

Behavioral evidence for a female-produced sex attractant in *Diaphorina citri*

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Abstract

Diaphorina citri Kuwayama (Hemiptera: Psyllidae) is an important worldwide pest of citrus. It vectors three phloem-restricted bacteria in the genus *Candidatus Liberibacter* that cause huanglongbing (citrus greening disease). Studies were conducted to examine the behavioral responses of male and female *D. citri* to conspecifics of the same and opposite sex, with and without associated citrus host plants, in both open-air arena choice assays and Y-tube olfactometer assays. Virgin and mated male *D. citri* colonized citrus plants that were currently or had been previously colonized by virgin or mated female *D. citri* in greater numbers than control plants without females. However, males or females did not accumulate more on plants colonized by conspecifics of the same sex compared to uninfested plants, and females showed no preference for plants pre-infested with males compared with uninfested controls. In complementary Y-tube olfactometer assays, virgin and mated males chose arms with odor sources from mated females compared with blank controls in the absence of associated citrus host plant volatiles. In both behavioral assays, mated female *D. citri* appeared more attractive than virgin females. The vibrational calling behavior of male *D. citri* was reduced when males were challenged by the odors of conspecific mated females relative to when males were challenged by the odor of other males. Collectively, our results provide behavioral evidence for a female-produced volatile sex attractant pheromone in *D. citri*. Future identification and synthesis of a sex attractant pheromone will be an important contribution to current monitoring and management practices for *D. citri*.

Introduction

Diaphorina citri Kuwayama (Hemiptera: Psyllidae) vectors three phloem-restricted bacteria in the genus *Candidatus Liberibacter*, the causative agents of huanglongbing (citrus greening disease) (Halbert & Manjunath, 2004; Hung et al., 2004; Bové, 2006). *Diaphorina citri*, which is not native to North America, was first identified in Florida in June 1998 (Tsai et al., 2000) and has since spread throughout the state's citrus-growing regions (Michaud, 2004). Feeding and/or

oviposition damage caused by immature and adult *D. citri* results in malformation or complete abscission of shoots and leaves (Michaud, 2004). Furthermore, the build-up of honeydew excretions produced by this insect promotes the development of sooty mold, which negatively affects the plants' photosynthesis capabilities (Tsai et al., 2000). However, the primary economic importance of the psyllid is transmission of huanglongbing, one of the world's most serious diseases of citrus (Bové, 2006). Huanglongbing was first discovered in southern Florida in August 2005, and only *Candidatus Liberibacter asiaticus* is known to occur in the state (Bové, 2006). Citrus trees infected by this disease may live only 5–8 years, during which they produce misshapen, poorly colored, bitter-tasting, and unmarketable fruit (Halbert & Manjunath, 2004; Bové, 2006). Despite the great economic importance of *D. citri* as a vector of huanglongbing, detailed investigations into the behavior of this pest have only recently begun (Wenninger & Hall, 2007, 2008).

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Management of *D. citri* and huanglongbing relies in part on frequent applications of chemical insecticides (Halbert & Manjunath, 2004). Excessive use of pesticides poses several potential problems, including increased development of physiological resistance in the target pest and reduction in populations of natural enemies that may otherwise contribute to control of *D. citri* (McFarland & Hoy, 2001; Michaud, 2002a,b, 2004). Various sampling techniques (including sampling leaves for adults and nymphs, tap sampling for adults) can be used for timing application of insecticides, but these techniques are labor intensive. The use of unbaited yellow sticky cards to monitor adult *D. citri* can be effective (Hall et al., 2007a), but less so when population levels are low unless large numbers of traps are deployed (DG Hall, unpubl.). Identification of volatile chemical attractants for this species, especially sex attractants, could allow for the development of new monitoring and management strategies that would reduce production costs and help to optimize spray intervals and/or reduce the dependence on insecticides.

Within the Psylloidea, little is known regarding how reproductively mature individuals attract or locate prospective mates. Substrate-borne vibrational signaling is involved in mate location for many psyllid species (Tishechkin, 1989, 2005, 2007; Percy et al., 2006), including *D. citri* (EJ Wenninger & RW Mankin, unpubl.). Visual cues may also be important for orientation to mates, at least at shorter distances, given that mating rates in some psyllid species are reduced when held in darkness (Krysan, 1990; Wenninger & Hall, 2007). Behavioral evidence for a female sex pheromone has been described in the pear-feeding psyllids *Cacopsylla bidens* (Šulc) (Soroker et al., 2004) and *Cacopsylla pyricola* (Förster) (Horton & Landolt, 2007; Horton et al., 2007), but a sex pheromone has yet to be identified within the Psylloidea. Visual and/or chemical cues from host plants may be involved in orientation to prospective mates as well. For example, males might orient to plants or specific parts of plants before searching locally for females (as in a leafhopper; Hunt & Nault, 1991), and/or feeding by females on plant tissue might alter the odors emitted by plants, resulting in male attraction (as in a scarab beetle; Heath et al., 2002).

Although the use of vibrational communication between the sexes has been established in the Psylloidea (see references above), including for *D. citri* (EJ Wenninger & RW Mankin, unpubl.), an increasing body of knowledge is accumulating on the use of both acoustic and olfactory signals in communication between the sexes in insects (e.g., Borges et al., 1987; Conner, 1999; Takács et al., 2003; Zhang et al., 2004; Nakano et al., 2006). Given that vibrational signaling in psyllids often does not precede successful copulation (Tishechkin, 2005, 2006, 2007; EJ Wenninger, pers. obs.), it

is likely that other signals in addition to substrate-borne vibrations are important for mate location. Moreover, there is some debate about whether small insects are able to locate the source of a vibrational signal without employing supplementary sensory modalities and associated long-range signals (see Virant-Doberlet et al., 2005).

