

Roles of Olfactory Cues, Visual Cues, and Mating Status in Orientation of *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) to Four Different Host Plants

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ABSTRACT *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) is an important worldwide pest of citrus that vectors bacteria (*Candidatus Liberibacter* spp.) responsible for huanglongbing (citrus greening disease). We examined the behavioral responses of mated and unmated *D. citri* of both sexes to odors from host plants in a Y-tube olfactometer, with and without visual cues. The host plants tested were 'Duncan' grapefruit (*Citrus paradisi* Macfayden), sour orange (*Citrus aurantium* L.), navel orange (*C. sinensis* L.), and *Murraja paniculata* L. Jack. Responses varied by plant species, psyllid sex and mating status, and the presence of a visual cue. Evidence of attraction generally was stronger in females and in mated individuals of both sexes relative to virgins. The presence of a visual cue typically enhanced attractiveness of olfactory cues; in no case did unmated individuals show evidence of attraction to host plant odors in the absence of a visual cue. In the absence of visual cues, mated females and males showed evidence of attraction only to odors from sour orange and navel orange, respectively. Psyllids exhibited anemotactic responses when assayed with plant odors alone but showed strong evidence of attraction only when olfactory and visual cues were combined, suggesting that olfactory cues facilitate orientation to host plants but may be insufficient alone. Antennal responses to citrus volatiles were confirmed by electroantennogram. The results reported here provide evidence that *D. citri* uses olfactory and visual cues in orientation to host plants and suggest the possibility of using plant volatiles in monitoring and management of this pest.

KEY WORDS Asian citrus psyllid, kairomones, citrus greening disease, attractants, olfaction

The psyllid *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) is a pest of worldwide importance that causes direct damage from feeding and/or oviposition (Michaud 2004) and transmits huanglongbing (citrus greening disease), one of the world's most serious diseases of citrus (Bové 2006). *D. citri* vectors three phloem-restricted bacteria in the genus *Candidatus Liberibacter*, which are the causative agents of huanglongbing (Halbert and Manjunath 2004, Hung et al. 2004, Bové 2006). Not native to the United States, *D. citri* was first discovered in Florida in June 1998 (Tsai et al. 2000) and has since spread throughout the state's citrus-growing regions (Michaud 2004). The disease was first discovered in southern Florida in August 2005 and only *Candidatus Liberibacter asiaticus* is known to occur in the state (Bové 2006). Infected citrus trees produce misshapen, poorly colored, bitter-tasting, and

unmarketable fruit, and trees may ultimately die from the disease (Halbert and Manjunath 2004, Bové 2006).

Although huanglongbing is not under completely successful management in any place in the world where the disease and vector co-occur (Halbert and Manjunath 2004), the most successful management efforts involve a combination of the use of clean nursery stock, prompt removal of inoculum sources, and aggressive pesticide sprays against the psyllids (Hung et al. 2004). Indeed, management of *D. citri* and huanglongbing in Florida currently relies on high inputs of broad-spectrum organophosphate and carbamate insecticides (Rogers et al. 2008), which almost certainly has adverse effects on natural enemies that may otherwise contribute to control of *D. citri* (McFarland and Hoy 2001; Michaud 2002a, 2002b, 2004). Timing of insecticide applications may be facilitated by monitoring for *D. citri*, but the monitoring methods developed thus far are labor intensive and/or not species specific (Hall et al. 2007a). The discovery of volatile chemical attractants for *D. citri* may allow for the development of new monitoring and management strategies that could reduce production costs, help to optimize spray intervals, and/or reduce insecticide use.

Within the Psylloidea, little is known regarding how individuals orient to their host plants. Visual cues are

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known to be involved in host-finding behavior in other species within the Sternorrhyncha, most notably in the Aphididae (Döring and Chittka 2007). The effectiveness of yellow sticky cards for monitoring psyllids (Adams et al. 1983, Samways 1987, Mensah and Maden 1992, Lapis and Borden 1995; Horton 1999) including *D. citri* (Hall et al. 2007a) suggests a visual role in orientation to host plants. The psyllids *Ctenarytaina eucalypti* Maskell and *Ct. spatulata* Taylor were both attracted to yellow-colored traps, but their responses to leaf colors suggest that leaf color alone is insufficient to explain the discrimination between leaf types exhibited by the two psyllids within their common host plant (Brennan and Weinbaum 2001). Similarly, Lapis and Borden (1995) showed attraction in *Heteropsylla cubana* Crawford to visual cues but suggested that olfaction is more important in host plant selection. Because leaf color is unlikely to provide by itself a sufficient cue by which herbivores might identify host plants (Prokopy and Owens 1983), it is likely that psyllids also rely on other sensory modalities to orient to their hosts.

Studies into the roles of olfactory cues in host finding and selection in psyllids have been rare. Moran and Brown (1973) showed that probing and walking behaviors in the African psyllid vector of huanglongbing, *Trioza erythrae* (Del Guercio), were influenced by host plant volatiles, but their study could not distinguish among olfactory, gustatory, or other functions of the antennae. Moran (1968b) reported a preference in *T. erythrae* among different host plants, but in olfactometer experiments observed no behavioral responses of adults to leaf extracts of the preferred host. To our knowledge, evidence of attraction to host plant volatiles has been shown explicitly in four species in the Psylloidea: *H. cubana* (Lapis and Borden 1993), *Cacopsylla bidens* (Šulc) (Soroker et al. 2004), *Ca. melanoneura* (Förster) (Gross and Mekonen 2005, Mayer and Gross 2007), and *Ca. picta* (Förster) (Gross and Mekonen 2005). In the former two species, only females showed clear evidence of attraction to host plant odors.

