Reversal of Insecticide Resistance in Florida Populations of Diaphorina citri (Hemiptera: Liviidae)

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Reversal of insecticide resistance in Florida populations of *Diaphorina citri* (Hemiptera: Liviidae)

Monique R. Coy[1,*], *Liu Bin*[2], *and Lukasz L. Stelinski*[1]

Abstract

We report the results from surveys conducted in 2013 and 2014 to monitor insecticide susceptibility in Florida field populations of the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae). These surveys are a component of the insecticide resistance management program for *D. citri,* which is the vector of '*Candidatus*' Liberibacter asiaticus. '*Candidatus*' Liberibacter asiaticus is the plant pathogen that causes citrus greening disease. The insecticides evaluated (carbaryl, chlorpyrifos, dimethoate, fenpropathrin, flupyradifurone, imidacloprid, and thiamethoxam) represent several modes of action and are among those that are currently used to manage *D. citri* in commercial groves in Florida. The 2013 and 2014 surveys revealed a decrease in the resistance ratios at the 50% response level (RR50) as compared with the 2009 survey, which was the last time this parameter was investigated. The results of the 2013 and 2014 surveys suggest a reversal to pre-2009 susceptibility levels in *D. citri* populations statewide for all modes of action tested. These results suggest that implementation of effective rotations and area-wide management of this pathogen vector may have contributed to insecticide stewardship.

Key Words: flupyradifurone; insecticide resistance management; monitoring program; neonicotinoid; psyllid

Resumen

Se presentan los resultados de los sondeos realizados en 2013 y 2014 para monitorear la susceptibilidad en poblaciones de campo de Florida del psílido asiático de los cítricos, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) a insecticidas. Estos sondeos fueron un componente del programa de manejo de resistencia a los insecticidas por *D. citri*, que es el vector de '*Candidatus*' Liberibacter asiaticus. '*Candidatus*' Liberibacter asiaticus es el patógeno de plantas que causa la enfermedad de enverdecimiento de los cítricos. Los insecticidas evaluados (carbaril, clorpirifós, dimetoato, fenpropatrina, flupyradifurone, imidacloprid y tiametoxam) representan varios modos de acción y se encuentran entre los que se utilizan actualmente para el manejo de *D. citri* en plantaciones comerciales en la Florida. Los sondeos de 2013 y 2014 revelaron una disminución en el ratio de resistencia en el nivel de respuesta del 50% (RR50) en comparación con el sondeo de 2009, que fue la última vez que este parámetro fue investigado. Los resultados de los sondeos de 2013 y 2014 sugieren una reversión al los niveles de susceptibilidad antes de 2009 en las poblaciones de *D. citri* en todo el estado para todos los modos de acción probados. Estos resultados sugieren que la implementación de rotaciones eficaces y el manejo de áreas amplias de este patógeno vector puede haber contribuido a la administración de insecticidas.

Palabras Clave: flupyradifurone; manejo de resistencia a los insecticidas; programas de monitoreo; neonicotinoides; psílido

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Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is an insect pest of citrus and the vector of the phloem-limited plant pathogen '*Candidatus*' Liberibacter asiaticus. '*Candidatus*' Liberibacter asiaticus is the presumptive causal agent of citrus greening disease or huanglongbing in Florida. Huanglongbing is a devastating disease of citrus, causing small and bitter fruit, aborted seeds, and rapid tree decline (Bovè 2006). Huanglongbing was first discovered in Miami-Dade County in 2005, and has since had a significant negative economic impact on the citrus industry in Florida (Hodges & Spreen 2012). Currently, there is no treatment for huanglongbing, and disease management relies heavily on the use of insecticides to reduce vector populations (Grafton-Cardwell et al. 2013). Repeated and intense use of insecticides can result in resistance, rendering them ineffective (Arthropod Pesticide Resistance Database 2015). The broad categories of resistance include (i) target site insensitivity, (ii) metabolic detoxification, (iii) reduced cuticular penetration, and (iv) increased sequestration or excretion. Modification of insect behavior in response to insecticide application, such as avoidance, has also been reported (Liu et al. 2006). The underlying mechanisms conferring resistance can be complex, often making the elucidation a time-consuming process. However, time is a critical factor in cases of resistance because action needs to be taken as quickly as possible. Therefore, regular surveillance of insecticide susceptibility in a treated insect population is a critical component of insecticide resistance management (McGaughey & Whalon 1992).

In 2008, a monitoring program was initiated to investigate insecticide susceptibility of *D. citri* to currently used insecticides (Boina et al. 2009). In 2009, the survey revealed decreased susceptibility in adult psyllids to several important insecticides, including up to a 35-fold decrease in susceptibility to imidacloprid, a critical insecticide for protecting young trees (Tiwari et al. 2011a). In 2010, the problem increased, with decreased susceptibility to the majority of insecticides used to manage *D. citri* throughout Florida (Tiwari et al. 2011a). Subsequent molecular investigations demonstrated the induction of a suite of *CYP4* genes in adult psyllids in response to contact exposure with imidacloprid (Tiwari et al. 2011b). *CYP* genes code for a major class of enzymes

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(cytochrome P450 monooxygenases) frequently associated with insecticide resistance in insects (Li et al. 2007). Subsequent molecular and biochemical studies using RNA interference further linked *CYP4* expression and imidacloprid by demonstrating that silencing of the *CYP4* genes in adult psyllids resulted in a decrease in P450 enzymatic activity with a concomitant increase in susceptibility to imidacloprid (Killiny et al. 2014).

