Odour Perception in the Codling Moth *Cydia pomonella* L.

– from Brain to Behaviour

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ABSTRACT

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The codling moth, *Cydia pomonella* L. (Lepidoptera: Tortricidae) is a renowned pest in apple, pear and walnut orchards, and its activities are in large guided by volatile odours as sensory cues. This thesis spans over a large part of the olfactory chain of events in the codling moth, from brain to behaviour.

The main emphasis was placed on the detection of plant odours, and some of the works presented are novel to codling moth research. The volatiles emitted by host-plant species were analysed, revealing variations in the odour profiles both between species of host plants and at different phenological stages of a host plant, which indicates that females are flexible in their behavioural response to host odours.

A first step was taken to map the antennal olfactory receptor neurons and their specificity, where several behaviourally active compounds were found to be detected by neurons housed in sensilla auricillica, one of the morphological types of sensilla found on the antenna of the moth.

In a study of the antennal lobe, the primary integration centre for odour processing in the insect brain, we describe the three dimensional structure of the array of olfactory glomeruli of both sexes.

Behavioural experiments show that both males and females are attracted to plant odours, and that host recognition in codling moths are encoded not by single compounds but by a blend of volatiles.

Taken together, the results presented in this thesis provide new insights into moth olfaction and odour-dependent behaviour in general, and into that of the codling moth in particular.

Keywords: Insecta, kairomone, host-plant volatiles, morphology, electrophysiology, sensillum, oviposition, attraction

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Appendix

Papers I–V

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

I. Bengtsson, M., Bäckman, A.-C., Liblikas, I., Ramírez, M.I., Borg-Karlson, A.-K., Ansebo, L., Anderson, P., Löfqvist, J., Witzgall, P., 2001. Plant odour analysis of apple: antennal response of codling moth females to apple volatiles during phenological development. *J. Agric. Food Chem.* 49, 3736–3741.

II. Ansebo, L., Coracini, M.D.A., Bengtsson, M., Liblikas, I., Ramírez, M., Borg-Karlson, A.-K., Tasin, M., Witzgall P., 2004. Antennal and behavioural response of codling moth *Cydia pomonella* to plant volatiles. *J. Appl. Entomol.* 128(7), 488–493.

III. Ansebo, L., Ignell, R., Löfqvist, J., Hansson, B.S. Responses to sex pheromone and plant odours by olfactory receptor neurons housed in *sensilla auricillica* of the codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae). Submitted.

IV. Ansebo, L., Ignell, R., Löfqvist, J., Hansson, B.S. Antennal lobe morphology of male and female codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae). Submitted.

V. Witzgall, P., Ansebo, L., Yang, Z., Angeli, G., Sauphanor, B., Bengtsson, M. Effect of plant volatile compounds on codling moth oviposition behaviour. Submitted.

Papers were reprinted with kind permission from the American Chemical Society (Paper I) and Blackwell Verlag (Paper II).

Objective

Dispersal of synthetic sex pheromones, causing mating disruption of male insects, has proven to be a successful tool to control orchard populations of the codling moth, *Cydia pomonella* L. (Lepidoptera: Tortricidae). As a testament to this, currently 50-60% of the apple orchard areas in Washington, USA, and in South Tyrol, Italy, are treated with synthetic sex pheromones causing mating disruption (Bengtsson et al., 2004). Due to the success of male mating disruption, focus is now being placed on improvements of this method and on finding new semiochemical-based methods aimed at the control of females. The objective of the present thesis was to study different aspects of the codling moth odour detection and odour-directed behaviour, with emphasis on the impact of host-related odours in these systems, odours that in the future may be used in more reliable control methods.

Introduction

The role of odours in insect behaviour

The atmosphere contains a complex mixture of millions of volatile compounds, and as such, it is difficult to comprehend how we can discern certain odours. The stimulus spectrum that the olfactory system has to deal with is radically different than that of our other senses. Both light and sound stimuli occur in very limited number of dimensions, whereas odour molecules are multi-dimensional in their nature, having different lengths, functional groups and chiralities. Thus, while e.g. the visual system functions with a low number of photo receptor types, the olfactory receptors constitute the largest gene family in the human genome, encompassing approximately 300 functional genes (Malnic et al., 2004). Still, the human sense of smell is in many ways surpassed by that of other animals. Insects have fewer receptors (≈50) but have developed an extreme sensitivity to certain odours (Keller and Vosshall, 2003). Many insect species rely to a high degree on odour cues in their search for food, mating partners, hosts and suitable oviposition sites. A wonderful example of this extreme sensitivity is provided by the noctuid moth, the cotton leaf worm, *Spodoptera littoralis*, whose heart frequency is affected by less than 10 molecules of odorant hitting the antennae (Angioy et al., 2003).

Semiochemicals

Chemical compounds that mediate interactions between organisms are called infochemicals or semiochemicals. If these signals are transmitted between individuals of different species they are called allelochemicals, while signals working between individuals of the same species are termed pheromones (Figure 1; Gullan and Cranston, 1994; Howse et al., 1998). This terminology can be compared to hormones, the chemical messengers within one individual. Allelochemicals are subdivided into three classes: allomones, kairomones and synomones. (Nordlund and Lewis, 1976, Gullan and Cranston, 1994). Allomones are signals that benefit the emitter while being negative or of no significance to the receiver. Examples of allomones are odours released by the plant dead horse arum, Helicodiceros muscivorus, mimicking a carcass to attract and trap flies for unrewarded pollination (Stensmyr et al., 2002). A kairomone, on the contrary, benefits the receiver, either evoking a behavioural or a physiological reaction. Generally, prey odours are typical kairomones, as well as plant odours used by herbivores in their search for suitable hosts. Synomones are odours that act as signals that benefit both the emitter and the receiver. Floral odours in general can be classified as synomones, as the insect receives nectar and pollen, while the flower becomes pollinated.

