

**Behavioral Responses of Two Parasitic Wasp Species with Different Degree of Host Specificity to Host-Related Plant Volatiles**

by

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A thesis submitted to the Graduate Faculty of  
Auburn University  
in partial fulfillment of the  
requirements for the Degree of  
Master of Science

Auburn, Alabama  
December 14, 2013

Keywords: *Microplitis croceipes*, *Cotesia marginiventris*, Specialist & Generalist, Host location, Plant volatiles, Cotton, Four-choice olfactometer

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## Abstract

As an indirect defense to herbivore attack, plants release many types of volatile organic compounds (VOCs), which guide parasitoids to their herbivore hosts. Plants may release constitutive volatiles or synthesize new ones as an induced response to herbivore damage. Several studies have tested the attraction of various natural enemies to both synthetic VOCs and natural plant odors, but few have compared the behavioral responses of specialist and generalist parasitoids with varying degree of host specificity to various plant odors. This study was conducted to test the attraction of two parasitoids, *Microplitis croceipes* (specialist) and *Cotesia marginiventris* (generalist) to synthetic VOCs and natural plant odors. The goal of the study was to address the evolutionary and mechanistic question of whether specialist and generalist parasitoids differ in their use of plant volatiles for host location. Both species are solitary larval endoparasitoids in the same family (Hymenoptera: Braconidae) and are important parasitoids of *Heliothis virescens* (Lepidoptera: Noctuidae) and other caterpillar pests of cotton.

In chapter II, VOCs were categorized as those released passively from undamaged plants (UD-VOC) and herbivore-induced plant volatiles (HIPVs). HIPVs were further categorized into: i) volatiles released by fresh damage plants (FD-VOC), and ii) volatiles released by old damage plants (OD-VOC).  $\alpha$ -pinene (UD-VOC), (Z)-3-hexenol (FD-VOC) and (Z)-3-hexenyl acetate (OD-VOC) were selected as representatives of the different VOC types based on GC-MS and behavioral results from previous studies. The attraction of both parasitoid species to synthetic VOCs and a binary mixture were tested in four-choice olfactometer bioassays. Female *M.*

*croceipes* (specialist) showed the greatest attraction to the HIPVs while female *C. marginiventris* could not discriminate among the VOC types. Comparing species, female *M. croceipes* were significantly more attracted than female *C. marginiventris* to (Z)-3-hexenol. In contrast, female *C. marginiventris* showed significantly greater attraction to  $\alpha$ -pinene compared to female *M. croceipes*. Conspecific males showed similar responses with a few exceptions. When presented with the choices;  $\alpha$ -pinene, (Z)-3-hexenol and a binary mixture (50:50v/v) of the two compounds, the specialist showed the greatest attraction to the mixture. The mixture did not elicit such an additive effect on the attraction of the generalist. Species and sexual (in the specialist) differences were recorded in the overall response latency (time taken to choose VOCs). The ecological significance and practical implications of these results are discussed.

In chapter III, the responses of both sexes of the two parasitoid species to VOCs emitted by cotton plants infested by host *H. virescens* larvae were investigated using a headspace volatile collection system coupled with-four choice olfactometer bioassay. The advantage of this set up is that it allows for direct bioassay of parasitoids to the headspace volatiles emitted by treatment plants and subsequent analysis by GC-MS, thus providing a possible direct explanation for the observed responses. The treatments tested were undamaged plants (UD), fresh (6 hr infestation) damage plants (FD), and old (24 hr infestation) damage plants (OD). Both sexes of *M. croceipes* showed a preference for VOCs from host-damaged plants (FD- and OD-plants) over UD-plants, In contrast, female *C. marginiventris* could not discriminate among UD-, FD- and OD-plants, whereas the males showed a preference for FD-plants. GC-MS analyses showed qualitative and quantitative differences in the VOC profiles of UD-, FD- and OD-plants which may explain the behavioral responses of the parasitoids. The ecological significance and practical implications of the results are discussed.

## Acknowledgements

I would like to thank my major professor, Dr. Henry Fadamiro, for giving me the opportunity to work on this interesting project. His patience, understanding, support and invaluable advisory role kept me going through the challenging phases of this study. He took a step further from being an advisor to becoming a mentor. From the very first time that I thought about having Dr. David Held and Dr. Arthur Appel on my committee, I knew they were the best people to consult when in doubt. I appreciate their accessibility and readiness to help all the time. When I came to Auburn, it was my first time travelling out of my home country but I give much thanks to Dr. Joseph Anikwe who made Auburn feel like home away from home. During my first few weeks of wondering around the lab, Dr. Clement Akotsen-Mensah took time to show me the basic steps in conducting four-choice olfactometer bioassays. I needed to master the art and science of using the olfactometer for this study so I thank him for introducing it to me. When I had to design a new olfactometer, I showed the prototype to Dr. Fadamiro and Dr. Rammohan Balusu who gave very helpful suggestions that led to the construction of the olfactometer used for this study. In the beginning when my attitude was not well aligned with the culture of graduate study, Kate Nangle was there to remind me of my expectations. I have also enjoyed working with Erica Williams, Matthew McTennan and Savannah Duke who were undergraduates that assisted in rearing the insects used for this study. In addition, I thank every member of Fadamiro's lab for their support and friendliness. I would not have been a balanced

student without the love shown by friends and family. In particular, I would like to thank Stephanie Daniels, my fiancée for her constant support and encouragement. Thank you all.

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# CHAPTER 1

## INTRODUCTION AND LITERATURE REVIEW

### 1.1 Parasitoids

Parasitoids are one of the most fascinating and widely studied group of natural enemies due to the variety of adaptive life strategies they exhibit. Some of these strategies include ovigeny (proportion of lifetime eggs that is matured on female emergence), host feeding (consumption of host tissue/hemolymph by adults) and egg resorption (reallocation of oocyte materials for body maintenance). Variations also exist in the developmental modes of parasitoids. These include idiobiont/ kionobiont, solitary/gregarious, and ectoparasitoid/ endoparasitoid (Jervis & Kidd 1986; Quicke 1997; Gilbert & Jervis 1998; Harvey & Strand 2002; Wiman & Jones 2013). A successful parasitization leads to the exploitation of host's internal resources, and ultimately death. Various developmental stages of insects can be utilized by different parasitoids. Furthermore, parasitoids can be classified as specialists (having a restricted host range) or generalists (having a broad host range). Clearly, some of these variations also influence their interactions with host plants and insects. Parasitoids possess a relatively efficient olfactory mechanism and have been considered good models for insect olfaction studies (Meiners *et al.* 2002; Rains *et al.* 2004; Harris *et al.* 2012). In the present study, the two parasitoids used as models are both solitary, koinobiotic, larval endoparasitoids. However, they differ in their degree of host specificity.

## 1.2 Plant Defense and Host Location in Parasitoids

In nature, plants and herbivorous insects engage in a constant arms race for survival (a classical example of coevolution). The detrimental effects of the feeding activities of herbivores have created a pressure on plants to defend themselves. In turn, herbivores have developed various means of evading or neutralizing some of these defenses. Thus, natural selection will favor the survival of plants that have developed effective defensive traits to contain herbivore infestation and associated damages (Agrawal & Rutter 1998; Heil *et al.* 2000; Ness 2003). Chemical defense is one of the most effective strategies used by plants (Mortenson 2013). More so, this strategy is useful both at short and long ranges. Although secondary plant metabolites such as volatile organic compounds (VOCs) are not considered important to essential metabolic processes, there is a general consensus that they play important roles in plant chemical defense (Berenbaum 1996; Rasmann & Agrawal 2009). These defenses can be direct (e.g., oviposition deterrence) or indirect (e.g., recruitment of natural enemies) (Kessler & Baldwin 2001).

At the third trophic level, natural enemies such as parasitoids have probably evolved to use plant VOCs as cues that guide them to their herbivore hosts. As a result of their role in biocontrol of insect pests, studies investigating host location strategies in parasitoids have gained attention in recent years (Price *et al.* 1980; Cortesero *et al.* 1997; Steidle *et al.* 2003). In parasitoids, host foraging begins with an active search of host habitat and the host, a selective process that is mostly mediated by odor cues from plants. However, interferences from other olfactory, visual and acoustic stimuli exist in natural environments. For effective host location, parasitoids must develop strategies to use the most reliable cues available during the time of the day that they are most active (Turlings *et al.* 2005). Once in the micro-habitat of the host, parasitoids rely on other host-specific chemicals and visual cues for recognition and acceptance

of hosts. Studies on host location and acceptance as well as identification of stimuli involved in behavioral responses of parasitoids remain active fields of research (Godfray 1994; Quicke 1997).

### **1.3 Parasitoid Host Specificity & Use of Plant Volatiles for Foraging and Host Location**

Parasitoids can be broadly categorized as specialists (utilizing one or relatively few host species) or generalists (utilizing several host species). The degree of host specificity in parasitoids may affect their use of various VOCs for host location (Smid *et al.* 2002; Chen & Fadamiro 2007; Ngumbi *et al.* 2009, 2010, 2012). Depending on several factors including plant species, herbivore species, type and duration of damage, the composition of plant VOC profiles can vary (Hilker & Meiners 2002; Dicke *et al.* 2009). Most VOCs involved in plant defenses are products of the lipoxygenase pathway, shikimic acid pathway and terpenoid pathway (Pichersky & Gershenzon 2002). Undamaged plants constitutively release small amounts of certain VOCs which may attract parasitoids and herbivores seeking food (Wackers 2004). For example, undamaged cotton releases a few monoterpenes such as  $\alpha$ -pinene,  $\beta$ -pinene and myrcene (Loughrin *et al.* 1994; Rose & Tumlinson 2004; Magalhaes *et al.* 2012).

The release of stored compounds and additional VOCs with new identities is induced by the effects of mechanical damage and elicitors from oral secretions in attacking herbivores (Pare & Tumlinson 1997; Boland *et al.* 1998; Turlings *et al.* 1998; Rose & Tumlinson 2004). Within several minutes to few hours of herbivore damage, the amount of constitutively released VOCs increases. In addition, fresh damage plants release green leaf volatiles (GLVs), six-carbon alcohols, aldehydes and ketones. In cotton and several similar plants, hexanal, (*Z*)-3-hexenal and (*Z*)-3-hexenol are common GLVs emitted (Ngumbi *et al.* 2009; Magalhaes *et al.* 2012; Hagenbucher *et al.* 2013). GLVs are released by many plants across several taxa in response to

mechanical injury and or herbivore damage. Therefore, the signals they transmit may not be considered very reliable for specialist parasitoids. Instead, generalist parasitoids may use them to compensate for a broad host range.

The latter stage of damage (from 16-24 hr) is often characterized by a delayed release of several compounds that are mostly synthesized *de novo*. These include several acyclic terpenoids, aromatic compounds and other VOCs belonging to different chemical groups (Pare & Tumlinson 1999). In cotton, corn and other similar plants, these HIPVs include (*E*)- $\beta$ -ocimene, (*E*)- $\beta$ -farnesene, nonatriene and tridecatetraene, indole, hexenyl acetates, isomeric hexenyl butyrates and 2-methyl butyrates (Loughrin *et al.* 1994; McCall *et al.* 1994; Rose *et al.* 1996, 1998; De Moraes *et al.* 1998; Pare & Tumlinson 1999; Rose & Tumlinson 2004; Hagenbucher *et al.* 2013). The signals transmitted by VOCs in this group are considered to carry more host specific information (Pare & Tumlinson 1999; Hagenbucher *et al.* 2013). Thus, specialist parasitoids may show relatively greater attraction to these HIPVs (Ngumbi *et al.* 2010, 2012)

Several studies have tested parasitoid attraction to single VOCs in order to identify the specific compounds responsible for the recruitment of parasitoids (Thaler 2002; James & Price 2004; James & Grasswitz 2005; Wei *et al.* 2007; Ngumbi *et al.* 2012). However, there is still an ongoing debate on whether certain single components or the entire natural suite of plant odors elicit complete behavioral responses in parasitoids (van Wijk *et al.* 2011). In the present study, attraction of parasitoids to single compounds as well as natural odors from live plants was tested.

