BIOLOGY AND BEHAVIOR OF *DIAPHORENCYRTUS ALIGARHENSIS* (HYMENOPTERA: ENCYRTIDAE) AN ENDOPARASITOID OF *DIAPHORINA CITRI* (HEMIPTERA: PSYLLIDAE)

By

ERIC ROHRIG

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To my parents and my wife, Mary
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In September of 2006, a population of *Diaphorencyrtus aligarhensis* (Shafee, Alam & Argarwal) (Hymenoptera: Encyrtidae) was collected in Guangdong province China and sent to Florida. *D. aligarhensis* is a host specific koinobiont parasitic wasp of *Diaphorina citri* Kuwayama (Homoptera: Psyllidae), the vector of citrus greening disease or Huanglongbing (HLB). This population is Thelytokous and tested positive for infection with the eubacteria endosymbiont *Wolbachia*. Relative humidity and temperature constraints on *D. aligarhensis* were determined and appropriate for release in Florida. Over 10,000 Adult parasitoids were released over 26 months in 7 Florida counties (Alachua, Collier, DeSoto, Hendry, Lake, Orange and Polk). Several adults were recovered but establishment could not be confirmed.

*D. aligarhensis* exhibited a weak olfactory response to host excretions and host infested plant volatiles during y-tube olfactometer experiments. Wasps held with access to nymphs prior to release maintained a higher egg load, parasitized more and were more efficient at host searching and discrimination than host-deprived individuals. The larval development of *D. alighahensis* passed through four instars, a prepupal and pupal...
stage as timed and recorded using digital imaging. A previously undescribed adaptation of the developing wasp was the observation of an anally secreted glue-like substance used to anchor 3\textsuperscript{rd} and 4\textsuperscript{th} instar larvae to internal host tissue.

During competition studies with \textit{Tamarixia radiata} (Waterston) (Hymenoptera: Eulophidae), an imported and established obligate ecto-parasitoid of \textit{D. citri}, both species expended a similar amount of time to examine and parasitize a nymph upon encounter and laid comparable numbers of eggs. Neither species discriminated for oviposition against a nymph that was previously parasitized by the other, regardless of the time interval between parasitism events. Up to 30\% multiparasitism was seen at low host densities with only a single parasitoid emerging from each host. If both wasps chose the same host for oviposition, \textit{T. radiata} always emerged unless \textit{D. aligarhensis} laid its egg at least 5 days prior. The wide distribution of \textit{T. radiata} and its clear competitive advantage may provide at least partial explanation for the difficulty in establishing \textit{D. aligarhensis}.
CHAPTER 1
INTRODUCTION

Literature Review

This chapter reviews the basic biology of (1) the Asian citrus psyllid (ACP) \textit{Diaphorina citri} Kuyawama, (Hemiptera: Psyllidae) which transmits the pathogen responsible for citrus greening disease, and (2) two obligate parasitoids of the psyllid: \textit{Tamarixia radiata} (Waterston) (Hymenoptera: Eulophidae) and \textit{Diaphorencyrtus aligarhensis} (Shafee, Alam & Agarwal) (Hymenoptera: Encyrtidae). Citrus greening disease and disease management strategies including ACP pest management practices are also discussed. The final segment of this chapter outlines my research objectives.

Asian Citrus Psyllid (ACP) Overview

The Asian citrus psyllid (ACP), \textit{Diaphorina citri} Kuyawama, (Hemiptera: Psyllidae) is a serious pest of citrus and close relatives of citrus. \textit{D. citri} [=\textit{Euphalalus citri} (Kuwayama)] was first described from citrus in Shinchiku, Taiwan in 1907 and later published in 1908 (Kuwayama, 1908). \textit{Diaphorina citri} is one of approximately 13 known species of psyllids that attack citrus (Halbert & Manjunath, 2004). Psyllid feeding activities cause direct damage to trees but more importantly, ACP is one of two species of psyllid that have been shown to be efficient vectors of \textit{Candidatus Liberibacter} species, bacterial pathogens responsible for citrus greening disease. The other species is \textit{Trioza erytreae} (del Guercio) (Sternorrhyncha: Triozidae) in Africa.

Morphology

Adult ACP measure 2- 4 mm long and are a patchy brown and tan color. Psyllid sex can be determined by slight differences in abdominal segments (Husain & Nath, 1927). Terminal abdominal segments of females are shorter than males and are in-line
horizontally with the rest of the abdomen. Male terminal abdominal segments are perpendicular to the rest of the abdomen. Adult abdominal colors can vary in daily and seasonal patterns between gray/brown, blue/green, and orange/yellow and are of little value when determining sexual maturity or mating status of females (Wenniger & Hall, 2008). Adults of both sexes reach reproductive maturity 2–3 d after eclosion (Wenninger & Hall, 2007) and are capable of laying over 800 eggs in a lifetime (Mead, 1977). Yellowish-orange, tear shaped eggs are laid on the unfolded leaves, flower buds, leaf axils and the tips of newly expanding flush (Yang et al., 2006). Red eye spots become visible prior to hatching. At 25°C, eggs begin to hatch 4 days post lay (Chavan & Summanwar, 1993). Nymphs pass through five instars (Mead, 1977), 0.25 to 1.7 mm in length (Liu & Tsai, 2000), requiring 11-15 days to become adults at 25°C (Chavan & Summanwar, 1993). Depending on temperature, between 16- 49 days are needed to complete one life cycle from egg to adult (Liu & Tsai, 2000). Liu & Tsai (2000) examined ACP biology at various temperatures and reported that a range of 25-28°C was most favorable for development. When held at 28°C, psyllids laid a maximum average of 748.3 eggs per female, had the shortest mean generation time (28.6 days) and population doubling time (3.5 days) as well as the highest net reproductive rate (292.2) (Liu & Tsai, 2000). Adult female longevity increased with decreasing temperatures with a maximum of 117 days at 15°C, 56 days at 25°C and 51 days at 30°C. Psyllids nymphs held at either 10°C or 33°C did not develop. Tsai and Liu (2000) reported that ACP does not diapause and population numbers decrease during the winter months when new flush is unavailable. Aubert (1987) reported that D. citri does not endure high humidity (90~100% RH) or cold, particularly freezes. However, ACP has been reported to
maintain high population numbers during very high humidity in summer months and has overwintered in North central Florida during cold spells as low as -5°C (Halbert & Manjunath, 2004).

Nymphs begin to feed on sap from the phloem of soft new flush and may feed on harder stems as they mature. Adults are capable of feeding on mature hardened leaves in the absence of new flush. Adults generally sit on the undersides of leaves while resting or feeding where they maintain their body at a 45° angle relative to the leaf surface (Yang et al., 2006). High densities of nymphs can cause new growth to wither and break off leading to the death of immatures. Nymphs are pale yellow to tan colored, posses red eyes wing pads which increase in size with each instar. Due to the high volume of plant sap ingested, nymphs produce large amounts clear sticky honeydew encased in white wax. Adults are poor fliers, and when disturbed, will jump or fly short distances (Yang et al., 2006). Nymphs spend their development to adulthood moving slowly up or down the flush they are feeding on (Mead, 1977).

**Geographical Distribution**

ACP has a native range of tropical and subtropical Asia and can also be found throughout many tropical and subtropical fruit producing regions of the world including India, Réunion and Mauritius islands, Saudi Arabia, South America (da Graca, 1991), Pakistan, Afghanistan, Thailand, Myanmar (Mead, 1977), Iran (Bové et al., 2000) and Venezuela (Cermeli et al., 2000). Hollis (1987) proposed that ACP originates from the Indian subcontinent where it then spread throughout Asia. It was reported from Brazil in 1942 by Lima (1942). In 1998 the psyllid was found in the Caribbean on Guadeloupe Island (Étienne et al., 1998). Later that same year ACP was found along Highway 1 on the east coast of Florida in Broward, Palm Beach and Martin Counties in South Florida.
by the Division of Plant Industry, Florida Department of Agriculture and Consumer services (Hoy & Nguyen, 1998). By 2001 the psyllid’s range had spread to all citrus growing counties in Florida (Halbert et al., 2002). ACP’s range continues to expand and has since been reported from Texas (French et al., 2001), Arizona, Alabama, Georgia, South Carolina, California, Louisiana, Hawaii, Mississippi, Guam (USDA-APHIS-DA-2009-58), the Cayman and Bahamian Islands, Jamaica, Dominican Republic, Cuba, Puerto Rico and Mexico (Halbert & Nunaz, 2004).

It is not known exactly when and where the psyllid first entered the US. USDA/APHIS/PPQ reports several hundred interceptions of live ACP in US ports originating from Asia and India (Halbert & Manjunath, 2004). Most psyllids were recovered from Murraya sp. plants as well as various citrus species. Additionally the psyllid may have migrated north from South America through Central America and the Caribbean (Halbert & Manjunath, 2004). If the latter were true the psyllid would likely have arrived in California before 2008, when it was first detected there.

**Damage**

ACP population levels can reach large numbers particularly when new tree flush is abundant. Immature and adult ACP feed on sap from the phloem of young soft shoots and buds while adults can also feed on mature, hardened leaves (Yang et al., 2006). ACP damage or destroy new growth by depleting sap as well as injecting toxic saliva (McFarland & Hoy, 2001). This toxin can cause shoot and leaf damage and distortion (Mead, 1977). High densities of ACP nymphs on new shoots often leads to the death of the meristem resulting in reduced flush which can limit tree growth (Michaud, 2004). Additionally, psyllids secrete large quantities of honeydew promoting the growth of sooty mold which covers leaf surfaces inhibiting photosynthesis as well as tarnishing fruit.
appearance (Chien & Chu, 1996; Wang et al., 2001). This type of direct feeding damage is minimal compared to tree infection with the greening bacterium which leads to poor fruit production and severe dieback (McClean & Schwarz, 1970).

**Citrus Greening Disease**

Citrus greening disease (CG) is the most serious citrus disease in the world (McClean & Schwarz, 1970), affecting most major citrus growing regions in Africa, China, Taiwan, Vietnam, Indonesia (Moll & Van Vuuren, 1977; Tsai & Liu, 2000), India (Mead, 1977), the Philippines (Martinez & Wallace, 1967), Thailand (Eng, 2007), Iran (Bové et al., 2000) and Saudi Arabia (Child & Roberts, 2005). Greening was first detected in the western hemisphere in Brazil by Coletta-Filho et al. (2004). After comparison with the Asian and African strains, Coletta-Filho et al. (2004) proposed an additional new species of Liberibacter “*Candidatus Liberibacter americanus*”. Greening was first detected in Florida in Miami-Dade County in September of 2005 and is now present in all citrus growing counties. Greening has since been detected in Louisiana, Georgia, South Carolina and Puerto Rico (USDA-APHIS-DA-2009-58).

Three bacterial species are currently known to cause citrus greening. All are phloem-limited, gram-negative bacteria, *Candidatus Liberibacter asiaticus* and *Candidatus Liberibacter americanus* (Jagoueix et al., 1994; Teixeira et al., 2005) both of which are vectored by *D. citri*. *Candidatus Liberibacter asiaticus* is the Asian form while *Candidatus Liberibacter americanus* is the South American form. The third is an African species *Candidatus Liberibacter africanus* vectored by the African citrus psyllid, *Trioza erytreae* (del Guercio) (Garnier et al., 2000). This African strain is sensitive to higher temperatures (27-30°C) and can be asymptomatic when temperatures exceed 27°C (Bové et al., 1974). Although the citrus greening pathogens transmitted by these two
psyllids are different, Lallemand et al. (1986) showed that either psyllid is capable of transmitting either the Asian or African forms under experimental conditions. Trees infected by either pathogen show similar symptoms but the Asian form causes greater dieback (Zhao, 1981). The “Candidatus” designation arrives from the fact that none of the three forms have been successfully cultured on artificial media (Garnier & Bove, 1993).

In Florida, ACP causes severe damage by vectoring the Asian form Candidatus Liberibacter asiaticus. This disease is commonly referred to as citrus greening disease or huanglongbing (yellow shoot disease) (van Vuuren, 1996). Mead (1977) noted the synonyms citrus chlorosis (Java), leaf-mottle (Philippines), yellow shoot (China) and decline (likubin) in Taiwan. Halbert and Manjunath (2004) recorded 19 genera of plants that host the greening pathogen. Symptoms include yellow leaf mottling along veins, blotchy chlorotic mottling, twig dieback, stunted growth and defoliation (da Graca, 1991). Affected fruit, which can drop prematurely, is generally misshapen, lopsided, sour or bitter tasting, poorly colored and may contain small dark colored aborted seeds (Capoor et al., 1974). Symptoms can vary depending on tree age as well as time of year.

HLB bacterium are acquired by adult and all stages of immature psyllids but only transmitted by 4\textsuperscript{th} and 5\textsuperscript{th} instars and adults (Xu et al., 1988). Greening pathogens are restricted to the sieve tubes of infected plants where they are then acquired by ACP through phloem feeding (Garnier & Bové, 1983). Transmission from ACP to healthy trees likely involves the salivary secretions of psyllids into the phloem (Aubert, 1987). Capoor et al. (1974) observed that ACP can acquire HLB after 30 minutes of feeding on
an infected plant. HLB can also be transmitted by vegetative propagation of infected plant material and dodder (Captor et al., 1974).

**Host Plants**

Hosts of ACP in China include 27 species within the following seven genera of Rutaceae: Atalantia, Citrus, Clausena, Euodia, Fortunella, Murraya, and Poncirus (He, 2000). Halbert and Manjunath (2004) listed 25 genera all within Rutaceae with *Citropsis*, *Citrus* and *Murraya* being the preferred hosts. Orange jasmine, *Murraya paniculata* (L.) Jack, is a common ornamental shrub used in landscaping throughout south Florida. *M. paniculata* as well as dooryard citrus and abandoned groves serve as reservoirs for adult psyllids to overwinter and avoid pesticides commonly used in commercial citrus groves.

In September 2005, USDA-APHIS imposed a federal order (DA-2005-30) setting up a quarantine area including most of Florida to regulate the movement of *Diaphorina citri* host plant material and *Candidatus Liberibacter asiaticus* (CG) host plant material (live plants, budwood and cuttings) within and out of most counties in Florida to the following areas: Alabama, American Samoa, Arizona, California, Guam, Hawaii, Louisiana, Northern Mariana Islands, Puerto Rico, Texas, and the US Virgin Islands. Shipments going to areas outside those previously mentioned must have been treated with insecticides prior to movement.

Regulated hosts of *Candidatus Liberibacter asiaticus* included in this order are as follows (in alphabetical order): *Aeglopsis chevalieri*, *Balsamocitrus dawei*, *Calodendrum capense*, x *Citrofortunella microcarpa*, _Citroncirus webberi*, *Citrus* spp., *Clausena indica*, *C. iansium*, *Fortunella* spp., *Limonia acidissima*, *Microcitrus australasica*, *Murraya paniculata*.
Murraya koenigii, Poncirus trifoliata, Severinia buxifolia, Swinglea glutinosa, Toddalia lanceolata and Triphasia trifolia.

Regulated hosts of *Diaphorina citri* include: Aegle marmelos, Afraegle gabonensis, Afraegle paniculata, Atalantia spp., Citropsis gilletiana, Citropsis schweinfurthii, Clausena anisum-olens, Clausena excavate, Eremocitrus glauca, Eromocitrus hybrid, Merrillia caloxylon, Microcitrus australis, Microcitrus papuana, Microcitronella, Murraya paniculata, M. koenigii, Naringi crenulata, Pamburus missionis, Toddalia asiatica, Vepris lanceolata, and Zanthoxylum fagara.

In November of 2007, this regulatory action was updated for the third time to further expand the host plant quarantine area from Florida only to include 32 counties in Texas as well as all islands of Hawaii and Guam and the entire commonwealth of Puerto Rico. Seeds and fresh fruits of any of the above mentioned plants were excluded from this order.

In November of 2009, a new federal domestic order (DA-2009-58) was issued to attempt to stop the further spread of ACP and citrus greening disease. This order states that in addition to the previously mentioned areas no ACP/CG host plant materials, including seeds, can be moved within or out of Georgia, portions of Louisiana and South Carolina, Alabama, California, and Mississippi.

**Managing Citrus Greening and ACP**

There is currently no completely successful control of greening disease in any of the citrus growing regions of the world where it occurs (Halbert & Manjunath, 2004). Current control of citrus greening involves the use of an integrated pest management program (Halbert & Keremane, 2004). These include chemical and biological controls to reduce or eliminate psyllid populations, as well as proper quarantine procedures and the
mechanical removal of infected trees (Wang et al., 2002). Additionally, the maintenance of clean nursery stocks and identification and/or development of disease resistant citrus species are essential (He, 2000).

Using pesticide sprays to control psyllids is common throughout the world but usage varies in regard to the timing of sprays and their mode of action. These include the use of mineral oils and synthetic insecticides (Beattie et al., 2002). Systemic pesticides have been shown to be effective against ACP (Buitendag & von Broembsen, 1993). Research in Florida has shown three soil-applied insecticide active ingredients aldicarb, imidacloprid and thiamethoxam, provide effective control of ACP (mostly on young trees), while several broad spectrum foliar insecticides including fenpropathrin and chlorpyrifos are effective for targeting adults before spring flush (Stansly & Rogers, 2006; Qureshi & Stansly, 2008). An application of a systemic insecticide prior to the spring flush, before densities of *D. citri* nymphs reach their highest levels, is crucial to reducing psyllid populations (Stansly & Rogers, 2006). The use of chemical control alone is not a suitable option, and heavy usage in China has demonstrated this (Huang et al., 1999). Citrus groves which relied solely on chemical control still suffered from whole orchard loss. Additionally, using large quantities of sprays can be expensive and have a negative impact on natural enemies as well as the environment.

Early detection through vigorous scouting and removal of infected trees has been recommended to reduce the spread of the disease (Manjunath et al., 2008). However, early detection can be complicated due to the fact that infected trees may remain asymptomatic for several years (da Graca, 1991). Additionally, early signs of infection can be mistaken for zinc deficiency (Roistacher, 1996). There is no known cure for HLB.
though some cultivars have shown tolerance to infection (Koizumi et al., 1993). In recent years polymerase chain reaction (PCR) technique improvements have led to a relatively quick and reliable means of detecting infection (Jagoueix et al., 1996). Although some studies show the use of heat in the form of hot water, hot humid air or a heat therapy chamber can destroy the greening pathogen in diseased budwood (Raychaudhuri et al., 1974) the only option for trees that test positive is whole tree removal.

Chemical control along with the use of natural enemies including imported biological control agents can effectively reduce psyllid populations (Rogers & Timmer, 2007). Complete eradication of \textit{D. citri} is not practical due to their wide distribution (Hoy & Nguyen, 1998).

**Natural Enemies of ACP**

Natural predators of ACP consist of several polyphagous insects including syrphids (Diptera: Syrphidae), ladybeetles (Coleoptera: Coccinellidae), lacewings (Neuroptera: Chrysopidae), ants (Hymenoptera: Formicidae), mantids (Mantodea: Mantidae) and spiders and mites (Acari) (Michaud, 2002, 2004; Yang et al., 2006). Chien and Chu (1996) reported 4 main indigenous predators of ACP in Taiwan \textit{Menochilus sexmaculatus} Fab., \textit{Mallada boninensis} (Okamoto), \textit{Serangium} sp. and \textit{Geocaris} sp..

Entomopathogens provide some control (Yang et al., 2006). In China these include \textit{Acrostalagmus aphidum} Oudem, \textit{Paecilomyces javanicus} (Friederichs & Bally) AHS Brown and G. Smith, \textit{Verticillium lecanii} (Zimm.) Viegas (Anamorphic Ascomycetes), and \textit{Beauveria bassiana} (Balsamo) Vuillemin (Anamorphic Clavicipitaceae) (Xie et al., 1988; Ye et al., 1994). Entomopathogens attacking ACP
recorded in Florida include *Hirsutella citriformis* and *Isaria fumosorosea* Wize (Ifr) (=Paecilomyces fumosoroseus) (Hypocreales: Cordycipitaceae) (Meyers et al., 2008). Halbert and Manjunath (2004) reported that only a very few ACP regulatory samples examined at the Florida Department of Agriculture and Consumer Services, Division of Plant Industry (DPI) were killed by an unknown fungus. A description of the fungus and the total number of individual specimens affected was not reported.

Michaud 2001, reported that the ash-gray ladybeetle, *Olla v-nigrum* (Mulsant) = *Olla abdominalis* (Say), a once rare coccinellid in Florida citrus before ACP’s arrival, has now become the dominate species where the psyllid is present. Additionally, the Asian multicolored ladybeetle, *Harmonia axyridis* Pallas, feeds on ACP nymphs in Florida (Michaud, 2001). Michaud (2004) identified two lacewing species *Ceraeochrysa* sp. and *Chrysoperla rufilabris* Burmeister, and a spider, *Hibana velox* (Becker) as natural predators in Florida citrus. Additional important predators of ACP in Florida include the metallic blue ladybeetle, *Curinus coeruleus* (Mulsant) and the blood red ladybeetle, *Cycloneda sanguinea* Linnaeus (Coleoptera: Coccinellidae); *Allograpta* sp. (Diptera: Syrphidae); and *Zelus longipes* L. (Hemiptera: Reduviidae) (Hall et al., 2008).

In a 2003 survey, Pluke et al. (2003) identified 8 species of coccinellids found in Puerto Rico amongst 180 surveyed citrus trees. Two species, *Coelophora inaequalis* F. and *Cycloneda s. limbifer* L., were the most abundant. All 8 species consumed ACP nymphs with varying degrees during no-choice lab feeding studies. In choice tests, *Cladus nitidula* F., *Chilocordus cacti* L., *Coleomegilla innonata* Mulsant, and *Cryptolaemus montrouzieri* Mulsant showed a stronger preference for ACP nymphs over the brown citrus aphid *Toxoptera citricida* (Homoptera: Aphidae) while C. s.
*C. limbifer* and *C. inaequalis* preferred the brown citrus aphid. The remaining two species, *Hippodamia convergens* Guerin and *Scymnus* sp. had no preference for either prey.

Also in 2003, Michaud and Olsen examined if six different species of coccinellids could develop on a diet of ACP nymphs. *Curinus coerules* Mulsant, *Exochomus childreni* Mulsant, *Harmonia axyridis* Pallas, *O. v-nigrum* Mulsant and *Cycloneda sanguinea* L. were able to develop on a diet of ACP nymphs while *Curinus coerules* Mulsant, *Exochomus childreni* Mulsant, *Harmonia axyridis* Pallas and *O. v-nigrum* Mulsant were able to reproduce normally after feeding on ACP as adults. *Coelophora inaequalis* (F.) did not develop or reproduce well. Through field studies they found that the coccinellids *Harmonia axyridis* Pallas, *Olla v-nigrum* Mulsant, *Cycloneda sanguinea* L., and *Exochomus childreni* Mulsant were the most important predators of ACP when ACP were abundant. These coccinellids were responsible for > 95% of mortality of immature stages of *T. radiata*. When exclusion cages were used to keep predators from feeding on immature psyllids, their survival rate increased by a factor of 120. In addition, these cages increased the survival of the parasitic wasp *Tamarixia radiata* by a factor of 40. Qureshi and Stansly (2009) provided evidence that the conservation of natural enemies of *D. citri* is vital for sustainable management of the pest.

Additionally two host specific parasitic wasps, *Tamarixia radiata* (Waterston) and *Diaphorencyrtus aligarhensis* (Shafee, Alam and Argarwal), were imported into Florida in 1998 for ACP control (Hoy & Nguyen, 1998). *D. aligarhensis* is an endoparasitoid originally recorded from India (Shafee et al., 1975), while *T. radiata* is an ectoparasitoid originating from Pakistan (Waterston, 1922). Both wasps were imported from Taiwan Agricultural Research Institute in Taiwan (McFarland & Hoy, 2001). An additional
collection of *T. radiata* was also imported from South Vietnam and later mixed with the Tiawanese colony. Parasitoids were screened for the presence of greening pathogen through the use of polymerase chain reaction (PCR) (Hoy et al., 1999, 2001). After quarantine lab testing was completed, both wasps were mass reared and released into Florida in late 1999 and early 2000. There is no evidence that either species of primary parasitoids are capable of developing on any host other then ACP (Aubert & Quilici, 1984).

Currently, *T. radiata* has established in most citrus growing regions of Florida providing varying levels of control (Michaud, 2002; Hoy et al., 2000; Quereshi et al., 2009), while *D. aligarhensis* has been less successful for unknown reasons. Although *T. radiata* has never been intentionally released in Texas, it was recovered from *D. citri* infested *M. paniculata* plants at a Texas nursery in 2001 (French et al., 2001) and later from ACP infested citrus in the lower Rio Grande valley (Michaud, 2004).

