J Chem Ecol (2016) 42:941–951
DOI 10.1007/s10886-016-0745-4

A Phagostimulant Blend for the Asian Citrus Psyllid

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Received: 19 April 2016 / Revised: 27 June 2016 / Accepted: 29 June 2016 / Published online: 19 August 2016

Abstract Chemical cues that elicit orientation by the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), are of interest because it is the primary vector of the causal pathogen of citrus greening disease. Non-pesticidal control methods for *D. citri* remain a high priority for the citrus industry. While searching for semiochemicals that may be involved in orientation to host plants, we previously identified a blend of formic and acetic acids that stimulated substrate probing by *D. citri*. Here, we applied geometric mixture designs and response surface modeling to identify and optimize a 3-component blend that further increased the number of salivary sheaths produced by *D. citri* on a wax substrate containing a 3.5:1.6:1 blend of formic acid, acetic acid, and *p*-cymene, respectively. No evidence was found for remote orientation by *D. citri* adults through olfaction to the phagostimulant blends. Increased probing in response to the presence of phagostimulants in the wax matrix occurred after contact with the substrate. Yellow wax beads always attracted more *D. citri* adults and received more probes compared with white wax beads. Yellow beads containing the 3-component blend of phagostimulants were probed by *D. citri* 2 to 3 times more often compared with yellow beads alone. The phagostimulant effect also was tested by covering wax beads containing the 3-component blend with a plastic film to minimize olfaction or contact chemoreception by antennation. The plastic film did not affect the probing response, thus suggesting that chemosensation was associated with mouthparts and not olfactory receptors. Salivary sheaths produced in wax beads containing the phagostimulant blend were 4.5 times longer than sheaths produced in beads without tastants. This phenomenon might be used to improve a trap, design an attract-and-kill product, or enhance other means of managing *D. citri* and citrus greening disease.

Keywords Insect-plant interactions · Salivary sheath · Psyllid probing behavior · Formic acid · Acetic acid · *p*-cymene · Hemiptera · Liviidae

Introduction

Production of oranges and grapefruit in Florida has declined by approximately 75 % since near-record production in 2000 (NASS 2016). This decline is attributed largely to crop loss and tree mortality resulting from citrus greening disease. The phloem-limited bacterial pathogen, '*Candidatus* Liberibacter asiaticus' (CLas) is transmitted by the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae). The pathogen produces symptoms referred to as citrus greening disease or Asiatic huanglongbing (HLB) that include mottled leaves as well as irregular and bitter fruit. Efforts by grove managers to control the spread of the pathogen have focused on intensive pesticide regimens to control the vector, but with minimal effect on the progress of the disease. Studies of plant and conspecific odors have failed to identify pheromones or kairomones for *D. citri* that could be used as attractants, although certain blends have shown some attraction under laboratory conditions (Aksenov et al. 2014; Coutinho-Abreu et al. 2014a; George et al. 2016; Wenninger et al. 2009a). Electroantennogram studies have failed to demonstrate antennal responses by adult *D. citri* to volatiles released by citrus, with the exception of some small molecules that result from

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degradation of certain compounds (George et al. 2016). Extensive recordings from single antennal sensilla have shown the potential for response to a wide variety of host plant volatiles (Coutinho-Abreu et al. 2014b). Movement toward volatile plant odors has been demonstrated by tests in olfactometers and cages (Mann et al. 2012; Patt and Sétamou 2010; Wenninger et al. 2009a), but field tests with odors have had mixed success. In residential citrus in southern California, scent baits failed to capture more *D. citri* in the field than unscented traps (Godfrey et al. 2013). A 3-component blend identified by Coutinho-Abreu et al. (2014a) attracted nominally more adult *D. citri* compared with a solvent control. The psyllid is consistently attracted to the color yellow (Sétamou et al. 2014). In addition to orientation to visual and, possibly, chemical odorants, close-range sexual communication has been shown to occur by substrate vibration (Rohde et al. 2013; Wenninger et al. 2009b). The apparent weak orientation at a distance by *D. citri* to host plant odors is consistent with the mechanism proposed to explain dispersal and orientation to host plants by small flighted insects such as aphids: visual perception of yellow wavelengths during flight, and gustatory perception of arrestants after alighting on a potential host followed by a limited number of shorter flights if the initial host is deemed unacceptable (Kennedy and Stroyan 1959). The suitability of a host plant for sustained feeding and oviposition in aphids has been shown to occur before the stylets reach the phloem, during their intercellular passage between and intracellular sampling of epidermal and parenchyma cells (Powell et al. 2006). The possibility that host selection or probing behavior by *D. citri* is mediated by chemosensory factors (tastants) perceived after settling on a potential host has not been studied.

Psyllids, like aphids, produce a gelling saliva to form a salivary sheath that surrounds the stylets during probing and feeding. Recently, George et al. (2016) reported that acetic and formic acids were singularly excitatory to antennae of male and female adult *D. citri.* They also demonstrated that adult *D. citri* responded to the presence of formic and acetic acids by increasing the number of probes into a wax substrate containing the two carboxylic acids. Those results suggested that acetic and formic acids (and perhaps others compounds) act as phagostimulants. George et al. (2016) also demonstrated that a 1.9:1 blend of formic:acetic acid was the optimal proportion of those compounds in a two-component blend for maximal probing by *D. citri* of a wax substrate. We investigated this phenomenon further by using mixture designs to study the effect of incorporating additional compounds into multiple component blends to increase the stimulatory effect of acetic and formic acids on psyllid probing behavior. We focused on compounds identified as stimulatory to olfactory receptors by single-cell recordings from neurons in the antennae of *D. citri* (Coutinho-Abreu et al. 2014b). We also tested the hypothesis that adult *D. citri* orient to these compounds from a distance and studied the interaction between visual attraction to yellow and perception of phagostimulants. Finally, we studied how probing behavior was affected by placement of a layer of plastic film between the substrate containing phagostimulants and the psyllid in an attempt to separate olfaction and contact chemoreception from gustation.

Methods and Materials

Asian Citrus Psyllids (ACPs) A healthy (CLas-free) colony of *D. citri* was maintained at the U.S. Horticultural Research Laboratory, Fort Pierce, FL, USA. The colony was tested routinely to confirm that it was HLB-negative by using PCR methods previously reported (Li et al. 2008). Psyllids were reared on seedlings of a susceptible host, *Citrus macrophylla* Wester, and maintained at 28 °C, 14:10 h L:D. All insects used for bioassays were adults between 8 and 10 d old; sex ratio was approximately 50:50.