One single compound may, however, act in more than one manner, depending on the circumstances. For example, the sesquiterpene (*E*)-ß-farnesene is a compound with multiple semiochemical functions. Aphids use it as an alarm pheromone in response to disturbance; the ladybird *Adalia bipunctata* use this alarm signal as a kairomone to locate its aphid prey; while the parasitoid *Aphidius ervi* use the increased plant release of the compound from *Vicia faba* due to aphid feeding, as a cue for the presence of its larval host, hence the plant signal (*E*)-ß-farnesene acts as a synomone (Bowers et al., 1972; Du et al., 1998; Francis et al. 2004).

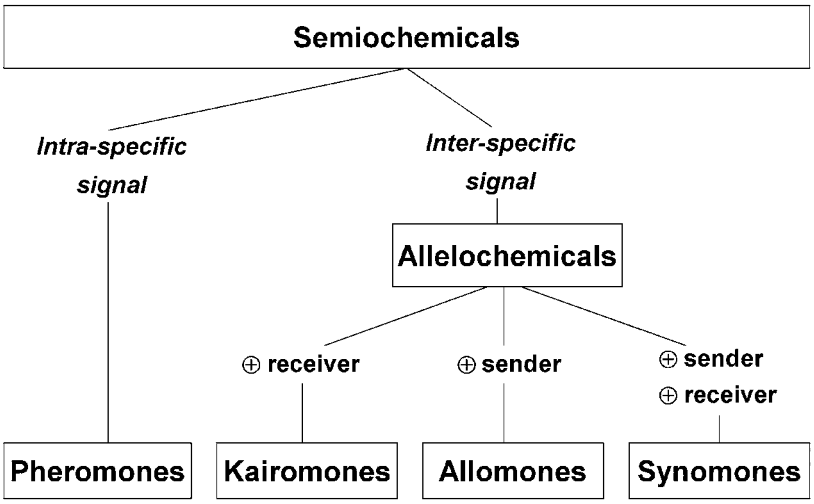

Figure 1. The classification of chemical compounds that mediate interaction between organisms, semiochemicals, according to the effect they have on the involved organisms. ⊕ = benefit.

Sex pheromones

Pheromones is the class of semiochemicals that has attracted most attention by researchers. Several types of pheromones have been demonstrated (e.g. Jutsum and Gordon, 1989; Wyatt, 2003), such as sex-, aggregation-, alarm- and trail-pheromones. Mate finding in moths is largely based on olfactory communication via sex pheromones. Sex pheromones are divided into long-range and close-range signals, which are involved in attraction of the opposite sex over long distances and in courtship behaviour, respectively (Hartlieb and Anderson, 1999). The long-range sex pheromones in moths are mainly released by female abdominal glands and usually consist of a blend of several compounds, mostly C10-C21 unsaturated straight-chain aldehydes, acetates and alcohols (Hartlieb and Anderson, 1999). The long-range sex pheromone is highly species-specific, but some sympatric species may share components. Specificity is in such cases accomplished by the addition of unique compounds, by the use of different ratios, or by other barriers such as geographic dispersal or differences in diurnal rythms or host plants (e.g. Hartlieb and Anderson, 1999; Löfstedt, 1993). Male moths are highly sensitive to the long-range sex pheromone, and even very low amounts can be detected in the characteristic odour filaments in the air and evoke upwind flight towards the source (Hartlieb and Anderson, 1999) On the contrary, if the pheromone level is too high, or too uniformly dispersed, the male will often abort its flight towards the source (Hansson, 1995). The close-range courtship involves male-released sex pheromone, which often is different from that of the long-range pheromones, being accompanied by visual and mechanosensory cues (e.g. Grant, 1987; Gullan and Cranston, 1994).

Plant semiochemicals

Most herbivorous insects are very selective, carefully choosing their host plants, and often also which particular part, when feeding and depositing their eggs (Schoonhoven et al., 1998). The host plant selection sequence can be divided into searching, which if successful ends with host finding; and contact-testing, which ends with acceptance or rejection (Schoonhoven et al., 1998). Orientation towards plants is commonly guided by an integration of both visual and olfactory cues, of which odours have a much larger variety of characteristica than the visual cues, e.g. air distribution and odour composition and ratios (Schoonhoven et al., 1998; Rojas and Wyatt, 1999; Raguso and Willis, 2002). However, attraction to host plants by odours, without optical stimuli, has been shown for many insect species (Bernays and Chapman, 1994; Schoonhoven et al., 1998).

The volatiles that plants emit are mainly secondary metabolites, including e.g. terpenoids, aromatic phenols, alcohols and aldehydes, which are released from both intact plants and upon plant tissue damage (Schoonhoven et al., 1998; see also Bäckman et al., 2001). Though liberated plant volatiles can exceed hundred different compounds, the blend is usually dominated by a few compounds. The profile of odours is species-specific, but significant intra-specific differences occur, depending on e.g. physiological, developmental and genotypic variations in the plant, herbivory, and abiotic factors such as season, light, and geographic location (Schoonhoven et al., 1998).

Semiochemicals for insect control

Soon after the first identification of a lepidopteran sex pheromone in 1959 (*Bombyx mori*; Butenandt et al.), it was hypothesised that synthetic pheromones could be suited to manipulate insect behaviours for pest control (Babson, 1963; Wright, 1963). Eight years later this potential was demonstrated (Gaston et al., 1967) and today synthetic pheromones are efficient in monitoring and controlling several pest insect species (Cardé and Minks, 1997). For example, mating disruption is a technique used for the manipulation of several moth species, in which the highly species-specific synthetic sex pheromone is evenly distributed over a treated area. This dispersal causes the males to be unable to locate the calling females, resulting in no mating and hence no larval damage (Ridgeway et al., 1990).

