UNIVERSITY OF SOUTHAMPTON

FACULTY OF NATURAL AND ENVIRONMENTAL SCIENCES

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Host Location in a Specialist Parasitoid Wasp via Olfactory Cues – A Physiological, Behavioural and Morphological Study

by

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ABSTRACT

FACULTY OF NATURAL AND ENVIRONMENTAL SCIENCES
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HOST LOCATION IN A SPECIALIST PARASITOID WASP VIA OLFACTORY CUES – A PHYSIOLOGICAL, BEHAVIOURAL AND MORPHOLOGICAL STUDY

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For successful host location, parasitoids are thought to have evolved different strategies to filter relevant olfactory cues which indicate the presence of the host. Because of their versatility in their ecology and behaviour, as well as their fine tuned olfactory system to volatile compounds of the host and host plant, they have gained increasing recognition as model organisms to study learning and behaviour in an adaptive ecological context. However, neural and cellular mechanisms of olfactory detection and processing in parasitoids are mainly unknown.

In this thesis physiological, behavioural and morphological experiments were used to determine neural and behavioural mechanisms of host location via olfactory cues in the specialist parasitoid *Cotesia vestalis*. *C. vestalis* showed significant antennal responses to a range of odour compounds. Behavioural experiments, however, have demonstrated that only the herbivore-induced plant volatile linalool attracts *C. vestalis* males and females, but 1-nonanol has a repulsive effect on females. A morphological study of the antennal lobe, the first brain area where olfactory information is processed, revealed 40 ordinary glomeruli in both males and females. In addition, a complex of 2-3 enlarged glomeruli (MGC) was found in males. The courtship behaviour observed in males and the MGC suggest that males could use sex pheromones to locate females. Finally, calcium imaging studies showed glomerular activity to olfactory stimulation in bees but not in parasitoids.

In conclusion, the degree of host specialisation in *C. vestalis* appears to influence olfactory learning in males and females, which favours learning of volatiles related to its host and host plant, as well as the morphological organisation of the antennal lobe. Larger, fewer and possibly specialised glomeruli could enhance processing of odour cues which are important for this parasitoid.

"I cannot persuade myself that a beneficent and omnipotent God would have designedly created parasitic wasps with the express intention of their feeding within the living bodies of Caterpillars."

Charles Darwin; 22 May 1860, Letter to Asa Gray

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Declaration of authorship

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Host Location in a Specialist Parasitoid Wasp via Olfactory Cues – A Physiological, Behavioural and Morphological Study

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Abbreviations

ACD	Actinomycin D
AL	Antennal lobe
AMPA	2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid
AN	Antennal nerve
ARM	Anesthesisa-resistant memory
C	Cumulus
cAMP	Cyclic adenosine monophosphate
CHX	Cycloheximide
CLSM	Confocal laser scanning microscopy
CREB	cAMP response element-binding
DBM	Diamondback moth
GLV	Green leaf cabbage volatile
GPCR	G-protein-coupled receptor
GR	Granular cell
HIPV	Herbivore- induced plant volatile
IP3	Inositol 1,4,5-trisphosphate
LFG	Large female glomerulus
LN	Interneuron
LTF	Long-term facilitation
LTM	Long-term memory
eLTM	early Long-term memory
lLTM	later Long-term memory
LTP	Long-term potentiation
MB	Mushroom body
MG	Macroglomerulus
MGC	Macroglomerular complex
M/T	Mitral/tufted cell
MTM	Intermediate memory
NHVs	Non-host volatiles
NMDA	*N*-methyl-D-aspartate
OB	Olfactory bulb

OBP	Water-soluble odorant-binding protein
OL	Optical lobe
OR	Olfactory receptor
ORN	Olfactory receptor neuron
ORP	Olfactory receptor protein
PG	Periglomerular cell
PN	Projection neuron
S	Sensillum
STM	Short-term memory
T	Toroid
VOC	Volatile organic compound

CHAPTER 1

GENERAL INTRODUCTION

Parasitoid wasps (parasitoids) are insects whose larvae feed on other arthropods (usually insects), eventually causing the death of the host (Godfray, 1994). To successfully locate a host, parasitoids use olfactory cues from the host plant, the host, or cues associated with the host, such as frass, pheromones or silk (Turlings et al., 1991; Potting et al., 1999; Wölfling and Rostás, 2009). Many of their hosts are crop pests, which makes parasitoids important organisms for use in biological pest control, contributing to enormous savings in agriculture (Simpson et al., 2011).

The influence of host specialisation on the parasitoid's ability to learn relevant olfactory cues has been widely studied in recent years (Smid et al., 2007; Van den Berg et al., 2010). Parasitoids vary strongly in their host specialisation, and even closely related wasps show differences in learning and memory. It is thought that these differences are an adaptation to optimise their fitness related to their host specialisation (Hoedjes et al., 2011). However, many of these studies have neglected the neuronal and cellular mechanisms behind learning, acquisition and storage of memory, and it is only recently that progress in this area has been made (Bleeker et al., 2006b; Collatz et al., 2006; Van den Berg et al., 2010). To understand how parasitoids use olfactory information for host location it is

essential to integrate knowledge from olfactory perception to processing and behavioural output.

For decades, scientists have tried to unravel the processes that are involved from perception, processing, learning and storing olfactory information (Firestein, 2001). One of the driving questions is to understand the cellular organisation and mechanism on which olfactory driven behaviour is based (Axel, 1995; Axel, 2005). Moreover, if neural and genetic mechanisms underlying olfactory perception and learning are similar among species, where and why do changes in inter-species variation in learning and memory formation occur? One way to understand these differences is to combine different areas of research to analyse the olfactory pathway and investigate learning and memory, and also consider the ecological relevance of a certain cue for an animal.

The overall aim of this thesis was to determine behavioural and neuronal mechanisms of host location in the specialist parasitoid *C. vestalis* by analysing olfactory perception and learning, and by investigating the anatomical structures underlying olfactory processing. Integrating behavioural, morphological and physiological methods will provide us with more insight into complex olfactory processing in a parasitoid wasp.

1.1. Host location in parasitoids via olfactory cues

To reproduce, parasitoid wasps oviposit into the host, which eventually leads to its death. Many of the parasitoid wasps are considered beneficial insects because they control populations of agricultural pests (Bianchi et al., 2006; Uefune et al., 2011). Research in parasitoids has mainly focussed on their ecology and their use in pest control (Bianchi and Wäckers, 2008; Kaplan, 2011). Due to the versatility of their ecology and behaviour, as well as their fine-tuned olfactory system to plant volatiles, they represent valuable animal models for the study of learning and behaviour in an adaptive ecological context (Hoedjes et al., 2011). Many of the beneficial parasitoids are from the family Braconidae, which are estimated to have between 50,000 and 150,000 different species. Depending on the species and the host stage they use, one can distinguish between egg-, larval-

or adult parasitoids. Parasitoids also differ in their host specialisation. Parasitoids can be considered specialists (one species) or generalists (several species), not only at the host level, but also at the host plant level. Vet and Dicke (Vet and Dicke, 1992) suggested four categories of specialisation: Group A are specialist parasitoids on a host and host plant level, meaning that these parasitoids only prefer one host species that can only be found on one host plant species; group B are generalists at a host level, but specialists at a plant level; group C are specialists on a host level, but generalists at a host plant level, and group D are generalists at a host and host plant level.

For successful reproduction, parasitoids have to locate their host. Host searching behaviour can be divided into several stages, and includes host habitat finding, host finding, host recognition, and host acceptance (Vinson, 1976). For successful host location, parasitoids are thought to have evolved different strategies to filter relevant olfactory cues which indicate the presence of the host (Vet and Dicke, 1992), often through the detection of chemical signals emitted by the host plant and host. Parasitoids of phytophagus hosts (hosts feeding on plant matter) face a dilemma to distinguish essential olfactory cues from a huge range of olfactory cues in the atmosphere (Fig. 1.1). Essential olfactory cues include those from the host habitat, the host plant and from the host (pheromones, faeces, saliva, or the host itself). These olfactory cues, however, differ in their composition, concentration, working distance and reliability (Bruce and Pickett, 2011).

In their reliability-detectability hypothesis, Vet and Dicke (Vet et al., 1991; Vet and Dicke, 1992) argued that olfactory cues released by the host may be reliable cues for natural enemies, but are characterised by a low detectability due to the herbivore needing to remain inconspicious, as well as the herbivore's smaller biomass. Olfactory cues from the plant may contain less specific information; however, they are released in a vast amount and can be detected over a greater range. These major constraints have led to adaptated strategies in the parasitoid to improve host location. Hence, over time plants have evolved different mechanisms (inducible and constitutive) as a defence against herbivores, while parasitoids have evolved strategies to distinguish between hosts (Van Valen, 1973; Allison and Hare, 2009), a situation often referred to as an "evolutionary arms race" (Ehrlich and Raven, 1964; Loxdale et al., 2011).

Parasitoids can use an "infochemical detour", where they orient towards host pheromones (Vet et al., 1991; Vet and Dicke, 1992). These are detectable over a greater range and give specific information. Another strategy is the detection of "herbivour induced synomones" such as volatiles emitted upon herbivore attack. As a third strategy parastoids learn to associate highly detectable cues with reliable ones, which will be explained in detail in section 1.2.

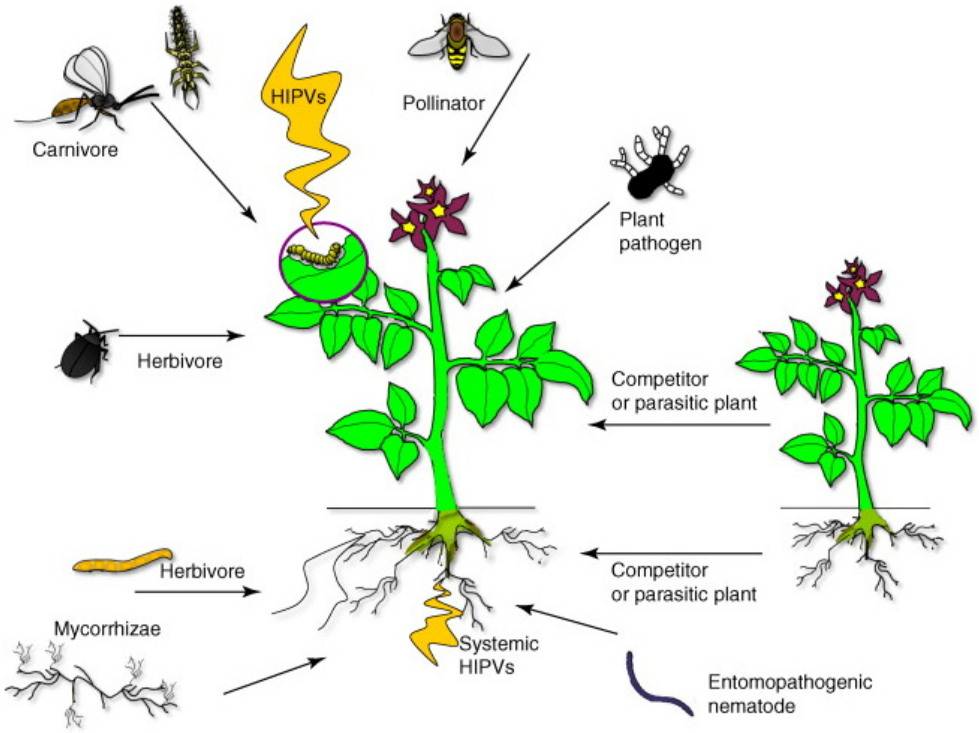

Fig. 1.1 Herbivore-induced plant volatile (HIPV) emission of a plant during herbivore attack. A plant can be damaged from below and above ground. While many laboratory studies recreate simplified scenarios involving one plant, one herbivore and one parasitoid, situations in the natural environment are usually more complex. There, a plant can be attacked by several herbivores, which in turn can attract a range of natural enemies. Taken from (Dicke and Baldwin, 2010).

1.1.1. Herbivore-induced plant volatiles

Plants emit volatile organic compounds (VOCs) as a herbivore-induced defence, defending the plant directly by inducing a repellent or a toxic effect (Kessler and Baldwin, 2001), or indirectly, by attracting natural enemies of the herbivore, for example parasitoids (Halitschke et al., 2008). Upon attack or egg deposition by herbivores, VOCs are either specifically induced as a herbivore response (qualitative changes), or emitted in a higher concentration (quantitative changes), or in different ratios (Hilker and Meiners, 2006; Dicke and Baldwin, 2010; Hilker and Meiners, 2010; Bruce and Pickett, 2011). Plant volatile blends can be very complex and consist of hundreds of compounds and contain information about the plant species (Mumm and Hilker, 2005), herbivore species (Shiojiri et al., 2010) and even the developmental stage of the herbivore (Takabayashi et al., 1995; Yoneya et al., 2009). Different biosynthesis pathways can be involved (Fig. 1.2), such as the octadecanoid pathway with the phytohormone jasmonic acid, the shikimic acid pathway with the phytohormone salicylic acid, and the ethylene pathway (Bruinsma et al., 2009; Bruce and Pickett, 2011).

Green leaf volatiles

Although mechanical damage can cause volatile emission, it differs from herbivore-induced volatile emission, due to the presence of bioactive compounds in insect saliva (Gatehouse, 2002). Constitutive compounds, including green leaf volatiles (GLVs) are usually fatty acid derived including C6 alcohols, esters, and aldehydes such as *cis*-3-hexanal, hexanal and *cis*-3-hexen-1-ol, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexenyl acetate, and are released immediately after mechanical or herbivore damage from plant organelles (Turlings et al., 1990; Fall et al., 1999; Allmann and Baldwin, 2010). In maize they are released 30 min post herbivore attack (Turlings et al., 1998).

Herbivore-induced plant volatiles

Herbivore-induced plant volatiles (HIPVs) are usually released as a delayed response to herbivore feeding (Dicke, 2009). HIPVs include various terpenes (mono-, sesqui- and homoterpenes) and phenylpropanoids (e.g., methyl salicylate, indole) which are released in an increased amount as a result of herbivore infestation (Vuorinen et al., 2004). Most of the HIPVs appear to be common to many plant species, and among the most commonly induced terpenes are the monoterpene linalool, the sesquiterpenes (E,E)-α-farnesene and β-caryophyllene and the homoterpenes DMNT (4,8-dimethylnona-1,3,7-triene) and TMTT (4,8,12-trimethyltrideca-1,3,7,11-tetraene) (Holopainen, 2004; Mumm and Dicke, 2010). Some monoterpenes, such as linalool, are *de novo* synthesised after herbivore damage and emitted from the whole plant while sesquiterpenes are emitted from the herbivore-damaged foliage of plants (Holopainen, 2004). Terpenoids appear to affect the behaviour of a large range of insects. Linalool, for example, was shown to be either attractive (Colazza et al., 2004; Yan and Wang, 2006; Gregg et al., 2010; Schmera and Guerin, 2012) or repellent (Jongsma, 2004; Zhang et al., 2004; McCallum et al., 2011) to insects. Transgenic plants with an enhanced linalool emission can thus have a positive effect on natural enemies, such as increasing the attractiveness of a plant (Kappers et al., 2005) or increasing the repellent effect on herbivores (Yang et al., 2008; McCallum et al., 2011). Enhanced linalool emission in plants can therefore be beneficial for biological pest control.

Glucosinolates

In addition, glucosinolates, which are secondary plant metabolites for defence (e.g. found in Brassicaceae species) that are released in response to herbivore feeding are hydrolysed into toxic substances (e.g., isothiocyanates or nitriles), have recently been shown to be beneficial for parasitoids (Kos et al., 2012). It was suggested that for *C. vestalis* glucosinolates including benzyl cyanide might be important for long-range orientation, while GLVs and terpenes might provide more information about the host at a shorter range (Pinto et al., 2007b). Glucosinolates might generally form reliable synomones for parasitoids of *Brassica* herbivores to indicate host presence (Agelopoulos and Keller, 1994), although it was found that in contrast to benzyl cyanide, traces of methyl

thiocyanate, nitrile and allyl nitrile were also detected in the emission of intact plants (Pinto et al., 2007b).

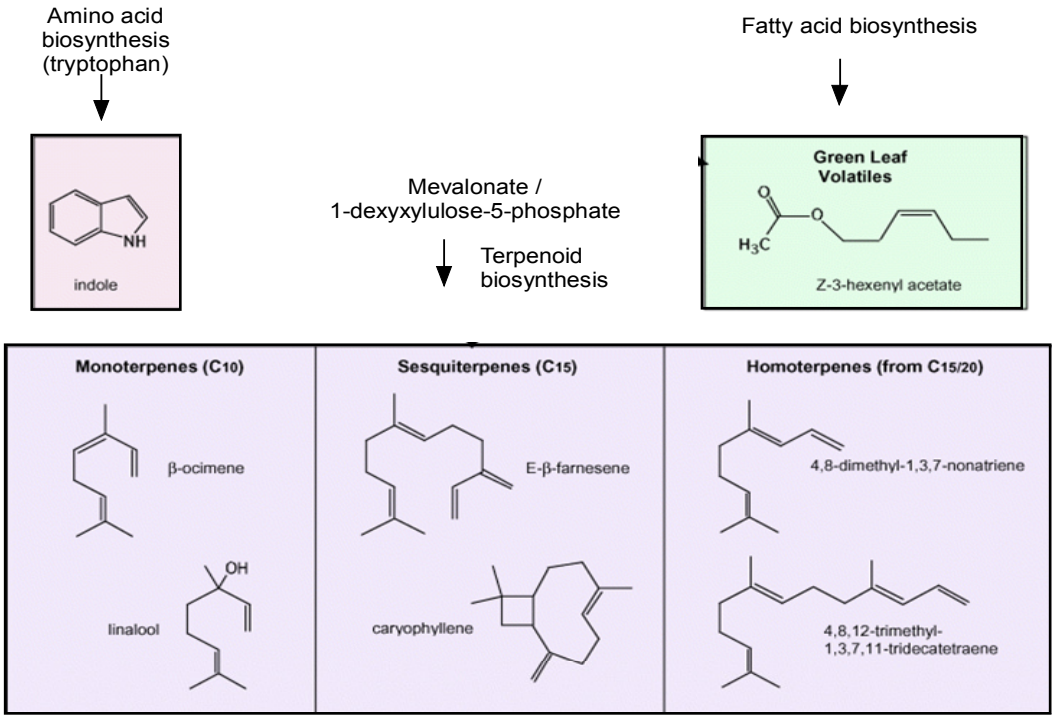

Fig. 1.2 Common plant volatiles as a response to herbivore attack. Upon herbivore infestation the plant releases certain odour compounds, which give the parasitoid specific information about the host. Two groups of volatiles can be found: green leave volatiles (GLVs), which are immediately released after mechanical or herbivore damage; and herbivore-induced plant volatiles (HIPVs), which are released at a later stage and give more specific information about the host. Modified from (Gatehouse, 2002).

1.1.2. Volatiles from undamaged plants

Many parasitoids are able to distinguish between mechanically damaged plants, host damaged plants, or undamaged plants, with host damaged plants eliciting the highest behavioural response in parasitoids (D'Alessandro and Turlings, 2005; Connor et al., 2007; Allison and Hare, 2009). For egg parasitoids, however, plant volatiles induced by host feeding are not necessarily an indicator for the presence of host eggs. Some studies have demonstrated that egg parasitoids, e.g. *Trichogamma* spp., respond to volatiles from undamaged plants

(Reddy et al., 2002). In some cases, egg parasitoids use plant volatiles induced by the oviposition of the host onto the host plant (Hilker and Meiners, 2006). Also, in many egg parasitoids, plant volatiles can induce an arrestment of the parasitoid after hatching, rather than an attraction (Fatouros et al., 2008).

1.1.3. Host cues

Host cues, such as faeces or saliva are more reliable, but less detectable over a longer range. Host pheromones, however, can be detected over longer distances and represent a very reliable cue (Vet and Dicke, 1992). Especially for egg parasitoids, sex pheromones, anti-sex pheromones and aggregation pheromones are a strong indicator for the egg stage of the host (Lewis et al., 1982; Fatouros et al., 2005; Huigens et al., 2010).

However, not only the recognition of suitable host plants or hosts is important, it is also necessary to recognise unsuitable ones (Zhang and Schlyter, 2010). Studies have demonstrated a repellent effect of non-host volatiles (NHVs) in several insect species (Nottingham et al., 1991; Mauchline et al., 2005; Zhang and Schlyter, 2010; Jactel et al., 2011), indicating, that the repellent effect and avoidance learning of NHVs are important to distinguish unsuitable habitats, hosts, or plants from suitable ones (Zhang and Schlyter, 2010).

1.1.4. The model species *Cotesia vestalis*

The parasitoid used in this study was *Cotesia vestalis* (Haliday) (former *plutellae* Kurdjumov) (Hymenoptera: Braconidae). It is a widely distributed, primary solitary larval specialist of the Diamondback Moth (DBM), *Plutella xylostella* (L.) (Lepidoptera: *Plutellidae*) (Velasco, 1982; Verkerk and Wright, 1996). The DBM is one of the most serious crop pests of cruciferous plants (plants of the family Brassicaceae) throughout the world (Talekar and Shelton, 1993; Sarfraz et al., 2005). The DBM is attacked in various developmental stages by parasitoids. Larval parasitoids are the most efficient and predominant ones, in particular *Diadegma insulare* (Cresson) and *C. vestalis*. *C. vestalis* has a

considerable potential as a biological control agent (Alizadeh et al., 2011), because it is the only larval parasitoid which can survive in lowland areas of the tropics and subtropics (Talekar and Shelton, 1993; Verkerk and Wright, 1996).

C. vestalis parasitoids lay one egg into their host larvae (2^{nd} and 3^{rd} instars - developmental stage) to ensure complete development of the parasitoid larva (Fig. 1.3). The parasitoid larva emerges during the 4^{th} instar phase and builds a cocoon, which it leaves as a fully grown parasitoid (3-7 mm) after two to four days. Females attack their hosts during their whole adult life span (5-35 d), although higher attack rates occur during the first 2 days after hatching (Nofemela, 2004). *C. vestalis* has a relatively high temperature tolerance, but prefers temperatures between 21-33°C, with the highest parasitisation rate at 21°C (Nofemela, 2004).

Several of the nearly 1000 *Cotesia* species are not only used for biological pest control of caterpillars, but also as model organisms in studies of host-parasitoid interaction, genetics, or behavioural ecology (Michel-Salzat and Whitfield, 2004). Research has been conducted into two related *Cotesia* species, *C. glomerata*, a generalist, and *C. rubecula*, a specialist (Smid et al., 2003; Bleeker et al., 2006a; Smid et al., 2007). Both parasitoids attack *Pieris* sp. caterpillars, but show great differences in learning and memory. Similarities or differences to each of these related *Cotesia* species will also be discussed in this study.

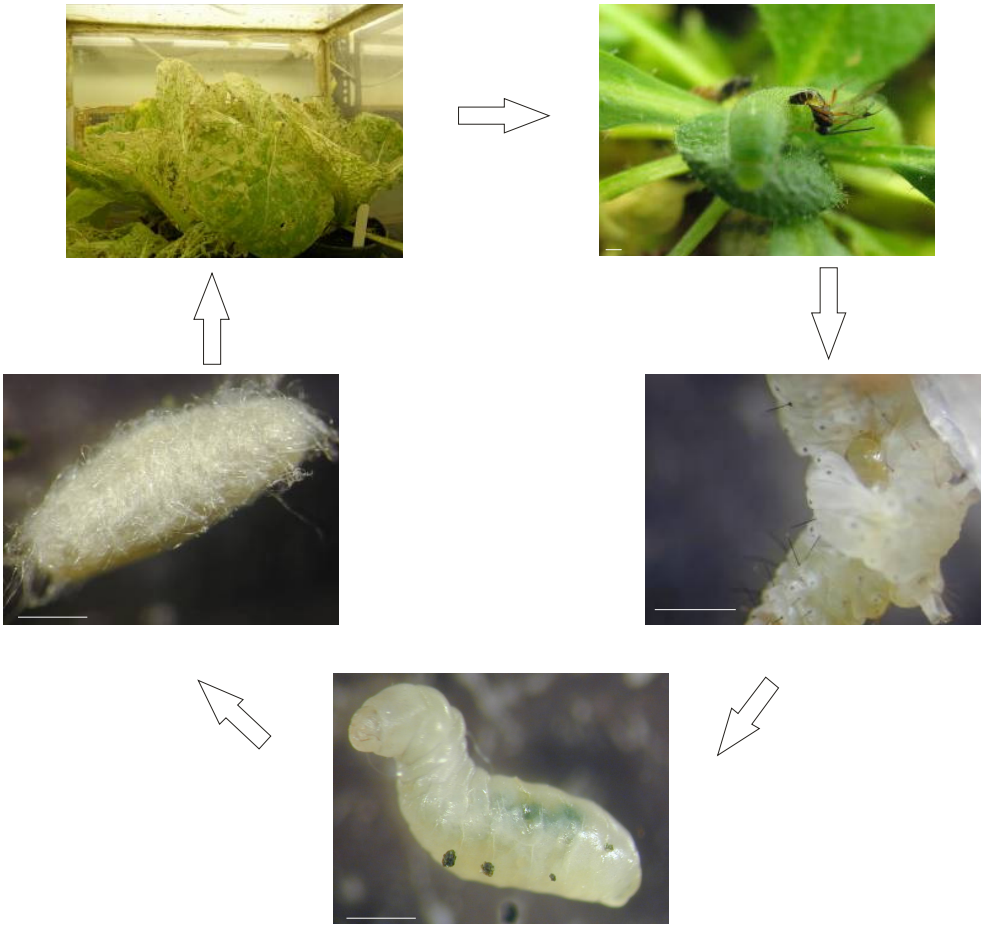

Fig. 1.3 The life cycle of *C. vestalis*. After infestation with the Diamondback Moth (DBM) the plant emits specific plant volatiles which are attractive to *C. vestalis*. The parasitoid inserts its egg via oviposition into the caterpillar, where the larva develops. To emerge from the host the parasitoid eats its way through the caterpillar, leading to its death. The parasitoid larva forms a cocoon, from which the adult emerges after 4-6 days. Scale bar= 500 µm. Picture of *C. vestalis* ovipositing DBM taken from (Center for Ecological Research, 2011)

1.2. Olfactory released behaviour and learning

While many of the behaviours in an animal are innate, other behavioural responses can be learned, or innate behaviour can be improved through learning (Lorenz, 1981). Animals can learn to associate and memorise stimuli from their environment, but not all forms of learning are similar. Depending on the nature and complexity of the information acquired, several learning phenomena can be distinguished.

1.2.1. Learning through conditioning

Habituation (a decrease of a response to a repeated stimulus that has no explicit outcome) and sensitisation (an increase of a response after stimulation with a relevant stimulus) are the simplest forms of learning (Kandel and Schwartz, 1982; Rankin et al., 2009; Thompson, 2009). Both can be described as non-associative learning, in which an animal changes its response as a consequence of its experience with a single stimulus.

Associative learning, on the other hand, implies the establishment of a new link between stimuli, or between a given action and its outcome. This comprises classical (Pavlovian) and operant conditioning, respectively. Classical (Pavlovian) conditioning, based on Ivan Petrovich Pavlov's work (Pavlov, 1927; Pavlov, 2010), describes a contingency between two stimuli; the unconditioned stimulus (US); and the conditioned stimulus (CS). Pavlov (Pavlov, 1927; Pavlov, 2010) showed this with an example of saliva production of a dog during food consumption. When presented with food, the dog shows an increase in saliva production (US), a spontaneous response to a biologically relevant stimulus. If the presence of food is then paired with an irrelevant stimulus such as the sound of a bell (CS), the dog will learn to associate the bell (CS) with food. As a result, ringing the bell alone will elicit increased saliva production. The previously neutral stimulus (CS) has become capable of evoking the same response as the unconditioned stimulus (US) initially did.

This type of learning is very important for survival in animals. Depending on the nature of the US (appetitive-positive reinforcement or aversive-negative

reinforcement) animals will learn to seek or avoid certain stimuli. This learning paradigm is widely used in research investigating principles of learning and memory in mammals as well as invertebrates (Kuwabara, 1957; Gormezano et al., 1962; Bitterman et al., 1983; Tully and Quinn, 1985; Colomb and Brembs, 2010).

1.2.2. Synaptic plasticity underlying learning and memory

Many invertebrates are capable of processing information in a flexible way and exhibit adaptive behaviour that is responsible for their evolutionary and ecological success (Menzel, 1999). Experiments with fruit flies, nematodes and snails have revealed specific pathways that are involved in learning, memory, and synaptic plasticity, short- and long-term changes in synaptic connections (Tully and Quinn, 1985; Grünbaum and Müller, 1998; Kandel, 2001; Comas and Petit, 2004; Amano and Maruyama, 2011). Invertebrates with a fully sequenced genome, such as *Drosophila* bring the advantage of a gene collection of mutant flies. This can be used in the research of learning and memory to study the consequences of mutations (Tully and Quinn, 1985; Kahsai and Zars, 2011).

Learning leads to memory, which is a dynamic and self-organizing process of information storage which requires time to develop, and proceeds through different phases (Menzel, 1999). It is not caused by an increase in neurons, but by an increase in synaptic strength (Cajal, 1894) and the formation of new connections (Hebb, 1949) between neurons. These connections can change in strength in response and either increase (potentiation) or decrease (depression), also known as synaptic plasticity. Temporary changes are referred to as short-term memory (STM) (short-term potentiation), while persistent changes lead to long-term memory formation (LTM) (long-term potentiation) (Bliss and Lømo, 1973; Kandel, 2001; Lømo, 2003).

Synaptic plasticity is an important process during learning and memory formation. Two mechanisms for Hebbian (or associative) plasticity involve the NMDA (*N*-methyl-D-aspartate) and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors, which are ionotropic glutamate receptors (Lamprecht and LeDoux, 2004). During synaptic transmission, the release of a neurotransmitter is triggered by an action potential in the presynaptic cell. The depolarisation of the presynaptic cell leads to an influx of Ca^{2+} through voltage-

dependent calcium selective ion channels. Ca^{2+} ions bind to proteins and in turn lead to transmitter release into the synaptic gap (Fatt and Katz, 1953).

A pre-synaptic glutamate release activates AMPA and NMDA receptors. Na^{+} ions enter through AMPA receptors into the postsynaptic cell, resulting in a depolarisation of the postsynaptic cell (Fig. 1.4) (Shepherd and Huganir, 2007). With pre-synaptic glutamate release and coinciding depolarisation of the post-synaptic cell, Mg^{2+} ions initially blocking the NMDA ion channels are displaced, allowing Ca^{2+} to enter the cell. This consequently improves the sensitivity of the post-synaptic cell to the neurotransmitter through initiating a cascade of processes leading to morphological changes at the synapses (Malenka and Bear, 2004).

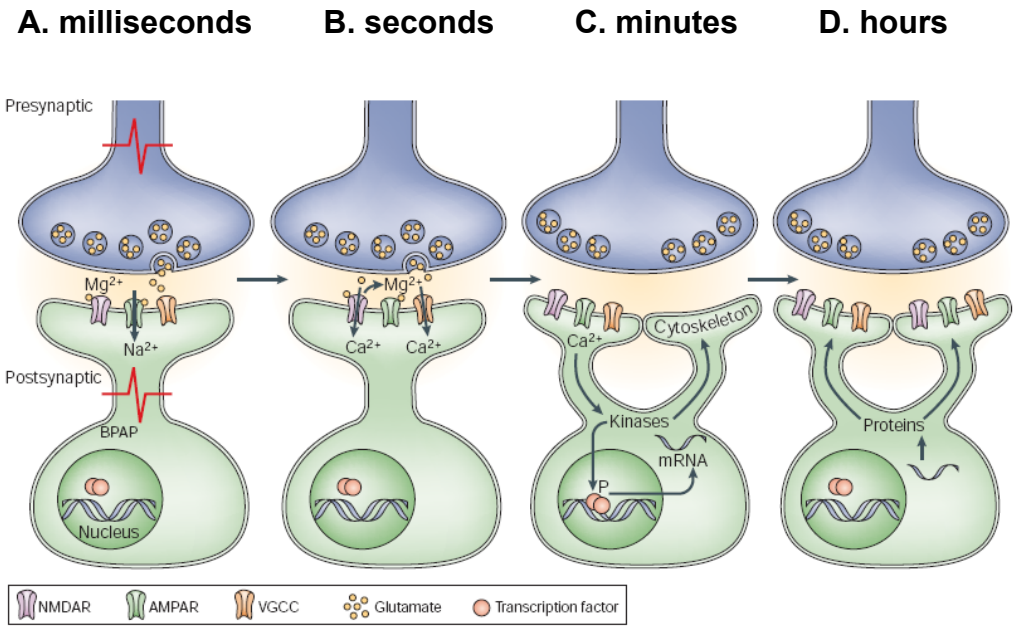

Fig. 1.4 Molecular mechanism involved in synaptic plasticity. A) Glutamate release from presynaptic neurons leads to activation of AMPAR (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor) and to the depolarisation of the postsynaptic membrane. **B)** The depolarisation leads to a displacement of Mg^{2+} on the NMDA (N-methyl-D-aspartate) channels, causing an influx of Ca^{2+}. **C)** Kinases activated by Ca^{2+} modulate the activity of their substrates, which lead to morphological changes at the synapse, or induced RNA transcription. **D)** Synaptic changes are stabilised through protein synthesis. Taken from (Lamprecht and LeDoux, 2004).

1.2.3. Memory dynamics

At least two different phases of memory formation can be distinguished in both vertebrate and invertebrate models (Table 1.1) (Menzel, 1999): a short-term memory (STM), induced by local synaptic changes and by a single conditioning trial, and a long-term memory (LTM), mediated by the activation of transcription factors to translate new proteins, which modify synaptic function. Intermediate forms of memory (MTM) can be found in several animal models, such as, for example the molluscs *Aplysia* or *Lymnea*, or vertebrates, and can require protein of existing proteins but not RNA synthesis of new proteins (Sutton et al., 2001; Sutton et al., 2002; Sangha et al., 2003; Grimes et al., 2011). In *Drosophila* memory phases coexist in parallel (Tully et al., 1990; Tully et al., 1994; Margulies et al., 2005). These consist of STM and MTM (lasting less than 24 h), which are formed in a serial order. Anaesthesia-resistant memory (ARM) (lasting 4 d) and LTM (lasting 7 d), however, are developed in parallel.

In ants, memory phases have been found to be similar to those found in bees (Josens et al., 2009; Guerrieri et al., 2011). In bees (Menzel, 1999), however, two phases of LTM were discovered. Early LTM (eLTM) depends on the translation of already existing mRNA and is induced by massed training), while late LTM (lLTM) depends on the transcription and translation of new proteins, and is induced by spaced conditioning (Schwärzel and Müller, 2006).

Table 1.1 Memory phases in different species. ARM= anaesthesia resistant memory, eLTM= early long-term memory, lLTM= late long-term memory, MTM= intermediate memory STM= short-term memory, ? = so far unknown

Species	STM	ARM	MTM	eLTM	lLTM	Reference
Bee	20 min	-	1 h - 12 h	1 d - 2 d	> 3 d	Menzel, 1999
Drosophila	1 h	1 d - 4 d	5 h	5 h - 7 d		Tully et al., 1994; Margulies et al., 2005
Ants	> 5 min	?	1 h - 12 h	1 d - 2 d?	> 3 d	Josens et al., 2009; Guerrieri et al., 2011
Lymnea	a few min		3 h	> 6 h		Sangha et al., 2003
Aplysia	30 min	-	1 h - 3 h	> 1 d		Sutton et al., 2001
Rat	1 - 3 h	-	5 h	> 1 d		Grimes etal., 2011

Protein synthesis is the process in which cells build new proteins, beginning with amino acid synthesis and the transcription of nuclear DNA into messenger RNA, followed by the translation into new proteins. Protein synthesis is essential for LTM formation and occurs within the cell body (transcription) and cytoplasm (translation) and concerns neurotransmitters, receptors and new synaptic pathways (Kandel, 2006). Synaptic responses such as LTP or long-term facilitation (LTF), which are long lasting changes in the strength of synaptic connections and "cellular correlates" of learning and memory, have also been shown to be dependent on protein synthesis (Lynch, 2004). Only LTM depends on the transcription and the translation of new proteins, while MTM depends on the translation of existing proteins (Sutton et al., 2001; Sutton et al., 2002; Sangha et al., 2003; Grimes et al., 2011).

Transcription consists of three phases (Alberini, 2009): initiation, in which the enzyme RNA polymerase binds to the double stranded DNA; elongation, in which nucleotides are added to the 3' end of the chain, and termination, which consist of the release of the RNA polymerase. Several of the transcription factors, proteins with which RNA polymerase needs to interact to function, are involved in synaptic plasticity and memory formation, e.g., CREB (cAMP response element-binding protein). LTM can thus also be inhibited by disrupting CREB (Sakamoto et al., 2011; Chen et al., 2012). Protein synthesis occurs in multiple waves, and several time windows for transcription and translation have been shown to exist, usually around the time of training and a few hours later (Igaz et al., 2002; Epstein et al., 2003; Bekinschtein et al., 2007).

1.2.4. Learning and memory in parasitoids

Parasitoids are thought to have developed associative learning to improve host finding strategies by linking reliable cues with detectable ones (Vet and Dicke, 1992). Host derived stimuli may be recognized innately, but during their life time parasitoids will associate them with surrounding stimuli (Turlings et al., 1993; Peñaflor et al., 2011).

Behavioural experiments have revealed that learning capacities of parasitoids are not necessarily restricted to a certain life period. Learning is known to take place during different life periods such as a pre-imago (Cortesero

and Monge, 1994), post emergence (van Emden et al., 2008), or as an adult (Du et al., 1997). It has also been shown that associative learning can occur not only after oviposition (Vet and Groenewold, 1990; Takasu and Lewis, 2003) and host-associated chemicals (Eller et al., 1992; Meiners et al., 2003), but also after contact experience with food (Takasu and Lewis, 1996; Wäckers et al., 2002).

Whereas some studies (Collatz et al., 2006) suggested that oviposition is not essential for associative learning, it has, however, been demonstrated that learning with an oviposition experience results in higher learning rates (Vet and Groenewold, 1990). Moreover, parasitoids are also able to learn and associate odours which they would not encounter in their natural environment (DeJong and Kaiser, 1991; Olson et al., 2003).

Parasitoids show a strong variability in their host searching behaviour/preference, indicating that strong differences in the acquisition and storage of memory (Table 1.2) are related to their ecological strategy (Bleeker et al., 2006a; Smid et al., 2007; Hoedjes et al., 2011). Generalists will encounter a variety of host related olfactory cues. Hence, an alteration of an innate response will benefit their reproductive success (Simons et al., 1992) and they appear to have a more flexible learning system than specialists (Bleeker et al., 2006a). Specialists on the other hand only need to improve their innate response to host related odour cues, as they will encounter less variability in host cues and HIPVs. In many specialists, memory consolidation seems to be slower and less flexible, and memory seems shorter than in generalists (Cortesero et al., 1995; Fujiwara et al., 2000; Fukushima et al., 2001; Kaiser et al., 2003).

Smid and colleagues (Smid et al., 2007) examined memory formation in two related *Cotesia* species. LTM formation differed in *C. glomerata* and *C. rubecula* depending on the number of conditioning trials. Protein synthesis dependent memory is already formed after 1 CT in *C. glomerata*, whereas *C. rubecula* needs three spaced trials. Memory consolidation is complete within 4 h in *C. glomerata* whereas it takes 2-3 d in *C. rubecula. C. glomerata's* memory trace is exclusively protein synthesis-dependent while *C. rubecula* has a protein synthesis-independent memory trace (ARM), which lasts about 48 h after spaced conditioning. In both wasps LTM is transcription and translation dependent. A molecular study (Van den Berg et al., 2010), however, showed that CREB expression in *C. glomerata* and *C. rubecula* was, with the exception of two low

abundant transcripts (which was higher in *C. glomerata*), similar in these two wasps.

Table 1.2 Memory duration in parasitoid species. G= generalist, HS= host specialisation, No. exp.= number of experience (conditioning trials) and type of conditioning, spaced= spaced conditioning, massed= massed conditioning.

Parasitoid	HS	No. exp.	Memory	Reference
Eupelmus vuilletti	G	massed	7d	Cortesero et al., 1995
Cotesia glomerata	G	1 or 3 spaced/massed	5 d	Smid et al., 2007
Lariophagus distinguendus	G	massed	6 d	Mueller et al., 2006
Cotesia marginiventris	G	1	> 2 d	Martin and Lewis unp.ex Turlings et al., 1993
Leptopilina heterotoma	G	1	≤ 2 d	Poolman-Simons et al., 1992
Nasonia vitripennis	G	1	4 d	Schurmann et al., 2009
Cotesia vestalis	S	massed	< 1 h	Chapter 2
Cotesia congregata	S	4 h exposure	7d	Kester and Barbosa, 1991
Cotesia kariyai	S	1	< 1 d	Fujiwara et al., 2000; Fukushima et al., 2001
Cotesia rubecula	S	3 spaced	5 d	Smid et al., 2007
Microplitis croceipes	S	1	1 d	Takasu and Lewis, 2003
Leptopolina boulardi	S	1	< 1 d	Kaiser and DeJong, 1993

1.3. The olfactory pathway

Animals have developed a highly sensitive system to detect and process olfactory information which enables them to search for food and mates, to identify predators and hosts, and to communicate within their species (Ache and Young, 2005). With their highly sensitive olfactory system, parasitoids can distinguish volatiles emitted by the plant upon herbivore attack from irrelevant "background" odours (De Bruyne and Baker, 2008).

1.3.1. Peripheral olfactory perception

On the antennae of insects, a variety of olfactory, gustatory, mechano-sensory and thermo-sensory sensilla can be found (Keil, 1999). The olfactory chemoreceptors are located in the antennae in innervated hair structures, the sensilla. Olfactory wall pore sensilla differ in their morphology and include sensilla trichodea, basiconica, coeloconica and placodea. A smaller number of olfactory sensilla can also be found on the maxillary and labial palpi, tarsi and on the ovipositor (Obonyo et al., 2011; Zhang et al., 2012). ORNs are embedded in the sensilla (Keil, 1999) (Fig. 1.5). Usually, an insect olfactory sensillum can consist of one or several bipolar ORNs (Chapman, 1982), but in bees, one sensillum placodeum is innervated by up to 30 ORNs (Esslen and Kaissling, 1976) and in locusts and sphecid wasps, up to 50 and 140 ORNs, respectively, innervate one sensillum basiconicum (Martini, 1986; Ochieng et al., 1998).

The usually bipolar insect ORN has one olfactory cilium ("outer dendritic segment") that is, depending on type of sensillum, either unbranched (e.g., sensillum trichodeum) or multiply branched (e.g., sensillum basiconicum) (Keil, 1999). Below the cuticle, ORN axons collect into bundles which unite in the antennal nerve.

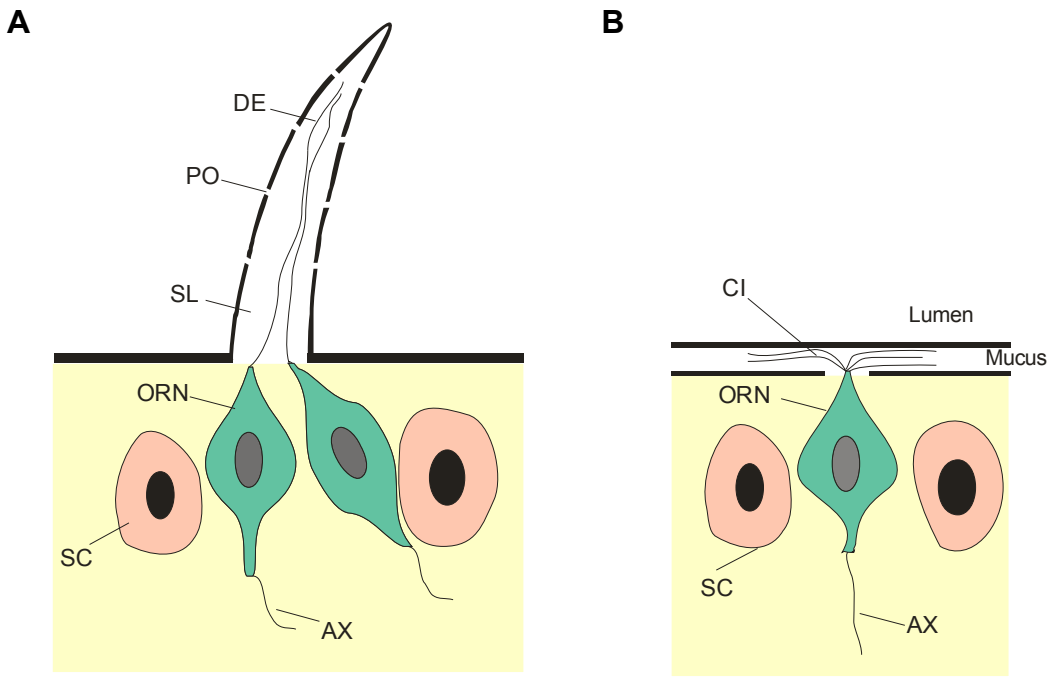

Fig. 1.5 Schematic representation of the general structure of ORNs in insects and vertebrates. A) ORNs are the first afferent connection to the antennal lobe. The sensillum located on the antennae consists of one or several ORNs. Below the cuticle ORN axons collect to bundles which merge in the antennal nerve which connects to the AL. The schematic drawing represents a single walled olfactory s. trichodea, which is usually innervated by one to three ORNs and numerous wall pores. **B)** In vertebrates, cilia of the ORN reach into the mucus layer. ORN axons bundle into the olfactory nerve which connects to the olfactory bulb. AL= Antennal lobe, AX= Axon, CI= Cilia, DE= Dendrite, ORN= Olfactory receptor neuron, PO= Pores, SC= Support cell, SL= Sensillum lymph. Adapted from (Eckert et al., 2000; Sanchez-Gracia et al., 2009).

Odour molecules diffuse through the pores into the sensillum lymph to water-soluble odorant-binding proteins (OBPs), which are thought to transport the odour molecules to the corresponding olfactory receptor (OR) on the olfactory receptor neurons (ORNs) (Galizia and Sachse, 2010). As the odour molecules pass through the pores into the cuticle, and through the sensillum lymph, they experience a range of transformation cascades (Lerner et al., 1990), while they transform from a chemical molecule into an electric signal.

ORs in insects have 7 trans-membrane domains and have long been assumed to be G-protein coupled receptors (GPCRs), that activate G-protein based signalling cascades (Buck and Axel, 1991; Axel, 2005; Buck, 2005), but recent studies have shown that they can act as ionotropic receptors (Sato et al., 2008; Wicher et al., 2008; Benton et al., 2009). Although ORs vary between species,

similarities have been observed between vertebrates. Invertebrates, on the other hand, have similar, but independently (non-homologous) expanded families of GPCRs (Ache and Young, 2005). The Or83b, for example, is conserved between insect species, and orthologes have been found in *Drosophila* (DmOr83b) (Vosshall, 2003), *Anopheles* (AgOr7) (Hill et al., 2002), moths (*HivrR2*) (Krieger et al., 2004) and the honeybee (AmOr2) (Robertson and Wanner, 2006).

GPCRs activate second messengers such as cAMP or inositol 1,4,5-trisphosphate (IP3), which opens ligand gated ion-channels. The influx of Na^+ into the cell leads to a depolarisation of the membrane, and if a certain threshold is reached, to the generation of action potentials (Galizia and Sachse, 2010). The ORNs thus respond to the chemical information with a receptor potential that generates action potentials. These action potentials contain information about odour quantity and quality, as well as the time course, and are sent to the first integration centre in the brain, the antennal lobe (AL).

The number of ORNs and OR set the limit to the range of odours that can be detected (De Bruyne and Baker, 2008). Each receptor is specific to a number of odour molecules (Keller and Vosshall, 2003) and ORNs express one or a small number of ORs (Su et al., 2009). ORNs respond to one or a few chemical molecules, and individual odorants activate subsets of ORs so that the identity of an odour is encoded in the activity pattern of different sets of ORN classes (Su et al., 2009). Receptors vary in their breadth of tuning and in moths, for example, a high sensitivity to a narrow range of molecules such as HIPVs, or pheromone components, has been demonstrated (Boeckh and Ernst, 1983; Rostelien et al., 2005). Often, ORNs for plant volatiles are located in the same olfactory sensillum (Stensmyr et al., 2003), which enhances the fine scale spatio-temporal resolution in the AL (Bruce et al., 2005). In *Drosophila* and mammals, axons of ORNs expressing the same receptor, converge in the same glomerulus in the antennal lobe (AL) (Mombaerts et al., 1996; Vosshall et al., 2000). Here, ORNs synapse with PNs, which transport the information into higher brain centres, such as the mushroom bodies (MB). Glomeruli are connected via LNs, which can often be inhibitory (Bargmann, 2006; Martin et al., 2011).

1.3.2. Central olfactory information processing

Once the olfactory signal is transformed into an electrical signal, it is transported via the antennal nerve into the first olfactory relay, the olfactory bulb in vertebrates, the antennal lobe (AL) in insects, and the olfactory lobe in crustaceans. The AL is a paired structure located at the front of the brain below the antennae, and is organised into functional synaptic units, the glomeruli. A key feature of the glomerular organisation of the olfactory system is that glomeruli do not receive topographically-organised input, but input from ORNs expressing the same receptor gene (Eisthen, 2000).

The number of glomeruli is known to be species dependent and influenced by evolutionary rank and it is thought to be greater in social hymenopterans (Rospars, 1988; Baier and Korsching, 1994). Contrary to vertebrates, invertebrates show a large variation in the number of glomeruli, ranging from 40 in *Drosophila* (Laissue et al., 1999), 60-70 in Lepidoptera (Rospars, 1983), 160 in honeybees (Arnold et al., 1985), to approximately 1000 in locusts (Rospars, 1983; Arnold et al., 1985; Laissue et al., 1999; Kurylas et al., 2008). Although a great deal is understood about the ecology of parasitoids, very little is known about olfactory integration, processing and pathway, and so far, only one study has investigated the anatomy of the first brain area processing olfactory information; the antennal lobe (Smid et al., 2003). They found around 188 glomeruli in the generalist parasitoid *C. glomerata* and 196 glomeruli in the specialist parasitoid *C. rubecula* (Smid et al., 2003).

Glomeruli contain synaptic connections of afferent receptor axons of the ORNs, local, often inhibitory interneurons (LNs), and projection neurons (PNs) (Fig. 1.6), and it has been shown that the volume and size of glomeruli also change with age and experience due to an increase in synaptic connections (Devaud and Masson, 1999; Hourcade et al., 2009). LNs create lateral connectivity of glomeruli, while PNs convey processed information to higher-order brain areas such as the MBs in insects and the olfactory cortex in vertebrates.

LNs are diverse in their morphology and pharmacology. Some LNs branch in all glomeruli, some in a subpopulation only, some receive input in certain glomeruli and form output in others. They can also vary in intraglomerular

arborisations, either innervating the cortex, the core, or both. They can be inhibitory or excitatory (Galizia and Rössler, 2010). A fundamental difference between vertebrates and insects is that lateral excitation is non-topographic in insects, while no such evidence has yet been found in vertebrates, and only excitation between mitral/tufted cells (M/T cells) in the same glomerulus has been observed. Furthermore, presynaptic inhibition of ORN axons is a major pathway for lateral cross talk between glomeruli in insects, while presynaptic inhibition is strictly intraglomerular in vertebrates (Wilson, 2008). Most of the PNs are uniglomerular (uPNs), branching in one glomerulus only. There are, however, multiglomerular PNs (mPNs), which are thought to be responsible for collecting combinatorial activity patterns (Galizia and Rössler, 2010).

The number of ORNs projecting into the AL depends on the species. In cockroaches and moths, for example, around 200,000 – 300,000 ORNs project into the AL (Sanes and Hildebrand, 1976; Boeckh and Selsam, 1984). In bees, the number depends on the cast. Worker bees have a much lower number (65,000) compared to drones (300,000) (Esslen and Kaissling, 1976). In vertebrates for example, 25,000 ORNs converge in one glomerulus (Allison, 1953).

The branching of the ORNs into the glomeruli is important for information coding. Only ORNs expressing the same receptor connect into the same glomerulus. In some arthropods, both uniglomerular and multiglomerular branching can be found (Schmidt and Ache, 1992). Each glomerulus receives highly convergent input from a large number of ORNs. Depending on the nature of an odour, a certain number of ORNs from across the antenna are active and converge upon and synapse with a certain number of interneurons.

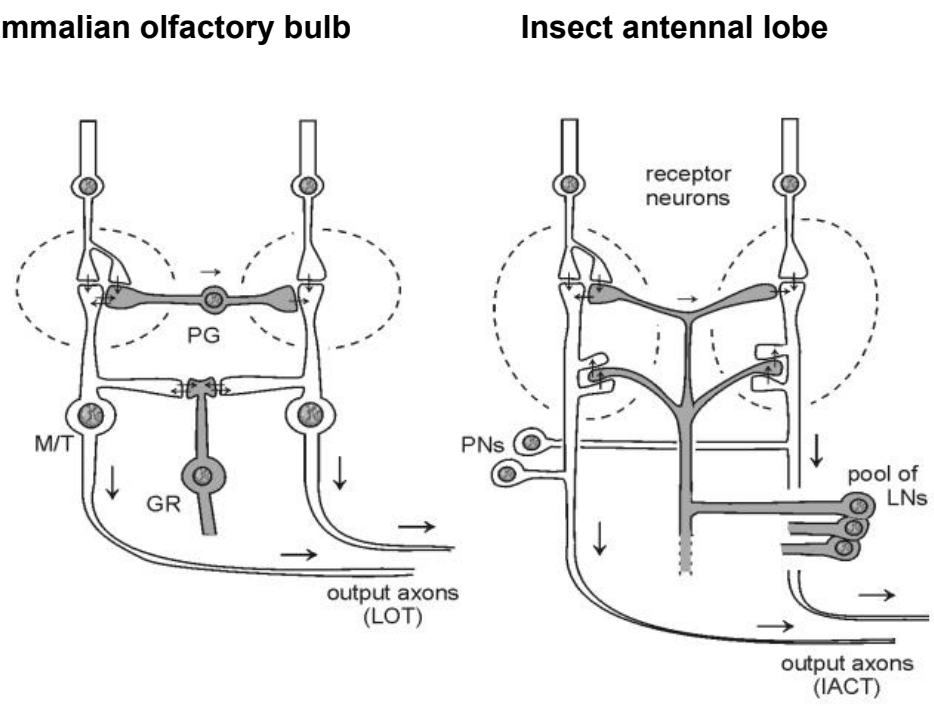

Fig. 1.6 Schematic view of the primary olfactory processing centre in vertebrates and insects. In the olfactory bulb, ORNs synapse with M/T and periglomerular (PG) cells. M/T cells further connect with granular cells (GR). In the AL a similar organisation is found, where ORNs synapse with PNs, and LNs. Taken from (Ache and Young, 2005).

1.3.3. Selective information coding in the antennal lobe

In addition to an analogous structure of the olfactory system, similarities in odour processing in the AL and OB have been found among the animal kingdom. As previously mentioned, spatially distributed ORNs expressing the same OR converge on one or a small number of glomeruli. This way, input from spatially separated ORNs is enhanced, which in turn increases the sensitivity of the system. This suggests that functionally similar input must be a key to how olfactory information is processed. Indeed, glomerular activity patterns have been found across the animal kingdom (Friedrich and Korsching, 1997; Joerges et al., 1997; Rubin and Katz, 1999). A glomerulus may thus be innervated as part of the code for different odours, but an odour is generally coded in a specific pattern of activated glomeruli (Cinelli et al., 1995; Christensen et al., 1996; Joerges et al., 1997). These patterns are equal between individuals within a species but differ between species, suggesting that these patterns have a strong genetic basis

(Friedrich and Korsching, 1997). However, responses can still be plastic and learning can modify the intensity of the active patterns (Faber et al., 1999). Many of the ORNs are broadly tuned, and glomeruli, which respond to a certain odour, are likely to respond to other odours with similar structure, carbon chain length or category (Laska et al., 1999; Rubin and Katz, 1999; Sachse et al., 1999). In female moths, a selectivity was shown for linalool, a common plant-produced volatile, which elicits a strong response in specific enhanced glomeruli, the large female glomeruli (LFGs) (King et al., 2000).

Furthermore, pheromone specific pathways have been found. ORNs in male moths have been shown to be tuned to female sex pheromones (Berg et al., 1998) and pheromone sensitive ORNs often branch widely within the macroglomerular complex (MGC) (Hansson et al., 1992), whereas ORNs responding to plant related odours connect within ordinary glomeruli (Boeckh et al., 1984; Christensen and Hildebrand, 1987b). This coding system is called "combinatorial labelled line system" (Fig. 1.7 B), where there is an anatomical as well as functional separation in the AL (Galizia and Rössler, 2010).

The macroglomerular complex found in moths usually consists of 2-3 enlarged glomeruli (Christensen and Hildebrand, 1987b; Hansson et al., 1991b) situated at the entrance of the AL, which is specialised for pheromone processing (Boeckh and Boeckh, 1979; Matsumoto and Hildebrand, 1981). Pheromone processing in moths has been intensively studied (Haupt et al., 2010; Martin and Hildebrand, 2010; Belmabrouk et al., 2011). It was shown that major pheromone components such as (E,Z)-10,12-hexadecadienol (bombykol) triggers mating behaviour in silkmoths (Kaissling et al., 1978). The antennae of male silkmoths has 17,000 sensilla trichodea (Steinbrecht, 1973), each containing two types of ORNs, which are sensitive to one of the two components (Kaissling et al., 1978).

Due to their shape, the three subdivisions of the MGC are often referred to as (cumulus, toroid, and horseshoe) (Kanzaki et al., 2003). The pheromone responsiveness of MGC-PNs correlates with their dendritic arborisations in the subdivisions of the MGC (Kanzaki et al., 2003). Macroglomeruli have also been found in other insects, such as ants, bees or cockroaches (Prillinger, 1981; Arnold et al., 1985; Kleineidam et al., 2005).

Similarities in the olfactory system between invertebrates and vertebrates can be found from the detection of odours by G-protein-coupled receptors (GPCRs) and olfactory receptor proteins (ORPs) with a specific affinity for an odour, to the convergence of olfactory receptor neurons (ORNs) into synaptic contacts located in the glomeruli, and central processing of olfactory formation in the glomeruli. This glomerular organisation can be found throughout the animal kingdom, which suggests that it is a successful way to integrate and process olfactory information. While glomeruli can be found in neopteran insects, they are not present in those which have secondarily lost odorant-sensitive antennae (Strausfeld et al., 1998), or insects of the division of Palaeoptera (primitive group of winged insects), such as mayflies, damselflies and dragon flies (Strausfeld and Hildebrand, 1999). On the other hand, glomeruli have been found in Onychophora (Schürmann, 1995), an ancient animal group (fossils more than 500 million years old have been found), but not in nematodes or basal hexapods such as bristletails, silverfish or firebrats (Strausfeld and Hildebrand, 1999). This suggests that the glomerular structure has evolved independently, as a functional adaptation for odour processing, either through convergent adaptations or homology, and is not due to a common ancestor of arthropods, molluscs and craniates (Strausfeld and Hildebrand, 1999; Eisthen, 2000; Ache and Young, 2005).

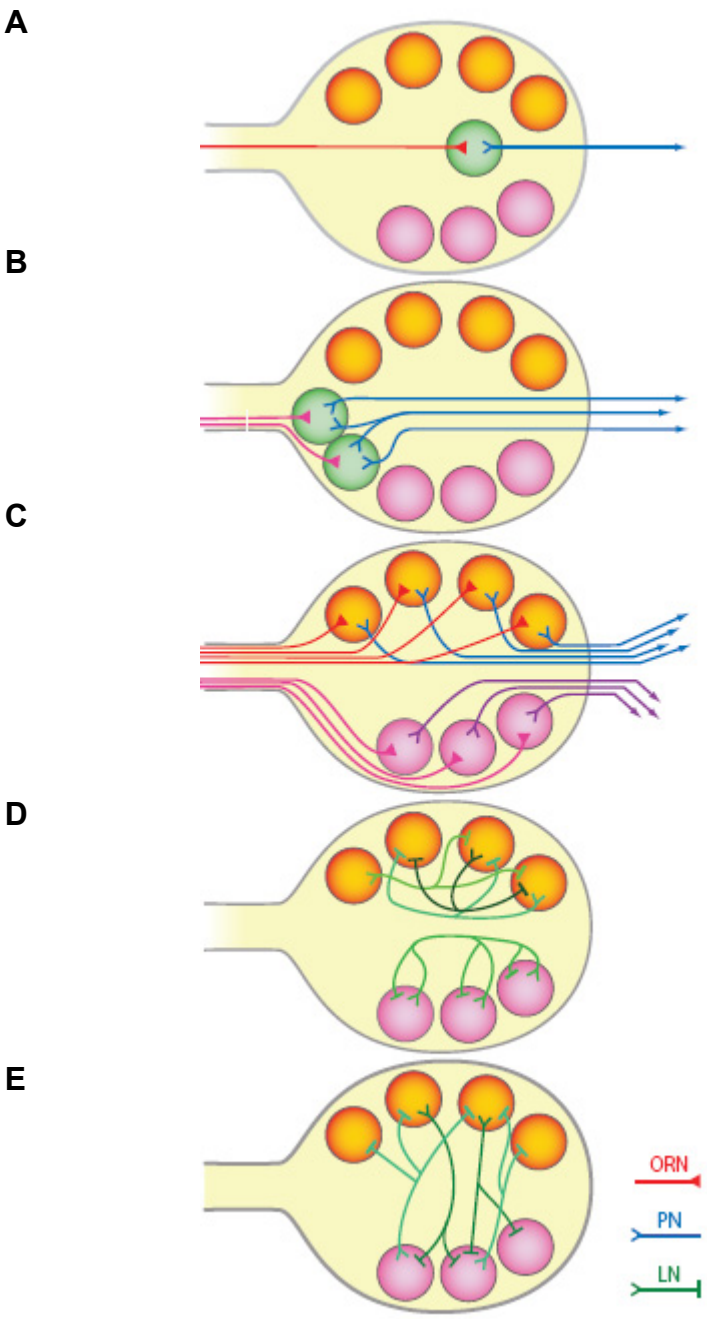

Fig. 1.7 Different parallel olfactory processing pathways. The AL is shown in yellow, different distinctive groups of glomeruli are shown in orange, and specialised glomeruli are represented in green. **A)** True labelled line system, which can be found in CO_2 detection in flies. **B)** Combinatorial labelled line system, e.g. sex pheromone detection in moths. **C)** Duplicated system, e.g. the l-APT and m-APT system in honeybees, where uPNs leave the AL in distinct output tracts to mediate different properties for odour perception. **D)** Separate coding system, where glomeruli are connected via specialised LNs, to extract odour quality information (bees, flies). **E)** Interactive separate processing system, a hypothetical network, where glomerular subsystems are connected to code for the same odours. Taken from (Galizia and Rössler, 2010).

1.4. Thesis aim, structure and outline

The overall aim of this thesis was to determine behavioural and neuronal mechanisms of host location in the specialist parasitoid *C. vestalis*. This was carried out by combining physiological, behavioural and morphological experiments to investigate how olfactory information is coded and processed in a parasitoid brain. This will contribute to the understanding of how and why inter-specific variations in learning and memory occur.

Chapter 2 Antennal and behavioural responses of the parasitoid *C. vestalis* to olfactory compounds

Electroantennogram recordings were used to investigate if there were antennal responses to odour compounds. Odour choice bioassays were applied to assess the importance of different plant volatiles. To determine the extent of learning in the parasitoid, behavioural experiments investigating memory formation were conducted. Furthermore, the attractiveness of olfactory cues and courtship behaviour in males was investigated.

Hypotheses:

- *C. vestalis* shows a stronger antennal response to linalool than to 1-nonanol
- *C. vestalis* can learn linalool but not 1-nonanol.
- *C. vestalis* does not form a long-term memory.
- *C. vestalis* males can learn HIPVs in order to locate females.

Chapter 3 Optimisation of a staining protocol for *C. vestalis* antennal lobes and glomeruli

Fluorescent staining protocols used in morphological and imaging studies were adapted for the use in a parasitoid's brain. Three fluorescent dyes and two types of microscopes, as well as different mounting and dissection procedures were tested to identify the optimum approach.

Chapter 4 The morphology of the antennal lobe of *C. vestalis*: a confocal microscopy study

Brain morphology was determined by the size, volume and number of glomeruli using staining methods established in Chapter 3.

Hypotheses:

- Due to differences in their host preference and specialisation, the number and size of glomeruli of *C. vestalis* is different compared to *Cotesia glomerata* and *C. rubecula*.
- There are no gender differences in ordinary glomeruli in *C. vestalis*.
- Males possess in addition to ordinary glomeruli macroglomeruli, enlarged glomeruli for pheromone detection.
- Females possess enlarged glomeruli for the detection of HIPVs.

