Chemical Ecology of Some Underexplored Aspects of Plant-Herbivore-Parasitoid Tritrophic Interactions

by

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Abstract

Plants, herbivorous insects and their natural enemies are involved in an intricate tritrophic interaction in a complex chemical environment. Herbivore infested plants, as well as plant-fed herbivores release volatile organic compounds (VOCs) that can be used by natural enemies such as parasitoids to locate their hosts. This study investigated the chemical ecology of some underexplored aspects of plant-herbivore-parasitoid tritrophic interactions, mostly from the parasitoid's perspective. These aspects include the synergy between plant-related experiences and herbivore-related experiences, parasitoid's use of plant-associated volatiles emitted by herbivores, the abundance-relevance relationship of volatile compounds, and the dual nature of certain defensive compounds as both allomone and kairomone.

In chapter I, I discussed existing literature on chemical ecology of parasitoids in tritrophic interactions, identified specific underexplored aspects of these interactions, and provided rationale for the current study.

In chapter II, I tested the prediction that specialist parasitoids that utilize generalist herbivores rely more on herbivore-derived cues than plant-derived cues using a model system consisting of the parasitoid *Microplitis croceipes* (Hymenoptera: Braconidae) and its herbivore host *Heliothis virescens* (Lepidoptera: Noctuidae), which is a pest of cotton and soybean. It was hypothesized that: i) naïve parasitoids will show innate responses to herbivore-emitted kairomones, regardless of host plant identity, and ii) herbivore-related experience will have a greater influence on intraspecific oviposition preference than plant-related experience.

Inexperienced (naïve) female *M. croceipes* did not discriminate between cotton-fed and soybean-fed *H. virescens* in oviposition choice tests, supporting the first prediction. Oviposition experience alone with either host group influenced subsequent oviposition preference while experience with infested plants alone did not elicit preference in *M. croceipes,* supporting the second prediction. Further, associative learning of oviposition with host-damaged plants facilitated host location. Interestingly, naïve parasitoids attacked more soybean-fed than cotton-fed host larvae in two-choice tests when a background of host-infested cotton odor was supplied, and vice versa. This suggests that plant volatiles may have created an olfactory contrast effect. I discussed ecological significance of the results and concluded that both plant- and herbivore-related experiences play important role in parasitoid host foraging.

In chapter III, I hypothesized that certain compounds play key roles in the attractiveness of host-associated odor blends to parasitoids. The larval parasitoid, *M. croceipes* and its herbivore host, *H. virescens,* a major pest of cotton plant were used as model species to identify key compounds mediating attraction of parasitoids to hosts. Comparative GC/MS analyses of cotton-fed versus artificial diet-fed hosts indicated that 12 of 17 compounds in the headspace of *H. virescens* larvae were exclusive to plant-fed hosts, and thus considered to be plant-associated. In order to identify key attractive compounds, a full blend of 15 commercially available synthetic compounds was modified by removing each of the 10 plant-associated compounds emitted by host larvae. In Y-tube olfactometer bioassays testing parasitoid responses to modified blends, 1-octen-3-ol, decanal, (*E*)-β-caryophyllene, α-humulene, α-farnesene and β-pinene were identified as key compounds contributing to attractiveness of the natural blend of VOCs emitted by cotton-fed hosts. The results showed that while various host-associated compounds act in concert to serve as useful host location cues, only a fraction of the natural blend mediates attraction in parasitoids. Furthermore, the role of a compound is better assessed in the context of other compounds, and odor blends are better perceived as a whole rather than as individual components.

In chapter IV, I investigated whether the relative abundance of the compounds emitted by cotton-fed *H. virescens* in chapter III is correlated with the level of antennal response in *M. croceipes*. In the present study, the olfactory response of female *M. croceipes* to synthetic versions of 15 previously identified compounds was tested in electroantennogram (EAG) bioassays. Female *M. croceipes* showed varying EAG responses to test compounds, indicating different levels of bioactivity in the insect antenna. Eight compounds, including decanal, 1-octen-3-ol, 3-octanone, 2-ethylhexanol, tridecane, tetradecane, α-farnesene and bisabolene, elicited EAG responses above or equal to the 50th percentile rank of all responses. Interestingly, decanal, which represented only 1% of the total amount of odors emitted by cotton-fed hosts, elicited the highest (0.82 mV) EAG response in parasitoids. On the other hand, (*E*)-β-caryophyllene, the most abundant (29%) blend component, elicited a relatively low (0.17 mV) EAG response. The results suggest that EAG response to host-related volatiles in parasitoids is probably more influenced by the ecological relevance or functional role of the compound in the blend, rather than its relative abundance.

In chapter V, I summarized the major findings of my dissertation research and discussed areas of future studies in insect olfaction research. The effect of atmospheric pollution on the fidelity of plant-plant, plant-insect and tritrophic interactions was identified as one of the areas that merits further consideration. Future studies should investigate the effect of atmospheric pollutants such as ozone on signaling and metabolomic responses in plants, and to understand how plants may cope with these widespread abiotic stressors. In addition, the underlying behavioral, physiological and molecular mechanisms by which insects respond to the complex of plant odors and air pollutants should be studied.

In the appendix, I introduced an on-going study that tracked behavioral responses of two parasitoids with varying degrees of host specificity, *M. croceipes* (relatively specialized) and *C. marginiventris* (generalist) to defensive secretion of a their larval host, *H. virescens*. Three hypotheses were tested: i) herbivore defensive secretion effectively deters parasitoids due to the presence of plant-derived chemicals, ii) *M. croceipes* (specialist) will show greater ability to circumvent host defensive secretion than *C. marginiventris* (generalist), and iii) self and conspecific defensive secretions will elicit behavioral responses in herbivores. A tracking software was used to analyze and visualize the behavioral responses of parasitoids to host defensive secretion.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Overview

Plants and herbivores are in a constant arms race for survival. As an indirect defense to herbivore damage, plants release many types of volatile organic compounds (VOCs), which guide natural enemies such as parasitoids to their herbivore hosts. Herbivores also emit plant-associated compounds after feeding on plants. These plant-associated cues can be used by parasitoids to locate their hosts. However, herbivore- and plant-related experiences influence how parasitoids respond to these host location odor cues. Regardless of the type of odor cue, parasitoids are usually not attracted to all the compounds in an odor blend. Some blend components are key to parasitoid attraction while others may provide context for host recognition. Further, the relative abundance of a blend component does not necessarily predict its relevance. While parasitoids have evolved efficient host location strategies, some herbivore species have also coevolved to deter their natural enemies by using defensive secretions. It is hypothesized that the extent to which parasitoids respond to, and manipulate these defensive secretions may depend on their degree of host specificity.

1.2 Parasitoid Life Strategy and Tritrophic Interactions

1.2.1 Parasitoid life strategy

Parasitoids and other natural enemies of pest insects have been the focus of several of previous studies, with potential practical application in integrated pest management systems.

Female parasitoids lay their eggs inside or on their hosts (parasitization). The eggs hatch and parasitoid larvae exploit host tissues, eventually killing the host after egression. Thus, parasitoids and their hosts share an intimate life history and their relationships often represent classic examples of species coevolution. Parasitoids exhibit a wide variety of adaptive life strategies. These include ovigeny – proportion of lifetime eggs matured on female emergence, host feeding – consumption of host tissue/hemolymph by adults, and egg resorption – reallocation of oocyte materials for body maintenance (Jervis and Kidd 1986; Quicke 1997). Parasitoids can be idiobiont or kionobiont, depending on whether they prevent or allow further development of host after initial parasitization, respectively (Harvey and Strand 2002). In solitary parasitoid species, only one progeny can successfully complete development in a host while several progenies can utilize a single host in gregarious species. Ectoparasitoids lay their eggs on the host while endoparasitoids deposit their eggs inside the host (Quicke 1997). Another important strategy is the degree of host specificity in parasitoids. Based on their host range, parasitoids can be broadly categorized as specialist (restricted host range) and generalist (broad host range). The degree of host specialization in parasitoids is a continuum, with only few species at the two extreme ends of the spectrum, across which parasitoids exhibit variation in use of host location cues associated with their herbivore and plant hosts. Parasitoids rely heavily on use of odor cues and are generally considered good models for studying insect olfaction and cognition studies (Meiners et al. 2002; Olson et al. 2003; Rains et al. 2006; Ngumbi et al. 2012).

1.2.2 Plant-herbivore-parasitoid tritrophic interactions

Herbivores feed on plants to obtain essential nutrients. In response to herbivore damage, plants have developed various defensive strategies against infestation (Agrawal and Rutter 1998; Heil et al. 2000; Ness 2003). Such strategies include chemical (toxins and repellents) and physical (spines, thorns, trichomes, etc) mechanisms. Herbivores have also developed countermeasures against plant defense in an evolutionary arms race (Winde and Wittstock 2011). Behavioral strategies include avoidance of toxin ingestion (Dussourd and Eisner 1987) while physiological strategies include detoxification, excretion or sequesteration of plant toxins (Ivie et al. 1983; Opitz and Muller 2009; Winde and Wittstock 2011; Mithöfer and Boland 2012).

Plants can also defend themselves indirectly by emitting VOCs that can attract parasitoids to their herbivore hosts (Figure 1). VOCs are secondary plant metabolites that are considered to play important roles in indirect defense by recruiting natural enemies of herbivores (Berenbaum 1996; Rasmann and Agrawal 2009). A recent study about hyperparasitoid host location revealed that plants show differences in metabolomic responses when infested by healthy versus parasitized herbivores (Zhu et al. 2015), indicating another level of intricacy in the plant-herbivore-parasitoid tritrophic interaction. Although secondary plant metabolites are used for indirect defense, specialist herbivores may exploit such compounds for locating host plants. In such cases, specialist herbivores become more adapted species than generalist herbivores, and use plant defensive compounds as kairomones for locating plant hosts. For instance, specialist herbivores use glucosinolates peculiar to cruciferous plants to find host plants (Visser 1986).

In general, successful parasitization requires at least three stages of selection: host habitat location, host location and host acceptance (Vinson 1976; Steiner et al. 2007). To optimize host foraging activities, parasitoids must use the most reliable cues available during the time of the day that they are most active (Turlings et al. 2005). While odor cues are most important to parasitoids for host location, other types of cues such as visual and tactile input may be integrated for host recognition and acceptance.

1.3 Odor Cues Used by Parasitoids for Host Location

In the plant-host complex, infochemical cues used for host location in parasitoids can be emitted by plants when infested by herbivores (De Moraes et al. 1998; Turlings and Wäckers 2004; Pare and Tumlinson 1999; Peñaflor et al. 2011; Uefune et al. 2012; Fürstenberg-Hägg et al. 2013) or emitted by herbivores and their frass after feeding on plants (Elzen et al. 1984; Alborn et al. 1995; Chuche et al. 2006; de Rijk et al. 2016). In addition, it has been proposed that traces of chemicals cues carried over by immature insects can affect their sensitivity to such chemicals at adult stage (chemical legacy hypothesis) (Corbet 1985). However, the validity of the hypothesis is still under debate (see Baron 2001).

1.3.1 Plant signalling cascade

Upon herbivore infestation, the combination of tissue damage and oral elicitors produced by insects, induce defense signaling cascade in plants. Within seconds, depolarization of plasma transmembrane potential (V_m) occurs, followed by increase in level of cytosolic calcium ions (Ca^{2+}) within minutes (Zebelo and Maffei 2014). Hydrogen peroxide (H_2O_2) is produced within minutes to hours due to an oxidative burst caused by herbivory (Fürstenberg-Hägg et al. 2013). This is followed by kinases and production of the phytohormones, jasmonic acid (JA) and salicylic acid (SA) within hours (Fürstenberg-Hägg et al. 2013; Zebelo and Maffei 2014). JA is usually associated with damage by chewing insects while SA is usually associated with damage by sucking insects or pathogens, with reports of crosstalk between the pathways of both phytohormones (Fürstenberg-Hägg et al. 2013). Phytohormone production is followed by activation of defense genes within hours and metabolic changes, including volatile production occurs within hours to days (Fürstenberg-Hägg et al. 2013; Zebelo and Maffei 2014) (Figure 2).

1.3.2 Plant volatiles and temporal dynamics

Plant volatiles are important long- and short-range odor cues for foraging parasitoids. The composition of plant VOCs can vary depending on several factors including plant species, herbivore species, type and duration of damage, and abiotic stresses (Hilker and Meiners 2002; Dicke et al. 2009; Morawo and Fadamiro 2014a; Ngumbi and Kloepper 2016). Most VOCs involved in plant defenses are products of the lipoxygenase pathway, shikimic acid pathway and terpenoid pathway (Pichersky and Gershenzon 2002). Plants constitutively release small amounts of certain compounds which may attract foraging insects (Wackers 2004). For instance, a few monoterpenes such as α-pinene, β-pinene and myrcene are emitted by undamaged cotton (Rose and Tumlinson 2004; Magalhaes et al. 2012; Morawo and Fadamiro 2014a). The combination of tissue damage caused by feeding and action of elicitors from insect oral secretions, induces increased emission of constitutive and newly synthesized volatiles (Pare and Tumlinson 1997; Boland et al. 1998; Turlings et al. 1998; Rose and Tumlinson 2004). Plants that are freshly damaged often release green leaf volatiles (GLVs) which are six-carbon alcohols, aldehydes and ketones (Ngumbi et al. 2009; Magalhaes et al. 2012; Hagenbucher et al. 2013; Morawo and Fadamiro 2014a). As duration of damage progresses, several host-induced plant volatiles (HIPVs) are synthesized *de novo*. In cotton, corn and other similar plants, HIPVs emitted include (*E*)-β-ocimene, linalool, (*E*)-β-farnesene, nonatriene and tridecatetraene, indole, hexenyl acetates, isomeric hexenyl butyrates and 2-methyl butyrates (Loughrin et al. 1994; De Moraes et al. 1998; Rose et al. 1998; Pare and Tumlinson 1999; Rose and Tumlinson 2004; Hagenbucher et al. 2013; Morawo and Fadamiro 2014a). It should be noted that temporal dynamics in the production of plant volatiles affect plant chemical profile at different stages of herbivory. It is possible that such variations lead to temporal partitioning of host resources in certain parasitoid species (Morawo and Fadamiro 2014a).

1.3.3 Herbivore-related odor cues

Herbivores can acquire plant chemicals in their diet (Despres et al. 2007). When herbivores emit certain plant-associated compounds, they can serve as kairomones used by parasitoids to locate their hosts. These kairomones may originate from body, frass or even trails left by host (Elzen et al. 1984; Alborn et al. 1995; Chuche et al. 2006; de Rijk et al. 2016). It has been demonstrated that herbivore diet affects olfactory responses of parasitoids to host-specific odors (Ed Sauls et al. 1979; Nettles 1980; Elzen et al. 1984; Hofstetter and Raffa 1997; Sullivan et al. 2000; Reis et al. 2003). Arguably, plants have larger biomass and produce more abundant volatiles than herbivorous insects (Turlings et al. 1995). However, once parasitoids find host plant patch and make appropriate landing on a plant, herbivore host-specific odors become critical short-range cues in the later phase of host location process (Afsheen et al. 2008; de Rijk et al. 2013; Colazza et al. 2014). This aspect of plant-herbivore- parasitoid tritrophic interaction has been largely underexplored. To the best of my knowledge, this is one of the first studies to identify compounds mediating attraction of a parasitoid to plant-fed host larvae, using a combination of chemical ecology techniques.

1.4 Infochemical Use in Complex Chemical Environments

1.4.1 Classification of semiochemicals

Insects live and interact with other components of the ecosystem in complex chemical environments. Like other insects, parasitoids are exposed to chemicals originating from other organisms, those originating from abiotic sources and those released into the environment as a result of human activities. Chemicals used for communication among various species are generally called semiochemicals. These chemicals can be broadly categorized as allelochemicals (for interspecific communication) or pheromones (for intraspecific communication).

Furthermore, allelochemicals can be classified as allomones, kairomones or synomones depending on whether the releaser, receiver or both benefit from the communication. Allomones affect the behavior of another species (receiver) in a way that only benefits the releaser e.g., plant chemicals that repel herbivores. Kairomones benefit the receiver, but not the releaser e.g., herbivore host-specific odor cues that attract parasitoids. Synomones benefit both releaser and receiver in a mutualistic-like relationship e.g., herbivore-induced plant volatiles that attract parasitoids to their hosts (Vet and Dicke 1992).

1.4.2 Complexity of chemical environments

Given the plethora of odorants encountered by parasitoids and their reliance on olfactory cues, they must develop efficient means to perceive, interpret and respond to various odors to ensure optimal foraging. Natural odors often exist in blends of different compounds with varying quantity and quality. Therefore, the ability to discriminate various odor blends is critical to host foraging in parasitoids. Consequently, only a fraction of host-associated odor blends are attractive or ecologically relevant to parasitoids, thus, the need to identify key compounds that mediate behavioral responses in these species (D'Alessandro and Turlings 2005; D'Alessandro et al. 2009; van Dam et al. 2010; Clavijo McCormick et al. 2014). The presence of ecologically irrelevant compounds and air pollutants further adds another level of complexity to the use of odor cues in parasitoids. There is evidence to suggest that atmospheric pollutants such as ground-level ozone ("bad ozone") can degrade or interfere with semiochemicals used in plant-insect communication systems (McFrederick et al. 2009; Blande et al. 2010; Li et al. 2016). It is likely that pollutants or products of plant odor degradation also affect odor perception and host location in parasitoids.

1.4.3 Host specificity and use of infochemicals

Arguably, odors emitted by plant and herbivore hosts are the most relevant host location cues for parasitoids. However, the two odor sources present a ‘detectability’ versus ‘reliability’ dilemma (Vet and Dicke 1992). Plant odors can be perceived from longer range and are considered to be more detectable while herbivore-specific odors are short-range cues considered to be more reliable indicators of host presence. According to the theory of dietary specialization and infochemical use (Vet and Dicke 1992), the diet breadth of parasitoids and their hosts dictate the type of cues needed for successful host foraging. Specialist parasitoids of specialist herbivores are expected to show strong innately fixed responses to herbivore cues and herbivore-induced plant volatiles. Specialist parasitoids of generalist herbivores are expected to rely more on herbivore-derived cues than plant-derived cues. Generalist parasitoids of specialist herbivores are expected to favor innately fixed responses to herbivore-induced plant volatiles. Generalist parasitoids of generalist herbivores are expected to exhibit more random searches and less adaptiveness to use of allelochemicals, especially when host resources are dispersed (Vet and Dicke 1992). Further, the degree of host specificity in parasitoids has been shown to affect their use of various VOCs for host location (Cortesero et al. 1997; Smid et al. 2002; Chen and Fadamiro 2007; Ngumbi et al. 2010, 2012; Harris et al. 2012; Sobhy et al. 2012; Morawo and Fadamiro 2014a, b).

1.5 Mechanisms of Odor Reception and Recognition in Insects

1.5.1 Odor reception

The antenna is the principal olfactory organ in insects. There are few to several hundreds of hair-like projections on the antenna which may or may not possess pores on their surfaces. Olfactory sensilla often possess pores (e.g. sensilla placodea in braconid parasitoids) (Das et al. 2011). Odorants reception occurs through the pores of olfactory sensilla. Odor molecules then bind to odorant-binding proteins (OBPs) in the sensillum lymph. In one signaling pathway hypothesis, the complex of odor and OBP activates odorant receptors (ORs). In another pathway hypothesis, odor molecules are released from OBP-odor complex and the ligand directly activates ORs in the dendrites of olfactory receptor neurons (ORNs, also called olfactory sensory neurons) (Laughlin et al. 2008). One or few ORs are typically expressed in each ORN, and neurons bearing similar receptors converge in distinct structures called glomeruli in the antennal lobe (a neuropil) which is located in the deutocerebrum. The glomeruli synapse with second-order neurons called projection neurons. The ORs are related to seven transmembrane G-protein-coupled receptors (GPCR) (Smart et al. 2008). An odor molecule activates the OR, which then activates the G-protein. There is still a debate on the exact role of G-proteins in the signal pathway (Galizia and Sachse 2010) but it is believed to activate other membrane proteins, which later leads to the opening of Ca^{2+} channel. The influx of Ca^{2+} into the cell depolarizes the system and creates an action potential which sends electrical signals to higher centers such as mushroom bodies in the brain (Galizia and Sachse 2010). The mushroom bodies (*corpora pedunculata*) are a pair of neuropils made up of specialized neuronal cells called Kenyon cells, and considered to be the center of integration for multimodal input, memory and olfactory learning in insects (Heisenberg 1998).

1.5.2 Odor recognition and context

In foraging insects, odor recognition may be achieved by one of three proposed mechanisms: (i) species-specific odor recognition in which a few compounds associated with a particular species or closely related species are used for host recognition (Visser 1986), (ii) ratio-specific odor recognition in which relative ratios of certain ubiquitous volatiles are used to identify hosts (Visser 1986), or (iii) whole blend odor recognition in which all of the blend components are recognized as a whole (an all-or-none situation) (Clavijo McCormick et al. 2012; Morawo and Fadamiro 2016) .

Chemical ecologists are interested in differentiating useful cues from ‘background noise’, with regards to insects’ use of host-associated odor blends. According to the olfactory contrast hypothesis, compounds in an odor blend that are not key to parasitoid attraction may still function as background odors to enhance detection of other important resource-indicating cues (Hilker and McNeil 2008). Thus, background odors may provide context by which a resource-locating cue is perceived, leading to better odor recognition and discrimination. The importance of background odors to odor perception and recognition in several insect species, including parasitoids (Mumm and Hilker 2005; Schröder and Hilker 2008; Wajnberg et al. 2008; Beyaert et al. 2010; Clavijo McCormick et al. 2012; Clavijo McCormick et al. 2014), house fly (Kelling et al. 2002) and *Drosophila* (Faucher et al. 2006) have been previously discussed (Morawo and Fadamiro 2016).

