To my family, for believing in me. To Ryan, for all those late night journeys to the lab and for supporting me when I was about to buckle. To my cats, for smudging the screen of my laptop as I wrote this, reminding me to scratch their ears and take a break from work every once in a while. Thank you all.
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Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

HOST LOCATION AND UTILIZATION BY THE PEPPER WEEVIL,
Anthonomus eugenii CANO

By

Karla Michele Addesso

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Chair: Heather J. McAuslane
Major: Entomology and Nematology

The pepper weevil, Anthonomus eugenii Cano, a major pest of all species of cultivated peppers (Capsicum spp.), is currently found throughout Central America, the Caribbean and the southern United States, including Florida. Pepper weevil larvae complete their development and pupate within the fruit, making adults the only stage susceptible to foliar pesticides and behavioral controls. I believe thorough investigation of ecological factors influencing pepper weevil behavior can lead to the improvement of current control methodologies. Three broad areas of study were undertaken to increase our knowledge of this crop pest; pepper weevil nutrition, oviposition and host location behavior.

An attempt to improve the Toba diet formulation resulted in several significant pieces of information: egg hatch was closest to natural levels on moist, sterilized paper towel; adult dry mass was heavier for those reared on the control Toba diet compared with ‘Jalapeno’ fruit reared weevils; and methyl paraben is not responsible for egg mortality. Oviposition was not successfully induced in artificial diet but spherical leaf sachets covered in Parafilm or netting were acceptable. The leaves used in these sachets could be pepper leaves or leaves from several other host and non-host plants.
In further oviposition studies, females laid fewer eggs in naturally infested pepper fruits in cage and small arena studies. A host marking pheromone responsible for the deterrence was identified in the oviposition plug and frass of female weevils. This compound or compounds are slightly polar with low volatility and are potential candidates for use in pest management programs.

An investigation into the response of pepper weevil to host plant volatiles found that 10-d-old males and 2 and 10-d-old females oriented to the volatiles of three host plants (pepper, American black nightshade, and eggplant). Only 10-d-old males and females could perceive differences in volatile plumes, ignoring volatiles from non-host plants outside the Solanaceae. Ten-d-old males and females were also strongly attracted to pepper plants with actively feeding female weevils. It is hoped that by combining this knowledge of the deterrent pheromone and attractive plant volatiles, a new tool for pepper weevil pest management can be developed.
CHAPTER 1
LITERATURE REVIEW

Economic Importance of Pepper Weevil

Pepper weevil is an important agricultural pest of peppers throughout Central America, Puerto Rico and the southern United States (Elmore et al., 1934; Andrews et al., 1986; Armstrong, 1994; Servin et al., 2002). It has been well established in the literature that the pepper weevil can cause severe losses to crop yields if left unchecked. Its hosts include cultivars of the genus Capsicum including *C. annuum*, *C. frutescens*, and *C. chinensis* (Elmore et al., 1934; Burke & Woodruff, 1980; Abreu & Cruz, 1985; Andrews et al., 1986; Bujanos, 1986; Patrock & Schuster, 1992).

In 2005, the top five states producing fresh market vegetables included California, Florida, Georgia, Arizona and Texas in descending order. The distribution of the pepper weevil includes all five states. Florida is the second largest producer of fresh market pepper in the country, accounting for 11.5% of the 2005 vegetable crop value in the state (Olson et al., 2006). In 2006 the total value of bell pepper production in Florida was $187,300,000. Pepper weevil infestation causes damage to the flowers, buds, and fruits from the feeding of adults and larvae. Feeding causes abscission of the fruit and lowers crop yields, with estimates ranging from 33% (Walker, 1905), to 50%, 75% (Elmore et al., 1934) or even 100% (Campbell, 1924; Genung & Ozaki, 1972).

Taxonomy

The pepper weevil, *Anthonomus eugenii* Cano was first described from specimens collected in Guanajuato, Mexico (Cano & Alacio, 1894); it was later described by Champion (1903) as *A. aeneotictus*. The pepper weevil is in the family Curculionidae, tribe Anthonomini, which contains 18 genera inhabiting the West Indies and North and Central America. There are
currently over 500 species of Curculionidae known to occur in Florida (Anderson & Peck, 1994), with over two dozen species of *Anthonomus* found in the state (O’Brien & Wibmer, 1982).

**Life History of the Pepper Weevil**

**Description and Life Cycle**

The pepper weevil was first described by Cano & Alacio (1894) as a glossy, black beetle with a robust, subovate body. Its body is covered with grey or ochraceous, scalelike hairs, densely covering the elytra intervals and scutellum but sparsely covering the legs, head, and prothorax. The beak is fairly stout, moderately curved, and slightly longer than the head and prothorax. Body length, exclusive of the beak, is 1.9 to 3.7 mm with a width of 1.1 to 2 mm. The size is variable according to the food available for larval development (Elmore et al., 1934). Characters of the male median lobe were found to be diagnostic for most Palearctic, Nearctic, and Neotropical *Anthonomus* species, although in some species groups there is insufficient variation to determine species differences (Burke, 1976). Males and females of *A. eugenii* can be separated by their metatibial mucrones. Male pepper weevils possess larger and more strongly curved metatibial mucrones than those of females (Eller, 1995). Characteristics used to determine sex in other *Anthonomus* species include the distance of antennal insertion from the tip of the rostrum and number of dorsal abdominal segments visible when the elytra are unfolded (Wilson, 1986). Also, Agee (1964) found male boll weevils (*Anthonomus grandis* Boheman) could be distinguished from females by a notch visible on the eighth tergum of the abdomen.

The egg is oblong oval, 0.53 mm in length with a diameter of 0.39 mm. It is pearly white when first laid but later turns yellow as the embryo develops. The outer surface is smooth, shiny, and flexible but tough (Elmore et al., 1934). Third instar larvae are legless, 6 mm long with a white, cylindrical body that appears grey when the digestive tract is filled. The head is yellow brown with dark brown mandibles (Elmore et al., 1934). Variation in the number, arrangement,
and relative lengths of the setae on the body and head are often used for systematic purposes (Burke, 1976). The pupae are 3.5 to 4 mm long, 2 mm wide, and uniformly white. The rostrum, antennae, and legs are closely folded against the underside of the body and the wings are pressed against the sides of the body (Elmore et al., 1934). The dorsal surface of the head, prothorax, and abdomen bear short, inconspicuous bristles. The number and arrangement of setae are the main characteristics in pupal systematics (Burke, 1976).

In the field, the life cycle of the pepper weevil can last anywhere from 20-30 d but can be faster during summer months or in the laboratory (Watson, 1935; Toba et al., 1969; Genung & Ozaki, 1972; Wilson, 1986; Gordon & Armstrong, 1990). Under laboratory conditions, up to eight generations of pepper weevil have been obtained in one year but three to five are common under natural conditions. Adults are long-lived (an average of 78.7 d under laboratory conditions) and produce overlapping generations, making exact counts of generations in the field difficult. Diapause has not been reported in the pepper weevil. The species overwinters where food is available (Elmore et al., 1934; Goff & Wilson, 1937).

Oviposition occurs within 2 d of mating, as early as 4 d after emergence. Eggs are laid singly in flower buds or fruit pods. The female creates a hole with her rostrum, deposits her egg, and seals the hole with a light brown anal secretion that hardens and darkens. This ‘plugging’ behavior has also been observed in the boll weevil, *A. grandis* Boheman (Stansly & Cate, 1984). The entire process takes between 2 to 4 minutes and the incubation period averages 4.3 d (Elmore et al., 1934; Goff & Wilson, 1937).

There are three larval instars: the first lasting 1.7 d, the second 2.2 d and the third 8.4 d (Elmore et al., 1934). About 4.9 d of the third instar are spent as a prepupa, which creates a cell for pupation from anal secretions. Larvae are aggressive if placed together on diet and have been
observed cannibalizing each other (personal observation). The pupal stage lasts for about 4 d and emergent adults escape from the pepper pod through a round hole (Elmore et al., 1934; Goff & Wilson, 1937). All life stages except the adult occur within the host flower buds or fruits. In the absence of food, adult pepper weevils survive an average of 6.8 d (Goff & Wilson, 1937).

**Oviposition**

Patrock & Schuster (1992) found that pepper weevil females preferred to lay eggs in the anthers (89.1%) over the ovary (10.9%) of bell pepper flowers, oviposition was three times as great in pepper fruit than in the flowers, and the oviposition rate was significantly slower from 2400-0800 hr than during four other time periods evaluated (0800-1200, 1200-1600, 1600-2000, 2000-2400). Females oviposit at a rate of 5 to 7 eggs/d with an average of 341 eggs deposited in a lifetime. The average ovipositional period is from 30 to 72 d (Elmore et al., 1934).

Rodriguez-Leyva (2006) examined the effects of crowding and sex ratios on eggs laid/female/d/fruit. Females caged individually laid significantly more eggs per fruit than females caged with a male. When two to 10 females were caged together, the number of eggs/female/d/fruit decreased with increasing density. While the number of eggs laid by females decreased with crowding, the percentage of eggs deposited without a plug covering increased due to interference by conspecifics or some other behavioral adaptation to overcrowding. When females were presented with pepper fruit and floral buds, the number of feeding punctures was significantly greater in the high nitrogen content buds while oviposition was greater in the fruit. The addition of high nitrogen floral buds to the diet of laboratory-reared weevils increased the total number of eggs laid in 7 d from 23.8 to 89.1. It was also demonstrated that females presented immature fruit produced more offspring than those presented fruit of marketable size (Rodriguez-Leyva, 2006).
Pepper weevil development in the laboratory is affected by both temperature and food supply. Generation time was estimated at 17.5 d at a temperature range of 23.9 to 26.7 °C and 60 to 85% humidity (Genung & Ozaki, 1972). This agrees closely with results from Toba et al. (1969), who found that nearly 90% of emerging adults fed a modified cabbage looper diet emerged by day 18. Gordon & Armstrong (1990) and Wilson (1986) estimated a generation time of 14.2 d at a temperature of 25.7-27.7°C at 40 to 100% relative humidity. Toapanta et al. (2005) studied development rates under different constant temperatures and found that weevils reared at 30°C and 60% r.h. under a 14:10 light: dark regime had the fastest development time with a mean of 12.9 d. Wilson (1986) and later Patrock & Schuster (1992) showed that development rate did not differ between weevils raised on bell pepper, Tabasco pepper, American black nightshade and Eastern black nightshade. However, the adult dry mass was significantly different among hosts, with the greatest mass being for bell pepper-reared insects and the lowest for American black nightshade. The other two plant hosts did not produce adults with significantly different dry mass. Rodriguez-Leyva (2006) performed a life history analysis for females fed on flower buds and immature fruit. The net reproductive rate ($R_o = 158.1$), the intrinsic rate of increase ($r_m = 0.14$), generation time ($T = 36.04$), doubling time ($D_t = 4.93$), and finite rate of increase ($\lambda = 1.15$) of the weevil were identified at 27°C and 14:10 light: dark regime. The demographic values based on fecundity were much higher than those found by Toapanta et al. (2005) when females were fed fruit alone with no flower buds ($R_o = 33.57$, $r_m = 0.11$, $T = 32.39$, $D_t = 6.35$, $\lambda = 1.11$), indicating the importance of adequate nutrition for optimal fecundity.
Ecology of the Pepper Weevil

Distribution

The pepper weevil is believed to have originated in Central America, specifically Mexico, due to the large number of naturally occurring parasitoids found there (Cortez et al., 2005) as well as the origin of the genus *Capsicum* (Hernandez-Verdugo, 1999). The current distribution of the weevil covers the southern United States (including Hawaii, California, Arizona, New Mexico, Texas, Georgia, Louisiana, and Florida), Mexico, El Salvador, Guatemala, Honduras, Puerto Rico and Costa Rica (O’Brien & Wibmer, 1982; Andrews et al., 1986). Walker (1905) first reported the pepper weevil in the United States in 1904 in Boerne, Texas. In 1923, the weevil was reported in southern California (Campbell, 1924) and in 1933 it was reported in Honolulu, Hawaii (Elmore et al., 1934). In April 1935 *A. eugenii* was reported attacking pepper fields in Manatee County, Florida (Watson, 1935; Goff & Wilson, 1937). Rolston et al. (1977) reported the weevil in Louisiana in 1971 and, in 1982, the pepper weevil was found in northern Puerto Rico (Abreu & Cruz, 1985). It has been collected as far north as Santa Barbara, California on the west coast as far north as New Jersey on the east coast (Burke & Woodruff, 1980). It can survive anywhere that environmental conditions are appropriate and host plants are available. The weevil was even reported infesting greenhouse peppers in British Columbia, Canada (Costello & Gillespie, 1993). It is believed the weevils may have arrived in Canada with the imported transplants on shipping palettes.

Host Plant Associations

The host plants of the pepper weevil belong to two genera of the family Solanaceae, *Capsicum* (pepper) and *Solanum* (nightshade) (Table 1-1). Common varieties of the pepper *C. annuum* L. (pimiento, Tabasco, chili, and bell pepper) and *C. frutescens* L. are susceptible to pepper weevil infestation, especially those varieties with thin-walled mesocarp (Elmore et al.,
Thick walled fruits are also utilized, but there may be a shift in preference for younger fruits. Host suitability studies have shown that *C. pubescens* Ruiz and Pavon, and *C. baccatum* L. are also acceptable hosts for the pepper weevil (Patrock & Schuster, 1992).

Although Elmore et al. (1934) stated that pepper weevil was common on black nightshade, *Solanum nigrum* L., he was likely referring to *S. americanum* Mill because *S. nigrum* L. is an introduced relative, rarely found in the southern United States (Ogg et al., 1981). Pepper weevil is often found on wild *Solanum* species growing around pepper fields. The following species of *Solanum* in Florida are reported in greenhouse studies to be acceptable as food sources and oviposition sites: *S. americanum* Mill., *S. ptycanthum* Dun., *S. pseudogracile* Heiser, *S. triquetrum* Cav., *S. pseudocapsicum* L., *S. melongena* L. (eggplant), *S. carolinense* L., *S. eleagnifolium* Cav., *S. dimidiatum* Fav., and *S. rostratum* Dunal (Patrock & Schuster, 1992; Wilson, 1986). *Solanum tuberosum* L. (potato) and representatives of the genera *Physalis, Lycopersicon, Datura, Petunia,* and *Nicotiana* were acceptable as food sources but not for oviposition. These studies were carried out under laboratory conditions using no-choice tests. It is unclear whether all of these plants would be utilized as adult and/or larval hosts under natural conditions. In the wild, pepper weevil has been collected on *S. hindsianum* Benth., a species found in the vicinity of pepper fields, and shown to support pepper weevil in field experiments conducted in Baja California Sur, Mexico (Aguilar & Servin, 2000). *Solanum americanum* var. *nodiflorum* was identified as a wild host in Puerto Rico (Gordon-Mendoza et al., 1991). Pepper weevil has been collected in the wild from *S. americanum* Mill., *S. xanti* Grey, *S. umbelliferum* Esch., *S. villosum* Mill. and *S. melongena* (var. *depressum* Bailey and var. *esculentum* Nees) as well as from two species of ornamental nightshade *S. glaucum* Dunal and *S. aviculare* Forst (Elmore et al., 1934). *Solanum elaeagnifolium* was identified as the most important alternative
host in Mexico (Tejada & Reyes, 1986). In Florida, Patrock & Schuster (1987) found adult pepper weevil for a seven-month period on *S. americanum* at six pepper farms. They concluded that this weed was capable of supporting the pepper weevil population between cropping seasons.

It has been the goal of many studies to identify pepper weevil-resistant varieties of pepper plants in order to produce the most peppers suffering the least damage. In a study of 12 commercial cultivars (jalapeno, bell, pimiento, serrano, yellow, cayenne, long chile, tabasco and cherry), and 23 multiple virus-resistant pepper breeding lines, only one jalapeno line, S’90 (J305), was selected as resistant due to its low number of scarred fruit and buds, higher number of fruit per plant and higher number of clean fruit per plant (Berdegue et al., 1994). A later study by Quinones & Favela (2002) evaluated 18 genotypes of jalapeno and found four that showed resistance to pepper weevil infestation. In another varietal resistance study, 11 commercial varieties were tested for pepper weevil resistance and only two, ‘Hot Chili’ and ‘Habanero,’ had significantly lower rates of fruit infestation (Seal & Bondari, 1999). It is important to note that even resistant varieties had some degree of infestation.

Interestingly, pepper weevil feeds and oviposits on both sweet and hot pepper. While other factors are certainly involved in plant resistance to pepper weevil, the studies by Berdegue et al. (1994), Seal & Bondari (1999) and Quinones & Favela (2002) show that hotter varieties are marginally less susceptible to pepper weevil damage. No studies to date specifically address the fitness of pepper weevil on individual varieties with different levels of capsaicinoids, the compounds responsible for fruit heat.

Identifying native flora susceptible to pepper weevil infestation is imperative if control measures are to be effective. In this regard, systematic studies of the *Capsicum* and *Solanum*
genera are of great importance. Such information would allow more directed work on host plant range by allowing researchers to focus on closely related species within the geographic distribution of known pepper weevil hosts. McLeod et al. (1979) conducted a preliminary biochemical systematic analysis of 12 species and varieties of pepper. Fifteen allozymes were used to determine the degree of relatedness among the species examined. The analysis identified the purple-flowered taxa (*C. pubescens*, two wild South American species, *C. eximium* Hunz. and *C. cardenasii* Heiser & Smith and an unnamed species) and white-flowered taxa (*C. baccatum* var. *baccatum*, *C. baccatum* var. *pendulum*, *C. praetermissum* Heiser & Smith, *C. chacoense*, *C. annuum* var. *aviculare*, *C. annuum* var. *annuum*, *C. frutescens* and *C. chinense*) as distinct species groups. The white-flowered taxa were further subdivided into two groups; subgroup one included both wild (*baccatum*) and domesticated (*pendulum*) varieties of *C. baccatum* and *C. praetermissum* Heiser & Smith. These two species were also said to possess identical flavonoids, further supporting their close relationship. Subgroup two contained the *C. annuum* complex and both *C. chinense* and *C. frutescens*. *Capsicum chacoense*, a white-flowered species, is ‘biochemically’ half way between the purple and white taxa groupings.

There are also a few systematic studies on the genus *Solanum*, but much work still needs to be done on this group to determine the relationship among species within the genus, which might explain why the pepper weevil will oviposit on some members of the genus and not others (Schilling, 1981; Bohs & Olmstead, 1997; Olmstead & Palmer, 1997).

**Chemical Ecology**

Understanding the effect of semiochemicals on the pepper weevil and its parasitoids is important for developing effective monitoring and mass rearing technology. In many species of weevil, males are known to produce pheromones that usually attract both sexes. These aggregation pheromones can function for considerable distances (up to 10 m for the boll weevil)
Females have also been recorded producing pheromones that attract males as well as oviposition-deterring pheromones that affect the behavior of conspecific females. 

Coudriet & Kishaba (1988) were the first to demonstrate that male pepper weevils were producing a chemical attractant. They tested the attractiveness of traps baited with live males and with dichloromethane extracts of pepper weevil males in the field. They found that both male and female weevils were equally attracted to traps baited with five live males and 40 male equivalents of extract. Eller et al. (1994) identified six components of the male-produced pepper weevil aggregation pheromone: (Z)-2-(3,3-dimethylcyclohexylidene) ethanol, (E)-2-(3,3-dimethylcyclohexylidene) ethanol, (Z)-(3,3-dimethylcyclohexylidene) acetaldehyde, (E)-(3,3-dimethylcyclohexylidene) acetaldehyde, (E)-3,7-dimethyl-2,6-octadienoic acid (geranic acid) and (E)-3,7-dimethyl-2,6-octadien-1-ol (geraniol). The field-tested formulation attracted more females than males, with trap captures averaging 84% female. Three of the six compounds released by the pepper weevil are shared with the boll weevil, but the concentrations emitted by the two species differ (Tumlinson et al., 1969; Eller et al., 1994). In 1998, a new commercial pheromone formulation (TRE 8420 + 8462) was tested in the field with yellow sticky traps. The new formulation lasted for five weeks and detected the presence of pepper weevils before field infestation was observed (Bottenberg & Lingren, 1998).

Laboratory observations of the pepper weevil’s response to the oviposition plug have suggested it may have a deterrent effect on conspecific females (personal communication, Phil Stansly). Stansly & Cate (1984) found that the boll weevil’s oviposition plug deterred conspecifics from depositing eggs in *Hampea nutricia* Fryxell flowers. In addition to the boll weevil, the production of a marking pheromone has been confirmed in other weevil species including members of the families Curculionidae (*Ceutorhynchus assimilis* Paykull),
Ceutorhynchus floralis (Paykull), and Bruchidae (Acanthoscelides obtectus (Say), Callosobruchus chinensis (L.), Callosobruchus rhodesianus (Picreaus), Callosobruchus subinnotatus (Picreaus), Zabrotes subfasciatus (Boheman)) (Anderson, 2002). The pepper weevil’s oviposition behavior is similar to the boll weevil. It is possible that the pepper weevil oviposition plug contains a marking pheromone similar to the one produced by its congener.

In addition to the male-produced aggregation pheromone and female marking pheromones, weevils also show behavioral and physiological responses to host plant volatiles. A body of literature exists on boll weevil attraction and physiological response to host plant and green leaf volatiles (McKibben et al., 1977; Dickens, 1984, 1986, 1989 and 1990; Dickens & Moorman, 1990). The apple blossom weevil (Anthonomus pomorum L.) and the strawberry blossom weevil (Anthonomus rubi (Herbst)) also respond to host plant volatiles in behavioral and electrophysiological studies (Kalinova et al., 2000; Bichão et al., 2005a, 2005b). It is reasonable to assume that the pepper weevil will also respond to the presence of host plant volatiles, though this hypothesis has not yet been tested. It has also been noted that boll weevils reared on artificial diet are less attractive than field-caught males (Hardee et al., 1971), suggesting that something missing from the artificial diet was essential for the production of the male attractant. Hardee et al. (1971) further demonstrated that water extracts of cotton squares increased the attractiveness of Grandlure®, an artificial boll weevil pheromone formulation. A careful look at the response of pepper weevils to host plant volatiles may prove useful if they synergize with the aggregation pheromone, making the combined lure more effective in field monitoring programs.

Semiochemicals produced by the host plants as well as those produced by the weevil affect not only the weevil itself, but could also play a vital role in the search and selection behaviors of pepper weevil parasitoids and predators. An in-depth study of the chemically-mediated...
relationships between the pepper weevil and its host plants are essential for understanding pepper weevil behavior in the field and may additionally aid in the development of effective mass rearing programs for the pepper weevil and its parasitoids.

**Agricultural Importance and Management**

**Integrated Pest Management**

Management of the pepper weevil relies on the integration of natural mortality factors, and applied cultural, insecticidal and biological controls. There are a number of natural environmental conditions that provide some measure of population control of the pepper weevil. Among these are freezing temperatures, overheating of fallen fruit, destruction of fallen fruit, and lack of host material. The pepper weevil does not appear to diapause and so cold weather is a limiting factor in the spread of pepper weevil (Elmore et al., 1934; Boswell et al., 1964). Fallen fruits desiccate in the hot weather, increasing mortality of pepper weevil larvae and pupae. Destruction of fallen fruit and lack of hosts are detrimental to the pepper weevil populations. Other natural mortality factors such as ant predation may also be important (Sturm et al., 1990). Cultural controls often work on increasing the impact of natural population controls. Weather conditions cannot be controlled in an open field agricultural setting, but other factors can be augmented by farmer intervention.

Cultural practices were some of the earliest methods suggested for control of pepper weevil populations in pepper crops. These methods include destruction of post-harvest pepper crops, as well as alternative solanaceous hosts, field sanitation practices, and use of uninfested transplants. As early as 1934, a campaign was undertaken that continues today in Orange County, California to have farmers disk and plow their pepper fields after harvest and destroy all solanaceous plants along fences, roads and other areas surrounding their fields (Elmore et al., 1934; Patrock & Schuster, 1987; Riley & Sparks, 1993). To be effective, however, all fields in
the community must be destroyed, otherwise, pepper weevil can survive the host free period in a neighboring field and reinfest fields next season. Because there is no observed diapause in the pepper weevil, removal of alternative hosts is imperative to reduce the number of adults surviving from one planting to another. During the cropping season it is also important to pick up fallen fruit immediately to prevent newly emerging pepper weevil from re-infesting the field (Elmore et al., 1934; Watson, 1935; Goff & Wilson, 1937). Transplants have also been implicated as sources of pepper weevil infestations (Elmore et al., 1934). Genung & Ozaki (1972) blamed poor handling of foreign pepper seedling imports as a contributing factor to the spread of pepper weevils to new areas. It was suggested early on that pepper plants should be purchased from pepper weevil-free areas or, if this is not possible, plants should be carefully inspected for pepper weevil infestation. Also, the search for resistant pepper strains has been attempted with limited success (Berdegue et al., 1994; Seal & Bondari, 1999, Quinones & Favela, 2002).

