Associative learning in two closely related parasitoid wasps: a neuroecological approach

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Contents

Summary

Insects are useful model organisms to study learning and memory. Their brains are less complex than vertebrate brains, but the basic mechanisms of learning and memory are similar in both taxa. In this thesis I study learning and subsequent memory formation in two parasitoid wasp species that differ in associative learning of the odours of plants on which they have encountered a host caterpillar. After ovipositing in a caterpillar on a certain plant species *C. glomerata* shifts its preference to the experienced plant odour, whereas *C. rubecula* does not shift plant odour preference after a similar experience. This difference in learning between these two closely related wasp species provides an attractive model to study physiological and ecological factors that could influence learning.

As a first step to analyse possible physiological differences that could influence learning, I describe morphological, anatomical and histochemical aspects of the neural pathways that mediate associative learning of odours in these wasps. The two wasp species display a high degree of similarity in morphology of the olfactory pathway at both the level of the sensilla, and the level of the glomeruli, the primary olfactory neuropile. I furthermore identify the octopaminergic neurons that could mediate the reward stimulus in the two wasp species, but the results did not allow us to distinguish possible dissimilarities between the species.

In addition I redefined the difference in preference learning between the two species in terms of associative and non-associative learning and analysed the temporal dynamics of the memory trace. Both wasps display associative learning after an oviposition reward conditioning, but the temporal dynamics differ. *C. glomerata* displays a stable memory for the experienced odour that lasts for at least five days, whereas in *C. rubecula* the memory starts to wane after one day.

Finally, I studied the effect of physiological and ecological traits of hosts as possible factors influencing memory formation. For this I used two geographically disjunct populations of *C. glomerata* that differ in their host use. Both populations only change preference after an oviposition reward on their preferred host species, suggesting that physiological factors exert a major influence on learning in these two populations. I discuss the ultimate factors that could have contributed to a difference in learning in *C. glomerata* and *C. rubecula*.

1

General Introduction

Insect learning and memory

However tiny an insect is, and however small its brain, insects are able to learn. Learning is the process of acquiring knowledge about the world and memory is the retention or storage of this knowledge (Kupfermann, 1991). Insects can use learning to adapt their behaviour, and are not, in contrast to popular belief, little programmed machines, but change their behaviour according to experience. The grasshopper *Schistocerca americana* can learn to avoid toxic food (Bernays, 1993). Honeybees learn how to handle complex flowers to extract nectar and pollen (Chittka et al., 1999), they learn odours, colours, shapes and patterns to recognize awarding flowers and they use landmark learning to locate foraging patches and their nest site (Gould, 1993). Parasitoid wasps can learn to associate plant odours with the presence of their hosts (Turlings et al., 1993).

Studies on insects have provided hypotheses on the adaptive value of learning and on the ultimate factors influencing learning (Gould, 1993; Roitberg, 1993; Stephens, 1993; Turlings et al., 1993; Vet et al., 1995), insight in cellular and molecular mechanisms involved in learning (Menzel, 1999; Waddell and Quinn, 2001), temporal dynamics of memory formation (Menzel 1999; Dubnau et al., 2003), sites of memory storage (Menzel, 1999; Waddell and Quinn, 2001) and genes involved in learning and memory (Waddell and Quinn, 2001). Comparisons of these insect studies with studies on learning that use other invertebrates or vertebrates as model organisms have led to the conclusion that at least at the cellular level, the basic mechanisms of learning and memory formation are highly conserved throughout the animal kingdom (Kandel, 2001). Hence, studies on insect learning provide us with insight in learning and memory in general and their less complex brains facilitate research in this direction.

The olfactory pathway

Odours contain vital information for the location of food, mating partners and oviposi-

tion sites and for a range of insect species the use of this information is optimized through learning. Consequently, learning in insects often features learning of odours. Hence, we first describe the insect olfactory pathway, along which the information contained in odours is processed.

The first level of odour processing occurs with the sensory detection of the odours. In insects, the odour receptors are predominantly located on the antennae (Schoonhoven et al., 1998). The receptor neurons, together with accessory cells and the cuticular structures in which the cells are located, form small sensory organs; the sensilla. Besides olfaction, other sensory modalities are also located in antennal sensilla, e.g. taste, mechanoreception and hygroreception. The olfactory sensilla can be recognized by the multiple pores in the wall of the sensillum (Keil, 1999; Steinbrecht, 1997). In the sensillum one or more olfactory receptor neurons are present (Hallem and Carlson, 2004). Different odorants activate a different subset of these neurons, depending on which type of olfactory receptor is expressed (Gao et al., 2000; Hallem and Carlson, 2004). The axons of the olfactory receptor neurons join to form the antennal nerve, which projects to the antennal lobe in the brain (Hallem and Carlson, 2004).

In the antennal lobe further processing of the olfactory information occurs before it is sent to the protocerebrum. The antennal lobe contains globular shaped structures called glomeruli, which are the projection fields of the olfactory receptor neurons onto the second order neurons, the projection neurons (Hallem and Carlson, 2004; Gao et al, 2000). Every olfactory axon terminates in only one glomerulus (Rospars, 1988) and axons of olfactory receptor neurons expressing the same olfactory receptor type project to the same glomerulus (Joerges *et al.*, 1997; Gao et al., 2000; Hallem and Carlson 2004). The number of glomeruli is constant in a given species and is normally in the range of 50 to 200 (Rospars, 1988). Besides constancy in number of glomeruli in a species, there is also constancy in shape and position of the glomeruli between individuals of the same species (Arnold *et al.*, 1988; Rospars, 1988). Glomeruli that process the information on similar odours are located together in the antennal lobe (Meijerink et al., 2003). Hence, the glomeruli do not represent a topographical map of the sensilla on the antennae, but a functional map of the olfactory information. Every projection neuron typically innervates only one glomerulus and their axons project to the mushroom bodies and the lateral horn of the brain (Hallem and Carlson, 2004).

The mushroom bodies are located in the protocerebrum and are the centres of higher order processing in insects (Menzel and Giurfa, 2001). In the mushroom bodies integration of the olfactory information with other sensory modalities occurs, e.g. visual information. The mushroom bodies are also involved in olfactory memory formation (Menzel and Giurfa, 2001; Heisenberg, 2003).

Associative learning

The experiments done by Pavlov (1927) on his dog provide well known examples of associative learning. He let his dog hear the sound of a bell, before he gave it food. After this training the dog began to salivate already at the sound of the bell. Thus the dog learned to react to the sound of the bell in preparation of receiving food. This form of associative learning is termed classical (or Pavlovian) conditioning. In classical conditioning the relationship between two stimuli, the unconditioned stimulus (US) and the conditioned stimulus (CS) is learned (Kupfermann, 1991). The US is a biologically meaningful stimulus, such as provided by food, which always results in an overt response, the unconditioned response (UR), such as salivation. The CS is an initially neutral stimulus that does not produce any overt responses. When the conditioned response has been (repeatedly) followed by the unconditioned stimulus, the conditioned stimulus will begin to elicit the conditioned response (CR). This response usually resembles the UR, but can also differ (Kupfermann, 1991). During classical conditioning an animal learns the predictive relationship between two stimuli. A rewarding US, like food, results in appetitive conditioning. A noxious stimulus can also be used as US, this results in defensive conditioning (Kupfermann, 1991). Another form of associative learning is operant conditioning (Kupfermann, 1991). In operant condition an animal learns to associate its own behaviour with a subsequent environmental event. A well known example is the rat in the Skinner box that learns to press a lever for food (Skinner, 1938).

Stimuli that are used as US in classical conditioning can also induce a general increase in sensitivity. When studying classical conditioning, it is therefore important to disentangle this sensitization effect from associative learning. Sensitisation is a non-associative form of learning and is defined as the general increase in response that follows after an intense or noxious stimulus (Kupfermann, 1991).

A well-studied example of classical conditioning in insects is the association of an odour stimulus to a reward stimulus, a sucrose solution, in the honeybee (Bitterman et al. 1983; Menzel, 1999). In this example the US is represented by the sugar and a neutral odour (an odour that does not induce an overt response) acts as the CS. Presenting sucrose to the antennae of the honeybee results in the unconditioned response, the extension of its proboscis (its mouthparts). This proboscis extension reflex (PER), can be conditioned by presenting an odour (the CS) to the antennae, followed by the presentation of sugar to the antennae and proboscis. After pairing, the odour alone is sufficient to induce proboscis extension.

Cellular mechanisms

At the neural level, the sucrose presented to the antennae and proboscis activates a re-

ward-sensitive neuron in the suboesophageal ganglion in the honeybee brain (Hammer, 1993). This facilitating neuron is octopaminergic (i.e. this neuron uses octopamine as neurotransmitter, Kreissl et al., 1994) and projects to the olfactory pathway of the honeybee brain (Hammer, 1993). Artificial activation of this neuron substitutes for the sugar reward (Hammer 1993). Octopamine is also important in olfactory reward learning in the fruit fly *Drosophila* (Dudai et al., 1987).

The molecular mechanisms underlying classical conditioning in the marine snail *Aplysia californica* have been studied extensively (Kandel, 1991). In this model system a touch of the siphon or mantle shelf can be associated with a strong shock to the tail. It is likely that in insects octopamine plays a similar role in appetitive conditioning as serotonin in defensive conditioning in *Aplysia*. In *Aplysia* the synapses that are activated by the CS and are subsequently targeted by serotonin form stronger connections with their targets. This synaptic plasticity can persist a few minutes to hours after a single trial, or days to hours after repeated trials. Short-term plasticity is the result of transmitter mobilization and an enhanced transmitter release. After repeated trials protein synthesis occurs, resulting in the formation of new synapses and long-term memory (Kandel, 1991).

Classical conditioning results in the formation of a memory for the experienced odour. This memory does not arise instantaneously, but develops gradually and different memory phases can be distinguished over time (Menzel, 1999; Dubnau et al., 2003). The strength of the memory formed after an experience depends on several factors. First, the strength of the US and CS are important; a stronger US or CS results in a stronger memory (Tully and Quinn, 1985; Vet et al. 1995). Second, the number of pairings between the US and CS influences the strength of the memory, with a higher number of trainings resulting in a stronger memory (Menzel, 1999; Tully et al., 1994). Third, the interval between the trainings is important; several trainings that quickly follow each other, i.e. massed training, result in a memory that lasts for hours or even days, but spaced trainings, i.e. trainings that are separated in time (e.g. 10 minutes between trainings) result in a stronger, longer-lasting memory (Menzel, 2001; Tully et al., 1994). In the honeybee five sequential memory phases can be distinguished (Menzel 1999). The duration of these memory phases seem to perfectly fit the duration of different aspects of foraging behaviour in which the memory is used. This has led to the idea that certain aspects of memory formation are adapted to the species and its species-typical behaviour (Menzel, 1999).

Tailor-made memory

The idea that properties of learning and memory are not purely dictated by their cellular and molecular mechanisms, but are also dependent on species-typical requirements has de-

veloped recently (Menzel, 1999; Dukas, 1998a; Shettleworth, 1998). This ecological view on learning holds not only that the duration of memory phases is adjusted to species-typical behaviour (Menzel, 1999), but also that memory capacity can differ between species according to ecological needs, e.g. the spatial memory in birds of food-storing and non-food-storing species (Dukas, 2004), and that learning ability might differ between species. For instance, some species might need only a few experiences to form long-term protein-synthesis dependent memory (LTM), whereas another species needs many experiences before LTM is formed. In the ecological view the lower learning ability of the slow learner is thought to be an adaptation to its environment and therefore 'smart in its own way' (Shettleworth, 1998).

So, when there is a difference in learning between species, and this is due to species-typical needs, what ultimate factors influence learning? Why is it useful for one species to learn fast, whereas it isn't or less so for another? Although the adaptive value of learning seems obvious, there are costs associated with learning. First, the formation and maintenance of memory involve energetic costs (Dukas, 1999; Mery and Kawecki, 2005). Second, there are ecological costs to learning; learning takes time and is vulnerable to mistakes (Dukas, 1998b). Hence, it is likely that when innate behaviour can suffice learning is not used.

Stephens (1993) suggests that learning is likely to be favoured when the predictability of the environment is low between generations, but high within generations. A completely unpredictable environment does not favour learning, as learning is based on predictability. On the other hand, a completely predictable environment is more likely to lead to innate behaviour than to learning. In addition, according to Roitberg (1993) learning is adaptive when a large number of decisions have to be made.

Comparative research can provide a powerful tool to study the above mentioned hypotheses. If we find that for a variety of species the presence or absence of a special ecological condition is reliably associated with the presence or absence of a specific characteristic, then it is logical to conclude that the characteristic is an adaptation to that condition (Williams 1975, cited in Thornhill and Alcock, 1983). In addition, comparing species that differ in their learning ability, i.e. the amount of experiences needed to form LTM, can provide insight in the underlying physiological mechanisms that result in fast learning and high memory performance. Comparative research with normal and mutant *Drosophila* fruit flies has elucidated genes that are important in learning and memory (Waddell and Quinn, 2001). Comparing closely related species is ideal in that it eliminates differences due to different ancestors. In this thesis we apply this comparative approach to two closely related parasitoid species that differ in learning.

The model system

Parasitoid wasps

Parasitoid wasps are Hymenopteran insects that use other arthropods as food source for their offspring (Godfray, 1994). Eggs are laid on or in the host (ectoparasitoids and endoparasitoids respectively) and the emerging larvae feed from the still living host. This eventually kills the host and results in the emergence of the adult parasitoids. The new adult wasps have to find new victims for their own offspring. The efficiency of host-searching behaviour is therefore directly linked to Darwinian fitness and strong evolutionary pressures are likely to have shaped this behaviour (Van Alphen and Vet, 1986). A major constraint on the evolution of efficient host-searching in parasitoids is the continuous selection on hosts for inconspicuousness to prevent becoming the victim of a predator or parasitoid. So although direct cues from hosts are highly reliable, they are difficult to detect (Vet and Dicke, 1992). In contrast, stimuli from plants are much easier to detect, but less reliable (Vet and Dicke, 1992). To deal with this reliability-detectability problem many parasitoids use the odours emitted by plants that are damaged by their herbivorous victims (Turlings et al., 1993). These odours can be innately attractive, or are learned by the parasitoid. It is likely that parasitoids that use hosts that are restricted to one, or several similar plant species, are innately attracted by the induced plant odours (Vet and Dicke, 1992). However, parasitoids that use hosts that can use several unrelated plants, that differ in odour composition and are variably available in space and time, are thought to learn the induced plant odour (Vet and Dicke, 1992). This hypothesis nicely fits the idea of Stephens (1993) that learning is favoured when within-generation predictability is high.

In summary, parasitoid host-searching is thought to have evolved under strong evolutionary pressures and parasitoids use learning in host-searching. In addition, use of learning in host-searching differs among species (e.g. Poolman Simons et al. 1992; Potting et al. 1997; Geervliet et al. 1998; Fujiwara et al. 2000; Fukushima et al. 2001). Hence, parasitoids are an ideal animal group to study ultimate questions on learning. The perfect model for this kind of research involves the comparison of closely related species that have the same ancestor and forage in the same environment, but differ in their use of learning during host-searching. We have found these conditions for two braconid species *Cotesia glomerata* L. and *Cotesia rubecula* Marshall (Hymenoptera: Braconidae).

Cotesia glomerata and *Cotesia rubecula*

The parasitoid wasps *C. glomerata* and *C. rubecula* are indigenous to Europe and lay their eggs in the caterpillars of *Pieris* butterflies. They find their hosts by the use of odours

emitted by the plants upon caterpillar feeding (Steinberg et al., 1993; Geervliet et al., 1994; Geervliet et al., 1996). The caterpillars of *Pieris* butterflies are restricted to plants that contain glucosinolates (Chew, 1980). Most of these plants belong to the crucifer family and herbivore-damaged plants of this family, in particular cabbage, are highly attractive to the parasitoids (Geervliet et al., 1996). However, several non-cruciferous plants that contain glucosinolates can be used by the butterflies as host-plant e.g. nasturtium (*Tropaeolum major*) (Feltwell, 1982; Geervliet et al., 1997). The parasitoids have to learn to recognize the odours of these other host plants as predictors of the presence of hosts. In this learning experience the innately unattractive odour, the CS, is associated with a successful oviposition in the hosts, the US. After encountering its host on such a new plant *C. glomerata* learns to prefer this new plant odour (Geervliet 1998). After an oviposition experience, the learned odour of the plant indicates the presence of hosts to the wasps and in a choice situation the newly learned plant odour is now preferred over the innately preferred cabbage odour. *C. rubecula* does not switch its plant odour preference after a similar experience (Geervliet 1998).

Cotesia rubecula is a solitary parasitoid; only one egg is laid in a single host. It is a specialist on the small cabbage white *Pieris rapae*. *Cotesia glomerata*, formerly known as *Apanteles glomeratus* is a gregarious species and approximately 20 eggs are laid in a single host (Vos and Vet, 2004). It can attack several host species of the Pieridae (Feltwell, 1982). However, different populations of *C. glomerata* can be more restricted in host range due to absence of several hosts, or competition. The Dutch population can parasitize *P. rapae*, *P. brassicae* and *P. napi*, but in the field mostly the large cabbage white *P. brassicae* is parasitized (Geervliet et al., 2000). In large parts of the USA and Japan, where *C. glomerata* is imported, *P. brassicae* is absent and *P. rapae* is the main host (Sato and Ohsaki, 1987; Vos and Vet, 2004). So although the species *C. glomerata* can use several species as host, different populations seem to be more restricted in host range. This difference in specialization at the species level compared to the specialization at the population level is an often encountered phenomenon (Fox and Morrow, 1981).

Aim and outline

This thesis is part of an elaborate project that studies the neural mechanisms that underlie a difference in learning behaviour in two parasitoid wasps. The main aim in this thesis is to (partly) map the CS and US pathways and to identify whether morphological differences in these pathways are present between the species. In addition, I redefine the learning behaviour displayed by these two wasp species in terms of associative and non-associative learning and study the temporal dynamics of the memory trace. Finally, I address the effect of the host species as a physiological and ecological factor influencing the learning experience and

subsequent memory formation.

Part 1: Morphological studies on learning. A difference in odour perception can influence odour learning. Therefore, I start with studying the olfactory pathway of both species. In **chapter 2** scanning and transmission electron microscopy are used to analyse the different types of sensilla present on the antennae of the two wasp species. I identify the olfactory sensilla and study whether there are any differences in these sensilla and in their numbers between the two species. In **chapter 3** I continue the description of the olfactory pathway with a study on the anatomy of the antennal lobe. A three dimensional map of the glomeruli of both wasp species is presented and differences between the species are identified.

Subsequently, I study the other pathway involved in the oviposition reward conditioning in these wasps, the US. In the honeybee a VUM neuron is responsible for the US during sugar reward learning. This neuron is octopaminergic. In **chapter 4** an antibody against octopamine is used to identify the octopaminergic cells in the brain of the two *Cotesia* species.

Part 2: Behavioural studies on learning. In **chapter 5** I reanalyse the difference in learning between the two species in such a way that it can be better compared to other model species used for learning. I use a no-choice wind tunnel bioassay to analyse the temporal dynamics of the memory trace for the experienced odour after oviposition reward conditioning.

In **chapter 6** I focus on an ecological factor that could have driven the difference in learning ability. A clear difference between the two wasp species is that *C. rubecula* is a specialist parasitoid on the solitary species *P. rapae*, whereas, in The Netherlands, *C. glomerata* is specialised on the gregarious species *P. brassicae*. I hypothesise that this difference in host species has driven the difference in learning and I test this by comparing the Dutch population of *C. glomerata* with a Japanese population that is specialised on *P. rapae*.

Finally, in **chapter 7** I summarize and discuss the results and propose directions for future research. Furthermore, I evaluate the contribution of this study to the understanding of the ultimate factors influencing learning in parasitoids in general.

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Part 1

Morphological studies

2

Antennal sensilla of two parasitoid wasps: a comparative scanning electron microscopy study

M.A.K. Bleeker, H.M. Smid, A.C. van Aelst, J.J.A. van Loon, L.E.M. Vet

Abstract

Two closely related parasitoid wasp species, *Cotesia glomerata* (L.) and *C. rubecula* (Marshall) (Hymenoptera: Braconidae), are different in associative learning of plant odours. To provide a solid basis for our research on the mechanisms that underlie this difference, we described the morphology of the antennal sensilla of these two species using scanning electron microscopy complemented with transmission electron microscopy. Female and male antennae of both species have the same six types of sensilla. We classified these sensilla as sensilla trichodea without pores, sensilla trichodea with a tip pore, sensilla trichodea with wall pores, sensilla coeloconica type I, sensilla coeloconica type II and sensilla placodea. We conclude that the morphology, numbers and distribution of the sensory receptors is highly similar in these two closely related wasp species. Differences between species and sexes occurred only in sensilla placodea numbers. *C. rubecula* has more sensilla placodea than *C. glomerata* and males of both species have a larger number and a higher density of sensilla placodea compared to females of the same species.

Introduction

Cotesia glomerata (L.) and *C. rubecula* (Marshall) (Hymenoptera: Braconidae) are two closely related parasitoid wasps (Smith and Khambhampati, 1999). They are endoparasitoids of *Pieris* larvae and coexist in the same habitats in the Netherlands (Geervliet et al., 2000). In these habitats they occupy partly overlapping niches: *C. rubecula* is a solitary parasitoid and a specialist on the solitary small cabbage white *Pieris rapae* (L.) (Lepidoptera: Pieridae). The gregarious *C. glomerata* is considered a generalist, it can parasitize *P. rapae* and other Pieridae, but prefers the gregarious large cabbage white *P. brassicae* (L.) (Lepidoptera: Pieridae) as a host (Geervliet et al., 2000). These two species find their hosts by the use of plant odours, which are induced by the feeding of caterpillars (Geervliet et al., 1994; Steinberg et al., 1993; Vet and Dicke, 1992). They differ in their use of olfactory associative learning for host searching (Geervliet et al., 1998b). Under standardized conditions, *C. glomerata* increases its preference for the odours of a particular plant species after an oviposition experience on that plant, whereas *C. rubecula* does not alter its innate preference (Geervliet et al., 1998a). The fact that these two species are closely related, live in the same habitats and have overlapping host-ranges but differ in their use of associative learning makes them a good model to investigate the neural mechanisms that underlie the differences in associative learning. In order to provide a solid basis for such neurobiological research, we need more knowledge of the organization of the wasp's olfactory pathway. For this purpose, we have analysed the olfactory receptive range of these two wasp species (Smid et al., 2002), and the three dimensional organization of the primary olfactory neuropil in the brain; the glomeruli in the antennal lobe (Chapter 3, Smid et al., 2003).

In the present study we describe the morphology, quantity and location of the sensilla present on the flagellum of males and females of *Cotesia glomerata* and *C. rubecula* using Scanning Electron Microscopy (SEM). Transmission Electron Microscopy (TEM) techniques were used to confirm the classification of the different types of sensilla based on SEM. We discuss to what extent these two species are comparable at the level of the antennal sensilla.

Methods

Plants and insects

Cotesia glomerata and *C. rubecula* were obtained from colonies that originated from individuals collected in Brussels sprouts fields in the vicinity of Wageningen, The Netherlands. The parasitoids were reared on *Pieris brassicae* and *P. rapae* respectively in a cli-

matic room at 20-22°C and a photoperiod of L16:D8 as described by Geervliet et al. (1998b). *Pieris* larvae were reared on Brussels sprouts plants (*Brassica oleracea gemmifera* cv. Cyrus), under the same climatic conditions.

Electron microscopy

Preparation for Scanning Electron Microscopy (SEM) was modified from Cuperus (1985). Male and female wasps of *C. glomerata* and *C. rubecula* were prepared free from their cocoons just before emergence to avoid debris accumulation on the antennae. Wasps were decapitated and heads were immersed overnight in CCl_4 (Aldrich) at room temperature and subsequently boiled three times for 1 minute, each time with new CCl_4. Preparations were then transferred to 100% ethanol, rinsed once, critical point dried and sputtered with 10nm platinum for observation with a JEOL JSM 6300F field emission scanning electron microscope. Alternatively, preparations were sputtered with 50/100nm gold/palladium for observation with a JSM 5200 scanning electron microscope for counting of sensilla.

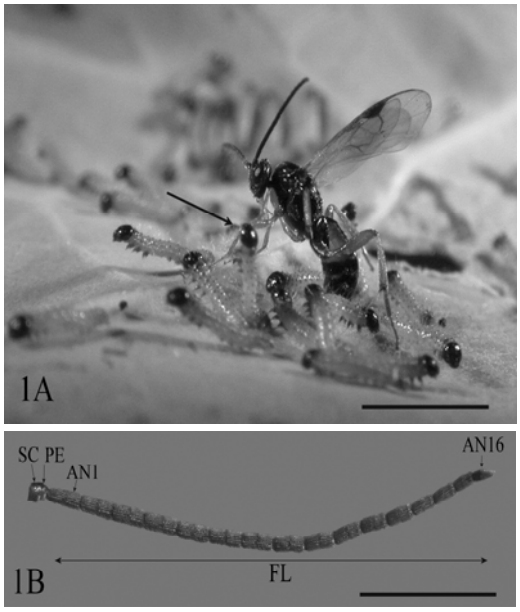


Fig. 1A. Macro photograph of a female *C. glomerata* laying her eggs in 1st instar larva of the large cabbage white, *P. brassicae*. Note the biting defense reaction of the caterpillars (arrow). Bar = 2 mm

Fig. 1B. Light microscopic photograph of the antenna of a *C. glomerata* female. Proximal is left, distal is right. SC, Scape; PE, Pedicel; AN1 (16) , Antennomere 1 (16); FL, 16 antennomeres together comprising the Flagellum. Bar = 1 mm.

For Transmission Electron Microscopy (TEM), antennae of both sexes of *C. glomerata* and *C. rubecula* were cut into pieces of approximately 2 antennomeres and immobilized on the bottom of an embryoblock with doublesided adhesive tape. They were fixed in 2% glutaraldehyde in 0.1M cacodylate buffer for 3 hr, briefly washed in 0.1M cacodylate buffer and then postfixed in 1% osmium tetraoxide in 0.1M cacodylate buffer for 2 hr, all at pH 7.0 and room temperature. After dehydration in a graded series of ethanol, they were embedded in epon. Epon was polymerized at 60°C for 48 hr. Serial sections of 80nm were cut with a Diatome diamond knife and collected on formvar-coated nickel grids. Sections were contrasted with uranylacetate and observed with a Philips CM12 electron microscope. For LM observations, 1-2 µm sections were mounted on poly-L-lysine coated slides and stained with 1% toluidine blue.

Table 1: Characteristics of antennae and sensilla placodea in males and females of *C. glomerata* and *C. rubecula*[1].

	C. glomerata		*C. rubecula*	
	male	female	male	female
Mean length of antenna (mm)	3.7 (SD 0.1)	2.8 (SD 0.1)	3.9 (SD 0.3)	3.8 (SD 0.1)
Length of antennomere (mm)	0.19-0.24	0.14-0.20	0.23-0.26	0.19-0.26
Length of s. placodeum (μm)	86-127	70-110	81-132	82-123
Density of s. placodea[2]	7.24 (SD 0.66)	5.24 (SD 0.57)	7.03 (SD 0.31)	5.64 (SD 0.72)

[1] For all groups $n = 5$

[2] Number of s. placodea on antennomere no 2 per 100μm circumference of the antennomere.

Results

The antennae of both species and sexes have the same flagellate shape (fig. 1). The mean length of the antennae is similar in both sexes of *C. rubecula*: 3.8mm (SD 0.1) in males and 3.9mm (SD 0.3) in females (table 1). In *C. glomerata* males have significantly longer antennae compared to females: 3.7mm (SD 0.1) in males and 2.8mm (SD 0.1) in females (table 1; $p < 0.001$, independent samples t-test for equality of means). The flagellum of both male and female *C. glomerata* and *C. rubecula* consists of 16 antennomeres (fig. 1). The length of the antennomeres decreases from proximal to distal and ranges from 0.26mm to 0.23mm in male *C. rubecula*, 0.26mm to 0.19mm in female *C. rubecula*, 0.24mm to 0.19mm in male *C. glomerata* and 0.20mm to 0.14mm in female *C. glomerata* (table 1). Thus in *C. glomerata*, but not in *C. rubecula*, there is a sexual dimorphism since the individual antennomeres of males are longer than those of females, which accounts for the clear difference in length between antennae of male and female *C. glomerata*.

Sensilla types

Six different types of sensilla were present on the flagellum of both sexes and species. They were classified into sensilla trichodea, sensilla coeloconica and sensilla placodea. Classification was done according to Zachuruk (1980; 1985) and Keil (1999). "Hairlike" structures with a diameter to length ratio less than 0.30 were classified as s. trichodea (Olson and Andow, 1993). Presence of pores was indicated by the addition NP for nonporous, TP for tip pore and WP for wall pores. The use of AP was avoided due to possible confusion between aporous and apical pore. The types and topographical arrangement of sensilla was the same in both sexes and species and is described below.

