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Research article

Neonicotinoid-Induced Mortality of *Diaphorina Citri* (Hemiptera: Liviidae) is Affected by Route of Exposure

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Abstract

The use of neonicotinoids in citrus (Rutaceae) has increased substantially to help manage the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), a vector of the devastating citrus disease, huanglongbing (HLB). In citrus pest management programs, neonicotinoids are most often applied to the soil as a drench and move through xylem channels from the roots into the foliage. We developed a novel assay to quantify the dose required to kill *D. citri* following ingestion and compare it with the dose required to kill by contact. The LC_{50} of the laboratory strain for ingestion of imidacloprid, thiamethoxam, and clothianidin were each approximately 10-fold greater than the respective LC_{50} by contact exposure. Four field populations were tested to validate comparative exposure of the laboratory strain to imidacloprid and determine the relative susceptibility of field populations to imidacloprid by exposure through ingestion and contact. The contact assay exhibited low (<10) RR_{50} values for the Vero Beach and Labelle populations when compared to the ingestion assay method. High (>10) RR_{50} values were observed for the Lake Placid and Lake Alfred populations using the contact and the ingestion method. This research demonstrates that the ingestion assay method described herein is more sensitive in detection of low-level resistance and should be the standard methodology used in monitoring for resistance to systemic insecticides for this global pest. We found *D. citri* populations with a lower than expected susceptibility to neonicotinoids in the field, which warrants the implementation of resistance management practices to preserve the utility of soil-applied neonicotinoids in citrus.

Key words: neonicotinoid, citrus, *Diaphorina citri*, ingestion, contact

The Asian citrus psyllid, *Diaphorina citri* (Kuwayama; Hemiptera: Liviidae), is a major pest of citrus (Rutaceae) throughout the world, negatively impacting productivity and yield (Halbert and Manjunath 2004; Bové 2006; Gottwald 2007; Ichinose et al. 2010a,b; Grafton-Cardwell et al. 2013). *D. citri* serves as the vector of the bacterium, *Candidatus* Liberibacter asiaticus (CLas), the presumed causal agent of huanglongbing (HLB), or citrus greening disease. CLas is a phloem-limited bacterium that negatively impacts the root system leading to a decline in the tree canopy, including twig dieback, mottled leaves, misshapen fruit, decreased fruit quality, increased fruit drop, and subsequent death of infected trees (Halbert and Manjunath 2004, Bové 2006, Grafton-Cardwell et al. 2013). *D. citri* was first discovered in Florida in 1998 (Halbert and Manjunath 2004), followed by HLB in 2005 (Halbert 2005). HLB was recently discovered in California (Kumagai et al. 2013). The Florida citrus industry was valued at nearly 9.9 billion dollars during 2014 and 2015 (Hodges and Spreen 2015) and is greatly threatened by the spread of HLB. Since HLB was discovered in Florida in 2005, the use of insecticides, particularly neonicotinoids, has increased substantially and plays a vital role in the management of the insect vector, and thus HLB (Rogers 2008).

Following the discovery of CLas in Florida, investigations of a wide array of management strategies to reduce the spread of HLB in Florida citrus was initiated. The use of biological control agents such as *Tamarixia radiata* (Waterston; Hymenoptera: Eulophidae), nursery sanitation, rogueing of infected trees in the field, and scouting-based sprays were each suggested as methods for management of HLB (Stansly and Rogers 2006, Hall and Albrigo 2007, Hall et al. 2008). Given the severity and potential impact of the disease, vector control through use of insecticides remained the fundamental tool for slowing the spread of HLB in Florida citrus (Rogers 2008, Boina et al. 2009, Qureshi and Stansly 2009).