Insecticidal control has a limited impact on pepper weevil damage. Immature stages develop within the flower bud or fruit and are protected from the insecticides. Insecticides are used to deal with adult infestations. Often, however, once adult pepper weevil infestation is obvious, it is too late to save the crop. A great deal of study has been focused on sampling methods and the identification of action thresholds for application of insecticides. The clumped pattern of pepper weevil infestation makes sampling fields a difficult prospect. Pepper weevils move into the field from surrounding nightshades so density is highest on the outer margins of the fields. Focusing sampling efforts along the borders of the fields will allow for earlier detection of an infestation. There are five major methods used for detection of pepper weevil damage including terminal bud or bud cluster inspection, yellow sticky traps, whole plant
inspections, scouting terminal bud feeding damage or egg laying, and a combination of yellow sticky traps baited with male pepper weevils or aggregation pheromone extracts (Riley & Sparks, 1993).

Traps can be used as a cheap and effective way to both monitor and remove adult pepper weevils from pepper fields, provided they are sufficiently attractive. The most efficient trapping method is yellow sticky traps baited with male-produced aggregation pheromone. This combination attracts both male and female adults to the traps (Eller et al., 1994; Riley & Schuster, 1994; Bottenberg & Lingren, 1998). The yellow sticky traps with a surface area of 300 cm² held 10 to 60 cm above the ground captured more pepper weevil than white, beige, ivory, blue, grey, maroon, black, red and brown (Riley & Schuster, 1994). One 375 cm² sticky trap captured as many adults as were detected by inspecting 50 terminal pepper buds. Eller et al. (1994) found that yellow sticky traps baited with synthetic male pheromones captured more pepper weevils of both sexes than unbaited control traps and pheromone-baited boll weevil traps. Pheromone-baited traps capture a greater percentage of females than males, accounting for 50-100% of weevils in the traps across all locations tested. Newer formulations of the pheromone (TRE8420 + 8462) showed superior longevity and sensitivity when compared to unbaited traps, the standard lure, and TRE 8420 + 8461. The new lure lasted up to 5 weeks in the field providing an economic means of pepper weevil monitoring (Bottenberg & Lingren, 1998).

The assembled knowledge of weevil aggregation pheromones, attractive host plant volatiles, and oviposition deterrents may be combined into a push-pull management strategy. The ‘push’ stimuli may include visual cues, synthetic repellents, non-host volatiles, host-derived semiochemicals, epideictic/alarm pheromones, antifeedants, or oviposition deterrents (Cook et al., 2007). ‘Pull’ stimulants may be a combination of visual stimuli, host volatiles,
sex/aggregation pheromones, gustatory or oviposition stimulants. Information on pepper weevil aggregation pheromone and trap preferences already exists. Investigation into attractive host plant volatiles and an effective ‘push’ stimulus are still required if this type of management system is to be developed for the pepper weevil.

**Biological Control and Artificial Rearing Methods**

Early biological control studies on pepper weevil were based on notes on boll weevil parasitoids that were also found to parasitize pepper weevil. Known natural enemies of the pepper weevil include several insect predators and parasitoids (Pratt, 1907; Cross & Chestnut, 1971; Genung & Ozaki, 1972; Wilson, 1986; Mariscal et al., 1998; Toapanta, 2001; Cortez et al., 2005). Two natural enemies in particular, the parasitic wasps *Catolaccus hunteri* Crawford and *Triaspis eugenii* Wharton and Lopez-Martinez have been studied as potential biological control agents of pepper weevil.

*Catolaccus hunteri* was first identified as a parasitoid of pepper weevil in studies focusing on boll weevil (Cross & Chestnut, 1971; Cross, 1973). It was the most abundant parasitoid found in association with pepper weevil in Florida (Genung & Ozaki, 1972; Wilson, 1986) and the only one found in Tabasco, Mexico. Rodriguez-Leyva et al. (2000) and Seal et al. (2002) described biological and developmental information on *C. hunteri*. *Catolaccus hunteri* was released in organic bell pepper fields and adjacent wild nightshade stands in Florida (Schuster, 2007). The author suggests that while the cost of *C. hunterii* releases may be prohibitive for conventional growers, organic farms may benefit from this biological control agent due to the higher market value of organic pepper.

The major disadvantage of *C. hunteri* as a biological control agent is that it is a larval parasitoid. The best biological control agent not only achieves the highest level of parasitism but also attacks its host early in its development. A study by Mariscal et al. (1998) in Nayarit,
Mexico identified nine species of pepper weevil parasitoids. The braconid *Triaspis eugenii* was the most abundant and caused up to 55% of the observed parasitism. Over a two-year period, Toapanta (2001) monitored pepper weevil parasitism in Nayarit, Mexico. Parasitism due to *T. eugenii* ranged from 2-41% with a sex ratio averaging 1: 1.4 (male: female). Out of the 1,210 parasitoids collected, 88.4% of all hymenopterous parasitoids obtained from pepper fruit were *T. eugenii*. This wasp is an endoparasitic koinobiont egg-larval parasitoid (Wharton, 1993). As an egg parasitoid it can attack the pepper weevil before the larvae migrate into the ovary, where *C. hunteri* cannot reach them. Finally, the entomophagous fungus *Beauveria bassiana* was also studied and its effectiveness as a biological control agent of *A. eugenii* evaluated (Carballo et al., 2001). The fungus did increase pepper weevil mortality, but its effectiveness as a biological control agent has yet to be verified.

To date, no research has been done that specifically addresses predation on pepper weevil. Although the egg and larval stages of the weevil are protected within the pepper fruit, the adult is susceptible to predation. In Tabasco, Mexico, ants were the main mortality factor for the boll weevil, which has a similar life history to the pepper weevil (Stansly, 1985). Pepper weevil adults may gain a defensive advantage over generalist predators due to the presence of the toxic capsaicin in the host plants and presumably in the weevil gut. Other weevil species may derive some measure of chemical defense through the consumption and sequestration of host plant allelochemicals including *Callosobruchus maculatus* Fabricius (Uchoa et al., 2006) and *Oxyops vitiosa* Pascoe (Wheeler et al., 2003).

Development of mass-rearing programs for the parasitoids is the next logical step in pepper weevil biological control. A laboratory method for rearing *C. hunteri* on a factitious host, the cowpea weevil (*Callosobruchus maculatus*), was developed as a cost effective alternative to
rearing pepper weevil (Vasquez et al., 2005). *Triaspis eugenii* has been reared on pepper weevil using immature ‘Jalapeño’ pepper fruit (Rodríguez-Leyva, 2006). Rearing parasitoids on pepper-reared weevils is an expensive and time consuming process and is the major current limitation in developing inundative biological control for the pepper weevil.

Some work has been done to develop an effective artificial diet for pepper weevil larval development and female oviposition, with poor results. Toba et al. (1969) used a modified cabbage looper diet and reported 1.4% egg mortality and 69% adult emergence. The commercial formulation pepper weevil diet sold by BioServ (Frenchtown, NJ) caused a low hatch rate of 0 to 14% and 96% of weevils died between the egg and first instar, indicating the diet was ovicidal (Toapanta, 2001). Preservatives in artificial diets have been shown to cause high egg mortality in cabbage looper (Kishaba et al., 1968). The modified cabbage looper diet used by Toba et al. (1969) contained 3/4 and 1/4 less of the preservatives sorbic acid and methyl $p$-hydroxybenzoate, respectively, compared to the unmodified cabbage looper diet. However, the Bio-Serv diet seems to have the same concentrations of both preservatives as Toba’s modified diet so some other factor, such as rough handling of eggs, may be responsible for the observations of Toapanta (2001). Improving the artificial diet by adding feeding stimulants may improve the rearing of pepper weevil and consequently, *T. eugenii*. Adding ground plant material increased feeding and oviposition response in other weevils (Trudel et al., 1994; Blossey et al., 2000). Including ground pepper, alternative protein sources or reducing the amount of preservatives may improve the acceptability of the pepper weevil artificial diet.

In addition to developing an acceptable artificial diet, a method for stimulating oviposition into an artificial substrate by the weevil is required for mass rearing to succeed. An effective method of achieving oviposition by *Catolaccus grandis* (Burks) used Parafilm bubbles
containing one third instar boll weevil larva (Cate, 1987). The boll weevil parasitoid oviposited through the Parafilm into the larva. It is believed this method of achieving parasitoid oviposition may work for other species that parasitize insects concealed within fruiting structures. Calderon-Limon et al. (2002) used a similar method to achieve pepper weevil oviposition in an artificial ‘fruit’ sachet. One-cm-diameter polyurethane spheres were covered with fresh pepper leaves to create an artificial fruit. The total number of eggs hatched was 67%. Identifying successful oviposition stimulants and techniques, in conjunction with an effective artificial diet may provide a cost-effective method for mass rearing *T. eugenii* with limited reliance on living plant material.

A thorough understanding of pepper weevil behavior and chemical ecology can help us understand the development of pepper weevil infestations in the field, and facilitate our designs of new laboratory rearing technology and integrated pest management programs. With those goals in mind, this dissertation will address the following five objectives:

1. To improve pepper weevil artificial diet for larval development and oviposition.
2. To characterize physical and plant oviposition stimulant(s).
3. To demonstrate discrimination of infested hosts plants and fruit by gravid females.
4. To isolate and identify the oviposition deterrent produced by female pepper weevils.
5. To quantify attractiveness of constitutive and induced host plant volatiles.
Table 1-1. Pepper weevil host plants acceptable for feeding, oviposition and development as reported in the literature

<table>
<thead>
<tr>
<th>Host</th>
<th>Feeding</th>
<th>Oviposition</th>
<th>Development</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. americanum</em> Mill</td>
<td>X</td>
<td>X</td>
<td>Field, Lab</td>
<td>6</td>
</tr>
<tr>
<td><em>S. eleagnifolium</em> Cav.</td>
<td>X</td>
<td>X</td>
<td>Field, Lab</td>
<td>7</td>
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<tr>
<td><em>S. pseudogracile</em> Heiser</td>
<td>X</td>
<td>X</td>
<td>Lab</td>
<td>6</td>
</tr>
<tr>
<td><em>S. ptycanthum</em> Dun</td>
<td>X</td>
<td>X</td>
<td>Lab</td>
<td>6</td>
</tr>
<tr>
<td><em>S. triquetrum</em> Cav.</td>
<td>X</td>
<td>X</td>
<td>Lab</td>
<td>4</td>
</tr>
<tr>
<td><em>S. pseudocapsicum</em> L.</td>
<td>X</td>
<td>X</td>
<td>Lab</td>
<td>6</td>
</tr>
<tr>
<td><em>S. melongena</em> L.</td>
<td>X</td>
<td>X</td>
<td>Lab</td>
<td>3,6</td>
</tr>
<tr>
<td><em>S. melongena</em> L.</td>
<td></td>
<td></td>
<td>Field, Lab</td>
<td>4</td>
</tr>
<tr>
<td><em>S. carolinense</em> L</td>
<td>X</td>
<td>X</td>
<td>Lab</td>
<td>6</td>
</tr>
<tr>
<td><em>S. dimidiatum</em> Fav.</td>
<td>X</td>
<td>X</td>
<td>Lab</td>
<td>6</td>
</tr>
<tr>
<td><em>S. rostratum</em> Dunal</td>
<td>X</td>
<td>X</td>
<td>Lab</td>
<td>6</td>
</tr>
<tr>
<td><em>S. hindsianum</em> Benth</td>
<td>X</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td><em>S. xanti</em> Grey</td>
<td>X</td>
<td></td>
<td></td>
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</tr>
<tr>
<td><em>S. umbelliferum</em> Esch.</td>
<td>X</td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td><em>S. villosum</em> Mill.</td>
<td>X</td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td><em>S. glaucum</em> Dunal</td>
<td>X</td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td><em>S. aviculare</em> Forst</td>
<td>X</td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td><em>S. americanum</em> var. nodiflorum</td>
<td>X</td>
<td></td>
<td>Field, Lab</td>
<td>5</td>
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<tr>
<td><em>S. tuberosum</em> L. (potato)</td>
<td>X</td>
<td></td>
<td>Field, Lab</td>
<td>2,6</td>
</tr>
<tr>
<td><em>C. annuum</em> L.</td>
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<td>X</td>
<td>Field, Lab</td>
<td>2,6</td>
</tr>
<tr>
<td><em>C. frutescens</em> L.</td>
<td>X</td>
<td>X</td>
<td>Field, Lab</td>
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<tr>
<td><em>C. pubescens</em> R. and P.</td>
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<td>X</td>
<td>Field, Lab</td>
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<tr>
<td><em>C. baccatum</em> L.</td>
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<td>X</td>
<td>Field, Lab</td>
<td>2,6</td>
</tr>
<tr>
<td>Physalis sp.</td>
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<td></td>
<td>6</td>
</tr>
<tr>
<td>Lycopersicon sp.</td>
<td>X</td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>Datura sp.</td>
<td>X</td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>Petunia sp.</td>
<td>X</td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>Nicotiana sp.</td>
<td>X</td>
<td></td>
<td></td>
<td>6</td>
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</tbody>
</table>

CHAPTER 2
MODIFICATION OF A PEPPER WEEVIL ARTIFICIAL DIET FOR LARVAL DEVELOPMENT AND OVIPOSITION

Introduction

The pepper weevil, *Anthonomus eugenii* Cano (Coleoptera: Curculionidae), is a pest of cultivated pepper (*Capsicum* spp.) throughout the southern United States, Central America and the Caribbean. The weevil also reproduces on a number of wild nightshades including American black nightshade (*Solanum americanum* Mill.) and silverleaf nightshade (*S. elaeagnifolium* Cav.), and has recently been found in eggplant fields (*S. melongena* L.) (Tejada & Reyes, 1986; Wilson, 1986; Diaz et al., 2004). Females oviposit preferentially in young fruit but will also utilize mature fruit or flower buds for oviposition, with larvae feeding on the seed and placental material within the fruit body (Elmore et al., 1934). The resulting infestation in pepper causes fruit drop and losses in yield. In order to conduct research on the weevil and its potential biological control agents, it is necessary to maintain a colony in the laboratory. The growth and maintenance of pepper plants for the harvesting of young fruit is a limiting factor in colony rearing. The development of an artificial or semi-artificial rearing system could allow the weevil to be reared without a dependence on fresh pepper fruit or the need for large amounts of field or greenhouse space.

A limited amount of attention has been paid to the development of an artificial diet for pepper weevil larvae. Toba et al. (1969) used a modified cabbage looper diet to rear pepper weevil. The authors reported 1.4% egg mortality and 69% adult emergence from the diet. The commercial formulation pepper weevil diet sold by BioServ (Frenchtown, NJ) caused a low hatch rate of 0 to 14% and approximately 96% of weevils died between the egg and first instar indicating the diet was ovicidal (Toapanta, 2001). Preservatives in artificial diets have been
shown to cause high egg mortality in cabbage looper (Kishaba et al., 1968). The modified cabbage looper diet used by Toba et al. (1969) contained 3/4 and 1/4 less of the preservatives sorbic acid and methyl \( p \)-hydroxybenzoate, respectively, compared to the unmodified cabbage looper diet. However, the Bio-Serv diet seems to have the same amount of both preservatives as Toba’s modified diet, so some other factor, such as rough handling of eggs, may be responsible for the observations of Toapanta (2001). It may be possible to improve hatch and survival by manipulating the amount of preservatives in the current diet formula.

Another simple way to improve the artificial diet is by adding feeding stimulants. Adding ground plant material increased feeding and oviposition response in other weevils (Trudel et al., 1994; Blossey et al., 2000). The standard boll weevil diets contain cottonseed meal as a ‘natural’ nutritional additive and feeding stimulant (Sterling et al., 1965). Including ground pepper, alternative protein and lipid sources and/or reducing the amount of preservatives may improve the acceptability of the pepper weevil artificial diet. The boll weevil artificial diet has also been shown to successfully elicit oviposition when presented in a cylindrical shape with rounded surfaces (Vanderzant and Davich, 1961). This method of diet presentation may also elicit oviposition in pepper weevil.

The purpose of the following assays is to identify artificial oviposition and diet substrates. In the first set of experiments, a series of agar-based artificial substrates were assayed to see if they would elicit oviposition by pepper weevils. In the second set of experiments, an artificial diet was modified by the addition of freeze-dried ground ‘Jalapeño’ pepper, the addition of lipid, the removal of methyl paraben and the substitution of cottonseed or pepperseed meal for the casein protein source.
Materials and Methods

Insects

Pepper weevils were collected in south Florida near the city of Clewiston in the spring of 2004, and a laboratory colony was established at the University of Florida, Gainesville. Additional field collections were made in Immokalee, Bradenton and Wimauma in the fall of 2005 and 2006 to maintain colony health. Insects were maintained in the laboratory (14:10 light:dark, approx. 27ºC and 30% r.h.) on excised greenhouse-grown ‘Jalapeño’ peppers (Capsicum annuum L.) with water and honey supplements. Gravid females were removed from the colony 10 d after emergence and transferred into oviposition containers made from 250 mL, 8.5 cm diameter waxed cardboard cans with screened lids (The Fonda Group, Inc., Union, NJ).

Oviposition

Artificial diet bubble assays. A commercially available pepper weevil diet from BioServ (F#9709B, Frenchtown, NJ) was prepared according to the packaged instructions. To prepare 1 liter of diet, agar (23.7 g) was added to 830 ml of cold water and brought to a full boil for 1 min. The agar and water were then poured into a Waring blender (New Hartford, CT) and 141.0 g of the dry mix was added followed by KOH (4.8 g) and formaldehyde (0.4 g). Diet was poured onto a sheet of bubbles formed by placing a length of Parafilm® (Pechiney Plastic Packaging Company, Chicago, IL) on a diet press (Fig. 2-1). The diet press was made from two components; a 1 cm thick plastic mould and a 1 cm thick aluminum plate. The plastic mould contained one hundred 8mm diameter cylindrical holes (spaced 1.25 cm center to center) in a 10 × 10 grid. The complimentary aluminum plate was fitted with one hundred 1-cm protruding lengths of cylindrical bar stock with hemispherical ends in the same 10 × 10 configuration. A stream of air was used to ‘blow out’ the Parafilm to form bubbles of approximately 0.25 ml volume. The diet was allowed to cool and was sealed with a second piece of Parafilm by placing
a moist cloth over the second sheet of Parafilm and passing a hot iron over the surface to melt the
two sheets together. All assays were conducted in Florida Reach-In chambers (Walker et al.,
1993) held at 27°C and approx 40% r.h.

In the first experiment, two diet bubble treatments were compared. In the first treatment,
plain diet was used. In the second treatment, 8 drops of green food dye (McCormick &
Company, Inc., Sparks, MD) was added to 250 ml of diet to see if a green color would improve
feeding response and oviposition. Ten to twenty females (> 10 d old) were placed in a 1.5-l
Tupperware ® box (Orlando, FL) with one sheet of 70 diet-encapsulating bubbles. The numbers
of feeding punctures and eggs deposited per female were recorded for the treatments after 24 h.
Twelve replications were performed.

In the second experiment, 20 female weevils were held with a sheet of 70 diet bubbles for
3, 5 or 7 h. The number of feeding punctures and eggs deposited per female was recorded for the
three time treatments. Four replications were performed.

In the third experiment, two treatments were compared. The first treatment contained a
sheet of diet bubbles and an empty 7-dram plastic vial with a screen lid. The second treatment
contained the diet bubbles as well as a plastic vial containing a young ‘Jalapeño’ pepper fruit to
provide a source of potentially stimulatory plant volatiles. The plastic vials were covered with
aluminum foil so the contents of the vial could not be seen. Ten to twenty females were placed
in a box and the number of feeding punctures and eggs deposited per female was recorded for the
treatments after 24 h. Four replications were performed.

**Cylindrical media assays.** Since the diet bubbles were unsuccessful at stimulating
oviposition, an alternative method of artificial egg collection was tested. Agar and artificial diet
media were formed into cylindrical pellets similar to those presented to the boll weevil
The following assays were performed using 8 different oviposition media (1) agar with Alphacel ® non-nutritive bulk (MP Biomedicals, Irvine, CA), (2) agar with Alphacel substituted with 5%, (3) 10%, or (4) 25% ground ‘Jalapeño’ pepper, (5) Bioserv diet, (6) Bioserv diet with dry mix substituted with 5% (7) 10% and (8) 25% ground ‘Jalapeño’ pepper. The media were made by bringing 250 ml of DI water and 5.93 g of agar to a boil and adding 1.2 g of KOH and 35.25 g of ‘dry mix’. The ‘dry mix’ consisted of either Alphacel for the agar cylinders or Bioserv diet mix for the diet cylinders. For the 5%, 10% or 25% pepper treatments, that percentage of alphacel or diet dry mix was substituted with ground ‘Jalapeño’ pepper. The media was dispensed into 48-well disposable Falcon ELISA plates (Sigma-Aldrich, St. Louis MO) and were stored at 4ºC. Cylinders (2 cm long, 1 cm diameter) were removed from the ELISA plates and divided into three treatments. Twenty cylinders remained as controls, twenty were wrapped in Parafilm and twenty were dipped into melted paraffin wax (Royal Oak Sales, Inc., Clover, SC) with sterilized forceps. Twenty females were presented individually with one media cylinder with no covering, covered in Parafilm, or dipped in paraffin wax in a wax cardboard oviposition container described above. Insects were held in an incubator at approximately 27ºC, 60% r.h. and egg counts were made after 24 h.

**Artificial Diets for Larval Development**

**Egg collection and larval handling.** Eggs were collected using artificial ‘fruit’ sachets made from several rolled up pepper leaves wrapped in Parafilm ® (Calderon-Limon et al., 2002). Sachets were placed in a cage with 50-100 gravid females overnight. Eggs were collected from the unrolled pepper leaves using a fine-tipped paintbrush (2/0) dipped in a 1% bleach solution. Eggs were placed in a Petri dish on Kleenex Brand Premiere ® paper towel (Kimberly-Clark Global Sales, Inc., Neenah, WI) moistened with 1% bleach solution. Eggs were incubated in a
rearing room until hatch approximately 2-3 d later (27°C, 30% r. h.). Neonates were transferred to diet with a fine-tipped paint brush sterilized by dipping in 1% bleach solution.

**Diet preparation and assay.** Two diet formulas were used in the following experiments: the commercially available pepper weevil diet described above and a modified version of the pepper weevil diet developed by Toba et al. (1969). The Toba diet was prepared by bringing agar and water to a boil for 1 min (Table 2-1). The agar mixture was then blended with the dry mix, followed by KOH, choline chloride, formaldehyde and vitamin mix. Sorbic acid and methyl paraben were added individually. When the temperature of the mixture dropped below 70°C, ascorbic acid and aureomycin were added.

Diets were dispensed immediately into 6.0 × 1.5 cm Fisherbrand Petri dishes (Fisher Scientific, St. Louis, MO) for the hatching assays or 48-well disposable Falcon ELISA plates for the rearing assays and were stored at 4°C until use. During the diet assays, one neonate pepper weevil (less than 6 h old) was placed in each well (or the 20 interior wells) of each ELISA plate. Five to 10 replicate plates were set up for each diet experiment. Upon emergence adult weevils were sexed then killed by freezing in wax paper envelopes. The dead weevils were placed in an oven to dry (45°C). Hatching success on the Petri dishes and development time from hatch to eclosion, sex ratio, adult dry mass and survival were recorded from the ELISA plates for each experiment, unless stated otherwise.

**5% Pepper-augmented BioServ diets.** Three diet treatments were compared using a commercially available BioServ pepper weevil diet. The treatments tested were: (1) BioServ diet, (2) BioServ diet with 5% of the dry mix substituted with an equal amount of freeze-dried Jalapeño pepper powder (Bulkfoods.com), and (3) BioServ diet with 5% of the dry mix substituted with an equal amount of non-nutritive filler (Alphacel®, MP Biomedicals, Irvine,
CA). Five replicate plates were set up for each diet treatment. Hatching analysis was not conducted for this experiment.

**Pepper-augmented Toba diets.** Seven treatments were compared using a modified version of the Toba diet (Table 2-1) and a Jalapeño fruit as a control. The Toba diet treatments included the Toba control and Toba diets modified with 5%, 10%, 20%, 50% or 100% of the dry mix substituted with freeze-dried Jalapeño pepper powder. For the hatching experiment, 10 eggs per replicate were placed directly onto the 6 diets in 6.0 × 1.5 cm Petri dishes and on Kleenex Brand Premiere ® paper towel moistened with 1% bleach solution for the ‘paper’ treatment. Eggs laid by weevils within ‘Jalapeño’ fruit were observed within the fruit by removing the oviposition plug. Fruit were obtained from colony oviposition cups and contained 4 or 5 eggs as is normal for colony rearing. Eggs were incubated in a rearing room until hatch approximately 2-3 d later (27ºC, 30% r. h.).

