Insect science

Insect Science (2012) 00, 1–12, DOI 10.1111/j.1744-7917.2012.01541.x

ORIGINAL ARTICLE

Chemical and behavioral analysis of the cuticular hydrocarbons from Asian citrus psyllid, *Diaphorina citri*

Rajinder S. Mann[1], Russell L. Rouseff[2], Jack Smoot[2], Nandikeswara Rao[3], Wendy L. Meyer[1], Stephen L. Lapointe[4], Paul S. Robbins[4], Dong Cha[5,†], Charles E. Linn[5], Francis X. Webster[6], Siddharth Tiwari[1] and Lukasz L. Stelinski[1]

[1]*Entomology and Nematology Department, Citrus Research and Education Center, University of Florida,* [2]*Flavor and Volatile Analysis Group, Citrus Research and Education Center, University of Florida, Lake Alfred, FL 33850, USA,* [3]*National Research Center for Citrus, Nagpur, Maharashtra, India,* [4]*USDA-ARS, US Horticultural Research Laboratory, Fort Pierce, FL 34945,* [5]*Department of Entomology, NYS Agricultural Experiment Station, Cornell University, Geneva, NY 14456,* [6]*Department of Chemistry, State University of New York College of Environmental Science and Forestry, Syracuse, NY 13210, USA*

Abstract Huanglongbing (HLB) is the most destructive disease of citrus worldwide. The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), is the vector of the phloem-inhabiting bacterium, *Candidatus* Liberibacter asiaticus, which is presumed to cause HLB in Florida citrus. Laboratory and field studies were conducted to examine the behavioral responses of male and female *D. citri* to their cuticular extracts. In olfactometer assays, more male *D. citri* were attracted to one, five, or 10 female cuticular extract equivalent units than blank controls. The results were confirmed in field studies in which clear or yellow traps baited with 10 female cuticular extract equivalent units attracted proportionately more males than clear traps baited with male cuticular extract or unbaited traps. Analyses of cuticular constituents of male and female *D. citri* revealed differences between the sexes in chemical composition of their cuticular extracts. Laboratory bioassays with synthetic chemicals identified from cuticular extracts indicated that dodecanoic acid attracted more males than clean air. Traps baited with dodecanoic acid did not increase total catch of *D. citri* as compared with blank traps at the dosages tested; however, the sex ratio of psyllid catch was male biased on traps baited with the highest lure loading dosage tested (10.0 mg).

Key words citrus, cuticular hydrocarbon, dodecanoic acid, Huanglongbing, semiochemical

Introduction

Huanglongbing (HLB) is the most economically important disease of citrus worldwide. HLB affects plant phloem, causing yellow shoots, mottling, chlorosis, and twig die back that result in rapid tree decline and may ultimately cause tree death. Fruit on diseased trees are misshaped, reduced in size, and sour in taste (Bové, 2006; Capoor, 1963; Dagulo *et al.*, 2010; Halbert & Manjunath, 2004). The disease is associated with either of three species of phloem-limited, noncultured, gram-negative bacteria: *Candidatus* Liberibacter asiaticus (Las), *Candidatus* Liberibacter africanus (Laf), or *Candidatus* Liberibacter americanus (Lam) (Bové, 2006; Gottwald, 2010). The pathogen is primarily transmitted worldwide by the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), as well as, by the African citrus

Correspondence: Lukasz L. Stelinski, Entomology and Nematology Department, Citrus Research and Education Center, University of Florida, 700 Experiment Station Road, Lake Alfred, FL, 33850, USA. Tel: 863 956 8851; fax: 863 956 4631; email: stelinski@ufl.edu

[†]Present address is USDA-ARS, Yakima Agricultural Research Laboratory, Wapato, WA 98951, USA.

psyllid, *Trioza erytreae* (Del Guercio), in Africa. Both psyllid species can transmit any of the three bacterial species (Gotwald, 2010). In North America, the disease is thought to be caused by Las and transmitted by *D. citri* (Bové, 2006; Gottwald, 2010; Gottwald *et al.*, 2007).

Current management for *D. citri* relies on application of broad-spectrum insecticides (Rogers, 2008) because resistant cultivars do not exist (Halbert & Manjunath, 2004), biological control is insufficiently effective (Qureshi & Stansly, 2009), and to date, cultural control strategies have not prevented proliferation of the disease (Childers & Rogers, 2005; Powell *et al.*, 2007). However, insecticide use has negatively affected populations of natural enemies and has led to development of insecticide resistance (Tiwari *et al.*, 2011). Therefore, identification of attractants may allow development of useful alternative tools for monitoring and control of *D. citri*. Recent investigations indicate that males of some psyllid species use volatile chemicals for mate location (Guédot *et al.*, 2009; Soroker *et al.*, 2004; Wenninger *et al.*, 2008). Behavioral evidence for a female-produced volatile sex attractant was reported for pear psylla, *Cacoppsylla bidens* (Šulc) (Soroker *et al.*, 2004). Post-diapausing female pear psylla, *Cacopsylla pyricola* (Förster), produce a cuticular hydrocarbon (13-methylheptacosane) that attracts opposite sex conspecifics (Guédot *et al.*, 2009). Also, there is behavioral evidence of a female-derived volatile attractant for male *D. citri* (Wenninger *et al.*, 2008). Furthermore, ultrastructure of olfactory sensilla on *D. citri* antennae suggests chemosensory functions (Onagbola *et al.*, 2008). The specific objectives of the current investigation were to determine if cuticular extracts from female *D. citri* attract conspecific male *D. citri* and to identify potential attractants that may have practical applications in the management of *D. citri*.

Materials and methods

Insect source

Adult *D. citri* used in behavioral bioassays were obtained from a laboratory culture maintained at the University of Florida Citrus Research and Education Center (Lake Alfred, FL, USA). The culture was established in 2000 from field populations in Polk Company (FL, USA) (28.00N, 81.90W) prior to the discovery of HLB in FL. The culture is maintained without exposure to insecticides on sour orange (*Citrus aurantium* L.) and "Hamlin" orange (*C. sinensis* [L.] Osb.) seedlings at $27 \pm 1^{\circ}$C, $63\% \pm 2\%$ relative humidity (RH), and under a L14 : D10 photoperiod.