In the current investigation, we examined behavioral responses of male and female *D. citri* to conspecifics of the same and opposite sex using open-air arena choice assays and Y-tube olfactometer assays. Our primary goals were to determine whether *D. citri* exhibits attraction to conspecifics and whether olfactory cues may be involved in mate location in this species. Our results suggest male *D. citri* respond to a volatile, female-produced sex attractant.

Materials and methods

Insect cultures

Psyllids used for open-air arena assays were drawn from a culture continuously reared at the University of Florida Citrus Research and Education Center (Lake Alfred, FL, USA) and established in 2000 from field populations in Polk Co., FL, USA (28.0°N, 81.9°W) prior to the discovery of huanglongbing in the state. This culture is maintained on sour orange (*Citrus aurantium* L.) and 'Hamlin' orange [*Citrus sinensis* (L.)] (Rutaceae) seedlings with flush (immature leaves as described by Hall & Albrigo, 2007) in a greenhouse at 27–28 °C, 60–65% r.h., and L14:D10 photoperiod. Fifth-instar nymphs were collected from this culture and placed in groups of 40–50 on similar seedlings previously uninfested by psyllids. These new plants were isolated from the parental culture within individual plastic cylinders with fine-mesh screen lids, but maintained in a greenhouse at otherwise identical conditions. The nymphs were checked daily to determine the day of adult eclosion. Emerging adult psyllids were segregated by sex and transferred either individually (to preclude mating) or in opposite sex pairs to flushing 'Hamlin' seedlings. These plants were isolated as described above. To obtain mated psyllids, opposite sex pairs of sexually mature psyllids (3–5 days old) were established and maintained on plants for 24–48 h prior to initiation of behavioral assays. Opposite sex pairings of psyllids ≥ 4 days of age under similar conditions are known to yield 100% mating (Wenninger & Hall, 2007). In this manner, psyllids of known age and mating status were established for use in the experiments described below.

Psyllids used in the Y-tube olfactometer experiments were obtained from a laboratory colony at the US Horticultural Research Laboratory (as described by Hall et al., 2007b) reared on *Murraya paniculata* (L.) Jack (Rutaceae). For assays in which virgins were used, 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 & 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. All virgin psyllids used in experiments were 4–8 days post-eclosion. When not using virgins, adults were collected from the laboratory colony at ca. 2–4 days post-eclosion and held for 4–6 days on *M. paniculata* in same-sex groups of 15–30 individuals inside a ventilated, 3.78-l plastic container. Psyllids collected as adults from the colony were presumed to be mated, and all females were gravid (i.e., exhibited an orange/yellow abdominal color, indicating the presence of eggs) when used in the olfactometer experiments. When maintained in culture with males, females with orange/yellow coloration of the abdomen are very likely mated, given the rarity of such coloration in virgin females (Wenninger & Hall, 2008). Until ready for use in experiments, adult psyllids were held in an environmental chamber at 26 °C, 60% r.h., and L14:D10 photoperiod, which resulted in 70–80% r.h. inside cages.

Open-air arena choice assays

The objective of this series of experiments was to determine whether *D. citri* of one sex colonize citrus plants that are infested with members of the same or opposite sex in greater numbers than paired uninfested plants (control). A flushing 22–24-month-old ‘Hamlin’ seedling approximately 50–60 cm in height was treated by infesting it with 10 psyllids (of the same sex and mating status) in a Plexiglas assay arena (70 × 70 × 60 cm). Psyllids were placed into a 3-cm diameter Petri dish mounted onto a clothes pin. Plants were infested with the psyllids by affixing the clothes pin to the plant stem. After the clothes pin was attached to the plant, the Petri dish cover was removed allowing psyllids to exit. The treatment plant intended for pre-infestation was subsequently isolated from the rest of the arena by covering it with a translucent plastic cylinder with a fine-mesh screen lid. Psyllids were allowed 24 h to settle and acclimate on this plant. A second plant, of matching size and age, was inserted into the assay arena 15 cm from the pre-infested plant and was left uninfested, serving as the control. Treatment and control plants were roughly equivalent in size and degree of flush in a given assay and between replicates. After 24 h, the plastic cover isolating the pre-infested plant was removed, and 10 new psyllids were introduced into the arena (in a Petri dish as described above) by placing them equidistantly (approximately 7 cm away from each plant stem) between the two plants on a

20-cm high plastic platform. The assay psyllids introduced into the arena 24 h later were marked with a bioluminescent powder (BioQuip Products, Rancho Dominguez, CA, USA) to distinguish them from the psyllids having pre-infested the treatment plant. Psyllids were marked by placing them into a 3-cm diameter Petri dish containing 20 mg of powder, which was gently shaken immediately prior to introduction into the assay arena. Four hours after introducing the marked psyllids, the bioassay was terminated and all marked and unmarked psyllids found on the treatment plant, control plant, and in the chamber but not on either plant were counted and sexed. Marked and unmarked psyllids were easily distinguished without microscopy. Behavioral assays were initiated at 10:00 hours and terminated at 14:00 hours, a period when *D. citri* are known to be sexually active (Wenninger & Hall, 2007). Assays were conducted under the temperature and humidity conditions described for the main culture. Light intensity within the arena ranged between 3 500 and 4 000 lux. The following combinations of treatments were tested: (i) virgin males assayed in response to plants pre-infested with virgin females, (ii) virgin males assayed in response to plants pre-infested with mated females, (iii) virgin males assayed in response to plants pre-infested with virgin males, (iv) virgin males assayed in response to plants pre-infested with mated males, (v) virgin females assayed in response to plants pre-infested with virgin females, (vi) virgin females assayed in response to plants pre-infested with mated females, (vii) virgin females assayed in response to plants pre-infested with virgin males, and (viii) virgin females assayed in response to plants pre-infested with mated males. Two more experimental treatment manipulations were conducted that were identical to experiments 1 and 2 above, except that all female psyllids used to pre-infest plants 24 h earlier that could be located were removed from plants and the assay arena immediately prior to the introduction of marked male assay psyllids into the arena. All virgin and mated psyllids were 5–7 days old when used for behavioral assays or as the pre-infestation treatment. Twenty replicates were conducted for each of the 10 treatment manipulations.