#### **1.4 Characterizing Behavioral Responses of Parasitoids to Host-Related Plant Volatiles**

Generally, behavioral responses of insects to olfactory stimuli can include attraction, repulsion or even neutrality. The Y- or T-tube olfactometer bioassays are among the techniques that have been in use for decades (Monteith 1955; Rotheray 1981; Wei & Kang 2006; Ngumbi *et*

*al.* 2012). They typically allow for comparing the response of parasitoids to a test odor from one arm and a control from the other arm. However, testing the preference of parasitoids between two or more treatment odors require the use of multi-choice olfactometers. Four-choice olfactometer (Patterson 1970) and six-choice olfactometer (Turlings *et al.* 2004) are commonly used in preference tests. More often, a constant airstream is supplied to carry the stimulus odors into a central chamber through the arms. To avoid mixing up of odors, air is sucked out of the system at a flow rate equal or greater than the sum of inlet flows. Discrete choices made by parasitoids are often recorded as counts or proportions.

In a Y- or T-tube olfactometer, the probability that an insect ends up in one of the arms by chance is 50 percent. The probability of this potential error is reduced to 25 percent in a four-choice olfactometer (Vet *et al.* 1983). The statistical advantages of using multi-choice olfactometer have been discussed by Vet *et al.* (1983) and Turlings *et al.* (2004). Furthermore, Davison & Ricard (2011) recently reviewed various models for analyzing data generated from olfactometer bioassays. Notwithstanding, the choice of olfactometer type to be used should depend on the objectives of the study. In the present study, modifications were made on existing four-choice olfactometer models to suite the innate behavior of test insects. In addition, both olfactometer and headspace volatile collection systems were coupled to allow for real time trapping of odors eliciting behavioral responses in parasitoids (see Turlings *et al.* 2004; Hoballah & Turlings 2005; Fontana *et al.* 2011). The advantage of this approach is that GC-MS analysis of headspace extracts may offer a direct chemical-based explanation for the behavioral responses observed in the parasitoids.



## 1.5 Model System

This study uses a tritrophic model which includes cotton (*Gossypium hirsutum* Var. Max 9), tobacco budworm, *H. virescens*, and two parasitoids with varying degree of host specificity, *M. croceipes* (specialist) and *C. marginiventris* (generalist). Cotton is an economically important crop in the United States and many other countries in the world. The crop plays host to several caterpillar pests, including *H. virescens*, a generalist herbivore on cotton, tobacco, flax, alfalfa, and many other field crops. Both *M. croceipes* and *C. marginiventris* are solitary larval endoparasitoids in the same family (Hymenoptera: Braconidae) and are important parasitoids of *H. virescens* (Lepidoptera: Noctuidae). So far, *M. croceipes* is known to naturally utilize only three host species, *H. virescens*, *Helicoverpa zea* and *H. subflexa* (Tillman & Laster 1995). This parasitoid species has shown remarkable capacity to discriminate various odors. At the finest level, Meiners *et al.* (2002) reported that *M. croceipes* was able to discriminate between aliphatic alcohols only differing in the length of carbon chain or position of functional group; and also between an alcohol and its corresponding aldehyde. On the other hand, *C. marginiventris* can utilize several noctuid host species including *H. virescens* (see Tillman 2001 for host range). Behavioral responses of both parasitoid species have been tested and characterized in various olfaction studies (Elzen *et al.* 1987; Navasero & Elzen 1989; Meiners *et al.* 2002; Turlings *et al.* 2004; Ngumbi & Fadamiro 2012; Sobhy *et al.* 2012; Harris *et al.* 2012).

## 1.6 Justification of the Study

In the past few decades, studies on host-parasitoid interactions have received significant attention. Parasitoids are promising biocontrol agents that can be incorporated into Integrated Pest Management (IPM) programs. Similar to other insects, parasitoids rely largely on the sense of olfaction for forging. However, certain aspects of olfactory communication in parasitoids are

yet to be fully elucidated. In particular, it has been shown that herbivore damaged plants release VOCs that guide parasitoids to their host as an indirect defense mechanism (Kessler & Baldwin 2001). However, there are still debates as to whether the whole suite of naturally emitted plant odors or certain single components in these blends elicit complete behavioral responses in parasitoids (van Wijk *et al.* 2011). In the present study, the attraction of parasitoids to single components, VOC mixtures and natural odors from plants was tested in separate bioassays to address this question.

With only few comparative studies on the behavioral responses of specialist and generalist parasitoids reported, further empirical studies are required to confirm the hypothesis that these broad groups of parasitoids have evolved divergent olfactory mechanisms. These studies have serious ecological and practical implications. The current evolutionary hypothesis is that host location tactics in parasitoids correlates with their degree of host specificity (Cortesero *et al.* 1997, Smid *et al.* 2002; Chen & Fadamiro 2007; Ngumbi *et al.* 2010, 2012). According to these studies, the species with a restricted host range (specialist) showed greater response to HIPVs, compared to the species with a broad host range (generalist). However, Geerveliet *et al.* (1996) and Smid *et al.* (2002) reported no difference in the responses of specialist and generalist parasitoids to host-related plant volatiles. Thus, further comparative studies are required.

According to Knudsen & Gershenson (2006), over 1700 VOCs have been identified from almost one hundred families of plants. Broadly, these VOCs can be categorized based on their chemical identity or functionality. Since the duration of herbivore infestation directly impacts the level of damage in plants, VOCs can be functionally grouped as those released by undamaged, fresh damage and old damage plants (Loughrin *et al.* 1994; McCall *et al.* 1994; Rose *et al.* 1996, 1998; De Moraes *et al.* 1998; Pare & Tumlinson 1999; Rose & Tumlinson

2004; Hagenbucher *et al.* 2013). However, very few studies have compared the responses of parasitoids to plants subjected to these treatment groups or their representative single VOCs. Hoballah & Turlings (2005) tested the attraction of naive *Microplitis rufiventris* and *C. marginiventris* to volatiles from fresh *versus* old damage maize. However, undamaged plants were not included in the treatment. Besides, ‘old damage’ plants were treated for only 6 hr and herbivore damage was simulated using caterpillar regurgitant in that study. Thus, the need for further studies extending the duration of ‘old damage’ to 24 hr using host larvae feeding to induce VOC emissions as would be expected in nature.

## **1.7 Thesis Goal and Outline**

The goal of this research was to study chemically-mediated, tritrophic interactions among plants, herbivores, and parasitoids. In particular, the tritrophic model system used consists of cotton, its key caterpillar pest, *H. virescens* and its parasitoids, *M. croceipes* (specialist) and *C. marginiventris* (generalist). This research seeks to characterize mechanisms of olfaction and behavioral responses to host-related plant volatiles in the two parasitic wasps. Behavioral and analytical techniques were used to answer the following key questions: i) is there a correlation between the degree of specialization in parasitoids and their behavioral response to host-related plant volatiles? ii) does the duration of plant damage by herbivore influence the attraction of specialist and generalist parasitoids to plant odors? iii) do male and female parasitoids respond differently to host-related plant volatiles? This study followed a stepwise order in the complexity of test odors presented to the parasitoids. First, attraction of parasitoids to single VOCs was tested. In the next set of experiments, a binary mixture of VOCs was included in the choices. Lastly, attraction to the entire suite of natural odors from plants was tested.

In chapter II, the attraction of both sexes of *M. croceipes* (specialist) and *C. marginiventris* (generalist) to  $\alpha$ -pinene (UD-VOC) and HIPVs [(*Z*)-3-hexenol (FD-VOC) and (*Z*)-3-hexenyl acetate (OD-VOC)] was tested in four-choice olfactometer bioassays. The goal of the experiments in chapter II was to test if *M. croceipes* and *C. marginiventris* differ in their use of various host-related plant volatiles for host location. In addition, a separate experiment was set up to test if the parasitoids will show preference for a binary mixture over single VOCs. In general, *M. croceipes* showed a preference for the HIPVs (FD-VOC and OD-VOC). On the other hand, *C. marginiventris* could not discriminate among the treatment odors. Numerically, (*Z*)-3-hexenol and  $\alpha$ -pinene were most attractive to *M. croceipes* and *C. marginiventris* respectively. Therefore, a binary mixture of  $\alpha$ -pinene and (*Z*)-3-hexenol (50:50 v/v) was included in a second set of experiments. When presented with the choices;  $\alpha$ -pinene, (*Z*)-3-hexenol and the binary mixture, the specialist showed the greatest attraction to the mixture. The mixture did not elicit such an additive effect on the attraction of the generalist. In general, *M. croceipes* (specialist) made choices faster than *C. marginiventris* (generalist). Female *M. croceipes* spent a longer time making choices than conspecific males.

In chapter III, the attraction of both parasitoid species to odors from undamaged cotton plants (UD), fresh (6 hr infestation) damage cotton plants (FD), and old (24 hr infestation) damage plants (OD) was tested in four-choice olfactometer bioassays. The olfactometer was coupled with headspace volatile collection chamber which allowed for simultaneous trapping of odors. The goal of the experiments reported in chapter III was to test if the duration of plant damage by *H. virescens* caterpillars influences the attraction of specialist and generalist parasitoids to odors from cotton. In general, the result showed that female *M. croceipes* (specialist) were more attracted to the host damaged (FD- and OD-) plants than to undamaged

(UD-) plants. On the other hand, *C. marginiventris* (generalist) could not significantly discriminate host damaged plants from undamaged ones. Interestingly, the two parasitoid species generally showed high attraction to FD-plant odors. Cotton headspace volatiles trapped from the different treatment plants were analyzed with GC-MS for identification and quantification of peaks. The analysis showed qualitative and quantitative differences in the VOC profiles of UD-, FD- and OD-plants, which offered possible chemical-based explanations for the observed responses in the parasitoids.

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## CHAPTER 2

### ATTRACTION OF TWO LARVAL PARASITOIDS WITH VARYING DEGREE OF HOST SPECIFICITY TO SINGLE COMPONENTS AND A BINARY MIXTURE OF HOST-RELATED PLANT VOLATILES

#### 2.1 Introduction

Natural enemies such as parasitoids, herbivore insects and their host plants interact in a complex tritrophic system in which herbivore infested plants release VOCs that can attract parasitoids. Host induced plant volatiles (HIPVs) are released by plants in response to herbivore infestation and may be used for host location by natural enemies such as parasitoids (De Moraes *et al.* 1998; Pare & Tumlinson 1999; Mumm & Hilker 2005; Wei & Kang 2006; Ngumbi & Fadamiro 2012). Plants may release constitutive volatiles or synthesize new ones as an induced response to attack (mechanical/ herbivore damage) (Pare & Tumlinson 1997; Boland *et al.* 1998; Rose & Tumlinson 2004). Only certain components of natural volatile blends are attractive or ecologically relevant to parasitic wasps, making the identification of specific VOCs that inform parasitoid behaviors a critical task (D'Alessandro & Turlings 2005; Hoballah & Turlings 2005; Schnee *et al.* 2006; van Dam *et al.* 2010). Therefore, parasitoids must fine tune their olfactory system to discriminate among several odors in order to exploit certain VOCs for host location. The degree of host specificity required may determine to what extent a parasitoid species may have to discriminate among plant VOCs. Studies have demonstrated attraction of some parasitoid

species to single components of plant VOCs both in the laboratory (Wei *et al.* 2007) and in the field (James & Grasswitz 2005). Others have reported the positive role of synthetic VOCs in recruiting natural enemies for plant defense (Thaler 2002; James & Price 2004). Indeed, most olfactory receptor neurons (ORNs) in insects only respond to one or very few chemical compounds (Kaissling 1986; Meiners *et al.* 2002; De Bruyne & Baker 2008).

However, natural odors from plants are rarely emitted as single compounds (Bargmann, 2006). VOCs that are not attractive to a parasitoid species may still contribute to the olfactory contrast that enhances attraction to other VOCs of interest in the mixture/blend. Thus, a mixture of plant VOCs may be more attractive than a single compound because it presents an odor context more similar to what obtains in nature (van Wijk *et al.* 2011). It is believed that the differences in various VOC blends may serve as important host recognition codes for natural enemies (De Moraes *et al.* 1998; Smith 1998; De Bruyne & Backer 2008). At the simplest level, the effect of natural plant volatile blends on the attraction of parasitoids can be demonstrated with binary mixtures of synthetic VOCs.

