UNIVERSITY OF CALIFORNIA
RIVERSIDE

House Fly (*Musca domestica* L.) Attraction to Insect Honeydew With Identification and Behavioral Studies of Honeydew Volatile Compounds.

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Entomology

by

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December 2015

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ACKNOWLEDGMENTS

Special thanks to Dr. Alec C. Gerry for his guidance and support

and to

Drs. Jocelyn G. Millar and Ring T. Cardé for their valued input.

DEDICATION

To Hong K. Lyu, Esq. for enduring the years of fly discussions
and to my family and friends.

ABSTRACT OF THE DISSERTATION

House Fly (*Musca domestica* L.) Attraction to Insect Honeydew With Identification and Behavioral Studies of Honeydew Volatile Compounds.

by

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Doctor of Philosophy, Graduate Program in Entomology
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House flies are common pests on cattle feedlots and dairies, where they develop in and feed on animal waste. By contacting animal waste, house flies can acquire human pathogenic bacteria such as *Escherichia coli* and *Salmonella* spp., in addition to other bacteria, viruses, or parasites that may infect humans and animals. The subsequent dispersal of house flies from animal facilities to nearby agricultural fields containing food crops may lead to pre-harvest food contamination with these pathogens. We hypothesized that odors from honeydew, the sugary excreta produced by sucking insects feeding on crops, or molds and fungi growing on honeydew, may attract house flies, thereby increasing the risk of food crop contamination. House fly attraction to honeydew-contaminated plant material was evaluated using a laboratory bioassay and attraction was evident for the following plant-pest-honeydew combinations: citrus mealybug on squash fruit, pea aphid on faba bean plants, whitefly on navel orange and grapefruit leaves, and

combined citrus mealybug and cottony cushion scale on mandarin orange leaves. Two fungal species, *Aureobasidium pullulans* and *Cladosporium cladosporioides*, were repeatedly isolated from field-collected honeydew samples. House flies were attracted to odors from *A. pullulans* cultures but not to those of *C. cladosporioides*. Gas chromatography-electroantennogram detection (GC-EAD) and gas chromatography-mass spectrometry (GC-MS) identified possible active compounds from pea aphid on faba bean plants, whitefly on navel orange, and whitefly on grapefruit leaves. 10 different compounds were identified from honeydew aeration samples that elicited a house fly antennal response. 4 of these compounds were identified from honeydew produced by whiteflies on navel orange and whiteflies on Marsh grapefruit plants, while one compound was identified from these two whitefly honeydews samples as well as honeydew from a laboratory colony of pea aphids. Two of these compounds were attractive to house flies in a blend and individually. These compound blends and single compounds were compared to fermented vinegar compounds previously found to be attractive to house flies. This dissertation presents the first study of house fly attraction to honeydew and specific honeydew odors. It contributes materially to our understanding of house fly responses to honeydew volatiles and demonstrates that insect honeydew is attractive to house flies, supporting our hypothesis that honeydew production by sucking insects infesting food crops may contribute to attraction of house flies to food crops.

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CHAPTER 1

INTRODUCTION

Background

House flies (*Musca domestica* L.) develop in animal and human waste, materials that may harbor an abundance of microorganisms that pose considerable risk to human health and food safety (Lindsay and Scudder 1956, Blazar et al. 2011). House flies are capable of carrying more than 65 pathogens that affect humans (Greenberg 1971, Olsen 1998), including bacteria such as *Escherichia coli* O157:H7 (Sasaki et al. 2000), *Salmonella enteritidis* (Mian et al. 2002, Wang, Chang, et al. 2011), *Yersinia pseudotuberculosis* (Zurek et al. 2001), *Helicobactor pylori* (Grubel et al. 1997), *Campylobacter jejuni* (Skovgård et al. 2011), and *Vibrio cholerae* (Fotedar 2001), as well as viruses such as rotavirus (Tan et al. 1997). House flies were the biotic factor most closely associated with the spread of pathogenic *E. coli* from livestock to children in Japan (Sasaki et al. 2000), and area-wide control of house flies has been associated with a concurrent reduction in human sickness due to enteric pathogens (Cohen et al. 1991, Levine and Levine 1991, Chavasse et al. 1999). Given widespread use of antibiotics in animal facilities, the dispersal of antibiotic-resistant bacteria from animal facilities to urban sites is particularly worrisome (Chakrabarti et al. 2010). With the digestive tract of house flies providing suitable conditions for the transfer of antibiotic-resistant genes from one bacteria species to another (Macovei and Zurek 2006), flies may also be responsible in part for the rapid spread of antibiotic resistance throughout the environment.

Flies amplify the risk of food-borne disease by transporting pathogens from places where the pathogens pose little risk to humans to places where the risk is greatly increased (Gorham 1989, Chakrabarti et al. 2010). Food-borne pathogens including *E. coli*, *Salmonella* spp., and *Campylobacter* spp., are persistent and widespread problems in food production and preparation (Greenberg 1973, Elder et al. 2000). The extent to which flies contribute to the maintenance and spread of pathogens within and between livestock operations and the community is unknown (Mead et al. 1999). However, it has been recently suggested that flies may play a key role in the movement of human pathogens from domestic livestock rearing facilities to human food crops (Talley et al. 2009).

In 2006, an outbreak of *Escherrichia coli* O157:H7 from spinach leaves, that traced back to a spinach field near a beef ranch, affected more than 200 people and sparked a national controversy between the desire for clean, pathogen-free food and practical efforts of growers to maintain sustainable agricultural practices (CDC 2006, Stuart et al. 2006). Initially, it was thought that contamination of leafy green vegetables with pathogens may have been caused by air movement of contaminated particulate matter from a nearby cattle pasture (Berry et al. 2015, McEachran et al. 2015), from wild animals moving through the leafy greens fields (Jay et al. 2007, Langholz and Jay-Russell 2013), or perhaps even through contaminated water sources, though the risk of contamination through water is probably low (Hutchison et al. 2008). Even higher ambient temperatures have been linked to greater disease incidences from the aggravation of contamination risks related to sewage water, manure, and particulate matter (Liu et al. 2013).

Preliminary field collections of insects in leafy greens fields near the outbreak area found filth flies carrying animal pathogens, particularly in areas where there were aphid infestations in the leafy greens crop (Talley et al. 2009). At each site, samples were collected during early May from two adjacent habitats: rangeland recently occupied by cattle (a potential source of *E. coli* O157:H7) and adjacent lettuce or spinach production areas. The leafy greens fields had the expected complement of plant-associated insects such as leafhoppers, aphids, whiteflies, and thrips. However, some of the lettuce plantings, particularly the mature lettuce infested with aphids, also contained moderate numbers of house flies and blow flies (Calliphoridae). A subsample of flies from one lettuce field tested positive for *E. coli* O157:H7 (Talley et al. 2009) suggesting that house flies may be responsible for *E. coli* O157:H7 contamination of leafy-green crops. Moreover, fly defecation/regurgitation on the fly-infested lettuce was evident and flies were observed to readily enter the lettuce whorls possibly contaminating the leaves (Wasala et al. 2013). While the origin of the flies remains unclear, it is likely that they originated from adjacent animal pastures.

In addition to the previously mentioned collection of filth flies near aphid infestations on lettuce crops, large numbers of house flies have been observed by the authors to associate with plants and trees heavily infested with honeydew-producing insects. For example, jacaranda trees heavily infested with an unidentified soft scale in Chino, CA harbored enormous numbers of house flies feeding on the available honeydew that had accumulated on the tree leaves and the ground below them trees (Figures 1.1 and 1.2). A possible source of flies was a dairy farm approximately 1 km from the infested

trees. Eucalyptus trees infested with lerp psyllid (Family: Psyllidae) in the Central Valley of California were similarly discovered to have numerous house flies feeding on the available honeydew which these insects excrete in the form of a protective "lerp" structure (Hung et al. 2015). Numerous house flies were also observed to be feeding and moving around alfalfa plants infested by aphids on a dairy facility in San Jacinto, CA. It was unclear in these examples whether the flies found in association with the honeydew had arrived at the plants through non-directed dispersal or orientation toward volatiles associated with the honeydew; however, some insects orient toward honeydew odors as an indicator of food or oviposition sites (Wang, Johnson, et al. 2011, Leroy et al. 2012). Because honeydew sugars are not volatile or odorous, we hypothesized that house flies detect and orient toward honeydew by following odor plumes associated with honeydew, and that fungal digestion of honeydew sugars may produce such odors. Better understanding of house fly interactions with their bacterial loads, dispersal behavior within and among agricultural facilities, and response to different natural odors encountered in their habitats may help to elucidate their role in contamination of foodstuffs with pathogens.

House fly association with microbes

While there are many ways that human food crops might become contaminated with animal pathogens, the dispersal of filth flies from development sites associated with livestock has been implicated following the recovery of pathogen-carrying flies resting or feeding on food crops in near proximity to grazing cattle (Brandl 2006, Talley et al.

2009). Newly emerged adult flies contact the contaminated substrates in which they developed and adult house flies frequent animal manure where they feed and lay eggs. Following contact with manure, they disperse throughout the environment, potentially distributing pathogens to locations where they pose a risk to humans and domestic animals (Lysyk and Axtell 1986, Wang, Chang, et al. 2011). Additionally, adult flies feed and lay eggs on animal feces, thereby contaminating external and internal body surfaces with fecal microbes. Pathogenic microbes acquired by flies are readily dispersed throughout the environment as flies disperse in search or feeding and oviposition sites (Ekdahl et al. 2005, Nichols 2005).

Flies can transmit bacteria by three possible routes. Bacteria pass through the gastrointestinal tract and are excreted with fly fecal droplets (Wasala et al. 2013) in numbers as high as 10^5 cells per droplet (Sasaki et al. 2000). Flies can also regurgitate previously consumed bacteria through the oral cavity during subsequent feeding events for up to 3 days after initial acquisition (Kobayashi et al. 1999). Furthermore, flies exhibit a behavior called "bubbling", where the fly excretes a liquid droplet through the mouth without necessarily depositing the droplet onto a surface (Larson and Stoffolano 2011). Bubbling may lead to deposition of bacteria if the bubbles are dropped. Finally, microbes on the fly body, particularly the mouthparts and the fine hairs on the legs, can be dislodged onto a food surface during fly feeding and grooming (Tan et al. 1997, Graczyk et al. 2001).

Depending on the strain of bacteria and the load amount, pathogens can persist in the digestive tract of flies anywhere between several hours to many days. With later

deposition of these pathogens through fly feces or regurgitant, pathogens are disseminated throughout the environment. For example, the bacterium *Aeromonas caviae* can multiply for up to 2 days and persist in flies up to 8 days post ingestion (Nayduch et al. 2002), and exotic Newcastle disease virus (ENDV) can persist in the fly digestive tract for at least 4 days after ingestion (Chakrabarti et al. 2008). *Enterococcus faecalis* proliferates in the crop and remains in the flies for at least 96 hours (Doud and Zurek 2012). Kobayashi (1999) and Sasaki (2000) observed that flies harbored *E. coli O157:H7* for up to 4 days after feeding. House flies harbored *Staphylococcus aureus* in the midgut for up to 6 hours (Nayduch et al. 2013). Further research is needed to understand the interactions among different bacterial strains, the fly digestive tract, and the fly immune system in order to anticipate the persistence of bacteria in the midgut and to evaluate house flies as potential vectors of various pathogens of livestock and humans.

House fly dispersal behavior

In addition to their role as vectors of human pathogens, flies are a nuisance pest. Their dispersal from animal facilities or other fly development sites (e.g. waste management facilities) can result in conflict between the animal operations and neighboring residential areas (Campbell and Thomas 1993, Thomas and Skoda 1993). House flies can disperse over a range of 0.5-6 kilometers (Schoof 1959, Iwasa et al. 1999, Winpisinger et al. 2005), and will readily enter buildings and domiciles (Lole 2005). Heavy fly populations can also cause economic losses in livestock production from litigation costs stemming from lawsuits citing flies as a nuisance (Campbell and Thomas

1993). Even off-farm applications of manure can be problematic, as evidenced by the 1.5 million house flies reported to have emerged from a hectare of poultry litter applied as pre-plant fertilizer (Cook et al. 1999).

Information on house fly dispersal behavior from animal facilities is limited. Flies may disperse in a random fashion and, due to their large numbers, many by chance may land in nearby vegetable crops, mechanically transmitting the pathogens attached to their body surfaces. Flies can disperse from one animal facility to neighboring facilities and have been recorded to travel as far as 20 km (Pickens et al. 1967, Lysyk and Axtell 1986, Nazni et al. 2005). The probability that house flies may transmit pathogens among animal facilities is dependent upon the bacterial loads they carry and the number of flies produced by and dispersing from the source.

House fly antennae

Filth flies use receptors on their tarsal pads, palps, and antennae to detect chemical cues from the environment around them (Dethier 1976, Kelling, Biancaniello, et al. 2002, Vosshall and Stocker 2007), leading them to exhibit certain behaviors associated with those cues. Detection of odors by antennal receptors is perhaps most important in eliciting movement in the environment. A filth fly has three antennal segments from proximal to distal: a scape, pedicel, and flagellum. Scanning electron microscope images show that filth flies have more sensilla on the flagellum than the rest of the antenna (Sukontason et al. 2004). The sensilla contains odorant receptors (OR) on the olfactory receptor neurons (ORNs). Binding of an odor with an odorant binding

protein (OBP) transmits a signal to the OR and triggers a signal transduction pathway through the neuron which signals the brain that an odor was detected (Jefferis 2005, Carey and Carlson 2011, Leal 2013).

Different concentrations of (±)-1-octen-3-ol as a background odor can increase or decrease antennal responses to 2-pentanone, (*R*)-limonene, and additional pulses of (±)-1-octen-3-ol (Kelling, Ialenti, et al. 2002). There were no apparent sex- (male versus female) or age-related (newly emerged versus >4 days old) differences in antennal sensitivity to previously tested odors including: 1-octen-3-ol, amyl acetate, 2-pentanone, 3-methylphenol, (*R*)-limonene, muscalure, and 6-methyl-5-hepten-3-one (Kelling, Biancaniello, et al. 2002, Kelling et al. 2003). The age range of these mature flies was large (4-28 days), suggesting that the antennal receptors do not undergo noticeable changes as they age. Conversely, house flies may respond differently to these odors depending on their physiological status and sex. Although house fly antennae may be equally sensitive to both 1-octen-3-ol and (*R*)-limonene (Kelling et al. 2003), this does not correlate with house fly attraction. Limonene, an essential oil extract, is a house fly repellent (Kumar et al. 2011) and toxicant (Geden 2012). 1-Octen-3-ol, identified from cattle odors, is an attractant for tsetse flies, stable flies, face flies, and horn flies (Hall et al. 1984, Birkett et al. 2004, Jeanbourquin and Guerin 2007, Oyarzun et al. 2009), but specific attraction for house flies has not been tested. Additionally, house flies exhibit low antennal responses to well-known attractants such as indole, skatole, and acetic acid (Kelling, Biancaniello, et al. 2002). Therefore the amplitude of antennal responses may

not be an indicator of attraction and behavioral studies are required to determine whether or not compounds that elicit antennal responses are indeed attractants.

House fly responses to attractive material

At a distance, we expect flies to use visual cues for food searching behaviors especially if food odors may not be easily detected in a vast environment with many competing odors. House flies respond to blue-colored surfaces and some reflective surfaces such as Alsynite traps (Geden 2006). However house fly visual cues in association to food attractants are not well known. Food search behaviors by flies have been limited to studies by Dethier (1954, 1976) examining sugar detection and feeding response as well as long-range wind-tunnel studies (Cossé and Baker 1996). Dethier (1976) noted that detection of sugar by the tarsi is followed by the fly orienting their body and extending the labellum toward the sugar source. Cossé and Baker (1996) observed that flies detected pig manure odors at a distance, moved upwind towards the odor source, and landed near the odor source, though they noted more flies moving upwind than landing. While many studies have examined house fly responses to odors, many of these studies used odors in combination with traps, which only indicate the behavioral endpoint of the flies arriving at the trap and not the movement behaviors prior to capture.

For over a century, scientists have understood the house fly's capacity to transmit pathogens and cause nuisance (Howard 1911). Morrill (1914) conducted one of the earliest recorded studies examining house fly attraction to various substances ranging from natural materials (bread, beer, sour milk, decayed and fresh banana, cheese, fresh

orange, cane sugar, decayed and fresh apple, dried blood) to synthetic compounds (formalin, alcohol, cobalt, bichromate) and mixtures of these materials. Many of the early works discussing house fly attraction to natural and chemical attractants and repellents have been reviewed by Frishman and Matthysse (1966). Since then, scientists and researchers have been motivated to investigate fly attraction in the interests of improved understanding of public health implications and pest management practices. Other materials studied for house fly attraction included malt (Brown et al. 1961), rotten eggs (Willson and Mulla 1973a), fermented grain (Pickens et al. 1973), fermenting yeast and milk (Mulla et al. 1977), animal feces (Cossé and Baker 1996), black-strap molasses (Quinn et al. 2007), and fermented vinegar (Qian et al. 2013). Synthetic mixtures and single chemical compounds that have been examined as house fly attractants include ethyl alcohol, acetic acid, lactic acid, amyl alcohol, carbohydrates (Richardson 1917, Brown et al. 1961), ammonia, carbon dioxide, ethanol (Wieting and Hoskins 1939), trimethylamine, ammonia, indole, and linoleic acid (Mulla et al. 1977). In the last few decades, scientists began describing the volatile profiles from attractive materials through identification of specific attractive compounds, including indole, skatole, 3-methylbutanoic acid, and dimethyltrisulfide identified from pig manure (Cossé and Baker 1996); ethanol from fermented sucrose (Hwang et al. 1978); and hexanoic acid, 2-phenylethanol, *p*-cresol, and furfural from fermented vinegar (Qian et al. 2013). Many of these compounds are offensive and difficult for humans to tolerate at high concentrations but are very effective in attracting house flies (Pickens and Miller 1987), and some of

these compounds comprise proprietary odor blends used in commercial fly traps such as attractants from Mulla et al. (1977) and Cossé and Baker (1996) (Warner 1991).

Many scientists have noted a "fly factor" in which flies tend to aggregate where one fly is already present (Barnhart and Chadwick 1953, Dethier and Rhoades 1954, Brown et al. 1961). This may be due to a combination of a visual component of seeing other flies on a surface and attraction to a sex pheromone (Chapman et al. 1999, Hanley et al. 2009), particularly for male flies (Rogoff et al. 1964). In (1973) Carlson and Beroza studied fly responses to (*Z*)-9-tricosene in the field and noted higher catches in traps baited with this putative fly pheromone than in controls. However, there is conflicting information as to whether this compound is effective as a long-range attractant (Hanley et al. 2004, Butler et al. 2007). The large variation of (*Z*)-9-tricosene (termed “muscalure”) levels in female flies from different fly populations may have been a factor in the unreliable attraction of this pheromone (Butler et al. 2009). A study examining male and female house fly cuticular hydrocarbons found several major components were female-specific, suggesting that the female-produced pheromone may actually consist of multiple components (Nelson et al. 1981).

House fly sugar-feeding behavior

Carbohydrate sources that flies may commonly encounter in natural environments include nectar, tree sap, open or rotting fruit, and honeydew. Honeydew is readily consumed by many dipterans including black flies (Simuliidae) (Burgin and Hunter 1997a, 1997b), horse and deer flies (Tabanidae) (Schutz and Gaugler 1989,

Janzen and Hunter 1998, Hunter and Ossowski 1999), mosquitoes (Culicidae) (Foster 1995, Russell and Hunter 2002), fruit flies (Tephritidae) (Wang, Johnson, et al. 2011), and sand flies (Psychodidae) (Moore et al. 1987, MacVicker et al. 1990). Honeydew consumption by filth flies have been suggested because the flies' sponging mouthparts may be well-adapted for consuming honeydew on plant surfaces (Downes and Dahlem 1987). Because many filth flies are also synanthropic, meaning they are closely associated with humans, they may also take advantage of any food source that may be exposed by human activity. Many dipterans, including *Drosophila melanogaster*, *Rhagoletis pomonella*, *Phlebotomus papatasi*, *Aedes albopictus*, *Stomoxys* sp., and *M. domestica*, are attracted to natural substrates which contain carbohydrates, such as overripe mango (Zhu et al. 2003), as well as various fruits and flowers (Nojima et al. 2003, Junnila et al. 2011, Müller, Revay, et al. 2011, Müller, Xue, et al. 2011, Zito et al. 2015). Feeding on complex sugars (such as those available in honeydew), may benefit flies by providing energy for flight (Stanfield and Hunter 2010) and could potentially extend insect longevity (Lee et al. 2004, Hardin et al. 2008). However, studies on the effects of utilizing honeydew sugars to extend house fly longevity and flight are lacking.

Field trapping of flies

Different trapping structures and methodologies have proven effective in capturing filth flies as part of integrated pest management systems. For example, large sticky traps lining the upper canopy of greenhouses reduced the number of stable flies collected on the dairy animals (Kaufman et al. 2005). Cone traps or Brundrett traps have

been used in the field for house fly collection (Bishopp 1921, Brundrett 1953, Qian et al. 2013) in which the bait is placed on a platform below the base of a cone that opens at the top into a cylinder which holds trapped insects until the device is removed. Additionally, cone traps are available commercially such as the Bioquip "Diptera/Hymentoptera Cone Trap" (Item #2826) or the Arbico Organics Solar Fly Trap (Item # 1256001) making them common methods for fly collections when using an odorous attractant. Cone traps have been used for the ease of collecting flies without the use of messy killing media, which makes identifying and counting the individual flies difficult. The cone trap also takes advantage of the flies' tendency to naturally move upwards after resting on a horizontal surface. Baited jars or buckets containing attractants have also been commonly used for fly management in poultry and dairy facilities (Willson and Mulla 1973b, Mulla et al. 1977). Sticky pyramid traps intercepted flies as they moved between sites because they were a visual target for flies, and baited traps were most efficient at breeding sites and fly-aggregating sites (Pickens and Miller 1987). Trap placement in the field is important as each trap has an effective trap radius, therefore trap position and density in the field should be considered in experimental design (Byers et al. 1989, Byers 2012). Effectiveness of fly capture deteriorates as the baits age and is affected by the combination of bait contents with the trap type (Geden 2005, Geden et al. 2009). Geden et al. (2009) also noted the importance of preventing fly access to external food resources in order for the sugar-based traps containing toxic baits to capture flies efficiently. As with all pest management programs targeting house flies or other filth flies, there is no one fool-proof method to reduce fly numbers and trapping alone has not been sufficient at

reducing filth fly numbers. Currently, consistent trapping and monitoring combined with aggressive manure management strategies are considered the most sustainable techniques for reducing fly numbers, but improvement of trapping techniques will further improve house fly pest management strategies.

Conclusion

Increasingly, the presence of house flies and other filth flies has been associated with transmission of pathogens to humans (Fukushima et al. 1979, Cohen et al. 1991, Graczyk et al. 2001). The widespread use of broad-spectrum insecticides to manage high fly densities has led to fly resistance and cross-resistance in house fly populations worldwide (Chapman et al. 1993, Scott 1995, Keiding 1999, Kristensen and Jespersen 2004, Srinivasan et al. 2008, Gerry and Zhang 2009, Kaufman et al. 2010). Growing concerns about insecticide resistance and the environmental impacts of chemical insecticides are driving the search for alternative control methods. “Attract and kill” or "push-pull" strategies have been suggested as methods to reduce local populations of some invasive insects and pests (Carlson and Hogsette 2007, Cook et al. 2007, El-Sayed et al. 2009, Pickett et al. 2014). Additionally, attract and kill strategies are advantageous for reducing human exposure to insecticides and abating potential insecticide resistance because the toxicants are used in a limited setting (El-Sayed et al. 2009). By limiting the toxicant to a specific application (only in the trap), we can reduce the chances that the flies will receive non-lethal doses, reducing opportunities for the population to develop tolerance and eventually resistance. Studying fly attraction to honeydew possibly leads to

a better understanding of house fly dispersal to a crop-field with honeydew accumulation and house fly pathogen transmission. Research on the response of house flies to volatile odors contributes to our basic knowledge of their biology and behavior, but we hope it will also contribute to the development of methods for long-term management of these flies, reduce pesticide use and resistance development, and minimize food-borne pathogen outbreaks.

The main objectives of this dissertation were:

1. Collect honeydew materials from the laboratory and the field and examine house fly attraction to these materials using a two-choice bioassay method (Chapters 2 and 3).
2. Analyze the volatiles profile of attractive honeydews, and identify these volatiles using gas chromatography coupled to electroantennogram detection (GC-EAD) and gas chromatography coupled to mass spectrometry (GC-MS) (Chapter 4).
3. Evaluate these volatiles individually or in blends to verify attraction in semi-field or field environments (Chapters 5 and 6).

Figures

Figure 1.1: House flies on a jacaranda tree. Flies were noted to be feeding on the scale honeydew

Figure 1.2: Flies captured on a sticky trap after 24 hours on a scale-infested jacaranda tree.

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CHAPTER 2

DEVELOPMENT OF A TWO-CHOICE OLFACTOMETER TO ASSESS HOUSE FLY ATTRACTION TO HONEYDEW ODORS

Abstract

In order to examine house fly (*Musca domestica*) attraction to insect-produced honeydew, developed a bioassay method that could assess house fly behavior quickly we consistently, and that could be replicated to provide data for robust statistical analyses. We developed a two-choice olfactometer to examine house fly orientation toward citrus mealybug (*Planococcus citri* Risso) honeydew, with additional observations of fly movement into the olfactometer arms as a function of fly age and period of starvation before testing, for various olfactometer designs. We determined that flies 3-5 days old and starved about 18 hours were most responsive and discriminatory. Overall, house flies were generally attracted to odors from citrus mealybug honeydew. However, results were not consistent among trials, and this method was ultimately abandoned in favor of a cage-choice assay described in the following chapter of the dissertation.

Introduction

Filth flies feed on animal and human waste (e.g. manure and garbage) where they can pick up and transmit pathogens into the environment as they disperse, posing a risk to

human health. They also are a nuisance to homeowners when present in large numbers, and often contribute to aesthetic damage of residences by deposition of regurgitation and defecation spots (Thomas and Skoda 1993). House flies (*Musca* domestica) have been implicated as mechanical vectors of over 65 pathogens, including bacteria, viruses, and protozoa (Greenberg 1973). They can also be responsible for pathogen transmission to humans and animals in areas where fly management is poor (Cohen et al. 1991, Levine and Levine 1991, Chavasse et al. 1994, 1999). A reduction in fly numbers following control efforts has been associated with reduction in human illness (Watt and Lindsay 1948, Lindsay et al. 1953, Cohen et al. 1991). Furthermore, the ability of flies to spread antibiotic resistant bacteria is particularly worrisome (Chakrabarti et al. 2010, Doud et al. 2014).

In 2006, an outbreak of *Escherrichia coli* O157:H7 from spinach leaves affecting over 200 people sparked national attention and controversy between the desire for clean, non-pathogenic food and practical efforts of growers to maintain sustainable agricultural practices (CDC 2006, Stuart et al. 2006). Preliminary field collections of insects in leafy-greens fields near the outbreak area found filth flies carrying animal pathogens, particularly in areas where there were aphid infestations in the leafy greens crop (Talley et al. 2009), leading us to hypothesize that insect honeydew may be a sugar source for filth flies and a possible cause for leafy greens contamination.