In this study, we examined behavioral responses of *D. citri* to odors from host plants with and without visual cues in Y-tube olfactometer assays. Experiments were conducted using individuals of both sexes that were either mated or unmated. We also used electroantennogram (EAG) assays to characterize antennal responses of male and female psyllids to host plant volatiles. Our primary goals were to determine whether (1) *D. citri* exhibits orientation to its host plants using olfactory cues, (2) visual cues play a role in host-plant finding, (3) mating status affects orientation to host plant cues, (4) responses differ for different host plants, and (5) psyllids exhibit antennal response to host plant volatiles.

Materials and Methods

Insect Cultures. Psyllids used in the Y-tube olfactometer experiments and EAG recordings were obtained from one of two cultures: (1) a laboratory colony at the U.S. Horticultural Research Laboratory (as described by Hall et al. 2007b) reared on *Murraya*

paniculata L. Jack (Rutaceae) or (2) a laboratory colony at the University of Florida Citrus Research and Education Center (as described by Wenninger et al. 2008) maintained on sour orange (*Citrus aurantium* L.) and 'Hamlin' orange (*C. sinensis* L.). To obtain adults of known age, fifth-instar nymphs were transferred individually to 'Duncan' grapefruit (*Citrus paradisi* Macfayden) seedlings (2–3 leaf stage) caged in plastic vial containers, which are described in Wenninger and Hall (2007). Briefly, each cage consisted of a 52-mm-tall vial, modified as an open-ended cylinder with a foam plug used to stopper the top opening and two ventilation holes on the sides; individual cages were slipped over a seedling grown in a cone-shaped planting container. Psyllids were either caged individually and maintained as virgins or, to obtain mated psyllids, opposite sex pairs of sexually mature psyllids (3–5 d old) were established and maintained on plants for 24–48 h before initiation of behavioral assays. Opposite sex pairings of psyllids ≥ 4 d of age under similar conditions have been shown to yield 100% mating (Wenninger and Hall 2007). In this manner, psyllids of known age and highly likely mating status were established for use in the experiments described below. All psyllids used in experiments, regardless of mating status, were 5–7 d posteclosion. Until ready for use in experiments, adult psyllids were held in an environmental chamber at 26°C, 60% RH, and 14:10 h L:D photoperiod, which resulted in 70–80% RH inside vial cages.

Behavioral Responses of *D. citri* to Olfactory and Visual Cues. The objective of this series of experiments was to test whether psyllids responded behaviorally to volatile odors from citrus host plants presented with or without visual cues, supplied in the form of a green or white light-emitting diode (LED). We tested the behavioral responses of psyllids to host plant odors in a glass Y-tube olfactometer (Analytical Research Systems, Gainesville, FL). The Y-tube 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. Charcoal-filtered, humidified air was metered through the two arms of the Y-tube through polytetrafluoroethylene (PTFE) tubing at 300 ml/min into each arm. A vacuum (600 ml/min) was applied at the glass plug at the base of the Y-tube using a modified rubber stopper; a smoke test showed that application of the vacuum markedly reduced mixing of air at the branching point of the Y-tube. The air exiting the wire screen plug at the base of the stem (with the vacuum temporarily removed) was 26–28°C and 70–85% RH; light intensity just above the branching point of the Y-tube was $\approx 1,700$ lux. Observations were made during photophase under bright light given that activity of *D. citri* is greatly reduced during scotophase (Wenninger and Hall 2007).

Odor sources were delivered to the arms of the Y-tube by routing the air source (through the PTFE tubing) through 1-liter glass canning jars upwind of the junction with the Y-tube. Individually potted 'Duncan' grapefruit plants were housed inside the jars, and Parafilm (Alcan Packaging, Nennah, WI) labora-

tory film was wrapped around the pot up to the base of the plant to limit any soil odors from entering the Y-tube. Trials were conducted using two 2- to 3-month-old plants ≈ 10 cm in height that were approximately equal in age and size and that had young flush (immature leaves as described by Hall and Albrigo 2007).

A green LED (www.plasmaled.com) was used to simulate a visual plant cue, and a white LED was used as a control. The green LED emitted a peak wavelength at ≈ 513 nm; the white LED emitted a main peak at ≈ 453 nm and a secondary peak at ≈ 532 nm. The bulbs were 5 mm wide and featured a 25° viewing angle. LEDs were powered with a nine V battery, and the green and white bulbs were of similar intensity: 11,000 and 9,000 millicandelas (www.plasmaled.com), respectively. The bulbs were positioned behind an organdy cloth screen (125 by 30 mm) that was placed within the crotch of the branching point of the Y-tube, touching the outside wall of the Y-tube. The bulbs were oriented horizontally and aimed parallel with the stem of the Y-tube, separated from each other by ≈ 22 mm. We assume that a green LED illuminating the organdy screen behind the Y-tube serves as an appropriate proxy for a visual cue from a green plant.

The olfactory and visual cue treatment combinations tested were as follows: grapefruit odors alone versus clean air alone; clean air + green LED versus clean air + white LED; grapefruit odors + green LED versus clean air + white LED; grapefruit odors + green LED versus clean air + green LED. The position (left or right) of the treatment odor source was randomly selected for the first of any set of observations and alternated thereafter; the position of the visual cue was alternated after every two observations. Plants were replaced after no more than six replicates. Between assays, glassware and tubing were soaked in hot soapy water for 30–60 min, rinsed thoroughly with tap water and deionized water, and held in a drying oven (150°C) for at least 2 h (and usually overnight) before reuse.

