OPTIMIZING THE USE OF SOIL-APPLIED NEONICOTINOIDS FOR CONTROL OF DIAPHORINA CITRI KUWAYAMA (HEMIPTERA: LIVIIDAE) IN YOUNG CITRUS TREES

By

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To my mom and dad

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5-OH	2-Chloro-5[(4,5 dihydro-2-nitroamino)-1-H imidazole-1-yl]-methyl-3-pyridinol
ACP	Asian citrus psyllid
*C*Las	*Candidatus* Liberibacter asiaticus
FL	Florida
HLB	Huanglongbing
$LC_{\#\#}$	Lethal concentration at the ## percent mortality level
L:D	Light:Dark
LOD	Limit of detection
LOQ	Limit of quantification
LS	Laboratory susceptible
Olefin	1-[(6-Chloro-3-pyidinyl)methyl]-1,3-dihydro-N-nitro-2H-imidazol-2-imine
QuEChERS	Quick easy cheap effective rugged safe
RH	Percent relative humidity
PCR	polymerase chain reaction
TZMU	N-(2-chlorothiazol- 5-ylmethyl)-N-methylurea
TZNG	N-(2-chlorothiazol-5- ylmethyl)-N-nitroguanidine
UHPLC-MS	Ultra high performance liquid chromatography – mass spectrometry

Abstract of Dissertation Presented to the Graduate School
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Diaphorina citri Kuwayama is the insect vector of *Candidatus* Liberibacter asiaticus (*C*Las), the presumed cause of huanglongbing (HLB) in citrus (Rutaceae). Following the 2005 discovery of HLB in Florida, management strategies were developed using soil-applied neonicotinoids to protect young trees from the deadly disease. In such programs, neonicotinoids are applied to the soil allowing uptake through xylem channels from the roots into the foliage. *Diaphorina citri* are exposed to the insecticide through feeding. Despite implementation of neonicotinoid-intensive management programs, infection continues to spread among even the most intensively managed groves. A series of studies conducted in the laboratory, greenhouse, and field attempted to: 1) Quantify the spatial and temporal distribution of neonicotinoids in citrus foliage following soil-application; 2) Determine how tree size and application rate affect expression and *D. citri* incidence and abundance; 3) Determine the concentration needed to kill *D. citri* by ingestion and compare with residues required to kill by contact; 4) Use electropenetrography to investigate how dosage administered in diet affects feeding behavior. Higher concentrations occurred in the bottom 10% of the canopy compared to other regions, yet

no difference occurred between the bottom and center, and levels peaked up to five weeks after application. Tree size and application rate affected titer in leaf tissue. Approximately 64.63 ppm thiamethoxam was required to reach a one percent probability of encountering a flush shoot with at least one adult *D. citri*. The ingestion LC_{50} of the laboratory strain was approximately 10-fold greater than the contact LC_{50}. Neonicotinoid resistance was found in various field populations. No effect on feeding behavior was observed after 0.55 ppm imidacloprid was administered, however 5.5 and 55 ppm imidacloprid reduced various probing, pathway, and salivation/ingestion behaviors. Overall, neonicotinoid titers observed in the field failed to reach lethal levels quantified in the lab. Exposure to sublethal dosages is of concern for HLB management, as well as development of neonicotinoid resistance. Based on comprehensive results, the use of neonicotinoids may be better applied by foliar rather than soil application to maintain the utility of this chemical class in future insecticide management programs in Florida citrus.

CHAPTER 1
INTRODUCTION

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hempitera: Liviidae) is the insect vector of *Candidatus* Liberibacter asiaticus (*C*Las), the presumed cause of huanglongbing (HLB), or citrus greening disease. Throughout the world, HLB reduces health and productivity of citrus trees (Rutaceae) (Halbert and Manjunath 2004, Bové 2006, Gottwald 2007, Ichinose et al. 2010a, 2010b; Grafton-Cardwell et al. 2013). *Candidatus* Liberibacter asiaticus is a phloem limited bacteria that negatively impacts root density and fuction, 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). *Diaphorina citri* was first discovered in Florida in 1998 (Halbert and Manjunath 2004), followed by HLB in 2005 (Halbert 2005). Since, HLB has spread through the southern United States and 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).

Diaphorina citri **Life History**

Diaphorina citri reproduce and develop rapidly, laying up to 800 eggs per female, primarily on young, unexpanded flush. The eggs hatch after two to four days, and the insect completes 5 nymphal stages within 11 to 15 days, continuously feeding on the phloem sap of the young, growing flush shoots. Between 15 to 47 days are required for completion of one life cycle (Liu and Tsai 2000, Grafton-Cardwell et al. 2013). Optimum temperature conditions for

development are 25-28°C, temperatures that occur often during the months of May, June, and September in Florida, which also coincide with peak populations of *D. citri* and tree flush availability (Liu and Tsai 2000, Tsai et al. 2002, Hall and Albrigo 2007, Hall et al. 2008). Both adult and nymphal stages actively feed on phloem sap (Boina et al. 2009). *Diaphorina citri* prefer to feed on young leaf tissue, however, they will feed on mature leaves in the absence of young flush (Tsai et al. 2002, Serikawa et al. 2012). When eggs are deposited on the flush of *C*Las infected trees, the developing nymphs ingest the bacteria upon feeding on bacteria-containing phloem sap (Pelz-Stelinski et al. 2010). Pelz-Stelinski et al. (2010) found that *C*Las acquisition was greater for *D. citri* nymphs that fed on *C*Las infected tissue than for *D. citri* adults feeding on infected tissue. When a newly infected nymph completes development, the adult disperses and inoculates uninfected trees. Bonani et al. (2010) determined that successful *C*Las acquisition by adults could occur after 1h of phloem ingestion (EPG waveform E2).

Florida Citrus and Management of *Diaphorina citri*

In Florida, mature trees typically follow a synchronized annual flush sequence characterized with a major flush in early spring, another major flush in summer, and minor flushes during late summer and fall. In contrast, young trees tend to flush asynchronously and more often throughout the year (Hall and Albrigo 2007). Young flush shoots emit volatiles attractive to *D. citri* adults (Patt and Setamou 2010). Serikawa et al. (2012) found that *D. citri* conducted more feeding probes and probed for a longer duration per event on young citrus leaves compared to mature leaves. This result indicates that *D. citri* adults prefer young leaf tissue to mature leaf tissue. Because young trees flush more frequently, they are presumably at greater risk of contracting *C*Las (Stansly and Rogers 2006). Although *D. citri* prefer young tissue, adults can survive on mature leaves (Tsai et al. 2002). Following the discovery of *C*Las in Florida, researchers attempted to develop more efficient sampling methods using flushing events to

predict *D. citri* population densities (Hall et al. 2008). As a result, the use of a year-round scouting program was recommended to justify control and align foliar insecticide sprays with *D. citri* egg hatch and nymph development. Stansly and Rogers (2006) suggested the use of clean nursery stock, a program of soil applied systemic insecticides for protection of growing nursery stock, and the rapid removal of any trees that displayed evidence of HLB infection. They also suggested using minimal foliar sprays in the field to reduce negative impacts on *Tamarixia radiata* Waterston (Hymenoptera: Eulophidae), a key parasitic wasp of *D. citri*. Despite early control measures, the incidence and severity of HLB in Florida increased rapidly and quickly warranted the evolution of new management recommendations. In addition to vector management, increased attention was given to the overall health of mature trees; the reduction of stressors associated with HLB was studied as a means of maintaining existing mature citrus groves (Inserra et al. 2003, Obreza and Morgan 2008, Graham et al. 2013, 2014; Gottwald et al. 2012). For example, *Phytophthora spp.* targeted fungicide applications and the use of resistant rootstocks were found essential to promoting fibrous root health (Graham et al. 2013, 2014). Also, the selection of nematode resistant rootstocks became more important in the management of nematode infestations (Inserra et al. 2003). Moreover, the use of macro-nutrient fertilizers were found to increase yields of previously stressed trees (Obreza and Morgan 2008). Each of these management strategies can play an important role in overall grove health as a component of circumventing the impact of HLB. However, some expressed concerns of increased disease spread following implementation of nutrient management strategies due to an increase in flushing frequency and abundance (Gottwald et al. 2012). Nonetheless, insecticidal control remains arguably the most important component of *D. citri* management, and thus, the management of HLB (Stansly and Rogers 2006, Rogers 2008, Boina et al. 2009, Qureshi and

Stansly 2009, Qureshi et al. 2011, Ichinose et al. 2010a, 2010b; Rogers 2012, Stansly et al. 2012, Hall et al. 2013a, 2013b; Qureshi et al. 2014).

As mature groves accumulated HLB infection over time, it was believed that to maintain grower production sustainability, unproductive, HLB infected citrus trees would need to be replaced with new trees (Rogers 2012). As a result, an emphasis was placed on young tree care to maintain the industry. Because young trees flush often, and because flush is preferred by *D. citri*, young trees were believed to require more protection than mature trees (Hall and Albrigo 2007, Serikawa et al. 2012, Rogers et al. 2015, Qureshi et al. 2014). Rogers (2012) noted that the goal of *D. citri* management in young trees is to prevent HLB until after the trees reach fruit bearing age, which generally occurs between two and five years after planting. If HLB is prevalent around a new planting or if *D. citri* control is lacking in surrounding groves, it can be difficult to protect young trees from the disease (Hall et al. 2013b).

An area-wide approach to vector control was implemented as the citrus health management area (CHMA) program in 2010 (Rogers et al. 2012). The impact of the CHMA program from a pest management point of view is two-fold: 1) To reduce the overall psyllid population in Florida citrus (Rogers et al. 2012), and; 2) To manage potential resistance problems from the repeated use of the same insecticide (Tiwari et al. 2011a). The area wide sprays are conducted between applications of neonicotinoid insecticides to the soil. Soil applied neonicotinoids have been a key management tool in the attempt to control *D. citri*, and thus help to mitigate the risk of HLB infection in young citrus (Rogers 2012, Rogers et al. 2015). The translocation of insecticides within plant tissue was first noted in 1947 (Jeppson et al. 1952). In 1950, researchers found that when the organophosphate insecticides, paraoxon and octamethyl pyrophosphoramide, were applied to the soil, each moved into the foliage and killed feeding

mites on citrus (Metcalf and Carlson 1950a, 1950b; Jeppson et al. 1952). A relatively new class of chemistry that is also highly systemic and mobile within plant tissue are neonicotinoids. The Insecticide Resistance Action Committee (IRAC) classifies neonicotinoids within the chemical sub-group 4A, which act on the Nicotinic acetylcholine receptor (nAChR) (IRAC 2009). Neonicotinoids mimic acetylcholine and bind to the nAChR, causing the nerve to continuously fire. Acetylcholinesterase cannot hydrolyze the acetylcholine mimic, resulting in over exhaustion of the insect nervous system, paralysis, and eventual death. Neonicotinoid insecticides can be applied to plant foliage and translocate throughout the plant, or applied to the soil, and transported through the xylem channels from the roots into the plant foliage (Elbert et al. 2008). While systemic insecticides are effective at controlling targeted pests, when applied to the soil, they help to minimize 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 six and eleven weeks after application (Qureshi and Stansly 2007, Qureshi and Stansly 2009, Ichinose et al. 2010a, Setamou et al. 2010, Byrne et al. 2012, Rogers 2012). The use of soil applied neonicotinoids on newly planted seedlings at a two month interval was found to reduce psyllid infestation and disease (Ichnose et al. 2010a). Moreover, thiamethoxam and imidacloprid each provided better *D. citri* control than other systemic insecticides; clothianidin was not tested as it was not registered in citrus at the time (Rogers and Shawer 2007). Transport of chemistry through the plant xylem is critical for

the effectiveness of neonicotinoids when applied to the soil. Water solubility of each chemistry can influence rate of ground leaching and successful uptake (Rogers 2012). Thiamethoxam is the most water soluble, imidacloprid is less water soluble, and clothianidin is the least water soluble, comparatively. Castle et al. (2005) found that the concentration of thiamethoxam in citrus tissue increased more rapidly than imidacloprid, and Qureshi and Stansly (2009) found that thiamethoxam provided rapid *D. citri* control compared to imidacloprid, which suggests that rapid uptake is directly related to water solubility. Rogers (2012) proposed the use of the most water soluble compound (thiamethoxam) during the dry winter months, and the least water soluble compound (clothianidin) during the rainy summer months to maximize uptake of each insecticide in Florida. Because the three effective soil applied insecticides comprise one mode of action, their use must be accomplished with great care, such to prevent resistance (Rogers 2012). One effective resistance management strategy is to rotate soil applied neonicotinoids with foliar sprays of a different mode of action (Rogers 2008), as done with the CHMA program. Tiwari et al. (2011a) reported neonicotinoid resistance in field collected *D. citri* populations in Florida in 2009, but subsequent studies conducted in 2013, after the implementation of the CHMA program did not detect resistance, indicating that reversion had occurred (Coy et al. 2016).

Even in the most intensively managed citrus groves, trees continued to accumulate HLB infection over time at an estimated rate of one to three percent each year (Rogers 2013). A more thorough understanding of the use of soil applied neonicotinoids is critical to further reduce the spread of HLB. Limited knowledge regarding the movement and distribution of soil applied neonicotinoids through citrus tissue subsists to date. Boina et al. (2009) proposed that uneven temporal and spatial distribution in citrus tissue may cause exposure of *D. citri* to sublethal doses of insecticide. Uneven uptake of systemic insecticides by the root system could make it possible

for *D. citri* to develop (Rogers 2012). Previous studies that attempted to quantify neonicotinoid concentration in citrus sampled either xylem fluid or entire leaves and quantified concentrations using enzyme-linked immunosorbent assay (ELISA) (Castle et al. 2005, Garlapati 2009, Setamou et al. 2010). Castle et al. (2005) stated that the potential for cross-reactivity with plant metabolites of imidacloprid was assumed as positive detection of the parent compound, imidacloprid. The use of Ultra-High Performance Liquid Chromatography - Mass Spectrometry (UHPLC-MS) is a more reliable method for quantifying the concentration of neonicotinoids in leaf tissue, as the method can distinguish metabolites from parent compounds. Neonicotinoid insecticides metabolize into various analytes over time, though the effect of any one resulting metabolite on *D. citri* mortality is unknown (Byrne et al. 2017). For example, thiamethoxam metabolizes into clothianidin (Nauen et al. 2003) and clothianidin further metabolizes into TZNG (N-(2-chlorothiazol-5- ylmethyl)-N-nitroguanidine) and TZMU (N-(2-chlorothiazol- 5-ylmethyl)-N-methylurea) (Kim et al. 2012). Likewise, imidacloprid metabolizes into 5-OH (2-Chloro-5[(4,5 dihydro-2-nitroamino)-1-H imidazole-1-yl]-methyl-3-pyridinol) and then 5-OH further metabolizes into olefin (1-[(6-Chloro-3-pyidinyl)methyl]-1,3-dihydro-N-nitro-2H-imidazol-2-imine) (Sur and Stork, 2003). Nevertheless, Castle et al. (2005) found no difference in the spatial distribution of the parent compounds of imidacloprid or thiamethoxam within a citrus tree as it related to the glassy-winged sharpshooter, *Homalodisca coagulata* (Say). The authors sampled xylem fluid from branch shoots, which is not consistent with the phloem-feeding patterns of *D. citri*. In addition, the authors applied insecticides through a micro-irrigation system, which evenly distributes material around the tree trunk. In contrast, Florida growers typically use a drench application device mounted to a four-wheel utility vehicle to apply a solution of approximately 237 mL (water + insecticide) to the soil below only one side of

each tree. This application method may result in an uneven distribution of insecticide within a tree canopy. Moreover, differences within a single leaf may occur, causing uneven distribution within a leaf. Whole leaf samples, as used in Garlapati (2009) and Setamou et al. (2010), quantified a mean titer within a leaf, which did not account for potential differences within a leaf. If the lowest concentration within a leaf coincide with where *D. citri* feeding occurs, and that concentration is sublethal, successful *C*Las acquisition and/or inoculation may result. In addition to potential differences in spatial distribution, temporal expression over time as it relates to residual control may result in sublethal dosages. Temporal expression of soil applied neonicotinoids may be influenced by factors such as soil type, leaf maturity, application volume, irrigation, tree age and size, addition of adjuvants, and environmental conditions. While six to eleven weeks of control have been reported following application of neonicotinoids to the soil (Qureshi and Stansly 2007, Qureshi and Stansly 2009, Ichinose et al. 2010a, Setamou et al. 2010, Byrne et al. 2012, Rogers 2012), studying factors that influence uptake and expression over time would allow great improvement to suggested management plans.

