IMPROVING THE EFFICIENCY OF MASS REARING *TAMARIXIA RADIATA* (HYMENOPTERA: EULOPHIDAE) BASED ON BEHAVIORAL ASPECTS OF THE FUNCTIONAL RESPONSE, HOST MARKING, AND ENDOSYMBIONT DETECTION

By

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To my beloved family and friends, without their support and love, none of this would have happened

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IMPROVING THE EFFICIENCY OF MASS REARING *TAMARIXIA RADIATA* (HYMENOPTERA: EULOPHIDAE) BASED ON BEHAVIORAL ASPECTS OF THE FUNCTIONAL RESPONSE, HOST MARKING, AND ENDOSYMBIONT DETECTION

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Tamarixia radiata is the primary parasitoid attacking *Diaphorina citri*, the Asian citrus psyllid (ACP). The parasitoid has been used to manage ACP in abandoned citrus, residential areas, and organic orchards, which requires mass rearing. The objective of the project was to determine the factors deterring the mass rearing efficiency of *T. radiata* through studies of functional response, oviposition behavior, endosymbiont analysis, and improvements in mass rearing protocols.

Functional response was first studied under laboratory conditions by releasing *T. radiata* to oviposit ACP nymphs at different densities. Results showed that wasps presented a type II response relationship with the increasing host density, and oviposition behaviors were assessed by direct observation. Observed handling time was estimated at 3.9 min, and the searching coefficient decreased when host density increased. These results were modelled by adding an additional parameter to the disk equation, resulting in better fit to experimental results. Searching area tended to be negatively correlated with searching duration implying the possible existence of a mark on the parasitized hosts to deter further oviposition.

Four experiments were set up to verify existence of the host mark, and evaluate its characteristics. Observation showed that *T. radiata* females used antennae to detect the mark on the parasitized hosts and mark was not volatile within 24 h. A “patch mark” protecting unparasitized neighbors was observed within 1 h, and probably persists for longer although a different experimental arena would need to be devised.

Rearing conditions were evaluated under greenhouse conditions to optimize wasp production. The mass rearing efficiency was optimized by releasing 60 female wasps per cage under a photophase of 12 h. Molecular detection suggested that the dominate eubacteria species in Gainesville colony was *Burkholderia* sp, whereas *S. saprophyticus* in Immokalee colony, which might lead to the fitness differences in these two colonies. An economic analysis was conducted to estimate costs and benefits of rearing and releasing *T. radiata*. The cost of rearing one wasp was estimated to average $0.11 under current conditions. An equation balancing the number of *T. radiata* released and the percent parasitism was proposed in order to provide guidance on releasing.

CHAPTER 1
INTRODUCTION

Tamarixia radiata is an arrhenotokous ectoparasitoid of *Diaphorina citri*, vector of citrus greening disease or huanglongbing (HLB). The psyllid was first found in Florida in 1998 and HLB detected 7 years later (Halbert & Manjunath 2004). By December 2007, the disease had been confirmed in 30 counties in the southern half of the state (Hall 2008). HLB is one of the most destructive diseases of citrus known (Halbert & Manjunath 2004, Teixeira et al. 2005, Bove 2006, Wang et al. 2006, Batool et al. 2007, Manjunath et al. 2008), and has been estimated to have cost Florida's economy $4.4 billion and resulted in 8,257 jobs lost from 2006- 2011 (Hodges & Spreen 2012).

Strategies to manage HLB include utilization of pathogen-free nursery stock, removal of infected trees, and an effective ACP control program for which biological control is a very important component (Grafton-Cardwell et al. 2013). *T. radiata* is the parasitoid species that has received the most attention for biological control of *D. citri*. *T. radiata* was first imported to Florida from Taiwan and Vietnam in 1998, released in 1999-2001 (Hoy et al. 2001), and is now established in citrus orchards throughout the state (Qureshi et al. 2009). *T. radiata* has been widely used to control the population of *D. citri* in abandoned citrus, residential areas, and organic groves due to constraints on the use synthetic insecticides (http://www.freshfromflorida.com/Divisions-Offices/Plant-Industry/Bureaus-and-Services/Bureau-Of-Methods-Development-Biological-Control/Biological-Control/Biological-Control-of-Asian-Citrus-Psyllid-in-Dooryard-Citrus-and-Ornamentals).

The success of parasitoids as biological control agents is related, in part, to the behaviors the parasitoids present in response to an increasing host density (Solomon

1949, Holling 1959a), referred to as “functional response”. Hollilng (1959 a) identified three basic forms of functional response. Type I response is a positive linear relationship between prey density and prey consumed per predator. Type II response is increasing numbers of prey consumed per predator at increasing prey density but at a constantly decreasing rate such that the propotion of prey consumed decreases with prey density. For a type III functional response, the propotion of prey consumed per predator increases at first, then decreases formng an S-shaped curve (Fig. 1-1). There is a upper limit for both type II and III, corresponding to the upper limit of parasitoid oviposition or predator consumption (Holling 1959a).

Tamarixia radiata was reported to express a type II functional response relationship with the increasing host density, but the unknown parameter handling time based on the type II functional response equation seemed to be overestimated (Chen 2013, Sule et al. 2013), moreover, functional response had not been studied using the *T. radiata* Florida population. To better understand the potential parasitization rate with the increasing host density, and explain the biological significance of the functional response equation, *T. radiata*’*s* functional response was studied in the lab under favorable conditions. Furthermore, to better estimate the handling time, *T. radiata* foraging behaviors were observed at six host densities for 30 min, and recorded at three host densities for 12 h to explain the unknown parameters in biological terms.

During the functional response study, I found that the percent parasitism was lower than expected even under favorable conditions, and especially in small experimental arenas. My hypothesis was that the existence of a host marking pheromone might be the cause. Many parasitoid species are known to avoid utilizing

already parasitized hosts to reduce the level of competition in the offspring (Prokopy 1981, Nufio & Papaj 2001). This behavior is mediated by deposition of chemical signals on utilized hosts after oviposition as a mark (Stelinski et al. 2007). Female *T. radiata* have been reported to discriminate between parasitized and unparasitized hosts to avoid superparasitism (Chien et al. 1991a). Husain & Nath (1923) observed superparasitism during December and January when hosts were scarce, but not when hosts were abundant. Chien et al. (1991a, 1991b) observed superparasitism rates of up to 5.6% when host density was low and active space was limited. Chen (2013) found superparasitism at host densities of 10 to 60 per parasitoid, and reported that the superparasitism rate was the highest (37.9%) at the lowest host density. Nufio & Papaj (2001) stated that the function of host marking pheromones produced by female parasitoids following oviposition was to be detected by contact chemoreception during subsequent encounters. Interestingly, some parasitoids mark not only the parasitized host, but also the patch they searched (Van Alphen & Visser 1990). Patch marking is a mechanism that may help to avoid waste of searching effort on resources that have been exploited already. However, deployment of signals may entail a cost if used by competitors or limiting the probability of finding suitable hosts (Gauthier et al. 2002). The existence of a host marking mechanism in *T. radiata*, as well as characteristics such as degree, range, duration and volatility of a putative host mark, could be factors that affect mass rearing efficiency. Host marking could impair host availability, since it wastes the parasitoid's time of detecting one host, and she may reject it later, which also influences the functional response.

Other factors that may reduce mass rearing efficiency include rearing methodology. Skelly & Hoy (2003) suggested a mass rearing method on orange jasmine *Murraya paniculata*, and basic rearing procedures. However, there remains a lack of information on optimal *T. radiata* release rates as a function of host density, sex ratio and photophase to maximize efficiency in mass rearing. Therefore, I evaluated different releasing rates of *D. citri* for optimal psyllid production, different releasing rates of *T. radiata* for optimal wasp production, effects of parental sex ratio on the progeny sex ratio, and the effect of photophase on optimal wasp production. I followed this with an economic analysis of mass rearing of *T. radiata* and management of *D. citri* in the field. This project will have great importance for improving the mass rearing efficiency of *T. radiata,* for instance in the mass rearing facility at the Florida Department of Agriculture, Division of Plant Industry (FDACS-DPI) in Gainesville and Dundee, and the University of Florida facility at the Southwest Florida Research and Education Center (SWFREC).

After over a decade of rearing *T. radiata* in these two facilities, it was observed that production at SWFREC was inferior to that at DPI despite similar methodology (personal communication), leading to the hypothesis of pathogen or beneficial symbiont effects on the fitness of the different populations. Meyer & Hoy (2008) surveyed endosymbionts in *T. radiata* (Gainesville colony) including Archaea, Eubacteria, *Candidatus* Liberibacter asiaticus, *Arsenophonus,* Wolbachia *wsp*A, *Caulobacter,* and *Methylobacterium.* They discovered the presence of *Caulobacter sp., Methylobacterium sp*., and species in the family Alcaligenaceae, and concluded that all except for *Caulobacter sp.* were likely acquired from the environment rather than vertically

transmitted. More importantly, they did not find *Ca.* L. asiaticus, the causal agent of HLB which would have prohibited release in the field. However, this research was published more than 8 years ago and only included *T. radiata* from the DPI colony. Furthermore, production of the SWFREC colony has lagged recently giving rise to suspicions of possible infection by a pathogen affecting fitness. Therefore, I compared the existence of different symbionts in the *T. radiata* populations from both colonies, and also determined the genome size and a single copy gene for future studies.

In summary, the main objective of this research project was to evaluate and improve the capacity of *T. radiata* to utilize *D. citri* hosts more efficiently, which involved studies of functional response, host marking, improved methodology of mass rearing, and molecular detection of endosymbionts that may affect fitness. The four specific objectives were as follows:

1. Evaluate functional response of *T. radiata* at different host densities and validate with behavioral observations.
2. Confirm the existence of and investigate the characteristics of *T. radiata* host discrimination and host marking
3. Optimizing methodology parameters to improve the mass rearing efficiency in the *T. radiata* colony
4. Utilize molecular detection of endosymbionts to investigate possible constraints to colony health in two *T. radiata* populations.

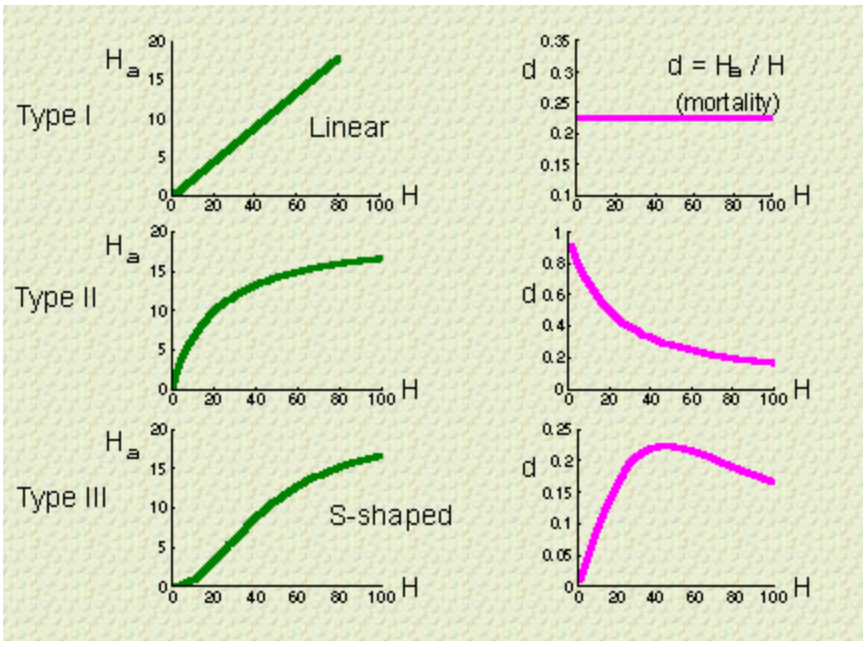


Figure 1-1. Type I, II, and III functional response relationships. Green lines represent the relationship between host density (H) and the number of hosts parasitized (Ha). Pink lines represent the relationship between host density and the proportion of host parasitized (d).

CHAPTER 2
LITERATURE REVIEW

Citrus greening or huanglongbing (HLB) is considered the world's most destructive citrus disease (Halbert & Manjunath 2004, Texieira et al. 2005, Bové 2006, Wang et al. 2006, Batool et al. 2007, Manjunath et al. 2008). The putative causative agents are three known vector-borne α-proteobacteria of which the most widespread and pervasive is '*Candidatus* Liberibacter asiaticus', vectored by the Asian citrus psyllid, *Diaphorina citri* (Bové 2006) (Hemiptera: Psylloidea). *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae, an ectoparasitoid) and *Diaphorencyrtus aligarhensis* (Shafee, Alam and Argarwal) (Hemiptera: Encyrtidae, an endoparasitiod) are the only known primary parasitoids of *D. citri* nymphs (Tang 1990). Both were first described from the northern Indian subcontinent (Waterston 1922, Shafee et al. 1975).

Tamarixia radiata has been successfully introduced in Réunion (Aubert & Quilici 1984), Taiwan (Chien et al. 1989), Mauritius (Quilici 1986), Philippines (Gavarra et al. 1990), Saudi Arabia (Aubert 1984), East Java, Indonesia (Nurhadi 1988), Guadalupe (Étienne et al. 2001), California (Hoddle 2012) and Florida (Skelley & Hoy 2004), where it spread throughout the state (Qureshi et al. 2009). *T. radiata* appeared inadvertently in Brazil (Gomez-Torres et al. 2005, Torres et al. 2006), Argentina (Lizondo et al. 2007), Venezuela (Cermeli et al 2007), Mexico (De León & Sétamou 2010), Puerto Rico (Pluke et al. 2008) and Texas (French et al. 2001). *T. radiata* has high host specificity on *D. citri*, although *Bactericera cockerelli* Sulc was found parasitized at low levels (5%) (Hoddle & Pandey 2014).

Given the rapidity with which *T. radiata* has established and spread, it is an obvious choice for augmentative biological control of *D. citri*. Such efforts are underway

in numerous states and countries in the Americas including Brazil, Costa Rica, Jamaica, Mexico, Florida, Texas, and California.

Morphology and Sensory Perception

Tamarixia radiata male and female are similar in color and body structure, except for antennae and a somewhat darker abdomen in the male. The female antenna has eight segments, both funicle and club with three segments covered with fine, short setae. The funicle is slender with the 1st segment longer than the 2nd and the 2nd segment longer than the 3rd. The length of the 3rd segment is almost equal to the width. The male antenna is more slender, and 9-segmented. The four-segmented funicle is covered with long, slightly curved hairs, with a ventral sensorium near the base of the scape (Tang & Aubert 1990). Male antennae possess a number of olfactory multiporous trichoid sensilla which could function for perception of mating-related volatile cues, whereas female antennae are characterized by more multiporous placoid sensillia suggesting greater sensitivity to host-related volatile cues (Onagbola et al. 2009). Behavioral assays conducted in an olfactometer verified that female wasps use volatiles emanating from *D. citri* nymphs for host location and that male wasps are attracted to a volatile pheromone emitted by female conspecifics (Mann et al. 2010).

Life Cycle

The four life stages of *T. radiata* were described by Chien et al. (1991a). The egg is translucent, ivory, and reniform, with one end adhering to the host. There are four larval instars, each distinguished by head capsule width: 0.06mm, 0.09mm, 0.14mm, and 0.22mm for 1st, 2nd, 3rd, and 4th instar larva respectively (Chien et al. 1991a). Development of the immature stages was reported by Xu & Tang (1993) and Chien et al. (1991a). The newly-enclosed larva sucks fluids externally from the site where it is

closely attached to the nymph's integument (Husain & Nath 1923, Tang & Huang 1991). The 3rd instar crawls to the ventral side of the host thorax to feed (Chien et al. 1991a). The parasitized nymph continues to live and secrete honeydew for some time (Husain & Nath 1923). All contents of the nymph are consumed by the time the parasitoid molts to the 4th instar and the nymph turns to a dark-brown mummy (Husain & Nath 1923, Chien et al. 1991a). The mature larva ceases to feed as it progresses to the prepupal stage which secures the mummy to the plant surface by means of silken threads (Chien et al. 1991a). After expelling the meconium, it molts to the pupal stage which turns yellow, with red ommatidia and ocelli (Chien et al. 1991a). As soon as the adult hardens, it makes its way out of the mummy by chewing a round hole of about 0.5 mm diameter in the region of thorax (Husain & Nath 1923, Chien et al. 1991a, Aubert1987). Over 80% of adults emerge between 5 a.m. to 10 am, with peak emergence at 7 a.m. to 8 a.m (Chien et al. 1991a). The male emerges 1.5 h earlier than the female on average.

Mating

Males use the antennae to locate a female. Once a receptive female was found, the male crawled onto the dorsum where he remained for 68 ± 7 s before actual mating for an additional 33 ± 3 s (Chien et al. 1991a). About 93% of females mated once and only once during the first day of emergence. The remaining 7% mated twice during the first 2 d following emergence. Fecundity and longevity of females were not affected by mating frequency. Males are capable of multiple matings over their lifetime (Chien et al. 1991a).

Oviposition

Eggs can be laid immediately after emergence by either mated or unmated females (Chien et al. 1991a). The period from 5 a.m. to 10 a.m. is the most active time

of d for oviposition (Chu & Chien 1991). Host volatiles mediate host location (Mann et al. 2010). The female moves actively among *D. citri* nymphs using her antennae to search for suitable hosts (Husain & Nath 1923). She deposits an egg or occasionally two on the underside of the chosen nymph, usually next to a mid or hind coxa (Husain & Nath 1923, Aubert 1987, Chien et al. 1991a, Hall 2008b, Tang & Huang 1991, Fig. 2-1). Oviposition took 3 to 4 min according to Husain & Nath (1923), but only 61 ± 8 s according to Chien et al. (1991a). Chien et al. (1991a) reported that the female *T. radiata* injects venom into the host nymph through the ovipositor, immobilizing it for 4 to 8 min. If the egg was removed, the host nymph still could not molt, and died 8 d later at 25 °C. The 1st, 2nd, or 3rd instar *T. radiata* larvae placed on an unparasitized 5th instar nymph could not attach, and dropped off when the nymph began crawling, highlighting the importance of the venom for paralyzing *D. citri* nymphs (Chien et al. 1991a).

Host Preference

Studies on host selection have produced varying results. Chien et al. (1991a) and Chu & Chien (1991) reported that 5th instar nymphs were preferred for oviposition. Survival rates were 85%, compared with 33% and 71% from 3rd and 4th instars, respectively (Chien et al. 1991a). Body length was also greater among offspring from 5th instar hosts compared to 4th instar hosts: 1.12 mm compared with 0.91 mm (female), 1.03 mm compared with 0.86 mm (male) respectively. This pattern was repeated with fecundity estimates: 215 compared to 120 eggs per female, and longevity: 18.0 d compared to 14.4 d (females), or 11.6 d compared to 7.2 d, (males) (Chien et al. 1991). However, Tang & Huang (1991) reported that 4th instar nymphs are parasitized significantly more frequently than 3rd or 5th instars.

Host Feeding

Both genders feed on honeydew from *D. citri* nymphs, and females use the ovipositor to puncture the nymphal cuticle for host feeding (Chien et al. 1991a, Skelley & Hoy 2004). Males also feed upon hemolymph from nymphs punctured by a female (X. Chen, personal observation). Like oviposition, host-feeding occurs during the daytime and takes an average of 21 ± 2 s (Chien et al. 1991a). Nymphs die once fed upon, and females avoid laying eggs and feeding on the same host (Chien et al. 1991a, Tang & Huang 1991). The ratio of host-feeding to oviposition for 4-18 day old females averages 5.6:1 and correlates with host density and parasitoid age (Chien et al. 1991a). Younger (< 4 d) or older (> 18 d) females laid one egg for an average 0.29 or 0.38 hosts fed upon respectively (Chien et al. 1991a). Approximately 80% of nymphal mortality in the laboratory results from parasitism with an additional 20% from host-feeding (Chien et al. 1991a, 1994a). Therefore, it has been estimated that a single female *T. radiata* could kill up to 500 nymphs during her lifetime (Chu & Chien 1991). However, temperature can influence the number of *D. citri* nymphs killed. Chien et al. (1993) estimated host-killing capacity (host feeding + parasitism) at 16, 25, 245, 196 nymphs per female at 15 °C, 20 °C, 25 °C and 30 °C, respectively. Skelley and Hoy (2004) reported that 36% of hosts were parasitized and 57% were fed upon in their colonies at 25± 2°C, 30- 65% RH and 18L: 6D photoperiod.

Sex Ratio

Tamarixia radiata is arrhenotokous, meaning unfertilized eggs produce males, while fertilized eggs develop into females. The average number of eggs deposited by virgin and mated females in one study was 209.2 and 215.4 respectively (Chu & Chien 1991). Progeny sex ratio is highly correlated with the age of female parasitoids (Tang &

Huang 1991, Chu & Chien 1991). The proportion of female progeny increased as the mother aged, from 0.5 (1 d-old female) to 0.77 (from 22 d-old females) (Chu & Chien 1991). Sex ratio is also correlated with host stage, although published results differ. Tang & Huang (1991) reported 88% females emerging from 5th instar hosts, 75% females from 4th instars and 41% from 3rd instars. However, Chu & Chien (1991) reported 67% females from 5th instar nymphs, compared with 16% from 4th instars. Skelley & Hoy (2004) found 64% and 67% females emerging from 4th instar nymphs in their Taiwanese and Vietnamese colonies of *T. radiata,* respectively.

Ovigeny

Tamarixia radiata has been characterized by an ovigeny index of 0.03, meaning that the parasitoid is synovigenic and that host- feeding is required to mature most eggs (Jervis et al. 2001). Like most synovigenic parasitoids, *T. radiata*, can resorb eggs when hosts are absent or scarce (Chien et al. 1994b), thereby maintaining reproductive resources and synchrony with the host population (Jervis et al. 2001). Egg resorption was observed in *T. radiata* at 15 °C and 25 °C, even when honey was provided, and occurred at rates positively correlated with host deprivation time (Chien et al. 1991a, 1994b, Chen & Stansly 2014).

Once suitable hosts are fed upon, new eggs can be matured and oviposition can occur (Chien et al. 1994b). Little or no effect on total fecundity was observed after host deprivation for 10 d at 25 °C with honey provided as food (204 eggs), but there was a loss of fecundity after host deprivation for 20 d (156 eggs) (Chien et al. 1994b). Wasps maintained for 10 to 20 d at 25 °C laid significantly more eggs (156 eggs) than wasps stored maintained 15 °C (98 eggs). Fecundity decreased greatly following host deprivation for 30 to 40 d at 15 °C (25~59 eggs).

Superparasitism

Female *T. radiata* can discriminate between parasitized and unparasitized hosts to avoid superparasitism (Chien et al. 1991a). Husain & Nath (1923) observed superparasitism during Dec and Jan when hosts were scarce, but not when hosts were abundant. Chien et al. (1991a) observed superparasitism rates of up to 10.4% when host density was low (20 hosts per parasitoids) and active space was limited. Chen (2013) found superparasitism rates averaging 37.9 ± 0.03% at a female: host density of 1:10 to less than 0.5% at host densities of 1:40 and above. However, only one larva made it to the adult stage, regardless of the superparasitism levels.

Other Host Density Effects

Longevity, fecundity, sex ratio and ratio of host feeding to oviposition all correlate with host density (Chu & Chien 1991, Chien et al. 1991a, 1995). The relationship between host density and parasitoid longevity (both males and females) was described as following a domed parabolic response, meaning that female longevity and fecundity ascend with host density to a peak and then decrease as host density increases (Chien et al. 1995). Average longevity increased from 15.9 to 18.6 to 20.3 d when host densities of 10, 20 and 30 nymphs respectively were provided daily to individual females. However, female longevity decreased from 23.6 to 17.2 to 11.2 d over a range of 40, 60, 80 hosts per day, respectively (Chien et al. 1995). Chu & Chien (1991) reported that females lived an average 23.6 d and males 14.8 d when 40, 5^{th} instars were provided at 25 °C, 14:10 h L: D photoperiod and 100% relative humidity (RH). Both daily and lifetime fecundity showed similar parabolic responses to host density, with the peak at 40 hosts per day (Chien et al. 1995). However, Chen (2013) found the average number of 4^{th} instars parasitized in 24h to increase from 4 to 11 over densities

of 10 to 40 hosts per female, and then to level off at higher densities, resulting in a type II functional response. Searching efficiency was estimated at 0.442± 0.036 d^{-1} (10.61± 0.84 h^{-1}), and handling time per host at 0.045± 0.008 d (1.08± 0.48 h). Sule et al. (2014) also reported a type II functional response but estimated searching efficiency at 39.99 h^{-1} and 34.04 h^{-1}, and handling time at s 0.60 h and 0.71 h for 4^{th} and 5^{th} instar *D. citri* nymphs respectively.

Influence of Food

Adults deprived of food or water survived from 1.0 to 1.7 d (Chien et al. 1994b). They found all food supplements tested improved fecundity, longevity and progeny survivorship compared to total deprivation, but observed no difference in longevity among female adults deprived of hosts and fed either honey alone (22.5 d), honey and pollen (23.0 d), or honey and yeast extract (23.4 d). Chen el al. (2013) observed a survival rate of 97% when *T. radiata* was stored at 25 °C with honey for 14 d, which agreed with the results from Hall & Klein (2014). Adults fed on a diet of honey and yeast extract significantly decreased host-feeding while maintaining or improving intrinsic rate of increase (0.2976 to 0.3014 progeny per day) and the net reproductive rate (140 to 187 female eggs per female), respectively (Chien et al. 1994b). Addition of hydrolyzed corn gluten (Nu-Lure ®) to a diet of honey alone increased egg load, as did substitution of this same proteinaceous liquid for honey to supplement host feeding (Chen & Stansly 2014).