Psyllids were released individually into the glass plug at the base of the stem of the Y-tube ($N \geq 30$ per experiment), and their behavior was observed over a 5-min period. Males and females were observed separately for each experiment, and only virgin individuals were used. We recorded the time to exit the glass plug at the base of the Y-tube (i.e., to enter the Y-tube stem), the time to make a first choice, and the time elapsed before vibrational calling behavior (identified by characteristic movement of the wings of an adult) was observed. *D. citri* exhibits acoustic communication between the sexes, and individuals call readily in an olfactometer environment (Wenninger et al. 2009). The first choice of each psyllid was recorded as the arm in which the psyllid first entered ≈ 1 cm into the arm beyond the branching point. The total duration of time spent in each arm as well as the number of vibrational calls that occurred over the 5-min observation period were also quantified.

Effect of Mating Status and Host Plant Variety on *D. citri* Behavioral Response to Olfactory and Visual Cues. The objective of this series of experiments was to determine the effect of mating status on the be-

havioral response of *D. citri* to host plant volatiles. In addition, we tested the effect of host-plant type on behavioral response of both male and female psyllids. The host plants chosen for testing were 'Duncan' grapefruit, sour orange, navel sweet orange (*C. sinensis* L.), and *M. paniculata*. These plants were chosen because they are all known host plant varieties of this specialist herbivore and span the most important potential citrus production and ornamental plant varieties that this herbivore infests worldwide. Odor sources were delivered to the arms of the Y-tube by routing the air source (through the PTFE tubing) through 5-liter glass jars upwind of the junction with the Y-tube. For each experiment, one jar was filled with 300 mg (fresh mass) of unexpanded and newly expanded leaf flush clipped from a particular test plant variety, whereas the control container was left empty. Cleaning procedures between test runs and randomization procedure were identical to those described above. In addition, Y-tube test conditions, psyllid rearing, and introduction procedures, as well as data collection, were identical to that described above except that only the first choice made by each tested psyllid and the total duration of time spent in each arm were recorded. Separate experiments were conducted for each sex and mating status combination. In addition, each sex and mating status combination was tested with or without presentation of a visual cue given that the first set of experiments indicated that behavioral responsiveness to grapefruit odor increased in the presence of a visual cue for unmated psyllids (see Results). In experiments with a visual cue, a yellow sticky card (Pherocon AM; Trécé, Adair, OK) was folded at a 45° angle to match the angle of the Y-tube junction and positioned within the crotch of the Y-tube junction 1 cm removed from the olfactometer. These yellow traps were slightly modified by removing the majority of sticky material and covering the remaining sticky surface with translucent plastic wrap. This visual cue was positioned such that there was no directionality associated with the color cue and thus directionality of psyllid behavioral response is ascribed to the odor stimulus alone within the context of the visual cue. The Pherocon AM sticky trap was used as a standard visual cue given that a previous study determined that such yellow traps are attractive to *D. citri* in a field setting (Hall et al. 2007a).

EAG Responses of *D. citri* to Citrus Volatiles

Collection of Volatiles. The objective of this study was to measure the antennal responses of *D. citri* to host plant volatiles by EAG. Grapefruit was chosen as the volatile collection source given that it was used as the stimulus for testing in both series of behavioral experiments described above. Volatiles from plants were collected using a push-pull system according to the methods of Rodriguez-Saona et al. (2001). The vegetative portion of 0.4-m-tall grapefruit seedlings (stem, branches, and leaves) was inserted into a 4-liter, 30.5-cm-tall, and 15.2-cm-diameter glass cylinder custom designed for collection of volatiles (Analytical

Research Systems, Gainesville, FL). A guillotine support base (Analytical Research Systems) closed the cylinder around the stem of the plant. Air was pushed through the system through a portable 2.0-HP, 75-liter tank (Westward air compressor; Grainger, Northbrook, IL). The air was purified before entering the collection chamber by pushing it through activated charcoal, and it entered over the plant at a rate of 2 liters/min. Volatiles were collected in Super-Q adsorbent traps (Alltech, Deerfield, IL), positioned 5 cm from the base of the cylinder by pulling 50% of the air from the chamber at a rate of 1 liter/min with the aid of a Gast 0.25-HP vacuum pump (Grainger, Houston, TX). The remainder of the air was vented from the system through an opening around the stem of the plant that was loosely closed with cotton to prevent abrasion. Volatile samples were collected at $28 \pm 0.5^\circ\text{C}$, 60–75% RH, and 16:8 h L:D.

Volatiles were collected from four plants at two time intervals for 24 h: during the day from 0900 to 1800 hours and at night from 1800 to 0900 hours. Empty chambers were sampled to test for contamination and yielded no detectable volatiles as determined by gas chromatography. Grapefruit volatiles were extracted from Super-Q traps with 150 μl pentane (Soroker et al. 2004).

Preparation of EAG Stimulus Cartridges. Grapefruit volatiles were extracted from Super-Q traps as described above, and four serial dilutions (log scale) in pentane were produced. EAG cartridges were prepared by pipetting 20 μl of each dilution of the grapefruit volatile solution onto 1.4 by 0.5-cm strips of Whatman No. 1 filter paper (Fisher, Pittsburgh, PA). We used 20 μl of solvent alone as the control. After 5 min in a fume hood for solvent evaporation, treated strips were inserted into disposable glass Pasteur pipettes, which were sealed with Parafilm before use. Cartridges were always used 20–90 min after preparation, and new cartridges were prepared for each recording session. For a separate investigation, EAG cartridges were loaded with synthetic (+)-limonene (Aldrich, Milwaukee, WI; $\geq 98\%$ pure by our gas chromatographic analysis). This synthetic standard was also tested as a comparison given that limonene is a primary component of citrus headspace volatiles (Ting and Rouseff 1986). Limonene-loaded EAG cartridges were prepared identically to those described for extracted grapefruit volatiles by diluting neat chemical in pentane on a log scale.