Three different types of sensilla trichodea were identified. The *s. trichodea NP* had a

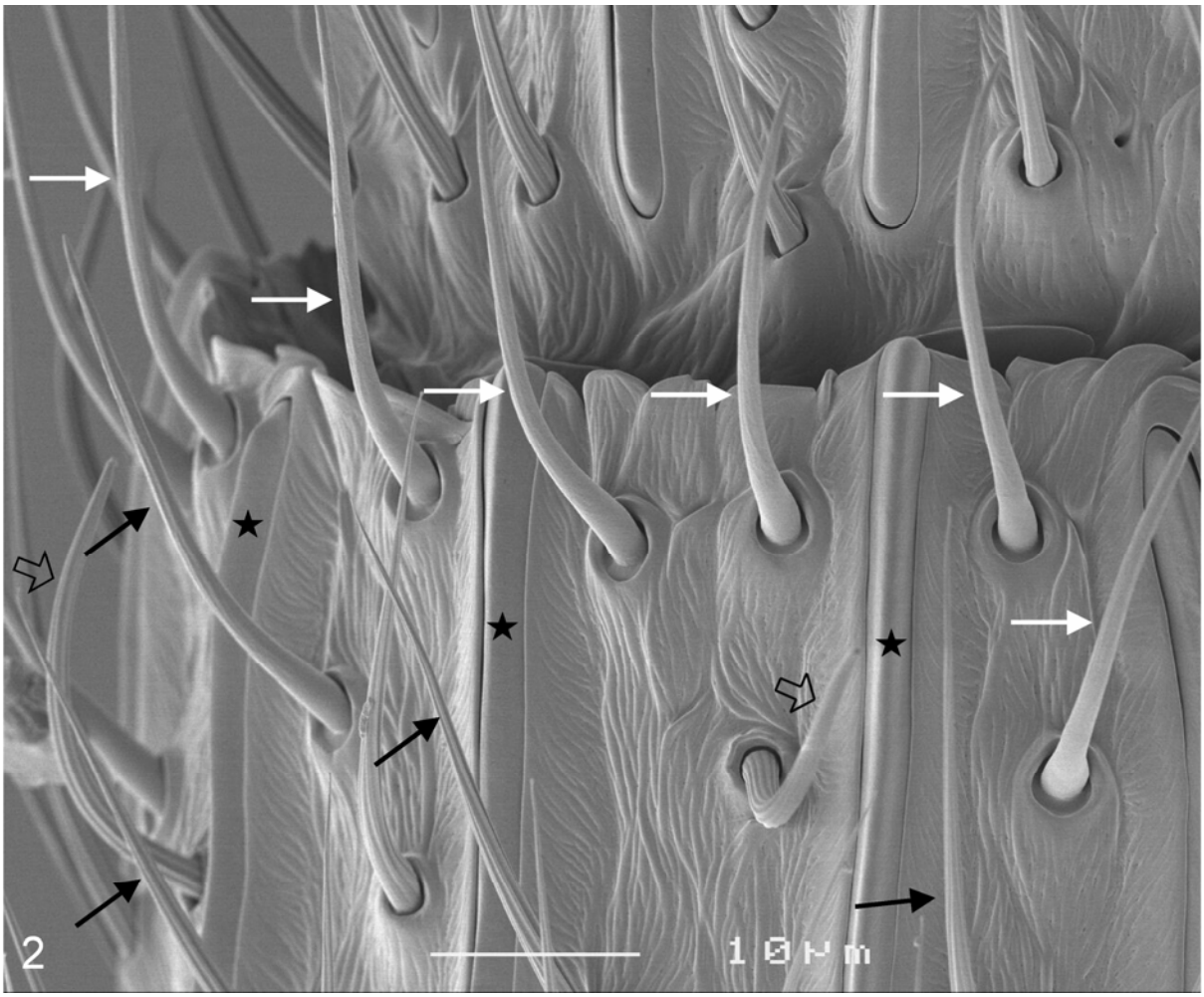
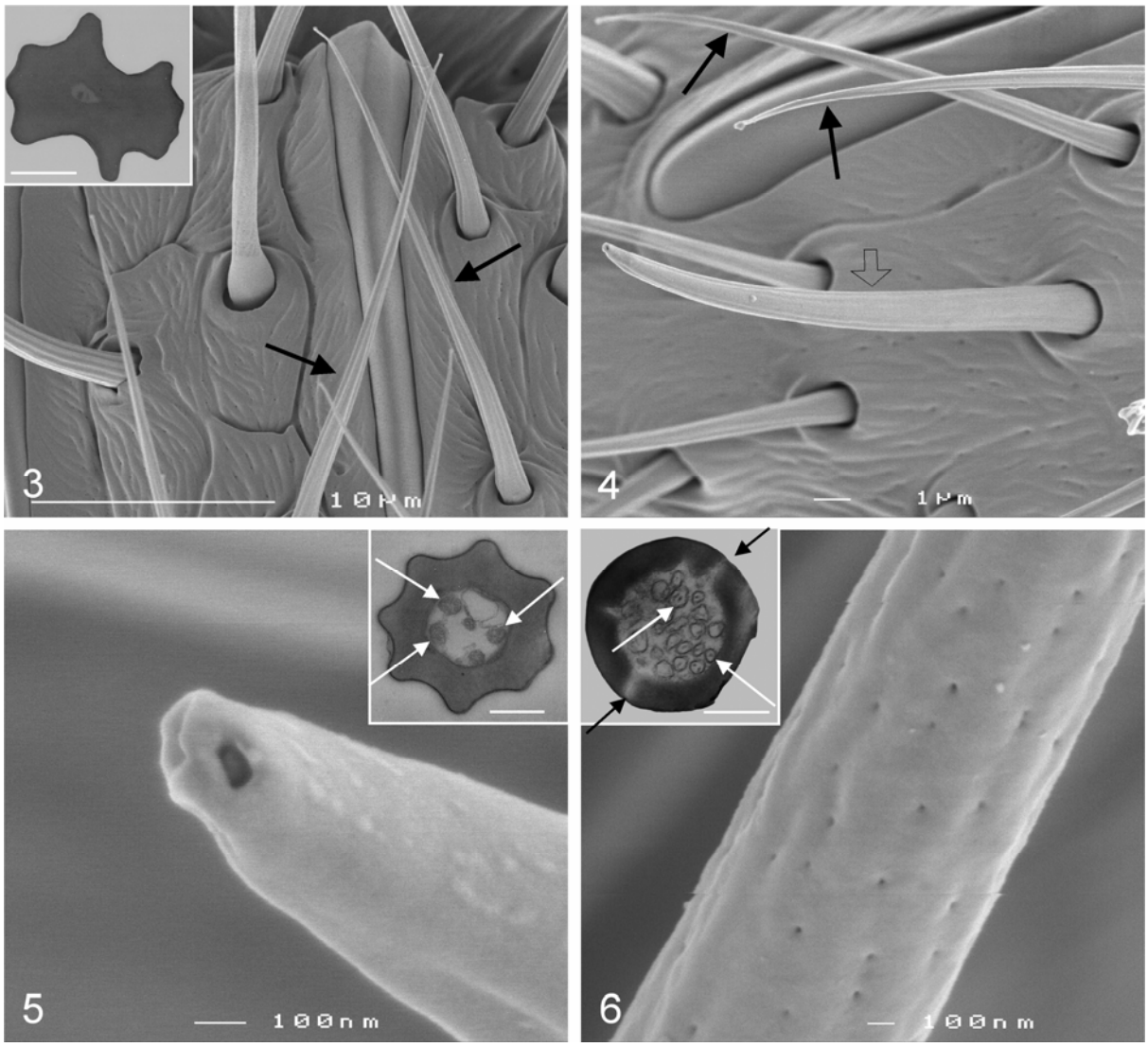


Fig. 2: Distal part of 5th antennomere of a female *C. glomerata* showing s. trichodea NP (black arrows), s. trichodea TP (open arrows), s. trichodea WP (white arrows) and s. placodea (asterisks). SEM.

grooved surface and a small bulbous structure at the tip (fig. 2, 3, 4). It was inserted in a socket and had a length of approximately 30µm with a diameter of approximately 2µm at the base. This type of sensillum had a thick solid wall without pores and the sensillum lymph was not innervated by dendrites of sensory neurons (fig. 3 inset). It was the most abundant hair-type present on all antennomeres, and was predominantly situated in between the sensilla placodea. These sensilla were slightly curved towards the apex of the antennomere and usually bent over the sensilla placodea.

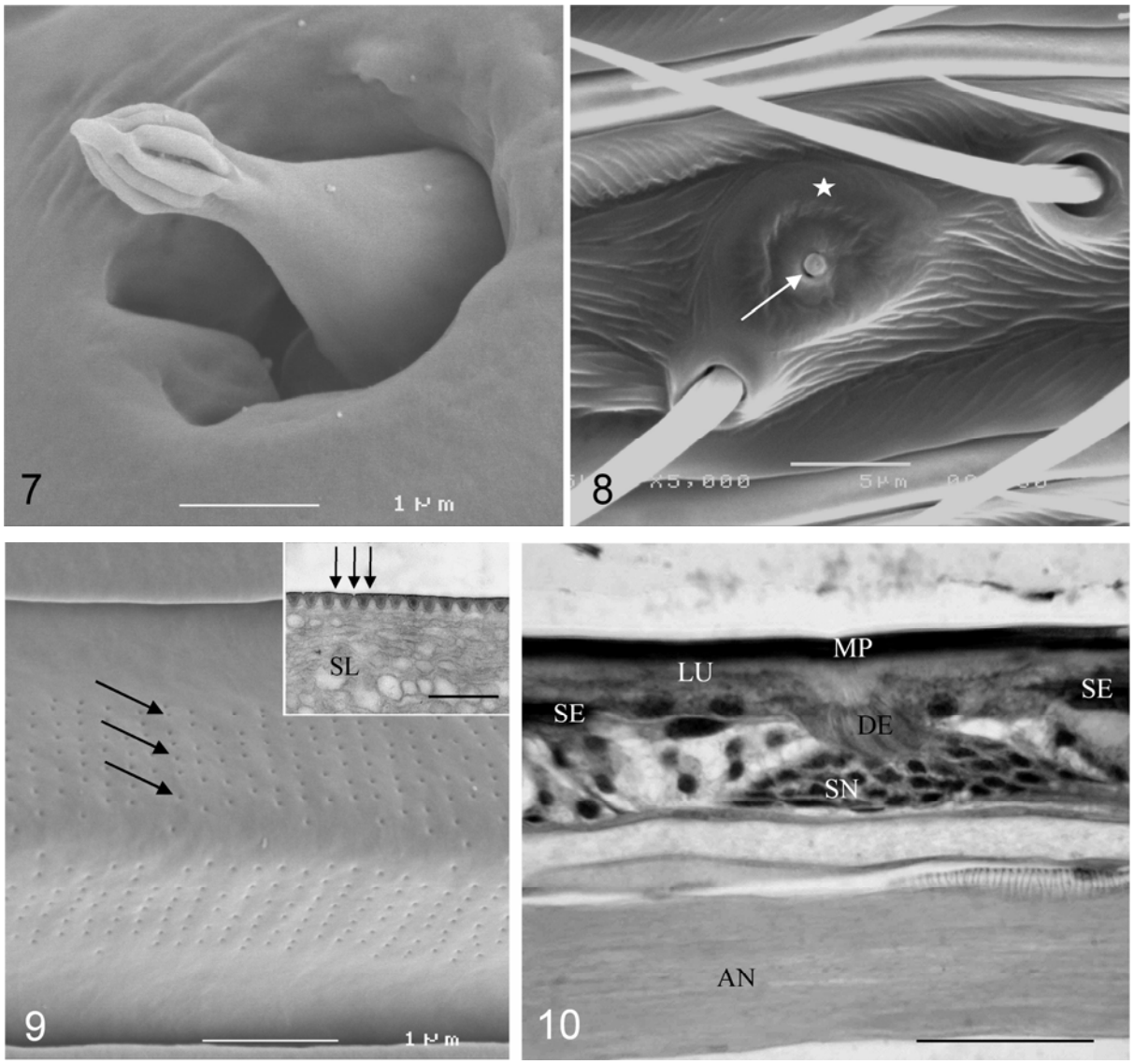
The second type of trichoid sensilla, the *s. trichodea TP*, had a grooved surface and a double pore on the apex (fig. 2, 4, 5). It was more perpendicular on the antenna than the other s. trichodea, had a socket and was approximately 30µm in length and approximately 2µm in diameter at the base. It was a little stouter than the s. trichodea NP (fig. 4). A single nonporous wall surrounded the inner lumen, which was usually innervated by 5 dendrites



Figs. 3-6. Fig. 3: Distal part of 15th antennomere of a female *C. glomerata* showing two s. trichodea NP (arrows). SEM. Inset: TEM transverse section of a sensillum trichodeum NP. Note the thick sensillum wall and the sensillum lymph, which is not innervated by dendrites. Bar = 0.5µm. Fig. 4: s. trichodeum NP (arrows) and s. trichodea TP (open arrow). SEM. Note the stout appearance of the s. trichodea TP compared to the s. trichodea NP. Fig. 5: Detail of s. trichodeum TP showing the apical double pore. SEM. Inset: TEM transverse section of a sensillum trichodeum TP, with a single non-porous sensillum wall. Within the sensillum lymph five unbranched dendrites are visible (arrows) indicating the presence of five sensory neurons at the base. Bar = 0.5µm. Fig. 6: Detail of the wall of a s. trichodeum WP, showing multiple pores. SEM. Inset: TEM transverse section of s. trichodeum WP. Note the numerous dendritic branches (white arrows) and the pores in the sensillum wall (black arrows). Bar = 0.5µm.

(fig. 5 inset). Several sensilla trichodea TP were present on each antennomere, predominantly at the apical side.

The third type of trichoid sensilla was the *s. trichodeum WP* (fig. 2, 6). This type had a



Figs 7-10. Fig 7: Detail of sensillum coeloconicum type I, showing the characteristic peg. Note the deep grooves between the fingerlike projections. SEM. Fig. 8: S. coeloconica type II, with bulbous structure (arrow) and donut shaped ring (asterisk). SEM. Fig 9: Detail of s. placodeum showing multiple pores arranged in rows (arrows). SEM. Inset: Longitudinal TEM section of s. placodeum showing the cuticular pores (arrows) and numerous dendritic processes in the sensillum lymph (SL). Bar = 0.5µm. Fig. 10: LM micrograph of longitudinal section through a s. placodeum. Note the antennal nerve (AN) and cell bodies of sensory neurons (SN) from which the dendrites (DE) run through the aperture in the septum (SE) into the lumen (LU) under the multiporous plate (MP). Bar = 25µm.

smooth surface with numerous pores of about 20nm in diameter (fig. 6). It had a socket and was 30µm in length and approximately 2µm in diameter and often had a slightly enlarged base (fig. 2). Numerous dendritic branches innervated the lumen. These sensilla were present in a circular arrangement along the distal end of each antennomere.

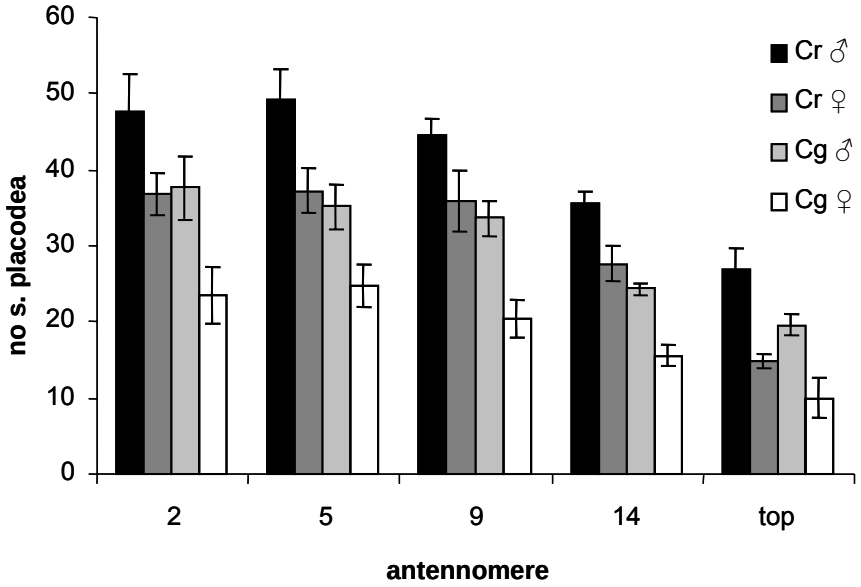
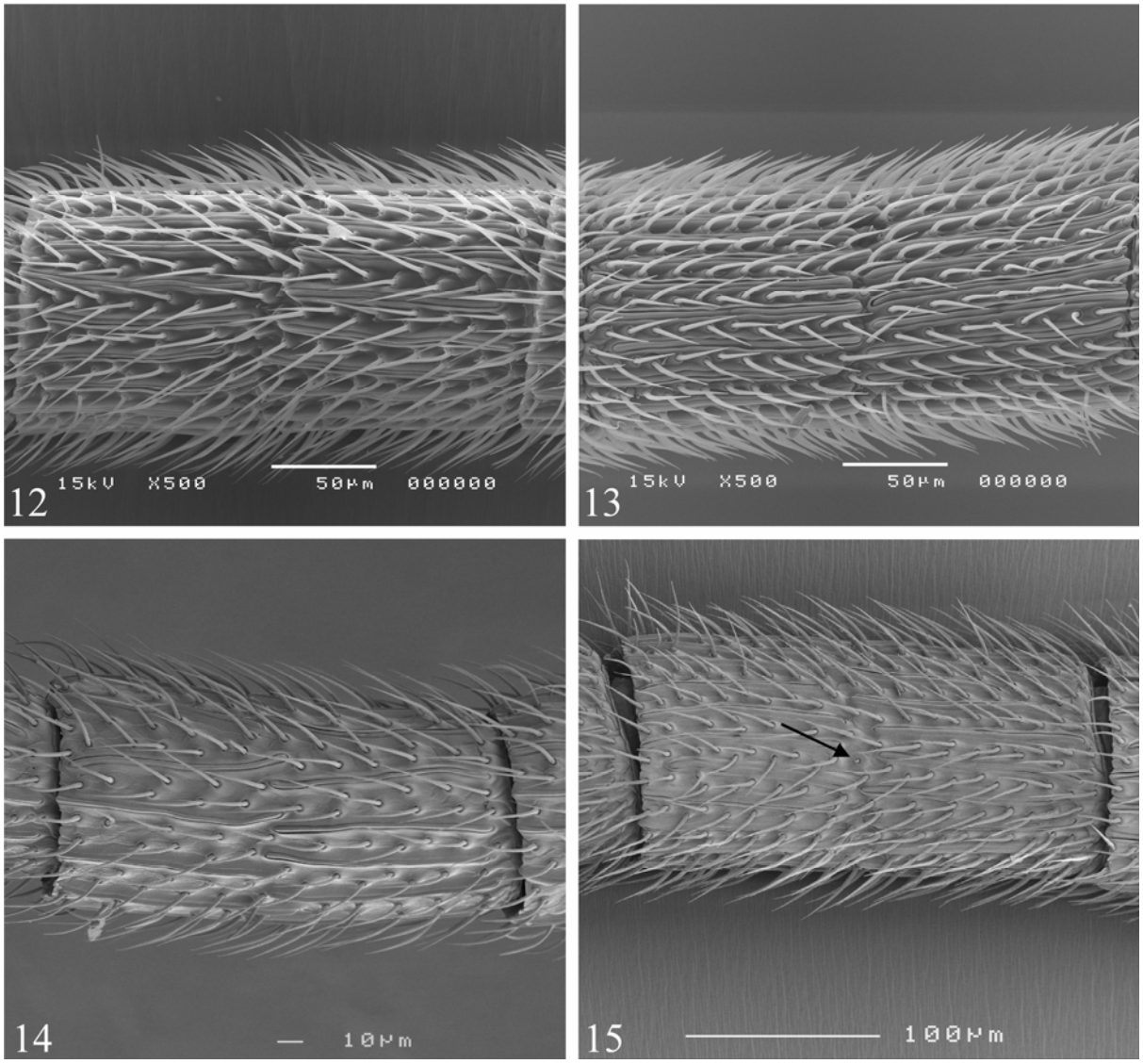


Fig. 11: Numbers of sensilla placodea specified for different antennomeres counted from proximal to distal. (*n* = 5). Cr is *Cotesia rubecula* and Cg is *C. glomerata*, top is antennomere 16.

Two types of *sensilla coeloconica* were present. *Sensillum coeloconicum type I* consisted of a peg of 2 to 3µm wide and 4µm long, protruding from a pit (fig. 7). The stalk of the peg gave rise to fingerlike projections that join at the tip. There were deep grooves between the fingers. A donut-shaped ring of 8µm in diameter surrounded the peg. A single sensillum of this type was present at the exterior-lateral side in the middle of each antennomere except for the most proximal and most distal antennomere.

Sensillum coeloconicum type II consisted of a donut-shaped ring of 9µm in diameter with a small bulbous structure of 1µm in the middle (fig. 8). A single sensillum of this type was present on the antennomeres 3, 5, 7, 9, 11 and 13, counted from proximal to distal. It was located distally from s. coeloconicum type I near the apex of the antennomere.

Sensilla placodea, also called multiporous plate sensilla (MPS), were the most prominent on the antennae. They consisted of an elongated plate of 2 to 4µm wide with multiple pores arranged in rows (fig. 2, 9). Sensillum lengths varied from 70µm to 132µm, depending of its position on the antenna, the sex and species (table 1). Within an individual sensilla could vary 30µm in length, with the smaller sensilla present on the distal segments. Sensilla placodea were significantly longer in males compared to females in both *C. glomerata* ($p < 0.001$) and *C. rubecula* ($p < 0.005$)(table 1; independent samples t-test for equality of means of length of s. placodea on antennomere no.2). Under the multiporous plate a lumen was present with numerous dendrites, which run in parallel with the sensillum. The dendrites originate from a large group of receptor neurons that were located under the septum (Fig. 10). The number of receptor neurons per sensillum was not systematically determined.



Figs. 12-15: Overview of antennomeres of different sexes and species. SEM. Figs. 12 and 13: Comparison of density of s. placodea between male and female waps. Fig. 12: Fifth antennomere of female *C. rubecula*. Fig. 13: Fifth antennomere of male *C. rubecula*. Note the higher density of sensilla placodea in the male. Figs. 14 and 15: Comparison of number of s. placodea between species. Fig 14: Fifth antennomere of female *C. glomerata*. Fig. 15: Sixth antennomere of female *C. rubecula*, showing the sensillum coeloconicum type I (arrow). Note the larger number of s. placodea and a larger diameter of the antennomere in *C. rubecula*.

The s. placodea were equally distributed around the antennomere and lie in a circular arrangement in parallel with the longitudinal axis of the antennae. There were two rows of s. placodea per antennomere, which were lying adjacent on the proximal segments but partly interweave on the distal segments. The total numbers of s. placodea per antennomere was significantly different between species as well as between the sexes (fig. 11). Males of both species had a higher number and density of s. placodea compared to females of the same

species (fig. 12 and 13, table 1; $p < 0.001$ for *C. glomerata* and $p < 0.01$ for *C. rubecula*, independent samples t-test for equality of means on density of s. placodea). This was due to a shorter inter-sensillum distance in males, with fewer s. trichodea NP lying in between the s. placodea. Furthermore, *C. rubecula* males and females had a larger number of placoid sensilla compared to *C. glomerata* males and females respectively (fig. 11, 14 and 15). However, there are no differences in the density of s. placodea between the two species (table 1); the larger diameter of the antennomeres in *C. rubecula* can accommodate a higher number of s. placodea in this species.

Discussion

We studied the antennal sensilla of the two parasitoid wasp species *C. glomerata* and *C. rubecula*. Based predominantly on SEM, we conclude that both species have the same six types of antennal sensilla. We compared our results with other studies on parasitoid wasps based on a detailed analysis of SEM microphotographs. Other species that belong to the same family (Braconidae) or superfamily (Ichneumonoidea) have sensilla that are very similar to the sensilla we found here (Barbarossa et al., 1998; Navasaro and Elzen, 1991; Norton and Vinson, 1974; Ochieng et al., 2000), although the nomenclature in these studies varied. *Sensilla trichodea TP* have been described as s. basiconica type I (Ochieng et al., 2000), s. basiconica A (Navasaro and Elzen, 1991), fluted basiconic sensilla (Norton and Vinson, 1974) and as curved trichoid formations with an apical pore (Barbarossa et al., 1998). *Sensilla trichodea WP* have been described as s. basiconica type 2 (Ochieng et al., 2000), s. basiconica B (Navasaro and Elzen, 1991) and as curved non-fluted basiconic sensilla (Norton and Vinson, 1974). The *s. coeloconicum type I* was first described as smooth basiconic sensillum (Norton and Vinson, 1974). *S. coeloconica type II* have only been described in *M. croceipe*s as s. campaniforma. The *sensillum placodeum* has also been described as multiporous plate sensillum (MPS) and occurs widely throughout the Hymenoptera (Barlin and Vinson, 1981a, b; Basibuyuk and Quicke, 1999). The external morphology of the placoid sensillum ranges from elongated like in *Cotesia* and other parasitoids (Amornsak et al., 1998; Baaren et al., 1996; 1999; Barbarossa et al., 1998; Barlin and Vinson, 1981a, b; Basibuyuk and Quicke, 1999; Borden et al., 1978a, b; Butterfield and Anderson, 1994; Navasaro and Elzen, 1991; Norton and Vinson, 1974; Ochieng et al., 2000; Olson and Andow, 1993) to circular in e.g. honeybees (Basibuyuk and Quicke, 1999; Wcislo, 1995). The precise reason for the divergent shape throughout the Hymenoptera remains unclear. In other groups (e.g. Chalcidoidea) antennal sensilla with a different external morphology are present (Amornsak et al., 1998; Baaren et al., 1996; 1999; Cave and Gaylor, 1987; Olson and Andow, 1993). This indicates that similarity of sensilla between species is highly dependent

on how closely related they are.

Function of sensilla

The putative function of sensilla can be deduced from the number of pores (Keil, 1999). *Sensilla trichodea NP* are putative mechanoreceptors (Keil, 1999). *Sensilla trichodea TP* have a gustatory function (Barbarossa et al., 1998). *Sensilla trichodea WP* are most likely to be olfactory sensilla (Keil, 1999; Steinbrecht, 1997). The function of the sensilla coeloconica is more difficult to assess. *Sensillum coeloconicum type I* is thought to be olfactory (Keil, 1999; Steinbrecht, 1997), whereas *S. coeloconica type II* might be a thermo- or hygroreceptor (Altner et al., 1983). *Sensilla placodea* have multiple pores and have an olfactory function in the honeybee (Akers and Getz, 1992), scarabid beetles (Hansson et al., 1999; Larsson et al., 2001) and the braconid wasp *Microplitis croceipes* (Ochieng et al., 2000). They seem to be specifically suited for the processing of odorant mixtures (Akers and Getz, 1993; Getz and Akers, 1994). In Hymenoptera these sensilla might be used to perceive plant odours (Ochieng et al., 2000).

Differences between the sexes

In *C. glomerata*, but not in *C. rubecula*, the length of the antennae differs between male and female. Male *C. glomerata* antennae are longer than female *C. glomerata* antennae. In *M. croceipes* (Navasaro and Elzen, 1991; Ochieng et al., 2000), two mymarid species (Baaren et al., 1999) and a pteromalid (Pettersson et al., 2001) antennae of males were also longer than those of females. This sexual dimorphism may be correlated with the difference in length of s. placodea between males and females. In *C. glomerata* the length of antennomeres is correlated with the length of s. placodea. Males have longer s. placodea, which might result in longer antennomeres and therefore longer antennae. This is consistent with *M. croceipes* where males have longer s. placodea, longer antennomeres and longer antennae (Navasaro and Elzen, 1991; Ochieng et al., 2000). In *C. glomerata* the difference in length of s. placodea between male and female is much more pronounced than in *C. rubecula*. We have no indication for the function of this difference, and some studies have also found that male s. placodea are shorter than female s. placodea (Amornsak et al., 1998; Borden et al., 1978a).

There is also a difference in the number of s. placodea between the sexes. Males have a larger number of s. placodea due to a higher density of s. placodea. Hymenopteran males often have a higher number of s. placodea (Baaren et al., 1999; Borden et al., 1978b; Navasaro and Elzen, 1991; Ochieng et al., 2000). Insect males in general often have a higher number of olfactory sensilla compared to females (see Chapman, 1982 for a review). In

most of these species males are attracted to females by sex-pheromones (Chapman, 1982), which is also true for *C. glomerata* and *C. rubecula* (Field and Keller, 1993; Tagawa, 1977; Tagawa and Kitano, 1981). Higher numbers of sensilla may indicate an increase in sensitivity (Chapman, 1982; Ignell et al., 1999). This could indicate a mate locating function of these sensilla in males and more specifically the detection of sex-pheromones. In the scarabid beetle *Anomala cuprea* males have a higher number of sensilla placodea than females, and these s. placodea are sensitive to sex-pheromones (Larsson et al., 2001; Leal and Mochizuki, 1993). The males of *C. glomerata* and *C. rubecula* may use the s. placodea to detect sex-pheromones, possibly in conjunction with host-plant odours. In a moth species a host plant volatile was discovered to increase the response of a sex-pheromone specific olfactory receptor neuron (Ochieng et al., 2002).

In both species no differences in type and topographical arrangement was found between males and females. In another Ichneumonoid, *M. croceipes,* this difference was also absent (Ochieng et al., 2000). Such differences in types and location of sensilla are present in other parasitoids that belong to the Chalcidoidea and Platygasteroidea, and these differences are thought to be associated with sex-specific differences in behaviour e.g. courtship and host recognition (Amornsak et al., 1998; Baaren et al., 1999; Cave and Gaylor, 1987).

Differences between the species

There is a significant difference in quantity of s. placodea between the two species. *C. rubecula* females possess a larger number of s. placodea compared to *C. glomerata* females and *C. rubecula* males have a larger amount of s. placodea compared to *C. glomerata* males. This might indicate a higher olfactory sensitivity for *C. rubecula* compared to *C. glomerata*. However, the number of sensilla placodea is positively correlated with body size in bees (Johnson and Howard, 1987) and such a correlation may also explain the higher number of s. placodea in the slightly larger *C. rubecula*.

We can conclude that these two species are morphologically similar at the sensory receptor level. This is in line with other studies where we found that the olfactory receptive range (Smid et al., 2002) as well as the antennal lobe structure are similar (Chapter 3, Smid et al., 2003). Therefore, these studies further emphasize the value of this model system for a comparative approach to study the neurobiological mechanism underlying a difference in associative learning. The morphological description presented here provides a basis for further electrophysiological research.

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3

Three-dimensional organization of the glomeruli in the antennal lobe of the parasitoid wasps *Cotesia glomerata* and *C. rubecula*

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Abstract

Two closely related parasitoid wasp species, *Cotesia glomerata* and *C. rubecula*, differ in their use of associative learning. To investigate the neural basis underlying these differences, it is necessary to describe the olfactory pathway of both wasp species. This paper focuses on the organization of the glomeruli in the antennal lobe. Glomeruli were stained by retrograde axon tracing of all axons in the antennal nerve and observed by confocal laser scanning microscopy. Stacks of optical sections were processed with AMIRA software, and 3D digital models of the glomeruli were produced. The combined use of 2D images and 3D surface models of the antennal lobes enabled the identification of a set of corresponding glomeruli in both wasp species. This offers unique opportunities for the study of subtle differences involved in synaptic plasticity that may occur at the glomerular level and factors regulating this plasticity.

Introduction

The primary olfactory neuropil of insects is located in the antennal lobe, situated in the deutocerebrum. It receives input from the olfactory receptor cells (ORC) located on the antenna and mouthparts. Its organization has been extensively described by several authors, as reviewed by Rospars (1988), Homberg et al. (1989), Masson and Mustaparta (1990), Boeckh and Tolbert (1993), Hildebrand (1996), Hildebrand and Shepherd (1997), Anton and Homberg (1999), and Hansson and Anton (2000). Briefly, the ORC axons terminate in distinct morphological units, the glomeruli, where they make synaptic contact with local interneurons, which interconnect subsets of glomeruli, and with projection neurons, which project to the higher brain centers. The size, number and position of glomeruli are rather invariant between individuals of the same species and sex; however, the degree of invariance differs between species (Rospars, 1983). This opens the possibility of identification of individual glomeruli. A detailed 3D map of glomeruli has been constructed for *Blaberus craniifer* (Chambille and Rospars, 1981), *Mamestra brassicae* (Rospars, 1983), *Apis mellifera* (Flanagan and Mercer, 1989; Galizia et al., 1999), *Drosophila melanogaster* (Pinto et al., 1988; Stocker et al., 1990; Laissue et al., 1999), *Manduca sexta* (Rospars and Hildebrand, 1992), *Helicoverpa assulta* and *Heliothis virescens* (Berg et al., 2002) and *Spodoptera littoralis* (Sadek et al., 2002). The number of glomeruli varies among species ranging from 35 in *Aedes aegypti* (Bausenwein and Nick, 1998) up to 1,000 in locusts (Ernst et al., 1977) and in the wasp *Vespa crabo* (Hanström, 1928), but most species investigated thus far have between 50 and 160 glomeruli.

The organization of the insect's antennal lobe has a striking similarity with the vertebrate glomerular organization, and for this reason insects are important model organisms for olfactory research (Hildebrand and Shepherd, 1997). Moreover, results from honeybee research show that the antennal lobe is one of the brain compartments that are involved in associative memory formation (Erber et al., 1980; Hammer, 1993, 1997; Hammer and Menzel, 1998). It is attractive to study associative memory formation at the level of the antennal lobe, because it is highly compartmentalized into identifiable glomeruli; this opens up the possibility of measuring, e.g., the number of synapses within one glomerulus before and after memory formation (Sigg et al., 1997). Faber et al. (1999) showed that the glomerular representation of odours is modified by associative learning, using calcium imaging techniques on honeybee brains. Furthermore, if individuals are available that are different in their learning ability, it would be possible to investigate which mechanisms underlie this difference, and again the level of the individual glomerulus is highly suitable for such an approach. Ideally, one would need two closely related species that are profoundly different in their use of associative learning, and we have found such a situation in some parasitoid

wasp species.