In 1983, *Tamarixia radiata* was imported into Taiwan from Réunion Island (Chien, 1988). Both parasitoids were successful in reducing psyllid populations in Réunion Island (Etienne & Aubert, 1980) and Taiwan (Chien & Chu, 1996). *D. aligarhensis* was imported into South Africa in 1983 to control African citrus psyllid, *Trioza erytreae* (Del Guercio) but was not released from quarantine after it was unable to reproduce using this host (Prinsloo, 1985).

Seventeen hyperparasitoids have been recorded attacking one or both of these primary parasitoids (Yang et al., 2006). Chien et al. (1989) reported 42.2% (n= 14,319) of *D. aligarhensis* mummies collected in Taiwan (1985-1988) were hyperparasitized by 10 indigenous species. Four species, responsible for 41.3% of the hyperparasitism of *D.
were as follows: *Pachyneuron concolor* (Forster) (Pteromalidae) (18.5%), *Chartocerus walkeri* Hayat (Signiphoridae) (13.5%), *Syrphophagus taiwanus* Hayat and Lin (Encyrtidae) (6.8%), and *Marietta leopardina* Motschulsky (Aphelinidae) (2.5%). Less than 1% (n= 11,342) of *T. radiata* mummies collected were hyperparasitized by 7 indigenous species. No hyperparasitoids have been reported attacking *T. radiata* in Florida (Michaud, 2002).

**Diaphorencyrtus aligarhensis Biology**


Adult wasps are small (~ 1–1.5 mm) and possess yellowish legs and antennae and a black head and thorax. Sexual dimorphism is exhibited in slight differences in the antennae and abdomen. Females possess a large, rounded yellow abdomen while the male abdomen is smaller, black, and more cylindrical. Female antennae are smooth and clubbed while male antennae are slightly longer, lack clubs and are covered with short hairs. This wasp generally reproduces by thelytoky which results in all female
offspring (Chien, 1995; Hoy, 2003). Imported wasps tested positive for infection with the endosymbiont *Wolbachia* through PCR analysis (Jeyaprakash & Hoy, 2000). *Wolbachia* infection can induce parthenogenesis as well as other reproductive anomalies in insects (Werren, 1997). Shafee et al. (1975) reported small numbers of males in Asian populations surveyed.

*D. aligarhensis* life cycle from egg to adult takes 18 days at 25°C (Chien, 1995). Females are reported to parasitize 2nd – 4th instar nymphs and host feed on 1st- 4th instars (Chien & Chu, 1996; Skelley & Hoy, 2003). Adult females are capable of killing up to 280 *D. citri* nymphs through host feeding and parasitism (Chien, 1995; Skelley & Hoy, 2004). Skelley and Hoy (2004) reported that there was no significant difference in the mean number of progeny or adult wasp size when reared from 2nd, 3rd or 4th instar nymphs. Females produced an average of 6.6 progeny per day during laboratory rearing and only 10% of adult *D. aligarhensis* could survive for 50 days at 25°C (Skelley & Hoy, 2004). Chein (1995) determined mean longevity average 20 days (Chien, 1995).

**Tamarixia radiata Biology**

*Tamarixia radiata*, previously designated as *Tetrastichus radiatus* (Waterson, 1922), is an obligate ectoparasitoid specific to *D. citri* (Aubert & Quilici, 1984). *T. radiata* has a high reproductive rate with a single female laying up to 300 eggs through her lifetime (Pluke et al., 2008). Chien, 1995 reported female wasps can kill up to 500 psyllids through host feeding and parasitism combined. Adult wasps as described by Waterston (1922) are small (1-3 mm) and have a black thorax and head with red eyes. *T. radiata* are sexually dimorphic with males being smaller with a solid black abdomen whereas female’s abdomens are a yellow-tan color with a black
posterior end. Additionally males possess antennae with longer hairs. *T. radiata* are arrhenotokous; unfertilized eggs are haploid resulting in males, and fertilized eggs are diploid, resulting in females (Hoy, 2003). Females lay a single egg on 2nd - 5th instar ACP nymphs, generally on the ventral side of the first abdominal segment (Aubert & Quilici, 1984). After egg hatch, larvae begin to feed and develop through 4 instars, a pre-pupal and pupal stage. Approximately 7 days post parasitism at 25°C, the hardened, darkened exoskeleton of the consumed psyllid host can be seen attached to plant material by silken threads produced by the wasp larva (Aubert & Quilici, 1984; Skelley & Hoy, 2004). Development time from egg to adult takes approximately 12 days at 25°C (Skelley & Hoy, 2004). Chein (1995) reported a female: male ratio of 3.2: 1.0 for a field population while Skelley and Hoy (2004) reported a female: male ratio of 1.8:1.0 for a Taiwan lab population and 2.0:1.0 in a Vietnam lab population. Skelley and Hoy (2004) reported that 10% of adult *T. radiata* could survive for 50 days at 25°C. Chein (1995) determined mean longevity to be 24 (♀) and 14 (♂) days (Chien, 1995).

**Research Objectives**

Presently there is limited information available on *D. aligarhensis* behavior, particularly host searching strategies and interactions/competition with *T. radiata*, a competing parasitoid. This is reflected in the minimal amount of published literature on this subject. The goals of this research were to import, rear, study, mass release and establish a population of *D. aligarhensis* from Mainland China with the hope that a new population from a different region would better adapt and compete in Florida as a biological control agent of ACP than the population that was previously released in Florida.
In September of 2006, I obtained 100 ACP nymphs collected by Ru Nguyen (FDACS-DPI Gainesville) from Guangzhou Guangdong province, China. Parasitoids where sent to Florida under APHIS permit number No.526-75827. Fifty adult parasitoids emerged in the maximum security room of the Florida Biological Control Laboratory quarantine facility at the Florida Department of Agriculture and Consumer Services, Division of Plant Industry compound in Gainesville, Florida. For the first three generations, males accounted for 5% of the population. No males have since emerged. Wasps were reared through more than 20 generations until the necessary permits for release were granted.

Chapter II focuses on the biology of this population of *D. aligarhensis*. These studies were conducted to determine *D. aligarhensis’* environmental constraints as a biological control agent as well as provide general information regarding wasp biology and development. Wasp survivability under different temperatures and relative humidities was examined. Wasp longevity feeding on various food sources was determined. I address the probable cause of thelytoky in this wasp population by using PCR to determine that this population of *D. aligarhensis* is infected with the *Wolbachia* endosymbiont. I attempted to use antibiotics to “cure” wasps of *Wolbachia* to produce a bi-parental population.

Chapter III examines the larval development of immature *D. aligarhensis* within their host nymphs. Vital dyes were used in an attempt to label wasp eggs to make successful ovipositions easier to identify and to track larval development without the fatal dissection of hosts. Digital photography and Scanning Electron Micrscopy (SEM) were used to capture images of all stages of wasp development.
Chapter IV examines the influence of age, host availability and supplemental food on egg load, oviposition behavior and host feeding of *Diaphorencyrtus aligarhensis*. Egg loads of wasps stored under different temperatures and feeding regimens over a 30 day period were examined. Host feeding and parasitism frequencies and survivorship of wasps held under various conditions, including suitability of different host instars utilized for host feeding and parasitism were determined. Which host instars utilized for host feeding and parasitism by *D. aligarhensis* was determined.

In Chapter V, I examine *D. aligarhensis'* proficiency at utilizing host and host plant volatiles to locate hosts through the use of a y-tube olfactometer. Volatile collection and analyzation (GC/MS) methods were employed to attempt to identify which volatile compound cues are used by *D. aligarhensis* to locate hosts.

Chapter VI examines the interactions and competition between *D. aligarhensis* and its competing parasitoid *Tamarixia radiata*. Experiments were conducted to determine whether these species discriminate for oviposition against a previously parasitized host. I also determined which species would complete development to adulthood when both wasps parasitize the same host whether before, after or at the same time as each other. Finally, I evaluated the percentage of multi- and superparasitism when both or one species have access to hosts at three different densities.

Chapter VII covers the field release of adult *D. aligarhensis* wasps through cage and open releases. Releases were made over 26 months in 7 Florida counties (Alachua, Collier, DeSoto, Hendry, Lake, Orange and Polk). *D. aligarhensis* were released into 3 commercial groves (2 organic), 1 research grove (SWFREC), 1 private
dooryard grove and several *Murraya paniculata* ornamental hedge sites. Following releases, field evaluation for the presence and effectiveness of the parasitoid was conducted by means of sampling for ACP mummies, parasitized nymphs, and adult wasps.
CHAPTER 2
DIAPHORENCYRTUS ALIGARHENSIS BIOLOGY

Introduction

Temperature and humidity requirements are important to evaluate suitability of a potential biological control agent in a particular location as well as to assess environmental constraints for field release and mass rearing. A previously imported and released (2001) Taiwanese population of *D. aligarhensis*’s survivability under different temperature and relative humidities was examined by McFarland and Hoy (2001). Their study was used as a guide to compare mainland China and Taiwan populations and provide benchmarks for any that may be imported in the future.

Efficiency as a biological control agent may be influenced by the presence of males within the population. Infection with the endobacterium *Wolbachia* can induce parthenogenesis as well as other reproductive anomalies in insects (Werren, 1997). Long PCR, using the *wsp* gene, has been shown to be an effective means of testing for *Wolbachia* infection in insects (Jeyaprakash & Hoy, 2000). Treating wolbachia infected insects with antibiotics has successfully induced the production of males as well as affected female sexual behavior in several hymenopteran and dipteran parasites (Puttaraju & Prakash, 2005; E. Zchori-Fein et al., 2001; Dobson & Rattanadechakul, 2001).

Longevity of an insect used as a biological control agent has important implications regarding mass rearing and the timing of releases. Wasps are often stored with an artificial diet before release. Knowing which diet will provide proper nutrition to maximize longevity can increase the number and quality of surviving wasps when held for long periods, when building numbers, or shipping insects before release. It is
important to release an organism with ample longevity remaining to maintain viability and effectiveness in the field.

**Materials and Methods**

**Colonies**

*D. citri* colonies were maintained on orange jasmine, *Murraya paniculata* plants in 3.78 L pots with a 30:70 mix of vermiculite (Jungle Growth Professional Growers Mix potting soil, Piedmont Pacific, Inc. Statham GA). Plants were maintained under natural sunlight in a greenhouse. Temperatures were maintained between 27-32° C and the relative humidity (RH) varied from 40-70%. Freshly flushing plants were moved from the greenhouse into a rearing room and held at 25° ± 1°C and 55 ± 5% RH. Plants were housed in 47.5 x 47.5 x 93.0 cm BugDorm-4180F Insect Rearing Cages (Bioquip, Rancho Dominguez, CA) and illuminated with 400 Watt EYE 4200K BT28 bulbs in Metal Halide Growlights (Hydrofarm, Petaluma, Ca.) for a 16:8 hour light:dark period. Approximately 100 psyllid adults were introduced into each cage and allowed to oviposit for 12-14 days until all 5 nymphal stages were present.

*D. aligarhensis* were maintained under the same conditions as above but located in a separate room to prevent contamination of the *D. citri* colony. After the 12-14 day psyllid exposure period, plants were moved to the parasitoid room and placed in a cage with 10 newly emerged female wasps. All wasps were removed after 1 week. Plants remained in the cage for an additional 2 weeks until all parasitoids had emerged. Emerging parasitoids were collected daily, labeled and held in 50 mL centrifuge tubes (Crystalgen, Long Island NY) with a honey-soaked cotton wick until used.
Molecular Analysis of *D. aligarhensis* Endosymbionts

100 adult newly emerged (<24 hr) female *D. aligarhensis* wasps were collected and stored in fresh 95% ethanol at -80° C. Two replicates of DNA extractions were performed. This analysis required the use of ethanol stored wasps rather than live in order to allow their removal from a quarantine facility.

**Purification**

Twenty-five adult *D. aligarhensis* wasps weighing a total of 2.8 mg (dry weight) were ground in 600 µl Puregene Cell Lysis Buffer using microfuge pestles made from melted and molded plastic pipette tips and purified using the Puregene® DNA Purification Kit.

**Polymerase Chain Reactions**

*Wolbachia* surface protein (*wsp*) fragments were amplified using a high fidelity polymerase chain reaction (PCR) protocol (Jeyaprakash and Hoy, 2000) and MJ Mini thermocycler (Bio-Rad). Instead of a single polymerase, a 5:1 mixture of Taq:Tgo polymerase was used, with a buffer consisting of (10X) 50mM Tris, 16mM ammonium sulfate, 1.75mM MgCl2. The primer for *wsp* (based on those used by Braig et al., 1998) had the following sequences:

(fwd) 5’TGGTCCAATAAGTGATGAAGAAAC-3’ and

(rev) 5’-AAAAATTAAACGCTACTCCA-3’

Five pmol of the primer were added to a 25 µl reaction volume containing 1 µl purified genomic DNA solution, 700 µM dNTPs, 2.5 µl 10x buffer (described above), and 1 µl Taq/Tgo polymerase mix (1U Taq polymerase, 0.2U Tgo polymerase) (Bio- Rad Laboratories, Inc., Hercules, CA; Roche Molecular Biochemicals, Indianapolis, IN). Cycling conditions were as follows. Initial denaturation at 94ºC for 5 min, followed by 10
cycles of 94°C for 10 sec, 56°C for 30 sec, and 68°C for 2 min, followed by 20 cycles of 94°C for 10 sec, 56°C for 20 sec, 68°C for 2 min + 20 sec/cycle. The PCR setup was done in a “PCR clean area” cleaned with DNAse Away before each reaction setup, and only filter tips were used for pipetting. Negative controls with identical reaction conditions except for the substitution of ddH2O for genomic DNA were run with each experimental amplification. Negative controls showed no evidence of contamination.

**Cloning, sequencing, and Sequence Analysis**

PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System according to the manufacturer’s instructions then cloned into the pCR®II-TOPO vector utilizing TOP10 chemically competent cells (Invitrogen). Plasmid DNA from cells containing inserts was prepared using the GenElute HP Plasmid Miniprep Kit (Sigma). Restriction digest was used to confirm the presence of inserts. Plasmids were sequenced by NorthWoods DNA (Solway, MN). Both strands of the inserts were sequenced and sequencing was repeated for each fragment. Sequences were determined from chromatograms using SeqMan software (DNAstar, Madison, WI). DNA sequences from this study and a comparison sequence from GenBank were aligned using Clustal X software (Thompson et al., 1997).

**Antibiotic “Curing” of *Wolbachia* from *D. aligarhensis***

*D. aligarhensis* adult females were collected within 24 hours of emergence and provided with a Tetracycline hydrochloride (Sigma Chemical Co., St Louis, MO) and pure clover honey mixture. Twenty mm long cotton wicks were soaked in the mixture and provided to adult female *D. aligarhensis* held in 50 mL centrifuge tubes at 25°C, 60% RH with a 16L: 8D photoperiod for 24 hours. A preliminary toxicity test consisting of various antibiotic/ honey concentrations ranging from 0-30 mg/ mL were conducted.
All concentrations were tested against a honey only control and the effect on the wasp’s longevity and general behavior was recorded. Each preliminary dose was tested using 30 wasps held together in a 50 mL centrifuge tube under the above conditions for 48 hours. Within 48 hours 70% of wasps treated with 15mg/mL and 100% of wasps that fed on 20, 25 or 30 mg/mL concentrations were dead. Approximately 80% of all wasps which fed on 5 or 10 mg/mL were alive and appeared normal. Comparatively, approximately 90% of the control wasps were alive after 48 hours. For this reason two separate doses (5 and 10mg/mL) were used for further testing.

After treatment with either a 5 or 10 mg/mL dose of antibiotics 3 wasps per dose were placed individually onto ~406.4 mm tall *M. paniculata* plants contained in 3.78 l pots. Each plant was infested with > 100 ACP nymphs (2\textsuperscript{nd}-4\textsuperscript{th} instars). Plants were held inside clear cast acrylic cylinders 127 mm diameter, 457.2 mm high with four 57.2 mm diameter opposing side holes covered with organdy to allow air exchange. All plant cylinders were held in a Powers Scientific (Pipersville, PA) level 2 incubation chamber 25°C, 60 % RH with a 16L: 8D photoperiod using standard fluorescent grow bulbs. Plants were held for 30 days and all progeny were collected daily. Each new offspring (up to 10 per parent per dose) were treated within 24 hours of emergence with the same dose and in the same manner as its parent. After treatment all dead wasps were discarded. Of the remaining live wasps, 3 were randomly chosen and placed individually on a new plant as above. This procedure was conducted for three consecutive generations or until no new progeny emerged. This entire experiment was repeated twice for each of the two doses.
Survivability under Different Temperatures and Relative Humidities

Newly emerged (<24 hrs) adult parasitoids were collected from a rearing cage and held in a pretreatment 30.5 cm³ clear acrylic cube with a 12.7 cm diameter circular opening for a period of 21 hours. The opening could be fitted with an acrylic cover to be completely sealed or with a fine mesh to allow air exchange. No food or water was provided. Humidity inside the cube was maintained at 56% RH using a [Mg(NO₃)₂ 6H₂O] magnesium nitrate hexahydrate salt solution at a temperature of 17°C (Winston & Bates, 1960; O'Brien, 1948; Carr & Harris, 1949). This pretreatment was to remove cuticular surface moisture and reduce ingestion, defecation and excretion effects (Arlian & Ekstrand, 1975). Pretreatment and treatment cubes were held in Powers Scientific (Pipersville, PA) level 2 incubation chambers with an 16L: 8D photoperiod. After 21 hours parasitoids were removed from the pretreatment cube and randomly placed, 20 females each, in one of 5 identical cubes of differing RH: 7, 33, 53, 75, and 97%. These five RH were held in one of two identical environmental chambers held at either 25° or 30° C. The various RHs were maintained using reagent grade salts according to Winston and Bates (1960). Temperature and RH was monitored using a HOBO 2-channel temperature and relative humidity data logger (Onset Computer Co. Bourne, MA). Salts were obtained from Spectrum Chemical Mfg Co. (CA, NJ). Salts used were as follows: sodium hydroxide (7% RH), magnesium chloride hexahydrate (33% RH), magnesium nitrate hexahydrate (53% RH), sodium chloride (75% RH), and potassium sulfate (97% RH). All salts were dissolved in reverse osmosis water to form a paste which was then poured into small shallow stainless steel pans. Pans were covered with a fine mesh that prevented parasitoids from falling into the solution while still allowing adequate air exchange. Three replicates of each RH were tested under each of the two
different temperatures. Cubes were checked every three hours and the number of dead specimens recorded.

**Longevity of *D. aligharensis* Provided with Various Food Sources at 25°C**

Adult newly emerged (<24 hrs) *D. aligharhensis* females were collected and randomly placed 50 each in 8 separate 30.5 cm³ clear acrylic cubes with 12.7 cm diameter circular openings. Cube openings were fitted with a fine mesh to allow air exchange. Cubes were held in a Powers Scientific (Pipersville, PA) level 2 incubation chambers maintained at 25°C, 60 % RH with a 16L: 8D photoperiod using standard fluorescent grow bulbs. Cages contained one of the following food sources: water, yeast/water mixture, flowering *Richardia scabra* L. (Florida pusley), ACP nymph honeydew/wax, ACP nymphs (1⁰ - 4⁰ instars), honey, honey/yeast (50:50) mixture or no food/water source. Water was provided in 5 mL plastic cups with a cotton wick. Yeast/water, honey/yeast and honey were provided in the form of a soaked cotton wick. Honeydew/wax was provided in clear 100 x 15mm sterilized polystyrene petri dishes (Thomas scientific, Swedesboro, NJ). Nymphal honeydew/wax was replaced every other day to maintain freshness. Nymphs on plants were replaced every two to three days to maintain a high enough density so that all wasps had access to live nymphs. The above procedure was repeated three times.

**Statistical Analysis**

Mean longevity (+ SEM) in hours was calculated for each survival % (0, 10, 20, 30...100%) for each temperature and RH block during the survivability under different temperatures and relative humidities experiment. LT50 (+ SEM) and survival curves were determined for each RH and temperature block and compared to the Taiwan population examined by McFarland and Hoy (2001).
Cages were monitored daily to record the number of surviving wasps during the longevity experiment. Longevity was measured in days until mortality. The mean number of days until mortality for each treatment was calculated, an analysis of variance was performed (SAS Institute, 9.1) and means were further compared using Tukey’s HSD Studentized Range test (SAS Institute, 9.1) at $\alpha = 0.05$.

**Results**

**Molecular Analysis of *D. aligarhensis* Endosymbionts**

Amplification products for the *wsp* gene of *Wolbachia* were detected in both of the DNA samples extracted from adult female *D. aligarhensis*. Sequences, when aligned using Clustal X software, were 100% identical in size (542 bp) and nucleotide composition to the *wsp* gene previously reported from the Taiwanese laboratory population of *D. aligarhensis*; accession number AF217715 (Jeyaprakash & Hoy, 2000).

**Antibiotic “Curing” of Wolbachia from *D. aligarhensis***

No males were produced as a result of antibiotic treatment. Although overall fecundity and longevity was not recorded the treatment appeared to have negative effects on both. Fewer offspring emerged from each successive generation under both treatment concentrations during both repetitions. Additionally, offspring of each successive generation survived for a shorter period on average than those previously tested.

**Survivability under Different Temperatures and Relative Humidities**

All wasps survived longer at 25° than 30° C and all were dead within 39 hours at either temperature (Figures 2:1-5). Actual data sets were not available for McFarland and Hoy’s (2001) testing of *D. aligarhensis* so differences with these values could not be statistically analyzed. However, comparison of summary data from both experiments
showed that the mainland China population appeared to survive longer before reaching LT<sub>50</sub> values over all temperature and RH treatments with the exception of 7% RH at 25°C (Table 2-1).

**Longevity of *D. aligharenis* Provided with Various Food Sources at 25°C**

Access to water, a yeast/water mixture or flowering Florida Pusley did not significantly increase lifespan over wasps held without food or water (P>.01) (Table 2-2). Unfed wasps survived one day on average while providing water prolonged mortality for an additional 0.5 days. Those provided with yeast/water mixture or flowering Florida pusley lived an average of 2 and 2.75 days respectively. In contrast, honey fed *D. aligarhensis* survived for over 35 days (Table 2-2). Adding yeast to the honey prolonged the average lifespan for an additional 2.5 days, however this was not significantly longer then those fed honey alone (df= 1, F= 3.29, P= 0.0707). Wasps lived for an average of 10 days when given nymphaal honeydew/wax which was significantly less than 27 days when provided with live nymphs (df= 1, F= 167.88, P= 0.0001). Access only to nymphs resulted in a significantly shorter lifespan then those held with honey only (df= 1, F= 29.14, P= 0.0001).

**Discussion**

Longer survival under almost all RH and temperature conditions suggests that the Mainland China population has a lower net water loss rate. However, when population survival curves for this population were compared with the Taiwanese population (McFarland & Hoy, 2001) overall similarities were evident suggesting both have relatively equivalent moisture requirements. This information provides evidence that both populations of *D. aligarhensis* are equally suited in regards to RH, to survive in Florida, perhaps with a slight advantage of this mainland China population to withstand
periods of lower moisture levels. This data can be used to evaluate and compare the moisture requirements of incoming shipments of *D. citri* parasitoids from various overseas locations.

Results from the molecular analysis of *D. aligarhensis* endosymbionts provides strong evidence that both populations of *D. aligharensis* tested (Taiwan and mainland China) share the same strain of *Wolbachia*.

During antibiotic “curing” experiments it is not clear if there was an increasing toxic sensitivity to the antibiotic in each new generation or perhaps a just a cumulative loss of longevity. While I was not successful in producing males, Meyer and Hoy (2007) did report success even though their produce was quite similar: Fifty adult females, through three generations were fed a 10mg/mL tetracycline/honey mixture for 24 hours then released together into a cage containing ACP nymphs. New plants were added to the cage each generation. Males were produced, tested negative through PCR for *Wolbachia* and were destroyed. Perhaps their success indicates a higher tolerance for tetracycline in the Taiwanese population. Unfortunately no comparison studies examining the general behavior and biology of “cured” wasps against the thelytokous strain were conducted.