Effect of Blends on Psyllid Probing Behavior A completely randomized choice assay was used to study the probing behavior of *D. citri* in response to chemosensory stimuli (odorants and/or tastants) as described (George et al. 2016; Patt et al. 2013). This assay measured insect orientation within a screened cage to combinations of color and odor, and subsequent probing behavior that may result from a combination of olfaction and gustation upon contact with a wax substrate containing odorants or tastants. Test compounds were incorporated into a slow-release wax matrix (SPLAT™, ISCA Technologies Inc., Riverside, CA, USA) and offered to caged *D. citri* adults in beads of yellow or white SPLAT. A yellow wax substrate was prepared by adding 6 μl of green food coloring (McCormick & Co., Inc., Hunt Valley, MD, USA) to 10 ml of white SPLAT provided by the manufacturer resulting in a yellow-green mixture. Yellow and white stocks were combined separately with odorant/tastant compounds in a vortex rotor for 5 min. One ml of the treatments (white or yellow SPLAT with or without odorants/tastants) was applied as narrow strips or beads (2.0 × 0.5 × 0.1 cm) to 6 glass cover slips (22 × 22 mm, Fisherbrand Microscope Cover Glass 12–542-B). Each cover slip received approximately 0.17 ml of wax and 0.67 μl of the chemosensory blends. The beads were air-dried for 2 h and placed in a completely randomized pattern with 6 replications on the floor of a cubical cage (60 × 60 × 60 cm, BioQuip, San Diego, CA, USA). Cages were replicated 4 times and treated as blocks. The same design was used in each cage but with unique randomizations of treatments within each cage. Cohorts of 200 5- to 8-d-old *D. citri* adults were starved for 6 h and then released into each cage and allowed to move freely and probe into the wax beads (Fig. 1a) within the cage for 21 h. Cages were held in a temperature and humidity controlled incubator at 26 °C, 75 % RH

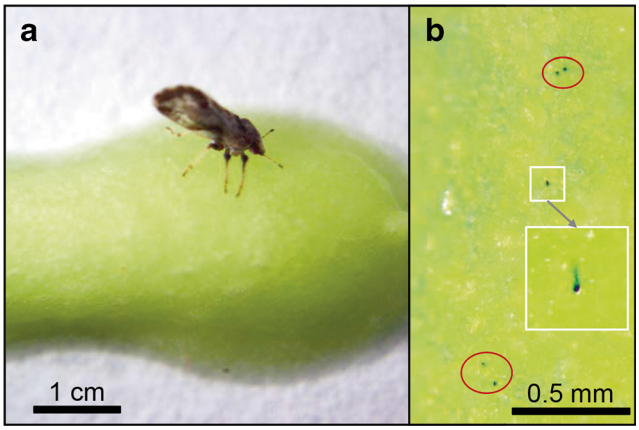

Fig. 1 An adult *Diaphorina citri* attempting to feed on a yellow wax bead (**a**) and salivary sheaths visualized by staining with Coomassie blue (**b**)

and continuous light. To visualize salivary sheaths produced by feeding attempts on the wax beads, the cover slips were removed from the cages, and beads were stained with Coomassie blue dye for one min, washed in water, and allowed to dry in air (Patt et al. 2013). The number of salivary sheaths (Fig. 1b) in each bead was counted under a stereomicroscope at 50 X magnification.

Response surface methods (RSM) (Anderson and Whitcomb 2005) were used to identify primary drivers in 5- and 3-component mixtures and to identify an optimal blend of primary drivers for maximum probing of the wax substrate by *D. citri*. Experiment designs were generated and analyzed with Design-Expert® software (v9, Stat-Ease, Inc., Minneapolis, MN, USA) to test the hypothesis that one or more of the components identified by Coutinho-Abreu et al. (2014a) would contribute to increased probing by *D. citri* in blends containing varying proportions of acetic and formic acids (George et al. 2016). For the mixtures, the total amount of odorant/tastant in stock preparations was constant at 4 μl/ml of SPLAT. In addition to acetic and formic acids, compounds tested included the three components (ethyl butyrate, *p*-cymene, and myrcene) of the blend reported by Coutinho-Abreu et al. (2014a) as attractive to adult *D. citri*.

I-optimal mixture designs were constructed sufficient to satisfy a Scheffé cubic or lower order polynomial response surface model (Cornell 2002). I-optimal designs were chosen because the algorithm picks design point that minimize the prediction variance across the design space (region of experimentation). Such designs are preferred for response surface models where prediction is important (Cornell 2002). An I-optimal 5-component mixture design consisting of 40 runs was used to identify primary drivers in blends of acetic acid, formic acid, *p*-cymene, ethyl butyrate, and myrcene (Table 1). Selected model points were replicated within each block (cage) sufficient to provide 10 degrees of freedom for lack of fit and 15 for pure error.

Table 1 Experiment design to test proportions of 5 odorant components varied in an I-optimal mixture design to study probing response of *Diaphorina citri*

Run	Formic acid	Acetic acid	*p*-Cymene	Ethyl butyrate	Myrcene
1	0.50	0.50	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	1.00
3	0.00	0.50	0.00	0.00	0.50
4	0.20	0.20	0.20	0.20	0.20
5	0.03	0.00	0.68	0.18	0.13
6	1.00	0.00	0.00	0.00	0.00
7	0.20	0.20	0.20	0.20	0.20
8	0.50	0.00	0.00	0.00	0.50
9	0.00	1.00	0.00	0.00	0.00
10	0.00	0.00	1.00	0.00	0.00
11	0.00	0.73	0.28	0.00	0.00
12	0.00	1.00	0.00	0.00	0.00
13	0.35	0.35	0.00	0.00	0.30
14	0.10	0.00	0.18	0.03	0.70
15	0.50	0.00	0.00	0.50	0.00
16	0.03	0.03	0.15	0.65	0.15
17	0.20	0.20	0.20	0.20	0.20
18	0.20	0.20	0.20	0.20	0.20
19	1.00	0.00	0.00	0.00	0.00
20	0.00	0.50	0.00	0.50	0.00
21	0.00	0.48	0.53	0.00	0.00
22	0.00	0.00	0.00	0.50	0.50
23	0.00	0.00	0.00	1.00	0.00
24	0.00	0.00	0.00	0.50	0.50
25	0.00	0.00	0.50	0.00	0.50
26	0.05	0.23	0.00	0.25	0.48
27	0.00	0.50	0.00	0.00	0.50
28	0.00	0.00	0.00	0.00	1.00
29	0.00	0.00	0.50	0.50	0.00
30	0.50	0.00	0.00	0.00	0.50
31	0.00	0.28	0.48	0.00	0.28
32	0.50	0.00	0.50	0.00	0.00
33	0.00	0.00	1.00	0.00	0.00
34	0.00	0.00	0.00	1.00	0.00
35	0.00	0.75	0.00	0.25	0.00
36	0.50	0.00	0.00	0.50	0.00
37	0.60	0.18	0.15	0.00	0.08
38	0.20	0.20	0.20	0.20	0.20
39	0.50	0.00	0.50	0.00	0.00
40	0.50	0.50	0.00	0.00	0.00

