

# Quantifying Dispersal of *Diaphorina citri* (Hemiptera: Psyllidae) by Immunomarking and Potential Impact of Unmanaged Groves on Commercial Citrus Management

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**ABSTRACT** *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) is an important pest of citrus. It is an efficient vector of three bacterial pathogens that are the presumptive causal agents of huanglongbing (HLB) or citrus greening disease. The movement patterns and dispersal capabilities of *D. citri* require study to better understand the spread of HLB and to improve management strategies for *D. citri*. A recently developed immunomarking technique that uses crude food proteins (chicken egg albumin, bovine casein, and soy protein) was evaluated for marking and tracking movement of *D. citri* in Florida citrus groves. In general, both egg and milk protein markers exhibited longer residual activity (35 d) than the soy protein marker (20 d) when applied to citrus leaves with a residual activity order of egg > milk > soy protein. However, residues of all three protein markers decreased with a simulated rain; this was more pronounced for soy protein than for egg and milk proteins. Temperature did not significantly affect acquisition of markers by adult *D. citri*. Egg, milk, and soy protein markers were detected on >90% of adult *D. citri* for up to 10, 10, and 5 d, respectively, after field application. Addition of tetrasodium ethylenediamine tetraacetic acid (water softener) and/or Silwet L-77 (wetting agent) to marker solutions did not affect longevity of detection. Each of the protein markers was detected on ≥80% of exposed *D. citri* for up to 30 d after direct application to adults. A field study was conducted to measure movement of *D. citri* between replicated pairs of 0.4 ha managed and unmanaged citrus plots separated by 60–100 m. Approximately 70% of captured *D. citri* were found marked 3 d after application of proteins in the field. Using two marker proteins, it was determined that *D. citri* moved bi-directionally between managed and unmanaged (abandoned) groves within 3 d with a greater number of *D. citri* adults moving from unmanaged into managed plots than from managed into unmanaged plots (net movement). These data indicate frequent movement by adult *D. citri* between groves and suggest that unmanaged groves may act as refuge sites for *D. citri*, leading to reinfestation of nearby managed groves.

**KEY WORDS** *Diaphorina citri*, immunomarking, mark-capture, dispersal behavior, huanglongbing

*Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) is one of the most important pests of citrus worldwide. Direct damage caused by this insect includes leaf notching and curling, and in severe infestations, death of new shoots (Halbert and Manjunath 2004). More importantly, *D. citri* vectors three phloem-limited bacteria in the genus *Candidatus Liberibacter*, the presumptive causal agents of huanglongbing (HLB), otherwise known as citrus greening disease (Halbert and Manjunath 2004). HLB-infected citrus plants show early symptoms of leaf mottling and yellowing, which results in appearance of yellow shoots (Halbert and Manjunath 2004, Bové 2006). In addition, diseased trees bear few fruits that are of reduced size, deformed, have undesirable organoleptic properties, and are without full coloration rendering them unmarket-

able (Halbert and Manjunath 2004, Bové 2006). HLB is the most serious threat to the future of the U.S. citrus industry. Florida alone accounts for ≈75% of the U.S. citrus acreage with estimated annual earnings of U.S. \$1.4 billion (Anonymous 2008a).

In Florida, *D. citri* was first discovered in June 1998 (Halbert 1998) and has now spread to all citrus production areas in the state (Halbert 2005). *D. citri* has also established in Texas (French et al. 2001) and has been detected in areas of Alabama, California, Georgia, Louisiana, Mississippi, and South Carolina (USDA-APHIS 2008). The presence of HLB in Florida was first confirmed in 2005 and by 2008 it spread to 30 counties within south and central Florida (Anonymous 2008b). Understanding both the local and long-range movement patterns of *D. citri* is of critical importance for effective management of HLB. Also, determining the impact of *D. citri* movement from unmanaged (aban-

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doned) and dooryard citrus into commercial groves requires study, given the prevalence of unmanaged and residential citrus acreage in Florida. The USDA estimates at least 53,230 ha of abandoned citrus acreage in Florida currently (Giles 2008).

To quantify the movement patterns of *D. citri*, an effective technique for marking and capturing natural populations directly in the field and over large areas is needed. Immunomarking techniques may prove highly effective for such studies (Hagler et al. 1992). Protein markers are acquired by insects either by direct contact during application or subsequently as insects walk on previously marked surfaces (Hagler et al. 1992, Jones et al. 2006). Insects that are "tagged" by protein markers can be easily captured with attractive traps (visual, olfactory, or passive) and subsequently analyzed for the specific marker protein by a highly sensitive and unambiguous enzyme-linked immunosorbent assay (ELISA) (Hagler et al. 1992; Hagler 1997, 1998; Hagler and Miller 2002; Hagler and Naranjo 2004). Recently, Jones et al. (2006) developed an inexpensive immunomarking technique with crude food proteins such as chicken egg albumin (as egg whites), bovine casein (as cows' milk), and soy protein (as soy milk) for mark-capture of insects in the field, including pear psylla, *Cacopsylla pyricola* Foerster, and codling moth, *Cydia pomonella* L. Crop type, environmental conditions, water quality, and spray additives may influence the stability of protein markers in the field (Jones et al. 2006). Therefore, we evaluated this immunomarking technique to determine its limitations and potential under Florida conditions.

The objectives of this study were to determine (1) the residual longevity of egg, milk, and soy protein markers on citrus leaves with or without spray additives; (2) the effect of temperature on acquisition of protein markers by *D. citri* adults walking on field-aged leaves with dried residues; (3) the residual longevity of protein markers on *D. citri* acquired by direct contact with protein sprays; and (4) to quantify the movement of *D. citri* between adjacent unmanaged and managed citrus groves using the most effective protein markers identified.