Concentration of neonicotinoid insecticide required to kill by ingestion is an additional major unknown. Serikawa et al. (2012) determined that *D. citri* can undergo phloem ingestion (EPG waveform E2) for at least 1h with imidacloprid in the plant tissue; one hour is enough time for successful *C*Las acquisition. Although eventual death did occur in *D. citri* individuals that ingested imidacloprid, it is possible for those individuals to move to an uninfected tree and salivate within the phloem before death, thus inoculating the new tree. In Florida, an estimated 80-100% of *D. citri* are *C*Las 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.

Quantifying uptake and distribution patterns of neonicotinoids in citrus leaf tissues when applied to the soil is paramount to understanding how to control *D. citri* with soil applied neonicotinoids and to further reduce or stop the spread of HLB in Florida citrus. Moreover, it is important to understand how tree size and application rate affects expression in citrus leaf tissues. By quantifying expression of neonicotinoids in citrus leaf tissues, we can map the pattern of sublethal neonicotinoid titers within a tree or leaf and develop strategies to minimize exposure to those dosages, or improve uptake efficiency to maximize expression when applied to the soil. Quantification of the lethal concentration of neonicotinoids by ingestion coupled with studying feeding behavior under exposure to neonicotinoids using electropenetrography will allow us to understand how dosages observed in field and greenhouse studies impact *D. citri* behavior and mortality, and will allow us to better understand the potential for *C*Las transmission under *D.*

citri managed systems. Moreover, understanding the relationship between neonicotinoid dosage and mortality levels will help identify the potential for development of resistance and help develop sound resistance management strategies. The overarching goal of this research is to allow us to refine current vector management programs which will help either maximize the reduction or perhaps optimistically, prevent the spread of *C*Las in Florida citrus.

CHAPTER 2
SPATIAL AND TEMPORAL DISTRIBUTION OF SOIL-APPLIED NEONICOTINOIDS IN CITRUS

Diaphorina citri Kuwayama is the insect vector of *Candidatus* Liberibacter asiaticus (*C*Las), the presumed cause of huanglongbing (HLB) in citrus (Rutaceae). Soil-applied neonicotinoids are used to manage vector populations and thus reduce the spread of HLB in Florida citrus. A series of studies conducted in the greenhouse and field attempted to quantify the spatial distribution within a single leaf and throughout the tree canopy, as well as temporal expression following soil application of three neonicotinoid insecticides. No difference in parent material titer was observed between leaf middle and leaf margin following application of Platinum 75SG or Belay 2.13SC, however, imidacloprid titer was higher in the leaf margin following application of Admire Pro in the field. TZMU and TZNG accumulated in leaf margins after application of Platinum and Belay. The bottom tree region contained higher levels of imidacloprid compared with other regions, but was not different from the spherical center. In the greenhouse, imidacloprid and clothianidin titers peaked at five weeks following application of Admire and Belay, respectively, and thiamethoxam titer peaked at three weeks after application of Platinum. We were unable to quantify temporal expression in the field due to existing imidacloprid titers at the time of application. Overall, titers observed in the field failed to reach lethal levels quantified in previous studies. Exposure to sublethal dosages is of concern for HLB management, as well as development of neonicotinoid resistance. Based on our results, subsequent work should realign focus on foliar use patterns to maintain the utility of neonicotinoids in citrus.

Justification

The Florida citrus (Rutaceae) industry has come under severe decline over the last decade, due to the combined introductions of the Asian citrus psyllid, *Diaphorina citri*

Kuwayama (Hemiptera: Liviidae), and the presumed causal agent of citrus greening disease, *Candidatus* Liberibacter asiaticus (*C*Las) (Halbert and Manjunath 2004, Bové 2006). Citrus greening disease, or Huanglongbing (HLB), was first detected in the state in 2005, seven years after the discovery of the insect vector, *D. citri* (Halbert and Manjunath 2004). The citrus industry in Florida was valued at 9.9 billion dollars during 2014 and 2015 and is the single largest agricultural commodity in the state (Hodges and Spreen 2015). When trees succumb to HLB through feeding by *C*Las-positive *D. citri*, the bacteria moves through the phloem from the infection site to the roots, which negatively impacts the root system. In turn, the tree canopy is starved for nutrients, causing leaf and fruit drop, thereby reducing yield in the near term, and eventually resulting in tree death (Halbert and Manjunath 2004, Bové 2006, Grafton-Cardwell et al. 2013). Various methods of HLB management have been investigated, including routine releases of the biological control agent, *Tamarixia radiata* Waterston (Hymenoptera: Eulophidae), nursery sanitation, and rogueing of infected trees in the field, among other strategies (Stansly and Rogers 2006, Hall and Albrigo 2007, Hall et al. 2008). Given the potential impact of the disease and an estimated 80-100 percent of *D. citri* in the state that are infected with *C*Las (Coy and Stelinski 2015), insecticides have become the primary method for slowing the spread of HLB throughout Florida grove space, particularly through use of soil-applied neonicotinoids in young tree plantings (Rogers 2008, 2013).

Young trees do not bear fruit and are typically categorized as those less than eight feet tall (Hall and Albrigo 2007, Rogers 2012). Unlike mature citrus trees, non-bearing trees flush often throughout the year, which places them at great risk of HLB infection (Stansly and Rogers 2006). *Diaphorina citri* adults are attracted to volatiles emitted by actively growing flush shoots, which are preferred for oviposition (Patt and Setamou 2010). Newly hatched nymphs feed on

phloem sap of the developing flush shoots where nymphs acquire the bacterium if on infected plant material (Pelz-Stelinski et al. 2010). As newly-infected nymphs reach adulthood, they disperse and inoculate uninfected citrus tissue. In attempt to intercept this cycle, Rogers (2008) developed a program approach of rotating between neonicotinoids applied to the soil and foliar sprays of alternate modes of action. Neonicotinoids are highly systemic, xylem-mobile insecticides within the Insecticide Resistance Action Committee (IRAC) sub-group 4A and are often applied to the soil for transport to the plant foliage (Elbert et al. 2008). Three neonicotinoid insecticides are labeled for use in non-bearing citrus in Florida: 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). Previous investigations documented residual *D. citri* adult and / or nymph control of six to eleven weeks after neonicotinoids were applied to the soil (Qureshi and Stansly 2007, 2009; Ichinose et al. 2010; Setamou et al. 2010; Byrne et al. 2012; Rogers 2012). However, even in the most intensively managed citrus groves, trees continued to accumulate HLB infection over time at an estimated rate of one to three percent each year (Rogers 2013).

Little is known regarding the movement and distribution of soil-applied neonicotinoids through citrus tissues. Boina et al. (2009) proposed that uneven temporal and spatial distribution in citrus tissue may cause exposure of *D. citri* to sublethal doses of insecticide. Furthermore, uneven uptake of systemic insecticides by the root system make it possible for *D. citri* to develop (Rogers 2012). Previous studies that quantified neonicotinoid concentration in citrus sampled either xylem fluid or entire leaves and quantified parent material concentrations using enzyme-linked immunosorbent assay (ELISA) (Castle et al. 2005, Garlapati 2009, Setamou et al. 2010).

When quantifying chemical constituents using ELISA, one cannot differentiate between parent material and resulting metabolites. Nevertheless, Castle et al. (2005) found no difference in the spatial distribution of imidacloprid or thiamethoxam throughout citrus tree canopy xylem fluid for control of the glassy-winged sharpshooter, *Homalodisca coagulata* (Say). They sampled xylem fluid from branch shoots, which is not consistent with the phloem-feeding patterns of *D. citri*. In addition, the authors applied insecticides through a micro-irrigation system, which evenly distributes insecticide around the tree trunk at the time of application. In contrast, Florida growers typically use a drench application device mounted to a four-wheel utility vehicle to apply a solution of approximately 237 mL (water + insecticide) to the soil below only one side of each tree. This application method may result in an uneven distribution of insecticide within a tree canopy. In the case of Florida citrus, quantifying the spatial distribution of insecticide within a tree, as well as within a single leaf, is essential to understanding the potential of *D. citri* exposure to sublethal dosages.

Setamou et al. (2010) correlated percentage control with imidacloprid concentration in citrus leaf tissues. They determined that between 200 and 250 parts per billion (ppb) was required to provide *D. citri* control in citrus. More recent studies found that 62.19 parts per million (ppm) imidacloprid was required to kill 90 percent of a laboratory *D. citri* population when administered by ingestion (Langdon and Rogers 2017). They suggested that control observed by Setamou et al. (2010) may have been a result of feeding deterrence at sublethal dosages. In addition, a series of more recent studies determined that 64.63 ppm thiamethoxam was required to achieve a one percent probability of encountering a flush shoot with at least one adult *D. citri* in the field, as determined by liquid chromatography mass spectrometry (LC-MS) (Langdon 2017). The highest mean thiamethoxam titer observed in their field study was 33.39

ppm, more than 30 ppm below the threshold for a tree predicted to be free of *D. citri* adults. The magnitude of difference between dosages required to achieve high mortality levels or perceived control, and observed thiamethoxam titer may explain why HLB infection incidence continues to rise despite deliberate soil-applied neonicotinoid use in the field. While spread of HLB is of great concern, sublethal dosages resulting from uneven spatial and temporal distribution is also likely to increase the potential for development of resistance to neonicotinoids. Tiwari et al. (2011a) documented resistance to neonicotinoids in the field in 2009, but no resistance was detected in subsequent studies conducted in 2014 (Coy et al. 2016). This shift was thought to be due to the implementation of area-wide spray programs that used non-neonicotinoid insecticides applied over broad landscapes (Rogers et al. 2012). However, resistance was again detected in 2016 to imidacloprid in various field populations throughout the state, albeit resistance appeared to be isolated to specific areas at the time; tests evaluating resistance to thiamethoxam or clothianidin were not conducted (Langdon and Rogers 2017).

Quantifying uptake and distribution patterns of neonicotinoids in citrus leaf tissues when applied to the soil is paramount to understanding how to control *D. citri* and to further reduce or stop the spread of HLB in Florida citrus. In addition, we must quantify exposure of insects to sublethal insecticide dosages to minimize the potential for resistance development to key insecticide classes, such as neonicotinoids. The purpose of this study was to quantify the spatial distribution and temporal expression of all analytes in citrus leaf tissues resulting from the soil-application of three neonicotinoid insecticides using an application method commonly implemented by Florida citrus growers. By quantifying expression of neonicotinoids in citrus leaf tissues, we can map the pattern of sublethal neonicotinoid titers within a tree or leaf and

develop strategies to minimize exposure to those dosages, or improve uptake efficiency for maximum expression when applied to the soil.

Materials and Methods

Spatial and Temporal Neonicotinoid Distribution

Greenhouse study. A greenhouse study was conducted to evaluate the uptake of three neonicotinoid insecticides following application to the soil. The distribution of insecticide residue within citrus leaves was evaluated. Small citrus trees (ca. 0.08m^3 canopy volume) were planted to 11.4L pots containing a blend of 50% sand and 50% potting media. Plots consisting of four trees were arranged in a randomized complete block design (RCBD) with 4 treatments and 4 replicates. Treatments consisted of an untreated control, Platinum 75SG, Admire Pro 4.6F, and Belay 2.13SC applied at the recommended rate for non-bearing citrus trees based on 346 trees / hectare (140 trees / acre) (Table 2-1). A single insecticide application was made by applying 237 mL of insecticide solution (deionized water + insecticide) into each pot. Leaf tissue samples were collected prior to the application of insecticides and then weekly for 13 weeks following the application. At each sample date, four leaves across each of the four trees within a plot were harvested. Each leaf was excised into two sections: 1. Middle (area inclusive of 0.5cm on either side of the mid-vein extending from leaf petiole to 0.5cm from leaf tip), and 2. Margin (remainder of leaf not associated with the 'middle' leaf section). Leaf material from each section within a plot was wrapped separately in labeled heavy duty aluminum foil and collectively stored by treatment in a plastic re-sealable bag at -20°C until residue analyses were conducted.

Two season by two location field study. A field study was conducted at two commercial grove locations across two seasons to evaluate the spatial and temporal distribution of three neonicotinoid insecticides in citrus trees following application to the soil. Non-bearing (v. Hamlin / r.s. Swingle) trees of similar size and age (ca. 1.3m^3 canopy volume and field planted

approximately 18 months prior to the first application) were identified in two commercial groves, each of which represent a major citrus production area of Florida (pine 'flatwoods' and 'central ridge'). The low lying flatwoods location was a continuous solid-set planting of trees of the same age. The trees were planted to sandy soils comprised of 96.4% sand, 2% clay, and 1.6% silt with 0.68% organic matter and cation exchange capacity (CEC) of 14.2 meq/100g. The central ridge location contained a mature grove with random spans of between two and fifteen young trees planted among the mature trees. This grove was comprised of sandy soils with 98.4% sand, 1.6% clay, and 0% silt with 0.59% organic matter and CEC of 4.1 meq/100g. The central ridge site was centrally located on the North-South running ridge through central Florida spanning from near Orlando to south of Lake Placid. At each site, plots were arranged in a randomized complete block design with 4 treatments and 4 replicates. Treatments consisted of an untreated control, Platinum 75SG, Admire Pro 4.6F, and Belay 2.13SC applied at the recommended rate for non-bearing citrus trees based on 346 trees / hectare (140 trees / acre) (Table 2-1). At each location, the first season insecticide application was made on 19-VIII-2015 and the second season application was made on 13-I-2016. At the time of application, 237 mL of insecticide solution (deionized water + insecticide) was applied to the soil at the base of each tree trunk. At the flatwoods location, tree rows were oriented north-south, and the application was made on the west side of the tree trunk. At the central ridge location, tree rows were oriented east-west, and the application was made on the south side of the tree trunk. Leaf tissue samples were collected prior to the application of insecticides and then weekly for 12 weeks following the application. Trees were divided into 7 tree regions: bottom (lower 10% of canopy), spherical center, top (upper 10% of canopy), and 4 cardinal sides (east, west, south, north). At each sample date, four leaves from each of the seven tree regions across each of the four trees within a plot were

harvested. Each leaf was excised into two sections: 1. Middle (area inclusive of 0.5cm on either side of the mid-vein extending from leaf petiole to 0.5cm from leaf tip), and 2. Margin (remainder of leaf not associated with the 'middle' leaf section). Leaf material from each leaf section and each tree region within a plot were wrapped individually in labeled heavy duty aluminum foil and stored collectively in a re-sealable plastic bag at -20°C until residue analyses were conducted. To evaluate distribution of analytes within a leaf, only leaf tissues from the 'top' tree region were used to confirm within-leaf residue distribution observed in the greenhouse. To evaluate temporal expression differences and to determine the distribution of analytes throughout the tree canopy, only leaf tissues from the 'middle' leaf section were used.

Effect of Leaf Maturity on Neonicotinoid Expression

Two season field study. A field study was conducted across two seasons to determine the effect of leaf maturity on expression of each of three neonicotinoids following application to the soil. Untreated, non-bearing citrus trees (v. Hamlin / r.s. Swingle) (ca. 1.5m^3 canopy volume) were used in the study. Trees were field planted approximately 22 months prior to the first insecticide application to sandy soil comprised of 96.8% sand, 1.6% silt, and 2% clay, with 1.04% organic matter and CEC of 6.7 meq/100g. Trees were planted using a 2.4m in-row spacing and 2.4m between-row spacing, which provided sufficient separation to eliminate uptake of insecticides applied to an adjacent tree, confirmed by analysis of trees in the untreated control. The study was arranged in a randomized complete block design with 8 treatments and 4 replicates. Treatments consisted of an untreated control, Platinum 75SG, Admire Pro 4.6F, and Belay 2.13SC applied at the recommended rate for non-bearing citrus trees based on 346 trees / hectare (140 trees / acre) (Table 2-1). Approximately 14d prior to each insecticide application, a gas-powered hedge trimmer was used to trim the tree canopy to a mean canopy volume (MCV) of approximately 1.3m^3 to promote flushing. The first season insecticide application was made

on 5-V-2017 and the second season application was made on 21-VI-2017, each when flush shoots were approximately 2.5cm long. At the time of application, 237 mL of insecticide solution (deionized water + insecticide) was applied to the soil at the base of each tree trunk. Leaf tissue samples were collected prior to the application of insecticides and then weekly for 4 weeks following the application. At each sample date, four mature leaves and four flush shoots were harvested across each of the four trees within a plot. Mature leaves and flush shoots were placed into separate labeled paper bags and collectively stored by treatment in a plastic re-sealable bag at -20°C until residue analyses were conducted. The same cohort of flush shoots were sampled each week to control for potential differences in expression values in flush that had not yet formed at the time of application in later sampling dates.