For parasitoid wasp species, there is a wealth of results on the significance of associative learning at the level of behavioural ecology. Many species are known to use associative learning to optimize their searching efficiency (reviewed in Turlings et al., 1993; Vet et al., 1995). Interestingly, some closely related wasp species can be very different in the level of behavioural plasticity, which makes them ideal subjects for a comparative approach (Poolman Simons et al., 1992; Potting et al., 1997; Geervliet et al., 1998). The neural basis underlying these differences has not been explored. Such research could gain immensely if compared with existing knowledge on the honeybee, also a Hymenopteran. In addition, it is necessary to compare results on, e.g., cognitive functions obtained from the honeybee model with other insect models, preferably in an evolutionary ecological context, since such functions may be strongly adapted to a species' particular niche (Menzel, 2001). In this light, Dukas (1998) proposed the term "cognitive ecology" as a research field focussing on the effects of (constraints on) information processing on animal behaviour, and ultimately on animal fitness.

In this paper, we will focus on two congeneric parasitoid wasp species, *Cotesia glomerata* (L.) and *C. rubecula* (Marshall) (Hymenoptera, Braconidae). They lay their eggs into larvae of pierid species such as cabbage white butterflies, *Pieris brassicae* (L.) and *P. rapae* (L.) (Lepidoptera, Pieridae). These *Cotesia* species are phylogenetically closely related (Dowton and Austin, 1994; Smith and Kambhampati, 1999). They find their hosts by the use of plant odours, which are induced by the feeding of caterpillars (Steinberg et al., 1993; Geervliet et al., 1994; Vet and Dicke, 1992). The two species are different in their use of associative learning for host location. Under standardized conditions, *C. glomerata* increases its preference for the odours of a particular plant species after an oviposition experience on that plant, whereas *C. rubecula* does not alter its innate preference (Geervliet et al., 1998).

In order to provide a solid basis for further neurobiological research, we need a comparative description of the morphology of the olfactory pathway of *C. glomerata* and *C. rubecula*. We give here a description of the gross morphology of the glomerular organization of the antennal lobe from female *C. glomerata* and *C. rubecula*. A digital, three-dimensional atlas of the relative size and position of the glomeruli is presented, which provides a set of landmark glomeruli that can be identified in both wasp species. A detailed description of the sexual dimorphism in the antennal lobe is in preparation and a description of the antennal sensilla has been published (Chapter 2, Bleeker et al., 2004).

Methods

Insects

Laboratory colonies of parasitoids and their host larvae originated from individuals collected in Brussels sprouts fields near Wageningen, The Netherlands, less than 1 year ago. *P. brassicae* and *P. rapae* larvae were obtained from colonies maintained under the same conditions on Brussels sprouts in a climate room at 20–22˚C, 50–70% RH, and L16:D8 photo/scotophase. *C. glomerata* and *C. rubecula* had been maintained on *P. brassica*e and *P. rapae* larvae, respectively. Parasitoid cocoons were kept in Petri dishes and emerged adults were transferred to cages and allowed to mate. Cages were placed in a climate room under the same conditions as given above, but devoid of host or plant odours. Honey and wet cotton wool was supplied and insects were used for experiments within 6 days after emergence.

Axonal tract tracing

Parasitoids were sedated by CO2 and immobilized in clay, leaving one antenna accessible. This antenna was cut at the level of the second proximal segment of the flagellum. A glass microcapillary tube, filled with 2.5% biotin dextran amide as axonal tracer (Sigma, MW 10 kDa) in distilled water, was placed over the antenna, and left for 4–5 h at room temperature. Animals were then decapitated and the brains were dissected under Ringer's solution. Antennal nerves were removed to prevent blocking of the fluorescent label in the underlying structures during confocal imaging. Brains were fixed overnight at room temperature in 4% formaldehyde in 0.1 M phosphate buffer, pH 7.0. After fixation, brains were dehydrated to 90% ethanol and immersed in heptane, in order to make the tissue more permeable for subsequent labelling procedures (Breidbach, 1990). Brains were then rehydrated, washed in four changes of phosphate-buffered saline (PBS, Oxoid, "Dulbecco A") and incubated in streptavidin conjugated to FluoroLinkCy2 (Amersham; further referred to as Cy2), diluted 1:500 in PBS with 1% bovine serum albumin and 0.25% Triton X-100. Brains were incubated at 4˚C for 24 h, washed in six changes of PBS for a total of 4 h, dehydrated in graded series of ethanol, cleared in xylene and mounted on glass microscope slides in Depex (Fluka). Due to the short working distance of the used microscope objective (see below) it was necessary to have the antennal lobe as close as possible under the cover glass. Therefore, we did not use spacers between the slides and cover glass. Care was taken to mount brains within a thin layer of Depex, so that the antennal lobe was as close as possible under the cover glass, but without applying pressure on the brains. A total of 37 brains were treated with this protocol, of which 27 yielded selective labelling of the glomeruli, and were digitized with confocal laser scanning microscopy. Two brains of each wasp species were selected for image segmentation and glomerular matching.

Confocal laser scanning microscopy

Preparations were examined with a Zeiss LSM 510 confocal laserscanning microscope, equipped with a krypton/argon laser. The 488-nm line of this laser was used for excitation of the Cy2 fluorophore, with a long-pass emission filter at 505 nm. Antennal lobes were imaged with a Zeiss x40 NA 1.3 oil-immersion objective, with a working distance of 200 µm, at a resolution of 512x512 pixels (size ranging from 135 to 145 µm). A stack of 80–100 optical sections covered the entire depth (ranging from 60 to 90 µm) of the glomerular part of the antennal lobe, with approximately 20% oversampling. This means that optical sections were 20% overlapping in the Z-direction to optimize subsequent volume rendering results. Immersion oil and mounting medium both have a refractive index N_d [20] of 1.5; therefore no correction was made for Z-axis refractive index mismatch.

Three-dimensional computer analysis

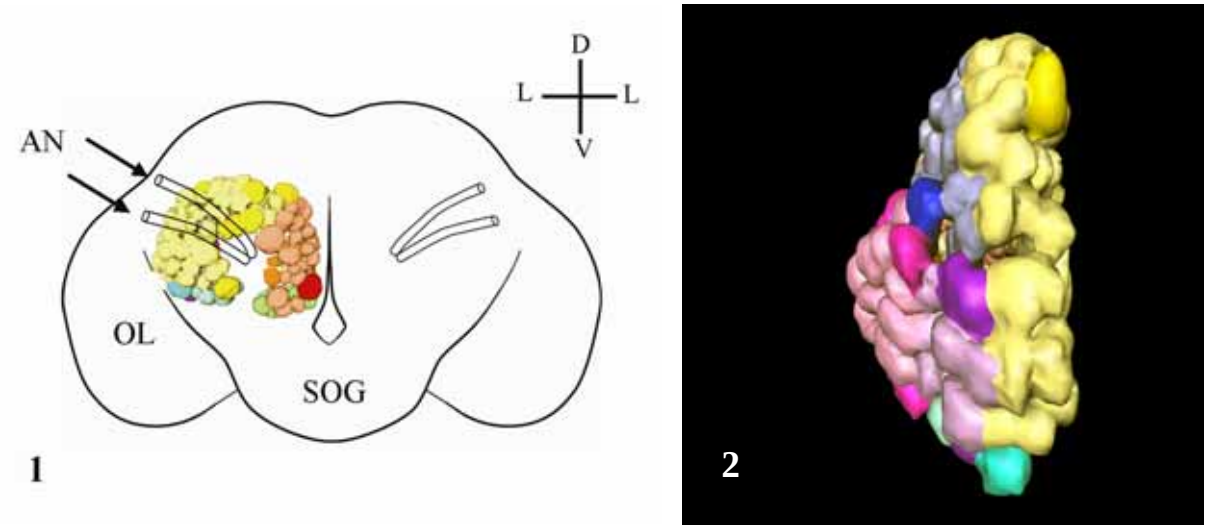
All image processing and 3D analysis was done on standard Windows 98 platforms. Scion-Image software (W. Rasband, freely available at http://www.scioncorp.com) was used to optimize contrast and brightness. It was decided to remove every other section from each stack to keep the workload reasonable for subsequent image segmentation (see below). This resulted in stacks of 40–50 optical sections, which were further analyzed with AMIRA for Windows v.2.2 (Indeed Visual Concepts GmbH, Berlin; TGS Inc., San Diego).

In each optical section, contours of glomeruli were demarcated by hand (i.e., image segmentation). Pixels that were within contours enclosing the same glomerulus in consecutive sections were given a randomly chosen unique number and colour. This resulted in a 3D array of voxels where each voxel was labelled as "background" or as belonging to one of the glomeruli. The volume of each glomerulus could easily be calculated from a segmented dataset, and a 3D surface model of the antennal lobe could be obtained by surface rendering. Such a 3D model showed surfaces of each glomerulus viewed from any position, and individual glomeruli could be removed to visualize underlying structures. In addition to 3D surface models we also used volume rendering to obtain 3D antennal lobe reconstructions. Two antennal lobes were analysed in detail from female *C. glomerata* as well as *C. rubecula* for comparison. Surface data were exported to Virtual Reality Modelling Language (VRML) in order to make datasets available on the internet.

Matching of glomeruli from different antennal lobes

In order to compare antennal lobes of different specimens, we identified the corresponding glomeruli. Datasets of con- as well as heterospecific females were matched and given corresponding glomerular codes. Three morphological characteristics were used as criteria

for identification: (1) size of glomerulus, (2) 2D morphology and typical innervation pattern, (3) 3D position (in surface reconstructions), and 3D morphology (as visible after volume rendering).



Figs. 1-2. Fig. 1: Schematic representation of the brain of *C. glomerata* (anterior view), with surface reconstruction of glomeruli (AN antennal nerves, OL optic lobe, SOG subesophageal ganglion).
Fig. 2: Three-dimensional surface projection of the antennal lobe of *C. rubecula*, lateral view, to show the Z-axis configuration. Note that the frontal surface is flattened.

Results

Orientation and size of the brains

A schematic drawing of the brain of *C. glomerata* or *C. rubecula* is given in Fig. 1. Brains of both wasp species have identical external morphology. Average width of *C. glomerata* brains is 702±24 µm, whereas *C. rubecula* brains measure 878±15 µm (means of five brains, fixed in 4% formaldehyde and mounted in Depex; some distortion due to chemical fixation after dissection will have occurred). A 3D surface reconstruction of glomeruli of a *C. glomerata* is included in the drawing to indicate the position of the glomeruli relative to the antennal nerve (Fig. 1). From 3D reconstructions, it appeared that the surface of the antennal lobe is flattened by the cover glass due to the shrinkage of the mounting medium, as visible in Fig. 2. Use of spacers having exactly the thickness of a wasp's brain is therefore recommended in order to overcome this problem, and still allow for the use of high-resolution objectives which have short working distances.

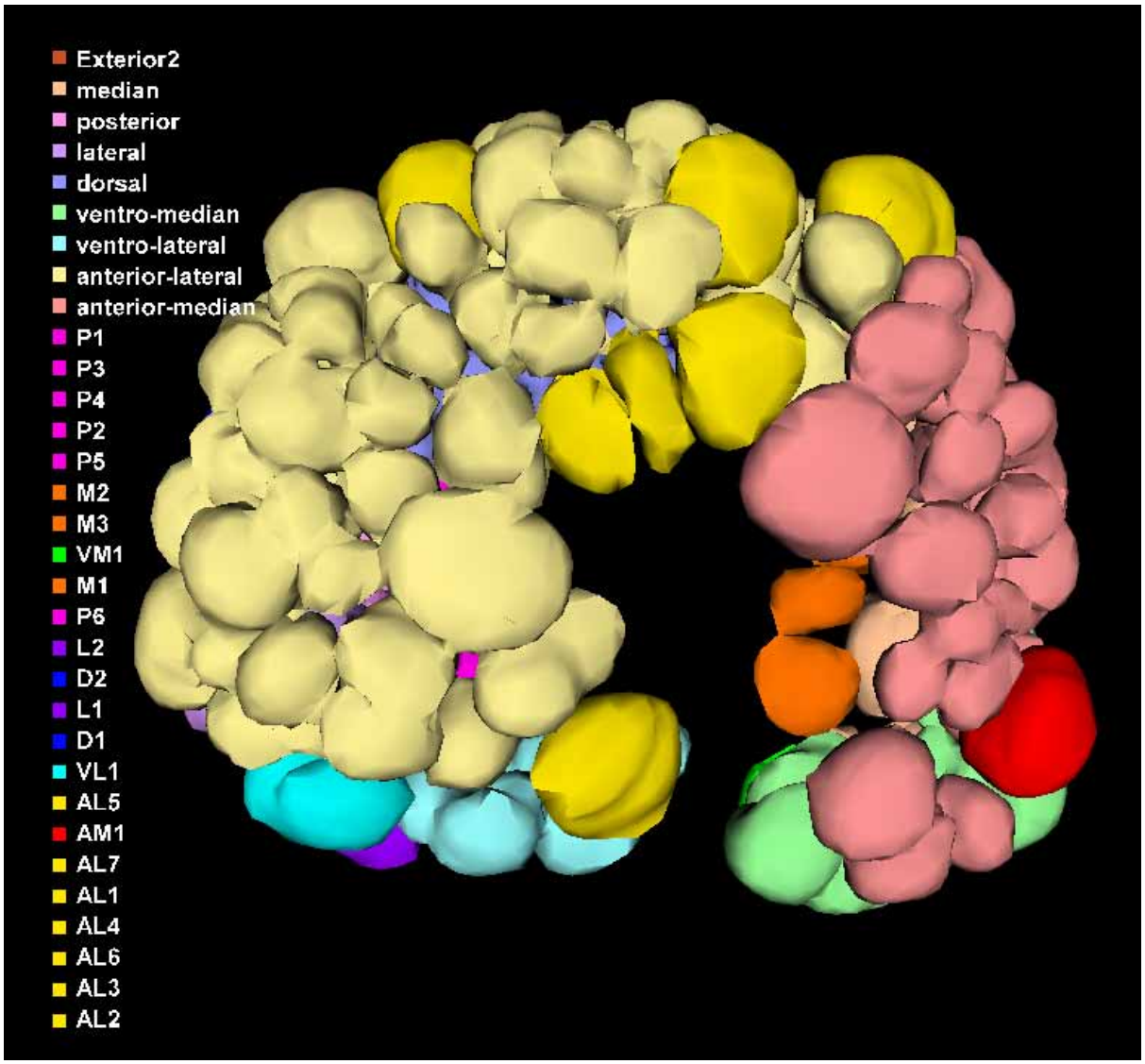


Fig. 3: VRML model of an antennal lobe of *C. rubecula*. See Table 1 for colours and codes of landmark glomeruli. Colour saturation is low in ordinary glomeruli, and high in landmark glomeruli. The VRML model can be rotated, and selective glomeruli can be removed on every current desktop computer using freely distributed software. For VRML export, it was necessary to reduce the number of different tissues (glomeruli) in the surface datasets by combining all glomeruli that were in the same compartment (see Table 1) into one tissue, except for the landmark glomeruli. This process causes such glomeruli to fuse during subsequent smoothing operations. To overcome this problem it was necessary to insert extra space between boundaries of glomeruli.

Organization of the antennal lobe neuropil

From a frontal view, the glomerular mass is ovally shaped, the dorsoventral axis being smaller than the median-lateral axis (Figs. 3, 4, 5, 6). The glomeruli are arranged in a peripheral layer of one to three glomeruli around a fibrous core. The medial half of the glomerular layer is smaller compared to the lateral half in all specimens. The medial and

Table 1: Subdivision of glomeruli and landmark glomeruli within these subgroups. Colour saturation is low in ordinary glomeruli and high in landmark glomeruli.

Compartment	Colour	Landmark glomeruli
Anterior lateral	Yellow	AL 1-7
Anterior-median	Red	AM1
Ventrolateral	Cyan	VL1, VL2
Lateral	Violet	L1, L2
Ventromedian	Green	VM1
Median	Orange	M1-M3
Dorsal	Blue	D1, D2
Posterior	Magenta	P1-P6

lateral halves are dorsally continuous with each other in all specimens, whereas they lie separated at the ventral side. A smaller subset of glomeruli lies posterior from the larger subgroup. This posterior group also has a central fibrous core (Fig. 7). A further subdivision of the larger, anterior subgroup can be made based on the innervation from the antennal nerve.

The antennal receptor neuron afferents are gathered into two antennal nerves, which fuse prior to their entrance into the antennal lobe. The fused antennal nerve penetrates the antennal neuropil frontally (Fig. 8) and runs through the antennal lobe core, so that all glomeruli are innervated from within the antennal lobe. There, it divides into two main tracts (Figs. 9, 10), as well as numerous small tracts; the latter innervate the anterior glomeruli. One of the main tracts, the lateral tract, runs in a ventrolateral direction, along the ventrolateral glomeruli, and then bends dorsally along the lateral glomeruli. It innervates the ventrolateral, lateral, and dorsal glomeruli as well as the posterior glomeruli. The other main tract, the median tract, runs in a ventromedian direction and then divides into two tracts, one innervating the mechanosensory and motor centre (see below, Fig. 11); the other, which is more diffuse than the lateral tract, runs along and innervates the ventromedian and median glomeruli.

Thus, the antennal lobe glomeruli can be subdivided into an anterior group, innervated by numerous small tracts, a ventromedian and median group, innervated by the median tract, a ventrolateral and lateral group, innervated by the lateral tract, and a dorsal and a posterior group, also innervated by the lateral tract (Fig. 3, Table 1). The resolution of the optical sections does not in all cases allow the determination of exact boundaries between the different groups, as all groups lie adjacent to one or more other groups, and the antennal nerve innervation is not visible to every single glomerulus.

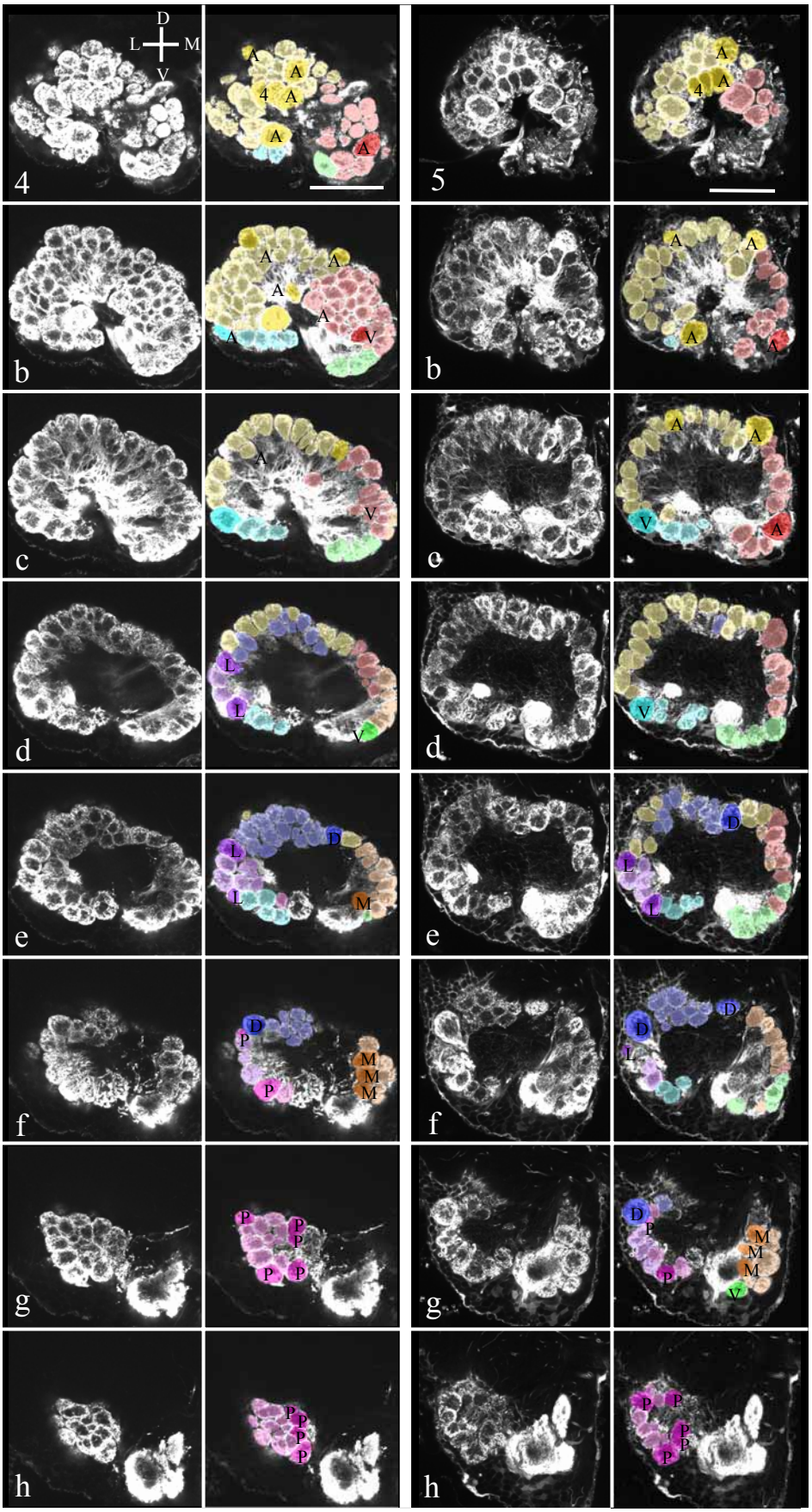
Apart from the glomeruli, there is a neuropil structure lying ventromedially from the posterior group and posterior to the ventromedian group, which is probably the mechanosensory and motor centre (AMMC) (Strausfeld, 1976). This structure is intensely labelled. The lateral part of the AMMC is fibrous and has a characteristic unlabelled, circular-shaped centre whereas the median part consists of intensely labelled small spherical units (Fig. 11). Furthermore, a median (Fig. 12) and lateral (not shown) antennoglomerular tract, as well as two ventral unpaired median (VUM) neurons in the subesophageal ganglion and part of their arborizations (Fig. 12), were typically labelled by the antennal nerve mass filling procedure. The cause of these labelling is unclear; we did not investigate this phenomenon further.

Glomerular shape

The glomeruli are globular to egg shaped, with the fluorescent label present mainly in the cortical layer, and in a few fibers in the centre. The cortical layer can be rather faint on the side facing towards the central fibrous core of the antennal lobe, from where the glomeruli are innervated by the antennal receptor neurons (the proximate side), and most intensely labelled on the distal side, facing towards the periphery of the antennal lobe. The intensity of the labelling differs between individual glomeruli, but in general the ventromedian and frontal glomeruli are most intensely labelled, whereas the dorsal glomeruli are relatively faint.

Two neuropil structures have a fibrous overall staining instead of a cortical labelling of the glomeruli. One of them, with an irregular shape, is situated medially to the posterior group (Fig. 7). The other, with a globular shape and in some individuals with a small non-fibrous core, is located ventrally from the other, medially to the ventrolateral glomeruli group (Fig. 13). It is not clear whether this fibrous structure, which is innervated by the lateral tract, is a glomerulus, and therefore we have omitted it from the 3D surface models and volume determinations.

The total volume of all glomeruli for *C. glomerata* was $192x10^3$ and $141x10^3$ μm^3 respectively, whereas for *C. rubecula* it was $341x10^3$ and $273x10^3$ μm^3. The volumes of single glomeruli ranged from 145 to 3,732 μm^3 in *C. glomerata* and from 95 to 6,920 μm^3 in *C. rubecula*. The frequency distribution of the glomerular volumes is given in Fig. 14. Volumes are given as percentages of the total glomerular volume to correct for differences in total brain volume.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← Fig. 4-5. Fig. 4a–h: Optical sections (left) and colour-marked glomeruli superimposed on optical sections (right), from anterior to posterior through the antennal lobe of *C. glomerata*. For colours and abbreviations see Table 1. Dimensions: stack size 53.9µm, 99 optical sections; 10-section interval. Bar = 50µm. Fig. 5a–h: As Fig. 4, but from *C. rubecula*. Dimensions: stack size 90.9 µm, 102 optical sections, 10-section interval. Bar = 50µm.

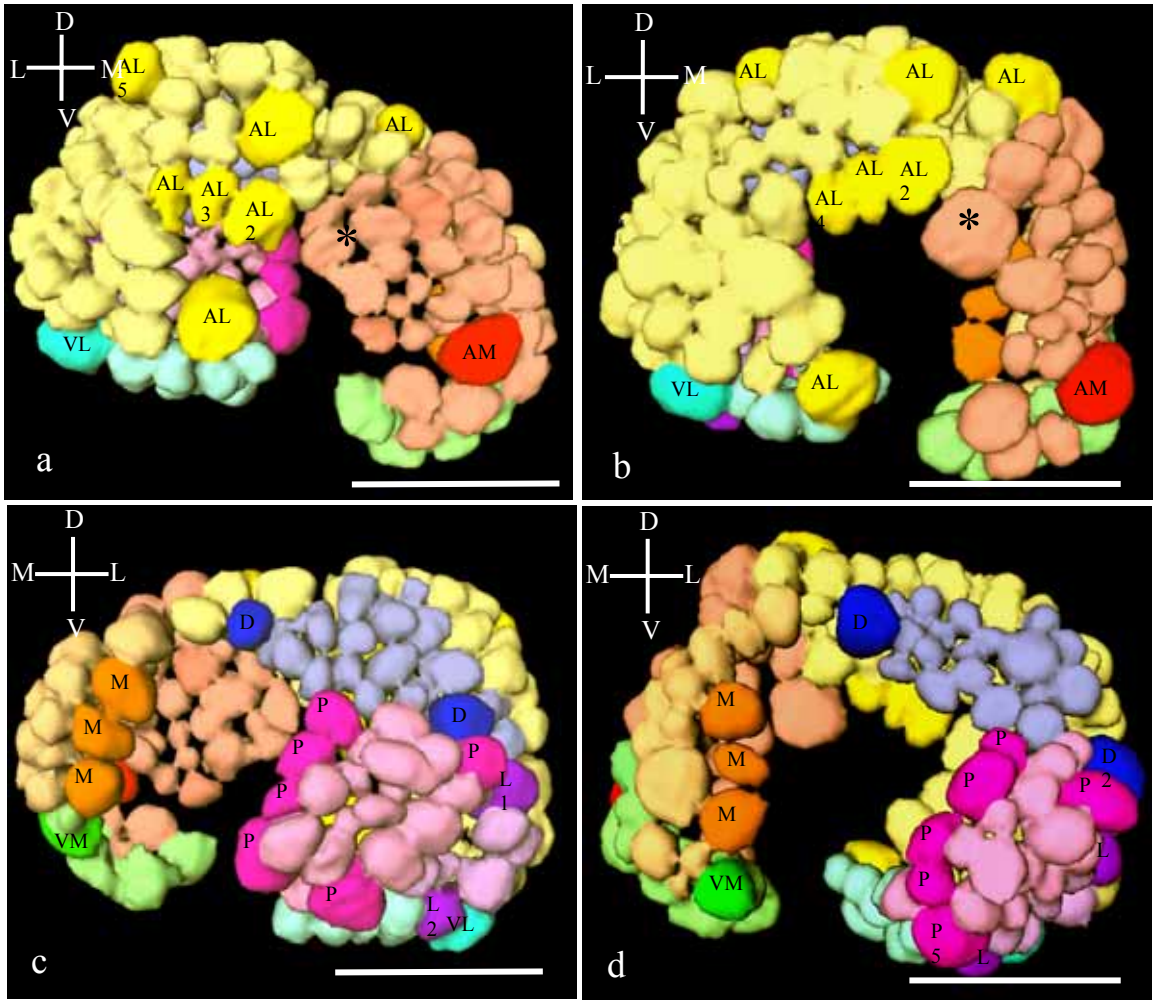
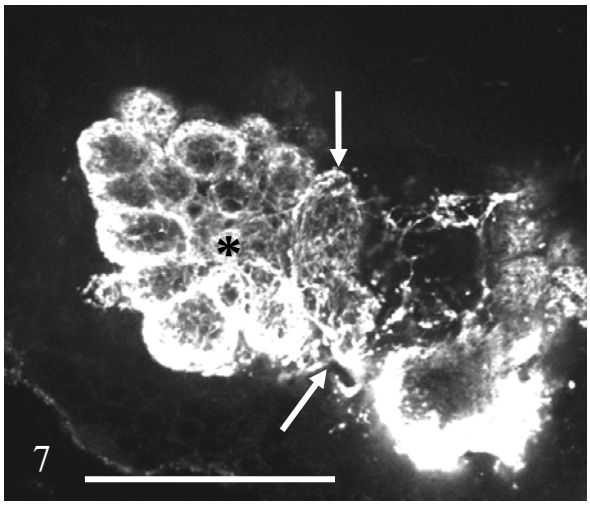
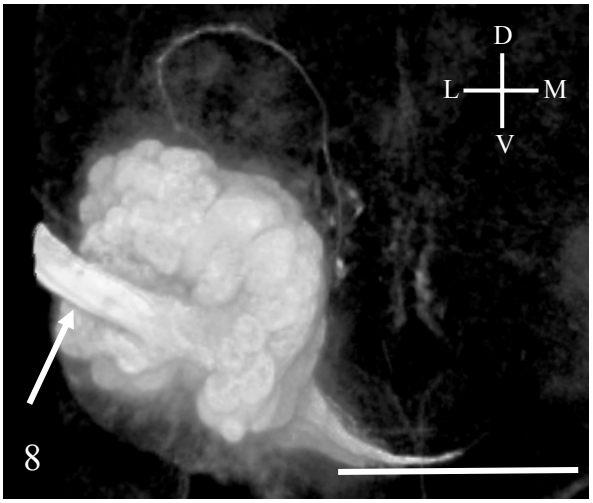


Fig. 6a–d: Surface reconstruction 3D models of the antennal lobe of *C. glomerata*: anterior view (a), posterior view (c); and *C. rubecula*: anterior view (b), posterior view (d). For colours and abbreviations see Table 1. An asterisk marks the location where four glomeruli in *C. glomerata* seem to be fused into one glomerulus in *C. rubecula*. Bar = 50 µm.

Glomerular invariance

The concept of glomerular invariance implies that the number, position and size of glomeruli is constant between individuals of the same sex (Chambille and Rospars, 1981; Rospars, 1983). The number of glomeruli is indeed fairly constant. We found that the glomerular numbers for *C. glomerata* differed by three between the two individuals (186





Figs. 7-8. Fig.7: Fibrous neuropil structure with irregular shape in posterior antennal lobe (arrow) in *C. rubecula*. Note the fibrous core corresponding to the posterior group of glomeruli (asterisk). Bar = 50 µm. Fig. 8 Three-dimensional volume projection (frontal view) of the antennal lobe with the antennal nerve (arrow) not removed, to show the entrance of the antennal nerve into the antennal lobe. Bar = 100 µm.

and 189, respectively), and by five for *C. rubecula* (193 and 198, respectively). We matched the images obtained from the two pairs of conspecific females in order to establish invariance in position of the glomeruli. It appeared that some glomeruli could be recognized in both specimens, by their specific innervation, morphology and position. These glomeruli were marked as landmark glomeruli. However, the majority of the glomeruli around such landmark glomeruli were more different in size and/or position or brightness, and we finally concluded that complete identification was not possible in a reliable manner. We have nevertheless matched the entire antennal lobes by assigning tentative identifications to variable glomeruli, in order to localize differences in glomerular organization. In this way, possible differences in organization could be localized. In conspecific females no obvious differences were found. Small differences occur because glomeruli can appear as one large glomerulus in one preparation, whereas in another preparation they appear as two separate glomeruli. In a heterospecific comparison, it appeared that female *C. rubecula* have some ten glomeruli more than *C. glomerata*, although this difference is not located in one particular area, but is due to small differences distributed over several locations. There is, however, one location where there is a clear difference between the species. *C. glomerata* has four glomeruli which are not present in *C. rubecula*, located anteromedially from the entrance of the antennal nerve, laterally from glomerulus AM1 (Fig. 6, see below). Overall, the antennal lobes of both wasp species were fairly similar in organization.