EAG Recordings. The EAG system and test protocols were similar to those described previously (Stelinski et al. 2003). EAGs were recorded using a data acquisition interface board (Type IDAC-02) and universal single-ended probe (Type PRS-1) from Syntech (Kirchzarten, Germany). The recording and indifferent electrodes were silver-coated wire in pulled glass micropipettes (10- μl micro-hematocrit capillary tubes) containing 0.5 M KCl. Psyllids were 4- to 6-d-old adults when used for EAG recording. The recording electrode was placed over the tip of one antenna that was not clipped (as is often done when recording EAGs with other insects), and the reference electrode

was inserted into the back of the head. Volatile stimuli were delivered through a glass Y-tube (each arm 2 cm in length, base 1 cm long, and 0.5 cm diameter) positioned ≈ 3 mm from the antenna. Carbon-filtered and humidified air was delivered at 30 ml/min into one arm of the Y-tube using Tygon tubing (Fisher). EAGs were measured as the maximum amplitude of depolarization elicited by 1-ml puffs of air through EAG cartridges directed over antennae of whole insect preparations. Stimulus puffs were generated through the cartridges with a clean, hand-held 20-ml glass syringe connected to the pipettes with a 1-cm piece of Tygon tubing. Preliminary testing ($N = 4$ psyllids of each sex) indicated no difference between EAG response to grapefruit volatiles collected during day and night time periods; therefore, these samples were combined for subsequent EAG recordings. The extracted grapefruit volatiles and (+)-limonene treatments were delivered in an ascending dosage order beginning with the solvent control to separate groups of 10 replicate male and 10 female *D. citri* antennae.

Statistical Analyses. For the olfactometer trials, the number of individuals that chose the treated arm versus the control arm was compared by χ^2 tests. To compare the duration of time spent in each arm, the time spent in the control arm was subtracted from the time spent in the treated arm, and the numerical difference was compared with zero by *t*-test. For the LED experiments, the additional behaviors recorded were compared (separately for males and females) among the four experimental treatments by analyses of variance (ANOVAs). The factors analyzed were time to enter the Y-tube from the exit plug (square-root transformed), time to make a first choice, time to initiate calling, and total number of calls (square-root transformed). When ANOVA showed a significant difference, Tukey's pairwise comparisons tests were used to discriminate among treatments. Additionally, to compare the proportion of individuals that entered the Y-tube and the proportion of individuals that called during the assay among the LED treatments, we ran (separately for males and females) a 2 by 4 χ^2 test or—for cases in which the expected count within one or more cells was < 5 —a Fisher exact test (Quinn and Keough 2002). A two-factor randomized complete block ANOVA was conducted on the EAG data. Differences in pairs of means between psyllid sex within each dosage tested and among dosages within each sex were separated using Fisher protected least significant difference (LSD) multiple comparison procedure. Data are presented as mean \pm SEM. The significance level was set at $\alpha = 0.05$.

Results

Behavioral Responses of *D. citri* to Olfactory and Visual Cues. Males and females generally responded similarly to olfactory and visual cues in the Y-tube olfactometer assays. In the LED experiments, neither virgin males nor virgin females showed a preference for the treated arm of the olfactometer—in terms of either the first choice made or the length of time spent

Table 1. Results of Y-tube olfactometer trials examining responses of virgin psyllids to treatment odor sources versus a clean air control, with and without visual cues (green or white light-emitting diodes)

Sex assayed	Treatment	N ^a	First choice			Number of minutes spent in arm			
			Treated ^b	χ^2	P	Treated	Control	t value ^c	P
Female	Grapefruit plants versus clean air	30 (27)	0.56	0.33	0.564	1.54 ± 0.3	1.12 ± 0.3	0.79	0.439
Female	Green LED versus white LED	30 (19)	0.74	4.3	0.039	1.01 ± 0.2	0.65 ± 0.2	1.01	0.324
Female	Grapefruit plants + green LED versus white LED	30 (29)	0.76	7.8	0.005	2.34 ± 0.3	0.82 ± 0.2	3.28	0.003
Female	Grapefruit plants + green LED versus green LED	30 (30)	0.73	6.5	0.011	1.98 ± 0.3	1.03 ± 0.3	1.82	0.079
Male	Grapefruit plants versus clean air	30 (25)	0.44	0.36	0.549	1.38 ± 0.2	1.09 ± 0.2	0.70	0.490
Male	Green LED versus white LED	37 (16)	0.75	4.0	0.046	0.97 ± 0.2	0.76 ± 0.3	0.49	0.633
Male	Grapefruit plants + green LED versus white LED	33 (28)	0.75	7.0	0.008	2.31 ± 0.3	0.81 ± 0.2	3.29	0.003
Male	Grapefruit plants + green LED versus green LED	34 (29)	0.51	0.03	0.853	1.66 ± 0.3	1.21 ± 0.2	1.08	0.291

^a Total sample size (with no. of individuals that made a choice in parentheses).
^b Proportion of individuals (of those that made a choice) that chose the treated arm first.
^c Compares numerical difference between treatment and control with zero.

in an arm—when assayed in response to an olfactory cue alone (Table 1). When assayed with a visual cue in the absence of citrus odors, significantly more individuals of both sexes chose the arm illuminated with a green LED, but neither sex showed a difference in the length of time spent in the treated versus control arm (Table 1). When both the olfactory and visual cues were presented simultaneously (green LED + citrus odors versus white LED), both sexes exhibited a significant response to the treated arm with respect to first choice; moreover, individuals of both sexes spent significantly more time in the treated versus control arm (Table 1). When both arms of the Y-tube were illuminated with an identical green LED, but only one arm (which was considered as the “treated”

arm) contained grapefruit odors, significantly more females entered the treated arm first, but the time spent in the treated versus control arms did not differ significantly (Table 1). In contrast, males showed no evidence of preference for the treated arm in this assay (Table 1).