## Materials and Methods

**Residual Longevity of Protein Markers on Citrus Leaves in the Field.** The objective of this experiment was to determine the residual longevity of protein markers on citrus leaves in the field. Also, we studied whether addition of a water softener, tetrasodium ethylenediamine tetraacetic acid (EDTA; Sigma-Aldrich, St. Louis, MO), or a wetting agent, Silwet L-77 (Helena Chemical, Collierville, TN), would increase retention of protein markers on leaves and acquisition of markers by *D. citri*. The protein markers studied were chicken egg albumin (All Whites; Papetti Foods, Elizabeth, NJ); bovine casein (All Natural Whole Milk; Publix Super Markets, Lakeland, FL); and soy protein (Plain Soy Milk Organic; White Wave, Boulder, CO), henceforth referred to as egg, milk, and soy proteins. Each protein marker was tested as four treat-

ments: (1) protein alone at 10, 20, and 20% in water for egg, milk, and soy proteins, respectively; (2) protein + 0.5 g/liter EDTA; (3) protein + 2000 ppm Silwet L-77; and (4) protein + 0.5 g/liter EDTA + 2000 ppm Silwet L-77. The experiments were conducted within an 11-yr-old sweet orange (*Citrus sinensis* L. 'Valencia') grove in Lake Alfred, FL. Two trees (1.5–2.0 m in height) were randomly selected for each protein formulation treatment. On each tree, two to three randomly selected branches were sprayed until runoff with the protein marker solution using a hand-held atomizer (The Bottle Crew, West Bloomfield, MI). Tree branches sprayed with water alone served as controls. Leaves from protein-treated and water-treated (control) tree branches were collected at 1, 5, 10, 20, and 35 d after application and transferred to the laboratory in plastic bags. Field-collected leaves were either analyzed immediately after collection from the field or sprayed thoroughly until run-off using a hand-held atomizer ( $\approx 5$  ml/leaf) to simulate rain and allowed to air dry. Subsequently, 16 1.2-cm-diameter leaf discs (one per leaf sample) were excised for each protein formulation treatment using a cork borer. Similarly, 16 leaf discs were excised from leaves that had been subjected to the rain simulation for each protein formulation treatment. Individual leaf discs were completely immersed in 1 ml of protein extraction buffer (Tris-buffered saline, pH 8.0 [Sigma-Aldrich, St. Louis, MO] + 0.5 g/liter EDTA) in separate 1.5-ml centrifuge tubes for 5 min. To determine the presence and intensity of the protein markers on treated leaves, all samples were analyzed by indirect ELISA (Crowther 2001) as described below.

**Effect of Temperature on Protein Marker Acquisition by *D. citri* Walking on Field-Aged Residues.** The objective of this experiment was to determine the ability of *D. citri* to acquire protein markers by walking on field-aged protein residues on leaves collected at various intervals after application. In addition, we studied the effect of temperature on acquisition of markers by *D. citri*. Two sets of six protein-treated leaves from each treatment described above were collected at 1, 5, 10, 20, and 35 d after application and were placed individually into 90-mm plastic disposable petri plates. Similar sets were prepared with water-treated leaves, which served as controls. Eight adult *D. citri* were released into each petri plate, which were subsequently sealed with Parafilm M (Structure Probe, West Chester, PA). One set of petri plates was placed in a growth chamber at 25°C and another set was placed at 35°C. After 24 h of exposure, *D. citri* within petri plates were killed by freezing and transferred immediately into 1 ml protein extraction buffer for 5 min and subjected to indirect ELISA as described below.

**Marking *D. citri* by Direct Contact With Protein Marker Spray Application and Residual Longevity.** The objective of this experiment was to determine the efficiency of protein marker acquisition by adult *D. citri* through direct contact with protein solution spray and to measure residual longevity. Rough lemon (*Citrus jambhiri* Lush) plants (25–30 cm in height) with

100–120 actively feeding 1- to 5-d-old adult *D. citri* per plant were placed individually into Plexiglas sleeve cages (40 by 40 by 40 cm). Two plants were sprayed with egg (10%), milk (20%) or soy (20%) protein solutions alone until runoff using a hand-held atomizer as described above. Plants with *D. citri* adults of the same age and sprayed with water alone served as controls. Cages with plants were maintained at  $25 \pm 1^\circ\text{C}$  and  $50 \pm 5\%$  RH in a walk-in growth room. Eight *D. citri* adults were collected from each sprayed plant at 1, 5, 10, 20, and 30 d after application and immediately killed by freezing. Killed *D. citri* were immediately transferred into individual 1.5-ml microcentrifuge tubes with 1 ml of the protein extraction buffer and subjected to indirect ELISA as described below.

**Movement Pattern of Adult *D. citri* Between Unmanaged and Managed Citrus Grove Plots.** A field study was conducted using the immunomarking technique described above to quantify the movement of *D. citri* adults between unmanaged and managed citrus grove plots. Four replicate 0.4-ha plots (110 trees on a 11 by 10-m spacing) of Valencia citrus trees were chosen in adjacent managed and unmanaged citrus groves in Lake Alfred, FL. Unmanaged plots did not receive chemical sprays for at least 5 yr before the initiation of the study. Commercially managed plots were maintained according to standard management practices in FL, which includes four to six insecticide applications for *D. citri* (Rogers et al. 2008). The replicated managed and unmanaged plots were separated by 60–100 m of mowed grass and replicate plots within groves were separated by at least 40 m.

