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Insecticide resistance in field populations of Asian citrus psyllid in Florida

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Abstract

BACKGROUND: Asian citrus psyllid (ACP), *Diaphorina citri*, is a major pest of citrus because it vectors the putative causal agent of huanglongbing disease. Insecticides are currently the basis of psyllid management programs, and the number of annual insecticide applications has increased significantly. In this paper, a series of investigations of insecticide resistance among field populations of adult and immature ACP in Florida is described.

RESULTS: In 2009, the highest level of resistance for adult ACP, as compared with the laboratory susceptible (LS) population, was found with imidacloprid with an LD_{50} resistance ratio (RR_{50}) of 35 in one population. This was followed by chlorpyriphos (RR_{50} = 17.9, 13.3, 11.8 and 6.9), thiamethoxam (RR_{50} = 15 and 13), malathion (RR_{50} = 5.4 and 5.0) and fenpropathrin (RR_{50} = 4.8). In 2010, mortality of adults from all five sites sampled was lower than with the LS population at three diagnostic concentrations of each insecticide tested. Among nymph populations, indications of resistance were observed with carbaryl (RR_{50} = 2.9), chlorpyriphos (RR_{50} = 3.2), imidacloprid (RR_{50} = 2.3 and 3.9) and spinetoram (RR_{50} = 4.8 and 5.9). General esterase, glutathione S-transferase and monooxygenase levels were also elevated in field-collected adult and nymph ACP as compared with the LS population.

CONCLUSION: The present results suggest that varying levels of insecticide susceptibility exist in ACP populations across the citrus-growing areas of Florida. Increased levels of detoxifying enzymes in these populations may partially explain these differences. The present results indicate that insecticide resistance may become an emerging problem for ACP control if effective resistance management is not practiced.

Supporting information may be found in the online version of this article.

Keywords: *Diaphorina citri*; general esterase; glutathione *S*-transferase; insecticide resistance; monooxygenase

1 INTRODUCTION

The Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), is one of the most serious pests of citrus because it is a vector of the putative causal agent of huanglongbing (HLB), *Candidatus* Liberibacter asiaticus.[1] HLB is one of the most economically important diseases of citrus and it is now present in most of the citrus-growing parts of the world.[1,2] The disease causes stunting, off-season bloom, premature fruit drop and small, misshapen, bitter fruit.[1] In Florida, the average infection rate of HLB in citrus groves is estimated to be 1.6%, reaching up to 100% in the southern and eastern parts of the state.[3] The nymph is the stage in which the highest rate of pathogen acquisition occurs, but inoculation of healthy plants with the pathogen is carried out by mobile adults.[4]

At present, the most common practice for management of HLB is aggressive use of insecticides to control the vector. Available effective insecticides include compounds of various chemistry and mode of action.[5–7] However, the number of available modes of action is limited, and in some cases repeated sequential use of the same insecticide or mode of action occurs in Florida owing to economic constraints or ignorance of the need to rotate between different modes of action, or because the large number of applications that growers are implementing makes effective rotation difficult. There is an urgent need to develop strategies that maintain effective use of currently labeled insecticides for sustainable ACP control into the future.

The present study was conducted to document resistance levels in Florida populations of ACP to commonly used insecticides. Baseline susceptibility data for both adult and immature ACP were developed using a laboratory susceptible population as a comparison. In addition, biochemical assays were performed to quantify differences in general esterase, glutathione *S*-transferase and monooxygenase levels between field populations and the laboratory susceptible strain to gain insight into the possible underlying mechanisms of resistance in ACP.

2 MATERIALS AND METHODS

2.1 Asian citrus psyllid culture

The laboratory susceptible culture (LS) of ACP was continuously reared at the Citrus Research and Education Center (CREC),

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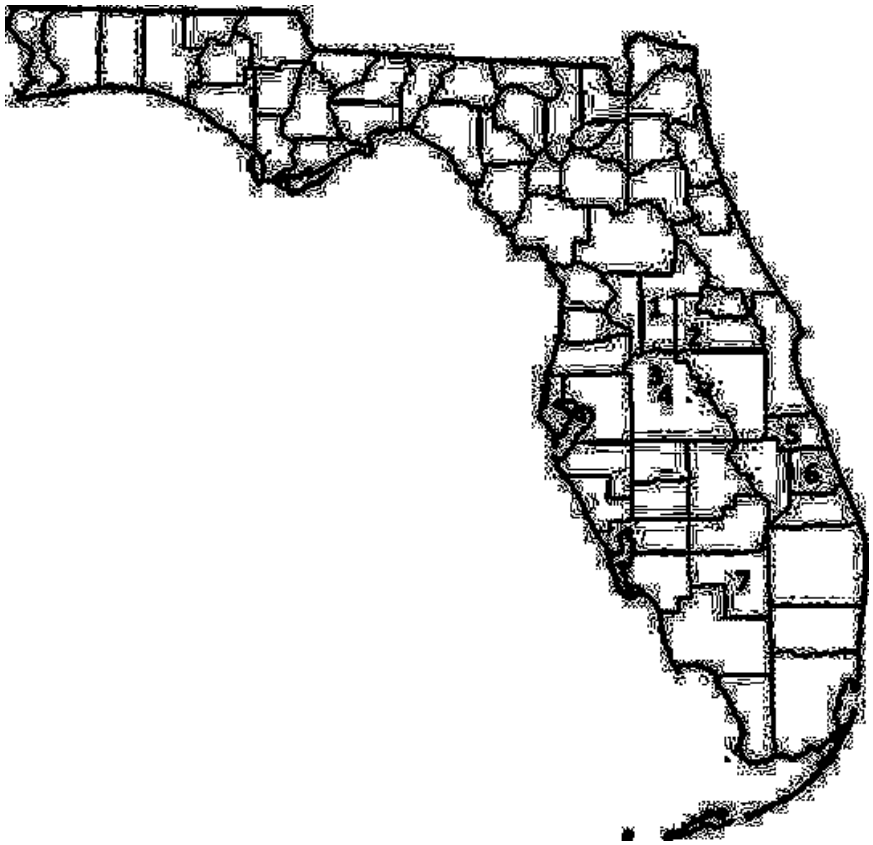

Figure 1. Location of five commercial citrus groves in Florida for evaluating susceptibility levels in adult and fourth-instar *Diaphorina citri* towards 12 and five insecticides respectively. 1: Groveland (adult and nymph); 2: Winter Garden (nymph); 3: Lake Alfred 1 (nymph); 4: Lake Alfred 2 (adult and nymph); 5: Vero Beach (adult); 6: Fort Pierce (adult); 7: La Belle (adult).

University of Florida, Lake Alfred, Florida. The culture was established in 2000 using field populations collected in Polk County, Florida (28.0′ N, 81.9′ W) prior to the discovery of HLB in the state. The culture was maintained on 'sour orange' (*Citrus aurantium* L.) seedlings without exposure to insecticides in a greenhouse at 27–28 °C and 60–65% RH with a 14 : 10 light : dark photoperiod.

