Behavioral Response of *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae) to Volatiles Emanating from *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) and Citrus

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Abstract Tamarixia radiata Waterston (Hymenoptera: Eulophidae) is an effective idiobiont ectoparasitoid of the psyllid Diaphorina citri Kuwayama (Hemiptera: Psyllidae), vector of the huanglongbing (HLB). We examined the olfactory responses of T. radiata to volatiles emanating from D. citri or plant volatiles using a custom designed T-maze olfactometer and open arena bioassays. We also examined the behavioral response of male and female T. radiata to conspecifics of the opposite sex to determine whether olfactory signals mediate mate location. T. radiata adults exhibited a sexually dimorphic response to volatiles emanating from D. citri and citrus. Female T. radiata responded positively to the odors emanating from *D. citri* nymphs in both olfactometer and open arena bioassays. However, female wasps showed no response to odors emanating from D. citri adults, D. citri honey dew secretions, intact citrus or orange jasmine leaves. Odors emanating from D. citri damaged citrus were not attractive to T. radiata females but stimulated attraction of wasps to D. citri on damaged plants. T. radiata females were not attracted to D. citri immatures when they were presented as visual cues. Male T. radiata did not show attraction to D. citri nymphs or other putative odors that were attractive to female T. radiata. In olfactometer bioassays, more male T. radiata responded to the odor of female conspecifics than blank controls in the absence of associated citrus host plant volatiles. Odors emanating from female T. radiata were not attractive to male T. radiata. Male or female T. radiata were not attracted to the

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odors emanating from same sex conspecifics. Both male and female *T. radiata* adults showed positive phototactic behavior. Collectively, our results provide behavioral evidence that: 1) female *T. radiata* use volatiles emanating from *D. citri* nymphs to locate its host and: 2) female *T. radiata* release a volatile pheromone that attracts male conspecifics.

Keywords *Tamarixia radiata* · Asian citrus psyllid · huanglongbing · citrus · sex pheromone

Introduction

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), is one of the most destructive pests of citrus because of its ability to vector the causal pathogen of huanglongbing (HLB) disease (Halbert and Manjunath 2004; Bové 2006). HLB infected trees produce bitter, inedible, and misshapen fruits and eventually die within 5-10 years of infection (Aubert 1990; Bové 2006). Currently all possible vector and disease control methods are being employed to manage this disease in Florida including biological control with Tamarixia radiata (Waterston) (Hymenoptera: Eulophidae) and Diaphorencyrtus aligarhensis (Shafee, Alam and Agarwal) (Hymenoptera: Encyrtidae) (Hoy et al. 2006; Qureshi and Stansly 2009; Mann and Stelinski 2010). T. radiata is an effective ectoparasitoid of D. citri. Release of this parasitoid revived the citrus industry in Reunion Island after its introduction from India in 1978 (Étienne et al. 2001). Furthermore, T. radiata caused substantial decline in D. citri populations in Guadeloupe Island within 1 yr of release (Aubert and Quilici 1984; Étienne et al. 2001). T. radiata was introduced into Florida in 1999 after D. citri was detected in 1998 (Hoy et al. 2006). The parasitoid dispersed quickly and established throughout the major citrus-growing regions of the State (Hoy et al. 1999; Hoy and Nguyen 2001). The parasitoid also dispersed to adjoining regions such as Puerto Rico where it was not released intentionally (Pluke et al. 2008). However, considerable variation has been reported in its potential effectiveness in controlling D. citri in Florida and other adjoining regions (Tsai et al. 2002; Michaud 2004; Pluke et al. 2008; Qureshi et al. 2009).

The adult female *T. radiata* lays one or occasionally two eggs beneath a *D. citri* nymph. A single *T. radiata* female can deposit up to 300 eggs over the course of its life (Hoy et al. 2006; Mann and Stelinski 2010). The newly hatched parasitoid larva feeds on hemolymph from the site of attachment, eventually killing the host. The adult parasitoid emerges from the thorax, leaving a distinctive emergence hole. *T. radiata* females parasitize all immature stages of *D. citri* and show a significant preference for 5th-instar nymphs (Chien et al. 1991; Hoy et al. 2006). The females also feed on honey dew excreted by psyllids (Hoy et al. 2006). Female *T. radiata* may obtain protein for egg development by feeding on hemolymph from psyllid nymphs, which is accessed through ovipositor-induced punctures (Hoy et al. 2006).