Largely due to the increased frequency of insecticide applications in citrus following the onset of HLB, it was recognized that growers could not rely solely on foliar applied insecticides to protect young trees (Rogers 2012). As growers removed infected trees for replanting, protection of young trees from HLB for the first 3 to 5 yr of growth to bearing-age became highly important (Rogers 2012). As a result, soil-applied neonicotinoids were identified as a very effective tool for reducing *D. citri* populations; they remain a key component of management programs that allow growers to mitigate the risk of

HLB infection in young citrus, typically defined as trees less than eight feet in height (Rogers and Shawer 2007, Rogers 2012, Rogers et al. 2015). University of Florida recommendations suggested an intensive program in which neonicotinoids are applied to the soil at 6-wk intervals, with supplemental non-neonicotinoid foliar applications made between soil application events (Rogers 2012). Neonicotinoids are characterized as highly systemic and mobile within plant tissue. The Insecticide Resistance Action Committee (IRAC) classifies neonicotinoids within the chemical sub-group 4A, which act on the nicotinic acetylcholine receptor (nAChR). Neonicotinoid insecticides often are applied to the soil where they are absorbed through the roots and transported to the foliage through xylem channels (Elbert et al. 2008). Systemic insecticides applied to the soil effectively target insect pests, while minimizing direct contact with pollinators and other beneficial insects (Stansly and Qureshi 2008). Currently, three neonicotinoid insecticides are labeled for use in Florida citrus: thiamethoxam (Platinum 75 SG—Syngenta Crop Protection, Inc., Greensboro, NC), imidacloprid (Admire Pro 4.6F—Bayer CropScience, Research Triangle Park, NC), and clothianidin (Belay 2.13 SC—Valent USA Corporation, Walnut Creek, CA) (Rogers et al. 2015).

A number of studies have addressed the use of neonicotinoids as a means of protecting young citrus trees from feeding with residual control effects reported between 6 and 11 wk after application (Qureshi and Stansly 2007, Qureshi and Stansly 2009, Ichinose et al. 2010a, Setamou et al. 2010, Byrne et al. 2012, Rogers 2012). Serikawa et al. (2012) used electropenetrography to demonstrate that adult *D. citri* exhibited a reduced number and duration of phloem-related feeding behaviors on citrus plants receiving soil applications of imidacloprid compared to untreated plants. Despite the use of soil-applied neonicotinoids, 2013 reports estimated 1–3% of trees becoming infected annually in intensively managed groves in Florida (Rogers 2013). Boina et al. (2009) proposed that uneven temporal and spatial distribution of imidacloprid in citrus tissue following a soil application may permit exposure of *D. citri* to sublethal doses of imidacloprid. Uneven uptake of systemic insecticides by the root system make it possible for *D. citri* to develop on treated trees (Rogers 2012). If *D. citri* feed on CLas-infected citrus tissue with sublethal imidacloprid concentrations which do not inhibit feeding, acquisition and/or inoculation of CLas is possible. In Florida, roughly 80–100% of *D. citri* are CLas positive (Coy and Stelinski 2015) and therefore, a single successful feeding event on an uninfected tree cannot be tolerated. Setamou et al. (2010) identified the lethal concentration of imidacloprid for *D. citri* as between 200 and 250 parts per billion (ppb). This lethal threshold was developed by correlating percentage control of *D. citri* and leaf tissue residue analysis using enzyme-linked immunosorbent assay (ELISA). When evaluating insecticides under field conditions, percentage control, or efficacy, is most often defined by the absence of a particular insect pest as compared to some untreated control. In the case of systemic insecticides, efficacy could be a result of mortality, repellency, feeding deterrence, or a combination thereof. In this case, repellence can be defined as olfactory avoidance behavior of aversive volatiles, associated with feeding sites and deterrence can be defined as gustatory avoidance of less or non-suitable feeding sources. Dosages of imidacloprid between 200 to 250 ppb associated with imidacloprid efficacy observed by Setamou et al. (2010) may have resulted from a combination of mortality, repellency, and/or feeding deterrence caused by imidacloprid rather than mortality only. Because mortality was not quantified in the aforementioned study, the concentration of imidacloprid required to kill *D. citri* through feeding remains unknown.

To date, resistance monitoring efforts in citrus utilize only contact-style assay methods for comparing susceptibility levels of field-collected populations to that of laboratory susceptible (LS) cultures (Tiwari et al. 2011a, 2013; IRAC 2009, 2011, 2014; Coy et al. 2016; Kanga et al. 2016). Three distinct methodologies are among the contact-style assay methods cited: 1) topical; 2) vial; and 3) leaf dip. Topical assays are used to evaluate only contact exposure by administration of a small volume of insecticide directly to the insect thorax (IRAC 2011; Tiwari 2011a, 2013; Coy et al. 2016). Vial assays are also used to evaluate only contact exposure by coating the inside walls of a glass vial with insecticide, aspirating insects into the treated vial, and allowing them to traverse the treated glass surface (Kanga et al. 2016). Unlike the topical and vial assays, leaf dip assays encompass both contact and ingestion routes of exposure, where insects are permitted to walk on and feed upon insecticide covered leaf material (IRAC 2009, 2014; Tiwari et al. 2011a). While contact assays are effective for determining shifts in susceptibility over time, and if resistance exists in some field population, contact values are not equivalent to ingestion concentrations required to kill *D. citri*. In the case of systemic insecticides, such as neonicotinoids applied to soil, ingestion is the primary route of insecticide exposure, and thus the concentration of insecticide required to cause mortality exclusively through ingestion should be quantified.