However, the use of plant volatiles in pest management is still in its infancy. Possible functions are e.g. to attract predators and parasitoids, to employ ‘push and pull’ techniques in which the pest insect is pushed out from the crop, and pulled into a more attractive, non-crop plant population, or to use non-host volatiles to mask the host plant odours (Schoonhoven et al., 1998; Khan et al., 2000, James, 2003). A good example of a binary semiochemical effect is the control of aphids with the aphid sex pheromones, cyclopentanoid nepetalactones, which also act as kairomones in attracting aphid parasitoids and predators (Birkett and Pickett, 2003). It is further known that plant volatiles can interact and synergise insect response to con-specific sex pheromones (e.g. Light et al., 1993; Yang et al., 2004), hence attention is paid to combinations of these in order to improve pest control programs (Reddy and Guerrero, 2004).

The detection of odours

Odours are mainly detected by sensory neurons on the insect antennae. The moth antenna is filiform and consists of smaller segments called flagellomeres. On each flagellomere there are sensory structures, the sensilla, that either protrude from the cuticle as cuticular hairs, have the shapes of porous plates on the cuticular surface, or are situated in grooves or pits in the cuticle. A schematic drawing in Figure 2 shows the sensillum types that are found on the codling moth antenna.

Each sensillum houses one to several olfactory receptor neurons (ORNs), whose cell-bodies are located in the tissue under the cuticle (Keil, 1999). Each ORN sends a dendrite into the sensillum. The dendrite can be more or less branched. The sensillum can be single- or double-walled, with pores or slits through which odour molecules can enter (Keil, 1999). In the lymph between the sensillum wall and the neuron dendrite, special binding proteins are thought to transport odour molecules from the inner openings of the sensillum wall, through the lymph, to the dendritic membrane where receptor proteins receive the odour stimuli and decide the physiological specificity of the ORN (Kaissling, 2001). It is still not totally clear how the odour binding proteins act and interact with the odour molecules and the receptor protein.

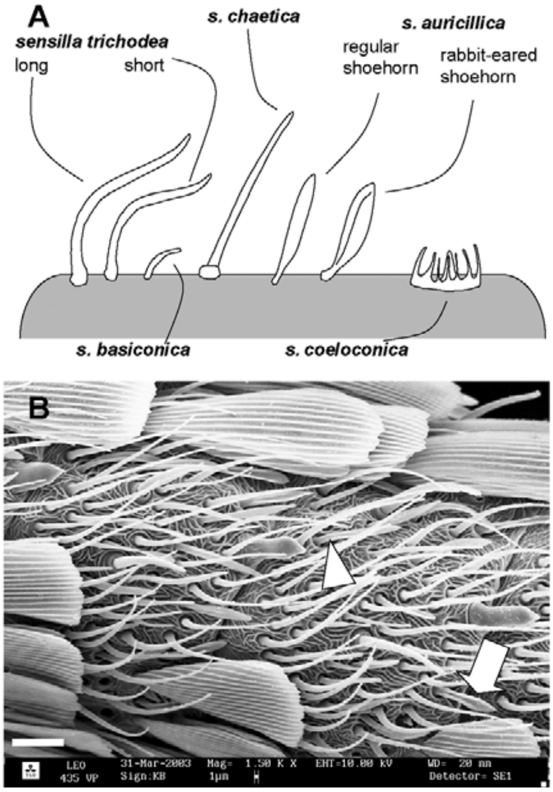

Figure 2. (**A**) Schematic drawing of the sensillum types found on the antenna of the codling moth, *Cydia pomonella*, the sensillum type *s. styloconica* is not included. (**B**) An SEM image at 1500x magnification of the scale-less section of the 4th and 5th flagellomere, counted from the base, of a female codling moth antenna. Right side – distal margin; left side – proximal margin. Scale bar = 10 µm.

It seems that each ORN carries at least one type of receptor that is specific for a limited number of ORNs; there are, in addition, a more general receptor type found in numerous neurons of e.g. *Heliothis virescens*, *Drosophila melanogaster* and *Anopheles gambiae*, and its function is yet unclear (Krieger et al., 2003; Vosshall et al., 2000). In *Drosophila melanogaster*, the most studied insect at the molecular level, approximately 40 different types of antennal receptors have been found (Vosshall et al., 2000). Each receptor is specific for a number of odour chemicals, and one odour can be detected by several receptors; this overlap is suggested to lead to that insects can detect a higher number of odours than dictated by the number of receptors (Elmore et al., 2003; Keller and Vosshall, 2003; Hallem et al., 2004). The odour molecule-receptor interaction initiates a chain-reaction that results in a receptor potential. This change in potential elicits bursts of action potentials from the neuron, with frequencies dependent on odour quality and quantity. However, an odorant-receptor interaction may also result in an inhibition of the neuron activity. The information is then sent via the ORN the axon through the antennal nerve (AN) to the brain (Stengl, 1999).

The brain

Primary integration of olfactory information in insects occurs at the antennal lobes (AL) of the brain (Anton and Homberg, 1999). The neuropil in the AL is organised in dense, more or less globular shapes called glomeruli. The number of glomeruli is species-specific and varies between e.g. 32 in the mosquito *Aedes aegypti* to over 1000 in the locust *Locusta migratoria* (Ernst et al., 1977; Bausenswien and Nick, 1998). A majority of the glomeruli are sexually isomorphic, i.e. found both in males and females, whereas a group of sexually dimorphic glomeruli are found in male moths at the entrance of the AN into the AL (Anton and Homberg, 1999). The ORN axons reach the glomeruli and connect to the vast network of interneurons that (a) interconnect glomeruli, and (b) relay the processed signal to higher olfactory centres. Signals are integrated by local interneurons, and the coded information is transported by projection neurons to higher centres of the brain for further processing, which may ultimately result in a behavioural or physiological reaction (de Belle and Kanzaki, 1999).