2.2 Field collection

Adult ACP were collected from five commercial citrus groves in Florida during 2009 and 2010 (Fig. 1). Collected adults were transferred to the laboratory in coolers and released on citrus plants in Plexiglas cages (40 × 40 × 40 cm) until use in bioassays. In addition, fourth-instar ACP were collected from four commercial groves in central Florida (Fig. 1) at two different times during the 2010 citrus-growing season. Two of the sites overlapped for adult and nymph collections. Nymphs were collected by cutting off an entire leaf flush from the main branch and were brought to the laboratory in an ice cooler. Flush were maintained by placing stems in water until nymphs were removed for bioassays. Nymphs were assayed on the same day of collection.

2.3 Insecticides

All bioassays were conducted with analytical-grade insecticides. The susceptibility of adult and immature ACP was tested against 12 insecticides and five insecticides, respectively, belonging to different chemistry classes and modes of action. The insecticides evaluated against ACP adults included abamectin (97.1%) (Sigma-Aldrich, St Louis, MO), acetamiprid (99.9%) (Sigma-Aldrich), aldicarb (99.9%) (Sigma-Aldrich), bifenthrin (99.0%) (ChemService, West Chester, PA), carbaryl (99.5%) (ChemService), chlorpyriphos (99.5%) (Sigma-Aldrich), cypermethrin (98.5%) (Sigma-Aldrich), dimethoate (99.4%) (Sigma-Aldrich), fenpropathrin (99.5%) (ChemService), imidacloprid (99.5%) (ChemService), malathion (98.4%) (ChemService), spinetoram (%) (Dow AgroSciences LLC, Indianapolis, IN, and ChemService) and thiamethoxam (99.7%) (Sigma-Aldrich). Insecticides evaluated against ACP nymphs included carbaryl, chlorpyriphos, fenpropathrin, imidacloprid and spinetoram.

2.4 Adult topical application bioassay

2.4.1 2009: Establishment of baseline susceptibilities

In 2009, a topical application technique was used for bioassays on adult ACP.[8] ACP adults of mixed gender were anaesthetized under CO_2, and a 0.2 µL droplet of technical-grade insecticide in analytical-grade acetone was administered to the dorsal side of the thorax using a 10 µL Hamilton syringe. The same amount of acetone alone was applied to adults as a negative control. For each insecticide concentration, at least 20 adult ACP were treated in three replicates, and each insecticide was tested at 5–6 concentrations. Concentrations used for each insecticide were either based on previous investigations or on preliminary results.[9,10] Treated insects were placed in 60 mm plastic disposable petri dishes with 60 mm citrus leaf disks placed over agar beds as food. Petri dishes with insects were kept at 25 ± 1 °C and 50 ± 5% RH with a 14 : 10 h light : dark photoperiod in a growth chamber

for 24 h. After 24 h, mortality was assessed. ACP adults that did not move upon prodding were considered dead. Mortality data were corrected for the control treatment using Abbott's formula[11] and analyzed using log-probit regression analysis to estimate the lethal dose for mortality in 50, 75 and 95% of the population (LD_{50}, LD_{75} and LD_{95}) with corresponding 95% confidence limits (CLs).[12] The LD_{50} values between the laboratory susceptible (LS) population and field populations for each insecticide were considered significantly different ($P > 0.05$) if their 95% confidence intervals did not overlap.

2.4.2 2010: Diagnostic dose bioassays

The susceptibility of ACP adults collected during 2010 was tested using three diagnostic doses of each insecticide obtained from the 2009 topical application bioassays conducted on the LS population. The lethal doses of each insecticide that resulted in 50, 75 and 95% mortalities of the LS population were used for topical application bioassays conducted on field-collected and LS adults. Bioassays were conducted as described above. A diagnostic dose for each insecticide was replicated 3 times on each population, using 20–30 adults of mixed gender per replicate. Bioassays were performed on field-collected and LS populations on the day of collection. Mortality was recorded after 24 h, as described above.

Percentage mortality data were analyzed by analysis of variance (ANOVA) using a general linear model (PROC GLM)[12] for each diagnostic dose, and using insecticides and collection sites as main effects. Based on a significant interaction between main effects, subsequent analyses were performed to determine whether significant differences occurred in percentage mortality between field-collected and LS populations within each diagnostic dose, followed by Fisher's protected LSD tests.

2.5 Petri dish bioassay

Considering the delicate nature and size (about 1.0 mm) of fourth-instar ACP, bioassays were performed using a leaf-dip bioassay method.[10,13] Bioassay arenas were prepared by pouring 3–5 mL of a 1.5% agar solution into 60 mm diameter plastic disposable petri dishes (Fisherbrand, Thermo Fisher Scientific, Waltham, MA) to form a solidified bed. Fresh citrus leaves collected from 'Valencia' orange trees maintained in a CREC greenhouse were used in bioassays. Leaf disks (60 mm diameter) were excised, dipped in test (insecticide) solutions made in acetone for 30 s and allowed to air dry in a fume hood for 1 h prior to bioassays. For the control treatment, leaf disks were dipped in acetone alone. After 1 h, leaf discs were placed on agar beds, and 20–25 fourth-stage nymphs were transferred into each dish using a camel hair brush. Petri dishes were wrapped with parafilm (Pechiney Plastic Packaging, Chicago, IL). Sealed petri dishes with ACP were transferred into a growth chamber (Percival Scientific, Inc., Perry, IA) set at $25 \pm 1\ ^{\circ}C$ and $50 \pm 5\%$ RH with a 14 : 10 light : dark photoperiod. Each concentration of insecticide was replicated 3 times ($n = 60–75$ nymphs per concentration). Bioassays were repeated 2 times for each population, corresponding to each collection date.

The mortality of ACP nymphs was assessed after 24 h. Nymphs found flaccid, dried, light colored and unable to move when probed with a camel hair brush were considered dead. All mortality data were corrected for the control treatment using Abbott's formula.[11] Mortality data were analyzed separately for each population. Mortality data for two collection dates were pooled for each site and subjected to probit regression analysis to calculate the LC_{50} for each insecticide with 95% corresponding confidence limits and slopes of regression lines.[12] The LC_{50} values between field-collected and LS populations were considered significantly different ($P > 0.05$) if their 95% confidence intervals did not overlap.

2.6 Enzyme preparation

Enzyme preparations were made according to established protocols, with some modifications from field-collected and LS populations of adults of mixed gender and nymphs during 2010.[14–16] Twenty adults or 50 fourth-instar ACP were homogenized in sodium phosphate buffer (500 µL; 0.1 M; pH 7.5) + 0.1% (v/v) Triton X-100 in 1.5 mL centrifuge tube(s) using a handheld homogenizer with a plastic pestle. Homogenized samples were centrifuged at 10 000 × g for 15 min at 4 °C. The supernatant was transferred to a 1.5 mL centrifuge tube and diluted appropriately with 0.1 M sodium phosphate buffer (pH 7.5) without Triton X-100. This served as an enzyme source for subsequent bioassays. Enzyme preparations were conducted separately on adult and nymph populations collected from each site in 2010. The total protein content of the enzyme preparation was determined by the bicinchoninic acid method using bovine serum albumin as a standard.[17] The absorbance of the reaction product was measured in a 96-well microplate reader at 562 nm and 25 °C.