A reliable laboratory bioassay setup is important for assessing house fly behaviors towards honeydew and odors of honeydew. Previous assay methods have used wind tunnels where a substance was released upwind of the flies and fly movement towards the

materials was observed (Cossé and Baker 1996), Y-tube choice olfactometers where the flies moved into one of the two arms either containing a treatment or control (Chaudhury et al. 1972, Bay and Pitts 1976, Machtinger et al. 2015), or a two-choice cage arena where two substances were released into a cubical arena (Wieting and Hoskins 1939, Brown et al. 1961, Frishman and Matthysse 1966).

Having reviewed the bioassays that have been used to assess house fly behavior in response to odors, we believed that developing a two-choice olfactometer would provide the most diagnostic responses, particularly when testing responses to honeydew, which, in addition to providing a sugar source, may have odors associated with it which could aid the orientation of flies to honeydew. We wanted an olfactometer that would allow the evaluation of fly responses to a potential attractant within a short amount of time and with a suitable number of repetitions before the test material was no longer emitting volatiles similar to those when collected from the field. Also, we reasoned that with the smaller arena needed for an olfactometer relative to a two-choice cage arena used by others, less test material would be needed, which is helpful when a large accumulation of honeydew is difficult to find. Here we use the term "attraction" to describe the orientation toward honeydew volatiles over short distances, either due to honeydew odors eliciting increased short range orientation and landing behaviors, or as a result of increased time spent near a source of test material, or both.

Methods

Insect colonies:

House flies were collected by sweep net from a southern California dairy facility in 2010 with the owner's permission and maintained in an insectary at constant conditions of 25 ± 2°C, 40% relative humidity (RH), and 12L:12D photoperiod. Larvae were fed a standard fly rearing medium (Mandeville et al. 1988), and adult flies were provided a 50:50 mixture of powdered milk and sucrose, and given water *ad libitum*. Citrus mealybugs (*Planococcus citri* Risso) were from an established UC Riverside colony maintained on butternut squash fruit (*Cucurbita moschata* Duchesne) at 25 ± 2°C, 40% RH and 14L:10D photoperiod in an insectary room (Figure 2.3). Pesticide-free butternut squash grown in the Agricultural Operations (AgOps) fields at University of California at Riverside (UCR) were harvested, thoroughly washed with a dilute solution of micro90® detergent, rinsed with water, dried for 1-2 days, and stored at 15.5°C until use (up to 1 year).

Starvation study

It was thought that it would be best to evaluate house fly attraction to honeydew odors (a potential signal for a carbohydrate food source) using starved flies which might be more motivated to respond to food odors. However, flies that are starved for too long may become moribund or even die prior to or during the assay, so it was important to evaluate the optimal starvation period for house flies reared in colony. Thus, 4 groups of

25, 2-3-day old female flies were placed into containers with water only. Mortality was observed beginning with 12 hour intervals followed by every 1-5 hour intervals when fly mortality was observed until over 90% of the flies were dead. Flies that were not walking, laid on their sides, or on their backs were considered dead for the purpose of this study. Flies were observed for at least a minute to ensure the immobile flies would not be upright again.

Two-choice olfactometer:

Citrus mealybug (CMB) honeydew was used as an attractive material based upon earlier unpublished work, during the development of the olfactometer to determine the optimal fly age, sex, and starvation period. Honeydew was collected from the bottom of the colony cages, thoroughly mixed with a spatula, and 0.5 g subsamples in 2 mL distilled water were used for each replicate. Distilled water (2 mL) was utilized as a negative control for each bioassay. External factors such as room temperature and light source (presence or absence, location relative to the experimental unit) were also varied and evaluated. Following a year of testing a number of olfactometer designs, an apparatus comprised of acrylic plastic with Teflon tubing was developed that produced fairly good results with the CMB honeydew. The olfactometer was easy to wash to remove volatiles between runs and Teflon tubing was used because it does not absorb volatiles, avoiding potential contamination between runs. Flow meters were utilized to ensure similar airflow entering the olfactometer from either side as the air was passed through a glass flask containing the attractant materials (or control) to be tested.

The olfactometer consisted of three compartments, two side arms and a center release chamber (Figure 2.1 and 2.2) where starved flies were released at the beginning of the assay. The side arms were joined to the center release chamber by plastic funnels to allow flies to move easily into the side arms with little movement back to the center. The olfactometer was connected by Teflon tubing to glass canning jars which contained either the treatment or a control. The jars had a Teflon lid with attachments for the Teflon tubing connecting to the olfactometer and to a source of charcoal-filtered air. Air was pulled through the system by an outlet in the center release point connected to a mesh-covered vacuum source, with flow controlled with a flow meter at each side of the olfactometer. The flow rate was set at 212 ml/min, which was high enough to pull air through the system but not so high so as to inhibit fly movement. The filtered air passed through the treatment jars and towards the center of the olfactometer where the flies could detect the odor plumes and respond. The olfactometer featured airtight seals to ensure that air entered the system only through a charcoal filter prior to passing over the test material held within a glass flask. The plastic funnels did inhibit some of the air movement but air was still able to pass through to the center. This was tested by connecting a vacuum to the olfactometer and adding artificial smoke generated from $TiCl_4$ in a fume hood.

Fly movement into the olfactometer arms and assay consistency was enhanced by placing the olfactometer inside a box with black interior with xenon lights placed equidistant from the edges on the left and right side of the box lid to sit directly above each olfactometer arm when the lid was closed. Flies generally exhibit positive

phototaxis and the light encouraged movement of the flies into the olfactometer arms. Flies are released into the center release chamber and can move upwind toward either arm after passing through a funnel to restrict their movement back into the olfactometer once they have moved into the arm. Within the 20 min assay period, flies would either move into one of the olfactometer arms (treatment or control) or would remain in the release chamber. Only flies moving into one of the olfactometer arms were considrered to have participated in the assay. Before reuse, the olfactometer was cleaned using micro90 (International Products Corp., Burlington, NJ) detergent, rinsed with deionized water, and air dried.

Prior to running bioassays, 3-to-5 day-old flies were aspirated from colony cages and anaesthetized by placing in the freezer for 3-5 minutes. Flies were sorted on a chill table into 8 groups of 25 female flies (males were discarded) and placed into a plastic container with a moist paper towel as a water source. A preliminary study evaluating house fly attraction to CMB honeydew at different starvation periods was performed using house flies starved for 18, 24, and 36 hours. For subsequent trials, flies were starved overnight (18 hours). The following day, each group of 25 female flies was briefly anesthetized with CO_2 and placed into an olfactometer to complete a single test replicate (n = 8 replicates per trial day). Each replicate was performed in the morning (8:30-12:00) and lasted for 20 minutes from the time that flies were introduced into the olfactometer. At the end of the assay, the entire olfactometer including the flies was placed in the freezer to kill the flies and fly numbers in each arm and in the release chamber were counted and recorded. Two olfactometers were used simultaneously for

these assays and the known attractive mealybug honeydew was alternated between olfactometer arms on sequential replicates. We did not allow the flies to recover from CO_2 exposure before putting them into olfactometer because recovery time may have permitted flies to move to the side chambers before the start of the bioassay. Consequently, we allotted more time than we intended (5-10 min) for the flies to respond.

Statistical Analysis

Flies collected in the treatment and control arms were recorded for each replicate. Counts were analyzed with a paired student t-test to assess significant preference for the honeydew treatment relative to the control with each bioassay condition tested. Flies remaining within the release chamber were recorded as making "no choice" but were otherwise not included in any analysis. When repeating assays on subsequent days, counts were analyzed using two-way ANOVA (SAS v9.4) to assess differences between days. Assumptions for normality were checked using the Shapiro-Wilk test and the Levene test was used to check for equal variance assumptions.

Results and Discussion

Starvation study

Flies began to die 44 hours after being denied food, with half of the flies dead by 52-69 hours and 90% of the flies dead by 93 hours without food (Table 2.1). The flies

tolerated up to 2 days of starvation prior to beginning a bioassay, but flies starved for 18 hours had the highest assay participation rates and demonstrated a greater preference for honeydew relative to flies starved for other periods of time (Table 2.2). Fly movements were observed to be more lethargic in flies starved longer than 48 hours compared with flies starved for less than 44 hours. Published studies using bioassays to examine house fly responses to food or feeding attractants have used starved flies, but the period of starvation varied among studies. House flies starved for 6 hours responded to baited traps more than satiated flies during a 24 hour test period (Geden et al. 2009). Flies that were starved 24 hours prior to testing only needed 3 minutes to elicit fly response in an olfactometer (Qian et al. 2013). Riley and Dill (2005) also starved flies 24 hours to observe male fly distribution among sugar sources with different predation risks. Darbro and Mullens (2004) starved flies 16-20 hours prior to performing 2-choice feeding assays to examine methomyl resistance. Larson and Stoffolano (2011) starved flies for 20-24 hours prior to offering flies different sugar solutions for observation of "bubbling" behaviors. Based upon the range of published results and the starvation test we performed, starving flies for 18 hours was expected to be suitable to observe house fly attraction to an odor source associated with nutrition within a 20 minute observation period.

Two-choice olfactometer

Overall, the olfactometer design demonstrated house fly attraction to honeydew, ($F=88.89$; $df=1,77$; $p<0.0001$), with flies being significantly attracted to CMB honeydew

on 4 out of 5 test dates when analyzed independently (Table 2.3). Unfortunately, the trials on days 6/24, 8/25, 8/30, 8/31 showed varying levels of fly response towards the honeydew and our preferred bioassay would show more consistency in fly response to the treatment from day to day. For statistical strength, 8 replicates should be completed within a single day because test materials containing honeydew collected in the field might lose attractive volatiles or change in volatile profile as new honeydew is no longer deposited onto the plant surface and older honeydew may support altered microbial activity.

The inconsistent responses from the flies may be due to the varying airflow and the pressure inside the olfactometer. Because the plastic funnels were handmade and there were many connections for the system, there may be variation in the airflow pattern, particularly if a connection was not sealed properly. The flow meters allowed for a consistent air speed moving through the olfactometer but the change in air pressure within the system may have altered fly behaviors as well. The olfactometer was in a controlled indoor environment with consistent temperature but the humidity was not regulated and the flies were experiencing ambient humidity. Although we do not expect a drastic fluctuation in ambient humidity during each trial, we cannot rule this out as a possible factor that may vary fly responses. The mealybugs were maintained in a continuous cycle of new squash placed onto old squash in the same colony cages with honeydew continuously accumulating on the cage floor. Honeydew utilized on each of the test dates was collected by scraping substantial honeydew from the cage floor on the same day, mixing the honeydew in a dish, and taking a subsample of the honeydew for

each replicate. Given the continuity of the rearing process with the cages, we expect little variation in the honeydew provided to the flies on each date. We also expect that there was little variation in the flies used among test dates, as flies were reared and handled similarly throughout testing.

In preliminary studies, males were noted to spend much of the assay time attempting to mate with females within the central chamber and were less likely than females to leave the center chamber of the olfactometer. Alternatively, the females may have been driven from the central chamber to the side arms because of the persistent mating attempts by the males. As a result, we used only female flies with the final olfactometer build. Female flies that were 3-5 days old were deemed most suitable for testing based on numerous observations during the olfactometer development period. In preliminary studies, flies older than 7 days were observed to remain within the release chamber in greater numbers compared to younger flies. Female flies older than 7 days are often gravid and were perhaps more interested in seeking an oviposition substrate than a food source like the honeydew provided in the olfactometer.

This olfactometer did not fulfill our requirements for a bioassay because we needed definitive and consistent results within 24 hours of honeydew collection. Honeydew was not reused on a subsequent day due to concern that the volatile profile would be changed. However, the olfactometer could potentially be used for future assays with materials that are not time sensitive and could be sampled over multiple days. The preferred bioassay was a device where enough flies participated by moving into one of the two arms to make a statistically significant response in a relatively short time (less

than 30 minutes). Fly responses towards the treatments and controls should remain consistent as much as possible among multiple trials using identical treatments, which was not evident in our bioassay. Other studies using olfactometer assays with flies that did not clearly indicate whether or not they included flies that made "no choice" in their analysis (Carlson et al. 1974, Bay and Pitts 1976). Bay and Pitts (1976) only included the flies that moved in their treatment or control arms in their analysis and did not include the number of flies that did not make a decision. Carlson et al. (1974) reported the percentage of flies attracted to the attractants and compared this to the percentage of flies trapped by their muscalure mixture, but the distinction between the flies that made "no choice" or trapped in the control. The bioassay should not have a bias of flies moving towards one side or the other when using the same treatment on both sides; therefore both of the arms in the olfactometer should be built evenly and allow air to move through the device in the same manner. With this in mind, the flies also should not indicate attraction to one of the treatments in the bioassay when using identical treatment on both sides, which was verified in preliminary studies using water on both sides. To control for any minor bias that may inadvertently exist for one arm or the other, future treatments were alternated between the two arms for each replicate. For a more reliable rapid assessment of house fly attraction, other testing methods will need to be evaluated. A two-choice bioassay within colony cages has been used before (Wieting and Hoskins 1939, Quinn et al. 2007) and might be better suited for our needs because of its larger area for flies to exhibit orientation or other odor response behaviors.

The olfactometer did not give the flies room to exhibit flight movements so flies were often walking around inside the arena so behaviors assessed may be those that occur naturally following landing. This olfactometer study provides some evidence that house flies can distinguish the treatment odors from the control at least over a short distance. It is possible that honeydew odors result in increased short-range, food-searching behavior that may increase the number of flies walking to one arm over the other. In spite of this, using water controls on both sides in preliminary studies showed no indication of reduced total fly captures in both arms. We used several materials not reported in this study in addition to the CMB honeydew to assess the olfactometer design including a fresh cantaloupe flesh, aged cantaloupe flesh, lerp psyllid on eucalyptus, aged milk/sugar/yeast solution, and commercial sugar baits, which are reportedly attractive for house flies. Because these materials did not seem to garner a strong preference compared with their respective controls, the CMB honeydew was used to assess the effectiveness of the olfactometer.

The results obtained using the many olfactometer designs tested during the study generally show house fly attraction to CMB honeydew. However, in the end the results even with our best design did not demonstrate consistent house fly responses to similar honeydew treatments, leading us to seek an alternative method to examine house fly responses to honeydew.

Tables

Table 2.1: House fly mortality by starvation period. Each treatment includes 4 replicates of 25 flies each.

Hours post-starvation	0	20	44	49	52	69	70	73	75	93
Mean # dead	0	0	0.8	4	6.8	14.3	15	16.5	18.3	23.8
% dead	0	0	3	16	27	57	60	66	73	95

Table 2.2: Number of flies (out of 25) in each chamber, testing citrus mealybug honeydew vs. water for different starvation periods. Each trial had n = 8 replicates. % at treatment is the number of flies captured in the treatment arm over the total flies captured.

Starvation period	Treatment (SEM)	Control (SEM)	% at treatment	p-value
18 h	14.6 (1.7)	5.0 (0.9)	73.1%	**0.006**
24 h	11.2 (1.6)	7.0 (1.3)	60.3%	0.13
36 h	11.5 (1.7)	5.9 (2.0)	67.9%	0.17

Table 2.3: Number of house flies (out of 25) captured in olfactometer test arms in 20 min, using the final olfactometer design. Results are shown by test day. Treatment was 0.5 g citrus mealybug honeydew; the control was 2 mL deionized H_2O. Each trial date had n = 8 replicates except 6/24 which had 7. Trials were repeated on 5 different days (n = 39 replicates total). P-values in bold indicate statistical significance. % at treatment is the number of flies captured in the treatment arm over the total flies captured.

Date	Treatment (SEM)	Control (SEM)	% at treatment	p-value
6/24/11	10.85 (1.7)	0.71 (0.2)	94.7	**0.001**
8/25/11	14.63 (1.7)	5.00 (0.9)	73.1	**0.02**
8/30/11	14.63 (2.0)	5.63 (1.4)	70.6	**0.029**
8/31/11	13.25 (0.9)	5.00 (0.7)	72.6	**0.0003**
9/1/11	10.38 (1.2)	6.75 (1.6)	62.1	0.182
Overall Mean	12.79 (0.7)	4.70 (0.6)	73.1	**<0.0001**

Figures

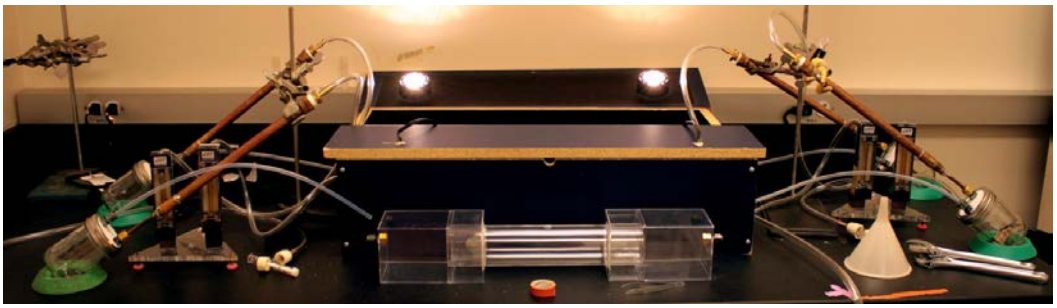

Figure 2.1: The final olfactometer design. A two–choice olfactometer comprised of an acrylic tube with funnels leading to terminal collection boxes. These were connected with Teflon tubing to a jar containing the odor source or control. Air was pulled through the system by a vacuum source connected to an outlet in the top of the central chamber.

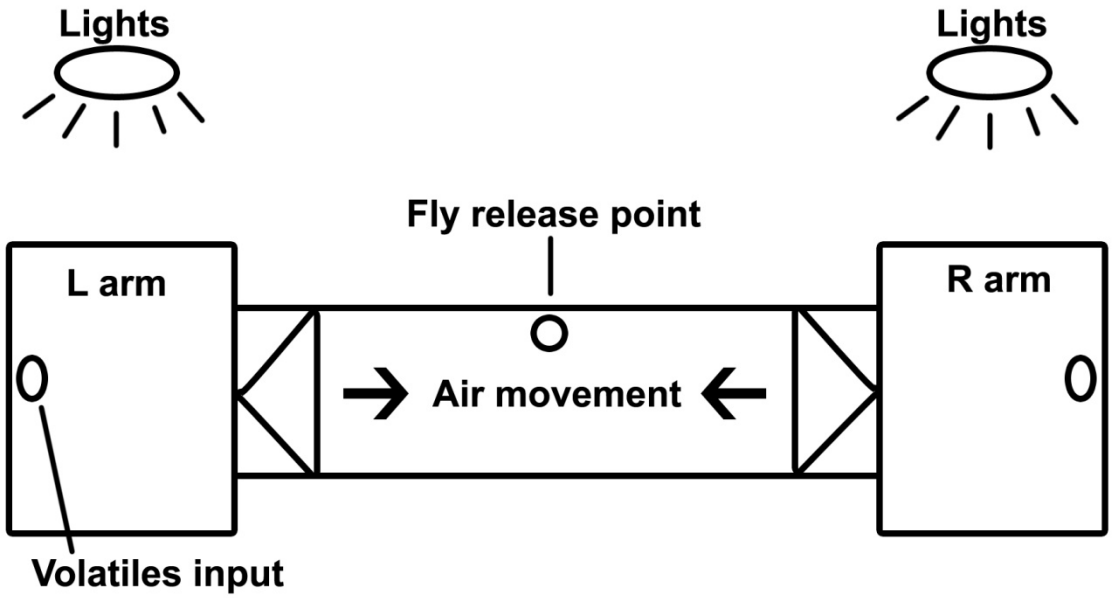

Figure 2.2: Diagram of olfactometer. The olfactometer was enclosed inside a dark box during the bioassay period. Lights were attached to the inside top of the box above each arm. The triangles in the diagram are the funnels that allowed the flies to move into the olfactometer arm but limited flies returning to the central chamber. Flies were placed inside the olfactometer through the fly release port, and a vacuum drew air from this port, pulling air from both arms towards the center. Each arm was connected to an odor source not displayed in the diagram.

Figure 2.3: Mealybugs on squash in colony. The length of time each squash was in the mealybug colony increases from the recently added squash on the left, to the squash most heavily colonized by mealybugs and contaminated with honeydew on the right. Honeydew accumulating on the floor of the cage beneath the squash was used to test fly responses.

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CHAPTER 3

HOUSE FLY (*MUSCA DOMESTICA* L.) ATTRACTION TO INSECT HONEYDEW

Abstract

House flies are of major concern as vectors of food-borne pathogens to food crops. House flies are common pests on cattle feedlots and dairies, where they develop in and feed on animal waste. By contacting animal waste, house flies can acquire human pathogenic bacteria such as *Escherichia coli* and *Salmonella* spp., in addition to other bacteria, viruses, or parasites that may infect humans and animals. The subsequent dispersal of house flies from animal facilities to nearby agricultural fields containing food crops may lead to pre-harvest food contamination with these pathogens. We hypothesized that odors from honeydew, the sugary excreta produced by sucking insects feeding on crops, or molds and fungi growing on honeydew, may attract house flies, thereby increasing the risk of food crop contamination. House fly attraction to honeydew-contaminated plant material was evaluated using a laboratory bioassay. House flies were attracted to the following plant-pest-honeydew combinations: citrus mealybug on squash fruit, pea aphid on faba bean plants, whitefly on navel orange and grapefruit leaves, and combined citrus mealybug and cottony cushion scale on mandarin orange leaves. House flies were not attracted to field-collected samples of lerp psyllids on eucalyptus plants or aphids on crepe myrtle leaves. Fungi associated with field-collected honeydews were isolated and identified for further study as possible emitters of volatiles attractive to house flies. Two

fungal species, *Aureobasidium pullulans* and *Cladosporium cladosporioides,* were repeatedly isolated from field-collected honeydew samples. Both fungal species were grown in potato dextrose enrichment broth and house fly attraction to volatiles from these fungal cultures was evaluated. House flies were attracted to odors from *A. pullulans* cultures but not to those of *C. cladosporioides*. Identification of specific honeydew odors that are attractive to house flies could be valuable for the development of improved house fly baits for management of this pest species.

Introduction

Food-borne pathogens including *Escherichia coli*, *Salmonella* spp., and *Campylobacter* spp. are persistent and widespread problems in food production and preparation (Greenberg 1973, Elder et al. 2000), and the Centers for Disease Control (CDC) estimates that there are 48 million cases of food-borne illness in the United States each year (Elder et al. 2000). Leafy vegetables were the second most common food commodity associated with Shiga toxin-producing *E. coli* outbreaks and are considered one of the top commodities for food-borne disease outbreaks (Elder et al. 2000). Flies magnify the risk of food-borne disease by contacting animal waste, carrying pathogens, and subsequently dispersing throughout the surrounding area, transporting pathogens from places where they pose little risk to humans, such as at animal rearing facilities, to places where the risk is greatly amplified (Lysyk and Axtell 1986, Wang, Chang, et al. 2011). However, the extent to which flies contribute to the maintenance and spread of

pathogens within and among livestock operations and the community is not well documented. Flies may play a key role in the distribution of human pathogens from domestic livestock to human food crops (Talley et al. 2009), but it is still unknown whether flies are attracted to human food crops or simply encounter them randomly during undirected movement through the environment.

Filth flies, including the house fly (*Musca domestica* L.), are closely associated with animal waste, from which they can acquire a variety of potentially pathogenic protozoa, viruses, and bacteria (Greenberg 1971, Olsen 1998, Graczyk et al. 2001). For example, in Japan, house flies were identified as the biotic factor most closely associated with the spread of pathogenic *E. coli* from livestock to children (Sasaki et al. 2000). Furthermore, area-wide reductions in house fly abundance have been correlated with concurrent reductions in human illness due to enteric pathogens (Watt and Lindsay 1948, Lindsay et al. 1953, Cohen et al. 1991, Levine and Levine 1991, Chavasse et al. 1999). The dispersal of antibiotic resistant bacteria from animal and human waste to the environment is also of increasing concern (Chakrabarti et al. 2010, Doud et al. 2014).

In 2006, an outbreak of *Escherichia coli* O157:H7 associated with bagged spinach affected over 200 individuals in 26 US states (CDC 2006). Subsequent studies in the region where the contaminated spinach originated resulted in the capture of filth flies carrying this pathogen on leafy greens (spinach and lettuce) in field crops adjacent to a cattle pasture (Talley et al. 2009). Filth flies may have contaminated pre-harvest leafy greens with human pathogens by deposition of fly feces and/or regurgitant onto the leaves (Wasala et al. 2013). However, filth flies are not typically associated with leafy

green crops, and flies that typically develop in crop waste would not be expected to harbor human or animal pathogens such as *E. coli* O157:H7. Therefore, it is likely that *E. coli*-contaminated flies captured in leafy green crop fields had previous contact with *E. coli*-contaminated animal feces in a nearby cattle pasture (Talley et al. 2009).

Within a field of leafy greens, filth flies are more abundant where honeydew-producing insects are present (Talley et al. 2009). The authors have observed house flies feeding on honeydew (Figures 3.2 and 3.3), a carbohydrate-rich excretion produced by phloem-feeding insects (Downes and Dahlem 1987), presumably to obtain the carbohydrates needed to sustain flight or other physiological functions (Stanfield and Hunter 2010). House fly dispersal behavior is poorly understood, but odors from honeydew and/or fungi growing on honeydew-contaminated crop plants may attract flies. Alternatively, flies may simply accumulate on honeydew-contaminated crop plants while feeding on the available sugars, i.e., arrestment rather than attraction. Some insects orient toward honeydew odors as an indicator of food or oviposition sites (Wang, Johnson, et al. 2011, Leroy et al. 2012); however, honeydew sugars are not volatile or odorous. Thus, we hypothesized that house flies detect and orient toward honeydew by following odor plumes associated with honeydew, and that fungal digestion of honeydew sugars may produce such odors. Thus, our project objectives were:

1. To assess house fly attraction to odors from several insect honeydew and host-plant combinations, and
2. To assess house fly attraction to odors produced by cultures of two fungi repeatedly isolated from field-collected honeydew.

Materials and Methods

Insect colonies:

House flies were collected by sweep net from a southern California dairy facility in 2010 with the owner's permission and subsequently maintained as a colony in an insectary at constant conditions of 25 ± 2°C, 40% relative humidity (RH), and 12L:12D photoperiod. Immature flies were fed a standard fly rearing medium (Mandeville et al. 1988), and adult flies were fed a 50:50 mixture of powdered milk and sucrose, and given water *ad libitum*. Citrus mealybugs (*Planococcus citri* Risso) were from an established UC Riverside colony maintained on butternut squash fruit at 25 ± 2°C, 40% RH and 14L:10D photoperiod in an insectary room. Pesticide-free butternut squash grown in the Agricultural Operations fields at UC Riverside were harvested, thoroughly washed with a dilute solution of micro90® detergent, rinsed with water, dried for 1-2 days, and stored at 15.5°C until use (up to 1 year). Pea aphids (*Acyrthosiphon pisum* Harris) were established at UC Riverside in 2009 from a colony maintained at Oklahoma State University. Pea aphids were reared in a greenhouse on faba bean plants (*Vicia faba* L.) and maintained under ambient daylight conditions.