1.6 Learning in Parasitoids

Insects have a relatively limited neural architecture with reduced number of neurons and small brains, yet they exhibit a remarkable level of behavioral plasticity, compared to other larger animals (Giurfa 2015). Insects are capable of modifying their behavior by learning from various experiences and storing the learned information (memory) for subsequent use. The Pavlovian conditioning (associating conditioned and unconditioned stimuli) and operant conditioning (associating a behavior with a reinforcement of the action) are the most common forms of associative learning (Giurfa 2015).

Parasitoids have been regarded as good models for cognition and olfaction studies in insects (Meiners et al. 2002; Rains et al. 2004; Harris et al. 2012; Ngumbi et al. 2012). In particular, parasitoids can associate an activity with a reward (positive association) or punishment (negative association). Such associations may reinforce learning of new or routine stimuli. Consequently, positive associative learning may lead to subsequent attraction to a stimulus while negative associative learning may lead to a subsequent avoidance behavior. Several previous studies have reported odor association in parasitoid species (Geervliet et al. 1998; Honda and Kainoh 1998; Olson et al. 2003; Takasu and Lewis 2003; Rains et al. 2006; Harris et al. 2012; Ngumbi et al. 2012).

Parasitoids may learn from various experiences during foraging activities and such experiences may shape subsequent host selection and location strategies. Therefore, studies that seek to model host selection in parasitoids should consider the effect of various experiences on their foraging decisions. In particular, associative learning with an unconditioned stimulus (e.g., food) and conditioned stimulus (e.g., plant odor) has been shown to enhance attraction of parasitoids to host-induced plant volatiles (Wackers et al 2002; Olson et al. 2003; Ngumbi et al 2012). It is likely that parasitoids learn plant-related experiences, herbivore-related experiences, or both to optimize their foraging activities.

1.7 Techniques in Chemical Ecology

The science of chemical ecology presents an opportunity to study the natural chemical communication among interacting organisms in the ecosystem, including plant-herbivore-parasitoid tritrophic systems. Chemical ecology mainly combines techniques in chemistry and ecology to: i) test biological activity and behavioral responses of interacting organisms to chemical signals, ii) isolate the chemical signals or compounds, iii) identify, quantify and characterize such chemicals, and iv) investigate the mechanisms of signal reception, processing and response in organisms. Some common methods used in insect chemical ecology includes olfactometer and flight tunnel bioassays, headspace volatile collection, electroantennogram (EAG) recording, single-sensillum recording, microscopy, odor mapping, gas chromatography (GC), mass spectrometry (MS), coupled GC-EAD, and coupled GC-MS. In recent years, molecular techniques have been developed for identifying and characterizing odorant receptors and associated genes. These include reverse transcription polymerase chain reaction (RT-PCR), *Xenopus* oocyte expression system, two-electrode voltage clamp electrophysiological recording, cDNA cloning, genomic techniques, etc. (Liu et al. 2017; Zhang et al. 2017). This study used a combination of behavioral, electrophysiological, analytical and tracking techniques to investigate certain underexplored aspects of plant-herbivore-parasitoid tritrophic interactions (see example in Figure 3).

1.8 Model System and Justification of the Study

1.8.1 Model system

This study uses a tritrophic model system which includes two plants, cotton (*Gossypium hirsutum* L.) and soybean [*Glycine max* (L.) Merrill]; an herbivore host, *Heliothis virescens* (F.) (Lepidoptera: Noctuidae); and two parasitoid species, *Microplitis croceipes* (Cresson) (Hymenoptera: Braconidae) and *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae). Cotton and soybean are important economic crops in the United States and many other countries in the world. Their production is limited by insect pest infestation including caterpillars of *H. virescens*, a generalist herbivore on several field crops (Fitt 1989). Both *M. croceipes* and *C. marginiventris* are solitary larval endoparasitoids of *H. virescens*. However, *M. croceipes* is a relatively specialized parasitoid of the *Heliothis*/*Helicoverpa* complex (Tillman and Laster 1995) while *C. marginiventris* is regarded as a generalist parasitoid of several noctuid host species (see Tillman 2001 for host range). Both parasitoid species have been shown to use host-related odor cues to locate their hosts (Elzen et al. 1987; Navasero and Elzen 1989; Meiners et al. 2002; Turlings et al. 2004; Ngumbi and Fadamiro 2012; Harris et al. 2012; Sobhy et al. 2012; Morawo and Fadamiro 2014a, b, 2016).

1.8.2 Justification of the study

A great deal of attention has been devoted to the chemical ecology of plant-insect interactions in the past few decades, and in particular, tritrophic interactions involving parasitoids have been widely studied. A quick search of the keyword “parasitoid” in the Journal of Chemical Ecology alone yielded over 600 articles as of 2017. Thousands more have been published on this area of research in other related scholarly journals. Yet, there are still aspects of plant-herbivore parasitoid chemical ecology that have been largely underexplored.

Several previous studies have compared the responses of specialist and generalist parasitoids to host-related plant volatiles (Elzen et al. 1987; Navasero and Elzen 1989; Meiners et al. 2002; Turlings et al. 2004; Ngumbi and Fadamiro 2012; Harris et al. 2012; Sobhy et al. 2012). Vet and Dicke (1992) proposed a relationship between dietary specialization and infochemical use by natural enemies. Although several studies have attempted to test this theory in parasitoids, few to none have considered the effect of experience type in the same matrix. Tested as a stand-alone factor, learning/experience has been shown to influence foraging decisions in parasitoids. In the present study, I asked whether the type of experience (herbivore- or plant-related) impacts the expected use of infochemical by a specialist parasitoid that attacks a generalist herbivore.

Among the more explored aspects of tritrophic interactions involving parasitoids is their olfactory and behavioral responses to herbivore induced plant volatiles (De Moraes et al. 1998; Pare and Tumlinson 1999; Turlings and Wäckers 2004; Peñaflor et al. 2011; Uefune et al. 2012;

Fürstenberg-Hägg et al. 2013). Parasitoid response to herbivore-specific cues such as frass and exuviae has also been reported (Elzen et al. 1984; Alborn et al. 1995; Chuche et al. 2006; de Rijk et al. 2016). However, plant-associated compounds acquired and subsequently emitted from herbivore body have been largely overlooked. While plants have larger biomass and evidently emit greater quantity of volatile compounds, herbivores emit a unique blend of plant-derived compounds that are probably better indications of host presence and identity (Turlings et al. 1995; Afsheen et al. 2008; Colazza et al. 2014; De Rijk et al. 2013). It is expected that these herbivore-emitted odor cues are useful in short-range host location and recognition in parasitoids. Therefore, the compounds were isolated, identified and characterized to ivestigate their relevance to host foraging in parasitoids.

In general, parasitoids encounter odor cues as a blend of various compounds in nature. Compounds in a blend often vary in quantity, and perform different roles in the blend (Clavijo McCormick et al. 2012, 2014; Morawo and Fadamiro 2016). It is assumed that the level of olfactory response of a parasitoid to a compound is informed by the ecological relevance of the compound rather than its physical properties (Ngumbi et al. 2010). Yet, few studies have tested this hypothesis. Following the characterization of volatile blend emitted by plant-fed *H. virescens*, I asked whether the level of antennal response of the parasitoid *M. crociepes* correlates with the relative abundance of constituent compounds.

Lastly, the vast majority of studies on plant-herbivore interactions have focused on host selection, feeding behavior, herbivore defensive strategies and induced defensive responses in plants. Herbivores can sequester plant chemicals for defensive purposes against natural enemies (Kazana et al. 2007; Opitz and Muller 2009; Agrawal et al. 2012). However, some natural enemies have also evolved avoidance tactics against herbivore host defenses, including defensive secretions (Goff and Nault 1974; Rotheray 1981; Gross 1993). In preliminary studies, I observed that the parasitoid, *M. croceipes* was initially deterred by *H. virescens* defensive secretion but later showed a strong attraction to the dried secretion. Allelochemicals produced by one species of organism that act as both allomone and kairomone to another species based on temporal dynamics is uncommon. It is possible that the degree of host specificity in parasitoids may influence their ability to manipulate herbivore host defensive secretions. Thus, the present study compared the behavioral responses of *M. croceipes* (relatively specialized) and *C. marginiventris* (generalist) to defensive secretion of their larval host, *H. virescens* using a tracking software.

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Figure lengend

Figure 1: Illustration of plant-herbivore-parasitoid tritrophic interactions.

Figure 2: Post-herbivory signaling cascades in plant (Modified from Fürstenberg-Hägg *et al.*, 2013).

Figure 3: An example of how chemical ecology techniques may be combined to identify semiochemicals. (A) Four-choice olfactometer coupled with headspace volatile collection from plant-fed host larvae. Behavioral response of parasitoids to odors host-related odors are tested in four-choice olfactometer bioassays while simultaneously trapping headspace volatiles from host larvae enclosed in airtight glass chamber using a Super-Q trap. (B) Gas chromatography (GC) coupled with mass spectrometry (MS). GC machine separates a mixture of compounds into individual components using a gaseous mobile phase and solid stationary phase (column). MS ionizes the compounds with a stream of electrons so that they can be sorted based on mass to charge ratio (M/Z).

Figure 1

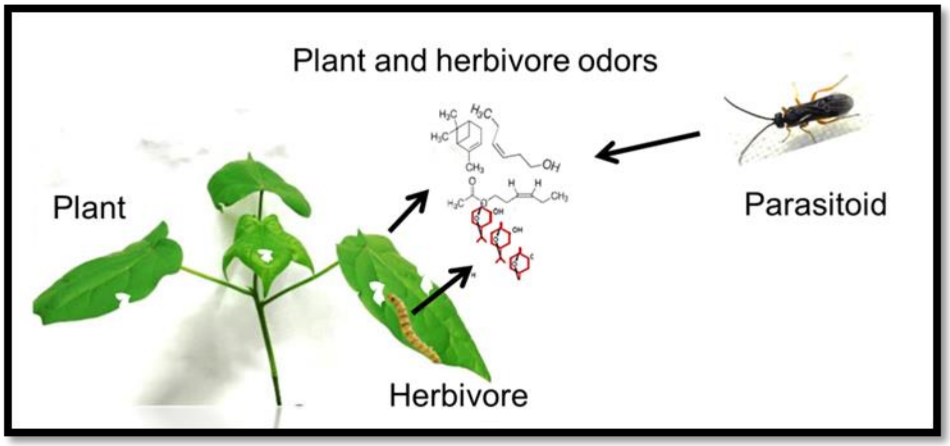


Figure 2

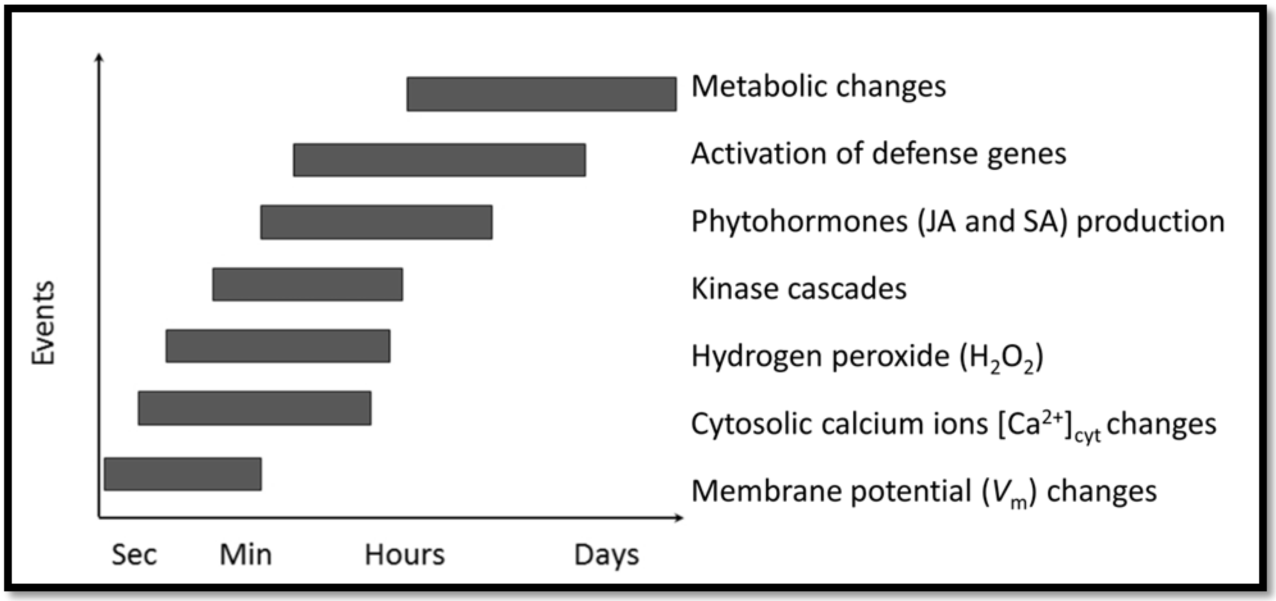
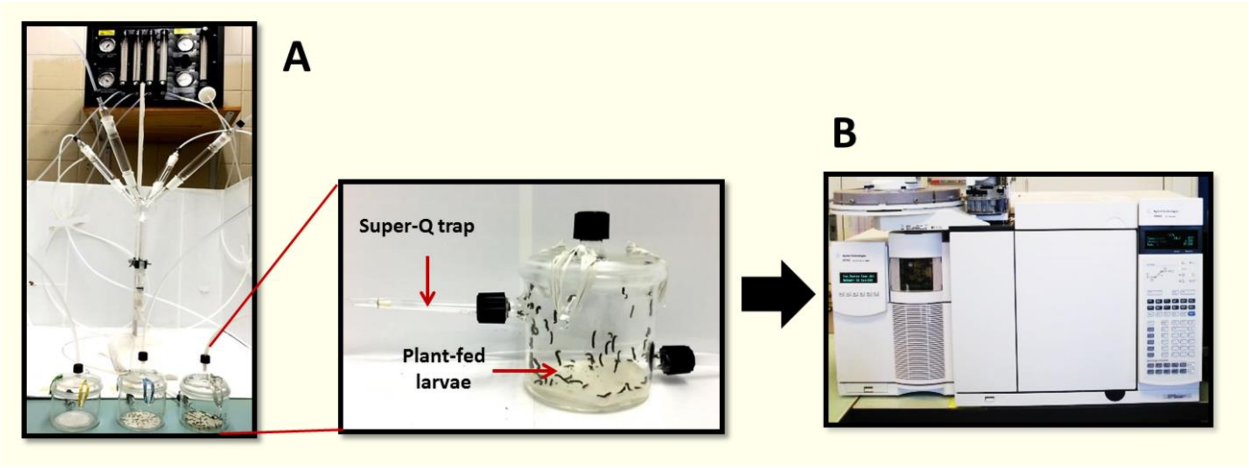

Metabolic changes
Activation of defense genes
Phytohormones (JA and SA) production
Kinase cascades
Hydrogen peroxide (H_2O_2)
Cytosolic calcium ions $[Ca^{2+}]_{cyt}$ changes
Membrane potential (V_m) changes
Events
Sec
Min
Hours
Days

Figure 3



CHAPTER 2

THE ROLE OF HERBIVORE- AND PLANT-RELATED EXPERIENCES IN INTRASPECIFIC HOST PREFERENCE OF A RELATIVELY SPECIALIZED PARASITOID

2.1 Introduction

Parasitoids are important agents of biological control against pest insects. They rely on olfactory cues to locate their herbivore hosts in a complex chemical environment. In general, successful parasitization requires at least three different but continuous stages of selection: host habitat location, host location and host acceptance (Vinson 1976; Steiner et al. 2007). The host location process is largely dependent on use of olfactory cue from plants, herbivore hosts or the interaction between plants and herbivore hosts (Elzen et al. 1984; De Moraes et al. 1998; Steidle and Van Loon 2003; Turlings and Wäckers 2004; Chuche et al. 2006; Fürstenberg-Hägg et al. 2013; de Rijk et al. 2016; Morawo and Fadamiro 2016). The role of plant-emitted volatiles has been the main focus of several previous studies, with less attention devoted to herbivore-emitted volatiles.

Plant volatiles are more detectable and considered important long-range cues for foraging parasitoids to locate host plant patch (Vet and Dicke 1992; Röse et al. 1997; Steidle and Van Loon 2003). However, at short-range, herbivore host cues are considered more reliable for host location in parasitoids (Vet and Dicke 1992; Alborn et al. 1995; De Rijk et al. 2013; Colazza et al. 2014; Morawo and Fadamiro 2016). Parasitoids likely resolve the detectability-reliability problem by using plant volatiles as long-range cues and herbivore odors as short-range cues (Colazza et al. 2014). Host acceptance is marked by oviposition, and is largely mediated by odor cues. Parasitoid species that are not egg-limited often seek out more hosts after their first oviposition experience (Mills and Wajnberg 2008; Hopper et al. 2013). Thus, parasitoids are exposed to both plant- and herbivore-related experiences in the course of foraging.

Based on their host range, parasitoids can be broadly categorized as specialist (restricted host range) and generalist (broad host range). Furthermore, their herbivore hosts can be specialists (monophagous) or generalists (polyphagous). These dietary specializations strongly influence the life strategy and foraging behavior of parasitoids. However, the degree of host specialization in parasitoids is a continuum, with only few species at the two extreme ends of the spectrum. According to the concept of dietary specialization and infochemical use proposed by Vet and Dicke (1992), the degree of polyphagy in a generalist herbivore should drive its specialist parasitoid to rely more on herbivore cues than plant cues. The foraging behavior of specialist parasitoids is likely complemented by a narrowly tuned olfactory mechanism, compared with generalist species (Cortesero 1997; Meiners et al. 2002; Chen and Fadamiro 2007; Ngumbi et al. 2009; Najar-Rodriguez et al. 2015). This strategy greatly reduces the number of different chemical patterns that a specialist parasitoid would have to recognize if it only depended on plant-emitted volatiles (Wajnberg et al. 2008).

Regardless of the source of olfactory cues used, several parasitoid species are able to modify their behavior by learning from various foraging experiences (Vet and Groenewold 1990; Olson et al. 2003; Hoedjes et al. 2011; Ngumbi et al. 2012; de Rijk et al. 2013). As such, studies that seek to model host selection in parasitoids should consider the effect of experience on their foraging decisions (Vet 1999). Parasitoids can associate an activity with a reward. Such reward may reinforce learning of new or routine stimuli. In female parasitoids, host-related odors (conditioned stimulus) may be associated with a reward (unconditioned stimulus) such as food or oviposition. In particular, associative learning with an unconditioned stimulus (e.g., oviposition) and conditioned stimulus (e.g., plant odor) has been shown to enhance attraction of the parasitoid, *Microplitis croceipes* (Cresson) (Hymenoptera: Braconidae) to host-induced plant volatiles (Lewis and Takasu 1990; Olson et al. 2003; Takasu and Lewis 2003; Ngumbi et al. 2012). Therefore, it is also important to investigate the combined effect of both plant- and herbivore-related experiences on the foraging behavior of parasitoids.

Parasitoids share an intimate life history with their hosts. According to the optimal foraging theory, female parasitoids are expected to select hosts that best support the development of their progeny (Pyke 1984; Goubault et al. 2003; Steiner et al. 2007; Chesnais et al. 2015). Parasitoid species with relatively higher longevity (less affected by time-limitation) are expected to discriminate hosts based on suitability and quality (Hopper et al. 2013). In turn, the quality of herbivore host depends on plant nutrients and phytotoxins, which have direct impact on the development of immature parasitoids (Harvey et al. 2013). Each plant species expresses a distinct chemical profile, even when infested by the same herbivore species (Van Den Boom et al. 2004). Furthermore, herbivores may retain and emit different plant-associated compounds, depending on their diet (Morawo and Fadamiro 2016). Therefore, intraspecific host preference may be expected in a specialist parasitoid that utilizes a generalist herbivore. However, such discriminatory ability is probably influenced by the experience of a female parasitoid (Hopper et al. 2013). Also, parasitoid experience likely compensates for the challenge of incomplete information in a complex chemical environment (Mills and Wajnberg 2008).

Microplitis croceipes is a relatively specialized larval endoparasitoid of *Heliothis virescens* (F.) (Lepidoptera: Noctuidae), which is a generalist herbivore on several crops including cotton and soybean (Fitt 1989). *Microplitis croceipes* is described as relatively specialized because it is restricted to very few host species in the *Heliothis*/*Helicoverpa* complex (Tillman and Laster 1995). In the present study, I investigated the role of certain plant- and herbivore-related experiences on short-range intraspecific host preference in *M. croceipes*. Parasitoids are generally considered good models for studying insect olfaction, and *M. croceipes* is especially considered an excellent model for cognition studies (Meiners et al. 2002; Olson et al. 2003; Rains et al. 2006; Ngumbi et al. 2012). Based on the concept of dietary specialization and infochemical use (Vet and Dicke 1992), the following predictions were tested about specialist parasitoids of generalist herbivores using *M. criceipes/H. virescens* as a model system: i) naïve parasitoids will show innate responses to herbivore-emitted kairomones, regardless of host plant identity and ii) herbivore-related experience will have greater influence on intraspecific oviposition preference than plant-related experience. The effect of various experiences/conditioning, as well as plant backgrounds on intraspecific host preference was tested in behavioral bioassays using cotton-fed and soybean-fed *H. virescens* larvae. I discussed the ecological significance of the results.