2.7 General esterase assay

General esterase activity was measured using α-naphthyl acetate (α-NA) (Sigma Aldrich) as a substrate. General esterase activity was measured following a protocol[14,18–20] based on the amount of naphthol produced from the hydrolysis of naphtholic ester. Six aliquots of 15 µL of the enzyme solution and 135 µL of the 0.3 mM substrate were added to each well of the 96-well microplate (NUNC PolySorp) (Fisher Scientific Co.). Control wells consisted of 15 µL of phosphate buffer and 135 µL of 0.3 mM substrate. Plates were covered with aluminum foil and incubated for 30 min at 37 °C. Following incubation, 50 µL of Fast Blue B Salt in 5% SDS solution was added to each well to stop the reaction. The mixture was set aside at room temperature for 15 min to develop color. General esterase activity was determined by reading the plate at 595 nm using a microplate reader (Spectramax 250; Sunnyvale, CA) at 25 °C. Mean general esterase activity was calculated and standardized per mg of protein measured for each ACP, as described above.

2.8 Glutathione *S*-transferase (GST) assay

GST activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma Aldrich) as the substrate.[21,22] Six aliquots of 10 µL of the enzyme solution, 2 µl of 200 mM CDNB [containing 0.1% (v/v) ethanol] and 188 µL of 10.35 mM GSH in phosphate buffer (0.1 M; pH 7.5; pH 7.5) were pipetted into separate wells of the 96-well microplate. GST activity was determined by the change in absorbance as measured continuously for 1 min at 340 nm and 25 °C. Control wells consisted of 2 µL of CDNB, 188 µL of GSH and 10 µL of phosphate buffer (0.1 M; pH 7.5; pH 7.5). Changes in absorbance per minute were converted into µmol CDNB conjugated min^{-1} mg^{-1} protein using the extinction coefficient of the resulting 5-(2, 4-dinitrophenyl)-glutathione: $\varepsilon_{340\ nm} = 9.6\ nM^{-1}\ cm^{-1}$.[21]

2.9 Monooxygenase (cytochrome P450) assay

Cytochrome P450 activity was estimated by measuring heme peroxidase activity.[22–24] As heme constitutes the majority of

cytochrome P450 in non-blood-fed insects, quantification of heme activity can be expressed as cytochrome P450.[23] Heme peroxidase activity was measured using the substrate 3,3′5,5′-tetra-methylbenzidine (TMBZ) (Sigma Aldrich). Four aliquots of 20 μL of enzyme solution, 80 μL of 0.625 M potassium phosphate buffer (pH 7.2), 200 μL of TMBZ solution and 25 μL of hydrogen peroxide (3%) were pipetted into separate wells of the 96-well microplate. Plates were incubated at room temperature for 2 h before reading at 450 nm as the endpoint in the microplate reader at 25 °C. Control wells consisted of 20 μL of distilled water, 80 μL of 0.625 M potassium phosphate buffer, 200 μL of TMBZ solution and 25 μL of hydrogen peroxide (3%). A standard curve for heme peroxidase activity was prepared using different concentrations of cytochrome C from horse heart (Sigma Aldrich). Monooxygenase levels obtained from plate reading were expressed as equivalent units (EUs) of cytochrome P450 mg^{-1} protein using the standard curve of cytochrome C.

Analysis of variance (ANOVA) followed by Fisher's protected LSD mean separation tests was used to determine the differences in the levels of detoxifying enzymes between field-collected and LS populations (PROC GLM).[12] Separate analyses were performed for adult and nymph populations.

3 RESULTS

3.1 Adult topical application bioassay

3.1.1 2009: Establishment of baseline susceptibilities

Five field-collected populations of ACP were tested to estimate susceptibility levels in response to 12 insecticides (Table 1). One or more field populations exhibited higher LD_{50} values to fenpropathrin, imidacloprid, malathion and thiamethoxam compared with the LS population. The highest level of resistance was displayed by the La Belle population, with resistance ratios of 35 and 13 to imidacloprid and thiamethoxam, respectively. Three populations displayed moderate levels of resistance to malathion (Ft Pierce: RR = 5.4; Lake Alfred: RR = 5.0; Groveland: RR = 3.7). The Vero Beach population displayed a moderate level of resistance to fenpropathrin, with an RR value of 4.8.

3.1.2 2010: Diagnostic dose bioassays

Three diagnostic doses corresponding to 50, 75 and 95% mortalities determined from the LS population in 2009 (Table 2) were chosen to assess the susceptibility of field populations in 2010. Susceptibilities of field populations were compared with the LS population. Based on a significant interaction between main effects of collection site and insecticide for each diagnostic dose [(LD_{50}: $F = 1.99$; df = 41, 118; $P = 0.0022$), (LD_{75}: $F = 2.57$; df = 41, 118; $P < 0.0001$) and (LD_{95}: $F = 1.89$; df = 41, 118; $P = 0.0042$)], differences in percentage mortalities were analyzed between different populations within each insecticide for each dose. The mean percentage mortalities obtained from LD50 and LD75 are presented in the supporting information of this paper (Tables S1 and S2). At the diagnostic dose of LD_{95} there was a significant difference in mean percentage mortality between various populations for bifenthrin, carbaryl, chlorpyriphos, fenpropathrin, imidacloprid, spinetoram and thiamethoxam (Table 3).

3.2 Nymph petri dish bioassay

The susceptibility levels of immature ACP from four field populations to five insecticides are presented in Table 4. The LC_{50} value for carbaryl obtained from the LS population was significantly lower than the highest value obtained from one of the field populations representing a resistance ratio of 2.88 at LC_{50} (RR_{50}). For chlorpyriphos, the highest LC_{50} (8.31) observed from a field population was significantly higher than that from the LS population (2.58), representing an RR_{50} of 3.22. The synthetic pyrethroid, fenpropathrin, yielded a range of LC_{50} values from 0.15 to 0.57, with the lowest value obtained from the LS population and the highest from the Groveland population. Two of the field populations tested exhibited significantly lower susceptibilities to imidaclopid than the LS population exhibiting RRs of 3.81 and 2.27 respectively. Two of the populations tested showed significantly lower susceptibilities to the microbial insecticide spinetoram than the LS population with RR_{50} values of 2.98 and 5.88.

3.3 Detoxifying enzymes

3.3.1 Adults

General esterase ($F = 4.88$; df = 5, 18; $P = 0.0054$), glutathione *S*-transferase ($F = 11.36$; df = 5, 18; $P < 0.0001$) and monooxygenase ($F = 5.31$; df = 5, 18; $P = 0.0036$) levels were higher in several field-collected populations than in the LS population (Fig. 2). Mean (± SEM) general esterase levels were significantly higher in adult populations from Vero Beach (40.29 ± 1.85), Lake Alfred (36.72 ± 1.17) and La Belle (34.44 ± 2.48) than from the LS population (28.79 ± 1.14). Mean glutathione *S*-transferase levels were significantly higher in populations from all sites than in the LS population. Mean monooxygenase levels were significantly higher in populations from Ft Pierce (1.25 ± 0.36) and Vero Beach (0.95 ± 0.27) than in the LS population (0.12 ± 0.04).