Chapter 5 Calcium imaging of imaging brain activity in the antennal lobe of *C. vestalis*

The aim was to identify regions glomerular activity while imaging at different depths in the antennal lobe. For calcium imaging, a method was adapted for the use in parasitoids to investigate spatio-temporal activity patterns in the AL.

Hypotheses:

- The odours elicit different activity patterns in the AL.
- These patterns are different to other insect species, but similar within individuals of *C. vestalis*.
- No gender differences in ordinary glomeruli exist.
- Learning leads to an increase in calcium and modifies the glomerular activity pattern.
- Plant odours do not elicit activity in the male MGC.

CHAPTER 2

ANTENNAL AND BEHAVIOURAL RESPONSES OF THE PARASITOID *COTESIA VESTALIS* TO VOLATILE COMPOUNDS

2.1. Introduction

Many parasitoid wasps locate their hosts via olfactory cues emitted by the plant and host. It is thought that associative learning of rewarding stimuli, amongst others, improves the ability of parasitoids to find their host (Vet and Dicke, 1992; Hoedjes et al., 2011). Parasitoids show a great variety in host specialisation, and their learning and memory abilities have been shown to vary greatly even between closely related species. These differences are thought to be caused by the parasitoid's host specialisation (Geervliet et al., 1998b; Tamo et al., 2006; Smid et al., 2007).

To understand how and at what level a parasitoid detects, selects and integrates olfactory cues for host location it is important to investigate peripheral perception at the antennae as well as behavioural responses to the stimuli. The aim of the work presented in this Chapter was to investigate antennal as well behavioural responses of *C. vestalis* to different olfactory cues to understand how *C. vestalis* uses olfactory cues for host location.

For parasitoid wasps, host location is essential for reproductive success. To find a host, parasitoids rely on chemical cues emitted from a host-infested plant, as well as on host cues, such as pheromones, host frass, silk or saliva (Webster et al., 2010; Bruce and Pickett, 2011). However, parasitoids face a "reliability-detectability" problem (Vet and Dicke, 1992), and while host cues are the most reliable indicator for host presence, they are not as effective and detectable over a long range as plant volatiles. To overcome this problem, parasitoids have evolved several strategies to detect hosts. The detection of plant volatiles released as a response to herbivore attack is one way to locate hosts. Among the compounds are green leaf volatiles (GLVs), released immediately after mechanical or herbivore damage, and herbivore-induced plant volatiles (HIPVs), released as a delayed response to herbivore feeding (Dicke et al., 2009). This information is detectable over a long distance, but is less reliable than, for example, host cues. However, parasitoids can improve their foraging strategy if they learn to associate detectable information with reliable ones. This way, HIPVs can become a more reliable indicator of host presence.

Parasitoids show a huge variation in their ability to learn, which appears to be strongly influenced by their host specialisation. Generalist species, which use a variety of hosts, usually demonstrate more flexibility in learning than a specialist species, which often displays a more rigid learning ability (Smid et al., 2007). A few studies have investigated orientation and foraging behaviour in *C. vestalis* (Liu et al., 2006; Pinto et al., 2007b; Roux et al., 2007; Pinto et al., 2008), but learning and memory in this parasitoid have been less frequently explored (Durrant, 2007). *C. vestalis* is highly specialised, and studies have shown that it selectively prefers host plants exclusively infested by its host (Shiojiri et al., 2006).

EAG studies have shown that related *Cotesia* species are able to detect many olfactory compounds (Smid et al., 2002) but not all compounds are expected to have an effect on their behaviour (Park et al., 2001; Gouinguen et al., 2005; Mumm and Dicke, 2010). The composition of volatiles contains herbivore-specific information (Turlings et al., 1990; Dicke, 1999; Ngumbi et al., 2009), which is processed differently in the brain of a parasitoid, depending on its life strategy (Geervliet et al., 1996). Indeed it has been shown that generalist parasitoids have a higher sensitivity to GLVs, whereas specialists demonstrate a

higher sensitivity to HIPVs on an antennal as well as a behavioural level (Vet and Dicke, 1992; Chen and Fadamiro, 2007).

One way to determine the impact of learning is to measure the ability of an animal to remember events (Hoedjes et al., 2011), which involves a complex cascade of biological processes (Menzel, 1999). Studies in parasitoids have so far revealed differences in the formation of LTM, depending on the degree of host specialism (Collatz et al., 2006; Smid et al., 2007). However, unlike other insect animal models such as the honeybee, or *Drosophila*, memory formation in parasitoids remains widely unexplored. Parasitoids, therefore, make a useful model organism to study species-specific adaptations of learning and memory due to ecological constraints.

The basis of neural and genetic mechanisms underlying learning ability and memory has been shown to be similar across vertebrate and invertebrate species. Different dynamic phases in memory have been distinguished: short-term memory (STM), or anaesthesia resistant memory (ARM), intermediate memory (MTM), which is not dependent on the synthesis of new proteins, and long-term memory (LTM), which requires protein synthesis (Tully et al., 1994). LTM is usually formed after multiple trials (Tully et al., 1994; Margulies et al., 2005), but it has been shown that in certain parasitoid species, one trial conditioning is sufficient to induce LTM (Collatz et al., 2006; Smid et al., 2007). Persistent molecular changes to create LTM are known to require gene expression and as a result the synthesis of new proteins (Kandel, 2001). Studies have demonstrated the necessity of protein synthesis in LTM in many animals by blocking transcription or translation of mRNA with a transcription blocker such as Actinomycin D (ACD), or translation blockers such as anisomycin (Agranoff et al., 1967; Squire and Barondes, 1970; Wüstenberg et al., 1998). ACD acts by binding to the pre-melted DNA within the transcription initiation complex and thus prevents elongation of RNA chains (Sobell, 1985).

Parasitoids use pheromones over a long range for attraction and over a short range for species recognition and courtship, although in many parasitoid species, pheromone components have not yet been identified (Eller et al., 1984; DeLury et al., 1999; Danci et al., 2006). Courtship behaviour mediated by female sex pheromones has been demonstrated in several species (Tagawa, 1977; Decker et

al., 1993; Steiner et al., 2006), including parasitoids (Keeling et al., 2004). It can be defined as a sequence of behaviour displayed by males, such as attraction, recognition, orientation, wingfanning vibration, mounting and antennation leading to copulation (Ruther et al., 2000; McClure et al., 2007) when they come into contact with females. Males initiate courtship behaviour long before antennal contact with the female, which suggests that females produce pheromones (Ruther et al., 2000; McClure et al., 2007). However, it was shown that males display courtship behaviour not only in the presence of females, but also when presented with female extracts only (Vinson, 1972; Tagawa, 1983). This has also been shown in *C. vestalis*, where female body parts or extracts elicited courtship behaviour in males (Tagawa, 1977; Tagawa, 1983). Morphological studies of the antennal lobe (AL) of moths revealed that they possess specialised enlarged glomeruli which form a macroglomerular complex (MGC) for pheromone processing (Masante-Roca et al., 2005; Jarriault et al., 2010), which have also been found in *C. vestalis* (Chapter 4).

The aim of this study was to investigate antennal as well behavioural responses of *C. vestalis* to different olfactory cues, to understand how *C. vestalis* uses olfactory cues for host location. Peripheral responses were measured using an electroantennogram and behavioural responses as well as memory dynamics of the specialist *C. vestalis* were investigated using behavioural bioassays. Both genders were used, as only a few studies have investigated the ability of males to detect and learn to recognise odours (Takasu et al., 2007; Villagra et al., 2008). A well documented HIPV (linalool) (Pinto et al., 2007a), as well as an odour cue (1-nonanol), which is not part of the HIPVs emitted from *Brassica rapae* var. *Wong bok* (Durrant, 2007) but learned in honeybees (Guerrieri et al., 2005a), were used. In addition, courtship behaviour was observed in males, to use as an indicator to determine if they use sex pheromones to locate females. This investigation was carried out because a MGC was found in males (Chapter 4), and previous studies have shown that MGC process pheromone information. Furthermore, to analyse to what degree olfactory information is learned and stored, memory dynamics were investigated in female wasps using the transcription blocker Actinomycin D. It was assumed that in a specialist parasitoid such as *C. vestalis* olfactory learning is limited to odours relevant for host location. It was further hypothesised that males use HIPVs and pheromones to locate females.

2.2. Materials and methods

2.2.1. Materials

Plants

Chinese cabbage (*Brassica rapa, Wong bok* cultivar) plants were grown in a glasshouse compartment at 24 ± 2°C with 60 ± 15% relative humidity. Seeds were sown in 0.3 l plastic pots filled with standard JS2 potting compost (Levingtons, Ipswich, Suffolk, UK) with no extra fertiliser added and grown under a L16:D8 light-dark photoperiod.

Insects

Adult *P. xylostella* were kept at a temperature of 19 ± 2°C with 60 ± 15% relative humidity at a L16:D8 photoperiod and were fed with Chinese cabbage. *C. vestalis* were kept at a temperature of 20 ± 1°C with 55 ± 5% humidity, and a L16:D8 photoperiod and were provided with honey water. For parasitisation, *C. vestalis* and *P. xylostella* larvae were kept in Perspex cages with nylon mesh covering two sides to enhance ventilation. Parasitisation was carried out up to three times a week. Second or third instar larvae were placed with female parasitoids and left for a maximum of 24 h to ensure successful oviposition behaviour of the parasitoid. Insects used in the following experiments were kept in a room free of plant volatiles to ensure that only "naïve" animals that have not previously been exposed to any host plant volatiles or the host and have not been given oviposition experience, were used. Environmental conditions in the room were maintained at 20 ± 1°C with 55 ± 5% relative humidity and a L16:D8 photoperiod. The photo phase was between 0600 – 2200 h.

Odours

For the experiments presented in Chapter 2 (and Chapter 5), linalool and 1-nonanol (1 mg/ml in hexane, Sigma-Aldrich) were used as a conditioned odour stimulus to determine whether *C. vestalis* can perceive and learn these individual chemicals. In a previous study (Durrant, 2007) with *C. vestalis*, (E)-β-caryophyllene and (Z)-3-hexenyl acetate, two highly emitted volatiles during

caterpillar infestation, have already been used as conditioned odours. Therefore, in the current study, linalool, a common attractant in plant interactions and by parasitoids well perceived HIPV was used in this study (Vuorinen et al., 2004; Pinto et al., 2007a; Unsicker et al., 2009; Egigu et al., 2010). Linalool is also frequently used in imaging studies in moths (Saveer et al., 2012). 1-nonanol occurs naturally in orange oil and grapefruit and was shown not to be part of the HIPVs emitted by *Brassica rapa* var. *Wong bok* upon attack with DBM (Durrant, 2007). Therefore, it should be a "novel" chemical for *C. vestalis* not associated with plant defence in the tritrophic system used in the present study. However, 1-nonanol is an odour well learned by bees, and therefore often used in behavioural experiments with bees (Guerrieri et al., 2005a). However, due to time restrictions, only one repeat was conducted for α-farnesene and limonene in females only. These HIPVs were therefore excluded from data analysis (Appendix 1).

For conditioning and bioassay 20 µl of the odour source was micropipetted onto a filter paper (3 cm^2) and placed into a clear Polystyrene plastic container (diameter: 8.65 cm, height: 4.5 cm) (ODOUR ONLY and HP conditioning), or onto a petridish (FOOD conditioning). For the bioassay the filter paper was placed near the orifice of each side of the arm of the Y-tube olfactometer (bioassay). In the bioassay 20 µl hexane was used in the second arm as the control odour.

2.2.2. Antennal responses of *Cotesia vestalis* to different odour compounds measured by EAG

Electrophysiology

To measure antennal responses of *C. vestalis* to olfactory cues, electroantennogram (EAG) recordings were conducted at Rothamsted Research (UK) under the supervision of Christine Woodcock. Upon interaction between a chemical compound and an olfactory receptor a change of voltage across the cell membrane results in depolarisation. The intensity of the receptor potential determines the frequency of action potentials travelling to the nervous system (Schneider, 1957). The summation of receptor potentials from various sensilla is measured with an electroantennogram (EAG) (Schneider, 1957). For the EAG, Ag-AgCl glass electrodes were filled with saline solution (composition as in Maddrell (Maddrell, 1969) but without glucose): NaCl (7.55 g/l), KCl (0.64 g/l),

CaCl (dihydrate) (0.22 g/l), MgCl, (1.73 g/l), $NaHCO_3$ (0.86 g/l), Na_3PO_4 (0.61 g/l). Male and female parasitoids were chilled for 1 min and the head excised. The indifferent electrode was placed within the head capsule, and the tips of both antennae were removed to ensure a good contact prior to being placed within the recording electrode (Suckling et al., 1996). The signals were passed through a high impedance amplifier (UN-06, Syntech, The Netherlands) and analysed using a customised software package (Syntech).

The stimulus (2 s duration) was delivered into a purified airstream (1 l/min) flowing continuously over the preparation, using a stimulus controller (Syntech CS-02). Samples (10 µl) of the standard solutions of test compounds (1 mg/ml and 5 mg/ml in hexane) were applied to filter paper strips and the solvent allowed to evaporate (30 s) before the strip was placed in the cartridge. The control stimulus was hexane (10 µl). Fresh cartridges were prepared for each stimulation and five replicates were conducted for each odour. The odour delivery system, which employed a filter paper strip in a disposable Pasteur pipette cartridge, has been described previously (Wadhams et al., 1982). Three compounds, decanal, 1-nonanol and linalool, were tested against both females and males, five replicates of each. To compensate for the naturally occurring loss of response during the course of the measurements, the antennal response to hexane (control) was measured before and after every run-through. The results were expressed as a percentage of the artificial 1 mV signal (i.e. 1 mV = 100%). Significant differences between test solutions and the hexane control were calculated using a Wilcoxon test. For the calculations of significance to the control the average of hexane stimulations before and after the treatment was taken. The different volatiles were not statistically compared due to differences in volatility and the relationship between the dose of a compound applied to filter paper and the amount of compound released.

2.2.3. Behavioural response of *Cotesia vestalis* to odour compounds

Conditioning

An attraction to green leaf volatiles (GLVs) and host-induced plant volatiles (HIPVs) for *C. vestalis* females has been previously shown (Potting et al., 1999; Pinto et al., 2007b). Learning a new odour via oviposition or food conditioning,

however, has received less attention (Durrant, 2007). This experiment tested whether *C. vestalis* can learn different olfactory cues, and whether there were gender differences in learning when animals were trained to linalool, and 1-nonanol. Conditioning consisted of three groups per gender, in which animals were exposed to a stimulus over a period of 10 min (Fig. 2.1 and 2.2). For conditioning, naive parasitoids were used 2 - 4 d after eclosion. Experiments were conducted over several days to account for any daily variability due to atmospheric conditions, which is known to influence the parasitoid's performance (Villagra et al., 2005; McClure et al., 2007; Villagra et al., 2008).

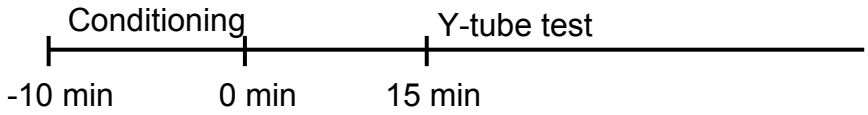

Fig. 2.1 Time line showing the experimental protocol used for the three different conditioning (treatment) groups. Animals were conditioned for 10 min and tested 15 min after conditioning.

a) HP conditioning

In the host/plant (HP) group, animals were kept in clear Polystyrene plastic containers (diameter: 8.65 cm, height: 4.5 cm) (Fig. 2.2 A). Animals were presented with the odour (linalool or 1-nonanol), the host plant (a cabbage leaf which has been previously infested with caterpillars 24 h before conditioning), as well as the host (caterpillar); males were presented 1 female and allowed to copulate with a female and females allowed to oviposit. During the conditioning it was verified if copulation took place. Only one male and one female were used at a time for the conditioning. As copulation was only observed in two cases for linalool, males experiencing copulation were excluded from data analysis.

b) FOOD conditioning

To use a paradigm where both genders had the same learning experience, males and females were food conditioned to the odours mentioned above (FOOD group). Individuals of the FOOD group were kept in 1 ml glass vials (8 x 40 mm polyethylene plug glass) with a string of tissue (Fig. 2.2 B). 20 µl sucrose (1 M)

was pipetted onto the string and the vial placed over a 3 cm^2 filter paper containing 20 µl of linalool or 1-nonanol on petridishes (10 x 1.5 cm diameter) (Wäckers et al., 2002; Wäckers et al., 2006).

c) ODOUR ONLY conditioning

In the control group (ODOUR ONLY) individuals were kept with the odour stimulus alone. ODOUR ONLY individuals were kept in clear Polystyrene plastic containers (diameter: 8.65 cm, height: 4.5 cm) (Fig. 2.2 C).

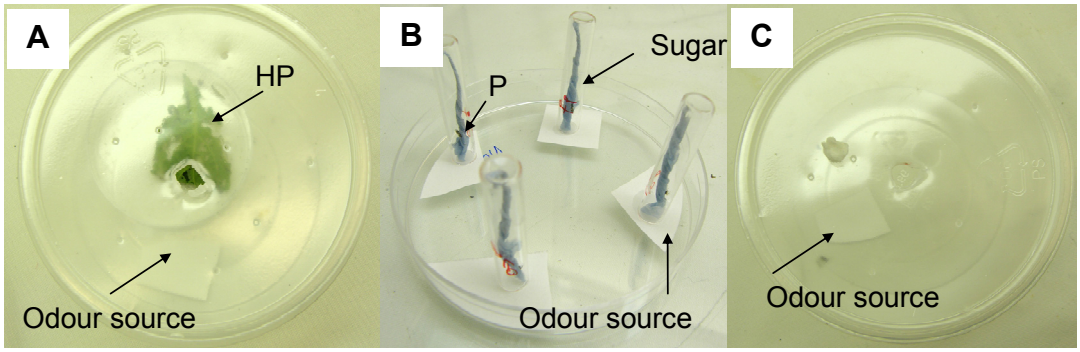

Fig. 2.2 The setup of the different conditioning groups. A) HP: males experienced the presence of a female, the host, the host plant and the conditioned odour. Females experienced the host, the host plant and were allowed to oviposition. **B) FOOD conditioning:** the odour was presented simultaneously with a food source (sucrose) applied on a tissue string. **C) ODOUR ONLY:** insects were exposed to the odour only presented on a filter paper without any associative stimulus. HP= host and host plant, P= Parasitoid.

Behavioural bioassay

Responses of parasitoids to linalool and 1-nonanol were tested in a Y-tube olfactometer (Fig. 2.3). This is an established, commonly used method for measuring choice responses in parasitoids to a control and trained odour (Du et al., 1996; Girling et al., 2011). The olfactometer had an internal diameter of 1.5 cm, a 9.5 cm stem and 10 cm arms at a 60° angle. Bioassays were conducted between the third and sixth hour of the insect's photo phase (McClure et al., 2007). A continuous air flow was created by an air-pump (Neuberger ICN F, Freiburg, Germany) and regulated with a flowmeter (Fig. 2.3). Air was delivered via Teflon tubing through an activated charcoal filter for air purification. The air was further passed through distilled water for humidification and through each of the Y-tube's arms, one containing 20 µl of hexane as a control, and the other containing 20 µl of linalool or 1-nonanol (1 mg/ml) as the conditioned odour. Air

was adjusted with a flowmeter (Airflow GPE limited, Leighton Buzzard UK) and kept constant at 200 ml/min. The airflow was started 15 min before the experiment began to allow the system to stabilise.

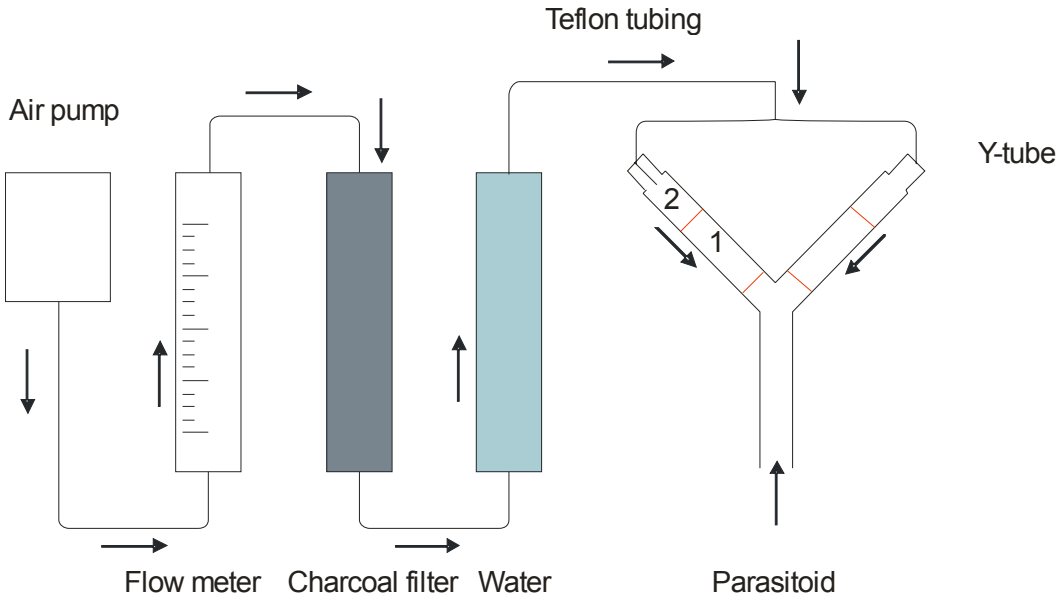

Fig. 2.3 The bioassay setup. Arrows indicate direction of air flow, which was regulated by a flow meter, and passed through a charcoal filter and distilled water via Teflon tubing. In the Y-tube, 1 indicates "first choice" and 2 indicates "final (second) choice".

Individual parasitoids were tested 15 min after conditioning for 5 min. Time measuring started with the individual entering the stem of the Y-tube. A choice was defined when the individual spent more than 30 s in the upper arm of the Y-tube. The time of first entry into the Y-tube, the first arm entered, first choice (after the cross junction), final choice (upper arm of the Y-tube), and the number of times entered in an arm were recorded. Behavioural traits associated with mating such as wingfanning were also recorded. Individuals which remained stationary for 2 min were excluded from data analyses. The olfactometer was reversed 180° after every animal to avoid side bias. The Y-tube was replaced after testing 8 individuals (or a conditioning group) to avoid any errors caused by possible odour traces from the parasitoid. Odour sources were replaced after each animal.

To standardise visual cues, the olfactometer was enclosed by white sheets. A light source comprising of two 15 W fluorescent tubes produced a light intensity of 1.7 klx. The tubes were covered with prismatic reflectors to ensure an even distribution of light and placed above the olfactometer. Preference for either control or experienced odour in the Y-tube was analysed with a goodness of fit Chi-square test. Comparison between each conditioning group was conducted using ANOVA. The set up followed designs previously established and used in bioassays (Girling et al., 2011).

2.2.4. Characteristics of memory formation in *Cotesia vestalis*

To test the impact of learning, an additional experiment was conducted to investigate long-term memory formation in female *C. vestalis*. The response to the conditioned odour 1-linalool was tested after 1 h, 1 d, 2 d and 3 d following conditioning (Collatz et al., 2006). In this experiment, female insects were trained to linalool in the HP group only. For conditioning, females were put in groups of 5 in the Polystyrene plastic containers previously described for 20 min. The longer exposure time ensured that the females had oviposition experience (Potting et al., 1999). Insects were trained in two different treatment groups: a control group (sugar group), which only received sugar water, and the ACD group, which received sugar water and the transcription blocker Actinomycin D (ACD) at a concentration of 0.5 mM (Smid et al., 2007). Actinomycin D (Sigma) was prepared as a 1 mM stock solution. This was obtained by mixing 3.68 mg ACD in 3 ml DMSO (6.4 mM) (Dimethyl sulfoxide, Sigma). For a concentration of 0.5 mM ACD, 2.5 ml of the stock solution was mixed with 5 ml of 2% sucrose (0.1 g sucrose per 5 ml water). Animals were fed 2 µl ACD 24 h before the experiment, and 2 h after testing (1 h for the 1 h group) (Tully et al., 1994; Wüstenberg et al., 1998; Collatz et al., 2006), to ensure that the drug had been consumed (Smid et al., 2007) and because it was shown that the effectiveness of the drug is dependent on the time of administration (Wüstenberg et al., 1998). In chicks, for example, two time windows for inhibition were found: at the time of training, and 3 – 4 h after training (Igaz et al., 2002). It was also shown that in bees, a critical time period of up to 6 h after training was found to be important for protein synthesis (Grünbaum and Müller, 1998; Wüstenberg et al., 1998) and often several time

windows for protein synthesis can take place (Agranoff et al., 1967; Bourtchouladze et al., 1998; Bekinschtein et al., 2007).

Parasitoids were subsequently tested in the Y-tube olfactometer after 1 h, 1 d, 2 d and 3 d following conditioning (Fig. 2.4). Preference for either control or experienced odour in the Y-tube was analysed with a goodness of fit Chi-square test. Comparison between each treatment group was conducted using ANOVA (Lunney, 1970). To account for any daily variability due to atmospheric conditions, experiments were conducted over several days and an even number of parasitoids of the sugar group and the ACD group was used for each treatment and test day (Marchand and McNeil, 2000).

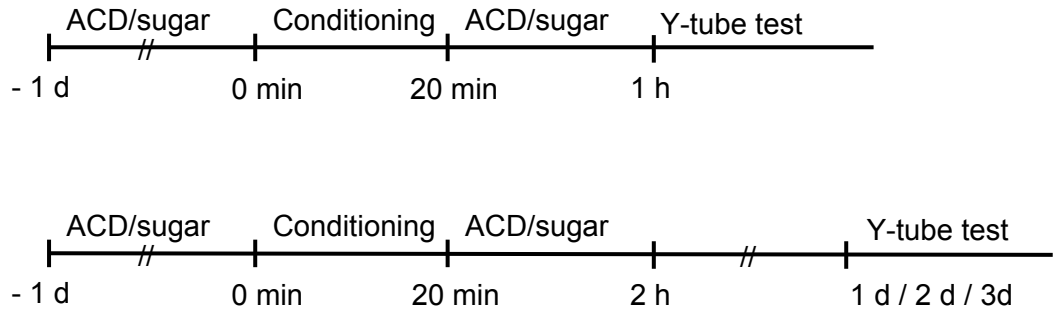

Fig. 2.4 Time line showing the experimental protocol used for investigating the memory dynamics in *C. vestalis*. Animals were fed with ACD and sugar or sugar only 24 h prior and 1 or 2 h after conditioning.

2.2.5. Courtship behaviour in the parasitoid *Cotesia vestalis*

Males initiate courtship behaviour long before antennal contact with the female, suggesting that females produce pheromones (Ruther et al., 2000; McClure et al., 2007). In Chapter 4, a macroglomerular complex (MGC) was found in males, which is known to process pheromone information. Therefore, in this experiment, courtship behaviour in male parasitoids in the presence of a female was used as an indicator for sex pheromone communication in *C. vestalis*. Thus, a complex investigation of courtship behaviour in this species was conducted to explore the possibility of pheromone communication in *C. vestalis*.

Behavioural essays were used in different combinations to test if courtship behaviour was displayed, which sex was responsible for the attraction of the opposite sex, and to determine if an attraction between individuals of the same sex or to a different species exists (males-only, females-only, males with females, and males with females of a different parasitoid species).

To observe whether courtship behaviour was influenced by mating status, males and females were paired as mated male and female, as well as virgin male and female. As it was shown that mating behaviour is influenced by time (McClure et al., 2007), copulation success was measured by the occurrence of courtship behaviour in male/female pairs, mornings - am (0800 h - 1000 h), and afternoons - pm (1200 h - 1600 h), for both mated and virgin individuals.

For observation, individuals were placed together in pairs in clear Polystyrene plastic containers (diameter: 8.65 cm, height: 4.5 cm). Behaviour was observed continuously over a period of 5 min and elements of courtship and other behaviour were noted (Table 2.1). Four elements of courtship behaviour were distinguished (Vinson, 1972; McClure et al., 2007): orientation was recorded when a male orientated towards another insect; antennal contact was defined as touching the body of another individual with the antennae; wingfanning in combination with abdominal raising was based on the number of times males displayed wing vibration; attempted copulation was recorded when a male mounted another insect and attempted copulation; copulation was recorded when successful copulation took place. Other behaviour was classified as walking, exploring (walking with antennae touching the ground), grooming (cleaning of extremities), antennal touch (the individual continuously moved its antennae while touching it with its front legs) and immobile (the individual remained completely still).

Six pairs of 2 d old individuals were observed in each experiment and behavioural assays were carried out over several days to account for any daily variability due to atmospheric conditions (Marchand and McNeil, 2000). Behaviour was noted as number of occurrences and was then transformed into percentage of the total behaviour per individual. For statistical analysis, the percentage of courtship behaviour per individual was arcsine transformed and different groups were analysed with a Student's t-test (McDonald, 2009).

Table 2. 1 Parameters of courtship- and other behaviour

Courtship behaviour	Orientation	Male is orienting towards other individual
	Antennal contact	The individual is touching the body of another individual with the antennae
	Wingfanning and abdominal raise	Male is displaying rapid wing vibration that is sometime accompanied by raising the abdomen
	Attempted copulation	Male is mounting another individual and attempts copulation
	Copulation	Male is mounting another individual and succeeds in copulation
Other behaviour	Walking	The individual is walking around the container
	Exploring	The individual is walking around the container while its antennae are touching the ground
	Grooming	The individual is cleaning its extremities
	Antennal touch	The individual is continuously moving its antennae while touching them with its front legs
	Immobile	The individual is remaining still without displaying any behaviour

Cleaning of plastic and glassware

After each experiment the glassware used was cleaned and dried thoroughly to eliminate residual volatiles which may otherwise affect the results of subsequent experiments. Equipment was first soaked with Decon 90 (Decon Laboratories Limited, UK) and rinsed with hot water. Glassware was then scrubbed with acetone and rinsed with distilled water before being dried in an oven at 200°C for a minimum of 3 h. Plastic containers used for conditioning procedures and courtship behaviour were only used once.

2.3. Results

Part 1 Plant volatile studies

2.3.1. Antennal responses of *Cotesia vestalis* to different odour compounds measured by EAG

This experiment was conducted to establish if male and female *C. vestalis* can detect different plant volatiles at a peripheral level. The antennal response of male and female *C. vestalis* to linalool and decanal, 1-nonanol and a control stimulus (hexane) was tested using an EAG.

Odours were presented in 5 runs in a different order each time, with hexane as a start and end stimulus after each run. Both genders detected the different volatiles and showed an EAG response to all test compounds with similar signal amplitudes (Fig. 2.5) and rate (Fig. 2.6). An example of one of the 5 runs is shown in Figure 2.5. The signal increased after odour onset. Linalool (5 mg/ml) had the longest response which lasted for 5-6 s before it returned to baseline.

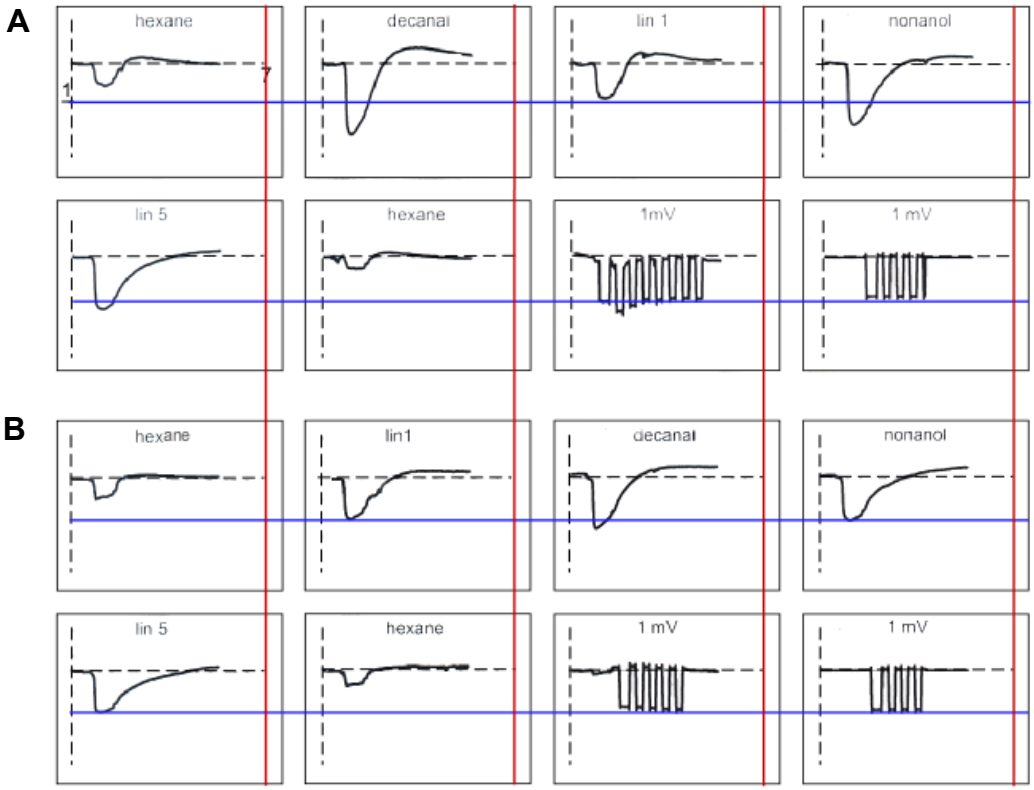

Fig. 2.5 Signal intensities of males (A) and females (B) for the different compounds. The signal amplitude of one run is shown for the different compounds for males and females. Signal intensities were similar in both genders (y-axis). Decanal and 1-nonanol elicited the highest response of the signals tested. Red line x axis: 7= 7 s, blue line y-axis: 1= 1 mV for all compounds. Lin 1= Linalool (1 mg/ml); Lin 5= Linalool (5 mg/ml). 1 mV= Amplitude of the artificial 1 mV.

The results shown in Figure 2.6 comprise responses to the compounds at 1 mg/ml (1-nonanol, linalool, decanal) and 5 mg/ml (linalool) and are expressed as a percentage of the artificial 1 mV signal (i.e. 1 mV = 100%). Responses to all compounds tested were significantly higher than responses to the hexane control ($p<0.05$). There was no significant difference between genders ($p>0.05$).

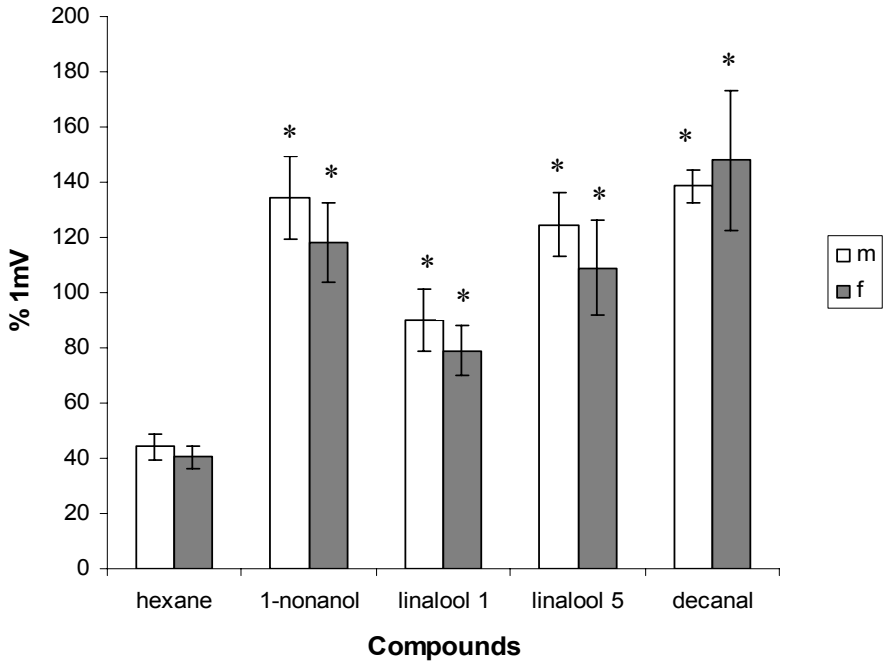

Fig. 2.6 EAG responses of *C. vestalis* to different odour compounds. 1-nonanol, linalool 1 and decanal had a concentration of 1 mg/ml. Linalool 5 had a concentration of 5 mg/ml. Data were normalised as a percentage of the 1 mV signal (1 mV signal is 100%). * indicates a significant difference between mean responses to the different compounds and mean response to hexane (control) at the level $p<0.05$ compared to hexane for both genders.

2.3.2. Behavioural response of *Cotesia vestalis* to a linalool

To determine if males and females use associative learning during host (female) location, animals were trained in different conditioning groups to linalool. In the HP group, animals were presented with the host, host plant and linalool. Females were allowed to oviposition, and males were allowed to mate with females. In the FOOD group, animals received sugar water in the presence of the odour, and in the ODOUR ONLY group, animals were exposed to the odour alone, without any additional stimulus. The animals' ability to learn was tested by placing them in a Y-tube olfactometer bioassay with one arm containing the conditioned odour (linalool) and the other a control odour (hexane). A choice was recorded if the animal spent more than 30 s in the upper half of an arm.

In both genders, animals trained in the HP group showed a higher preference for linalool in the Y-tube olfactometer when presented with the choice of a conditioned stimulus (linalool) and a control stimulus (hexane) (Fig. 2.7 A, B). Linalool was preferred by 50% of males and 76% of females. Only 14% of females went to the control instead of linalool and none of the males chose the control odour. To compare the response between control and conditioned stimulus within each conditioning group and gender, a heterogeneity Chi-square test (goodness of fit) was used. Significantly more males ($\chi^2 = 11$, df= 1, $p<0.05$) and females ($\chi^2 = 7.2$, df= 1, $p<0.05$) subjected to the HP group chose linalool rather than the control odour in the Y-tube.

Both genders showed a lower preference for linalool in the FOOD group and ODOUR ONLY group and no significant difference was found for the other conditioning groups for either of the genders. The percentage of males that did not respond in all the conditioning groups was extremely high (over 50%), suggesting that linalool is less attractive to males than it is to females. However, looking at the actual percentage of animals (excluding the no choice animals), males showed a high preference for linalool in all conditioning groups (over 60%).

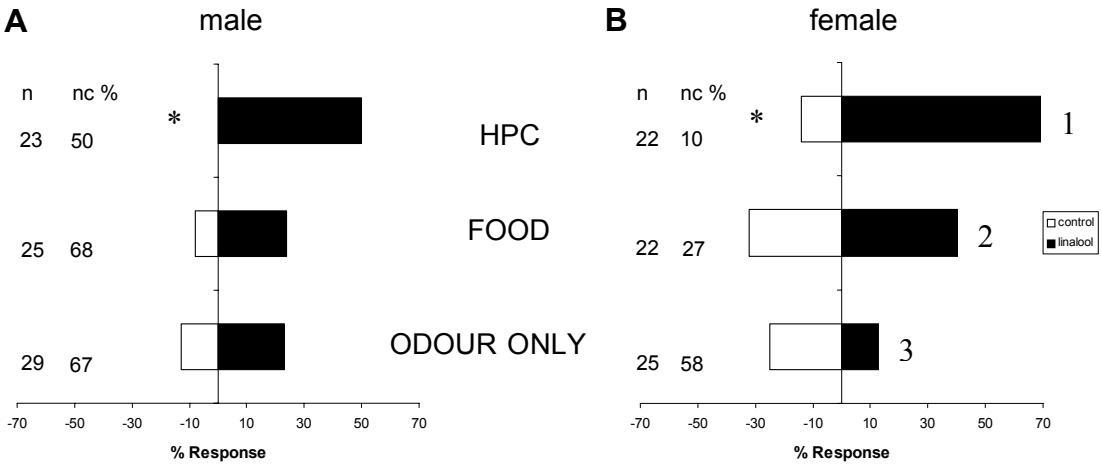

Fig. 2.7 Responses of *C. vestalis* in a Y-tube olfactometer to a control stimulus (hexane) or trained stimulus (linalool). Individuals were subjected to 3 conditioning groups (Host/plant (HP); FOOD and ODOUR only). **A)** Response to male parasitoids trained with linalool. **B)** Response of female parasitoids trained with linalool. * indicates a significant difference between control and linalool at the level $p<0.05$. Contrast 1-3 indicates a significant difference between conditioning groups at the level $p<0.05$. The percentage of wasps that failed to respond within the 5 min time period are displayed as nc-no choice and were excluded from data analysis. The total number of tested animals (n) for each conditioning group is displayed to the left of the corresponding bars.

To compare the conditioning groups and both genders with each other, a 1-way ANOVA was used. The only significant difference could be found in females between the HP and ODOUR ONLY group ($F_{2,44}= 4.050$, $p<0.05$). This suggests that an associative connection between the conditioned odour and the HP was stronger in females.

To summarise, when males and females were trained with linalool, both genders significantly preferred linalool in the HP group. Comparison between treatments, however, only revealed a significant difference between the HP and ODOUR ONLY group in females. The generally high percentage of males making no choice and the lack of significant difference between the conditioning groups in males, suggests that, although males prefer linalool in the HP conditioning, a stronger associative trace is formed in females.

2.3.3. Behavioural response of *Cotesia vestalis* to a 1-nonanol

EAG measurements (section 2.3.1) showed that animals responded to linalool, decanal and 1-nonanol. To investigate whether *C. vestalis* could also learn an odour which is not part of their tritrophic system, animals were trained in different conditioning groups to 1-nonanol, as previously described in section 2.3.2.

Overall, response to the conditioned stimulus was lower when conditioned to 1-nonanol (Fig. 2.8 A, B). Males showed the highest response in the HP group, but more individuals chose the control odour (35%) over 1-nonanol (25%). In females a high percentage (43%) of individuals chose the control odour over 1-nonanol (5%).

Furthermore, both genders had a lower response rate in the FOOD group. Only 14% of males chose 1-nonanol and 9% the control. In females 29% (67% actual percentage excluding no choice animals) preferred 1-nonanol and only 14% chose the control.

The response in the ODOUR ONLY group was also very low. In males, the percentage choosing 1-nonanol and the control was equal (15%). Only 14% of females chose 1-nonanol and 24% the control. In all groups and genders the percentage of individuals not making a choice was higher when they were treated with 1-nonanol. The only significant difference was found in the HP group, where females significantly preferred the control stimulus over 1-nonanol ($\chi^2 = 5.4$, df= 1, $p<0.05$).

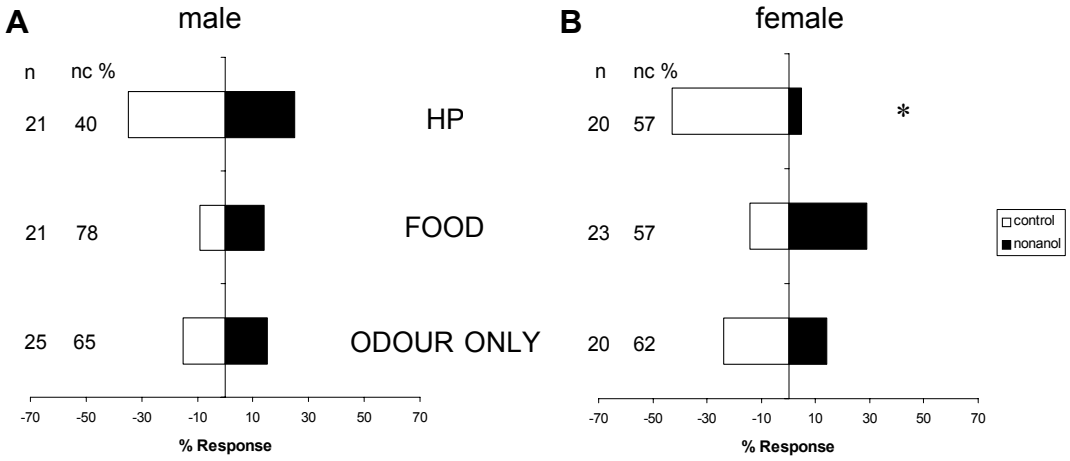

Fig. 2.8 Responses of *C. vestalis* in a Y-tube olfactometer to a control stimulus (hexane) or trained stimulus (1-nonanol) Individuals were subjected to 3 conditioning groups (Host/plant complex (HP); FOOD and ODOUR only). **A)** Response to male parasitoids trained with 1-nonanol. **B)** Response of female parasitoids trained with 1-nonanol. ***** indicates a significant difference between control and 1-nonanol at the level $p<0.05$. The percentage of wasps that failed to respond within the 5 min time period are displayed (nc-no choice) and were excluded from data analysis. The total number of tested animals (n) for each conditioning group is displayed to the left of the corresponding bars.

A 1-way ANOVA showed no significant gender ($_1F_{60}= 3.314$, $p<0.05$) or conditioning difference ($F_{1,60}= 0.952$, $p<0.05$) for 1-nonanol. Generally, the percentage of insects not eliciting a response within the 5 min time interval for 1-nonanol was high with a no choice rate over 50% in all groups.

A statistical comparison (uGLM) between linalool and 1-nonanol showed a significant difference between gender ($F_{1,136}= 7.382$, $p<0.05$), odour ($F_{1,136}= 13.696$, $p<0.05$) and conditioning and odour ($F_{2,136}= 5.248$, $p<0.05$). In summary, experience with 1-nonanol in the HP group resulted in avoidance learning in females, but was not learned in any of the other conditioning procedures in females or males.

Part 2 Memory dynamics

2.3.4. Characteristics of memory formation in *Cotesia vestalis*

Experiments were conducted to determine whether *C. vestalis* would form long-term memory after experience with linalool during HP conditioning. To assess memory dynamics, females were fed the irreversible transcription blocker Actinomycin D (ACD), known to inhibit protein synthesis and consequently the formation of LTM. Females were fed either sucrose only (sugar group), or a transcription blocker diluted in sucrose (ACD group), 24 h before training and up to 2 h after training and tested in a Y-tube 1 h, 1 d, 2 d and 3 d following training. In the Y-tube their choice between a control stimulus (hexane) and the conditioned stimulus (linalool) was recorded. Like previous experiments, a choice was recorded when the individual spent more than 30 s in the upper half of an arm.

Female parasitoids in the sugar group (Fig 2.9 A) showed the highest response 1 h after conditioning, with 76% of the animals choosing linalool in the Y-tube. Over time, their response decreased to 21% and was lowest after 3 d. The number of individuals with no choice increased with time, suggesting that the odour became increasingly unattractive to the females. Comparison within each testing time using a Chi-square test (goodness of fit) revealed no significant difference between responses to control or linalool at any time point. Therefore, despite an initially higher response to linalool when tested 1 h after training, female parasitoids failed to establish an associative connection to the learned odour, indicating no LTM was formed.

Individuals of the ACD group (Fig 2.9 B) showed similar responses to linalool when tested after 1 h, 2 d and 3d. The number of females preferring linalool over the control decreased after 1 h from 57% to 15% tested 3 d after training. After 1 d, females failed to respond to the learned odour, and while a high percentage of females made no choice (67%), 33% preferred the control over linalool. A Chi-square test (goodness of fit), comparing responses between the control odour and linalool within each group, only revealed a significant

difference for females tested 1 day after training (χ^2= 5.4, df= 1, p<0.05) (Fig. 2.9), where it led to an avoidance reaction in females.

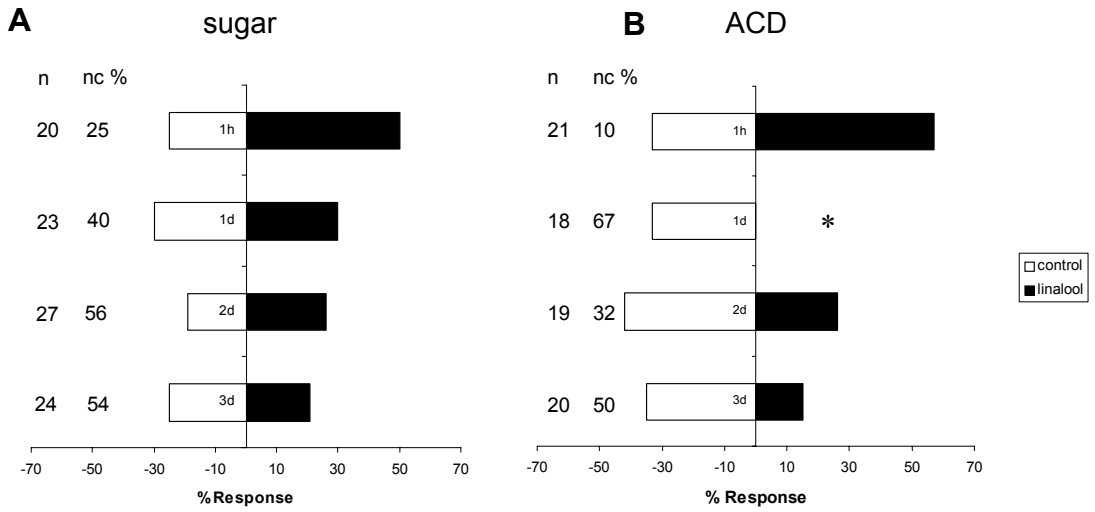

Fig. 2.9 Responses of *C. vestalis* in a Y-tube to a control odour and linalool at different time points following conditioning. A) The sugar group received sugar water only. There was no significant difference between control and linalool at any of the time points. **B)** Animals were fed with ACD 24 h before and 1/2 h after training. At 1 d, animals only responded to the control odour and avoided linalool. * indicates a significant difference between control and linalool at the level p<0.05. The percentage of wasps that failed to respond within the 5 min time period is also displayed (nc-no choice).

A 1-way ANOVA comparing the different time points (Fig. 2.10) within the sugar group showed no significant difference between the different time points ($_3F_{51}$= 0.821, p>0.05). By comparison, within the ACD group, a significant difference between times could be found ($_3F_{47}$= 3.161, p<0.05); however only between 1 h group and 1 d ($F_{1,24}$= 9.463, p<0.05).

A comparison between sugar and ACD groups showed a significant difference between time ($F_{3,99}$= 2.914, p<0.05) as well as group ($F_{1,99}$= 6.174, p<0.05), but no group and time interaction ($F_{3,99}$= 1.007, p>0.05). With the exception of animals tested after 1 h ($F_{1,20}$= 10.857, p<0.05) and 1 d ($F_{1,19}$= 5.400, p<0.05), no significant difference was found between individuals of the ACD and sugar group.

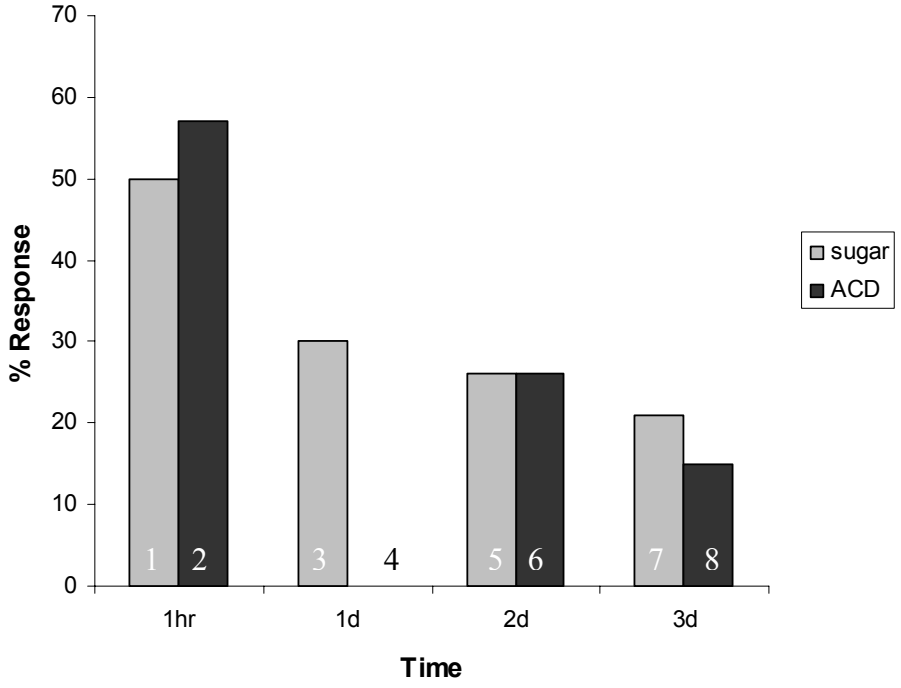

Fig. 2.10 Responses of *C. vestalis* females to linalool at different time points following conditioning. Parasitoids in the sugar group only received sugar water, while parasitoids in the ACD group were fed with sugar water containing the transcription blocker ACD. Contrasts 1-4, 2-4 and 3-4 were significantly different at the level $p<0.05$.

Part 3 Pheromone studies

2.3.5. Courtship behaviour in the parasitoid *Cotesia vestalis*

Although males learned linalool in the HP group, the formed association was not as strong as in females (Section 2.3.2). This result and the MGC found in Chapter 4 could indicate that males use sex pheromones in combination with HIPVs to locate females. The courtship behaviour experiments presented in this section investigated whether males display courtship behaviour in the presence of a female. To account for the influence of time and mating status, virgin and mated individuals were observed in the morning and the afternoon. Animals were placed together in different combinations, such as males-only, females-only, mated males and females, virgin males and females, as well as males with females of a different species.

In the presence of a female of the same species, males initiated courtship behaviour (n= 24). This included a chain of consecutive behaviours (Fig. 2.11). The male started orienting towards the female and pursuing it (Fig. 2.11 A, B) which was followed by wingfanning (Fig. 2.11 C). The wingfanning behaviour was sometimes accompanied by raising the abdomen (Fig. 2.11 D). Locomotory activity increased and contact with the female was attempted (Fig. 2.11 E, F).

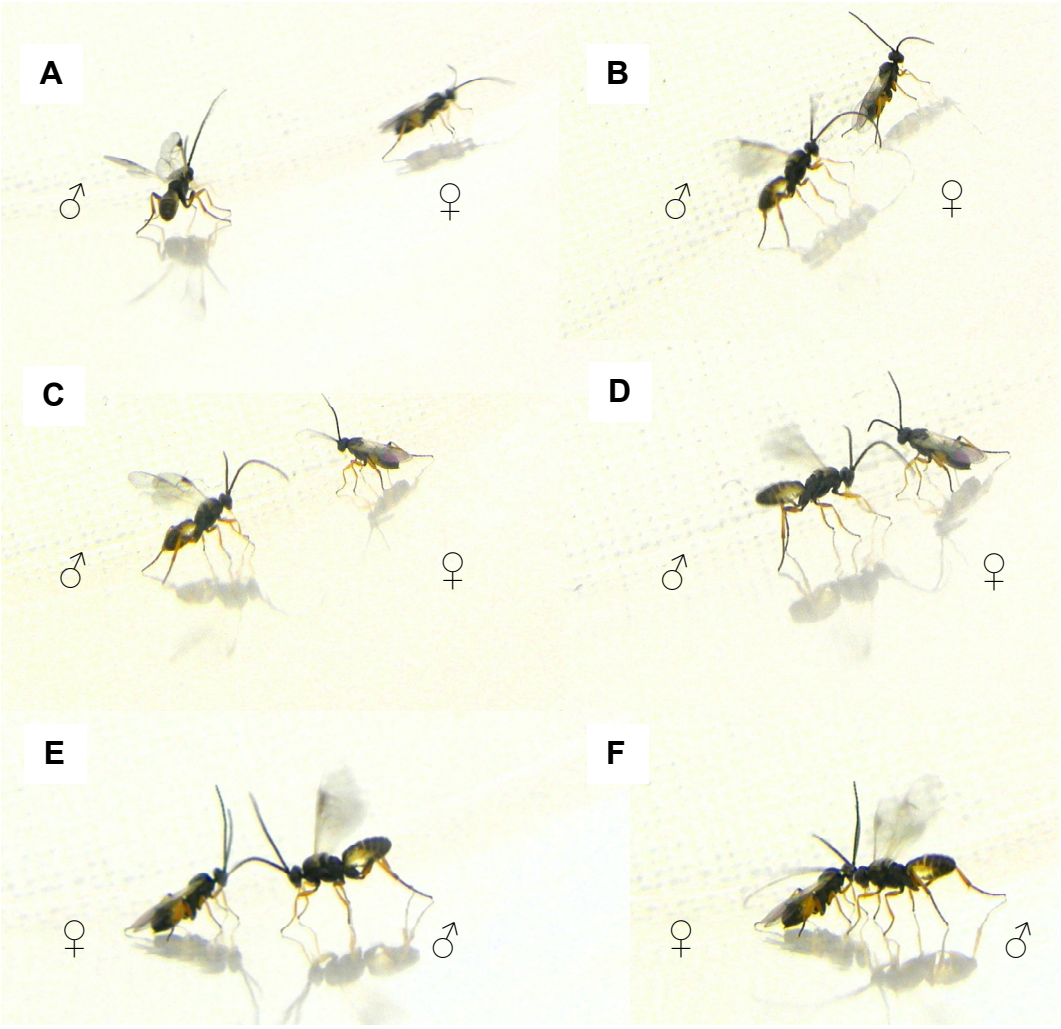

Fig. 2.11 Behavioural elements of *C. vestalis* courtship behaviour. **A)** Orientation: in the presence of a female, males initiated orientation. **B)** Chase: after orientation, males were often observed following the female. **C)** Wingfanning: wingfanning often already occurred during orientation and chasing the female. During this behaviour the male was fanning its wings at a high frequency. **D)** Abdominal raise and wingfanning: wingfanning was often accompanied by an abdominal raise. **E)** Antennal contact: once the female remained still, the male tried to approach the female and initiate antennal contact. **F)** Contact and attempted copulation: in 94% of the observations, the female refused the male and walked off after antennal contact. Total number of pairings observed = 24.

The receptive female allowing copulation did not display any behaviour that could be linked with the release of a pheromone, although it was observed that in the case of accepting the male, the female stopped and lifted the abdomen slightly

and allowed copulation. Copulation only occurred in one case. Hence, in 94% of the observations, the female refused copulation and walked off after attempted contact by the male.

Courtship behaviour was observed in mated and virgin males, although virgin males displayed more courtship behaviour (80%) (Fig. 2.12). Within both groups, no diel effect was found (Mated: $t= 0.813$, $df= 5$, $p>0.05$; Virgins: $t= 5.801$, $df= 5$, $p>0.05$). However, between-group comparison showed that mated males in the morning displayed significantly less courtship behaviour than virgin males ($t= -2.721$, $df= 5$, $p<0.05$). Furthermore, virgin males spent most of their time displaying courtship than other behaviour, such as for example, walking, grooming or remaining immobile ($t= 6.164$, $df= 5$, $p<0.05$).

In summary, courtship behaviour was found in mated and virgin males, tested at any time. Only virgins, however, displayed significantly more courtship behaviour than other behaviour.

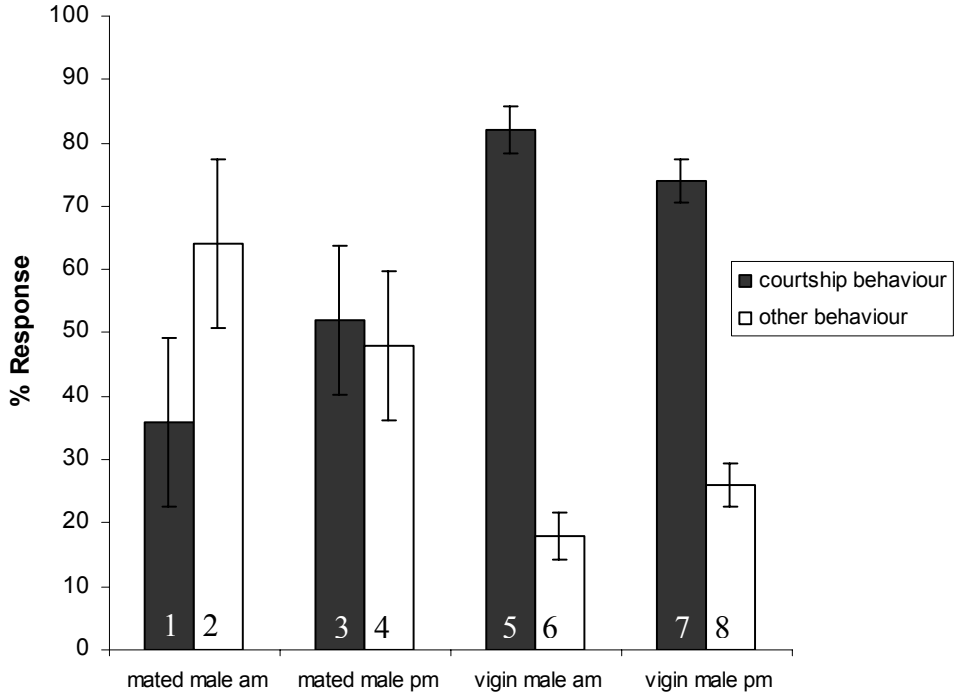

Fig. 2.12 Behavioural responses of mated and virgin males tested in the morning (am) and afternoon (pm). Courtship behaviour was significantly higher in virgin males than in mated males tested in the morning. Virgin males showed significantly more courtship behaviour than other behaviour. n= 6 per group. Contrasts 1-5; 5-6 and 7-8 and are significantly different at the level $p<0.05$.

The different behavioural elements of courtship behaviour revealed differences depending on mating status and time (Fig. 2.13). Mated males in the morning displayed significantly less orientation than virgin males in the morning ($t= -3.697$, $df= 5$, $p<0.05$). Also, virgin males showed more orientation in the morning than virgin males in the afternoon ($t= -3.697$, $df= 5$, $p<0.05$).

Wingfanning behaviour in virgin males made up about 50% of the behaviour. Virgin males tested in the morning and afternoon displayed significantly more wingfanning than mated males tested in the afternoon ($t= -3.006$, $df= 5$, $p<0.05$; $t= -2.812$, $df= 5$, $p<0.05$). Antennal contact did not depend on mating status or time.

In summary, wingfanning was the most frequently used element of courtship behaviour, followed by orientation, suggesting that wingfanning is the most important element in the courtship sequence.

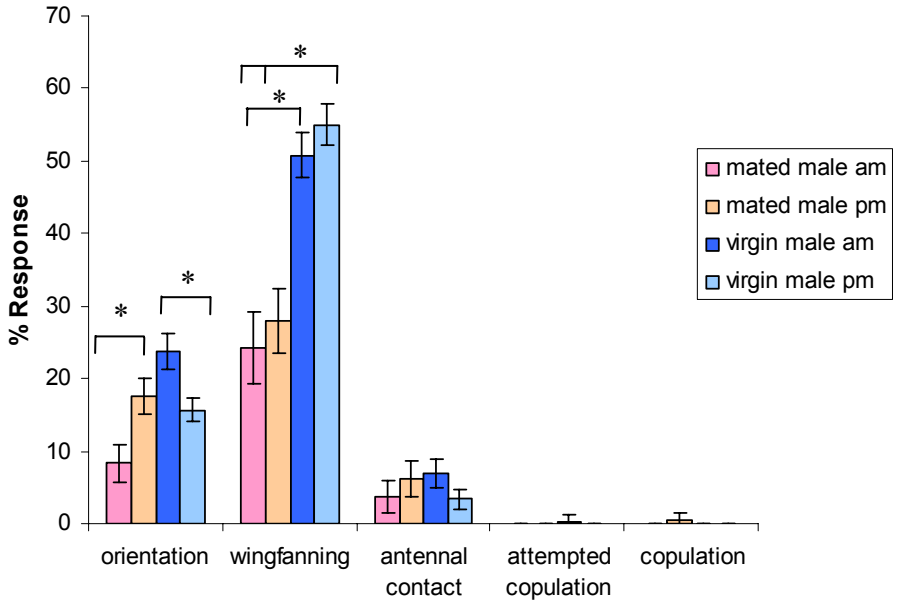

Fig. 2.13 Courtship elements of mated and virgin males tested in the morning (am) and afternoon (pm). Wingfanning behaviour was the dominant element in courtship behaviour, which was significantly higher in virgin males. Orientation, antennal contact and copulation were less frequent. n= 6 per group. * indicates a significant difference at the level $p<0.05$.

In the absence of females, no courtship behaviour was observed (Fig. 2.14). Males spent their time displaying other behaviour, such as antennal movement, grooming, walking, exploring, or remaining immobile. In females-only observations, no courtship behaviour could be observed. In addition, males displayed significantly less courtship behaviour in the presence of females of a different species (*Asobaria tabida*) than mated and tested in the afternoon ($t= 2.182$, $df= 5$, $p<0.05$), as well as virgin males tested in the morning ($t= 8.370$, $df= 5$, $p<0.05$) and afternoon ($t= 7.864$, $df= 5$, $p<0.05$). However, the only courtship behaviour observed was orientation towards the female of a different species, and one male initiated antennal contact, but no wingfanning occurred. Furthermore, their general activity increased. This suggests that orientation and antennal contact were in this case used as a means of exploring the female of a different species.

In summary, *C. vestalis* males display courtship behaviour in the presence of a female of the same species which could be an indicator for pheromone communication in this species. Courtship behaviour in *C. vestalis* does not appear to be restricted to a certain time period in the day and wingfanning appears to be the dominant element in courtship behaviour.