Total amount of odorant was constant at 0.7 μl incorporated into each bead of wax substrate

Three-component mixture designs consisting of 27 runs (Table 2) were used to separately determine the contribution of ethyl butyrate, myrcene, or *p*-cymene to the blend of acetic and formic acids previously identified as stimulatory to

Table 2 Design to test proportions of 3 odorant components varied in a I-optimal mixture design to study probing response of *Diaphorina citri*

Run	Formic acid	Acetic acid	Component#3
1	0.00	0.00	1.00
2	0.00	0.00	1.00
3	0.00	0.33	0.67
4	0.00	0.33	0.67
5	0.00	0.50	0.50
6	0.00	0.67	0.33
7	0.00	1.00	0.00
8	0.00	1.00	0.00
9	0.17	0.17	0.67
10	0.17	0.42	0.42
11	0.17	0.67	0.17
12	0.17	0.67	0.17
13	0.33	0.00	0.67
14	0.33	0.00	0.67
15	0.33	0.33	0.33
16	0.33	0.67	0.00
17	0.33	0.67	0.00
18	0.42	0.17	0.42
19	0.42	0.42	0.17
20	0.50	0.00	0.50
21	0.67	0.00	0.33
22	0.67	0.00	0.33
23	0.67	0.17	0.17
24	0.67	0.33	0.00
25	0.67	0.33	0.00
26	1.00	0.00	0.00
27	1.00	0.00	0.00

Total amount of odorant was constant at 0.7 μl incorporated into each bead of wax substrate. Odorants tested as component #3 were ethyl butyrate, myrcene, and *p*-cymene

probing (George et al. 2016) and to validate the results of the 5-component blend experiment. For each experiment, the third component (ethyl butyrate, myrcene, or *p*-cymene) was varied in a 3-component blend sufficient to satisfy a cubic or lower order Scheffé polynomial. For each of the 3-component blends, the entire experiment (cage) was replicated 4 times. The same design was used for each odorant and in each cage but with unique randomizations of treatments within each cage.

All cages were kept in the same environmental chamber at 26 ± 1 °C and populated simultaneously with cohorts of 200 adult *D. citri*. In addition to those needed to satisfy model terms, points (odorant/tastant combinations) were added to estimate lack of fit, and several points were duplicated to attain sufficient degrees of freedoms to estimate pure error across the design space, provide estimates of block effects, and to attain a near uniform leverage for all points (Weisberg 1985).

The statistical methods used to evaluate blend designs follow Lapointe et al. (2008). For the probing assays, models were calculated with Design-Expert. Model selection then was based on a lack of aliased terms, low residuals, a low *P* value, nonsignificant lack of fit, a low SD, high R^2, and a low PRESS (prediction error sum of squares) value. Selected models were evaluated by adequacy tests (Anderson and Whitcomb 2005). A Box-Cox plot (Box and Cox 1964) was used to select transformation of count data as appropriate. For the 5-component screening design, Cox or Piepel trace plots of the contribution of each component to the response variable (number of probes) were used (Cox 1971; Piepel 1982). The optimal 3-component blend of formic acid, acetic acid, and *p*-cymene was calculated with Design-Expert using a simplex hill-climbing algorithm (Anderson and Whitcomb 2005) in a multi-dimensional pattern search to converge at a stationary point. The parameter for optimization was maximum number of probes within the range of odorant/tastant proportions tested.

The control (no odorant or tastant) was, by definition, not included in the 3-component mixture design space because mixture designs have no intercept. To test the hypothesis that the best blend predicted by the response surface model resulted in a greater number of probes than the unscented control, the number of probes on the control was compared by ANOVA, with the number of probes observed on the actual blend design point that fell closest to the predicted optimum blend. The prediction was validated in the two-factor color x blend interaction experiment wherein the predicted optimum was compared with the control treatments to determine if increased probing resulted from inclusion of odorants/tastants in the wax substrate.

Color x Blend Interaction A two-factor interaction factorial design was used to evaluate the interaction of color and semiochemical blend on probing behavior on a wax substrate as described above. Main effects were color (white or yellow) and semiochemical blend (0, 1, 2, or 3 components). Treatments included a control (no odorant or tastant), formic acid alone, a 2-component blend of formic and acetic acids, and a 3-component blend of formic acid, acetic acid, and *p*-cymene in the proportions predicted as optimal here (Fig. 2) and by George et al. (2016). The predicted optimal 2-component blend ratio was 1.9:1.0 formic:acetic acid. The 3-component blend was 3.5:1.6:1.0 formic acid:acetic acid:*p*-cymene. The design consisted of 8 treatment combinations (2 colors × 4 blends) replicated 3 times in each cage (block) (Table 3). The entire experiment (cage) was replicated 4 times simultaneously. Cover slips with SPLAT beads were arrayed in a completely randomized pattern on the floor of cages. Cages were kept in the same environmental chamber at 26 °C and provisioned with cohorts of 200 adult *D. citri* in