3.3.2 Nymphs

For ACP nymphs, general esterase ($F = 48.83$; df = 4, 35; $P < 0.0001$), glutathione *S*-transferase ($F = 9.13$; df = 4, 35; $P < 0.0001$) and monooxygenase ($F = 22.93$; df = 4, 35; $P < 0.0001$) levels were significantly higher in certain field populations than in the lab population (Fig. 3). The mean (± SEM) general esterase level was significantly higher in nymphs from one field population (Winter Garden) (67.01 ± 3.18) than in the LS population (53.40±2.48). Also, the mean glutathione *S*-transferase level was significantly higher in two populations (214.14 ± 28.16) (Winter Garden) and (145.50 ± 31.02) (Groveland) than in the LS population (69.74 ± 22.83). The mean monooxygenase level was significantly higher in one population (3.54 ± 0.34) (Winter Garden) than in the LS population (1.11 ± 0.15).

4 DISCUSSION

The present investigation provides baseline susceptibility data for several Florida ACP populations to commonly used insecticides and verifies reduced susceptibility to several insecticides among geographically separated populations. In general, reduced susceptibility to the insecticides tested was more widespread in the second year of the study. In 2009 there was reduced susceptibility to fenpropathrin, imidacloprid, malathion, and thiamethoxam, as compared with the laboratory susceptible strain, in populations from one to three sites. However, in 2010, ACP adults were less susceptible to each insecticide tested, as compared with the LS (lab susceptible) population, for one or more of the diagnostic doses tested.

In 2009 there were instances when LD_{50} values of field populations were lower than for the LS population; however,

Insecticides		LD_{50} (ng AI insect^{-1}) LS	Groveland	Lake Alfred	Ft Pierce	Vero Beach	La Belle
Abamectin	LD_{50}	0.70 a	0.91 a	1.34 a	1.33 a	1.62 a	0.30 a
	(95% CL)	(0.031–0.56)	(0.151–0.36)	(0.03–2.02)	(0.07–1.97)	(0.93–2.24)	(0.01–0.75)
	LD_{95}	4.01 a	1.95 a	4.64 a	4.40 a	3.27 a	4.12 a
	(95% CL)	(1.73–1.2×10^4)	(1.32–493.62)	(2.79–14×10^3)	(2.72–1×10^3)	(2.34–11.39)	(1.19–2×10^3)
	χ^2	27.55	30.03	18.01	17.40	23.60	27.02
	Slope ± SE	2.17 ± 0.58	4.95 ± 1.40	3.06 ± 0.87	3.17 ± 0.87	5.44 ± 1.15	1.34 ± 0.31
	RR_{50}	–	1.30	1.91	1.90	2.31	0.43
	RR_{95}	–	0.49	1.16	1.10	0.82	1.03
Acetamiprid	LD_{50}	3.36 a	4.10 a	3.74 a	3.41 a	4.55 a	3.40 a
	(95% CL)	(2.36–4.20)	(2.23–5.91)	(1.01–6.30)	(0.70–5.63)	(1.15–8.55)	(1.78–4.88)
	LD_{95}	9.36 a	19.63 a	14.39 a	15.37 a	17.79 a	9.28 a
	(95% CL)	(7.01–17.21)	(12.20–59.71)	(7.94–319.0)	(8.34–478.6)	(9.17–2×10^3)	(6.03–54.70)
	χ^2	8.84	10.55	20.91	17.25	26.73	19.54
	Slope ± SE	3.70 ± 0.49	2.42 ± 0.36	2.81 ± 0.65	2.52 ± 0.60	2.78 ± 0.69	3.77 ± 0.79
	RR_{50}	–	1.22	1.11	1.01	1.35	1.01
	RR_{95}	–	2.10	1.54	1.64	1.90	0.99
Aldicarb	LD_{50}	1.57 a	3.38 a	2.98 a	3.62 a	2.30 a	1.25 a
	(95% CL)	(0.32–2.36)	(0.89–5.27)	(0.04–5.08)	(2.27–4.78)	(1.42–3.42)	(0.51–2.10)
	LD_{95}	4.83 a	8.95 a	7.08 a	10.60 a	17.83 a	10.72 a
	(95% CL)	(2.94–361.6)	(5.60–429.5)	(4.40–6×10^5)	(7.14–37.90)	(9.32–83.71)	(5.33–68.11)
	χ^2	21.17	27.35	38.19	11.46	16.02	24.07
	Slope ± SE	3.37 ± 0.88	3.89 ± 1.01	4.38 ± 1.30	3.52 ± 0.64	1.86 ± 0.30	1.76 ± 0.33
	RR_{50}	–	2.15	1.90	2.31	1.46	0.80
	RR_{95}	–	1.85	1.47	2.19	3.69	2.22
Bifenthrin	LD_{50}	0.03 a	0.10 a	0.05 a	0.10 a	0.02 a	0.01 a
	(95% CL)	(0.01–0.06)	(0.05–0.17)	(0.016–0.11)	(0.02–0.23)	(0.01–0.03)	(6×10^{-3}–0.01)
	LD_{95}	0.26 a	0.71 a	1.07 a	2.20 a	0.30 a	0.20 a
	(95% CL)	(0.10–4.89)	(0.36–3.24)	(0.41–7.56)	(0.74–34.17)	(0.12–1.72)	(0.12–0.37)
	χ^2	16.79	10.32	8.65	11.63	7.30	3.64
	Slope ± SE	1.64 ± 0.29	1.94 ± 0.27	1.23 ± 0.16	1.22 ± 0.19	1.36 ± 0.16	1.17 ± 0.10
	RR_{50}	–	3.33	1.67	3.33	0.67	0.33
	RR_{95}	–	2.73	4.12	8.46	1.15	0.77
Carbaryl	LD_{50}	3.92 a	11.47 a	5.62 a	6.71 a	4.39 a	2.53 a
	(95% CL)	(0.01–17.81)	(7.16–16.22)	(7×10^{-7}–16.14)	(0.03–17.43)	(0.02–22.83)	(1.31–4.26)
	LD_{95}	81.54 a	36.23 a	59.04 a	69.01 a	80.69 a	28.10 a
	(95% CL)	(18.01–7.1×10^{13})	(23.03–144.44)	(19.05–1×10^{23})	(23.02–3×10^{10})	(17.81–2×10^{11})	(13.62–120.81)
	χ^2	31.90	12.82	27.64	23.37	38.35	17.91
	Slope ± SE	1.25 ± 0.36	3.29 ± 0.58	1.61 ± 0.49	1.63 ± 0.47	1.30 ± 0.37	1.57 ± 0.22
	RR_{50}	–	2.93	1.43	1.71	1.12	0.65
	RR_{95}	–	0.44	0.72	0.85	0.99	0.34
Chlorpyriphos	LD_{50}	0.25 a	0.30 a	2.95 a	3.32 a	4.48 a	1.73 a
	(95% CL)	(0.01–2.21)	(0.02–1.71)	(0.08–5.92)	(0.30–5.73)	(1.50–8.34)	(0.81–2.57)
	LD_{95}	4.81 a	4.28 a	8.73 a	8.94 a	10.93 a	5.10 a
	(95% CL)	(0.90–1×10^7)	(1.19–383.8)	(4.89–2×10^8)	(5.38–6×10^5)	(6.73–6×10^3)	(3.21–34.84)
	χ^2	41.61	23.58	39.49	30.81	32.16	17.59
	Slope ± SE	1.28 ± 0.33	1.42 ± 0.28	3.49 ± 1.04	3.83 ± 1.12	4.25 ± 1.18	3.50 ± 0.74
	RR_{50}	–	1.20	11.80	13.28	17.92	6.92
	RR_{95}	–	0.44	0.72	0.85	0.99	0.34
Cypermethrin	LD_{50}	0.14 a	0.17 a	0.23 a	0.18 a	0.20 a	0.04 a
	(95% CL)	(0.06–0.27)	(0.06–0.36)	(0.10–0.43)	(0.15–0.22)	(0.08–0.44)	(0.02–0.08)
	LD_{95}	1.04 a	1.91 a	1.83 a	1.43 a	1.63 a	0.83 a
	(95% CL)	(0.59–13.14)	(0.73–33.43)	(0.81–19.88)	(1.07–2.06)	(0.65–30.00)	(0.33–6.74)
	χ^2	14.18	15.31	12.91	5.88	19.70	8.49