A carbohydrate and protein mixture in the form of pure clover honey and yeast provided the longest longevity, far beyond that with water alone. Honey and yeast are both readily available, inexpensive and require little effort compared to providing live nymphs for nutrition. *D. aligarhensis* given a honey/yeast or honey soaked cotton wick can be stored for several weeks with minimal mortality until an appropriate release time. However it is also important that they be in good condition at the time of release.
Flowering Florida pusley does not appear to be an important food source for *D. aligharhensis* though it did appear to extend longevity in the absence of hosts. Although the average longevity when feeding on flowers was extended only 2 days over water alone, many wasps survived 4 or more days, several up to 9 days, which was at least 3 times longer than all wasps given water only (Figure 2-6). This provides evidence that *D. aligharhensis* are capable of floral feeding. However, it is not known if *D. aligarhensis* will actively seek flowers for food so further testing in the form of olfactory bioassays is warranted. Additionally, identification and testing of other flowering plants found in Florida citrus groves is justified.
Table 2-1. Survival of two populations of *D. aligarhensis* at five different relative humidities (RH) at two temperatures 25°C and 30°C.

<table>
<thead>
<tr>
<th>LT-50 values ± SEM at different RH (in hours)</th>
<th>7%</th>
<th>33%</th>
<th>53%</th>
<th>75%</th>
<th>97%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1998 Taiwan population</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25°C</td>
<td>21.3 ± 1.8</td>
<td>21.8 ± 1.6</td>
<td>24.2 ± 1.5</td>
<td>26.1 ± 1.8</td>
<td>25.5 ± 2.1</td>
</tr>
<tr>
<td>2006 mainland China population</td>
<td>21.1 ± 1.3</td>
<td>22.3 ± 1.2</td>
<td>25.8 ± 1.7</td>
<td>27.3 ± 1.2</td>
<td>25.9 ± 1.8</td>
</tr>
<tr>
<td>30°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1998 Taiwan population</td>
<td>12.1 ± .8</td>
<td>14.3 ± 1.1</td>
<td>18.0 ± 1.2</td>
<td>18.6 ± 1.2</td>
<td>22.3 ± 1.8</td>
</tr>
<tr>
<td>2006 mainland China population</td>
<td>12.8 ± 1.2</td>
<td>16.2 ± 1.3</td>
<td>20 ± 1.8</td>
<td>20.3 ± .8</td>
<td>23.5 ± .7</td>
</tr>
</tbody>
</table>
Table 2-2. Longevity of *D. aligharensis* provided with various food sources at 25°C. Means in the same column followed by the same letter are not significantly different (Tukey’s Studentized range HSD at α= 0.05).

<table>
<thead>
<tr>
<th>Food source</th>
<th>Longevity (days until mortality)</th>
<th>Range (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>no food/water</td>
<td>$1.05 \pm 0.02$ A</td>
<td>0 - 2</td>
</tr>
<tr>
<td>water only</td>
<td>$1.43 \pm 0.05$ A</td>
<td>0 - 3</td>
</tr>
<tr>
<td>yeast/water</td>
<td>$2.05 \pm 0.1$ A</td>
<td>0 - 4</td>
</tr>
<tr>
<td>Florida pusley (flowering)</td>
<td>$2.75 \pm 0.1$ A</td>
<td>0 - 9</td>
</tr>
<tr>
<td>ACP honeydew/wax</td>
<td>$10.5 \pm 0.6$ B</td>
<td>0 - 26</td>
</tr>
<tr>
<td>ACP nymphs (1-4th instars)</td>
<td>$27.5 \pm 1.2$ C</td>
<td>0 - 61</td>
</tr>
<tr>
<td>honey</td>
<td>$35.4 \pm 1.2$ D</td>
<td>0 - 62</td>
</tr>
<tr>
<td>honey/yeast</td>
<td>$38.1 \pm 0.9$ D</td>
<td>0 - 71</td>
</tr>
</tbody>
</table>
Figure 2-1. Survival curve of *D. aligarhensis* held at 7% RH at 25° and 30°C.
Figure 2-2. Survival curve of *D. aligarhensis* held at 33% RH at 25° and 30°C.
Figure 2-3. Survival curve of *D. aligarhensis* held at 53% RH at 25° and 30°C.
Figure 2-4. Survival curve of *D. aligarhensis* held at 75% RH at 25° and 30°C.
Figure 2-5. Survival curve of *D. aligarhensis* held at 97% RH at 25° and 30°C.
Figure 2-6. Box and whisker diagrams showing longevity data for D. aligarhensis provided with various food sources at 25°C. Boxes enclose upper and lower quartiles, horizontal line inside box indicates median value and cross mean value, vertical lines extend to the data range.
CHAPTER 3
LARVAL DEVELOPMENT OF DIAPHORENCYRTUS ALIGARHENSIS
(HYMENOPTERA: ENCYRTIDAE) AN ENDOPARASITOID OF DIAPHORINA CITRI
(HEMIPTERA: PSYLLIDAE).

Introduction

The Asian citrus psyllid (ACP), Diaphorina citri Kuyawama, (Hemiptera: Psyllidae) is a serious pest of citrus and its close relatives (McClean & Schwarz, 1970). Immature D. citri feed on sap from the phloem of young soft shoots and buds, causing leaf damage and distortion or death of new shoots (Mead, 1977). Additionally, psyllids secrete large quantities of honeydew, promoting the growth of sooty mold which can inhibit photosynthesis as well as tarnish fruit appearance (Wang et al., 2001). However, damage from direct feeding is of little consequence compared with the transmission of the phloem-limited gram-negative bacterium Candidatus Liberibacter asiaticus Jagoueix, Bove´ and Garnier (α-Proteobacteria) (Jagoueix et al., 1994) which results in a systemic tree infection leading to poor fruit production and tree decline (McClean & Schwarz, 1970). This disease is commonly referred to as citrus greening due to the characteristic of affected fruit to remain green at the peduncular end (Bove, 2006). The disease is also known as Huanglongbing (HLB), Chinese for “yellow shoot disease” in reference to the initial foliar symptoms (van Vuuren, 1996). Greening was first reported in Florida in Miami-Dade County in September of 2005 and has since been confirmed in over 30 counties (Morris & Muraro, 2008) as well as Georgia, South Carolina, and Louisiana (NAPPO 2008, 2009b, a).

Two host specific koinobiont parasitic wasps, Tamarixia radiata (Waterston) and Diaphorencyrtus aligarhensis (Shafee, Alam & Argarwal), were imported from Taiwan into Florida in 1998 (Hoy & Nguyen, 1998) as biological control agents for ACP. Both
parasitoids were released in a successful effort to reduce psyllid populations in Reunion Island (Étienne & Aubert, 1980) and has been attributed with a significant role in biological control of the psyllid in Taiwan (Chien & Chu, 1996). Both species were released into Florida beginning in 1999. Currently, *T. radiata* has established in many regions of Florida providing varying levels of control (Qureshi et al., 2009), while *D. aligarhensis* has not yet established.

In September of 2006, an additional population of *D. aligarhensis* was collected in Guangdong province, China (R. Nguyen personal communication), and sent to Florida where a colony was initiated in the Maximum Security Room of the Florida Biological Control Laboratory located at the Florida Department of Agriculture and Consumer Services, Division of Plant Industry compound in Gainesville, Florida. Parasitoids were held in quarantine through 20 generations before release permitting was complete. The goal of this importation from a different region of China was to rear, study, mass-release and establish this population of *D. aligarhensis* which may better adapt and compete in Florida as a biological control agent of ACP.

PCR analysis proved both populations of *D. aligarhensis* to be positive for *Wolbachia* (Jeyaprakash & Hoy, 2000; Chapter 1) which is the most likely cause of thelytoky in these wasps (Stouthamer et al., 2002; Hagimori et al., 2006). *D. aligarhensis* reportedly utilizes 2nd – 4th instars for oviposition and host feeds on 1st – 4th instars (Skelley & Hoy, 2004). However to date, there has been no published study on larval development of *D. aligarhensis*. This report compares the development times of *D. aligarhensis* embryos and larvae oviposited in 2nd, 3rd and 4th instar nymphs of *D. citri*. Eggs and larvae were imaged using a digital stereoscope or a Scanning Electron
Microscope (SEM). This information regarding wasp development provides a foundation to improve the culturing of this wasp for augmentative releases as well as add to the general knowledge of *D. aligarhensis* and *D. citri* biology.

**Materials and Methods**

**Colonies**

*D. citri* colonies were maintained on orange jasmine, *Murraya paniculata* plants in 3.78 L pots with a 30:70 mix of vermiculite (Jungle Growth Professional Growers Mix potting soil, Piedmont Pacific, Inc. Statham GA). Plants were maintained under natural sunlight in a temperature controlled greenhouse. Temperatures were maintained between 27-32°C and the relative humidity (RH) varied from 40-70%. Freshly flushing plants were moved from the greenhouse into a rearing room and held at 25° ± 1°C and 55 ± 5% RH. Plants were kept in 47.5 x 47.5 x 93.0 cm BugDorm-4180F Insect Rearing Cages (Bioquip, Rancho Dominguez, CA) and illuminated with 400 Watt EYE 4200K BT28 bulbs in Metal Halide Growlights (Hydrofarm, Petaluma, Ca.) for a 16:8 hour light:dark period. Approximately 100 adult psyllids were released per cage and allowed to oviposit for 12-14 days until all 5 nymphal stages were present.

*D. aligarhensis* were maintained under the same conditions as above but located in a separate room to prevent contamination of the *D. citri* colony. After the 12-14 day psyllid exposure period, plants were moved to the parasitoid room and placed in a cage with 10 newly emerged female wasps. All wasps were removed after 1 week. Plants remained in the cage for an additional 2 weeks until all parasitoids had emerged. Emerging parasitoids were collected daily, labeled and held in 50 mL centrifuge tubes (Crystalgen, Long Island NY) with a honey-soaked cotton wick until they were used.
**Vital Dyes**

Fluorescent and vital dyes have been successfully used as internal markers in various parasitic Hymenoptera and Diptera (Strand et al., 1990; Hagler & Jackson, 2001). In an attempt to label parasitoid eggs and perhaps the emerging larvae to facilitate detection and tracking, possibly without dissection, parasitoids were fed various vital dyes. Acridine orange, blue dextran, Congo red, fluorescein isothiocyanate-dextran or trypan blue (Sigma, St. Louis, MO) were added to a 50:50 honey:distilled water mixture at varying concentrations. Acridine orange and fluorescein isothiocyanate-dextran (average molecular weight of 150,000) concentrations were tested at 0.05%, 0.1%, 0.5%. Blue dextran, Congo red and trypan blue were initially tested at a concentration of 0.1%, 2% and 5%. All doses were tested against a control consisting of a 50:50 honey:distilled water mixture. Adult wasps were fed the dyes by applying 7 µl of each solution to a 6 mm diameter Whatman # 1 filter paper disc (Whatman, Bridgestone, England). A treated disc was placed in a 5 mL glass screw top vial with a single newly eclosed adult (< 12 hrs.) female wasp. A total of ten wasps were exposed to each concentration for each of the 5 dyes. Vials were kept in the dark to prevent photo degradation and were held for 24 hours at 25°C and 50% RH. After the 24 hour feeding period, the wasps were observed for effects on behavior or survival. Wasps were dissected under the Leica MZ FLIII and dyes were detected using a GFP/DsRed dual filter set (Excitation 470/30 nm and 565/20 nm; Barrier 510 nm and 580; Emitter 535/45 nm and 610/60 nm), a Texas Red filter set (Excitation 560/40 nm; Barrier 610 LP nm; Emitter 630/60 nm) or a GFP Plus filter set (Excitation 480/40 nm; Barrier 510 nm; Emitter 525/50 nm) and photographed.
Larval Development

Newly eclosed adult wasps (<12 hours old) were collected and placed directly into one of three identical BugDorm cages (described above) with one orange jasmine plant containing either 2\textsuperscript{nd}, 3\textsuperscript{rd} or 4\textsuperscript{th} instar psyllid nymphs. One set each of 5.5 ± 0.5, 6.5 ± 0.5 and 7.5 ± 0.5 day old 4\textsuperscript{th} instar nymphs were evaluated. Clean freshly flushing plants were placed into a psyllid rearing cage for 24 hours to permit oviposition. After 24 hrs, all adult psyllids were removed and each plant was isolated in a separate cage until nymphs reached the desired instar. Between 20 and 30 wasps were placed in each cage for 24 hours and then removed. All plants were held under the above rearing conditions for the duration of the experiment. The developmental stage of psyllid instars was determined using previously published criteria (Liu & Tsai, 2000), and instars were confirmed by head capsule width (Bioquip, Rancho Dominguez, CA) based on a published scale (Nava et al., 2007).

Approximately 10-30 nymphs of each instar were dissected until at least 5 parasitoid larvae were collected. The duration of each developmental stage of *D. aligarhensis* was determined by conducting dissections at 24 hr intervals following the removal of the wasps so that the starting population of embryos was 0 – 24 h old at first observation. At each dissection, the time when the first members of a specific larval stage appeared and when the last members were present was noted. The median of that period was then taken as the estimated duration for that developmental stage. Dissections were conducted using a Leica MZFLIII stereomicroscope with fluorescent capability and equipped with a Leica DC500 digital camera operated with Leica IM50 software. *D. aligarhensis* eggs, larvae, or pupae were photographed, measured using
the microscope’s reticule calibrated with a stage micrometer (Unico, 0-2 mm, 0.01mm divisions), and their physical position within the nymph noted. In the case of superparasitism, the developmental stage of all larvae was recorded. Each of the three nymphal instar development experiments was repeated three times.

To determine if the reduced development time (12 d vs 16 d) observed when rearing wasps from 6.5 d old 4\textsuperscript{th} instar nymphs affected wasp fertility or lifespan, the fecundity and longevity of wasps recovered from short period development were examined. Three newly emerged wasps reared from 6.5 d old 4\textsuperscript{th} instar nymph and three reared from 3 - 4 d old 2\textsuperscript{nd} - 3\textsuperscript{rd} instar nymphs were randomly selected and individually isolated on infested (2\textsuperscript{nd} - 4\textsuperscript{th} instar nymphs) \textit{M. paniculata} plants held under the above rearing conditions. Each wasp was located and transferred every five days to a new plant infested with psyllids for oviposition until the wasp died. Starting 10 days after first wasp exposure, the number of resulting progeny from each wasp was recorded daily.

Freshly dissected larvae were placed in a 2.5\% Glutaraldehyde solution for up to 1 week until prepared for SEM imaging. Larvae were double rinsed in double distilled water, dehydrated by passage from water through 25\%, 50\%, and 100\% ethanol in succession over <1 hr, and stored in 100\% ethanol. Specimens were subsequently air dried then sputter coated with Gold-Palladium using a Baltec SCD-005 sputter coater, and examined using a Hitachi S-450 scanning electron microscope operating at 10 kV.

**Statistical Analysis**

The Chi-square Chi square analysis analyzing the difference in the proportion of nymphal instars superparasitized. To compare the difference in lifespan and fertility
between adult wasps emerging from nymphs parasitized as 2\textsuperscript{nd} or 3\textsuperscript{rd} instars against those emerging from mid (6.5 day old) 4\textsuperscript{th} instars were analyzed using PROC GLM (SAS institute, 9.1). To determine if there was a significant difference between the length of development for eggs oviposited in 6.5 d 4\textsuperscript{th} instar nymphs, the immature wasp life stages (egg – adult) seen per day were given a numerical value (1-8) and totaled. Daily totals were divided by the number of stages seen per day to create a mean. Means per development day per host stage were analyzed using PROC GLM (SAS Institute, 9.1).

\textbf{Results}

\textbf{Vital Dye Transfer}

Green fluorescence was observed through the abdominal cuticle of all live \textit{D. aligarhensis} wasps fed acridine orange (AO) or fluorescein isothiocyanate-dextran (FID) regardless of concentration. Higher concentrations led to brighter fluorescence (data not shown). No color was seen through the abdomen of any live wasp fed Congo red (CR), blue dextran (BD) or trypan blue (TB) regardless of concentration. All wasps fed AO or FID at all concentrations survived with no measurable effect on behavior and they retain some level of fluorescence through 21 days post dye ingestion. No detectable effect on behavior or survivorship resulted from a 24h period in the presence of honey containing AO or FID at 0.05– 0.5% or from 1\% and 2\% doses of CR, BD, and TB. On the other hand, \textit{D. aligarhensis} wasps fed the 5\% doses of BD and TB were alive but appeared sluggish and exhibited reduced searching behavior while all wasps fed the 5\% dose of CR died within the 24h feeding period which is consistent with previously observed negative effects of these dyes (Barbosa & Peters, 1970). Nevertheless, upon dissection, no dye passed through the insect’s gut wall and into the hemolymph, regardless of
concentration. Isolated parasitoid guts were colored but no other tissues including ova were stained.

**Larval Development, Morphology and Axial Orientation**

*D. aligarhensis* development included an embryonic stage, four larval instars, a prepupal and a pupal stage that occurred within the *D. citri* nymphs. Newly laid eggs were a milky white color but cleared within 12 hours after oviposition (Figure 3-1A). Eggs were oval with an axial length of 130 ± 10 µm and diameter of 100 ± 1µm and the structures of the developing embryo easily discernable within older eggs (Figure 3-2B). The oval eggs of *D. aligarhensis* were not seen to share the typical respiratory specialized dumb-bell shape of other Encyrtidae parasitoids (Quicke, 1997). The chorion of the eggs was smooth, thin and flexible with no apparent anchoring structures observable, and the eggs were not found adhering to any host tissues. Eggs were located randomly throughout the host’s abdomen and thorax with the majority localized within the abdomen and exhibiting no apparent target site or tissue preference. Encapsulation of the eggs was observed in only 1% of the parasitized hosts (5 of 500 parasitism events examined). Encapsulated eggs were uniformly dark in color and completely covered in clear soft tissue. One encapsulation event was seen in both 2nd and 3rd instar hosts while three were observed in 4th instar *D. citri*.

All larval stages of *D. aligarhensis* were hymenopteriform and possessed a translucent integument and a weakly developed head capsule (Figure 3-1C-F). Larvae tapered to a rounded tip at the posterior end which became less pronounced as larvae entered their 3rd and 4th instars. All larval stages possessed short, small, reddish-orange mandibular hooks that were consistently about 1/5 (20%) of total body width (Figure 3-
2D). No other external appendages, including spiracles or hairs, were observed on 1st-4th instars (Figure 3-2C). Gut contents were clearly visible through the transparent cuticle (Figure 3-1C-F).

First instar larvae were 410 ± 7 µm long and 100 ± 5 µm in diameter (Figure 3-1C). These larvae were unanchored and free moving. Most were found in the fore-abdomen or thorax, although several were located in the head region. The orientation of the larval head to the major anterior-posterior axis within the host appeared random.

Second instar larvae measured 620 ± 50 µm in length and 180 ± 2 µm in diameter (Figure 3-1D). The majority of these larvae instar were predominantly oriented with the head towards the rear of the host. Larvae were centrally located in the abdomen and/or thorax.

Third instar larvae measured 860 ± 70 µm in length and 200 ± 8 µm in diameter (Figure 3-1E). The majority of these larvae still maintained their orientation in opposition to the axis of the host with only a few exceptions observed. Larval mouthparts were generally oriented toward the host’s ventral surface. Larvae were centrally located within the abdomen and thorax with the apical tergites often extending into the host head region. Most (80%) had the tail anchored to host tissue by anal secretions which remained attached when the larva was removed (Figure 3-2C, D).

Fourth instar larvae measured 1190 ± 10 µm in length and 400 ± 20 µm in diameter (Figure 3-2D) and all were oriented in opposition to the host with their anterior towards the posterior of the host. The larval mouthparts were generally oriented toward the host’s ventral surface. The majority (80%) of those observed were anchored by anal secretions to various host tissues. The last instar larvae were centrally located and
extended through all 3 body segments of the host nymph. All 4th instar larvae were contained within a fully hardened, darkened mummy by the time they became prepupae. Mummies did not contain any internal host fluids or tissues and were attached to plant surfaces by their ventral surface between the fore-abdomen and the head. As the host’s exoskeleton began to harden, the wasp larva chewed a small hole in the ventral side of the mummy in the thorax to fore-abdomen region. This area remained soft as the rest of the exoskeleton hardened. Liquid drained from this hole and hardened forming a glue-like substance holding the mummy to the plant surface. To test if host fluids alone were sufficient to glue the mummy to the substrate, hardened mummies of various ages were removed from host plants, rinsed in distilled water, and allowed to air dry. The ventral surface was covered in internal fluids from unparasitized 4th and 5th instar nymph mummies’, held to plant tissue and allowed to air dry. None were unable to stay attached regardless of the drying time. This suggested that an additional component from the D. aligarhensis larva was essential to produce the “glue” that attached the parasitized mummy to the plant surface.

Prepupating 4th instar larvae shortened by constriction to a length of 1070 ± 40 µm and a diameter of 510 ± 12 µm, forming uniform segments often utilizing only 70-80% of the length of the psyllid mummy (Figure 3-1F). All pupae were exarate and positioned in the opposite direction to the host axis, with the head still turned downward facing the host’s ventral surface. The structural definition of the head and abdomen was observed within 2 days of pupation with the legs, mouthparts and antennae becoming distinguishable by day 4 of the pupal stage (Figure 3-3A). Subsequently, the thorax, eyes and ocelli had begun to melanize within 5 days which was complete in the
abdomen, legs, and lastly the antennae by 7 days after pupation. In addition to increased pigmentation, wing venation and sensilla development had occurred by this time.

Three events of adult eclosion were observed during this study. The pharate adult began eclosion with the ventral surface oriented towards the ventral surface of the nymphal mummy. The eclosing adult underwent rotational movements to reorient 180° so that they finally faced upward towards their host’s dorsal surface. The eclosing adult then chewed through the dorsal abdominal integument of the *D. citri* mummy leaving a round emergence hole (Figure 3-3B). Once the emergence hole was excavated, the eclosing adult was able to crawl out onto the plant surface where it groomed and flexed its wings, rested and then began walking about.

**Concomitant Host and Parasitoid Development**

Liu & Tsai (Liu & Tsai, 2000) determined that *D. citri* nymphs reared at 25°C enter the 4th instar on average 5.5 d after egg hatch and enter the 5th instar approximately 4.5 d later on day 9. This is in comparison with a duration of 1.5 d for both the 2nd and 3rd instar nymphs. Although the fifth instar has the longest duration of approximately 5 d, the 5th instar *D. citri* nymphs are not utilized by *D. aligarhensis* for parasitism or feeding purposes.

Following a parasitism event by *D. aligarhensis*, *D. citri* nymphs continued to feed, develop and molt through to the 5th instar regardless of which instar (2nd - 4th) was parasitized. Parasitized nymphs remained as 5th instars until they began to harden to form a mummy.
For *D. aligarhensis* that were oviposited in 2\textsuperscript{nd} or 3\textsuperscript{rd} instar *D. citri* nymphs, the embryonic, 1\textsuperscript{st} and 2\textsuperscript{nd} larval instar stages lasted an estimated 2 d each (Figure 3-4 A). The duration of the 3\textsuperscript{rd} and 4\textsuperscript{th} instar larvae and prepupal stages was 1 d each followed by a 7 d pupal stage. Nymphal mummies formed at 8 d after parasitism which coincided with the beginning of the prepupal stage for the *D. aligarhensis* larvae, and adult eclosion occurred 16 d after oviposition.

On the other hand, when oviposition occurred in 4\textsuperscript{th} instar hosts, development time was reduced depending on host age. When 5.5 d old 4\textsuperscript{th} instar *D. citri* nymphs were parasitized, development time from the egg to the 4\textsuperscript{th} instar was slightly shortened (1 d) when compared to those developing from parasitized 2\textsuperscript{nd} or 3\textsuperscript{rd} instar nymphs with no discernable difference in the duration of the prepupal or pupal stages (Figure 3-4B). Mummies were seen 7-8 d post-parasitism with adult eclosion on day 15, one day less than for 2\textsuperscript{nd} or 3\textsuperscript{rd} instars (Figure 3-4B). However, when parasitization occurred in 6.5 d old 4\textsuperscript{th} instar *D. citri* nymphs, mummification was observed within 5-6 d and adult eclosion occurred on day 12 (Figure 3-4C). Developmental (egg – pupa) of immature *D. aligarhensis* was clearly shortened when oviposition occurred in these older nymphs.

