**Plasticity of Olfactory Response to Host-Related Plant Volatiles in
The Parasitoid *Microplitis croceipes***

by

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Abstract

Plants release blends of volatile organic compounds (VOCs) in response to herbivore damage. Parasitoids use certain VOCs as indirect cues to locate their herbivore hosts. However, response of parasitoids to these chemical cues can be plastic, depending on their physiological state. Previous studies have demonstrated that various factors such as nutritional status, age, and mating may modulate response of parasitoids to host-related volatile compounds. In this study, the parasitoid *Microplitis croceipes* was used to test the hypothesis that the physiological state of parasitoids affects their response to host-related plant volatiles. *Microplitis croceipes* is a relatively specialized endoparasitoid of larvae of *Heliothis virescens*, which is a major pest of cotton. Specifically, the effect of nutritional status, age, and mating on olfactory response of *M. croceipes* to select cotton plant volatiles was investigated in a series of electroantennogram (EAG) and behavioral bioassays. The following VOCs were tested in this study since they have been previously shown to elicit olfactory response in *M. croceipes*: cis-3-hexenol (a green leaf volatile or GLV), α-pinene (a constitutive monoterpene), a 50/50 v/v binary mixture of cis-3-hexenol and α-pinene, and a complex natural odor blend collected from the headspace of *H. virescens*-damaged cotton plants.

In chapter II, the effect of nutritional status on *M. croceipes* olfactory response to plant VOCs was investigated. Parasitoids were fed different diet treatments (i.e. 40%, 20%, 10%, or 0% sucrose/water solution (w/v)) using EAG recording and Y-tube olfactometer bioassays. Antennal and behavioral responses to select synthetic VOCs, 50:50 binary VOC mixture, and

natural VOC blend were measured relative to their nutritional status. Parasitoids fed 20% or 10% sucrose solution showed significantly higher EAG response to the binary odor mixture (cis-3-hexenol + α-pinene) than sugar-starved wasps. Also, parasitoids fed 40% sucrose solution showed a significantly higher EAG response to *H. virescens*-infested cotton extract than sugar-starved parasitoids. In Y-tube olfactometer bioassays, female *M. croceipes* fed 40% sucrose solution were significantly more attracted to the natural odor blend than to solvent control. In general, *M. croceipes* showed relatively lower response to the test single compounds (cis-3-hexenol and α-pinene) compared to the binary odor mixture and the complex natural odor blend. These results suggest that nutritional status may modulate olfactory plasticity in *M. croceipes*, with consequences for its performance as a biological control agent.

In Chapter III, effect of age and mating status of *M. croceipes* on their olfactory response to VOCs was investigated. Female parasitoids were first separated based on mating status and were either introduced into a cage with males or left in a cage with other females. Parasitoids were then arranged into age groups of 1-3, 4-6, 7-9, or 10-12 days old. Similar to chapter II, EAG recording and Y-tube olfactometer bioassays were conducted. No significant difference in EAG response was recorded relative to parasitoid age. With regard to mating status, only mated parasitoids aged 7-9 days old showed significant EAG response to single compound α-pinene and the binary odor mixture. In Y-tube olfactometer bioassays, mated female *M. croceipes* aged 4-6 days old were significantly more attracted to the binary mixture than to solvent control. In addition, mated female *M. croceipes* aged 1-3 days old were significantly more attracted to the natural odor blend than to control. The implications of these findings are discussed.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Parasitoids

Parasitoids are some of the most compelling and complex insects with regard to their unique physiology and behavior, which includes oviposition on hosts. Consequently, many studies during the past half-century have attempted to study the life history and physiology of parasitoids (Takasu and Lewis, 1993). For example, parasitoids can be solitary- meaning only one individual can complete development in each host, or gregarious- meaning several immatures can successfully share a single host. Parasitoids are classified with regard to development within the host as either koinobiont, allowing development of host after parasitization, or idiobiont, ceasing further development of the host upon parasitization. Some parasitoids are capable of parasitizing different life stages of their host (egg, larvae, pupae, adult), but most are only able to parasitize a particular life stage (Harvey and Strand, 2002). Parasitoids are accounted for in the order Diptera in families such as Tachinidae and Cryptochaetidae, the order Coleoptera in families such as Meloidae and Staphylinidae, and the order Hymenoptera within several families including Braconidae and Trichogrammatidae (Eggleton and Belshaw, 1993). All parasitic Hymenoptera undergo haploid parthenogenesis and display one of three variations of reproduction: arrhenotoky- unfertilized eggs produce males and fertilized eggs produce females, deuterotoky- unmated females produce both males and females, or thelytoky- only females are produced (Cook 1993).

Parasitoids can also be classified as either generalists or specialists with regard to the range of hosts parasitized. Generalists have a wider range of hosts suitable for oviposition while the range is much narrower for specialists. Parasitoids have a specialized olfactory system allowing them to gain a selective advantage by utilizing host-related plant volatiles. Upon herbivory, plants release odors that guide parasitoids to the herbivore, and therefore are capable of finding their herbivore hosts associated with these odors. Parasitic wasps in particular, are considered good models for studying this interaction. Parasitoids are common biological control agents due to their fine-tuned olfactory efficiency in locating and parasitizing hosts with varying degrees of specificity. Thus, the mechanisms of parasitoid olfaction should be investigated with respect to their physiology, behavior, and chemical ecology.

1.2   Volatile Organic Compounds and Host Location in Parasitoids

Herbivore-induced plant volatiles play a major role in the foraging behavior of predatory and parasitic arthropods. In response to feeding damage, plants release volatile organic compounds (VOCs) as both a direct and indirect defense against herbivores. VOCs can guide parasitoids to their hosts (De Moraes et al., 1998; Pare and Tumlinson, 1999; Mumm and Hilker, 2005; Wei and Kang, 2006). VOCs consist of different functional groups and structures including short, straight carbon chains, and multi-ring sesquiterpenes (Knudsen et al., 1993; Meiners et al., 2002). Plants, upon damage by herbivores, release volatile compounds which can be classified into groups. Green leaf volatiles (GLVs), which are usually 6-carbon alcohols and aldehydes, are released from plants immediately following mechanical damage or immediately after initiation of herbivore damage (Knudsen et al., 1993; Meiners et al., 2002). Cotton is the plant system used in this series of studies. GLVs known to be released from cotton include hexanal, (*Z*)-3-hexanal, and (*Z*)-3-hexenol among others. Herbivore induced plant volatiles

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(HIPVs) are released as a delayed response to herbivore damage. HIPVs commonly released from cotton after mechanical or herbivore damage are (E)-β-ocimene, (E)-β-farnesene, nonatriene, tridecatetraene, indole, hexenyl acetates, isomeric hexenyl butyrates, and 2-methyl butyrates (DeMoraes et al., 1998; Pare and Tumlinson, 1999; Rose and Tumlinson, 2004; Ngumbi et al., 2009).

Plants emit several different compounds regardless of the herbivore species causing damage. These compounds can be released constitutively or induced, following mechanical or herbivore damage (Turlings et al., 1998). However, there is variation in the composition of VOC emission for different plant species depending on the identity of the attacking herbivore (De Moraes et al., 1998; Hoballah et al., 2002; Ngumbi et al., 2009), plant species, and mode and duration of damage (Morawo and Fadamiro, 2014). VOCs typically occur in nature as blends of different compounds at different concentrations, although some studies have investigated the possibility of a single compound to elicit a response in parasitoids (Morawo and Fadamiro, 2014). Furthermore, only certain components of natural volatile blends are considered attractive or ecologically advantageous to parasitic wasps (Schnee et al., 2006; van Dam et al., 2010; Morawo and Fadamiro, 2014). Typically, parasitoids perceive odor mixtures better than single compounds. Furthermore, compounds that elicit low attraction as single components may still affect parasitoid attraction when perceived in the context of a blend. In laboratory settings, a plant VOC mixture may be more attractive than an individual compound because it represents an odor mixture comparable to what a parasitoid would perceive in a natural setting (van Wijk et al., 2011, Morawo and Fadamiro, 2014). Differences in various VOC blends may in fact act as host specific cues for parasitoids and other natural enemies for locating oviposition sites (Smith, 1998; De Bruyne and Backer, 2008; Morawo and Fadamiro, 2014).

1.3.   Methods for Characterizing Olfactory Response of Parasitoids to VOCs

During the past few decades, researchers have attempted to fine-tune existing technology and introduce advanced tools for recording olfactory response of parasitoids to chemical stimuli. Electroantennogram (EAG) has been used in previous olfaction studies and has proven to be useful in investigating electrophysiology and chemical ecology of parasitoids. Recently, EAG has been an important tool in studies involving parasitoid-host relationships mediated by host related plant volatiles. EAG measures the sensitivity of olfactory receptor neurons located across the insect antenna and records the sum DC potential of all olfactory receptor neurons on the antenna following the introduction of a chemical stimulus (Park et al., 2001; 2002). However, behavioral bioassays may also be necessary to include in studies because parasitoid EAG response does not guarantee the presence of a behavioral response (Park et al., 2001). Therefore, olfactometer studies are carried out in order to characterize behavioral responses of insects to odors. The Y-tube olfactometer is used for indicating attraction of an insect to a particular odor or odor mixture. One arm is designated for the test odor while the other arm holds the control containing a solvent or air alone, depending on the chemical composition of the test odor. Y-tube olfactometer has been shown in previous studies to be a reliable tool for measuring behavioral response of parasitoids to host related plant volatiles.

1.4.   Plasticity and Physiological State

Plasticity of insect response to host-related plant volatiles can be affected by their physiological state, consequently affecting host and food-foraging behavior (Takasu and Lewis, 1993; Rivero and Casas, 1999). Insect physiological states such as mating status, nutritional status and age can change the response of an insect to perform in certain ways or search for

particular resources. Physiological state has been documented as likely the most critical intrinsic factor that influences resource-adapted behavior (Brown 1993; Martel et al., 2009). Consequently, olfactory plasticity is frequently observed in insects and depends on survival requirements such as nutritional needs and mating partners. As a result, insects have developed efficient strategies for host location including development of behavioral and physiological adaptations (DeMoraes et al., 2000). For example, honeybee workers switch from in-hive (nursing) to outside-hive (foraging) tasks depending on their age, colony demand, and outside conditions (Farooqui, 2014). In addition, parasitoids switch from host searching behaviors to food searching, and use different visual and olfactory cues accordingly, providing evidence that innate preferences can adjust due to parasitoid physiological state (Wackers, 1994; Lewis et al., 1997). Olfactory responses of natural enemies and parasitoids to host related plant volatiles can be manipulated to optimize the release of natural enemies with an exclusive physiological state in which their responses are most sensitive (Vet and Dicke, 1992). Furthermore, pest management utilizing biological control can be improved by the evolution of plasticity of parasitoid responses to host-related plant volatiles.

1.4.1   Effect of Nutritional Status

Parasitoid reproduction and parasitism can be accomplished successfully once certain essential requirements are met. Food, shelter and hosts are central among those requirements and are necessary for survival (Stapel et al., 1997). Some parasitoids are known to utilize host-related food sources such as hemolymph through concurrent and non-concurrent feeding behavior (Jervis and Kidd, 1986; Heimpel and Collier, 1996; Heimpel and Jervis, 2005. Others use sugar sources as their primary and often only means of nourishment (Stapel et al., 1997). In nature, sugar can be obtained from nectar and homopteran honeydew (Evans 1986; Jervis and Kidd,

1996; Olson et al., 2000), which consist mainly of sucrose, fructose and glucose (van Handel et al., 1972; Magnarelli 1979; Harborne 1988). Nectar can be produced by floral or extrafloral nectaries and nectar composition can vary depending on the species, variety, and age of the plant producing it (Stapel et al., 1997). Parasitoids are able to discriminate between sugars in floral and extra-floral nectars. These nectars are either sucrose-rich or hexose-rich and their sugar composition can vary. When provided at equal concentrations, parasitoids consume larger quantities of high quality sugars such as glucose, fructose, and sucrose compared to low-quality sugars (Wackers 2001; Makatiani et al., 2014). Previous analyses of the composition of major sugars found in nectar of several flowering plant species located in the southern USA show that sugar concentration varies widely relative to plant species, and can contain sucrose concentrations of 20-50% (van Handel et al., 1972; Fadamiro et al., 2005). Makatiani et al., (2014) reported that *M. croceipes* gustatory response to maltose feeding was inhibited compared with higher quality sugars fructose, glucose and sucrose. This implies that if provided all four sugars, *M. croceipes* will give up the lesser quality food for the more nutritionally sufficient sugars (Makatiani et al., 2014).

The variation of parasitoid feeding responses to different sugars at various concentrations could be explained by optimal foraging theory. The theory states that foraging insects will choose diet components that maximize their net energy intake or net fitness benefits (Charnov, 1976; Stephens and Krebs, 1986; Sih and Christensen, 2001). This theory could also verify the possibility that when parasitoids are fed higher concentrations of sucrose/water mixture, they will have a higher response to VOCs compared to parasitoids that are sugar-starved or fed no sucrose. However, additional optimal foraging models are necessary for parasitoids that must allocate their energy between foraging and host searching such as *M. croceipes*.

Carbohydrate-based resources are carried over from immature parasitoid life stages, however a sugar diet supplements these resources. Thus, they can generate energy for metabolic processes or stored as an alternative energy source by conversion to trehalose and glycogen (Friedman 1985; Olson et al., 2000; Rivero and Casas, 1999). It has been established that sugar is a requirement for physiological processes such as maturing eggs (Olson and Andow, 1998; Jervis et al., 2001; Fadamiro et al., 2005; Heimpel and Jervis, 2005), preventing egg resorption (Heimpel et al., 1997; Fadamiro and Chen, 2005), and adult longevity and fecundity (Wackers 2001, Chen and Fadamiro, 2006; Winkler et al., 2006; Wäckers et al., 2008; Desouhant et al., 2010; Harvey et al. 2012). Most studies have focused particularly on parasitoid longevity and fecundity relative to receiving nectar subsidies. Typical parasitoid lifespan ranges between 1 and 5 days for sugar-starved subjects and between 2 and 8 weeks for sugar-fed parasitoids (Jervis et al., 1996; Thompson 1999).