Insecticides		LD_{50} (ng AI insect^{-1})					
		LS	Groveland	Lake Alfred	Ft Pierce	Vero Beach	La Belle
	Slope ± SE	1.64 ± 0.26	1.56 ± 0.27	1.82 ± 0.31	1.85 ± 0.14	1.81 ± 0.33	1.29 ± 0.18
	RR_{50}	–	1.21	1.64	1.29	1.57	0.80
	RR_{95}	–	1.84	1.76	1.38	1.57	0.79
Dimethoate	LD_{50}	0.49 a	1.66 a	1.49 a	1.46 a	0.74 a	0.25 a
	(95% CL)	(0.12–2.18)	(0.09–2.87)	(0.27–2.19)	(0.14–2.14)	(0.46–1.18)	(0.05–0.66)
	LD_{95}	7.67 a	3.87 a	4.51 a	4.27 a	9.66 a	7.32 a
	(95% CL)	(1.79–2.6 × 10^3)	(2.43–4 × 10^4)	(2.82–227.7)	(2.70–8 × 10^2)	(4.54–40.43)	(2.26–127.7)
	χ^2	28.04	38.98	19.27	20. 06	11.68	14.74
	Slope ± SE	1.41 ± 0.30	4.46 ± 1.31	3.42 ± 0.89	3.54 ± 0.97	1.47 ± 0.18	1.12 ± 0.18
	RR_{50}	–	3.39	3.04	2.98	1.51	0.51
	RR_{95}	–	0.50	0.59	0.56	1.26	0.95
Fenpropathrin	LD_{50}	0.30 b	0.64 ab	0.32 b	1.32 ab	1.44 a	0.37 b
	(95% CL)	(0.16–0.55)	(0.36–0.85)	(0.26–0.40)	(0.08–1.92)	(0.74–2.59)	(0.18–0.66)
	LD_{95}	2.71 a	1.74 a	4.46 a	4.33 a	7.45 a	4.56 a
	(95% CL)	(1.21–17.97)	(1.24–4.48)	(3.00–7.60)	(2.80–933.32)	(3.63–1 × 10^2)	(2.02–26.63)
	χ^2	11.08	12.17	5.27	14.02	37.84	19.16
	Slope ± SE	1.73 ± 0.25	3.81 ± 0.67	1.45 ± 0.12	3.29 ± 0.90	2.31 ± 0.52	1.51 ± 0.23
	RR_{50}	–	2.13	1.10	4.40	4.80	1.23
	RR_{95}	–	0.64	1.65	1.60	2.75	1.68
Imidacloprid	LD_{50}	0.004 b	0.06 ab	0.03 ab	0.04 ab	0.04 ab	0.14 a
	(95% CL)	(5 × 10^{-4}–0.05)	(4 × 10^{-5}–0.20)	(5 × 10^{-3}–0.07)	(6 × 10^{-3}–0.08)	(7 × 10^{-3}–0.15)	(0.07–0.24)
	LD_{95}	2.00 a	0.30 a	1.18 a	0.48 a	1.09 a	0.82 a
	(95% CL)	(0.11–2.9 × 10^5)	(0.13–526.0)	(0.31–75.57)	(0.18–22.63)	(0.26–4 × 10^2)	(0.43–4.72)
	χ^2	18.05	44.80	13.01	13.26	23.38	13.08
	Slope ± SE	0.62 ± 0.12	2.40 ± 0.78	1.01 ± 0.18	1.50 ± 0.31	1.17 ± 0.24	2.18 ± 0.37
	RR_{50}	–	15.00	7.50	10.00	10.00	35.00
	RR_{95}	–	0.15	0.59	0.24	0.55	0.41
Malathion	LD_{50}	1.04 c	3.85 a	5.20 a	5.60 a	3.15 abc	1.23 b
	(95% CL)	(0.22–2.06)	(3.50–4.17)	(3.85–6.09)	(3.83–6.86)	(1.65–5.03)	(0.52–2.05)
	LD_{95}	9.97 a	11.41 a	8.97 a	10.34 a	11.16 a	10.60 a
	(95% CL)	(4.52–94.19)	(9.99–13.55)	(7.36–16.60)	(8.04–28.16)	(6.38–1 × 10^2)	(5.33–61.99)
	χ^2	16.17	3.42	11.57	15.91	38.58	23.18
	Slope ± SE	1.69 ± 0.31	3.48 ± 0.27	6.96 ± 1.28	6.19 ± 1.28	3.00 ± 0.71	1.76 ± 0.32
	RR_{50}	–	3.70	5.00	5.38	3.03	1.18
	RR_{95}	–	1.14	0.90	1.04	1.12	1.06
Spinetoram	LD_{50}	0.16 a	–	–	–	0.32 a	0.21 a
	(95% CL)	(0.08–0.30)				(0.13–0.77)	(0.13–0.31)
	LD_{95}	2.00 a	–	–	–	2.43 a	1.28 a
	(95% CL)	(0.82–15.15)				(0.94–66.35)	(0.72–4.25)
	χ^2	11.88	–	–	–	21.61	6.75
	Slope ± SE	1.49 ± 0.21				1.88 ± 0.36	2.09 ± 0.27
	RR_{50}	–	–	–	–	2.00	1.31
	RR_{95}	–	–	–	–	1.22	0.64
Thiamethoxam	LD50	0.01 c	0.15 ab	0.01 c	0.02 c	0.04 b	0.13 a
	(95% CL)	(3 × 10^{-3}–0.04)	(0.09–0.21)	(1 × 10^{-3}–0.08)	(4 × 10^{-3}–0.044)	(0.02–0.09)	(0.12–0.14)
	LD95	0.61 a	0.72 a	1.68 a	0.77 a	0.58 a	0.33 a
	(95% CL)	(0.15–14.96)	(0.44–2.01)	(0.20–4 × 10^3)	(0.20–17.02)	(0.20–6.82)	(0.28–0.41)
	χ^2	11.50	8.14	19.57	10.28	12.04	4.17
	Slope ± SE	0.96 ± 0.14	2.39 ± 0.31	0.78 ± 0.15	0.97 ± 0.14	1.38 ± 0.19	4.00 ± 0.37
	RR_{50}		15.00	1.00	2.00	4.00	13.00
	RR_{95}		1.18	2.75	1.26	0.95	0.54

[a] LD_{50} and LD_{95} values followed by different letters within each row were significantly different from one another, based on non-overlap of 95% confidence intervals. LD_{50} and LD_{95} values were calculated using 300–360 adults for each insecticide and population.