Y-tube olfactometer assays

The objective of this series of experiments was to test whether responses of male psyllids to females were due to a volatile chemical rather than potential non-volatile contact cues on seedling plants that could have influenced the results of assays in the open-air arena. Also, the Y-tube experiments tested the hypothesis that an olfactory signal from female *D. citri* without associated host-plant odors was sufficient to induce a behavioral response from males. To accomplish this, we tested the attraction responses of

psyllids in a glass Y-tube olfactometer (Analytical Research Systems, Gainesville, FL, USA). The Y-tube consisted of a 14-cm long stem and two 10-cm long arms, each with a 2-cm i.d. A screened glass plug at the base of the stem was used to introduce insects into the Y-tube. Upwind, at the end of each arm was a glass tube (2 cm i.d. \times 10 cm long) within which odor sources were placed (except when plants with psyllids were used as odor sources; see below); a wire screen prevented psyllids from entering these chambers and contacting odor sources. The fine mesh screen also precluded visual cues from influencing orientation behavior of the assayed psyllids. Given that vibrational signals may not provide a directional cue for psyllids on plants (see Discussion), we assume that any possible acoustic signals emitted by psyllids used as the odor source did not influence orientation behavior in the Y-tube. Charcoal-filtered, humidified air was metered through the two arms of the Y-tube via polytetrafluoroethylene (PTFE) tubing at 500 ml min⁻¹. The air exiting the wire screen plug at the base of the stem was 26–28 °C and 70–85% r.h.; light intensity just above the branching point of the Y-tube was ca. 7 000 lux. When plants were used as odor sources, air was routed via the PTFE tubing through 1-l glass canning jars upwind of the junction with the Y-tube. Individual potted 'Duncan' grapefruit plants were housed inside the jars, and a plastic bag was wrapped around the pot up to the base of the plant to limit any soil odors from entering the Y-tube. Each trial was conducted using 2–3-month-old plants 10 cm in height that were approximately equal in age and size and that had flush.

The position (left or right) of the test odor source was randomly selected for the first of any set of observations and alternated thereafter. 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 re-use.

Psyllids of various sex and mating status ($n \geq 30$ per experiment) were released individually into the glass plug at the base of the stem of the Y-tube, and their behavior was observed over a 10-min period. 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. With the exception of two experiments, the total duration of time spent in each arm as well as the number of vibrational calls (identified by characteristic movement of the wings of an adult) that occurred over the 10-min observation period was quantified.

Statistical analysis

Data on psyllid responses in the open air bioassay arena were transformed to $\ln(x + 1)$, which normalized the

distributions and homogenized variances, and then were subjected to a two-factor analysis of variance (ANOVA) (SAS Institute, 2000). The two factors for the ANOVA were: (i) the pre-infestation treatment manipulation for a particular experimental run (psyllid sex, mating status, and presence or absence of pre-infestation psyllids) and (ii) the final location of psyllid settlement (on pre-infested plant, control plant, or in cage but not on either plant). Following identification of a significant global ANOVA, orthogonal contrasts were used to compare cell means. Data for behaviorally assayed psyllids and those used as the pre-infestation treatment were analyzed separately. In all cases, significance level was $\alpha = 0.05$.

The number of individuals that chose the treated arm vs. the control arm was compared by χ^2 -tests ($\alpha = 0.05$). 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 ($\alpha = 0.05$). Data are presented as mean \pm SEM.

Results

Open-air arena choice assays

Final location of assayed psyllids. Final settlement location of assayed psyllids ($F_{2,570} = 16.3$, $P < 0.0001$) as well as the pre-infestation treatment manipulation ($F_{9,570} = 117.8$, $P < 0.0001$) were both statistically significant factors. Also, there was a significant ($F_{18,570} = 88.6$, $P < 0.001$) interaction between these two main effects. Significantly more assayed male psyllids were found on plants that had been pre-infested with female psyllids than on uninfested control plants, irrespective of whether the pre-infesting female psyllids were removed from treated plants prior to initiation of the bioassay (Table 1). When pre-infesting female psyllids were virgin and not removed, significantly more assayed males were found on the walls of the open-air bioassay cage than on either plant; there was no difference between the numbers of male psyllids found in these two locations in the experiment in which pre-treatment females were removed prior to the behavioral assay (Table 1). However, when pre-infesting female psyllids had been mated prior to the assay, significantly more assayed male psyllids were found on the pre-treated plants than on the walls of the cage, irrespective of whether the pre-infesting female psyllids were removed prior to the bioassay (Table 1). In cases where psyllids were assayed using conspecifics of the same sex as the pre-infestation treatment, significantly more assayed psyllids were found in the bioassay arena than on either plant and there were no differences between the numbers found on pre-treated plants vs. control plants; mating status of pre-infesting psyllids did not affect