One of the aims of this study was to identify corresponding glomeruli in the two wasp species, for use in future comparative research. Therefore we decided to select a set of land-

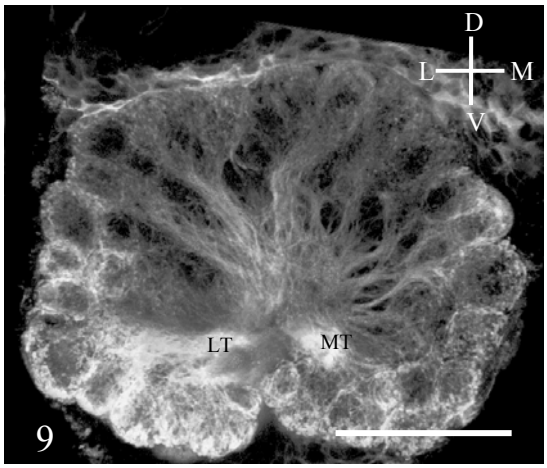
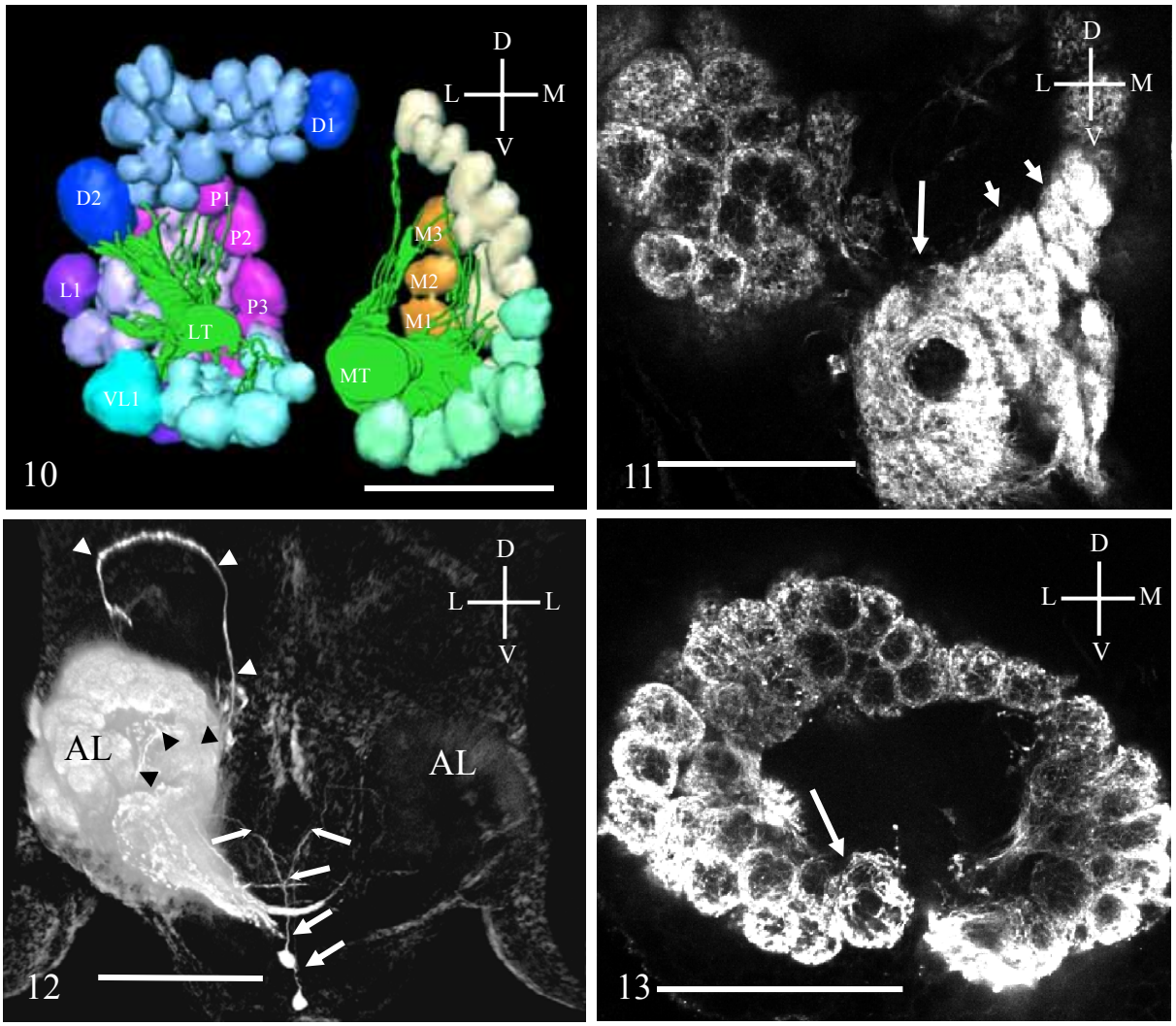


Fig. 9 Three-dimensional volume projection of the innervation pattern by numerous tracts in *C. glomerata*, which innervate the AM and AL glomeruli (LT lateral tract, MT median tract). Bar = 50 µm.

mark glomeruli, limited to those that could be recognized in both individuals of each wasp species (Figs. 4, 5, 6). In addition to these landmark glomeruli, it is possible to identify more glomeruli in an intraspecific comparison, but for this comparative study we limited the identification to those glomeruli which could be identified in both wasp species. A glomerular subdivision was made, based on the innervation from the different tracts from the antennal nerve, and further by using the selected landmark glomeruli. The boundaries of the subgroups are also consistent with the tentative matching of con- as well as heterospecific females. In the 3D models, each subgroup has a different colour (Table 1).

The anterior glomeruli, innervated by numerous small tracts, were further subdivided into a median and lateral anterior group (AM, AL). The border between these two groups is marked by landmark glomeruli AL2 and AL7. The ventromedian (VM) and median (M) glomeruli are innervated by the median tract, and separated by landmark glomerulus VM1. The border between AM and VM is set by landmark glomerulus AM1; albeit three glomeruli which are situated ventrolaterally from landmark glomerulus AM1 are still within the AM glomeruli. The ventrolateral (VL) and lateral (L) glomeruli are innervated by the lateral tract, and separated by landmark glomerulus VL2. Separation from the AL glomeruli is by landmark glomeruli VL1 and L1. The dorsal glomeruli are innervated by the lateral tract, and are located between landmark glomeruli D1 and D2. They are separated from the L glomeruli by landmark glomerulus L1. It is not clearly visible whether all D glomeruli are in fact innervated by the lateral tract; some may be innervated by the numerous small tracts. The border with the AL glomeruli is based on the fact that glomeruli in the dorsal group are arranged in up to three layers, whereas in the dorsal part of the AL glomeruli there is only one layer. The posterior glomeruli are in most preparations well separated from the other groups, and the border with the L glomeruli is by landmark glomeruli P5 and P6.



Figs. 10-13. Fig. 10: Three-dimensional surface reconstruction of the innervation pattern by the median and lateral tracts (bright green), which innervate the VM, M and VL, L, D and P glomeruli respectively. AM and AL glomeruli are removed (LT lateral tract, MT median tract); for other abbreviations see Table 1. Fig. 11: Optical section through the posterior antennal lobe of *C. rubecula*, showing the mechanosensory and motor centre. Note the fibrous lateral part with a characteristic unlabelled, circular-shaped centre (arrow) and the median part with intensely labelled small spherical units (small arrows). Bar = 50 µm. Fig. 12: Three-dimensional volume projection, caudal view, showing the labelled antennal lobe (AL white), the unlabelled contralateral antennal lobe (AL black), the median antennoglomerular tract (arrowheads) and two VUM neuron cell bodies (large arrows) and their arborizations (small arrow). Note that the VUM neuron arborizations are only partly labelled. Bar = 100 µm. Fig. 13 Fibrous neuropil structure with a globular shape and with a small non-fibrous core (arrow) in the posterior antennal lobe in *C. rubecula*. Bar = 50 µm.

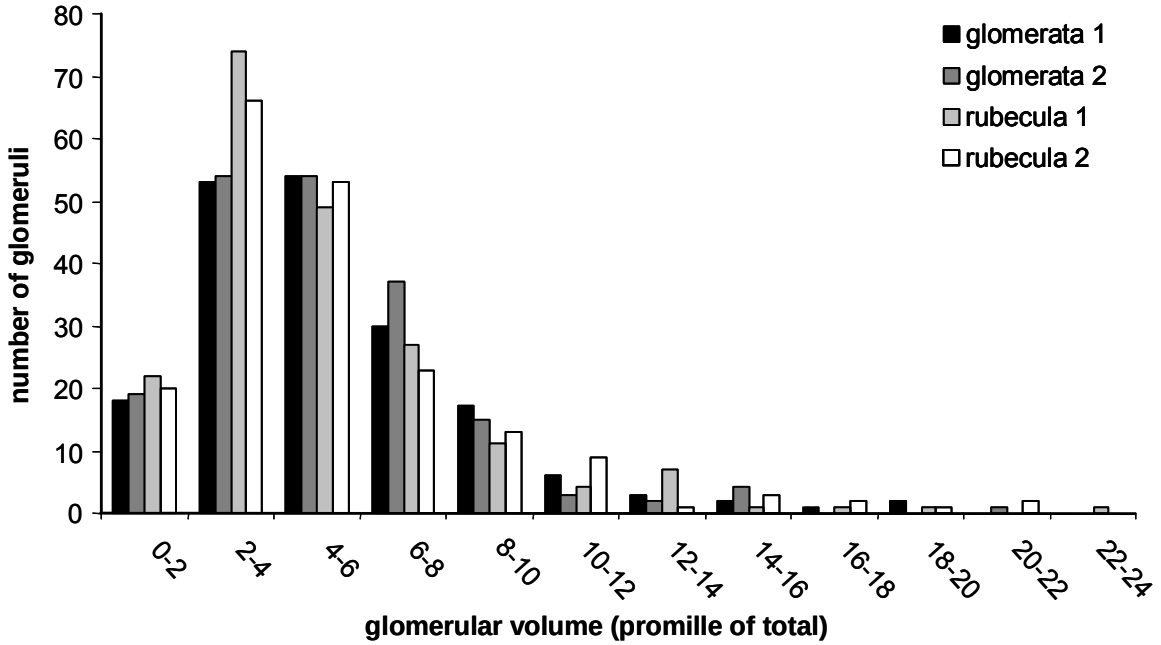


Fig. 14 Frequency distribution of glomerular volumes from two individuals of *C. glomerata* and two individuals of *C. rubecula*. The values are represented as percentages of total glomerular volume. Frequency distributions are not different between the two species (chisquare test).

Discussion

Previous anatomical studies of the antennal lobe of Hymenopteran species include the bees *Apis mellifera* (Suzuki, 1975; Arnold et al., 1984, 1985, 1988; Flanagan and Mercer, 1989; Galizia et al., 1999), *A. florea* (Brockmann and Brückner 2001) and *Bombus hypnorum* (Fonta, 1984); the ants *Camponotus vagus* (Masson and Strambi, 1977), *Formica pratensis* (Goll, 1967) and *Mesoponera caffraria* (Masson, 1972); and the wasps *Polistes gallicus* (Masson and Strambi, 1977), *Acromyrmex octospinosus* (Delabie, 1984) and *Vespa crabro* (Hanström 1928). From these studies, it appears that the number of glomeruli ranges from 92 or 104 in drones of *A. florea* and *A. mellifera*, respectively, to 166 in worker *A. mellifera*, 200 in *Camponotus vagus* and *Formica pratensis* and up to 1,000 in *Vespa crabro*. The two wasp species described in this study are within this range (except *V. crabo*); *C. glomerata* has 186–189 glomeruli and *C. rubecula* has 193–198 glomeruli.

Like the other investigated Hymenopteran species, the glomerular arrangement in *C. glomerata* and *C. rubecula* can be divided into subcompartments. There is a large anterior lobe with glomeruli arranged around a fibrous central core, and a small posterior lobe, with a small fibrous central core. For the ant *C. fagus* and the wasp *P. gallicus*, a similar subdivi-

sion has been described (Masson and Strambi, 1977). In the honeybee, by far the most extensively described Hymenopteran species, a different subdivision is made based upon the branching pattern of the antennal nerve in the antennal lobe. The antennal nerve divides into six tracts, four of which, T1–T4, innervate the glomeruli. T5 and T6 innervate the AMMC. T1 and T2 pass through the central fibrous core, T1 innervates 70 dorsofrontal glomeruli and T2 innervates 7 medial glomeruli. T3 and T4 run ventrocaudally outside of the glomerular mass, along with T5 and T6. T3 innervates 70 ventral-caudal glomeruli, and T4 innervates 7 posterior glomeruli which have ORN terminals within the entire neuropil, and not only in the cortex. This situation is different in *C. glomerata* and *C. rubecula*. All antennal nerve branchings run through the central fibrous core, and not outside of the glomerular mass like T3–T6 in the honeybee. There were no glomeruli with ORN terminals within the entire neuropil in female *C. glomerata* and *C. rubecula*, although a few fibers were present in the core of most glomeruli. There are, however, two non-glomerular fibrous structures in the posterior region, one of them with irregular shape and one ventrally from these two with a globular shape; such structures were also mentioned by Arnold et al. (1985) near the T4 glomeruli. In *Cotesia* numerous small tracts arise from the entrance of the antennal nerve, to innervate the frontal glomeruli (including a macroglomerulus in the male specimens; Smid, unpublished observations) and dorsofrontal glomeruli. These tracts are probably best compared to the honeybee T1 and T2 tracts. The *Cotesia* medial tract which innervates the ventromedial and medial glomeruli and then runs to the AMMC can best be compared to the T3 tract and the T5 tract. The *Cotesia* lateral tract innervates the ventrolateral, lateral and dorsal glomeruli, and the posterior glomeruli. This tract can best be compared to the T4 tract.

Variability of the glomeruli

The concept of glomerular invariance implies that the number, shape, size and position of glomeruli is constant between conspecific individuals. This study comprises only two specimens of two species, but it can already be concluded that the glomerular organization is more variable than that described for, e.g., *Mamestra brassicae* (Rospars, 1983) or *Blaberus craniifer* (Chambille and Rospars, 1981), and comparable to the variability described for *Pieris brassicae* (Rospars, 1983). The number of glomeruli differs by three in *C. glomerata* and by five in *C. rubecula*. Such a small variation has been reported for several other species; and may be caused by glomeruli which are fused in one individual, whereas they can be separate in another individual (Galizia et al., 1999).

The size, shape and position of glomeruli were variable for both wasp species at several locations. This variability is a problem for identification also because the boundaries between the subgroups are not always clear. Nevertheless, there are several glomeruli that can be recognized in two individuals of each species, listed in Table 1. Visual identification of

the glomeruli surrounding these landmark glomeruli is difficult due to considerable variation in location and size of these glomeruli. Furthermore, the larger the total numbers of glomeruli (numbers in *Cotesia* are at the high end of the range found in Hymenopterans), the more problems are caused by this variation. By assigning the best corresponding glomerulus in cases where visual identification is difficult, the entire antennal lobes could be tentatively matched. In this way, differences between individuals could be localized. This approach was also applicable for interspecific matching of glomeruli of both wasp species. After matching of all glomeruli, it became clear that the difference in the number of glomeruli was not due to the presence of an extra group of glomeruli in *C. rubecula*, but that small differences are spread over the entire antennal lobe. Furthermore it was found that there is a group of four small glomeruli present in *C. glomerata* which is absent in *C. rubecula* (Fig. 6). This group is located frontomedially relative to the entrance of the antennal nerve, ventral from the AL2 glomerulus. In *C. rubecula*, this group seems to be fused in the glomerulus that is located ventromedially from landmark glomerulus AL2. We have no explanation for this difference. Interestingly, this is the location were there is a macroglomerulus instead in male individuals of both wasp species (Smid, unpublished). In conclusion, the variation in the number of glomeruli is small, but the variation in location, shape and size of the glomeruli is considerable within and between these two wasp species. Nevertheless, a list of corresponding glomeruli for both species could be made. By using these landmarks, it is possible to identify more glomeruli in conspecific females. Future functional analyses, or antibodies that selectively stain subsets of glomeruli (Mistry et al., 2000), may further improve the number of identified glomeruli.

Applications of the 3D map

The availability of a digital 3D map of the antennal lobe has been of primary importance for a number of recent studies towards the understanding of the olfactory code in the antennal lobe and higher brain centres in, e.g., *Spodoptera littoralis* (Sadek et al., 2002), *Drosophila* (Vosshall et al., 2000; Gao et al., 2000; Marin et al., 2002; Rein et al., 2002; Wong et al., 2002), and the honeybee (Sachse et al., 1999; Galizia and Menzel, 2001). This study presents a three-dimensional map of corresponding glomeruli in two closely related wasp species, which are different in their use of associative learning for host searching. Although the mushroom bodies have an important role in associative learning (Davis, 1993; De Belle and Heisenberg, 1994), there is increasing evidence that the antennal lobes are involved in associative learning as well (Hammer and Menzel, 1998). The availability of a list of identifiable glomeruli in two closely related wasp species offers unique possibilities for the study of possible differences in the regulation of plasticity at the glomerular level. For instance, it has been shown that glomeruli change in size, depending on age and behavioural tasks in *Dro-*

sophila (Mistry et al., 2000) and honey bee (Sigg et al., 1997; Winnington et al., 1996). Such changes in glomerular volume may be the result of long-term synaptic plasticity. Our parasitoid wasps offer a good model system to study this phenomenon further. Another option is to analyse innervation in identified glomeruli, of interneurons which are involved in associative learning, such as the octopaminergic honeybee ventral unpaired median mx1 (VUMmx1) neuron (Hammer, 1993, 1997). This neuron innervates the glomeruli in the antennal lobe, the mushroom body calyces and the lateral protocerebral lobe, and mediates the reinforcing stimulus (reward) in the honeybee. When a sucrose reward is applied on antenna and proboscis, the VUMmx1 neuron responds with an excitation and releases octopamine, which results in olfactory conditioning if an odour is applied directly before (but not after) sucrose stimulation. This olfactory conditioning can also be achieved by substitution of the sucrose reward with depolarization of the VUMmx1 neuron alone, or with local octopamine injections (Hammer and Menzel, 1998). Our hypothesis is that a difference in learning may be reflected in the density of the arborization pattern of the octopaminergic VUMmx1neuron homologue in *Cotesia*. Other interneurons that are candidates for comparison in our *Cotesia* model system are peptidergic neurons containing tachykinins as described in the blowfly (Lundquist et al., 1994) and the mosquito (Meola et al., 2000).

Another advantage of having a digital 3D map is that such models can be made available on the internet. The surface-rendered data in AMIRA format can be transferred into VRML format (virtual reality modelling language), which is accessible for all current desktop computers using freely distributed software (Fig. 3). After downloading the VRML model from the internet, it can be viewed and manipulated (e.g., rotation, zooming, removal of tissues). We have transferred the 3D glomeruli models described in this paper into VRML, available from the homepage of H.M. Smid at our website http://www.dpw.wau.nl/ento/personeel/personeel.htm. Future neuroanatomical data can be merged into an existing model (Chiang et al., 2001; Rein et al., 2002). Online sources for the *Drosophila* central nervous system are available on www.flybrain.org (Armstrong et al., 1995), and for the honeybee (Galizia et al., 1999) and *Helicoverpa assulta* and *Heliothis virescens* (Berg et al., 2002) on www.neurobiologie.fu-berlin.de/honeybeeALatlas/. By making neuroanatomical data accessible in interactive 3D format, visualization can be significantly improved.

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4

Octopamine-like immunoreactivity in the brain and suboesophageal ganglion of two parasitic wasps, *Cotesia glomerata* and *Cotesia rubecula*

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Abstract

Two closely related parasitoid wasp species, *Cotesia glomerata* L. and *C. rubecula* Marshall (Hymenoptera: Braconidae) differ in their display of associative learning and memory during host searching. As octopamine is involved in learning and memory in insects we investigated the octopaminergic pathways in the brain and suboesophageal ganglion (SOG) of the two wasps. We used an anti-octopamine antibody and subsequent wholemount analysis using a confocal laserscanning microscope and pertinent software. Three groups of octopaminergic cells were located in the brain and suboesophageal ganglion. One group was located near the antennal lobes and consisted of six to eight cell bodies. A second group was located ventrally in the SOG and was most likely formed by ventral unpaired median (VUM) and VCBN (ventral cell body neurite) neurons. A third group was located in the pars intercerebralis and consisted of four to six cells. Octopamine-like immunoreactivity was furthermore present in the central body, protocerebral bridge, the SOG, antennal lobe, near the alpha and beta lobes of the mushroom bodies and in the mushroom body calyces. Due to the used methods and a high variability in staining intensity it was not possible to detect if there were any differences in the number of neurons, in arborisation patterns or in labelling intensity between the two wasp species.

Introduction

Octopamine is a biogenic amine that occurs in both vertebrates and invertebrates and can function as a neurotransmitter, neurohormone and neuromodulator (David and Coulon, 1985). In insects octopamine has a variety of effects on peripheral and central tissues, ranging from modulation of sensory and endocrine organs to induction of rhythmic behaviours and action as stress hormone (reviewed in Roeder, 1999).

Octopamine also effects learning and memory in insects. In *Drosophila*, learning ability is reduced after blocking of octopamine and possibly other aminergic receptors with formamidines (Dudai et al., 1987). More specifically, octopamine seems to contribute to appetitive learning, but not to aversive learning in *Drosophila* (Schwaerzel et al., 2003). In honeybees, injection of octopamine in the brain can replace a sucrose reward and induce associative learning (Hammer and Menzel, 1998). *In vivo*, a specific octopaminergic neuron located in the suboesophageal ganglion in the brain, the VUMmx1, mediates the association of an odour with sucrose (Hammer, 1993). This neuron responds to sucrose and makes synaptic contacts with the olfactory pathway. Artificial stimulation of this single neuron could entirely substitute for the sucrose reward in a proboscis extension reward conditioning experiment (reviewed in Hammer and Menzel, 1995). Furthermore, selective inhibition of an octopamine receptor in the antennal lobe of the honeybee, by injections with either an OA-receptor antagonist or by double stranded OA-receptor RNA, inhibited olfactory memory acquisition and recall (Farooqui, 2003).

We study learning in the two closely related parasitoid wasp species *Cotesia glomerata* and *C. rubecula* (Fig. 1). These species differ in their display of associative learning of odours during host searching behaviour. *C. glomerata* changes its preference for the odour of a particular plant species after an oviposition experience on that plant (Geervliet et al., 1998). A single oviposition experience in the presence of a specific odour induces a long-lasting memory trace for the experienced odour that lasts for at least five days (Chapter 5, Bleeker et al., in press). *C. rubecula* also forms an associative memory for the odour experienced during an oviposition (Chapter 5, Bleeker et al., in press). This memory lasts for one day only and is not strong enough to alter the innate odour preference (Geervliet et al., 1998; Chapter 5, Bleeker et al., in press). Thus, these two species are closely related, but differ in their use of learning during host searching. This provides us with an excellent opportunity to study the physiology of learning.

To provide a solid basis for further research on this difference in olfactory learning we first studied the olfactory pathway. Of both species we described the antennal sensilla (Chapter 2, Bleeker et al., 2004), analysed the olfactory receptive range (Smid et al., 2002) and made a three-dimensional map of the primary olfactory neuropil in the brain; the

glomeruli in the antennal lobe (Chapter 3, Smid et al., 2003). In the present study we describe the distribution of the putative octopaminergic neurons in the brain and suboesophageal ganglion. These data give us further insight in the possible pathways involved in learning in these parasitoid wasp species. We suspect that the difference in learning between the two wasps may be reflected in differences in the characteristics of the octopaminergic VUM neurons. A number of candidate octopaminergic neurons were identified in this study that could be involved in oviposition reward learning.

Methods

Insects

Cotesia glomerata (Fig. 1) and *Cotesia rubecula* were obtained from colonies that originated from individuals collected in Brussels sprouts fields in the vicinity of Wageningen, The Netherlands. The parasitoids were reared on *Pieris brassicae* (Fig. 1) and *P. rapae* respectively, as described by Geervliet et al. (1998) in a climatic room at 20-22°C and a photoperiod of L16:D8. *Pieris* larvae were reared on Brussels sprouts plants (*Brassica oleracea gemmifera* cv. Cyrus), under the same climatic conditions. Recently emerged (0 – 4 h old) and 1 week old wasps were used for subsequent octopamine labelling.

Octopamine labelling

Female wasps were caught in glass vials and sedated by cooling on ice for 5 minutes. In order to achieve a rapid, "perfusion-like" fixation, wasps were then injected ventrally in anterior direction in the thorax using a syringe with a gauge #32 needle (Hamilton), with a mixture of 1 part 25% glutaraldehyde and 3 parts saturated picric acid with 0.1% glacial acetic acid (GPA) until pressure build-up was monitored by extension of the neck membrane. To relief pressure from the head capsule and improve accessibility of the brain to the fixative, the antennae were cut at the base. Wasps were killed instantaneously by this injection procedure. After 5 minutes, the wasps were decapitated and the cut head was immersed in GPA for 30 minutes. Brains (Fig. 2) were dissected from the headcapsules in Ca-free ringer, postfixed for 2 to 4 hrs in GPA, rinsed several times in 70% ethanol and kept overnight at 4 ºC in 70% ethanol. Brains were dehydrated to 90 % ethanol and immersed for 20 s in heptane, in order to make the tissue more permeable for subsequent labelling procedures (Breidbach, 1990) and rehydrated to phosphate buffered saline (PBS, Oxoid, 'Dulbecco A'). Brains were subsequently incubated for 20 min in 0.5% sodiumborohydride (Merck) to reduce oxidized octopamine, rinsed several times in PBS, incubated for 1 h in collagenase (0.5 mg/ml PBS), rinsed in PBS-T (PBS + 0.5% Triton X-100), preincubated for 1 h in 10%

normal goat serum in PBS-T (PBS-T-NGS) and incubated for 20 h at RT in rabbit anti-octopamine (Mobitec #1003GE, Goettingen, Germany, identical to serum #AP007 from GEMAC, Talence, France) produced against an octopamine-glutaraldehyde-bovine serum albumin complex (Mons, 1987), diluted 1:500 in PBS-T-NGS, under mild agitation. Brains were washed in 6 changes of PBS-T for a total of 3 h, incubated for 4 h at RT in goat-anti-rabbit conjugated to FluoroLink™Cy™2 (Amersham) 1:200 in PBS-T-NGS, rinsed in several washes of PBS-T for 4 h, subsequently rinsed in several washes of PBS for 4 h or overnight, dehydrated in a graded series of ethanol, cleared in xylene and mounted on glass microscope slides in Depex (Fluka). Control experiments were performed without octopamine antiserum in the first incubation. These preparations did not yield any fluorescent signal. Specificity of the antiserum for glutaraldehyde-conjugated octopamine has been determined by ELISA competition tests as specified by the manufacturer (www.mobitec.de) and by pre-adsorption tests in earlier studies using this antiserum (Strausfeld, 2003).

Confocal laser scanning microscopy

Preparations were examined with a Zeiss LSM 510 confocal laser-scanning microscope, equipped with a krypton/argon laser. The 488 nm line of this laser was used for excitation of the Cy™2 fluorophore, with a long-pass emission filter at 505 nm. Brains were imaged with a 25x oil-immersion objective (N.A 0.8) at a resolution of 1024x1024 pixels. The field diameter of this objective allows that the entire brain can be digitised except for the optic lobes. A stack of 60 optical sections covered the entire depth of the brain. In total we used 36 preparations that showed octopamine-like immunoreactivity **(**OA-IR) for further analyses, 21 for *C. rubecula* and 15 for *C. glomerata*. Immunoreactive labelling is given here as single optical sections, or as 3D projections from several adjacent sections made with the 3D software module belonging to the Zeiss LSM 510 microscope. Neural axis was used to indicate positional directions.

Results

General labelling results

Consistent OA-IR in both *C. glomerata* and *C. rubecula* brains was only obtained after perfusion fixation, as described in Material and methods. Experiments in which we dissected unfixed brains in ice-cold ringer solution followed by subsequent immersion fixation identical to the methods described in this paper never yielded any positive result. We did not systematically determine whether perfusion fixation is an absolute prerequisite for successful staining, but our results suggest that during the process of dissection or fixation, part of

the OA-IR may vanish. Preparations obtained after perfusion fixation were still variable in labelling intensity. In general, the labelling procedure resulted in intense labelling of cell bodies, whereas the axons of the labelled neurons were only partly visible or unlabelled. Axon terminals were occasionally immunolabelled. Due to the incomplete labelling of axons and axon terminals it was not or only partially possible to define the projection pathways of the immunoreactive cells.

Octopaminergic neurons

Three groups of neurons consistently showed octopamine-like immunoreactivity. The results of the morphological pattern observed in *C. glomerata* and *C. rubecula* were similar and will below be described jointly. As we found a lower number of OA-IR neuron groups than were described previously for the honeybee by Kreissl et al (1994), we used a different nomenclature to preserve consecutive numbering.

Group 1: Neurons near the antennal lobe.

One cluster of cell bodies was located medial to the antennal lobe neuropil in both hemispheres. This group of cells is stained most consistently and with the highest intensity (Fig. 3). Usually there is some variability in the intensity of staining within this group of cells, and the labelling intensity is not bilaterally symmetrical within individual animals. The cells are located within a depth of 30 µm to 90 µm from the anterior surface of the antennal lobe. The diameter of the somata of these cells is between seven and ten µm. Each cluster consists of six to eight cells (Fig. 3). Some of these neurons project in posterior direction (not shown).

Group 2: Ventral neurons in the SOG:

A second group of cells that is labelled consistently is located in the suboesophageal ganglion (SOG) (Fig. 4 - 6). The diameter of the cell bodies in this group was between seven and ten µm. The cells were located in the mandibular, maxillary and labial neuromere. Most immunoreactive cells are located at the ventral side of the SOG, but occasionally a cluster is located more dorsally. The number of labelled cells is highly variable but was in the range of 14 to 20 cells. A single axon typically emanated from some of these cell bodies projecting along the midline of the SOG and bifurcating just below the oesophagus (Fig 4). In four preparations, one pair of cells was located in a more lateral position (Fig. 5) Their neuronal arborisations were not visible.

Group 3: Cells in the pars intercerebralis.

A group consisting of 4 to 6 separate neurons was present in the dorsal protocerebrum, in the pars intercerebralis (fig. 3, fig. 7). Cell bodies of this group had a diameter of approximately 5 µm. Their neuronal arborisations were invisible.