For the larval development experiment, neonates were transferred onto the diets using a fine tipped brush and placed in an incubator (27ºC, 30% r. h.). Hatching analysis, development time from hatch to eclosion, sex ratio, adult mass and survival were recorded. Ten replicate plates were set up for each hatching and diet treatment.

**Methyl paraben and lipid modified diets.** Eight diet treatment combinations were evaluated using the Toba diet as the base: (1) Toba control diet, (2) Toba diet with methyl paraben removed, (3) Toba diet with Wesson corn oil added (10 ml/l diet), (4) Toba diet with methyl paraben removed and Wesson corn oil added, (5) Toba diet with the replacement of 20% of the dry mix with ground Jalapeño pepper powder (= 20% pepper diet), (6) 20% pepper diet with methyl paraben removed, (7) 20% pepper diet with Wesson corn oil added, and (8) 20% pepper diet with methyl paraben removed and Wesson corn oil added. For the hatching
experiment, 10 eggs per replicate were placed directly onto the 8 diets in 6.0 × 1.5 cm Petri dishes. Eggs were incubated in a rearing room until hatch approximately 2-3 d later (27°C, 30% r. h.).

For the larval development experiment, neonates were transferred onto the diets using a fine tipped brush and placed in an incubator (27°C, 30% r. h.). Hatching analysis, development time from hatch to eclosion, sex ratio, adult mass and survival were recorded. Ten replicate plates were set up for each diet treatment.

**Protein alternative diets.** Six different preparations of the Toba diet were compared as follows: 1) control diet, (2) cottonseed meal (Traders Protein, Memphis, TN) substituted for casein, (3) ground ‘Jalapeño’ pepper seeds (Dorsing Seeds, Inc., Parma, ID) substituted for casein, (4) 20% pepper diet (5) 20% pepper diet with cottonseed meal substituted for casein, and (6) 20% pepper diet with ground pepper seeds substituted for casein. Pepper seed was dried in a drying oven and ground in a Wiley Mill, (size 20 mesh; Thomas Scientific, Swedesboro, NJ).

For the hatching experiment, 10 eggs per replicate were placed directly onto the 5 diets in 6.0 × 1.5 cm Petri dishes. Eggs were incubated in a rearing room until hatch approximately 2-3 d later (27°C, 30% r. h.).

For the larval development experiment, neonates were transferred onto the diets using a fine tipped brush and placed in an incubator (27°C, 30% r. h.). Hatching analysis, development time from hatch to eclosion, sex ratio, adult mass and survival were recorded. Ten replicate plates were set up for each diet treatment.

**Data Analysis**

Data were analyzed using ANOVA (PROC GLM). Non-normal data were log or square root transformed. If transformation did not achieve normality, data were analyzed using ANOVA under a Poisson distribution (PROC GLIMMIX) and means were compared using
LSD. Male and female adult dry mass was pooled if there was no significant interaction of sex and diet. Regression analysis was performed with mass and development time for each diet (PROC REG).

**Results**

**Oviposition**

**Artificial diet bubble assays.** In the first experiment, slightly more feeding punctures were made in the green diet bubbles as compared to the control ($F_{1,20} = 4.44, P = 0.0473$) but no difference was observed in the number of eggs laid (control = 3, green = 5; $F_{1,20} = 0.24, P = 0.6293$).

There was no difference in the number of feeding punctures (mean ± SE) ($F_{2,9} = 0.00, P = 0.9996$; 3 h = 0.11 ± 0.05, 5 h = 0.21 ± 0.05, 7h = 0.24 ± 0.07) or eggs laid between the three time intervals ($F_{2,9} = 1.36, P = 0.3042$; 3 h = 0, 5 h = 0, 7 h = 0.03 ± 0.01). Only one egg was laid in the four replicates so the experiment was discontinued.

There was no difference in the number of feeding punctures ($F_{1,6} = 2.01, P = 0.2059$) or eggs laid ($F_{1,6} = 0.00, P = 0.9837$) in the control and volatile treatments. Only one egg was laid in the four replicates so the experiment was discontinued.

**Cylinder media assays.** Females laid no eggs in any of the media whether covered with Parafilm, paraffin wax or left uncovered. The media lacked sufficient stimuli to induce oviposition.

**Artificial Diets for Larval Development**

**5% Pepper-augmented BioServ diets.** Overall adult mass was different by diet treatment ($F_{2,222} = 3.58; P = 0.0295$; Table 2-2) and sex ($F_{1,222} = 12.74, P = 0.0004$; female mass = 1.54 ± 0.02, male mass = 1.67 ± 0.03) but there was no interaction of diet and sex ($F_{2,222} = 0.07, P = 0.9330$). Weevil mass was greater in pepper-augmented diet than alphacel-augmented diet in
pair-wise comparisons. There was no difference in days to 50% emergence ($F_{2, 12} = 0.72$, $P = 0.5087$), sex ratio ($F_{2, 12} = 0.30; P = 0.7431$) or survival ($F_{2, 12} = 1.65; P = 0.2325$) across the three diet treatments. There was no relationship between mass and emergence time in the three BioServ diet treatments (all P-values above 0.05).

**Pepper-augmented Toba diets.** Total egg hatch differed significantly among treatments ($F_{7, 72} = 19.74, p < 0.0001$; Figure 2-2), as did hatch at 2 d ($F_{7, 72} = 12.87, p < 0.0001$). There was no difference in hatch observed at 3 d ($F_{7, 72} = 0.57, P = 0.7803$). Percent hatch was highest in pepper fruit and on moist paper towel (98.2 and 93.0% respectively). In the artificial diet treatments, egg hatch was highest in the 20% pepper treatment (69.5%).

When mass was analyzed there was no significant interaction between sex and diet ($F_{5, 417} = 0.58, P = 0.7141$) but there were significant differences in diet ($F_{5, 417} = 2.77, P = 0.0180$) and sex ($F_{1, 417} = 10.00, P = 0.0017$). Males were heavier than females (female mass = 1.31 ± 0.02, male mass = 1.40 ± 0.02). Male and female mass was pooled for pair-wise comparisons across diets. Weevils reared on control, 5%, 10% and 20% pepper-augmented artificial diet were heavier than weevils reared on pepper fruit in pair-wise LSD tests. There was no difference in the number of days to 50% emergence among diet treatments ($F_{5, 54} = 1.08, P = 0.3796$; Table 2-3). Sex ratio ($F_{5, 54} = 0.88, P = 0.5016$) and survival were the same among the treatments ($F_{5, 54} = 0.60, P = 0.70$).

There was no relationship between mass and emergence time for the control, 10%, 50%, and fruit reared weevils (all P-values above 0.05). Mass and emergence time showed a positive relationship in the 5% ($F_{1, 63} = 10.74, P = 0.0017$) and 20% ($F_{1, 85} = 6.13, P = 0.0152$) diets. Despite the significance of these two regressions, the R-squared values were low for both the 5%
(Rsq = 0.1457) and 20% (Rsq = 0.0673) diets meaning only 15 and 7% of the variation in mass could be explained by emergence time.

**Methyl paraben and lipid modified diets.** The total number of eggs hatching on each diet treatment did not differ (F7, 56 = 1.14; P = 0.3523; Figure 2-3). Egg hatch was similar for all treatments on day two (F7, 48 = 1.38, P = 0.2351) but differed on day three (F7, 48 = 4.88; P = 0.0003).

Adult dry mass was not different between diet treatments (F7, 327 = 0.74, P = 0.6403) and there was no interaction between diet and sex (F7, 327 = 0.67, P = 0.7012). Males were heavier than females (female mass = 1.35 ± 0.02, male mass = 1.43 ± 0.02; F1, 327 = 7.62, P = 0.0061). There was no difference in days to 50% emergence (F7, 50 = 0.87, P = 0.5370) across treatments (Table 2-4). Sex ratio was not different across diet treatments (F7, 42 = 0.94, P = 0.4886). Survival was different across treatments (F6, 49 = 6.24, p < 0.0001) and low overall, ranging from 6.9% (pepper treatment without methyl-paraben) to a high of 36.9% (control diet). In this experiment only control diet showed a predictive relationship between mass and development time (F1, 53 = 5.35, P = 0.0247) with development time being responsible for approximately 9% of the variation in mass (Rsq = 0.0916).

**Protein alternative diets.** There was a difference in total (F5, 54 = 4.13, P = 0.0030; Figure) hatch rate and also in hatch rate at 2 (F5, 54 = 2.58, P = 0.0364) and 3 d (F5, 54 = 9.20, p < 0.0001) (Figure 2-4). No larvae survived in the control and pepper diets augmented with ground pepper seed (Table 2-5). Survival was extremely low for the remaining diets. Control and pepper diets were not different but survival was low compared to other experiments. Diets containing cottonseed and pepperseed had low or no survival (2% and 0%, respectively). Weevil mass was different across diet treatments (F3, 113 = 10.67, P < 0.0001) and by sex (female mass =
1.27 ± 0.04, male mass = 1.46 ± 0.04; F_{1,113} = 14.13, P = 0.0003) but there was no interaction of
sex and diet treatment (F_{3,113} = 0.51, P = 0.6741).

There was no difference in days to 50% emergence (F_{1,18} = 0.03, P = 0.8655) across
treatments (Table 2-5), however, only control and pepper diets had sufficient survival for data
analysis. The overall difference in mass was significant across diet treatments (F_{3,117} = 9.70, p <
0.0001). Survival was different (F_{5,54} = 47.39, P < 0.0001) across treatments with extremely low
levels of survival in the pepper and cottonseed augmented diets compared to controls. Sex ratio
was not different (F_{1,15} = 0.12, P = 0.7384) but only control and pepper diets could be analyzed.
Regression analysis was performed on the control and 20% pepper diets. There was no
relationship between mass and development time in either treatment (P-values above 0.05).

Discussion

The artificial diet and agar-based media tested here were not successful in stimulating
pepper weevil oviposition. Instead, the ‘leaf sachet’ method developed by Calderon-Limon et al.
(2002) was used to collect eggs for further diet studies when it became clear that diet bubbles
and cylinders were not acceptable.

The augmentation of the standard pepper weevil diet shows somewhat conflicting results
when compared to previous diet experiments. Egg hatch, in particular, was substantially higher
than observed by Toapanta (0-14%) but nowhere near as good as that observed by Toba et al.
(98.6%) (Toba et al., 1969; Toapanta, 2001). This is curious, because the control diet used in our
hatch assays was the same formula developed by Toba et al. (1969). Our best hatch rates were
between 65-70% and were found in the control and 20% pepper diets. Mean percent hatch in the
control diet varied in the three separate diet assays, ranging from 52.8-65.0 %. I was able to
obtain a 93% hatch rate when eggs were incubated on moist paper towel. If eggs can be
permitted to incubate or hatch on paper before being transferred to diet, this will increase the
overall productivity of the artificial rearing system by lowering egg mortality. It should also be noted that larvae hatching on paper cannibalized nearby eggs and larvae if they were not transferred to diet immediately after hatch. Removing methyl paraben from the Toba diet did not have a significant impact on egg hatch, making the antimicrobial an unlikely cause of egg mortality. One likely factor impacting egg and neonate mortality is water content. The Toba diet was very moist and it is possible that drowning was a mortality factor for early stages. Increasing agar or decreasing water content may improve survival on the diet.

Male and female weevils reared on control Toba diet and those with the addition of ground ‘Jalapeño’ pepper weighed more than weevils reared in Jalapeño fruit. This may indicate that the diets (including the control) are nutritionally superior to the fruit. Conversely, the higher weight might be due to nutritional inadequacies causing the weevils to compensate for missing nutrients by consuming more. There are a few instances of a positive relationship between development time and mass and in one of the experiments the control diet showed heavier weevils emerged later but this was not consistent across treatments. Males were consistently heavier than females, significantly so on several diets (control, 5% alphacel, 5% and 20% pepper). Male and female mass converged when reared on poor diets. The remaining diet modifications failed to improve the control diet.

It appears that a simple method for improving oviposition and developmental parameters of the pepper weevil artificial diet requires further investigation. The addition of pepper material did not improve survival or oviposition; in contrast incorporation of plant material improved these performance measures for the white pine weevil (*Pissodes strobe* (Peck)) (Trudel et al., 1994) and the purple loosestrife control agent *Hylobius transversovittatus* Goeze (Blossey et al., 2000). Survival of the white pine weevil increased from 18% in a 1% white pine diet to 62% in
a 5% diet. Survival of *H. transversovittatus* increased from 0 to approximately 60% when the control diet was augmented with 20% purple loosestrife root powder. Pepper weevil survival hovered at around 50% in all diets from 0-50% pepper powder.

In addition to improving survival, a 5% white pine bark diet was sufficient to stimulate feeding in the white pine weevil but a 10% white pine bark diet was required to stimulate oviposition. The authors suggested that a different set of compounds stimulated pine weevil feeding and oviposition and a larger percentage of plant material was required before oviposition stimulants were in a high enough concentration for the weevil to detect. I exposed the pepper weevil to artificial media containing 0 to 25% freeze-dried ‘Jalapeño’ powder without any observed oviposition. If contact with pepper ‘surface compounds’ are required to achieve oviposition, they may not be in a high enough concentration for the weevils to detect.

The major problems encountered with these diets were molding and desiccation. The assays were carried out in ELISA cell culture plates so that larvae could be reared individually. The 48-well ELISA plates held more than enough diet to sustain a single larva and prevented the neonates from cannibalizing one another. Unfortunately, wells at the edge of the plates were subject to desiccation while the central wells would become moldy if condensation built up in the plates. Additionally, some weevils became ‘trapped’ in the diet cylinders upon eclosing. These weevils may have skewed emergence data if they took a day or two to chew their way to the top of the plate.

A different approach must be taken if a more efficient pepper weevil diet and rearing system are to be developed. First, a thorough investigation of pepper oviposition stimulants should be undertaken. Pepper surface compounds can be tested for stimulatory properties. If those compounds do not elicit a response, this can be followed by an investigation of the fruit
wall compounds. Larval feeding stimulants may be isolated from the placental material and seeds where the larvae do the majority of their feeding. The addition of pepper weevil specific feeding and oviposition stimulants at sufficient levels may improve both larval survival and female oviposition.
Table 2-1. Amounts and sources of chemicals used to produce 1 liter of pepper weevil artificial diet, modified from Toba

<table>
<thead>
<tr>
<th>Diet Ingredients</th>
<th>Amount</th>
<th>Source</th>
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<tbody>
<tr>
<td><strong>Dry mix</strong></td>
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<td></td>
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<tr>
<td>Casein</td>
<td>33.2 g</td>
<td>BioServ, Frenchtown, NJ</td>
</tr>
<tr>
<td>Sucrose</td>
<td>33.2 g</td>
<td>BioServ, Frenchtown, NJ</td>
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<td>Wheat germ</td>
<td>28.5 g</td>
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<td>Alfalfa meal</td>
<td>14.2 g</td>
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<td>Wesson salt mix</td>
<td>9.5 g</td>
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<td>Alphacel®</td>
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<td>MP Biomedicals, Irvine, CA</td>
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<tr>
<td>Agar, USP</td>
<td>23.7 g</td>
<td>BioServ, Frenchtown, NJ</td>
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<tr>
<td>Vanderzant vitamin mix</td>
<td>19.2 g</td>
<td>BioServ, Frenchtown, NJ</td>
</tr>
<tr>
<td>Choline chloride (10% aq.)</td>
<td>9.5 ml</td>
<td>BioServ, Frenchtown, NJ</td>
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<td>Formaldehyde, 37% w/w</td>
<td>1.08 ml</td>
<td>Fisher Scientific, St. Louis, MO</td>
</tr>
<tr>
<td>KOH, 4M</td>
<td>4.8 ml</td>
<td>Fisher Scientific, St. Louis, MO</td>
</tr>
<tr>
<td>Methyl paraben</td>
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<td>BioServ, Frenchtown, NJ</td>
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<td>Sorbic acid</td>
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<tr>
<td>Ascorbic acid, 97%</td>
<td>4.0 g</td>
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<tr>
<td>Aureomycin, 14.1%</td>
<td>0.13 g</td>
<td>BioServ, Frenchtown, NJ</td>
</tr>
</tbody>
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These ingredients were added to 825 ml of water.
Figure 2-1. Diet press used to form bubble sheets

Table 2-2. Developmental fitness parameters for pepper weevil neonates reared on BioServ diet substituted with ‘Jalapeño’ pepper powder and alphacel

<table>
<thead>
<tr>
<th>Diet treatment</th>
<th>Adult dry mass</th>
<th>Days to 50% emergence</th>
<th>Survival %</th>
<th>Sex ratio (f:m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.57 ± 0.03 ab</td>
<td>18.2 ± 0.86</td>
<td>24.2 ± 4.8</td>
<td>1.1 ± 0.39</td>
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<tr>
<td>5% alphacel</td>
<td>1.57 ± 0.03 b</td>
<td>17.1 ± 0.40</td>
<td>37.1 ± 7.2</td>
<td>1.0 ± 0.17</td>
</tr>
<tr>
<td>5% pepper</td>
<td>1.66 ± 0.03 a</td>
<td>17.5 ± 0.63</td>
<td>34.6 ± 3.3</td>
<td>1.3 ± 0.19</td>
</tr>
</tbody>
</table>

1Means having the same lowercase letter are not significantly different using LSD test at P = 0.05
Figure 2-2 Percent hatch for pepper weevil eggs laid on the control (con), pepper-augmented Toba diets (5, 10, 20, 50, 100%), paper towel (pa) and those deposited naturally in pepper fruits (fr). Total hatch bars with different letters are different by LSD (P = 0.05)
<table>
<thead>
<tr>
<th>Diet treatment</th>
<th>Adult dry mass</th>
<th>Days to 50% emergence</th>
<th>Survival %</th>
<th>Sex ratio (f:m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fruit</td>
<td>1.26 ± 0.04 c</td>
<td>12.40 ± 0.22</td>
<td>58.4 ± 6.8</td>
<td>1.35 ± 0.55</td>
</tr>
<tr>
<td>Control</td>
<td>1.38 ± 0.03 ab</td>
<td>14.60 ± 0.40</td>
<td>50.6 ± 4.5</td>
<td>1.12 ± 0.24</td>
</tr>
<tr>
<td>5% pepper</td>
<td>1.39 ± 0.04 ab</td>
<td>14.40 ± 0.43</td>
<td>47.3 ± 4.4</td>
<td>1.81 ± 0.46</td>
</tr>
<tr>
<td>10% pepper</td>
<td>1.40 ± 0.03 a</td>
<td>15.50 ± 0.82</td>
<td>49.6 ± 4.4</td>
<td>1.94 ± 0.43</td>
</tr>
<tr>
<td>20% pepper</td>
<td>1.40 ± 0.03 a</td>
<td>14.60 ± 0.22</td>
<td>53.9 ± 4.4</td>
<td>1.19 ± 0.20</td>
</tr>
<tr>
<td>50% pepper</td>
<td>1.30 ± 0.03 bc</td>
<td>16.10 ± 0.43</td>
<td>54.1 ± 5.8</td>
<td>1.05 ± 0.23</td>
</tr>
<tr>
<td>100% pepper</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

1Means having the same lowercase letter are not significantly different using LSD at P = 0.05
Figure 2-3. Percent hatch for pepper weevil eggs on Toba diets with methyl paraben removed and lipid added; control (c), lipid-augmented (cl), methyl paraben removed (cp), lipid-augment + methyl paraben removed (clp), 20% pepper (p), 20% pepper + lipid-augmented (pl), 20% pepper + methyl paraben removed (pp), 20% pepper + lipid-augmented + methyl paraben removed (plp)
Table 2-4 Developmental fitness parameters for pepper weevil neonates reared on Toba diets with methyl paraben removed and the addition of lipid

<table>
<thead>
<tr>
<th>Diet treatment</th>
<th>Adult dry mass</th>
<th>Days to 50% emergence</th>
<th>Survival %</th>
<th>Sex ratio (f:m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.41 ± 0.04</td>
<td>17.6 ± 1.0</td>
<td>36.9 ± 3.9 a</td>
<td>1.9 ± 0.8 a</td>
</tr>
<tr>
<td>Lipid</td>
<td>1.39 ± 0.04</td>
<td>14.0 ± 0.4</td>
<td>24.4 ± 5.0 bc</td>
<td>1.2 ± 0.2 ab</td>
</tr>
<tr>
<td>No methyl paraben</td>
<td>1.34 ± 0.03</td>
<td>14.8 ± 0.5</td>
<td>35.0 ± 3.5 ab</td>
<td>1.4 ± 0.3 ab</td>
</tr>
<tr>
<td>Lipid + no methyl paraben</td>
<td>1.36 ± 0.03</td>
<td>14.6 ± 0.6</td>
<td>36.6 ± 4.4 ab</td>
<td>0.9 ± 0.2 ab</td>
</tr>
<tr>
<td>20% pepper</td>
<td>1.42 ± 0.05</td>
<td>17.4 ± 1.3</td>
<td>23.8 ± 3.9 bc</td>
<td>1.8 ± 0.5 a</td>
</tr>
<tr>
<td>20% pepper + lipid</td>
<td>1.41 ± 0.04</td>
<td>16.1 ± 0.7</td>
<td>33.8 ± 5.3 ab</td>
<td>0.7 ± 0.2 bc</td>
</tr>
<tr>
<td>20% pepper + no methyl paraben</td>
<td>1.45 ± 0.05</td>
<td>17.0 ± 0.8</td>
<td>6.9 ± 3.5 d</td>
<td>0.7 ± (n/a) bc</td>
</tr>
<tr>
<td>20% pepper + lipid + no methyl paraben</td>
<td>1.42 ± 0.04</td>
<td>15.4 ± 0.4</td>
<td>16.9 ± 4.4 cd</td>
<td>1.7 ± 0.8 ab</td>
</tr>
</tbody>
</table>

1Means having the same lowercase letter are not significantly different using LSD at P = 0.05
Figure 2-4  Percent hatch (± SE) for pepper weevil eggs incubated on Toba diets with casein (controls), pepperseed and cottonseed meal as protein sources; control (c), cottonseed augmented (cc), pepperseed augmented (cp), 20% pepper (p), 20% pepper + cottonseed augmented (pc), 20% pepper + pepperseed augmented (pp). Total hatch bars with different letters are different by LSD at (P = 0.05).
Table 2-5. Developmental fitness parameters for pepper weevil neonates reared on Toba diets with casein, cottonseed or pepperseed meal as protein sources

<table>
<thead>
<tr>
<th>Diet treatment</th>
<th>Adult dry mass</th>
<th>Days to 50% emergence</th>
<th>Survival %</th>
<th>Sex ratio (f:m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caesin</td>
<td>1.32 ± 0.04 b</td>
<td>15.7 ± 0.4</td>
<td>30.5 ± 4.6 a</td>
<td>1.36 ± 0.4</td>
</tr>
<tr>
<td>Cottonseed</td>
<td>0.90 ± 0.10 c</td>
<td>n/a</td>
<td>2.0 ± 1.1 b</td>
<td>n/a</td>
</tr>
<tr>
<td>Pepperseed</td>
<td>n/a</td>
<td>n/a</td>
<td>0 b</td>
<td>n/a</td>
</tr>
<tr>
<td>20% pepper + caesin</td>
<td>1.47 ± 0.04 a</td>
<td>15.6 ± 0.4</td>
<td>31.5 ± 2.6 a</td>
<td>1.19 ± 0.3</td>
</tr>
<tr>
<td>20% pepper + cottonseed</td>
<td>0.93 ± 0.05 c</td>
<td>n/a</td>
<td>2.0 ± 1.1 b</td>
<td>n/a</td>
</tr>
<tr>
<td>20% pepper + pepperseed</td>
<td>n/a</td>
<td>n/a</td>
<td>0 b</td>
<td>n/a</td>
</tr>
</tbody>
</table>

*Means having the same lowercase letter are not significantly different using LSD at P = 0.05*
CHAPTER 3
PEPPER WEEVIL OVIPOSITION IN LEAF SACHETS

Introduction

Phytophagous insects utilize an array of signals and cues to identify appropriate oviposition sites (Schoonhoven et al. 1998). Long range cues may include visual and olfactory stimuli that draw the insect into the vicinity of the host. Short range cues can include physical and chemical characteristics of the targeted oviposition site. Understanding what cues an insect of pest or biological control relevance utilizes when choosing an optimal oviposition site can lead to improved mass rearing and behavioral manipulation.

The pepper weevil, *Anthonomus eugenii* Cano, oviposits in the flower buds and small fruits of plants in the genera *Capsicum* and *Solanum*. Mass rearing of the weevil for research and as hosts for production of biological control agents is limited by the costly and time-consuming need for young pepper fruit as oviposition sources. While there is an artificial diet currently available commercially for larval development, attempts to induce weevil oviposition into the diet were unsuccessful (Chapter 2).