Insecticides	ng AI in 1 μL of acetone		
	LD_{50}	LD_{75}	LD_{95}
Abamectin	3.50	7.16572	20.05
Acetamiprid	16.80	36.18891	46.80
Aldicarb	7.85	12.43936	24.15
Bifenthrin	0.15	0.33719	1.30
Carbaryl	19.60	68.47558	407.70
Chlorpyriphos	1.25	4.17052	24.05
Cypermethrin	0.70	1.79112	5.20
Dimethoate	2.45	7.42639	38.35
Fenpropathrin	1.50	3.7165	13.55
Imidacloprid	0.02	0.26604	10.00
Malathion	5.20	22.75688	49.85
Spinetoram	0.80	2.23301	10.00
Thiamethoxam	0.05	0.30234	3.05

owing to overlapping 95% confidence intervals of such LD_{50} values, susceptibility levels between the LS population and respective field populations were not statistically different. Furthermore, the differing susceptibility levels between geographically discrete populations could be a result of inherent genetic differences between ACP populations, differential insecticide exposures, host plant differences or variation in other environmental conditions. In 2010, ACP populations were significantly lower at some of the collection sites than in 2009, which may be attributed to intensified insecticide use by growers on an area-wide scale, as well as an unusually cold winter in 2009. This prevented the collection of a sufficient number of ACP adults to conduct all the tests on the La Belle population in 2010.

Surprisingly, there were indications of reduced susceptibility to spinetoram in some field-collected populations of ACP, even though its use in Florida began in 2008, which serves as an early warning for judicious use of this insecticide. Spinetoram is considered as a possible replacement to organophosphate insecticides. Significantly lower mortality of ACP adults after treatment with spinetoram was observed in three field populations at two of the diagnostic doses tested, as compared with the lab susceptible population. Low levels of resistance to spinosad (8–10-fold), toxicologically identical to spinetoram, has been reported in Colorado potato beetle, *Leptinotarsa decemlineata*.[25] In another study conducted on obliquebanded leafroller, *Choristoneura rosaceana*, resistance to spinetoram was found to be correlated with spinosad resistance, suggesting possible cross-resistance.[26] In general, comparisons made at the LD_{75} and LD_{95} were greater indicators of resistance of ACP field populations against various insecticides than at the LD_{50} diagnostic dose.

In Florida, the Asian citrus psyllid was first reported in June 1998 from three southeastern counties, Palm Beach, Broward and Martin.[1] It is possible that aggressive area-wide management of ACP in southern Florida has resulted in greater selection pressure for resistance among southern populations. In 2009 there were six instances when field populations displayed significantly higher LD_{50} values than the LS population, and four of them were from southern Florida (Vero Beach, Ft Pierce and La Belle). Likewise, in 2010, reduced susceptibility of field populations was more prevalent in southern populations (25 out of 43 instances) from Vero Beach, Ft Pierce and La Belle than in northern populations.

General esterase, glutathione *S*-transferase and monooxygenase levels were lower in adults and nymphs from the LS population than in those from field-collected populations, suggesting that insecticide resistance is positively correlated with levels of detoxifying enzymes. Insecticide resistance levels have been positively correlated with levels of detoxifying enzymes in several insect pests. In such cases, detoxifying enzymes have been explained as a mechanism of resistance.[20,27,28] Although resistant populations of field-collected adults and nymphs displayed significantly higher levels of detoxifying enzymes, detoxifying enzymes may not be the only mechanism of resistance in this case. Other mechanisms of resistance, such as reduced penetration, target-site

Insecticides	LS	Groveland	Lake Alfred	Ft Pierce	Vero Beach	La Belle
Abamectin	90.00 (±10.00) a	83.33 (±8.82) a	80.00 (±0.00) a	66.67 (±3.33) a	85.00 (±2.89) a	–
Acetamiprid	90.00 (±2.89) a	73.33 (±8.82) a	75.00 (±10.41) a	63.33 (±14.53) a	81.67 (±4.41) a	85.00 (±2.89) a
Aldicarb	91.67 (±4.41) a	78.33 (±4.41) a	70.00 (±0.00) a	65.00 (±7.64) a	70.00 (±11.55) a	–
Bifenthrin	98.33 (±1.67) a	76.67 (±7.26) bc	90.00 (±0.00) ab	75.00 (±8.66) c	85.00 (±2.89) abc	81.67 (±1.67) bc
Carbaryl	96.67 (±3.33) a	85.00 (±2.87) a	90.00 (±5.77) a	61.67 (±7.26) b	86.67 (±4.41) a	93.33 (±1.67) a
Chlorpyriphos	95.00 (±1.67) a	91.67 (±4.41) a	80.00 (±5.77) ab	71.67 (±15.90)	66.67 (±12.02) b	95.00 (±1.67) a
Cypermethrin	95.00 (±1.67 (a	83.33 (±4.41) a	83.33 (±6.67) a	71.67 (±9.28) a	76.67 (±3.33) a	93.33 (±4.41) a
Dimethoate	91.67 (±1.67) a	83.33 (±1.67) a	83.33 (±3.33) a	63.33 (±13.64) a	83.33 (±1.67) a	–
Fenpropathrin	95.00 (±5.00) a	86.67 (±4.41) ab	73.33 (±3.33) b	46.67 (±7.26) c	71.67 (±1.67) b	91.67 (±6.01) a
Imidacloprid	98.33 (±1.67) a	78.33 (±1.67) bc	86.67 (±3.33) ab	63.33 (±14.24) cd	55.00 (±2.89) d	78.33 (±3.33) bc
Malathion	93.33 (±1.67) a	90.00 (±2.87) a	81.67 (±1.67) a	88.33 (±4.41) a	85.00 (±2.87) a	–
Spinetoram	91.67 (±1.67) a	76.67 (±3.33) b	80.00 (±1.67) ab	46.67 (±7.26) c	80.00 (±2.87) ab	80.00 (±2.87) ab
Thiamethoxam	98.33 (±1.67) a	93.33 (±3.33) ab	83.33 (±3.33) b	35.00 (±5.00) c	83.33 (±3.33) b	93.33 (±4.41) ab

[a] Mean percentage mortality followed by different letters within each insecticide (row) were significantly different ($P < 0.05$). Mean percentage mortality was calculated using 60–90 adults for each insecticide and population.
[b] LD_{95} as reported in Table 2 was selected as the diagnostic dose for each insecticide.
[c] Statistical details are given in the supporting information (Table S3).

