Mechanisms of Olfaction in Parasitic Wasps: Neurophysiological and Neuroanatomical Studies of Olfaction in a Specialist (*Microplitis croceipes*) and a Generalist (*Cotesia marginiventris*) Parasitoid

by

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Abstract

The success of parasitic wasps (parasitoids) in controlling pest populations depends on their ability to locate their hosts in a complex olfactory environment. Parasitic wasps are known to utilize various types of host-related volatile signals as host location cues. These volatile signals could be green leaf volatiles (GLVs), herbivore induced plant volatiles (HIPVs) or host specific odors. The olfactory system plays a major role for odor detection, processing and relaying information to higher centers of the insect brain which helps in recognition and decision making. Despite the intense interest in host-parasitoid interactions, the underlying mechanism of olfactory communication in this group of insects is not well understood. This study was conducted to characterize mechanisms of olfaction and response to host-related odor in two parasitic wasps (Hymenoptera: Braconidae) with different degrees of host specificity, *Microplitis croceipes* (Cresson) (specialist) and *Cotesia marginiventris* (Cresson) (generalist), utilizing an integration of neurophysiological and neuroanatomical techniques. Specifically, this research characterized species and sexual differences in olfactory mechanisms in parasitic wasps by: (1) comparing abundance of olfactory sensilla in the antennae of *M. croceipes* and *C. marginiventris* ; (2) characterizing single sensillum responses of olfactory receptor neurons in the sensilla placodea of *M. croceipes* and *C. marginiventris*to host-related volatiles; (3) characterizing antennal lobe architecture and glomerular organization in *M. croceipes* and *C. marginiventris*; and (4) identifying glomerular projections of olfactory receptor neurons in the antennal lobes of both species.

In chapter II, studies were conducted to compare the abundance of antennal sensilla types in both sexes of *M. croceipes* and *C. marginiventris* to determine if there is a correlation between abundance of olfactory sensilla and host specificity. Five major sensilla types were recorded in both species: sensilla chaetica (non-porous), s. trichodea (non-porous), s. placodea (multiporous), s. basiconica (two types, type 1 with terminal opening and type 2 with wall pores), and s. coeloconica (non-porous). The putative chemosensilla types, s. placodea and s. basiconica, were more abundant in *M. croceipes* (specialist) than in *C. marginiventris* (generalist), and this was true for both sexes. Comparing the sexes, s. placodea and s. trichodea were significantly more abundant in *M. croceipes* males compared with females. In contrast, s. placodea was relatively more abundant in female *C. marginiventris* than in males. In Chapter III, I characterized the responses of olfactory receptor neurons (ORNs) housed in the sensilla placodea of both parasitoid species to host-related plant volatiles. The extracellular activity showed presence of two neurons in the olfactory sensilla of these two parasitoids. In *M. croceipes*, single neuron elicited response to green leaf volatile (GLV) *cis*-3-hexenol and herbivore-induced plant volatile (HIPV) *cis*-3-hexenyl butyrate. The rest of the compounds elicited response in both ORNs in the sensilla. Mixtures of GLVs and HIPVs showed excitation in the ORNs, however, mixture of *cis*-3-hexenol & hexanal (GLVs) with linalool inhibited the ORNs. In *C. marginiventris*, *cis*-3-hexenol (GLV) and *cis*-3-hexenyl acetate (HIPV), elicited in single olfactory neuron. The other GLVs and HIPVs elicited responses in both neurons similar to *M. croceipes*. All mixtures of GLVs and HIPVs enhanced responses in the ORNs in *C. maginiventris*. The most significant finding is the inhibitiory effect of linalool in *M. croceipes* in a mixture with *cis*-3-hexenol and hexanal; however, ORNs in *C. marginiventris* showed enhanced response. The results show that

ORNs in *M. croceipes* have specific responses to compounds as compared to *C. marginiventris*. This difference in neuronal activity might suggest that, olfactory system in *M. croceipes* (specialist) have evolved with specific response to host-related volatiles, which might provide an olfactory code for volatiles damaged by its hosts for specific host recognition and location.

In Chapter IV, I reconstructed the antennal lobe morphology and glomerular organization of both parasitoid species. In *M. croceipes*, the medial half of the antennal lobe is larger with greater number of glomeruli compared to the lateral half, whereas in *C. marginiventris* the lateral half is larger than the median half. The most striking sexual difference was the presence of an enlarged glomerulus (macroglomerulus or MG) at the entrance of the antennal nerve in males of both species. In addition, a complex of 3-4 macro-glomeruli (complex of macro-glomeruli or CMG) was observed in the posterior region of the antennal lobe of males of both species. The average volume of the antennal lobe is similar between the sexes but ~ 2.5 greater in *M. croceipes* compared to *C. marginiventris*. In Chapter V, I conducted studies to understand how olfactory stimuli are processed in the brains of both species by characterizing glomerular projections of olfactory receptor neurons (ORNs) responding to single odors and mixtures of host-related plant volatiles in their antennal lobes. The ORNs responding to the tested host-related plant volatiles send projections to glomeruli in the medial half of the AL in *M. croceipes*, versus the lateral half of the AL in *C. marginiventris*. In *M. croceipes*, *cis*-3-hexenol (a green leaf volatile or GLV) and *cis*-3-hexenyl butyrate (a herbivore-induced plant volatile or HIPV) both activated a distinct glomerulus, whereas hexanal (HIPV) activated two adjacent glomeruli in the antero-median section. In contrast, *cis*-3-hexenol (GLV) and *cis*-3-hexenyl acetate (HIPV) activated distinct glomeruli in *C. marginiventris*. Hexanal (HIPV) activated one glomerulus and

the ORN projects to another glomeruli in ventro-lateral region, suggesting a connection in the odor processing between these two glomeruli. In *M. croceipes*, an odor mixture (blend) of *cis*-3-hexenol (GLV) and *cis*-3-hexenyl butyrate (HIPV) showed enhanced activation in the same glomerulus activated by the single components. However, no activation was recorded with a mixture of *cis*-3-hexenol (GLV) and linalool (HIPV), suggesting inhibition by linalool. In *C. marginiventris*, a mixture of *cis*-3-hexenol (GLV) and *cis*-3-hexenyl acetate (HIPV) exhibited intense labeling in their respective glomeruli. These results suggest that odor mixtures activate the same glomeruli as their individual components. No remarkable sexual differences were recorded for both species in glomerular projections of ORNs. Like females, host-related plant volatiles are processed in ordinary glomeruli in males rather than in the male-specific complex of macro-glomeruli.

The results of this research advance our understanding of the olfactory system and neurophysiological mechanisms of olfaction in parasitic wasps and may explain species and sexual differences in the response of *M. croceipes* and *C. marginiventris* to host-related plant volatiles.

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DEDICATION

This work is dedicated to my father the late Mr. Dilip Kumar Das

and my mother Ms. Krishna Das

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Host Related Volatiles and Parasitic Wasps

The success of parasitic wasps (parasitoids) in controlling pest populations depends on their ability to locate their hosts in a complex olfactory environment. Parasitoids are known to utilize various types of host-related volatile signals as host location cues (Dicke and Sabelis, 1988; Turlings et al. 1990; McCall et al. 1993). These host-related volatile signals can be plant-based, originate from the herbivore host or produced from an interaction between herbivores and their plant hosts (Turlings et al. 1990; McCall et al. 1993; De Moraes et al. 1998). Plant based volatile signals are green leaf volatiles (GLVs) and/or herbivore induced plant volatiles (HIPVs) (Turlings et al. 1990; McCall et al. 1993; De Moraes et al. 1998). Green leaf volatiles (GLVs) are passively emitted by plants. In cotton, GLVs include *cis*-3-hexenol, hexanal, hexenal, *trans*-2-hexenal, and *cis*-3-hexenal (Loughrin et al. 1994; McCall et al. 1994). On the other hand, herbivore induced plant volatiles (HIPVs), which are emitted as a delayed response to herbivore damage, are considered more dependable cues for foraging parasitoids if the released compounds are specific to the host species (Dicke and Vet 1999; Chen and Fadamiro, 2007). Examples of HIPVs induced by caterpillar feeding on cotton plants include *cis*-3-hexenyl butyrate, *cis*-3-hexenyl acetate, linalool,

indole, *trans*-2-hexenyl butyrate, (E, E)-α-farnesene and (E)-β-caryophyllene (Loughrin et al. 1994; McCall et al. 1994).

The olfactory system in insects includes structures that detect (i.e. sensilla and olfactory receptor neurons) and process odors (i.e. antennal lobe). Although, the olfactory system and mechanisms have been studied in a few insects including moths, *Drosophila*, honey bees, many aspects of olfactory communication in parasitic wasps remain poorly understood, despite the increasing interest in their use as biological pest control agents.

1.1.1 Olfactory System

Olfaction is an important sensory modality for insects and vertebrates, influencing their search for mates, food and host location. The framework of the olfactory system is well known in many species. Though the arrangement of the olfactory epithelia in vertebrates (upper nasal cavity) is different than in insects (in sensilla on the antennae), the primary anatomy of the two systems has striking similarity (Steinbrecht 1992, Hildebrand and Shepherd 1997). The axons of the olfactory receptor neurons (ORNs) are projected directly to the primary olfactory center of the brain, the antennal lobe (AL) in insects and the olfactory bulb (OB) in vertebrates. In these centers, the primary axon terminals synapse with the second order of neurons in the typical spherical units called glomeruli (Boeckh et al. 1990, Boeckh and Tolbert 1993), which are considered mostly invariant in quantity and location within conspecific individuals (Rospars 1988; Meisami 1991). Structurally, there are two separate olfactory systems, one for species-specific chemical signals or signature compounds (e.g. pheromones) and the other for general odorants (e.g. food odors). The

easily accessible olfactory system in insects and its similarity with the vertebrate olfactory system make insects suitable model organisms for studying the underlying mechanisms of olfaction at different levels.

1.1.2 Olfactory System in Insects

Insects have a pair of antenna which is superficially covered with diverse types of chemosensilla, primarily for olfaction and gustation. Odor detection in insects is performed by olfactory receptor neurons (ORNs) housed in olfactory sensilla, most of which are located on the antennae. Olfactory sensilla responding to odors have been categorized into different types in insects. The olfactory sensilla on the antennae of, 1) fruit fly has three major sensilla types, sensilla basiconica, sensilla trichodea, and sensilla coeloconica (Stocker, 2001); 2) mosquitoes have small and large sensilla coeloconica, sensilla ampullaceae, grooved pegs and sensilla trichodea as major types; 3) moths have sensilla trichodea, sensilla ceoloconica and sensilla basiconica (Lee and Strausfeld, 1990; Pophof, 1997); 4) honey bee has sensilla placodea (Akers and Getz, 1993, 1994), and 5) parasitic wasps has sensilla placodea (Norton and Vinson, 1974; Navasero and Elzen, 1991). Odor molecules enter through the cuticular pores of these sensilla located on the antennae and are detected by olfactory receptor neurons (ORNs). The antennal receptor neurons (RNs) are bundled into antennal nerves and projects directly to the deutocerebrum. The two-sided deutocerebrum consist of two distinct antennal lobes (ALs), and antennal mechanosensory and motor center (AMMC, also called the dorsal lobe in other species) (Strausfeld, 1976). The ORNs send their axons into the AL, whereas the AMMC receives axons from the mechanosensory

neurons (Homberg et al. 1989). The terminals of receptor neurons (RNs) ceases in discrete morphological units called glomeruli (Rospars, 1988; Boeckh and Tolbert, 1993), in the antennal lobe. The ORNs make synaptic contacts with local interneurons, which interconnect different glomeruli and with projection neurons (PNs). The projection neurons connect antennal lobe glomeruli to the higher brain center.

Olfactory system in insects has been studied mainly using moths (Lepidoptera), *Drosophila* (Diptera) and honey bees (Hymenoptera) as model systems. The peripheral structures on antennae, morphology of antennal lobe, and arborization of projection neurons responding to host-related volatiles and pheromones have been characterized in *Manduca sexta* (Reisenman et al. 2004; Rospars and Hildebrand 2000; Shields and Hildebrand 1999), heliothine species (Almaas and Mustaparta 1990; Berg et al. 2002; Hansson et al. 1995; Hillier et al. 2007) and *Spodoptera littoralis* (Ochieng et al 1995; Carlsson et al. 2002). Studies on the fruit fly, *Drosophila melanogaster* olfactory system revealed similar structures of olfactory hardware (system) and physiological mechanism (software) (Clyne et al. 1999; Laissue et al. 1999; Jefferis et al. 2002; Stocker et al. 1983; Stocker 2001). The structure of antennal lobe has also been studied in honey bees and a few species in the order hymenoptera (Masson and Strambi 1977; Flanagan and Mercer 1989; Galizia et al. 1999). However, relatively little information is available on the mechanisms of odor detection and processing in parasitic wasps.

1.2 Techniques Used to Study Olfactory System and Mechanism in Insects

Scanning electron microscopy (SEM) technique is performed to identify the major type of

olfactory sensilla. The most important techniques include single sensillum recording (SSR) technique to characterize olfactory receptor neuron (ORN) responses and confocal laser scanning microscopy (CLSM) to map the pathway of ORN projections in the antennal lobe. Single sensillum recording (SSR) is a robust neurophysiological technique to measure the extracellular activity of ORNs housed in olfactory sensilla and determines the response of ORNs to different odorants (Larsson et al. 1999**;** Shields and Hildebrand, 2001; Stensmyr et al. 2003). This technique has been used to determine differential responses from pheromone and general odor receptor neurons in moths and fruit flies (Rostelien et al. 2000; Stensmyr et al. 2003). Differences in the SSR responses have been used as a diagnostic tool to relate differences in odor detection for genetic differences between *Heliothis virescens* and *H. subflexa* Guenée (Lepidoptera: Noctuidae). Studies have also characterized responses of ORNs present in olfactory sensilla placodea to various compounds in insects like, honey bee (Aker & Getz, 1992, 1993), which responded to a range of plant volatiles, and scarab beetles to conspecific sex pheromone and plant volatiles (Leal & Mochizuki, 1993; Bengtsson et al. 2011). Recently, responses from whole antenna using electroantennogram (EAG) technique have characterized sexual differences and antennal specificity of a specialist (*Microplitis croceipes*) and generalist (*Cotesia marginiventris*) parasitoids to different host-related volatiles (Chen and Fadamiro 2007; Ngumbi et al. 2010). However, no attempts have been made to characterize the specificity of olfactory receptor neurons (ORNs) in parasitic wasps to host-related volatiles, which is one of the objectives of this study.

Confocal laser scanning microscopy (CLSM) has been used as a precise and reliable technique to reconstruct the structure of antennal lobe (AL) and to determine the ORN projections to destination glomeruli processing volatiles in mosquitoes, honey bees, ants and moths (Ghaninia

et al. 2007; Kelber et al. 2009; Varela et al. 2011). Confocal microscopy has also been utilized to reconstruct antennal lobe model in two parasitic wasps, *Cotesia glomerata* and *C. rubecula* (Smid et al. 2003). Confocal scanning microscopy is a robust technique to trace ORN projections to destination glomeruli in the antennal lobe of insects. This helps in determining specificity of glomerular units processing volatiles in the primary olfactory center in insects. Several studies have been conducted to reconstruct the morphology and glomerular organization of antennal lobes in moths (Berg et al. 2002), and honey bees (Kelber et al. 2006). In a recent study, Ghaninia et al (2007) used florescent dyes to trace ORNs responding to specific volatile compounds and utilize confocal microscopy to capture digital images to determine ORN projections in mosquito *Aedes aegypti*. The CLSM technique was utilized in this study to trace the ORN projections to glomerular units in two parasitic wasps to determine differences in odor processing that might elucidate the underlying mechanism for their host-specificity.

1.3 Model System

This study uses a model system consisting of two parasitoids, *Microplitis croceipes* and *Cotesia marginiventris*. Both parasitoids belong to the same family (Hymenoptera: Braconidae). *Microplitis croceipes* is a relatively specialist parasitoid specific to the caterpillars of corn earworm, *Heliothis* spp., while *C. marginiventris* is a generalist parasitoid of caterpillars of a wide range of lepidopteran species, including *H. zea, H. virescens, S. exigua* and several other moth species (Jalali et al. 1987; Turlings et al. 1990; Röse et al. 1998). Both parasitoids were selected as experimental models for this comparative study because they have served as models in previous

studies of parasitoid olfaction, and several aspects of their responses to host-related volatiles have been characterized (e.g., Li et al. 1992, Cortesero et al. 1997, Röse et al. 1998, Park et al. 2002, Gouinguené et al. 2005, Chen and Fadamiro 2007).

1.4 Justification of Study

Despite the intense interest in host-parasitoid interactions, certain aspects of olfactory processing in this group of insects are not well understood. A current paradigm regarding the evolution of parasitoid foraging and host location is that the degree of specificity of the signals needed by a parasitoid to successfully locate its host correlates with its level of specialization (Vet and Dicke 1992, Cortesero et al. 1997, Smid et al. 2002). It is hypothesized that specialist parasitoids utilizing relatively few numbers of hosts have a highly efficient host detection system (high olfactory sensitivity to host related cues) compared to generalist parasitoids. There are, however, very few comparative studies in the literature that have examined olfaction in a specialist and generalist parasitoid. Some studies showed that specialist parasitoids exhibit greater electrophysiological and behavioral responses than generalist parasitoids to host-related odor (Elzen et al. 1987, Vet et al. 1993, Chen and Fadamiro 2007, Ngumbi et al., 2009, 2010). In addition, sexual differences were also recorded with females of both species showing relatively greater responses than conspecific males to most of the tested host-related volatiles. In contrast, Geervliet et al (1996) found no noticeable differences in behavioral responses between the generalist parasitoid, *C. marginiventris*, and the specialist, *Cotesia rubecula* (Marshall), to host and non-host volatiles. Smid et al (2002) also reported no difference in the antennal responses of *Cotesia rubecula* (specialist) and *C. glomerata* (generalist) to a wide variety of host-related

volatiles. Together, these results suggest that diverse species of specialist or generalist parasitoids may respond differently to different types of host-related volatiles. This provides relevant justification to investigate the underlying mechanism of olfaction and odor processing in parasitoids with different degrees of host specificity.

1.5 Dissertation Outline, Goals and Objectives

The overall goal of this research is to characterize the olfactory system and physiological mechanisms of olfaction in parasitic wasps (Hymenoptera: Braconidae) using an integration of morphological, electrophysiological, and neuroanatomical techniques. Specifically, this dissertation uses a model system consisting of two parasitoid species with different degrees of host specificity, *Microplitis croceipes* (a specialist parasitoid) and *Cotesia marginiventris* (a generalist parasitoid) to investigate the neurophysiological mechanisms mediating host-specificity in parasitoids. The broad research questions are: i) Are there notable morphological differences in the olfactory hardware (antennal sensilla and antennal lobe) of both parasitoid species? ii) Are there differences in the olfactory sensitivity of both parasitoid species to host-related odors? and iii) Is there a sexual dimorphism in the olfactory hardware and olfactory neuron sensitivity of the parasitoids to host-related odors?

My dissertation comprised of an introductory chapter and four research chapters. In chapter II, studies were conducted to compare the abundance of antennal sensilla types in both sexes of *M. croceipes* and *C. marginiventris* to determine if there is a correlation between abundance of olfactory sensilla and host specificity. Five major sensilla types were recorded in both species: sensilla chaetica (non-porous), s. trichodea (non-porous), s. placodea (multiporous),

s. basiconica (two types, type 1 with terminal opening and type 2 with multiple pores on wall), and s. coeloconica (non-porous). The putative chemosensilla types, s. placodea and s. basiconica, were more abundant in *M. croceipes* (specialist) than in *C. marginiventris* (generalist), and this was true for both sexes. Comparing the sexes, s. placodea and s. trichodea were significantly more abundant in *M. croceipes* males compared with females. In contrast, s. placodea was relatively more abundant in female *C. marginiventris* than in males. In Chapter III, I characterized the responses of olfactory receptor neurons (ORNs) housed in the sensilla placodea of both parasitoid species to host-related plant volatiles. The extracellular activity showed presence of two neurons in the olfactory sensilla of these two parasitoids. In *M. croceipes*, single neuron elicited response to green leaf volatile (GLV) *cis*-3-hexenol and herbivore-induced plant volatile (HIPV) *cis*-3-hexenyl butyrate. The rest of the compounds elicited simultaneous activity in both ORNs in the sensilla. In *C. marginiventris*, *cis*-3-hexenol (GLV) and *cis*-3-hexenyl acetate (HIPV), elicited in single olfactory neuron. The other GLVs and HIPVs elicited responses in both neurons similar to *M. croceipes*. The most significant finding is the inhibitiory effect of linalool in *M. croceipes* in a mixture with *cis*-3-hexenol and hexanal; however, ORNs in *C. marginiventris* showed enhanced response. The results show that ORNs in *M. croceipes* have specific responses to compounds as compared to *C. marginiventris*. This difference in neuronal activity might suggest that, olfactory neurons in *M. croceipes* (specialist) have evolved with specific response to host-related volatiles, which might provide an olfactory code for volatiles damaged by its hosts for specific host recognition and location.

In Chapter IV, I reconstructed the antennal lobe morphology and glomerular organization of both parasitoid species. In *M. croceipes*, the medial half of the antennal lobe is larger with

greater number of glomeruli compared to the lateral half, whereas in *C. marginiventris* the lateral half is larger than the median half.The most striking sexual difference was the presence of an enlarged glomerulus (macroglomerulus or MG) at the entrance of the antennal nerve in males of both species. In addition, a complex of 3-4 macro-glomeruli (complex of macro-glomeruli or CMG) was observed in the posterior region of the antennal lobe of males of both species. The average volume of the antennal lobe is similar between the sexes but ~ 2.5 greater in *M. croceipes* compared to *C. marginiventris*. In Chapter V, I conducted studies to understand how olfactory stimuli are processed in the brains of both species by characterizing glomerular projections of olfactory receptor neurons (ORNs) responding to single odors and mixtures of host-related plant volatiles in their antennal lobes. The ORNs responding to the tested host-related plant volatiles send projections to glomeruli in the medial half of the AL in *M. croceipes*, versus the lateral half of the AL in *C. marginiventris*. In *M. croceipes*, *cis*-3-hexenol (a green leaf volatile or GLV) and *cis*-3-hexenyl butyrate (a herbivore-induced plant volatile or HIPV) each activated a single (but distinct) glomerulus, while hexanal (HIPV) activated two adjacent glomeruli in the antero-median section. In *C. marginiventris*, *cis*-3-hexenol (GLV) and *cis*-3-hexenyl acetate (HIPV) activated a single (but distinct) glomerulus, whereas hexanal (HIPV) activated one glomerulus in the ventro-lateral region and the ORN projects to another glomeruli in the same region, suggesting a connection in odor processing between these two glomeruli. In *M. croceipes*, an odor mixture (blend) of *cis*-3-hexenol (GLV) and *cis*-3-hexenyl butyrate (HIPV) showed enhanced activation in the same glomerulus activated by the single components. However, no activation was recorded with a mixture of *cis*-3-hexenol (GLV) and linalool (HIPV), suggesting inhibition by linalool. In *C. marginiventris*, a mixture of *cis*-3-hexenol (GLV) and *cis*-3-hexenyl acetate (HIPV) exhibited

intense labeling in their respective glomeruli. These results suggest that odor mixtures activate the same glomeruli as their individual components. No remarkable sexual differences were recorded for both species in glomerular projections of ORNs. Like females, host-related plant volatiles are processed in ordinary glomeruli in males rather than in the male-specific complex of macro-glomeruli.

My dissertation provides the first comparative study of olfactory apparatus and mechanisms in specialist versus generalist parasitoids. The outcomes from my dissertation will advance our understanding of the underlying mechanism of olfaction that facilitates parasitoids to forage to specific hosts.

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CHAPTER 2

ABUNDANCE OF ANTENNAL CHEMOSENSILLA IN TWO PARASITOID WASPS WITH DIFFERENT DEGREE OF HOST SPECIFICITY, *MICROPLITIS CROCEIPES* AND *COTESIA MARGINIVENTRIS* MAY EXPLAIN SEXUAL AND SPECIES DIFFERENCES IN THEIR RESPONSE TO HOST-RELATED VOLATILES

2.1 Introduction

The parasitic wasps, *Microplitis croceipes* (Cresson) and *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) are two endoparasitoids of lepidopteran larvae with different degree of host specificity. *Microplitis croceipes* is a relatively specialist parasitoid specific to larvae of *Helicoverpa* spp. and *Heliothis* spp., whereas *C. marginiventris* is a generalist parasitoid of larvae of a wide range of lepidopteran genera including *Helicoverpa* spp., *Heliothis* spp., and *Spodoptera* spp. (Turlings et al., 1990; Lewis et al., 1991; Röse et al., 1998). Like most other parasitoids, both species use host-related volatiles for foraging and host location (Drost et al., 1986; Eller et al., 1988; Turlings et al., 1990; Cortesero et al., 1997). Several studies have reported electroantennogram (EAG) and/or behavioral responses of both parasitoid species to host-related volatiles (Chen and Fadamiro, 2007; Cortesero et al., 1997; Gouinguene´ et al., 2005; Loughrin et al., 1994; Park et al., 2002; Röse et al., 1998; Roux et al., 2007; Shalit et al., 2003; Ngumbi et al., 2009, 2010).