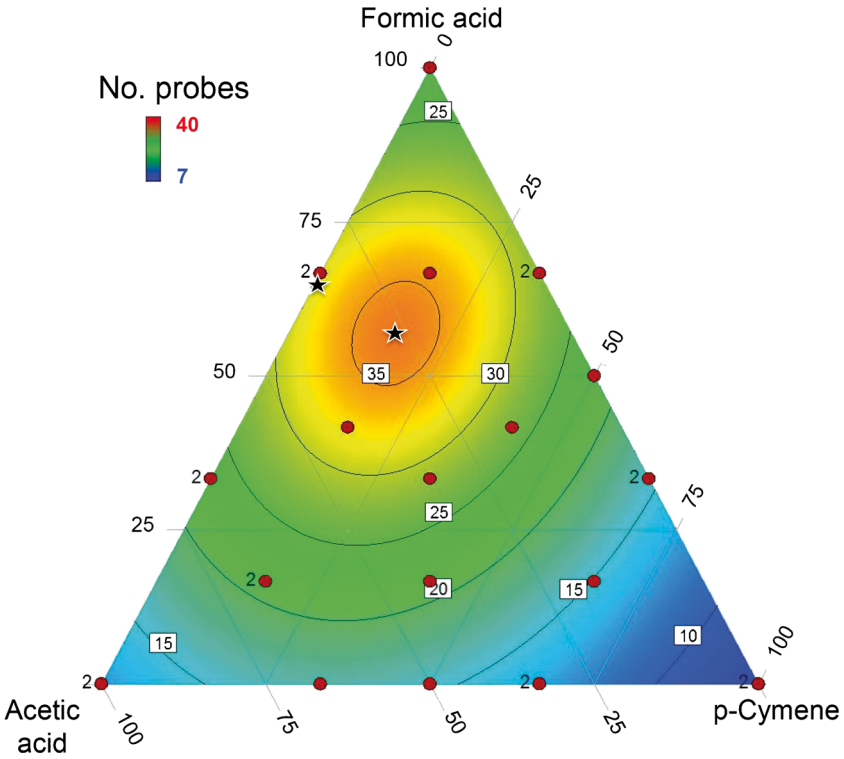

Fig. 2 Predicted surface response ternary plot generated by a 3-component mixture design. The response surface is plotted in color; contour lines indicate the number of individual salivary sheaths (probes) by *Diaphorina citri* into a wax substrate containing varying proportions of formic acid, acetic acid, and *p*-cymene. Axes scales are plotted as percent; vertices are single component blends, axial points are 2-component blends and interior points are 3-component blends. Red circles are design points. Stars indicate the predicted 2-component and 3-component optimal blends for maximum number of probes

each cage. Wax beads were removed after 21 h and stained for analysis.

Attraction to Odorant Blends A vial assay (Hall et al. 2015) was conducted to test the hypothesis that *D. citri* adults are attracted by olfaction to blends of acetic acid, formic acid, and *p*-cymene. Clear plastic 40 dram vials (50 mm I.D. × 85 mm, were capped with opaque white plastic lids (Thornton Plastic Co., Salt Lake City, UT, USA) with a 11 mm diam hole to allow psyllids to enter each vial. Vials were wrapped with light grey paper so that the vial contents were not visible to psyllids outside of the vials. Each vial received 30 ml of a dilute detergent solution to capture psyllids that entered the vials and to prevent escape. Vials were provisioned with wax beads on cover slips attached to the underside of each lid. Treatments consisted of yellow wax beads containing one of four odorant blends (0, 1, 2, or 3 components) as described in the previous section. Odorants were tested at two concentrations: 4 and 40 μl/ml. Twelve vials (3 replicates of 4 treatments) were placed in each cage (30 × 30 × 30 cm, BugDorm 1, BioQuip Products Inc., Rancho Dominguez, CA, USA). Cages were provisioned with 50 adult *D. citri* each. The experiment was replicated in 4 cages; cages were treated as blocks. Cages were kept in an environmental chamber at 27 ± 0.6 °C, 42 ± 1.5 % RH, 14:10 h L:D. The number of psyllids captured in each vial was determined after 24 h and compared by ANOVA with odorant blend, amount of odorant, and block as main effects. The term odorant is appropriate in the context of this experiment because the assay was designed to test attraction through olfaction only.

Close Range Orientation: Effect of Adjacent Treatments To further elucidate the relative influence of color and blend on probing behavior and to address the hypothesis that close-range orientation may involve olfaction, an experiment was conducted using adjacent SPLAT beads on cover slips. In this experiment, each bead was divided into two so that each cover slip received two beads, each measuring 1.0 × 0.5 × 0.1 cm. Only the 3-component blend of acetic acid, formic acid, and *p*-cymene and an unscented control were included. All possible combinations of color [white (W) or yellow (Y)] and odorant (+) or control (−) were tested resulting in 10 unique treatment combinations: Y + Y+, Y-Y-, Y-Y+, W + W+, W-W-, W + W-, W + Y+, Y-W-, W-Y+, and W + Y-. Reciprocal combinations, e.g., Y-Y+ and Y + Y-, were considered identical. Each treatment was replicated 3 times in a cage so that each cage received 30 cover slips in a completely randomized array. The entire experiment was replicated in 3 cages simultaneously. Cages were held in an environmental chamber at 26 ± 1 °C. The number of salivary sheaths in the SPLAT beads was visualized after 21 h in cages with 200 adult *D. citri.* The mean number of probes in each color/odorant half-bead was compared by Tukey's HSD ($\alpha = 0.05$) when preceded by a significant ANOVA term. The number of probes on the adjoining color/odorant treatment was similarly analyzed to test the effect of treatments on probing response to the respective adjacent treatments.

Table 3 Treatment combinations for a two-factor interaction factorial design to test the effect of substrate color and odorant blend on probing of a wax substrate by *Diaphorina citri*

Run	Blend	Color
1	0	W
2	0	W
3	0	W
4	1	W
5	1	W
6	1	W
7	2	W
8	2	W
9	2	W
10	3	W
11	3	W
12	3	W
13	0	Y
14	0	Y
15	0	Y
16	1	Y
17	1	Y
18	1	Y
19	2	Y
20	2	Y
21	2	Y
22	3	Y
23	3	Y
24	3	Y

Odorant blends were 0 (no odorant), 1 (formic acid), 2 (formic + acetic acids), or 3 (formic acid, acetic acid, and *p*-cymene). Total amount of odorant was constant for all odorant blends except the control

No-Choice Test To estimate the number of probes per insect and confirm our observation of increased probing by individual psyllids in response to the 3-component blend, we conducted a no-choice test. Fifty *D. citri* adults were placed into cages for 21 h containing 5 cover slips with beads of wax (SPLAT). Cages contained 5 beads each of one of the following: yellow wax without odorant, white wax without odorant, yellow wax containing the 3-component blend of acetic acid, formic acid, and *p*-cymene as above, or white wax containing the 3-component blend. Each treatment was replicated 4 times. The wax beads were collected, stained with Coomassie blue, and scored for salivary sheaths. The number of psyllids alive at the end of the 21 h test was recorded. A 2 × 2 factorial design was used; results were analyzed by ANOVA.

