

Research Paper

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Fitness costs associated with thiamethoxam and imidacloprid resistance in three field populations of *Diaphorina citri* (Hemiptera: Liviidae) from Florida

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Abstract

Insecticide resistance is an increasing problem in citrus production. The Asian citrus psyllid, *Diaphorina citri* Kuwayama, is recognized as one of the most important citrus pests worldwide and it has developed resistance in areas where insecticides have been overused. The development of insecticide resistance is often associated with fitness costs that only become apparent in the absence of selection pressure. Here, the fitness costs associated with resistance to thiamethoxam and imidacloprid were investigated in three agricultural populations of *D. citri* as compared with susceptible laboratory colonies. Results showed that all field populations had greater resistance than laboratory susceptible colonies. For both thiamethoxam and imidacloprid, a *Candidatus Liberibacter asiaticus*-positive (CLas⁺) colony was more susceptible than the CLas⁻ colony. Resistance ratios ranged from 7.65–16.11 for imidacloprid and 26.79–49.09 for thiamethoxam in field populations as compared with a susceptible, CLas⁻ laboratory strain. Among three resistant field populations, a significantly reduced net reproductive rate and finite rate of population increase were observed in a population from Lake Wales, FL as compared to both susceptible strains. The fecundity of field populations from Lake Wales, FL was statistically lower than both laboratory susceptible populations. Certain changes in morphological characteristics were observed among resistant, as compared, with susceptible strains. Our data suggest fitness disadvantages associated with insecticide resistance in *D. citri* are related to both development and reproduction. The lower fitness of *D. citri* populations that exhibit resistance to neonicotinoid insecticides should promote recovery of sensitivity when those populations are no longer exposed to thiamethoxam and/or imidacloprid in the field. The results are congruent with a strategy of insecticide rotation for resistance management.

Introduction

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is a global economic pest of citrus. It is a vector of the bacterium *Candidatus Liberibacter asiaticus* (CLas), the putative causal agent of the disease huanglongbing (HLB) (Halbert and Manjunath, 2004; Bové, 2006; Gottwald, 2010; Grafton-Cardwell *et al.*, 2013; Vázquez-García *et al.*, 2013; Kanga *et al.*, 2016; Stockton *et al.*, 2017). Vector suppression with insecticides is one of the primary current management practices for this disease (Grafton-Cardwell *et al.*, 2013; Kanga *et al.*, 2016; Chen *et al.*, 2018). The short generation time and high fecundity of *D. citri* have resulted in the development of resistance to several insecticides (Tsai and Liu, 2000; Kanga *et al.*, 2016; Chen and Stelinski, 2017; Chen *et al.*, 2018).

The development of insecticide resistance is an increasing problem for citrus production (Grafton-Cardwell *et al.*, 2013; Chen *et al.*, 2017). Resistance has been documented for organophosphates (Tiwari *et al.*, 2013; Vázquez-García *et al.*, 2013; Kanga *et al.*, 2016), pyrethroids (Tiwari *et al.*, 2013; Kanga *et al.*, 2016), neonicotinoids (Vázquez-García *et al.*, 2013; Chen *et al.*, 2018), and carbamates (Kanga *et al.*, 2016). Neonicotinoid insecticides have been an important tool in pest control in citrus for well over a decade (Alyokhin *et al.*, 2007). The importance of these insecticides is in part due to their effectiveness against a broad spectrum of insect pests including *D. citri*. Neonicotinoids are highly systemic and mobile within citrus tissue and used as soil applications for young trees and foliar applications for mature trees. The insecticide resistance action committee classifies neonicotinoids within the chemical subgroup 4A, which acts on the nicotinic acetylcholine receptor (nAChR), thus hindering

nerve impulse transmission as a result of a depolarizing effect within the central nervous system of insects (Elbert *et al.*, 2008; Jeschke *et al.*, 2011; Oliveira *et al.*, 2011; Salgado, 2016).

Fitness is a measure of the expected reproductive potential of an individual (Kilot and Ghanim, 2012). Differences in the biological parameters affecting the net reproductive rate are of particular interest for insecticide resistance management (Haunbrugge and Arnaud, 2001). It is critical to understand the fitness consequences associated with the development of insecticide resistance. This issue has fundamental implications for evolutionary responses to stress and immediate applications for efforts to manage resistance (Hollingsworth *et al.*, 1997). Because resistant insects are typically not present at high frequency before frequent use of insecticides, it is generally assumed that resistance genotypes must have a reproductive disadvantage in the absence of selection pressure (Haubruge and Arnaud, 2001). If resistance genes have associated negative consequences on fitness in the absence of insecticide, then the frequency of resistant phenotypes should decline when insecticide pressure is reduced (Arnaud and Haubruge, 2002). Fitness costs associated with insecticide resistance have been reported for many classes of insecticides and insect species, including *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae) (Puinean *et al.*, 2010), *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) (Feng *et al.*, 2009), *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) (Chen *et al.*, 2006; Ribeiro *et al.*, 2014), *Musca domestica* (L.) (Diptera: Muscidae) (Abbas *et al.*, 2015, 2016a, 2016b), *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) (Abbas *et al.*, 2012, 2014), *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae) (Sayyed *et al.*, 2008), and *Phenacoccus solenopsis* (Tinsley) (Hemiptera: Pseudococcidae) (Afzal *et al.*, 2015).