The reproductive success of parasitoids is determined by their ability to effectively locate mates and hosts, and to avoid natural enemies and unsuitable environmental conditions (Dicke and Grostal 2001). Hymenopteran insects exhibit behavioral complexity and great diversity in their dispersal and mating behavior, and

in the chemistry of their exocrine secretions (Ayasse et al. 2001). Chemical signaling is the most common method employed in sex attraction, mate finding and host location by hymenopteran parasitoids (Vinson 1991; Ayasse et al. 2001). Wasps perceive pheromones via specialized olfactory sensilla located on their antennae (Isidoro et al. 1996; Ayasse et al. 2001). Recently, Onagbola et al. (2009) described possible pheromone and plant volatile detecting sensilla on the antennae of *T. radiata*.

Given that *T. radiata* is a classical biological control agent for *D. citri*, substantial information is available on its rearing methodology, distribution, parasitization success, and developmental biology (McFarland and Hoy 2001; Étienne et al. 2001; Michaud 2002; Pluke et al. 2008; Qureshi et al. 2009). However, information on its mate finding and host recognition behavior is lacking. In the current investigation, we examined the behavioral responses of *T. radiata* to olfactory stimuli emanating from *D. citri* nymphs and adults as well as citrus to determine the mechanisms of host location. In addition, we examined the behavioral response sex to determine the olfactory cues used by *T. radiata* for mate location.

Materials and Methods

Insects

Tamarixia radiata adults were reared on *D. citri* maintained on *Murraya paniculata* (orange jasmine). Insects were reared in a glass house and maintained at $25-27^{\circ}$ C, 60-80% RH, and 14 : 10 (L : D) h photocycle. Prior to bioassays the adult wasps were segregated into $40 \times 40 \times 40$ cm Plexiglas cages at $26\pm1^{\circ}$ C, $60\pm5\%$ RH, under 14:10 (L:D) h photocycle and provided with 25% sugar solution. Adult and immature *D. citri* used in behavioral bioassays were obtained from a laboratory colony described in Wenninger et al. (2008). The culture was established in 2000 from field populations in Polk Co. FL, USA (28.0'N, 81.9'W) prior to the discovery of HLB in FL. The culture was maintained without exposure to insecticides on sour orange (*Citrus aurantium* L.) and 'Hamlin' orange [*C. sinensis* (L.)] seedlings at $27\pm1^{\circ}$ C, $63\pm2\%$ RH, and L14:D10 photoperiod.

Behavioral Response of T. radiata to Geotactic and Phototactic Cues

Prior to evaluation of olfactory responses, the geotactic and phototactic responses of *T. radiata* were examined using a glass Y-tube olfactometer (Analytical Research Systems, Gainesville, FL, USA). The Y-tube olfactometer consisted of a 14-cm long stem and two 10 cm long arms, each with a 2-cm inner diameter. A screened glass plug at the base of the stem was used to introduce insects into the Y-tube. To ensure chemical free ambient air supply, both the arms of the olfactometer received charcoal purified and humidified air from a custom made air delivery system (ARS, Gainesville, FL). For determining the geotactic response, the Y-tube olfactometer was placed in either vertical or horizontal positions under a fluorescent 1600 lux light source (GE Lighting, Cleveland, OH) mounted within a $1.0 \times 0.6 \times 0.6$ m fiber board box for uniform light diffusion. When the Y-tube was positioned vertically, the

light intensities at the point of insect release, junction of two arms, and exit point were ~700, 900 and 1000 lux, respectively. When the olfactometer was positioned horizontally, the measured light intensity was ~700 lux throughout the entire Y-tube. *T. radiata* adults of mixed sex were released individually ($n \ge 60$) at the base of the Y-tube and allowed 300 s to exhibit a behavioral response. The treatments compared were: 1) vertical and 2) horizontal position of the Y-tube. The number of wasps leaving the release point was recorded. A positive response was recorded when a wasp moved from the stem and entered into either arm of the olfactometer. All assays were conducted at $26\pm1^{\circ}$ C and $60\pm2\%$ RH.