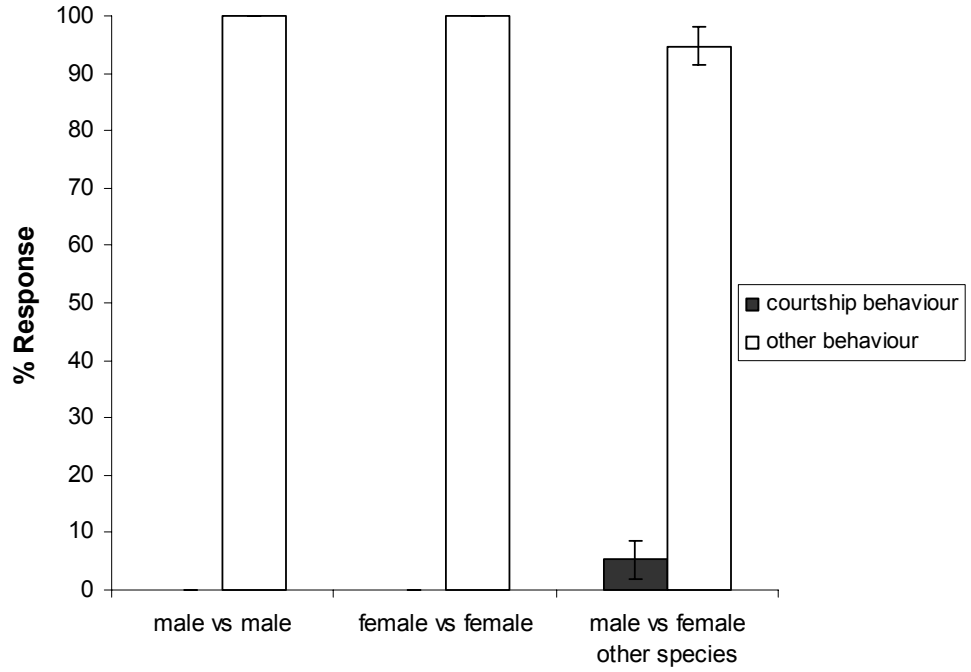

Fig. 2.14 Behavioural responses of males only, females only and males presented with a female of a different parasitoid species. There was no courtship behaviour in males or females only groups. Males paired with a female of another parasitoid species sometimes approached the female. n= 6 per gender and group.

2.4. Discussion

The aim of this study was to investigate antennal as well as behavioural responses of *C. vestalis* to different olfactory cues, to understand how *C. vestalis* uses olfactory cues for host location. Therefore, EAG studies and behavioural bioassays were carried out to investigate antennal and behavioural responses of the parasitoid to olfactory cues. The hypothesis was that in a specialist parasitoid learning is more restricted to odours relevant for host location. Learning in females through oviposition is well known (Kaiser et al., 2009; Costa et al., 2010). However, little work has been carried out to investigate the ability of male parasitoids to detect and learn odours. It was argued that because *C. vestalis* is a solitary specialist, HIPVs are also important for males, to locate females. Also, pheromones emitted by the female may play an important role to attract males over longer distances.

2.4.1. Antennal responses of male and female *C. vestalis* to linalool and 1-nonanol

Antennal responses to olfactory cues as an indicator for specialisation?

In this experiment parasitoids responded to all odours tested. Measurements of EAG responses in *C. vestalis* revealed significant responses to the control for all compounds tested, in males and females. EAGs are useful to screen for receptive ORNs upon odour stimulation, however, some studies have shown contrasting conclusions (Park et al., 2001; Sen et al., 2005), indicating that EAG activity alone is not an exact indicator of the "relevance" of certain compounds to an animal (Suckling et al., 1996). Ngumbi (Ngumbi et al., 2009; Ngumbi et al., 2010) demonstrated that specialists had greater EAG responses to host specific odours and HIPVs, while generalists demonstrated greater antennal responses to a variety of odour compounds (Chen and Fadamiro, 2007). However, in moths and other parasitoid species, similar EAG responses of specialists and generalists have been found (Smid et al., 2002; Gouinguen et al., 2005). This suggests that although EAGs can be an indicator to test which odours can be perceived, they are

less suitable as an indicator for host specialisation and differences in response levels might also depend on the volatility of compounds (Germinara et al., 2009).

Antennal responses of males and females

EAG studies in males and females also show a great variety of responses depending on the species. Although some studies revealed no gender differences (Li et al., 1992; Park et al., 2001; Dweck et al., 2010), others have shown that in some cases, males are prone to respond more strongly to certain volatiles (Germinara et al., 2009). In the current study, males had a slightly higher antennal response to the compounds tested, but no significant gender differences could be found. In both genders the compound concentration influenced the antennal response. A higher concentration of linalool elicited higher EAG responses than the lower concentration. Previous studies (Whitman and Eller, 1990; Jyothi et al., 2002) have found that females have a lower threshold and a higher sensitivity to certain compounds, for example, it has been shown that *Microplitis croceipes* and *Cotesia marginitventris* females respond more to GLVs and males more to inducible compounds such as terpenoids which are released later (Chen and Fadamiro, 2007).

Response differences to the odour compounds could be explained by the difference in the number of receptor neurons tuned to compounds, the number and type of sensilla and the volatility of an odour. In *C. vestalis*, seven types of sensilla have been identified in males and females, three of which are thought to be olfactory sensilla; sensilla placodea, sensilla trichodea WP and sensilla coelonica I (Keil, 1999; Ochieng et al., 2000; Roux et al., 2005). In total, 771 and 621 olfactory sensilla were found in males and females, respectively, which have also been identified across insect species (Akers and Getz, 1992; Larsson et al., 2001). In species where pheromone detection plays a bigger role it was shown that males had more sensilla, especially sensilla placodea (Hansson et al., 1989; Anderson et al., 1995; Ansebo et al., 2005). It was thought that these sensilla could contain ORNs stimulated by sex pheromones and plant volatiles, or volatile compounds that stimulate pheromone receptors (Hansson et al., 1989; Anderson et al., 1995; Ansebo et al., 2005), and thus increase the response of a sex pheromone specific ORN (Ochieng et al., 2002). Morphological studies of the antennal sensilla in *C. vestalis*, *C. rubecula* and *C. glomerata* showed that males in these

species had a higher number of sensilla placodea (Bleeker et al., 2004; Roux et al., 2005). In summary, male and female *C. vestalis* showed antennal responses to the compounds tested, indicating that they have receptors for these odour compounds.

2.4.2. Behavioural responses of male and female *C. vestalis* to linalool and 1-nonanol

Is olfactory learning related to the "ecological relevance" of an odour to improve host location?

To overcome the problem of identifying the right cues for host location, it is thought (reliability-detectability hypothesis) that parasitoids have learned to associate reliable cues from the host with HIPVs, which are detectable over a greater distance than odour cues from the host (Vet and Dicke, 1992). Hence, learning to recognise HIPVs can be seen as an evolutionary adaptation to improve host location and thus fitness.

In the current study, learning increased the responsiveness of *C. vestalis* to a generic HIPV linalool, while 1-nonanol, an odour not associated with their tritrophic system (Durrant, 2007), resulted in avoidance learning. It has previously been shown that generalists are more "flexible" in changing their preference than specialists (Geervliet et al., 1998b). Generalists, contrary to specialists, have a variety of hosts, and learning different cues of their host-plant complex would therefore be beneficial. For a specialist it is essential to be able to discriminate HIPVs from non-HIPVs and thus, learning of non-HIPVs would be irrelevant and costly for them. This suggests that their olfactory system is fine tuned for specific HIPVs of their host-plant complex, which remain constant. In turn, innate preference to "relevant" HIPVs may also be relatively high and improved through learning.

However, there have been cases in which specialist parasitoids were shown to learn new or "ecologically irrelevant" odours (DeJong and Kaiser, 1991). This has also been demonstrated in *C. vestalis*, where females were able to learn vanillin (Durrant, 2007). On the other hand, it has been previously discovered that HIPVs can elicit avoidance behaviour too (Durrant, 2007; Snoeren et al., 2010). This suggests that olfactory learning in parasitoids might not only depend on host specialisation and the "ecological relevance" of an odour, but also on the

functional group, type and concentration of odour (Smith and Menzel, 1989; Getz and Smith, 1990; Daly et al., 2001; Meiners et al., 2002; Ngumbi et al., 2009).

Furthermore, it has been argued that the degree of host specialisation alone might not be a good indicator to what extent animals rely on innate cues (Steidle and Van Loon, 2003). Indeed, it was found that innate responses can also be important for generalists (Steidle and Van Loon, 2003; Peñaflor et al., 2011).

Responses to HIPVs: a cue for hosts and reproduction?

It has been shown in the past that female parasitoids are able to learn odours through oviposition (Potting et al., 1999). Learning in males, however, has received less attention (Goh and Morse, 2010). In this study, EAG and behavioural studies were used in both genders to test the hypothesis whether HIPVs are important for males as well as females. Indeed, measurements of antennal responses of *C. vestalis* have shown that males perceived all of the compounds tested (section 2.3.1). Furthermore, males learned linalool, but not 1-nonanol in the HP conditioning group. Copulation did not appear to be essential for learning, as males did not succeed in copulation. Hence, just the presence of the female was sufficient to induce learning in males. However, a high number of animals did not make a choice, which suggests that linalool might be less attractive to males (Girling et al., 2006) than to females. This might be caused by the quality of reward (the lack of copulation), and it can be hypothesised that the association would have been stronger if copulation had taken place.

In summary, it can be assumed that HIPVs play a different role for males and females. The reproductive success of males is dependent on when and where females are available, while females need to locate the host for successful reproduction. Especially in solitary specialist species, the chance to encounter females is less than in generalists. Therefore, being able to detect and associate an odour with the presence of a female (in the case of the male), or the presence of a host (in the case of the female); or avoid those which are not an indicator for female/host presence can be of an advantage. From these results it can be suggested that *C. vestalis* males use HIPVs as a cue to locate females and females prefer volatiles indicating host presence.

Food conditioning: differences in neural pathways depending on conditioning paradigm?

Appetitive food conditioning is widely used in the insect world (Guerrieri et al., 2011; Roussel et al., 2011; Salloum et al., 2011; Sanford and Tomberlin, 2011; Simões et al., 2011). Successful food conditioning has been shown in previous studies in other parasitoid species (Lewis and Takasu, 1990; Wäckers et al., 2002; Wäckers et al., 2006). In this study, however, neither males nor females were able to learn via food conditioning, regardless of the odour. Animals showed more response to linalool, but both odours failed to induce a significant association in any of the conditioning groups. A lack of learning the odour 1-nonanol has also been observed in previous studies (Meiners et al., 2002). Interestingly, females trained to 1-nonanol in the FOOD group showed a higher preference to the odour than females in the HP group. This reinforces the possible aversive effect of 1-nonanol on females in the HP group. One explanation might be that the odour needs to be relevant to the natural context. Hence, HIPVs could be better learned with oviposition conditioning, and floral odours with food conditioning. This might also indicate different pathways for food and oviposition conditioning, which differs to the suggestion of Bleeker (Bleeker et al., 2006b), who suggested that oviposition learning could be mediated by the VUMmx1 neuron, which is known to mediate PER (proboscis extension conditioning) in bees (Hammer, 1993).

2.4.3. Temporal dynamics and memory formation

To determine the impact of learning, experiments investigating LTM formation were conducted. It has been shown, that memory dynamics in parasitoid are different to, for example, memory dynamics in honeybees, and so far, in certain parasitoid species LTM formation is completed after already 1 d (Collatz et al., 2006; Smid et al., 2007). In *C. vestalis*, however, the memory trace began to wane after only 1 h (in the sugar group). Although the response rate was still around 60%, no significant differences were found compared to responses to the control odour (hexane) at any of the time points tested. Additionally, responses to linalool at day 2 and 3 were only 25% and 15%, respectively, which is similar to the response rate of parasitoids experiencing a non-associative

exposure (ODOUR ONLY) to an odour (Section 2.3.2). These results indicate that no LTM formation occurred. This is different to other studies of the related specialist species, *C. rubecula,* where LTM was formed despite a decline of responses to the learned stimulus after 1 day (Bleeker et al., 2006a). Responses of *C. vestalis* to the odour at 1 h,1 d, 2 d and 3 d could be due to a combination of non-associative and associative learning (Bleeker et al., 2006a).

Learning and the influence of the inter-trial interval (ITI)

One explanation for this result could be the ITI. In the experiment investigating LTM formation, females were placed with the conditioned odour for 20 min during the HP treatment. This relates to a training protocol classified as massed learning (Smid et al., 2007), in which the female has a number of oviposition experiences in sequence, but is not removed from the leaf in between the trials. This resembles a natural situation in which the parasitoid would encounter a cluster of caterpillars on a plant. However, it has been shown that in some species, spaced learning trials are needed to establish LTM (Menzel, 2001; Giurfa et al., 2009).

Generalist parasitoid species, such as *Eupelmus vuillet, C. glomerata* and *Lariophagus distinguendus,* however, showed a memory stable trace up to 7 d after training, regardless of the training procedure (Cortesero et al., 1995; Müller et al., 2006; Smid et al., 2007). In the specialist *Leptopilina boulardi* (Kaiser et al., 2003) massed conditioning lead to a memory trace lasting about 48 h and in *Microplitis croceipes* memory lasted about 24 h despite spaced conditioning (Drost et al., 1986). In a few specialist species, on the other hand, spaced learning was needed for memory formation. In *C. rubecula*, for example, three massed learning trials failed to induce learning after 24 h (Smid et al., 2007), which is similar to results in *C. vestalis*. Thus, massed learning in *C. vestalis* might not induce LTM formation and spaced learning trials could be needed for LTM formation.

Memory formation and host distribution pattern

In the natural environment insects will not encounter a controlled ITI. Spaced trials could reflect a longer interval between encounters of scarce hosts or food sources and therefore require memory formation. Massed learning, on the

other hand, could reflect a situation where the parasitoid encounters the host on a regular basis in bigger batches. Hence a memory lasting more than 24 h would be of little use for a species that experiences regular encounters with a host (Kaiser et al., 2003). Bleeker (Bleeker et al., 2006) argued that differences in the host distribution led to differences in memory dynamics between solitary parasitoids (foraging on a solitary feeding host) and gregarious parasitoids (foraging on a gregariously feeding host). A solitary parasitoid would encounter many single CT over a period of time. A gregarious one however, is more likely to encounter one massed CT, as it will encounter the host in high number patches (Hoedjes et al., 2011).

Host specialisation and the variability of odour cues

The lack of memory formation in *C. vestalis* could also be related to a high innate preference for certain HIPVs. Although a learning experience enhances the parasitoid's performance over a brief period, memory formation over a long time would be too costly. Stephens (Stephens, 1991; Stephens, 1993) argued that the variability of the environment can influence the value of an innate response. If between generations variability is low, as it would be for a specialist species, the value of an innate response would be high. This would result in a low learning rate, which has also been shown in bumblebees (Ings et al., 2009). Furthermore, if the innate preference to an odour is important it would be too costly to maintain LTM formation for a specialist, as the variability in olfactory cues will remain low.

Costs involved in memory formation have been demonstrated in *Drosophila*, where animals producing LTM died earlier than ones that only produced ARM (Mery and Kawecki, 2005). Likewise, it has been argued that learning has less impact on initially strong responses (Vet and Dicke, 1992). Therefore, the attractiveness of initially preferred odours may be enhanced through learning; while it will be neglected for "ecological irrelevant" odours (which are not part of volatiles of the host-plant complex).

For generalists on the other hand, environmental stimuli may vary or change. Therefore, learning different odour cues would increase their host searching efficiency and allow them to alter their behaviour if necessary. For generalists it would be more useful to store olfactory information. Depending on

the variability of odour cues, information could be stored in a more accessible memory, in which information can easily be modified (such as STM or MTM). This would also suggest that LTM which has been observed under laboratory conditions (with a low variability) (Collatz et al., 2006; Smid et al., 2007) might not be formed under more natural conditions. The degree of foraging specialisation can therefore not solely be responsible for, but may nevertheless influence memory formation. It appears that due to its specialisation (and thus a lower variability in HIPVs), *C. vestalis* does not rely on memory formation, and it can be argued that massed learning does not lead to an associative LTM formation in this species.

Side effects of antibiotics blocking protein synthesis

Evidence suggests that LTM formation is dependent on the synthesis of new proteins (Giurfa et al., 2009; Guerrieri et al., 2011; Chen et al., 2012). The control experiment (sugar group) did not provide evidence for a LTM memory formation in *C. vestalis*, thus, the lack of response in the parasitoids in the ACD group tested 1 d after conditioning cannot be explained by a disruption of transcription and thus inhibition of memory formation.

The process of protein synthesis has been shown to occur in critical time-windows following conditioning (Fulton et al., 2005; Fulton et al., 2008). Inhibitors of transcription and translation are thus thought to be most effective in blocking LTM when administered just before or immediately after training (Davis and Squire, 1984). In the ACD group, animals were therefore fed ACD 24 h before the experiment, and 2 h after testing (1 h for the 1 h group), to ensure that the drug had been consumed in the time frame sensitive to protein synthesis disruption (Grünbaum and Müller, 1998; Wüstenberg et al., 1998; Igaz et al., 2002), following the protocol of other studies (Tully et al., 1994; Wüstenberg et al., 1998; Collatz et al., 2006).

With the exception of testing 1 d after training, responses to the learned stimulus were similar to the sugar group at other time points. After 1 d, however, animals showed an avoidance response to the learned odour. These results were unexpected for a number of reasons. The lack of memory formation after 1 d is similar to findings in other parasitoids (Collatz et al., 2006; Smid et al., 2007), but due to the decrease in response to the learned odour over the course of time, one

would have expected no LTM memory formation and thus no dependency on RNA synthesis. Previous experiments in parasitoids (Collatz et al., 2006; Smid et al., 2007) did not test the effect of ACD at time points beyond 24 h, however, if ACD was blocking protein synthesis, responses after 2 d and 3 d should be affected as well, as the effects of this drug are irreversible (Igaz et al., 2002).

The fact that the memory impairment only took place at one time point could suggest that (ACD) had a toxic effect on the animal. Actinomycin D, like other protein synthesis inhibitors, is a highly toxic (Waksman and Woodruff, 1940, 1941; Philips et al., 1960) DNA binding molecule (Kersten et al., 1960), which has been shown to inhibit the growth of tumours (Sugiura, 1960; Malogolowkin et al., 2008; Ferrari et al., 2010), gram-positive bacteria (Waksman and Woodruff, 1942) and even reverse transcription in the HIV virus (Paramanathan et al., 2011) and fungi (Schoenian et al., 2011). Apart from blocking RNA synthesis (Kirk, 1960) by inhibiting polymerase A, an enzyme essential for DNA replication, it was further shown that ACD also inhibits cell growth, RNA loss, causes shrinking of cytoplasm and inhibits biosynthesis (Reich et al., 1962). ACD can intercalate (via a planar tricyclic phenoxazone ring) between double stranded DNA (dsDNA) base pairs (SOBELL et al., 1971) and bind to single-stranded DNA (ssDNA) (Zhou et al., 2007), thus preventing elongation by RNA polymerase. A recent study (Paramanathan et al., 2012) has found that by binding to pre-melted dsDNA, as found in transcription bubbles (destabilised duplexes, where duplex DNA is unwound at the forward end of RNA polymerase and rewound at its rear end) (Berg JM et al., 2002), ACD stops RNA synthesis and thus leads to cell death (Paramanathan et al., 2012).

Toxic effects of the drug after 1 d have been discussed in several papers (Squire and Barondes, 1970). Squire (Squire and Barondes, 1970) suggested that an impaired memory retention 1 d after training is not due to ACD inhibiting RNA synthesis needed for LTM formation, but caused by toxic effects of ACD. They have also been found to occur after 1 d in rat neurons (Rizzuto and Gambetti, 1976). Rizzuto (Rizzuto and Gambetti, 1976) demonstrated that intracranial injection of ACD resulted in a status spongiosus (fluid-filled spaces) of the white matter in the cerebrum, cerebellum, brain stem and optic nerve within 48 h after injection, caused by vacuole formation within the myelin sheath. Although it was suggested that this was a result of the inhibitiory effect of ACD, a

direct toxic effect of the drug can nevertheless not be excluded. Barondes and Cohen (Barondes and Jarvik, 1964; Cohen and Barondes, 1966) argued that the effectivity of ACD as a protein synthesis inhibitor is difficult to interpret, as concentrations suitable for affecting protein synthesis are accompanied by toxic side effects of the drug. Even smaller doses of the drug led to chromatolysis, necrosis and/or electrical abnormality (Nakajima, 1969). Hence, toxic effects could cause avoidance learning observed in the current study.

Aversive conditioning

The avoidance reaction observed at 1 d post treatment could also be caused by a taste aversive reaction (Garcia and Kimeldorf, 1957). Application of the drug in the 1 h or 2 h following treatment with the HP and the odour could have caused an aversive form of STM which was not affected by the drug's disruption of protein synthesis. This could have been induced by classical forward conditioning, as the CS (the odour) was preceding the drug. Studies in rodents or snails (Garcia et al., 1955; Sugai et al., 2007) have shown that taste aversion learning can usually be induced by only one conditioning trial and does not necessarily require a short spacing between CS and US, leading to a long-lasting avoidance.

Taste avoidance STM would, however, not explain the lack of avoidance response at 1 h testing, as so far, a delayed response in aversive taste conditioning has not been shown yet. However, the exposure of 1 h might have not been sufficient to cause an aversion, contrary to an exposure of 2 h. In this case, it could be suggested, that 2 h post exposure to ACD induced aversive STM lasting 1 d, as only long-term taste aversion memory depends on protein synthesis (Rosenblum et al., 1993; Gal-Ben-Ari and Rosenblum, 2011) and consequently the degradation of proteins (Rodriguez-Ortiz et al., 2011), but not STM (Houpt and Berlin, 1999). Tully (Tully et al., 1994) demonstrated in his study about memory formation in *Drosophila* that feeding 35 mM of the proteinsynthesis inhibitor Cycloheximide (CHX) 12-15 h before training and until 24 h after training did not lead to aversive behaviour or any deleterious effects of the drug. Deleterious effects of CHX such as death of the animal, increased after 24 h from 19% dead animals to 100% dead animals 96 h later, contrary to feeding with sucrose, which resulted in 3% and 12% dead animals, respectively (Tully et al., 1994).

In summary, the avoidance response observed in the ACD group at 1 d following conditioning could be due to an aversive STM formation. However, considering the toxicity of the drug, it is likely to be due to toxic effect occurring at 1 d, which has also been shown in other studies (Squire and Barondes, 1970).

2.4.4. Courtship behaviour

A macroglomerular complex for pheromone processing was found in *C. vestalis* males (Chapter 4). Bioassays were conducted to determine if males exhibit courtship behaviour, which can be an indicator for pheromone communication. It was further investigated if courtship behaviour is dependent on time and mating status of the animal.

C. vestalis displayed a sequence of courtship behaviour, including orientation towards the female, abdominal raise and wingfanning, and antennal contact. Courtship behaviour often includes the extensive use of the antennae and antennal contact is thought to be important for transferring a contact pheromone to the female to initiate mating (Bin et al., 1999; Romani et al., 2008). In the current study, wingfanning was the most dominant element observed during courtship behaviour and males initiated wingfanning before antennal contact with the female. It has been found that wingfanning occurs as a response to pheromone detection and increases the airflow and consequently the rate of interception of pheromone on the male antennae (Loudon and Koehl, 2000). Wingfanning can be elicited from a distance of up to 120 m from the pheromone source in male gypsy moths (Elkinton et al., 1987). This suggests that the high percentage of wingfanning behaviour could be an indicator for pheromone detection in males. Moreover, behavioural elements such as wingfanning and antennation could involve acoustical communication (Joyce et al., 2008; Villagra et al., 2011). Wingfanning produces a courtship song, which contains specific information about the male (Simmons et al., 2010; Villagra et al., 2011). The courtship song can even induce molecular changes in the female (Immonen and Ritchie, 2011). It has been shown that the material in which the animals are held can influence mating success to some extent (Joyce et al., 2008), which might have caused the low copulation rate observed in the current study. Another explanation for the low copulation rate observed could be the quality of the male. Inbreeding as a result of

laboratory culturing leads to a higher number of diploid males (De Boer et al., 2007a; De Boer et al., 2008), which produce less offspring but still exhibit courtship behaviour (De Boer et al., 2007b).

It was shown that courtship behaviour is influenced by time (McClure et al., 2007). Female parasitoids, such as, for example, the egg parasitoid *Anaphes listronoti*, have been shown to be most active during the early hours of their photo phase (Cormier et al., 1998). In the current study, however, courtship behaviour was observed in the morning as well as in the afternoon.

In the current study, courtship behaviour was influenced by mating status. Although courtship behaviour was observed in mated and virgin males, only virgin males displayed more courtship behaviour than other behaviour. Mated males have less need to find new females, so their courtship activity is lower compared to virgin males. Although courtship behaviour was less frequent in males, mated males did, however, not avoid mated females, as it has been found in fruit flies (Dukas, 2005). Virgin males showed a higher response to virgin females. This is in accordance with other studies, which found a male preference towards virgin females (King et al., 2005; McClure et al., 2007).

Antennal sensilla for pheromones

It has been shown that certain sensilla are associated with sex pheromones. In moths, sensilla trichodea have been found to contain pheromone receptors (Kaissling et al., 1978). Similar observations have been made in parasitoids. In *Trichogamma* sp, sensilla trichodea Type 4 were the most abundant and were possibly involved in pheromone detection (Barlin and Bradleigh Vinson, 1981; Hansson et al., 1991a; Olson and Andow, 1993; Amornsak et al., 1998).

In *C. vestalis* males, s. trichodea are less abundant than in females. Sensilla placodea, however, are more numerous in males (Roux et al., 2005). In *Microplitis*, it was argued that sensilla placodea could be important for mate location in males (Ochieng et al., 2000; Bleeker et al., 2004). Both parasitoid species, *C. vestalis* and *M. croceipes*, are solitary specialists and could therefore encounter females less frequently. Studies have shown that s. placodea are sensitive to HIPVs in males and females, leading to the hypothesis that males may use HIPVs in conjunction with pheromones to locate females (Vareschi, 1971).

Sensilla placodea contain several dozens of receptor neurons (Hansson, 1999), some of which might therefore be a receptor for pheromones in males and a receptor for kairomones and HIPVs in females.

In conclusion, male *C. vestalis* are likely to use a combination of sex pheromones and HIPVs to locate females. This suggest that *C. vestalis* might posses glomeruli specifically for pheromone processing. Indeed, a MGC for pheromone processing was found in male *C. vestalis* (Chapter 4).

To summarise, it has been found that in the specialist parasitoid *C. vestalis*, peripheral detection of an odour does not always lead to a behavioural response. While linalool and 1-nonanol elicited antennal responses, only linalool was learned in a positive associative context, while 1-nonanol induced avoidance behaviour in females. Olfactory information of an odour might thus be filtered according to "ecological relevance and context", and only volatiles associated with the host-plant complex induce preference learning. It was further shown that males can learn to recognise HIPVs such as linalool. The results of this study suggest that males use HIPVs to locate females. Sex pheromones could play an additional role in the detection of females. The temporal nature of memory formation in *C. vestalis* remains inconclusive. Further studies will be needed to clarify whether the lack of response after 1 d was due to toxic effects of ACD, or aversive learning.

CHAPTER 3

OPTIMISATION OF A STAINING PROTOCOL FOR *COTESIA VESTALIS* ANTENNAL LOBES AND GLOMERULI

3.1. Introduction

One central question in olfactory neurobiology is how olfactory information is represented in the glomerular activity pattern in the glomeruli in primary olfactory processing centres, such as the olfactory bulb (OB) in vertebrates and the antennal lobe (AL) in invertebrates (Christensen et al., 1996). Experiments described in Chapter 2 have shown that despite a similar antennal response to the herbivore induced plant volatile linalool, a common plant volatile (Pinto et al., 2007a), and 1-nonanol, which is not part of the HIPVs emitted from *Brassica rapae* var. *Wong bok* (Durrant, 2007), behavioural responses differed between genders. While both genders learned linalool, 1-nonanol was avoided by females avoided and not learned by males. This suggests that olfactory information in a specialist parasitoid is integrated in the brain according to ecological relevance. As a first step in determining how olfactory information is processed and stored in the brain of *C. vestalis* it is crucial that we have a clear understanding of the anatomical and morphological organisation of the AL in this species.

Numerous studies have been devoted to the exploration of neural networks in the olfactory bulb in vertebrates (Buck, 1996; Leon and Johnson, 2003) and the

AL in invertebrates (Hildebrand and Shepherd, 1997; Hansson and Anton, 2000; Galizia and Rössler, 2010). Many different approaches have been taken to reveal their organisation, including morphological (Matsumoto and Hildebrand, 1981; Flanagan and Mercer, 1989a), molecular (Dudai et al., 1976; Buck and Axel, 1991; Rützler and Zwiebel, 2005), physiological (Cook and Milligan, 1972; Kanzaki et al., 1989; Galizia and Kimmerle, 2004), and behavioural methods (von Frisch, 1919; Kuwabara, 1957; Vareschi, 1971). The aim of the current study was to identify suitable staining methods for morphological description of the AL by adapting staining methods used in other insects for the use in *C. vestalis*.

Histology

Much of our knowledge about the structure and organisation of the nervous system has been derived from histological staining techniques (Cajal, 1906; Golgi, 1906). These staining techniques are generally used to reveal the morphology of single neurons, or groups of sensory or motor neurons and sections through the nervous system. A common approach is the cobalt staining method where the dye is injected intracellularly or backfilled through cut axons (Pitman et al., 1972; Newland, 1990). This method is sufficient to reveal larger fibres or soma, but is often not able to produce a more detailed resolution of dendritic branches (Davis, 1982). Staining intensity can be enhanced by additional silver staining, a method that was first established by Timm (Timm, 1958). Initially adapted from Strausfeld (Strausfeld and Obermayer, 1976) and Bacon (Bacon and Altman, 1977), this combined method has been frequently used to stain neurons (Tyrer and Bell, 1974; Bacon and Altman, 1977; Pflüger, 1980; Davis, 1982; Pflüger et al., 1988; Todd et al., 1995; Strausfeld and Okamura, 2007).

Fluorescence

Fluorescent staining complements the fundamental power of light microscopy in the ability to analyse spatial and functional distribution of cellular components (Agard et al., 1989). The great advantage of fluorescence labelling is the possibility of simultaneously observing cellular components in live tissue, which have been stained with markers of different emission wavelength to identify their spatial and temporal distribution (Stemmer et al., 2008), as well as intense and selective staining of neurons in live tissue (Strausfeld and Hausen,

1977). Fluorescent staining procedures have developed from the use of proteins and lipids as fluorescent markers (Taylor et al., 1984) to immuno-fluorescence methods using tagged antibodies (Leitch and Laurent, 1996; Laissue et al., 1999; Berg et al., 2002; Skiri et al., 2005). Fluorescence is used widely, from morphological studies (Smid et al., 2003; Skiri et al., 2005) to measuring membrane potential (Ross et al., 1977), or intracellular pH (Paradiso et al., 1987). Since the 1970s, axon tracing has been carried out using dyes such as Lucifer Yellow (Stewart, 1981), the dialkylcarbocyanine dye Dil (1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate) (Honig and Hume, 1989) or Procion dyes (Stretton and Kravitz, 1968) .

Fluorescent dyes such as the voltage sensitive dye RH795 or Neutral Red have been frequently used to label glomeruli in ALs in bees (Galizia et al., 1997; Sachse et al., 1999; Dupuy et al., 2010). They are also applied after calcium imaging with Calcium Green 1-AM, as Calcium Green 1-AM does not stain glomerular borders and an identification of glomeruli with this dye alone would not be possible (Galizia and Vetter, 2005).

Microscopy

The fluorescence widefield microscope brought new possibilities to morphological studies (Fonta et al., 1993). Its principle is to excite the fluorescently labelled preparation with a short wave length from a light source and filter the emitted light (Lichtman and Conchello, 2005). Software products associated with widefield microscopy made it possible to create 3D images of the stack sections. However, focus problems due to the thickness of the preparation as well as fluorescence from other parts of the preparation can result in unclear structure and loss of detail (Amos and White, 2003), especially for morphological studies. This means that regardless of where the microscope was focussed, illumination causes the entire preparation to emit light. This problem can be overcome by using construction of the confocal laser scanning microscope. Although it was invented by Minsky in 1955 (Minsky, 1988), it was only in the late 1970s that it was investigated again by Brackenhoff (Brakenhoff et al., 1986; Brakenhoff et al., 1989), as in earlier years, confocal laser scanning microscopy (CLSM) was limited by the lack of fast processors, storage devices and also by expensive lasers (Amos and White, 2003).

The principal of CLSM is the illumination of an object with a laser beam, with only signals from this spot and the focal area being detected. Illumination is further regulated by a pinhole narrowing the fluorescent light. This reduces the out of focus structure interference experienced with widefield microscopy (Sheppard and Wilson, 1982) resulting in a clearer visualisation. Furthermore, the thickness of the sections can be regulated as needed (Földes-Papp et al., 2003), and the same preparation can be sectioned at different intervals. Optical sections can then be fused into digitised 3D reconstructions with the help of software programmes (Földes-Papp et al., 2003).

3D reconstructions

Advances in microscopy and software have also created more possibilities in the 3D reconstruction of optical sections of the brain, which enables the construction of 3D standard brain maps. These are a suitable tool to study the spatial relationship between neurons innervating different brain structures. Morphological maps of the brain structure of invertebrates were created to identify glomeruli and compare physiological results at a glomerular level between individuals and across species, including two parasitoid species, *Cotesia glomerata* and *C. rubecula* (Flanagan and Mercer, 1989a; Galizia et al., 1999a; Laissue et al., 1999; Berg et al., 2002; Sadek et al., 2002; Smid et al., 2003; Greiner et al., 2004; Kurylas et al., 2008; El Jundi et al., 2009).

Signal measurements in the brain

A thorough understanding of brain function not only requires morphological characterisation of neurons but also of their physiological properties. Electrophysiological recordings followed by dye labelling of neurons for light microscopy have brought great insight into the functional properties and morphology of neurons (Matsumoto and Hildebrand, 1981; Tolbert et al., 1996; Newland et al., 2000). Recordings have been conducted in many studies analysing olfactory information processing in insects, measuring signals at the sensilla on the antennae (Anderson et al., 1995; Todd et al., 1995), olfactory receptor neurons (Rostelien et al., 2000; De Bruyne et al., 2001; Marshall et al., 2010), projection neurons (Flanagan and Mercer, 1989b; Kanzaki et al., 1989; King et al., 2000; Sadek et al., 2002), or interneurons (Suzuki and Tateda, 1974; Burrows et al.,

1982; Fonta et al., 1993). Recordings have further been conducted in other brain areas such as the MBs (Homberg, 1984; Schildberger, 1984; Gronenberg, 1986), the optical lobes (Okamura and Strausfeld, 2007; Strausfeld and Okamura, 2007; Strausfeld et al., 2007), the unpaired median neuron maxillare 1 (VUMmx1) in honeybees (Bräunig, 1991), the dorsal unpaired median neuron (DUM) in locusts (Burrows et al., 1982), or motor neurons (Galizia et al., 1997).

In studies of primary olfactory centres, imaging has found increasing use, as it allows the analysis of the spatiotemporal patterns of signalling pathways in several glomeruli simultaneously. This is based on detecting either changes in trans-membrane voltage, using dyes such as RH795 (Galizia et al., 1999c; Sachse and Galizia, 2002; Galizia and Vetter, 2005; Deisig et al., 2006), or measuring changes in intracellular ions such as calcium using calcium sensitive dyes (Wachowiak and Cohen, 2001). Calcium imaging studies investigating glomerular activity patterns in olfactory integration centres such as the olfactory bulb, or the AL during odour application found a wide use, from vertebrates (Kauer, 1988; Friedrich and Korsching, 1997; Rubin and Katz, 1999; Wachowiak and Cohen, 2001; Wachowiak et al., 2002; Wachowiak and Shipley, 2006) to invertebrates (Joerges et al., 1997; Galizia et al., 1999b) and have been frequently combined with electrophysiological analyses (Cayre et al., 1999; Stosiek et al., 2003; Galizia and Kimmerle, 2004; Carlson and Coulter, 2008). Calcium sensitive dyes, in particular, have gained in popularity for studies in vertebrates (Kauer, 1988; Cinelli et al., 1995; Friedrich and Korsching, 1997; Cossart et al., 2003; Cossart et al., 2005; Ohki et al., 2005), as well as invertebrates (Joerges et al., 1997; Faber et al., 1999; Galizia et al., 1999b; Galizia et al., 1999c; Galizia and Vetter, 2005). Bath applied calcium sensitive dyes can be loaded into neurons without injection, providing a less invasive method than electrophysiological studies (Aston-Jones and Siggins, 1995).

The aim of the current study was to optimise the methods used for dissection, mounting and staining, as well as choosing the right microscope and dye, to find the optimum approach to investigate the morphology of the AL and its functional units, the glomeruli (Chapter 4). A further aim was to optimise the loading protocol for a dye used in calcium imaging studies (Chapter 5).

3.2. Materials and methods

3.2.1. Insect preparations

Mounting of Asobara tabida

At the start of the project *C. vestalis* were not available, as the *C. vestalis* and *P. xylostella* culture first had to be established. The parasitoid species *A. tabida* was therefore used to become acquainted with handling methods. Insects were anesthetised with CO_2 and inserted through a 100 µl pipette tip (the base of the tip was cut off so that the top end was reduced to the size of 1 cm) and their mouth parts fixed to the tip with super glue (Loctite, Precision Superglue, Henkel Loctite, UK). Once the glue was dry, the insect was placed under a dissecting microscope, and the cuticle of the head was carefully cut off to expose the brain. For this procedure, different instruments were tested, including tweezers, needles and a scalpel. A small blade fixed in a blade holder (World Precision Instruments, USA) proved to be the most efficient tool for removal of the cuticle.

Mounting of C. vestalis

To serve as an insect holder, the base of a 1 ml pipette tip was cut off so that the top end was reduced to the size of 1 cm. After insects were anesthetised with CO_2 they were pushed through the tip. The antennae were gently extracted from the tip and the mouthparts fixed with super glue (Loctite) to the tip to stabilise the head (Fig. 3.1 A). Once the glue was dry the insect was placed on the stage of an upright dissecting microscope, the cuticle of the head carefully removed and the ALs exposed (Fig. 3.1 B, D). Complete removal of the cuticle revealed the ALs, optical lobes, and mushroom bodies (Fig. 3.1 C).

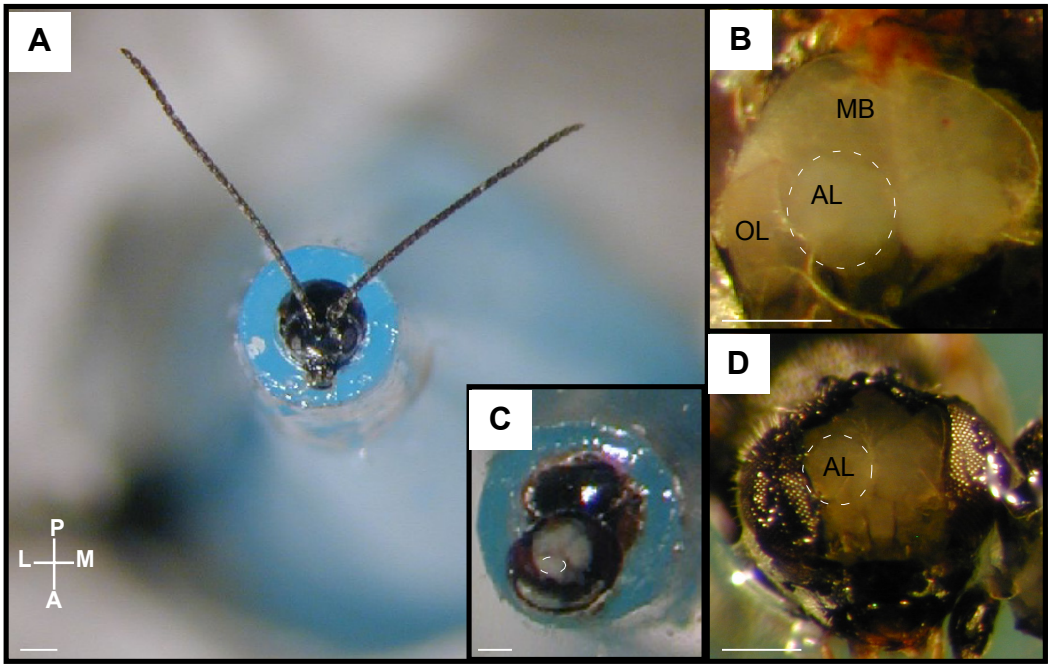

Fig. 3.1 Mounting procedure for *C. vestalis*. **A)** *C. vestalis* was fixed in a pipette tip. This fixation method ensures a successful dissection of the brain, as the head remains stable (glued to the tip). **B)** *C. vestalis* with removed cuticle, the two ALs became visible (circled areas in B, C and D indicate the right AL). **C+D)** View onto the brain. OL= Optical lobe, MB= Mushroom body. Scale bar= 200 µm.

Plexiglas stage for insect mounting

Two different types of acrylic blocks (2 x 2 x 2 cm) were constructed to position the mounted animal during *in vivo* imaging, following previous designs of mounting blocks used for *Drosophila* (Fiala and Spall, 2003) (Fig. 3.2). The first version provided an acrylic block, with a hole (5 mm diameter, 7 mm depth) in the middle of the block, into which the animal mounted in the pipette tip was placed (Fig. 3.2 A, C). The second version consisted of a block with two thin grooves onto which the animal was glued with abdomen facing downwards (Fig. 3.2 B, D). In both cases the head was covered with a cover slip to prevent desiccation of the brain.

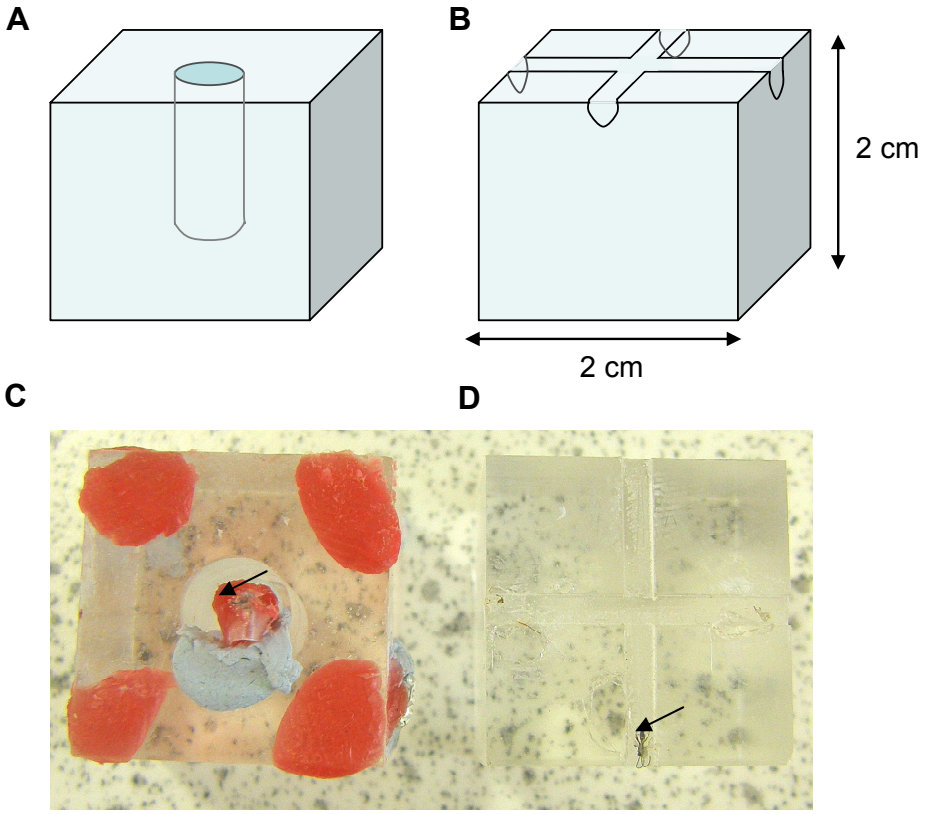

Fig. 3.2 The Plexiglas stages serving as parasitoid holder. Acrylic blocks with **A)** A hole into which the insect mounted into a pipette tip was placed. **B)** 2 grooves. The parasitoid was glued into one of the groove, abdomen downwards. **C)** Dorsal view onto the stage with the hole. Inside is the insect mounted in a tip (black arrow), stabilised with Blu-Tack. **D)** Dorsal view of the holder containing grooves with the parasitoid (black arrow).

3.2.5. Fixing the antennae

For calcium imaging experiments it is essential to keep the antennae intact, dry, and restrained in their movement. First, soft wax was placed onto the side of the pipette tip containing the mounted insect. A parafilm (1-2 mm width) strip was gently placed over the head, holding the antennae down to the front and its ends fixed onto the soft wax. The parafilm was gently pulled to the front over the antennae to reveal the cuticle just behind the base of the antennae. Gaps between the preparation and the parafilm were filled with soft wax to prevent saline reaching the antennae. This created a small incubation chamber for the dye surrounding the head capsule.

3.2.6. Exposing the brain

To expose the brain an incision was made at the base of the antennae, followed by cuts to the side of the compound eyes. The final cut was made parallel to the antennae, just above the ocelli, and the cuticle removed to expose the brain. Saline was immediately applied to the brain to prevent desiccation. The brain was rinsed several times to remove excess enzymes, which could destroy brain structures as suggested (Galizia and Vetter, 2005). Tracheal tissue in the brain cavity had to be carefully removed from the ALs. All dissections were conducted using a blade fixed to a blade holder.

3.2.7. Staining procedures

The dyes used in this study were selected because of their great success in experiments with other insects (Galizia and Vetter, 2005). However, due to the much smaller size and different anatomy of *C. vestalis* the staining procedure had to be optimised for this species. Three different staining methods were investigated in this study: the calcium sensitive dye Calcium Green 1-AM, the cell membrane dye RH795 and the intracellular dye Neutral Red.

For dye loading experiments, as well as the brain morphology, the head was cut off and placed in an incubation chamber which consisted of a slide with a small chamber made of Blu-Tack (Fig. 3.3). The dyes were bath applied to the brain and a cover slip placed onto it. Afterwards, the preparation was incubated in the dark for 1 h to prevent bleaching and to allow the dye to penetrate.

Staining with Calcium Green 1-AM

The protocol was adapted from a dye loading procedure applied in bees (Galizia and Vetter, 2004) using the green fluorescent dye Calcium Green 1-AM (Calcium Green™-1-AM, cell permeant, Molecular Probes, Invitrogen, UK).

The dye (50 µg) was diluted in 50 µl Pluronic F-127 (Molecular Probes, Invitrogen, UK), which is a non-ionic detergent useful for solubilising large dye molecules to facilitate cell loading. After vortexing for 1 min, the dye was then diluted in 800-900 µl saline solution (Locust saline: 140 mM NaCl, 10 mM KCl, 4 mM $CaCl_2$, 4 mM $NaHCO_3$, 6 mM NaH_2PO_4 in 5 l) and vortexed for 1 min. The

dye (50 µl; 40 µM) was bath applied to the brain and a cover slip placed over it. The preparation was placed in a humid polystyrene box and placed in an incubator at 13°C for 45 min to enable the dye to penetrate deep into the brain. The low temperature is thought to prevent intracellular esterase activity (Galizia and Vetter, 2005). Following this, the preparation was placed at room temperature for 15 min to promote cleavage of the acetoxymethyl from the dye. Finally, the brain was rinsed three times with 50 µl saline solution to remove excess dye.

Staining with RH795

To increase dye penetration (Sachse and Galizia, 2002) protease (Type XIV, Sigma-Aldrich, UK) dissolved in saline solution was applied to the brain for 3 min after removing the cuticle. The head was removed from the body and placed onto a cover slide holding a small incubation chamber made of Blu-Tack. Before applying the dye RH795 (20 µM), the brain was washed with saline. During an incubation period of 45 min to 1 h, the preparation was placed in the dark to prevent bleaching. Brains were washed three times with 50µl saline solution before viewing under a microscope.

Staining with Neutral Red

After removing a patch of cuticle on the head, insects were stored in 4% paraformaldehyde (PAF) or 2-4% glutaraldehyde over night at 4°C. The preparation was rinsed after 24 h with PBS (0.1 M) three times (5 min each) and was then placed in Neutral Red (Sigma-Aldrich, UK) (on a shaker) for 3 h. After the staining procedure the preparation was again washed in PBS three times (5 min each). This was followed by dehydration in an alcohol series: (50%, 70% 5 min each; 90%, 95% each 10 min and 100% with molecular sieve granules (UOP Type 3A, Fluka-Sigma-Aldrich, UK), three times for 5 min each). The insects were then placed into methyl salicylate for at least 48 h at -18°C to clear the brain tissue (Skinner, 1986).

The dissected brain was then mounted in DPX (Pathlore, UK) or Fluoromount (Sigma-Aldrich, UK) on a cover slide, and a cover slip separated by imaging spacers was placed over the preparation to avoid crushing the brain.

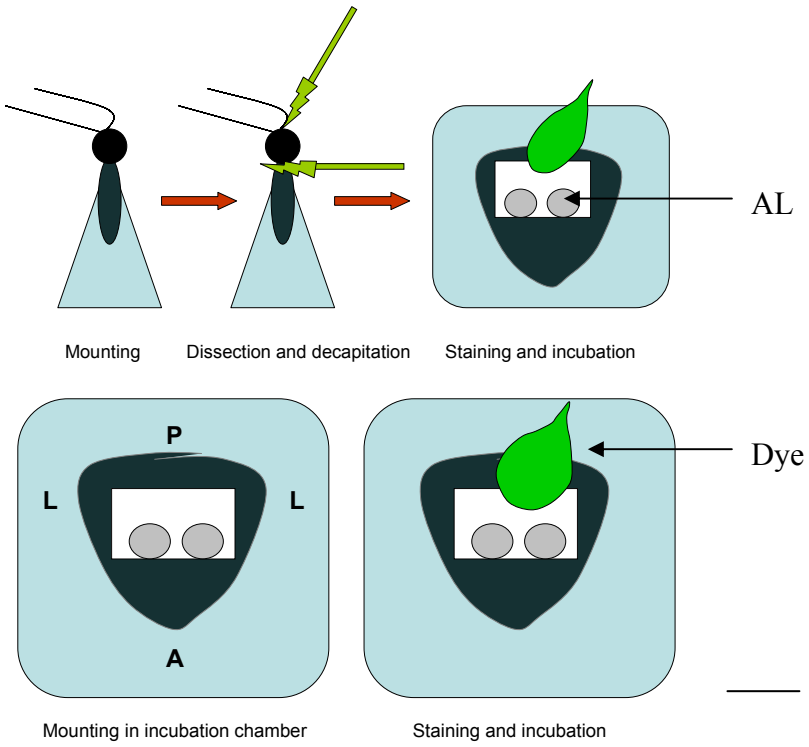

Fig. 3.3 Mounting and staining procedure of *C. vestalis*. After the insect was mounted in the top end of a pipette tip, the cuticle was removed and the head separated from the body. The open head capsule was placed into an incubation chamber and the dye applied. Brains are always viewed from dorsal. A= Anterior, L= Lateral, P= Posterior. These orientation markers are indicating the orientation of the AL in brain images obtained through microscopy in Chapter 3, 4 and 5. Scale bar= 200 µm.

3.2.8. Microscopy

Widefield microscopy

Images were taken with a 12 bit high-resolution camera (Roper Scientific, USA) attached to an upright microscope (Nikon E800) in the epi-fluorescence mode (Excitation: 460-500 nm, Emission: 510 nm long pass filter). Images and stack series were processed using MetaMorph software (Version 6.2., Molecular Devices, UK).

Confocal laser scanning microscopy

A confocal microscope (Leica TCS SP2 FCS MP) was used for the imaging procedure (Table 3.1). For preparations stained with Calcium Green 1-AM, an argon laser line with 488 nm excitation was used (Emission: 500-600 nm). Images were processed using the Leica LCS Software program. For brains stained with

RH795 an argon laser with an excitation of 514 nm was used (Emission: 590-720 nm). For brains stained with Neutral Red a solid state laser with an excitation of 561 nm was used for the imaging procedure (Emission: 550-650 nm).

For a comparison of the staining quality between the dyes using wide field and confocal microscopy the relative frequency of visibility was calculated by counting in how many preparations glomeruli were identifiable. For a quantitative dye analysis, mean, minimum, and maximum Δ-fluorescence for each dye was calculated from the brain stack series. To calculate the Δ-fluorescence the mean of the background fluorescence was subtracted (obtained through selecting a region of interest outside the preparation) and divided from the stack series using the Leica software tool Arithmetic and Boolean logic – Image and constant/Arithmetic with world coordinates.

Table 3.1 Excitation and emission rates in nanometre (nm) of fluorescent dyes used in the experiments.

Dye	Excitation	Emission
Calcium Green 1-AM	488 nm	500-600 nm
RH795	514 nm	590-720 nm
Neutral Red	561 nm	550-650 nm

3.2.9. Survival times of insects mounted on acrylic blocks

To investigate the viability of animals with a dissected brain their survival over time was measured. Therefore, insects were mounted on a Perspex stage as previously described (Section 3.2.4.) and their brain dissected. Survival was verified every 5 min by observing antennal movements, as well as reaction to the mechanical stimulus of touching the antennae with a pin. This served as a preliminary experiment to produce a protocol to promote the viability of the parasitoid during *in vivo* calcium brain imaging experiments (Chapter 5). For statistical analysis a general linear model (GLM) was conducted (SPSS Inc. 17.0).

3.3. Results

3.3.1. Mounting and dissection methods

Calcium imaging experiments are performed over a period of approximately 2 h, thus it is important to ensure the viability of animals. The mean survival time of insects mounted in the block with a hole was 129 ± 30 min (mean ± SEM; n= 10), while those mounted on a block with grooves had an average survival of only 50 ± 15 min (mean ± SEM; n= 11) (Fig. 3.4). There was a significant difference between the two methods (GLM: $_1F_{20}$=5.413, $p<0.05$) (Fig. 3.4). Therefore, Perspex stages with a hole became the preferred choice for further calcium imaging experiments (Chapter 5) due to the extended survival period.

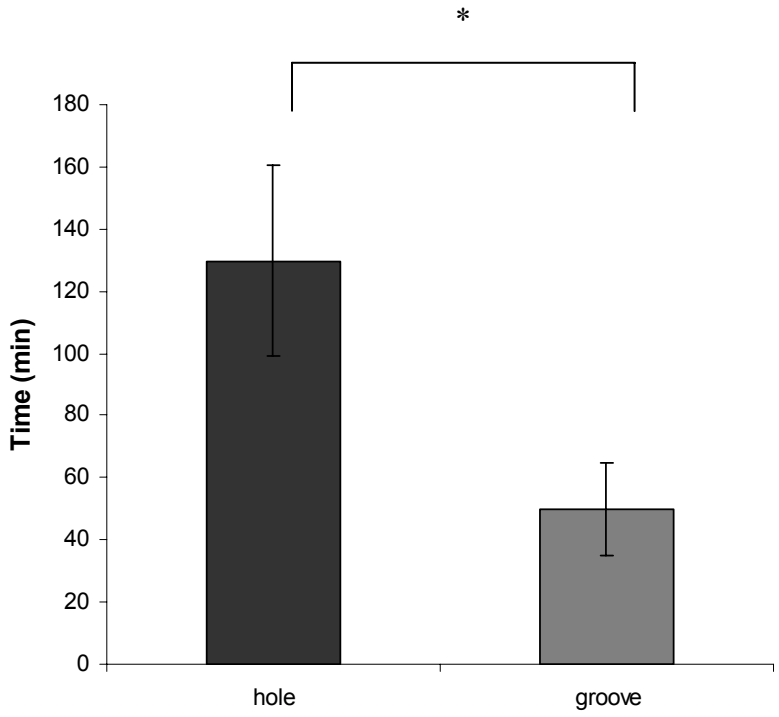

Fig. 3.4 Survival time of parasitoids mounted in acrylic blocks containing a hole or groove. (hole: n= 10; groove: n= 11). There was a significant difference between the two methods (* indicates a significant difference of $p<0.05$).

3.3.2. Evaluation of fluorescent dyes using confocal and fluorescence widefield microscopy of the antennal lobe of *C. vestalis*

The suitability of fluorescence widefield and confocal microscopy was compared using three fluorescent dyes for brain morphological studies.

3.3.2.1. Evaluation of fluorescence widefield microscopy and CLSM using Calcium Green 1-AM

Calcium Green 1-AM is widely used during *in vivo* imaging studies in insects (Galizia et al., 1997; Joerges et al., 1997; Galizia et al., 1999c). To ensure that dye loading with the current protocol was successful in parasitoid ALs, differences between stained and unstained preparations were investigated.

With fluorescence widefield microscopy the ALs, as well as the mushroom body complex, were readily identifiable without staining (Fig. 3.5 A), due to the autofluorescent nature of the brain tissue. However, the preparation showed a weaker fluorescence (n= 5) compared to preparations labelled with a fluorescent dye.

Initial experiments using the dye Calcium Green 1-AM without the addition of the non-ionic detergent Pluronic F-127 proved to be unsuccessful and were similar to unstained preparations (n= 4) (Fig. 3.5 B). Calcium Green 1-AM dissolved in Pluronic F-127 resulted in fluorescent staining of the brain in which clear anatomical structures were present (Fig. 3.5 C). Thus, Pluronic F-127 appeared to be essential when using Calcium Green 1-AM and was used for all following brain stainings.

The ALs were located below the antennae and detectable as round structures. They were distinguishable from the other brain parts. Its sub-units, the glomeruli, were small, round structures. Boundaries of glomeruli were only visible in 17 out of 65 preparations (relative frequency 22%; n= 65). Additionally, some preparations required a higher exposure time (up to 10 s) to obtain an illuminated image of the brain. Furthermore, because of the small working distance of the objective, it was impossible to measure the complete depth, and sections into deeper regions of the AL resulted in a blurred image. Hence, a full morphological description of the AL was not possible.

With CLSM, glomeruli were only visible in 23% of preparations (relative frequency; n= 23) (Fig. 3.5 D) using the dye Calcium Green 1-AM; a similar outcome as obtained with fluorescence widefield microscopy. However, using CLSM enabled depth measurements of the ALs by optical sectioning, though a detailed description of the AL was not possible, due to a lack of detail in the glomerular structures.

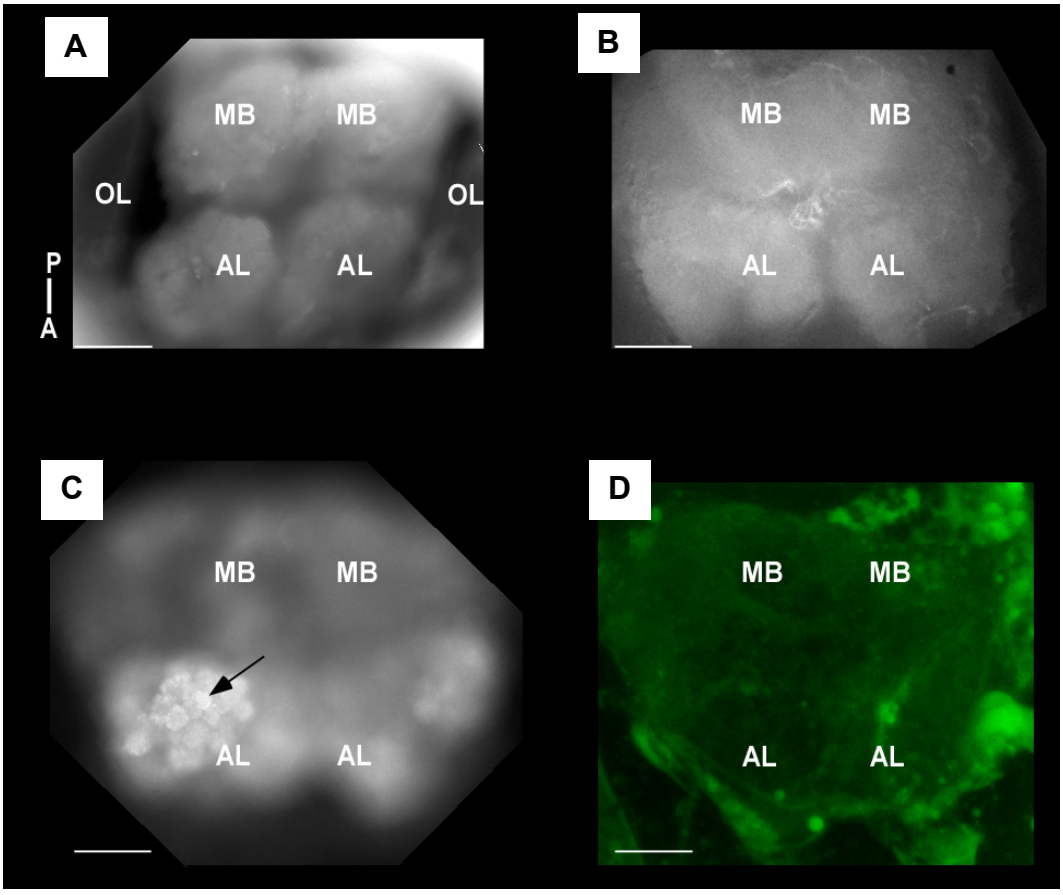

Fig. 3.5 Comparison of widefield microscopy and CLSM using Calcium Green 1-AM. A) View of a male brain of *C. vestalis* without staining using widefield microscopy. The ALs are visible, however the glomeruli are less defined. The mushroom body complex is easily distinguishable in the posterior area and does not consist of distinct structures. **B)** Calcium Green 1-AM staining without Pluronic F-127 using widefield microscopy. The AL outlines and the mushroom body complex are visible. However, no glomerular borders can be seen. **C)** Staining with Calcium Green 1-AM diluted in Pluronic F-127 using widefield microscopy. Glomeruli (arrow) are very distinctive in the upper regions of the AL. Due to the thickness of the preparation it was not possible to get clear images simultaneously of both sides of the brain. **D)** Average stack projection of a brain stained with Calcium Green 1-AM using confocal microscopy. Mushroom body and AL borders are hard to locate and glomeruli are not identifiable. A= Anterior, P= Posterior, AL= Antennal lobe, MB= Mushroom body, OL= Optical Lobe. A-C) Scale bar= 100 µm. D) Scale bar= 50 µm.

3.3.2.2. Evaluation of fluorescence widefield microscopy and CLSM using RH795

Using fluorescence widefield microscopy to evaluate preparations stained with the dye RH795 resulted in a higher visibility of glomeruli than staining with Calcium Green 1-AM (relative frequency: 72%; n= 18). However, due to the thickness of the preparation, as well as blurred image resolution in deeper regions of the AL, it was not possible to determine the actual depth of the preparation, nor borders of glomeruli. Furthermore, background fluorescence, caused by fluorescence from the deeper regions of the preparation (Fig. 3.6 B), made it more difficult to distinguish glomerular borders.

With CLSM, 57% of glomeruli were visible (relative frequency; n= 61). Figure 3.6 shows images of the same preparation, using confocal and widefield microscopy. Using CLSM provided better images in terms of contrast of the glomerular borders, as well as out-of-focus interference. The image resolution with CLSM (Fig. 3.6 A) showed a clear improvement of structure detail around the glomerular borders in both ALs simultaneously, and background fluorescence was eliminated with a confocal microscope. The glomerular borders were more definite, especially when focussing along the z-axis (Fig. 3.7 A-D). Moreover, some preparations showed more detailed staining (Fig. 3.12 C, D), where the outlines of some glomeruli appear brighter. This is probably caused by more intensely stained interneurons, which absorbed more of the dye. CLSM had the advantage that depth measurements obtained through optical sections of the AL could be performed. This provides a 3D description of the brain structures for future experiments (Chapter 4).