Bioassays to assess house fly attraction:

Bioassays were conducted in a room maintained at 25 ± 2ºC, 40% RH, and 14L:10D photoperiod in the UC Riverside Insectary building. The ventilation system in the room provided a slight negative pressure with air pulled through a centrally

positioned ceiling vent creating airflow throughout the room. Prior to initiating bioassays, the entire room was scrubbed with unscented soap and water, and floor drains were sealed to reduce outside odors. Eight screen-cages (45 × 45 × 45 cm, Product no. 1450D, BioQuip Products, Rancho Dominquez, CA, USA) were set up on wire shelving with a 57-watt incandescent bulb within an 20.3-cm aluminum reflector housing placed 25 cm above each cage to provide light that was evenly distributed across each cage. Each cage contained two 2-L glass beakers to hold test materials. Beakers were washed with micro90® detergent and rinsed thoroughly with deionized water to remove odors prior to use in bioassays. For each bioassay period, treatment and control materials were randomly assigned to a beaker position (left or right side of the cage) in cage #1, and the position of the treatment and control material was then alternated in cages #2-8 to minimize within-cage position effects. The beakers were placed approximately 10 cm apart from each other and covered with mesh netting held in place with a rubber band to prohibit fly access to material held within the beaker. White sticky cards (Pherocon 1C Liner, Trécé, Adair, OK, USA) were folded into a tent shape, with the sticky surface on the inside, and fixed above each beaker with a metal wire (Figure 3.1).

Flies (3-5 d-old) were removed by aspirator from colony cages, sorted on a chill table (Bioquip Products, Rancho Dominquez, CA, USA) into 8 groups of 50 female flies (400 flies total), and each group was placed into a release chamber (947 ml clear plastic food cup, First Street™, Amerifoods Trading Co., Los Angeles, CA, USA) covered by a plastic lid with many small holes for air exchange and a 2.5 cm diameter hole blocked with a cork that could be easily removed allowing flies to exit. The release chamber

contained a 30 mL plastic cup (First Street™, Amerifoods Trading Co., Los Angeles, CA, USA) with water-soaked paper towel to provide moisture for the flies. Flies were starved in the bioassay room for 40-44 hr before use. The release chamber was used to slow the release of flies into the bioassay cage because early trials showed that a rapid release of flies into the assay cage resulted in substantial undirected flight and similar capture of flies on both treatment and control sticky cards within the first few minutes of the assay start time. An assay period thus began by placing a release chamber positioned equidistant from the treatment and control beakers into each of the 8 assay cages, followed by removal of the cork to allow starved flies to individually emerge from the release chambers during the 24 hr bioassay period, after which the number of flies captured on each sticky card was recorded.

House fly attraction to honeydew from colony insects:

Prior to use in an assay, clean butternut squash fruit were introduced into the citrus mealybug colony for at least 4 wk resulting in infestation of squash fruit by large numbers of nymph and adult mealybugs. This exposure period ensured sufficient production and deposition of mealybug honeydew onto the squash. Faba bean plants 30-45 cm tall were infested with ~ 50 pea aphids of different life stages and then held in the colony room for an additional exposure period of 2-3 wk to allow for sufficient production and deposition of aphid honeydew onto leaf surfaces. Uninfested faba bean plants were grown in a separate colony room under similar conditions and for the same 2-3 wk period. Following the aphid exposure period, both infested and uninfested faba bean

plants were cut near the base of the stem for placement into beakers within the bioassay cages.

The following bioassay comparisons were made using colony insects: (1) Infested squash were compared to an empty beaker during 4 separate bioassay periods to evaluate variation in fly response by cage and by assay period; (2) Mealybug-infested squash were compared to uninfested squash during two bioassay periods, with both infested and uninfested squash incubated in the citrus mealybug colony room for the same 4 wk exposure period; (3) Uninfested squash were compared to empty beakers to evaluate fly attraction to the squash alone; (4) Uninfested squash punctured 30× with a needle to simulate insect feeding damage and then incubated in the mealybug colony room for 2 wks until signs of decay were obvious were compared to intact, uninfested squash to evaluate whether house flies were attracted to damaged and decaying fruit alone; 5) Pea aphid-infested faba bean plants were compared to uninfested faba bean plants (Table 3.1). Four cages were removed from comparison (1) and one cage was removed from comparison (4) due to flies accessing the beaker materials through gaps or tears in the mesh covering.

Collection of honeydew and associated fungi from field sites:

Plant stems and leaves with accumulated honeydew and without honeydew (control) were collected using latex or nitrile gloves to avoid contamination with human skin odors. All honeydew-contaminated and control samples were pruned from the plant with sterile shears, stored in oven bags (Terinex Ltd., Bedford, England) to maintain

odors, and placed on ice for transport back to the laboratory for bioassays. Transport of plant material and honeydew was approved by the California Department of Food and Agriculture (permit #2776). Navel orange (*Citrus sinensis* Osbeck) and marsh grapefruit (*Citrus paradisi* Macfadyen) leaves infested with whiteflies (Family: Aleyrodidae) were collected from a single site in Riverside, CA. These two types of citrus plants were maintained as separate samples for fungal isolation but were combined together in equal amounts by mass for house fly attraction bioassays. Similarly, cottony cushion scale (*Icerya purchasi* Maskell) and citrus mealybugs on honey mandarin orange trees (*Citrus reticulata* Blanco) were collected from a single site at the UC Riverside Agricultural Operations citrus groves and maintained as separate samples for fungal isolation but were combined together for bioassay. Eucalyptus leaves (tentatively river red gum, *Eucalyptus camaldulensis* Dehnh) infested with lerp psyllids (*Glycaspis brimblecombei* Moore) were collected from a eucalyptus grove on the UC Riverside campus. Lerp psyllid honeydew was also collected on leaves from a red ironbark eucalyptus tree (*Eucalyptus sideroxylon* A. Cunn.) in Riverside, CA. Data from one cage was not included in analyses due to flies accessing the beaker materials. A second collection of foliage from the same red ironbark eucalyptus with lerp psyllid honeydew received ~20 mL of water in the collection bag and was incubated at 60% relative humidity and 25°C in the insectary room for one day before evaluation in the bioassay. Crepe myrtle (*Lagerstroemia* sp.) cuttings infested with an unidentified aphid species were collected from a residential yard with the owner's permission in Riverside, CA. Because plant material and honeydew dries out quickly after collection, attraction of house flies to each of these field-collected honeydews was

evaluated within 48 hr of collecting material from the field. If available, uninfested material from the same plant or a nearby plant of the same species was used in control beakers, otherwise control beakers contained nothing (Table 3.2).

For each honeydew-contaminated and uncontaminated (control) sample taken from the field, a small portion (13-33 g) was removed and washed with 15-30 mL deionized water (dH_2O), where wash volume depended upon the volume of the field sample. Similar methods were used for host plant materials from citrus mealybug and pea aphid colonies, except that 8 week-old butternut squash infested with citrus mealybugs and clean butternut squash controls were gently washed with 30 mL of dH_2O and the runoff was collected. Following their use in bioassays and for fungal collection, all plant material and honeydew residues were autoclaved before disposal.

The wash water from each sample was frozen and shipped overnight on ice to the Kearney Agricultural Research and Extension Center (KAREC), where 100 µl of leaf wash was diluted in sterile water as needed (1:10 - 1:1000) to obtain isolated colonies on a plate of growth medium. Thus, 100 µl of the dilution was applied to 4 Petri dishes containing acidified potato dextrose agar (APDA; 39 g potato dextrose agar acidified with 2.4 ml of a 25% v:v lactic acid per L medium) and incubated at 25°C for 5 d. The colony forming units (CFU) for each fungal species on the plates were counted, and the mean CFUs per plate were used to calculate the original concentration (CFU/100µl) in each wash sample by multiplying the colony counts by the dilution factor.

Fungi were identified to genus or species when possible by morphology and color. Polymerase chain reaction (PCR) and sequencing was used to verify species

identification of *Aureobasidium* sp., *Cladosporium* sp., and *Rhodotorula* sp. Single-spore isolates were grown on 2% potato dextrose agar (PDA) for 7–10 d at 25±3 °C for DNA extraction. Pure culture mycelia were scraped directly from the medium using a sterile scalpel and the total genomic DNA was extracted using a FastDNA Kit (BIO 101, Inc., Vista, CA, USA). The internal transcribed spacer (ITS) regions, including ITS1, ITS2, and the 5.8S rRNA gene of the ribosomal DNA (rDNA), were amplified using primers ITS1 and ITS4 (White et al. 1990). The PCR of ITS regions were conducted according to previous studies (Slippers et al. 2004, Chen et al. 2011). An UltraClean PCR Clean-Up Kit (MO BIO Laboratories, Inc., Solana Beach, CA, USA) was used to purify the PCR products. The resulting amplicons were sequenced in both directions using the same primers used for the PCR reactions. Sequence reactions were run on an automated sequencer by the University of California-Davis, Division of Biological Sciences sequencing facility and the sequences were compared with the ex-type isolates of the fungal species for definitive identification. *Fusarium* sp. from the citrus mealybug colony was handled similarly as above except the DNA was extracted using an AllPrep DNA/RNA/miRNA Universal Kit (Cat. No. 80224, Qiagen Sciences, Germantown, MD, USA) and purified using Diffinity RapidTip (Cat. No. RT025-008, Diffinity Genomics, Inc., West Henrietta, NY, USA). The resulting amplicons were sequenced at the University of California, Riverside, Genomics Core sequencing facility using the ITS1 primer. The sequence was input into the NCBI's BLAST sequence database and identified to 99% identity. Identified fungi were isolated and grown on individual APDA

plates and stored at 4°C up to 6 mo or placed in 25% glycerol at -80°C for longer term storage.

In preparation for use in bioassays, isolated fungi from the honeydew-contaminated plant washes were plated onto APDA and shipped overnight to UC Riverside, then stored at 4°C until use. Autoclaved potato dextrose broth (PDB) media (250 mL) was inoculated with either *Aureobasidium pullulans* or *Cladosporium cladosporioides* and grown at 25°C under otherwise sterile conditions. *Cladosporium cladosporioides* was incubated for 2 wks while stirring constantly to achieve some degree of homogeneity throughout the media. *Aureobasidium pullulans* was incubated for 4 wks and allowed to grow as a layer on the surface of the media. About 30 mL aliquots of the *C. cladosporioides* culture or equal sized sections of the *A. pullulans* surface layer were placed into 8 glass petri dishes (9 cm diameter) that had been washed with micro90® detergent, rinsed with tap water, deionized water and acetone, then baked at 140°C to remove any residual odors. Petri dishes containing either the fungal cultures or sterile PDB (uninoculated and held under sterile conditions for the same incubation time) were placed into treatment or control glass beakers, respectively, for the bioassay as described above. House fly attraction to *C. cladosporioides* was evaluated during one bioassay period while attraction to *A. pullulans* was evaluated over two bioassay periods to confirm the significant attraction noted in the first bioassay period.

Data Analysis

For bioassay comparison #1 (mealybug-infested squash vs. nothing) the number of flies captured on each sticky trap (treatment and control) during each bioassay period was log transformed to normalize the data and then analyzed for differences among assay cage, assay period, and treatment (and for interactions among these factors) using multi-factorial ANOVA with treatment means separated using Fisher's least significant difference (LSD) test. All remaining bioassay comparisons were analyzed by calculating the difference between the number of flies captured on the sticky cards above the treatment and the control in each cage (diff = treatment – control) and using a one sample t-test to compare the observed difference values to an expected value of zero, if the treatment and control were not different in their attraction to flies. The Shapiro-Wilk test was used to verify normality of all data sets prior to analysis. All analyses were performed using SAS statistical software v9.4 (Cary, NC, USA).

Results

House fly attraction to laboratory-reared honeydew-producing insects:

House flies were significantly more attracted to mealybug-infested squash relative to the empty beaker control ($F = 72.87$; $df = 1,55$; $p < 0.0001$) (Table 3.1). There were significant differences in the total number of flies captured among assay periods ($F = 12.6$; $df = 3,55$; $p = 0.0001$) indicating variable participation by each cohort of flies used,

but no differences among assay cages ($F = 1.43$; df = 7,55; $p = 0.26$) and no interactions between treatment and assay period ($F = 2.53$; df = 3,55; $p = 0.092$) or treatment and assay cage ($F = 1.42$; df = 7,55; $p = 0.26$). Thus, we deemed the bioassay suitable for evaluation of house fly responses to odors.

House flies were significantly attracted to mealybug-infested squash relative to uninfested squash ($t = 3.56$; df = 1,15; $p = 0.0028$), but not to uninfested squash relative to an empty beaker ($t = 0.51$; df = 1,7; $p = 0.62$) or to needle-damaged, decaying squash relative to uninfested/undamaged squash ($t = 0.28$; df = 1,6; $p = 0.79$). Flies were also attracted to aphid-infested faba bean plants relative to uninfested faba bean plants ($t = 3.39$; df = 1,7; $p = 0.012$).

House fly attraction to field-collected host plants infested with sucking insects:

House flies were attracted to combined samples of whitefly-infested orange and grapefruit leaves ($t = 2.73$; df = 1,7; $p = 0.029$) and to citrus mealybug and cottony cushion scale-infested mandarin orange leaves ($t = 3.58$; df = 1,7; $p = 0.009$) (Table 3.2). In contrast, flies were not attracted to lerp psyllid-infested foliage of red ironbark eucalyptus ($t = 1.7$; df = 1,7; $p = 0.13$). Wetting the lerps on red ironbark eucalyptus foliage followed by incubation for 24 hr under humid conditions did not increase fly attraction ($t = 1.6$; df = 1,7; $p = 0.15$). Flies were also not attracted to aphid-infested crepe myrtle ($t = 0.85$; df = 1,7; $p = 0.43$) or to lerp psyllid-infested red river gum eucalyptus leaves ($t = 0.00$; df = 1,6; $p = 1.0$).

House fly attraction to volatiles of fungi isolated from honeydew-contaminated plants:

Aureobasidium pullulans and *C. cladosporioides* were identified in higher concentrations from most of the field collected honeydew samples relative to the corresponding uninfested (control) plant material. Neither fungus was detected from the laboratory cultures of citrus mealybug on squash, where *Fusarium solani* was predominant (Table 3.3). Other fungi isolated from wash water samples of field-collected, honeydew-contaminated plant materials included *Rhodotorula* sp., *Penicillium* sp., *Alternaria alternata*, *Fusarium* sp., *Aspergillus niger*, and *Rhizopus stolonifer*. However the isolation of these fungi was inconsistent across honeydew samples or their concentrations in the honeydew samples were similar to the concentrations in wash samples from uninfested plant material. House flies were significantly attracted to odors from *A. pullulans* cultured in PDB ($t = 3.62$; $df = 1,15$; $p = 0.0025$) but not to odors from *C. cladosporioides* in PDB ($t = 0.64$; $df = 1,7$; $p = 0.54$) relative to sterile broth (Table 3.4).

Discussion:

House flies were attracted to odors associated with honeydew produced by a range of honeydew-producing insects, including citrus mealybug, pea aphid, whitefly, and cottony-cushion scale. To our knowledge, this is the first study to demonstrate house fly attraction to odors associated with honeydew, although house fly feeding on honeydew has previously been reported (Downes and Dahlem 1987). The honeydew-

contaminated plant materials that were attractive to house flies were generally wet and sticky, with microbial growth on the honeydew deposits. In contrast, the non-attractive lerp psyllid-infested eucalyptus leaves were dry and showed no visible evidence of fungal or microbial growth on the leaves or on the crystallized honeydew forming the “lerps”. Whereas the authors have observed house flies feeding on lerps in the field (Figure 3.3), the flies may have encountered the lerps during undirected movement rather than being attracted to them from a distance. Strong odors from the eucalyptus leaves may also repel flies or disrupt their detection of honeydew odors. Incubating lerps on eucalyptus leaves under high humidity conditions for 24 hr prior to conducting bioassays did not increase fly attraction, possibly due to insufficient time for fungi or other microorganisms to colonize the recently wetted lerp honeydew. We made no attempt to test fly attraction to lerp honeydew wetted for a longer period of time given the focus of this study on attraction to honeydews recently obtained from a field site. Although the aphids infesting crepe myrtle leaves released wet, sticky honeydew and leaves had some visible mold growth, the amount of honeydew and level of aphid infestation was relatively low, which may explain the lack of house fly attraction to this test substrate.

It is difficult to separate attraction to honeydew from attraction to honeydew-producing insects when evaluating house fly responses to field collected honeydews, because these samples often included some honeydew-producing insects or insect detritus. However, attraction solely to host plant volatiles was not noted in this study. In the absence of any honeydew, house flies were not attracted to volatiles of either intact squash or needle-damaged, decaying squash as a surrogate for an insect-damaged host

plant. Furthermore, most bioassays used uninfested plant material as the control suggesting that flies were attracted to odors from honeydew-producing insects, honeydew, fungi or other honeydew colonizers, or to some combination of these rather than to plant volatiles alone. Nevertheless, enhancement of house flies attraction to honeydew odors as a result of the presence of host plant volatiles cannot be excluded.

Honeydew is rich in simple and complex carbohydrates (Wäckers 2001), and is known to be a food source for dipteran species (Moore et al. 1987, Schutz and Gaugler 1989, MacVicker et al. 1990, Foster 1995, Burgin and Hunter 1997, Hunter and Ossowski 1999). However, attraction of insect species in general to odors associated with honeydew has received relatively little study. Aphid honeydew is known to attract some aphid predators including the Asian lady beetle, *Harmonia axyridis* (Pallas), (Leroy et al. 2012) and the aphidophagous gall midge, *Aphidoletes aphidimyza* (Rondani) (Choi et al. 2004). Honeydew also stimulates oviposition by the aphidophagous hoverfly, *Episyrphus balteatus* (De Geer) (Leroy et al. 2010). Thus, for aphid parasitoids and predators at least, honeydew or odors associated with honeydew appear to serve as general kairomones for predation or oviposition. On the other hand, honeydew was not attractive to wild *Aedes albopictus* (Skuse) mosquitoes when tested under field conditions (Müller et al. 2011) despite observations of mosquitoes feeding on honeydew in the field (Foster 1995).

Two fungi (*A. pullulans* and *C. cladosporioides*) were repeatedly isolated from field-collected plant materials contaminated with honeydew that were attractive to house flies in laboratory bioassays. Volatile odors from one of these fungi (*A. pullulans*) grown on PDB media were attractive to house flies in the absence of any honeydew or insect

odors, suggesting that house fly attraction to honeydew may be due, at least in part, to odors produced by this fungus. These odors are likely byproducts from the metabolic breakdown of the honeydew by the fungi (Davis et al. 2013). In a related example, odors produced by bacteria isolated from honeydew have been shown to attract syrphid flies (Leroy et al. 2011).

Aureobasidium pullulans is a yeast-like fungus which is commonly found in soil and on plant surfaces. It also constitutes the dark sooty mold that colonizes leaves and is aptly named "black yeast" (Cooke 1959). Odors from *A. pullulans* have been previously shown to attract *Vespula* spp. wasps (Davis and Landolt 2013) and increase the number (particularly Diptera) and diversity of insects captured in an agricultural field (Davis et al. 2012). Because of the ubiquity of this fungus as a colonizer of insect honeydews, odors resulting from metabolism of honeydew by *A. pullulans* might signal the availability of a carbohydrate-rich food source. Fermentation odors produced by *A. pullulans* may therefore be general insect attractants, as suggested by Davis and Landolt (2013).

The lack of house fly attraction to odors from the fungus *C. cladosporioides* grown on PDB does not prove that house flies would not be attracted to volatiles produced by this fungus when growing on honeydew under field conditions. In particular, odors produced as a byproduct of metabolism may vary depending upon the media on which microbial species are grown (Beltran et al. 2008). Interestingly, the concentration of *C. cladosporioides* was low in the non-attractive honeydew from aphids on crepe myrtle leaves and from lerp psyllids on eucalyptus leaves, whereas the concentration of *C. cladosporioides* was very high in the attractive honeydew from whiteflies on marsh

grapefruit and on navel orange, cottony cushion scale and citrus mealybugs on honey mandarin, and pea aphids on faba bean. This suggests that odors produced by *C. cladosporioides* may act synergistically with *A. pullulans* odors, or that *C. cladosporioides* needs specific substrates or different growing conditions in order to produce odors attractive to house flies.

In contrast, butternut squash infested with citrus mealybugs that attracted flies in laboratory bioassays did not yield either *A. pullulans* nor *C. cladosporioides*, but was heavily colonized by *F. solani*. It remains to be determined whether the attraction seen was due to *F. solani* producing a volatile profile similar to *A. pullulans*, or whether the two fungal species have markedly different, but still attractive, odor profiles.

Overall, the results from this study support our hypothesis that honeydew production by sucking insects infesting food crops may contribute to attraction of house flies to those crops, particularly when they are grown in proximity to animal rearing facilities or other sites that produce large numbers of house flies. Thus, managing honeydew-producing insects on food crops may reduce house fly visitation, and consequently the risk of crop contamination with food-borne pathogens.

Growing concerns about insecticide resistance and the environmental impact of chemical insecticides are driving the search for alternative pest control methods, including “attract and kill” strategies using attractive odors to draw pest insects into traps (El-Sayed et al. 2009). A few studies have identified odors that are attractive to house flies, most of which are odors associated with animal feces or are products of fermentation or decay (Wieting and Hoskins 1939, Brown et al. 1961, Frishman and

Matthysse 1966, Cossé and Baker 1996, Quinn et al. 2007, Qian et al. 2013). Some of these odors are unpleasant to humans and may target female flies seeking a protein source or oviposition site rather than a sugar-based food source. Although levels of fly attraction to plant materials contaminated with insects and honeydew versus controls in this study was modest, with 60-65% of flies captured at honeydew, the isolation and identification of specific honeydew odors attractive to house flies could provide useful alternate attractants for comparison with currently known attractants.

Acknowledgments:

We thank the UC Cooperative Extension Farm Advisors, commercial farm managers, and local gardeners who assisted with honeydew collection. We thank Dr. Greg Walker for providing the pea aphid colony and Dr. Mark Hoddle for providing the citrus mealybug colony. Ryan Puckett and ShuaiFei Chen assisted with enumerating and identifying fungi from honeydew samples. DOI: 10.1371/journal.pone.0124746

Table 3.1: **House fly attraction to plant materials infested with honeydew-producing insects.** Fifty female flies were used per replicate, with 8 replicates per 24 hr bioassay period. Data were analyzed with ANOVA for the first comparison and by paired t-test for the remaining comparisons within each row. "% Response to treatment" is the total number of house flies captured on the treatment over the total flies captured on both treatment and control.

Treatment	Control	Treatment mean captures ± SEM	Control mean captures ± SEM	P-value	% Response to treatment	Reps
Citrus mealybug on squash	Nothing	13.68 ± 0.99	7.21 ± 0.48	$p < 0.0001$	65.5	28*
Citrus mealybug on squash	Uninfested squash	19.1 ± 1.34	12.6 ± 0.87	$p = 0.0028$	60.3	16
Uninfested squash	Nothing	8.75 ± 1.29	9.88 ± 1.38	$p = 0.62$	47.0	8
Needle-damaged squash	Uninfested squash	9.57 ± 2.77	8.43 ± 1.65	$p = 0.79$	53.2	7*
Pea aphid on faba bean plant	Faba bean plant, not infested	18.4 ± 1.34	10.3 ±1.26	$p = 0.012$	64.1	8

*Indicates that 1 or more replicates were discarded due to a cage failure allowing flies to access the test material within a beaker.

Table 3.2: **House fly attraction to field-collected plant materials contaminated with honeydew.** Fifty female flies were used per replicate, with a maximum of 8 replicates per 24 hr assay period. Data were analyzed by paired t-test for comparisons within each row. "% Response to treatment" is the total number of house flies captured on the treatment over the total flies captured on both treatment and control.

Treatment	Control	Treatment mean ± SEM	Control mean ± SEM	Paired t-test	% Response to treatment	Reps
Citrus mealybug and cottony cushion scale on honey mandarin	Honey mandarin cuttings, not infested	12.5 ± 0.63	8.5 ± 1.09	p = 0.009	59.5	8
Whitefly on marsh grapefruit and navel orange	Beaker only	11.6 ± 1.22	7.6 ±1.12	p = 0.029	60.4	8
Lerp psyllid on red ironbark eucalyptus	Red ironbark eucalyptus, not infested	14.9 ± 1.04	12.3 ±0.92	p = 0.13	54.8	8
Lerp psyllid on red ironbark eucalyptus incubated under high humidity 24 hr	Beaker only	7.9 ±0.77	6.0 ± 0.96	p = 0.15	56.8	8
Lerp psyllid on river red gum eucalyptus	River red gum eucalyptus, uninfested	7.7 ± 0.70	7.7 ±0.76	p = 1.0	50.0	7*
Aphids on crepe myrtle	Crepe myrtle, uninfested	20.3 ±2.68	16.9 ±1.64	p = 0.43	54.5	8

*One or more replicates were discarded due to a cage failure allowing flies to access the test material within a beaker.

Table 3.3: **Fungi collected from honeydew-contaminated plant material.** Numbers are presented as colony forming units/100 µl or CFU/100ul. Rows above the double line are the honeydew-contaminated plant materials that were attractive to house flies. Rows below the double line were materials that were not attractive to flies. "Tmt" is treatment leaves or fruit containing insects and honeydew. "Ct" is control leaves or fruit with no visible insects or honeydew.

		Aureobasidium pullulans		***Cladosporium cladosporioides***		***Rhodotorula spp.***		***Penicillium spp.***		***Alternaria alternata***		***Fusarium spp.***		***Aspergillus niger***	
Host plant	**Insect**	**Tmt**	**Ct**	**Tmt**	**Ct**	**Tmt**	**Ct**	**Tmt**	**Ct**	**Tmt**	**Ct**	**Tmt**	**Ct**	**Tmt**	**Ct**
Butternut squash fruit	Citrus mealybug colony	0	0	0	0	0	0	0	0	0	0	55000	0	0	0
Faba bean leaves	Pea aphid colony	26	0	109	15	11	0	5	0.25	0	0	7.5	0	6	1
Navel Orange Leaves	Whitefly	6490	450	1030	12	0	0	70	6	30	18	30	8	0	0
Grapefruit Leaves	Whitefly	>30000	680	>10000	128	0*	0	0*	0	0*	20	0*	0	0*	0
Mandarin leaves	Citrus Mealybug	4400	0	556	44	0	0	0	0	0	0	0	0	0	0
Mandarin leaves	Cottony cushion scale	9872	0	1294	44	0	0	0	0	0	0	0	0	0	0
Eucalyptus, red ironbark leaves	Lerp psyllid	347	58	38	0	0	0	256	0	13	15	0	0	0	0
Eucalyptus, river red leaves	Lerp psyllid	3625	1195	6	5	0	0	0	0	0	1	0	0	0	0
Crepe myrtle leaves	Aphid	>225000	207	2	1	0	0	0	0	1	0	0	1	0	0

* Indicates there was overgrowth of the *Aureobasidium* and *Cladosporium* so that other specimens could not be identified**.**

Table 3.4: **House fly attraction to odors from fungi isolated from honeydew and cultured on potato dextrose broth (PDB).** Fifty female flies were used per replicate, with 8 replicates tested per 24 hr-assay period. Data were analyzed by paired t-test for comparisons in each row. "% Response to treatment" is the total number of house flies captured on the treatment over the total flies captured on both treatment and control.