Behavioral bioassays

A custom-designed, two port divided T-olfactometer from Analytical Research Systems (ARS, Inc., Gainesville, FL, USA) as described by Mann *et al.* (2011) was used to evaluate the behavioral response of *D. citri* to odors emanating from live adults or cuticular extracts. The olfactometer consisted of a 30 cm glass tube that was bifurcated into two equal halves with a Teflon strip forming a T-maze. Each half served as an arm of the olfactometer enabling the psyllids to make a choice between two potential odor fields. The olfactometer arms were connected to odor sources placed in solid-phase microextraction chambers (SPMECs) (ARS, Inc.) through Teflon glass tube connectors. A SPMEC consisted of a straight glass tube (17.5 cm long × 3.5 cm wide) supported with an inlet and outlet valve for incoming and outgoing air streams, respectively (Mann *et al.*, 2011). The test samples were wrapped in laboratory tissue (Kimwipes, Kimberly-Clark, Roswell, GA, USA) and placed into the SPMEC. Purified and humidified air was pushed through the SPMEC via two pumps connected to an air delivery system (ARS, Inc.). A constant airflow of 0.1 L/min was maintained through both arms of the olfactometer. The olfactometer was housed within a temperature controlled room and positioned vertically under a fluorescent 900 lx light bulb mounted within a $1.0 \times 0.6 \times 0.6\,m^3$ fiber board box for uniform light diffusion. This position took advantage of the negative geotactic and positive phototactic response of *D. citri* (Mann *et al.*, 2011). The olfactometer inlet adapter was covered with black cloth to facilitate insect movement toward odor sources by exploiting the positive phototactic response of *D. citri* (Mann *et al.*, 2011).

Absence of positional bias on psyllid behavior was confirmed prior to assays with chemical treatments by exposing *D. citri* adults to clean air versus clean air. An odor source was then randomly assigned to one of the arms of the olfactometer at the beginning of each bioassay and was reversed after every 25 insects to further control for positional bias. *D. citri* adults were released individually into the inlet adapter at the base of the olfactometer. Adults were given 5 min to exhibit a behavioral response by entering either olfactometer arm. The numbers of adults entering the treatment arm, control arm, or remaining in the release port or below the T-maze division were recorded. A treatment or control arm choice was recorded when an insect moved into either olfactometer arm by crossing the division in the T-maze. A no response (release arm) choice

was recorded when an insect remained in the release port or below the T-maze division. The choice for treatment arm, control arm, or release arm was scored as attraction, repulsion, or not responding, respectively. All experiments were conducted at $26 \pm 1^{\circ}$C and $60\% \pm 2\%$ RH. The olfactometer and connecting tubes were thoroughly cleaned with 2% soap solution, rinsed with acetone, and baked at 60°C for 24 h between each treatment run. Bioassays were performed using a minimum of 100 *D. citri* per treatment and sex combination.

Behavioral response of D. citri *to cuticular extract of opposite or same sex conspecifics*

Cuticular extractions were prepared according to the procedures outlined by Guédot *et al.* (2009). Briefly, for each extraction, 500 male or female *D. citri* adults or nymphs (3rd–5th instar) were transferred into separate 11 mL glass vials containing 500 μL pentane and the vial was shaken by hand for 10 min. The solution was subsequently transferred to a clean glass vial and defined as the stock solution. The solution was partitioned into defined equivalent units. For example, 1 μL of solution obtained from 500 μL of female extract was defined as one female equivalent unit, while 5 or 10 μL of solution was defined as five or 10 female equivalent units, respectively. Solutions of male cuticular extract were prepared identically. Simultaneously with each extraction, a control treatment of 500 μL of pentane only was prepared. A new extract was prepared every 15 days throughout the investigation. The extracts were stored in a freezer at 0°C. Odor sources for the olfactometer bioassasys were prepared by dissolving the appropriate insect equivalent dose in 100 μL of pentane. This was then pipetted onto a 2 cm Richmond cotton wick (Petty John Packaging, Inc. Concord, NC, USA). The control treatment consisted of a cotton wick impregnated with 100 μL of pentane only. The solvent from both treatments was allowed to evaporate in a fume hood for 30 min prior to assays. The treated and control cotton wicks were wrapped in laboratory tissue (Kimwipes, Kimberly-Clark) and placed randomly into one of the SPMEC chambers. The response of male and female *D. citri* to one, five, or 10 male or female cuticular extract equivalent units was recorded. We tested the response of (i) males to female cuticular extract versus clean air, (ii) males to male cuticular extract versus clean air, (iii) females to male cuticular extract versus clean air, and (iv) females to female cuticular extract versus clean air.

To determine if the compounds extracted from the cuticular surface of the psyllids were the only source of behaviorally active chemicals, the cadavers of the insects used for the extractions were used as a source in one of the SPMEC chambers. The treatment combinations were (i) response of males to 50 dead females versus clean air or (ii) response of females to 50 dead males versus clean air. Each assay was conducted in the T-maze olfactometer according to the methods described above. Preliminary tests confirmed previous data (Wenninger *et al.*, 2009a) that recently killed, but nonextracted female *D. citri* were attractive to live males.

Field trials with male and female cuticular extract

A field-trapping test evaluating the attractiveness of male and female *D. citri* cuticular extracts was conducted. The experiment was deployed in a 10.2 ha commercial orange (*Citrus sinensis* [L.] var. "Valencia") orchard. Trees were 12 years old, planted on $3 \times 6\,m^2$ spacing and averaged 4 m in height. No insecticides were applied at this site in the year of testing. The traps used were yellow sticky cards (Pherocon AM; Trécé, Adair, OK, USA) and clear panel traps made from clear Plexiglass ($23 \times 14 \times 0.625\,cm^3$) that were coated with thin layer of Tangle-Foot glue (Great Lakes IPM Inc., Vestaburg, MI, USA). The coating on the clear traps was applied to obtain a sticky surface area ($18 \times 23\,cm^2$) identical to the yellow traps. Clear traps were used since it is known that *D. citri* adults are highly attracted to the yellow color (Hall *et al.*, 2007). Gray halo butyl rubber septa (West Pharmaceutical Services, Lionville, PA, USA) were used as dispensers. Septa were pre-extracted with pentane, and then aired for 24 h at 24°C in a fume hood before use. Septa were loaded with one, five, or 10 male or female *D. citri* cuticular extract equivalent units dissolved in 100 μL of pentane. Control lures were treated with 100 μL of pentane only. Lures were aired at room temperature in fume hoods for 0.5 h prior to deployment. Lures were attached to the center of traps using safety pins. Each trap was hung near the exterior of a tree canopy approximately 1.5 m above ground with 30 cm of wire. One trap was deployed per tree and treatments were separated by 6 m. The experiment was arranged as a randomized complete block design with five replicates and with 12 m spacing between blocks. Each replication consisted of 14 treatments in total: one, five, or 10 equivalents of male or female cuticular extract units on either yellow or clear sticky traps and one unbaited control treatment for each trap type. The lures were removed from each trap and placed on a new trap at 1, 2, and 4 days to determine the longevity of the lure. The traps were removed after 7 days in the field. All *D. citri* captured on each trap were counted in the laboratory with separate counts for males and females. The field experiment was repeated

identically in July and September 2011 with the same number of replicates and treatments.