Recent and ongoing studies by our group (e.g., Chen and Fadamiro, 2007; Ngumbi et al., 2009, 2010) have focused on the use of *M. croceipes* and *C. marginiventris* as experimental models to test a current paradigm regarding parasitoid foraging and host location strategies which predicts that specialist and generalist parasitoids will show differential response to different suites of host-related volatiles (Chen and Fadamiro, 2007; Geervliet et al., 1996; Stilmant et al., 2008; Vet et al., 1993). Specifically, our research aims to identify possible differences in the use of host-related volatiles for host location by specialist and generalist parasitoids by comparing the responses of both parasitoid species to different suites of host-related volatiles including green leaf volatiles (GLVs), herbivore-induced plant volatiles (HIPVs), and host-specific volatiles (i.e. host sex pheromones, and extracts of host larva body and frass), using a combination of chemical ecology techniques including EAG, coupled gas chromatography electroantennogram detection (GC-EAD), single sensillum electrophysiological recordings, and olfactometer bioassays. The results have revealed intriguing species and sexual differences in the responses of both parasitoids to different suites of host-related volatiles. In general, *M. croceipes* (specialist) showed relatively greater response than *C. marginiventris* (generalist) to HIPVs and host-specific volatiles, whereas the generalist showed greater response than the specialist to GLVs (Chen and Fadamiro, 2007; Ngumbi et al., 2009, 2010). In addition, sexual differences were also recorded with females of both species showing relatively greater responses than conspecific males to most of the tested host-related volatiles.

The antennae play a major role in the detection of odor by parasitic wasps (Hays and Vinson, 1971; Weselow, 1972), and both parasitoid species are known to use antennal chemoreceptors to detect host-related volatiles, as demonstrated in EAG studies (Chen and

Fadamiro, 2007; Ngumbi et al. 2009, 2010; Ngumbi and Fadamiro, unpublished data). Thus, it is likely that a comparative study of antennal morphology and abundance of chemosensilla in both sexes of *M. croceipes* and *C. marginiventris* will reveal important differences that may explain the observed differential response of both sexes and species to host-related volatiles. Although, a few studies have characterized antennal sensilla types in *M. croceipes* (Navasero and Elzen, 1991; Norton and Vinson, 1974; Ochieng et al., 2000), we are not aware of any published information on antennal structure and chemosensilla of *C. marginiventris*. Thus, a comparative study of antennal morphology and abundance of chemosensilla in both species is warranted. The aims of the present study were to 1) investigate antennal morphology of both sexes of *M. croceipes* and *C. marginiventris* using scanning electron microscopy (SEM) and silver staining techniques with the goal of characterizing putative chemosensilla types that may be involved in host location, and 2) compare abundance of putative antennal chemosensilla in both sexes of both species. We tested two key hypotheses based on the results of our electrophysiological and behavioral studies (Chen and Fadamiro, 2007; Ngumbi et al., 2009, 2010): (1) females of both species will have greater number of chemosensilla putatively involved in the detection of host-related/host-specific volatiles than conspecific males, and (2) *M. croceipes* (specialist) will have greater number of chemosensilla putatively involved in the detection of host-related/host-specific volatiles than *C. marginiventris* (generalist). It is anticipated that this study will support our ongoing research on the neural mechanisms of host location in parasitoids and provide the basis for the reported differential response of both parasitoid species to host-related volatiles.

2.2 Materials and Methods

2.2.1. Insects. The parent cultures of *M. croceipes* and *C. marginiventris* were provided by the USDA-ARS, Crop Protection and Management Research (Tifton, Georgia) and the Department of Entomology, University of Georgia (Tifton campus, Contact: John Ruberson), respectively. *Microplitis croceipes* was reared on larvae of *Heliothis virescens* Fab., whereas *C. marginiventris* was reared on larvae of *Spodoptera exigua* (Hübner). Colonies of both parasitoid species were maintained in the laboratory as previously reported (Lewis and Burton, 1970; Ngumbi et al., 2009). Adult parasitoids (2-3 days old) were first anesthetized by chilling for ~15-20 min at 4°C and then processed for electron microscopy or silver staining.

2.2.2. Scanning electron microscopy (SEM). Preparation for SEM was modified after procedures described by Sukontason et al. (2003, 2007) and Chen and Fadamiro (2008) with minor modifications. For each parasitoid species, 10 individuals per sex were decapitated and the antennae (n = 20) were carefully excised from the antennal socket under a stereomicroscope (National Microscope, Model DC 3-420, Meiji, Japan). The antennae were first pre-fixed in 2.5% glutaraldehyde mixed with phosphate buffer solution (PBS) at a pH of 7.4 at 4^{o}C for 24 h. This was followed by post-fixation in 1% osmium tetroxide overnight. Specimens were then rinsed with PBS and dehydrated in a graded ethanol series of 30, 50, 70, 80, 90, 95, and 99.9% in each case for 1 h, and then in absolute alcohol for 15-20 min. The dehydration process was followed by critical point drying (EMX 850). The specimens were then mounted to carbon coated double-sticky tapes on aluminum stubs, and sputter coated with gold (in EMX 550X auto sputter coater). Samples were examined with an EVO 50 SEM (Carl Zeiss, Jena, Germany) and micrographs were taken of

the antennae, flagellum antennomeres (flagellomeres) and sensilla types.

To visualize pores on the antennal sensilla, the procedure by Cuperus (1985) was followed with minor modifications. For each species, antennae were excised from five individuals per sex (10 antennae per sex per species) and submerged in tetrachloromethane (CCl_4) at room temperature overnight. Later, the antennae were transferred in a vial also containing CCl_4, which was brought to a boil. The boil time was 20 s, and the fluid was renewed and boiled again. This was repeated 3-4 times, after which the antennae were dehydrated in series of ethanol, rinsed in xylene, mounted to double sticky tapes on aluminum stubs, and sputter coated with gold. FE-SEM (JEOL JSM-7000F) was utilized to confirm peripheral pores on the different sensilla types. Micrographs of the flagellomeres and sensilla types were taken and the dimensions were measured. Abundance and distribution of antennal sensilla types were compared between the sexes and between species (n = 5 antennae per sex per species).

2.2.3. Silver nitrate staining technique. Silver staining was used to examine sensilla porosity by penetration of dye into porous sensilla. The procedure for silver staining described by Nayak and Singh (1983) was followed with minor modifications. Briefly, antennae from 10 individuals of each species (20 antennae per sex per species) were kept in 10% KOH solution for 48 h to soften the cuticle, and then immersed for 48 h in 70% ethanol containing 0.1% silver nitrate. The antennae were then dehydrated in ethanol series, cleared in xylene, and mounted in DPX for observation under light microscopy at 400 X.

2.2.4. Statistical Analyses. Sensilla types on the dorsal and ventral surfaces of the

antennae were identified and counted. Measurements (µm) obtained from photomicrographs of at least five antennae or sensilla were used to calculate means. Data was not normally distributed, and transformations did not adequately correct this anomaly. Thus, the data was analyzed using Wilcoxon signed-rank (nonparametric) test to establish significant sexual and species differences in the abundance of each sensilla type ($P < 0.05$; JMP® 7.0.1; SAS Institute, 2007).

2.3 Results

2.3.1. General description of antennae of *M. croceipes* and *C. marginiventris*. The antennae of *M. croceipes* and *C. marginiventris* are flagellate in shape and consist of the basic antennal segments, scape, pedicel and flagellum (Fig. 1A). The mean antennal length (mm) was different in both sexes of *M. croceipes* and *C. marginiventris*, with males of both species having longer antennae than conspecific females (Table 3).

2.3.2. Scape and pedicel. The scape is the first and shortest antennal segment and fits into the antennal socket in both species (Fig. 1A). The pedicel, the second antennal segment is short, barrel-shaped, and divided into two subsegments in both species (Fig. 1A). The cuticular surface of the pedicel in both species was densely covered with hair-like s. chaetica, which possess blunt tips.

2.3.3. Flagellum. The flagellum of both sexes of *M. croceipes* (Table 1) and *C. marginiventris* (Table 2) consists of 16 flagellomeres. The length of the flagellomeres gradually decreases from proximal to distal and ranged from 0.32 to 0.27 mm in male *M. croceipes,* 0.29 to 0.15 mm in female *M. croceipes*, 0.21 to 0.15 mm in male *C. marginiventris*, and 0.19 to 0.13 mm in female *C. marginiventris*. Furthermore, both species exhibited sexual dimorphism in the length

of flagellomeres, which were generally longer in males than in conspecific females. Four major sensilla types were recorded on the flagellum of male and female *M. croceipes* and *C. marginiventris*: sensilla trichodea, s. placodea, s. basiconica, and s. coeloconica (Fig. 1B). In this paper, sensilla types were classified according to the nomenclature used by Ochieng et al. (2000). All sensilla are oriented toward the tip of the antenna, with the exception of s. placodea and s. coeloconica. The average numbers of the different sensilla types found on each flagellomere of *M. croceipes* and *C. marginiventris* are shown in Tables 1 and 2, respectively.

2.3.4. Sensilla chaetica. Sensilla chaetica (Fig. 1C) occur only on the scape and pedicel of both species. These bristle like structures are inserted in cuticular depression. They are similar to s. trichodea but are shorter in length and have blunt tips instead of pointed tips in s. trichodea. These sensilla are distributed on the dorsal and ventral surfaces of the pedicel, and on the dorsal and latero-dorsal areas of the scape. In this paper, no efforts were made to compare abundance of s. chaetica in both sexes and species, since this sensilla type was not recorded on the flagellum and is not known to play a role in host location by parasitoids.

2.3.5. Sensilla trichodea. Sensilla trichodea are the most numerous sensilla type found on the flagellum of both sexes of *M. croceipes* and *C. marginiventris*. The base of this sensilla type is inserted into a socket, which is elevated above the cuticle. Each s. trichodea rises from a conspicuous base and gradually tapers to a pointed tip in both species. Sensilla trichodea are fairly uniformly distributed on all flagellomeres and all sides of the antennae of both species. These

sensilla occur perpendicular to the length of the flagellum (Fig. 1D). The s. trichodea of M. croceipes are longer than that of *C. marginiventris*. Under high magnification (~5,000 X), s. trichodea in *M. croceipes* and *C. marginiventris* have longitudinal grooves that spiral around its surface, and with no pores penetrating the walls of the sensilla (Fig. 1D). In addition, the sensilla did not exhibit silver staining (Figs. 2G and 2H), indicating lack of pores. In *M. croceipes*, the length of s. trichodea ranged from 12.5 to 16.8 lm in both sexes and were significantly more abundant in males compared to females (χ^2 = 6.82, df = 1, *P* = 0.009; Table 3). In *C. marginiventris*, the length of s. trichodea varied from 7.4 to 12.2 μm in both sexes. In contrast to *M. croceipes*, *s. trichodea* were significantly more abundant in female *C. marginiventris* than in males (χ^2 = 6.82, *df* = 1, *P* = 0.009; Table 3). Comparing the two species, *s. trichodea* were significantly more abundant in *M. croceipes* males compared to *C. marginiventris* males (χ^2 = 6.82, *df* = 1, P = 0.009). In contrast, *C. marginiventris* females had relatively more *s. trichodea* than *M. croceipes* females (χ^2 = 6.82, *df* = 1, *P* = 0.009).

2.3.6. Sensilla placodea. Sensilla placodea are widely distributed on all flagellomeres of *M. croceipes* and *C. marginiventris*. These sensilla are embedded on the surface of flagellomeres in both species and are slightly elevated above the antennal surface (Fig. 1E). In *M. croceipes,* s. placodea were equally distributed around the flagellar segments parallel with the longitudinal axis of the antenna, and arranged roughly in four circular rows which are interwoven mostly on the later distal segments. The sensilla were more densely arranged in males than in females. In *C. marginiventris,* s. placodea were equally distributed around the flagellomeres in two circular rows in the proximal segments and partly interwoven in the distal segments. In contrast to *M. croceipes*,

s. placodea was more densely arranged in *C. marginiventris* females compared with conspecific males. The pores on s. placodea in *M. croceipes* are mostly abundant around the central region of the sensilla (Fig. 2A), and their abundance fades toward both ends. In *C. marginiventris*, the pores are mostly found in rows distributed throughout the sensilla (Fig. 2B).

In *M. croceipes*, s. placodea varied in length from 72.3 to 161.5 μm. Lengths of s. placodea in males (92.3 to 161.5 μm) were comparatively greater than in females (72.3 to 107.7 μm). Similarly, s. placodea in *C. marginiventris* varied in length from 91.3 to 113.0 μm. Due to the wide variation in length, s. placodea in both species were distinguished into short (70 – 100 μm) and long (101 – 170 μm) subtypes. The long subtype s. placodea were relatively more abundant in *M. croceipes* males (~ 9%) compared with females (~ 2%), but relatively more abundant in *C. marginiventris* females (~ 28%) than in conspecific males (~ 21%) (Table 3). When the absolute counts of s. placodea (both subtypes) on the dorsal and ventral surfaces were compared between the sexes, s. placodea were significantly more abundant in *M. croceipes* males than females (χ^2 = 6.82, *df* = 1, *P* = 0.0090; Table 3). In contrast, s. placodea were more abundant in *C. marginiventris* females compared with males (χ^2 = 4.81, *df* = 1, *P* = 0.0283; Table 3). Comparing the two species, s. placodea were significantly more abundant in *M. croceipes* males compared to *C. marginiventris* males (χ^2 = 6.82, *df* = 1, *P* = 0.0090; Fig. 3). Similarly, s. placodea were more abundant in *M. croceipes* females compared to *C. marginiventris* females (*F* = 4.81, *df* = 1, *P* = 0.0283; Fig. 3). However, the density of s. placodea was higher in *C. marginiventris* females than in *M. croceipes* (Table 3). This higher density of s. placodea in *C. marginiventris* is possibly due to the relatively shorter flagellomere length in *C. marginiventris* (Table 3), which resulted in the more densely packing of sensilla per unit area compared to *M. croceipes*.

2.3.7. Sensilla basiconica. Sensilla basiconica (Figs. 1F & 1G) occur on all flagellomeres of both species. In both sexes of both species, s. basiconica were distinguished into two types based on ultrastructural features, s. basiconica type 1 and s. basiconica type 2. Sensilla basiconica type 1 (Fig. 1F) is differentiated by the grooved surface projecting slightly more perpendicular to the antennae, as compared to s. basiconica type 2 and s. trichodea. Under higher magnification, the tip of the sensilla exhibited terminal openings in both species (Figs. 2C & 2D). Sensilla basiconica type 1 also exhibited silver staining on its tip in both species (Figs. 2G & 2H, black arrowheads), suggesting the presence of openings on the tip. At least one s. basiconica type 1 was found at the tip of terminal flagellomere in both species. The second type, s. basiconica type 2 (Fig. 1G) is gradually curved with blunt tip. The smooth cuticular surfaces are penetrated by numerous cuticular pores which are uniformly distributed on the surface (wall) of the sensilla (Figs. 2E & 2F). Positive silver staining also confirmed presence of pores throughout the sensilla in both species (Figs. 2G & 2H, black arrowheads).

Sensilla basiconica (both types) ranged in length from 7.3 to 10.9 μm in *M. croceipes* with no noticeable difference between the sexes. Similarly, s. basiconica varied in length from 6.1 to 10.9 μm in *C. marginiventris*. The total counts of s. basiconica (both types) on the dorsal and ventral surfaces were similar in both sexes of *M. croceipes* (χ^2 = 0.53, *df* = 1, *P* = 0.4647; Table 3). Similarly, no significant sexual differences were recorded in the abundance of s. basiconica in *C. marginiventris* (*F* = 0.09, *df* = 1, *P* = 0.7540; Table 3). Comparing the two species, s. basiconica were approximately three times more abundant in *M. croceipes* males compared to *C. marginiventris* males (*F*= 6.82, *df* = 1, *P* = 0.0090; Fig. 3), and also three times more abundant in *M. croceipes* females compared to *C. marginiventris* females (*F*= 4.81, *df* = 1, *P* = 0.0283; Fig. 3).

2.3.8. Sensilla coeloconica. The least abundant sensilla type in *M. croceipes* and *C. marginiventris*, s. coeloconica occur on the dorsal and ventral surfaces of the flagellomeres (Fig. 1H). In both species, only one or two *s. coeloconica* were found per segment. They were located in depressions and surrounded by a doughnut-shaped ring (Fig. 1H). These sensilla terminate in a bulb-like tip and with no peripheral wall pores.

2.4 Discussion

The main goal of this study was to compare the abundance of antennal sensilla types in both sexes of *M. croceipes* (specialist) and *C. marginiventris* (generalist). No remarkable differences were recorded between the sexes and species in antennal structure, sensilla types and topographical arrangement, however, important sexual and species differences were recorded in the abundance of key sensilla types.

2.4.1. Sensilla types and their putative functions. Five main types of sensilla were recorded on the antennae of both sexes of both species: sensilla chaetica, s. trichodea, s. placodea, s. basiconica (two types, type 1 and type 2), and s. coeloconica. These sensilla types have also been reported on the antennae of other parasitic wasps in the same family (Braconidae) or superfamily (Ichneumonidae) (Gao et al., 2007; Isidoro et al., 1996; Navasero and Elzen, 1991; Ochieng et al., 2000; Roux et al., 2005; Van Baaren et al., 2007), although many of the studies used different nomenclature to describe the sensilla types.

Sensilla chaetica were found only on the scape and pedicel in both species. These non-

porous, bristle-like hairs are consistent in their location and number, and have been proposed as putative mechanoreceptors and possibly proprioreceptors perceiving antennal movements in *M. croceipes* (Ocheing et al., 2000) and *M. pallidipes* Szepligeti (Gao et al., 2007). In honey bee antennae, s. chaetica at the two proximal joints were shown to be phasic-tonic mechanoreceptors presumably acting as proprioreceptors perceiving antennal position (Schneider, 1964). The hair-like s. trichodea, which were widely distributed on the antennae of *M. croceipes* (Ochieng et al., 2000) and *C. marginiventris*, have also been reported in various other species of parasitic wasps (Bleeker et al., 2004; Keil, 1999; Navasero and Elzen, 1991; Olson and Andow, 1993; Onagbola and Fadamiro, 2008; Roux et al., 2005). In both species, s. trichodea have socket-like insertion into the antennal cuticle and are non-porous. In general, non-porous s. trichodea have been described in many parasitic wasp species as having putative mechanoreceptive functions (Alm and Kurczewski, 1982; Lane et al., 1988). The occurrence of the articulating membrane at the base of s. trichodea may also indicate involvement in proprioreception (Navasero and Elzen, 1991). In the present study, absence of apical or peripheral pores on s. trichodea in *M. croceipes* and *C. marginiventris* as confirmed by their inability to exhibit silver nitrate staining, suggest that s. trichodea may indeed function as mechanoreceptors or proprioreceptors in both species. Ochieng et al. (2000) also proposed a mechanoreceptive function for s. trichodea in *M. croceipes*.

The elongated s. placodea, also termed as multiporous placoid sensilla, have been reported in many parasitic wasp species (Amornsak et al. 1998; Barlin and Vinson, 1981a, b; Hallberg and Hansson, 1999; Olson and Andow, 1993; Pettersson et al. 2001; Richerson et al. 1972; Van Baaren et al. 1999; Wibel et al. 1984). Sensilla placodea are elongate-like and arranged in

alternate rings around the antennomeres (Barlin and Vinson, 1981a; Pettersson et al. 2001). The multiple wall pores on s. placodea of both species, also confirmed with silver staining, suggest an olfactory function (Barlin and Vinson, 1981b; Bleeker et al. 2004; Gao et al. 2007; Marques Silva et al. 2006; Ochieng et al. 2000; Roux et al. 2005). Ochieng et al. (2000) successfully recorded responses from single olfactory receptor neurons in s. placodea of *M. croceipes* to several host-related plant volatiles and anthropogenic compounds, confirming an olfactory function for s. placodea.

Sensilla basiconica type 1 with terminal pore and grooved surface have previously been described with different names including s. trichodea terminal pore (Bleeker et al. 2004), s. trichodea with tip pore (Roux et al. 2005), s. basiconica A (Navasero and Elzen, 1991), fluted basiconic sensilla (Norton and Vinson, 1974), and curved trichoid formation with an apical pore (Barbarossa et al. 1998), and have been considered as contact chemosensilla (Barbarossa et al. 1998). Sensilla basiconica type 2 with peripheral pores have also been called different names such as multiporous sensilla trichodea with pore (Bleeker et al. 2004; Pettersson et al. 2001; Ryan, 2002; Wibel et al. 1984), s. basiconica B (Navasero and Elzen, 1991), curved non-fluted basiconic sensilla (Norton and Vinson, 1974), and multiporous pitted sensilla trichodea C (Olson and Andow, 1993). In agreement with our silver staining results which confirmed multiple peripheral pores on s. basiconica type 2 in both species, transmission electron microscopy also revealed multiple pores on s. basiconica type 2 in *M. croceipes* (Ochieng et al. 2000). In general, s. basiconica type 2 is presumed to function as olfactory receptors in many insects (Bleeker et al. 2004; Hansson et al. 1991; Steinbrecht, 1987, 1997). Hansson et al (1991) confirmed using

electrophysiological recordings the sex pheromones receptor function of s. basiconica type 2 in *Neodiprion sertifer* Geoffroy (Hymenoptera: Diprionidae). Thus, we consider s. basiconica type 2 as putative olfactory sensilla in both species, as previously proposed for *M. croceipes* (Ochieng et al. 2000). Sensilla coeloconica were the least abundant sensilla type on both sexes of *M. croceipes* and *C. marginiventris*, and have been previously described as "pit organs" because they recessed into deep pits (Wcislo, 1995), and as s. coeloconica type I (Bleeker et al. 2004). This non-porous sensilla type is generally presumed to be associated with thermo-hygro perception, having a triad of neuron for cold, dry and moist detection (Altner et al. 1983; Bleeker et al. 2004).

2.4.2. Sexual differences in antennal length and abundance of key sensilla types. The results which showed significantly longer antennae in males of *M. croceipes* and *C. marginiventris* compared with conspecific females are in agreement with some previous reports on other parasitic wasp species (Bleeker et al. 2004; Gao et al. 2007; Ocheing et al. 2000; Onagbola and Fadamiro, 2008; Pettersson et al 2001). In contrast, Norton and Vinson (1974) reported that females of the braconid wasps, *Cardiochiles nigriceps* Viereck and *Campoletis sonorensis* (Cameron), had slightly longer antennae than conspecific males. Interestingly, the number of flagellomeres is the same in both sexes (16 flagellomeres) of *M. croceipes* and *C. marginiventris*, however the flagellomeres are generally longer in males than in females, resulting in relatively longer male antennae.

Comparing abundance of the three key sensilla types, s. trichodea, s. basiconica and s. placodea, between the sexes, no significant sexual differences were recorded in the abundance of s.

basiconica in both species. However, sexual differences were recorded in the abundance of s. trichodea and s. placodea in both species. Sensilla trichodea were significantly more abundant in *M. croceipes* males than in females. An opposite trend was recorded for *C. marginiventris* in which s. trichodea was significantly more abundant in females compared with males. We are not aware of any published studies which investigated sex-specific differences in the abundance of s. trichodea in parasitic wasps. Our results which showed sexual differences in the abundance of s. trichodea in both species are not easily explainable given the common notion that the non-porous s. trichodea are associated with mechanoreceptive functions in insects. Nevertheless, the results may suggest sexual differences in the role of mechanoreception in the behavioral ecology of both species.

Perhaps more intriguing are the sexual differences recorded in the abundance of s. placodea in *M. croceipes* and *C. marginiventris*. In *M. croceipes*, the density and total number of s. placodea per flagellomere is almost twice in males compared with females resulting in an overall greater abundance of s. placodea on male antennae than on female antennae. These results are in agreement with previous reports on *M. croceipes* by Ochieng et al. (2000), and a similar trend has been reported for several other parasitic wasp species (Baaren et al. 1999; Bleeker et al. 2004; Borden et al. 1978b; Navasero and Elzen, 1991; Roux et al. 2005). In contrast, s. placodea were more abundant and with greater density in *C. marginiventris* females compared with conspecific males. This is in contrast to a study of antennal sensilla in two related species, *C. rubecula* (Marshall) and *C. glomerata* (L.) by Bleeker et al (2004), which reported greater abundance of s. placodea in males of both species compared with conspecific females (Bleeker et al. 2004). Thus, it is unlikely that the higher abundance of s. placodea in males of *M. croceipes* compared with

females is solely a manifestation of the comparatively longer male antennae, as a similar trend would have been expected for *C. marginiventris*, which also had comparatively longer male antennae. The higher abundance of s. placodea in *C. marginiventris* females compared to males is not surprising. As a generalist, *C. marginiventris* females might have evolved the ability to detect a wide range of GLVs and plant volatiles induced by various hosts, which may explain the greater abundance of s. placodea in females, since higher numbers of sensilla may indicate greater sensitivity (Chapman, 1982). These results are also in agreement with our EAG and olfactometer studies which showed that *C. marginiventris* females were more responsive to host-related volatiles than the males (Chen and Fadamiro, 2007; Ngumbi et al. 2009, 2010). On the other hand, females of the specialist, *M. croceipes*, are relatively less responsive to GLVs but more responsive to HIPVs (Ngumbi et al. 2009, 2010).

In general, males of parasitic wasps often have higher number of olfactory sensilla than females, as males of many species are attracted to females by sex pheromones (Chapman, 1982; Field and Keller, 1993; Tagawa, 1977; Tagawa and Kitano, 1981). Although no sex pheromones have been reported for *M. croceipes* and *C. marginiventris*, higher abundance of s. placodea in males of *M. croceipes* may suggest the involvement of this sensilla type in mate location, as proposed by Ochieng et al (2000). Furthermore, s. placodea have been reported to function in the detection of sex pheromones in some other insects (Bin et al. 1989; Hansson et al. 1999; Lacher and Schneider, 1963, Larsson et al. 1999). Of particular interest is the putative role of the long s. placodea which were found mostly on the proximal and mid flagellomeres of both species. In *M. croceipes*, the long s. placodea were proportionally more abundant in males but rarely found in females. In contrast, long s. placodea were relatively and proportionately more abundant in *C.*

marginiventris females compared with conspecific males. Based on the above results we propose the following hypotheses relating to the sensitivity of the two subtypes of s. placodea in both species to different suites of odor: i) long s. placodea in *M. croceipes* males are likely to specialize in the detection of sex pheromones and thus should show greater sensitivity to sex pheromone components than to GLVs or HIPVs, and ii) short s. placodea are likely to play a greater role in the detection of host-related volatiles in both sexes of *M. croceipes* and *C. marginiventris* and thus should show greater sensitivity to GLVs and HIPVs than to sex pheromone components. Future studies on identification of sex pheromones of both species followed by single sensillum recording experiments are necessary to test the above hypotheses on the relative sensitivity of the short and long subtypes of s. placodea to different suites of odor.

