Effects of Cold-Acclimation, Pathogen Infection, and Varying Temperatures on Insecticide Susceptibility, Feeding, and Detoxifying Enzyme Levels in Diaphorina citri (Hemiptera: Liviidae)

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Effects of cold-acclimation, pathogen infection, and varying temperatures on insecticide susceptibility, feeding, and detoxifying enzyme levels in *Diaphorina citri* (Hemiptera: Liviidae)

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Abstract

Infection of Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), with '*Candidatus*' Liberibacter asiaticus (Las), the causal pathogen of citrus greening disease or huanglongbing, increases psyllid susceptibility to insecticides. *Diaphorina citri* populations in citrus occur year-round in tropical and sub-tropical habitats, and thus insecticide applications for managing this plant disease vector occur over a wide temperature range (10–40 °C). During the winter season, *D. citri* is occasionally exposed to periods of freezing temperatures, when temperatures fall below −6.5 °C. In this investigation, we compared insecticide susceptibility of uninfected and Las-infected *D. citri* at various temperatures (20–37 °C). Cold-acclimated (6 ± 1 °C) *D. citri* adults were less susceptible to neonicotinoid insecticides as compared with non-acclimated controls, but this trend was not observed for other insecticides tested. A positive correlation between temperature and percentage mortality caused by chlorpyriphos, imidacloprid, spinetoram, and thiamethoxam was found irrespective of infection status when evaluated at temperatures ranging between 20 and 37 °C. In contrast, a negative correlation between temperature and percentage mortality was observed for fenpropathrin for both infected and uninfected psyllids. Glutathione S-transferase levels were negatively correlated with temperature, whereas levels of cytochrome P450 and general esterase were not correlated with temperature fluctuations. These results indicate that altered insecticide susceptibility due to temperature may not be related to glutathione S-transferase, cytochrome P450, and general esterase levels. *Diaphorina citri* adults that carried the Las bacterium had reduced *CYP4* transcript and protein levels, and ingested less than uninfected counterparts, as measured by the production of honeydew. *Diaphorina citri* adult feeding was greatest at 32 °C within the temperature range tested. Overall, annual temperature fluctuation does not appear to be a major factor impacting management of *D. citri*.

Key Words: citrus greening; detoxifying enzymes; feeding; honeydew; huanglongbing; insecticide susceptibility

Resumen

La infección del psílido asiático de los cítricos, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), con '*Candidatus*' Liberibacter asiaticus (Las), el patógeno que causa Huanglongbing, aumenta la susceptibilidad del psílido a los insecticidas. Poblaciones de *D. citri* en cítricos se producen durante todo el año en los hábitats tropicales y subtropicales, de ahi que las aplicaciones de insecticida para el manejo de este vector de enfermedades de las plantas ocurren en un amplio rango de temperaturas (10–40 °C). Durante la temporada de invierno, *D. citri* ocasionalmente se expone a períodos de congelación, cuando las temperaturas caen por debajo de −6.5 °C. En esta investigación, se comparó la susceptibilidad a insecticidas de *D. citri* infectados y no infectados de Las a diversas temperaturas (20–37 °C). Los adultos de *D. citri* aclimatadas al frío (6 ± 1 °C) fueron menos susceptibles a los insecticidas neonicotinoides, en comparación con los controles no aclimatados, pero esta tendencia no se observó para otros insecticidas probados. Se encontró una correlación positiva entre la temperatura y el porcentaje de mortalidad a clorpirifos, imidacloprid, spinetoram y tiametoxam independente del estado de la infección cuando se evalúa a 20–37 °C. En contraste, se observó una correlación negativa entre la temperatura y el porcentaje de mortalidad para fenpropatrin tanto en los psílidos infectados y no infectados. Los niveles de glutatión S- transferasa se correlacionaron negativamente con la temperatura, mientras que los niveles de citocromo P450 y esterasa en general no se correlacionaron con los cambios de temperatura. Estos resultados indican que la susceptibilidad a los insecticidas alterados debido a la temperatura puede no estar relacionada con la glutatión S-transferasa, citocromo P450, y los niveles de esterasa generales. Los adultos de *D. citri* que llevan la bacteria Las tenian el transcripto *CYP4* y niveles de proteína reducidos, y se alimenta menos de sus contrapartes no infectados, en base a la medida de la producción de mielcilla. La alimentación de los adultos de *D. citri* fue mayor a los 32 °C dentro del rango de temperaturas probadas. En general, la fluctuación anual de la temperatura no parece ser un factor importante que afecte el manejo de *D. citri*.

Palabras Clave: enverdecimiento de los cítricos; enzimas desintoxicantes; alimentación; mielcilla; Huanglongbing; susceptibilidad a los insecticidas

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), and huanglongbing (HLB) are the greatest threats to commercial citrus production worldwide. Direct feeding by *D. citri* nymphs and adults destroys new flush, causes fully developed leaves to curl, and

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promotes growth of sooty mold. *Diaphorina citri* transmits the putative causal agent of HLB, '*Candidatus*' Liberibacter asiaticus (Las) in the U.S. (Halbert & Manjunath 2004; Manjunath et al. 2008; Grafton-Cardwell et al. 2013). HLB causes stunting, off-season bloom, premature fruit drop, and the production of small, misshapen, and bitter fruit (Halbert & Manjunath 2004).

Currently, the chemicals available for management of *D. citri* include insect growth regulators/antifeedants, microbials, neonicotinoids, organophosphates, and pyrethroids (Srinivasan et al. 2008; Boina et al. 2009; Sétamou et al. 2010; Tiwari et al. 2011a, 2012a,b, 2013a,b). The efficacy of insecticides under field conditions is known to vary depending on environmental factors such as temperature, rainfall, and humidity, and non-environmental factors such as insecticide coverage, host plants, host infection status, and color morphotypes (Wood et al. 1981; Scott 1995; Verkerk & Wright 1996; Musser & Shelton 2005; Rogers & Stansly 2007; Satpute et al. 2007; Boina et al. 2009; Tiwari et al. 2011b,c,d, 2013b). The toxicity of an insecticide at a given temperature depends on its class, the target pest, spray coverage, and application method (Musser & Shelton 2005). The wide range of annual temperatures (10–40 °C) in tropical and sub-tropical areas where *D. citri* occurs causes variation in toxicity of insecticides (Boina et al. 2009). During winter in Florida, *D. citri* is occasionally exposed to periods of freezing weather, when temperatures fall below −6.5 °C (Miller & Glantz 1988). However, a large proportion of *D. citri* adults and nymphs survive during these freezes (Hall et al. 2011).