Development Time

Chien et al. (1991a) found the duration of one generation for *T. radiata* on orange jasmine (egg to adult emergence) to be around 11.4 d at 25 °C, 14:10 h L: D photoperiod, and 100% RH. Development times for individual life stages were 45 h for

the egg, 24 + 24 + 22 + 26 = 96 h for the 1st through 4th instars, respectively, and 14.4 h and 117.6 h for the prepupal and pupal stages, respectively. Xu & Tang (1993) reported a generation time of 12.6 d at 25 ± 1 °C, 14:10 h L: D photoperiod, and 75 to 85% RH, using 3rd and 4th instar nymphs. This corresponded to 40 h for the egg and 119 h = 25, 28, 32 and 34 h for the 1st through 4th larval instar respectively, 24 h for the prepupa and 120 h for the pupa. These differences could reflect the different humidity conditions under which the experiments were conducted.

Temperature and Humidity Effects

Tamarixia radiata completed development at 15 to 32 °C with an optimum temperature of 25 °C (Chien et al. 1993). Gomez-Torres et al. (2012) found parasitism rates to be highest at 25 and 30 °C (85.5 and 72.8%, respectively) compared to 23.1 and 40.2% at 15 and 35 °C respectively. They also found emergence rates to be highest, 86.7 and 88.3%, at 25 and 30 °C respectively, compared to about 50% in the 15 to 20 °C range. At 70 ± 10% RH and 14:10 H L: D, they estimated maximum parasitism rates of 77.2% at 26.3 °C, whereas emergence was greatest (89.9%) at an estimated 30.8 °C. Pre-imaginal development was longer for females, varying from 489.6 h at 15 °C to 247.2 h at 35 °C compared to males from 343.2 h to 146.4 h over this same range.

Longevity with access to pure honey was negatively correlated with temperature between 8 °C to 30 °C (Quilici & Fauvergue 1990). These authors found that adult longevity decreased from 34 d at 20 °C to 22 d at 22 °C, 10 d at 30 °C and 8 d at 35 °C. Chien et al. (1993) found longevity increased from 45.5 to 59.5 at 8 and 15 °C, but decreased to 22.5 and 9.6 d at 25 and 30 °C, respectively. Only 10% of *T. radiata* adults

survived for 50 d when stored at 25 °C with access to honey and yeast extract (Skelley & Hoy 2004).

McFarland & Hoy (2001) reported that *T. radiata* adults from Vietnam survived longer without food and water compared to parasitoids from Taiwan over a range of RH from 7% to 97% at 25 °C and especially at 30 °C. They attributed this difference to greater moisture requirements for *T. radiata* from Taiwan. Host-killing capacity also increased to a peak at 25 °C and then decreased at higher temperature as noted above. Gomez-Torres (2012) estimated intrinsic rate of increase (r_m), net reproductive rate (R_0), and mean generation time (T) for pairs of *T. radiata* provided 5th instar *D. citri* nymphs reared on orange jasmine. Results differed considerably, especially at low temperatures (Table 2-1). Chien et al. (1993) did their study at a host density of 20 per female and 100% RH for five replications, whereas Gomez-Torres et al. (2012) conducted their study at a host density of 30 per female at 70 ± 10% RH for 10 replications. Thus, different experimental conditions may have led to differing results, although inherent differences in the races of *T. radiata* tested from Taiwan and Brazil, respectively, cannot be ruled out.

Skelley & Hoy (2004) showed that *T. radiata* stored for up to 35 d at 17 °C with honey and yeast suffered less than 5% mortality. Chien et al. (1994a) reported that females stored for 20 d at 25 °C laid a total of 156 eggs, compared with 98 eggs when stored at 15 °C. We may conclude that less mortality was experienced at low temperature but that reproductive fitness suffered. Therefore, ideal storage temperature should be determined according to specific objectives (establishment or augmentation) and conditions (host availability).

Genetics

Barr et al. (2009) used sequence analysis of the internal transcribed spacer (ITS) region 1, ITS-2, and the 5' end of the cytochrome oxidase subunit 1 (CO1) gene to evaluate relatedness among laboratory colonies and field collections of *T. radiata* from China, Vietnam, Pakistan, Florida, Puerto Rico, Guadeloupe and Texas. They determined that all were the same species and identified six haplotypes, one of which was shared between some Florida collections and Texas, whereas other haplotypes were unique to a colony originating from Vietnam, one from south China, and collections made on the Caribbean islands of Puerto Rico and Guadeloupe. DeLeon & Setamou (2010) also analyzed sequences from ITS-1 and CO1 but found no common haplotypes between Texas and Florida, concluding, therefore, that their origins were different. McFarland & Hoy (2001) suggested that populations from Vietnam and Taiwan were different ecotypes based on response to relative humidity. However, the expected relationship was reversed given that the less drought tolerant wasps from Taiwan actually originated in the Punjab (now Pakistan) by way of Reunion Island where they had been established for biological control of *D. citri* (Aubert 1987). Given these origins, wasps from Taiwan would be expected to show better adaptation to low RH compared to the Vietnam population. Founder effects from the two introductions might provide a better explanation for the apparent lack of tolerance to low RH among wasps sourced from Taiwan. Pena-Garrillo et al. (2015) reported that *T. radiata* collected in northeast and west Mexico showed morphological and genetic variations, and molecular analysis revealed the presence of two haplotypes. Haplotype H_2 was found in both regions, but H1 was only found in northeast, suggesting the possibility of gene flow from the USA.

Symbionts

Meyer & Hoy (2008) found molecular evidence of three bacterial symbionts in *T. radiata*: *Caulobacter* sp., *Methylobacterium* sp. and a species of Alcaligenaceae. However, none were found in eggs suggesting no vertical (transovarial) transfer, and thus only transient associations with the host parasitoid. A study of symbionts is included in this dissertation.

Impact of Toxic Chemicals and Biological Control Agents

Hall & Nguyen (2010) reported that the following pesticides were found toxic to adult *T. radiata* when sprayed directly: abamectin, carbaryl, chenopodium oil, chlorpyrifos, fenpropathrin, imidacloprid, fenpyroximate, phosmet, pyridaben and 435 spray oil with 80- 100% mortality within 24 h and 100% within 72 h. Mortality from 80% to 100% within 24 h was observed on leaves with fresh dry residues of abamenctin, carbaryl, chlorpyrifos, fenpropathrin, imidacloprid, fenpropathrin, fenpyroximate, phosmet and 435 spray oil. Cocco & Hoy (2008) reported that, survival rate of *T. radiata* was significantly influenced by imidacloprid (95% mortality) and abamectin (91% mortality) through residual effects.

Ethanolic extracts from *Annona mucosa* Jacq. (Magnoliales: Annonaceae) seeds, which has the acetogenin rolliniastatin-1 as its major compound, caused high mortality in *D. citri* 3rd instar nymphs and adults (LC_{50}= 429.43, and 5359.00 mg/L for nymphs and adults in 24 h, respectively), and it also significantly influenced *T. radiata* adult emergence rate when applied at LC_{90}= 4463.00 mg/L to hosts parasitized by *T. radiata* in the larval stage (Ribeiro et al. 2015). Santos et al. (2015) reported that azadirachtin-based biopesticide showed high efficacy (around 90%) in controlling

nymphs, which was similar to the achieved results of imidacloprid as positive control; however, it caused 77.8% *T. radiata* adult mortality after a 24 h exposure period.

Entomopathogenic fungus *Isaria fumosorosea* Wize (Hypocreales: Cordycipitaceae) applied on *D. citri* also induced disruptive effects on *T. radiata* (Chow et al. 2016). Studies showed that topical application of a blastospore formulation of *I. fumosorosea* onto the dorsal surfaces of ACP nymphs parasitized with 2nd-instar *T. radiata* larva reduced host mummification by 50% and parasitoid emergence by 85%. Parasitoid emergence rate was reduced by 80% following application of the blastospore formulation to hosts containing a 4th instar wasp larva (Chow et al. 2016).

The use of acaricides targeting pest mites can also alter the efficacy of *T. radiata* (Lira et al. 2015). Results showed that acrinathrin, bifenthrin, carbosulfan, and fenpropathrin caused high acute toxicity (mortality > 77%) to *T. radiata*. Abamectin, diflubenzuron, etoxazole, fenbutatin oxide, fenpyroximate, flufenoxuron, hexythiazox, propargite, spirodiclofen, and sulfur caused low acute toxicity and affected the parasitism rate and emergence rate of adults, and were considered slightly harmful to *T. radiata*. Dicofol and pyridaben did not affect the survival and action of *T. radiata*, and were considered harmless (Lira et al. 2015).

Psyllaphycus diaphorinae (Hymenoptera: Encyrtidae) emerged from *D. citri* nymphs was collected and introduced to University of California, riverside under quarantine. Studies showed that *P. diaphorinae* was an obligate hyperparasitoid attacking *T. radiata* and *Diaphorencyrtus aligarhensis* (Shafee, Alam, and Agarwal) (Hymenoptera: Encyrtidae) pupae, with *D. aligarhensis* being a more favorable host (Bistline et al. 2016).

Table 2-1. Demographic parameters of *Tamarixia radiata* estimated by Chien et al. (1993) and Gomez-Torres et al. (2012).

Parameter* at various temperatures, °C	Chien et al. (1993)	Gomez-Torres et al. (2012)
Generation time, $T_{15\ °C}$	39.9 days	20.3 days
Generation time, $T_{20\ °C}$	22.8 days	18.8 days
Generation time, $T_{25\ °C}$	16.1 days	15.5 days
Generation time, $T_{30\ °C}$	12.3 days	11.8 days
Generation time, $T_{35\ °C}$	NA	10.4 days
Net reproductive rate, $R_{15\ °C}$	2	9.9
Net reproductive rate, $R_{20\ °C}$	6	23.6
Net reproductive rate, $R_{25°C}$	140	126.8
Net reproductive rate, $R_{30\ °C}$	90	58.6
Net reproductive rate, $R_{35\ °C}$	NA	21.3
Intrinsic rate of increase per day, $r_{15\ °C}$	0.0011 progeny per day	0.18 progeny per day
Intrinsic rate of increase per day, $r_{20\ °C}$	0.0081 progeny per day	0.25 progeny per day
Intrinsic rate of increase per day, $r_{25\ °C}$	0.31 progeny per day	0.37 progeny per day
Intrinsic rate of increase per day, $r_{30\ °C}$	0.37 progeny per day	0.34 progeny per day
Intrinsic rate of increase per day, $r_{35\ °C}$	NA	0.25 progeny per day

*T = generation time in days, R_0 = net reproductive rate as larvae per female, and r = intrinsic rate of increase per day, i.e., the daily rate at which the population increased in size in the absence of density-dependent forces regulating the population.

CHAPTER 3
FUNCTIONAL RESPONSE OF *TAMARIXIA RADIATA* AT DIFFERENT HOST DENSITIES AND BEHAVIOR VALIDATION

Tamarixia radiata, is an arrhenotokous ectoparasitoid of *Diaphorina citri*, vector of citrus greening disease or huanglongbing (HLB). HLB was discovered in Florida in August 2005 (Halbert & Manjunath 2004), and by December 2007, it had spread through 30 counties located south of Marion County. HLB is one of the most destructive diseases of citrus in the world (Bove 2006, Halbert & Manjunath 2004). HLB is estimated to have cost Florida's economy $4.4 billion and resulted in 8,257 jobs lost from 2006 through 2011 (Hodges & Spreen 2012). *T. radiata* has been credited with controlling *D. citri* in Reunion Island (Aubert & Quilici 1983) and providing good levels of suppression in India (Husain & Nath 1927), Puerto Rico (Pluke et al. 2008), and Guadeloupe (Etienne et al. 2001), even though it is not clear how much *D. citri* levels were reduced in these locations (Hall et al. 2013). *T. radiata* was first imported to Florida from Taiwan and Vietnam in 1998 and released in 1999-2001 (Hoy et al. 2001). It has become established in citrus orchards throughout the state according to a survey in 2006-7 (Qureshi et al. 2009, Qureshi & Stansly 2009). However, success of *T. radiata* as an augmentative biocontrol agent in the field has not been reported.

The success of parasitoids as biological control agents is thought to be related to their response to increasing host density, known as functional response (Huffaker et al. 1971, Hassell 2000, Fernandez-Arhex & Corley 2003, Jones et al. 2003). Functional response is important to the process of predation and parasitism (Hassell 2000), and considered critical for selecting biocontrol candidates by testing their capacity to search for hosts at low host densities (Burnett 1958, Takahashi 1968, van Lenteren & Bakker 1978, Walde & Murdoch 1988, vanAlebeek et al. 1996).

Functional response of predators to prey was first described by Holling (1959b), who characterized three types based on a linear (type I), decelerating (type II) or sigmoid (type III) response between increasing host (prey) density and number consumed. Both type II and type III functional responses reach an upper asymptotic limit. Holling (1965) considered type II functional response to be typical for invertebrate predators and parasitoids, and type III for vertebrate predators which tend to concentrate their efforts on abundant prey. Hassell et al. (1977) challenged this generalization by showing that type III functional response may be found more commonly in invertebrate predators and insect parasitoids than previously thought. This result had implications for biological control because type III functional responses, in contrast to Type II, are considered stabilizing due to increasing searching efficiency with increased host density.

Holling (1959a) derived the so-called disc equation to describe the Type II functional response:

$$\mathrm{Enc} = \frac{\acute{a}\,\mathrm{N}\,\mathrm{T_t}}{1+ a'\mathrm{N}\,T_h} \tag{3-1}$$

where Enc= number of encounters; N = prey density; T_t = total experimental time available; T_h= handing time per prey and “á” was termed the “instantaneous rate of discovery” also known as the “attack coefficient” (Rogers 1972). For type II functional response, á and T_h are constant (Holling 1959a). Rogers (1972) defined a’ for parasitoids as rate of search or the area covered per unit of the time, and is therefore limited by the available searching area. He suggested a refinement of the disk equation by assuming a random search:

$$\mathrm{N_p} = \mathrm{N}\left(1 - \exp\left(-\frac{\acute{a}\,\mathrm{T_t}}{1+\acute{a}\,T_h\mathrm{N}}\right)\right) \tag{3-2}$$

where N_p= the number of hosts parasitized. This model assumes that, unlike the predator- prey model, parasitized hosts are not removed and therefore hosts remain to be subsequently re-encountered.

Equations for type III sigmoid functional response were proposed by Hassell et al. (1977) and Hassell (1978). They observed that, for type III functional response, the attack coefficient (a') increases with increasing host densities.

$$\mathrm{a} = \frac{b\mathrm{N}}{1+c\mathrm{N}} \qquad \text{(Hassell 1978 equation 3.9) (3-3)}$$

where b and c are constants. In this case, the equation of type III functional response for parasitoid is:

$$\mathrm{N}_a = \mathrm{N}\left(1 - \exp\left(-\frac{b\mathrm{TN}}{1+c\mathrm{N}+bT_h\mathrm{N}^2}\right)\right) \text{(Hassell 1978 equation 3.11) (3-4)}$$

It has been reported that the fecundity of *T. radiata* is highly correlated with host density (Chien et al. 1991a, 1995). Chien et al. (1995) stated that the relationship between both daily and lifetime fecundity and host density showed similar parabolic responses, whereby female fecundity ascends with host density to a peak of 40 hosts/day, then decreases as host density increases up to 80 hosts/ female wasp. However, these experiments were conducted at 100% RH which could increase honeydew deposits that might interfere with search at high host densities. Furthermore, the late 5th instar nymphs used may not have been suitable hosts, especially if they are about to emerge as adults.

Chen (2013) also found fecundity of *T. radiata* to increase in response to densities of 10 to 40, 4th instar nymphs on shoots of *M. paniculata* in a growth chamber at 24±4 C°, RH 60-80%, with no increase in fecundity at greater densities. Data fitted to the Holling's disk equation gave estimates of á (attack coefficient) =0.0184±0.0015 h^{-1}

and T_h (handling time) = 0.045±0.008 hour per host. Sule et al. (2013) also evaluated functional response of *T. radiata* under similar conditions and also saw a leveling off of fecundity at 4^{th} instar densities above 40 per day, but reported a quite different searching efficiency of 39.99 h^{-1} as calculated using the disk equation. Given the importance that these results are purported to have in predicting success of biological control (Barlow and Goldson 1993), we repeated these studies under favorable conditions of relative humidity and host availability, included lower host densities than Chen (2013) and compared results using both Holling's (1959) Equation 3-1 and Rogers's (1972) Equation 3-4 to make the calculations. An additional term, adjusted searching efficiency (ASE) is proposed to express attack coefficient as a function of searching area and thus serve as a useful term to reconcile disparities in results among different studies.

Estimates for handling time based on previous studies seemed to be overestimates in comparison to casual observations of behavior. A possible explanation is that other time-consuming activities were not included in the calculation (Chong & Oetting 2007). Both Holling (1959 b) and Rogers (1972) stated that functional response equations may explain the data mathematically, but not necessarily the actual behaviors. Reconciling functional response with behavioral observations is a goal yet to be realized (Fernandez-Arhex & Corley 2003). I aimed to record and quantify the activities of *T. radiata* females ovipositing with a view toward reconciling actual behaviors with functional response parameters as previously described. Observations of *T. radiata* were made continuously at six different host densities for 30 min and at three different host densities for 10 sec intervals from 12 h recordings. In both scenarios, a

"searching coefficient" (á) was calculated as the area searched per unit time, and defined as

$$á = \frac{Enc}{T_s * N} \quad (3\text{-}5)$$

where Enc is the number of hosts encountered (whether parasitized or not), T_s = searching time, and N = host density (Rogers 1972). Searching time was further defined as

$$T_s = T - Enc * T_h \quad (3\text{-}6)$$

where T = the total exposure time, and T_h = the handling time which in behavioral terms was considered to include probing, ovipositing and/or host feeding.

Validation with behavioral observations should be useful to better understand the biological meaning of functional response parameters, provide practical comparisons among biological control agents, and help optimize the rearing conditions in the laboratory as well as releasing conditions in the field.

Materials and Methods

Colonies

Rearing methods generally followed those of Skelley & Hoy (2004). *Murraya paniculata* was grown from seed and transplanted into 3.92 liter Air-Pots™ (TerraHydro, Carmichael, CA) filled with 40% Canadian sphagnum peat plus bark, vermiculite, perlite, dolomitic limestone, and a wetting agent (Fafard 4P Professional Growing Mix, Sun Gro Horticulture, Agawam, MA.). Plants were grown in an unheated naturally ventilated hoop style trussed greenhouse covered with insect screen mesh plus an additional polyethylene roof and raised sides used during cool weather. Each selected plant had at least eight shoots of new growth, with each new shoot about 3 cm in length with

terminal bud not fully open. Plants were sprayed as needed four or more days before use with 1% M-pede soap (Dow AgroSciences LLC) to control unwanted psyllids and other pests.

Diaphorina citri were reared in BugDorm-2400 Insect Rearing Tents (75x75x115 cm, MegaView Science Co. Ltd., Taichung, Taiwan) inside an air-conditioned glass greenhouse maintained at 22-32 °C, 50-80% RH monitored with a HOBO® RH/ Temp/ Light/ External Data Logger- H08-004-02 (Onset Computer Corp. Onset Computer Corporation, Bourne, MA). Nine flushing *M. paniculata* plants were placed into each cage and approximately 1500 *D. citri* were released and held for 72 h to oviposit. Plants were then moved to a similar clean cage for about 10 days until eggs hatched and nymphs developed to the 4th instar.

Six *M. paniculata* plants infested with 4th nymphal instar psyllids were transferred to a wooden frame cage with polyacrylic sides inside a 4 x 4 m air-conditioned polycarbonate greenhouse maintained at 26±1 °C, RH 55-85%. One hundred twenty females and 60 male *T. radiata* were released inside for 7 days and progeny were collected from day eight until no more were found (around day 22).

Experiment 1. Functional Response Studies

Arena setups. Experimental arenas were prepared from conical polypropylene 50 ml centrifuge tubes (28 x 115 mm, Fisher Scientific, Pittsburg PA), into which were placed a single 7 cm young shoot of *M. paniculata* infested with 1, 3, 6, 10, 20, 30, 40, 50, or 60, 4th instar psyllid nymphs. Healthy young shoots with an excess of nymphs were chosen and a small paintbrush was used to remove extra nymphs to achieve the

desired densities. This method was preferred to adding nymphs which then tended to crawl off the shoots.

Six days after *T. radiata* were released into the *D. citri* colony, small (2 cm) portions of shoots containing one parasitized nymph with the wasp close to pupal stage were excised and placed individually into a glass tube 75 mm long × 12 mm diam (Fisher Scientific, Hampton, NH). Tubes were checked frequently, and wasps removed immediately upon emergence (Chen & Stansly 2014) to a petri dish with 4th instar *D. citri* nymphs, where they were kept for 72 h with host nymphs changed every 24 h.

One randomly-chosen 3-d old parasitoid pair was released into each centrifuge tube sealed with Parafilm® (Bemis NA, Neenah, WI) ventilated by small holes made with a No.1 insect pin. The total searching area included the interior surface of the centrifuge tube (103 cm^2) plus the two sides of the leaves, giving a total of approximately 147cm^2. Arenas were placed in a Percival® model I-36LL-1 incubator with two fluorescent lights on each of two shelves and set for 25± 1 °C, L: D=14:10. RH ranged from 55-85%. Shoots were replaced every 24 h for 5 d. Exposed nymphs were then inverted under a stereoscopic microscope to check for parasitoid eggs. Six replications were completed for each host density.

Scotophase activity. Single young shoots of *M. paniculata* infested with 10, 4th instar *D. citri* nymphs were placed in 50 ml centrifuge tubes as above. One randomly-chosen 3-d-old *T. radiata* pair was released into each centrifuge tube and maintained in incubators as above from 8:00 p.m. to 6:00 a.m. at 25± 1 °C, RH 55-85% without light. *T. radiata* pairs in the control groups were held under the same conditions except in

light for the same time period. Nymphs were then checked for parasitization. There were 10 replications.

Experiment 2. Behavior Observations

Behavior studies based on 30 min direct observation. A young *M. paniculata* shoot with five leaflets was attached to a sterile polystyrene petri dish (Fisher Scientific, product#: 0875712, Pittsburgh, PA) of diameter 14 cm (area 154 cm^2) using double sided Scotch cellophane tape (Scotch®, product #:MMS000039327)**.** Six host densities were prepared consisting of 10, 20, 30, 40, 50, and 60 4^{th} instar *D. citri* nymphs. Nymphs were evenly divided among the five leaflets, and randomly distributed on each leaflet. A 3-d-old *T. radiata* female was released into this arena for 10 min to adjust to the environment. The wasp was then tracked using a stereoscopic microscope for 30 min and all behaviors recorded using Observer XT 11.0 (2013) behavior coding and analysis software (Noldus, Wageningen, The Netherlands). Behaviors recorded included antennation, grooming, resting, host searching, probing, oviposition (verified later by carefully inverting the host with a probe), and host feeding. Duration and frequency of each behavior were recorded. There were 10 replications at each host density.

Behavior studies based on 12 h recordings. The observation arena was prepared in a similar way as described above. A young *M. paniculata* shoot with three leaflets was attached to a sterile polystyrene petri dish (Fisher Scientific, product#: 0875713A, Pittsburgh, PA) of diameter 5.3 cm (area 67 cm^2) using double sided Scotch cellophane tape (Scotch®, product #:MMS000039327). The different size Petri dish was required to accommodate the focal range of the camera. Three host densities using 4^{th}

instar *D. citri* nymphs were prepared: 10, 30, and 60, with the 4th instar nymphs evenly divided among the three leaflets, and randomly distributed on each leaflet. A 3-d-old *T. radiata* female was released into this arena for 10 min to adjust to the environment. Then she was tracked and recorded for 12 h using Dino-Lite Edge Digital Microscope attached to the computer (The Microscope Store, LLC, Roanoke, VA). There were six replicates at each host density. Recordings were checked by fast forwarding every 10 s, and the number of hosts encountered and parasitized, searching duration, non-searching (grooming and resting) duration, and handling (probing and parasitizing) duration were noted. Additionally, the first 30 min of each recording were monitored completely and tracked with Observer software to compare with results from the 30-min study done in the larger (154 cm^2) arena.

Statistical Analysis

Experiment 1. Functional Response Studies

Functional Response. A two-factor factorial analysis was used to determine the significant effects of age and density. Fecundity and superparasitism as proportions were subjected to ANOVA with mean separation determined by Fisher's LSD ($P < 0.05$). Values were first subjected to a square root transformation to satisfy the equal-variance assumption of ANOVA (JMP software, SAS Institute Inc. 2013).

Model selection. We considered the parasitization outcome to be binary, and so the number of hosts parasitized should follow a binomial distribution with the probability of being parasitized given by the corresponding expressions in the appropriate functional response equations. To choose among these, we fitted our data using maximum likelihood to expressions (2) and (4). The best fitted model was chosen as the

one with smallest Bayesian information criterion (BIC) based on the likelihood function (Schwarz 1978). Attack coefficient and handling time were estimated using the appropriate model equation. Attack coefficient is defined as a function of the area a parasitoid covers in a unit of time (Rogers 1972), therefore, we proposed using the term searching efficiency (ASE) calculated as: ASE= attack coefficient * searching area available (e.g.,147cm^2 in experiment 1). Data were also fitted to Equation 3-1 in order to compare estimates of the unknown parameters with those of Sule et al. (2013).

Experiment 2. Behavioral Observations

Behavior analysis. The number of hosts parasitized (N_p), parasitization duration per host (T_p), searching duration (T_s), other behavior durations, including time interval between two ovipositions (OI) among host densities were compared using ANOVA with means separated by Fisher's LSD ($P< 0.05$).

Frequencies of all behaviors at each host density were visualized using behavior diagrams (Rohrig 2010). Frequency of each behavior at the six host density levels was tested using regression analysis. Fractions of all behaviors among six host densities were compared using multinomial Chi-squared test ($P<0.05$). Probing rejection rates leading to activities other than oviposition, were compared using Kruskal-Wallis test due to non-normal distribution. The relationship between searching duration with exposure time was compared using linear regression analysis assuming a positive correlation. Data were analyzed using JMP software (SAS Institute Inc. 2013).