Here we report the results from surveys conducted in 2013 and 2014 in which we observed an increase in insecticide susceptibility among populations of *D. citri* throughout Florida as compared with 2009. We also report the baseline LD50 estimates in these populations for a new butenolide insecticide, flupyradifurone, which targets the nicotinic acetylcholine receptor. Flupyradifurone targets the same receptor as neonicotinoids; however, the difference in structure between these insecticides may prevent cross resistance. Specifically, flupyradifurone is not metabolized by recombinant CYP6CM1, a P450 associated with resistance to imidacloprid in whiteflies (Karunker et al. 2008; Nauen et al. 2013, 2015). Further, no cross-resistance has been found to date between flupyradifurone and imidacloprid in several sap-sucking pests with high degrees of resistance to imidacloprid (Nauen et al. 2015). This insecticide was evaluated in the 2013 survey for both the laboratory strain and field populations of *D. citri*.

Materials and Methods

INSECTS FOR BIOASSAYS

The laboratory strain of *D. citri* was established from psyllids collected in Polk County in 2000 prior to the detection of huanglongbing within Florida. The laboratory strain is maintained on *Citrus sinensis* (L.) 'Valencia' (Sapindales: Rutaceae) without exposure to insecticides in a greenhouse with controlled conditions of 27 ± 1 °C, 60 ± 5% RH, and a 14:10 h L:D photoperiod. The laboratory strain was used as the susceptible strain for comparative analysis, and these insects were collected by mouth aspiration from the greenhouse. Adult psyllids were collected in the field using a D-Vac vacuum (Rincon-Vitova Insectaries, Ventura, California) with permission from grove managers. Based on sensitivity ratios, preliminary studies showed no difference in insecticide susceptibility between mouth-aspirated and vacuum-collected psyllids for fenpropathrin and imidacloprid (see below for description of data analysis). The sensitivity ratio was 1.15 (95% CI: 0.93–1.33) for fenpropathrin and 0.93 (95% CI: 0.80–1.08) for imidacloprid. Psyllids were transported to the laboratory in coolers and released into 40 × 40 × 40 cm Plexiglas cages. Psyllids were provided with five 35 to 40 cm tall 'Kuharski Carrizo' (*C. sinenesis* (L.) 'Osbeck' ´ *Poncirus trifoliata* (L.) 'Raf') saplings, with approximately 500 individuals per enclosure. Psyllids were kept in controlled conditions as described above and allowed to acclimate to laboratory conditions for 24 h prior to use in assays. All assays were completed within 3 d of collection. For testing the laboratory colony, psyllids were moved from the main laboratory colony into Plexiglas cages and maintained under the same conditions as the field psyllids prior to testing. The sites and dates for field collection are listed in Table 1.

INSECTICIDES AND TOPICAL ASSAYS

Insecticides tested were of analytical grade and included carbaryl, chlorpyrifos, dimethoate, fenpropathrin, flupyradifurone, imidacloprid, and thiamethoxam, representing several insecticide classes (Table 2). With the exception of flupyradifurone, all active ingredients are currently used in rotation schedules to manage psyllid populations in Florida. Insecticides were obtained from Sigma-Aldrich (St. Louis, Missouri). Flupyradifurone was provided by Bayer CropScience (Research Triangle Park, North Carolina). New lots of insecticides were obtained for both years. The number of doses tested ranged from 7 to 10, with 3 to 5 replicates of 10 insects per dose, based on the number of insects collected per site and on preliminary tests to determine dose placement. The range of mortality was 5–10% to 90–95% for the dose range selected for each insecticide. Psyllids were anesthetized with a short puff of CO_2 and topically treated by applying insecticide dissolved in analytical grade acetone. Applications were made in a 0.2 µL volume onto the lateral thorax using a 26 gauge needle with an AS tip (7786-02) affixed to a 701RN 10 µL Hamilton syringe (80330; Hamilton Co., Reno, Nevada) mounted onto a Hamilton PB-600 repeat dispenser. After treatment, psyllids were kept in 35 mm Petri dishes on Valencia citrus leaves cut to size, laid abaxial side up, over a 1.5% solidified agar bed. Dishes were wrapped in Parafilm M, inverted, and housed in controlled conditions of 27 ± 1 °C, 60 ± 5% RH, and a 14:10 h L:D photoperiod. Insects were scored as dead or alive, with dead considered to be total lack of movement with prodding, 24 h post-treatment.

