiments in paper II showed one or two glomeruli that were only activated by β-ocimene and not by β-myrcene, which might be one underlying mechanism for this discrimination. Although there were differences in the response patterns for these two odorants in the AL, the overlap in the sensitive odour specific glomeruli was significant. It was therefore interesting to find a relative large degree of confusion between the two odorants in the generalisation tests. For instance, with β-myrcene rewarded and *racemic* linalool unrewarded during the training, the following generalisation test with β-ocimene elicited PER. Altogether the results obtained in these appetitive learning tests have shown a correlation between the response patterns in the AL and the odour discrimination ability of this moth species.

Another interesting aspect of these studies was the finding of a difference in salience for two of the odorants. This was demonstrated by a higher discrimination ability when β-myrcene was rewarded and *racemic* linalool unrewarded as compared to the opposite combination. This asymmetry indicated a higher salience for β-myrcene than for *racemic* linalool. Furthermore, these results implies that a high sensitivity to an

odorant, as for *racemic* linalool, does not necessary mean that this odorant has a higher salience, when the concentration effect is eliminated. Thus, the use of compensated concentrations of odorants is important when searching for odour differences in salience.

The results in paper III also showed that the learning ability of the moths increased with increased concentration of all three odorants. This is not surprising since the learning ability is measured here as conditioning of PER during appetitive learning. This kind of learning takes place when the insect is sitting on the flower, exposed to high odour concentrations. Increased learning success with increasing odour concentrations during appetitive learning has also been shown in studies of the moth *M. sexta* and the honeybee *A. mellifera* (Pelz *et al.*, 1997; Bhagavan and Smith, 1997; Daly *et al.*, 2001; Wright and Smith, 2004). Interestingly, the conditioning experiments of this study also showed concentration dependent odour differences in learning ability, where much lower concentrations were needed for *racemic* linalool than for the other two odorants. These results correlated well with the RN sensitivity measured as electroantennograms in paper III and as calcium responses in paper II. Also the somewhat higher sensitivity to β-ocimene than to β-myrcene correlated with the results of electroantennogram and optical imaging recordings as well as of previous electrophysiological recordings (Røstelien *et al.*, 2000b; Stranden *et al.*, 2003b). Thus, the results from these experiments have shown that improved learning success in PER conditioning is well correlated with a higher activity in the sensory olfactory system.

Altogether this study has shown that the moths can be used as a model to address questions related to learning, discrimination, generalisation and salience of olfactory stimuli. The results have opened up for more studies to be performed, where additional odorants recently identified, should be included. Further studies should also be performed on odorants involved in different(Stranden *et al.*, 2002; Stranden *et al.*, 2003a) contexts, like egglaying and nectar feeding.

# CONCLUSIONS AND PROSPECTS

The studies included in this thesis were the first step to investigate the functional organisation of the ordinary glomeruli in heliothine moths. The anatomical atlases of the glomeruli in the AL of *H. armigera* and *H. assulta* (female), was a complement to the previously made atlases of *H. assulta* (male) and *H. virescens* (Berg *et al.*, 2002), and showed constancy in glomerular number, size and positions across individuals within a species, as well as similarities and differences across species. The optical imaging study was the first to address specific questions about how biologically relevant plant odour qualities were represented in the AL of heliothine moths. The results showed activation of odorant specific glomeruli as expected from the knowledge about RN-types. However a few glomeruli were also found that were activated by more than one odorant. These patterns were constant across individuals of *H. virescens*. Future studies may show whether the functional organisation is similar across species, both by using calcium imaging and electrophysiology combined with neurone staining. The comparison between polyphagous (*H. armigera* and *H. virescens*) and oligophagous species (*H. assulta*) are especially interesting. A crucial step in this process is to develop methods enabling identification of the activated glomeruli according to the available atlases. Helpful in this process will be the standard atlases of the whole brain and the ALs, which is under reconstruction in our lab.

The results of the olfactory learning experiments have demonstrated that the moths can be used as a model for studying olfactory learning and discrimination. Interesting in future studies will be to combine in the same preparation learning experiments with measurements of activity in the brain, both the ALs and the mushroom bodies, in order to investigate changes of responses during associative learning. Future studies should therefore emphasise on establishing experimental procedures for combining single cell recordings and PER conditioning or optical imaging and PER conditioning.

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