Previous studies have investigated parasitoid host searching ability relative to nutritional status (Takasu and Lewis, 1993; Wackers 1994; Takasu and Lewis, 1995; Stapel et al., 1997). Stapel et al., (1997) observed detectability and searching time of *M. croceipes* for its caterpillar host *Helicoverpa zea* in relation to feeding on extrafloral nectar, honeydew, and sucrose, and discovered enhanced searching time and parasitism in the presence of extrafloral nectar and sucrose. After finding a food source, parasitoids allocated more time to host searching (Takasu and Lewis, 1995; Stapel et al., 1997). Other studies on the effect of parasitoid nutrition on host and food foraging have been investigated (Lewis and Takasu, 1990; Takasu and Hirose, 1991 Takasu and Lewis, 1993; Wackers 1994) and found similar results.

Although the effect of nutritional state on parasitoid performance has been previously highlighted, few studies have investigated the effect of adult parasitoid nutritional state on

olfactory response to host-related plant volatiles. A previous study by Wackers (1994) showed that behavioral responses of the parasitoid, *Cotesia rubecula* to rape seed leaf versus rape seed flower odors were affected by food deprivation. Sugar-fed parasitoids preferred leaf damage odors over flower odors while sugar-starved parasitoids preferred flower odors. Wackers (1994) demonstrated the propensity for sugar-fed female parasitoid response to VOCs to be higher than unfed parasitoids. An additional study (Takasu and Lewis, 1995), showed a strong correlation between sugar-fed versus unfed parasitoids and time allocation in corn and soybean plots with and without access to food. In plots with access to food, unfed *M. croceipes* flew to food sources (honey), while fed parasitoids flew to damaged corn or soybean leaves. Sugar-fed parasitoids allocated a greater amount of time to host searching on damaged leaves than undamaged leaves. Unfed parasitoids, once introduced to a food source, engaged in host searching quickly (Takasu and Lewis, 1995). Takasu and Lewis, (1993) reported similar results involving host searching efficiency and hunger state. Findings showed that *M. croceipes* foraging was dependent upon the physiological state, or hunger state of the parasitoid. Well-fed *M. croceipes* visited and spent more time on host-plants than food-plants, while starved *M. croceipes* preferred food plants. Oviposition was also affected by hunger state. Well-fed parasitoid oviposition events were significantly higher than starved parasitoid oviposition events. Den Otter et al., (1991) reported EAG responses to host-related odors in several species of Tsetse fly and recorded increased EAG response in starved flies *Glossina morsitans* and *G. tachinoides* but no starvation effects in *G.austeni* and *G. fuscipes* (den Otter et al., 1991). In addition, at the mammalian level of classification, modulation of olfactory performance by nutritional status mediated by leptin receptors in rats was documented in a previous study (Prud'homme et al., 2009). The study discovered modulation of olfactory response in satiated vs fasted rats in behavioral bioassays and

immunocytochemistry of the olfactory bulbs of rats, implying that nutritional status may affect olfactory response of animals across phyla.

1.4.2 Effect of Age

Plasticity of olfactory response to chemical stimuli has been linked to age of insects in previous studies (Roelofs and Comeau, 1971; Den Otter et al., 1991; Steinberg et al., 1992; Ray and Ferneyhough, 1997; Gemeno and Hayes, 2000; Laloi et al., 2001). Age can influence the antennal and behavioral response of insects either through maturation and senescence, or by the motivation to search for resources or needs caused by limited time and energy for resource investment and searching (Martel et al., 2009). Some studies have documented age-dependent variations in odor learning abilities and memory specifically in *Apis mellifera* (Ray and Ferneyhough, 1997; Laloi et al., 2001). Other studies have investigated age-related variation of olfactory response to pheromones in Lepidoptera (Roelofs and Comeau 1971; Gemeno and Haynes, 2000), and Coleoptera (Dickens and Moorman, 1990). Additional studies involve host-related odors in the order Diptera (Den Otter et al., 1991). In EAG recording, olfactory response has shown to decrease with age in *Argyrotaenia velutinana* (Roelofs and Comeau, 1971) and *Glossina morsitans* (Den Otter et al., 1991). In other studies, EAG response increased accordingly with an increase in age in *Phormia regina* (Crnjar et al., 1990), *Anthonomus grandia* (Dickens and Moorman, 1990), and *Apis mellifera* (Allan et al., 1987). EAG response has also been reported to peak at specific ages in *Pseudoletia unipunctata* (Seabrook et al., 1979), *Ostrinia nubilalis* (Fescemyer and Hanson, 1990), *Apis mellifera* (Masson and Arnold, 1984), and *Anastrepha suspense* (Kendra et al., 2005).

Due to the complexity of parasitoid olfaction involving a plant-host complex, plasticity of parasitoid olfactory response to VOCs is a subject that is widely unknown. Few studies have

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documented a lack of influence of age on EAG response. Park et al., (2001) tested the effect of age on *M. croceipes* EAG response to single VOCs, cis-3-hexenol, linalool, 2-diisopropyl aminoethanol, and cyclohexanol. The authors reported no significant effect of age on antennal response. With regard to age-dependence, the maintenance of stable antennal neuronal sensitivity in parasitoids of increasing age would provide maximal opportunity for successful host location for the duration of the parasitoid life (Takasu and Lewis, 1996; Park et al., 2001). In a wind tunnel study (Steinberg et al., 1992), relatively older *Cotesia glomerata* females (4-5 and 6-9 days old) showed greater response to VOCs emitted from host-infested cabbage, compared with younger parasitoids (1-2 days old). In the glasshouse, no significant difference in response was recorded between age groups. In a study by Herard et al., (1988) wind tunnel flight response of experienced *M. demolitor* to cowpea-*Heliothis zea* complex exhibited a low response at age 1-2 and 3-4 days old, a gradual increase at age 5-6, leading to a peak at age 7-8, and then a gradual drop and leveling out of response by day 14. Additional studies (Drost et al., 1985), involve the effect of maturation (age) on *M. croceipes* flight behavior to headspace volatiles released from *Helicoverpa zea*-infested cowpea, which found no significant effect of age on flight response in wind tunnel bioassays.

Other studies have investigated the influence of age on parasitization rate of parasitoids. The age of the parasitoid is crucial for successful parasitism due to the higher fecundity of younger parasitoids versus older parasitoids (Amalin et al., 2005). Parasitoid oviposition record may occur in phases with regard to age (Bellows 1985). Bellows (1985) reported that *Lariophagus distinguendus* oviposition occurred in four phases, starting with a period of moderate oviposition directly following emergence, which is likely associated with feeding and maturation of eggs. The second phase represents a relatively constant plateau of relatively high

ovarian egg production and oviposition. The third phase is accompanied by a decrease in ovarian

egg production and oviposition. The final phase is a plateau of moderate oviposition. Parasitoids

aged 1-7 days old laid approximately twice as many eggs as parasitoids aged 8-14. Parameters

quantifying search behavior efficiency were also recorded in this study. Search efficiency was

low on the first day of the adult life of *L. distinguendus* and in the later phase of its adult life,

while searching efficiency was highest on the second day following adult emergence (Bellows

1985). Similar results were found on *Ceratogramma etiennei* in which parasitoids aged 1-2 days

old had higher parasitism rates than older wasps (Amalin et al., 2005). Similarly, in *Cotesia*

marginiventris the optimum age for successful parasitism of *Spodoptera frugiperda* larvae was

between 48 and 96 hours post-emergence (Rajapakse et al., 1992).

1.4.3   Effect of Mating Status

Haplodiploid female parasitoids such as *M. croceipes* may encounter a tradeoff between

searching for hosts versus searching for mates (Godfray 1990, 1994; Godfray and Hardy, 1993).

This tradeoff can be considered a physiological shift which may result in plasticity of olfactory

responses with regard to mating status. Fauvergue et al., (2008) reported that virgin female

Lysiphlebus testaceipes, when introduced to hosts, spent more time motionless, not in pursuit of

hosts, compared to mated females that attacked hosts at a higher parasitization rate. In another

study (Michaud, 1994), 80% of aphid parasitoids in different genera parasitized pea aphid hosts

at a significantly higher rate when mated compared to unmated. Most studies showing mating

status as a factor causing variation of response to host-related plant VOCs involve moths due to

the characterization of some moth pheromone blends. Ding et al., (1997) reported that mated

female *Helicoverpa armigera* showed greater EAG response to cotton volatiles compared to

virgin females. Another study (Xiao et al., 2002), reported no significant effect of mating on

EAG response of *H. armigera* females to volatiles of a non-host plant, Chinese wing-nut tree. Shu et al., (1997) recorded a general increase in the response of mated female *Dioryctria abietivorella* to plant produced monoterpenes compared to unmated females. In another study (Martel et al., 2009), contrasting results were reported. EAG responses of *Spodoptera littoralis* to plant odors were tested. It was reported that virgin females were more sensitive to plant odors than mated females (Martel et al., 2009).

Studies using parasitoids as a model system have focused specifically on parasitization rate relative to mating status (Tagawa 1987; Fauvergue et al., 1998; Antolin 1989; Michaud 1994; Michaud and Mackauer, 1995; Guertin 1996; King 2002; Fauvergue et al., 2008). Some studies have reported parasitization rate of mated parasitoids to be higher than that of unmated parasitoids (Tagawa et al., 1987; Antolin 1989; Michaud 1994; Michaud and Mackauer, 1995). Findings from other studies show no significant difference between mated and unmated female parasitization rate (Guertin et al., 1996; Fauvergue et al., 1998; King 2002; Fauvergue et al., 2008). Plasticity of parasitoid response to host-related plant volatiles relative to mating status has gained little attention in literature. Chen and Fadamiro, (2007) reported no significant difference in the EAG responses of mated versus unmated female *M. croceipes* and *C. marginiventris* to synthetic VOCs. There are no known published studies linking parasitoid mating status and response to VOCs using both EAG recording and behavioral bioassays. It is hypothesized that mated females will be in search of oviposition sites rather than mates and thus, olfactory response will be tuned to host-related plant odors. Unmated females response is expected to be less tuned to plant odors and expect mating to take priority over host searching.

Based on a thorough literature search, this appears to be the first study to investigate plasticity of antennal or behavioral response of parasitoids to VOCs with regard to a diet of

various sugar concentrations. Since sugar sources vary in quality, quantity, and composition in nature, the chances of finding an amount of sugar sufficient to increase longevity from an individual feeding opportunity can be highly demanding for parasitoid success and fitness (Jervis and Kidd, 1999; Lavandero et al., 2005). Thus, parasitoids must maximize the amount of time they are searching for hosts Therefore, determining the effect of the nutritional state of parasitoids on response to VOCs could have substantial impact on explanation of increased performance of sugar-fed parasitoids. In addition, findings of this study can provide further reinforcement of physiological state as a factor playing an important role in tritrophic interactions between plant and parasitoid.

1.5   Model System

This study uses a model system consisting of the parasitoid, *Microplitis croceipes* and its caterpillar host, *Heliothis virescens*. *M. croceipes* (Hymenoptera:Braconidae) is a relatively specialized parasitoid of three highly polyphagous larval hosts, *Heliocoverpa zea*, *Heliothis virescens,* and *Heliothis subflexa* (Lepidoptera: Noctidae) (Lewis and Tumlinson, 1988; Turlings et al., 1989; DeMoraes et al., 1998; Olson et al., 2003). *H. virescens*, the model caterpillar host in the current study is also known as the tobacco budworm and is an economic pest of row crops such as tobacco, cotton, and soybean. Other crops affected are alfalfa, cabbage, lettuce, okra, pea, pepper, squash, and tomato. *H. virescens* bore into buds or blossoms and destroy the fruiting structures of these economically important crops. In the U.S., *H. virescens* occurs mostly in the southeast and along the east coast and north to Canada. *M. croceipes* has been used in many parasitoid olfaction studies and is considered a favorable model for studying tritrophic interactions involving parasitoid and host. *M. croceipes* EAG and behavioral responses to host-

related plant volatiles have been characterized in previous studies which has expanded the opportunity to further investigate parasitoid responses to host and plant-associated odors.

1.6   Justification for Study

Testing insect responses to VOCs is an idea that has gained attention lately due to the increasing need for novel sustainable pest management practices. Integrated pest management (IPM) has arrived at the forefront of sustainable pest management and seeks to utilize a combination of compatible management practices to achieve the common goal of suppressing pest populations. Both herbivore pests and natural enemies, such as parasitoids use plant-related volatile cue to forage. Parasitic wasps play an important role in sustainable agriculture due to the ability to control herbivore pest populations (Wackers, 2004). While parasitoid response to VOCs has been studied widely in the past, few studies have focused on how response to VOCs may be affected by physiological state. Effects of parasitoid age, mating status, nutritional status, and others could be essential in determining the most effective and reliable parameters for releasing parasitoids as biological control agents.

Most studies regarding parasitoid response to VOCs have been performed with the purpose of investigating learning ability, characterizing responses to various synthetic or natural VOCs, or recently, comparing responses to VOCs in parasitoids of varying degree of host specificity. Studies on parasitoids continue to reveal the complexity of the tritrophic interactions between plant, herbivore, and parasitoid. This complexity further initiates questions and concerns about how parasitoids are successfully utilized as biological control agents.

Success in biological control relies on the utilization of natural enemies and manipulation of natural enemy populations to shift pest-populations to non-pest levels (Caltagirone, 1981). Parasitoids and other natural enemies are limited by the physiological state and nutritional value

of their hosts (Vinson 1975; Brodeur 2012). However, the efficacy of the control agent may also be limited or intensified due to its own physiological state which could introduce additional constraints to a developing control strategy. In order for parasitoids to be used as effectively as possible in prospective IPM programs, studies are necessary to test for possible improvements in their use.