The purpose of this study was to determine the concentration of systemic insecticide within citrus tissue required to kill *D. citri* through ingestion and to validate the lethal concentration using various field populations within citrus production areas of Florida. By determining the lethal concentration of systemic insecticide by ingestion, we will advance our understanding of the interaction between *D. citri* as a vector of CLas and citrus treated with soil-applied systemic neonicotinoid insecticides.

Materials and Methods

Lab Culture

The LS strain was reared in continuous culture at the University of Florida Citrus Research and Education Center in Lake Alfred on *Murraya koenigii* maintained at 27°C with RH 65% with a photoperiod of 14:10 (L:D) h. The LS strain was maintained CLas-free, confirmed by routine testing of the colony using rt-PCR, and did not receive any exposure to insecticides following establishment of the colony in 2005. Adult *D. citri* were collected directly from plants through oral aspiration. Adult *D. citri* were collected and used during the same day to minimize negative effects from storage and to reduce unintended mortality.

Field Collection

Four citrus groves were sampled for *D. citri*, each representing a major citrus production area in the state: 1) Vero Beach, east coast flatwoods, collected 24-VIII-2016; 2) Lake Placid, southern central ridge, collected 6-IX-2016; 3) Lake Alfred, northern central ridge, collected 19-IX-2016; and 4) Labelle, southern pine flatwoods, collected 21-IX-2016. Adult *D. citri* were collected by two methods: 1) aspiration directly from citrus foliage, or 2) by sweep net and aspiration of trapped adults. *D. citri* adults were transported from the field within labeled plastic aspirator vials placed into a small cooler containing one cold pack wrapped in paper towels. *D. citri* collected from the field were assayed during the same day to minimize negative effects of storage and to reduce unintended mortality. In the case of the Labelle, FL population, a limited number of adult *D. citri* were available in the grove at the time of collection.

Instead of collecting adults during the grove visit, flush infested with fourth and fifth instar *D. citri* nymphs were collected into small paper bags and transported to the lab. Flush stems were inserted into floral foam, placed in a plastic tray, and wetted with deionized water. Each plastic tray containing foam and flush was held in a small mesh insect cage with two *Murraya koenigii* plants. The cage was stored in a greenhouse cubicle set to 27°C under ambient lighting and humidity conditions. After 9 d, adult *D. citri* were abundant and thus collected for assay as done with the direct field-collected populations.

Adult Ingestion Assay

The ingestion assay method used was a modification to that described in Huseth et al. (2016). A 30% sucrose solution similar to that described in Hall et al. (2010) was prepared to achieve a final volume of 600 ml in the following order of mixture steps: 300 ml deionized water, 180 g sucrose (30% w/v; Sigma Life Science, St. Louis, MO, Cat. No.: S0389-5KG), 0.6 ml green food dye (0.1% v/v; McCormick & Co., Inc. Hunt Valley, MD), and 2.4 ml yellow food dye (0.4% v/v; McCormick & Co., Inc.). This mixture was lightly heated to dissolve sucrose. Once the sucrose was in solution, deionized water was added to reach a final volume of 600 ml. Aliquots of the stock sucrose solution were then used to perform a serial dilution of one of three formulated neonicotinoid insecticides of seven to eight doses: Admire Pro 4.6F (550 g imidacloprid L^{-1}, Bayer CropScience, Research Triangle Park, NC), Platinum 75SG (750 g thiamethoxam kg, Syngenta Crop Protection, Greensboro, NC), or Belay 2.13 SC (255 g clothianidin L^{-1}, Valent USA Corporation). The cap was removed from 5 ml snap-cap centrifuge tubes (Eppendorf Tubes, Hamburg, Germany, Cat. No.: 0030119401) and appropriately labeled by treatment. Each centrifuge tube cap was filled with 0.7 ml sucrose solution with or without insecticide. A 2 cm^2 piece of Parafilm M (Bemis, Neenah, WI, Cat. No.: PM-992) was stretched and placed over the diet-filled cap and excess was wrapped around the cap. Depending on availability of insects, four to six adult *D. citri* were aspirated into individual centrifuge tubes and a diet-filled cap was reinstalled for feeding through the thin Parafilm M membrane. Tubes were placed upright in a tube tray and held at 27°C, 70% relative humidity, with a photoperiod 14:10 (L:D) for 72 h. One replicate consisted of one tube and 10 replicates were used for each of seven to eight doses in each ingestion assay. A total of 40 to 60 adults were tested for each dose. Insects were assessed at 72 h for mortality. Insects were scored as alive (full function), moribund (insects lacking coordinated movement), or dead (no movement upon disturbing). Moribund insects were classified as dead for data analysis. The lab susceptible culture was tested against each of the three insecticides and each field population was tested against only imidacloprid due to the lack of availability of field-collected insects.