Understanding the biological parameters associated with insecticide resistance can improve integrated resistance management; however, this information has been lacking for *D. citri*. Therefore, we compared fitness and morphological characteristics between three populations of *D. citri* from commercially managed citrus groves in central Florida that exhibited resistance to neonicotinoid insecticides (thiamethoxam and imidacloprid) and two susceptible populations reared either in the absence (CLas⁻) or presence (CLas⁺) of the CLas pathogen.

Materials and methods

Insects

A susceptible laboratory population of *D. citri* was reared in a greenhouse at the Citrus Research and Education Center (CREC), University of Florida, Lake Alfred, FL. The culture was established in 2000 from field-collected insects in Polk County, Florida (27°86' N, 81°69' W) prior to the discovery of HLB in the state. This strain has been reared without exposure to insecticides or subsequent input of field-collected *D. citri* for approximately 320 generations. CLas⁻ *D. citri* were collected from a subculture free of CLas that was tested monthly using a quantitative real-time polymerase chain reaction (qPCR) assay for confirmation (Pelz-Stelinski *et al.*, 2010). The colony was maintained on sweet orange (*Citrus sinensis* (L) Osbeck) 'Valencia' in a temperature controlled greenhouse at 27–28°C, with 60–65% relative humidity and a 14:10 h (light:dark) photoperiod. CLas⁺ *D. citri* were obtained from a population reared on CLas-infected *C. sinensis* 'Valencia' plants housed in a separate facility at the University of Florida, CREC. The infection status of

these insects was confirmed as described below and the infection rate was 40%. All plants were 2–4 years of age.

The field populations of adult *D. citri* were collected from commercial citrus orchards in central Florida located in: Davenport (N: 28°09'933"; W: 81°37'907"), Clermont (N: 28°26'986"; W: 81°34'951"), and Lake Wales (N: 27°57'759"; W: 81°34'951") in 2017. About 3000 adults were collected from each location. Growers sprayed insecticides monthly for psyllid control. Insects were collected using a D-Vac insect suction sampler (Rincon-Vitova Insectaries, Ventura CA). Both the Davenport and Lake Wales populations had a CLas infection rate of 17%, while the infection rate in the Clermont population was 20% as determined by the qPCR. Tested insecticides were commercial formulations and included Admire Pro 4.6F (imidacloprid, LC) and Actara (thiamethoxam, SG). Admire pro 4.6F was obtained from Bayer Crop Science, USA and Actara was obtained from Syngenta, USA.

Adult feeding bioassay

An artificial diet (Hall *et al.*, 2010; Langdon and Rogers, 2017) was made by using 100 ml deionized water, 60 g sucrose (w/v; Fisher Scientific, Fair Lawn, NJ, Cat. No: S5-500), 0.2 ml green food dye (0.1% v/v; McCormick & Co., Inc. Hunt Valley, MD), and 0.8 mL yellow food dye (0.4% v/v; McCormick & Co., Inc. Hunt Valley, MD). The food solution was heated and mixed with a magnetic stirrer. When the sucrose was completely dissolved, deionized water was added to bring the final volume up to 100 ml. Aliquots of the stock 30% sucrose solution were then used to make serial dilutions of the insecticides. Caps were removed from 8 ml centrifuge tubes (Eppendorf Tubes, Hamburg, Germany, Cat. No: 033381D); the approximate dimensions of the 8 ml tubes were 1.3 cm × 5.5 cm. Five hundred and fifty microliters of the sucrose solution were added to each centrifuge tube cap and a 1.5 cm² piece of Parafilm M® (Bemis®, Neenah, WI) was stretched in both directions and placed over the treatment cap. While stretching the Parafilm tight, the excess Parafilm was wrapped over the back side of the cap forming a feeding membrane. Four to six adult *D. citri* were aspirated into the individual centrifuge tubes and the treatment filled cap was then pressed onto the centrifuge tube to allow feeding. The tubes were placed upright in a tray and held at 25°C on a 14:10 h light:dark photoperiod for 72 h. Each insecticide was tested over a range of concentrations (0, 0.001, 0.01, 0.1, 1, 10, 100, and 1000 ng μl⁻¹) and each concentration was replicated four times. Approximately 300 *D. citri* adults were tested from each resistant and susceptible population for each insecticide using the feeding bioassay. The mortality was determined 72 h after treatment. An insect was considered dead if there was no movement after being touched with a probe.