Calculation of parameters based on behavioral observations. Encounter rates at six host densities were compared using ANOVA with means separation by Fisher's LSD ($P<0.05$). Searching coefficient (á) was calculated using Equation 3-5 with T_s estimated two ways: (1) by substituting 30 min for T, and observed values for Enc

(2.18 eggs) and T_h (3.65 min) in Equation 6, and (2) based on the mean observed searching time T_s. The searching coefficient was modified based on a searching area of 154 cm^2 and 67 cm^2 for 30 min observation and 12 h recording, respectively.

Functional response models provide a theoretical alternative for the calculation of the searching coefficient. Results of the experiment 1 indicated that a type II random parasitoid equation is appropriate:

$$N_p = N(1 - \exp\left(-\frac{\tilde{a}\, T_t}{1+\tilde{a}T_h N}\right)) \quad (3\text{-}7)$$

Where ã denotes the theoretical searching coefficient. Based on the two behavior scenarios, we obtained the value T_h = 0.065 h. N_p and N were those obtained based on experiment 1. The best-fit value for the searching coefficient over all host densities ($\tilde{a}_1$) was obtained through nonlinear least squares (NLS) analysis. We also calculated searching coefficients separately for each host density ($\tilde{a}_2$). These are compared with values from behavioral observations. Quality of fit was assessed both visually as well as by comparing the residual sum of squares (RSS).

Computations for all analyses were carried out in JMP (SAS Institute Inc. 2012) and R software (R Development Core Team 2011).

Results

Functional Response

Fecundity did not vary significantly within host densities over the 5-d study period in experiment 1 (F = 1.5118, df = 4, P = 0.1991). In contrast, the effect of density on fecundity was highly significant (F = 150.6340, df = 8, P < 0.0001) as it was for average fecundity among densities over all days (F = 330.51; $df_{(model, error)}$= 8, 45; P < 0.0001). The effect of density on fecundity was still significant even after the low densities (1, 3,

and 6) were eliminated (F = 75.3679; df = 5, 30; P < 0.0001). Fecundity increased with host density from 1 to 40 hosts to a maximum of 11 eggs per day with no significant differences among host densities of 40, 50, and 60 (Table 3-1, Fig. 3-1).

Percent parasitism was highest at 73.3% at the lowest (1 host) density and least at 18.6% at the highest (60 hosts) density, again with no differences among the top 3 densities (F = 87.5; df = 8, 45; P < 0.0001, Table 3-1, Fig. 3-2A). Percent superparasitism (the number of superparasitized hosts over parasitized hosts) varied significantly among densities (F = 4.68; df = 8, 45; P < 0.0001) from 16.2% at 6 hosts per female to one or less at 30 hosts per female or greater with no differences among the highest four densities (Table 3-1, Fig. 3-2B).

Parasitization During Scotophase

No oviposition during scotophase was observed at any of the 10 replications demonstrating that parasitization does not take place in darkness. The mean (± SE) oviposition in the control group was 3.4 ± 0.03. Consequently, the 10 hours' dark period was eliminated when fitting the functional response; that is, the total experimental period (T) relevant for functional response analysis was assumed to be 14 h.

Model Selection

The calculated BIC values for the Rogers (1972) type II Equation 3-2 and Hassell (1978) type III (4) were -978.1 and -972.5, respectively, so the type II equation was chosen as the best fit model. The fitted parameters using type II functional response Equation 3-2 were a'=0.0864 h^{-1} (SE=0.0082), and T_h = 0.873 h per host (SE=0.044). Given a total searching area of 147 cm^2, ASE was estimated at 12.7± 1.21 cm^2/h (0.21± 0.02 cm^2/min).

Estimates obtained by fitting our data to Holling's disc Equation 3-1, which unlike Rogers's (1972) equation, takes unit parameters into consideration, were a'= 7.45 cm^2/h (SE= 0.53), and T_h= 0.85 h per host (SE= 0.04) per host.

Host Density Effects on Behavior Frequency Based on 30 Min Observation

Behaviors at six different densities showed similar patterns (S1-5, Appendix A). As an example, wasps presented with 40 hosts executed 756 behavioral events observed over the 30-min observation period (Fig. 3-3). Of the 264 host-searching events, 211 (80%) led to antennating of a nymph. Ninety nine (33.6%) out of the 295 antennations led to host probing, five to host feeding, and two to honeydew feeding; the rest (64.1%) resulted in rejection and searching again for hosts. Twenty-one out of the 99 (21.2%) probing events led to successful oviposition and 78.8% led to rejection, followed either by host searching or antennating. Sixty four grooming events were observed of which 70.3% led to host searching. The remaining behavior diagrams at each host density are available as supplementary Figs. S1-S5 (Appendix A).

Searching frequency increased from 19.7±1.2 (Mean ± SEM) at density 10 to 36.2±4.3 at density 60, with significant differences among host densities ($F = 10.05$, $df = 1, 58$, $P = 0.0024$). Antennation frequency also varied significantly among densities ($F = 9.31$, $df = 1, 58$, $P= 0.034$), increasing from 23.6±2.2 to 40.9±5.5 over densities of 10 to 60. In contrast, frequency of probing (mean 10.7±0.76), grooming (mean 5.88±0.37), resting (0.5±0.13), host feeding (0.38±0.08) honeydew feeding (0.2±0.07) and oviposition (1.48±0.2) showed no significant effect of host density (S6, Appendix A).

The multinomial Chi-square test showed that fractions of frequencies of individual behaviors over the frequency of all behaviors observed were not independent of host density ($X^2 = 69.43$, $df = 36$, $P =0.0007$). The largest deviations from expected fractions

under the null hypothesis of independence were seen in the probing fraction ($X^2_{probing}$=28.1, 40.5% of the total X^2 statistic), which showed an overall decreasing trend from 0.21 to 0.12 as host density increased from 10 to 60. This decrease was offset by modest increases in the observed fractions of searching events (0.31 at density 10, 0.37 at density 60) and antennating events (0.37 at density 10, 0.42 at density 60). Fractions of other behaviors remained relatively constant.

Host Density Effect on Behavior Durations

No significant host density effect was observed on N_p (number of hosts parasitized) and T_p (time spent probing and parasitizing) during the 30 min observation time. Mean T_p was estimated at 3.65±0.24 min (0.06±0.004 h) ($F = 0.52$, $df = 5, 42$, $P = 0.76$), and mean N_p at 2.18±0.15 parasitized hosts ($F = 0.55$, $df = 5, 54$, $P = 0.74$).

The time interval between ovipositions (OI) ranged from 3.68±0.72 to 5.57±0.90 min, with mean estimate 4.85 ±0.41 min and no significant effect of host density ($F = 0.49$, $df = 5, 58$, $P = 0.78$). Searching duration (including antennation) and resting duration were not significantly influenced by host densities (searching: $F = 1.28$, $df = 5, 54$, $P = 0.28$; resting: $F = 1.7273$, $df = 5, 54$, $P = 0.14$), and the mean durations (min) were 10.71±0.52, and 10.54±4.71, for searching and resting respectively. Host density did affect grooming duration ($F = 18.31$; $df = 5, 54$; $P < 0.0001$), with the mean increasing from 3.75±0.93 to 15.67±1.31 min as host density increase from 10 to 60. There was no significant host density effect on probing rejection rate (57.0±0.04 %, $X^2 = 1.85$, $df = 5$, $P = 0.87$).

Behavior Based on 12 H Recordings

Number of hosts parasitized (N_p) increased from 5.3±0.42, 6.67±0.76 to 7.17±0.60 at increasing host densities of 10, 30, to 50 respectively but differences were

not significant (F = 2.41; df = 2, 15; P = 0.12). T_p averaged 0.07 h over 12 h (F = 0.35, df =2, 15; P = 0.71) and OI varied from 1.9±0.23, to 1.64±0.17, to 1.66± 0.13 h, from 10, 30, and 50 hosts respectively (mean 1.75±0.1, χ^2 = 2.37, df =2, P = 0.31).

Mean percentage of time spent searching (T_s) varied significantly with ranges of 21.7 to 43.5%, with 10 hosts, 11 to 22.8% with 30 hosts and 36.4 to 89.4% at 50 hosts (F = 3.83, df = 1, 70, P = 0.05; F = 15.62, df = 1, 70, P = 0.0002; F = 6.54, df= 1, 70, P = 0.02 respectively). Probing rejection rate increased significantly with increasing host density (F = 9.55, df = 1, 16, P= 0.008), from 60.2±7.1%, to 70.0±4.2% to 80.5±3.4% at densities of 10, 30, and 50 respectively.

The mean number of hosts parasitized (N_p) during the first 30 min in the 67 cm^2 area averaged 1.4±0.24, with no significant differences among host densities (F = 3.66, df = 2, 15, P = 0.06). This result was at variance with an N_p of 2.18±0.15 reported above for the 30 min study in the larger arena. Mean rejection rate was 67.3± 6.2% for the first 30 min of the 12 h study, likewise with no host density effect (χ^2 = 1.28, df = 2, P = 0.53). This result was roughly consistent with a rejection rate of 57.0±0.04 % for the 30 min study.

Parameters Based on Behavioral Studies

When calculations were based on searching time as the difference between exposure time (30 min) and total handling time Equation 3-6, the searching coefficient ($á_1$) showed a decreasing pattern ranging from 122.7 cm^2/h at 10 hosts to 21.2 cm^2/h at 60 hosts based on Equation 3-5 (Table 1). However, a larger searching coefficient ($á_2$) resulted if observed values for searching time (T_s) were used which did not include resting and grooming. A decreasing trend was again observed, but from 202.7 to 53.4 cm^2/h over the same host density range (Table 3-4).

Searching coefficients were similarly calculated in these two ways for the 12 h experiment (yielding á$_3$ and á$_4$), and for the first 30 min of observation time in the 12 h experiment (yielding á$_5$ and á$_6$), respectively (Table 3-5). These values also showed decreasing patterns with increasing host densities. However, searching coefficients were considerably less than those obtained in the larger arena (154 cm^2), especially for the full 12 h observation period (Table 3-5).

Best Fit Type II Functional Response Equation

The best-fit searching coefficient using Equation 3-7 across all host densities obtained through NLS, with T_h fixed at 0.065h was ã$_1$ = 6.32 cm^2/h (SE=0.32). However, this functional response model provided a poor fit to the original data (Fig. 3-4, dotted line). The residual sum of squares (RSS) was 17.6, and the lack of fit was evident from the plot. In contrast, if we allow searching coefficient to vary for each host density, again with T_h= 0.065 h fixed, the result is a deceasing pattern with the increasing host density (Table 3-6, ã$_2$). The RSS in this case was 9.8.

Holling (1959b) suggested that the disk equation could also be used if searching coefficient decreased with increasing host density by hypothesizing two constants b and c with the following relationship to ã:

$$\tilde{a} = \frac{b}{c+N} \qquad (3\text{-}8)$$

yielding estimates b= 1.23± 0.07, and c= 14.22± 1.99, based on NLS. This leads to a new functional response model Equation 3-5) by replacing ã in type II random parasitoid Equation 3-7 with Equation 3-8:

$$N_p = N[1 - \exp\left(-\frac{b\,T_t}{c+N+b\,T_h N}\right)] \qquad (3\text{-}9)$$

Substituting b= 1.23, and c= 14.22, this model gave a better fit to the original dataset (Fig. 3-4, dash line) that effectively captured the decreasing pattern in $\tilde{a}_2$, and resulted in a RSS of 10.00, which was very close to 9.82 reported above.

Discussion

Chien et al. (1995) reported peak fecundity of about 25 eggs/female/day at a host density of 40 but a decrease at higher host densities up to 80. Sule et al. (2014) reported a maximum of 21.2 nymphs (4^{th} instar) parasitized per female during a single 24h exposure to 80 nymphs beyond which incidence of parasitism leveled off. We found number of hosts parasitized, percent parasitism and percent superparasitism were all significantly influenced by host density over a 3-8 day old range. The number parasitized increased to a maximum of 11.2/day from the lowest density through 40 hosts per female, then remained relatively constant through 60 hosts per wasp, indicating an upper limit had been reached. Percent parasitism was highest (73%) at the lowest host density and declined thereafter. Host-feeding may have been the reason that a 100% percent parasitism was not reached even at low host densities. Chien et al. (1995) and Sule et al. (2014) used larger arena areas than we did to test host density effects which might explain the higher fecundities they observed.

Considerable levels of superparasitism were observed at host densities ranging from 1 to 20, but became negligible at densities above 30. Chien et al. (1991b) reported that *T. radiata* females were able to discriminate between parasitized and non-parasitized hosts, although superparasitism still occurred when nonparasitized hosts were available. Superparasitism is wasteful because only one egg can mature to the adult stage. Any others will be killed by the first hatched larva (Chien et al. 1991a, Harvey et al. 2013). We found percent parasitism to be greater at a host:parasitoid ratio

1:30 than at 1:40 whereas superparasitism was not significantly different at the two host densities. On the other hand, more hosts were parasitized at a 1:40 parasitoid:host ratio. Therefore, while the 1:30 ratio may result in more efficient use of hosts, the 1:40 ratio may result in more efficient use of other resources of time or space for mass rearing.

Experiment 1 suggested a type II functional response by *T. radiata* to host density over five days. However, there are many reasons given why type III responses could be difficult to detect, including exposure to the hosts in a fixed area (Hassell et al. 1977, van Lenteren & Bakker 1978) and fixed periods of search (Huffaker et al. 1971, van Lenteren & Bakker 1978, Collins et al. 1981). Furthermore, an accelerating trend leading to a sigmoid (type III) response could occur at lower densities but be masked over a wide host density range. While the type III functional response is thought to have a stabilizing effect and therefore more likely to lead to population regulation (Hassel et al. 1977), the shape of the functional response curve has yet to be linked to success of biocontrol (Fernandez-Arhex & Corley 2003).

Attack coefficient is considered to define the steepness of the curve approaching the upper fecundity asymptote and estimates the proportion of area searched during the experimental period (Gitonga et al. 2002). In experiment 1, *T. radiata* was able to search 12.7±1.21 cm^2/h based on Rogers's (1972) Equation 3-2 and 7.45±0.53 cm^2/h based on Holling's Equation 3-1. Holling's equation takes units into consideration and generates attack coefficient with units of cm^2/h directly. A comparable attack coefficient (cm^2/h) was obtained from Rogers's (1972) equation by calculating ASE.

Sule et al. (2014) also reported a type II functional response of *T. radiata* when a mature female was introduced for oviposition to densities of 2 to 120, 4th instar *D. citri* nymphs for 24 h under lab conditions. Using Equation 3-1, they reported handling time (T_h) 0.6 h^{-1} for 4th instars which is close to our estimate of 0.85h^{-1}. However, they also reported searching efficiency for 4th instar hosts of 39.99 h^{-1} which is over 400-fold higher than our estimate of 0.86. Using their estimated value of T_h = 0.6 h^{-1}, the calculated values for a' came out much closer to ours than the published value (Table 3-2). Chien et al. (1995) reported an attack coefficient of 0.78/ day (0.03/h) which was similar to our results. The available searching area in their experiment was approximately 229.5 cm^2, resulting in an ASE = 6.885 cm^2/h, which was also very close to our result (Table 3-3).

Handling time is defined as the time needed by a parasitoid to pursue, subdue, oviposit and recover (Holling 1959a). Estimates for handling time also vary among studies. Chien et al. (1995) calculated handling times for *T. radiata* that varied from 26.2 to 13.1 to 4.3 min depending on whether total time was considered as 24 h, 12 h (active time) or 4 h (peak oviposition time) respectively. Sule et al. (2014) calculated a handling time for 4th instar hosts of 0.6 h over 24 h total time which is not far from the 0.87 h estimate from our research calculated on the basis of 14 h total time. All estimates of handling time greatly exceed 61 s estimated by Chien et al. (1995) based on behavioral observations.

My behavioral observations revealed that *T. radiata* females took approximately 3.6-4.2 min to probe and parasitize a host (T_p) with no significant effect of host density seen over 30 min or 12 h. Oviposition interval (OI) averaged 4.85 min during the 30 min

observation period, and 1.75 h over 12 h. The result is not surprising as the wasps could hardly lay an egg every 5 min for 12 h. Nevertheless, the oviposition interval accounted for a similar 16.2% and 14.3% of total exposure times of 30 min and 12 h respectively.

Searching and antennating durations also did not change with host density, in contrast to greater frequency of these activities with more abundant hosts. Thus, more total time was spent searching and antennating at high host density, leaving less time for other activities. A likely candidate of such activity was probing, the frequency of which relative to all other activities decreased from 21% to 12% over the range 10 to 60 hosts, even though absolute frequency did not increase significantly with host density.

Grooming duration increased significantly with increasing host density, probably in response to increased amounts of honeydew. Even so, searching time was not prolonged at greater host densities with hosts at greater proximity.

An average of 5 nymphs were probed over the course of 30 min observations, of which a mean 2.18 were parasitized and the remaining 57% rejected with no effect of host density on rejection rate. This result contrasted with probing rejection rates over 12 h which increased significantly from 60.2% to 70.0% to 80.5% at host densities of 10, 30, to 50 respectively. The resulting maximum fecundity of 7.17 eggs was at variance with 11.4 obtained at a density of 50 per female for a 14 h light cycle in a petri dish with a searching area of 147 cm^2.

Given that all conditions were similar, the lower fecundity in the 12 h experiment could be due to the smaller (67 cm^2) searching area. To further evaluate this hypothesis, we compared the first 30 min of the 12 h recording with the 30 min

observations done in the larger (154 cm^2) arena. As we hypothesized, the probing rejection rate averaged 67.3% with no density effects in small area, which was markedly higher than 57.0% under 30 min observation experiment at larger arena. This finding further confirmed the hypothesis that rejection rates and thus fecundity depend a great deal on searching area.

Another apparent effect of small arena size was the observation that the proportion of time spent searching was significantly greater with 50 hosts (36.4 to 89.4%) than with 10 hosts (21.7 to 43.5%). No such density effect on searching time was seen in the larger arena. Experiment 1 revealed that the rate of parasitization decreases over a prolonged exposure time, presumably as the supply of mature eggs is exhausted. Nevertheless, we would expect about 9.2 more eggs to be laid between the first 30 min (2.18 eggs) and a total of 11.4 from the previous study in the 157 cm^2 arena. Instead, we found only an increase of about 5.8 eggs from an initial 1.4 eggs during the first 30 min to a final 7.17 after 12 h in the 67 cm^2 arena. The negative effect of searching area on fecundity as well as increased searching time and rejection rate in the small arena are all consistent with potential patch effects of a host mark left by the parasitizing *T. radiata* female to deter further oviposition. Such a “patch” mark would exert its strongest effect on surrounding hosts under conditions of high host density, limited searching area, and long exposure to the same hosts. Further work is needed to verify this hypothesis.

These behavioral observations indicate a constant handling time per parasitization of 0.065 h (3.9 min) over two exposure times and searching areas, in concert with Holling’s (1959b) basic assumption of constant T_h. This result is at variance

with a handling time of 0.87 h estimated by Equation 3-4 in experiment 1. Chong & Oetting (2007) suggested that such overestimation was due to neglect of other time-consuming activities. If we consider handling time as all time-consuming activities minus searching, then its mean was approximately 1.37 h based on the 12 h recording, which was the oviposition interval reported. However, this number is still not close to 0.87. The oviposition interval based on 30 min observation was 4.85, which illustrates that the handling time thus calculated is a function of exposure time rather than host density.

Holling (1959b) defined searching time (T_s) as the difference between the entire exposure duration and handling time for all parasitized hosts. However, there are clearly other time-consuming activities besides handling and searching such as grooming and resting. This explains why estimates for searching efficiency based on handling time (T_h) as total time minus searching time ($á_1$, $á_3$ and $á_5$) were notably smaller than their counterparts ($á_2$, $á_4$ and $á_4$, respectively) calculated based on T_h as total time minus searching time plus all other observed behaviors (Tables 3-4 and 3-5). Given that the most realistic estimate of T_h is one that includes all observed behaviors, we conclude that $á_2$, $á_4$ and $á_6$ are the best estimates of searching coefficients under 30 min, 12 h, and the first 30 min in 12 h respectively. Interestingly, the estimates of $á_2$, $á_4$ and $á_6$ all showed a decreasing pattern with increasing host density. We suspected that $á_2$, $á_6$ might not be good estimates of the searching coefficient, because the higher fecundity limit was not reached with the exposure time of 30 min. Therefore, based strictly on 30 minute observations, one may suspect that the decreasing pattern of $á_2$ and $á_4$ was solely due to the increasing host density. However, the decreasing trend of $á_4$ based on 12 h recording confirmed that the searching coefficient did indeed decrease with the

increasing host density. The conclusion is logical since the wasps do not have to search as far at high host densities to find the hosts they need for oviposition. Furthermore, searching time increased with the increasing host density, possibly due to host marking. However, this contradicted the basic assumption of the type II functional response, namely that the theoretical ã is constant at all host densities. To better resolve this question, we calculated ã using our best estimate of T_h= 0.065 in Equation 3-7, which also resulted in a decreasing pattern. If we then fix ã to be constant as 6.32 cm^2 based on NLS for all host densities, the result was a poor fit to the equation (RSS=17.56).

Holling (1959b) suggested that the disk equation could also be used if searching coefficient decreased with increasing host density by hypothesizing two constants b and c with the following relationship to ã, which led to Equation 3-5 .This provided a good fit to the data (RSS=10.00), which was very close to RSS=9.82 when ã varied individually at each host density. Therefore, we conclude that searching coefficient should decrease with the increasing host density, from 57.3 to 8.0 over a host density range of 10 and 50 per 67cm^2 under our experimental conditions.

However, one question remains, if á$_2$ and á$_6$ were both estimates of the searching coefficient based on 30 min, what resulted in their discrepancy? In this case, the different searching area was the only factor that varied. A possible explanation was that *T. radiata* females would spend more time searching the smaller areas as a result of the greater deterring effects of host marking. Since á has a negative relationship with T_s, the estimate of á in small searching areas would be smaller than in large areas.

In conclusion, the searching coefficient á for *T. radiata* should decrease with the increasing host density instead of being constant. The ã based on functional response

equation was underestimated, because other time consuming activities were ignored. The biological meaning of á in the functional response equation should be the area of parasitoid searching and resting per time unit, instead of searching alone. A modified functional response Equation 9 is suggested for future research.

Table 3-1. Mean ± SE number of parasitized hosts, percent parasitism and percent superparasitism at nine different host densities from 1 to 60, 4th instar nymphs per arena per day.

Host Density	Parasitized Hosts	Percent parasitism (%)	Percent superparasitism (%)
1	0.73 ± 0.08 G	73.33 ± 8.21 G	10.00 ±5.57 A
3	1.97 ± 0.13 F	65.56 ± 4.37 F	14.44 ±4.56 A
6	3.43 ± 0.19 E	57.22 ± 3.26 E	16.22 ±4.27 A
10	4.63 ± 0.25 D	46.33 ± 2.56 D	13.17 ± 2.52 A
20	6.86 ± 0.40 C	34.41 ± 1.98 C	9.25 ± 2.65 AB
30	9.40 ± 0.50 B	31.33 ± 1.67 B	1.03 ± 0.72 BC
40	11.20 ± 0.46 A	28.00 ± 1.16 A	0.35 ± 0.25 C
50	11.43 ± 0.37 A	22.90 ± 0.75 A	0.43 ± 0.43 C
60	11.13 ± 0.42 A	18.55 ± 0.70 A	0.27 ± 0.27 C

Means in the same column followed by the same letter are not significantly different (Fisher's LSD, $P < 0.05$).

Table 3-2. Attack coefficient (a') of *T. radiata* female attacking 4th instar *D. citri* nymphs at increasing density using T = 24 h , T_h = 0.6 h^{-1} and an estimated search area of 190 cm^2 (Sule et al. 2014)

Density (N)	Hosts parasitized (Enc)	a' (h^{-1})	a' (cm^2/h)
15	13.2	0.055	10.40
20	16.8	0.06	11.47
40	20.2	0.04	8.08
80	21.2	0.023	4.46
100	22.0	0.02	3.87

Table 3-3. Comparisons of handling time (T_h), attack coefficient (a') and ASE among Chien et al. (1995), Sule et al. (2014) and my results

Comparisons	T_h (h)	a' (/h)	ASE (cm^2/h)
Chien et al. (1995)	0.437 (T=24h) 0.218 (T=12h) 0.072 (T=4)	0.03	6.885
Sule et al. (2014)	0.6	0.06 - 0.02[1]	11.47 - 3.87
Equation (1) in experiment 1	0.85	--	7.45
Equation (2) in experiment 1	0.873	0.0864	12.7

[1]Our calculation. Sule et al. (2014) reported [1]=39.99

Table 3-4. Mean (± SE) encounter rates and searching coefficients at six host densities using Equation 5, either with Ts based on Equation 6 (á1) or on real searching time (á2) as observed over 30 min and a searching area of 154 cm^2.

Density	Encounter rate (Hosts/min)	$á_1$ (cm^2/h)[1]	$á_2$ (cm^2/h)[2]
10	0.07±0.01	122.72±26.78	202.73±38.50
20	0.05±0.01	45.27± 10.35	124.54±30.97
30	0.09±0.02	50.88±9.70	210.82±98.75
40	0.07±0.01	37.13±7.53	149.71±47.06
50	0.08±0.01	35.00±6.10	93.05±17.15
60	0.07±0.01	21.16±4.46	53.40±14.79
Mean	0.07±0.01 [NS]		

[1] $á_1$ calculated based on Equation 1 with T_s= 30 min minus total handling time
[2] $á_2$ calculated based on real searching time, where T_s= 30 min minus total handling time minus resting minus grooming time.
[NS] No significant effect of density (ANOVA, $P < 0.05$).