Previous attempts at alternative egg collection methods resulted in a successful ‘leaf sachet’ collection method (Calderon-Limon et al., 2002). Pepper leaves wrapped around 1-cm-diameter polyurethane spheres and covered with Parafilm® were presented to gravid females. The females chewed through the thin layer of Parafilm into the leaves and deposited eggs within the sachet. Eggs could then be collected by removing the external covering, unrolling the leaf within and removing the eggs. In the wild, pepper weevils oviposit only in fruit and flower buds, never in leaves. There are multiple hypotheses as to why leaves presented in sachets are stimulatory to oviposition whereas normally they are not. I hypothesize that the shape of the oviposition surface (i.e. spherical vs. flat) changes the stimulatory properties of the substrate.
Also, physical damage to the leaves occurring when leaves are rolled into sachets may release enough stimulatory volatiles to induce oviposition.

Pepper weevil has been known to oviposit in the fruit of a number of solanaceous species including peppers (*Capsicum annuum* L.), American black nightshade (*Solanum americanum* Mill.), and eggplant (*Solanum melongena* L.) while other plants within the family, such as tomato (*Solanum lycopersicum* Mill.), potato (*Solanum tuberosum* L.), and ornamental tobacco (*Nicotiana alata* Link & Otto) are not used as oviposition hosts in laboratory studies (Patrock & Schuster 1992). I hypothesize that leaves of host plants will show more stimulatory activity than leaves of non-host plants both within and outside the Solanaceae.

The goal of this study is to evaluate physical and plant-associated oviposition stimulants with the dual purpose of developing more effective egg collection technologies to assist in weevil mass production and as a starting point for a deeper understanding of plant-specific oviposition stimuli. I will investigate whether weevil oviposition is affected by the size, shape and covering of sachets and the species of leaf within the sachet.

**Materials and Methods**

**Insects and Plant Material.**

Pepper weevils in the following experiments came from a colony maintained at the University of Florida, Gainesville, Florida. The colony was initiated with wild insects which were collected in the spring of 2004 from pepper fields in southern Florida and was supplemented with additional collections during the fall of 2005 and 2006 from pepper fields in southern Florida and introduced into the colony to maintain colony health. Insects were reared under a 14:10 light:dark regime at 27°C and 30% r.h. on ‘Jalapeño’ pepper fruit (*Capsicum annuum* L.). Mated females (> 10-day-old) were held individually in oviposition cups (250 ml, 8.5 cm diameter) made from waxed cardboard cans (The Fonda Group, Inc., Union, NJ) and
were provided with a clean pepper fruit every 2 d. Infested fruit were transferred to emergence boxes (1.5-l Tupperware® containers). Weevils emerged 12-20 d later and were transferred to the colony cage where they were fed ‘Jalapeño’ fruit and provided with water and honey supplements.

Plant material in the following experiments came from greenhouse-grown ‘Jalapeño’ pepper, ‘Ghostbuster’ eggplant (*Solanum melongena* L.), ‘Better Boy’ tomato (*Solanum lycopersicum* L.), ‘Fordhook 242’ bush lima bean (*Phaseolus lunatus* L.), cotton DPL90 (*Gossypium hirsutum* L.), tropical soda apple (*Solanum viarum* Dunal) and jasmine tobacco (*Nicotiana alata* Link & Otto) grown from seed, and American black nightshade (*Solanum americanum* Mill.) grown as wild transplants obtained from Gainesville, FL. All plants were grown under greenhouse conditions (approx. 34ºC daytime temperature) and fertilized using Osmocote® 14-14-14 slow release pellets (The Scotts Company, Marysville, OH).

**General Bioassay Conditions.**

Mated females (> 10 days old) taken directly from the colony cage were used in the following experiments. Weevils were held individually for 24 h prior to assay on a clean pepper fruit. All assays were performed in small plastic boxes with screen lids (10 × 10 × 8 cm). Sachets were made by rolling a young leaf into a ball and wrapping the ball in Parafilm® (Pechiney Plastic Packaging Company Inc., Chicago, IL), unless otherwise noted. Individual females were offered an artificial fruit ‘sachet’ for feeding and oviposition in no-choice tests. Assays were run for 6 h with 15 different females (= replicates), unless otherwise noted. The numbers of feeding punctures and eggs deposited in the sachets were recorded.

**Effect of physical factors on acceptance of sachets for oviposition.** In the first experiment, weevils were presented with sachets of varying diameter made of pepper leaves.
These diameters correspond to a range of sizes available in the small fruit preferred by weevils produced by wild nightshade and ‘Jalapeno’ pepper. Sachets were divided into three size classes to determine the effect of small changes in size on feeding and oviposition: < 1 cm, 1 to < 2 cm, and 2 to 2.5 cm in diameter. The fresh weight of leaf material for the three size classes was approximately 0.4 g, 0.9 g and 1.1 g.

In a second experiment, weevils were presented with pepper leaf sachets wrapped in Parafilm that were either formed into spheres (1-2 cm in diameter) or flattened (approximately 2 × 2 cm square and 1 cm thick) to investigate the influence of shape on feeding and oviposition. Both sachet treatments contained a similar mass of leaves, and leaves to be used in the flattened treatment were first rolled into spheres to ensure an equivalent level of tissue damage.

In a third experiment, two sachet-covering options were compared to the standard Parafilm covering in separate bioassays. In the first assay, weevils were presented with 1-cm-diameter pepper leaf-rolled balls contained in cheesecloth (John L. Lyman Co., Chicopee, MA). The second assay examined pepper leaf sachets covered in bridal netting (1 mm aperture).

**Effect of plant leaves on acceptance of sachets for oviposition.** In the first series of bioassays, leaves from eight plant species were examined as feeding and oviposition stimulants: two non-host plants outside the Solanaceae (cotton and bean), two known solanaceous host plants (American black nightshade and eggplant), and four non-host plants within the Solanaceae (potato, jasmine tobacco, tomato, and tropical soda apple). Leaves were rolled into Parafilm sachets approximately 1 cm in diameter. For each assay of the eight assays, pepper leaf sachets were run as a control alongside the alternative plant species.

In the second experiment, ‘Jalapeño’ pepper fruit (4-5 cm long) and 1-cm-diameter Parafilm sachets of pepper, eggplant, tomato, or bean leaves were presented to females in no-
choice tests for an extended oviposition period. Females were permitted to oviposit for 24 h to
determine whether results from long-term exposure to the sachets would be consistent with the
results from the 6 h studies. Twenty replications were performed.

**Data Analysis.**

The number of feeding punctures and eggs laid were recorded for all assays, unless
otherwise noted. Data were analyzed by the Mann-Whitney U Test and the Kruskal-Wallis Test
for multiple treatment comparisons (SAS Institute 2006).

**Results**

**Effect of Physical Factors on Acceptance of Sachets for Oviposition.**

There was no difference in the number of feeding punctures between the three size classes
of pepper leaf sachets ($\chi^2 = 0.0363$, df = 2, $P = 0.9820$) (< 1 cm: $1.53 \pm 0.26$ [SE], 1 to < 2 cm:
1.6 $\pm$ 0.29, and 2 to 2.5 cm: 1.8 $\pm$ 0.33). Neither were there differences in the number of eggs
laid in the sachets ($\chi^2 = 0.7530$, df = 2, $P = 0.6863$) (< 1 cm: 0.87 $\pm$ 0.22, 1 to < 2 cm: 0.67 $\pm$
0.21, and 2 to 2.5 cm: 0.87 $\pm$ 0.19)

There was no difference between the number of feeding punctures in flat (0.93 $\pm$ 0.46) and
spherical sachets (1.53 $\pm$ 0.40) ($Z = 1.5590$, $P = 0.1190$). However, weevils laid more eggs in
the sphere-shaped sachets (1.07 $\pm$ 0.27) than in the flat sachets (0.2 $\pm$ 0.11) ($Z = 2.7044$, $P$
= 0.0068).

Females made more feeding punctures ($Z = -2.9791$, $P = 0.0029$; Table 3-1) and laid
more eggs ($Z = -3.9177$, $P < 0.0001$) in the Parafilm sachets. Sachets made from netting were
just as stimulatory as those made with Parafilm. Equal numbers of feeding punctures were made
in the netting and Parafilm treatments ($Z = 0.8885$, $P = 0.3743$). Females also laid equivalent
numbers of eggs in the two sachet types ($Z = 0.4129$, $P = 0.6797$).
Effect of Plant Leaves on Acceptance of Sachets for Oviposition.

Leaves of the two non-solanaceous plants, cotton and bean, did not stimulate feeding or oviposition. Females made fewer feeding punctures in cotton ($Z = 3.1435, P = 0.0017$; Figure 3-1) and bean ($Z = 2.5931, P = 0.0095$) sachets than in sachets with pepper leaves. Fewer eggs were also laid in cotton ($Z = 2.6474, P = 0.0081$) and bean ($Z = 2.7379, P = 0.0062$) sachets compared with pepper sachets. The two known larval host plants, in addition to pepper, stimulated feeding and oviposition. Females made equal numbers of feeding punctures in nightshade ($Z = 1.5283, P = 0.1264$) and eggplant ($Z = 0.7610, P = 0.4466$) sachets as compared to pepper sachets. They also laid equivalent numbers of eggs in nightshade ($Z = 1.5517, P = 0.1207$) and eggplant ($Z = -0.3628, P = 0.7167$) as in pepper sachets. The four solanaceous non-host plants differed in their results. Sachets made from the leaves of potato and jasmine tobacco, accumulated an equal number of feeding punctures (potato, $Z = -0.1487, P = 0.8818$; wild tobacco, $Z = 0.0433, P = 0.9655$) and eggs (potato, $Z = 0.7628, P = 0.4456$; wild tobacco, $Z = 1.1697, P = 0.2421$) compared to pepper sachets. Tomato and tropical soda apple leaves did not stimulate feeding or oviposition. Females made fewer feeding punctures in tomato ($Z = 3.4051, P = 0.0007$) and tropical soda apple ($Z = 3.3924, P = 0.0007$) sachets than in pepper sachets. They also laid fewer eggs in tomato ($Z = 2.0143, P = 0.0440$) and tropical soda apple ($Z = 2.8254, P = 0.0047$) as compared to pepper sachet controls.

There was an overall difference in egg deposition in the experiment where females were allowed a 24-h exposure period to pepper fruit or to sachets containing bean, tomato, eggplant, or pepper leaves ($\chi^2 = 30.9720, df = 4, P < 0.0001$). Females laid the same number of eggs in pepper fruit as in pepper leaf sachets ($Z = 0.6508, P = 0.5151$; Fig. 3-2). Tomato and eggplant sachets contained fewer eggs than pepper leaf sachets but the counts were not significantly different at $\alpha = 0.05$ level (tomato, $Z = 1.9106, P = 0.0561$; eggplant, $Z = 1.7832, P = 0.0746$).
There was no difference between the number of eggs laid in tomato \((Z = -1.5768, P = 0.1148)\) or eggplant sachets \((Z = -1.2163, P = 0.2239)\) and pepper fruit. Females also laid fewer eggs in bean sachets than in pepper sachets \((Z = -4.9803, P < 0.0001)\) and pepper fruit \((Z = -4.9771, P < 0.0001)\) just as they did in the 6 h assay.

**Discussion**

The range of sachet sizes used here \((< 1 \text{ cm}, 1 \text{ to } < 2 \text{ cm} \text{ and } 2-2.5 \text{ cm})\) did not influence feeding or oviposition by pepper weevils. The presence of feeding punctures and eggs should not be surprising given the range of fruit and flower bud sizes the weevil is accustomed to utilizing but one might have expected a higher number of eggs in larger sachets if the weevil is somehow measuring food availability by circumference or some other measure of host size, but, in fact, the pepper weevil may show the opposite trend. A recent study conducted by Porter et al. (2007) demonstrated that the weevil prefers smaller ‘Jalapeño’ pepper fruits when given the choice between three size classes \((\text{small } = 1.0-1.4, \text{ medium } = 1.9-2.2 \text{ and large } = 2.6-3.1 \text{ cm in length})\) In no choice situations the weevils still laid more eggs on the two smaller classes than on the larger fruit. This was not observed for the narrow range of sachet sizes used in our study.

The shape of the substrate influenced the number of eggs laid but not feeding initiation. In the field, pepper weevil adults will feed on terminal buds, leaves and even stem tissue of host plants, but will only deposit eggs in the spherical flower buds and fruiting bodies (Elmore et al., 1934; Patrock & Schuster, 1992). These field observations of the weevil support our results; equivalent numbers of feeding punctures in the flat and spherical sachets, but five times more eggs deposited in spherical sachets.

The three alternative covering types presented different textures to the weevil. Three times more feeding punctures were made through Parafilm covering than through cheesecloth while the netting treatment was similar to Parafilm. I believe this difference is due to a lack of
mechano- or chemoreceptor stimulation when females are in contact with the cheesecloth. When presented with cheesecloth sachets, females were sometimes observed tapping their tarsi against the surface while waving their antennae, suggesting the weevils could smell pepper leaves but were reluctant to initiate feeding due to the tactile quality of the materials. The netting treatment allowed the weevils to make physical contact with the leaf surface providing sufficient stimulation. This leads us, however, to a curious question; why will females chew through Parafilm to reach pepper leaves and not cheesecloth? The answer may have something to do with the waxy surface compounds of pepper fruit and leaves. Plant waxes can act as insect arrestants, feeding or oviposition stimulants alone or in concert with host-specific compounds. When the diamondback moth, *Plutella xylostella* (L.), was presented with a mixture of sinigrin and paraffin waxes (n-alkanes), they deposited more eggs than when they encountered sinigrin or waxes alone. This synergistic effect was due to the decrease in movement, greater turning and longer time spent on the substrate (Spencer et al., 1999). Additionally, Parafilm has been used to present *Brassica napus* L. leaf surface compounds to the diamondback moth in oviposition assays by pressing the Parafilm against the leaf surface for 30 s (Justus et al., 2000). Parafilm may therefore act as a suitable arrestant as well as an absorbent for pepper leaf surface compounds causing the females to come into contact with plant-derived oviposition stimulants. This hypothesis awaits further testing.

When different host and non-host plant leaves were presented to the pepper weevil, I observed a mixture of results. Females were stimulated to feed and oviposit on leaf sachets made from its known host plants, pepper, eggplant and American black nightshade. Two non-host plants within the Solanaceae, potato and jasmine tobacco, had the same number of feeding punctures and eggs as pepper sachets. This occurred despite the fact that Patrock and Schuster
(1992) observed no feeding on the leaves of jasmine tobacco in laboratory studies and no oviposition on either species’ leaves and flowers (potato and tobacco) or fruit (tobacco only, potato fruit were not available). Again, I suggest the physical shape of the sachet may have been enough to induce oviposition on leaves not normally stimulatory. Aside from the change in presentation, there is no way to explain why the tobacco leaves in our study were fed upon. Unlike potato and tobacco, tomato and tropical soda apple were unacceptable hosts in the sachet experiments. It would be interesting to discover which characteristics of the leaves ultimately determine acceptance (surface chemistry, leaf volatiles or a combination of the two) contradict previous oviposition studies. It is well known that plants within the genera *Solanaceae* produce a wide array of alkaloids which may affect palatability. Examples form the genera *Solanum* and *Capsicum* include solasodine, solasonine and capsaicin (Aldana & Lima, 1999; Supalkova et al., 2007).

In the 24-h oviposition study, I observed two differences from the 6-h study. After 24 h, females continue to lay fewer eggs in tomato sachets than pepper, but the counts are just above the significance level ($P = 0.0561$) compared with the 6-h assay ($P = 0.0440$). The eggplant sachets displayed a different trend. After 24 h, oviposition in eggplant sachets was decreased compared to that in pepper leaves while at 6 h they were relatively comparable. There are several explanations as to why we see a difference over time. First, the small number of eggs deposited in the 6 h assays can make it difficult to observe differences and so the differences may simply be due to statistical error. If, however, the changes we observe over time are real, this may indicate that the stimulatory properties of a particular leaf species can change over time depending on how long the weevil is exposed to the sachet. Alternately, changes may be the result of a decrease in stimulatory signals, an increase of repellent or deterrent signals, or a
combination of the two. Furthermore, females may be willing to deposit one or two eggs in a less than ideal substrate but will only continue to oviposit for an extended period in an ideal substrate. Alternatively, an oviposition substrate that is not accepted initially may be ‘settled for’ if no other option is available. In such a case, egg deposition would increase over time as it did with tomato. A comparison of the 6 and 24 h assays demonstrate that the plant species presented and the length of time exposed to the sachet can alter female decision making and the acceptability of a sachet for feeding or oviposition. There are many possible avenues for further investigation including a look at acceptance by females at different physiological states (i.e. starved vs. well fed).

It is clear from our results that the shape, type of covering and species of plant used to make sachets is important in their success as an artificial egg collection medium. In the Parafilm covered sachets, females laid an equal number of eggs as in ‘Jalapeño’ fruit and eggs could be detected without dissection by the presence of an oviposition plug deposited over the oviposition hole. In an artificial rearing system, eggs could be collected for further experimentation or for mass rearing on an artificial diet by presenting cages of females to pepper leaf sachets. Eggs can be collected from unrolled sachet leaves with a fine tipped paintbrush or by immersing the leaves in water, gently agitating them and collecting the fallen eggs in a filter paper funnel. This method was used for egg collection in the artificial diet studies in Chapter 2.

The results of the plant leaf assays raise questions about plant-specific feeding and oviposition stimulants. A detailed investigation of leaf surface chemistry may explain why females accepted the leaves of some plant species over others. Such knowledge will not only add to the body of literature on feeding and oviposition stimulation in phytophagous insects, but
may also facilitate the development of a completely artificial system for rearing of the pepper weevil.
Table 3-1. Number of feeding punctures and eggs laid by pepper weevil females in net or cheesecloth sachets compared with Parafilm sachets (mean ± SE).

<table>
<thead>
<tr>
<th></th>
<th>Parafilm</th>
<th>Cheesecloth</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feeding punctures</td>
<td>3.2 ± 0.07</td>
<td>1.07 ± 0.48</td>
<td>0.0029</td>
</tr>
<tr>
<td>Egg deposition</td>
<td>1.07 ± 0.21</td>
<td>0.07 ± 0.07</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Parafilm</th>
<th>Netting</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feeding punctures</td>
<td>3.00 ± 0.41</td>
<td>3.73 ± 0.46</td>
<td>0.3743</td>
</tr>
<tr>
<td>Egg deposition</td>
<td>1.07 ± 0.21</td>
<td>1.2 ± 0.14</td>
<td>0.6797</td>
</tr>
</tbody>
</table>
Figure 3-1. Average number of feeding punctures and eggs laid by pepper weevil females in sachets containing leaves of host and non-host plant species in no-choice tests with ‘Jalapeño’ pepper leaf sachets during a 6-h exposure period (mean ± SE).
Figure 3-2. Average number of eggs laid by pepper weevil females in sachets containing leaves of host and non-host plant species or in intact 4 to 5 cm pepper fruit during a 24-h exposure period in no-choice situations (mean ± SE). Pepper, bean, eggplant and tomato were leaf sachets. ‘Fruit’ is ‘Jalapeno’ pepper fruit.
CHAPTER 4
HOST MARKING BY FEMALE PEPPER WEEVILS

Introduction

Insect oviposition behavior can be modified by cues and signals associated with the presence of conspecific immature stages. Cues associated with the presence of immatures can come directly from eggs, larvae, or the response of hosts to infestation (Rausher, 1979; Blaakmeer et al., 1994; Fatouros et al., 2005; Schröder et al., 2005). In addition to brood-associated cues, many insect herbivores and parasitoids have evolved specific pheromone signals to convey information about host quality (Nufio & Papaj, 2001). These signals, referred to as marking pheromones, may be used by females to reduce offspring competition (Prokopy, 1981).

Currently, more than 100 species in the orders Coleoptera, Diptera, Hymenoptera, Lepidoptera, and Neuroptera are known to produce marking pheromones (Anderson, 2002). Many of these species oviposit on discrete hosts that must support complete larval development.

Marking pheromones may be produced by female insects or by larvae that co-occur with them. Female marks may be applied externally or internally, as in the case with some parasitoids. Deposition may be simultaneous with or following oviposition. For example, in the genus *Ostrinia*, extracts of egg masses contain a marking pheromone (Li & Ishikawa, 2005) while female *Rhagoletis* flies deposit marking pheromones by dragging their ovipositor over the fruit following oviposition (Prokopy, 1972; Prokopy et al., 1976). *Trissolcus basalis* (Wollaston), an egg parasitoid of the southern green stink bug, *Nezara viridula* (L.), marks its hosts on the surface (Rosi et al., 2001) while the aphid parasitoid, *Ephedrus cerasicola* Stary, uses both an internal and external marker (Hofsvang, 1988).

A marking pheromone will only be selected for when it increases the relative fitness of marking individuals. Marking can improve the search efficiency of an individual by allowing
the female to avoid previously infested patches in addition to decreasing competition with conspecifics. In some cases, females may choose to ignore the marking pheromone. Reasons for ignoring the pheromone may include a lack of unexploited patches (Messina & Renwick, 1985) or differences in female egg load (Höller & Hörmann, 1993). Marking pheromones may also differ between individuals within a species, allowing females to discriminate between self and conspecific marks (van Dijken et al., 1992; Ueno, 1994; Agboka et al., 2002; McKay & Broce, 2004).

The pepper weevil, *Anthonomus eugenii* Cano (Coleoptera: Curculionidae), is a Neotropical pest of cultivated pepper, *Capsicum* spp., and also reproduces on wild American black nightshade (*Solanum americanum* Mill.), silverleaf nightshade (*S. elaeagnifolium* Cav.), and in rare cases, Old World eggplant (*S. melongena* L.) (Tejada & Reyes, 1986; Wilson, 1986; Diaz et al., 2004). Females oviposit preferentially in young fruit but will also utilize mature fruit or flower buds for oviposition. They deposit eggs individually in feeding punctures and then cover the hole with a clear anal secretion often mixed with frass that hardens into a plug. Similar oviposition behavior has been observed in the boll weevil, *Anthonomus grandis* Boheman, and their oviposition plugs were found to deter other females from reusing staminate *Hampea nutricia* Fryxell flower buds (Stansly & Cate, 1984). Prior to this study, other authors noted the deterrent properties of previously infested cotton squares, female frass, and anal secretions (Everett & Earle, 1964; Mitchell & Cross, 1969; Hedin et al., 1974).

Pepper fruit and flower buds are discrete hosts, highly susceptible to overcrowding. An examination of boll weevil oviposition found that females offered 10, 15, and 20 cotton squares per day had a greater estimated percent egg hatch and survival of offspring to adulthood than females offered 5 or 1 square per day, presumably due to larval competition for host resources.
It is not clear if competition or cannibalism plays a role in pepper weevil offspring success. If it does, female pepper weevils would benefit from avoiding previously exploited hosts.

Nufio & Papaj (2001) outlined four categories of evidence required to document the presence of marking pheromones. The first category includes behavioral assays demonstrating a response to the marking pheromone. These studies quantify rejection patterns of marked hosts, distinguish between responses to the mark and other potential cues, and determine the chemical nature and composition of the pheromone. The second category is the observation and description of a distinct host-marking behavior. The third category of evidence involves identifying the mechanisms of marking pheromone production and detection, and the final category is documentation of the ecological consequences of the marking pheromone, assessed under natural conditions.

A putative host marking behavior has already been described in the pepper weevil; the deposition of an anal secretion that hardens into a plug (Elmore et al., 1934). This paper will address the behavioral response of pepper weevil to infested pepper fruit in order to confirm the presence of a marking pheromone. Through the use of naturally infested and artificially manipulated hosts, I will answer the following three questions: (1) Are female pepper weevils deterred by previous oviposition? (2) What components of oviposition confer deterrence? (3) Are the deterrent effects observed in small scale arena experiments also seen when insects are forced to search larger patches within and among plants?

Materials and Methods

Insects and Plants

Pepper weevils were collected in south Florida near the city of Clewiston (26°45′12″N, 80°56′1″W) in the spring of 2004, and a laboratory colony was established at the University of
Florida, Gainesville, FL, USA. Additional field collections were made in Immokalee, Bradenton, and Wimauma, FL, USA in the fall of 2005 and 2006 to maintain colony health. Insects were maintained in the laboratory (L14:D10, approximately 27°C, and 30% r.h.) on excised greenhouse-grown ‘Jalapeño’ peppers \( \text{Capsicum annuum} \) L. (Solanaceae) with water and honey supplements. Gravid females were removed from the colony cage 10 d after emergence and transferred singly into oviposition containers made from waxed cardboard cans with screened lids of 250 ml, 8.5 cm in diameter (The Fonda Group, Inc., Union, NJ, USA). Gravid females were provided with a single pepper, which was replaced every 2 d with a new one. Infested fruit were held in plastic emergence boxes (1.5-l Tupperware® containers, Orlando, FL, USA) for 3 weeks or until all weevils emerged.

Weevils were assayed on immature ‘Jalapeño’ peppers (4-5 cm in length) collected from pepper plants grown in a glass greenhouse in Gainesville, FL. Peppers were collected from plants the day prior to bioassay to ensure freshness. Infested fruit used in the bioassays were prepared by presenting a single pepper to a gravid female in an oviposition container. The female was permitted to oviposit overnight and the following morning the pepper was collected. Oviposition scars were counted on the collected fruit and only those fruit containing 3-4 eggs, as indicated by oviposition plugs, were used in the assays as this represents the average number of eggs deposited by one weevil per day. Gravid females, at least 10-d-old, used in the following assays were isolated from the colony 24 h prior to the assay and allowed to feed and oviposit on a single clean pepper fruit during that period.