1.7 Thesis Goal and Outline

The goal of this series of studies was to investigate areas of insect olfactory mechanisms that may change with the physiological status of insects. Parasitoids rely on olfactory cues released during plant defense in order to locate their hosts for oviposition. This tritrophic interaction involves the use of blends of many different compounds that parasitoids such as *M. croceipes* use for host location. However, olfactory response to these compounds by parasitoids may be plastic and modulated by their physiological state. Plasticity is the capacity for organisms to change their behavior depending on changes in physiological state, environmental factors, or other elements. Investigating these areas of plasticity that may affect responses of parasitoids to host-related plant odor has significant impact on utilization of parasitoids in biological control of pests. The overall objective tested in this study was to test if olfactory response of *M. croceipes* to plant VOCs is plastic and dependent on its physiological state.

In chapter I, the effect of nutritional status on response of *M. croceipes* to host-related plant volatiles was tested in electroantennogram (EAG) recording and Y-tube olfactometer bioassays. Responses to single VOCs (cis-3-hexenol, α-pinene), a binary VOC mixture (cis-3-hexenol + α-pinene, and caterpillar-damaged cotton headspace volatiles (natural odor) were recorded in parasitoids fed different diet treatments (i.e. 40%, 20%, 10%, or 0% sucrose/water solution (w/v)). The specific hypothesis for this study was sugar-fed parasitoids would show

higher response to host-related plant volatiles than sugar-starved individuals. EAG response to the binary VOC mixture was significantly higher in 10% and 20% sucrose-fed wasps compared to the sugar-starved control. In addition, EAG response to the caterpillar-damaged cotton headspace volatiles was significantly higher in 40% sucrose-fed wasps compared to the sugar-starved control. In Y-tube bioassays, attraction to the natural odor was significantly higher than solvent control in 40% sucrose-fed wasps.

In chapter II, the effect of parasitoid age and mating status on *M. croceipes* response to VOCs was tested. Parasitoids either mated or unmated and aged 1-3, 4-6, 7-9, or 10-12 were used in EAG recording and responses to VOCs were tested. In Y-tube olfactometer mated and unmated females of age groups 1-3, 4-6, and 7-9 days old were included. There were two specific hypotheses for this study: younger wasps would show higher response to host-related plant volatiles than older wasps, and mated wasps would show higher response to plant volatiles than unmated wasps. EAG responses of wasps at different age groups were not significantly different from one another. With regard to mating status, EAG responses to α-pinene and binary VOC mixture in mated *M. croceipes* aged 7-9 days old were significantly higher than response of unmated wasps. In Y-tube olfactometer bioassays, attraction to binary VOC mixture was significantly higher than solvent control in 4-6 day old mated wasps. In addition, attraction to the natural odor was significantly higher than solvent control in aged 1-3 day old mated wasps. The implications of the current findings are discussed.

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CHAPTER 2

SUGAR FEEDING AFFECTS RESPONSE OF *MICROPLITIS CROCEIPES* TO HOST-RELATED PLANT VOLATILES: IMPLICATIONS FOR BIOLOGICAL CONTROL

2.1. Introduction

Several species of parasitoids rely on sugar resources as their primary sources of nourishment, and maximization of their fitness is dependent on availability of sugar. In the field, energetic demands can be met through utilization of nectar and homopteran honeydew (Jervis et al., 1993; Jervis and Kidd, 1996; Olson et al., 2000; Wackers et al., 2008) and typically contain between 10-50% sucrose (van Handel et al., 1972; Fadamiro et al., 2005;). Parasitoids rely on odor cues to locate food and host resources (Takasu and Lewis, 1993; Wackers 1994; Kugimaya et al., 2010). However, parasitoid olfactory response to such resources is greatly influenced by their physiological state, in particular nutritional status (Browne 1993; Takasu and Lewis 1993; Martel et al., 2009). Consequently, parasitoids may show plasticity in response to odors depending on the level of nutrition and type of diet they have received. Changes in nutritional status of parasitoids can cause a shift in their innate preferences between food odors and host-associated odors (Lewis et al., 1990; Wackers 1994). For instance, poor nutrition may cause parasitoids to switch from host searching behavior to food searching behavior (Takasu and Lewis, 1993; Wackers 1994; Takasu and Lewis, 1995; Lewis et al., 1998; Wackers 2004).

The effect of nutrition on host searching ability of the parasitoid, *Microplitis croceipes* has been previously studied (Wackers 1994; Takasu and Lewis, 1995; Stapel et al., 1997; Lewis et al., 1998; Wackers 2004; Heimpel and Jervis, 2005). Stapel et al., (1997) investigated effect of extrafloral nectar, honeydew, and sucrose feeding on detectability and searching time of *M. croceipes* for its caterpillar host, *Helicoverpa zea*. The authors reported enhanced searching time and parasitism in parasitoids that had access to extrafloral nectar and sucrose. Other studies have investigated attraction of parasitoids to host-related odors versus food odors (Takasu and Lewis, 1993; Wackers 1994; Lewis et al., 1998; Kugimaya et al., 2010). Wackers (1994) reported that sugar-fed parasitoids preferred odors associated with rape seed leaf damage (host-related) while sugar-deprived parasitoids preferred flower odors (food-related). It has been demonstrated that *M. croceipes* modulates their foraging behavior by decreasing sensitivity to host-related odors or increasing sensitivity to food-associated odors (Takasu and Lewis, 1993). Optimal foraging and energetic considerations in female parasitoids often involve foraging tradeoff between food and hosts. The less time and energy parasitoids spend searching for sugar resources, the more time they can allocate towards host searching (Sirot and Bernstein, 1996; Tenhumberg et al., 2006). Lack of sugar can negatively impact searching efficiency and parasitism in parasitoids (Takasu and Lewis, 1993; Wackers 1994; Takasu and Lewis, 1995; Stapel et al., 1997; Wackers 2004) but it is not clear whether this is related to reduced perception and response to host-related odor.

Plants release blends of volatile organic compounds (VOCs) in response to herbivore damage (Loughrin et al., 1994; McCall et al., 1994; De Moraes et al., 1998; Ngumbi et al., 2009; Ngumbi et al., 2012). Plant VOCs can guide parasitoids to their herbivore hosts. VOCs emitted from plants can be functionally categorized into two groups: constitutive compounds and herbivore-induced plant volatiles (HIPVs). Constitutive compounds are released regardless of

herbivore damage and often increase in emission following herbivore infestation. HIPVs are

released from plants in response to herbivore damage (Ngumbi et al., 2009; Magalhaes et al.,

2012; Ngumbi and Fadamiro, 2012; Morawo and Fadamiro, 2014).

In this study, we tested the hypothesis that nutritional status of *M. croceipes* females

affects their olfactory response to plant VOCs. *Microplitis croceipes* (Hymenoptera:Braconidae)

is a relatively specialized larval endoparasitoid of *Heliothis*/*Heliocoverpa* spp, which are

important pests of cotton and several other crops. Previous studies have reported attraction of *M.

croceipes* to VOCs released from herbivore-damaged cotton, including cis-3-hexenol and α-

pinene (McCall et al., 1993; Rose et al., 1998; Wei et al., 2007; Yu et al., 2010; Ngumbi and

Fadamiro, 2012; Morawo and Fadamiro, 2014). Here, the effect of varying sucrose diet on

plasticity of olfactory response in *M. croceipes* to select cotton VOCs was investigated using

electroantennogram (EAG) and Y-tube olfactometer bioassays. Cis-3-Hexenol and α-pinene

were chosen as test VOCs because they have been detected consistently in the headspace of

cotton host plants (Rose and Tumlinson, 2004; Ngumbi et al., 2009; Magalhaes et al., 2012;) and

have elicited olfactory response in *M. croceipes* (Chen and Fadamiro, 2007; Ngumbi et al., 2012;

Morawo and Fadamiro 2014). Cis-3-Hexenol is a host-induced green leaf volatile (GLV) emitted

by cotton. α-Pinene is a constitutive monoterpene that is passively released from cotton plant but

also emitted in higher amounts in caterpillar-damaged cotton plants. In addition, a binary mixture

of α-pinene and cis-3-hexenol, as well as odors from the headspace *H. virescens*-infested cotton

were tested. Previous studies have shown that VOC mixtures may be more attractive than

individual compounds because they are more representative of the natural context in which

parasitoids encounter host-related odors (Mumm and Hilker, 2005; van Wijk et al., 2011;

Morawo and Fadamiro, 2014). We are not aware of previous studies that have focused on

olfactory plasticity of parasitoid response to VOCs in response to varying concentration of sucrose diet. The physiological and ecological implications of the present results are discussed.

2.2.   Materials and Methods

2.2.1.   Insects. *M. croceipes* were reared in our laboratory (Auburn University AL, USA) on 2nd-3rd instar larvae of *H. virescens*. The rearing procedures were similar to those described by Ngumbi et al., (2009) and Morawo and Fadamiro, (2014) and Upon emergence, adult wasps were transferred to aerated plastic cages (30 x 30 x 30 cm) and supplied with 10% sucrose/water solution (w/v). Mated, naive (untrained) parasitoids (aged 1-3 days old) were used in electroantennogram (EAG) and behavioral bioassays. Most behavioral studies have used mated parasitoids because mating is known to increase the probability of host searching behavior in parasitoids (Chen and Fadamiro, 2007). Larvae of *H. virescens* were reared on pinto bean artificial diet (Shorey and Hale, 1965). The general rearing conditions for all insects were 25 ± 1 °C, 75 ± 5 % RH and 14:10 h (L:D) photoperiod.

2.2.2.   Plants. Cotton (*Gossypium hirsutum*, var. max 9) plants were grown in individual pots (9 cm high, 11 cm diameter) in a growth chamber (Auburn University) at 26.6 °C day, 25.6 °C night, 60% RH 16:8 h (L:D) photoperiod. Seeds were planted in a top soil/vermiculate mixture. Plants used for headspace volatile collections were 4–6 weeks old.

2.2.3.   Diet Treatments. Parasitoids used for EAG bioassays were transferred into 9 cm aerated petri dishes upon emergence. Petri dishes were supplied with sugar treatments: 0% (water only), 10%, 20%, or 40% sucrose/water solution (w/v) on two cotton balls placed at the bottom of the petri dish and by smearing the sugar treatment on the underside of the lid. Parasitoids used for Y-tube olfactometer bioassays were transferred to aerated plastic cages (30 x 30 x 30 cm) upon emergence. A larger containment was used for parasitoids in the olfactometer

bioassays in order to avoid crowding of insects, which may affect behavioral responses of parasitoids. Each cage was supplied with different sugar treatments and administered by smearing sucrose solution on the inner top of the cage. In addition, a petri dish containing cotton balls soaked in the sucrose solution treatment was placed in each cage. Female parasitoids used in all experiments were age 1-3 days old and assumed mated after interacting with males for at least 24 h within their designated treatment cages/petri dishes. Parasitoids were allowed to feed for at least 24 h. Feeding was assumed to have occurred because sugar-starved parasitoids did not live beyond 3 days old (personal observation)

      2.2.4. Odor Stimuli. cis-3-Hexenol and α-pinene are two components detected in the headspace of *H. virescens*-infested cotton (Loughrin et al., 1994; DeMoraes et al., 1998; Rose and Tumlinson, 2004; Ngumbi et al., 2009; Magalhaes et al., 2012; Ngumbi et al., 2012), and have been demonstrated to elicit olfactory response in *M. croceipes (*Rose et al., 1998; Chen and Fadamiro, 2007; Ngumbi et al., 2012; Morawo and Fadamiro, 2014). Thus, both compounds were tested in this study. Synthetic compounds (purity 95-99%) were purchased from Sigma® Chemical Co. (St. Louis, Missouri). A binary mixture of both compounds (cis-3-hexenol + α-pinene) was also formulated at 50/50 v/v as the third odor stimulus. Synthetic compounds were formulated in hexane (HPLC-grade) at 1 µg/µl concentration and delivered as 10 µl samples (10 µg dose). In addition, an odor extract collected from the headspace of *H. virescens*-infested cotton was tested as the fourth odor stimulus in EAG and Y-tube bioassays.

      2.2.5. Headspace Volatile Collection. Headspace volatile extract was collected from *H. virescens*-infested cotton plants using the methodology and protocols reported by Ngumbi et al., (2009). To induce release of HIPVs from plants, 30 2nd-3rd instar larvae of *H. virescens* were allowed to feed on a cotton plant for 24 h during volatile collection. Pots with soil were wrapped

with aluminum foil to minimize contamination. The plant was then placed in a volatile collection chamber (Analytical Research Systems, Inc., Gainesville, FL, USA) consisting of a 5-L glass jar. A purified air stream of 500 ml/min was passed through the jar at room temperature for 24 h. Headspace volatiles were collected with a trap containing 50 mg of Super-Q (Alltech Associates, Deerfield, IL, USA) and eluted with 300 µl of methylene chloride.

2.2.6. EAG Recording. The EAG protocol was similar to that previously described by Chen and Fadamiro, (2007) and Ngumbi et al., (2009). A reference electrode consisting of a glass capillary (1.1 mm ID) filled with Ringer solution was connected to the back of the head of a female *M. croceipes*. The recording electrode consisted of another glass capillary connected to the cut tip of the terminal segment of the antenna. Chlorinated silver–silver junctions maintained electrical conduct between the electrode and input of the preamplifier. The analog signal was detected through a probe (INR-II, Syntech1, the Netherlands), and was captured and processed with a data acquisition controller (IDAC-4, Syntech1, the Netherlands) and analyzed using EAG 2000 software (Syntech1, the Netherlands) on a computer. cis-3-Hexenol, α-pinene and a 50:50 binary mixture were diluted in hexane and delivered as 10-µl samples on a filter paper (7 mm 40 mm, Whatman1 No. 1). Headspace volatile extract of *H. virescens*-infested cotton eluted with methylene chloride was also delivered as 10-µl samples on filter paper. A Pasteur pipette (14 cm in length, Fisher Scientific, Pittsburgh, PA, USA) was used as an odor cartridge and a filter paper strip impregnated with the test odor was inserted. Hexane (control for synthetic stimuli) or methylene chloride (control for *H. virescens*-infested cotton extract) were introduced in the same manner. A different control was used for natural odor extract because this odor was eluted in methylene chloride. The tip of the pipette was placed about 3 mm into a small hole in the wall of a glass tube (13 cm long, 8 mm diameter) oriented towards the antennal preparation (0.5 cm

away from the preparation). The stimuli were delivered as 0.2 s puffs of air (2 ml) into continuous humidified air stream at 1000 ml/min generated by an air stimulus controller (CS-55, Syntech1, the Netherlands). A time interval of 2 min was allowed between odor puffs in order to account for antennal recovery. For each antennal preparation, the following stimuli were presented: hexane (control), methylene chloride (control), cis-3-hexenol, α-pinene, binary mixture, headspace volatile extract. Hexane and methylene chloride (controls) were applied at the beginning and end of each recording series while the position of other test stimuli was randomized across replicates. For analysis, EAG response to the solvent control was deducted from the EAG amplitudes elicited by the test stimuli.