Extraction and Leaf Tissue Analysis

Leaf material from each plot was ground to a fine powder using liquid nitrogen and morter and pestal. A ca. five gram subsample of leaf powder was weighed and transferred to a 20 mL glass vial with a Teflon™-lined cap and stored at -20°C until extraction; the exact weight of each sample was recorded for conversion of analyte concentration to the fresh leaf weight basis. Extraction was conducted using QuEChERS in 15 mL acetonitrile using pre-weighed reagent sachets (United Chemical Technologies, #ECQUEU7-MP). A cleanup step was then conducted in which chlorophyll was removed from the acetonitrile extract using ChloroFiltr® polymeric based sorbent tubes (United Chemical Technologies, # ECMPSGG15CT). The supernatant from cleanup was then filtered through a 20 µm Teflon™ filter into an auto sampler vial. Separation and quantification of analytes was accomplished using Ultra-High Performance Liquid Chromatography with a C-18 column coupled to a Thermo TSQ Quantum™ mass spectrometer. The aqueous mobile phase was 0.1% formic acid in water and the polar modifying phase was 0.1% formic acid in acetonitrile. Samples were run against standards to construct a five point

linear curve in a concentration range of 0.5-50 ppm, and then against a five point standard curve in the range of 5-300 ppb. The concentration represented by the curve (in extract solution) was then converted back to µg/g leaf tissue using the exact sample weight.

Statistical Analyses

In-leaf distribution of neonicotinoids. Chemical titer data for greenhouse leaf section means were averaged over replicate and subjected to a general linear mixed model to account for the experimental design using SASv9.4 (Proc GLIMMIX, SAS Institute, 2013) to test for sample date by leaf section interactions. For leaf section field data, only chemical titer leaf section means from the 'top' tree region were used and averaged over replicate, then subjected to a general linear mixed model to account for experimental design using SASv9.4 (Proc GLIMMIX, SAS Institute, 2013) to test for sample date by leaf section interactions; location was treated as a random effect and the model was adjusted for cumulative rainfall. Mean separations indicate differences between leaf sections at $\alpha \leq 0.05$.

Temporal expression of neonicotinoids. Chemical titer data for greenhouse means were averaged over replicate and subjected to a general linear mixed model to account for experimental design using SASv9.4 (Proc GLIMMIX, SAS Institute, 2013) to test for sample date by leaf section interactions. For field data, chemical titer means from only the 'middle' leaf section were averaged over replicate and subjected to a general linear mixed model to account for experimental design using SASv9.4 (Proc GLIMMIX, SAS Institute, 2013) to test for sample date by location and sample date by tree region interactions; the model was adjusted for cumulative rainfall. Means were square root transformed prior to analysis to achieve homogeneity of variance and meet the assumptions of the model. Mean separations indicate differences between sample date at $\alpha \leq 0.05$.

Spatial distribution of Admire Pro analytes throughout the tree canopy. Chemical titer data were averaged over replicate and subjected to a general linear mixed model to account for experimental design using SASv9.4 (Proc GLIMMIX, SAS Institute, 2013) to test for location by tree region and sample date by tree region interactions; the model was adjusted for cumulative rainfall. Means were square root transformed prior to analysis to achieve homogeneity of variance meeting the assumptions of the model. Mean separations indicate differences between tree region at $\alpha \leq 0.05$.

Effect of Leaf Maturity on Neonicotinoid Expression. Chemical titer data were averaged over replicate and subjected to a general linear mixed model to account for experimental design using SASv9.4 (Proc GLIMMIX, SAS Institute, 2013) to test for sample date by leaf maturity interactions; season was treated as a random effect and the model was adjusted for cumulative rainfall. Means were square root transformed prior to analysis to achieve homogeneity of variance meeting the assumptions of the model.

Results

In-Leaf Distribution of Neonicotinoids

Following the application of Admire Pro in the greenhouse, no sample date by leaf section interaction was observed for imidacloprid ($F_{11, 1} = 2.14$; $p = 0.4914$), 5-OH ($F_{12, 4.107} = 1.2$; $p = 0.4682$), or olefin ($F_{12, 18.4} = 2.42$; $p = 0.0532$). Furthermore, no significant difference in titer was observed between leaf margin and leaf center for imidacloprid ($F_{1, 8.654} = 1.97$; $p = 0.1950$; Table 2-2), 5-OH ($F_{1, 7.393} = 1.82$; $p = 0.2175$; Table 2-2), or olefin ($F_{1, 12.57} = 1.37$; $p = 0.2643$; Table 2-2). When Admire Pro was applied to the soil in the field, no sample date by leaf section interaction was observed for imidacloprid ($F_{9, 276} = 0.19$; $p = 0.9948$), 5-OH ($F_{9, 275.9} = 0.27$; $p = 0.9832$), or olefin ($F_{9, 275.9} = 0.64$; $p = 0.7631$). A significant difference in titer between leaf sections was observed for imidacloprid ($F_{1, 276} = 4.19$; $p = 0.0415$; Table 2-2) and 5-OH ($F_{1,}$

$_{275.9}$ = 12.27; p = 0.0005; Table 2-2) where the leaf margin contained a higher concentrations than the leaf center. No difference in titer was observed between leaf sections for olefin ($F_{1, 275.9}$ = 1.22; p = 0.2699; Table 2-2).

Following the application of Platinum 75SG in the greenhouse, no sample date by leaf section interaction was observed for thiamethoxam ($F_{12, 1}$ = 1.38; p = 0.5894), clothianidin ($F_{12, 1}$ = 6.04; p = 0.3088), or TZMU ($F_{12, 27.2}$ = 0.94; p = 0.5267). No significant difference was observed in titer between leaf margin and leaf center for thiamethoxam ($F_{1, 23.67}$ = 1.05; p = 0.3158; Table 2-3) or for clothianidin ($F_{1, 2.981}$ = 4.11; p = 0.1363; Table 2-3), however, the leaf margin contained more TZMU than the leaf center ($F_{1, 17.12}$ = 12.44; p = 0.0026; Table 2-3). A sample date by leaf interaction was observed for TZNG ($F_{12, 14.4}$ = 4.52; p = 0.0042), but the order between leaf sections remained constant over time with the exception of 11 weeks following application. Nevertheless, no significant difference was observed in titer between leaf margin and leaf center for TZNG ($F_{1, 12.83}$ = 4.41; p = 0.0561). Following application of Platinum 75SG to the soil in the field, no sample date by leaf section interaction was observed for thiamethoxam ($F_{7, 191.1}$ = 0.08; p = 0.9992) or for clothianidin ($F_{7, 189.9}$ = 0.02; p = 1.000). Furthermore, no significant difference in titer was observed between leaf margin and leaf center for thiamethoxam ($F_{1, 191.1}$ = 0.16; p = 0.6938; Table 2-3) or for clothianidin ($F_{1, 189.9}$ = 0.33; p = 0.5668; Table 2-3). In contrast to observations from the greenhouse, TZMU and TZNG were not detected in the field.

Following the application of Belay 2.13SC in the greenhouse, no sample date by leaf section interaction was observed for clothianidin ($F_{9, 1}$ = 3.49; p = 0.3944) and no significant difference in leaf section was observed for clothianidin ($F_{1, 6.418}$ = 2.28; p = 0.1785; Table 2-4). A sample date by leaf section interaction was observed for TZMU ($F_{11, 12.9}$ = 3.01; p = 0.0315), yet

the order between leaf sections remained constant over time with the exception of five weeks following application. However, a significant difference in TZMU titer was observed between leaf margin and leaf center ($F_{1, 1.29} = 81.54$; $p = 0.0402$; Table 2-4) where the leaf margin had higher TZMU concentrations than the leaf center. Likewise, a sample date by leaf section interaction was observed for TZNG ($F_{12, 3.657} = 8.12$; $p = 0.0356$), but the order between leaf section concentration remained constant across all sample dates. Furthermore, a significant difference in TZNG titer was observed ($F_{1, 5.897} = 102.05$; $p < 0.0001$; Table 2-4) where the leaf margin had a higher TZNG titer than the leaf center. When Belay 2.13SC was applied to the soil in the field, no sample date by leaf section interaction was observed for clothianidin ($F_{7, 146} = 0.64$; $p = 0.7256$) or for TZNG ($F_{7, 145.9} = 1.21$; $p = 0.3019$). No difference was detected between leaf margin and leaf center for clothianidin ($F_{1, 146} = 3.37$; $p = 0.0685$; Table 2-4), yet higher levels of TZNG occurred in the leaf margin than the leaf center ($F_{1, 145.9} = 10.05$; $p = 0.0019$; Table 2-4). Dissimilar to observations in the greenhouse, TZMU was not detected in the field.

Temporal Expression of Neonicotinoids

When Admire Pro was applied to the soil in the greenhouse, a significant effect of sample date was observed for imidacloprid ($F_{12, 78} = 7.4$; $p < 0.0001$; Table 2-5), 5-OH ($F_{12, 17.25} = 10.71$; $p < 0.0001$; Table 2-5), and olefin ($F_{12, 18.4} = 11.6$; $p < 0.0001$; Table 2-5). The titer of each analyte peaked at five weeks following application and persisted through 13 weeks following application. The highest mean imidacloprid titer observed was 192.060 ppm, while the highest mean titer for 5-OH and olefin was 33.673 ppm and 8.134 ppm, respectively. Following the application of Admire Pro in the field, a location by sample date interaction was observed for imidacloprid ($F_{9, 935} = 18.54$; $p < 0.0001$), and imidacloprid titer was affected by sample date ($F_{9, 935} = 48.84$; $p < 0.0001$; Table 2-6). The highest mean imidacloprid titer was observed one week following application at the flatwoods location (1.052 ppm) and just before application at the

central ridge location (1.246 ppm) (Table 2-6). Low levels (< 0.090 ppm) of imidacloprid were detected up to eight weeks following application at the flatwoods location and 10 weeks following application at the central ridge location. A location by sample date interaction was also observed for 5-OH following the application of Admire Pro to the soil in the field ($F_{9, 935}$ = 15.26; $p < 0.0001$), and a significant effect of 5-OH titer was observed by sample date ($F_{9, 935}$ = 45.85; $p < 0.0001$; Table 2-6) where at the flatwoods location, 5-OH titer remained relatively constant to two weeks following application before decreasing, while the 5-OH titer at the central ridge location was highest prior to application and continuously decreased over time. Furthermore, a location by sample date interaction was observed for olefin ($F_{9, 935}$ = 2.52; p = 0.0076) following the application of Admire Pro, and olefin titer was affected by sample date ($F_{9, 935}$ = 23.66; $p < 0.0001$; Table 2-6). At each location, olefin persisted for up to six weeks following application of Admire Pro.

When Platinum 75SG was applied to the soil in the greenhouse, sample date had a significant effect on thiamethoxam titer ($F_{12, 5.146}$ = 7.94; p = 0.015; Table 2-7), where thiamethoxam expression was highest at three weeks (271.140 ppm) following application. Similarly, sample date had a significant effect on clothianidin titer ($F_{12, 5.068}$ = 6.16; p = 0.0274; Table 2-7) and TZMU titer ($F_{12, 34.62}$ = 5.92; $p < 0.0001$; Table 2-7) following the soil application of Platinum 75SG, which also peaked at 3 weeks (99.379 ppm and 3.019 ppm, respectively) following application. While sample date significantly affected TZNG expression ($F_{12, 12.65}$ = 134.66; $p < 0.0001$; Table 2-7) following the soil application of Platinum 75SG, no clear TZNG peak was observed at a single time point; TZNG titer fluctuated over the weeks following application. In contrast to expression levels observed in the greenhouse, when Platinum 75SG (0.37g / tree) was applied to the soil in the field, limited quantifiable thiamethoxam titers, or

resulting metabolite titers were detected in citrus leaf tissues, thus residue analyses for these citrus leaf tissues were ceased.

Following the soil-application of Belay 2.13SC in the greenhouse, a significant effect was observed by sample date for clothianidin ($F_{12, 12.61} = 14.25$; $p < 0.0001$; Table 2-8), TZMU ($F_{11, 14.9} = 3.86$; $p = 0.0087$; Table 2-8), and TZNG ($F_{12, 15.67} = 25$; $p < 0.0001$; Table 2-8). The maximum mean clothianidin titer (62.226 ppm) was observed at five weeks following application. The mean TZMU titer exhibited two distinct peaks, the first (0.543 ppm) at four weeks following application, and the second (0.833 ppm) at 11 weeks following application. A continual increase was observed for TZNG through eight weeks (peak mean 11.430 ppm) after application. Like following the application of Platinum 75SG to the soil in the field, limited quantifiable analytes were observed after application of Belay 2.13SC to the soil (1.27 mL / tree) in the field, therefore residue analyses for these citrus leaf tissues were discontinued.

Spatial Distribution of Admire Pro Analytes throughout the Tree Canopy

When Admire Pro was applied to the soil in the field, we observed no sample date by tree region interaction ($F_{54, 935} = 0.61$; $p = 0.9877$) and no location by tree region interaction for imidacloprid ($F_{6, 84} = 2.16$; $p = 0.0555$). Tree region had a significant effect on imidacloprid titer ($F_{6, 84} = 8.86$; $p < 0.0001$; Fig. 2-1A), in which the bottom tree region contained a significantly higher mean imidacloprid titer than the top or four cardinal side regions; no difference was observed between the spherical center region and the bottom region. Likewise, the spherical center region contained a higher mean imidacloprid titer than the top, north, or east tree regions, but was not different from the west or south tree regions. Furthermore, no difference was observed between the top tree region and the four cardinal side regions. No sample date by tree region interaction ($F_{54, 935} = 1.04$; $p = 0.3982$), or location by tree region interaction ($F_{6, 84} = 0.32$; $p = 0.9249$), was observed for olefin following application of Admire Pro to the soil in the field.

Furthermore, a significant effect of tree region was observed for olefin ($F_{6, 84} = 7.41$; $p < 0.0001$; Fig. 2-1B) in which the bottom tree region contained a higher mean olefin titer than the top tree region or the four cardinal side regions; no difference was observed between the bottom tree region and the spherical center region. No difference was observed in mean olefin titer between the top tree region and the four cardinal side regions, and no difference was observed between the spherical center region and the west and top tree region. In contrast, for the analyte 5-OH, no sample date by tree region interaction ($F_{54, 935} = 1.3$; $p = 0.0777$) was observed, yet a location by tree region interaction was observed ($F_{6, 84} = 3.94$; $p = 0.0016$). Tree region had a significant effect on mean 5-OH titer ($F_{6, 84} = 16.65$; $p < 0.0001$; Fig. 2-2) at the flatwoods location, where the bottom tree region contained higher 5-OH levels than all other tree regions. No difference in 5-OH titer was observed between the spherical center, west, and south tree regions, and no difference was observed between the top and four cardinal side regions. At the central ridge location, no difference was observed between the bottom, spherical center, west, north or east tree regions, and no difference was observed between the spherical center, top, and four cardinal side tree regions.