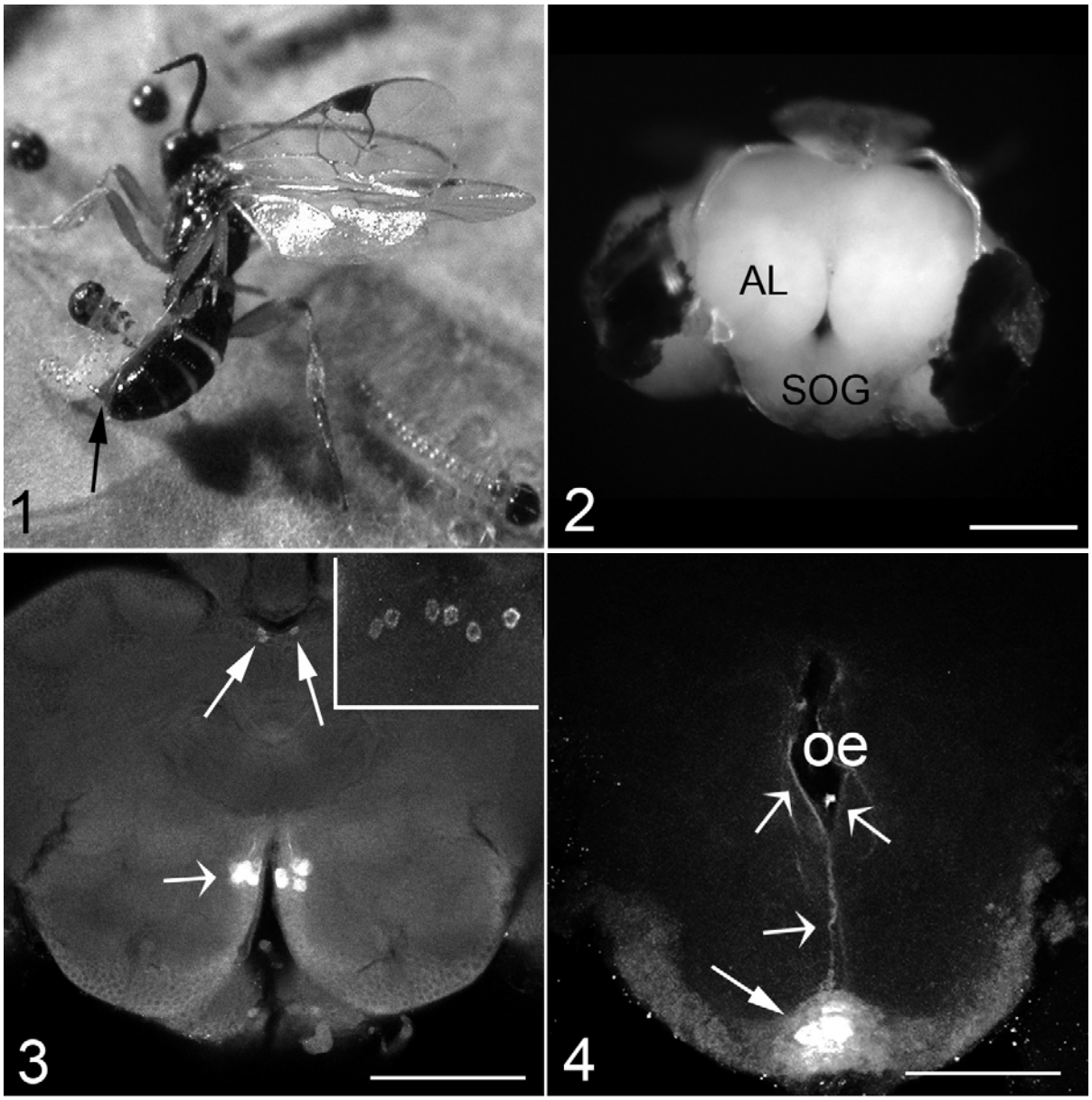

Fig. 1 - 4: Fig. 1: Photograph of a *C. glomerata* female wasp inserting her ovipositor into a 1st instar larva of the large cabbage white, *P. brassicae.* Arrow indicates the ovipositor. Fig. 2: Photomicrograph of a dissected brain of *C. glomerata* (frontal view, top = dorsal, bottom = ventral). AL = antennal lobe; SOG = suboesophageal ganglion. Bar = 100 µm. Fig. 3: Optical section of a brain of *C. rubecula*, showing paired cluster of group 1 somata medial to the antennal lobe neuropil (arrow with concave arrowhead), and 2 somata of group 3 neurons in the pars intercerebralis (arrow with triangular arrowhead). Bar = 100 µm. Inset: detail 3D projection from stack of adjacent sections showing 6 somata of group 3 in the pars intercerebralis. Fig 4: 3D Projection of a SOG of *C. glomerata*, showing the projections of the ventral unpaired median neurons (group 2). Note the somata ventral in the SOG (arrow with triangular arrowhead), the typical projection along the midline of the SOG, and the bifurcation near the oesophageal canal (OE) (arrow with concave arrowhead). Bar = 50µm.

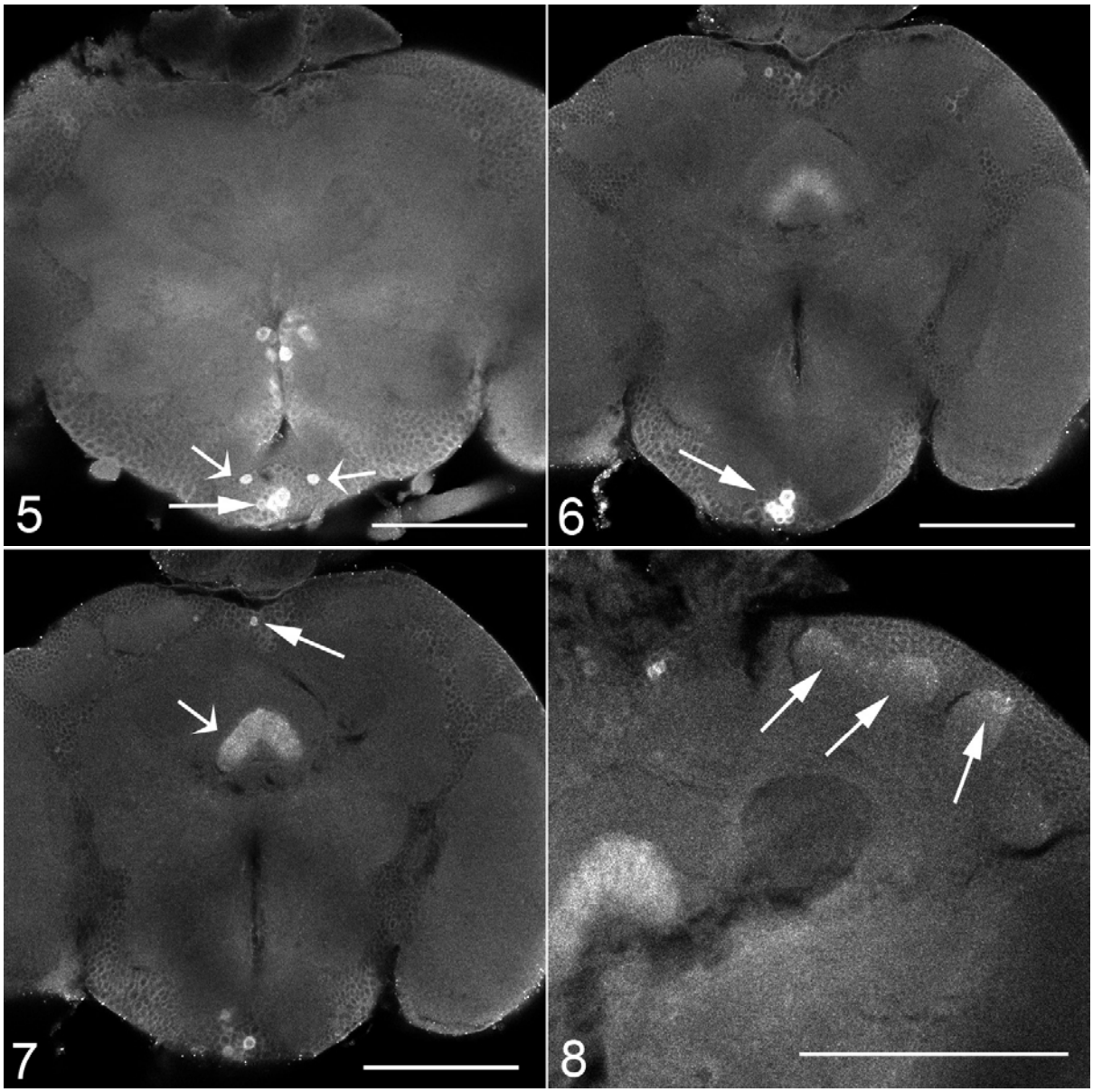

Fig. 5 - 8. Fig. 5: Optical section of a brain of *C. glomerata*, showing somata of VUM neurons (arrow with triangular arrowhead) and of one pair of lateral neurons (arrow with concave arrowhead). Bar = 100µm. Fig 6: Optical section of a brain of *C. rubecula*, showing somata of VUM neurons in the SOG (arrow). Bar = 100µm. Fig 7: Optical section of a brain of *C. glomerata*, showing immunoreactive ventral body complex (arrow with concave arrowhead) and a somata of group3 in the pars intercerebralis (triangular arrowhead). Bar = 100 µm. Fig. 8: Optical section of a brain of *C. rubecula,* showing immunoreactive varicosities in the mushroom body calyces (arrow with triangular arrowhead). Bar = 100µm.

Octopaminergic axons and axon terminals

In addition to the three groups of cell bodies, OA-IR was present throughout the brain in axons and axon terminals. The ventral neurons in the SOG project into the mandibular and maxillary midline tract in the SOG described above (Fig. 4). Clearly labelled tracts were furthermore present on both sides of the oesophagus (Fig 4). Few preparations contained incompletely immunostained fibers that project to the optic lobes at the posterior part of the protocerebrum, to the antennal lobes and on either side of the midline of the protocerebrum (not shown). Strong labelling occurred in the lower part of the central body (Fig. 7). Octopaminergic varicosities were found in the SOG, antennal lobe, around the alpha and beta lobes of the mushroom bodies and in the mushroom body calyces (Fig. 8). Connections of these varicosities to primary neurites were never visible and therefore it remains obscure from which of the above described cells these varicosities originate.

Discussion

Octopamine labelling

There was a high variability in the intensity of the immunoreactivity. Consistent immuno-reactivity was only achieved when dissection of the brains was done after perfusion fixation. Apparently dissection has a great influence on the octopamine reactivity, even when done in ice cold Ringer solution. Even after perfusion fixation there is variability in the labelling intensity. With this method it was not possible to map entire arborisation patterns of octopaminergic cells, because labelling of axons and axon terminals was incomplete. This incomplete labelling of axons is not likely due to antibody penetration problems, as the labelling of some of the cell bodies is very intense while their primary axons are entirely invisible. Furthermore, experiments using an antiserum against serotonin in a similar whole mount protocol revealed immunoreactive fibers appropriately in *Cotesia* brains (Smid, unpublished). Apparently, the density of octopamine-like immunoreactivity in the axons is below detection limits. Nevertheless, the sensitivity of the antiserum may be improved by using vibratome sections, instead of the whole mount labelling methods used in this study. Variability of OA-IR has been described before and might be related to variety in the behavioural state of the animals (Kreissl et al., 1994 and references therein; Spivak *et al*., 2003). In order to gain a more complete labelling of octopaminergic cells and axons a different labelling method might be helpful. In *Drosophila* the tyramine β-hydroxylase (TBH) immunoreactivity pattern is highly similar to the octopamine immunoreactivity pattern, but shows more cell bodies (Monastirioti et al., 1996). As TBH catalyses the last step in the octopamine biosynthesis this could indicate that it is a more sensitive method to detect OA neurons (Monastirioti et al., 1996). In addition, studying the distribution of OA-

receptors is necessary to understand which are the targets of the octopaminergic neurons (Grohmann, 2003).

Octopamine immunoreactivity

The most consistently stained group of cells was located medial of the antennal lobe neuropil. In *Cotesia* this group consists of six to eight cells per cluster. This group of octopaminergic cells was also found in the honeybee where it consists of eight to nine cells per cluster (group 3 cells, Kreissl et al, 1994; Spivak et al., 2003). A comparable group consisting of four to five cells per cluster is present in *Drosophila* (Monastirioti et al., 1995). Although these cells are located near the antennal lobe, we could not detect any fibers running directly towards the glomeruli, which is also the case in the honeybee and *Drosophila* (Kreissl et al., 1994, Monastirioti, *et* al.,1995). Rather, at least some of these cells project in the opposite direction to the oesophagus, where the tract bends and continues posteriorly. A similar course was found in *Drosophila* and the honeybee (Kreissl et al., 1994; Monastirioti et al., 1995).

The second group of 14-20 OA-IR neurons was located ventral in the SOG. This group consists of ventral unpaired median (VUM) neurons and one pair of neurons in a more lateral position. The latter group was found in only four preparations. In the honeybee, a single pair of OA-IR neurons is described at a similar location, the paired ventral cell body neurite (VCBN) neurons (group 6, Kreissl et al., 1994). These cells do not send their axons via the median tracts, as do the VUM neurons, but via the ventral cell body neurite tracts (Schröter, 2002). They project to the neuropil next to the oesophagus, the neuropil surrounding the alpha lobe and to the upper part of the central body (Schröter, 2002). In *Cotesia* we found one pair of octopaminergic neurons that was clearly separated from the median cluster of VUM neurones. Their projections were not visible. Possibly, they are similar to the VCBN neurons described for the honeybee.

The VUM neurons are unpaired. Similar groups are found in the honeybee and in *Drosophila* (Group 7, Kreissl et al., 1994; Monastirioti et al., 1995). In the honeybee there are seven to ten cells per neuromere. In *Drosophila* a total of 12 to 14 cells have been reported. In the honeybee there are 10 types, 4 of which project to peripheral nerves, whereas 6 project centrally (Schröter, 2002). Two of the peripheral VUM neurons project to the antennal nerve, whereas the other two project to the mandibular nerve (Schröter, 2002). The combined projection pattern of the central VUM neurons includes the SOG, the neuropil along the oesophagus, the tritrocerebral neuropil ventral of the dorsal lobe, the deutocerebral neuropil at the base of the efferent tract of the antennal lobe, the antennal lobe, the lip and basal ring of the calyces, the lateral protocerebrum and the anterior protocerebral neuropil around the alpha lobe (Schröter, 2002). In *Cotesia* the cells in the

SOG are located in the same position as in the honeybee, project via the median tracts and display the same typical bifurcation and projection patterns. In a previous study, two VUM neurons were visualised by mass fills of the antennal nerve with a fluorescent tracer, indicating the presence of two VUM neurons that project into the antennal nerve, similar to the honeybee (Smid et al 2003). Also, VUM neurons were visualised in *Cotesia* wasps by injection of fluorescent tracers into the antennal lobe (Smid, unpublished). We conclude that a number of VUM neurons are also present in *Cotesia*.

One of the VUM neurons in the honeybee, the VUMmx1, innervates the AL, MB's and lateral protocerebrum (Hammer, 1993). This reward-sensitive neuron mediates associative learning of odours with a sugar reward (Hammer, 1993). Associative learning of odours with sugar also occurs in parasitoids (e.g Wäckers et al., 2002; Tertuliano et al., 2004) and it is likely that a similar VUM neuron is present in *Cotesia*, mediating sugar learning. In our conditioning procedure we use an oviposition as a reward, not sugar. To be able to mediate the association of odours with an oviposition a candidate reward-sensitive neuron should converge with the odour-processing pathway in much the same way as the VUMmx1 neuron. In host conditioning, contact of the ovipositor with the host haemolymph constitutes the reward, at least partially (Takasu and Lewis, 2003). The oviposition reward-sensitive neuron should therefore be activated by presenting host haemolymph to the ovipositor. The VUM neurons can be activated by several different stimuli (Schröter, 2002). Therefore, it could be possible that the same neuron is responsible for sugar conditioning and for host conditioning. However, it could also be that a different VUM, with a similar branching pattern, is activated by oviposition. More studies are needed to pinpoint this neuron.

The third group of OA-IR cells consists of a few single cells, located in the pars intercerebralis. Cells in this region project to the corpora cardiaca, a major release site for neuropeptides into the haemolymph, but projections of the OA-IR cells were not immunoreactive. In the honeybee OA-IR cells in the pars intercerebralis were only visible in starved animals (Group 1 cells, Kreissl et al., 1994). In our wasps this was not a prerequisite, as we did not specifically starve animals. The number of OA-IR cells in the wasps was much lower than in the starved honeybees (4-6 vs 45 cells respectively).

In the honeybee a number of other groups of OA-IR cells have been described (Kreissl et al., 1994). These cells are located mediodorsally to the antennal lobes (group 2 cells), on either side of the central body (group 4 cells), and between the lateral protocerebral lobes and the dorsal lobes of the deutocerebrum (group 5 cells). No immunoreactivity was found in any of these cells in our parasitoid wasps.

Large parts of the brain contain OA-IR fibers. In the honeybee OA-IR was also widespread throughout the brain. Only the pedunculi and β-lobes of the mushroom bodies were devoid of octopaminergic fibres (Kreissl et al., 1994). Our results show a similar picture, but

labelling of axons was not consistent enough to provide a complete overview. It can be expected that the OA-IR originates mainly from the VUM neurons. These neurons display an elaborate innervation pattern in the honeybee (Schröter, 2002), and are likely to do so in our wasps as well.

The current study provides us with a number of candidate neurons that could be involved in the associative learning pathway in our wasp species. Based on analogy with the honeybee we consider one of the VUM neurons most promising in fulfilling this role. It might be possible to get a more complete picture of the octopaminergic cells and their arborisation pattern with use of anti-TBH, so we could pinpoint a neuron with an arborisation pattern similar to the VUMmx1 neuron in the honeybee. This might also allow us to identify whether there are any differences in arborisation pattern or in octopamine quantity in this neuron in our two wasp species.

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Part 2

Behavioural studies

5

Differences in memory dynamics between two closely related parasitoid wasp species

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Abstract

The two closely related parasitoids *Cotesia glomerata* and *C. rubecula* (Hymenoptera: Braconidae) coexist in The Netherlands where they occupy slightly different niches. In search for their caterpillar hosts, they use host-plant odours that are released upon feeding by the caterpillars. The species differ in their preference for plant odours during host searching after an associative learning experience. *C. glomerata* changes its preference for the odour of a particular plant species after an oviposition experience on that plant, whereas *C. rubecula* does not alter its naive preference. Using no-choice wind tunnel bioassays we tested for both species to what extent an oviposition experience induces memory formation and whether this resulted from associative learning. In experiment 1 we characterised the temporal dynamics of the memory trace. In both species an oviposition experience induced increased response levels compared to naive wasps. Memory dynamics differed between the species. A single associative learning experience induced a stable long-lasting memory trace that persisted for at least five days in *C. glomerata*. In *C. rubecula* a memory trace for the experienced odour is present during the first day after experience, but wanes over the following days. From a second experiment we concluded that the increased response could be attributed to a combination of non-associative and associative learning. We furthermore formulate the learning paradigm for the parasitoids and hypothesise that adaptation to different spatial distributions of the preferred host species has led to the observed differences in memory dynamics.

Introduction

Insects have small and relatively simple brains, but display a rich behavioural repertoire and are therefore good models for neurobiological research (Menzel and Giurfa 2001). Studies on the formation and structure of memory in insects have mostly concentrated on *Drosophila* and the honeybee (see Menzel 1999; Dubnau et al. 2003 for reviews). To be able to separate general mechanisms of learning from species-typical adaptations, however, comparative studies are needed (Menzel 2001). Parasitoids are well suited for comparative approaches. The ability of learning is common among parasitoid wasps and has been a fruitful study area for ecologists since the 1980's (see Turlings et al. 1993; Vet et al. 1995 for reviews). These studies have addressed the effect of experience on the response to specific stimuli, and yielded hypotheses on the adaptive value of learning in parasitoids. Only recently have researchers begun to use parasitoids to study the mechanistic aspects of learning (Kaiser et al. 2003; Takasu and Lewis 2003).

Parasitoids are an ecologically diverse group, and differences in the expression of learning occur between species (e.g. Poolman Simons et al. 1992; Potting et al. 1997; Geervliet et al. 1998a; Fujiwara et al. 2000; Fukushima et al. 2001). This allows the study of important ecological questions about the adaptive value of behavioural plasticity in different species. In addition, these differences create excellent opportunities to study species-typical mechanisms of learning, especially by comparing closely related species that differ in their learning ability. We find such an opportunity in our model system of two parasitoid species.

The model system

Cotesia glomerata (L.) and *Cotesia rubecula* (Marshall) (Hymenoptera: Braconidae) are closely related parasitoid wasp species (Michel-Salzat and Whitfield 2004). They are endoparasitoids of *Pieris* (Lepidoptera: Pieridae) larvae and coexist in The Netherlands, where they occupy slightly different niches (Geervliet et al. 2000). The gregarious *C. glomerata* is considered a generalist, it can successfully develop in several host species, but mainly parasitises the large white butterfly *P. brassicae* in The Netherlands (Geervliet et al. 2000). *C. rubecula* is a solitary parasitoid and a specialist on the small cabbage white butterfly *P. rapae*. Parasitoids of both species find their caterpillar hosts by the use of plant odours, the production of which is induced by feeding of the caterpillars (Vet and Dicke 1992; Steinberg et al. 1993; Geervliet et al. 1994). The parasitoid species differ in their use of olfactory learning in host searching (Geervliet et al. 1998a). Naive *C. glomerata* and *C. rubecula* females are attracted to the odour of damaged Brussels sprouts, a common host plant of *Pieris* butterflies (Geervliet et al. 1996). Both parasitoid species prefer Brussels sprouts to red cabbage or nasturtium although these are all suitable host plants for *Pieris* cat-

erpillars (Geervliet et al. 1996). However, after experiencing hosts and host products on red cabbage or nasturtium *C. glomerata* females change their preference in favour of the experienced plant odour. In this conditioning procedure the wasp learns to associate the plant odour with the presence of a reward, the caterpillars. When a similar conditioning and testing procedure is used for *C. rubecula* females, however, this species still prefers Brussels sprouts odour (Geervliet et al. 1998a). Thus, in *C. glomerata*, but not in *C. rubecula*, an oviposition experience causes a shift in preference towards the experienced plant odours. In *C. glomerata* the preference for nasturtium wanes gradually and four days after the experience Brussels sprouts again becomes the preferred host plant (Geervliet et al. 1998b). So, *C. glomerata* and *C. rubecula* clearly differ in oviposition learning.

To provide a framework for further studies on the neural mechanisms of this difference in learning we first studied the organisation of the wasps' olfactory pathway. We have described the antennal sensilla (Chapter 2, Bleeker et al. 2004), analysed the olfactory receptive range (Smid et al. 2002) and mapped the three-dimensional organisation of the primary olfactory neuropil in the brain, the glomeruli in the antennal lobe (Chapter 3, Smid et al. 2003). In the present study we analyse the memory dynamics of these two parasitoid wasp species.

Due to the dual choice set-up used by Geervliet et al. (1998a), the difference in learning between the species was described as a difference in preference shift after oviposition learning, as determined by a two-choice wind tunnel assay. This method is very useful to study the effect of experience during host searching in a natural context. However, to understand the differences in learning and memory in these species, in this study we use a no-choice wind tunnel bioassay to measure the flight response to the conditioned stimulus after oviposition reward conditioning. This allows us to measure the response to the conditioned odour, unbiased for the response to the naively preferred cabbage odours, which may be different between both wasp species. We analysed the temporal dynamics of the memory trace formed after oviposition conditioning and separated associative from non-associative learning forms in the two species. We furthermore provide a description of the learning paradigm of our model system in the discussion.

Methods

Insects

Cotesia glomerata and *C. rubecula* were obtained from colonies that originated from individuals collected in Brussels sprouts fields in the vicinity of Wageningen, The Netherlands. The parasitoids were reared on *Pieris brassicae* L. and *P. rapae* L. (Lepidoptera:

Pieridae) respectively, in a climatic room at 20-22 °C and a photoperiod of L16:D8 as described by Geervliet et al. (1998a). Pupae were collected from the colony, transferred to emergence cages, provided with water and honey and kept at 23 ºC and L16:D8. Female wasps were collected from the emergence cages for training when they were 3 to 9 days old.

Pieris larvae were reared on Brussels sprouts plants (*Brassica oleracea* var. *gemmifera* L. cv. Cyrus), under the same climatic conditions. For the experiments eggs and caterpillars were used. Eggs that were about to hatch as indicated by their dark coloration were selected for experiment 1 with *C. glomerata* (temporal dynamics of memory). For all other experiments caterpillars that had just emerged and had not yet fed from the cabbage leaf were used, hereafter called unfed caterpillars.

Odour sources

Nasturtium plants (*Tropaeolum majus* L. cv. Glorious Gleam) were reared in the greenhouse at 20-25 °C, 50-70 % RH and a L16:D8 photoperiod. Two plants were planted in a single black square pot of 11 by 11 cm. Three to four week old plants, bearing 7 to 10 leaves per plant, were used in the experiments. For *C. glomerata* in experiment 1 (temporal dynamics of the memory trace), single leaves of one plant were infested with 20 to 30 *Pieris* eggs. Two days after infestation, the infested leaves carrying about 20 caterpillars were cut and used to train the wasp (see Wasp training procedure) or used as an odour source in the wind tunnel. In all other experiments, due to rapid decline of the quality of cut leaves, whole plants were used. For these experiments two leaves of each of the two plants in a pot were each infested with 20 unfed caterpillars (see Insects) resulting in 80 caterpillars in total. For *C. glomerata* experiments larvae of the gregarious *P. brassicae* were added in clusters (20 individuals per cluster). For *C. rubecula* 20 caterpillars of the solitary *P. rapae* were divided over each of the four leaves. Plants were used one to three days after infestation.

Wasp training procedure

Experiment 1: Temporal dynamics of the memory trace

We investigated the memory dynamics after an associative learning experience, by measuring the response of a female wasp to the experienced odour. To this end we created two groups:

Naive wasps: Wasps were collected from the emergence cage in a glass vial and transferred to a small glass cage (15x15x15 cm) provided with water and honey and kept at 23 ºC and a L16:D8 photoperiod. This group served as a control group for the experienced wasps (see Statistical analysis).

Experienced wasps: Wasps, originating from the same batch as the control group, were collected from the emergence cage in a glass vial and transferred to an infested leaf

of a nasturtium plant. They were released in close vicinity of the caterpillars to ensure that the parasitoids can immediately perceive host stimuli, such as faeces and silk. This resulted in immediate host-searching behaviour. The wasps were given the opportunity to oviposit once in a single caterpillar. A low number of wasps did not oviposit within 2 minutes and they were excluded from the experiment. After the oviposition experience, wasps were removed from the leaf by keeping a vial in front of them during the last moments of the oviposition. Almost all wasps walked into this vial and could thereby be removed gently. Afterwards, wasps were transferred to a small glass cage (15x15x15 cm) provided with water and honey and kept at 23 ºC and a L16:D8 photoperiod.

C. glomerata females were tested in the wind tunnel for their response to infested nasturtium after 1 min, 5 min, 10 min, 30 min, 1 hr, 2 hrs, 4 hrs, 8 hrs, 16 hrs, 1 day, 3 days and 5 days. We tested 10 to 26 naive and 25 to 35 experienced wasps per time period, adding up to a total of 524 *C. glomerata* wasps. Cut leaves were used as an odour source (see Odour sources). *C. rubecula* females were tested after 10 min, 1 hr, 4 hrs, 1 day, 2 days, 3 days and 5 days. We tested 21 to 71 *C. rubecula* wasps per treatment per time period, adding up to a total of 539 *C. rubecula* wasps. Whole plants were used as an odour source (see Odour sources). Each wasp was only tested once in the wind tunnel.

Experiment 2: Associative learning versus non-associative learning

An increase in responsiveness after an oviposition experience on a nasturtium plant could be attributed to associative learning or non-associative learning processes. To distinguish between these effects we used the following set-ups: Initially we added an extra group to experiment 1 for *C. glomerata* (Experiment 2a). These “oviposition-only control” wasps were of the same batches as the naive and experienced wasps. They were allowed an oviposition in a single unfed caterpillar without faeces and silk. They were tested from 1 minute to 2 hours after experience. Per time point 25 “oviposition-only control” wasps were tested adding up to 150 experimental wasps in total. Thereafter we designed a set-up that allowed us to better compare the effects of exposure to the leaf odour only, the oviposition reward only, which included contact with silk, and a complete experience (see below). We prepared three Petri dishes as follows: A clean nylon gauze with mesh size 0.5 mm, was placed over the bottom half of a Petri dish, which was closed with the lid. On the gauze we introduced caterpillars, while on the bottom of the Petri dish we introduced the odour source. Female wasps were collected from the emergence cage with a glass vial and divided over four treatments:

Naive wasps: see above (Experiment 1).

Odour experience only: An infested leaf was taken from an infested nasturtium plant by

cutting the petiole just below the leaf. Caterpillars and silk were carefully removed and the leaf was placed at the bottom of a gauze-covered Petri dish. A wasp was then introduced onto the gauze and was kept in the nasturtium odour for 1 minute.

Oviposition experience only: Twenty unfed caterpillars (*P. brassicae* for *C. glomerata* and *P. rapae* for *C. rubecula*) were transferred onto the gauze of a Petri dish. The caterpillars were left for at least 30 minutes to have a certain amount of silk produced. Wasps were released on the gauze and allowed one oviposition experience in one caterpillar. No leaves were offered underneath the gauze.

Complete experience: Twenty caterpillars were put on the gauze of a Petri dish as described for 'oviposition experience only'. Then an infested leaf was put at the bottom of the Petri dish as described for 'odour experience only'. A wasp was then introduced onto the gauze and allowed one oviposition experience in a single caterpillar in the presence of the nasturtium odour.

After the experience, wasps were transferred to a small glass cage (15 x 15 x 15 cm), provided with water and honey and kept at 23 ºC and a L16:D8 photoperiod, until they were tested in the wind tunnel after 1 day. Whole plants were used as an odour source (see Odour sources). We tested 29 to 34 wasps per treatment per time period, adding up to 121 wasps for *C. rubecula* wasps and 125 wasps for *C. glomerata*.

Wind tunnel bioassay

We used the wind tunnel described previously by Geervliet et al. (1994). Wind speed was set at 0.1 m/s and light intensity in the wind tunnel varied from 7 to 13 mmol/m^2s (518 to 962 lux). Wasps were tested at a relative humidity above 40% and temperatures between 22 and 28 ºC. Each wasp was collected from the glass cage with a glass vial and transferred to the wind tunnel. A female was released in the middle of a release cylinder at approximately 70 cm from the odour source (a pot with two infested nasturtium plants). Wasps that did not initiate flight within 5 minutes were scored as a no-response. If a wasp initiated flight within 5 minutes and landed on the plant this was considered a positive response. Landings anywhere else in the wind tunnel were considered a no-response. Wasps were given only one opportunity to show a positive response in experiment 1 for *C. glomerata.* For *C. rubecula*, we adapted the wind tunnel protocol to compensate for overall low response levels, by allowing one intermediate landing elsewhere in the wind tunnel. In experiment 2 all wasps that initiated flight but did not land on the plant were captured with the vial and released again immediately in the release cylinder for a second chance to initiate flight within the 5 minute time period.

Experimental design

Females of different treatments were always trained and/or tested on the same day. For each data point the corresponding wind tunnel experiments were carried out on at least two different days, to reduce the possible influence of a day effect (Steinberg et al. 1992).

Statistical analysis

The fraction of individuals responding in the bioassay set-up was the response variable. The data analysis was carried out by using generalised linear modelling (GLIM) procedures for data with a binomial distribution of error variance and a logit-link function (McCullagh and Nelder 1989).