2.4.3. Comparison between species. Although the same sensilla types were found in *M. croceipes* and *C. marginiventris*, important species differences were recorded in the abundance of the two putative chemosensilla, s. placodea and s. basiconica. In general, s. placodea were significantly more abundant in *M. croceipes* compared with *C. marginiventris*, and this was true for both sexes. A similar trend was also recorded for s. basiconica which were more abundant in *M. croceipes* than *C. marginiventris*. While it is tempting to link these results to the degree of host specificity of both parasitoid species, it is possible that the results may simply reflect differences in the size and phylogeny of both species. For instance, the number of s. placodea is positively correlated with body size in bees (Johnson and Howard, 1987). *Microplitis croceipes* is almost twice as large as *C. marginiventris*, however size alone may not explain our results given that

abundance of sensilla in the sexes was not always correlated with antennal length in both species. Thus, higher abundance of putative chemosensilla in the specialist, *M. croceipes* compared to the generalist may have important functional or ecological significance. In general, these results may explain the reported species and sexual differences in the responses of both parasitoids to host related volatiles (Chen and Fadamiro, 2007; Ngumbi et al. 2009, 2010). Furthermore, the results at least in part, support our hypotheses that the specialist, *M. croceipes* will have greater number of chemosensilla putatively involved in the detection of host-related/host-specific volatiles than the generalist, *C. marginiventris*, and that females of both species will have greater number of chemosensilla putatively involved in the detection of host-related/host-specific volatiles than conspecific males. Functional electrophysiological studies are necessary to confirm the function of the putative chemosensilla identified in this study and compare their sensitivity to different suites of odor.

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Table 1. Distribution of key sensilla types on the flagellum of male and female *M. croceipes*

Sensilla type	Sex	Flagellomere															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
S. placodea	Male	78±	82±	80±	80±	77±	82±	75±	78±	74±	75±	72±	73±	68±	61±	55±	49±
		1.1	0.9	1.3	1.4	1.4	2.0	2.1	1.3	1.0	1.5	0.9	1.4	0.9	2.1	1.3	2.0
	Female	39±	43±	49±	48±	48±	52±	48±	51±	46±	48±	44±	45±	38±	36±	34±	32±
		0.9	0.5	1.0	1.0	0.9	0.9	0.5	0.8	1.1	1.1	0.5	0.4	0.5	0.5	0.2	0.3
S. basiconica	Male	45±	50±	50±	58±	63±	75±	70±	81±	81±	75±	83±	71±	73±	71±	55±	72±
(types 1 & 2)		1.9	2.5	3.4	4.1	3.5	2.3	2.9	2.2	3.0	1.5	0.8	2.6	2.4	2.2	2.1	5.7
	Female	38±	38±	42±	54±	57±	72±	78±	82±	81±	85±	91±	98±	88±	89±	89±	84±
		1.6	1.9	0.8	0.7	2.3	1.8	3.6	3.3	3.0	4.2	2.6	2.6	1.8	1.3	1.8	2.7
S. trichodea	Male	530±	527±	545±	524±	521±	532±	532±	538±	532±	499±	464±	474±	463±	423±	425±	449±
		7.3	13.3	13.6	12.7	8.2	17.5	13.4	19.6	17.8	17.9	20.4	16.2	15.9	17.1	17.6	17.9
	Female	436±	366±	387±	362±	341±	330±	322±	313±	284±	291±	269±	257±	264±	250±	243±	335±
		8.9	3.1	3.9	9.5	4.4	5.6	5.2	4.5	5.2	4.6	3.7	4.4	4.6	4.6	3.4	8.6

*Values are average numbers (n = 5) of the different types of sensilla on each flagellomere**.**

Table 2. Distribution of key sensilla types found on the flagellum of male and female *C. marginiventris*

Sensilla type	Sex	Flagellomere															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
S. placodea	Male	21±	24±	23±	26±	27±	25±	25±	25±	25±	25±	25±	24±	21±	20±	17±	17±
		1.2	0.7	1.1	0.9	1.4	1.3	1.3	1.3	1.3	1.0	1.0	1.0	0.6	0.8	0.7	1.1
	Female	39±	40±	40±	38±	39±	39±	38±	38±	36±	36±	33±	34±	33±	31±	27±	24±
		1.4	0.8	1.1	1.3	1.1	1.1	0.8	1.3	1.3	1.2	1.1	1.0	1.2	1.1	1.1	1.2
S. basiconica (types 1 & 2)	Male	20±	19±	21±	19±	21±	22±	22±	25±	27±	27±	29±	31±	35±	33±	29±	29±
		1.0	0.8	1.5	0.8	0.9	1.2	1.1	1.5	1.1	1.6	1.9	1.1	0.9	1.0	1.5	2.1
	Female	25±	25±	20±	22±	21±	22±	24±	26±	26±	26±	29±	29±	30±	30±	28±	33±
		2.1	2.0	1.9	1.3	2.4	1.2	1.1	2.0	2.1	1.7	2.8	2.7	2.6	2.3	3.0	2.1
S. trichodea	Male	278±	289±	302±	298±	287±	289±	279±	289±	282±	284±	262±	258±	243±	233±	223±	240±
		5.0	3.9	6.3	4.5	2.8	4.3	6.0	2.3	4.9	5.6	4.7	4.5	4.0	5.1	2.4	4.3
	Female	342±	391±	395±	394±	374±	366±	354±	366±	341±	337±	320±	321±	310±	307±	272±	293±
		3.1	8.2	6.8	9.7	8.2	2.4	4.0	6.2	4.0	2.6	6.3	4.5	1.3	3.5	3.6	4.8

*Values are average numbers (n = 5) of the different types of sensilla on each flagellomere

Table 3. Morphometric data of the antenna and key sensilla types in both sexes of *M. croceipes* and *C. marginiventris*.

	M. croceipes		*C. marginiventris*	
	Male	Female	Male	Female
Mean length of antenna (mm ± SE)	4.9 ± 0.04	3.4 ± 0.06	3.06 ± 0.01	2.71 ± 0.12
Length of flagellomeres (mm)	0.27 - 0.32	0.15 - 0.29	0.15 - 0.21	0.13 - 0.19
Length of s. trichodea (µm)	12.5 - 16.8	12.5 - 16.8	7.7 - 12.2	7.4 - 12.1
Length of s. basiconica (µm)	7.6 - 10.9	7.3 - 10.7	6.5 - 10.9	6.1 - 10.9
Length of s. placodea (µm)	92.3 - 161.5	72.3 - 107.7	91.3 - 113.0	94.6 -106.6
Total number of s. trichodea[a]	7978 ± 532	5050 ± 149	4336 ± 121	5612 ± 107
Total number of s. basiconica (1 and 2)[a]	1073 ± 73	1165 ± 149	410 ± 36	416 ± 24
No. of short s. placodea (70-100 µm)	1053	690	294	408
No. of long s. placodea (101-70 µm)	107	11	77	156
Total number of s. placodea[a]	1160 ± 36	701 ± 12	371 ± 35	564 ± 39
Density of s. placodea[b]	5.3	3.7	4.6	6.1

Values are average numbers (n = 5) of the different types of sensilla on each flagellomere.

[a]Number of sensilla. Data are presented as mean ± SE; (n = 5).

[b]Density of s. placodea estimated by counting number per 5×10^3 μm^2 area of the second flagellomere.

Figure Legend

Figure 1. SEM micrograph of adult *M. croceipes* showing the antenna and sensilla types: **(A)** male (A1) and female (A2) antenna; **(B)** middle part of a flagellomere showing three types of sensilla; **(C)** s. chaetica; **(D)** s. trichodea; **(E)** s. placodea; **(F)** s. basiconica type 1; **(G)** s. basiconica type 2; and **(H)** s. coeloconica. Sc, scape; Pd, pedicel; FL, flagellum; TR, s. trichodea; PL, s. placodea; BS, s. basiconica. Note: similar sensilla types were recorded on *C. marginiventris*.

Figure 2. High-resolution SEM/silver staining micrographs of different sensilla types identified on the flagellum of *M. croceipes* and *C. marginiventris*. **(A)** s. placodea of *M. croceipes* showing scattered distribution of multiple pores (white arrows); **(B)** s. placodea of *C. marginiventris* showing pore distribution in rows (white arrows); **(C)** s. basiconica type 1 of *M. croceipes* showing terminal opening with finger-like projections at the tip (arrow); **(D)** s. basiconica type 1 of *C. marginiventris* with finger-like projections; **(E)** s. basiconica type 2 of *M. croceipes* showing multiple pores on wall (arrows); **(F)** s. basiconica type 2 of *C. marginiventris* showing multiple pores on wall (arrows); **(G)** *M. croceipes* flagellomere showing silver stained s. basiconica (1 & 2) (black arrow heads) and s. placodea (white arrows); **(H)** *C. marginiventris* flagellomere showing silver stained *s.* basiconica (1 & 2) (black arrow heads) and s. placodea (white arrows).

Figure 3.Abundance of antennal chemosensilla types in both sexes of *M. croceipes* and *C. marginiventris.*

Fig. 1

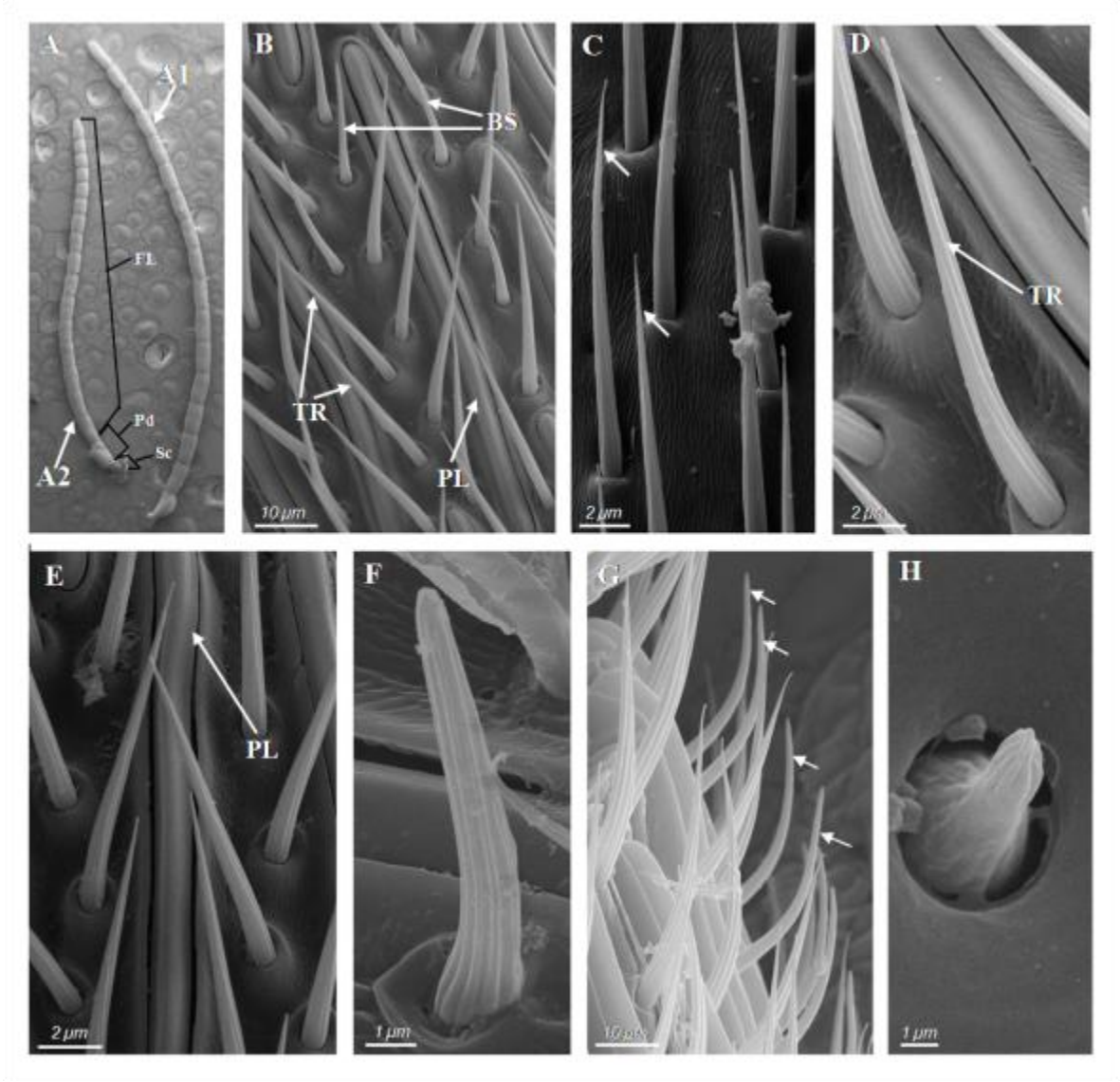


Fig. 2

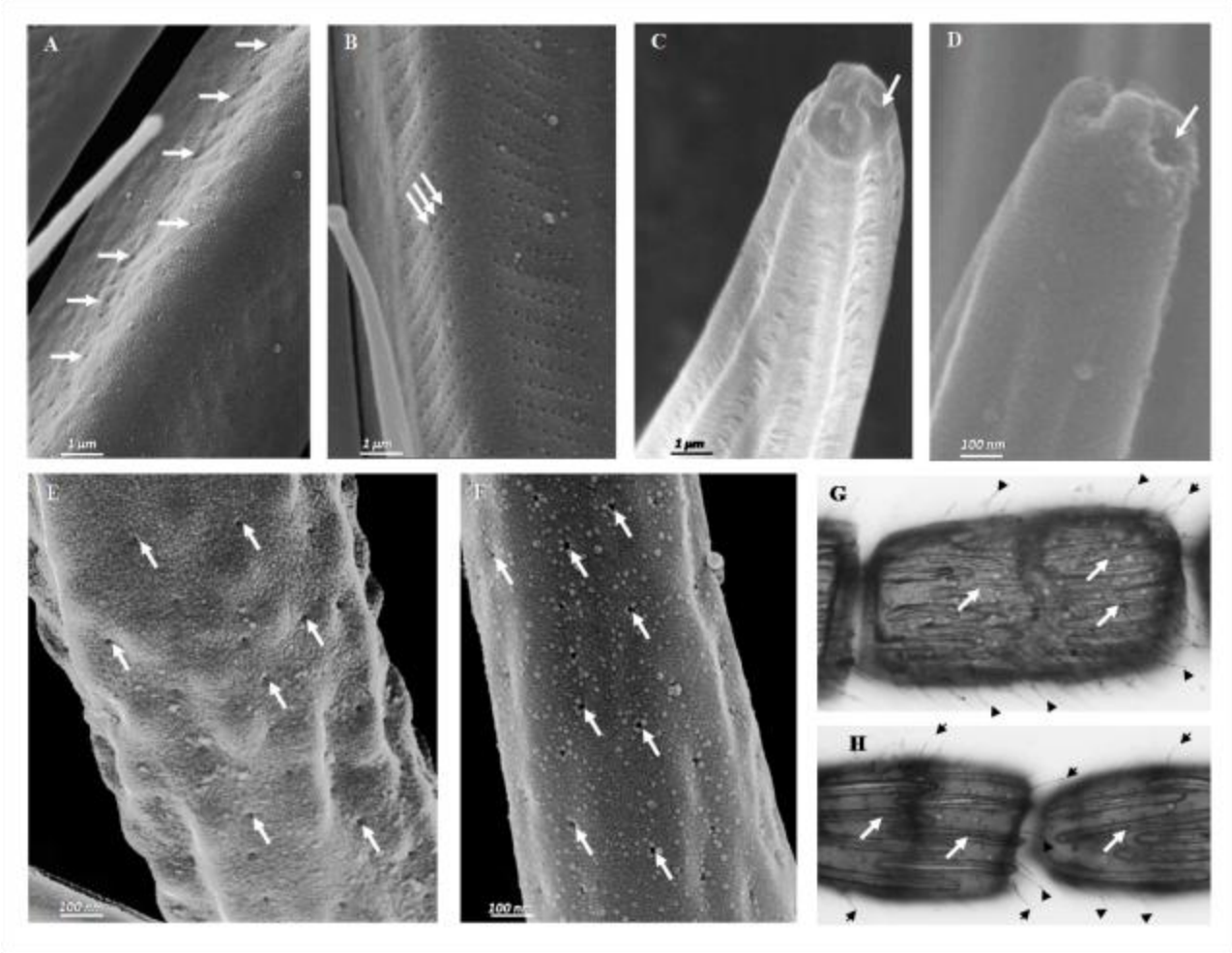
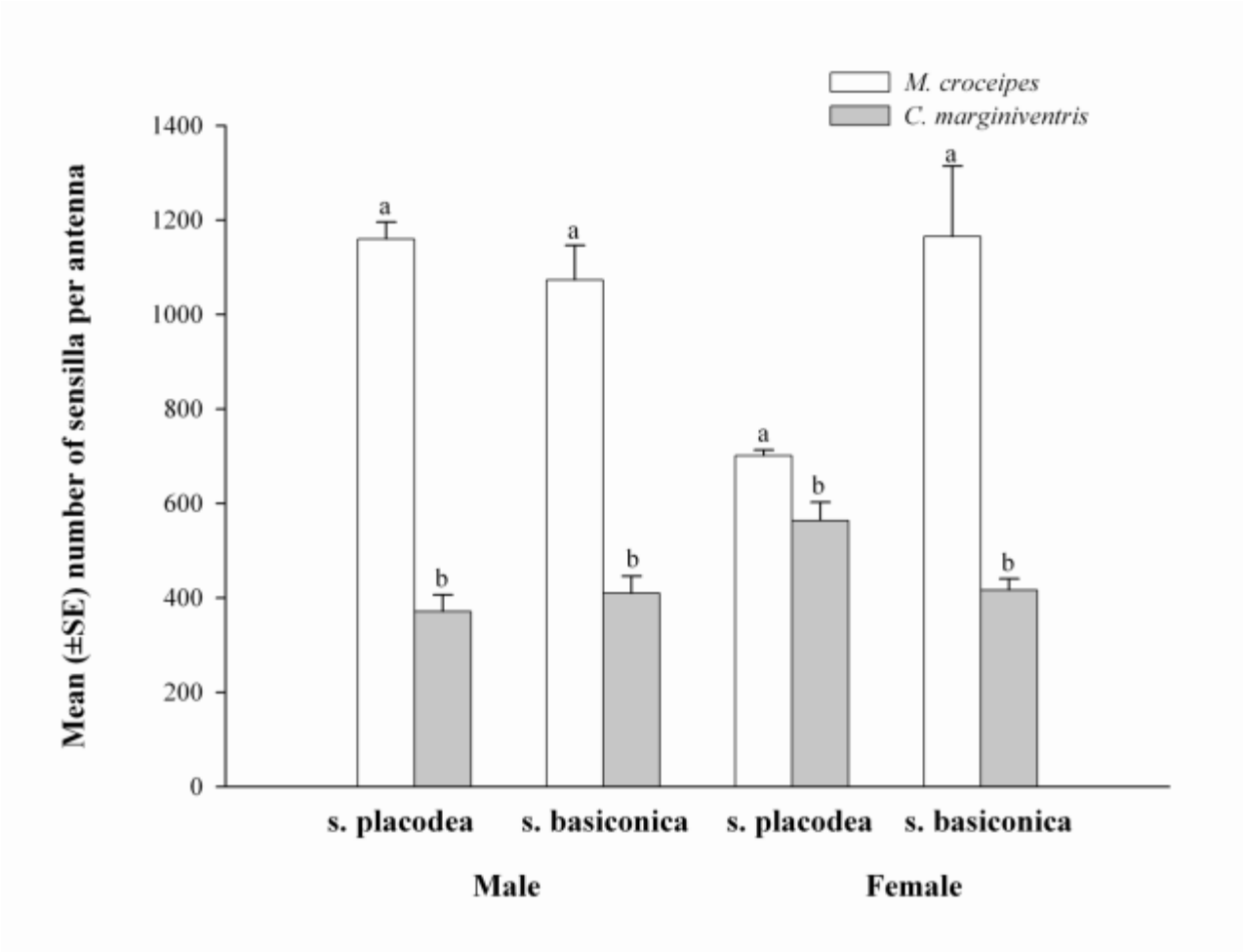


Fig. 3


M. croceipes
C. marginiventris
Mean (±SE) number of sensilla per antenna
1400
1200
1000
800
600
400
200
0
a
b
a
b
a
b
a
b
s. placodea
s. basiconica
s. placodea
s. basiconica
Male
Female

CHAPTER 3

SINGLE SENSILLUM OLFACTORY RESPONSES OF A SPECIALIST (*MICROPLITIS CROCEIPES*) AND A GENERALIST (*COTESIA MARGINIVENTRIS*) PARASITOID TO HOST-RELATED VOLATILES

3.1 Introduction

Long range detection of odor signals offers vital cues for an insect to forage and locate suitable hosts, habitat, oviposition site and mate. Olfaction is an important sensory modality and at the peripheral level olfactory receptor neurons (ORNs) detect odors released by oviposition sites/hosts, conspecifics and food sources. The ability of ORNs to detect odors, housed in olfactory sensilla, is characterized based on the electrophysiological activity obtained in response to diverse odor molecules. ORN responses to different odor types have been explored in many insect species like, moths, honey bees, mosquitoes, fruit fly and locusts (Mustaparta et al. 1980; Aker & Getz, 1992, 1993; Hansson et al.1999; Stensmyr et al. 2003; Qiu et al. 2006; Raman et al. 2010). In insects, ORNs that are tuned to a broad range of odors are "generalist" compared to "specialists" which detects a narrow range of odors (Mustaparta et al. 1980; Wojtasek et al. 1998; Larsson et al. 1999; Qiu et al. 2006; Bengtsson et al. 2011).

Parasitic wasps (parasitoids) are known to deploy different types of host-related odor signals for host location (Dicke and Sabelis, 1988; Turlings et al. 1998). Like other insects, parasitoids have major olfactory sensilla (sensilla placodea) innervated by olfactory receptor

neurons (ORNs) (Ochieng et al. 2000; Das et al. 2011) that detect host related odors. *Microplitis croceipes* (Cresson) is a relatively specialist parasitoid specific to larvae of *Helicoverpa* spp. and *Heliothis* spp., whereas *Cotesia marginiventris* (Cresson) is a generalist parasitoid which has larval hosts for a wide range of lepidopteran including *Spodoptera* spp. and *Heliothis* spp. (Turlings et al. 1990; Lewis et al. 1991; Röse et al. 1998). Studies have shown behavioral and antennal responses in specialist and generalist parasitoids to different host-related volatiles (Elzen et al. 1987; Vet et al. 1993; Cortesero et al. 1997; De Moraes & Lewis, 1999). Despite growing interests in parasitoid host-specificity, no published literature has characterized the ORN responses from olfactory sensilla that might justify their host-specificity. Research has used *M. croceipes* (a specialist) and *C. marginiventris* (a generalist) as model insects (e.g., Chen and Fadamiro, 2007; Ngumbi et al. 2009, 2010) to test an existing paradigm of parasitoids' host finding strategies which predict that, specialist and generalist parasitoids will have differences in their degrees of responses to diverse group of host-related volatiles (Vet et al. 1993; Geervliet et al. 1996; Chen and Fadamiro, 2007; Stilmant et al. 2008). The results of recent studies have shown that, antennal responses of generalist parasitoid (*C. marginiventris*) are relatively greater to plant volatiles than specialist (*M. croceipes*), whereas the specialist showed greater response to host specific cues than the generalist (Chen and Fadamiro, 2007; Ngumbi et al. 2010).

I hypothesizd that ORNs in *C. marginiventris* (a generalist) would allow detection of a broad range of compounds that might facilitate the ability to recognize and locate wide range of larval hosts. With this information, it is important to investigate the responses from ORNs in a specialist and generalist parasitoid which might shed light on the underlying differences in host specificity at the peripheral level. Here, I investigated the above prediction by comparing

extracellular responses of ORNs to green leaf volatiles (GLVs), herbivore-induced plant volatile (HIPVs), host sex pheromone, and ecologically irrelevant compound using single sensillum recording technique (SSR). The aim of this study was to compare ORN responses in *M. croceipes* and *C. marginiventris* with a goal to characterize the ability to detect volatiles by different ORN types housed in the olfactory sensilla placodea.

3.2 Materials and Methods

3.2.1 Insects. The parent cultures of *M. croceipes* and *C. marginiventris* were provided by the USDA-ARS, Crop Protection and Management Research (Tifton, Georgia) and the Department of Entomology, University of Georgia (Tifton campus, Contact: John Ruberson), respectively. *Microplitis croceipes* was reared on the larvae of *Heliothis virescens* Fab, and *C. marginiventris* was reared on *Spodoptera exigua* (Hübner). Colonies of both parasitoid species were maintained in the laboratory as previously reported (Lewis and Burton, 1970; Das et al. 2011). Caterpillars of *H. virescens* and *S. exigua* were reared on laboratory prepared pinto bean diet (Shorey and Hale, 1965) and were maintained at 25 ± 1 °C, 75 ± 5% r.h. and 14:10 L:D photoperiod. Newly emerged adults were collected and both sexes were kept together in cages for mating. Ten percent sugar solution and water soaked in cotton balls were provided in small transparent cups for food. For moth species, sugar solution and water was provided to the newly emerged female and male moths in 25ml conical flasks with distilled water and placing a cotton absorbal wick (Wheat Ridge, CO, USA) at the center. To avoid moths being trapped inside the flask, the mouth was sealed with Parafilm. The parent cultures of *M. croceipes* and *C. marginiventris* were provided by the USDA-ARS, Crop Protection and Management Research

(Tifton, Georgia) and the Department of Entomology, University of Georgia (Tifton campus, Contact: John Ruberson), respectively. *Microplitis croceipes* was reared on the larvae of *Heliothis virescens* Fab, and *C. marginiventris* was reared on *Spodoptera exigua* (Hübner). Colonies of both parasitoid species were maintained in the laboratory as previously reported (Lewis and Burton, 1970; Das et al. 2011). Caterpillars of *H. virescens* and *S. exigua* were reared on laboratory prepared pinto bean diet (Shorey and Hale, 1965) and were maintained at 25 ± 1 °C, 75 ± 5% r.h. and 14:10 L:D photoperiod. Newly emerged adults were collected and both sexes were kept together in cages for mating. Ten percent sugar solution and water soaked in cotton balls were provided in small transparent cups for food. For moth species, sugar solution and water were provided to the newly emerged female and male moths in 25ml conical flasks with distilled water and a cotton absorbal wick (Wheat Ridge, CO, USA) placed at the center. To avoid moth being trapped inside the flask, the mouth was sealed with Parafilm.