Effect of Leaf Maturity on Neonicotinoid Expression

After application of Admire Pro to the soil, there was no sample date by leaf maturity interaction for expression of imidacloprid ($F_{4, 55} = 0.84$; $p = 0.5053$), olefin ($F_{4, 55} = 0.77$; $p = 0.5500$), or 5-OH ($F_{4, 55} = 1.00$; $p = 0.4178$). Moreover, no difference in titer was observed between flush shoots and mature leaves for imidacloprid ($F_{1, 7} = 0.74$; $p = 0.4191$), olefin ($F_{1, 7} = 1.95$; $p = 0.2057$), or 5-OH ($F_{1, 7} = 2.55$; $p = 0.1543$). Following application of Platinum 75SG to the soil, no sample date by leaf maturity interaction was observed in expression of thiamethoxam ($F_{4, 55} = 2.14$; $p = 0.0879$), clothianidin ($F_{4, 55} = 1.07$; $p = 0.3823$), or TZNG ($F_{4, 55} = 0.21$; $p = 0.9318$). Furthermore, no difference was observed between flush shoots and

mature leaves in expression of thiamethoxam ($F_{1, 7} = 2.08$; $p = 0.1929$), clothianidin ($F_{1, 7} = 0.01$; $p = 0.9419$), or TZNG ($F_{1, 7} = 0.04$; $p = 0.8531$). No TZMU was detected following the application of Platinum 75SG to the soil in this study. In contrast, a sample date by leaf maturity interaction was observed in clothianidin titer following application of Belay 2.13SC to the soil ($F_{4, 55} = 3.36$; $p = 0.0156$). Although an interaction did occur, no difference was observed in clothianidin titer between flush shoots and mature leaves during each sample date or when sample date data were pooled ($F_{1, 7} = 5.26$; $p = 0.0554$). No sample date by leaf maturity interaction was observed in TZNG titer following application of Belay 2.13SC to the soil ($F_{4, 55} = 1.28$; $p = 0.2908$) and no difference was observed between flush shoots and mature leaves ($F_{1, 7} = 3.16$; $p = 0.1189$). No TZMU was detected following the application of Belay 2.13SC to the soil in this study.

Discussion

The goal of this study was to quantify the spatial distribution and temporal expression of three currently labeled neonicotinoid insecticides in the citrus tree canopy to elucidate why trees continue to succumb to HLB infection despite intensive management efforts by growers. Previous studies have discussed the possibility of uneven expression of neonicotinoids in citrus resulting in potential exposure of *D. citri* to sublethal dosages (Boina et al. 2009, Rogers 2012). This study was the first to use UHPLC-MS to quantify the temporal expression and spatial distribution of neonicotinoids and resulting metabolites in citrus following application to the soil. High parent material titers were observed following applications of Admire Pro (imidacloprid), Platinum 75SG (thiamethoxam), and Belay 2.13SC (clothianidin) in the greenhouse (max. mean 192 ppm imidacloprid; max. mean 240 ppm thiamethoxam; max. mean 62 ppm clothianidin). In contrast, low parent material titers (max. mean 1.246 ppm) of imidacloprid and very low titers (thiamethoxam max. mean 0.008; clothianidin max. mean 0.159) of thiamethoxam and

clothianidin were detected after application in the field. As a result, only the Admire Pro treatment in the field was used to evaluate expression of neonicotinoids and resulting metabolites over time and space; the 'top' tree region was used to evaluate in-leaf distribution for all three insecticides in the field, despite very low detection levels of thiamethoxam and clothianidin.

While we did not find a difference in imidacloprid concentration between leaf sections in the greenhouse, we did discover that the leaf margin contained elevated levels of imidacloprid following application of Admire Pro in the field. This difference was inconsistent with our findings for thiamethoxam and clothianidin following the application of Platinum 75SG and Belay 2.13SC, respectively; no difference in active ingredient expression was observed between leaf sections for either insecticide. Mendel et al. (2000) found low levels of ^{14}C-labeled imidacloprid around leaf vascular bundles when compared with the leaf margins. While we found one event that exhibited a difference between leaf sections, it is possible that our excision method failed to fully account for intricate vascular-bundle related expression patterns within a citrus leaf, resulting in a failure to detect differences. A number of the metabolites were detected at a higher concentration in the leaf margin compared with the leaf middle, however, because the concentration of each metabolite is directly dependent on the concentration of the associated parent material, we cannot determine how any one metabolite may affect *D. citri* mortality in this study. Understanding the role of each metabolite is beyond the scope of this investigation, though it is possible that a combination of multiple metabolites has an additive effect on *D. citri* mortality. Furthermore, because radiolabeled parent material cannot be differentiated from metabolites through radiographic imaging, it is possible that patterns related to the vascular bundles observed by Mendel et al. (2000) were actually accumulations of metabolites (metabolized imidacloprid constituents) carrying the ^{14}C marker instead of accumulations of the

parent material, imidacloprid. Nevertheless, inconsistent expression within a leaf remains of concern as it relates to potential expression of sublethal neonicotinoid dosages.

Understanding how neonicotinoids are expressed over time is important for a number of reasons: 1) determining when a subsequent non-neonicotinoid foliar spray must be applied, 2) determining the time it takes to reach peak expression levels as related to application timing, and 3) understanding the persistence of neonicotinoids in leaf tissues at sub-lethal levels. While lethal levels of each compound, as determined by Langdon and Rogers (2017), were observed in the present greenhouse study, sublethal levels of all analytes were detected in citrus leaf tissues in the field following insecticide application to the soil. Between six and eleven weeks of *D. citri* control have been reported following soil-application of neonicotinoids to young trees in the field (Qureshi and Stansly 2007, 2009; Ichinose et al. 2010; Setamou et al. 2010; Byrne et al. 2012; Rogers 2012). Percentage control is often quantified by comparing a mean number of insects across replicates in a given treatment with a mean number of insects across replicates within an untreated control. In this case, for percentage control to be reduced, insects must re-infest the treated area and develop to stages that make detection possible. Given the life cycle of *D. citri*, there is a presumed delay between when the expression level drops below the oviposition deterrence threshold (currently unknown) and the time that nymphs are detected in insect counts. The result is that while some number of weeks control may have been observed in the field, titers likely fell below lethal or deterrent levels before drops in percentage control were detected. Nevertheless, at the flatwoods location, we found a peak mean titer of 1.098 ppm imidacloprid at one week following application of Admire Pro, and at the central ridge location, a peak mean titer of 1.246 ppm imidacloprid at the time of application of Admire Pro. Because trees at each location contained imidacloprid at the time of application, we cannot draw

definitive conclusions based on temporal expression observed in the current study. We allowed seven weeks from the last known soil application of Admire Pro and the start of our field studies each season. Given the current state of HLB infection in commercial groves, we opted to not allow more time due to the risk of developing HLB infection in cooperator groves. Interestingly, the levels of imidacloprid and metabolites observed in the pre-application samples were not statistically different from the highest mean titer observed following the application for our study. Nevertheless, while the temporal expression objective was compromised, the objective outcomes addressing within-leaf concentration gradient and spatial canopy distribution remain valid.

Mapping the distribution of neonicotinoids throughout the tree canopy following application to the soil is critical to identifying likely inoculation sites and for determining where to sample on the tree in future research studying systemic neonicotinoids in citrus. We found higher imidacloprid concentrations in the bottom 10% of the tree canopy compared to other tree canopy regions, although no difference was observed between the lower canopy and the spherical center. Neonicotinoids are highly systemic and move through the plant xylem (Elbert et al. 1991, Maienfisch et al. 2001). A common characteristic of xylem mobile herbicides applied to the soil is injury accumulation in the oldest leaves. This is in contrast to phloem mobile herbicides, which cause injury near the growing point of the plant, or in the newest leaf tissue. Triazine herbicides, which like neonicotinoid insecticides are xylem mobile, result in higher concentrations within older leaves compared to new leaves when applied to the soil (Stoller 1970). The lower tree canopy contains the oldest set of leaves and our findings are consistent with movement patterns and accumulation of known xylem mobile herbicides. Castle et al. (2005) sampled xylem fluid to study spatial distribution of thiamethoxam and imidacloprid in

citrus tree canopies, but found no spatial difference in expression. They divided the tree into upper and lower halves and divided each half into quadrants. They sampled xylem fluid from two branches within each quadrant. The sampling technique used may have contributed to the differential outcome of their study compared to the present study. In addition, we wanted to determine if our one-sided application technique influenced distribution within the tree canopy. Insecticide solution was applied to the west side of the tree trunk at the flatwoods location, and the south side of the tree trunk at the central ridge location. We did not observe a location by tree region interaction for imidacloprid expression, which indicates that the pattern of spatial distribution was not different between locations. This suggests that the drench application site did not affect the distribution of imidacloprid within the tree canopy. Because we found no difference between the four tree canopy sides and the tree top, subsequent studies evaluating citrus leaf tissues should sample from this area to achieve a consistent sampling pattern.

Young, non-bearing trees flush more often than mature trees throughout the year and gravid adults are attracted to the volatiles emitted by flush shoots (Stansly and Rogers 2006, Patt and Setamou 2010). Nymphs are also more likely to acquire *C*Las as they develop on flush shoots (Pelz-Stelinski et al. 2010). Although *D. citri* are more attracted to flush shoots, much of the leaf tissue subjected to analytical evaluation of chemical titers to date have utilized only mature leaves, largely due to the constant availability of leaves within the same cohort over a long period of time following a single application (Langdon 2017). It is important to determine whether insecticide expression levels differ between mature leaves and flush shoots to allow one to predict whether better or worse control would be expected in flush shoots based on known titers in mature leaves. We found no difference in titer between flush shoots and mature leaves for any chemical evaluated following application of each of the three neonicotinoids included in

our study. At the time insecticide applications were made, flush shoots had already emerged and were actively growing, and the same cohort was sampled across the four post-application sample events. Subsequent studies should be conducted to evaluate the relation between bud break timing and timing of application to the soil, such that resultant titers are maximized in flush shoots. Perhaps if one were to apply neonicotinoids to the soil two weeks prior to bud break, expression levels in flush shoots would have been less than in mature leaves due to limited availability of insecticide by the time flush shoots emerged. Nevertheless, in the present study, neonicotinoid expression in mature leaves did not differ from expression in the more attractive flush shoots when the application was made after bud break.

The overarching goal of this research was to identify the temporal expression and spatial distribution of neonicotinoids in citrus leaf tissues following application to the soil. While we successfully mapped the distribution of three neonicotinoids and resulting metabolites within individual leaves, and one neonicotinoid and resulting metabolites throughout the tree canopy, we unexpectedly observed low levels of expression of all compounds tested, much lower than what was identified as lethal through ingestion by Langdon and Rogers (2017). Contributing factors to low-level expression observed in our study may have been that application rates were not high enough for the size of tree tested or while unlikely, it is possible that HLB infection negatively impacted uptake of our treatment applications as trees were not tested for *C*Las infection. Tree size and application rate have been shown to directly impact uptake and expression of thiamethoxam following application to the soil in citrus (Langdon 2017). Furthermore, Langdon (2017) found that 0.55 ppm imidacloprid did not reduce in any feeding behavior including probing, pathway, or salivation / ingestion activities when monitoring using electropenetrography. However, they did find that 5.5 ppm imidacloprid caused significant

reductions in pathway and salivation / ingestion behaviors. Nevertheless, it is likely that the highest mean titer observed for any neonicotinoid in the present study offered no reduction in feeding activity, and thus no interception of *C*Las inoculation. Because non-neonicotinoid foliar sprays were routinely applied to the study area, no attempt was made to correlate insect incidence or abundance with neonicotinoid titer levels.

In addition to potential risk of the spread of HLB, neonicotinoid resistance following exposure to sublethal dosages is of significant concern. *Diaphorina citri* resistance to neonicotinoids was recently detected in Florida (Langdon and Rogers 2017), which may have been exacerbated by sublethal neonicotinoid expression like that observed in the present study. Tiwari et al. (2011a) originally discovered *D. citri* resistance to neonicotinoids in Florida in 2009, but likely due to intensive area-wide spray programs implemented in 2010, reversion was believed to have occurred by 2013 (Coy et al. 2016). Tiwari et al. (2011a) found that imidacloprid resistant *D. citri* populations expressed higher levels of detoxifying enzymes, including general esterase, glutathione *S*-transferase, and cytochrome P_{450} monooxygenases. Subsequent research identified five family 4 cytochrome P_{450} genes that were induced by exposure to imidacloprid (Tiwari et al. 2011b). Although elevated levels of detoxifying enzymes were found in insecticide resistant populations, Tiwari et al. (2011a) suggested that reduced penetration, target-site insensitivity, and mutations in detoxifying enzymes may also impact development of neonicotinoid resistance. Nevertheless, development of resistance to neonicotinoids by *D. citri* has occurred in the field, and therefore, applications of neonicotinoids must be carefully administered such that *D. citri* exposure to sublethal dosages is minimized.

The present study quantifies the concentration of imidacloprid, thiamethoxam, and clothianidin in citrus leaf material in space and over time following application to the soil. While

imidacloprid accumulated at higher levels in the lower portion of the tree canopy, we did not find titers near the lethal range quantified by Langdon and Rogers (2017), therefore even the bottom tree region, which exhibited the highest imidacloprid levels, would expose *D. citri* to sublethal dosages. Langdon and Rogers (2017) found that lethal activity from contact exposure to neonicotinoid insecticides occurs at very low concentrations compared with ingestion. To potentially maximize the activity of neonicotinoids and permit the longevity of their use, subsequent work should investigate neonicotinoid residues over time following foliar application. Presumably, foliar application would result in much higher acute residues following application, with a more rapid residue degradation, which is more suitable within the scope of insecticide resistance management. Given the recent findings of neonicotinoid resistance in various field populations of *D. citri*, future implementation of neonicotinoid insecticides in the field should focus on reducing the likelihood of increasing the incidence and severity of resistance.

Table 2-1. Neonicotinoid product description and use rates for greenhouse and field studies.

Product	Rate per hectare applied (rate per acre)	Rate per tree (based on 346 trees per hectare or 140 trees per acre)	Grams active ingredient per tree	Resulting analytes
Admire Pro 4.6F	511.09 mL/ha (7 fl oz/ac)	1.48 mL/tree	0.814 g/tree	imidacloprid* 5-OH olefin
Platinum 75SG	128.10 g/ha (1.83 oz wt/ac)	0.37 g/tree	0.324 g/tree	thiamethoxam* clothianidin TZMU TZNG
Belay 2.13SC	438.07 mL/ha (6 fl oz/ac)	1.27 mL/tree	0.278 g/tree	clothianidin* TZMU TZNG

*****Active ingredient of listed formulated product.

Table 2-2. Chemical titer (ppm) in citrus leaf tissue across two leaf sections following application of Admire Pro (1.48 mL per tree) to the soil in the greenhouse and in the field.

Study	Leaf section	imidacloprid		5-OH		Olefin	
		Mean	95% CI	Mean	95% CI	Mean	95% CI
greenhouse	center	109.930a	(44.819 - 175.041)	15.399a	(8.777 - 22.021)	3.571a	(1.738 - 5.404)
	margin	129.310a	(64.199 - 194.421)	21.217a	(14.595 - 27.839)	4.959a	(3.126 - 6.791)
		p-value = 0.1950		p-value = 0.2175		p-value = 0.2643	
field	center	0.412b	(0.295 - 0.528)	0.078b	(0.062 - 0.095)	0.015a	(0.009 - 0.022)
	margin	0.528a	(0.406 - 0.650)	0.110a	(0.092 - 0.127)	0.019a	(0.012 - 0.026)
		p-value = 0.0415		p-value = 0.0005		p-value = 0.2699	

Values sharing the same letter do not differ significantly at $\alpha \leq 0.05$.

Table 2-3. Chemical titer (ppm) in citrus leaf tissue across two leaf sections following application of Platinum 75SG (0.37g per tree) to the soil in the greenhouse and in the field.

Study	Leaf Section	thiamethoxam		clothianidin		TZMU		TZNG	
		mean	95% CI	mean	95% CI	mean	95% CI	mean	95% CI
Green-house	center	94.162a	(54.080 - 134.244)	45.201a	(29.866 - 60.536)	0.796a	(0.081 - 1.512)	3.637a	(2.875 - 4.399)
	margin	104.660a	(64.578 - 144.742)	54.230a	(38.895 - 69.565)	1.052b	(0.340 - 1.765)	4.776a	(4.014 - 5.538)
		p-value = 0.3158		p-value = 0.1363		p-value = 0.0026		p-value = 0.0561	
field	center	0.006a	(0.000 - 0.012)	0.002a	(0.000 - 0.004)	0	-	0	-
	margin	0.008a	(0.002 - 0.014)	0.003a	(0.000 - 0.005)	0	-	0	-
		p-value = 0.6938		p-value = 0.5668		-		-	

Values sharing the same letter do not differ significantly at $\alpha \leq 0.05$.

