FEEDING ECOLOGY AND OVIPOSITIONAL CUES OF THREE PARASITOIDS;
DIACHASMIMORPHA LONGICAUDATA, DORYCTOBRACON AREOLATUS, UTETES
ANASTREPHA (HYMENOPTERA: BRACONIDAE) ATTACKING NEOTROPICAL
ANASTREPHA SPP. (DIPTERA: TEPHRITIDAE)

By

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To my family, friends and acquaintances: for without them, I would not be the man I am today
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Polyphagous generalist tephritids are often pests in both their native ranges and when introduced to other tropic and subtropic areas. *Anastrepha suspensa* (Lowe) commonly known as the Caribbean fruit fly (Caribfly), indigenous to the Greater Antilles, infests over 100 fruit species. There is a potential to use argumentatively released parasitoids, particularly opine braconids, to biologically control this and other tephritids pests worldwide. While adult ability to find both food and hosts is critical to the success of such programs little is known about the feeding and chemical ecology of this group of insects.

Three opine fruit fly parasitoids: *Diachasmimorpha longicaudata* (Ashmead), *Doryctobracon areolatus* (Szepligeti) and *Utetes anastrephae* (Viereck) have been used in the control of *A. suspensa* in Florida and in other locations. Adult parasitoids feed on extrafloral nectars, hemipteran-honeydews and on fruit juices as they seep from injured fruit. The ability to exploit fruit juice would allow the parasitoids to efficiently forage for hosts and food sources simultaneously.
Chemical cues from the parasitoids’ hosts and the substrates in and on which the hosts develop are known, or are hypothesized to be, fundamental components of orientation during foraging for adult food, oviposition opportunities and perhaps mating sites.

Compounds produced by the larvae have not been previously described nor their significance to parasitoid foraging determined. Volatiles collected from four species of tropical and subtropical Tephritidae were identified. Para-ethylacetophenone, an analog of a known tephritid parasitoid attractant, was a major constituent of all four species and was not associated with larvae of non tephritid flies.

Mass-rearing and augmentative release of tephritid fruit fly parasitoids has great promise, particularly in conjunction with sterile males and in areas where insecticides cannot be widely applied. Currently there are no means to monitor the survival and dispersal of fruit fly parasitoids following release. Isolation of specific volatile components in fruit juices and the larval host that stimulates long range attraction would be a first step in the identification of effective synthetic attractants. A reliable trap that uses an effective attractant, is efficient over time, and will restrain the trapped insects for monitoring needs to be developed.
CHAPTER 1
GENERAL INTRODUCTION

Literature Review

This chapter provides an introduction to the 1) methodology of bioassays used in this research, 2) the description of the three Opiine Fruit Fly Parasitoids (Hymenoptera: Braconidae) that were utilized in these studies: *Diachasmimorpha longicaudata* (Ashmead), *Doryctobracon areolatus* (Szepligeti) and *Utetes anastrephae* (Viereck) (Hymenoptera: Braconidae), 3) and discusses the fruit fly *Anastrepha suspensa* (Loew) (Diptera: Tephritidae) and current management practices. The final segment of this chapter outlines the research objectives.

The introduction of exotic insect natural enemies for the permanent suppression of insect pests was first used by H. S. Smith in 1919; he termed this “biological control”. This term has since been employed to cover practically all biologically based pest control measures such as plant breeding for resistance to pests, autocidal controls, application of semiochemicals and cultural controls (Waage et al., 1998). There are a few polyphagous generalist species of Tephritidae (true fruit flies) that are often pests in both their native ranges and when introduced to other tropic and subtropic areas. *Anastrepha suspensa* (Lowe) is one in this group. *A. suspensa* commonly known as the Caribbean fruit fly (Caribfly), was first detected in the United States in Key West in 1930 (Weems, 1966), and was accidently introduced into mainland Florida in 1965. It is now established in central and southern Florida (Nation, 1972). This species of tephritid fruit fly is indigenous to the West Indies and infests over 100 fruits (Swanson and Baranowski, 1972). The preferred hosts are *Psidium guajava* (common guava), *Eugenia uniflora* (Surinam cherry), *Prunus persica* (peach), *Eriobotrya japonica* (loquat),
Syzygium jambos (rose apple), and Terminalia catappa (tropical almond) (Weems 1966, Nguyen et al., 1992). Like many pest fruit flies, A. suspensa is long lived, highly mobile and exhibits high reproductive potential (Zwölfer, 1983).

Although A. suspensa usually infests only overripe commercial citrus, it is a threat to the Florida citrus industry. Due to strict importation regulations of exports to Japan, citrus growers have spent millions of dollars monitoring and managing this pest (Nigg et al., 2004).

The use of insecticide+bait sprays are the standard procedure for the maintenance of fly-free export zones, such as Florida’s, and low-prevalence production zones (Enkerlin, 2005), as well as eradication programs where they typically precede sterile male releases (Krafsur, 1998). However, there are situations, such as urban and conservation areas, where chronic insecticide use is impractical and because of this there is an ongoing search for more environmentally-friendly techniques (Godfray, 1998).

The use of parasitoids as a pest control has been employed worldwide as alternative to labor intensive and environmentally hazardous practices (Aluja, 1994). As with many other agriculturally important insects, there is a long history of fruit fly parasitoid introductions/establishments (Ovruski et al., 2000) and more recently a growing emphasis on the mass-rearing and augmentative release of several of these species (e.g., Sivinski et al., 1996). Conservation biological control of tephritid parasitoids, the providing of food, shelter and alternative hosts in order to concentrate and increase numbers of natural enemies is in its early stages (Rohrig et al., 2006; Aluja et al., unpublished data). In all these forms of biological control, monitoring the survival
and dispersal of natural enemies is critical to their efficient practice and sometimes even to their success. However, there is relatively little known about the chemical cues used by fruit fly parasitoids to forage for food, mates and hosts, and which could be adopted as lures for traps or as stimulants in mass-rearing and the manipulation of populations in the field. At present there are no simple, cost-effective and efficient attractants for the major tephritid biological control agents (Messing and Wong, 1992).

Most animals and many plants use chemicals either as signals (semiochemicals; e.g., pheromones) to communicate with con- and heterospecifics or as cues (kairomones) to obtain information about their environment. Chemicals in the environment, both of con- and heterospecific origin, are important among Hymenoptera and are essential to social organization and host, nutrient and mate location (Ayasse, 2007). For example, it has been demonstrated that fruit-associated chemicals are used for host location by certain tephritid parasitoids. Chemicals released by fungi within rotten fruits are attractive to females of the opiine braconid *Diachasmimorpha longicaudata* (Ashmead) (Greany et al., 1977a, Messing & Jang, 1992). Odors of fresh cucumber and decaying pumpkin elicit similar responses in another fruit fly attacking opiine *Psyttalia fletcher* (Silvestri) (Hymenoptera: Braconidae) (Messing et al., 1996). The larvae within the fruit may also contribute chemical cues used by female natural enemies searching for oviposition opportunities (Stuhl, 2011a; chapter 2). In addition, host fruit of tephritid fruit flies may be used to locate adult feeding sites for both males and females (Sivinski et al., 1997, Stuhl et al., 2011a; chapters 3 and 4).

This research investigated two potentially important sources of chemical cues for the location of hosts and adult food, tephritid larvae and the fruit they occupy, as they
apply to three opine parasitoids, *Diachasmimorpha longicaudata* (Ashmead), *Doryctobracon areolatus* (Szepligeti) and *Utetes anastrephae* (Viereck) attacking *A. suspensa* and a number of other agriculturally important tephritids.

**Nutrition**

Knowledge of feeding behavior is an important factor in the study of insect nutrition. Many insect species will not feed or only feed poorly in the absence of specific chemical stimuli (Zwölfer and Harris, 1971). When investigating survival, growth and reproduction, it is difficult to determine if poor performance is due to a failure of their diet or due to starvation. For many animals the common nutrients themselves are enough to stimulate feeding behavior (Forbey et al., 2009). Others will initiate feeding activity in response to volatile chemical signals that are not necessarily emitted by nutrients, but which indicate the presence of nutrients. These volatiles often arise from “secondary products” of metabolism and sometimes from what would otherwise be chemical defenses produced by the plant (Glendinning, 2007).

The dietary requirements for adult *Anastrepha* spp. for proper development, reproduction, and survival are amino acids, vitamins, minerals, carbohydrates, and water (Hagen, 1952). Natural sources of these essential nutrients can be located in liquids oozing from overripe or damaged fruit, bird feces and the surface of leaves and fruit. An excellent source of water can be found in rain drops (Aluja and Birke, 1993). Because adult fruit flies and fruit fly parasitoids occur in the same habitats and exhibit similar diel patterns of activity, they may also exploit some of the same nutritional resources, principally fruit juices oozing from wounded or infested fruit (Eijs et al., 1998, Sivinski et al., 2006). Feeding by adult hymenopteran parasitoids can have a considerable effect on their longevity, fecundity, and movement (Leius, 1961; Wäckers
Swaans, 1993; Morales-Ramos et al., 1996; Jervis & Kidd, 1999). Wasps feed on many different carbohydrate sources, such as floral and extrafloral nectar, honeydew, host fluids and fruit juices (Jervis et al. 1993, Eijs et al., 1998). While floral feeding will increase the longevity, fecundity and parasitism rates of some species (Idris and Grafius 1995; Zhao et al., 1992); there is little evidence that such sources are important to the opiine braconids under consideration (Sivinski et al., 2006). Neither is there any evidence of host feeding which is not surprising given the sequestered nature of the hosts.

**Semiochemicals: Kairomones and Attraction**

Plant odors are typically composed of small organic molecules that readily evaporate. Chemicals that express this property are described as volatile compounds (Niinemets et al., 2004). Insect-plant interaction involves detection of volatiles through the antennae, maxillary palps, tarsi and the ovipositor (Martin, 2008), in which the surfaces contain specialized cells that recognize and bind to specific volatiles. Each cell may only contain one receptor type, but a single compound can be recognized by more than one receptor (Visser, 1986). The result is a pattern of neuronal firing that is elicited by a specific compound or a mixture that will be unique to the individual species. This is an extremely sensitive system that allows for the receptors to detect an airborne volatile at concentrations of a few parts per billion (Dudareva et al. 1996, Pare and Tumlinson, 1999, Dicke and van Loon, 2000, Kessler and Baldwin, 2001, Dudareva et al. 2004).

Host plant odors and kairomones from their host are significant cues in parasitoid foraging (Turlings et al. 1990), and there are a large number that have been identified as attractants (Metcalf and Metcalf, 1992). Plant-produced semiochemicals act to benefit the insect perceiver with rewards such as nectar and pollen. In some cases
plants under attack by herbivores release semiochemicals that attract predators and parasitoids (Tumlinson et al., 1993). Fruits are prolific in the production of volatile compounds that serve as both signals and cues (Averill et al., 1988).

**Parasitoids**

Three Opiine Fruit Fly Parasitoids (Hymenoptera: Braconidae) were utilized in these studies: *Diachasmimorpha longicaudata*, *Doryctobracon areolatus* and *Utetes anastrephae* (Hymenoptera: Braconidae). All three parasitoid species are used in the control of *Anastrepha suspensa* (Diptera: Tephritidae) and other fruit fly pests (Tables 1-1, 1-2, 1-3). Almost 60% of the 46 parasitoid species recorded from *Anastrepha* belong to the family Braconidae, and of these, 81.5% of the parasitoid species are in the Opiinae (Ovruski et al., 2000).

**Diachasmimorpha longicaudata**

*Diachasmimorpha longicaudata* (Ashmead, 1905) is a larval-prepupal endoparasitic koinobiont larval parasitoid. It is a synovigenic species, thus requiring a source of nutrition to maximize female fecundity. It was collected in Southeast Asia from mangos and guava infested with the Oriental fruit fly *Bactrocera dorsalis* (Hendel) (Bess et al., 1961, Clausen et al., 1965). *Diachasmimorpha longicaudata* was introduced into Hawaii for control of fruit flies of economic importance in 1948 (Bess et al., 1961) and in 1954 *D. longicaudata* was introduced into Mexico to control the Mexican fruit fly *Anastrepha ludens* (Wharton, 1989). This has been the most frequently studied fruit fly parasitoid due to its control potential and ease of rearing (Purcell, 1998). Following the introduction of *A. suspensa* populations into Florida, *D. longicaudata* was imported into Florida and released in 1972 (Baranowski, 1987). It has since become well established in areas with high *A. suspensa* populations (Eitam et al., 2004).
species forages for late instar larvae in ripe fruit in the tree canopy and particularly on
fallen fruit (Purcell et al., 1994).

*Diachasmimorpha longicaudata* has one of the broadest host ranges and inflicts
among the highest mortalities of any species used in Neotropical tephritid biocontrol
(Ovruski et al., 2000). The average adult female body length excluding the ovipositor is
~ 4.5 mm, and the antennae are longer than the body length (Sivinski and Aluja, 2003).
The length of the male is ~3.4 mm. Identification is aided by coloration as they have a
reddish-brown body and brown eyes and the antennae become black from the fourth
segment outward (Wharton and Gilstrap, 1983). The female’s gaster has a dorsal
central black band, while the male has dark brown to black dorsal posterior segments
(Wharton and Marsh, 1978). Definitive identification, within Florida, can be
accomplished by observing wing venation (Wharton and Gilstrap, 1983). The Cu2
submarginal cell of the forewing is short and five sided (Figures 1-1, 1-2) (Wharton and
Gilstrap, 1983).

*Doryctobracon areolatus*

*Doryctobracon areolatus* (Szépligeti, 1911) is the most widely distributed
Neotropical/subtropical native larval-prepupal parasitoid of *Anastrepha* (Ovruski et al.,
2000, López et al., 1999). Like *D. longicaudata*, this species is a synovigenic,
endoparasitic koinobiont, although it develops particularly well in 2nd instar larvae
(Wharton and Marsh, 1978). It forages for larvae in ripe fruit on the tree and seldom
over fallen fruit (Sivinski et al. 1997). It is distributed from Florida (where it was
introduced in 1969) deep into South America (Sivinski et al., 1998).

The adult body coloration is yellow to orange with clear wings, and the apical
abdominal tergites in males are often black (Wharton and Marsh, 1978). They have
very distinctive banding pattern on the hind tibia. The labrum is usually visible and the clypeus is relatively short compared to some of the other *Doryctobracon* species with an ovipositor length of ~3.77 mm (Sivinski and Aluja, 2003). The Cu2 submarginal cell of fore wing is 4-sided (Figures 1-3, 1-4) (Wharton and Marsh, 1978; Sivinski et al., 2001).

*Utetes anastrephae*

*Utetes anastrephae* (Viereck, 1913) is widespread and the only species of *Utetes* recorded repeatedly from several species of *Anastrepha* (Ovruski et al. 2000). This species is also a synovigenic solitary endoparasitic koinobiont. Like *D. areolatus*, it forages for late instar larvae in ripe fruit hanging on tree branches. It is also the only *Anastrepha* attacking *Utetes* species native to the continental US (Wharton, 1997). *Utetes anastrephae* may be a complex of closely related species, with perhaps subtle differences in ovipositor length, body sculpture, and host preferences (Wharton, 1997). It is a color-variable species, and different color morphs have been obtained from the same collections. The Cu2 submarginal cell of the forewing is five sided and relatively long compared to *D. longicaudata* (Figures 1-5, 1-6) (Wharton and Marsh, 1978).