Table 3-5. Mean (± SEM) searching coefficients at three densities as observed in a 67cm^2 arena, calculated using Equation 1 and based on 12 h recordings or the first 30 min of same, respectively. Searching efficiencies á3 and á5 assume total time (Tt) as composed only of handling time (T_h) + observed

searching time (Ts), whereas á4 and á6 assume T_h as equal to Tt minus all observed behaviors (handling, resting and grooming) .

Host density	Calculation based on 12 h (cm^2/h)		Calculation based on first 30 min (cm^2/h)	
	$á_3$*	$á_4$**	$á_5$*	$á_6$**
10	4.25±0.54	23.77±10.25	21.28±7.40	57.26±18.13
30	1.68±0.19	10.93±2.22	5.51±2.44	25.29±8.40
50	1.05±0.12	5.59±1.20	3.96±1.02	7.98±2.28

* $á_3$ and $á_5$ calculated using handling time T_h= T_t minus observed searching time (T_s)
** $á_4$ and $á_6$ calculated using handling time T_h = T_t minus (observed handling + resting + grooming time).

Table 3-6. Searching efficiency (mean ± SE) obtained from type II functional response equation (2) with T_h = 0.065 h, for each host density.

Density	$ã_2$ (cm^2/h)
1	13.82±3.23
3	11.32±1.32
6	9.11±0.88
10	6.62±0.59
20	4.56±0.29
30	4.12±0.29
40	3.53±0.15
50	2.94±0.15
60	2.21±0.15

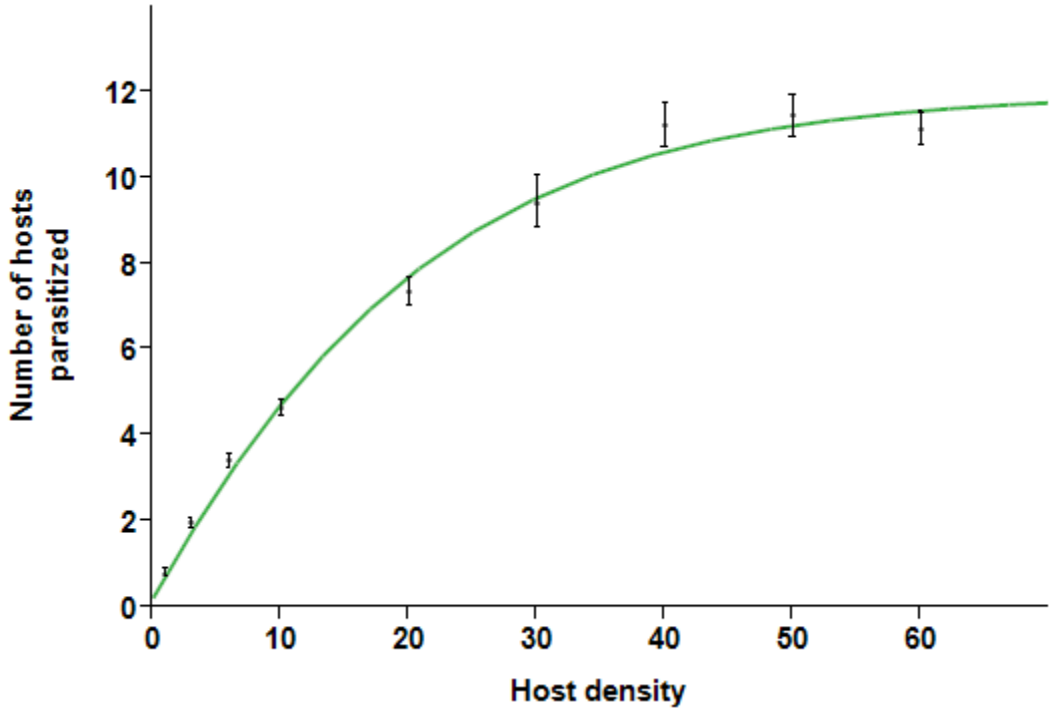


Figure 3-1. Mean (SE error bars) number of parasitized hosts per day by one 3-d-old *T. radiata* female at nine host densities. A smoothed curve based on non-linear least squares has been superimposed.

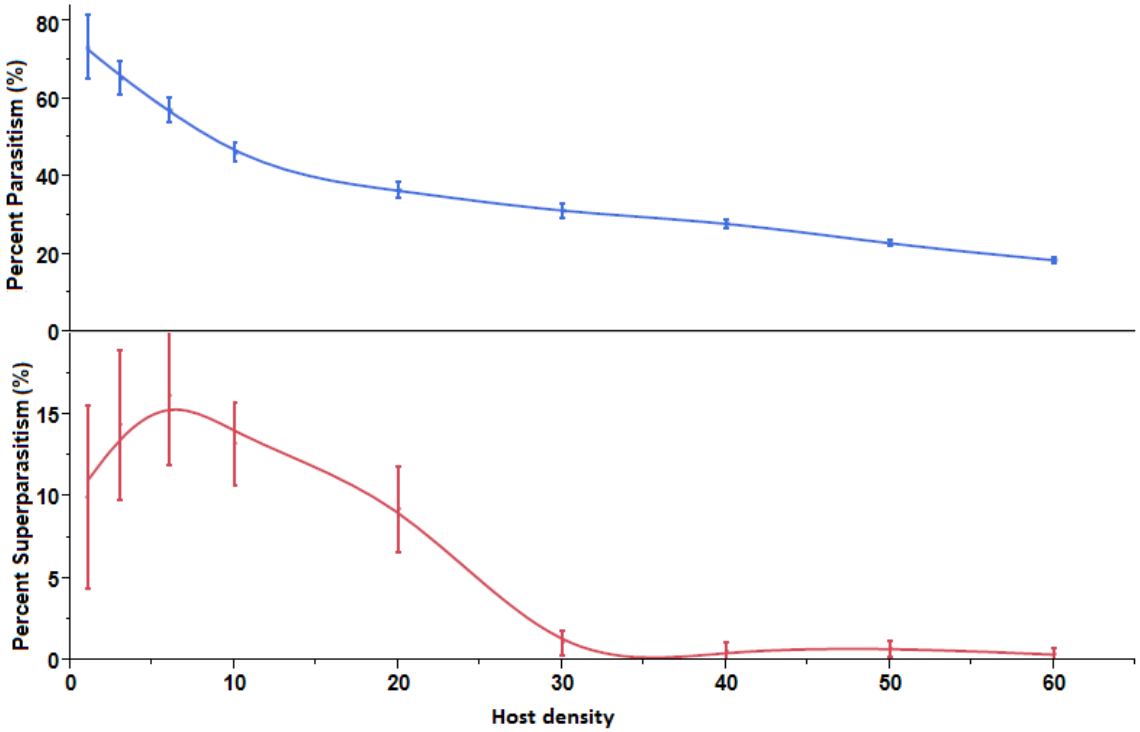


Figure 3-2. A smoothed curve of mean (%) parasitism and superparasitism at nine host densities. Error bars at each point represent the standard error of the mean.

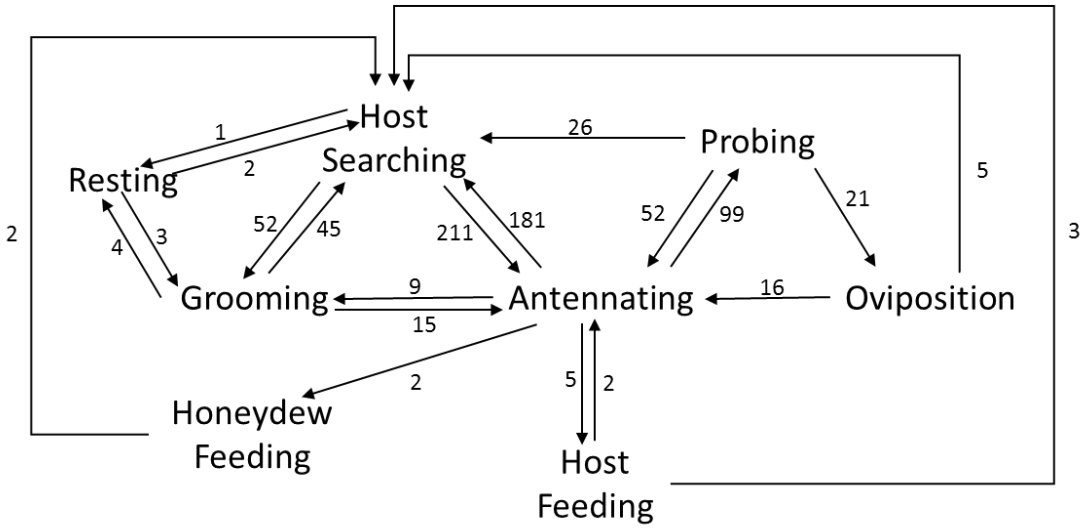


Figure 3-3. Behavior diagram for a 3-d-old mated female *T. radiata* at density 40. Numbers refer to frequency of the behavior over a 30 min observation period (N=10). Arrows indicate preceding or subsequent behaviors.

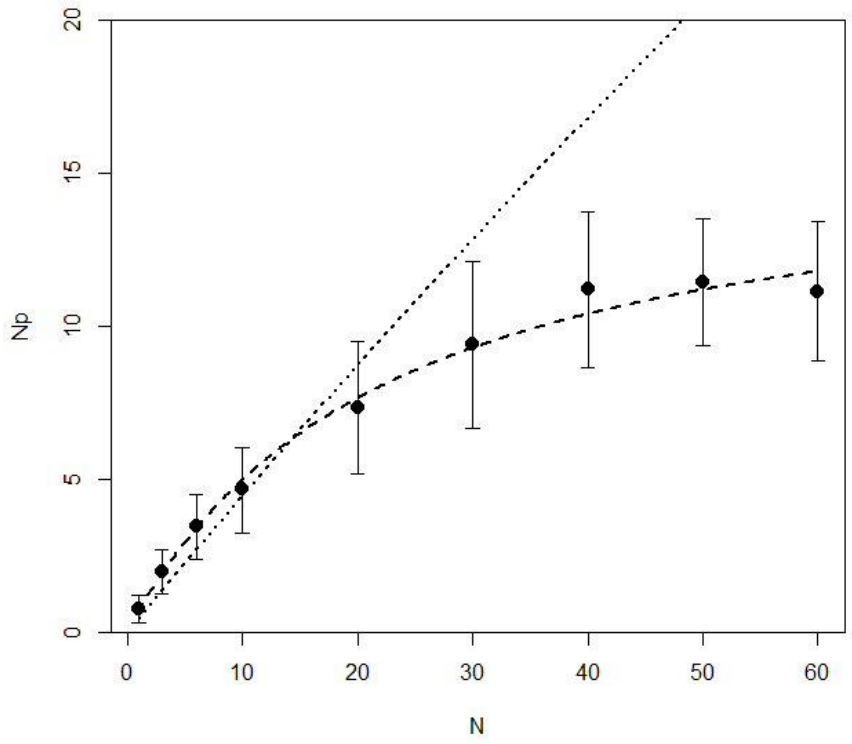


Figure 3-4. Type II functional response curves fitted to the original dataset. N is the host density, and Np is the number of hosts parasitized. Black points

indicated the mean parasitized host numbers, and the error bars indicated 95% confidence intervals. The dotted line used $\tilde{a}_1$= 6.32 cm2/h obtained through NLS, and T_h= 0.065 h from direct observations. Dashed line is Equation 3-9.

CHAPTER 4
HOST MARKING AND HOST DISCRIMINATION BY *TAMARIXIA RADIATA*

Many insects are known to avoid already infested or parasitized insect hosts to reduce the level of competition among offspring, often by responding to chemical signals deposited previously by an ovipositing female (Prokopy 1981, Nufio & Papaj 2001). These signals permit females to distinguish utilized and unutilized hosts by contact chemoreception (Seeley 1989). Such marking pheromones have been found in Coleoptera, Diptera, Hymenoptera, Lepidoptera, and Neuroptera (Prokopy 1981). For hymenopteran parasitoids, 150 to 200 species in nearly every family have demonstrated the ability to discriminate between parasitized and unparasitized insect hosts (van Lenteren 1981).

It has also been reported that hymenopteran females are less likely to self-superparasitize than superparasitize another conspecific (Vandijken et al. 1992; Danyk & Mackauer 1993; Vanbaaren et al. 1994). The rationale would be that, whereas superparasitizing another conspecific might eliminate a potential competitor, superparasitizing herself would only increase competition among siblings (Van Alphen & Visser 1990).

Hymenopteran females may use either, or both, internal or external markers to indicate the utilized hosts (Van Lenteren 1976, Hofsvang 1990). Whether the host marking pheromone is deposited on or in the host depends on the host stages attacked. Egg parasitoids usually mark the hosts externally, whereas parasitoids attacking other stages usually mark the hosts internally. Most marking pheromones are non-volatile and can be detected by contact chemoreceptors (Kouloussis & Katsoyannos 1991) on antennae (Ferguson et al. 1999), mouthparts or tarsi (Prokopy & Spatcher 1977,

Messina et al. 1987) or ovipositor (Van Lenteren 1972, Valencia & Rice 1982). A vertical T-maze choice olfactometer can be used to determine whether the marking pheromone is volatile. The T-maze takes advantage of the negative geotaxis shown by many insects, which can be reinforced by placing a light source directly above the olfactometer to take advantage of positive phototaxis (Stelinski & Tiwari 2013). An external host marking pheromone can be detected by antennae which is easier than using the ovipositor inside the host, however ,the external marker will disappear when the host molts (Bosque & Rabinovich 1979). The expectation is that the marker will last as long as it benefits the parasitoid (Van Alphen & Visser 1990). Differences in female preference may be due to a chemical change on/in the host after infestation (Nufio & Papaj 2001). Eggs themselves may serve as the source of marking pheromone (Ganesalingham 1974).

Some parasitoids mark not only the parasitized host, but also the patch they search (Vanalphen & Visser 1990). Patch marking is a mechanism that helps the marker to avoid wasting search effort on resources that have been exploited already (Gauthier et al. 2002). Patch marking is likely to cost more, since a larger surface will be marked. However, if hosts occur in poorly defined patches, or they can move a long distance, patch marking is not feasible (Van Alphen & Visser 1990).

Previous studies reported that female *T. radiata* wasps discriminate between parasitized and non-parasitized hosts to avoid superparasitism (Chien et al. 1991a). Husain & Nath (1923) observed superparasitism by *T. radiata* in the field during December and January when hosts were scarce, but not at times when hosts were abundant. Chien et al. (1991a, b) observed superparasitism rates of up to 5.6% by *T.*

radiata when host density was low and active space was limited. Chen (2013) found superparasitism to decrease with increasing host density over a range from 10 to 60 per parasitoid, with the highest rate of 37.9% at the lowest host density. However, whether the generally low rate of superparasitism at higher host densities was due to host marking remained unknown. Host marking may influence mass rearing and control efficiency especially with the possible existence of patch marking, which may reduce the parasitization on the hosts surrounding the parasitized nymph. The objective of this study was to determine whether *T. radiata* female wasps can avoid superparasitism by identifying hosts parasitized by herself or by a conspecific, the characteristics of the mark, and the possible existence of a patch marking mechanism.

Materials and Methods

Colonies

Tamarixia radiata colonies were maintained at the Southwest Florida Research and Education Center in Immokalee, Florida, generally following methods of Skelley & Hoy (2004) using *Murraya paniculata* (L.) Jacq. (Sapindales: Rutaceae) as host plant, and they have been grown in airpots (height= 35 cm, diameter= 16cm) for 5 years. Nine newly pruned plants with healthy flushes were held in a BugDorm™ 2400 insect rearing tent (75 × 75 × 115 cm) (MegaView Science Co. Ltd., Taichung, Taiwan) inside an air-conditioned glass greenhouse controlled during spring 2014 to approximately 27 °C, 65% RH (HOBO® RH/Temp/Light/External Data Logger H08-004-02, Onset Computer Corp., Bourne, MA). About 1,500 adult psyllids were introduced into the cage for 72 h to oviposit. Adults were then removed, and plants were held in the same cage for about 10 d until nymphs hatched and developed to 4^{th} instars. Six of these plants infested with 4th instar psyllid nymphs were transferred into a wooden framed cage (60 × 80 × 90 cm)

with polyacrylic sides maintained in an air-conditioned polycarbonate greenhouse at 26 ± 1 °C, 55 to 85% RH, and a 14:10 h L:D photoperiod. Two hundred female and 60 male *T. radiata* adults were then released into the cage and removed after 7 d. The *T. radiata* progeny were collected from day 8 until no more could be found.

In order to obtain 3-d-old *T. radiata*, six days after *T. radiata* adults were released into the colony, small (2 cm) portions of shoots containing one parasitized nymph with the wasp close to the pupal stage were excised and placed individually into a glass tube 75 mm long × 12 mm in diameter (Fisher Scientific, Hampton, NH). Tubes were checked several times per day, and wasps were removed immediately upon emergence (Chen & Stansly 2014).

Experiment 1. Avoidance of Parasitized Hosts by Female *T. Radiata*

Possible behavioral responses to parasitized hosts were investigated using a marking technique to readily distinguish among nymphs identified as parasitized or not parasitized. Ten 4th instar *D. citri* nymphs were randomly placed on a young *M. paniculata* shoot with six leaflets in a Petri dish (Fisher Scientific, product#: 0875712, Pittsburgh, PA) of diameter 14 cm. A Pigma® marker (Micro® Archival) was used to randomly mark five hosts with a small dot on the thorax. One 24-h-old *T. radiata* female wasp was released into this arena (N = 20) to evaluate possible response to the mark. After 24 h, all hosts were inverted under a stereoscopic microscope to check for *T. radiata* eggs indicating parasitization.

To evaluate preference for self-parasitized versus unparasitized hosts, one 5-cm-long *M. paniculata* shoot infested with thirty 4th instar *D. citri* nymphs was placed in a Petri dish, and a single mated 24-h-old *T. radiata* female wasp was released into this arena for oviposition. After 24 h, nymphs were checked under the microscope for

parasitization, and 6 randomly chosen parasitized hosts were transferred to a new arena where a young uninfested *M. paniculata* shoot with six leaflets was attached at the bottom of a Petri dish with double-sided cellophane tape. One parasitized and one non-parasitized host were placed at a random location on each of six leaflets. Parasitized nymphs were identified by a drawing small dot on the thorax made with a Pigma® marker for five replicates, and unparasitized nymphs were marked for another five of the replicates. The same female parasitoid which had parasitized the six hosts was released into this Petri dish and maintained under constant observation for 1 h by using a stereoscopic microscope to note the number of hosts contacted or probed. Nymphs were then inverted under the stereoscopic microscope to assess the number of parasitized or superparasitized nymphs as indicated by the presence of one or more eggs, respectively (N = 10). The same procedure was used for conspecific-discrimination except that a different experienced 24-h-old *T. radiata* female wasp was released (N = 10).

A 3rd experiment was conducted to evaluate superparasitism on conspecific- versus self-parasitized hosts. The same setup was used as described before. However, as preliminary results indicated little parasitism was likely to occur on parasitized eggs in 1 h, exposure time was lengthened to 24 h. Therefore, the Petri dishes were placed in the growth chamber at 25 °C, 70 to 85% RH, and a 14:10 h L: D photoperiod. The numbers of superparasitized hosts were checked under a stereoscopic microscope after 24 h (N = 20).

A 4th experiment was conducted to further confirm that *T. radiata* females use the antennae to discriminate parasitized from unparasitized hosts. The same setup as the

2nd experiment was used, with 6 parasitized hosts and 6 clean hosts randomly distributed on the leaves in a petri dish into which was introduced at 3-day-old *T. radiata* female. The number of times each host was antennated, probed, or parasitized within 1 h was noted (N=10).

Experiment 2. Association of Marking with Oviposition and/or Probing

Experimental set ups were similar to the above except that the objective was to evaluate preference for unprobed versus conspecific-probed-nonparasitized (PNP) hosts. One host of each type was placed at random on each of six leaflets of a *M. panaculata* shoot attached to a petri dish. One 24-h-old *T. radiata* female was released into this arena for 1 h under observation and the number of probed hosts and parasitized hosts for these two host categories was noted (N=10). The same procedure was used to determine the preference of PNP hosts over parasitized hosts (N=10).

Experiment 3. Volatility of the Host Mark

The vertical T-maze choice olfactometer was used to determine the volatility of the marking pheromone. The olfactometer is composed of four parts including the releasing chamber, where the *T. radiata* was released; T-maze chamber, which allows the *T. radiata* to crawl and to choose one odor source delivered from the source chamber linked by tubes; source chamber, where odor source was placed; and an air delivery system. Fluorescent 900 lux light bulb was provided on top of the T-maze chamber (Stelinski & Tiwari 2013). After the connection of the whole system, the air delivery system was turned on at 10 psi for five min to obtain a constant air pressure (Fig. 4-1). Three scenarios were tested using the olfactometer by placing different samples in the order source chamber: 1) clean air vs clean air (N= 10), 2) clean air vs parasitized hosts (N= 10), 3) unparasitized hosts vs parasitized hosts (N= 10). Ten 24-

h-old *T. radiata* females were released into the releasing chamber. After 10 min, the subjects displayed three types of behaviors: 1) remained in release chamber, 2) entered either one of the T-maze ports, 3) left the T-maze and entered the odor source chamber. A subject was considered as making a positive response if it moved 0.5 cm into a specific side of the division (Stelinski & Tiwari 2013). Parasitized hosts were placed into each odor source chamber randomly for each replication, and the tubes were cleaned using 2% soap solution after each replication. The number of *T. radiata* exhibiting each of the three behaviors, and the number of the females making choices were noted.

Experiment 4. Influence of Egg Presence on Host Marking

To evaluate the preference between unparasitized hosts and hosts with eggs attached, *T. radiata* infested flushes were collected from the colony, and hosts were inverted to check for parasitoid eggs. Parasitoid eggs were removed from parasitized nymphs using a small paint brush, and collected in a small petri dish for future use. Methyl cellulose was used as an adhesive agent to attach parasitoid eggs onto the thorax of unparasitized hosts. The adhesive was made from methyl cellulose powder (viscosity 4000 cPs, Alfa Aesar, Ward Hill, MA) following directions provided by the supplier (http://pdfs.carriagehousepaper.com/CarriageHousePaper-MethylCellulose.pdf). Briefly, 30 g of powder was dissolved in 120 ml boiling water, and stirred thoroughly until all granules absorbed water, and the solution turned thick. Then 240 ml cold water was added, and mixed thoroughly until it was ready to use.

Thirty 4th instar clean *D. citri* nymphs were randomly distributed on a 5-cm-long *M. paniculata* flush in a petri dish. Fifteen nymphs were randomly chosen to receive a small drop of the methyl cellulose glue on the ventral side of metathorax using a small

paint brush, and the other 15 nymphs received a drop of glue, and one parasitoid egg attached to it. Nymphs were left lying on their dorsum to allow the glue dry, then turned over after 10 min. One 24-h-old experienced *T. radiata* female, which has been exposed to *D. citri* nymphs was then released in the petri dish for a 24 h oviposition period during which the petri dishes were held in a growth chamber at 25 °C, 70 to 85% RH, and a 14:10 h L: D photoperiod (N=10). The nymphs were then checked under the microscope, and the number parasitized of each host type was noted.

Experiment 5. Existence of a Patch Marking Mechanism

Two scenarios were set up to determine the existence of patch mark: one was under 24 h, and the other was under 1 h. The basic set up was that a young *M. paniculata* shoot infested with 30-40, 4th instar ACP nymphs was inserted in a 5-ml centrifuge tube filled with water, sealed using parafilm and placed in a Petri dish (Fisher Scientific, product#: 0875712, Pittsburgh, PA) of diameter 14 cm. One 24-h-old *T. radiata* female was released in the Petri dish and held for 24 h in a growth chamber under the same conditions as above. After 24 h, the shoot was checked for parasitization under the microscope by flipping over all nymphs. Once a parasitized nymph was found, the nymphs that were in close proximity of around 1 cm (usually 5-6) were checked with minimum manipulation trying to maintain their original locations, and confirmed to be unparasitized. These nymphs were marked with a black dot on the thorax using a Pigma marker. A new 4th instar nymphs infested *M. paniculata* flush of similar length was then obtained from the ACP colony. Extra nymphs were removed using a small paint brush to control the number remaining to the same as were on the parasitized flush. Those nymphs on the unparasitized flush were marked as above but using red color. The two flushes were placed side by side in a petri dish into which a 24-

h-old *T. radiata* female was released and held in the growth chamber. The number of nymphs parasitized in these two treatments were checked and noted after 24 h (N=10).

The second scenario was conducted in the same way except that the *T. radiata* female was observed under the microscope for 1 h after which the number of parasitized nymphs was noted (N=10).

Statistical Analysis

Statistical analyses were done using JMP software (SAS Institute 2013). Preference among the two host types was assessed using one-way Anova ($P < 0.05$) to compare parasitization of Pigma®-marked hosts versus u hosts, probing and parasitization between parasitized hosts and unparasitized hosts or between conspecific-parasitized and self-parasitized hosts. Antennating rejection rate, which was the rate of antennating that did not lead to probing, was compared using Chi square test ($\alpha < 0.05$). The Kruskal-Wallis test was used to analyze the experiment of antennation preference due to non-normal distribution. Incidence of probing on PNP hosts vs unprobed hosts, and PNP hosts vs parasitized hosts were compared using one-way ANOVA ($P < 0.05$). The incidence of parasitization on the same was compared using the Kruskal-Wallis test due to non-normality of the data. Probing rejection rate, which was the rate of probing that did not lead to oviposition, was compared using Chi square test ($\alpha < 0.05$). Proportional choices made by *T. radiata* females in the olfactometer among odor sources were compared using Chi square test ($\alpha< 0.05$). Incidence of parasitization between glue only hosts versus glue+ egg hosts was compared using one-way ANOVA ($P < 0.05$) based on a normal distribution. Frequency of parasitization in clean patches vs patches with parasitized hosts was compared using one way

ANOVA ($P < 0.05$), and the parastization preference within 1 h was compared using Chi square test ($\alpha < 0.05$) due to non-normal distribution.