PARAMETERS FOR INSECTICIAL SUSCEPTIBILITY

LD50 estimates and their 95% confidence intervals were determined with PoloPlus (LeOra Software 2006; Robertson et al. 2007) using the probit model (Finney 1971). Control mortality was corrected for by the PoloPlus program. Chi-square tests (χ^2) were used to estimate how well the data fit the probit model. Resistance ratios (RR50 estimates) were calculated in comparison with the laboratory strain and the most susceptible field population for each insecticide. Confidence intervals for RR50 estimates were calculated using regression lines as described in Robertson & Preisler (1992). LD50 estimates were considered significantly different if the null value of '1' did not fall within the confidence interval for the ratio (Robertson et al. 2007). The overlap test, where significance is determined based on the overlap of the LD50 95% confidence intervals, was not used because it lacks statistical power (Wheeler et al. 2006).

Results

The 2013 and 2014 LD50 estimates and resistance ratios, along with respective 95% confidence intervals and slopes, are presented in Tables 3 and 4, respectively. Results from the χ^2 tests (χ^2 and *P* values) used to determine how well the data fit the assumption of the probit model are also presented. For the majority of the assays, the predicted values of the probit model did not differ significantly from the observed data obtained from the topical assays ($P > 0.05$), demon-

Table 1. Sites and dates of *Diaphorina citri* field collections.

Site Location	County	2013 collection date	GIS coordinates	2014 collection date	GIS coordinates
LaBelle	Hendry	16 Sep	26.6897222°N, 81.4600000°W	14 Jul	26.6936111°N, 81.4391667°W
Lake Alfred	Polk	10 Sep	28.1216667°N, 81.7533333°W	11 Aug	28.1233333°N, 81.7525000°W
Port St. Lucie	St. Lucie	19 Aug	27.3686111°N, 80.5505556°W	8 Jun	27.3686111°N, 80.5505556°W
Winter Garden	Orange	18 Jul	28.4658333°N, 81.6591667°W	5 May	28.4691667°N, 81.6530556°W

Table 2. Classification and biochemical targets of the insecticides evaluated.

Insecticide	IRAC Group[a]	Class	Biochemical target	Action
Carbaryl	1A	carbamate	acetylcholine esterase	inhibitor
Chlorpyriphos	1B	organophosphate	acetylcholine esterase	inhibitor
Dimethoate[b]	1B	organophosphate	acetylcholine esterase	inhibitor
Fenpropathrin	3A	pyrethroid	voltage-gated sodium ion channel	modulator
Flupyradifurone[c]	4D	butenolide	nicotinic acetylcholine receptor	agonist
Imidacloprid	4A	neonicotinoid	nicotinic acetylcholine receptor	agonist
Thiamethoxam	4A	neonicotinoid	nicotinic acetylcholine receptor	agonist

[a] Insecticide Resistance Action Committee mode-of-action group number.
[b] Evaluated in 2014.
[c] Evaluated in 2013.

strating that the probit model was suitable for the calculation of LD50 estimates. The data that did not fit the probit model include the 2013 data for carbaryl at Winter Garden, the 2013 data for flupyradifurone at LaBelle, and the 2013 and 2014 data for carbaryl at LaBelle. This is in contrast to the data obtained in the survey for 2009, in which the data from 68 out of the 75 assays conducted did not fit the probit model (Tiwari et al. 2011a). The improvement in the fit of the data in the 2013 and 2014 surveys is likely because more insects were used per

Table 3. LD50 estimates and resistance ratios for select insecticides against *Diaphorina citri* adults in 2013.[c]