To quantify the phototactic response of *T. radiata*, one arm of the olfactometer was covered with black cloth and the other arm was left uncovered. The Teflon tubing connecting the air delivery system to the olfactometer arm was also covered with black cloth to avoid light penetration into the shaded arm. A minimum of 60 *T. radiata* adults of mixed sex were released individually at the base of the stem and given 300 s to exhibit a behavioral response by entering either olfactometer arm. The number of adults entering the covered or uncovered arm was recorded. The olfactometer was positioned within the fiber board box in vertical or horizontal positions as described above for the geotactic response assays.

Behavioral Response of T. radiata to Host Related Olfactory Cues

A custom designed two-port divided T-maze olfactometer [Analytical Research Systems (ARS), Inc., Gainesville, FL] thoroughly described in Mann et al. (2010) was used to evaluate the behavioral responses of *T. radiata* to odors emanating from *D. citri* or citrus. The T-maze olfactometer consisted of a 30 cm glass tube that was bifurcated into two equal halves with a Teflon strip forming a T-maze. Each half served as an arm of the olfactometer enabling *T. radiata* to make a choice among two potential odor fields. The odor sources were placed into solid-phase micro-extraction chambers (SPMEC) (ARS, Gainesville, FL) connected to two olfactometer arms through Teflon[®]-glass tube connectors (Fig. 1). The SPMEC was comprised of a straight glass tube (17.5 cm long×3.5 cm wide) supported with an inlet and outlet valve for incoming and outgoing air streams, respectively. Movement of *D. citri* nymphs and adults from the SPMEC into the connecting tubes was



restricted by a metal mesh filter which was glued to the connecting tube. Odors were pushed through the olfactometer arms using purified and humidified air via two pumps connected to the air delivery system (ARS, Gainesville, FL). A constant airflow of 0.1 L/min was maintained through both arms of the olfactometer. Given that *T. radiata* adults were positively phototactic and numerically more adults responded when the olfactometer was positioned vertically (see results), the olfactometer was positioned in this manner under a fluorescent 1600 lux light bulb within the fiber board box described above for uniform light diffusion. An odor source was randomly assigned to one arm of the olfactometer at the beginning of each bioassay and was reversed every 30 insects to eliminate positional bias.

Prior to odor testing, T. radiata adults were exposed to clean air vs. clean air in the olfactometer to verify the absence of positional bias. Thereafter, the following odor source combinations were tested: 1) intact citrus vs. clean air, 2) D. citri damaged citrus vs. clean air, 3) orange jasmine vs. clean air, 4) D. citri nymphs (4-5th instar) vs. clean air, 5) D. citri male adults vs. clean air, 6) D. citri female adults vs. clean air, 7) D. citri nymphs+citrus vs. clean air, 8) D. citri nymphs + orange jasmine vs. clean air, and 9) D. citri honey dew (wax) secretions vs. clean air. To obtain plants that received D. citri damage or infestation, potted 4-6 week old citrus or orange jasmine plants were placed into a greenhouse containing an existing D. citri culture. Adults psyllids naturally infested experimental plants and 10–12 week old plants used for testing were highly infested by D. citri nymphs. Corresponding non-infested control plants were maintained under similar conditions but in greenhouse without D. citri infestation. Because D. citri are exclusively associated with new growth (Catling 1970), we used ~ 2.0 g of fresh growing shoots for citrus samples. D. citri damaged citrus was comprised of ~ 2.0 g of young citrus flush as defined by Hall and Albrigo (2007) from which D. citri nymphs were removed carefully with a camel hair brush. For D. citri nymphs + citrus, ~2.0 g of young citrus flush with approximately 20 fourth and fifth instar living D. citri nymphs was used. The younger nymphs were removed carefully with a camel hair brush. The male and female adult D. citri samples were comprised of 20 sexually mature adults. The honey dew secretions (10 mg) were collected on filter paper from D. citri-infested citrus flush. A minimum of 120 T. radiata male and female adults were examined per treatment combination. T. radiata adults were released individually into the inlet adapter at the base of the olfactometer. Adults were given 300 s to exhibit a behavioral response by choosing either of the olfactometer arms. The number of adults entering the treatment arm or control arm (clean air) or remaining below the T-maze division (release arm) was recorded. A positive response was recorded when a wasp moved into either olfactometer arm by crossing the division of the T-maze olfactometer. Between treatments, the equipment and connecting tubes were thoroughly cleaned with 2% soap solution and baked at 94°C for ~16 hrs. Male and female T. radiata were tested separately.