In the unmanaged plots, trees were sprayed with 10% egg protein + 2,000 ppm Silwet L-77, whereas trees in managed grove plots were sprayed with 20% milk protein + 2,000 ppm Silwet L-77 using a hand gun sprayer (model 5275016; Fimco Industries, North Sioux City, SD) at 20–30 psi with 1–2 liters of spray fluid per tree. All trees within each plot were sprayed. Spray applications were made on 11 April 2008. Eighteen unbaited Pherocon AM yellow sticky traps (Trécé, Adair, OK) were hung in each plot to capture flying adult *D. citri* (Hall et al. 2007). Traps were placed on the 1st (border), 5th (central), and 10th (interior) tree of every other row in each plot relative to the plot border. Three days after application of protein markers, the traps were removed from the field. Adult *D. citri* from each trap were carefully removed using forceps and placed in 1 ml protein extraction buffer as described earlier. After removal of each psyllid, forceps were thoroughly rinsed with acetone and subsequently washed under tap water to prevent cross-contamination. Trap location and number of *D. citri* captured per trap were recorded. Adult *D. citri* obtained from a laboratory greenhouse colony described in Wenninger et al. (2008) served as controls. Extracts of captured *D. citri* adults were assayed for both egg and milk proteins by indirect ELISA as described below.

**Indirect ELISA.** An indirect ELISA (Crowther 2001) was performed on the protein solutions col-

lected from treated leaves and adult *D. citri* to determine the presence and quantify the intensity of protein markers. Eighty-microliter aliquots of marker protein solutions (milk, egg, or soy) in extraction buffer were transferred from each 1.5-ml centrifuge tube into individual wells of 96-well microplates (Nunc Polysorp; Fisher, Pittsburgh, PA). Similarly, 80- $\mu\text{l}$  aliquots of extraction buffer from the control treatments as well as extraction buffer alone (blank) were transferred into individual microplate wells, which served as negative controls and blanks, respectively.

Microplates with protein solutions were covered with aluminum foil and incubated for 2 h at  $37^\circ\text{C}$ . After 2 h of incubation, milk and egg protein plates were washed five times with 300  $\mu\text{l}$  of phosphate-buffered solution (PBS; Sigma-Aldrich, St. Louis, MO), pH 7.4, + 0.09% Triton X-100 (PBST; Sigma-Aldrich) per well. Soy protein plates were washed three times with 300  $\mu\text{l}$  PBS + 2.3 g/liter sodium dodecyl sulfate (SDS; Sigma-Aldrich) (PBS-SDS) per well followed by two washes with the same volume of PBS. After washing, 300  $\mu\text{l}$  of blocking solution (10% ethanalamine; Sigma-Aldrich) in PBS for milk and StartingBlock (37538; Pierce Biotechnology, Rockford, IL) for egg and soy proteins was added per well and incubated for 1 h at  $37^\circ\text{C}$ . After 1 h of incubation, plates were washed once with 300  $\mu\text{l}$  of PBST per well. Eighty microliters of the appropriately diluted milk, egg, or soy primary antibody (see below) was added to each well of their respective plates and incubated for 30 min for egg and soy and 1 h for milk at  $37^\circ\text{C}$ . After incubation, the primary antibodies were discarded, and the plates were washed five times with 300  $\mu\text{l}$  of PBST per well. Thereafter, 80  $\mu\text{l}$  of the appropriately diluted milk, egg, or soy secondary antibody (see below) was added to each well of their respective plates and incubated for 2 h at  $37^\circ\text{C}$ . After 2 h of incubation, the plates were washed three times with 300  $\mu\text{l}$  of PBS-SDS per well and two times with 300  $\mu\text{l}$  of PBS. Eighty microliters of TMB (ImmunoPure, Ultra TMP substrate kit 34028; Pierce Biotechnology, Rockford, IL) solution was added to each well. Thereafter, the plates were covered with aluminum foil and placed on a shaker for 20 min at room temperature. A blue color development indicated presence of proteins in the solution. After 20 min on a shaker, 80  $\mu\text{l}$  of 2 N  $\text{H}_2\text{SO}_4$  was added to each well to stop the reaction, and the wells with protein turned yellow in color. Optical density (OD) from each well was read at 450 nm, with 490 nm as a reference standard on an Emax microplate reader (Molecular Devices, Sunnyvale, CA).

**Antibodies.** The primary antibody used for egg protein was rabbit anti-chicken egg albumin (Sigma-Aldrich), which was diluted in StartingBlock + 1300 ppm Silwet L-77 in a 1:4,000 ratio. The primary antibody for milk protein was sheep anti-casein (Biodesign International, Saco, ME) and was mixed in 20% bovine serum albumin (HyClone, Logan, UT) in PBS + 1300 ppm Silwet L-77 in a 1:500 ratio. For soy protein, rabbit anti-soy (R-Biopharm, South Marshall, MI) was used as the primary antibody and was mixed

**Table 1.** Mean  $\pm$  SD ELISA optical density values of marker protein-treated leaves (averaged across four treatments) collected from a citrus grove in Lake Alfred, FL, with or without simulated rainfall