Effect of insecticide resistance on Asian citrus psyllid fertility and survivorship

One hundred and fifty to 200 mature nymphs reared from each test strain were collected and caged on potted plants. Adults that emerged within four days were aspirated into cylindrical cages (diameter: 110 mm; height: 130 mm). For each experiment, 100 mixed sex adults from the above colonies were transferred to a group of eight potted 'Swingle' *C. aurantifolia* (Chrism) plants (1 year old) with new leaf flush (12 cm × 11 cm) for a 24-h oviposition period. At the end of this period, the adults were removed from the plants and the number of eggs was counted using a

stereomicroscope (Leica, Wild M3C, Leica Microsystems Inc, Buffalo Grove, IL, 6.4X). Following egg hatch, the fertility of each strain was determined at the onset of the experiment. The plants were returned to the growth chamber at 25°C, 60% RH, and a 14:10 h light:dark photoperiod. Thereafter, eggs and nymphs were counted daily. The nymphal instars were identified according to the size of the insect body and development of wing pads (Tsai and Liu, 2000). Survival of psyllids was recorded for each life stage: eggs, first-second-instar nymphs, third-instar nymphs, fourth-fifth-instar nymphs, and adults to determine cumulative mortality (K -value) of psyllids in response to pathogen infection and insecticide resistance. This was calculated as:

$$K = -\ln(s) \text{ and } K = \sum k_i$$

where k was the negative natural logarithm of survival (s) for each life stage and K was the sum of all k -values for the entire life cycle. The magnitude of the K -value reflects the risk of mortality for a treatment group, such that mortality of a group increases as k -values increase (Pelz-Stelinski and Killiny, 2016).

Effect of insecticide resistance on fecundity and longevity

The objective of this experiment was to determine the effect of insecticide resistance or CLas infection on *D. citri* fecundity and longevity. Five colonies of *D. citri* were compared; two susceptible laboratory cultures (CLas⁻ and CLas⁺) and three field populations exhibiting insecticide resistance and varying levels of CLas infection. Twenty pairs of virgin adults from each population were sexed and then transferred as male and female pairs onto CLas⁻ 'Swingle citrumelo' (*Citrus paradisi* Macf. × *Poncirus trifoliata* L. Raf) plants. Citrus plants with psyllids were held in an environmental chamber at 25°C and 60% RH under a 14:10 h (light:dark) photoperiod to reflect typical field conditions. Egg production was determined by counting the total number of eggs laid by each female for 70 days. Eggs were counted and adults were transferred to new plants at 3–5 days intervals to promote feeding and oviposition on new flush throughout the experiment. To determine total egg deposition per female, leaf flush was removed with a sterile scalpel and the number of eggs was counted using a stereomicroscope.

Population growth and reproductive rate

Data were analyzed as stage dependent life tables (Birch, 1948; Pelz-Stelinski and Killiny, 2016; Chen *et al.*, 2017). Life tables were constructed from the cohort of eggs laid by the same females on the same day. Twenty females were used for each treatment. The net reproductive rate (R_0) was calculated as the number of female progeny produced per female per generation, assuming a 1:1 sex ratio, as per the following equation:

$$R_0 = \sum (l_x m_x)$$

x : time (days); l_x : proportion of females alive at time x , and; m_x : age-specific fecundity (average daily number of eggs laid by females per treatment divided by 2 to compensate for the 1:1 sex ratio of progeny).

The intrinsic rates of population increase for CLas⁺ and CLas⁻, susceptible psyllids, as well as, field-collected, insecticide resistant

populations of varying infection status were calculated as described by Birch (1948) and Chen *et al.* (2017) as: $r_m = \ln R_0/T$
 T is the generation time (in days) calculated as:

$$T = \frac{\sum x l_x m_x}{\sum l_x m_x}$$

The finite rate of population increase representing the number of females produced per female per day was calculated (Birch, 1948; Pelz-Stelinski and Killiny, 2016) as:

$$\lambda = \exp(r_m)$$

$$\lambda = \frac{R_0^1}{\sum x(l_x m_x)/R_0}$$

The relative fitness (Rf) was calculated by the following method (Cao and Han, 2006; Afzal *et al.*, 2015):

Rf = R_0 of experimental population/ R_0 of susceptible population.

Adult weight

The weight of male and female adults from the three field populations, as well as, the CLas⁻ and CLas⁺, laboratory susceptible populations were recorded using a Mettler AE 160 (Mettler-Toledo Columbus OH USA) balance. *D. citri* were sampled at random from each population obtaining the following sample sizes: laboratory CLas⁻ (male: 28; female: 41), laboratory CLas⁺ (male: 20; female: 20), Clermont (male: 68; female: 65), Davenport (male: 43; female: 45), and Lake Wales (male: 16; female: 6).

Morphological measurements

Morphological measurements for adult *D. citri* were made using a stereomicroscope. Insects were killed by freezing (−20°C for 30 min) and held using a piece of double-sided sticky tape. Body length, abdominal length, wing length, femur length, and head width were measured for two laboratory susceptible cultures and three field populations at 10× magnification with a 1.2 mm ocular ruler. At least 40 individuals from each population were measured.