The odor sources that were found attractive to *T. radiata* in the olfactometer assays were also tested in an open arena assay. Given that females were more responsive than males in the olfactometer tests, only female adults were tested in open arena experiments. Bioassays for this series of experiments were carried out on a Whatman[®] No.1 filter paper disc (14.0 cm diameter) placed in a Petri dish (14 cm

inner diameter). A 4.0 cm diameter circle was drawn in the center of each filter paper. The filter paper disc was divided into 4 equal quadrants originating from the central circle. Each quadrant was assigned with one of the following treatments: 1) D. citri nymphs (4-5th instar), 2) D. citri nymphs+citrus (~ 20, 4-5th instar, undisturbed D. citri nymphs attached to ~2.0 g of citrus flush), 3) ~2.0 g of young citrus flush (never exposed to D. citri), and 4) an empty quadrant (control). Each treatment was placed within the center of each quadrant along the outer margin of the filter paper. The visual stimulus associated with each treatment was separated for each quadrant by positioning 2.0×5.0 cm filter paper strips along the boundary of each quadrant, which acted as barriers. These strips were held in place with transparent cellophane tape. The side walls of each Petri dish were also covered with filter paper strips to eliminate external visual stimuli. Each complete assay chamber was placed in a temperature controlled room under a fluorescent light bulb (1600 lux, GE Lighting, LLC, OH), fixed within a 1.0×0.6×0.6 m fiber board box for uniform light diffusion. T. radiata adults were released individually at the central circle of each Petri dish and allowed 300 s to exhibit a choice between the four quadrants. A positive choice was recorded when a wasp crossed the circle and entered into one of the quadrants. The number of wasps entering each quadrant was recorded. Four replications of 60 female T. radiata per rep were examined.

Behavioral Response of T. radiata to Host Related Visual Cues

The objective of this series of experiments was to determine whether *T. radiata* adults use visual cues to locate their host. The bioassay procedures were similar to those described for the Petri dish arena evaluation of olfactory cues. However, in this case, treatments were placed within transparent air tight gelatin capsules to eliminate release of odors associated with treatments. The treatments compared were: 1) 20 *D. citri* nymphs (4–5th instar), 2) a male adult *D. citri*, 3) a female adult *D. citri* and, 4) an empty gelatin capsule (control). Each gelatin capsule was placed centrally within each quadrant along the outer margin of the filter paper as described previously. *T. radiata* adults were released individually at the inner circle of the Petri dish chamber and allowed 300 s to exhibit a behavioral response. A response was recorded when a wasp entered one of the possible quadrants. The number of wasps entering each quadrant was recorded. Four replications of 60 female *T. radiata* per rep were examined.

Behavioral Response of T. radiata to Sex Related Olfactory Cues

In this series of experiments, we tested the hypothesis that *T. radiata* use olfactory signals for mate location. We examined the response of males to females vs. clean air as well as to other males vs. clean air. We also examined the responses of females to males, vs. clean air as well as to other females vs. clean air. Each assay was conducted in the T-maze olfactometer according to the methods described above. The olfactometer procedures were identical to those described for the experiment examining response to host related olfactory cues. A minimum of 120 *T. radiata* adults were examined per treatment combination.

Data Analyses

The numbers of wasps leaving versus remaining at the stem of the Y-tube in the geotaxis experiment were compared by χ^2 tests. The numbers of wasps entering the treatment or control arm in the Y-tube or T-maze olfactometer assays were also compared by χ^2 tests. The number of wasps entering quadrants in the Petri dish arena assays were compared with one-way analysis of variance followed by mean separation using Tukey's HSD test. In all cases, the significance level was α =0.05.