In experiment 1, we analysed if experience and time elapsed after experience influenced the response level of parasitoids by fitting the GLIM. Time points were calculated in hours or fractions of hours. We did not compare the species directly, as the odour sources differed (*P. brassicae* versus *P. rapae* damaged plants and cut leaves versus whole plants) and species performance in the wind tunnel differed. We used the response of naive wasps as internal control for each species and analysed flight performance of the experienced wasps over time and compared this to the response levels of naive wasps over time. In experiment 2, the main factor tested was the type of experience with four levels (i.e. complete experience, oviposition only, odour only and naive) and the interaction term of the parasitoid species and factor level. When the main factor was found to produce a significant effect, the comparison of individual treatment means was carried out by fitting linear contrasts. In all comparisons the significance level was $\alpha = 0.05$. Calculated significance was based on Likelihood Ratios. Analysis was carried out using SAS 8.02 (PROC GENMOD, SAS© Inc.).

Results

Experiment 1: Temporal dynamics of the memory trace

Naive and experienced *C. glomerata* wasps responded differently to the nasturtium odour (GLIM: Experience: $\chi^2 = 93.999$, df = 1, $P < 0.0001$). A larger proportion of experienced wasps showed a response to nasturtium, compared to naive wasps (Fig. 1). When offered nasturtium odour, 0 to 20 % of the naive wasps responded whereas from 2.2 minutes after the experience until 5 days after experience 50 to 75 % of experienced wasps responded (Fig. 1). The response to nasturtium odour did not change over time for both the experienced and the naive wasps (Fig. 1), as the response level was not influenced by the time after experience and there was no interaction between time after experience and experience (GLIM: Time: $\chi^2 = 1.990$, df = 1, $P = 0.158$ and Time * Experience: $\chi^2 = 1.868$, df = 1,

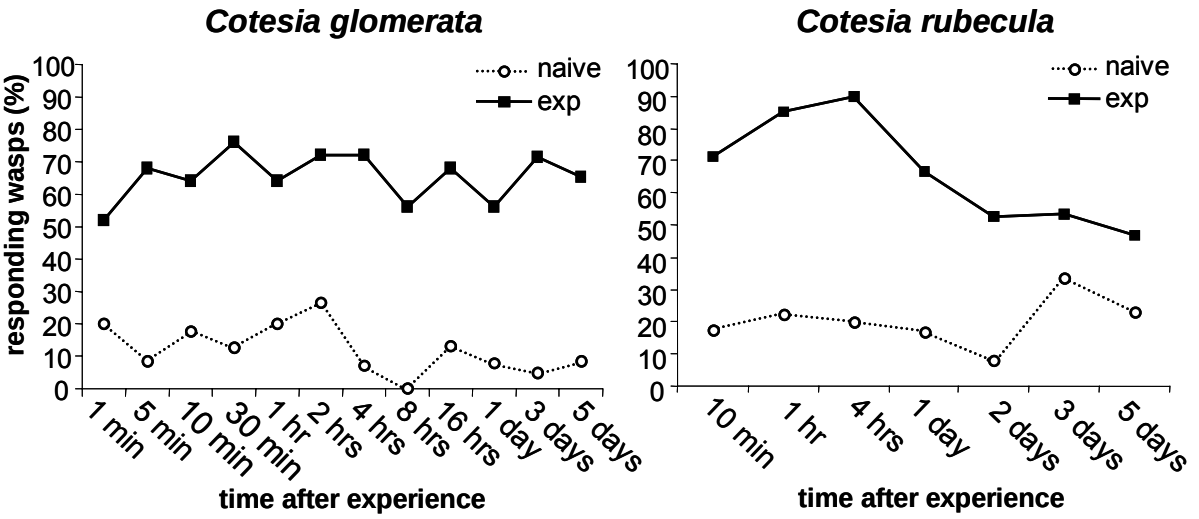

Fig. 1: Response levels of naive and experienced *C. glomerata* females at different times after experience. Naive = naive wasps. Exp = experienced wasps, i. e. wasps that were allowed an oviposition experience in a single *P. brassicae* caterpillar in the presence of nasturtium odour. N = 10 to 26 per time point for naive wasps and N = 25 to 35 per time point for experienced wasps.

Fig. 2: Response levels of naive and experienced *C. rubecula* females at different times after experience. Naive = naive wasps. Exp = experienced wasps, i. e. wasps that were allowed an oviposition experience in a single caterpillar in the presence of nasturtium odour. N = 24 to 71 per time point for naive wasps and N = 21 to 71 per time point for experienced wasps.

P = 0.172).

A larger proportion of experienced *C. rubecula* females responded to the nasturtium odour than of naive females (Fig. 2). The time course of responsiveness differed between naive and experienced females as an interaction between experience and time after experience occurred. Experienced females initially show a high response to the nasturtium odour, but the response level decreases after a few hours (Fig. 2). A low proportion of naive females responded to nasturtium odour, which does not decrease over time. (GLIM: Experience: $\chi^2 = 90.095$, df = 1, $P < 0.0001$; Time: $\chi^2 = 0.772$, df = 1, P = 0.380; Experience * Time: $\chi^2 = 12.756$, df = 1, P = 0.0004).

Experiment 2: Associative vs. non-associative learning

We tested which factors during the experience contributed to the increased response level of experienced wasps. Initially we tested *C. glomerata* wasps that were given an oviposition in a caterpillar without the presence of silk or faeces (oviposition-only control wasps) from two minutes to two hours after the experience (Experiment 2a). These wasps showed response levels that were significantly lower compared to experienced wasps and

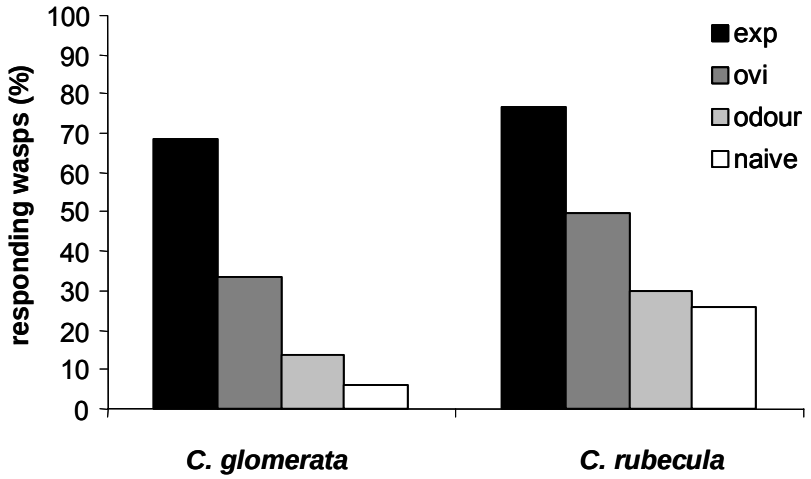

Fig. 3: Response levels of *C. glomerata* and *C. rubecula* females one day after different experimental treatments. Exp = complete experience, ovi = oviposition experience only, odour = odour experience only and naive = no experience. N = 29 to 34 per treatment per species.

did not significantly differ from naive wasps (Chi^2-test, data not shown).

We furthermore tested whether the difference between the conditioned response and the unconditioned response as revealed for both wasp species in experiment 1, is caused by differences in occurrence of associative learning or non-associative learning one day after the conditioning experience. Parasitoids that received different treatments responded differently to the nasturtium odour (GLIM: Treatment: $\chi^2 = 44.280$, df = 3, $P < 0.0001$). Both species react similarly to the different treatments, as there was no interaction between species and experience (Fig. 3).

C. glomerata and *C. rubecula* females that received a complete experience i.e. an oviposition experience in the presence of nasturtium odour, showed a higher response level compared to naive wasps (GLIM: $\chi^2 = 31.56$, df = 1, $P < 0.0001$ for *C. glomerata* and $\chi^2 = 16.55$, df = 1, $P < 0.0001$ for *C. rubecula*). *C. glomerata* and *C. rubecula* females with an oviposition experience only had an increased response compared to the naive females (GLIM: $\chi^2 = 8.37$, df = 1, P = 0.004 for *C. glomerata* and $\chi^2 = 3.85$, df = 1, P = 0.0499 for *C. rubecula*), but responses were lower compared to females with a complete experience (GLIM: $\chi^2 = 7.95$, df = 1, P = 0.0048 for *C. glomerata* and $\chi^2 = 4.67$, df = 1, P = 0.031 for *C. rubecula*; fig. 3). *C. glomerata* and *C. rubecula* females that had experienced the damaged nasturtium odour alone do not show a higher response towards this odour compared to naive wasps (GLIM: $\chi^2 = 1.14$, df = 1, P = 0.285 for *C. glomerata* and $\chi^2 = 0.13$, df = 1, P = 0.715 for *C. rubecula*; fig. 3).

Discussion

The parasitoid oviposition learning paradigm

Historically, parasitoid learning studies were driven by ecological questions. In contrast, many studies on learning in other species were driven by behavioural questions. Combining these two approaches can provide much insight in the function of species-typical adaptations. For this purpose we describe the learning paradigm of our parasitoid wasps from a behaviourist viewpoint in the following paragraphs. We compare the learning paradigm of our parasitoids to the proboscis extension reflex (PER) conditioning in bees (Menzel 1993).

The conditioning of the wasps in our learning paradigm (Experiment 1) occurs in an ecologically relevant, yet controlled manner. Wasps are released onto the surface of a leaf infested with their host caterpillars, in a way that the parasitoids immediately perceive host stimuli, such as faeces and silk. Perception of these host-derived stimuli have three different effects, comparable to the effects induced by antennal contact with sugar in the PER conditioning in the honeybee as described by Menzel (1993). First, they induce searching behaviour of the wasp, including probing of the leaf surface with the antenna and ovipositor (Van Alphen and Vet 1986; Lewis and Tumlinson 1988). Second, these stimuli sensitize the wasp; it becomes more responsive to plant odours (termed priming by Turlings et al. 1993). Third, these stimuli are known to act as reinforcer; the unconditioned stimulus that acts as a reward in Pavlovian conditioning of the wasps to the leaf odours (Lewis and Tumlinson 1988; Geervliet et al. 1998a). Contact with the host by-products initiates host-searching behaviour in the parasitoid. This behaviour can be considered to be comparable to the proboscis extension reflex occurring in bees upon stimulation of the antennae with sugar. The host-search is completed by the oviposition in the host. In the honeybee it is a droplet of sugar offered to the extended proboscis which completes the conditioning trial. Putative stimuli that serve as reinforcer during oviposition are host haemolymph (Takasu and Lewis 2003), or host cuticular compounds and mechanoreception of the release of the eggs. Even if no hosts are found, the plant odour is learned as a predictor of host presence as a consequence of the perception of host by-products as reinforcer, and a memory trace is formed that can last for several hours, as can be shown by a plant odour preference shift in a wind tunnel assay (Geervliet et al. 1998a). However, if the wasp finds and parasitizes a host, a stronger memory trace is formed that lasts for several days. Thus, perception of host-derived substances followed by an oviposition constitutes a stronger reinforcement than perception of host-derived substances alone (Vet et al. 1995; Takasu and Lewis 2003). Directly after the oviposition is finished, the wasp is removed from the leaf, ending a single conditioning trial.

In this learning paradigm, the approach to the plant (flight and walking) that occurs un-

der natural circumstances is excluded. Thus, the stimulation by host-derived cues and oviposition is not reinforcing a behavioural response to the plant odours, but purely the association of host-derived stimuli to the plant odour stimulus. The behavioural response of the wasp to the host cues can be considered as the unconditioned response (UR), that occurs irrespective of the odour environment, comparable to the proboscis extension reflex occurring in bees upon stimulation of the antennae with sugar. Therefore, we consider this learning paradigm as classical (Pavlovian) conditioning, not as operant conditioning. The odours of the plant constitute the conditioned stimulus (CS), whereas the host-related cues and oviposition, as described above, constitute the unconditioned stimulus (US).

Wasps are tested for acquisition of memory for the conditioned odour in an operant context. We measure the effect of conditioning on an ecologically relevant behavioural response, odour-mediated orientation in flight, which is studied in a wind tunnel set-up. Here we can test the wasp's response to the CS by offering it a choice between a plant of which the odours have been paired to an oviposition reward and a plant of which the odours are naively preferred over the CS. The level of conditioning is then shown by a shift in preference after conditioning. Alternatively, we test the response levels to the conditioned odour in a no-choice wind tunnel assay, as was done in this study. This set-up demonstrates that memory established during Pavlovian conditioning is transferred to an operant context. The wasps change their flight responses to plant odours based on memory established during oviposition behaviour. A similar phenomenon has been described for the honeybee, where information acquired during proboscis extension learning is transferred to an instrumental context (Sandoz et al. 2000). This method provides us with an efficient, well reproducible conditioning procedure that allows us to test its effect on subsequent foraging behaviour.

In this study we show that the two parasitoids *C. glomerata* and *C. rubecula* increase their response to infested nasturtium after a single oviposition experience in the presence of nasturtium odour. This increase in response level is due to both associative and non-associative learning. The temporal dynamics of the memory trace differs between the species.

Associative and non-associative learning

Both *C. glomerata* and *C. rubecula* display an increased flight response to nasturtium after an oviposition reward conditioning with this odour. For both species this increased response is partly due to associative learning when tested one day after the experience (Experiment 2). In a previous study with both wasp species, Geervliet et al. (1998a) showed that only *C. glomerata* and not *C. rubecula* shows a preference shift after a learning experience. Here we conclude that absence of a shift in preference behaviour after a learning ex-

perience does not exclude the presence of a memory trace caused by associative learning.

Exposure to nasturtium odour alone does not increase response levels in either species (Experiment 2). In other parasitoid wasps, exposure to a neutral stimulus did not affect response levels either (Lewis and Tumlinson 1988; De Jong and Kaiser 1991; Fukushima et al. 2001). In contrast, in a study by Kaiser and Cardé (1992), *C. rubecula* increased its response level to an infested Brussels sprouts plant after exposure to odours of a Brussels sprouts plant infested with *P. rapae* caterpillars. During the exposure, which was done at close range of the plant, the wasps displayed host-searching behaviour, indicating that they could perceive the presence of the hosts. These host-derived stimuli most likely will have served as a reward stimulus, even if no direct contact with the host has been made. Thus, the difference with our results can be explained by the fact that in our study, host related substances were removed from the leaf (and hence the wasp did not initiate search and probing behaviour).

An oviposition alone in a caterpillar with silk induced an increased flight response in both wasp species (Experiment 2). The increase of response levels after an oviposition alone has been shown more often in parasitoids (McAuslane et al. 1991; Takasu and Lewis 2003). This non-associative learning effect usually lasts for only a short period of time. An increase in response level was demonstrated half an hour after experience (Takasu and Lewis 2003), but was not present after 2 hours (Kaiser et al. 2003) or 1 day (De Jong and Kaiser 1991; Kaiser and Cardé 1992; Kaiser et al. 2003; Takasu and Lewis 2003). Most likely this short term increase in response is comparable to sensitization in other insects (Menzel 1999). Interestingly, in both *C. glomerata* and *C. rubecula* oviposition alone in a single caterpillar induced an increase in response levels that was still present after 1 day (Fig 1). We think that this long-lasting effect might be due to an increased motivational state after the first oviposition. In *C. glomerata* an oviposition alone in a clean caterpillar without the presence of silk and faeces did not result in an increased response level (Experiment 2a). Apparently, the perception of host-derived cues such as silk is needed to induce a sensitization effect of oviposition. This effect can not be explained by contamination of the silk by odours released by damaged plants, as we used caterpillars that had not yet fed from the leaf and had spinned the silk on a filter paper in the petri-dish (experiment 2).

Temporal dynamics and memory structure

In *C. glomerata* one associative learning experience results in a memory trace for the experienced odour that lasts for at least five days and is constant throughout this period (Experiment 1). This contrasts with the study by Geervliet et al. (1998b), which demonstrated that *C. glomerata* changes her preference for cabbage odours after an oviposition experience on a nasturtium plant. This effect was, after three massed oviposition experiences,

not permanent, as the wasp's preference returned towards its naive preference for cabbage plants four days after the learning experience. Thus, in *C. glomerata*, the preference shift has different temporal dynamics than the memory trace we analysed here in a no-choice test by measuring the increase in response levels to the experienced plant odours. Although the study by Geervliet et al. (1998b) showed that the preference change had waned after four days, this study reveals that the memory trace for the experienced odour is intact after five days. Apparently, the increased responsiveness to nasturtium remains at the same level for five days, whereas the response to this stimulus relative to the response to cabbage odours has waned at four days after the experience.

The stable, long-lasting memory in *C. glomerata* that lasts at least five days suggests that the single oviposition learning experience in our experiment might cause long term, protein synthesis-dependent memory (LTM). According to studies with honeybees and fruit-flies, LTM requires multiple, spaced conditioning trials (Menzel 1999; DeZazzo and Tully 1995). We are planning to test whether the memory formed in *C. glomerata* after a single oviposition experience is protein-synthesis dependent by using protein synthesis inhibitors.

In *C. rubecula* a memory trace develops that begins to wane after 1 day and has further diminished by 3 and 5 days (Experiment 1). At one day after the experience the response level is due to a combination of non-associative and associative learning (Experiment 2). Currently we do not know to what extent the two forms of learning contribute to the memory curve displayed in *C. rubecula*. The response to nasturtium 5 days after the oviposition experience is still higher than that of the naive wasps, but there is a significant decline in response level. The non-associative effect of oviposition present at 1 day after experience (Experiment 2) may still persist after 5 days. A more elaborate study on sensitization by oviposition is required to quantify the contribution of sensitisation to memory formation in *C. rubecula*. The temporal dynamics of the memory trace in *C. rubecula* would be indicative of a weaker memory trace than in *C. glomerata*, e.g. of medium term memory (MTM) in accordance with the memory formed in other species after a single rewarding experience (DeZazzo and Tully 1995; Menzel 1999). Using anaesthesia we can block MTM and thereby see whether MTM is present in both species.

In conclusion, the two wasps differ in their temporal memory dynamics after a similar oviposition conditioning. Whether this difference is due to a difference in occurrence of the memory phases MTM and LTM, and/or a difference in the occurrence of sensitization should be addressed in additional studies that focus on the sensitization effect and use different methods to interfere with the specific memory phases, such as application of anaesthesia and protein-synthesis inhibitors (Xia et al. 1998, 1999; Menzel 1999).

Possibly, the observation that choice behaviour changes after an oviposition experience in one species but not the other corresponds with the observed difference in temporal mem-

ory dynamics between the two species. The long-lasting memory trace in *C. glomerata* seems to be more robust than the memory trace formed in *C. rubecula*, and such robust memory may be required to change the naive preference for plant odours. It is an interesting phenomenon that the memory trace for nasturtium in *C. glomerata* lasts longer than the effect of this memory trace on preference behaviour of this species.

Ecology and memory

Different species are thought to display differences in memory formation and memory structure due to different ecological needs (Menzel 1999). *C. glomerata* and *C. rubecula* are closely related (Michel-Salzat and Whitfield 2004), coexist in the same habitats in The Netherlands (Geervliet et al. 2000) and are both endoparasitoids of *Pieris* larvae. They differ only in a few aspects: 1) *C. rubecula* is a solitary parasitoid and lays only one egg per host, whereas *C. glomerata* is gregarious and lays about thirty eggs in a single host during oviposition. Thus the reward represented by a single oviposition event could have different effects in the two species. *C. glomerata* produces on average 25 eggs in a single oviposition bout whereas *C. rubecula* oviposits a single egg in one host. This could affect the strength of the reinforcement. As the strength of the reinforcement can influence the strength of the memory, this could explain the long-lasting memory in *C. glomerata* compared to the weaker memory trace in *C. rubecula*. However, an oviposition of *C. glomerata* in a *P. rapae* caterpillar, in which they also lay a clutch of eggs, does not induce a preference shift (Chapter 6). This suggests that oviposition by a gregarious species does not automatically lead to a shift in preference behaviour after an oviposition learning experience. 2) *C. rubecula* is a specialist on the small cabbage white *Pieris rapae*, whereas *C. glomerata* prefers *P. brassicae* as its host. We hypothesise that adaptation to different hosts may have led to differences in preference behaviour after oviposition learning in these two species. *C. glomerata* can parasitize several Pieridae but in The Netherlands it is mainly associated with *P. brassicae* (Geervliet et al. 2000), which is a superior host for the Dutch population of *C. glomerata* wasps (Brodeur et al. 1998; Harvey 2000). *P. brassicae* selects a stand of plants and lays its eggs in large clutches on a few of them (Rothschild 1987; Le Masurier 1994) and *C. glomerata* is adapted to this spatial distribution in host searching (Wiskerke and Vet 1994; Vos et al. 1998). For *C. glomerata* an encounter with a *P. brassicae* caterpillar indicates the presence of many more suitable host caterpillars in which she can lay additional eggs. For *C. glomerata* it would therefore be adaptive to temporarily specialize on the plant odour on which she has previously located a *P. brassicae* caterpillar. In contrast, *P. rapae* lays its eggs singly on plants and selects isolated host plants for oviposition (Root and Kareiva 1984; Hern et al. 1996). So, the presence of a *P. rapae* caterpillar on a host plant does not necessarily indicate the presence of more hosts in the direct vicinity in the same odorous

environment. Hence, it would be maladaptive for *C. rubecula* to concentrate on the odour of a particular plant species during host searching after a single encounter with a suitable host. We are currently testing the hypothesis that specialization on the two different host species *P. rapae* and *P. brassicae* could have selected for differences in learning of the parasitoids, due to the differences in spatial distribution of the hosts.

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6

Oviposition reward learning in two populations of a parasitoid wasp: effect of host species

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Abstract

A Dutch population of the parasitoid wasp *Cotesia glomerata* L. uses mainly the young caterpillars of the large cabbage white *Pieris brassicae* L. as its host. In Japan, however, *P. brassicae* is absent and *C. glomerata* attacks the small cabbage white *Pieris rapae* L.. In search for the caterpillars, the wasp uses host-plant odours that are released upon feeding by the caterpillars. This wasp species easily learns to respond to odours from plant species where it has encountered host caterpillars. In the current study we investigate whether the host species affects this odour learning. Females from a Dutch and a Japanese population were tested for their plant odour preference reciprocally in a dual choice test one day after an oviposition experience in either a *P. brassicae* or a *P. rapae* caterpillar, on either Brussels sprouts or nasturtium. In both populations only an oviposition experience on the preferred host could significantly influence choice distributions in favour of the experienced odour. The two caterpillar species most likely represent different rewards for the two wasp populations. Only an oviposition in the preferred host constitutes a reinforcement that is strong enough to result in a difference in plant odour choice behaviour. We discuss the results from an ecological viewpoint.

Introduction

Parasitoid wasps find their caterpillar hosts by the use of plant odours that are induced by feeding of the caterpillars (Vet and Dicke, 1992; Vinson, 1998). Many parasitoids use associative learning of these plant odours during host searching and change their preference to the experienced plant odour after finding their hosts on that plant species. This behavioural plasticity is thought to increase host-searching efficiency (see Turlings et al., 1993; Vet et al., 1995 for reviews). However, some parasitoids do not change their preference for plant odours after a host encounter on that plant (e.g. Geervliet et al., 1998; Potting et al., 1997). In these parasitoids associative learning can still occur, but apparently not to the degree that it induces a shift in preference (Chapter 5, Bleeker et al., in press). In parasitoids, host-searching efficiency is directly linked to Darwinian fitness (Van Alphen and Vet, 1986). It is therefore likely that differences in the occurrence of preference shifts during host searching reflect adaptations to different host-searching strategies.

A Dutch population of the parasitoid wasp species *Cotesia glomerata* L. (Hymenoptera: Braconidae) uses associative learning during host-searching (Geervliet et al., 1998; Chapter 5, Bleeker et al., in press). Naive wasps are attracted to the odour of herbivore-damaged Brussels sprouts, a common host plant of their *Pieris* caterpillar hosts (Geervliet et al., 1996). Naive *C. glomerata* prefer Brussels sprouts odour to the odour of red cabbage or nasturtium, plants that are all suitable host plants for *Pieris* caterpillars (Geervliet et al., 1996). The parasitoids can learn to associate other, innately less attractive plant odours with the presence of caterpillars by means of associative learning (Geervliet et al., 1998; Chapter 5, Bleeker et al., in press). Geervliet et al. (1998) showed that *C. glomerata* changes its preference towards the experienced nasturtium odour, after a single oviposition experience in its host that was feeding on nasturtium. Thus this population of *C. glomerata* easily learns to respond to odours that have been associated with its host through an oviposition experience. In contrast, the species *Cotesia rubecula* (Marshall) does not change its plant odour preference after an oviposition experience in its host on an innately less attractive plant (Geervliet et al., 1998).

We aimed at understanding why these two species differ in their use of associative learning during host searching and subsequent preference behaviour. The two species are closely related (Michel-Salzat and Whitfield, 2004) and coexist in the same habitats (Geervliet et al., 2000). We first hypothesized that the differences in preference behaviour are adaptations to differences in the degree of host specialization (Geervliet et al., 1998). *C. rubecula* is a specialist on the small cabbage white *P. rapae*, whereas *C. glomerata* is a more generalist parasitoid that can parasitize a few *Pieris* species. It is often posed that it is adaptive for gen-

eralist species to use associative learning, whereas specialist species might be more successful using innate cues (Vet and Dicke, 1992; Steidle and van Loon, 2003). However, *C. glomerata* is fairly specialized on *P. brassicae* in The Netherlands (Geervliet et al., 2000) and since both *P. brassicae* and *P. rapae* have similar host plant ranges, there is no clear difference in specialization at the plant level either. Hence, the generalist/specialist theory does not provide us with a satisfactory explanation for the difference in learning between *C. glomerata* and *C. rubecula*. As both species use different host species, we then hypothesised that it is the host species that determines preference behaviour after an oviposition reward experience.

A shift in plant odour preference after an oviposition reward experience can only be adaptive if the newly preferred plant odour reliably indicates the presence of additional hosts in the same odorous environment. *Pieris brassicae* is a gregarious species of which always more caterpillars are present on the same plant. In contrast, *P. rapae* is a solitary caterpillar, and the presence of a single caterpillar on a specific plant does not indicate the presence of additional hosts in the same odorous environment. Thus, there may be a link between the distribution of hosts, resulting from the species-specific oviposition behaviour of the butterfly species, and the value of the reward as a reliable predictor of host presence. We hypothesised that an oviposition reward experience in a solitary *P. rapae* caterpillar does not result in a shift in preference behaviour whereas an oviposition in a gregarious *P. brassicae* caterpillar does.

Here we study whether, and to what extent, the host offered as reward in an oviposition reward experience affects the subsequent plant odour preference behaviour of the parasitoid. We do this by comparing two populations of *C. glomerata*; a Dutch population and a Japanese population that differ in their host use. The Dutch population mainly uses *P. brassicae* as host and has extensively been used in studies on oviposition reward conditioning (see above). On the main island of Japan, Honshu, *P. brassicae* does not occur and Japanese *C. glomerata* attack *P. rapae* (Sato and Ohsaki, 1987). We test 1) whether host species affects preference behaviour after an oviposition reward experience and 2) whether different parasitoid populations that are adapted to different host species are affected differently by the same experiences.

Methods

Insects

The Dutch *C. glomerata* population was obtained from colonies that originated from in-

dividuals collected in Brussels sprouts fields in the vicinity of Wageningen, The Netherlands during the summer previous to the experiments. The parasitoids were reared on *Pieris brassicae*, in a climate room at 20-22°C and a photoperiod of L16:D8 as described by (Geervliet et al., 1998). Pupae were collected from the colony, transferred to emergence cages, provided with water and honey and kept at 23ºC and L16:D8. Japanese *C. glomerata* were kindly provided by Dr. Y. Kainoh and Dr. J. Tagawa. They were collected from Okyama, Japan from *P. rapae* in May 2004. They were reared for one generation on *P. rapae* in Japan. Pupae were sent to the Netherlands, and they were further reared on Dutch *P. rapae* under the same climatic conditions as described for the Dutch *C. glomerata*. The original and subsequent generations were used for the experiments. *Pieris* larvae were reared on Brussels sprout plants (*Brassica oleracea gemmifera* cv. Cyrus), under the same climatic conditions.

Odour sources

Nasturtium plants (*Tropaeolum majus* cv. Glorious Gleam) were reared in the greenhouse at 20-25°C, 50-70%RH and a L16:D8 photoperiod. Two plants were planted in a single black square pot of 11 by 11 cm. Three to four weeks old plants, bearing 7 to 10 leaves per plant, were used in the experiments. Brussels sprouts plants (*Brassica oleracea gemmifera* cv. Cyrus) were reared under the same conditions. A single plant was grown in a black square pot of 11 by 11 cm. Plants bearing 15 to 18 leaves were used.

We standardized any quantitative differences in plant odour and reward strength by using mechanically damaged plants treated with caterpillar regurgitant, which are similar to caterpillar damaged plants in terms of parasitoid attraction (Mattiaci et al., 1994), to avoid uncontrollable amounts of feeding damage. Two leaves per plant were artificially damaged by treating 3 cm^2 with a pattern wheel, resulting in a grid of holes (0.5 mm^2) 3 mm apart. Subsequently the damaged area was treated with 5 ml regurgitant of 4-5th instar larvae of either *P. rapae* or *P. brassicae* (Fatouros et al., 2005). After one day, these treated plants were used for training of the wasps and as an odour source in the wind tunnel. Whole plants were used in the training of the wasps (see Wasp training procedure). For the wind tunnel two damaged Brussels sprouts leaves and six damaged nasturtium leaves were cut from the plant and their petioles were put in two separate glass flasks filled with water.

Wasp training procedure

A single 1-day-old caterpillar was transferred to a damaged leaf of a nasturtium or Brussels sprouts plant. Caterpillars that were put on Brussels sprouts originated directly from the *Pieris* rearing colony. Caterpillars that were put on nasturtium had been transferred to a nasturtium plant 1 day before, when they had emerged from the egg up to 4 hours before, and

had not yet eaten from the Brussels sprouts leaf, to avoid rejection of nasturtium as host plant.

The conditioning of the wasps in our learning paradigm occurs in an ecologically relevant, yet controlled manner. Wasps are trained in a classical conditioning paradigm (Chapter 5, Bleeker et al., in press). Female wasps no older than 7 days were collected from the emergence cages. A female wasp was gently released from a glass vial onto the damaged area of the leaf near the caterpillar. The wasp was allowed to oviposit in the caterpillar. The host constitutes the unconditioned stimulus (US), and contact with the host-derived cues (frass, silk) and the subsequent oviposition in the host serve as the reinforcer for learning of the plant odour. After the oviposition experience, wasps were removed from the leaf by keeping a vial in front of them during the last moments of the oviposition. Wasps walked into this vial and could thereby be removed gently. A new caterpillar was used for each conditioning.

We used four different treatments: a) an oviposition experience in a *P. rapae* caterpillar on damaged Brussels sprouts, b) an oviposition experience in a *P. rapae* caterpillar on damaged nasturtium, c) an oviposition experience in a *P. brassicae* caterpillar on damaged Brussels sprouts and d) an oviposition experience in a *P. brassicae* caterpillar on damaged nasturtium. Plants that had been treated with the regurgitant of *P. rapae* or *P. brassicae* were used for training of the wasps on *P. rapae* and *P. brassicae* respectively.

After the experience, wasps were transferred to a small glass cage (15 x 15 x 15 cm), provided with water and honey and kept at 23ºC and L16:D8 photoperiod, until they were tested in the wind tunnel (see below) the next day.