DNA isolation and real time PCR assays

Deoxyribonucleic acid (DNA) was isolated from *D. citri* using a DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) according to the manufacturer's protocol with a modification for the isolation of bacterial DNA from arthropods (Li *et al.*, 2006; Pelz-Stelinski *et al.*, 2010). A Las specific 16S ribosomal DNA probe (5'-FAMAGACGGGTGAGTAACGCG-3BHQ-3') and primers (LasF: 5'TCGAGCGCGTATGCAATACG-3'; LasR: 5'-GCGTTATCCCGTAGAAAAAGGTAG-3') were used in qPCR assays to detect CLas (Li *et al.*, 2006; Pelz-Stelinski and Killiny, 2016). In addition to target DNA, internal control primers were used in multiplex qPCR amplification of samples, as described previously (Pelz-Stelinski *et al.*, 2010). Each reaction tube contained a primer and probe set to amplify the psyllid wingless gene (Wg) [(WgF: 5'GCTCTCAAAGATCGGTTTGACGG-3'; WgR: 5'-GCTGCCACGA ACGTTACCTTC-3'), Wg probe (5'-JOE-TTACTGACCATCAC TCTGGACGC-3BHQ2-3')]. The quantitative PCR scheme for all assays consisted of 2 min at

50°C, 10 min at 95°C, and 3 min 40 cycles with 15 s at 95 and 60°C 1 min. Samples were considered positive for CLAs if a product was amplified within the 40 amplification cycles used for reactions.

Statistical analysis

Mortality data of *D. citri* were subjected to Probit analysis using SAS software (SAS Institute Inc, Cary, 9.4, 2002–2012, NC, USA) for the determination of LC₅₀ values and their 95% fiducial limits (FLs) (Finney, 1971). The data were corrected for control mortality using Abbott's formula (Abbott, 1925). In order to determine the best response metric for calculating RRs, the relative precision of 95% FLs for LC₅₀ values was calculated as the width of the 95% FL divided by the LC₅₀ values obtained from the CLAs⁻ population. The RRs and their 95% FL were calculated for each field-collected and laboratory population according to Robertson and Preisler (1992) and Bilbo *et al.* (2019) using the laboratory CLAs⁻ colony as the susceptible reference.

Insect survival was analyzed using the Kaplan–Meier method and pairwise comparisons between treatment groups were made using the log rank (Mantel Cox) test. The psyllid weight abdominal length, femur length, head width, and wing length were analyzed using an analysis of variance (ANOVA) with sex and colony as fixed effects (PROC GLM; SAS institute, 2002–2012).

Female longevity, number of eggs per female, finite rate of population increase, and net reproductive rate were compared between groups of the three insecticide resistant strains and the CLAs⁻ and CLAs⁺ susceptible strains using a one-way ANOVA ($\alpha = 0.05$) followed by Tukey's mean separation (PROC GLM; SAS Institute, 2002–2012). Population growth rate, reproductive rate, fecundity, fertility, and morphological measurements were dependent variables in linear regression models with the resistance ratio as the independent variable (PROC GLM; SAS Institute, 2002–2012). The models' assumption of equality of variance was assessed using a plot of residuals vs. predicted values. The assumption of normality in the residuals was assessed

using a q–q plot. The independent variable was log transformed for ANOVA and both the dependent and independent variables were log transformed for regression.

Results

Susceptibility of laboratory and field populations to thiamethoxam and imidacloprid

All field populations exhibited more resistance than either of the laboratory susceptible colonies (Lab CLAs⁻: 0.22–1.52 ng μL^{-1} ; Lab CLAs⁺: 0.11–0.68 ng μL^{-1}) except for the Clermont population to imidacloprid (0.9–1263 ng μL^{-1}) (table 1 and fig. 1). The Lake Wales, Clermont, and Davenport populations exhibited resistance to imidacloprid, with resistance ratios ranging from 7.65–16.11. For thiamethoxam, resistance ratios in the field populations ranged from 26.79–49.09 compared with the susceptible CLAs⁻ laboratory population.

Fecundity and fertility

There was a significant relationship between resistance ratio and fecundity, with a reduction in eggs with increasing resistance ratio for both insecticides (table 2). There was a significant relationship between resistance ratio and fertility, with a reduction in egg survival with increasing resistance ratio for both insecticides (table 2).

Survival

Survival of first ($F = 0.33$; $df = 4, 35$; $P = 0.86$), second ($F = 0.33$; $df = 4, 35$; $P = 0.53$), third ($F = 0.53$; $df = 4, 35$; $P = 0.99$), and fourth ($F = 0.99$; $df = 4, 35$; $P = 0.42$) instar nymphs did not differ between the three field populations and the susceptible populations (table 3). Survival of fifth instar nymphs from the Davenport population was significantly higher ($F = 2.74$; $df = 4, 35$; $p = 0.044$) than that observed in all of the other populations measured (table 3).