Adult Contact Assay

To test contact activity, the vial roll method similar to that described in Kanga et al. (2016) was used due to similar insecticide exposure properties to that of a foliar spray, while excluding the possibility of ingestion activity. Analytical-grade insecticides (>99.5% purity) of each imidacloprid, thiamethoxam, and clothianidin were obtained from Chem Service (Chem Service, Inc, West Chester, PA). An initial stock insecticide solution was prepared using acetone (Fisher Scientific, Fair Lawn, NJ, Cat. No.: A929-4). A serial dilution was utilized to achieve seven to eight doses for each assay. Individual pre-labeled 16 ml glass vials (Wheaton, Millville, NJ, Cat. No.: 224746) were each treated with 1.5 ml insecticide solution and placed onto an electric hot-dog roller within a fume hood. Vials were rolled for 1–2 h or until all acetone evaporated from within the glass vial. Control vials were treated with acetone only and subjected to the same rolling process. Treated vials were stored in a dark cardboard container at room temperature conditions for no more than 24 h until use in an assay. Depending on availability of insects, eight to twelve adult *D. citri* were aspirated into individual vials using a small medical vacuum (Invacare, Elyria, OH, Model: IRC1135) and a cap was installed. Tubes were placed horizontally onto a cafeteria tray and held at 27°C, 70% relative humidity, with a photoperiod 14:10 L:D for 24 h. One replicate consisted of one vial and five replicates were used for each of seven to eight doses in each contact assay. A total of 40 to 60 adults for each dose were tested. Insects were assessed at 24 h for mortality. Insects were scored as alive (full function), moribund (insects lacking coordinated movement), or dead (no movement upon disturbing). Moribund insects were classified as dead for data analysis. The lab susceptible culture was tested against each of the three insecticides and each field population was tested against only imidacloprid due to the lack of availability of field-collected insects.

Statistical Analyses

Concentration mortality data were subjected to Probit analysis using SAS v9.4 (Proc Probit, SAS Institute, 2013). Mean separations between *D. citri* populations within each exposure route were based on mortality at the mean dose level using Tukey-Kramer Least Squares Means where means differed significantly at $\alpha \leq 0.05$.

Results

A fully susceptible laboratory *D. citri* strain (LS) was tested to determine baseline susceptibilities to imidacloprid, thiamethoxam, and clothianidin when exposed to each insecticide by ingestion and contact (Table 1). The LC_{50} for ingestion was 0.39, 0.11, and 0.09 parts per million (ppm) for imidacloprid, thiamethoxam, and clothianidin, respectively. In contrast, the LC_{50} for contact exposure was 0.04, 0.01, and 0.01 ppm for imidacloprid, thiamethoxam, and clothianidin, respectively. The relative difference in LC_{50} values were compared using a ratio of LC_{50} via ingestion divided by the LC_{50} via contact for each insecticide and is described as IC_{50} in Table 1. The IC_{50} for imidacloprid indicates that the LC_{50} by ingestion was 9.75-fold greater than by contact; the IC_{50} for thiamethoxam 11-fold greater and the IC_{50} for clothianidin ninefold greater.