3.2.2 Synthetic test odor stimuli. Seven synthetic volatile compounds were tested in this study: *cis*-3-hexenol, hexanal, *cis*-3-hexenyl butyrate, *cis*-3-hexenyl acetate, linalool, benzaldehyde and Z11-16Ald. The aim of choosing these specific compounds were to compare responses from ORNs housed in sensilla placodea in these two model parasitoids (Das et al. 2011). The first two compounds, *cis*-3-hexenol and hexanal, are components of green leaf volatiles (GLVs) emitted passively by plants, like cotton, which are potential host for herbivores attacked by these two parasitoids. While the other three compounds, *cis*-3-hexenyl butyrate, *cis*-3-hexenyl acetate and linalool, are herbivore-induced plant volatiles (HIPVs) (Dicke, 1994; McCall et al. 1994; DeMoraes et al. 1998; Hoballah et al. 2002, Ngumbi et al. 2009) emitted by plants after an insect

attack. All selected compounds have been shown to elicit behavioral and antennal responses in both parasitoids (Park et al. 2001; Chen and Fadamiro 2007; Ngumbi et al. 2009, 2010). One ecologically irrelevant compound, benzaldehyde, was tested in this study. Ecologically irrelevant compounds are classified in this category because they are not produced passively by plant hosts or after insect damage which would benefit the parasitoids for host location. Of all ecologically irrelevant compounds tested recently (Ngumbi et al. 2010), benzaldehyde elicited comparatively greater antennal response in these two parasitoids. Therefore, benzaldehyde was chosen to determine the versatality of ORNs in these two parasitoids. All synthetic test compounds were purchased from Sigma® Chemical Co. (St Louis, MO) with purity >97% as indicated on the labels. One major sex-pheromone component, Z11-16Ald, was tested to determine if any specific neuron type responded to host signature compound in these parasitoids, as most studies have focused on the response of male moths to female sex-pheromones. The versatility of antennal response to host pheromone components had been shown in these two species (Ngumbi et al. 2010). The pheromone component of *H. virescens* was purchased from Bedoukian Research (Dantbury, Ct) with 98% purity.

The plant volatiles appear as blends/mixtures to parasitoid antenna rather than single compounds in the real world. With this assumption, mixtures of selected compounds were utilized to test ORN responses after obtaining responses to individual plant volatiles in these two parasitoids. The compounds for blends were selected between GLVs and HIPVs which had comparatively greater response in both parasitoids. Dual mixtures of GLV and HIPV, *cis*-3-hexenol & *cis*-3-hexenyl butyrate for *M. croceipes* and *cis*-3-hexenol & *cis*-3-hexenyl acetate for *C. marginiventris* were tested. Linalool is an herbivore-induced plant volatile (HIPV) and known

to elicit excitatory or inhibitory responses in different insects. To determine the effect on olfactory neuron activity, a mixture of linalool with GLVs (*cis*-3-hexenol or hexanal) and HIPVs (*cis*-3-hexenyl butyrate & *cis*-3-hexenyl acetate) was tested on *M. croceipes* and *C. marginiventris*. Also, a mixture of three compounds, GLVs and HIPVs, with linalool as a common component in the mixture was chosen to test the ORN response. The trio compounds tested to characterize ORN responses were *cis*-3-hexenol, linalool and *cis*-3-hexenyl butyrate for *M. croceipes,* and *cis*-3-hexenol, linalool and *cis*-3-hexenyl acetate for *C. marginiventris* to characterize ORN responses. Each compound was diluted in hexane (HPLC grade) to give 100 µg/µl solutions. Further dilutions were made to 0.1 µg/µl solution. Low dilutions of 0.05 µg/µl were made for dose corrections with mixtures. The solutions were kept in a freezer at < – 20 °C until used.

3.2.3 Single sensillum recordings. We used a standard protocol demonstrated by Stensmyr et al (2003) with modification for insect preparation. The parasitoid body was immobilized by dental wax fixed on a stage. The head was immobilized with dental wax leaving the eye to access for placing the reference electrode, and the antenna was fixed with a little elevation in order to access for sensilla. Two tungsten microelectrode were electrolytically sharpened (to 1µm tip diameter) by repeated immersion in potassium nitrite solution. The tip of the recording electrode was gently inserted into the base of a sensilla placodea until successful electrical activity from the ORNs was obtained. The reference electrode was placed in the eye of the parasitoid. The recording set up was viewed at 400x magnification under an upright Leica stereomicroscope (Z6 APO). The recorded extracellular action potentials obtained were amplified by a high-impedance preamplifier, fed into an IDAC4-USB, carried to a PC and analyzed using Autospike v. 3.9 (Syntech, Germany) software. The recorded ORN activity from sensilla placodea

was obtained from 3-5 days old parasitoids.

A continuous humidified air stream (0.5m/ s) delivery was passed through a glass tube over the insect preparation. A 10µl aliquot of each solution of odor stimulus was applied to a piece of filter paper and placed inside a Pasteur pipette for odor delivery. The control stimulus was a similar pipette containing a filter paper with 10 µl aliquot hexane. The tip of the pipette was placed into a small hole in the glass tube (with humidified air) oriented towards the antennal preparation. Using a stimulus controller (Syntech, Germany), a 0.5-s air puff was passed into the stimulus pipette into the humidified continuous air flow to apply odors on the antenna. At least 2 min was allowed between successive stimulation for olfactory neurons to recover. The compounds were randomly applied starting with low doses (0.1µg/µl) and ending with high dose (100µg/µl) to avoid neuron adaptation at high doses. One hundred microgram of hexanal was presented to an antenna at the beginning and end of a recording to confirm receptor neuron activity. For data analysis, spike counts (spikes/s) to solvent control and spontaneous activity were deducted from the number of spikes recorded when stimulated by compounds.

3.2.4 Statistical analysis. For analysis of net response (spikes) to compounds, the number of spikes/second in the onset of response to compounds was deducted from the total number of spikes in the spontaneous activity and to the solvent control. Data were not normally distributed and thus transformed by using the square-root ($\sqrt{x} + 0.5$) transformation method for analysis. Net SSR responses (spikes) to each odor stimuli were compared between species and between sexes of each species using Student's *t*-test ($P < 0.05$; JMP Version 7.01, SAS Institute, 2007).

3.3 Results

3.3.1 Single unit recording. The short sensilla placodea are the most abundant olfactory sensilla type located on the antenna of *M. croceipes* and *C. marginiventris* (Das et al. 2011). Hence, they were the focus of this study. The spontaneous activity of ORNs recorded extracellularly from sensilla placodea showed two distinct neurons firing in these parasitoids (Figs. 1a & 2a). The different ORN types were interpreted by spike amplitude, waveform and polarity for affirmation. In *M. croceipes*, the ORN with larger amplitude was termed A, and those with smaller amplitude was termed B. Similarly, the larger amplitude ORN in *C. marginiventris* is C, and the smaller neuron as D. Here onwards, the ORN types in both species will be addressed as larger (neuron A & C) and smaller (neuron B & D) neuron in this chapter. A phasic-tonic activity was observed in the neurons after stimulation by compounds, and the activity generally began 150-250 milliseconds after the onset of stimulation. The neurons exhibited wide response spectrum to individual host-related compounds and mixtures. The ORNs housed in sensilla placodea of both species elicited simultaneous stimulation by most compounds, both as single compounds (GLVs & HIPVs) and mixtures, with few ligands (compounds) eliciting response in only single neuron in these two species. However, linalool (HIPV) inhibited neuronal activity in *M. croceipes* when presented as mixtures with green leaf volatiles (GLVs) (Figs. 3b & 3d). A few green leaf volatile (*cis*-3-hexenol) and herbivore induced plant volatiles (*cis*-3-hexenyl butyrate & *cis*-3-hexenyl acetate) elicited response in single neuron in *M. croceipes* (large amplitude neuron A), and *C. marginiventris* (large amplitude neuron C), although, both neurons in parasitoid species elicited simultaneous response to the rest of the compounds, including a major component of host sex pheromone and ecologically irrelevant compound (Figs. 1 & 2).

Single sensillum recordings were obtained from 784 sensilla, out of which neurons from 367 sensilla responded to the compounds tested. Therefore, more than 50 percent of the ORNs in the sensilla attempted for recording detected these compounds. Green leaf volatile (GLV) *cis*-3-hexenol and herbivore induced plant volatile (HIPV) *cis*-3-hexenyl butyrate elicited response in larger amplitude neuron in *M. croceipes* (Figs. 1b & d). In *C. marginiventris*, neuron with larger amplitude was stimulated by *cis*-3-hexenol (GLV) and *cis*-3-hexenyl acetate (Figs. 2b & d). However, *cis*-3-hexenyl acetate elicited simultaneous activity in both ORNs in *M. croceipes*, and *cis*-3-hexenyl butyrate in *C. marginiventris*. The ORNs in these two species elicited simultaneous activity to rest of the compounds like, specifically hexanal (GLV) and linalool (HIPV), host sex pheromone (Z11-16 Ald), and benzaldehyde (ecologically irrelevant compound) (Figs. 1 & 2). To compare the physiological activity of olfactory neurons to different volatile groups released by plants before and after herbivore attack, mixtures of selected GLVs and HIPVs are presented to observe ORN activities. Olfactory neurons in both species when stimulated with mixtures showed excitation and enhanced responses to most compounds (Figs. 3 & 4). When stimulated with mixtures of two GLVs, *cis*-3-hexenol and hexanal, ORNs in both parasitoids showed enhanced activity. When a mixture of two HIPVs, linalool and *cis*-3-hexenyl butyrate, was presented, the olfactory neurons in *M. croceipes* showed enhanced and highest prolonged activation (59 ± 6 seconds) (Fig. 3c) compared to other mixtures attempted in this study. Similarly in *C. marginiventris*, HIPVs linalool and *cis*-3-hexenyl acetate showed excitation in the ORNs (Figure 4b).

Interestingly, the ORN activity in *M. croceipes* was inhibited when GLVs, *cis*-3-hexenol and hexanal, were presented with linalool as dual mixtures (Figs. 3b & d). It was observed that

linalool suppressed the ORN activity responding to the two GLVs in *M. croceipes*. To confirm the inhibitory effect of linalool on GLVs, a mixture of α- pinene (another GLV) and linalool was presented. However, linalool did not appear to reduce the ORNs activity elicited by α- pinene in a mixture (data not shown), compared to responses to dual mixtures of linalool with *cis*-3-hexenol and hexanal (Fig. 3). Linalool isconsidered an induced plant volatile and showed inhibition when mixed with selected GLVs (*cis*-3-hexenol & hexanal) in *M. croceipes*. To authenticate the responses obtained at 0.1 µg dose of each compound for a mixture, corrections with lower doses (0.05 µg) of each compound were presented to confirm the effect of linalool (data not shown). Similar results were obtained with linalool inhibiting the activity of ORNs responding to *cis*-3-hexenol and hexanal in this study. When linalool was presented in combination with induced plant volatiles, *cis*-3-hexenyl butyrate and *cis*-3-hexenyl acetate, the ORNs elicited extended response both in *M. croceipes* and *C. marginiventris*. Linalool showed no inhibitory effect on the ORN activity in generalist *C. marginiventris* in a mixture with green leaf volatile (*cis*-3-hexenol) (Figure 4a) or herbivore induced plant volatiles (*cis*-3-hexenyl acetate) (Figure 4b). The selected three compounds, which elicited maximum response as single compound was presented as a mixture of three to record ORN activity to different compound groups (GLVs & HIPVs) in both species. In *M. croceipes*, mixture of *cis*-3-hexenol, linalool and *cis*-3-hexenyl butyrate showed less enhanced activity (17 ± 4 seconds) (Fig. 3e), whereas *C. marginiventris* showed an enhanced activity to the mixture of *cis*-3-hexenyl acetate, linalool and *cis*-3-hexenol (25 ± 9 seconds) (Figure 4c).

3.3.2 ORN responses between parasitoid species. Table 1 shows the results of Student's *t*-test comparison of SSR responses between *M. croceipes* and *C. marginiventris* to seven

compounds tested. Female in *C. marginiventris* elicited greater responses to the two GLVs, *cis*-3-hexenol and hexanal at low dose (0.1 µg) (Figure 5a). In contrast, *M. croceipes* showed significantly greater responses at high doses to *cis*-3-hexenol (100 µg: $t = 95.73$, df = 1, $P = 0.0006$; Fig. 5a) compared to *C. marginiventris*. Similarly, hexanal also elicited greater response in *M. croceipes* female at high dose (100 µg). *Cis*-3-hexenyl butyrate at high dose elicited significantly greater response in *M. croceipes* (100 µg: $t = 40.35$, df = 1, $P = 0.0031$; Fig. 5a) female compared to *C. marginiventris*. Linalool (HIPV) elicited greater response in *C. marginiventris* females at low dose (0.1 µg) (Fig. 5a). In male, hexanal elicited significantly greater SSR response in *M. croceipes* (100 µg: $t = 76.66$, df = 1, $P = 0.0009$; Fig. 5b) at high dose (100 µg). In contrast, male in *C. marginiventris* elicited significantly greater response to linalool at high dose (100 µg: $t = 241.78$, df = 1, $P = 0.0001$; Fig. 5b). Similarly, *C. marginiventris* showed greater response to *cis*-3-hexenyl butyrate than *M. croceipes* at high dose (100 µg). In response to host sex pheromone (Z11-16Ald), both parasitoids showed significantly different responses at both doses (0.1 & 100 µg). *Microplitis croceipes* male showed significantly greater response to Z11-16Ald at low dose (0.1µg; $t = 128.79$, df = 1, $P = 0.0003$; Fig. 5b). In contrast, at high dose *C. marginiventris* (100µg; $t = 198.9$, df = 1, $P = 0.0001$; Fig. 5b) showed significantly greater response to host sex pheromone component.

3.3.3. Sexual comparison of SSR responses. The results of Student's *t*-test comparison of SSR responses between sexes in both parasitoids are represented in table 2. In *M. croceipes*, female showed greater SSR responses to GLV *cis*-3- hexenol and HIPV *cis*-3-hexenyl butyrate athigh dose (100 µg) (Figure 6a). In contrast, male in *M. croceipes* elicited significantly greater response to host sex pheromone (Z11-16Ald) (0.1µg; $t = 129.59$, df = 1, $P = 0.0003$; Fig. 6a) at

low dose (0.1 µg). In *C. marginiventris,* female showed greater responses to two GLVS, *cis*-3-hexenol and hexanal, at low dose (0.1µg) (Fig. 6b). *Cis*-3-hexenyl butyrate (HIPV) also elicited greater response in females at low dose (0.1µg) compared to male (Figure 6b); however, male showed significantly greater response at high dose (100µg; $t = 49.01$, df = 1, $P = 0.0022$; Fig. 6b). Similarly, male elicited significantly greater response to linalool at high dose (100µg; $t = 181.86$, df = 1, $P = 0.0002$; Fig. 6b). In *C. marginivetris,* female elicited greater ORN responses to benzaldehyde at low dose (0.1 µg), whereas, male showed greater response at high dose (100µg) (Figure 6b). At low dose (0.1 µg), host sex pheromone Z11-16 Ald elicited significantly greater response in female (1µg; $t = 185.70$, df = 1, $P = 0.0002$; Fig. 6b), whereas, male responded greater at high dose (100µg) (Fig. 6b).

3.4 Discussion

The results in this study revealed intriguing differences in the response spectrum of ORNs stimulated by green leaf volatiles (GLVs), herbivore induced plant volatiles (HIPVs) and mixtures in a specialist and generalist parasitoid. Similar to other insects reported, the ORNs exhibited different degrees of specificity both as specialist and generalist neurons in these two parasitoids. In the present study, activation of large amplitude neuron (neuron A) by *cis*-3-hexenol (GLV) and *cis*-3-hexenyl butyrate (HIPV) is revealed in *M. croceipes*. The other compounds elicited greater response in one neuron type as compared to the second neuron in the sensilla, hence eliciting simultaneous activity in both neurons. However, in *C. marginiventris*, *cis*-3-hexenol (GLV) and *cis*-3-hexenyl acetate (HIPV) elicited greater activity in larger amplitude neuron (neuron C) but also showed simultaneous activity in the second neuron in some cases. The other compounds

elicited excitation in both neurons leading to asynchronous firing. In comparison between two parasitoid species, ORNs in the short sensilla placodea in generalist parasitoid *C. marginiventris* responded to compounds tested in this study and showed elevated response to plant volatiles (GLVs & HIPVs), host sex pheromone (Z11-16Ald) and ecologically irrelevant compound (benzaldehyde) compared to specialist. In *M. croceipes*, dual mixture of *cis*-3-hexenol and hexanal (GLV) with linalool (HIPV) inhibited the ORNs which showed excitation to the GLVs. However, these mixtures enhanced the ORNs in *C. marginiventris*. It might be conceivable that ORNs in the generalist parasitoid (*C. marginiventris*) detect a wide range of volatiles owing to the fact that they attack a broad range of hosts feeding on diverse plant varieties. In contrast, specialist parasitoid (*M. croceipes*) might have evolved with an olfactory system to detect a narrow range of hosts. The plants emit volatiles after damaged by specific host might release compounds which excites or inhibits the ORNs to facilitates specific host recognition.

3.4.1. Species comparison of ORN responses. The selected GLVs and HIPVs elicited ORN activity in the specialist and generalist parasitoids. ORNs in female *C. marginiventris* elicited greater response to all compounds compared to *M. croceipes* at low doses. However, *M. croceipes* females elicited greater ORN response at high doses to GLVS and HIPVs. This may signify that ORNs in generalist are more responsive to the low doses of volatiles in the present study. Similarly, ORNs in male elicited greater firing rate at high doses for hexanal (GLV) and *cis*-3-hexenyl acetate (HIPV). Males of *M. croceipes* (specialist) which showed greater response to other tested volatile compounds may have developed an efficient peripheral odor detecting system to facilitate successful mate finding in the arena of females searching for their hosts. In these

parasitoid species, *cis*-3-hexenol (both species), *cis*-3-hexenyl butyrate (in *M. croceipes*) and *cis*-3-hexenyl acetate (in *C. marginiventris*) elicited response in single ORN. This might suggest that specific compounds emitted before and after host damage might relay important odor signals for these parasitoids, and more than 50 percent of the sensilla (attempted in this study) have the ability to detect these compounds. Previous and recent electrophysiological studies have solely focused on the olfactory responses from the whole antenna (Geervliet et al. 1996; Smid et al. 2002, Chen and Fadamiro, 2007; Ngumbi et al. 2009, 2010). The results from the present study are in agreement with the previous comparison of olfactory responses between these two specialist and generalist parasitoids (Ngumbi et al. 2009, 2010).

Very few studies have characterized and compared ORN responses of a specialist versus generalist parasitoid from sensilla placodea which are the major olfactory sensilla in parasitic wasps. Smid et al (2002) demonstrated no difference in the olfactory responses from the antenna of specialist parasitoid, *Cotesia rubecula*, and a generalist, *C. glomerata*, to diverse host related compounds. Ochieng et al (2000) performed functional characterization of sensilla placodea in olfaction in *M. croceipes*, which is the only published work that reported ORN responses from placoids in parasitic wasps. Few functional characterizations of placoids in hymenopteran species were performed in honey bees (Akers and Getz, 1992, 1993) to certain common floral scents, where the ORNs were generalists and had a wide range of compound detection. Also, studies on scarab beetle, *Anomala cuprea* Hope, have characterized ORNs responding to sex-pheromone components in sensilla placodea (Leal and Mochizuki, 1993; Larsson et al. 1999). A recent study has distinguished ORNs in sensilla placodea as generalist and specialist in two congeneric scarab beetles, *Pachnoda interrupta* and *P. marginata* (Bengtsson et al. 2011). Therefore, earlier studies

performed on different insects and the present study in parasitic wasp suggests that, ORNs housed in sensilla placodea might be generalist and/or tuned to detect specific compounds.

3.4.2. Responses of olfactory neurons between sexes. Sexual differences in the ORN responses were not vast for all compounds, but females showed greater ORN response than males to compounds at both doses in these parasitoids. In *M. croceipes,* female had greater responses to two GLVs (*cis*-3-hexenol & hexanal), and two HIPVs (linalool & *cis*-3-hexenyl butyrate) at low and high doses. The other compounds (*cis*-3-hexenyl acetate, Z11-16 Ald and benzaldehyde) showed greater response in males. It may be plausible that these two GLVs and two HIPVs might be few of the important compounds that specialist parasitoid, *M. croceipes*, detects and rely on specific host location. In *C. marginiventris*, females showed greater response at low doses to most compounds in this study. Except for linalool, there was no difference in the response between sexes. At high dose, the response to compounds was reversed, in which male showed greater response. The results are in agreement with the prediction that males might use volatiles at higher concentration as long range cue for mate location, since no sex pheromone have been reported for both parasitoids. These results are also in agreement with previous electroantennogram responses of female and male parasitoids (Chen and Fadamiro, 2007; Ngumbi et al. 2009, 2010). However, extracellular activity of olfactory receptor neurons from individual sensilla does not fully corroborate with the summated response of diverse chemosensory (olfactory and gustatory) receptors housed in the antenna. However, it may provide a clue on the role of differences in response that might supply essential temporal coding in the higher centers of insect brains, which might help parasitoids in decision making for host finding and mate location.

Comparing the abundance of major olfactory sensilla placodea between sexes (Das et al. 2011) with the ORN responses, females in *C. marginiventris* (generalist) have a greater number of placoids than males. In generalist, females might have evolved the ability to detect a wide range of plant volatiles induced by various hosts, which may explain the greater abundance of olfactory sensilla (s. placodea) showing greater responses, with the theory that higher number of sensilla may indicate greater sensitivity (Chapman, 1982). However, s. placodea in *M. croceipes*, were more abundant on male antenna than females. The females had a greater response to majority of the compounds supporting our hypothesis that specialist may have developed an efficiently host-related odor detecting machinery which might help them for host location and finding in the complexity of nature.

Olfactory receptor neurons (ORNs) in insects (or other animals) are often described as ‘‘specialist’’ (usually pheromone-sensitive cells) or ‘‘generalist’’ (detecting plant odors) due to specificity in activity (Kaissling, 1974). In this study, ORNs were generalist in nature in both parasitoids and responded to plant odors, host sex pheromone and ecologically irrelevant compound. This might suggest that these two parasitoid species have ORNs which have the ability to detect wide variety of compounds, with the ORNs in generalist parasitoid having greater neuronal responses than specialist parasitoid. A few studies on honey bees also demonstrated that ORNs present in s. placodea are tuned to detect general odors (Akers and Getz, 1992, 1993). Olfactory neurons present in s. placodea of certain scarab beetle are known to respond to signature compounds like species sex-pheromones (Leal and Mochizuki, 1993; Larsson et al. 1999). A few beetles have been reported to have specialized ORNs for certain compounds or for a narrow range of food odors (Bengtsson et al. 2011). Studies on moths have mainly focused on sex-pheromones,

but response of general ORNs to diverse plant volatiles have been reported (Rostelien et al. 2000; Shields and Hildebrand, 2001).

Different mixtures have been reported as ligands which enhance or inhibit neuronal activity in different insects. Similar trend have been observed in this study with ORN excitation or inhibition in presence of certain compounds. In *M. croceipes*, a blend of *cis*-3-hexenol, linalool and *cis*-3-hexenyl butyrate had less response in comparison to the mixture of linalool and *cis*-3-hexenyl butyrate. This might suggest that reduced or enhanced activity of either blend (two or three compounds) might provide *M. croceipes* (a specialist) a specific code that indicates plant in attack by certain host (s). A recent study on mosquito neuronal responses evaluated that mixture of certain compounds confuses the CO_2 detecting machinery making it difficult for mosquitoes to choose the real source, as the mixtures were detected by CO_2 neurons (Turner et al. 2011). Hence, the mixtures in our study might encode similar signals of host related volatiles which might facilitate parasitoids in identifying specific hosts, similar to the findings described in mosquito species for CO_2 source. Mixture of two GLVs, *cis*-3-hexenol and hexanal, and linalool with *cis*-3-hexenyl butyrate (HIPV) showed excitation in these two parasitoids. Interestingly, linalool elevated the firing frequency of ORNs as a single compound, but inhibited the neuronal activity in *M. croceipes* (specialist) when mixed with green leaf volatiles, *cis*-3-hexenol and hexanal, separately. These results may suggest that inhibition of ORNs responding to certain GLVs after detection of linalool released by damaged plants might be a signal to a specialist parasitoid, which may provide a specific temporal code and may be an important signal relayed to the higher center of the brain to recognize specific host-related signals. Conversely, *C. marginiventris* (a generalist) has elevated neuron activity to compound mixtures with linalool. In generalist parasitoid, enhanced

responses of ORNs upon perceiving volatiles may deliver a signal code to help the parasitoid recognize its wide range hosts.