Table 1. Susceptibility of *D. citri* adults to thiamethoxam and imidacloprid as measured by artificial diet feeding bioassay

Insecticides	Population	χ^2	<i>df</i>	<i>P</i>	Slope \pm SE	LC ₅₀ (95% FL) (ng μL^{-1})	RR ₅₀ (95% FL)
Thiamethoxam							
	Lab CLAs ⁻	1.66	5	0.36	0.60 \pm 0.09	0.66 (0.22–1.52)	1 (0.22–4.50)
	Lab CLAs ⁺	3.28	5	0.35	0.72 \pm 0.10	0.32 (0.11–0.68)	0.48 (0.11–1.99)
	Clermont	2.42	5	0.79	0.33 \pm 0.04	17.76 (2.50–26.05)	26.79 (4.40–163.16)
	Davenport	3.99	5	0.55	0.34 \pm 0.05	32.54 (10.10–151)	49.09 (7.13–338.10)
	Lake Wales	7.34	5	0.20	0.32 \pm 0.04	21.77 (6.95–92.11)	32.84 (6.42–168.06)
Imidacloprid							
	Lab CLAs ⁻	3.16	5	0.68	0.40 \pm 0.04	0.82 (0.30–2.16)	1 (0.25–4.06)
	Lab CLAs ⁺	3.57	5	0.61	0.39 \pm 0.04	0.67 (0.24–1.78)	0.83 (0.20–3.42)
	Clermont ^a	2.19	5	0.87	0.18 \pm 0.05	11.44 (0.9–1263)	14.10 (0.61–325.57)
	Davenport	1.04	5	0.90	0.27 \pm 0.04	6.22 (1.66–30.58)	7.65 (1.40–44.97)
	Lake Wales	1.96	5	0.85	0.32 \pm 0.04	13.09 (4.00–57.71)	16.11 (2.88–90.25)

^aLC₅₀ values generated by SAS exceed the maximum concentration while actual mortality was lower.

^bResistance ratios calculated using LC₅₀ from field populations or CLAs⁺ colony divided by LC₅₀ of CLAs⁻ susceptible laboratory colony. Robertson and Preisler (1992), Bilbo *et al.* (2019)

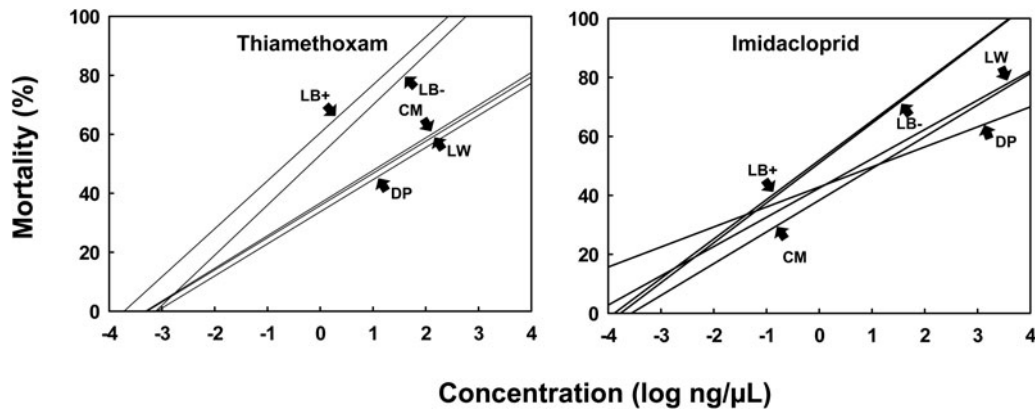


Figure 1. Dose-response curves for *D. citri* populations treated with serial dilutions of thiamethoxam or imidacloprid in water. Abbreviations identify geographic origins of each population (LB⁻, Laboratory CLas⁻; LB⁺, Laboratory CLas⁺; CM, Clermont; DP, Davenport; LW, Lake Wales).

Table 2. Regressions of fecundity and fertility against the thiamethoxam and imidacloprid resistance ratio.

Parameter ^a	Insecticide	Intercept				Resistance ratio			
		Estimate	SE	t-Value	P-value	Estimate	SE	t-Value	P-Value
Fertility									
	Thiamethoxam	-0.42	0.02	-22.21	<0.0002	0.05	0.01	7.90	0.0042
	Imidacloprid	-0.43	0.03	-16.66	<0.0005	0.08	0.01	6.02	0.0092
Fecundity									
	Thiamethoxam	13.43	0.49	27.40	<0.0001	-0.64	0.18	-3.63	0.03596
	Imidacloprid	13.61	0.43	32.02	<0.0001	-0.100	0.22	-4.58	0.00195

^aModels with fertility as dependent variable were log transformed. Models with fecundity as dependent variable were square root transformed; fertility: $df=1, 38$; fecundity: $df=1, 98$.

Population growth and reproductive rate

The net reproductive rate of the Lake Wales field population was significantly lower ($F=6.1$; $df=4, 93$; $P<0.001$) than that for all other field and laboratory populations (table 4). Similarly, the finite rate of population increase was significantly lower ($F=5.67$; $df=4, 93$; $P<0.001$) for the Lake Wales population as compared to all others measured (table 4). There were no significant differences between the populations for adult longevity ($F=0.86$; $df=5, 58$; $P=0.50$); however, CLas⁻ and CLas⁺ laboratory females laid significantly more ($F=3.26$; $df=5, 58$; $P=0.01$) eggs than females from the Lake Wales field population (table 4).