Treatment	**Control**	Treatment mean captures ± SEM	Ctrl mean captures± SEM	Paired t-test	% Response to treatment	**Reps**
Aureobasidium pullulans	PDB only	9.75 ± 0.78	6.06 ± 0.61	p = 0.0025	61.7	16
Cladosporium cladosporioides	PDB only	13.25 ± 1.76	11.63 ± 1.03	p = 0.54	53.3	8

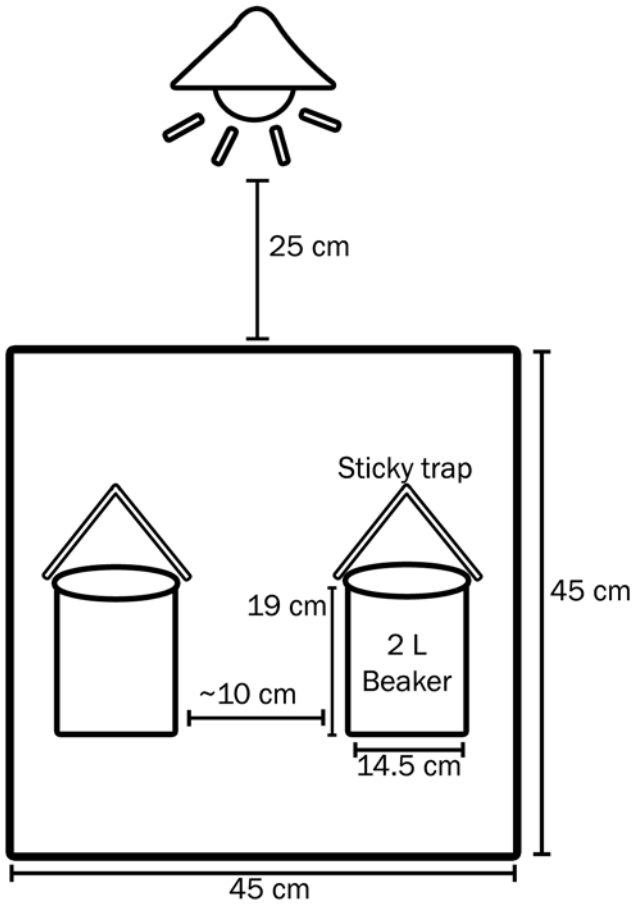

Figure 3.1: **A diagram of a cage bioassay set up.** Beakers containing either honeydew and plant material or a control were placed about 10 cm apart in a 45 × 45 × 45 cm cage. Sticky traps (sticky surface down) placed above each beaker captured flies near the beaker's opening.

Figure 3.2: **House flies feeding on soft scale honeydew**. Honeydew was produced by soft scales infesting jacaranda trees in Chino, CA. This location is less than 1 mile from an agricultural animal facility. Image was taken by ACG.

Figure 3.3: **House flies feeding on "lerp" honeydew**. Honeydew was produced by lerp psyllids infesting unidentified eucalyptus trees in Bakersfield, CA. These trees were in the proximity of an animal agricultural facility. Image was taken by ACG.

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CHAPTER 4

IDENTIFICATION OF HONEYDEW VOLATILES ELICITING A HOUSE FLY ANTENNAL RESPONSE

Abstract

House flies (*Musca domestica* L.) are mechanical vectors of food-borne pathogens including *Salmonella* spp., *Eschirichia coli* O157:H7, and *Shigella* spp., resulting in increased risk of diarrheal disease in areas where flies are abundant. House flies were previously found to be attracted to honeydew-infested plant material, potentially increasing the risk of pathogen transfer to food crops infested by honeydew-producing insects. We used gas chromatography-electroantennogram detection (GC-EAD) and gas chromatography-mass spectrometry (GC-MS) to identify possible active compounds from honeydew materials known from previous research to be attractive to house flies. Volatile compounds identified from honeydew aeration samples that elicited antennal responses from house flies included benzaldehyde, butyl hexanoate, β-caryophyllene, Δ3 carene, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (*Z*)-3-hexenyl acetate, myrcene, limonene, linalool, and naphthalene. Benzaldehyde, β-caryophyllene, (*Z*)-3-hexenyl acetate, and myrcene were identified from honeydew produced by whiteflies on both navel orange and Marsh grapefruit trees under natural field conditions, whereas benzaldehyde was identified from these whitefly honeydews as well as honeydew from a

laboratory colony of pea aphids. Further experiments are necessary to identify whether house fly antennal responses are indicative of attraction to these volatiles.

Introduction

Filth flies, aptly named due to their close association with animal feces and other decaying organic matter, include flies in the families Muscidae (house flies) and Calliphoridae (blow flies). House flies (*Musca* domestica L.) are known to be mechanical vectors of bacteria, viruses, and protozoans that affect human and animal health (Greenberg 1973, Olsen 1998, Graczyk et al. 2001). Area-wide management of house fly populations has reduced incidence of enteric disease in human populations, implicating house flies as mechanical vectors of these pathogens (Watt and Lindsay 1948, Lindsay et al. 1953, Lindsay and Scudder 1956, Cohen et al. 1991, Levine and Levine 1991, Chavasse et al. 1999). House flies can mechanically transmit pathogens from locations where they pose little risk to human health to locations where human health might be at considerable risk (Lysyk and Axtell 1986, Chakrabarti et al. 2010, Wang et al. 2011). Recently, filth flies were implicated as a factor in *Escherichia coli* O157:H7 contamination of lettuce and other leafy greens produced for human consumption (Talley et al. 2009). These fields were adjacent to cattle pasture where flies likely acquired the pathogen through contact with cattle manure prior to dispersal into the food crop. House flies contaminated with *E. coli* might be expected to deposit contaminated regurgitant or

feces onto plants when resting or feeding on these plants (Sasaki et al. 2000, Wasala et al. 2013).

Leafy vegetables such as spinach and lettuce may be infested with honeydew-producing insects including aphids and whiteflies, leading to accumulation of honeydew, the sugary excretion produced by sucking insects, on the leaves. Heavy infestations of honeydew-producing insects or honeydew production over a long time may lead to an accumulation of the sticky material on the infested plants. A previous study found that house flies were attracted to plants contaminated with insect honeydew (Hung et al. 2015). Therefore, flies may play a role in contamination of leafy greens through their attraction to insect honeydew on those plants.

Previous studies have identified a number of natural materials which are attractive to house flies, including rotten eggs (Willson and Mulla 1973), animal feces (Cossé and Baker 1996), fermenting yeast and milk (Mulla et al. 1977), fermented grain (Pickens et al. 1973), malt (Brown et al. 1961), vinegar (Qian et al. 2013), dried blood (Pickens et al. 1973), and alcohol (Wieting and Hoskins 1939, Brown et al. 1961). Volatile compounds produced by these attractant sources include urea, skatole, indole, benzyl alcohol, butyl alcohol, trimethylamine, ammonia, linoleic acid, n-butyric acid, butanoic acid, 3-methylbutanoic acid, and ethanol (Frishman and Matthysse 1966, Mulla et al. 1977, Hwang et al. 1979, Cossé and Baker 1996, Quinn et al. 2007). Pure, sterile aqueous sugar solutions are not attractive to house flies (Richardson 1917) because the sugars are completely nonvolatile; however, house flies are attracted to sugar-based substances such as malt extract (Frishman and Matthysse 1966), fermented sucrose (Hwang et al. 1978),

molasses (Brown et al. 1961, Hwang et al. 1978, Quinn et al. 2007, Geden et al. 2009) and honeydew (Hung et al. 2015), perhaps due to the release of volatile compounds produced by the breakdown of the constituent sugars by thermal or microbial activity. The identification of attractive odors from honeydew may lead to a better understanding of the underlying mechanism of attraction to this commonly available food source for insects.

House fly management practices typically include the use of toxic baits containing attractive odors identified from animal feces (e.g., Cossé and Baker 1996), many of which are malodorous to humans. Identification of honeydew odors that attract house flies would be useful for fly management, particularly as these odors are unlikely to be as noxious to humans as volatiles from animal feces, and can be formulated into baits attractive to house flies of both sexes and over a wide physiological age range, all of which require carbohydrates for survival and energy for flight. Thus, the aim of this study was to collect and identify volatile compounds that elicited house fly antennal responses from honeydew-infested plant material. Synthetic compounds were compared to the original volatiles to confirm the identities.

Materials and Methods

Insect colonies:

House flies were collected by sweep net from a southern California dairy in 2010 and subsequently maintained in an insectary at constant conditions of 25°C, 40% relative

humidity (RH), and 12L:12D photoperiod. Larvae were fed a standard fly rearing medium (Mandeville 1988) while adult flies were provided a 50:50 dry milk/sugar mixture and given water *ad libitum*. Only adult female flies from 3-5 days old were utilized in these studies. Pea aphids (*Acyrthosiphon pisum* Harris) were established at the University of California, Riverside (UCR) in 2009 from a colony maintained at Oklahoma State University. Pea aphids were reared in a greenhouse on faba bean plants (*Vicia faba* L.) maintained under natural daylight conditions.

Collection of honeydew materials

Plant stems and leaves with accumulated honeydew and without honeydew (control) were collected using latex or nitrile gloves to avoid contamination with human skin odors. All honeydew-contaminated and control samples were pruned from the plant with sterile shears, stored in oven bags (Terinex Ltd., Bedford, England) to maintain odors, and placed on ice for transport back to the laboratory for bioassays. Transport of plant material and honeydew was approved by the California Department of Food and Agriculture (permit #2776). Navel orange (*Citrus sinensis* Osbeck) and Marsh grapefruit (*Citrus paradisi Macfadyen)* leaves infested with whiteflies (Family: Aleyrodidae) were collected from a single site in Riverside, CA. A clean faba bean plant grown in the greenhouse was placed inside the pea aphid colony cage for 1 week for the insects to establish on the plant. A plant of the same age without insects was used as the control.

Collection of honeydew odors

Volatile odors were captured from honeydew samples by pulling air at 2 L/min through a sealed, glass aeration chamber containing the samples, with odors subsequently captured in glass collection tubes containing activated charcoal held in place by glass wool plugs. Another glass fitting with a charcoal filter was connected to the chamber inlet to remove contaminants from the incoming air. Aerations were performed in an insectary room under fluorescent lighting at 27°C and 60% relative humidity. Volatile compounds were eluted from collection tubes with dichlormethane (DCM) into screw cap vials with Teflon cap-liners and stored at -20°C until use.

Honeydew odors were collected from 2-3 whitefly-infested citrus cuttings about 30 cm long (navel orange and Marsh grapefruit) for 48 hr before the collectors were eluted. These cuttings included leaf material and no flowers or fruit. Honeydew volatiles were collected from a 25-35 cm tall faba bean plant heavily infested with pea aphids (50-100 insects) over 7 d with the collection tube changed once after 3 d resulting in 2 continuous collections. The same type of aeration chamber was utilized for all samples, however because the faba bean plants were potted, the pots were tightly wrapped with clean aluminum foil to minimize collection of volatiles from the pot and soil. Plants without any insects or honeydew on the leaves were aerated with the same methods for the control. Two pea aphid honeydew elutions were analyzed by GC, with volatiles subsequently identified and tested for fly antennal responses from the second elution because it contained a greater number of volatile compounds than the first sample. For each honeydew sample, respective plants or cuttings without insects or honeydew were simultaneously aerated and volatiles eluted for subsequent evaluation.

Coupled Gas Chromatography-Electroantennography (GC-EAD)

Aliquots of elutions (1 µl) were injected into a Hewlett-Packard (Palo Alto, CA, USA) 5890 Series II gas chromatograph equipped with a DB-17 column (30 mm long × 0.25mm internal diameter, 0.25 µm film thickness (122-1732 Agilent Technologies, Santa Clara, CA, USA). Helium was used as the carrier gas. The column effluent was split 50:50 between the flame ionization detector (FID) and an electroantennogram detector (EAD). The GC oven was programmed from 30 °C for 1 min, then 10º/min to 275 ºC, hold for 5 min.

The elutions from honeydew-infested samples were tested for fly antennal responses using antennae from 5-10 female house flies (Table 4.1), with control samples being tested with antennae from 3-5 female house flies. Heads of female house flies were removed and inserted between two saline-filled (7.5 g NaCl, 0.21 g $CaCl_2$, 0.35 g KCl, 0.20 g $NaHCO_3$ in 1 L distilled H_2O) capillary glass electrodes with gold wires down the center. A compound was considered active when it elicited a response from at least 50% of the antennae tested. A 1 µl injection of 3-methylindole, butanoic acid, 3-methylbutanoic acid, dimethyltrisulfide, indole, phenylethyl alcohol, and phenol at 500 ng/µl or 1-octen-3-ol at 100 ng/µl was used with the GC-EAD to confirm that the antennal preparations were responsive to these positive standards prior to the first injection of honeydew volatiles. An elution from each honeydew aeration sample was injected into the GC-EAD at least twice with each antenna to confirm consistent antennal responses to the sample. Extracts were injected into the GC-EAD after spiking with

straight chain hydrocarbon standards (C_8-C_{28}:H mix, 25ng/µl in heptane except C_{10}:H, C_{15}:H, C_{20}:H, and C_{25}:H at 50ng/µl and C_{28}:H at 10ng/µl) to calculate the Kovats retention indices. The compounds that elicited a fly antennal response were then identified by GC-MS. Control samples of the odors of uninfested plants were also tested with the GC-EAD to identify the plant compounds that elicited antennal responses.

Coupled Gas Chromatography-Mass Spectrometry (GC-MS)

Aliquots of extracts were analyzed with a Hewlett-Packard (Palo Alto, CA, USA) 6890 gas chromatograph equipped with a DB-17 split column as described above, using an oven temperature program of 40 °C for 1 min, then 5º/min to 280 ºC, held for 5 min. The GC was coupled to a Hewlett-Packard 5973 mass selective detector. Compounds which had elicited antennal responses in the GC-EAD analyses were located in the extracts from their retention indices, and their mass spectra were recorded.

Confirmation of compound identities

Benzaldehyde (CAS 100-52-7), Δ3-carene (CAS 13466-78-9), and Ocimene as a mixture of isomers (W353901) were purchased from Sigma-Aldrich (St. Louis, MO). (*S*)-(-)-limonene (CAS 5989-54-8) was purchased from Alfa Aesar (Ward Hill, MA). (*D*)-(+)-limonene (CAS 5989-27-5) and myrcene (CAS 123-35-3) were purchased from Spectrum Chemical (New Brunswick, NJ). Butyl hexanoate (CAS 626-82-4), β-caryophyllene (CAS 87-44-5),(±)- linalool (CAS 78-70-6), and farnesene as a mixture of isomers were purchased from TCI America (Portland, OR). (*Z*)-3-hexenyl acetate (CAS

3681-71-8) was purchased from Penta International (West Coldwell, NJ). Naphthalene was acquired from crushed mothballs from a staff member at the UCR Entomology Museum.

(*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) was synthesized using a two-step method (Figure 4.1) adapted from Leopold (1986) and Lebel and Paquet (2004). Thus, a solution of oxalyl chloride (2.8 mL, 32.4 mmol) in anhydrous CH_2Cl_2 (70 mL) was cooled to -78 °C under Ar. A solution of DMSO (4.8 mL, 67.5 mmol) in anhydrous CH_2Cl_2 (15 mL) was added dropwise. After 30 min, a solution of geraniol (4.16 g, 27.0 mmol) in anhydrous CH_2Cl_2 (10 mL) was added and the mixture was stirred for 1 h. Et_3N (18.8 mL, 135 mmol) then was added and stirring continued for 30 min. The cold bath was removed and the mixture was stirred for 1.5 h while warming to room temperature. The mixture was poured into water, and extracted with hexanes. The combined organic layer was washed with 1 M HCl, saturated aqueous $NaHCO_3$, and brine, then dried and concentrated. Kugelrohr distillation (1 torr, 80 – 85 °C) of the crude product gave geranial as a yellow liquid (3.69 g, 90%).

Sodium hexamethyldisilazide (2M in THF, 13.2 mL, 26.4 mmol) was added to a solution of methyltriphenylphosphonium bromide (9.43 g, 26.4 mmol) in THF (80 mL) at 0 °C. The resulting yellow mixture was stirred for 1 h at room temperature, then geranial (3.65 g, 24 mmol) in THF (10 mL) was added and the solution was stirred at room temperature. After 3 h, the mixture was poured into saturated NH_4Cl solution and extracted with hexane. After concentration, the residue was taken up in hexane to precipitate most of the triphenylphosphine oxide byproduct. The supernatant solution was

decanted and washed with 1 M HCl (to remove hexamethyldisilazine), saturated aqueous $NaHCO_3$, and brine, then dried and concentrated. The crude product was purified by vacuum flash chromatography on silica gel (hexane, $R_f = 0.73$) to give (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) as a colorless liquid (2.79 g, 78%).

Solutions containing 50, 100, or 200 ng/µl of authentic standards in DCM, depending on the relative amount of material detected in 1 µl injections of the original honeydew extracts, were prepared to confirm the retention times and the antennal responses of the tentatively identified honeydew volatiles on the GC-EAD. Aliquots of the various solutions were analyzed by GC-EAD as described above.

Results

Seventeen compounds in the extracts of whitefly honeydew odors elicited reproducible responses from house fly antennae, of which 10 compounds were identified by GC-MS and confirmed with authentic standards (Table 4.2). Benzaldehyde, β-caryophyllene, (*Z*)-3-hexenyl acetate, and myrcene were identified from both honeydew from whitefly on navel orange cuttings and honeydew from whitefly on Marsh grapefruit cuttings, with the remaining six volatiles identified from only one of the two honeydew samples. Ocimene and farnesene isomers were not fully confirmed because the authentic standards of the isomers that were available did not match those from the extract of whitefly honeydew on navel orange. Similarly, three volatiles from the whitefly honeydew on Marsh grapefruit were not fully identified because the available ocimene

and α-farnesene standards did not match the isomers in the sample, and an authentic standard of (*Z*)-2-hexenal did not elicit a response from fly antennae in GC-EAD trials. Both of the control aerations from uninfested plants contained fewer compounds than their respective whitefly honeydew aerations, but the control aerations did have some compounds that elicited antennal responses (Figures 4.2-4.7). These compounds were not identified due to the low amounts present in the collections.

Only one compound (benzaldehyde) in the volatiles collected from honeydew of pea aphids on faba bean elicited a response from house fly antennae (Figure 4.8). No EAD responses were elicited by volatiles from the faba bean control (Figure 4.9), which contained fewer compounds than the honeydew aeration (Figure 4.10).

Discussion

We identified several volatile compounds from insect honeydew that elicited electroantennogram responses from antennae of female house flies. Blends of volatiles that included butyl hexanoate, β-caryophyllene, or linalool were previously shown to be attractive to house flies (Qian et al. 2013, Zito et al. 2013, 2015), with β-caryophyllene noted to be relatively unimportant (Zito et al. 2015). Benzaldehyde, β-caryophyllene, (*Z*)-3-hexenyl acetate, limonene, and linalool are all common plant constituents that are attractive to a variety of insects (e.g., Bruce and Pickett 2011, Zito et al. 2013). However, they also have been previously identified from decaying organic matter which might be attractive to house flies, including benzaldehyde from a rodent carcass (Johansen et al.

2014), butyl hexanoate from fermented vinegar (Qian et al. 2013), Δ3-carene from pig manure (Cossé and Baker 1996), β-caryophyllene from cattle rumen contents and from cattle and horse dung (Jeanbourquin and Guerin 2007a, 2007b), limonene from animal feces (Cossé and Baker 1996, Jeanbourquin and Guerin 2007a, 2007b), and linalool from fermented sugar and molasses (El-Sayed et al. 2005). However, the attraction of house flies to any of these odors has not been tested.

Limonene, linalool, and myrcene are common components of plant essential oils and these compounds have been reported to be repellent to house flies and other filth flies (Maganga et al. 1996, Hieu et al. 2014). Additionally, linalool and naphthalene, identified from cattle odors, individually reduced two Muscid fly, *Musca autumnalis* (de Geer) and *Haematobia irritans* (L.), responses in wind-tunnel assays (Birkett et al. 2004). However, it is easily possible that these compounds may be part of an attractant when presented in the right proportions as part of a blend with other components (Bruce and Pickett 2011).

Benzaldehyde was the only volatile from the aphid honeydew that elicited a consistent antennal response. This was unlike other honeydew elutions which contained many compounds that elicited antennal responses. While studies revealed multiple volatiles identified from honeydew played a role in eliciting insect responses (Leroy et al. 2011, 2012), it is possible that benzaldehyde alone may be enough to elicit house fly attraction to pea aphid honeydew.

The GC-EAD antennal responses indicate only detection of an odor compound and cannot predict a behavioral response. The house fly antennae may detect compounds that elicit an attractive, repellant, or even an oviposition response. Therefore, behavioral

assays are needed to determine house fly attraction toward a volatile compound relative to a suitable control. Of the 10 compounds identified, benzaldehyde, Δ3-carene, DMNT, and (*Z*)-3-hexenyl acetate were shown to elicit responses from house fly antennae, and none of these have been associated with house fly attraction. Studying house fly attraction toward these compounds, especially benzaldehyde, is of high interest. Benzaldehyde, β-caryophyllene, (*Z*)-3-hexenyl acetate, and myrcene were collected from two attractive honeydew materials, and future behavioral assays should focus on examining these compounds as a blend for potential house fly attraction.

Although we collected volatile compounds from the control samples, we did not analyze them. The control plant samples may release some of the same compounds as the honeydew infested materials; however, the relative ratios or release rate of the compounds and inclusion of other compounds in the blend may signify to the flies that they are detecting honeydew on a plant rather than solely a plant. There may be a benefit in subtracting the compounds found in the control to possibly increase rate of fly capture, but as discussed earlier, we cannot predict *a priori* which blends and ratios of compounds may be optimal. Some of these compounds have not previously been observed to play a role in house fly attraction and it would be intriguing to see any of these compounds added to the growing list of fly attractants.

Acknowledgments

We thank the UC Cooperative Extension Farm Advisors, commercial farm managers, and local gardeners who assisted with honeydew collection; Dr. Greg Walker for providing the pea aphid colony; and Dr. Mark Hoddle for providing the citrus mealybug colony. Dr. Yunfan Zou synthesized DMNT. This project was funded by United States Department of Agriculture National Integrated Food Safety Initiative (USDA-NIFSI) # 2009-51110-05856. Support for KYH was also provided by the National Science Foundation Graduate Research Fellowship Program (NSF GRFP).

Table 4.1: Summary of samples of honeydew on plants and plant controls, the number of antennae of female house flies tested, and the number of compounds identified from each sample of volatiles.

Sample	# antennae tested	# Compounds
Whitefly honeydew on navel orange	7	8
Navel orange control	5	-
Whitefly honeydew on Marsh grapefruit	10	6
Marsh grapefruit control	3	-
Pea aphid honeydew on faba bean	5	1
Faba bean control	4	-

Table 4.2: Volatile compounds identified from aeration samples of whiteflies on navel orange or whiteflies on Marsh grapefruit trees. Compounds marked with an asterisck were tentatively identified but the EAD response was not confirmed**.** The remaining compounds were verified by matching mass spectra and retention times with those of known standards, and by confirmation that they elicited responses from antennae of female houseflies. Numbers in the first and second columns refer to the EAD responses in Figures 4.2 and 4.3 respectively. KI is the Kovats Index calculated on a DB-17 column.

Whitefly Navel Orange	Whitefly Marsh Grapefruit	Volatile	Ret. Time	KI
	1	**(*Z*)-2-Hexen-1-al***	5.5	0981
1	2	**Myrcene**	6.8	1053
2		**Δ3 Carene**	7.14	1073
	3	**Unknown**	7.0	1078
3		**Limonene**	7.6	1100
4	4	**(*Z*)-3-hexenyl acetate**	8.03	1130
5	5	**Benzaldehyde**	8.4	1156
	6	**Unknown**	8.8	1194
	7	**Linalool**	8.97	1204
6		**DMNT**	9.05	1201
	8	**Ocimene***	9.56	1244
7		**Ocimene***	9.67	1244
8		**Butyl hexanoate**	10.5	1300
	9	**Naphthalene**	11.98	1413
9		**Farnesene***	12.2	1416
	10	**α-farnesene***	13.37	1513
10	11	**β-caryophyllene**	13.9	1540

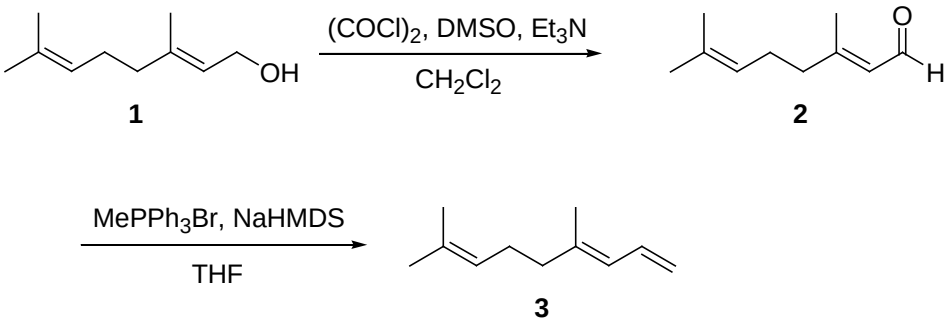

Figure 4.1: Synthesis of (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT).