The LC_{50} values for various insecticides, as a function of temperature variation, have been determined previously for *D. citri* (Boina et al. 2009). However, this has not been investigated with formulated insecticides used in the field. In addition, the effect of cold-acclimation on insecticide toxicity has not been investigated for *D. citri*. In the present study, insecticide susceptibility of cold-acclimated *D. citri* (exposed to 6 ± 1 °C for 1 or 2 wk) was compared with non-acclimated (exposed to 27–28 °C for 1 or 2 wk) controls. Additionally, experiments were conducted to quantify changes in feeding behavior and changes in the expression levels of general esterase, glutathione S-transferase, and cytochrome P450 of *D. citri* at various temperatures. General esterase, glutathione S-transferase, and cytochrome P450 are the detoxifying enzyme systems that have been implicated previously with insecticide resistance in *D. citri* (Tiwari et al. 2011a,c). We also investigated the effect of Las infection status on temperature–toxicity correlations of various insecticides against *D. citri*, and the effect of infection status on *CYP4* (cytochrome P450 Family 4, hemoprotein) transcript and protein levels.

Materials and Methods

Laboratory susceptible (LS) colonies of uninfected or Las-infected *D. citri* were continuously reared at the Citrus Research and Education Center (CREC), University of Florida, Lake Alfred, Florida, USA. The original colony was established in 2000 from field populations in Polk County, Florida, USA (28.0°N, 81.9°W) prior to the discovery of HLB in the state. The colonies were maintained on sour orange (*Citrus aurantium* L.; Sapindales: Rutaceae) seedlings with no insecticide exposure in greenhouses at 27 to 28 °C, 60 to 65% RH, and a 14:10 h L:D photoperiod. In addition, *D. citri* was collected in the field from a commercial citrus grove in Lake Alfred, Florida, USA, that is known historically to have high levels of HLB infection. Adults were collected using aspirators, transferred to the laboratory in coolers, and maintained on citrus plants in Plexiglass cages (40 × 40 × 40 cm) prior to use in the bioassays. Details on the bioassay are provided in the following subsections. After the bioassays were performed, *D. citri* was tested for Las using quantitative real-time PCR (qPCR) as described in Tiwari et al. (2010). Five insecticides were used in this study, and the modes of action and rates are provided in Table 1. Formulated products for each insecticide were used in the bioassays at the respective manufacturer's labeled rate. A mean rate was used when a manufacturer recommended a range of rates.

Insecticide Susceptibility in Cold-Acclimated *D. citri*

Field-collected adults were transferred to the laboratory in coolers and released onto citrus plants in Plexiglas cages (40 × 40 × 40 cm). The Plexiglas cages containing *D. citri* were transferred into a cold room set at 6 ± 1 °C, 50 ± 5% RH, and a 14:10 h L:D photoperiod, or were maintained at room temperature (27–28 °C) for 1 or 2 wk. After these time intervals, adults from both temperatures were evaluated using a leaf-dip Petri dish method developed by Prabhaker et al. (1989) and slightly modified as described in Hall et al. (2010) and Tiwari et al. (2011a). The bioassay arena consisted of 60-mm diameter plastic disposable Petri dishes (Fisherbrand, Thermo Fisher Scientific, Waltham, Massachusetts, USA) containing a 2 to 3 mm thick solidified bed of 1.5% agar solution. Leaf discs (60 mm diameter) from fresh citrus leaves were excised, dipped for 30 s in insecticide solutions made in water, and allowed to air dry in a fume hood for 1 h prior to bioassays. For the control treatment, leaf discs were dipped in distilled water alone.

After 1 h, leaf discs were placed on agar beds, and 20 to 30 adults were transferred into each dish using a camel hair brush. Adults were anesthetized briefly with CO_2 to facilitate handling and transfer. Petri dishes were wrapped with parafilm (Pechiney Plastic Packaging, Chicago, Illinois, USA) to prevent escape of psyllids. Sealed Petri dishes with adults were transferred into a growth chamber (Percival Scientific, Inc., Perry, Iowa, USA) set at 25 ± 1 °C, 50 ± 5% RH, and a 14:10 h L:D photoperiod. The mortality of adults was assessed 48 h after placement into the growth chamber. Adults that were found on their side or back and that were unable to move when probed with a camel hair brush were considered dead. All bioassays were repeated twice. The mean percentage mortality among adults exposed to various insecticides was compared using 3-way factorial analysis of variance (ANOVA)

Table 1. Insecticides tested against *Diaphorina citri* to determine the effects of cold-acclimation, pathogen infection, and different temperatures on physiology and biochemistry of this pest.

Common name	Trade name	Manufacturers' recommended rate for field application ha^{-1}	Class	Mode of action	Manufacturer/supplier
Chlorpyriphos	Lorsban 4E	5.86 L	Organophosphate	Acetylcholinesterase inhibitor	Dow AgroSciences LLC, Indianapolis, IN
Fenpropathrin	Danitol 2.4EC	1.16 L	Synthetic pyrethroid	Sodium channel modulator	Valent USA Corp., Walnut Creek, CA
Imidacloprid	Provado 1.6F	0.74 L	Neonicotinoid	Nicotinic acetylcholine receptor agonist	Bayer CropScience LP, Research Triangle Park, NC
Spinetoram	Delegate WG	0.28 kg	Microbial	Nicotinic acetylcholine receptor modulator	Dow AgroSciences LLC, Indianapolis, IN
Thiamethoxam	Actara 25WG	0.28 kg	Neonicotinoid	Nicotinic acetylcholine receptor agonist	Syngenta Crop Protection, Inc., Greensboro, NC

and contrast analyses were conducted using exposure period (2 levels), insecticide (5 levels), and cold-acclimation (2 levels) as main effects (PROC GLM) (SAS Institute 2004) ($P < 0.05$).

Effect of Temperature on the Feeding Activity of Las-Infected and Uninfected *D. citri*

Feeding activity of LS uninfected and Las-infected adults was measured by quantifying honeydew excretion during exposure to various temperatures. Given that certain insecticide formulations require ingestion by feeding, temperature-related variation in feeding activity may influence observed toxicity. Single adults were placed in a Petri dish with a leaf disc over an agar bed for 24 h. The Petri dish was sealed with a lid lined with 60 mm Whatman filter paper (Whatman International Ltd, Kent, United Kingdom). Petri dishes were wrapped with parafilm (Parafilm "M"®, Pechiney Plastic Packaging, Chicago, Illinois, USA), turned upside down, and transferred into temperature-controlled growth chambers (Percival Scientific, Inc., Perry, Iowa, USA) set at one of the following temperatures: 20 ± 1, 24 ± 1, 28 ± 1, 32 ± 1, or 37 ± 1 °C. All growth chambers were maintained at 50 ± 5% RH and a 14:10 h L:D photoperiod. Filter papers were collected and subjected to a ninhydrin (Sigma-Aldrich, St. Louis, Missouri, USA) test to count honeydew droplets (Nauen & Elbert 1997). Each treatment (*D. citri* type) was replicated 20 times at each temperature.