Insecticide		LC_{50} (ng AI μL^{-1})				
		LS	Lake Alfred	Winter Garden	Lake Alfred 2	Groveland
Carbaryl	LC_{50}	17.59 b	41.02 ab	50.71 a	20.84 b	45.17 ab
	(95% CL)	(3.78–29.04)	(25.65–76.11)	(32.72–67.20)	(15.25–26.29)	(23.75–63.56)
	LC_{95}	64.38 a	173.38 a	244.17 a	308.77 a	226.98 a
	(95% CL)	(36.55–1.6 × 10^3)	(86.42–1.2 × 10^4)	(144.37–1.1 × 10^3)	(192.92–661.37)	(128.38–1.7 × 10^3)
	χ^2	14.37	10.14	6.78	3.48	9.38
	Slope ± SE	2.92 ± 0.70	2.63 ± 0.61	2.41 ± 0.40	1.40 ± 0.18	2.35 ± 0.44
	RR_{50}	–	2.33	2.88	1.19	2.57
	RR_{95}	–	2.69	3.80	4.80	3.53
Chlorpyriphos	LC_{50}	2.58 b	3.16 ab	5.32 a	8.30 a	2.97 b
	(95% CL)	(0.34–5.62)	(0.80–10.56)	(2.45–7.09)	(6.00–11.29)	(2.31–3.67)
	LC_{95}	14.95 a	48.09 a	10.78 a	16.46 a	65.43 a
	(95% CL)	(6.34–5.2 × 10^6)	(12.55–3.13 × 10^{11})	(7.78–110.64)	(11.81–114.25)	(36.09–172.05)
	χ^2	12.79	10.48	21.73	11.62	5.17
	Slope ± SE	2.15 ± 0.60	1.39 ± 0.39	5.36 ± 1.34	5.54 ± 1.28	1.22 ± 0.16
	RR_{50}	–	1.23	2.07	3.22	1.15
	RR_{95}	–	3.22	0.73	1.10	4.38
Fenpropathrin	LC_{50}	0.13 a	0.32 a	0.50 a	0.16 a	0.57 a
	(95% CL)	(0.004–0.47)	(0.21–0.52)	(0.21–0.87)	(1.5 × 10^{-3}–0.73)	(0.11–3.00)
	LC_{95}	38.22 a	92.71 a	5.84 a	3.09 a	20.63 a
	(95% CL)	(4.04–7.3 × 10^7)	(22.25–1.2 × 10^3)	(2.34–153.64)	(0.68–1.6 × 10^{11})	(3.50–2 × 10^8)
	χ^2	8.91	2.74	8.32	15.34	14.75
	Slope ± SE	0.67 ± 0.16	0.67 ± 0.10	1.54 ± 0.29	1.27 ± 0.36	1.05 ± 0.27
	RR_{50}	–	2.38	3.73	1.16	4.23
	RR_{95}	–	2.43	0.15	0.08	0.54
Imidacloprid	LC_{50}	0.22 b	0.49 ab	0.50 a	0.84 a	0.47 ab
	(95% CL)	(0.19–0.24)	(0.03–1.32)	(0.44–0.57)	(0.52–6.22)	(0.17–0.71)
	LC_{95}	0.58 b	1.80 ab	2.88 a	17.56 a	1.39 a
	(95% CL)	(0.49–0.74)	(0.88–8 × 10^8)	(2.12–4.46)	(3.46–3.4 × 10^4)	(0.81–47.63)
	χ^2	1.56	28.79	1.48	1.22	23.81
	Slope ± SE	3.84 ± 0.41	2.89 ± 0.86	2.17 ± 0.21	1.25 ± 0.41	3.30 ± 0.80
	RR_{50}	–	2.25	2.32	3.90	2.04
	RR_{95}	–	3.11	4.98	30.35	2.40
Spinetoram	LC_{50}	0.66 b	1.27 ab	3.88 a	3.15 ab	3.15 ab
	(95% CL)	(0.48–0.85)	(0.40–4.00)	(1.84–6.14)	(0.29–15.71)	(0.29–15.71)
	LC_{95}	6.04 b	69.46 a	26.75 a	234.71 a	234.71 a
	(95% CL)	(3.99–11.04)	(13.06–3.3 × 10^4)	(14.18–141.01)	(31.28–1.3 × 10^9)	(31.27–1 × 10^9)
	$\chi\chi$	0.94	8.55	8.78	16.86	16.86
	Slope ± SE	1.70 ± 0.19	0.95 ± 0.18	1.96 ± 0.31	0.88 ± 0.22	0.88 ± 0.22
	RR_{50}	–	1.94	5.92	4.81	4.81
	RR_{95}	–	11.49	4.43	38.84	38.84

[a] LC_{50} and LC_{95} values followed by different letters within each row were significantly different from one another based on non-overlap of 95% confidence intervals. LC_{50} and LC_{95} values were calculated using 600–900 fourth instars for each insecticide and population.

insensitivity and mutations within detoxifying enzymes, may also be involved in resistance development among field populations of ACP.[29]

Baseline susceptibility data on fourth-instar ACP from the present study provide a reference point for future evaluations of resistance in immature ACP. Considering the differences in morphology and feeding behavior between adult and immature stages, it is possible that stage-specific differences in insecticide susceptibilities occur. Insecticide susceptibility is known to vary among various developmental stages of the same insect species.[13,30–33] Immature stages often exhibit greater resistance than adult counterparts.[30,33] For example, fourth-instar greenhouse whitefly, *Trialeurodes vaporariorum*, are more resistant to abamectin, buprofezin, imidacloprid, acetamiprid, fenpropathrin and profenofos than corresponding adults.[30] Similarly, late-instar German cockroach, *Blattella germanica* (L.), nymphs are more resistant to bendiocarb, chlorpyriphos and cypermethrin than male adults.[33] The above results are congruent with the present investigation and a previous study[10] which indicate that fourth-instar ACP exhibit higher insecticide resistance than adults to carbaryl, chlorpyriphos, fenpropathrin, imidacloprid and spinetoram. LC_{50} values for all of these insecticides observed for adults in the previ-