      2.2.7. Y-tube Olfactometer. A Y-tube olfactometer (Analytical Research Systems, Inc., Gainesville, FL, USA) was used to test attraction of female *M. croceipes* to the four odor stimuli. The Y-tube setup and protocol was similar to that reported by Ngumbi and Fadamiro, (2012). The inlet air was pushed at a constant rate of 250 ml/min into each arm of the olfactometer. Air was simultaneously removed by suction via a vacuum pump through the central arm of the olfactometer at the rate of 500 ml/min. Parasitoids were introduced individually into the central arm of the Y-tube olfactometer. The choice of a parasitoid was recorded after walking into one of the arms of the Y-tube and remaining in that arm for 15 s. If a parasitoid did not make a choice within 5 min of being inserted into the Y-tube, it was removed and recorded as making no choice. After three individual parasitoids had been tested, the olfactometer arms were flipped around 180º to minimize positional effect. In between diet treatments, the olfactometer was cleaned with soap, rinsed with water, dried and then rinsed with acetone. Each synthetic compound (1 µg/µl cis-3-hexenol, α-pinene, or 50:50 binary mixture) was delivered as a 10 µl sample placed on No. 1 filter paper strips (7 mm x 40 mm, Whatman No. 1). After allowing for

10 seconds of solvent evaporation, the filter paper strip was inserted into a designated arm of the olfactometer. Another filter paper strip containing hexane (control) was inserted into the other arm of the olfactometer.

Attraction of parasitoids to volatiles released from *H. virescens*-infested cotton was tested using a protocol similar to Balusu and Fadamiro, (2011) and Chen et al., (2009). One arm of the olfactometer was connected to an air-tight glass jar (5-L) containing *H. virescens*-infested cotton. The other arm was connected to a similar glass jar containing a pot of soil covered with aluminum foil, which served as control. Cotton plant was infested with 30 2nd-3rd instar larvae of *H. virescens* that were allowed to feed for 24 hr. Caterpillars remained on the cotton plant while conducting bioassay. Humidified and purified (charcoal filtered) air was let into the jars at a constant rate of 300 ml/min. Air was continuously pushed through the jars and into the arms of the olfactometer and simultaneously removed by a vacuum suction through the central arm of the olfactometer at the rate of 600 ml/min. Olfactometer data was recorded from 20 replicates per diet treatment per odor stimulus.

2.2.8. Data Analyses. Absolute EAG data met the key assumptions of parametric tests and were not transformed prior to analysis. EAG responses of parasitoids fed different diet treatments were compared using one-way analysis of variance (ANOVA) for each odor stimulus. Means were separated using Tukey-Kramer HSD multiple comparison test. For olfactometer data, deviation of parasitoid response to stimulus versus control from a 50:50 ratio was analyzed using a Chi square goodness of fit test. All analyses were performed with SAS 9.2 ($P < 0.05$).

2.3 Results

2.3.1. Effect of Diet on EAG Response. Diet had a significant effect on the response of female *M. croceipes* to certain (but not all) test VOCs. While nutrition did not affect EAG

response to individual synthetic compounds cis-3-hexenol (Fig. 1A) and α-pinene (Fig. 1B), parasitoids fed 10% or 20% sucrose solution showed significantly higher EAG response to the binary mixture (cis-3-hexenol + α-pinene) than sugar-starved wasps ($F = 4.58$, $df = 3$, $P = 0.0081$) (Fig. 1C). Similarly, parasitoids fed 40% sucrose solution showed significantly greater EAG response to *H. virescens*-infested cotton volatile extract (natural odor) compared to the sugar-starved control ($F = 3.11$, $df = 3$, $P = 0.0383$) (Fig.1D).

2.3.2. **Effect of Diet on Olfactometer Response.** Diet had a variable effect on the behavioral response of *M. croceipes* to the test odor stimuli. In general, sugar-fed parasitoids appeared to show higher response to the test odor stimuli compared to sugar-starved individuals (Fig. 2), although a significant difference was only recorded in response to the natural odor treatment (*H. virescens*-infested cotton volatiles) in which parasitoids fed 40% sucrose solution were significantly ($\chi 2 = 6.06$, $df = 1$, $P = 0.0138$) more attracted to *H. virescens*-infested cotton than to solvent control (Fig. 2D).

2.4.1. Discussion

The present results indicate that sugar feeding impacts olfactory response in *M. croceipes*. Sugar-fed *M. croceipes* had significantly higher EAG response than sugar-starved wasps to some of the test stimuli, specifically a binary mixture of cis-3-hexenol and α-pinene and a natural odor blend (*H. virescens*-infested cotton headspace volatile extract). In Y-tube olfactometer bioassays, *M. croceipes* fed 40% sucrose solution showed significant attraction to the natural odor blend while sugar-starved wasps could not distinguish between the natural odor blend and solvent control.

Together, our results suggest that parasitoids fed sucrose-rich diet are more likely to respond to host-related odors than sugar-deprived parasitoids. This finding is in agreement with previous

studies showing a strong connection between sugar-feeding and parasitoid foraging for hosts (Takasu and Lewis, 1993; Wackers 1994; Takasu and Lewis, 1995; Lewis et al., 1998; Kugimaya et al., 2010). Takasu and Lewis, (1993) reported that sucrose-fed *M. croceipes* visited and spent more time on leaves containing *Heliothis zea* frass extract than leaves with sucrose and vanilla extract, while starved *M. croceipes* preferred food plants. Parasitoid oviposition was also affected by starvation in studies conducted by Takasu and Lewis, (1993) and Mitsunaga et al., (2004). Takasu and Lewis, (1993) reported that sucrose-fed *M. croceipes* oviposit at a higher rate than unfed *M. croceipes* on its caterpillar host *Heliothis zea*. Similarly, *Cotesia plutellae* oviposition on *Plutella xylostella* was suppressed by lack of sugar availability (Mitsunaga et al., 2004). These studies suggest a switch from host searching to food searching behavior in response to food deprivation.

Little is known about the hormonal and neuronal mechanisms modulating the observed effect of nutrition on parasitoid olfactory response. Nutritional status and appetite affects food-searching behavior in animals via the activity of neuropeptides (Root et al., 2011). In *Drosophila*, hunger increases the expression of a neuropeptide receptor due to a reduction in insulin signaling. The outcome triggers food searching behaviors. Insulin acts as an indicator of hunger in *Drosophila* and mediates olfactory sensory neuron (OSN) activity through variable signaling of neuropeptides (Root et al., 2011). Insulin can also work in concert with neuropeptides to produce variable behavioral responses in insects, depending on their physiological state (Wu et al., 2005; Root et al., 2011). The effect of nutritional state on chemoreceptor activity has also been documented at the peripheral level in the mosquito *Aedes aegypti* (Davis 1984).

Various optimal foraging models can lend support to the tradeoff between food foraging and host searching in parasitoids (Charnov 1976; Stephens and Krebs, 1986; Sirot and Bernstein, 1996; Tenhumberg et al., 2006). For example, the TSK model predicts that parasitoids will choose to search for either food or hosts, but not both concurrently (Tenhumberg et al., 2006). Host-searching increases the chance of locating hosts for oviposition, however, energy reserves are constantly depleted during the process. Food-foraging delays the initial opportunity to oviposit, but maximizes the time and energy that a parasitoid may put forth to host-searching. This theory may explain our findings in which sugar-starved *M. croceipes* showed lower response to host-related volatiles compared with sugar-fed individuals.

A second major finding from the current study is the relatively lower parasitoid response to the test single VOC components (cis-3-hexenol and α-pinene) compared to the binary mixture and the more complex natural odor blend (*H. virescens*-infested cotton headspace volatile extract). In nature, foraging parasitoids typically encounter odor cues as blends rather than as single compounds (Hoballah and Turlings, 2005; Schnee et al., 2006; van Dam et al., 2010; Morawo and Fadamiro, 2014). Thus, this may explain the greater EAG and behavioral responses to the binary mixture and natural odor blend. Similar results have been reported in the literature. (Riffell et al., 2009; van Wijk et al., 2011). For instance, single VOC components of *Tetranychus urticae*-infested lima bean were not attractive to the mite *Phytoseiulus persimilis*, whereas a 5-component blend elicited high attraction (van Wijk et al., 2011). In another study, food-associated odorant mixtures elicited a stronger behavioral response than single odorants in the hawk moth, *Manduca sexta* in flight tunnel bioassays (Riffell et al., 2009; van Wijk et al., 2011).

In general, our EAG data appear to support the results from the behavioral bioassays, although not always significant. This is noteworthy given that EAG activity does not always

translate to a behavioral response in insects (Blaney et al., 1986; Park et al., 2001; Ngumbi et al., 2010). While EAG is a useful tool for measuring antennal sensitivity and summed activity of olfactory receptor neurons, behavioral bioassays are necessary to determine the functional and ecological relevance of odor stimuli (Birch 1971; Ngumbi et al., 2010).

In summary, our findings suggest a positive effect of sugar feeding on parasitoid olfaction may have implications for their use as biological control agents. First, olfactory response of parasitoids to host-related plant volatiles can be manipulated to optimize their release in the field (Vet and Dicke 1992). Furthermore, availability of supplemental sugar sources in the field either in the form of habitat manipulation (i.e. farmscaping) or artificial sugar provisioning may increase parasitoid longevity and fecundity, as well as enhance their response to host-related volatiles and consequently their foraging behavior. However, sugar provisioning may not always lead to increased parasitism because specialist parasitoids only parasitize a select number of hosts and thus sugar-feeding may have limited effect. Future studies are needed to decipher the neuronal and hormonal mechanisms by which sugar feeding can enhance olfactory response in parasitoids.

2.5.   Acknowledgements

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Figure Legend

Figure 1.1 Effect of diet on the electroantennogram (EAG) response of *Microplitis croceipes* to various host-related odor treatments. Absolute EAG responses (mV± SE, *n* = 10) of *M. croceipes* females fed 40%, 20%, 10% or 0% sucrose solution (w/v) to cis-3-hexenol (A), α-pinene (B), a 50:50 binary mixture of cis-3-hexenol and α-pinene (C), and a complex natural odor blend (*Heliothis virescens*-infested cotton headspace volatile extract) (D). All stimuli were presented at 10 µg dose. Means for each odor stimulus having no letter in common are significantly different (ANOVA, Tukey HSD test, $P < 0.05$).

Figure 1.2 Effect of diet on the behavioral response of *Microplitis croceipes* to various host-related odor treatments. Response of *M. croceipes* females fed 40%, 20%, 10% or 0% sucrose solution (w/v) in a Y-tube olfactometer when given a choice between hexane (solvent control) and cis-3-hexenol (A), α-pinene (B), a 50:50 binary mixture of cis-3-hexenol and α-pinene (C), and a choice between control jar and *Heliothis virescens*-infested cotton (D). All stimuli were presented at 10 µg dose (*n* = 20 parasitoids per choice test). Asterisk (*) indicates significant differences between stimulus and control (χ^2 test, $P < 0.05$).