Table 1 Final location of virgin male or female psyllids, *Diaphorina citri*, following behavioral assay in response to citrus plants pre-infested with psyllids (virgin or mated) or uninfested control plants in open-air assay arena

Assayed psyllids	Pre-infestation psyllids		Number of marked psyllids recovered following behavioral assay ¹		
	Sex	Mating status	Control plant	Pre-infestation plant	Cage
Male	Female	Virgin	0.10 ± 0.07Ac	2.60 ± 0.26Bb	4.70 ± 0.34Aa
Male	Female	Mated	0.00 ± 0.00Ac	4.55 ± 0.46Aa	2.65 ± 0.36Bb
Male	Male	Virgin	0.05 ± 0.05Ab	0.20 ± 0.09Cb	5.70 ± 0.49Aa
Male	Male	Mated	0.10 ± 0.07Ab	0.01 ± 0.07Cb	5.85 ± 0.34Aa
Female	Female	Virgin	0.00 ± 0.00Ab	0.05 ± 0.05Cb	6.40 ± 0.24Aa
Female	Female	Mated	0.05 ± 0.05Ab	0.00 ± 0.00Cb	6.65 ± 0.37Aa
Female	Male	Virgin	0.00 ± 0.00Ab	0.15 ± 0.08Cb	5.90 ± 0.30Aa
Female	Male	Mated	0.15 ± 0.08Ab	0.00 ± 0.00Cb	6.30 ± 0.35Aa
Male	Female ²	Virgin	0.10 ± 0.07Ab	2.95 ± 0.29Ba	3.30 ± 0.45Ba
Male	Female ²	Mated	0.10 ± 0.07Ac	4.95 ± 0.35Aa	1.95 ± 0.27Bb

¹Means within a column followed by the same upper case letter and within a row by the same lower case letter are not significantly different (two-way ANOVA followed by orthogonal contrasts: $P > 0.05$).

²All female psyllids used to pre-infest plants in this treatment that could be located were removed from plants and the bioassay arena prior to the behavioral assay.

this result (Table 1). Similarly, when female psyllids were assayed in response to plants pre-infested with unmated or mated male psyllids, the majority of females were found in the assay arena and on neither plant; there was no difference in the numbers found on pre-treated vs. control plants (Table 1).

When comparing among the 10 experimental treatment manipulations, there was no significant difference among the numbers of psyllids alighting on uninfested control plants (Table 1). Significantly more assayed psyllids were found on pre-infested plants in experiments in which male psyllids were released into cages pre-infested with female psyllids than in experiments in which same-sex releases were tested or in those in which females were released into cages pre-infested with males (Table 1). Moreover, significantly more male psyllids were found settled on plants that were pre-infested with mated female psyllids than on plants pre-infested with unmated female psyllids (Table 1). When males were assayed in response to plants pre-infested with mated females, significantly fewer assayed psyllids were found settled on the walls of the bioassay arena and not on either plant than for treatments (i) in which males were released into cages pre-infested with unmated females, (ii) in which same-sex pairs were tested, or (iii) in which females were released in cages with plants pre-infested with males.

Final location of psyllids used to pre-infest plants. Final settlement location of psyllids used to pre-infest plants ($F_{2,570} = 68.6$, $P < 0.0001$) as well as the pre-infestation treatment manipulation ($F_{9,570} = 113.7$, $P < 0.0001$) were both statisti-

cally significant factors. Also, there was a significant ($F_{18,570} = 27.4$, $P < 0.001$) interaction between these two main effects. For each of the 10 treatment manipulations tested, very few psyllids used to pre-infest treatment plants were found settled on control plants (mean ≤ 0.3 /plant) following the behavioral assay (Table 2). In the majority of the treatments tested in which pre-infesting psyllids were not removed, the numbers of pre-treatment psyllids found on the treated plant were not significantly different from the numbers found on the walls of the cage (Table 2). Thus, although some pre-treatment psyllids moved off of the treatment plant after the isolating container was removed, very few of these psyllids settled on the control plant. In the experiments in which unmated or mated female psyllids were used to pre-infest treated plants, significantly more of these psyllids were counted on the pre-treated plant than on the walls of the cage (Table 2). In the two experiments in which the female psyllids used to pre-infest plants were removed prior to the behavioral assay, two of the females for the unmated female assay and one of the females for the mated female assay were found remaining on the treatment plant when the assay was terminated (Table 2).

Comparing among the 10 treatments tested, there was no significant difference between the numbers of pre-infestation psyllids that settled on control plants (Table 2). When comparing among the eight experiments in which pre-infestation psyllids were not removed prior to introduction of the marked assay psyllids, there was no significant difference among the numbers of pre-infestation psyllids found on the treatment plant or among those found in the cage but not on either plant (Table 2). However,

Table 2 Final location of psyllids, *Diaphorina citri*, used to pre-infest plants prior to introduction of behaviorally assayed psyllids