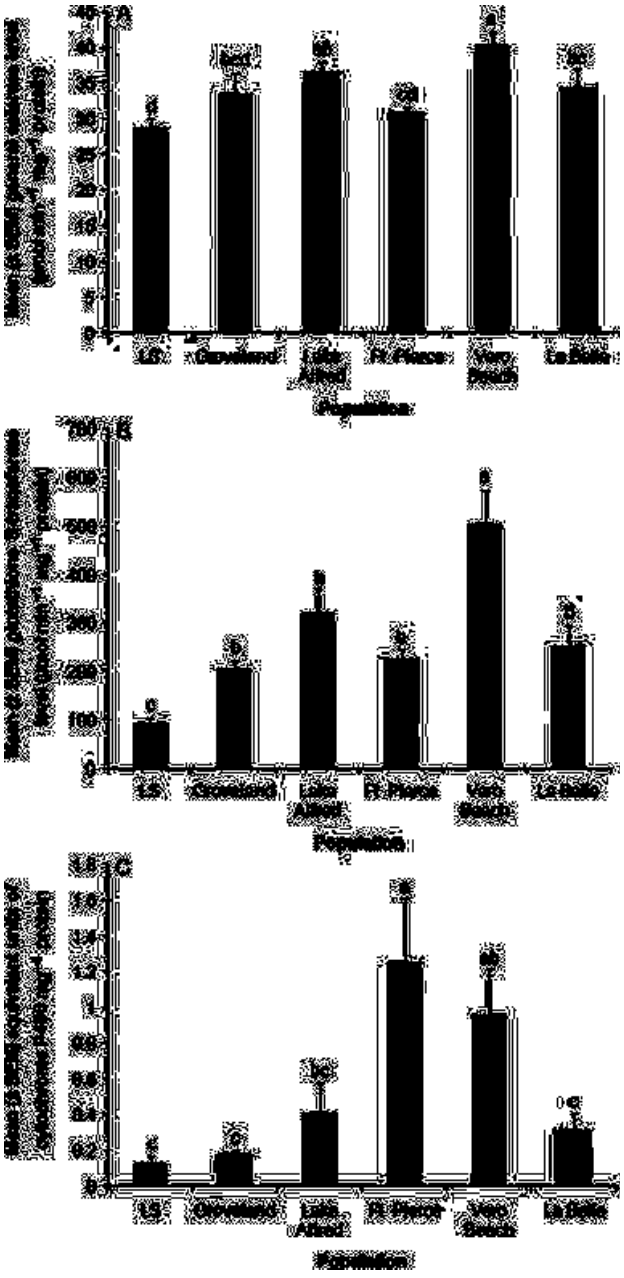

Figure 2. Comparison of (A) general esterase, (B) glutathione *S*-transferase (CDNB as the substrate) and (C) monooxygenase levels between laboratory susceptible (LS) and five field populations of *Diaphorina citri* adults.

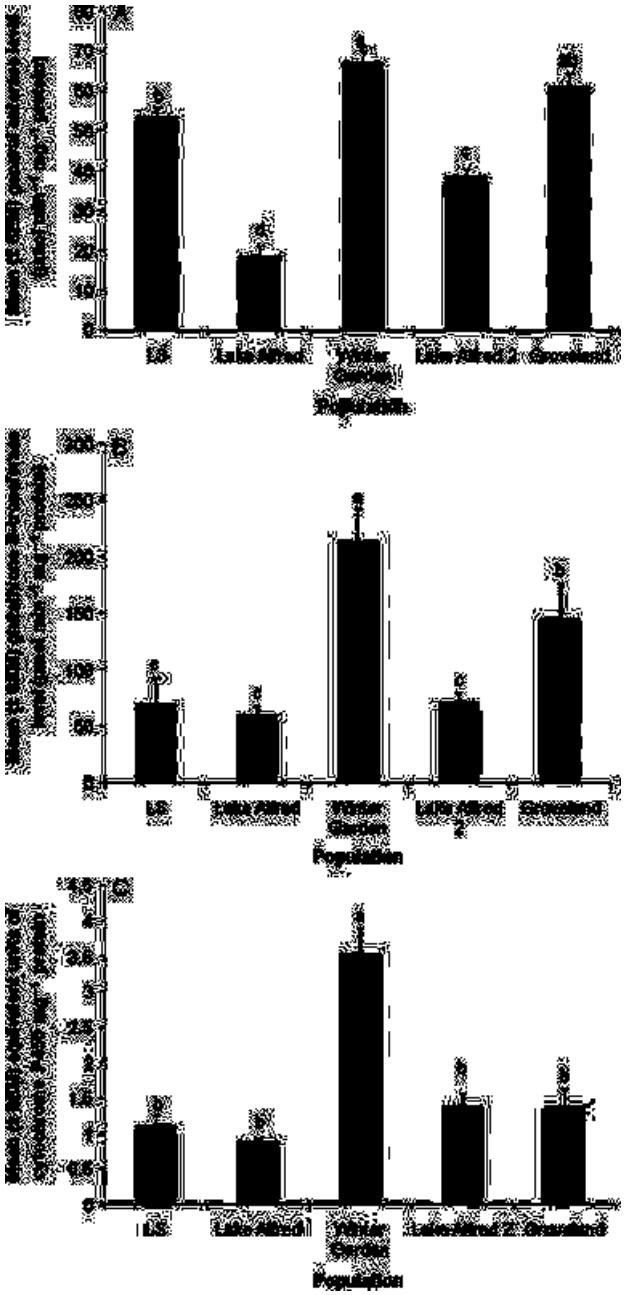

Figure 3. Comparison of (A) general esterase, (B) glutathione *S*-transferase (CDNB as the substrate) and (C) monooxygenase levels between laboratory susceptible (LS) and four field populations of fourth-instar *Diaphorina citri*. Each population mean presented is derived from pooled data obtained during two collection times from each site.

ous study[10] were lower than those obtained for fourth-instar ACP in the present study. Bioassays on fourth-instar ACP in the present study and on adults in the previous study were performed on the LS population, with no shift in insecticide susceptibility levels between the two study periods. Greater resistance in ACP nymphs than in adults to certain insecticides may be due to reduced penetration associated with a thicker wax barrier as proposed for *T. vaporariorum*.[30] Furthermore, higher levels of detoxifying enzymes in nymphs than in adults may explain this difference among resistance levels. Levels of both general esterase and monooxygenase were higher in nymphs than in adults of the LS population. Levels of detoxifying enzymes vary among developmental stages; cytochrome P450 activity is greater in larval *Helicoverpa armigera* and prepupal and pupal *Manduca sexta* than in other stages.[34,35] Likewise, general esterase levels were found to vary among the developmental stages of *Lygus hesperus*. Different levels of esterases between adults and nymphs are thought to be due to differences in body weight and total protein content.[36]

Differing levels of detoxifying enzymes and resistance among the various populations of ACP nymphs sampled could be a result of differential selection pressure imposed by insecticide spray schedules among the various sites sampled. Nymphs from the Winter Garden site exhibited the highest general esterase, glutathione *S*-transferase and monooxygenase levels, which could

be correlated with the relatively high resistance levels to carbaryl, imidacloprid and spinetoram. In at least one population of ACP nymphs (Lake Alfred 2) (Table 4) there did not appear to be a correlation between enzyme levels and resistance levels to chlorpyriphos, imidacloprid and spinetoram. This suggests that resistance in this population could be a result of other mechanisms. However, in the majority of cases, elevated enzyme levels were correlated with greater resistance levels. Use of enzyme inhibitors, such as piperonyl butoxide, diethyl maleate and triphenyl phosphate, for cytochrome P450, gluthione *S*-transferase and carboxylesterase, respectively, may be useful in cases where increased enzymatic detoxification is contributing to resistance.[37,38]

Several currently labeled insecticides for ACP target both adults and nymphs. Therefore, understanding resistance levels among the various developmental stages is needed to determine the most appropriate field dose for both adults and nymphs. The present results show that levels of resistance among populations of nymphs can be equal to or greater than the levels in adults. Continued resistance monitoring of both immature and adult stages of ACP is needed to keep track of whether resistance levels will continue to increase, as intense use of insecticides for ACP management is likely to continue for several years. Rotation of existing registered modes of action as well as incorporation of new modes of action and/or pesticide alternatives will be crucial to maintain effectiveness of currently available insecticides against ACP.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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