Insecticide (IRAC)[a]	*n*[b]	χ^2 (df)	Slope ± SE	LD50 ng/µL (95% CI)	RR50 (95% CI) laboratory strain[c]	RR50 (95% CI) most susceptible field population[c]
Carbaryl (1A)						
Laboratory strain	358	6.31 (4)	2.58 ± 0.43	23.03 (6.89–34.33)	1	0.71 (0.49–1.05)
LaBelle	505	20.58 (7)	1.68 ± 0.26	33.59 (14.00–59.85)	1.46 (0.95–2.24)	1.04 (0.74–1.47)
Lake Alfred	508	11.61 (7)	2.38 ± 0.30	32.25 (22.31–43.27)	1.40 (0.96–2.05)	1
Port St. Lucie	486	4.32 (7)	1.69 ± 0.19	37.55 (29.82–47.75)	1.63 (1.09–2.43)*	1.16 (0.86–1.58)
Winter Garden	487	18.37 (7)	2.16 ± 0.27	35.49 (19.94–57.79)	1.54 (1.03–2.31)*	1.10 (0.80–1.51)
Chlorpyrifos (1B)						
Laboratory strain	427	2.77 (5)	6.22 ± 0.56	9.56 (8.93–10.22)	1	1.02 (0.89–1.17)
LaBelle	399	1.32 (5)	5.67 ± 0.74	9.38 (8.15–10.43)	0.98 (0.85–1.13)	1
Lake Alfred	422	6.31 (5)	5.40 ± 0.72	12.56 (9.98–14.56)	1.31 (1.15–1.51)*	1.34 (1.13–1.59)*
Port St. Lucie	400	8.55 (5)	3.03 ± 0.39	12.70 (9.14–16.34)	1.33 (1.12–1.57)*	1.36 (1.11–1.65)*
Winter Garden	207	3.70 (4)	2.18 ± 0.64	13.68 (8.75–19.29)	1.43 (1.02–2.00)*	1.46 (1.03–2.07)*
Fenpropathrin (3A)						
Laboratory strain	267	2.86 (6)	2.31 ± 0.25	16.35 (13.00–20.06)	1	1.46 (1.05–2.04)*
LaBelle	509	8.84 (6)	3.66 ± 0.50	16.61 (12.12–20.66)	1.02 (0.77–1.34)	1.49 (1.11–1.98)*
Lake Alfred	471	6.87 (6)	2.06 ± 0.31	39.18 (30.29–59.38)	2.40 (1.73–3.12)*	3.51 (2.51–4.91)*
Port St. Lucie	203	1.68 (4)	3.24 ± 0.55	11.17 (8.40–14.00)	0.68 (0.49–0.96)*	1
Winter Garden	241	3.86 (5)	3.09 ± 0.37	21.81 (17.93–26.59)	1.33 (0.99–1.80)	1.95 (1.43–2.67)*
Flupyradifurone (4D)						
Laboratory strain	462	8.50 (6)	1.96 ± 0.19	14.30 (9.96–19.45)	1	1.81 (1.35–2.43)*
LaBelle	449	14.61 (6)	2.32 ± 0.23	8.33 (5.07–12.16)	0.58 (0.43–0.79)*	1.05 (0.79–1.40)
Lake Alfred	455	6.89 (6)	1.83 ± 0.16	7.93 (5.99–10.22)	0.55 (0.41–0.74)*	1
Port St. Lucie	441	9.12 (6)	1.60 ± 0.16	11.34 (7.07–16.60)	0.79 (0.56–1.12)	1.43 (1.03–2.00)*
Winter Garden	454	11.52 (6)	1.87 ± 0.25	18.70 (9.34–28.78)	1.31 (0.90–1.88)	2.36 (1.66–3.35)*
Imidacloprid (4A)						
Laboratory strain	349	5.99 (4)	8.72 ± 1.26	1.30 (1.04–1.49)	1	1.97 (1.53–2.54)*
LaBelle	503	6.68 (7)	2.97 ± 0.34	0.66 (0.50–0.81)	0.51 (0.39–0.66)*	1
Lake Alfred	495	4.59 (7)	3.61 ± 0.37	0.89 (0.73–1.03)	0.68 (0.56–0.82)*	1.34 (1.00–1.79)
Port St. Lucie	290	1.54 (7)	1.80 ± 0.38	2.35 (1.85–2.85)	1.80 (1.45–2.25)*	1.24 (0.99–1.56)
Winter Garden	299	9.09 (7)	4.62 ± 0.51	1.90 (1.60–2.16)	1.46 (1.27–1.67)*	2.87 (2.21–3.72)*
Thiamethoxam (4A)						
Laboratory strain	509	4.43 (7)	3.55 ± 0.36	1.31 (1.10–1.49)	1	3.40 (2.73–4.24)*
LaBelle	506	10.33 (7)	4.07 ± 0.58	0.39 (0.29–0.48)	0.29 (0.24–0.37)*	1
Lake Alfred	539	13.50 (8)	2.90 ± 0.61	0.61 (0.15–0.92)	0.47 (0.32–0.67)*	1.59 (1.09–2.30)*
Port St. Lucie	267	7.67 (6)	2.79 ± 0.44	0.59 (0.28–0.83)	0.45 (0.31–0.64)*	1.52 (1.05–2.19)*
Winter Garden	298	10.44 (7)	4.73 ± 0.53	2.11 (1.78–2.40)	1.61 (1.34–1.93)*	5.47 (4.52–6.62)*

[a] Insecticide Resistance Action Committee (IRAC) mode-of-action group number.
[b] Number of insects tested per insecticide per site.
[c] Asterisks indicate that LD50 estimates are significantly different from those of the susceptible population ($P \leq 0.05$).

Table 4. LD50 estimates and resistance ratios for select insecticides against *Diaphorina citri* adults in 2014.