*Anastrepha suspensa*

This species range occurs in the southern US, but is native to the Greater Antilles (Cuba, Jamaica, Hispaniola, and Puerto Rico) (Aluja, 1994). *Anastrepha suspensa* females place a single egg at a time in color-breaking to mature fruits. The eggs hatch in two to three days and develop over a period occupies of 10 to 14 days dependent upon temperature. Larvae undergo three instars and feed exclusively on fruit pulp (Aluja, 1994). The larvae are cylindrical and elongated with the anterior end usually recurved ventrally and a flattened caudal end. The last instar is ~8-10 mm in length; this instar leaves the fruit and pupates in the soil. In addition to parasitoids, this larvae
and pupae are attacked by an array of predators such as stapylinid beetles and ants (Aluja, 1994).

The taxonomy of most *Anastrepha* species is based on female morphology (Norr bom, 1985). Color patterns on the body and wings are useful characters in taxonomy of many *Anastrepha* species. Adults are yellowish-brown with rather long patterned wings (Stone, 1942). The female has a prominent ovipositor and can be distinguished by a black posterior spot on the thorax. Wing bands (Figure 1-7) are yellow brown to brown with costal and S bands touching or narrowly separated at the second longitudinal vein (Stone, 1942). In males the inverted V band is always distinctly connected at its apex with the S band (Stone, 1942).

**Fruit Fly Management and Control**

Currently, the emphasis in area-wide fruit fly management is on insecticide (increasingly Spinosad) + protein bait sprays followed by the release of sterile males (Sterile Insect Technique=SIT) (Enkerlin, 2005). The later necessitates rearing many millions of the target flies in large facilities where they are reproductively sterilized by irradiation and released into nature. The released males then mate with wild females, and the eggs fertilized by the released males fail to develop (Knipling, 1955). Use of these methods can lead to eradication and so greatly reduce the continued need for potentially environmentally hazardous pesticides (Kahn et al., 1990).

Certain fly populations can be monitored through traps baited with lures based either on male-pheromone precursors (methyl eugenol), food-cues (multillure) or the often difficult to categorize “parapheromones” (trimedlure) (Heath et al., 1995). Monitoring fruit flies is important to determine their presence or arrival to an area which allows growers, or more likely government agricultural agencies, to decide when and
where treatment is needed. Lures can be added to McPhail-type traps or sticky traps (USDA-APHIS, 2011).

As noted earlier, the control of pest flies through classical biological control and augmentative releases has recently been given increasing attention (Sivinski, 1996). Biological control would benefit from reliable methods to accurately monitor the survival and dispersal of fruit fly parasitoids following initial and augmentative releases but no such lures presently exist (Messing, 1992).

Specific Research Objectives

The overall goal of this research was to lay a foundation for the development of a device that would allow the monitoring of parasitoid populations in a field environment. The general approach followed was to isolate specific volatile compounds that act as kairomones and stimulate a behavioral response in the form of long range attraction and at the same time minimize the capture of non-target insects. This research investigated the host and fruit-associated chemicals which initiate host location, oviposition and feeding by *D. longicaudata*, *D. areolatus* and *U. anastrephae*. Also studied was the nutritional qualities of fruits that the parasitoids might use an adult food and if adult food quality was related to volatile attractiveness.

Chapter 2 focused on the following hypothesis: 1) that *D. areolatus* and *U. anastrephae* would find the historically sympatric guava (*Psidium guajava*) a superior food to an Old World fruit such as orange (*Citrus sinensis*); 2) that *D. longicaudata* would survive equally well on either fruit, or possibly longer on the more roughly sympatric orange; 3) that *D. longicaudata* should be better adapted to a diet of fruit juice because of its ground-based foraging habits and would live longer on a fruit diet than honey or a water control than would *D. areolatus* or *U. anastrephae*. The parasitoids
Doryctobracon crawfordi (Viereck) and Opius hirtus (Fisher) which are not found in North America north of Mexico, and thus unavailable to study, were examined in confirmatory experiments in Mexico. Results suggested the presence of a toxic component in guava and through assays there was a search for toxic sugars in the fruits. This in turn led to the exposure of D. longicaudata to diets containing a candidate compound.

In Chapter 3, bioassays were performed to compare the attraction of the three parasitoid species to the juice of two fruit species, both known tephritid hosts with volatiles known to attract other frugivorous insects, but with significantly different food values. An evaluation of the attraction of D. longicaudata, D. areolatus and U. anastrephae females to fruit juices and fruit juice volatiles introduced into in a flight tunnel and then compared their behavior to the various volatile components through neural responses measured by EAG.

In Chapter 4, the composition of volatiles emitted by four species of tephritid larvae were described and compared with non-tephritid Diptera. The larval volatiles were then compared to those derived from two artificial diets prior to and following their use as developmental substrates. The volatiles from Eugenia uniflora L. fruit occupied by A. suspensa larvae were analyzed to discover if larval volatile components escape the surface of infested fruit. Electroantennograms and electroovipositorgrams were used to measure the responses of sensillae on the antennae and ovipositor of D. longicaudata to para-ethylacetophenone, a compound apparently unique to tephritid larvae. A flight tunnel bioassay was then used to determine if para-ethylacetophenone attracted either male or female D. longicaudata. The capacity of para-ethylacetophenone to act as an
arrestant / oviposition stimulus in *D. longicaudata*, both by itself and in the presence of host fruit was tested. This research examined if *D. longicaudata* was stimulated by para-ethylacetophenone to oviposit into a device used to mass-rear opiine braconids destined for augmentative release.
Table 1-1. Some fruit fly host plant species and fruit fly hosts of *Diachasmimorpha longicaudata* (Aluja et al., 2000, Aluja et al., 2003).

<table>
<thead>
<tr>
<th>Fruit fly host</th>
<th>Fruit fly host fruit</th>
</tr>
</thead>
</table>
| *Anastrepha fraterculus* | *Psidium guajava* L.  
*Eugenia uniflora* L.  
*Syzgium jambos* L.  
*Prunus persica* L.  
*Terminalia catappa* L.  
*Syzgium jambos* L. |
| *Anastrepha obliqua* | *Mangifera indica* L.  
*Spondias mombin* L.  
*Spondias purpurea* L.  
*Spondias radkolferti* Donn. Sm.  
*Spondias sp* L.  
*Tapirira mexicana* Marchand |
| *Anastrepha serpentina* | *Calocarpum mammosum* (L.) Pierre  
*Manilkara zapota* (L.)  
*Bumelia sebolana* Lundell  
*Chrysophyllum cainito* L.  
*Pouteria sp* Aubl.  
*Mangifera indica* L. |
| *Anastrepha suspensa* | *Psidium guajava* L.  
*Eugenia uniflora* L.  
*Syzgium jambos* L.  
*Prunus persica* L.  
*Terminalia catappa* L. |
| *Anastrepha ludens* | *Citrus sinensis* (L.) Osbeck  
*Citrus aurantium* L.  
*Casimiroa edulis* La Llave & Lex.  
*Mangifera indica* L. |
| *Anastrepha striata* | *Psidium guajava* L.  
*Psidium guineense* Sw.  
*Psidium sartorianum* (O. Berg) Nied. |
| *Rhagoletis spp.* | *Crataegus mexicana* DC.  
*Crataegus rosei rosei* Eggel. |
Table 1-2. Some fruit fly host plant species and fruit fly hosts of *Doryctobracon areolatus* (Aluja et al., 2000, Aluja et al., 2003).

<table>
<thead>
<tr>
<th>Fruit fly host</th>
<th>Fruit fly host plant</th>
</tr>
</thead>
</table>
| *Anastrepha serpentina* | *Bumelia sebolana* Lundell  
 Calocarpum mammosum (L.) Pierre  
 Chrysophyllum cainito L.  
 *Pouteria* sp Aubl.  
 *Manilkara zapota* (L.) P. Royen  
 *Mangifera indica* L. |
| *Anastrepha obliqua* | *Mangifera indica* L.  
 *Spondias mombin* L.  
 *Spondias purpurea* L.  
 *Spondias radkolfieri* Donn. Sm.  
 *Spondias sp.* L.  
 *Tapirira mexicana* Marchand |
| *Anastrepha suspensa* | *Psidium guajava* L.  
 *Eugenia uniflora* L.  
 *Syzygium jambos* L.  
 *Prunus persica* L.  
 *Terminalia catappa* L. |
| *Anastrepha ludens* | *Citrus aurantium* L.  
 *Citrus paradisi* Macfad.  
 *Citrus sinensis* (L.) Osbeck  
 *Mangifera indica* L. |
| *Anastrepha fraterculus* | *Ampelocera holtte* Standl.  
 *Psidium guajava* L.  
 *Syzygium jambos* L. |
| *Rhagoletis spp.* | *Crataegus mexicana* DC.  
 *Crataegus rosei* rosei Eggl. |
| *Anastrepha bahiensis* | *Myrciaria floribunda* (H. West ex Willd.) O. Berg  
 *Brosimum alicastrum* Sw. |
| *Anastrepha aphelocentema* | *Pouteria hypoglauc* (Standl.) Baehni |
| *Anastrepha striata* | *Psidium guajava* L. |
| *Anastrepha cebra* | *Quararibea funebris* (La Llave) Visher |
| *Anastrepha spatulata* | *Schoepfia schreberi* J.F. Gmel. |
| *Anastrepha alveata* | *Ximenia americana* L. |
Table 1-3. Some fruit fly host plant species and fruit fly hosts of *Utetes anastraphae* (Aluja et al., 2000, Aluja et al., 2003).

<table>
<thead>
<tr>
<th>Fruit fly host</th>
<th>Fruit fly host plant</th>
</tr>
</thead>
</table>
| *Anastrepha suspensa* | *Psidium guajava* L.  
*Eugenia uniflora* L.  
*Syzygium jambos* L.  
*Prunus persica* L.  
*Terminalia catappa* L. |
| *Anastrepha obliqua* | *Ampelocera hottle* Standl.  
*Mangifera indica* L.  
*Spondias mombin* L.  
*Spondias sp.* L.  
*Tapirira mexicana* Marchand |
| *Anastrepha fraterculus* | *Psidium guajava* L.  
*Psidium guineense* Sw.  
*Psidium sartorianum* (O. Berg) Nied.  
*Syzygium jambos* L. |
| *Anastrepha serpentina* | *Crategus gracilior* J. B. Phipps  
*Bumelia sebolana* Lundell  
*Manilkara zapota* L. |
| *Rhagoletis spp.* | *Crataegus mexicana* DC.  
*Crataegus rosei parayana* (Eggl.) J. B. Phipps  
*Crategus gracilior* J. B. Phipps |
| *Anastrepha striata* | *Psidium guajava* L.  
*Psidium guineense* Sw.  
*Psidium sartorianum* (O. Berg) Nied. |
| *Anastrepha bahiensis* | *Malmea gaumeri* (Greenm.) Lundell |
| *Anastrepha ludens* | *Mangifera indica* L. |
| *Anastrepha cebra* | *Quararibea funebris* (La Llave) Vischer |
| *Anastrepha alveata* | *Ximenia americana* L. |
Figure 1-1. Forewing of *Diachasmimorpha longicaudata*. Cu1, Cu2, Cu3 = 1st, 2nd and 3rd cubital cells.

Figure 1-2. Forewing and hindwing of *Diachasmimorpha longicaudata*.
Figure 1-3. Forewing of *Doryctobracon areolatus*. Cu1, Cu2, Cu3 = 1st, 2nd and 3rd cubital cells.

Figure 1-4. Forewing and hindwing of *Doryctobracon areolatus*.
Figure 1-5. Forewing of *Utetes anastrephae*. Cu1, Cu2, Cu3 = 1\textsuperscript{st}, 2\textsuperscript{nd} and 3\textsuperscript{rd} cubital cells.

Figure 1-6. Forewing and hindwing of *Utetes anastrephae*.
Figure 1-7. Wing of *Anastrepha suspensa* male. Costal (C) and S band (S) touching the second longitudinal vein. In males the inverted V band (V) is always distinctly connected at its apex with the S band.
CHAPTER 2
LONGEVITY OF MULTIPLE SPECIES OF TEPHRITID (DIPTERA) FRUIT FLY
PARASITOID (HYMENOPTERA: BRACONIDAE: OPIINAE) PROVIDED EXOTIC
AND SYMPATRIC-FRUIT BASED DIETS

Adult hymenopteran parasitoids consume a variety of foods including flower
nectar (Jervis et al., 1996), extrafloral nectar (Koptur, 1989), host fluids (Jervis and
Kidd, 1999) and hemipteran honeydew (Wäckers, 1999, 2001). Fruit or fruit juices,
while an obvious food source for a “fruit fly” parasitoid, are only rarely reported as
feeding substrates. The tephritid-attacking opiine braconid Diachasmimorpha
longicaudata (Ashmead) consumes juices seeping from injured citrus and other fruits
(Sivinski et al. 2006) and a parasitoid of drosophilids, Asobara spp. (Braconidae) feeds
on fermenting fruit (Eijs et al., 1998). Since adult tephritid fruit fly parasitoids occur in
the same habitats as adult flies (Aluja and Burke, 1993), there may be widespread
opportunities to exploit the same nutritional resources, particularly juice expelling from
ovipositor-wounded or infested fruit. Since parasitoids incur costs and risks foraging for
adult food separate from hosts (Bernstein and Jervis, 2008), a competitive advantage
could accrue to individuals that concentrate their feeding and breeding within the same
resource patches (Sivinski et al., 2006). However, fruit juices, just as other plant-
produced substances, vary in nutritional quality and some contain compounds
particularly detrimental to Hymenoptera, including toxic sugars (Barker, 1977).

Opiine braconids are typically the most abundant/diverse frugivorous-tephritid
parasitoids (López et al., 1999; Sivinski et al., 2000), and can be mass-reared and
inundatively released for area-wide control (Sivinski et al., 1996; Montoya et al., 2000).
This research compared the longevity of three opiine species, all larval-prepupal
koinobionts that attack Anastrepha spp. fruit flies in Mexico and throughout much of the
neotropics, when provided with the pulp and juice of two different fruits, *Psidium guajava* L. (guava; Myrtaceae) and *Citrus sinensis* L. (orange; Rutaceae). The parasitoids *Doryctobracon areolatus* (Szepligeti) and *Utetes anastrephae* (Viereck) are Latin American natives and share an evolutionary history with guava (Wharton and Marsh, 1978; Morton, 1987) but not with the exotic and highly domesticated orange. *D. longicaudata* was originally collected in the Indo-Philippine region attacking *Bactrocera* spp. and has only been established in the native range of guava over the last half century (Ovruski et al., 2000). Unlike *D. areolatus* and *U. anastrephae*, whose host-searches are concentrated in fruit tree canopies where fruit is less likely to be damaged (Sivinski et al., 1997), *D. longicaudata* forages extensively in fallen fruit (Purcell et al., 1994) and so might have more immediate access to injured and oozing fruit.