Parasitic wasps have been considered good models for insect olfaction studies (Meiners *et al.* 2002; Rains *et al.* 2004; Harris *et al.* 2012). Based on their relative host range, they can be broadly categorized as specialist or generalist. The question of whether the degree of host specificity affects odor discriminatory ability in parasitoids is yet to be fully answered. This question has serious ecological and evolutionary significance as it concerns the fitness of the two groups of parasitoids. In this study, the specialist parasitoid, *Microplitis croceipes* (Cresson) and the generalist parasitoid, *Cotesia marginiventris* (Cresson) were used as models to test the hypothesis that specialist and generalist parasitoids differ in their use of VOCs for host location. Both wasps are koinobiont, solitary larval endoparasitoids (Hymenoptera: Braconidae) of

*Heliothis virescens* (Fab.) (Lepidoptera: Noctuidae), an important pest of cotton. *M. croceipes* and *C. marginiventris* have been used in many behavioral olfactometer bioassays to study parasitoid attraction to plant VOCs (Navasero & Elzen 1989; Meiners *et al.* 2002; Olson *et al.* 2003; Turlings *et al.* 2004; Sobhy *et al.* 2012; Ngumbi & Fadamiro 2012).

In the present study, VOCs were categorized as those released passively from undamaged plants (UD-VOC) and herbivore-induced plant volatiles (HIPVs). HIPVs were further categorized into: i) volatiles released by fresh damage plants (FD-VOC), and ii) volatiles released by old damage plants (OD-VOC). In making the selection of test VOCs, results from previous studies (Loughrin *et al.* 1994; McCall *et al.* 1994; Rose *et al.* 1996, 1998; De Moraes *et al.* 1998; Rose & Tumlinson 2004; Ngumbi *et al.* 2009; Magalhaes *et al.* 2012) that have collected, identified and quantified VOCs from cotton headspace were considered.  $\alpha$ -pinene (UD-VOC), (Z)-3-hexenol (FD-VOC) and (Z)-3-hexenyl acetate (OD-VOC) were selected as representatives of broader categorizations of plant volatiles.  $\alpha$ -pinene is a constitutive monoterpene of cotton. During the earliest stages of herbivore damage, the quantity of  $\alpha$ -pinene emission increases (Loughrin *et al.* 1994). (Z)-3-hexenol is generally considered host induced in cotton. Like many GLVs, this VOC is usually released by cotton starting during the early stages (2- 6 hr) of herbivore damage (Mc Call *et al.* 1994; Penafior *et al.* 2011). (Z)-3-hexenyl acetate is also induced by herbivore damage in cotton. Mc Call *et al.* (1994) reported that (Z)-3-hexenyl acetate was the only GLV that was significantly detected in cotton during the late stages (16-24 hr) of host infestation. The three compounds have been associated with the attraction of parasitoids (Wei *et al.* 2007; Ozawa *et al.* 2008; Luzano *et al.* 2000; Yu *et al.* 2010; Ngumbi & Fadamiro 2012; Uefune *et al.* 2012, 2013). In addition to testing parasitoid attraction, the time taken to choose different VOCs (response latency) was also recorded in this study. The concept

of behavioral response latency to semiochemicals in insects has only been investigated in a few studies (Baker & Vogt, 1988; Ngumbi *et al.* 2012).

In this study, parasitoid attraction to select synthetic VOCs and a binary mixture of cotton volatiles was tested. Based on previous studies from our group (Chen & Fadamiro 2007; Ngumbi *et al.* 2009, 2010, 2012), it is hypothesized that the two parasitoid species will discriminate among single VOCs to varying extent, and that binary mixtures will generally be more attractive than single VOCs. The ecological significance and practical application of the results are discussed.

## **2.2 Materials and Methods**

**2.2.1 Insects.** *M. croceipes* and *C. marginiventris* were reared in our laboratory (Auburn University AL, USA) on *Heliothis virescens* larvae. The rearing procedures were similar to those described by Lewis and Burton (1970) and Ngumbi *et al.* (2009). Upon emergence, adult wasps were transferred to aerated plastic cages (~ 30 × 30 × 30 cm) and supplied with 10% sugar water. For parasitization, female wasps (2-5 days old) were supplied with 2<sup>nd</sup>-3<sup>rd</sup> instar larvae (caterpillars) of *H. virescens* in the ratio 1 female to 20 larvae. Mated, naïve (untrained) parasitoids (aged 2-5 days old) were used in the behavioral bioassays. Larvae of *H. virescens* were reared on pinto bean artificial diet (Shorey & Hale 1965). The general rearing conditions for all insects were 25 ± 1°C, 75 ± 5% RH and 14:10 h (L:D) photoperiod.

**2.2.2 Four-Choice Olfactometer.** The setup of the four choice olfactometer used for behavioral bioassays is as shown in Fig. 1. Consideration for the new design was partly due to studies by Turlings *et al.* (2004) and Ngumbi & Fadamiro (2012). The olfactometer used was made of glass and supported with a retort stand. The main piece has a spherical bulb 75 mm diameter from which four horizontally inclined arms 10 cm long projected upwards. At the base



of the bulb, a central tube 17 cm long extends downwards to form the entry route for insects. A 30 mm diameter hemispherical depression on top of the bulb (decision-making area) created a vantage position from which insects were evenly exposed to odor streams from all four arms. The VOCs tested were placed on filter paper strips (odor source) and inserted into the small connector tubes from which insects were physically excluded to avoid contamination. A white light bulb (20W, 250 lux) hung about 40 cm above the olfactometer provided illumination. The entire set up was placed in a white box (80 cm × 60 cm × 60 cm) to minimize visual distraction. An air delivery system (Analytical Research Systems, Gainesville, FL) passed humidified and purified air through Teflon® tubes into the olfactometer arms.

**2.2.3 Behavioral Bioassays.** Humidified and purified air was passed into each of the olfactometer arms at 200 ml/min while the vacuum pump was set at 800 ml/min to avoid a mix-up of volatiles in the chamber. The synthetic VOCs used (purity 95-99%) were purchased from Sigma® Chemical Co. (St. Louis, Missouri). The compounds were formulated in hexane (HPLC-grade) at 1 µg/µl concentration and delivered as 10 µl samples (10 µg dose) on Watman No.1 filter paper strips (25 × 7mm). This dose was selected based on the results of a preliminary experiment and previous studies by our group (Ngumbi & Fadamiro 2012). The solvent was allowed to evaporate from the filter paper for about 10 s before insertion into the olfactometer arm.

In the first experiment, each sex of *M. croceipes* (specialist) and *C. marginiventris* (generalist) was presented with  $\alpha$ -pinene, (Z)-3-hexenol, (Z)-3-hexenyl acetate and hexane (control) in separate tests.  $\alpha$ -pinene elicited the greatest attraction in the generalist while (Z)-3-hexenol elicited the greatest attraction in the specialist. Consequently, a second experiment was set up in which the parasitoids were presented with four choices:  $\alpha$ -pinene, (Z)-3-hexenol, a

binary mixture of the both compounds, and hexane (control). The binary mixture tested was made by mixing equal volume (50:50 v/v) of  $\alpha$ -pinene and (*Z*)-3-hexenol at the same concentration (1  $\mu\text{g}/\mu\text{l}$ ). Individual insects were tested for odor preference and response latency. Response latency was defined as the duration from the time of insect release to the time insect crosses into the extension tube of an arm. After testing four insects, the odor sources were replaced and the olfactometer was rotated 90° to avoid any error due to position effect, and the entire set-up was cleaned (with acetone) after testing 20 insects. Wasps were used only once and discarded. A wasp that did not make a choice after 15 min of exposure was recorded as ‘No choice’ and not included in the data analysis (< 10% in all experiments). A parasitoid was recorded to have made a clear choice for the odor offered through an arm when it enters into the extension tube and remains there for at least 15 s. Bioassays of different sexes and species were carried out in a randomized block design on different days between 0900 hr and 1700 hr.

**2.2.4 Data Analyses.** Attraction of parasitoids to each VOC was modeled as a binary response count and treatments were compared using Logistic Regression Analysis. The model adequacy for each set of experiment was confirmed with a Likelihood Ratio (Wajnberg & Haccou 2008). Slopes were separated using Proc Logistic Contrast in SAS. For data presentation, parasitoid attraction to VOCs was represented on charts as percentages of total wasps that responded due to varying sample sizes. Sexual difference in overall response latency was analyzed using two-sided Wilcoxon-Mann-Whitney test. All analyses were performed using SAS 9.2 with 0.05 level of significance.

## **2.3 Results**

**2.3.1 Attraction to Single VOCs.** Female *M. croceipes* (specialist) were significantly ( $\chi^2= 18.17$ ;  $P < 0.0004$ ;  $N = 59$ ) more attracted to the two HIPVS, (*Z*)-3-hexenol (FD-VOC) and

(Z)-3-hexenyl acetate (OD-VOC) than to  $\alpha$ -pinene (UD-VOC) and hexane (control) (Fig. 2a). Males were also significantly ( $\chi^2= 10.97$ ;  $P < 0.01$ ;  $N =49$ ) more attracted to (Z)-3-hexenol than to the other treatments (Fig. 2b). Both sexes of *C. marginiventris* (generalist) could not significantly discriminate among the three VOCs (Fig. 3). These results suggest that the specialist parasitoid showed greater attraction to herbivore-damaged VOCs, whereas the generalist did not show preference among the VOCs.

**2.3.2 Effect of Binary VOC Mixture.** When females of *M. croceipes* (specialist) were presented with a choice of  $\alpha$ -pinene, (Z)-3-hexenol and a mixture (50:50v/v) of both compounds, the mixture elicited the highest attraction (40% of wasps) ( $\chi^2 =6.31$ ;  $P < 0.01$ ;  $N= 80$ ) (Fig. 4a). Similarly, conspecific males showed a significantly ( $\chi^2 =8.99$ ;  $P < 0.0027$ ;  $N= 85$ ) greater attraction to the mixture, compared to the single VOCs (Fig. 4b). In contrast, female *C. marginiventris* (generalist) showed no preference among the three treatments (Fig. 5a), while males showed the greatest attraction to  $\alpha$ -pinene (Fig. 5b).

**2.3.3 Response Latency to Single VOCs.** Overall, a significantly shorter response latency ( $Z= 5.91$ ;  $P < 0.0001$ ;  $N= 108$ ) was recorded for males (68.1 s) than for females (128.6 s) of *M. croceipes* (Fig. 6a). No significant sexual difference in overall response latency was recorded for *C. marginiventris* (Fig. 6b). Comparing the species, mean response time was significantly ( $Z= 2.48$ ;  $P < 0.01$ ;  $N= 116$ ) shorter for female *M. croceipes* (128.6 s) compared to female *C. marginiventris* (231.2 s).

## 2.4 Discussion

The attraction of *M. croceipes* (specialist) to (Z)-3-hexenol and (Z)-3-hexenyl acetate (both HIPVs) was consistent with the findings of van Poecke *et al.* (2003), Penafior *et al.* (2011) and Ngumbi & Fadamiro (2012), which showed that specialist parasitoids were more attracted to

induced plant volatiles than to constitutive volatiles. Arguably, there is a greater chance that HIPVs will provide more specific host recognition cues than constitutive plant volatiles. On the hand, *C. marginiventris* (generalist) showed no preference among the tested constitutive plant volatile ( $\alpha$ -pinene) and the two HIPVs ((*Z*)-3-hexenol and (*Z*)-3-hexenyl acetate). The results are in support of the findings of Fontana *et al.* (2011) in which *C. marginiventris* was attracted to constitutive volatiles of maize. Although constitutively released in cotton,  $\alpha$ -pinene is also released in higher amounts during early stages of herbivore damage (Loughrin *et al.* 1994). Ozawa *et al.* (2008) and Uefune *et al.* (2012, 2013) have also reported the attraction of other parasitoids in the genus *Cotesia* to  $\alpha$ -pinene.