Probing Response through Plastic Film To support the hypothesis that increased probing was due to phagostimulants and not olfactory cues, a no-choice experiment was performed to study the probing behavior of *D. citri* adults through plastic film into wax beads. Yellow beads were prepared without tastants or with the 3-component phagostimulant blend (3.5:1.6:1 formic acid:acetic acid:*p*-cymene). Beads of SPLAT were covered with a polyethylene/polybutylene plastic film (Saran™Cling Plus® Wrap, S. C. Johnson & Son, Inc., Racine, WI, USA) to minimize the potential for olfaction or contact chemoreception associated with antennal tapping behavior (antennation). The film is impermeable to water and presumably to the larger molecules of the phagostimulant blend. Fifty *D. citri* adults were placed in each of four cages for 21 h. Each cage contained 5 cover slips with yellow wax beads prepared with or without tastants and with or without a covering of plastic film. The wax beads were collected, stained with Commassie Blue, and scored for salivary sheaths. The experiment was analyzed as a 2 × 2 factorial with 5 replications. Stained salivary sheaths were removed from the wax substrate by gently lifting the plastic film and separating it from the substrate. The length of the stained salivary sheaths adhering to the plastic film was measured under a stereo microscope. Ten salivary sheaths were measured for each treatment (plastic film over wax beads without tastants and plastic film over wax beads with the 3-component tastant blend).

Results

Effect of Blends on Psyllid Probing Behavior The 5-component blend experiment produced a highly significant linear mixture model (Table 4). The lack-of-fit test was not significant ($P > F = 0.23$). The inverse square root transformation was applied to the data (number of probes) as per the Box-Cox analysis. Model diagnostics were within acceptable limits. The R^2 statistics were clustered with a difference < 0.12. The model was adequate to navigate the design space. Trace plots of the Cox effects (Fig. 3) estimated the effects of

Table 4 *P*-values, regression coefficients, and response surface model fitting diagnostic statistics for a linear mixture model of probing by *Diaphorina citri* in response to varying proportions of 4 odorants

	Sum of Squares	*df*	Mean Square	*F*	*P*-value	Coefficient Estimate (SE)
Model	373.69	4	93.42	5.3	0.002	
Formic acid		1				21.4 (2.0)
Acetic acid		1				16.6 (2.0)
p-Cymene		1				14.3 (2.1)
Ethyl butyrate		1				11.1 (2.1)
Myrcene		1				8.8 (2.0)
Lack of Fit	406.71	20	20.34	1.45	0.232	

Model terms and coefficient estimates are untransformed data to show coefficients in terms of real components; R^2 values are from model applying inverse square root transformation. Model $R^2 = 0.45$; $R^2_{adj} = 0.39$; $R^2_{pred} = 0.27$

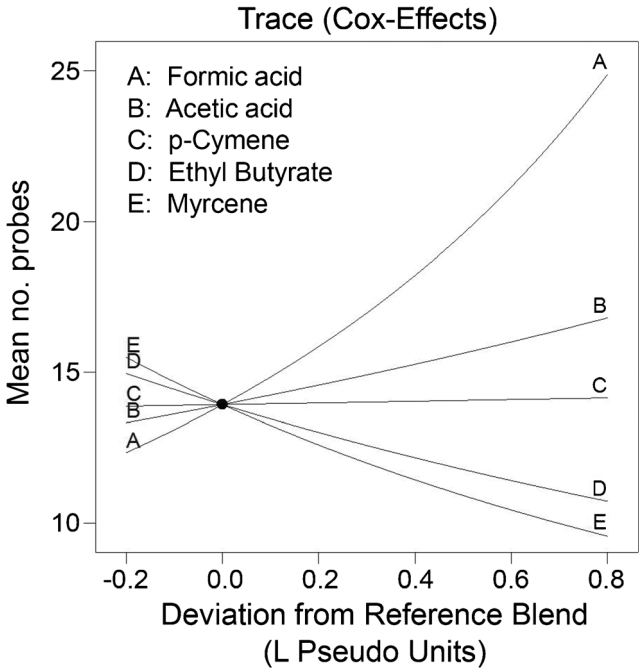

Fig. 3 Trace plot of main effects (Cox-effects) estimating the effects of increasing the proportion of individual components in relation to a reference blend (equal proportions of all 5 components) while relative proportions of remaining components are kept constant. A positive slope indicates a positive effect on the response variable (number of probes)

Table 5 *P*-values, regression coefficients, and response surface model fitting diagnostic statistics for a quadratic mixture model of probing by *Diaphorina citri* in response to varying proportions formic acid, acetic acid, and *p*-cymene

	Sum of Squares	df	Mean Square	*F*	*P*-value	Coefficient Estimate (SE)
Model	0.016	5	0.0033	12.5	<0.001	
Linear Mixture	0.0096	2	0.0048	18.3	<0.001	
Formic acid (A)						0.051 (0.011)
Acetic acid (B)						0.084 (0.011)
p-Cymene (C)						0.13 (0.011)
AB	0.0015	1	0.0015	5.8	0.025	-0.11 (0.046)
AC	0.0038	1	0.0038	14.6	0.001	-0.16 (0.043)
BC	0.0033	1	0.0033	12.5	0.002	-0.16 (0.044)
Residual	0.0055	1	0.0052			
Lack of Fit	0.0035	12	0.0003	1.31	0.348	
Pure Error	0.0020	12	0.0002			

The inverse transformation was applied to normalize count data. Number of probes =1/coefficient estimates. Model R^2 = 0.75; R^2_{adj} = 0.69; R^2_{pred} = 0.54

increasing the proportion of the individual components in relation to the reference blend (equal amounts of each of the 5 components). The slope of the Cox direction was highly significant and positive for formic acid ($P > t < 0.001$) and significant but negative for ethyl butyrate and myrcene ($P > t = 0.03$ and 0.002, respectively). The effects for Cox direction for acetic acid and *p*-cymene were less pronounced ($P > t = 0.14$ and 0.90, respectively) but prior knowledge of the contribution of acetic acid (George et al. 2016) and the lack of a negative Cox direction for *p*-cymene justified inclusion of these two components in additional 3-component blends.

The contributions of *p*-cymene, ethyl butyrate, and myrcene in 3-component blends with formic and acetic acids were evaluated separately. The effects of myrcene and ethyl butyrate on the response variable (number of probes) were negative. The Cox directions for myrcene and ethyl butyrate were negative, and the numerical optimization algorithm identified only 2-component (axial) blends of formic and acetic acids. A 1.9:1 axial blend of formic acid:acetic acid was predicted by the algorithm in both 3-component blend experiments. In contrast, the 3-component blend of formic acid, acetic acid, and *p*-cymene resulted in a significant quadratic mixture model with significant blending terms for all 3 components (Table 5). The optimization algorithm suggested two solutions: a 3-component blend (3.5:1.6:1 formic acid:acetic acid:*p*-cymene, desirability =0.80) and a 2-component blend (1.9:1 formic:acetic acid, desirability =0.68). Based on these results, validation of the contribution of *p*-cymene and a comparison of the predicted 3-component and 2-component optimal blends were carried out in the color x blend interaction test.