Results

Behavioral Response of T. radiata to Phototactic and Geotactic Cues

The number of *T. radiata* leaving the release point of the Y-tube olfactometer in response to clean air only was statistically equivalent (χ^2 =1.58, df=1, p=0.21) between vertical (74%) and horizontal (61%) Y-tube orientations. In experiments that investigated the behavior of *T. radiata* in response to light, 100% of tested wasps entered the uncovered arm of the olfactometer as opposed to the shaded arm. This occurred when the olfactometer was positioned both vertically and horizontally.

Behavioral response of T. radiata to Host Related Olfactory Cues

Significantly more *T. radiata* female adults entered the arm containing *D. citri* nymphs (χ^2 =5.97, df=1, p=0.01), *D. citri* nymphs+citrus (χ^2 =8.05, df=1, p=0.004) and *D. citri* nymphs+orange jasmine (χ^2 =7.45, df=1, p=0.006) than the arm receiving clean air only (Fig. 2a). The behavior of female *T. radiata* was not affected by volatiles emanating from male *D. citri* adults (χ^2 =0.21, df=1, p=0.65), female adults (χ^2 =2.51, df=1, p=0.11), intact citrus (χ^2 =0.32, df=1, p=0.57), damaged citrus (χ^2 =1.56, df=1, p=0.21), orange jasmine (χ^2 =0.31, df=1, p=0.58), and *D. citri* honey dew secretions (χ^2 =0.21, df=1, p=0.64) (Fig. 2a).

The behavior of male *T. radiata* was not affected by volatiles emanating from *D. citri* nymphs ($\chi^2=0.82$, df=1, p=0.36), *D. citri* male adults ($\chi^2=0.22$, df=1, p=0.64), female adults ($\chi^2=2.03$, df=1, p=0.15), intact citrus ($\chi^2=0.06$, df=1, p=0.80), *D. citri* damaged citrus ($\chi^2=1.31$, df=1, p=0.25), orange jasmine ($\chi^2=0.36$, df=1, p=0.55), and *D. citri* honey dew secretions ($\chi^2=0.17$, df=1, p=0.68), *D. citri* nymphs+orange jasmine ($\chi^2=2.07$, df=1, p=0.15) and *D. citri* nymphs+citrus ($\chi^2=0.44$, df=1, p=0.51) (Fig. 2b).

D. citri nymphs+citrus attracted significantly (F=3.71, df=3,12; p<0.05) more *T. radiata* adults than *D. citri* nymphs alone, intact citrus or the control treatment in open arena bioassays (Fig. 3). However, *D. citri* nymphs attracted significantly more *T. radiata* adults than the intact citrus or control treatments (Fig. 3). There were no significant differences between the response of *T. radiata* to citrus alone and the control treatment (Fig. 3).



Fig. 2 Responses of female (a) or male (b) *Tamarixia radiata* when presented with volatiles emanating from *D. citri* and/or citrus. Black bars followed by * are significantly different from white bars (χ^2 test, p < 0.05)

Behavioral Response of T. radiata to Host Related Visual Cues

Overall, 77% of *T. radiata* female adults left the release point and entered one of the four quadrants. However, roughly equivalent numbers of *T. radiata* entered each of the quadrants and there was no significant difference between any of the visual treatments and the control (F=0.71, df=3,12; p<0.56) (Data not shown).



Behavioral Response of T. radiata to Sex Related Olfactory Cues

Significantly (χ^2 =4.49, df=1, p=0.03) more male *T. radiata* adults chose the olfactometer arm containing *T. radiata* females than clean air (Fig. 4a). However, there was no significant difference in male response to other males versus clean air (χ^2 =0.18, df=1, p=0.66) (Fig. 4a). There was no significant difference between the response of female *T. radiata* to males versus clean air (χ^2 =0.31, df=1, p=0.57) or to other females versus clean air (χ^2 =0.49, df=1, p=0.48) (Fig. 4b).