Results

Avoidance of Parasitized Hosts by Female *T. Radiata*

Incidence of parasitism was not significantly different between clean hosts and Pigma®-marked hosts (mean ± SE: 2.05 ± 0.98 and 2.10 ± 1.12, respectively) ($F = 0.0$; $df_{(model, error)} = 1, 38$; $P = 0.8823$). This result indicated that the Pigma® mark had no effect on host choice.

Significantly fewer self-parasitized hosts were probed ($F = 9.1$; df = 1, 18; $P = 0.0093$) and parasitized ($F = 27.8$; df = 1, 18; $P < 0.0001$) compared to unparasitized hosts (Table 4-1). Likewise, incidence of probing ($F = 58.8$; df = 1, 18; $P < 0.0001$) and parasitism ($F = 59.7$; df = 1, 18; $P < 0.0001$) was significantly less on conspecifically parasitized compared to unparasitized hosts (Table 4-1).

The incidence of antennating on clean hosts and parasitized hosts did not differ significantly ($X^2 = 0.87$, df = 1, P = 0.35). However, there were significant preferences for unparasitized hosts over parasitized hosts as evidenced by 4-fold more probing ($X^2 = 9.44$, df = 1, P = 0.0021) and 10-fold more parasitism ($X^2 = 6.08$, df = 1, P = 0.01). In addition, antennating rejection rate was four times higher on the parasitized hosts than on unparasitized hosts ($X^2 = 12.60$, df = 1, P = 0.0004) (Table 4-2).

Association of Marking with Oviposition and/or Probing

Probing incidence was not significantly different between unprobed versus PNP hosts ($F = 0.6429$, $df_{(model, error)} = 1,18$, $P = 0.43$), whereas parasitized hosts were probed less than PNP hosts ($F = 15.82$, df=1,18, P= 0.0009). However unprobed hosts were parasitized more than PNP hosts and PNP hosts more than parasitized ($X^2 = 3.79$, df =

1, P = 0.05; X^2 = 11.17, df = 1, P = 0.0008 respectively). The probing rejection rate was signigicantly different between clean hosts vs PNP hosts, and between PNP hosts vs parasitized hosts (X^2 = 3.0, df = 1, P = 0.08; X^2 = 3.82, df = 1, P = 0.05) (Table 4-3).

Volatility of the Host Mark

Most females (85%) chose one or another of the arms of the olfactometer with no difference between them when the source tubes were empty (X^2 = 21.11, df = 1, p < 0.0001). This indicated that the olfactometer had no positional bias, and was ready for use. Signicantly more females chose the arm with parasitized hosts compared to the arm with no host odor (X^2 = 4.01, df = 1, P = 0.045) with a 96% response rate. However, females showed no preference between the arms with unparasitized hosts or parasitized hosts (X^2 = 0.246, df = 1, P = 0.62), even though 93% of the test subjects made a choice.

Influence of Egg Presence on Host Marking

No preference was observed between hosts with glue only and hosts with eggs glued on (F = 0.48; df = 1, 18; P = 0.4973). Mean incidence of parasitization was 3.4±0.4, and 3.8±0.42 for glue hosts and glue+egg hosts respectively.

Existence of Patch Mark

Similar numbers of nymphs were parasitized on orange jasmine flushes containing a parasitized host (3.6±0.52), and on flushes containing no parasitized hosts (3.6±0.37, F = 0; df = 1, 18; P = 1.0) after 24 h. It was observed that after 24 h nymphs on the shoot containing the parasitized nymph moved from their original locations after the disturbance of being flipped to check for parasitization, and unparasitized nymphs from the infested flush and the uninfested flush were mixed up, and most of the nymphs were on the uninfected flush. However, preference was shown for the clean patch (1.3±

0.26) over the patch containing a parasitized host (0.2 ± 0.13) during the 1 h observation (X^2 = 8.28, df = 1, P = 0.004).

Discussion

Tamarixia radiata females were able to discriminate between parasitized and unparasitized hosts under the experimental conditions. They were observed to ignore parasitized hosts or probe briefly, only to move off and rest. They avoided probing or parasitizing hosts that the same or a conspecific female wasp had already parasitized indicating that a host mark was released on the parasitized hosts although the response to the conspecific-made mark was stronger than the mark made by herself. A significantly high antennating rejection rate was observed in response to parasitized hosts versus unparasitized hosts. Detection with the antennae indicated that the host mark was deposited externally.

Three possible mechanisms have been proposed to explain how parasitoids might guard against self- and conspecific-superparasitism at different intensities. First, there could be a two-component marking system with one short-lived component that guards against self-superparasitism and a 2nd, longer-lived component guarding against conspecific-superparsitism (Field & Keller 1999). Second, there may be perceivable differences among individuals in one or more marking pheromone components (Nufio & Papaj 2001). Third, individuals may learn to discriminate between and remember recently parasitized hosts (Ueno & Tanaka 1996). However, Van Alphen et al. (1987) reported that inexperienced parasitoids are capable of discriminating parasitized from non-parasitized hosts, indicating that host discrimination ability does not have to be gained through learning, even though superparasitism may be influenced by experience (Van Alphen & Visser 1990).

Tamarixia radiata females probed a similar number of unprobed hosts and PNP hosts, but parasitized significantly more unprobed hosts, which lead to a high probing rejection rate on the PNP hosts. These results indicated that females were able to discriminate between PNP and unprobed hosts using the ovipositor but not the antennae. These results indicated that no host mark was released on the PNP hosts, and the host mark must be released after oviposition. Since the PNP hosts were the hosts that got rejected originally, *T. radiata* females may use physical cues to avoid parasitizing them instead of chemical cues.

It is interesting that females showed a similar level of attraction to the unparasitized hosts vs the parasitized hosts using the T-maze olfactometer, and prefer the tube containing parasitized hosts over the empty tube. It was reported that *T. radiata* females were attracted to ACP nymphs using the same olfactometer setup (Mann et al. 2010). These results indicate that nymphs release one or more volatiles, which could be termed kairomone, are attractive to *T. radiata* females, but that the mark left by the ovipositing female is not volatile within 24 h.

Parasitoid eggs alone did not serve as the source of the mark within 24 h. This was not surprising, since the eggs were small comparing with the size of the nymphs. However, when eggs develop into later larval instars, they may yet help to prevent superparstization either by direct effect of the larva or changes it causes in the host.

We were not able to detect the existence of a patch mark in a 24 h scenario. The reason may be because nymphs on the shoot containing the parasitized host moved from their original locations after the disturbance of being flipped to check for parasitization. It was observed that when we checked the hosts after 24 h, the

unparasitized nymphs on the infested shoot and on the un-infested shoot were mixed up, and most of the hosts were on the un-infested flush. However, *T. radiata* females preferred the purely unparasitized nymphs over the unparasitized nymphs on the infested shoot within 1 h of observation since there was less time for movement of nymphs during this short period. Under these conditions, females did not show a preference to walk directly to the clean flush when they were just released in the petri dish.

Based on these results, it is reasonable to speculate that either the mark is lost if the nymph moves, or the movement away from the parasitized host removes neighbors from the protection of the mark. Vanalphen & Visser (1990) reported that if nearby hosts move a long distance, patch marking would not be feasible. The apparent existence of patch mark might be the reason why percent parasitism is low under mass rearing conditions.

Recent studies focused on the functions of marking pheromones and how they influence parasitoid decisions (Nufio & Papaj 2001). One effect of marking pheromone is to decrease the possibility of a female wasting time or progeny on an alaready utilized host (Nufio & Papaj 2001). Another effect can be to increase dispersal of females away from the marked host or a patch of hosts (Roitberg et al. 1984). The rejection response changes over time after the host has been utilized (Van Lenteren 1976), leading to different possibilities. Parasitoids showed increased levels of host rejection over time, or strong rejection initially but more ready acceptance over time depending on the species of parasitoid studied (Nufio & Papaj 2001). However, the pattern of increased levels of rejection over time is more likely due to the changes in the host or parasitoid than a

response to marking pheromone (Strand 1986). A decreasing rejection pattern often indicates the degradation of the marking pheromone over time (Nufio & Papaj 2001). The amount of marking pheromone released may increase with the number of eggs laid and thus help a subsequent female to assess the amont of brood in the host (Nufio & Papaj 2001). In some insects, the same marking pheromone can exert more than one effect on female behavior (Nufio & Papaj 2001). Studies on Mediteranean fruit fly *Ceratitis capitata* showed that, after a recent contact with marking pheromone, the fly tends to terminate some stationary behaviours, including resting, grooming, or oviposition, in favor of walking or flying (Papaj et al. 1989).

In the future, a modified scenario which will minimize the movement of the parasitized host or its nearby hosts should be designed to detect the patch marking mechanism. Additionally, it would be interesting to know the source of the host mark. For hymenopterans, Dufour's gland (Harrison et al. 1985, Abdalla & Cruz-Landim 2001, Rosi et al. 2001) and the poison gland (Yamaguchi 1987) have been reported as the sources of marking pheromone. Interestingly, the ratio of the hydrocarbons found in Dufour's gland and cuticular extracts is similar, which suggests that, they may derive from a common source, possibly oenocytes, secretary cells found under the larval epidermis (Howard & Baker 2003). The fact that there is no association between tcuticular hydrocarbons and hosts suggests that parasitoids synthesize their own hydrocarbons rather than acquiring them from the hosts (Howard 2001).

Table 4-1. Mean ± SE number of non-parasitized and self- or conspecific-parasitized ACP nymphs probed and parasitized by a 24-h-old *T. radiata* female wasp during a 1-h observation time.

Experiment	Host type	Probing	Parasitization
Self-identification	Self-parasitized	1.51 ± 0.43	0.25 ± 1.53
	Non-parasitized	3.63 ± 0.53	2.63 ± 0.42
Conspecific-identification	Conspecific-parasitized	0.25 ± 0.16	0.13 ± 0.13
	Non-parasitized	2.63 ± 0.26	2.13 ± 0.22

Table 4-2. Mean ± SE number of hosts antennated, probed, and parasitized in the clean and parasitized hosts categories by a 24-h-old *T. radiata* female wasp during a 1-h observation time.

	Clean	Parasitized hosts
Antennating	2.7±0.40	2.3±0.37
Probing	2.0±0.30	0.5±0.22
Parasitization	1.1±0.31	0.1±0.1
Rejection rate	0.21±0.07	0.81±0.08

Table 4-3. Mean ± SE number of probed but non parasitized hosts (PNP), clean and parasitized hosts probed, parasitized, and the rejection rate by a 24-h-old *T. radiata* female wasp during a 1 h observation time.

	PNP vs Clean hosts		PNP vs Parasitized hosts	
	clean	PNP	PNP	Para
Probing	1.6± 0.34	2.0± 0.37	3.80± 0.49	1.50± 0.31
Parasitization	0.6±0.31	0.1±0.1	1.40± 0.16	0.30± 0.15
Rejection rate	0.63±0.15	0.96±0.04	0.59± 0.05	0.79± 0.12

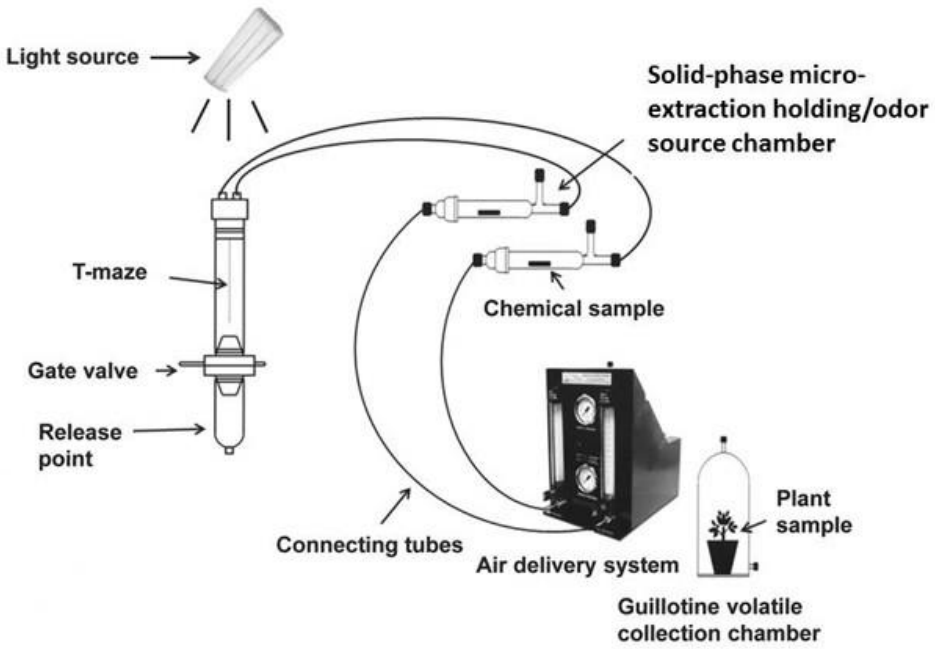


Figure 4-1. Vertical T-maze olfactometer connected with odor source and air delivery system for marking pheromone determination (Stelinski & Tiwari 2013)

CHAPTER 5
IMPROVING EFFICIENCY OF MASS REARING *TAMARIXIA RADIATA* AND ECONOMIC ANALYSIS OF FIELD RELEASE

Asian citrus psyllid (ACP) is the principal vector of huanglongbing (HLB) or citrus greening disease, caused by *Candidatus* Liberibacter asiaticus (Halbert and Manjunath, 2004). HLB was discovered in Florida in August 2005 (Halbert 2005), and by December 2007, had spread to 30 southern counties of the state (Hall 2008). HLB is one of the most destructive known diseases of citrus (Halbert & Manjunath 2004, Teixeira et al. 2005, Bove 2006, Wang et al. 2006), and is estimated to have cost Florida's economy $4.4 billion including 8,257 lost jobs from 2006- 2011 (Hodges & Spreen 2012).

Strategies to manage HLB include the utilization of pathogen- free nursery stock, removal of infected trees and most importantly, effective *D. citri* control including dormant (overwintering) insecticidal sprays (Belasque et al. 2010, Grafton-Cardwell et al. 2013). Biological control is recognized as a necessary component of integrated pest management (IPM), with a present focus on abandoned citrus, residential areas, and organic orchards (Florida Department of Agriculture and Consumer Services web information).

Diaphorina citri has been reported to be attacked by various predators, but only two primary parasitoid species are known, of which *T. radiata* has received the most attention. *Tamarixia radiata* is an arrhenotokous ectoparasitoid that has been credited with controlling *D. citri* in Reunion Island (Aubert & Quilici 1983), and providing good levels of suppression in India (Husain & Nath 1927), Puerto Rico (Pluke et al. 2008), and Guadeloupe (Etienne et al. 2001), although questions remain on actual degree of reduction of ACP (Hall et al. 2013). *Tamarixia radiata* has been released in Florida, and

is an obvious choice for augmentative biological control given its rapid establishment and spread (Qureshi et al. 2009, Chen & Stansly 2014a).

An efficient mass rearing system is a prerequisite for successful augmentative biological control (Sorensen et al. 2012, Chen & Stansly 2014b, Shimoda et al. 2015). One way of achieving efficiency is to maximize the proportion of parasitized hosts. The effect of host density on predation rate termed “functional response” has been considered an essential component affecting biological control (Holling 1959). Functional response in regard to parasitoids relates to rates of parastisim and host feeding (Holling 1959). *T. radiata* females have been studied under laboratory conditions (Chien et al. 1995, Sule et al. 2014, Chen et al. 2016b), but not related to mass rearing under colony conditions. Based on their results, Chen et al. (2016b) suggested that a ratio of 30:1 4th instar ACP: female *T. ratiata* would be the most efficient use of hosts although production of *T. radiata* was greater at the 40:1 ratio which might optimize resources of time and space. Agreement with results from the present study would indicate the value of small scale laboratory studies applied to mass rearing conditions.

Skelley & Hoy (2004) reported a mass rearing system based on *Murraya paniculata* (L.) Jack (Rutaceae) and maintenance procedures for plants, *D. citri* and *T. radiata*. This system became the standard at the Florida Department of Agriulture and Consumer Services, Division of Plant Industry (DPI) in Gainesville and Dundee, the University of Florida’s Southwest Florida Research and Education Center (SWFREC) in Immokalee (Chen et al. 2016b), as well as in Brazil (Gomez-Torres et al. 2014) and Mexico (Palomares-Perez et al. 2015). However, there remains a lack of information on

optimal *T. radiata* release rates as a function of host density, sex ratio and photophase to maximize efficiency in mass rearing. These parameters vary among protocols employed at the different locations mentioned above. Therefore, the first objective of this research was to improve mass rearing efficiency by optimizing release rates, sex ratio, and photophase. A second objective was to compare laboratory and mass rearing scale estimates of functional response parameters, to better understand the large area effect on the functional response.

Mass rearing and releasing *T. radiata* necessitate evaluating the value of *T. radiata*. Therefore, the cost of rearing and of releasing *T. radiata* in the field were calculated, and the benefit was estimated as the difference between the reduction of revenue loss incurred in the presence of *T. radiata*, and the cost of releasing *T. radiata*. Releasing (control) was estimated based on an Economic Injury Level (EIL) model proposed by Monzo & Stansly (2016, under review), to better understand the economic value of *T. radiata* in the field.

Materials and Methods

Colonies

Methodology for maintaining colonies was based on Skelley and Hoy (2004). *D. citri* was maintained on *M. paniculata* grown in 3.92 L Air-Pots (TerraHydro, Carmichael, CA) using 40% Canadian sphagnum peat + bark, vermiculite, perlite, dolomitic limestone and wetting agent (Fafard 4P Professional Growing Mix, Sun Gro Horticulture, Agawam, MA). Plants were sprayed with 1% M-pede soap (Dow AgroSciences LLC) as needed 4 or more days before use to control unwanted psyllids and other pests. Plants were grown in a clear single wall polycarbonate greenhouse (T= 12-33°C, RH= 52-97%), (HOBO RH/ Temp/ Light/ External Data Logger- H08-004-02,

Onset Computer Corporation, Bourne, MA) provided with fans and pad evaporative pad cooling protected on one end by a filtered screen room, supplemental propane heat, and air-curtained anteroom covered with insect screen mesh (United Greenhouse Systems, Edgerton WI).

Newly trimmed plants with at least eight new shoots suitable for colonization were used to rear *D. citri* in BugDorm-2400 Insect Rearing Tents (75x75x115 cm) (MegaView Science Co. Ltd., Taichung, Taiwan) inside an air-conditioned glass greenhouse maintained at 22-32 °C, 50-80% RH. Nine flushing *M. paniculata* plants were placed in each cage, into which approximately 1,500 ACP adults were released and held for 72 h to oviposit. Adults were then removed, and plants kept in the same cage for about 10 d until eggs hatched and nymphs developed to 4th instar.

Six *M. paniculata* plants infested with 4th instar psyllids were transferred into a wooden framed cage with polyacrylic sides maintained at 26±1°C, RH 55-85%, 14:10 (L:D) inside a separate 3 x 3 meters air-conditioned clear single wall polycarbonate greenhouse. Two hundred female and 60 male *T. radiata* were released inside for seven d and progeny were collected from day eight until no more could be collected.

Release Rates of *D. Citri* Adults for Optimal Psyllid Production

Nine randomly chosen *M. paniculata* plants with at least eight new shoots were placed in an ACP rearing tent in the glass greenhouse. An acceptable shoot was approximately 3 cm long with the terminal bud not fully opened. The number of ACP adults released per shoot was controlled at 10, 20 and 30 (N=6). Adults were removed after three d and the plants were held in the same cage until progeny adults had all emerged, and the number of adults calculated. The cohort producing the most psyllids was selected for further experimentation.

Release Rates of *T. Radiata* for Optimal Wasp Production

Plants were infested as above by releasing 20 ACP per shoot. The resulting cohort was allowed to develop to the 4th instar (about 10 d) and then transferred to the *T. radiata* rearing colony. Six randomly chosen plants were placed in one of the wooden framed cages providing approximately 48 young shoots per cage. Cages were randomly assigned to receive one of four levels of *T. radiata* females: 16, 30, 60 and 120 per cage at a 2:1 female: male ratio. Wasps were allowed to parasitize for 10 days after which progeny were collected daily from day 10 until no more could be collected. Number of *T. radiata* females, males and *D. citri* adults were recorded. Host density was estimated as the net number *T. radiata* + *D. citri* obtained, i.e total emerged adults. Apparent parasitism, the ratio of net *T. radiata* divided by host density, was calculated for each cage (McAuslane et al. 1993) as:

$$P = \frac{P_e}{H_e + P_e}$$

where P is the apparent percent parasitism, P_e is the number of parasitoids emerged, and H_e is the number of psyllids emerged.

Net production was calculated as the difference between wasp production and number of wasps released (females + males). The procedure began Nov. 2013 with successive replicates for a total of seven (N=7) replicates until Mar. 2014.

Effects of Release Sex Ratio on Progeny Sex Ratio of *T. Radiata*

Colonies were maintained as above and the female *T. radiata* level corresponding to the highest production from the previous experiment was used. Normal sex ratio for *T. radiata* is 2:1 female: male (Chien et al. 1991). Therefore, four sex ratios were evaluated: female: male= 4:1, 2:1, 1:1, and 1:3 (N=10) with the objective

of determining whether male interference played a role in fecundity. Six plants infested with 4th instar nymphs infested with 60 females per shoot were held in an acrylic cage and wasps at each sex ratio level obtained from the main colony were released into a randomly chosen cage and held for 7 d for oviposition. Progeny were collected daily starting from day 10 until no more progeny could be collected and the number of female and males were counted. This experiment was conducted from Mar. until Jun. 2014.

Effects of Photophase on *T. Radiata* Production

An individual mature *T. radiata* female was placed in a petri dish provided with one young *M. paniculata* shoot infested with 40, 4th instar nymphs and stuck to the bottom with double stick cellophane tape. Petri dishes were held in a growth chamber (Percival Scientific, model I36LLC8, Perry Iowa) at 24°C and 75-85% RH for a single light exposure of 0h,4h, 8h, 12h, 14h, or 24h with 0h (no light exposure) considered as control. There were 20 replications of each light exposure duration. The number of hosts parasitized was verified by inverting nymphs under a stereoscopic microscope.

Economic Analysis of *T. Radiata* Controlling *D. Citri* in the Field

The cost of mass rearing *T. radiata* was based on the mass rearing facilities belonging to the Division of Plant Industry in Gainesville, and Dundee, FL. Cost categories included mass-rearing staff salary, utility bills, shipping bills, supply bills (such as soil, pots, pesticides, fertilizers, light bulbs etc.). The cost analysis was based on the number of *T. radiata* produced and cost generated from September 2013 to August 2014.

The gross income recovered from the field receiving *T. radiata* releases was based on Tansey et al. (2016). Briefly, *T. radiata* was released in a 5.2 ha block of citrus located in Collier County, Florida, planted in 2001 with *Citrus sinensis* (L.) Valencia. The

block was divided into 16 plots in a randomized complete block (RCB) design consisting of two levels, with and without two factors: insecticide and foliar nutrient. Therefore, there were four untreated plots, and approximately 3,000 *T. radiata* were randomly released on psyllids infested flushes every other week in half of each plot, with the other half considered as the control. Population of ACP adults was monitored approximately every 2 weeks by conducting two stem tap samples per tree from six randomly selected trees at two randomly selected points (stops) per plot. For each stem tap sample, a white plastic clipboard (22 cm × 28 cm) was held horizontally under a randomly chosen branch that was struck three times with a 60 cm length of PVC pipe. ACP adults that fell on the clipboard were assessed visually. Gross income was estimated based on three growing seasons from Jan. 2012 to Jan. 2013, Feb. 2013 to Jan 2014, and Feb. 2014 to Jan. 2015 respectively (Tansey et al. 2016). Randomly chosen *D. citri* infested flushes in these four *T. radiata* release plots were collected every month, reared in clear acrylic cylinders, and the number of ACP, and *T. radiata* emerged were noted in order to calculate the percent parasitism.

Statistical Analysis

Release Rates of *D. Citri* Adults for Optimal Psyllid Production

D. citri production with no transformation at the three infestation levels were compared using one-way ANOVA in JMP software (SAS Institute Inc., Cary NC), with mean separation by LSD ($P<0.05$) upon a significant F value for treatment.

Release Rates of *T. Radiata* for Optimal Wasp Production

Total *T. radiata* production, production per female, percent parasitism and sex ratio at four treatments were compared using ANOVA, LSD ($P< 0.05$). The relationship between the number of females released and progeny produced per female was

analyzed using nonlinear regression analysis. The relationship between the host density per released female and the progeny produced per female (functional response) were fitted into type II and type III functional response equations respectively as follows:

$$N_p = N(1 - \exp\left(-\frac{a' T_t}{1 + a' T_h N}\right)) \quad \text{(Rogers 1972) (5-1)}$$

$$N_p = N\left(1 - \exp\left(-\frac{bT_t N}{1 + cN + bT_h N^2}\right)\right) \quad \text{(Hassell 1978) (5-2)}$$

Where N_p= the number of hosts parasitized; N=host density; T_t= total time available; a'=searching coefficient, i.e area covered per unit of time; and T_h= handing time per host (Holling 1959). In type II functional response, a' and T_h are constant (Rogers 1972), whereas in type III equation, a' decreases with increasing host densities in a pattern of a= bN/(1+cN), and b, c and T_h are constant (Hassell 1978). The unknown parameters a' and T_h in type II equation, and b, c, and T_h in type III equation were estimated through nonlinear least squares (NLS). Bayesian information criteria (BIC) were calculated for both equations with the lower BIC as criterion for best fit.

Effects of Release Sex Ratio on Progeny Sex Ratio of *T. Radiata*

The logit transformation was applied to sex ratio data. *T. radiata* sex ratio and progeny at four levels were then compared using ANOVA, LSD ($P<0.05$).

Effects of Photophases on *T. Radiata* Production

The number of hosts parasitized under different light durations was compared using ANOVA, LSD ($P<0.05$).