Among females, a smaller percentage of individuals entered the Y-tube arena from the glass plug inserted at the stem of the Y-tube when presented with a visual cue in the absence of citrus odors (Table 2). Females that did enter the Y-tube when assayed with a visual cue alone took significantly longer to enter the Y-tube relative to females assayed with citrus odors with or without a visual cue (Table 2). Similarly, females presented with only a visual cue took significantly longer

Table 2. Comparison of responses among Y-tube olfactometer trials examining responses of virgin female or male psyllids to treatment odor sources versus a clean air control, with and without visual cues (green or white light-emitting diodes)

Sex assayed	Treatment	Percent that entered Y-tube ^a	Time to enter Y-tube (min) ^{b,c}	Time to first choice (min) ^b	Percent that called	Time to start calling (min) ^b	Total no. of calls ^c
Female	Grapefruit plants versus clean air	100	0.65 ± 0.2a	1.89 ± 0.3a	53.3	2.67 ± 0.2ab	2.75 ± 0.5
Female	Green LED versus white LED	76.7	1.73 ± 0.3b	2.95 ± 0.3b	33.3	3.43 ± 0.4b	3.00 ± 0.8
Female	Grapefruit plants + green LED versus white LED	96.7	0.61 ± 0.1a	1.38 ± 0.2a	46.7	2.39 ± 0.3ab	2.07 ± 0.4
Female	Grapefruit plants + green LED versus green LED	100	0.84 ± 0.2a	1.67 ± 0.2a	63.3	2.14 ± 0.3a	3.68 ± 0.6
	Test statistic	Fisher exact test	F = 5.80	F = 6.89	$\chi^2 = 5.7$	F = 3.12	F = 1.35
	P	0.001	0.001	<0.001	0.127	0.033	0.267
Male	Grapefruit plants versus clean air	93.3	1.06 ± 0.3	2.00 ± 0.3a	93.3	1.84 ± 0.3	7.04 ± 0.8
Male	Green LED versus white LED	62.2	1.07 ± 0.3	2.89 ± 0.3b	63.3	1.80 ± 0.3	6.68 ± 1.0
Male	Grapefruit plants + green LED versus white LED	97.0	0.98 ± 0.2	1.45 ± 0.2a	81.8	1.86 ± 0.2	7.85 ± 1.0
Male	Grapefruit plants + green LED versus green LED	88.2	0.91 ± 0.2	1.74 ± 0.2a	100	1.78 ± 0.2	5.74 ± 0.6
	Test statistic	$\chi^2 = 20.0$	F = 0.10	F = 5.10	$\chi^2 = 31.3$	F = 0.03	F = 1.23
	P	<0.001	0.960	0.0026	<0.001	0.995	0.302

^a The proportion of individuals that entered the Y-tube was compared among treatments by χ^2 test, except when the expected count within one or more cells was <5, mandating the use of the Fisher exact test.
^b Means within a column that share the same letter differ significantly based on Tukey’s pairwise comparisons test (data were analyzed separately for males and females).
^c Data were square-root transformed for analysis, but untransformed values are shown here.

Table 3. Results of Y-tube olfactometer trials examining responses of unmated and mated psyllids to treatment odor sources versus a clean air control, with and without visual cues (yellow-colored sticky card)