Wind tunnel bioassay

Dual choice (preference) tests were carried out in the wind tunnel as previously described by Geervliet et al. (1994). Wind speed was set at 0.1 m/s and light intensity in the wind tunnel varied from 7 to 13 mmol/m^2s (518 to 962 lux). Wasps were tested at a relative humidity above 40% and temperatures between 23 and 29 ºC. Wasps were given a choice between nasturtium and Brussels sprouts treated with either *P. brassicae* or *P. rapae* regurgitant. The two flasks with either Brussels sprouts or nasturtium leaves (see Odour sources) were placed at the upwind side of the wind tunnel, 20 cm apart. The positions of the odour sources alternated in such a way that half of the females of each treatment were tested with Brussels sprouts on the left and the other half with Brussels sprouts on the right. Wasps that had received an experience on *P. rapae* or *P. brassicae* caterpillars were offered *P. rapae* and *P. brassicae* treated leaves respectively. Each wasp was collected from the glass cage with a glass vial and released in the wind tunnel in the middle of a release cylinder at approximately 70 cm from the odour sources. Wasps were allowed 5 or 10 minutes to land on a plant. If the wasp had not landed on the plant within this time frame, or had not initiated

flight, this was considered as a no-response. Dutch *C. glomerata* received 5 minutes to initiate flight. Due to low response levels, Japanese *C. glomerata* were allowed 10 minutes to initiate flight. This did not influence the choice distribution, but increased overall response levels.

Statistical analysis

Choices between odour sources were analysed with a two-tailed binomial test with continuity correction. Differences between the groups were tested with a χ^2, 1 degree of freedom and continuity correction.

Results

We tested whether two allopatric populations of *C. glomerata* show differences in host plant odour choice after an oviposition reward conditioning on different hosts. Dutch wasps that had oviposited in a *P. brassicae* larva on Brussels sprouts showed a significant preference for the Brussels sprouts odour (fig. 1). Dutch wasps that had oviposited in a *P. brassicae* larva on nasturtium did not show a significant preference for either nasturtium or Brussels sprouts (fig. 1). These two groups, i. e. wasps that had oviposited in a *P. brassicae* larva on either Brussels sprouts or nasturtium, displayed significantly different preferences. Dutch wasps that had oviposited in a *P. rapae* caterpillar on Brussels sprouts preferred Brussels sprouts odour to nasturtium odour (fig. 1). Dutch wasps that had oviposited in a *P. rapae* caterpillar on nasturtium did not prefer either Brussels sprouts or nasturtium odour (fig.1). These two groups, i. e. wasps that had oviposited in a *P. rapae* larva on either Brussels sprouts or nasturtium, showed similar choice distributions (fig. 1).

Japanese *C. glomerata* showed a different response. Wasps that had oviposited in a *P. brassicae* on Brussels sprouts preferred the Brussels sprouts odour to the nasturtium odour (fig. 2). Wasps that had oviposited in a *P. brassicae* larva on nasturtium did not display a preference for either Brussels sprouts or nasturtium (fig. 2). These two groups, i.e. wasps that had oviposited in a *P. brassicae* caterpillar on either Brussels sprouts or nasturtium did not differ significantly in their preference behaviour (fig. 2). Japanese wasps that had oviposited in a *P. rapae* caterpillar on Brussels sprouts displayed a significant preference for this odour (fig. 2). Wasps that had oviposited on a *P. rapae* caterpillar on nasturtium did not prefer either Brussels sprouts or nasturtium. These two groups, i.e. wasps that oviposited in a *P. rapae* caterpillar on either nasturtium or Brussels sprouts differed significantly in their preference behaviour.

The two populations respond differently to the different treatments. In Dutch *C. glomerata* wasps only an experience on a *P. brassicae* caterpillar could significantly influence

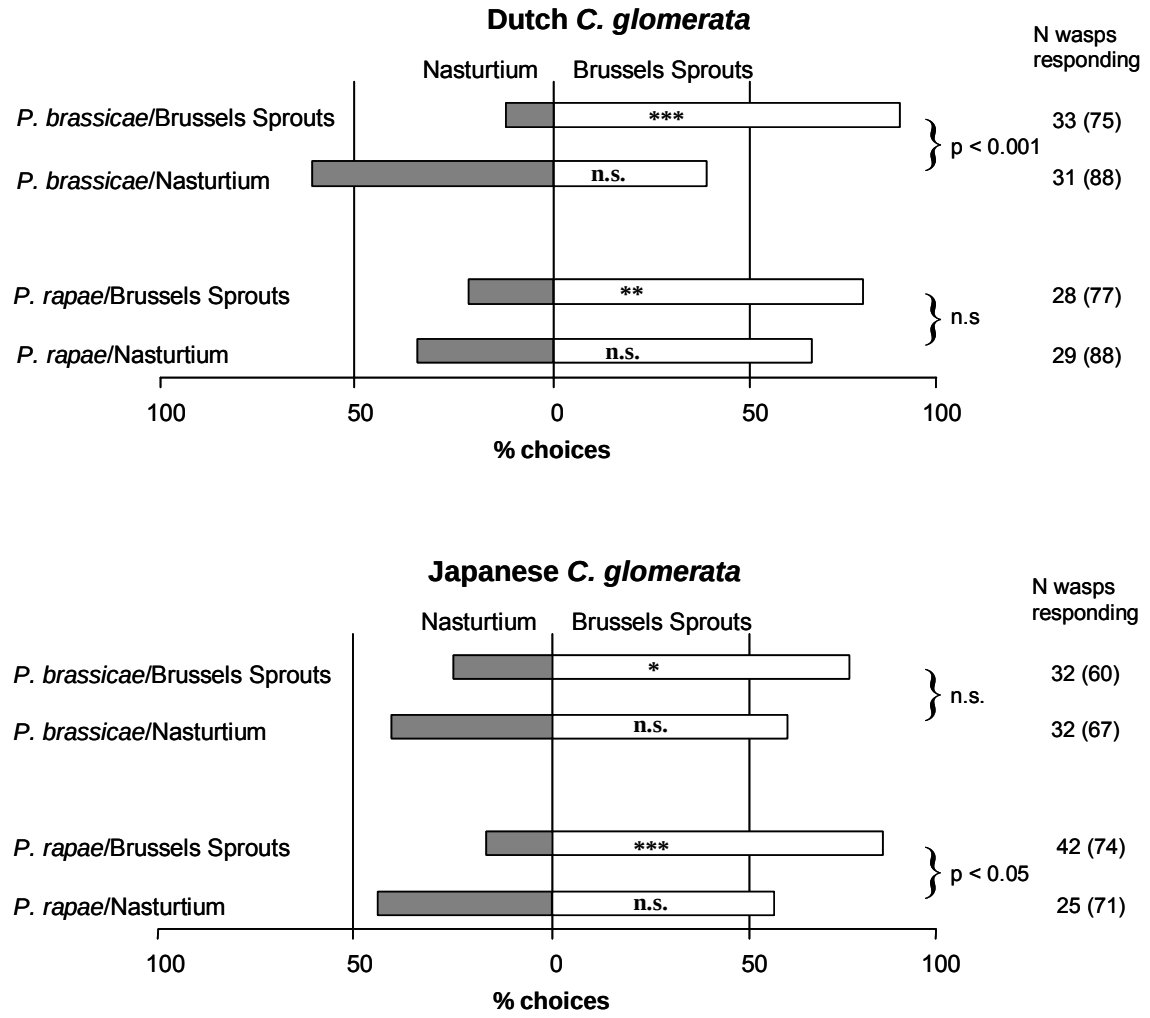

Fig. 1 - 2: Responses of Dutch and Japanese *C. glomerata* wasps to nasturtium and Brussels Sprouts odour after different oviposition experiences. *P. brassicae*/nasturtium: wasps oviposited in a single *P. brassicae* larva on a damaged nasturtium plant. *P. brassicae*/Brussels Sprouts: wasps oviposited in a single *P. brassicae* larva on a damaged Brussels Sprouts plant. *P. rapae*/nasturtium: wasps oviposited in a single *P. rapae* larva on a damaged nasturtium plant. *P. rapae*/Brussels Sprouts: wasps oviposited in a single *P. rapae* larva on a damaged Brussels Sprouts plant. The percentage of wasps responding to nasturtium is represented by a grey bar on the left-hand-side of the graph. The percentage of wasps responding to Brussels Sprouts is represented by a white bar on the right-hand-side of the graph. Total numbers of responding wasps are noted on the right, with the total of wasps tested in brackets. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

choice distributions in favour of the experienced odour, whereas in Japanese wasps only an experience on a *P. rapae* caterpillar significantly influenced choice distribution in favour of the experienced odour.

Discussion

We studied whether different host species can affect the formation of plant odour preference behaviour in a parasitoid wasp. To disentangle physiological and ecological effects, we used two allopatric populations of the same parasitoid species *C. glomerata* that, in the field, use different hosts.

We show that in both populations the host species, which was offered during an oviposition reward conditioning, affects the formation of subsequent preference behaviour. In the Dutch population of *C. glomerata* an experience with a *P. brassicae* caterpillar could influence preference behaviour, whereas an experience with a *P. rapae* caterpillar did not significantly influence the choice distributions. *P. brassicae* is a better quality host for the Dutch *C. glomerata*. Dutch *C. glomerata* achieves a higher percentage of successful parasitism in *P. brassicae* compared to *P. rapae* (Brodeur et al., 1998). Furthermore, *P. brassicae* supports larger egg clutches and results in bigger individuals than *P. rapae* (Harvey, 2000). Wasps reared on *P. brassicae* reject *P. rapae* significantly more frequently than *P. brassicae* (Vos and Vet, 2004). An oviposition in a *P. brassicae* larva could therefore have a stronger rewarding effect than an oviposition in a *P. rapae* caterpillar.

In Japanese *C. glomerata* only an experience with a *P. rapae* caterpillar influences preference behaviour, whereas experience with a *P. brassicae* caterpillar did not influence choice distributions. In Japan *P. brassicae* is not present and *P. rapae* is the main host. Japanese *C. glomerata* have likely adapted to *P. rapae* and *P. brassicae* might be a suboptimal host. Another population of *C. glomerata* that is restricted to *P. rapae* occurs in the USA. These American *C. glomerata* have adapted their host-searching strategy to *P. rapae* (Vos and Hemerik, 2003) and have a low acceptance for *P. brassicae* larvae (Vos and Vet, 2004). Recently *P. brassicae* has invaded the northern island of Japan, Hokkaido. Parasitism of these caterpillars by *C. glomerata* is low in the field, and *C. glomerata* females reject these *P. brassicae* larvae in the laboratory (Sato and Ohsaki, 2004). In our study typically less than 50% of the Japanese *C. glomerata* females accepted *P. brassicae* (Bleeker, unpublished). So, Dutch *C. glomerata* are adapted to *P. brassicae* and Japanese *C. glomerata* are adapted to *P. rapae*. Both populations only chose more often for the experienced odour after an oviposition experience in their preferred host. This indicates that the host species can significantly influence plant odour preference behaviour in parasitoid wasps after an oviposition reward conditioning.

We assumed from an evolutionary perspective, that the shift in preference behaviour after an encounter with a host should be adaptive for the parasitoid. So only if the odour ex-

perienced during the encounter with the caterpillar would reliably predict the presence of more caterpillar hosts, would it be adaptive to shift preference towards this odour. For the Dutch *C. glomerata* this condition is met. First of all, the caterpillars of *P. brassicae* are gregariously feeding. In addition, *P. brassicae* butterflies select stands of the same host plant (Le Masurier, 1994; Rothschild, 1987). Hence the chance of finding a second caterpillar nearby on the same host plant or a clutch nearby on the same host plant species is high.

In contrast, *P. rapae* butterflies select isolated host plants for the oviposition of single eggs (Hern et al., 1996; Root and Kareiva, 1984). So the odour emitted by the plant on which a *P. rapae* caterpillar is feeding, is not a reliable indicator for more *P. rapae* caterpillars in the same odorous environment. In concordance with this, in the Dutch *C. glomerata* an oviposition reward conditioning on *P. rapae* does not influence plant odour preference. However, in the Japanese *C. glomerata* a single experience in a *P. rapae* caterpillar does influence plant odour preference, albeit not to the same degree as in the Dutch *C. glomerata* after an experience with a *P. brassicae* larvae. So in this study physiological factors of the host seem to play a dominant role in influencing the occurrence of a preference shift after an oviposition reward conditioning.

We addressed the question whether the difference in preference behaviour after an oviposition reward conditioning in the two species *C. glomerata* and *C. rubecula* could be due to different host distributions. The Dutch *C. rubecula*, a specialist on the solitary *P. rapae*, does not display changes in preference behaviour even after multiple experiences, whereas the Dutch *C. glomerata* changes preference after a single oviposition in the gregarious *P. brassicae* (Geervliet et al., 1998). To be able to study the effect of host species, we used two populations of *C. glomerata* that use *P. brassicae* or *P. rapae* as host to allow a reciprocal test. Our study suggests that an oviposition reward conditioning in the preferred host induces a shift in preference behaviour. However, this result does not explain the lack of preference shift in *C. rubecula* after an oviposition reward conditioning in a *P. rapae* caterpillar, as *C. rubecula* is a specialist on *P. rapae*. This suggests that: 1) Japanese wasps have not yet completely adapted to using the uniformly distributed *P. rapae* as its host. 2) In Japan shifting preference after an experience on a *P. rapae* caterpillar may be more adaptive due to a different, more clustered, distribution of *P. rapae* in Japan compared to The Netherlands, or due to a lower number of available cruciferous host plants. 3) Factors other than host species play a role in the occurrence of a shift in preference behaviour.

Although we did not answer the question we originally set out to address, we value this study because it shows the importance of host identity, in terms of preferred host species, in oviposition reward learning. We conclude that detailed knowledge on the host range, host

plant range, preferred host and host distribution are needed to gain insight in the adaptive value of a shift in preference behaviour after an oviposition reward conditioning.

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7

General discussion

Two closely related parasitoid wasp species differ in learning during host-searching behaviour (Geervliet et al., 1998). After ovipositing in a caterpillar on a certain plant species *C. glomerata* shifts its preference to the experienced plant odour, whereas *C. rubecula* does not shift plant odour preference after a similar experience. This thesis is part of an elaborate project that studies the neural mechanisms that underlie this difference in learning. In this thesis I described morphological, anatomical and histochemical aspects of the neural pathways that underlie associative learning of odours in these wasps, as a first step to analyse possible differences in physiology. I furthermore redefined the difference in preference learning between the two species in terms of associative and non-associative learning and analysed the temporal dynamics of the memory trace. Finally, I studied the effect of host species as a physiological and ecological factor influencing memory formation in these two wasps.

The olfactory pathway

The two parasitoid species differ in learning of plant odours. To determine whether the observed differences in the olfactory learning behaviour were due to differences in olfactory processing, (part of) the olfactory processing pathway of both wasps was analysed in **chapter 2** and **chapter 3**.

The antennae

In insects, the olfactory receptor neurons are located in sensilla and the majority of these olfactory sensilla can be found on the antennae (Schoonhoven et al., 1998). Besides olfac-

tory receptor neurons the antennal sensilla can also house neurons for the perception of movement, humidity, taste and pressure. In **chapter 2** I analysed the external morphology of the antennal sensilla of both parasitoid wasp species, to identify whether there are any qualitative or quantitative morphological differences between the two species at the level of odour perception. In both wasp species the same six types of sensilla were present. Other parasitoid species that belong to the same family (Braconidae) or superfamily (Ichneumonoidea) have sensilla that are very similar to the sensilla of our *Cotesia* wasps (Barbarossa et al., 1998; Navasaro and Elzen, 1991; Norton and Vinson, 1974; Ochieng et al., 2000), whereas sensillar morphology in other groups (e.g. Chalcidoidea) was clearly different (Amornsak et al., 1998; Van Baaren et al., 1996; 1999; Cave and Gaylor, 1987; Olson and Andow, 1993). This suggests that phylogenetic origin is an important factor in determining sensillar morphology.

Three of the six types of sensilla that were found in both *Cotesia* wasps have a putative olfactory function. The sensillum coeloconicum type I is thought to be olfactory (Keil, 1999; Steinbrecht, 1997), sensilla trichodea WP (wall pores) are most likely olfactory sensilla (Keil, 1999; Steinbrecht, 1997) and sensilla placodea have an olfactory function in the honeybee (Akers and Getz, 1992), scarabid beetles (Hansson et al., 1999; Larsson et al., 2001) and the braconid wasp *Microplitis croceipes* (Ochieng et al., 2000). From these three types of sensilla, the sensilla placodea were present in the highest number and dominated the appearance of the antennae. These sensilla seem to be specifically suited for the processing of odorant mixtures (Akers and Getz, 1993; Getz and Akers, 1994) and they respond in a dose-dependent manner to plant volatiles (Ochieng et al., 2000). In parasitoids they may be used specifically for the processing of host-plant odours. Parasitoids use the odours of damaged host-plants to locate their hosts, and the odour bouquet of these host-damaged plants differs from undamaged plants or mechanically damaged plants in the relative amounts of odour compounds (Mattiacci et al., 1994). Hence, these sensilla that are specifically suited to process odour mixtures seem likely candidates for the processing of host-plant odours.

The number of sensilla placodea differed between the species and sexes. *C. rubecula* has more sensilla placodea than *C. glomerata* and males of both species have a larger number and a higher density of sensilla placodea compared to females of the same species. The larger number of sensilla placodea in *C. rubecula* could be related to the larger size of *C. rubecula* compared to *C. glomerata*, as the number of sensilla placodea is positively correlated with body size in bees (Johnson and Howard, 1987). However, it could also signify a functional difference. A higher number of sensilla can lead to an increased sensitivity (Chapman, 1982; Ignell et al., 1999). For *C. rubecula* this might be important, as it is specialized on a solitary caterpillar that only induces small amounts of damage resulting in a low amount of herbivore-induced volatiles. In contrast, *C. glomerata* predominantly uses the gregarious *P.*

brassicae as host, which feeds in groups and induces larger amounts of damage, resulting in a higher amount of herbivore-induced volatiles. In behavioural tests *C. rubecula* often displays a higher response level to low amounts of damage, which might indicate a higher olfactory sensitivity (Bleeker, unpublished results). Alternatively, the higher number of sensilla placodea in *C. rubecula* might be used to distinguish more different odour blends. The sensilla placodea house about 30 olfactory receptor neurons (Smid, unpublished results) that could express different olfactory receptor molecules. A higher number of sensilla could imply more combinations of olfactory neurons expressing different ranges of olfactory receptor molecules. A combination of a higher sensitivity and more combinations might also be possible.

Males possess a higher number and density of sensilla placodea compared to females of the same species. Males of Hymenoptera, and insects in general, often have a higher number of sensilla (Chapman, 1982; Van Baaren et al., 1999; Borden et al., 1978; Navasaro and Elzen, 1991; Ochieng et al., 2000) and in most of these insects the males are attracted by sex-pheromones (Chapman, 1982). *C. glomerata* and *C. rubecula* also use sex-pheromones to find females (Field and Keller, 1993; Tagawa, 1977; Tagawa and Kitano, 1981). In males of the scarabid beetle *Anomala cuprea* the sensilla placodea are sensitive to sex-pheromones (Larsson et al., 2001; Leal and Mochizuki, 1993). It is therefore possible that males of both *Cotesia* species use the sensilla placodea for the detection of sex-pheromones. Males of both species posses a macroglomerulus in the antennal lobe (**Chapter 3**), that is used for the processing of sex-pheromones (Rospars, 1988; Hildebrand, 1996; Hansson, 2002). The sexual dimorphism at the level of the antennae is more obvious in *C. glomerata* than in *C. rubecula*. This could indicate that males of *C. glomerata* are more specialized for detecting the sex-pheromone, whereas males of *C. rubecula* more resemble the female in olfactory processing. Interestingly, at the level of the glomeruli, there is also a stronger sexual dimorphism in *C. glomerata* compared to *C. rubecula* (Smid et al., 2001). *C. glomerata* males have much less glomeruli compared to the females, whereas in males and females of *C. rubecula* the numbers of glomeruli are more similar. This difference in sexual dimorphism between the species might be related to the different life-styles of the two *Cotesia* species. *C. rubecula* is a solitary species and the males have to search for the females. A possible strategy might be to use herbivore-induced plant odours in conjunction with the sex-pheromones to locate the females. So, males of this species should be able to process host-plant odours as well as the sex-pheromone. *C. glomerata* is a gregarious species, and in a cluster of parasitoid cocoons males emerge before the females and are arrested by the sex-pheromone that is produced by the females, already before emergence (Tagawa and Kitano, 1981; Tagawa, 1977). So, for *C. glomerata* males it may be less useful to be able to process host-plant volatiles.

It would be interesting to disentangle the olfactory processing at the antennal level in these species. Are the different olfactory sensilla used for the processing of functional different odours? How do the sensilla placodea, with their numerous olfactory receptor cells, process odours? Is the different number of sensilla placodea in *C. glomerata* and *C. rubecula* due to a functional difference? How do males of both species process the sex-pheromone, is there a difference between the species?

Summarizing, the two wasp species are highly similar in sensilla morphology and sensilla distribution and only display differences in the number of sensilla placodea. Although the presence of this difference raises some interesting questions, it is unlikely to explain the observed differences in learning between the two species.

The antennal lobe

We continued the analysis of the olfactory pathway with a study on the next level of olfactory processing. The olfactory receptor cells send the information to the primary olfactory neuropile; the antennal lobe (Hallem and Carlson, 2004). The antennal lobe is composed of spherical structures; the glomeruli (Hallem and Carlson, 2004; Gao et al, 2000). In **chapter 3** an analysis of the antennal lobe was made. The antennal lobes of two female wasps of each species were analysed. The variation in number of glomeruli was small within and between the species. The antennal lobes of the two *C. glomerata* females consisted of 186 and 189 glomeruli, and those of *C. rubecula* of 193 and 198 glomeruli. A difference in number of glomeruli between conspecifics has been described more often and may be caused by the fusion of glomeruli (Galizia et al., 1999). The higher number of glomeruli in *C. rubecula* is not due to the presence of an extra group in this species, but to small differences that are spread over the entire antennal lobe. However, there is one group consisting of four small glomeruli that is present in *C. glomerata*, but absent in *C. rubecula*.

Variability was observed in location, size and shape of the glomeruli between and within the two wasp species, but several ‘landmark’ glomeruli can be recognised in both individuals of the two species. The other glomeruli were tentatively matched and a digital three dimensional map of the antennal lobe could be constructed for females of both species. With future functional analysis and the use of antibodies that selectively stain a subset of glomeruli (Mistry et al., 2000), the identification of particular glomeruli may be improved further.

So, although some differences occur in the antennal lobes of the two species, it is unlikely that these differences would contribute to a difference in learning. Overall morphology was highly similar between the species and several glomeruli could be identified that are present in both species. The antennal lobe plays an important role in the formation of an associative memory in the honeybee (Hammer and Menzel, 1998) and the 3D maps

allow us to further study the role of the antennal lobes in associative learning in our two wasp species. Glomeruli can change size depending on age and behavioural tasks in *Drosophila* and the honeybee (Mistry et al., 2000; Sigg et al., 1997; Winnington et al., 1996). These volume changes might be the result of long-term synaptic plasticity. The 3D maps of the antennal lobes of both wasp species provide us with the excellent opportunity to study possible differences between the two wasp species in long-term synaptic plasticity in the antennal lobe after an associative learning experience. In addition, the 3D maps allow the analysis of the innervation of identified glomeruli by reward-sensitive interneurons in both species (**Chapter 4**).

From **chapter 2** and **chapter 3** we conclude that both species have a highly comparable anatomy of the olfactory processing pathway. This suggests that both species are similar in their perception and processing of odours. This is supported by an electrophysiological study (Smid et al, 2002), which shows that the species' receptive range of odours is not really different. This similarity of odour processing in these two wasp species emphasizes the value of this model system for comparative research on the neurobiological mechanisms underlying the difference in associative learning of odours. We hypothesise that the difference in learning between the two species is not due to an initial difference in the perception or processing of the conditioned stimulus (the odours), but to a difference in the processing of the unconditioned stimulus (the reward). This could be expressed by a difference in the neurons mediating the reward, or by a difference in sensitivity of the olfactory pathway to the reward.

Octopamine

In honeybee sugar learning, the unconditioned stimulus is mediated by an identified interneuron that is located in the suboesophageal ganglion of the honeybee brain and innervates neuropiles involved in the processing of odours, including the antennal lobes and the mushroom bodies (Hammer 1993). This VUMmx1 neuron is activated by presenting sugar to the antennae and proboscis of the honeybee and activation of this neuron can substitute for the sugar reward. This neuron is octopaminergic (Kreissl et al., 1994) and pairing octopamine injections with the CS can induce associative learning (Hammer and Menzel, 1998). Octopamine is also important in appetitive learning in *Drosophila* (Dudai et al., 1987; Schwaerzel et al., 2003). In the honeybee a difference in the amount of octopamine has been found between individual bees, related to the expression of hygienic behaviour, during which the bees detect diseased brood based on olfactory cues (Spivak et al., 2003). We hypothesise that the difference in learning between the two *Cotesia* species might be reflected

in a difference in the density of the arborisation pattern of this octopaminergic VUM neuron homologue in *Cotesia*.

In **chapter 4** we therefore analysed the octopaminergic neurons in the brain of the *Cotesia* wasps. Both species display similar complements of octopaminergic cells. We found several neurons that are similar to the VUM neurons in the honeybee. However, variability in staining intensity was high, and the used method was insufficient to reveal the number of VUM neurons and their arborisation patterns. We therefore were unable to identify possible differences between the species or to identify neurons with a similar morphology as the VUMmx1 neuron in the honeybee. However, preliminary studies involving dye injections in the antennal lobe revealed labelled VUM neurons, indicating the presence of VUM neurons projecting to the antennal lobes (Smid, unpublished). It is therefore likely that VUM neurons innervating the olfactory pathway, such as the VUMmx1 in he honeybee, are present in *Cotesia* as well.

In the honeybee the VUMmx1 neuron mediates the sugar reward in PER learning (Hammer, 1993). Parasitoids also display sugar reward learning (e.g Wäckers et al., 2002; Tertuliano et al., 2004) and it is likely that a homologue of the honeybee VUMmx1 neuron is mediating this learning in *Cotesia*. In our learning paradigm we use an oviposition as reward, not sugar. In oviposition reward learning contact of the ovipositor with host haemolymph constitutes, at least partially, the reward (Takasu and Lewis, 2003). It is possible that the same VUM neuron that is responsible for sugar learning also mediates oviposition learning, as the VUM neurons can be activated by several different stimuli (Schröter, 2002). However, it could also be that a different VUM neuron, with a similar branching pattern, is activated during an oviposition. It would be interesting to know whether *C. glomerata* and *C. rubecula* also differ in sugar reward learning. If the difference in oviposition learning is mediated by a difference in VUM neuron morphology, and there is no difference in sugar learning between the species, this would imply that oviposition learning and sugar learning are mediated by different VUM neurons.

Future studies should focus on revealing the VUM neuron responsible for mediating oviposition learning in these two *Cotesia* species. The candidate neuron should converge with the olfactory processing pathway and be activated during oviposition. Intracellular recordings or morphological studies using activity dependant dyes could pinpoint the neurons that are activated during oviposition. Further morphological studies using vibratome sections, or whole mount studies using anti-tyramine β-hydroxylase (Monastirioti et al., 1996) could reveal the arborisations of the VUM neurons better than the method used in our study and possibly reveal differences between the two wasp species.

In conclusion, the two wasp species are highly similar in morphology at the level of the

olfactory processing pathway (**Chapter 2** and **Chapter 3**), and are also alike in their olfactory receptor range. It is therefore unlikely that differential olfactory processing could account for the difference in learning between the two species. A possible mechanism for the difference in learning is variation in the processing of the unconditioned stimulus (the reward). We have identified a number of neurons that could mediate the reward stimulus in the two wasp species, but the results did not allow us to distinguish possible dissimilarities between the species (**Chapter 4**). We hypothesise that in *C. rubecula* a lower amount of octopamine might be released during an oviposition experience resulting in the formation of a weaker memory compared to *C. glomerata*.

Alternatively, the sensitivity to octopamine of the neurons in the olfactory pathway that are targets of the octopaminergic VUM neurons may vary. The formation of long-term memory requires the synthesis of new proteins, which can be induced by binding of the transcription factor CREB (cAMP responsive element binding protein) to the CRE (cAMP responsive element) region in the DNA (Kandel, 1991). There are several different isoforms of CREB, some of which activate transcription, whereas others suppress transcription (Yin et al., 1994). We hypothesise that the difference in learning ability between *C. glomerata* and *C. rubecula* might be related to a difference in tissue specific expression of the activating CREB isoforms and the suppressing CREB isoforms (Smid, in press).

In the next part of the discussion we leave the levels of molecules and cells and move up to the level of the organism. With use of behavioural studies we reanalyse the difference in learning and memory formation between the two species in such a way that it can be better compared to other model species. Subsequently we address the ultimate factors that could have driven this difference in learning between the two parasitoid species and whether these factors could help explain the difference in learning in parasitoids in general.

The difference in learning redefined

The difference in learning between *C. glomerata* and *C. rubecula* was defined as a difference in preference learning (Geervliet et al., 1998). Geervliet et al. (1998) used a choice-test, in which parasitoids could choose between two odour sources. Naive *C. glomerata* wasps prefer herbivore-damaged Brussels sprouts odour, but wasps that had an oviposition experience on nasturtium prefer the newly experienced odour; they display preference learning. *C. rubecula* does not change its preference; it does not display plant odour preference learning (Geervliet et al., 1998). The term preference learning is often used in parasitoid studies that focus on ecological functions of learning (Turlings et al., 1993; Fujiwara et al., 2000; Fukushima et al., 2001). However, in the study by Geervliet et al. (1998), it is a rela-

tive measure of learning, since the response to the learned odour is compared to the response to another, innately preferred odour. In **chapter 5** a no-choice test was used in which the response to the experienced odour was measured *per se*, without interference of an innately attractive odour. We found that *C. glomerata* displays a stable memory for the experienced odour that lasts for at least five days. This finding suggests that long-term protein synthesis dependent memory (LTM) occurs after only a single training in *C. glomerata*. According to studies with honeybees and fruit flies, LTM requires multiple conditioning trials (DeZazzo and Tully, 1995; Menzel, 1999). It would be remarkable if LTM is formed in *C. glomerata* after one single experience only. In future research protein synthesis inhibitors can be used to test whether the memory formed in *C. glomerata* after a single oviposition experience is protein-synthesis dependent. *C. rubecula* also displays associative learning for the experienced odour, but this memory diminishes after one day, resembling memory traces of other species after a single experience (DeZazzo and Tully, 1995; Menzel, 1999). So, although *C. rubecula* does not display preference learning, it does display associative learning after an oviposition reward conditioning. The difference in preference learning between the two species measured in a two-choice test (Geervliet et al., 1998) corresponds with a temporal difference in memory trace in the no-choice experiment. The long-lasting memory trace in *C. glomerata* seems to be more robust than the memory trace formed in *C. rubecula*, and such robust memory may be required to change the naive preference for plant odours.

Ultimate factors

What has caused the difference in learning between *C. glomerata* and *C. rubecula*? What are the ultimate factors that have driven its evolution? The two species are closely related (Michel-Salzat & Whitfield 2004), and they co-occur in the same habitats (Geervliet et al., 2000). They differ only in a few aspects, one of which is their use of host species: *C. rubecula* is a specialist on the small cabbage white *P. rapae*, whereas *C. glomerata* can parasitize *P. rapae*, *P. napi* and *P. brassicae*, but in The Netherlands mainly *P. brassicae* is used as host (Geervliet, 2000).