Economic Analysis of *T. Radiata* Controlling *D. Citri* in the Field

Apparent percent parasitism of *T. radiata* on the collected flushes was calculated as the number of *T. radiata* emerged divided by the total of *T. radiata* and ACP emerged (McAuslane et al. 1993). The accumulative number of ACP recorded by tap sampling

per tree per season (*k*) in the *T. radiata* released plots (N_1) was calculated based on the tap sampling data, and the *k* in the control plots where *T. radiata* was not released (N_2) was estimated based on N1 increased by the percent parasitism. The cost of releasing *T. radiata* in the field ($/ha) was calculated as the number of *T. radiata* released per ha times the estimated cost per wasp.

The gross income under insecticide treatment in 2013, 2014, and 2015 was $5,455, $7,410, and $11,360/ha (Tansey et al. 2016), and the difference among plots in accumulative number of ACP under this treatment was negligible, therefore, these yield were considered as the baseline ($P*Y_m$). The yield loss with the corresponding number of ACP was estimated using the EIL equation (Monzo and Stansly 2016, under review):

$$E = P^* Y_m * \frac{3.029*k}{1+\frac{3.029*k}{32.76}} *100^{-1} \quad (5\text{-}3)$$

Where E was the revenue loss ($/ha), k was the cumulative number of ACP adults per tree per season, P was the market price per kg of solids, and Y_m was the yield (kg/ha). The real gross income in the *T. radiata* released plots was based on Tansey et al. (2016). In order to confirm the accuracy of revenue loss equation above, the yield loss ($/ha) in the *T. radiata* treated plots was estimated based on this equation, and compared with the real gross income.

Results

Release Rates of *D. Citri* Adults for Optimal Psyllid Production

A significant effect of infestation level on *D. citri* production per flush shoot was determined ($F = 54.7$, df = 2, 15, $P < 0.0001$). Production increased with each *D. citri*

infestation level, but with no significant difference between 20 and 30 ACP per shoot (Table 5-1).

Release Rates of *T. Radiata* for Optimal Wasp Production

Progeny production increased over release rates of 16 through 60 female wasps per cage, although without significant differences among the highest three densities (Table 5-2). Net production followed a similar pattern as gross production although with a trend toward decrease at the highest release rate. Apparent parasitism increased with increasing number of females released (F = 13.1, df = 3, 24, P < 0.0001) although differences between 30 and 60 or 60 and 120 females were not significant. Production per female decreased from 68.7 to 15.4 over the release range (F= 13.4, df = 3, 24, P < 0.0001) although differences between the two lowest or the two highest densities were not significant. There was no significant effect of release rate on sex ratio (F= 0.30, df = 3, 24, P = 0.82).

Bayesian information criterion (BIC) values were estimated at -51.6 and -42.5 when production per female (N_p) and host density per released female (N) data were fit to Equations 5-1 and 5-2 respectively. Therefore, type II response equation provided the better fit model (Fig. 5-1). The estimated parameters obtained from NLS were a'= 0.09 h^{-1} (SE=0.0085), and T_h= 0.08 h (SE= 0.016).

Effect of Release Sex Ratio on Progeny Sex Ratio of *T. Radiata*

Progeny production averaged 2,361.5±470.5, and progeny sex ratio female: male 2.75±0.14 over the range of release sex ratios with no significant treatment effect detected (Production: F = 0.54, df = 3, 36, P = 0.65; Sex ratio: F = 1.21, df = 3, 36, P = 0.32).

Effects of Photophase on *T. Radiata* Production

The number of hosts parasitized increased with increasing photophase. *Tamarixia radiata* females were able to parasitize 12.1±1.1 hosts during the 24 h photophase which was significantly more than 8h (Table 5-3). The wasps laid more than 1/3 of the total amount within the first four h, another 1/3 in the following four h, after which the rate of parasitization slowed considerably (Fig.5- 2).

Economic Analysis of *T. Radiata* Controlling *D. Citri* in the Field

The total expenses for mass rearing *T. radiata* per year was around $359,520 (Table 5-4). Dundee produced approximately 150,000 *T. radiata* per month, and Gainesville produced around 125,000 per month, which generated 3.3 million from these two facilities per year. Therefore, it cost approximately $0.11 to produce one *T. radiata*.

An average of 81,300 *T. radiata* were released at an estimated cost of $ 8,943/ha (Table 5-5). The gross income ($/ha) of the *T. radiata* released plots were $3,938.92, $6094.36, and $10050.07/ha in 2013, 2014 and 2015, respectively (Table 5-6) (Tansey et al. 2016). The *k* in *T. radiata* treated plots (N_1) were 80.18, 11.22, and 6.18, in 2013, 2014, and 2015 respectively. Percent parasitism in 2013, 2014, and 2015 based on random sampling were 0.09, 0.008, and 0.105, respectively. The EIL Equation 5-5 provided a good estimate of the yield loss, and the estimated gross income, which were the difference between baseline gross income and estimated yield loss, were$ 3,880.63, $6,173.58 and $10,006.75, versus $3,938.92, $6,094.36, and $10,050.07/ha in 2013, 2014, and 2015, respectively (Table 5-7). Therefore, EIL equation was used for further analysis.

In theory, growers would profit from *T. radiata* releases if the value of *T. radiata*, as measured by the reduced revenue loss is greater than the cost of releasing *T. radiata*.In order to provide a guidance to estimate the amount of *T. radiata* releases, the following equation was developed:

$$E_{N2} - E_{N1} \geq C \quad (5\text{-}4)$$

Where C ($/ha) was the releasing cost, which equaled the number of *T. radiata* releases* $0.11. E_{N2} was the yield loss ($/ha) at control, and ACP density *k* was N_2. E_{N1} was the yield loss when *T. radiata* was released, and the ACP density *k* was N_1. The relationship between N_1 and N_2 was: $N_2 = N_1 * (1+P)$, where P was the percent parasitism when *T. radiata* was released. E_{N1} and E_{N2} were estimated using EIL equation. Therefore, Equation 5-2 was transformed into:

$$P\ Y_m * 100^{-1} \left(\frac{3.029 * N_2}{1+\frac{3.029 * N_2}{32.76}} - \frac{3.029 * N_1}{1+\frac{3.029 * N_1}{32.76}}\right) \geq M * 0.11 \quad (5\text{-}5)$$

Where M was the accumulative number of *T. radiata* released yearly. Equation 5-5 provided a relationship between the mean percent parasitism per year and the number of *T. radiata* released yearly based on the known accumulative number of ACP at *T. radiata* released plots (N_1), and the baseline gross income (Y_m*P). For instance, the baseline gross income in 2012-2013 was $5,455.40, and the AAY ($N_1$) was 80.18, then if we release 5,000 *T. radiata*, the mean percent parasitism need to be at least 81% in order to make a profit by releasing *T. radiata*.

Discussion

ACP production was greatest when the number of adults released was increased from 10 to 20 per flush shoot, which also doubled the number of progeny from about 50

to about 100 per shoot. ACP production did not increase significantly when the number of adults was increased to 30 per shoot. Furthermore, plants receiving 30 ACP adults per shoot were sticky with honey dew, which could make it difficult for *T. radiata* to search. Therefore, a density of 20 ACP per shoot was chosen as the optimal release rate and used for further experiments.

Host density based on net production of adult *D. citri* and *T. radiata* decreased with increasing parasitoid release rates (Table 5-2). This increased the estimate of apparent parasitism and could be partially due to increased host feeding at the higher release rates.

Sixty *T. radiata* females per cage, or a little more than one female per infested shoot, could be considered optimal since the higher release rate (120 wasps per cage) did not improve progeny production. Additionally, net production was greatest when 60 females were released. Assuming the cost of obtaining one *T. radiata* was the same at each releasing density, the most economically efficient rearing method would be the one achieving the highest net production. Therefore, releasing 60 females per cage (one per infested shoot) could be recommended.

The stocking number of ACP nymphs in the beginning of the study was estimated at approximately 4,800 per cage, including 4^{th}, 3^{rd} and even younger instars. Since plants were infested by ACP adults for 3 d, the number of 4^{th} instar nymphs upon *T. radiata* release was around 1,600. This release rate would provide 4^{th} instar host: parasitoid ratio of 27:1, which is close to the 30-40:1 ratio considered optimal at the Petri-dish level (Chen et al. 2016b).

While production per wasp (Table 5-2) was less at 60 than at 30 females per cage, there was a trend toward fewer progeny per cage and lower apparent parasitism at the lower release rate. These results also mirrored those seen in the laboratory and indicated that under the conditions of this experiment, only about half the hosts were utilized, regardless of how many wasps were released. The reason for this is not clear, although male interference cannot be blamed since sex ratio over a range of 1:3 to 4:1 female: male had no effect on progeny production. Nevertheless, the apparent percentage of hosts parasitized in cages was greater than the true parasitism rate of 31-19% obtained in the laboratory over a host: parasitoid range of 30-60-:1 (Chen et al. 2016b). It is likely that competition among females is somehow preventing more complete host utilization, possibly through physical altercations or a marking mechanism that affects nearby hosts. Such competition might be expected to have greater effect in the smaller space afforded by the Petri-dish arena compared to much larger the cage.

Host density per female and hosts parasitized per female followed a type II functional response, with an estimated searching coefficient (a') of 0.09 ± 0.0085 h^{-1}, and handling time (T_h) = 0.08 ± 0.016 h. The estimate for handling time was close to that obtained in a previous study (Chen et al., 2016b) of T_h = 0.06 h when 3-d-old *T. radiata* females were observed at 6 host densities for 30 min. Estimated a' for that study decreased from 0.095 h^{-1} to 0.015 h^{-1} over all the host densities when T_h=0.06 was fitted into the type II functional response equation (Rogers, 1972). The two parameters for the present study fell within previous estimation ranges, predicting that *T. radiata* takes

approximately 0.08 h (4.8 min) to parasitize a host, while searching 0.09 standard areas per hour.

The searching area in the previous study was 147 cm^2, which resulted in a real searching coefficient of 2.01-13.97 cm^2/h. The searching area in this study was estimated using this value divided by a' = 0.09 h^{-1}, giving a searching area within the range of 24.5-155.2 cm^2. This study showed that despite the scale of the experiment, searching area was within the range of that estimated in the laboratory. However, a smaller searching area could be rationalized by the likelihood that local host density was greater in the cage experiment.

Progeny sex ratio was not influenced by sex ratio of released wasps ranging from 1:3 to 4:1 female: male. Therefore, we can expect a nonbiased progeny sex ratio of about 2.75 regardless of initial sex ratio. However, it has been reported that 93% of females mated once and only once during the first day of emergence (Chien et al. 1991) so females may have already been mated when they were selected for release.

Our result showed that *T. radiata* is strictly diurnal in regard to oviposition, since no eggs were laid in the dark. Fecundity under 24 h of light was significantly greater than 8:16 L: D but not 12:12 or 14:10. More research is needed to determine whether there may be deleterious effects of long term exposure to 24 h of light.

It costs approximately $0.11 to produce one *T. radiata* adult in these mass rearing facilities. The EIL Equation 5-5 provided a good estimation of the yield loss, and could be used for further analysis. An estimated relationship between the percent parasitism and the number of *T. radiata* released yearly was proposed, and it will

provide a guidance of releasing *T. radiata* and of monitoring the percent parasitism at the same time.

In summary, the efficiency of mass rearing under our conditions was maximized by releasing 20 psyllids per flush to achieve the optimal amount of host density, releasing one *T. radiata* female per *D. citri* infested flush shoot, at a photophase at 12 hours per day. This new mass rearing methodology improved the *T. radiata* production in Immokalee colony by 3-fold. However, we were not able to improve incidence of parasitism beyond about 60%. Further studies of oviposition behavior are needed to improve rearing efficiency which may also reflect efficiency of parasitism in the field, and an economic analysis of utilizing the improved rearing method would be interesting to know.

Table 5-1. *D. citri* adult production (mean ± SEM) at infestation levels of 10, 20, and 30 per shoot

D. citri released per shoot	*D. citri* progeny produced per shoot
10	48.2±2.0A[z]
20	109.06±6.7B
30	119.39±5.7B

[z] Means followed by the same letter are not significantly different LSD (P< 0.05).

Table 5-2. Mean± SEM host density*, T. radiata* gross and net production, sex ratio, percent parasitism, and production per *T. radiata* female at four host densities (female: male= 2:1)

Density (females/cage)	Host density	Production (Wasps/cage)	Net Production	Production per female (Wasps/female)	Apparent Parasitism	Sex ratio Female: male
16	3733.3±396.4A[z]	1099.3±168.4B	1075.3±168.4B	68.7±10.5A	0.3±0.0C	2.2±0.2A
30	3696.0±289.9A	1652.6±166.4AB	1592.6±166.4AB	55.1±5.6A	0.4±0.0B	2.1±0.2A
60	3429.4±529.0A	2004.6±234.0A	1884.6±234.0A	33.4±3.9B	0.5±0.0AB	2.4±0.2A
120	3218.6±666.5A	1843.1±364.7A	1603.1±364.7AB	15.4±3.0B	0.6±0.03A	2.4±0.4A

z Means in the same column followed by the same letter are not significantly different LSD (P< 0.05).

Table 5-3. Number of hosts parasitized (Mean ± SEM) under different photophases

Photophase (h)	Hosts parasitized
0	0C
4	4.5± 0.6C
8	8.8± 1.0B
12	10.6± 0.8AB
14	10.6± 0.81AB
24	12.1± 1.1A

Means followed by the same letter are not significantly different (LSD, P <0.05).

Table 5-4. Total expenses of mass rearing *T. radiata* in Gainesville and Dundee from Sep. 2013 to Aug. 014

Cost categories (per year)	DPI ($)	Dundee($)
Mass-rearing staff salary	129,000	147,000
Utility bills	20,000	20,795.8
Shipping	6,000	6,000
Supply expenses (soil, pots, pesticides, fertilizers, light bulbs etc)	15,000	15,000
Phone bills	--------	724.29

Table 5-5. Average cost of releasing *T. radiata* in the field in season 2013, 2014, and 2015

	# Releasing per ha	Application cost ($/ha)
2012-2013	70185.19	7720.37
2013-2014	92140.74	10135.48
2014-2015	81574.07	8973.15

Table 5-6. Mean gross income of the *T. radiata* released plots in season 2013, 2014, and 2015 (Tansey et al. 2016)

	# trees per ha	# box per ha[1]	kg of solid per ha[2]	Juice price ($ /kg solid)	gross income ($/ha)
2012-2013	377	415.58	1115.84	3.53	3938.92
2013-2014	377	641.92	1608.01	3.79	6094.36
2014-2015	377	821.66	2175.34	4.62	10050.07

1. # box per ha[1]= (# boxes/ tree)* (# tress/ha)
2. kg of solid per ha=(kg solid/ box)* (# box/ ha)

Table 5-7. Yield loss estimated using the EIL Equation 5-5

	N_1	Baseline gross income ($P*Y_m$)	Estimated yield loss with *T. radiata* released	Estimated gross income with *T. radiata* released*
2012-2013	80.18	5455.40	1574.77	3880.63
2013-2014	11.22	7409.54	1235.96	6173.58
2014-2015	6.18	11360	1353.25	10006.75

*Estimated gross income= baseline gross income- estimated yield loss

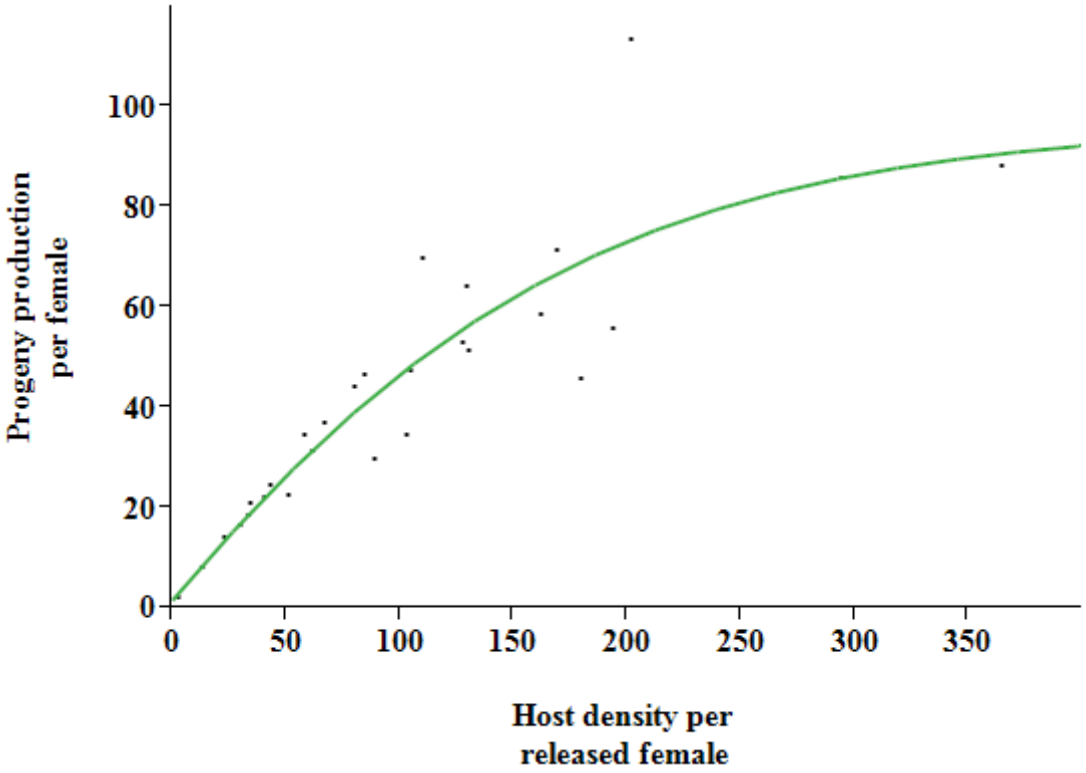


Figure 5-1. Type II functional response of the progeny production per female and host density per released female.

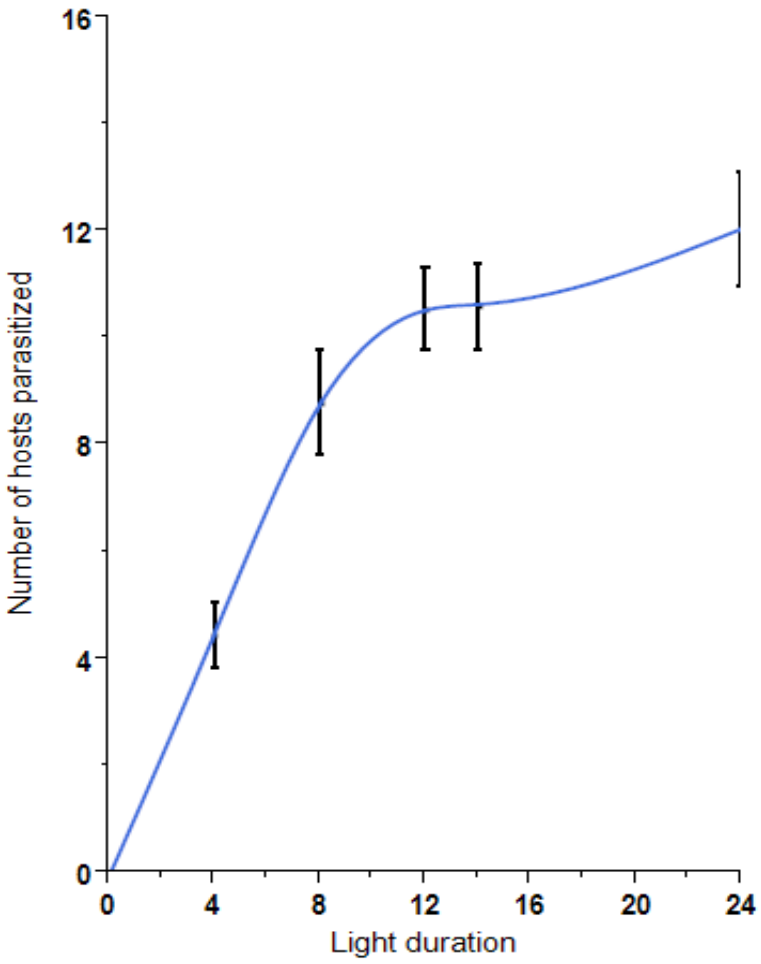


Figure 5-2. Number of hosts parasitized under different photophase duration (h). Error bars indicate the standard error of the means.

CHAPTER 6
MOLECULAR DETECTION OF ENDOSYMBIONTS IN *TAMARIXIA RADIATA*

Diaphorina citri is the vector of Huanglongbing (HLB) or citrus greening disease, which is estimated to have cost Florida's economy $4.4 billion and resulted in 8,257 jobs lost from 2006-2011 (Hodges & Spreen 2012). *D. citri* has been reported to be attacked by various predators, but only two parasitoid species, of which *Tamarixia radiata* has received the most attention. *T. radiata* is an arrhenotokous ectoparasitoid that has been credited with controlling *D. citri* in Reunion Island (Aubert & Quilici 1983), and providing good levels of suppression in India (Husain & Nath 1927), Puerto Rico (Pluke et al. 2008), and Guadeloupe (Etienne et al. 2001), even though it is not clear how much *D. citri* levels were reduced in these locations (Hall et al. 2013). *T. radiata* has been introduced in Florida, and it is an obvious choice for augmentative biological control given its rapid establishment and spread in Florida (Chen & Stansly 2014a).

Any program aimed at augmentation of a natural enemy would require an efficient system of mass production (Chen & Stansly 2014b, Sorensen et al. 2012). Mass rearing facilities of the Florida Department of Agriculture and Consumer Service's Division of Plant Industry (DPI) facility in Gainesville and University of Florida's Southwest Florida Research and Education Center (SWFREC) in Immokalee were initiated independently and have been maintained for more than 10 years. The *T. radiata* population in DPI was collected neat to Gainesville, and those in Immokalee colony was collected in Collier and Hendry Counties near Immokalee. A larger capacity DPI facility in Dundee was recently initiated from the Gainesville colony. However, production at SWFREC is inferior to that at DPI despite similar methodology, raising the possibility of pathogen or symbiont effects on fitness.

Meyer & Hoy (2008) surveyed endosymbionts in *T. radiata* (UF, Gainesville colony) including Archaea, Eubacteria, *Candidatus* Liberibacter asiaticus, *Arsenophonus,* Wolbachia *wsp*A, *Caulobacter,* and *Methylobacter.* They discovered the presence of *Caulobacter sp., Methylobacterium sp*., and species in the family Alcaligenaceae, and concluded that all except for *Caulobacter sp.* were likely acquired from the environment rather than vertically transmitted. More importantly, they did not find *Ca.* L. asiaticus, the causal agent of HLB which would have obviated release in the field. However, this research was published more than 8 years ago and only included *T. radiata* from the UF Gainesville colony.

This study followed up on Meyer & Hoy (2008) by identifying eubacteria species in the present DPI Gainesville and UF Immokalee colonies. Our objective was to survey eubacteria species present in *T. radiata* from both colonies, and more importantly, confirm the absence of *Ca.* L. asiaticus in the two *T. radiata* populations. In order to do so, we sequenced a single copy gene from *T. radiata* DNA for quantification in order to confirm an equal bacterial infection level in the two populations, and also estimated genome size of *T. radiata*, which has not been reported previously. It was suggested that insect genome size was related to parasitism and development, and estimation of insect genome size is of great importance in understanding insect evolution (Gregory 2002).

Materials and Methods

Collection of Leaf Samples, Psyllids and *T. Radiata*

HLB infected leaves were used as positive controls in detecting the existence of *Ca.* L. asiatius DNA using real time PCR. Infected/positive leaves were collected from known HLB positive citrus trees in SWFREC, and confirmed by the HLB diagnostic lab

in SWFREC (http://www.imok.ufl.edu/programs/citrus-path/hlb-lab/). Negative/non-infected leaves were collected from healthy Valencia citrus trees in a well-maintained greenhouse at SWFREC, inspected monthly for ACP infestation and tested by PCR every 6 months by the Florida Department of Agriculture and Consumer Services, Division of Plant Industries.

Psyllid nymphs and *T. radiata* adults were collected from the mass rearing facilities in Gainesville (DPI), and in Immokalee (UF-SWFREC), respectively. Psyllid adults and young flushes infected by psyllid nymphs were also collected from a HLB-positive citrus grove in Dundee, FL and SWFREC Field 10. Infected shoots were checked under stereoscopic microscope for parasitized nymphs. Once found, the shoot was held in a 1.5 ml centrifuge tube with purified water covered with a small cylinder (12 cm tall, 1.5 cm in diameter) in a growth chamber maintained at 26.8±2 °C, 70% RH, and a photoperiod of 14: 10 (L: D). Water in 1.5 ml centrifuge tubes were replaced every 2 d, and emerged *T. radiata* adults were collected using an aspirator once emerged.