For treatments using Las-infected *D. citri*, each adult was transferred into a sterile 1.5 mL microcentrifuge tube containing 80% ethanol and stored at −20 °C for DNA extraction to confirm infection with Las using methods described below. A *D. citri* sample was considered positive for Las if the cycle quantification (Cq) value determined by the ABI 7500 real-time software was 35 or less (Tiwari et al. 2010). If a *D. citri* sample was found negative for the Las gene, the treatment was repeated until 20 Las-positive samples were obtained for each temperature. The effect of temperature and infection status on *D. citri* feeding activity was determined by a 2-way ANOVA, with *D. citri* type and temperature as main effects, followed by a Fisher's protected LSD mean separation test (PROC GLM) (SAS Institute 2004) ($P < 0.05$). A honeydew droplet of 2 × 2 mm was considered standard. Droplets larger than the 2 × 2 mm size were adjusted accordingly; for example, a 2 × 4 mm droplet was counted as 2 droplets. Likewise, droplets smaller than 2 × 2 mm were adjusted accordingly.

Effect of Temperature on Detoxifying Enzymes

The effect of temperature on expression levels of 3 detoxifying enzymes was investigated using the uninfected LS *D. citri* colony. Treatments consisted of imidacloprid- or spinetoram-treated adults maintained at 5 temperature regimes, described above, for 48 h. Each insecticide and temperature combination was replicated 3 times, and each combination was tested with 100 to 120 adults. Imidacloprid and spinetoram were prepared as solutions in distilled water and used at the manufacturers' label rates of 1.5 L/ha and 0.27 kg/ha, respectively. *Diaphorina citri* adults of mixed gender were applied onto leaves dipped in the above-described insecticide solutions in distilled water using the Petri dish method described above. About 30 to 40 adults were transferred to each Petri dish. After 48 h, surviving adults were subjected immediately to detoxifying enzyme assays.

The enzyme preparations were performed according to established protocols (Zhu & Gao 1999; Gao & Zhu 2000) with slight modifications. The total protein content of the enzyme preparation was determined with the bicinchoninic acid method using bovine serum albumin as a standard (Smith et al. 1985). The absorbance of the reaction product was measured in a 96-well microplate reader at 562 nm and 25 °C.

General esterase activity was measured using α-naphthyl acetate (α-NA) (Sigma-Aldrich, St. Louis, Missouri, USA) as a substrate (Srigiriraju et al. 2009; Tiwari et al. 2011b). Glutathione S-transferase activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma-Aldrich, St. Louis, Missouri, USA) as the substrate (Habig et al. 1974; Tiwari et al. 2011c). Cytochrome P450 activity was estimated by measuring heme peroxidase activity (Brogdon et al. 1997; Tiwari et al. 2011c). As heme constitutes the majority of cytochrome P450 in non-blood-feeding insects, the quantification of heme activity can be used to compare the levels of cytochrome P450 (Brogdon et al. 1997; Casimiro et al. 2006; Penilla et al. 2007). Heme peroxidase activity was measured using the substrate 3,3',5,5'-tetra-methylbenzidine (TMBZ) (Sigma-Aldrich, St. Louis, Missouri, USA). The effect of temperature on enzyme levels was determined separately for each insecticide by 1-way ANOVA followed by a Fisher's protected LSD mean separation test (PROC GLM) (SAS Institute 2004) ($P < 0.05$). Correlation analyses between temperature and enzyme levels were performed separately for each insecticide and enzyme combination (PROC CORR) (SAS Institute 2004).

CYP4 Gene Expression Analysis and CYP4 Protein Levels from Uninfected and Las-Infected *D. citri* Populations

The relative transcription levels of 5 *CYP4* genes, *CYP4C67*, *CYP4DA1*, *CYP4C68*, *CYP4G70*, and *CYP4DB1*, were determined using qPCR from uninfected and Las-infected *D. citri* populations. Methods for RNA isolation, cDNA synthesis, and qPCR, and primers for the 5 *CYP4* genes and the reference gene *actin* were as described in Tiwari et al. (2011b). RNA was isolated from groups of 25 adult *D. citri* from 5 uninfected and Las-infected populations (5 biological replicates). The infection rates ranged from 70% to 90% for Las-infected populations. Paired *t*-tests were conducted to compare the relative expression of each gene between the 2 populations. Values were considered statistically different at $P < 0.05$.

To determine potential differences in *CYP4*-associated protein expression levels between uninfected and Las-infected *D. citri*, subcellular, microsomal protein fractions were prepared as described in Wheeler et al. (2010). Protein concentrations were estimated with a QuickStart (Bio-Rad Laboratories, Hercules, California, USA) Bradford protein assay (Bradford 1976) with ovalbumin as the standard. Twenty-five µg of microsomal protein was electrophoresed through a sodium dodecyl sulphate–polyacrylamide gel and then transferred to a polyvinylidene fluoride membrane, and the membrane was blocked as described previously in Tiwari et al. (2013a,b). The membrane was then probed for *CYP4*-related protein in a western blot analysis as described by Tiwari et al. (2013a,b). Briefly, the membrane was incubated with 1:1,000 primary antibody in Tris-buffered saline (TBS) (polyclonal rabbit antibody, Anti-Cytochrome P450 19A1, Sigma-Aldrich, St. Louis, Missouri, USA) with shaking for 1 h. The membrane was washed 3 times with phosphate-buffered saline–Tween and subsequently incubated with 1:10,000 secondary antibody (Anti-Rabbit IgGs-Alkaline phosphatase, Cat. # A3937, Sigma-Aldrich, St. Louis, Missouri, USA) in TBS for 1 h. After washing, the membrane was developed using 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium chloride solution. Four independent samples from different rearing cages were used for both uninfected and Las-infected *D. citri* to represent 4 discrete populations.