A significant difference in the overall developmental time between host stages was confirmed for eggs oviposited in 6.5 d old 4\textsuperscript{th} instar *D. citri* nymphs when compared with developmental times observed for eggs oviposited in all younger *D. citri* nymphs (df = 1, F = 8.74, P = 0.0044). If oviposition occurred in 7.5 d old 4\textsuperscript{th} instar nymphs, no wasps were recovered. Eggs were found up to 6 d after parasitism in these *D. citri* nymphs but appeared darkened with little or no embryonic development. No *D. aligarhensis* larvae were found in any of the 7.5 d old 4\textsuperscript{th} instar *D. citri* nymphs.
No significant difference was seen in either lifespan (df= 1, F= 0.04, P= 0.85) or
fertility (df= 1, F= 0.08, P= 0.79) between wasps emerging from 6.5-day old 4th instars
and thus with 12 d development times and those emerging from younger stages and
taking 16 days to develop. Wasps reared from 6.5 d old 4th instar nymphs lived for an
average of 26.3 ± 2.9 d and produced an average of 185.3 ± 31.7 offspring. Those
reared from 3 - 4 d old 2nd - 3rd instar nymphs lived for an average of 25.6 ± 1.48 d and
produced 175 ± 21.5 offspring.

Superparasitism

Of the more than 500 wasp eggs, larvae or pupae located in D. citri nymphs, only
22 events of superparasitism (4%) were observed (Table 3-1). Ten were observed in 2nd
instar nymphs, four in 3rd instar nymphs, and eight in 4th instar nymphs (4 in 5.5 day old
nymphs, 4 in 6.5 day old nymphs). Chi square analysis showed no significant difference
in the proportion of 2nd- 4th instars that were superparasitized ($\chi^2= 2.5$, df= 2, P= 0.28).

Of the ten events in 2nd instar hosts, six consisted of two eggs in one host (all
within the abdomen) and four were instances of two larvae in one host. In all four
events, one 1st instar larva along with one 2nd, 3rd or 4th instar larva was observed.
Superparasitism of 3rd instar nymphs consisted of two eggs in 2 different hosts, and one
instance each of one egg plus one 1st instar larva and one 1st and 3rd instar larva. Two
5.5 day and two 6.5 day 4th instar hosts contained two eggs each and another two hosts
each contained a 1st instar and a 3rd instar wasp larva. No superparasitism was noted in
7.5 day old 4th instar hosts. In all cases where two larvae where found in one host the
more advanced larvae appeared healthy while the smaller appeared sluggish or
motionless, though alive.
Discussion

The immature stages of *D. aligarhensis* progressed through a larval/pupal development that is typical for many endoparasitoids (Quicke, 1997). The larvae were soft-bodied with no hairs, bristles or external appendages, such as anal vesicles, observable in any instar. The number of encapsulated eggs was low (1%) indicating that the wasp has probably evolved a means of suppressing such a host response. Initially eggs and larvae were free-floating within the hemocoel but by the 3\(^{rd}\) instar, larvae had begun attaching to nympha! tissues via anal secretions. This attachment provided a means of orientation which was in opposition to the host nymph and face down with the posterior end of 3\(^{rd}\) and 4\(^{th}\) larval instars anchored to the host thoracic region. During the 4\(^{th}\) instar, the host nymph hardened into a mummy glued to a plant surface, apparently with the aid of secretions from the wasp larva. Development of *D. aligarhensis* from egg to adult took approximately 16 days when oviposition occurred in 2\(^{nd}\) through early 4\(^{th}\) instar nymphs but was shortened by four days (25%) when the wasps oviposited in mid-4\(^{th}\) instar *D. citri* nymphs.

The observation that the *D. aligarhensis* larvae were anchored to tissues of the *D. citri* nymphs by secretions originating from the anus of the larvae is the first report of this manipulation of the host environment and represents a unique modification within the hymenopteran family Encyrtidae. Anal secretions have been observed in other parasitoids but they have been associated with other functions. A clear, sticky anal secretion was identified as a means of fastening the freshly-ecdysed pupa of *Elachertus scutellatus* Howard (Hymentoptera: Eulophidae) an ectoparasitoid of *Calpodes ethlius* Stoll (Lepidoptera: Hesperiidae) to its leafy substrate (MacDonald & Caveney, 2004). The larva of the endoparasitic wasp *Pimpla turionellae* L. (Hymenoptera:
Ichneumonidae), produces an anal secretion which directly inhibits the growth of bacteria and fungi (Fuhrer & Willers, 1986). Additionally, many chalcidoids, including some endoparasitoids, secrete a protective silk cocoon-like structure from the anus of the final instar (Quicke, 1997). In contrast to the anal secretions, the anal vesicles protruding from early instars of some braconids and ichneumonids are responsible for various secretory and uptake functions including respiration, nutrition, water and ion balance, and excretion (Kaeslin et al., 2006; Yu et al., 2008).

The total time that this population of *D. aligarhensis* completed development took 16 days at 25°C when the eggs were oviposited in 2nd and 3rd *D. citri* nymphs. In comparison, the previously imported Taiwan population of *D. aligarhensis* required 18 days at 25°C (Skelley & Hoy, 2004). The ectoparasite, *Tamarixia radiata* (Hymenoptera: Eulophidae), another imported biological control agent of ACP in Florida, requires 12 days to complete development at 25°C (Liu & Tsai, 2000). However when oviposition occurred in 6.5 day (mid) 4th instar *D. citri* nymphs, adult eclosion of *D. aligarhensis* occurred only 12 days after oviposition which shortened development by 25%.

Importantly, the reduced developmental period did not have a negative effect on either the fertility or lifespan of the wasp. This finding suggests that commercial production of *D. aligarhensis* for augmentive release could be enhanced by timing oviposition to mid 4th instar *D. citri* nymphs.

Wasps were unable to effectively parasitize 7.5 day old 4th instar hosts. By late 4th instar the cuticle thickens and hosts become more efficient at defensive twitching. These changes make it more difficult for wasps to successfully oviposit and are likely responsible for the low number of eggs located in these nymphs (n=10). Additionally, all
eggs found oviposited in late 4\textsuperscript{th} instar \textit{D. citri} nymphs were dead which suggested there may be a physiological restriction to wasp development in older nymphs. It was not determined whether older 4\textsuperscript{th} instar hosts are capable of suppressing egg hatch, or if some chemical or hormone in host hemolymph needed for development is lacking.

This population of \textit{D. aligarhensis} exhibited low levels of superparasitism (<5\%) despite large numbers of wasps (20-30 per plant per instar) being released into the same cage. However, the exposure period was short (24hrs) and superparasitism rates would likely increase with increased exposure times. No more than 2 larvae were observed in any one host nymph. In all superparasitism events, one larva had advanced in development while the appeared to have remained as a 1\textsuperscript{st} instar. Supernumerary conspecific larvae occurring in superparasitism are subject to competitive elimination, either by physical attack or by physiological suppression of development (Fischer, 1971). Typically, 1\textsuperscript{st} instar larvae of other endoparasitic species are equipped with large mandibles capable of destroying conspecifics (Salt, 1961; Quicke, 1997). However, the mandibles of \textit{D. aligarhensis} are small and short, and no physical damage was observed in any coexisting supernumerary larvae, suggesting a physiological suppression of limiting supernumerary development.

Several forms of physiological suppression have been proposed, including larval release of toxins or cytolytic enzymes, anoxia, nutritional deprivation by older instars, changes in nutrient availability, or inhibition by substances injected by the adult parasitoid during oviposition (Fischer, 1971; Vinson & Iwantsch, 1980). Unfortunately, there was no transference of orally administered vital/fluorescent dyes to the eggs so non-invasive observation of superparasitism was not possible. Because dissection was
the only means of determining superparasitism events, it was impossible to determine if the larger of the two larvae would successfully complete development to adulthood. However, one superparasitism event showed a healthy prepupa coexisting with a living 1\textsuperscript{st} instar larva indicating that development of the more developed wasp could most likely have gone to completion.

As the 4\textsuperscript{th} instar \textit{D. aligarhensis} larva transformed to the prepupa, the parasitized nymph began to harden into a mummy. The wasp larva must also attach the dead host’s exoskeleton to the plant tissue and does this by chewing a hole through the host’s ventor, through which host body fluids and likely the larva’s anal secretions drain and harden forming a glue-like substance that secures the mummy to the plant surface. The use of anal secretions as a glue has been observed previously in \textit{Elachertus scutellatus} Howard (Hymenoptera: Eulophidae) an ectoparasitoid of \textit{Calpodes ethlius} Stoll (Lepidoptera: Hesperiidae) where newly-ecdysed pupa uses a clear, sticky anal secretion as a means of fastening to its leafy substrate (MacDonald & Caveney, 2004). In contrast, the prepupa of \textit{T. radiata} spins a silk webbing to secure the mummy to the substrate (Patil et al., 1993; P. Stansly unpublished data).

Once pharate adult development has completed, the eclosing adult \textit{D. aligarhensis} wasp must exit the mummy by chewing a round hole through the dorsal surface of the host’s abdominal cuticle similar to the behaviors of other endoparasites (Liu & Stansly, 1996). This exit hole can be used to distinguish between a mummy that has been parasitized by \textit{D. aligarhensis} from \textit{T. radiate} which exits dorsally through the thorax (Hoy, 2005) rather than the abdomen.
The observations on development of *D. aligarhensis* provide a foundation to developing informed strategies for rearing and augmentive release of this endoparasite for the control of the Asian citrus psyllid, *D. citri*. That the temporal period for completing development can be shortened by 25% may facilitate a more efficient rearing program for the production of *D. aligarhensis* wasps.
Table 3-1. Superparasitism events observed in dissected 2nd-4th instar ACP nymphs. Each + symbolizes the supernumerary stage encountered (egg-4th instars) and the host age. No more than 2 immature wasps were found in any dissected nymph.

<table>
<thead>
<tr>
<th>Nymphal instar (# events observed)</th>
<th>D. aliagrhensis immature life stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Egg</td>
</tr>
<tr>
<td>2nd (10)</td>
<td>++  (6)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd (4)</td>
<td>++  (2)</td>
</tr>
<tr>
<td></td>
<td>+</td>
</tr>
<tr>
<td>4th (8)</td>
<td>++  (2)</td>
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<tr>
<td>- 5.5 days post egg hatch (4)</td>
<td>+</td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>- 6.5 days post egg hatch (4)</td>
<td>+</td>
</tr>
</tbody>
</table>
Figure 3-1. Egg and larval stages of *Diaphorencyrtus aligarhensis*. Specimens are shown oriented with the anterior (head) to the left and posterior (anus) to the right in transmitted light micrographs. The panels show an egg isolated from a host within 12 hr after parasitization (Panel A), an egg isolated from a host within 48 hr after parasitization (Panel B), first instar larva (Panel C), a second instar larva (Panel D), a third instar larva (Panel E), and a fourth instar in the prepupal stage (Panel F).
Figure 3-2. Detailed structure of fourth instar larvae. The mouth and anterior region of a larva were examined in a scanning electron micrograph (Panel A). The black arrow points to the position of the mouth. The anus and posterior region of a larva were observed in a scanning electron micrograph (Panel B). The black arrow points to the position of the anus. The entire ventral surface of the larva was examined in a scanning electron micrograph (Panel C). The black arrow points to the position of the mouth, the white arrow points to the position of the anus and the white arrowhead points to the attached anal secretions. The ventral surface and internal structures of a larva were observed in a transmitted light micrograph and demonstrate the position of the mandibular hooks (white arrowhead) and the anal secretions (Panel D). The posterior region was cleared of anal secretions and observed in a transmitted light micrograph (Panel E). The black arrow points to the position of the anus. All micrographs show the specimen with the anterior to the left.
Figure 3-3. Ventral view of a pharate adult wasp (Panel A) with the head (anterior) oriented to the left. The mummified remains of a Diaphorina citri larva was observed to show the exit hole in the dorsal abdominal cuticle through which emerged the eclosing Diaphorencyrtus aligarhensis adult (the mummy is oriented with the anterior to the left; the black arrowhead points to the anterior edge of the hole).
Figure 3-4. The estimated temporal pattern for the developmental stages of the wasp, *Diaphorencyrtus aligarhensis* oviposited into various stages of host nymphs. The developmental periods for wasp progeny whose eggs were oviposited in third instar *Diaphorina citri* nymphs (Panel A). The profile for eggs deposited in second instar nymphs was not included as there was no discernable difference between the data for second and third nymph oviposition. The developmental periods for wasp progeny whose eggs were oviposited into early (day 5.5) fourth instar *Diaphorina citri* nymphs (Panel B). The developmental periods for wasp progeny whose eggs were oviposited in mid (day 6.5) fourth instar *Diaphorina citri* nymphs (Panel C). The curve representing the number of embryos observed are dark blue, first instar larvae are brown, second instar larvae are green, third instar larvae are purple, fourth instar larvae are blue, prepupae are orange, pupae are light blue, and wasps are violet.
CHAPTER 4
INFLUENCE OF AGE, HOST AVAILABILITY AND SUPPLEMENTAL FOOD ON EGG LOAD, OVIPOSITION BEHAVIOR AND HOST FEEDING OF DIAPHORENCYRTUS ALIGARHENSIS (HYMENOPTERA: ENCYRTIDAE) AN ENDOPARASITOID OF DIAPHORINA CITRI (HEMIPTERA: PSYLLIDAE).

Introduction

The Asian citrus psyllid (ACP), Diaphorina citri Kuwayama, is a key pest of citrus, causing substantial economic damage throughout citrus growing regions of the world (da Graca, 1991; Halbert & Manjunath, 2004). Feeding causes direct damage to trees (McFarland & Hoy, 2001) but more importantly, ACP can vector a phloem-limited gram-negative bacterium Candidatus Liberibacter asiaticus Jagoueix, Bove´ & Garnier (a-Proteobacteria) (Jagoueix et al., 1994). This disease is commonly referred to as citrus greening disease or “huanglongbing” meaning “yellow shoot disease” in Chinese.

Two parasitic wasps, Tamarixia radiata (Waterston) and Diaphorencyrtus aligarhensis (Shafee, Alam and Argarwal), have been reported as primary parasitoids of ACP. T. radiata was successful in reducing psyllid populations when imported from India to Reunion Island and from there to Taiwan (Etienne & Aubert, 1980). D. aligarhensis is an endoparasitoid, while T. radiata is an ectoparasitoid. Both wasps were imported from Taiwan (McFarland & Hoy, 2001) into the US in 1998 (Hoy & Nguyen, 1998). An additional population of T. radiata was also imported into Florida from Vietnam and mixed with the colony from Taiwan. After quarantine lab testing was completed and permits obtained, both species of wasps were released into Florida in late 1999 and early 2000. Currently T. radiata has established throughout of the citrus growing region of Florida, providing varying levels of control (Qureshi et al., 2009), while D. aligarhensis has not established for unknown reasons.
In September of 2006, 100 *D. citri* nymphs on *Citrus* sp. cuttings were sent by R. Nguyen from Guangdong province, China into the Maximum Security facility of the Florida Biological Control Laboratory located at the Florida Department of Agriculture and Consumer Services, Division of Plant Industry compound in Gainesville, Florida. Fifty adult *D. aligarhensis* emerged and were reared in quarantine through 20 generations until the necessary permits for release were granted. The goal of this importation was to rear, study, mass release and establish *D. aligarhensis* from mainland China, with the hope that it would better adapt than the Taiwanese population and compete successfully in Florida as a biological control agent of ACP.

Colonies of *D. aligarhensis* from both these origins are thelytokous. The first three generations of the Chinese population produced only 5% males after which only females emerged. Both the Taiwanese and Mainland colonies have been tested using PCR analysis and proved positive for Wolbachia (Jeyaprakash & Hoy, 2000; E. Rohrig, Chapter 1).

Presently, there is little published information on the biology of *D. aligarhensis*, particularly regarding host searching and oviposition behavior. One of our goals was to assess oviposition and host feeding preferences of *D. aligarhensis* for different instars of *D. citri*. Another was to evaluate the influence of access to hosts or supplemental food on oviposition and feeding behavior. Because *D. aligarhensis* is being mass reared and released, it is often necessary to hold wasps for a week or more until appropriate numbers are collected for release. The accepted practice when holding parasitic hymenoptera for this purpose is to provide an artificial diet of honey soaked in a cotton wick (Skelley & Hoy, 2004). This holding period, isolated from nymphs for host feeding,
could affect the wasp’s efficiency as a biological control agent. Parasitoids learn cues that are associated with their hosts through experience, and use these cues to forage more efficiently (Papaj & Lewis, 1993). Additionally, lack of proteins and other nutrients provided through host feeding likely leads to egg resorption, further limiting oviposition rates once the wasp is released. For these reasons we compared the behavior and egg load of wasps held with and without honey and/or host nymphs.

### Materials and Methods

**Colonies**

*D. citri* colonies were maintained on orange jasmine, *Murraya paniculata* plants in 3.78 L pots filled with Jungle Growth Professional Growers Mix potting soil (Piedmont Pacific, Inc. Statham GA). Plants were maintained under natural sunlight in a greenhouse at the USDA, CMAVE complex in Gainesville, FL. Temperatures were held at 25-30°C and humidity levels varied from 40-70%. Freshly flushing plants were moved from the greenhouse into a rearing room with temperature and humidity control at the same USDA complex. Plants were held in 47.5x47.5x93.0 cm BugDorm-4180F Insect Rearing Cages (Bioquip Rancho Dominguez, CA). EYE 4200K BT28 400 Watt bulbs in Hydrofarm Metal Halide growlights (Petaluma, Ca.) illuminated the cages for a 16:8 hour light: dark period. Humidity was maintained at 50-60% at a temperature of 25°C. Approximately 100 adult psyllids were released into each cage and allowed to oviposit for 12-14 days until all 5 nymphal stages were present and available for experimentation or for rearing.

*D. aligarhensis* were maintained under the same conditions as above but located in a separate room to prevent contamination of the *D. citri* colony. After the 12-14 day exposure period to adult psyllids, infested plants were moved to the parasitoid room and
placed in a cage with 10 newly emerged female wasps. All wasps were removed after 1 week. Plants remained in the cage for an additional 2 weeks until all parasitoids had emerged.

**Egg-load**

We compared the egg-load of newly emerged female wasps with those held for 5, 10, 15, 20, 25 and 30 days with access to either psyllid nymphs and honey or honey only. Wasps with access to honey were held at either 25°C or 17°C. It is not uncommon for biological control practitioners to hold wasps at a relatively low temperature to slow metabolism and increase longevity until an appropriate release time. All wasps used in this experiment were collected when newly emerged (<12 hrs old). Ten wasps were immediately dissected and eggload recorded. All mature eggs located in the common oviduct and ovarioles were counted. Remaining wasps were placed 120 each into one of three identical cages described above. The average lifespan of *D. aligarhensis* was 28 days under these conditions (ER, unpublished data). Therefore, a larger number of wasps (n= 120) were placed into each cage to have sufficient numbers available for dissection over the 30-day period. Caged wasps were provided with either a honeysoaked cotton strip alone, or accompanied by ~500 nymphs on orange jasmine plants for host feeding and oviposition experience (2nd-4th instars). Old plants and nymphs were removed and replaced each morning. Caged wasps held at 17°C were located inside a Forma Scientific environmental chamber with a 16:8 L: D light cycle and 50-60% RH. The cage was removed every other day and held at 23-25°C, 50% RH for one hour to allow the wasps to feed. Without this feeding period, wasp longevity was reduced to an average of 15 days (ER, unpublished). Cages were held under the above rearing conditions for the remainder of the experiment. At 5, 10, 15, 20, 25, and 30 days
post emergence, 10 live female wasps per treatment were dissected and the numbers of mature eggs noted. This procedure was repeated 5 times for a total of 50 adults dissected per holding time. All dissections were made using a Leica MZFLIII stereomicroscope. Egg loads at dissection under various holding conditions were recorded and compared.

Observational Arenas

Behavioral observations were made with three distinct sets of wasps: (1) newly emerged wasps (<12 hrs), (2) held 7 days with access to honey only (naïve) or (3) held 7 days with access to all stages of nymphs for oviposition and host feeding (experienced). Approximately 8 days post-parasitism, nymphs hardened and darkened, forming a mummy which remained for an additional 8 days until adult wasp emergence (Chapter 1). At fourteen days post parasitism, mummies were carefully collected directly from the plant and placed individually into 50 mL Crystalgen polypropylene centrifuge vials with 50.8 mm x 50.8 mm fine mesh screens placed over the cap end and held in place by a rubber band. Seven day-old wasps were held with either a honey-soaked cotton strip, or were provided with 40 nymphs of four different growth stages daily. *D. aligarhensis* are reported to utilize 2nd, 3rd and 4th instars (of 5 total) for ovipostion and 1st-4th instars for host feeding (Skelley & Hoy, 2004). Ten each of 1st-4th instars were carefully transferred onto small, newly cut young shoots 101.6 mm long using a fine sable hair brush and placed into the 50mL vials. Each morning, the old shoots and nymphs were removed and replaced with fresh ones. All vials were held under the above cage conditions until they were used in experiments. Parasitoids and nymphs were used only once in these experiments.
Parasitoid feeding and oviposition behavior were observed in clear 100 x 15mm sterilized polystyrene petri dishes (Thomas scientific, Swedesboro, NJ). One 70-90 mm long freshly cut young shoot of *M. paniculata* containing 6-8 leaves was placed in the center of the petri dish. Scotch® double sided cellophane tape was used to affix the underside of the leaves to the bottom of the petri dish, preventing nymphs from moving under the cuttings and out of sight. Forty *D. citri* nymphs, 10 each of 1st - 4th instar, were transferred from a rearing plant onto the dorsal surface of the cut shoot. Two hours before experiments began, nymphs were randomly placed, 5-7 per leaf, onto the cuttings and allowed to adjust and begin feeding. Placement was random to make certain that the order of encounters of the parasitoid was not affected by the location of the host. Nymphs on leaves were numbered 1–40 with a Sharpie nontoxic black marker. Numbers were placed directly on the leaf next to the nymph and a diagram was created to help keep track of each nymph if it moved while being investigated by a wasp. Each number (1-40) correlated with a specific nymph for identification during the observational period and dissection. Observations were made for a period of one hour using an Olympus SZ trinocular stereo microscope illuminated with a Southern Micro Instruments EKE Light Source. Activities were recorded using a Sony DCR-TRV10 Mini DV Camcorder connected to the microscope's trinocular eyepiece. Room temperature was 23 ± 1°C with an RH of 50 ± 5%. Time was marked from the initiation of a probing event using an Avalon Coach Series digital stop watch with extra large display. Five replicates of each of the three sets of wasps were observed and recorded. The wasp was removed at the end of the one hour observational period. All nymphs were dissected and the presence of eggs noted.
Frequency and duration of 8 behavioral events were recorded:

1) **Searching.** The wasp moved along drumming the surface with its antennae held forward and apart. Generally the wasp moved forward until reaching a leaf edge or stem end, then turned and moved forward again until locating a nymph or some wax. Often the wasp left the plant cutting and moved around all surfaces of the petri dish until encountering the plant cutting.

2) **Antennating.** The wasp drummed the antennae over the entire surface area of a nymph (particularly the anus). In many cases, the parasitoid would mount the nymph and use her legs to lift one side of it to drum part of the ventral surface. Whether or not the nymph was lifted to drum was not recorded. Lifting was nearly always observed during encounters with older, larger nymphs (3rd-4th instars). Antennae were held forward and apart but moved more rapidly then while searching.

3) **Probing.** The wasp arched its back, pulling the abdomen slightly down and forward. The ovipositor was inserted into the nymph’s abdomen or thorax. Antennae were held together anteriorly and in a downward position.

4) **Host feeding.** The wasp lapped host haemolymph from a hole in the dorsal surface of the abdomen previously created by the ovipositor during a probing event.

5) **Wax feeding.** The wasp fed on a piece of host wax, generally not connected to a nymph.

6) **Oviposition.** The wasp successfully inserted an egg into the nymph’s abdomen. Verified by nymphal dissection.

7) **Grooming.** The wasp stopped moving and rubbed its fore-tarsi over its antennae and head and/or rubbed its hind legs together and over the wings.
8) Resting. The wasp sat motionless with its antennae apart and forward off of the surface.

Repeat visits to previously probed nymphs for oviposition or feeding were recorded and nymphs dissected and checked for superparasitism. Mean duration of each behavior was calculated. Each individual nymph that was antennated and then rejected, probed, probed once then rejected, fed upon, repeatedly probed, oviposited into or superparasitized was recorded for each of the three sets of wasps. *D. citri* nymphs often twitched violently from side to side upon an encounter with *D. aligarhensis*. This was considered to be a form of host defense and all such occurrences were recorded as either negative when the wasp succeeded in probing the host, or positive when the wasp was deterred from probing.