This study may suggests an association between olfactory neuron responses of the parasitoids and their degrees of host specificity, a finding that supports our assumption that specialist parasitoid *M. croceipes* might have an efficient volatile detetcting system compared to generalist *C. marginiventris*. The ORNs showed specific responses to the compounds in this study. The most significant finding is the inhibitiory effect of linalool in *M. croceipes* in a mixture with *cis*-3-hexenol and hexanal, however, ORNs in *C. marginiventris* showed enhanced response. Morphology of the antennal lobe is necessary to understand the glomerular organization of the primary odor processing center (antennal lobe) of these two parasitoids. This will help to trace the ORNs responding to host-related volatiles to identify specific glomeruli processing host-related compounds to the antennal lobe of both parasitoids

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Table 1. Results of Student's *t*-test analysis to compare net olfactory responses (spikes) of *M. croceipes* and *C. marginiventris* to selected host-related plant volatiles, host sex pheromone and ecologically irrelevant volatile at two doses.

Compound		Female		Male	
	Dose (µg)	***t***	***P***	***t***	***P***
Host-related plant volatiles					
cis-3-Hexenol	0.1	51.69	0.0020	6.11	0.0688
	100	95.73	0.0006	1.06	0.3614
Hexanal	0.1	8.62	0.0425	0.12	0.7442
	100	45.21	0.0025	76.66	0.0009
cis-3-Hexenyl butyrate	0.1	0.02	0.8919	3.85	0.1212
	100	40.35	0.0031	33.73	0.0044
Linalool	0.1	11.34	0.0281	12.96	0.0227
	100	1.88	0.2422	241.78	0.0001
cis-3-Hexenyl acetate	0.1	27.90	0.0062	0.06	0.8079
	100	7.23	0.0547	1.46	0.2927
Ecologically irrelevant plant volatiles					
Benzaldehyde	0.1	5.23	0.0840	11.84	0.0263
	100	0.78	0.4250	8.54	0.0431
Sex pheromones					
Z11-16 Ald (*H. Virescens*)	0.1	128.79	0.0003	198.90	0.0001
	100	8.40	0.0441	11.49	0.0275

df = 1

Table 2. Results of Student's *t*-test analysis to compare net olfactory responses (spikes) in female and male of *M. croceipes* and *C. marginiventris* to selected host-related plant volatiles, host sex pheromone and ecologically irrelevant volatile at two doses.

		M. croceipes		*C. marginiventris*	
Compound		**Female vs male**		**Female vs male**	
	Dose (µg)	***t***	***P***	***t***	***P***
Host-related plant volatiles					
cis-3-Hexenol	0.1	0.36	0.5780	52.19	0.0019
	100	47.23	0.0023	13.56	0.0211
Hexanal	0.1	4.65	0.0972	21.00	0.0102
	100	2.27	0.2062	12.53	0.0240
cis-3-Hexenyl butyrate	0.1	3.35	0.1409	18.64	0.0125
	100	28.20	0.0060	49.01	0.0022
Linalool	0.1	1.36	0.3082	0.07	0.8028
	100	0.44	0.5420	181.86	0.0002
cis-3-Hexenyl acetate	0.1	2.02	0.2280	12.64	0.0237
	100	0.63	0.4712	0.76	0.4321
Ecologically irrelevant plant volatiles					
Benzaldehyde	0.1	6.04	0.0697	10.66	0.0309
	100	4.34	0.1055	21.38	0.0098
Sex pheromones					
Z11-16 Ald (*H. virescens*)	0.1	129.59	0.0003	185.70	0.0002
	100	5.27	0.0833	23.25	0.0085

df= 1

Figure Legend

Fig. 1. Extracellular single unit recording from short sensilla placodea revealed the presence of 2 ORNs with distinct response in *M. croceipes*. **(a)** Spontaneous activity of the ORNs housed in the sensillum reveals neuron A and neuron B with different spike amplitude. **(b)** Response to a 0.5s stimulation by *cis*-3-hexenol, a green leaf volatile, exhibited excitation of neuron A followed by a little response in neuron B **(c)** Activation of both neuron A and B by hexanal followed by asynchronous firing pattern . **(d)** Stimulation of neuron A by *cis*-3-hexenyl butyrate, a herbivore induced volatile **(e)** Stimualation of both neurons by linalool, a HIPV **(f)** Ecologically irrelevant compound revealed excitation in both neuron (A and B).

Fig. 2. Extracellular single unit recording from short sensilla placodea revealed the presence of 2 ORNs with distinct responses in *C. marginiventris*. **(a)** Spontaneous activity of the ORNs housed in the sensillum reveals neuron C with largeand neuron D with smaller spike amplitude. **(b)** Response to a 0.5 s stimulation by *cis*-3-hexenol, a green leaf volatile, exhibited excitation of neuron C. **(c)** Activation of both neuron C and D by hexanal, a GLV, shows asynchronous firing pattern **(d)** Stimulation of neuron C by *cis*-3-hexenyl butyrate, a herbivore induced volatile **(e)** Activation of both neurons by linalool, a HIPV **(f)** Stimulation of neuron A and B by host sex pheromone Z11-16Ald.

Fig. 3. Extracellular single unit recording from short sensilla placodea to different mixture of compounds in *M. croceipes* **(a)** Mixture of *cis*-3-hexenol (GLV) and *cis*-3-hexenyl butyrate (HIPV) showed enhanced activity in neurons **(b)** Linalool (HIPV) inhibited the neurons activated by *cis*-3-hexenol (GLV) in a mixture **(c)** Linalool and *cis*-3-hexenyl butyrate stimulated enhanced activity **(d)** Linalool (HIPV) inhibited the neurons activated by hexanal (GLV) in a mixture **(e)** A mixture of cis-3-hexenol, linalool and cis-3-hexenyl stimulated the neurons.

Fig. 4. Extracellular single unit recording from short sensilla placodea to different mixtures of compounds in *C. marginiventris* **(a)** *Cis*-3-hexenol (GLV) with linalool elicited enhanced activity in neurons **(b)** *Cis*-3-hexenyl acetate and linalool exhibited enhanced response in both the neurons **(c)** a mixture of three compounds, *cis*-3-hexenyl acetate, linalool and *cis*-3-hexenol showed enhanced activity in both neurons in *C. marginiventris*.

Fig. 5. Net single sensillum responses (spikes/s ± SE, n= 3) of *M. croceipes* and *C. marginiventris* to tested volatiles in (a) female and (b) male at two doses (0.1µg and 100 µg). * Significant difference between two species (*t* test, $P< 0.05$).

Fig. 6. Net single sensillum responses (spikes/s ± SE, n = 3) of female and male to tested volatiles in (a) *M. croceipes* and (b) *C. marginiventris* at two doses (0.1µg and 100 µg). *Significant difference between sexes (*t* test, $P< 0.05$).

Fig. 1

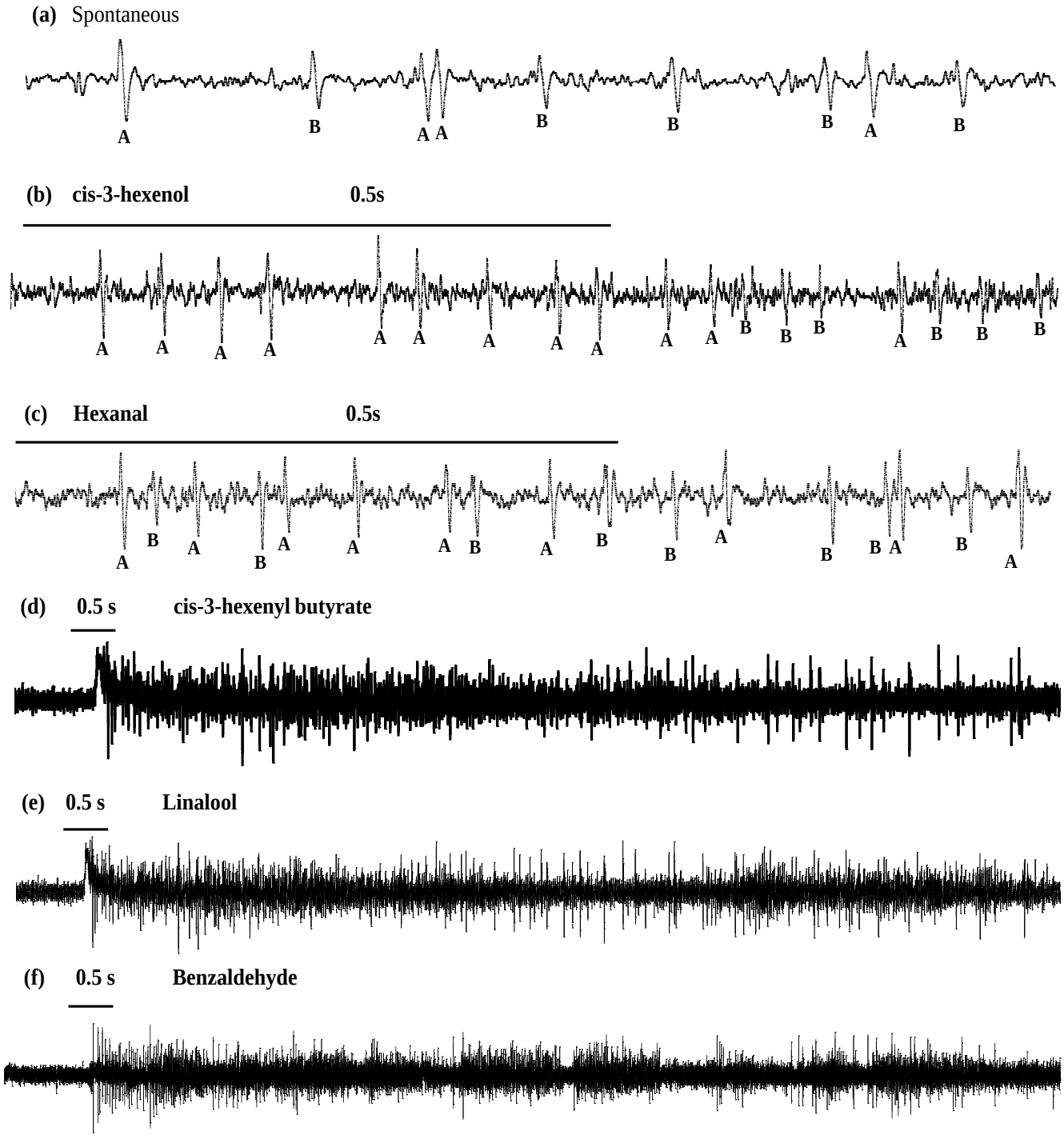

(a) Spontaneous
(b) cis-3-hexenol 0.5s
(c) Hexanal 0.5s
(d) 0.5 s cis-3-hexenyl butyrate
(e) 0.5 s Linalool
(f) 0.5 s Benzaldehyde

Fig. 2

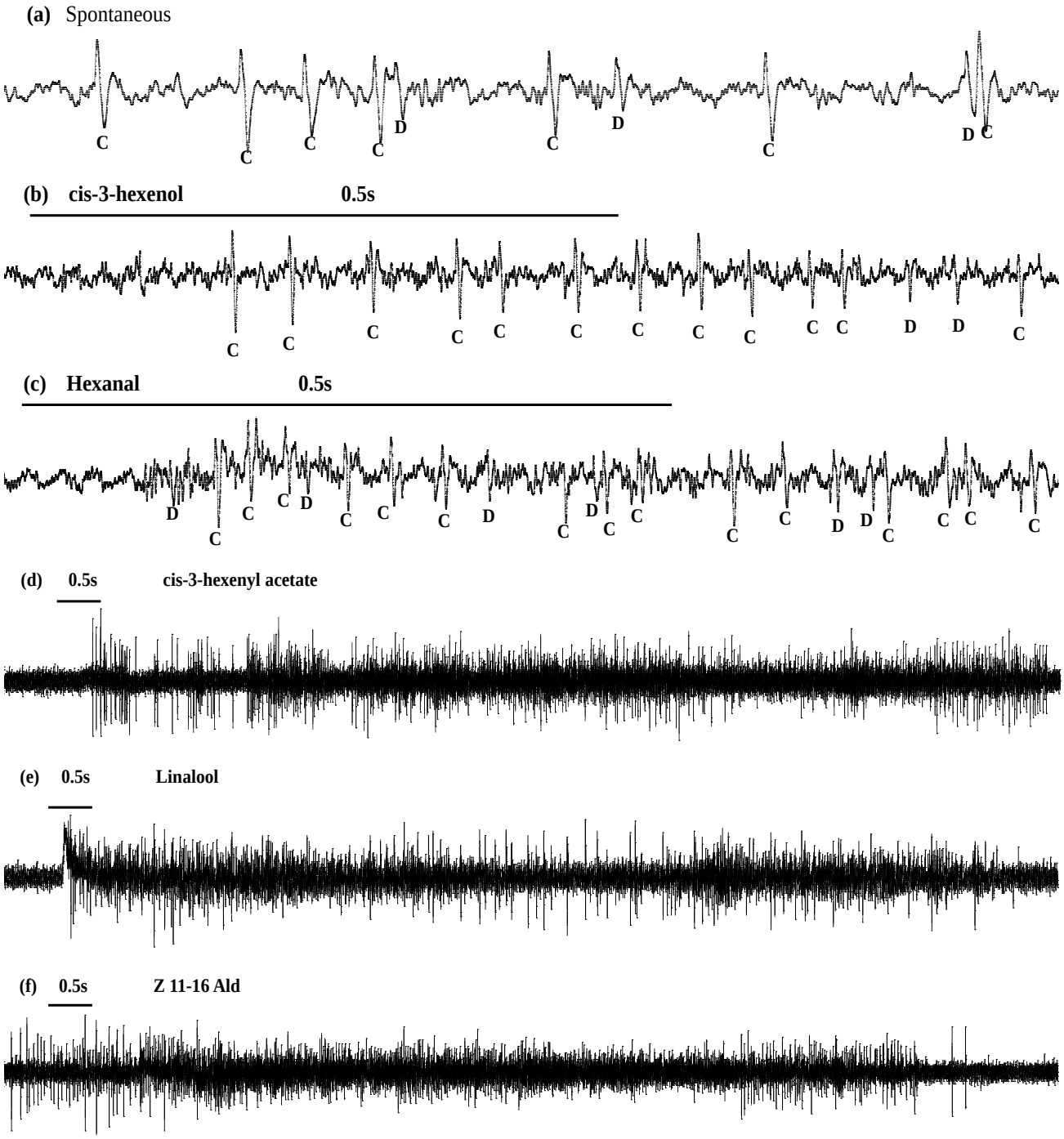

(a) Spontaneous
(b) cis-3-hexenol 0.5s
(c) Hexanal 0.5s
(d) 0.5s cis-3-hexenyl acetate
(e) 0.5s Linalool
(f) 0.5s Z 11-16 Ald

Fig. 3

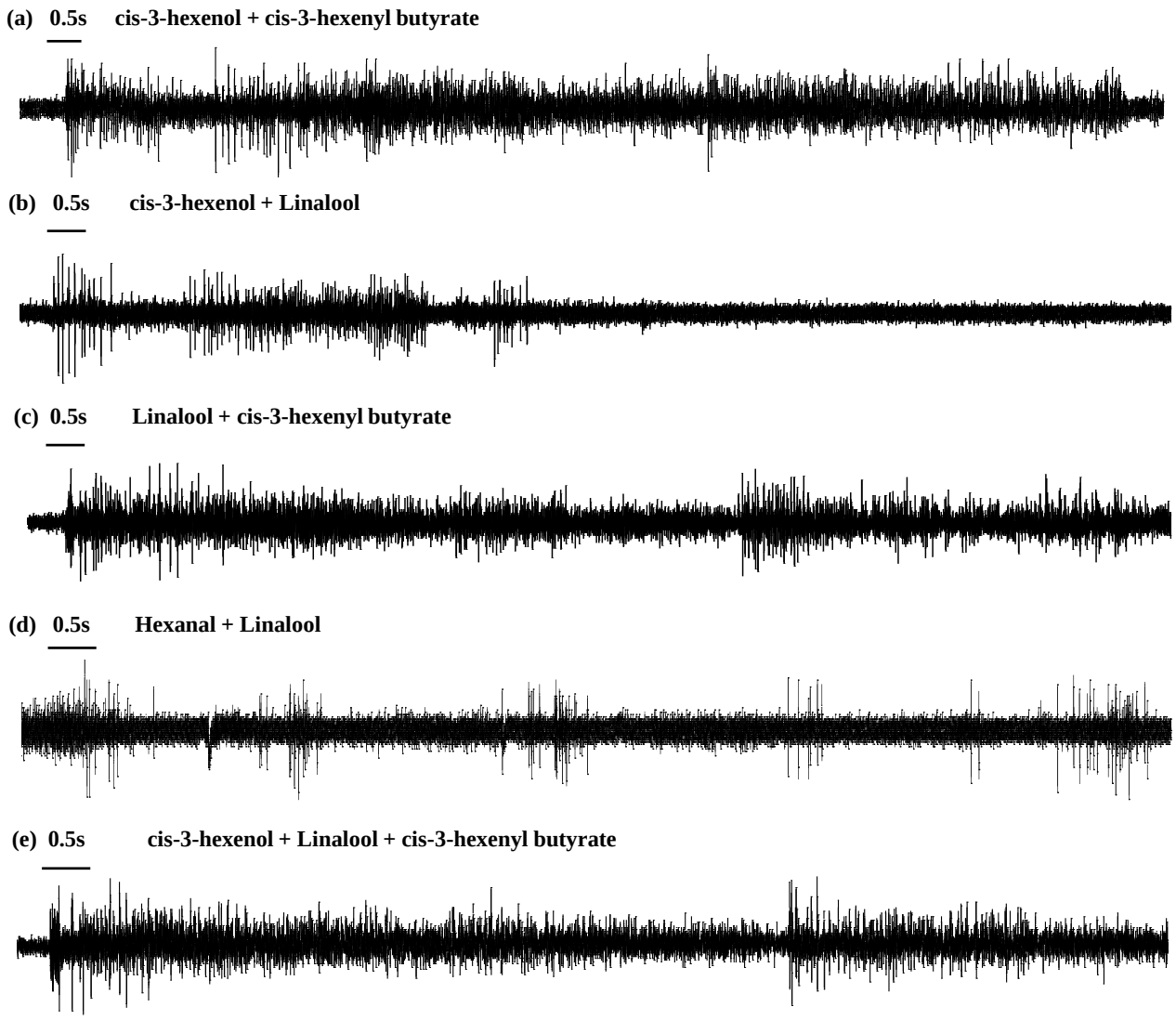

(a) 0.5s cis-3-hexenol + cis-3-hexenyl butyrate
(b) 0.5s cis-3-hexenol + Linalool
(c) 0.5s Linalool + cis-3-hexenyl butyrate
(d) 0.5s Hexanal + Linalool
(e) 0.5s cis-3-hexenol + Linalool + cis-3-hexenyl butyrate

Fig. 4

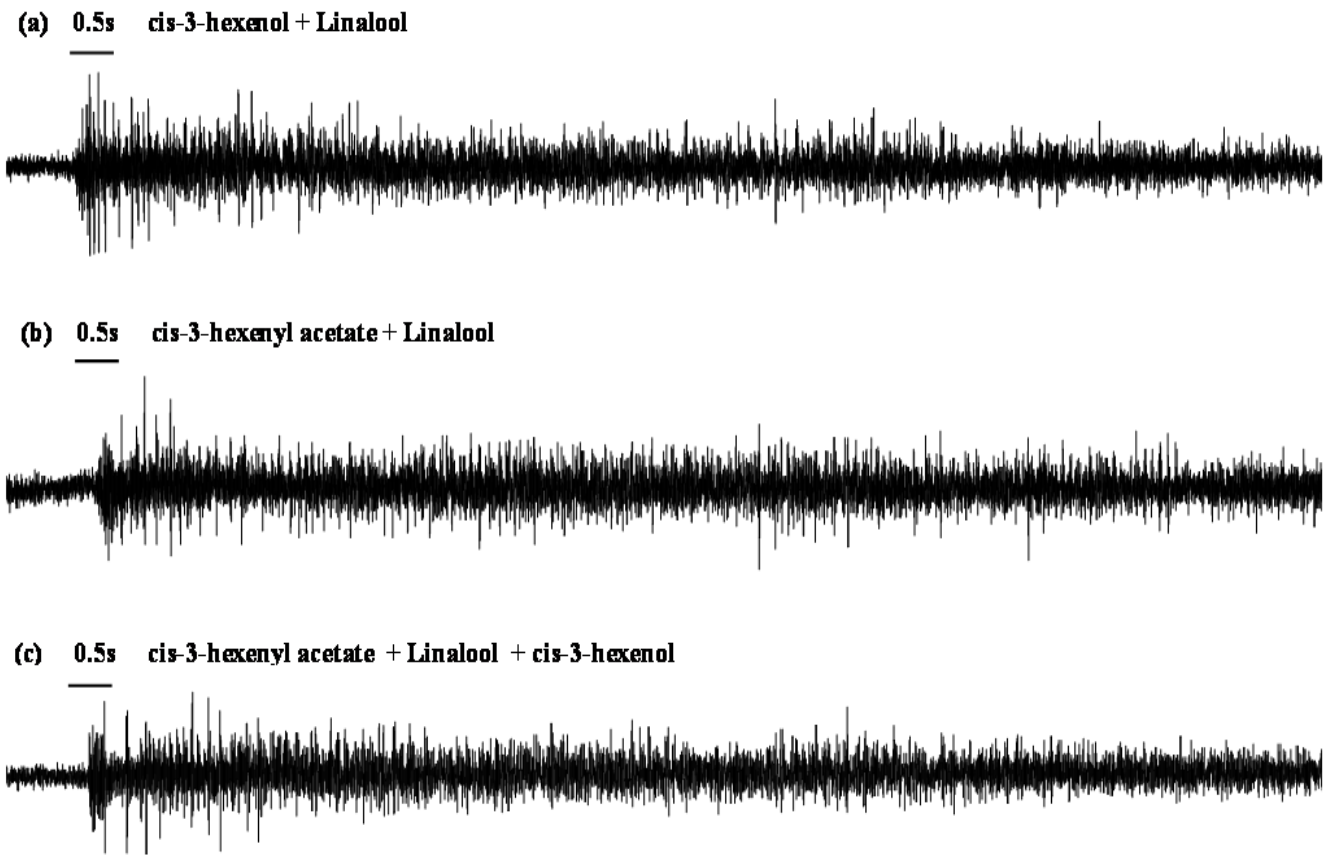

(a) 0.5s cis-3-hexenol + Linalool
(b) 0.5s cis-3-hexenyl acetate + Linalool
(c) 0.5s cis-3-hexenyl acetate + Linalool + cis-3-hexenol