Insecticide Susceptibility of Las-Infected and Uninfected *D. citri* Exposed to Varying Temperatures (20–37 °C)

Insecticide bioassays were conducted using a leaf-dip Petri dish method as described above. Leaf discs 60 mm in diameter were excised, dipped in the test insecticide solutions for 30 s, and allowed to air dry in a fume hood for 1 h prior to use in the bioassays. For the control treatment, leaf discs were dipped in distilled water alone. After 1 h, the leaf discs were placed in Petri dishes, and 20 to 30 adults of mixed gender were transferred into each dish using a camel hair brush. Petri dishes were wrapped with parafilm and transferred into temperature-controlled growth chambers (Percival Scientific, Inc., Perry, Iowa, USA) set at one of the following temperatures: 20 ± 1, 24 ± 1, 28 ± 1, 32 ± 1, or 37 ± 1 °C. All growth chambers were set at 50 ± 5% RH and a 14:10 h L:D photoperiod. For all insecticides, each concentration was replicated 3 times (n = 60–90 adults per insecticide). Bioassays for all insecticides and the control were repeated twice for each of the following *D. citri* treatment types: 1) uninfected laboratory colony, 2) field-collected and uninfected, and 3) field-collected and Las-infected. The mortality of adults was assessed 48 h after transfer into the growth chamber. Adults found on their sides or backs and unable to move when probed with a camel hair brush were considered dead. Percentage mortality in each treatment was corrected using Abbott's formula (Abbott 1925).

For bioassays using field-infected *D. citri*, each live or dead psyllid was transferred into a sterile 1.5 mL microcentrifuge tube (Fisher Scientific Co., Pittsburg, Pennsylvania, USA) containing 80% ethanol at −20 °C after the mortality data were recorded and prior to DNA extraction to confirm infection with Las by quantitative real-time PCR according to the protocol described in Tiwari et al. (2010). Adults found positive for Las comprised the field-collected and Las-infected treatment, and those found negative for Las comprised the field-collected and uninfected treatment. A *D. citri* sample was considered positive for Las if the cycle quantification (Cq) value determined by the ABI 7500 real-time software was 35 or less (Tiwari et al. 2010). Mortality data obtained from the 2 bioassays conducted for each *D. citri* treatment were pooled for subsequent analyses. The mean percentage mortality of *D. citri* was subjected to a 3-way ANOVA (PROC GLM) using *D. citri* treatment (uninfected LS, field-collected and uninfected, and field-collected and Las-infected), insecticide, and temperature as main effects (SAS Institute 2004). If a significant interaction was observed between main effects, subsequent analyses were performed to inspect for differences in mean percentage mortality among significant main effects (PROC GLM), followed by a Fisher's protected LSD mean separation test. Correlation analyses between temperature and percentage mortality were performed separately for each insecticide and *D. citri* treatment (PROC CORR) (SAS Institute 2004) ($P < 0.05$). Correlation coefficients obtained for each insecticide and *D. citri* treatment were compared using Fisher's Z transformation (PROC CORR) (SAS Institute 2004).

Results

Insecticide Susceptibility in Cold-Acclimated *D. citri*

A 3-way factorial ANOVA involving cold-acclimation, insecticide, and exposure time as main effects revealed that cold-acclimation (F = 8.16; df = 1, 100; P = 0.0052); insecticide (F = 3.32; df = 4, 100; P = 0.0134), interactions between cold-acclimation and insecticide (F = 2.51; df = 4, 100; P = 0.0467), and interactions between exposure time, cold-acclimation, and insecticide (F = 2.24; df = 8, 100; P = 0.0306) all had significant effects on the susceptibility of *D. citri*. However, insecticide susceptibility was not affected by exposure time (F = 2.90; df = 1, 100; P = 0.0918) and interactions between cold-acclimation and exposure time (F = 0.64; df = 1, 100; P = 0.4239). Comparisons of percentage mortality were performed for cold-acclimated versus control *D. citri* for each time period and for each insecticide (Table 2). *Diaphorina citri* that were cold-acclimated for 1 wk were less susceptible to imidacloprid than *D. citri* maintained at room temperature (Table 2). Likewise, *D. citri* that were cold-acclimated for 2 wk were less susceptible to thiamethoxam than *D. citri* maintained at room temperature (Table 2). Comparable mortality was found between cold-acclimated and control *D. citri* for the other insecticides tested (Table 2).

Effect of Temperature on the Feeding Activity of Las-Infected and Uninfected *D. citri*

PCR results showed that Las-infection among the *D. citri* analyzed ranged between 50 and 90%. A 2-way ANOVA indicated a significant effect of temperature (F = 5.71; df = 4, 288; $P < 0.0002$) and *D. citri* infection status (F = 6.09; df = 1, 288; $P < 0.0001$) on the number of honeydew droplets produced by an adult. The mean (± SE) number of honeydew droplets produced by Las-infected adults (3.2 ± 0.3) was significantly smaller than that produced by uninfected adults (4.9 ± 0.4). Based on the overall number of honeydew droplets recorded, 32 °C was the optimal temperature for feeding, resulting in production of significantly more honeydew droplets than the other temperatures examined (Table 3).

Effect of Temperature on Detoxifying Enzymes

According to ANOVA, treatment of *D. citri* with imidacloprid (F = 2.01; df = 4, 10; P = 0.1683) or spinetoram (F = 0.64; df = 4, 10; P = 0.6447) had no effect on cytochrome P450 activity (Fig. 1A). Likewise, correlation analysis revealed no significant relationship between temperature and cytochrome P450 activity for *D. citri* treated with either imidacloprid (r = −0.3761, P = 0.1671) or spinetoram (r = 0.0200, P = 0.9437). Temperature had no significant effect on general esterase activity levels for *D. citri* treated with imidacloprid (F = 0.76; df = 4, 10; P = 0.5457) or spinetoram (F = 1.28; df = 4, 10; P = 0.3394) (Fig. 1B). There was no significant relationship between temperature and general esterase activity for *D. citri* treated with either imidacloprid (r = 0.0954, P = 0.7348) or spinetoram (r = −0.2587, P = 0.3518). In contrast to the observations for cytochrome P450 and general esterase activities, temperature significantly affected the activity level of GST enzymes for *D. citri* treated with either imidacloprid (F = 3.63; df = 4, 10; P = 0.0446) or spinetoram (F = 12.23; df = 4, 10; P = 0.0007) (Fig. 1C). Temperature was negatively correlated with GST activity for *D. citri* treated with either imidacloprid (r = −0.7031, P = 0.0035) or spinetoram (r = −0.5857, P = 0.0218).

Table 2. Mean percentage mortality of cold-acclimated and control *Diaphorina citri* when exposed to various insecticides.

	Mean percentage mortality (± SE)		
Insecticide	Cold-acclimated (6 ± 1 °C)	Control (27–28 °C)	P value
	One-week exposure period		
Chlorpyriphos	99.2 ± 0.8	93.3 ± 3.1	0.4463
Fenpropathrin	76.1 ± 7.6	86.5 ± 6.7	0.1774
Imidacloprid	62.6 ± 14.1	90.8 ± 3.5	0.0004*
Spinetoram	84.2 ± 2.7	89.6 ± 1.6	0.4792
Thiamethoxam	87.7 ± 3.5	93.9 ± 1.6	0.4226
	Two-week exposure period		
Chlorpyriphos	97.5 ± 1.8	98.0 ± 1.2	0.9392
Fenpropathrin	94.9 ± 3.3	86.2 ± 8.0	0.2594
Imidacloprid	89.1 ± 4.6	98.2 ± 1.1	0.2362
Spinetoram	81.6 ±5.0	87.4 ± 5.6	0.4531
Thiamethoxam	77.1 ± 5.4	95.2 ± 2.4	0.0202*

*P values less than 0.05 represent a significant difference. P values were derived from the orthogonal contrast of variables involving interactions between exposure period (2 levels), insecticide (5 levels), and temperature (2 levels).