Table 2-4. Chemical titer (ppm) in citrus leaf tissue across two leaf sections following application of Belay 2.13SC (1.27 mL per tree) to the soil in the greenhouse and in the field.

Study	Leaf section	clothianidin		TZMU		TZNG	
		mean	95% CI	mean	95% CI	mean	95% CI
greenhouse	center	38.425a	(27.890 - 48.960)	0.340a	(0.191 - 0.488)	6.595a	(5.536 - 7.653)
	margin	48.306a	(37.771 - 58.841)	0.522b	(0.374 - 0.669)	9.649b	(8.592 - 10.707)
		p-value = 0.1785		p-value = 0.0402		p-value < 0.0001	
field	center	0.138a	(0.115 - 0.160)	0	-	0.033a	(0.020 - 0.046)
	margin	0.159a	(0.137 - 0.182)	0	-	0.055b	(0.042 - 0.068)
		p-value = 0.0685		-		p-value = 0.0019	

Values sharing the same letter do not differ significantly at $\alpha \leq 0.05$.

Table 2-5. Chemical titer (ppm) in citrus leaf tissue during the weeks following application of Admire Pro (1.48 mL per tree) to the soil in the greenhouse.

Weeks following	imidacloprid		5-OH		Olefin	
application	mean	95% CI	mean	95% CI	mean	95% CI
0	00.000d	-	0cd	-	0bc	-
1	60.148cd	(00.000 - 128.178)	3.548c	(00.147 - 6.948)	0.269b	(0.137 - 0.402)
2	153.770ab	(85.279 - 222.261)	14.474abc	(10.940 - 18.007)	1.796b	(1.402 - 2.191)
3	167.060ab	(87.238 - 246.882)	25.178a	(20.784 - 29.571)	4.375ab	(3.523 - 5.227)
4	171.690a	(102.554 - 240.826)	27.001a	(21.031 - 32.972)	5.191ab	(4.033 - 6.349)
5	192.060a	(122.523 - 261.597)	33.673a	(25.500 - 41.845)	8.134a	(5.917 - 10.350)
6	164.640ab	(91.245 - 238.035)	26.616a	(19.821 - 33.411)	5.091ab	(3.286 - 6.897)
7	137.020abc	(61.172 - 212.868)	22.544ab	(14.249 - 30.838)	5.339ab	(2.891 - 7.786)
8	101.190abcd	(31.965 - 170.415)	16.796abc	(10.968 - 22.625)	4.896ab	(2.853 - 6.940)
9	102.810abcd	(33.698 - 171.922)	18.309ab	(11.883 - 24.734)	5.920ab	(2.776 - 9.064)
10	64.766bcd	(00.000 - 134.007)	9.376bc	(04.023 - 14.729)	2.390b	(1.642 - 3.138)
11	105.670abcd	(07.877 - 203.463)	14.260abc	(03.686 - 24.834)	1.956b	(0.952 - 2.961)
12	93.438abcd	(10.572 - 176.303)	18.015abc	(05.753 - 30.277)	6.250ab	(0.600 - 11.900)
13	40.810d	(00.000 - 108.649)	8.219bc	(03.094 - 13.343)	3.834ab	(1.197 - 6.470)
	p-value < 0.0001		p-value < 0.0001		p-value < 0.0001	

Values sharing the same letter do not differ significantly at $\alpha \leq 0.05$.

Table 2-6. Chemical titer (ppm) in citrus leaf tissue during the weeks following application of Admire Pro (1.48 mL per tree) to the soil in the field at two commercial Florida citrus groves.

Location	Weeks following application	Imidacloprid		5-OH		Olefin	
		Mean	95% CI	Mean	95% CI	Mean	95% CI
Flatwoods	0	0.926bc	(0.777 - 1.075)	0.440bcd	(0.375 - 0.505)	0.142abc	(0.091 - 0.193)
	1	1.098ab	(0.989 - 1.207)	0.436bcd	(0.388 - 0.483)	0.096bcd	(0.059 - 0.133)
	2	1.052ab	(0.958 - 1.147)	0.447bc	(0.405 - 0.488)	0.143ab	(0.111 - 0.175)
	3	0.902bc	(0.812 - 0.992)	0.390cd	(0.351 - 0.429)	0.055cd	(0.024 - 0.085)
	4	0.539e	(0.449 - 0.630)	0.270ef	(0.231 - 0.310)	0.106abc	(0.076 - 0.137)
	5	0.491ef	(0.400 - 0.583)	0.223efg	(0.183 - 0.263)	0.103bcd	(0.072 - 0.134)
	6	0.301fg	(0.208 - 0.394)	0.174fgh	(0.133 - 0.215)	0.048cd	(0.016 - 0.079)
	8	0.090gh	(0.000 - 0.185)	0.045ij	(0.004 - 0.087)	0.000efg	-
	10	0.000h	-	0.000ij	-	0.000efg	-
	12	0.000h	-	0.000j	-	0.000fg	-
Central Ridge	0	1.246a	(1.097 - 1.395)	0.606a	(0.541 - 0.672)	0.210a	(0.160 - 0.261)
	1	1.117ab	(0.988 - 1.246)	0.539ab	(0.482 - 0.595)	0.126abc	(0.082 - 0.169)
	2	0.885bc	(0.788 - 0.982)	0.434bcd	(0.391 - 0.476)	0.141abc	(0.109 - 0.174)
	3	0.784cd	(0.694 - 0.873)	0.393cd	(0.354 - 0.432)	0.014def	(0.000 - 0.045)
	4	0.561de	(0.470 - 0.653)	0.296de	(0.256 - 0.335)	0.096abc	(0.065 - 0.127)
	5	0.484ef	(0.390 - 0.577)	0.255efg	(0.214 - 0.296)	0.066bcd	(0.035 - 0.098)
	6	0.398ef	(0.302 - 0.494)	0.289de	(0.246 - 0.331)	0.038cde	(0.006 - 0.071)
	8	0.149gh	(0.036 - 0.262)	0.157gh	(0.108 - 0.207)	0.000fg	-
	10	0.006h	(0.000 - 0.125)	0.069hi	(0.018 - 0.121)	0.000g	-
	12	0.000h	-	0.000ij	-	0.000g	-
		p-value < 0.0001		p-value < 0.0001		p-value < 0.0001	

Values sharing the same letter do not differ significantly at $\alpha \leq 0.05$.

Table 2-7. Chemical titer (ppm) in citrus leaf tissue during the weeks following application of Platinum 75SG (0.37g per tree) to the soil in the greenhouse.

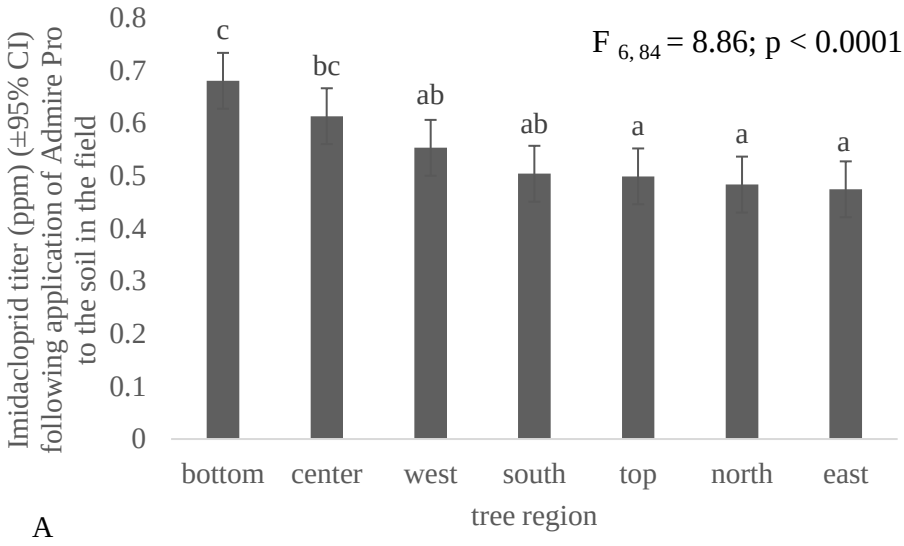
Weeks following application	thiamethoxam		clothianidin		TZMU		TZNG	
	mean	95% CI	mean	95% CI	mean	95% CI	mean	95% CI
0	00.000cd	-	00.000cd	-	0.000b	-	0.000bc	-
1	69.801abc	(26.922 - 112.679)	12.293c	(00.000 - 27.877)	0.000b	-	0.943b	(0.762 - 1.123)
2	240.070ab	(152.601 - 27.539)	66.470abc	(36.160 - 96.780)	1.039b	(0.149 - 1.928)	4.028ab	(1.873 - 6.182)
3	271.140a	(180.863 -361.417)	99.379a	(74.223 - 124.534)	3.019a	(2.129 - 3.908)	5.358a	(5.121 - 5.594)
4	92.293abc	(51.673 - 132.912)	40.939abc	(24.869 - 57.008)	0.701b	(0.000 - 1.591)	2.503b	(2.249 - 2.756)
5	118.970abc	(68.429 - 169.511)	58.515abc	(37.710 - 79.320)	0.855b	(0.000 - 1.744)	3.830ab	(2.864 - 4.796)
6	86.693abc	(47.322 - 126.063)	51.415abc	(35.267 - 67.563)	0.478b	(0.000 - 1.367)	2.795ab	(2.233 - 3.357)
7	114.820abc	(61.219 - 168.421)	68.703ab	(46.374 - 91.031)	0.692b	(0.000 - 1.581)	5.228ab	(4.014 - 6.441)
8	58.246abc	(17.836 - 98.656)	61.495abc	(31.895 - 91.095)	1.025b	(0.136 - 1.914)	5.313ab	(3.636 - 6.989)
9	62.636abc	(23.276 - 101.997)	43.769abc	(25.739 - 61.799)	0.886b	(0.000 - 1.775)	2.690ab	(1.268 - 4.112)
10	57.736abc	(18.481 - 96.992)	39.940abc	(24.144 - 55.736)	0.969b	(0.079 - 1.858)	2.728ab	(1.664 - 3.791)
11	44.153bc	(00.517 - 87.788)	27.114bc	(11.430 - 42.797)	1.176b	(0.287 - 2.066)	3.528ab	(2.760 - 4.295)
12	49.399abc	(07.219 - 91.579)	43.935abc	(19.780 - 68.090)	1.011b	(0.121 - 1.900)	4.940ab	(1.777 - 8.103)
13	26.421c	(26.421 - 65.464)	32.336bc	(16.303 - 48.370)	0.453b	(0.000 - 1.342)	3.400ab	(2.344 - 4.456)
	p-value = 0.0150		p-value = 0.0274		p-value < 0.0001		p-value < 0.0001	

Values sharing the same letter do not differ significantly at $\alpha \leq 0.05$.

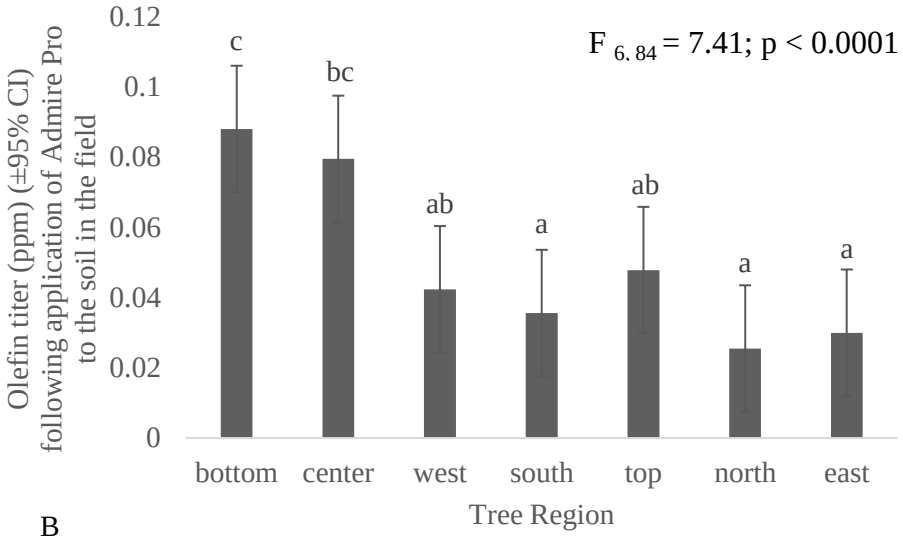
Table 2-8. Chemical titer (ppm) in citrus leaf tissue during the weeks following application of Belay 2.13SC (1.27 mL per tree) to the soil in the greenhouse.

Weeks following application	clothianidin		TZMU		TZNG	
	mean	95%CI	mean	95%CI	mean	95%CI
0	00.000cd	-	0.000bc	-	0.000de	-
1	12.714c	(8.009 - 17.418)	0.000bc	-	1.019d	(0.000 - 2.465)
2	39.171ab	(33.239 - 45.103)	0.084b	(0.000 - 0.332)	2.813cd	(1.313 - 4.312)
3	49.430ab	(42.742 - 56.118)	0.419ab	(0.171 - 0.667)	6.349bc	(4.894 - 7.803)
4	55.218a	(47.285 - 63.150)	0.543ab	(0.295 - 0.790)	5.903bc	(4.445 - 7.360)
5	62.225a	(46.816 - 77.634)	0.460ab	(0.212 - 0.708)	9.045ab	(7.551 - 10.539)
6	51.076ab	(42.718 - 59.434)	0.232b	(0.000 - 0.480)	9.605ab	(8.135 - 11.075)
7	54.576a	(43.322 - 65.831)	0.129b	(0.000 - 0.377)	10.933a	(9.361 - 12.504)
8	48.741ab	(39.853 - 57.629)	0.451ab	(0.203 - 0.699)	11.430a	(9.880 - 12.980)
9	42.701ab	(35.428 - 49.975)	0.564ab	(0.316 - 0.812)	9.848ab	(8.329 - 11.366)
10	36.338abc	(21.613 - 51.062)	0.613ab	(0.365 - 0.860)	7.733abc	(5.042 - 10.423)
11	45.959ab	(35.325 - 56.593)	0.833a	(0.585 - 1.080)	10.649a	(9.141 - 12.157)
12	36.048abc	(23.594 - 48.501)	0.545ab	(0.266 - 0.824)	10.267ab	(8.044 - 12.489)
13	29.558bc	(17.487 - 41.628)	0.295ab	(0.034 - 0.556)	9.994ab	(6.920 - 13.067)
	p-value < 0.0001		p-value = 0.0087		p-value < 0.0001	

Values sharing the same letter do not differ significantly at $\alpha \leq 0.05$.



A



B

Figure 2-1. Comparison of chemical titer between seven tree regions during 2015 and 2016 field seasons. A. Imidacloprid titer in citrus leaf tissues resulting from soil-application of Admire Pro in the field. B. Olefin titer in citrus leaf tissues resulting from soil-application of Admire Pro in the field. Bars sharing the same letter do not differ significantly at $\alpha \leq 0.05$.