Figure 1.1
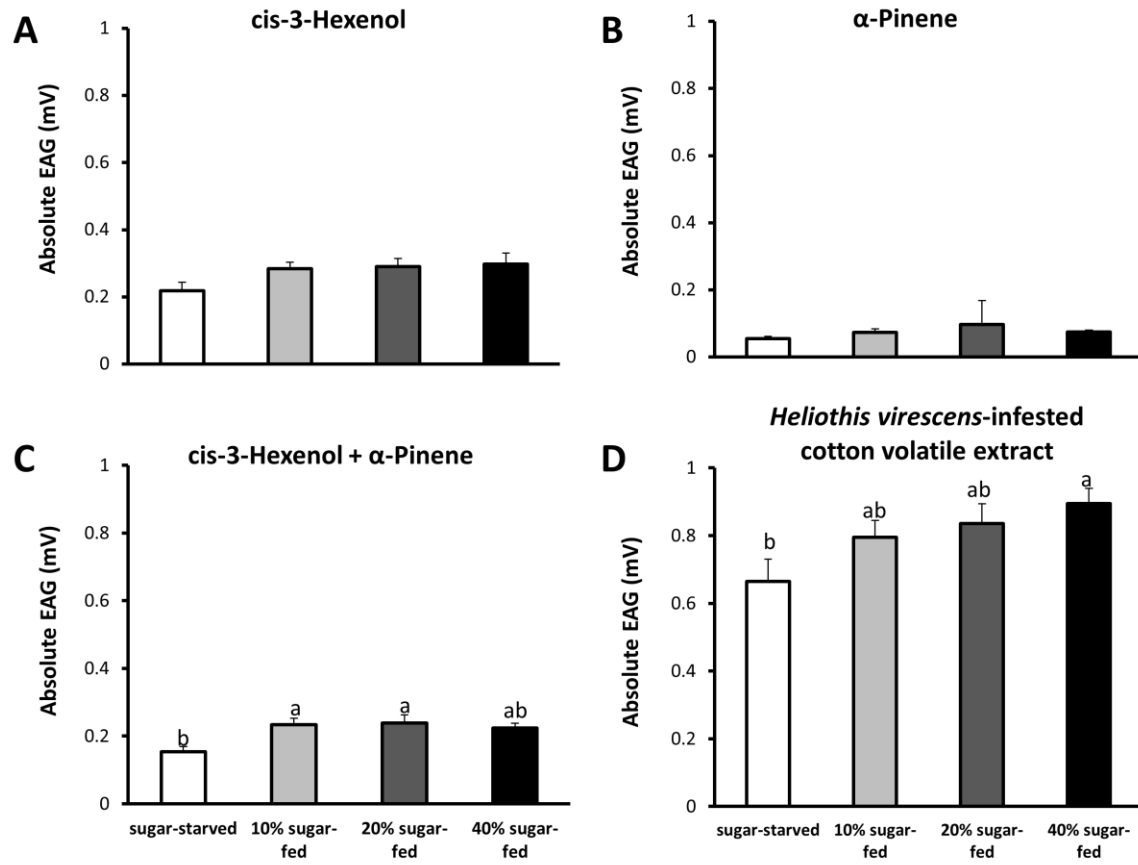
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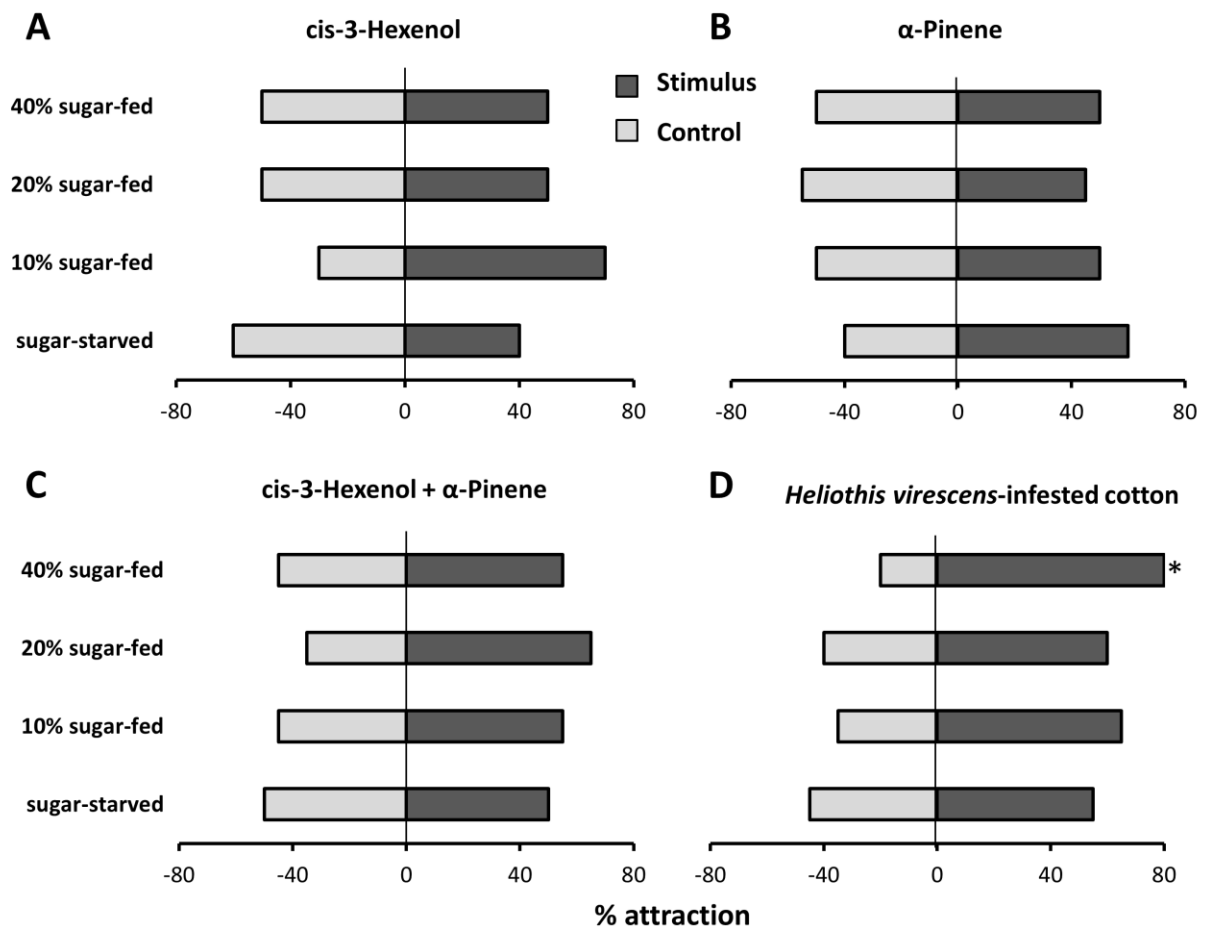
CHAPTER 3

EFFECT OF AGE AND MATING OLFACTORY RESPONSE TO HOST-RELATED PLANT VOLATILES IN *MICROPLITIS CROCEIPES*

3.1.   Introduction

Various natural enemies and parasitoids rely on chemical stimuli in order to maximize foraging and host searching abilities. Chemical stimuli often serve as important indicators useful for insects that rely heavily on their sense of olfaction, such as those that interact with plants. Parasitoids use volatile organic compounds (VOCs) released from plants as indirect cues for locating hosts for oviposition (De Moraes et al., 1998; Pare and Tumlinson, 1999; Mumm and Hilker, 2005; Wei and Kang, 2006). VOCs can be released from herbivore-damaged, mechanically-damaged, or undamaged plants. Plant VOC blends may also differ relative to the attacking herbivore (De Moraes et al., 1998; Hoballah et al., 2002; Ngumbi et al 2009), plant species, mode and duration of damage (Morawo and Fadamiro, 2014), and plant VOC profile composition (Hilker and Meiners, 2002, Dicke et al 2009). Only certain components of natural volatile blends are considered attractive or ecologically important to parasitic wasps (Schnee et al., 2006; van Dam et al., 2010; Morawo and Fadamiro, 2014). Furthermore, parasitoids do not rely on perception of a single attractant in odor mixtures when searching for hosts. However, individual VOC components that are not considered attractive to parasitoids may still affect

attraction to other VOCs present in a blend (Schnee et al., 2006; van Dam et al., 2010; Morawo and Fadmairo, 2014).

Olfactory response to VOCs may not remain consistent throughout the duration of the adult parasitoid life. Changes in physiological state are known to affect response to VOCs in parasitoids and other insects (Brown 1993; Takasu and Lewis, 1993; Rivero and Casas, 1999; Martel et al., 2009). Olfactory plasticity has been reported frequently in insects, and caused by changes in age, mating status, and nutritional status to name a few (Roelofs and Comeau 1971; Seabrook et al., 1979; Crnjar et al., 1990; Takasu and Lewis, 1993; Ding et al., 1997; Stapel et al., 1997; Ray and Ferneyhough, 1997; Gemeno and Hayes, 2000; Fauvergue et al., 2008). The focus of this study will examine age and mating status specifically.

Aging has been consistently shown to affect olfaction in insects. The effect of aging on olfactory mechanisms in insects is often termed as senescence. Behavioral senescence of tasks and responses involved with locomotion, olfaction, and learning have been consistently documented in fruit flies (Cook-Wiens and Grotewiel, 2002; Wang et al., 2003; Grotewiel et al., 2005). In addition, senescence of odorant responsiveness has been recorded and attributed to changes in response of olfactory receptor neurons (Ayer and Carlson, 1992; Grotewiel et al., 2005). Senescence of response to odors in insects has also been demonstrated as a function of neuromodulator and neurotransmitter activity in both the peripheral and central nervous systems in honeybees (Farooqui 2007). Age-related plasticity of response to VOCs has been documented in previous studies using parasitoids (Steinberg et al., 1992; Herard et al., 1998) while other studies have not demonstrated a significant age effect (Drost et al., 1985; Steinberg 1992; Park et al., 2001). Parasitoid parasitization rate of hosts decreased with age in previous studies (Bellows 1985; Rajapakse et al., 1992; Amalin et al., 2005), which can be attributed to senescence of

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response to odors and/or decreased mobility as a result of age. These findings can be explained by the increased need and impulse to find resources as the likelihood of mortality increases with age due to limited longevity (Martel et al., 2009).

Some parasitoids are haplodiploid organisms in which virgin females produce unfertilized eggs and are haploid while mated females produce fertilized eggs and are diploid. Unmated females produce male offspring while mated females are able to adapt the sex ratio of offspring according to environmental conditions and thus produce females (Godfray 1990; Fauvergue et al., 2008). Consequently, parasitoids may choose to search for hosts, or instead search for mates and thus encounter tradeoffs due to optimal allocation of time and energy between the two foraging behaviors (Godfray 1990; Godfray and Hardy, 1993; Fauvergue et al., 2008). In order to further describe such tradeoffs, optimal foraging models have been developed which attempt to explain how parasitoids balance host and reproductive needs (Godfray 1990; Guertin et al., 1996; Kugimaya et al., 2010).

Studies involving parasitoids have demonstrated an effect of mating status on parasitization rate of hosts with mated females showing a higher rate than unmated females (Antolin 1989; Michaud 1994; Michaud and Mackauer, 1995; Tagawa et al., 1987; Fauvergue et al., 2008). Equal rates of parasitism were reported between unmated and mated females in other studies (Ramadan et al., 1995; Guertin et al., 1996; Fauvergue et al., 1998; King 2002). However, plasticity of response of parasitoids to host-related plant volatiles relative to mating status is an area that has gained little attention despite its implications and relevance to host searching and parasitization potential. A previous study using *M. croceipes* (Chen and Fadamiro 2007) investigated the effect of mating status on electroantennogram (EAG) responses to host-related plant volatiles and found no significant effect. The authors concluded that differences in

behavioral response of parasitoids of different mating status to VOCs could be due to variation in higher-order processing of olfactory cues (VOCs) between mated and unmated females.

This study tests the hypothesis that age and mating status of *M. croceipes* females affects their response to host-related plant volatiles. *Microplitis croceipes* (Hymenoptera:Braconidae) is a larval endoparasitoid of *Heliothis/Helicoverpa* spp., which are pests of cotton and other crops of economic importance. Previous studies have reported attraction of *M. croceipes* to select VOC components released from herbivore-damaged cotton, including cis-3-hexenol and α-pinene (McCall et al., 1993; Rose et al., 1998; Wei et al., 2007; Yu et al., 2010; Ngumbi and Fadamiro, 2012; Morawo and Fadamiro, 2014). Olfactory response of *M. croceipes* to select cotton VOCs was investigated using two different methods. Electroantennogram (EAG) was used to record antennal response of *M. croceipes* to test VOCs and Y-tube olfactometer bioassays for observing behavioral response of parasitoids to the chosen stimuli. cis-3-Hexenol and α-pinene were chosen as test VOCs because previous studies have reported detection of both compounds in the headspace of *H. virescens*-infested cotton (Rose and Tumlinson, 2004; Ngumbi et al., 2009; Magalhaes et al., 2012.). cis-3-Hexenol and α-pinene have also been reported to elicit olfactory response in *M. croceipes* (Chen and Fadamiro, 2007; Ngumbi et al., 2012; Morawo and Fadamiro 2014). cis-3-Hexenol is characterized as a green leaf volatile (GLV), released following herbivore damage in cotton while α-Pinene is a constitutive monoterpene passively released from both undamaged and damaged cotton plants. In addition, a binary mixture of α-pinene and cis-3-hexenol, and a VOC blend collected from the headspace of *H. virescens*-infested cotton were tested. Currently there are few studies investigating the effects of age and mating status on EAG and behavioral responses of parasitoids to host-related plant volatiles. The implications of these findings are discussed in a physiological and ecological context.

3.2. Materials and Methods

3.2.1 Insects. *M. croceipes* were reared in our laboratory (Auburn University AL, USA) on 2nd-3rd instar larvae of *H. virescens*. The rearing procedures were similar to those described by Ngumbi et al., (2009) and Morawo and Fadamiro, (2014). Upon emergence, adult wasps were transferred to aerated plastic cages (30 x 30 x 30 cm) and supplied with 10% sucrose/water solution (w/v). Mated and unmated, naive (untrained) parasitoids of age groups 1-3, 4-6, 7-9, and 10-12 days old were used in electroantennogram (EAG) recording while the 10-12 day old age group was not included in behavioral bioassays. Larvae of *H. virescens* were reared on pinto bean artificial diet (Shorey and Hale, 1965). The general rearing conditions for all insects were 25 ± 1 °C, 75 ± 5 % RH and 14:10 h (L:D) photoperiod.

3.2.2 Plants. Cotton (*Gossypium hirsutum*, var. max 9) plants were grown in individual pots (9 cm high, 11 cm diameter) in a growth chamber (Auburn University) at 26.6 °C day, 25.6 °C night, 60% RH 16:8 h (L:D) photoperiod. Seeds were planted in a top soil/vermiculate mixture. Plants used for headspace volatile collections were 4–6 weeks old.

3.2.3 Age and Mating Status Treatments. Parasitoid cocoons were isolated into plastic cups (Solo®, 37.0 ml) until emergence. Upon emergence, parasitoids were separated into either mated or unmated cages. Mated cages were smaller (19 x 13 x 10 cm) contained males and females at a 2:1 male:female ratio while unmated cages contained females only. Females were allowed to mate for at least 24 h before use in bioassays and thus were assumed mated. Both mated and unmated females were further separated into cages designated by age group. Assigned age groups of 1-3, 4-6, 7-9, and 10-12 days-old were included in electroantennogram (EAG) recording. Age groups of 1-3, 4-6, and 7-9 days-old were included in behavioral bioassays. Wasps aged 10-12 days old were excluded from behavioral bioassays due to significant mortality

beginning with parasitoids at that age. Adult *M. croceipes* emergence from cocoons peaks at days 2 and 3 of emergence (Lewis and Burton 1970), therefore, cocoons were placed in a refrigerator (4ºC) in order to delay adult emergence from cocoons to occur across all age groups.