Table 3. Mean number of honeydew droplets produced by '*Candidatus*' Liberibacter asiaticus–infected and uninfected *Diaphorina citri* adults at various temperatures.

	Number (± SE) of honeydew droplets per adult		
Temperature (°C)	Las-infected	Uninfected	Mean[a]
20	2.8 ± 0.5	4.2 ± 1.1	3.7 ± 0.7 b
24	3.8 ± 0.7	4.1 ± 0.9	3.7 ± 0.6 b
28	2.7 ± 0.6	3.0 ± 0.7	2.9 ± 0.5 b
32	5.2 ± 1.0	7.9 ± 1.1	7.0 ± 0.8 a
37	2.4 ± 0.7	4.7 ± 0.9	4.1 ± 0.7 b

[a] Means followed by different lowercase letters for each temperature are significantly different from one another ($P < 0.05$).

Correlation of Las-Infection Status and *CYP4* Transcript Levels and *CYP4*-Related Protein Expression in Las-Infected *D. citri*

With the exception of *CYP4DA1*, which remained similar between uninfected and Las-infected *D. citri* populations, the relative abundance values of the remaining 4 *CYP4* transcripts were significantly downregulated in Las-infected compared with uninfected *D. citri* populations ($P < 0.05$; Fig. 2A). Western blot analysis showed a strong signal of a band corresponding to a 45 kDa protein in uninfected *D. citri* populations (Fig. 2B). This band corresponded with the *CYP450* proteins that cross-reacted with the anti-cytochrome P450 19A1 antibody (Tiwari et al. 2013a,b). Previously, a positive correlation between the expression of this protein and insecticide resistance has been shown (Tiwari et al. 2013a). This band was reduced significantly in Las-infected *D. citri* populations, demonstrating a drop in protein expression levels that was concomitant with reduced transcript levels in Las-infected compared with uninfected *D. citri* populations (Figs. 2A and 2B).

Insecticide Susceptibility of Las-Infected and Uninfected *D. citri* Exposed to Different Temperatures (20–37 °C)

Diaphorina citri treatment (infection status), insecticide, temperature, and main-effect interactions between *D. citri* treatment and insecticide, *D. citri* treatment and temperature, and insecticide and temperature significantly affected mean percentage mortality of *D. citri* adults (Table 4). Consequently, separate ANOVAs and mean separation tests were performed within each *D. citri* treatment to determine the effects of temperature and insecticide on mean percentage mortality of *D. citri*. Additionally, separate ANOVAs and mean separation tests were performed for each temperature and insecticide to determine the effects of *D. citri* treatment on mean percentage mortality. The mean Las infection rate found in field-collected *D. citri* ranged from 5 to 10%.

For the uninfected LS *D. citri* colony, ANOVA indicated that the main effects insecticide ($F = 22.59$; df= 4, 125; $P < 0.0001$) and temperature ($F = 3.62$; df = 4, 125; $P = 0.0079$) and the interaction between main effects ($F = 10.25$; df = 16, 125; $P < 0.0001$) had significant effects on mortality. ANOVA performed for each insecticide indicated that temperature had a significant effect on *D. citri* mortality for fenpropathrin ($F = 11.66$; df = 4, 25; $P < 0.0001$), imidacloprid ($F = 16.23$; df = 4, 25; $P < 0.0001$), spinetoram ($F = 12.68$; df = 4, 25; $P < 0.0001$), and thiamethoxam ($F = 16.25$; df = 4, 25; $P < 0.0001$), but no effect was observed for chlorpyriphos ($F = 1.62$; df = 4, 25; $P = 0.2011$) (Table 5). There was a

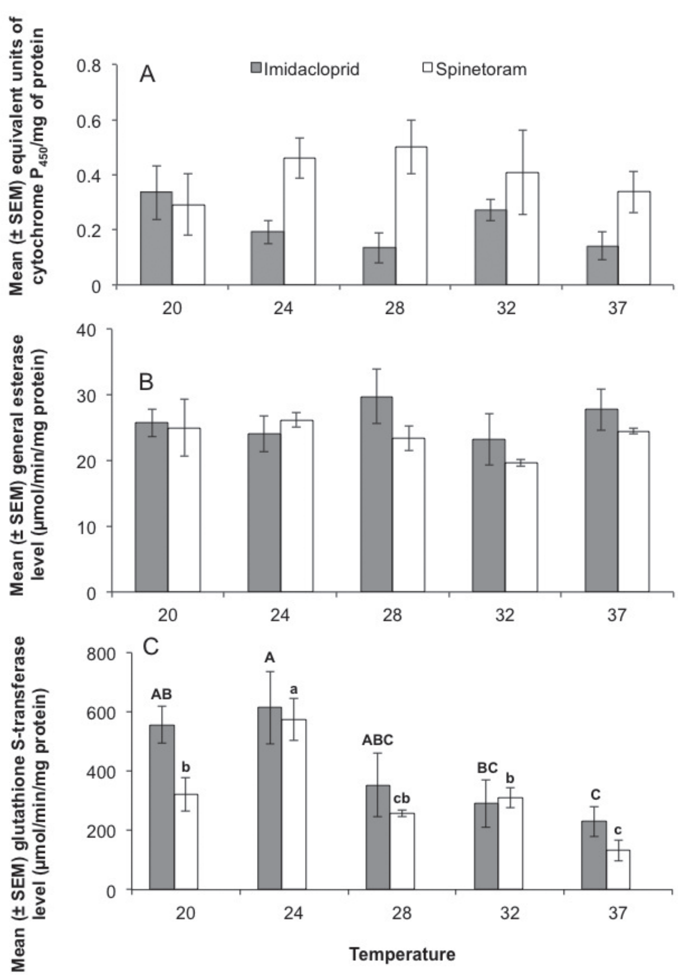

Fig. 1. Comparison of cytochrome P450 (A), general esterase (B), and glutathione S-transferase (C) activity levels in laboratory susceptible *Diaphorina citri* adults at 5 temperatures. For glutathione S-transferase, means with the same uppercase letters are not significantly different from one another for imidacloprid-treated *D. citri.* Means with the same lowercase letters are not significantly different from one another for spinetoram-treated *D. citri*.