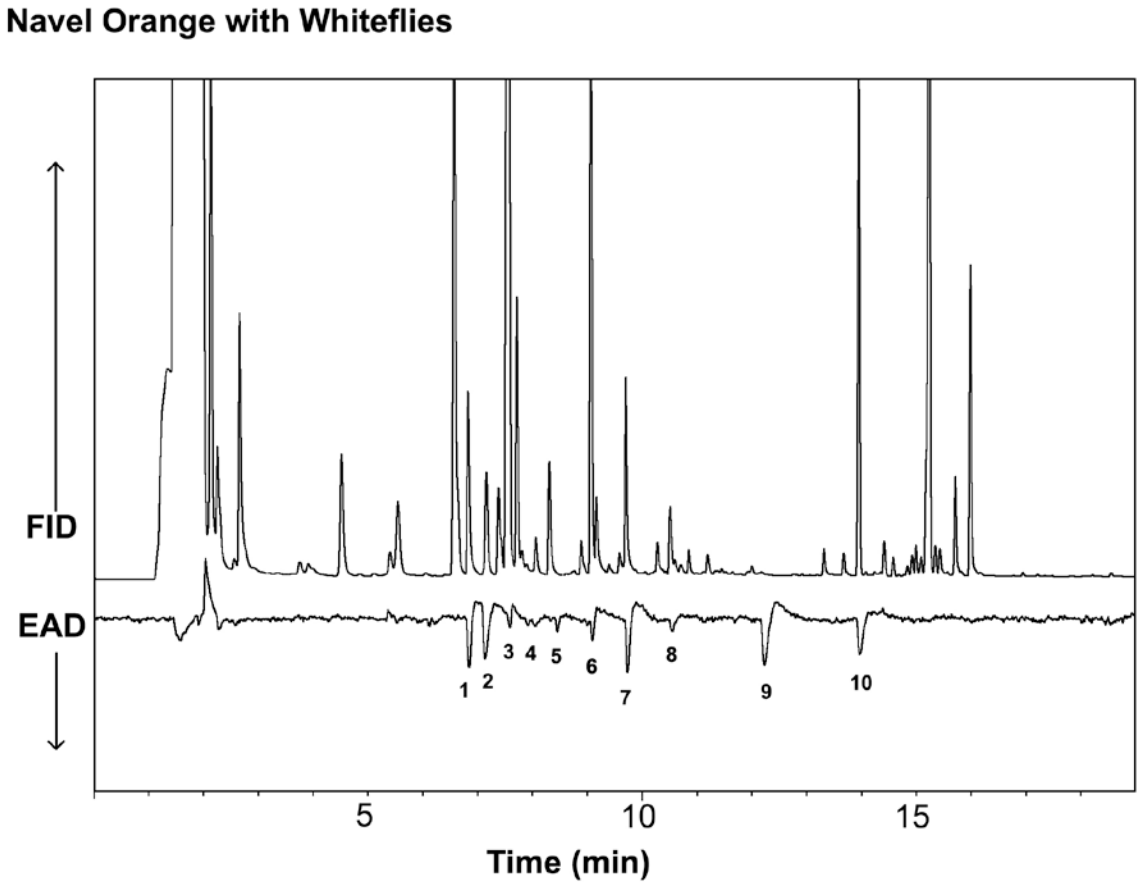

Figure 4.2: Coupled gas chromatography-electroantennogram analysis of volatiles from whitefly-infested navel orange foliage, with a female house fly antenna. Top trace is the GC trace and the bottom inverted trace is the electroantennogram trace. Numbered peaks correspond to the compounds indicated in Table 4.2.

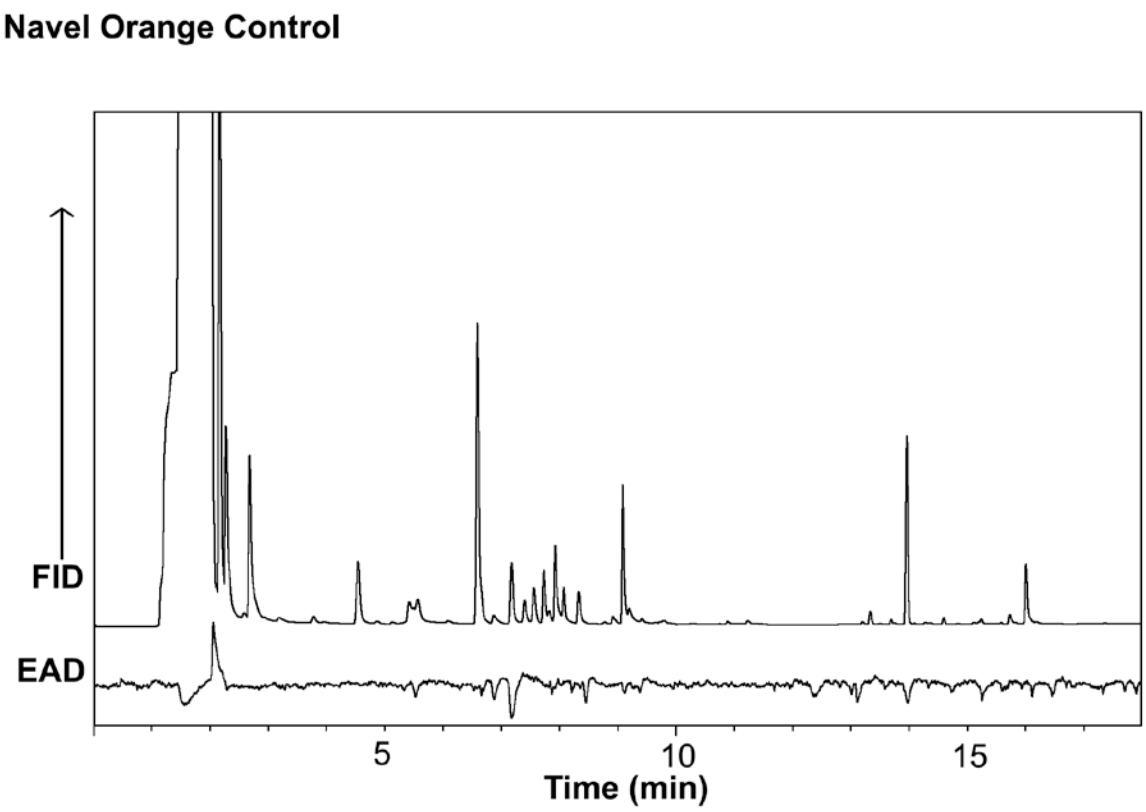

Figure 4.3: Coupled gas chromatography-electroantennogram analysis of volatiles from uninfested navel orange foliage, with a female house fly antenna. Top trace is the GC trace and the bottom inverted trace is the electroantennogram trace. Peaks in the control were not identified.

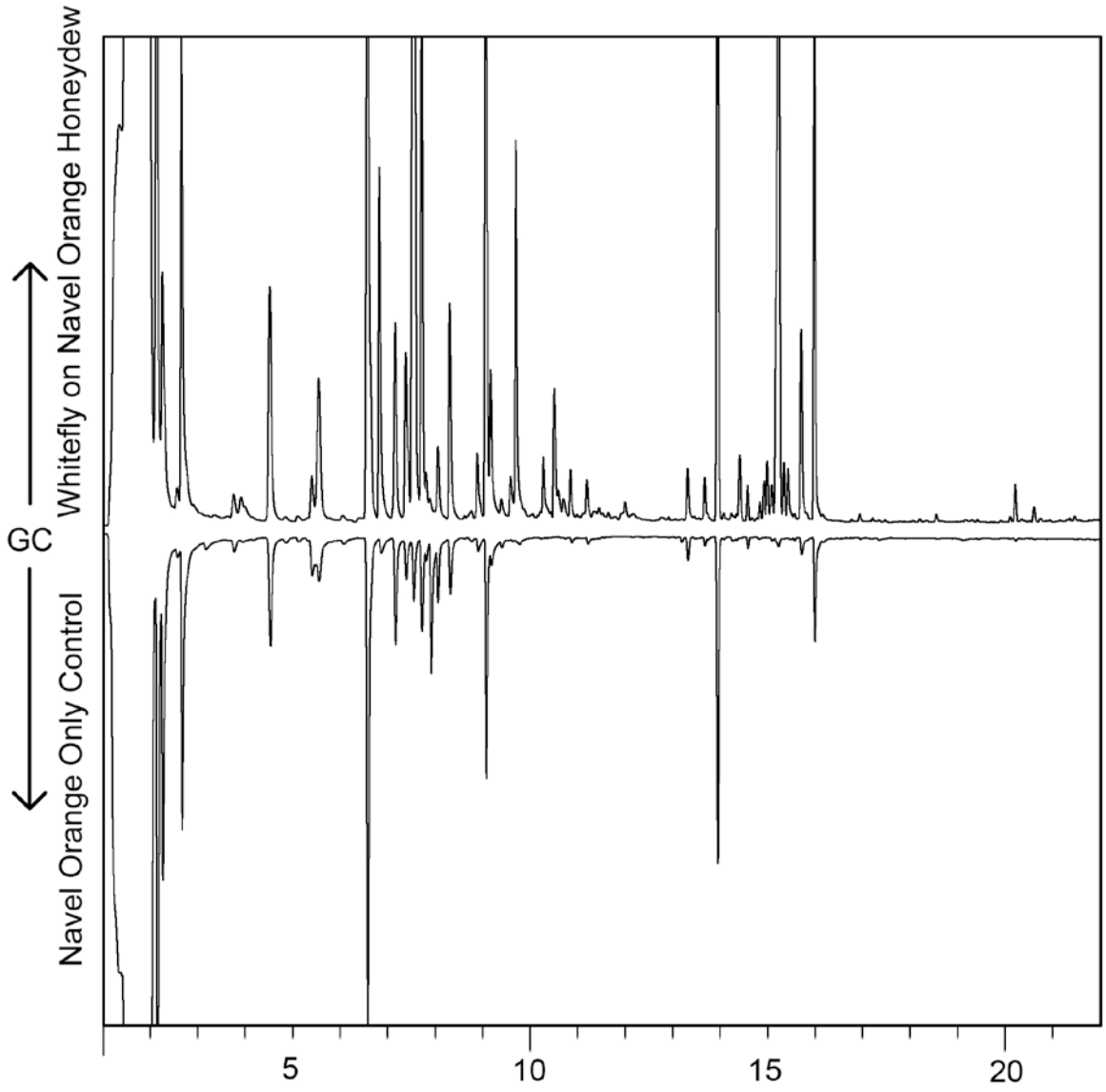

Figure 4.4: GC traces of volatiles from navel orange foliage infested with whiteflies (top trace) vs uninfested navel orange foliage control (inverted bottom trace).

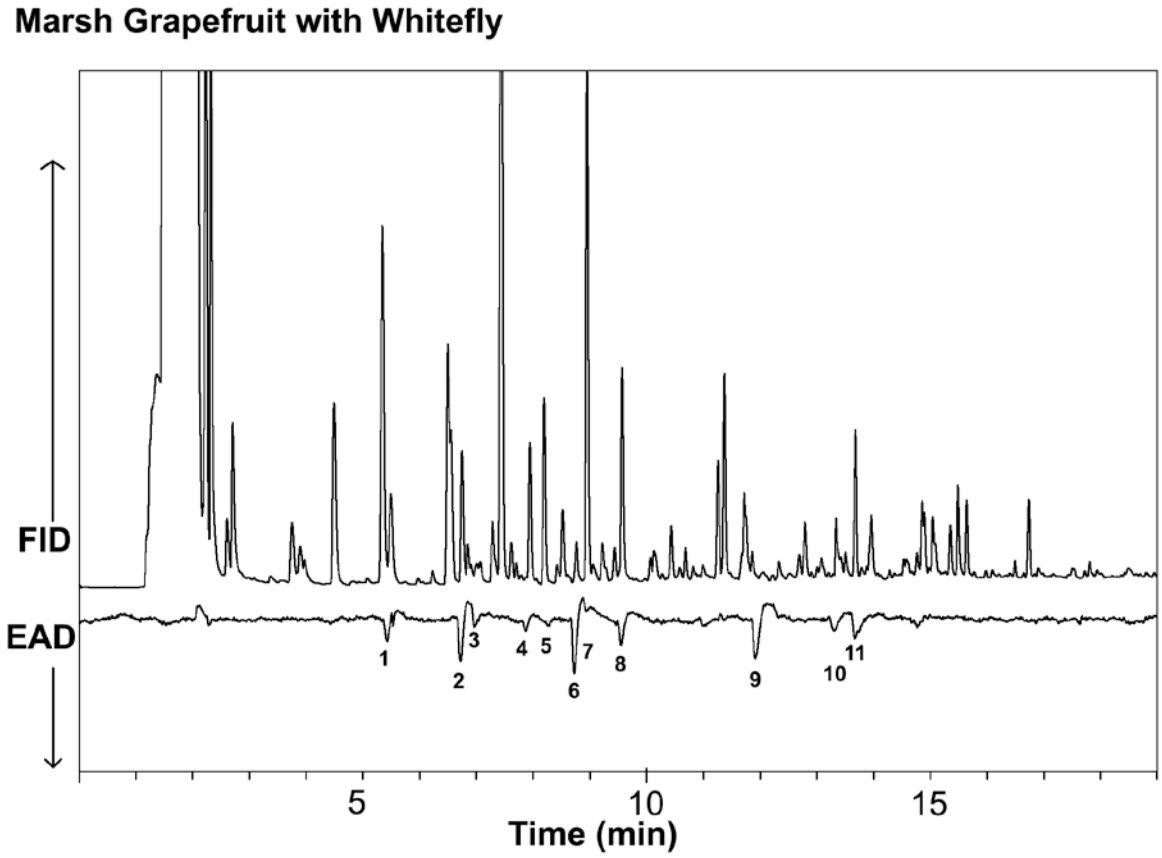

Figure 4.5: Coupled gas chromatography-electroantennogram analysis of volatiles from whitefly-infested Marsh grapefruit foliage, with a female house fly antenna. Top trace is the GC trace and the bottom inverted trace is the electroantennogram trace. Numbered peaks correspond to the compounds listed in Table 4.2.

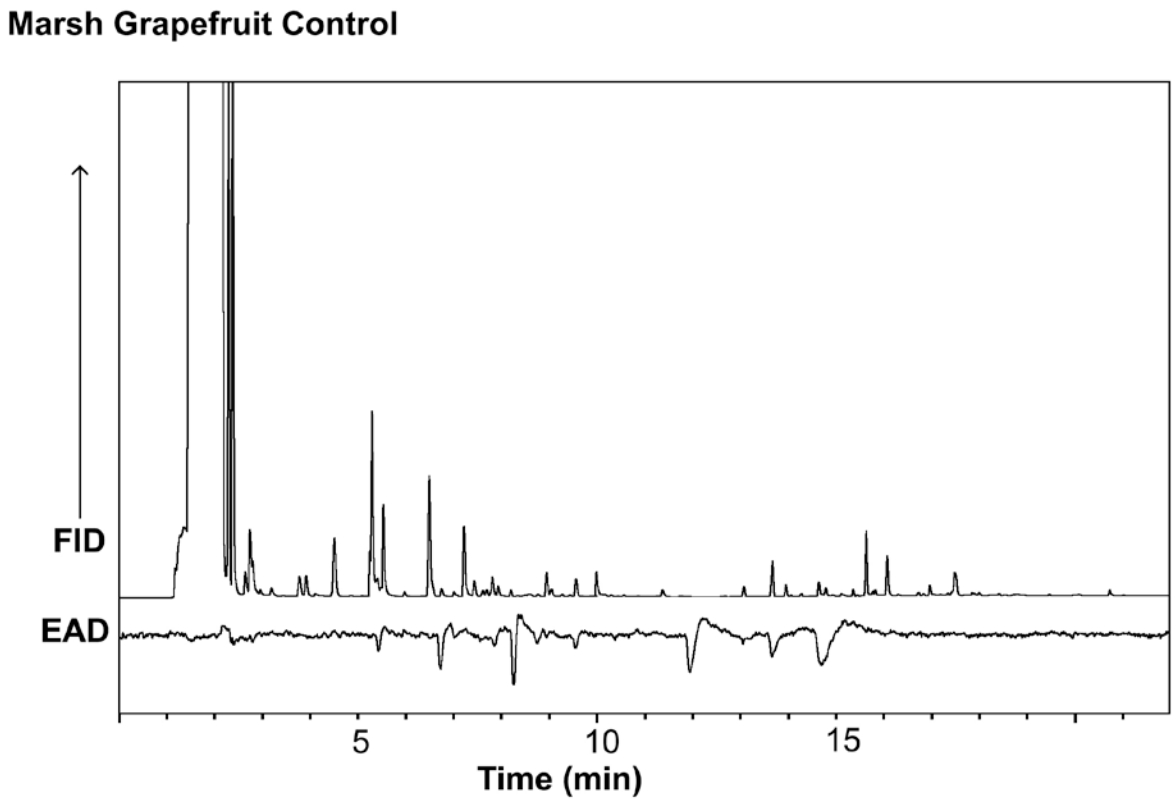

Figure 4.6: Coupled gas chromatography-electroantennogram analysis of volatiles from uninfested Marsh grapefruit foliage, with a female house fly antenna. Top trace is the GC trace and the bottom inverted trace is the electroantennogram trace. Peaks in the control were not identified.

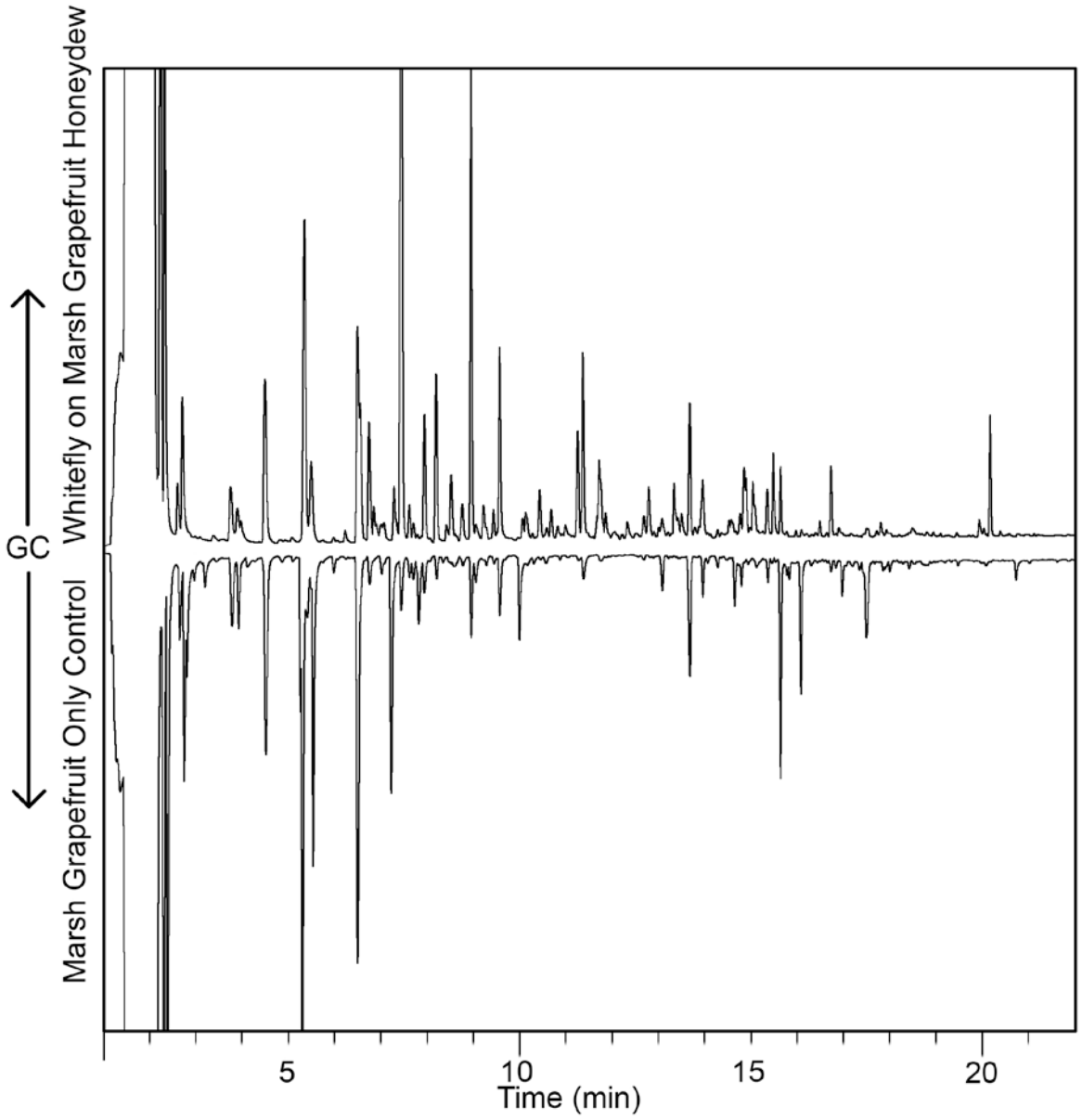

Figure 4.7: GC traces of volatiles from whitefly-infested Marsh grapefruit foliage (top trace) vs uninfested Marsh grapefruit foliage control (inverse bottom trace).

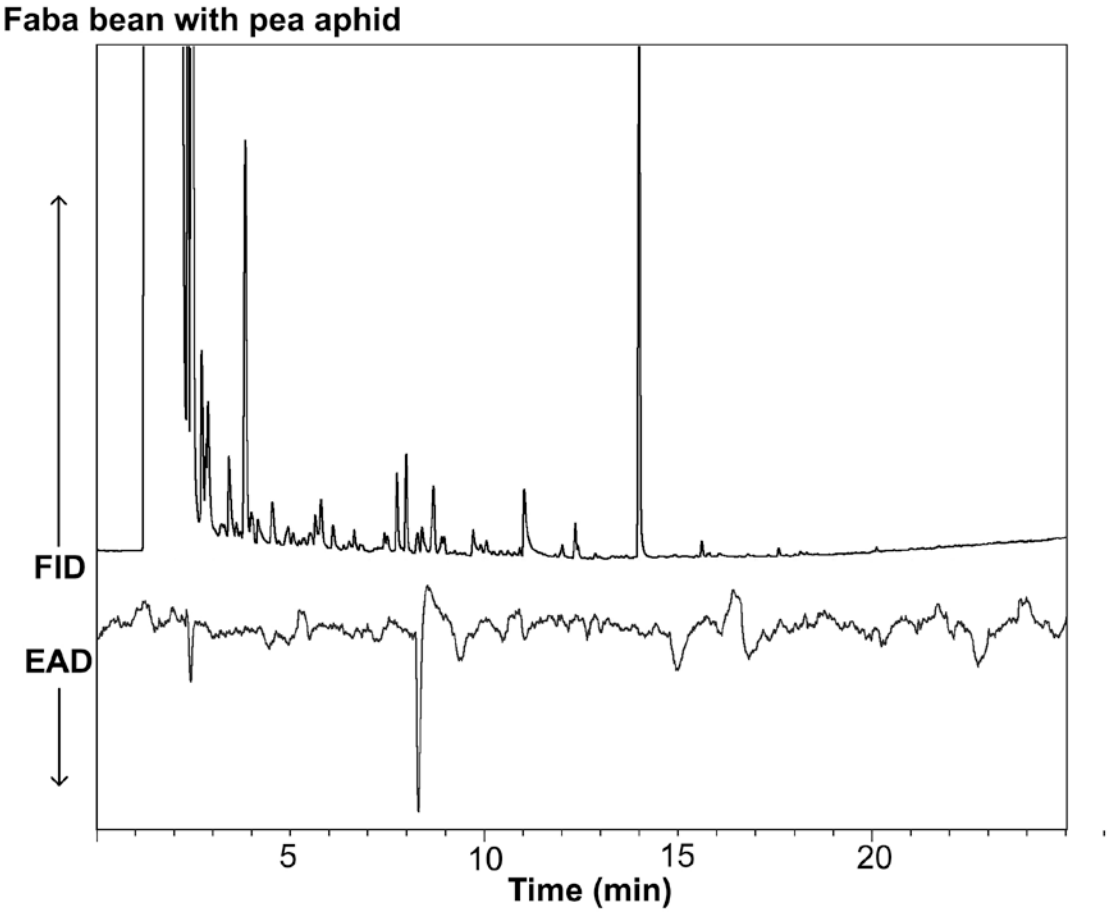

Figure 4.8: Coupled gas chromatography-electroantennogram analysis of volatiles from pea aphid infested fava bean, with a female house fly antenna. Top trace is the GC trace and the bottom inverted trace is the electroantennogram trace. The large EAD peak near 8.3 minutes is benzaldehyde.

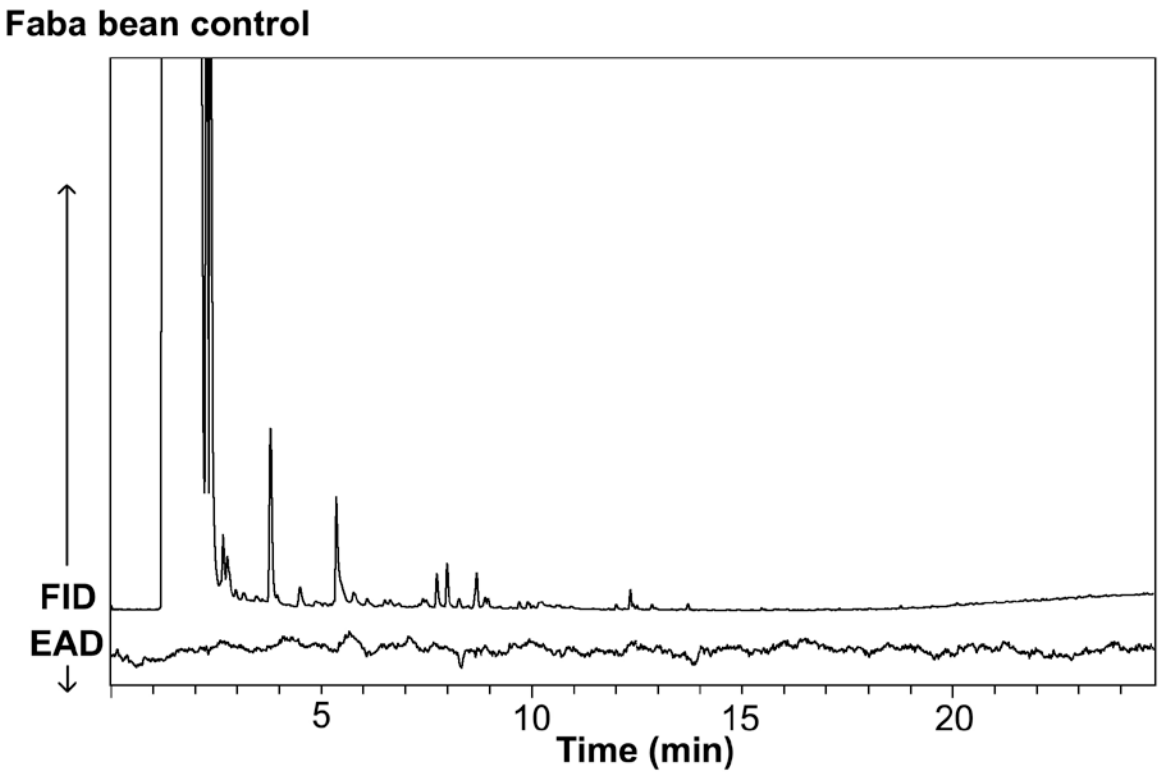

Figure 4.9: Coupled gas chromatography-electroantennogram analysis of volatiles from uninfested fava bean, with a female house fly antenna. Top trace is the GC trace and the bottom inverted trace is the electroantennogram trace.