Insecticide (IRAC)[a]	*n*[b]	χ^2 (df)	Slope ± SE	LD50 ng/µL (95% CI)	RR50 (95% CI) laboratory strain[c]	RR50 (95% CI) most susceptible field population[c]
Carbaryl (1A)						
Laboratory strain	523	2.20 (7)	1.68 ± 0.24	79.73 (62.13–101.74)	1	1.54 (1.10–2.17)*
LaBelle	493	15.53 (7)	2.15 ± 0.22	120.95 (88.21–195.62)	1.52 (1.10–2.09)*	2.34 (1.71–3.21)*
Lake Alfred	432	13.35 (7)	2.22 ± 0.20	67.68 (51.51–94.08)	0.85 (0.63–1.14)	1.31 (0.98–1.76)
Port St. Lucie	495	7.10 (7)	2.48 ± 0.41	51.65 (34.68–65.52)	0.65 (0.46–0.91)*	1
Winter Garden	515	2.09 (7)	2.61 ± 0.33	78.37 (66.02–91.95)	0.98 (0.73–1.32)*	1.52 (1.14–2.03)*
Chlorpyrifos (1B)						
Laboratory strain	506	1.75 (7)	2.05 ± 0.22	24.21 (20.58–29.82)	1	2.50 (1.47–4.25)*
LaBelle	493	4.89 (7)	3.51 ± 0.28	14.66 (13.34–16.15)	0.61 (0.49–0.75)*	1.51 (0.91–2.51)
Lake Alfred	252	0.75 (3)	3.36 ± 0.93	9.68 (3.46–13.44)	0.40 (0.24–0.68)*	1
Port St. Lucie	501	6.34 (7)	2.27 ± 0.25	18.88 (16.28–22.24)	0.78 (0.61–0.99)*	1.95 (1.16–3.28)*
Winter Garden	476	5.53 (7)	3.20 ± 0.26	10.84 (9.75–12.01)	0.45 (0.36–0.55)*	1.12 (0.67–1.86)
Dimethoate (1B)						
Laboratory strain	512	2.64 (7)	3.83 ± 0.31	9.48 (8.53–10.45)	1	1.34 (1.14–1.57)*
LaBelle	499	19.03 (7)	3.73 ± 0.29	11.25 (9.27–13.48)	1.19 (1.03–1.36)*	1.59 (1.36–1.85)*
Lake Alfred	478	10.28 (7)	4.21 ± 0.37	7.85 (6.73–8.98)	0.83 (0.72–0.95)*	1.11 (0.95–1.30)
Port St. Lucie	504	4.46 (7)	3.21 ± 0.29	7.08 (6.21–7.94)	0.75 (0.64–0.88)*	1
Winter Garden	523	7.83 (7)	2.05 ± 0.23	20.32 (16.64–25.73)	2.14 (1.76–2.61)*	2.59 (2.13–3.14)*
Fenpropathrin (3A)						
Laboratory strain	492	0.84 (7)	2.14 ± 0.31	44.87 (37.20–57.02)	1	3.92 (3.01–5.12)*
LaBelle	503	5.38 (7)	3.47 ± 0.28	25.66 (23.34–28.37)	0.57 (0.46–0.72)*	2.24 (1.85–2.72)*
Lake Alfred	475	6.34 (7)	3.65 ± 0.44	26.62 (23.40–29.84)	0.59 (0.47–0.75)*	2.33 (1.90–2.86)*
Port St. Lucie	513	5.84 (7)	2.46 ± 0.26	11.44 (9.48–13.27)	0.26 (0.20–0.33)*	1
Winter Garden	464	3.84 (7)	3.22 ± 0.30	18.44 (16.01–20.93)	0.41 (0.32–0.53)*	1.61 (1.30–1.10)*
Imidacloprid (4A)						
Laboratory strain	478	2.48 (7)	3.04 ± 0.26	2.03 (1.83–2.27)	1	1.76 (1.51–2.04)*
LaBelle	501	4.10 (7)	2.55 ± 0.28	3.81 (3.28–4.67)	1.87 (1.53–2.30)*	3.29 (2.68–4.03)*
Lake Alfred	514	5.61 (7)	3.09 ± 0.26	1.16 (1.04–1.28)	0.57 (0.49–0.66)*	1
Port St. Lucie	498	1.46 (7)	1.64 ± 0.35	8.22 (5.60–18.50)	4.04 (2.38–6.86)*	7.10 (4.18–12.04)*
Winter Garden	467	2.61 (7)	2.08 ± 0.28	2.46 (2.03–3.02)	1.21 (0.97–1.51)	2.12 (1.70–2.65)*
Thiamethoxam (4A)						
Laboratory strain	513	4.29 (7)	1.28 ± 0.22	4.22 (3.12–6.89)	1	3.82 (2.44–5.99)*
LaBelle	511	9.44 (7)	1.85 ± 0.22	3.15 (2.50–4.47)	0.75 (0.49–1.14)	2.86 (2.70–3.94)*
Lake Alfred	476	4.23 (7)	1.67 ± 0.22	2.18 (1.79–2.71)	0.52 (0.34–0.79)*	1.97 (1.42–2.73)*
Port St. Lucie	510	1.21 (7)	1.21 ± 0.20	1.11 (0.82–1.39)	0.26 (0.17–0.41)*	1
Winter Garden	472	3.73 (7)	1.13 ± 0.21	2.48 (1.85–3.70)	0.59 (0.36–0.96)*	2.25 (1.49–3.40)*

[a] Insecticide Resistance Action Committee (IRAC) mode-of-action classification number.
[b] Number of insects tested per insecticide per site.
[c] Asterisks indicate that LD50 estimates are significantly different from those of the susceptible population ($P \leq 0.05$).

dose, more doses were used per insecticide, and dose placement was optimized to determine the lethal dose response at 50% (Robertson et al. 2007), as compared with the 2009 survey. This was possible because we were able to capture more insects by the vacuum collection method as compared with mouth aspiration. In addition, we investigated a smaller subset of insecticides in 2013 and 2014 as compared with 2009, focusing primarily on the broad-spectrum insecticides used for management of *D. citri*. This allowed for testing of more psyllids for each assay. The failure of data for carbaryl to fit the probit model well for *D. citri* collected from LaBelle during 2 consecutive years of the survey suggests that the dose placement for this insecticide requires further optimization. However, the data obtained can still provide a valuable estimate of susceptibility for this population.