Axons from antennal ORNs expressing a specific type of dendritic receptor protein target the same glomerulus (Vosshall, 2000; Keller and Vosshall, 2003; Hallem et al., 2004). Many ORNs involved in host plant coding are characterised by a relatively wide response spectrum, and their response spectra often overlap, hence several glomeruli may be activated by one odorant signal. This creates a specific pattern of activated glomeruli when the ORNs detect odours, and these patterns have been shown to depend on odour quality as well as quantity in moths (Carlsson, 2003 and references therein). This complex mechanism is referred to as ’across-fibre’ information coding, and allows the insect to identify and quantify a vast number of odorants (Hansson and Christensen, 1999).

The pathways of sex pheromone and non-pheromone information are to a large degree separated (Hildebrand, 1996). Male moths possess an extra set-up of glomeruli, called the macroglomerular complex (MGC), which is situated at the entrance of the AN. It usually consists of a large, irregularly shaped glomerulus, along with some satellite glomeruli. It has been shown for several moth species that the number of MGC sub-units is equal to the number of components involved in pheromone communication (Hansson, 1997). ORNs detecting pheromone information are highly specific and sensitive. Information about pheromone signals from a specific type of ORN reaches a certain glomerulus of the MGC, and, theoretically, the information is directly passed on to projection neurons for transmission to higher brain centres. Such straightforward information channels are referred to as ’labelled-lines’ (Hansson and Christensen, 1999). However, the anatomical labelled-lines in its strict sense have been shown to be not true in some species (Anton and Hansson, 1999). The output from the antennal lobe in e.g. *Trichoplusia ni* have been shown to not match the input of information on pheromone signals, as a result from interglomerular activity via local neurons, and pheromone-responding projection neurons have been found to also respond to plant-associated odours (Anton and Hansson, 1999).

Figure 3. The codling moth, *Cydia pomonella* (above; photo by Peter Valeur), and its larva (arrowhead) inside an apple with typical tunnels and larval frass (arrow; photo by Lena Ansebo).

The target insect – *Cydia pomonella*

The codling moth, *Cydia pomonella* L. (Lepidoptera: Tortricidae) is ca. 10 mm long, with wings that are marmorated in different shades of grey (Figure 3). Towards the distal end of each forewing there is a dark and coppery-brown patch. Codling moths prevail in temperate regions, and their host plants, all members of the family Rosaceae, are mainly apple (*Malus domestica* L.), but also pear (*Pyrus communis*), walnut (*Juglans* spp.) and quince (*Cydonia oblonga*). Codling moths over-winter as larvae hidden under bark, pupate in early spring and emerge as adults in late spring. In cooler regions such as Scandinavia codling moths are generally univoltine, but in warm summers two generations can be observed.

The reproductive activity of codling moths occurs at dusk, when males actively fly around host tree canopies in search for females. Calling females reside on trees, and release a blend of sex pheromone compounds from a gland at the tip of the abdomen. This blend is suggested to comprise six components, of which codlemone, (*E,E*)-8,10-dodecadienol, is the major (e.g. Roelofs et al., 1971; Arn et al., 1985; Bäckman et al., 1997; El-Sayed et al., 1999; Witzgall et al., 2001). The males are highly sensitive to the pheromone, and can detect odour filaments in the air at a long distance from the calling female. The pheromone triggers males to fly upwind to find the female to mate.

The females initiate oviposition 12–to-24 hours after mating. Plant odours are known to be involved as cues in the search for oviposition sites suitable for larval development (Wildbolz.,1958; Wearing et al., 1973; Yan et al., 1999). The eggs are dispersed singly on leaves close to the fruit, occasionally on the fruitlet (Wildbolz, 1958). The hatched larvae seek up and bore into the fruit, normally one larva per fruit, and feed during the entire larval period from seeds and flesh within the fruit (Figure 3). The larvae finally leave the fruit for diapause or pupation.

Results

To fully understand the process of plant volatile detection and host odour-directed behaviour of the codling moth, efforts must be directed towards (a) identifying what plant compounds are emitted from the hosts, (b) which of the odorants can be detected by the moth, (c) in what way are they detected, and (d) how they affect the moths. The following summarises the results of the findings in Paper I-V according to the Appendix of this thesis.

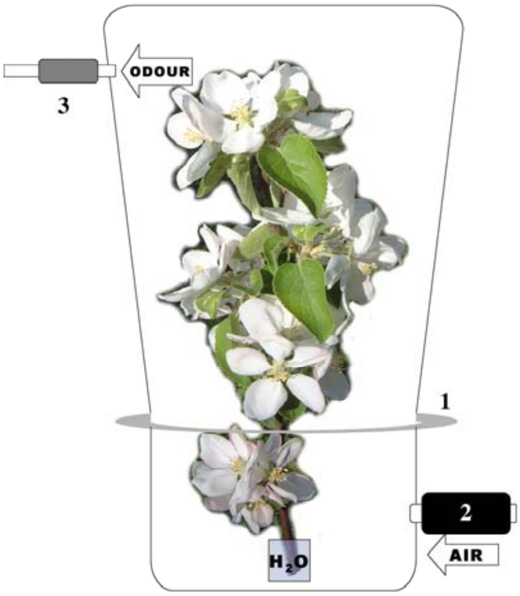

Figure 4. Schematic drawing of the odour collection apparatus for headspace collections of odour volatiles. The plant is placed inside a glass-jar, consisting of two parts inter-sealed with an airtight ground-glass fitting (1). Air is pulled into the jar through a char-coal filter (2), passed over the plant and pulled out through a filter that traps emitted volatiles (3).

The host (Papers I and V)

Identifying compounds released by the host trees of the codling moth, at the time for reproductive activity of the insect, would effectively narrow the list of compounds that are potentially used by the moths for host recognition. To achieve this, we collected released volatiles from branches with leaves and flowers or fruit in filters (Figure 4). The filters were rinsed with an organic solvent, thus extracting the host-plant compounds. The extracts were analysed by means of a gas chromatograph (GC) and mass spectrometer (for further details on Method and Materials, see Paper I).