2.2 Materials and Methods

2.2.1 Plants. Cotton (*Gossypium hirsutum* L., var. max 9) and Soybean (*Glycine max* (L.) Merrill, var. Pioneer P49T97R-SA2P) plants were grown according to Morawo and Fadamiro (2014) in growth chambers (Auburn University) at 26.6 °C day, 24.6 °C night, and 60% RH. Illumination was provided using daylight fluorescent tubes (270 PAR) with a 16:8 h (L/D) photoperiod. Seeds were planted in a top soil/vermiculite/peat moss mixture in plastic pots. Plants used in bioassays were 4-5-week-old. In order to ensure that “plant-fed” *H. virescens* larvae used in each bioassay fed on a substantial amount of leaf tissues, plants were laid down horizontally in clear aerated plastic bowls (28 × 16 × 8 cm). Cotton and soybean were selected for this study partly because they are economically important crops in the southern United States, and partly because they are expected to have relatively different chemical profiles. For instance, herbivory significantly induces gossypol in cotton (Zebelo et al. 2017) while methyl salicylate is induced in soybean (Zhu and Park 2005).

2.2.2 Insects. *Heliothis virescens* larvae were initially reared on pinto bean artificial diet and adult moths fed 10% sugar water *ad libitum*. Larvae were removed from artificial diet and starved for 6 h in a plastic bowl. Afterwards, the larvae were cleaned of frass and artificial diet crumbs with moistened paper towel. Then, they were separated into two groups: one group was fed cotton foliage (cotton-fed) while the other group was fed soybean foliage (soybean-fed). Before use in oviposition choice bioassays, 3rd instar larvae of *H. virescens* were removed from plant materials and cleaned with moistened paper towel. *Microplitis croceipes* were reared on 2nd-3rd instar larvae of *H. virescens* and adult wasps were supplied 10% sugar water *ad libitum* upon emergence. The rearing procedures for *M. croceipes* were similar to those described by Lewis and Burton (1970). Parasitoids used in behavioral bioassays were 3–4 day–old females that were presumed mated after interaction with males for at least 48 h. The general rearing conditions for all insects were 25±1 °C, 75±5 % RH and 14:10 h (L:D) photoperiod.

2.2.3 Feeding choice tests for host larvae. Feeding bioassays were conducted in no-choice and two-choice experiments to test the preference of *H. virescens* larvae for cotton or soybean. Larvae were initially fed artificial diet and cleaned as previously described. For these experiments, the leaf (cotton) or trifoliate leaf (soybean) of an infested plant was clipped at the petiole, close to the leaf blade. In no-choice tests, a leaf from either plant was placed centrally in an aerated plastic bowl (22 × 16 × 6 cm) inlayed with moistened paper towel. Ten 3rd instar larvae were placed on the leaf to feed for 24 h. In two-choice tests, one cotton leaf and one soybean trifoliate leaf were placed at opposite ends of a similar bowl, separated by at least 5 cm. Ten 3rd instar larvae were randomly placed in the space between the leaves of both plants to feed for 24 h. Leaf pictures were taken before and after infestation for leaf area analyses in ImageJ software. Experiments were performed in a randomized complete block design using each of four plant replicates on separate days.

In initial feeding tests, *H. virescens* larvae showed preference for cotton over soybean, and consumed about 2 fold cotton foliage compared with soybean foliage within 24 h (Fig. 1). The feeding preference of *H. virescens* larvae informed the duration or number of larvae that should infest cotton or soybean to avoid possible bias in subsequent oviposition choice tests with parasitoids. Thus, for oviposition choice experiments with parasitoids, a group of 40 *H. virescens* larvae was fed cotton for 24 h while a similar group was fed soybean for 48 h.

2.2.4 Treatments for oviposition choice tests. In two-choice oviposition tests with cotton-fed and soybean-fed hosts, female parasitoids received one of seven treatments: i) No experience with oviposition/ conditioning with host-damaged plant/ plant background (Naïve), ii) Experience with oviposition in cotton-fed hosts only (OV-Cotton), iii) Experience with oviposition in soybean-fed hosts only (OV-Soybean), iv) Conditioned with host-damaged cotton only (DG-Cotton), v) Conditioned with host-damaged soybean only (DG-Soybean), vi) Inexperienced parasitoids tested with a background of host-damaged cotton odors (BG-Cotton), and vii) Inexperienced parasitoids tested with a background of host-damaged soybean odors

(BG-Soybean). Parasitoids with treatments BG-Cotton and BG-Soybean had no previous oviposition experience. See Fig. 2 for illustration.

For OV-Cotton and OV-Soybean treatments, a female parasitoid was presented with a cotton-fed or soybean-fed host in a training petri dish (25 × 10 mm) and allowed a single oviposition. The host larva was immediately removed while the wasp remained in isolation for about 5 min. Then, the procedure was repeated a second time with a new host larva of the same type. A previous study (Eller et al. 1992) showed that a single experience with plant-host complex was insufficient to elicit subsequent preference in *M. croceipes*. Thus, parasitoids were trained two times. For DG-Cotton and DG-Soybean treatments, a plant was infested with 20 (for cotton) or 40 (for soybean) third instar *H. virescens* larvae for 24 h to compensate for host feeding preference. Each infested plant was placed in a 5 L glass jar (Analytical Research Systems, Inc., Gainesville, FL, USA). Host larvae and frass were removed and a female parasitoid was introduced into the jar. The parasitoid was observed to antennate on the leaves (contact leaf with antenna in a probing manner) 4-5 times before it was removed. The parasitoid was left in isolation for about 5 min and the procedure was repeated a second time using the same plant. After conditioning treatments, female parasitoids were left to acclimatize in arena conditions for 5 min before use in a bioassay.

For BG-Cotton and BG-Soybean treatments, plants were infested and placed in a glass jar as previously described. The petri dish oviposition arena was perforated with 10 holes (2mm diameter each) uniformly spread on the base. The perforated base was then placed above a second base that was connected to the glass jar through Teflon® tube. A purified (charcoal filtered) and humidified air stream of 200 ml/min was passed through the jar at room temperature into the petri dish arena so that plant volatiles can serve as background odors.

2.2.5 Treatments for oviposition no-choice tests. In no-choice tests with cotton-fed or soybean-fed hosts, parasitoids received one of two treatments: i) Associative conditioning with oviposition in cotton-fed hosts in the presence of host-infested cotton odors (AC-Cotton) and ii) Associative conditioning with oviposition in soybean-fed hosts in the presence of host-infested soybean odors (AC-Soybean). Treatment controls (parasitoids with no conditioning) were also included for comparison. For associative conditioning, volatiles emitted by infested plants were passed into a training petri dish as previously described. A parasitoid was then allowed to associate oviposition experience (unconditioned stimulus) with host-infested plant odors (conditioned stimulus). The procedure was repeated two times, similar to conditioning treatments.

2.2.6 Oviposition bioassays. Two-choice oviposition tests were conducted in medium size petri dish (100 × 15 mm) arena to investigate intraspecific host preference in *M. croceipes*. One cotton-fed and one soybean-fed 3rd instar host larvae of similar size were randomly positioned in the middle of the arena before introducing each wasp. A female parasitoid was released at one of 12, 3, 6 or 9' o clock positions in the arena to avoid bias. Once introduced into the petri dish, the parasitoid was allowed a maximum of 10 min to parasitize one host larva. A parasitoid that did not make a choice within 10 min was scored as 'no-choice', removed from the arena and replaced with another parasitoid that had received the same treatment. Parasitoids, host larvae and petri dishes were used once and discarded. Oviposition choice experiments were arranged in a randomized complete block design with equal number of replicates tested for each treatment group (Naïve, OV-Cotton, OV-Soybean, DG-Cotton, DG-Soybean, BG-Cotton and BG-Soybean) on each day between 0900 and 1700 h. Oviposition choice tests were repeated two times with 30 responding parasitoids per test. Experiments testing effect of plant backgrounds were repeated three times to confirm consistency of the results. In oviposition choice bioassays, the number of parasitoids scored as ‘no-choice’ was relatively low (range 0-3) and thus excluded from data analyses.

No-choice oviposition tests were conducted with parasitoids that received treatments AC-Cotton and AC-Soybean to investigate the effect of associative learning on host location in *M. croceipes*. The experimental design and procedure was similar to the one previously described for oviposition choice tests, with some exceptions. A relatively larger petri dish (140 × 20 mm) arena was used to ensure considerable distance between host larva and parasitoid at the time of wasp release into the arena. The petri dish was perforated with 20 holes (2mm diameter each) and connected to a jar containing host-infested plant. One host larva (cotton-fed or soybean-fed) was positioned at the center of the arena before a parasitoid was released into the petri dish at one of 12, 3, 6 or 9’ o clock positions. Time taken for a conditioned parasitoid to locate and parasitize a cotton-fed host in an arena supplied with host-infested cotton odors was recorded. A similar experiment was performed with soybean-fed host/infested soybean combination. For control experiments, time taken for unconditioned parasitoids to locate and parasitize hosts in similar arenas was also recorded. Only parasitoids that responded within 10 min were included in the analyses but the number of individuals that did not respond within 10 min (range 0-5) was also recorded. No-choice oviposition tests were repeated two times with 30 replicates per test.

2.2.7 Data analyses. Feeding preference of *H. virescens* larvae between cotton and soybean was analyzed by comparing leaf areas consumed using ImageJ software (v. 1.50i). Data on *H. virescens* feeding preference satisfied normality and equal variance requirements, thus, there was no need for transformation. In two-choice feeding tests for *H. virescens*, the infested leaf areas were compared using Paired *T-test*. In no-choice feeding tests for *H. virescens*, the infested leaf areas were compared using Two independent samples *T-test*. For *M. croceipes* two-choice oviposition tests, the deviation of parasitized hosts from a 50:50 % distribution between cotton-fed and soybean fed host groups was analyzed using a *Chi-square* test. In no-choice oviposition tests with *M. croceipes*, significant differences between time taken by conditioned and unconditioned parasitoids to parasitize hosts were analyzed using Two independent samples *T-test*. Time data satisfied normality and equal variance requirements, thus, there was no need for transformation. All analyses were conducted using SAS 9.2 with 0.05 level of significance.

2.3 Results

2.3.1 Two-choice oviposition tests: Intraspecific host preference in *M. croceipes*.

Intraspecific oviposition preference in female *M. croceipes* was influenced by experience, conditioning or plant background provided. Results of oviposition choice tests are shown in Fig. 3. Naïve parasitoids did not discriminate between cotton-fed and soybean-fed hosts ($\chi^2 = 0.53$, $P = 0.47$). However, parasitoids that only had oviposition experience with cotton-fed hosts subsequently showed preference for cotton-fed over soybean-fed hosts ($\chi^2 = 8.53$, $P = 0.004$). Similarly, parasitoids that only had oviposition experience with soybean-fed hosts subsequently showed preference for soybean-fed over cotton-fed hosts ($\chi^2 = 6.53$, $P = 0.01$). Conditioning with host-infested plant (cotton or soybean) only did not significantly elicit oviposition preference in *M. croceipes*. Interestingly, inexperienced parasitoids showed preference for soybean-fed over cotton-fed hosts when host choices were presented with background of host-infested cotton odors ($\chi^2 = 4.80$, $P = 0.03$). Similarly, inexperienced parasitoids showed preference for cotton-fed over soybean-fed hosts when host choices were presented with background of host-infested soybean odors ($\chi^2 = 6.53$, $P = 0.01$).

2.3.2 No-choice oviposition tests: Effect of associative learning on host location in *M. croceipes*. Oviposition no-choice tests were conducted to investigate the effect of associative learning on host location in *M. croceipes*. Conditioned parasitoids took significantly (t = -6.63, $P < 0.0001$) less time to locate and parasitize cotton-fed hosts, compared to control (unconditioned parasitoids) (Fig. 4A). Similarly, conditioned parasitoids took significantly (t = -6.10, $P < 0.0001$) less time to locate and parasitize soybean-fed hosts, compared to control (Fig. 4B). Moreover, most of the parasitoids that received training took less than 5 min to locate host larvae while untrained parasitoids took between 5-10 min to locate host larvae (Fig 4).

2.4 Discussion

Microplitis croceipes is a relatively specialized parasitoid of *H. virescens*, which is a generalist herbivore. According to the concept of dietary specialization and infochemical use proposed by Vet and Dicke (1992), parasitoids with such diet restrictions are predicted to rely more on herbivore-derived cues than plant-derived cues. Although widely accepted, only few empirical studies have tested this concept (Steidle and Van Loon 2003), especially in dynamics with the role of parasitoid experience. In the present study, naïve (inexperienced) female *M. croceipes* did not discriminate between cotton-fed and soybean-fed *H. virescens* in oviposition choice tests, supporting the first prediction that this category of parasitoids shows innate responses to herbivore-emitted kairomones, regardless of host plant identity. Furthermore, oviposition experience alone strongly influenced subsequent oviposition preference, whereas, conditioning with host-infested plants alone did not elicit host preference in parasitoids. This supports the second prediction that herbivore-related experience has greater influence on intraspecific host preference than plant-related experience.

Plant chemicals acquired by herbivores can be used for metabolism and defense (Despres et al. 2007). When such chemicals are emitted by their herbivore hosts, they may also serve as kairomones for parasitoids (Morawo and Fadamiro 2016). In a previous study, it was reported that plant-fed *H. virescens* larvae retained and emitted plant-associated compounds that attracted *M. croceipes* (Morawo and Fadamiro 2016). In the present study, *H. virescens* larvae showed a clear feeding preference for cotton over soybean, both in no-choice and two-choice experiments. However, naïve *M. croceipes* did not discriminate between *H. virescens* larvae that fed on different plants but oviposition experience influenced subsequent intraspecific host preference.

Although oviposition in soybean-fed hosts appears to be the optimal choice due to higher survival rates of host larvae (Nadgauda and Pitre 1983), herbivore-related experience (with cotton-fed hosts) may override such considerations. This contradicts the ‘mother knows best’ hypothesis (Henry et al. 2005; Chesnais et al. 2015). In fact, preference-performance hypothesis may not adequately explain host selection; possible conflicts between the needs of progeny and behavioral dynamics of mother insects should also be considered (Scheirs and De Bruyn 2002; McCormick et al. 2017). When hosts are abundant, optimal host use in parasitoids tend to be incremental, such that oviposition increases the probability of staying in a host patch to seek more hosts (Mills and Wajnberg 2008; Kugimiya et al. 2010). In general, these results suggest that herbivore-related experiences such as oviposition play important role in host discrimination in specialist parasitoids.

Nevertheless, the results also showed that plant-related experience play an important role in shaping the foraging decisions made by parasitoids. Besides serving as highly detectable long-range cues for locating host habitat (Vet and Dicke 1992), plant volatiles may play other unique roles in short-range host location. In the present study, associative learning of infested plant odors with oviposition facilitated short-range host location in parasitoids. Such associative learning is a classic example of plant- and herbivore-related experiences combining to produce a synergistic effect. Although present results from laboratory experiments showed that oviposition experience alone influenced subsequent host preference in *M. croceipes*, herbivores are less likely to be encountered outside the context of their host plants. Comparing the volatiles emitted by cotton-fed *H. virecens* larvae (Morawo and Fadamiro 2016) and those emitted by *H. virecens*-infested cotton (Morawo and Fadamiro 2014a), it can be deduced that host-emitted volatiles are mostly plant-derived but the profile differs from that of the host plant. Therefore, in addition to the effect of associative learning, it is also possible that plant odors served as background odors to facilitate recognition of herbivore host odors (Hilker and McNeil 2008).

To further investigate the effect of background odors, naïve parasitoids were tested in oviposition choice bioassays with a background of host-infested cotton or soybean odors. Parasitoids that did not receive prior conditioning or training showed preference for soybean-fed over cotton-fed hosts when host choices were presented with background of host-infested cotton odors, and vice versa. The result is noteworthy because naïve parasitoid did not discriminate host groups when a plant background was not supplied in previous tests. The result is also interesting because parasitoids showed preference for the host group that did not feed on the plant background presented. A plausible explanation is that a background of soybean odors probably created more contrast with cotton-fed hosts than with soybean-fed hosts, and vice versa. The result further lends support to the olfactory contrast hypothesis proposed by Hilker and McNeil (2008). The more difference in the chemical profiles of an herbivore host and its plant background, the more olfactory contrast generated, and the more detectable a host larva becomes (Hilker and McNeil 2008). Similar observations have been reported for male moths (Light et al.

1993; Deng et al. 2004; Yang et al. 2004; von Arx et al. 2012) and parasitoids (Xu et al. 2017) in which plant volatiles synergistically enhanced the detection of female sex pheromones. The relevance of plant volatiles as background odors in parasitoid host location has been discussed in previous studies (Mumm and Hilker 2005; Schröder and Hilker 2008; Beyaert et al. 2010).

The enemies hypothesis predicts that pest population is reduced in mixed stands (polycultures) because natural enemies thrive in complex vegetation (Root 1973). Here, I also suggest that the olfactory contrast effect may have serious fitness consequences for generalist herbivore species such as *H. virescens* larvae in polycultures. This may also apply to more mobile generalist herbivores such as beetles that are capable of switching host plants across monoculture systems. Thus, among other factors, host switching may render a generalist herbivore more detectable to natural enemies because of the olfactory contrast generated with odors of the newly infested plant species. This leads to an opportunistic fitness benefit for specialist parasitoids of generalist herbivore species.

In summary, the present study investigated the role of plant- and herbivore-related experiences in intraspecific host preference in a relatively specialized parasitoid, *M. croceipes*. Evolution should favor an olfactory mechanism that is highly sensitive and tuned to herbivore host cues in specialist parasitoids (Vet and Dicke 1992; Steidle and Van Loon 2003; Wajnberg et al. 2008). While the results partly confirm the prediction that herbivore-related experience has greater influence than plant-related experience on intraspecific host preference in *M. croceipes*, the results also indicate a possible synergistic effect of the two types of experiences during associative learning. Further, plant odors probably serve as a contrasting background with herbivore odors (Hilker and McNeil 2008), thus facilitating host location and leading to intraspecific host discrimination in *M. croceipes*. To the best of my knowledge, this is one of the few studies to confirm the olfactory contrast hypothesis in a similar tritrophic system. Overall, these results suggest that both plant- and herbivore-related experiences play important roles that affect foraging decisions in parasitoids, especially at short-range (Röse et al. 1997). While the present study focused on odor cues, other inputs such as tactile and visual information may contribute to host discrimination in parasitoids (Turlings et al. 1993; Heipel and Casas 2008). Future studies should compare the role of experience and other physiological factors such as nutrition and mating status affecting host discrimination in specialist versus generalist parasitoid species.

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Figure Legend

Figure 1: Feeding preference of *Heliothis virescens* larvae for cotton and soybean in (A) No-choice feeding experiments and (B) Two-choice feeding experiments. Leaf area (cm^2) consumed by ten 3rd instar larvae was visualized using ImageJ (v. 1.50i) software. Difference between mean (± SEM) leaf area of cotton and soybean consumed in no-choice feeding tests was analyzed using Two independent samples *T-test* (N= 4). Difference between leaf area consumed in two-choice feeding tests was analyzed using Paired *T-test* (N= 4). Asterisks (*) indicate significant differences ($P < 0.05$; *T-tests*).

Figure 2: An illustration showing different plant- and herbivore-related experiences received by female *Microplitis croceipes* tested in oviposition choice experiments: No experience with oviposition, no conditioning with host-damaged plant and no plant background (Naïve); experience with oviposition in cotton-fed hosts only (OV-Cotton); experience with oviposition in soybean-fed hosts only (OV-Soybean); conditioned with host-damaged cotton only (DG-Cotton); conditioned with host-damaged soybean only (DG-Soybean); inexperienced parasitoids tested with a background of host-damaged cotton odors (BG-Cotton); and inexperienced parasitoids tested with a background of host-damaged soybean odors (BG-Soybean). Parasitoids with treatments BG-Cotton and BG-Soybean had no previous oviposition experience. Individual parasitoids were presented with a choice of one cotton-fed and one soybean-fed *Heliothis virescens* larva in petri dish arena.

Figure 3: Oviposition preference of female *Microplitis croceipes* for cotton-fed and soybean-fed *Heliothis virescens* (*H.v*) larvae in a petri dish arena. Parasitoids received one of the following treatments: No experience with oviposition, no conditioning with host-damaged plant and no plant background (Naïve); experience with oviposition in cotton-fed hosts only (OV-Cotton);

experience with oviposition in soybean-fed hosts only (OV-Soybean); conditioned with host-damaged cotton only (DG-Cotton); conditioned with host-damaged soybean only (DG-Soybean); inexperienced parasitoids tested with a background of host-damaged cotton odors (BG-Cotton); and inexperienced parasitoids tested with a background of host-damaged soybean odors (BG-Soybean). Individual parasitoids were presented with a choice of one cotton-fed and one soybean-fed *Heliothis virescens* larva in petri dish arena. N = 30 responding wasps per test. Numbers inside bars indicate actual number of parasitized hosts. Numbers in parenthesis indicate number of parasitoids that made no choice within 10 min. Asterisks (*) indicate significant deviation from a 50:50 % distribution for total number of ovipositions made into the two types of host ($P < 0.05$; *Chi-square* test).

Figure 4: Effect of associative learning on host location in *Microplitis croceipes*. Parasitoids tested in a petri dish oviposition arena received two treatments: (A) Associative conditioning with oviposition in cotton-fed hosts in the presence of host-infested cotton (AC-Cotton) and (B) Associative conditioning with oviposition in soybean-fed hosts in the presence of host-infested soybean (AC-Soybean). A parasitoid was subsequently presented with a *Heliothis virescens* larva that fed on the conditioning plant. Asterisks (*) indicate significant differences between time taken (sec) by trained parasitoids and unconditioned parasitoids (Control) to locate and parasitize hosts ($P < 0.05$; Two independent samples *T-test*). Proportions outside bars indicate number of responders that located hosts within 10 min / total number of replicates per test. Percentages inside bars represent proportions of responders that located hosts under 5 min and between 5-10 min durations.