**Small Arena Experiments**

Female weevils were placed individually in 10 × 10 × 8 cm plastic boxes with screen lids for all experiments (20 replicates for each experiment). In no-choice tests, a single pepper was
offered to a female. In choice tests, one pepper of each treatment was offered to a female. Peppers were laid in the containers on their sides. Eggs were counted after a 12-h oviposition period during the light phase.

**Influence of maternal source of eggs in infested peppers on oviposition preference.**
In the first choice and no-choice experiments, females were presented with clean peppers and/or peppers containing eggs laid during the previous night by a different female. In the second experiments, females were presented in choice and no-choice experiments with clean peppers and/or peppers containing eggs they had laid during the previous night. In a third experiment, females were offered a choice between two infested peppers, one containing their own eggs and a second containing the eggs of a different female. In this choice assay, both peppers contained the same number of eggs (either three or four).

**Effects of potential cues associated with oviposition on oviposition preference.** All bioassays performed for this series of experiments were conducted under choice situations only. Stimuli associated with oviposition were either added to clean fruit or removed from infested fruit to determine their importance in oviposition deterrence. In the first experiment, fruit were punctured with a sterilized metal probe to mimic the mechanical damage that would be associated with oviposition. Females were given a choice between a clean fruit and a fruit with four mechanical punctures around the calyx, the preferred oviposition site. The punctures were made in the fruit moments before being placed in the arena.

In a second experiment, females were presented with peppers contaminated with male or female frass, as might occur naturally during feeding and oviposition. Separate 1.5-l square plastic boxes (18 × 18 × 6 cm) with screen lids were set up containing either male or female weevils. Weevils were permitted to feed on pepper fruit for 7 days before frass was collected
from the cages. Frass was collected by first lightly scraping the container with a sterilized metal probe to loosen the fecal spots. A small paintbrush (2/0, 1 cm bristle length) was dipped in deionized water and rolled in the frass. Frass was then smeared around the calyx of the pepper fruit. Approximately 0.30 mg of frass was applied to each fruit. Females were presented with choices of fruit contaminated with male frass versus clean fruit, fruit contaminated with female frass versus clean fruit, or fruit contaminated with male frass versus contaminated with female frass.

In a third experiment, females were presented with clean peppers and clean peppers to which oviposition plugs were attached with deionized water. Oviposition plugs were dissected from infested peppers using an insect pin and paintbrush (2/0, 1 cm bristle length). Plugs were taken from fruit that had been infested the previous night. Four plugs were applied around the calyx of the fruit using deionized water.

In the fourth and fifth experiments, components of the oviposited fruit were removed with cotton swabs moistened with deionized water and the effect of their removal on oviposition quantified. In the fourth experiment, females were presented with infested fruit and one of the following: (1) an infested pepper with plugs removed, (2) an infested pepper with frass removed, or (3) an infested pepper with both plugs and frass removed. In the fifth experiment, females were given a choice of a clean fruit and an infested fruit with both frass and plugs removed.

**Whole Plant Cage Experiments**

The purpose of the two whole plant cage experiments was to determine how females distribute their eggs within and between plants with different levels of initial infestation. In the first experiment, single ‘Jalapeño’ plants at the flowering stage (2-3 months old) were contained in plastic cylinder cages (60 cm tall × 15 cm in diameter) with screen lids and three 10 × 10 cm
screen windows to provide ventilation. Artificial branches were constructed from a 45-cm green bamboo stake capped by two 12-cm wooden craft sticks crossed at right angles to each other. Four ‘Jalapeño’ peppers were hung from the ends of the wooden stick ‘branches’ with green twist ties. Two treatments were tested; the clean treatment had four clean fruit and the mixed treatment had three clean fruit and one infested fruit containing 3-4 eggs. The infested fruit were placed systematically in each of the four positions (front, back, left, and right) to control for position effects. Two females were added to each enclosure through a corked hole just above the base of the cylinder and were held in a rearing room under L14:D10 regime at 27°C. Eggs were counted in each fruit after 48 h. Twenty-two replications were performed.

In a second experiment, two ‘Jalapeño’ plants at the flowering stage were placed in a chiffon mesh cage (30 × 30 × 30 cm) in a glass greenhouse (L11:D13, average temperature 24°C, and 60% r.h.). Artificial branches as described above held four clean fruit on one plant and four infested fruit containing 3-4 eggs on the second plant in the cage. Two females were placed in each cage. Eggs in each fruit were counted 48 h later. Twenty replications were performed.

Data analysis

Egg counts were analyzed using the normal approximation of a one-sided Wilcoxon Rank Sum analysis for choice tests with two treatments and the Kruskal-Wallis test for multiple treatments (SAS, 2006). No-choice data were analyzed by Mann-Whitney test (Conover, 1980). Data are presented as means ± SE.

Results

Small Arena Experiments

Influence of maternal source of eggs in infested peppers on oviposition preference.

Females laid more eggs in clean fruit than in fruit previously infested by conspecifics in both no-choice (U = -3.06, P = 0.0031) and choice tests (Z = -3.21, P = 0.0007) (Table 4-1). They also
laid more eggs in clean fruit than in their own previously infested fruit in both no-choice (U = -3.33, P = 0.0008) and choice tests (Z = -5.15, P < 0.0001). Females did not discriminate between peppers containing their own or conspecific eggs, laying equivalent numbers of eggs in both treatments (Z = 0.07, P = 0.4706).

**Effects of potential cues associated with oviposition on oviposition preference.**

Mechanical punctures did not deter oviposition (Z = -0.56, P = 0.2867). Females laid equivalent numbers of eggs when given a choice of clean peppers and peppers containing mechanical punctures (clean = 0.90 ± 0.18; mechanical damage = 0.80 ± 0.21). In addition, two females laid eggs within the artificial punctures and covered the punctures with oviposition plugs.

Male frass added to clean fruit did not deter oviposition (Z = 0.51, P = 0.3061), but female frass was deterrent (Z = -3.61, P = 0.0002) (Table 4-2). Females chose to lay more eggs on peppers with male frass than female frass when given a choice between the two (Z = 2.49, P = 0.0065), indicating that deterrent compounds present in the weevil frass are female-specific in origin. The source of frass did not influence the total number of eggs laid in the three choice experiments ($\chi^2 = 1.14, P = 0.5660$).

Females laid fewer eggs on clean peppers to which oviposition plugs were added than on uninfested peppers (Z = -2.04, P = 0.0209; clean = 1.25 ± 0.20; plugs = 0.65 ± 0.20). The tiny amount of plug material (four plugs) required to decrease oviposition marginally compared to the amount of frass used in these experiments (0.30 mg of frass = ~150 plugs) suggested that the active compound(s) in the plug were more concentrated than in the frass.

Females laid more eggs in infested peppers with frass removed (Z = -1.86, P = 0.0311), plugs removed (Z = -3.30, P = 0.0005), and frass and plugs removed (Z = -2.41, P = 0.0080) than in infested fruit contaminated with all female-deposited material (Table 4-3). In addition,
females preferred clean peppers over peppers with plugs and frass removed (Z = 4.36, P<0.0001; clean = 1.6 ± 0.15; removed = 0.40 ± 0.13).

**Whole Plant Cage Experiments**

There were no branch position effects observed in the clean ($\chi^2 = 3.12$, d.f. = 3, P = 0.7749) and mixed treatments ($\chi^2 = 0.96$, d.f. = 3, P = 0.8096). There was no difference in total eggs laid in the clean and mixed treatments (Z = -1.35, P = 0.0879) (Table 4-4). Females laid more eggs per clean fruit in the mixed treatment than in the clean treatment (Z = -1.75, P = 0.0399). In the mixed treatment, females laid more eggs per fruit in the clean pepper than in the infested peppers (Z = 2.96, P = 0.0015), indicating a shift in the distribution of new egg deposition rather than a decrease in overall eggs laid. In the second cage experiment, where females were caged with two plants, one with infested peppers and one with clean peppers, females moved between plants, laying more eggs on plants with clean peppers than on plants with infested peppers (Z = 2.41, P = 0.0081).

**Discussion**

This study clearly shows that oviposition plugs are involved in deterrence. The deterrent effect of plugs was extremely high given that they covered only 0.01- 0.04% of the pepper surface area. Female frass was also deterrent, but much more frass was required to observe deterrent activity, suggesting the frass may be contaminated by the anal secretion and that frass alone is not the source of the marking pheromone. Male frass, on the other hand, showed no deterrent or stimulatory properties. Males are known to produce an aggregation pheromone, one that is excreted in the frass (Eller et al., 1994). The potential presence of this pheromone on peppers contaminated with male frass did not appear to influence female oviposition. The presence of the aggregation pheromone could act as a deterrent if it signals a high probability that the patch is occupied and other females have already laid eggs in the fruit.
In addition to the female-specific deterrent, pepper fruit damage and eggs may also have some deterrent effects, although the deterrence in fruit with plugs and frass removed could have been due to incomplete removal of these materials. Females do not normally come into direct contact with eggs as they are laid in cavities below the fruit surface. However, the presence of eggs may itself alter the chemistry of the fruit, thus providing additional cues to the presence of brood (see review by Hilker & Meiners, 2006).

The production of host-marking pheromones is known to occur in closely related species. Some well-studied genera include *Rhagoletis* (15 spp.), *Telenomus* (6 spp.), *Anastrepha* (5 spp.), *Callosobruchus* (4 spp.), *Chrysopa* (4 spp.), *Ephestia* (3 spp.), and *Anaphes* (3 spp.) (Agboka et al., 2002; Anderson, 2002; Aluja & Diaz-Fleischer, 2006). The only other species of *Anthonomus* known to possess an oviposition deterrent is another member of the *mexicanus* group, the cotton boll weevil, *A. grandis* (Stansly & Cate, 1984). Other well-known pest species within the genus include the strawberry blossom weevil (*A. rubi* Herbst) and the apple blossom weevil (*A. pomorum* (L.)) both found primarily in Eurasia, and the North American cranberry weevil (*A. musculus* Say). *Anthonomus rubi* and *A. pomorum* do not exhibit the same plugging behavior as the boll weevil and pepper weevil (Jerry Cross, pers. com.). The cranberry weevil does cover its oviposition scars with a plug (Anne Averill, pers. com.) but whether the plug contains an oviposition deterrent is unknown. In addition to pest species, *Anthonomus tenebrosus* Boheman, a potential biological control agent of the tropical soda apple (*Solanum viarum* Dunal), also exhibits plugging behavior (Bobby Jo Davis, pers. com.). It is currently unclear how widespread ‘plugging’ behavior is within the genus, whether it occurs randomly throughout *Anthonomus*, or if it is a characteristic of particular species groups. More research is
needed to determine if other ‘plugging’ species within *Anthonomus* also deposit marking pheromones with their plugs.

It is important to note that pepper weevils did lay eggs in the presence of oviposition plugs and female frass, though in smaller numbers. One major question of interest when studying marking pheromones is: when should females ignore the signal? Some possible reasons females lay eggs in the presence of a deterrent include genetic variation in detecting the deterrent, differences in egg load, and habituation. Egg load may have affected female decision-making. Females laid anywhere from one to seven eggs during the 12 h assay period (no females laid no eggs in any of the replicates). It is quite possible that females with higher egg loads were less discriminating in their choice of oviposition sites. In the aphid hyperparasitoid, *Dendrocerus carpenteri* (Curtis), females with low egg loads spent less time in previously explored patches as compared to females with large egg loads (Höller & Hörmann, 1993). In addition, females with low egg load continuously applied the marking pheromone while walking, presumably in an attempt to increase its deterrent effect.

It is also important to point out that the mechanical damage, frass, and plugs were applied solely around the calyx of the fruit, where the majority of oviposition takes place. If a female decided to oviposit elsewhere on the fruit, she may never have encountered the deterrent. It is also possible that rather than assessing the presence or absence of eggs, pepper weevils may be measuring the level of competition their offspring will encounter in a given host patch, altering the number of eggs deposited based on pepper infestation level. The seed bruchid, *C. maculatus*, laid more eggs in seeds with small egg loads and fewer eggs in seeds with high egg loads, maintaining a uniform egg distribution within the seeds, as well as indicating that females can detect small differences in egg density (Messina & Renwick, 1985). The walnut fly, *R.*
*juglandis*, known for reusing oviposition sites, determines the level of competition in a fruit by the amount of pheromone detected (Nufio & Papaj, 2004). Another reason female weevils might ignore the marking pheromone is that they may become insensitive to the deterrent after repeated exposure as seen in *R. cerasi* (Boller & Aluja, 1992). For all fruit assays, females were confined to one or two fruit for 12 h. Females who deposited eggs on infested fruit may have lost sensitivity to the deterrent after being exposed to the marking pheromone for such a long period of time.

In some species, particularly parasitoids, females have been shown to discriminate between their own marks and those of conspecifics (van Dijken et al., 1992; Ueno, 1994; McKay & Broce, 2004). Discrimination is expected if superparasitism of their own offspring decreases female fitness, while depositing additional eggs in conspecific-parasitized hosts may increase fitness. The pepper weevil does not appear capable of discriminating between self and conspecific marks, suggesting that the fitness consequences of reusing a particular host are independent of the identity of developing larvae.

One major danger of using host marking pheromones is eavesdropping by predators and parasitoids. Previous studies have demonstrated that insect pheromones can be used by predators and parasitoids to locate potential hosts (Prokopy & Webster, 1978; Roitberg and Lalonde, 1991; Wiskerke et al., 1993; Aldrich, 1995; Hoffmeister & Gienapp, 1999; Hoffmeister et al., 2000; Kumazaki et al., 2000; Onodera et al., 2002). A recent study of the pepper weevil egg-larval parasitoid, *Triaspis eugenii* Wharton and Lopez-Martinez, showed parasitism success decreased by 2.5-3 times when weevil oviposition plugs were removed (Rodriguez-Leyva, 2006). Further studies are required to determine whether the same compounds involved in weevil oviposition deterrence are used by *T. eugenii* to identify hosts.
Information on pepper weevil oviposition deterents adds to the small but growing body of evidence that intraspecific chemical communication is a vital part of female decision making during the host selection process. Such knowledge may lead us to new and targeted ways of controlling or suppressing weevil pest populations by augmenting current chemical, behavioral, and cultural control methods. In order for practical use of the pepper weevil oviposition deterring pheromone to be possible, it must first be isolated and identified from female frass and oviposition plugs. We will then have a better understanding of the nature of the pheromone and whether it can be a useful tool in integrated pest management programs.
Table 4-1. Mean number of eggs laid by female pepper weevils in choice and no-choice tests in clean peppers or peppers oviposited in by themselves or other females

<table>
<thead>
<tr>
<th>Comparisons</th>
<th>Mean eggs laid per pepper ± SE</th>
<th>Choice test</th>
<th>No-choice test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Clean</td>
<td>Infested P</td>
</tr>
<tr>
<td>Clean vs. other-infested</td>
<td>1.35 ± 0.28</td>
<td>0.30 ± 0.11</td>
<td>0.0007</td>
</tr>
<tr>
<td>Clean vs. self-infested</td>
<td>1.82 ± 0.21</td>
<td>0.23 ± 0.09</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Other-infested vs. self-infested</td>
<td>1.05 ± 0.26</td>
<td>0.85 ± 0.15</td>
<td>0.4706</td>
</tr>
</tbody>
</table>
Table 4-2. Mean number of eggs laid by female pepper weevils in choice tests with uninfested pepper fruit contaminated with male and female frass

<table>
<thead>
<tr>
<th>Comparisons</th>
<th>Mean eggs laid per pepper ± SE</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clean</td>
<td>Frass</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>Clean vs. male frass</td>
<td>1.20 ± 0.31</td>
<td>1.00 ± 0.27</td>
<td>0.3061</td>
<td></td>
</tr>
<tr>
<td>Clean vs. female frass</td>
<td>1.95 ± 0.29</td>
<td>0.55 ± 0.15</td>
<td>0.0002</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>Male frass vs. female frass</td>
<td>1.45 ± 0.26</td>
<td>0.60 ± 0.20</td>
<td>0.0065</td>
<td></td>
</tr>
</tbody>
</table>
Table 4-3. Mean number of eggs laid by female pepper weevils in choice tests between infested peppers and peppers with frass and/or oviposition plugs removed

<table>
<thead>
<tr>
<th>Comparisons</th>
<th>Mean eggs laid per pepper ± SE</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infested</td>
<td>Female-produced material removed</td>
<td>P</td>
</tr>
<tr>
<td>Infested vs. frass removed</td>
<td>0.75 ± 0.19</td>
<td>1.35 ± 0.24</td>
<td>0.0311</td>
</tr>
<tr>
<td>Infested vs. plugs removed</td>
<td>0.50 ± 0.17</td>
<td>1.30 ± 0.18</td>
<td>0.0005</td>
</tr>
<tr>
<td>Infested vs. frass &amp; plugs removed</td>
<td>0.63 ± 0.17</td>
<td>1.42 ± 0.24</td>
<td>0.0080</td>
</tr>
</tbody>
</table>
Table 4-4. Mean number of eggs laid by female pepper weevils in peppers in whole plant experiments

<table>
<thead>
<tr>
<th>Comparisons</th>
<th>Mean eggs laid per pepper ± SE</th>
<th>Clean treatment</th>
<th>Mixed treatment</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1&lt;sup&gt;1&lt;/sup&gt; - Clean treatment vs. mixed treatment (overall eggs laid)</td>
<td></td>
<td>2.16 ± 0.31</td>
<td>2.77 ± 0.34</td>
<td>0.0879</td>
</tr>
<tr>
<td>Experiment 1 - Clean treatment vs. mixed treatment (clean fruit only)</td>
<td></td>
<td>2.16 ± 0.31</td>
<td>3.21 ± 0.40</td>
<td>0.0399</td>
</tr>
<tr>
<td>Clean peppers vs. infested peppers (mixed treatment)</td>
<td></td>
<td>3.21 ± 0.40</td>
<td>1.45 ± 0.36</td>
<td>0.0015</td>
</tr>
<tr>
<td>Clean plant vs. infested plant</td>
<td></td>
<td>4.50 ± 0.89</td>
<td>1.45 ± 0.36</td>
<td>0.0081</td>
</tr>
</tbody>
</table>

<sup>1</sup> In experiment 1, females were presented with a single plant with four clean peppers (clean treatment) or a single plant with one infested pepper and three clean peppers (mixed treatment).

<sup>2</sup> In experiment 2, females were presented a choice between two plants, one with all four peppers infested and one with no peppers infested.
CHAPTER 5
ISOLATION AND CHARACTERIZATION OF THE PEPPER WEEVIL HOST MARKING PHEROMONE

Introduction

Host marking is a phenomenon commonly found in insects that develop on or in a single host and is seen in parasitoids, fruit-feeding insects and other species with low larval motility (Nufio & Papaj, 2001). The marking of a previously exploited host can act to reduce intra- and interspecific competition by allowing gravid females to judge the level of crowding in a host prior to making an oviposition decision. While behavioral studies indicate the presence of a marking pheromone in more than a hundred species of insects, the chemical identity of most marking pheromones remains unknown (Anderson, 2002).

The production of a putative marking pheromone has been demonstrated in only three members of the weevil family Curculionidae, *Ceutorhynchus assimilis* (Paykull), *Ceutorhynchus floralis* (Paykull), and the boll weevil, *Anthonomus grandis* Boheman (Stansly & Cate, 1984; Kozlowski, 1989; Mudd et al., 1997). All of these species feed and oviposit on discrete hosts that are susceptible to overcrowding. The pepper weevil (*Anthonomus eugenii* Cano), a congener of the boll weevil, oviposits exclusively in the flower buds and small fruits of some solanaceous species including cultivated pepper (*Capsicum* spp.), wild nightshades (*Solanum* spp.) and on rare occasions, eggplant (*Solanum melongena* L.). Females chew a cavity into the fruiting body, lay a single egg within the hole and cover the opening with a clear yellow anal secretion that hardens into a plug (Elmore et al., 1934). The plug may protect the egg from desiccation and predation and deters female oviposition (Chapter 4).

Identification of marking pheromones for use in pest management has seriously lagged behind that of other types of pheromones (ex. aggregation and sex pheromones). Only one marking pheromone, produced by the cherry fruit fly (*Rhagoletis cerasi* Loew) has been both
chemically identified and applied in the field (Aluja & Boller, 1992). The application of the fly’s marking pheromone reduced fruit infestation ten-fold when the entire tree canopy was covered. These encouraging results suggest that the use of a marking pheromone may be an effective control measure in other agricultural systems. I believe that the pepper weevil marking pheromone, in combination with an attractant (such as the commercially available aggregation pheromone traps and/or host plant volatiles) in a push-pull strategy has the potential to improve integrated pest management of the pepper weevil in the field.

Before the potential of the marking pheromone can be tested, it must first be chemically identified. Therefore, the purpose of this chapter was to isolate the active compounds present in the oviposition plugs through differential solvent extraction and high performance liquid chromatography (HPLC). Compounds in active fractions will be identified by gas chromatography/mass spectrometry (GC/MS).

**Materials and Methods**

**Insects and Plants**

Female pepper weevils used in this study came from the colony maintained at the University of Florida, Entomology and Nematology Department and collected from Clewiston, FL. The colony was initiated from individuals collected in the spring of 2004 and individuals were added to the colony in the fall of 2005 and 2006 to maintain colony genetic variation. The colony was maintained under a 14:10 light:dark regime at 27°C and 30% r.h. on ‘Jalapeño’ pepper fruit (*Capsicum annuum* L.) with water and honey supplements. The ‘Jalapeño’ fruit used in the following assays were grown outdoors in pots (25 l) and fertilized weekly with Peters Professional ® 14-14-14 (Scotts International B.V., Geldermalsen, Netherlands). Peppers used in all assays were 4-5 cm in length and were collected the same day as the experiments. Females
used in the assays were held individually overnight prior to the experiment on a clean pepper fruit and were allowed to feed and oviposit at will.

**Oviposition Deterrence Bioassay**

Fresh oviposition plugs (1-2 d old) were dissected with a size ‘0’ insect pin from pepper fruit into 1- or 5-ml glass vials and stored at -80°C until use. Crude extracts were made by dissolving 100 plugs in 1 ml of commercial solvent (all solvents used were Optima HPLC grade, Fisher Scientific, Pittsburgh, PA) or solvent: deionized water combinations (see below). The solvent and plugs were sonicated for 20 min. After the undissolved plug material settled to the bottom of the vial, the supernatant was transferred to a clean vial and evaporated under a flow of nitrogen to 20 μl (five plug equivalents/μl). Four μl (20 plug equivalents) of extract or solvent control was applied evenly around the calyx of a pepper fruit (between 4-5 cm long). The solvent was applied in this manner to maximize the likelihood that females would make contact with the pheromone since females prefer to oviposit in this region (Rodriguez-Leyva, 2006). The solvent was permitted to evaporate prior to presenting the pepper to a female in either choice or no-choice situations, as noted below. Individual females and treated fruit were placed in small plastic boxes with screened lids (10 × 10 × 8 cm) and allowed to oviposit for 12 h at ~27°C and 30% r.h. Egg counts were recorded after 12 h.

**Solvent extraction assays.** The effectiveness in removing deterrent compounds of solvents with a range of polarities was examined. Eleven treatments were analyzed under no-choice conditions: (1) uninfested fruit, (2) uninfested fruit with five plugs attached with deionized water, (3) water control, (4) methanol:water (50:50) control, (5) methanol:water (80:20) control, (6) methylene chloride control, (7) water plug extract, (8) methanol: water (50:50) plug extract, (9) methanol: water (80:20) plug extract, (10) methylene chloride plug extract and (11) a recombined extract made by taking 250 μl of each extract treatment (water,
50:50, 80:20, and methylene chloride) and evaporating it to 20 μl (= 4 reps). This assay was carried out under no choice conditions due to the large number of treatments and comparisons required for this assay and to identify solvent extracts that were strong enough to deter oviposition when females were give no other option. Twenty replications were performed.

Based on the results of the first test, plugs were first extracted in methylene chloride and then in methanol: water (80:20) to determine if the same active compounds were extracted by methylene chloride and methanol: water (80:20). Extract-treated fruit were this time presented to individual females in choice tests with solvent control-treated fruit. Twenty replications were performed.

The effectiveness of methylene chloride and pentane to extract active compounds was assessed. Plugs were extracted in methylene chloride or pentane and then extract-treated fruit were presented to individual females in choice tests with solvent control-treated fruit. Twenty-four replications were performed.

To determine if the same active compounds were extracted by pentane and methylene chloride, plugs were first extracted in pentane followed by methylene chloride. Pentane and methylene chloride extract-treated fruit were presented in choice tests with their solvent control-treated fruit. Twenty replications were performed.