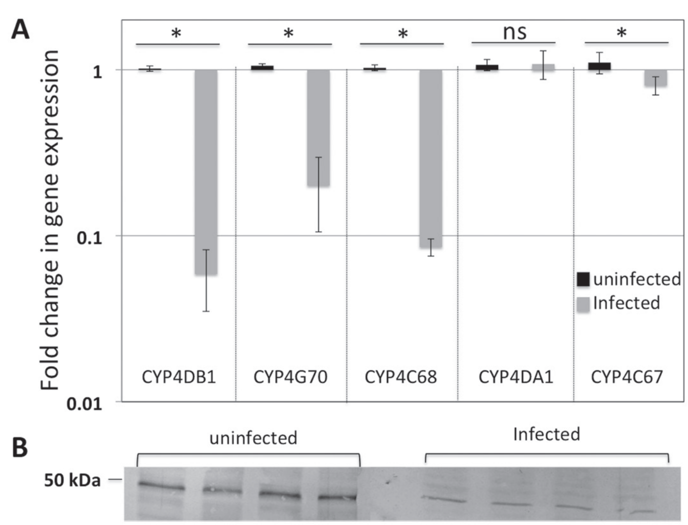

Fig. 2. Quantification of the transcription levels of 5 *CYP4* genes from uninfected and Las-infected *Diaphorina citri* populations (A). Cq values were first normalized to the endogenous control gene *actin*. Standard deviations were calculated on the basis of 5 independent samples with 3 technical replicates each. Asterisks indicate statistically significant differences ($P < 0.05$). Western blot of CYP4 microsomal proteins from 4 independent samples isolated from uninfected and Las-infected *D. citri* (B).

Table 4. ANOVA results for the effect of temperature on the susceptibility of *Diaphorina citri* to insecticides.

Treatment	df effect, error	*F* value	*P* value
D. citri treatment type	2, 225	26.81	< 0.0001
Temperature	4, 225	20.20	< 0.0001
Insecticide	4, 225	17.39	< 0.0001
D. citri treatment type × temperature	8, 225	2.22	0.0269
D. citri treatment type × insecticide	8, 225	6.88	< 0.0001
Temperature × insecticide	16, 225	21.94	< 0.0001
D. citri treatment type × temperature × insecticide	32, 225	0.66	0.9228

significant positive correlation between temperature and *D. citri* mortality for chlorpyriphos (Pearson correlation coefficient $r = 0.4397$, $P = 0.0150$), imidacloprid ($r = 0.8230$, $P < 0.0001$), spinetoram ($r = 0.8117$, $P < 0.0001$), and thiamethoxam ($r = 0.8030$, $P < 0.0001$). A significant negative correlation was observed between temperature and *D. citri* mortality for fenpropathrin ($r = -0.8006$, $P < 0.0001$).

For the field-collected uninfected *D. citri*, ANOVA indicated that the main effects insecticide ($F = 4.36$; df = 4, 50; $P = 0.0042$) and temperature ($F = 14.74$; df = 4, 50; $P < 0.0001$), and the interaction between main effects ($F = 6.58$; df = 16, 50; $P < 0.0001$), had significant effects on mortality of *D. citri*. ANOVA performed for each insecticide indicated that temperature had a significant effect on *D. citri* mortality for chlorpyriphos ($F = 8.49$; df = 4, 10; $P = 0.0030$), fenpropathrin ($F = 11.01$; df = 4, 10; $P = 0.0011$), imidacloprid ($F = 6.76$; df = 4, 10; $P = 0.0067$), spinetoram ($F = 9.20$; df = 4, 10; $P = 0.0022$), and thiamethoxam ($F = 8.06$; df = 4, 10; $P = 0.0036$) (Table 5). There was a significant positive correlation between temperature and *D. citri* mortality for chlorpyriphos ($r = 0.7743$, $P = 0.0007$), imidacloprid ($r = 0.8445$, $P < 0.0001$), thiamethoxam ($r = 0.8649$, $P < 0.0001$), and spinetoram ($r = 0.7582$, $P = 0.0011$). A significant negative correlation was observed between temperature and *D. citri* mortality for fenpropathrin ($r = -0.9017$, $P < 0.0001$).

For the field-collected Las-infected *D. citri*, ANOVA indicated that the main effects insecticide ($F = 69.45$; df = 5, 60; $P < 0.0001$) and temperature ($F = 20.30$; df = 4, 60; $P < 0.0001$) had a significant effect on

Table 5. Effect of temperature on the toxicity of various insecticides against 3 treatment types of *Diaphorina citri.*

	Mean % mortality (± SE)[a]		
Temperature (°C)	Laboratory susceptible	Field-collected uninfected	Field-collected Las-infected
		Imidacloprid	
20	68.3 ± 2.8 d	57.6 ± 1.6 c	80.8 ± 1.5 c
24	76.7 ± 2.1 c	63.3 ± 9.5 bc	81.0 ± 1.5 c
28	86.7 ± 2.1 b	72.1 ± 7.1 bc	91.9 ± 1.8 b
32	86.7 ±4.4 b	81.0 ± 3.0 ab	100.0 ± 0.0 a
37	95.0 ± 2.6 a	94.7 ± 2.7 a	100.0 ± 0.0 a
		Thiamethoxam	
20	79.2 ± 1.5 c	59.0 ± 9.0 c	71.5 ± 8.5 b
24	80.0 ± 2.6 c	70.2 ± 3.7 c	75.0 ± 4.8 b
28	90.0 ± 3.2 b	76.1 ± 5.2 bc	88.9 ± 11.1 ab
32	95.0 ± 0.0 ab	91.7 ± 4.8 ab	100.0 ± 0.0 a
37	95.8 ± 0.8 a	97.2 ± 2.8 a	100.0 ± 0.0 a
		Spinetoram	
20	77.5 ± 3.8 c	71.8 ± 0.9 c	65.9 ± 2.6 c
24	80.0 ± 1.8 c	77.0 ± 6.0 bc	75.4 ± 5.9 bc
28	86.7 ± 1.1 b	85.5 ± 1.2 b	84.8 ± 6.0 ab
32	90.0 ± 2.2 b	86.0 ± 2.9 b	89.6 ± 9.1 ab
37	97.5 ± 1.1 a	97.6 ± 2.4 a	100.0 ± 0.0 a
		Chlorpyriphos	
20	85.8 ± 4.7 a	65.0 ± 2.3 b	78.6 ± 3.6 b
24	85.8 ± 7.5 a	65.2 ± 3.3 b	80.7 ± 9.6 b
28	92.5 ± 4.2 a	72.0 ± 3.9 b	97.0 ± 3.0 a
32	94.2 ± 4.0 a	72.9 ± 5.2 b	97.3 ± 2.7 a
37	100.0 ± 0.0 a	92.9 ± 4.1 a	100.0 ± 0.0 a
		Fenpropathrin	
20	94.2 ± 3.3 a	91.4 ± 1.9 a	100.0 ± 0.0 a
24	83.3 ± 5.4 ab	83.0 ± 2.2 ab	91.2 ± 4.6 a
28	69.2 ± 5.7 bc	75.1 ± 1.0 bc	91.3 ± 5.9 a
32	61.7 ± 6.5 cd	68.9 ± 5.1 cd	61.5 ± 5.9 b
37	51.7 ± 2.8 d	60.8 ± 5.4 d	65.5 ± 1.2 b