For a number of insecticides investigated, the laboratory strain did not produce the lowest LD50 estimate. Given this result, RR50 estimates were also calculated with the field population having the lowest LD50 estimate. Overall, there was a decrease in RR50 estimates for all insecticides tested from all field populations surveyed during 2013 and 2014 as compared with the 2009 survey (Tiwari et al. 2011a).

2013 SURVEY

For the 2013 survey, 4 field populations (Port St. Lucie, LaBelle, Lake Alfred, Winter Garden; Table 2) and a laboratory strain of *D. citri* were tested for their susceptibility to 6 insecticides: carbaryl, chlorpyrifos, fenpropathrin, flupyradifurone, imidacloprid, and thiamethoxam. The insects collected from the grove in Winter Garden exhibited the highest LD50 estimates for chlorpyrifos, flupyradifurone, and thiamethoxam. In contrast, the psyllids collected from LaBelle exhibited the lowest LD50 estimates for chlorpyrifos, imidacloprid, and thiamethoxam (Table 3). Many field populations showed statistically greater LD50 estimates than the laboratory strain ($P \leq 0.05$; Table 3). However, most of the resulting RR50 estimates were under 3. The highest RR50 estimate was obtained for fenpropathrin against psyllids collected at Lake Alfred with a ratio of 2.40, which is a statistically significant difference (CI 95%: 1.73–3.12; Table 3). For most of the field populations tested, this represents a decrease in RR50 estimates as compared with the 2009 survey (Tiwari et al. 2011a). The 2009 RR50 estimate for chlorpyrifos in Lake Alfred, Port St. Lucie, and LaBelle were 11.80, 13.28, and 6.28, respectively (Tiwari et al. 2011a). Imidacloprid RR50 estimates were also high, at 7.50, 10.00, and 35.00 for Lake Alfred, Port St. Lucie, and LaBelle, respectively, in 2009 (Tiwari et al. 2011a). For the remaining two insecticides, fenpropathrin and thiamethoxam, the 2009 survey revealed two RR50 estimates above 3; these were 4.40 for fenpropathrin in Port St. Lucie, and 13.00 for thiamethoxam in LaBelle (Tiwari et al. 2011a). The results for the 2009 and 2013 survey for carbaryl were comparable, with all RR50 estimates below 2 (Tiwari et al. 2011a). In a few cases, the estimated LD50 for the laboratory strain was greater than that observed for field populations (Table 3). Given this result, the most susceptible field population for each insecticide was used to calculate RR50 estimates in addition to the laboratory strain. For these comparisons, 2 ratios were above 3 (Table 3). There was a ratio difference of 5.47 (95% CI: 4.52–6.62) for thiamethoxam between Winter Garden and LaBelle, with the latter population being the more susceptible. The other was for fenpropathrin with a RR50 estimate of 3.51 (95% CI: 2.51–4.91) between Lake Alfred and Port St. Lucie, with insects from the latter being more susceptible. Even when we used the most susceptible field population to calculate RR50 estimates, there was a decrease in resistance ratios for most of the insecticides tested for field populations of *D. citri* during 2013 as compared with 2009.

Flupyradifurone, a new nicotinic acetylcholine receptor agonist, was evaluated in the 2013 survey and compared with the 2 neonicotinoids imidacloprid and thiamethoxam. The laboratory strain LD50 estimate was 14.30 ng/µL (CI 95%: 9.96–19.45 ng/µL), which was approximately 10 times greater than that for thiamethoxam (1.31 ng/µL, CI 95%: 1.10–1.49 ng/µL) and imidacloprid (1.30 ng/µL, 95% CI: 1.04–1.49 ng/µL). LD50 estimates for field populations ranged from 7.93 ng/µL (95% CI: 5.99–10.22 ng/µL) in Lake Alfred to 18.70 ng/µL (95% CI: 9.34–28.78 ng/µL) in Winter Garden (Table 3). Interestingly, resistance ratios for flupyradifurone mirrored those for imidacloprid and thiamethoxam, except in the case where higher resistance ratios were observed for thiamethoxam in the laboratory strain and the Winter Garden population (Fig. 1). In these 2 cases, the RR50 estimates for flupyradifurone remained similar to that observed for imidacloprid.

2014 SURVEY

In 2014, the same sites and insecticides were tested, except for flupyradifurone, which was replaced with the organophosphate dimethoate. Insects from Port St. Lucie exhibited the lowest LD50 estimates for 4 out of the 6 insecticides tested: carbaryl, dimethoate, fenpropathrin, and thiamethoxam (Table 4). In 2014, the laboratory strain exhibited the highest LD50 estimates for 3 out of the 6 insecticides tested: chlorpyrifos, fenpropathrin, and thiamethoxam ($P \leq 0.05$; Table 4). Aside from the laboratory strain, the highest LD50 estimate for the remaining insecticides tested were similar across the field sites tested, with LaBelle having the highest for carbaryl and thiamethoxam (Table 4). The only RR50 over 3 was for imidacloprid at Port St. Lucie (4.04, CI 95%: 2.38–6.86) using the laboratory strain as a comparison. Using the LD50 estimate from Lake Alfred as a comparison, an RR50 of 7.10 (CI 95%: 4.18–12.04; Table 4) was obtained for the Port St. Lucie population in 2014. Also, the RR50 estimate for imidacloprid for psyllids collected from LaBelle in 2014 was over 3 (3.29, CI 95%: 2.68–4.03), as compared with Lake Alfred.