Furans such as 2-Furoic acid have hypolipidemic properties and are found in some fruits such as guava, papaya and kiwi (European Food Safety Authority Journal, 2005). The furan 5-hydrodymethylfurfufal inhibits growth and development in the lepidopteran *Diatraea grandiosella* (Popham and Chippendale, 1996), and regulates fatty acids and cholesterol in mammals (Hall et al. 2006). Hydroxymethylfurfural (HMF), a heat-formed derivative of furan, derived from dehydration of sugars, is a common toxicant of *Apis mellifera* L (LeBlanc et al., 2009). It has been identified in a wide variety of heat-processed foods including fruit, fruit juices and honey (US Food and Drug Administration, 2009).

Given the differences in parasitoid foraging and the possibility that fruits might differ both nutritionally and in terms of unfavorable compounds, it was hypothesized that: 1) *D. areolatus* and *U. anastrephae* would find the historically sympatric guava a
superior food to orange; 2) *D. longicaudata* would survive equally well on either fruit, or possibly longer on the more roughly sympatric orange; 3) *D. longicaudata* would be better adapted to a diet of fruit juice because of its ground-based foraging habits and would live longer on a fruit diet relative to a honey and water control than would *D. areolatus* or *U. anastrephae*; and 4) in confirmatory experiments in Mexico, *D. areolatus* and *U. anastrephae*, as well as the native tephritid parasitoids *Doryctobracon crawfordi* (Viereck) and *Opius hirtus* (Fisher) would survive at least as well on a guava diet as on a honey+water control. Early results suggested the presence of a toxic component in guava, which subsequently initiated a search for toxic sugars in the fruits. This led to the exposure of *D. longicaudata* to diets containing a candidate compound.

Studies of foods that enhance adult parasitoid survival in the field could improve the diets of parasitoids mass-reared for augmentative releases and help direct natural enemy introduction and conservation (Sivinski et al., 2006). In addition, volatiles from these natural foods may be attractive and could be incorporated into traps that would help monitor the survival and dispersal of released parasitoids.

**Materials and Methods**

**Source of USA Parasitoids**

*D. longicaudata, D. areolatus* and *U. anastrephae*, were obtained from colonies the USDA-ARS, Center for Medical, Agricultural and Veterinary Entomology (USDA-ARS, CMAVE), Gainesville, Florida, USA. *D. longicaudata* had been in colony for approximately 10 years, and *D. areolatus* and *U. anastrephae* for approximately 4 years. All parasitoids were reared on larvae of the Caribbean fruit fly, *Anastrepha suspensa* (Lowe) that developed in an artificial diet (FDACS, 1995) obtained from the Florida Department of Agriculture and Consumer Services Division of Plant Industries,
Gainesville, Florida, USA. Insects were reared in a temperature controlled chamber at 23± 5°C, 60% RH, and photoperiod of 12:12 (L:D) h. Breeding colony adults were fed a diet of 10% honey/sugar solution.

**Survival on Alternative Fruit-Based Diets**

Parasitoids were provided with a food source and/or water within 24 hours of eclosion. Ripe guava was collected from a small orchard organically grown at USDA-ARS, CMAVE, Gainesville, Florida, USA. Fruits were cut into quarters and a single quarter (~40 g) was presented as a source of moist pulp. Guava juice was extracted from peeled ripe fruit by pressing the pulp through a mesh to remove the seeds. The pulp and an equal amount of water were blended together. Juice was collected by filtering the blend through cloth to separate the solids from the juice. Orange pulp was prepared from organic fruit purchased from a local market then peeled, sectioned (~60 g) and sliced crosswise to expose pulp. Juice was manually extracted.

Each repetition consisted of three 20 x 20 x 20 cm mesh and Plexiglas® cages containing 25 male and 25 female parasitoids. In one cage the insects were presented with either guava or orange juice or pulp and a 118 ml water cup with a protruding cotton wick. Juices were presented in saturated cotton balls inside a 60 ml cup. Another cage contained a 118 ml cup of water and ~1 ml of honey (pure unfiltered, uncooked) smeared on 5.5 cm dia. filter paper and placed in a 9 cm dia. Petri dish. The third cage contained only a 118 ml cup of water. Food sources were replaced daily to ensure freshness. Each treatment was replicated 5 times. Because insects in one treatment cage were not independent from one another, the survival was quantified as the total lifetimes of all insects in one treatment cage. Analyses of variance (ANOVA)
followed by a Dunnett’s test were used to determine differences among the total lifetimes for all treatments for both sexes of each species.

Survival on Guava Relative to Honey (Mexico)

To confirm the generality of Florida results, *D. longicaudata* and *U. anastrephae* from colonies at the Instituto de Ecología, A. C. in Xalapa, Veracruz, México were exposed to guava, as were additional non-native opiine fruit fly parasitoids (*Doryctobracon crawfordi* [Viereck] and *Opius hirtus* [Fischer]). All were reared on larvae of the Mexican fruit fly, *Anastrepha ludens* (Lowe) that had developed in an artificial diet (FDACS, 1995). A single newly emerged female parasitoid was placed in a 2 cm Petri dish that contained water in a 5ml vial with a cotton wick and one of three diet treatments: 1) honey *ad libitum*, 2) honey every second day and 3) organic guava pulp/juice (purchased from a local market). Guava was replaced daily to guarantee freshness. Different cohorts of each parasitoid (~15 females) species/food treatment were examined at 3, 6, 9, 12, and 15 days and the proportions that had survived that length of time were calculated. Survival rates were compared on a pair wise basis using repeated regressions in which time and diet served as independent variables (Zar, 1974). Proportional data were log-arsine transformed prior to analysis.

Survival on Mixed Guava/Orange Juice Compared To Diluted Orange Juice

Preliminary results found significantly shorter lifespans in parasitoids fed guava. To determine if guava was innutritious or harmful, *D. longicaudata* were provided with various orange and/or guava diets within 24 hours of emergence. The treatments consisted of guava juice; orange juice; 1:1 mixture of guava/orange juice; 1:1 orange juice/water dilution and a water control. This allowed us to determine if any decrease in longevity occurred due to substance present in guava juice (guava juice + orange juice
= guava juice alone) or innutritious (guava juice + orange juice > guava juice alone; orange juice dilution = guava juice + orange juice). For each treatment, 10 male and 10 female \( D. \ longicaudata \) were individually placed in 470 ml clear polypropylene containers. A 7.5 cm hole was placed in the center of the lid and replaced with organdy cloth. A 12 mm dia. hole was placed in the side of the container to introduce the insect and blocked with a foam plug. Two 10 mm dia. holes were placed in the bottom of the container to allow cotton wicks to protrude into the cup (one for treatment, one for water). Treatment solutions were held below in a 30 ml cup. The bioassay ended when all of the insects fed the guava juice + orange juice had died. Separation of means was accomplished by ANOVA followed by Waller’s test (SAS Inst., 2002). To ensure the treatments were ingested by the insects, in a separate test, juices were dyed using 2 µl/ml of green food dye (McCormick & Co., Inc., Hunt valley, MD). This enabled us to visually confirm the treatment within the gut of the insect.

**Identification of Sugar Constituents and Survival on a Potentially Toxic Sugar**

The toxicity of guava fruit and juice prompted us to investigate potential toxins. Certain sugars, arabinose, mannose, raffinose, lactose and rhamnose, can be nutritious to mammals but poisonous to honey bees (\( A. \ mellifera \)) and \( Cotesia \ glomerata \) (Barker, 1977; Wäckers, 2001). A commercial carbohydrate analysis of guava fruit (EMSL Analytical, Inc., Westmont, NJ), identified sugars at concentrations greater than 0.022 mg/g. Only fructose 37 mg/g, glucose 20 mg/g and sucrose 38 mg/g were listed in the analysis. Orange juice contains: fructose 18.4 g/l, glucose 15.3 g/l and sucrose 37.8 g/l (Muntean, 2010), although higher fructose levels, 20-38 mg/ml (Villamiel et al., 1998 and citations) have been reported. Thus, fructose, a known opiine nutrient and the
positive control in the experiments described below, occurs in roughly similar levels in orange.

Since toxic sugars might have been present at undetectable levels, a more sensitive analysis using gas chromatography-mass spectrometry (GC-MS) of guava and orange pulp was performed. Mannose has been previously detected in guava but not in the peel and membranes from orange (Grohmann et al., 1995).

GC-MS is only useful for monosaccharides, so large oligo- or polysaccharides must first be hydrolyzed to their component monosaccharides through an acylation technique (Price, 2004). Fruit samples were dried using a food dehydrator (Mr. Coffee FD53196) then pulverized to a fine powder. A 0.50 mg sample was placed in a 3 ml reaction vial with 100 μl of aqueous 2M trifluoroacetic acid (TFA) and heated in a water bath at 110 °C for 30 minutes. The sugars in solution were then evaporated to dryness. 0.5 ml of hydroxylamine hydrochloride in pyridine (33 mg/ml) was added and reacted in a water bath at 60 °C for 30 minutes. The sample was cooled, and without evaporating, 200 μl of acetic anhydride was added and heated in a water bath at 60 °C for 30 minutes thus completing the peracetylation. The reaction was quenched after 30 minutes by the addition of 3 ml of mili-Q water. The sugars were extracted by partitioning with 2 ml ethyl acetate. The upper organic layer was used directly for GC/MS analysis. Mass spectra were recorded over the range m/z 50-550 in positive ion mode. It was important to have the low range cutoff set at 50 otherwise the spectra are dominated by an acetate ion at m/z 43.

**Mannose Bioassay**

Assays described above found the potentially toxic sugar mannose in guava, but not in oranges. Consequently, *D. longicaudata* was exposed to mannose (Sigma-
Aldrich) to examine its effect on parasitoid survival. The treatments consisted of a 5% mannose + 10% fructose solution, a 5% mannose solution, a 10% fructose solution and a water control. This allowed us to determine if any decrease in longevity in the presence of mannose was due to toxicity (mannose + fructose = mannose alone) or innutrition (mannose + fructose > mannose alone and mannose alone = water control). Sugar concentrations of 1 molar (1M) have been shown to increase parasitoid longevity. A 10% fructose solution can maintain *D. longicaudata* for over 30 days (Stuhl et al., 2011a). For each treatment, five male and five ~2 day old female *D. longicaudata* were individually placed in 470 ml clear polypropylene containers with a lid replaced with organdy cloth. A 10 mm diameter hole was placed in the bottom of the container to allow a saturated cotton wick containing the treatments to protrude into the cup. Ten individuals (five males and five females), for a total of five replications were performed. The bioassay ended when all of the insects died. Separation of means was accomplished by ANOVA followed by Waller-Duncan means separation test (SAS Inst., 2002).

**Survival on solutions of furoic acid.**

To discover if tested fruits contained potentially toxic furans, guava and another myrtaceous fruit, Surinam cherry (*Eugenia uniflora* L.) were collected from the CMAVE orchard. Organic oranges were purchased commercially. Whole ripe fruit were macerated in a blender. A 1 ml sample of the puree was placed in a 5 ml vial to which was added 3 ml of methanol, and the sample agitated for 2 minutes. Supernatant was removed and added to an additional 3 ml of methanol. From this a 50 µl sample was removed and analyzed. Analysis was performed by positive ion electron impact gas chromatography-mass spectrometry (EI GS-MS) on an HP 6890 gas chromatograph.
coupled to an HP 5973 MS detector. One µl of the sample was injected (240°C) onto an Agilent HP-5MS dimethylpolysiloxane column (30 m×250 µm (i.d.) × 0.25 µm, Agilent Technologies, Palo Alto, CA, USA) and separated by temperatures programmed from 35°C (1.0 min hold) to 230°C at 10°C/min. Helium was used as a carrier gas at 1.2 ml/min. Compounds were identified by comparison of mass spectra (a) with mass spectra libraries (NIST and Department of Chemical Ecology, Göteborg University, Sweden) and (b) with mass spectra and retention times of authentic standards. Guava contained furan compounds, including furoic acid, as did Surinam cherry. However, they were not present in orange. Berenbaum (1978) stated that the normal concentrations of furan compounds in plants range from 0.1-1.0 percent. Spodoptera eridania (Cramer) exposed to these concentrations proved to be fatal.

We subsequently exposed D. longicaudata to dilutions of 1%, 0.5% and 0.1% 2-furoic acid (Sigma-Aldrich) in a 10% fructose solution and examined their effect on parasitoid longevity. Although the exact percentage of furoic acid present in the fruit was not known, the dilutions were chosen as per Berenbaum (1978). For each dilution, five male and five female D. longicaudata ~2 days old were individually placed in a 470 ml clear polypropylene container (RD16, Placon Products, Madison, WI) as described earlier. The bioassay ended when all of the insects died. Statistical analysis was through Analysis of Variance with subsequent mean comparisons by Waller’s test (SAS Inst., 2002).

**Results**

**Survival on Alternative Fruit-Based Diets**

Mean longevities of both sexes of all three species provided with the pulp and juice of guava were indistinguishable from those obtained on water alone (Figures 2-1
thru 2-6), this in spite of its repeatedly observed ingestion (Figure 2-7). When provided with orange juice, but not orange pulp, male *D. areolatus* and *U. anastrephae* life spans were similar to those given guava juice. In both sexes of *D. areolatus* and *U. anastrephae* survival rate on orange pulp was higher than on juice, but for male and female *D. longicaudata* there was no difference in survival between pulp and juice. In general, orange diets were superior to either water alone or guava diets and typically insects with orange diets lived as long as those with a honey diet.

**Survival on Guava Compared to Honey (Mexico)**

Survival rates were lower on diets of guava than those fed either honey or honey every other day for all four species of parasitoids (Figure 2-8). For *D. longicaudata* and *U. anastrephae* insects fed honey every day survived at a higher rate than those fed honey every other day.

**Survival on Guava /Orange Juice Mixtures Relative to Diluted Orange Juice**

Both male and female *D. longicaudata* fed with guava juice died at a greater rate than those fed with orange juice. Survival on a 1:1 orange juice + guava juice mixture was no greater than that obtained on guava juice alone (Figures 2-9, 2-10) and was less than a 1:1 mixture of orange juice and water.

**Identification of and Survival on Potentially Toxic Sugars**

GC-MS analysis of guava utilizing an acylation technique showed the presence of mannose in our samples. Mannose was not detected in orange. The amount was not quantified, only its presence verified.
Survival on Solutions of Mannose

Mannose was not toxic at a level that exceeds its reported concentration in guava either in combination with fructose or alone (Figure 2-11). In fact mannose alone was slightly nutritious as it extended life significantly beyond a water-alone diet.

Survival on Solutions of Furoic Acid.