Comparing species, *M. croceipes* females were significantly more attracted to (*Z*)-3-hexenol (HIPV) than *C. marginiventris* females, suggesting that the specialist may depend more on induced volatiles for host location. More importantly, the specialist was able to discriminate HIPVs from constitutive VOC of cotton while the generalist could not, possibly indicating a more specialized olfactory mechanism. In contrast, *C. marginiventris* females were significantly more attracted to  $\alpha$ -pinene (UD-VOC) than *M. croceipes* females, suggesting the likelihood of the generalist to frequent plants more. A narrowly-tuned olfactory mechanism has the advantage of saving valuable energy resources while searching for specific hosts. However, when extrinsic interspecific competition exists, a broadly-tuned olfactory mechanism may present an ecological edge. The results corroborate the prediction of previous studies (Smid *et al.* 2002; Chen & Fadamiro 2007; Ngumbi *et al.* 2009, 2010, 2012) that the degree of host specificity in parasitoids may affect their use of various plant volatiles for host location. Generally, similar trends were recorded for conspecific males (as their females), suggesting that male parasitoids may be able to

exploit certain VOCs as cues to enhance mate location (Chen & Fadamiro 2007; Ngumbi & Fadamiro 2012).

In the bioassays with *M. croceipes*, the mixture of  $\alpha$ -pinene and (*Z*)-3-hexenol elicited a greater attraction than either compound - an additive effect that was not recorded in bioassays with *C. marginiventris*. There are two general models that may explain how an animal's olfactory system processes odor mixtures, leading to behavioral responses: the elemental and the configural models (Erickson *et al.* 1990; Alvarado & Rudy 1992; Kay *et al.* 2005). A classic review of the central processing of odor blends in insects was provided by Lei & Vickers (2008). In the simplest terms, the elemental model holds that responses to odor mixtures resemble that of individual components while the configural model holds that odor blends present an entirely new identity and they elicit responses that are different from those of individual components. In this study, the binary mixture used has highly dissimilar components [ $\alpha$ -pinene and (*Z*)-3-hexenol]. The components differ in chemical class, pathway of production (terpenoid and lipoxygenase pathways), and the timing of release by plants. Linster & Cleland (2004) explained that the more dissimilar the components of an odor mixture, the less overlap the signals generated, and the more the response to the mixture becomes a linear summation of the responses to both components (elemental processing). Thus the greater attraction elicited by the mixture suggests an elemental processing of the binary mixture in the specialist. However, the mixture did not elicit an additive effect in the attraction of the generalist. A possible explanation is that the generalist could not discriminate among the component VOCs of the mixture in the initial bioassays with single compounds. Conceivably, the less apparent the difference in the components, the less likely it is for the odor mixture to elicit an additive effect (Linster & Cleland 2004). It should be noted that the above is considered a possible explanation of the

present results from the perspective of neural processing, and that other factors may influence insect behavior. Another plausible explanation is that the specialist may have evolved an olfactory mechanism that is more tuned to VOC mixtures than to single components, as would be expected in nature.

There was no correlation between response latency and attraction of parasitoids to each VOC, suggesting that response latency to VOCs may be more related to a species' olfactory architecture rather than to functional behavioral responses. Furthermore, Ngumbi *et al.* (2012) reported no significant differences in the response latencies of trained *versus* untrained *M. croceipes* and *C. marginiventris* to various host-related plant volatiles, indicating that response latency may be innate in these parasitoids. In the present study, *M. croceipes* (specialist) generally made choices faster than *C. marginiventris* (generalist) in the olfactometer, similar to the report of Ngumbi *et al.* (2012). Adult parasitoids have limited ability to synthesize lipids. Thus, a reduced activity rate in some female parasitoids has been linked to energy conservation (Denis *et al.* 2013). Further studies with other parasitoids are needed to establish if host specificity affects the response latency of parasitoids to host-related plant volatiles.

In summary, results of the present study showed that key differences exist in the responses (attraction and response latency) of *M. croceipes* and *C. marginiventris* to select synthetic VOCs and mixture. Previous studies, including from our group, have already established that parasitic wasps use olfactory cues from plant volatiles to locate their hosts (Smid *et al.* 2002; Chen & Fadamiro 2007; Ngumbi *et al.* 2009, 2010, 2012). Both wasp species used as models are larval endoparasitoids belonging to the same family Braconidae. In addition, they are both solitary and koinobionts. Thus, they share a great deal of behavior and life strategy, but they differ in the degree of host specificity. This key difference is believed to affect parasitoids' odor

discriminatory ability as well as the use of various VOCs for host location. Further studies investigating the attraction of various parasitoids to plant VOCs, based on other differences in life strategy are needed. These studies are expected to yield results that could inform the identification of attractive VOCs and mixtures that may enhance the performance of the parasitoids as biocontrol agents.

## **2.5 Acknowledgements**

I thank Erica Williams, Matthew McTernan and Savannah Duke for rearing the insects used for this study.

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## Figure Legend

**Figure 1.** Major parts of the four choice olfactometer: Retort stand (A), Entry area for insects (B), Central tube (C), Bulb (D), Hemispherical depression (E), Olfactometer arm (F), Extension tube (G), and Connector tube (H).

**Figure 2.** Attraction of *Microplitis croceipes* to different types of VOCs: females (A), and males (B). Values (%) having no letter in common are significantly different ( $P < 0.05$ ; Proc. Logistic Regression). Attraction to VOCs was modeled as binary response counts and represented on the chart as percentage of total responding wasps.

**Figure 3.** Attraction of *Cotesia marginiventris* to different types of VOCs: females (A), and males (B). Values (%) having no letter in common are significantly different ( $P < 0.05$ ; Proc. Logistic Regression). Attraction to VOCs was modeled as binary response counts and represented on the chart as percentage of total responding wasps.

**Figure 4.** Attraction of *Microplitis croceipes* to single VOCs and a binary mixture: females (A), and males (B). Values (%) having no letter in common are significantly different ( $P < 0.05$ ; Proc.



Logistic Regression). Attraction to VOCs was modeled as binary response counts and represented on the chart as percentage of total responding wasps.

**Figure 5.** Attraction of *Cotesia marginiventris* to single VOCs and a binary mixture: females (A), and males (B). Values (%) having no letter in common are significantly different ( $P < 0.05$ ; Proc. Logistic Regression). Attraction to VOCs was modeled as binary response counts and represented on the chart as percentage of total responding wasps.

**Figure 6.** Overall response latency (time taken to choose all VOCs) of both sexes of *M. croceipes* (A) and *C. marginiventris* (B). For each parasitoid, mean ( $\pm$ SEM) values for the two sexes having no letter in common are significantly different ( $P < 0.05$ ; Wilcoxon-Mann-Whitney test).

**Figure 1**

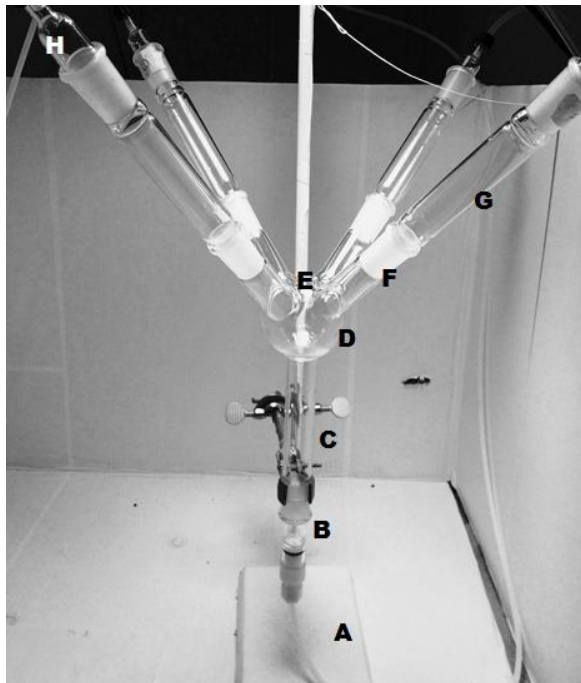


Figure 2.

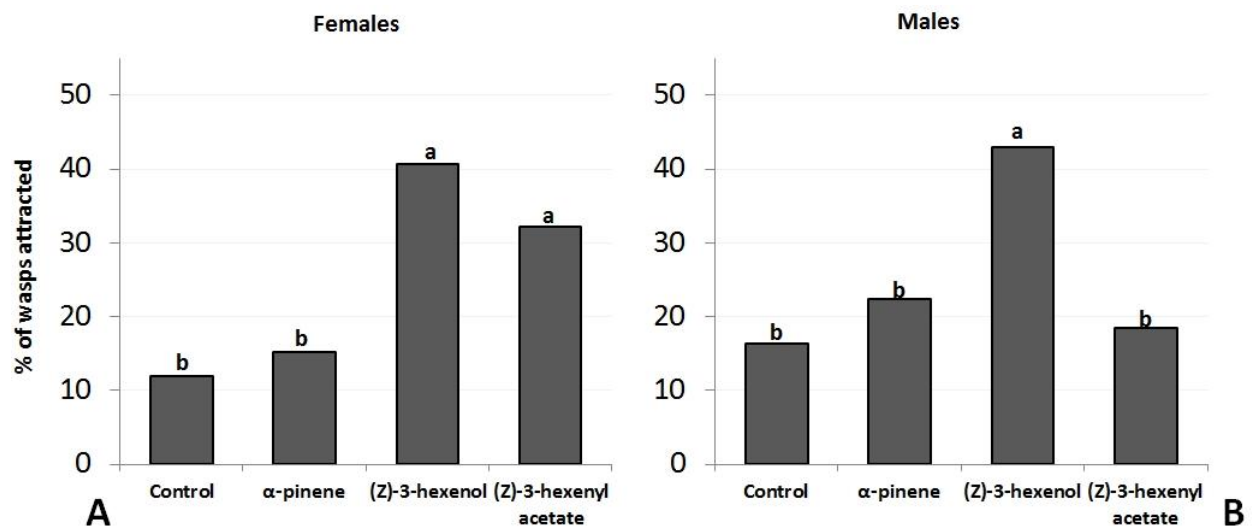


Figure 3.

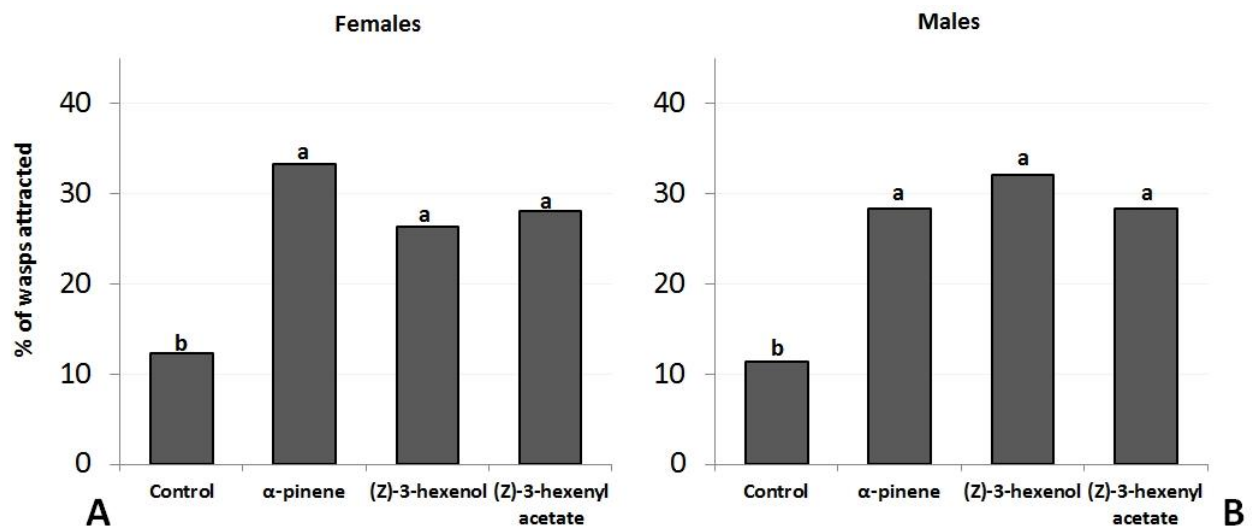


Figure 4.