Figure 1

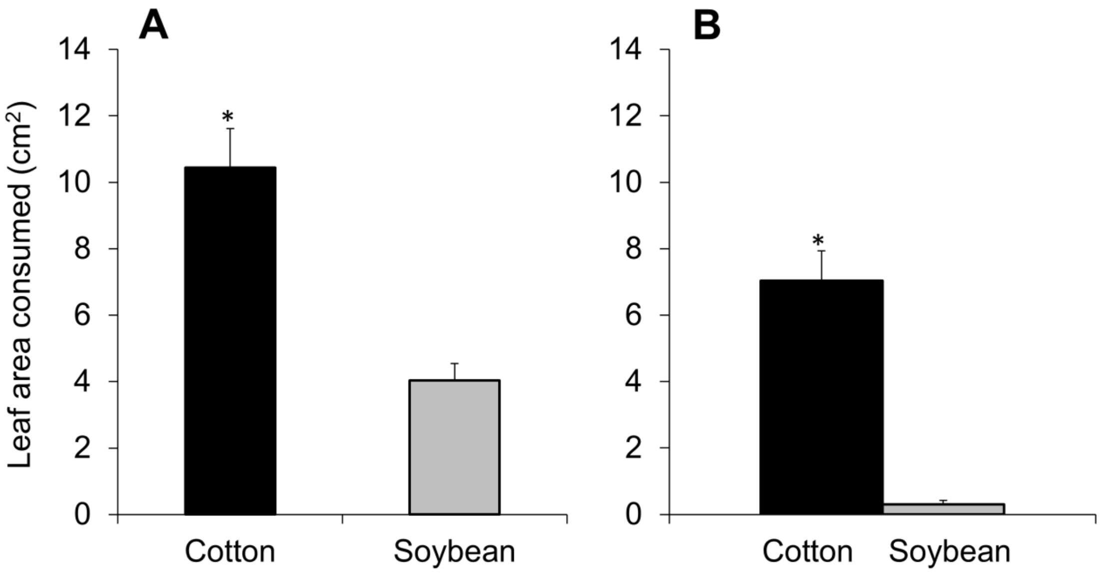


Figure 2

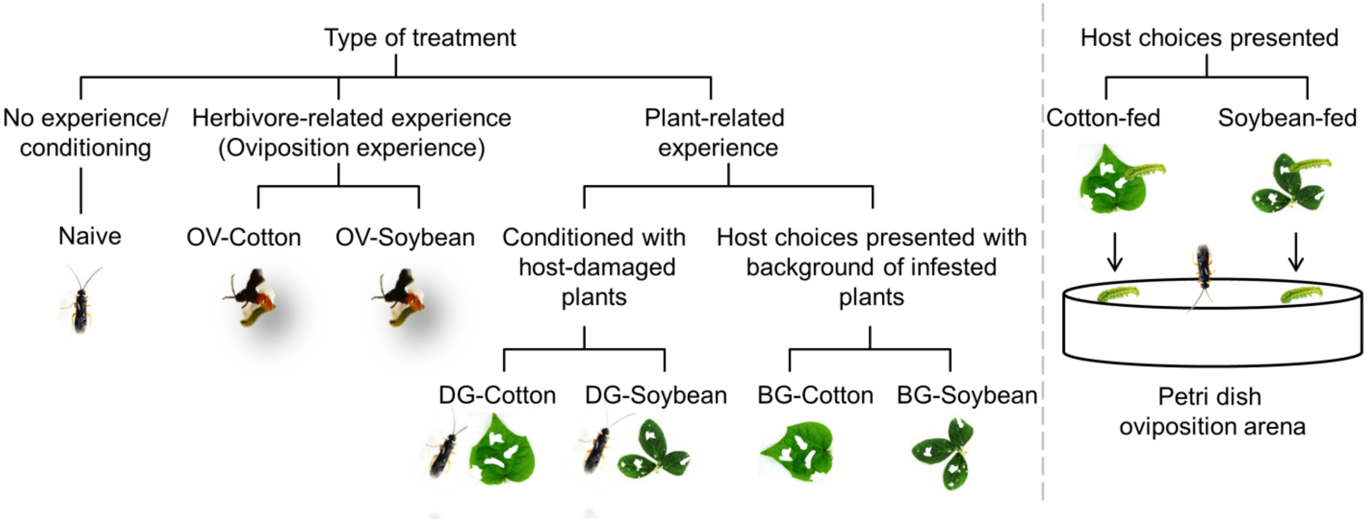

Type of treatment
No experience/ conditioning
Herbivore-related experience (Oviposition experience)
Plant-related experience
Naive
OV-Cotton
OV-Soybean
Conditioned with host-damaged plants
Host choices presented with background of infested plants
DG-Cotton
DG-Soybean
BG-Cotton
BG-Soybean
Host choices presented
Cotton-fed
Soybean-fed
Petri dish oviposition arena

Figure 3

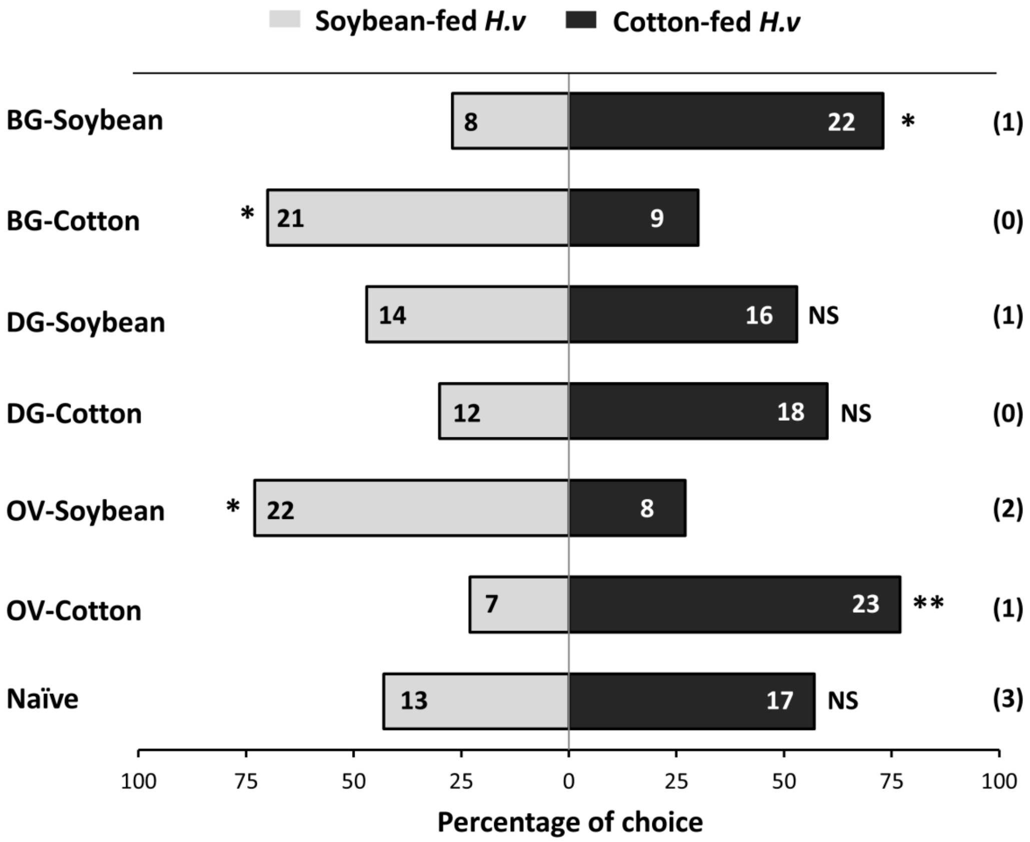
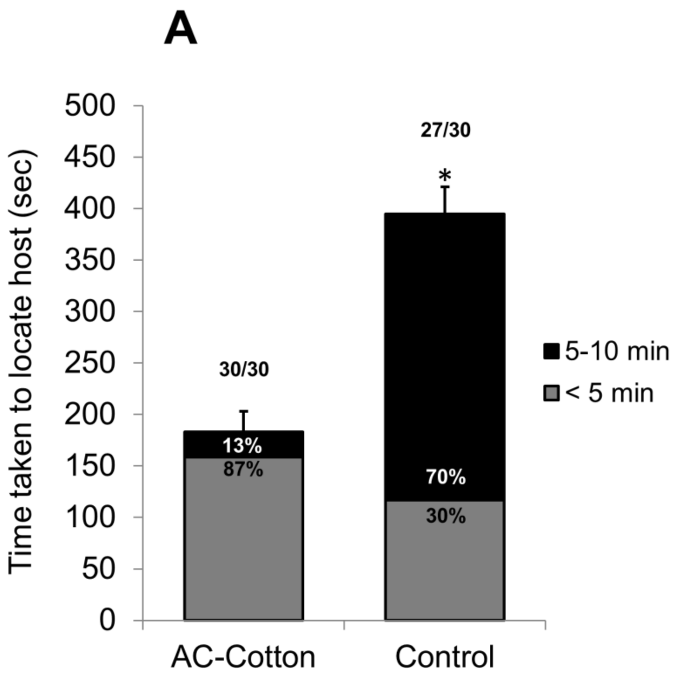
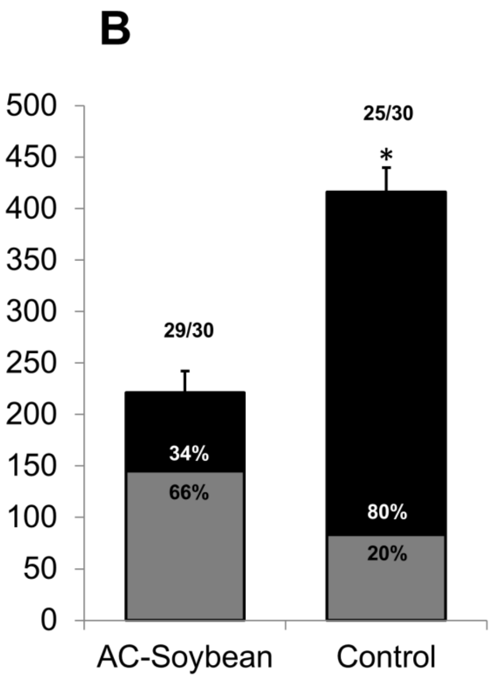

Soybean-fed *H.v*
Cotton-fed *H.v*
BG-Soybean
8
22
*
(1)
BG-Cotton
*
21
9
(0)
DG-Soybean
14
16
NS
(1)
DG-Cotton
12
18
NS
(0)
OV-Soybean
*
22
8
(2)
OV-Cotton
7
23
**
(1)
Naïve
13
17
NS
(3)
100
75
50
25
0
25
50
75
100
Percentage of choice

Figure 4


A
500
450
400
350
300
250
200
150
100
50
0
Time taken to locate host (sec)
30/30
13%
87%
27/30
*
70%
30%
AC-Cotton
Control
■ 5-10 min
▨ < 5 min


B
500
450
400
350
300
250
200
150
100
50
0
29/30
34%
66%
25/30
*
80%
20%
AC-Soybean
Control

CHAPTER 3

IDENTIFICATION OF KEY PLANT-ASSOCIATED VOLATILES EMITTED BY *Heliothis virescens* LARVAE THAT ATTRACT THE PARASITOID, *Microplitis croceipes*: IMPLICATIONS FOR PARASITOID PERCEPTION OF ODOR BLENDS

3.1 Introduction

Foraging natural enemies rely on a plethora of semiochemicals from plant-host complex for location, recognition and acceptance of hosts. However, only a fraction of host-associated odor blends are attractive or ecologically relevant to natural enemies, thus, the need to identify key compounds that mediate behavioral responses in these species (Clavijo McCormick et al. 2014; D'Alessandro and Turlings 2005; D'Alessandro et al. 2009; van Dam et al. 2010). In the plant-host complex, informative volatile cues for parasitoid host location can be emitted by plants when infested by herbivores (De Moraes et al. 1998; Pare and Tumlinson 1999; Turlings and Wäckers 2004; Peñaflor et al. 2011; Uefune et al. 2012; Fürstenberg-Hägg et al. 2013) or emitted by herbivores and their frass after feeding on plants (Elzen et al. 1984; Alborn et al. 1995; Chuche et al. 2006; de Rijk et al. 2016). Arguably, plants have larger biomass and produce more abundant volatiles than herbivorous insects (Turlings et al. 1995). However, once parasitoids find host plant patch and make appropriate landing on a plant, herbivore host-specific odors become critical short-range cues in the later phase of host location process (Afsheen et al. 2008; de Rijk et al. 2013; Colazza et al. 2014). The choice between the use of plant volatiles versus host-specific odors presents a ‘detectability’ versus ‘reliability’ dilemma to foraging natural enemies (Vet and Dicke 1992). Host-specific cues are more important to relatively specialized parasitoids whose hosts are generalist herbivores. This is due to the high number of chemical profile patterns that such parasitoids would have to detect and recognize if solely depending on host plant cues (Wajnberg et al. 2008). According to optimal foraging theory, parasitoids would be expected to select hosts that show the greatest fitness attribute so as to maximize their chances of progeny survival (Goubault et al. 2003; Steiner et al. 2007; Chesnais et al. 2015). In the field, herbivore hosts that recently fed on plants may be momentarily deemed more suitable by parasitoids than those that did not recently feed on plants.

Herbivores can acquire plant chemicals in their diet for further physiological or metabolic processes, or sequester toxic ones for defensive purposes (Despres et al. 2007). However, certain plant-associated compounds emitted by herbivores can serve as kairomones when used by parasitoids as odor cues to locate their hosts. In fact, it has been demonstrated that herbivore diet affects olfactory responses of parasitoids to host-specific odors (Ed Sauls et al. 1979; Elzen et al. 1984; Hofstetter and Raffa 1997; Nettles 1980; Sullivan et al. 2000; Reis et al. 2003). For instance, the parasitoid, *Microplitis croceipes* showed greater attraction to cowpea-fed *Helicoverpa zea* larvae compared with artificial diet-fed hosts (Ed Sauls et al. 1979). In a similar study, *Campoletis sonorensis* showed greater attraction to cotton-fed *Heliothis virescens* larvae compared with artificial diet-fed hosts (Elzen et al. 1984). In both studies, the increased attraction of parasitoids was attributed to plant-associated odors emitted by their hosts. Similarly, Nettles (1980) reported that *H. virescens* larvae that were otherwise not attractive to the parasitoid, *Eucelatoria* sp. (Tachinidae) became attractive after feeding on okra leaves. However, not all the components of such odor blends mediate attraction in parasitoids; demonstrating the role of each compound in odor mixtures can be tasking (Schnee et al. 2006). The important question of which particular volatile compounds play key roles in the attraction of parasitoids to plant-fed hosts remains to be answered in several plant-herbivore-parasitoid systems that have been studied. Yet, chemical ecologists are interested in differentiating useful cues from ‘background noise’, with regards to insects’ use of host-associated odor blends. Previous studies have used a combination of chemical ecology techniques (Nojima et al. 2003; Logan et al. 2008; Beyaert et al. 2010; Cha et al. 2012; Molnár et al. 2015; Isberg et al. 2016), molecular techniques (Kappers et al. 2005; Schnee et al. 2006) and bioinformatics approach (van Dam et al. 2010) to identify key compounds or blend fractions mediating attraction of foraging insects to host odors. Isolation and functional identification of specific compounds mediating attraction of parasitoids to host-associated odors will increase our knowledge of odor perception and discrimination in parasitoids. In addition, this serves as a critical step in the quest for improved monitoring strategies and effectiveness of parasitoids as biocontrol agents.

In the present study, I hypothesized that certain plant-associated compounds play key roles in the attraction of parasitoids to VOC blends emitted by herbivores that recently fed on plant. *Microplitis croceipes*, a relatively specialized larval endoparasitoid of *Heliothis virescens*, which is an important pest of cotton and other economic crops in the US, was used as model species to identify key compounds mediating its attraction to plant-fed hosts. Using a combination of headspace volatile collection coupled with four-choice olfactometer bioassay, gas chromatography-mass spectrometry (GC/MS), and Y-tube olfactometer bioassays, it was established that only a fraction of the VOC blend emitted from the body and frass of cotton-fed *H. virescens* larvae mediates attraction of *M. croceipes*. To the best of my knowledge, this is the first study to identify compounds mediating attraction of *M. croceipes* to plant-fed *H. virescens*, using a combination of chemical ecology techniques. Here, I present a reductionist approach whereby a full blend is reduced by a single component in order to determine the relevance of the missing compound. Furthermore, it is proposed that the role of a compound is better assessed in the context of other compounds in an odor blend. The implication of the current results to perception and use of host-associated odors in parasitoids is discussed.

3.2 Materials and Methods

3.2.1 Insects. Cocoons of *M. croceipes* were provided by the USDA-ARS Insect Biology and Population Management Research Laboratory (Tifton, Georgia, USA). The rearing procedures were similar to those described by Lewis and Burton (1970) with modifications described by Morawo and Fadamiro (2014a). Briefly, *M. croceipes* were reared on 2nd-3rd instar larvae of *Heliothis virescens* and adult wasps were supplied with 10 % sugar water upon emergence. The two host groups, cotton-fed and artificial diet-fed *H. virescens* larvae, were initially reared on pinto bean artificial diet (Shorey and Hale 1965). The cotton-fed group was removed from diet, cleaned and placed on a cotton plant to feed for 24 h. Larvae were initially cleaned by soft camel hair brush and then allowed to walk over a moistened paper towel to remove diet crumbs and frass. The artificial diet-fed group was allowed to feed continuously on artificial diet. Before use in bioassays, host larvae from both groups were cleaned of frass, leaf or diet crumbs as described above. All parasitoids used for behavioral bioassays were 2–5 day-old females that were presumed mated but inexperienced with oviposition. 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 according to Morawo and Fadamiro (2014a) in growth chambers (Entomology & Plant Pathology, Auburn University) at 26.6 °C day, 25.6 °C night, and 60 % relative humidity. Care was taken to avoid fungal infection or insect infestation prior to use of plants in experiments. Illumination was provided using daylight fluorescent tubes (270 PAR) with a 16:8 h (L/D) photoperiod. Seeds were planted in a top soil/vermiculite/peat moss mixture in plastic pots. Plants deliberately infested with *H. virescens* were 4-5- weeks-old.

3.2.3 Oviposition choice tests. Oviposition choice tests were conducted in two separate experiments (1 and 2) using 9 cm petri dish arena. In experiment 1, each female parasitoid was presented with 5 cotton-fed and 5 artificial diet-fed hosts in a petri dish. *H. virescens* larvae were randomly positioned before introducing a single parasitoid into the center of the arena, and a new petri dish was used for each replicate. Once introduced into the petri dish, a female parasitoid was allowed 15 min to oviposit (parasitize) 10 times, and the frequency of successful oviposition made in either host group was recorded. There was no need to physically mark the larvae to differentiate plant-fed hosts from artificial diet-fed hosts because *H. virescens* larvae that recently fed on foliage took up the green color. This visual method was also used to ensure that each *H. virescens* larvae considered plant-fed actually consumed plant tissues. In order to confirm that female *M. croceipes* were not showing affinity for already parasitized hosts, a second experiment, similar to the one described by Elzen et al (1984) was conducted. In experiment 2, one cotton-fed and one artificial diet-fed host larva was presented to each parasitoid in a petri dish. After one type of host was stung, it was replaced with another one of its kind until the wasp had oviposited 10 times. The results of the two oviposition choice experiments were compared. Experiments were arranged in a randomized complete block design. Each test was replicated 10 times (1 wasp per replicate) and repeated at least 2 times.

3.2.4 Headspace volatile collection coupled with four-choice olfactometer bioassays. In order to identify plant-associated volatiles emitted by *H. virescens* larvae, headspace VOCs were collected from cotton-fed and artificial diet-fed larvae for profile comparison. Headspace volatile collection was coupled with four-choice olfactometer so as to match behavioral responses of parasitoids to the chemistry of headspace volatiles. The set-up was similar to the one described by Morawo and Fadamiro (2014a) with slight modifications (Fig. 1). A group of cotton-fed hosts was cleaned and placed inside a volatile collection chamber (400 ml). Similarly, a group of artificial diet-fed hosts was also cleaned and placed inside a second chamber. The set-up included a third (control) chamber containing only moistened filter paper. The fourth arm of the olfactometer was connected directly to the air delivery system (air control). Purified (charcoal-filtered) and humidified air was let into each chamber at 300 ml/min. Each chamber had two outlets; one outlet was connected to an olfactometer arm while the other was connected to a trap containing 50 mg of Super-Q (Alltech Associates, Deerfield, IL, USA). To avoid mix-up of odors in the olfactometer, air was drawn by a vacuum pump from the bottom of the olfactometer at 1300 ml/min.

The set-up was arranged such that odors from both host groups were passed through opposite arms, separated by each of the two control arms. Parasitoids were released individually through the bottom of the olfactometer and given up to 10 min to make a choice. For instance, after a female *M. croceipes* made a choice, the insect was removed and one minute elapsed before replacing with another one. A parasitoid was recorded to have made a choice for the odor offered through an arm when it moved into an extension of the arm and remained there for at least 15 s. The olfactometer was rotated 90° after testing four insects. The apparatus was cleaned with acetone after testing 12 insects. Parasitoids were used once and non-responders (< 6%) were excluded from data analyses. In a separate set-up, volatiles were trapped from 250 mg of frass produced by cotton-fed larvae so as to differentiate compounds exclusive to host body from those present in frass. In all experiments, headspace volatiles were collected for 6 h after it was determined in preliminary studies that this duration was optimum for trapping VOCs from host larvae and frass without subjecting them to desiccation. Experiments were completely randomized and repeated at least four times on different days between 0900 and 1700 h.