Furoic acid was toxic to both male and female *D. longicaudata*. Survival on 10% fructose solutions containing 1% and 0.5% furoic acid was no greater than that obtained on water alone (Figure 2-12). Only when furoic acid concentrations fell to 0.1% was there a significant increase in longevity relative to water alone.

Discussion

Carbohydrates and other nutrients are critical for the survival and continued fecundity of synovigenic parasitoids, and *D. longicaudata* shows an ability to acquire these from a variety of sources. One source, juices that seep from certain injured fruits, provides a high quality diet and minimizes the need to separately forage for oviposition sites and adult food (Sivinski et al., 2006). However, fruits, as well as flower nectars and other plant exudates vary in nutritional quality and some may contain detrimental compounds, including toxic sugars (Barker, 1977; Wäckers, 2001). Because of these potential problems with fruit-foods, the hypothesis was that the native Mexican opines, *D. areolatus* and *U. anastrephae* being sympatric with guava would be adapted to its use and find it particularly suitable. On the other hand, it was postulated that the exotic *D. longicaudata* would not fare as well on guava but be the best able to exploit a fruit of Old World derivation, *C. sinensis*, or at least do as well on this highly modified fruit as the New World parasitoids. In addition, it was hypothesize that another two Mexican species, *D. crawfordi* and *O. hirtus* would find guava nutritious and life-sustaining
relative to a honey solution. Because of a host-foraging strategy that brings *D. longicaudata* into closer contact with fallen, overripe and damaged fruit than either *D. areolatus* or *U. anastrephae* it was predicted that it might be better adapted to feed on fruit juices. If so, the expected *D. longicaudata* life spans on fruit-based diets to be the highest of the three species relative a honey solution. These suppositions were almost all incorrect.

Both sexes of *D. longicaudata*, from USA and Mexican-derived cultures died at similar rates when provided guava pulp or juice or a water-only control. In the USA *D. areolatus* and *U. anastrephae*, presumably adapted to the nutrient/chemical constituents of guava, also died at a rate similar to that of the water-control. Survival of all three species on orange pulp was greater than on water and longevity often equaled that obtained on a honey positive control. In Mexico, *D. areolatus* and *U. anastrephae*, as well as the tephritid parasitoids *D. crawfordi* and *O. hirtus*, died at significantly higher rates when provided guava in comparison to two feeding schedules of a honey diet. This general *P. guajava*-induced mortality could be due to guavas being 1) unlocatable/repellent, 2) innutritious or 3) toxic (*sensu lato*). *Diachasmimorpha longicaudata* clearly consumed guava juice tagged with a colored dye. Dilutions of orange and guava juice resulted in shorter lifespans than dilutions of orange juice and water demonstrating that while diluted orange juice provided nutrition the addition of guava created toxicity. Mannose, a sugar known to be toxic to some Hymenoptera (De la Fuente et al., 1986), occurred in guava but was not detected in citrus. It was slightly nutritious to female *D. longicaudata*, leaving the toxic compound(s) unidentified.
However, furoic acid, a constituent of guava but not fresh citrus, was toxic to *D. longicaudata* and might be the cause of mortality.

Given the differences in fruit-food quality, adult opiine food would not be obtainable at all oviposition sites and more foraging for food than previously postulated may be required (Sivinski et al., 2006). This would be a particular handicap for *D. areolatus* which is more likely than *U. anastrephae* to be recovered from hosts infesting guava (López et al., 1999; Sivinski et al., 2000). *Psidium* specialists, or near specialists, occur among Mexican endemic tephritid parasitoids outside of the Opiinae. The figitid *Odontosema albinerve* Kieffer (=*Odontosema anastrephae*, sensu Kieffer) is largely associated with guava and its congener, as is to a lesser extent another figitid, *Aganaspis pelleranoi*. It remains to be seen if the compounds toxic to the braconid members of the parasitoid guild also effect specialist adult Figitidae.

It is unknown why multiple species of braconids, some sympatric with a host fruit and the flies that inhabit it over evolutionary time, have not evolved a tolerance to an abundant and convienent food source. Since the fruits tested were uninfested the harmful compound is not a host-product designed to discourage natural enemies. Neither was it obvious why guavas would produce a compound harmful to the parasitoids of larvae that might damage seeds or comprimise the attractiveness of fruit to seed dispersers. Perhaps opiine mortality is simply a by-product of selection in some other context such as discouraging an unidentified seed-predator.

Several species of opiine braconid parasitoids of pest tephritids are mass-reared for augmentative release and are typically fed with honey or honey solutions (Sivinski et al., 1996; Wong and Ramadan, 1993). But little is known about their dietary
requirements, and even less about potential food sources in the field. Since the sustained efficacy of a release might be impacted by the availability of adult food, it is important to determine the nutritional landscape parasitoids might encounter. It is now clear that guava is not an exploitable resource. Ultimately, examination of natural foods might suggest diet improvements that lead to longer lived, more fecund and healthier mass-reared parasitoids for more efficacious biological control.
Figure 2-1. Survival of *Diachasmimorpha longicaudata* males on various diets. The Y axis measures the sum of days lived by all 25 individuals (i.e. 100/25 = 4 days); the X axis corresponds to the treatments. The box represents the middle 50% of the data. The upper boundary of the box locates the 75th percentile of the data set, while the lower boundary indicates the 25th percentile. The mean is represented by a plus sign (+) in the center of each box. The line in the box represents the median value. The whiskers represent the minimum and maximum values of the data set. An (*) denotes a significant difference.
Figure 2-2. Survival of *Diachasmimorpha longicaudata* females on various diets. The Y axis measures the sum of days lived by all 25 individuals (i.e. 100/25 = 4 days); the X axis corresponds to the treatments. The box represents the middle 50% of the data. The upper boundary of the box locates the 75th percentile of the data set, while the lower boundary indicates the 25th percentile. The mean is represented by a plus sign (+) in the center of each box. The line in the box represents the median value. The whiskers represent the minimum and maximum values of the data set. An (*) denotes a significant difference.
Figure 2-3. Survival of *Doryctobracon areolatus* males on various diets. The Y axis measures the sum of days lived by all 25 individuals (i.e. 100/25 = 4 days); the X axis corresponds to the treatments. The box represents the middle 50% of the data. The upper boundary of the box locates the 75th percentile of the data set, while the lower boundary indicates the 25th percentile. The mean is represented by a plus sign (+) in the center of each box. The line in the box represents the median value. The whiskers represent the minimum and maximum values of the data set. An (*) denotes a significant difference.
Figure 2-4. Survival of *Doryctobracon areolatus* females on various diets. The Y axis measures the sum of days lived by all 25 individuals (i.e. $100/25 = 4$ days); the X axis corresponds to the treatments. The box represents the middle 50% of the data. The upper boundary of the box locates the 75th percentile of the data set, while the lower boundary indicates the 25th percentile. The mean is represented by a plus sign (+) in the center of each box. The line in the box represents the median value. The whiskers represent the minimum and maximum values of the data set. An (*) denotes a significant difference.
Figure 2-5. Survival of *Utetes anastrephae* males on various diets. The Y axis measures the sum of days lived by all 25 individuals (i.e. 100/25 = 4 days); the X axis corresponds to the treatments. The box represents the middle 50% of the data. The upper boundary of the box locates the 75th percentile of the data set, while the lower boundary indicates the 25th percentile. The mean is represented by a plus sign (+) in the center of each box. The line in the box represents the median value. The whiskers represent the minimum and maximum values of the data set. An (*) denotes a significant difference.
Figure 2-6. Survival of *Utetes anastrephae* females on various diets. The Y axis measures the sum of days lived by all 25 individuals (i.e. 100/25 = 4 days); the X axis corresponds to the treatments. The box represents the middle 50% of the data. The upper boundary of the box locates the 75th percentile of the data set, while the lower boundary indicates the 25th percentile. The mean is represented by a plus sign (+) in the center of each box. The line in the box represents the median value. The whiskers represent the minimum and maximum values of the data set. An (*) denotes a significant difference.
Figure 2-7. Visual confirmation that a female *Diachasmimorpha longicaudata* has fed on guava juice mixed with 2 µl/ml green food dye.
Figure 2-8. *Doryctobracon areolatus*, *Diachasmimorpha longicaudata*, *Opius hirtus* and *Utetes anastrephae* cohorts maintained on guava and honey. The graphs show the proportions of parasitoids survival on guava (*Psidium guajava*), honey every day or honey every other day that was alive after various lengths of time. Regressions sharing letters are not significantly different.
Figure 2-9. Proportion of *Diachasmimorpha longicaudata* males that survived on solutions of fruit juice. Treatments were a solution of orange juice, 1:1 dilution of orange juice and water, guava juice, 1:1 mixture of orange juice and guava juice and a water control over time.
Figure 2-10. Proportion of *Diachasmimorpha longicaudata* females that survived on solutions of fruit juice. Treatments were a solution of orange juice, 1:1 dilution of orange juice and water, guava juice, 1:1 mixture of orange juice and guava juice and a water control over time.
Figure 2-11. Mean days of survival for male and female *Diachasmimorpha longicaudata* survival on mannose. Treatments were 5% solutions of fructose, mannose and fructose, mannose and water.
Figure 2-12. The mean days of survival of male and female *Diachasmimorpha longicaudata* on furoic acid solutions. Parasitoids were provided with fructose solutions containing different proportions of furoic acid or only water. Means sharing letters are not significantly different.
CHAPTER 3
RESPONSE OF MULTIPLE SPECIES OF TEPHRITID (DIPTERA) FRUIT FLY PARASITOIDS (HYMENOPTERA: BRACONIDAE: OPIINAE) TO GUAVA AND ORANGE JUICE VOLATILES

Chemical cues from the parasitoids’ hosts and the substrates in and on which the hosts develop are known, or are hypothesized to be, fundamental components of orientation during foraging for adult food, oviposition opportunities and perhaps mating-convention-sites (Stuhl et al., 2011b, Vet and Dicke, 1992, Godfray, 1994; Sivinski and Petersson, 1997). However, within any particular guild of parasitoids not all chemicals may be of interest to all the species or even the sexes of the same species. For example, males participating in mating systems that do not center on oviposition site “guarding” might not sense or respond to volatiles emitted by larval-development substrates (Sivinski and Petersson, 1997). Females confronted with exotic hosts might not orient towards their chemical bouquets, even if they are otherwise suitable for their offspring. Even within a native guild exploiting the same host a specialist female might search for a cue that uniquely identifies the oviposition site (Vet and van Alphen, 1985), while a generalist species might react to chemicals that all its various hosts share (Godfray, 1994). Thus the gender of an insect, its evolutionary history with a potential developmental substrate and its degree of specialization could all influence its behavior in the presence of a particular host-associated bouquet.

In the parasitoid guild attacking Anastrepha spp. in the New World, species of opiine braconids are the most abundant and diverse elements (López et al., 1999; Sivinski et al., 2000). In addition to pest population suppression performed by native species, various opiines have been introduced to attack invasive flies and these are sometimes mass-reared and inundatively released for area-wide control (Sivinski et al.,
Of these opiines, two native and one introduced species are the most common. While all are relative generalist koinobionts, attacking the late instar larvae of several *Anastrepha* spp. in a wide variety of fruits (Sivinski et al., 1996, Aluja et al., 2008), they do differ in regions of origin and there are potential sexual and species differences in their responses to fruits based on their relative value as either an adult food or as host-larval-substrates (Stuhl et al., 2011a). A species by species set of predictions of their responses to two fruit species follows. These fruits were *Psidium guajava* L. (guava; Myrtaceae) and *Citrus sinensis* L. (orange; Rutaceae). The first originated in Meso America (Popenoe, 1974) while the later comes from Asia (Scora, 1975). Oranges and other citrus are infested by *Anastrepha ludens* and their juice/pulp is a high quality food for all the adults of all three parasitoid species (Wharton and Marsh, 1978; Morton, 1987). While guava is a major host of *Anastrepha fraterculus, striata, suspensa* and *obliqua* it is toxic when consumed by all the adult parasitoids of both sexes (Stuhl et al., 2011a).

*Diachasmimorpha longicaudata* (Ashmead): This species was originally collected in the Indo-Philippine region attacking *Bactrocera* spp. and has only been established in the New World over the last half century (Ovruski et al., 2000). While it is a major parasitoid of *Anastrepha* spp. in both fruits, much of its foraging is over fallen fruit (Garcia-Medel et al., 2007) and it is known to be attracted to by-products of fungal decomposition such as ethanol and ethyl acetate (Greany et al, 1977a) as well as infested and uninfested fruit odors (Eben et al., 2000). Those matings that have been observed in nature occurred on and under host trees (J. Sivinski, unpublished data). Males appear to emit a pheromone from the upper surface of a leaf which they
seemingly defended from rival males. They periodically leave the foliage to investigate the ground from which virgin females might emerge. Given this natural history, it was predicted that 1) females in search of oviposition sites will respond to the volatiles of both fruits, but not to compounds unique to guava since there is no history of sympatry; 2) males and females will be attracted by the volatiles of the high quality food source, orange, with which they also share a history of sympatry; 3) males will not recognize volatiles unique to guava since they lack a history of sympatry.

*Doryctobracon areolatus* (Szepligeti): This is a wide spread Neotropical native koinobiont parasitoid of late-instar *Anastrepha* spp. larvae and rarely *Rhagoletis* spp. (Wharton et al., 1981, Aluja et al., 1990). Thus its host range, while broad, is not as broad as that of *D. longicaudata*. *Doryctobracon areolatus*, unlike *D. longicaudata*, does not forage over fallen fruit but only attacks larvae in fruit still on the tree (Garcia-Medel et al., 2007). The few observations of mating in the field suggest that its mating system resembles that of *D. longicaudata* with aggregated males defending leaf-territories from which they emit a pheromone and periodically sally to the ground to search for emerging females (J. Sivinski, unpublished data). This natural history would suggest the following responses to the fruit volatiles: 1) Females would respond to chemicals emitted by mature fruit but be less likely to use decomposition odors to locate oviposition sites. 2) Males would use fruit decomposition products to locate sites of female emergence. 3) Neither sex would respond to volatile compounds unique to oranges since they have no history of sympatry. 4) Males would not respond to compounds unique to fresh guava since it is neither an adult food nor an environmental cue to the emergence of virgin females.
*Utetes anastrephae* (Viereck): This is also a wide-spread neotropical-native koinobiont parasitoid of late-instar larvae, and one that often occurs in close sympatry with *D. areolatus*. The two species apparently coexist through asymmetries in larval-larval intrinsic competition and differences in ovipositor length (Sivinski and Aluja, 2003). *Utetes anastrephae* also attacks larvae in hanging fruit, but because of a relatively short ovipositor is restricted to parasitizing hosts in smaller fruit (Sivinski et al., 2001). For all practical purposes, it thus has the narrowest host range of the three species. Nothing is known on its mating system, although glandular development suggests a male-produced pheromone (Wharton, 1997). Predicted responses to guava and orange volatiles would be similar to those of *D. areolatus*.