Adult weight

The source of collected insects (laboratory susceptible and field collected populations) and sex were significant independent variables in a model predicting $\ln(\text{weight})$ ($F=11.2$; $df=9, 342$; $P<0.001$). Adult weight ($F=9.23$; $df=4, 342$; $P<0.001$) and sex ($F=58.25$; $df=1, 342$; $P<0.001$) varied among colonies with a non-significant interaction term ($F=1.09$; $df=4, 342$; $P=0.362$) that was removed from the model (fig. 2). While males were smaller than females and size differed between populations, the size difference between males and females did not vary depending on source population. Examining population differences for males using a Tukey test, we found that males from the CLas⁻, laboratory population weighed more than CLas⁺, laboratory

males, and those from the Clermont population. There were no statistical differences in female weight between the populations examined.

Morphological characteristics

There were no significant differences between the populations for either head width or femur length (table 5). The CLas⁺ laboratory population had significantly shorter wings than the Clermont population, but there were no other differences in the wing length. Psyllids from Lake Wales had shorter body length than any other population, but *D. citri* from both Lake Wales and Clermont had shorter abdomens than the other four populations. There was a significant effect of sex for all measurements except femur length and in all cases, females were larger than males (table 5).

Discussion

We detected reduced susceptibility to neonicotinoids among the tested *D. citri* field populations (table 1 and fig. 1). In general, resistance was more pronounced with thiamethoxam than with imidacloprid and occurred at all sampled locations. Furthermore, the CLas⁺ susceptible colony was more sensitive to the insecticides evaluated than the CLas⁻ laboratory population. This is consistent with earlier surveys of *D. citri* populations in Florida (Chen *et al.*, 2017; Langdon and Rogers, 2017). *Candidatus Liberibacter asiaticus* infection increased the

Table 3. Survival rate of immature stages of *D. citri* from three field resistant and laboratory susceptible populations.

Population ^a	Laboratory CLas ⁻	Laboratory CLas ⁺	Clermont	Lake Wales	Davenport
1st instar					
Survival rate	78.30a	82.72a	68.14a	42.43a	65.39a
K-Value	0.25	0.19	0.38	0.86	0.43
2nd instar					
Survival rate	86.30a	89.38a	81.67a	78.72a	83.33a
K-Value	0.15	0.11	0.20	0.24	0.18
3rd instar					
Survival rate	85.10a	87.20a	84.06a	81.25a	74.58a
K-Value	0.16	0.14	0.17	0.21	0.29
4th instar					
Survival rate	91.96a	90.28a	87.50a	87.50a	80.63a
K-Value	0.08	0.10	0.13	0.13	0.21
5th instar					
Survival rate	88.42a	93.75a	85.42a	87.50a	100b
K-Value	0.12	0.07	0.16	0.13	0
Egg to adult					
Survival rate	38.68a	54.43a	24.25a	14.92a	15.97a
K-Value	0.94	0.60	1.41	1.90	1.83
K	1.7	1.21	2.45	3.34	4.26

^aMeans followed by the same letter within a row are not significantly different.

Table 4. Fitness parameters (\pm SE) of thiamethoxam and imidacloprid resistant field populations of *D. citri* as compared with laboratory controls

Analyzed parameter	Lab CLas ⁻	Lab CLas ⁺	Clermont	Davenport	Lake Wales
Insecticide susceptibility ^a	SS	SS	RR	RR	RR
Finite rate of increase (λ) ^b	1.90 \pm 0.27a	2.01 \pm 0.32a	1.41 \pm 0.32a	1.38 \pm 0.91a	1.09 \pm 0.28b
Longevity of female (day)	50.35 \pm 3.24a	52.70 \pm 3.63a	50.75 \pm 3.43a	52.50 \pm 3.38a	51.95 \pm 3.84a
Egg number of per female	182.40 \pm 27.46a	192.35 \pm 22.29a	138.30 \pm 23.17a	133.15 \pm 21.02a	101.95 \pm 23.53b
Net reproductive rate (R_0)	91.20 \pm 11.72a	96.18 \pm 11.15a	89.15 \pm 11.58a	66.58 \pm 11.51a	50.98 \pm 11.75b
Relative fitness ^c	1	1.05	0.98	0.73	0.56

^aSS: susceptible line; RR: insecticide resistant line.

^bMeans followed by the same letter within a row are not significantly different.