3.2.4   Odor Stimuli. Cis-3-Hexenol and α-pinene are two components detected in the headspace of *H. virescens*-infested cotton (DeMoraes et al., 1998; Loughrin et al., 1994; Magalhaes et al., 2012; Ngumbi et al., 2009; Ngumbi et al., 2012; Rose and Tumlinson, 2004), and have been demonstrated to elicit olfactory response in *M. croceipes (*Rose et al., 1998; Chen and Fadamiro, 2007; Morawo and Fadamiro, 2014; Ngumbi et al., 2012). Thus, both compounds were tested in this study. Synthetic compounds (purity 95-99%) were purchased from Sigma® Chemical Co. (St. Louis, Missouri). A binary mixture of both compounds (cis-3-hexenol + α-pinene) was also formulated at 50/50 v/v as the third odor stimulus. Synthetic compounds were formulated in hexane (HPLC-grade) at 1 µg/µl concentration and delivered as 10 µl samples (10 µg dose). In addition, an odor extract collected from the headspace of *H. virescens*-infested cotton was tested as the fourth odor stimulus in EAG and Y-tube bioassays.

3.2.5   Headspace Volatile Collection. Headspace volatile extract was collected from *H. virescens*-infested cotton plants using the methodology and protocols reported by Ngumbi et al., (2009). To induce release of HIPVs from plants, 30 2nd-3rd instar larvae of *H. virescens* were allowed to feed on a cotton plant for 24 h during volatile collection. Pots with soil were wrapped with aluminum foil to minimize contamination. The plant was then placed in a volatile collection chamber (Analytical Research Systems, Inc., Gainesville, FL, USA) consisting of a 5-L glass jar. A purified air stream of 500 ml/min was passed through the jar at room temperature for 24 h. Headspace volatiles were collected with a trap containing 50 mg of Super-Q (Alltech Associates, Deerfield, IL, USA) and eluted with 300 µl of methylene chloride.

3.2.6 EAG Recording. The EAG protocol was similar to that previously described by Chen and Fadamiro, (2007) and Ngumbi et al., (2009). A reference electrode consisting of a glass capillary (1.1 mm ID) filled with Ringer solution was connected to the back of the head of a female *M. croceipes*. The recording electrode consisted of another glass capillary connected to the cut tip of the terminal segment of the antenna. Chlorinated silver–silver junctions maintained electrical conduct between the electrode and input of the preamplifier. The analog signal was detected through a probe (INR-II, Syntech1, the Netherlands), and was captured and processed with a data acquisition controller (IDAC-4, Syntech1, the Netherlands) and analyzed using EAG 2000 software (Syntech1, the Netherlands) on a computer. cis-3-Hexenol, α-pinene and a 50:50 binary mixture were diluted in hexane and delivered as 10-µl samples on a filter paper (7 mm 40 mm, Whatman1 No. 1). Headspace volatile extract of *H. virescens*-infested cotton eluted with methylene chloride was also delivered as 10-µl samples on filter paper. A Pasteur pipette (14 cm in length, Fisher Scientific, Pittsburgh, PA, USA) was used as an odor cartridge and a filter paper strip impregnated with the test odor was inserted. Hexane (control for synthetic stimuli) or methylene chloride (control for *H. virescens*-infested cotton extract) were introduced in the same manner. The tip of the pipette was placed about 3 mm into a small hole in the wall of a glass tube (13 cm long, 8 mm diameter) oriented towards the antennal preparation (0.5 cm away from the preparation). The stimuli were delivered as 0.2 s puffs of air (2 ml) into continuous humidified air stream at 1000 ml/min generated by an air stimulus controller (CS-55, Syntech1, the Netherlands). A time interval of 2 min was allowed between odor puffs in order to account for antennal recovery. For each antennal preparation, the following stimuli were presented: hexane (control), methylene chloride (control), cis-3-hexenol, α-pinene, binary mixture, headspace volatile extract. Hexane and methylene chloride (controls) were applied at the beginning and end

of each recording series while the position of other test stimuli was randomized across replicates. For analysis, EAG response to the solvent control was deducted from the EAG amplitudes elicited by the test stimuli.

3.2.7   Y-tube Olfactometer. A Y-tube olfactometer (Analytical Research Systems, Inc., Gainesville, FL, USA) was used to test attraction of female *M. croceipes* to the four odor stimuli. The Y-tube setup and protocol was similar to that reported by Ngumbi and Fadamiro, (2012). The inlet air was pushed at a constant rate of 250 ml/min into each arm of the olfactometer. Air was simultaneously removed by suction via a vacuum pump through the central arm of the olfactometer at the rate of 500 ml/min. Parasitoids were introduced individually into the central arm of the Y-tube olfactometer. The choice of a parasitoid was recorded after walking into one of the arms of the Y-tube and remaining in that arm for 15 s. If a parasitoid did not make a choice within 5 min of being inserted into the Y-tube, it was removed and recorded as making no choice. After three individual parasitoids had been tested, the olfactometer arms were flipped around 180º to minimize positional effect. In between age treatments, the olfactometer was cleaned with soap, rinsed with water, dried and then rinsed with acetone. Each synthetic compound (1 µg/µl cis-3-hexenol, α-pinene, or 50:50 binary mixture) was delivered as a 10 µl sample placed on No. 1 filter paper strips (7 mm x 40 mm, Whatman No. 1). After allowing for 10 seconds of solvent evaporation, the filter paper strip was inserted into a designated arm of the olfactometer. Another filter paper strip containing hexane (control) was inserted into the other arm of the olfactometer.

Attraction of parasitoids to volatiles released from *H. virescens*-infested cotton was tested using a protocol similar to Balusu and Fadamiro, (2011) and Chen et al., (2009). One arm of the olfactometer was connected to an air-tight glass jar (5-L) containing *H. virescens*-infested

cotton. The other arm was connected to a similar glass jar containing a pot of soil covered with aluminum foil, which served as control. Cotton plant was infested with 30 2nd-3rd instar larvae of *H. virescens* that were allowed to feed for 24 hr. Caterpillars remained on the cotton plant while conducting bioassay. Humidified and purified (charcoal filtered) air was let into the jars at a constant rate of 300 ml/min. Air was continuously pushed through the jars and into the arms of the olfactometer and simultaneously removed by a vacuum suction through the central arm of the olfactometer at the rate of 600 ml/min. Olfactometer data was recorded from 20 replicates per diet treatment per odor stimulus.

3.2.8 Data Analyses. Absolute EAG data for age met the key assumptions of parametric tests and were not transformed prior to analysis. EAG responses of parasitoids of different age groups were compared using one-way analysis of variance (ANOVA) for each odor stimulus. Means were separated using Tukey-Kramer HSD multiple comparison test. EAG responses of parasitoids in different mating status were compared using a Wilcoxon rank sums test. For olfactometer data, deviation of parasitoid response to stimulus versus control from a 50:50 ratio was analyzed using a Chi square goodness of fit test. All analyses were performed with SAS 9.2 ($P < 0.05$).

3.3 Results:

3.3.1 Effect of Age on EAG Response. The age of *M. croceipes* did not have a significant effect on EAG response. However, *M. croceipes* did show a steady decrease in EAG response to *H. virescens*-infested cotton extract after age 4-6 days old but the trend was not statistically significant (Fig. 1D)

3.3.2 Effect of Mating on EAG Response. EAG response of *M. croceipes* to VOCs was relatively unaffected by mating status. Mating status only had a significant effect on EAG

response to α-pinene and binary VOC mixture at age 7-9 days old (Figs. 3C, 4C). In both occurrences, EAG response of mated *M. croceipes* was significantly (Z=2.83, df=1, P=0.0046), (Z=2.1544, df=1, P=0.0443) higher than that of unmated females. EAG responses to *H. virescens*-infested cotton volatile extract were similar between unmated and mated females except at age 4-6 days old where response was higher in mated wasps however the difference was not statistically significant (Fig. 5B).

        3.3.3   Effect of Age and Mating on Olfactometer Response. Age and mating status had a combined effect on behavioral response of *M. croceipes.* Aged 4-6 day old mated *M. croceipes* were significantly (χ^2 = 7.20, df = 1, P =0.0073) more attracted to the binary VOC mixture compared to hexane control (Fig. 6C). In addition, aged 1-3 day old mated *M. croceipes* were significantly (χ^2 = 5.00, df = 1, P =0.0253) more attracted to *H. virescens*-infested cotton VOCs compared to control jar (Fig. 6D). Response of *M. croceipes* to *H. virescens*-infested cotton VOCs (Fig. 6D) followed a similar trend in EAG recording (Fig. 1D), both of which showed higher response in younger parasitoids.

3.4   Discussion:

        Age did not have a significant effect on *M. croceipes* EAG response to host-related plant volatiles. However, behavioral response to host-related volatiles was significantly affected. *M. croceipes* attraction to *H. virescens*-infested cotton in Y-tube olfactometer was significantly higher than the solvent control at age 1-3 days old. These findings are confirmed in EAG where *M. croceipes* EAG response was highest (not significant) at relatively younger ages. In addition, *M. croceipes* attraction to the binary mixture was significantly higher than the control at age 4-6 days old. Overall, mating status did not have a significant effect on *M. croceipes* EAG response to VOCs. At age 7-9 days old, mated *M. croceipes* responded significantly higher than unmated

wasps to α-pinene and binary VOC mixture (cis-3-hexenol + α-pinene). No other significant findings were recorded in EAG. In Y-tube olfactometer, mated wasps aged 4-6 days old were significantly more attracted to the binary VOC mixture compared to solvent control. In addition, mated wasps aged 1-3 days old showed significantly higher attraction to *H. virescens*-infested cotton than to control jar.

Previous studies have documented age-related plasticity of parasitoid EAG or behavioral responses to odor (Steinberg et al., 1992; Herard et al., 1988) while others have not documented a significant effect of age (Park et al., 2001; Drost et al., 1986). Other studies have focused on age-related parasitization rate of parasitoids on their caterpillar hosts (Bellows 1985; Rajapakse et al., 1992; Amalin et al., 2005; Ayvaz et al., 2008). Relatively younger parasitoids tend to parasitize at a significantly higher rate than older parasitoids. Higher parasitization potential at a certain age is likely due to higher fecundity within a particular age group (Ayvaz et al., 2008). Although factors leading to successful parasitism include both long range (plant odors) and short range cues (host body and frass odors), response to volatile compounds such as those included in the current study are known to be of importance in host searching (McCall et al., 1993; Rose et al., 1998; Wei et al., 2007; Yu et al., 2010; Ngumbi and Fadamiro, 2012; Morawo and Fadamiro, 2014).

Age-related plasticity of insect olfactory response at the peripheral level has been attributed to a number of different physiological and neuronal mechanisms. Odor reception in insects is controlled by stimulation of olfactory receptor neurons (ORNs) located across the insect antenna. Stimulation of ORNs often depends on the physiological state of the insect (Kumar et al., 1998; Kovacs 2003; Grotewiel et al., 2005). Senescence of ORN response is likely due to a combination of different physiological and neuronal factors (Kumar et al., 1998).

Previous studies on Drosophila have reported ORN dendrite deterioration with increasing age due to elevated calcium levels of neurons (Kovacs 2003; Grotewiel et al., 2005). However, hormonal and environmental factors may also contribute to desensitization of ORNs (Kumar et al., 1998; Shulz and Robinson, 1999; Wagener-Hulme et al., 1999; Farooqui 2003, 2012). A previous study on the moth *Agrotis ipsilon* showed the neuromodulator octopamine is involved in the responsiveness of male moths to sex pheromone. Octopamine is present in relatively high concentrations in neuronal tissues of many invertebrates and is involved in desensitization of sensory inputs and modulation of initiation/impulse behaviors (Farooqui 2012).

Neurotransmitters and neuromodulators also have the ability to modify sensory inputs important in control of muscles involved with locomotion and peripheral and sense organs (Farooqui 2003, 2012). Octopamine and other biogenic amines have been indicated to be involved in learning and memory in honeybees (Schulz and Robinson, 1999; Farooqui 2003). Octopamine also affects response thresholds for stimuli associated with foraging (Mercer and Menzel, 1982; Bicker and Menzel, 1989; Hammer et al., 1993; Shulz and Robinson, 1999). In addition, Mercer and Menzel, (1982) reported enhancement of responses to olfactory stimuli with brain treatment of octopamine. Previous studies have reported that honeybee age affects brain levels of neurotransmitters dopamine, serotonin, and octopamine (Harris and Woodring, 1992; Taylor et al., 1992; Wagener-Hulme et al., 1999; Shulz and Robinson, 1999), showing that older bees had higher brain levels of the three hormones than younger bees. Other studies have reported no significant change in octopamine concentration relative to aging (Woodring et al., 1988; Seid and Traniello, 2005 Jarriault et al., 2009). Seid and Traniello, (2005) reported concentrations of octopamine did not change with age but ratios of octopamine:serotonin and octapamine:dopamine were significantly higher in younger *Pheidole dentate* worker ants. A

combination of these findings suggests that octopamine and other neuromodulators have modulatory effects on insect sensory systems including olfaction.

Other studies point to juvenile hormone (JH) as a factor influencing glomerular activity in the antennal lobe of male moths in response to sex pheromone (Jarriault et al., 2009; Martel et al., 2009). The moth peripheral olfactory system may not be fully mature around the time of adult emergence and a few days may be necessary for full effectiveness of odor reception within the peripheral nervous system (Martel et al., 2009). Parasitoids may incur similar hormonal effects on their perception of odors with regard to adult development. In the current study, it is unclear whether 1 day old *M. croceipes* differ in their response to VOCs than 3 day old *M. croceipes*. It is also unknown to what extent JH titers differ between ages and age groups in parasitoids. Although the role of hormones in parasitoids is relatively unknown, some hormones may be implicated in behaviors such as host searching and may modulate responses to olfactory stimuli.

Previous studies have reported mixed findings regarding plasticity of response of insects to chemical stimuli relative to their mating status. Xiao et al., (2002) reported no significant effect of mating status on EAG responses of *Helicoverpa armigera* to plant volatiles. Other studies have reported EAG responses unaffected by mating status (Gadenne et al., 2001, De Cristofaro et al., 2004, Yang et al., 2011). Most studies investigating olfactory response to host-related plant volatiles have used mated female parasitoids as test subjects because of the assumption that mated females are more likely to engage in host searching behavior than unmated females (Chen and Fadamiro, 2007).