Cuticular extract analyses

The cuticular extracts of male and female psyllids were analyzed with a Perkin/Elmer® Clarus 500 quadrupole gas chromatograph–mass spectrometer (GC–MS) equipped with Turbo Mass software (Perkin/Elmer, Shelton, CT, USA) and a 60 m × 0.25 mm, inner diameter. × 0.50 μm film thickness, Restek (Stabilwax) capillary column (Restek Inc., Bellefonte, PA, USA). Ten microliter aliquots (10 male or female equivalents) were concentrated down to 1 μL aliquots under nitrogen gas and then injected into the GC–MS in the splitless mode. Helium was used as the carrier gas at a constant flow of 2 mL/min. The source was kept at 180°C, and the transfer line and injector were maintained at 240°C. The oven was programmed from 40 (2 min initial hold time) to 240°C at 7°C/min with a 9.5 min final hold. Observed mass spectra from selected chromatographic peaks were identified using NIST 2005 version 2.0 standard spectra (NIST, Gaithersburg, MD, USA). Only identifications with spectral fit values equal to or greater than 800 were considered positive. Identifications were confirmed by matching chromatographic retention time and spectra with those from authentic standards.

The identified chemicals that were detected in female cuticular extracts only were further analyzed for their presence in male cuticular extract using the more analytically selective extracted ion chromatogram mode. Two unique and characteristic *m/z* ions (171 and 200) were monitored to selectively detect dodecanoic acid and other peaks of interest.

Behavioral response to synthetic chemicals

We also examined the responses of male *D. citri* adults to authentic synthetic standards obtained from Sigma–Aldrich (St. Louis, MO, USA) (Table 1) versus clean air at 0.1, 1.0, 10.0, and 100.0 μg dosages. All chemicals were >95% pure. The procedures for assaying synthetic chemicals were identical to those described above for cuticular extract treatments and employed clear Plexiglass traps that were coated with thin layer of Tangle-Foot glue as described above. The chemicals were dissolved in pentane. Acetone and ethanol were used as solvents for chemicals insoluble in pentane. Appropriate solvents were used as controls for each chemical. No differences were observed between nonresponses to each solvent and therefore these were pooled for statistical analyses.

Field trials with dodecanoic acid

A field trial was conducted to evaluate the attractiveness of dodecanoic acid to *D. citri*. It was deployed in the above-described commercial citrus orchard. Gray halo butyl rubber septa loaded with 0.1, 1.0, and 10.0 mg of dodecanoic acid dissolved in 100 μL of pentane were used as dispensers. Control lures were treated with 100 μL of pentane only. Ten female *D. citri* cuticular extract equivalent units dissolved in pentane were used as a positive control. Lures were prepared and deployed as described above. The experiment was arranged as a randomized complete block design with five replicates. Treatments were separated by 12 m. The traps were removed 72 h after deployment and all *D. citri* captured per trap were counted by sex in the laboratory. The entire experiment was replicated five times.

Data analysis

The numbers of *D. citri* entering the treatment or control arm in the T-maze olfactometer assays were compared by χ^2 square tests. For the field assays evaluating cuticular extracts, the analysis was performed on the total number of psyllids captured on each trap using a two factor (trap type, treatment) repeated measures analysis of variance (ANOVA). Given that the number of psyllids captured per trap was low (<5 psyllids per trap) 1 day after deployment, the catch data from the initial 2 days were combined for analysis. There was a significant interaction between trap color and cuticular extract treatments. Therefore, the cuticular extract data were analyzed separately for each trap color (see results). For field assays involving dodecanoic acid, data were analyzed using one-way ANOVA to compare the total number of male psyllids captured per trap. The analysis was performed on log $n+1$ transformed means to normalize the data. The means were compared using Tukey's HSD test. χ^2 square tests were used to determine the sex ratio of psyllids captured per trap. χ^2 square test was performed on the total number of psyllids captured from all replicates ($\alpha = 0.05$). All data were analyzed in PROC GLM option of SAS Version 9.1 for Windows (SAS Institute, 2003).

Results

Behavioral response of D. citri *to cuticular extract of opposite or same sex conspecifics*

Significantly ($\chi^2 = 10.31$, df = 1, $P = 0.001$) more male *D. citri* adults chose the olfactometer arm containing

Table 1 GC–MS identification of constituents from cuticular extract of male and female *Diaphorina citri*.

Sex	Chemical†	CAS no.	Observed column linear retention index	Literature linear retention index	Retention time
Female	Acetic acid	64-19-7	1 470	1 477	17.17
	Ethyl undecanoate	627-90-7	1 761	1 737	22.34
	Hexanoic acid	142-62-1	1 868	1 872	24.05
	Geranyl acetone	141-12-8	1 771	1 838	24.38
	1-Dodecanol	112-53-8	1 988	1 970	25.86
	Decanoic acid	34-48-5	2 297	2 296	30.14
	Isopropyl tetradecanoate	110-27-0	2 049	2 048	26.86
	1-methylpropyl Dodecanoate	6 937-42-4	2 235	NA‡	29.43
	Pentadecanoic acid	112-80-1	2 500	2 821	39.10
Male					
	Hexadecane	1 560-92-5	1 605	1 600	19.66
	1-Dodecanol	112-53-8	1 988	1 970	25.86
	Dodecanoic acid	143-07-7	2 517	2 517	33.19
	Tetradecanoic acid	822-12-8	2 500	2 724	37.61

†Identification was based on comparisons of retention times with authentic standard and spectral data from Nist05 Libraries.
‡Literature index unavailable.