**Statistical Analysis**

Analysis of Variance (SAS Institute, 9.1) was used to compare egg loads of dissected females at 5-day increments under the three treatments, to compare mean event durations from the behavioral experiments and to compare newly emerged, naïve, and experienced wasps in terms of numbers of nymphal instars antennated, probed, fed upon and oviposited into. When statistically significant relationships were found, further comparisons of means were made using Tukey’s studentized range (HSD) tests. Chi-square (SAS Institute, 9.1) was used to compare the proportion of host instars superparasitized as well as the proportion of instars parasitized. Ethograms of behavioral pathways and a time budget analysis were completed for each set of wasps. All events that occurred <4 times were left off to simply the ethogram.
Results

Egg-load

*D. aligarhensis* possess paired ovaries, each consisting of 3 polytrophic ovarioles. Newly emerged females contained 8-44 mature eggs each, with an average of 18 ± 0.35 eggs per female. Significant differences in egg load among all three treatments were observed at every 5 day increment (P < .01) except between 17°C with honey only and 25°C with access to hosts at 15 d (F= 3.94, df= 1, P= 0.567). Egg load decreased gradually to an average 5.4 ± 0.27 after 25 days in females fed honey alone at 17°C but more rapidly to less than one egg after 25 days at 25°C (Table 4-1). Females provided with nymphs at 25°C carried a greater egg load at each five day increment than those held with honey only at 25°C. Nymph-fed wasps maintained nearly twice as many eggs as honey-fed wasps until 20 days post emergence. Egg loads differed by as much as 3 – 11x by the end of the 30 day experiment. Egg load averages of wasps held with hosts at 25°C were similar to those held with honey at 17°C for 20 days post emergence but then decreased more rapidly.

Ethograms

A total of 1415 behavioral events were recorded for *D. aligarhensis* over all three treatments. The 5 newly emerged (<12 hrs) wasps observed exhibited 486 behavioral events (Figure 4-1). Of the 150 searching events, 69 (46%) led to antennating of a nymph of which 51% continued on to a probe. Thirty probes (38%) led to host feeding, 30 to a search and 18 (23%) resulted in oviposition. Frass feeding was observed 3 times. Twenty-four (80%) of host feeding events resulted in a repeat probe while 6 (20%) led to a search. Fifty-four (66%) of 81 total grooming events resulted in a search.
The 5 experienced 7 day old wasps exhibited 392 behavioral events (Figure 4-2). Of 100 searching events, 75% led to antennating of a nymph 81% of which continued on to a probe. Twenty-eight probes (28%) led to host feeding, 24 (24%) led to a search and 16 (16%) resulted in oviposition. Frass feeding was observed 4 times. Twenty-one (75%) of host feeding events resulted in a repeat probe while 7 (25%) led to a search. Twenty-five (69%) of the 36 total grooming events resulted in a search.

Naïve 7 day old wasps exhibited 537 behavioral events (Fig. 4-3). Of the 144 searching events, 90 (62%) led to antennating of a nymph of which 63% went on to probe. Fifty-seven probes (50%) led to host feeding, 39 (34%) to a search and 10 (8%) resulted in oviposition. Frass feeding was not observed. Forty-eight (84%) host feeding events resulted in a repeat probe while 3 (5%) led to a search. Fifty-four (69%) of the 78 total grooming events resulted in a search. The following refer to events that individual nymphs were subjected to (ie. excludes repeat visits).

Antennation  Naïve wasps tended to antennate the most individual nymphs (n= 97, 48.5% of total nymphs) followed by newly emerged wasps (n= 81, 40.5% of total nymphs) compared to (n= 78, 39% of total nymphs) for experienced wasps although overall differences were not significant (F= 0.48, df= 2, P= 0.629) by analysis of variance.

Antennation followed by rejection  Experienced and newly emerged wasps rejected the same number of individual nymphs antennated (n=18, 23% of those antennated; n= 18, 22% of those antennated respectively) followed by naive wasps (n= 11, 11% of those antennated). No significant difference was seen between treatments (F= 1.69, df= 2, P= 0.226).
Single Probing  Experienced wasps tended to probe more individual nymphs a single time (n = 50, 25% of total nymphs) followed by newly emerged (n = 48, 24% of total nymphs) than naïve wasps (n = 40, 20% of total nymphs) although no significant difference was seen among all treatments (F = 0.41, df = 2, P = 0.672). Wasps most often probed the center of the dorsal surface of the nymph’s abdomen. In cases where a nymph was ‘lifted’ the host was often probed in the side of the abdomen, usually penetrating the intersegmental membrane.

Probing once then rejected  Rejection rate tended to be highest for experienced wasps (n = 40, 80% of nymphs probed once) followed by newly emerged wasps (n = 33, 68%) and then naïve wasps (n = 24, 60%) although overall differences were not significant (F = 1.88, df = 2, P = 0.194).

Repeat probing  Naïve wasps repeated a probe on significantly more individual nymphs (n = 36) than newly emerged (n = 15) or experienced wasps (n = 8) (F = 15.93, df = 2, P = .0004). Repeat probes varied between 2-19 probes per individual nymph.

Host feeding  Naïve wasps fed on the most individual nymphs (n = 12), followed by experienced (n = 9) and then newly emerged (n = 7). Naïve wasps host fed on one 1st instar, six 2nd, three 3rd and two 4th instar nymphs. Experienced wasps did not feed on any 1st or 4th instars, but fed on six 2nd, and three 3rd. Newly emerged wasps also fed only on 2nd and 3rd instars, 4 and 3 respectively. No significant difference was seen in the number of nymphs host fed on between treatments (F = 1.65 df = 2, P = 0.232).

Oviposition  Newly emerged wasps parasitized the most nymphs (n = 18) followed by experienced (n = 16) than naïve wasps (n = 10). Newly emerged wasps parasitized seven 2nd, ten 3rd and one 4th instar nymphs. Experienced wasps parasitized seven 2nd
and nine 3\textsuperscript{rd} instars nymphs while naïve wasps parasitized five 2\textsuperscript{nd}, four 3\textsuperscript{rd}, and one 4\textsuperscript{th}. However, no significant difference was seen in the number of nymphs oviposited in between treatments (F= 1.39, df= 2, P= 0.287). Upon dissection, all eggs were found in the middle to anterior abdomen with no apparent target site or tissue.

*Superparasitism* Superparasitism where two eggs where found in one host was observed 3 times in naïve wasps (three different specimens) and once amongst newly emerged wasps. No superparasitism was seen from experienced wasps. No significant difference was seen in the number of nymphs superparsitized between treatments ($x^2$= 3.50, df= 2, P= 0.1738).

In addition to superparasitism, naïve wasps probed plant tissue and nymphal wax excretions. Normal plant (stem) tissue was probed 6 times and waxes 3 times. All plant tissue and wax probe durations lasted 2-3 seconds and were followed by resumed searching, grooming or resting. Plant and wax probes were excluded from all analysis.

Host defense by violent twitching back and forth was exhibited only by third and fourth instar nymphs. This behavior was observed a total of 47 times among all three treatments. In this way, 3rd instar nymphs successfully escaped 6 out of 16 probing attacks (37%). Fourth instar nymphs escaped 25 of 31 or 83.9% of probing attacks.

**Oviposition Preference**

*D. aligarhensis* exhibited a preference for utilizing 2\textsuperscript{nd} and 3\textsuperscript{rd} instars for host feeding, probing and parasitism (Figure 4-5). When the number of nymphs utilized for either host feeding, probing or parasitism for each instar where compared among the three wasp treatments, no significant difference was seen (p >.05) so data was pooled. Chi square analysis of the total number of nymphs utilized by the three pooled treatments showed wasps host fed, probed and parasitized significantly more 2\textsuperscript{nd} and
3rd instars ($\chi^2 = 20.86$, df = 3, $P = 0.0001$; $\chi^2 = 95.11$, df = 3, $P < 0.0001$; $\chi^2 = 37.27$, df = 3, $P < 0.0001$ respectively). No difference was seen between 2nd and 3rd instars or between 1st and 4th instars ($p > .05$) used for host feeding, probing or parasitism. Significantly more 2nd, 3rd and 4th instars were probed host fed upon or parasitized ($\chi^2 = 32.72$, df = 2, $P < 0.0001$; $\chi^2 = 58.53$, df = 2, $P < 0.0001$; $\chi^2 = 12.5$, df = 2, $P = 0.0019$ respectively). There was no significant difference in the number of 1st instars used for host feeding, probing or parasitism ($\chi^2 = 1$, df = 2, $P = 0.61$).

**Time Budget Construction**

Searching and grooming occupied the majority of newly emerged wasp’s time (51% and 16% respectively) while the remaining 5 events combined occupied 32% of their time (Table 4-2). Experienced wasps spent the majority of their time searching and probing (37% and 20% respectively) while similar amounts of time were spent host feeding, grooming, and resting (12%, 12%, 11% respectively) with the remaining 7% spent antennating. Naïve wasps spent a little more than half their time searching and probing (31% and 23% respectively). Resting and host feeding (16% and 16%) occupied another 33% while anntenating and grooming occupied 3% and 7% of their time.

Newly emerged wasps spent significantly more time per average antennating event ($F = 4.66$, df = 2, $P = 0.01$) than experienced or naïve parasitoids (Table 4-2). Naïve wasps spent significantly more time on average per probing event ($F = 18.68$, df = 2, $P = 0.0001$) compared to newly emerged or experienced wasps. Mean durations of probing events that led to successful oviposition by newly emerged, experienced or naïve wasps were shorter than those that did not lead to oviposition and it took significantly longer for naïve wasps to successfully lay an egg ($F = 5.32$, df = 2, $P=$
Naïve wasps host fed more often and for significantly longer durations ($F = 7.56, df = 2, P = 0.0008$) than both newly emerged and experienced wasps. Experienced wasps groomed much less frequently than newly emerged or naive wasps; however the duration of grooming was significantly shorter for naïve wasps ($F = 8.01, df = 2, P = 0.0005$) than those newly emerged or experienced. Newly emerged wasps rested more often than experienced or naïve wasps; however, naïve wasps spent significantly more time per average resting event ($F = 152.85, df = 2, P = 0.0001$) than newly emerged or experienced parasitoids. Experienced wasps exhibited far fewer searches than newly emerged or naïve wasps, and average search durations were significantly shorter ($F = 5.24, df = 2, P = 0.0057$) compared to newly emerged or naïve parasitoids.

**Discussion**

Parasitoids that are mass reared for biological control are often stored for several weeks prior to augmentive or inoculative release. Additionally, many practitioners store insects at ~17°C to slow down metabolism, thus extending life span and helping maintain eggload by reducing egg resorption in the absence of hosts. We saw that egg load decreased much more rapidly in wasps held at 25°C than at 17°C when only honey was offered. This would indicate that egg resorption is more rapid at the higher temperature. On the other hand, wasps provided access to hosts at 25°C maintained about the same egg load as wasps maintained at 17°C without hosts, indicating that host feeding provided the nourishment necessary to replace eggs lost through oviposition.

Wasps denied access to hosts resorbed their eggs and at a faster rate than wasps with access to hosts, and were unable to regenerate eggs without host feeding. Lower temperature reduced the rate of egg resorption and is likely the best storage condition if
egg load is the major concern. Presumably, these parasitoids would increase their egg load once they found hosts and began feeding, although this would take time and might delay parasitism events after release. Further research is warranted to determine how long wasps can be stored at low temperatures and how quickly they recuperate.

*D. aligarhensis* exhibited a clear preference for attacking 2nd and 3rd instars for host feeding and parasitism (Figure 4-5). As nymphs develop, the cuticle begins to thicken, commensurate with increased mobility and ability to “twitch” defensively. Fourth instars were more successful at preventing a probe in this way than 3rd instars (83.9%, 37.5% respectively). It is likely that increased defensive behavior combined with physiological and internal morphological changes occurring as the nymphs near their 5th and final instar, all contribute to the avoidance of 4th instars. However, it has been reported that the previously imported Taiwan population exhibited no significant difference in the number of progeny produced from 2nd, 3rd or 4th instars in no-choice tests (Skelley & Hoy, 2004). Further research would be required to determine whether avoidance of the 4th instar we observed translates into reduced suitability of this host stage or represents an innate difference between the Chinese mainland and Taiwanese populations.

Although host feeding and parasitism can be concurrent (Jervis & Kidd, 1986), wasps were rarely observed feeding and parasitizing the same host. Additionally, the vast majority of nymphs that were used for host feeding were continually probed and fed upon until death. The low incidence of wax feeding indicates nymphal wax and the sugars it contains do not provide an important source of nutrition for *D. aligarhensis* in the presence of hosts. However, during laboratory feeding and longevity studies, *D.*
aligarhensis was able to survive for an average of 15 days when feeding solely on fresh (<5 days old) waxy exudate (E. Rohrig, unpublished). Wax was fed on 3 times by newly emerged wasps for an average of 0.6s ± 0.09s which accounted for only 0.001% of their total time. Experienced wasps fed on wax 4 times for an average of 1.50s ± 0.565s which was 0.02% of their total time. No naïve wasps fed on wax. In addition to nutrition, parasitoids may be reinforcing their familiarity of host chemical cues used for aiding host identification by antennating and chewing on wax.

In these experiments, naïve wasps fed on 2x as many hosts as newly emerged wasps or experienced wasps (Table 4-2). Additionally, naïve wasps spent 2.8x and 1.7x more time per feeding event than newly emerged or experienced wasps respectively. Clearly, naïve wasps are hungry and need to make up for a nutrition deficit before they can develop eggs efficiently. It is important to note that host feeding destroys the nymph which in turn increases the parasitoids host killing capacity. Nevertheless, naïve wasps parasitized fewer nymphs than newly emerged or experienced wasps. It is arguable that the higher parasitism rates would be achieved if the time spent host feeding to acquire the nutrients necessary to rebuild egg supplies by naïve wasps following field release were spent on oviposition. Supplying wasps with hosts prior to field release would allow them to begin parasitizing immediately upon release. This may be of particular importance if there is a limited window of opportunity for released parasitoids to oviposit before detrimental conditions occur, such as nymphal development past an exploitable instar, or unfavorable weather.

It is often impractical to immediately release a parasitoid the same day it emerges. Wasps may need to be driven or shipped to the site of release or held until appropriate
numbers are available. Storage of biological control agents in the absence of hosts prior
to release is the most common method of simplifying the process and reducing time and
space needed. However, this isolation period can have negative effects on parasitoid
efficiency. Adult parasitoids improve their efficiency over time by experience with hosts
and through associative learning (Turlings et al., 1993). The experiments described
here provide evidence that D. aligarhensis conform to this model by becoming more
efficient with experience and consequently, their ability to evaluate host quality
improves. Experienced wasps’ antennated fewer individual nymphs and rejected more
of those antennated than naïve wasps. Experienced wasps spent 2.5x less time per
average probing event compared to naïve wasps and required 2x less time to oviposit
than naïve parasitoids. Additionally, searches by experienced wasps were fewer and
shorter than newly emerged or naïve wasps. Experienced wasps likely become better
able to locate hosts through detection and/or utilization of host chemical cues, therefore
reducing average search time.

Host deprived wasps may temporary lose their ability to recognize a parasitized
host, but can relearn once they are reintroduced to hosts (Klomp et al., 1980). Van
lentern (1976) showed that naïve Leptopilina heterotoma (Eulocidae) wasps
superparsitized hosts more often than experienced individuals. In experiments reported
here, naïve D. aligarhensis superparasitized three nymphs, while newly emerged wasps
superparasitized only one. Experienced wasps did not superparasitize any nymphs.
Although these are small numbers, they do provide evidence that lack of experience
may result in higher rates of superparasitism.
Parasitoids often chemically mark a nymph, depositing excretions from the ovipositor immediately following oviposition as a cue that a particular host has already been parasitized. Although we did not observe any indicative behavior, if *D. aligarhensis* is nevertheless marking hosts, experienced wasps would have more likely to recognize a parasitized nymph. Newly emerged wasps and naïve wasps have yet to begin the learning process and so are more likely to make mistakes. As an example, naïve wasps probed plant tissue 6 times (3 individuals) and nymphal wax 3 times (2 individuals). Neither newly emerged or experienced wasps probed any plant tissue or wax. The 7 day isolation period from plants, hosts and their chemical cues likely led to “confusion” where the wasps had not yet learned how to utilize these signals to locate a suitable host.

Results from these experiments are important in several aspects. As stated in the introduction, *Tamarixia radiata* is a competing wasp in Florida citrus that has established and currently provides varying levels of ACP control. *D. aligarhensis* has not yet established for unknown reasons. However, it is known that the two species coexist in different parts of Asia (Qing, 1988; P. Stansly unpublished). It would be instructive to repeat these experiments with *T. radiata* and compare behavioral pathways, frequencies and durations expressed by *D. aligarhensis* as well as observe both wasp species interacting together. Comparitive studies may provide clues into why only one species has established despite many releases of *D. aligarhensis*. *T. radiata* has established throughout Florida including most, if not all, of the areas used as release sites for *D. aligarhensis* (Qureshi et al, 2009, E. Rohrig, unpublished data). It is likely
that *T. radiata* is outcompeting *D. aligarhensis* for hosts and comparative studies are warranted.

Beyond the scope of these two parasitoid species and their effect on populations of ACP, these experiments provide evidence that associative learning may be manipulated to enhance biological control. Holding wasps in the presence of hosts until release may condition them to become more efficient biological control agents. In these experiments, experienced parasitoids maintained a higher egg load, parasitized more and were more efficient at host searching and discrimination than host deprived individuals. Field studies are warranted to determine how preconditioning will affect parasitism rates outside of the laboratory. Additional laboratory studies may help determine how long learned cues/behaviors are maintained once a previously experienced wasp is isolated from hosts and then reintroduced to them. Periods of host deprivation in the lab prior to field release may be equivalent to host scarcity and natural fluctuations of pest population densities.
Table 4-1. Egg-loads of *D. aligarhensis* held with honey or ACP nymphs at two temperatures (17°C and 25°C) over 5 day increments. Averages in the same column followed by the same letter are not significantly different (Tukey’s Studentized Range HSD, P < 0.05%).

<table>
<thead>
<tr>
<th>Days old at time of dissection</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>17°C honey only</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>range</td>
<td>10-24</td>
<td>8-20</td>
<td>2-20</td>
<td>4-22</td>
<td>6-14</td>
<td>2-10</td>
</tr>
<tr>
<td>average</td>
<td>17.5 B</td>
<td>12.4 A</td>
<td>9.9 A</td>
<td>9.2 A</td>
<td>10.4 A</td>
<td>5.4 A</td>
</tr>
<tr>
<td><strong>25°C honey only</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>range</td>
<td>6-22</td>
<td>6-20</td>
<td>0-35</td>
<td>0-6</td>
<td>0-8</td>
<td>0-4</td>
</tr>
<tr>
<td>average</td>
<td>9.4 C</td>
<td>6.4 C</td>
<td>5.1 B</td>
<td>2.4 C</td>
<td>0.69 C</td>
<td>0.34 C</td>
</tr>
<tr>
<td><strong>25°C host fed</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>range</td>
<td>10-32</td>
<td>10-24</td>
<td>8-22</td>
<td>8-18</td>
<td>6-12</td>
<td>6-10</td>
</tr>
<tr>
<td>average</td>
<td>18.2 A</td>
<td>12 B</td>
<td>10.5 A</td>
<td>8 B</td>
<td>6 B</td>
<td>4 B</td>
</tr>
</tbody>
</table>

Eggs per female: 92
Table 4-2. Total number of events per treatment, mean duration ± SE in seconds for various behavioral events, percentage of total time spent in each behavior and the proportion of each event. Behavioral event totals and means in the same column followed by the same letter (per event) are not significantly different (Tukey’s Studentized Range HSD, P < 0.05%).

<table>
<thead>
<tr>
<th>Behavioral event</th>
<th>Total frequency</th>
<th>Mean duration of behavior ± SE in seconds</th>
<th>Percentage of total time</th>
<th>Proportion of total events</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anntenating</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Newly emerged</td>
<td>99 A</td>
<td>8.0 ± 2.5 A</td>
<td>8.8% A</td>
<td>20.4% A</td>
</tr>
<tr>
<td>Exp. 10 d</td>
<td>94 A</td>
<td>4.9 ± 0.5 B</td>
<td>7.0% A</td>
<td>23.9% A</td>
</tr>
<tr>
<td>Naïve 10 d</td>
<td>108 A</td>
<td>5.4 ± 9 B</td>
<td>3.2% A</td>
<td>20.1% A</td>
</tr>
<tr>
<td><strong>Probe</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Newly emerged</td>
<td>78 A</td>
<td>13.4 ± 3.9 A</td>
<td>9.6% A</td>
<td>16.0% A</td>
</tr>
<tr>
<td>Exp. 10 d</td>
<td>100 A</td>
<td>13.9 ± 1.9 A</td>
<td>19.4% A</td>
<td>25.5% A</td>
</tr>
<tr>
<td>Naïve 10 d</td>
<td>114 A</td>
<td>33.8 ± 8.4 B</td>
<td>23.7% A</td>
<td>21.2% A</td>
</tr>
<tr>
<td><strong>Host feed</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Newly emerged</td>
<td>30 A</td>
<td>242 ± 76 A</td>
<td>6.8% A</td>
<td>6.2% A</td>
</tr>
<tr>
<td>Exp. 10 d</td>
<td>28 A</td>
<td>23.3 ± 5.7 A</td>
<td>12.0% A</td>
<td>7.1% A</td>
</tr>
<tr>
<td>Naïve 10 d</td>
<td>57 B</td>
<td>50.2 ± 13.9 B</td>
<td>16.8% A</td>
<td>16.8% A</td>
</tr>
<tr>
<td><strong>Wax feed</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Newly emerged</td>
<td>3 A</td>
<td>.6 ± .1 A</td>
<td>.001% A</td>
<td>.01% A</td>
</tr>
<tr>
<td>Exp. 10 d</td>
<td>4 A</td>
<td>1.5 ± .6 A</td>
<td>.02% A</td>
<td>1.0% A</td>
</tr>
<tr>
<td>Naïve 10 d</td>
<td>0 A</td>
<td>0 A</td>
<td>0% A</td>
<td>0% A</td>
</tr>
<tr>
<td><strong>Groom</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Newly emerged</td>
<td>81 A</td>
<td>23.7 ± 7.0 A</td>
<td>16.3% A</td>
<td>16.7% A</td>
</tr>
<tr>
<td>Exp. 10 d</td>
<td>36 B</td>
<td>24.1 ± 2.4 A</td>
<td>12.9% A</td>
<td>9.2% A</td>
</tr>
<tr>
<td>Naïve 10 d</td>
<td>78 A</td>
<td>18.1 ± 5.4 B</td>
<td>7.9% A</td>
<td>14.5% A</td>
</tr>
<tr>
<td><strong>Rest</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Newly emerged</td>
<td>45 A</td>
<td>19.2 ± 8.7 A</td>
<td>6.9% A</td>
<td>9.3% A</td>
</tr>
<tr>
<td>Exp. 10 d</td>
<td>30 A</td>
<td>24.9 ± 9.2 A</td>
<td>11.2% A</td>
<td>7.6% A</td>
</tr>
<tr>
<td>Naïve 10 d</td>
<td>36 A</td>
<td>83.8 ± 17.7 B</td>
<td>16.8% A</td>
<td>6.7% A</td>
</tr>
<tr>
<td><strong>Search</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Newly emerged</td>
<td>150 A</td>
<td>40.2 ± 9.3 A</td>
<td>51.4% A</td>
<td>30.9% A</td>
</tr>
<tr>
<td>Exp. 10 d</td>
<td>100 B</td>
<td>25.3 ± 3.9 B</td>
<td>37.3% A</td>
<td>25.5% A</td>
</tr>
<tr>
<td>Naïve 10 d</td>
<td>144 A</td>
<td>37.8 ± 5.9 A</td>
<td>31.6% A</td>
<td>26.8% A</td>
</tr>
</tbody>
</table>
Figure 4-1. Ethogram of the behavioral pathway of newly emerged (<12 hrs) wasps.
Figure 4-2. Ethogram of the behavioral pathway of experienced (host fed) wasps.
Naïve (Honey only) 7d

Figure 4-3. Ethogram of the behavioral pathway of naïve (honey only) wasps.
Figure 4-4. Average ± SE of individual nymphs subjected to parasitoid behavioral events per wasp.
Average ± SE of nymphal instars attacked per wasp
(3 treatments combined)

Figure 4-5. Average ± SE of instars utilized for host feeding, probing and oviposition per wasp (3 wasp treatments combined).
CHAPTER 5
ORIENTATION TO PLANT AND HOST VOLATILES BY *DIAPHORENCYTRUS ALIGARHENSIS* (HYMENOPTERA: ENCYRTIDAE) AN ENDPARASITOID OF *DIAPHORINA CITRI* (HEMIPTERA: PSYLLIDAE).