Color x Blend Interaction The effect of block (cage, $N = 4$) was not significant ($P > F = 0.62$, ANOVA). The total number of probes (estimated by the number of individual salivary sheaths visualized by staining) ranged from 277 to 324 probes/cage over 21 h of the test. The main effects of color ($F_{1,44}$ = 128.67, $P > F < 0.001$), blend ($F_{3,44}$ = 7.96, $P > F < 0.001$), and the interaction between color and blend ($F_{3,44} = 5.02$, $P > F = 0.0045$) were significant (Fig. 4). Within the white treatment, the effect of blend was not significant ($P > F = 0.14$). Within the yellow treatment, there was no effect of block or interaction between block and blend ($\alpha = 0.05$). Within the yellow SPLAT treatment, the number of salivary sheaths observed in individual beads increased with increasing number of blend components. The effect of blend was significant ($P > F = 0.005$); beads containing the 2-component blend (1.9:1 formic:acetic acids) or the 3-component blend (3.5:1.6:1 formic acid:acetic acid:*p*-cymene) received more probes compared with beads containing only formic acid or no odorant (yellow SPLAT control) (Tukey's HSD, Fig. 4). All comparisons between color treatments within blends were highly significant ($P > F < 0.001$). A greater number of salivary sheaths was always present on yellow compared with white SPLAT beads. The mean numbers of salivary sheaths observed in yellow SPLAT containing the 2-component (formic + acetic acids) or 3-component (formic acid, acetic acid, and *p*-cymene) blends were significantly greater than the number of sheaths observed in the

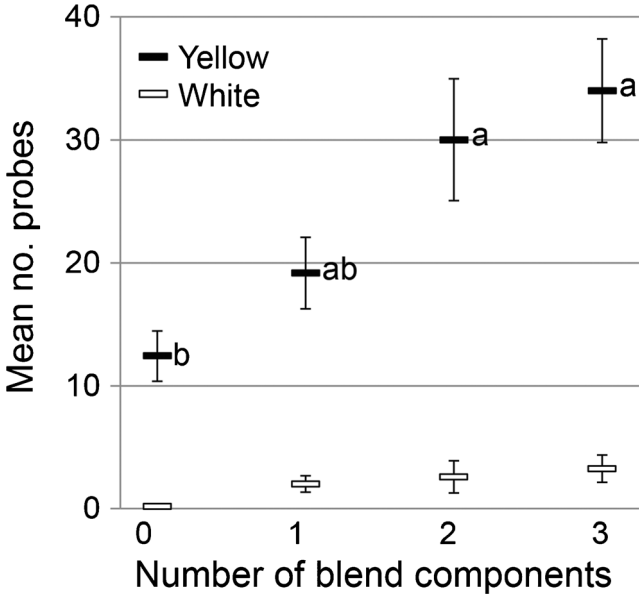

Fig. 4 Mean ± SEM ($N = 12$) number of probes by adult *Diaphorina citri* into white or yellow wax substrate containing 0, 1, 2, or 3 blend components. 1 = Formic acid, 2 = 1.9:1 blend of formic:acetic acids, 3 = 3.5:1.6:1 blend of formic acid:acetic acid:*p*-cymene. Means labeled with the same letter are not significantly different ($\alpha = 0.05$, Tukey's HSD). There were no significant differences between means within the white treatment ($\alpha = 0.05$, ANOVA). All comparisons between pairs within blend treatments were significant ($P > F < 0.001$)

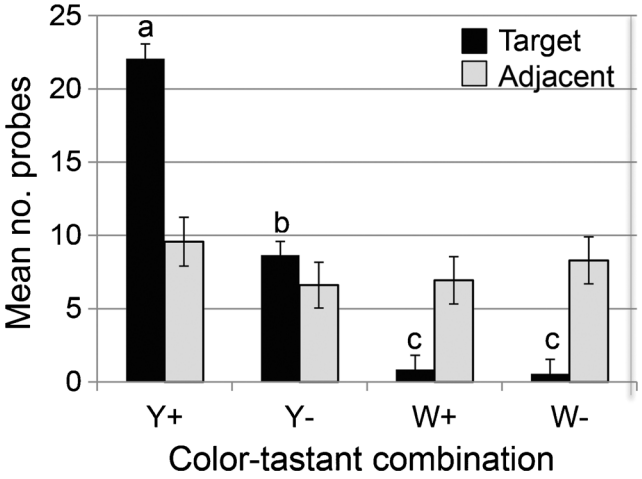

Fig. 5 Mean ± SEM ($N = 45$) number of probes by adult *Diaphorina citri* into wax beads (target) and the corresponding adjacent beads arranged in pairs of white (W) or yellow (Y) wax substrate combined with (+) or without (−) a 3-component blend (3.5:1.6:1) of formic acid:acetic acid:*p*-cymene (+). Mean number of probes observed on treatments (*black columns*) with the same letter are not significantly different ($\alpha = 0.05$, Tukey's HSD). There was no significant difference ($P > F = 0.55$) between means of the number of probes observed on treatments adjacent to the labeled treatment (*grey columns*)

yellow wax control (no odorant/tastant) ($\alpha = 0.05$, Tukey's HSD).

Attraction to Odorant Blends There were no significant differences in the number of ACPs captured in vials containing different odorant blends or unscented controls after 24 h either with 4 μl total odorant/ml of wax ($F_{3,44} = 0.81$, $P > F = 0.50$) or with 40 μl/ml ($F_{3,44} = 0.13$, $P > F = 0.94$). For both concentrations, there was no effect of odorant blend (0, 1, 2, or 3 components) or block and no interaction between the main effects ($P > F > 0.37$). The mean number (± SEM, $N = 48$) of psyllids captured in the vials was 3.1 ± 0.3 and 3.4 ± 0.3 at 4 and 40 μl/ml, respectively. The mean (± SEM, $N = 8$) total number of psyllids of the 50 introduced into each cage that were captured in the vials was 78 ± 3 %.