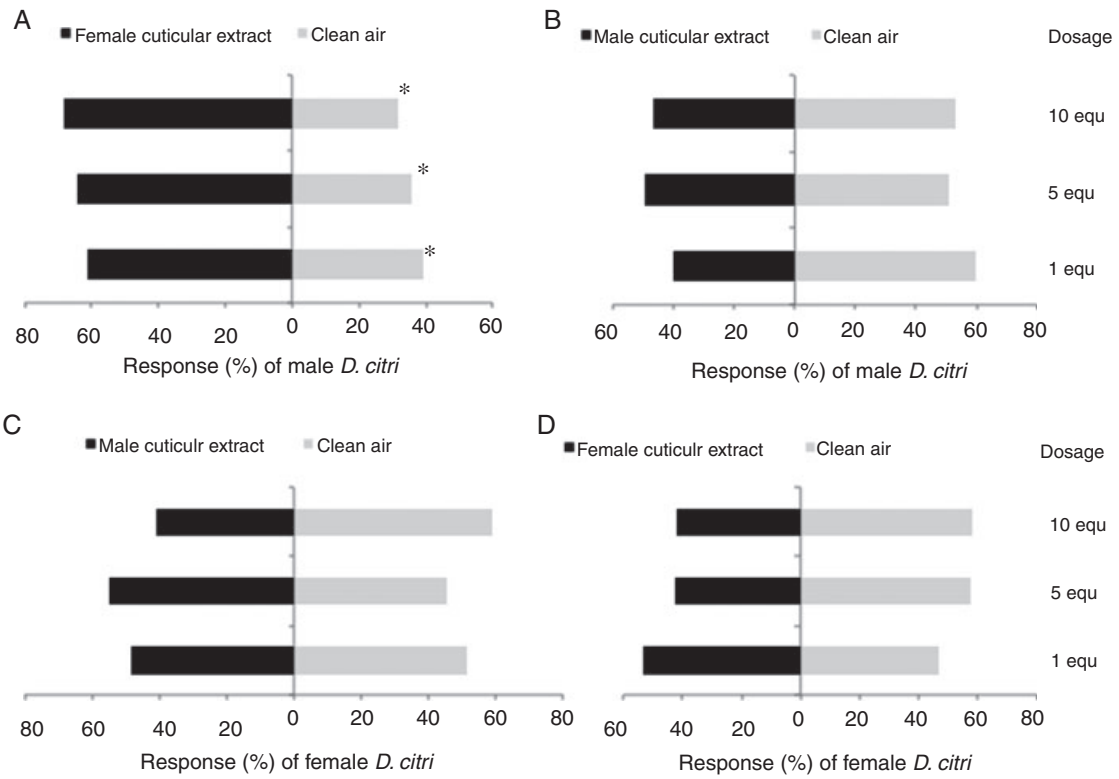

Fig. 1 Behavioral response of (A) male to female, (B) male to male, (C) female to male, and (D) female to female *Diaphorina citri* to one, five, or 10 female cuticular extract equivalent units (equ) compared with clean air in laboratory olfactometer.

one, five, or 10 female equivalent units of cuticular extract than clean air (Fig. 1A). However, males did not show a preference for male cuticular extract compared with clean air ($\chi^2 = 0.25$, df = 1, $P = 0.61$) (Fig. 1B). Females were not attracted to male or female cuticular extracts when they were given a choice between male extract versus clean air ($\chi^2 = 2.31$, df = 1, $P = 0.12$) (Fig. 1C) or female extract versus clean air ($\chi^2 = 1.75$, df = 1, $P = 0.18$) (Fig. 1D). Adult male and female *D. citri* did not exhibit attraction to solvent-extracted cadavers of conspecific males ($\chi^2 = 2.48$, df = 1, $P = 0.11$ and $\chi^2 = 0.41$, df = 1, $P = 0.52$, respectively) or females ($\chi^2 = 0.58$, df = 1, $P = 0.44$ and $\chi^2 = 0.25$, df = 1, $P = 0.61$, respectively) directly after the cuticular extract procedure. It has been established that killed female *D. citri* attract conspecific males (Wenninger *et al.*, 2009b) and preliminary tests here confirmed this result (data not shown).

Field trials with male and female cuticular extract

Repeated measure analysis indicated significant effects of trap color ($F = 22.10$; df = 1.56; $P < 0.0001$), cuticular extract treatment ($F = 5.37$; df = 6.56; $P = 0.0002$), and their interaction ($F = 2.55$; df = 6.56; $P = 0.0298$). Analysis of data performed separately for the two trap types indicated that clear traps baited with 10 *D. citri* male or female cuticular extract units captured significantly more total psyllids than unbaited clear control traps 2 days after deployment ($F = 4.24$; df = 6.28; $P = 0.0037$). Clear traps baited with one, five, or 10 female cuticular extract equivalent units attracted more males than females (Table 2). Yellow traps baited with 10 female cuticular extract equivalent units captured more males than females (Table 3). However, the total number of psyllids captured on yellow traps baited with 10 female equivalents was not different from the unbaited yellow control (Table 3). Yellow traps baited with 10 male cuticular extract equivalent units captured more total psyllids than unbaited control traps after 2 days of deployment ($F = 3.81$; df = 6.28; $P = 0.0067$) (Table 3). The total number of *D. citri* captured on traps baited with any of the cuticular extract treatments was not different from the control beyond the second day of field deployment (data not shown).

Cuticular extract analyses

Acetic acid, ethyl undecanoate, isopropyl tetradecanoate, 1-methylpropyl dodecanoate, hexanoic acid, geranyl acetate, decanoic acid, and pentadecanoic acid were constituents detected only in female extracts; hexadecane was detected only in male extracts; and 1-dodecanol, dedecanoic acid, and tetradecanoic acid were detected in both male and female extracts (Table 1 and Fig. 2). As shown in Figure 2, the highly selective extracted ion mode chromatograms (EIC), employing only the masses unique to dodecanoic acid, revealed that dodecanoic acid and tetradecanoic acid were present in male extract in very low quantities, compared with females. Additionally, very low levels of three esters, ethyl undecanoate, isopropyl tetradecanoate, and 1-methylpropyl dodecanoate were revealed in female extracts using EIC mode. Shown in Figure 3 is a demonstration of the selectiveness in using only those ions characteristic of dodecanoic acid compared with the normal total ion chromatogram (TIC). The mass at *m*/*z* 200 was chosen because it is the molecular ion for dodecanoic acid. As seen from the MS fragmentation pattern for standard dodecanoic acid, there is also an appreciable peak at *m*/*z* 171, which corresponds to the loss of an ethylene group. The relatively clean baseline shown in the EIC chromatogram in Figure 3 compared with the erratic baseline in the same sample shown in the normal TIC mode is because both of the chosen masses are relatively unique and there are few if any interferences in the EIC mode.

Behavioral response to synthetic chemicals

Behavioral bioassays with synthetic chemicals identified from the female cuticular extract indicated that more male *D. citri* entered the arm receiving dodecanoic acid ($\chi^2 = 5.08$; df = 1; $P = 0.02$) at the 100 μg dosage than the arm receiving clean air (Table 4). No other test dosage tested elicited *D. citri* response to dodecanoic acid. None of the other chemicals tested elicited a positive response as compared with the control (data not shown).

Field trials with dodecanoic acid

Clear traps baited with septa loaded with 10 mg of dodecanoic acid attracted significantly more *D. citri* males than females ($\chi^2 = 6.00$; df = 1; $P = 0.01$) (Table 5). The sex ratio of captured psyllids was not affected by dodecanoic acid at any of the other dosages tested (Table 5). The total catch of all psyllids was not increased by any of the treatments tested as compared with the blank control (Table 5).

Discussion

Male *D. citri* were attracted to the cuticular extracts of conspecific females. Furthermore, removal of cuticular

Table 2 Capture of male and female *Diaphorina citri* on clear traps baited with 1, 5, or 10 *D. citri* cuticular extract equivalent units after 2 days of deployment.