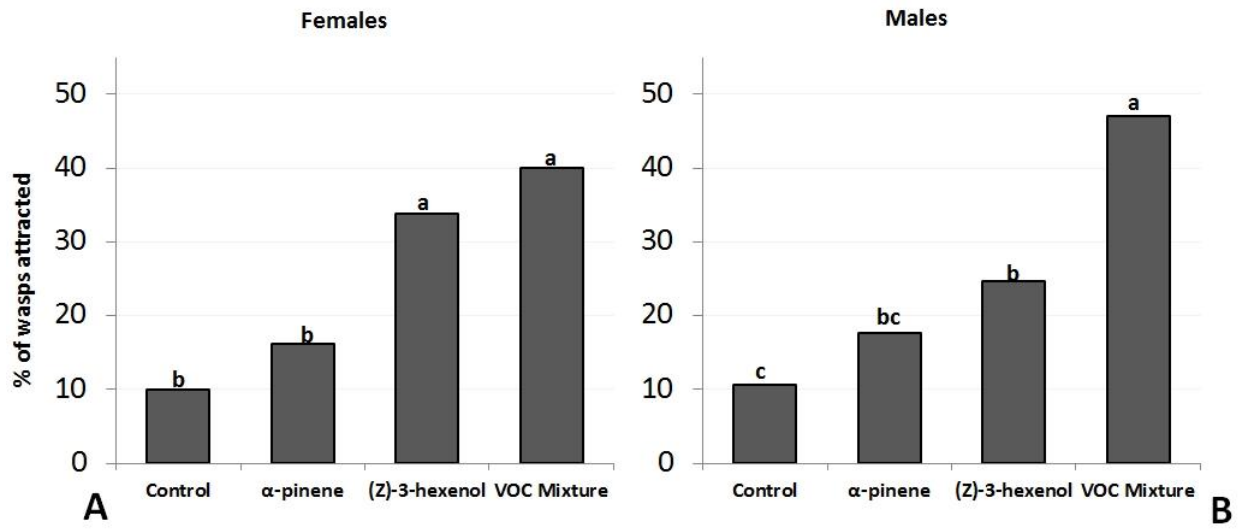


Figure 5.

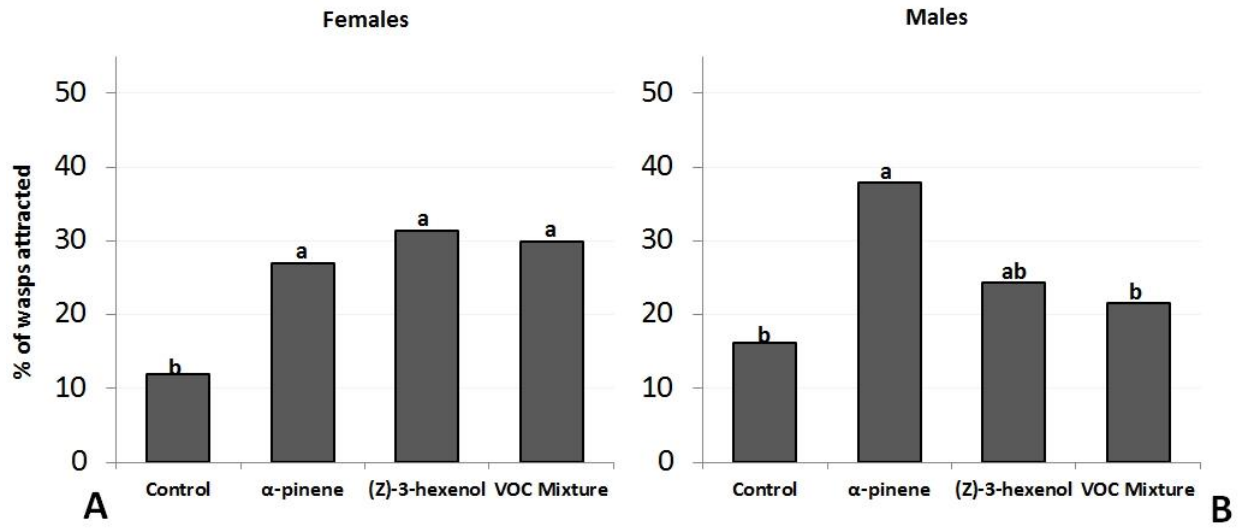
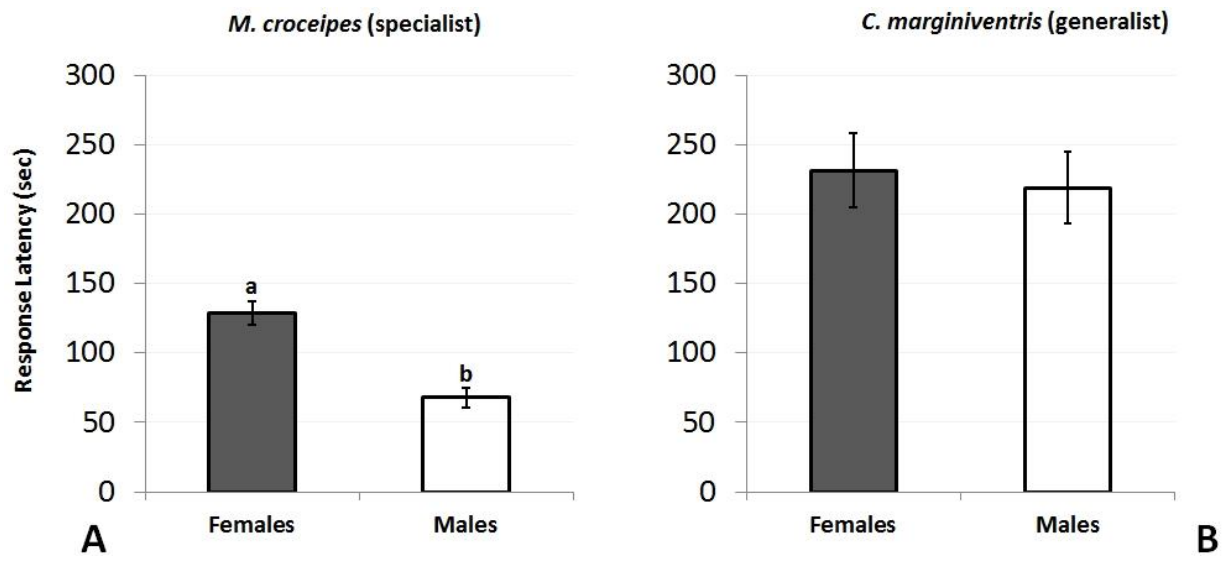


Figure 6.



## CHAPTER 3

# DURATION OF PLANT DAMAGE BY *HELIOTHIS VIRESCENS* CATERPILLARS AFFECTS ATTRACTION OF TWO PARASITOIDS WITH VARYING DEGREE OF HOST SPECIFICITY (*MICROPLITIS CROCEIPES* AND *COTESIA MARGINIVENTRIS*) TO COTTON

### 3.1 Introduction

Herbivore-damaged plants emit odors that often guide parasitoids to their hosts. Undamaged plants constitutively release small amounts of certain volatile organic compounds (VOCs), whose emissions often increase during herbivore damage. Additional VOCs with new identities are released as infestation proceeds (Pare & Tumlinson 1997; Boland *et al.* 1998; Rose & Tumlinson 2004). The composition of VOC profiles depend on several factors including plant and pest species; pest density; type and duration of damage (Dicke *et al.* 2009). These qualitative and quantitative differences are believed to generate important host recognition codes for natural enemies (De Moraes *et al.* 1998; Smith 1998; De Bruyne & Backer 2008). Of particular interest are the differences between the odors emitted by the same plant species at different stages of herbivore damage. The question of whether these differences influence the recruitment of specific natural enemies is ecologically important in tritrophic interactions. Both plants and herbivores strongly influence the release of plant odors, but plants ultimately dictate the relevance of the signals transmitted (Turlings *et al.* 1995). At the third trophic level, parasitoids have probably evolved to use these plant-related signals for host location.

Based on their relative host range, parasitoids can be broadly categorized as specialist (species with a restricted host range) or generalist (species with a broad host range). In the present study, *Microplitis croceipes* (Cresson) (specialist) and *Cotesia marginiventris* (Cresson) (generalist), were used as models. Both wasps (Hymenoptera: Braconidae) are koinobiont larval endoparasitoids of *Heliothis virescens* (Fab.) (Lepidoptera: Noctuidae), an important pest of cotton. Previous studies (Smid *et al.* 2002; Chen & Fadamiro 2007; Ngumbi *et al.* 2009, 2010, 2012) have reported that specialist and generalist parasitoids differ in their olfactory responses to various plant VOCs. These studies showed that specialist parasitoids were generally more attracted to host induced plant volatiles (HIPVs) than generalist parasitoids. In cotton and several other plants, considerable qualitative and quantitative differences exist in their volatile profiles based on the duration of herbivore damage (McCall *et al.* 1994; Rose *et al.* 1998; Rose & Tumlinson 2004; Hoballah & Turlings 2005; Magalhaes *et al.* 2012).

Specifically, undamaged cotton is known to constitutively release a few stored (constitutive) terpenes such as  $\alpha$ -pinene and myrcene. Few hours after herbivore infestation, constitutive terpenes are released in greater amounts. In addition, the green leaf volatiles (GLVs) such as hexanal, (*Z*)-3-hexenal and (*Z*)-3-hexenol are also released. The latter stages of damage ( $\geq 24$  hr) is characterized by the release of several acyclic terpenes such as (*E*)- $\beta$ -ocimene, (*E*)- $\beta$ -farnesene, nonatriene and tridecatetraene. In addition, indole (aromatic compound), hexenyl acetates, isomeric hexenyl butyrates and 2-methyl butyrates are also released (Loughrin *et al.* 1994; McCall *et al.* 1994; Rose *et al.* 1996, 1998; De Moraes *et al.* 1998; Pare & Tumlinson 1999; Rose & Tumlinson 2004; Magalhaes *et al.* 2012).

Herbivore feeding activities can inflict substantial amount of damage within a short time, leading to reduced plant growth/development and even mortality (Coley 1987). To avoid

excessive damage, plants are expected to initiate or reinforce various defense strategies early on herbivore attack. The question of whether the duration of herbivore damage determines the type of parasitoids recruited to plants concerns the fitness of both plants and parasitoids. Where interspecific competition between parasitoids exists, the arrival time (largely determined by attractiveness of plant odors) may very well determine who dominates the competition (Tillman & Powell 1992; De Moraes & Mescher 2005; De Moraes *et al.* 1999; Mohamad *et al.* 2011). De Moraes & Mescher (2005) reported that *C. marginiventris* (generalist) dominated intrinsic competitions with *M. croceipes* (specialist) when the generalist oviposited first or simultaneously with the specialist.

In a related study, Hoballah and Turlings (2005) tested the attraction of two parasitoids, *Microplitis rufiventris* and *C. marginiventris* to odors from fresh *versus* old damage maize plants. The authors reported that inexperienced *C. marginiventris* showed preference for fresh damage maize while inexperienced *M. rufiventris* showed no preference. In that study, plants treated with *Spodoptera littoralis* regurgitant for 6 hr were regarded as old damage. However, several studies (McCall *et al.* 1994; Rose & Tumlinson 2004; Magalhaes *et al.* 2012) have showed that most HIPVs are released from 16-24 hr of herbivore damage in many plants. Thus, further studies extending the duration of damage to at least 24 hr (for old damage) using real caterpillar hosts (as would be expected in nature) are needed.