The mass-rearing and augmentative release of tephritid fruit fly parasitoids has great promise, particularly in conjunction with sterile males and in areas where insecticides cannot be widely applied. However there are challenges associated with its adoption. There are few practical means at present to monitor the survival and dispersal of fruit fly parasitoids following release (Messing, 1992). Comparisons of fruit-volatile attractiveness and of the volatile components present in the fruit juices such as described above would be a first step in the preliminary identification of effective synthetic attractants.

**Materials and Methods**

**Source of Parasitoids**

Parasitoids were reared on larvae of the Caribbean fruit fly, *Anastrepha suspensa* (Loew) that developed in an artificial diet (FDACS, 1995) obtained from the Florida Department of Agriculture and Consumer Services Division of Plant Industries, Gainesville, Florida, USA. *D. longicaudata*, *D. areolatus* and *U. anastrephae*, were
obtained from colonies the USDA-ARS, Center for Medical, Agricultural and Veterinary Entomology (USDA-ARS, CMAVE), Gainesville, Florida, USA. *D. longicaudata* had been in colony for ~10 years, *D. areolatus* ~2 years and *U. anastrephae* for ~1 year. Insects were reared in a temperature controlled chamber at 23± 5°C, 60% RH, and photoperiod of 12:12 (L:D) h. Breeding colony adults were fed a diet of 10% sucrose solution.

**Occupancy of Feeding Stations: Choice and No-Choice Tests**

To determine the response of *D. areolatus*, *D. longicaudata* and *U. anastrephae* to various food sources, parasitoids were observed in a flight tunnel. Ten 4-6 day old male and ten female parasitoids were starved for 24 h prior to the experiment, then introduced to a flight tunnel constructed of clear acrylic sheets 128 cm long by 31.8 cm by 31.8 cm and located inside a laboratory at CMAVE. Illumination was provided two 120 cm fluorescent bulbs suspended above the flight tunnel. The light source and the light emitted by the room lighting produced an illumination within the tunnel of ~1600 lux. The room temperature ranged from 28.7-28.8 °C and humidity between 37.6 – 38.1% RH. Outside air was pulled into the flight tunnel by using a Shaded Pole Blower (Dayton Electric Mfg. C., Niles, IL). This produced air flow within the tunnel through a charcoal filter and exhausted it into a hood. The exhaust was covered with screen to prevent insects from entering the tube. A baffle inside a tube that connected the downwind end of the tunnel with the exhaust system of the hood allowed for air flow adjustment. Air speed was maintained at 0.2 m/s. Previous studies performed by Messing et al. (1997) determined this to be the speed that most stimulated flight in *D. longicaudata*. All tests were performed between 0900 and 1400 hours. Guava and orange juice were prepared as previously described. Three treatments consisting of
guava juice, orange juice or water were utilized in this study. Saturated cotton balls containing 10 ml of liquid were suspended inside 60 ml soufflé cups (SoloHP47, Solo Cup Co., Lake Forest, IL) ~10 cm from the top of the flight tunnel using wire. There was a total of six suspension sites on the ceiling of the flight tunnel spaced 15 cm apart along the short axis and 30 cm apart along the long axis. The placement of the treatments was rotated over consecutive replicates and repetitions were rotated between the tunnels to account for positional effect. A total of six replicates were performed for each parasitoid species. Parasitoids were introduced into the flight tunnel through an opening in the top at the extreme downwind portion. Observations were made every half hour beginning at 0900 hours and ending at 1400 hours. When an observation was made and an insect was noted on a treatment, it was removed and replaced with another of the same sex. Following this experiment, in which parasitoids had a choice of fruit juices, a similar set of experiments was conducted to compare responses to guava juice or water. Comparisons of guava juice, orange juice and water were made through Analysis of Variance with subsequent mean comparisons by Waller’s test (SAS Inst., 2002). A T-Test procedure was performed for the analysis of guava juice and water (SAS Inst., 2002).

**Volatile Collection**

Volatile Collection were collected using a head space collection technique (Heath and Manukian, 1992). Ripe guava was collected from a small orchard organically grown at USDA-ARS, CMAVE, Gainesville, Florida, USA. Guava juice was extracted from peeled ripe fruit by pressing the pulp through a mesh to remove the seeds. The pulp and an equal amount of water were blended together. Juice was collected by filtering the blend through cloth to separate the solids from the juice. Orange juice was
prepared from organic fruit purchased from a local market. Juice was manually extracted. A 10 ml juice sample was placed in a 50 ml Pyrex beaker. Juices were then placed in a cylindrical stainless steel chamber (18.5 cm tall and 15 cm OD) with a stainless steel lid and a single union bulkhead inlet and a single outlet. Dry charcoal filtered air was pushed into the bottom end of the chamber and over the fruit and exited the chamber near the top via a vacuum system. The air passed through a volatile collection filter containing 50 mg of HayeSepQ (Hayes Separation Inc., Bandera, TX). Filters were eluted with 175µl methylene dichloride to remove volatile components.

**Identification of Fruit Juice Volatiles**

Volatile analysis was performed by electron impact gas chromatography-mass spectrometry (EI GS-MS) on an HP 6890 gas chromatograph coupled to an HP 5973 MS detector. One µl of the sample was injected (240°C) onto an Agilent HP-5MS dimethylpolysiloxane column (30 m×250 µm (i.d.)× 0.25 µm, Agilent Technologies, Palo Alto, CA, USA) and separated by temperatures programmed from 35°C (1.0 min hold) to 230°C at 10°C/min. Helium was used as a carrier gas at 1.2 ml/min. Volatiles were identified by comparison of mass spectra (a) with mass spectra libraries (NIST and Department of Chemical Ecology, Göteborg University, Sweden) and (b) with mass spectra and retention times of authentic standards.

**Electroantennogram (EAG) Measured the Parasitoids to Major Fruit Juice Volatiles**

GC-EAD is a useful tool in identifying active compounds (Cha et al., 2011) and a way to avoid evaluating compounds from fruit and fruit juices that do not initiate biological activity. To determine if the parasitoids had a sensory response to specific compound isolated from fruit juice volatiles, 25, 50,100 and 200 ng/µl dilutions of
synthetic compounds in dichloromethane (Sigma-Aldrich) were prepared. The natural compounds were collected from orange and guava juice prepared as previously stated. Male and female parasitoid antennal responses were measured using an electroantennographic detector (EAD). Extracts were analyzed with a GC interfaced to both flame ionization (FID) and EAD. In this manner, antennal responses were matched with FID signals for compounds eluting from the GC. Volatile extracts were prepared in the manner described above, and 1μl aliquots were analyzed on a Hewlett-Packard (HP) 5890 Series II gas chromatograph equipped with an HP-5 column (30 m×0.32 mm ID× 0.25 mm) (Agilent, Palo Alto, CA, USA). The oven temperature was held at 40 °C for 5 min, then programmed to increase to 10°C /min to 220°C and held at this temperature for 5 min. Helium was used as a carrier gas at a flow rate of 2.0 ml/min. A charcoal filtered humidified air stream was delivered over the antenna is at 1 ml/min.

The parasitoids antennae were excised by grasping the scape at its base with a jeweler’s forceps (No. 5, Miltex Instrument Company Inc, Switzerland). The antennae were held between electrodes (Syntech, Germany) at the extreme distal and proximal ends of in conductivity gel (Parker labs, Fairfield, NJ). The EAD and FID signals were concurrently recorded with a GC-EAD program (Syntech EAGPro, Germany), which analyzed the amplified signals on a PC.

**Attraction to Fruit Juice Volatiles: Choice and No-Choice Tests**

Since initial comparisons of fruit juice volatiles did not distinguish between attraction and arresting, a flight tunnel bioassay was developed to determine the longer-distance (~1m) parasitoid responses. Ten 4-6 day old male and ten female parasitoids were placed in a flight tunnel constructed as previously described. Treatments
consisted of fruit juice of *Psidium guajava* L. (guava; Myrtaceae) and *Citrus sinensis* L. (orange; Rutaceae) and a water control. Two cylindrical stainless steel chambers (18.5 cm tall and 15 cm OD) with a stainless steel lid, a single union bulkhead inlet and a single outlet were used to contain our samples. To measure the response to a single fruit juice, one chamber contained the juice while the other held a blank control. For the comparison of fruit juices, each chamber contained a fruit juice. The treatments were placed in a saturated cotton ball containing 10 ml of liquid inside a 60 ml soufflé cup (SoloHP47). Clean air was passed over the two odor sources and emerged separately in the flight tunnel. Air flow into the fruit containers was controlled by an adjustable flow meter (Aalborg Instruments, Monsey, NY) set at ~0.5 liters/min. Treated air emerged into two insect traps located at the upwind end of the tunnel and placed midway between its ceiling and floor. These were constructed from two clear polypropylene round-bottom *Drosophila* stock bottles (82005-718, VWR International, LLC, Radnor, PA). A 10 mm hole was placed in the center of the cap to allow insects to enter the trap. The flight tunnel and its traps were checked every 30 minutes from 0900 to 1400 hours. A positive response was recorded when there was a parasitoid inside the trap. The insect was removed from the trap and replaced with a naive insect (i.e., never exposed to fruit odors) from a stock cage where the original insects had been obtained. The position of the treatment and control were changed after each replication to prevent positional effects. There were five replicates each of type of fruit.

**Results**

**Occupancy of Feeding Stations Containing Fruit Juices or Water**

When provided with feeding stations containing guava juice, orange juice or water, both sexes of all species were significantly more likely to be located at the orange
stations than at those containing guava (Figure 3-1). When given the more limited choice of water or guava juice, females of *D. longicaudata* and *D. areolatus* were observed more often at guava stations. However, female *U. anastrephae* and males of all three species had no preference for guava juice over water (Figure 3-2).

**Identification of Fruit Juice Volatiles**

Representative chromatograms collected from orange juice (Figure 3-3) and guava juice (Figure 3-4) is shown. The identified compounds are listed in Table 3-1. The three most abundant compounds were common to both fruit juices. These were: hexanal, ethyl butanoate, 3-hexen-1-ol and limonene. The GC-MS chromatogram of 200 ng/μl synthetic standards is shown (Figure 3-5).

**Electroantennogram (EAG) Measurements to Major Fruit Volatiles**

Sensillae on male antennae and the female antenna (Figure 3-4) of all three parasitoids responded to fruit juice volatiles and synthetic standards. A concentration of 200 ng/μl dilutions of the synthetic compounds in dichloromethane elicited particularly unambiguous response and are illustrated (Figures 3-6 thru 3-11).

**Attraction to Fruit Juice Volatiles: Choice and No-Choice Tests**

When presented the volatiles of orange juice or water, both sexes of all three species significantly selected those of orange juice (Figure 3-12). When provided a choice of guava juice volatiles or water, all females were attracted to guava juice volatiles (Figure 3-13). *D. longicaudata* and *U. anastrephae* males also showed preference for guava juice while *D. areolatus* males chose equally amongst the treatments. Males and females of all 3 species were more attracted to the volatiles of orange juice than to those from guava juice or water (Figure 3-14).
Discussion

Both males and females of all three species were attracted to orange juice when presented a choice of orange juice, guava juice and water. When guava juice and water were the only choice, female *D. longicaudata* and *D. areolatus* were significantly more likely to be located at the guava stations. The females of *U. anastrephae* and males of all three species had no significant preference for juice over water. The results support previous observations that *D. areolatus* and *U. anastrephae* are less likely to locate damaged fruit in fruit tree canopies where their host-searches are concentrated (Sivinski et al., 1997). Fallen fruit is the foraging location of choice for *D. longicaudata* (Purcell et al., 1994). This may account for a difference in attraction to fruit juice stations. Juice expelling from ovipositor-wounded or infested fruit may afford widespread opportunities to exploit a conventional nutritional resource.

Likewise, both males and females of all three species were significantly attracted to orange juice volatiles when presented a choice of orange juice or water and when compared to guava juice and water. However, all females were attracted to guava juice volatiles when provided only a choice of guava juice volatiles or water. *Diachasmimorpha longicaudata* and *U. anastrephae* males also showed preference for guava juice volatiles. Only *D. areolatus* males chose equally amongst the treatments.

What might account for the evolution of such a pattern of responses? It was suggested that history of sympatry, degree of specialization, mating system and fruit food value might all influence response to a particular volatile. Some of these influences, sympatry and specialization, are not obviously supported by the results. Perhaps the most consistent explanation lies in a combination of oranges’ superior food value with different sexual motivations. The argument is as follows: 1) all insects are
attracted to orange volatiles, with the exception of *D. areolatus* males, to the volatiles of guava; 2) but none of the males nor *U. anastrephae* females occupied guava juice-stations because they may sense guava’s toxicity through short-range or contact cues; 3) however, females of *D. longicaudata* and *D. areolatus* remain at guava juice stations not because of food, but because of the suggestion of oviposition opportunities.

How were the responses to the fruits mediated? Again, responses to orange juice and orange juice volatiles were universally positive while those to guava were mixed. Presumably there were either unique compounds observed from the chromatograms in the two fruits or different amounts of common compounds that acted as cues and these ultimately accounted for the differences in attractiveness/juice-site-residency. While there were large numbers of minor difference in the constituency of the 2 volatile profiles, major components, those that accounted for 50% of the volatiles emitted, were similar (Table 3-1).

Of these similar compounds, hexanal, an aldehyde with a sweet grassy odor found in many flowering plant and fruit volatiles, was 4X more abundant in guava juice (~400 ng/µl) than in orange juice samples (~100 ng/µl). Natural and synthetic hexanal initiated an antennal response in females of all three parasitoids, however, males showed little or no response. Thus its relative abundance cannot entirely account for the parasitoids’ behavioral response patterns particularly the attraction of males to volatiles fruit juice volatiles.

Two other of the major volatile constituents were also unlikely to explain the response pattern. 3-hexen-1-ol (leaf alcohol) is used by some predaceous insects as a cue to detect insect feeding (Dickens, 1999). This compound occurred in the guava
juice samples at ~50 ng/µl and at ~25 ng/µl in orange juice. There was no antennal
response from any of the parasitoids to this compound, whether in its natural form or to
various doses of a synthetic. Likewise, the parasitoids did not exhibit an antennal
response to natural or synthetic limonene. Limonene is the main odor constituent of
plants in the family Rutaceae (Sun, 2007), and its concentration was greater in orange
juice volatiles (~400 ng/µl) than in guava juice volatiles (~200 ng/µl). It has however
been shown to be an attractant in some insects, such as the White Pine Cone Beetle
(Coleoptera: Scolytidae) (Miller, 2007), and has been used as an insecticide and a
repellent (Gleiser et al., 2011).

On the other hand, both sexes of all the parasitoid species exhibited an antennal
response to natural and synthetic ethyl butanoate, although the response tended to be
greater in females than males. The concentration of ethyl butanoate in orange juice
volatiles was significantly higher (~200 ng/µl) than in guava juice volatiles (~25 ng/µl).
Ethyl butanoate is the odor most associated with that of fresh orange juice and is often
added to commercial orange juice (Ahmed et al., 1978). Thus of the major constituents
only ethyl butanoate is in a position to account for the differences in behavioral
responses (i.e., is sensed by the all of the insects).