Assayed psyllids	Pre-infestation psyllids		Number of psyllids recovered that were used to preinfest plant ¹		
	Sex	Mating status	Control plant	Pre-infestation plant	Cage
Male	Female	Virgin	0.10 ± 0.07Ab	3.65 ± 0.29Aa	3.85 ± 0.34Aa
Male	Female	Mated	0.00 ± 0.00Ab	3.60 ± 0.37Aa	3.05 ± 0.33Aa
Male	Male	Virgin	0.30 ± 0.11Ab	3.40 ± 0.29Aa	3.10 ± 1.52Aa
Male	Male	Mated	0.00 ± 0.00Ab	3.30 ± 0.36Aa	3.75 ± 0.34Aa
Female	Female	Virgin	0.20 ± 0.09Ac	4.15 ± 0.25Aa	2.95 ± 0.29Ab
Female	Female	Mated	0.05 ± 0.05Ac	4.25 ± 0.37Aa	3.10 ± 0.33Ab
Female	Male	Virgin	0.00 ± 0.00Ab	3.90 ± 0.36Aa	3.25 ± 0.38Aa
Female	Male	Mated	0.20 ± 0.09Ab	3.50 ± 0.37Aa	3.20 ± 0.31Aa
Male	Female ²	Virgin	0.05 ± 0.05Aa	0.05 ± 0.05Ba	0.00 ± 0.00Ba
Male	Female ²	Mated	0.05 ± 0.05Aa	0.00 ± 0.00Ba	0.00 ± 0.00Ba

¹Means within a column followed by the same upper case letter and within a row by the same lower case letter are not significantly different (two-way ANOVA followed by orthogonal contrasts: $P > 0.05$).

²All female psyllids used to pre-infest plants in this treatment that could be located were removed from plants and the bioassay arena prior to the behavioral assay.

significantly fewer pre-infestation psyllids were recovered on the treatment plant and in the cage, but not on either plant following the bioassays in the two experiments in which pre-infestation psyllids were removed prior to the assay than in the eight experiments in which pre-infestation psyllids were not removed (Table 2).

Y-tube olfactometer assays

In response to odors introduced by direct attachment of an odor source chamber to the Y-tube, mated males spent

more time in the arm containing four mated females than the control arm (Table 3). In no other case did the assayed psyllids show a difference in the length of time spent in the treated vs. control arm. Virgin males did not show a preference for the arm receiving the odors from four or 15 virgin females; however, significantly more virgin males chose the arm containing 15 mated females compared to the control arm (Table 3). In no other case did psyllids exhibit a significant first choice preference for the treatment arm vs. the control arm (Table 3).

Table 3 Results of Y-tube olfactometer trials examining responses of psyllids, *Diaphorina citri*, to treatment odor sources vs. a clean air control

Sex assayed	Treatment odor source	Odor source attachment ¹	n ²	First choice			Number of minutes spent in arm ⁴			
				Treated ³	χ^2	P-value	Treated	Control	t-value ⁵	P-value
Mated male	4 mated females	Direct	34 (28)	0.46	0.14	0.706	3.53 ± 0.4	2.11 ± 0.3	2.27	0.032
Mated male	4 mated males	Direct	32 (27)	0.33	3.0	0.083	2.19 ± 0.4	2.89 ± 0.4	-1.05	0.305
Virgin male	4 virgin females	Direct	30 (27)	0.37	1.8	0.178	1.85 ± 0.5	2.58 ± 0.6	-0.82	0.418
Virgin male	15 virgin females	Direct	30 (30)	0.60	1.2	0.271	–	–	–	–
Virgin male	15 mated females	Direct	30 (27)	0.70	4.5	0.034	–	–	–	–
Mated female	4 mated females	Direct	30 (30)	0.57	0.53	0.465	3.06 ± 0.4	2.54 ± 0.3	0.73	0.470
Mated female	4 mated males	Direct	30 (30)	0.60	1.2	0.273	3.45 ± 0.4	2.61 ± 0.4	1.10	0.281
Virgin male	6–7 virgin females on grapefruit	Tubing	30 (27)	0.37	1.8	0.178	2.07 ± 0.5	3.20 ± 0.5	-1.25	0.223

¹Odor sources were held upwind of the Y-tube assembly in chambers that were either directly attached to the Y-tube (direct) or attached to the Y-tube with 60 cm of tubing (tubing).

²Total sample size (n, number of individuals that made a choice in parentheses).

³Proportion of individuals (of those that made a choice) that chose the treated arm first.

⁴Number of minutes spent in arm was not recorded for the two assays using 15 females as the treatment odor source.

⁵Compares numerical difference between treatment and control with zero.

For mated males observed in the Y-tube with direct attachment of the odor source chamber, vibrational calling frequency of individual males exposed to odors from four mated females (2.59 ± 0.7) was significantly ($t = -3.51$, d.f. = 62, $P < 0.001$) lower than when exposed to the odors from four mated males (7.31 ± 1.2). Calling frequency of individual virgin males assayed in response to odors of four virgin females directly attached to the Y-tube (17.8 ± 2.6) did not differ ($t = -1.65$, d.f. = 57, $P = 0.105$) from that of individual virgin males exposed to odors from 6–7 virgin females on grapefruit in the separated odor chamber (23.6 ± 2.4). Females assayed in the Y-tube rarely exhibited vibrational calling behavior, and presentation of an olfactory stimulus in the Y-tube did not affect calling frequency.