In the present study, a four choice olfactometer was coupled with a headspace volatile collection system such that the actual plant odors that elicited behavioral responses in parasitoids were analyzed real time. Based on the results of previous studies on olfactory mechanisms in the two parasitoid models (Chen & Fadamiro 2007; Ngumbi *et al.* 2009, 2010, 2012) and because herbivore-damaged plants are expected to provide more information on host presence and

suitability than undamaged plants, it is hypothesized that the specialist species (*M. croceipes*) will show a preference for VOCs from host-damaged plants [i.e., fresh (6 hr infestation) damage plants (FD), and old (24 hr infestation) damage plants (OD)] compared to the generalist species, *C. marginiventris*. Finally, GC-MS analysis of headspace volatiles from undamaged (UD), fresh damage (FD) and old-damage (OD) plants was conducted to offer possible chemical explanations for the observed responses of the parasitoids.

## **3.2 Materials and Methods**

**3.2.1 Insects.** *M. croceipes* and *C. marginiventris* were reared in our laboratory (Auburn University AL, USA) on *Heliothis virescens* larvae. The rearing procedures were similar to those described by Lewis and Burton (1970). Upon emergence, adult wasps were transferred to aerated plastic cages (~ 30 × 30 × 30 cm) and supplied with 10% sugar water. For parasitization, female wasps (2-5 days old) were supplied with 2<sup>nd</sup>-3<sup>rd</sup> instar larvae (caterpillars) of *H. virescens* in the ratio 1 female to 20 larvae. Mated, naïve (untrained) parasitoids (aged 2-5 days old) were used in the behavioral bioassays. Larvae of *H. virescens* were reared on pinto bean artificial diet (Shorey & Hale 1965). The general rearing conditions for all insects were 25 ± 1°C, 75 ± 5% RH and 14:10 h (L:D) photoperiod.

**3.2.2 Plants.** Cotton (*Gossypium hirsutum*, var. max 9) plants were grown in growth chambers (Entomology & Plant Pathology, Auburn University) at 26.6°C day, 25.6°C night, and 60 % relative humidity. Illumination was provided using daylight fluorescent tubes (270 μmol m<sup>-2</sup> s<sup>-1</sup>) with 16:8 h (L/D) photoperiod. Seeds were planted in a top soil/vermiculite/peat moss mixture. Plants deliberately infested with *H. virescens* were 4-5 weeks old.

**3.2.3 Infestation.** For each trial, three treatment plants were tested: undamaged cotton plants (UD), fresh (6 hr infestation) damaged cotton plants (FD), and old (24 hr infestation)



damage plants (OD). To induce VOC emissions from plants, 20 second instar larvae of *H. virescens* were allowed to feed on cotton plants for the previously stated time durations. Each plant, with the feeding larvae (as would be expected in nature) was placed in a 5 L volatile collection jar (Analytical Research Systems, Inc., Gainesville, FL.). To reduce contamination, the plant pot and soil was wrapped with aluminum foil.

**3.2.4 Coupled Headspace Volatile Collection-Olfactometer.** Headspace VOCs from undamaged (UD-plants) and host-damaged cotton [FD (6 hr infestation) and OD (24 hr infestation)-plants] were collected according to the protocol used by Ngumbi *et al.* (2009), but with few modifications. The collection was commenced after caterpillar infestation of FD- and OD-plants had continued for 6 hr and 24 hr, respectively. Each of the three treatment plants were placed in separate jars. A fourth jar with no plant (control) was included in the set-up. Coupling of headspace volatile collection and olfactometer bioassay was according to Turlings *et al.* (2004) with slight modifications. The four-choice olfactometer used has been previously described in chapter II. Each jar has two air outlets: one outlet was connected to an olfactometer arm, and the other outlet was connected to a trap containing 50 mg of Super-Q (Alltech Associates, Deerfield, IL, USA). A purified and humidified air stream of 400 ml/min was passed through all jars at room temperature for a collection period of 2 hr. Preliminary experiments and previous studies showed that 2 hr was a sufficient time to trap VOCs from cotton plants, especially since infestation had earlier proceeded for some hours. Air carrying plant odor (olfactory stimulus) from the jars was passed into each of the four arms of the olfactometer at 400 ml/min through Teflon® tubes while the vacuum suck was set at 1600 ml/min to avoid a mix-up of odors.

Naive mated wasps of each sex of both parasitoid species (2-5 days old) were released individually into the olfactometer from the bottom of the central tube. After testing four insects, the olfactometer was rotated 90° to avoid any error from position effect, and the entire set-up was cleaned (with acetone) after testing 20 insects. Wasps were used only once and discarded. A wasp that did not make a choice after 15 min of exposure was recorded as ‘No choice’ and was not included in the data analysis (< 10% in all experiments). A parasitoid was recorded to have made a clear choice for the odor offered through an arm when it gets into the extension tube and remains there for at least 15 s. Bioassays of different sexes and species were carried out in a randomized block design on different days between 0900 hr and 1700 hr.

**3.2.5 GC-MS Analyses.** The trapped headspace volatiles of cotton were eluted with 200 µl of methylene chloride and the resulting extracts were stored in a freezer (at -20°C) until use. Identification and quantitation of headspace volatiles was done using an Agilent 7890A GC coupled to a 5975C Mass Selective Detector, with a HP-5ms capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness) according to the protocol used by Ngumbi *et al.* (2009). For each headspace volatile extract, 1 µl was injected into the GC-MS in splitless mode. The GC was programmed as follows: inject at 40°C, hold at 40°C for 2 min, and then increased by 5°C/min to 200°C for a total of 40 min. The temperature of both injector and detector was set at 200°C. Mass spectra were obtained using electron impact (EI, 70 eV). Identification of peaks was done by using NIST 98 library (National Institute of Standards and Technology, Gaithersburg, Maryland) and by comparing with published GC profiles of cotton head space volatiles (Loughrin *et al.* 1994; McCall *et al.* 1994; Ngumbi *et al.* 2009). Compounds were identified according to their retention times and mass spectra, in comparison with a NIST library (Agilent)

and commercially available synthetic standards (purity 95-99%) obtained from Sigma® Chemical Co. (St. Louis, Missouri).

**3.2.6 Data Analyses.** Attraction of parasitoids to each VOC was modeled as a binary response count and treatments were compared using Logistic Regression Analysis. The model adequacy for each set of experiment was confirmed with a Likelihood Ratio test (Wajnberg & Haccou 2008). Slopes were separated using Proc Logistic Contrast in SAS. For data presentation, parasitoid attractions to VOCs were represented on charts as percentages of total wasps that responded due to varying sample sizes. Significant differences in the amounts of each volatile component emitted by treatment plants were established using Kruskal-Wallis one-way analysis of variance, followed by Sidak's multiple comparison test. The significance level was adjusted by the Sidak method to:  $\alpha' = 0.0169$  [ $\alpha' = 1 - (1 - \alpha)^{1/k}$ ;  $\alpha' = 1 - (1 - 0.05)^{1/3} = 0.0169$ ] (Rose & Tumlinson 2004). All analyses were performed using SAS 9.2 with 0.05 level of significance.

### 3.3 Results

**3.3.1 Effect of Duration of Caterpillar Damage on Attraction of Parasitoids.** Female *M. croceipes* (specialist) were significantly ( $\chi^2 = 13.71$ ;  $P < 0.0002$ ;  $N = 85$ ) more attracted to odors from herbivore-damaged plants (FD- and OD-plants) than to odors from uninfested plants (UD-plants) or the control (Fig. 1). Numerically, more females chose fresh damage (FD) plants than old damage (OD) plants. Males were significantly ( $\chi^2 = 22.77$ ;  $P < 0.0001$ ;  $N = 106$ ) more attracted to herbivore-damaged and undamaged plants than to the control, but could not discriminate the plant treatments (Fig. 2). For *C. marginiventris* (generalist), females showed significantly greater attraction to UD- and FD-plants than to the control, ( $\chi^2 = 8.71$ ;  $P < 0.03$ ;  $N = 95$ ) (Fig. 1). Male also showed a similar trend with conspecific females (Fig. 2).

**3.3.2 Species Differences in Response.** Comparing species, female *M. croceipes* (specialist) were significantly ( $\chi^2 = 4.18$ ;  $P < 0.041$ ;  $N = 167$ ) more attracted than female *C.*

*marginiventris* (generalist) to OD-plant odors (Fig. 1). In contrast, female *C. marginiventris* showed significantly ( $\chi^2 = 3.88$ ;  $P < 0.048$ ;  $N=167$ ) greater attraction to UD-plant odors, compared to female *M. croceipes* (Fig. 1). Similarly, male *M. croceipes* were significantly ( $\chi^2 = 4.16$ ;  $P < 0.041$ ;  $N=201$ ) attracted than male *C. marginiventris* to odors from OD-plants (Fig. 2).

**3.3.3 GC-MS Analyses.** Analyses of headspace volatiles emitted from UD-, FD- and OD-plants simultaneously trapped during olfactometer bioassays revealed qualitative and quantitative differences in composition. Generally, more compounds were detected, and at relatively greater amounts in the headspace of OD-plants than in FD-plants or UD-plants (Fig. 3). The headspace of UD-plants contained the least number of VOCs, usually at the lowest amounts. In total, twenty-four VOC components were identified in this study. These included several terpenes such as  $\alpha$ -pinene,  $\beta$ -pinene, myrcene, (*E*)- $\beta$ -caryophyllene, (*E*)- $\beta$ -ocimene, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (*Z,E*)- $\alpha$ -farnesene,  $\alpha$ -humulene,  $\beta$ -elemene; GLVs [e.g., (*Z*)-3-hexenal, (*Z*)-3-hexenol and (*Z*)-3-hexenyl acetate]; and aromatic compounds such as indole (Table 1). Comparing the treatments,  $\alpha$ -pinene and myrcene were prominent components detected in the headspace of UD-plants. GLVs were hardly detectable in the headspace of UD-plants. The amount of  $\alpha$ -pinene and myrcene emitted increased in herbivore-damaged plants (FD and OD). In addition, (*Z*)-3-hexenal, (*Z*)-3-hexenol,  $\beta$ -pinene, limonene, (*E*)- $\beta$ -caryophyllene and  $\alpha$ -humulene were detected in the headspace of FD-plants. Certain components including (*Z*)-3-hexenyl acetate, (*E*)- $\beta$ -ocimene, DMNT, (*Z*)-3-hexenyl butyrate, (*Z*)-3-hexenyl-2-methyl butyrate, indole, (*Z,E*)- $\alpha$ -farnesene and  $\beta$ -elemene, were only found in significant amounts in the headspace of OD-plants (Table 1).

### 3.4 Discussion

Results of the present study showed that *M. croceipes* (specialist) and *C. marginiventris* (generalist) differ in their attraction to odors from undamaged and herbivore-damaged cotton. According to Turlings *et al.* (2005), clarity, specificity and timing are the yardsticks for measuring the suitability of signals that would serve as effective host location cues for natural enemies. Plant odors can be such effective host location cues for parasitic wasps, especially considering their role in long range attraction. In the present study, female *M. croceipes* (specialist) were significantly more attracted to odors from herbivore-damaged plants (FD- and OD-plants) than to odors from undamaged plants (UD-plants), supporting our initial hypothesis. Conspecific males could not significantly discriminate among odors from the treatment plants. Since the female sex parasitizes the host, it is expected that they possess a greater ability to discriminate between odors from damaged and herbivore-damaged plants, compared to males. Unlike the specialist, female *C. marginiventris* (generalist) could not significantly discriminate among the various plant odors, suggesting the use of general odor cues in host location. Odors from old damage (OD) plants attracted the least number of females and males of the generalist, suggesting that *C. marginiventris* was less attracted to host specific cues. Since generalist parasitoids have a broad host range, they may have evolved to use more general host location cues (UD- and FD-plant odors) from plants.