Sex assayed	Mating status	Treatment	N ^a	First choice			Number of minutes spent in arm			
				Treated ^b	χ^2	P	Treated	Control	t value ^c	P
Female	Unmated	Navel without visual cue	35 (31)	0.45	0.29	0.590	1.78 ± 0.1	2.22 ± 0.4	-1.24	0.342
Female	Unmated	Navel with visual cue	30 (30)	0.63	2.1	0.144	1.43 ± 0.4	0.92 ± 0.3	0.98	0.397
Female	Mated	Navel without visual cue	30 (29)	0.62	1.7	0.194	1.54 ± 0.2	1.33 ± 0.4	1.11	0.352
Female	Mated	Navel with visual cue	32 (29)	0.76	7.8	0.005	1.98 ± 0.3	0.71 ± 0.3	2.94	0.005
Female	Unmated	Sour Orange without visual cue	38 (36)	0.56	0.44	0.505	1.17 ± 0.2	1.22 ± 0.2	-0.81	0.402
Female	Unmated	Sour Orange with visual cue	35 (33)	0.64	2.5	0.117	1.11 ± 0.3	0.98 ± 0.3	0.66	0.545
Female	Mated	Sour Orange without visual cue	41 (35)	0.83	15.1	0.0001	2.11 ± 0.2	0.76 ± 0.2	3.11	0.004
Female	Mated	Sour Orange with visual cue	33 (30)	0.73	6.5	0.011	2.32 ± 0.2	0.61 ± 0.2	3.23	0.002
Female	Unmated	Grapefruit without visual cue	45 (41)	0.63	3.0	0.086	1.42 ± 0.2	1.72 ± 0.2	-1.08	0.358
Female	Unmated	Grapefruit with visual cue	30 (28)	0.68	3.6	0.058	2.38 ± 0.3	0.67 ± 0.3	2.77	0.005
Female	Mated	Grapefruit without visual cue	34 (30)	0.43	0.53	0.465	0.97 ± 0.2	1.11 ± 0.2	-1.23	0.343
Female	Mated	Grapefruit with visual cue	40 (35)	0.74	8.3	0.004	1.75 ± 0.2	0.51 ± 0.2	3.02	0.004
Female	Unmated	<i>Murraya</i> without visual cue	31 (30)	0.40	1.2	0.273	1.22 ± 0.2	1.56 ± 0.2	-0.89	0.399
Female	Unmated	<i>Murraya</i> with visual cue	33 (31)	0.77	9.3	0.002	1.73 ± 0.2	0.48 ± 0.2	2.98	0.005
Female	Mated	<i>Murraya</i> without visual cue	30 (30)	0.57	0.53	0.465	1.16 ± 0.3	1.28 ± 0.3	-0.76	0.511
Female	Mated	<i>Murraya</i> with visual cue	31 (29)	0.72	5.8	0.016	1.21 ± 0.3	0.69 ± 0.2	1.21	0.344
Male	Unmated	Navel without visual cue	35 (31)	0.42	0.81	0.369	1.62 ± 0.2	1.81 ± 0.2	-0.78	0.509
Male	Unmated	Navel with visual cue	36 (32)	0.63	2.0	0.157	1.55 ± 0.2	1.12 ± 0.2	1.04	0.359
Male	Mated	Navel without visual cue	33 (31)	0.71	5.5	0.0196	1.82 ± 0.2	1.95 ± 0.2	-0.61	0.556
Male	Mated	Navel with visual cue	30 (28)	0.75	7.0	0.0008	2.48 ± 0.2	0.74 ± 0.2	2.89	0.005
Male	Unmated	Sour Orange without visual cue	34 (31)	0.39	1.6	0.209	0.98 ± 0.3	0.65 ± 0.2	1.13	0.351
Male	Unmated	Sour Orange with visual cue	32 (29)	0.55	0.31	0.577	1.31 ± 0.2	1.42 ± 0.2	-0.69	0.544
Male	Mated	Sour Orange without visual cue	35 (32)	0.53	0.13	0.724	1.57 ± 0.2	1.13 ± 0.3	1.22	0.344
Male	Mated	Sour Orange with visual cue	31 (29)	0.69	4.2	0.041	1.25 ± 0.3	0.88 ± 0.2	0.88	0.400
Male	Unmated	Grapefruit without visual cue	30 (30)	0.47	0.13	0.715	0.89 ± 0.3	0.67 ± 0.2	0.65	0.546
Male	Unmated	Grapefruit with visual cue	32 (30)	0.63	2.1	0.144	1.46 ± 0.2	1.78 ± 0.2	-0.72	0.513
Male	Mated	Grapefruit without visual cue	33 (31)	0.39	1.6	0.209	1.15 ± 0.2	0.97 ± 0.2	0.68	0.544
Male	Mated	Grapefruit with visual cue	31 (30)	0.67	3.3	0.068	1.37 ± 0.3	1.51 ± 0.2	-1.27	0.340
Male	Unmated	<i>Murraya</i> without visual cue	30 (30)	0.50	0.0	1.0	1.57 ± 0.2	1.41 ± 0.2	0.95	0.399
Male	Unmated	<i>Murraya</i> with visual cue	32 (30)	0.63	2.1	0.144	1.07 ± 0.3	1.32 ± 0.2	-1.19	0.345
Male	Mated	<i>Murraya</i> without visual cue	30 (30)	0.47	0.13	0.715	1.49 ± 0.2	1.35 ± 0.2	0.79	0.509
Male	Mated	<i>Murraya</i> with visual cue	31 (30)	0.60	1.2	0.273	0.89 ± 0.3	1.02 ± 0.2	-1.26	0.341

^a Total sample size (with no. of individuals that made a choice in parentheses).

^b Proportion of individuals (of those that made a choice) that chose the treated arm first.

^c Compares numerical difference between treatment and control with zero.

to make a first choice relative to females presented with citrus odors with or without a visual cue (Table 2). The proportion of females that exhibited calling behavior in the Y-tube did not differ among the different experimental treatments (Table 2). Of the females that exhibited calling behavior, calling was observed significantly earlier for females assayed with citrus odors and a green LED in both arms of the olfactometer relative to females assayed with a visual cue alone; latency to initiation of calling behavior did not differ among the other pairwise comparisons of treatments (Table 2). The total number of calls observed in females did not differ among treatments (Table 2).

Relative to the other treatments, males were also less likely to enter the Y-tube arena when assayed with a visual cue alone; however, of the individuals that entered the Y-tube, the time that males took to enter did not differ among treatments (Table 2). As with females, males presented with only a visual cue took significantly longer to make a first choice relative to males presented with citrus odors with or without a visual cue (Table 2). The proportion of males that exhibited calling behavior in the Y-tube differed significantly among the different experimental treatments; fewer males called when assayed with a visual

cue alone relative to the other treatments that included grapefruit plants (Table 2). The latency to initiation of calling behavior in males did not differ among treatments, nor did the total number of calls observed over the 5-min observation period (Table 2).