3.2.5 GC-MS analyses. Headspace volatiles trapped from host larvae complex (larvae and frass) as well as frass alone was analyzed by GC/MS as described by Morawo and Fadamiro (2014a). Super-Q traps were eluted with 200 µl of methylene chloride and the resulting extracts were stored in a freezer (at −20 °C) until use. Identification and quantification of headspace volatiles was done using an Agilent 7890A GC coupled to a 5975C Mass Selective Detector, with an HP-5 ms capillary column (30m × 0.25mm i.d., 0.25 µm film thickness). 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). Compounds were identified according to their retention times and mass spectra, in comparison with a NIST 98 library (National Institute of Standards and Technology, Gaithersburg, MD, USA) and commercially available synthetic standards (purity 95–99 %) obtained from Sigma-Aldrich® Chemical Co. (St. Louis, MO, USA) and Alfa Aesar® (Ward Hill, MA, USA) (Table 1). Synthetic standard of α-bergamotene was not commercially available so its identity was confirmed by comparing with published GC profiles of cotton headspace volatiles (Loughrin et al. 1994; Mc Call et al. 1994; Ngumbi et al. 2009). For quantification, external calibration curves were made with standard solutions of select representative compounds (α-pinene, 1-octen-3-ol and (*E*)-β-caryophyllene) (Zebelo et al. 2014).

3.2.6 Y-tube olfactometer bioassays. Attraction of parasitoids to synthetic blends and single VOCs was tested in a Y-tube olfactometer (Analytical Research Systems Inc., Gainesville, FL, USA) set-up similar to the one previously described by Ngumbi and Fadamiro (2012). A full synthetic blend of 15 compounds identified in the headspace of cotton-fed *H. virescens* was formulated in hexane at 0.01, 0.05 and 0.1 μg/μl concentration, according to component proportions in natural blend. Odor blends were delivered on Whatman No. 1 filter paper strips (25 × 7 mm) at 0.1, 0.5 and 1 μg doses. The doses were selected as ecologically relevant based on GC/MS analyses results of total amount of volatiles emitted by cotton-fed *H. virescens* larvae in 6 h, and in consideration of the maximum time (10 min) given each parasitoid to make a choice. The full blend (at the most attractive dose of 1 μg) was then modified by removing each of the 10 compounds exclusive to cotton-fed hosts to make separate blends. In addition, attraction of parasitoids to select single compounds (1-octen-3-ol and decanal) was tested at 0.01 and 0.1 μg doses. Single compounds were tested at relatively lower doses compared to the full blend to simulate ecologically relevant concentrations. The experimental conditions and procedure for testing individual wasps were similar to those earlier described for four-choice olfactometer bioassays. After synthetic compounds were applied to filter paper strips, 10 s elapsed to allow solvent evaporation and avoid initial steepness in release rate of odor stimuli. For Y-tube olfactometer bioassays, the apparatus was rotated 180° and filter paper strips were changed after testing 4 insects. Parasitoids were used once and non-responders (< 7% in all experiments) were excluded from data analyses. Insects chose between one arm carrying odor from synthetic blend or single compound, and the other arm carrying hexane (control).

3.2.7 Data analyses. The deviation of oviposition choice of parasitoids from a 50:50% distribution between two different groups of hosts was analyzed using a *Chi-square* goodness of fit test. Four-choice olfactometer data was modeled as a response count with *Logistic Regression* and slopes separated by Proc Logistic Contrast in SAS. The model adequacy was confirmed with a *Likelihood Ratio* test (Wajnberg and Haccou 2008). For visual comparison, both oviposition and olfactometer data were presented as percentages on charts. Amount of volatile compounds trapped from groups of 50 host larvae was expressed in ng while amount of volatiles trapped from frass produced by cotton-fed hosts was expressed in ng/50 mg of frass. Significant differences in the amounts of each compound emitted by cotton-fed and artificial diet-fed larvae were established using *Wilcoxon-Mann-Whitney* test. A two-sided *binomial* test was used to compare parasitoid responses to stimulus versus control in Y-tube olfactometer. *Chi-square* goodness of fit test was used to analyze the deviation of responses to modified blends from the 79:21% (stimulus: control) recorded for full blend. The test was conducted in order to determine if the removal of a compound significantly affects attraction of parasitoids to the full blend. All analyses were performed using SAS 9.2 with 0.05 level of significance.

3.3 Results

3.3.1 Oviposition choice of *M. croceipes*. In oviposition choice tests, female *M. croceipes* parasitized more cotton-fed larvae than artificial diet-fed larvae. In experiment 1, each female parasitoid was presented with 5 cotton-fed and 5 artificial diet-fed hosts. In experiment 2, one cotton-fed and one artificial diet-fed host larva was presented to each parasitoid. Sixty-two percent ($\chi^2 = 5.76$; $P = 0.016$) of total oviposition was made into cotton-fed larvae when parasitoids were simultaneously presented with 5 *H. virescens* larvae from each of two host groups (Experiment 1) (Fig. 2). Similarly, 68% ($\chi^2 = 12.96$; $P = 0.0003$) of total oviposition was made into cotton-fed larvae when parasitoids were simultaneously presented with one *H. virescens* larva from each of two host groups (Experiment 2) (Fig. 2). A comparison of results obtained from both experiments indicates that incidence of superparasitism in experiment 1 did not significantly influence oviposition choice of parasitoids.

3.3.2 Parasitoid preference between cotton-fed and artificial diet-fed hosts in four-choice olfactometer. In four-choice olfactometer bioassays, female *M. croceipes* showed a clear preference for cotton-fed hosts over artificial diet-fed hosts ($\chi 2$= 11.79, *df*= 1, *P*= 0.0006) (Fig. 3). In total, 58% of parasitoids chose the arm offering odors from cotton-fed hosts, 23% of parasitoids chose artificial-diet fed hosts, and 19% chose the control arms (combined). This result suggests that *M. croceipes* can distinguish between host larvae fed different diets, and are more attracted to *H. virescens* larvae that recently fed on cotton.

3.3.3 GC-MS analyses of host headspace VOCs. A comparison of headspace volatile profiles of cotton-fed larvae complex (larvae and frass), artificial diet-fed larvae complex, and frass of cotton-fed larvae showed qualitative and quantitative differences in composition of compounds. In total, 17 compounds (1 unknown) were consistently detected in the headspace of *H. virescens* larvae but not found in control (moistened filter paper). These included the monoterpenes, α-pinene, β-pinene, myrcene and limonene; the alcohols, 1-octene-3-ol and 2-ethylhexanol; the carbonyl compounds, 3-octanone and decanal; the alkanes, tridecane and tetradecane; the sequiterpenes, (*E*)-β-caryophyllene, α-bergamotene, α-humulene, α-farnesene, bisabolene and α-bisabolol (Table 2). More compounds (all 17) were detected in the headspace of cotton-fed larvae complex (larvae + frass), and generally in greater amounts compared with artificial diet-fed larvae. Only five compounds, 3-octanone, limonene, 2-ethylhexanol, tridecane and tetradecane, were consistently detected in the headspace of artificial diet-fed larvae complex. Thus, the remaining 12 compounds were exclusive to cotton-fed larvae. Comparing VOC profiles of cotton-fed larvae complex to that of their frass alone, it can be inferred that 1-octen-3-

ol, myrcene and decanal were exclusively released from the body of cotton-fed *H. virescens* larvae.

3.3.4 Attraction of parasitoids to synthetic VOC blends and single compounds in Y-tube olfactometer. Attraction of parasitoids to formulated odor blend was dose-dependent. Compared to control (hexane), parasitoids showed the greatest attraction to full (15-component) blend at the highest dose of 1 µg (79%, $P = 0.007$, *Binomial* test). Parasitoids showed reduced attraction to full blend at the lower doses of 0.5 µg (71%, $P = 0.06$, *Binomial* test) and 0.1 µg (63%, $P = 0.31$, *Binomial* test) (Fig. 4). Thus, the 1 µg dose was considered optimum and used in subsequent bioassays with volatile blends. Parasitoids could not significantly discriminate odor blend from control when 1-octen-3-ol (42% of parasitoids chose blend), decanal (42%), (*E*)-β-caryophyllene (46%), α-humulene (46%), α-farnesene (50%), β-pinene (58%), α-pinene (67%) or α-bisabolol (67%) were removed (Fig. 5). Furthermore, a goodness of fit test indicated that attraction of parasitoids to blends from which 1-octen-3-ol ($\chi^2 = 20.16$, $df = 1$, $P < 0.0001$), decanal ($\chi^2 = 20.16$, $df = 1$, $P < 0.0001$), (*E*)-β-caryophyllene ($\chi^2 = 15.91$, $df = 1$, $P < 0.0001$), α-humulene ($\chi^2 = 15.91$, $df = 1$, $P < 0.0001$), α-farnesene ($\chi^2 = 12.17$, $df = 1$, $P = 0.0005$) or β-pinene ($\chi^2 = 6.18$, $df = 1$, $P < 0.01$) had been removed was significantly less than the level of attraction elicited by full blend (79: 21%; blend: control %). In contrast, removal of myrcene and bisabolene had no significant effect on attraction of parasitoids. When presented as a single compound, decanal did not elicit significant attraction in parasitoids at 0.01 and 0.1 µg doses. However, parasitoids showed significantly (75%, $P = 0.02$, *Binomial* test) greater attraction to 0.01 µg dose of 1-octen-3-ol, compared to control (Fig. 6).

3.4 Discussion

Based on the results from a combination of techniques used, it was evident that only a fraction of the VOCs emitted by cotton-fed *H. virescens* larvae mediates attraction in *M. croceipes*. In agreement with a previous study (Ed Sauls et al. 1979), *M. croceipes* preferred cotton-fed *H. virescens* larvae to artificial diet-fed larvae in oviposition choice tests. Similarly, effect of herbivore plant diet on olfactory responses of various parasitoids to their hosts has been previously reported (Elzen et al. 1984; Hofstetter and Raffa 1997; Sullivan et al. 2000; Reis et al. 2003). In the present study, parasitoids showed the greatest attraction to plant-fed hosts in four-choice olfactometer bioassays, indicating that odor played a role in their oviposition preference. Furthermore, comparative GC/MS analyses showed that certain VOCs were exclusively emitted by plant-fed larvae and their frass, thus highlighting the role of plant-associated compounds in the attraction of parasitoids to their herbivore hosts.

As expected, the full 15-component blend of volatiles emitted by *H. virescens* elicited the greatest attraction in Y-tube olfactometer, supporting the claim that odor perception in insects is optimized with blends (Bruce and Pickett 2011; van Wijk et al. 2011; Takemoto and Takabayashi 2015). The contribution of a compound to the attractiveness of an odor blend can be more accurately determined in the context of other blend components. Thus, a reductionist approach was used to determine the contribution of each compound in the present study. In general, attraction of parasitoids to the full blend was reduced when a component was removed. Removal of 1-octen-3-ol, decanal, (*E*)-β-caryophyllene, α-humulene, α-farnesene or β-pinene significantly reduced attraction of parasitoids to the full blend. These results highlight the important roles played by these compounds in mediating attraction of *M. croceipes* to *H. virescens* larvae. In contrast, the removal of myrcene, bisabolene, α-bisabolol and α-pinene had no significant effect on attractiveness of full blend, suggesting that they may not be critical components of the attractive blend or that they only play minor roles.

(*E*)-β-Caryophyllene, α-humulene, α-farnesene, β-pinene and decanal have been detected in the headspaces of uninfested and/or herbivore-infested cotton in previous studies (Loughrin et al. 1994; Turlings et al. 1995; De Moraes et al. 1998; Röse et al. 1998; Röse and Tumlinson 2004; Ngumbi et al. 2009; Magalhães et al. 2012; Morawo and Fadamiro 2014a; Sobhy et al. 2015). However, these compounds are ubiquitous and not restricted to cotton plant. In fact, the roles of these compounds have been investigated in other plant-herbivore-parasitoid tritrophic interactions. For instance, β-pinene has been identified as chemical cues from larval frass of Cerambycids used for host location by the parasitoid, *Dastarcus helophoroides* (Wei et al. 2013). Similarly, (*E*)-β-caryophyllene was identified as the key volatile emitted by stink bug-infested bean (Colazza et al. 2004) and aphid-infested tomato (Sasso et al. 2007) that mediates attraction of the parasitoids, *Trissolcus basalis* and *Aphidius ervi*, respectively. In another study, where transgenic *Arabidopsis* was used to express production of maize sesquiterpenes, farnesene was identified as one of two compounds mediating attraction of the parasitoid, *Cotesia marginiventris* (Schnee et al. 2006). I am not aware of any previous study in which α-humulene was identified as a key volatile mediating attraction of parasitoids. However, α-humulene has been previously reported as a component of cotton-fed *H. virescens* odor that elicits attraction in *Campoletis sonorensis* (Elzen et al. 1984).

Removal of decanal or 1-octen-3-ol diminished the attractiveness of the full blend to the greatest extent, suggesting that the two compounds play a major role in attraction of *M. croceipes* to host-emitted odor blend. Comparative GC/MS analyses showed that the two compounds were exclusively emitted from the body of cotton-fed host larvae, suggesting their use as reliable cues for host discrimination in parasitoids. Previous studies have reported decanal as a component in the blend of VOCs released from the greater wax moth (Dweck et al. 2010) and scales of codling moth (Delury et al. 1999) that attracts the parasitoids, *Bracon hebetor* and *Ascogaster quadridentata*, respectively. In addition, it has been reported that decanal mediates attraction (Isberg et al. 2016) or host discrimination (Logan et al. 2008) in certain hematophagous insects, further suggesting its ubiquitous nature. 1-Octen-3-ol is another ubiquitous compound commonly associated with fungal odors (Steiner et al. 2007). In the present study, care was taken to avoid fungal infection on plants, and no visible moldiness was observed. Regardless, comparative GC/MS analyses suggest that 1-octen-3-ol emitted by cotton-fed host larvae was plant-associated. The present results indicate that 1-octen-3-ol is a key component of the attractive kairomone used by *M. croceipes* to locate cotton-fed *H. virescens*. In general, more parasitoids chose 1-octen-3-ol or decanal over control when tested as single compounds but parasitoids only showed significant attraction to 0.01 μg dose of 1-octen-3-ol. Conversely, 0.1 μg dose of 1-octen-3-ol, as well as 0.01 and 0.1 μg doses of decanal did not elicit significant attraction in parasitoids. Previous studies have also reported instances in which a compound was not attractive to an insect when tested alone, but elicited attraction when presented in the context of a blend (Beyaert et al. 2010; Webster et al. 2010; Morawo and Fadamiro 2014b).

Furthermore, the present results suggest the relevance of background odors and importance of contextual perception of key compounds in attractive odor blends. Three mechanisms of odor recognition have been proposed for foraging insects: (i) species-specific odor recognition in which a few compounds associated with a particular species or group of closely related species are used to establish presence of host (Visser 1986), (ii) ratio-specific odor recognition in which insects detect certain ubiquitous volatiles and use their relative ratios to discriminate hosts (Visser 1986), and (iii) whole blend odor recognition in which all of the blend components are perceived as a whole in what seems to be an all-or-none situation (Clavijo McCormick et al. 2012). In the present study, behavioral responses of *M. croceipes* to full and modified blends in Y-tube olfactometer bioassays suggest a ratio-specific odor recognition pattern. Odor discrimination in *M. croceipes* was affected by presence/absence of six ubiquitous compounds which were formulated in ratios that mimic the natural blend. Two of the six compounds, 1-octen-3-ol and decanal affected attractiveness of the full blend to the greatest extent.

However, the relevance of key compounds was most apparent in the context of other blend components. Other plant-associated compounds not considered key to parasitoid attraction may serve as background odors to enhance olfactory contrast of key volatiles. Similarly, five other compounds, 3-octanone, limonene, 2-ethylhexanol (widespread contaminant), tridecane and tetradecane, whose relevance was not directly investigated in the present study, may play a role in providing context to the blend as a whole. Such roles may include masking the effect of another repellent component or enhancing the contrast of an attractive component. The importance of background odors to odor perception and recognition in several insect species, including parasitoids (Mumm and Hilker 2005; Schröder and Hilker 2008; Wajnberg et al. 2008; Beyaert et al. 2010; Clavijo McCormick et al. 2012, 2014), house fly (Kelling et al. 2002) and *Drosophila* (Faucher et al. 2006) have been discussed.

In conclusion, natural enemies of herbivores often face the dilemma of choosing between use of plant odors (more detectable) and host-specific odors (more reliable) (Vet and Dicke 1992). It is possible that this dilemma is optimally resolved by using long-range cues from plant volatiles to find host patch, and short-range cues from plant-associated volatiles emitted by hosts to locate and recognize suitable hosts (Colazza et al. 2014). Certain compounds may contribute in a major way to the attractiveness of host-associated odor blend. However, a complete blend, including less attractive components serving as background or masking odors, is often required for optimum perception and recognition of odors in parasitoids. The more an attractive component contrasts with the background, the greater the effectiveness of the cue (Wajnberg et al. 2008). In the present study, cotton plant, *H. virescens* and *M. croceipes* were used as model species to identify and characterize the role of key compounds emitted by plant-fed hosts in mediating attraction of parasitoids. Future studies are recommended to investigate interspecific differences in emission of plant-associated VOCs by herbivores, and possible effect on host preference in natural enemies.

3.5 Acknowledgements

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Table 1. Information on purity and supplier of the synthetic compounds used in this study

Compound	Purity (%)	Supplier
α-Pinene	98	Sigma-Aldrich [a]
β-Pinene	99	Sigma-Aldrich [a]
1-Octen-3-ol	98	Alfa Aesar [b]
3-Octanone	98	Alfa Aesar [b]
Myrcene	>95	Sigma-Aldrich [a]
Limonene	97	Sigma-Aldrich [a]
2-Ethylhexanol	>99	Sigma-Aldrich [a]
Decanal	96	Alfa Aesar [b]
Tridecane	>99	Sigma-Aldrich [a]
Tetradecane	>99	Alfa Aesar [b]
(*E*)-β-Caryophyllene	>98.5	Sigma-Aldrich [a]
α-Humulene	>96	Sigma-Aldrich [a]
α-Farnesene [c]	-	Sigma-Aldrich [a]
Bisabolene [c]	-	Alfa Aesar [b]
α-Bisabolol	>95%	Sigma-Aldrich [a]

[a] Sigma-Aldrich® Chemical Co. St. Louis, MO, USA
[b] Alfa Aesar®, Ward Hill, MA, USA
[c] Compounds are mixture of isomers

Table 2. Composition of headspace volatile organic compounds (VOCs) emitted by artificial diet-fed *Heliothis virescens* larvae, cotton-fed larvae and frass of cotton-fed larvae

ID[a]	Compound	Artificial Diet-fed larvae complex[b]		Cotton-fed larvae complex[b]		Frass (Cotton-fed larvae) only[c]	
		Amount[d] (ng)	Rel. %	Amount[d] (ng)	Rel. %	Amount[e] (ng)	Rel. %
1	α-Pinene [f]	0	0	863.5±68.6 *	15.1	33.3±15.1	12.1
2	β-Pinene [f]	0	0	91.3±10.5 *	1.6	2.8±1.7	1.1
3	1-Octen-3-ol [f, g]	0	0	79.5±29.2	1.4	0	0
4	3-Octanone	43.4±15.8	7.6	42.8±14.9	0.8	0	0
5	Myrcene [f, g]	0	0	153.0±34.0 *	2.7	0	0
6	Unknown [f]	0	0	72.4±13.5 *	1.2	10.5±5.6	3.8
7	Limonene	246.6±24.0	41.5	519.4±147.2 *	9.1	0	0
8	2-Ethylhexanol [h]	24.4±9.4	4.2	126.5±23.1 *	2.2	3.3±1.1	1.3
9	Decanal [f, g]	0	0	54.2±19.1 *	1.0	0	0
10	Tridecane	169.2±60.1	28.8	355.0±106.5	6.2	0	0
11	Tetradecane	106.8±40.2	17.8	140.5±39.2	2.4	0	0
12	(*E*)-β-Caryophyllene [f]	0	0	1673.5±221.9 *	29.2	124.5±13.6	45.0
13	α-Bergamotene [f]	0	0	39.6±9.0 *	0.7	2.9±0.5	1.1
14	α-Humulene [f]	0	0	367.6±51.6 *	6.5	28.8±4.1	10.5
15	α-Farnesene [f]	0	0	44.0±7.7 *	0.8	4.0±0.8	1.4
16	Bisabolene [f]	0	0	496.6±55.2 *	8.6	41.2±7.6	14.8
17	α -Bisabolol [f]	0	0	450.9±71.6 *	7.9	24.4±7.0	8.9

[a] In order of elution during gas chromatography

[b] Volatiles were collected for 6 h from 50 third instar *Heliothis virescens* larvae that were continuously fed artificial diet (artificial diet-fed) or fed cotton foliage 24 h prior to VOC collection (cotton-fed). It is assumed that trapped volatiles were released from host body and frass produced during the 6 h of volatile collection.

[c] Volatiles were collected for 6 h from 250 mg of frass excreted by cotton-fed larvae that had been separated from larvae, exuviae or leaf crumbs

[d] Amounts (ng) are mean ± SEM of four replicates

[e] Amounts (ng/50 mg of frass) are mean ± SEM of four replicates

[f] Compounds detected only in cotton-fed larvae and or their frass

[g] Compounds detected in the complex of cotton-fed larvae (larvae + frass) but not in their frass

[h] Compound was detected in all three treatments

*Asterisks indicate significant difference between mean amount of each compound emitted by artificial diet-fed and cotton-fed host complexes ($P < 0.05$; *Wilcoxon-Mann-Whitney* test)

The identity of all compounds was confirmed by NIST library and comparison with synthetic standards (purity 95–99 %), with one exception. Identity of compound 13 was confirmed by NIST library and comparison with published GC profiles of cotton headspace volatiles

Figure Legend

Figure 1: A simplified overview of headspace volatile collection coupled with four-choice olfactometer set-up. Each of three chambers A, B, C have one air inlet and two outlets. One outlet is connected to an arm of the olfactometer as an odor source while the other arm is connected to a volatile trap. Purified air is directly passed into the fourth arm of the olfactometer. Description of parts; ads, air delivery system; tct, Teflon connecting tube; ao, air outlet; vp, vacuum pump; as, air source; vcc, volatile collection chamber; ai, air inlet; afh, artificial diet-fed hosts; mfp, moistened filter paper; oa, olfactometer arm; cma, choice making area; vt, volatile trap; ct, central tube; cfh, cotton-fed hosts; irp, insect release point.