Protein marker	Leaf type	Days after application				
		1	5	10	20	35
Egg	Without washing	3.536 $\pm$ 0.953a (64) <sup>a</sup>	1.344 $\pm$ 0.494b (64)	0.728 $\pm$ 0.560a (64)	0.136 $\pm$ 0.121a (64)	0.033 $\pm$ 0.021a (64)
	Simulated rain wash	0.441 $\pm$ 0.228c (61)	0.180 $\pm$ 0.099d (63)	0.053 $\pm$ 0.032b (64)	0.009 $\pm$ 0.007b (49)	0.013 $\pm$ 0.016b (24)
Milk	Without washing	3.214 $\pm$ 1.050b (64)	1.539 $\pm$ 0.660a (64)	0.435 $\pm$ 0.297c (64)	0.103 $\pm$ 0.064c (64)	0.016 $\pm$ 0.019b (64)
	Simulated rain wash	0.360 $\pm$ 0.239c (64)	0.214 $\pm$ 0.245d (64)	0.114 $\pm$ 0.128cd (61)	0.020 $\pm$ 0.014c (18)	0.014 $\pm$ 0.006b (14)
Soy	Without washing	3.326 $\pm$ 1.028ab (64)	0.937 $\pm$ 0.445c (64)	0.146 $\pm$ 0.092cd (58)	0.027 $\pm$ 0.022c (62)	—
	Simulated rain wash	0.229 $\pm$ 0.166c (64)	0.038 $\pm$ 0.042e (64)	0.020 $\pm$ 0.012d(44)	0.005 $\pm$ 0.003c (47)	—
	F-statistic	323.80	171.42	60.52	41.27	12.11
	df	5,375	5,377	5,349	5,298	3,162
	P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

<sup>a</sup> No. leaf discs from which mean  $\pm$  SD OD values were calculated.

Mean  $\pm$  SD OD values followed by a different letter in a column are significantly different from one another according to LSD ( $P < 0.05$ ).

in StartingBlock in a 1:4,000 ratio. The secondary antibody used for egg and soy proteins was donkey anti-rabbit IgG (H + L) with a peroxidase conjugate (Pierce Biotechnology, Rockford, IL), which was mixed in StartingBlock in 1:6,000 and 1:8,000 ratios, respectively. For milk protein, donkey anti-sheep IgG with a peroxidase conjugate (Sigma-Aldrich) was used as the secondary antibody and was mixed in StartingBlock in a 1:1,500 ratio.

**Calculations and Statistical Analyses.** OD readings from all wells, including controls, for each microplate were corrected for their respective blank mean OD reading. Mean ( $\pm$ SD) OD readings for control wells in each microplate were calculated. If the OD value measured for a treatment well was higher than the mean plus 4 SD values of the control (positive threshold), it was considered as positive for the protein under detection (Jones et al. 2006). All OD readings are presented as mean ( $\pm$ SD). OD value data obtained from leaves marked with the four formulation treatments were separately tested by one-way analysis of variance (ANOVA) for samples not subjected to rain simulation as well as those receiving rain simulation for each of the three marker proteins compared (PROC GLM; SAS Institute 2005). Given that ANOVAs were not significant for the four formulation treatments tested, with or without rain simulation, for each marker protein (egg, milk, soy) tested, data from the four formulations were combined for each marker protein and subjected to one-way ANOVA to test for differences between marker proteins. When significant ANOVAs were identified, differences among means were separated using least significant difference (LSD) tests at  $\alpha = 0.05$ . The percentage of *D. citri* acquiring protein markers at the two temperatures tested were compared using a *t*-test (PROC TTEST; SAS Institute 2005) at  $\alpha = 0.05$ . For the field experiment, the percentage of *D. citri* marked with egg, milk, or both proteins was calculated. For the purpose of statistical analysis, *D. citri* that were found marked with both proteins and captured in unmanaged grove plots were considered marked with milk protein. Similarly, *D. citri* adults that were marked with both proteins and captured in managed grove plots were considered marked with egg protein. Differences between the percentages of marked *D. citri* captured on border, central, and interior traps were compared by

ANOVA (PROC GLM; SAS Institute 2005) and mean separation was performed LSD tests at  $\alpha = 0.05$ . The numbers of adult *D. citri* captured in the managed grove plots that were marked with egg protein and those captured in the unmanaged grove plots that were marked with milk protein were compared using  $\chi^2$  analysis (PROC FREQ; SAS Institute 2005) at  $\alpha = 0.05$ .

**Results**

**Residual Longevity of Protein Markers on Citrus Leaves in the Field.** There were no significant differences in the average OD values between the four marker formulation treatments compared for each marker protein tested with or without rain simulation [(without rain simulation: egg protein  $F = 1.37-2.73$ ;  $df = 3,60$ ;  $P = 0.07-0.26$ ; milk protein  $F = 1.48-2.91$ ;  $df = 3,60$ ;  $P = 0.06-0.23$ ; and soy protein  $F = 0.06-2.04$ ;  $df = 3,58-60$ ;  $P = 0.12-0.98$ ) (with rain simulation: egg protein  $F = 0.28-2.28$ ;  $df = 3,20-60$ ;  $P = 0.09-0.84$ ; milk protein  $F = 0.41-1.04$ ;  $df = 3,10-60$ ;  $P = 0.38-0.75$ ; and soy protein  $F = 0.07-2.74$ ;  $df = 3,40-60$ ;  $P = 0.08-0.97$ ]. Therefore, data from the four formulation treatments were combined for each marker protein, with or without rain simulation, and analyzed for significant differences between the three protein markers. Leaves collected from egg protein-treated citrus trees showed significantly higher OD values than OD values of milk and soy protein-treated leaves with or without rain simulation or egg protein-treated leaves with rain simulation throughout the study period (Table 1;  $F = 12.11-323.80$ ;  $df = 5,162-378$ ;  $P = <0.0001$ ). Leaves collected from egg, milk, and soy protein-treated citrus trees 1 d after application exhibited higher OD values (3.536, 3.214, and 3.326, respectively) for their respective proteins than leaves collected on subsequent days (Table 1; Figs. 1a-c). OD values from leaves gradually decreased on every successive collection date reaching the lowest reading 35 d after application for egg and milk proteins and 20 d after application for soy protein (Table 1; Fig. 1a-c). Rainfall occurring on the 4th, 21st, and 28th d after application (6.22, 96.00, and 3.27 mm, respectively) could have con-