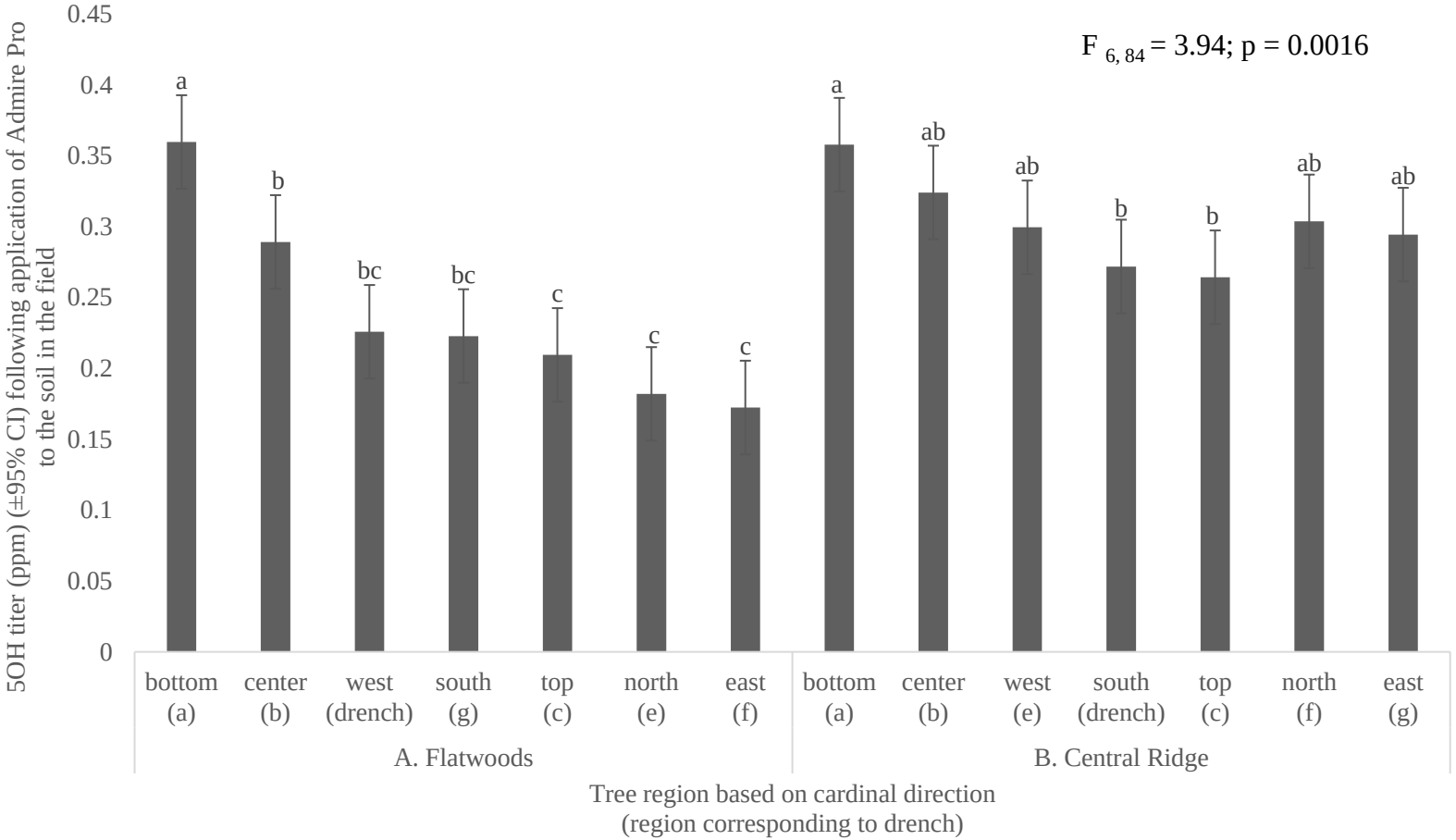


Figure 2-2. Comparison of 5-OH titer between seven tree regions at two locations during 2015 and 2016 field seasons. A. Tree region comparison at the flatwoods location following soil-application of Admire Pro in the field. B. Tree region comparison at the central ridge location following soil-application of Admire Pro in the field. Bars sharing the same letter do not differ significantly at $\alpha \leq 0.05$.

CHAPTER 3
INFLUENCE OF TREE SIZE AND APPLICATION RATE ON EXPRESSION OF THIAMETHOXAM IN CITRUS AND ITS EFFICACY AGAINST *DIAPHORINA CITRI* (HEMIPTERA: LIVIIDAE)

Neonicotinoids are a key group of insecticides used to manage *Diaphorina citri* Kuwayama, in Florida citrus. *Diaphorina citri* is the vector of *Candidatus* Liberibacter asiaticus (*C*Las), the presumed causal agent of huanglongbing (HLB), a worldwide disease of citrus. A two-season field study was conducted to evaluate the effect of tree size and application rate on expression of thiamethoxam in young citrus following application to the soil. *Diaphorina citri* adult and nymph abundance was also correlated with thiamethoxam titer in leaves. Tree size and application rate each significantly affected thiamethoxam titer in leaf tissue. The highest mean thiamethoxam titer observed (33.39 ppm) in small trees (mean canopy volume = $0.08m^3$) occurred after application of the high rate (0.74 g Platinum 75SG per tree) tested. There was a negative correlation between both nymph and adult abundance with increasing thiamethoxam titer in leaves. A concentration of 64.63 ppm thiamethoxam was required to reach a one percent probability of encountering a flush shoot with at least one adult *D. citri*, while 19.05 ppm was required for the same probability of encountering nymphs. The LC_{99} for the field population was 147.91 ppm by ingestion and 0.33 ppm by contact. Because thiamethoxam titer failed to reach a lethal level (>147.91 ppm), *D. citri* were presumably exposed to sublethal thiamethoxam doses, likely exacerbating resistance potential. Based on our results, we suggest the use of neonicotinoids by foliar rather than soil application to maintain the utility of this chemical class in future insecticide management programs in Florida citrus.

Justification

Citrus (Rutaceae) is the largest agricultural commodity in Florida; approximately one-quarter million hectares valued at nearly 9.9 billion dollars were cultivated in 2015 (Hodges and

Spreen 2015). This crop has come under severe decline in recent years due to the spread of the devastating citrus disease, huanglongbing (HLB). Huanglongbing is presumably the result of infection by the phloem-limited bacterium, *Candidatus* Liberibacter asiaticus (*C*Las), transmitted by the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) (Halbert and Manjunath 2004, Bové 2006, Grafton-Cardwell et al. 2013). Following inoculation into a tree by a feeding *C*Las-infected psyllid, bacteria move from the infection site, through the vascular phloem to compromise the root system, which in turn deprives the tree canopy and reduces fruit yield (Halbert and Manjunath 2004, Bové 2006, Grafton-Cardwell et al. 2013). Huanglongbing was first detected in Florida in 2005 (Halbert 2005), just seven years after the discovery of *D. citri* (Halbert and Manjunath 2004). Vector management using insecticides quickly became the key strategy to reduce *D. citri* populations and consequent spread of HLB (Rogers 2008).

Diaphorina citri develop and reproduce rapidly, requiring as little as 15 days to complete the egg to adult life cycle under optimal environmental conditions (25-28°C) (Liu and Tsai 2000, Grafton-Cardwell et al. 2013). Adults typically seek volatile-emitting flush shoots as sites for oviposition (Patt and Setamou 2010). Eggs hatch in two to four days, where newly emerged nymphs feed on phloem sap, thus acquiring *C*Las from the infected tree (Pelz-Stelinski et al. 2010). Because the probability of successful *C*Las acquisition by *D. citri* is higher for nymphs than for adults, the greatest risk of spread is from insects that acquire the bacteria during the nymph stage (Pelz-Stelinski et al. 2010). As nymphs become adults, they disperse and spread the bacteria to uninfected trees. An estimated 80-100 percent of *D. citri* found in Florida are *C*Las positive (Coy and Stelinski 2015); therefore, both nymphs developing on infected citrus tissue, as well as, infected adults feeding on new, uninfected plant material must be targets for successful vector suppression.

Young trees are defined as less than eight feet in height that flush asynchronously and more often than mature trees (Hall and Albrigo 2007, Rogers 2012). Because young trees produce attractive flush shoots often throughout the year, they are at high risk of becoming infected with *C*Las throughout the year (Stansly and Rogers 2006). Insecticides have been important in mitigating *C*Las infection of young trees, especially before trees reach fruit-bearing age (Rogers 2012). Extension recommendations by the University of Florida suggest a rotation between soil-applied neonicotinoids and non-neonicotinoid foliar sprays to reduce *D. citri* populations in young tree groves (Rogers 2012, Rogers et al. 2015). Neonicotinoids are within the Insecticide Resistance Action Committee (IRAC) sub-group 4A, and act on the insect nicotinic acetylcholine receptor (nAChR) (IRAC 2017). Neonicotinoids are highly systemic and when applied to the soil, are taken up by the root system, and transported to the foliage via xylem channels (Elbert et al. 2008).

Three neonicotinoid insecticides are currently labeled for use in non-bearing citrus in Florida: 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). Between six and eleven weeks of *D. citri* control have been documented following the application of neonicotinoids to the soil (Qureshi and Stansly 2007, Qureshi and Stansly 2009, Ichinose et al. 2010, Setamou et al. 2010, Rogers 2012). Residual activity of insecticides applied to the soil are likely influenced by factors such as soil type, application volume, irrigation/rainfall, tree age and size, and environmental conditions. Moreover, neonicotinoid insecticides metabolize into various analytes, though the effect of any one resulting metabolite on *D. citri* mortality is unknown (Byrne et al. 2017). For example, thiamethoxam metabolizes into clothianidin (Nauen et al.

2003) and clothianidin further metabolizes into TZNG and TZMU (Kim et al. 2012). Nevertheless, to date, there has been scant information on movement, distribution, and persistence of soil-applied neonicotinoids or metabolites in citrus tissue in the field.

Previous insecticide trials evaluating soil-applied neonicotinoids used percent control relative to the untreated check or mean number of *D. citri* per sample size to assess efficacy (Qureshi and Stansly 2007, Qureshi and Stansly 2009, Ichinose et al. 2010, Setamou et al. 2010, Byrne et al. 2012, Rogers 2012). Additional studies specifically quantified the concentration of neonicotinoids in leaf tissue following soil application using enzyme-linked immunosorbent assay (ELISA) (Castle et al. 2005, Garlapati 2009, Setamou et al. 2010, Byrne et al. 2017). However, only one attempted to compare percent control with chemical titer within citrus leaf tissues. The lethal concentration of imidacloprid for *D. citri* was estimated between 200 and 250 parts per billion (ppb) by correlating percentage control of *D. citri* with imidacloprid titer (Setamou et al. 2010). Because virtually all *D. citri* in Florida are assumed to be *C*Las positive, growers cannot tolerate any feeding on uninfected trees. In 2013, an estimated one to three percent of trees succumb to HLB infection annually in Florida groves, despite deliberate use of soil-applied neonicotinoids (Rogers 2013). Langdon and Rogers (2017) found that 62.19 ppm of imidacloprid was required to kill 90 percent of *D. citri* from a laboratory susceptible population through ingestion and hypothesized that the 200 to 250 ppb efficacy threshold set by Setamou et al. (2010) may have been the result of sublethal feeding deterrence as opposed to lethal activity. Nevertheless, uneven uptake or distribution of neonicotinoids in citrus tissue may also result in exposure of *D. citri* to sublethal doses (Boina et al. 2009, Rogers 2012), which may aid in development of resistance to this particular chemistry. The problem of uneven distribution is compounded as trees grow, requiring increasing application rate. As rates are increased to match

tree age and size, annual use limits become increasingly restricting for the development of effective year-long management strategies. Resistance to neonicotinoids was discovered in the field in 2009 (Tiwari et al. 2011a); however, reversion to susceptibility was reported by 2014 (Coy et al. 2016). This was attributed to increased rotations with foliar applied insecticides through area-wide spray programs. A more thorough understanding of the use of soil-applied neonicotinoids and factors that may stimulate the development of resistance is critical to maintaining effective use of this mode of action for management of HLB in Florida citrus.

The purpose of this study was to quantify thiamethoxam expression over time within citrus tissue following soil application, to evaluate efficacy of Platinum 75SG applied at two rates to young citrus trees of two sizes against *D. citri*, and to quantify susceptibility of the *D. citri* field population to thiamethoxam by exposure through ingestion and contact. By quantifying the temporal distribution of thiamethoxam in citrus leaf tissue with distinct tree sizes and application rates, we can more effectively refine management recommendations for the use of neonicotinoids in young citrus within the context of resistance management.

Materials and Methods

Insecticide Application and Citrus Leaf Sampling

A two-season field study was conducted during 2016 and 2017 to determine the concentration of thiamethoxam and resulting metabolites in citrus leaves following application of Platinum 75SG to the soil over time, based on application rate and tree size. We also determined the influence of thiamethoxam concentration on incidence and abundance of *D. citri* on treated trees over time. Untreated citrus (v. Hamlin / r.s. Swingle) trees of two non-bearing size classes were used in the study and defined as: large (mean canopy volume (MCV) approx. 1.34m^3) and small (MCV approx. 0.08m^3) in size. Large trees used in the study were field planted approximately 18 months prior to the first insecticide application and small trees were field

planted approximately 1 month prior to the first insecticide application. The same cohort of trees used during 2016, were used during 2017. Trees were planted to sandy soil comprised of 96.8% sand, 1.6% silt, and 2% clay, with 1.04% organic matter and cation exchange capacity (CEC) of 6.7 meq/100g. Although rate calculations were based on the most common plant density of 140 trees per acre, due to space constraints, trees for this study were planted using a 2.4m in-row spacing and 2.4m between-row spacing, which provided sufficient separation to eliminate uptake of insecticides applied to an adjacent tree, confirmed by analysis of trees in the untreated control. The study was arranged in a randomized complete block design with six treatments and four replicates and each plot consisted of four trees. Prior to each insecticide application, tree canopy volume was measured, but no attempt was made to account for differences in individual tree size when identifying treatment plots. The first season insecticide application was made on 8-IX-2016 and the second season application was made on 20-I-2017. At the time of application, 237 mL of insecticide solution (deionized water + insecticide) was applied to the soil at the base of each tree trunk, which is the common application volume in the commercial setting. The high application rate was 0.74 g Platinum 75SG per tree (equiv. 3.67 oz wt product/ac on 140 trees/ac) and the low application rate was 0.37 g Platinum 75SG per tree (equiv. 1.83 oz wt product/ac on 140 trees/ac). Leaf tissue samples were collected prior to the application of insecticides and then weekly for 12 weeks following the application. At each sample date, four mature, fully expanded leaves (ca. 5 - 15 grams) were randomly harvested from the outer canopy across each of the four trees within a plot. Leaves were placed into labeled paper bags and collectively stored by treatment in a plastic re-sealable bag at -20°C until residue analyses were conducted. Additionally, trees were evaluated weekly for the incidence and abundance of *D. citri* nymphs and adults. At each sample date, the number of *D. citri* nymphs and adults across ten

flush terminals per plot, and the number of nymph and adult infested terminals within each plot were counted and reported separately.

Extraction and Leaf Tissue Analysis

Leaf material from each plot was ground to fine powder using liquid nitrogen with morter and pestle. A ca. five gram subsample of leaf powder was weighed and transferred to a 20 mL glass vial with a Teflon-lined cap and stored at -20°C until extraction; the exact weight of each sample was recorded for conversion of analyte concentration to fresh leaf weight. Extraction was conducted with QuEChERS (Anastassiades 2003) in 15 mL acetonitrile using pre-weighed reagent sachets (United Chemical Technologies, #ECQUEU7-MP). A cleanup step was then conducted in which chlorophyll was removed from the acetonitrile extract using ChloroFiltr® polymeric based sorbent tubes (United Chemical Technologies, Horsham, PA, # ECMPSGG15CT). The supernatant from cleanup was filtered through a 20µm Teflon filter into an auto sampler vial. Separation and quantification of analytes was accomplished using Ultra-High Performance Liquid Chromatography with a C-18 column coupled to a Thermo TSQ Quantum mass spectrometer (UHPLC-MS). The LOQ was 5 ng/g for imidacloprid, 10 ng/g for clothianidin, thiamethoxam, and 5-OH, and 25 ng/g for olefin, TZMU, and TZNG. The LOD was 1.5 ng/g for imidacloprid, 3.2 ng/g for clothianidin and thiamethoxam, 3.0 ng/g for 5-OH, 8.0 ng/g for olefin, and 8.3 ng/g for TZMU and TZNG. The aqueous mobile phase was 0.1% formic acid in water and the polar modifying phase was 0.1% formic acid in acetonitrile. Samples were run against standards to construct a five point linear curve in a concentration range of 0.5-50 ppm, and then against a five point standard curve in the range of 5-300 ppb. The concentration represented by the curve (in extract solution) was then converted back to µg/g leaf tissue using the exact sample weight. The standards were matrix matched to compensate for signal suppression effects of the matrix. Plant tissue free of all four analytes was extracted using

QuEChERS as outlined above in order to obtain a blank matrix for mixing working standards. Primary standards were made using technical grade material (97.6-99.9%) in acetonitrile; technical grade material was obtained from either Syngenta Crop Protection, Inc., Greensboro, NC, or Valent USA Corporation, Walnut Creek, CA. A set of working standards encompassing the linear range of concentrations was prepared from the primary standards, again in acetonitrile. To prepare a range of working standards in blank plant matrix, a 1000 µL aliquot of blank plant matrix was dry evaporated under nitrogen. The residue was then reconstituted with a 1000 µL aliquot of standard acetonitrile. The solution was sonicated to ensure a homogeneous product and passed through a 0.2 µm PTFE filter prior to injection, as were unknowns.