Sex	*D. citri* cuticular extract dosage (equivalent units)	Mean number of *D. citri*/ trap captured†	Percent of male *D. citri* captured	χ^2 value	*P* value‡
Male	1	2.6 ± 0.3 bc	35.9	3.10	0.08
Male	5	4.0 ± 0.4 ab	54.0	0.40	0.53
Male	10	4.9 ± 0.3 ab	46.0	0.49	0.48
Female	1	3.3 ± 0.4 bc	65.2	6.39	0.01*
Female	5	4.6 ± 0.3 ab	61.2	4.59	0.03*
Female	10	6.4 ± 0.6 a	60.4	4.17	0.04*
Control	0	1.9 ± 0.2 c	48.3	0.03	0.85

†Means labeled with the same letters in rows are not significantly different (Tukey's HSD Test, $\alpha = 0.05$).
‡Values labeled with * indicate a significant difference (χ^2 test, $\alpha = 0.05$).

Table 3 Capture of male and female *Diaphorina citri* on yellow traps baited with 1, 5, or 10 *D. citri* cuticular extract equivalent units after 2 days of deployment.

Sex	*D. citri* cuticular extract dosage (equivalent units)	Mean number of *D. citri*/trap captured†	Percent of male *D. citri* captured	χ^2 value	*P* value§
Male	1	5.9 ± 0.3 ab‡	57.6	2.41	0.12
Male	5	7.1 ± 2.8 ab	41.6	0.14	0.70
Male	10	7.4 ± 1.5 a	51.7	0.10	0.75
Female	1	7.3 ± 1.3 a	56.2	2.72	0.10
Female	5	11.2 ± 1.3 a	47.7	0.23	0.63
Female	10	5.6 ± 0.7 ab	61.6	3.96	0.04*
Control	0	2.7 ± 0.2 b	42.5	0.90	0.34

†Pooled mean of three trials.
‡Means labeled with the same letters in rows are not significantly different (Tukey's HSD Test, $\alpha < 0.05$).
§Values labeled with * indicate a significant difference (χ^2 test, $\alpha = 0.05$).

Table 4 Responses of male *Diaphorina citri* when assayed to dodecanoic acid versus clean air in an olfactometer.

Dodecanoic acid dosage (μg)	Percent of *D. citri* responding to treatment arm	χ^2 value	*P* value†
0.1	46.9	0.25	0.61
1.0	56.9	1.24	0.26
10.0	61.4	3.66	0.05
100.0	63.4	5.08	0.02*

†Values labeled with * indicate a significant difference (χ^2 test, $\alpha = 0.05$).

hydrocarbons by solvent washing rendered females nonattractive to males, while normally, recently killed females are known to attract male psyllids (Wenninger *et al.*, 2009b). The primary role of insect cuticular hydrocarbons is to provide a hydrophobic barrier to minimize transpiration and desiccation (Howard & Blomquist, 1982; Lockey, 1988; Nelson, 1978). However, cuticular hydrocarbons are also known to mediate chemical communication in many insect species, including psyllids (Guédot *et al.*, 2009; Howard & Bloomquist, 2005; Yew *et al.*, 2009).

The behavioral and chemical laboratory results indicated a sex-specific function of female cuticular hydrocarbons, but our field results did not exclude a possible aggregative function. Sex pheromone-mediated mate finding is known in several sternorrhynchan families (Campbell *et al.*, 2003; Doane, 1966; Yin & Maschwitz, 1983), including psyllid species in the genus *Cacopsylla* (Guédot *et al.*, 2009;Horton & Landolt, 2007; Horton *et al.*, 2007; Soroker *et al.*, 2004). Male *D. citri* are known to colonize citrus plants that are currently or had been previously colonized by virgin or mated female *D. citri* in greater numbers than control plants without females, suggesting the presence of a sex attractant (Wenninger

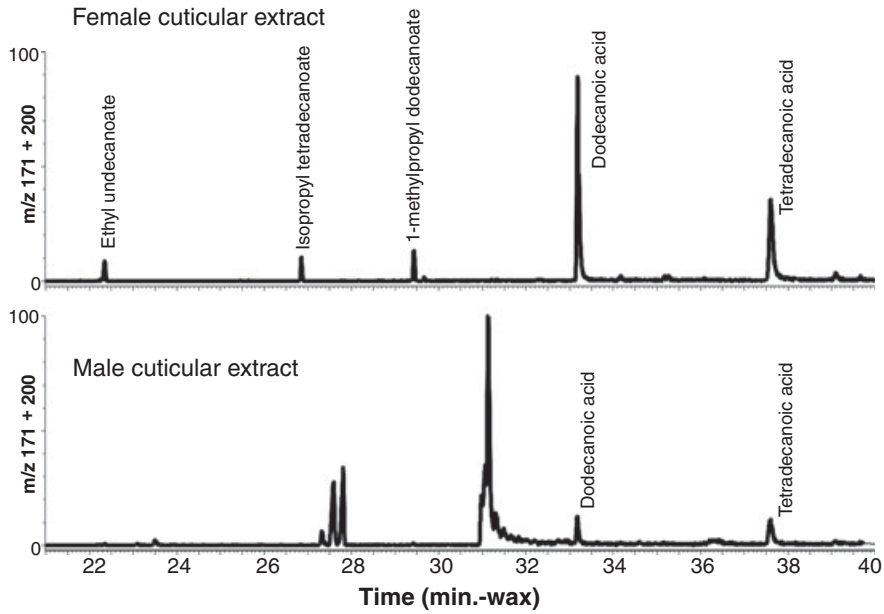

Fig. 2 Comparison of female and male psyllid pentane cuticular extracts as assessed by extracted ion mode chromatograms (EIC).

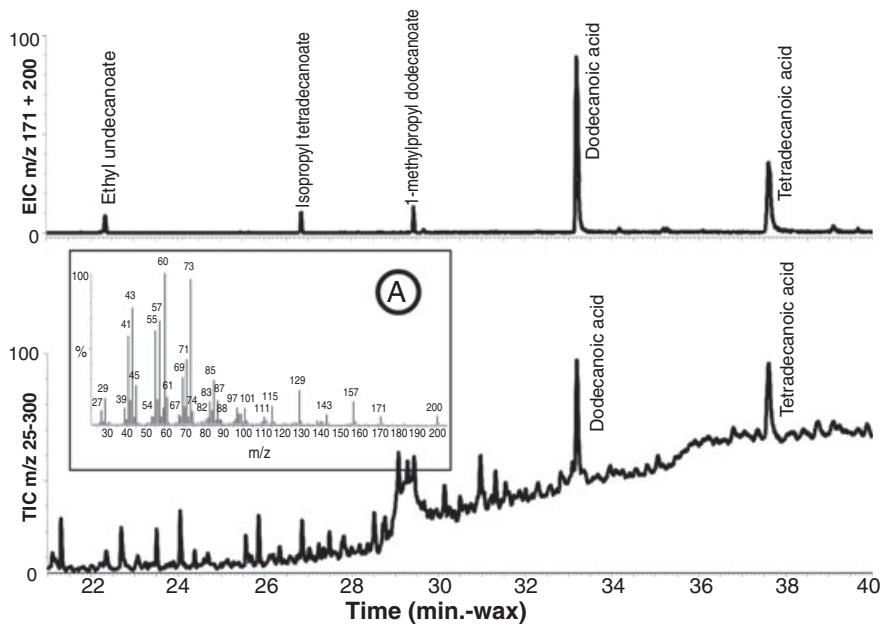

Fig. 3 Comparison of EIC versus TIC chromatograms from 1 μL injection of female psyllid cuticular extract. The insert a depicts MS fragmentation pattern of standard dodecanoic acid. Note the small but unique masses at *m*/*z* 171 and the molecular ion at *m*/*z* 200.

et al., 2008). Furthermore, aggregation of *D. citri* within citrus trees was recently observed by Hall and Hentz (2010) in trapping experiments.