Four field populations of *D. citri* were tested to validate comparative exposure observations of the laboratory *D. citri* strain to imidacloprid exposure and to determine the relative susceptibility of field populations to imidacloprid by exposure through ingestion and contact (Table 2). LC_{50} values were greater by ingestion than by contact in each field population investigated. Resistance ratios were also generated to compare susceptibility levels of field populations to the LS strain within each exposure route. Resistance ratios at the 50% mortality level (RR_{50}) were calculated by dividing the LC_{50} of the field population by the LC_{50} of the LS strain. All field populations tested expressed some level of resistance as compared to the LS strain. The contact assay exhibited low level RR_{50} values for the Vero Beach and Labelle populations (3.06 and 5.77, respectively) when compared with RR_{50} values generated using the ingestion assay method (10.57 and 26.36, respectively). High RR_{50} values were observed for the Lake Placid and Lake Alfred populations using the contact method (18.75 and 42.21, respectively), and the ingestion method (20.39 and 33.43, respectively).

Table 1. Response of laboratory susceptible *D. citri* strain to three neonicotinoid insecticides by ingestion and contact

Insecticide	Assay method	Strain	N[a]	Slope + SE	LC_{50}[b]	95% CL	LC_{90}[b]	95% CL	X^2	IC_{50}[c]	NC[d]
Imidacloprid	Ingestion	LS	546	0.25 + 0.03	0.39	(0.19–0.72)	62.19	(30.36–164.74)	96.21	9.75	100
	Contact	LS	320	1.03 + 0.10	0.04	(0.03–0.04)	0.13	(0.10–0.18)	100.80	—	
Thiamethoxam	Ingestion	LS	404	0.34 + 0.04	0.11	(0.05–0.21)	4.94	(2.63–11.75)	73.58	11.00	100
	Contact	LS	405	0.75 + 0.12	0.01	(0.01–0.02)	0.05	(0.04–0.11)	38.69	—	
Clothianidin	Ingestion	LS	402	0.28 + 0.03	0.09	(0.03–0.19)	9.35	(4.55–25.15)	69.74	9.00	100
	Contact	LS	393	0.51 + 0.07	0.01	(0.01–0.02)	0.16	(0.10–0.34)	46.59	—	

[a]Number of adult *D. citri* tested.
[b]Parts per million (ppm) active ingredient.
[c]Ratio of ingestion LC_{50} divided by contact LC_{50}.
[d]Percent mortality in negative control containing no diet at 72 h.

Table 2. Response of laboratory and field collected *D. citri* to imidacloprid by ingestion and contact in 2016

Method	Population	N[a]	Slope + SE	LC_{50}[b,c]	95% CL	LC_{90}[c]	95% CL	X^2	$RR_{50\ Lab\ Susc}$	$RR_{50\ Field\ Susc}$	IC_{50}[d]	NC[e]
Ingestion	LS	546	0.25 + 0.03	0.39a	(0.18–0.71)	62.19	(30.36–164.74)	96.21	—	0.09	9.75	100
	Vero Beach	284	0.39 + 0.04	4.13b	(2.43–6.77)	109.19	(55.27–284.22)	83.96	10.57	—	37.55	97.5
	Lake Placid	282	0.31 + 0.04	7.97bc	(4.35–14.42)	522.58	(204.31–2150)	69.85	20.39	1.93	11.72	95
	Lake Alfred	440	0.29 + 0.03	13.10c	(8.04–21.61)	1077	(455.13–3622)	104.01	33.54	3.17	8.51	98.3
	Labelle	359	0.34 + 0.03	10.28bc	(6.36–16.60)	425.46	(201.83–1206)	99.76	26.36	2.49	48.95	98.0
Contact	LS	320	1.03 + 0.10	0.04*a*	(0.03–0.04)	0.13	(0.10–0.18)	100.80	—	0.36	—	—
	Vero Beach	418	0.34 + 0.03	0.11*b*	(0.06–0.18)	4.87	(2.59–11.11)	116.08	3.06	-	—	—
	Lake Placid	320	0.33 + 0.03	0.68*c*	(0.40–1.17)	31.81	(14.65–92.08)	102.44	18.75	6.18	—	—
	Lake Alfred	496	0.19 + 0.02	1.54*c*	(0.80–3.07)	1232	(313.60–9480)	83.67	42.21	14.00	—	—
	Labelle	408	0.30 + 0.03	0.21*b*	(0.12–0.35)	14.61	(6.96–39.69)	111.49	5.77	1.91	—	—