Insect Biological Assays

Lab Culture. The laboratory susceptible (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. The colony did not receive any exposure to insecticides following establishment in 2005 and routine quantitative real time (qPCR) testing as described in Pelz-Stelinski et al. (2010) was used to confirm the colony was *C*Las-free. Adult *D. citri* were aspirated from host rearing plants and used in laboratory bioassays during the same day to reduce unintended mortality.

Field Collection of *D. citri*. *Diaphorina citri* adults were collected prior to the first field application to establish baseline susceptibility of the field population to thiamethoxam in the lab. Adults were aspirated from citrus foliage in the field and transported to the lab within labeled plastic aspirator vials. Laboratory assays were conducted during the same day that *D. citri* were collected from the field to reduce unintended mortality.

Ingestion and Contact Bioassays. The ingestion and contact assays have been comprehensively described in Langdon and Rogers (2017). Briefly, for the ingestion assay, a

30% sucrose solution was used as the base artificial diet. Serial dilution was conducted to form eight doses of spiked diet using formulated Platinum 75SG (750 g thiamethoxam kg, Syngenta Crop Protection, Greensboro, NC). The caps of 5 mL snap-cap centrifuge tubes (Eppendorf Tubes®, Hamburg, Germany, Cat. No.: 0030119401) were filled with 0.7 mL of each prepared dose. Parafilm M® (Bemis®, Neenah, WI, Cat. No.: PM-992) was stretched over each diet-filled cap. Four to six adult *D. citri* were loaded into each centrifuge tube and the diet-filled cap was reinstalled. Tubes were held upright in a tube tray at 27°C, 70% relative humidity, with a 14:10 L:D photoperiod for 72h. One replicate was defined as a single tube and 10 replicates were used for each dose. Between 40 and 60 adults were tested for each dose. After 72h, 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.

A serial dilution using analytical-grade thiamethoxam (> 99.5% purity) (Chem Service, Inc, West Chester, PA) and acetone (Fisher Scientific, Fair Lawn, NJ, Cat. No.: A929-4) was used to prepare eight doses for the contact assay. A 1.5 mL aliquot of each dose, including the acetone control, was pipetted into individually labeled 16 mL glass vials (Wheaton, Millville, NJ, Cat. No.: 224746) and vials were rolled on an electric hot-dog roller until all acetone evaporated (approx. 1-2hr). Treated vials were stored at room temperature conditions in a closed cardboard container overnight. Eight to twelve adult *D. citri* were aspirated into each vial and a cap was installed. Tubes were held horizontally at 27°C, 70% relative humidity, with a photoperiod 14:10 L:D for 24h. Each replicate consisted of one vial and five replicates were used for each dose. Between 40 and 60 adults were tested for each dose. After 24h, 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.

Statistical Analyses

Chemical titer data were averaged over replicate and subjected to a general linear mixed model using SASv9.4 (Proc GLIMMIX, SAS Institute, 2013) to test for year by treatment interactions. Means were square root transformed prior to analysis to achieve homogeneity of variance meeting the assumptions of the model, as checked by visual examination of the residuals to ensure constant variance and normality. Additionally, chemical titer data were averaged over replicate and year and subjected to a general linear mixed model using SASv9.4 (Proc GLIMMIX, SAS Institute, 2013) to test for tree size by application rate interactions. Means were square root transformed prior to analysis to achieve homogeneity of variance and meet the assumptions of the model. For tests of differences between treatments, data were subjected to a non-parametric multiple comparisons test where mean separations indicate differences between treatments within the same sample week at $\alpha \leq 0.05$.

Insect count data were in monotonic distribution; therefore, data were subjected to Spearmans rank-order correlation using JMP (JMP Version 13, SAS Institute, 2007) to determine if concentration of thiamethoxam, or the metabolites clothianidin, TZNG (N-(2-chlorothiazol-5-ylmethyl)-N-nitroguanidine), or TZMU (N-(2-chlorothiazol- 5-ylmethyl)-N-methylurea) influenced *D. citri* incidence on leaves. Correlations were estimated using the Restricted Maximum Likelihood (REML) method. Additionally, nymph and adult incidence data were subjected to Probit analysis using SAS v9.4 (Proc Probit, SAS Institute, 2013) to determine the probability of encountering a flush terminal containing at least one nymph or one adult based on thiamethoxam titer.

Results

Chemical Titer in Leaf Tissue

No year by treatment interaction was observed for thiamethoxam ($F_{1, 5}$ = 0.9982; p-value = 0.4328), clothianidin ($F_{1, 5}$ = 1.4132; p-value = 0.2429), or TZMU ($F_{1, 5}$ = 2.1454; p-value = 0.0822); however, a year by treatment interaction was observed for TZNG ($F_{1, 5}$ = 3.5969; p-value = 0.0097). For TZNG, a larger magnitude of difference was observed in 2016 compared with 2017; however, the order of the treatment effects was the same across years. Therefore, data were combined across years for each of the four chemicals to evaluate the effect of treatment on chemical titer.

A tree size by application rate interaction was observed for thiamethoxam ($F_{1, 27}$ = 15.11; p-value = 0.0006), clothianidin ($F_{1, 27}$ = 11.66; p-value = 0.0020), and TZMU ($F_{1, 27}$ = 43.60; p-value < 0.0001); however, no tree size by application rate interaction was observed for TZNG ($F_{1, 27}$ = 0.8936; p-value = 0.3529). Main effects of tree size and application rate were therefore analyzed separately. Tree size influenced thiamethoxam titer ($F_{1, 27}$ = 180.5; p-value < 0.0001), where higher thiamethoxam titers were expressed in small trees compared to large trees. Tree size also influenced titer of clothianidin ($F_{1, 27}$ = 187.2; p-value < 0.0001), TZMU ($F_{1, 27}$ = 233.6; p-value < 0.0001), and TZNG ($F_{1, 27}$ = 263.3; p-value < 0.0001). Additionally, rate of application affected thiamethoxam titer ($F_{1, 27}$ = 44.24; p-value < 0.0001), where application of the high rate resulted in significantly more thiamethoxam in leaf tissue than at the low rate. Application rate also affected clothianidin ($F_{1, 27}$ = 39.35; p-value < 0.0001), TZMU ($F_{1, 27}$ = 58.02; p-value < 0.0001), and TZNG ($F_{1, 27}$ = 27.03; p-value < 0.0001) by increasing measured titer with increased application rate.

The high rate (0.74g Platinum 75SG per tree) applied to the small tree size resulted in the highest thiamethoxam titer observed during each week following application; the peak mean

concentration occurred two and three weeks following application at 33.39 and 33.29 ppm thiamethoxam, respectively (Table 3-1). Mean thiamethoxam titer peaked at 12.53 ppm by three weeks after application following the low rate application (0.37g) of Platinum 75SG per small tree; however, no significant difference in titer was observed between rates in small trees three weeks following application ($X^2 = 36.53$, $p < 0.0001$, Table 3-1). Peak thiamethoxam titer was observed at two (2.86 ppm) and four (2.47 ppm) weeks after the high rate (0.74g Platinum 75SG per tree) application to large trees, and at two weeks (0.69 ppm) following the low rate (0.37g Platinum 75SG per tree) application to large trees. At two and four weeks after application, significantly more thiamethoxam was found after the high rate rather than the low rate application to large trees ($X^2 = 40.27$, $p < 0.0001$ and $X^2 = 43.91$, $p < 0.0001$, respectively, Table 3-1).

The thiamethoxam metabolite, clothianidin, peaked at five weeks post application of both the high rate (15.44 ppm) and the low rate (6.29 ppm) of Platinum 75SG in trees of the small size (Table 3-2). Following application to the large size trees, clothianidin titer peaked at four weeks with the high rate (1.69 ppm) and the low rate (0.48 ppm). Very low levels (<2 ppm) of the clothianidin metabolites, TZMU (Table 3-3) and TZNG (Table 3-4) were detected in leaf tissues from trees following the application of Platinum 75SG to the soil.

Baseline Susceptibility of Field *D. citri* Population

The lethal concentration required to kill half of the field collected (Vero Beach) population (LC_{50}) by ingestion was 0.20 ppm of thiamethoxam, while the LC_{50} was 0.11 ppm for the lab susceptible population (Table 3-5). A comparison of these results indicates that the field population investigated during this experiment was fully susceptible to thiamethoxam, exhibiting a resistance ratio (RR_{50}) of 1.82. Furthermore, the lethal concentration required to kill 99 percent of the field-collected population by ingestion (LC_{99}) was 147.91 ppm thiamethoxam. In contrast,

the LC_{50} of the field population when exposed to thiamethoxam through contact was determined at 0.01 ppm, which was identical to that of the laboratory susceptible population. Moreover, the LC_{99} by contact was 0.33 ppm, approximately 450-fold less than by ingestion.

Relationship Between *D. citri* Incidence and Chemical Titer

Adult incidence was negatively correlated with increasing thiamethoxam (Spearmans ρ = -0.6440; Table 3-6), clothianidin (Spearmans ρ = -0.6320; Table 3-6), TZMU (Spearmans ρ = -0.5429; Table 3-6), and TZNG (Spearmans ρ = -0.6117; Table 3-6) titer. Likewise, nymph incidence was also negatively correlated with increasing thiamethoxam (Spearmans ρ = -0.7010; Table 3-6), clothianidin (Spearmans ρ = -0.6913; Table 3-6), TZMU (Spearmans ρ = -0.6051; Table 3-6), and TZNG (Spearmans ρ = -0.6655; Table 3-6) titer. An estimated 64.63 ppm (95% CL: 34.40 - 147.16) of thiamethoxam was required to achieve a one percent probability of encountering a flush terminal with one *D. citri* adult (Fig. 3-1A). Additionally, a thiamethoxam titer of 0.329 ppm (329 ppb) yielded a 50 percent probability of encountering a flush terminal with one *D. citri* adult (Table 3-7). In contrast, an estimated 19.05 ppm of thiamethoxam was required to achieve a one percent probability of encountering a flush terminal with one *D. citri* nymph (Fig. 3-1B); 0.715 ppm of thiamethoxam (715 ppb) yielded a 50 percent probability of encountering a flush terminal with one *D. citri* nymph (Table 3-8).

Approximately five weeks of nymph and adult control was observed in 2016 and 2017, respectively (Figs. 3-2A and 3-2B, respectively), following application of the low rate of Platinum 75SG to the soil beneath small trees, where ‘control’ is defined 100 percent compared to the untreated check. In contrast, ten and nine weeks of nymph and eight and seven weeks of adult control was observed in 2016 (Fig. 3-2C) and 2017 (Fig. 3-2D), respectively, following the high rate application to trees of the small size. The low rate applied to the large tree size did not offer complete control of nymphs or adults at any time during 2016 or 2017 (Figs. 3-3A and 3-

3B, respectively). During 2016, complete adult control was observed only during weeks one through three following the high rate application to trees of the large size (Fig. 3-3C), but the same level of adult control was not observed during 2017 (Fig. 3-3D); nymph control reached 100 percent only at two weeks after application in 2016 (Fig. 3-3C).

Discussion

The goals of this study were to quantify uptake and expression of thiamethoxam in young citrus trees of two sizes following the application of Platinum 75SG to the soil at two rates, as well as, to evaluate residual efficacy against *D. citri* nymphs and adults, and quantify the inherent susceptibility of the field population of *D. citri* to thiamethoxam to compare lethal concentration values with perceived control. This was the first formal investigation to use UHPLC-MS for quantification of the four analytes of Platinum 75SG (thiamethoxam, clothianidin, TZNG, and TZMU) in citrus leaf tissues following soil application in the field. Thiamethoxam is a neonicotinoid precursor to clothianidin (Nauen et al. 2003) and clothianidin is known to metabolize into TZNG and TZMU (Kim et al. 2012). While clothianidin is effective against *D. citri*, little is known about the influence of specific dosage on *D. citri* feeding behavior or mortality (Byrne et al. 2017). Langdon and Rogers (2017) found that the LC_{50} of thiamethoxam and clothianidin by ingestion was 0.11 and 0.09 ppm, respectively, while the LC_{90} was 4.94 and 9.35 ppm, respectively. Because a higher dose of clothianidin was required to achieve the same level of mortality at the 90 percent lethal concentration, it is unlikely that mortality observed in the current study is the result of exposure to the metabolite, clothianidin alone. Furthermore, the concentration of each of the three metabolites is directly dependent upon the concentration of thiamethoxam. Consequently, we cannot determine how any one metabolite, including clothianidin, affects *D. citri* mortality in this study. While understanding the role of each metabolite is beyond the scope of this investigation, it is possible that the combination of

multiple metabolites has an additive effect on *D. citri* mortality as suggested by Byrne et al. (2017). Byrne et al. (2017) demonstrated a strong correlation between thiamethoxam and the metabolite, clothianidin, but were unable to determine whether mortality was the result of either thiamethoxam or clothianidin, or a combination of the two. Nevertheless, earlier studies used ELISA to quantify expression of neonicotinoids, but did not quantify metabolites of thiamethoxam nor manipulate tree size or application rate to study effects on thiamethoxam titer (Castle et al. 2005, Garlapati 2009, Setamou et al. 2010).

The target concentration threshold of imidacloprid following application to the soil was 200 to 250 ppb based on the report by Setamou et al. (2010). Since a correlation between *D. citri* abundance and clothianidin or thiamethoxam titer did not exist, 200 to 250 ppb became the assumed efficacy threshold concentration for all neonicotinoids (K. W. Langdon, personal observation). In the current investigation, peak levels of nearly 3 ppm (3000 ppb) and 0.7 ppm (700 ppb) of thiamethoxam were measured when the respective high and low rates tested were applied to trees of the large size. When the high and low rates were applied to trees of the small size, peak concentrations were nearly 18 and 11-fold, respectively, higher than when applied to the large tree size. Each titer was well above the 250 ppb upper threshold set for imidacloprid, suggesting that efficacy should be expected in both tree sizes at the rates tested. However, despite the high titers observed in our study, we failed to reach a mean dose high enough to provide a 95 percent confidence (34.40 ppm) of only a one percent probability of encountering a flush shoot with at least one adult *D. citri*. We did, however, observe a mean concentration high enough in only the small tree size at both rates, to achieve a 95 percent confidence (12.17 ppm) of only a one percent probability of encountering a flush shoot with at least one nymph, albeit

that titer was reached only during the third week of the investigation in the low rate treatment; the high rate treatment exceeded 12.17 ppm between weeks one and six.

The LC_{99} for the field population was 147.91 ppm. This was 4.5-fold higher than the highest mean dose observed when the high rate was applied to trees of the small size. Given that nearly 100 percent control of adults was observed for up to eight weeks following application and that the highest observed concentration was 4.5 times lower than the lowest lethal dose (LC_{99}), a dose of less than 34.40 ppm remains likely to deter *D. citri* from feeding and therefore, may offer non-lethal control of *D. citri* in young citrus trees. Langdon and Rogers (2017) defined insecticide-mediated feeding deterrence of *D. citri* as "gustatory avoidance of less or non-suitable feeding sources." As adults search for a suitable host plant and are exposed to citrus tissue containing less than 34.40 ppm of thiamethoxam, adults may move to find a leaf surface containing a lower concentration within the same plant or move to a new host plant that contains a concentration sufficiently low to be suitable for feeding. This movement away from the treated foliage or tree may result in a perception of control upon visual assessment, but should not be confused with mortality. While control may be perceived at titers less than 34.40 ppm, any concentration below 147.91 ppm should be assumed as sublethal and therefore exposure to a dose below 147.91 ppm is likely to result in exposed survivors, which may promote the development of resistance in populations of *D. citri* to thiamethoxam and likely other chemistries within the same mode of action. Thiamethoxam is known to metabolize into clothianidin, which acts on the same receptor site as imidacloprid (Nauen et al. 2003), supporting a high likelihood of cross resistance. Imidacloprid resistant *D. citri* express higher levels of detoxifying enzymes, including general esterase, glutathione *S*-transferase, and cytochrome P_{450} monooxygenases (Tiwari et al. 2011a). Reduced penetration, target-site insensitivity, and mutations in detoxifying

enzymes may also play a role in resistance (Tiwari et al. 2011a). Tiwari et al. (2011b) found five family 4 cytochrome P_{450} genes induced by imidacloprid exposure. Imidacloprid and thiamethoxam are within the same chemical sub-group (4A); therefore, cross resistance between the two chemistries is of concern. Additionally, thiamethoxam persisted at very low levels (ca. 0.05 – 0.80 ppm) during the final evaluation of this investigation (12 weeks following application). The duration at which sublethal doses in this range will persist is unknown; if doses in this range do not inhibit feeding activity it may further increase the likelihood and rate of resistance development.