Genome Size of *T. Radiata*

The genome size of both *T. radiata* females and males were analyzed using flow cytometry by measuring the fluorescence emission indirectly (Kamentsky et al. 1965, Dolezel and Bartos 2005). Briefly the procedure was to place 20 unsexed *T. radiata* obtained from the mass rearing colony at DPI in Gainesville in a medium sized polystyrene antistatic weight boat (Fisher Scientific, Pittsburgh, PA, #08732115) with 1 ml chopping buffer (MOPS, 0.5 M, 4.0 ml; $MgCl_2$, 0.5 M, 9.0 ml; $Na_3C_6H_5O_7$, 0.5 M, 6.0 ml; Triton X-100, 10%, 1.0 ml; RNase, 10 mg/ml, 0.1 ml; Water 79.9 ml)to be finely diced with a razor blade. The homogenate was then filtered successively through a 50 um and then 15 um nylon mesh into a clean weight boat. Filtrate was transferred to a

1.5 ml microcentrifuge tube and centrifuged at 1,000 rpm for 3 min. The pellet was re-suspended in 300 µl staining buffer (chopping buffer 50.0 ml; propidium I 5 mg/ml, 0.5 ml) and again centrifuged at 1,000 rpm for 3 min. The pellet was resuspended in 200 µl of staining buffer and analyzed in a flow cytometer (Union Biometrica BioSorter, Holliston, MA). The same procedure was used to test the genome size of common bean *Phaseolus vulgaris*, which was used as a standard to calculate the genome size of *T. radiata* using the following equation.

$$\text{Sample DNA content} = \frac{\text{Relative Fluorescence of sample}}{\text{Relative Fluorescence of Standard}} * \text{standard DNA content} \quad (6\text{-}1)$$

DNA Extraction and Purification

DNA extraction from citrus leaves. DNA from approximately 2.5 g of positive and negative citrus leaves was extracted according to Vallejos (2007). Briefly, leaves were ground in liquid nitrogen and transferred to 50 ml Polypropylene tube containing 45 ml sample resuspension buffer (SRB-B) (Tris.HCl 1 M, pH=8.0, 10.0 ml; EDTA 0.5 M, pH=8.0, 1.0 ml; NaCl 5 M, 4.0 ml; water added to 100.0 ml). After spinning at 1,500 rpm for 15 min, supernatant was discarded, and 15 ml nuclei resuspension buffer (NRB) (Tris.HCl 1 M, pH=8.0, 10.0 ml; EDTA 0.5 M, pH=8.0, 1.0 ml + water to 100.0 ml) was added followed by incubation at room temperature for 20 min. Then 10 ml of 2X lysis buffer (Tris.HCl 1 M, pH=7.8, 10.0 ml; EDTA 0.5 M, pH=8.0, 1.0 ml; NaCl 5 M, 28.0 ml; CTAB 10%, 20.0 ml; water 41.0 ml) was added and the mixture incubated at 65°C for one h. Nucleic acids were extracted using 15 ml phenol, followed by a second extraction with 15 ml chloroform, and the supernatant was saved in a new tube after spinning at 4,500 rpm for 20 min. DNA was precipitated using an equal volume of isopropanol and spinning at 4,500 rpm for 20 min. The DNA pellet was washed using 20 ml EtOH

(100%) and dissolved in 40 µl of 0.1X TE buffer (Tris.HCl 1 M, pH=8.0, 1.0 ml; EDTA 0.5 M, pH=8.0, 0.2 ml; water added to 100 ml). The quality of DNA was assessed via a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE) (Desjardins & Conklin 2010) and electrophoretic analysis using 1% agarose gel. Nanodrop is an absorbance of measurement made on spectrometers, which measures the absorbance of all molecules in the sample that absorb at different wavelength. The ratio of absorbance at 260 nm and 280 nm was used to test the purity of DNA and RNA. A ratio of approximately 1.8 is considered pure DNA, a ratio larger than 1.8 or close to 2.0 is accepted as contamination of RNA (http://www.nanodrop.com/Library/T009-NanoDrop%201000-&-NanoDrop%208000-Nucleic-Acid-Purity-Ratios.pdf). The DNA was compared using either lambda hind III molecular marker, or pstl marker (Thermo Fisher Scientific Inc. Gainesville, FL) depending on the size of the DNA.

Extracting DNA from psyllids and *T. radiata* DNA from 30 *T. radiata* or 20 psyllid adults were extracted according to DeBarro et al. (1995) and Nachappa et al. (2011) respectively. Briefly, insects were finely ground in liquid nitrogen in a 1.5 ml centrifuge tube using a pellet pestle, incubated for 2 h at 55 °C in 200 µl TES (100 mM Tris, pH=8.0; 10 mM EDTA, 2% SDS) with 2 µl Proteinase K (Promega Corporation, Madison, WI) followed by a 10 min incubation at 65 °C. After being dissolved in 56 µl of 5 M NaCl and 25 µl 10% CTAB to denature protein, the nucleic acids were extracted using 280 µl of SEVAG (chloroform: isoamylalcoho, 24:1), and the supernatant was transferred to another 1.5 ml centrifuge tube containing 90 µl 5 M NH4Ac, followed by centrifuging for 10 min at 15,000 rpm. DNA was precipitated by adding 315 µl isopropanol to the supernatant. The resulting DNA was dissolved in 30 µl TE buffer. The

DNA of *T. radiata* and psyllids from both lab colonies and the field were extracted separately. The quality of DNA was assessed by Nanodrop and electrophoretic analysis using 1% agarose gel.

DNA purification Insect DNA extracted based on the protocol above was contaminated with RNA, so a DNA purification procedure was employed. Briefly, TE buffer was used to bring the DNA solution up to 90 µl, then 10 µl 0.5 M NaCl and 1 µl RNase (Qiagen, Valencia, CA) were added and incubated at room temperature for 15 min. One hundred µl of solution A (Tris.HCl, pH= 8.0, 0.1M; NaCl 1M) was applied to the tube prior to DNA extraction using 100 µl phenol: chloroform (4:1). Supernatant was extracted again using 100 µl chloroform. DNA was precipitated with 200 µl isopropanol and washed with EtOH 76%/0.2 M NaOAc and 100% EtOH successively. The resulting DNA was dissolved in 40 µl TE buffer. The quality of DNA was assessed by Nanodrop and electrophoretic analysis using 1% agarose gel.

Sequencing One Single Copy Gene from *T. Radiata*

Eukaryotic 28s rDNA primers (forward primer: CCCTGTTGAGCTTGACTCTAGTCTGGC; reverse primer: AAGAGCCGACATCGAAGGATC) (de Leon & Setamou 2010, Werren et al. 1995) were used to detect the presence of *T. radiata* and psyllid genomic DNA. The target size was around 550 bp. In order to obtain a single-copy-gene for DNA quantification in *T. radiata*, primers (forward primer: GCCATGGTTCAAGGGATG, reversed primer: TGGTGCATCTCCACAGACTT) based on Eulophidae wasp *Quadrastichus erythrinae* elongation factor 1 alpha (EF) (Rubinoff et al. 2010) were used for detection in *T. radiata*, given that elongation factor is considered as a highly conserved and a putative single-copy gene (Cho et al. 1995; Clouse et al. 2013; Rubinoff et al. 2010). Extracted

T. radiata DNA was used for conventional PCR amplification using the pair of EF primers. The reaction mixture contained 2 µl of the template DNA, 2 µl of the primers 1 uM, 2 µl of the 10 X buffer, 2 µl of the $MgCl_2$ 15 mM, 2 µl of dNTPs, 2 µl of Taq DNA polymerase and 8 µl of PCR water in a reaction volume of 20 µl. The PCR cycling conditions were: 95 °C for 2 min followed by 40 cycles of 95 °C for 30 sec, 65 °C for 30 sec, and 72 °C for 60 sec, and a finishing temperature of 72 °C for 5 min. The same conditions were used for amplification of 28s rDNA except for an annealing temperature of 55 °C. Agarose gel (2 %) electrophoresis was used to analyze the PCR products.

The PCR reaction from EF primers was cloned using StrataClone Blunt PCR Cloning Kit (Agilent Technologies, La Jalla, CA) and sequenced at the University of Florida, Institute of Interdisciplinary Center for Biotechnology Research (ICBR). Two sequences were obtained, analyzed and blasted using GeneBank and ClustalX (Thompson et al. 1997). One turned out to be an artifact based on the poor blasting results. One pair of primers was designed based on the first exon of this EF sequence. The primers (TEF) were forward: TTGAGCGCAAAGAAGGCAAA, reverse: GAGGATGGCATCGAGAGCTTC. They were later used for *T. radiata* DNA quantification.

Real-time PCR

Real-time PCR was used to quantify *T. radiata* DNA by amplifying a single copy gene from both colonies to examine the possibility that differences in fitness were due to different bacterial infection levels. We assumed that DNA extracted from *T. radiata* populations contained *T. radiata* DNA and all symbiont DNA. By adjusting to the same concentration and loading equal volumes of DNA solution to the PCR reaction,

differences in resulting Ct values between the two populations would suggest that symbionts infection levels were also different.

The primers TEF were used to run real-time PCR with DNA from each colony. The amplification reactions were 10 µl, containing 1 µl buffer 10X, 1 µl $MgCl_2$, 1 µl dNTPs, 0.1 µl Taq DNA polymerase, 2 µl primers, 4 µl template DNA, 0.5 µl Syle 82, and 0.4 µl water.

Bacterial Detection

Specific primers were used in conventional PCR reactions to detect Eubacteria, Archaea, *Ca*. L. asiaticus, *Arsenophonus*, Wolbachia *wsp*A, *Caulobacter* and *Methylobacter* (Myers & Hoy 2008, Table 6-1). PCR cycling conditions were similar to the conditions amplifying Eukaryotic 28s rDNA, but each bacterium has its specific annealing temperature (Table 6-1). PCR reactions were assessed using a 2% agarose gel electrophoresis (Fig. 6-5).

Eubacteria Cloning and Sequencing

Eubacteria PCR amplification products were ligated to Puc18 plasmid using StrataClone Blunt PCR Cloning Kit (Agilent Technologies, La Jalla, CA), and cloned into competent *Escherichia coli* cells, incubated in 96-well-plates. Plasmid DNA was purified from *E. coli* colonies with QIAGEN Plasmid Mini Columns (Valencia, CA). The presence of the inserted DNA was confirmed by agarose gel electrophoresis. DNA was sequenced at the University of Florida, Interdisciplinary Center for Biotechnology Research (ICBR). Sequences were analyzed and blasted in GeneBank and ClustalX (Thompson et al. 1997).

Search for *Ca.* L. Asiatius Using Real-time PCR

Ca. L. asiatius specific primers (Li et al. 2006) were used in real-time PCR to detect *Ca.* L. asiatius in psyllids and *T. radiata* collected from both lab colonies, and the field. Detection procedures were conducted in the HLB diagnostic lab at SWFREC following the USDA New Pest Response Guidelines of Citrus Greening Disease, and samples were considered positive if the Ct value was less than 36, negative if the Ct value was greater or equal to 36, not detectable if no increase in fluorescent signal above background was detected after 40 cycles of PCR. (https://www.aphis.usda.gov/plant_health/plant_pest_info/citrus_greening/downloads/pdf_files/cg-nprg.pdf). Two DNA samples were conducted from each population.

Results

Genome Size of *T. Radiata*

Based on a genome size of 637 Mbp for *Phaseolus vulgaris* (Arumuganathan and Earle 1991), the female genome size was calculated as 420 Mbp, whereas the male genome size was 210 Mbp based on Equation 6-1.

DNA Extraction

The DNA initially extracted from the insect samples was contaminated with RNA as indicated by significant decreased $A_{260/280}$ absorbance ratio after purification expected to be 1.8 for pure DNA (Desjardins & Conklin 2010). Both Nanodrop (Table 6-2) and the agarose gels (Fig. 6-1) showed that the purification procedure eliminated most RNA contamination.

Sequencing One Single Copy Gene

DNA amplified using Eukaryotic 28s rDNA primers and EF primers (Fig. 6-2) showed that *T. radiata* DNA extraction was successful. However, two clear bands

appeared when amplified using EF primers. Sequencing indicated that the upper band was an artifact based on the poor blasting results and the sequence of the lower band of 462 bp in total showed 86% identity to *Quadrastichus erythrinae* elongation factor 1 alpha (EF) (Rubinoff et al. 2010). Thus the elongation factor sequenced could be considered as single copy gene which could be used for DNA quantification with sequence. The elongation factor sequence was:

GCCTGGTTCAAGGGATGGGCTGTTGAGCGCAAAGAAGGCAAAGCCGACGGCAAA
TGCCTCATCGAAGCTCTCGATGCCATCCTCCCACCCAGCAGACCCACTGACAAAG
CCCTTCGTCTTCCCCTCCAGGTGATTACAATGATGCATCTTTCATGCTATTTACTCC
AAATTACTATTAAATATGATGAGAACCCTAAAACTTCATTCGCGGTTTCCACCGGAG
GATCTTATCCAGTGACGGCCGAAGGCGTCTGACTCAATATTGTTGAAAGAATATAG
TTCACCTTTTTCTTTAACATCAAACTAATGTAACTTTTTTCTCTTGCAGGACGTGTAC
AAGATTGGTGGTATCGGAACGGTACCCGTCGGTCGTGTCGAAACTGGTGTTCTGA
AGCCCGGTATGGTCGTTACCTTCGCTCCAGTCGGTCTGACCACCGAAGTCAAGTC
TGTGGAGATGCACCA,

Real-Time PCR

DNA from the two colonies required the same number of cycles to achieve an equal DNA concentration level, confirming that the initial quantity of *T. radiata* DNA tested was the same for both populations. This result was consistent with the conclusion that symbiont infestation levels in both colonies were comparable (Fig. 6-3).

Detecting Different Bacteria

All bacteria detected in both colonies showed multiple bands (Fig. 6-4), which may include the target sequence, other stains of the bacteria, or artifacts. However, the

bands of each bacterial category showed different patterns between the two colonies, for instance Wolbachia (5th lane on gels in Fig. 6-4).

Eubacteria Coloning and Sequencing

A total of 96 clones from each *T. radiata* colony were analyzed. Sequences of the eubacteria amplification from the Gainesville colony indicated eight different groups with each matched sequence showing 96%- 100% identity (Fig. 6-6). Sequences of eubacteria amplification from the Immokalee colony indicated four different groups, with each matched sequence achieving 97%- 100% identity (Fig. 6-7). The dominant Eubacteria species in Gainesville colony was *Burkholderia* sp, which consisted of 75% of the entire bacteria species detected, whereas the dominant species in Immokalee colony was *Staphylococcus saprophyticus* 61%.

Detection of *Ca.* L. Asiatius Using Real-Time PCR

Results showed that *D. citri* from SWFREC, Dundee, Gainesville colony and Immokalee colony were positive with an average Ct value of 31.77, 15.47, 34.88 and 32.66 respectively. *Ca.* L. asiatius was not detected in *T. radiata* populations from either colonies or the field using *Ca.* L. asiatius specific primers (Li et al. 2006), even if Fig 6-4 showed otherwise, since there was no detectable increase in fluorescent signal above background in the 40 cycles of PCR.

Discussion

It was reported that insects have a moderate sized genome, and the average genome size was 504 Mbp (He et al. 2016). The *T. radiata* female genome was estimated at 420 Mbp, exactly twice that of males at 210 Mbp. This was the expected result in that *T. radiata* is an arrhenotokous ectoparasitoid with haploid males and

diploid females. *T. radiata* has a medium genome size because hymenoptera genome size was reported to vary from 160 Mbp to 654 Mbp (Hanrahan & Johnston 2011).

DNA extracted from psyllids in both lab colonies showed multiple bands (approximately 2000- 4000 bp), which were removed by an RNase-based purification procedure indicating that the multiple bands were double-stranded RNAs, whose identities need further study. The presence of a single copy gene based on the *Q. erythrinae* elongation factor 1 alpha of 462 bp was verified and will be a useful tool for future research i.e. quantitative real-time PCR of *T. radiata*.

Real-time PCR suggested that the bacterial infestation levels in general were similar in both *T. radiata* colonies. The observed difference in fitness was not due to the amount of bacterial DNA. However, the dominant Eubacteria species detected in the Gainesville colony was *Burkholderia* sp, which was 75% of the entire bacteria species detected, whereas, it only accounted for 18% of the bacteria species found in the Immokalee colony. *Burkholderia* sp. has been reported to significantly improve the fitness of the broad-headed bug *Riptortus clavatus*, which acquired the bacteria from the environment. Body length and weight were reported to be significantly greater in *Burkholderia* sp. infected *R. clavatus* than uninfected insects, and the *Burkholderia* sp. infection is the principle factor (Kikuchi et al. 2007).

In contrast to the Gainesville colony, the dominant species in the Immokalee colony was *S. saprophyticus,* which was 61% of the entire bacteria species detected. This species was not detected in Gainesville colony although the sample size of 96 was small. *S. saprophyticus* specific primers could be designed later to determine its presence in the Gainesville colony. *S. saprophyticus* was less frequently reported in

insects but from other living systems (Goja et al. 2013). It was reported to digest cellulose in termite guts (Paul et al. 1986), and was also isolated from B and Q types of *Bemisia tabaci* (Indiragandhi et al. 2010), from the gut of red imported fire ant *Solenopsis invicta* (Peloquin & Greenberg 2003), and from the mealy bug species *Rhizoecus amorphophalli* (Sreerag et al. 2014). Unfortunately, the effects of *S. saprophyticus* on insects are not well known.

Achromobacter sp. found in the Immokalee colony may include many species, but *Achromobacter nematophilus* was reported to be extremely lethal to the larvae of *Galleria mellonella* (Poinar & Thomas 1967).

Tamaraxia radiata from both colonies were not infected with *Ca.* L. asiatius in this study. Future research will focus on developing *Ca.* L. asiatius specific conventional PCR primers to detect its presence, which will be cheaper and more convenient than the real time PCR.

Table 6-1. Primers used to detect different microorganisms.

Microorganism	Gene name	Left primer Right primer	PCR product size	Annealing temperature	Citation
Archaea	16S rRNA	5'-TTCCGGTTGATCCYGCCGGA-3' 5'-YCCGGCGTTGAMTCCAATT-3'	915	64 °C	(Delong, 1992)
Eubacteria	16S rRNA	5'-GAGAGTTTGATCCTGGCTCAG-3' 5'-CTACGGCTACCTTGTTACGA-3'	1400	60 °C	(Weisburg et al., 1991)
Ca. L. asiaticus	16S rRNA	5'-GTGCCAGCAGCCGCGGTAATAC-3' 5'-CACCTGTGTAAAGGTCTCCG-3'	535	55 °C	(Subandiyah et al., 2000)
Arsenophonus	16S rRNA	5'-GTGCCAGCAGCCGCGGTAATAC-3' 5'-CACCTGTCTCAGCGCTCCCG-3'	535	64 °C	(Subandiyah et al., 2000)
Wolbachia *wsp*A	16S rRNA	5'-TGGTCCAATAAGTGATGAAGAAAC-3' 5'-AAAAATTAAACGCTACTCCA-3'	601	64 °C	(Braig et al., 1998)
Caulobacter	16S rRNA	5'-CTGGACCGCCACAGAGAT-3' 5'-CCTTCGGGTAAAGCCAACTC-3'	435	60 °C	(Meyer & Hoy, 2008)
Methylobacter	16S rRNA	5'-GAGATCCAGGGTCCTCTTCG-3' 5'-CCGTCGGGTAAGACCAACT-3'	422	64 °C	(Meyer & Hoy, 2008)

Table 6-2. Psyllids and *T. radiata* DNA before and after purification assessed by Nanodrop

	Psyllids DNA		*T. radiata* DNA	
	Before	After	Before	After
$A_{260/280}$	1.98- 2.21	1.87- 1.94	2.18- 2.21	1.85- 1.98
Concentration (ng/ul)	1446- 1895	363- 566	968- 1335	160- 244

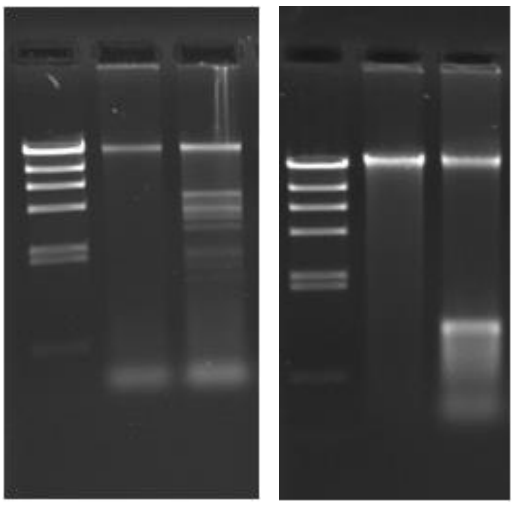


Figure 6-1. A. Agarose gel electrophoresis of psyllids DNA before and after purification. B. Agarose gel electrophoresis of *T. radiata* DNA before and after purification. On each gel picture, lane 1 was lambda DNA/Hind III molecular marker, lane 2 was DNA after purification, and lane 3 was DNA before purification

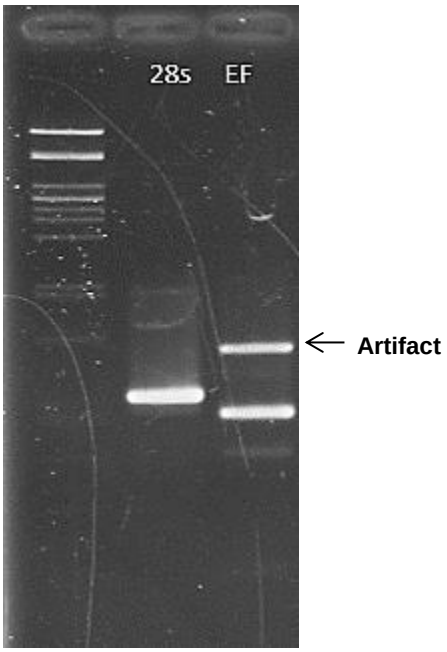


Figure 6-2. PCR results analyzed using 2% agarose gel electrophoresis of DNA that was extracted from a *T. radiata* lab colony in SWFREC. Amplification of DNA extracted from a Gainesville lab colony and the field showed the same pattern, so they were not presented here. Lane 1 was the lambda DNA

digested by PstI, lane 2 was the amplification using Eukaryotic 28s rDNA primers, and lane 3 was the amplification using EF primers

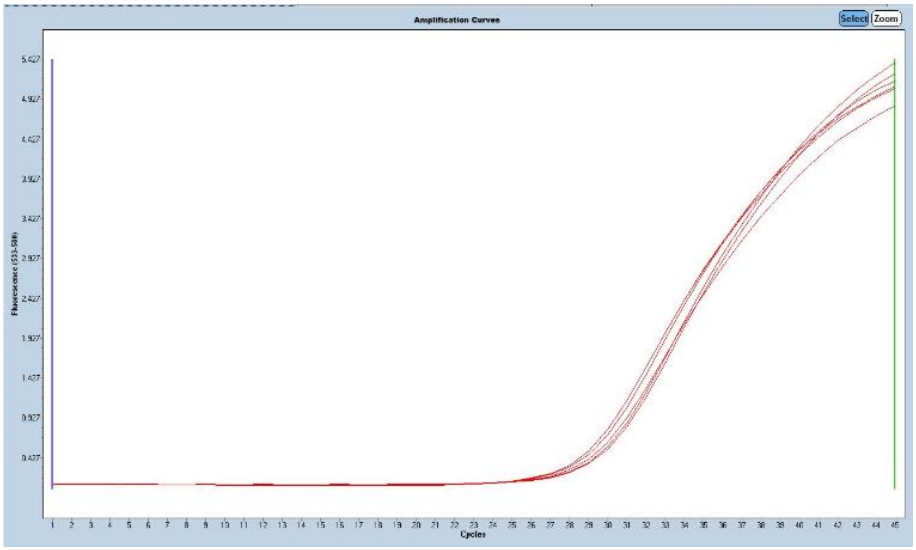
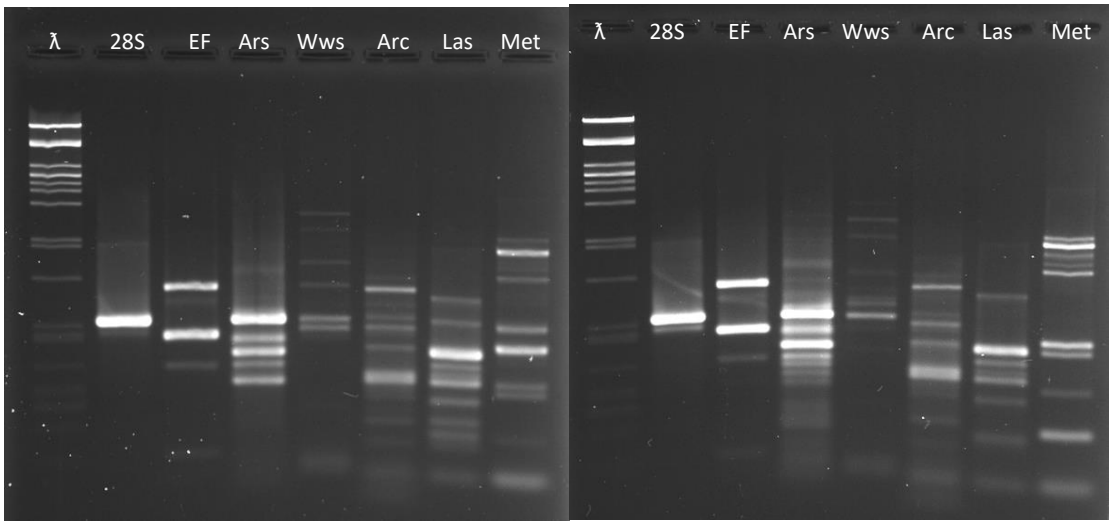


Figure 6-3. Real-time PCR of *T. radiata* from both lab colonies using TEF primers



A B

Figure 6-4. A. Bacteria detected in *T. radiata* DNA from the Immokalee lab colony. B. Bacteria detected in *T. radiata* from the Gainesville lab colony. On each gel, lane 1 was the lambda DNA digested by PstI, lane 2 was amplification of eubacteria 28S rDNA, lane 3 was amplification of elongation factor, lane 4 was amplification of *Arsenophonus,* lane 5 was amplification of Wolbachia *wsp*A, lane 6 was amplification of Archaea, lane 7 was amplification of Las, and lane 8 was amplification of *Methylobactor*.