The odour profiles apple branches and fruit at different phenological stages of apple were analysed (Paper I). Results showed that branches with flowers release a characteristic bouquet of odorants, more specifically benzenoid compounds, especially benzyl alcohol, not found in other developmental stages. Volatiles from small green apples were found to contain several terpenoid compounds, but also some alcanes and aldehydes and one alcohol. Emissions from leaves were similar to green apples. Mature apples differed from green apples by releasing very few terpenoids. Instead they released esters.

Apple, pear and walnut serve as larval hosts for codling moths, and in Paper V the question addressed was whether these trees share a common or different odour profiles. Odours from the three hosts and one non-host (cherry) were collected and analysed. Several compounds were found to be shared by all species including the non-host, whereas other compounds were exclusive to some of them, e.g. α–caryophyllene was exclusively released by pear. In addition, ratios between compounds were found to be different between different species of hosts and the non-host. However, further analyses were needed to make relevant comparisons, see next chapter.

These studies provided a valuable overview of the odour bouquets that are emitted from host-plants and a non-host. The next step was to identify which of these compounds were biologically relevant to the codling moths.

The detection (Papers I,II and III)

With the list of odorants emitted by the plants, we needed to identify which of them that the codling moths could detect. Insect odour detection can be studied by two electrophysiological methods. Both involve studying ORN responses but at two ’resolution’ levels, the antennal and the sensillar level. Studies at the antennal level, electroantennography (EAG), is thought to be a measurement of the entire antennal ORN-population, and is a time-efficient method to screen antennal ORNs. However, low responses to a compound caused for example by a low number of responding ORNs, may be overseen. Studies at the sensillar level, through single sensillum recordings (SSR), measure responses by the ORNs housed in a single sensillum, and although it is more time-consuming, this method gives a more refined picture of and provides more information about the odour detection (Wibe, 2004).

Host odours detected by the moth (Papers I and II)

Electroantennographic (EAG) recordings provided a list of the antennally detectable odorants in the plant extracts. The antenna was placed between two electrodes and coupled to an amplifier (Figure 5). When an odour was presented and ORNs responded, the change in electric potential was visualised as a peak protruding from the constant noise of the baseline. The size of a specific EAG response was shown to be dose dependent and probably relate to the number of responding ORNs, and their sensitivity to the odorant (R. Ignell, personal comm.). In order to screen for antennal specificity to the compounds in the plant odour extracts, a technique linking flame ionisation and Electro-Antennographic Detection in the outlet of a Gas Chromatograph (GC-EAD) was used. The odorants collected from the hosts and the non-host were separated in the GC column and split in two, one part leading to the FID and the other over the antenna (see Material and Methods in Papers I,II).

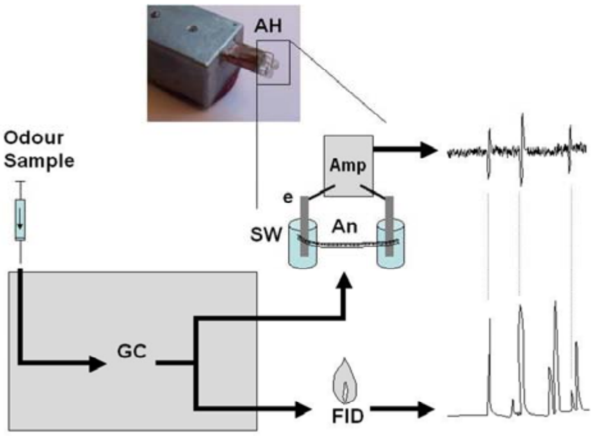

Figure 5. Schematic drawing of simultaneous recordings of signals from a flame ionisation detector (**FID**) and an insect antenna (**An**). The odour sample is injected on a gas chromatograph (**GC**) with a split column and an extra outlet. The antenna is placed with its cut ends in saline-filled wells (**SW**) on the antenna holder (**AH**), connected to an amplifier (**Amp**) and a voltmeter. (Photo by L. Ansebo)

In Paper I, compounds in the extracts of apple volatiles of different phenological stages were screened for EAD activity and 17 out of 64 compounds elicited EAD responses in the female codling moth antenna (Table 1). One of the EAD-active compounds was exclusively emitted by flowers, while seven were emitted by ripe apples only. Six compounds were released by flowers, leaves and small green fruit. Three compounds were released at all phenological stages. The nine EAD-active compounds that were present in odours of green apple and leaves were considered as candidate components of the odour bouquet that female codling moths recognise as indicative of a suitable host.

Table 1. Volatile compounds in headspace collections from apple ('Discovery') at different phenological stages (flower-emitted volatiles excluded) that elicited antennal responses by female codling moths, *Cydia pomonella*, as determined by GC-EAD. Compound amounts are relative to (*E,E*)-α-farnesene. Data are extracted from Table 1 in Paper 1.

	1-3 weeks after bloom			ripe
	leaves	leaves,fruit	fruit	fruit
esters				
butyl butanoate				36
propyl hexanoate				4
hexyl propanoate				7
butyl hexanoate				29
hexyl butanoate				21
hexyl 2-methyl butanoate				112
hexyl hexanoate				25
alcohols				
(*Z*)3-hexenol	71	8	11	
benzenoids				
methyl salicylate		3	1	
terpenoids				
4,8-dimethyl-1,3,(*E*)7-nonatriene	13	26	21	34
(±)-linalool	2	2	2	
ß-caryophyllene	29	13	7	
(*E*)-ß-farnesene	79	1	3	
germacrene D	21	27	7	
(*Z,E*)-α-farnesene	4	2	2	7
(*E,E*)-α-farnesene	100	100	100	100

In Paper V, the odour profiles of three host species (apple, pear and walnut) and one non-host (cherry) were analysed. Before any comparisons were made, the odours in the extracts were screened for EAD activity. Only EAD-active compounds were included in the comparisons, which showed that compounds present in the host species were not found in the non-host, however there were variation in both quality and quantity between hosts.