[a]Number of adult *D. citri* tested.
[b]Test of differences in mortality at the mean dose level where means not sharing the same letter differ significantly at $\alpha \leq 0.05$ (Contact: 19.5 ppm; Ingestion: 97.7 ppm).
[c]Parts per million (ppm) active ingredient.
[d]Ratio of ingestion LC_{50} divided by contact LC_{50} by location.
[e]Percent mortality in negative control containing no diet at 72 h.

Discussion

This study is the first to quantify the lethal concentration of neonicotinoid insecticides required to effectively kill *D. citri* when ingested in the absence of contact exposure. All lethal concentrations developed to date utilized only an assay method that permits physical contact between the insect and insecticide where insects cannot escape exposure (Tiwari et al. 2011a, 2013; IRAC 2009, 2011, 2014; Coy et al. 2016; Kanga et al. 2016). Neonicotinoid insecticides are most often applied to young citrus trees as a soil drench, absorbed by the roots and expressed in leaf tissue. Because *D. citri* are only exposed to these insecticides by ingesting insecticide-inclusive plant sap, there was a need to determine insecticide concentrations required to kill *D. citri* upon ingestion. This research is also the first to document the magnitude of difference in mortality between ingestion and contact exposure. A concentration of nine to 11-fold higher, depending on active ingredient, was required to kill 50% of the LS strain through ingestion when compared to contact for imidacloprid, thiamethoxam, and clothianidin. Similarly, the lowest imidacloprid concentration difference between ingestion and contact for the field populations tested was 8.51-fold higher. These results document that a higher neonicotinoid concentration is required to kill the same number of *D. citri* individuals through ingestion than by contact. The observed difference between mortality by ingestion and by contact may be explained by the following factors: 1) Volume of diet consumed determines the amount of insecticide exposure; 2) a portion of ingested insecticide is evacuated through the digestive tract and rendered unavailable to the insect before absorption into the body occurs; and 3) higher metabolic activity in the gut may impact insecticide toxicity compared with absorption through the cuticle via contact. High mortality observed in the negative control (no available diet) suggests that observed survivors within the ingestion assay did successfully feed, therefore complete avoidance of the insecticide diet was unlikely. The ingestion assay also likely better approximates field exposure of adult *D. citri* to systemically occurring imidacloprid, since these hemipterans must alight on plant material and initiate feeding prior to exposure. Upon insertion of stylets into the plant material, *D. citri* can choose whether or not to feed. Individuals that do not feed in the field can move to new host plants in search of more acceptable food sources. Presumably, if feeding deterrence occurred in the ingestion assay, those individuals would have died prior to evaluation, further reducing the LC_{50} values for ingestion. This would reduce the magnitude of difference between insecticidal activity with the ingestion and contact assays. In previously published studies, between 200 and 250 ppb (0.2–0.25 ppm) of imidacloprid was determined as the (presumed) lethal concentration needed to kill *D. citri* by correlating insecticide efficacy with imidacloprid titer (Setamou et al. 2010). In the present study, a concentration of 0.39 ppm (390 ppb) imidacloprid was required to kill half (LC_{50}) of the LS strain by ingestion, and 62.19 ppm (62190 ppb) imidacloprid was required to kill 90% (LC_{90}) of the LS strain by ingestion. The higher-than-expected values observed indicates that the imidacloprid

concentration threshold required to kill *D. citri* in the field is likely much higher than previously assumed.

Because Setamou et al. (2010) found that 200–250 ppb of imidacloprid provide strong efficacy against *D. citri* field populations, and the current study found 62.19 ppm to kill just 90% of the LS population, it is likely that 200–250 ppb corresponds to a sublethal dose as a result of feeding deterrence rather than mortality. In the case of systemic insecticides where feeding is required for insecticide exposure, insect mortality is likely not required to achieve perceived high levels of control. Additional work is warranted to investigate the feeding behavioral response of *D. citri* when exposed to various neonicotinoid concentrations.