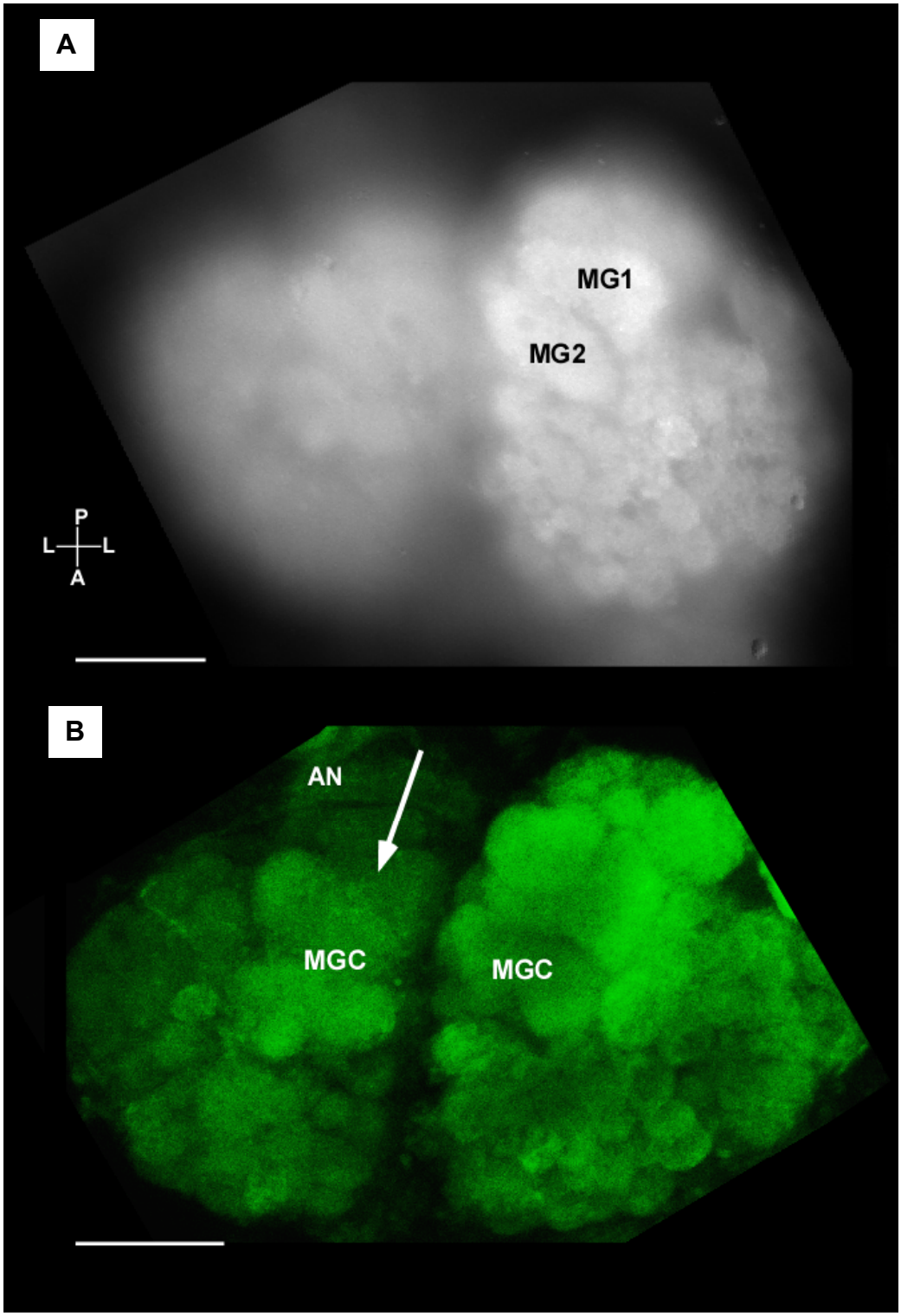

Fig. 3.6 Left and right male ALs stained with RH795. A) Widefield microscopy image of the same preparation in the upper region of the AL. Two enlarged glomeruli, so called macroglomeruli (MG1, MG2) are visible in the left AL. However, due to focus problems, the right AL is blurred and glomeruli can not be identified. A= Anterior, P= Posterior. Scale bar= 50 µm. **B)** CLSM image of the average stack projection of the AL shows a better resolution and glomerular borders are better emphasised in the left and right side. There is no out-of-focus halo as seen in B. Additionally, the antennal nerve entrance (AN) into the AL is visible on the right AL above the macroglomerular complex (See Chapter 4 for a more detailed description of the AL) (MGC; indicated by the white arrow).

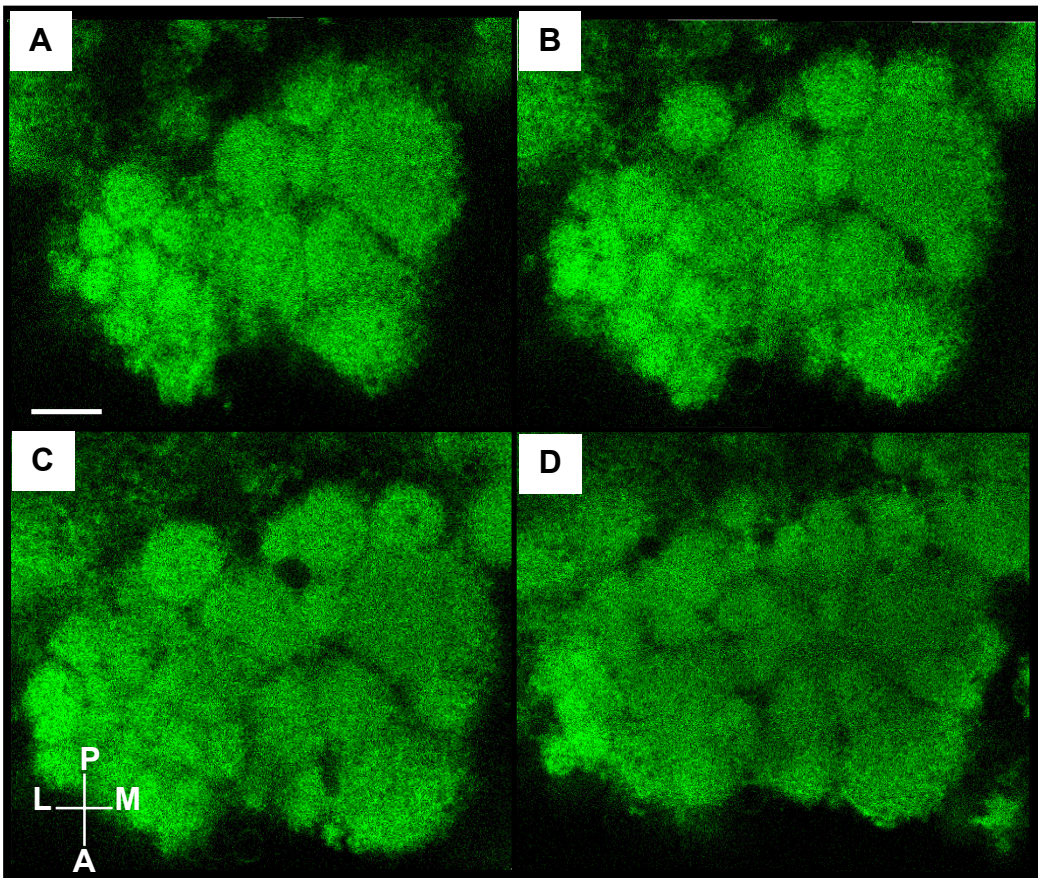

Fig. 3.7 Evaluation of CLSM using RH795. Optical sections through a right male AL. Glomerular borders remain visible even in deeper planes. Sections were taken at different depths from top **(A)** to bottom **(D)** of the AL. **A)** 16.2 µm. **B)** 19.8 µm. **C)** 25.2 µm. **D)** 32.4 µm. A= Anterior, L= Lateral, M= Medial, P= Posterior. Scale bar= 20 µm.

3.3.2.3. Evaluation of CLSM using Neutral Red

The dye Neutral Red was tested with CLSM only, as this microscope has been identified as the better option for morphological analyses (Section 3.3.2.2.). Neutral Red is a fluorescent dye used for intracellular staining of neurons (Evans and O'Shea, 1978; Matsumoto and Hildebrand, 1981; Honegger et al., 2002; Sandoz, 2006; Dupuy et al., 2010). Glomeruli were visible with a relative frequency of 31% (n= 13). Fluorescence signals were detected throughout the AL; however, glomerular borders were only obvious in the upper sections of the preparation (Fig. 3.8 A, B). With increasing depth, no glomerular borders were distinguishable (Fig. 3.8 C, D). The AL appeared as one fused structure, which might be caused by the fixation process, rather than the actual staining procedure

(Fig. 3.8 D). Moreover, in some preparations the fixation process caused ripping of the AL in upper and deeper sections (Fig. 3.9 A-D).

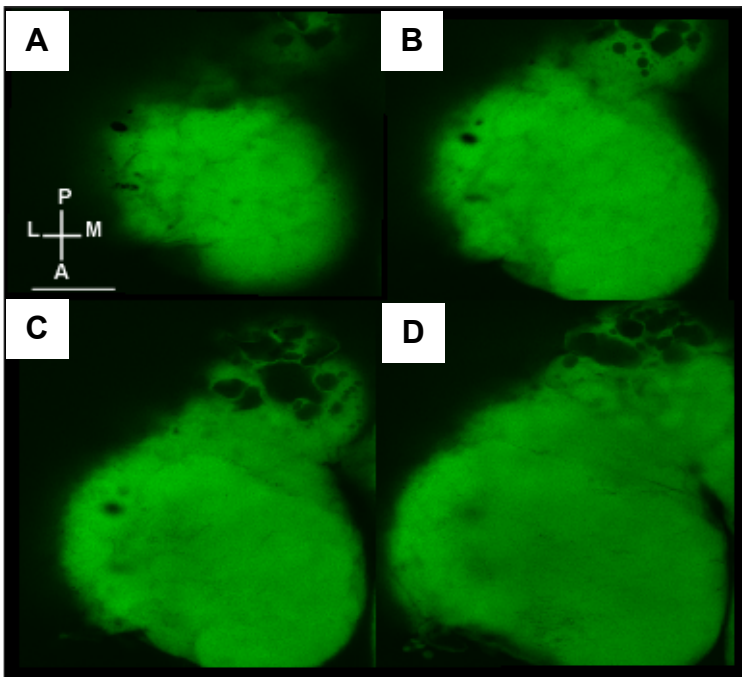

Fig. 3.8 Fluorescent staining with Neutral Red of a female right AL from top (A) to bottom (D). The outlines of glomeruli were in general less clear with this dye and became blurred with increasing depth (C, D). Sections were taken at different depths from top (A) to bottom (D) of the AL. **A)** 7 µm. **B)** 9 µm. **C)** 12 µm. **D)** 18 µm. A= Anterior, L= Lateral, M= Medial, P= Posterior. Scale bar= 50 µm.

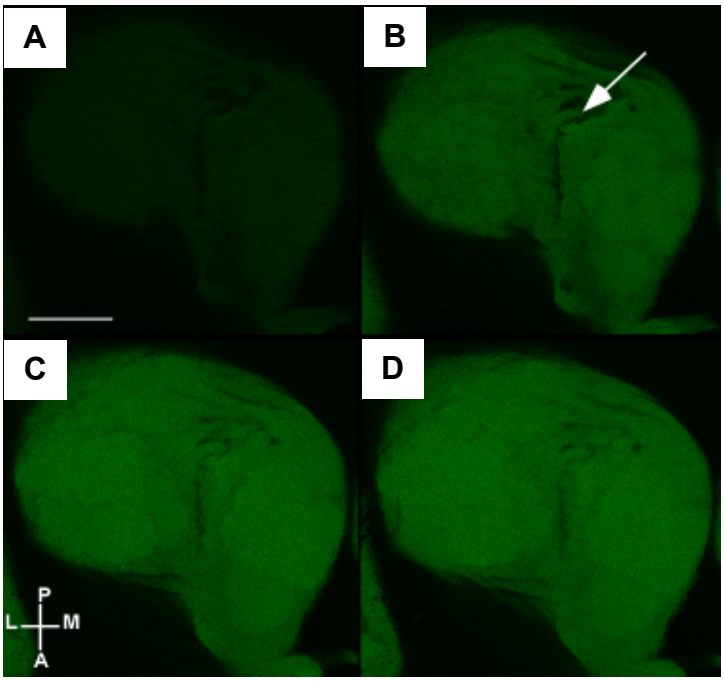

Fig. 3.9 Fluorescent staining with Neutral Red of a male right AL from top (A) to bottom (D). The preparation shows a fissure in the tissue in some parts of the AL. Sections were taken at different depths from top (A) to bottom (D) of the AL. **A)** 1 µm. **B)** 3 µm. **C)** 5 µm. **D)** 9 µm. A= Anterior, L= Lateral, M= Medial, P= Posterior. Scale bar= 50 µm.

3.3.2.4. Quantitative analyses of the dyes Calcium Green 1-AM, RH795, and Neutral Red

Size comparison

To compare the three dyes with each other, brain measurements obtained using confocal microscopy were analysed. The results showed that preparations stained with Neutral Red were smaller compared to those stained with Calcium Green 1-AM or RH795. Both Calcium Green 1-AM and Neutral Red had less fluorescence in deeper regions and therefore a smaller depth size. A statistical comparison of the size of the brain structures revealed that preparations stained with Neutral Red were significantly smaller than those stained with RH795 (Fig. 3.10). It showed a significant difference between the RH795 and Neutral Red in AL length (mGLM: $F_{2,61}$= 5.415, $p<0.05$) and depth (mGLM: $F_{2,61}$= 12.887, $p<0.05$). No significant difference in the dimensions of glomeruli was found.

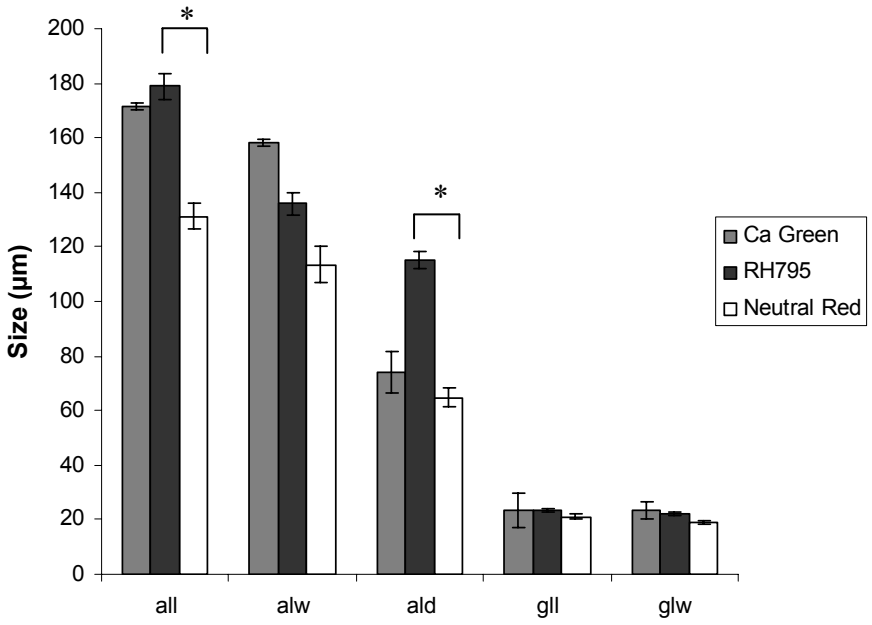

Fig. 3.10 Comparison of brain structures viewed with confocal microscopy and stained with Calcium Green 1-AM, RH795, and Neutral Red. Calcium Green 1-AM (Ca Green): all/alw: n= 9, d: n= 14; gll/glw: n= 4; RH795: all/alw: n= 50, d: n= 85; gll/glw: n= 98; Neutral Red: all/alw: n= 17, d: n= 29; gll/glw: n= 10. * indicates a significant difference in AL length and depth between RH795 and Neutral Red ($p<0.05$). all= antennal lobe length; alw= antennal lobe width; ald= antennal lobe depth; gll= glomerular length; glw= glomerular width.

Fluorescence labelling intensity

To determine the efficiency of fluorescence labelling, the relative fluorescence value changes (Δ-fluorescence (ΔF/F)) of preparations stained with Calcium Green 1-AM, RH795 and Neutral Red were analysed. The mean and minimum intensities were very similar for Calcium Green 1-AM and RH795 when observed through fluorescence widefield microscopy. (Fig. 3.11 A). With CLSM, the mean (ANOVA: $F_{2,32}$= 8.714, $p<0.05$), minimum (ANOVA: $F_{2,32}$= 7.555, $p<0.05$) and maximum (ANOVA: $F_{2,32}$= 9.658, $p<0.05$) intensities between the dyes were significantly different. The minimum intensity was lower in all dyes compared to fluorescence widefield microscopy (Fig. 3.11 B). From all dyes, RH795 showed the highest mean and peak intensity with both microscopes. In confocal microscopy, only one image plane at a time is scanned. Hence no additional fluorescence from other regions interferes with the scanned focus plane. It is also possible to scan deeper into the preparation where fluorescence of the preparation is lower. Therefore, the minimum intensity with confocal microscopy was lower than with fluorescence widefield microscopy.

In summary, RH795 in combination with CLSM provided better quality images in terms of visibility of glomeruli and depth analysis of the AL (Fig. 3.12), and will be used in the following Chapter (Chapter 4) for a more detailed description of the brain morphology of *C. vestalis.*

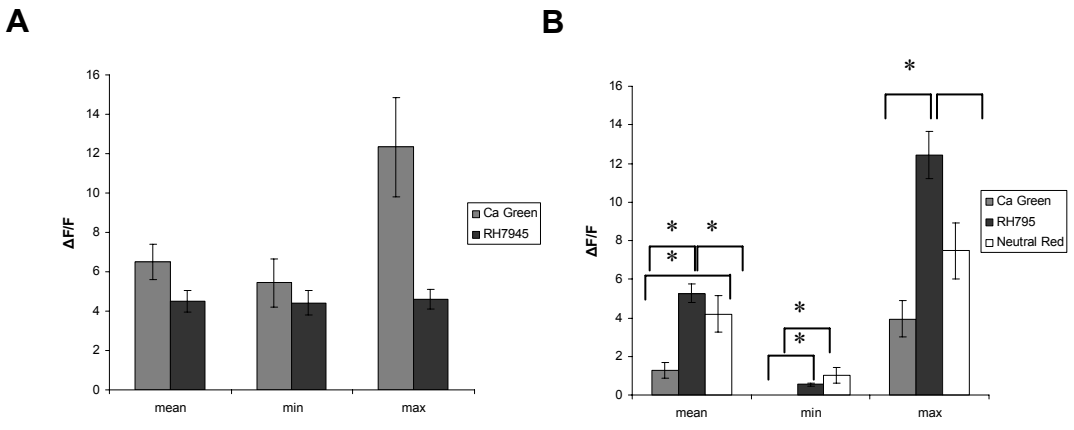

Fig. 3.11 Comparison of ΔF/F intensity between different dyes. A) Fluorescence widefield microscopy: Calcium Green 1-AM (Ca Green): n= 3; RH795: n= 2 and **B)** CLSM: Calcium Green 1-AM (Ca Green): n= 7; RH795: n= 18; Neutral Red: n= 8. ΔF/F was calculated by subtracting and dividing the mean amplitude of background fluorescence from the stack series.

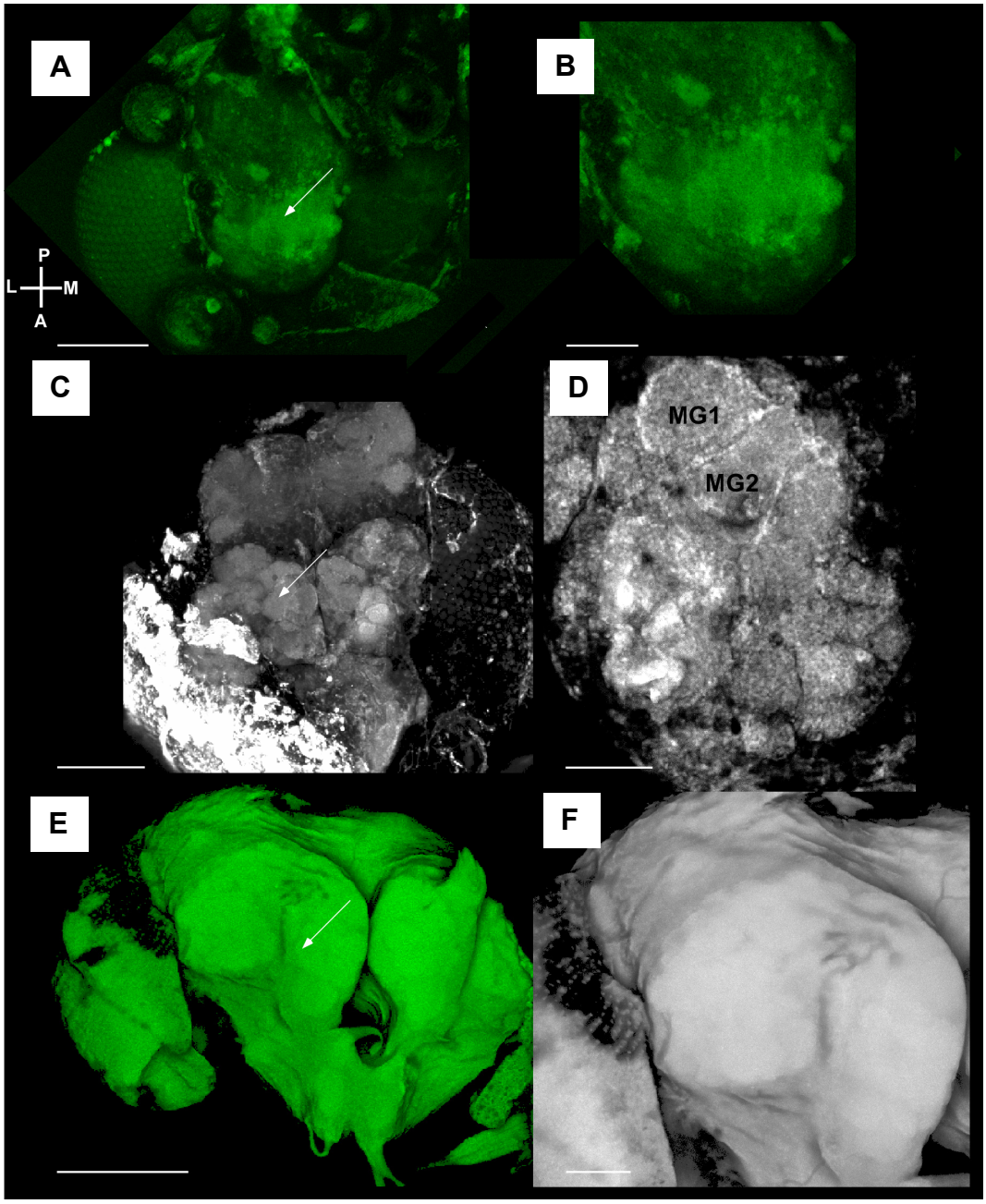

Fig. 3.12 Comparison between different fluorescent dyes using CLSM. Left row: **(A, C, E)** View of the exposed brain, white arrow indicates the AL. Scale bar= 150 µm. Right row **(B, D, F)** zoom into the AL. Scale bar= 50 µm. **A, B)** Calcium Green 1-AM. Glomerular outlines are not identifiable. **C, D)** Male AL and glomeruli stained with RH795. Glomerular borders are very distinctive. MG1 and MG2 indicate two macroglomeruli. **E, F)** Brain fixed with glutaraldehyde and stained with Neutral Red. Glomeruli were less identifiable in fixed tissue. White arrow indicates damages to the tissue. A= Anterior, L= Lateral, M= Medial, P= Posterior.

3.4. Discussion

Results presented in this Chapter have shown that the use of RH795 in combination with CLSM resulted in a higher fluorescence and visibility of glomeruli, and therefore proved to be the most suitable method for a brain morphology study of *C. vestalis* (Chapter 4). This is further supported by the ability to make optical sections through the preparation and to analyse its depth.

Calcium Green 1-AM and Neutral Red were less suitable for investigating the morphology of the AL. However, this experiment demonstrated that dye loading (Calcium Green 1-AM) is possible in parasitoids and can thus be used during *in vivo* imaging studies (Chapter 5).

Preparation and mounting

The aim of this study was to develop a mounting and preparation procedure for a small insect to allow dissection and staining of brain structures. A stable fixed positioning of the insect was essential for dissection as well as for *in vivo* imaging as movement could result in false signals during imaging. It was further important to create a small incubation chamber around the brain for dye incubation. This ensured that the brain was kept moist so that sufficient dye could penetrate brain cells.

Exposing the brain was a difficult procedure in a small parasitoid. First, it was crucial not to damage the antennal nerve or the AL. Second, the cut had to be big enough to reveal the AL structures. Third, non-neural tissue had to be removed to obtain access to the AL and to allow the dye to penetrate the neurons. Non-neural tissue not only blocks the access to the AL, but it is also highly autofluorescent, which could lead to signal interference during *in vivo* imaging (Galizia and Vetter, 2005).

A mounting procedure was developed following a method used for *Drosophila* (Fiala and Spall, 2003). The version of an acrylic block containing grooves proved to be less useful for mounting parasitoids, since the ventral part of the body was covered in glue to keep it restrained within the groove on the Perspex stage. Their survival time was considerably reduced during these experiments. Cyanoacrylate, the basis of superglue reacts with water to form a polymer, which can lead to desiccation and death in the case of an animal of such

a small size (Cary et al., 2000; Galizia and Vetter, 2005). Covering the body may not only cause desiccation of the preparation but could also affect respiration, restricting tracheal pumping movement as well as closure of spiracles (Weis-Fogh, 1967; Slama, 1988, 1999). An adapted version, where the preparation was placed into a Perspex stage containing a hole, provided a useful alternative. The parasitoid was first mounted in a pipette tip and the glue was only put near the mouthparts to stabilize the head. The glue did not cover the whole body and therefore did not block the spiracles. Hence, desiccation and restriction in breathing was less of an issue. The mean survival time of the parasitoid at around 2 h was sufficient for the duration of calcium imaging experiments.

Many studies have reported that access to the AL is restricted, and *in vivo* imaging could only be conducted in some parts of the AL (Faber et al., 1999; Dupuy et al., 2010). Although the AL is always located below the antennae (section 1.3.2), the actual position of the antennae varies between insect species (Fig. 3.13). Hence, the method used to dissect the preparation needs to be adapted for each insect species, including *C. vestalis*. The current method provided a view over most of the AL. In addition, a cover slip could easily be stabilised onto the stage and thus prevent desiccation of the open brain.

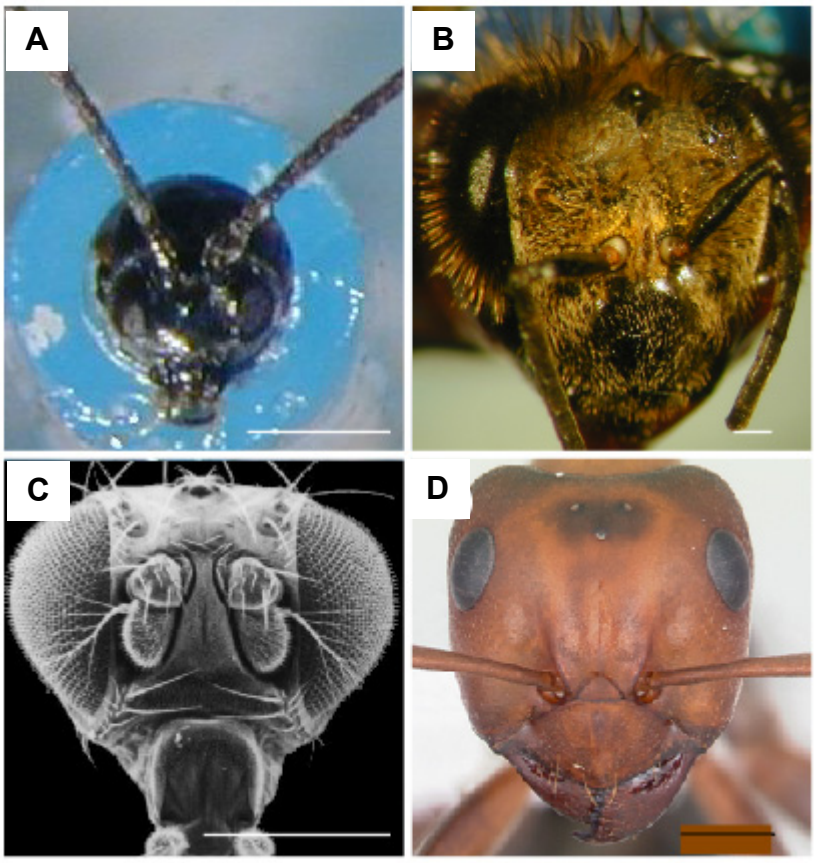

Fig. 3.13 The position of the antennae in different insects. A) *C. vestalis.* **B)** *A. mellifera*. **C)** *D. melanogaster* (Moorhouse, 2005). **D)** *F. exsectoides* (Nobile, 2011). Scale bar in all pictures= 0.5 mm.

Staining

To characterise the morphology of the *Cotesia* brain and conduct imaging studies, three fluorescent dyes were tested and staining methods modified where applicable. In the past, brain morphology was studied using histological methods. To reveal structures, histological sections had to be conducted. Considering the small head size of *Cotesia* (about 500 µm), less invasive procedures such as optical sectioning could be advantageous.

With technical advances, brain studies were conducted using fluorescent probes (Amos and White, 2003). Fluorescent dyes have been shown to possess certain advantages over dyes used in histological studies in some animals, such as more distinct staining of fine structures like e.g. dendritic arborisations and imaging in live tissue (Masante-Roca et al., 2005; Helge et al., 2007; Løfaldli et al., 2010; Seki et al., 2010). The fluorescent dyes RH795 (Galizia et al., 1997;

Sachse et al., 1999; Malun et al., 2002) and Neutral Red (Matsumoto and Hildebrand, 1981; Sandoz, 2006; Dupuy et al., 2010) are frequently used for morphological staining in insects. In the present study, staining with RH795 provided the best results over the other dyes tested. The glomerular borders were visible in most of the preparations and the fluorescence was highest when using RH795. In the current study, some glomerular borders, especially around the MGC, were more intensely labelled as RH795 is a voltage sensitive dye specifically staining cell membranes (Galizia et al., 1997; Le Calvez and Ulfendahl, 2000; Wang et al., 2008).

Using Neutral Red resulted in several disadvantages such as glomeruli were less distinguishable and glomerular borders were hard to locate. With increasing depth the preparations were fused to one structure, making it impossible to identify glomeruli. Furthermore, some preparations were damaged. The AL dimensions but not glomerular dimensions were also significantly smaller using Neutral Red. These problems could be related more to the fixation process than the dye itself. During fixation, the brain is stored in a fixative over night, stained, and dehydrated in an alcohol series followed by clearing in methyl salicylate. Formaldehyde penetrates tissue quickly, but reactions with proteins occur slowly (Kiernan, 2000). A brief fixation process can promote hardening of the specimen due to reduced or stopped autolysis. Proteins that are not completely fixed can coagulate. This could explain the hardened surface of the AL (Kiernan, 2000).

During the dehydration process, ethanol was used to remove water from the preparation. This led to shrinkage of the material. It was shown that at higher alcohol concentrations the tissue also begins to harden and shrink (Puchtler and Meloan, 1985). Shrinkage of the tissue due to osmotic processes has also been described in other studies using glutaraldehyde as a fixative (Hopwood, 1967; Drobysheva et al., 2008). An explanation for the shrinkage of the AL but not the glomeruli could be that glomeruli are neuropils containing dense synaptic contacts of neurons. The AL, however, can contain other tissue, such as fat cells and other cell bodies, which may be more susceptible to loss of water and substance during the fixation procedure and this could result in a greater shrinkage of the tissue surrounding the glomeruli, causing the volume of the AL to diminish.

Shrinkage of certain parts of the brain therefore makes Neutral Red less informative, as the position of the AL and consequently glomeruli could change. An identification of landmark glomeruli may thus become more difficult.

The use of Calcium Green 1-AM for morphological purpose has reportedly resulted in a lack of labelling glomeruli (Sachse et al., 1999; Galizia and Vetter, 2005). However, the dye is a reliable indicator for cell activity, as Calcium Green 1-AM binds to intracellular calcium. Once split from the AM esters, it remains inside the cell (Galizia and Vetter, 2005). Olfactory stimulation of the antennae results in depolarisation of the cell, followed by an influx of calcium (Cossart et al., 2005), which can be detected with this dye. Hence, Calcium Green 1-AM was used in calcium imaging experiments (Chapter 5), but not for morphological studies (Chapter 4).

Microscopy

Using fluorescence widefield microscopy, glomerular borders were detectable but it led to some limitations in describing the anatomy of the AL. Focal clarity throughout the preparation was limited due to depth of the preparation as well as out-of-focus fluorescence, which has been reported in other cases (Amos and White, 2003). Moreover, clarity was reduced due to fluorescence from deeper regions of the preparation, resulting in a greater amount of out-of-focus fluorescence. This in turn can complicate a description of the AL and glomerular dimensions.

With CLSM, clearer images of the brain could be obtained, similar to the findings in other studies (White et al., 1987; Amos and White, 2003; Svoboda and Yasuda, 2006). Using the CLSM resulted in a more detailed image of the brain structures for several reasons. Due to the illumination of one focal plane at a time there was no out-of-focus fluorescence of the preparations. A depth measurement could be obtained by a laser beam scanning through the preparation at variable section intervals. Imaging in deeper regions of the preparation resulted in a better resolution of the glomeruli, but thicker preparations could cause a problem even for CLSM. The deeper the focus into a preparation, the smaller the fraction of photons reaching the plane, and the higher the possibility of beam scattering, resulting in a loss of resolution. This however, can be compensated for by higher

laser power, which is adjustable, emitting more light in deeper regions and less in upper regions (Svoboda and Yasuda, 2006). There is also less bleaching and optical sections can be merged into a 3D reconstruction.

In conclusion, the current study described a mounting and dissection protocol for a characterisation of the morphology of the AL in *C. vestalis*. The protocol permits stabilised mounting of the parasitoid to conduct staining experiments and image acquisition. The current study further demonstrated the successful use of CLSM in combination with the fluorescent dye RH795 for future anatomical studies (Chapter 4) and Calcium Green 1-AM for calcium imaging studies (Chapter 5).

CHAPTER 4

THE MORPHOLOGY OF THE ANTENNAL LOBE OF *COTESIA VESTALIS*: A CONFOCAL MICROSCOPY STUDY

4.1. Introduction

Parasitoids rely on the detection of volatiles to find a suitable host. This requires a very sensitive olfactory system to detect and filter relevant information. Due to their different host specialisation and resulting differences in learning and memory abilities (Smid et al., 2007), they represent ideal animal models for a comparative investigation of olfactory processing. However, very little research has focused on the investigation of central olfactory processing mechanisms in parasitoids (Smid et al., 2003; Bleeker et al., 2006b).

The results presented in Chapter 2 have shown that males and females of the parasitoid species *C. vestalis* can detect a well-documented HIPV (linalool) (Pinto et al., 2007a), as well as an odour cue (1-nonanol), which is not part of the HIPVs emitted from *Brassica rapae* var. *Wong bok* (Durrant, 2007) at a peripheral level. The behavioural output, however, differed between genders. While females were able to learn linalool in a positive association, 1-nonanol elicited an avoidance reaction. Males learned linalool but did not learn 1-nonanol.

Optical imaging studies in insects have shown a broad spatio-temporal activity pattern in the glomeruli (Joerges et al., 1997). For a better understanding

of olfactory processing in a parasitoid and to investigate whether behavioural differences are also represented in different glomerular activity patterns it is first necessary to describe the morphology of the brain structures underlying olfactory processing.

Glomeruli are the functional units of the antennal lobe (AL). They are spheroid-like structures, which are often surrounded and separated by glial cells (Homberg et al., 1989), and their morphology and physiology has been reviewed in the past (Christensen and Hildebrand, 1987a; Homberg et al., 1989; Galizia and Rössler, 2010). In the glomeruli, olfactory receptor neurons (ORNs) synapse with projection neurons (PNs) and local interneurons (LNs) (Homberg et al., 1989; Hildebrand and Shepherd, 1997; Galizia and Rössler, 2010). Synapses are mainly found in the cortical layers of the glomeruli, but bees also show synaptic connections in the central regions. In most cases, the synaptic connection consists of one presynaptic and two or more postsynaptic elements (Hansson, 1999). Most ORNs expressing the same receptor, project into one or two specific glomeruli. The theory that glomeruli are functional units has further been supported by different sensilla mapping onto specific groups of glomeruli in *Drosophila* (Couto et al., 2005; Fishilevich and Vosshall, 2005). Furthermore, in Hymenoptera, distinctly clustered glomeruli are innervated by different antennal sensory tracts (Kirschner et al., 2006; Zube et al., 2008). This principle is found throughout the animal kingdom (Strausfeld and Hildebrand, 1999) and reinforces the theory that glomeruli are functional units involved in coding odour quality.

In parasitoids, only one morphological study has investigated the anatomy of the antennal lobe so far (Smid et al., 2003). In this study (Smid et al., 2003), axon tracing was used to visualise the glomeruli in the AL. Smid and collegues (Smid et al., 2003) found a similar number of glomeruli in the two related *Cotesia* species, *C. glomerata* (~188 glomeruli) and *C. rubecula* (~196 glomeruli). The organisation of the glomeruli within the AL of the related *Cotesia* species (Smid et al., 2003) was similar to that found in other Hymenoptera (Zube et al., 2008).

In insect species which use sex-pheromone communication, a sexual difference in glomerular structure has been observed (Christensen and Hildebrand, 1987b). In moths, it has been shown that some glomeruli code specific olfactory information, and that there are gender differences in the glomerular organisation

of the AL. Male moths have a sexually dimorphic cluster of enlarged glomeruli forming the macroglomerular complex (MGC) which exclusively processes information about pheromones (Rospars, 1983; Christensen and Hildebrand, 1987b). The MGC is innervated by local LNs (Matsumoto and Hildebrand, 1981) and each sub-unit encodes specific pheromone component information (Hansson et al., 1992). In the male sphinx moth, the male MGC is characterised by three enlarged glomeruli, the cumulus (C), the toroid 1 (T1) and toroid 2 (T2) (Heinbockel et al., 1998). Four types of MGC-PNs have been characterised (Kanzaki et al., 2003) (toroid-PNs, cumulus-PNs, horseshoe-PNs, and c_t-PNs) and it was shown that the pheromone responsiveness of these PNs correlates with their dendritic arborsation in the subdivisions of the MGC (Kanzaki et al., 2003). PNs innervating the cumulus were shown to respond to the minor pheromone component and PNs innervating the toroid responded to the major pheromone component (Hansson et al., 1991b; Kanzaki et al., 2003). The pheromone input is modified by LNs and responses in the MGC are more broadly tuned than in the ORNs (Jarriault et al., 2010).

In some lepidopteran species, like the sphinx moth *Manduca sexta*, a female homologous to the male MGC, so called "large female glomeruli" (LFGs), can be found (King et al., 2000; Reisenman et al., 2004; Kalberer et al., 2010). They consist of two relatively large glomeruli in the dorsolateral region of the AL, close to the entrance of the antennal nerve (AN). They are unique for females and are approximately the same size as the largest ordinary glomeruli (Rössler et al., 1998).

The variety host specialisation in parasitoids suggests that differences in olfactory information processing exist not only at an antennal or behavioural level, but also at a central level in the brain. The aim of this study was to analyse the morphology of *C. vestalis* to determine whether the general organisation of glomeruli in the AL of *C. vestalis* is similar to other Hymenoptera. Also, male and female brains were compared to determine if differences in behaviour (Chapter 2) could be due to differences in the morphology. The morphology of the AL was investigated using the methods established in Chapter 3. It is thought that due to their differences in host specialisation, the size and number of glomeruli differs from those in *C. glomerata* and *C. rubecula* (Smid et al., 2003). Furthermore, *C. vestalis* show courtship behaviour in the presence of females (Chapter 2), which

suggests that males have specialised glomeruli for pheromone processing. Therefore, the current study also investigated whether there are segregated regions for pheromone and non-pheromone processing, as well as specialised glomeruli for the detection of HIPVs.

4.2. Materials and methods

To investigate the morphology of the AL, the brain was stained with RH795 (Invitrogen) as described in Chapter 3. Optical sections were taken through the brain from dorsal to ventral with a confocal microscope. An argon laser with an excitation wavelength of 514 nm was used for the imaging procedure (Emission wavelength: 590-720 nm).

For quantitative analysis, brain dimensions were first analysed using manual measurements (manual data). In order to compare male and female brains, measurements of brain structures were conducted along the length and the width of the AL and glomeruli (Fig. 4.1 A, B). The AL and glomeruli were measured in the average projection of the stack series. Two to four glomeruli were measured per preparation. With the exception of the macroglomeruli, which could be easily identified as "landmark glomeruli", different glomeruli were selected for the measurements each time. The dimensions of each structure were statistically compared using a univariate/multivariate general linear model (GLM) and ANOVA (SPSS 17.0).

For three dimensional (3D) analyses, the AMIRA software package (Visage Imaging GmbH, Version 5.3) was used to calculate the volume of the AL and glomeruli. This was carried out by tracing every third slide with the "lasso tool" (Fig. 4.2). This ensured that changes in shape could be accounted for throughout the slides. After the last slide the stack series was interpolated to combine the stacks and to determine the volume. The number of glomeruli was calculated by counting manually through each of the stack series. This number was then compared with results obtained from using AMIRA software.

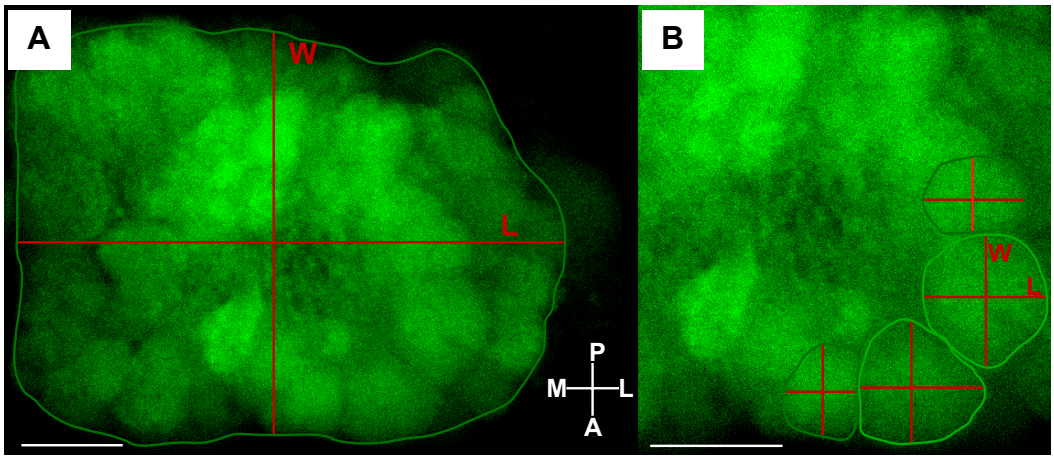

Fig. 4.1 Dimension measurements of the AL and glomeruli. A) The AL was measured in the stack average projection along the length and width for each side in both genders. **B)** Two to four glomeruli were measured along the length and width (as in the AL) per preparation in the stack average projection. Scale bar= 30 µm. L= Length, W= Width, A= Anterior, L= Lateral, M= Medial, P= Posterior.

Calculating volumes from manual measurements

To compare manual and 3D analyses the volume of AL and glomeruli had to be modelled with measurements obtained from manual calculations. The AL was modelled as an ellipsoid as this best fitted its shape (Fig. 4.1 A). Its volume (V_e) was calculated using

$$V_e = \frac{4}{3}\pi abc$$

where a, b and c are width, length and height axis radii respectively. The glomerular shape equates to a sphere (Rospars and Hildebrand, 2000; Berg et al., 2002). Therefore, the glomeruli were modelled as spheres (Fig. 4.1 B); their volume (V_s) was calculated using

$$V_s = \frac{4}{3}\pi r^3$$

where r is the radius.

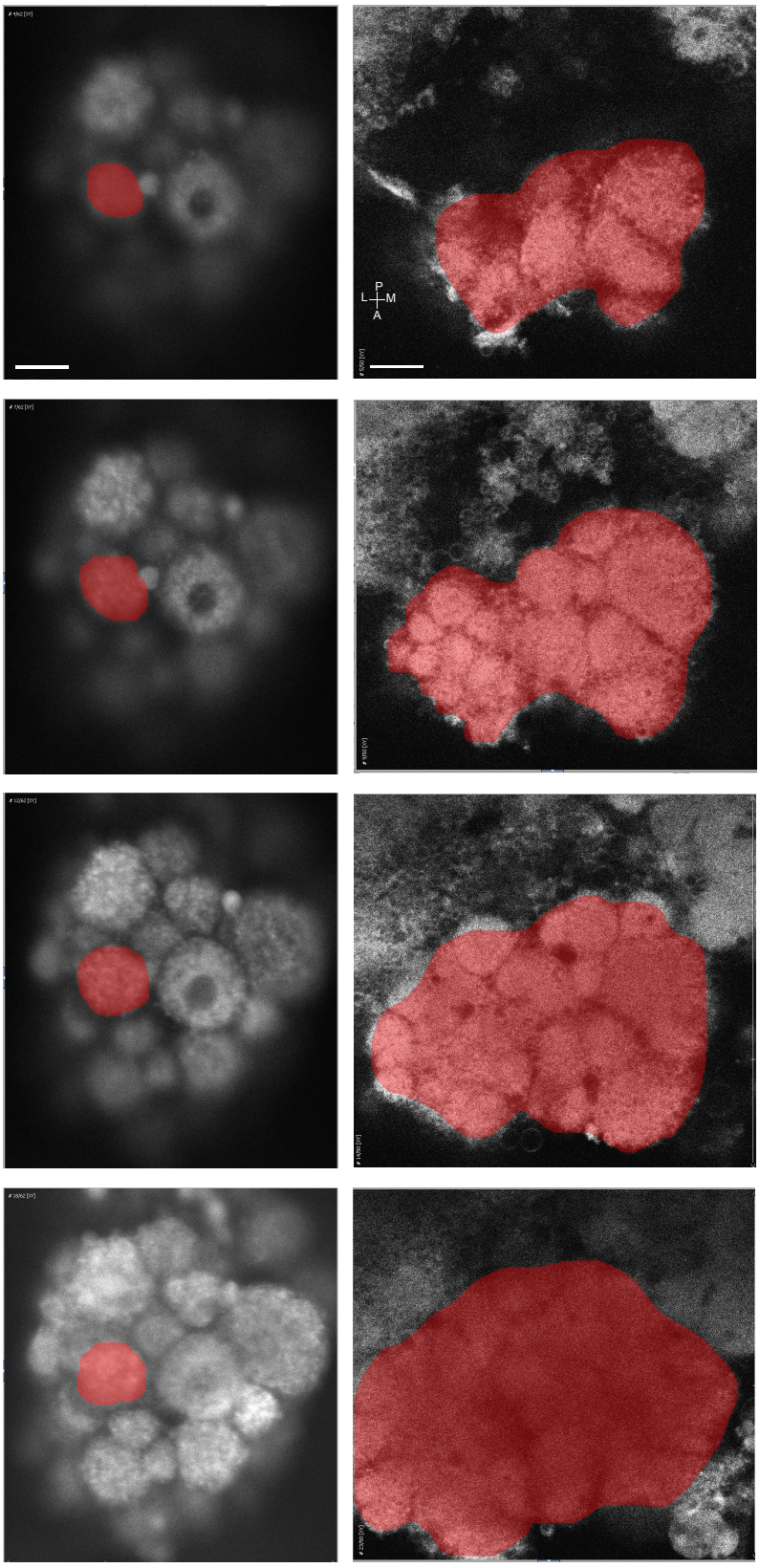

Fig. 4.2 3D measurements of glomeruli and the AL. 3D measurements enabled a more exact volume analysis as they could account for changes in shape through depth, which is shown in the figure top to bottom. The red colour indicates the selected areas of glomeruli (left) or the AL (right) (outlined using the lasso tool) through different depths of the preparation. Scale bar= 30 µm.

4.3. Results

In order to investigate the morphological structure of the AL in *C. vestalis* to detect possible gender differences, male and female brains were dissected, stained, and imaged using confocal microscopy. To analyse the data, measurements of the AL were first conducted manually, and the number of glomeruli were identified through counts of the stack series. Secondly, the AL and glomerular volume was calculated using AMIRA software.

4.3.1. Description of the AL and glomeruli through manual measurements

Antennal lobe

To identify the structures underlying olfactory processing, the AL dimensions were first measured over two dimensions (length and width). To calculate the AL volume, depth measurements were taken from the stack series data. These measurements were then used as a reference to determine whether the number of counted glomeruli was accurate.

Overall, a total of 85 ALs were obtained from male and female parasitoids. The AL is a round to ellipsoid structure consisting of single units, the glomeruli, and is located in the dorsal area below the antennae. It measured 179 ± 5 µm (mean ± SEM; n= 50) x 136 ± 4 µm (mean ± SEM; n= 50) x 115 ± 3 µm (mean ± SEM; n= 85) (L x W x D – length x width x depth; left-right; male-female fused data) (Fig. 4.3). An ANOVA analysis showed a significant gender difference in the left AL length ($F_{1,23}$= 5.156, $p<0.05$) and left AL depth ($F_{1,42}$= 6.330, $p<0.05$). Based on these measurements, the AL volume was 13 x 10^5 μm^3 and 18 x 10^5 μm^3, for males and females, respectively.

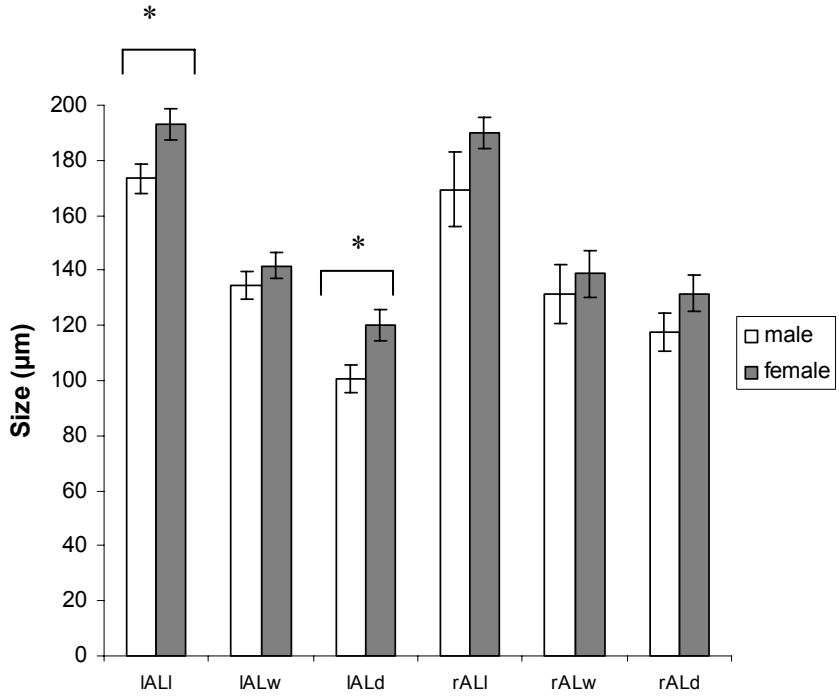

Fig. 4.3 Comparison between male and female mean AL size of left (lAL) and right (rAL) side. Female structures were in general larger; a significant difference, however, was only found in the lALl and d. l= length, w= width, d= depth. Male lAL: l, w: n= 15; d: n= 27; rAL: l, w: n= 16, d: n= 28. Female lAL: l, w: n= 8, d: n= 15; rAL: l, w: n= 11, d: n= 15. * indicates a significant difference at $p<0.05$.

Glomeruli

To determine if *C. vestalis* had a similar glomerular organisation to other insects and if peripheral and behavioural differences (Chapter 2) were due to a difference in the morphology, the number and size of glomeruli were analysed in both genders.

In both genders, ordinary glomeruli were arranged in several layers around a central core devoid of glomeruli (Fig. 4.4 A-C). The central core did not extend through the entire AL (Fig. 4.4 D-F). In addition to the three sex-specific glomeruli in males, an average of 32 ± 3 (mean ± SEM; n= 23) ordinary glomeruli could be identified in males and females (Fig. 4.5).

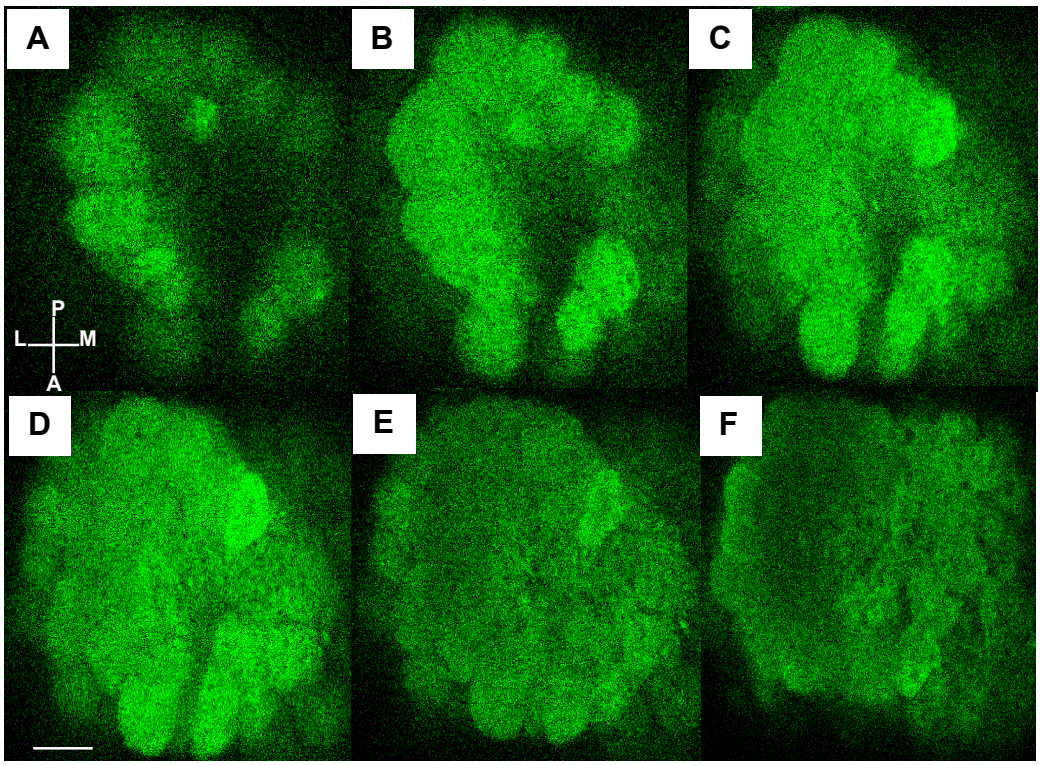

Fig. 4.4 Optical sections through the female right AL from top (A) to bottom (D). A central core devoid of glomeruli is visible (A-C). **A)** 16.15 µm depth. **B)** 19 µm depth. **C)** 20.9 µm depth. **D)** 22.8 µm depth. **E)** 24.7 µm depth. **F)** 32.3 µm depth. Scale bar= 30 µm. A= Anterior**,** L= Lateral, M= Medial, P= Posterior.

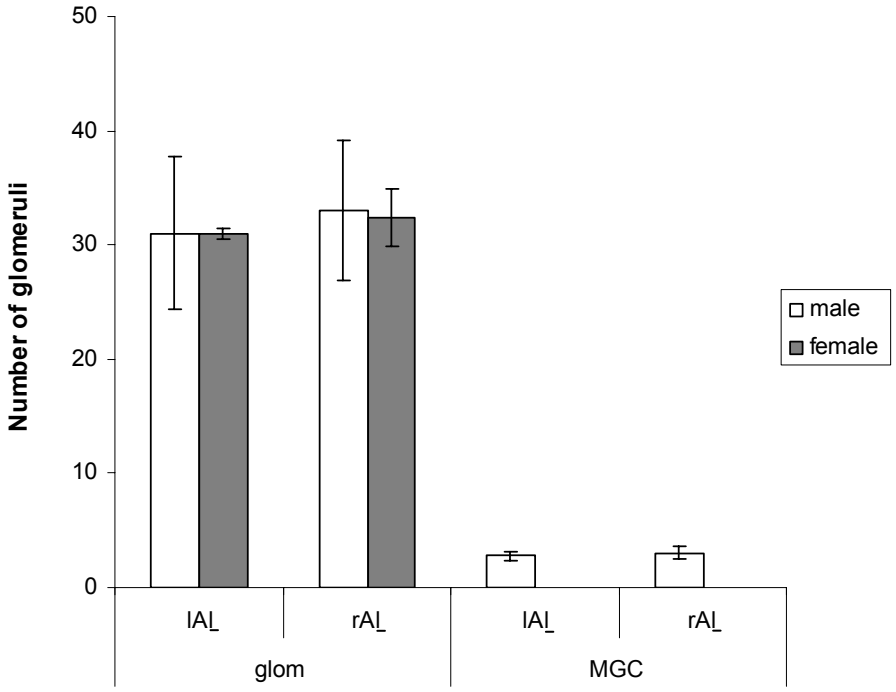

Fig. 4.5 Number of ordinary glomeruli (glom) and the macroglomerular complex (MGC) in left (lAL) and right AL (rAL) for males and females. Male glom lAL: n= 8, rAL: n= 7; MGC lAL: n= 22, rAL: n= 8; female glom lAL: n= 3, rAL: n= 5. There was no significant gender or side difference in the number of ordinary glomeruli.

Glomerular size remained constant throughout the different individuals, with an average size of 23 x 22 ± 0.6 µm (mean ± SEM; n= 98; length x width). Statistical analysis showed that there was no significant difference between left and right side ($F_{1,97}$= 1.537, p>0.05), or gender difference ($F_{1,97}$= 2.322, p>0.05), nor an interaction between side and gender ($F_{1,97}$= 1.317, p>0.05). This is shown in Figure 4.6 and suggests a similar organisation of ordinary glomeruli in males and females. The estimated volume of a glomerulus was 5, 575 μm^3. Therefore, based on the average number of glomeruli, the AL should measure 1.8 x 10^5 μm^3. Due to the discrepancy of estimated AL volume and the estimated total volume of glomeruli, a 3D analysis was carried out, the results of which are described in section 4.3.2.

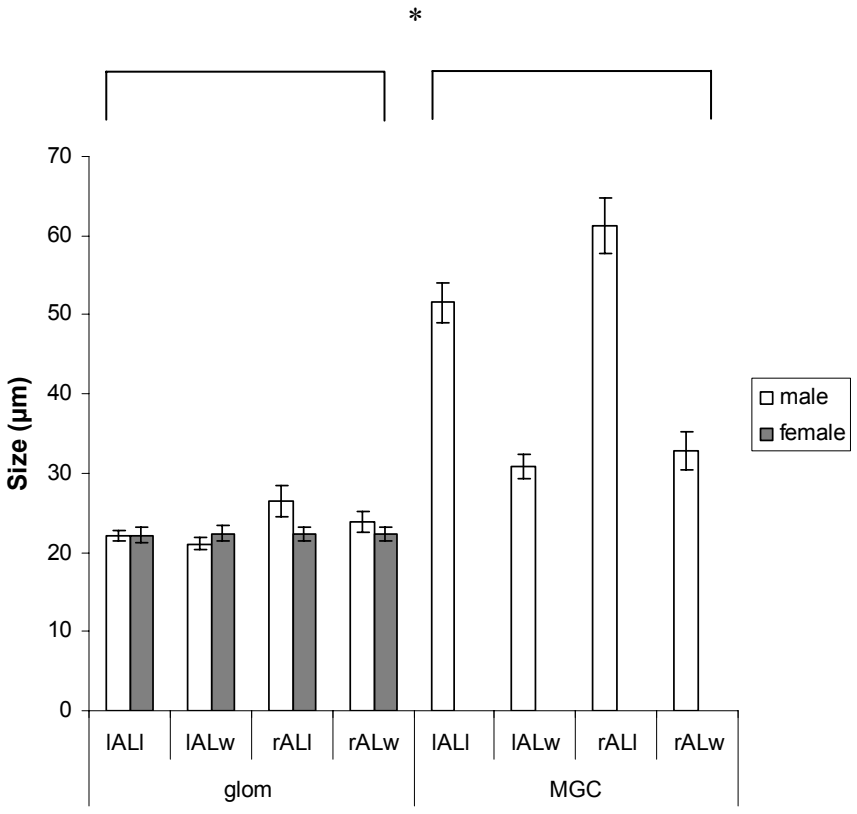

Fig. 4.6 Average size of glomeruli in males and females. Left (lAL) and right (rAL) means were fused together. MGC lAL: n= 22, rAL: n= 8; male glom lAL: n= 31, rAL: n= 25; female glom lAL: n=18, rAL: n= 24. * indicates a significant difference at p<0.05 between MGC and ordinary glomeruli. MGC = Macroglomerular complex, glom= glomeruli, l= length, w= width.

Macroglomerular complex (MGC)

Moths have enlarged glomeruli, so called macroglomeruli, for pheromone processing (Christensen and Hildebrand, 1987b). So far, no gender differences in parasitoids have been described. Courtship behaviour is known to be mediated by pheromones (Tagawa, 1977; Decker et al., 1993; Steiner et al., 2006). In *C. vestalis* males courtship behaviour was induced by the presence of a female (Chapter 2), suggesting that male *C. vestalis* detect sex pheromones. Therefore, male parasitoids could use sex pheromones (in addition to HIPVs) to locate females. It was thus hypothesised that macroglomeruli can be found in *C. vestalis* males.

Indeed, enlarged and long shaped glomeruli, so called macroglomeruli, were found in males in the current study. The macroglomerular complex (MGC) consisted of 3 ± 0.3 (mean ± SEM; n= 15) enlarged glomeruli (54 ± 2 µm x 31 ± 1µm; mean ± SEM; n= 22) near the entrance of the antennal nerve. Contrary to ordinary glomeruli, which appeared to be of a round shape, macroglomeruli were of an elongated shape (Fig. 4.7 A-D). The first and second most distal macroglomerulus (MG1, MG2) were of an elongated shape, with the MG2 adjacent to MG1. The third macroglomerulus (MG3) was smaller and more roundly shaped than MG1 and MG2 (Fig. 4.8) and was located closer to the MG1. There was a significant difference in length (ANOVA; $F_{3,85}$= 75.897; $p<0.05$) and width (ANOVA; $F_{3,85}$= 16.823; $p<0.05$) between the MGC and ordinary male glomeruli (Fig. 4.6).

In summary, this part of the study showed that the AL of *C. vestalis* had a similar glomerular organisation to that found in other hymenopterans (Zube et al., 2008). The glomeruli were organised peripherally around a central core, which did not contain glomeruli. No gender differences were found in size and number of ordinary glomeruli. An average of 32 glomeruli was identified. Hence, it would appear that behavioural differences (Chapter 2) were not due to a different morphology of the AL. In addition, males possess a MGC, similar to that found in moths suggesting that *C. vestalis* males can detect and process pheromones.

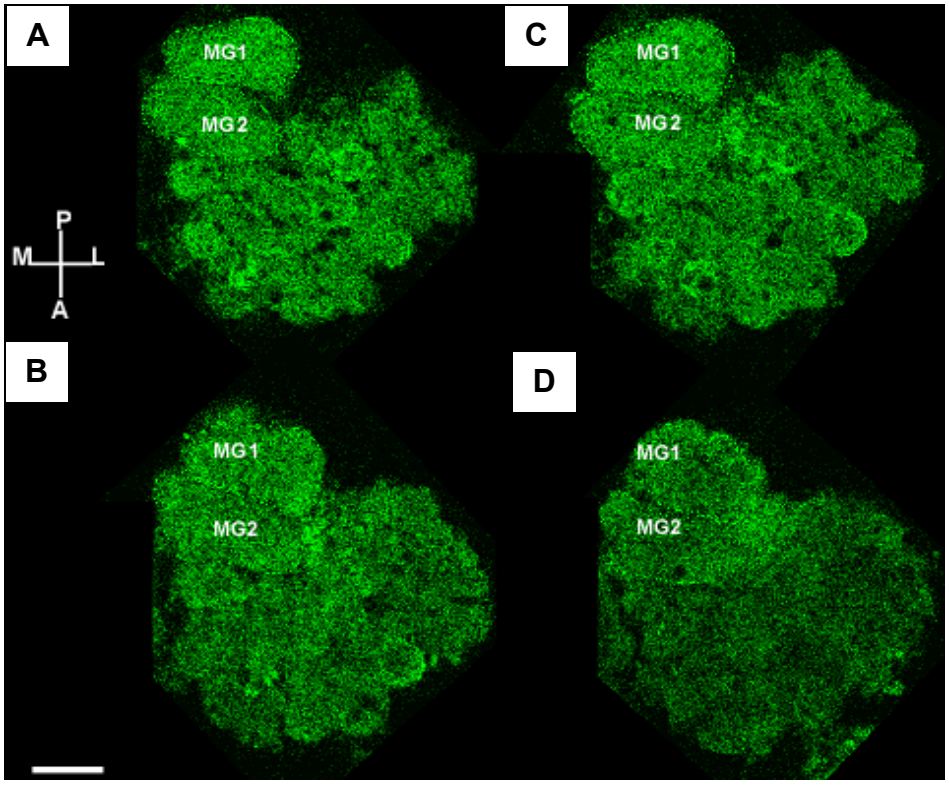

Fig. 4.7 Optical sections through the male left AL from top (A) to bottom (D). The progression of ordinary as well as two macroglomeruli (green outline and marked with MG1, MG2) is shown at different depths in the AL. Their size differed significantly from ordinary glomeruli. **A)** 7 µm depth. **B)** 10 µm depth. **C)** 12 µm depth. **D)** 16µm depth. Scale bar=30 µm. A= Anterior, L= Lateral, M= Medial, P= Posterior.