These results have important implications for the practice of fruit fly biological
control. While augmentative parasitoid releases have promise as a means of area-wide
control, particularly in urban environments and protected natural sites where
insecticides cannot be used repeatedly applied, there are problems associated with its
use. One of these is the inability to easily and cheaply monitor the survival and
dispersal of the released parasitoids. This need might be met by an effective trap baited with synthetic feeding and/or ovipositional cues.

The next step in the development of such a lure would be to compare and then combine ethyl butanoate with previously reported compounds that mediate the orientation of an opiine fruit fly parasitoid such as ethanol, acetaldehyde and acetic acid (Greany, 1977a).
Table 3-1. GC-MS of volatile compounds identified in guava juice (*Psidium guajava*)
and orange juice (*Citrus sinensis*).

<table>
<thead>
<tr>
<th>Peak</th>
<th>Compound</th>
<th>ng/µl</th>
<th>Peak</th>
<th>Compound</th>
<th>ng/µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>hexanal</td>
<td>400</td>
<td>1</td>
<td>hexanal</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>ethyl butanoate</td>
<td>25</td>
<td>2</td>
<td>ethyl butanoate</td>
<td>200</td>
</tr>
<tr>
<td>3</td>
<td>(Z) 3-Hexen-1-ol</td>
<td>50</td>
<td>3</td>
<td>(Z) 3-Hexen-1-ol</td>
<td>25</td>
</tr>
<tr>
<td>4</td>
<td>D-limonene</td>
<td>200</td>
<td>4</td>
<td>D-limonene</td>
<td>400</td>
</tr>
</tbody>
</table>
Figure 3-1. Parasitoid attraction to orange juice, guava juice and water in a flight tunnel. Probability values are associated with the various sets of bars and separations of means are indicated by letters (those sharing a letter are not significantly different).
Figure 3-2. Parasitoid attraction to guava juice and water in a flight tunnel. Graphs to the right represent no choice to guava juice [Psidium guajava] and those to the left choice of water.
Figure 3-3. Representative chromatogram of volatile compounds identified in orange juice (Citrus sinensis). Number on peak refers to compounds listed in Table 3-1.
Figure 3-4. Representative chromatogram of volatile compounds identified from guava juice (*Psidium guajava*). Number on peak refers to compounds listed in Table 3-1.
Figure 3-5. GC-MS representative chromatogram of 200ng/µl synthetic standards identified from guava juice and orange juice. Number on peak refers to compounds listed in Table 3-1.
Figure 3-6. Gas Chromatography (GC) - Electroantennogram (EAD) response of a *Diachasmimorpha longicaudata* male to synthetic compounds. Compounds consisted of 200 ng/μl of synthetic: 1) hexanal, 2) 3-hexen-1-ol, 3) ethyl butanoate, 4) limonene. The top peak represents the flame ionization detection (FID) of the synthetic compounds; the bottom peak represents the EAD response of *Diachasmimorpha longicaudata* antenna to the presence of the synthetic compounds.
Figure 3-7. Gas Chromatography (GC) - Electroantennogram (EAD) response of a *Diachasmimorpha longicaudata* female to synthetic compounds. Compounds consisted of 200 ng/μl of synthetic: 1) hexanal, 2) 3-hexen-1-ol, 3) ethyl butanoate, 4) limonene. The top peak represents the flame ionization detection (FID) of the synthetic compounds; the bottom peak represents the EAD response of *Diachasmimorpha longicaudata* antenna to the presence of the synthetic compounds.
Figure 3-8. Gas Chromatography (GC) - Electroantennogram (EAD) response of a *Doryctobracon areolatus* male to synthetic compounds. Compounds consisted of 200 ng/μl of synthetic: 1) hexanal, 2) 3-hexen-1-ol, 3) ethyl butanoate, 4) limonene. The top peak represents the flame ionization detection (FID) of the synthetic compounds; the bottom peak represents the EAD response of *Doryctobracon areolatus* antenna to the presence of the synthetic compounds.
Figure 3-9. Gas Chromatography (GC) - Electroantennogram (EAD) response of a *Doryctobracon areolatus* female to synthetic compounds. Compounds consisted of 200 ng/μl of synthetic: 1) hexanal, 2) 3-hexen-1-ol, 3) ethyl butanoate, 4) limonene. The top peak represents the flame ionization detection (FID) of the synthetic compounds; the bottom peak represents the EAD response of *Doryctobracon areolatus* antenna to the presence of the synthetic compounds.
Figure 3-10. Gas Chromatography (GC) - Electroantennogram (EAD) response of a *Utetes anastrephae* male to synthetic compounds. Compounds consisted of 200 ng/μl of synthetic: 1) hexanal, 2) 3-hexen-1-ol, 3) ethyl butanoate, 4) limonene. The top peak represents the flame ionization detection (FID) of the synthetic compounds; the bottom peak represents the EAD response of *Utetes anastrephae* antenna to the presence of the synthetic compounds.
Figure 3-11. Gas Chromatography (GC) - Electroantennogram (EAD) response of a *Utetes anastrephae* female to synthetic compounds. Compounds consisted of 200 ng/μl of synthetic: 1) hexanal, 2) 3-hexen-1-ol, 3) ethyl butanoate, 4) limonene. The top peak represents the flame ionization detection (FID) of the synthetic compounds; the bottom peak represents the EAD response of *Utetes anastrephae* antenna to the presence of the synthetic compounds.
Figure 3-12. *Diachasmimorpha longicaudata*, *Doryctobracon areolatus* and *Utetes anastrephae* exposed to guava juice volatiles and a water control.
Figure 3-13. *Diachasmimorpha longicaudata*, *Doryctobracon areolatus* and *Utetes anastrephae* exposed to orange juice volatiles and a water control.
Figure 3-14. *Diachasmimorpha longicaudata*, *Doryctobracon areolatus* and *Utetes anastrephae* exposed to orange juice and guava juice volatiles.
CHAPTER 4
A COMPOUND PRODUCED BY FRUGIVOROUS TEPHRITIDAE (DIPTERA) LARVAE PROMOTES OVIPOSITION BEHAVIOR BY THE BIOLOGICAL CONTROL AGENT DIACHASMIMORPHA LONGICAUDATA (ASHMEAD) (HYMENOPTERA: BRACONIDAE)

Host location in parasitoid Hymenoptera relies on a variety of visual, tactile, and chemical cues (Godfray, 1994). The range at which these cues are perceived varies from many meters to actual contact (Vinson, 1984), but hosts sequestered inside plant tissue, where they cannot be seen and chemical signatures might be blocked or masked, create a special set of foraging problems. However, parasitoids have evolved a number of tactics to deal with these difficulties, including searching visually for associated damage (Faeth, 1990), sensing synomones [chemical signals produced by plants under attack] (Dutton et al., 2000), pinpointing infrared emissions from fruit (Richerson and Borden, 1972), orienting to larval feeding sounds (Lawrence, 1981) and even echolocate through antennal vibrations that place the inactive pupae of stem-boring Lepidoptera (Wäckers et al., 1998). Moreover, not all substrates are completely impervious to host-derived chemical cues, and in some cases host-refugia might be probed with the ovipositor for chemical evidence of occupancy (Vet and van Alphen, 1985).

Tephritid fruit fly larvae are hosts of the parasitoid, and the larvae are sequestered inside the fruit. While a variety of chalcidoids, diapriids, figitids and ichneumonoids manage to parasitize Tephritidae (López et al., 1999), braconids of the subfamily Opiinae are typically the most numerous and diverse members of the guilds attacking frugivorous species (Purcell, 1998). Opiines are solitary, koinobiont, larval/egg-prepupal, mostly endoparasitoids of Cyclorrhapha Diptera (Wharton and Marsh, 1978). Several species are considered important regulators of fruit fly populations and have
been introduced, and frequently established throughout the world. *Diachasmimorpha longicaudata* (Ashmead), the species used in our experiments, is one of the most widely employed (Ovruski et al., 2000). Adult females use their relatively long ovipositor to parasitize a number of second and third-instar larvae of various fruit fly species infesting a wide range of host fruits (Sivinski et al., 2001). The species was originally discovered in the Indo-Philippine region, where it attacks *Bactrocera* spp. (Wharton and Marsh, 1978), and in 1947 was introduced into Hawaii for the control of oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Clancy et al., 1952). In 1972, *D. longicaudata* was established in Florida to control the Caribbean fruit fly, *Anastrepha suspensa* (Loew), and subsequently reduced populations by ~40% in a five year period of releases (Baranowski et al., 1993).

*D. longicaudata* and other fruit fly parasitoids use both chemical attractants (host – location cues) and arrestants (host-habitat cues). *D. longicaudata* is attracted to acetaldehyde, ethanol, and acetic acid released by a fungus that grows on decaying fruit (Greany et al., 1977a), to unidentified volatiles emitted by uninfested fruit (Eben et al., 2000) and acetophenone, a chemical originally identified from a floral volatile (Rohrig et al., 2008). Females of other opiines, such as *Fopius arisanus* (Sonan), are also attracted to fruit volatiles (Messing and Jang, 1992, Eben et al., 2000, Altuzar et al., 2004), and *Pysttalia fletcheri* Silvestri, to decaying fruits and leaves of pumpkins and cucumbers (*Cucurbita* spp; Cucurbitaceae) (Messing et al., 1996). Nishida (1956) had earlier found that stem tissues of cucurbits are attractive to *P. fletcheri*, and Messing et al. (1996) suggested that the basis of the attraction was “green leaf volatiles”, a suite of
common leaf-derived compounds known to be attractive to other braconid species (Whitman and Eller, 1990).

The opiine *Utetes canniculatus* (Gahan) (= *Opius lectus*), as well as the pteromalid fruit fly parasitoid *Halticoptera rosae* Burks, are arrested by the oviposition deterring pheromone of their *Rhagoletis* hosts and concentrate their searching on marked fruit (Prokopy and Webster, 1978, Roitberg and LaLonde, 1991). Other apparent arrestants are produced by uninfested fruit and can be used to stimulate oviposition in opiines such as *Doryctobracon areolatus* (Szepligeti) (Eitam et al., 2003).

It is apparent that fruit infested with host larvae are sources of semiochemicals important to parasitoid foraging. However, there is a source of kairomones not previously considered, tephritid larvae themselves. The research in this chapter 1) described the composition of volatiles emitted by four species of tephritid larvae in three genera (*Anastrepha* [subfamily Trypetinae], *Bactrocera* and *Ceratitis* [subfamily Dacinae]) and compared them to non-tephritid Diptera. 2) compares the above larval volatiles to those derived from two artificial diets prior to and following their use as developmental substrates. 3) analyzed the volatiles from Surinam cherry (*Eugenia uniflora* L.) fruit occupied by *A. suspensa* larvae to discover if larval volatile components escape the surface of infested fruit; 4) examined through electroantennograms and electroovipositorgrams the responses of sensillae on the antennae and ovipositor of *D. longicaudata* to para-ethylacetophenone, a major larval volatile component and chemical analog of the known floral-derived attractant acetophenone; 5) determined, in a flight tunnel, if para-ethylacetophenone attracted either male or female *D. longicaudata*; 6) tested the capacity of para-ethylacetophenone to act as an *D. longicaudata*.
**longicaudata** arrestance / oviposition stimulus, both by itself and in the presence of host fruit and 7) examined if **D. longicaudata** was stimulated by para-ethylacetophenone to oviposit into a device used to mass-rear of opiene braconids destined for augmentative release.

**Materials and Methods**

**Volatile Collection**

*Anastrepha suspensa* larvae were obtained from a mass reared colony derived from wild stock several years previously and maintained at the Florida Department of Agriculture and Consumer Services, Division of Plant Industry (FDACS DPI), Gainesville, Florida, USA. Volatiles were collected from 10 ml of third instar larvae, 25 ml of artificial diet (FDACS DPI, 1995) prior to use by larvae, and 25 ml of diet subsequent to larval development. In addition, volatiles from 0.75 l of field-obtained host fruit, *E. uniflora* L., were collected and subsequently a total of 5 ml of mature larvae was removed from the same fruit and their volatiles collected. Volatile collections were also taken from intact / uninfested individual *Psidium guava* L. and *E. uniflora* (Myrtaceae) host fruits. All *A. suspensa* related collections were performed at the USDA-ARS, Center for Medical, Agricultural and Veterinary Entomology, Gainesville, Florida (CMAVE). *Bactrocera dorsalis* and *Ceratitis capitata* (Wiedemann) larvae were also taken from mass-rearing artificial diets (FDACS, 1995), and as above volatiles were collected from larvae outside of diet and then from diet both prior to and subsequent to its use as a rearing medium. In the case of *Bactrocera cucurbitae* (Coquillett) larval volatiles were collected from larvae, used artificial diet, and infested fruit (zucchini, *Cucurbita pepo* L.) gathered from the field in the vicinity of Honolulu,
Hawaii. Volatiles from *B. dorsalis*, *B. cucurbitae* and *C. capitata* were all collected at the USDA-ARS, Tropical Plant Pests Research Unit, Honolulu, HI. Volatiles were collected onto a HayeSepQ filter and later analyzed using gas chromatography-mass spectrometry at CMAVE. In addition to *Tephritidae*, volatiles were collected from 5 ml of another acalypterate fly larvae, *Drosophila melanogaster* Meigen (Drosophilidae) and 10 ml of a calypterate fly larvae, *Musca domestica* L. (Muscidae) both obtained from long-maintained colonies at the CMAVE.

Volatiles were collected using a head space collection technique (Heath and Manukian, 1992). This technique was used for volatiles collected from larvae and fruit. Samples were placed in a glass volatile collection chamber (34 cm long and 4 cm outside diameter) with a glass frit inlet and a glass joint outlet and a single port collector base. Dry charcoal filtered air was pushed into one end the chamber and over the larvae and exited the chamber via a vacuum system. The air then passed through a volatile collection filter containing 50 mg of HayeSepQ (Hayes Separation Inc., Bandera, TX). Filters were eluted with 175 µl methylene dichloride to remove volatile components. Individual larvae were selected from the artificial diet fruit using soft forceps until a volume of 10 ml was obtained. Larvae were kept moist in ~2 ml of deionized water. There were 5 replicates for each species/rearing medium.

**Identification of Larval Volatiles**

Volatile analysis was performed by electron impact gas chromatography-mass spectrometry (EI GS-MS) using an HP 6890 gas chromatograph coupled to an HP 5973 MS detector. One µl of the sample was injected using a splitless injector (injector purge at 0.5min) onto an HP-5MS dimethylpolysiloxane column (30 m×250 µm (i.d.) × 0.25 µm
film, Agilent Technologies, Palo Alto, CA, USA). The GC oven was programmed from 35°C (1.0 min hold) to 230°C at 10°C/min. Helium was used as a carrier gas at 1.2 ml/min. Volatiles were identified by comparison of mass spectra (a) with mass spectra libraries (NIST and Department of Chemical Ecology, Göteborg University, Sweden) and (b) with mass spectra and retention times of authentic standards.