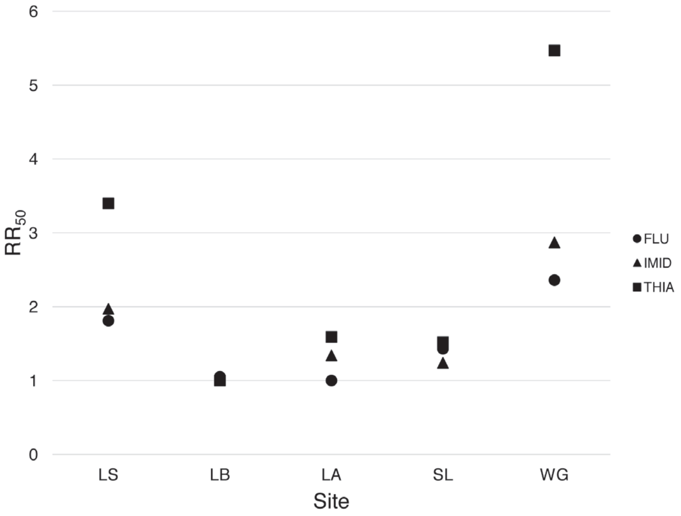

Fig. 1. Resistance ratios at the 50% response level using the most susceptible population as a comparison for the 3 nicotinic acetylcholine receptor agonists evaluated in this study. Abbreviations for insecticides are FLU, flupyradifurone; IMID, imidacloprid; THIA, thiamethoxam. Abbreviations for sites are LB, LaBelle; LA, Lake Alfred; SL, Port St. Lucie; WG, Winter Garden.

Discussion

Over the course of the surveys conducted between 2008 and 2010, a steady decrease in insecticide susceptibility was observed in *D. citri* field populations in Florida to numerous insecticides (Boina et al. 2009; Tiwari et al. 2011a). The major finding from the 2013 and 2014 annual surveys reported here is a reversal of insecticide resistance among *D. citri* populations across Florida based on RR50 estimates. Evidence of resistance was observed within a short time span (Boina et al. 2009; Tiwari et al. 2011a), and it appears to have decreased in a likewise relatively short period of time. This suggests that susceptibility to insecticides among populations of *D. citri* is dynamic and should be subject to regular monitoring when relying on insecticides in managing this pest. Low RR50 estimates were observed for both years, but the underlying reason(s) for this remain in question in contrast to the recent trend towards reduced susceptibility in the previous years.

The Florida citrus industry has witnessed significant improvement in insecticide use since the first report of huanglongbing in 2005 through the development of insecticide rotation schedules specific for citrus and the coordination of insecticidal treatments among groves. In 2008, a coordinated spray proposal was introduced to the citrus industry. In 2008 to 2009, the first coordinated insecticide sprays were attempted. In 2010, the National Academy of Sciences proposed Citrus Health Management Areas (National Research Council 2010). These are groups of commercial citrus groves located in close proximity, and where growers work cooperatively to reduce the spread of huanglongbing through the coordination of insecticide treatments. The coordination of treatments is meant to enhance insecticide efficacy against *D. citri* by preventing rapid re-infestation by psyllids from non-sprayed areas. By the end of 2010, 10 Citrus Health Management Areas were formalized and as of this writing, there are 52. In fall of 2011, the monitoring program for *D. citri* counts began, and reports became available online (http://www.crec.ifas.ufl.edu/extension/chmas/index.shtml). It is during this time when Citrus Management Health Areas became established that the 2010 survey was conducted, and perhaps it is not coincidental that between 2010 and 2013, there has been a decrease in resistance ratios among *D. citri* populations. Given that insecticide resistance is often associated with fitness costs and that Citrus Management Health Area programs may optimize insecticide use, it is possible that this program has had a positive impact on managing insecticide resistance. However, this hypothesis requires further investigation. Populations of *D. citri* do appear to become rapidly resistant to insecticides at up to 4,000-fold resistance ratios when modes of action are not rotated (Vázquez-García et al. 2013).

Given that the RR50 estimates for both 2013 and 2014 were consistently low as compared with 2009 in several field populations of *D. citri*, a long-term trend may be occurring. Also, not all of the data from the 2009 survey adequately fit the probit model, in contrast to this investigation, and this may affect the assessment of change in RR50 estimates. Future surveys of resistance in populations of *D. citri* will answer these questions.