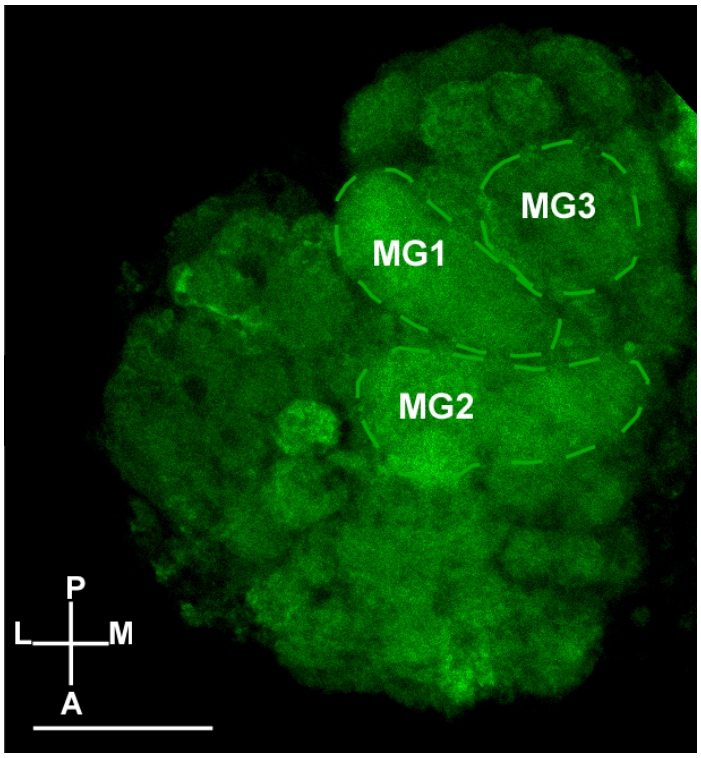

Fig. 4.8 View of the three macroglomeruli, MG1, MG2 and MG3 in an average projection. MG1 and MG2 were characterised by an elongated shape, whereas MG3 was usually smaller and round. Scale bar= 50 µm. A= Anterior, L= Lateral, M= Medial, P= Posterior.

4.3.2. 3D analysis of the AL and glomeruli using AMIRA software

Manual measurements have shown that male and female *C. vestalis* possess an average of 32 ordinary glomeruli (section 4.3.1). However, a previous study (Smid et al., 2003) which investigated the anatomy of the AL of two related *Cotesia* species, *C. glomerata* (~188 glomeruli) and *C. rubecula* (~196 glomeruli), found a higher number of ordinary glomeruli in these two species (Fig. 4.9).

In the current study, volume calculations of the AL and the total glomerular volume were not identical. The AL volume was considerably higher and this could be explained by the limitations of manual measurements, which did not account for changes of the shape of the AL with depth of the preparation.

In order to obtain more accurate measurements to validate results described in section 4.3.1, 3D volume calculations were conducted using the software program AMIRA. Optical sections obtained using confocal microscopy were analysed with AMIRA, and AL volume and total glomerular volume were calculated for direct comparison with manual measurements. This was done by tracing the entire AL and individual glomeruli with AMIRA and interpolating every third optical stack. The AL volume served as a volume guideline. In addition, the volume range of ordinary and gender-specific glomeruli was given. An evaluation of manual data and 3D data using one female AL as well as the average value of all preparations was conducted to compare the two methods (Appendix 2).

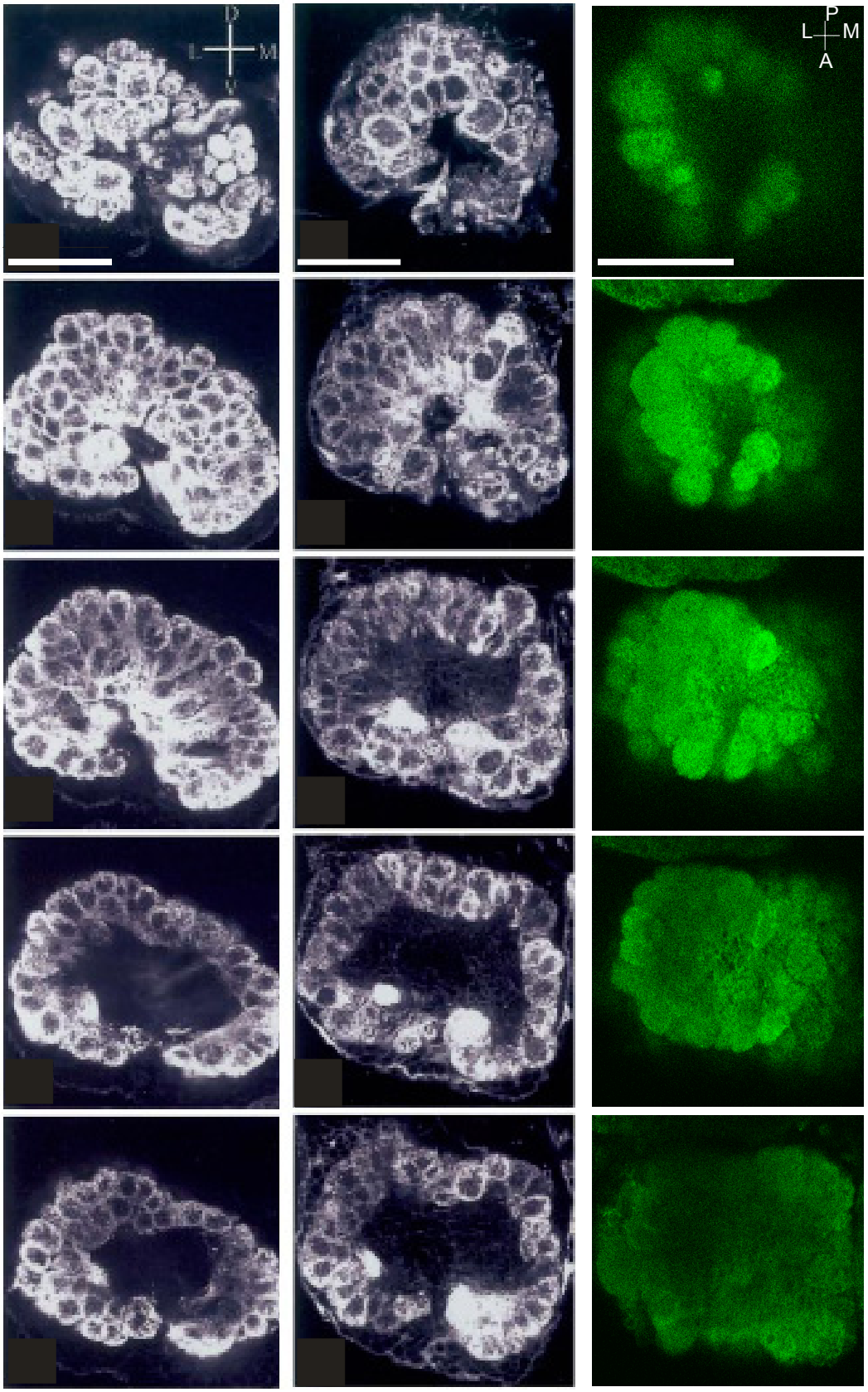

Fig. 4.9 Comparison of brain sections of *C. glomerata*, *C. rubecula* and *C. vestalis*. Brain stack series from top to bottom of *C. Glomerata* (left column), *C. rubecula* (middle column) modified from (Smid et al., 2003). *C. vestalis* (right column). Scale bar= 50 µm. A= Anterior, D= Dorsal, L= Lateral, M= Medial, P= Posterior, V= Ventral.

Antennal lobe

As with manual measurements, the AL volume was calculated as a reference volume. The AL volume measured 4.3 x 10^5 ± 3.5 x 10^4 µm^3 (mean ± SEM; n= 15) for males and 4.3 x 10^5 ± 4.6 x 10^4 µm^3 (mean ± SEM; n= 9) for females (Fig. 4.10 A, C). Measurements excluded the central core in the AL, but could not take into account any intra-glomerular space. The core itself measured 8.3 x 10^4 ± 1.5 x 10^4 µm^3 for males (mean ± SEM; n= 13) and 1 x 10^5 ± 2.3 x 10^4 µm^3 for females (mean ± SEM; n= 12). It made up 15% of the AL volume in males and 23% in females. In summary, volume measurements of the AL using AMIRA more precise as it allowed the volume of the central core to be removed and changes in shape with depth to be considered.

Glomeruli

For a more precise volume and number of glomeruli, glomeruli were traced with AMIRA. An average of 40 ± 3 (mean ± SEM; n= 9) ordinary glomeruli for males and 41 ± 6 (mean ± SEM; n= 8) for females could be identified. The mean total volume of glomeruli per AL was 1.5 x 10^5 ± 2 x 10^4 µm^3 for males (mean ± SEM; n= 7), excluding the MGC, and 2.6 x 10^5 ± 4.8 x 10^4 µm^3 including the male MGC (mean ± SEM; n= 7) (Fig. 4.10. D; Fig. 4.11). For females, the total volume of ordinary glomeruli was 1.3 x 10^5 ± 3 x 10^4 µm^3 (mean ± SEM; n= 8) (Fig. 4.10. B; Fig. 4.11). Compared to the AL volume, the total glomerular volume was smaller by 2.8 x 10^5 µm^3 and 2.9 x 10^5 µm^3 for males and females, respectively. An uGLM showed no significant difference between genders ($F_{1,569}$= 3.680, $p>0.05$) or side ($F_{1,569}$= 0.022, $p>0.05$). There was no significant interaction between gender and side ($F_{1,569}$= 1.269, $p>0.05$).

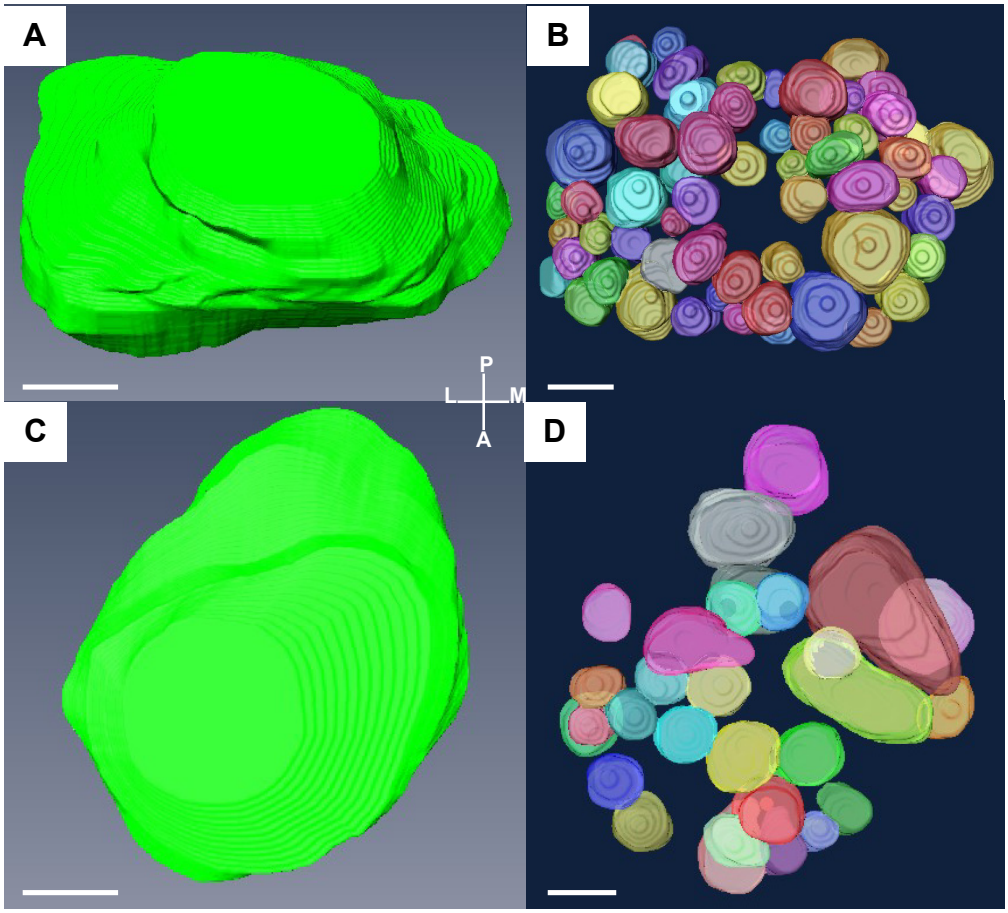

Fig. 4.10 Antennal lobe (AL) volume and total volume of glomeruli. A) 3D reconstruction of a female AL from which the AL volume was calculated. The central core was excluded from volume calculations but the gaps between the glomeruli could not be removed. **B)** 3D reconstruction of glomeruli (in a certain stack plane) of the female AL from which the total glomerular volume was calculated. The glomerular volume was smaller than the AL volume. **C)** 3D reconstruction of a male AL. **D)** 3D reconstruction of glomeruli of the male AL (in a certain stack plane). Scale bar= 30 µm. A= Anterior, L= Lateral, M= Medial, P= Posterior.

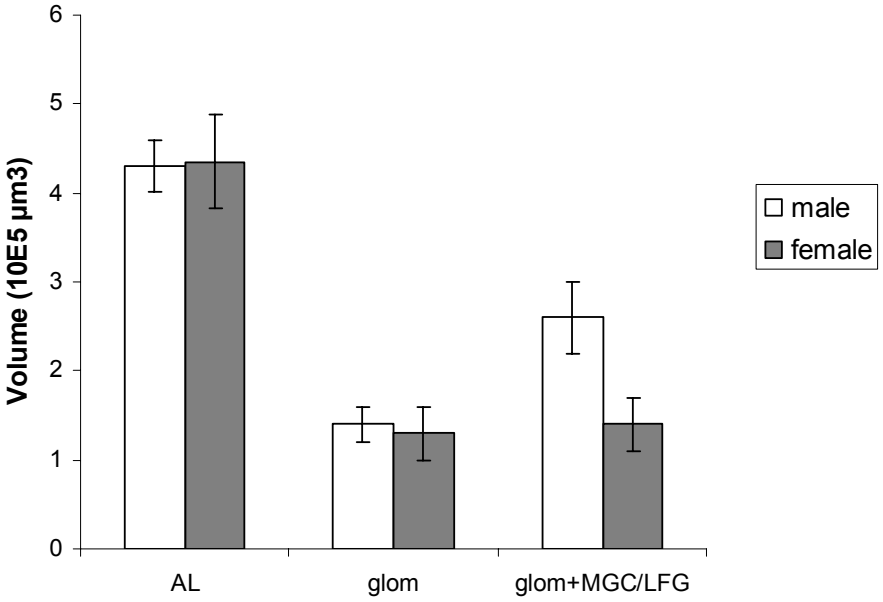

Fig. 4.11 Comparison between AL volume and total glomerular volume in males and females. The glomerular volume (glom) of males and females is given without the volume of macroglomeruli and large female glomeruli, respectively. Ordinary glomeruli and macroglomeruli/large female glomeruli (glom+MGC/LFG) comprise the total glomerular volume in males and females including the macroglomerular complex (MGC) and large female glomeruli (LFG), respectively.

Arnold (Arnold et al., 1985) suggested that glomeruli occupy about 40% of the AL. Based on the AL volume and total volume of ordinary glomeruli, glomeruli make up 35% of the AL volume in males, and 33% in females. In males ordinary glomeruli and MGC made up 65% of the AL. In total, the core and glomeruli made up 50% (80% including MGC) of the AL in males, and 56% in females. However, measurements did not take into account spaces between the glomeruli as well as other structures such as cell bodies.

In two related species, *Cotesia glomerata* and *C. rubecula* the volume of glomeruli was found to be $1.7 \times 10^5 \pm 2.5 \times 10^4$ µm^3 (mean ± SEM; n= 2) and $3.1 \times 10^5 \pm 3.4 \times 10^4$ µm^3 (mean ± SEM; n= 2), respectively (Fig. 4.12 A) (Smid et al., 2003). Despite the similar total glomerular volume, the number of glomeruli was much higher in *Cotesia glomerata* (188 ± 1.5; n= 2) and *C. rubecula* (196 ± 2.5;

n= 2) (Fig. 4.12 B). The size rank of ordinary glomeruli, however, was considerably smaller, ranging from 145 to 3,732 and 95 to 6,920 μm^3, in *C. glomerata* and *C. rubecula,* respectively. Based on these values, the average volume of an ordinary glomeruli is 1,939 ± 1,794 μm^3 (mean ± SEM; n=2) for *C. glomerata* and 3,508 ± 3,413 μm^3 (mean ± SEM; n=2) for *C. rubecula*. In *C. vestalis,* the volume of single glomeruli ranked between 416 to 16,212 μm^3 in males, and between 272 to 21,185 μm^3 in females. The average volume of an ordinary glomerulus was 3,719 ± 528 μm^3 (mean ± SEM; n= 7) in males, and 3,777 ± 288 μm^3 (mean ± SEM; n=7) in females. There was no significant gender difference between ordinary glomeruli (uGLM: $F_{1,13}$ = 0.509, p>0.05). The frequency distribution of the glomerular volumes is given in Figure 4.13 and 4.14. Volumes are expressed as percentages of the total glomerular volume to correct for differences in total brain volume and size of individuals.

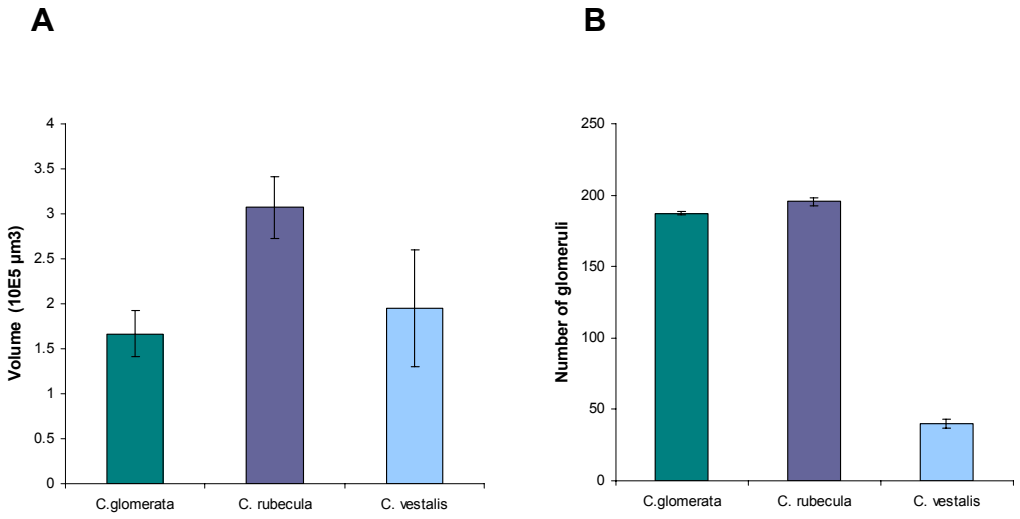

Fig. 4.12 Comparison of total glomerular volume and number of glomeruli between *Cotesia* species. A) Total glomerular volume. *C. glomerata* had a similar volume to *C. vestalis*, whereas the volume of *C. rubecula* was double. **B)** The number of glomeruli. Both *C. glomerata* and *C. rubecula* had nearly five times more glomeruli than *C. vestalis*.

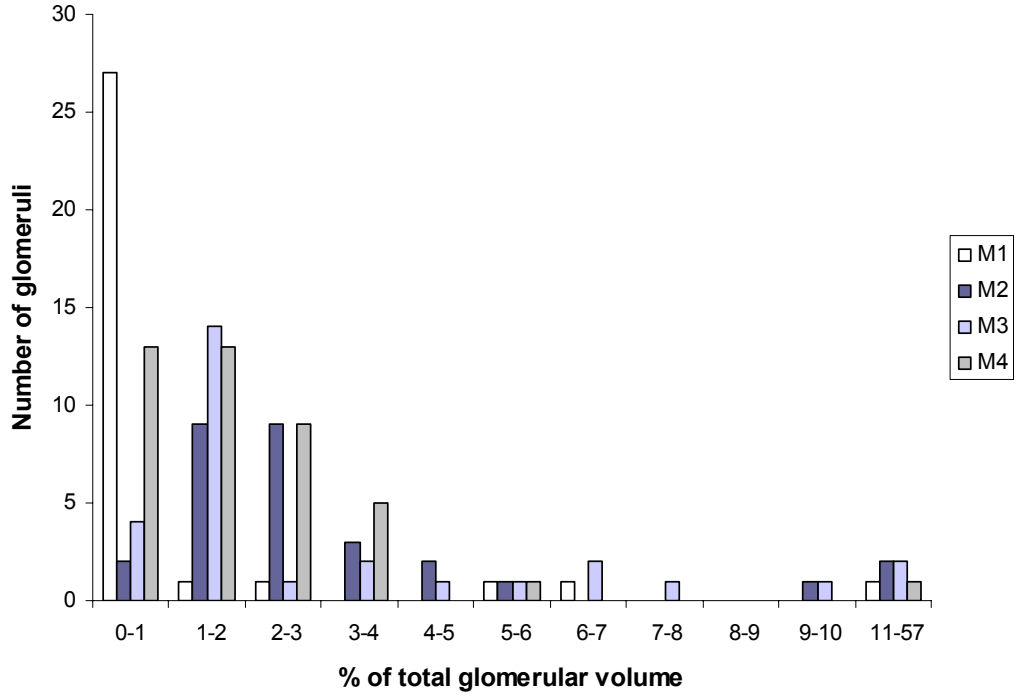

Fig. 4.13 Frequency distribution of glomeruli in male *C. vestalis*. The values of four males are represented as percentages of total glomerular volume. M1-4= male 1-4.

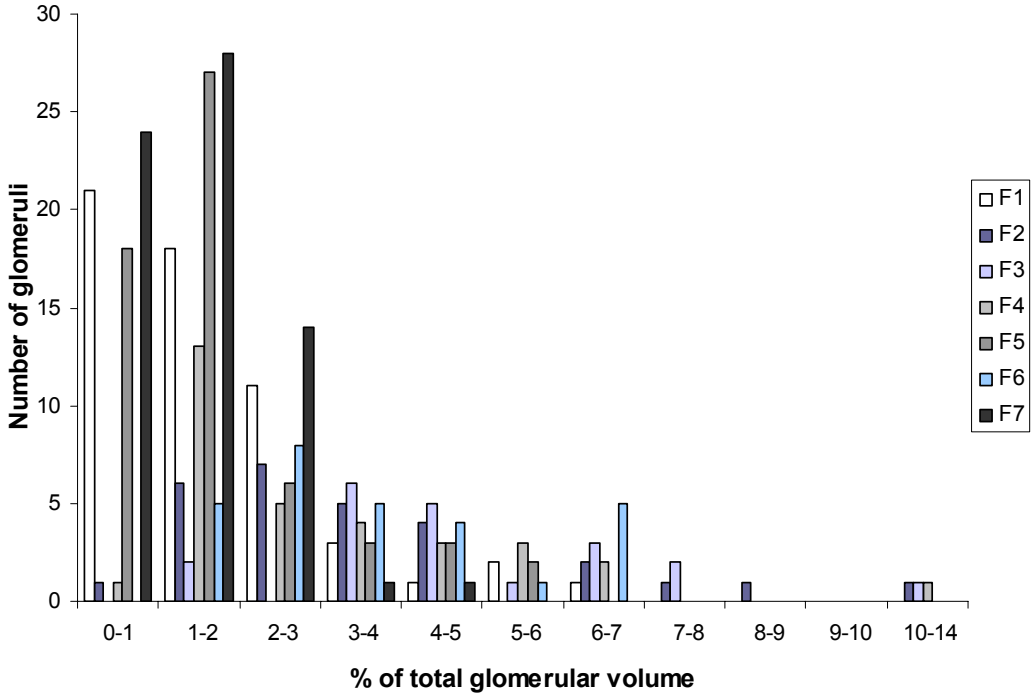

Fig. 4.14 Frequency distribution of glomeruli in female *C. vestalis*. The values of seven females are represented as percentages of total glomerular volume. F1-7= female 1-7.

Macroglomerular complex (*MGC)*

To confirm that the enlarged glomeruli found through manual measurements were indeed macroglomeruli, volume measurements were conducted. The MGC had a volume ranging from 18,123 to 91,583 μm^3. The average volume of a macroglomerulus was 38,267 ± 528 μm^3 (mean ± SEM; n= 8) (Fig. 4.15), which was about ten times larger than an ordinary glomerulus. There was a significant difference between ordinary male glomeruli (uGLM: $_1F_{13} = 9.512$, $p<0.05$) and the MGC.

To verify that the larger size of macroglomeruli was not caused by a size difference in the individuals, a correlation analysis (Pearson's) was conducted between the volume of three male brains and their body sizes (Kuebler et al., 2010). The correlation coefficient was 0.28 ($r^2= 0.28$, $p>0.05$), indicating no correlation between the individual's size and the size of the glomeruli. Hence, macroglomeruli were not influenced by the animal's size, suggesting that *C. vestalis* males indeed use pheromone detection and therefore have enlarged glomeruli designated to pheromone processing.

Large female glomeruli (LFG)

With manual measurements, no significant differences in glomerular size in females could be found. With AMIRA measurements, however, in three out of seven female preparations, larger glomeruli were noticeable. When analysing their volume it was found that they were about four times larger than ordinary glomeruli. A statistical comparison revealed a significant difference between ordinary glomeruli and large female glomeruli (LFGs) (ANOVA: $F_{1,128}= 144.753$, $p<0.05$) (Fig. 4.15). Ordinary glomeruli had a volume of 3,166 ± 360 μm^3 (mean ± SEM; n= 7), and LFGs a volume of 11,901 ± 976 μm^3 (mean ± SEM; n= 3). Unlike the MGC, however, the LFG could not be identified at a certain position in the AL. Also, they did not have a characteristic shape which would facilitate their identification. It can therefore only be speculated that in *C. vestalis* the enlarged glomeruli correspond to the LFG.

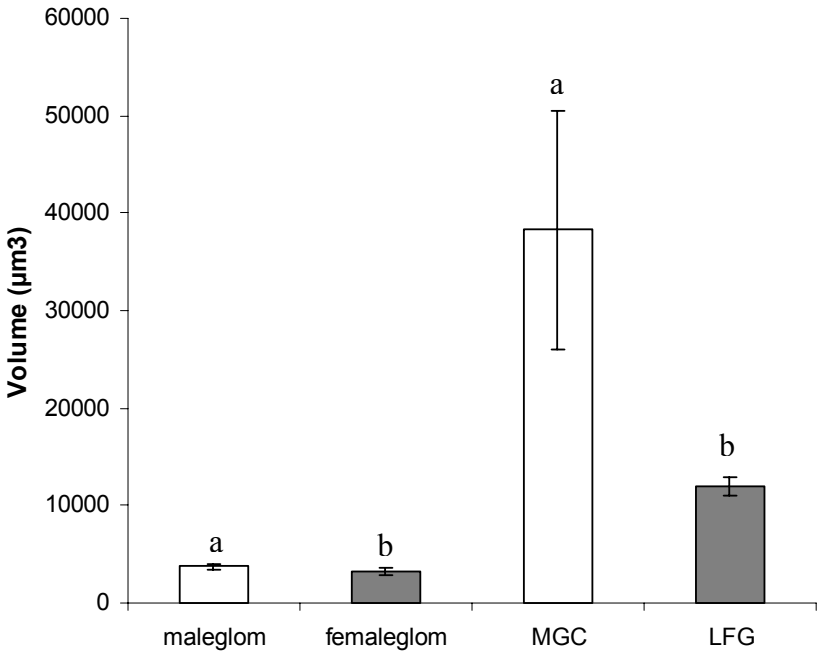

Fig. 4.15 The average volume of ordinary male and female glomeruli and macroglomeruli (MGC). The MGC was about ten times larger than ordinary glomeruli, whereas the LFG was only four times larger. a and b indicate a significant difference at $p<0.05$ between ordinary glomeruli and macroglomeruli/LFGs. Maleglom: n= 9, femaleglom: n= 8, MGC: n= 7, LFG: n= 3.

In summary, 3D calculations of the AL and its glomeruli showed no significant gender difference in the ordinary glomeruli. The average number of glomeruli was 40. In males, significantly enlarged glomeruli were found, which are thought to form the MGC. In addition, enlarged glomeruli were found in certain female preparations, which could correspond to the LFGs responsible for processing HIPVs found in moths. Moreover, these results enabled a more direct comparison with the results of Smid's study (Smid et al., 2003). The current study has shown that *C. vestalis* had fewer but larger glomeruli than *C. glomerata* and *C. rubecula*.

4.4. Discussion

The aim of the current study was to investigate the morphology of the AL in *C. vestalis*. Therefore, the organisation of the male and female *C. vestalis* AL was analysed by describing size, number and volume of the glomeruli, and the volume of the AL. Several hypotheses were formulated and tested. Firstly, the number of glomeruli could explain behavioural differences (Chapter 2). Secondly, the segregated regions for pheromone and non-pheromone processing found in other insect species also occur in *C. vestalis*. This would therefore result in enlarged glomeruli in males. Thirdly, females could possess LFGs, which have been previously found in moths (King et al., 2000). These LFGs process certain host-plant volatiles (HIPVs), such as linalool. Finally, it was expected that *C. vestalis* might differ from its related *Cotesia* species, *C. glomerata* and *C. rubecula*, due to a different host specialisation.

Gender comparison of C. vestalis

Similar to other species, *C. vestalis* showed common morphological characteristics; for example, glomerular organisation was located in the peripheral layer of the AL around a central core devoid of glomeruli (Zube et al., 2008). In many hymenopteran species, males are known to exhibit a smaller behavioural repertoire than females, and as a consequence, an often lower number of glomeruli (Zube et al., 2008; Kuebler et al., 2010; Stieb et al., 2011). This is contrary to findings in the current study, which has demonstrated that there were no significant gender differences in size and number of ordinary glomeruli. However, in the related *Cotesia* species, generalist *C. glomerata* males had fewer glomeruli, while in *C. rubecula* no gender differences were found (Smid et al., 2003). This suggests that differences in learning are less likely to be due to morphology of the AL, but more to differences in central processing of an odour. Furthermore, these results support the hypothesis (Chapter 2) that HIPVs play an important role for males to locate females. Processing of HIPVs could thus take place in both genders, which will be described in the following Chapter (Chapter 5).

Macroglomeruli in males

Macroglomeruli are well documented in other species such as moths, bees, cockroaches and ants. In most cases it was shown that macroglomeruli are involved in the detection of sex pheromones (Arnold et al., 1985; Rospars, 1988). In social insect species showing a division into casts (e.g. ants) macroglomeruli process, for example, trail pheromones instead of sex pheromones (Kleinadam, 05; Kelber, 09).

It was hypothesised that *C. vestalis* males use sex pheromones in addition to HIPVs to locate females (Chapter 2). This study revealed for the first time the evidence of a MGC in parasitoid males, which consisted of 2 to 3 significantly enlarged glomeruli. Their shape and position are similar to those found in other species (Kuebler et al., 2011). Macroglomeruli can be identified by their location, shape, size, or volume. They are usually located near the antennal nerve, and are often of a distinctive elongated cylindrical shape. In Heliothide moths, three distinctive macroglomeruli have been identified (Huetteroth and Schachtner, 2005); the cumulus, the toroid and the horseshoe. The cumulus is the first and most distal located MG and is elongated and cylindrical. The toroid is sometimes also U-shaped, while the horseshoe gets its name from its characteristic shape.

In *C. vestalis*, two macroglomeruli were of an elongated shape. A third was more variable in size and of a rounder shape. The average volume of a macroglomerulus was 38,267μm^3. It was about ten times larger than the average volume of an ordinary glomerulus (3,719μm^3). This is in accordance with findings in leaf-cutting ants, where it was found that the macroglomerulus can be approximately 9-10 times the volume of ordinary glomeruli (Kleineidam et al., 2005). In two species *Atta vollenweideri* and *A. sexdens* the macroglomerulus had a volume of 70,181 μm^3 and 73,589 μm^3, respectively, whereas ordinary glomeruli had an average volume of 7,577 μm^3 and 7,994 μm^3, respectively (Kleineidam et al., 2005).

In Lepidoptera, it is assumed that the number of MGC compartments is correlated to the number of behaviourally active components in the pheromone blend (Huetteroth and Schachtner, 2005). Its increased size results from a higher number of ORNs, and it therefore has a lower threshold to odours (Vickers and Baker, 97; Berg et al 98). This enables males to locate females over longer

distances, and in the case of parasitoids could increase their sensitivity to female pheromones amongst the numerous environmental odours.

In summary, size, volume, shape, as well as position of the enhanced glomeruli found in males indicate the existence of a MGC in *C. vestalis*. The MGs and the observed courtship behaviour (Chapter 2) provide further evidence for pheromone communication in *C. vestalis*. The necessity for a MGC could further be related to the host specialisation of this parasitoid species. The location of the host can vary; hence, males rely on the efficient detection of relevant odour cues to locate females for mating. Females thus need to be able to attract males from a distance (Fauvergue et al., 1995). This could be important for solitary species such as *C. vestalis*. Hence, enlarged glomeruli solely for the detection of pheromones, might be important for male *C. vestalis*.

Large female glomeruli

In some female moths, female equivalents to the MGC, called "large female glomeruli" (LFGs) have been found (Rössler et al., 1998; King et al., 2000; Rospars and Hildebrand, 2000) which are approximately the same size as the largest ordinary glomeruli (Rospars and Hildebrand, 2000; Kleineidam et al., 2005).

In this study, it can only be speculated that specialised LFGs can be found in females. LFGs are reportedly not substantially larger than ordinary glomeruli (in moths LFGs range between 92-100 µm in diameter, while ordinary glomeruli range between 45-100 µm in diameter) (Rospars and Hildebrand, 2000). In *C. vestalis*, larger glomeruli have been found using AMIRA. In certain preparations a few enlarged glomeruli, which were about four times larger than the average glomerulus, could be identified. These glomeruli could represent LFGs, which have been found in moths (King et al., 2000). However, their shape and position was variable, which made them less easy to identify compared to the MGC in males.

It was shown, that certain LFGs respond uniquely or more specifically to HIPVs such as linalool (King et al., 2000). Central neurons with arborisation in the lLFG respond more to linalool and other monoterpenoids, and may be involved in processing information about host plants or courting males (King et

al., 2000; Shields and Hildebrand, 2001). This has also been supported by King (King et al., 2000), who found that the corresponding ORNs of type-A trichoid sensilla are tuned to terpenoids and aromatic esters, which are HIPVs. LFGs might also process information about male pheromones, which often contain a mixture of volatiles associated with plants; for example, linalool (Ljungberg et al., 1993). Hence, due to its host specialisation, and also the observation that the female's presence induces courtship behaviour in males (Chapter 2), it is possible that *C. vestalis* females have functionally specialised glomeruli. In *C. rubecula*, a related specialist parasitoid, fused glomeruli were found at the location where a MGC was found in males (Smid et al., 2003), which supports the results of the current study that specialists have LFGs for processing HIPVs. Calcium imaging studies can visualise the response pattern of those glomeruli to, for example, linalool (Chapter 5).

Antennal lobe

To determine the accuracy of glomerular measurements, the AL volume was analysed as a reference volume using manual measurements, as well as with the software program AMIRA. With both methods, the AL volume was larger than the total glomerular volume. Manual measurements differed greatly, as they could not take into account any intra-glomerular spaces, areas devoid of glomeruli (central core), or changes in shape of the AL with depth.

The average AL volume was 4.3×10^5 μm^3 when analysed with a software program and did not differ between sides or genders. Manual measurements, however, showed a gender difference in the length and depth of the left AL. Brain asymmetry has mainly been shown in vertebrates but a few studies have referred to differences in invertebrates (Bisazza et al., 1998; Vallortigara et al., 1999). Asymmetry can occur on a morphological level, as differences in gene expression, or on a functional level. A study in *Drosophila* suggests that asymmetry is important for long term memory formation (Pascual et al., 2004). Indeed, studies in bees confirmed this theory (Rogers and Vallortigara, 2008; Frasnelli et al., 2010b). Research has shown that the right antenna appears to be important for olfactory learning, and bees with a covered right antenna did not recall any odours, contrary to bees with the left antenna covered (Letzkus et al., 2006). These functional differences suggest that differences would also occur in their

morphology. It was shown that the right antenna of bees contained more sensilla placodea than the left (Letzkus et al., 2006; Frasnelli et al., 2010a; Anfora et al., 2011). A morphological study of the antennae of *C. vestalis*, however, has shown that while females had a higher number of s. trichodea WP (olfactory) and trichodea TP I and II (chemosensory-gustatory), males had a higher number of sensilla placodea (olfactory). However, there was no difference in side distribution (Roux et al., 2005). Hence, the difference found in the left AL length is more likely to be caused by experimental handling differences than lateralisation.

The number of ordinary glomeruli is species specific

The number of glomeruli is known to be species-specific and vary accordingly (Rospars, 1988). For a better understanding of odour coding in the glomeruli of *C. vestalis* it is essential to know their distribution and number. An average of 40 glomeruli could be identified in *Cotesia* males and females. This is similar to the number of glomeruli found in *Drosophila* and other insects. Insects show, contrary to vertebrates, a great variation in the number of glomeruli (Fig. 4.16). In vertebrates, on the other hand, an increased number of glomeruli from lower to "higher origin" can be found (zebrafish 80, rodents 2000) (Baier and Korsching, 1994). In invertebrates, the number of glomeruli can range from as low as 40 in *Drosophila* (Laissue et al., 1999), 50 in mosquitoes (Ignell et al., 2005), up to 1000 in locusts and hornets (Hanström, 1928). Some insects such as Hemiptera (Homoptera) have no glomeruli (Kristoffersen et al., 2008).

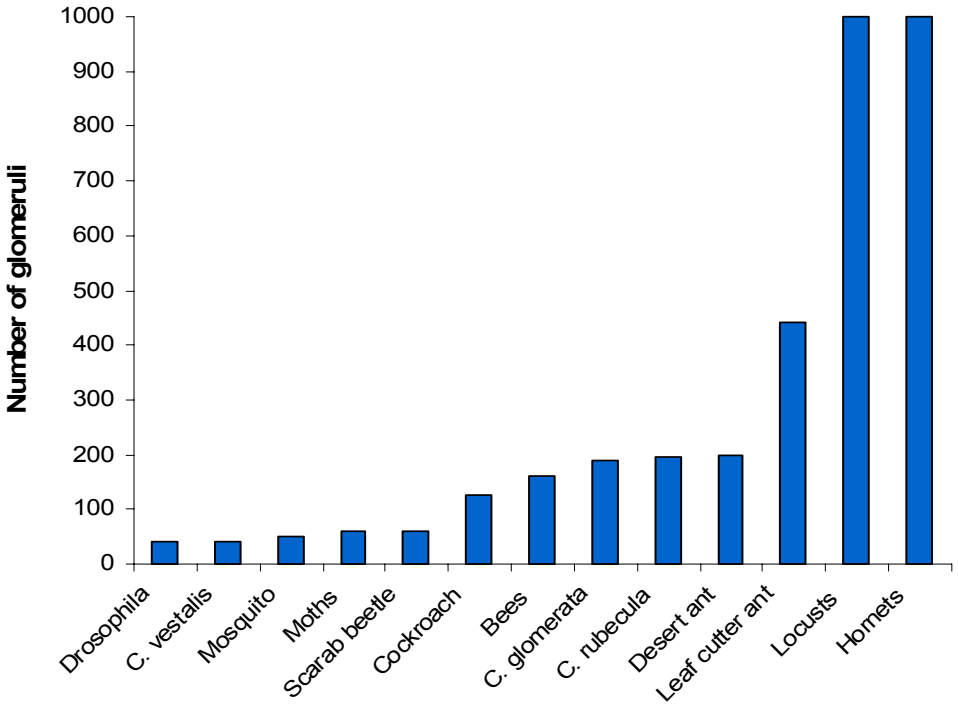

Fig. 4.16 Species-specific variation in the number of glomeruli. The number of glomeruli is species-specific, and generally higher in social Hymenoptera.

Compared to *C. glomerata* (188 glomeruli) and *C. rubecula* (196 glomeruli) (Smid et al., 2003), however, the number of identified glomeruli in *C. vestalis* is surprisingly small. This could be explained by the following arguments:

a) Body size

One explanation could be that body size and length of the antennae play a role in the number of glomeruli. Both *C. glomerata* and *C. rubecula* are larger than *C. vestalis*, and have longer antennae and larger sensilla (Table 4.3) (Bleeker et al., 2004; Roux et al., 2005).

Table 4.3 Comparison of body size, the length of antennae and glomeruli between *C. glomerata*, *C. rubecula* and *C. vestalis*. Modified from (Bleeker et al., 2004; Roux et al., 2005) and the current study (number of glomeruli in *C. vestalis*). The number of glomeruli is given as the mean number found in these species (*C.g* and *C.r* n= 2; *C.v* n= 9). Body size and length of antennae give values of females (first value) and males (second value).

	C. glomerata	*C. rubecula*	*C. vestalis*
Body size	6-7 mm	6-7 mm	4-5 mm
Length of antennae	2.8-3.7 mm	3.8-3.9 mm	2.1-2.9 mm
Number of glomeruli	188	196	40

C. rubecula has, as the biggest parasitoid of these three related *Cotesia* species, the highest number of glomeruli (Smid et al., 2003). Although size has generally been ruled out as a factor influencing the number of glomeruli (Masante-Roca et al., 2005), a positive correlation between body size and number of antennal sensilla was previously suggested (Bleeker et al., 2004), and has also been found in bees (Johnson and Howard, 1987). However, based on the values presented in Table 4.3, the number of glomeruli in female *C. vestalis* would be between 108-141, and 134-147 in males. However, based on the body size, or size of antennae, these estimated numbers would also result in a higher number in *C. glomerata* as well as *C. rubecula*. Therefore, body size alone cannot be an indicator for the number of glomeruli.

b) The number of sensilla and ORN

It has been demonstrated that in some insects the number of glomeruli is also closely related to the number of receptors. In *Drosophila*, the number of glomeruli corresponds approximately to the number of olfactory receptor molecules (Vosshall et al., 2000). Consequently, there could be a positive correlation between the number of sensilla, the number of ORNs, and the number of glomeruli. Thus, the size and number of glomeruli could be influenced by the amount and kind of odours that need to be distinguished (Couto et al., 2005) and may thus be related to the complexity of plant volatiles. In the case of *C. vestalis*, only certain host specific HIPVs might be relevant and thus result in a lower number of ORs/ORNs and glomeruli. Studies of the antennal structure in *C. glomerata*, *C. rubecula* and *C. vestalis* have shown that the length of sensilla was similar in *C. glomerata* and *C. rubecula*, but shorter in *C. vestalis* (Bleeker et al.,

2004; Roux et al., 2005). The exact number of olfactory sensilla is only known in *C. vestalis*; however, s. coeloconica I were less abundant in *C. vestalis* than in *C. glomerata* and *C. rubecula*. However, s. trichodea TP II were only present in *C. vestalis*. Therefore, differences in the number of olfactory sensilla could lead to differences in the number of glomeruli and might be not only influenced by the degree of host specialisation but also the type of host.

c) Host specialisation and phylogenetic relations

Although it was suggested that the degree of specialisation is not linked to the number of antennal sensilla (Roux et al., 2005), it is so far unknown whether it could influence the number of glomeruli. In fact, studies have shown that *C. glomerata* and *C. vestalis* are more closely related than *C. rubecula* (Michel-Salzat and Whitfield, 2004). Despite being closely related, *C. glomerata*, *C. rubecula* and *C. vestalis* differ not only in the degree of specialisation, but also in the preferred host species. *C. glomerata* parasitises several *Pieris* species, but prefers *P. brassicae*, while *C. rubecula* only parasitises *P. rapae*. *C. vestalis's* host, on the other hand, is *Plutella xylostella*. Due to parasitoids' great variation in learning and memory it may be possible that the number of glomeruli could vary considerably. Hence, in parasitoids, the number of glomeruli might correlate with their degree of host specialisation, rather than the degree of phylogenetic relation, contrary to findings in moths (Skiri et al., 2005).

Not only the host, but also the degree of host specialisation might influence the number and size of glomeruli. Specialists like *C. vestalis* and *C. rubecula* could have more synaptic connections and thus larger glomeruli to amplify relevant signals and discriminate between specific HIPVs. This was also suggested in previous studies, which stated that specialists have to adapt to find hosts that occur with a lesser density (Geervliet et al., 1998a). Indeed, behavioural studies in *C. vestalis* have shown that specialists are more responsive to HIPVs (Chapter 2). This in turn helps them to target their host plants more efficiently. Hence, more synaptic connections could be needed to filter specific volatiles, rather than showing a great responsiveness to a variety of odours as has been shown in generalists. Generalists need to distinguish between more relevant HIPVs, and could, as a consequence have a higher number of ORNs and OR and thus a higher number of glomeruli.

d) Other explanations

Differences in the number of glomeruli can also be influenced by the role of social behaviour. As the number of glomeruli is higher in social insects than in solitary ones (Galizia et al., 1999a; Kleineidam et al., 2005; Kleineidam and Rössler, 2009), one could assume that solitary wasps possess fewer glomeruli. This would support findings in *C. vestalis* but contradict those in *C. rubecula*.

Another explanation for the low number of glomeruli could be that visual cues play an additional role for *C. vestalis*. It has been shown that species with a lower number of olfactory glomeruli have a more developed visual sense (Nishikawa et al., 2008; Kuebler et al., 2010; Stieb et al., 2011). A few studies have investigated visual learning in parasitoids, and suggested that olfactory, as well as visual cues, play a role in their foraging behaviour (Turlings et al., 1993). Although olfactory cues are more reliable and detectable over longer distances, visual cues could enhance their foraging success at shorter distances (Wäckers and Lewis, 1994). A study using *C. vestalis* suggested that this species does indeed use visual cues to locate food sources (Kugimiya et al., 2010). Therefore, this parasitoid might use olfactory cues to locate the host from a greater distance, and visual cues could become important at a closer range.

e) Methodological differences

Methodological differences could provide an explanation for the observed difference between the numbers of glomeruli found in *C. vestalis* compared to *C. rubecula* and *glomerata.* In the current study, a bath applied dye has been used, rather than axon tracing, which was the method used by Smid (Smid et al., 2003). The dye RH795 has been frequently and successfully applied to label glomeruli in other Hymenoptera (Galizia et al., 1997; Sachse et al., 1999; Malun et al., 2002). It is a voltage sensitive dye which specifically stains cell membranes (Galizia et al., 1997; Le Calvez and Ulfendahl, 2000; Wang et al., 2008). RH795 is bath applied with an incubation period of usually not more than 1 hr, and its penetration into the tissue is increased by adding protease (Sachse and Galizia, 2002). One disadvantage of using a bath applied dye, however, is that it does not guarantee that all glomeruli will be labelled with the same intensity (unequal labelling) (Galizia and Vetter, 2005). This could be caused by a lack of penetration of the dye into the tissue, in particular in deeper regions or layers

within the AL, resulting in weaker tissue labelling. Therefore, glomeruli which were only partially stained may not have been seen and this could have led to their number being underestimated.

In Smid's study, however, (Smid et al., 2003), glomeruli were labelled using biotin dextran amide as axonal tracer. With this method, axonal projections reaching into the glomeruli were traced from their source (the cell body or soma) to their point of termination (the synapse; anterograde tracing), and in the case of the suboesophageal ventral unpaired median neurons (VUM), axonal projections were stained from their point of termination (the synapse) to their source (the cell body or soma; retrograde tracing) (Smid, pers. communication). This method ensures a more targeted labelling of the synaptic connections within the glomeruli. In addition, brains of *C. glomerata* and *C. rubecula* were incubated in heptane to increase dye permeability and subsequently incubated in streptavidin for 24 hr (conjugated to FluoroLinkCy2 and bovine serum albumin and 0.25% Triton X-100) to visualise biotin dextran. Such a way of labelling ORN axons is a suitable method to study structure, distribution, physiology and development of olfactory glomeruli (Braubach et al., 2012) and it is very likely that with this method, all glomeruli were equally labelled. Consequently, more evenly stained glomeruli can be counted more accurately. This technique, involves, however, fixation of the tissue, which was found to cause a disadvantage for morphological studies in *C. vestalis* (Chapter 3).

In summary, the glomerular organisation in *C. vestalis* was similar to other hymenopterans. No gender differences were found in ordinary glomeruli. Therefore, gender differences in behaviour (Chapter 2) were not due to a different morphological organisation of the AL. In addition to 40 ordinary glomeruli, gender-specific glomeruli have been identified. The MGC and the courtship behaviour observed in males (Chapter 2) could indicate that females produce pheromones which are detected by the male.

The low number and larger volume of ordinary glomeruli raises speculation that for a specialist parasitoid like *C. vestalis*, the detection of specific HIPVs is essential for successful host location. Fewer and larger glomeruli could amplify

the relevant odour cues and facilitate their detection. In addition, enlarged glomeruli found in females could be responsible for processing specific HIPVs. Moreover, a comparison with two closely related *Cotesia* species revealed that the number of glomeruli can differ even between closely related species, depending on host type and degree of host specialisation.

CHAPTER 5

CALCIUM IMAGING OF BRAIN ACTIVITY IN THE ANTENNAL LOBE OF *COTESIA VESTALIS*

5.1. Introduction

For parasitoids, volatile cues from the host-habitat, the host-plant and the host are important for host location and hence reproduction (Vet and Dicke, 1992). Experiments described in Chapter 2 have shown that for the specialist parasitoid *Cotesia vestalis* the peripheral (antennal) perception of odours is similar in males and females. However, gender differences were found at a behavioural level. While both genders learned linalool, a common plant volatile (Pinto et al., 2007a), gender differences were found for 1-nonanol, which is not part of the HIPVs emitted from *Brassica rapae* var. *Wong bok* (Durrant, 2007). Females avoided 1-nonanol and males did not learn it, suggesting that for this specialist parasitoid, the "ecological relevance" of an odour could be important. These results raise the question of how olfactory information is represented at a central level and whether males and females show similar or different activation patterns of glomeruli.

Previous studies in vertebrates as well as invertebrates have shown that odours evoke specific spatio-temporal activity patterns in glomeruli which are conserved within a species (Friedrich and Korsching, 1997; Joerges et al., 1997; Galizia et al., 1999b; Rubin and Katz, 1999). Although the functional organisation

in the honeybee appears to be genetically pre-determined, odour coding is also experience-dependent and can be altered via learning experiences (Faber et al., 1999; Rath et al., 2011). To date, very little is known about neural processing in a parasitoid brain (Smid et al., 2003). Brain imaging can provide a valuable insight into selective odour processing influenced by host specialisation of a parasitoid.

Spatio-temporal properties of odour processing

Every odour generates a specific glomerular activity pattern and every glomeruli has a characteristic response, which can be tonic, phasic tonic or slow phasic (Galizia and Menzel, 2001). Odour representation is symmetrical in both sides of the AL suggesting that the functional identity of a glomerulus is genetically encoded (Faber et al., 1999). Guerrieri (Guerrieri et al., 2005b) demonstrated that only differential or side-specific, but not absolute conditioning, evoke changes in glomeruli patterns in the antennal lobe. Absolute conditioning, as an elementary form of learning, involves only the association of one odour to a reward, whereas in differential conditioning the animal learns to distinguish between a rewarded and one non rewarded odour (Guerrieri et al., 2005b). This could cause differences in neural processing and therefore lead to a different glomerular activity pattern.

Functional odour processing

In many moth species, research has shown that in males, plant odours are processed in ordinary glomeruli (Galizia et al., 2000b; Skiri et al., 2004) while the MGC is responsible for coding pheromone information. Non-pheromones have been shown to elicit higher inter-individual differences in glomerular activity patterns than pheromones (King et al., 2000; Reisenman et al., 2004). The functional division of pheromone and odour processing pathways has recently been challenged by a study in moths which found that ordinary glomeruli did process plant odours in addition to pheromones (Varela et al., 2011). However, in the species used by Varela (Varela et al., 2011), both sexes can detect sex pheromones emitted by females and pheromone and plant-odour processing pathways seem to be less separated than in other moths. In *C. vestalis* males it was shown that with the exception of the pheromone processing macroglomerular

complex (MGC), the glomerular structure of the AL was the same in males and females (Chapter 4).

Plant odours have been shown to elicit combinatorial activity patterns in ordinary glomeruli in both males and females (Hansson et al., 2003; Reisenman et al., 2005). In many female moths, so called large female glomeruli (LFGs) have been identified which process plant related odours such as linalool (King et al., 2000; Kalberer et al., 2010). Linalool is emitted from floral and vegetative parts of many plants, including *Brassicaceae* plants, which are host plants of *C. vestalis* (Knudsen et al., 1993).

The aim of the current study was to understand how olfactory cues are processed in a parasitoid brain and to investigate neural mechanisms of host location. Therefore, calcium imaging based on measurements of neural activity using fluorescent imaging was used in this study. To test the suitability of the protocol and equipment, several control experiments were carried out before using the parasitoid as a model. This involved adapting the calcium imaging protocols currently used in bees for use with the parasitoid *C. vestalis*.

5.2. Materials and methods

Animals

For brain dissection, parasitoids were fixed in a pipette tip as previously described in Chapter 3. To summarise, *C. vestalis* was mounted in an acrylic block and the cuticle on the dorsal side of the head removed to reveal the ALs. To ensure the antennae remained dry throughout the experiment, the antennae were shielded with parafilm and holes between the brain cavity and antennae were sealed with hard wax.

Calcium imaging has often been used to investigate spatio-temporal activity patterns of the glomeruli in bees, using a fluorescence microscope (Joerges et al., 1997; Deisig et al., 2010). In the current study, a confocal microscope was chosen due to the lack of availability of a fluorescence microscope and also because of its better spatial resolution. Hence, bees were used as a reference model to test the suitability of a confocal microscope for calcium imaging studies in the parasitoid. Bees were captured at the entrance of a hive and cooled on ice for approximately 4 min, to allow mounting into a 1 ml pipette tip. The tip had been cut on both sides to fit the size of the bee. To immobilise the bee it was mounted in the tube and insulating tape was placed around its neck to prevent movement of the head (Fig. 5.1 A). The head was further restrained by gently applying hot wax around the tape (Fig. 5.1 B). Both antennae were held back with a fine wire to prevent antennal movement. To reveal the ALs a small window behind the antennae was cut into the cuticle and tracheal tissue and air sacs were removed (Fig. 5.1 B-D). For imaging a 10x objective was used for both bees and parasitoids.

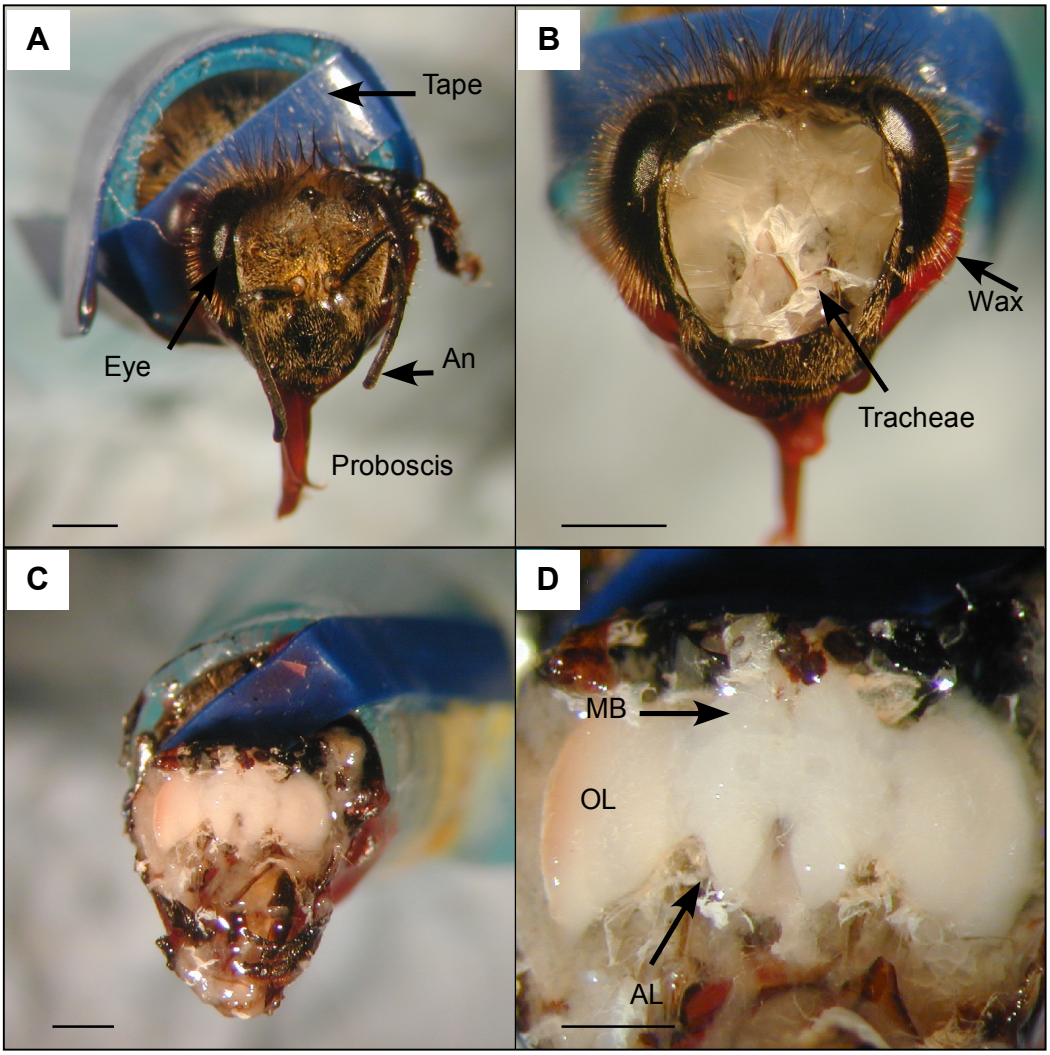

Fig. 5.1 The honeybee preparation. **A)** The honeybee was secured in a pipette tip and fixed with tape. The head was additionally fixed with hot wax to prevent movement. **B)** Top view of the restrained bee after an incision in the cuticle is made, exposing the bee's brain covered with tracheae. **C)** Exposed brain of the bee with completely removed tracheae. **D)** Higher power view of the exposed bee brain. AL= antennal lobe, AN= antenna, MB= mushroom body, Tracheae = tracheal tissue. Scale bar= 100µm.

Dye preparation

Following preliminary studies (Chapter 3), the fluorescent dye Calcium Green 1-AM (Invitrogen, UK) was used for all experiments. Calcium green 1-AM is a calcium-sensitive dye bound to an acetoxymethyl group (AM) and is frequently used in calcium imaging studies (Galizia and Vetter, 2005). Inactive in its AM-bound form, it only becomes active inside cells, when esterases cleave the AM group and leave the dye active and cell-impermeant within the cell (Galizia and Vetter, 2005). During imaging, the dye responds to changes in calcium levels brought about by changes in neuronal activity in synaptic signalling. The dye

mainly measures a compound signal of the activity of olfactory receptor neurons (ORNs), but also of local interneurons (LNs), projection interneurons (PNs) and possibly glial cells (Galizia and Menzel, 2001).

A detailed description of dye preparation can be found in Chapter 3. In summary, 50 µg dye was diluted with 50 µl Pluronic F-127 and vortexed for 1 min. It was then added to 950 µl saline solution and again vortexed for 5 min. Several control experiments were conducted to test the suitability of the protocol developed in the current study for use with parasitoids.

Staining protocol for potassium chloride experiments

a) Rat hippocampal neurons

The suitability of Calcium Green 1-AM for parasitoids in combination with a confocal microscope was first tested in rat hippocampal cells (supplied by Angela Cheung). A cell culture of 6 d old neurons was stained with 200 µl dye and 1000 µl saline solution. During a 45 min incubation period the cell culture was placed in an incubator kept at 37°C (Hajieva et al., 2009). Cells were washed with locust saline solution (Chapter 3) three times before imaging. Imaging was conducted with a 20x water dipping lens using the same parameters as described in Chapter 3. To determine if a calcium increase could be detected with a confocal microscope, potassium chloride (KCl) was used as a depolarisation agent. Potassium chloride depolarises neurons, causing an influx of Ca^{2+} via voltage-gated calcium channels. The neurons remained in the well used for cell culturing and were covered with 1000 µl saline solution. The experiment did not consist of a constant saline flow with changing concentration but a bathing chamber containing 1000 µl saline solution, into which, during imaging, 40 µl KCl were pipetted. To test at which concentration KCl was effective, different concentrations of KCl were used (60 mM, 2 M and 4 M). This resulted in a final KCl concentration of 2.4 mM, 80 mM and 160 mM respectively. For the time series, images were taken over 100 frames at 247 ms/frame (4 frames/s) in a 256 x 256 pixel frame, using the same laser settings as described in Chapter 3.

b) Parasitoid AL

To test the suitability of Calcium Green 1-AM for calcium imaging with a confocal microscope in a parasitoid brain, insects were mounted and dissected as described in Chapter 3. After removal of the cuticle, the head was removed and placed into the incubation chamber. After the incubation period, the brain was washed with saline solution to remove residual dye traces. To determine if a change in fluorescence and thus an increase in calcium could be detected, 20 µl KCl was added into the bathing chamber containing 500 µl saline solution. KCl was applied in different concentrations (2 M, 4 M), resulting in a final KCl concentration of 80 mM and 160 mM, respectively. Imaging was conducted with a 20x water dipping lens. Images were taken over 100 frames at 247 ms/frame in a 256 x 256 pixel frame, using the same laser settings as described in Chapter 3.

Staining protocol for calcium imaging experiments during odour stimulation

The insect brain was washed with saline solution several times before 50 µl of Calcium Green 1-AM (40 µM) was applied. A cover slip was placed above the brain to prevent desiccation (Fig. 5.2). During incubation, the preparation was placed into a fridge (13°C) for 45 min, followed by 15 min incubation time at room temperature. During incubation the preparation was placed in a box to avoid photo bleaching of the dye. Before imaging, the brain was washed once more with saline solution to remove residual dye traces.

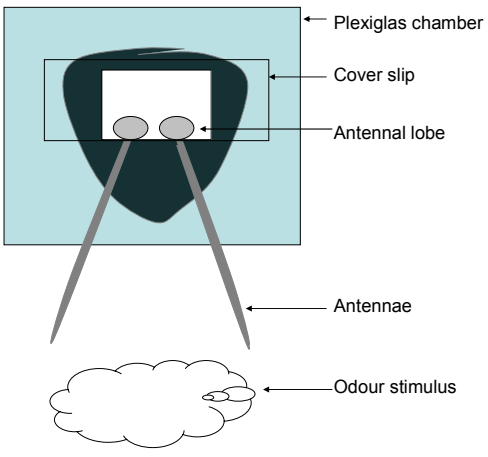

Fig. 5.2 Dorsal view of the parasitoid placed under the microscope. The cuticle on the dorsal part of the brain was removed prior to staining to reveal the ALs. The brain cavity was filled with saline solution and a cover slip was placed over the exposed brain structures to prevent desiccation. The preparation was placed at a 2 cm distance from the odour source.

Odour application with a syringe in preliminary imaging experiments

a) Bee imaging

In preliminary experiments, different odours (citronella oil, clove oil, 1-nonanol) were applied manually with a 10 µl syringe during calcium imaging. The odour (40 µl) was pipetted on filter paper (1 cm). For the final experiments only 1-nonanol was used, as it elicits conspicuous responses in the AL in other studies (Deisig et al., 2006). Imaging with no stimulus was used as a control. Three repeats per position in the AL were conducted with an inter-stimulus interval (ISI) of 2 min within one repeat set (no stimulus, 1-nonanol) and an inter-trial interval (ITI) of 5 min during each repeat set.

b) Parasitoid imaging

For preliminary imaging experiments with *Cotesia*, linalool (an important HIPV (Pinto et al., 2007a); 5 mg/ml ether) and 1-nonanol were used. For parasitoids, the same protocol was used as in bees and odours were presented in a set of "no air", "air" and "odour" over 3 repeats.

Controlled odour delivery set up

To avoid possible mechanical stimulation during odour application with a syringe, an improved set up consisting of a continuous airflow into which the odour was introduced was developed. The airflow was regulated with a flowmeter (0.8 l/min) and connected to a stimulus controller (Syntech, The Netherlands) (Fig. 5.3). A Y-tube (internal diameter of 1.5 cm, a 11.5 cm stem and 9 cm arms at a 60° angle) was connected to the stimulus device via Teflon tubing. One arm was connected to the continuous airflow (0.8 l/min) and the other arm was connected to the odour source flow. The odour (10 µl) was applied onto 1 cm^2 filter paper at the base of the arm connected to the stimulus device. The animal was placed at a 2 cm distance from the stem of the Y-tube and odours were applied for 4 s with an ISI of 2 min and an ITI of 5 min. Imaging was conducted at several depths in the AL per preparation and per depth (position) three to five stimuli repeats were carried out.