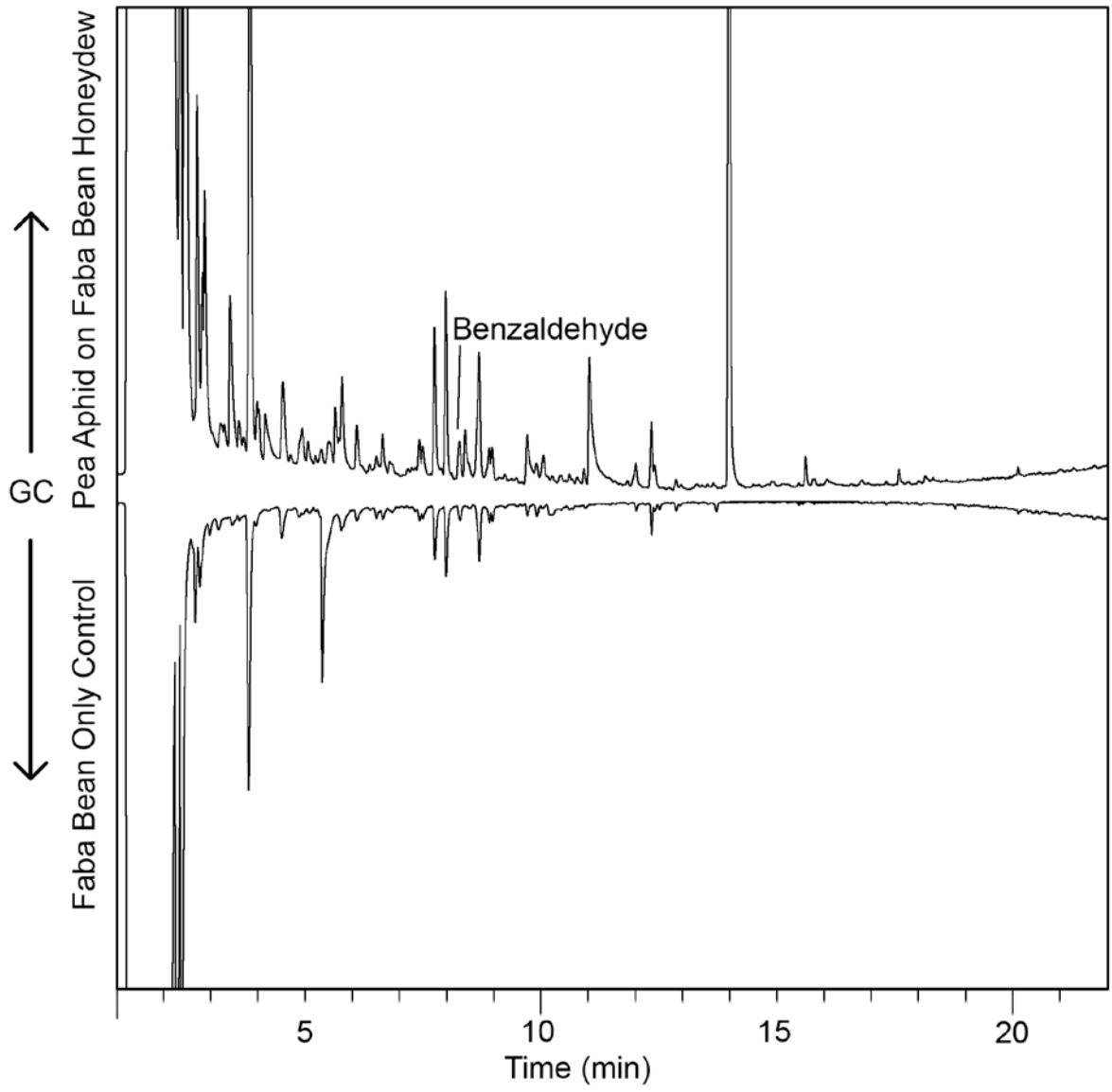

Figure 4.10: GC traces of pea aphid infested faba bean (top trace) vs uninfested faba bean control (inverted bottom trace).

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CHAPTER 5

FIELD ASSESSMENT OF ATTRACTION OF HOUSE FLIES TO ODORS FROM INSECT HONEYDEW

Abstract

House flies (*Musca domestica*) are pests in both urban and agricultural environments due to their nuisance and their ability to mechanically transmit pathogens. House flies can carry pathogens such as *Eschirichia coli, Salmonella* spp., and *Campylobacter* spp. that cause food-borne illnesses, and may deposit these pathogens onto leafy-green food crops through regurgitation and defecation onto leaf surfaces. Movement of house flies into food crops may be increased by the presence of honeydew produced by sucking insects feeding on these crops. A cone trap design to evaluate house fly attraction to previously published house fly attractants was tested at a commercial dairy facility. House flies were captured in greater numbers in cone traps baited with a volatile blend derived from pig feces relative to a control. Two other published attractants, citrus mealybug honeydew (*Planococcus citri* Risso) and fermented vinegar, were not attractive to house flies in field trials. Odors from honeydew produced by whiteflies on navel orange, whiteflies on Marsh grapefruit, and pea aphids on faba bean, that elicited antennal responses from house flies in electroantennogram assays, were subsequently evaluated using the cone traps at the same dairy facility. Honeydew volatiles, as individual compounds and blends, were not attractive to house flies under the tested conditions.

Introduction

House flies are vectors of numerous human and animal pathogens including *Eschirichia coli, Salmonella* spp., and *Campylobacter* spp.(Greenberg 1973, Ahmad et al. 2007, Blazar et al. 2011). These pathogens pose public and animal health risks when flies disperse from breeding sites in human or animal waste from which they may acquire pathogens of concern. Flies are common in areas where animal waste and manure are abundant, and when present in large numbers can cause nuisance in residential areas (Olsen 1998, Graczyk et al. 2001). Managing fly populations can lead to reduction of gastrointestinal illness for nearby human residents (Watt and Lindsay 1948, Lindsay et al. 1953, Cohen et al. 1991, Levine and Levine 1991, Chavasse et al. 1999).

Filth fly presence is a factor in *Escherichia coli* O157:H7 contamination of leafy-green crops (Talley et al. 2009) and contaminated flies can transmit pathogens by depositing regurgitation and fecal spots onto plant surfaces (Sasaki et al. 2000, Wasala et al. 2013). Plants infested with sucking insects such as aphids and whiteflies are likely to have leaf surfaces covered with sticky, sugary excretions called honeydew. House flies were captured in greater numbers near plants contaminated with honeydew and honeydew-producing insects relative to uninfested plants (Hung et al. 2015). The increased capture of flies near honeydew-contaminated plants suggested that honeydew presence on food crops could increase contamination of these crops with pathogens carried by house flies. Here we use the term "attraction" to denote the orientation toward honeydew volatiles at least over short distances, perhaps due to honeydew odors eliciting

increased short-range orientation and landing or simply increased time spent near the odor source. Volatile compounds were identified from these fly-attractive honeydew samples using gas chromatography-electroantennogram detection (GC-EAD) and gas chromatography-mass spectrometry (GC-MS) (Hung Chapter 4). Whether the identified volatiles or their blends are attractive to house flies under field conditions needs to be evaluated because fly antennae can respond to compounds that are attractive, repellent, or have no effect on orientation.

This study first investigated house fly attraction to known attractive baits to evaluate the efficiency of a cone trap in a natural field setting, and then examined house fly attraction to individual honeydew odor components and a 9-compound blend.

Materials and Methods

Field site and trapping design

Studies were conducted at a large commercial dairy (SB Dairy) surrounded by planted fields and undeveloped hills in Riverside County, California. The nearest neighboring dairy was about 1.5 km away. The study utilized a randomized block design with cone traps placed in four blocks called East, West, Manure, and Calf representing the areas of the dairy where each trapping block was set up. The east block was on the east side of the dairy parallel to a perimeter fence line. The west block was on the west side of the dairy parallel to an adjacent alfalfa field. The manure block was about 0.5 km

from the dairy center and was positioned between a large manure storage pile and an alfalfa field. The calf block was in a run in the middle of the dairy near calf hutches.

Attraction of house flies to various bait materials, including volatiles identified from insect honeydews, was determined by capturing flies attracted to baits placed beneath a 1-m-high cylindrical cone trap (e.g., see Qian et al. 2013) constructed with charcoal-gray window screen and with an internal cone to direct flies to an upper trap chamber. Baits were held on a wood platform (30.5 × 30.5 cm) suspended 10 cm below the opening of the cone trap (Figure 5.1). Bait materials were applied to a sanitary pad (Always Proctor & Gamble, SKU 37000-31484-4) cut to fit inside a glass Petri dish (9-cm diam.) which was previously washed with Micro90® soap, rinsed with distilled water and then acetone, and baked at 120 °C. Petri dishes were covered with a mesh top held by a rubber band to prevent flies from accessing the test material. House flies were captured in the cone traps if they flew upward after leaving the bait. Traps were placed 10 m apart. The trials were performed from July to September of 2013 and 2014 during the morning from 7:30 to 10:30. Trapping was not conducted on days with rain or high wind (> 3 m/s). Typical environmental conditions consisted of clear mornings with ample sunlight in the blocks except the Calf block where shade was provided by an awning.

Evaluation of trap design

Compounds established by others to be attractive to house flies were initially used to show that the trap design was suitable for demonstrating attraction. Attractive compounds identified from pig manure (40 µg each of 3-methylbutanoic acid,

dimethyldisulfide, 3-methylindole, indole, butanoic acid, 2-phenylethanol, phenol, and dimethyl tetrasulfide) (Cossé and Baker 1996) were added to 20 ml 50% ethanol (EtOH). Volatile compounds identified from fermented vinegar (9.7 mL acetic acid, 92 µl furfural, 61 µl 2-phenylethanol, 51 µl isovaleric acid, 14 µl hexanoic acid, 11 µl butanoic acid, and 8 mg *p*-cresol) were added to 10 mL distilled water for a total volume of 20 mL in the same ratio as given by Qian et al. (2013). Both of these solutions were added to a Petri dish with a sanitary pad immediately before use. Ten grams of citrus mealybug honeydew, known to attract more flies than a plant-only control to house flies in laboratory bioassays (Hung et al. 2015), were collected as scrapings from the squash and colony cage and added to cover the bottom surface of a Petri dish without a sanitary pad, and 20 mL water was added immediately before use. These three baits were compared with a Petri dish with only a sanitary pad as the control. Trials were performed using a randomized block design with 4 traps per block and 4 trapping days. Baits were changed daily.

In a second test, the pig manure volatile blend described above was compared with > 4 wk-old butternut squash infested with citrus mealybug and covered with mealybug honeydew, 20 ml of 50% EtOH control, whole uninfested butternut squash control, and a Petri dish with sanitary pad negative control. Butternut squash with honeydew and the uninfested control squash were placed in 2.5 L paper buckets (5T1-N0195, Solo, Lake Forest, IL) covered with a mesh held by a rubber band and placed below the cone trap. Trials were performed using a randomized block design with 5 traps per block and 5 trapping days. Baits were changed daily.

House fly response to honeydew volatiles

Volatiles identified from honeydew that were attractive to house flies and which had elicited responses from fly antennae in GC-EAD analyses (Hung Chapter 4) were evaluated using methods similar to those used for the pig manure volatiles described above. Benzaldehyde (CAS 100-52-7) and Δ3-carene (CAS 13466-78-9) were purchased from Sigma-Aldrich (St. Louis, MO). (*Z*)-3-hexenyl acetate (CAS 3681-71-8) was purchased from Penta International (West Coldwell, NJ). (*S*)-(-)-limonene (CAS 5989-54-8) was purchased from Alfa Aesar (Ward Hill, MA). (*D*)-(+)-limonene (CAS 5989-27-5) and myrcene (CAS 123-35-3) were purchased from Spectrum Chemical (New Brunswick, NJ). Butyl hexanoate (CAS 626-82-4), β-caryophyllene (CAS 87-44-5), and (±)- linalool (CAS 78-70-6) were purchased from TCI America (Portland, OR). Naphthalene was acquired from crushed mothballs from a staff member at the UCR Entomology Museum. A blend of benzaldehyde, butyl hexanoate, β-caryophyllene, Δ3-carene, (*Z*)-3-hexenyl acetate, (±)-limonene, (±)-linalool, myrcene, and naphthalene in equal amounts of 40 μg in 20 ml 1% EtOH (1% blend) and a blend of the same 9 compounds at the same concentration in 50% EtOH solvent (50% blend) were used to test honeydew compounds blends. Both of these solutions were added to a Petri dish lined with a sanitary pad immediately before use. Petri dishes with 20 mL of 1% EtOH, 50% EtOH, and a sanitary pad only (no solvent) were used as controls. Trials were performed using a randomized block design with 5 traps per block and 6 trapping days, except one trial date used only 3 of the 4 blocks. Baits were changed daily.

To determine a dose appropriate for capturing flies in the field, blends of the same 9 compounds at 0.4 mg, 4 mg, and 40 mg in 20 ml of 50% EtOH were compared over 3 days in the same randomized block design as described above, with a Petri dish without bait and a Petri dish with 50% EtOH solvent as controls.

As a preliminary test to assess whether individual volatiles identified from insect honeydew (Hung Chapter 4) attract flies, butyl hexanoate, benzaldehyde, β-caryophyllene, Δ3-carene, (*Z*)-3-hexenyl acetate, limonene, linalool, myrcene, and naphthalene were tested as single compounds at 0.04, 4, and 40 mg in 20 mL hexane solvent. All three concentrations of the same volatile, along with a hexane only and a unbaited control, were randomly assigned to one of five trap positions at one of the 4 block locations for direct comparison among concentrations during a single test date. Based upon results from a single test date, benzaldehyde, butyl hexanoate, (*Z*)-3-hexenyl acetate, β-caryophyllene, and Δ3-carene were evaluated again on a subsequent single test date at the same 3 concentrations using 4 separate treatment blocks, with 2 blocks each on the east and west borders of the dairy (5 traps per block). The manure and calf blocks were not used for this trial because of low fly capture at these blocks in earlier trials and limited space, respectively. Evaluation of benzaldehyde and butyl hexanoate was repeated at the same 4 treatment blocks for an additional test date to confirm results for these compounds.

Statistical Analysis

Differences in male, female, and total fly captures for each treatment were analyzed using ANOVA, with block and trial date as independent variables. The least-significant-difference (LSD) test was used for post-hoc analysis. There were no interactions between date and response to treatment or block/treatment or block/date. Normality and variance assumptions were verified by examining the residual plots. Trap counts from the evaluation of trap design studies were normalized with a $\log_{10}$ transformation prior to analysis. All other trial data met the ANOVA assumptions and so were analyzed without transformation. Statistical analyses were performed using SAS v9.4 (SAS institute Cary, NC)

Results

Evaluation of trap design

In the first test, more flies were captured in the traps with pig manure volatiles compared to the unbaited control or to the honeydew treatment but not compared to the vinegar treatment ($df=3,61$; $F=5.21$; $p=0.0032$) (Table 1). More flies were captured in the Calf and East blocks relative to the other two blocks ($df=3,61$; $F=50.67$; $p<0.0001$) (Table 2), but there was no interaction between block and treatment outcome ($p>0.05$). There was no difference in total fly captures among the test dates ($df=3,61$; $F=2.35$;

p=0.08), but the number of male (df=3,59; F=3.02; p=0.038) and female flies (df=3,54; F=4.36; p=0.0088) captured differed among test dates.

In the second test, more flies were captured in the traps with pig manure volatiles than the negative control, honeydew treatments, or the squash control but not the 50% EtOH solvent control (df=4,85; F=7.30; p=<0.0001) (Table 3). The 50% EtOH-baited traps did not capture more flies than the unbaited control. Manure and East locations captured more flies than the other blocks (df=3,85; F=13.35; p=<0.0001). Male (df=4,81; F=5.15; p=0.0011) and female flies (df=4,81; F=6.75; p=0.0001) showed similar responses.

House fly response to honeydew volatiles

The study comparing 9-compound blends in 1% and 50% ethanol solvents collected more flies in traps baited with the 50% blend and 50% EtOH treatments than traps baited with 1% blend and 1% EtOH (df=4,113; F=7.67; p<0.0001) and this response was similar for both male (df=4,113; F=5.3; p=0.0006) and female flies (df=4;113 F=7.56; p<0.0001) (Table 4). Fly captures in each trap using the 9-compound honeydew volatile blend at 0.4 mg, 4 mg, and 40 mg doses in 20 ml 50% EtOH solvent were too low to analyze (Table 5).

The preliminary tests using individual volatiles identified from insect honeydews suggested that benzaldehyde, butyl hexanoate, β-caryophyllene, Δ3-carene, and (*Z*)-3-hexenyl acetate might attract more flies than the controls, at least at one of the tested concentrations (Table 6). These compounds were therefore tested again on 1-2

subsequent dates at four different treatment blocks to provide enough repetition for analysis of fly responses to these odors. None of the individual honeydew volatiles attracted house flies in greater numbers than the control, regardless of the dose tested (Table 7). In fact, the only significant differences in fly captures were for butyl hexanoate, which captured flies in greater numbers at the lower doses (0.04 mg and 4 mg) relative to the higher dose (40 mg) and the hexane control ($df=4,39$; $F=3.22$; $p=0.026$). However, with no differences between the low doses of butyl hexanoate and the negative control ($p>0.05$), there was no compelling evidence for attraction.

Discussion

The cone traps were efficient enough to demonstrate significant attraction of house flies to reconstructed blends of pig manure volatiles versus other treatments in the initial tests (Table 1), but it appeared that EtOH had a greater effect on fly capture than the pig manure volatiles (Table 3). After considering that flies might actually be attracted to the EtOH solvent rather than the volatile being tested, I evaluated house fly responses to volatile blends in 1% or 50% EtOH as well as to EtOH alone (Table 4). A significant increase in fly capture with the increase in EtOH concentration, regardless of the addition of honeydew volatiles, suggests that EtOH is attractive to house flies. EtOH has long been known as a fly attractant (Richardson 1917, Wieting and Hoskins 1939); therefore, it was not unexpected that EtOH increased fly captures. Because of the house flies' responses to EtOH in the baits, the remaining tests examining individual volatiles utilized hexane as the solvent, which is not known to affect fly responses as evidenced by other

studies, although those authors did not specifically assess house fly responses to the solvent alone with a no-solvent control (Quinn et al. 2007, Qian et al. 2013).

Overall fly captures in the traps were low for a 3 h interval with at most a mean of ~30 flies in a trap and this perhaps limited our ability to identify attraction of house flies to volatiles besides ethanol. In part, the low fly captures may have been due to reduced fly abundance at the dairy during the trial period perhaps due to the aggressive manure management strategies the dairy was utilizing, especially in 2014. Manure piles at the dairies were present for no longer than a week, reducing the opportunity for fly larval development to reach completion. However, low captures in a field setting is not unusual, particularly when using baited cone traps. Qian et al. (2013) collected at most ~200 flies in 24 h at a dairy facility using a similar cone trap baited with fermented vinegar, and Willson and Mulla (1973) recorded at most ~150 house flies collected in 24 h at a commercial poultry ranch using a sugar-toxicant with egg attractants in a jar trap. Historically, cone-type traps have been used for fly monitoring and bait trapping (Bishopp 1921, Brundrett 1953, Pickens et al. 1973, Qian et al. 2013), but these reports do not address the actual efficiency of the traps in capturing flies that encounter the traps. There was considerable variation in captures among the blocks in the study, with blocks on the east and west side of the dairy consistently capturing more flies than the other two blocks. For this reason, I relocated all trapping blocks to the east and west side of the dairy for the later evaluations of honeydew volatiles.

None of the individual honeydew volatiles, honeydew blends, or whole honeydew treatments were attractive to house flies in this study, despite showing house fly attraction

to citrus mealybug honeydew in the laboratory. These materials may need an increased load to increase the attractiveness of the lures. The dairy facility had abundant food and oviposition sources, including widespread manure and animal feed containing molasses, which are attractive to flies (Quinn et al. 2007). With the abundance of these alternate food sources such as manure, spilled milk, or sweet feed, as well as other potentially attractive odor sources one might expect on an active dairy facility where flies are typically quite numerous, the concentration of honeydew odors offered in this study may have been too low or are simply not attractive in a context in which other more attractive odors were present. Removing possible repellent compounds may also increase the attractiveness of the honeydew blends. Limonene, linalool, and myrcene have been reported to be repellent to house flies and other filth flies (Maganga et al. 1996, Hieu et al. 2014). Additionally, linalool and naphthalene individually reduced *Musca autumnalis* (de Geer) and *Haematobia irritans* (L.) responses in wind-tunnel assays (Birkett et al. 2004).

The combination of the trap type and the bait may need to be optimized to improve fly captures. Geden et al. (2009) evaluated two commercial fly traps (Terminator® and FliesBeGone®) and discovered that fly capture rates were reduced when each trap system was used with the attractant blend of the competitor's trap rather than the attractant blend supplied. This may be due to a visual or physical factor that may be aiding fly captures differently with the attractants used for these commercial traps.

In this study, the cone traps were comprised of a dark-colored mesh barrel supported by white PVC pipe legs. This design may have aided the collection of flies by

providing a visual cue attractive to flies for resting in the sun to warm up on cool mornings, or as a shaded spot to shelter from the sun during hotter times of the day. The white PVC poles are also a desired house fly resting spot as vertical posts in the animal facilities tend to collect flies (Gerry et al. 2011). The relatively large number of flies captured in some of the unbaited control traps is likely due to the visual attraction of flies to these cone traps. Some flies were observed to land on the traps, either at the poles or on the trap themselves, and walk to the platform with the lure. However, many did not move from the top portion of the traps where they first landed.

Honeydew volatiles did not increase house fly captures over the solvent controls in the field with these methodologies. Further studies evaluating the volatiles in a more controlled setting, such as a semi-field setting to eliminate competing dairy odors, are needed to determine if flies will respond to these volatiles. If semi-field studies show house fly attraction to any of the honeydew volatiles, then additional field studies may be considered but overall, house flies were not responding to the whole honeydew, honeydew blends, or individual compounds. Future studies should avoid use of ethanol as a solvent for volatiles to be tested, given the attraction of house flies to EtOH alone. Should volatiles prove attractive, then EtOH might be considered as an additional attractant that might be tested for an additive effect with other attractive volatiles.

Acknowledgments

We thank the SB Dairy for permission to use their property for this study. David Theuret and Levi Zahn assisted with construction and deployment of the cone traps. Dr. Mark

Hoddle provided the citrus mealybug colony. Funding for this study was provided by United States Department of Agriculture National Integrated Food Safety Initiative (USDA-NIFSI) # 2009-51110-05856. Support for KYH was also provided by the National Science Foundation Graduate Research Fellowship Program (NSF GRFP).

Tables and Figures

Table 5.1: Number of total flies and flies by sex captured in traps baited with previously reported house fly attractants. N=16 for all treatment rows. **Within a column, different letters indicate significant difference between treatments.**

Treatment	Total (SEM)	Male (SEM)	Female (SEM)
Pig manure	28.9 (8.8)a	14.8 (4.8)a	14.2 (4.2)a
Vinegar	19.7 (5.2)ab	12.9 (3.3)a	6.8 (2.0)b
Control	12.9 (3.4)b	9.6 (2.8)a	3.8 (1.0)b
Honeydew	12.9 (2.7)b	9.1 (2.1)a	3.4 (0.9)b
p-value	0.0032	0.20	<0.0001

Table 5.2: Fly captures at each block for the baits in Table 1. N=16 for all block rows. Within each column, different letters indicate significant differences among treatments.

Block	Total (SEM)	Male (SEM)	Female (SEM)
Calf	32.4 (6.9)a	22.2 (3.5)a	14.8 (3.4)a
East	31.5 (6.0)a	17.7 (3.8)a	9.3 (2.9)a
West	6.8 (1.4)b	4.1 (0.9)b	2.6 (0.7)b
Manure	3.8 (0.7)b	2.3 (0.4)b	1.4 (0.4)b
p-value	<0.0001	<0.0001	<0.0001

Table 5.3: Mean fly response to citrus mealybug honeydew vs. pig manure volatiles. N=18 for all treatment rows. Within each column, different letters indicate significant differences among treatments. Data from the Calf block on two trial dates were excluded due to lack of access to the block location.

Treatment	Total (SEM)	Male (SEM)	Female (SEM)
Pig manure	17.9 (3.0)a	10.1 (1.8)a	7.8 (1.5)a
50% EtOH	12.3 (2.8)ab	7.6 (1.8)ab	4.7 (1.1)b
Unbaited	8.2 (1.9)bc	5.6 (1.4)bc	2.6 (0.6)bc
Honeydew	6.6 (1.4)c	4.2 (1.0)c	2.4 (0.5)bc
Uninfested squash control	5.5 (0.8)c	3.9 (0.6)c	1.6 (0.4)c
p-value	<0.0001	0.001	0.0001

Table 5.4: House fly responses to 9-compound blend of honeydew volatiles in different concentrations of ethanol (EtOH). Concentration of the compound blends were 40 µg / 20 mL. Within each column, different letters indicate significant differences among treatments. N=23 for all treatment rows except the treatment indicated by a * had N=22 due to a lost sample.

Treatment	Total (SEM)	Male (SEM)	Female (SEM)
50% blend	34.2 (7.0)a	19.3 (4.1)a	14.9 (3.1)a
50% EtOH	27.6 (5.7)a	14.9 (2.9)ab	11.7 (2.9)ab
1% blend	17.3 (3.8)b	10.5 (2.3)b	6.8 (1.9)b
1% EtOH*	12.9 (2.7)b	8.5 (1.9)c	4.4 (1.0)c
control	13.8 (3.2)b	8.4 (1.7)c	5.4 (1.6)c
p-value	<0.0001	0.0006	<0.0001

Table 5.5: Mean house fly captures with 9-compound blend at different doses per 20 ml EtOH. Fly captures were too low to statistically analyze. N=14 for all treatment rows.

Treatment	Total (SEM)	Male (SEM)	Female (SEM)
40 mg	1.2 (0.47)	0.4 (0.20)	0.8 (0.32)
4 mg	2.6 (0.83)	1.1 (0.45)	1.6 (0.47)
0.4 mg	2 (0.78)	0.8 (0.35)	1.2 (0.52)
control	6.9 (3.8)	2.8 (1.6)	4.1 (2.2)

Table 5.6: Preliminary study examining fly responses to single honeydew volatile compounds. Doses are per 20 mL of hexane, and traps with each concentration of a single compound were placed in only one block on a single date.

Total (Male and Female)

Compound	0.02 mg	2 mg	20 mg	Hexane	Negative	Block
Benzaldehyde	19	10	2	5	0	Calf
Butyl hexanoate	4	10	25	12	19	East
(*Z*)-3-hexenyl acetate	4	13	4	3	1	Calf
(±)-Limonene	0	1	0	1	0	West
(±)-Linalool	7	9	5	6	4	Calf
Myrcene	1	0	5	10	0	West
Naphthalene	0	0	1	4	3	Manure
β-Caryophyllene	18	10	3	2	22	East
Δ3-carene	4	5	11	24	16	East

Male

Compound	0.02 mg	2 mg	20 mg	Hexane	Negative	Block
Benzaldehyde	9	8	1	3	0	Calf
Butyl hexanoate	4	8	15	8	13	East
(*Z*)-3-hexenyl acetate	0	5	2	2	1	Calf
(±)-Limonene	0	1	0	1	0	West
(±)-Linalool	5	9	2	5	3	Calf
Myrcene	0	0	4	9	0	West
Naphthalene	0	0	1	4	2	Manure
β-Caryophyllene	10	9	1	2	15	East
Δ3-carene	3	3	10	19	11	East

Female

Compound	0.02 mg	2 mg	20 mg	Hexane	Negative	Block
Benzaldehyde	10	2	1	2	0	Calf
Butyl hexanoate	0	2	10	4	6	East
(*Z*)-3-Hexenyl acetate	4	8	2	1	0	Calf
(±)-Limonene	0	0	0	0	0	West
(±)-Linalool	2	0	3	1	1	Calf
Myrcene	1	0	1	1	0	West
Naphthalene	0	0	0	0	1	Manure
β-Caryophyllene	8	1	2	0	7	East
Δ3-Carene	1	2	1	5	5	East

Table 5.7: House fly responses to single honeydew compounds at East and West blocks. Doses are per 20 mL of hexane. Different letters next to the standard error indicate a significant difference within a row.