Effect of Mating Status and Host Plant Variety on *D. citri* Behavioral Response to Olfactory and Visual Cues. For treatments that featured a visual cue, a significantly greater proportion of mated female *D. citri* chose the arm of the Y-tube releasing volatiles from navel orange or grapefruit compared with the blank control; these females also spent significantly more time in the treatment arm than in the control arm for these plant types (Table 3). Unmated females assayed with grapefruit odors in the presence of the visual cue spent significantly more time in the treatment arm; the first choice recorded for these females showed a tendency to select the treatment arm, but the difference was not significant (Table 3). Mated females responded significantly to the volatiles of sour orange, in terms of first choice and time spent in the treatment arm, irrespective of the presence of the visual cue (Table 3). Mated and unmated females responded to *M. paniculata*, in terms of first choice, but only in the presence of the visual cue; only unmated females spent significantly more time in the treatment

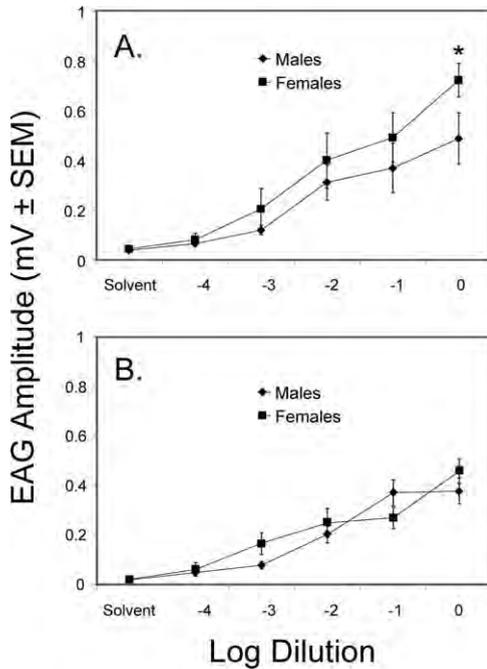


Fig. 1. Dosage-response relationships for male and female *D. citri* live-insect antennal preparations in response to a log dilution series of (A) grapefruit volatiles extracted with Super-Q adsorbent traps and (B) synthetic (+)-limonene. *Significant ($P < 0.05$) differences between mean response of male and female *D. citri* at a particular log dilution.

arm containing flush of this host plant compared with the blank control (Table 3). Mated male *D. citri* responded significantly to the odors of both navel orange and sour orange in terms of first choice, but—in the case of sour orange—only in the simultaneous presence of the visual cue (Table 3). When testing navel orange as the odor source, males also spent significantly more time in the treatment arm than the control, but not in the case of sour orange (Table 3). In no other case did the recorded responses indicate a significant treatment effect.

EAG Responses of *D. citri* to Citrus Volatiles. EAG responses of both male ($F = 17.8$; $df = 5,180$; $P < 0.001$) and female ($F = 12.3$; $df = 5,180$; $P = 0.01$) *D. citri* to the extracts of grapefruit volatiles were significantly higher than to the solvent control at dilutions ranging from -3 log through the undiluted grapefruit extract obtained directly from Super-Q traps (Fig. 1A). The EAG response of female *D. citri* to the undiluted extract was significantly ($F = 11.5$; $df = 1,180$; $P < 0.01$) higher than that of males, but not so for any of the dilution treatments (Fig. 1A).

The EAG responses of male ($F = 5.4$; $df = 3,180$; $P = 0.03$) and female ($F = 8.7$; $df = 3,135$; $P = 0.002$) *D. citri* to synthetic (+)-limonene were significantly higher than to the solvent control at dilutions ranging from -3 log through the 20-mg neat material dosage (Fig. 1B). There was no difference in responses between the sexes at any of the cartridge dosages for this syn-

thetic standard. It seemed that EAGs in response to the extracted grapefruit volatiles (average maximum ≈ 0.7 mV) were slightly greater than those elicited by (+)-limonene (average maximum ≈ 0.4 mV), but these were not directly compared statistically because they were obtained using different groups of insects on different days. In general, millivolt readings in the EAG assays were low, not reaching 1 mV on average for any of the treatments tested.

Discussion

The results presented here provide evidence that *D. citri* uses both olfactory and visual cues to orient to its host plants and confirm that *D. citri* exhibits antennal detection of odors from citrus. The importance of visual cues in influencing behavior is well known for the Psylloidea (see Introduction), as well as for other hemipterans, most notably the Aphididae (Döring and Chittka 2007). Moreover, enhanced responses in insects to olfactory and visual cues presented in combination versus individually have been reported in other hemipterans, including the glassy-winged sharpshooter *Homalodisca vitripennis* (Germar) (Patt and Sétamou 2007) and the Western tarnished plant bug *Lygus hesperus* Knight (Blackmer and Cañas 2005). The interaction of visual and olfactory cues in orientation to host plants has yet to be explicitly evaluated in other psyllids. In our results with *D. citri*, in no assay using unmated individuals did psyllids show evidence of attraction to host plant odors in the absence of a visual cue. In contrast, psyllids did exhibit evidence of attraction to visual cues in the absence of host plant odors. However, relative to psyllids that were assayed in response to both olfactory and visual cues, psyllids that were exposed only to visual cues were less likely to enter the Y-tube, and those that did took longer to make a choice. These results suggest that olfactory cues facilitate anemotactic orientation to host plants in *D. citri*, but in the absence of visual cues, olfactory cues generally may be insufficient. More explicit tests will be required to evaluate the full suite of behaviors involved in host-plant orientation and selection in *D. citri*. Vibrational calling behavior was also reduced to some extent in both sexes when assayed in the absence of olfactory cues, which may indicate that psyllids are less inclined to use vibrational signaling when held under more artificial conditions. Vibrational signaling is involved in communication between the sexes in *D. citri* (Wenninger et al. 2009), but might also have other roles in insects, including locating food resources or a group of conspecifics (Cocroft 2001). The biological importance of variation in calling behavior with respect to odor treatment remains to be examined.