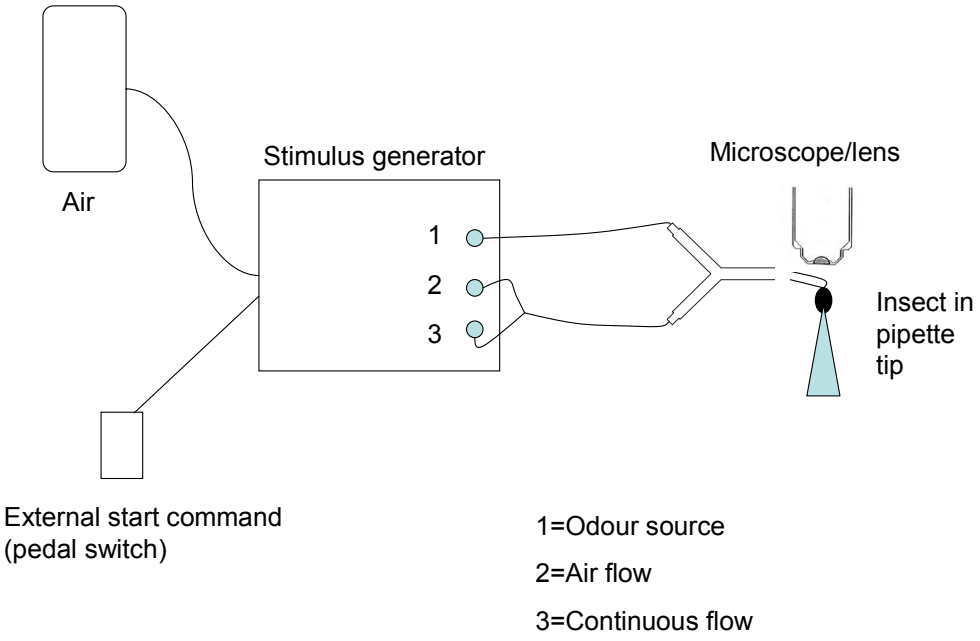

Fig. 5.3 Schematic drawing of the controlled odour delivery setup. A continuous airflow (0.8 l/min) was passed through a stimulus generator, which delivered a constant airflow into which the odour stimulus was inserted. The odour stimulus was pipetted onto filter paper and placed into the Y-tube arm connected to the odour source channel (No. 1 in the figure). The other arm was connected to the airflow and continuous flow (No. 2 and 3). Once the olfactory stimulus was activated, the airflow stopped. This ensured that the continuous airflow remained constant throughout the experiment.

Confocal microscope

Calcium imaging was conducted using a confocal microscope (Leica TCS SP2, Leica Microsystems) in a xyt - time sampling mode (2 spatial and 1 temporal dimension). For excitation, a 488 nm Argon Laser with an excitation rate of 520-650 nm was used. Pinhole range and intensity were adjusted for each preparation. Imaging with bees was conducted over 24.04 s, 400 ms/frame (2.5 frames/s) and an image size of 512 x 512 pixels with 8 bit resolution. Odour application lasted 4 s. In KCl experiments and imaging with parasitoids, image size was 256 x 256 pixels with 8 bit resolution. The sampling rate of the laser was 800 Hz. A bi-directional scanning mode was used to increase the speed. Images were taken over a 24.5 s period at 247 ms/frame (4 frames/s).

Data processing

Data processing followed well established examples of calcium imaging studies in Hymenoptera (Galizia and Vetter, 2005) using Calcium Green 1-AM as a fluorescence marker. Data were analysed using custom written software (provided by Oliver Dewhirst) (MATLAB, R2011b, MathWorks, UK). Each image consisted of 2 spatial dimensions (x, y) and one temporal dimension (t). Raw data were filtered in the two spatial dimensions using a median filter with a size of 5 pixels in order to reduce photon noise. To correct for bleaching in the temporal dimension, the median value of all pixels of each frame was subtracted from each pixel in each frame (baseline correction). To detect changes in fluorescence, relative fluorescence value changes ($\Delta F/F$) were calculated as (F-F_0)/F_0. Background fluorescence (F_0) was defined as an average of 11 frames before odour or KCl stimulation (frames 5-15) (Sandoz, 2006). Regions of interest (ROIs) and their data points were constructed using the same software and ROIs inside and outside the AL were selected for data analysis. To facilitate the identification of active regions, images presented in the results section are shown in false colour coding (F-F_0) with dark blue indicating lowest fluorescence and red highest fluorescence.

Statistical analysis

Odour stimulation was repeated 3 – 5 times per preparation and the average intensity before, during and after odour onset was statistically compared. To see whether a calcium increase shown by an increase in fluorescence occurred during odour stimulation, several regions in and outside the AL were selected. To ensure the recorded signal was only due to neuronal activity, strict criteria were set, such as no activity outside the AL and increase of fluorescence inside the AL during odour stimulation, followed by a decrease after stimulation.

Means of intensity before, during and after odour application were compared using a paired t-test. In experiments with bees, frames before (5-15), during (20-30) and after (40-50) odour stimulation corresponded to 2 s – 6.1 s, 8.1 s – 12.2 s and 16.3 s – 20.4 s, respectively. In imaging experiments with the parasitoid, frames before (5-15), during (31-49) and after (50-60) odour stimulation corresponded to 1.2 s – 3.7 s, 7.7 s – 12.1 s and 12.4 s – 14.9 s, respectively.

5.3. Results

5.3.1. Temporal pattern of odour evoked activity in the honeybee antennal lobe

In order to determine whether the dissection and staining method was successful and suitable for imaging experiments with parasitoids, imaging experiments were first conducted in the honeybee. The honeybee is a well-studied model organism and considerably larger than a parasitoid. From three out of seven individuals a response to olfactory stimulation was recorded (Table 5.1), from which the one with the best signal was chosen as an example (Fig. 5.4 A).

In bees, no fluorescence increase was observed before olfactory stimulation (Fig. 5.4 B). During olfactory stimulation, however, changes in fluorescence level became apparent (Fig. 5.4 C). A maximum increase was found 2 s following odour onset (Fig. 5.5 A). The response returned to baseline after 4 s (n= 3). The maximum response amplitude was approximately 0.06% $\Delta F/F$. No signal increase was detected in the ROI outside the AL (Fig. 5.5 B) and in three ROIs inside the AL (Fig. 5.5 C).

The average of frames before (5-15), during (20-30) and after (40-50) odour stimulation over 3 stimuli repeats was determined and statistically compared (Fig. 5. 5 E). In ROI 2, ROI 3, ROI 5, ROI 7, and ROI 9 the amplitude of the signal increased during odour stimulation and then decreased once stimulation had ceased. Statistical tests, however, showed no significant difference between before and during odour onset, nor during and after odour onset. However, comparison between before and after odour onset revealed a significant difference for ROI 2, ROI 3, ROI 5, ROI 7, and ROI 9 (ROI 2: $T= 6.089$, $df= 2$, $p<0.05$; ROI 3: $T= 16.118$, $df= 2$, $p<0.05$; ROI 5: $T= -10.949$, $df= 2$, $p<0.05$; ROI 7: $T= -12.859$, $df= 2$, $p<0.05$; ROI 9: $T= -4.311$, $df= 2$, $p<0.05$).

Comparison of signals from regions inside the AL with the region outside the AL (ROI 8) only revealed significant differences before stimulation in ROI 3 (ROI 3: $T= -4.554$, $df= 2$, $p<0.05$) and after stimulation in ROI 2, 3, 5 and 7 (ROI 2: $T= 6.463$, $df= 2$, $p<0.05$; ROI 3: $T= -7.757$, $df= 2$, $p<0.05$; ROI 5: $T= 8.407$, $df= 2$, $p<0.05$; ROI 7: $T= -5.135$, $df= 2$, $p<0.05$). A control trial in which a time

series was recorded without olfactory stimulation (Sachse and Galizia, 2003) did not show any increase in fluorescence levels (Fig. 5.5 D, F).

Therefore, it can be said that the observed fluorescence increase during olfactory stimulation is due to changes caused by calcium increase as a result of olfactory detection. In summary, an increase in fluorescence levels was measurable during olfactory stimulation, suggesting glomerular activity during olfactory stimulation can be detected in the honeybee AL using confocal microscopy.

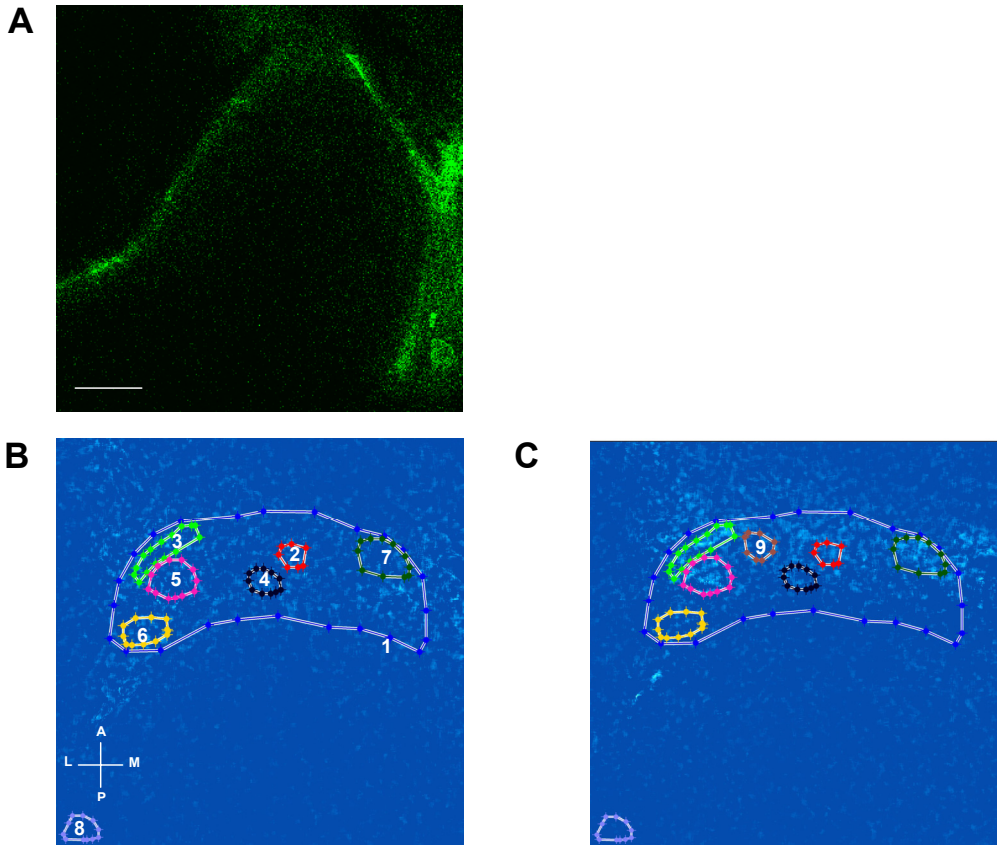

Fig. 5.4 Calcium imaging in the honeybee. A) View onto the AL. **B)** Fluorescence intensity remained at the baseline before olfactory stimulation. **C)** Olfactory stimulation led to an increase in fluorescence. L= Lateral, M= Medial, P= Posterior, V= Ventral. Scale bar = 30 µm.

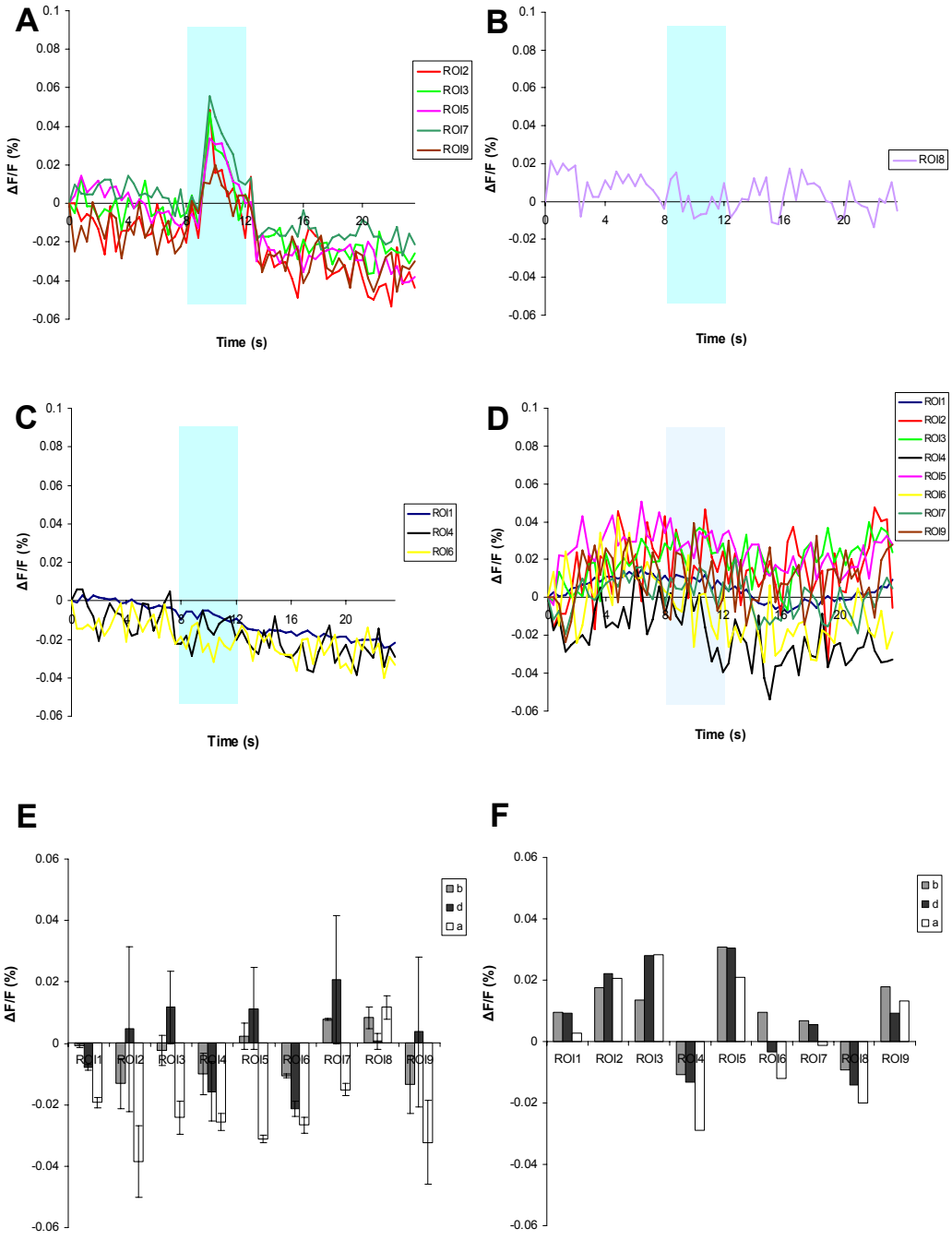

Fig. 5.5 Stimulation with 1-nonanol leads to fluorescence changes in the honeybee. A) During olfactory stimulation, an increase in fluorescence of 0.06% ΔF/F was observed. Shaded areas indicate odour onset. **B)** There was no increase in the ROI outside the AL. **C)** ROIs inside the AL with no fluorescence increase during olfactory stimulation. **D)** During a time series without stimulation inside the AL, no change in fluorescence was observed. **E)** Odour stimulation was repeated 3–5 times per preparation and the average intensity before, during and after odour onset was statistically compared. The average intensities increased during odour stimulation and decreased after odour stimulation. **F)** Average intensities of a time series with no stimulus did not show the characteristic pattern of increase followed by a decrease. Shaded areas in A, B and C indicate odour stimulation. Lighter shaded areas in D highlight the frames corresponding to the time of stimulation in A, B and C. b= before, d= during, a= after odour onset. Frame interval= 2.5 frames/s.

Tab. 5.1 Summary of the experiments measuring neuron responses to KCl and olfactory stimulation. The table shows the number of recorded antennal lobe (No. ALs), the total number of recordings (No. rec), and the number of interpretable results (No. interpret. results). The comments section provides additional information about the experiments. For experiments using KCl in hippocampal neurons (Hip. KCl) and in the AL of *C. vestalis* (AL KCl) one recording was conducted. For experiments using olfactory stimulation, recordings were conducted at 3-5 positions in the AL (positions at different depth levels), and per position the olfactory stimulus was repeated 3-5 times. Prelim.= preliminary.

Experiment	No. ALs	No.rec	No. interpret. results	Comments
Hip. KCl	10	10 (60mM: 5; 4M: 5)	5 (60mM: 1; 4M: 4)	2 prep. no signal, 3 prep. frame loss
AL KCl	29	29 (60mM: 7; 2M: 5; 4M: 17)	8 (2M: 3; 4M: 5)	8 prep. signal loss or unsufficient dye loading, 13 prep. no signal
Bee imaging prelim.	7	36	0	Preliminary experiments were conducted using manual odour stimulation and could not be interpreted due to the movement of the preparation caused by breathing
Bee imaging controlled	7	36	3	Only 1 bee fulfilled the set criteria (no signal outside the AL, increase during odour application, followed by decrease)
***Cotesia* imaging prelim.**	10	59	0	Preliminary experiments were conducted using manual odour stimulation but no signal could be detected.
***Cotesia* imaging controlled**	34	337	0	No signal could be detected. The preparation in Fig. 5.9 shows an increase during odour stimulation in ROI 8, but this is not significantly different from the ROI outside the AL.

5.3.2. Stimulation with KCl in hippocampal neurons

To test the suitability of calcium imaging in parasitoids, control experiments using KCl as a stimulant in hippocampal neurons, as well as in the AL, were conducted. Subsequently, calcium imaging of the AL during odour stimulation in the parasitoid was carried out.

In this experiment, 6 d old hippocampal neurons were stained with Calcium Green 1-AM and stimulated with KCl to determine whether an increase in calcium was detectable as a change in fluorescence (n= 10; Fig. 5.6). Five preparations showed an increase in fluorescence upon stimulation with KCl, while no effect was observed in two preparations. Three preparations experienced a signal loss upon KCl application and were excluded from data analysis. Figure 5.6 A shows the average stack projection of three neurons connected via axons. Regions of interest (ROI) 1-3 contained the three neurons, ROI 4 defined the axons, and ROI 5 was a region outside the neurons.

No change in fluorescence was observed in the frames before stimulation with KCl (Fig. 5.6 B, D). Upon stimulation with KCl an increase in fluorescence levels could be observed (Fig. 5.6 C, D). Peak fluorescence intensity in the different ROIs was between 0.3-0.5% $\Delta F/F$. Fluorescence intensity was highest approximately 6 s after stimulation (Frame 70) and reached maximum response amplitude of 0.5% $\Delta F/F$ in ROI 3. In the region outside the neurons (ROI 5) no change in fluorescence was observed (Fig. 5.6. E). Hence, KCl increases calcium and thus the fluorescence intensity in hippocampal neurons to a level which is detectable using Calcium Green 1-AM in combination with a confocal microscope.

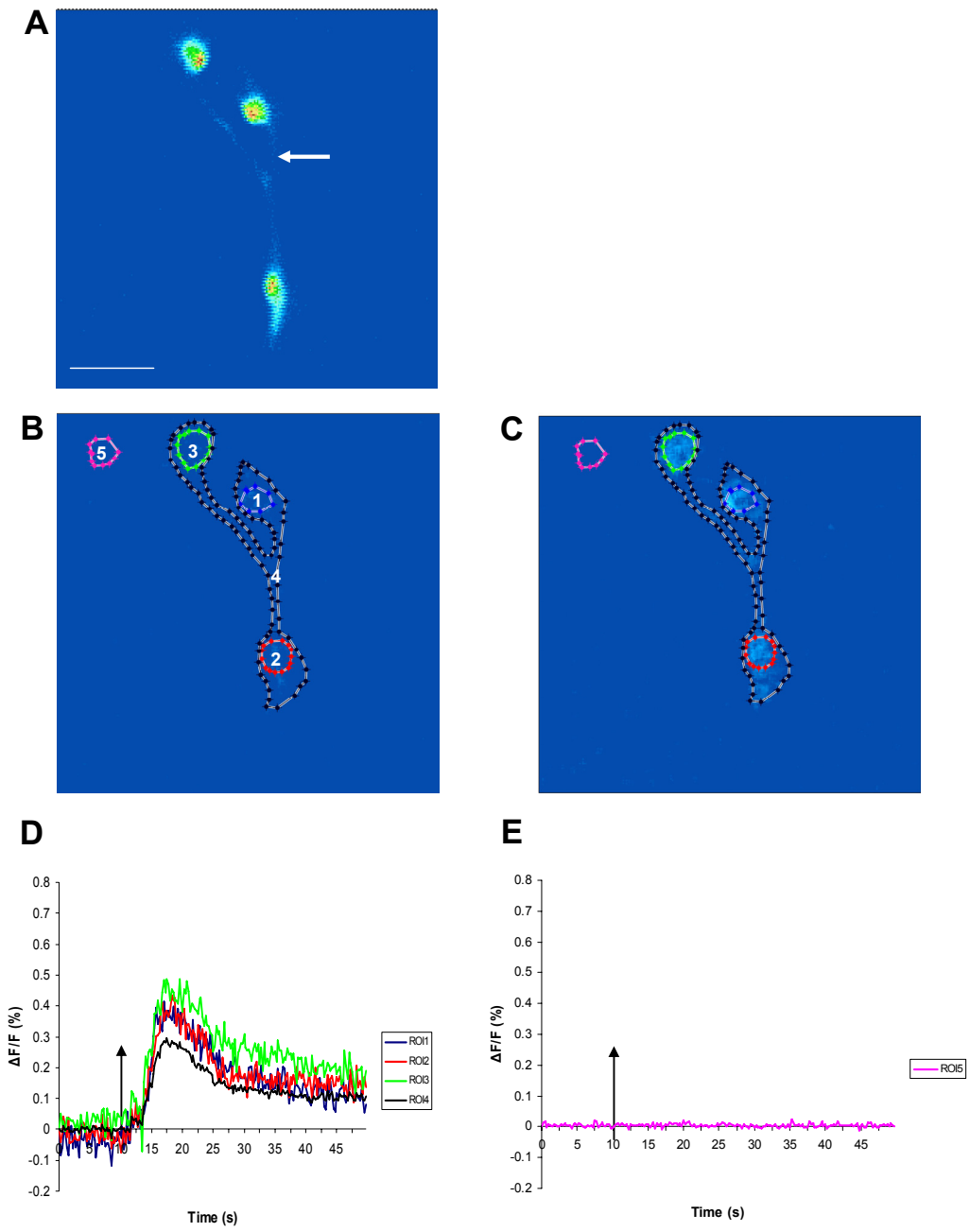

Fig. 5.6 Stimulation with KCl increases fluorescence in hippocampal neurons. A) Average stack projection of the time series showing 3 neurons connected via axons (white arrow). **B)** Frame 5 showing the neurons before stimulation. No fluorescence activity could be detected. **C)** Frame 70 showing the neurons after stimulation, in which an increase in fluorescence in ROI 1-4 is visible. **D)** The graph shows the ΔF/F intensity values before, during and after stimulation with 60 mM KCl. The maximum response amplitude was 0.5% ΔF/F. **E)** No changes in fluorescence were observed in the ROI outside the neurons. Black arrow indicates stimulus onset. Frame rate= 4 frames/s. Scale bar = 100µm.

5.3.3. Stimulation with KCl in the AL of *C. vestalis*

This experiment was designed to determine if the current dye loading protocol was suitable to detect changes in calcium and thus fluorescence in the AL (Fig. 5.7 A) of *C. vestalis*. Eight out of 20 preparations showed an increase upon stimulation with KCl. A further 16 preparations were excluded from data analysis because either no signal was detected or a complete signal loss occurred.

To see whether changes in fluorescence could be detected in the glomeruli, different ROI were selected, covering glomeruli in different areas in the AL (ROI 2-6, ROI 7-9), while ROI 5 comprised an area outside the AL.

Before stimulation, no increase in fluorescence was found (Fig. 5.7 B). When stimulated with KCl, a visible increase in fluorescence and thus calcium increase in the glomeruli could be measured (Fig. 5.7 C, D). Fluorescence intensity reached a maximum 4 s after stimulation with a maximum amplitude of 0.6% $\Delta F/F$ in ROI 3. Fluorescence levels eventually returned to baseline 13 s to 30 s after stimulus onset. There were differences in fluorescence level and time of excitation in different glomeruli and some glomeruli responded less or later to KCl (Fig. 5.7 D). In the region outside the AL no increase was observed (Fig. 5.7. E). In twelve cases a signal loss/drop in fluorescence was observed due to a change in the volume of solution between brain and objective (Perisse et al., 2009) when adding KCl.

To summarise, stimulation with KCl leads to an increase in Ca^{2+} in the glomeruli which is shown as an increase in fluorescence. Changes in fluorescence were thus measurable using Calcium Green 1-AM and a confocal microscope and the amount of Ca^{2+} released into the cell during action potentials is detectable with the equipment used in these experiments.

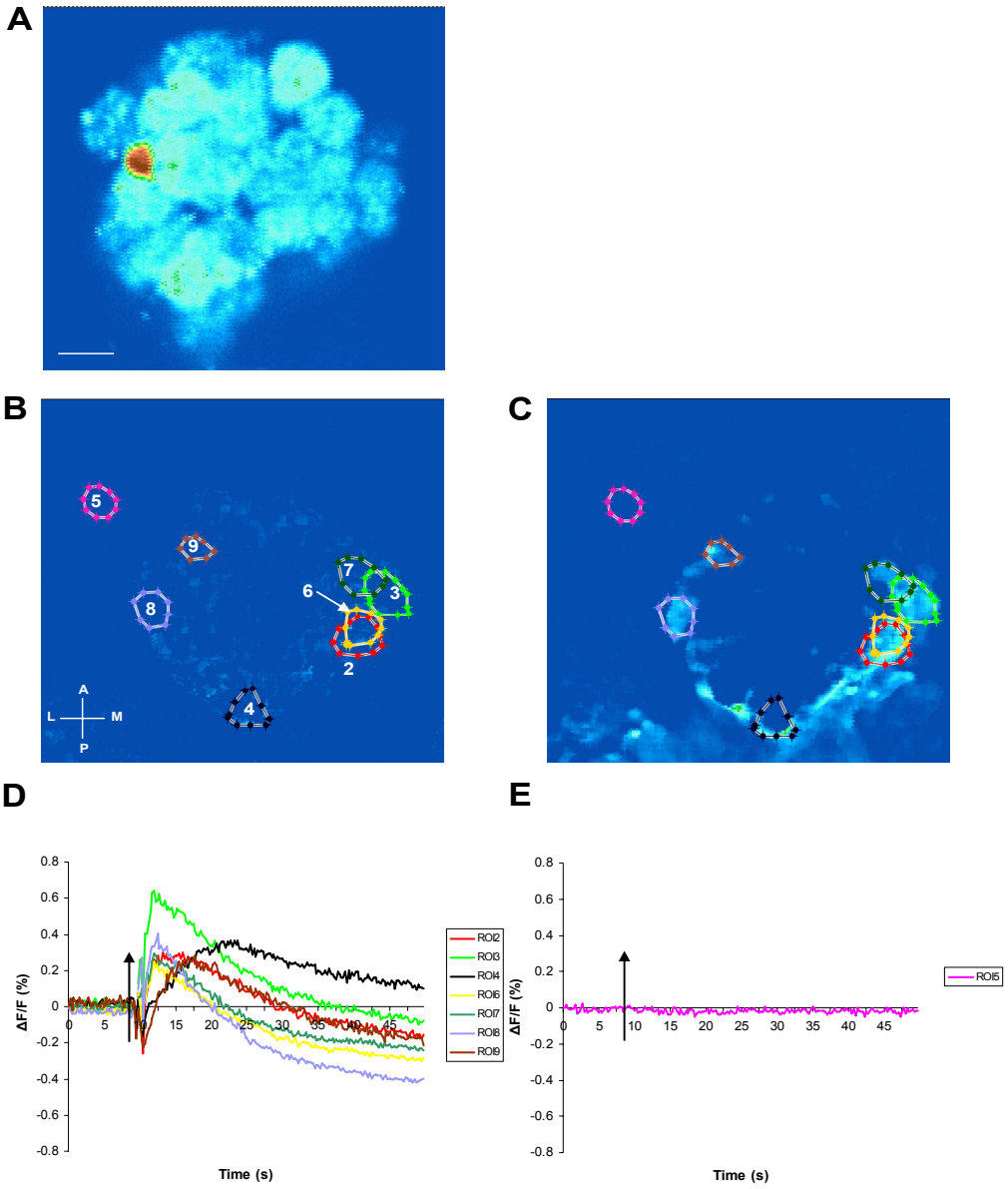

Fig. 5.7 Stimulation with KCl increases fluorescence in the glomeruli of *C. vestalis*. A) View onto the average stack projection of the time series of the AL and its units, the glomeruli. **B)** The AL and the ROI before stimulation with KCl. **C)** During stimulation with KCl an increase in fluorescence was visible in the different regions of the AL. **D)** The graph shows intensity changes before (Frames 0-38), during and after KCl (4 M) stimulation. Black arrow indicates stimulus onset. For a better data representation Frame 38 is not presented in ROI 2, 3, 6-8. L= Lateral, M= Medial, P= Posterior, V= Ventral. Frame rate= 4 frames/s. Scale bar = 30 µm. Note ROI labelling starts at 2.

5.3.4. Calcium imaging during odour stimulation in the AL of *C. vestalis*

Preliminary experiments in this study (sections 5.3.1, 5.3.2.1) suggest that the staining protocol works sufficiently well to detect calcium changes in the AL. To investigate if stimulation with a HIPV and non-HIPV would generate different activity patterns in the AL and whether these patterns were different between males and females, calcium activity in the brain was measured with live imaging during olfactory stimulation.

In the current study 337 recordings from 34 different ALs were made. Figure 5.8 shows one example of imaging studies in the left AL (Fig 5.8 A) using linalool as an olfactory stimulus. There was no difference in fluorescence intensity before and during odour stimulation (Fig. 5.8 C, D). During olfactory stimulation, intensity changes in regions inside and outside the AL remained below 0.05% $\Delta F/F$ and did not increase during stimulation with the odour inside or outside the AL (Fig. 5.8 E, F). Analysis of the average (n= 3) intensity values from before, during and after olfactory stimulation (Fig. 5.8 B) suggests that intensity values increase during and post stimulation. Analysis of the data, however, revealed no significant difference between the time points for linalool. Furthermore there was no difference between regions inside and outside the AL.

In summary, stimulation with the HIPV linalool did not lead to an increase in calcium and therefore no specific spatial activity patterns in the glomeruli could be observed in any of the preparations.

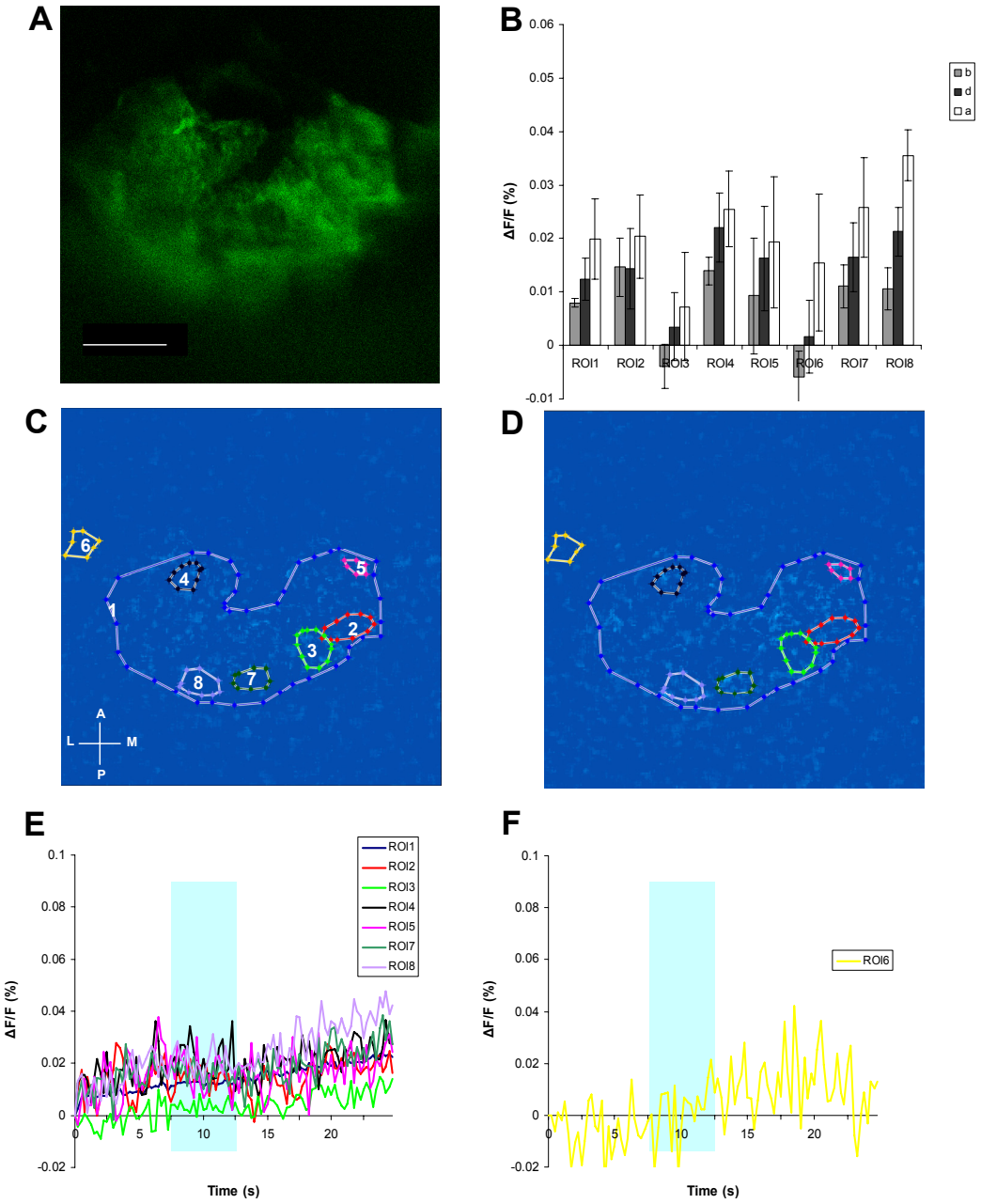

Fig. 5.8 Stimulation with linalool does not lead to changes in fluorescence in *C. vestalis*. A) View onto the AL. **B)** The histograms demonstrate the intensity averages before and during odour stimulation (given as an average of 3 repeats). **C)** and **D)** No increase in fluorescence was found before and during odour stimulation. **E)** The graph shows average intensities of 3 repeats over the course of the imaging period for ROIs inside the AL. **F)** There were no changes in fluorescence in a ROI outside the AL. Shaded areas indicate stimulation with linalool. b= before, d= during, a= after odour onset. L= Lateral, M= Medial, P= Posterior, V= Ventral. Frame rate= 4 frames/s. Scale bar = 30 µm.

Figure 5.9 shows an example of imaging in the left AL (Fig. 5.9 A). No difference in fluorescence intensity could be found before and during stimulation with 1-nonanol (Fig. 5.9 C, D). Intensity changes lay below 0.05% ΔF/F in ROIs inside and outside the AL (Fig. 5.9 E, F). A comparison of average responses before, during, and after odour stimulation showed a slightly higher fluorescence signal during odour stimulation followed by a decrease after stimulation in ROI 2-4, and ROI 8 (Fig. 5.9 B). A significant difference before and during odour stimulation was only found in ROI 8 (ROI 8: $T= -4.849$, $df= 2$, $p<0.05$). However, there was no significant difference between ROI 8 and the region outside the AL (ROI 6). Therefore, stimulation with 1-nonanol did not lead to changes in fluorescence intensity over time.

To summarise, olfactory stimulation with either a HIPV or a non-HIPV did not result in measurable changes of fluorescence in the AL, suggesting that no glomerular activity during olfactory stimulation could be detected.

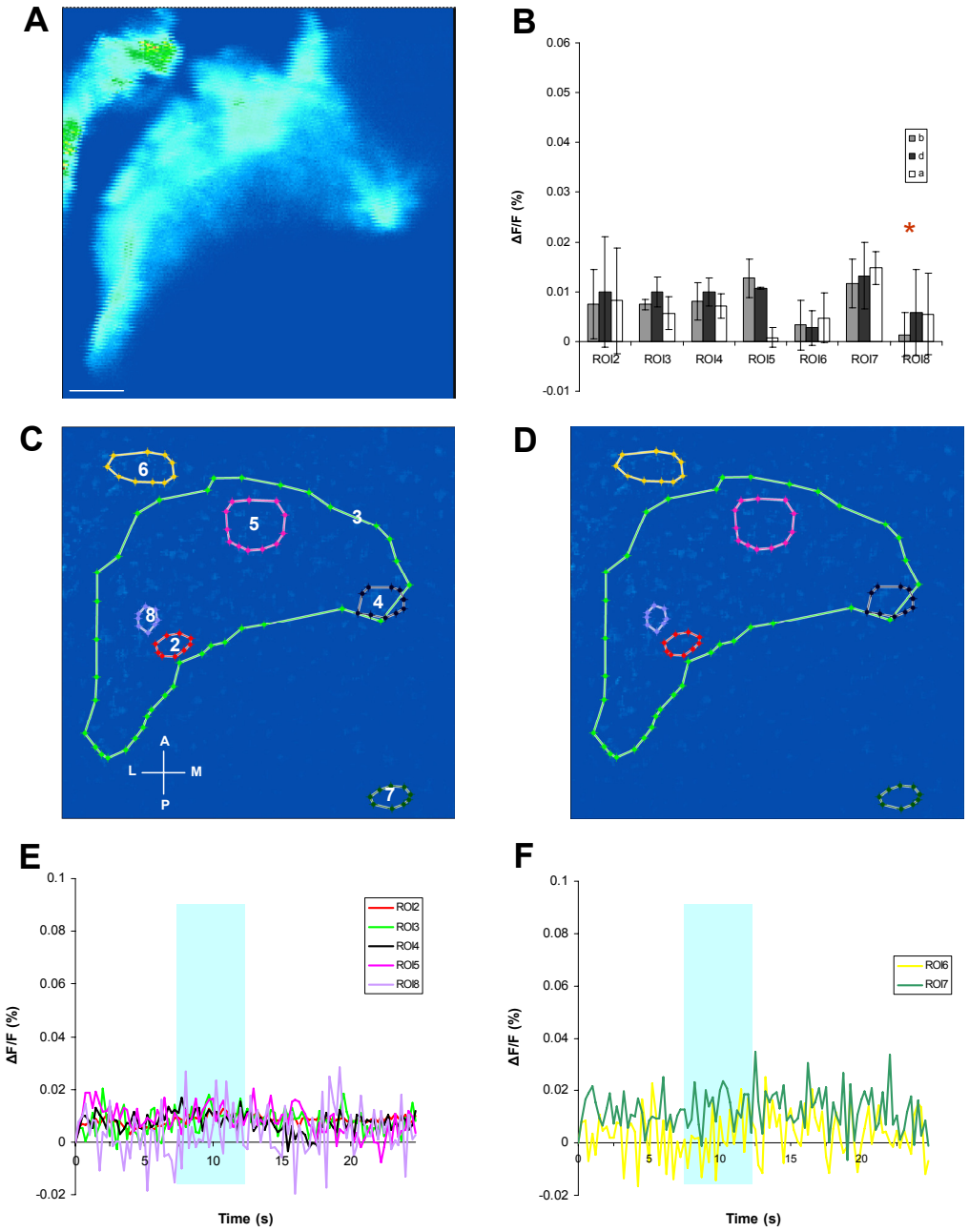

Fig. 5.9 Stimulation with 1-nonanol does not lead to changes in fluorescence in *C. vestalis*. A) View onto the AL. **B)** The histograms demonstrate the intensity averages before, during and after odour stimulation (given as an average of four repeats). **C)** and **D)** There was no difference in fluorescence before and during odour stimulation. **E)** Time curves for an average of four repeats over the course of the imaging period for significant areas inside the AL. **F)** Time curves for an average of four repeats over the course of the imaging period for significant areas outside the AL. Shaded areas indicate stimulation with 1-nonanol. b= before, d= during, a= after odour onset. L= Lateral, M= Medial, P= Posterior, V= Ventral. Frame rate= 4 frames/s. Scale bar = 30 µm. * indicates a significant difference at $p<0.05$. Note ROI labelling starts with 2.

5.4. Discussion

The aim of this study was to investigate how *C. vestalis* processes olfactory stimuli in the first neuropil responsible for olfactory coding (the AL). Olfactory stimulation in bees caused an increase in calcium in the AL which was measured as an increase in fluorescence. While stimulation with a depolarisation agent (KCl) led to detectable changes in calcium level in hippocampal neurons and neurons of the parasitoid AL, olfactory stimulation in parasitoids did not lead to detectable changes in calcium.

5.4.1. Development of calcium imaging methodology

In the current study a staining protocol and imaging method used in other insects (Fiala and Spall, 2003; Galizia and Vetter, 2005) was adapted for investigating glomerular activity in a parasitoid AL. The transferability of a method from one species to another is often not a trivial matter (Galizia et al., 1999b). Therefore, a series of control experiments were conducted to aid this process. Firstly, the suitability of the procedure was tested in hippocampal neurons. Experiments using Calcium Green 1-AM in conjunction with a confocal microscope and KCl as a depolarisation agent resulted in a fluorescence increase upon stimulation in hippocampal neurons. Secondly, the same experiments were conducted in the AL of *C. vestalis*. Stimulation with KCl led to a measurable increase in calcium and thus fluorescence intensity in the glomeruli. In both experiments a maximum response amplitude of approximately 0.6 % $\Delta F/F$ was observed.

Calcium is involved in many biochemical cascades as well as changes in the mediation of synaptic potentials or gene transcription. In neurons, such changes in intracellular calcium concentration can occur due to neuronal depolarisation, and thus serve as an indicator of neuronal activity (Fiala and Spall, 2003). To analyse whether the observed fluorescence during KCl stimulation and olfactory stimulation is due to neuronal activity, the calcium concentration can be

calculated from the fluorescence intensity. This estimated calcium concentration can thus be used to see whether these values equate to calcium levels found during neuronal activity.

Based on the data sheet of Calcium Green 1-AM (Johnson and Spence, 2010), at 39.8 μM free Ca^{2+}, the fluorescence emission is 1.35% ΔF/F. With a fluorescence emission of 0.5% ΔF/F, as observed in hippocampal neurons under KCl stimulation, a concentration of 14.21 μM free Ca^{2+} could be assumed. With an emission of 0.6% ΔF/F as observed in the AL of *C. vestalis* during KCl stimulation, a maximum of 17.7 μM free Ca^{2+} was estimated to be present. Generally, the concentration of Ca^{2+} during an action potential increases from 100 nM to 100 μM within a few hundred microseconds (Zimmerberg, 2000). Stimulation with KCl leads to a large depolarisation; hence a higher fluorescence signal can be expected compared to experiments measuring neuronal activity during olfactory stimulation.

Imaging studies of glomerular activity during olfactory stimulation in Hymenoptera (Galizia et al., 1999b; Sachse et al., 1999; Galizia et al., 2000b; Carlsson et al., 2002), however, showed relatively low fluorescence levels. In bees, fluorescence intensities from below 0.1% ΔF/F up to 0.2% ΔF/F have been measured (Sachse et al., 1999), whilst slightly higher intensities have been found in studies in ants and moths (Galizia et al., 1999b; Galizia et al., 2000b; Carlsson et al., 2002). Calcium Green 1-AM is especially suited for imaging at lower calcium concentrations, as it has a higher fluorescence at lower calcium concentrations compared to other dyes, and therefore facilitates the detection of calcium changes (Johnson and Spence, 2010). In the current study, a maximum of 1.71 μM free Ca^{2+} during olfactory stimulation was calculated based on the peak fluorescence intensity observed in bees. Similar levels have also been measured in the lobster, where odour stimulation led to an increase of 1 μM Ca^{2+} (Ukhanov et al., 2011). This is in contrast to imaging studies in parasitoids, conducted in the current study (section 5.4.4), where only 0.14 μM free intracellular Ca^{2+} was calculated to be present during olfactory stimulation. This concentration corresponds to a resting potential, suggesting no glomerular activity occurred during olfactory stimulation.

5.4.2. Calcium imaging in bees

Calcium imaging studies are frequently conducted in the honeybee; however, these experiments are usually carried out with a fluorescent microscope (Galizia and Vetter, 2005). For the current study, however, a confocal microscope was chosen due to its availability and higher spatial resolution. Therefore, to investigate if imaging on a confocal microscope was possible, bees were used to test the suitability of the equipment. During olfactory stimulation, a calcium increase was observed which can be described as an intracellular calcium increase from the extracellular medium, directly related to neuronal activity (Galizia and Kimmerle, 2004; Roussel et al., 2010). In the AL, the measured signal results from a compound signal of LNs, PNs and glial cells (Galizia and Vetter, 2005), but most likely it is caused by presynaptic ORNs (Galizia et al., 1998).

The signal intensities of up to 0.06 % $\Delta F/F$ found in the current study were in a similar range to other calcium imaging studies in honeybees (Sachse et al., 1999; Sandoz, 2006). In general, glomerular activity patterns are dynamic and not all glomeruli respond simultaneously at the odour onset. Patterns usually evolve within 2 s after stimulus onset (Galizia and Menzel, 2000). This is consistent with the results found in the current study, where a peak in fluorescence is observed around 2 s following olfactory stimulation. When 1-nonanol was used as an olfactory stimulus, a fluorescence peak 2 s after odour application was observed, which returned to the baseline after about 4 s after odour onset.

Olfactory signals have slow temporal dynamics compared to other senses (Galizia et al., 2000a). A recent study (Mota et al., 2011) showed that excitatory signals in visual processing reached a maximum amplitude around 1 s post stimulation. It has been suggested (Galizia et al., 2000a) that bees recognise an odour more precisely after 2 s than directly after olfactory stimulation. Although they can respond to an odour after only 0.5 s, differential conditioning requires odour pulses of at least 1 s duration. This is a relatively long processing time in comparison to, for example, pheromone pulses in moths, where a response is recorded within milliseconds (Kaissling, 1996; Bau et al., 2002). This suggests that it is not temporal, but the quality of floral odour processing that is more important for bees and consequently, spatial odour representation in the AL becomes more unique within 2 s after odour onset (Galizia et al., 2000a). This

would make more sense in an ecological point of view, where fine tuned odour discrimination for a bee is important at a closer distance to the food source. Therefore, odour quality and thus perception differs when a bee is hovering over a flower compared to a free flight situation.

5.4.3. Calcium imaging in the parasitoid AL

The calcium imaging method developed in the current study worked successfully in bees and with KCl stimulation in hippocampal and parasitoid AL neurons. However, when applied to parasitoids to measure glomerular activity patterns during olfactory stimulation with a HIPV and a non-HIPV, no change in fluorescence was observed. One explanation for this result could be the choice of microscope type. Calcium imaging studies are usually conducted with a fluorescence microscope and a CCD camera system (Galizia and Vetter, 2005). With a CCD camera, pixels are recorded at the same time and also have less noise (Galizia and Vetter, 2005). Recent studies, however, have demonstrated imaging using a 2-photon system (Haase et al., 2011a; Haase et al., 2011b). The advantage of a 2-photon system is that it provides better spatial resolution; however, because it is a scanning device, different points in space are imaged at a different time. Another explanation for the lack of response could be that imaging was conducted at a depth in the AL where the glomeruli were not activated during stimulation by the odours used in this study.

It has been shown that not all dyes are suitable for certain insects (Galizia and Vetter, 2005). An explanation for the lack of signal during olfactory stimulation could be that although Calcium Green 1-AM was suitable to detect higher changes of calcium increase during KCl stimulation, a calcium increase during odour stimulation did not result in a high enough concentration to elicit an increase in fluorescence. In this case, a different fluorescent dye, or a combination of fluorescent dyes could be used for the future (Galizia and Vetter, 2005; Deisig et al., 2010; Rath et al., 2011). Using double staining can be used to observe the input-output function of olfactory glomeruli, as dextran dyes selectively stain PNs, while AM dyes mainly measure the signal of ORNs (Sachse and Galizia, 2003; Galizia and Vetter, 2005).

Differences in the cytoarchitecture of glomeruli in *C. vestalis* could also cause differences in imaging responses. The innervation pattern in this species could be different to other insects, such as bees, moths or ants. It was suggested that in vespide wasps and locusts the cytoarchitecture was different compared to other insects, and that they might not form functional units in those species (Galizia and Vetter, 2005). Locusts and vespide wasps have around 1000 very small not individually identifyable glomeruli (Ernst and Boeckh, 1983; Hansson and Anton, 2000). In two related *Cotesia* species, it was shown that no glomeruli with ORN terminals were found in *C. glomerata* and *C. rubecula* (Smid et al., 2003). If this is the case in *C. vestalis*, a different fluorescent dye might be more appropriate, as Calcium Green 1-AM records signals mainly from ORNs, but also from PNs and LNs.

Whilst calcium imaging using bath applied dyes has been successful in insects such as moths, bees or ants, previous studies have shown that the successful detection of signals can be as low as 25% (Sachse and Galizia, 2003; Skiri et al., 2004). This demonstrates the difficulty of this procedure even in animals a minimum of 10 times larger the size of *C. vestalis*. It can be summarised that with the current methodology, no glomerular activity in the AL of *C. vestalis* could be detected during olfactory stimulation. However, calcium imaging in bees as well as KCl experiments in hippocampal neurons and AL neurons of parasitoids were successful using confocal microscopy.

CHAPTER 6

GENERAL DISCUSSION

6.1. Key findings

6.1.1. Peripheral odour input and behavioural output

- Results presented in **sections 2.3.1 – 2.3.3** show that there are differences between olfactory input and behavioural output in the specialist parasitoid *C. vestalis*. Although parasitoids (males and females) could detect HIPVs (linalool) and an odour not associated with their tritrophic system (1-nonanol), learning differed between genders. Both genders could learn linalool, but only females learned to avoid 1-nonanol. This suggests that odours are filtered in the brain according to their ecological relevance and host location can be improved by learning relevant odour cues.
- When female parasitoids were tested at several time points following training, a decline in the response to the conditioned odour was already present after 1 h **(section 2.3.4)**. Individuals fed with a transcription blocker to prevent LTM formation showed a similar trend, with the exception of animals tested 1 d following conditioning. At 1 d, the response to the conditioned odour was completely inhibited. These results suggest that under the current training protocol, no memory formation was

occured in *C. vestalis*; however, more experiments will be needed to verify this.

6.1.2. The olfactory pathway

- Similar to other hymenopteran species, *C. vestalis* showed common characteristics of AL morphology; for example, glomerular organisation was located in a peripheral layer of the AL around a central core devoid of glomeruli (**Chapter 4**). The variation in the number of glomeruli was small within individuals and genders. An average of 40 ordinary glomeruli was found. The lifestyle of a parasitoid, whether solitary or social; their ecological strategy, whether generalist or specialist; as well as the number of olfactory receptors (the higher the number, the greater the number of glomeruli) could therefore influence brain morphology. It can be argued that for *C. vestalis*, the degree of specialisation, type of host and strong innate preference (**Chapter 2**) determines the number of glomeruli, suggesting that fewer odours need to be processed.
- In addition to ordinary glomeruli, a MGC, consisting of 2-3 enlarged glomeruli, was found in males. This, in combination with their courtship behaviour (**Chapter 2**), could be an indicator for sex pheromone communication in this species.

6.1.3. Central processing

- Calcium imaging experiments in bees revealed glomerular activity during olfactory stimulation (**section 5.3.1**). Experiments in hippocampal neurons and the parasitoid AL using KCl as a depolarisation agent suggested that this dye is suitable for calcium imaging in parasitoids (**sections 5.3.2**, **5.3.3**). However, no glomerular activity was found when the parasitoid was stimulated with a HIPV or a non-HIPV.

6.2. General discussion - From peripheral perception and central processing to behavioural output

The aim of the present research was to determine the behavioural and neuronal mechanisms of host location in a specialist parasitoid wasp, *Cotesia vestalis*, a parasitoid of *Plutella xylostella*, which is one of the major crop pests of Brassicaceae plants. To achieve this, physiological, behavioural and morphological studies were combined to investigate the neural processes and pathways underlying olfactory processing, learning and memory in this parasitoid.

The olfactory system of insects is designed not only to detect and discriminate amongst a huge range of odour molecules but also to detect changes in their concentration. Lower concentrations attract an animal over long distances but at the source, the insect will be exposed to higher concentrations (Pelz et al., 1997; Rains et al., 2004). Therefore, the animal must possess fine-tuned olfactory sensory organs to detect and process odour molecules which vary greatly in their nature, functional groups, chain lengths, or chirality (Malnic et al., 2004). This explains the high number of olfactory receptors, for example 62 OR in *Drosophila* (Bargmann, 2006), in comparison to other senses, such as vision, where only three photoreceptors are required (Wald, 1953; Nathans et al., 1986).

How do parasitoids overcome the challenge of identifying the relevant odour cues to enhance their ability to detect hosts and hence improve their fitness? Does the reliability-detectability dilemma apply to all parasitoids? Can we predict learning abilities from known host specialisation? Can we predict form from function and vice versa?

Results presented in this study (Chapter 2) have demonstrated that for *C. vestalis*, in an environment with a low variability of odour compounds, HIPVs can be a reliable source for host location, which can be improved through learning. Host specialisation, therefore, may provide an indication of the learning and memory abilities of a parasitoid, but not necessarily for the detection of odour compounds. The broad detection of a variety of odour compounds, however, could still be linked to the evolution of parasitoids from a generalist to a specialist species. In this sense, the reliability-detectability dilemma might present a bigger problem for generalists, which need to learn to distinguish between a variety of

odour cues, than for specialists. Specialism could thus represent an adaptation of the parasitoid to improve host location. Adaptations in the olfactory system are likely to have evolved to favour innate "reliable" cues, suggesting changes in their morphology in favour of enhancing these signals.

Can we predict morphological brain structures from an insect's behaviour? From the results of this study it can be suggested that for *C. vestalis*, the degree of host specialisation, the type of host, and the innate preference for a HIPV are an indicator for the number of glomeruli, suggesting that fewer glomeruli are needed to process fewer odours. The lifestyle of a parasitoid (whether solitary or social) and their ecological strategy (whether generalist or specialist) could therefore influence the brain morphology. Thus, if ecology influences brain morphology, we can expect other insects with similar ecological and lifestyle patterns to show a similarity in brain morphology.

The current study and Smid's study (Smid et al., 2003) are the only morphological studies of the AL of parasitoids. They demonstrate that even closely related wasps can show differences in the size and number of glomeruli. Differences in host type and host specialisation of parasitoids could therefore not only cause differences in learning and memory, but also differences in the morphology of the AL.

Social insects are thought to have a higher number of glomeruli than solitary insects (Rospars, 1988). Hence, it could be hypothesised that the number of glomeruli correlates with levels of social organisation in insect colonies. Indeed, a high variability in the number of glomeruli can be found within the different casts of social insects, indicating that the number (and size) of glomeruli is related to the complexity of odour processing. In social insects, the ecology or behavioural richness can indeed be an indicator for the number of glomeruli. Just as behaviour could be an indicator for an either high or low number of glomeruli, the number of glomeruli could indicate the richness of the behavioural repertoire of an animal. The example of the parasitoid wasps *C. glomerata* and *C. rubecula* demonstrates, however, insects do not necessarily need to be social insects in order to have a high number of glomeruli.

Experiments presented in Chapter 2 suggest that males can learn relevant plant volatiles and therefore host specialisation, like in females, could influence

their learning ability. In addition to HIPVs, males could use sex pheromones to locate females. The courtship behaviour observed in males did predict form and function of the glomeruli, as they possess a MGC, which is a complex of several enlarged glomeruli for pheromone processing (Chapter 4). Specialised glomeruli have been found in many insects, either for detecting female pheromones, intraspecific pheromones, or glomeruli specialised for the detection of plant volatiles for oviposition and food sites (King et al., 2000; Ibba et al., 2010; Kuebler et al., 2010; Hu et al., 2011). This suggests to a certain degree that form can be predicted by function; and the way the morphology is related to the physiology and behaviour comes back to explain how glomeruli are linked to their function in processing odours.

The ability of insects to become highly specialised, which allows them to adapt and thrive within their environment, is likely to be one of the reasons to explain their success. The adaptation of the olfactory system in insects living in such diverse ecological niches shows how form is dependent on function and vice versa. Whilst much progress in understanding the behavioural, physiological, neural and genetic mechanisms underlying olfaction has been made by scientific research, there is still a lack of understanding as to how the neural circuitry can adapt. None of the different disciplines alone, however, can be a sole indicator for behaviour, morphology and physiology. Combining and integrating findings of these different disciplines and perspectives is therefore essential for a complementary understanding of olfactory processing in parasitoids and other insect species. This study has shown that in addressing a question using different approaches, such as physiological, behavioural and morphological ones, can improve our understanding of how structure and function underlying olfactory processing are related.

Parasitoids could thus represent new model organisms in order to understand a range of biological phenomena, such as olfactory processing, learning abilities or memory formation, and more importantly, how adaptations shaped by evolution and ecological strains have modified and specialised neural circuits underlying those phenomena.

6.3. Further implications

Olfaction plays an essential role not only in the life of insects, but also in insect behaviour relevant to humans. Beneficial insects such as natural enemies of other insects, or pollinators, play an important role in the ecosystem (Van Naters and Carlson, 2006; Carey and Carlson, 2011). Diseases mediated by insect vectors such as malaria, dengue fever or Chagas disease, a drastic decline of major pollinators such as the honeybee, or famine which occurs as a result of insect crop pests, make investigations into the neurobiology of olfaction more important than ever. For these insects, olfactory cues in host location play a crucial role.

The discovery of infochemical use by natural enemies together with learning abilities of parasitoids created the idea of enhancing natural responses of parasitoids, and their application as biological pest control (Vet and Dicke, 1992). Parasitoids would represent a useful model organism, not only for their functional use as a biological pest control, but because of the vast range of diverse ecological environments they inhabit, and also because of differences in their ability to learn and memorise information. These properties make them suitable models to investigate learning and memory in a multi-disciplinary approach, combining ecological, evolutionary and physiological studies.

The reduction of scientific findings and possible explanations to only one level, also known as the reductionist approach, can only provide limited understanding. Mechanisms of olfactory processing and host detection are influenced by a number of causes and events. In breaking it down into its essential parts it might be possible to understand the whole although the latter cannot be understood merely as the sum of its parts. Thus, a combination of reductionist (neurophysiology, genetics) and ecological approaches will be most productive in devising appropriate measures to control, monitor, and predict insect-borne diseases.

Comparison of a wide range of insects will also help us to understand insect olfaction to its whole extent. Insects have evolved over hundreds of millions of years and are extremely diverse. Taking this into account will help us to understand how evolution has shaped olfactory pathways over time in different species to meet their needs (Carey and Carlson, 2011). Because of the conserved genetic pathways underlying learning and memory, parasitoids provide a wealth

of information, contributing to a better understanding of learning and memory in higher animals. This can be applied in the research of neurodegenerative diseases (Dubnau, 2003).

In addition, greater understanding of the capability of parasitoids to detect very low concentrations of odours could lead to improvements in electronic chemical detectors such as the E-nose (Cyranose) (Rains et al., 2004). Artificial noses (E-noses) mimic an animal's olfaction to detect odours and chemical components. These devices have many applications, ranging from medical diagnostic systems (Dent et al., 2011), the food and cosmetic industries (Nakamoto et al., 1993; Ampuero and Bosset, 2003; Zheng et al., 2009), environmental protection (Baby et al., 2000; Henderson et al., 2010), to the detection of harmful substances (Romain et al., 2009).

A biomedical interest in olfaction could not only help in the development of artificial sensors, but also in the treatment of neurodegenerative disorders, as there is a link between these disorders and a loss of smell (Hawkes, 2003). Furthermore, people suffering from anosmia (complete olfactory dysfunction) experience a decrease in life quality which can result in mood changes, decreased appetite, sexual dysfunction and a lack of awareness of dangerous toxins (Hoover, 2010). Parasitoids could thus represent new model organisms to study various applications and thereby lead to an improvement in the quality of life for humans.

6.4. Conclusion

This study has shown that learning in *C. vestalis* could depend on the "ecological relevance" of an odour. When analysing the morphological structure of the AL, a glomerular organisation similar to that in other insects was found. Whilst further study is required to investigate central odour processing in parasitoids in more detail, the morphological experiments carried out in this study demonstrate that in males and possibly in females, a functional division of odour processing takes place. Males had a MGC to process pheromone information, whereas larger glomeruli were found in females (possible LFGs), which could be involved in processing information about HIPVs. By using different approaches, this study has contributed to the understanding of how the specialisation of *C.*

vestalis can influence olfactory processing, as well as the brain structures underlying olfactory processing.

6.5. Future work - what next

The results of this study provide a number of insights for future research. In the current study, differences between oviposition and food learning were found. To determine whether different neuronal pathways are involved in oviposition and food learning, future experiments could compare whether different odours elicit different responses depending on the conditioning procedure. For a better comparison between the two paradigms, the same number of conditioning trials and inter-trial intervals could be used.

In the current study it was found that a non-HIPV had an aversive effect on females. To test if this effect was truly aversive, the role of neurotransmitters in appetitive and aversive learning could be explored. Octopamine is involved in appetitive learning, while dopamine is known to be involved in aversive learning (Schwärzel et al., 2003; Vergoz, 2007; Agarwal et al., 2011). Dopamine could further be used to test whether non-HIPVs have an aversive effect in females. Moreover, antennal as well as behavioural responses to a greater range of HIPVs and non-HIPVs could be tested. A study by Sarfraz (Sarfraz et al., 2005) suggested that *C. vestalis* might not be as specialised as had been previously assumed, and it showed that the parasitoid preferred noxious weed ragwort (*Senecio jacobaea* L.) over cabbage plants. Future experiments could therefore explore antennal responses to more odour compounds, such as for example non-crucifer plant volatiles and cabbage volatiles. To determine if different olfactory sensilla are used for processing functional odours, single cell recordings from specific ORNs could be made.

The experiments investigating the memory dynamics of *C. vestalis* showed different results compared to the results of other studies. The question of whether LTM formation occurs in *C. vestalis* still remains unanswered. An understanding of the memory dynamics will provide a better insight into olfactory learning in this specialist species. Therefore, future experiments could repeat the LTM experiment using different concentrations of the transcription blocker, as well as

using translation blocker. Moreover, the animals could be tested after shorter time intervals. Furthermore, to investigate if the number of trials influences memory formation in *C. vestalis*, different conditioning paradigms such as 1 CT, massed and spaced learning could be applied.

In the current study, courtship behaviour in males indicated the possibility of pheromone communication in *C. vestalis*. More studies are required, however, to confirm that pheromone communication effectively occurred in the present case. Evidence from both physiological and behavioural perspectives will be necessary to support this claim. For example, behaviour of males to female extracts could be observed, and EAG measurements could be conducted to test antennal responses to female extracts. A lack of copulation was observed in males. It is possible that this might have been caused by the material of the container in which the insects were tested (Joyce et al., 2008). Future experiments could use different materials, for example glass, to determine if this enhances copulation success in males. It can be assumed that associative learning would be stronger if males experienced a full reward (copulation).

For the future, a comparison between more parasitoid species will provide new insight into how the organisation of the AL is related to biological constraints (e.g. host specialisation). Furthermore, selective neuron staining could be conducted for a better understanding of the connectivity of neurons within the glomeruli of *C. vestalis* and to characterise the morphology of different types of AL neurons. Anterograde (Stieb et al., 2011) or retrograde dye fills (Dacks et al., 2006) could be conducted to label ORN axons, to investigate, whether ORNs in *C. vestalis* innervate the entire volume of the glomerulus, as found in sawflies (Dacks and Nighorn, 2011); the outer rind only, as found in bees (Kirschner et al., 2006); or the distal part of the glomerulus, as found in moths (Oland et al., 1990). Via backfill staining, the output tracks of PNs in to higher brain centres could be labelled, to analyse how many antennoprotocerebral tracts (APT) can be found in *C. vestalis*, to allow comparison to other *Cotesia* species (Smid et al., 2003) and other insect species (Homberg et al., 1988; Kirschner et al., 2006; Zube et al., 2008; Dacks and Nighorn, 2011). To visualise pre- and postsynaptic connections in the glomeruli, glomeruli could be double labelled with an antibody to synapsin and f-actin-phalloidin staining (Rössler et al., 2002).

In addition, volume comparisons of glomeruli before and after learning could be made. It has been shown that learning increases glomerular volume (Devaud and Masson, 1999; Hourcade et al., 2009). Therefore, if long-term synaptic plasticity (Chapter 2) existed in *C. vestalis*, it would result in increasing glomerular volume.