Electroantennogram (EAG) and Electroovipositorgram (EOG) Measured Response in *D. longicaudata* to a Major Larval Volatile

*Diachasmimorpha longicaudata* were obtained from consecutive generations reared in colonies at CMAVE, Gainesville, Florida in conjunction with USDA-APHIS/PPQ, Gainesville, Florida, USA. The colony had been maintained for approximately 10 years with occasional (~yearly) introductions of feral individuals collected throughout southern Florida. Parasitoids were reared on *A. suspensa* larvae obtained from FDACS DPI, Gainesville, Florida, USA.

To determine if female *D. longicaudata* had a sensory response to para-ethyl acetophenone, 25 ng/μl of para-ethylacetophenone (Sigma-Aldrich) in 100% ethanol (USI Chemical Company, Tuscola, IL) and the natural compounds collected from late instar *A. suspensa* larvae were exposed to both the parasitoids antennae and ovipositor using an electroantennographic detector.

Extracts were analyzed with a GC interfaced to both flame ionization (FID) and electroantennograph detectors. In this manner, antennal and ovipositor responses were matched with FID signals for compounds eluting from the GC. Volatile extracts were prepared in the manner described above, and 1-μl aliquots were analyzed on a Hewlett-Packard (HP) 5890 Series II gas chromatograph equipped with an HP-5 column (30
m×0.32 mm ID× 0.25 mm) (Agilent, Palo Alto, CA, USA). The oven temperature was held at 40 °C for 5 min, then programmed to increase to 10°C /min to 220°C and held at this temperature for 5 min. Helium was used as a carrier gas at a flow rate of 2.0 ml/min. A charcoal filtered humidified air stream was delivered over the antenna is at 1 ml/min.

Antennae from female wasps were excised by grasping the scape at its base with a jeweler’s forceps (No. 5, Miltex Instrument Company Inc, Switzerland). The extreme distal and proximal ends of the antennae were held between electrodes (Syntech, Germany) in conductivity gel (Parker labs, Fairfield, NJ). Ovipositors were excised by grasping the base with the same jeweler’s forceps. The valvulae were pulled away from the abdomen, leaving a nerve bundle exposed. Ovipositors were placed between the electrodes as described above, with the proximal portion placed on one fork but the distal tip protruded past the other. This prevented sensory structures from being encased in conductivity gel and allowed exposure to the volatile compounds. The electroantennal detector (EAD) and FID signals were concurrently recorded with a GC-EAD program (Syntech EAGPro, Germany), which analyzed the amplified signals on a personal computer.

**Ovipositor Insertion into Treated and Untreated Cattley Guava Fruit**

*Anastrepha suspensa* pupae previously parasitized by *D. longicaudata* were placed in a 30.5 x 30.5 x 30.5 cm cage constructed of sheet acrylic and screen (13 x 13 lines per square centimeter). The newly eclosed parasitoids were provided with a 10% raw, unfiltered and uncooked honey and water solution presented in a 118 ml plastic cup. The insects obtained moisture on a cotton dental wick (Braided roll, Richmond) inserted through the lid. A 118 ml cup provided water by the same method. Each
repetition consisted of one cage containing 100 male and 100 female parasitoids ~4
days old with no prior exposure to fruit or larvae. Twenty-four h prior to fruit exposure
the insects were transferred to 20 x 20 x 20 cm² cages constructed of sheet acrylic and
screen (13 x 13 lines per square centimeter). Food and water was provided by the
same method as previously described.

Ripe cattley guava (Psidium cattleianum Sabine), a principal host of A. suspensa
in Florida and one in which A. suspensa is heavily parasitized by D. longicaudata
(Sivinski 1991), was collected from trees grown at CMAVE, Gainesville, Florida, USA.
Each repetition utilized two mature fruits. One fruit was treated with 25ng/μl of para-
ethylacetophenone (Sigma-Aldrich), a major component of larval volatiles, in 100%
ethanol (USI Chemical Company, Tuscola, IL). This concentration was chosen on the
basis of the positive response by D. longicaudata to volatile acetophenone, a floral
compound with a similar chemical structure (Rohrig et al. 2008). The para-
ethylacetophenone in ethanol was applied to the entire circumference of the fruit with
fine painter’s brush and allowed to dry before presentation. This allowed for the ethanol
to evaporate. The second fruit was brushed with 100% ethanol applied in the same
manner. Each fruit was presented to the parasitoids on top of an inverted 60 ml plastic
cup placed 8 cm apart on the floor of the cage. Each repetition consisted of one fruit
treated with para-ethylacetophenone in ethanol and another treated with ethanol alone.
The insects were observed for a period of ten min; complete insertion of the ovipositor
into the fruit was noted as a positive response. Ten replicates were performed.
Numbers of insertions in the two types of fruit were compared by Wilcoxon paired
sample test (Zar, 1974).
Oviposition Device Choice Test

Parasitoids were allowed to oviposit into one of five differently treated “oviposition devices” commonly used for *D. longicaudata* mass-rearing. The oviposition device contained third instar *A. suspensa* larvae with treated Parafilm® (American National Can, Menasha, WI). The devices were based on a 7.5 cm diameter plastic embroidery ring (1004-W025, Westex Corp.), with a 15 x 15 cm piece of organdy cloth placed on one half of the open ring. Five ml of third instar *A. suspensa* larvae, thoroughly washed with water to remove diet particles, were placed in the center of the cloth. An additional piece of organdy cloth along with a 15 x 15 cm piece of Parafilm® was placed over the larvae. The ring was then assembled, sandwiching the larvae inside.

When the treatment included exposure to compounds from the surface of a fruit, a ripe commercially produced “Bartlett” pear, *Pyrus communis* L. subsp. *communis*, was used as the source. *Pyrus communis* is the standard fruit used to provide oviposition cues for rearing the opiine tephritid parasitoid *D. areolatus* in the laboratory. The fruit was wrapped with Parafilm® 24 h prior to allow for absorption of odors (Eitam et al. 2003). Other treatments were applied directly to the Parafilm® and the five treatments were as follows: no treatment; fruit compounds; 20 μl of para-ethylacetophenone solution (25 ng/μl of para-ethylacetophenone [Sigma-Aldrich] in 100% ethanol [USI Chemical Company, Tuscola, IL]); fruit compounds and 20 μl of para-ethylacetophenone + ethanol solution; and 20μl 100% ethanol.

Ten female parasitoids ~3-10 days old were placed in a 473 ml clear polypropylene container (RD16, Placon Products, Madison, WI). A 7.5 cm diameter hole was placed in the center of the lid and replaced with Organdy cloth hot-glued into place. This allowed for the parasitoids to oviposit through the top of the container. An
oviposition device with one of the Parafilm® treatments was placed face down on the organdy lid. Observations of the number of insects on the underside of the lid and parasitoids probing with their ovipositors were recorded as separate data points at one min intervals for a period of 15 min.

After 15 min the larvae from each treatment were placed in moistened vermiculite inside a 15mm plastic Petri dish (3488-B28, Thomas Scientific). After ~12 days, 100 pupae were transferred to a 473 ml plastic container with an organdy lid to allow air circulation and held for 5 weeks or until eclosion. Percent parasitism (= number of parasitoids / number of A. suspensa pupae) was then calculated. There were 19 replicates. Percent data were transformed by taking the arcsines of their square roots. Statistical analysis was through Analysis of Variance with subsequent mean comparisons by Waller’s test (SAS Inst., 2002).

**Flight Tunnel Bioassays**

A flight tunnel bioassay was developed to determine the longer-distance response of D. longicaudata to fruit treated with either a para-ethylacetophenone solution or a fruit with ethanol alone. The flight tunnel was constructed of clear acrylic sheets and measured 128 x 31.8 x 31.8 cm and located inside a laboratory at the CMAVE, Gainesville, Florida, USA. Illumination was provided by two 120 cm fluorescent bulbs suspended above the flight tunnel. The light source and the light emitted by the room lighting produced an illumination within the tunnel of ~1600 lux. The room temperature ranged from 28.7-28.8 ºC and humidity between 37.6 -38.1% RH. Air flow within the tunnel was produced by a Shaded Pole Blower (Dayton, Niles, IL) which pulled outside air into the tunnel through a charcoal filter and exhausted into a hood. The exhaust end was screened to prevent insects from entering the tube. Airflow could be adjusted by
the use of a baffle inside a tube that connected the downwind end of the tunnel with the exhaust system of the hood. Air speed was maintained at 0.2 m/s. Previous studies performed by Messing et al. (1997) determined this to be the speed that most stimulated flight in *D. longicaudata*.

Two 3.8 l glass jars fitted with a metal lid containing two brass hose fittings contained the fruit and allowed air to pass over 2 odor sources and emerge separately in the flight tunnel. Air flow into the fruit containers was controlled by an adjustable flow meter (Aalborg Instruments, Monsey, NY) set at ~0.5 liters/min. Treated air emerged into two insect traps located at the upwind end of the tunnel and placed midway between its ceiling and floor. These were constructed from 40 dram clear plastic snap cap vials (Thornton Plastics, Salt Lake City, UT). A 10 mm hole was placed in the center of the cap to allow insects to enter the chamber. The wind tunnel was checked every half h from the period of 0900 to 1400 hours. A positive response was recorded when there was a parasitoid inside the trap. The insect was removed from the trap and replaced with a naive insect from a stock cage where the original insects had been obtained. The position of the treatment and control were changed after each replication to prevent positional effects. There were five replicates each of 2 different para-ethylacetophenone concentrations (25 and 50 ng/μl of para-ethylacetophenone in 100% ethanol) applied to two different, but related, host fruits both heavily parasitized by *D. longicaudata* in the field (*P. cattleianum* and *P. guajava* L. [common guava]). Dilutions were chosen on the basis of concentrations used with positive results in previous oviposition experiments, (this paper) and experiments with a related compound (acetophenone; Rohrig et al. 2008). All were compared to species of fruit to which it
was applied. Analysis of data began with multi-variant ANOVA (SAS 2002). When variables proved to have an insignificant effect on numbers of males and females captured, data were pooled and pair-wise comparisons of responses to treated and control fruit accomplished with the Wilcoxon paired-sample test (Zar, 1974).

Results

Identification of Larval Volatiles

Para-ethylacetophenone was consistently a major volatile component released by all tephritid larvae, regardless of species or larval diet (Figure 4-1a). The compound was not found in either the larvae of a non-tephritid acalypterate species (*D. melanogaster*) (Figure 4-1b) or a calypterate species (*M. domestica*). Para-ethylacetophenone was detected in both the used artificial diets and from *E. uniflora* fruit infested with *A. suspensa* larvae but with intact skin. It was not detected in either the unused artificial diets or the uninfested *P. guajava* or *E. uniflora*. In summary, all tephritid larvae and used larval developmental-substrates emitted para-ethylacetophenone, but neither of the other two flies or the unoccupied larva-substrates tested did so.

Electroantennogram and Electroovipositorgram Responses of Parasitoid Antennae and Ovipositor to a Major Larval Volatile Component

The sensillae on the antennae (Figure 4-2) and ovipositor (Figure 4-3) of *D. longicaudata* responded to synthetic para-ethylacetophenone.

Ovipositor Insertion in Treated and Untreated Fruit

Female *D. longicaudata* inserted their ovipositors significantly more frequently into *P. cattleianum* treated with a dilution of para-ethylacetophenone than controls brushed with ethanol alone (Figure4-4).
Oviposition Device Choice Test

There were no significant differences in either the number of females observed landing on the variously treated oviposition devices ($F= 0.39; df (model) = 4; df (error) = 70; p= 0.81$). However, there were significant differences in the number of oviposition insertions/female landed ($F= 2.67; df (model) = 4; df (error) = 70; p < 0.04$; Figure 4-5), with para-ethylacetophenone + ethanol, para-ethylacetophenone + ethanol + fruit, and ethanol alone all having higher values than the control. “Fruit” (Parafilm wrapped for 24 h around a ripe pear) did not differ significantly from the control.

In terms of actual oviposition, as determined by the mean numbers of eclosing adult parasitoids in each treatment, only para-ethylacetophenone + ethanol + fruit was significantly different from the untreated control ($F= 1.74; df (model) = 4; df (error) = 90; p= 0.15$; Figure 4-6). Interestingly, it was found that ethanol-treated Parafilm continued to emit volatiles for at least 18 h. This was confirmed by volatile collection and GC-MS analysis of the Parafilm after the 18 h period (Stuhl et al., 2011a). This long period of emission was unlikely to be the case in the previous experiment where 100% ethanol was applied directly to the fruit and quickly evaporated.

Flight Tunnel

Neither male nor female captures were significantly influenced by the dose response of para-ethylacetophenone or the species of fruit it was applied to. Capture data were thus pooled and compared solely on the basis of whether or not para-ethylacetophenone was applied. In neither males nor females were there significantly different responses to treated or control fruit. Given the lack of significance, the response data were further pooled and male and female captures compared across fruit species, treatment and dilution of para-ethylacetophenone (mean [male]= 3.5 [SE=
0.43] vs. mean [female]= 11.0 [SE= 0.50]). Significantly more females than males were captured (T= 0, n= 40, p < 0.001).

Discussion

Para-ethylacetophenone is a chemical analog of acetophenone, a floral-derived compound that is attractive to female, but not male, *D. longicaudata*. In addition it was a major constituent of the volatiles produced by larvae of three genera from two subfamilies, Dacinae and Trypetinae, of tropical/subtropical Tephritidae and thus a chemical that might be expected to mediate female opiine behavior. It is associated with fruit flies since it was not found in larvae of another acalypterate fly, *Drosophila melanogaster*, or in the calypterate *Musca domestica*. However, it was not produced as a consequence of fruit consumption, since it was detected from *A. suspensa* larvae that developed in both fruits and artificial diet and also from *B. dorsalis* and *C. capitata* reared on artificial diets or from guava fruit.

While collected in the present work as a volatile, para-ethylacetophenone may be present in the fluid surrounding larvae inside fruit and so could act as a cue to foraging parasitoids identifying a suitable fruit-microhabitat in which to search. If a chemical gradient occurs within infested fruit it could lead ovipositor-probing parasitoids to the larvae themselves. Since para-ethylacetophenone was emitted across the surface of intact but infested *E. uniflora*, it could in principle serve as a longer-distance attractant as well. There was no evidence that this was the case, but it did act as an oviposition stimulant when applied to *P. cattleianum* fruit.

In a yet smaller experimental arena, where the entire ceiling consisted of a treated artificial-oviposition device, there were no differences in the numbers of females present on the various devices but the numbers of oviposition insertions per female
were greater in treatments that included both para-ethylacetophenone and an ethanol dilutant. Since ethanol is known to attract *D. longicaudata* to decaying fruit, (Greany et al., 1977a), para-ethylacetophenone may not have played any important role in these this particular result. When comparing the number of parasitoids emerging from the different cohorts of exposed larvae, ethanol alone did not differ significantly from the control while ethanol + para-ethylacetophenone + fruit showed significance. Taken together, these experiments suggest that para-ethylacetophenone is not a powerful attractant but might be characterized as a cue that stimulates ovipositor probing and oviposition, perhaps mediated in part by sensillae on the ovipositor.