Fig. 5

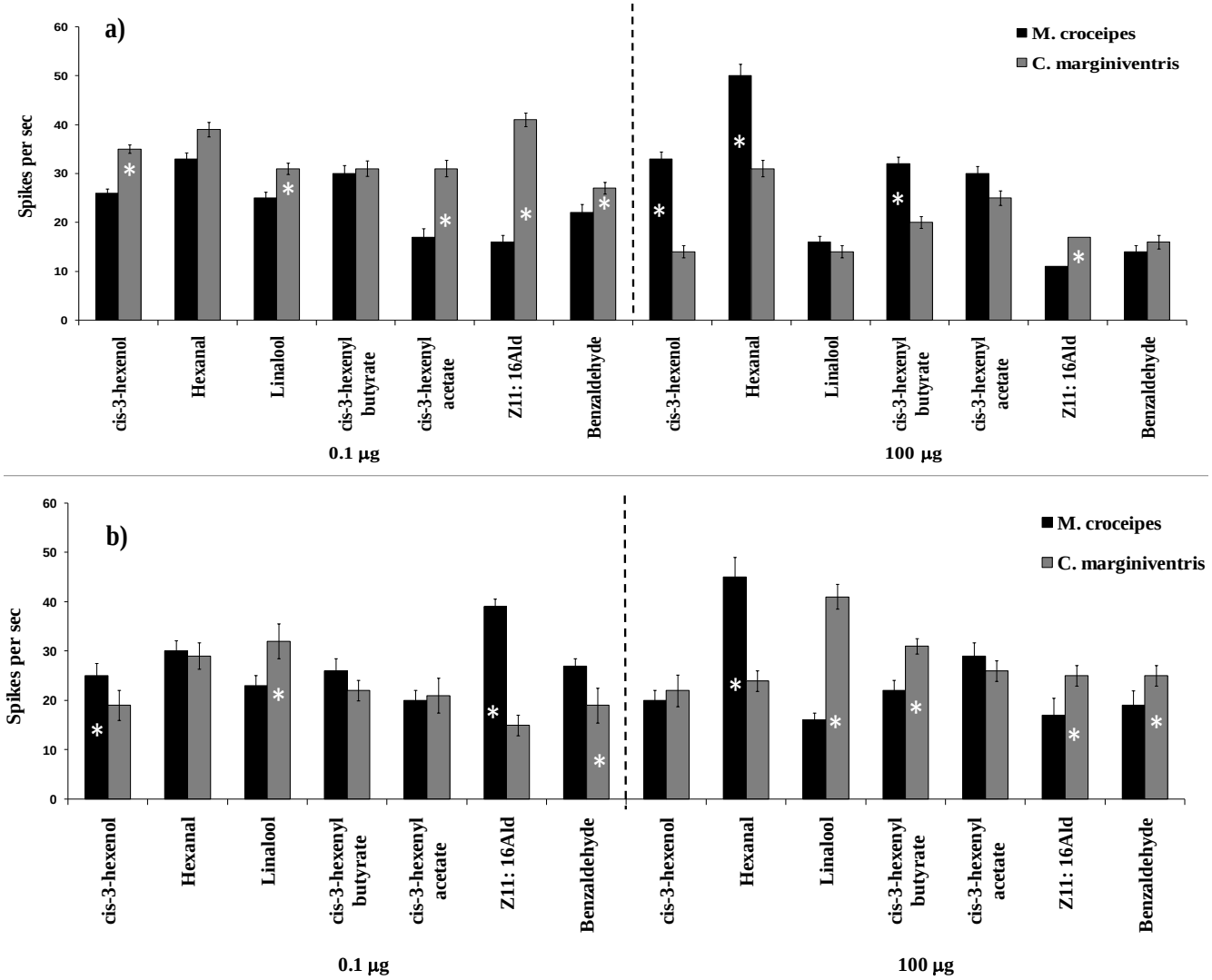
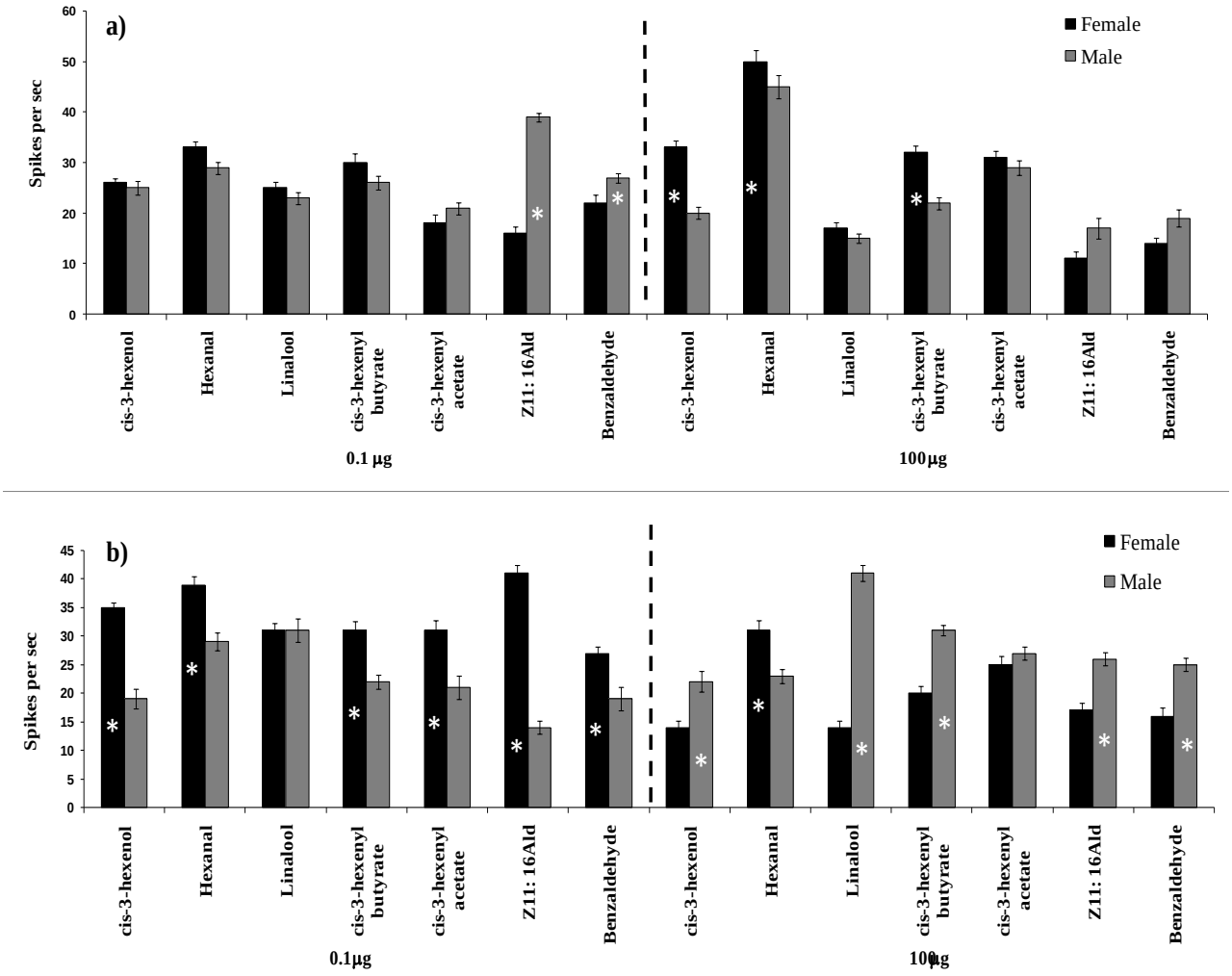

a)
Spikes per sec
M. croceipes
C. marginiventris
cis-3-hexenol
Hexanal
Linalool
cis-3-hexenyl butyrate
cis-3-hexenyl acetate
Z11: 16Ald
Benzaldehyde
0.1 µg
100 µg
b)
Spikes per sec
M. croceipes
C. marginiventris
cis-3-hexenol
Hexanal
Linalool
cis-3-hexenyl butyrate
cis-3-hexenyl acetate
Z11: 16Ald
Benzaldehyde
0.1 µg
100 µg

Fig. 6


a)
Female
Male
Spikes per sec
0
10
20
30
40
50
60
cis-3-hexenol
Hexanal
Linalool
cis-3-hexenyl butyrate
cis-3-hexenyl acetate
Z11: 16Ald
Benzaldehyde
0.1 µg
100µg
b)
Female
Male
Spikes per sec
0
5
10
15
20
25
30
35
40
45
cis-3-hexenol
Hexanal
Linalool
cis-3-hexenyl butyrate
cis-3-hexenyl acetate
Z11: 16Ald
Benzaldehyde
0.1µg
100µg

CHAPTER 4

SPECIES AND SEXUAL DIFFERENCES IN ANTENNAL LOBE ARCHITECTURE AND GLOMERULAR ORGANIZATION IN TWO PARASITOIDS WITH DIFFERENT DEGREE OF HOST SPECIFICITY, *MICROPLITIS CROCEIPES* AND *COTESIA MARGINIVENTRIS*

4.1 Introduction

The antennal lobe is the primary olfactory information processing center in the insect brain. Because the insect antennal lobe (AL) is remarkably similar to the vertebrate olfactory bulb (OB), insects are attractive model organisms for investigating neuronal architecture and mechanisms of olfaction in animals (Hildebrand and Shepherd 1997; Aungst and Spehr, 2005). Both the AL and OB consist of distinct morphological units called glomeruli, which receive input from olfactory receptor neurons (ORNs) expressing the same receptor type (Mombaerts et al. 1996; Vosshall et al. 2000). Briefly, the AL receives inputs from the olfactory receptor neurons (ORNs) housed within olfactory sensilla located on the antenna, the primary olfactory organ in insects. The ORNs from olfactory sensilla terminate in the glomeruli, where they make synaptic contact with local interneurons, which interconnect subsets of glomeruli, and with projection neurons, which project to the higher brain centers.

The morphology of the AL and glomerular organization has been described in some insect taxa including moths (Lepidoptera) (Rospars, 1988; Homberg et al. 1989; Boeckh and Tolbert,

1993; Hildebrand and Shepherd, 1997; Anton and Homberg, 1999; Hansson and Anton, 2000), ants (Hymenoptera) (Zube et al. 2008; Kelber et al. 2009), bees (Hymenoptera) (Flanagan and Mercer, 1989; Galizia et al. 1999), and flies (Diptera) (Vosshall et al. 2000; Wong et al. 2002). Also, Smid et al. (2003) described antennal lobe architecture in two species of parasitic wasps (Hymenoptera). In many of these studies, it was showed that axon terminals from ORNs expressing a specific membrane receptor innervate a particular glomerulus or glomeruli in the AL responsive to a specific set of odorants (Gao et al. 2000; Vosshall et al. 2000; Couto et al. 2005; Hallem and Carlson, 2006). In moths, plant odor processing occurs mainly in the ordinary glomeruli (Christensen and Hildebrand, 2002; Christensen and White, 2000), whereas sex pheromone processing in the male occurs in a distinct complex of glomeruli, the macroglomerular complex (Hansson et al. 1991; Anton and Homberg, 1999). These two systems are, however, not entirely separate because pheromone responses have also been found in neurons within ordinary glomeruli (Kanzaki et al. 1989; Anton and Hansson, 1995).

Like moths, parasitic wasps (Hymenoptera) or parasitoids use various types of plant based volatiles as cues for foraging and location of their herbivore hosts (Dicke and Sabelis 1988; Turlings et al. 1990; McCall et al. 1994; De Moraes et al. 1998; Chen and Fadamiro 2007). However, many aspects of olfactory communication in this important group of insects remain poorly understood, despite the increasing interest in their use as biological pest control agents. Research by our group (Chen and Fadamiro, 2007; Ngumbi et al. 2009, 2010, Das et al. 2011) has focused on olfactory communication in two parasitoids (Hymenoptera: Braconidae) with different degrees of host specificity, *Microplitis croceipes* (Cresson) and *Cotesia marginiventris* (Cresson). *Microplitis croceipes* is a specialist larval parasitoid of *Heliothis* spp., whereas, *C. marginiventris* is

a generalist larval parasitoid of several caterpillar genera including *Heliothis* spp. and *Spodoptera* spp. In particular, a recent study of the antennal morphology of both species has revealed important differences in abundance of antennal chemosensilla: the putative chemosensilla types, sensilla placodea and s. basiconica, were more abundant in *M. croceipes* (specialist) than in *C. marginiventris* (generalist) (Das et al. 2011).

In furtherance of my research on mechanisms of olfaction in both parasitoids species, the present study was conducted to characterize the parasitoids' antennal lobe morphology and glomerular organization. A comparative three-dimensional description of the organization, relative size, position, and number of glomeruli in both sexes of *M. croceipes* and *C. marginiventris* is presented, and key species and sexual differences are discussed.

4.2 Materials and Methods

4.2.1 Insects. The parent cultures of *M. croceipes* and *C. marginiventris* were provided by the USDA-ARS, Crop Protection and Management Research (Tifton, Georgia) and the Department of Entomology, University of Georgia (Tifton campus, Contact: John Ruberson), respectively. *Microplitis croceipes* was reared on larvae of *Heliothis virescens* Fab., whereas *C. marginiventris* was reared on larvae of *Spodoptera exigua* (Hübner). Colonies of both parasitoid species were maintained in the laboratory as previously reported (Lewis and Burton, 1970; Ngumbi et al. 2009). Adult parasitoids (2−3 days old) were first anesthetized by chilling for ~15 – 20 min at 4°C and then processed for tissue staining for confocal microscopy.

4.2.2. Antennal lobe staining. The neuroanatomical procedures used in this study were

modified after Smid et al (2003) and Zube et al (2008). Parasitoids were sedated on ice and immobilized by dental wax, leaving the antennae accessible. The antenna was cut at the level of the third or fourth proximal segment of the flagellum under a stereomicroscope (National Microscope, Model direct current 3−420, Meiji, Japan). The axon tracer was prepared as 3.5% biotin dextran amide (Molecular Probes, Oregon, USA, MW 10 kDa,) in distilled water. Biotin amide solution was filled in a pointed open end glass microcapillary to fill the antenna. The cut end of the antenna was slightly inserted into the glass capillary, and left for 4 – 5 hours at room temperature to allow the tracer to enter through the antennal nerves. The heads were removed and the brains were dissected in Ringer's solution using a stereomicroscope, and were fixed overnight at room temperature in 4% formaldehyde in 0.1 M phosphate buffer, pH 7.0, as performed by Smid et al (2003). Post-fixation, brains were dehydrated in ethanol series to 90% and then immersed in n-heptane. The brains were washed four times in phosphate buffer saline for rehydration, and then incubated in streptavidin conjugated to Alexa 488 (S − 11223, Molecular Probes). The incubation mixture was prepared by diluting streptavidin (conjugated to Alexa 488) with 1:500 in phosphate buffer with 1% bovine serum albumin and Triton X-100. The brains were refrigerated (incubated) for 24 hours, and washed six times in 0.1 M phosphate buffer for 4 hours. Post washing of brain tissues was followed by dehydration in ethanol series, cleared in xylene and mounted in Depex (Fluka) as mounting media. The mounting of delicate brains tissues in Depex was critical, so it is very crucial that care must be taken to avoid tissue damage. To avoid the pressure of cover glass on brains, spacers were used to mount *M. croceipes* brains, as they are comparatively larger than the brains of *C. marginiventris*. Of the 64 brains (total for both species and sexes) treated with this protocol, 39 yielded selective labeling of the glomeruli, and were

digitized with confocal laser scanning microscopy. Three brains per sex of each species were selected for image segmentation and glomerular matching.

4.2.3. Confocal microscopy and 3D reconstruction. The brain samples were examined with a BioRad MRC-1024 confocal laser-scanning microscope, equipped with argon/krypton laser. The Alexa fluorophore (conjugated to streptavidin) was excited with a wavelength of 488 nm, to obtain digital image stacks from laser scanning. Images from brain tissues were obtained using Zeiss ×40 NA 1.3 oil-immersion objectives, by resolution of 512 × 512 pixels. The optical sections from antennal lobe were obtained in a stack of 100 − 200 images (80 − 120µm). Complete stacks of images were imported in the 3D analysis software AMIRA 5.3.3 (Visage Imaging Inc., San Diego, CA). To maintain similarity in glomerular identification, we used the nomenclatures of Smid et al (2003) to describe different landmark glomeruli in *C. marginiventris*. However, glomeruli nomenclatures were different in *M. croceipes* since the glomerular organization and positions are different compared to *C. marginiventris*. A contour enclosing different glomeruli was given a specific color to incorporate the glomerulus in the same glomerular layer. A randomly chosen number was provided to each landmark glomerulus. The volume of antennal lobe mass was calculated and the 3D surface model was constructed manually from optical section stacks using AMIRA for analysis. Images of slices from confocal scans and Amira reconstructions were further processed with Adobe Photoshop 7.0 software (Adobe Systems, San Jose, CA) to adjust for brightness and contrast. For 3D reconstruction, three antennal lobe image stacks, each from an individual wasp/sex were analyzed for comparison.

To identify glomerular variance in each species, antennal lobe specimens were compared to

match different glomeruli in each layer. Optical image stacks were matched to confirm species and sexual differences in antennal lobe organization. The size of each glomerulus, morphology of each confocal section and position of glomeruli in 3D reconstruction of antennal lobe were utilized to provide identification.

4.3 Results

4.3.1. Antennal lobe architecture. An image of the full brain of *M. croceipes* was superimposed with a 3D antennal lobe model (Fig. 1) to indicate the position of the antennal lobe, and antennal nerves innervating the glomerular mass. The antennal lobes of both wasp species have different external morphology and glomerular organization. Visually distinct sections/layers of the antennal lobes for both species are depicted with different colors in Figs. 2 & 3. The average width of *M. croceipes* brains is 1320 ± 45 µm (n = 3), compared to 852 ± 25 µm (n = 3) in *C. marginiventris.* The antennal lobe is oval shaped and the height is smaller compared to its width (Fig. 4). Antennal lobe volume ranged from $867 \times 10^3 - 897 \times 10^3$ μm^3 in *M. croceipes*, compared to $347 \times 10^3 - 384 \times 10^3$ in *C. marginiventris*. This species difference in antennal lobe volume is likely due to the fact that *M. croceipes* has comparatively larger head size and hence greater antennal lobe volume than *C. marginiventris*. Similarly, the glomerular number per antennal lobe ranged from 219 − 222 (females) and 220 − 224 (males) in *M. croceipes*, compared to 192 − 194 (female) and 193 −196 (male) in *C. marginiventris*.

The antennal lobe in both species is separated into two halves, the medial and lateral. Adjacent glomeruli in these two halves are either attached or overlap each other (Figs. 2 & 3). The medial and lateral halves are fairly continuous in all specimens, however, they are separated at the

ventral and partly at the posterior side. The receptor neuron axons form two antennal nerves before entering each antennal lobe. The two antennal nerves innervate the glomerular mass anteriorly and project posteriorly through the antennal lobe as two main tracts, one median and one lateral. Each tract then branches into the subgroups of glomeruli within its respective region (half) of the antennal lobe (medial or lateral). A confocal image obtained dorsally from *C. marginiventris* (Fig. 5) antennal lobe shows major division of the tracts.

Key morphological differences were observed between the two species. In *M. croceipes*, the medial half is larger compared to the lateral half (Figs. 2, 4a, c). In contrast, the lateral half in *C. marginiventris* is larger than the medial half (Figs. 3, 4b, d). As described for *Cotesia rubecula* and *C. glomerata* (Smid et al. 2003), a small group of glomeruli attached to the posterior region from the larger subgroup was also observed in *C. marginiventris*, but not in *M. croceipes*. In *M. croceipes*, the median tract innervates the medial half with a greater number of glomeruli, goes through the ventromedian direction, and innervates the ventro-median, median, and dorsal glomeruli. The lateral tract, on the hand, branches into two, one penetrates the ventro-lateral and the other to the lateral glomeruli. The presence of a greater number of glomeruli in the medial half suggests that this region may process more odors than the lateral half in *M. croceipes*. The innervating pattern is reversed in *C. marginiventris*. The lateral tract enters the lateral, ventro-lateral, and dorsal glomeruli, and reaches the posterior glomeruli. The lateral tract might have ORN axons projecting greater number of glomeruli, as it is innervating the lateral glomerular mass, which has a greater number of glomeruli than in the medial half. The median tract in *C. marginiventris* innervates into the ventro-median and median glomeruli. In contrast to *M. croceipes*, more glomeruli are present in the lateral half of the antennal lobe of *C. marginiventris*

that in the medial half. This suggests that the lateral half may process more odors in *C. marginiventris*.

4.3.2. Glomerular organization in the antennal lobe. The glomeruli which were consistent in different image stacks were considered landmark glomeruli in both species. The anterior glomerular mass was divided into median (antero-median or AM) and lateral (antero-lateral or AL) anterior glomerular clusters. In both species, the ventro-median (VM) and median (M) glomeruli were separated by landmark glomeruli VM1 (Figs. 4c, d). The ventro-lateral (VL) and lateral (L) glomeruli were separated by landmark glomeruli VL1. Each glomerular cluster was further divided into different subgroups and landmark glomeruli within the subgroups depending on the position (Fig. 4) in different regions. In both species, the antero-lateral mass had a set of landmark glomeruli grouped according to their position (AL 1−7) and the antero-median mass had one landmark glomeruli AM1. In contrast, the ventro-lateral region had single glomeruli VL1, and the ventro-median had one glomerulus VM1. The lateral glomerular mass had two landmark glomeruli (L1 & L2) and three glomeruli in the median region (M1, M2 & M3). In addition, dorsal layer had one landmark glomeruli D1, and the posterior had a group of three glomeruli (P1, P2 & P3).

Glomerular organization in *C. marginiventris* is similar to that in *Cotesia rubecula* and *C. glomerata* (Smid et al. 2003), but different in *M. croceipes*. Differences were recorded between both species in the size, shape and position of glomeruli at several locations (Figs. 2 & 3). In general, *M. croceipes* has larger comparative glomeruli than *C. marginiventris*. Remarkable sexual differences were also recorded. In males of both species, a prominently enlarged macroglomerulus

(MG) was found close to the entrance of antennal nerves (Fig. 6). Analyses of three complete confocal image stack confirmed that the MG is larger than the ordinary glomeruli in all specimens. In addition to the MG, a complex of 3 − 4 macro-glomeruli (complex of macro-glomeruli or CMG) was observed in the posterior region of the antennal lobe of males of both species (Fig. 7). A glomerulus, which was consistently found (in all observed antennal lobe specimens) adjacent to the MG in the anterior glomerular region of males in both parasitoid species, was designated as "putative satellite glomerulus" (Fig. 6). Qualitative inspection of the MG and the associated satellite glomeruli in other individuals revealed a similar spatial arrangement. Interestingly, the antennal lobes of females of both species completely lacked both the MG at the entrance of the antennal nerves and the complex of macro-glomeruli (CMG) at the posterior region of antennal lobe.

4.4 Discussion

The results revealed important species and sexual differences in antennal lobe morphology and glomerular organization in *M. croceipes* and *C. marginiventris*. In *M. croceipes*, the medial half of antennal lobe is larger enclosing greater number of glomeruli compared to the lateral half, whereas in *C. marginiventris*, the lateral half is relatively larger with greater glomeruli number than the medial half. The innervation pattern of median and lateral tracts in *C. marginiventris* is similar to the patterns described for *Cotesia glomerata* and *C. rubecula* (Smid et al. 2003), suggesting that closely related species may have evolved similar antennal lobe architecture. The average volume of the antennal lobe is similar between the sexes but three times greater in *M. croceipes* compared to *C. marginiventris*. The observed difference in antennal lobe volume is

likely related to size: *M. croceipes* is relatively bigger and has a comparatively larger brain size and hence greater antennal lobe mass than *C. marginiventris*.

Despite the significant species difference in antennal lobe volume, the number of glomeruli per antennal lobe is only slightly higher in *M. croceipes* (219 − 224) than in *C. marginiventris* (192 − 196), and not different between the sexes. Results from a previous study showed that sensilla placodea, the main antennal olfactory sensilla in both parasitoid species, is significantly more abundant in *M. croceipes* than in *C. marginiventris* (Das et al. 2011). Thus, my results suggest no relationship between abundance of olfactory sensilla and number of glomeruli.

The most striking sexual difference observed is the presence of two remarkable glomerular structures in males of both species which are missing in conspecific females: i) an enlarged macroglomerulus (MG) at the entrance of the antennal nerve, and ii) a complex of macro-glomeruli (CMG) in the posterior region of the antennal lobe. To our knowledge, this is the first report of both structures (MG and CMG) in parasitic wasps. However, similar structures have been reported in other insects such as leaf-cutting ant workers (Kleineidam et al. 2005), fungus growing ant workers (Kelber et al. 2009), male fruit fly (Laissue et al. 1999), and male moths (Berg et al. 2002; Varela et al. 2011). In many insects such as moths, honey bees, and flies, pheromone is processed in specific glomeruli such as the macroglomerular complex in male moths or by groups of ordinary glomeruli (Galizia et al. 2000; Sache and Galizia 2002; Varela et al. 2011). Although sex pheromones have not been reported in *M. croceipes* and *C. marginiventris*, the MG and CMG may function in the detection of female odors, such as pheromones. Future identification of sex pheromones for both parasitoid species will aid confirmation of the function of the MG and CMG.

The slight variation observed in this study in the number of glomeruli per antennal lobe

among individuals of the same species is not uncommon. Many authors have also recorded individual variations in the number of glomeruli in many insect species (Galizia et al. 1999; Laissue et al. 1999; Berg et al. 2002; Smid et al. 2003, Couton et al. 2009). In many cases, this variation is due to the difficulty in separating apart fused glomeruli from two different layers (Galizia et al. 1999; Laissue et al. 1999; Berg et al. 2002; Smid et al. 2003, Couton et al. 2009). In the present study, the glomeruli identified in both parasitoid species ranged from smaller to larger glomeruli, as reported also for carpenter ant, *Camponotus floridanus* (Zube et al. 2008), fungus growing ant, *A. cf. mayri* (Kelber et al. 2009), and several species of stink bugs (Kristoffersen et al. 2008). The smaller size glomeruli are sometimes difficult to separate and differentiate. This variability represents a challenge in glomeruli identification and separation due to unclear boundaries between different glomerular layers and subgroups.

Among the hymenopterans, the highest number of glomeruli per antennal lobe (630 glomeruli) was reported in the fungus growing ant, *Apterostigma cf. mayri* (Kelber et al. 2009), which is ~ 2.8 and 3.3 times greater than recorded in the present study for *M. croceipes* and *C. marginiventris*, respectively, and also ~ 3.3 times greater than in *C. glomerata* and *C. rubecula* (Smid et al. 2003). Clearly, size alone cannot fully account for the above differences in number of glomeruli since the head width of the fungus growing ant, *A. cf. mayri* is only slightly larger than that of the above parasitoid species. In a comparative study of antennal lobe morphology of several species of fungus growing (Attini) ants, Kelber et al. (2009) reported a correlation between head width of the ants and antennal lobe volume; however number of glomeruli was not positively correlated with head width. Similarly, the number of glomeruli in the antennal lobe of moths ranges between 60 - 66 (Berg et al. 2002; Løfaldli et al. 2010). This is less than the number of

glomeruli in the fungus growing ant and the two parasitoids species investigated in the present study; but antennal lobe size in moth is greater than both parasitoid species. Thus, differences in number of glomeruli between insect families or taxa may be related to phylogeny or have functional significance.

This study presents a three-dimensional map comparing the antennal lobe morphology in *M. croceipes* (specialist) and *C. marginiventris* (generalist). Despite a marked species difference in antennal lobe architecture, only a slight difference in the number of glomeruli was recorded between the two species. The most significant finding is the presence of an enlarged glomerulus (MG) and a complex of 3 − 4 macro-glomeruli (CMG) in males of both species, which are lacking in the females. Functional characterization of different glomeruli through neuronal mapping studies should lead to identification of individual or groups of glomeruli responsible for processing different types of odor in both parasitoid species.

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Figure legends

Fig. 1. Representation of *M. croceipes* brain structure with superimposed 3D model of antennal lobe (anterior view) (AN = antennal nerves, OL = Optic lobe)

Figs. 2 a-f. Optical sections showing different glomerular layers by color from anterior to posterior through the antennal lobe of female *M. croceipes*. Dimensions: stack size 109 µm, 204 optical sections; 15-section interval. Landmark glomeruli have been named in subgroups on their positions: Antero-lateral (AL 1-7), antero-median (AM1), dorsal (D1), median (M 1-3), ventro-lateral (VL 1), ventro-median (VM 1), lateral (L 1-2), & posterior (P 1-3). Colors indicate glomeruli in subgroups. Orientation of AL sections: Dorsal (D), Ventral (V), Median (M), Lateral (L). *Bar* 50 µm.

Figs 3 a-f. Confocal sections of color-marked glomeruli from anterior to posterior through the antennal lobe of female *C. marginiventris* showing different glomerular layers. Abbreviations for landmark glomeruli are same as Fig. 2. Dimensions: stack size 94 µm, 130 optical sections; 15-section interval. *Bar* 50 µm.