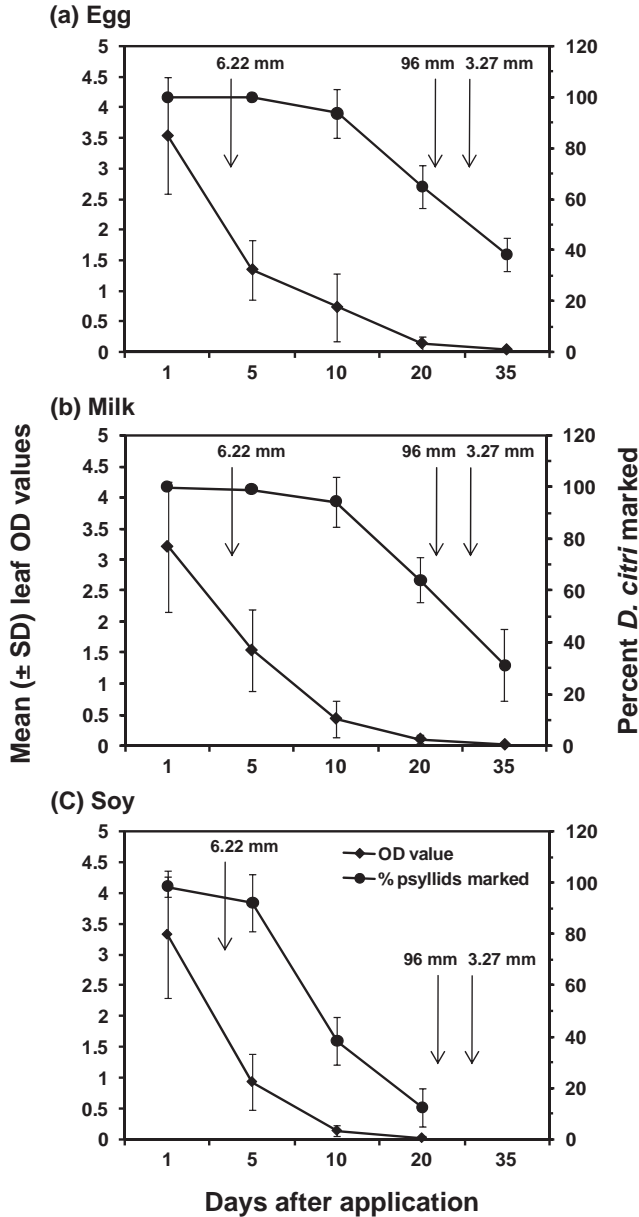


Fig. 1. Mean ( $\pm$ SD) OD values from leaves treated with marker proteins (averaged across four treatments for given a protein; OD value  $y$ -axis) and mean ( $\pm$ SD) percentage of *D. citri* adults marked (averaged across two temperatures for all four treatments; percentage *D. citri*  $y$ -axis) with proteins when confined on field-aged leaves over a 4-wk period. (a) Egg protein; the positive threshold range for leaf and adult *D. citri* samples was 0.002–0.009. (b) Milk protein; the positive OD threshold range for leaf and adult *D. citri* samples was 0.001–0.005. (c) Soy protein; the positive threshold range for leaf and adult *D. citri* samples was 0.001–0.006. Rainfall of 6.22, 96.0, and 3.27 mm occurred 4, 21, and 28 d after application, respectively, during the experiment as indicated by arrows.

tributed to the observed decrease in OD values from leaves over time (Table 1; Fig. 1a–c). The OD values for egg and milk proteins decreased by 3- to 15-fold and 1- to 9-fold, respectively, after a simulated rain wash of leaves collected from the field compared with nonwashed leaves (Table 1). The decrease in OD value for soy protein after simulated rain wash

(5- to 26-fold compared with nonwashed leaves) was higher than that for egg and milk proteins (Table 1).

**Effect of Temperature on Protein Marker Acquisition by *D. citri* Walking on Field-Aged Residues.** There were no significant differences between the mean percentages of *D. citri* acquiring a protein mark

at the two temperatures tested across the four treatments for each protein marker on each day of field aging (egg protein  $t = 0.77$ – $1.46$ ;  $df = 3$ ;  $P = 0.24$ – $0.56$ ; milk protein  $t = 0.29$ – $1.48$ ;  $df = 3$ ;  $P = 0.23$ – $0.78$ ; and soy protein  $t = 1.00$ – $1.73$ ;  $df = 3$ ;  $P = 0.18$ – $0.39$ ). Thus, the data across the four treatments at each temperature were averaged for each protein. The percentages of adult *D. citri* marked with detectable egg or milk protein were  $\geq 93.8\%$  after 24 h of confinement on 1-, 5-, or 10-d field-aged leaves (Fig. 1a and b); however, this decreased to nearly 65% after 20 d of field aging. The percentage of *D. citri* marked with egg or milk protein decreased thereafter (Fig. 1a and b). As observed with egg and milk proteins, the percentage of adult *D. citri* marked with detectable soy protein was  $\geq 92.2\%$  when confined on 1- and 5-d field-aged leaves for 24 h (Fig. 1c). The decrease in the percentage of marked *D. citri* with increasing leaf aging was more pronounced with soy protein than with milk or egg proteins, reaching the lowest level (12.5%) on leaves aged for 20 d (Fig. 1c).