While the foremost goal of this study was to compare the difference between ingestion versus contact mortality, our results indicate a second event of reduced susceptibility to neonicotinoids in field populations of *D. citri* at our selected study sites not unlike that documented for populations in similar regions of Florida in 2010 (Tiwari et al. 2011a). Resistance ratios generated using the contact assay suggest that low levels of resistance exist in the Vero Beach and Labelle populations. Interestingly, resistance ratios calculated using the ingestion assay method for the same populations are higher, demonstrating that the ingestion assay method is more sensitive in detection of low-level resistance development. Populations from Lake Alfred and Lake Placid exhibited high resistance ratios by both the contact assay method and the ingestion assay method. Perceived product failures have been observed at or near the Lake Alfred and Lake Placid collection sites in previous years (M. E. Rogers, personal observation). Results from this study illustrate the importance of matching each specific insecticide with the route of insecticide exposure in the field when undertaking resistance monitoring efforts. This match of exposure is especially important in the detection of low-level resistance in the field before product failures occur. Tiwari et al. (2011a) found that imidacloprid resistant field populations of *D. citri* expressed higher levels of detoxifying enzymes, including general esterase, glutahione *S*-transferase, and cytochrome P_{450} monooxygenases. Later work discovered five family 4 cytochrome P_{450} genes that were induced by imidacloprid exposure (Tiwari et al. 2011b). Tiwari et al. (2011a) advised that despite elevated levels of detoxifying enzymes in insecticide resistant populations, other mechanisms of resistance may play a role in the development of resistance in *D. citri* populations. Suggested mechanisms were reduced penetration, target-site insensitivity, and mutations in detoxifying enzymes. Nonetheless, because *D. citri* are most often exposed to neonicotinoids in citrus through ingestion and that *D. citri* likely encounter sub-lethal concentrations of this insecticide more frequently than lethal ones (Boina et al. 2009), it is possible that behavioral resistance as a single mechanism has thus far been incorrectly ignored as possibly a primary concern given the need for ingesting neonicotinoids by *D. citri* following soil-applied treatments. The most recent resistance monitoring work to occur in Florida reported a reversion of insecticide resistance to imidacloprid and thiamethoxam in 2013 and 2014 *D. citri* populations (Coy et al. 2016). This work was completed using a topical contact assay and reemphasizes the dynamic susceptibility shifts described by Tiwari et al. (2013). Nevertheless, resistance monitoring efforts that utilize contact assay methods may underestimate neonicotinoid resistance or fail to detect mechanisms specific to neonicotinoid resistance that are related to ingestion exposure pathways.

The present study quantifies the concentration of imidacloprid, thiamethoxam, and clothianidin in citrus leaf material required to effectively kill *D. citri* and identifies the utility of an ingestion assay in monitoring for neonicotinoid resistance in field populations of *D. citri*. Although we determined the lethal dose required to kill *D. citri* upon feeding, this study did not determine the insecticide concentration threshold at which feeding is deterred relative to pathogen transmission disruption. Serikawa et al. (2012) demonstrated that a small portion of *D. citri* tested were able to undergo phloem ingestion (E2) for more than 1 h on citrus tissue assumed to contain lethal levels of imidacloprid. While 1 h of ingestion (E2) is sufficient for CLas acquisition to occur (Bonani et al. 2010), Serikawa et al. (2012) explained that subsequent inoculation of nearby uninfected citrus plants following CLas acquisition was not likely due to lethal effects of imidacloprid. While lethal levels of imidacloprid may prevent successful CLas transmission, sublethal levels that do not deter feeding may allow successful CLas acquisition from infected tissue and subsequent inoculation into new, uninfected trees. The dose required to deter feeding, as it relates to pathogen transmission, remains unknown. Future work should utilize tools such as electropenetrography to determine the dose at which feeding activity is interrupted to determine the minimum neonicotinoid dose required to significantly reduce pathogen transmission. Since 2009, insecticide resistance to neonicotinoids has been a reoccurring phenomenon in *D. citri* (Tiwari et al. 2011a, 2013; Coy et al. 2016; Kanga et al. 2016). Because of these acute shifts in susceptibility to neonicotinoids, growers must remain cognizant of the potential for resistance. Furthermore, our finding of potentially neonicotinoid resistant *D. citri* populations in the field in 2016 warrants the development and implementation of resistance management practices directly aimed to preserve the utility of soil-applied neonicotinoids in citrus.

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