Both (Male & female)							
Compound	**0.04 mg (SEM)**	**4 mg (SEM)**	**40 mg (SEM)**	**Hexane (SEM)**	**Neg (SEM)**	**p-value**	**N**
Benzaldehyde	7.0 (2.1)	7.6 (2.1)	10.9 (2.6)	8.1 (2.0)	6.3 (1.6)	0.22	8
Δ3-carene	18.8 (5.9)	13.3 (4.9)	16.5 (2.5)	20.5 (2.9)	13.8 (3.7)	0.33	4
β-Caryophyllene	16.5 (5.3)	17.0 (3.5)	17.0 (3.3)	19.3 (1.9)	17.8 (4.2)	0.96	4
Butyl Hexanoate	19.9 (5.9)a	19.6 (6.1)a	8.1 (2.6)b	11.9 (2.4)b	16.6 (5.5)ab	0.026	8
(*Z*)-3-hexenyl acetate	15.8 (6.3)	16.5 (2.9)	18.0 (3.2)	18.3 (4.8)	10.3 (3.8)	0.31	4
Male							
	0.04 mg (SEM)	**4 mg (SEM)**	**40 mg (SEM)**	**Hexane (SEM)**	**Neg (SEM)**		**N**
Benzaldehyde	5.0 (1.6)	4.6 (1.0)	5.9 (1.3)	5.4 (1.2)	3.6 (1.1)	0.093	8
Δ3-carene	11.5 (3.3)	9.8 (3.6)	11.0 (1.1)	13.0 (1.4)	8.0 (2.0)	0.33	4
β-Caryophyllene	13.0 (4.2)	11.0 (1.7)	10.5 (2.1)	10.8 (1.8)	9.3 (2.2)	0.75	4
Butyl Hexanoate	12.3 (4.0)	11.8 (3.5)	5.5 (2.0)	8.1 (1.9)	10.5 (3.7)	0.13	8
(*Z*)-3-hexenyl acetate	10.3 (5.4)	11.5 (1.9)	13.5 (2.1)	13.0 (3.81)	6.8 (3.0)	0.29	4
Female							
	0.04 mg (SEM)	**4 mg (SEM)**	**40 mg (SEM)**	**Hexane (SEM)**	**Neg (SEM)**		**N**
Benzaldehyde	2.0 (0.8)	3.0 (1.2)	5.0 (1.5)	2.8 (0.9)	2.6 (0.8)	0.26	8
Δ3-carene	7.3 (2.7)	3.5 (1.3)	5.5 (1.6)	7.5 (1.7)	5.8 (2.3)	0.39	4
β-Caryophyllene	3.5 (1.2)	6.0 (2.3)	6.5 (1.3)	8.5 (1.2)	8.5 (3.0)	0.28	4
Butyl Hexanoate	7.6 (2.1)a	7.9 (2.6)a	2.6 (0.8)b	3.8 (0.7)b	6.1 (1.9)ab	0.019	8
(*Z*)-3-hexenyl acetate	5.5 (1.6)	5.0 (1.7)	4.5 (1.7)	5.3 (1.1)	3.5 (0.9)	0.81	4

Figure 5.1: Trap placement in the field. Traps in this figure were in the West block and placed 10 m apart in a line. Traps in the other blocks were placed in similar fashion.

Figure 5.2: Field site 2013. Blue pins indicate the field sites used for this season. The top left pin is the West block. To the right, in the middle of the facility, is the Calf block. The top right pin is the East block. The bottom pin is the Manure block, where there was typically a manure pile. For 2014 only the East and West blocks of the dairy were used for trials.

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CHAPTER 6

ATTRACTION OF HOUSE FLIES TO HONEYDEW VOLATILES IN A SEMI-FIELD ENVIRONMENT

Abstract

House flies (*Musca domestica*) are pests in both urban and agricultural environments due to their nuisance and their ability to mechanically transmit pathogens. House flies can carry pathogens that cause food-borne illnesses such as *Eschirichia coli, Salmonella spp.,* and *Campylobacter spp.*, and may deposit these pathogens onto leafy-green food crops through regurgitation and defecation onto leaf surfaces. Movement of house flies into food crops may be increased by the presence of honeydew produced by sucking insects feeding on these crops. Odors from honeydew produced by whiteflies on navel orange, whiteflies on Marsh grapefruit, and pea aphids on faba bean that previously elicited antennal responses from house flies in electroantennogram assays were evaluated as sequentially reductive blends of compounds and as individual compounds from attractive blends in an outdoor environment to determine their attractiveness to house flies. Blends including the most common and abundant volatiles identified from the sampled honeydew (8-compound and 7-compound blends) were not attractive to flies, perhaps due to the presence of repellent compounds in these blends. However, a reduced 4-compound blend of benzaldehyde, (*Z*)-3-hexenyl acetate, myrcene, and β-caryophyllene was attractive to house flies, as was a further reduced blend of benzaldehyde and (*Z*)-3-

hexenyl acetate, but not a blend of myrcene and β-caryophyllene. (*Z*)-3-Hexenyl acetate and benzaldehyde also were attractive as single components.

Introduction

House flies (*Musca domestica*) can transmit more than 65 different bacteria and viruses of major concern to human and animal health, particularly where manure and waste management is lacking (Greenberg 1973, Olsen 1998, Graczyk et al. 2001). They may pick up pathogens from contact with manure and waste and disperse them, posing a threat to public health (Lysyk and Axtell 1986, Chakrabarti et al. 2010, Wang et al. 2011). Filth fly presence is a factor in *Escherichia coli* O157:H7 contamination of leafy-green crops (Talley et al. 2009) and contaminated flies can transmit pathogens by depositing regurgitation and fecal spots onto plant surfaces (Sasaki et al. 2000, Wasala et al. 2013). Plants infested with sucking insects such as aphids and whiteflies are likely to have leaf surfaces covered with sticky, sugary excretions called honeydew. House flies were captured in greater numbers near plants contaminated with honeydew and honeydew-producing insects relative to uninfested plants (Hung et al. 2015). The increased capture of flies near honeydew-contaminated plants suggests that honeydew presence on human food crops could increase contamination of these food crops with pathogens carried by house flies.

Using coupled gas chromatography-electroantennogram detection (GC-EAD) and coupled gas chromatography-mass spectrometry (GC-MS), we identified volatile

compounds from insect honeydew that elicited responses from house fly antennae (Hung et al., in preparation). Identified compounds included β-caryophyllene, myrcene, limonene, Δ3-carene, (*Z*)-3-hexenyl acetate, benzaldehyde, butyl hexanoate, and (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT). The GC-EAD antennal responses indicate only detection of an odor compound and cannot predict a behavioral response. Therefore, behavioral assays are needed to determine house fly attraction toward a volatile compound relative to a suitable control.

Previous studies have identified odors associated with putrefaction or microbial decay of manure and other proteinaceous materials that were reported to attract house flies or other filth flies (Morrill 1914, Richardson 1917, Brown et al. 1961, Frishman and Matthysse 1966, Pickens et al. 1973, Mulla et al. 1977, Cossé and Baker 1996). Many volatiles from protein decay may be repulsive to humans at concentrations that that are attractive to flies, and some of these compounds are known to attract mostly parous or protein-seeking female flies (Willson and Mulla 1973). Additional sugar-based materials purported to be attractive to house flies include molasses and honeydew (Quinn et al. 2007, Geden et al. 2009, Hung et al. 2015), where their volatiles are a byproduct of molecular or microbial breakdown.

The objective of this study was to assess attraction of house flies to single compounds and reconstructed blends of volatiles from honeydew in an outdoor setting. Here we define the term "attraction" as orientation toward honeydew volatiles at least over short distances, or honeydew odors eliciting short range orientation and landing behaviors, or simply increased time spent near the odor source. Our overall goal was to

identify compounds or blends of compounds that could be used to increase house fly capture in odor-baited traps or increase house fly feeding on toxic fly baits. In particular, we hoped to identify alternative odors to those currently used in fly baits formulated with odors of putrefaction, or fly baits based on the putative fly pheromone (*Z*)-9-tricosene (Muscalure), which alone may not be effective (Butler et al. 2007).

Materials and methods

Insect colonies

House flies were collected by sweep net from a southern California dairy in 2014 and subsequently maintained as a colony in an insectary at constant conditions of 25°C, 40% relative humidity (RH), and 12L:12D photoperiod. Larvae were fed a standard fly rearing medium (Mandeville et al. 1988) while adult flies were fed a 50:50 dry milk/sugar mixture and given water *ad libitum*. When late-stage larvae were close to pupation, the medium in which they were developing was saturated with cool tap water to drive larvae from the rearing container into an outer container where they were allowed to pupate in dry bran.

For each trial, a small subset of mixed sex pupae was randomly selected, weighed and then counted to develop an estimate of number of pupae per unit weight. Then approximately 500 pupae (by weight) were placed into individual 118 mL cups for emergence in separate release chambers (4.9 L paper buckets, Item # 10T1-N0198 Solo, Lake Forest, IL) with a mesh top held in place with a rubber band. Each release chamber

had a 118 mL cup with fly colony food and a 118 mL lidded cup with water and a dental wick pushed through the lid. The food cup from each fly release chamber was removed 40-44 h before the beginning of the experiment to starve the flies. Aged honeydew from citrus mealybugs (*Planococcus citri* Risso) was used to confirm that house flies would respond with the bioassay described below. Mealybugs were from an established UC Riverside colony maintained on butternut squash fruit (*Curcurbita moschata* Duchesne) at 25 ± 2°C, 40% RH and 14L:10D photoperiod in an insectary room using the same methods as Hung et al (2015).

Field site procedures

Flies were released within camping tents (2.4 x 2.4 x 1.4 m, SKU: 3742202 Rugged Exposure, Big 5 Corp., Los Angeles, CA) that served as the experimental unit for the trials to contain flies in the vicinity of the volatiles being tested. The tents were enclosed to minimize flies exiting and foreign organisms entering the tent. The majority of the tent was comprised of mesh that permitted air movement within the tent. Nine tents were placed in three 10 m rows of 3 tents ~10 m apart on a grassy area shaded by trees at the University of California, Riverside (UCR) Agricultural Operations (AgOps) (Figure 6.1). Within each tent, the volatile compound(s) being tested and an appropriate control were placed into separate glass Petri dishes covered with a mesh top to prevent flies from accessing the compounds. White sticky traps (Pherocon 1C trap liners, Trécé Inc., Adair, OK), were placed on top of the Petri plates in a folded upside-down V shape with the sticky side facing down (Figure 6.2). The interior of each tent was divided into 4 equal

quadrants, with each quadrant containing a treatment or control when testing 3 volatile treatments, or only the two rear quadrants away from the tent entryway containing a treatment or control placed ~1 m apart when testing a single volatile treatment. A dish of water with a paper towel was placed in the middle of the tent to provide water to released flies for the duration of the test. At the start of each trial, a fly release chamber was placed in the center of the tent and turned upside down to expose a 5-cm-diam. covered circular opening on the bottom panel of the release chamber from which the flies could emerge during the trial. Following a minimum 5 min acclimation period, flies were released through the opening in the release chamber by removing the cover of the release port. Following a 4 h exposure period, traps were removed and flies counted by sex. Trials were performed from 10 am – 2 pm during November 2014-May 2015 and 9 am – 1 pm during June-July 2015.

To ensure the tent bioassay methods were effective in evaluating fly attraction to volatiles, preliminary tests were conducted using two known house fly attractants, aged (> 4 wk-old) squash infested with citrus mealybug covered with honeydew (Hung et al. 2015) and a fermented vinegar blend created similar to Qian et al. (2013) from 9.7 mL acetic acid, 92 µl furfural, 61 µl 2-phenylethanol, 51 µl isovaleric acid, 14 µl hexanoic acid, 11 µl butanoic acid, and 8 mg *p*-cresol added to 10 mL of distilled water. In the first test, aged citrus mealybug honeydew-infested squash and an uninfested squash control were offered to flies released in the tents as indicated above. Both squash treatments were placed inside 4.9 L paper buckets with a mesh cover and the sticky fly traps were placed on top of the paper bucket. After 6 h, captured flies (sex not determined) were counted

while observing traps from outside the tent enclosure. After 24 h, the traps were collected and the flies were counted and sexed.

The second test evaluated the best release method for volatile compounds formulated into a liquid. A fermented vinegar blend was created as mentioned above. Release methods tested were: 1) 2 mL of test solution applied to an unscented sanitary pad (Always, Proctor & Gamble, SKU 37000-31484-4) cut to fit inside a glass Petri dish, 2) 2 mL of test solution in an open 4 dram vial, and 3) 10 µl of test solution without water applied to a 11 mm × 5 mm rubber sleeve septum (West Pharma, Lititz, PA). A sticky trap with no bait material was used for the control. After 4 h, captured flies (sex not determined) were counted while observing traps from outside the tent enclosure. After 24 h, the traps were collected and the flies counted and sexed.

House fly attraction to honeydew volatiles

Following statistically significant attraction of house flies to both the citrus mealybug honeydew and the fermented vinegar blend when applied to sanitary pads, honeydew volatiles identified by Hung et al. (in prep) were subsequently tested as attractants for house flies using tent enclosures, and with volatiles applied to a sanitary pad as described above.

Volatiles evaluated for fly attraction were: benzaldehyde (CAS 100-52-7) and Δ3-carene (CAS 13466-78-9) purchased from Sigma-Aldrich (St Louis, MO); (*D*)-(+)-limonene (CAS 5989-27-5) and myrcene (CAS 123-35-3) purchased from Spectrum Chemical (New Brunswick, NJ); butyl hexanoate (CAS 626-82-4), β-caryophyllene

(CAS 87-44-5), and (±)-linalool (CAS 78-70-6) purchased from TCI America (Portland, OR); (*Z*)-3-hexenyl acetate (CAS 3681-71-8) purchased from Penta International (West Coldwell, NJ). (*E*)-4,8-dimethyl-1,3,7-nonatriene was synthesized as described in Hung et al. (in prep).

Before each trial, the Petri dishes were washed with micro-90 detergent (International Products Corp., Burlington, NJ), rinsed with tap and distilled water, and dried at 120°C for at least 10 h. Blends of volatiles or individual volatiles were formulated in 1 ml hexane (control was hexane only), with the blend subsequently applied to a sanitary pad placed into a Petri dish as indicated above. Following application of the volatiles to be tested, sanitary pads were held for 5 min in a fume hood to let the hexane solvent evaporate. Immediately before use in the field, 2 mL of distilled water was added to each pad as a carrier solvent.

The following blends of honeydew volatiles were evaluated as indicated in Table 6.1: (a) 8-compound blend containing 10 mg each of β-caryophyllene, myrcene, limonene, Δ3-carene, (*Z*)-3-hexenyl acetate, benzaldehyde, butyl hexanoate, and DMNT; (b) 7-compound blend containing 10 mg β-caryophyllene, 5 mg myrcene, 5 mg limonene, 2 mg Δ3-carene, 2 mg (*Z*)-3-hexenyl acetate, 2 mg benzaldehyde, and 2 mg butyl hexanoate to roughly mimic the ratio of volatiles recovered from an extract of whitefly honeydew on navel orange that was attractive to house flies; (c) 7-compound blend containing the same volatiles as above at the same relative ratio, but at 1/10th of the dose, to evaluate whether a reduced dose might be more or less attractive relative to the blend above; (d) 4-compound blend containing 10 mg each of myrcene, (*Z*)-3 hexenyl acetate,

benzaldehyde, and β-caryophyllene; (e) 4-compound blend containing the same volatiles as above except all the compounds at 1 mg dose; (f) A blend of the 4 compounds in blend d but using 1:1:1:4 ratio of myrcene, (*Z*)-3 hexenyl acetate, benzaldehyde, and β-caryophyllene; g) a 2-compound blend containing (*Z*)-3-hexenyl acetate and benzaldehyde; h) a 2-compound blend containing myrcene and β-caryophyllene; i) benzaldehyde only; and j) (*Z*)-3-hexenyl acetate only.

Following evaluation of the more complex blends (a-c), a reductive process was used to formulate new blends to be tested, with a new blend containing half the volatiles comprising a parent blend. Blends that were attractive to house flies were further reduced in this fashion until individual component volatiles were tested alone. Blends or individual volatiles that were attractive to house flies on a first trial date were subsequently tested again on 1-2 separate days, with a new batch of flies used on each date to confirm the result.

After evaluating house fly attraction to blend d, it was hypothesized that the release ratio from this blend applied to a sanitary pad during the 4 h exposure period did not match the ratio of volatiles loaded. To determine the ratio of the volatiles released from the sanitary pad, a sample of blend d applied to the pad as described above was aerated for 4 h by pulling air at 2 L/min through a sealed, glass aeration chamber containing the bait, with odors subsequently captured in glass collection tubes containing activated charcoal held in place by glass wool plugs. The aeration was performed at room temperature (~25°C) with fluorescent lighting. The collected volatiles were then eluted in dichloromethane (DCM) into screw cap vials with Teflon cap-liners and stored at -20°C

until use. To measure the release ratio, 1 µl of the resulting extract was analyzed by GC using methods described previously (Hung et al. 2015). Of the four volatiles comprising this blend, three were eluted at a similar amount, but the fourth (β-caryophyllene) had a peak area that was only 25% of the peak area of the other three volatiles (Figure 6.3). To determine whether the attractiveness of this blend to house flies could be enhanced, a new blend was created (blend f) that increased the dose of β-caryophyllene by 4-fold to 40 mg per lure, so that each volatile in the blend was released in approximately equal amounts.

Lastly, house fly attraction to (*Z*)-3-hexenyl acetate, individually and in a blend with benzaldehyde, was compared to the fermented vinegar blend described above and a solvent control using the tent enclosure system, with all 4 quadrants in each tent utilized as described above.

Statistical analysis

All statistical analyses were performed using SAS v9.4 (SAS institute Cary, NC) with statistical significance defined by $p<0.05$. Fly captures (total, female, and male) for each compound or blend tested were checked for normality and equal variances by examining the residual plots prior to further analysis. The 6-hour counts assessing house fly attraction to citrus mealybug honeydew, female fly counts from blend d, and total counts from (*Z*)-3-hexenyl acetate only were $\log_{10}$ transformed and total fly counts from blend b were square transformed to normalize the data. Multi-factorial ANOVA (proc GLM) with

treatment/control and position within the tent test arena as independent factors was used to analyze the number of flies captured above each of the baited traps. Test date was included in the analysis if the same volatile blend was tested on multiple days. There were no statistical differences in total fly capture among the tent rows or columns at the field site, nor were there any interactions among tent rows or columns for any of the trials, meaning the traps within each tent were capturing an equivalent number of flies. There were also no interactions between treatment and position of treatments within the tent. With no significant differences or interactions, these factors were not included in the final analyses. The frequencies of male and female flies captured on the treatment or control traps were analyzed using a chi-squared test to evaluate differences in attraction by sex.

Results

Assessment of test methodology

Mean fly captures were greater on traps above the citrus mealybug honeydew than the control baits at 6 h ($df=1,17$; $F=27.18$; $p=0.0001$) and at 24 h ($df=17,1$ $F=19.98$; $p=0.0004$), confirming fly attraction to honeydew in the semi-field setting (Table 6.2). Both male and female flies were attracted to the mealybug honeydew ($df=17,1$; $F=12.79$; $p=0.0028$ and $df=17,1$; $F=0.17$; $p=0.0005$, respectively).

More flies were captured at 4 h ($df=3,35$; $F=47.05$; $p < 0.0001$) and at 24 h ($df=3,35$; $F=28.86$; $p<0.0001$) when fermented vinegar was released from a sanitary pad

relative to other volatile release methods tested (Table 6.3), with both male and female flies also captured in significantly greater numbers in the vinegar-baited sanitary pad traps than the other traps ($df=3.35$; $F=12.84$; $p<0.0001$ and $df=3,35$; $F=2.78$; $p< 0.0001$, respectively). In contrast to the mealybug honeydew test, an effect of trap position within the tent was not evident at 4 h ($df=3,35$; $F=2.41$; $p=0.087$), but was significant for total flies at 24 h ($df=3,35$; $F=4.44$; $p = 0.011$). There was no position effect for separate male or female fly counts at 24 h.

Assessment of honeydew volatiles

Results for the flies captured above the reconstructed blends of honeydew odors are summarized in Table 6.4. Overall, the same relative proportions of male and female flies were captured in both the treatments and controls for all trials.

Significantly more flies were captured at the 4-compound blend d than its control ($df=1,31$; $F=6.75$; $p=0.015$), with increased capture of females ($df=1.31$; $F=4.78$; $p=0.037$) but not to males ($df=1,31$; $F=4.1;2$ $p=0.052$). Of the 2-compound blends derived from blend d, the (*Z*)-3-hexenyl acetate/benzaldehyde blend was significantly attractive to house flies compared to the control ($df=1,53$; $F=17.67$; $p=0.0001$) with increased capture of both males ($df=1,53$; $F=24.32$; $p<0.0001$) and females ($df=1,53$; $F=5.65$; $p=0.021$). The other 2-compound blend, myrcene/β-caryophyllene was not attractive, and in fact, possibly repellent, with less than 40% of the flies captured at the treatment relative to the control. All other blends were not attractive to house flies ($p>0.05$). The two single compounds tested, (*Z*)-3-hexenyl acetate and benzaldehyde,

were both attractive to house flies compared to controls (df=1,35; F=22.4; p<0.0001 and df=1,33; F=19.81; p=0.0001, respectively), with traps baited with (*Z*)-3-hexenyl acetate capturing greater numbers of both male and female flies (df=1,35; F=11.55; p=0.0018 and df=1,35; F=16.52; p=0.0003, respectively), while traps baited with benzaldehyde captured greater numbers of males (df=1,33; F=19.97; F=0.0001) but not females. Traps baited with the tested odor blends and individual volatiles each captured approximately 60% of the total flies that were captured in the treatment and control traps combined.

In a 4-choice test, the (*Z*)-3-hexenyl acetate/benzaldehyde blend attracted more flies than (*Z*)-3-hexenyl acetate only, fermented vinegar, or the control (F=7.41; df=3,103; p=0.0002). The vinegar compounds and the single compounds were not more attractive than the control. There were more captures of male flies in traps baited with the 2-compound blend than traps with the other baits (F=7.25; df=3,103; p=0.0002), and female flies showed no difference in captures among the treatments (F=2.47; df=3,103; p=0.067).

Five of the ten 2-choice trials evaluating honeydew odors showed a bias for greater fly capture on the traps positioned on the right (northwest) side of the tent, and the 4-choice test showed a bias for increased fly capture in trap positions near the rear of the tent (southwest). Four of the trials performed on multiple dates showed a significant difference in the number of flies captured among the dates, but there was no interaction between date and treatment except for the trial with the (*Z*)-3-hexenyl acetate and benzaldehyde blend. This interaction was due to a difference in the magnitude of the number of flies attracted to the volatile blend rather than a loss of attraction to the blend,

so this interaction was also not considered in the final analysis. Natural variations in temperature, light intensity, air movement, humidity, along with variation among fly cohorts undoubtedly affected fly participation rates in these studies.

Discussion

In this study, some odor blends and individual odors resulted in greater captures of flies on traps positioned near these odors, even though flies had no access to the odor source. The increased fly capture near these odors suggests that flies either orient toward these honeydew odors at least over short distances, or that these honeydew odors elicit increased short range orientation and/or landing behaviors, or that fly detection of these odors simply increases time spent near the odor source through arrestment or increased localized searching behavior. While it is unknown how flies are specifically responding to these odors, the length of time required to run the assay (4 h), the low proportion (<10%) of ~500 flies released that were captured at a trap and relatively low proportion (<60%) of flies caught in the treatment traps over the control traps, suggests that flies may not be orienting and moving toward odor sources (anemotaxis) over distances of several meters.

It is possible that the doses of baits need to be greater for high fly response, given that blend d, with a 10-fold greater dose than blend e, was attractive to flies whereas blend e was not (Table 6.4, treatments d and e). On the other hand, a 10 mg dose on a sanitary pad is a relatively large dose of material considering that nanogram amounts of

compounds elicited responses from fly antennae in GC-EAD analyses (Hung et al. In prep). Other field studies (Willson and Mulla 1973, Qian et al. 2013) also recorded relatively low fly captures and low response rates in the field considering the fly load in the commercial facilities where the field studies were conducted should be noticeably higher than what was released in a tent. Willson and Mulla (1973) recorded at most ~150 house flies collected in a 24 h collection period at a commercial poultry ranch using sugar bait with rotten eggs as attractants in a jar trap and noted a 3:1 ratio of flies collected in traps baited with the test attractant versus an unbaited control. Qian et al. (2013) also collected low numbers (at most ~200 in the vinegar-baited trap) of house flies at a dairy in a 24 h collection period. However, the ratio of fly captures in vinegar-baited traps to control traps was very high at 20:1. A 25% solution of fresh molasses in water seems to perform better in an outdoor cage compared with our honeydew bait, with about 25% of flies responding to the baited traps in 6 h and about 10:1 ratio of flies in the molasses-baited traps compared to the control traps (Geden et al. 2009). Geden et al. (2009) noted that physical trap features may affect the efficiency of collections, but no indications were given in their study whether the flies were responding to the bait by attraction or arrestment.

Flies in this semi-field study were captured in greater numbers on traps near citrus mealybug honeydew on squash than on traps near uninfested butternut squash controls, verifying house fly attraction to honeydew in an outdoor setting (Table 6.2) (Hung et al. 2015). A reconstructed blend of volatiles mimicking fermented vinegar and released from a sanitary pad captured a greater number of flies than when the same volatiles were

released from rubber septum lures or open vials, perhaps because of the greater volatilization rate due to the large surface area of the sanitary pad. Qian et al. (2013) effectively used sanitary pad lures in cone traps to catch flies in the field.

More house flies were captured in traps baited with the 4-compound blend d, than in traps baited with the two-compound blend [(*Z*)-3-hexenyl acetate/benzaldehyde] derived from this blend, or in traps baited with (*Z*)-3-hexenyl acetate and benzaldehyde individually compared to their respective controls (Table 6.4, treatments d, g, i, and j). We selected the particular compounds to constitute blend d based on the fact that they were identified from at least two separate honeydew samples. Traps baited with (*Z*)-3-hexenyl acetate and benzaldehyde as a blend captured more flies than the fermented vinegar bait or the (*Z*)-3-hexenyl acetate individually, suggesting that these two compounds may act synergistically (Table 6.5).