[a]Means followed by different lowercase letters within a column for each insecticide are significantly different from one another ($P < 0.05$). Mean % mortality within each *D. citri* treatment type was corrected using Abbott's formula (Abbott 1925).

mortality of *D. citri*, whereas the interaction between main effects ($F = 0.95$; df = 20, 60; $P = 0.5304$) was not significant. ANOVA performed for each insecticide indicated that temperature had a significant effect on mortality of *D. citri* for chlorpyriphos ($F = 4.25$; df = 4, 10; $P = 0.0289$), fenpropathrin ($F = 18.71$; df = 4, 10; $P > 0.0001$), imidacloprid ($F = 58.57$; df = 4, 10; $P < 0.0001$), thiamethoxam ($F = 4.15$; df = 4, 10; $P = 0.0310$), and spinetoram ($F = 5.38$; df = 4, 10; $P = 0.0142$) (Table 5). There was a significant positive correlation between temperature and *D. citri* mortality for chlorpyriphos ($r = 0.7311$, $P = 0.0020$), imidacloprid ($r = 0.9284$, $P < 0.0001$), spinetoram ($r = 0.8223$, $P = 0.0002$), and thiamethoxam ($r = 0.7603$, $P = 0.0010$). A significant negative correlation was observed between temperature and *D. citri* mortality for fenpropathrin ($r = -0.8426$, $P < 0.0001$).

A significant positive correlation between percentage mortality and temperature was found for chlorpyriphos, imidacloprid, spinetoram, and thiamethoxam for all 3 *D. citri* treatments. For fenpropathrin, however, there was a significant negative correlation between temperature and *D. citri* mortality. A comparison of the correlation coefficients for uninfected and Las-infected *D. citri* revealed no significant difference between the 2 treatments for chlorpyriphos ($z = 0.24$, $P = 0.8103$), fenpropathrin ($z = -0.62$, $P = 0.5353$), imidacloprid ($z = -1.00$, $P = 0.3173$), spinetoram ($z = 0.34$, $P = 0.7339$), and thiamethoxam ($z = 0.77$, $P = 0.4413$) (Fig. 3).

Discussion

Cold-acclimated *D. citri* were up to 1.5-fold more tolerant to neonicotinoid insecticides than non-acclimated controls, suggesting possible lower efficacy of this mode of action during winter temperatures. However, cold acclimation did not affect susceptibility to chlorpyriphos, fenpropathrin, and spinetoram. Further investigations are needed to determine the mechanism underlying decreased susceptibility of cold-acclimated *D. citri* to neonicotinoids. However, this level of decreased susceptibility is unlikely to influence management efficacy in the field.

In general, temperature affects the binding of a substrate to the enzyme and the rate of enzymatically catalyzed reactions (Hochachka & Somero 1984; Hoffmann 1985). Therefore, we hypothesized that varying levels of insecticide susceptibility in *D. citri* due to temperature fluctuations may involve altered levels of detoxifying enzyme activities. However, temperature did not affect cytochrome P450 and general esterase activity levels. Glutathione S-transferase was the only group of enzymes influenced by variations in temperature, with reduced levels at 37 °C in both imidacloprid- and spinetoram-treated *D. citri*. However, the reduced activity of glutathione S-transferase does not explain the lower mortality of *D. citri* when treated with fenpropathrin at 37 °C because reduced levels of glutathione S-transferase enzymes would be expected to increase insecticide susceptibility rather than decreasing susceptibility as observed at high temperatures. Glutathione S-transferase enzymes contribute to pyrethroid insecticide resistance (Grant & Matsumura 1989; Tiwari et al. 2011a,c). Our results indicate that changes in the toxicity levels of several insecticides in *D. citri* in response to temperature fluctuations are not associated with corresponding changes in activity of 3 detoxifying enzyme groups. Therefore, temperature-influenced fluctuations in toxicity may be caused by other mechanisms such as reduced penetration, transport to the target site, and/or altered membrane permeability. Although several investigations have proposed that detoxifying enzymes alter insecticide toxicity as a result of temperature fluctuations, this hypothesis has not directly been investigated previously (Chandler et al. 1991; Wadleigh et al. 1991; Hodjati & Curtis 1999).

We also found reduction in transcript levels of 4 out of 5 *CYP4* genes: *CYP4C67*, *CYP4C68*, *CYP4G70*, and *CYP4DB* in Las-infected *D. citri* populations compared with uninfected populations. This result extends the work reported by Tiwari et al. (2011d), demonstrating a concomitant reduction in CYP4 protein expression levels corresponding to reduced *CYP4* transcript levels. A few minor differences were observed in the present study compared with that of Tiwari et al. (2011d) and may be due to the use of mixed gender insects in the present study, whereas males and females were analyzed separately in Tiwari et al. (2011d). For example, *CYP4DA1* expression levels were comparable between the 2 populations in the present study, whereas in the previous study, a statistically significant drop in transcript levels was observed in male *D. citri* for this gene transcript. The reduction of *CYP4* transcript and CYP4 protein levels in Las-infected *D. citri* suggests possible molecular and biochemical interactions between *D. citri* and '*Candidatus*' Liberibacter asiaticus that may influence insecticide susceptibility in *D. citri* and that remain to be resolved.

Herein, we investigated whether *D. citri* carrying '*Candidatus*' Liberibacter asiaticus responded differently to temperature fluctuations than uninfected psyllids with respect to insecticide susceptibility. Temperature is known to affect insecticide susceptibility of *D. citri* that do not harbor the Las bacterium (Boina et al. 2009). As the occurrence of HLB has increased in Florida (Morris et al. 2009), the proportion of *D. citri* carrying Las, the putative causal agent of HLB, is in some instances 100% (Coy & Stelinski 2015). Although *D. citri* infected with Las is more susceptible to insecticides than uninfected counterparts (Tiwari et al. 2011b), our current findings indicate that this temperature-related change in susceptibility is the same irrespective of whether or not *D. citri* carries the Las bacterium. In general, there was a positive correlation between temperature and percentage mortality for both uninfected and Las-infected *D. citri* for chlorpyriphos, imidacloprid, spinetoram, and thiamethoxam, and a negative correlation for fenpropathrin. The current results are congruent with a recent investigation that established LC_{50} values for various insecticides for uninfected *D. citri* at various temperatures (Boina et al. 2009).