**Marking *D. citri* by Direct Contact With Protein Marker Spray Application and Residual Longevity.** There were no significant differences between the percentages of *D. citri* marked by a given protein at each time interval post application ( $F = 1.00$ – $3.50$ ;  $df = 2,3$ ;  $P = 0.16$ – $0.46$ ). Proteins were detectable on all *D. citri* for up to 20 d after directly spraying *D. citri* with protein marker solutions, with the exception of soy protein at 20 d after application when only 87.7% of *D. citri* exhibited a detectable OD value (Fig. 2a and b). The percentage of *D. citri* marked with egg and soy proteins decreased slightly (93.75 and 81.25%, respectively) at 30 d after application, whereas it remained at 100% for milk protein (Fig. 2a). Both egg and milk proteins showed similar stability (OD values) on *D. citri* throughout the study with OD values of  $>3.0$  at 1 and 5 d after marking and  $>2.0$  and  $>1.0$  at 10 and 20 d after marking, respectively (Fig. 2b). For the same time intervals, OD values obtained with soy protein were similar until 10 d after application and slightly lower at 20 d (Fig. 2b).

**Movement Pattern of Adult *D. citri* Between Unmanaged and Managed Citrus Grove Plots.** A total of 129 and 149 adult *D. citri* were captured on yellow sticky traps in managed and unmanaged grove plots, respectively, 3 d after application of marker protein solutions. Of those captured in the milk protein sprayed grove plots (managed), the majority (69%) were found marked with proteins. Similarly, of those captured in the egg protein sprayed grove plots (unmanaged), 73% were marked with proteins. No detectable proteins were found on the remaining *D. citri* captured in managed (31%) and unmanaged (27%) grove plots. In the managed plots, 80% of the marked *D. citri* adults were positive for milk protein alone (Fig. 3). However, 17% were marked with egg protein and 3% with milk and egg, indicating movement from the unmanaged groves into the managed groves (Fig. 3). Similarly, the majority of the marked *D. citri* captured in the unmanaged grove plots were marked with egg protein (88%), whereas those that had moved

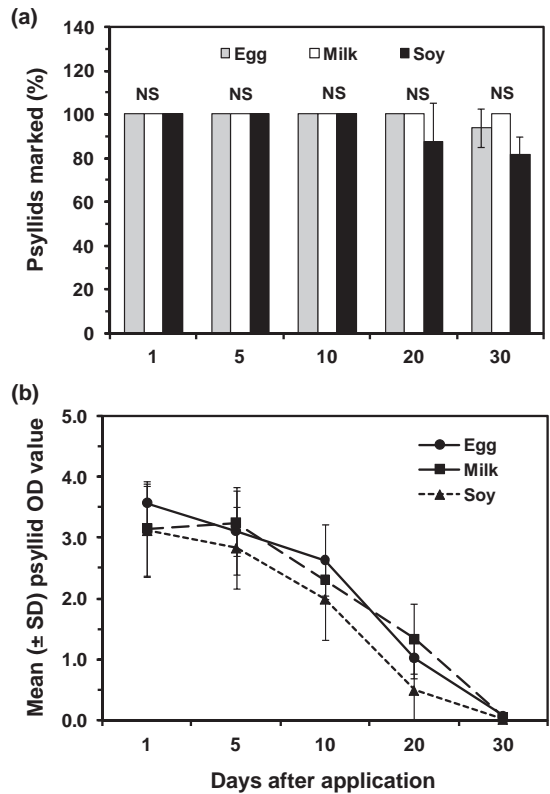


Fig. 2. Adult *D. citri* labeled with marker proteins by direct contact of protein solution sprays applied to adult *D. citri*-infested rough lemon plants in the laboratory and collected on five sampling dates post application. (a) Mean ( $\pm$ SD) percentage of adult *D. citri* marked with proteins. (b) Mean ( $\pm$ SD) OD values of marked adult *D. citri*; the positive threshold range for adult *D. citri* samples was 0.002–0.004. NS, no significant differences between treatments according to ANOVA ( $P > 0.05$ ).

from the managed plots into the unmanaged plots were marked with milk (8%) or both milk and egg (4%) (Fig. 3). There was no statistically significant difference between the numbers of egg-protein marked *D. citri* captured in managed grove plots and milk-protein marked *D. citri* captured in unmanaged plots ( $\chi^2 = 0.8$ ;  $df = 1$ ;  $P = 0.36$ ).