FLUPYRADIFURONE

Flupyradifurone has systemic properties and therefore may be important for future management of *D. citri*. As more diseased trees are removed and re-planted, there will be greater need for use of the neonicotinoids in order to bring newly planted trees into production. Although the biochemical target of imidacloprid and flupyradifurone is the same, the 2 molecules differ in chemical structure. Imidacloprid is a neonicotinoid and flupyradifurone is a butenolide. Data generated thus far suggest that cross-resistance is not likely between these 2 insecticides based on known mechanisms of resistance (Nauen et al. 2013, 2015). It remains to be demonstrated experimentally, but given the difference in chemical structure, it is unlikely the CYP4 P450 monooxygenases associated with imidacloprid exposure in *D. citri* will also be responsive to flupyradifurone (Tiwari et al. 2011b; Killiny et al. 2014). Interestingly, the RR50 estimates for flupyradifurone clustered with those of imidacloprid and thiamethoxam, except in 2 cases where thiamethoxam was higher and the RR50 estimate for flupyradifurone remained similar to that observed for imidacloprid (Fig. 1). It is unknown whether this is indicative of differences in the general response to these insecticides between field populations due to natural variability or previous insecticide exposure, and this should be investigated further as flupyradifurone receives greater field use. An additional systemic insecticide would be an asset if it proved effective against *D. citri* without cross-resistance to imidacloprid or similar neonicotinoids.

FUTURE DIRECTIONS FOR FIELD INVESTIGATION OF *D. CITRI* RESPONSE TO INSECTICIDES

The use of our current laboratory susceptible strain of *D. citri* as a baseline comparison against field populations requires further consideration. Although this colony is maintained without exposure to insecticides, the laboratory strain had greater LD50 estimates as compared with the field populations for 10 out of 30 assays in 2013 (Table 3), and for 18 out of 30 in 2014 (Table 4). Although this did not affect the overall trend of lower RR50 estimates as compared with the 2009 survey, in a number of cases it resulted in lower RR50 estimates compared with using the most susceptible field population (Tables 3 and 4). In most cases, the difference between the RR50 estimates calculated using the different populations was minimal. However, for future surveys, if the LD50 estimate for the laboratory strain is sufficiently high, then resulting RR50 estimates may be inappropriately low. For field populations that have exhibited signs of increased tolerance, the level at which action should be taken could be underestimated. Because LD50 estimates for the laboratory strain were higher for some insecticides as compared with the field populations, this could indicate natural variability in susceptibility between populations of *D. citri*, and it warrants further investigation. Even though the colony is maintained under controlled conditions, it remains possible that it could have been contaminated by psyllids from outside. Also, plants used to maintain the colony are periodically replaced with new plants. Even though every effort is taken to eliminate the residual insecticides used on these plants through repotting, holding in quarantine, and testing prior to being placed in the colony, it is possible that undetected residual insecticide remains.

There is significant natural variation among populations of *D. citri* in terms of insecticide susceptibility. For example, color morphotype is correlated with insecticide susceptibility among populations of *D. citri* (Tiwari et al. 2013), and there is significant variation among color morphs within local populations of *D. citri* (Wenninger & Hall 2008). Also, susceptibility of *D. citri* to insecticides increases among those carrying the pathogen causing huanglongbing, as compared with uninfected counterparts (Tiwari et al. 2011c). There is significant variation in the rate of infection of *D. citri* across Florida currently (Coy & Stelinski 2015). This variation across the state may affect how resistance ratios should be interpreted. The RR50 cutoff of 3 was used in this investigation because the majority of RR50 estimates for both years were below this value. In terms of insecticide resistance management, the cutoffs should indicate true changes in insecticide susceptibility above natural variation. For example, it is unknown if the RR50 difference of 5.47 between Winter Garden and LaBelle in 2013 was within the range of natural variation, or whether the population in Winter Garden actually exhibited some level of resistance to thiamethoxam (Table 3). Also, the difference in RR50 between the laboratory strain and the LaBelle population for thiamethoxam was 3.40 (LaBelle more susceptible; Table 3). Statistically, the LD50 estimates for these populations were different ($P \leq 0.05$), but considering potential natural variation, the biological significance of this difference needs further scrutiny (Boina et al. 2009). These cutoffs are easier to establish for higher levels of reduced susceptibility, especially if they can be correlated with product failure. Furthermore, use of diagnostic dosages as a method of measuring resistance is a potentially faster alternative that requires fewer *D. citri* (Tiwari et al. 2011a); however, it may not account for natural variation between populations as well because fewer insects are used. We are currently developing a diagnostic dose vial assay (Brogdon & McAllister 1998), which may allow for more extensive sampling across the state of Florida and elsewhere for resistance in *D. citri*.

In conclusion, the results of the 2013 and 2014 surveys indicate that some field populations of *D. citri* have become more susceptible to all insecticides tested, as compared with results from 2009. Whether this is due to recent changes in insecticide management practices, which also occurred during this time period, and/or other factor(s), is unknown. Although some of the LD50 estimates in 2013 and 2014 were considered to be statistically different from one another, the practical significance of most of the RR50 estimates is unclear because they were all low and could be due to natural variability in the response of *D. citri* to insecticides. Based on our recent surveys, it is unlikely that a current insecticide application failure for *D. citri* is due to insecticide resistance.

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