**Silica column separation of active pentane extract.** A crude pentane extract was separated by passing through a silica column constructed from a glass Pasteur pipette (15 cm, Fisher Scientific, Inc., Pittsburgh, PA), silica (300 mg of silica, grade 12, 80/100, Alltech Associates, Inc., Deerfield, IL), and glass wool (Alltech Associates, Inc., Deerfield, IL) and eluting the compounds with 3-ml volumes of pentane, pentane:ethyl acetate (95:5), and pentane:ethyl acetate (70:30). Five treatments were presented to females in a choice test with
pentane solvent controls: 1) the crude extract, 2) pentane fraction, 3) pentane: ethyl acetate (95:5) fraction, 4) pentane: ethyl acetate (70:30) fraction, and 5) the recombined fractions. Sixteen replications were performed.

**HPLC separation of active pentane fraction.** A crude pentane extract of 400 oviposition plug equivalents was passed through a 900 mg Maxi-Clean Silica Cartridge (50 μm particle size, 60 Å pore size; Alltech Associates, Inc., Deerfield, IL) and eluted with pentane, pentane: ethyl acetate (95:5), and pentane: ethyl acetate (70:30). The pentane and pentane:ethyl acetate (95:5) fractions were stored at -80ºC and discarded when activity in the pentane: ethyl acetate (70:30) fraction was confirmed. The 70:30 elution was fractionated by normal-phase HPLC on a 5-μm silica column (Zorbax Rx-SIL 4.6 × 250 mm; Agilent Technologies, Santa Clara, CA, USA). The 70:30 elution was concentrated to 50 μl and injected onto the column and eluted with a solvent gradient of 100% pentane to 50% pentane: 50% ethyl acetate at 1 ml/min. One-minute fractions were collected for 30 min. The 1-min fractions were recombined into 11 treatments: ‘1’ (0-5 min), ‘2’ (6-8 min), ‘3’ (9-10 min), ‘4’ (11-12 min), ‘5’ (13-14), ‘6’ (15-16), ‘7’ (17-18), ‘8’ (19-20), ‘9’ (21-23), ‘10’ (24-25), and ‘11’ (26-29). Minutes were pooled this way to focus on minutes 9-20 where the solvent mixture approached and exceeded 70:30 pentane: ethyl acetate. The fractions were concentrated under a flow of N gas to 5 plug equivalents/ μl. Twenty plug equivalents were applied to the calyx of a pepper fruit. The fraction treatments were compared to a control pepper fruit and a 70:30 elution control under no-choice conditions. This assay was also carried out under no choice conditions due to the large number of treatments required for this assay and to identify fractions that were strong enough to deter oviposition when females were given no other option. Twelve replications were performed.
**Characterization of Active Fraction 6.** Normal phase HPLC fractions were analyzed directly by cold on-column GC/MS (6890/5975 GC/MS, Agilent Palo Alto, CA) in both EI and isobutane CI mode. Samples (5 μl) were injected into a 10 m deactivated retention gap connected to a methyl silicone column, (HP5, 30 m x 0.25 mm I.D. x 0.1μm film thickness, Agilent Technologies, Santa Clara, CA, USA). The injector and column were kept at 30 °C for 5 min and then temperature programmed at 10 °C/min to 280 °C. The He carrier gas flow rate was 30 cm/sec. (constant flow) and the transfer line temperature was 260 °C. The ion source temperature was 220 °C in EI mode and 250 °C in CI mode. Spectra library search was performed using a floral sent database compiled at the Department of Chemical Ecology, Göteborg Sweden, the Adams terpenoid/natural product library (Allured Corporation) and the NIST05 library.

**Data Analysis**

Egg counts were analyzed using Mann-Whitney U and Kruskal-Wallis tests for no choice data and Wilcoxon signed-rank tests for choice tests (SAS Institute, 2006).

**Results**

**Solvent Extraction Assays**

There were no significant differences among the controls (clean fruit and four solvents) in the number of eggs laid ($\chi^2 (4) = 4.0804$, P = 0.3952; Fig. 5-1). More eggs were laid in clean fruit than in fruit with plugs attached (Z = -1.6912, P = 0.0454). Females laid fewer eggs in peppers treated with the methanol:water (80:20) extract (Z = -1.7697, P = 0.0384) and the methylene chloride extract (Z = 1.4130, P = 0.0788) at the 0.10 level as compared to their respective controls. Females also laid fewer eggs in the recombined extract (Z = 1.6050, P =
0.0542) at the 0.10 level as compared to clean fruit. No deterrent activity was seen in the water 
(Z = 0.5840, P = 0.2796) or methanol:water (50:50) extracts (Z = 0.3489, P = 0.3636).

When plugs were first extracted with methylene chloride followed by methanol: water 
(80:20), the methanol:water extract exhibited no deterrent activity (S = -10.5, P = 0.6052) 
indicating that all active compounds were removed by methylene chloride (S = -45; P = 0.0462).

Comparison of pentane extracts and methylene chloride extract indicated that pentane 
was a more effective solvent of the deterrent compounds. Weevils laid over three times more 
eggs in the pentane control than in the fruit treated with the pentane extract (control = 28 eggs, 
pentane = 8 eggs; S = 46, P = 0.0065) but laid only twice as many eggs in the methylene chloride 
control as the extract treated fruit (control = 30 eggs, MeCl = 15; S = -28.5, P = 0.0757).

When plugs were first extracted with pentane followed by methylene chloride, pentane 
removed all the deterrent activity (S = 63, P = 0.0169), leaving the methylene chloride extract 
inactive (S = 19.5, P = 0.5341).

Silica Column Separation of Active Pentane Extract

The pentane:ethyl acetate (70:30) fraction (S = -39, P = 0.0005; Fig. 5-2d) contained active 
compounds. The crude extract (S = -32.5, P = 0.0841; Fig. 5-2a) and recombined fraction (S = - 
22, P = 0.0933; Fig. 5-2e) were also active at the 0.10 level. The pentane fraction (S = 3.5, P = 
0.8608; Fig. 5-2b) and pentane:ethyl acetate (95:5) fraction (S = 8.5, P = 0.5283; Fig. 5-2c) 
showed no deterrent activity.

HPLC Separation of Active Pentane Fraction

There were significant differences in the number of eggs laid in the 13 treatments \(\chi^2_{(12)} = \)
25.8067, P = 0.0114; Fig. 5-3). Peppers treated with fractions 6 (min 15-16) and 7 (min 17-18) 
had significantly fewer eggs than the clean pepper treatment (fraction 6, Z = 2.686, P = 0.0072; 
fraction 7, Z = 2.3492, P = 0.0188) while fraction 10 had marginally fewer than the clean
treatment ($Z = -1.6194, P = 0.0527$). More eggs were laid in the clean treatment than the 70:30 elution treatment ($Z = 2.5809, P = 0.0049$). Fractions 6, 7 and 10 were just as deterrent to oviposition as was the 70:30 pentane: ethyl acetate elution (fraction 6: $Z = -0.6223, P = 0.5338$; fraction 7: $Z = 0.9693, P = 0.3324$; fraction 10: $Z = 0.5450, P = 0.5858$). The remaining fractions did not deter oviposition (all $P$-values greater than 0.05), containing the same number of eggs as the clean treatment.

**Characterization of Active Fraction 6**

Two compounds were observed in the active fraction (Fig. 5-4). The first compound was present in minute 15 (Fig. 5-4b) with a $R_t = 16.456$ and an $m/z = 180.1$. It is a substituted aromatic with at least 2 alcohol groups. The NIST05A database identified 2, 4-dihydroxy-3-methylpropiophenone as a close match. The second compound was present in minute 16 (Fig. 5-4c) with an $R_t = 13.570$ and an $m/z = 152.0$. It is also a substituted aromatic with one alcohol group. The NIST05A database identified 3-phenoxy-1-propanol as a close match. The structures need to be verified with $^1$H and $^{13}$C-NMR.

**Discussion**

The active compounds in the pepper weevil oviposition plug can be extracted with solvents of low to moderate polarity (pentane, relative polarity = 0.009; ethyl acetate, relative polarity = 0.228; methylene chloride, relative polarity = 0.309). Fraction 6 is slightly more active than fraction 7, the same active compound(s) are present in both fractions, eluting from the column somewhere between min 15 and 18, when the solvent gradient was between 60:40 and 54:46 pentane:ethyl acetate. The final active fraction, fraction 10, eluted between 24 and 25 min, when the solvent gradient was held at 50:50 pentane:ethyl acetate residual activity of the fraction 6 and 7 compound(s) being pulled off the column. The boiling point of 3-phenoxy-1-propanol boiling ranges from 83-85°C making it thermally stable.
The chemical compositions of several marking pheromones have been identified in the orders Diptera, Lepidoptera, Orthoptera and Coleoptera. Despite the increased interest in insect pheromone production over the past few decades, the cabbage seed weevil (C. assimilis) is the only weevil whose pheromone has been characterized (Mudd et al., 1997). The active fraction of this pheromone contained a mixture of iso and n-alkanes, dimethylalkanes, alkenes, fatty acids, 15-nonacosanone, 15-nonacosanol, and cholesterol. It is unclear if all or only some of these compounds are responsible for the pheromone activity. Unfortunately, the marking pheromone of the boll weevil has not been chemically identified. An investigation into the chemical composition of the boll weevil marking pheromone as well as more information on marking behavior within the genus may give us some insight into the relationship of these species to one another.

In nature, the marking pheromone allows female pepper weevils to make informed choices about where to deposit their eggs so they can maximize reproductive success. This may be done by avoiding infested fruit altogether or by altering the number of eggs deposited in previously infested fruit. The ability to manipulate pepper weevil behavior with this pheromone on a small scale has already been demonstrated with the use of infested sentinel fruit in cage bioassays (Chapter 4). However, in order for this pheromone to be effective as a management tool, it must first be demonstrated to effect behavioral changes under field and commercial greenhouse conditions in the absence of plant damage. Additionally, the cost of the pheromone formulation must be reasonable in relation to other currently available control measures. Ultimately, the development of a push-pull management system combining aggregation pheromone traps and foliar sprays of the marking pheromone may prove to be an effective
suppression system for the weevil in conventional and organic cropping systems (Cook et al., 2007).

While use of the marking pheromone in the field may assist growers in controlling pepper weevil populations, it may simultaneously interfere with the searching behavior of pepper weevil parasitoids. An examination of oviposition behavior by the pepper weevil parasitoid, *Triaspis eugenii* Wharton & Lopez-Martinez, demonstrated a link between the oviposition plug and rate of parasitism (Rodriguez-Leyva, 2006). In choice tests, *T. eugenii* successfully parasitized 50.7% of the plugged eggs but only 18.6% of the eggs with plugs removed. In no-choice tests, when oviposition plugs were intact, *T. eugenii* successfully parasitized 63.6% of the eggs present whereas only 20.7% of the uncovered eggs were parasitized. While it is clear the presence of the oviposition plug increases parasitism success rate, it is not known if the compounds forming the pepper weevil marking pheromone are the signal used for host detection by the parasitoid. Further chemical and behavioral analysis is required to determine if this is indeed a case of pheromone ‘eavesdropping’ by the parasitoid or if some other aspect of the oviposition plug is responsible for *T. eugenii*’s response.
Figure 5-1. Mean number (+/- SE) of eggs laid by pepper weevil in no-choice tests with uninfested fruit, fruit with 5 plugs applied, solvent controls, water extract, methanol: water (50:50) extract, methanol: water (80:20) extract, MeCl extract, and the combined fraction. * indicate fractions that are significantly different from their respective solvent controls at P < 0.05.
Figure 5-2. Mean number (+/− SE) of eggs laid by pepper weevil in fruits to which silica column-separated fractions were applied: (a) pentane solvent vs. crude extract, (b) pentane solvent vs. pentane elution, (c) 95:5 solvent vs. 95:5 elution, (d) 70:30 solvent vs. 70:30 elution, (e) pentane solvent vs. recombined elutions. *** indicate fractions that are significantly different from the respective control at P < 0.001.
Figure 5-3. Mean number (+/- SE) of eggs laid by pepper weevil in uninfested fruit (un), fruit treated with a 70:30 pentane:ethyl acetate extract of oviposition plugs, and HPLC fractions of the 70:30 pentane:ethyl acetate extract (1, 0-5 min; 2, 6-8 min; 3, 9-10 min; 4, 11-12 min; 5, 13-14 min; 6, 15-16 min; 7, 17-18 min; 8, 19-20 min; 9, 21-23 min; 10, 24-25; 11, 26-29 min). * indicate fractions that are significantly different from the uninfested control (* = P < 0.05, ** = P < 0.01).
Figure 5-4. GC spectra of (a) active fraction 6 and minutes (b) 15 and (c) 16 in EI mode
CHAPTER 6
RESPONSE OF PEPPER WEEVIL TO CONSTITUTIVE HOST AND NON-HOST PLANT VOLATILES

Introduction

Phytophagous insects utilize a wide range of general and host-specific cues to locate their host plants within a heterogeneous environment (Schoonhoven et al., 1998). An insect, moving randomly through space, will use long-range visual and/or olfactory cues to lead it to the vicinity of the plant. Once the insect makes contact with a potential host, short range olfactory, mechanical and chemical cues verify the plant’s suitability as a feeding or oviposition site. Other factors, such as species-specific pheromone signals, add to the complexity of host plant selection patterns as these signals may also work to both draw an insect into a habitable patch as well as aid in mate location.

Determining the sequence of events leading to host acceptance is particularly important for phytophagous insect pests. The pepper weevil, Anthonomous eugenii Cano, commonly infests cultivated pepper (Capsicum spp.) fields in the southern United States, Central America and the Caribbean (Goff and Wilson, 1937; O’Brien and Wibmer, 1982; Abreu and Cruz, 1985). In addition to feeding on Capsicum spp., the weevil is also capable of reproducing in the southern United States on a number of wild and cultivated plants in the genus Solanum, including eggplant, Solanum melongena L. (Diaz et al., 2004), and American black nightshade, Solanum americanum Mill. (Patrock & Schuster, 1987). The ability of the pepper weevil to survive the fallow season on wild nightshades makes this insect difficult to eradicate since nightshade-residing populations are able to reinfest pepper fields the following season. While these generalized migratory patterns of the weevil have been described by previous authors (Patrock & Schuster, 1987), no one has yet addressed how pepper weevils initially locate their cultivated and wild host plants.
While no research describing the pepper weevil’s response to host plant volatiles exists, there is a body of literature on the volatile attractants of three of its congeners, the boll weevil (*Anthonomus grandis* Boheman), the apple blossom weevil (*Anthonomus pomorum* L.), and the strawberry blossom weevil. Previous behavioral bioassays and electroantennogram studies have demonstrated that these congeners can detect and orient to host plant volatiles. The boll weevil displayed attraction to cotton plant volatile oils and cotton square extracts (Hardee et al., 1971; McKibben et al., 1977) in behavioral bioassays. Later, Dickens (1984, 1986, 1989, 1990) verified boll weevil response and attraction to six-carbon ‘green leaf volatiles’ and host-specific volatiles using electroantennography (EAG) and single cell recording (SCR) techniques. An examination of apple blossom weevil response to volatile blends of various apple cultivars also suggests that this species uses volatiles as cues to locate its host plants (Kalinova et al., 2000). More recently, examinations of the olfactory neurons of the strawberry blossom weevil, identified 15 receptor types that detected a total of 54 host and non-host plant volatiles (Bichão et al., 2005a), as well as five additional receptors detecting volatiles from strawberry plants that are induced by weevil feeding (Bichão et al., 2005b).

The goal of this research is to better understand the complexities of pepper weevil host plant selection. I did this by first addressing the response of weevils to constitutive volatile cues from host and non-host plants in the absence of visual cues or pheromone signals. Removing these alternate sources of information will allow us to focus solely on the importance of plant volatiles on the weevil’s ability to locate a host plant. In addition, any differences between male and female response to plant volatiles can be identified without confounding differences in sensitivity to the male-produced aggregation pheromone (Eller et al., 1994). I will also be able to identify changes in volatile discrimination by females at different ages. From a life-history
perspective, newly emerged females should spend the majority of their time seeking mates and feeding locations while older, previously mated females should be more interested in oviposition sites. These changes in female behavior over time may lead to changes in volatile preferences if plant hosts sufficient for mating and adult feeding are not necessarily optimal sites for larval development.

In order to begin unraveling the complexities of pepper weevil host plant selection I seek to answer the following questions: (1) Do pepper weevils orient to host plant volatiles? (2) Do pepper weevils orient to general (non-host) plant volatiles? (3) Do pepper weevils show a preference for the volatiles of particular host plants? (4) Do males and females respond differently to plant volatiles? (5) Do newly emerged females respond differently than gravid females to host plant volatiles?

**Materials & Methods**

**Insects and Plants**

A pepper weevil colony was established at the University of Florida in the spring of 2004 from insects collected from pepper fields in Clewiston, FL. Additional wild insects from Immokalee, Bradenton and Wimauma were introduced into the colony in the fall of 2005 and 2006 to maintain colony health. The colony was maintained under a 14:10 light: dark regime at 27ºC and 30% r.h. Weevils were reared on greenhouse-grown ‘Jalapeño’ peppers (*Capsicum annuum* L.), with water and honey supplements. Gravid females (> 10-day-old) were placed in oviposition cups with a single pepper fruit, which was replaced every 2 d. Oviposition cups with screened lids were made from waxed cardboard cans (250 ml, 8.5 cm diameter) (The Fonda Group, Inc., Union, NJ). Infested fruit were held in emergence containers (1.5-l Tupperware® containers) until all weevils emerged. Newly emerged weevils were collected and transferred into a colony cage for use in assays. Colony insects were fed ‘Jalapeño’ pepper fruit.
‘Jalapeño’ pepper, ‘Ghostbuster’ eggplant (*Solanum melongena* L.), American black nightshade (*Solanum americanum* Mill.), ‘Better Boy’ tomato (*Solanum lycopersicum* L.), and ‘Fordhook 242’ bush lima bean (*Phaseolus lunatus* L.) plants (Illinois Foundation Seeds, Champagne, IL) were grown under greenhouse conditions. Plants were fertilized using Osmocote® 14-14-14 slow release pellets (The Scotts Company, Marysville, OH).

**Y-tube Olfactometer Experimental Design**

Bioassays were conducted in a glass Y-tube olfactometer (Analytical Research Systems, Gainesville, FL; Fig. 6-1) with Teflon tubing connections. Breathing quality compressed air was pushed through a charcoal filter and humidified with deionized water prior to splitting into two holding chambers. Three types of holding chambers were used in our assays: (1) Plexiglas® chambers (60 cm tall, 15 cm internal diameter), (2) large glass chambers (21 cm length, 3 cm internal diameter), and (3) small glass chambers (6 cm length, 2 cm internal diameter). Airflow was maintained at 250 ml/min by two inline flowmeters (Manostat, New York, NY). The glass Y-tube (12 cm common tube, 10 cm arms, 2.5 cm internal diameter) was held at a 60º angle above horizontal inside a cardboard enclosure (46 cm × 28 cm × 42 cm). Holding chambers were placed outside the enclosure to eliminate visual cues. The Y-tube assembly was illuminated by a fluorescent light fixture (four 85-W bulbs) suspended 70 cm above the table surface. The assay room was maintained between 25º- 27ºC and 40% r.h.

Weevils were sexed according to Eller (1995). Male and female weevils were starved overnight prior to assay without access to water. Two age classes of females (newly emerged and > 10-day-old) and one male age class (> 10-day old) were tested under each set of experimental conditions. Older females had been confined with males in the colony cage since their emergence and were presumed to have mated. Forty insects in each of the three age/sex classes were assayed in each experiment. Insects were given 15 min to make a choice of arms in the
olfactometer. Weevils that passed halfway or further into one arm of the Y-tube were recorded as making a choice and then removed from the system. Weevils were assayed individually or in groups of five, depending on the assay. Ten weevils in each of the three classes were assayed each day. After five assays, the airflow was reversed to the opposite side to control for right- or left-handed bias. After one sex/age class of weevils was assayed, the Y-tube was cleaned with soapy water, rinsed with ethanol and dried on the bench before the next class was assayed. The starting class was randomly selected each day and assays were run within the previously established activity period for oviposition of 10:00 – 17:00 h (Patrock & Schuster, 1992).

**Y-tube Bioassays**

**Host plant and non-host plant volatile attraction assays.** Three host plants (pepper, eggplant and nightshade) and two non-host plants, one solanaceous (tomato) and one non-solanaceous (lima bean) were evaluated in one-way choice tests against a purified air control. Plants were presented in Plexiglas cylinders. Flowering plants used in these assays were between 2 and 3 months old and insects were assayed individually. Forty insects in each age/sex class were assayed ten per day on four separate days.

**Host plant preference assays.** Three known pepper weevil host plants (pepper, eggplant, nightshade) were evaluated in pair-wise choice tests to determine preference among the plant odors. Flowering plants used in these assays were between 2 and 3 months old and insects were assayed individually. Plants were presented in Plexiglas cylinders. Forty insects in each age/sex class were assayed ten per day on four separate days.

**Flower volatile attraction assays.** Pepper, eggplant, and nightshade flowers were assayed in one-way choice tests against a purified air control. Flowers were picked immediately before use and stems were wrapped with moistened cotton ball swabs to prevent wilting. Five pepper flowers, two eggplant flowers or 40 nightshade flowers were placed in small glass
(pepper) or large glass (eggplant and nightshade) chambers. Different numbers of flowers were presented to approximate equivalent floral mass. Ten insects in each age/sex class were assayed per day, for a total of forty insects. Five weevils were placed in the Y-tube at a time and the response of each insect was recorded.

**Fruit volatile attraction assays.** Pepper, eggplant, and nightshade fruit were assayed in one-way choice tests against a purified air control. Fruit were picked immediately before use. Two pepper fruit (< 5 cm long), two eggplant fruit (< 2 cm diameter) or 25 nightshade fruit (~0.5 cm diameter) were placed in small glass (nightshade) or large glass (pepper and eggplant) chambers. Different numbers of fruit were presented to approximate similar mass. Ten insects in each age/sex class were assayed per day, for a total of forty insects. Five weevils were placed in the Y-tube at a time and the response of each insect was recorded.

**Data Analysis**

Data were analyzed as percent response to each arm of the Y-tube using chi-squared analysis (SAS 2006). The percentage of 10-day male, 10-day female and 2-day old female insects assayed that did not respond was also compared using chi-squared analyses among the three sex/age class within each experiment.

**Results**

**Y-Tube Bioassays**

**Host plant and non-host plant volatile attraction assays.** In one-way choice tests, 10-day-old males and females preferred pepper, eggplant, nightshade, and tomato volatiles over purified air but showed no preference for lima bean (Fig. 6-2). Two-day-old females were attracted to pepper, eggplant, nightshade, tomato, and lima bean.

**Host plant preference assays.** In pair-wise choice tests, 10-d-old females preferred pepper volatiles over nightshade, while 10-d-old males and 2-d-old females displayed no
preference (Fig. 6-3). Ten-day-old males and females also preferred nightshade volatiles to eggplant volatiles. Two-day-old females showed no preference between volatiles of the two plants. Ten-day-old males preferred pepper over eggplant but 2-d and 10-d-old females showed no preferences.

**Flower volatile attraction assays.** Ten-day-old males, and 2- and 10-day-old females showed no preference for pepper floral odor over purified air (Fig. 6-4). Ten-day-old males and females were attracted to volatiles from eggplant flowers. Two-day-old females were only slightly attracted to eggplant flowers ($\chi^2(1) = 3.24, P = 0.0719$). All three age/sex categories were attracted to nightshade flowers.

**Fruit volatile attraction assays.** Ten-day-old males, and 2-d and 10-day-old females were attracted to pepper fruit volatiles (Fig. 6-5). Only 10-day-old males and females were attracted to nightshade and eggplant fruit volatiles.

**‘No-choice’ data.** Some insects remained at the base of the Y-tube or did not make a choice in the time allotted. Across assays, each sex/class had the same percentage of weevils in the ‘no-choice’ category ($\chi^2(13) = 10.68, P = 0.6378$). However, a significant difference was observed between sex/classes, with 2-d-old females having the lowest percentage of weevils failing to make a choice ($\chi^2(2) = 20.54, P < 0.0001$; 10-day-old males = 22.9% ± 2.1, 10-day-old females = 26% ± 2.7, and 2-day-old females = 8.1% ± 1.7).