Overall, both male and female flies equally showed attraction towards the citrus mealybug honeydew as well as the (*Z*)-3-hexenyl acetate/benzaldehyde blend over the controls. Male flies were more numerous on both the treatment and control traps (>60%) compared to the females possibly due to higher male adult emergences than female flies or greater male fly activity which lead to their greater numbers. Fly captures were also male-biased in the field study (Chapter 5), perhaps due to their exhibiting mating or dispersal behaviors as well as food seeking behavior. We expect honeydew to be a food attractant, not an oviposition or breeding site; therefore, both male and female flies should respond to honeydew volatiles.

Benzaldehyde is produced from sources as diverse as a decaying mouse carcass (Johansen et al. 2014) to volatiles from healthy plants (Natale et al. 2003, Webster et al. 2010), but attraction of house flies to benzaldehyde has not previously been evaluated. (*Z*)-3-hexenyl acetate is a common plant volatile identified from healthy plant volatiles [reviewed in (Bruce and Pickett 2011)] and herbivore-stressed plants (Holopainen and Gershenzon 2010) , but no evidence of this compound have been found in decaying organic matter nor has it been evaluated for house fly attraction. Zito (2015) demonstrated house fly attraction to flowers which produce "sweet" terpenoids, including β-caryophyllene, as well as odors mimicking decaying organic matter such as indole and dimethyl trisulfide, but found that a mixture with the terpenoids and indole did not increase fly captures compared to indole. In contrast, another study by Zito (2013) examined a blend of three terpenes, including linalool, which was attractive to house flies. Although linalool was identified from our honeydew volatile collections (Hung et al. in prep.), we did not include it in this study because it was suspected to be repellent for muscid flies (Maganga et al. 1996, Birkett et al. 2004, Hieu et al. 2014).

Overall, house flies were attracted to honeydew volatiles that we identified from three different honeydew samples. The response rate to the honeydew volatiles was not as high as seen in other studies testing different baits, and was lower compared to the crude citrus mealybug honeydew, suggesting other components may contribute to fly attraction. Whether house flies are attracted from a distance to these volatiles, or they spend increased time investigating the sources of volatiles once they have found them, needs further investigation. Additional studies examining house fly responses to honeydew

volatiles may provide more information on the extent of filth flies as factors in food-borne contamination of leafy-greens crops contaminated with honeydew.

Acknowledgments

We thank Dr. Mark Hoddle for providing the citrus mealybug colony. Dr. Yunfan Zou synthesized DMNT. Jesse Montoya, Levi Zahn, and Caleb Hubbard assisted with the semi-field set up and colony care. This project was funded by United States Department of Agriculture National Integrated Food Safety Initiative (USDA-NIFSI) # 2009-51110-05856. Support for KYH was also provided by the National Science Foundation Graduate Research Fellowship Program (NSF GRFP).

Tables and Figures

Figure 6.1: Semi-field tent enclosure set-up in shaded area of UCR Agricultural Operations. Tents were placed in 3 rows of 3 each (9 tents in total).

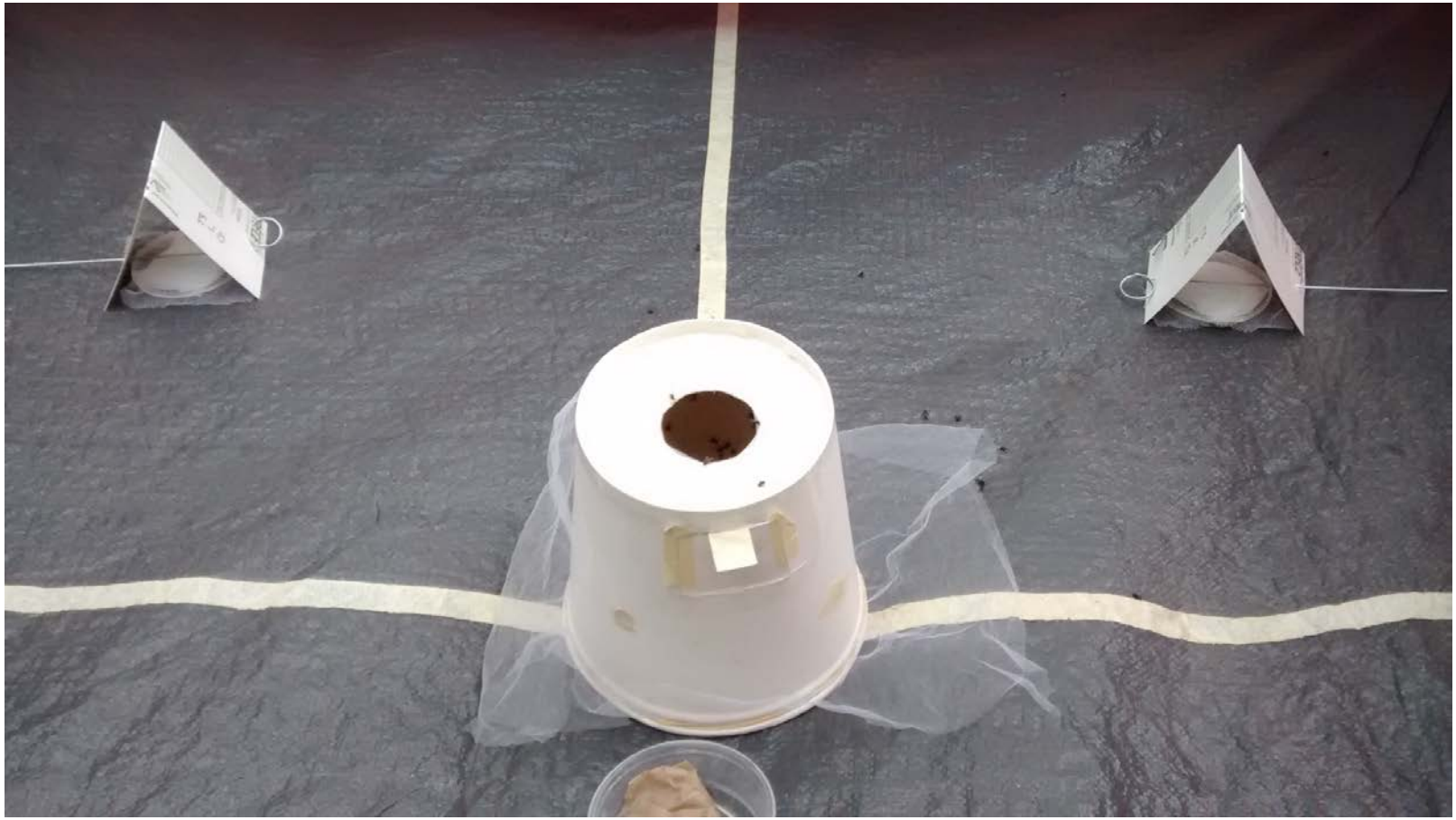

Figure 6.2: Inside the tent set-up showing the release chamber and volatile materials with sticky trap positioned above each volatile treatment.

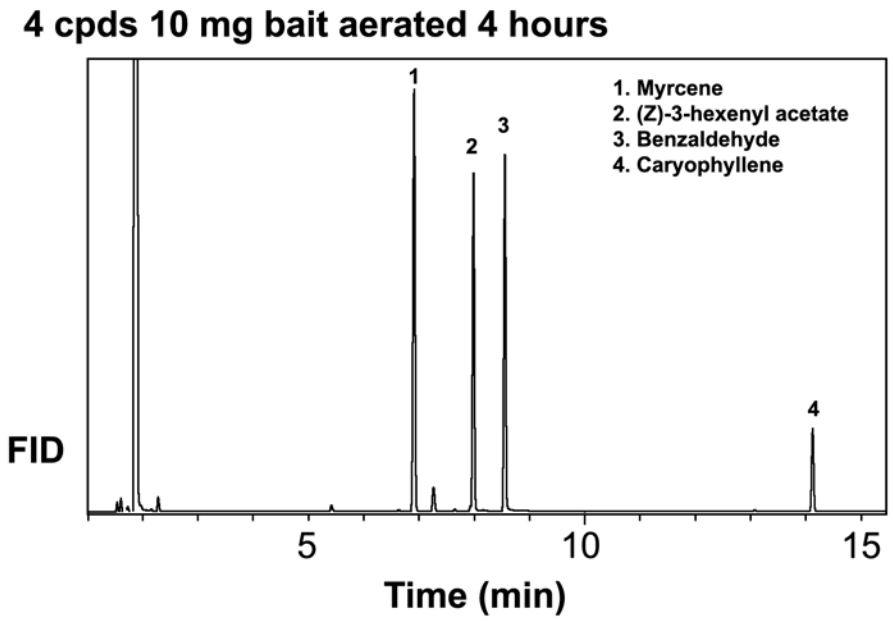

Figure 6.3: Gas chromatogram of volatiles collected by aeration of blend d for 4 hours. β-Caryophyllene (4) had a considerably reduced rate of release compared to the other three compounds despite the blend having equal 10 mg doses of each compound.

Table 6.1: Summary of blends and single compounds tested for house fly attraction

Treatment	P< 0.05	β-Caryophyllene	Myrcene	(*Z*)-3-Hexenyl acetate	Benzaldehyde	Limonene	Δ 3 Carene	Butyl hexanoate	DMNT
a	No	10 mg	10 mg	10 mg	10 mg	10 mg	10 mg	10 mg	10 mg
b	No	10 mg	5 mg	2 mg	2 mg	5 mg	2 mg	2 mg	
c	No	1 mg	0.5 mg	0.2 mg	0.2 mg	0.5 mg	0.2 mg	0.2 mg	
d	**Yes**	10 mg	10 mg	10 mg	10 mg				
e	No	1 mg	1 mg	1 mg	1 mg				
f	No	40 mg	10 mg	10 mg	10 mg				
g	**Yes**			10 mg	10 mg				
h	No	10 mg	10 mg						
i	**Yes**				10 mg				
j	**Yes**			10 mg					

Table 6.2: Mean numbers of flies (± SEM) from 500 flies released into the tent that were attracted to butternut squash covered with citrus mealybug honeydew (Tmt) versus clean squash (Ct).

	Male		Female		Both	
	Tmt	Ct	Tmt	Ct	Tmt	Ct
Mean	11.2 (2.0)	4.0 (1.0)	15.4 (2.4)	3.9 (1.0)	26.7 (4.2)	7. 9 (1.0)
Resp to tmt	73.7%		79.9%		77.2%	
ANOVA	0.0028		0.0005		0.0004	

Table 6.3: Mean numbers of houseflies (±SEM) from 500 flies attracted to a reconstructed odor blend mimicking fermented vinegar using different release devices. Means within columns followed by the same letters are not statistically different.

	4 h count	24 h count mean		
		Male	Female	Both
Pad	25.89 (2.03) a	12.11 (1.16) a	20.11 (2.60) a	32.22 (3.03) a
Vial	8.67 (1.15) b	5.56 (1.18) b	10.33 (1.29) b	15.89 (1.85) b
Septum	7.22 (1.09) b	4.67 (0.80) b	7.00 (1.08) bc	11.67 (1.29) b
Control	7.67 (0.91) b	5.22 (0.60) b	6.00 (0.75) c	11.22 (1.28) b

Table 6.4: Mean fly captures in two-choice assays for honeydew volatile compounds. Blend names are explained in Table 1. Tmt is the volatile compound treatment Ct is the solvent control. *Number of flies in a container were too low for the study and was removed from the analysis. [a] Values show significant number of fly captures on the right treatment position. [b] Statistically different among trial dates [c] An interaction effect was observed between the date and the number of flies captured at the treatment but was not included in the final analysis.

Treatment	Male		P-value	Female		P-value	Total Flies		P-value	% Capture at Tmt	N
	Tmt (SEM)	Ct (SEM)		Tmt (SEM)	Ct (SEM)		Tmt (SEM)	Ct (SEM)			
a	8.3 (1.5)	8.8 (1.2)	0.56[a]	5.7 (0.7)	6.2 (1.5)	0.60	14 (2.0)	15 (2.4)	0.51[a]	48.2%	9
b	6.3 (1.2)	7.8 (0.9)	0.28	4.8 (0.6)	7.4 (1.4)	0.12	11 (1.4)	15.1 (1.6)	0.058	42.1%	8*
c	11.3 (1.3)	13.2 (1.6)	0.27	4.6 (0.8)	4.8 (2.0)	0.86	15.6 (1.6)	18.0 (2.0)	0.35	46.9%	8*
d	7.4 (1.0)	5.1 (0.8)	0.052[b]	9.1 (1.5)	5.6 (0.8)	**0.037**	16.6 (2.3)	10.6 (1.2)	**0.015[ab]**	61%	18
e	5.9 (1.1)	6.6 (1.3)	0.76	7.1 (1.7)	9.0 (1.4)	0.47	15.6 (2.3)	13 (2.4)	0.53	45.6%	9
f	8.8 (0.8)	7.4 (0.7)	0.16[a]	6.9 (1.4)	7.7 (1.4)	0.13[ab]	15.6 (2.0)	15.1 (1.9)	0.90[ab]	50.9%	17*
g	16.9 (2.0)	8.8 (1.0)	**<0.0001[bc]**	13.1 (2.4)	8.3 (1.1)	**0.021[bc]**	30.0 (4.2)	17.1 (1.9)	**0.0001[bc]**	63.7%	27
h	13.5 (2.3)	18.5 (3.1)	0.061[b]	7.8 (1.3)	10.2 (1.7)	0.15[b]	21.3 (3.3)	28.7 (4.6)	0.055[b]	39.5%	9
i	24.8 (1.6)	14.9 (1.7)	**0.0001[a]**	5.1 (0.7)	3.6 (0.5)	0.14	29.8 (2.0)	18.5 (1.7)	**0.0001[a]**	61.7%	17*
j	26.4 (2.0)	19.5 (1.4)	**0.0018[a]**	29.1 (2.1)	20.6 (1.4)	**0.0003[ab]**	55.6 (3.4)	40.1 (2.5)	**<0.0001[a]**	58.1%	18

Table 6.5: Numbers of flies (±SEM) from 500 flies attracted to honeydew compounds or fermented vinegar. Means within columns followed by the same letters are not statistically different.

	Male	Female	Both
(*Z*)-3-hexenyl acetate and benzaldehyde blend	9.0 (1.9)a	8.9 (1.1)a	17.9 (2.1)a
(*Z*)-3-hexenyl acetate	5.1 (1.1)b	7.6 (0.7)ab	12.5 (1.2)b
Vinegar	4.2 (1.0)b	7.4 (0.9)ab	11.8 (1.2)b
Control	3.7 (0.6)b	6.3 (0.8)b	10.0 (0.9)b

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CONCLUDING REMARKS

House flies (*Musca domestica*) have been pests of humans and animals for eons due to their ability to transmit diseases by dispersing throughout their environment and depositing pathogens at sites where humans and animals may acquire them. Of particular concern is contamination of human foods by pathogens carried by flies (Talley et al. 2009). In the Salinas Valley of California, filth flies (Muscidae and Calliphoridae) were found in fields of leafy greens adjacent to cattle feedlots, especially where honeydew-producing insects were present on the crops (Talley et al. 2009). Honeydew comprises the sugar-rich excreta of plant-sucking insects such as scales, aphids, whiteflies, and mealybugs. The filth flies collected from these sites were contaminated with *Escherichia coli* O157:H7, and it had been suggested that filth flies may have played a role in the 2006 food-borne *E. coli* outbreak in bagged spinach that affected over 200 individuals and generated nationwide attention. While it is common to associate filth flies with outbreaks of food-borne diseases (Lindsay and Scudder 1956, Blazar et al. 2011), the possible correlation of fly prevalence with honeydew presence was intriguing. Filth flies had not previously been associated with honeydew as a factor leading to food-borne contamination, nor had honeydew been considered as a possible source of filth fly attractants.

We hypothesized that honeydew is a house fly attractant, leading to the accumulation of flies on the plants, thereby increasing the chance of food-borne

contamination. To address this hypothesis, we examined house fly responses to honeydew in laboratory bioassays, followed by the identification of potential attractants using gas chromatography-electroantennogram detection (GC-EAD), gas chromatography-mass spectrometry (GC-MS), field assays, and semi-field assays. In our behavioral studies, our methods did not directly analyze the movement of flies towards odor sources, but rather “attraction” as the increased capture of flies on traps placed near honeydew or honeydew volatiles. Therefore, honeydew as an "attractant" was defined by the behavioral endpoint of house flies captured in greater numbers near the attractive material, but with likely contributions from some or all of upwind odor-mediated anemotaxis, chemotaxis at close range, and/or increased landing at or near the odor source.

We first examined house fly attraction to honeydew with a two-choice olfactometer, where flies placed in the middle of the olfactometer were allowed to move into one of two side chambers with airstreams containing either the volatile being examined or no odor as a control. Overall, the olfactometer demonstrated house fly attraction toward citrus mealybug honeydew, a material that was expected to be attractive to house flies based upon unpublished preliminary trials. The olfactometer was advantageous in that we only needed enough material to fit into two canning jars through which the airstreams would pass prior to entering the olfactometer. However, the small central arena in the olfactometer restricted fly movement and the air flow may not have been consistent among different trial days using identical treatments. Other studies have used a two-choice olfactometer to successfully assess filth fly attraction to feces and (*Z*)-

9-tricosene (Chaudhury et al. 1972, Carlson et al. 1974, Bay and Pitts 1976), but, unlike our olfactometer, their system was open to the external air at the central chamber where the flies were released and air movement was pushed through the device rather than pulled with a vacuum. Another advantage we expected from our olfactometer was the short time needed for trials, which allowed for enough replicates to be completed during a single day for robust statistical testing. Because we expect the plant and honeydew-contaminated materials to change volatile production over time after collection, we desired to evaluate house fly attraction to honeydew on cuttings within 24-48 h of field collection to ensure that honeydew volatiles would still be present in amounts similar to when they were first collected. Unfortunately, in preliminary trials with a homogenous honeydew source (citrus mealybug honeydew), house fly responses toward the honeydew odors were inconsistent among replicates. While more flies moved into the olfactometer arm containing honeydew odors in general, and over all testing days (and replicates) this attraction was significant, on some individual days even with eight replicates, house fly responses to honeydew odors were not significantly different than responses to controls. Because of this lack of consistency between days, the olfactometer was eventually abandoned as a tool to demonstrate house fly attraction to honeydew, and instead we utilized a cage choice bioassay method.

The cage bioassay used a larger arena and allowed ambient air to move through the cage. The flies could behave more normally than in the olfactometer, which provided limited space for flight. The cage assays did require considerably more honeydew-contaminated material (enough to fit in a 2 L beaker) for the house flies to respond and a

longer response time (24 h) was also required than with the two-choice olfactometer (20 min). Nevertheless, statistically consistent responses were deemed more important than the speed of the assay and we ultimately decided the cage assay was a better method to examine house fly attraction to honeydew than the two-choice olfactometer.

Overall, house flies were attracted to odors from honeydew-producing insects, honeydew, fungi or other honeydew colonizers, or to some combination of these rather than to plant volatiles alone. Honeydew produced by citrus mealybugs on butternut squash, whiteflies on navel orange foliage, whiteflies on Marsh grapefruit foliage, and pea aphids on faba bean plants captured more flies compare to their respective controls. These honeydew-contaminated materials were collected from the field and the laboratory. House flies were attracted to honeydew produced by a number of different sucking insects (whiteflies, aphids, mealybugs) feeding on various plants (navel orange foliage, Marsh grapefruit foliage, faba bean foliage, butternut squash fruit). These honeydews were abundant on the collected plants, with leaf surfaces being sticky to the touch and with visible molds growing on the honeydews. Honeydews were colonized by several fungal species (Chapter 3). The attraction of house flies to honeydew excreta from different insect groups infesting different plants suggests that house flies may be attracted to honeydew generally, as long as the honeydew is abundant enough on the leaves and with enough fungal growth. In contrast, house flies were not attracted to the honeydew of lerp psyllids on eucalyptus leaves, perhaps because the odors produced by the eucalyptus cuttings were repellent, or because the lerp honeydew was not visibly moldy. House flies

also were not attracted to honeydew produced by aphids feeding on crepe myrtle, perhaps due to the relatively low number of insects and level of honeydew accumulation.

Many of the honeydews collected in the field during these studies were colonized by two fungal species, *Aureobasidium pullulans* and *Cladosporium cladosporioides* (Hung et al. 2015). Additionally, *Fusarium* spp. were identified in fly-attractive honeydew from laboratory maintained citrus mealybugs on butternut squash. *Aureobasidium pullulans* cultured in a potato dextrose broth was attractive to house flies, indicating fungi may produce volatiles that are attractive to flies. *Aureobasidium pullulans* has been associated with attraction of insects generally (Davis et al. 2012, Davis and Landolt 2013), but it was previously unknown that *A. pullulans* would also colonize honeydew and perhaps play a similar role there. Bacteria associated with honeydew were not examined in these studies, but bacterial volatiles may also be important as attractants. For example, the volatiles produced by one bacteria identified from honeydew were attractive to *Episyrphus balteatus* (Syrphidae), an aphid natural enemy (Leroy et al. 2011). Further studies examining the volatiles produced by *A. pullulans* colonizing honeydew may show that fungi play a role in house fly attraction by producing attractive volatiles as byproducts of their metabolism.

Analyses of the headspace odors of various honeydews showed that they contained numerous compounds and so it was necessary to narrow down compounds of interest to house flies through the use of GC-EAD, followed by identification of the volatiles that elicited responses from house fly antenna using GC-MS. Benzaldehyde, β-

caryophyllene, (*Z*)-3-hexenyl acetate, and myrcene were identified from multiple samples, which may indicate that flies only need to detect a few compounds out of the variety of volatiles produced by honeydew in order to interpret the volatile blend as a possible food source. For example, indole was identified from pig manure (Cossé and Baker 1996), but sapromyiophilous plants also produce this compound, in the absence of other volatiles common to pig manure, as a fly lure to aid in flower pollination (Zito et al. 2015). Thus, other compounds found in honeydew odors may help house flies to distinguish between different honeydews. However, my studies did not focus on the degree of attraction among the different honeydews, and so I cannot remark on whether some honeydews were more attractive than others.

We repeatedly used the honeydew from citrus mealybug feeding on butternut squash as a honeydew attractant for testing the olfactometer, cage bioassay, field assay, and semi-field assay to demonstrate house fly attraction to honeydew and verify the viability of the assay methods. Unfortunately, identification of volatile odors from the citrus mealybug honeydew has not been identified. This honeydew was easily produced in large amounts in laboratory colonies of mealybugs, unlike the other honeydew samples which were only available in the field during certain periods of the year. Thus, we prioritized identifying the honeydew volatiles from the more limited honeydew samples (Chapter 4). With additional work, given that honeydew produces a complex suite of volatile compounds, we may discover new volatile blends from this honeydew that may attract flies more effectively than the compounds or blends described in this thesis.

Given that electroantennogram measurements only show the ability of the fly to detect compounds and not the behavioral responses that those compounds may elicit, semi-field and field studies were used to assess whether house flies were attracted to the identified compounds. Because I was interested in developing attractants for field use, I focused on using field or semi-field assays to examine house fly behaviors towards selected honeydew volatiles, rather than using laboratory bioassays that might not translate well to field contexts.

The cone traps I utilized in field bioassays demonstrated house fly attraction to ethanol, but overall, the traps did not capture enough flies to distinguish between the different lures that I tested. The pig manure volatiles compared to the 50% ethanol solvent control only increased captures for the female house flies but not males, which is not unusual considering that pig manure volatiles can signal an oviposition site for female flies. While trap placement may have improved the number of flies captured, suitable locations around the dairy were constrained by the number of traps and amount of space. Traps were mostly located in areas around the perimeter of the animal pens. A different trap type might have increased captures; for example Geden et al. (2009) demonstrated that the same lure when coupled with different trap types can result in dramatic differences in capture rates. An increased amount of material may have captured more flies, but we based the dose of the volatiles tested on prior studies (Cossé and Baker 1996) or on expectations based upon the GC-MS data. Finally, perhaps placing the bait closer to the cone or placing the trap directly on the ground rather than elevating the cone

trap off the ground may have taken advantage of the short hopping flight behavior of flies to increase their capture in the cone trap.

Ethanol clearly interfered with fly responses to the reconstituted honeydew blend. Ethanol initially was used as solvent for the volatiles to be tested because not all the compounds were water soluble. However, following field bioassays that demonstrated house fly attraction to ethanol, hexane was used to solubilize test compounds for all remaining studies.

Because the field trapping studies did not demonstrate house fly attraction to honeydew or honeydew odors, we elected to continue with semi-field studies conducted in a location where the quantity and concentration of competing odors was expected to be reduced. Semi-field assays were similar to the laboratory bioassays in that flies were offered choices in a cage-type arena. In this case, the "cage" was a tent set up at UC Riverside's Agricultural Operations area to see whether flies would respond to volatiles outdoors. Testing of the volatiles produced by the attractive honeydew showed that a blend of benzaldehyde and (*Z*)-3-hexenyl acetate was attractive to flies in this bioassay, and these compounds had not previously been reported as possible house fly attractants. Overall, any future field studies should test a range of doses of these materials in order to try and capture enough flies to distinguish attractiveness of different individual honeydew volatiles or volatile blends.

Attractive, and even repellent, volatiles may be used to improve trapping systems for pest management, and volatile semiochemicals have been suggested for possibly reducing pests in plant and animal agriculture as well as reducing vectors that transmit

pathogens (Pickett et al. 1997, 2010). Studying house fly behaviors toward potential attractants might lead to improved management strategies and better understanding of house fly contributions to pathogen dispersal. With the benefits of push-pull systems being a frequently reviewed subject in pest management (Aldrich et al. 2003, Cook et al. 2007, Hassanali et al. 2008, Pickett et al. 2014) and the numerous successful applications of semiochemicals for pest monitoring and control (e.g., Agelopoulos et al. 1999, Cox 2004), attractant-based trapping should be further investigated as a practical method of controlling filth flies, thereby reducing area-wide pesticide use, and limiting development of insecticide resistance in house flies. Although house fly attraction behaviors have been studied for more than a century (Morrill 1914, Richardson 1917, Wieting and Hoskins 1939, Brown et al. 1961, Frishman and Matthysse 1966), specific attractants have only been identified over the past few decades (Mulla et al. 1977, Cossé and Baker 1996, Quinn et al. 2007, Qian et al. 2013).

This dissertation presents the first study of house fly attraction to honeydew and specific honeydew odors. It contributes to our understanding of house fly responses to honeydew volatiles and demonstrates that insect honeydew is attractive to house flies, supporting our hypothesis that honeydew production by sucking insects infesting food crops may contribute to attraction of house flies to food crops. There are still areas to explore to better understand house fly attraction to honeydew, such as investigating the role of fungi in production of volatiles from honeydew, and investigating additional honeydew volatiles to increase the attractiveness of the honeydew volatile blend. Reduction of contamination of food crops by fly-borne pathogens may also be aided by

changes in agricultural policy, such as expanding buffer zones between leafy-green food crops and sources of fly production, and reduction of honeydew-producing insects on food crops. This study integrates areas of food safety and public health, aiming to develop a better understanding of fly attraction to honeydew volatiles. We expect this knowledge to be useful for developing improved house fly and filth fly management strategies.

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