The results from Papers I and V showed considerable variation in the content of detectable odours between different phenological stages of a host and between different host species. These results indicate that females are quite flexible in their response to host volatile blends and, furthermore, able to adapt to new conditions, e.g. shift between host species and between phenological stages of the host, from the first moth generation to the second during a single season.

Screening antennal sensitivity and selectivity (Paper II)

In paper II we studied male but mainly female antennal odour detection by screening the antennal response to a set of host-plant odours, the major sex pheromone component (codlemone) and one aldehyde, (*E,E*)-2,4-decadienal, which is found in the larval defence secretion of the European apple sawfly, *Hoplocampa testudinea* (Boevé et al., 1996), at two doses, 10 and 100 ng. By using GC-EAD, instead of EAG in combination with the traditional dose-response procedure that includes odour application on filter papers, we circumvented problems with possible impurities and different volatilities of the compounds. This was accomplished due to the separation of the odours in the column, resulting in that volatiles were eluted in the same amounts from the GC onto the antenna.

We found differences in the female dose-response pattern to the test odorants; some evoked strong responses at both doses, whereas responses to other compounds could be low or zero at 10 ng but strongly increased at 100 ng, indicating different ORN response thresholds to different odorants. Male antennal EAD-responses to eight plant odours revealed similar response patterns as the females, indicating that plant odours are of relevance also for males. However, a striking difference was found in the male response to plant odours versus codlemone, as both doses of codlemone elicited more than five times stronger responses, probably due to a higher number and sensitivity of male ORNs tuned to codlemone. Interestingly, female EADs showed strong responses to codlemone at 100 ng. This kind of female auto-detection has only been reported for a few species, e.g. female *Spodoptera littoralis* were demonstrated to have pheromone-tuned ORNs as sensitive and specific as the males (Ljungberg et al., 1993; Ochieng et al., 1995). One can only speculate about the purpose of female auto-detection, e.g. to monitor the female's own pheromone-release, or detect other calling females that compete in male attraction.

Peripheral detection (Paper III)

Studies on the specificity of ORNs in single sensilla are required in the attempt to understand the mechanisms underlying detection of odours. Such studies need a morphological description of the antennal sensilla, and for this purpose scanning electron microscopy is an excellent tool. The morphological observations in Paper III were largely in accordance with Ebbinghaus et al. (1998). We did, however, observe a different distribution of the long-type *s. trichodea* as well as a previously non-described sensillum-type with basiconic features. A scanning electron microscope image of two flagellomeres and a schematic drawing of the codling moth sensillum types are shown in Figure1. Of the six sensillum types found on the antenna, *s. trichodea* have received most of the attention and have been shown to contain ORNs tuned to codlemone (Lucas et al., 1994; Ebbinghaus et al., 1998). In paper III, the target sensillum type was instead *s. auricillica* with its two sub-types, rabbit-eared and regular shoehorns. The function of this sensillum type has been unknown until recently when it was shown that *s. auricillica* in the herald moth, *Scoliopteryx libatrix*, and in the beet armyworm, *Spodoptera exigua*, house ORNs involved in plant odour detection (Mochizuki et al.,1992; Anderson et al., 2000).

The electrophysiological technique SSR allowed us to 'connect' to a single sensillum instead of the whole antenna, by means of an extremely sharp tungsten electrode, which was inserted through the cuticle of the sensillum base, close to the ORN cell bodies (Figure 6). The spontaneous activity and the excitation or inhibition of an ORN caused by specific test odorants were recorded. Several ORNs housed in the same sensillum could be discriminated by their action potential (spike) shapes and/or amplitudes (de Bruyne at al., 2001). (See Material and Methods in Paper III.)

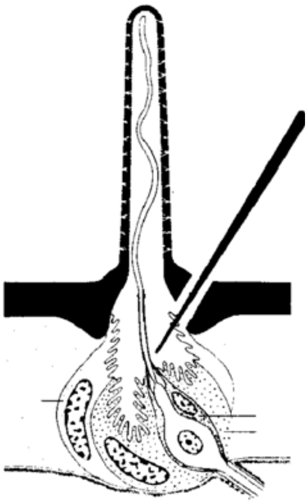

Figure 6. Schematic drawing of a sensillum, housing one neuron with its dendrite protruding up into the lumen of the hair-like sensillum. A sharpened tungsten electrode is inserted adjacent to the cell body of the receptor neuron for recordings of the neural activity. (Picture from P. Anderson).

A set of eleven plant compounds, four sex pheromone compounds, and one bimodal synergist / antagonist were tested. As a result, both males and females were found to have similar set-ups of 3-to-4 ORNs per sensillum (Figure 7B). All but one of the tested compounds elicited a response in one or several ORNs. These ORNs responded to 1-to-3 odorants, sometimes with overlapping response spectra, whereas other ORNs did not respond to any of the tested odorants. An example of a recorded ORN response is shown in Figure 7A. Codlemone evoked a response in male *s. auricillica* ORNs, showing that the detection of the major pheromone component is not restricted to the highly sensitive trichoid ORNs. The findings in Paper III indicate that codling moths possess a more complex odour detection system than expected.