Although both sexes of *D. citri* were attracted to host plant odors, females generally showed stronger evidence of attraction. For example, in the LED assays, both sexes were attracted to grapefruit odors in combination with the green visual cue; however, when the visual cue was present in both arms of the Y-tube, only females continued to choose the arm with

host plant odors. Moreover, in the assays using yellow cards as the visual cue, females showed some evidence of attraction to all four types of host plant tested (at least in combination with the visual cue), whereas males showed evidence of attraction only to two of the host plant varieties tested. These results are not surprising given that females require host plants on which to lay eggs (Husain and Nath 1927, Yasuda et al. 2005), and males may use olfactory cues from females (perhaps in combination with plant odors) to assist in finding their host plants (Wenninger et al. 2008). Furthermore, males on a plant seem to orient to flush by positive phototaxis and/or negative geotaxis, whereas females evidently use other cues (Yasuda et al. 2005), with plant odors possibly playing some role. Stronger responses for females to host plant odors have been shown in other psyllid species as well. Lapis and Borden (1993) and Soroker et al. (2004) reported that females of *H. cubana* and *Ca. bidens*, respectively, were attracted to host plant odors, but found no such attraction in males. Gross and Mekonen (2005) reported that both sexes of *Ca. melanoneura* orient to host plant odors, but only females discriminated between different summer host species.

Mating status had a pronounced effect on the orientation of *D. citri* to olfactory cues. In the experiments using a yellow sticky card as a visual cue, unmated females showed evidence of attraction only to two host species (grapefruit and *M. paniculata*) and only with the presence of the visual cue. In contrast, mated females were attracted to all four plant species tested, at least when the visual cue was also present. Similarly, males only showed evidence of attraction to host plants when mated. In the absence of a visual cue, mated females and males were attracted only to sour orange and navel orange, respectively. For mated females, the importance of locating host plants on which to lay eggs is obvious; however, it is unclear why males should be more attracted to host plants after mating. We speculate that males might benefit from searching for mates locally (within a tree) shortly after adult eclosion before engaging in longer distance dispersal to find additional mates, which may be more risky.

Tsai and Liu (2000) reported a higher intrinsic rate of increase for *D. citri* reared on grapefruit relative to *M. paniculata* and sour orange. Thus, the differences in responses of *D. citri* to the different hosts tested do not necessarily reflect which species represent the most suitable host. Similarly, Moran (1968b) reported that adult *T. erythrae* preferentially select rough lemon for feeding and oviposition sites despite more favorable nymphal development on other hosts (Moran 1968a). The variation in responses of *D. citri* to the different host plants tested could be influenced by pre-exposure to certain plant varieties in our psyllid cultures. The psyllids used in the experiments featuring yellow cards were reared on sour orange, which is the only species to which females were attracted without a visual cue. However, males showed no such attraction to sour orange, but rather were attracted to navel orange in the absence of a visual cue. The psyllids used in the experiments featuring LEDs were reared to the fifth-instar stage on *M. paniculata*, followed by grapefruit.

These psyllids did show stronger evidence of attraction to grapefruit (the only species tested in these assays) relative to the psyllids in the yellow card experiments. These differences might be simply attributable to the different visual cues used; however, it is noteworthy that in *Ca. melanoneura*, exposure to a different host plant for only 2–4 d was sufficient to alter preferences for odors in an olfactometer (Mayer and Gross 2007).

Relative to the Aphididae, dispersal mechanisms in the Psylloidea are poorly developed, which may be a reflection of the type of host plant typically exploited: perennial dicotyledenous plants on which the psyllid is able to complete its life cycle with little need to disperse (Hodkinson 1974). Certain temperate species (e.g., the pear psylla *Cacopsylla pyricola* Förster [Purcell and Suslow 1984], *Ca. melanoneura* [Mayer and Gross 2007], and *Trioza apicalis* Förster [Kristoffersen and Anderbrant 2007]) do move onto shelter plants to overwinter. Such species might be generally more adept at orienting to their hosts using olfactory cues alone relative to species like *D. citri* and *T. erythrae* that do not shift hosts. However, the present data are too limited to test this hypothesis.

Although both sexes of *D. citri* exhibited electrophysiological responses to headspace extracts of grapefruit volatiles, the responses were generally weak and <1 mV in amplitude on average (Fig. 1a). This level of EAG response to extracts of host plant volatiles is similar to that previously obtained with *Ca. bidens* (Šulc) to volatile extracts of its host plant (Soroker et al. 2004) and are likely explained by the paucity of plant volatile-detecting sensilla on the antennae of *D. citri* (Onagbola et al. 2008). Only 11 olfactory sensilla have been characterized per antenna for each sex of *D. citri* (Onagbola et al. 2008). The sparseness of olfactory sensilla seems to be characteristic of psyllid species and has been suggested to be an adaptation to high stimulus levels (Kristoffersen et al. 2006). Our results confirm that GC-EAD studies will be possible with *D. citri*, facilitating the identification of behaviorally active plant volatiles.

To our knowledge, this study presents the first explicit test of the interaction between visual and olfactory cues in host location in a species within the Psylloidea. Although male responses were generally less pronounced than those of females, we also present one of the first reports of male psyllids exhibiting behavioral responses to host plant odors in the absence of females. Our results showed that attractiveness of host plant odors to *D. citri* is dependent on sex, mating status, and plant species. Examination of headspace odors of different host species of *D. citri* is currently underway in an effort to identify volatile compounds that might be used in monitoring and management strategies for this worldwide pest of citrus.

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