Figure 2: Oviposition choice of female *Microplitis croceipes* between cotton-fed and artificial diet-fed *Heliothis virescens* larvae in a petri dish arena. Experiment 1: Each parasitoid was presented with 5 cotton-fed and 5 artificial diet-fed hosts and allowed to parasitize 10 times. Experiment 2: Each parasitoid was presented with 1 cotton-fed and 1 artificial diet-fed host and allowed to parasitize once, after which the parasitized host was replaced with another one of its type until the parasitoid had parasitized 10 times. N = 10 wasps per method. Numbers inside bars indicate actual number of parasitized hosts. Asterisks (*) indicate significant deviation from a 50:50% distribution for total number of ovipositions made into the two types of host ($P < 0.05$; *Chi-square* goodness of fit test).

Figure 3: Preference of female *Microplitis croceipes* between cotton-fed and artificial diet-fed *Heliothis virescens* larvae in four-choice olfactometer. Attraction to treatments was modeled as binary response counts and represented on the chart as percentage of total responding wasps. Numbers inside bars indicate actual number of wasps attracted to each arm of olfactometer. N =

48 wasps. Values (%) having no letter in common are significantly different ($P < 0.05$; Proc *Logistic Regression* Contrast).

Figure 4: Attraction of female *Microplitis croceipes* in Y-tube olfactometer when given a choice between hexane (control) and full (15-component) synthetic blend of volatiles emitted by cotton-fed *Heliothis virescens* larvae. Volatile blend was tested at three doses (0.1, 0.5 and 1 μg). N = 24 wasps per test. Asterisks (*) indicate significant difference between stimulus and control ($P <$ 0.05; *Binomial* test).

Figure 5: Attraction of female *Microplitis croceipes* in Y-tube olfactometer when given a choice between hexane (control) and the full (15-component) or modified (14-component) synthetic blend of volatiles emitted by cotton-fed *Heliothis virescens* larvae. A blend was modified by removing one of ten plant-associated compounds from the full blend. Blends were tested at optimum dose of 1 μg. N = 24 wasps per test. Asterisks (*) *outside* bars indicate significant difference between stimulus and control ($P < 0.05$; *Binomial* test). Asterisks (*) *inside* bars indicate significant deviation from the 79:21% (stimulus: control) response recorded for full blend ($P < 0.05$; *Chi-square* goodness of fit test).

Figure 6: Attraction of female *Microplitis croceipes* in Y-tube olfactometer when given a choice between hexane (control) and 1-octen-3-ol or decanal. Single compounds were tested at two doses (0.01 and 0.1 μg). N = 24 wasps per test. Asterisks (*) indicate significant difference between stimulus and control ($P < 0.05$; *Binomial* test).

Figure 1

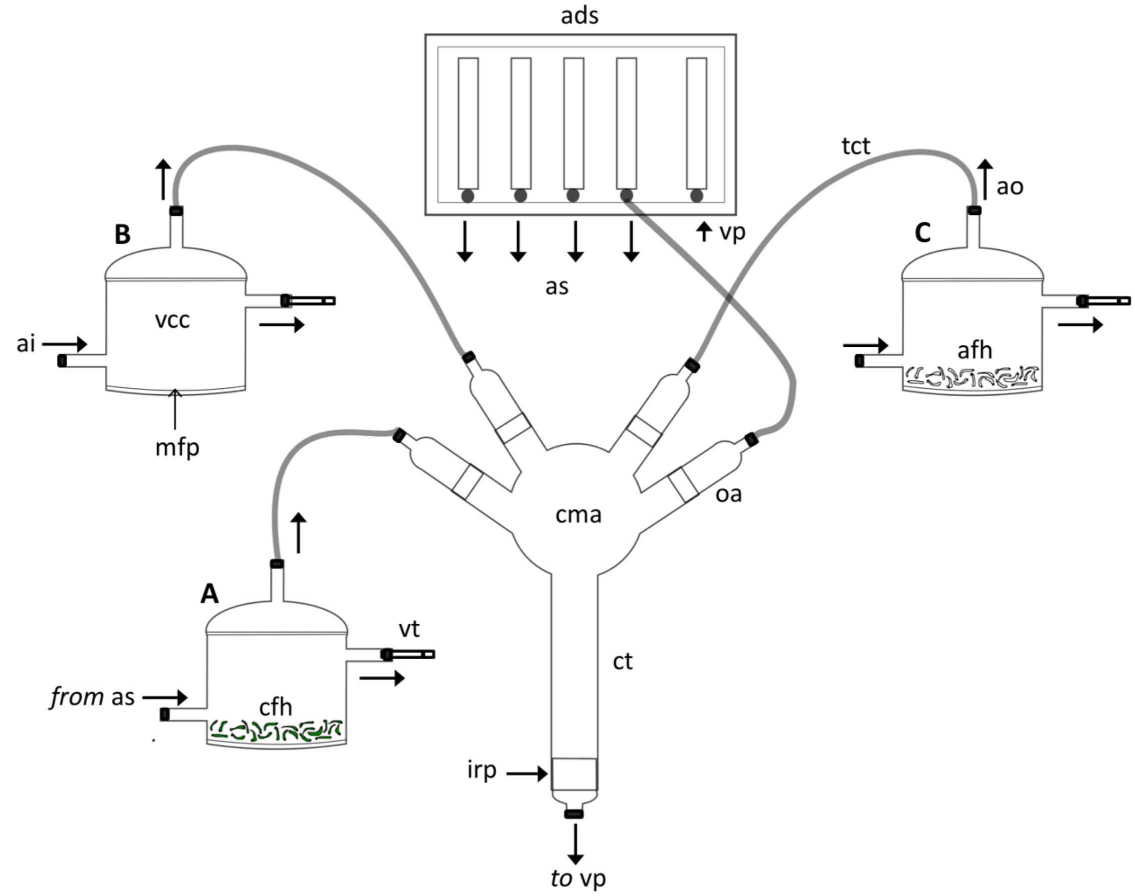

ads
tct
ao
B
vp
C
as
vcc
ai
afh
mfp
cma
oa
A
vt
ct
from as
cfh
irp
to vp

Figure 2

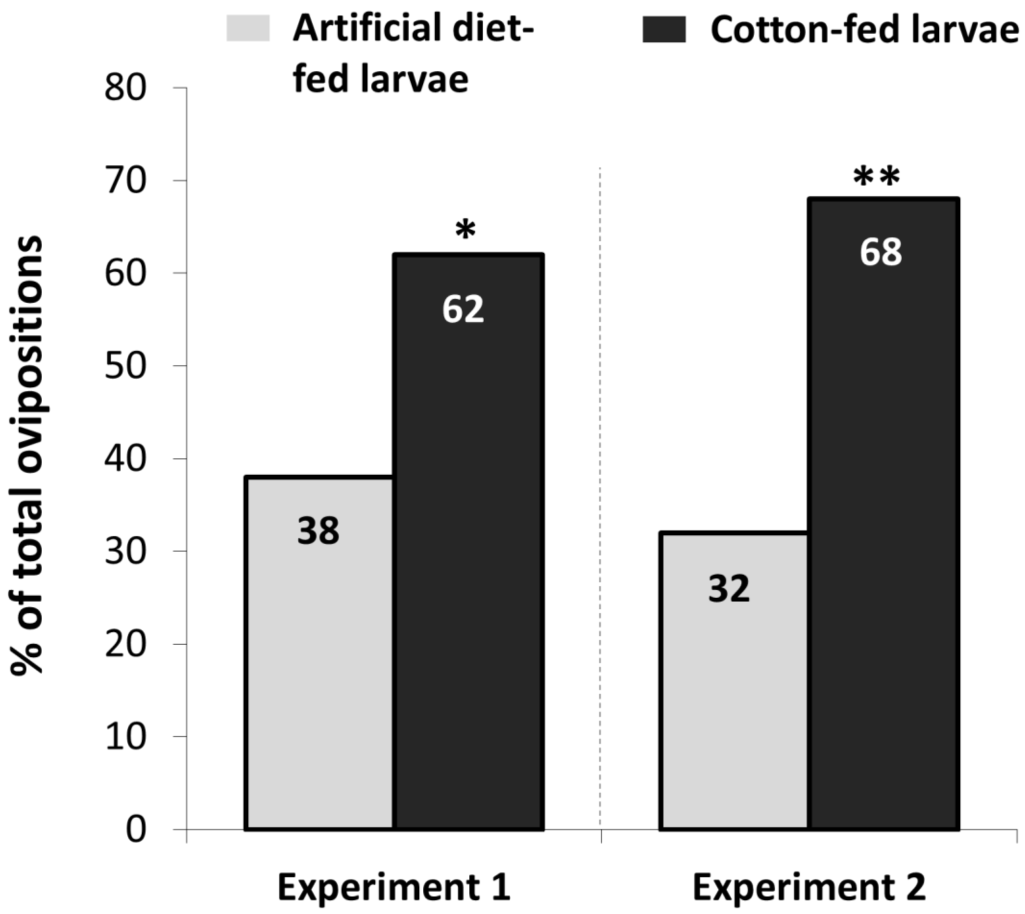
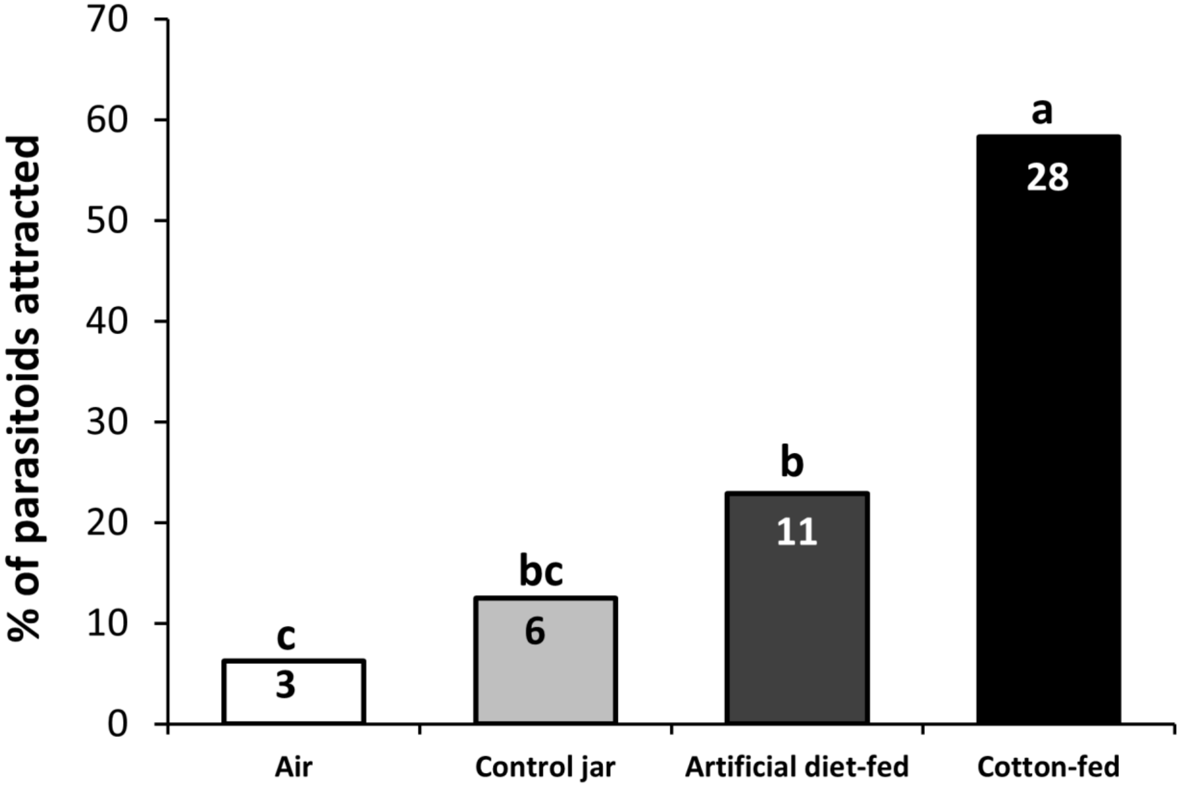

Artificial diet-fed larvae
Cotton-fed larvae
% of total ovipositions
80
70
60
50
40
30
20
10
0
38
62
*
32
68
**
Experiment 1
Experiment 2

Figure 3



% of parasitoids attracted
70
60
50
40
30
20
10
0
c
3
bc
6
b
11
a
28
Air
Control jar
Artificial diet-fed
Cotton-fed

Figure 4

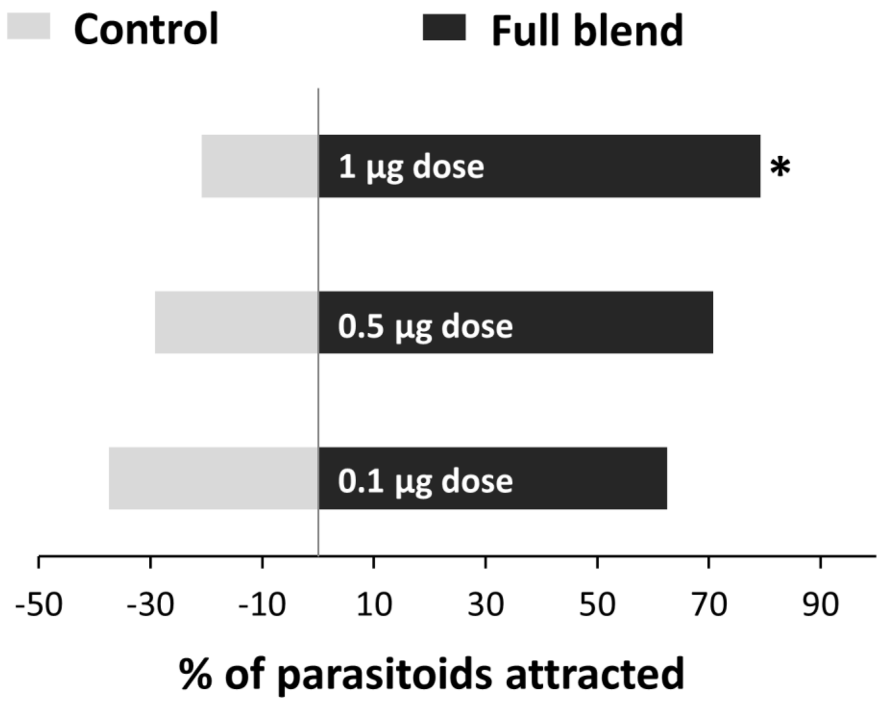

Control
Full blend
1 µg dose
*
0.5 µg dose
0.1 µg dose
-50
-30
-10
10
30
50
70
90
% of parasitoids attracted

Figure 5

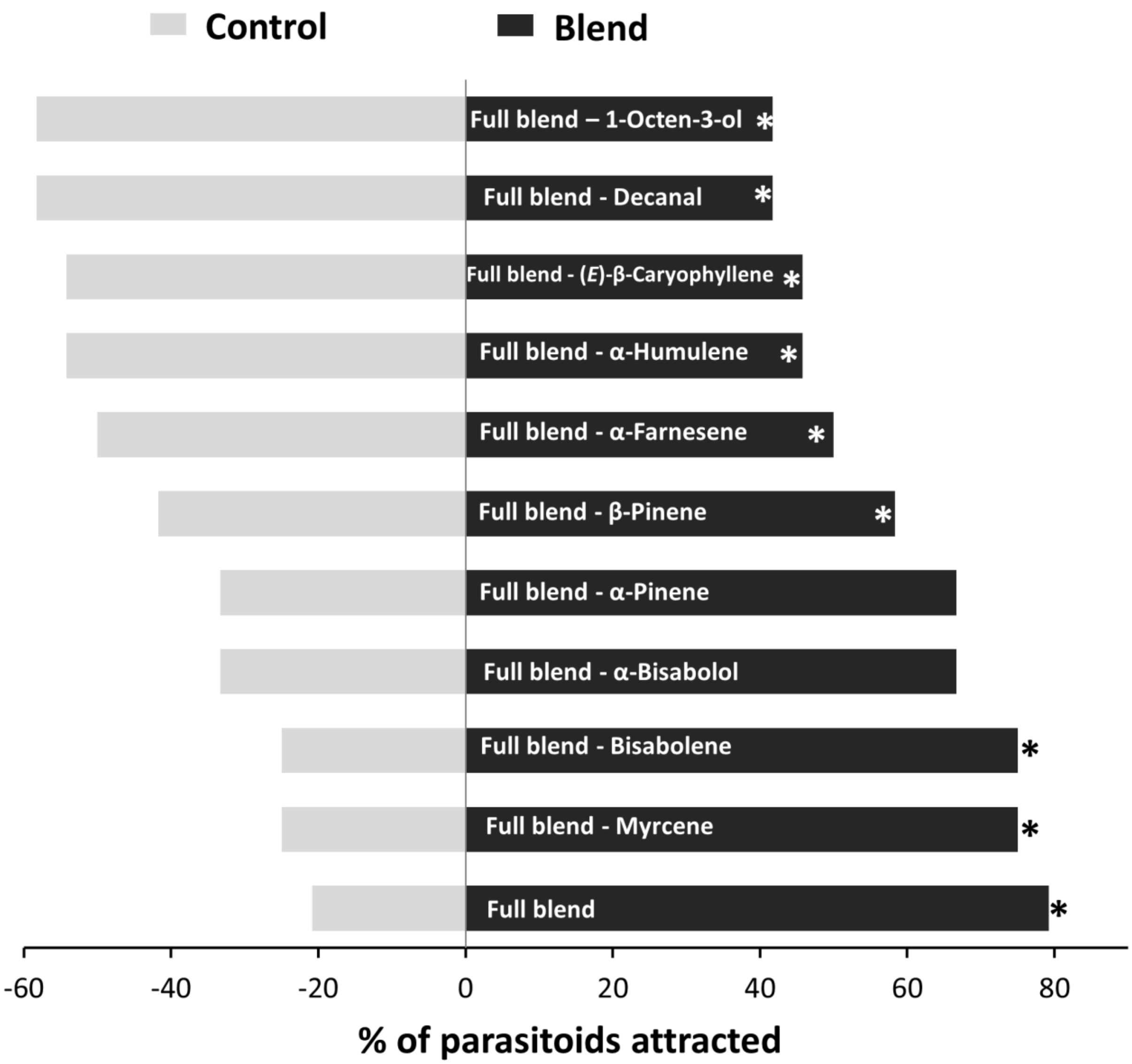
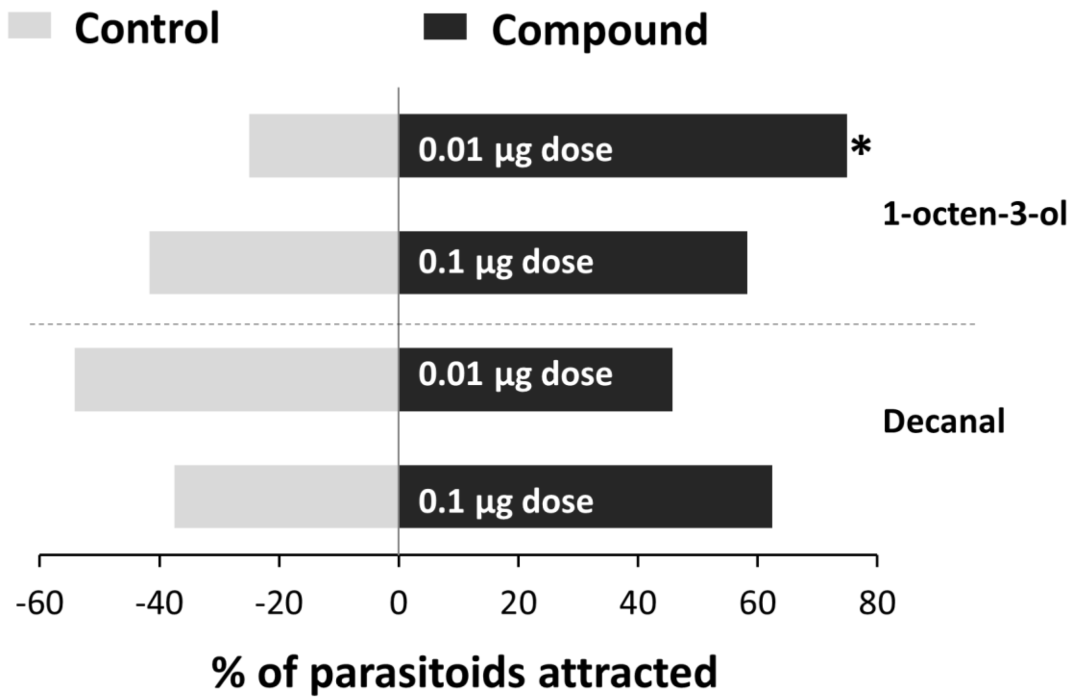

Control
Blend
Full blend – 1-Octen-3-ol *
Full blend - Decanal *
Full blend - (E)-β-Caryophyllene *
Full blend - α-Humulene *
Full blend - α-Farnesene *
Full blend - β-Pinene *
Full blend - α-Pinene
Full blend - α-Bisabolol
Full blend - Bisabolene *
Full blend - Myrcene *
Full blend *
-60
-40
-20
0
20
40
60
80
% of parasitoids attracted

Figure 6


Control
Compound
0.01 µg dose
*
1-octen-3-ol
0.1 µg dose
0.01 µg dose
Decanal
0.1 µg dose
-60
-40
-20
0
20
40
60
80
% of parasitoids attracted

CHAPTER 4

ELECTROANTENNOGRAM RESPONSE OF THE PARASITOID, *Microplitis croceipes* TO HOST-RELATED ODORS: THE DISCREPANCY BETWEEN RELATIVE ABUNDANCE AND LEVEL OF ANTENNAL RESPONSE TO VOLATILE COMPOUND

4.1 Introduction

Infested plants emit volatile organic compounds (VOCs) as an indirect defense against herbivore damage (De Moraes et al. 1998; Turlings and Wackers 2004). Informative volatile cues used by parasitoids for host location can be emitted by plants infested with herbivores (De Moraes et al. 1998; Turlings and Wackers 2004) or emitted by herbivores that fed on plants (Elzen et al. 1984; de Rijk et al. 2016). Although plant volatiles may initially lead parasitoids to the host patch, herbivore host-specific odors are important short-range cues used in the later stages of host location (Morawo and Fadamiro 2016). The specific mechanism by which plant-fed host larvae emit these volatiles is not fully understood. However, it is evident that parasitoids use these plant-associated VOCs in the host location process (Morawo and Fadamiro 2016). Such odor cues are usually released as a blend of various compounds in nature. Consequently, differentiating useful cues from ecologically irrelevant odors can be challenging for foraging parasitoids. Therefore, it is expected that antennal sensitivity of parasitoids will vary in response to different compounds. Antenna sensitivity in insects can be measured with electroantennogram

(EAG) recording. EAG measures the summed activity of olfactory receptor neurons in the antenna and indicates the level of biological activity elicited by various compounds.