In field trials, female cuticular extracts attracted more male *D. citri* on clear traps; however, this difference did not occur on yellow traps. Visual cues are known to strongly influence the orientation of *D. citri* to olfactory cues (Wenninger *et al.*, 2009a). *D. citri* adults are strongly attracted to yellow color (Hall, 2009; Hall & Albrigo, 2007; Hall *et al.*, 2007). Therefore, at the dosage of cuticular extracts tested here, it is possible that response to an olfactory cue was obscured by the visual

Table 5 Capture of male and female *Diaphorina citri* on clear traps baited with dodecanoic acid loaded in gray halo butyl rubber septa.

Synthetic chemical	Dosage (mg)	Number of male *D. citri*/trap	Percent of male *D. citri* captured	χ^2 value	*P* value†
Dodecanoic acid	0.1	2.8 ± 0.9	43.8	0.5	0.48
	1.0	4.0 ± 1.3	55.5	0.44	0.51
	10.0	3.6 ± 1.1	75.0	6.0	0.01*
Pentane (control)	0	5.2 ± 1.7	57.5	1.04	0.30

†Values labeled with *indicate a significant difference (χ^2 test, $\alpha = 0.05$).

stimulus of yellow traps. Host location and mate finding in *D. citri* is likely mediated by several modalities, including an integration of olfactory, visual, and vibrational cues. Cuticular hydrocarbons may stimulate production of shorter range vibrational signals that are used by males to locate females once they are on the same plant or substrate (Miklas *et al.*, 2003). *D. citri* are known to produce substrate borne vibrational signals for mate finding (Wenninger *et al.*, 2009c).

Dodecanoic acid was one of the few components detected in higher amounts from female than male extracts that also elicited a behavioral response from males in laboratory assays. Although dodecanoic acid alone did not increase total catch of psyllids on traps as compared with a blank control, it did skew the sex ratio of captured psyllids toward male bias at the highest dosage tested. The lack of attraction to dodecanoic acid in the field may have been due to the design of our dispenser. Because of the low volatility of this compound, the dosage range tested may have been insufficient to elicit response in the field. However, it is possible that other minor components are needed for full behavioral activity (Innocenzi *et al.*, 2004). Several hemipterans are known to use sex pheromone blends rather than single compounds for communication and mating (McBrien *et al.*, 2002; Millar *et al.*, 1997; Leal *et al.*, 1998; Tillman *et al.*, 2010). For example, a blend of (*E*)- and (*Z*)-1,2-epoxides of (*Z*)-α-bisabolene (44% and 15%, respectively), (*E*)-nerolidol (1.4%), and *n*-nonadecane (7.4%) is attractive to *Nezara viridula* (L.) (Aldrich *et al.*, 1987), while a blend of hexyl butyrate and (*E*)-4-oxo-2-hexenal is more attractive to *Lygus rugulipennis* Poppius than individual constituents (Innocenzi *et al.*, 2004; 2005). Furthermore, incongruence between field trapping experiments and laboratory assays is known to occur (Hedin *et al.*, 1985; Ho & Millar, 2002; Innocenzi *et al.*, 2004; Silk *et al.*, 2011). Dodecanoic acid is known to influence the behavior of at least 18 insect species; however, by itself, it is not known to be a sex attractant for any insect species (El-Sayed, 2011). It may be interesting to test the effect of synthetic dodecanoic acid on the response of *D. citri* to authentic cuticular extracts to determine potential additive or synergistic effects.

Both clear and yellow traps baited with five or 10 equivalent units of either male or female cuticular extract captured more total *D. citri* than unbaited controls. These results suggest a possible aggregative function of the cuticular hydrocarbons. Typically, males produce aggregation pheromones, but there are several exceptions (Jiang *et al.*, 2002; Judd & Borden, 1992; Wertheim *et al.*, 2005). Insect response to an aggregation pheromone is known to be largely affected by physiological state (e.g., hunger, mating condition, age, and molting cycle) and extrinsic factors (e.g., time of day, season, density of conspecifics of same or opposite sex, and temperature) (Aller & Cladwell, 1979; Bartelt & Jackson, 1984; Joosse, 1970; Lorenzo Figuerias *et al.*, 1994; Mayhew & Phillips, 1994; Schaner *et al.*, 1989). Many species are known to respond to aggregation pheromones when they are accompanied by odors of food or breeding substrates or other coattractants (Phillips and Burkholder, 1981; Ramirez-Lucas *et al.*, 1996; Wertheim *et al.*, 2005). Furthermore, release of aggregation pheromones is also influenced by presence of conspecific eggs, larvae, or pupae as well as adult density at the release site (Wertheim *et al.*, 2005). Aggregation of *D. citri* adults within citrus trees or on flush shoots has been previously reported (Dharajothi *et al.*, 1989; Hall & Hantz, 2010; Sétamou *et al.*, 2008). However, it is also possible that yellow traps may have attracted females initially and subsequently attracted males via an olfactory attractant from the captured females. Attraction to same sex extracts was not observed in the laboratory assays, suggesting a sex-specific function of the attractant.

Collectively, our results from both the laboratory and field suggest that one or more cuticular hydrocarbons from female *D. citri* may be an attractant for conspecific males. Investigations are in progress to identify further attractive components and to determine if a specific blend of cuticular hydrocarbons functions as a *D. citri* pheromone.

Acknowledgments

We thank Angel Hoyte and Michael Flores for help with insect maintenance and bioassays. This work was supported by a grant from the Citrus Research and Development Foundation, Florida, USA.

Disclosure

The authors have no conflicts of interest.

References

Aldrich, J.R., Oliver, J.R., Lusby, W.R., Kochansky, J.P. and Lockwood, J.A. (1987) Pheromone strains of the cosmopolitan pest, *Nezara viridula* (Heteropetera, Pentatomidae). *Journal of Experimental Zoology*, 244, 171–175.