Discussion

The current results provide evidence that *T. radiata* adults are attracted to volatile cues emitted by *D. citri* immatures to locate its host. In addition we provide evidence that male *T. radiata* are attracted to female-produced volatiles suggesting use of a long range sex pheromone. The establishment of *T. radiata* in Florida was rapid (Hoy and Nguyen 2001) suggesting efficient host searching behavior by *T. radiata*. Parasitism levels of *D. citri* nymphs have been reported to reach up to 70% in Réunion Island (Aubert and Quilici 1984). However, parasitism rates in Florida have been variable, averaging less than 20% during spring and summer and increasing to



Fig. 4 Responses of male (a) and female (b) *Tamarixia radiata* to conspecifics of the same or opposite sex in T-maze olfactometer. Black bars followed by * are significantly different from white bars (χ^2 test, p < 0.05)

39–56% in the fall (Qureshi et al. 2009). The current behavioral data indicate that female *T. radiata* primarily rely on olfactory cues for host location. It appears that *D. citri* nymphs are the primary source of attractant for *T. radiata* and only *D. citri* nymphs induce the release of plant volatiles that attract this parasitoid to citrus plants.

T. radiata has been reported to possess mechanosensory sensillae (Type I aporous sensilla trichoidea) on its antennae that are known to detect acoustic or vibrational signals generated by the host during feeding or courtship (Onagbola et al. 2009). Vibrational signaling as a means of communication between the sexes was recently confirmed for *D. citri* (Wenninger et al. 2009). It is possible that *T. radiata* exploit these vibration signals to locate adult *D. citri* to forecast availability of oviposition sites or for direct feeding on *D. citri* adults. It is known that female *T. radiata* may penetrate the cuticle of *D. citri* nymphs with their ovipositor to access hemolymph for feeding. However, it is unknown whether female *T. radiata* also feed on *D. citri* adults in this manner.

Volatiles from intact citrus or *D. citri*-damaged citrus did not attract *T. radiata* suggesting that the parasitoid does not use plant volatiles alone for host location. However, feeding *D. citri* nymphs on citrus leaves were more attractive to wasps than nymphs without citrus leaves suggesting release of attractive volatiles due to an interaction between the herbivore and plant. However, active feeding was necessary to observe this response since uninfested leaves that were previously damaged by *D. citri* nymphs were not attractive to *T. radiata*. Stimuli derived directly from herbivores are generally the most reliable sources of information for host location by parasitoids (Vet and Dicke 1992). However, these chemicals can also emanate from host by-products, food plants or from the interaction between these sources (Vinson 1991, 1997; Vet and Dicke 1992; Steiner et al. 2007). Furthermore, parasitoids exploit herbivore-induced plant volatiles during host location, which are produced to offset the fitness cost associated with herbivore damage (Turlings et al. 1990, 1995; Sabelis et al. 1999; Drukker et al. 2000; Dicke and vanLoon 2000; Carroll et al. 2006; Dicke 2009).

Female T. radiata were attracted to D. citri nymphs, but males were not. These results suggest that males do not exploit the chemical signals associated with host location to locate rendezvous sites for mating. This behavioral difference between males and females is consistent with sexual dimorphism of the antennal sensory structures of T. radiata (Onagbola et al. 2009). There are significantly more multiporous placoid sensilla (MPS) on the antennae of females than males, which are associated with detection of host related chemicals (Onagbola et al. 2009). Male T. radiata were attracted to the odors of females indicating use of a volatile sex attractant. These results are congruent with recent morphological investigations of T. radiata antennae (Onagbola et al. 2009). Male T. radiata possess a greater number of large multiporous sensilla trichoidea than females (Onagbola et al. 2009), which are known to function as detectors of female-produced sex pheromones (Pettersson et al. 2001; Onagbola and Fadamiro 2008). Light had a significant effect on behavior of T. radiata and thus light intensity should be considered when designing future bioassays or rearing facilities for this parasitoid. Our current efforts are focusing on identification of this putative sex-attractant pheromone as well as the nymph or plant-related host attractants. Our goal is to develop an attractant for T. radiata to

monitor population establishment as a result of classical biological control programs and to develop recruitment stations to possibly increase population densities of *T. radiata* in treated areas for enhanced biological control.

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