^cRf = R_0 of experiment population/ R_0 of laboratory susceptible CLas⁻ population.

susceptibility of *D. citri* to insecticides relative to CLas⁻ *D. citri* and this result is also consistent with previous investigations (Tiwari *et al.*, 2010). nAChRs are ligand-gated ion channel receptor complexes that mediate fast cholinergic synaptic transmission. nAChRs play a central role in the mediation of fast excitatory synaptic transmission in the insect central nervous system and are also the targets of commercially important classes of insecticides (Salgado and Saar, 2004; Oliveira *et al.*, 2010). Neonicotinoids are agonists that mimic the action of acetylcholine and therefore have widespread use against a broad spectrum of sucking and certain chewing insect pests (Oliveira *et al.*, 2011; Salgado, 2016). Following instances of misuse or overuse, nicotinic insecticide

resistance can contribute to potentially reduced performance of these insecticides (Oliveira *et al.*, 2011; Salgado, 2016).

Several biological parameters were compared between *D. citri* that exhibited resistance to thiamethoxam and imidacloprid in the field vs. their counterparts from known laboratory susceptible cultures. There was a significant relationship between resistance ratio and fecundity with egg production reduced proportionally with increasing resistance ratio for both insecticides (table 2). As indicated in tables 3 and 4, both laboratory susceptible populations exhibited higher egg to adult survival than field resistance populations. Furthermore, the egg to adult survival rate in the Lake Wales population was between 2.6 and 3.4-fold lower than

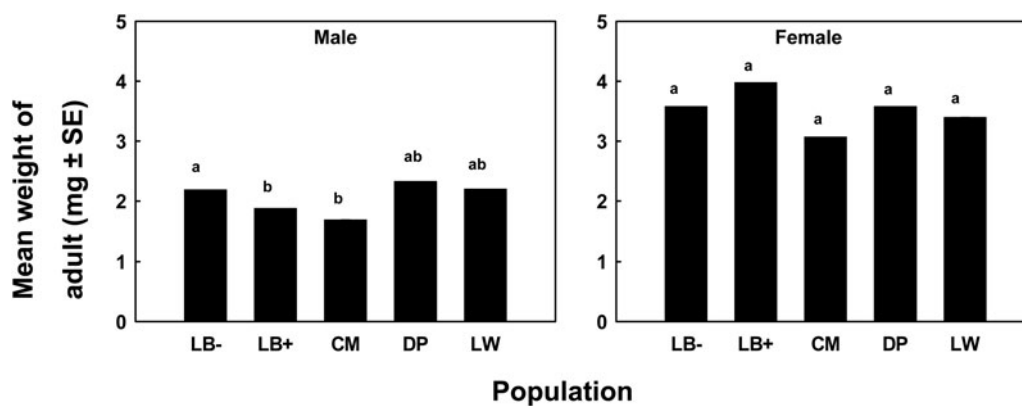


Figure 2. Mean weight (\pm SE) of adult *D. citri* from laboratory susceptible and three insecticide resistant field populations (LB⁻, Laboratory CLas⁻; LB⁺, Laboratory CLas⁺; CM, Clermont; DP, Davenport; LW, Lake Wales) (SE < 0.0009).

Table 5. Morphometric comparison of five adult body characteristics (\pm SE) of *D. citri* from three field resistant and two susceptible populations

Populations	Abdomen length (mm)	Body length (mm)	Femur length (mm)	Head width (mm)	Wing length (mm)
Lab CLas ⁻	1.40 \pm 0.020a	2.93 \pm 0.018a	0.36 \pm 0.008a	0.54 \pm 0.009a	2.00 \pm 0.021ab
Lab CLas ⁺	1.39 \pm 0.020a	2.95 \pm 0.018a	0.36 \pm 0.008a	0.51 \pm 0.009a	1.99 \pm 0.021b
Clermont	1.27 \pm 0.020b	2.95 \pm 0.018a	0.36 \pm 0.008a	0.52 \pm 0.009a	2.08 \pm 0.021a
Davenport	1.42 \pm 0.020a	2.96 \pm 0.018a	0.37 \pm 0.008a	0.53 \pm 0.009a	2.02 \pm 0.021ab
Lake Wales	1.28 \pm 0.020b	2.85 \pm 0.018b	0.36 \pm 0.008a	0.53 \pm 0.009a	2.01 \pm 0.021ab
Model	0.0001	0.0001	0.9437	0.0301	0.0007
Sex	0.0001	0.0438	0.3158	0.034	0.0001
Population	0.0001	0.0002	0.99	0.2326	0.025
Sex \times Population	0.0191	0.0238	0.7152	0.0699	0.6704

Means for each colony followed by a different letter are significantly different by the Tukey test. ANOVA model *P*-values with sex and population as fixed effects are given below the LS means with *df* = 9, 200. The *P*-values for each variable follow.

that of the susceptible populations. Higher mortality of resistant than susceptible *D. citri* is an indicator of a fitness disadvantage. In this case, both fecundity and egg survival were lower in populations exhibiting insecticide resistance. Collectively, our results indicate that resistance to thiamethoxam and imidacloprid in *D. citri* corresponded with a developmental disadvantage and suggest a trade off between the distribution of resources toward detoxification and fitness. Analogous fitness costs associated with insecticide resistance have been reported previously (Liu and Han, 2008; Feng *et al.*, 2009; Abbas *et al.*, 2012; Kilot and Ghanim, 2012).