GC-MS analysis of UD- and FD-plant headspaces confirmed the presence of certain terpenes and GLVs, which are ubiquitous compounds also released by mechanically-damaged plants (Cortesero *et al.* 1997; D'Alessandro & Turlings 2005; Hoballah & Turlings 2005; Ngumbi *et al.* 2012). Although GLVs have been associated with the attraction of several parasitoid species, they may not serve as reliable host finding cue for specialist parasitoids

(Ngumbi *et al.* 2012). Instead, they may be more useful for host-seeking generalist parasitoids. On the other hand, odors from OD-plants are expected to transmit more host specific signals. In the present study, (Z)-3-hexenyl acetate, (Z)-3-hexenyl butyrate, (Z)-3-hexenyl-2-methyl butyrate, (E)- $\beta$ -ocimene, DMNT, (Z,E)- $\alpha$ -farnesene,  $\beta$ -elemene and indole were the headspace components almost exclusive to OD-plants. Previous studies using  $^{13}\text{CO}_2$  labeling and timed collection of headspace volatiles have showed that most of these compounds are synthesized *de novo* as a delayed response to herbivore damage, offering a possible explanation about the relatively high attraction of the specialist parasitoid elicited by OD-plants (Loughrin *et al.* 1994; Pare & Tumlinson 1997; Boland *et al.* 1999; Rose & Tumlinson 2004; Dudareva *et al.* 2007; Magalhaes *et al.* 2012). Moreover, some of these HIPVs have been reported to elicit a strong attraction in *M. croceipes* (Chen & Fadamiro 2007; Ngumbi *et al.* 2010, 2012).

At the third trophic level, parasitoids are subjected to intra- and inter-specific competitions in the same niche. *M. croceipes* and *C. marginiventris* share a very similar life history and strategy, and are both larval parasitoids of *H. virescens*, a generalist herbivore on cotton, tobacco, flax, alfalfa, and many other field crops (Graham & Robertson 1970). Chances are that interspecific competition may occur between the two parasitoid species. Since female *C. marginiventris* showed the greater attraction to undamaged plants, they may frequent intact plants more randomly, possibly making the first contact with caterpillar hosts on the plant. On the other hand, female *M. croceipes* may arrive much later if cues from OD-plants were used. According to De Moraes & Mescher (2005), *C. marginiventris* (generalist) dominated intrinsic competitions with *M. croceipes* (specialist) when the generalist oviposited first or simultaneously with the specialist. Surprisingly, females of both parasitoid species showed the greatest attraction to FD-plant odors, further indicating the possibility of a direct interference (extrinsic

competition) during the early stages of herbivore damage. According to Mohamad *et al.* (2011), the size of females, egg load status and abundance of hosts are key factors that may determine the outcome of such competitions. Here, we suggest that the timing of parasitoid recruitment, largely dependent on the relative attractiveness of the plant odors, should also be considered a key factor that may determine the outcome of interspecific competitions.

In the present study, results of GC-MS analysis of cotton headspace volatiles from OD-plants showed the abundance of certain terpenes such as (*E*)- $\beta$ -ocimene, DMNT, (*Z,E*)- $\alpha$ -farnesene and  $\beta$ -elemene. The synthesis of such compounds is mediated by the terpenoid pathway and requires extensive chemical reduction reactions (Gershenzon 1994). Thus, substantial amount of energy is used by plants to drive their production. This may explain why many plants release several compounds in this group as a delayed response, but not early on herbivore attack.

In conclusion, results of the present study showed both differences and similarities in the attraction of the model parasitoid species to odors from undamaged and herbivore damaged plants. The implications of the odor preferences and possible interspecific competition between the model parasitoids used in this study should be considered in integrated pest management strategies that seek to optimize the use of various parasitoids as biocontrol agents.

### **3.5 Acknowledgements**

I thank Savannah Duke for rearing the insects used for this study.

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**Table 1.** Composition of headspace volatiles emitted by undamaged cotton plants vs. fresh damage (6 hr infestation) and old damage (24 hr infestation) cotton plants infested by *Heliothis virescens* caterpillars

ID	Compound <sup>a</sup>	Undamaged <sup>b</sup>		Fresh Damage <sup>b</sup>		Old Damage <sup>b</sup>	
		Amount <sup>c</sup> ng/g of fwt.	Rel. %	Amount <sup>c</sup> ng/g of fwt.	Rel. %	Amount <sup>c</sup> ng/g of fwt.	Rel. %
1	(Z)-3-hexenal <sup>j</sup>	0 <sup>b</sup>	0	7.5±6.1 <sup>ab</sup>	0.7	29.2±6.4 <sup>a</sup>	0.3
2	(E)-2-hexenal <sup>j</sup>	0 <sup>c</sup>	0	2.4±0.8 <sup>b</sup>	0.2	18.8±5.0 <sup>a</sup>	0.2
3	(Z)-3-hexenol <sup>j</sup>	0 <sup>b</sup>	0	10.3±7.3 <sup>a</sup>	1.0	60.8±33.1 <sup>a</sup>	0.7
4	α-pinene <sup>i</sup>	7.4±4.8 <sup>b</sup>	10.8	630.0±208.1 <sup>a</sup>	59.8	3264.3±923.2 <sup>a</sup>	36.4
5	Benzaldehyde <sup>i</sup>	6.8±1.7 <sup>a</sup>	10.0	3.4±1.2 <sup>a</sup>	0.3	2.8±0.9 <sup>a</sup>	0.03
6	β-pinene <sup>j</sup>	0 <sup>c</sup>	0	65.1±26.2 <sup>b</sup>	6.2	454.9±134.4 <sup>a</sup>	5.1
7	Myrcene <sup>i</sup>	47.9±38.3 <sup>b</sup>	70.0	128.0±65.2 <sup>ab</sup>	12.2	714.7±258.8 <sup>a</sup>	8.0
8	α-phellandrene <sup>i</sup>	3.6±2.1 <sup>a</sup>	5.3	3.5±2.1 <sup>a</sup>	0.3	4.3±2.5 <sup>a</sup>	0.1
9	(Z)-3-hexenyl acetate <sup>j</sup>	0 <sup>b</sup>	0	6.7±6.7 <sup>b</sup>	0.6	1585.9±757.9 <sup>a</sup>	17.7
10	Limonene <sup>j</sup>	0 <sup>b</sup>	0	52.2±19.8 <sup>a</sup>	5.0	206.2±62.8 <sup>a</sup>	2.3
11	(E)-β-ocimene <sup>j</sup>	0 <sup>b</sup>	0	5.7±3.9 <sup>b</sup>	0.5	602.1±209.9 <sup>a</sup>	6.7
12	(E)-4,8-dimethyl-1,3,7-nonatriene <sup>k</sup>	0 <sup>b</sup>	0	0 <sup>b</sup>	0	169.3±46.0 <sup>a</sup>	1.9
13	(E)-2-hexenyl butyrate <sup>k</sup>	0 <sup>b</sup>	0	0 <sup>b</sup>	0	2.8±1.5 <sup>a</sup>	0.03
14	(Z)-3-hexenyl butyrate <sup>k</sup>	0 <sup>b</sup>	0	0 <sup>b</sup>	0	70.0±55.7 <sup>a</sup>	0.8
15	(Z)-2-hexenyl butyrate <sup>k</sup>	0 <sup>b</sup>	0	0 <sup>b</sup>	0	15.0±8.7 <sup>a</sup>	0.2
16	(Z)-3-hexenyl-2-methyl butyrate <sup>k</sup>	0 <sup>b</sup>	0	0 <sup>b</sup>	0	100.4±75.4 <sup>a</sup>	1.1
17	Indole <sup>k</sup>	0 <sup>b</sup>	0	0 <sup>b</sup>	0	125.2±97.2 <sup>a</sup>	1.4
18	β-caryophyllene <sup>j</sup>	0 <sup>c</sup>	0	103.5±55.1 <sup>b</sup>	9.8	1043.2±244.0 <sup>a</sup>	11.6
19	(Z,E)-α-farnesene <sup>k</sup>	0 <sup>b</sup>	0	0 <sup>b</sup>	0	7.6±3.3 <sup>a</sup>	0.1
20	α-humulene <sup>j</sup>	0 <sup>c</sup>	0	18.3±10.8 <sup>b</sup>	1.7	270.7±59.1 <sup>a</sup>	3.0
21	β-elemene <sup>j</sup>	0 <sup>b</sup>	0	0.3±0.3 <sup>b</sup>	0.03	15.1±6.0 <sup>a</sup>	0.2
22	γ-bisabolene <sup>j</sup>	0 <sup>c</sup>	0	10.0±8.0 <sup>b</sup>	1.0	195.4±61.6 <sup>a</sup>	2.2
23	(E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene <sup>k</sup>	0 <sup>a</sup>	0	0 <sup>a</sup>	0	2.2±1.6 <sup>a</sup>	0.02
24	β-bisabolol <sup>i</sup>	2.8±2.8 <sup>a</sup>	4.1	6.3±6.3 <sup>a</sup>	0.6	16.0±5.3 <sup>a</sup>	0.2

<sup>a</sup>In order of elution during gas chromatography

<sup>b</sup>Volatiles were collected for 2 hr; *Heliothis virescens*-damaged plants were infested with 20 second instar larvae

<sup>c</sup>Amounts (ng/g of plant fresh weight) are mean ± SEM of four replicates

<sup>i</sup>Compounds were detected in undamaged plant as well as *Heliothis virescens*-damaged plants

<sup>j</sup>Compounds were only detected in fresh and old damage plants

<sup>k</sup>Compounds were only detected in old damage plants

Means across the same row followed by different letters are significantly different (P < 0.05; Kruskal-Wallis test followed by Sidak's multiple comparison test)

## Figure Legend

**Figure 1.** Species differences in the attraction of females of *Microplitis croceipes* and *Cotesia marginiventris* to odors released by undamaged (UD-plants) cotton plants *versus* plants damaged by *Heliothis virescens* caterpillars for 6hr (FD-plants) or 24 hr (OD-plants). Values (%) having no letter in common are significantly different ( $P < 0.05$ ; Proc Logistic Regression). Attraction to VOCs was modeled as binary response counts and represented on the chart as percentage of total responding wasps. Asterisks (\*) indicate significant differences between species for each odor source. ( $P < 0.05$ ; Proc. Logistic Regression Contrast).

**Figure 2.** Species differences in the attraction of males of *Microplitis croceipes* and *Cotesia marginiventris* to odors released by undamaged (UD-plants) cotton plants *versus* plants damaged by *Heliothis virescens* caterpillars for 6hr (FD-plants) or 24 hr (OD-plants). Values (%) having no letter in common are significantly different ( $P < 0.05$ ; Proc Logistic Regression). Attraction to VOCs was modeled as binary response counts and represented on the chart as percentage of total responding wasps. Asterisks (\*) indicate significant differences between species for each odor source. ( $P < 0.05$ ; Proc. Logistic Regression Contrast).

**Figure 3.** Typical chromatograms of headspace volatiles released by undamaged, fresh damage (6 hr infestation) and old damage (24 hr infestation) cotton plants. 20 second instar larvae of *Heliothis virescens* were used for infestation. Volatiles were trapped for 2 hr. Peak identities: **1**, (Z)-3-hexenal; **2**, (Z)-3-hexenol; **3**,  $\alpha$ -pinene; **4**, Benzaldehyde; **5**,  $\beta$ -pinene; **6**, Myrcene; **7**,  $\alpha$ -phellandrene; **8**, (Z)-3-hexenyl acetate; **9**, Limonene; **10**, (E)- $\beta$ -ocimene; **11**, (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT); **12**, (Z)-3-hexenyl butyrate; **13**, (Z)-3-hexenyl-2-methyl butyrate; **14**, Indole; **15**, (E)- $\beta$ -caryophyllene; **16**, (Z,E)- $\alpha$ -farnesene; **17**,  $\alpha$ -Humulene; **18**,  $\beta$ -elemene; **19**,  $\gamma$ -bisabolene; **20**,  $\beta$ -bisabolol. Peaks labeled (a) and (b) are unidentified.

Figure 1.