Microplitis croceipes (Hymenoptera: Braconidae) is an endoparasitoid of *Heliothis virescens* (Lepidoptera: Noctuidae), which is an important pest of cotton plant. In a previous related study (Morawo and Fadamiro 2016), female *M. croceipes* showed attraction to the odor blend emitted by cotton-fed *H. virescens* larvae in Y-tube olfactometer bioassays (Morawo and Fadamiro 2016). The blend components were identified and quantified using gas chromatography-mass spectrometry (GC-MS). Furthermore, the compounds in the attractive blend occurred in varying proportions (Table 1). However, the relative abundance of a blend component does not necessarily indicate its relevance to resource location in insects (Clavijo McCormick et al. 2014). In the present study, olfactory response of *M. croceipes* to synthetic versions of 15 previously identified compounds was tested in EAG bioassays. Comparing EAG results in the present study and GC/MS analyses in a previous study (Morawo and Fadamiro 2016), I showed the discrepancy between relative abundance of a volatile blend component and the level of antennal response in parasitoids.

4.2 Materials and Methods

4.2.1 Insects. *Microplitis croceipes* was reared on 2nd-3rd instar larvae of *Heliothis virescens*. The larvae of *H. virescens* used to rear *M. croceipes* were fed pinto bean artificial diet. Thus, parasitoids had no experience with plant odors. Adult wasps were supplied with 10% sugar water upon emergence in our laboratory at Entomology and Plant Pathology Department, Auburn University. For more details about rearing protocol, see Lewis and Burton (1970). Female parasitoids used for EAG bioassays were 2–3 days-old, presumed mated (after at least 24 h of interaction with males), and inexperienced with oviposition or plant material. The general rearing conditions for all insects were 25±1 °C, 75±5 % relative humidity and 14:10 h (light: dark) photoperiod.

4.2.2 EAG Recording. EAG responses of *M. croceipes* to 15 synthetic compounds (Table 1), previously identified in the headspace of cotton-fed *H. virescens* larvae (Morawo and Fadamiro 2016), were recorded according to the method described by Ngumbi et al.(2010) with modifications. Two compounds, α-bergamotene (not commercially available) and an unidentified compound reported in the previous study (Morawo and Fadamiro 2016) were not tested in the present study. α-Pinene, β-pinene, myrcene, limonene, 2-ethylhexanol, tridecane, (*E*)-β-caryophyllene, α-humulene, α-farnesene and α-bisabolol with purity 95–99% were purchased from Sigma-Aldrich ® (St. Louis, MO, USA). 1-Octen-3-ol, 3-octanone, decanal, tetradecane and bisabolene with purity 96–99% were purchased from Alfa Aesar ® (Ward Hill, MA, USA). Test compounds were formulated in hexane at 0.1 µg/µl and delivered onto Whatman ®No.1 filter paper strips at an optimum dose of 1 µg. Mass/volume concentration was used correct for differences in purity of synthetic compounds. The dose was selected as ecologically relevant based on GC-MS analyses results of total amount of volatiles emitted by cotton-fed *H. virescens* larvae (Morawo and Fadamiro 2016). Impregnated filter papers were placed inside glass Pasteur pipettes and stimulus was introduced as 0.2 s odor puffs. A glass capillary reference electrode filled with 0.1 M KCl was attached to the back of the wasp head, and a similar recording electrode was connected to the excised tip of the wasp antenna. The analog signal was detected through a probe and processed with a data acquisition controller (IDAC-4, Syntech, The Netherlands). Data was assessed using EAG 2000 software (Syntech, The Netherlands). EAG responses to the 15 compounds and control (hexane) were sequentially recorded for each of 15 insect replicates. Each compound was presented at positions 1 through 15 across replicates to minimize positional bias. For instance, 1-octen-3-ol and 3-octanone were introduced to the first insect as the 3rd and 4th compounds, respectively, but introduced to the second insect as the 4th and 5th compounds, respectively.

4.2.3 Data Analyses. Differences in absolute EAG values (EAG response to compound minus response to solvent control) of synthetic compounds were analyzed using the *Kruskal-Wallis* test, followed by *Sidak's* multiple comparison test. The relationship between EAG response and relative abundance was analyzed with *Proc Corr* (correlation) procedure in SAS. All analyses were performed in SAS v9.2 (SAS Institute Inc., Cary, NC, USA) with P=0.05 level of significance.

4.3 Results

Female *M. croceipes* showed varying EAG responses to test compounds (range: 0.05–0.82 mV; Fig. 1). Decanal elicited the highest EAG response (0.82 mV; $\chi 2$ = 134.13; *df* = 14; $P<0.0001$), while β-pinene elicited the lowest response (0.05 mV) in parasitoids. Decanal, tridecane, 3-octanone, 2-ethylhexanol, 1-octen-3-ol, bisabolene, tetradecane and α-farnesene elicited EAG responses ≥0.22 mV (50th percentile rank). Four of the top bioactive compounds: decanal, 3-octanone, 1-octen-3-ol and 2-ethylhexanol, were emitted in quantities ≤2.2% of the total blend (Table 1). On the other hand, (*E*)-β-caryophyllene, the most abundant (29.2% of total blend) component, elicited a relatively low EAG response (0.17 mV) in parasitoids (Fig. 1). However, the negative correlation between EAG response and relative abundance of compounds was not statistically significant (r = -0.33; *N* = 15; P=0.23).

4.4 Discussion

EAG responses of *Micropiltis croceipes* in the present study indicated variation in biological activity elicited by test compounds at the peripheral level, and revealed a discrepancy between relative abundance and level of antennal responses in parasitoids. High EAG response elicited by decanal in *M. croceipes* agrees with previous reports on olfactory responses of the parasitoids, *Microplitis mediator* (Yu et al. 2010) and *Bracon hebetor* (Dweck et al. 2010). Furthermore, decanal is a key attractant for host-seeking *M. croceipes* (Morawo and Fadamiro 2016). Although compounds are emitted in different quantities in natural blends, minor components can have a profound effect on resource location in parasitoids (Beyaert et al. 2010; Clavijo McCormick et al. 2014). Interestingly, decanal constituted only 1% of the total blend emitted by cotton-fed *H. virescens* (Morawo and Fadamiro 2016) but elicited the highest EAG response in *M. croceipes*, supporting the “little peaks-big effects” concept (Clavijo McCormick et al. 2014). On the other hand, (*E*)-β-caryophyllene, the most abundant blend component, elicited a relatively low EAG response in parasitoids.

Therefore, it is more likely that the ecological relevance of a compound, rather than its relative abundance determines the level of olfactory response in foraging insects. For instance, small amounts of isothiocyanates in the volatile blend of brassica plants serve as host location cues for parasitoids of brassica herbivores (Wajnberg et al. 2008; Najar-Rodriguez et al. 2015). More importantly, blend components act in concert to provide parasitoids with complete information (van Wijk et al. 2011). Consequently, certain compounds function as background odors to enhance detectability (olfactory contrast) of other attractive components in a blend (Mumm and Hilker 2005; Wajnberg et al. 2008). It is possible that (*E*)-β-caryophyllene serves as a background odor in the blend emitted by cotton-fed *H. virescens*. Finally, it should be noted that while EAG measures the level of bioactivity, behavioral bioassays are usually needed to establish the functional role of various compounds (Tamiru et al. 2015; Morawo and Fadamiro 2016). In addition, several species of parasitoids can be conditioned to respond to diverse odor cues, regardless of the relevance of such odor cues to their ecology.

4.5 Acknowledgements

I thank Matthew Burrows for helping with EAG recordings and Brandice Kopishke for rearing the insects used for this study.

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Table 1. Composition of headspace volatile organic compounds emitted by cotton-fed *Heliothis virescens* larvae. This table was modified from Morawo and Fadamiro (2016), with permission from the authors.

ID [1]	**Compound**	**Relative abundance (%)**	**Chemical category**
1	α-Pinene	15.1	Monoterpene
2	β-Pinene	1.6	Monoterpene
3	1-Octen-3-ol	1.4	Alcohol
4	3-Octanone	0.8	Ketone
5	Myrcene	2.7	Monoterpene
6	*Unknown* [2]	1.2	-
7	Limonene	9.1	Monoterpene
8	2-Ethylhexanol	2.2	Alcohol
9	Decanal	1.0	Aldehyde
10	Tridecane	6.2	Alkane
11	Tetradecane	2.4	Alkane
12	(*E*)-β-Caryophyllene	29.2	Sesquiterpene
13	α-Bergamotene [2]	0.7	Sesquiterpene
14	α-Humulene	6.5	Sesquiterpene
15	α-Farnesene	0.8	Sesquiterpene
16	Bisabolene	8.6	Sesquiterpene
17	α-Bisabolol	7.9	Sesquiterpene

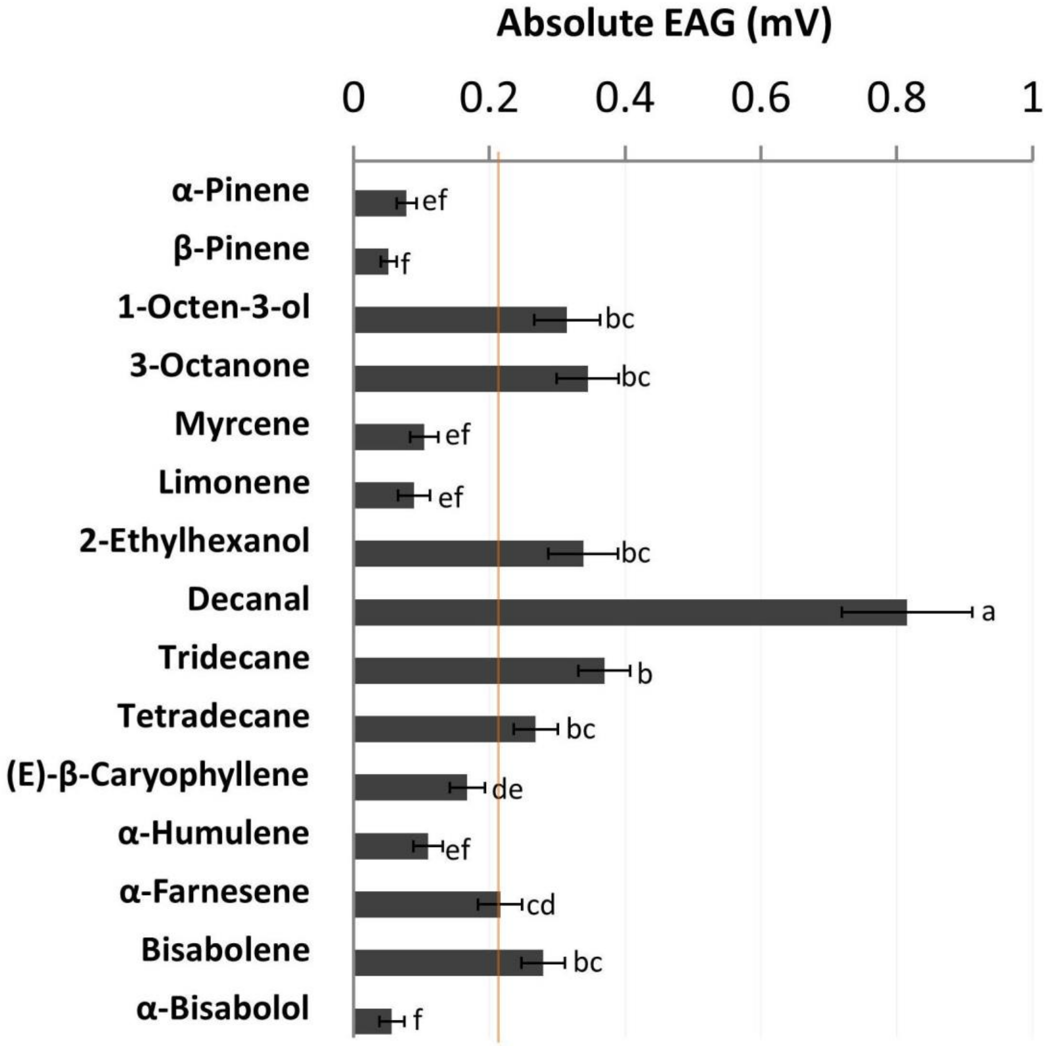
[1] In order of elution during gas chromatography

[2] Compounds that were not tested in the present study

Figure Legend

Figure 1: Mean absolute Electroantennogram (EAG) responses (mV ± SEM; *N* = 15) of female *Microplitis croceipes* to 15 volatile compounds identified in the headspace of cotton-fed *Heliothis virescens* larvae (Morawo and Fadamiro 2016). Synthetic compounds were formulated in hexane (solvent control) and tested at an optimum dose of 1 μg. Orange line indicates the arbitrary response threshold of 0.22 mV (50th percentile rank). Bars with no letters in common are significantly different ($P<0.05$; *Kruskal-Wallis* test followed by *Sidak's* multiple comparison test).

Figure 1


Absolute EAG (mV)
0
0.2
0.4
0.6
0.8
1
α-Pinene
β-Pinene
1-Octen-3-ol
3-Octanone
Myrcene
Limonene
2-Ethylhexanol
Decanal
Tridecane
Tetradecane
(E)-β-Caryophyllene
α-Humulene
α-Farnesene
Bisabolene
α-Bisabolol
ef
f
bc
bc
ef
ef
bc
a
b
bc
de
ef
cd
bc
f

CHAPTER 5

SUMMARY AND FUTURE STUDIES

5.1 Summary and Future Studies

This project investigated some aspects of the chemical ecology of plant-herbivore-parasitoid tritrophic interactions that were considered underexplored. The first study investigated the role of plant- and herbivore-related experiences in intraspecific oviposition preference in a relatively specialized parasitoid, using *M. croceipes-H. virescens* as model system. The results indicate a possible synergistic effect between the two types of experiences during associative learning of host-related odor cues. Further, the results suggest that plant odors probably serve as a contrasting background with herbivore odors to facilitate host location at short-range. On one hand, plant volatiles are better long-range cues and more detectable than herbivore-emitted volatiles. On the other hand, herbivore-specific cues are mostly short-range but considered more reliable indicators of host presence and identity. Although it was predicted that herbivore-related cues are more useful to specialist parasitoids of generalist herbivores than plant-related cues, evolution should favor strategies that use both sources of cues in a complementary manner. Future studies should also investigate how physiological factors such as nutrition, mating status, ovigeny, egg limitation and time limitation affect the choice of host-related cues used by parasitoids, with emphases on optimal foraging and energetic considerations.

The second study confirmed that intraspecific oviposition preference in *M. croceipes* reported in the first study was odor-mediated. Therefore, a mechanistic study was conducted to identify and characterize the role of key compounds mediating attraction of *M. croceipes* to its host, *H. virescens* larvae fed on cotton. The results support the prediction that only a fraction of the odor blend emitted by herbivore hosts mediates attraction in parasitoids. Further, testing modified synthetic blends with a reductionist approach showed that six of ten plant-associated compounds contributed the most to the attractiveness of the odor blend, with two of those compounds, 1-octen-3-ol and decanal being key to attraction of parasitoids. Regardless, a complete blend, including less attractive components serving as background or masking odors, is often required for optimum perception and recognition of odors in parasitoids. In complex chemical environments with host-related cues, non-host cues, ecologically irrelevant compounds and atmospheric pollutants, parasitoids must learn to differentiate between resource-indicating compounds and background noise. It is possible that certain background odors create an olfactory contrast effect to increase detectability of key attractive compounds.

Although most insect species have evolved relatively efficient means of differentiating host-related cues from non-host cues, man-made atmospheric pollutants may present a serious challenge for foraging insects. Plants emit volatile organic compounds that may attract foraging herbivores, pollinators and natural enemies, depending on the type of odor cues released. Further, herbivore infested plants often release induced volatiles that prime induced defenses in other plants and can serve as host/prey location cues for natural enemies. However, there is increasing evidence that atmospheric pollutants can degrade or interfere with semiochemicals used in plant-insect communication systems, with consequences for pollination services and biological control (Gate et al. 1995; Moraes et al. 2004; McFrederick et al. 2009; Blande et al. 2010; Farre-Armengol et al. 2016; Fuentes et al. 2016; Li et al. 2016). For instance, ground-level ozone (O_3) is a pollutant that can be formed when nitrogen dioxide (NO_2) from fuel combustion reacts with plant volatiles in the presence of sunlight. Besides human health issues caused by ozone, it can also disrupt the dynamics and fidelity of plant-insect interactions. Ozone degradation of plant odors can significantly increase the foraging time of honey bees (Farre-Armengol et al. 2016; Fuentes et al. 2016) and adversely affect host searching behavior in parasitoids (Gate et al. 1995). Other important air pollutants include nitrate radicals (NO_3) and sulphur dioxide (SO_2). Mechanistic studies on how atmospheric pollutants affect plant signaling and insect olfaction are only starting to unravel.

There is evidence that air pollutants degrade plant odors (Farre-Armengol et al. 2016; Fuentes et al. 2016; Li et al. 2016) but is not clear whether these pollutants also affect internal signaling, plant-to-plant signaling and other metabolomic responses in plants. The effect of pollution on plant signaling and induced defense merits further investigation. In insects, odor molecules are perceived by olfactory sensory neurons (OSNs) associated with olfactory sensilla on the antenna. Each OSN expresses one or few types of odorant receptor (ORs) associated with processing of specific odor molecules. Future studies should investigate the underlying behavioral, physiological and molecular mechanisms by which insects respond to atmospheric pollutants and plant odor-pollutant complexes. EAG, SSR and odorant mapping studies will reveal the mechanisms of perception, coding and processing of plant odor-pollutant complexes in herbivores and beneficial insects.

In addition, identification of target odorant receptors and characterization of expression patterns of putative chemosensory receptor genes will further unravel the molecular basis of olfaction in select pest and beneficial insect species. Besides increasing our knowledge of the mechanisms by which atmospheric pollutants interfere with olfaction in foraging insects, silencing the genes expressing ORs that bind with pollutants can mitigate the adverse effect of such antagonist molecules on optimal resource location in beneficial insects. This represents a potential practical application of such study down the road, and may lead to more efficient pollination services rates and biocontrol in urban and sub-urban areas.

Identification and quantification of plant-associated compounds emitted by cotton-fed *H. virescens* larvae in the second study revealed significant variation in the relative abundance of blend components. Also, it was established in behavioral bioassays that certain blend components were more attractive to parasitoids than others. Therefore, a third study was conducted to investigate whether the relative abundance of volatile compounds in the attractive odor blend correlates with the level of antennal response (odor perception) in *M. croceipes*. The results suggest a discrepancy between the two parameters. For instance, decanal constituted only 1% of the total blend but elicited the highest EAG response in *M. croceipes*. On the other hand, (*E*)-β-caryophyllene, the most abundant blend component (29%), elicited a relatively low EAG response in parasitoids. Therefore, the ecological relevance of a compound, rather than its relative abundance likely dictates the level of olfactory response in foraging insects. While this study investigated the correlation between odorant perception and one physical property of volatile compounds (relative abundance), future studies should correlate temporal processing of compounds in the insect olfactory system with properties such as volatility, molecular weight and compound configuration. Possible interactions between molecular processing (roles of odor binding proteins, odorant receptors and olfactory receptor genes) and odorant configurations such as polarity and chirality should be investigated to better understand why insects may show varying response latencies to different odorants (Morawo and Fadamiro 2014).

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APPENDIX I

TRACKING BEHAVIORAL RESPONSES OF TWO PARASITOIDS WITH VARYING DEGREES OF HOST SPECIFICITY TO THE DEFENSIVE SECRETION OF THEIR LARVAL HOST

6.1 Introduction

Parasitoids, herbivore and plants are involved in an intricate tritrophic interaction. Plants infested by herbivores defend themselves directly through chemical (toxins and repellents) and physical (spines, thorns, trichomes, etc) mechanisms. They can also defend themselves indirectly by emitting volatile organic compounds (VOCs) that can attract parasitoids to their herbivore hosts. However, herbivores have also developed countermeasures against plant defense in an evolutionary arms race (Winde and Wittstock 2011). Among other strategies, herbivores have developed efficient physiological means to evade plant defenses. In particular, herbivores can detoxify, excrete or sequester plant chemicals to avoid its detrimental effect (Ivie et al. 1983; Opitz and Muller 2009; Winde and Wittstock 2011; Mithöfer and Boland 2012). Further, sequestered plant chemicals may be used for defensive purposes against natural enemies (Opitz and Muller 2009). Thus, certain herbivores produce defensive compounds that deter and paralyze (Gullan and Cranston 2010), or even kill (Dietrick and van der Bosch 1957) natural enemies. For instance, the cabbage aphid (*Brevicoryne brassicae*) sequesters glucosinolates from its cruciferous plant hosts to make a lethal toxin that significantly impacts the survival of a ladybird predator (Kazana et al. 2007). Similarly, several specialist herbivores on milkweed sequester cardenolides for defensive purposes (Agrawal et al. 2012). On the other hand, some natural enemies have also evolved to circumvent host defenses through various behavioral mechanisms, most notably by avoidance tactics (Goff and Nault 1974; Rotheray 1981; Gross 1993).