The finite rate of increase provides an estimate of insect population growth potential (Stark and Banks, 2003), which provides broader insight than individual life history parameters. Net reproduction is not, however, the only component needed to assess the potential of population growth because the finite rate of increase depends on fecundity, percentage egg hatch, and growth (Stark and Banks, 2003). In this study, the finite rate of increase in resistant populations was 23–43% lower than that of the laboratory susceptible (CLas⁻) population. This type of difference in net reproductive and finite rate of increase has also been observed between susceptible and resistant populations of *Tetranychus urticae* Koch (Acari: Tetranychidae) and is known to contribute the instability of milbemectin resistance (Nicastro *et al.*, 2010). Similarly, imidacloprid resistance is associated with a lower net

reproductive rate in *Spodoptera litura* (Fabricius) compared with laboratory susceptible counterparts (Abbas *et al.*, 2012). The decline in population growth of *D. citri* observed here appeared to be mainly due to the decreased fecundity and hatch rate.

Decreased *R_f* associated with insecticide resistance has been reported previously in various insect taxa and for various chemistries including thiamethoxam resistant *B. tabaci* (*R_f* = 0.53) (Feng *et al.*, 2009); imidacloprid resistant *S. litura* (*R_f* = 0.38) (Abbas *et al.*, 2012) and two populations of *N. lugens* (*R_f* = 0.17 and 0.10) (Liu and Han, 2008) and acetamiprid resistant *P. solenopsis* (*R_f* = 0.22) (Afzal *et al.*, 2015). The present study indicated that the *R_f* of the resistant Lake Wales population was 0.56 of the laboratory susceptible (CLas⁻) population (table 4). Our results are thus congruent with the general convention that insecticide resistant populations exhibit reduced fitness compared with susceptible counterparts (Denholm and Rowland, 1992).

In contrast, there are instances where limited fitness costs have been associated with insecticide resistance (Haubruege and Arnaud, 2001; Chen and Nakasuji, 2004; Baker *et al.*, 2007; Bielza *et al.*, 2008) or certain modifiers can compensate for resistance costs (Coustau *et al.*, 2000). The lack of quantifiable fitness costs related to resistance typically occurs under specific environmental conditions that would not be detected under experimental

laboratory conditions (Bourguet *et al.*, 2004). Also, certain pleiotropic effects might not be detected with current methods to measure costs dependent on body size and reproduction (Fry, 1993).

Given the interaction between *D. citri* and CLAs and its associated consequences on insect fitness, the Rf costs associated with insecticide resistance cannot be investigated in *D. citri* without considering this additional interaction. Acquisition of phytopathogens by herbivorous insect vectors can have population scale fitness consequences. Several leafhopper vectors exhibit enhanced fitness following acquisition of plant pathogens. Beanland *et al.* (2000) and Ebbert and Nault (2001) reported that leafhoppers exposed to plant pathogens live longer than their uninfected counterparts. Pelz-Stelinski and Killiny (2016) indicated that fecundity of CLAs⁺ *D. citri* is greater than that of uninfected counterparts and that there is a consequential trade off associated with CLAs acquisition, which decreases life span compared with uninfected counterparts. Our results were congruent with previous investigations in that more adult female *D. citri* were produced per day by CLAs⁺, as compared, with CLAs⁻ counterparts (Pelz-Stelinski and Killiny, 2016). Propagative pathogens can impose metabolic or immune costs on their hosts associated with multiplication (Nault, 1997). The hormonal regulation of immune function and metabolic allocation likely underlie life history trade-offs (Harshman and Zera, 2007). Our results confirm that acquisition of CLAs by *D. citri* causes a fitness costs, which should be compounded by the negative fitness consequences associated with insecticide resistance in this species.

In summary, our investigation indicates that CLAs infection increased susceptibility of *D. citri* to both thiamethoxam and imidacloprid. We then showed that field-collected populations of *D. citri* exhibited resistance to these insecticides. The net reproductive rate of resistant populations declined with increasing resistance ratio. An additional cost of resistance appears to be smaller body size in *D. citri*. The smaller size of resistant *D. citri* may result in reduced maximum dispersal distance and reduced reproduction (Langellotto *et al.*, 2000; Baguette and Schtickzelle, 2006; Lewis-Rosenblum *et al.*, 2015; Pelz-Stelinski and Killiny, 2016; Ejsmond *et al.*, 2018; Villa *et al.*, 2018). Further research is needed to identify cause and effect mechanisms vs. correlations between insecticide resistance in *D. citri* and fitness costs. Our investigation also suggests that the current widespread problem of insecticide resistance to neonicotinoids among populations of *D. citri* across Florida (Chen *et al.*, 2018) should be effectively mitigated by ceasing thiamethoxam and imidacloprid use for a period of at least 6 months (Chen *et al.*, 2018). Additional resistance management should include rotations of at least six insecticide modes of action to diversify selection forces and complicate the evolution of resistance (Chen and Stelinski, 2017).

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