Discussion

Collectively, our results suggest that male *D. citri* are attracted to a volatile female-produced pheromone. In the open-air arena, male *D. citri* colonized citrus plants that were currently or had been previously colonized by female conspecifics in greater numbers than identical uninfested plants. The ratio of responding males to presumably 'calling' females was approximately 1:1 in both cases. However, in experiments in which psyllids were assayed in response to same-sex conspecifics or in which females' responses to male-infested plants were tested, no evidence of attraction was observed. These results suggest that females may not only actively attract males while on plants, but also leave behind the source of an attractant chemical(s) following removal. Our results could be explained by several possible non-mutually exclusive hypotheses, including (i) females produce volatiles that attract or arrest males, (ii) female feeding and/or oviposition causes plants to release volatiles that attract or arrest males, (iii) female feeding and/or oviposition induces systemic changes in plants that arrest males, and (iv) females leave chemical and/or physical cues on the plant surface that attract or arrest males. Female *D. citri* release and leave behind a waxy honeydew secretion when feeding, and it is possible that this may serve as a source of a volatile male attractant; adult males excrete honeydew without wax (EJ Wenninger, pers. obs.). Our results are similar to those recently reported for the pear psylla, *C. pyricola* (Förster) (Horton & Landolt, 2007). The authors of that study suggested that male attraction to female-infested pear shoots may have been due to a female-produced volatile pheromone or due to an interaction between the female psyllid and the plant (Horton & Landolt, 2007). Given that psyllid feeding induces the production of volatile chemicals (Scutareanu et al., 1996, 1999) that are attractive to predators of pear psylla (Drukker et al., 2000), it was postulated that female psyllids feeding or egg-laying

could induce emission of volatiles attractive to conspecific males. Alternatively, it is also possible that a pheromone deposited by females during oviposition could function as a male attractant (Anderson, 2002).

Our Y-tube assays were designed to control for both the possibilities that male psyllids responded to non-volatile contact cues or cues resulting from the female psyllid-plant interaction as a result of feeding and/or oviposition. Specifically, psyllids were challenged only with an olfactory cue and in certain treatments the source of volatiles was comprised only female *D. citri* in the absence of a host plant. Both virgin and mated males exhibited significant responses to the odors of mated, but not virgin females, in the absence of a host plant. As in the open-air arena assays, males did not respond to other males nor did females respond to other females over a clean-air control arm in the Y-tube olfactometer. These results indicate that the odors emanating from females alone were sufficient for attracting male *D. citri*. A similar result has been reported for a pear-feeding psyllid, *C. bidens* (Šulc); males responded to females alone as well as female psyllid-infested pear shoots compared with blank control arms in a Y-tube olfactometer (Soroker et al., 2004).

Both behavioral assay techniques employed in this investigation indicated that mated female *D. citri* are more attractive to virgin or mated males than are virgin females. Although a small proportion of mated females was likely 24–48 h older than virgins in the Y-tube experiment, female age was held constant among mated and virgin females in the open-air chamber assays, suggesting that mating status rather than age accounted for this effect. Furthermore, male response to 15 mated females was greater than to four mated females in the Y-tube, suggesting that a greater number of females produced a greater total amount of olfactory signal. Female *D. citri* require multiple matings in order to maintain high and consistent reproductive output and appear to have the capacity for storing multiple spermatophores (EJ Wenninger, unpubl.); thus, it is possible that females release more pheromone or call more frequently after an initial mating bout in order to consistently attract subsequent males for re-mating. Females that do not re-mate lay a large number of infertile ova, whereas virgin females lay very few ova before mating (EJ Wenninger, unpubl.). To our knowledge, there are no previous studies investigating the effect of mating status on sex pheromone-mediated behavior in the Psylloidea, and thus we cannot say whether this result is unique to the biology of *D. citri*.

Among psyllids, acoustic communication by substrate-borne vibrations is characterized by male-calling followed by a response from a stationary receptive female (Tishechkin, 2005, 2007). This call and response behavior is also

characterized by male close-range searching behavior as he homes in on the responding female (Tishechkin, 2005). In addition, male vibrational singing stimulates similar signaling in other nearby males eventually resulting in a fused chorus of male calling (Tishechkin, 2007). However, the amplitude of vibratory oscillations in the plant stem do not show a regular pattern of weakening with distance traveled (Michelsen et al., 1982) and thus males cannot simply follow an increasing gradient of vibratory amplitude to find receptive females (Tishechkin, 2005, 2007; but see Cocroft et al., 2000 and Virant-Doberlet et al., 2005). In addition, males are frequently observed finding and copulating with females without previous vibrational calling behavior (Tishechkin, 2007), suggesting that other sensory modalities may also be involved in psyllid mate-finding behavior. In our Y-tube olfactometer assays, male vibrational calling behavior appeared to be suppressed when males were challenged by the odors emanating from mated female *D. citri*, but not when males were challenged by odors from other mated males. Given that there was no behavioral evidence for a male-produced olfactory signal in any of the experiments, the results imply that the female-produced olfactory signal suppressed calling behavior in conspecific males. It is possible that male *D. citri* that are actively calling females by substrate-borne vibrations cease this behavior when detecting a conspecific female's air-borne pheromone and instead initiate searching behavior toward this putative long-range signal. Once in closer range, the male may then switch back to vibrational signaling awaiting a substrate-borne vibratory response from a nearby stationary female. In this fashion, volatile pheromones may function as long-range attractants (e.g., between trees) while vibrational signaling (and possible visual cues) may come into play in close-range mate-finding (e.g., on the same branch or set of new flushing shoots). More detailed research will be required to verify the above hypothesis and describe this putative dual-modality mate-finding behavior in *D. citri*.