One of the more important unresolved questions in this study was how olfactory information is processed in the AL. The limitations of this thesis lay in the investigation of central processing. Three unresolved key questions are whether calcium imaging is possible in a parasitoid brain, how it could be achieved and what it would show us. A morphological study in two related *Cotesia* species has shown that no glomeruli with ORN terminals were found in *C. glomerata* and *C. rubecula* (Smid et al., 2003). If this is the case in *C. vestalis*, a different fluorescent dye might be more appropriate, as Calcium Green 1-AM records signals mainly from ORNs, but also from PNs and LNs. For future experiments, Dextran dyes such as Fura-dextran which specifically stains PNs, or a combination of Fura-dextran and Calcium Green 1-AM could be applied (Galizia and Vetter, 2005; Deisig et al., 2010; Rath et al., 2011), as it has been shown that not all dyes are successful in certain insects (Galizia and Vetter, 2005).

Imaging brain activity during olfactory stimulation could show us in which spatio-temporal patterns odours are encoded in a parasitoid brain, and if these patterns change depending on experience or relevance of an odour. It could give as answers to the question of whether responses to HIPV are genetically determined (innate). If specialists really had a stronger innate preference to certain HIPVs it could be expected that, similar to pheromone coding, its glomerular activity pattern would not be modified (or only marginally) through associative learning and there would also be less inter-individual differences in the pattern. Specialised glomeruli for HIPV processing could be identified. Furthermore, pheromone processing in the MGC could be investigated to confirm the division into functionally segregated pathways and glomeruli in *C. vestalis*.

References

ACHE, B. W. & YOUNG, J. M. 2005. Olfaction: diverse species, conserved principles. *Neuron,* 48**,** 417-430.

AGARD, D. A., HIRAOKA, Y., SHAW, P. & SEDAT, J. W. 1989. Fluorescence microscopy in three dimensions. *Methods Cell Biol,* 30**,** 353-377.

AGARWAL, M., GUZMÁN, M. G., MORALES-MATOS, C., DÍAZ, R. A. D. V., ABRAMSON, C. I. & GIRAY, T. 2011. Dopamine and octopamine influence avoidance learning of honey bees in a place preference assay. *PLoS One,* 6**,** e25371.

AGELOPOULOS, N. G. & KELLER, M. A. 1994. Plant-natural enemy association in tritrophic system, *Cotesia rubecula-Pieris rapae*-Brassicaceae (Cruciferae). III: collection and identification of plant and frass volatiles. *J Chem Ecol,* 20**,** 1955-1967.

AGRANOFF, B. W., DAVIS, R. E., CASOLA, L. & LIM, R. 1967. Actinomycin D blocks formation of memory of shock-avoidance in goldfish. *Science,* 158**,** 1600-1601.

AKERS, R. P. & GETZ, W. M. 1992. A test of identified response classes among olfactory receptor neurons in the honey-bee worker. *Chem Senses,* 17**,** 191-209.

ALBERINI, C. M. 2009. Transcription factors in long-term memory and synaptic plasticity. *Physiol Rev,* 89**,** 121-145.

ALIZADEH, M., RASSOULIAN, G., KARIMZADEH, J., HOSSEINI-NAVEH, V. & FARAZMAND, H. 2011. Biological study of *Plutella xylostella* (L.)(Lep: Plutellidae) and its solitary endoparasitoid, *Cotesia vestalis* (haliday)(Hym. Braconidae) under laboratory conditions. *PAK J Biol Sci,* 14**,** 1090-1099.

ALLISON, A. C. 1953. The morphology of the olfactory system in the vertebrates. *Biol Rev,* 28**,** 195-244.

ALLISON, J. D. & HARE, J. D. 2009. Learned and naïve natural enemy responses and the interpretation of volatile organic compounds as cues or signals. *New Phytologist,* 184**,** 768-782.

ALLMANN, S. & BALDWIN, I. T. 2010. Insects betray themselves in nature to predators by rapid isomerization of green leaf volatiles. *Science,* 329**,** 1075-1078.

AMANO, H. & MARUYAMA, I. N. 2011. Aversive olfactory learning and associative long-term memory in *Caenorhabditis elegans*. *Learn Memory,* 18**,** 654-665.

AMORNSAK, W., CRIBB, B. & GORDH, G. 1998. External morphology of antennal sensilla of *Trichogramma australicum* Girault (Hymenoptera: Trichogrammatidae). *Int J Insect Morphol Embryol,* 27**,** 67-82.

AMOS, W. B. & WHITE, J. G. 2003. How the confocal laser scanning microscope entered biological research. *Biol Cell,* 95**,** 335-342.

AMPUERO, S. & BOSSET, J. 2003. The electronic nose applied to dairy products: a review. *Sens Actuators B,* 94**,** 1-12.

ANDERSON, P., HANSSON, B. S. & LÖFQVIST, J. 1995. Plant-odour-specific receptor neurones on the antennae of female and male *Spodoptera littoralis*. *Physiol Entomol,* 20**,** 189-198.

ANFORA, G., RIGOSI, E., FRASNELLI, E., RUGA, V., TRONA, F. & VALLORTIGARA, G. 2011. Lateralization in the invertebrate brain: left-right asymmetry of olfaction in bumble bee, *Bombus terrestris*. *PLoS One,* 6**,** e18903.

ANSEBO, L., IGNELL, R., LÖFQVIST, J. & HANSSON, B. S. 2005. Responses to sex pheromone and plant odours by olfactory receptor neurons housed in sensilla auricillica of the codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae). *J Insect Physiol,* 51**,** 1066-1074.

ARNOLD, G., MASSON, C. & BUDHARUGSA, S. 1985. Comparative study of the antennal lobes and their afferent pathway in the worker bee and the drone (*Apis mellifera*). *Cell Tissue Res,* 242**,** 593-605.

ASTON-JONES, G. S. & SIGGINS, G. R. 1995. Electrophysiology. *In:* BLOOM, F. E. & KUPFER, D. J. (eds.) *Psychopharmacology - the fourth generation of progress.* Raven Press, New York.

AXEL, R. 1995. The molecular logic of smell. *Sci Am,* 273**,** 154-159.

AXEL, R. 2005. Scents and sensibility: a molecular logic of olfactory perception (Nobel lecture). *Angew Chem Int Ed* 44**,** 6110-6127.

BABY, R., CABEZAS, M. & WALSÖE DE RECA, E. 2000. Electronic nose: a useful tool for monitoring environmental contamination. *Sens Actuators B,* 69**,** 214-218.

BACON, J. P. & ALTMAN, J. S. 1977. A silver intensification method for cobalt-filled neurones in wholemount preparations. *Brain Res,* 138**,** 359-363.

BAIER, H. & KORSCHING, S. 1994. Olfactory glomeruli in the zebrafish form an invariant pattern and are identifiable across animals. *J Neurosci,* 14**,** 219-230.

BARGMANN, C. I. 2006. Comparative chemosensation from receptors to ecology. *Nature,* 444**,** 295-301.

BARLIN, M. R. & BRADLEIGH VINSON, S. 1981. Multiporous plate sensilla in antennae of the Chalcidoidea (Hymenoptera). *Int J Insect Morphol Embryol,* 10**,** 29-42.

BARONDES, S. H. & JARVIK, M. E. 1964. The influence of actinomycin D on brain RNA synthesis and on memory. *J Neurochem,* 11**,** 187-195.

BAU, J., JUSTUS, K. A. & CARDÉ, R. T. 2002. Antennal resolution of pulsed pheromone plumes in three moth species. *J Insect Physiol,* 48**,** 433-442.

BEKINSCHTEIN, P., CAMMAROTA, M., IGAZ, L. M., BEVILAQUA, L. R. M., IZQUIERDO, I. & MEDINA, J. H. 2007. Persistence of long-term memory storage requires a late protein synthesis- and BDNF- dependent phase in the hippocampus. *Neuron,* 53**,** 261-277.

BELMABROUK, H., NOWOTNY, T., ROSPARS, J.-P. & MARTINEZ, D. 2011. Interaction of cellular and network mechanisms for efficient pheromone coding in moths. *Proc Natl Acad Sci USA,* 108**,** 19790-19795.

BENTON, R., VANNICE, K. S., GOMEZ-DIAZ, C. & VOSSHALL, L. B. 2009. Variant ionotropic glutamate receptors as chemosensory receptors in *Drosophila*. *Cell,* 136**,** 149-162.

BERG, B. G., ALMAAS, T. J., BJAALIE, J. G. & MUSTAPARTA, H. 1998. The macroglomerular complex of the antennal lobe in the tobacco budworm moth *Heliothis virescens*: specified subdivision in four compartments according to information about biologically significant compounds. *J Comp Physiol A,* 183**,** 669-682.

BERG, B. G., GALIZIA, C. G., BRANDT, R. & MUSTAPARTA, H. 2002. Digital atlases of the antennal lobe in two species of tobacco budworm moths, the oriental *Helicoverpa assulta* (male) and the american *Heliothis virescens* (male and female). *J Comp Neurol,* 446**,** 123-134.

BERG JM, TYMOCZKO JL & L, S. 2002. Transcription is catalyzed by RNA polymerase. *Biochemistry.* 5 ed. New York: W H Freeman.

BIANCHI, F., BOOIJ, C. & TSCHARNTKE, T. 2006. Sustainable pest regulation in agricultural landscapes: a review on landscape composition, biodiversity and natural pest control. *Proc R Soc B,* 273**,** 1715-1727.

BIANCHI, F. J. J. A. & WÄCKERS, F. L. 2008. Effects of flower attractiveness and nectar availability in field margins on biological control by parasitoids. *Biol Control,* 46**,** 400-408.

BIN, F., WÄCKERS, F., ROMANI, R. & ISIDORO, N. 1999. Tyloids in *Pimpla turionellae* (L.) are release structures of male antennal glands involved in courtship behaviour (Hymenoptera: Ichneumonidae). *Int J Insect Morphol Embryol,* 28**,** 61-68.

BISAZZA, A., J. ROGERS, L. & VALLORTIGARA, G. 1998. The origins of cerebral asymmetry: a review of evidence of behavioural and brain lateralization in fishes, reptiles and amphibians. *Neurosci Biobehav R,* 22**,** 411-426.

BITTERMAN, M. E., MENZEL, R., FIETZ, A. & SCHÄFER, S. 1983. Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). *J Comp Psychol,* 97**,** 107-119.

BLEEKER, M. A. K., SMID, H. M., STEIDLE, J. L. M., KRUIDHOF, H. M., VAN LOON, J. J. A. & VET, L. E. M. 2006a. Differences in memory dynamics between two closely related parasitoid wasp species. *Anim Behav,* 71**,** 1343-1350.

BLEEKER, M. A. K., SMID, H. M., VAN AELST, A. C., VAN LOON, J. J. A. & VET, L. E. M. 2004. Antennal sensilla of two parasitoid wasps: a comparative scanning electron microscopy study. *Microsc Res Tech,* 63**,** 266-273.

BLEEKER, M. A. K., VAN DER ZEE, B. & SMID, H. M. 2006b. Octopamine-like immunoreactivity in the brain and suboesophageal ganglion of two parasitic wasps, *Cotesia glomerata* and *Cotesia rubecula*. *Anim Biol,* 56**,** 247-257.

BLISS, T. V. P. & LØMO, T. 1973. Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *J Physiol,* 232**,** 331-356.

BOECKH, J. & BOECKH, V. 1979. Threshold and odor specificity of pheromone-sensitive neurons in the deutocerebrum of *Antheraea pernyi* and *A. polyphemus* (Saturnidae). *J Comp Physiol A,* 132**,** 235-242.

BOECKH, J. & ERNST, K. 1983. Olfactory food and mate recognition. *Neuroethology and behavioral physiology. Springer, Berlin Heidelberg New York*, 78-94.

BOECKH, J., ERNST, K., SASS, H. & WALDOW, U. 1984. Anatomical and physiological characteristics of individual neurones in the central antennal pathway of insects. *J Insect Physiol,* 30, 15-26.

BOECKH, J. & SELSAM, P. 1984. Quantitative investigation of the odour specificity of central olfactory neurones in the American cockroach. *Chem Senses,* 9, 369-380.

BOURTCHOULADZE, R., ABEL, T., BERMAN, N., GORDON, R., LAPIDUS, K. & KANDEL, E. R. 1998. Different training procedures recruit either one or two critical periods for contextual memory consolidation, each of which requires protein synthesis and PKA. *Learn Memory,* 5, 365-374.

BRAKENHOFF, G. J., VAN DER VOORT, H. T., VAN SPRONSEN, E. A. & NANNINGA, N. 1989. Three-dimensional imaging in fluorescence by confocal scanning microscopy. *J Microsc,* 153, 151-159.

BRAKENHOFF, G. J., VAN DER VOORT, H. T. M., VAN SPRONSEN, E. A. & NANNINGA, N. 1986. Three-dimensional imaging by confocal scanning fluorescence microscopya. *Ann NY Acad Sci,* 483, 405-415.

BRAUBACH, O. R., FINE, A. & CROLL, R. P. 2012. Distribution and functional organization of glomeruli in the olfactory bulbs of zebrafish (*Danio rerio*). *J Comp Neurol,* published before print, DOI: 10.1002/cne.23075.

BRÄUNIG, P. 1991. Suboesophageal DUM neurons innervate the principal neuropiles of the locust brain. *Phil Trans Biol Sci,* 332, 221-240.

BRUCE, T. J. A. & PICKETT, J. A. 2011. Perception of plant volatile blends by herbivorous insects - finding the right mix. *Phytochemistry,* 72, 1605-1611.

BRUCE, T. J. A., WADHAMS, L. J. & WOODCOCK, C. M. 2005. Insect host location: a volatile situation. *Trends Plant Sci,* 10, 269-274.

BRUINSMA, M., POSTHUMUS, M. A., MUMM, R., MUELLER, M. J., VAN LOON, J. J. A. & DICKE, M. 2009. Jasmonic acid-induced volatiles of *Brassica oleracea* attract parasitoids: effects of time and dose, and comparison with induction by herbivores. *J Exp Bot,* 60, 2575-2587.

BUCK, L. & AXEL, R. 1991. A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell,* 65, 175-187.

BUCK, L. B. 1996. Information coding in the vertebrate olfactory system. *Ann Rev Neurosci,* 19**,** 517-544.

BUCK, L. B. 2005. Unraveling the sense of smell (Nobel lecture). *Angew Chem Int Ed,* 44**,** 6128-6140.

BURROWS, M., BOECKH, J. & ESSLEN, J. 1982. Physiological and morphological properties of interneurones in the deutocerebrum of male cockroaches which respond to female pheromone. *J Comp Physiol A,* 145**,** 447-457.

CAJAL, S. 1906. The structure and connexions of neurons. *Nobel Lecture, December 12, 1906*.

CAJAL, S. R. Y. 1894. The Croonian Lecture: la fine structure des centres nerveux. *Proc R Soc London,* 55**,** 444-468.

CAREY, A. F. & CARLSON, J. R. 2011. Insect olfaction from model systems to disease control. *Proc Natl Acad Sci USA,* 108**,** 12987-12995.

CARLSON, G. C. & COULTER, D. A. 2008. In vitro functional imaging in brain slices using fast voltage-sensitive dye imaging combined with whole-cell patch recording. *Nat Protoc,* 3**,** 249-255.

CARLSSON, M. A., GALIZIA, C. G. & HANSSON, B. S. 2002. Spatial representation of odours in the antennal lobe of the moth *Spodoptera littoralis* (Lepidoptera: Noctuidae). *Chem Senses,* 27**,** 231-244.

CARY, R., EVANS, P., COCKER, J., KEEN, C. & DYNE, D. 2000. Methyl cyanoacrylate and ethyl cyanoacrylate risk assessment document EH72/13. *Concise International Chemical Assessment Document 36*.

CAYRE, M., BUCKINGHAM, S. D., YAGODIN, S. & SATTELLE, D. B. 1999. Cultured insect mushroom body neurons express functional receptors for acetylcholine, GABA, glutamate, octopamine, and dopamine. *J Neurophysiol,* 81**,** 1-14.

CENTER FOR ECOLOGICAL RESEARCH, K. U. 2011. *Center for Ecological Research, Kyoto University* [Online]. Kyoto: Center for Ecological Research, Kyoto University. Available: http://www.ecology.kyoto-u.ac.jp/~junji/Publication/publication.htm [Accessed 06.12.2011 2011].

CHAPMAN, R. F. 1982. Chemoreception: the significance of receptor numbers. *Adv Insect Physiol,* 16**,** 247-356.

CHEN, C. C., WU, J. K., LIN, H. W., PAI, T. P., FU, T. F., WU, C. L., TULLY, T. & CHIANG, A. S. 2012. Visualizing long-term memory formation in two neurons of the *Drosophila* brain. *Science,* 335**,** 678-685.

CHEN, L. & FADAMIRO, H. Y. 2007. Differential electroantennogram response of females and males of two parasitoid species to host-related green leaf volatiles and inducible compounds. *Bull Entomol Res,* 97**,** 515-522.

CHRISTENSEN, T. A., HEINBOCKEL, T. & HILDEBRAND, J. G. 1996. Olfactory information processing in the brain: encoding chemical and temporal features of odors. *J Neurobiol,* 30**,** 82-91.

CHRISTENSEN, T. A. & HILDEBRAND, J. G. 1987a. *Functions, organization and physiology of the olfactory pathways in the lepidopteran brain,* New York, John Wiley and Sons

CHRISTENSEN, T. A. & HILDEBRAND, J. G. 1987b. Male-specific, sex pheromone-selective projection neurons in the antennal lobes of the moth *Manduca sexta*. *J Comp Physiol A,* 160**,** 553-569.

CINELLI, A. R., HAMILTON, K. A. & KAUER, J. S. 1995. Salamander olfactory bulb neuronal activity observed by video rate, voltage-sensitive dye imaging. III. Spatial and temporal properties of responses evoked by odorant stimulation. *J Neurophysiol,* 73**,** 2053-2071.

COHEN, H. D. & BARONDES, S. H. 1966. Further studies of learning and memory after intracerebral actinomycin D *J Neurochem,* 13**,** 207-211.

COLAZZA, S., MCELFRESH, J. S. & MILLAR, J. G. 2004. Identification of volatile synomones, induced by *Nezara viridula* feeding and oviposition on bean spp., that attract the egg parasitoid *Trissolcus basalis*. *J Chem Ecol,* 30**,** 945-964.

COLLATZ, J., MÜLLER, C. & STEIDLE, J. L. M. 2006. Protein synthesis-dependent long-term memory induced by one single associative training trial in the parasitic wasp *Lariophagus distinguendus*. *Learn Memory,* 13**,** 263-266.

COLOMB, J. & BREMBS, B. 2010. The biology of psychology: "Simple" conditioning? *Commun Integr Biol,* 3**,** 142-145.

COMAS, D. & PETIT, F. 2004. *Drosophila* long-term memory formation involves regulation of cathepsin activity. *Nature,* 430**,** 460-463.

CONNOR, E. C., ROTT, A. S., SAMIETZ, J. & DORN, S. 2007. The role of the plant in attracting parasitoids: response to progressive mechanical wounding. *Entomol Exp Appl,* 125**,** 145-155.

COOK, D. J. & MILLIGAN, J. V. 1972. Electrophysiology and histology of the medial neurosecretory cells in adult male cockroaches, *Periplaneta americana*. *J Insect Physiol,* 18**,** 1197-1201.

CORMIER, D., ROYER, L., VIGNEAULT, C., PANNETON, B. & BOIVIN, G. 1998. Effect of female age on daily cycle of sexual pheromone emission in gregarious egg parasitoid *Anaphes listronoti*. *J Chem Ecol,* 24**,** 1595-1610.

CORTESERO, A. M. & MONGE, J. P. 1994. Influence of pre-emergence experience on response to host and host plant odours in the larval parasitoid *Eupelmus vuilleti*. *Entomol Exp Appl,* 72**,** 281-288.

CORTESERO, A. M., MONGE, J. P. & HUIGNARD, J. 1995. Influence of two successive learning processes on the response of *Eupelmus vuilleti* Crw (Hymenoptera: Eupelmidae) to volatile stimuli from hosts and host plants. *J Insect Behav,* 8**,** 751-762.

COSSART, R., ARONOV, D. & YUSTE, R. 2003. Attractor dynamics of network UP states in the neocortex. *Nature,* 423**,** 283-288.

COSSART, R., IKEGAYA, Y. & YUSTE, R. 2005. Calcium imaging of cortical networks dynamics. *Cell Calcium,* 37**,** 451-457.

COSTA, A., RICARD, I., DAVISON, A. C. & TURLINGS, T. C. J. 2010. Effects of rewarding and unrewarding experiences on the response to host-induced plant odors of the generalist parasitoid *Cotesia marginiventris* (Hymenoptera: Braconidae). *J Insect Behav,* 23**,** 1-16.

COUTO, A., ALENIUS, M. & DICKSON, B. J. 2005. Molecular, anatomical, and functional organization of the *Drosophila* olfactory system. *Curr Biol,* 15**,** 1535-1547.

D'ALESSANDRO, M. & TURLINGS, T. C. J. 2005. *In situ* modification of herbivore-induced plant odors: a novel approach to study the attractiveness of volatile organic compounds to parasitic wasps. *Chem Senses,* 30**,** 739-753.

DACKS, A. M., CHRISTENSEN, T. A. & HILDEBRAND, J. G. 2006. Phylogeny of a serotonin-immunoreactive neuron in the primary olfactory center of the insect brain. *J Comp Neurol,* 498**,** 727-746.

DACKS, A. M. & NIGHORN, A. J. 2011. The organization of the antennal lobe correlates not only with phylogenetic relationship, but also life history: a basal hymenopteran as exemplar. *Chem Senses,* 36**,** 209-220.

DALY, K. C., CHANDRA, S., DURTSCHI, M. L. & SMITH, B. H. 2001. The generalization of an olfactory-based conditioned response reveals unique

but overlapping odour representations in the moth *Manduca sexta*. *J Exp Biol,* 204**,** 3085-3095.

DANCI, A., GRIES, R., SCHAEFER, P. W. & GRIES, G. 2006. Evidence for four-component close-range sex pheromone in the parasitic wasp *Glyptapanteles flavicoxis*. *J Chem Ecol,* 32**,** 1539-1554.

DAVIS, H. P. & SQUIRE, L. R. 1984. Protein synthesis and memory: a review. *Psychol Bull,* 96**,** 518-559.

DAVIS, N. T. 1982. Improved methods for cobalt filling and silver intensification of insect motor neurons. *Biotech Histochem,* 57**,** 239-244.

DE BOER, J., ODE, P., VET, L., WHITFIELD, J. & HEIMPEL, G. 2007a. Complementary sex determination in the parasitoid wasp *Cotesia vestalis* (*C. plutellae*). *J Evolution Biol,* 20**,** 340-348.

DE BOER, J., ODE, P., VET, L., WHITFIELD, J. & HEIMPEL, G. 2007b. Diploid males sire triploid daughters and sons in the parasitoid wasp *Cotesia vestalis*. *Heredity,* 99**,** 288-294.

DE BOER, J. G., ODE, P. J., RENDAHL, A. K., VET, L. E. M., WHITFIELD, J. B. & HEIMPEL, G. E. 2008. Experimental support for multiple-locus complementary sex determination in the parasitoid *Cotesia vestalis*. *Genetics,* 180**,** 1525-1535.

DE BRUYNE, M. & BAKER, T. 2008. Odor detection in insects: volatile codes. *J Chem Ecol,* 34**,** 882-897.

DE BRUYNE, M., FOSTER, K. & CARLSON, J. R. 2001. Odor coding in the *Drosophila* antenna. *Neuron,* 30**,** 537-552.

DECKER, U., POWELL, W. & CLARK, S. 1993. Sex pheromones in the cereal aphid parasitoids *Praon volucre* and *Aphidius rhopalosiphi*. *Entomol Exp Appl,* 69**,** 33-39.

DEISIG, N., GIURFA, M., LACHNIT, H. & SANDOZ, J. C. 2006. Neural representation of olfactory mixtures in the honeybee antennal lobe. *Europ J Neurosci,* 24**,** 1161-1174.

DEISIG, N., GIURFA, M. & SANDOZ, J. C. 2010. Antennal lobe processing increases separability of odor mixture representations in the honeybee. *J Neurophysiol,* 103**,** 2185-2194.

DEJONG, R. & KAISER, L. 1991. Odor learning by *Leptopilina boulardi*, a specialist parasitoid (Hymenoptera: Eucoilidae). *J Insect Behav,* 4**,** 743-750.

DELURY, N. C., GRIES, G., GRIES, R., JUDD, G. J. R. & BROWN, J. J. 1999. Sex pheromone of *Ascogaster quadridentata*, a parasitoid of *Cydia pomonella*. *J Chem Ecol,* 25**,** 2229-2245.

DENT, A., BOWMAN, R., ZIMMERMAN, P., YANG, I. & FONG, K. 2011. Electronic nose distinguishes lung cancer from healthly smoking controls. *In:* ONCOLOGY, S. T. J. O. T. (ed.) *3rd Australian Lung Cancer Conference (ALCC).* Melbourne, Australia, S18-S18. 6-9

DEVAUD, J. M. & MASSON, C. 1999. Dendritic pattern development of the honeybee antennal lobe neurons: a laser scanning confocal microscopic study. *Develop Neurobiol,* 39**,** 461-474.

DICKE, M. 1999. Are herbivore-induced plant volatiles reliable indicators of herbivore identity to foraging carnivorous arthropods? *Entomol Exp Appl,* 91**,** 131-142.

DICKE, M. 2009. Behavioural and community ecology of plants that cry for help. *Plant Cell Environ,* 32**,** 654-665.

DICKE, M. & BALDWIN, I. T. 2010. The evolutionary context for herbivore-induced plant volatiles: beyond the "cry for help". *Trends Plant Sci,* 15**,** 167-175.

DICKE, M., VAN LOON, J. J. A. & SOLER, R. 2009. Chemical complexity of volatiles from plants induced by multiple attack. *Nat Chem Biol,* 5**,** 317-324.

DROBYSHEVA, D., AMEEL, K., WELCH, B., ELLISON, E., CHAICHANA, K., HOANG, B., et al. 2008. An optimized method for histological detection of dopaminergic neurons in *Drosophila melanogaster*. *J Histochem Cytochem,* 56**,** 1049-1063.

DROST, Y. C., LEWIS, W. J., ZANEN, P. O. & KELLER, M. A. 1986. Beneficial arthropod behavior mediated by airborne semiochemicals. *J Chem Ecol,* 12**,** 1247-1262.

DU, Y. J., POPPY, G. M. & POWELL, W. 1996. Relative importance of semiochemicals from first and second trophic levels in host foraging behavior of *Aphidius ervi*. *J Chem Ecol,* 22**,** 1591-1605.

DU, Y. J., POPPY, G. M., POWELL, W. & WADHAMS, L. J. 1997. Chemically mediated associative learning in the host foraging behavior of the aphid

parasitoid *Aphidius ervi* (Hymenoptera: Braconidae). *J Insect Behav,* 10**,** 509-522.

DUBNAU, J. 2003. Neurogenetic dissection of conditioned behavior: evolution by analogy or homology? *J Neurogenet,* 17**,** 295-326.

DUDAI, Y., JAN, Y. N., BYERS, D., QUINN, W. G. & BENZER, S. 1976. Dunce, a mutant of *Drosophila* deficient in learning. *Proc Natl Acad Sci USA,* 73**,** 1684-1688.

DUKAS, R. 2005. Experience improves courtship in male fruit flies. *Anim Behav,* 69**,** 1203-1209.

DUPUY, F., JOSENS, R., GIURFA, M. & SANDOZ, J. C. 2010. Calcium imaging in the ant *Camponotus fellah* reveals a conserved odour-similarity space in insects and mammals. *BMC Neurosci,* 11**,** 1-13.

DURRANT, L. F. 2007. *Olfactory learning in a parasitic wasp.* MPhil, University of Southampton.

DWECK, H., SVENSSON, G., GÜNDÜZ, E. & ANDERBRANT, O. 2010. Kairomonal response of the parasitoid *Bracon hebetor* Say, to the male-produced sex pheromone of its host, the greater wax moth, *Galleria mellonella* (L.). *J Chem Ecol,* 36**,** 171-178.

ECKERT, R., BURGGREN, W., FRENCH, K. & RANDALL, D. 2000. *Tierpysiologie,* Stuttgart, New York, Georg Thieme Verlag.

EGIGU, M. C., IBRAHIM, M. A., YAHYA, A. & HOLOPAINEN, J. K. 2010. Yeheb (*Cordeauxia edulis*) extract deters feeding and oviposition of *Plutella xylostella* and attracts its natural enemy. *Biocontrol,* 55**,** 613-624.

EHRLICH, P. R. & RAVEN, P. H. 1964. Butterflies and plants: a study in coevolution. *Evolution,* 18**,** 586-608.

EISTHEN, H. L. 2000. Why are olfactory systems of different animals so similar? *Brain Behav Evol,* 59**,** 273-293.

EL JUNDI, B., HUETTEROTH, W., KURYLAS, A. E. & SCHACHTNER, J. 2009. Anisometric brain dimorphism revisited: implementation of a volumetric 3D standard brain in *Manduca sexta*. *J Comp Neurol,* 517**,** 210-225.

ELKINTON, J., SCHAL, C., ONOT, T. & CARDÉ, R. 1987. Pheromone puff trajectory and upwind flight of male gypsy moths in a forest. *Physiol Entomol,* 12**,** 399-406.

ELLER, F. J., BARTELT, R. J., JONES, R. L. & KULMAN, H. M. 1984. Ethyl (Z)-9-hexadecenoate a sex pheromone of *Syndipnus rubiginosus*, a sawfly parasitoid. *J Chem Ecol,* 10**,** 291-300.

ELLER, F. J., TUMLINSON, J. H. & LEWIS, W. J. 1992. Effect of host diet and preflight experience on the flight responses of *Microplitis croceipes* (Cresson). *Physiol Entomol,* 17**,** 235-240.

EPSTEIN, H. T., CHILD, F. M., KUZIRIAN, A. M. & ALKON, D. L. 2003. Time windows for effects of protein synthesis inhibitors on Pavlovian conditioning in *Hermissenda*: behavioral aspects. *Neurobiol Learn Mem,* 79**,** 127-131.

ERNST, K. D. & BOECKH, J. 1983. A neuroanatomical study on the organization of the central antennal pathways in insects. *Cell Tissue Res,* 229**,** 1-22.

ESSLEN, J. & KAISSLING, K. E. 1976. Zahl und Verteilung antennaler Sensillen bei der Honigbiene (*Apis mellifera* L.). *Zoomorphol,* 83**,** 227-251.

EVANS, P. D. & O'SHEA, M. 1978. The identification of an octopaminergic neurone and the modulation of a myogenic rhythm in the locust. *J Exp Biol,* 73**,** 235-260.

FABER, T., JOERGES, J. & MENZEL, R. 1999. Associative learning modifies neural representations of odors in the insect brain. *Nat Neurosci,* 2**,** 74-78.

FALL, R., KARL, T., HANSEL, A., JORDAN, A. & LINDIGER, W. 1999. Volatile organic compounds emitted after leaf wounding - online analysis by proton-transfer-reaction mass spectrometry. *J Geophys Res,* 104**,** 15963-15974.

FATOUROS, N. E., DICKE, M., MUMM, R., MEINERS, T. & HILKER, M. 2008. Foraging behavior of egg parasitoids exploiting chemical information. *Behav Ecol,* 19**,** 677-689.

FATOUROS, N. E., HUIGENS, M. E., VAN LOON, J. J. A., DICKE, M. & HILKER, M. 2005. Chemical communication: butterfly anti-aphrodisiac lures parasitic wasps. *Nature,* 433**,** 704-704.

FATT, P. & KATZ, B. 1953. The electrical properties of crustacean muscle fibres. *J Physiol,* 120**,** 171-204.

FAUVERGUE, X., HOPPER, K. R. & ANTOLIN, M. F. 1995. Mate finding via a trial sex pheromone by a parasitoid wasp. *Proc Natl Acad Sci USA,* 92**,** 900-904.

FERRARI, S., PALMERINI, E., ALBERGHINI, M., STAALS, E., MERCURI, M., BARBIERI, E., et al. 2010. Vincristine, doxorubicin, cyclophosfamide, actinomycin D, ifosfamide, and etoposide in adult and pediatric patients with nonmetastatic Ewing sarcoma. Final results of a monoinstitutional study. *Tumori,* 96**,** 213-218.

FIALA, A. & SPALL, T. 2003. In vivo calcium imaging of brain activity in *Drosophila* by transgenic cameleon expression. *Sci STKE,* 174**,** PL6.

FIRESTEIN, S. 2001. How the olfactory system makes sense of scents. *Nature,* 413**,** 211-218.

FISHILEVICH, E. & VOSSHALL, L. B. 2005. Genetic and functional subdivision of the *Drosophila* antennal lobe. *Curr Biol,* 15**,** 1548-1553.

FLANAGAN, D. & MERCER, A. R. 1989a. An atlas and 3-D reconstruction of the antennal lobes in the worker honey bee, *Apis mellifera* L. (Hymenoptera: Apidae). *Int J Insect Morphol Embryol,* 18**,** 145-159.

FLANAGAN, D. & MERCER, A. R. 1989b. Morphology and response characteristics of neurones in the deutocerebrum of the brain in the honeybee *Apis mellifera*. *J Comp Physiol A,* 164**,** 483-494.

FÖLDES-PAPP, Z., DEMEL, U. & TILZ, G. P. 2003. Laser scanning confocal fluorescence microscopy: an overview. *Int Immunopharmacol,* 3**,** 1715-1729.

FONTA, C., SUN, X. J. & MASSON, C. 1993. Morphology and spatial distribution of bee antennal lobe interneurones responsive to odours. *Chem Senses,* 18**,** 101-119.

FRASNELLI, E., ANFORA, G., TRONA, F., TESSAROLO, F. & VALLORTIGARA, G. 2010a. Morpho-functional asymmetry of the olfactory receptors of the honeybee (*Apis mellifera*). *Behav Brain Res,* 209**,** 221-225.

FRASNELLI, E., VALLORTIGARA, G. & ROGERS, L. J. 2010b. Response competition associated with right-left antennal asymmetries of new and old olfactory memory traces in honeybees. *Behav Brain Res,* 209**,** 36-41.

FRIEDRICH, R. W. & KORSCHING, S. I. 1997. Combinatorial and chemotopic odorant coding in the zebrafish olfactory bulb visualized by optical imaging. *Neuron,* 18**,** 737-752.

FUJIWARA, C., TAKABAYASHI, J. & YANO, S. 2000. Oviposition experience on a host-infested plant affects flight and antennal searching behavior of

Cotesia kariyai toward the host-plant complex. *Entomol Exp Appl,* 97**,** 251-256.

FUKUSHIMA, J., KAINOH, Y., HONDA, H. & TAKABAYASHI, J. 2001. Learning of host-infested plant volatiles in the larval parasitoid *Cotesia kariyai*. *Entomol Exp Appl,* 99**,** 341-346.

FULTON, D., KEMENES, I., ANDREW, R. J. & BENJAMIN, P. R. 2005. A single time-window for protein synthesis-dependent long-term memory formation after one-trial appetitive conditioning. *Europ J Neurosci,* 21**,** 1347-1358.

FULTON, D., KEMENES, I., ANDREW, R. J. & BENJAMIN, P. R. 2008. Time-window for sensitivity to cooling distinguishes the effects of hypothermia and protein synthesis inhibition on the consolidation of long-term memory. *Neurobiol Learn Mem,* 90**,** 651-654.

GAL-BEN-ARI, S. & ROSENBLUM, K. 2011. Molecular mechanisms underlying memory consolidation of taste information in the cortex. *Front Behav Neurosci,* 5**,** 87.

GALIZIA, C. G., JOERGES, J., KUTTNER, A., FABER, T. & MENZEL, R. 1997. A semi-in-vivo preparation for optical recording of the insect brain. *J Neurosci Method,* 76**,** 61-69.

GALIZIA, C. G. & KIMMERLE, B. 2004. Physiological and morphological characterization of honeybee olfactory neurons combining electrophysiology, calcium imaging and confocal microscopy. *J Comp Physiol A,* 190**,** 21-38.

GALIZIA, C. G., KUTTNER, A., JOERGES, J. & MENZEL, R. 2000a. Odour representation in honeybee olfactory glomeruli shows slow temporal dynamics: an optical recording study using a voltage-sensitive dye. *J Insect Physiol,* 46**,** 877-886.

GALIZIA, C. G., MCILWRATH, S. L. & MENZEL, R. 1999a. A digital three-dimensional atlas of the honeybee antennal lobe based on optical sections acquired by confocal microscopy. *Cell Tissue Res,* 295**,** 383-394.

GALIZIA, C. G. & MENZEL, R. 2001. The role of glomeruli in the neural representation of odours: results from optical recording studies. *J Insect Physiol,* 47**,** 115-130.

GALIZIA, C. G., MENZEL, R. & HÖLLDOBLER, B. 1999b. Optical imaging of odor-evoked glomerular activity patterns in the antennal lobes of the ant *Camponotus rufipes*. *Naturwiss,* 86**,** 533-537.

GALIZIA, C. G., NAGLER, K., HOLLDOBLER, B. & MENZEL, R. 1998. Odour coding is bilaterally symmetrical in the antennal lobes of honeybees (*Apis mellifera*). *Europ J Neurosci,* 10**,** 2964-2974.

GALIZIA, C. G. & RÖSSLER, W. 2010. Parallel olfactory systems in insects: anatomy and function. *Annu Rev Entomol,* 55**,** 399-420.

GALIZIA, C. G. & SACHSE, S. 2010. Odor coding in insects. *In:* MENINI, A. (ed.) *The neurobiology of olfaction.* Boca Raton, London, New York: CRC.

GALIZIA, C. G., SACHSE, S., RAPPERT, A. & MENZEL, R. 1999c. The glomerular code for odor representation is species specific in the honeybee *Apis mellifera*. *Nat Neurosci,* 2**,** 473-478.

GALIZIA, C. G. & VETTER, R. S. 2005. Optical methods for analyzing odor-evoked activity in the insect brain. *In:* CHRISTENSEN, T. A. (ed.) *Methods in insect sensory neuroscience.* Boca Raton; London; New York; Washington, D.C: CRC Press.

GALIZIA, G. C., SACHSE, S. & MUSTAPARTA, H. 2000b. Calcium responses to pheromones and plant odours in the antennal lobe of the male and female moth *Heliothis virescens*. *J Comp Physiol A,* 186**,** 1049-1063.

GARCIA, J. & KIMELDORF, D. J. 1957. Temporal relationship within the conditioning of a saccharine aversion through radiation exposure. *J Comp Physiol Psychol,* 50**,** 180-183.

GARCIA, J., KIMELDORF, D. J. & KOELLING, R. A. 1955. Conditioned aversion to saccharin resulting from exposure to gamma radiation. *Science,* 122**,** 157-158.

GATEHOUSE, J. A. 2002. Plant resistance towards insect herbivores: a dynamic interaction. *New Phytol,* 156**,** 145-169.

GEERVLIET, J. B. F., ARIENS, S., DICKE, M. & VET, L. E. M. 1998a. Long-distance assessment of patch profitability through volatile infochemicals by the parasitoids *Cotesia glomerata* and *C. rubecula* (Hymenoptera: Braconidae). *Biol Control,* 11**,** 113-121.

GEERVLIET, J. B. F., VET, L. E. M. & DICKE, M. 1996. Innate responses of the parasitoids *Cotesia glomerata* and *C. rubecula* (Hymenoptera: Braconidae) to volatiles from different plant-herbivore complexes. *J Insect Behav,* 9**,** 525-538.

GEERVLIET, J. B. F., VREUGDENHIL, A. I., DICKE, M. & VET, L. E. M. 1998b. Learning to discriminate between infochemicals from different

plant-host complexes by the parasitoids *Cotesia glomerata* and *C. rubecula*. *Entomol Exp Appl,* 86**,** 241-252.

GERMINARA, G. S., DE CRISTOFARO, A. & ROTUNDO, G. 2009. Antennal olfactory responses to individual cereal volatiles in *Theocolax elegans* (Westwood) (Hymenoptera: Pteromalidae). *J Stored Prod Res,* 45**,** 195-200.

GETZ, W. & SMITH, K. 1990. Odorant moiety and odor mixture perception in free-flying honey bees (*Apis mellifera*). *Chem Senses,* 15**,** 111–128.

GIRLING, R., HASSALL, M., TURNER, J. & POPPY, G. 2006. Behavioural responses of the aphid parasitoid *Diaeretiella rapae* to volatiles from *Arabidopsis thaliana* induced by *Myzus persicae*. *Entomol Exp Appl,* 120**,** 1-9.

GIRLING, R. D., STEWART-JONES, A., DHERBECOURT, J., STALEY, J. T., WRIGHT, D. J. & POPPY, G. M. 2011. Parasitoids select plants more heavily infested with their caterpillar hosts: a new approach to aid interpretation of plant headspace volatiles. *Proc R Soc B,* 278**,** 2646-2653.

GIURFA, M., FABRE, E., FLAVEN-POUCHON, J., GROLL, H., OBERWALLNER, B., VERGOZ, V., ROUSSEL, E. & SANDOZ, J. C. 2009. Olfactory conditioning of the sting extension reflex in honeybees: memory dependence on trial number, interstimulus interval, intertrial interval, and protein synthesis. *Learn Memory,* 16**,** 761-765.

GODFRAY, H. C. J. 1994. *Parasitoids: behavioral and evolutionary ecology,* New Jersey, Princeton Univ Pr.

GOLGI, C. 1906. The neuron doctrine: theory and facts. *Nobel Lecture, December 12, 1906*.

GORMEZANO, I., SCHNEIDERMAN, N., DEAUX, E. & FUENTES, I. 1962. Nictitating membrane: classical conditioning and extinction in the albino rabbit. *Science,* 138**,** 33-34.

GOUINGUEN, S., PICKETT, J. A., WADHAMS, L. J., BIRKETT, M. A. & TURLINGS, T. C. J. 2005. Antennal electrophysiological responses of three parasitic wasps to caterpillar-induced volatiles from maize (*Zea mays mays*), cotton (*Gossypium herbaceum*), and cowpea (*Vigna unguiculata*). *J Chem Ecol,* 31**,** 1023-1038.

GREGG, P. C., DEL SOCORRO, A. P. & HENDERSON, G. S. 2010. Development of a synthetic plant volatile-based attracticide for female noctuid moths. II. Bioassays of synthetic plant volatiles as attractants for

the adults of the cotton bollworm, *Helicoverpa armigera* (Hübner)(Lepidoptera: Noctuidae). *Aust J Entomol,* 49**,** 21-30.

GREINER, B., GADENNE, C. & ANTON, S. 2004. Three-dimensional antennal lobe atlas of the male moth, *Agrotis ipsilon*: a tool to study structure-function correlation. *J Comp Neurol,* 475**,** 202-210.

GRIMES, M. T., SMITH, M., LI, X., DARBY-KING, A., HARLEY, C. W. & MCLEAN, J. H. 2011. Mammalian intermediate-term memory: new findings in neonate rat. *Neurobiol Learn Mem,* 95**,** 385-391.

GRONENBERG, W. 1986. Physiological and anatomical properties of optical input-fibres to the mushroom body in the bee brain. *J Insect Physiol,* 32**,** 695-704.

GRÜNBAUM, L. & MÜLLER, U. 1998. Induction of a specific olfactory memory leads to a long-lasting activation of protein kinase C in the antennal lobe of the honeybee. *J Neurosci,* 18**,** 4384-4392.

GUERRIERI, F., LACHNIT, H., GERBER, B. & GIURFA, M. 2005a. Olfactory blocking and odorant similarity in the honeybee. *Learn Memory,* 12**,** 86-95.

GUERRIERI, F., PORTELLI, G., SANDOZ, J. C. & GIURFA, M. 2005b. *Absolute appetitive conditioning does not modify odour-induced calcium signals in the antennal lobes of honey bees in: une etude integrative de la perception olfactive chez l'abeille Apis mellifera: comportement et imagerie calcique.* Phd, CNRS - Universit, Paul Sabatier Toulouse.

GUERRIERI, F. J., D'ETTORRE, P., DEVAUD, J.-M. & GIURFA, M. 2011. Long-term olfactory memories are stabilised via protein synthesis in *Camponotus fellah* ants. *J Exp Biol,* 214**,** 3300-3304.

HAASE, A., RIGOSI, E., FRASNELLI, E., TRONA, F., TESSAROLO, F., VINEGONI, C., ANFORA, G., VALLORTIGARA, G. & ANTOLINI, R. 2011a. A multimodal approach for tracing lateralisation along the olfactory pathway in the honeybee through electrophysiological recordings, morpho-functional imaging, and behavioural studies. *Eur Biophys J,* 40**,** 1247-1258.

HAASE, A., RIGOSI, E., TRONA, F., ANFORA, G., VALLORTIGARA, G., ANTOLINI, R. & VINEGONI, C. 2011b. *In-vivo* two-photon imaging of the honey bee antennal lobe. *Biomedical Opt Exp,* 2**,** 131-138.

HAJIEVA, P., KUHLMANN, C., LUHMANN, H. J. & BEHL, C. 2009. Impaired calcium homeostasis in aged hippocampal neurons. *Neurosci letters,* 451**,** 119-123.

HALITSCHKE, R., STENBERG, J. A., KESSLER, D., KESSLER, A. & BALDWIN, I. T. 2008. Shared signals -'alarm calls' from plants increase apparency to herbivores and their enemies in nature. *Ecol Lett,* 11**,** 24-34.

HAMMER, M. 1993. An identified neuron mediates the unconditioned stimulus in associative olfactory learning in honeybees. *Nature,* 366**,** 59-63.

HANSSON, B., PERS, J., HÖGBERG, H. E., HEDENSTRÖM, E., ANDERBRANT, O. & LÖFQVIST, J. 1991a. Sex pheromone perception in male pine sawflies, *Neodiprion sertifer* (Hymenoptera: Diprionidae). *J Comp Physiol A,* 168**,** 533-538.

HANSSON, B. S. 1999. *Insect olfaction,* Berlin, Heidelberg, New York, Springer.

HANSSON, B. S. & ANTON, S. 2000. Function and morphology of the antennal lobe: new developments. *Ann Rev Entomol,* 45**,** 203-231.

HANSSON, B. S., CARLSSON, M. A. & KALINOVÀ, B. 2003. Olfactory activation patterns in the antennal lobe of the sphinx moth, *Manduca sexta*. *J Comp Physiol A,* 189**,** 301-308.

HANSSON, B. S., CHRISTENSEN, T. A. & HILDEBRAND, J. G. 1991b. Functionally distinct subdivisions of the macroglomerular complex in the antennal lobe of the male sphinx moth *Manduca sexta*. *J Comp Neurol,* 312**,** 264-278.

HANSSON, B. S., LJUNGBERG, H., HALLBERG, E. & LOFSTEDT, C. 1992. Functional specialization of olfactory glomeruli in a moth. *Science,* 256**,** 1313-1315.

HANSSON, B. S., VAN DER PERS, J. N. C. & LÖFQVIST, J. A. N. 1989. Comparison of male and female olfactory cell response to pheromone compounds and plant volatiles in the turnip moth, *Agrotis segetum*. *Physiol Entomol,* 14**,** 147-155.

HANSTRÖM, B. 1928. *Vergleichende Anatomie des Nervensystems der wirbellosen Tiere: unter Berücksichtigung seiner Funktion,* Berlin, J. Springer.

HAUPT, S. S., SAKURAI, T., NAMIKI, S., KAZAWA, T. & KANZAKI, R. 2010. Olfactory information processing in moths. *In:* MENINI, A. (ed.) *The Neurobiology of Olfaction.* Boca Raton (FL): CRC Press.

HAWKES, C. 2003. Olfaction in neurodegenerative disorder. *Mov Disord,* 18**,** 364-372.

HEBB, D. O. 1949. *The organization of behavior: a neuropsychological theory,* New York, John Wiley.

HEINBOCKEL, T., KLOPPENBURG, P. & HILDEBRAND, J. G. 1998. Pheromone-evoked potentials and oscillations in the antennal lobes of the sphinx moth *Manduca sexta*. *J Comp Physiol A,* 182**,** 703-714.

HELGE, R., MÜLLER, D. & MUSTAPARTA, H. 2007. Anatomical organization of antennal lobe projection neurons in the moth *Heliothis virescens*. *J Comp Neurol,* 500**,** 658-675.

HENDERSON, W. G., KHALILIAN, A., HAN, Y. J., GREENE, J. K. & DEGENHARDT, D. C. 2010. Detecting stink bugs/damage in cotton utilizing a portable electronic nose. *Comput Electron Agr,* 70**,** 157-162.

HILDEBRAND, J. G. & SHEPHERD, G. M. 1997. Mechanisms of olfactory discrimination: converging evidence for common principles across phyla. *Ann Rev Neurosci,* 20**,** 595-631.

HILKER, M. & MEINERS, T. 2006. Early herbivore alert: insect eggs induce plant defense. *J Chem Ecol,* 32**,** 1379-1397.

HILKER, M. & MEINERS, T. 2010. How do plants "notice" attack by herbivorous arthropods? *Biol Rev,* 85**,** 267-280.

HILL, C. A., FOX, A. N., PITTS, R. J., KENT, L. B., TAN, P. L., CHRYSTAL, M. A., et al. 2002. G protein-coupled receptors in *Anopheles gambiae*. *Science,* 298**,** 176-178.

HOEDJES, K. M., KRUIDHOF, H. M., HUIGENS, M. E., DICKE, M., VET, L. E. M. & SMID, H. M. 2011. Natural variation in learning rate and memory dynamics in parasitoid wasps: opportunities for converging ecology and neuroscience. *Proc R Soc B,* 278**,** 889-897.

HOLOPAINEN, J. K. 2004. Multiple functions of inducible plant volatiles. *Trends Plant Sci,* 9**,** 529-533.

HOMBERG, U. 1984. Processing of antennal information in extrinsic mushroom body neurons of the bee brain. *J Comp Physiol A,* 154**,** 825-836.

HOMBERG, U., CHRISTENSEN, T. A. & HILDEBRAND, J. G. 1989. Structure and function of the deutocerebrum in insects. *Annu Rev Entomol,* 34**,** 477-501.

HOMBERG, U., MONTAGUE, R. A. & HILDEBRAND, J. G. 1988. Anatomy of antenno-cerebral pathways in the brain of the sphinx moth *Manduca sexta Cell Tissue Res,* 254**,** 255-281.

HONEGGER, H. W., MARKET, D., PIERCE, L. A., DEWEY, E. M., KOSTRON, B., WILSON, M., CHOI, D., KLUKAS, K. A. & MESCE, K. A. 2002. Cellular localization of bursicon using antisera against partial peptide sequences of this insect cuticle-sclerotizing neurohormone. *J Comp Neurol,* 452**,** 163-177.

HONIG, M. G. & HUME, R. I. 1989. Dil and DiO: versatile fluorescent dyes for neuronal labelling and pathway tracing. *Trends Neurosci,* 12**,** 333-341.

HOOVER, K. C. 2010. Smell with inspiration: The evolutionary significance of olfaction. *Am J Phys Anthropol,* 143**,** 63-74.

HOPWOOD, D. 1967. Some aspects of fixation with glutaraldehyde. A biochemical and histochemical comparison of the effects of formaldehyde and glutaraldehyde fixation on various enzymes and glycogen, with a note on penetration of glutaraldehyde into liver. *J Anat,* 101**,** 83-92.

HOUPT, T. A. & BERLIN, R. A. 1999. Rapid, labile, and protein synthesis–independent short-term memory in conditioned taste aversion. *Learn Memory,* 6**,** 37-46.

HOURCADE, B., PERISSE, E., DEVAUD, J.-M. & SANDOZ, J.-C. 2009. Long-term memory shapes the primary olfactory center of an insect brain. *Learn Memory,* 16**,** 607-615.

HU, J. H., WANG, Z. Y. & SUN, F. 2011. Anatomical organization of antennal-lobe glomeruli in males and females of the scarab beetle *Holotrichia diomphalia* (Coleoptera: Melolonthidae). *Arthropod Struct Dev,* 40**,** 420-428.

HUETTEROTH, W. & SCHACHTNER, J. 2005. Standard three-dimensional glomeruli of the *Manduca sexta* antennal lobe: a tool to study both developmental and adult neuronal plasticity. *Cell Tissue Res,* 319**,** 513-524.

HUIGENS, M. E., WOELKE, J. B., PASHALIDOU, F. G., BUKOVINSZKY, T., SMID, H. M. & FATOUROS, N. E. 2010. Chemical espionage on species-specific butterfly anti-aphrodisiacs by hitchhiking *Trichogramma* wasps. *Behav Ecol,* 21**,** 470-478.

IBBA, I., ANGIOY, A. M., HANSSON, B. S. & DEKKER, T. 2010. Macroglomeruli for fruit odors change blend preference in *Drosophila*. *Naturwissenschaften,* 97**,** 1059-1066.

IGAZ, L. M., VIANNA, M. R. M., MEDINA, J. H. & IZQUIERDO, I. 2002. Two time periods of hippocampal mRNA synthesis are required for memory consolidation of fear-motivated learning. *J Neurosci,* 22**,** 6781-6789.

IGNELL, R., DEKKER, T., GHANINIA, M. & HANSSON, B. S. 2005. Neuronal architecture of the mosquito deutocerebrum. *J Comp Neurol,* 493**,** 207-240.

IMMONEN, E. & RITCHIE, M. G. 2011. The genomic response to courtship song stimulation in female *Drosophila melanogaster*. *Proc R Soc B,* 279**,** 1359-1365

INGS, T. C., RAINE, N. E. & CHITTKA, L. 2009. A population comparison of the strength and persistence of innate colour preference and learning speed in the bumblebee *Bombus terrestris*. *Behav Ecol Sociobiol,* 63**,** 1207-1218.

JACTEL, H., BIRGERSSON, G., ANDERSSON, S. & SCHLYTER, F. 2011. Non-host volatiles mediate associational resistance to the pine processionary moth. *Oecologia,* 166**,** 703-711.

JARRIAULT, D., GADENNE, C., LUCAS, P., ROSPARS, J. P. & ANTON, S. 2010. Transformation of the sex pheromone signal in the noctuid moth *Agrotis ipsilon*: from peripheral input to antennal lobe output. *Chem Senses,* 35**,** 705-715.

JOERGES, J., KUTTNER, A., GALIZIA, C. G. & MENZEL, R. 1997. Representations of odours and odour mixtures visualized in the honeybee brain. *Nature,* 387**,** 285-288.

JOHNSON, I. & SPENCE, M. T. Z. 2010. *Molecular Probes Handbook, A Guide to Fluorescent Probes and Labeling Technologies*, Molecular probes.

JOHNSON, L. K. & HOWARD, J. J. 1987. Olfactory disc number in bees of different sizes and ways of life (Apidae: Meliponinae). *J Kansas Entomol Soc,* 60**,** 380-388.

JONGSMA, M. 2004. Novel genes for control and deterrence of sucking insect pests. *ISB News Report***,** 1-4.

JOSENS, R., ESCHBACH, C. & GIURFA, M. 2009. Differential conditioning and long-term olfactory memory in individual *Camponotus fellah* ants. *J Exp Biol,* 212**,** 1904-1911.

JOYCE, A. L., HUNT, R. E., BERNAL, J. S. & BRADLEIGH VINSON, S. 2008. Substrate influences mating success and transmission of courtship

vibrations for the parasitoid *Cotesia marginiventris*. *Entomol Exp Appl,* 127**,** 39-47.

JYOTHI, K., PRASUNA, A., SIGHAMONY, S., KUMARI, B. K., PRASAD, A. & YADAV, J. 2002. Electroantennogram responses of *Apanteles obliquae* (Hym., Braconidae) to various infochemicals. *J Appl Entomol,* 126**,** 175-181.

KAHSAI, L. & ZARS, T. 2011. *Learning and memory in Drosophila: behavior, genetics, and neural systems,* London, Oxford, Amsterdam, Waltham, San Diego, Academic Press, Elsevier.

KAISER, L., COUTY, A. & PEREZ-MALUF, R. 2009. Dynamic use of fruit odours to locate host larvae: individual learning, physiological state and genetic variability as adaptive mechanisms. *Adv Parasit,* 70**,** 67-95.

KAISER, L., PEREZ-MALUF, R., SANDOZ, J. C. & PHAM-DELEGUE, M. H. 2003. Dynamics of odour learning in *Leptopilina boulardi*, a hymenopterous parasitoid. *Anim Behav,* 66**,** 1077-1084.

KAISSLING, K., KASANG, G., BESTMANN, H., STRANSKY, W. & VOSTROWSKY, O. 1978. A new pheromone of the silkworm moth *Bombyx mori*. *Naturwissenschaften,* 65**,** 382-384.

KAISSLING, K. E. 1996. Peripheral mechanisms of pheromone reception in moths. *Chem Senses,* 21**,** 257-268.

KALBERER, N. M., REISENMAN, C. E. & HILDEBRAND, J. G. 2010. Male moths bearing transplanted female antennae express characteristically female behaviour and central neural activity. *J Exp Biol,* 213**,** 1272-1280.

KANDEL, E. R. 2001. The molecular biology of memory storage: a dialogue between genes and synapses. *Science,* 294**,** 1030-1038.

KANDEL, E. R. 2006. *In search of memory: the emergence of a new science of mind,* New York, Norton

KANDEL, E. R. & SCHWARTZ, J. H. 1982. Molecular biology of learning: modulation of transmitter release. *Science,* 218**,** 433-443.

KANZAKI, R., ARBAS, E. A., STRAUSFELD, N. J. & HILDEBRAND, J. G. 1989. Physiology and morphology of projection neurons in the antennal lobe of the male moth *Manduca sexta*. *J Comp Physiol A,* 165**,** 427-453.

KANZAKI, R., SOO, K., SEKI, Y. & WADA, S. 2003. Projections to higher olfactory centers from subdivisions of the antennal lobe macroglomerular complex of the male silkmoth. *Chem Sens,* 28**,** 113-130.

KAPLAN, I. 2011. Attracting carnivorous arthropods with plant volatiles: the future of biocontrol or playing with fire? *Biol Control,* 60**,** 77-89.

KAPPERS, I. F., AHARONI, A., VAN HERPEN, T. W. J. M., LUCKERHOFF, L. L. P., DICKE, M. & BOUWMEESTER, H. J. 2005. Genetic engineering of terpenoid metabolism attracts bodyguards to *Arabidopsis*. *Science,* 309**,** 2070-2072.

KAUER, J. S. 1988. Real-time imaging of evoked activity in local circuits of the salamander olfactory bulb. *Nature,* 331**,** 166-168.

KEELING, C. I., PLETTNER, E. & SLESSOR, K. N. 2004. Hymenopteran semiochemicals. *Top Curr Chem,* 239**,** 133-178.

KEIL, T. A. 1999. Morphology and development of the peripheral olfactory organs. *In:* HANSSON, B. S. (ed.) *Insect olfaction.* Berlin, Heidelberg: Springer.

KELLER, A. & VOSSHALL, L. B. 2003. Decoding olfaction in *Drosophila*. *Curr Op Neurobiol,* 13**,** 103-110.

KERSTEN, W., KERSTEN, H. & RAUEN, H. 1960. Action of nucleic acids on the inhibition of growth by actinomycin of *Neurospora crassa*. *Nature,* 187**,** 60-61.

KESSLER, A. & BALDWIN, I. T. 2001. Defensive function of herbivore-induced plant volatile emissions in nature. *Science,* 291**,** 2141-2144.

KIERNAN, J. A. 2000. Formaldehyde, formalin, paraformaldehyde and glutaraldehyde: what they are and what they do. *Microsc Today,* 00-1**,** 8-12.

KING, B. H., SAPORITO, K. B., ELLISON, J. H. & BRATZKE, R. M. 2005. Unattractiveness of mated females to males in the parasitoid wasp *Spalangia endius*. *Behav Ecol Sociobiol,* 57**,** 350-356.

KING, J. R., CHRISTENSEN, T. A. & HILDEBRAND, J. G. 2000. Response characteristics of an identified, sexually dimorphic olfactory glomerulus. *J Neurosci,* 20**,** 2391-2399.

KIRK, J. M. 1960. The mode of action of actinomycin D. *Biochim Biophysi Acta,* 42**,** 167-169.

KIRSCHNER, S., KLEINEIDAM, C. J., ZUBE, C., RYBAK, J., GRÜNEWALD, B. & RÖSSLER, W. 2006. Dual olfactory pathway in the honeybee, *Apis mellifera*. *J Comp Neurol,* 499**,** 933-952.

KLEINEIDAM, C. J., OBERMAYER, M., HALBICH, W. & RÖSSLER, W. 2005. A macroglomerulus in the antennal lobe of leaf-cutting ant workers and its possible functional significance. *Chem Senses,* 30**,** 383-392.

KLEINEIDAM, C. J. & RÖSSLER, W. 2009. *Adaptations in the olfactory system of social hymenoptera,* Cambridge, London, Harvard University Press.

KNUDSEN, J. T., TOLLSTEN, L. & BERGSTRÖM, L. G. 1993. Floral scents--a checklist of volatile compounds isolated by head-space techniques. *Phytochemistry,* 33**,** 253-280.

KOS, M., HOUSHYANI, B., WIETSMA, R., KABOUW, P., VET, L. E. M., VAN LOON, J. J. A. & DICKE, M. 2012. Effects of glucosinolates on a generalist and specialist leaf-chewing herbivore and an associated parasitoid. *Phytochemistry*.

KRIEGER, J., GROSSE-WILDE, E., GOHL, T., DEWER, Y., RAMING, K. & BREER, H. 2004. Genes encoding candidate pheromone receptors in a moth (*Heliothis virescens*). *Proc Natl Acad Sci USA,* 101**,** 11845-11850.

KRISTOFFERSEN, L., HANSSON, B. S., ANDERBRANT, O. & LARSSON, M. C. 2008. Aglomerular hemipteran antennal lobes - basic neuroanatomy of a small nose. *Chem Senses,* 33**,** 771-778.

KUEBLER, L., KELBER, C. & KLEINEIDAM, C. 2010. Distinct antennal lobe phenotypes in the leaf cutting ant (*Atta vollenweideri*). *J Comp Neurol,* 518**,** 352-365.

KUEBLER, L. S., OLSSON, S. B., WENIGER, R. & HANSSON, B. S. 2011. Neuronal processing of complex mixtures establishes a unique odor representation in the moth antennal lobe. *Front Neural Circuits,* 5**,** 1-16.

KUGIMIYA, S., UEFUNE, M., SHIMODA, T. & TAKABAYASHI, J. 2010. Orientation of the parasitic wasp, *Cotesia vestalis* (Haliday) (Hymenoptera: Braconidae), to visual and olfactory cues of field mustard flowers, *Brassica rapa* L. (Brassicaceae), to exploit food sources. *App Entomol Zool,* 45**,** 369-375.

KURYLAS, A. E., ROHLFING, T., KROFCZIK, S., JENETT, A. & HOMBERG, U. 2008. Standardized atlas of the brain of the desert locust, *Schistocerca gregaria*. *Cell Tissue Res,* 333**,** 125-145.

KUWABARA, M. 1957. Bildung des bedingten Reflexes von Pavlovs Typus bei der Honigbiene, *Apis mellifica*. *J Fac Sci Hokkaido Univ Ser VI Zool,* 13**,** 458-464.

LAISSUE, P. P., REITER, C., HIESINGER, P. R., HALTERS, S. & FISCHBACH, K. F. 1999. Three-dimensional reconstruction of the antennal lobe in *Drosophila melanogaster*. *J Comp Neurol,* 405**,** 543-552.

LAMPRECHT, R. & LEDOUX, J. 2004. Structural plasticity and memory. *Nature Rev Neurosc,* 5**,** 45-54.

LARSSON, M. C., LEAL, W. S. & HANSSON, B. S. 2001. Olfactory receptor neurons detecting plant odours and male volatiles in *Anomala cuprea* beetles (Coleoptera: Scarabaeidae). *J Insect Physiol,* 47**,** 1065-1076.

LASKA, M., GALIZIA, C. G., GIURFA, M. & MENZEL, R. 1999. Olfactory discrimination ability and odor structure–activity relationships in honeybees. *Chem Senses,* 24**,** 429-438.

LE CALVEZ, S. & ULFENDAHL, M. 2000. An in vitro preparation to access cellular and neuronal components in the mouse inner ear. *J Neurocytol,* 29**,** 645-652.

LEITCH, B. & LAURENT, G. 1996. GABAergic synapses in the antennal lobe and mushroom body of the locust olfactory system. *J Comp Neurol,* 372**,** 487-514.

LEON, M. & JOHNSON, B. A. 2003. Olfactory coding in the mammalian olfactory bulb. *Brain Res Rev,* 42**,** 23-32.

LERNER, M. R., GYORGYI, T. K., REAGAN, J. & ROBY-SHEMKOVITZ, A. 1990. Peripheral events in moth olfaction. *Chem Senses,* 15**,** 191-196.

LETZKUS, P., RIBI, W. A., WOOD, J. T., ZHU, H., ZHANG, S. W. & SRINIVASAN, M. V. 2006. Lateralization of olfaction in the honeybee *Apis mellifera*. *Curr Biol,* 16**,** 1471-1476.

LEWIS, W., NORDLUND, D. A., GUELDNER, R., TEAL, P. & TUMLINSON, J. 1982. Kairomones and their use for management of entomophagous insects. XIII. Kairomonal activity for *Trichogramma spp*. of abdominal tips, excretion, and a synthetic sex pheromone blend of *Heliothis zea* (Boddie) moths. *J Chem Ecol,* 1**,** 349-360.