Sensillae on both the antennae and ovipositor responded to para-ethylacetophenone. While host location and/or determination of host suitability through ovipositor chemosensation has been indirectly supported (Le Ralec et al., 1996), only recently has a gustatory response of an inserted ovipositor to host haemolymph been unequivocally demonstrated (van Lenteren et al., 2007). Two types of sensillae have been located on the ovipositor of *D. longicaudata* (Greany et al., 1977b). Lawrence (1981) examined oviposition behavior in *D. longicaudata* (then *Biosteres longicaudatus*) and concluded that dead or anesthetized larvae in the laboratory did not elicit ovipositor probing by foraging females. She suggested vibrations produced by larval activity were the principal means of host location. Dead or anesthetized larvae were approached even more frequently than active larvae and at the beginning of “non-random searching” following host encounter the ovipositor “quivers” from side to side. This behavior might be performed in order to sample an extended airspace for volatiles. While vibration may
play an important role in host location, *D. longicaudata* probed fruit in the complete absence of larvae, particularly if para-ethylacetophenone was present.

Para-ethylacetophenone, other than that derived from host larvae, sporadically occurs in the chemical environment of some tephritid flies and their parasitoids. It has been identified as a component of the volatiles from intact oranges (*Citrus sinensis* L.) where it elicits an electroantennogram response from *C. capitata* (Hernandez et al., 1996). Its role, if any, in the biology of fruit fly larvae is unknown, although a number of ketones are components of insect defensive secretions and semiochemicals (Forney and Markovetz, 1971).

While it is possible, perhaps likely, that chemosensillae on the ovipositor would be gustatory in function, sensing compounds present in fluids such as insect hemolymph or fruit juice; it cannot be discounted that they sense volatiles escaping from the surface of infested fruit. A positive volatile response would be consistent with a positive gustatory response, but a negative volatile response would require an additional experiment exposing the ovipositor to para-ethylacetophenone in a fluid.

Compounds that arrest foraging parasitoids and stimulate oviposition have several potential uses in control of pest tephritids, particularly in terms of mass rearing. While *D. longicaudata* readily accepts host larvae presented in a number of substrates and enclosed under numerous types of coverings, not all opines are equally obliging. For example, *D. areolatus* is the most widespread, and typically the most abundant, endemic fruit fly parasitoid in Latin America (Ovruski et al., 2000). With the recent spread of the West Indian fruit fly, *Anastrepha obliqua* (Macquart), to new islands in the Caribbean there has been an effort to introduce natural enemies as part of an integrated
management scheme (Palanchar et al., 2009). *D. areolatus* was a leading candidate since it develops well in *A. obliqua*, and in mainland habitats commonly co-occurs naturally with another opine, *U. anastrephae*, a species often already present in Caribbean environments (López et al., 1999). This frequently encountered sympatry suggested that the new species would not displace the original and that *D. areolatus* would be suited to the new conditions (Sivinski unpublished). While *D. areolatus* was eventually introduced into Puerto Rico and the Dominican Republic the effort expended by USDA-APHIS to produce the parasitoids was relatively high (JS unpublished). If there were a synthetic suite of chemicals that could be quickly and economically applied to the rearing devices, much of the cost and labor required to rear *D. areolatus* could be eliminated. Perhaps such a treatment would include para-ethylacetophenone as well as fruit volatiles and be used for the rearing of still other species, such as some tephritid attacking Figitidae (Aluja et al., 2008), that attain higher rates of parasitism or are easier to colonize in the presence of chemical cues associated with fruit and larvae.
Figure 4-1. Chromatogram showing the presence of para-ethylacetophenone. The arrow indicates the 12.1 minute retention time among the constituents of a volatile sample taken from *Anastrepha suspensa*. Para-ethylacetophenone is not present in the chromatogram from a sample taken from *Drosophila melanogaster*. 
Figure 4-2. GC - EAD of *Diachasmimorpha longicaudata* female to synthetic para-ethylacetophenone. A concentration of 200 ng/μl of the synthetic compound was used. The top peak represents the flame ionization detection (FID) of para-ethylacetophenone; the bottom peak represents the EAD response of *D. longicaudata*’s antenna to the presence of para-ethylacetophenone.
Figure 4-3. GC-EOD response of *Diachasmimorpha longicaudata*’s ovipositor to para-ethylacetophenone. A concentration of 200 ng/μl of the synthetic compound was used. The top peak represents the flame ionization detection (FID) of para-ethylacetophenone; the bottom peak represents the EOD response of *D. longicaudata*’s ovipositor to the presence of para-ethylacetophenone in 100% ethanol.
Figure 4-4. Mean number of ovipositional attempts of *Diachasmimorpha longicaudata* into treated *Psidium cattleianum* fruit. The fruit was treated with 25 ng/μl of synthetic para-ethylacetophenone + ethanol and ethanol alone (mean [treated] = 18.1 (SE= 2.3) vs. mean [control] = 5.5 (SE= 1.7); (T= 7.64; df= 10; \( p < 0.0001 \)).
Figure 4-5. Proportions of females present on artificial oviposition devices containing larvae of *Anastrepha suspensa* that were inserting their ovipositors. Parafilm sheets included in the devices were treated in the following manners: 1- untreated control; 2- exposure to pear fruit (*Pyrus communis*); 3- application of para-ethylacetophenone + ethanol; 4- application of para-ethylacetophenone + ethanol + exposure to *P. communis*; 5- application of ethanol alone. Means with shared letters are not significantly different.
Figure 4-6. Percent parasitism of larvae presented to females on artificial oviposition devices containing larvae of *Anastrepha suspensa*. Parafilm sheets included in the devices were treated in the following manners: 1- untreated control; 2- exposure to pear fruit (*Pyrus communis*); 3- application of para-ethylacetophenone + ethanol; 4- application of para-ethylacetophenone + ethanol + exposure to *P. communis*; 5- application of ethanol alone. Included in the above described oviposition devices. Means with shared letters are not significantly different.
The absence of information about fruit fly parasitoid behavior ecology and their impact on the non-target insects are all obstacles that face the successful pursuit of biological control (Duan and Messing, 1997, Aluja, 1994). Because the economic threshold for fly damage is very low, parasitoid use in the suppression of fruit flies has not always been regarded as promising when measured by economic returns, particularly in terms of exported commodities (Enkerlin, 2005). A predictable way to ensure that there is a high level of parasitoids in the field is to artificially increase the population. This is done by augmenting the numbers of parasitoids at critical times and places particularly when it can be combined with other techniques such as SIT (Sivinski, 1996, Enkerlin, 2005). However, there is an associated cost with this method, such as rearing large numbers of the hosts and the parasitoids, and then transporting them for release. At present, there is no reliable method to accurately monitor the survival and dispersal of fruit fly parasitoids following initial and augmentative releases (Messing and Wong, 1992). The eventual development of a means of monitoring in the field was the goal of this research.

Although the economic damage caused by *Anastrepha suspensa* has been relatively small, it is one of several species of fruit flies in which the larvae attack numerous tropical and subtropical fruits. Some preferred hosts are *Psidium guajava* (common guava), *Eugenia uniflora* (Surinam cherry), *Prunus persica* (peach), *Eriobotrya japonica* (loquat), *Syzygium jambos* (rose apple), and *Terminalia catappa* (tropical almond) and occasionally citrus, which gives rise to trade restrictions. For
example, the state of Florida has organized and monitors fly-free zones that allow citrus exports to major customers such as Japan (Sivinski et al. 1996).

With consumers’ rising demand for organic and "green" commodities and, biological control agents finding a niche in many agricultural production systems, proper monitoring techniques and sustaining natural and augmented populations would allow us to better understand the parasitoid density and distribution and substantially improve their effectiveness.

Many assumptions have been made concerning the carbohydrates and other nutrients needed for survival and reproduction by synovogenic parasitoids. *Diachasmimorpha longicaudata, D. areolatus* and *U. anastrephae* utilized in these studies showed an ability to acquire these from a variety of sources. Juices from injured fruit, extrafloral nectaries and hemipteran honeydew can provide them with a high quality diet. But consumption of fruit juices would minimize their need to separately forage for oviposition sites and food (Sivinski et al., 2006). The nutritional quality and chemical composition of certain fruits they encounter vary in quality and may contain detrimental compounds, including toxins (Barker, 1977; Wäckers, 2001).

Given the differences in parasitoid foraging and the possibility that fruits might differ both nutritionally and in terms of unfavorable compounds, the original hypothesis was that *D. areolatus* and *U. anastrephae* would find guava fruit a superior food to orange. It was also postulated that the other two Mexican species, *Doryctobracon crawfordi* and *Opius hirtus* would also find guava nutritious and life-sustaining relative to a honey solution. However, survival of all three species on orange pulp often equaled that obtained on a honey positive control. Both sexes of *D. longicaudata*, from USA and
Mexican-derived cultures died at similar rates when provided guava pulp or juice or a water-only control as did *D. areolatus* and *U. anastrephae*, who were presumably adapted to the nutrient/chemical constituents of guava. It was also noted that in Mexico, *D. areolatus* and *U. anastrephae*, as well as the tephritid parasitoids *D. crawfordi* and *O. hirtus*, died at significantly higher rates when provided guava in comparison to two feeding schedules of a honey diet. These results suggest that guava-induced mortality might have been due to guavas being either, innutritious, toxic or repellent. There is no question that *D. longicaudata* consumed guava juice tagged with a colored dye. Dilutions of orange and guava juice resulted in shorter lifespans than dilutions of orange juice and water. Thus while diluted orange juice provided nutrition, the addition of guava created toxicity. While investigating the possibility of a toxin, an assay was developed to search for a toxic sugar. The sugar mannose was detected, which is known to be toxic to some hymenoptera (De la Fuente et al., 1986). This sugar was found in guava samples, but was not detected in citrus. Even though it is known to be toxic in hymenoptera, in the specific case of female *D. longicaudata* it was found slightly nutritious. A definitive conclusion on mannose as a toxin is undetermined. Furoic acid was identified in guava but not fresh citrus. It was toxic to *D. longicaudata* and might be the cause of mortality. Further investigation into the composition of guava is needed.

In order to better understand the feeding behavior of the parasitoids, they were provided with fruit juice. It was assumed that while foraging, they are able to exploit this easily accessible resource. When the insects were presented a choice of orange juice, guava juice and water, both males and females of all three species came to occupy
orange juice. *D. longicaudata* and *D. areolatus* were significantly more likely to select the guava stations when guava juice and water were the only choice. *Utetes anastrephae* females and males of all three species had no significant preference for guava juice over water. The attraction to guava juice over water may be due to favorable volatile compounds emanating from the juice treatments and not necessarily its nutritional content.

Having discovered a difference in fruit-food quality, a suitable source of food may not be obtainable at all oviposition sites. This would imply that the insects may sometimes need to forage elsewhere to meet their nutritional needs (Sivinski et al., 2006).

The volatiles from fruit known to be highly or less nutritious and likely to attract/arrest the Opiine parasitoids were investigated. Both males and females of all three species preferentially selected orange juice volatiles when presented a choice of orange juice or water and when compared to guava juice and water. All females were attracted to guava juice volatiles when provided a choice of guava juice volatiles or water. The males of *D. longicaudata* and *U. anastrephae* also showed preference for guava juice while *D. areolatus* males chose equally amongst the treatments. The results suggest that the guava may be only suitable as an ovipositional site for *D. areolatus* and not a suitable feeding site for males.

While guava (unattractive as juice) and orange (attractive as juice) volatiles differed in a number of minor ways, they shared 4 major constituents, albeit in substantially different amounts: Hexanal, 3-hexen-1-ol, ethyl butanoate and limonene. Natural and synthetic hexanal initiated an antennal response in females of all three
parasitoids, however, males showed little or no response. Neither 3-hexen-1-ol nor limonene, the main odor constituent of citrus, produced antennal responses from any of the parasitoids to this compound. Only natural and synthetic ethyl butanoate elicited an antennal response in both sexes of all three species and so was a candidate to explain the pattern of response to both juice itself and the volatile they emitted. Ethyl butanoate thus has potential as a tephritid fruit fly parasitoid lure component.

Chapter 4 investigated the possibility that the larvae themselves produced an ovipositional cue that was being exploited by the female parasitoids. Greany et al. (1977b) photographed purported sensory structures on the ovipositor of *D. longicaudata* and suggested they were employed by ovipositing females to locate hosts sequestered within fruit. Larvae from several genera of tephritid larvae produced para-ethylacetophenone, but other flies such *Musca domestica* (L.) and *Drosophila melanogaster* (Meigen) did not. This chemical was emitted across the surface of intact but infested Surinam cherry (*E. uniflora*). Furthermore, it was detected from *A. suspensa* larvae that developed in both fruits and artificial diet, and also from *B. dorsalis* and *C. capitata* reared on artificial diets or from guava fruit. Therefore, this compound was not produced as a byproduct of fruit consumption. Thus it is a chemical that might be expected to mediate female opiine behavior.

Para-ethylacetophenone attracted/arrested female, but not male *D. longicaudata*, and also elicited an electroantennogram and electroovipositorgram response. Although para-ethylacetophenone was collected as a volatile, it may have also been present in the fluid surrounding larvae inside fruit. It did act as an oviposition stimulant when
applied to *Psidium cattleianum* (Sabine) fruit. There was no evidence that this compound serves as a longer-distance attractant.

The investigation into the volatile components of the fruit and larval host may broaden the understanding of the volatile components within the fruit juices used in feeding and possibly host location. Although the parasitoids did respond to single volatile constituents, it may be that an array of volatile compounds may elicit much greater response.

The utilization of these experimental results require further testing in a controlled field environment and eventually tested in a commercial fruit orchard. A reliable trap that uses an effective attractant that is effective over time, and will restrain the trapped insects for monitoring, still needs need to be developed.


BIOGRAPHICAL SKETCH

Charles J. Stuhl was born on Long Island, New York. He enlisted in the US Army in 1986 and spent a three-year tour of duty as a combat medic in Germany. Upon completion of his military duty, he attended Gupton-Jones College of Funeral Service in Atlanta, Georgia, receiving an Associate of Science degree in mortuary science. He relocated to Florida to work in the funeral industry. He returned to college to pursue a Bachelor of Science degree in entomology at the University of Florida, and was awarded his degree in 2000.

He began working at the US Department of Agriculture, Agriculture Research Service as biological science aid and was later hired as a biological science technician. He then pursued a Master of Science degree at the University of Florida and was awarded the degree in 2004. For his degree, he studied the behavioral differences in *Spodoptera frugiperda* host strains. Since 2009 he has served as a support scientist in the Insect Behavior and Biological Control research group.

He entered the PhD program in the entomology and nematology department, University of Florida in 2005. His doctoral research was focused on the chemical ecology of host fruits and ovipositional cues of three Tephritid fruit fly parasitoids. He received his PhD in entomology and nematology in Fall 2011.