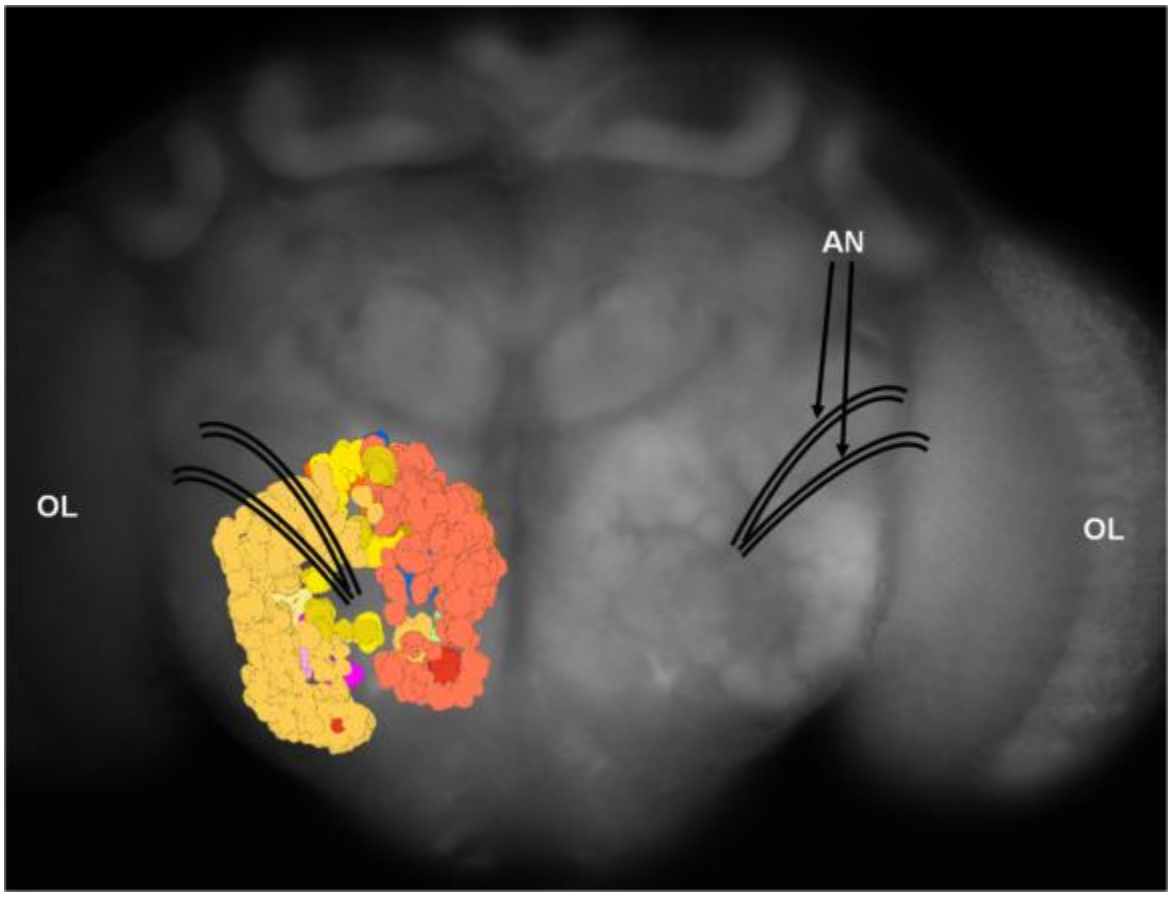
Figs. 4a–d. Surface reconstruction 3D models of the antennal lobe of female *M. croceipes* (Fig. 4a = anterior view, 4c = posterior view, left column); and female *C. marginiventris* (Fig. 4b = anterior view, 4d = posterior view, right column). *Bar* 50 µm

Fig. 5. Optical image section with dorsal view showing innervations pattern of median (white arrows) and lateral (blue arrows) tracts in *C. marginiventris* which innervate the anterior through the posterior of medial and lateral half. The median tract (MT, white arrows) innervates the median (M) and ventro-median (VM). The lateral tract (LT, blue arrows) innervates the dorsal (D), lateral (L), ventro-lateral (VL) and posterior (P) glomeruli. The dimension of the image shows anterior, posterior, dorsal and lateral regions. *Bar* 50 µm

Fig. 6. Frontal optical sections showing a macroglomerulus (MG, indicated by arrow) at the entrance of the antennal nerve (AN) and a putative satellite glomerulus (consistently found adjacent to the MG, asterisk) in male *M. croceipes*. *Bar* 50 µm

Fig. 7. Posterior optical section showing a complex of four macro-glomeruli (CMG) (asterisks) in male *M. croceipes*. Note the enlarged fibrous core corresponding to the posterior group of glomeruli. *Bar* 50 µm

Fig. 1



Figs. 2 & 3

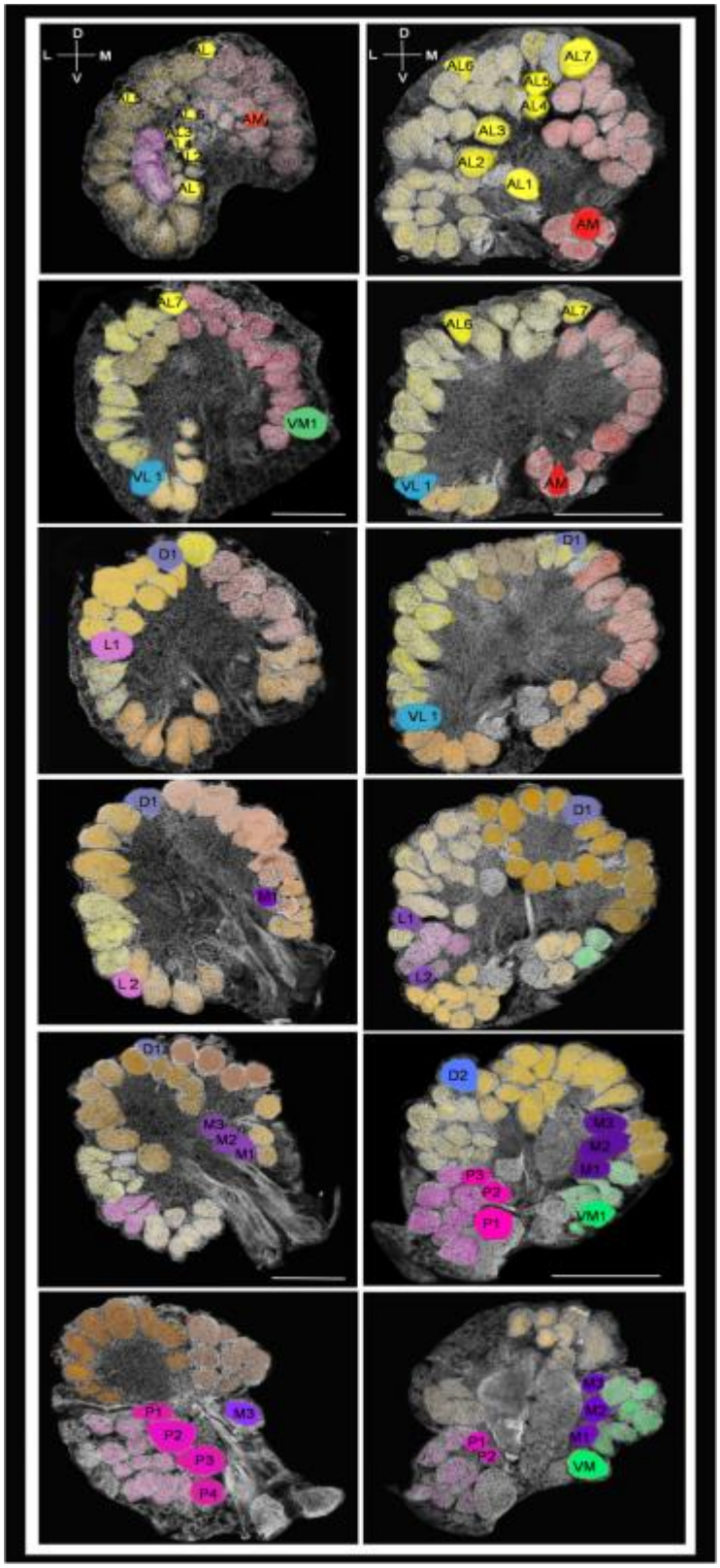

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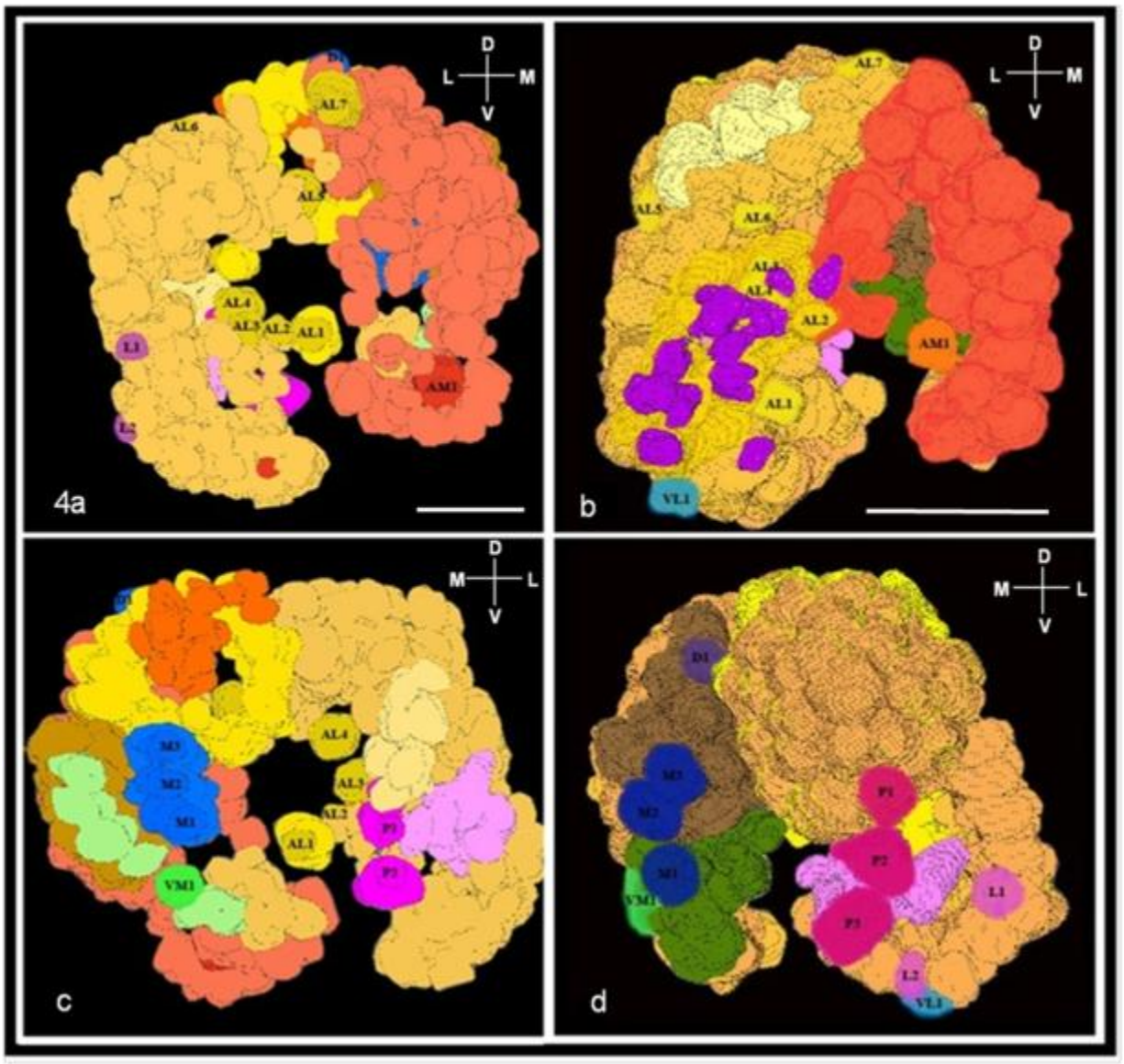
Fig. 4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P1
P2
P3

Fig. 5

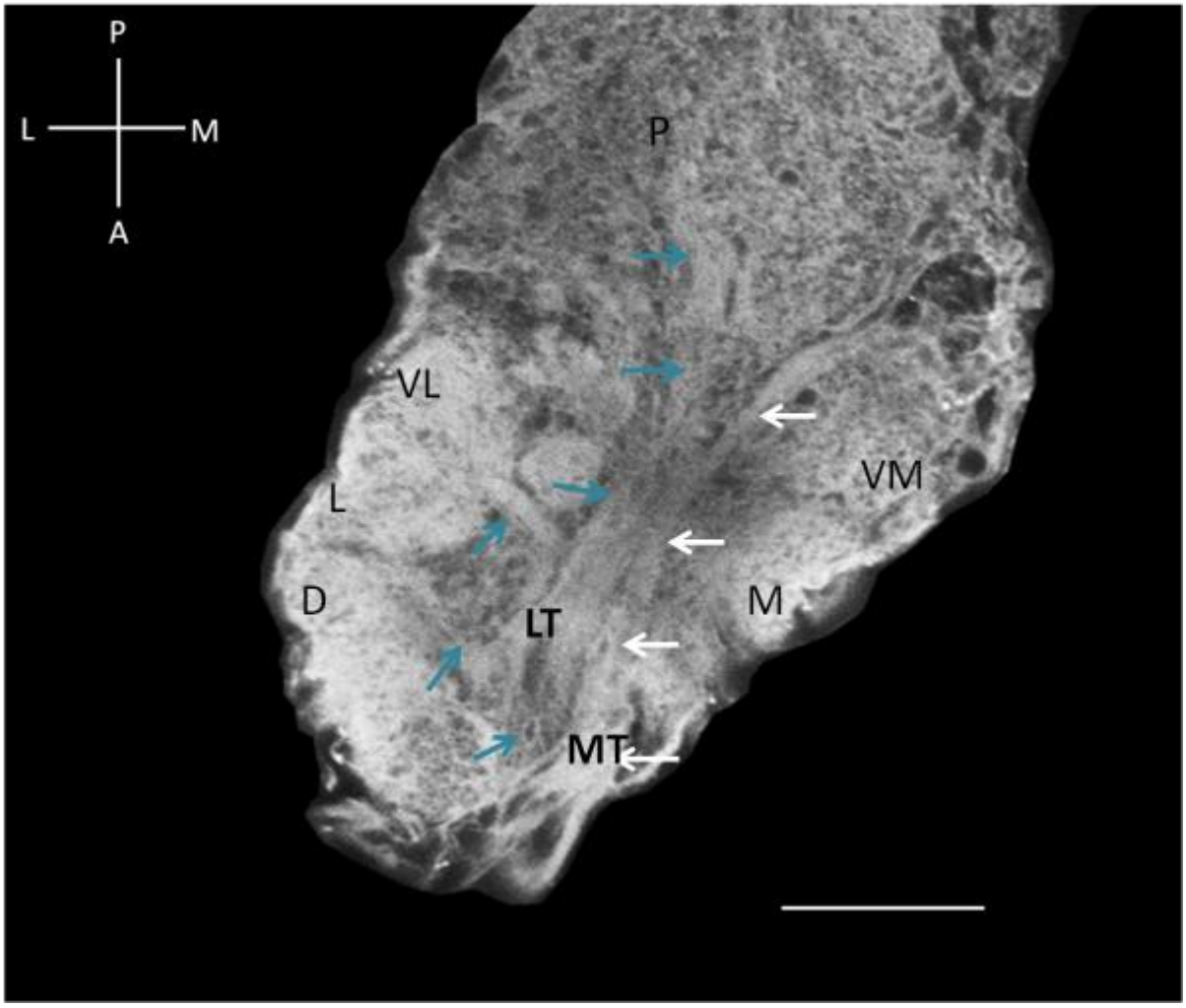


Fig. 6

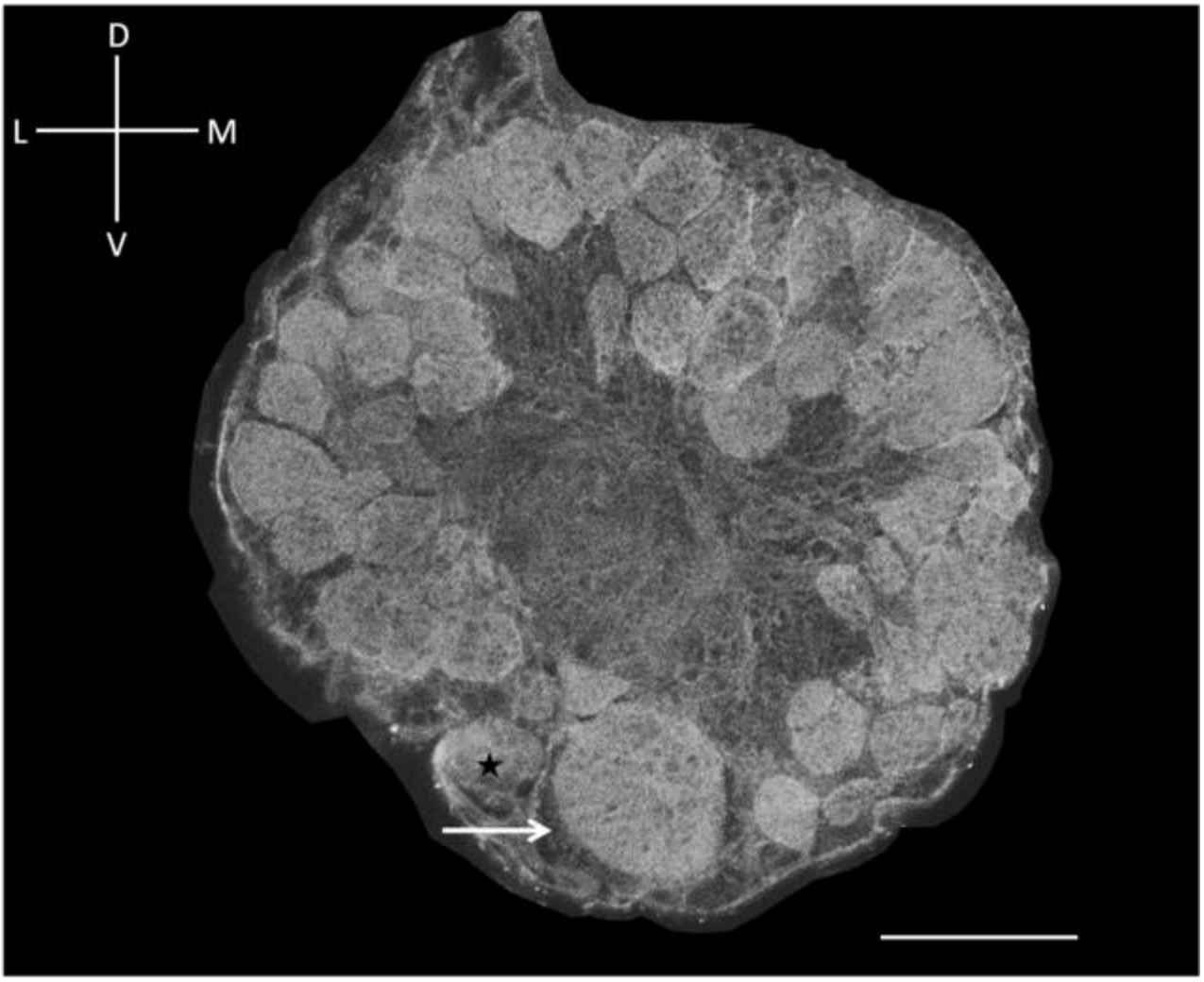
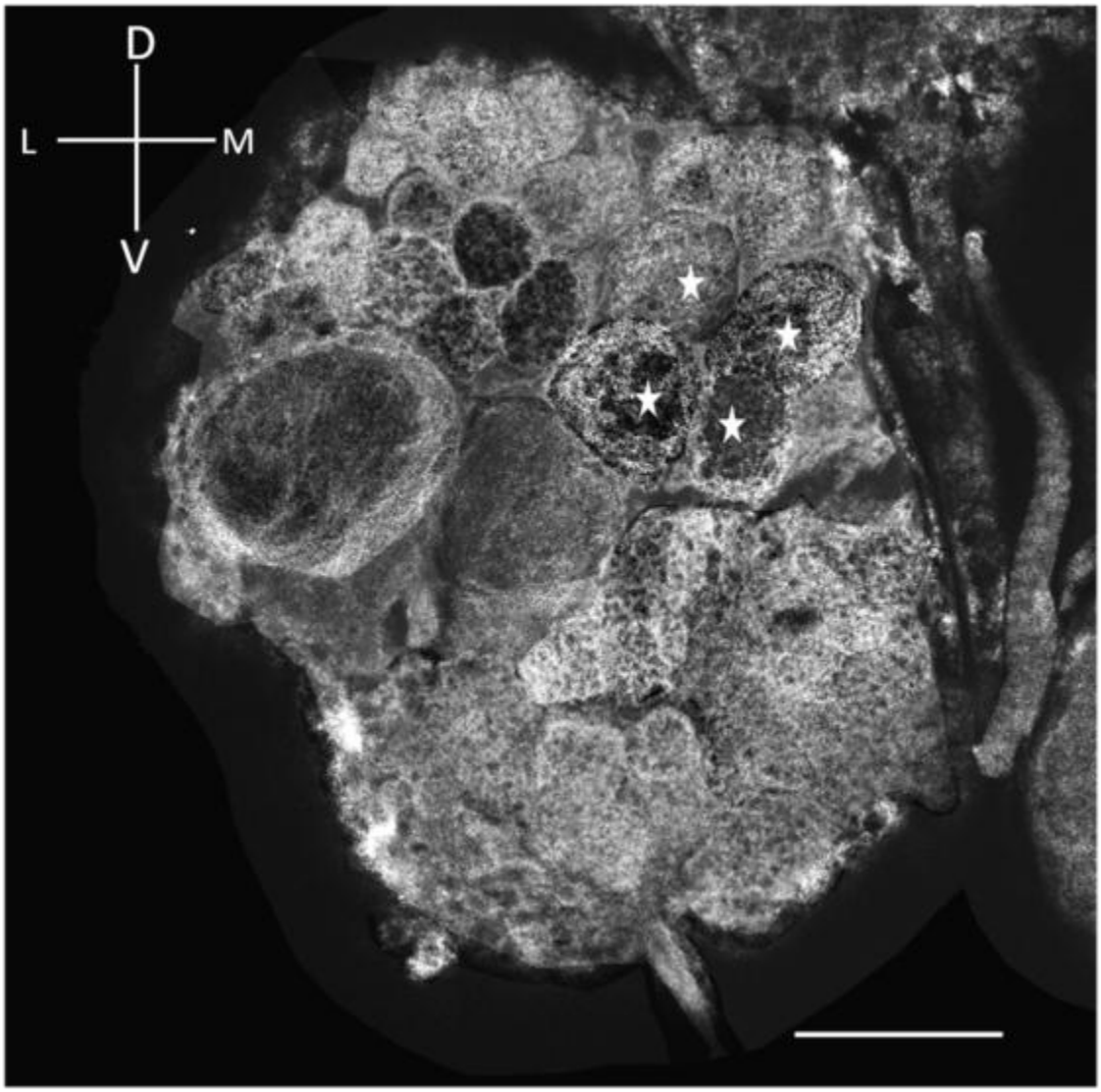


Fig. 7



CHAPTER 5

PROCESSING OF ODOR IN PARASITIC WASPS: GLOMERULAR PROJECTIONS OF OLFACTORY RECEPTOR NEURONS RESPONDING TO SINGLE AND MIXTURES OF HOST-PLANT VOLATILES IN THE ANTENNAL LOBE OF *MICROPLITIS CROCEIPES* AND *COTESIA MARGINIVENTRIS*

5.1. Introduction

Perception of a complex odor blend is important and is essential for the remarkably specific food odor, mate finding and host specificity in insects. Olfaction is an important sensory modality and olfactory system help in detecting odors released by oviposition sites/hosts, conspecifics and food sources. Olfactory system in insect consists of a pair of antenna and olfactory sensilla on the antenna play a major role in perceiving odors. Odor detection in insects is performed by olfactory receptor neurons (ORNs) housed in olfactory sensilla. The antennal receptor neurons (RNs) form antennal nerves and projects to the antennal lobes (AL) or primary odor processing center. The glomeruli in the antennal lobe receive inputs from ORNs and each glomerulus is considered to process single odor or specific group of odorants. ORNs express one or few membrane receptors responsive to specific set of odorants and innervate a glomerulus or set of glomeruli in the antennal lobe (Gao et al. 2000; Vosshall et al. 2000; Couto et al. 2005; Hallem et al. 2006; Hallem and Carlson, 2006).

Most studies on mechanism of odor processing have been conducted in moth, fruit fly and honey bee has shown that, individual odors are processed in different glomeruli in the antennal

lobe (Christensen and Hildebrand, 2002; Christensen and White, 2000; Hansson et al. 1991; Anton and Homberg, 1999). Parasitic wasps (Hymenoptera) or parasitoids use various types of plant based volatiles as cues for foraging and location of their herbivore hosts (Dicke and Sabelis, 1988; Turlings et al. 1990; McCall et al. 1994; De Moraes et al. 1998; Chen and Fadamiro, 2007). Like other insects, the antennal lobe (AL) is the primary odor processing center in the parasitoid brain. However, the mechanism of odor processing in the antennal lobe of this important group of insects remain poorly understood, despite the increasing interest in their use as biological pest control agents. A current evolutionary paradigm regarding host-related odor detecting machinery is that how signals processed by a parasitoid to successfully locate its host associates with its level of specialization (Chen and Fadamiro, 2007; Ngumbi et al. 2009; 2010). Research by our group (Chen and Fadamiro, 2007; Ngumbi et al. 2009, 2010) has focused on olfactory communication in two parasitoids (Hymenoptera: Braconidae) with different degrees of host specificity, *Microplitis croceipes* (Cresson) and *Cotesia marginiventris* (Cresson). *Microplitis croceipes* is a specialist larval parasitoid of *Heliothis* spp., whereas *C. marginiventris* is a generalist larval parasitoid of several caterpillar genera including *Heliothis*, *Spodoptera* and Pieridae spp. The antennal morphology of both species has revealed important differences in abundance of antennal olfactory sensilla (Das et al. 2011). In a recent study, the morphology of antennal lobe and glomerular number has indicated differences between these two parasitoid species (Das and Fadamiro, in press), and the olfactory receptor neurons (ORNs) responding to host-related volatiles exhibited differential responses in these two parasitoids (Das and Fadamiro, unpublished). Thus, it is important to identify the ORN projections targeting glomeruli in the AL, and the activity of individual glomeruli processing volatiles in these two parasitoids.