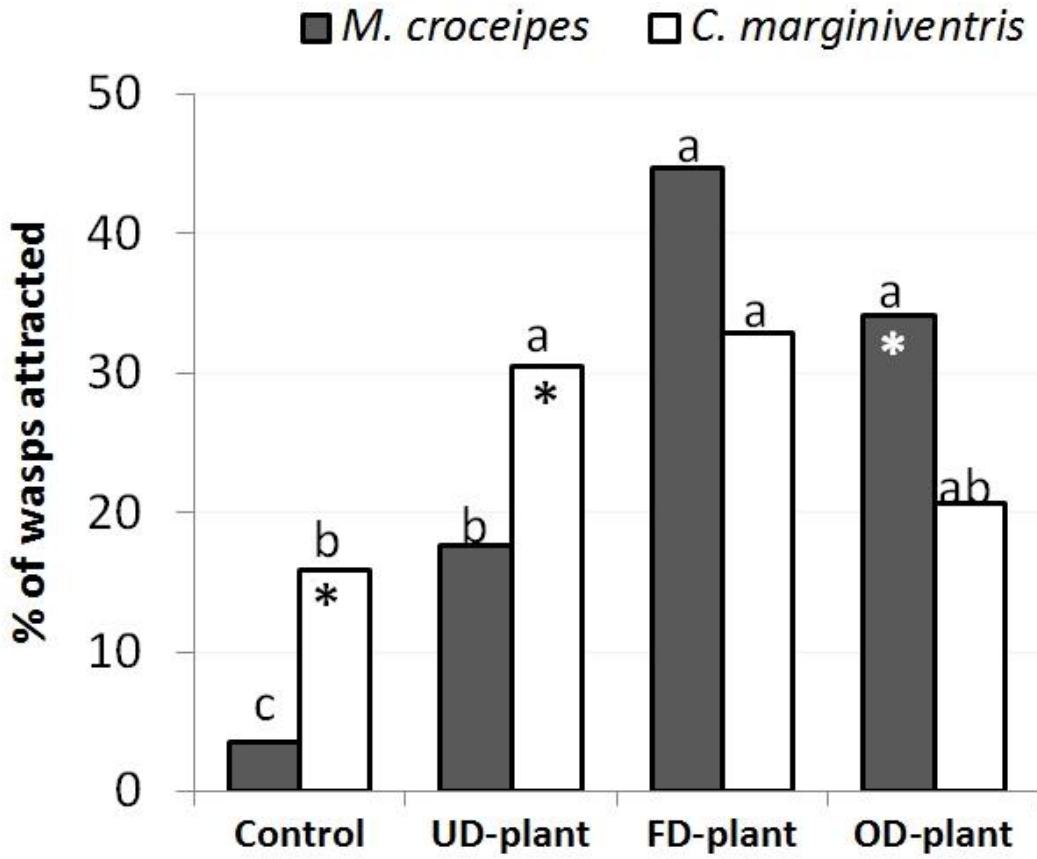


Figure 2.

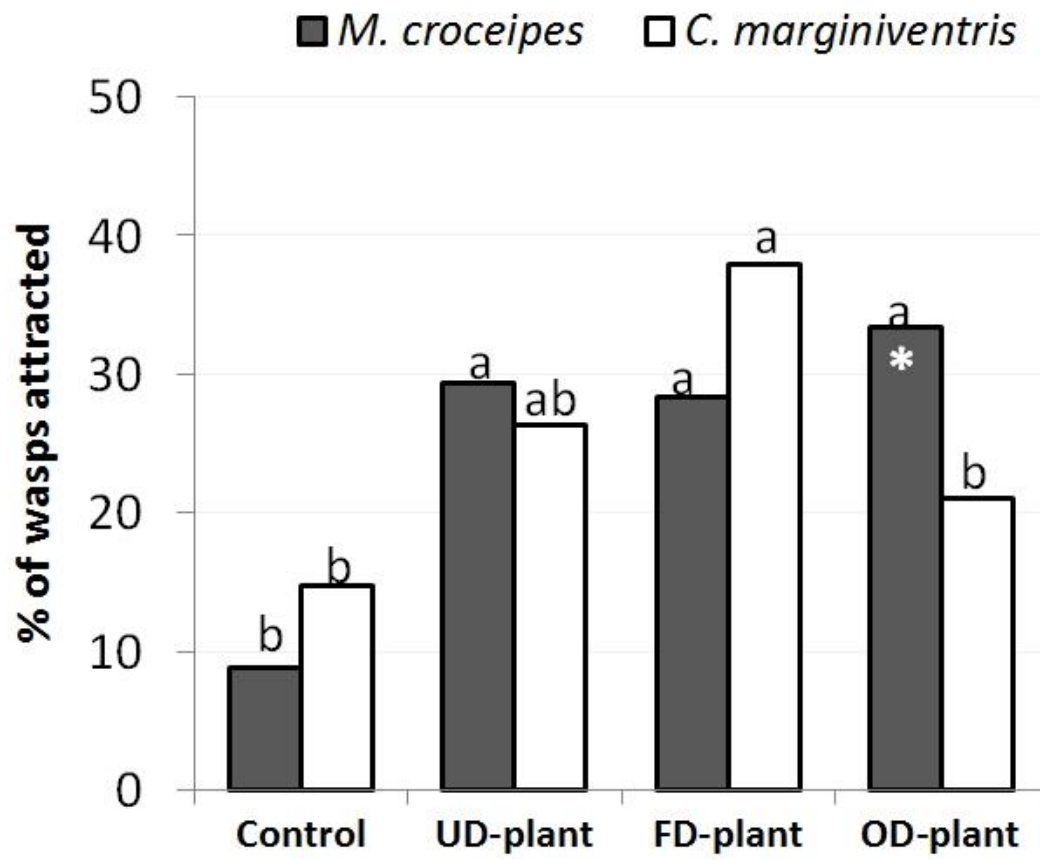
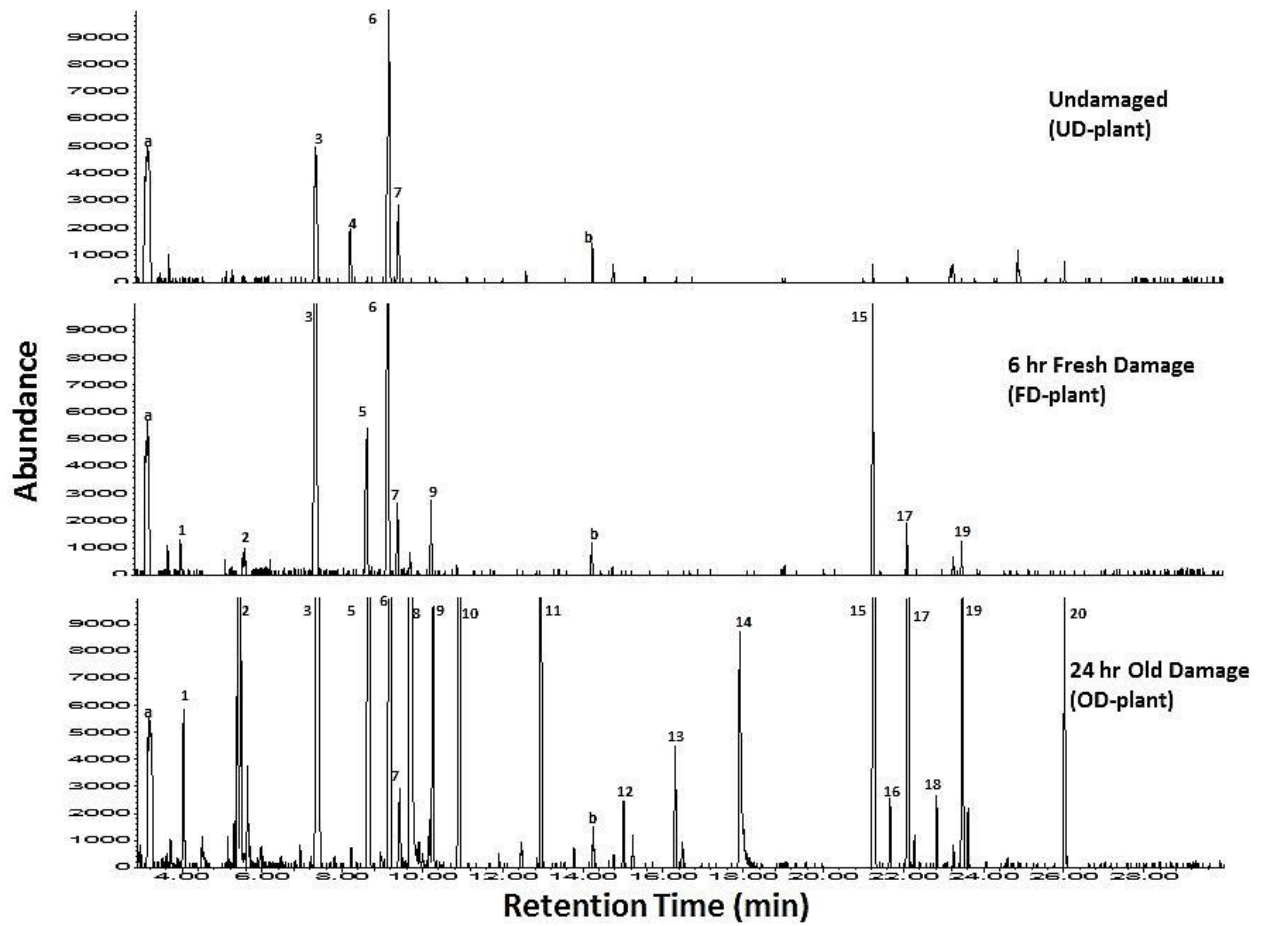




Figure 3.



## Conclusions

In nature, plants release a suite of VOCs whose composition may vary depending on the duration of herbivore damage. These variations affect the attraction of natural enemies such as parasitoids. In the present study, a stepwise approach was employed in the complexity of the odor stimuli presented to *M. croceipes* (specialist) and *C. marginiventris* (generalist). In the first set of four-choice olfactometer bioassays, attraction of parasitoids to select single components (synthetic VOCs) was tested. In the second set of bioassays, a binary mixture of VOCs was included in the choices presented to the parasitoids. Lastly, attraction of parasitoids to natural odors of undamaged and *H. virescens*-damaged (fresh damage and old damage) cotton was tested.

The results reported in chapter II (attraction to single components) were consistent with the results reported in chapter III (attraction to natural plant odors). In general, the specialist parasitoid (*M. croceipes*) showed a preference for host induced plant volatiles (HIPVs)/ *H. virescens*-damaged cotton (FD- and OD-plant) over undamaged plant volatile (UD-VOC)/ undamaged cotton (UD-plant). In addition, the specialist showed a greater attraction to a binary VOC mixture than to single components. On the other hand, the generalist parasitoid (*C. marginiventris*) could not discriminate among the single components/ treatment plants. Likewise, the generalist did not show preference for the binary VOC mixture over single components.

Comparing species, *M. croceipes* females were more attracted to old damage cotton than *C. marginiventris* females, who were more attracted to undamaged cotton compared to *M. croceipes* females. Interestingly, both parasitoid species showed a high attraction for fresh damage plant odors. Results of GC-MS analyses of cotton headspace volatiles showed qualitative and quantitative differences in the volatile profiles of cotton at various stages of host

damage. This may offer a chemical-based explanation for the differences observed in the responses of the specialist and the generalist parasitoids. The olfactory mechanism of the specialist parasitoid is probably tuned to HIPVs or odors from host-damaged plants which may provide cues for locating specific hosts. On the other hand, a broadly-tuned olfaction to various odors may enable the generalist parasitoid to locate a broader range of hosts.

The results of the present study agree with previous studies from our group suggesting that the degree of host specificity in parasitoids affects their use of host-related plant volatiles for host location. In addition, these results highlight the possibility of interspecific competition between the two parasitoid species. Here, we suggested that the timing of parasitoid recruitment, largely dependent on the relative attractiveness of the plant odors, should be considered as one of the key factors that may determine the outcome of interspecific competitions.

In future studies, the knowledge obtained from using this model in laboratory experiments will be transferred to field and semi-field conditions where other biotic and abiotic interactions must be accounted for. Further studies using other select compounds that will address the question of whether single compounds or the entire suite of natural odors from plants elicit complete behavioral responses in parasitoids are also required. The present study has a broader impact on the existing body of knowledge about olfaction in parasitoids. Considerations about odor preferences and possible interspecific competitions among parasitoids will be useful to IPM specialists in field monitoring as well as enhancing the use of parasitoids as biocontrol agents.