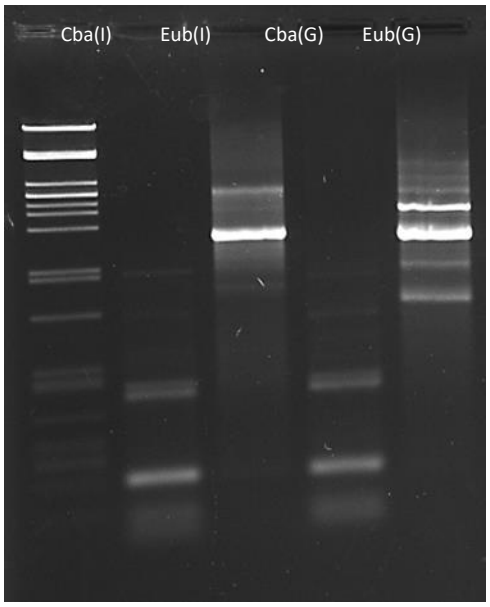


Figure 6-5. *Caulobacter* and eubacteria amplification in *T. radiata* DNA extracted from Immokalee and Gainesville lab colonies respectively. Lane 1 was the lambda DNA digested by PstI, lane 2 was *Caulobacter* amplification in the Immokalee *T. radiata* lab colony, lane 3 was Eubacteria amplification in the Immokalee *T. radiata* lab colony, land 4 was *Caulobacter* amplification in the Gainesville *T. radiata* lab colony, lane 3 was Eubacteria amplification in the Gainesville *T. radiata* lab colony.

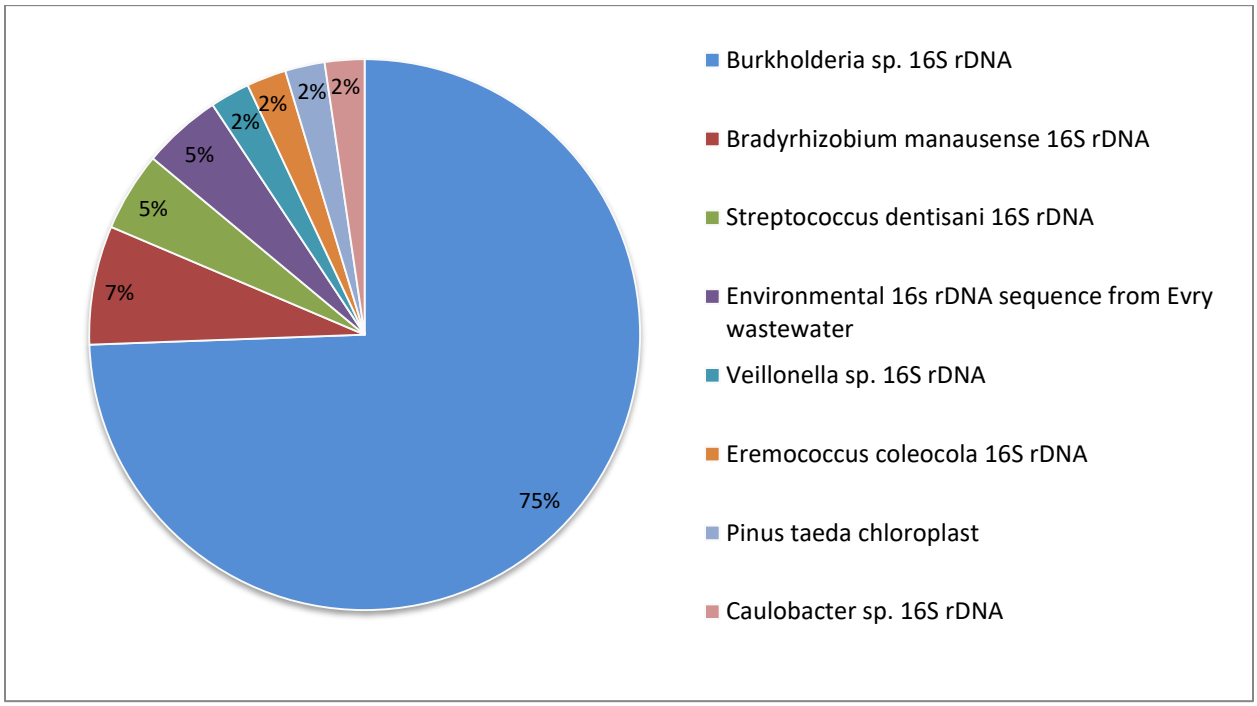


Figure 6-6. Sequences of eubacteria amplification in the *T. radiata* Gainesville lab colony

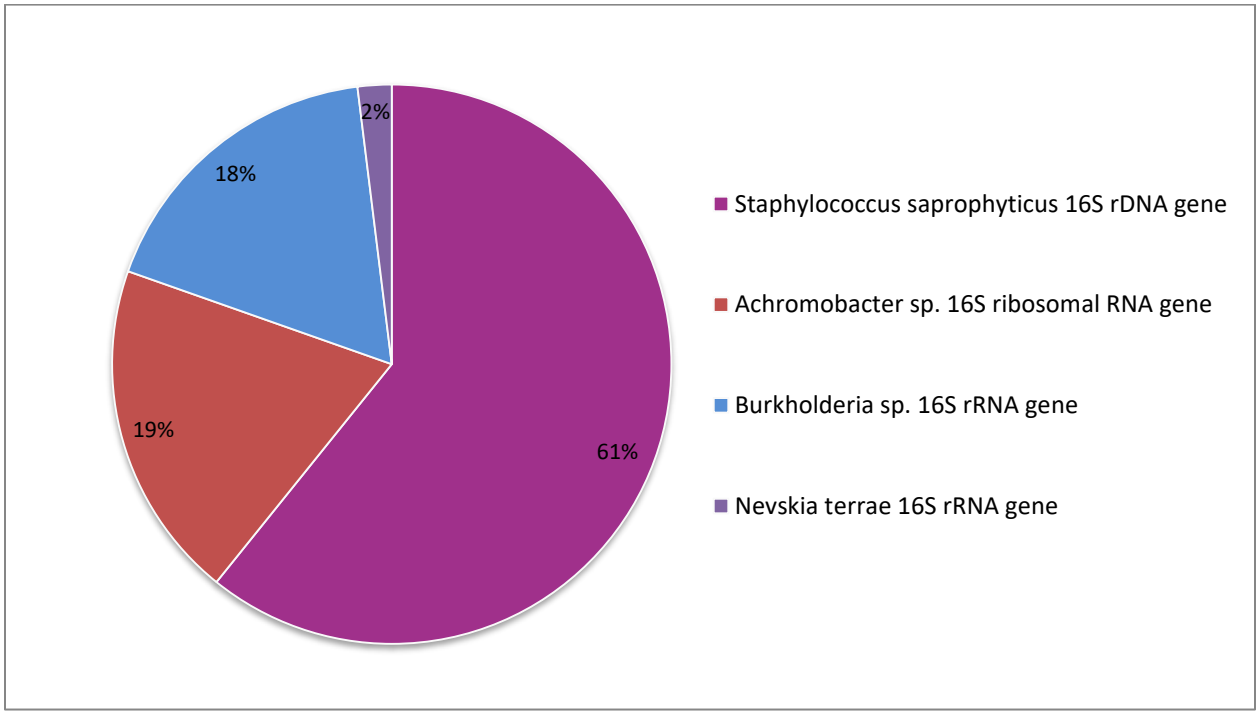


Figure 6-7. Sequences of eubacteria amplification in the *T. radiata* Immokalee lab colony

CHAPTER 7
CONCLUSION

The main objective of my dissertation was to determine and evaluate different factors that may impact efficiency of *T. radiata* as a parasitoid of *D. citri* under mass rearing and field releasing conditions. I concentrated on the following approaches: (1) evaluated the functional response of *T. radiata* to different host densities and validated the biological significance the functional response model based on behavioral observations, (2) confirmed the existence and evaluated the biological function of a host marking pheromone released by the parasitizing wasp that serves to distinguish it from unparasitized hosts, (3) evaluated the different endosymbionts in two *T. radiata* colonies, with the hope parsing out possible fitness effects on population fitness, and (4) assessed releasing rates, releasing sex ratio, and photoperiods to optimize mass production of *T. radiata*, and assess the costs and benefits of an augmentative biological control program.

The functional response study showed a typical type II relationship with the increasing host density meaning a reduced proportion hosts were parasitized when the ACP host density increased. The number parasitized increased to a maximum of 11.2/day from the lowest density through 40 hosts per female, then remained relatively constant through 60 hosts per wasp, indicating an upper limit had been reached. *T. radiata* was able to search 12.7±1.21 cm^2/h based on the random parasitoid equation, which was very close to previous research results. Handling time was over estimated as 0.87 h. Behavioral observations indicated that females spent approximately 3.9 min to probe and parasitize a host, and attack coefficient decreased with the increasing host density, which was inconsistent with a basic assumption of functional response equation

that attack coefficient remains constant regardless of host densities. I hypothesized that this inconsistency occurred because the functional response equation only took handling and searching time for consideration, ignoring other time consuming activities such as grooming and resting. Therefore, a modified functional response equation was proposed to better define the relationship between parastisim rate and host density which took into consideration of the decreasing attack coefficient. This resulting model fit the data better and was suggested for use other parasitoid species which also typically spend time grooming and resting besides simply searching and parasitizting or host feeding. Interestingly, it was also discovered that reducing searching area resulted in a higher host rejection rates, and that proportion of time spend searching in smaller areas was significantly greater. This result suggested that the existence of a host marking pheromone left by the parasitizing *T. radiata* female to deter further oviposition that, in addition to protecting an individual from superparasitism also exerted an effect on surrounding hosts in the “patch” under conditions of high host density and limited searching area.

Five experiments were set up to confirm the existence of the host mark and elucidate its function. Results confirmed that *T. radiata* females released an external host mark upon oviposition to discriminate parasitized hosts from unparasitized hosts. By tapping the antennae on the surface of the host the female wasps were able to detect the existence of the host which could not be detected at a distance within 24 h and thus was not volatile. The ‘mark’ was considered to be a chemical cue, since no physical changes were observed on the parasitized hosts, and the parasitoid eggs did not serve as the source of the mark. A patch marking effect was observed within 1 h,

but could no longer be detected after 24 h because most of the hosts have moved off the flush. Therefore, it is still possible that a patch marking effect released on the surface of where the hosts located instead of or in addition to on the hosts directly. Such a patch mark could reduce the proportion of host parasitized in a colony.

Another factor that might deter the *T. radiata* production was loss of insect fitness due to a pathogen or lack of a symbiont. It was observed that the production at SWFREC is inferior to that at Gainesville despite similar methodology, raising the possibility of pathogen or symbiont effects on fitness. Molecular research was conducted to compare bacteria present in wasps from the two colonies. Results showed that the dominate eubacteria species detected in Gainesville colony was *Burkholderia* sp, which was 75% of the entire bacteria species detected, whereas, it only accounted for 18% of the bacteria species found in Immokalee colony. *Burkholderia* might be considered beneficial since it was reported as a gut symbiont that significantly improved the fitness of the bean bug *Riptortus clavatus* (Heteroptera: Alydidae). Also in contrast to the Gainesville colonly, the dominate species in Immokalee colony was *S. saprophyticus,* which was 61% of the entire bacteria species detected. This speices was not detected in Gainesville colony, and its effects on insects fitness remained unclear although it is reported as a human pathogen. Importantly, *T. radiata* from both colonies were not infected with *Ca.* L. asiatius in this study.

I was able to improve the methodology of mass rearing *T. radiata* by optimizing the release rates, sex ratio, and photophase. Based on my research, releasing 20 ACP adults per flush was able to provide a good nymph density for *T. radiata* production. Sixty *T. radiata* females per cage, or a little more than 1 female per infested shoot, was

optimal to produce the most amount of *T. radiata*, and a photophase of 12 h was need to achieve the highest fecundity. However, progeny sex ratio was not influenced by sex ratio of released wasps ranging from 1:3 to 4:1 female: male. The cost of rearing a single *T. radiata* using current methods as estimated at approximatly $0.11 to produce one *T. radiata* in the two mass rearing facilities in Florida. An equation (Equation 5-5) originally derived to predict production loss based on cumulated number of *D. citri* adults obtained in a season by tap sampling was used to estimate the economic benefit of *T. radiata* based on the average proportion parasitized over the same time period and at the same location. These estimates were used for an economic analysis of cost and benefit which could be used to indicate level of percent parasitism necessary to justify the release of a given number of *T. radiata*.

APPENDIX
SUPPLEMENTARY MATERIALS OF CHAPTER 3

When 10, 4th instar nymphs were provided, the 10 observed female wasps exhibited 654 behavioral events (Fig. A-1). Of the 189 host-searching events, 140 (74.1%) led to antennating of a nymph. One hundred and twenty eight (49.2%) out of the 260 antennation events led to host probing, and 130 (50%) led to rejection and searching for hosts again. Twenty one (16.4%) out of the 128 probing events led to a successful oviposition, and 83.6% led to rejection followed either by host searching or antennating. Host feeding was observed twice. Forty eight grooming events were observed and 95.8% led to host searching.

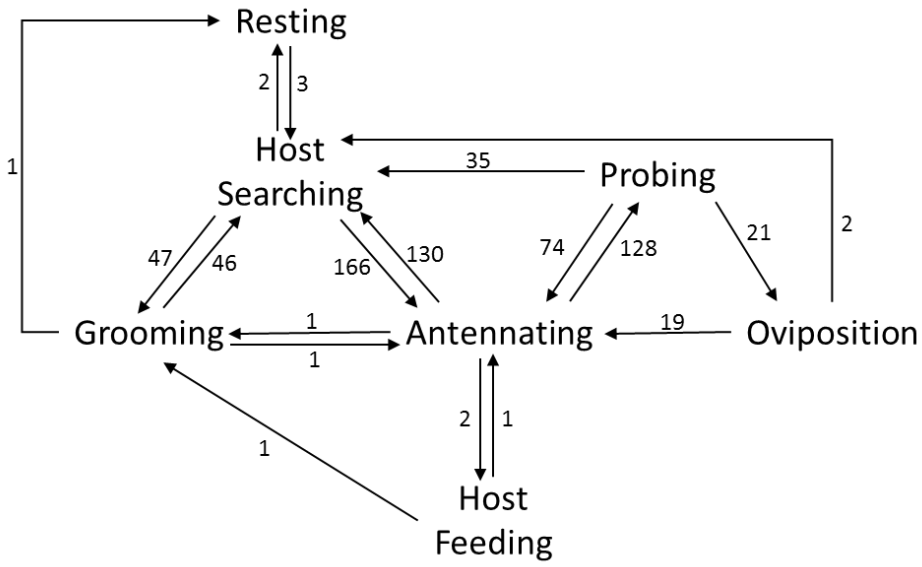


Figure A-1. Behavior diagram at density 10

When 20, 4th instar hosts were provided, the observed wasps exhibited 673 behavioral events (Fig. A-2). Of 224 host-searching events, 171 (76.3%) led to antennating of a nymph. One hundred and seven (42.6%) out of the 251 antennation led to host probing, four led to host feeding, and 2 led to honeydew feeding, the rest

55% resulted in rejection and searching for hosts again. Fifteen out of the 107 (14%) probing events led to successful oviposition, and 86% led to rejection followed either by host searching or antennating. Sixty grooming events were observed and 78.3% led to host searching.

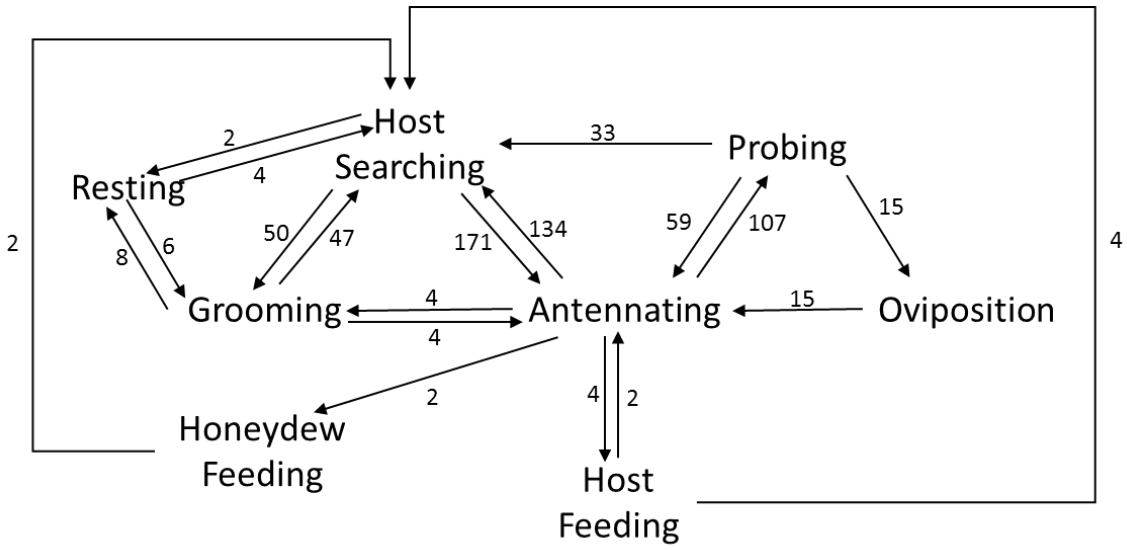


Figure A-2. Behavior diagram at density 20

When 30, 4th instar nymphs were provided, the observed wasps exhibited 783 behavioral events (Fig. A-3). Of the 288 host-searching events, 247 (85.8%) led to antennating of a nymph. Eighty eight (28%) out of the 314 antennation led to host probing, two led to host feeding, and 2 led to honeydew feeding, the rest 70.7% resulted in rejection and searching for hosts again. Twenty six out of the 88 (29.5%) probing events led to successful oviposition, and 69.3% led to rejection followed either by host searching or antennating. Fifty five grooming events were observed and 69.1% led to host searching.

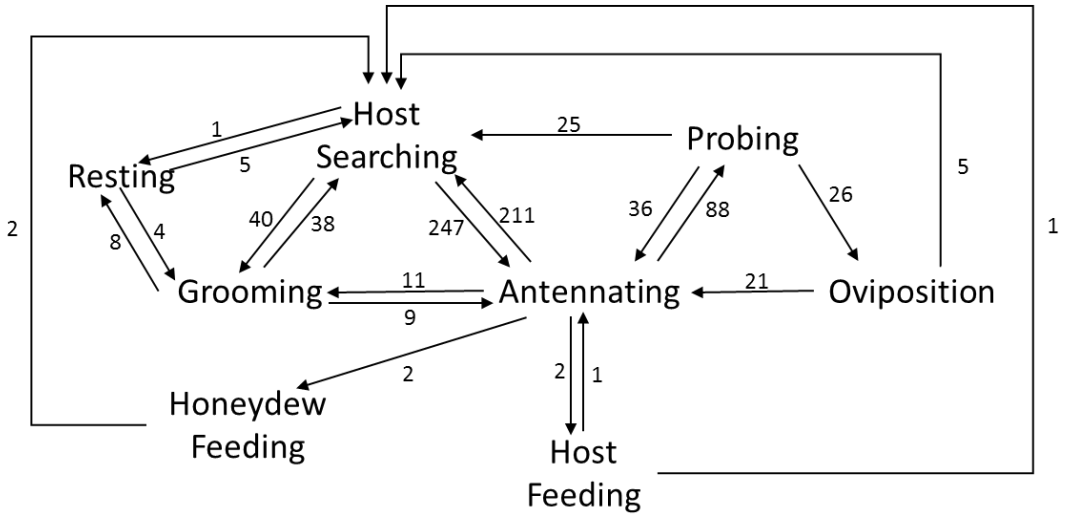


Figure A-3. Behavior diagram at density 30

When 50, 4th instar nymphs were provided, the observed wasps observed exhibited 843 behavioral events (Fig. A-4). Of the 302 host searching events, 250 (82.8%) led to antennating of a nymph. One hundred and twelve (32.8%) out of the 341 antennation led to host probing, four led to host feeding, and 1 led to honeydew feeding, the rest 65.7% resulted in rejection and searching for hosts again. Twenty three out of the 112 (20.5%) probing events led to successful oviposition, and 79.5% led to rejection followed either by host searching or antennating. Fifty nine grooming events were observed and 88.1% led to host searching.

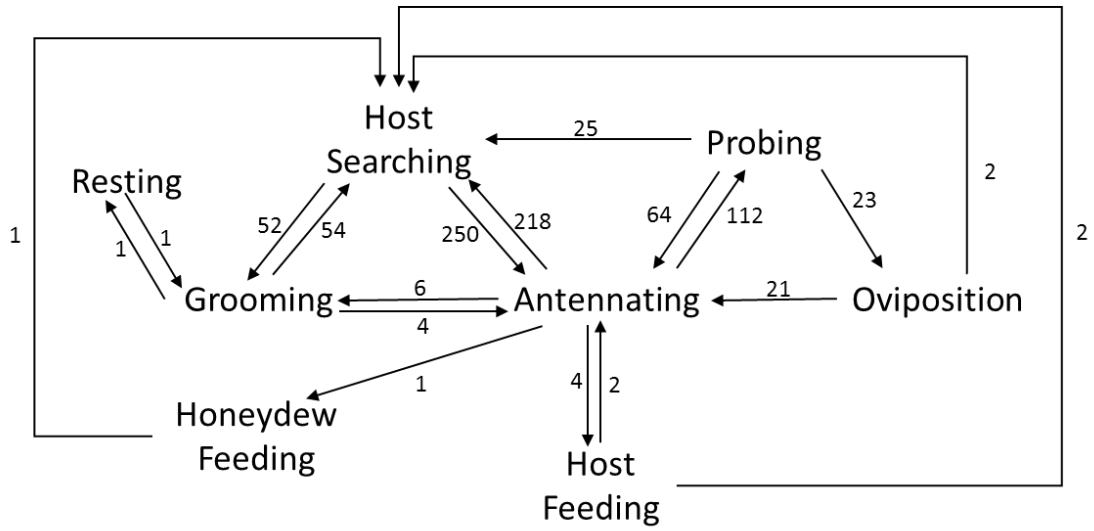


Figure A-4. Behavior diagram at density 50

When 60, 4th instar nymphs were provided, the observed female wasps observed exhibited 996 behavioral events (Fig. A-5). Of the 359 host searching events, 307 (85.5%) led to antennating of a nymph. One hundred and twenty five (30.3%) out of the 413 antennating events led to host probing, four led to host feeding, and 2 led to honeydew feeding, the rest 68.3% resulted in rejection and searching for hosts again. Twenty three out of the 125 (18.4%) probing events led to successful oviposition, and 81.6% led to rejection followed either by host searching or antennating. Fifty nine grooming events were observed and 71.2% led to host searching.

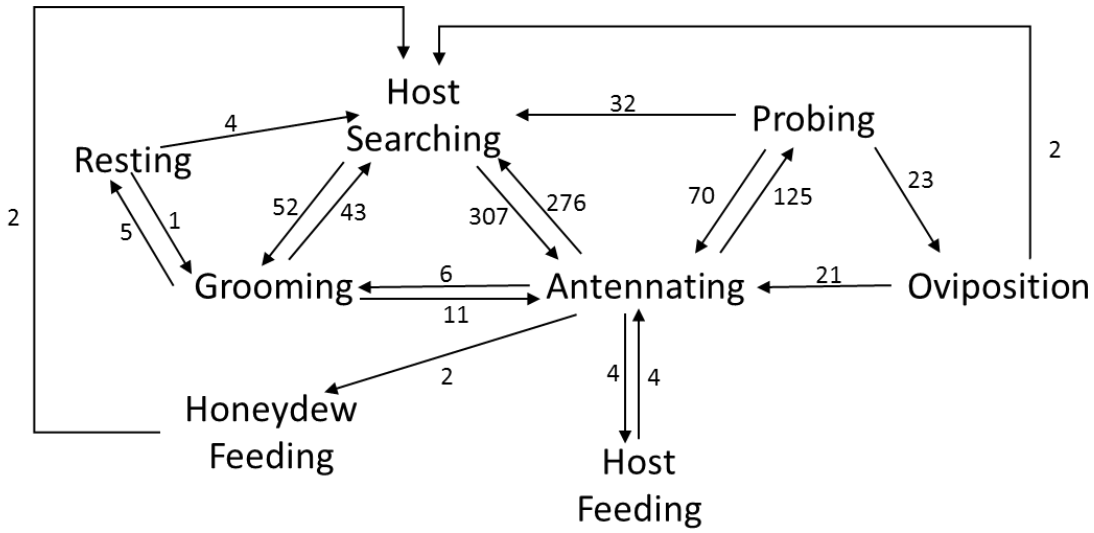


Figure A-5. Behavior diagram at density 60

Table A-1. Frequency (Mean±SEM) of each behavior at six host densities

Density	Searching	Antennation	Probing	Grooming	Resting	Host feeding	Honeydew feeding	Oviposition
10	19.7±1.2 B	23.6±2.2B	13.0±2.4	4.9±0.9	0.3±0.2	0.2±0.1	0	1.7±0.5
20	21.8±2.4 B	23.3±2.8 B	9.8±2.3	6.0±0.9	1.0±0.5	0.8±0.2	0.2±0.1	1.0±0.4
30	28.4±5.1 AB	31.5±5.5 AB	8.3±1.6	6.0±0.9	0.7±0.2	0.2±0.1	0.2±0.2	1.9±0.6
40	26.0±4.8 AB	29.3±5.3 AB	10.2±1.5	6.3±0.9	0.5±0.4	0.4±0.2	0.3±0.2	1.4±0.4
50	28.8±4.0 AB	32.3±4.0 AB	11.4±1.7	5.9±1.0	0.1±0.1	0.3±0.2	0.1±0.1	1.6±0.4
60	36.2±4.3 A	40.9±5.5 A	11.7±1.5	6.2±1.0	0.4±0.2	0.4±0.2	0.4±0.2	1.3±0.4

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BIOGRAPHICAL SKETCH

Xulin Chen focused her research on Integrated Pest Management on the Asian citrus psyllid. She graduated with a bachelor's degree in plant quarantine from Shandong Agriculture University in China. She obtained a Master of Science degree in entomology and nematology under the supervision of Dr. Phil Stansly at the University of Florida. She continued her graduate studies at the same university, beginning her Doctor of Philosophy degree program in entomology and nematology in summer 2013. Xulin received her Ph.D. from the University of Florida in the spring of 2016.