To possibly shed light in the underlying mechanisms of host-specificity in a specialist and generalist parasitoid, the present study was conducted by mapping the pathway of olfactory receptor neurons (ORNs) targeting glomeruli in the antennal lobe. With differential ORN responses housed in sensilla placodea of these two parasitoids, we hypothesized that there may be a dissimilarity in spatial arrangement of glomeruli processing odors and difference in the activity of individual glomeruli processing odors between these parasitoids. In the present study, the findings are discussed in relation to the ORN responses to host-related odors (Das and Fadamiro, unpublished) between these two parasitoid species.

5.2 Materials and Methods

5.2.1 Insects. The parasitoids *M. croceipes* and *C. marginiventris* were provided by the USDA-ARS, Crop Protection and Management Research (Tifton, Georgia) and the Department of Entomology, University of Georgia (Tifton campus, Contact: John Ruberson), respectively. *Microplitis croceipes* was reared on the larvae of *Heliothis virescens* Fab, while *C. marginiventris* was reared on *Spodoptera exigua* (Hübner) larvae. Colonies of both parasitoid species were maintained in the laboratory as previously reported (Lewis and Burton, 1970; Das et al. 2011). Caterpillars of *H. virescens* and *S. exigua* were reared on laboratory prepared pinto bean diet (Shorey and Hale, 1965) and maintained at 25 ± 1 °C, 75 ± 5% r.h. and 14:10 L:D photoperiod. Newly emerged adult parasitoids were collected and both sexes were kept together in cages for mating. Ten percent sugar solution and water soaked in cotton balls were provided in small transparent cups as food. For moth species, sugar solution and water was provided to newly emerged moths in 25ml conical flasks by placing a cotton absorbal wick (Wheat Ridge, CO, USA)

at the center. To avoid moth being trapped inside the flask, the mouth was sealed with Parafilm.

5.2.2 Host-related compounds for backfilling of ORNs and glomeruli with neurobiotin.

The olfactory receptor neurons housed in sensilla placodea in *M. croceipes* and *C. marginiventris* were characterized using single sensillum recordings by stimulating with volatiles (Das and Fadamiro, unpublished data). A few selected volatiles and mixtures are used for tracing AL glomeruli in this study. In *M. croceipes*, green leaf volatiles (GLVs) *cis*-3-hexenol and hexanal, herbivore induced plant volatile (HIPVs) *cis*-3-hexenyl butyrate and linalool, host major sex pheromone component Z11-16Ald and ecologically irrelevant compound benzaldehyde, elicited response in the ORNs in sensilla placodea. A mixture of *cis*-3-hexenol and *cis*-3-hexenyl butyrate enhanced the activity of the ORNs present in the sensillum. Linalool, a herbivore induced plant volatile (HIPV), as a single compound showed excitatory effect in ORNs. However, ORN response to GLV *cis*-3-hexenol was inhibited by linalool in a mixture (*cis*-3-hexenol + linalool), and hence suppressed the ORN activity in *M. croceipes*. In *C. marginiventris*, *cis*-3-hexenol and hexanal (both GLVs), *cis*-3-hexenyl acetate and linalool (both HIPVs), host major sex-pheromone component Z11-16Ald, and ecologically irrelevant compound benzaldehyde elicited response in the olfactory neurons as individual compound. The ORN activities in *C. marginiventris* were enhanced by a mixture of *cis*-3-hexenol (GLV) and *cis*-3-hexenyl acetate (HIPV). To trace the target glomeruli of ORNs responding and to observe the activity of different glomeruli in response to different volatiles, individual compounds and mixtures were used in this study to stimulate the ORNs to backfill using neurobiotin.

To map the pathway of olfactory receptor neurons (ORNs) responded to the selected

volatiles, anterograde backfilling using neurobiotin was performed. Anterograde backfills of single sensilla was modified after Ghaninia et al (2007). After obtaining successful responses from ORNs in small sensilla placodea to different odors, the reference and recording electrodes were removed and a glass capillary recording electrode was placed on the electrode holder attached to the micromanipulator. The glass capillary was one-fourth filled with 1% neurobiotin in 0.25 m KCl from the tip. The electrode was placed into the base of a sensillum. To stain the ORNs with neurobiotin, individual compounds (10µg dose) and mixtures that elicited characteristic response was applied on the antenna at intervals to stimulate (0.05 s pulse duration) the ORNs in the sensillum. Parasitoid was then removed from the setup and kept at 4 °C for 3-4 h, to permit the neurobiotin to diffuse into the olfactory neuron (s). The head was then detached from the insect body and fixed overnight at 4 °C in 4% formaldehyde in Millonig's buffer containing 0.25% Triton X-100 (M-0.25Tx). The parasitoid brain was dissected out in M-0.25Tx, and then washed four times for 10 min each in M-0.25Tx. After washing, the tissue was incubated in Alexa avidin 488, Alexa 546 Phalloidin (Molecular Probes) and M-0.25Tx (5: 3: 200) at 4 °C for 2 days. The brain sample was then rinsed 4 times, 10 min each in M-0.25Tx and mounted in Depex (Fluka). Spacers were used to protect the brain tissue from pressure of cover slip.

5.2.3 Confocal imaging. The brain samples were examined with Nikon A1 confocal laser-scanning microscope, equipped with argon/krypton laser. The Argon laser 488nm excited Alexa 488 molecules and signals from structures labeled were detected by 505nm long-pass filter. The 543 HeNe laser excites the Alexa 546 Phalloidin and signals were detected using 560 nm high pass filter to visualize the Phalloidin labelled structures. The images of antennal lobe slices were

obtained in stack of 100-150 images (0.5 µm thick) scanned using 60x 1.4 oil immersion DIC objective lens with a resolution of 1024 x 1024 pixels.

5.3 Results

In order to map the odor pathway of olfactory neuron projections to target glomeruli in the antennal lobe, 124 brains were attempted for anterograde staining of ORNs to single compounds and mixtures. Out of attempted staining, 29 preparations successfully showed ORNs and glomeruli labeling. ORNs which are stimulated by compounds showed staining and activated specific glomeruli. In *M. croceipes* (a specialist), glomeruli stained are located in the medial half of the antennal lobe. Single compound *cis*-3-hexenol (GLV) and *cis*-3-hexenyl butyrate (HIPV) elicited strong stimulation in one ORN in sensilla placodea (Das and Fadamiro, unpublished). The ORN responding to *cis*-3-hexenol sends projection and activated single glomeruli VM2 (Fig. 1a) in the ventro-median region, and ORNs stimulated by *cis*-3-hexenyl butyrate activated PM1 in the postero-median (Fig. 1d). Hexanal elicited strong stimulation in two neurons and two adjacent glomeruli are activated, AM1 and AM2 (Fig. 1b), in the antero-median region in *M. croceipes*. In response to linalool, one neuron is specifically stained that sends projection to glomerulus PD1 in the postero-dorsal layer (Fig. 1c). In contrast, glomeruli in *C. marginiventris* are stained in the lateral half of the antennal lobe. Green leaf volatile (GLV) *cis*-3-hexenol and herbivore-induced plant volatile (HIPV) *cis*-3-hexenyl acetate stimulated single neuron. Glomerulus VL1 in the ventro-lateral region (Fig. 1e) is activated by *cis*-3-hexenol and VL4 by *cis*-3-hexenyl acetate (Fig. 1h). Stimulation by hexanal activated one ventro-lateral glomerulus VL2 (Fig. 1f), and further the neuron showed projection into glomeruli VL4 which is stimulated by *cis*-3-hexenyl acetate;

however, VL4 is not activated to hexanal. Linalool activated glomeruli VL5 in the ventro-lateral region (Fig. 1g) in *C. marginiventris*.

A mixture of GLV *cis*-3-hexenol and HIPV *cis*-3-hexenyl butyrate are chosen to elucidate the activity and to locate if any different glomeruli are recruited for processing of mixture in *M. croceipes*. The ORN projects to the respective glomerulus for these two compounds (VM2 & PM1), and showed enhanced activation by the mixture (Fig. 2c). There was no recruitment of different glomeruli. However, the excitation by *cis*-3-hexenol (GLV) in glomerulus VM2 is inhibited (Fig. 3c) in the presence of linalool (HIPV) in a mixture in *M. croceipes*. In contrast, in *C. marginiventris*, a mixture of *cis*-3-hexenol and *cis*-3-hexenyl acetate exhibited intense labeling of glomeruli VL1 followed by VL4, activating their respective glomeruli (Fig. 4c); no different glomeruli were activated for this mixture.

5.4 Discussion

The results revealed differences in odor processing and spatial arrangement of different glomeruli stimulated by green leaf volatiles (GLVs), herbivore induced plant volatiles (HIPVs) and mixtures. The major difference was the processing of volatiles in different glomeruli located in opposite regions of the antennal lobe in these two parasitoids. In *M. croceipes* (specialist), GLVs, HIPVs, and mixtures are processed in the medial half of the antennal lobe, whereas in *C. marginiventris* (generalist), the host-related compounds are processed in the lateral half. Due to the differences observed in the olfactory neuron responses (using single unit electrophysiology) to host-related volatiles (Das and Fadamiro, unpublished), an assumption is made with the possibility of differential processing of GLVs and HIPVs in the antennal lobe inboth parasitoid. We found no

specific region in the antennal lobe separately processing GLVs and HIPVs in these two parasitoid species.

This is the first study which has compared the olfactory neuron projections targeting different glomeruli in the antennal lobes of parasitoids with different degrees of host-specificity (specialist and generalist). In *M. croceipes* (specialist), stimulation by *cis*-3-hexenol (GLV) stained one neuron which sends projection to single glomerulus (VM2) in the ventro-median region. Similarly, *cis*-3-hexenyl butyrate (HIPV) elicited strong stimulation in ORN (Das and Fadamiro, unpublished) and activated PM1 glomerulus in the postero-median region. The odor processing in the antennal lobe of these two parasitoids are in association with the reports on the other insects where individual odorant is processed in distinct glomerular units (Gao et al. 2000; Vosshall et al. 2000; Couto et al. 2005; Hallem and Carlson, 2006). Green leaf volatile (GLV) hexanal elicited strong stimulation in two neurons and two adjacent glomeruli are activated, AM1 and AM2, in the antero median region in *M. croceipes*. Among other known GLVs, hexanal might play an important in *M. croceipes* for specific host-volatile recognition which is processed in two adjacent glomeruli. A suggestion may be made that these two glomeruli might be responsible for associative processing of diverse ratios of hexanal emitted by plants damage by hosts. The perceived odor quality may depend on odor concentration as known from experiments in *Drosophila* (Stensmyr et al. 2003). Further investigation to measure the activity of hexanal ratios on the respective glomeruli is necessary for affirmation. Linalool activated glomerulus PD1 in the postero-dorsal layer of the antennal lobe in the medial half in *M. croceipes*. On the contrary, volatiles are processed in the lateral half in both sexes of *C. marginiventris* and in the opposite region of the antennal lobe compared to *M. croceipes*.

Cis-3-hexenol (GLV) and *cis*-3-hexenyl acetate (HIPV) activated single glomeruli VL1 and VL4 in the ventro-lateral region. The most striking feature was observed in *C. marginiventris*. A single neuron with strong stimulation by hexanal was specifically stained which sends projection to ventro-lateral glomerulus VL2, and the neuron further innervated another glomerulus VL4. Interestingly, VL4 in the ventro-lateral region was activated by *cis*-3-hexenyl acetate. Therefore, a neuron innervating two glomeruli highlight a clear connection and might suggest a possible interaction of odor processing between these two glomeruli, VL2 (for hexanal) & VL4 (for *cis*-3-hexenyl acetate). Linalool activated single glomerulus VL5 in the ventro-lateral region in *C. marginiventris*.

Olfactory neurons showed enhanced response to a mixture of *cis*-3-hexenol and *cis*-3-hexenyl butyrate in *M. croceipes* (Das and Fadamiro, unpublished data). The mixture of these two volatiles enhanced the activation of glomerulus VM2 (Fig. 2c) processing *cis*-3-hexenol in *M. croceipes*. Similarly, in *C. marginiventris*, *cis*-3-hexenol and *cis*-3-hexenyl acetate enhanced the activity of glomeruli VL1 (Fig. 4c) which process *cis*-3-hexenol. Mixture of two compound interactions at the glomerular level did not recruit any new glomeruli for mixture processing in both parasitoid species. In another mixture, linalool inhibited the neuronal response to green leaf volatile *cis*-3-hexenol in specialist *M. croceipes*, and hence the activity of glomerulus VM2 showed suppression with this mixture. Therefore, it is clear that linalool had an inhibitory effect on *cis*-3-hexenol at neuronal and at glomerular level in the specialist *M. croceipes*. Inhibition may provide necessary information for signal processing, *M. croceipes* may facilitate to detect volatiles induced by hosts from their host plants upon inhibition by linalool. These dissimilarities in the spatial arrangement of glomeruli processing individual odors and mixtures of host-related

compounds might explain the underlying mechanism for host specificity in a specialist and generalist parasitoid which help to locate and identify specific hosts.

Odor-specific activation patterns in primary olfactory center (antennal lobe) were illustrated for a number of insects (Rodrigues 1988; Vickers et al. 1998; Hansson et al. 2003; Carlsson et al. 2005), but no studies have been attempted in parasitic wasps. Activation pattern of different glomeruli did not exhibit any overlapping to different host-related volatiles in this study. My findings suggests that among all single compounds and mixtures, each glomerulus is a functional recruitment for processing specific volatile compound in the antennal lobe in these two parasitoids, with an exception of ORNs detecting hexanal (GLV) that send projections to two adjacent glomeruli in *M. croceipes*. Similar findings has been reported in moths that ORNs responding to sex pheromone components terminates in macroglomerular complex to distinct glomerular units (Hillier et al. 2005; Lee et al. 2006), in fruit fly *Drosophila* for specific food odors (Gao et al. 2000) to distinct glomeruli, and also in mosquito *Aedes aegypti* (Ghaninia et al. 2007). The difference in spatial representation and odor processing (excitation or inhibition) of the compounds in different glomeruli and possible inter-glomerular interaction in the antennal lobe of the specialist and generalist parasitoid might be essential to facilitate coding of complex host-related volatile blends to identify and locate specific hosts.

This study presents a pathway of odor processing in *M. croceipes* (a specialist) and *C. marginiventris* (a generalist) to investigate ORN arborizations to destination glomeruli in the antennal lobe, which might shed light on the differences in odor processing at glomerular level to correlate with difference in host specificity. The results support my prediction showing dissimilarities in compound representation in different glomerular region of antennal lobe, and

differential processing of volatile mixtures might help a specialist and generalist parasitoid to identify blends induced by specific host plants after an insect attack. Mapping the projections of olfactory neurons targeting specific glomeruli for selected host-related volatiles is a preliminary attempt to understand the spatial representation of volatiles in the antennal lobe of these two parasitoids. Functional characterization of other glomeruli in the antennal lobe is essential to understand the processing of compounds, which might help to understand how parasitoids make narrow and broad host preferences.

5.5 Acknowledgement

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Figure Legends

Fig 1. Olfactory receptor neurons staining by anterograde backfilling in sensilla placodea. **a-d** Neuronal projection patterns of ORNs housed in sensilla placodea responding to GLVs and HIPVs activating different glomeruli in *M. croceipes*. **(a)** *cis*-3-hexenol elicited response in one ORN projecting to VM2 glomerulus in the ventro-median region **(b)** stimulation by hexanal resulted in staining of two neurons projecting to adjacent glomeruli, AM1 and AM2, and HIPVs **(c)** linalool activated single glomerulus PD1 **(d)** cis-3-hexenyl butyrate stimulated single neuron and activated single glomerulus PM1; **e-h** Olfactory neuron projections in sensilla placodea responding to GLVs and HIPVs, and activation of different glomerulus in *C marginiventris*. **(e)** glomerulus VL1 was activated with stimulation of single neuron by cis-3-hexenol **(f)** hexanal stimulated one neuron innervated glomeruli VL2 and VL4, glomeurlus VL2 was labeled **(g)** Linalool stained one neuron and stimulated single glomerulus VL5 **(h)** HIPV cis-3-hexenyl acetate activated single glomerulus VL4.

Fig 2. Comparing glomerular activity of single compounds and mixture in *M. croceipes*. **(a)** GLV *cis*-3-hexenol stimulated glomerulus VM2 **(b)** HIPV *cis*-3-hexenyl butyrate activated glomerulus PM1 **(c)** mixture of *cis*-3-hexenol and *cis*-3-hexenyl butyrate activated same glomeruli VM2 and PM1; no different glomeruli was recruited for processing of mixture.

Fig 3. In *M. croceipes*, comparing glomerular activity of single compound and mixture **(a)** GLV *cis*-3-hexenol stimulated glomerulus VM2 **(b)** HIPV linalool activated glomerulus PD1, and **(c)** mixture of *cis*-3-hexenol and linalool suppressing the activity of glomeurlus VM2 processing *cis*-3-hexenol.

Fig 4. In *C. marginiventris*, glomerular activity of single compound and mixture using anterograde staining with neurobiotin **(a)** GLV *cis*-3-hexenol stimulated glomerulus VL1 **(b)** HIPV *cis*-3-hexenyl acetate activated glomerulus VL4, and **(c)** mixture of *cis*-3-hexenol and *cis*-3-hexenyl acetate activated same glomeurli VL1 and VL4; VL1 showed more activation with differential labeling.

Fig. 1

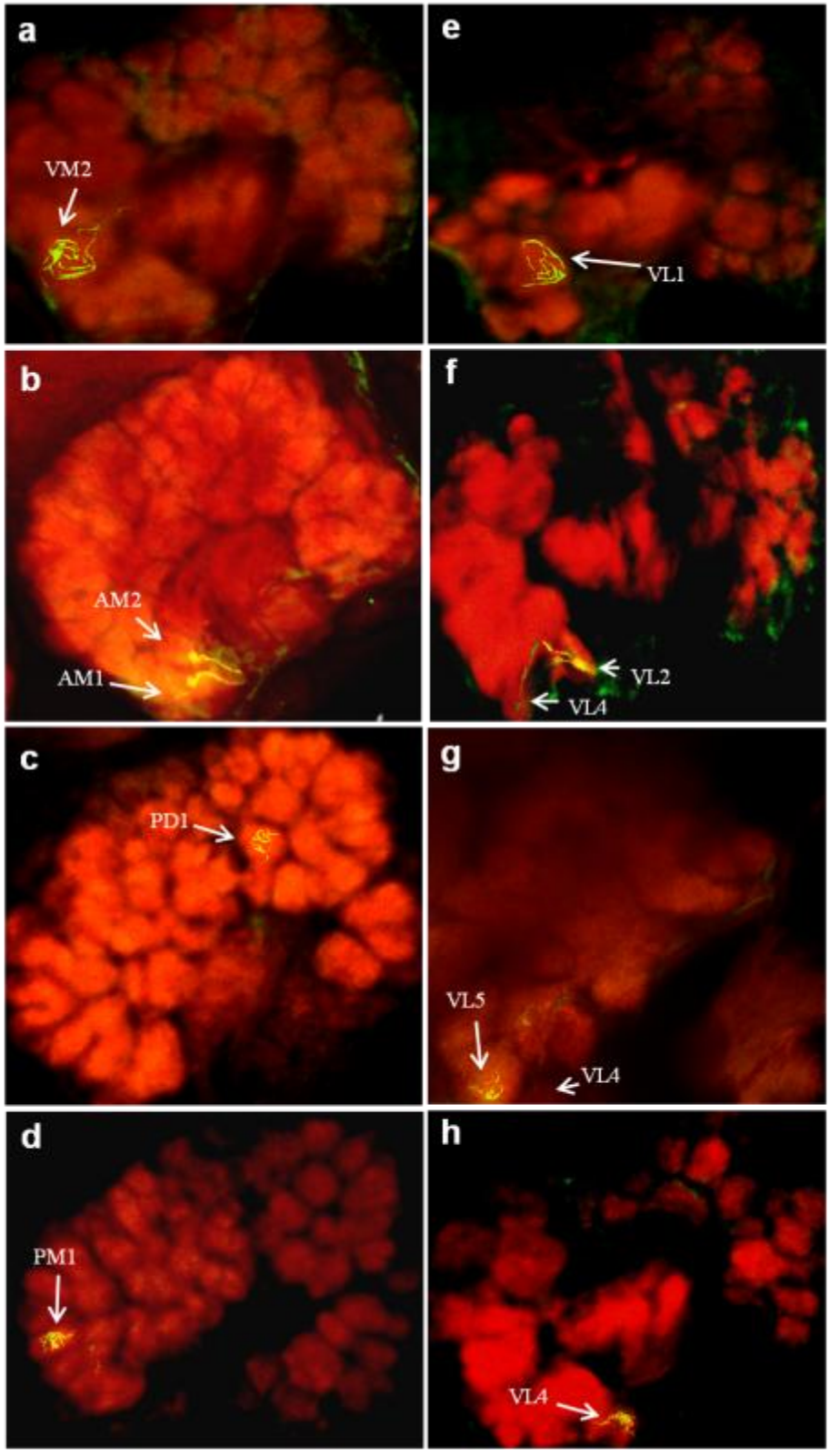

Fig. 2

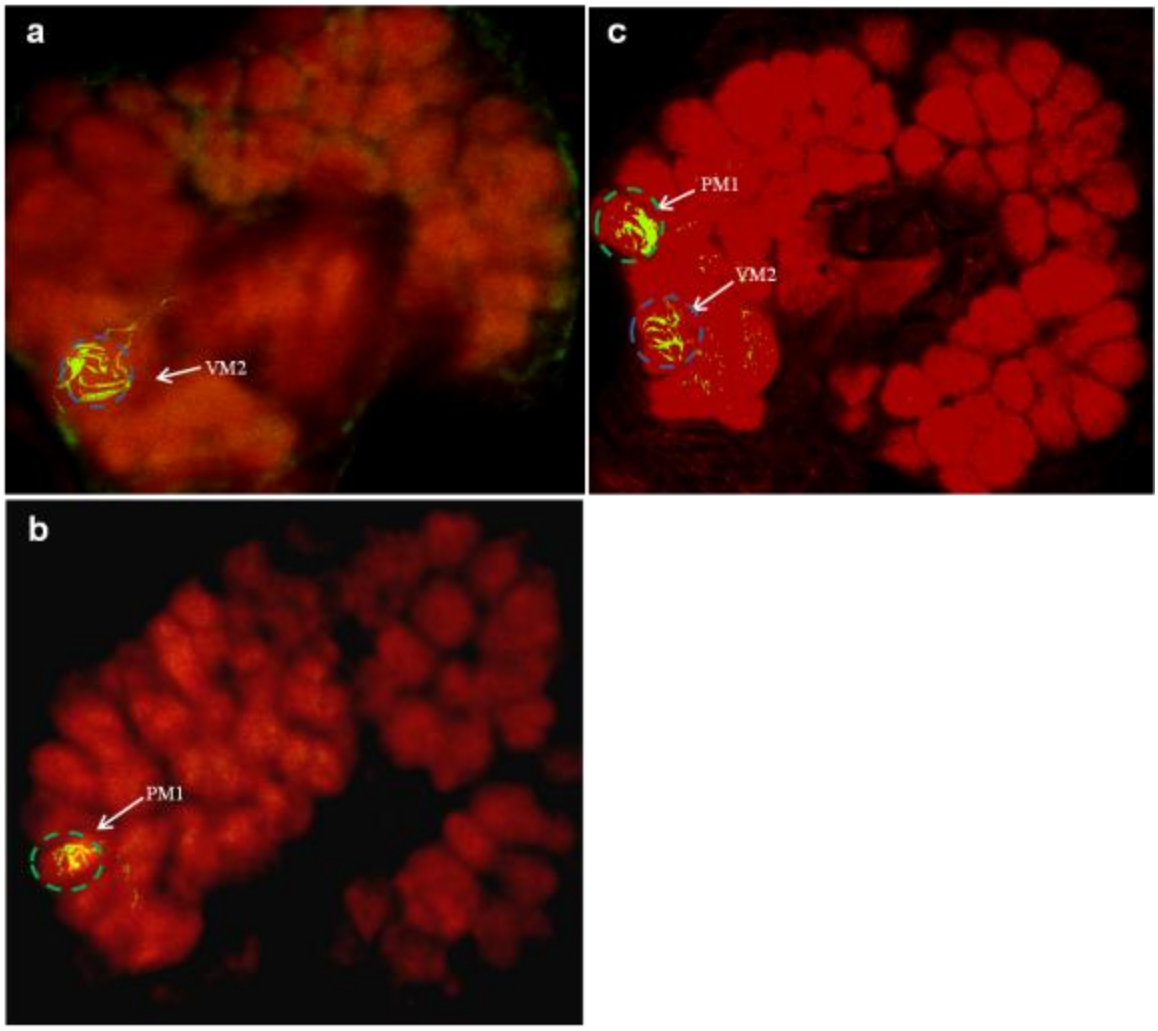

Fig. 3

Fig. 4