LEWIS, W. J. & TAKASU, K. 1990. Use of learned odours by a parasitic wasp in accordance with host and food needs. *Nature,* 348**,** 635-636.

LI, Y., DICKENS, J. & STEINER, W. 1992. Antennal olfactory responsiveness of *Microplitis croceipes* (Hymenoptera: Braconidae) to cotton plant volatiles. *J Chem Ecol,* 18**,** 1761-1773.

LICHTMAN, J. W. & CONCHELLO, J. A. 2005. Fluorescence microscopy. *Nat Methods,* 2**,** 910-919.

LIU, S. S., LI, Y. H. & LOU, Y. G. 2006. Non-host plant extracts reduce oviposition of *Plutella xylostella* (Lepidoptera: Plutellidae) and enhance parasitism by its parasitoid *Cotesia plutellae* (Hymenoptera: Braconidae). *Bull Entomol Res,* 96**,** 373-378.

LJUNGBERG, H., ANDERSON, P. & HANSSON, B. 1993. Physiology and morphology of pheromone-specific sensilla on the antennae of male and female *Spodoptera littoralis* (Lepidoptera: Noctuidae). *J Insect Physiol,* 39**,** 253-260.

LØFALDLI, B. B., KVELLO, P. & MUSTAPARTA, H. 2010. Integration of the antennal lobe glomeruli and three projection neurons in the standard brain atlas of the moth *Heliothis virescens*. *Front Sys Neurosci,* 4**,** 1-12.

LØMO, T. 2003. The discovery of long-term potentiation. *Philos Trans R Soc B,* 358**,** 617-620.

LORENZ, K. Z. 1981. Mechanisms exploiting instant information. *In:* LORENZ, K. & KICKERT, R. (eds.) *The foundations of ethology.* Wien, New York: Springer Verlag.

LOUDON, C. & KOEHL, M. 2000. Sniffing by a silkworm moth: wing fanning enhances air penetration through and pheromone interception by antennae. *J Exp Biol,* 203**,** 2977-2990.

LOXDALE, H. D., LUSHAI, G. & HARVEY, J. A. 2011. The evolutionary improbability of 'generalism' in nature, with special reference to insects. *Biol J Linn Soc,* 103**,** 1-18.

LUNNEY, G. H. 1970. Using analysis of variance with a dichotomous dependent variable: an empirical study. *J Educ Meas,* 7**,** 263-269.

LYNCH, M. 2004. Long-term potentiation and memory. *Physiol Rev,* 84**,** 87-136.

MADDRELL, S. 1969. Secretion by the malpighian tubules of *Rhodnius*. The movements of ions and water. *J Exp Biol,* 51**,** 71-97.

MALENKA, R. C. & BEAR, M. F. 2004. LTP and LTD: an embarrassment of riches. *Neuron,* 44**,** 5-21.

MALNIC, B., GODFREY, P. A. & BUCK, L. B. 2004. The human olfactory receptor gene family. *Proc Natl Acad Sci USA,* 101**,** 2584-2589.

MALOGOLOWKIN, M., COTTON, C. A., GREEN, D. M., BRESLOW, N. E., PERLMAN, E., MISER, J., et al. 2008. Treatment of Wilms tumor relapsing after initial treatment with vincristine, actinomycin D, and doxorubicin. A report from the National Wilms Tumor Study Group. *Pediatr Blood Cancer,* 50**,** 236-241.

MALUN, D., GIURFA, M., GALIZIA, C. G., PLATH, N., BRANDT, R., GERBER, B. & EISERMANN, B. 2002. Hydroxyurea-induced partial mushroom body ablation does not affect acquisition and retention of olfactory differential conditioning in honeybees. *J Neurobiol,* 53**,** 343-360.

MARCHAND, D. & MCNEIL, J. N. 2000. Effects of wind speed and atmospheric pressure on mate searching behavior in the aphid parasitoid *Aphidius nigripes* (Hymenoptera: Aphidiidae). *J Insect Behav,* 13**,** 187-199.

MARGULIES, C., TULLY, T. & DUBNAU, J. 2005. Deconstructing memory in *Drosophila*. *Curr Biol,* 15**,** 700-713.

MARSHALL, B., WARR, C. G. & DE BRUYNE, M. 2010. Detection of volatile indicators of illicit substances by the olfactory receptors of *Drosophila melanogaster*. *Chem Senses,* 35**,** 613-625.

MARTIN, J. P., BEYERLEIN, A., DACKS, A. M., REISENMAN, C. E., RIFFELL, J. A., LEI, H. & HILDEBRAND, J. G. 2011. The neurobiology of insect olfaction: sensory processing in a comparative context. *Prog Neurobiol,* 95**,** 427-447.

MARTIN, J. P. & HILDEBRAND, J. G. 2010. Innate recognition of pheromone and food odors in moths:a common mechanism in the antennal lobe? *Front Behav Neurosci,* 4**,** 159.

MARTINI, R. 1986. Fine structure and development of the large sensilla basiconica on the antennae of sphecid wasps. *Tissue Cell,* 18**,** 143-151.

MASANTE-ROCA, I., GADENNE, C. & ANTON, S. 2005. Three-dimensional antennal lobe atlas of male and female moths, *Lobesia botrana* (Lepidoptera: Tortricidae) and glomerular representation of plant volatiles in females. *J Exp Biol,* 208**,** 1147-1159.

MATSUMOTO, S. G. & HILDEBRAND, J. G. 1981. Olfactory mechanisms in the moth *Manduca sexta*: response characteristics and morphology of central neurons in the antennal lobes. *Proc R Soc B,* 213**,** 249-277.

MAUCHLINE, A. L., OSBORNE, J. L., MARTIN, A. P., POPPY, G. M. & POWELL, W. 2005. The effects of non-host plant essential oil volatiles on the behaviour of the pollen beetle *Meligethes aeneus*. *Entomol Exp Appl,* 114**,** 181-188.

MCCALLUM, E. J., CUNNINGHAM, J. P., LÜCKER, J., ZALUCKI, M. P., DE VOSS, J. J. & BOTELLA, J. R. 2011. Increased plant volatile production affects oviposition, but not larval development, in the moth *Helicoverpa armigera*. *J Exp Biol,* 214**,** 3672-3677.

MCCLURE, M., WHISTLECRAFT, J. & MCNEIL, J. 2007. Courtship behavior in relation to the female sex pheromone in the parasitoid, *Aphidius ervi* (Hymenoptera: Braconidae). *J Chem Ecol,* 33**,** 1946-1959.

MCDONALD, J. H. 2009. *Handbook of biological statistics*, Sparky House Publishing.

MEINERS, T., WACKERS, F. & LEWIS, W. J. 2002. The effect of molecular structure on olfactory discrimination by the parasitoid *Microplitis croceipes*. *Chem Senses,* 27**,** 811-816.

MEINERS, T., WACKERS, F. & LEWIS, W. J. 2003. Associative learning of complex odours in parasitoid host location. *Chem Senses,* 28**,** 231-236.

MENZEL, R. 1999. Memory dynamics in the honeybee. *J Comp Physiol A,* 185**,** 323-340.

MENZEL, R. 2001. Massed and spaced learning in honeybees: the role of CS, US, the intertrial interval, and the test interval. *Learn Memory,* 8**,** 198-208.

MERY, F. & KAWECKI, T. J. 2005. A cost of long-term memory in *Drosophila*. *Science,* 308**,** 1148.

MICHEL-SALZAT, A. & WHITFIELD, J. B. 2004. Preliminary evolutionary relationships within the parasitoid wasp genus *Cotesia* (Hymenoptera: Braconidae: Microgastrinae): combined analysis of four genes. *Syst Entomol,* 29**,** 371-382.

MINSKY, M. 1988. Memoir on inventing the confocal scanning microscope. *Scanning,* 10**,** 128-138.

MOMBAERTS, P., WANG, F., DULAC, C., CHAO, S. K., NEMES, A., MENDELSOHN, M., EDMONDSON, J. & AXEL, R. 1996. Visualizing an olfactory sensory map. *Cell,* 87**,** 675-686.

MOORHOUSE, A. 2005. Gene identified in flies may shed light on human sleep patterns. *J Young Investigators,* 12.

MOTA, T., YAMAGATA, N., GIURFA, M., GRONENBERG, W. & SANDOZ, J. C. 2011. Neural organization and visual processing in the anterior optic tubercle of the honeybee brain. *J Neurosci,* 31**,** 11443-11456.

MÜLLER, C., COLLATZ, J., WIELAND, R. & STEIDLE, J. L. M. 2006. Associative learning and memory duration in the parasitic wasp *Lariophagus distinguendus*. *Anim Biol,* 56**,** 221-232.

MUMM, R. & HILKER, M. 2005. The significance of background odour for an egg parasitoid to detect plants with host eggs. *Chem Senses,* 30**,** 337-343.

MUMM, R. M. R. & DICKE, M. D. M. 2010. Variation in natural plant products and the attraction of bodyguards involved in indirect plant defense. *Can J Zool,* 88**,** 628-667.

NAKAJIMA, S. 1969. Interference with relearning in the rat after hippocampal injection of actinomycin D. *J Comp Physiol Psychol,* 67**,** 457-461.

NAKAMOTO, T., FUKUDA, A. & MORIIZUMI, T. 1993. Perfume and flavour identification by odour-sensing system using quartz-resonator sensor array and neural-network pattern recognition. *Sens Actuators B,* 10**,** 85-90.

NATHANS, J., THOMAS, D. & HOGNESS, D. S. 1986. Molecular genetics of human color vision: the genes encoding blue, green, and red pigments. *Science,* 232**,** 193-202.

NEWLAND, P. L. 1990. Morphology of a population of mechanosensory ascending interneurones in the metathoracic ganglion of the locust. *J Comp Neurol,* 299**,** 242-260.

NEWLAND, P. L., ROGERS, S. M., GAABOUB, I. & MATHESON, T. 2000. Parallel somatotopic maps of gustatory and mechanosensory neurons in the central nervous system of an insect. *J Comp Neurol,* 425**,** 82-96.

NGUMBI, E., CHEN, L. & FADAMIRO, H. 2010. Electroantennogram (EAG) responses of *Microplitis croceipes* and *Cotesia marginiventris* and their lepidopteran hosts to a wide array of odor stimuli: correlation between EAG response and degree of host specificity? *J Insect Physiol,* 56**,** 1260-1268.

NGUMBI, E., CHEN, L. & FADAMIRO, H. Y. 2009. Comparative GC-EAD responses of a specialist (*Microplitis croceipes*) and a generalist (*Cotesia marginiventris*) parasitoid to cotton volatiles induced by two caterpillar species. *J Chem Ecol,* 35**,** 1009-1020.

NISHIKAWA, M., NISHINO, H., MISAKA, Y., KUBOTA, M., TSUJI, E., SATOJI, Y., OZAKI, M. & YOKOHARI, F. 2008. Sexual dimorphism in the antennal lobe of the ant *Camponotus japonicus*. *Zool Sci,* 25**,** 195-204.

NOBILE, A. 2011. *Formica exsectoides* [Online]. AntWeb.org / CC-BY-SA-3.0. Available: http://www.wildlifenorthamerica.com/Insect/Allegheny-mound-ant/Formica/exsectoides.html [Accessed 27.12.2011 2011].

NOFEMELA, S. R. 2004. *Studies on parasitoids of the diamondback moth, Plutella xylostella (L.)(Lepidoptera: Plutellidae), in South Africa.* MSc MSc Thesis, Rhodes University.

NOTTINGHAM, S. F., HARDIE, J., DAWSON, G. W., HICK, A. J., PICKETT, J. A., WADHAMS, L. J. & WOODCOCK, C. M. 1991. Behavioral and electrophysiological responses of Aphids to host and nonhost plant volatiles. *J Chem Ecol,* 17**,** 1231-1242.

OBONYO, M., SCHULTHESS, F., CHIMTAWI, M., MASCAREL, G., AHUYA, P. O., LE RU, B., VAN DEN BERG, J., SILVAIN, J. F. & CALATAYUD, P. A. 2011. Sensilla on antennae, ovipositor and tarsi of the larval parasitoids, *Cotesia sesamiae* (Cameron 1906) and *Cotesia flavipes* Cameron 1891 (Hymenoptera: Braconidae): a comparative scanning electron microscopy study. *Ann Soc Entomol Fr,* 47**,** 119-127.

OCHIENG, S., PARK, K. & BAKER, T. 2002. Host plant volatiles synergize responses of sex pheromone-specific olfactory receptor neurons in male *Helicoverpa zea*. *J Comp Physiol A,* 188**,** 325-333.

OCHIENG, S., PARK, K., ZHU, J. & BAKER, T. 2000. Functional morphology of antennal chemoreceptors of the parasitoid *Microplitis croceipes* (Hymenoptera: Braconidae). *Arthropod Struct Dev,* 29**,** 231-240.

OCHIENG, S. A., HALLBERG, E. & HANSSON, B. S. 1998. Fine structure and distribution of antennal sensilla of the desert locust *Schistocerca gregaria* (Orthoptera: Acrididae). *Cell Tissue Res,* 291**,** 525-536.

OHKI, K., CHUNG, S., CH'NG, Y. H., KARA, P. & REID, R. C. 2005. Functional imaging with cellular resolution reveals precise micro-architecture in visual cortex. *Nature,* 433**,** 597-603.

OKAMURA, J. Y. & STRAUSFELD, N. J. 2007. Visual system of calliphorid flies: motion-and orientation-sensitive visual interneurons supplying dorsal optic glomeruli. *J Comp Neurol,* 500**,** 189-208.

OLAND, L. A., ORR, G. & TOLBERT, L. P. 1990. Construction of a protoglomerular template by olfactory axons initiates the formation of olfactory glomeruli in the insect brain. *J Neurosci,* 10**,** 2096-2112.

OLSON, D. M. & ANDOW, D. A. 1993. Antennal sensilla of female *Trichogramma nubilale* (Ertle and Davis) (Hymenoptera: Trichogrammatidae) and comparisons with other parasitic Hymenoptera. *Int J Insect Morphol Embryol,* 22**,** 507-520.

OLSON, D. M., RAINS, G. C., MEINERS, T., TAKASU, K., TERTULIANO, M., TUMLINSON, J. H., WACKERS, F. L. & LEWIS, W. J. 2003. Parasitic wasps learn and report diverse chemicals with unique conditionable behaviors. *Chem Senses,* 28**,** 545-549.

PARADISO, A. M., TSIEN, R. Y. & MACHEN, T. E. 1987. Digital image processing of intracellular pH in gastric oxyntic and chief cells. *Nature,* 325**,** 447-450.

PARAMANATHAN, T., VLADESCU, I., MCCAULEY, M. J., ROUZINA, I. & WILLIAMS, M. C. 2012. Force spectroscopy reveals the DNA structural dynamics that govern the slow binding of Actinomycin D. *Nucleic Acids Res,* doi: 10.1093/nar/gks069**,** 1-8.

PARAMANATHAN, T., VLADESCU, I. D., MCCAULEY, M. J., ROUZINA, I. & WILLIAMS, M. C. Year. Actinomycin D binding mode reveals the basis for its potent HIV-1 and cancer activity. *In:* B Am Phys Soc, 2011 Dallas, Texas. H39.39.

PARK, K. C., ZHU, J., HARRIS, J., OCHIENG, S. A. & BAKER, T. C. 2001. Electroantennogram responses of a parasitic wasp, *Microplitis croceipes*, to host-related volatile and anthropogenic compounds. *Physiol Entomol,* 26**,** 69-77.

PASCUAL, A., HUANG, K. L., NEVEU, J. & PRÉAT, T. 2004. Neuroanatomy: brain asymmetry and long-term memory. *Nature,* 427**,** 605-606.

PAVLOV, I. P. 1927. *Conditioned reflexes: an investigation of the physiological activity of the cerebral cortex* London, Oxford University Press.

PAVLOV, I. P. 2010. Conditioned reflexes: an investigation of the physiological activity of the cerebral cortex. *Ann Neurosci,* 17**,** 136-141.

PELZ, C., GERBER, B. & MENZEL, R. 1997. Odorant intensity as a determinant for olfactory conditioning in honeybees: roles in discrimination, overshadowing and memory consolidation. *J Exp Biol,* 200**,** 837-847.

PEÑAFLOR, M. F. G. V., ERB, M., MIRANDA, L., WERNEBURG, A. & BENTO, J. M. S. 2011. Herbivore-induced plant volatiles can serve as host location cues for a generalist and a specialist egg parasitoid. *J Chem Ecol,* 37**,** 1304-1313.

PERISSE, E., RAYMOND-DELPECH, V., NEANT, I., MATSUMOTO, Y., LECLERC, C., MOREAU, M. & SANDOZ, J. C. 2009. Early calcium increase triggers the formation of olfactory long-term memory in honeybees. *BMC Biol,* 7**,** 30.

PFLÜGER, H. J. 1980. Central nervous projections of sternal trichoid sensilla in locusts. *Naturwiss,* 67**,** 316-317.

PFLÜGER, H. J., BRÄUNIG, P. & HUSTERT, R. 1988. The organization of mechanosensory neuropiles in locust thoracic ganglia. *Phil Trans R Soc B,* 321**,** 1-26.

PHILIPS, F. S., SCHWARTZ, H. S., STERNBERG, S. S. & TAN, C. T. C. 1960. The toxicity of actinomycin *Ann NY Acad Sci,* 89**,** 348-360.

PINTO, D. M., BLANDE, J. D., NYKÄNEN, R., DONG, W. X., NERG, A. M. & HOLOPAINEN, J. K. 2007a. Ozone degrades common herbivore-induced plant volatiles: does this affect herbivore prey location by predators and parasitoids? *J Chem Ecol,* 33**,** 683-694.

PINTO, D. M., HIMANEN, S. J., NISSINEN, A., NERG, A. M. & HOLOPAINEN, J. K. 2008. Host location behavior of *Cotesia plutellae* Kurdjumov (Hymenoptera: Braconidae) in ambient and moderately elevated ozone in field conditions. *Environ Pollut,* 156**,** 227-231.

PINTO, D. M., NERG, A. M. & HOLOPAINEN, J. K. 2007b. The role of ozone-reactive compounds, terpenes, and green leaf volatiles (GLVs), in the orientation of *Cotesia plutellae*. *J Chem Ecol,* 33**,** 2218-2228.

PITMAN, R. M., TWEEDLE, C. D. & COHEN, M. J. 1972. Branching of central neurons: intracellular cobalt injection for light and electron microscopy. *Science,* 176**,** 412-414.

POTTING, R. P. J., POPPY, G. M. & SCHULER, T. H. 1999. The role of volatiles from cruciferous plants and pre-flight experience in the foraging behaviour of the specialist parasitoid *Cotesia plutellae*. *Entomol Exp Appl,* 93**,** 87-95.

PRILLINGER, L. 1981. Postembryonic development of the antennal lobes in *Periplaneta americana* L. *Cell Tissue Res,* 215**,** 563-575.

PUCHTLER, H. & MELOAN, S. N. 1985. On the chemistry of formaldehyde fixation and its effects on immunohistochemical reactions. *Histochem Cell Biol,* 82**,** 201-204.

RAINS, G. C., TOMBERLIN, J. K., D'ALESSANDRO, M. & LEWIS, W. J. 2004. Limits of volatile chemical detection of a parasitoid wasp,

Microplitis croceipes, and an electronic nose: a comparative study. *Trans ASAE,* 47**,** 2145-2152.

RANKIN, C. H., ABRAMS, T., BARRY, R. J., BHATNAGAR, S., CLAYTON, D. F., COLOMBO, J., et al. 2009. Habituation revisited: an updated and revised description of the behavioral characteristics of habituation. *Neurobiol Learn Mem,* 92**,** 135-138.

RATH, L., GIOVANNI GALIZIA, C. & SZYSZKA, P. 2011. Multiple memory traces after associative learning in the honey bee antennal lobe. *Europ J Neurosci,* 34**,** 352-360.

REDDY, G. V. P., HOLOPAINEN, J. K. & GUERRERO, A. 2002. Olfactory responses of *Plutella xylostella* natural enemies to host pheromone, larval frass, and green leaf cabbage volatiles. *J Chem Ecol,* 28**,** 131-143.

REICH, E., FRANKLIN, R., SHATKIN, A. & TATUM, E. 1962. Action of actinomycin D on animal cells and viruses. *Proc Natl Ac Sci USA,* 48**,** 1238–1245.

REISENMAN, C. E., CHRISTENSEN, T. A., FRANCKE, W. & HILDEBRAND, J. G. 2004. Enantioselectivity of projection neurons innervating identified olfactory glomeruli. *J Neurosci,* 24**,** 2602-2611.

REISENMAN, C. E., CHRISTENSEN, T. A. & HILDEBRAND, J. G. 2005. Chemosensory selectivity of output neurons innervating an identified, sexually isomorphic olfactory glomerulus. *J Neurosci,* 25**,** 8017-8026.

RIZZUTO, N. & GAMBETTI, P. 1976. Status spongiosus of rat central nervous system induced by Actinomycin D. *Acta Neuropathol,* 36**,** 21-30.

ROBERTSON, H. M. & WANNER, K. W. 2006. The chemoreceptor superfamily in the honey bee, *Apis mellifera*: expansion of the odorant, but not gustatory, receptor family. *Genome Res,* 16**,** 1395-1403.

RODRIGUEZ-ORTIZ, C. J., BALDERAS, I., SAUCEDO-ALQUICIRA, F., CRUZ-CASTAÑEDA, P. & BERMUDEZ-RATTONI, F. 2011. Long-term aversive taste memory requires insular and amygdala protein degradation. *Neurobiol Learn Mem,* 95**,** 311-315.

ROGERS, L. J. & VALLORTIGARA, G. 2008. From antenna to antenna: lateral shift of olfactory memory recall by honeybees. *PLoS One,* 3**,** e2340.

ROMAIN, A. C., DEGRAVE, C., NICOLAS, J., LOR, M., VAUSE, K., DINNE, K., MAES, F. & GOELEN, E. Year. Olfactory, chemical and e-nose measurements to characterize odors emission of construct materials for the implementation of the European construction products directive (CPD) on

a Belgian level. *In:* Olfaction and electronic nose: proceedings of the 13th International symposium on olfaction and electronic nose, 15–17 April 2009 Brescia (Italy) AIP Conf Proc, 527-528.

ROMANI, R., ROSI, M. C., ISIDORO, N. & BIN, F. 2008. The role of the antennae during courtship behaviour in the parasitic wasp *Trichopria drosophilae*. *J Exp Biol,* 211**,** 2486-2491.

ROSENBLUM, K., MEIRI, N. & DUDAI, Y. 1993. Taste memory: the role of protein synthesis in gustatory cortex. *Behav Neural Biol,* 59**,** 49-56.

ROSPARS, J. P. 1983. Invariance and sex-specific variations of the glomerular organization in the antennal lobes of a moth, *Mamestra brassicae*, and a butterfly, *Pieris brassicae*. *J Comp Neurol,* 220**,** 80-96.

ROSPARS, J. P. 1988. Structure and development of the insect antennodeutocerebral system. *Int J Insect Morphol Embryol,* 17**,** 243-294.

ROSPARS, J. P. & HILDEBRAND, J. G. 2000. Sexually dimorphic and isomorphic glomeruli in the antennal lobes of the sphinx moth *Manduca sexta*. *Chem Senses,* 25**,** 119-129.

ROSS, W. N., SALZBERG, B. M., COHEN, L. B., GRINVALD, A., DAVILA, H. V., WAGGONER, A. S. & WANG, C. H. 1977. Changes in absorption, fluorescence, dichroism, and birefringence in stained giant axons: optical measurement of membrane potential. *J Membr Biol,* 33**,** 141-183.

RÖSSLER, W., KUDUZ, J., SCHÜRMANN, F. W. & SCHILD, D. 2002. Aggregation of F-actin in olfactory glomeruli: a common feature of glomeruli across phyla. *Chem Senses,* 27**,** 803-810.

RÖSSLER, W., TOLBERT, L. P. & HILDEBRAND, J. G. 1998. Early formation of sexually dimorphic glomeruli in the developing olfactory lobe of the brain of the moth *Manduca sexta*. *J Comp Neurol,* 396**,** 415-428.

ROSTELIEN, T., BORG-KARLSON, A. K., FALDT, J., JACOBSSON, U. & MUSTAPARTA, H. 2000. The plant sesquiterpene germacrene D specifically activates a major type of antennal receptor neuron of the tobacco budworm moth *Heliothis virescens*. *Chem Senses,* 25**,** 141-148.

ROSTELIEN, T., STRANDEN, M., BORG-KARLSON, A. K. & MUSTAPARTA, H. 2005. Olfactory receptor neurons in two Heliothine moth species responding selectively to aliphatic green leaf volatiles, aromatic compounds, monoterpenes and sesquiterpenes of plant origin. *Chem Senses,* 30**,** 443-461.

ROUSSEL, E., PADIE, S. & GIURFA, M. 2011. Aversive learning overcomes appetitive innate responding in honeybees. *Anim Cogn,* 15**,** 135-141.

ROUSSEL, E., SANDOZ, J. C. & GIURFA, M. 2010. Searching for learning-dependent changes in the antennal lobe: simultaneous recording of neural activity and aversive olfactory learning in honeybees. *Front Behav Neurosci,* 4**,** 155.

ROUX, O., GERS, C., TENE-GHOMSI, J. N., ARVANITAKIS, L., BORDAT, D. & LEGAL, L. 2007. Chemical characterization of contact semiochemicals for host-recognition and host-acceptance by the specialist parasitoid *Cotesia plutellae* (Kurdjumov). *Chemoecology,* 17**,** 13-18.

ROUX, O., VAN BAAREN, J., GERS, C., ARVANITAKIS, L. & LEGAL, L. 2005. Antennal structure and oviposition behavior of the *Plutella xylostella* specialist parasitoid: *Cotesia plutellae*. *Microsc Res Tech,* 68**,** 36-44.

RUBIN, B. D. & KATZ, L. C. 1999. Optical imaging of odorant representations in the mammalian olfactory bulb. *Neuron,* 23**,** 499-511.

RUTHER, J., HOMANN, M. & STEIDLE, J. L. M. 2000. Female derived sex pheromone mediates courtship behaviour in the parasitoid *Lariophagus distinguendus*. *Entomol Exp Appl,* 96**,** 265-274.

RÜTZLER, M. & ZWIEBEL, L. J. 2005. Molecular biology of insect olfaction: recent progress and conceptual models. *J Comp Physiol A,* 191**,** 777-790.

SACHSE, S. & GALIZIA, C. G. 2002. Role of inhibition for temporal and spatial odor representation in olfactory output neurons: a calcium imaging study. *J Neurophysiol,* 87**,** 1106-1117.

SACHSE, S. & GALIZIA, C. G. 2003. The coding of odour-intensity in the honeybee antennal lobe: local computation optimizes odour representation. *Europ J Neurosci,* 18**,** 2119-2132.

SACHSE, S., RAPPERT, A. & GALIZIA, C. G. 1999. The spatial representation of chemical structures in the antennal lobe of honeybees: steps towards the olfactory code. *Europ J Neurosci,* 11**,** 3970-3982.

SADEK, M. M., HANSSON, B. S., ROSPARS, J. P. & ANTON, S. 2002. Glomerular representation of plant volatiles and sex pheromone components in the antennal lobe of the female *Spodoptera littoralis*. *J Exp Biol,* 205**,** 1363-1376.

SAKAMOTO, K., KARELINA, K. & OBRIETAN, K. 2011. CREB: a multifaceted regulator of neuronal plasticity and protection. *J Neurochem,* 116**,** 1-9.

SALLOUM, A., COLSON, V. & MARION-POLL, F. 2011. Appetitive and aversive learning in *Spodoptera littoralis* larvae. *Chem Senses,* 36**,** 725-731.

SANCHEZ-GRACIA, A., VIEIRA, F. G. & ROZAS, J. 2009. Molecular evolution of the major chemosensory gene families in insects. *Heredity,* 103**,** 208-216.

SANDOZ, J. C. 2006. Odour-evoked responses to queen pheromone components and to plant odours using optical imaging in the antennal lobe of the honey bee drone *Apis mellifera* L. *J Exp Biol,* 209**,** 3587-3598.

SANES, J. R. & HILDEBRAND, J. G. 1976. Structure and development of antennae in a moth, *Manduca sexta*. *Dev Biol,* 51**,** 282-299.

SANFORD, M. R. & TOMBERLIN, J. K. 2011. Conditioning individual mosquitoes to an odor: sex, source, and time. *PLoS One,* 6**,** e24218.

SANGHA, S., SCHEIBENSTOCK, A., MCCOMB, C. & LUKOWIAK, K. 2003. Intermediate and long-term memories of associative learning are differentially affected by transcription versus translation blockers in *Lymnaea*. *J Exp Biol,* 206**,** 1605-1613.

SARFRAZ, M., KEDDIE, A. B. & DOSDALL, L. M. 2005. Biological control of the diamondback moth, *Plutella xylostella*: a review. *Biocontr Sci Technol,* 15**,** 763-789.

SATO, K., PELLEGRINO, M., NAKAGAWA, T., NAKAGAWA, T., VOSSHALL, L. B. & TOUHARA, K. 2008. Insect olfactory receptors are heteromeric ligand-gated ion channels. *Nature,* 452**,** 1002-1006.

SAVEER, A. M., KROMANN, S. H., BIRGERSSON, G., BENGTSSON, M., LINDBLOM, T., BALKENIUS, A., et al. 2012. Floral to green: mating switches moth olfactory coding and preference. *Proc R Soc B,* 279**,** 2314-2322

SCHILDBERGER, K. 1984. Multimodal interneurons in the cricket brain: properties of identified extrinsic mushroom body cells. *J Comp Physiol A,* 154**,** 71-79.

SCHMERA, D. & GUERIN, P. M. 2012. Plant volatile compounds shorten reaction time and enhance attraction of the codling moth (*Cydia pomonella*) to codlemone. *Pest Manag Sci,* 68**,** 454-461.

SCHMIDT, M. & ACHE, B. W. 1992. Antennular projections to the midbrain of the spiny lobster. II. Sensory innervation of the olfactory lobe. *J Comp Neurol,* 318**,** 291-303.

SCHNEIDER, D. 1957. Electrophysiological investigation on the antennal receptors of the silk moth during chemical and mechanical stimulation. *Cell Mol Life Sci,* 13**,** 89-91.

SCHOENIAN, I., SPITELLER, M., GHASTE, M., WIRTH, R., HERZ, H. & SPITELLER, D. 2011. Chemical basis of the synergism and antagonism in microbial communities in the nests of leaf-cutting ants. *Proc Natl Acad Sci USA,* 108**,** 1955-1960.

SCHÜRMANN, F. 1995. Common and special features of the nervous system of Onychophora: a comparison with Arthropoda, Annelida and some other invertebrates. *In:* BREIDBACH, O. & KUTSCH, W. (eds.) *The Nervous System of Invertebrates: an Evolutionary and Comparative Approach.* Basel: Birkhaeuser.

SCHWÄRZEL, M., MONASTIRIOTI, M., SCHOLZ, H., FRIGGI-GRELIN, F., BIRMAN, S. & HEISENBERG, M. 2003. Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in *Drosophila*. *J Neurosci,* 23**,** 10495-10502.

SCHWÄRZEL, M. & MÜLLER, U. 2006. Dynamic memory networks: dissecting molecular mechanisms underlying associative memory in the temporal domain. *Cell Mol Life Sci,* 63**,** 989-998.

SEKI, Y., RYBAK, J., WICHER, D., SACHSE, S. & HANSSON, B. S. 2010. Physiological and morphological characterization of local interneurons in the *Drosophila* antennal lobe. *J Neurophysiol,* 104**,** 1007-1019.

SEN, A., RAINA, R., JOSEPH, M. & TUNGIKAR, V. 2005. Response of *Trichogramma chilonis* to infochemicals: an SEM and electrophysiological investigation. *Biocontrol,* 50**,** 429-447.

SHEPHERD, J. D. & HUGANIR, R. L. 2007. The cell biology of synaptic plasticity: AMPA receptor trafficking. *Annu Rev Cell Dev Biol,* 23**,** 613-643.

SHEPPARD, C. J. R. & WILSON, T. 1982. The image of a single point in microscopes of large numerical aperture. *Proc R Soc A,* 379**,** 145-158.

SHIELDS, V. D. C. & HILDEBRAND, J. G. 2001. Responses of a population of antennal olfactory receptor cells in the female moth *Manduca sexta* to plant-associated volatile organic compounds. *J Comp Physiol A,* 186**,** 1135-1151.

SHIOJIRI, K., OZAWA, R., KUGIMIYA, S., UEFUNE, M., VAN WIJK, M., SABELIS, M. W. & TAKABAYASHI, J. 2010. Herbivore-specific, density-dependent induction of plant volatiles: honest or "cry wolf" signals? *PLoS One,* 5**,** e12161.

SHIOJIRI, K., OZAWA, R., MATSUI, K., KISHIMOTO, K., KUGIMIYA, S. & TAKABAYASHI, J. 2006. Role of the lipoxygenase/lyase pathway of host-food plants in the host searching behavior of two parasitoid species, *Cotesia glomerata* and *Cotesia plutellae*. *J Chem Ecol,* 32**,** 969-979.

SIMMONS, L. W., TINGHITELLA, R. M. & ZUK, M. 2010. Quantitative genetic variation in courtship song and its covariation with immune function and sperm quality in the field cricket *Teleogryllus oceanicus*. *Behav Ecol,* 21**,** 1330-1336.

SIMÕES, P., OTT, S. R. & NIVEN, J. E. 2011. Associative olfactory learning in the desert locust, *Schistocerca gregaria*. *J Exp Biol,* 214**,** 2495-2503.

SIMONS, M. T. T. P., SUVERKROPP, B. P., VET, L. E. M. & DE MOED, G. 1992. Comparison of learning in related generalist and specialist eucoilid parasitoids. *Entomol Exp Appl,* 64**,** 117-124.

SIMPSON, M., GURR, G. M., SIMMONS, A. T., WRATTEN, S. D., JAMES, D. G., LEESON, G., NICOL, H. I. & ORRE-GORDON, G. U. S. 2011. Attract and reward: combining chemical ecology and habitat manipulation to enhance biological control in field crops. *J Appl Ecol,* 48**,** 580-590.

SKINNER, R. A. 1986. The value of methyl salicylate as a clearing agent. *J Histotechnol,* 9**,** 27-28.

SKIRI, H. T., GALIZIA, C. G. & MUSTAPARTA, H. 2004. Representation of primary plant odorants in the antennal lobe of the moth *Heliothis virescens* using calcium imaging. *Chem Senses,* 29**,** 253-267.

SKIRI, H. T., RO, H., BERG, B. G. & MUSTAPARTA, H. 2005. Consistent organization of glomeruli in the antennal lobes of related species of heliothine moths. *J Comp Neurol,* 491**,** 367-380.

SLAMA, K. 1988. A new look at insect respiration. *Biol Bull,* 175**,** 289-300.

SLAMA, K. 1999. Active regulation of insect respiration. *Ann Entomol Soc Am,* 92**,** 916-929.

SMID, H. M., BLEEKER, M. A., VAN LOON, J. J. & VET, L. E. 2003. Three-dimensional organization of the glomeruli in the antennal lobe of the parasitoid wasps *Cotesia glomerata* and *C. rubecula*. *Cell Tissue Res,* 312**,** 237-248.

SMID, H. M., VAN LOON, J. J. A., POSTHUMUS, M. A. & VET, L. E. M. 2002. GC-EAG-analysis of volatiles from Brussels sprouts plants damaged by two species of Pieris caterpillars: olfactory receptive range of a specialist and a generalist parasitoid wasp species. *Chemoecology,* 12**,** 169-176.

SMID, H. M., WANG, G., BUKOVINSZKY, T., STEIDLE, J. L. M., BLEEKER, M. A. K., VAN LOON, J. J. A. & VET, L. E. M. 2007. Species-specific acquisition and consolidation of long-term memory in parasitic wasps. *Proc R Soc B,* 274**,** 1539-1546.

SMITH, B. H. & MENZEL, R. 1989. The use of electromyogram recordings to quantify odourant discrimination in the honey bee, *Apis mellifera*. *J Insect Physiol,* 35**,** 369-375.

SNOEREN, T. A. L., MUMM, R., POELMAN, E. H., YANG, Y., PICHERSKY, E. & DICKE, M. 2010. The herbivore-induced plant volatile methyl salicylate negatively affects attraction of the parasitoid *Diadegma semiclausum*. *J Chem Ecol,* 36**,** 479-489.

SOBELL, H. M. 1985. Actinomycin and DNA transcription. *Proc Natl Acad Sci USA,* 82**,** 5328-5331.

SOBELL, H. M., JAIN, S., SAKORE, T. & NORDMAN, C. 1971. Stereochemistry of actinomycin–DNA binding. *Nature,* 231**,** 200-205.

SQUIRE, L. R. & BARONDES, S. H. 1970. Actinomycin-D: effects on memory at different times after training. *Nature* 225**,** 649-650.

STEIDLE, J. L. M. & VAN LOON, J. J. A. 2003. Dietary specialization and infochemical use in carnivorous arthropods: testing a concept. *Entomologia Experimentalis et Applicata,* 108**,** 133-148.

STEINBRECHT, R. A. 1973. Der Feinbau olfaktorischer Sensillen des Seidenspinners (Insecta, Lepidoptera). *Cell Tissue Res,* 139**,** 533-565.

STEINER, S., HERMANN, N. & RUTHER, J. 2006. Characterization of a female-produced courtship pheromone in the parasitoid *Nasonia vitripennis*. *J Chem Ecol,* 32**,** 1687-1702.

STEMMER, A., BECK, M. & FIOLKA, R. 2008. Widefield fluorescence microscopy with extended resolution. *Histochem Cell Biol,* 130**,** 807-817.

STENSMYR, M. C., GIORDANO, E., BALLOI, A., ANGIOY, A. M. & HANSSON, B. S. 2003. Novel natural ligands for *Drosophila* olfactory receptor neurones. *J Exp Biol,* 206**,** 715-724.

STEPHENS, D. 1991. Change, regularity, and value in the evolution of animal learning. *Behav Ecol,* 2**,** 77-89.

STEPHENS, D. W. 1993. Learning and behavioral ecology: incomplete information and environmental predictability. *In:* PAPAJ D.R. & LEWIS A.C. (eds.) *Insect Learning: Ecological and Evolutionary Perspectives.* New York Chapman & Hall.

STEWART, W. W. 1981. Lucifer dyes - highly fluorescent dyes for biological tracing. *Nature,* 292**,** 17-21.

STIEB, S. M., KELBER, C., WEHNER, R. & RÖSSLER, W. 2011. Antennal-lobe organization in desert ants of the genus *Cataglyphis*. *Brain Behav Evol,* 77**,** 136-146.

STOSIEK, C., GARASCHUK, O., HOLTHOFF, K. & KONNERTH, A. 2003. In vivo two-photon calcium imaging of neuronal networks. *Proc Natl Acad Sci USA,* 100**,** 7319-7324.

STRAUSFELD, N. J., HANSEN, L., LI, Y., GOMEZ, R. S. & ITO, K. 1998. Evolution, discovery, and interpretations of arthropod mushroom bodies. *Learn Memory,* 5**,** 11-37.

STRAUSFELD, N. J. & HAUSEN, K. 1977. The resolution of neuronal assemblies after cobalt injection into neuropil. *Proc R Soc B,* 199**,** 463-476.

STRAUSFELD, N. J. & HILDEBRAND, J. G. 1999. Olfactory systems: common design, uncommon origins. *Curr Opin Neurobiol,* 9**,** 634-639.

STRAUSFELD, N. J. & OBERMAYER, M. 1976. Resolution of intraneuronal and transynaptic migration of cobalt in the insect visual and central nervous systems. *J Comp Physiol A,* 110**,** 1-12.

STRAUSFELD, N. J. & OKAMURA, J. Y. 2007. Visual system of calliphorid flies: organization of optic glomeruli and their lobula complex efferents. *J Comp Neurol,* 500**,** 166-188.

STRAUSFELD, N. J., SINAKEVITCH, I. & OKAMURA, J. Y. 2007. Organization of local interneurons in optic glomeruli of the dipterous visual system and comparisons with the antennal lobes. *Develop Neurobiol,* 67**,** 1267-1288.

STRETTON, A. O. W. & KRAVITZ, E. A. 1968. Neuronal geometry: determination with a technique of intracellular dye injecion. *Science,* 162**,** 132-134.

SU, C. Y., MENUZ, K. & CARLSON, J. R. 2009. Olfactory perception: receptors, cells, and circuits. *Cell,* 139**,** 45-59.

SUCKLING, D. M., KARG, G., GIBB, A. R. & BRADLEY, S. J. 1996. Electroantennogram and oviposition responses of *Epiphyas postvittana* (Lepidoptera: Tortricidae) to plant volatiles. *New Zeal J Bot,* 24**,** 323-333.

SUGAI, R., AZAMI, S., SHIGA, H., WATANABE, T., SADAMOTO, H., KOBAYASHI, S., et al. 2007. One-trial conditioned taste aversion in Lymnaea: good and poor performers in long-term memory acquisition. *Journal of experimental biology,* 210**,** 1225-1237.

SUGIURA, K. 1960. The effect of actinomycin D on a spectrum of tumors. *Ann NY Acad Sci,* 89**,** 368-372.

SUTTON, M. A., IDE, J., MASTERS, S. E. & CAREW, T. J. 2002. Interaction between amount and pattern of training in the induction of intermediate- and long-term memory for sensitization in *Aplysia*. *Learn Memory,* 9**,** 29-40.

SUTTON, M. A., MASTERS, S. E., BAGNALL, M. W. & CAREW, T. J. 2001. Molecular mechanisms underlying a unique intermediate phase of memory in *Aplysia*. *Neuron,* 31**,** 143-154.

SUZUKI, H. & TATEDA, H. 1974. An electrophysiological study of olfactory interneurones in the brain of the honey-bee. *J Insect Physiol,* 20**,** 2287-2299.

SVOBODA, K. & YASUDA, R. 2006. Principles of two-photon excitation microscopy and its applications to neuroscience. *Neuron,* 50**,** 823-839.

TAGAWA, J. 1977. Localization and histology of the female sex pheromone-producing gland in the parasitic wasp, *Apanteles glomeratus*. *J Insect Physiol,* 23**,** 49-56.

TAGAWA, J. 1983. Female sex pheromone glands in the parasitic wasps, genus *Apanteles*. *Appl Entomol Zool,* 18**,** 416-427.

TAKABAYASHI, J., TAKAHASHI, S., DICKE, M. & POSTHUMUS, M. 1995. Developmental stage of herbivore *Pseudaletia separata* affects production of herbivore-induced synomone by corn plants. *J Chem Ecol,* 21**,** 273-287.

TAKASU, K. & LEWIS, W. 1996. The role of learning in adult food location by the larval parasitoid, *Microplitis croceipes* (Hymenoptera: Braconidae). *J Insect Behav,* 9**,** 265-281.

TAKASU, K. & LEWIS, W. J. 2003. Learning of host searching cues by the larval parasitoid *Microplitis croceipes*. *Entomol Exp Appl,* 108**,** 77-86.

TAKASU, K., RAINS, G. C. & LEWIS, W. J. 2007. Comparison of detection ability of learned odors between males and females in the larval parasitoid *Microplitis croceipes*. *Entomol Exp Appl,* 122**,** 247-251.

TALEKAR, N. S. & SHELTON, A. M. 1993. Biology, ecology and management of the diamondback moth. *Annu Rev Entomol,* 38**,** 275-301.

TAMO, C., RICARD, I., HELD, M., DAVISON, A. C. & TURLINGS, T. C. J. 2006. A comparison of naive and conditioned responses of three generalist endoparasitoids of lepidopteran larvae to host-induced plant odours. *Anim Biol,* 56**,** 205-220.

TAYLOR, D., AMATO, P. A., LUBY-PHELPS, K. & MCNEIL, P. 1984. Fluorescent analog cytochemistry. *Trends Biochem Sci,* 9**,** 88-91.

THOMPSON, R. F. 2009. Habituation: a history. *Neurobiol Learn Mem,* 92**,** 127-134.

TIMM, F. 1958. Zur Histochemie der Schwermetalle: Das Sulfid-Silberverfahren. *Int J Legal Med,* 46**,** 706-711.

TODD, J. L., ANTON, S., HANSSON, B. S. & BAKER, T. C. 1995. Functional organization of the macroglomerular complex related to behaviourally expressed olfactory redundancy in male cabbage looper moths. *Physiol Entomol,* 20**,** 349-361.

TOLBERT, L. P., SUN, X. J. & HILDEBRAND, J. G. 1996. Combining laser scanning confocal microscopy and electron microscopy in studies of the insect nervous system. *J Neurosci Methods,* 69**,** 25-32.

TULLY, T., BOYNTON, S., BRANDES, C., DURA, J., MIHALEK, R., PREAT, T. & VILLELLA, A. 1990. Genetic dissection of memory formation in *Drosophila melanogaster*. *Cold Spring Harbor Symp Quant Biol*
55**,** 203-211.

TULLY, T., PREAT, T., BOYNTON, S. C. & DEL VECCHIO, M. 1994. Genetic dissection of consolidated memory in *Drosophila*. *Cell,* 79**,** 35-47.

TULLY, T. & QUINN, W. G. 1985. Classical conditioning and retention in normal and mutant *Drosophila melanogaster*. *J Comp Physiol A,* 157**,** 263-277.

TURLINGS, T. C. J., LENGWILER, U. B., BERNASCONI, M. L. & WECHSLER, D. 1998. Timing of induced volatile emissions in maize seedlings. *Planta,* 207**,** 146-152.

TURLINGS, T. C. J., TUMLINSON, J. H., ELLER, F. J. & LEWIS, W. J. 1991. Larval-damaged plants: source of volatile synomines that guide the parasitoid *Cotesia marginiventris* to the micro-habitat of its hosts. *Entomol Exp Appl,* 58**,** 75-82.

TURLINGS, T. C. J., TUMLINSON, J. H. & LEWIS, W. J. 1990. Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science,* 250**,** 1251-1253.

TURLINGS, T. C. J., WÄCKERS, F. L., VET, L. E. M., LEWIS, W. J. & TUMLINSON, J. H. 1993. Learning of host-finding cues by hymenopterous parasitoids. *In:* LEWIS, A. & PAPAJ, D. (eds.) *Insect Learning: Ecological and Evolutionary Perspectives.* New York: Chapman and Hall.

TYRER, N. M. & BELL, E. M. 1974. The intensification of cobalt-filled neurone profiles using a modification of Timm's sulphide-silver method. *Brain Res,* 73**,** 151-155.

UEFUNE, M., KUGIMIYA, S. & TAKABAYASHI, J. 2011. Herbivore-induced carnivore attractants enhance the residence time of carnivores on a host food plant. *J Plant Interact,* 6**,** 165-165.

UKHANOV, K., BOBKOV, Y. & ACHE, B. 2011. Imaging ensemble activity in arthropod olfactory receptor neurons in situ. *Cell Calcium,* 49**,** 100-107.

UNSICKER, S. B., KUNERT, G. & GERSHENZON, J. 2009. Protective perfumes: the role of vegetative volatiles in plant defense against herbivores. *Curr Opin Plant Biol,* 12**,** 479-485.

VALLORTIGARA, G., ROGERS, L. J. & BISAZZA, A. 1999. Possible evolutionary origins of cognitive brain lateralization. *Brain Res Rev,* 30**,** 164-175.

VAN DEN BERG, M., VERBAARSCHOT, P., HONTELEZ, S., VET, L., DICKE, M. & SMID, H. 2010. CREB expression in the brains of two closely related parasitic wasp species that differ in long term memory formation. *Insect Mol Biol,* 19**,** 367-379.

VAN EMDEN, H. F., STORECK, A., DOULOUMPAKA, S., ELEFTHERIANOS, I., POPPY, G. & POWELL, W. 2008. Plant chemistry and aphid parasitoids (Hymenoptera: Braconidae): imprinting and memory. *Europ J Entomol,* 105**,** 477-483.

VAN NATERS, W. G. & CARLSON, J. R. 2006. Insects as chemosensors of humans and crops. *Nature,* 444**,** 302-307.

VAN VALEN, L. 1973. A new evolutionary law. *Evol Theor,* 1**,** 1-30.

VARELA, N., AVILLA, J., GEMENO, C. & ANTON, S. 2011. Ordinary glomeruli in the antennal lobe of male and female tortricid moth *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae) process sex pheromone and host-plant volatiles. *J Exp Biol,* 214**,** 637-645.

VARESCHI, E. 1971. Duftunterscheidung bei der Honigbiene: Einzelzell-Ableitungen und Verhaltensreaktionen. *J Comp Physiol A,* 75**,** 143-173.

VELASCO, L. R. I. 1982. The life history of *Apanteles plutellae* Kurdj. (Braconidae), a parasitoid of the diamondback moth. *Philipp Entomol,* 5**,** 385-399.

VERGOZ, V. 2007. Aversive learning in honeybees revealed by the olfactory conditioning of the sting extension reflex. *PLoS One,* 2**,** e288.

VERKERK, R. H. J. & WRIGHT, D. J. 1996. Multitrophic interactions and management of the diamondback moth: a review. *Bull Entomol Res,* 86**,** 205-216.

VET, L. E. M. & DICKE, M. 1992. Ecology of infochemical use by natural enemies in a tritrophic context. *Ann Rev Entomol,* 37**,** 141-172.

VET, L. E. M. & GROENEWOLD, A. W. 1990. Semiochemicals and learning in parasitoids. *J Chem Ecol,* 16**,** 3119-3135.

VET, L. E. M., WACKERS, F. L. & DICKE, M. 1991. How to hunt for hiding hosts - the reliability-detectability problem in foraging parasitoids. *Neth J Zool,* 41**,** 202-213.

VILLAGRA, C., PINTO, C., PENNA, M. & NIEMEYER, H. 2011. Male wing fanning by the aphid parasitoid *Aphidius ervi* (Hymenoptera: Braconidae) produces a courtship song. *Bull Entomol Res,* 1**,** 1-7.

VILLAGRA, C. A., VASQUEZ, R. A. & NIEMEYER, H. M. 2005. Associative odour learning affects mating behaviour in *Aphidius ervi* males (Hymenoptera: Braconidae). *Eur J Entomol,* 102**,** 557-559

VILLAGRA, C. A., VÁSQUEZ, R. A. & NIEMEYER, H. M. 2008. Olfactory conditioning in mate searching by the parasitoid *Aphidius ervi* (Hymenoptera: Braconidae). *Bull Entomol Res,* 98**,** 371-377.

VINSON, S. B. 1972. Courtship behavior and evidence for a sex pheromone in the parasitoid *Campoletis sonorensis* (Hymenoptera: Ichneumonidae). *Environ Entomol,* 1**,** 409-414.

VINSON, S. B. 1976. Host selection by insect parasitoids. *Ann Rev Entomol,* 21**,** 109-133.

VON FRISCH, K. 1919. Über den Geruchssinn der Bienen und seine blütenbiologische Bedeutung (The bee's sense of smell and its significance during blooming). *Zool Jahrb,* 37**,** 1-238.

VOSSHALL, L. B. 2003. Diversity and expression of odorant receptors in *Drosophila*. *Pharmacol Toxicol,* 40**,** 97-132.

VOSSHALL, L. B., WONG, A. M. & AXEL, R. 2000. An olfactory sensory map in the fly brain. *Cell,* 102**,** 147-159.

VUORINEN, T., NERG, A. M., IBRAHIM, M. A., REDDY, G. V. P. & HOLOPAINEN, J. K. 2004. Emission of *Plutella xylostella* - induced compounds from cabbages grown at elevated CO_2 and orientation behavior of the natural enemies. *Plant Physiol,* 135**,** 1984-1992.

WACHOWIAK, M. & COHEN, L. B. 2001. Representation of odorants by receptor neuron input to the mouse olfactory bulb. *Neuron,* 32**,** 723-735.

WACHOWIAK, M., FALK, C. X., COHEN, L. B. & ZOCHOWSKI, M. R. 2002. Voltage and calcium imaging of brain activity: examples from the turtle and the mouse. *In:* ARTHUR, W. T. & JOHN, C. M. (eds.) *Brain Mapping: The Methods (Second Edition).* San Diego: Academic Press.

WACHOWIAK, M. & SHIPLEY, M. T. 2006. Coding and synaptic processing of sensory information in the glomerular layer of the olfactory bulb. *Semin Cell Dev Biol,* 17**,** 411-423.

WÄCKERS, F., BONIFAY, C., VET, L. & LEWIS, J. 2006. Gustatory response and appetitive learning in *Microplitis croceipes* in relation to sugar type and concentration. *Anim Biol,* 56**,** 193-203.

WÄCKERS, F. L., BONIFAY, C. & LEWIS, W. J. 2002. Conditioning of appetitive behavior in the hymenopteran parasitoid *Microplitis croceipes*. *Entomol Exp Appl,* 103**,** 135-138.

WÄCKERS, F. L. & LEWIS, W. J. 1994. Olfactory and visual learning and their combined influence on host site location by the parasitoid *Microplitis Croceipes* (Cresson). *Biol Control,* 4**,** 105-112.

WADHAMS, L., ANGST, M. & BLIGHT, M. M. 1982. Responses of the olfactory receptors of *Scolytus scolytus* (F.) (Coleoptera: Scolytidae) to the stereoisomers of 4-methyl-3-heptanol. *J Chem Ecol,* 8**,** 477-492.

WAKSMAN, S. A. & WOODRUFF, H. B. 1940. The soil as a source of microorganisms antagonistic to disease-producing bacteria. *J Bacteriol,* 40**,** 581-600.

WAKSMAN, S. A. & WOODRUFF, H. B. 1941. Actinomyces antibioticus, a new soil organism antagonistic to pathogenic and non-pathogenic bacteria. *J Bacteriol,* 42**,** 231-249.

WAKSMAN, S. A. & WOODRUFF, H. B. 1942. Selective antibiotic action of various substances of microbial origin. *J Bacteriol,* 44**,** 373-384.

WALD, G. 1953. The biochemistry of vision. *Ann Rev Biochem,* 22**,** 497-526.

WANG, S., SATO, K., GIURFA, M. & ZHANG, S. 2008. Processing of sting pheromone and its components in the antennal lobe of the worker honeybee. *J Insect Physiol,* 54**,** 833-841.

WEBSTER, B., BRUCE, T., PICKETT, J. & HARDIE, J. 2010. Volatiles functioning as host cues in a blend become nonhost cues when presented alone to the black bean aphid. *Anim Behav,* 79**,** 451-457.

WEIS-FOGH, T. 1967. Respiration and tracheal ventilation in locusts and other flying insects. *J Exp Biol,* 47**,** 561-587.

WHITE, J. G., AMOS, W. B. & FORDHAM, M. 1987. An evaluation of confocal versus conventional imaging of biological structures by fluorescence light microscopy. *J Cell Biol,* 105**,** 41-48.

WHITMAN, D. W. & ELLER, F. J. 1990. Parasitic wasps orient to green leaf volatiles. *Chemoecology,* 1**,** 69-76.

WICHER, D., SCHÄFER, R., BAUERNFEIND, R., STENSMYR, M. C., HELLER, R., HEINEMANN, S. H. & HANSSON, B. S. 2008. *Drosophila* odorant receptors are both ligand-gated and cyclic-nucleotide-activated cation channels. *Nature,* 452**,** 1007-1011.

WILSON, R. I. 2008. Neural and behavioral mechanisms of olfactory perception. *Curr Opin Neurobiol,* 18**,** 408-412.

WÖLFLING, M. & ROSTÁS, M. 2009. Parasitoids use chemical footprints to track down caterpillars. *Commun Integr Biol,* 2**,** 353-355.

WÜSTENBERG, D., GERBER, B. & MENZEL, R. 1998. Long- but not medium-term retention of olfactory memories in honeybees is impaired by actinomycin D and anisomycin. *Eur J Neurosci,* 10**,** 2742-2745.

YAN, Z. G. & WANG, C. Z. 2006. Identification of *Mythmna separata* induced maize volatile synomones that attract the parasitoid *Campoletis chlorideae*. *J Appl Entomol,* 130**,** 213-219.

YANG, L., DICKE, M., JONGSMA, M., PANG, B., FANG, Z. & VAN LOON, J. 2008. *Integration of host plant resistance and biological control: using Arabidopsis-insect interactions as a model system-effect of constitutive emission of linalool by transgenic plants on parasitoid attraction and survival.* PhD thesis, University of Wageningen.

YONEYA, K., KUGIMIYA, S. & TAKABAYASHI, J. 2009. Can herbivore-induced plant volatiles inform predatory insect about the most suitable stage of its prey? *Physiol Entomol,* 34**,** 379-386.

ZHANG, G. N., HU, F., DOU, W. & WANG, J. J. 2012. Morphology and distribution of sensilla on tarsi and ovipositors of six fruit flies (Diptera: Tephritidae). *Ann Entomol Soc Am,* 105**,** 319-327.

ZHANG, M.-X., LING, B., CHEN, S.-Y., LIANG, G.-W. & PANG, X.-F. 2004. Repellent and oviposition deterrent activities of the essential oil from *Mikania micrantha* and its compounds on *Plutella xylostella*. *Insect Sci,* 11**,** 37-45.

ZHANG, Q.-H. & SCHLYTER, F. 2010. Inhibition of predator attraction to kairomones by non-host plant volatiles for herbivores: a bypass-trophic signal. *PLoS One,* 5**,** e11063.

ZHENG, X., LAN, Y., ZHU, J., WESTBROOK, J., HOFFMANN, W. & LACEY, R. 2009. Rapid identification of rice samples using an electronic nose. *J Bionic Eng,* 6**,** 290-297.

ZHOU, X., SHEN, Z., LI, D., HE, X. & LIN, B. 2007. Study of interactions between actinomycin D and oligonucleotides by microchip electrophoresis and ESI-MS. *Talanta,* 72**,** 561-567.

ZIMMERBERG, B. 2000. *Neurotransmitter release* [Online]. Williamstown, Massachusetts, United States: Williams College. Available: http://web.williams.edu/imput/synapse/index.html [Accessed 25.10.2011 2011].

ZUBE, C., KLEINEIDAM, C. J., KIRSCHNER, S., NEEF, J. & RÖSSLER, W. 2008. Organization of the olfactory pathway and odor processing in the

antennal lobe of the ant *Camponotus floridanus*. *J Comp Neurol,* 506**,** 425-441.

Appendices

Appendix 1: Additional volatiles tested in an EAG

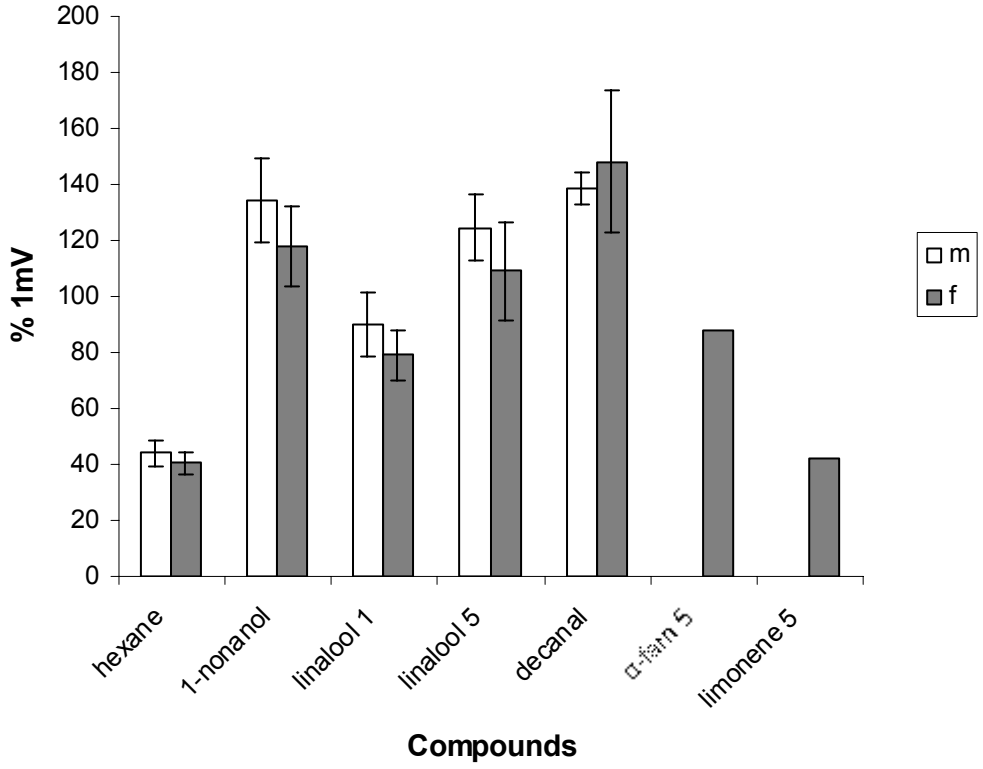

Fig. A.1 EAG responses to different volatiles including α-farnesen and limonene (5mg/ml).

Appendix 2: Evaluating manual and 3D data

To better illustrate differences and similarities between manual and AMIRA measurements, results obtained by the two methods are compared in the following section. One female AL, which showed clear glomerular structures, as well as the mean values of all preparations were used to compare AMIRA data and manual measurements data.

Evaluation of one female AL

When measured with AMIRA, the AL volume was 4.3 x 10^5 µm^3, and the total glomerular volume 1.1 x 10^5 µm^3. The average volume of a glomerulus was 1,890 ± 187 µm^3. The core volume measured 16,856 µm^3. Hence, the glomeruli made up 26% of the AL, and the central core 4% (Table A.1).

Table A.1 Comparison between manual measurements and AMIRA of one female AL. The table shows volume measurements for one female AL and its glomeruli measured with AMIRA and manually. The percentage values (%) relative to the AL volume are given.

	AMIRA	**Manual**	**AMIRA**	**Manual**
	Vol in µm^3	Vol in µm^3	%	%
Number of glomeruli	59	37	-	-
AL volume	4.3 x 10^5	7.6 x 10^5	100	100
Total glomerular volume	1.1 x 10^5	1.5 x 10^5	26	20
Average glomerular volume	1890	4189	0.44	0.53
Core volume	16857	5721	4	0.75

Using the manual measurement data taken from one female (Fig. 4.4) gave an estimated AL volume of 7.6 x 10^5 µm^3. The central core measured approximately 5,721 µm^3. A total of 37 glomeruli could be identified. With the measured average size of a glomerulus (20 x 20 µm) the average glomerular volume would be 4,189 µm^3. The total glomerular volume would therefore be 1.5 x 10^5 µm^3. In this case, the glomerular volume would make up 20% of the AL, and the central core only 0.75%.

Manual measurements, however, could not consider the volume difference of single glomeruli, as it was shown that single glomeruli can range between 272 to 21,185 µm^3. In this preparation, the size rank of glomeruli lay between 274 to 6,375 µm^3. In summary, both methods gave a similar glomerular volume (1.1 x 10^5 µm^3 and 1.5 x 10^5 µm^3), but not AL volume.

Evaluation of average data

Using the average values of manually and software obtained data, 32 and 40 glomeruli could be identified (n=23; 9) (Table A.2). As previously shown, with both methods volume measurements of the AL were considerably larger with both measurements, as they included spaces between the glomeruli and other

structures, such as cell bodies. The average volume of a glomerulus obtained through software measurements (3,777 ± 288 μm^3) was smaller than the estimated volume of 5,575 μm^3, which was based on manual measurements. The glomerular volume ranged between 1.3 x 10^5 μm^3 with software calculations to 1.8 x 10^5 μm^3, with manual measurements.

Comparing AMIRA and manual measurements data with one preparation and the average data (Table 4.1, 4.2), showed that AMIRA calculations had less variation between the volume measurements of the AL. In terms of glomerular size and AL volume, AMIRA measurements had the advantage that changes in dimension and shape through depth were taken into account.

Table A.2 Comparison between manual measurements and AMIRA using the average data of female preparations. The table shows the volume measurements for the mean values of AL and glomeruli measured with AMIRA and manually. The percentage values (%) relative to the AL volume are given.

	AMIRA	**Manual**	**AMIRA**	**Manual**
	Vol in μm^3	Vol in μm^3	%	%
Number of glomeruli	40	32	-	-
AL volume	4.3 x 10^5	18 x 10^5	100	100
Total glomerular volume	1.3 x 10^5	1.7 x 10^5	30	9.4
Average glomerular volume	3777	5575	0.86	0.31
Core volume	97447	-	23	-

In summary, with both methods (manual and AMIRA), no gender difference in ordinary glomeruli was found and the total glomerular volume was similar in both methods. Furthermore, with both methods, segregated regions for pheromone and non-pheromone processing were found in males. The macroglomeruli were significantly larger than ordinary glomeruli. In addition, analysis with AMIRA also found a few enlarged glomeruli in females.

With both methods, the AL volume was considerably larger than the total glomerular volume, which suggests that the AL volume can only be used as a rough guideline to estimate the number of glomeruli. Contrary to manual measurements, no side differences were found in the AL with AMIRA.