Close Range Orientation: Effect of Adjacent Treatments Results for this test were similar to results from previous experiments. The number of probes observed in yellow wax substrate containing the 3-component blend was significantly greater than the number observed in yellow wax alone, and both yellow treatments had more probes compared with white wax beads with or without the odorant blend ($\alpha = 0.05$, Tukey's HSD) (Fig. 5). The effect of color and blend combination on the number of probes on the adjacent bead was not significant ($F_{3,179} = 0.70$, $P > F = 0.55$) (Fig. 5). This result suggests that the discrimination between the substrate containing the 3-component phagostimulant blend and substrate without the blend occurred only after contact with the substrate. As was shown in the completely randomized comparison of blends and the color x blend interaction experiment, color was the sole basis for attraction of *D. citri*. The combination of yellow and the 3-component blend resulted in the greatest number of probes (Fig. 5).

No-Choice Test Results of the no-choice test (Table 6) were congruent with the results of the choice test (color x blend interaction). In the no-choice test, there was a significant interaction ($F_{1,12} = 12.60$, $P > F = 0.004$) between color and

Table 6 Effect of color (C) and 3-component odorant blend (O) on probing by 50 *Diaphorina citri* adults on white (W) or yellow (Y) wax beads containing a 3-component blend (B) of odorants or no odorant (N) in a no-choice 2 × 2 factorial experiment

			Contrasts[a]			
Color	Odorant	No. probes	O[W]	O[Y]	C[B]	C[N]
White	Blend	14 + 4	a		a	
White	None	13 + 3	a			a
Yellow	Blend	402 + 62		a	b	
Yellow	None	182 + 6		b		b
F ratio	0.003	12.70	39.77	645.89		
$P > F$	0.96	0.012	<0.001	<0.001		

There was a significant interaction between color and odorant blend ($P = 0.004$). ANOVA results are presented for four orthogonal contrasts

[a] Means within contrast columns with the same letter do not differ ($\alpha = 0.0125$, ANOVA)

blend in the total number of probes per cage. The main effects of color ($F_{1,12}$ = 80.84, $P > F$ = 0.004) and blend ($F_{1,12}$ = 12.65, $P > F < 0.001$) also were significant. The comparison of individual means showed no effect of the 3-component blend when incorporated into white SPLAT beads (Table 6). Yellow wax beads containing the 3-component blend received more probes compared with yellow beads without the odorants; yellow beads containing the 3-component blend received more probes compared with white beads containing the odorant blend; and yellow beads without the odorant blend received more probes compared with white beads without the odorant blend (Table 6). The number of *D. citri* adults per cage that were alive at the end of the 21 h test did not vary between treatments ($P > F = 0.06$, ANOVA) and ranged from 15 in cages with yellow beads without odorant to 23 in cages with yellow beads containing the 3-component odorant blend. ANOVA analyses performed on the total number of probes/cage and the number of probes/psyllid alive at the end of the experiment produced equivalent results. The mean ± SEM ($N = 4$) number of probes per psyllid calculated using the number of psyllids (50) initially introduced into each cage ranged from 0.3 ± 0.1 probes/psyllid on white beads to 3.6 ± 0.1 probes/psyllid on yellow beads without odorant and 8.0 ± 1.2 probes/psyllid on yellow beads containing the 3-component odorant blend.

Probing Response through Plastic Film There was no interaction between the main effects of plastic film and phagostimulant blend on the number of salivary sheaths observed ($P > F = 0.73$). There was no effect of plastic film ($P > F = 0.44$) on the number of salivary sheaths observed in the yellow wax beads. The effect of the 3-component tastant blend was significant ($P > F < 0.001$). The mean number (± SEM, $N = 10$) of salivary sheaths observed on yellow wax beads without tastants was 47.5 ± 5.3 and on yellow wax beads containing the 3-component blend was 94.5 ± 8.5 sheaths. Observation of the salivary sheaths that adhered to the plastic film (Fig. 6) revealed differences between the sheaths recovered from the wax substrate containing the 3-component blend of tastants (Fig. 6b) and sheaths recovered from the wax substrate without tastants (Fig. 6c). Salivary sheaths recovered from the wax beads containing the tastants were 4.5 times longer (208 ± 22 μm) compared with sheaths recovered from the wax substrate without tastants (46 + 7 μm) ($P > F < 0.001$). Sheaths recovered from the beads with tastants (Figs. 6a, b) were longer with filamentous portions extending beyond the thicker initial sheath. Sheaths from the beads without tastants most often consisted simply of short and thick portions without filamentous extensions (Fig. 6c).

Discussion

Phagostimulants are chemicals that induce feeding (Nation 2002). Compounds that stimulate feeding may be essential nutrients or token stimuli. Increased probing by adult *D. citri* in response to formic and acetic acids (George et al. 2016) was confirmed here, and a 3-component blend was optimized consisting of the two acids and *p*-cymene. Although there is no known nutritional role of these compounds, they are associated with metabolic processes in citrus plants. Catabolism and degradation of volatile organic compounds (VOCs) to smaller

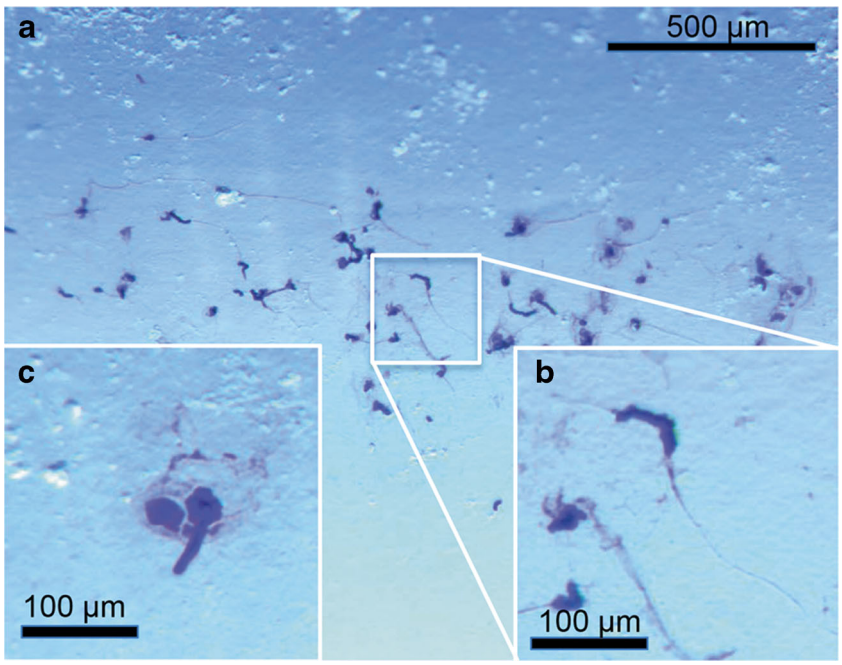

Fig. 6 Salivary sheaths produced by adult *Diaphorina citri* probing through plastic film into a wax substrate containing a 3-component blend of tastants (**a** and **b**), or into wax beads without tastants (**c**)

molecules such as acetic and formic acids can result from plant stress, environmental stress, herbivore attack, or plant-atmosphere interactions, and they may be involved in plant-plant signaling and insect-plant interactions (Fares et al. 2012; George et al. 2016; Oikawa and Lerdau 2013). Adult *D. citri* have been shown to be attracted to citrus plants stressed by infection with CLas (Mann et al. 2012; Martini et al. 2014). Additionally, it is known that young expanding foliage, a source of acetic acid produced during primary metabolism or as a product of enzyme-mediated oxidation (Kreuzwieser et al. 2001), is required for larval development of *D. citri*. Therefore, these carboxylic acids may be token stimuli to signal the presence of infected hosts or young, emergent leaf tissue.