Optimal foraging models such as that depicted in Godfray (1990) document the costs and benefits of constrained oviposition (females remaining unmated). Unmated females obtain an

instant fitness benefit due to the quick production of offspring. However, the progeny are males

of lesser quality than females. As a result, the likelihood of finding a mate is reduced because

time and energy have now been dedicated to finding hosts, and thus the waste of eggs and energy

sources on low quality offspring (males). Parasitoids that choose instead to search for a mate,

increase their chances of producing higher quality offspring (females), but are also suffering the

costs of time and energy put forth toward mate searching that instead could be used toward host

searching (Godfray 1990; Fauvergue 2008). Therefore, the tradeoff lies in the decision to either

search for hosts immediately in order to produce offspring, or to use energy and resources to find

a mate in order to produce offspring of higher quality (females). There are few studies on the

optimal balance between host and mate searching (Kugimaya et al., 2010; Guertin et al., 1996).

According to Kugimaya et al., (2010) there are benefits of unmated female oviposition. For

example, immediate oviposition can limit the risks involved with mate searching such as

starvation or mortality risk of predation or other causes. Findings in the current study suggest

that EAG response of *M. croceipes* remains unaffected by mating status. This is not entirely

unforeseen because for virgin and mated females to show similar antennal sensitivity to VOCs

would keep responses constant in order to successfully search for hosts regardless of their mating

status (Chen and Fadamiro, 2007).

In the current study, age-related EAG and behavioral responses to the natural stimulus

followed a similar trend. Parasitoids respond to odors of increasing complexity due to similarities

between odor mixtures and odors encountered in natural settings (Hoballah and Turlings, 2005;

Schnee et al., 2006; van Dam et al., 2010; van Wijk et al., 2011; Morawo and Fadamiro, 2014).

In addition, although age-related EAG responses were not statistically significant, we can

possibly deduce that senescence of ORNs may still occur but to a more limited extent than

expected. However, previous studies have documented that EAG may not always translate to behavioral response and vice versa (Blaney et al., 1986; Park et al., 2001; Ngumbi et al., 2010) There are other factors at a higher level order of the insect central nervous system that initiate orientation, walking, or flying which are necessary in initiating behaviors associated with attraction (Chen and Fadamiro, 2007).

In summary, these findings showing a marginal effect of age and mating status on behavioral response of parasitoids to plant odors may have an impact on their utilization as biological control agents. The results of this study may suggest that younger, mated parasitoids typically show the highest response to host-related plant volatiles. These findings can be coupled with others that have demonstrated higher parasitism potential in younger aged, mated wasps. Olfactory response of parasitoids to plant VOCs can be adjusted in this way in order to optimize their release in the field as biological control agents (Vet and Dicke, 1992). Combining these findings with others that have demonstrated physiological state effects in parasitoids can provide insight into further optimization of parasitoids in pest management systems.

3.5   Acknowledgements

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Figure legend

Figure 2.1 Effect of age on the electroantennogram (EAG) response of mated *Microplitis croceipes* to various host-related odor treatments. Figure shows absolute EAG responses (mV± SE, $n = 10$) of *M. croceipes* females age 1-3, 4-6, 7-9, or 10-12 days old to cis-3-hexenol (A), α-pinene (B), a 50:50 binary mixture of cis-3-hexenol and α-pinene (C), and a complex natural odor blend (*Heliothis virescens*-infested cotton headspace volatile extract) (D). All stimuli were presented at 10 µg dose. Means for each odor stimulus having no letter in common are significantly different (ANOVA, Tukey HSD test, $P < 0.05$).

Figure 2.2 Effect of mating status on the electroantennogram (EAG) responses of *Microplitis croceipes* to single VOC cis-3-hexenol at 10 µg dose. Figure shows absolute EAG responses (mV± SE, $n = 10$) of *M. croceipes* unmated and mated females of different age groups 1-3 (A), 4-6 (B), 7-9 (C), and 10-12 days old (D). Wilcoxon rank sums test ($P < 0.05$).

Figure 2.3 Effect of mating status on the electroantennogram (EAG) responses of *Microplitis croceipes* to single VOC α-pinene at 10 µg dose. Figure shows absolute EAG responses (mV± SE, $n = 10$) of *M. croceipes* unmated and mated females of different age groups 1-3 (A), 4-6 (B), 7-9 (C), and 10-12 days old (D). Wilcoxon rank sums test ($P < 0.05$).

Figure 2.4 Effect of mating status on the electroantennogram (EAG) responses of *Microplitis croceipes* to 50:50 binary VOC mixture (cis-3-Hexenol + α-pinene) at 10 µg dose Figure shows absolute EAG responses (mV± SE, $n = 10$) of *M. croceipes* unmated and mated females of different age groups 1-3 (A), 4-6 (B), 7-9 (C), and 10-12 days old (D). Wilcoxon rank sums test ($P < 0.05$).

Figure 2.5 Effect of mating status on the electroantennogram (EAG) responses of *Microplitis croceipes* to *Heliothis virescens*-infested cotton volatile extract 10 µg dose. Figure shows

absolute EAG responses (mV± SE, $n = 10$) of *M. croceipes* unmated and mated females of different age groups 1-3 (A), 4-6 (B), 7-9 (C), and 10-12 days old (D). Wilcoxon rank sums test ($P < 0.05$).

Figure 2.6 Effect of age and mating status on the behavioral response of *Microplitis croceipes* to various host-related odor treatments. Figure shows response of mated versus unmated *M. croceipes* females of ages 1-3, 4-6, and 7-9 days old in a Y-tube olfactometer when given a choice between hexane (solvent control) and cis-3-hexenol (A), α-pinene (B), a 50:50 binary mixture of cis-3-hexenol and α-pinene (C), and a choice between control jar and *Heliothis virescens*-infested cotton (D). All stimuli were presented at 10 µg dose ($n = 20$ parasitoids per choice test). Asterisk (*) indicates significant differences between stimulus and control (χ^2 test, $P < 0.05$).
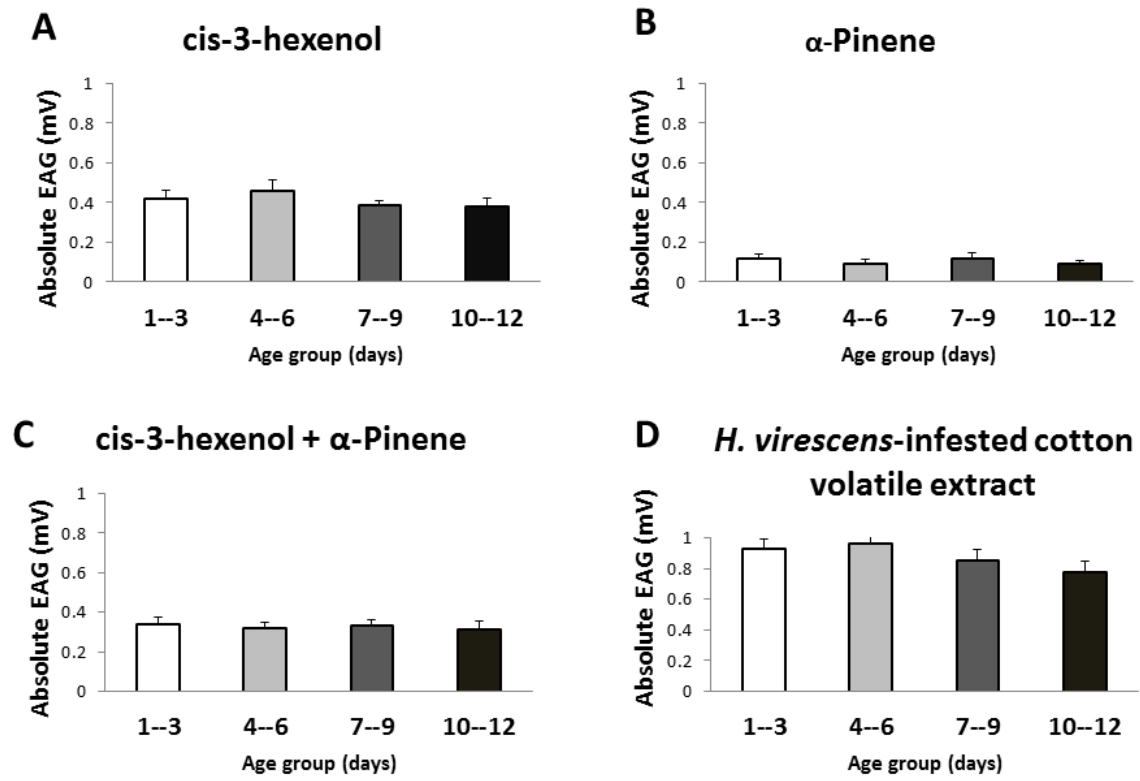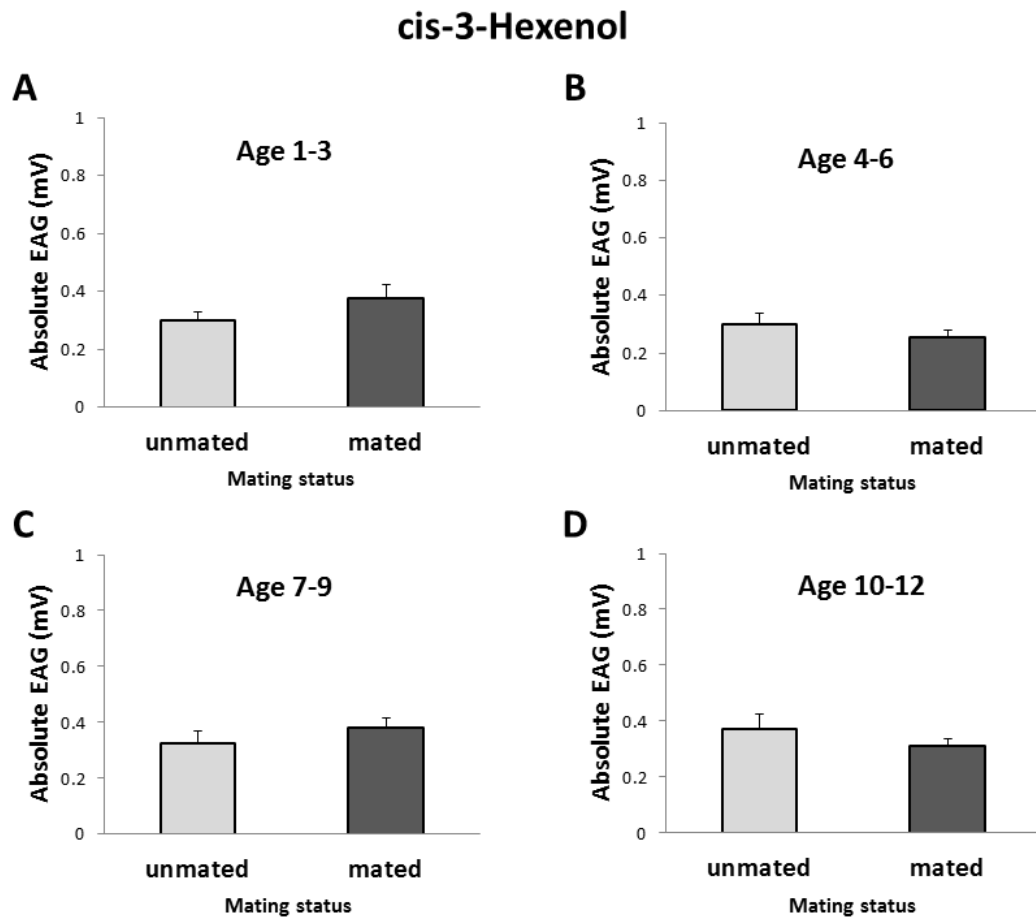
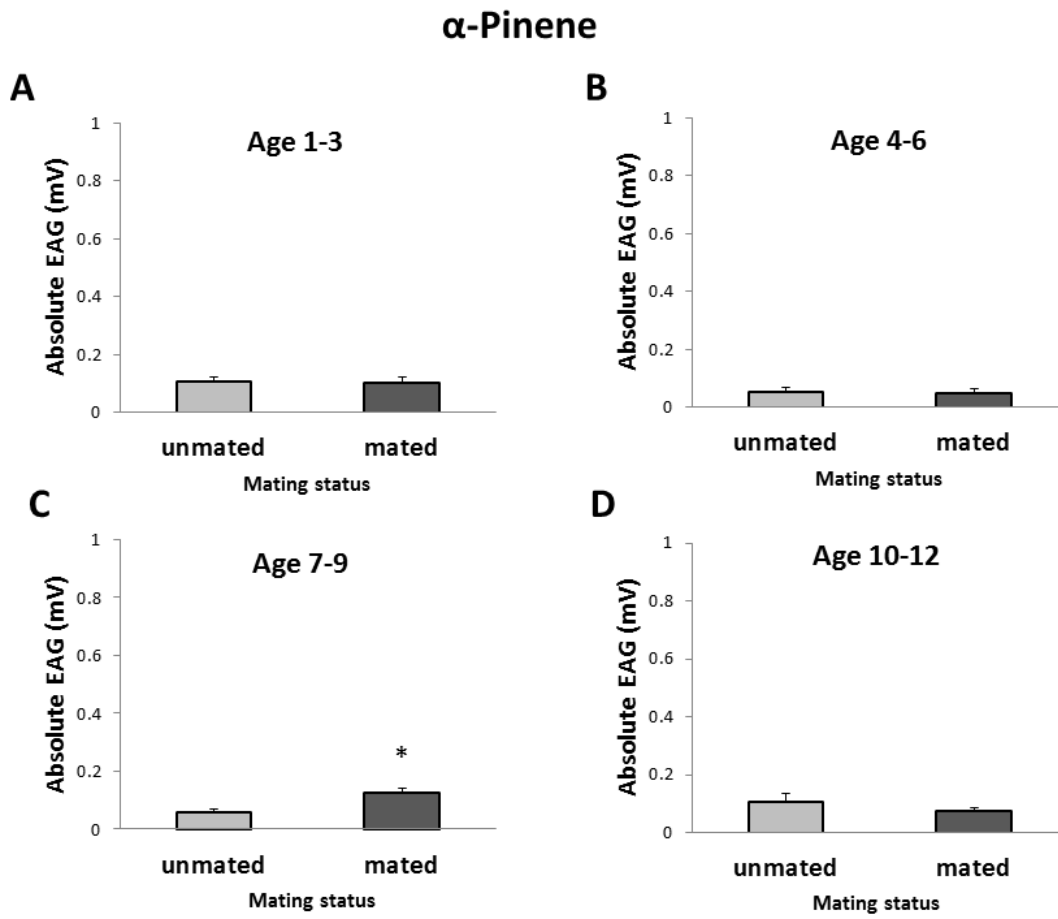**Figure 2.1**