The dietary specialization of herbivores may influence the degree to which they can tolerate plant toxins or sequester plant chemicals for defensive purposes. For instance, the larvae of *Heliconius* butterflies feed solely on leaves of passion vine (Genus *Passiflora*), which contains cyanogens (Engler-Chaouat and Gilbert 2007). However, monophagous species sequestered multiple folds of cyanogenic glycosides from specific host plants, compared with polyphagous (generalist) species (Engler-Chaouat and Gilbert 2007). It is also possible that the degree of host specificity in parasitoids may influence their ability to manipulate herbivore host defensive secretions. This is because parasitoid species with a restricted host range (specialists) are expected to have coevolved more closely with their hosts, compared with those with broader host range (generalists). Unlike predators that must capture and feed directly on their prey, adult parasitoids spend far less time interacting with their hosts during an oviposition encounter, usually within a fraction of a second (Gross 1993). Parasitoids of aphids (Goff and Nault 1974) and syrphid larvae (Rotheray 1981) have been reported to avoid defensive secretion of their hosts, which would have affected most predators. Although parasitoid contact with small amounts of host defensive secretion is not likely to be fatal in most cases [although see Dietrick and van der Bosch (1957)], it may have other negative impacts on foraging activities female parasitoids. In particular, prolonged grooming may lead to a disruption in normal host foraging activity (Hays and Vinson 1971).

Microplitis croceipes (Cresson) (Hymenoptera: Braconidae) is a relatively specialized larval parasitoid of *Heliothis virescens* (F.) (Lepidoptera: Noctuidae) while *Cotesia* *marginiventris* (Cresson) (Hymenoptera: Braconidae) is a generalist larval parasitoid of several noctuid species, including *H. virescens*. *Heliothis virescens* is a serious pest of several economic crops, including cotton and tobacco. Larvae of *H. virescens* feed on cotton leaves, inducing VOCs that mediate host location in both species of parasitoid (Ngumbi and Fadamiro 2012; Morawo and Fadamiro 2014a, b). During parasitization encounters, plant-fed *H. virescens* larvae often deploy a defensive oral secretion with a head strike. Contact with the freshly secreted fluid repels parasitoids and further interferes with normal foraging activities due to prolonged grooming, similar to the effect on *Cardiochiles nigriceps* Viereck (Hymenoptera: Braconidae) reported by Hays and Vinson (1971). Soon after the fluid was secreted, it becomes dry and solid. *Microplitis croceipes* and *C. marginiventris* seemed to show varying behavioral responses to dry *H. virescens* defensive secretion (personal observation). Moreover, the defensive secretion of *H. virescens* larvae that fed on artificial diet did not appear to be potent against parasitoids.

Therefore, I tested the assumption that herbivore defensive secretion effectively deters parasitoids due to the presence of plant-derived chemicals. To test this assumption, the grooming behavior of parasitoids was compared among individuals that contacted defensive secretion from cotton-fed hosts, artificial diet-fed hosts and those squirted with distilled water. Furthermore, the hypothesis that *M. croceipes* (specialist) will show greater attraction than *C. marginiventris* (generalist) to dried *H. virescens* defensive secretion was tested by tracking their behavioral responses in a petri dish arena. Finally, the behavioral response of *H. virescens* larvae to self and conspecific secretions was tested to know whether the secretion also elicits escape behavior in *H. virescens*.

6.2 Materials and Methods

6.2.1 Insects. Larvae of *Heliothis virescens* were initially reared on pinto bean artificial diet. A group of larvae was removed from artificial diet, cleaned with moistened paper towel and allowed to feed on cotton plant (plant-fed) for 24 h. *Heliothis virescens* larvae that fed on foliage take up the green color of leaves, thus, we ensured that individuals used in the bioassays were plant-fed. Oral/defensive secretion was obtained from 3rd instar larvae of *H. virescens*. *Microplitis croceipes* were reared on 2nd-3rd instar larvae of *H. virescens* according to the procedures described by Lewis and Burton (1970). Upon emergence, adult wasps were supplied 10% sugar water *ad libitum* in plastic cages. Parasitoids used in behavioral bioassays were 3–4 day–old females that were presumed mated after interaction with males for at least 48 h. The general rearing conditions for all insects were 25±1 °C, 75±5 % RH and 14:10 h (L:D) photoperiod.

6.2.2 Plants. Cotton (*Gossypium hirsutum*, var. max 9) plants were grown in growth chambers in our lab (Entomology & Plant Pathology, Auburn University) at 26.6°C day, 25.6°C night, and 60 % relative humidity (Morawo and Fadamiro 2014a). Daylight fluorescent tubes (270 PAR) with 16:8 hr (L/D) photoperiod provided illumination in the growth chamber. Seeds were planted in a top soil/vermiculite/peat moss mixture. Plants used to feed *H. virescens* larvae (plant-fed group) were 4-5 week old.

6.2.3 Behavioral response of parasitoids to host defensive secretion. The modified methods of Ulrich et al. (2016) were used to test the behavioral responses of parasitoids to defensive secretion of *H. virescens* larvae. Tops (lids) of sterile petri dishes (100 mm diameter) were lined with weighing paper (Fisherbrand®, Fisher Scientific, Pittsburg, PA, USA) and covered with a sheet of glass. Using the petri dish top reduced the height if the arena to 8 mm so that parasitoids were mostly walking on the weighing paper below, and not upside down.

Preliminary tests showed that physical properties of the defensive secretion on leaf are better mimicked on weighing paper than on filter paper (also suggested by Li Chen, pers. comm.). Weighing paper was used as substrate in place of cotton leaf to avoid parasitoid distraction due to plant volatiles.

A third instar larva of *H. virescens* was removed from cotton plant or artificial diet and put into a petri dish. Herbivores obtain defensive chemicals from plants, thus, secretion from artificial diet-fed larvae served as control. The larva was poked at the rear with the tip of a Pasteur pipette to elicit the head strike defensive behavior and subsequently production of defensive secretion. The secretion was immediately collected using a pipette and applied to the weighing paper in the petri dish arena. Third instar cotton-fed larvae produced an average of 0.3 µl while artificial diet-fed larvae produced an average of 0.2 µl of secretion at full discharge. In general, all larvae showed head strike defensive behavior. Over 90% of cotton-fed larvae produced defensive secretion while fewer (about 65%) artificial diet-fed larvae produced defensive secretion when poked 1-2 times.

For each replicate, the secretion was applied 1 cm from the perimeter of the arena at 3, 6, 9 or 12 o’ clock equidistant from each other to minimize possible positional effect (Fig. 1). A female parasitoid (*M. croceipes* or *C. marginiventris*) was left in a dummy arena to acclimatize for at least 3 min before being transferred to the assay arena. Individual parasitoids were introduced to the opposite end of the arena from the position of the secretion. For instance, a parasitoid was introduced at the 12 o’ clock position if the secretion was applied to the 6 o’ clock position. Once introduced, parasitoid behavior was recorded for 10 min. Each parasitoid and petri dish was used once while the glass cover was cleaned with acetone after testing each parasitoid.

Experiments were arranged in randomized complete block design with equal number of replicates tested for each parasitoid species and secretion (from cotton-fed or artificial diet-fed larvae) on different days between 0900 and 1700 h. Twenty insect replicates were tested for each bioassay.

6.2.4 Video recording and analyses. Movement of parasitoids and host larvae was monitored using a FireWire digital camera with image processing macro lens (Computar® model MLH-10X, Mag. 0.084-0.84X, F5.6; Computar, Cary, NC, USA). The camera was connected to a computer with a USB adapter and controlled through Video ToolBox Pro software (Zarbeco LLC, Randolph, NJ, USA). The camera was hung 35 cm above the arena while illumination was provided by daylight fluorescent lamp (approx. 260 PAR). The recording room was kept at 25±1 °C and 75±5 % RH. Video tracking was analyzed using Ethovision® XT software (Version 13; Noldus Information Technology Inc., Leesburg, VA, USA) according to Ulrich et al. (2016), with modifications. Briefly, the arena was calibrated in Ethovision® to get actual distance and speed parameters. Furthermore, the arena was divided into four equal zones: one zone contains the defensive secretion (secretion spot) at 3, 6, 9 or 12 o' clock position while the other three zones are untreated. A circular subzone (radius 1 cm) around the secretion spot was also marked (Fig. 1).

The following variables were considered in Ethovision®: total time spent by parasitoids in zones, total time spent in secretion subzone, total time spent on secretion spot, time taken to first visit of secretion zone, number of times zones were visited, number of times secretion spot was visited, and average speed in zones. Other variables recorded manually are total time spent by parasitoids while antennating on the secretion spot and total time spent probing secretion spot with ovipositor. In addition to other data output generated by Ethovision®, the software also provided visual representations of insect tracks and heat maps (see samples in Fig. 2). Tracks represented the actual walking path of insects while heat maps showed the relative amount of time spent in different locations in the arena.

6.2.5 Behavioral response of *H. virescens* larvae to their defensive secretion.

Experiments were set up to track behavioral response of cotton-fed *H. virescens* larvae to defensive secretion from self and conspecifics. A relatively large leaf from a healthy cotton plant was clipped at the petiole, close to the node, and placed in an aerated plastic bowl (inside dimension 19 × 12 × 5 cm). The petiole was inserted into an Eppendorf tube partially filled with water to keep the leaf in a near-living state (Fig. 3). For treatment 1, a larva was poked so that it produces its defensive secretion at the center of the leaf surface. Then a clean pair of forceps was used to position the larvae so that it makes contact with the secretion. If the larva contacts the secretion by itself, no further manipulation was done. For treatment 2, another larva was placed on the defensive secretion from a conspecific larva. For treatment control, a larva was gently placed in the center of a cotton leaf (i.e., no defensive secretion). Preliminary trials showed that *H. virescens* larvae removed from active feeding move in a random manner, and are not distracted by feeding for up to 5 min when placed on non-infested cotton leaf. Therefore, behavior of larvae was recorded for 5 min with the camera previously described. Twenty insect replicates were tested for each treatment, using a new cotton leaf per replicate.

The behavior of *H. virescens* larvae in response to self and conspecific defensive secretion was manually scored from analyses of video clips. To compensate for slight differences in leaf area across replicates, I recorded time taken for larva to exit the secretion zone i.e., walk a distance of 35 mm away from the secretion spot in any direction. The secretion zone was digitally marked using Video ToolBox Pro software (Zarbeco LLC, Randolph, NJ, USA).

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Figure Legend

Figure 1: Layout of petri dish arena (10 cm diameter) for tracking behavioral response of female *M. croceipes* and *C. marginiventris* to the defensive secretion of *H. virescens* larvae. The arena was divided into four equal sectors (zones) on a weighing paper substrate. For each trial, three zones were without secretion while secretion was applied to one zone (secretion zone). The secretion subzone is the area of circle 1 cm around the secretion spot. For each replicate, the secretion was applied 1 cm from the perimeter of the arena at 3, 6, 9 or 12 o' clock position. Individual parasitoids were introduced to the opposite end of the arena from the position of the secretion.

Figure 2: Visual representation of parasitoid movement in in the arena. (A) Typical insect tracks and (B) Typical heat map. Tracks represented the actual walking path of insects while heat maps showed the relative amount of time spent in different locations in the arena.

Figure 3: A simplified diagram of the experimental set up for scoring behavioral response of *H. virescens* larvae to defensive secretion from self and conspecifics. To test response to self-defensive secretion, a larva is poked so that it produces its defensive secretion at the center of the leaf surface. To test response to conspecific defensive secretion, the secretion was applied to the center of the leaf surface and a larva was placed on top of it. The secretion spot is approximately in the center of the leaf while the secretion zone is the area 35 mm radius from the secretion spot.

Figure 1

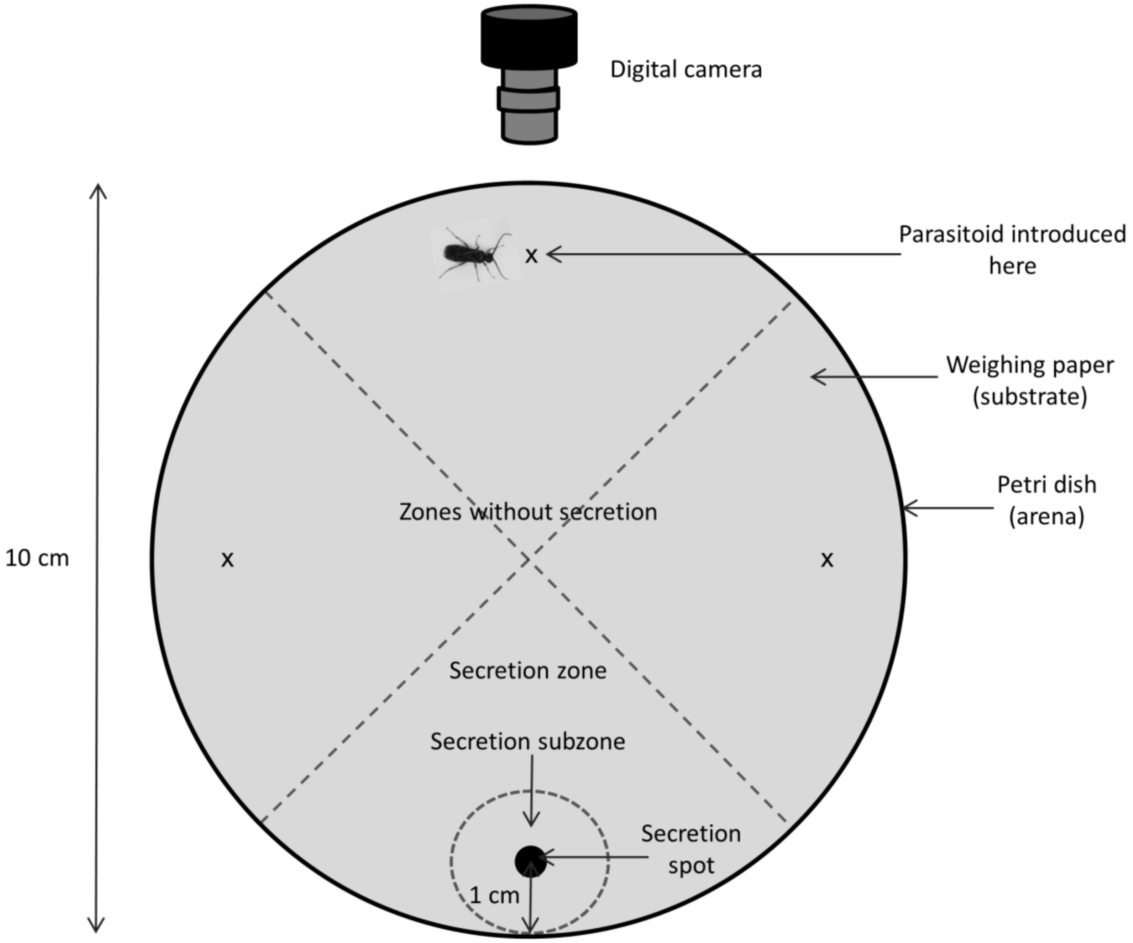

Digital camera
Parasitoid introduced here
x
Weighing paper (substrate)
Petri dish (arena)
Zones without secretion
10 cm
x
x
Secretion zone
Secretion subzone
Secretion spot
1 cm

Figure 2

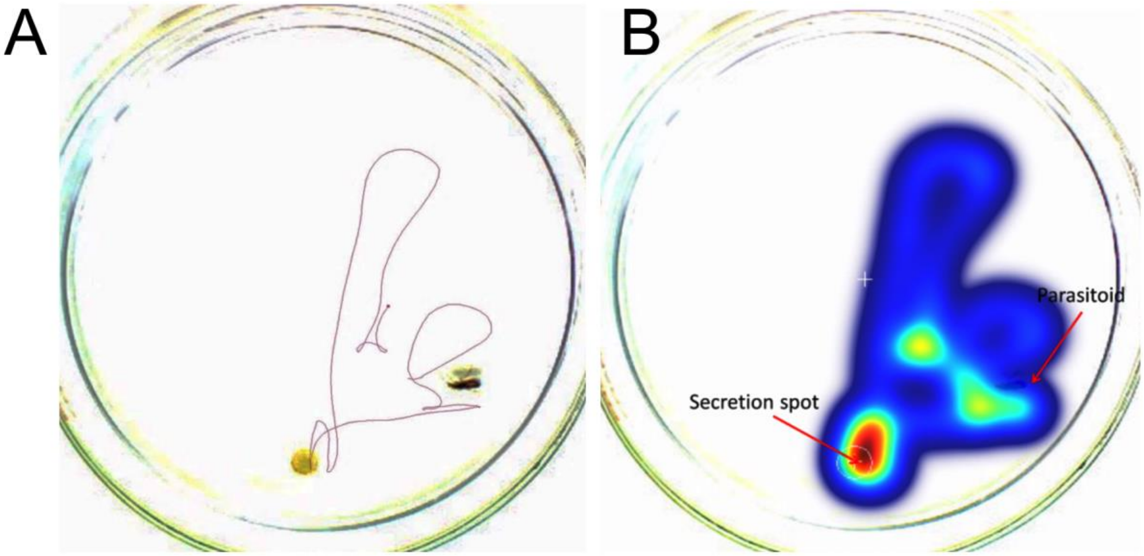
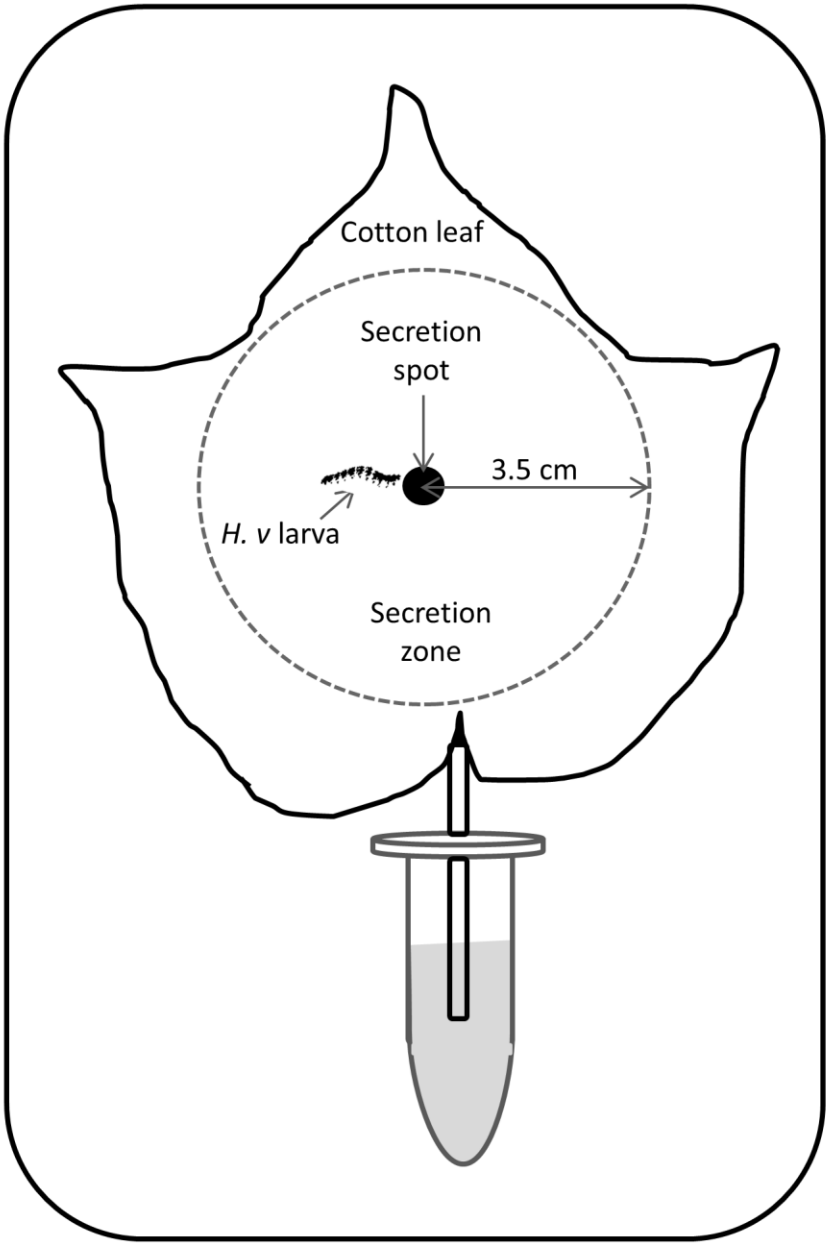


Figure 3



Disclosure

Chapters 2 – 4 of this dissertation are published in peer-reviewed journals as listed below:

Morawo T, Fadamiro H (2017) The role of herbivore- and plant-related experiences in intraspecific host preference of a relatively specialized parasitoid. Insect Science. DOI: 10.1111/1744-7917.12537.

Morawo T, Fadamiro H (2016) Identification of key plant-associated volatiles emitted by *Heliothis virescens* larvae that attract the parasitoid, *Microplitis croceipes*: implications for parasitoid perception of odor blends. Journal of Chemical Ecology 42: 1112–1121. DOI: 10.1007/s10886-016-0779-7.

Morawo T, Burrows M, Fadamiro H (2016) Electroantennogram response of the parasitoid, Microplitis croceipes to host-related odors: the discrepancy between relative abundance and level of antennal responses to volatile compound [version 2; referees: 4 approved]. F1000Research 5: 2725. DOI: 10.12688/f1000research.10104.1.