**Discussion**

Our bioassays reveal that pepper weevils do, in fact, orient to host plant volatiles in the absence of visual and pheromone stimuli. Males and females were attracted to the volatiles of known host plants but also responded to tomato, a non-host plant within the genus *Solanum*. In a series of feeding and oviposition assays, Patrock & Schuster (1992) observed pepper weevil feeding on tomato plants and other species within the family Solanaceae; the weevils oviposited
on only a small subset of the *Solanum* spp. (which did not include tomato) but oviposited on all *Capsicum* spp. tested. This suggests that there may be volatiles specific to plants in the family that can draw weevils in from a distance but that further contact or short range cues determine the acceptability of the plant species for oviposition. Interestingly, 10-day-old males and females were not attracted to the general plant volatiles of lima bean but 2-day-old females did respond to the non-host plant. The reasons for the difference in response may be due to experience or developmental differences in neurological response. In addition to showing a positive response to a non-host plant, 2-day-old females also failed to demonstrate the same discriminatory patterns as older females in pair-wise comparisons. All weevils used in these assays were reared on ‘Jalapeño’ pepper fruit and none had previous experience with the alternative host and non-host plants or pepper plant and flower volatiles. Therefore, naivety should not be a factor affecting the differences observed in the behavior of 2-day-old females since 10-day-old males and females were also unfamiliar with the alternative plant volatiles. It is therefore most likely that developmental differences in olfactory neurons were responsible for the observed differences in response. Dickens & Moorman (1990) came to the same conclusion when addressing the changing response of both male and female boll weevils to host plant volatiles with age. Between 0 and 4 days post emergence, the weevils showed a significant increase in sensitivity to the general host plant volatile 1-hexanol and the cotton-specific volatile β-bisabolol. It may be that while newly emerged pepper weevils can detect the presence of plant volatiles, their receptor neurons are not yet sensitive enough to differentiate between two complex odor plumes.

Ten-day-old males and females did display preferences for different host plants in the Y-tube, and these preferences changed depending on sex and which two plants were offered. It is
important to note that while 10-day-old females had a strong affinity for American black nightshade volatiles in the single-choice assays, when put in competition with pepper volatiles, the females overwhelmingly chose pepper. This occurred even though a small percentage of females walked to the air-laden arm in the one-way choice test with pepper in the alternative arm, while 100% of females walked to nightshade in the same series of assay. It is likely the difference in preference is due to the larger size and presumably better quality of pepper fruit as a larval host source. Females are rarely observed depositing more than one egg in nightshade fruit while the weevils are known to deposit many more in the larger ‘Jalapeño’ fruit (personal observation).

Since pepper weevils feed primarily and oviposit solely on flower buds and small fruits, we might have expected the weevils to be attracted to volatiles from host plant flowers and fruits. This does not appear to be true in all cases. While ten-day-old male and female weevils oriented toward the immature fruit of pepper, nightshade and eggplant, they only responded to nightshade and eggplant flowers. No attraction to pepper flower volatiles was observed. Further analysis of flower volatile profiles, GC-EAD, and behavioral bioassays are required to determine whether the attractive compounds present in the flowers of the two Solanum spp. are absent from C. annuum.

The experiments described in this chapter were specifically designed to exclude alternative cues often used in host plant location. Other volatile attractants excluded include the male-produced pepper weevil aggregation pheromone (Coudriet & Kishaba, 1988; Eller at al., 1994) as well as plant volatiles induced by weevil feeding and/or oviposition. While arguably, the very first weevil to arrive at an uninfested plant must rely solely on constitutive plant cues to locate the host, subsequent arrivals may be orienting to a combination of plant and insect-
specific volatiles. If the first arrival is female, her feeding or oviposition may induce changes in the volatile plume of the plant, which may potentially increase the plant’s attractiveness to other weevils. If the first arrival is male, a combination of plant volatiles and male-produced pheromone will be available to aid other weevils in locating the plant. Chapter 7 describes a series of wind tunnel experiments designed to address the effects of feeding damage and aggregation pheromone on the attractiveness of pepper plants.

As has been noted, more work is needed in order for us to understand the intricacies of pepper weevil host plant location and, more specifically, the role plant volatiles play in this complex chain of events. More interesting is the relationship between alternative host plants and the volatile cues that cause male and female weevils to select one species over another and allow populations to survive eradication by migrating back and forth between cultivated and closely related wild species. Our ultimate goal is to use GC-EAD and behavioral bioassays to identify host-specific volatile attractants common to the pepper weevil’s host plants in an effort to improve the attractiveness of the currently commercially available aggregation pheromone lures, thereby allowing growers to more accurately monitor and control pest populations in the field.
Figure 6-1. Y-tube olfactometer used to test the attractiveness to pepper weevils of volatile compounds released by host and non-host plants
Figure 6-2. Pepper weevil attraction to host and non-host plant volatiles in a Y-tube olfactometer. Data are represented as percentage responding to air (white bars) vs. (a) pepper (green striped bars), (b) nightshade (black bars), (c) eggplant (purple striped bars), (d) tomato (red bars), and (e) lima bean (blue bars). * = P ≤ 0.05, ** = P ≤ 0.01 *** = P ≤ 0.001 in χ² analysis.
Figure 6-3. Pair-wise comparison of pepper weevil host plant volatiles in a Y-tube olfactometer. Data are represented as percent responding. (a) nightshade (black bars) vs. pepper (green striped bars), (b) eggplant (purple striped bars) vs. pepper and (c) nightshade vs. eggplant. * = $P \leq 0.05$, ** = $P \leq 0.01$ *** = $P \leq 0.001$ in $\chi^2$ analysis.
Figure 6-4. Pepper weevil attraction to host plant flower volatiles in a Y-tube olfactometer. Data are represented as percent responding to air (white bars) vs. (a) pepper (green striped bars), (b) nightshade (black bars), (c) eggplant (purple striped bars). * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001 in χ² analysis.
Figure 6-5. Pepper weevil attraction to host plant fruit volatiles in a Y-tube olfactometer. Data are represented as percent responding to air (white bars) vs. (a) pepper (green striped bars), (b) nightshade (black bars), and (c) eggplant (purple striped bars). * = $P \leq 0.05$, ** = $P \leq 0.01$ ***= $P \leq 0.001$ in $\chi^2$ analysis.
CHAPTER 7
INFLUENCE OF VISUAL CUES, FEEDING DAMAGE AND MALE AGGREGATION
PEROMONE ON ATTRACTION OF PEPPER WEEVILS TO HOST PLANTS IN A WIND
TUNNEL

Introduction

The pepper weevil, *Anthonomus eugeni*i Cano, is a common pest of cultivated pepper
(*Capsicum* spp.) fields in the southern United States, Central America and the Caribbean region
(Goff and Wilson, 1937; O’Brien and Wibmer, 1982; Abreu and Cruz, 1985). The weevil is also
capable of reproducing on a number of wild and cultivated plants in the genus *Solanum*. In the
southern United States, these include other agricultural species like eggplant, *Solanum*
*melongena* L. (Diaz et al., 2004), and the ubiquitous weed, American black nightshade, *Solanum*

The ability of the pepper weevil to move from cultivated fields to surrounding
nightshades during crop-free periods and back again (Patrock & Schuster, 1987) stimulated our
interest in the potential use of volatile cues by pepper weevils during host plant location.
Previously, short-range volatile attraction was examined in a Y-tube olfactometer (Chapter 6).
Constitutive volatiles from undamaged pepper, eggplant and American black nightshade
attracted both male and female pepper weevils. The weevils also displayed preferences when
given a choice between the three host plants in pair-wise comparisons. While short-range assays
like those performed in a Y-tube olfactometer give us some information on weevil preferences, a
larger scale wind tunnel allows the insects more freedom to make decisions and to exhibit more
natural host location behavior.

The goal of this research is to further investigate weevil attraction to host plants by
observing their response to constitutive and induced host plant volatiles. The following four
questions were investigated: Do weevils move upwind in a wind tunnel without host plant
volatiles? Will weevils move upwind to constitutive volatiles with and without visual cues? Do weevils prefer plants with or without feeding damage? And finally, do weevils respond differently to plants with active feeding by males and females?

**Materials and Methods**

**Insects and Plants**

Pepper weevils used in the assays came from a colony maintained at the University of Florida in Gainesville. The colony was established in 2004 from weevils collected in pepper field in southern Florida near the city of Clewiston. Additional insects were introduced in 2005 and 2006 from fields in Immokalee, Bradenton and Wimauma to maintain colony health. The colony was maintained in a rearing room under a 14:10 light:dark regime at 27°C and 30% r.h. The insects were maintained on ‘Jalapeño’ peppers (*Capsicum annuum* L.) grown at the University of Florida with water and honey supplements. Gravid females (> 10-day-old) were placed in wax cardboard oviposition cans (250 ml, 8.5 cm diameter) (The Fonda Group, Inc., Union, NJ) with screen lids and offered a single pepper fruit every 2 d. Infested fruit were placed in emergence containers (1.5-l Tupperware® plastic boxes) for 3 weeks or until all weevils emerged. Newly emerged weevils were transferred into a colony cage or held in dated emergence containers for use in assays. Colony insects were fed ‘Jalapeño’ pepper fruit, water and honey supplements.

‘Jalapeño’ pepper and American black nightshade (*Solanum americanum* Mill.) plants were grown from seed in 12 cm pots in Metro-Mix 200 (SunGro, Bellevue, WA). Plants were watered as needed and fertilized using Osmocote® 14-14-14 slow release pellets (The Scotts Company, Marysville, OH). Plants used in these assays were approximately 3-4 months old and at the flowering stage.
Wind Tunnel Design

Bioassays were conducted in two sizes of Plexiglas® wind tunnels at the USDA-ARS CMAVE facility in Gainesville, FL (Fig. 7-1). The large wind tunnel (160 × 45 × 45 cm) was housed in a small greenhouse (approx. temp 30 ºC, 75% r.h.). For assays in the large wind tunnel, airflow was pulled by a vacuum at 0.2 m/s. Air came from a plant placed directly inside the tunnel. No traps were used in these assays. Once weevils made contact with the plant source, they did not leave.

Four small wind tunnels (120 × 30 × 30 cm) were housed in a separate greenhouse (approx temp 30 ºC, 66-75% r.h.) (Fig. 7-1). In these assays, plant volatiles were passed through two Plexiglas cylinders (60 cm tall, 15 cm internal diameter; Fig. 7-2) that contained plants or that were empty, depending on the experiment. Air from each chamber was split with Teflon tubing into four streams, which were attached to the volatile ports of four wind tunnels. Air entered the wind tunnels at 0.6 l/min, maintained by inline flowmeters, and airflow in the tunnels was pulled by a vacuum at 0.2 m/sec. All assays were carried out between 0900 and 1500 h.

In the small wind tunnels, traps were placed at the upwind end to capture any weevils attempting to contact the odor port. Traps were of two types (Fig. 7-3). The first type of trap was constructed from two 25-dram plastic vials (Bioquip, Gardena, CA) placed end to end (5.0 cm diameter, 8.5 cm height) fitted with a 1-ml plastic centrifuge tube to allow weevils to enter the trap but not escape. Traps were placed 25 cm above the floor and attached to the volatile ports. The second type of trap was made from white TOMCAT glue boards (10.0 × 24.0 cm) (Motomco, Clearwater, FL, USA) hung from the vial traps and making contact with the ground.

Wind Tunnel Bioassays

Male and mated female weevils (10-20 d old) were used in the following assays. Weevils were sexed and held separately in groups of 10 in 7-dram plastic vials with air holes. Weevils
were held overnight (approximately 15 h) with no food or water prior to assay. The same vials were placed into the downwind end of the wind tunnel unless stated otherwise (120 cm or 60 cm from the odor source in the large or small wind tunnel respectively). Weevils were released in groups of ten by removing the vial lid at the start of each assay (Fig. 7-4).

Weevil location was categorized and their upwind orientation to pepper volatiles was recorded after 15, 30, 60 and 300 min unless stated otherwise. Weevil locations recorded were: 1) weevils that passed halfway up the tunnel (60 cm upwind in the large tunnel, 45 cm in the small tunnel), 2) weevils that reached the vicinity of the source (120 cm upwind in the large tunnel, 90 cm in the small tunnel), and 3) those that made contact with the odor source (SC). All experiments were replicated on four days for a total of 40 weevils per sex per treatment.

**Orientation of pepper weevils in the absence of odors.** The first assay was conducted in the large wind tunnel to determine weevil orientation in a wind tunnel in the absence of plant odors. Weevils were placed in the center of the wind tunnel with no olfactory stimuli but with wind speed set at 0.2 m/s. Weevil orientation was observed 15 and 30 min after their introduction to determine if the insects prefer to move upwind or downwind in the absence of odors. Weevils were recorded as having oriented in one direction or the other if they moved 60 cm or more from the center of the tunnel. This distance was chosen because it represented the halfway distance of the large tunnel (the distance used to define orientation in the volatile assays). This assay will allow me to observe whether weevils will walk 60 cm upwind in the absence of volatiles. Differences in upwind and downwind orientation were compared within sex and between sexes.

**Orientation of weevils to constitutive volatiles released from undamaged host plants.** Three experiments were conducted to quantify weevil orientation toward constitutive host plant
volatiles. The first experiment was carried out in the large wind tunnel. In this assay, a 3-month old pepper plant was placed upwind inside the large wind tunnel to see if male and female weevils would move upwind in the presence of both visual and olfactory stimuli. Male or female weevils were released in groups of 10 at the downwind end of the tunnel. Their location in the wind tunnel was recorded 15, 30 and 60 min after their release. Three orientation classes were recorded: weevils that passed halfway up the tunnel (60 cm upwind), weevils that reached the vicinity of the source (120 cm upwind) and those that made contact with the plant (SC). Male and female response to the visual and olfactory stimulus was compared.

In the second and third experiments, the small wind tunnels were used. In the second experiment, plant volatiles were piped in through both of the two olfactory ports in each chamber to remove visual stimuli. Ten males or females were released at the downwind end of the wind tunnel. Weevil location was recorded at the four time intervals and male and female response to the plant volatiles without visual stimuli was compared.

Trap catches were low in the assay without visual stimuli so, in the third experiment, one white trap cap was covered with yellow labeling tape presenting the weevils with a choice between a white (unattractive) trap and a yellow (potentially attractive) trap. Yellow has been previously shown to be an attractive color to pepper weevils in field trap assays (Riley & Schuster, 1994) and was investigated as a way to improve trap capture in the wind tunnel assays. In this assay, American black nightshade was used as the volatile source due to a disease infestation of our pepper plants at that time. Nightshade volatiles were found to be attractive to male and female pepper weevils in Y-tube assays (Chapter 6). Male and female overall response and response to the two trap colors was compared within and between sexes.
Orientation of weevils to induced volatiles released from pepper plants damaged by female pepper weevils. Two experiments were performed to examine the effect of plant damage on attraction to pepper volatiles. Assay conditions were changed to no-choice tests with glue board traps after previous assays with vial traps resulted in low numbers of weevils entering the traps. In the first experiment, two treatments were presented to weevils in a no-choice assay: weevils were presented with 1) volatiles from an undamaged pepper plant or 2) volatiles from a pepper plant that had sustained 72 h of feeding damage by female weevils. To inflict the damage, five female weevils were confined to a single branch of the plants using 4-l Ziploc® vegetable bags (S. C. Johnson & Son, Inc., Racine, Wisconsin) closed with a twist tie (Fig. 7-2). Insects were removed 1 h prior to use in the assay to allow plant wounds to close. Male and female overall response and trap captures were compared between the two treatments and sexes.

In the second experiment, both plants had sustained 72 h of feeding damage by five female weevils. In one treatment, the female weevils were removed 1 h prior to the start of the assay. In the other treatment, females were permitted to continue to actively feed on the plants. Again, male and female weevils were assayed in no-choice tests, and overall response and trap captures were compared within and between sexes.

Orientation of weevils to induced volatiles released from pepper plants damaged by male or female pepper weevils. Male weevils are known to produce an aggregation pheromone that attracts other weevils in the field (Coudriet & Kishaba, 1988; Patrock et al., 1992; Eller et al., 1994). Therefore, I wished to evaluate male and female response to male or female active feeding to determine if the sex of weevils feeding on a plant alters its attractiveness. Weevils were presented a pepper plant with males feeding or a plant with females feeding. Five male or female weevils were confined to a single branch of the plants using 4-l Ziploc® vegetable bags
closed with a twist tie for 24 h. Bags were removed prior to use in the assay but insects were allowed to continue feeding. Male and female response to male feeding and female feeding was compared within and between sexes.

A separate feeding assay was conducted to compare the amount of feeding damage produced by male and female weevils. Twelve male and female weevils were placed individually into a 1.5 × 8.5 cm diameter Petri dish (Fisher Scientific, St. Louis, MO) with a screen lid. The weevils were presented with a leaf sachet made from one rolled up pepper leaf wrapped in Parafilm® (Pechiney Plastic Packaging Company, Chicago, IL). Weevils were allowed to feed for 24 h and the number of feeding punctures made by males and females were compared.

**Data Analysis**

Wind tunnel data were analyzed as percent response using chi-squared analysis (SAS 2006). Feeding puncture counts were analyzed with a Wilcoxon-rank sum.

**Results**

**Wind Tunnel Orientation in the Absence of Odors**

Pepper weevils occasionally made short distance flights from the release point and sometimes from floor to ceiling (or vice versa); however, the majority of movement in the absence of odors was through walking. A significantly greater percentage of females had moved downwind (27%) rather than upwind (5%) at 15 min (χ²(1) = 15.12, P < 0.0001) and 30 min (47.5% vs. 5%; χ²(1) = 34.40, P < 0.0001) after release. There was no difference in male location 15 min after release (12.5% downwind vs. 7.5% upwind; χ²(1) = 1.25, P < 0.2636), but after 30 min a larger percentage of males had moved downwind (25%) than had moved upwind (12.5%) (χ²(1) = 4.17, P < 0.0412).
Orientation of Weevils to Constitutive Volatiles Released from Undamaged Pepper Plants

When a pepper plant was placed in the large wind tunnel, female total upwind response increased over time with significant differences observed among the 15, 30 and 60 min time intervals ($\chi^2(2) = 9.13$, $P < 0.0104$; Table 7-1). The upwind response of males also increased with time ($\chi^2(2) = 10.77$, $P < 0.0046$). There was no difference between male and female total responses to pepper volatiles at 15 ($\chi^2(1) = 0.11$, $P < 0.7416$), 30 ($\chi^2(1) = 3.46$, $P < 0.0628$) and 60 min ($\chi^2(1) = 1.45$, $P < 0.2280$). Slightly more males than females made contact with the volatile source at 60 min (SC) ($\chi^2(1) = 3.67$, $P < 0.0555$).

In small wind tunnels, total response of female ($\chi^2(3) = 53.06$, $P < 0.0001$; Table 7-2) and male weevils ($\chi^2(3) = 58.11$, $P < 0.0001$) to pepper volatiles increased with time. There was no difference in total response of males and females after 300 min ($\chi^2(1) = 0.03$, $P = 0.8651$). However, more females than males were in contact with the odor source at 300 min ($\chi^2(1) = 4.26$, $P = 0.0391$).

When weevils were given a choice between two trap colors (yellow and white), both male ($\chi^2(3) = 73.34$, $P < 0.0001$; Table 7-3) and female ($\chi^2(3) = 59.97$, $P = < 0.0001$) total response increased with time and there was no difference between sexes in total response at 300 min ($\chi^2(1) = 0.00$, $P = 0.9499$). Females preferred yellow traps ($\chi^2(1) = 8.69$, $P = 0.0032$) while males showed no preference ($\chi^2(1) = 0.00$, $P = 1.0$). Similar percentages of males and females made contact with white traps ($\chi^2(1) = 0.02$, $P = 0.8848$), but more females made contact with yellow traps ($\chi^2(1) = 9.43$, $P = 0.0021$). These results must be taken with caution due to the very low number of weevils that actually made contact with the traps (nine females and four males).
Orientation of Weevils to Induced Volatiles Released from Pepper Plants Damaged by Female Pepper Weevils.

There was no difference in total upwind response at 300 min to the damaged and undamaged treatments by females ($\chi^2_{(1)} = 2.19, P = 0.1389$; Table 7-4) but a higher percentage of males responded to damaged plants ($\chi^2_{(1)} = 6.04, P = 0.0139$). However, more females contacted the odor source in the damaged treatment than in the undamaged treatment ($\chi^2_{(1)} = 10.51, P = 0.0012$) but no difference was observed for males ($\chi^2_{(1)} = 0.97, P = 0.3237$). More females than males contacted the odor source in the damaged treatment ($\chi^2_{(1)} = 6.58, P = 0.0103$) but equal numbers made source contact in the undamaged treatment ($\chi^2_{(1)} = 0.07, P = 0.7913$).

In the second experiment, males and females did not differ in total response to the damaged ($\chi^2_{(1)} = 1.15, P = 0.2831$; Table 7-5) or active-feeding treatments ($\chi^2_{(1)} = 2.28, P = 0.1306$) at 300 min. More males than females made source contact in the active-feeding treatment ($\chi^2_{(1)} = 5.89, P = 0.0152$) but unlike the previous assay, more males also made source contact in the damaged treatment ($\chi^2_{(1)} = 4.87, P = 0.0274$). There was a significantly greater total upwind response for both females ($\chi^2_{(1)} = 7.06, P = 0.0079$) and males ($\chi^2_{(1)} = 9.55, P = 0.0020$) in the active-feeding treatment as compared to the damaged treatment with insects removed. Trap capture was also significantly higher for both males ($\chi^2_{(1)} = 6.11, P = 0.0135$) and females ($\chi^2_{(1)} = 5.07, P = 0.0244$) in the active-feeding treatment when compared to the damaged treatment. The lower overall response of weevils in this assay compared to others might be attributable to unfavorable weather conditions. For two of the four days of this experiment, a storm front resulted in poor ambient light and low pressure, which may have reduced the weevils’ willingness to walk.
Orientation of Weevils to Induced Volatiles Released from Pepper Plants Damaged by Male or Female Pepper Weevils.

Female total response at 300 min was the same for both the ‘female feeding’ and ‘male feeding’ treatments ($\chi^2_{(1)} = 0.17, P = 0.6798$; Table 7-6) and increased over time for both treatments ($\chi^2_{(3)} = 85.12, P < 0.0001$). Male total response at 300 min was the same for both the ‘female feeding’ and ‘male feeding’ treatments ($\chi^2_{(1)} = 0.77, P = 0.3803$) and increased over time for both treatments ($\chi^2_{(3)} = 77.16, P = < 0.0001$). More females, and marginally more males, contacted the odor source in the ‘female feeding’ treatment than the ‘male feeding’ treatment (females: $\chi^2_{(1)} = 8.26, P = 0.0040$; males: $\chi^2_{(1)} = 3.48, P = 0.0622$). Females and males made source contact at the same frequency in the ‘female feeding’ ($\chi^2_{(1)} = 0.01, P = 0.9347$) and ‘male feeding’ treatments ($\chi^2_{(1)} = 1.24, P = 0.2651$). At 300 min, there was no difference between male and female total response in both the ‘male feeding’ ($\chi^2_{(1)} = 0.00, P = 0.9539$) and ‘female feeding’ treatment ($\chi^2_{(1)} = 0.17, P = 0.6838$). The feeding puncture experiment confirmed equivalent levels of feeding damage produced by male and female weevils (female feeding punctures = $7.58 \pm 1.1$ SE, male feeding punctures = $7.83 \pm 0.9$ SE; $Z = -0.0875, P = 0.9302$).

Discussion

Previous investigations into short-range volatile attraction demonstrated that the pepper weevil can discriminate between the volatiles of different host and non-host plants (Chapter 6). While short-range studies offer us some information about pepper weevil preferences, a wind tunnel gives us the opportunity to observe weevil behavior on a larger scale. One concern about insect behavior in a wind tunnel is whether the insects are cueing in on attractive volatiles or if they are merely positively anemotactic. I demonstrate here that in the absence of plant volatiles, both male and female weevils are more inclined to move downwind or remain stationary than to
move upwind. Therefore, the upwind response observed in the wind tunnel assays with plants can be attributed to attraction to plant volatiles.

In the three constitutive volatile assays, both males and females oriented upwind whether the plant source was visible or not. There was no difference in the total numbers of males and females orienting to the plant volatiles. However, males made source contact when the volatile source (pepper plant) was inside the wind tunnel, while more females made contact when the source of plant volatiles was not visible.

We already know that pepper weevil behavior can be manipulated using visual cues. An investigation into the influence of color on trap efficiency found that more pepper weevils were caught on yellow sticky traps (Riley & Schuster, 1994). Since the weevils did not fly in the wind tunnel, the hanging vial traps were difficult for them to reach. Weevils were observed climbing down the volatile port tube from the ceiling and walking onto the trap. Sometimes the weevils made it into the trap but often they remained ‘sitting’ on the outside. Often the weevils would fly back up to the ceiling after failing to locate the source of the volatiles. An attempt was made to improve trap capture by placing yellow tape on one of the two traps inside the tunnel. While the results of that assay appear to indicate females have a greater affinity for the yellow traps, this conclusion must be taken with caution due to extremely low numbers of weevils captured in the assays. Riley & Schuster (1994) also observed more females than males captured by yellow sticky traps relative to females and males counted in terminal buds. The authors offered three hypotheses for female-biased capture using the yellow traps including (1) greater female attraction to the trap color, (2) female response to males on traps (i.e., hypothesizing that females are attracted to the male-produced aggregation pheromone), and (3) greater flight activity of females, causing them to encounter traps at a higher frequency. I can discount attraction by
males in this study as insects were assayed separately by sex. Also, as stated previously, weevils spent the majority of time in the wind tunnel walking. Since no differences in male and female total upwind response were observed, their ‘activity levels’ are arguably similar. The only difference between the sexes appears to be in their ability to locate the source volatile trap. Since the same number of males and females entered the white traps while more females than males entered the yellow trap, I can conclude that female attraction to that color is the reason for the observed differences. Subsequent assays were conducted using white traps and glue boards due to concern over the unequal stimulatory properties of the color yellow to the different sexes.