Introduction

Host location is crucial to parasitoid wasp reproduction and different species use different cues including olfactory, visual, vibrational and tactile. By examining host location strategies we may better understand why some parasitoids are more effective biological control agents than others. Volatile odors act as chemical attractants to many Hymenoptera, advertising available food (Dobson et al., 1996) and hosts (Steidle et al., 2001). Research suggests that chemical cues may be more important to searching insects than visual cues (Skubatz et al., 1996) and are often the dominant means of long distance attraction (Dudareva & Pichersky, 2000). Infochemicals used as cues by parasitoids may be plant volatiles, herbivore damage induced volatiles or direct and indirect host chemicals (Vet et al., 1995). When herbivorous insects feed on plants they induce damage, prompting the release of synomone compounds such as terpenes and sesquiterpenes (Turlings et al., 1990). These compounds can be highly attractive to parasitoid wasps (Tumlinson et al., 1993). The objective of this study was to use of y-tube bioassays and GC/MS analysis of host and host plant volatiles to determine if the parasitoid *D. aligarhensis* uses volatile cues as olfactory stimuli to locate its host, the Asian citrus psyllid (ACP) *Diaphorina citri* hosts and its habitats.

Materials and Methods

Colonies

*D. citri* colonies were maintained on orange jasmine, *Murraya paniculata* plants in 3.78 L pots filled with Jungle Growth Professional Growers Mix potting soil (Piedmont
Pacific, Inc. Statham GA). Plants were maintained under natural sunlight in a greenhouse at the USDA, CMAVE complex in Gainesville, FL. Temperatures were held at 25-30°C and humidity levels varied from 40-70%. Freshly flushing plants were moved from the greenhouse into a rearing room with temperature and humidity control at the same USDA complex. Plants were held in 47.5x47.5x93.0 cm BugDorm-4180F Insect Rearing Cages (Bioquip Rancho Dominguez, CA). EYE 4200K BT28 400 Watt bulbs in Hydrofarm Metal Halide growlights (Petaluma, Ca.) illuminated the cages for a 16:8 hour light:dark period. Humidity was maintained at 50-60% at a temperature of 25 ± 1°C. Plants were exposed to psyllids for 12-14 days until all 5 nymphal stages were present and available for experimentation or for rearing.

*D. aligarhensis* was maintained under the same conditions as above but located in a separate room to prevent contamination of the *D. citri* colony. After the 12-14 day exposure period to adult psyllids, infested plants were moved to the parasitoid room and placed in a cage with 10 newly emerged female wasps. All wasps were removed after 1 wk. Plants remained in the cage for an additional 2 weeks until all parasitoids had emerged.

**Choice Tests**

The y-tube olfactometer was constructed of 5 cm O.D. clear glass tubing. The body of the Y-tube measured 58.0 cm long and the arms measured 15.2 cm. Room temperature was maintained at 25°C with 60-70% RH. Air entering the olfactometer passed through a stainless steel column of activated charcoal. Airflow entering each arm of the Y-tube was set at 0.30 L/min using an adjustable flow meter (Aalborg Instruments, Monsey, NY) after preliminary testing found this rate to elicit the most movement by *D. aligarhensis*. *D. aligarhensis* exhibit a strong negatively geotropic
response so the y-tube was positioned at a 30° angle so that wasps were required to move forward and upward to reach the odor source or blank air control. Two separate chambers containing either an odor source or a blank air control were housed below the y-tube and out of sight to prevent wasps from using any possible visual cues. Plant chambers were clear 3.8 l-glass jars while ACP nymphs or their honeydew/wax secretions were contained in clear 5cm O.D. x 15.2 cm long glass tubes fitted with an air port on each end. Plant chamber lids were modified to allow airflow to enter into the bottom of the jar, rise upward over the plant material and exit through the lid and carry into the arms of the olfactometer.

Plants were contained in 18.9 liter plastic pots with Jungle Growth Professional Growers Mix potting soil (Piedmont Pacific, Inc. Statham GA). Blank air control chambers during ACP infested plant experiments contained an 18.9 liter pot with the same Jungle Growth Professional Growers Mix potting soil. Glass jars, tubes and the y-tube were rinsed with acetone between each use and allowed to air dry. Additionally, glass tubes and the y-tube were placed into a scientific glassware oven (100°C) for several hours-days until needed. Airflow exiting the Y-tube was pulled by a vacuum set to a flow of 0.60 L/min., twice the intake flow. A 61 cm double bulb VHO tube fluorescent light held 30 cm above the y-tube provided illumination (2000 Lux).

Twenty-five newly emerged (<48 hr) female D. aligarhensis were placed into the posterior end of the y-tube olfactometer which was fitted with a fine screen cap to prevent insects from escaping. A yellow colored sticky card was placed inside the anterior end of each branch of the y-tube (treatment or blank). Any wasp stuck on the sticky card was recorded as a response to that volatile source. Volatile sources included
ACP infested (1st-4th instars) (~400 nymphs per plant) or non-infested *Citrus paradise* or *Murraya paniculata* plants, ACP nymphs (1st-4th instars) or nymphal honeydew/wax excretions. Each volatile source was tested against a blank air control. Volatile source chambers were alternated for each replicate to avoid positional effects. Wasps had free movement within the olfactometer and were not timed. Each experimental run ended when all wasps had chosen a branch of the y-tube. Twenty repetitions using each volatile source were conducted.

**Volatile Collection/Identification**

ACP infested (1st-4th instars) or non-infested *Murraya paniculata* plants, ACP nymphs (1st-4th instars) and nymphal honeydew/wax excretion volatiles were collected using methods previously described by Heath and Manukian (1994). Plant volatile collection chambers were of constructed of glass with a HDPE guillotine bottom allowing for collection from undamaged potted plants by isolating soil filled pots below the glass chamber. Each chamber had multiple collection ports located around the base which allowed for multiple collections throughout the day. Charcoal filtered air passed over the plant and was pulled through 50 mg Super Q® adsorbent traps to collect and hold any volatiles. Chambers were held in an insect free greenhouse maintained at 25°C with a RH of 60%. The greenhouse was illuminated by natural sunlight as well as numerous 400 watt metal halide (MH) and 400 watt high pressure sodium (HPS) bulbs running for 14 hours daily (6am-8pm). Infested and non-infested plants were placed into the chambers and allowed to adjust to greenhouse conditions for 48 hours prior to volatile collection. Collections were made during 3 consecutive 8 hour blocks — 6am-2pm, 2pm-10pm and 10pm-6am — using an automated vacuum system. During each 24 hour
collection period, 1 blank air control, 2 infested and 1 non-infested *M. paniculata* plants were tested. This procedure was repeated 5 times.

ACP nymphs (1st-4th instars), nymphal honeydew/wax excretions or *M. paniculata* plant clippings were placed in a volatile collection system consisting of a glass chamber (30 cm long x 4 cm OD) constructed of Pyrex glass with a glass frit inlet and a glass joint outlet with a single port collector base. Collection chambers were held at 23-24°C under overhead fluorescent lighting. Each collection was made over a four hour period. Five collections each of ACP nymphs, nymphal honeydew/wax, and newly expanding ACP nymph-infested and non-infested *M. paniculata* shoots were examined. Humidified charcoal filtered air was pushed into one end of each chamber and over the volatile source. Air was then pulled out of the other end via a vacuum system. Air exiting the chamber passed through a volatile collection filter containing 50 mg of Super-Q®. All collection filters were eluted with 150 ul methylene dichloride to remove odor molecules.

**Volatile Analysis**

Volatile samples extracted from the filter were analyzed by capillary gas liquid chromatography (GC) using a Hewlett Packard 5890 equipped with a split/splitless injector operated in the splitless mode (injector purge at 30 seconds). The column was a 30 m x 0.25 mm (i.d.) SE-30 capillary column (Alltech Assoc.). Helium (linear flow velocity 18 cm/sec) was used as a carrier gas. The oven temperature and injector temperatures were programmed from 60°C (hold for 5 min) to 200°C at 10°C/min. The GC was interfaced to a HP 5973 mass spectrometer operated in the electron impart mode (70 eV). Tentative identifications were made by comparison of fragmentation patterns with patterns available in the NIST-MS library and libraries developed at our location.
Statistical Analysis

Chi square analysis ($\chi^2$) was performed on y-tube olfactometer captures (SAS Institute, 9.1).

Results

Choice Tests

Significantly more wasps chose to move toward ACP-infested plants (*Murraya paniculata* and *Citrus paradisi*), or ACP nymphal honeydew/wax excretions alone compared to blank air controls as indicated by sticky card captures at the end of the respective y-tube branch (df= 1, $\chi^2= 11.86$, P= 0.0006), (df= 1, $\chi^2= 6.96$, P= 0.0083), (df= 1, $\chi^2= 7.44$, P= 0.0064) respectively (Table 5.1). However, ACP nymphs alone unaccompanied by honeydew secretions were not attractive (df= 1, $\chi^2= .1$, P= 0.7518), nor was non-infested *M. paniculata* or non-infested *C. paradisi* odor source traps (df= 1, $\chi^2= 1.06$, P= 0.3032; df= 1, $\chi^2= 0.1$, P= 0.7518) respectively.

Volatile Collection/Chemical Analysis

Only sesquiterpene hydrocarbons were tentatively identified from head space collections of clippings of both ACP infested and non-infested newly expanding shoots of *M. paniculata* based on spectral comparisons. The following: $\alpha$-Copaene (13.95 minutes), E-Caryophyllene (14.51 minutes), $\alpha$-Humulene (14.92 minutes), $\beta$-Cubene (15.23 minutes), Germacrene B (15.42 minutes), and D-Cadinene (15.69 minutes) were identified.

When compounds identified from blank air controls were subtracted from those found in ACP nymphs or their waxy excretions few compounds remained. When compounds identified with less than a quality rating of 80 (0-100) by the fragmentation
pattern libraries were removed only 2-5 compounds remained of which none could be identified.

**Discussion**

ACP infested plants (*M. paniculata* and *C. paradise*) caught significantly more *D. aligarhensis* than blank air controls. The same plants, without nymphs, failed to capture more wasps than blank air indicating that host plant volatiles from uninfested plants are not likely used by *D. aligarhensis* to locate hosts. Nymphs without wax failed to catch significantly more wasps than a blank (n= 8) while nymphal honeydew/wax captured significantly more (n= 42) than a blank air control. This suggests that it is a volatile from the nymphal excretion that is attractive to *D. aligarhensis* rather than the immature host psyllid itself. This is not surprising given that cues directly from hosts are often non-volatile contact kairomones which can only be perceived by contact chemoreception (Hare, 1996). The lack of cues from attacked host stages often leads parasitoids to rely on those emanating from sources associated with host presence such as frass and other excretions (Vet et al., 1995). Host frass has been shown to be the dominate host searching cue for several hymenopteran parasitoids (Godfray, 1994; Vet & Dicke, 1992). In many cases, it is unclear if the volatiles emanating from frass are a chemical product of host metabolism, the host plant or both.

During the ACP infested plant experiments, plants were struck repeatedly to remove excess nymphal excretions that fall over the plant surfaces as the nymphs feed. Despite this tapping, nymphal honeydew/wax was always present due to the high number of nymphs feeding on each plant and the rapid pace of excretion. It is unclear whether these wasps were attracted solely to nymphal excretions remaining on the
plants or perhaps also to volatiles released from the plant in response to nymphal feeding.

Although a statistically significant number of wasps were caught using infested plant and nymph excretions compared to blank air controls actual differences were not great (Fig 5-1). In all three cases less than 60% (54.2%, 56%, 57.8%) of the wasps tested per odor source (n= 500) were captured in the odor trap verses the blank. One could expect a 50% division of the total number of wasps tested to be caught in each end of the y-tube if the odor source and the blank were equally (un)attractive. The low percentage of wasps caught in the odor trap during each experiment indicates that *D. aligarhensis* are not highly attracted to any of the odors under the conditions of the experiment.

When compounds identified from volatile collections of whole host plants, using the guillotine volatile collection chamber described above, were subtracted from those found in their blank air controls few compounds remained and none could be identified. When samples were concentrated using nitrogen gas and reanalyzed by GC/MS analysis similar results were obtained. This may indicate that not enough volatile material was present inside the large collection chambers. This prompted analysis of ACP infested and non-infested shoots of *M. paniculata* using the much smaller and less complex volatile collection chambers outlined above. This system required that plant shoots be clipped with scissors thus inducing stem damage. Six common plant volatile organic compounds were identified from both ACP infested and non infested *M. paniculata* shoots: α-Copaene, E-Caryophyllene, Humulene, β-cubene, Germacrene B, and D-cadinene. Amounts of these compounds were always several times greater in
non-infested clippings than in infested clippings. This may indicate that the psyllid nymphs or their feeding activities are down-regulating the release of sesquiterpene hydrocarbons from damaged plant tissue so further analysis is warranted. The fact that no volatile compounds were positively identified from host nymphs or their wax excretions may indicate that only minute quantities of volatiles are released from either source or that a different collection/analyzation technique is needed.

_Tamarixia radiata_ (Waterston) (Hymenoptera: Eulophidae), a competing ACP parasitoid, quickly established and spread throughout Florida suggesting they are efficient host searchers (Hoy & Nguyen, 2001). Olfactory experiments utilizing _T. radiata_ were conducted by Mann et al., (2010 under review). Female _T. radiata_ positively responded to odors emanating from _D. citri_ nymphs in olfactometer and open arena bioassays. Wasps did not respond to odors emanating from _D. citri_ adults or intact citrus. Unlike _D. aligarhensis_, female _T. radiata_ were not attracted to _D. citri_ honeydew secretions.

Host location behavior is often a chain of responses to several different cues including non-volatile tactile infochemicals as well as visual cues. During these experiments, wasps often moved back and forth within the y-tube for long periods of time before choosing one of the traps. Although the time it took for all wasps to enter either trap was not recorded it generally took 5-8 hours per 25 wasp run. _T. radiata_ possess mechanosensory antennae sensillae (Type I aportous sensilla trichoidea) that are known to detect acoustic or vibrational signals generated by hosts (Onagbola et al., 2009). It is possible that _D. aligarhensis_ requires tactile and/or visual cues to effectively locate hosts. During y-tube assays, odor sources were kept below the y-tube out of
sight of the wasps. Examination of the *D. aligarhensis* antennal morphology and the use by the wasp of tactile or visual cues as stimuli sources is warranted.

It is interesting to note that through three years of lab wasp and host rearing, *T. radiata* consistently contaminated host psyllid colonies despite being located in separate rooms ~15 meters apart, in spite of strict rearing/cleaning protocols to prevent contamination. Comparatively, *D. aligarhensis* rarely (1-2 occasions) found their way into the host rearing room.
Table 5-1. Y-tube catches of *D. aligarhensis* using various olfactory stimulus sources.

<table>
<thead>
<tr>
<th>Material tested</th>
<th># wasps captured (n=500)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACP infested <em>Murraya paniculata</em></td>
<td>289</td>
<td>*0.0006</td>
</tr>
<tr>
<td>- Blank air control</td>
<td>211</td>
<td></td>
</tr>
<tr>
<td>ACP infested <em>Citrus paradise</em></td>
<td>280</td>
<td>*0.0083</td>
</tr>
<tr>
<td>- Blank air control</td>
<td>220</td>
<td></td>
</tr>
<tr>
<td>ACP nymph wax/ honeydew</td>
<td>281</td>
<td>*0.0064</td>
</tr>
<tr>
<td>- Blank air control</td>
<td>219</td>
<td></td>
</tr>
<tr>
<td>ACP nymphs</td>
<td>254</td>
<td>0.7518</td>
</tr>
<tr>
<td>- Blank air control</td>
<td>246</td>
<td></td>
</tr>
<tr>
<td>Non-infested <em>Citrus paradise</em></td>
<td>246</td>
<td>0.7518</td>
</tr>
<tr>
<td>- Blank air control</td>
<td>254</td>
<td></td>
</tr>
<tr>
<td>Non-infested <em>Murraya paniculata</em></td>
<td>238</td>
<td>0.3032</td>
</tr>
<tr>
<td>- Blank air control</td>
<td>262</td>
<td></td>
</tr>
</tbody>
</table>
Table 5-2. Box and wisker diagram showing the average number of *D. aligarhensis* captured per y-tube run(+), median (horizontal line inside box), 25-75% quantiles (box) and range (extended lines, n=25).
CHAPTER 6
INTER-SPECIFIC COMPETITION BETWEEN DIAPHORENCYRTUS ALIGARHENSIS (HYMENOPTERA: ENCYRTIDAE) AND TAMARIXIA RADIATA (HYMENOPTERA: EULOPHIDAE), PARASITOIDS OF THE ASIAN CITRUS PSYLLID DIAPHORINA CITRI (HEMIPTERA: PSYLLIDAE)

Introduction

The Asian citrus psyllid (ACP), Diaphorina citri Kuwayama, is an important pest of citrus, causing substantial economic damage in many citrus growing regions of the world (da Graça, 1991; Halbert & Manjunath, 2004). Psyllid feeding activities cause direct damage emerging foliage (McFarland & Hoy, 2001) as well as vectoring the phloem-limited gram-negative bacterium Candidatus Liberibacter asiaticus Jagoueix, Bove´ & Garnier (a-Proteobacteria) (Jagoueix et al. 1994), presumed causative agent of huanglongbing (HLB) or citrus greening disease.

Two parasitic wasps, Tamarixia radiata (Waterston) and Diaphorencyrtus aligarhensis (Shafee, Alam and Argarwal) are reported to attack ACP throughout its native range (Chien, 1995; Qing, 1988; Yang et al., 2006). D. aligarhensis is an endoparasitoid originally recorded from India (Shafee et al., 1975), while T. radiata is an ectoparasitoid first described as Tetrastichus radiata from Pakistan (Waterston, 1922). T. radiata was reported to have been successful in reducing psyllid populations after it was imported into Réunion Island (Étienne & Aubert, 1980). Both species were first imported into Florida in 1998 from Taiwan (Hoy & Nguyen, 1998; McFarland & Hoy, 2001). An additional population of T. radiata was also imported into Florida from Vietnam and mixed with the colony from Taiwan. Both species were released into Florida from 1999 to early 2001. Currently T. radiata is established throughout the citrus growing region of Florida providing varying levels of control (Qureshi et al., 2009), while D. aligarhensis has not established for unknown reasons. With the exception of these
two imported wasps, there are no known parasitoids of ACP in the United States. Additionally, there are no reported hyperparasitoids of either *D. aligarhensis* or *T. radiata* in the U.S.

In September of 2006, 100 ACP nymphs on *Citrus* sp. cuttings were sent by R. Nguyen from mainland China (Guangdong province), with the hope that they would better adapt than the Taiwanese population and compete in Florida as a biological control agent of ACP. Fifty adult *D. aligarhensis* emerged in the maximum security facility of the Florida Biological Control Laboratory located at the Florida Department of Agriculture and Consumer Services, Division of Plant Industry compound in Gainesville, Florida. Wasps were held in quarantine and reared through 20 generations until the necessary permits for release were granted.

Releases from the Chinese colony of *D. aligarhensis* began in August of 2007. To date over 10,000 wasps have been released in six Florida counties (Alachua, Collier, DeSoto, Hendry, Orange and Polk). This population of *D. aligarhensis* has apparently not established despite several initial recoveries of next generation wasps in spite of the fact that these two species coexist in different parts of Asia (Qing, 1988; P. Stansly & R. Nguyen unpublished). In contrast, *T. radiata* is established throughout Florida (Qureshi et al., 2009) including at all of the *D. aligarhensis* release sites. Therefore, it is possible that the presence of *T. radiata* is at least partially responsible for the inability of *D. aligarhensis*. However, there have been no published reports examining inter-specific competition between the two species. Competition studies may provide insight into why only *T. radiata* has established in Florida despite numerous releases of two populations
of *D. aligarhensis*. It is possible that *T. radiata* out-competed *D. aligarhensis* for hosts or actively interfered with its establishment.

The following experiments were conducted with 3 main goals in mind: (1), determine if either species discriminates for oviposition against a host that has been parasitized by the other species, (2) determine which species would complete development to adulthood when both wasps parasitize the same host at the same time or at different times, and (3) determine the percentage of multi- and superparasitism when both species have equal access to hosts at three different densities.

*T. radiata* requires 12 days to complete development at 25°C and utilizes 3rd-5th instar nymphs for oviposition, although older instars are preferred (Chu and Chien, 1991). The previously imported (1999) Taiwan population of *D. aligarhensis* required 18 days to complete development at 25°C and utilizes 2nd – 4th instar nymphs for oviposition (Skelley & Hoy, 2004). In comparison, the mainland China population of *D. aligarhensis* used in the following experiments completes development in 16 days at 25°C and utilizes 2nd-4th instar hosts for oviposition (Chapter 2).

**Materials and Methods**

*D. citri* colonies were maintained on plants of orange jasmine, *Murraya paniculata* (L.) Jack growing in 3.78 L pots filled with Jungle Growth Professional Growers Mix potting soil (Piedmont Pacific, Inc. Statham GA). Plants were maintained under natural sunlight in a greenhouse at the USDA, CMAVE complex in Gainesville, FL. Temperatures were held at 25-30°C and humidity levels varied from 40-70%. Newly flushing plants were moved from the greenhouse into a rearing room at the same USDA complex with temperature and humidity control. Plants were held in 47.5 x 47.5 x 93.0 cm BugDorm-4180F Insect Rearing Cages (Bioquip Rancho Dominguez, CA). EYE
4200K BT28 400 Watt bulbs in Hydrofarm Metal Halide growlights (Petaluma, Ca.) illuminated the cages for a 16:8 hour light:dark period. Humidity was maintained at 50-60% at a temperature of 25°C. Plants were exposed to psyllids for 12-14 days until all 5 nymphal stages were present and available for experimentation or continued rearing. The developmental stage of psyllid instars was determined using previously published criteria (Liu & Tsai 2000) confirmed by head capsule width (Bioquip, Rancho Dominguez, CA) based on a published scale (Nava et al. 2007).

*D. aligarhensis* and *T. radiata* were maintained under the same conditions as above but in a separate room to prevent contamination of the ACP colony. After a 12-14 day psyllid exposure period, plants were moved to the parasitoid room and placed in a cage with ten female wasps of one of the two species. All wasps were removed after 1 wk. Plants remained in the cage for an additional 2-3 weeks until all parasitoids had emerged.

**Interspecific Host Discrimination**

The oviposition behavior of a single female wasp of each species was observed and compared in observational arenas. Wasps were presented with a single ACP nymph that was either previously parasitized by the other species or never previously exposed to any wasp. Female wasps were three days post emergence, had no prior oviposition experience and were mated in the case of *T. radiata*. *D. aligarhensis* are thelytokous and thus do not produce male offspring. Observational arenas consisted of clear 25.4 x 12.2 mm sterile polystyrene petri dishes (Thomas scientific, Swedesboro, NJ). Each petri dish contained a single freshly clipped orange jasmine leaf and an early 4th instar nymph. Nymphs were transferred using a fine sable hair brush and a 0 point pin microscopy tool. Arenas were observed under an Olympus SZ trinocular stereo microscope.
microscope illuminated by a Southern Micro Instruments EKE fiber optics Light Source. Room conditions were 25°C with 50% RH. A single female *T. radiata* wasp was placed into the petri dish and observed until she parasitized the nymph. *T. radiata* eggs are easy to locate clinging to the ventral surface of the nymph on the thorax or anterior abdomen. After successful oviposition, the wasp was removed and discarded. Either immediately, one or three hours later, a single female *D. aligarhensis* was placed into the same petri dish and observed. Once the wasp antennated the nymph, the time taken to examine and oviposit was recorded. If oviposition did not occur within 10 minutes of the initial antennation, the event was recorded as discrimination and both wasp and nymph were discarded. After an oviposition event, the parasitoid was removed and discarded and the nymph dissected under a Leica MZFLIII stereomicroscope to verify the presence of a *D. aligarhensis* egg. If what appeared to be an oviposition event by *D. aligarhensis* was shown to be a probe with no egg deposited, the event was discarded from analysis and repeated with a new nymph and wasp. This step was to account for observational error in identifying oviposition by *D. aligarhensis* which can only be verified by dissecting the host nymph. This procedure was repeated 10 times per holding period (immediately, 1 hr., 3 hrs.) for a total of 30 observations and dissections. The entire procedure was then repeated, reversing the order of wasps so that *D. aligarhensis* had first access and then *T. radiata*. Additionally, a 4th instar nymph was presented to each wasp species in the arena to determine the average time required to examine and parasitize a nymph previously unexposed to another wasp. The time it took for the wasp to oviposit after an initial antennation event was recorded. This procedure was repeated 10 times for each wasp species.
The time spent from antennation to oviposition by females offered nymphs not parasitized (previously not exposed to a parasitoid) or parasitized nymphs (nymphs previously exposed to a parasitoid) were compared between and within the two parasitoid species using analysis of variance (P = 0.05).