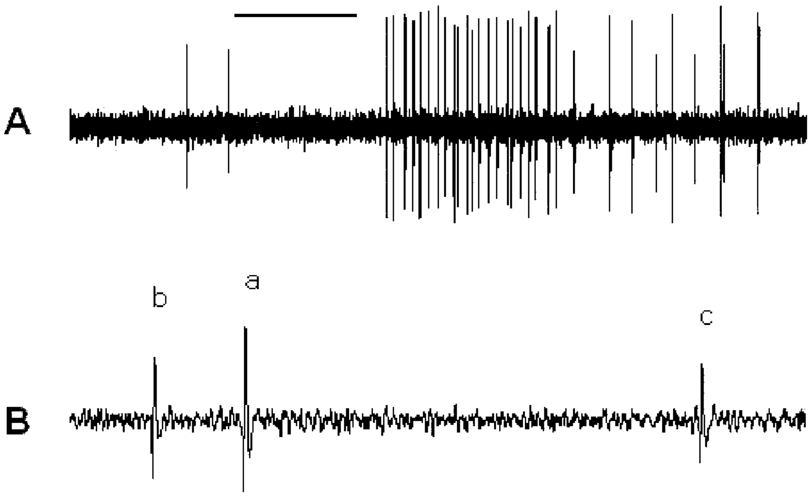

Figure 7. Single sensillum recording from a female *s. auricillica* rabbit-eared shoehorn in *C. pomonella*. (**A**) activation of the large-spiking neuron a to stimulation by ethyl (*E*,*Z*)-2,4-decadienoate (pear ester) formulated at 10 µg on a piece of filter-paper. Horizontal bar: stimulus duration (500 ms). (**B**) Examples of spike amplitudes of the three neurons housed in the sensillum: large spike, neuron *a*; medium spike, neuron *b*, and small spike, neuron *c*.

The centra of primary information integration (Paper IV)

Information from ORNs is transported to the primary olfactory centre of the brain, the AL, where the information is integrated, coded and transmitted to higher brain centres (Anton and Homberg, 1999; Hansson and Christensen, 1999). In order to investigate some of the complexity of the ALs, a first step is to study the morphology of these centres. The use of confocal microscopy in morphological studies of brain tissue has a great advantage in that organs can be kept intact, while laser beams scan through the tissue, section by section. These beams excite fluorescent markers that certain elements in the tissue have been labelled with, resulting in a stack of 2-dimensional images of the tissue.

We dissected male and female brains, and treated them with three fluorescent markers: α-synapsin, in conjunction with a fluorescent secondary antibody, which labels synapses inside neuropil tissue (Klagges et al., 1996); phalloidin, which labels F-actin in axons of ORNs (Rössler et al., 2002); and TOTO-3, which binds to the DNA and RNA of cell bodies. The three labels worked well together, and the major part of each AL was clearly visualised (Figure 8A-B).

Paper IV presents the morphology of the ALs as digital 3-dimensional (3D) reconstructions of the glomeruli (Figure 8C). Female and male ALs consisted of 52 (±2) and 50 (±1) ordinary glomeruli respectively. An additional prominent glomerulus, the cumulus, together with four satellite glomeruli were found in males, and were suggested to comprise the male-specific macro-glomerular complex, the MGC. The observed mismatch in the number of MGC glomeruli and the number of suggested pheromone components is apparent. This discrepancy may be explained by the fact that the cumulus is divided into subregions, each which may receive input from different ORN types. This has has been shown in the cabbage looper moth, *Trichoplusia ni*, where ORN types tuned to one of the six sex pheromone components or one behavioural antagonist specifically target one of seven regions in the five-glomerular MGC (Todd et al., 1995). Another explanation for the mismatch could be that the estimated number of MGC satellite glomeruli is incorrect, or that the pheromone composition is still not completely understood.

The position, size and shape of individual glomeruli varied substantially, especially in females, and it was not possible to assign enough number of glomeruli as landmarks that could facilitate further mapping of the AL. Such great variance has not been observed in most other moth species except for *T. ni*; it has also been observed in the hymenopteran parasitoids *Cotesia glomerata* and *C. rubecula* (Todd and Baker, 1996; Smid et al., 2003). This was a first study on the central nervous system in the codling moth, and further investigations of ORN specificity and their glomerular targets will add important clues to the mechanisms of codling moth sex pheromone and plant odour detection and processing.

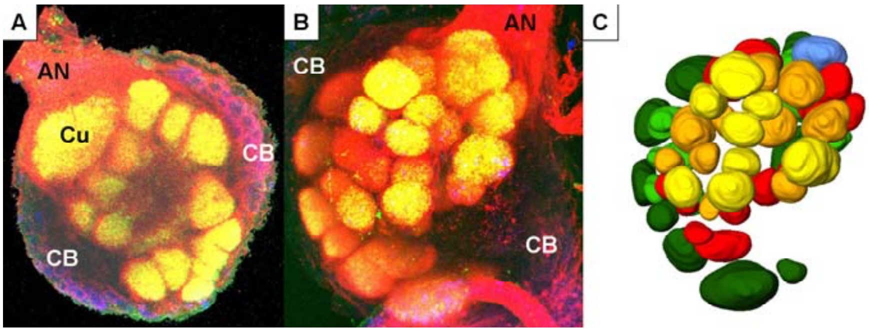

Figure 8. (**A**) A confocal section through the male antennal lobe in the codling moth, *Cydia pomonella*, viewed frontally, showing spherical glomeruli in a cluster. (**B**) A projection of 200 confocal sections of a female antennal lobe viewed frontally. (**C**) A 3D reconstruction of the glomeruli in **A**. Yellow: anterior; orange and red: medial; green: posterior; blue: a putative female homologue to the male Cu. AN: antennal nerve; Cu: the large irregularly shaped gomerulus that is part of the male-specific macroglomerular complex; CB: cell bodies of antennal lobe interneurons.

The behaviour (Papers I,II and V)

Orientation towards plant volatiles

It is well known that codling moth females orient towards apple odour, a behaviour also observed in our experiments (Paper I,V; e.g. Wildbolz, 1958; Yan et al., 1999). It has also been shown that (*E,E*)-a-farnesene increase oviposition and attracts females in laboratory experiments (Hern and Dorn, 1999; Yan et al., 2003). This compound is, however, commonly found in many non-host plants and cannot alone account for female host identification. In order to find the odours that may be involved in the host recognition process, plant volatiles that elicited antennal responses (see above) were chosen and screened for attractiveness, formulated singly in field traps (Paper II). However, no females were trapped, and the most probable explanation is that females are not attracted to single compounds but to multi-component blends. Another explanation for these results may be that the trap design was not suited for captures of females. Surprisingly, males were caught instead, in traps baited with (*E*)-ß-farnesene.