Collectively, the olfaction-mediated mate-finding behavior in *D. citri* has the following emerging set of characteristics: (i) males are attracted to volatile odors of females, (ii) mated females appear to be more attractive than virgin females to both mated and unmated males, (iii) there is no odor-mediated behavioral response between same-sex conspecifics, (iv) females do not respond to the odors of males, and (v) substrate-borne vibrational signaling in males appears to be suppressed in response to odors of conspecific females. The current results do not conclusively support or refute the hypothesis that feeding or oviposition by female *D. citri* on citrus host plants induces production of plant volatiles attractive to males or that plant volatiles enhance attractiveness of a female-produced olfactory

signal. Host plant volatiles are known to affect host-finding behavior in psyllids (Moran & Brown, 1973; Lapis & Borden, 1993; Soroker et al., 2004) and it has been postulated that female feeding and/or oviposition activity may induce production of host plant volatiles attractive to males (Horton & Landolt, 2007). The response of male and female *D. citri* to host plant volatiles and the effect of female plant colonization on production of inducible volatiles are the topics of currently ongoing research.

Sex pheromone-mediated mate-finding is known to occur in several sternorrhynchan families (Doane, 1966; Yin & Maschwitz, 1983; Campbell et al., 2003), and behavioral evidence for a sex-attractant has been described for two closely related psyllid species in the genus *Cacopsylla* (Soroker et al., 2004; Horton & Landolt, 2007; Horton et al., 2007). To our knowledge, this is only the third psyllid species in which evidence for a female-produced sex attractant has been described, and given its world-wide economic significance, identification and synthesis of a sex attractant pheromone will be an important contribution to current management practices. The input of broad-spectrum organophosphate and carbamate insecticides for pest management in Florida citrus has increased dramatically since the discovery of huanglongbing in 2005. Prior to the spread of this devastating disease, management sprays were dominated by horticultural oils, and biological control was a cornerstone of effective pest control (Michaud, 2002b). Identification of a sex pheromone and development of an effective synthetic lure for monitoring male *D. citri* occurrence should improve forecasting of management decisions.

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References

- Anderson P (2002) Oviposition pheromones in herbivorous and carnivorous insects. *Chemoecology of Insect Eggs and Egg Deposition* (ed. by M Hilker & T Meiners), pp. 235–264. Blackwell, Berlin, Germany.
- Borges M, Jepson PC & Howse PE (1987) Long-range mate location and close-range courtship behaviour of the green stink bug, *Nezara viridula* and its mediation by sex pheromones. *Entomologia Experimentalis et Applicata* 44: 205–212.

- Bové JM (2006) Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. *Journal of Plant Pathology* 88: 7–37.
- Campbell CAM, Cook FJ, Pickett JA, Pope TW, Wadhams LJ & Woodcock CM (2003) Responses of the aphids *Phorodon humuli* and *Rhopalosiphum padi* to sex pheromone stereochemistry in the field. *Journal of Chemical Ecology* 29: 2225–2234.
- Cocroft RB, Tieu TD, Hoy RR & Miles RN (2000) Directionality in the mechanical response to substrate vibration in a treehopper (Hemiptera: Membracidae: *Umbonia crassicornis*). *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology* 186: 695–705.
- Conner WE (1999) 'Un chant d'appel amoureux': acoustic communication in moths. *Journal of Experimental Biology* 202: 1711–1723.
- Doane CC (1966) Evidence for a sex attractant in females of the red pine scale. *Journal of Economic Entomology* 59: 1539–1540.
- Drukker B, Bruin J & Sabelis MW (2000) Anthocorid predators learn to associate herbivore-induced plant volatiles with presence or absence of prey. *Physiological Entomology* 25: 260–265.
- Halbert SE & Manjunath KL (2004) Asian citrus psyllids (Sternorrhyncha: Psyllidae) and greening disease of citrus: a literature review and assessment of risk in Florida. *Florida Entomologist* 87: 330–353.
- Hall DG & Albrigo LG (2007) Estimating the relative abundance of flush shoots in citrus, with implications on monitoring insects associated with flush. *HortScience* 42: 364–368.
- Hall DG, Hentz MG & Ciomperlik MA (2007a) A comparison of traps and stem tap sampling for monitoring adult Asian citrus psyllid (Hemiptera: Psyllidae) in citrus. *Florida Entomologist* 90: 327–334.
- Hall DG, Lapointe SL & Wenninger EJ (2007b) Effects of a particle film on biology and behavior of *Diaphorina citri* (Hemiptera: Psyllidae) and its infestations in citrus. *Journal of Economic Entomology* 100: 847–854.
- Heath JJ, Williams RN & Phelan PL (2002) Aggregation and male attraction to feeding virgin females in *Macrodactylus subspinosus* (F.) (Coleoptera: Scarabaeidae: Melolonthinae). *Environmental Entomology* 31: 934–940.
- Horton DR, Guédot C & Landolt PJ (2007) Diapause status of females affects attraction of male pear psylla, *Cacopsylla pyricola*, to volatiles from female-infested pear shoots. *Entomologia Experimentalis et Applicata* 123: 185–192.
- Horton DR & Landolt PJ (2007) Attraction of male pear psylla, *Cacopsylla pyricola*, to female-infested pear shoots. *Entomologia Experimentalis et Applicata* 123: 177–183.
- Hung TH, Hung SC, Chen CN, Hsu MH & Su HJ (2004) Detection by PCR of *Candidatus Liberibacter asiaticus*, the bacterium causing citrus huanglongbing in vector psyllids: application to the study of vector–pathogen relationships. *Plant Pathology* 53: 96–102.
- Hunt RE & Nault LR (1991) Roles of interplant movement, acoustic communication and phototaxis in mate-location behavior of the leaf-hopper *Graminella nigrifrons*. *Behavioral Ecology and Sociobiology* 28: 315–320.
- Krysan JL (1990) Laboratory study of mating behavior as related to diapause in overwintering *Cacopsylla pyricola* (Homoptera: Psyllidae). *Environmental Entomology* 19: 551–557.
- Lapis EB & Borden JH (1993) Olfactory discrimination by *Heteropsylla cubana* (Homoptera: Psyllidae) between susceptible and resistant species of *Leucaena* (Leguminosae). *Journal of Chemical Ecology* 19: 83–90.
- McFarland CD & Hoy MA (2001) Survival of *Diaphorina citri* (Homoptera: Psyllidae) and its two parasitoids, *Tamarixia radiata* (Hymenoptera: Eulophidae) and *Diaphorencyrtus aligarhensis* (Hymenoptera: Encyrtidae), under different relative humidities and temperature regimes. *Florida Entomologist* 84: 227–233.
- Michaud JP (2002a) Biological control of Asian citrus psyllid, *Diaphorina citri* (Hemiptera: Psyllidae) in Florida: a preliminary report. *Entomological News* 113: 216–222.
- Michaud JP (2002b) Classical biological control: a critical review of recent programs against citrus pests in Florida. *Annals of the Entomological Society of America* 94: 531–540.
- Michaud JP (2004) Natural mortality of Asian citrus psyllid (Homoptera: Psyllidae) in central Florida. *Biological Control* 29: 260–269.
- Michelsen A, Fink K, Gogala M & Traue D (1982) Plants as transmission channels for insect vibrational songs. *Behavioral Ecology and Sociobiology* 11: 269–281.
- Moran VC & Brown RP (1973) The antennae, host plant chemoreception and probing activity of the citrus psylla, *Trioza erytrae* (Del Guercio) (Homoptera: Psyllidae). *Journal of the Entomological Society of South Africa* 36: 191–202.
- Nakano R, Ishikawa Y, Tatsuki S, Surlykke A, Skals N & Takahashi T (2006) Ultrasonic courtship song in the Asian corn borer moth, *Ostrinia furnacalis*. *Naturwissenschaften* 93: 292–296.
- Percy DM, Taylor GS & Kennedy M (2006) Psyllid communication: acoustic diversity, mate recognition and phylogenetic signal. *Invertebrate Systematics* 20: 431–445.
- SAS Institute (2000) SAS/STAT User's Guide, Version 6, 4th edn, Vol. 1. SAS Institute, Cary, NC, USA.
- Scutareanu P, Drukker B, Bruin J, Posthumus MA & Sabelis MW (1996) Leaf volatiles and polyphenols in pear trees infested by *Psylla pyricola*. Evidence of simultaneously induced responses. *Chemoecology* 7: 34–38.
- Scutareanu P, Ma Y, Claeys M, Dommissie R & Sabelis MW (1999) Induction of a *p*-coumaroyl trihydroxy triterpene acid in *Psylla*-infested and mechanically damaged pear trees. *Journal of Chemical Ecology* 25: 2177–2191.
- Soroker V, Talebaev S, Harari AR & Wesley SD (2004) The role of chemical cues in host and mate location in the pear psylla *Cacopsylla bidens* (Homoptera: Psyllidae). *Journal of Insect Behavior* 17: 613–626.
- Takács S, Mistal C & Gries G (2003) Communication ecology of webbing clothes moth: Attractiveness and characterization of male-produced sonic aggregation signals. *Journal of Applied Entomology* 127: 127–133.
- Tishechkin DY (1989) Acoustic communication in the psyllids (Homoptera, Psyllinea) of the Moscow Province. *Vestnik Moskovskogo Universiteta Seriya XVI Biologiya* 4: 20–24.