**Within Host Competition**

A single female wasp was presented with a single ACP nymph in an observational arena described above. ACP nymphs had not been previously exposed to any wasp. Female wasps were three days post emergence, had no prior oviposition experience and were mated in the case of *T. radiata*. Second instar ACP were utilized to allow for a longer nymphal development period so *D. aligarhensis* would have 2nd-4th instars available for parasitism over a 7 day test period. Time for these parasitism events was not recorded. *T. radiata* prefer older instars for oviposition and were often reluctant to parasitize a 2nd instar. If the nymph was not parasitized within 5-10 minutes, the wasp was removed and replaced with another until oviposition occurred. Parasitized nymphs were then carefully transferred five each to the new tender flush of an orange jasmine seedling contained in a 3.78 l pot for a total of nine plants. Plants were individually caged using a clear cylindrical acrylic tube resting on the soil surface. Tops of the cylinders were covered with a fine organdy screening to isolate the plants and allow air exchange. White coffee filters were placed around the base of the plant stem, on the soil surface, to facilitate detection of dead or fallen wasps. Caged plants were held under the above rearing conditions for the remainder of the experiment. Immediately following parasitism by *T. radiata* and every 24 hours for an additional seven days, one set of ten caged nymphs were removed one at a time and placed individually into an observational arena (described above). A female *D. aligarhensis* was then introduced.
into each arena and allowed to parasitize a nymph. Nymphs were then transferred back onto their original orange jasmine seedling and held for an additional 20 days to check for adult emergence. The number and species of emerging wasps were counted and recorded. This experiment was then repeated reversing the order of wasps so that *D. aligarhensis* had first access then *T. radiata*. This experiment was repeated twice.

**Intra- and Interspecific Competition Under Different Host Densities**

To examine the frequency of parasitism, multiparasitism and superparasitism events exhibited between and within wasp species under varying host densities the following experiment was conducted. Newly flushing orange jasmine plants approximately 0.30 meters high were contained in 3.78 L pots enclosed in acrylic cylindrical cages and held under the above rearing conditions for the duration of the experiment. White coffee filters were placed around the base of the plant stem to facilitate detection of dead or fallen wasps. Late 3\textsuperscript{rd} and early 4\textsuperscript{th} instar nymphs (1:1) were transferred at: 20, 40, and 80 per caged plant. Female wasps used were 3 days post emergence, had no prior ovipositional experience and were mated in the case of *T. radiata*. Three treatments were examined. One female wasp of each of the two species was placed alone or together with a female of the other species in a cage for a period of eight hours, then removed. Twenty-four hours later all nymphs were removed, examined and dissected under a Leica MZFLIII stereomicroscope to check for parasitism, multiparasitism and superparasitism. This procedure was repeated three times under each psyllid density. Numbers of eggs, multiparasitism events and superparasitism events recorded during the competition studies were compared between the two species using analysis of variance.
Data Analyses

All analyses of variance were conducted using PROC GLM (SAS Institute, 9.1) at P = 0.05. When statistically significant differences were found, mean separations were investigated using Tukey’s studentized range (HSD) test (α=.05). Fischer’s exact test was used to analyze the number of ovipositional discriminations of a nymph previously parasitized by the competing wasp species.

Results

Interspecific Host Discrimination

Time from first antennation to oviposition into a previously unexposed 4\textsuperscript{th} instar ACP nymph did not significantly differ between the two parasitoid species (df=1, F= 0.56, P= 0.46) (Table 6-1). Likewise time spent by \textit{D. aligarhensis} and \textit{T. radiata} from antennation to oviposition of all hosts previously parasitized by the other species did not differ (df= 5, F= 1.04, P= 0.41). Time from antennation to oviposition into all nymphs, previously parasitized or not, by both wasp species did not significantly differ (df= 7, F= 0.96, P= 0.47). No significant difference was seen in the total number of ovipositional discriminations made by either wasp species against a host already parasitized by the other wasp species (P= 0.20 Fischer’s exact test) (Table 6-1).

Within Host Competition

\textit{T. radiata} completed development in any host to which it had first access (Table 6-3). Seventy-four \textit{T. radiata} adults emerged from the 80 nymphs first parasitized by this species, while 6 nymphs died or were unable to be located. \textit{T. radiata} out-competed \textit{D. aligarhensis}, even when it had access 4 days after \textit{D. aligarhensis}. Numbers of \textit{T. radiata} progeny decreased beginning 5 days after \textit{D. aligarhensis} parasitized first. \textit{D. aligarhensis} adults emerged only from hosts they had parasitized 5-7 days before
access by *T. radiata*. Eleven *T. radiata* and 17 *D. aligarhensis* emerged from 30 nymphs parasitized over those three days. Two nymphs died or were unable to be located.

**Intra- and Interspecific Competition Under Different Host Densities**

**Interspecific Competition**

An average of 6.0 ± 0.33 (30%) of nymphs were multiparasitised by both species when host density was 20 nymphs per plant (Table 6-2). Multiparasitism events for both *T. radiata* and *D. aligarhensis* dropped to 2.7 ± 0.19 (6.8%) and 2.3 ± 0.19 (2.9%) nymphs when host density was increased to 40 or 80 nymphs per cage, respectively. Multiparasitism events occurring from the 20 hosts per plant were significantly greater than at 40 or 80 hosts per plant (df= 2, F= 22.2, P= 0.0017, Tukey’s HSD, Table 6-2).

**Intraspecific Competition**

The number of intraspecific competition events for both wasp species when both had access decreased as the host density increased (Table 6-2). At 20 nymphs per plant, *T. radiata* superparasitized an average of 2.3 ± 0.19 (11.5%) nymphs while *D. aligarhensis* superparasitized 3.3 ± 0.19 (16.5%) nymphs. Intraspecific competition by *T. radiata* and *D. aligarhensis* decreased to 1.7 ± 0.19 and 2.7 ± 0.19 nymphs (4.25% and 6.75%, respectively) when psyllid density was increased to 40 nymphs. *T. radiata* and *D. aligarhensis* superparasitism rates further decreased to 0.3 ± 0.19 and 1.7 ± 0.38 (0.37% and 4.25%, respectively) when host density was 80 per plant. Intraspecific competition rates under all 3 nymph densities were significantly different (df= 5, F= 6.40, P= 0.004). Further comparison using Tukey’s HSD showed that *T. radiata*’s intraspecific competition at 80 nymphs per plant was significantly less than either *T. radiata* and *D. aligarhensis* at a density of 20 nymphs and less than *D. aligarhensis*’ at 40 nymphs per cage (Table 6-2).
Superparasitism rates for both parasitoid species also tended to drop with increasing host numbers when only one wasp species had access to nymphs (Table 6-2). However, when only one wasp species had access to nymphs, the number of superparasitism events for *D. aligarhensis* and *T. radiata* were not significantly different within species (df= 2, F= 2.63, P= 0.15) and (df=2, F= 1.40, P= 0.32) respectively or between species (df= 5, F= 2.11, P= 0.13).

**Total Oviposition**

In the dual presence of both wasp species, *T. radiata* and *D. aligarhensis* laid an average of 10.7 ± 0.51 and 9.3 ± 0.19 eggs respectively (Table 6-2) when host density was 20 nymphs per plant. When host density increased to 40 nymphs per plant, *T. radiata* and *D. aligarhensis* both laid a similar number of eggs; 10.0 ± 0.66 and 10.0 ± 0.58, respectively. At a host density of 80 per plant *T. radiata* egg lay increased to 12.3 ± 0.38 eggs, while *D. aligarhensis* decreased to 9.6 ± 0.69 eggs. No significant difference was seen in the number of eggs laid across all 3 densities (df= 5, F= 1.36, P= 0.3068).

*T. radiata* and *D. aligarhensis* laid an average of 10.0 ± 0.33 and 8.7 ± 0.38 eggs, respectively, during experiments where one wasp species had access to each set of nymphs and the host density was 20 per plant (Table 6-2). At a density of 40 nymphs per plant *T. radiata* and *D. aligarhensis* laid 9.3 ± 0.51 and 9.7 ± 0.19 eggs, respectively. When the density of nymphs was increased to 80 nymphs per plant, *T. radiata* laid 11.3 ± 0.69 eggs while *D. aligarhensis* laid 9.33 ± 0.51 eggs. No significant difference was seen in the number of eggs laid (df= 5, F= 1.26, P= 0.34).
Discussion

Although there were a few instances of possible discrimination during observational arena experiments, neither *D. aligarhensis* nor *T. radiata* significantly discriminated ovipositionally against a nymph that was already parasitized by the other species, regardless of the time interval between wasps access. However, of the five possible discrimination events recorded, four were by *D. aligarhensis* and one was by *T. radiata* after examining a nymph parasitized by the other species. This may indicate that *D. aligarhensis* has a higher propensity to avoid *T. radiata* than vice versa which would not be surprising due to the fact that *D. aligarhensis* will not complete development to adulthood if *T. radiata* has already oviposited. Additionally, the lack of discrimination may indicate that the two species did not evolve together.

Once a nymph has become a mummy, neither wasp species is capable of parasitizing it. During caged seedling experiments *T. radiata* oviposited on all nymphs previously parasitized by *D. aligarhensis* through the 7 days tested. *D. aligarhensis* was unable to oviposit in nymphs 6 -7 days post parasitism by *T. radiata*. During this time nymphs have little internal fluids remaining and begin to harden and darken forming a mummy. *D. aligarhensis* larvae form mummies 8 days post parasitism. The lack of significant ovipositional discrimination against a host already parasitized by the other species provides evidence that if either parasitoid encountered a 2\(^{nd}\) - 4\(^{th}\) instar nymph, even if it is parasitized by the competing species, they are both as likely to parasitize. However, *T. radiata* has two advantages over *D. aligarhensis*. First, *T. radiata* is capable of parasitizing early 5\(^{th}\) instar nymphs which *D. aligarhensis* is not. Second and more importantly, a *T. radiata* wasp will always emerge if it oviposits first and can still successfully produce some offspring if it parasitizes within 5-7 days after *D.*
*aligarhensis.* *D. aligarhensis* can only produce progeny if it oviposits at least 5 days before *T. radiata.* This is a major disadvantage when releasing *D. aligarhensis* into an area where *T. radiata* is already present.

When presented with a nymph not previously exposed to any wasp, the time spent by both *D. aligarhensis* and *T. radiata* until parasitism after an initial antennation event did not significantly differ. When given a nymph parasitized by the competing species, regardless of the holding time between wasps access, there was no significant difference in the time spent examining and ovipositing by either wasp. Although differences were not statistically different, *D. aligarhensis* averages were always at least twice that of *T. radiata.* The resulting standard errors were much higher in *D. aligarhensis,* indicating a greater variance in the time spent between individual wasps. Fecundity of both species of wasps did not differ significantly regardless of the presence or absence of a competing wasp species or the density of nymphs provided. These results indicate that both wasps can parasitize a similar number of nymphs in the same amount of time regardless of whether the nymph has already been parasitized by the competing species. However, *T. radiata* tended to lay the most eggs when given access to the greatest nymph density, while *D. aligarhensis* laid fewer eggs at the 80 nymph host densities than the 40 host density. This indicates *T. radiata* may perform better as a biological control agent under higher host densities.

During interspecific competition experiments, the number of hosts multiparasitized by both wasp species was the highest at the lowest nymph density. Thirty percent of nymphs were multiparasitized by both species at the lowest (20 nymph) host density, significantly more than seen at the 40 or 80 nymph densities. As
host density increased to 40 or 80 per cage the number of multiparasitizism events dropped by more than 50% down to 6.75% and 2.88% of nymphs respectively. Under low host densities, it is clear that there is a high level of competition between the two species. *D. aligarhensis* exhibited a higher rate of superparasitism on caged seedlings at every host density. This was true regardless of whether one or both wasp species had access to hosts. Superparasitism of both species decreased as host density increased, indicating reduced levels of intraspecific competition.

There are several issues that are likely influencing the establishment of *D. aligarhensis* in Florida. First, *T. radiata* is present throughout citrus growing regions of Florida (Qureshi et al. 2009) including all of the Florida sites where this mainland China population of *D. aligarhensis* has been released (E. Rohrig, unpublished). Any hosts parasitized by *T. radiata* before or within 5 days of *D. aligarhensis* will result in *T. radiata* offspring. *T. radiata* are capable of parasitizing 2nd – 5th instar nymphs (Chu & Chien, 1991) which are present for more than a ~10.5 day developmental period at 25°C (Liu & Tsai, 2000). *D. aligarhensis* can parasitize 2nd – 4th instars which are only available for ~5.5 days at 25°C. This gives *T. radiata* an ovipositional window that is nearly twice as long. Additionally, *T. radiata* has a shorter generation time (12 vs. 16-18 days) and a higher reproductive rate (Skelley & Hoy, 2004). Furthermore, a single female *T. radiata* can kill nearly twice as many psyllids in her lifetime than *D. aligarhensis* (Chu & Chien, 1991; Chien, 1995). Both wasps induce psyllid mortality through the combined effect of parasitism and host feeding. A single female *T. radiata* is capable of killing up to 500 nymphs through her lifetime (Chu & Chien, 1991) while *D. aligarhensis* can kill 280 (Chien, 1995). Regardless, both species coexist in parts of Asia (Qing, 1988; P. Stansly
and R. Nguyen unpublished) and further research in that region is warranted. Closer examination in Asia of natural enemies, including hyperparasitoids which are not present in Florida, might show reasons why the two species may sometimes coexist.
Table 6-1. Average time ± SE (in seconds) required by *T. radiata* or *D. aligarhensis* to examine and parasitize an unparasitized nymph or nymph previously parasitized by the other species immediately, or 1 or 3 hours prior.

<table>
<thead>
<tr>
<th></th>
<th>Parasitized by the other species:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>unparasitized</td>
<td>Immediately after</td>
</tr>
<tr>
<td><em>D. aligarhensis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>52.7 ± 15.93</td>
<td>86.5 ± 47.7</td>
</tr>
<tr>
<td></td>
<td>1 discrimination</td>
<td>2 discriminations</td>
</tr>
<tr>
<td><em>Tamarixia radiata</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>37.5 ± 12.7</td>
<td>32.2 ± 12.5</td>
</tr>
<tr>
<td></td>
<td>no discrimination</td>
<td>no discrimination</td>
</tr>
</tbody>
</table>

* Discrimination when a single parasitoid failed to oviposit a host previously parasitized by the other species within 10 minutes of an initial antennation event.
### Table 6-2. Multi- and superparasitism events ± SE when *T. radiata* and *D. aligarhensis* had equal or sole access to ACP nymphs at 3 host densities.

<table>
<thead>
<tr>
<th>ACP nymph density</th>
<th>Interspecific competition (multiparasitism)</th>
<th>Intraspecific competition (superparasitism)</th>
<th>eggs laid (total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td><em>T. radiata</em> + 2.3 ± .19 A</td>
<td>10.7 ± .51 A</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>D. aligarhensis</em> + 3.3 ± .19 A</td>
<td>9.3 ± .19 A</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td><em>T. radiata</em> + 1.7 ± .19 A</td>
<td>10.0 ± .66 A</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>D. aligarhensis</em> + 2.7 ± .19 B</td>
<td>10.0 ± .58 A</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td><em>T. radiata</em> + 0.3 ± .19 B</td>
<td>12.3 ± .38 A</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>D. aligarhensis</em> + 2.3 ± .19 B</td>
<td>9.6 ± .69 A</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td><em>T. radiata</em> —</td>
<td>1.7 ± .19 A</td>
<td>10.0 ± .33 A</td>
</tr>
<tr>
<td></td>
<td><em>D. aligarhensis</em></td>
<td>2.7 ± .38 A</td>
<td>8.7 ± .38 A</td>
</tr>
<tr>
<td>40</td>
<td><em>T. radiata</em> —</td>
<td>1.0 ± .33 A</td>
<td>9.3 ± .51 A</td>
</tr>
<tr>
<td></td>
<td><em>D. aligarhensis</em></td>
<td>1.3 ± .19 A</td>
<td>9.7 ± .19 A</td>
</tr>
<tr>
<td>80</td>
<td><em>T. radiata</em> —</td>
<td>.66 ± .19 A</td>
<td>11.3 ± .69 A</td>
</tr>
<tr>
<td></td>
<td><em>D. aligarhensis</em></td>
<td>1.0 ± .33 A</td>
<td>9.33 ± .51 A</td>
</tr>
</tbody>
</table>

Means in the same column followed by the same letters are not significantly different (Tukey’s Studentized Range HSD, α = 0.05%).
Table 6-3. Resulting progeny emerging from *T. radiata* or *D. aligarhensis* parasitized nymphs previously parasitized by the competing wasp species immediately-7 days prior.

| Wasp with first access to nymph (interval between wasp sp. access) | Resulting progeny |
|---|---|---|
| | *T. radiata* | *D. aligarhensis* | dead / missing nymph |
| *T. radiata* | | | |
| (immediately) | 10 | 0 | 0 |
| (1 day) | 10 | 0 | 0 |
| (2 day) | 9 | 0 | 1 |
| (3 day) | 10 | 0 | 0 |
| (4 day) | 8 | 0 | 2 |
| (5 day) | 9 | 0 | 1 |
| (6 day) | 8 | 0 | 2 |
| (7 day) | 10 | 0 | 0 |
| *D. aligarhensis* | | | |
| (immediately) | 9 | 0 | 1 |
| (1 day) | 8 | 0 | 2 |
| (2 day) | 9 | 0 | 1 |
| (3 day) | 10 | 0 | 0 |
| (4 day) | 9 | 0 | 1 |
| (5 day) | 6 | 3 | 1 |
| (6 day) | 3 | 7 | 0 |
| (7 day) | 2 | 7 | 1 |
CHAPTER 7
FIELD RELEASE OF DIAPHORENCYTRUS ALIGARHENSIS (HYMENOPTERA: ENCYRTIDAE).

Introduction

The Asian citrus psyllid (ACP), Diaphorina citri Kuyawama, (Homoptera: Psyllidae) is a serious pest of citrus and its close relatives (McClean & Schwarz, 1970). Immature ACP feed on sap from the phloem of young soft shoots and buds, causing leaf damage and distortion or death of new shoots (Mead, 1977). Additionally, psyllids secrete large quantities of honeydew, promoting the growth of sooty mold which can inhibit photosynthesis as well as tarnish fruit appearance (Wang et al., 2001). However, damage from direct feeding is of little consequence compared with the transmission of the phloem-limited gram-negative bacterium Candidatus Liberibacter asiaticus Jagoueix, Bove´and Garnier (α-Proteobacteria) (Jagoueix et al., 1994) which results in a systemic tree infection leading to poor fruit production and tree decline (McClean & Schwarz, 1970). This disease is commonly referred to as citrus greening due to the characteristic of affected fruit to remain green at the peduncular end (Bove, 2006). The disease is also known as Huanglongbing (HLB), Chinese for “yellow shoot disease” in reference to the initial foliar symptoms (van Vuuren, 1996).

Two host specific koinobiont parasitic wasps, Tamarixia radiata (Waterston) and Diaphorencyrtus aligarhensis (Shafee, Alam & Argarwal), were imported from Taiwan into Florida in 1998 (Hoy & Nguyen, 1998) as biological control agents for ACP. T. radiata was successful in reducing psyllid populations in Reunion Island (Étienne & Aubert, 1980) and Taiwan (Chien & Chu, 1996). Both species were released into Florida beginning in 1999. Currently, T. radiata has established in many regions of Florida providing varying levels of control (Qureshi et al., 2009), while D. aligarhensis has not
yet established. The objectives of this study were to release and establish *D. aligarhensis* as a biological control agent of ACP in Florida. It is hoped that *D. aligarhensis* will complement *T. radiata* and lead to increased suppression of ACP populations and the spread of citrus greening disease.

**Materials and Methods**

In September of 2006, I obtained 100 ACP nymphs collected by Ru Nguyen (FDACS-DPI Gainesville) from Guangzhou Guangdong province, China. Parasitoids were sent to Florida under APHIS permit number No.526-75827. *Citrus* sp. cuttings containing 100 *D. citri* nymphs were sent to Florida and the package opened in the maximum security room of the Florida Biological Control Laboratory located at the Florida Department of Agriculture and Consumer Services, Division of Plant Industry compound in Gainesville, Florida. Fifty adult wasps emerged and a colony was initiated. Males (~5%) emerged for the first three consecutive generations, then only females emerged. Parasitoids were held in quarantine through 20 generations until a release permit was obtained from USDA-APHIS. Field releases and evaluations were conducted from August of 2007 through October of 2009.

Newly emerged adult wasps were aspirated 50 each into clear 50 mL centrifuge tubes (Crystalgen Inc, New York, NY) with fine mesh covers and a cotton wick moistened with pure clover honey. Tubes were held in an incubation chamber (Powers Scientific Pipersville, PA) maintained at 25°C 70% RH for up to two weeks until release. Up to 24 hrs prior to release, mesh screens were replaced with plastic caps and sealed with parafilm to maintain humidity. Tubes were transported to the field in Styrofoam coolers containing an icepack wrapped in paper towels held under a 2.5 cm thick
sponge to prevent overcooling. Holding tubes were removed from the cooler and allowed to adjust to ambient temperature prior to release.

Wasps were either tapped out of holding tubes directly onto emerging shoots infested with immature ACP or were released into exclusion cages consisting of fine mesh bags tied around new shoots infested with nymphs. Shoots were carefully inspected and predators removed prior to tying cages closed. Mesh size allowed parasitoid wasps to move freely in or out of the cage while preventing predaceous insects from entering (Qureshi et al., 2009).

Post release sampling was conducted through visual inspection for the presence of adult *D. aligarhensis* or host mummies and by observing adult insect emergence from host plant cuttings. When exclusion cages were used, they were removed the following visit. After removal, host plant shoots were checked for the presence of *D. aligarhensis* parasitized host mummies. *D. aligarhensis* exits through the dorsal surface of the host’s abdominal cuticle similar to the behaviors of other endoparasites (Liu & Stansly, 1996). This exit hole can be used to distinguish between a mummy that has been parasitized by *D. aligarhensis* and *T. radiata* which exits dorsally through the thorax (Aubert & Quilici, 1984) rather than the abdomen. Additionally, *T. radiata* parasitized host mummies are attached to plant tissue by silk threading spun by the wasp larvae (Aubert & Quilici, 1984) which is not seen in *D. aligarhensis*. ACP nymph infested shoots were clipped directly from host plants and brought back to the laboratory. Shoots with highest ACP nymphal infestation were clipped unless flush or ACP incidence was so low that removing them would leave no host or host plant material at the release site. Clippings were placed into water filled 250 mL Pyrex glass beakers held inside a clear acrylic
cylinder (15.24 cm OD) fitted with a fine mesh top for air exchange. Cylinders were held at 25°C 60-70% RH for at least 3 weeks. Emerging adult ACP, D. aligarhensis and T. radiata wasps were removed and recorded daily.

**Release Sites.** Releases were made at 10 sites in 6 Florida counties (Alachua, Collier, DeSoto, Hendry, Orange and Polk). Sixty-seven releases were conducted totaling approximately 11,100 wasps. The following sites were utilized:

**Alachua County:** Marty Wertz Citrus - A privately owned organic grove located in Melrose, Florida (N 29° 42' 94.93", W 82° 76' 68"). This grove consisted of ~3 acres of red and white grapefruits, Satsuma oranges, sunburst tangerines, navels, Orlando tangelos, and Myer lemons varying in age from new sets to mature trees. Pine forest surrounded three sides and an abandoned citrus grove of ~3 acres was located on the north side.

**Collier County:** Silver Strand - A commercial citrus grove located in Immokalee, Florida. Releases were conducted in the 12 acre B9 block within the 4,431 acre North Grove (N 26° 29' 46", W 81° 23' 45"). This block consisted of 'Valencia' orange trees budded on 'Carrizo' citrange rootstock planted June 12, 2001.