In parasitoids, a difference in specialization is thought to underlie a difference in learning (Vet and Dicke, 1992; Steidle and van Loon, 2003). For generalists learning is thought to be necessary to cope with the variability in host availability, whereas for specialists the use of innate cues is expected (Vet and Dicke, 1992; Steidle and van Loon, 2003). However, *C. glomerata* is fairly specialized on *P. brassicae* in The Netherlands; it mainly uses *P. brassicae* as host in the field (Geervliet et al., 2000), *P. brassicae* is a superior host (Brodeur et al. 1998; Harvey 2000) and *C. glomerata* is more adapted to *P. brassicae* in host searching (Wiskerke and Vet 1994; Vos et al. 1998). Furthermore, since both *P. brassicae* and *P.*

rapae have similar host plant ranges, there is no clear difference in specialization at the plant level either (Geervliet, 1997). Hence, the generalist/specialist theory does not provide us with a satisfactory explanation for the difference in learning between *C. glomerata* and *C. rubecula*. As both species use different host species with striking differences in their distribution pattern, we hypothesise that it is this aspect that caused the variation in learning in our wasps.

P. rapae females spread their eggs widely in the habitat; 1) they usually lay a single egg per plant, 2) they tend to follow linear flight paths and 3) they fly over many suitable host plants (Root & Kareiva 1984). This results in widely dispersed *P. rapae* caterpillars, occurring on different host plant species. In contrast, *P. brassicae* females lay their eggs in large clutches of up to 150 eggs on a plant (Rothschild 1987; Le Masurier 1994). In addition, *P. brassicae* females select a stand of host plants (Rothschild 1987; Le Masurier 1994), which is likely to attract more ovipositing females. As a result, *P. brassicae* caterpillars display a clustered distribution, in which a patch of the same plant species probably carries several batches of *P. brassicae* caterpillars. The total fecundity of *C. glomerata* varies between 500 and 2200 eggs, and about 20 eggs are laid in a single host (Vos and Vet, 2004). Therefore, the location of a patch of host plants in which several batches of *P. brassicae* caterpillars are present represents a 'jackpot' reward for *C. glomerata*, in which she can lay all of her eggs (Vos and Vet, 2004). Hence, for *C. glomerata* the encounter with a single *P. brassicae* caterpillar might represent this 'jackpot' reward, and the immediate induction of a preference for the host-plant on which the caterpillar is encountered is likely to be adaptive. For *C. rubecula* a single encounter with a *P. rapae* caterpillar does not indicate the presence of additional caterpillars in the same odorous environment, and it would therefore be maladaptive to switch preference towards the experienced plant odour for *C. rubecula*.

In **chapter 6** I have tested the hypothesis that the difference in learning is the result of the difference in host distribution of the two host species. This was done by comparing two populations of *C. glomerata*; a Dutch population that uses the gregarious *P. brassicae* as host, and a Japanese population that uses the solitary *P. rapae* as host. *C. glomerata* is thought to have been naturalized in Japan a few hundred years ago. On the main island of Japan, Honshu, *P. brassicae* does not occur and Japanese *C. glomerata* attack *P. rapae* (Sato and Ohsaki, 1987). Hence we could compare learning in two populations of the same species that are adapted to different host species.

Our results showed that the Dutch population only changes its preference after an oviposition reward experience on a *P. brassicae* larva, but not after an experience on a *P. rapae* caterpillar. In contrast, the Japanese population changes its preference after an oviposition reward conditioning on a *P. rapae* caterpillar, but not after experience on a *P. brassicae* caterpillar. So, both populations only change preference after an oviposition reward on their

preferred host species. This difference can be explained by a physiological factor: a different reward representation of the two hosts for the two wasp populations. The Dutch population is adapted to *P. brassicae* (Wiskerke & Vet 1994; Vos et al. 1998; Brodeur et al. 1998; Harvey 2000; Geervliet et al., 2000), and therefore this host represents a stronger reward than a *P. rapae* caterpillar, resulting in the formation of a stronger memory. The Japanese population is adapted to using *P. rapae* as host, and therefore a *P. rapae* caterpillar represents a stronger reward than a *P. brassicae* caterpillar for this population.

However, coming back to our original question, this purely physiological explanation can not provide an answer to the lack of preference learning in *C. rubecula*, as this wasp is specialized on *P. rapae* and this caterpillar therefore should represent an optimal reward. There might be a difference in the occurrence of *P. rapae* in Japan and The Netherlands, making learning on *P. rapae* adaptive in Japan, but not in The Netherlands. Possibly, the distribution of *P. rapae* in Japan is be more clustered than in The Netherlands or the number of available cruciferous host plant species is lower. Another explanation could be that although Japanese *C. glomerata* have adapted physiologically to using *P. rapae* as host, adaptation of learning is slower, and they have not yet adapted to the distribution of the host.

Future studies should elucidate the effect of host-distribution on learning. The fitness effect of learning in environments with different host distributions could be calculated with the use of parasitoid foraging models (see e.g. Vos and Hemerik, 2003). Field studies on the distribution of *P. rapae* in Japan could validate whether there is a difference in *P. rapae* caterpillar distribution between The Netherlands and Japan.

In conclusion, *C. glomerata* and *C. rubecula* differ in the memory dynamics displayed after a similar oviposition experience (**Chapter 5**). *C. glomerata* forms a long-lasting stable memory that suggests LTM, whereas *C. rubecula* forms a memory for the experienced odour that wanes after one day, and suggests the formation of a weaker memory compared to *C. glomerata* (**Chapter 5**). This difference in memory strength corresponds with the difference in preference learning described by Geervliet et al. (1998). We hypothesise that the difference in memory strength between the two species is adaptive for both species, related to a difference in distribution of their respective main host species (**Chapter 6**).

According to Stephens (1993) hypothesis, predictability should be the key to explaining whether learning is adaptive. Learning is likely to be favoured when the predictability of the environment is low between generations, but high within generations. For *C. glomerata* and *C. rubecula* there *is* a difference in predictability for the two species. For a host-searching *C. rubecula* the encounter of a caterpillar on a specific host-plant does not predict the presence of the next host in any way (**Chapter 5** and **Chapter 6**), so within generation predictability

is low for *C. rubecula* and learning is not favoured. For *C. glomerata* the encounter of a *P. brassicae does* reliably predict the occurrence of numerous other hosts, probably allowing her to lay all her available eggs (**Chapter 5** and **Chapter 6**), so within generation predictability is high. Furthermore, the plant species on which the cluster of caterpillars is present is not predictable between generations, so learning is favoured. How much of our knowledge can be generalized to other parasitoid species? If learning is indeed related to the predictability of the environment and influenced by the degree of host preference we will need detailed knowledge on these ecological factors before we can predict whether parasitoids use learning in host-searching. At the same time we need to know the neurobiological and physiological mechanisms that could underlie differences in learning.

This knowledge is scarce for parasitoids in general. It will be necessary to test hypotheses with selective, comparative studies on related species. My study is a first step to reach this goal and integrate the still separate fields of behavioural ecology and neurobiology.

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Nederlandse samenvatting

Associatief leren in twee nauw verwante sluipwespsoorten: Een neuro-ecologische benadering

Leren in insecten

Ook al zijn insecten maar klein, ze hebben wel degelijk hersenen, waarmee ze kunnen leren. Met leren bedoelen we het proces waarbij kennis van de wereld wordt opgebouwd, de verworven kennis wordt daarbij opgeslagen in het geheugen. Bij insecten gaat het om het opslaan van informatie over de omgeving. Insecten zijn dus geen kleine geprogrammeerde machientjes, zoals vaak wordt aangenomen, maar kunnen hun gedrag aanpassen naar gelang ervaring. Zo kunnen sprinkhanen bijvoorbeeld leren om giftig eten te vermijden. Honingbijen moeten eerst leren hoe ze met complexe bloemen om moeten gaan om bij de nectar te komen, en ze leren de geuren, kleuren en vorm van bloemen die veel nectar bevatten herkennen. Ook de plaats van het nest of van groepjes bloemen waar ze nectar verzamelen slaan ze op in hun geheugen. Sluipwespen gebruiken leren om de insecten te vinden waar ze hun eitjes in leggen.

Door het bestuderen van leergedrag bij insecten is meer inzicht verkregen in hoe en waarom insecten leren. Als we het proces van leren en geheugenvorming van insecten en andere dieren vergelijken, blijkt dat het basale mechanisme van leren sterk overeenkomt. Het bestuderen van leren in insecten kan ons dus ook inzicht verschaffen in hoe leren werkt in andere dieren, inclusief de mens, terwijl de kleinere en minder complexe hersenen van insecten het bestuderen vergemakkelijken.

Sluipwespen

In dit proefschrift heb ik het leergedrag van de twee sluipwespsoorten *Cotesia glomerata* en *Cotesia rubecula* bestudeerd. Sluipwespen lijken niet op de geel-zwart gestreepte sociale wesp, en ze steken mensen ook niet. Veel soorten sluipwespen zijn ook veel kleiner dan deze bekende wespen; de soorten waar ik mee gewerkt heb zijn maar een paar millimeter groot. Sluipwespen zijn echter wel gevaarlijk voor andere insecten, ze gebruiken ze namelijk om hun eitjes in te leggen. *Cotesia glomerata* en *C. rubecula* gebruiken hiervoor de rupsen van koolwitjes. De jonge sluipwesplarven eten vervolgens hun nog levende slachtoffer langzaam op. Dit lijkt misschien gruwelijk, maar sluipwespen leveren op deze manier een

belangrijke bijdrage aan het in toom houden van schadelijke insecten. Ze worden dan ook veel gebruikt in de biologisch bestrijding.

Wanneer de *Cotesia* larven genoeg gegeten hebben verpoppen ze zich en komen de volwassen sluipwespen te voorschijn. Na het paren is het vinden van nieuwe jonge rupsen om haar eitjes in te leggen de voornaamste taak van het vrouwtje. Hoe echter zo'n kleine rups te vinden? De sluipwesp heeft daar wat op gevonden: de plant, waar de rupsen van eten, geeft geurstoffen af en deze worden door de sluipwesp gebruikt om de rupsen te vinden. Deze geuren zijn specifiek voor de plantensoort en kunnen zelfs specifiek zijn voor de rupsensoort die ervan eet. Het is dus een zeer betrouwbare informatiebron voor de sluipwesp. Sluipwespen zijn vaak gespecialiseerd op een bepaalde rups, en deze is op zijn beurt weer gespecialiseerd op een bepaalde plantensoort. Koolwitjes komen bijvoorbeeld voornamelijk op koolsoorten voor. De vrouwtjes *Cotesia* hebben dan ook een aangeboren voorkeur voor de geur van kool. Maar soms zijn de rupsen ook op andere plantensoorten te vinden, waar de *Cotesia* wespen van nature niet in zijn geïnteresseerd. Door de geuren van deze andere planten te leren, kunnen de sluipwespen ook de rupsen vinden die van deze planten eten.

Associatief leren in *Cotesia*

De sluipwespen kunnen de nieuwe plantengeur leren door middel van associatie. Bij associatief leren wordt de relatie tussen verschillende dingen geleerd. Een bekend voorbeeld van associatief leren is de Pavlov hond. Pavlov was een wetenschapper aan het begin van de 20ste eeuw, die zijn hond elke keer, vlak voordat hij te eten kreeg een bel liet horen. Na dit een aantal keer gedaan te hebben ging de hond al kwijlen bij het geluid van de bel. De hond had het geluid van de bel leren associëren met het eten. Deze vorm van associatief leren wordt klassieke conditionering genoemd. Bij deze vorm van associatief leren worden twee stimuli met elkaar gekoppeld, een initieel neutrale stimulus (het geluid van de bel), met een betekenisvolle stimulus (het eten). Onze sluipwespen kunnen de initieel neutrale nieuwe plantengeur koppelen met de aanwezigheid van rupsen door middel van associatief leren.

Uit eerder onderzoek is gebleken dat de ene soort, *C. glomerata*, de geuren van deze nieuwe planten inderdaad leert door een associatieve leerervaring. Bij een dergelijke ervaring vindt de sluipwesp, bij toeval, een rups op de nieuwe, aangevreten, plant en legt haar eitjes hierin. Tijdens deze ene ervaring leert *C. glomerata* de geur van de nieuwe plant te associëren met de aanwezigheid van rupsen. Hierna is het vrouwtje *C. glomerata* meer geïnteresseerd in de nieuwe plantengeur dan in de koolgeur. Maar na een soortgelijke ervaring vertoont de andere soort, *C. rubecula*, dit veranderde gedrag niet, deze soort vindt de koolgeur nog steeds aantrekkelijker. De twee soorten verschillen dus in hun gebruik van leren tijdens het zoeken naar rupsen. De ene soort, *C. glomerata*, leert snel, de andere, *C. rubecu-*

la, niet. Dit natuurlijke leerverschil kan uitstekend gebruikt worden om te onderzoeken welke onderliggende neurologische mechanismen een snel leervermogen mogelijk maken. Resulterende inzichten zouden wellicht ook leerstudies in de mens vooruit kunnen helpen. Dergelijk vergelijkend onderzoek, waarbij een natuurlijk verschil in leergedrag wordt gebruikt om de neurologische mechanismen van leren te achterhalen, wordt neuro-ecologisch onderzoek genoemd. In dit proefschrift beschrijf ik (gedeeltelijk) de zenuwbanen die betrokken zijn bij het associatief leren in deze twee sluipwespen. Wat zijn de verschillen tussen de soorten en in hoeverre zouden deze verschillen het leerverschil kunnen veroorzaken? Tevens heb ik me bezig gehouden met het nauwkeuriger omschrijven van het leerverschil tussen de twee sluipwespen, en vraag ik me af *waarom* de twee in leren verschillen. Wat is de ecologische, evolutionaire verklaring van het leerverschil?

De verwerking van geuren

Bij de sluipwespen gaat het om een specifieke geur die wordt geassocieerd met de aanwezigheid van rupsen. Na de associatie betekent het ruiken van die geur voor de sluipwesp dat er rupsen in de buurt zijn. Door de leerervaring is er dus iets veranderd in de betekenis, en daarmee de verwerking, van de geur voor dat specifieke sluipwespvrouwtje.

Het is daarom belangrijk om de geurverwerkingsbaan van de twee sluipwespen in kaart te brengen. Zijn er verschillen tussen de twee soorten? Verder is een kaart van deze zenuwbaan belangrijk om te gebruiken bij verder onderzoek. We kunnen hierdoor nauwkeurig bepalen wat bij beide soorten het effect van een leerervaring op de geurverwerking is, en de verschillen tussen de soorten definiëren.

Geuren worden bij insecten voornamelijk waargenomen door de antennes. De zenuwcellen die de geuren waarnemen bevinden zich in structuren in de cuticula (het uitwendige skelet van insecten); dit worden sensilla genoemd. Behalve sensilla met geurreceptoren, zijn er ook sensilla die smaak, luchtvochtigheid, druk, of beweging waarnemen. Geursensilla kunnen herkend worden door de aanwezigheid van poriën in de wand van het sensillum. In zo'n geursensillum bevinden zich één of meer zenuwcellen die op specifieke geuren reageren. Door welke geurstof de zenuwcel geactiveerd wordt hangt af van welk receptor molecuul in het celmembraan tot expressie is gebracht. In **hoofdstuk 2** heb ik de sensilla op de antennes van beide soorten in kaart gebracht. Beide soorten beschikken over dezelfde zes soorten sensilla in ongeveer dezelfde hoeveelheden. Daarvan zijn drie soorten waarschijnlijk betrokken bij de verwerking van geuren, waarvan de plaatsensilla het meest prominent aanwezig zijn. Plaatsensilla zijn zeer geschikt voor het verwerken van complexe geurmengsels en zouden dus gebruikt kunnen worden voor het verwerken van plantengeuren. Het totaal aantal plaatsensilla was groter bij *C. rubecula* dan bij *C. glomerata*, wat erop zou kunnen duiden dat *C.*

rubecula gevoeliger voor geuren is. Het groter aantal plaatsensilla van *C. rubecula* zou echter ook verklaard kunnen worden doordat *C. rubecula* iets groter is dan *C. glomerata*. Mannetjes van beide soorten hebben een groter aantal en een hogere dichtheid van plaatsensilla. Aangezien de mannetjes sex-feromonen gebruiken om de vrouwtjes te vinden, zou dit een aanwijzing kunnen zijn dat ze de plaatsensilla gebruiken bij de verwerking van deze geuren. Hoewel we een interessant verschil tussen de soorten vinden op het niveau van de antennes, is het onwaarschijnlijk dat dit verschil bijdraagt aan het leerverschil. Concluderend kunnen we stellen dat op het niveau van geurwaarneming de twee soorten sterk op elkaar lijken.

De geurcellen op de antennes projecteren naar de antennale lob, het geurverwerkingscentrum in de hersenen van insecten. De antennale lob is opgebouwd uit een groot aantal bolvormige structuren, de glomeruli, en lijkt daarmee op het geurverwerkingscentrum in de hersenen van mensen. Het aantal glomeruli is grotendeels constant binnen een soort, en varieert binnen de insecten normaal gesproken tussen de 50 en 200. Behalve het aantal, is ook de locatie van de specifieke glomeruli onderling constant. Geurcellen op de antennes die geactiveerd worden door dezelfde geurstoffen, projecteren naar hetzelfde glomerulus in de antennale lob, en glomeruli die geurstoffen verwerken die erg op elkaar lijken liggen bij elkaar in de buurt. De organisatie van de glomeruli in de antennale lob is dus niet topografisch, waarbij sensilla op een bepaalde locatie op de antennes naar een specifiek glomerulus projecteren, maar functioneel, waarbij specifieke geurstoffen bepaalde glomeruli activeren. In **hoofdstuk 3** is van elke soort de antennale lob van twee vrouwtjes geanalyseerd. De hoeveelheid glomeruli bij *C. glomerata* betrof 186 en 189 glomeruli, bij *C. rubecula* 193 en 198 glomeruli. Hoewel er binnen een soort nog een behoorlijke variatie in locatie, vorm en grootte van glomeruli was, kon met behulp van bepaalde glomeruli die erg opvielen door hun vorm of grootte, van beide soorten een driedimensionale kaart gemaakt worden. Het geringe verschil in aantal glomeruli binnen een soort lijkt te worden veroorzaakt doordat er soms twee of meer glomeruli bij een van de twee individuen zijn samengesmolten. Ook konden de kaarten van de twee soorten met elkaar vergeleken worden, met behulp van dezelfde 'herkenningspunt' glomeruli. Het verschil in aantal tussen de soorten wordt veroorzaakt door kleine verschillen, die over de gehele antennale lob verspreid zijn. Ook op het niveau van de antennale lob zijn dus interessante verschillen tussen de soorten, maar ook deze verschillen lijken niet van dien aard dat ze het leerverschil tussen de soorten kunnen verklaren.

Vanuit de glomeruli wordt de informatie doorgestuurd naar de paddestoelvormige lichamen, de hogere integratiecentra in de hersenen van insecten. Hier wordt de geurinformatie geïntegreerd met bijvoorbeeld visuele informatie.

Octopamine

Bij onze sluipwespen is behalve de geurverwerkingsbaan nog een zenuwbaan betrokken bij het vormen van de associatie, namelijk degene die het waarnemen van de rups registreert. Het waarnemen van de rups is voor de sluipwesp een zeer belangrijke gebeurtenis. Het geeft het succes aan van het vinden van een plaats om haar eitjes te leggen en vormt een soort beloning, net zoals het vinden van voedsel een beloning zou zijn. We weten nog niet precies hoe deze stimulus bij sluipwespen verwerkt wordt. We moeten daarom kijken naar een ander insect, waarbij al meer onderzoek is gedaan naar het associatief leren van geuren.

De honingbij is nauw verwant met onze sluipwespen, en zijn hersenen lijken ook erg op die van *Cotesia*. Honingbijen kunnen de aanwezigheid van nectar met geuren leren associëren. Bij de bij is de nectar dus de beloning, en het waarnemen van nectar activeert een speciale zenuwcel in de hersenen. Deze zenuwcel projecteert naar de geurverwerkingsbaan. Als tijdens de conditionering geen nectar wordt aangeboden, maar deze zenuwcel kunstmatig wordt geactiveerd, wordt de associatie ook gevormd. Deze zenuwcel kan dus de nectarbeloning vervangen en is verantwoordelijk voor het leggen van de associatie van de geur met de nectar. Deze 'beloningsgevoelige' zenuwcel geeft de stof octopamine af in de geurverwerkingsbaan als hij geactiveerd wordt. We denken dat in *Cotesia* bij het waarnemen van een rups ook zo'n 'beloningsgevoelige' zenuwcel actief wordt.

In **hoofdstuk 4** zijn daarom de cellen in de hersenen van *Cotesia* in kaart gebracht, die de stof octopamine bevatten. We vonden drie groepen octopamine bevattende cellen bij beide *Cotesia* soorten. Eén van die groepen komt overeen met de groep cellen in de honingbij waarin de zenuwcel zich bevindt die gevoelig is voor nectar. We denken dat de zenuwcel die reageert op het waarnemen van een rups tot deze groep behoort. Helaas was de methode die we hebben gebruikt onvoldoende gevoelig om te bepalen of beide sluipwespsoorten evenveel octopaminerge cellen bevatten, of om te zien of de cellen verschillen in hun projectiepatroon. Het zou kunnen zijn dat *C. rubecula* niet meteen de plantengeur leert associëren met de rups omdat bij *C. rubecula* minder octopamine wordt afgegeven bij het waarnemen van een rups in vergelijking met *C. glomerata*.

Geheugen

Het verschil in leren tussen *Cotesia glomerata* en *C. rubecula* is aangetoond in een twee-keuze test. Na de associatieve leerervaring mochten beide soorten wespen kiezen tussen de initieel aantrekkelijke koolgeur en de nieuw ervaren plantengeur. Zoals hiervoor al verteld, kiest *C. glomerata* na een dergelijke ervaring voor de nieuw ervaren geur, terwijl *C. rubecula* de koolgeur blijft prefereren. Door deze test is het duidelijk dat *C. glomerata* asso-

ciatief leren vertoont. Ook is het duidelijk dat een dergelijke ervaring minder effect heeft bij *C. rubecula*. Het wil echter niet zeggen dat *C. rubecula* helemaal niet associatief leert. Het kan immers zo zijn dat de nieuwe geur wel wordt geleerd, maar niet in een zo grote mate dat hij de voorkeur voor kool kan verdringen.

Om erachter te komen of *C. rubecula* de nieuwe geur helemaal niet leert, of alleen in mindere mate dan *C. glomerata* gebruik ik in **hoofdstuk 5** een geen-keuze test. In deze test geef ik de wespen een eileg-ervaring in een rups op de nieuw te leren plant en test vervolgens in hoeverre ze aangetrokken worden door deze geur. Ik vergelijk ze hierbij met wespen die geen leerervaring hebben ondergaan. Uit deze test blijkt dat *C. rubecula* wel degelijk een geheugen voor de ervaren plantengeur ontwikkeld. Vervolgens heb ik getest hoelang het geheugen voor de ervaren plantengeur stand hield. Het blijkt dat *C. glomerata* na een dergelijke ervaring een stabiel geheugen vormt dat minstens 5 dagen aanhoudt. Bij *C. rubecula* neemt het geheugen na een dag af. Dus, hoewel *C. rubecula* de geur wel leert, leert ze hem niet zo goed dat ze hem dagen onthoudt, en niet in zodanige mate dat het de voorkeur voor kool verdringt.

Evolutie en ecologie

De vraag was nu *waarom* dit verschil in leren tussen de soorten bestaat. De evolutietheorie leert ons dat dieren zich aanpassen aan hun omgeving, en datgene ontwikkelen, of in stand houden, wat ze tot voordeel is. Zou het kunnen zijn dat het voor *C. rubecula* gewoon niet voordelig is om van voorkeur te veranderen na een dergelijke leerervaring, terwijl het voor *C. glomerata* wel voordelig is? Wat zijn dan de verschillen tussen de soorten die een dergelijke variatie in leren kunnen verklaren? Een belangrijk verschil tussen de soorten is dat *C. glomerata* voornamelijk op de rupsen van het Groot koolwitje parasiteert, terwijl *C. rubecula* haar eitjes in het Klein koolwitje legt. De rupsen van het Groot koolwitje leven in groepjes bij elkaar, en de Grote koolwitjes vlinders zoeken vaak een groepje van dezelfde plantensoort uit om hun eitjes op te leggen. Een dergelijke groepje van planten trekt waarschijnlijk meerdere Grote koolwitjesmoeders aan, waardoor er een situatie ontstaat waarbij op een groepje van dezelfde planten een paar clusters Grote koolwitjesrupsen zitten. In een dergelijke omgeving zou het voordelig zijn voor *C. glomerata* om de geur van de plant waarop zij een Grote koolwitjesrups vindt meteen in te prenten. Het is immers zeker dat er nog meer rupsjes in de buurt zitten.

Aan de andere kant, de rupsen van het Klein koolwitje leven alleen. Bovendien heeft moeder Klein koolwitje de neiging om haar eitjes juist te verspreiden over alleenstaande planten. Als *C. rubecula* dus een rups tegenkomt wil dat helemaal niet zeggen dat er nog een rups te vinden is op dezelfde plantensoort. Voor *C. rubecula* is het dan dus helemaal niet

voordelig om zich na een ervaring met een enkele rups te richten op die geur die ze net geleerd heeft.

In **hoofdstuk 6** toets ik de bovenstaande theorie door middel van twee populaties van *C. glomerata*. De ene populatie is degene die in Nederland voorkomt, en waarmee alle bovenstaande experimenten gedaan zijn. De andere populatie is afkomstig uit Japan, waar geen Grote koolwitjes voorkomen en *C. glomerata* het Kleine koolwitje parasiteert. Elke populatie heb ik in vier groepen verdeeld, waarbij elke groep een eileg-ervaring krijgt op óf een Klein koolwitje op kool óf een Groot koolwitje op kool óf een Klein koolwitje op de nieuwe plantensoort (Oost-Indische kers) óf een Groot koolwitje op Oost-Indische kers. Na de ervaring konden de sluipwespen kiezen of ze kool of Oost-Indische kers prefereerden. Op deze manier kon ik onderzoeken in welke mate de identiteit van de rups (Groot of Klein koolwitje) invloed heeft op hoe goed de geur geleerd wordt.

Uit dit experiment bleek dat de Nederlandse *C. glomerata* alleen haar voorkeur veranderde in de richting van de ervaren geur na een ervaring op het Groot koolwitje, maar niet na een ervaring op het Klein koolwitje. Dit is dus in overeenstemming met de verwachting. Maar de Japanse wespen veranderen hun voorkeur juist na een ervaring op het Klein koolwitje, en niet op het Groot koolwitje. In dit experiment lijkt het er dus op dat niet de identiteit van de rups (de soort) de doorslaggevende factor is, maar in welke rups de sluipwesp het liefst haar eitjes legt. Beide populaties veranderen alleen hun voorkeur na een ervaring op de rups waar ze in de natuur hun eitjes het liefst in leggen.

Dit kan echter niet verklaren waarom *C. rubecula* niet leert op het Klein koolwitje. Het Klein koolwitje is immers haar favoriete rups om haar eitjes in te leggen. Het zou kunnen dat de Kleine koolwitjes rupsen in Japan een ander verspreidingspatroon hebben dan in Nederland, waardoor het voor de Japanse *C. glomerata* sluipwespen wel voordelig is om te leren op het Klein koolwitje.

Conclusie

In dit proefschrift heb ik een start gemaakt met het in kaart brengen van de zenuwbanen die betrokken zijn bij het associatief leren van geuren in sluipwespen. Het is gebleken dat de geurverwerkingsbaan van beide soorten erg op elkaar lijkt. De verschillen die gevonden zijn tussen de soorten zijn gering, en kunnen het leerverschil tussen de twee soorten niet verklaren. De analyse van de geurverwerkingsbaan kan gebruikt worden bij vervolgonderzoek, waarbij het effect van een ervaring op de geurverwerkingsbaan wordt bekeken en de verschillen hierin worden onderzocht tussen beide soorten. De grote gelijkenis tussen de twee soorten in de geurverwerkingsbaan vergemakkelijkt deze vergelijking. Tevens heb ik een

aantal cellen zichtbaar gemaakt die verantwoordelijk kunnen zijn voor de associatie van het eitjes leggen met de nieuwe plantengeur.

Door de nauwkeurigere omschrijving van het leerverschil tussen de twee soorten, kan het onderzoek beter vergeleken worden met studies naar leren in andere soorten. De beschrijving van de duur van het geheugen biedt verdere aanknopingspunten voor verder onderzoek naar het geheugen.

Als laatste heb ik gekeken in hoeverre een simpel ecologisch verschil, de identiteit van de rups, een verklaring zou kunnen vormen voor de vraag *waarom* de soorten verschillen in leren. Hieruit blijkt dat het onwaarschijnlijk is dat een dergelijke enkele ecologische factor een complete verklaring zou kunnen leveren.

Met dit onderzoek heb ik een bijdrage geleverd aan het ontrafelen van het hoe en waarom van leren in sluipwespen. Deze kennis kan gebruikt worden om de efficiëntie van sluipwespen in de biologische bestrijding te vergroten. Mogelijk kan kennis over leren in sluipwespen in de toekomst ook leiden tot een beter inzicht in leren en geheugen in dieren in het algemeen, inclusief de mens.

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Maartje
Nijmegen, November 2005

Curriculum vitae

Ik, Maartje Anne Kathelijn Bleeker, ben geboren op 2 februari 1976 te Nijmegen. Binnen een jaar verhuisden we van Nijmegen naar Tiel, en toen ik negen was verhuisden we naar Kerk-Avezaath, een klein dorpje even buiten Tiel. Hier bracht ik menig uurtje bij de sloot door en ontdekte ik dat ik bioloog wilde worden. Nadat ik in 1994 mijn VWO diploma had gehaald op het GSG Lingecollege in Tiel besloot ik daarom Biologie te gaan studeren aan de Katholieke Universiteit van Nijmegen (KUN). Ik koos een breed vakkenpakket, met ecologische, fysiologische en celbiologische vakken en liep stage bij Organismale Dierfysiologie van de KUN, waar ik onderzoek deed naar de stressrespons van regenboogforel op de ectoparasiet *Argulus foliaceus* en een scriptie schreef over de fysiologische aanpassingen van insecten aan extreme kou. Hieruit blijkt al mijn interesse voor insecten, en voor mijn tweede stage koos ik dan ook voor de vakgroep Entomologie aan de Wageningen Universiteit, waar ik een driedimensionale analyse maakte van de antennale lob van twee sluipwespsoorten die verschilden in leren. In augustus 1999 legde ik mijn doctoraal examen met goed gevolg af. Van april 2000 tot juni 2005 werkte ik aan mijn promotie onderzoek op de vakgroep Entomologie aan de Wageningen Universiteit onder leiding van Hans Smid, Joop van Loon en Louise Vet aan het leerverschil in de twee sluipwespsoorten waar ik tijdens mijn stage al kennis mee gemaakt had. Dit onderzoek staat beschreven in dit proefschrift.

List of publications

Smid, H. M., Bleeker, M. A. K., Van Loon, J. J. A. and Vet, L. E. M. 2003. Three-dimensional organization of the glomeruli in the antennal lobe of the parasitoid wasps *Cotesia glomerata* and *C. rubecula*. *Cell and Tissue Research*, **312**, 237-248.

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