Aller, T. and Caldwell, R.L. (1979) An investigation of the possible presence of an aggregation pheromone in the milkweed bugs, *Oncopeltus fasciatus* and *Lygaeus kalmii*. *Physiological Entomology*, 4, 287–290.

Bartelt, R.J. and Jackson L.L. (1984) Hydrocarbon component of the *Drosophila virilis* (Diptera, Drosophilidae) aggregation pheromone, (*Z*)-10-heneicosene. *Annals of Entomological Society of America*, 77, 364–371.

Bové, J.M. (2006) Huanglongbing, a destructive, newly-emerging century-old disease of citrus. *Journal of Plant Pathology*, 88, 7–37.

Campbell, C.A.M., Cook, F.J., Pickett, J.A., Pope, T.W., Wadhams, L.J. and Woodcock, C.M. (2003) Responses of the aphids *Phorodon humuli* and *Rhopalosiphum padi* to sex pheromone stereochemistry in the field. *Journal of Chemical Ecology*, 29, 2225–2234.

Capoor, S.P. (1963) Decline of citrus trees in India. *Bulletin of National Institute of Science India*, 24, 48–64.

Childers, C.C. and Rogers, M.E. (2005) Chemical control and management approaches of the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera, Psyllidae) in Florida citrus. *Proceedings of Florida State Horticultural Society*, 118, 49–53.

Dagulo, L., Danyluk, M.D., Spann, T.M., Valim, F., Schneider, R.G., Sims, C. and Rouseff, R.L. (2010) Chemical characterization of orange juice from trees infected with citrus greening (Huanglongbing). *Journal of Food Science*, 75, 199–207.

Dharajothi, B., Verghese, A. and Tandon, P.L. (1989) Ecological studies on citrus psylla, *Diaphorina citri* Kuwayama (Hemiptera, Psyllidae) with special reference to its spatial distribution and sampling plan. *Entomon*, 14, 319–324.

Doane, C.C. (1966) Evidence for a sex attractant in females of the red pine scale. *Journal of Economic Entomology*, 59, 1539–1540.

El-Sayed, A.M. (2011) The pherobase: database of pheromones and semiochemicals. Available at: http://www.pherobase.com (accessed 5 October, 2011).

Gottwald, T.R. (2010) Current epidemiological understanding of citrus huanglongbing. *Annual Review of Phytopathology*, 48, 1–21.

Gottwald, T.R., DaGraca, J. and Bassanezi, R. (2007) Citrus huanglongbing: The pathogen and its impact. *Plant Health Progress*, doi: 10.1094/PHP-2007-0906-01-RV.

Guédot, C.J., Millar, G., Horton, D.R. and Landolt, P.J. (2009) Identification of a sex attractant pheromone for male winter-form pear psylla, *Cacopsylla pyricola*. *Journal of Chemical Ecology*, 35, 1437–1447.

Halbert, S.E. and Manjunath, K.L. (2004) Asian citrus psyllids (Sternorrhyncha, Psyllidae) and greening disease of citrus, a literature review and assessment of risk in Florida. *Florida Entomologist*, 87, 330–354.

Hall, D.G. (2009) An assessment of yellow sticky card traps as indicators of the abundance of adult *Diaphorina citri* (Hemiptera, Psyllidae) in citrus. *Journal of Economic Entomology*, 102, 446–452.

Hall, D.G. and Albrigo, L.G. (2007) Estimating the relative abundance of flush shoots in citrus, with implications on monitoring insects associated with flush. *Hort Science*, 42, 364–368.

Hall, D.G. and Hentz, M.G. (2010) Sticky trap and stem–tap sampling protocols for the Asian citrus psyllid (Hemiptera, Psyllidae). *Journal of Economic Entomology*, 103, 541–549.

Hall, D.G., Hentz, M.G. and Ciomperlik, M.A. (2007) A comparison of traps and stem tap sampling for monitoring adult Asian citrus psyllid (Hemiptera, Psyllidae) in citrus. *Florida Entomologist*, 90, 327–334.

Hedin, P.A., Parrott, W.L., Tedders, W.L. and Reed, D.K. (1985) Field responses of the tarnished plant bug to its own volatile components. *Journal of Mississippi Academy of Science*, 30, 63–66.

Ho, H.Y. and Millar, J.G. (2002) Identification, electroantennogram screening, and field bioassays of volatile chemicasl from *Lygus hesperus* Knight (Heteroptera, Miridae). *Zoological Studies*, 41, 311–320.

Horton, D.R., Guédot, C. and Landolt, P.J. (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, D.R. and Landolt, P.J. (2007) Attraction of male pear psylla, *Cacopsylla pyricola*, to female-infested pear shoots. *Entomologia Experimentalis et Applicata*, 123, 177–183.

Howard, R.W. and Blomquist, G.J. (1982) Chemical ecology and biochemistry of insect hydrocarbons. *Annual Review of Entomology*, 27, 149–172.

Howard, R.W. and Blomquist, G.J. (2005) Ecological, behavioral, and biochemical aspects of insect hydrocarbons. *Annual Review of Entomology*, 50, 371–393.

Innocenzi, P.J., Hall, D.R., Cross, J.V., Masuh, H., Phythian, S.J., Chittamuru, S. and Guarino, S. (2004) Investigation of long-range female sex pheromone of the European tarnished plant bug, *Lygus rugulipennis*, chemical, electrophysiological and field studies. *Journal of Chemical Ecology*, 30, 1509–1529.

Innocenzi, P.J., Cross, J.V., Hesketh, H. and Hall, D. (2005) Attraction of male European tarnished plant bug, *Lygus rugulipennis* to components of the female sex pheromone in the field. *Journal of Chemical Ecology*, 31, 1401–1413.

Jiang, Y.C., Lei, L., Niu, C.Y., Fang, Y.L., Xiao, C. and Zhang, Z.N. (2002) Semiochemicals from ovaries of gravid females attract ovipositing female houseflies, *Musca domestica*. *Journal of Insect Physiology*, 48, 945–950.

Joosse, E.N.G. (1970) The formation and biological significance of aggregations in the distribution of Collembola. *Netherland Journal of Zoology*, 20, 299–314.

Judd, G.J.R. and Borden, J.H. (1992) Aggregated oviposition in *Delia antiqua* (Meigen), a case for mediation by semiochemicals. *Journal of Chemical Ecology*, 18, 621–635.

Leal, W.S., Kuwahara, S., Shi, X., Higuchi, H., Marinao, C.E.B., Ono, M. and Meinwald, J. (1998) Male-released sex pheromone of the stink bug *Piezodorus hybneri*. *Journal of Chemical Ecology*, 24, 1817–1829.

Lockey, K.H. (1988) Lipids of the insect cuticle, origin, composition, and function. *Comparative Biochemistry and Physiology B*, 89, 595–645.