**University of Florida South West Florida Research and Education Center (UF-SWFREC)** - A research facility consisting of crop land and ~ 50 acres of mixed citrus located in Immokalee, Florida (N 26° 28' 00.4", W 081° 26' 36.1"). Releases were conducted in a ~60 acre research block where various chemical, nutrient and biological control experiments are conducted by students and researchers.

**McDonalds** - A chain restaurant located in Marco Island, Florida (N 25° 57’ 20.26", W 081° 43’ 64.56”). Two large, mature *M. paniculata* hedges each measuring
23 x 2.5 x 1.2 meters ran the length of the east and west side parking lot. No citrus is
grown on the island. Numerous condominium buildings on the island maintain *M. paniculata* hedges as landscaping.

**DeSoto County:** Chapman - A 100 acre commercial citrus grove located in Arcadia, Florida (N 27° 01' 33", W 81° 46' 85"). Releases were conducted in a ~10 acre mature tree organic orange plot on the west edge of the grove. Farmland and citrus groves surrounded the property for several kilometers on all sides.

**Hendry County:** Clewiston City Hall - Government building in Clewiston, Florida (N 26° 45' 8.45", W 80° 56' 8.30"). Two mature *M. paniculata* hedges measuring approximately 6 x 1 x 2 meters were located on either side of the front entrance. Residential properties, farmland and Lake Okeechobee were located within several kilometers.

John Boy Auditorium - A public administrative office and auditorium located in Clewiston, Florida (N 26° 4' 52.89", W 80° 56' 4.71"). Two mature *M. paniculata* hedges measuring approximately 5 x 1 x 2 meters were located on either side of the front entrance. Residential properties, farmland and Lake Okeechobee were located with several kilometers.

**Orange County:** Chuck’s residence - A private residence in Apopka, Florida (N 28° 39' 6.43", W 081° 29' 21.36"). One large, mature *M. paniculata* bush ~2 x 1 x 1.5 meters was located on the side of the house near the front door. No citrus is grown within at least 3 kilometers in any direction.

Kathy’s residence - A private 1.5 acre dooryard grove located in Eustis, Florida (N 28° 51' 24.58", W 081° 38' 20.67"). This grove consisted of red and white grapefruit
and Hamlin orange trees varying in age from new sets to mature trees. Several 2-20 acre abandoned citrus groves were located within 2 kilometers in most directions.

**Polk County:** Coyote- A 20 acre commercial organic citrus grove located in Lake Wales, Florida (N 27° 52’ 77.72”, W 081° 32’ 86.1”). The grove was predominantly Hamlin orange trees along with a few other assorted citrus trees all planted in 1990. Citrus groves surrounded the property on all sides for 2-4 kilometers.

**Results**

Two adult female *D. aligarhensis* were recovered from *Citrus* sp. clippings taken from 2 sites in Collier County in 2007 (Table 7-1). One wasp was recovered from Silver Strand clippings taken on 10/8/07 the other from SWFREC clippings taken on 11/6/07.

Twelve adult female *D. aligarhensis* were recovered from *M. paniculata* clippings taken from Chuck’s dooryard in Orange County in 2008 (Table 7-1). Three emerged from clippings taken on 5/21/08 and 9 emerged from clippings taken on 6/11/08. Additionally, 3 wasps were seen foraging on the hedge on 5/21/08 and 2 were seen on 6/11/08.

Parasitized host mummies, left behind after *D. aligarhensis* emergence, were seen at several sites. Six mummies were observed at Silver Strand, 3 on 10/8/07 and 3 on 12/4/07. Five were observed at SWFREC, 3 on 18/8/07 and 2 on 11/6/07. Four were observed at Kathy’s dooryard citrus grove on 7/17/08. Twelve were observed at Marty Wertz citrus, 2 on 6/25/08, 6 on 7/21/08, and 4 on 5/30/09.

*T. radiata* adults or parasitized mummies were seen at all sites utilized for release of *D. aligarhensis*. Additionally, *T. radiata* adults emerged from host clippings taken from all release sites (Table 7-1).
Discussion

ACP and host plant flush incidence varied greatly according to location and time of year perhaps due to cultural practices and weather conditions. All citrus groves used for wasp release, including certified organic, utilized pesticide sprays to some extent during the release period. Overall ACP incidence in general appeared to decline over the >2 year release period, likely from an increased insecticide use to reduce spread of citrus greening disease. The use of insecticides targeting *D. citri* likely reduced both ACP and natural enemy populations (Qureshi & Stansly, 2007). During many trips to various locations for parasitoid release there was little to no host plant flush or immature psyllids present, particularly during late summer and fall. Mature Florida citrus trees generally produce a large flush during early spring followed by a smaller flush in summer and sometimes in fall (Hall & Albrigo, 2007). Adult psyllids were usually present at all locations throughout the release period.

In early 2009, releases were focused in Marty Wertz organic citrus in Alachua County. This sight was subjected to minimal certified organic pesticide sprays, maintained good cultural practices and was in close proximity to the rearing laboratory allowing more frequent visits. Despite good cultural practices, tree flush was sporadic and limited, even during the growing season. ACP incidence in the grove appeared to be low to medium in general and *T. radiata* wasps were consistently present.

*M. paniculata* release sights were not subjected to any form of insect control, however occasional plant trimming by the property owners did occur. Trimming always removed the new soft growth that ACP utilizes for feeding and oviposition, consequently leaving no immature hosts for *D. aligarhensis* reproduction.
Released wasps did successfully parasitize nymphs in the field and second generation wasps emerged. Evidence of this includes *D. aligarhensis* parasitized mummies found on shoots with exclusion cages (Table 1), the emergence of adult wasps from field clippings (Table 1) and the several adult wasps seen foraging on host *M. paniculata* plants. Twelve of the fourteen *D. aligarhensis* recovered emerged from *M. paniculata* clippings taken from the same dooryard hedge in Apopka. The initial release was on 4/30/08 with wasps emerging from clippings taken on 5/21/08 and 6/11/08. No *D. aligarhensis* adults or parasitized host mummies were observed at this site after 6/11/08. Psyllid populations were high during the first three releases but then dropped dramatically. Coinciding with the decrease in all life stages of psyllids was a large increase in the numbers of coccinellids present on the hedge, and an increased incidence of *T. radiata*.

*T. radiata* wasps were present at all release sites chosen for *D. aligarhensis* in this study (Table 1). This is not surprising as *T. radiata* is established throughout the citrus growing region of Florida (Qureshi et al., 2009). *T. radiata*’s presence likely impeded establishment of *D. aligarhensis*. Through the combined effect of host feeding and parasitism, a single female *T. radiata* can kill nearly twice (500 vs. 280) as many psyllids in her lifetime as can *D. aligarhensis* (Chu & Chien, 1991; Chien, 1995). Additionally, *T. radiata* has a shorter generation time (12 vs. 16-18 days) and a higher reproductive rate (Skelley & Hoy, 2004). Furthermore, any hosts parasitized by *T. radiata* before or within 5 days of *D. aligarhensis* will result in *T. radiata* offspring (E. Rohrig, unpublished).

Coccinellids predators were seen at most release sites, although incidence was low except during the late spring and summer months when psyllid populations were
high. *Curinus coeruleus* Mulsant, *Cycloneda sanguinea* (L.), *Harmonia axyridis* Pallas, and *Olla v-nigrum* Mulsant were the most abundant while the lacewing *Ceraeochrysa* sp. was occasionally seen. Generalist predators can induce high levels of psyllid mortality especially during the spring flush (Michaud, 2004; Qureshi & Stansly 2009). During laboratory observations *Curinus coeruleus* Mulsant, *Cycloneda sanguinea* (L.) and *Harmonia axyridis* Pallas readily consumed both ACP nymphs parasitized by *D. aligarhensis* and unparasitized nymphs (E. Rohrig, unpublished) indicating they may also be responsible for inducing wasp mortality.

Despite the release of thousands of wasps in 2000-2002 (Tawain population) (Skelley & Hoy, 2004) and over 11,000 in 2007-2009, (mainland China population) in numerous counties throughout the citrus growing regions of Florida, *D. aligarhensis* has not established to date. The combined effects of increased/more efficient use of pesticides to control ACP, inconsistent populations of immature ACP, the superiority of *T. radiata* as a biological control agent of ACP, and predation of parasitized hosts by generalist predators likely impeded the establishment of *D. aligarhensis*. 
Table 7-1. Total number of *D. aligarhensis* released and of adult ACP and parasitoids recovered from host plant clippings.

*D. aligarhensis* release and sampling summary

<table>
<thead>
<tr>
<th>Cutting emergence</th>
<th>Date</th>
<th># Released</th>
<th>Adult ACP</th>
<th><em>D. aligarhensis</em></th>
<th>T. radiata</th>
</tr>
</thead>
</table>

**Citrus:**

<table>
<thead>
<tr>
<th>Site</th>
<th>Date</th>
<th># Released</th>
<th>Adult ACP</th>
<th><em>D. aligarhensis</em></th>
<th>T. radiata</th>
</tr>
</thead>
<tbody>
<tr>
<td>Private dooryard (Eustis)</td>
<td>4/30/08</td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>6/11/08</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6/26/08</td>
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* Indicates parasitized mummies (with adult wasp emergence holes) were observed in exclusion cages remaining on tree shoots from the previous release.
CHAPTER 8
CONCLUSION

Greening disease, or huanglongbing, is the most serious citrus disease in the
world affecting most major citrus growing regions throughout the world. The Asian citrus
psyllid (ACP), *Diaphorina citri* Kuyawama, (Hemiptera: Psyllidae) is the sole vector of
citrus greening in the United States. Greening is widespread in Florida and has
threatened the long term viability of the citrus industry. Many citrus groves have become
unproductive or even abandoned. There is currently no completely successful control of
greening disease in any of the citrus growing regions of the world where it occurs.
Current control involves the use of an integrated pest management program including
chemical and biological controls to reduce or eliminate psyllid populations, quarantine
procedures, disease scouting and mechanical removal of infected trees, maintenance of
clean nursery stocks and identification and development of disease resistant citrus
species.

Two parasitic wasps, *Tamarixia radiata* (Waterston) and *Diaphorencyrtus
aligarhensis* (Shafee, Alam & Argarwal) were imported and released in Florida from
1999 to early 2001. Currently *T. radiata* is established throughout the citrus growing
region of Florida providing varying levels of control, while *D. aligarhensis* has not
established for unknown reasons.

In September of 2006, I obtained 100 ACP nymphs from Guangdong province,
China. Fifty adult parasitoids emerged in the maximum security room of the Florida
Biological Control Laboratory quarantine facility at the Florida Department of Agriculture
and Consumer Services, Division of Plant Industry compound in Gainesville, Florida.
Wasps were reared through more than 20 generations until the necessary permits for release were obtained.

The goals of this research were to import, rear, study, mass release and establish a population of *D. aligarhensis* from Mainland China with the hope that a new population from a different region will better adapt and compete in Florida as a biological control agent of ACP than the population that was previously released in Florida. Presently there is little published information regarding *D. aligarhensis* biology, particularly host searching strategies and interactions/competition with *T. radiata*.

For the first three generations, males accounted for 5% of the population. No males have since emerged. When examined through PCR analysis, wasps proved positive for infection with the bacterial endosymbiont Wolbachia. Wolbachia is known to affect reproduction in some insects. Antibiotic treatments intended to induce males were unsuccessful. A bi-parental population of *D. aligarhensis* may be capable of higher reproductive or parasitism rates and further investigation is warranted.

During feeding longevity studies *D. aligarhensis* were relatively long lived surviving for an average of 38.1 ± 0.9 days (25°C) when provided with a honey/yeast mixture. Wasps survived 1.05 ± 0.02 days without food or water, 10.5 ± 0.6 days on nymphal wax excretions, and 27.5 ± 1.2 days on host nymphs.

During laboratory studies *D. aligarhensis* exhibited a clear preference for attacking 2\textsuperscript{nd} and 3\textsuperscript{rd} instars for both feeding and oviposition. Wasps were capable of parasitizing early (5.5 d old at 25°C) and mid (6.6 d) 4\textsuperscript{th} instar hosts but were unable to successfully complete development from late (7.5d old) 4\textsuperscript{th} instar nymphs. Wasps
generally host fed on individual nymphs until mortality as opposed to utilizing the same host for feeding and parasitism.

The larval development of *D. aligarhensis* was examined and characterized. Eggs and early larvae were found free-floating within host hemocoel. Larvae were soft-bodied with no observable hairs, bristles or external appendages in any instar. By the 3rd instar, larvae had begun attaching to nympha tissues by anal secretions which provided a means of orienting within the host nymph. Prior to the beginning of the prepupal stage, the host nymph was turned into a mummy and glued to a plant surface apparently requiring some secretions from the wasp larva. Development from oviposition to adult eclosion of *D. aligarhensis* took approximately 16 d at 25°C when oviposition occurred in 2nd through early 4th (5.5 day old) instar nymphs, although this time was shortened by four days (25%) when the wasps oviposited in mid-4th (6.5 day old) instar *D. citri* nymphs. This reduction in developmental time did not affect wasp fertility or lifespan and may offer a significant approach to improved rearing of this wasp for augmentative releases to control the Asian citrus psyllid.

Parasitoids that are mass reared for biological control are often stored for several weeks prior to augmentive or inoculative release. Additionally, many practitioners store insects at ~17°C rather than in the mid 20’s (°C) to slow down wasp metabolism, thus extending life span and helping to maintain eggload by reducing egg resorption in the absence of hosts. I saw that egg load decreased much more rapidly in wasps held at 25°C than at 17°C when only honey was offered. This would indicate that egg resorption is more rapid at the higher temperature. However, wasps provided access to hosts at 25°C maintained a similar egg load as wasps maintained at 17°C without hosts,
indicating that host feeding provided the nourishment necessary to replace eggs lost through oviposition. Additionally, wasps held in the presence of hosts parasitized more, superparasitized less, antennated fewer individual nymphs and rejected more of those antennated, spent 2.5x less time per average probing event and required 2x less time to oviposit than naïve parasitoids during behavioral examination experiments. This provides evidence that associative learning may be manipulated to enhance biological control. Holding wasps in the presence of hosts until release may condition them to become more efficient biological control agents upon release.

Volatile odors act as chemical attractants to many Hymenoptera, advertising available food and hosts. Research suggests that chemical cues may be more important to searching insects than visual cues and are often the dominant means of long distance attraction. Through the use of y-tube bioassays and GC/MS analyzation of host and host plant volatiles I examined if *D. aligarhensis* uses volatile cues as olfactory stimuli to locate *D. citri* hosts and host habitats. ACP infested plants (*M. paniculata* and *C. paradise*) caught significantly more *D. aligarhensis* than blank air controls. The same plants, without nymphs, failed to capture more wasps than blank air indicating that undisturbed ACP host plant volatiles are not likely used by *D. aligarhensis* to locate hosts. Nymphs themselves failed to catch significantly more wasps than a blank (n= 8) while nymph honeydew/wax captured significantly more (n= 42) than a blank air control. This suggests that it is a volatile from the nymphal excretion that is attractive to *D. aligarhensis* rather than the immature host psyllid itself. This is not surprising given that cues directly from hosts are often non-volatile contact kairomones which can only be perceived by contact chemoreception. The lack of cues from attacked host stages often
leads parasitoids to rely on those emanating from sources associated with host presence such as frass and other excretions. Host frass has been shown to be the dominate host searching cue for several hymenopteran parasitoids. In many cases it is unclear if the volatiles emanating from frass are a chemical product of host metabolism, the host plant or both.

Although a statistically significant number of wasps were caught using infested plants (*M. paniculata* and *C. paradise*) and nymph excretions over blank air controls actual differences were not great. In all three cases less than 60% (54.2%, 56%, 57.8%) of the wasps tested per odor source (n= 500) were captured in the odor trap verses the blank indicating *D. aligarhenisis* was not highly attracted to any of the odor sources under these experimental parameters. *T. radiata* possess mechanosensory antennae sensillae (Type I asexual sensilla trichoidea) that are known to detect acoustic or vibrational signals generated by hosts. It is possible that *D. aligarhensis* also require tactile and/or visual cues to effectively locate hosts. Further testing examining *D. aligarhensis* antennae morphology and the use of tactile or visual cues as stimuli sources is warranted.

In spite of the fact that *T. radiata* is established throughout Florida including all of the *D. aliagarhensis* release sites and that these species coexist in different parts of Asia there have been no published reports examining inter-specific competition between the two species. When presented with a nymph not previously exposed to any wasp, the time spent by both *D. aligarhensis* and *T. radiata* until parasitism after an initial antennation event did not significantly differ. When given a nymph parasitized by the competing species, regardless of the holding time between wasps access, there was no
significant difference in the time spent examining and ovipositing by either wasp. Although this time did not statistically differ, *D. aligarhensis* averages were always at least twice that of *T. radiata*. The resulting standard errors were much higher in *D. aligarhensis* indicating a greater variance in the time spent between individual wasps. Egg lay totals from both species of wasps did not significantly differ regardless of the presence or absence of a competing wasp species or the density of nymphs provided. These results indicate that both wasps can parasitize a similar number of nymphs in the same amount of time even if the nymph has already been parasitized by the competing species. However, *T. radiata* always laid its highest number of eggs at the highest nymph density, while *D. aligarhensis* laid fewer eggs at the highest host density. This indicates *T. radiata* may perform better as a biological control agent under higher host densities.

During interspecific competition experiments, the number of hosts multiparasitized by both wasp species was the highest at the lowest nymph density. A significantly higher number of multiparasitism events were seen under the lowest nymph density (n=20). Thirty percent of nymphs were multiparasitized by both species. As host density increased to 40 or 80 per cage the number of multiparasitizism events dropped by more than 50% down to 6.75% and 2.88% of nymphs respectively. Under low host densities it is clear that there is a strong level of competition between the two species. During caged seedling experiments, regardless if one or both wasp species had access to nymphs, both wasps’ rate of intraspecific competition decreased as the nymph density increased. However, *D. aligarhensis* always exhibited a higher rate of superparasitism under each nymph density.
Although there were a few instances of discrimination during observational arena experiments, neither *D. aligarhensis* nor *T. radiata* significantly discriminated ovipositionally against a nymph that was already parasitized by the other species, regardless of the time interval between wasps’ access. The lack of significant ovipositional discrimination against a host already parasitized by the other species provides evidence that if either parasitoid encountered a 2<sup>nd</sup>–4<sup>th</sup> instar nymph, even if it is parasitized by the competing species, they are both as likely to parasitize. However, *T. radiata* has two advantages over *D. aligarhensis*. First, *T. radiata* is capable of parasitizing 5<sup>th</sup> instar nymphs (the longest developmental stage) which *D. aligarhensis* is not. Second and more importantly, a *T. radiata* wasp will always emerge if it oviposits first and can still successfully produce some offspring if it parasitizes within 5-7 days after *D. aligarhensis*. *D. aligarhensis* can only produce some progeny if it oviposits at least 5 days before *T. radiata*. This is a major disadvantage when releasing *D. aligarhensis* into an area where *T. radiata* is already present.

Field releases and evaluations were conducted from August of 2007 through October of 2009. Post release sampling was conducted through visual inspection for the presence of adult *D. aligarhensis* or host mummies and by observing adult insect emergence from host plant cuttings. Releases were made at 10 sites in 6 Florida counties (Alachua, Collier, DeSoto, Hendry, Orange and Polk). Sixty-seven releases were conducted totaling approximately 11,100 wasps. Two adult female *D. aligarhensis* were recovered from *Citrus* sp. clippings taken from 2 sites in Collier County in 2007. Twelve adult female *D. aligarhensis* were recovered from *M. paniculata* clippings taken from Orange County in 2008. Parasitized host mummies, left behind after *D. aligarhensis*.
aligarhensis emergence, were seen at several sites. T. radiata adults or parsitized mummies were seen at all sites utilized for release of D. aligarhensis. Additionally, T. radiata adults emerged from host clippings taken from all release sites. ACP and host plant flush incidence varied greatly according to location and time of year perhaps due to cultural practices and weather conditions. All citrus groves used for wasp release, including certified organic, utilized pesticide sprays to some extent during the release period. Overall ACP incidence in general appeared to decline over the >2 year release period, likely from an increased insecticide use to reduce spread of citrus greening disease. In early 2009, releases were focused in Marty Wertz organic citrus in Alachua County. This sight was subjected to minimal certified organic pesticide sprays, maintained good cultural practices and was in close proximity to the rearing laboratory allowing more frequent visits. Despite good cultural practices, tree flush was sporadic and limited, even during the growing season. ACP incidence in the grove appeared to be low to medium in general and T. radiata wasps were consistently present. M. paniculata release sights were not subjected to any form of insect control though occasional plant trimming by the property owners did occur. Trimming always removed the new soft growth that ACP utilizes for feeding and oviposition, consequently leaving no immature hosts for D. aligarhensis reproduction.

Released wasps did successfully parasitize nymphs in the field and second generation wasps emerged. Evidence of this includes D. aligarhensis parasitized mummies found on shoots with exclusion cages and the emergence of adult wasps from field clippings. Coccinellids predators were seen at most release sites, although incidence was low except during the late spring and summer months when psyllid
populations were high. *Curinus coeruleus* Mulsant, *Cycloneda sanguinea* (L.), *Harmonia axyridis* Pallas, and *Olla v-nigrum* Mulsant were the most abundant while the lacewing *Ceraeochrysa* sp. was occasionally seen. Generalist predators can induce high levels of psyllid mortality especially during the spring flush. During laboratory observations *Curinus coeruleus* Mulsant, *Cycloneda sanguinea* (L.) and *Harmonia axyridis* Pallas readily consumed both ACP nymphs parasitized by *D. aligarhensis* and unparasitized nymphs indicating they may also be responsible for inducing wasp mortality.

There are several issues that are likely influencing the establishment of *D. aligarhensis* in Florida. First, *T. radiata* is present throughout citrus growing regions of Florida including all of the Florida sites where this mainland China population of *D. aligarhensis* has been released. Any hosts parasitized by *T. radiata* before or within 5 days of *D. aligarhensis* will result in *T. radiata* offspring. *T. radiata* are capable of parasitizing 2\(^{nd}\) – 5\(^{th}\) instar nymphs which are present for more than a ~10.5 day developmental period at 25°C. *D. aligarhensis* can parasitize 2\(^{nd}\) – 4\(^{th}\) instars which are only available for ~5.5 days at 25°C. This gives *T. radiata* an ovipositional window that is nearly twice as long. Additionally, *T. radiata* has a shorter generation time (12 vs. 16-18 days) and a higher reproductive rate. Furthermore, a single female *T. radiata* can kill nearly twice as many psyllids in her lifetime than *D. aligarhensis*. Both wasps induce psyllid mortality through the combined effect of parasitism and host feeding. A single female *T. radiata* is capable of killing up to 500 nymphs through her lifetime while *D. aligarhensis* can kill 280. Regardless, both species coexist in parts of Asia and further research in that region is warranted. Closer examination in Asia of natural enemies,
including hyperparasitoids which are not present in Florida, might show reasons why the two species may sometimes coexist.

Despite the release of thousands of wasps in 2000-2002 (Tawain population) (Skelley and Hoy, 2004) and over 10,000 in 2007-2009, (mainland China population) in numerous counties throughout the citrus growing regions of Florida, *D. aligarhenisis* has not established to date. The combined effects of increased/more efficient use of pesticides to control ACP, inconsistent populations of immature ACP, the superiority of *T. radiata* as a biological control agent of ACP, and predation of parasitized hosts by generalist predators likely impeded the establishment of *D. aligarhensis*. 
LIST OF REFERENCES


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BIOGRAPHICAL SKETCH

Eric Rohrig was born in Canton, Ohio. He began his undergraduate studies in 1995 at the State University of New York at Oswego. After graduating with his bachelor’s in zoology he moved to Florida to attend the Santa Fe Teaching Zoo where he earned an associate’s in zoo technology in 1998. He was employed by a public zoo and aquarium before returning to Gainesville, Florida to take a position in entomology for the Florida Department of Agriculture in 2002. In 2004, he began his master’s work at the University of Florida under the supervision of Dr. John Sivinski with the United States Department of Agriculture-Agricultural Research Services. Also in 2004, he began his employment with the USDA-ARS where he is still currently employed. He completed his master’s in entomology in 2006 and enrolled in the PhD program under the supervision of Dr. Phil Stansly with the University of Florida.