Our primary interest was to identify volatile compounds capable of modifying the behavior of *D. citri*. Previously, a 1.9:1 blend of formic acid:acetic acid was identified as the optimal blend of those compounds to maximize the number of feeding attempts (probes) into a wax substrate (George et al. 2016). In an attempt to find additional compounds with similar activity, we studied the potential contribution of 3 components of a blend suggested by Coutinho-Abreu et al. (2014a) as attractive to *D. citri*: *p*-cymene, ethyl butyrate, and myrcene. Analysis of a 5-component blend suggested that ethyl butyrate and myrcene had no effect or a negative effect on probing behavior alone or in blends containing formic and/or acetic acids. *p*-Cymene was judged to have had a small but possibly positive effect (Fig. 3). The results of the 5-component blend study were confirmed in 3 experiments that sampled the ternary design space delineated by 3-component blends of formic acid, acetic acid and either ethyl butyrate, *p*-cymene, or myrcene. Of the three components identified as attractants of *D. citri* by Coutinho-Abreu et al. (2014a), only *p*-cymene contributed to the probing behavior of *D. citri* as a component of a 3-component blend (Fig. 2). The response surface identified this blend (3.5:1.6:1 formic acid:acetic acid:*p*-cymene) as the optimal 3-component phagostimulant blend of these compounds for *D. citri*. It should be noted that the actual ratio of compounds in the wax matrix experienced by the insect may differ from the ratio of compounds present when the matrix blend was prepared and before the 2-h drying period. Additionally, differential evolution of the three components may result in different ratios over the time course of an experiment. Nonetheless, we have demonstrated and confirmed that preparation of the wax substrate and the 3.5:1.6:1 ratio resulted in an optimal behavioral response.

We were unable to demonstrate any orientation by adult *D. citri* from a distance to the phagostimulant blends other than the probing that occurred after alighting upon or walking onto a wax bead. In the absence of a visual stimulus, the psyllids did not move preferentially to a source of the 3-component blend. The color yellow was highly attractive and movement of *D. citri* to yellow was consistent regardless of the compounds contained in the yellow substrate (Fig. 4). However, once the adult psyllids contacted the wax beads, probing behavior was modified resulting in more feeding attempts and a greater number of salivary sheaths deposited in the wax beads containing the phagostimulant blends. This was further demonstrated by our study of adjacent wax beads wherein the odorant blend contained in a given bead had no effect on the number of probes observed in the adjacent bead (Fig. 5). If olfaction mediated by an airborne cue were involved, one would expect the effect to occur at some distance from the immediate source of odorant. If that were true, it would be expected that more probes would be observed on beads adjacent to an attractive bead such as the yellow bead containing the optimal 3-component blend. We did not observe that. Therefore, we conclude that the phagostimulatory effect occurs upon contact, presumably by chemosensory perception mediated by chemosensory receptors on the antennae, labial palps, tarsi, or other body parts.

To isolate gustation from chemosensory input from antennae or other body parts in contact with the wax substrate, we covered the substrate containing the phagostimulant blend with plastic film and observed the same effect as occurred without the film. Adult *D. citri* probed more often and produced more and larger and more developed salivary sheaths on wax beads containing the 3-component blend with or without the plastic film. This result suggests that perception of the phagostimulants was mediated by external contact chemosensory sensillae at the tip of the labium (Garzo et al. 2012) or by contact with gustatory receptors within the alimentary canal of the insect. The viscosity of SPLAT would argue against the possibility of actual uptake through the food canal within the stylets, leaving gustation by labial tip sensillae as the most obvious source of sensory input associated with the insect's response to the phagostimulants. However, we can't rule out the possibility that phagostimulants may act by interaction with chemoreceptors located within the alimentary canal by salivation and by subsequent ingestion of saliva containing the phagostimulatory compounds.

The excellent scanning electron micrographs of the mouthparts of *D. citri* produced by Garzo et al. (2012) show a set of eight sensillae, four on either side of the labial tip. Similar structures are present in aphids but they are presumed to be mechanoreceptors with no chemosensory role (Powell et al. 2006; Tjallingii 1978; Wensler 1977). A chemosensory role for the labial sensilla has not been demonstrated but our results suggest this may occur in *D. citri*.

The 3-component phagostimulant blend increased probing activity by *D. citri* on yellow wax by a factor of ~3 compared with the yellow control in the interaction study (Fig. 4) and by a factor of ~2 in the no-choice test (Table 6). The challenge ahead is to determine how this phenomenon might be used to improve a trap, design an attract-and-kill product, or enhance other means of managing *D. citri*. The significance of formic acid in the ecology of *D. citri* may be related to mutualistic

interaction with ants. Tending and collection of the solid honeydew from *D. citri* nymphs by the Argentine ant, *Linepithema humile* has been observed in citrus orchards (Tena et al. 2013). Formic acid is known to be produced in the poison gland of some ants although its function has been thought to be exclusively defensive (Wilson 1971). It is intriguing to consider the possibility that formic acid produced by ants may play a role in their mutualistic relationships with phloem-feeding hemipterans by stimulating feeding and increasing the production of honeydew.

Acknowledgments We thank Larry Markle, Evan Koester, Bill Sauveur, and Matt Hentz for technical assistance and Anna Sara Hill (all USDA-ARS, Ft. Pierce, FL) for insect rearing. The manuscript was improved by comments from Joseph Patt (USDA-ARS, Ft. Pierce, FL). Significant funding was provided by the Citrus Research and Development Foundation. ISCA Technologies Inc., Riverside, CA graciously provided SPLAT™ products used in these studies. USDA is an equal opportunity provider and employer. Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

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