We therefore performed a wind tunnel experiment, using a sprayer apparatus as a dispenser, which release the test odours at known amounts without isomerisation (El-Sayed et al., 1998). We tested odorants known to elicit antennal responses in Papers I and II. The compounds were presented singly as well as in binary or multi-component blends, of which one roughly mimicked a green apple according to the extract contents presented in Paper I. The blends that attracted males to fly upwind towards the source were the apple mimic as well as the binary blends consisting of (*E,E*)-α-farnesene and (*E*)-ß-farnesene or ethyl (*E,Z*)-2,4-decadienoate (pear ester). The best attraction, however, was to a three-component blend of these three odorants. The pear ester has in field trials in some apple-growing regions been shown to be highly attractive to both sexes (Light et al., 2001), but it did not elicit upwind flights in the wind tunnel. It is not clear why males are attracted to plant compounds, but one reason may be that the probability to find females is higher in the host trees. In later studies it has been shown that male attraction to the major pheromone component codlemone is synergised by (*E*)-ß-farnesene, (±)-linalool and (*Z*)-3-hexenol (Yang et al., 2004). This is another indication that plant volatiles may be used by moths in optimising mating opportunities (see review by Reddy and Guerrero 2004).

Effect of larval origin on behaviour

The insects used in our laboratory experiments were reared on artificial diet for several generations and wild insects were regularly introduced. It was observed that the wild females were reluctant to oviposit in the absence of apple odours. This led us to investigate the role of larval origin on adult reproductive behaviour (Paper V). Batches of 10 pairs of males and females originating from populations in apple, walnut, pear, as well as from the laboratory, were placed in apple and walnut trees; non-host cherry trees; a nethouse at <50 m distance from nearest host tree; and in the lab with or without apple. Wild females oviposited significantly more on the host trees, except females originating from apple that were not stimulated by walnut odours, whereas very few eggs were laid without the presence of host odours. It was clear that host odours alone, without any other stimuli such as tactile cues, had an important influence on the oviposition choice of wild females, but not on lab-reared females as they willingly laid eggs wherever they were placed. The experimental set-up did, however, not allow us to establish whether all wild females responded to the odour cues, or if there only were a few that laid many eggs.

A subsequent experiment was therefore performed in which wild and lab-reared males and females were studied in single pairs, either with or without the presence of apple odours (see more details in Paper V). The results showed that most wild females were highly selective and thus reluctant to oviposit if the conditions were not right. However, a low number of wild females were not selective and laid many eggs. The presence of non-selective individuals have earlier been observed e.g. in a study on the privet hawkmoth, *Sphinx ligustri* (Schoonhoven et al., 1998). Larvae were transferred from its host to a non-host, and a few individuals did not have the behavioural barrier to feed from this non-host plant. This observation was commented by Schoonhoven et al. (1998): “The presence of ‘abnormal individuals’,..., probably reflects the built-in flexibility that enables a species to cope with changing conditions in its environment.” Our results clearly show that codling moths are able to adapt to new conditions. When eggs from such non-selective individuals are used to build up a laboratory culture a selection occurs resulting in females that are not as responsive to odour cues as wild females. This is of great general importance for future oviposition studies.

Concluding remarks and future perspectives

In a time when the awareness of environmental and human health is increasing, it is more important than ever to push research forward towards a goal of environmentally-safe methods to control pest insects. Hence, focus is now shifting towards mechanisms underlying odour detection and processing with a special interest in plant odours, which have been shown to possess great potentials to be used in the improvement of insect control techniques.

While it seems straight forward that codling moths orient towards apples, our results show the complexity of the olfactory cues, as the host odour profiles varied greatly. And the question can be asked, is it just plant odours in the right combination and ratios, or is there more? Besides olfaction, other sensory cues are most likely involved in codling moth orientation towards plant odours. Vision has for example been shown to influence host search and reproductive behaviour of many species (Shoonhoven et al., 1998).

Insect detection of odours is a complex and refined system, and as such one of the keys to the success of insects (Gullan and Cranston, 1994). This study was a further step to map the antennal sensillar ORNs and their odour specificity. The next step will also include the non-investigated short-type *s. trichodea*, *s. coeloconica* and the novel basiconic sensillum type that was observed. *Sensilla basiconica* have in other species been shown to be involved in both pheromone and plant odour detection, and *s. coeloconica*, studied in the silk moth *Bombyx mori*, in the desert locust *Schistocerca gregaria* and in *Drosophila melanogaster*, housed ORNs responding to host plant volatiles (Hansson, 1995; Pophof, 1997; Ochieng and Hansson, 1999; Ignell, unpublished data). These future studies on ORN detection will be performed in parallel with continued investigations of the ALs, as odour-specific ORN axons can be traced to their target glomeruli. This is especially interesting concerning the sex pheromone blend components, the MGC sub-divisions, and the possible female homologue to the MGC.

Electrophysiology also goes hand in hand with behavioural studies. The studies on behaviour towards plant compounds has obtained a new dimension due to the fact that males are attracted to some of the plant odorants in the wind tunnel. The specific plant compounds that elicited upwind flights by males in the wind tunnel are also likely to be relevant to females. However, future behavioural studies should focus on females, as their reproductive behaviour relies on odours for the host choice. In the long run, a control method that includes females is more efficient than if only males are affected.

Although the work in this thesis has not been aimed to find ‘The Odour Blend’ that mimics the host and attracts codling moths, several important pieces of information have been added to the increasing knowledge on the role of plant odours and odour perception by codling moths. In this way the thesis provides a solid foundation for further and deeper investigation of the olfaction system in *Cydia pomonella*.

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