A cis-3-hexenol

B α-Pinene

C cis-3-hexenol + α-Pinene

D *H. virescens*-infested cotton volatile extract

Figure 2.2

cis-3-Hexenol

Figure 2.3

α-Pinene

Figure 2.4

cis-3-hexenol + α-Pinene
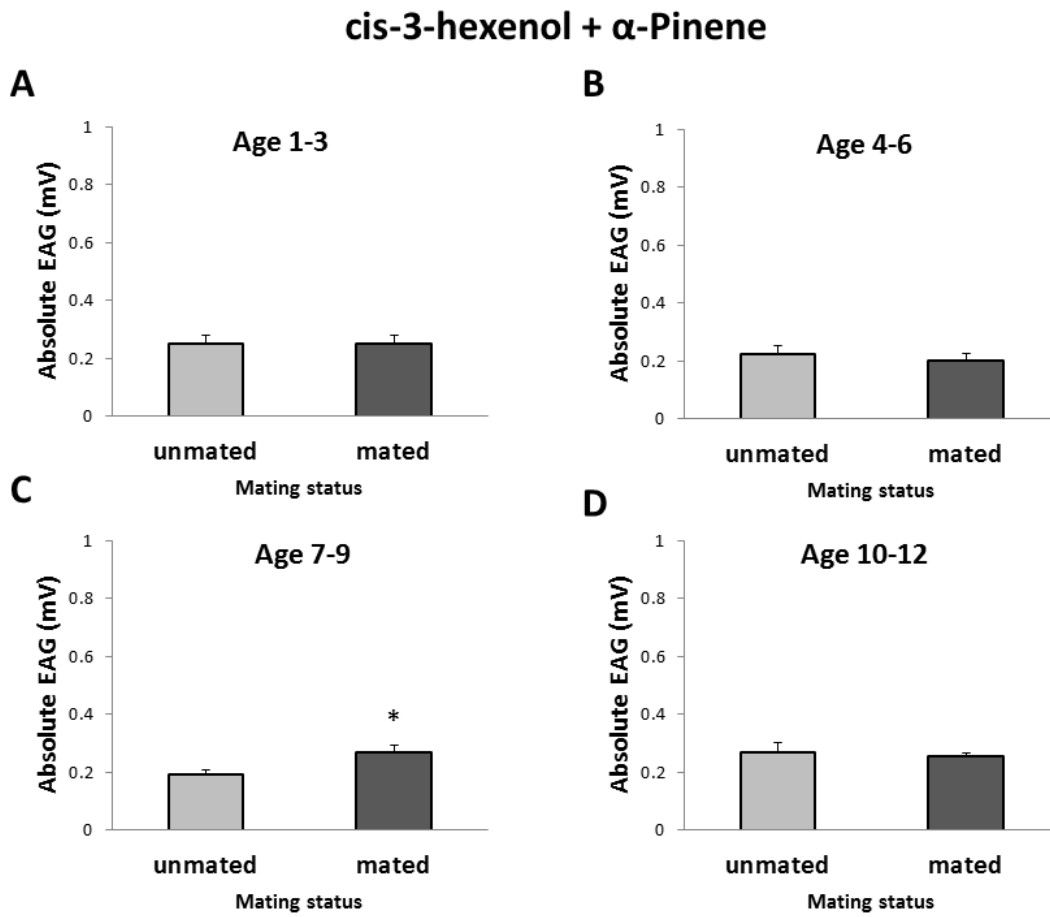
Figure 2.5

H. virescens-infested cotton volatile extract

Figure 2.6

A cis-3-Hexenol

Age 7-9: mated, unmated
Age 4-6: mated, unmated
Age 1-3: mated, unmated

-80   -30   20   70
% attraction

B α-Pinene

stimulus
control

Age 7-9: mated, unmated
Age 4-6: mated, unmated
Age 1-3: mated, unmated

-80   -30   20   70
% attraction

C cis-3-Hexenol + α-Pinene

Age 7-9: mated, unmated
Age 4-6: * mated, unmated
Age 1-3: mated, unmated

-80   -30   20   70
% attraction

D *H. virescens*-infested cotton

Age 7-9: mated, unmated
Age 4-6: mated, unmated
Age 1-3: * mated, unmated

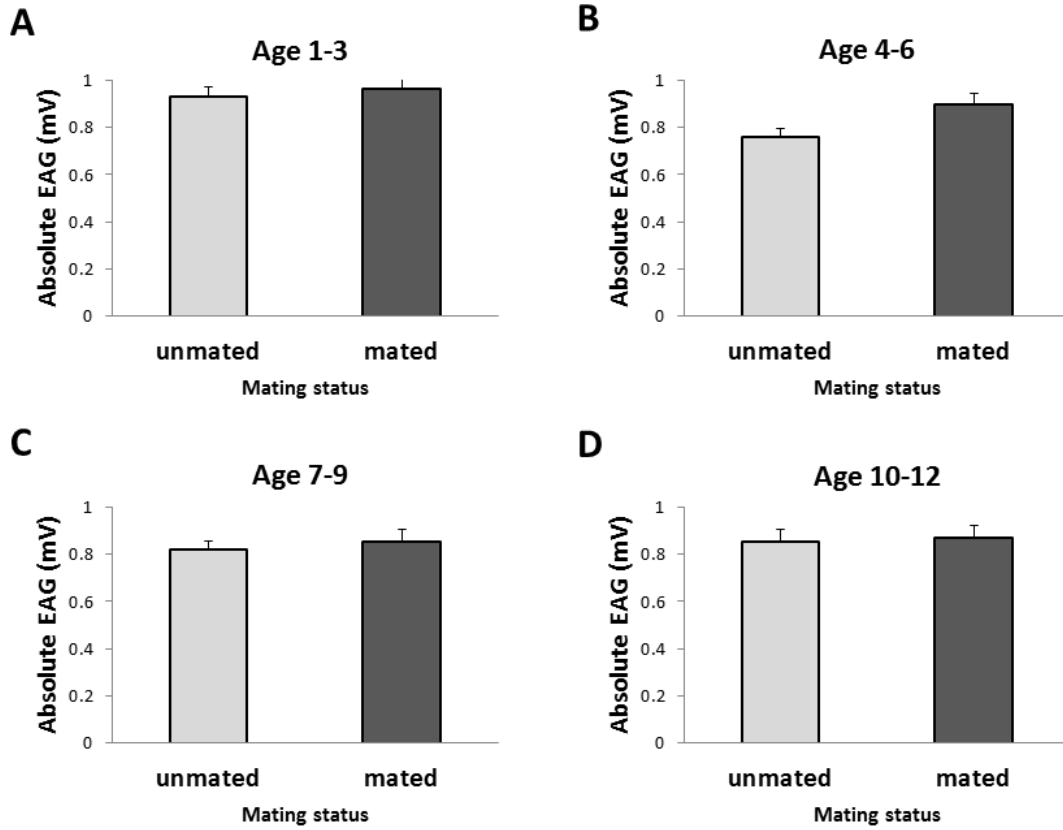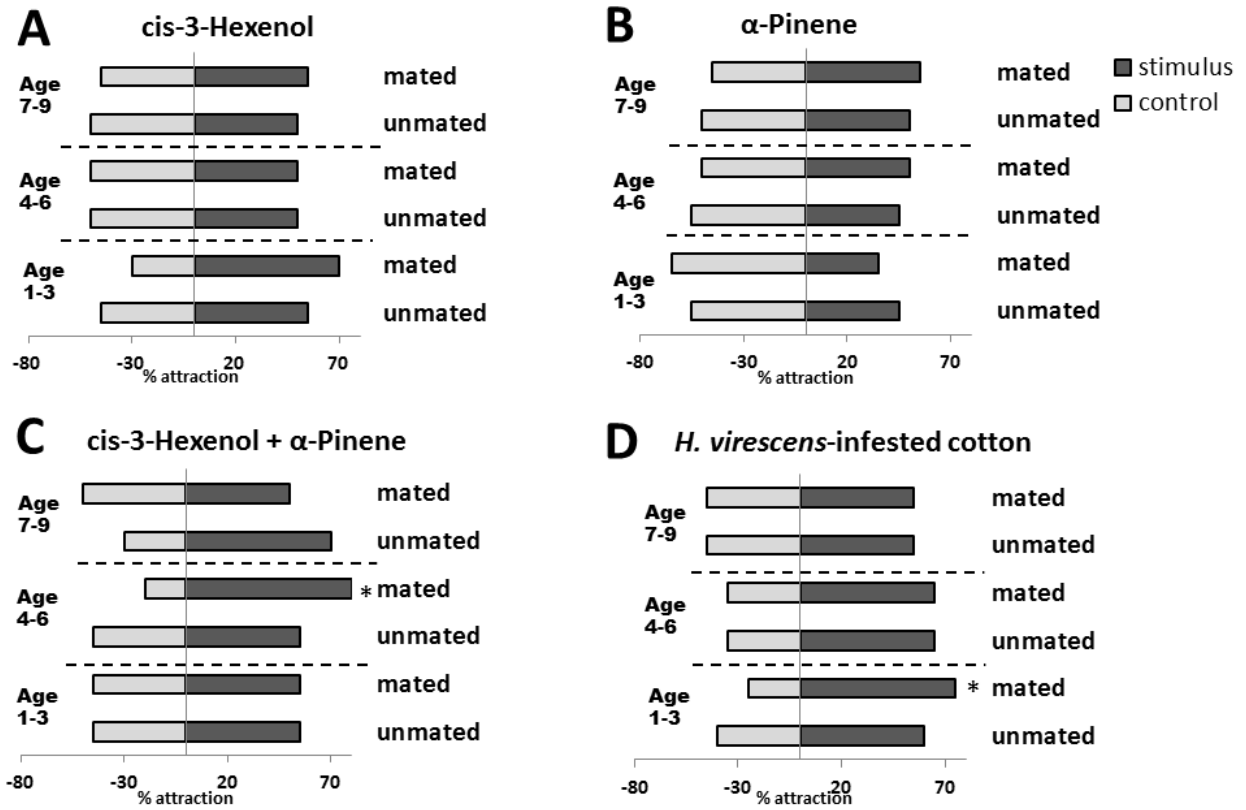-80   -30   20   70
% attraction

Conclusions

Parasitoids rely on chemical stimuli associated with plant defense in order to locate their hosts. Parasitoid response to such chemical stimuli may be plastic and dependent on physiological state of the insect. In this study, plasticity of response to host-related plant volatiles in the parasitoid *Microplitis croceipes* was tested in EAG and Y-tube olfactometer bioassays. Responses to single VOCs, binary mixture and host-infested plant odor were included in this study. cis-3-Hexenol, α-pinene, a binary mixture (cis-3-hexenol+α-pinene) and VOCs released from *Heliothis virescens*-infested cotton were chosen as test stimuli. The overall objective was to test if olfactory response of *M. croceipes* to VOCs is plastic and dependent on their physiological state.

In chapter II, the effect of nutritional status on response of *M. croceipes* to plant VOCs was tested in EAG and olfactometer bioassays. Parasitoids were fed different diet treatments: 0% (sugar-starved), 10%, 20%, or 40% sucrose/water solution (w/v). EAG responses to the binary mixture (cis-3-hexenol+α-pinene) and *H. virescens*-infested cotton extract were significantly affected by diet. Behavioral response to *H. virescens*-infested cotton was also significantly affected by diet. In EAG and Y-tube bioassays, sugar-fed parasitoids responded higher than sugar-starved parasitoids, suggesting that their response to VOCs is dependent on the sugar diet they received. These findings may have implications on nectar provisioning of sugar resources close to herbivore host patches in field settings. In this study, sugar-feeding enhanced response of *M. croceipes* to VOCs. Other studies have reported that sugar-feeding may also enhance parasitism rate. Taken together, these findings can provide optimal usage of parasitoids in field as biological control agents.

In chapter III, the effects of age and mating status on response of *M. croceipes* to test plant VOCs was tested in EAG and olfactometer bioassays. Female parasitoids were categorized as either mated (previously introduced to males) or unmated. They were also separated into age groups: 1-3, 4-6, 7-9 and 10-12 days old. In EAG recording, parasitoid age did not have a significant effect on EAG response. Mating status had a marginal effect on EAG response to VOCs. Mated females aged 7-9 days old showed significantly higher EAG response to α-pinene and the binary mixture than unmated wasps. In Y-tube bioassays, parasitoid behavior was affected by both age and mating status. Mated parasitoids aged 4-6 days old were significantly more attracted to the binary mixture than to the solvent control. In addition, mated *M. croceipes* aged 1-3 days old were significantly more attracted to *H. virescens*-infested cotton than to control. These findings suggest that age and mating status affect response of *M. croceipes* to VOCs to a limited extent.

In order to optimize the use of parasitoids as biological control agents, aspects of their physiological state (nutritional status, age, mating status) may be used to enhance their effectiveness. Parasitoids typically perceive VOCs as blends of many different compounds rather than individual components in a blend. The current findings reinforce this idea. In addition, this study reveals more areas of interest that can be integrated into current optimal foraging models.