Male and female weevils were clearly attracted to constitutive host plant volatiles as observed in previous Y-tube assays (Chapter 6) but short-range assays were not useful for determining how pepper weevils would respond to induced plant volatiles or a combination of induced volatiles and male-produced aggregation pheromone (unpublished data). When female-damaged and undamaged plants were compared in no-choice tests, male and female weevils were attracted to damaged plants. Both sexes preferred plants with actively feeding female weevils over plants with prior feeding damage. Despite a similar overall response to the ‘male’ and ‘female feeding’ treatments, females were trapped more often in the ‘female feeding’ treatment, not the treatment with males presumably producing aggregation pheromone. Males were also captured in slightly greater numbers in the ‘female feeding’ treatment. There are several possible explanations for these results. First, it is possible that the males were not producing pheromone and that more plant damage was present in the ‘female feeding’ treatment, making it more attractive. I did not analyze the headspace volatiles during these experiments; however, I did collect volatiles from pepper plants with male and female weevils feeding under the same experimental conditions on a separate occasion and could not detect the aggregation
pheromone. This could mean that the weevils were not producing it at all or that not enough frass had built up over the 24 h period to be detected by our GC analysis. Additionally, the feeding assay using leaf sachets showed no differences in the number of feeding punctures made by male and female weevils, suggesting that feeding damage was not the cause of the observed differences. A second possibility is that the weevils used in this study may no longer be sensitive to the aggregation pheromone. If, for example, the pheromone is used by females solely to locate mates in the field, the mated females (10 – 20 d old) used in these assays may not be attracted to a male aggregation, preferring the patch without males. Jang (1995) observed that female Mediterranean fruit flies, *Ceratitis capitata* (Wiedemann), preferred male pheromone over host volatiles prior to mating and then shifted olfactory preferences to host volatiles after mating. Male pepper weevils on the other hand, showed only a slight preference for the ‘female feeding’ treatment (P = 0.0622). If feeding damage in the ‘female feeding’ treatment had been significantly greater, males might be expected to show a stronger response. Finally, our experimental design means that the 10 weevils assayed in each replication were held together overnight in a small vial. If these males were exposed to pheromone contaminated frass during this period, they may have become habituated to the pheromone and therefore unable to respond to it when released in the wind tunnel.

While it is clear that males and females prefer some treatments over others, comparing male and female responses to the same treatment is a bit more difficult. In some instances, females made source contact more often than males and vice versa, but these results were not consistent. More males made contact with a pepper plant sitting inside a wind tunnel while more females made contact with the olfactory port traps in the small wind tunnel. When offered a damaged plant under no-choice conditions, females made more contact with the volatile source
in the first assay (damaged or undamaged plants) but more males made contact in the second assay (damaged plant or active feeding). These shifts in apparent sensitivity of males and females to the same treatment conditions may have a biological cause or be due to statistical error. Increasing the number of weevils assayed should decrease the amount of variation in the data.

If plant volatiles induced by active female feeding increase attraction to a source, the identification of these compounds may aid in improving currently available traps. A commercial pepper weevil trap comprised of a combination yellow sticky trap and aggregation pheromone lure (Pherocon®, Trécé Inc, Adair, OK) is currently available. A trap with a combination (or choice) of attractive plant volatiles and pheromone might be a more effective and flexible monitoring tool, especially if gravid females are found to ignore the aggregation pheromone. These traps would work best early in the cropping season or around fields during the fallow season. More investigation is needed to understand the changes in attraction to plant volatiles and aggregation pheromone with insect age before a targeted trapping program can be developed.

Pepper weevil damage to plants appears to aid both pepper weevils and their parasitoids in host location. In a recent field cage study of the pepper weevil parasitoid, *Triaspis eugenii* Wharton and Lopez-Martinez, the wasp parasitized more eggs when infested sentinel fruit were hung from plants with weevil feeding damage (57.6%) than when infested fruit were hung from undamaged plants (26.4%) (Rodriguez-Leyva, 2006). If new traps baited with induced plant volatiles are developed, careful attention must also be paid to changes in the behavior of biological control agents. While the presence of such traps in the field may increase the number
of parasitoids in the field, it may also cause those same insects to become trapped alongside their weevil hosts.

There are three areas of investigation that are sorely lacking in the pepper weevil literature. First, a greater understanding of pepper weevil movement and behavior in the field is necessary to develop new and accurate trapping techniques. Second, stimulatory plant volatiles must be identified using GC-EAD and behavioral assays must be developed to identify new attractants for use in the field. Finally, a clearer understanding on the role of weevil age and mating status in response to aggregation pheromone and plant volatiles is needed if we are to know when such attractants should be employed. The combination of a strong attractant such as a combination pheromone/plant volatile lure and a strong deterrent (e.g., the oviposition deterring pheromone identified in Chapter 4) can be used together in a push-pull strategy for pepper weevil management.
Figure 7-1. Large and small wind tunnels used to determine response of pepper weevil to plant volatiles

Figure 7-2. Feeding damage treatment and plant cylinder setup
Figure 7-3. Wind tunnel traps (a) cylinder traps and (b) sticky board trap

Figure 7-4. Weevils released from vial
Table 7-1. Percent upwind response of male and female pepper weevils in the large wind tunnel containing an uninfested pepper plant

<table>
<thead>
<tr>
<th>Observation time&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Orientation class&lt;sup&gt;2&lt;/sup&gt; (percent response)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
</tr>
<tr>
<td></td>
<td>60 cm</td>
</tr>
<tr>
<td>15 min</td>
<td>10.0</td>
</tr>
<tr>
<td>30 min</td>
<td>2.5</td>
</tr>
<tr>
<td>60 min</td>
<td>15.0</td>
</tr>
</tbody>
</table>

<sup>1</sup> Weevils were recorded into various orientation classes at different time intervals after release of 10 individuals from the holding vial.

<sup>2</sup> Weevils were recorded as having moved 60 or 120 cm upwind. Source contact (SC) was recorded if weevils made contact with the plant. For both males and females, n = 10, rep = 4.
Table 7-2. Upwind orientation response of male and female pepper weevils in a small wind tunnel to pepper volatiles without visual plant stimuli

| Observation time | Female | | | | Male | | | | 
|------------------|--------|---|---|---|--------|---|---|---|---|
|                  | 45 cm  | 90 cm | SC | Total response | 45 cm | 90 cm | SC | Total response |
| 15 min           | 15.0   | 12.5  | 2.5 | 30.0            | 15.0   | 7.5   | 0  | 22.5            |
| 30 min           | 12.5   | 7.5   | 0   | 20.0            | 17.5   | 12.5  | 0  | 30.0            |
| 60 min           | 15.0   | 10.0  | 2.5 | 27.5            | 7.5    | 7.5   | 2.5 | 17.5            |
| 300 min          | 5.0    | 15.0  | 57.5| 77.5            | 9.3    | 28.6  | 37.4| 75.4            |

1 Weevils were recorded into various orientation classes at different time intervals after release of 10 individuals from the holding vial.

2 Weevils were recorded as having moved 45 or 90 cm upwind. Source contact (SC) was recorded if weevils entered the vial traps. For both males and females, n = 10, rep = 4.
Table 7-3. Upwind response of pepper weevil to nightshade volatiles and capture in traps with either yellow or white lids in small wind tunnel

<table>
<thead>
<tr>
<th>Observation time(^1)</th>
<th>Orientation class(^2)</th>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>45 cm</td>
<td>90 cm</td>
</tr>
<tr>
<td>15 min</td>
<td></td>
<td>10.0</td>
<td>7.5</td>
</tr>
<tr>
<td>30 min</td>
<td></td>
<td>5.0</td>
<td>12.5</td>
</tr>
<tr>
<td>60 min</td>
<td></td>
<td>0</td>
<td>12.5</td>
</tr>
<tr>
<td>300 min</td>
<td></td>
<td>2.8</td>
<td>35.2</td>
</tr>
</tbody>
</table>

\(^1\) Weevils were recorded into various orientation classes at different time intervals after release of 10 individuals from the holding vial.

\(^2\) Weevils were recorded as having moved 45 or 90 cm upwind. Source contact (SC) was recorded if weevils entered the vial traps. For both males and females \( n = 10 \), rep =
Table 7-4. Upwind response of pepper weevil to volatiles released from undamaged pepper plants or those damaged by female weevils in no-choice tests in small wind tunnels

<table>
<thead>
<tr>
<th>Observation time¹</th>
<th>Orientation class²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
</tr>
<tr>
<td></td>
<td>Undamaged</td>
</tr>
<tr>
<td></td>
<td>45 cm 90 cm SC</td>
</tr>
<tr>
<td></td>
<td>Total response</td>
</tr>
<tr>
<td>15 min</td>
<td>15.0 15.0 0</td>
</tr>
<tr>
<td></td>
<td>30.0</td>
</tr>
<tr>
<td>30 min</td>
<td>12.5 12.5 0</td>
</tr>
<tr>
<td></td>
<td>25.0</td>
</tr>
<tr>
<td>60 min</td>
<td>7.5 15.0 10.0</td>
</tr>
<tr>
<td></td>
<td>32.5</td>
</tr>
<tr>
<td>300 min</td>
<td>25.5 20.9 14.7</td>
</tr>
<tr>
<td></td>
<td>61.1</td>
</tr>
</tbody>
</table>

|                   | Male              |
|                   | Undamaged         | Damaged         |
|                   | 45 cm 90 cm SC    | 45 cm 90 cm SC |
|                   | Total response    | Total response  |
| 15 min            | 15.0 2.5 2.5      | 22.5 2.5 2.5   |
|                   | 20.0              | 27.5            |
| 30 min            | 12.5 2.5 2.5      | 15.0 12.5 0    |
|                   | 17.5              | 27.5            |
| 60 min            | 7.5 7.5 5.0       | 17.5 17.5 0    |
|                   | 20.0              | 35.0            |
| 300 min           | 16.0 8.1 13.3     | 13.1 29.9 18.9 |
|                   | 37.4              | 61.9            |

¹ Weevils were recorded into various orientation classes at different time intervals after release of 10 individuals from the holding vial.

² Weevils were recorded as having moved 45 or 90 cm upwind. Source contact (SC) was recorded if weevils entered the vial traps or were captured on the glue board. For both males and females, n = 10, rep = 4.
Table 7-5. Upwind response of pepper weevil to volatiles released from female weevil-damaged plants with insects removed or insects actively feeding in no-choice tests in small wind tunnels

<table>
<thead>
<tr>
<th>Observation time¹</th>
<th>Orientation class²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
</tr>
<tr>
<td></td>
<td>Damage</td>
</tr>
<tr>
<td></td>
<td>45 cm 90 cm SC</td>
</tr>
<tr>
<td>15 min</td>
<td>5.0 12.5 0</td>
</tr>
<tr>
<td>30 min</td>
<td>7.5 17.5 0</td>
</tr>
<tr>
<td>60 min</td>
<td>2.5 12.5 0</td>
</tr>
<tr>
<td>300 min</td>
<td>7.5 7.5 7.5</td>
</tr>
</tbody>
</table>

|                   | Male              |
|                   | Damage            | Active feeding |
|                   | 45 cm 90 cm SC |  Total response | 45 cm 90 cm SC | Total response |
| 15 min            | 10.0 10.0 0     | 20.0           | 15.0 10.0 0    | 25.0           |
| 30 min            | 15.0 10.0 2.5   | 27.5           | 12.5 17.5 0    | 30.0           |
| 60 min            | 10.0 7.5 10.0   | 27.5           | 12.5 20.0 2.5  | 35.0           |
| 300 min           | 5.0 5.0 20.3    | 30.3           | 10.0 12.2 37.4 | 59.6           |

¹ Weevils were recorded into various orientation classes at different time intervals after release of 10 individuals from the holding vial

² Weevils were recorded as having moved 45 or 90 cm upwind. Source contact (SC) was recorded if weevils entered the vial traps or were captured on the glue board. For both males and females, n = 10, rep = 4.
Table 7-6. Upwind response of pepper weevils to volatiles released from females or males feeding on pepper plants feeding in no-choice tests in small wind tunnels

<table>
<thead>
<tr>
<th>Observation time¹</th>
<th>Orientation class²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
</tr>
<tr>
<td></td>
<td>Female feeding</td>
</tr>
<tr>
<td></td>
<td>45 cm 90 cm SC Total response</td>
</tr>
<tr>
<td>15 min</td>
<td>10.0 15.0 0 25.0</td>
</tr>
<tr>
<td>30 min</td>
<td>12.0 20.0 2.5 34.5</td>
</tr>
<tr>
<td>60 min</td>
<td>15.0 20.0 10.0 45.0</td>
</tr>
<tr>
<td>300 min</td>
<td>8.1 10.9 59.9 78.9</td>
</tr>
</tbody>
</table>

¹ Weevils were recorded into various orientation classes at different time intervals after release of 10 individuals from the holding vial

² Weevils were recorded as having moved 45 or 90 cm upwind. Source contact (SC) was recorded if weevils entered the vial traps or were captured on the glue board. For both males and females, n = 10, rep = 4.
Small modifications to the standard pepper weevil artificial diet did not improve important factors like survival or egg hatch. Despite these disappointing results, a few important pieces of information came out of the diet experiments. In our egg hatch assays, weevil eggs had poor hatch rates when placed on artificial diet, however, hatch on sterilized paper towels (93%) was close to that of naturally laid eggs (98%) and well above the best diet (70%). If eggs can be permitted to incubate for a day or two on a paper towel and then laid on an artificial rearing media it could significantly improve early stage mortality rates. Another interesting result was that weevils fed on artificial diet (control, 5%, 10% and 20% pepper) were heavier than those emerging from pepper fruit and appeared healthy. This would suggest that the nutritional quality of the diet, though possibly low in some essential nutrients, was not the cause of the high mortality. Other factors such as diet texture may be affecting survival to adulthood. Both eggs and larvae were observed floating in pockets of liquid on the surface of the diet. Decreasing the moisture content of the diet might improve survival of the weevils in these early stages.

Pepper weevils preferred to oviposit in spherical substrates covered in Parafilm or netting. Feeding and oviposition were induced in the laboratory in artificial fruit made from pepper leaves or the leaves of some alternate solanaceous plants (American black nightshade, eggplant, potato and jasmine tobacco). Other solanaceous (tomato and tropical soda apple) and non-solanaceous (bean and cotton) plants were rejected as food and oviposition sites. The reason why some non-host plants in the Solanaceae elicited oviposition while others did not is not known but it is possible that plant chemistry plays a role in the weevil’s decision making. The overall lack of feeding initiation in bean and cotton sachets suggest that the volatile cues required
to recognize a potential food or oviposition source were not present in these species. The solanaceous host plants. Differences in surface waxes, alkaloids or other solanaceae-specific secondary metabolites may explain the acceptance of some of the species tested and the rejection of others.

**Host Marking Pheromone**

Females pepper weevils have been observed depositing a clear anal secretion that hardens into an “oviposition plug” over their oviposition hole. In choice and no-choice tests, females preferred clean fruit to fruit that were infested with four eggs, whether the fruit contained conspecific eggs or their own eggs. Further bioassays demonstrated that the presence of female frass or oviposition plugs alone, in the absence of eggs or any fruit damage, was sufficient to deter oviposition. In addition, females given the choice between an infested fruit with the oviposition plug removed or an unaltered infested fruit preferred the fruit with no plugs, even when eggs, frass and feeding damage were still present. To determine whether females would avoid infested peppers under more natural conditions, I quantified oviposition on infested and uninfested sentinel pepper fruit within individually caged plants and on clean and infested plants caged together. Females consistently laid more eggs on clean fruit than on infested fruits and moved within and among pepper plants to search for more acceptable oviposition sites. I concluded that oviposition plugs, along with contaminated female, but not male, frass contain a deterrent which, in the absence of any other cue, is enough to alert a female that a patch is occupied. This allows the females to make an informed decision on where to deposit eggs and to judge the level of competition her offspring will experience within the fruit. The rate of encounter with the marking pheromone may also give the weevil an idea of the level of infestation in a field, allowing her to alter her egg laying strategies. If the female has a low rate of encounter with the marking pheromone she may be more willing to space out her eggs on
separate hosts. A higher rate of encounter can increase the number of eggs she deposits in a clean host. The female may also be more willing to deposit eggs in a previously infested fruit if no unmarked hosts are available. The type of information female pepper weevils gain from the marking pheromone and how it alters behavior in the field needs to be determined before the pheromone can be used in pest management.

Once I determined that the marking pheromone was present in the oviposition plug, I proceeded to isolate and identify the active components of the pheromone. The compounds were isolated by bioassay-directed differential solvent extraction, silica column separation, HPLC fractionation, and GC-MS. Active fractions were obtained by passing a pentane extract of the oviposition plugs through a silica column and eluting the active compounds with a 70:30 ratio of pentane and ethyl acetate. Three active fractions were isolated by HPLC gradient elution. These active fractions were eluted at 15-16 min (fraction 6), 17-18 min (fraction 7) and 24-25 min (fraction 10).

**Host Plant Volatiles**

A series of Y-tube assays were carried out to investigate the importance of plant volatile cues in host location by pepper weevil. Ten-day-old males and gravid females as well as 2-day-old virgin females were assayed in single choice tests with three host plants (pepper, nightshade and eggplant) and two non-host plants (tomato and lima bean). All three classes of insects oriented to pepper, nightshade, eggplant and tomato. Attraction to tomato volatiles is interesting since females did not accept rolled up tomato leaves in the feeding/oviposition assay nor was any oviposition observed by Patrock & Schuster’s (1992) host plant survey. This points to a similarity in the volatile plumes of both host and non-host plants within the genus *Solanum*. Interestingly, only 2-day-old females oriented to lima bean—a non-host plant outside the solanaceae. Ten-day-old males and females were not attracted to bean volatiles, suggesting that
newly emerged weevils cannot yet discriminate between volatile plumes of various plant species, while older weevils will ignore the volatiles from a non-host plant unrelated to its hosts. In pair-wise assays comparing the three host plants, 10-day-old males preferred pepper over eggplant and 10-day-old females preferred pepper over nightshade. Two-day-old females displayed no preferences for any plant in the multiple pair-wise tests, strengthening the hypothesis that newly emerged weevils are not able to discriminate between volatile plumes. All classes of insects were attracted to fruit volatiles from the three host plants but only eggplant and nightshade flowers were attractive.

I conclude that pepper weevils do detect and orient to host plant volatiles, males and females have different preferences for the volatiles of certain plants, and that newly emerged virgin females differ in their response to plant volatiles than 10-day-old gravid females. The differences in weevil response by sex may be due to the different resource requirements of males and females. The change in female response with age may be attributed to different life history requirements or developmental changes. Newly emerged females are primarily seeking mates and feeding locations while older, mated females are in search of oviposition sites. Over time, maturation and functional changes in olfactory neurons may alter the insect’s sensitivity to different types of volatiles as was observed in the boll weevil.

In order to further investigate the influence of plant volatiles and male-produced aggregation pheromone in a more natural environment, a series of wind tunnel assays was conducted. Male and female weevils moved downwind when no olfactory stimulus was present. Both sexes oriented upwind to constitutive and induced plant volatiles, but movement to the source of odors at the most upwind end of the tunnel and capture in traps there differed, depending on treatment conditions. More males than females made source contact when a
pepper plant was present inside the wind tunnel, adding a visual stimulus and a more dispersed source of volatiles. Females made source contact more often than males when no visual stimulus was provided, when vial traps had yellow caps, and when exposed to damaged plants. Both males and females made source contact more often and had a greater upwind response to plants with actively feeding females when compared to plants with old feeding damage but no active feeding. Females also made source contact more often than males in the ‘female feeding’ treatment (no male-produced aggregation pheromone) in the pheromone assay. Males showed a slight preference for the ‘female feeding’ over the ‘male feeding’ treatment but the difference was not significant. These results conflict with previous research on the attractiveness of the aggregation pheromone in the field. There are several reasons why I might see different results in the wind tunnel assay. First, males in the treatment may not have been producing the aggregation pheromone or the level of pheromone production was too low for the weevils in the assay to detect, if this is the case, the ‘female feeding’ treatment may have simply had more feeding damage making that treatment more attractive. A second option is that the pheromone was present, but the insects in the assay were ignoring the signal or repelled by it. Field tests of the aggregation pheromone did not evaluate the age/mating status of the weevils reaching the traps. If female pepper weevils are only attracted to the pheromone prior to mating, it would explain why field tests caught many females (those seeking mates) while our assay showed a deterrent effect on females (females previously mated). In fact, it may be in the best interest for gravid females to seek out plants without an aggregation as there is a better chance of finding uninfested hosts. The response of males, on the other hand, is more difficult to explain. Males showed no significant preference for the ‘male’ or ‘female feeding’ treatments. This could be due to the absence of pheromone or an artifact of the experimental design. The male weevils
were held overnight in a container in groups of ten and so may have been habituated to the pheromone if the frass produced by the weevils in the container contained pheromone. Alternately, if no pheromone was produced, the slight preference for the ‘female feeding’ treatment may be due to a difference in the level of feeding damage. A more detailed understanding of weevil response to the aggregation pheromone is required to determine which of these explanations is correct.

**Future Directions**

There is still a great deal of research needed on pepper weevil ecology. If an artificial rearing system is to be developed for this weevil, more work needs to be done to identify feeding and oviposition stimulants. One approach is to obtain a chemical profile of the pepper seed and placental material normally fed on by the weevil larvae. Despite the fact that weevils emerging from the control diet were heavier than those reared from peppers, the diet may still lack a key feeding stimulant responsible for the limited survival on diet. Therefore, a deeper investigation into chemical stimulants for feeding and oviposition may be necessary to increase diet acceptance by larvae and adults. We already know that pepper weevils will oviposit on the rolled up leaves of some solanaceous plants, despite the fact that the pepper weevil does not lay eggs on leaves. The acceptance of these leaves indicates the presence of stimulatory compounds in these plant parts that, together with a spherical shape, induce oviposition. The first step would be to analyze the stimulatory effects of leaf surface compounds. If the surface waxes have no effect on oviposition, an analysis of whole leaf extracts can address the effect of internal chemistry. Solanaceous plants are known for producing a wide array of alkaloids that may enable the weevils to recognize its host, and/or stimulate feeding and oviposition.

The identification of the marking pheromone components is only the first step in the development of the pheromone as a management tool. Once the chemical structure of the
pheromone is known with some certainty, a synthetic version must be tested to verify its activity. When the active compound(s) have finally been verified, they must then be evaluated in larger scale greenhouse and field trials to determine if the pheromone can be used to manipulate weevil behavior. Further, the formulation may need to be altered to improve stability through the addition of adjuvants. Finally, an application protocol must be developed to maximize the pheromone’s effectiveness in an integrated pest management program.

Attractive host plant volatiles offer another potential area of research that may improve pepper weevil pest management. Gas chromatography-electroantennography (GC-EAD) can be used to identify active plant volatiles. The attractiveness of these compounds can then be evaluated in a Y-tube or wind tunnel. These plant attractants can then be tested alone or in combination with the aggregation pheromone as a trapping lure. The ultimate goal is to combine the oviposition deterrent with an improved pheromone/host plant volatile lure into a push-pull trapping system. This management tool has potential to prevent weevil movement into fields early in the season. A stronger lure may also be helpful in trapping weevils spending the fallow season in nightshades surrounding pepper fields.
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BIOGRAPHICAL SKETCH

Karla Michele Addesso was born in 1980, in Edison, New Jersey, USA to Janet and Louis Addesso. She earned a Bachelor of Science degree in Biology from The College of New Jersey (formerly Trenton State College), Ewing, NJ. After graduation, she worked 1 year as a Biological Aide at the New Jersey Department of Plant Industry, Phillip Alampi Beneficial Insect Rearing Laboratory in Trenton, NJ. In fall 2003, she began a PhD in the Entomology and Nematology Department at the University of Florida. She began work on pepper weevil, under the supervision of Dr. Heather J. McAuslane. Her research focused on pepper weevil artificial diets, oviposition stimulants and deterrents as well as host plant volatile attraction. She won several awards for papers presented at the Florida Entomological Society, the Entomological Society of America and the Southeastern Branch of the Entomological Society of America. She was also active in departmental and campus clubs, serving as President for the Entomology and Nematology Student Organization, as well as the Campus Unitarian Universalists. She also volunteered as co-chair for the Graduate Student Council’s Committee for Financial Affairs. She plans to continue working in the fields of chemical ecology and pest management.