Lorenzo Figueiras, A.N., Kenigsten, A. and Lazzari, C.R. (1994) Aggregation in the haematophagous bug *Triatoma infestans*, chemical signals and temporal pattern. *Journal of Insect Physiology*, 40, 311–316.

Mann, R.S., Smoot, J.M., Rouseff, R.L., Castle, W.J. and Stelinski, L.L. (2011) Sulfur volatiles from *Allium* spp. affect Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera, Psyllidae) response to citrus volatiles. *Bulletin of Entomological Research*, 101, 89–97.

Mayhew, T.J. and Phillips, T.W. (1994) Pheromone biology of the lesser grain borer, *Rhyzopertha dominica* (Coleoptera, Bostrichidae). *Proceedings of 6th International Workshop and Conference on Stored-Production and Protection*, 1, 541–544.

McBrien, H.L., Millar, J.G., Rice, R.E., McElfresh, J.S., Cullen, E. and Zalom, F.G. (2002) Sex attractant pheromone of the red-shouldered stink bug *Thyana pallidovirens:* a pheromone blend with multiple redundant components. *Journal of Chemical Ecology*, 28, 1797–1818.

Miklas, N., Lasnier, T. and Renou, M. (2003) Male bugs modulate pheromone emission in response to vibratory signals from conspecifics. *Journal of Chemical Ecology*, 29, 561–574.

Millar, J.G., Rice, R.E. and Wang, Q. (1997) Sex pheromone of the mired bug *Phytocoris relativus*. *Journal of Chemical Ecology*, 23, 1743–1754.

Nelson, D.R. (1978) Long-chain methyl-branched hydrocarbons: occurrence, biosynthesis, and function. *Advances in Insect Physiology*, 13, 1–33.

Onagbola, E.A., Meyer, W., Boina, D.R. and Stelinski, L.L. (2008) Morphological characterization of the antennal sensilla of the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera, Psyllidae), with reference to their probable functions. *Micron*, 39, 1184–1191.

Phillips, J.K. and Burkholder, W.E. (1981) Evidence for a male-produced aggregation pheromone in the rice weevil. *Journal of Economic Entomology*, 74, 539–42.

Powell, C.A., Burton, M.S., Pelesi, R.A., Ritenour, M.A. and Bullock, R.C. (2007) Effects of insecticide on Asian citrus psyllid (Hemiptera, Psyllidae) populations in a Florida citrus grove. *Plant Health Progress*, doi:10.1094/PHP-2007-1101-01-RS.

Qureshi, J.A. and Stansly, P.A. (2009) Exclusion techniques reveal significant biotic mortality suffered by Asian citrus psyllid *Diaphorina citri* (Hemiptera, Psyllidae) populations in Florida citrus. *Biological Control*, 50, 129–136.

Ramirez-Lucas, P., Rochat, D. and Zagatti, P. (1996) Field trapping of *Metamasius hemipterus* with synthetic aggregation pheromone. *Entomologia Experimetalis et Applicata*, 80, 453–460.

Rogers, M.E. (2008) General pest management considerations responses to greening and its vectors have modified pest management programs. *Citrus Industry*, 89, 12–17.

SAS Institute, INC (2003) *SAS/STAT User's Guide*. Version 9.1. SAS Institute, Inc., Cary, NC.

Schaner, A.M., Graham, K.J. and Jackson, L.L. (1989) Aggregation pheromone characterization and comparison in *Drosophila ananassae* and *Drosophila bipectinata*. *Journal of Chemical Ecology*, 15, 1045–1055.

Sétamou, M., Flores, D.F., French, J.V. and Hall, D.G. (2008) Dispersion patterns and sampling plans for *Diaphorina citri* (Hemiptera, Psyllidae) in citrus. *Journal of Economic Entomology*, 101, 1478–1487.

Silk, P.J., Ryall, K., Mayo, P., Lemay, M.A., Grant, G., Crook, D., Cossé, A., Fraser, I., Sweeney, J.D., Lyons, D.B., Pitt, D., Scarr, T. and Magee, D. (2011) Evidence for a volatile pheromone in *Agrilus planipennis* Fairmaire (Coleoptera, Buprestidae) that increases attraction to a host foliar volatile. *Environmental Entomology*, 40, 904–916.

Soroker, V., Talebaev, S., Harari, A.R. and Wesley, S.D. (2004) The role of chemical cues in host and mate location in the pear psylla *Cacopsylla bidens* (Homptera, Psyllidae). *Journal of Insect Behavior*, 17, 613–626.

Tillman, P.G., Aldrich, J.R., Khrimian, A. and Cottrell, T.E. (2010) Pheromone attraction and cross-attraction of *Nezara,*

Acrosternum, and *Euchistus* spp. (Heteroptera: Pentatomidae) in the field. *Environmental Entomology*, 39, 610–617.

Tiwari, S., Mann, R.S., Rogers, M.E. and Stelinski, L.L. (2011) Insecticide resistance in field populations of Asian citrus psyllid in Florida. *Pest Management Science*, 67, 1258–1268.

Wenninger, E.J., Stelinski, L.L. and Hall, D.G. (2008) Behavioral evidence for a female-produced sex attractant in *Diaphorina citri* Kuwayama (Hemiptera, Psyllidae). *Entomologia Experimentalis et Applicata*, 128, 450–459.

Wenninger, E.J., Stelinski, L.L. and Hall, D.G. (2009a) Roles of olfactory cues, visual cues, and mating status in orientation of *Diaphorina citri* Kuwayama (Hemiptera, Psyllidae) to four different host plants. *Environmental Entomology*, 38, 225–234.

Wenninger, E.J., Stelinski, L.L. and Hall, D.G. (2009b) Relationships between adult abdominal color and reproductive potential in *Diaphorina citri* (Hemiptera, Psyllidae). *Annals of the Entomological Society of America*, 102, 476–483.

Wenninger, E.J., Hall, D.G. and Mankin, R.W. (2009c) Vibrational communication between the sexes in *Diaphorina citri* (Hemiptera: Psyllidae). *Annals of the Entomological Society of America*, 102, 547–555.

Wertheim, B., van Baalen, E.J.A., Dicke, M. and Vet, L.E.M. (2005) Pheromone-mediated aggregation in nonsocial arthropods, an evolutionary ecological perspective. *Annual Review of Entomology*, 50, 321–346.

Yew, J.Y., Dreisewerd, K., Luftmann, H., Muthing, J., Pohlentz, G. and Kravitz, E.A. (2009) A new male sex pheromone and novel cuticular cues for chemical communication in *Drosophila*. *Current Biology*, 19, 1245–1254.

Yin, L.T. and Maschwitz, U. (1983) Sexual pheromone in the green house whitefly *Trialeurodes vaporariorum*. *Journal of Applied Entomology*, 95, 439–446.

Accepted May 21, 2012