Significantly more *D. citri* were captured on border traps than those placed 40 (central traps) and 90 m (interior traps) away from plot borders (Fig. 4a) (milk protein  $F = 13.19$ ;  $df = 2,9$ ;  $P = 0.002$ ; milk + egg proteins  $F = 29.9$ ;  $df = 2,9$ ;  $P \leq 0.0001$ ; and unmarked  $F = 37.4$ ;  $df = 2,9$ ;  $P \leq 0.0001$ ) in managed plots. Similarly, significantly more ( $F = 4.88$ ;  $df = 2,9$ ;  $P = 0.03$ ) milk protein marked *D. citri* were captured on border traps than on central and interior traps in unmanaged plots (Fig. 4b). However, captures of egg protein marked and unmarked *D. citri* on border traps were significantly higher than on interior traps (egg protein:  $F = 7.50$ ;  $df = 2,9$ ;  $P = 0.01$ ; and unmarked  $F = 3.99$ ;  $df = 2,9$ ;  $P = 0.05$ ). In general, more adult *D. citri* were captured on border traps

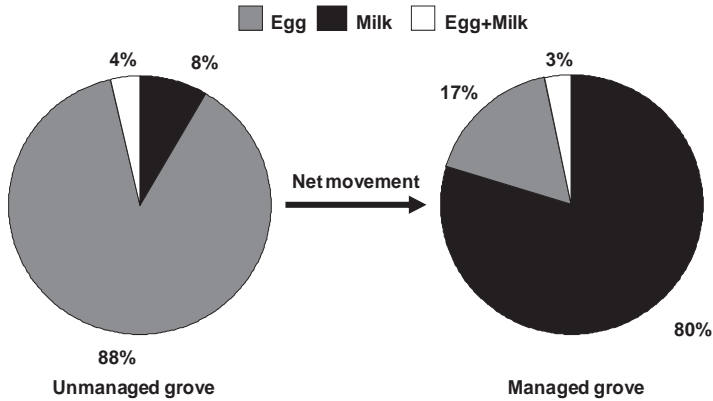


Fig. 3. Percentage of *D. citri* positive for egg, milk, or egg + milk proteins captured in adjacent managed and unmanaged citrus plots showing net movement of *D. citri* from unmanaged into managed grove plots.

than on traps placed 40 and 90 m from plot borders in both unmanaged and managed citrus plots (Fig. 4a and b).

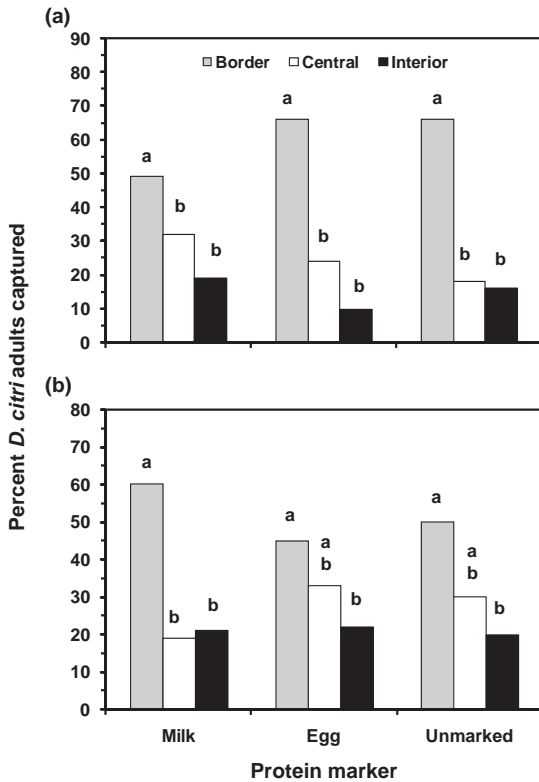


Fig. 4. Mean percentage of marked or unmarked *D. citri* adults captured on border, central, and interior yellow sticky traps in (a) managed (milk) and (b) unmanaged (egg) Valencia citrus grove plots. Border, central, and interior traps were placed on the 1st, 5th (40 m from border), and 10th (90 m from border) tree relative to the plot border. Bars with different letters for a given protein marker (or unmarked) are significantly different from one another according to LSD ( $P < 0.05$ ).

Discussion

To quantify the movement patterns of *D. citri* and determine its dispersal capacity in the field, a reliable in situ technique for marking *D. citri* in natural habitats and over a large areas is required. Characteristics of effective insect marking techniques are durability, low cost, low toxicity to the insect and environment, ease of application, and clear and unambiguous detection capability of the marker (Hagler and Jackson 2001). Techniques such as physical tags, mutilation markings, and paints/inks are inconvenient and perhaps unsuitable for use with *D. citri* because of its small size. Also, such techniques could affect this insects' natural behavior. Dust marking with fluorescent dyes may be effective for *D. citri* but would be laborious in the field, unless the dust could be sprayed with conventional spray equipment. Also, such dusts could be washed off given the frequent rain in Florida; our initial studies proved that such dust powders were of limited effectiveness for this very reason. Another problem that might occur with dusts is cross-marking, because the particles could be carried large distances by wind. This would confound data if more than one marker was used, which is necessary to determine the directionality of movement as was done in this study. Finally, dusts have been shown to be toxic to certain insects or interfere with normal dispersal behavior (Hagler and Jackson 2001).

Protein markers that are either sprayed directly onto insects or acquired by insects walking on a marked surface area overcome the many drawbacks of the other insect marking techniques discussed above (Jones et al. 2006). Among the three food proteins evaluated to mark adult *D. citri*, egg and milk were the most effective. Residues of each protein marker tested on citrus leaves decreased over time with detection of egg and milk proteins lasting longer (35 d) than soy protein (20 d). Jones et al. (2006) did not observe a significant decrease in egg and milk protein detection over a 20-d period on apple leaves, whereas soy protein detection remained stable for 8 d after application. The decrease in detection of marking proteins

after 5 d of field aging in our study may have been caused by rainfall, which occurred on 3 separate d after application throughout the collection period. In addition, differences in leaf characteristics and temperature between these two studies may have contributed to the observed differences. Application of simulated rainfall to leaves marked in the field decreased our ability to detect protein markers, which was congruent with the field-aging study. Irrespective of the marker used or age of the residue, temperature did not influence acquisition of protein markers by *D. citri*. Similarly, addition of a water softener, EDTA, or a wetting agent, Silwet L-77, did not increase either the retention of protein makers on citrus leaves or acquisition by *D. citri*. Therefore, use of either of these two additives with protein marker solutions is not needed in future mark-capture studies of *D. citri*.