The mechanisms underlying altered insecticide toxicity due to temperature fluctuations are not clearly understood. However, several attempts have been made to explain the effect of temperature on insecticide susceptibility (Pradhan et al. 1952; Narahashi 1985; Narahashi et al. 1995; Song & Narahashi 1996; Wellmann et al. 2004). Temperature is known to alter permeability, by directly affecting the lipids of neuronal membranes (Pradhan et al. 1952). In addition, temperature influences the binding affinities of toxic molecules with the lipid-rich nervous system of insects (Narahashi 1985; Wellmann et al. 2004). After treatment with the pyrethroid tetramethrin, repetitive nerve firing is decreased at higher (30–35 °C) compared with lower (15–20 °C) temperatures (Narahashi et al. 1995; Song & Narahashi 1996). These results may partially explain the negative correlation between temperature and *D. citri* mortality as a result of fenpropathrin treatment. The reduced toxicity of chlorpyriphos, imidacloprid, spinetoram, and thiamethoxam at lower temperatures might be a result of slower penetration and reduced transport of these insecticides to the target site as compared with higher temperatures (Tyler & Binns 1982). Although susceptibility to various insecticides was higher in Las-infected than uninfected *D. citri*, the correlation coefficients between temperature and percentage mortality were not affected by Las infection.

Currently, chemical control is the most effective tool available for management of *D. citri* and HLB; therefore, an understanding of interactions between biotic and abiotic factors that may influence insecticide toxicity may help improve management of this pest. Our results indicate changes in insecticide susceptibility of *D. citri* as a function of temperature fluctuation that are not related to changes in detoxifying enzymes levels. Our results also indicate that cold-acclimated *D. citri* are slightly less susceptible to neonicotinoid insec-

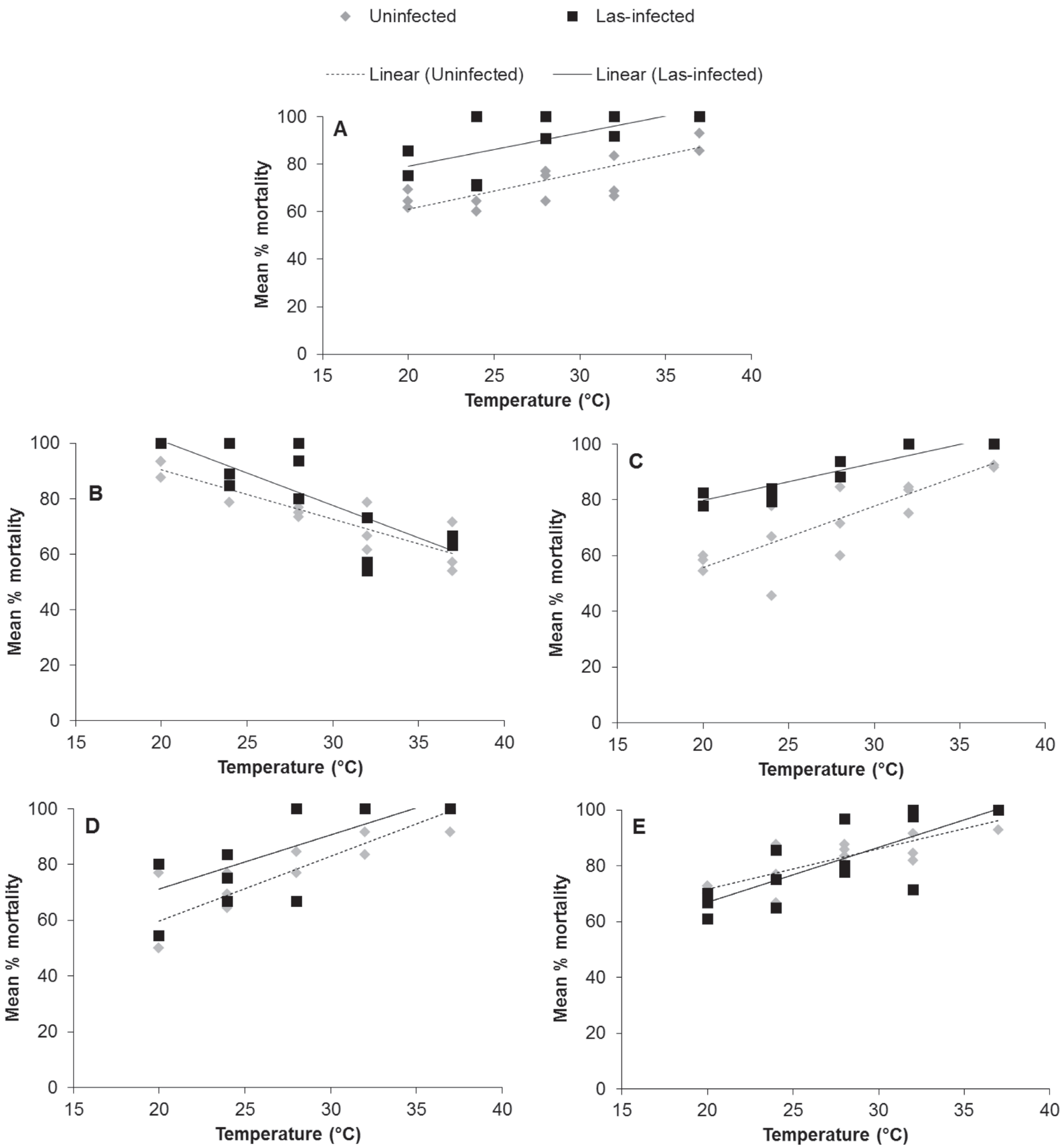

Fig. 3. Correlations between mean percentage mortality of *Diaphorina citri* and temperature for field-collected and uninfected *D. citri* and field-collected and '*Candidatus*' Liberibacter asiaticus–infected *D. citri*, when exposed to chlorpyriphos (A), fenpropathrin (B), imidacloprid (C), thiamethoxam (D), and spinetoram (E).

ticides than non-acclimated controls. Las-infected *D. citri* fed less than uninfected counterparts, as measured indirectly by honeydew production, which should be confirmed by electrical penetration graph studies. Maximum feeding by *D. citri* adults occurred at 32 °C, which suggests that efficacy of insecticides requiring ingestion may be temperature dependent. Overall, annual temperature fluctuations should not have a major impact on management of *D. citri* with insecticides.

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