- Tishechkin DY (2005) Vibratory communication in Psylloidea (Hemiptera). *Insect Sounds and Communication: Physiology, Behaviour, Ecology, and Evolution* (ed. by S Drosopoulos & MF Claridge), pp. 357–363. Taylor & Francis, Boca Raton, FL, USA.
- Tishechkin DY (2006) On the structure of stridulatory organs in jumping plant lice (Homoptera: Psyllinea). *Russian Entomological Journal* 15: 335–340.
- Tishechkin DY (2007) New data on vibratory communication in jumping plant lice of the families Aphalaridae and Triozidae (Homoptera, Psyllinea). *Entomological Review* 87: 394–400.
- Tsai JH, Wang JJ & Liu YH (2000) Sampling of *Diaphorina citri* (Homoptera: Psyllidae) on orange jessamine in southern Florida. *Florida Entomologist* 83: 446–459.
- Virant-Doberlet M, Čokl A & Zorović M (2005) Use of substrate vibrations for orientation: from behaviour to physiology. *Insect Sounds and Communication: Physiology, Behaviour, Ecology, and Evolution* (ed. by S Drosopoulos & MF Claridge), pp. 81–97. Taylor & Francis, Boca Raton, FL, USA.
- Weninger EJ & Hall DG (2007) Daily timing of mating and age at reproductive maturity in *Diaphorina citri* (Hemiptera: Psyllidae). *Florida Entomologist* 90: 715–722.
- Weninger EJ & Hall DG (2008) Daily and seasonal patterns in abdominal color in *Diaphorina citri* (Hemiptera: Psyllidae). *Annals of the Entomological Society of America* 101: 585–592.
- Yin LT & Maschwitz U (1983) Sexual pheromone in the green house whitefly *Trialeurodes vaporariorum*. *Zeitschrift für Angewandte Entomologie* 95: 439–446.
- Zhang Q-H, Chauhan KR, Erbe EF, Vellore AR & Aldrich JR (2004) Semiochemistry of the goldeneyed lacewing *Chrysopa oculata*: attraction of males to a male-produced pheromone. *Journal of Chemical Ecology* 30: 1849–1870.