Egg and milk protein markers were detected on  $\geq 90\%$  of *D. citri* confined on marked leaves 1, 5, and 10 d after application of proteins. This level of detection was observed for the soy protein marker on leaves aged 1 and 5 d after treatment. In contrast, 80–100, 8–38, and 0–5% of *C. pyricola* were marked with egg, milk, and soy proteins, respectively, after confinement on marked apple leaves (Jones et al. 2006). This discrepancy may be due to the use of different experimental arenas for marking insects with field-aged leaves. In our study, 90-mm-diameter petri plates were used to confine adult *D. citri* on marked leaves that could have resulted in more frequent contact between *D. citri* and protein residues than in the 0.5-liter plastic containers used by Jones et al. (2006). Furthermore, under sealed petri plate conditions at 25 and 35°C, contact of protein markers by *D. citri* could be greater because of increased moisture resulting from leaf transpiration. Also, leaf characteristics (apple versus citrus) and behavioral differences between the two psyllid species could have also contributed to the observed differences.

Detected residue levels of the three protein markers over time were similar when markers were directly sprayed onto *D. citri* and were longer lasting than when insects were exposed to dried proteins on leaf surfaces. Egg, milk, and soy protein markers were detected on  $>90\%$  of sprayed adult *D. citri* for up to 30, 30, and 20 d, respectively. In contrast, detection of egg, milk, and soy proteins was  $>90\%$  for only up to 10, 10, and 5 d, respectively, for adult *D. citri* that acquired markers by walking across residues on field-aged leaves. Therefore, in the event of rain, use of egg or milk protein would likely allow for a minimum of 10 d of reliable detection, whereas use of soy protein would likely allow for a minimum of 5 d of reliable detection after marker application with our current protocol. Also, *D. citri* that are directly sprayed with markers in the field will be tagged for up to 20 d longer than those acquiring markers by walking across dry protein residues on leaves.

A thorough understanding of insect dispersal such as range limits, frequency of movement, and seasonality should improve development of pest management strategies, particularly for plant disease vectors.

In an effort to track the movement of *D. citri*, fluorescent dyes were recently evaluated (Nakata 2008). Although nontoxic and detected on up to 20% of marked *D. citri* in the field for up to 40 d, fluorescent dyes appeared to inhibit flight activity for at least the first 4 h after application. Also, as mentioned previously, this technique does not allow for reliable marking of feral insects in their natural habitat. The African citrus psyllid, *Trioza erytreae* (Del Guercio), is the only other vector of the HLB pathogen, but does not occur in New World habitats. To quantify the dispersal range of *T. erytreae*, van den Berg and Deacon (1988) circumvented the need for a marker by releasing 25,000 adults in an open area of plowed land without nearby host plants and recaptured adults on yellow sticky traps as was done in this study. The authors found that *T. erytreae* were capable of dispersing up to 1.5 km from the release site with females moving longer distances than males. However, from the standpoint of disease epidemiology, it is difficult to relate such data to more natural dispersal behavior within and among host plants (i.e., a citrus orchard). The dispersal behavior of *T. erytreae* in an open field may have been influenced by lack of surrounding host plants given that psyllid movement may be influenced by the availability of food and/or oviposition sites. Therefore, the nonobtrusive immunomarking technique tested herein, which labels *D. citri* in their natural habitat, overcomes the drawbacks of the above studies allowing for direct tracking of *D. citri* movement in the field. In future studies, we plan to quantify the characteristics of *D. citri* dispersal including frequency, distance, speed, and seasonality as well as correlating these factors with disease spread by tracking movement with immunomarking.

In our field experiment, we detected protein markers on 69% of captured adult *D. citri* in the managed grove (treated with milk protein) and 73% in the unmanaged grove (treated with egg protein). Of the marked *D. citri* captured in managed plots, 80% were marked with milk and 20% were marked with egg. Because the source of the egg protein was the spray applied to the unmanaged grove plots, this suggests movement from the unmanaged plots into the managed plots. By the same logic, adult *D. citri* captured in the unmanaged grove plots that were marked with milk likely originated from the commercially managed grove plots sprayed with this protein. These findings indicate that a portion of *D. citri* moved bi-directionally between unmanaged and managed groves separated by 60–100 m within 3 d. Also, our results suggest a net directed movement from the unmanaged into the managed plots. Although the underlying reasons for this frequent and highly active dispersal behavior are not yet understood, this is the first direct evidence for rapid, short distance movement of *D. citri* between unmanaged and managed groves. Studies are needed to determine whether unmanaged groves are reservoirs of HLB and whether this contributes to disease spread into commercial citrus. In such case, direct management or potential destruction of unmanaged groves may be required for effective management of



*D. citri* and HLB in Florida. Second, direct proof of rapid short range psyllid movement between adjacent groves suggests that *D. citri* may be capable of rapidly reinfesting a managed citrus grove from a neighboring grove where psyllid management practices are lax. This suggests that areawide management strategies may be needed or supplemental border sprays may prove helpful in cases where *D. citri* are invading from infested neighboring areas. Consistently higher captures of *D. citri* on traps located on plot borders further suggests that citrus grove borders may require more intense management than interior portions of the grove.

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