Diaphorina citri resistance to neonicotinoids has been recently documented in Florida (Tiwari et al. 2011a, Kanga et al. 2016); therefore, a deeper understanding of soil-applied neonicotinoids was warranted for development of future resistance management strategies. We observed a number of effects that are of significant concern regarding use of neonicotinoids by soil application in Florida citrus: 1) failure to achieve lethal concentrations (those that exceed LC_{99}) in leaf tissue following application to the soil; 2) persistence of thiamethoxam concentrations at low levels (less than 1 ppm) through 12 weeks following application; 3) failure of the highest allowable annual rate to achieve acceptable *D. citri* control following application to trees 18 months of age (MCV = $1.34m^3$); 4) expression level relative to dose applied (e.g. high rate of 0.74g Platinum 75SG in 237 mL water per tree is equivalent to 2370 ppm thiamethoxam applied to the soil, and low rate of 0.37g Platinum 75SG in 237 mL water per tree is equivalent to 1185 ppm thiamethoxam applied to the soil); and 5) higher sensitivity of *D. citri* to thiamethoxam through contact exposure compared to ingestion.

The foremost strategy for stewardship and future implementation of neonicotinoids in citrus must be resistance management. Therefore, the current results suggest that foliar use of

neonicotinoids may be more appropriate than their applications to the soil, particularly in trees with a canopy larger than 0.08m^3 to mitigate resistance development and preserve efficacy of this mode of action. Because contact sensitivity in our field population was approximately 450-fold greater than by ingestion at the LC_{99} level, labeled rates of thiamethoxam applied to the foliage would be sufficient (foliar applied dose of 68.65 ppm at 1400 L/ha; Contact LC_{99} = 0.33 ppm; Table 3-5) to effectively kill *D. citri*. Subsequent investigations should evaluate factors including coverage uniformity, peak residue levels, persistence / degredation, and resulting efficacy following foliar applications of neonicotinoid insecticides. The significant reduction in titer between small trees and large trees after soil application may simply be a result of dilution of chemical due to an increase in canopy size, application method in relation to root distribution under the canopy, or perhaps due to compromise of the root system caused by *C*Las infection resulting in reduced uptake of available compound. If the latter is true, soil-applied neonicotinoids may work better when applied to trees not compromised by HLB. Because the trees used in this study were insecticide-free prior to each application, it is likely that all trees, particularly of the large size, had some level of *C*Las infection, which may have negatively influenced uptake efficiency.

Follow up comparative investigations quantifying the concentration of thiamethoxam, imidacloprid, and clothianidin required to interrupt and manipulate feeding behavior of *D. citri* that utilize electropenetrography are warranted to further improve use of these management tools for *D. citri* and HLB in citrus. Moreover, alternative soil application methods should be investigated that attempt to increase uptake efficiency, particularly in trees 18 months and older. Nonetheless, future work on foliar application of neonicotinoids should investigate temporal residue and breakdown/metabolism as related to the probability of sublethal exposure of *D. citri*.

Given the dynamic nature of susceptibility of *D. citri* to insecticides, we must remain diligent in research efforts with a keen focus on resistance management and be willing to adjust insecticide use patterns to ensure the longevity of each available chemical class.

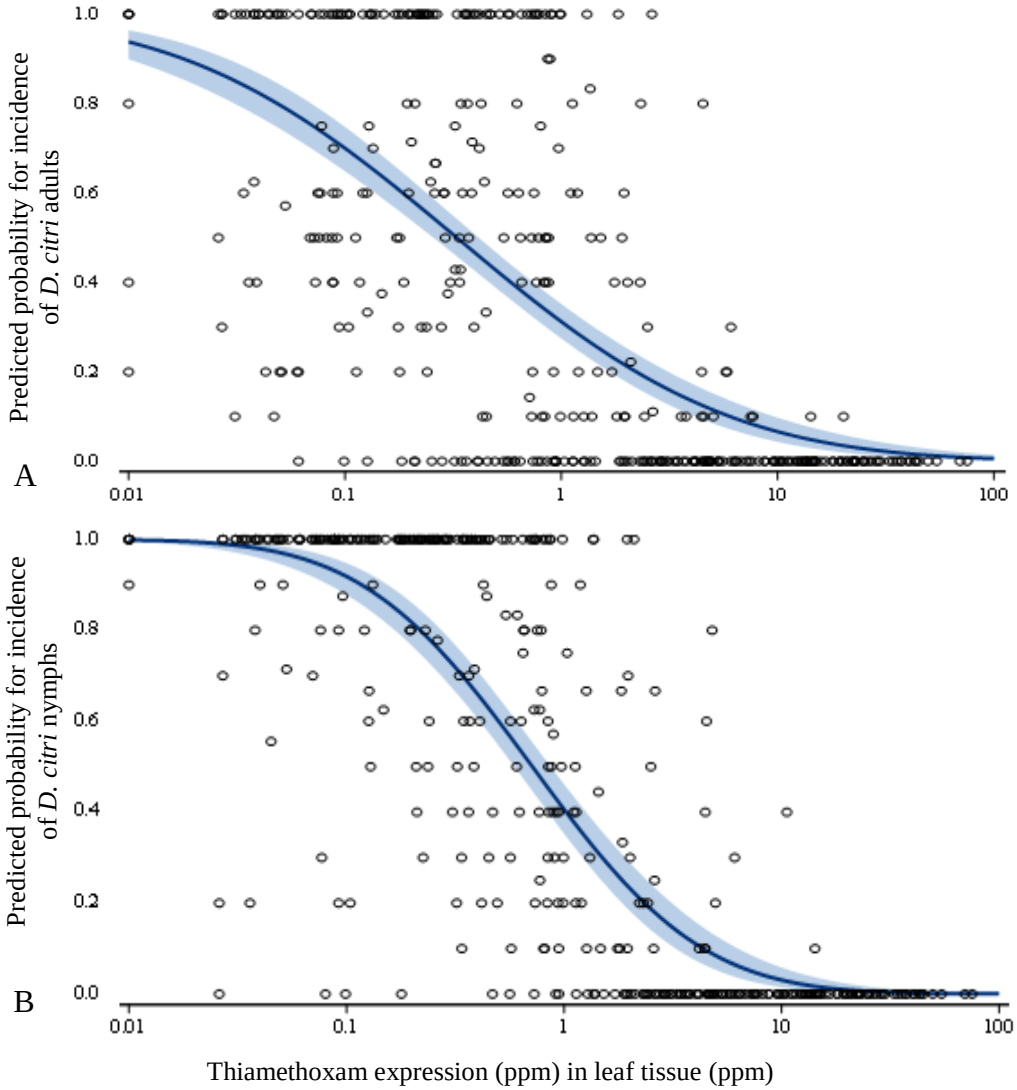


Figure 3-1. Predicted probability for incidence of insects based on thiamethoxam titer (ppm) in citrus leaf tissue. A. Incidence of *Diaphorina citri* adults B. Incidence of *Diaphorina citri* nymphs

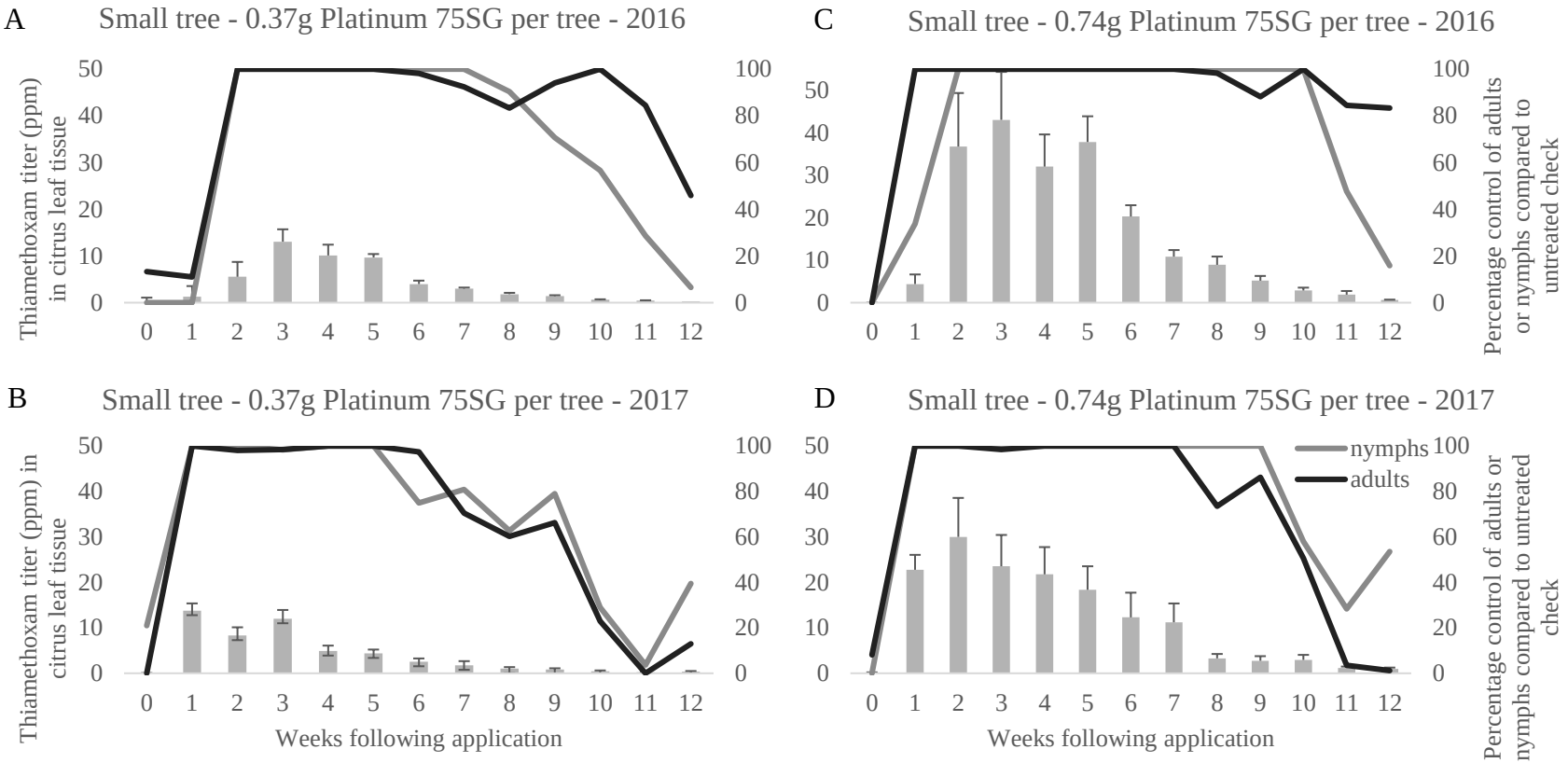


Figure 3-2. Comparison of thiamethoxam titer (ppm) and percentage insect control in trees of the large size during 2016 and 2017 field seasons. A. Low rate applied to small (0.08m^3) trees in 2016. B. Low rate applied to small (0.08m^3) trees in 2017. C. High rate applied to small (0.08m^3) trees in 2016. D. High rate applied to small (0.08m^3) trees in 2017.

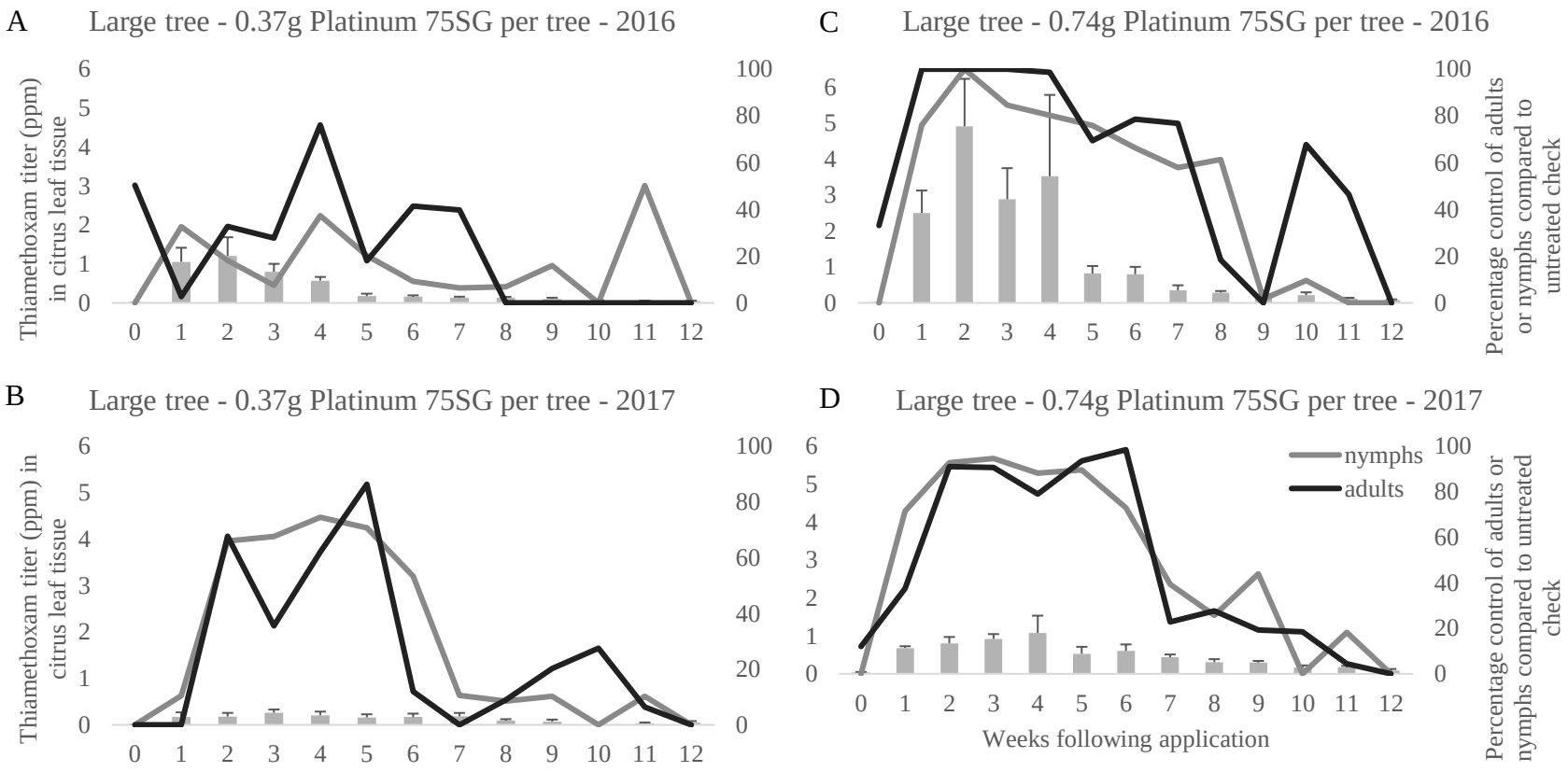


Figure 3-3. Comparison of thiamethoxam titer (ppm) and percentage insect control in trees of the large size during 2016 and 2017 field seasons. A. Low rate applied to large (1.34m^3) trees in 2016. B. Low rate applied to large (1.34m^3) trees in 2017. C. High rate applied to large (1.34m^3) trees in 2016. D. High rate applied to large (1.34m^3) trees in 2017.

Table 3-1. Mean parts per million (ppm) of thiamethoxam (95% CI) found in citrus leaf tissue during 2016 and 2017 field experiments.

Tree Size	Application rate per tree	Weeks after application												
		0	1	2	3	4	5	6	7	8	9	10	11	12
	Untreated	0	0	0	0	0	0	0	0	0	0	0	0	0
Small 0.08m^3 MCV[a]	0.37g Platinum 75SG	0	7.53bc (3.46-11.61)	6.92b (3.20-10.63)	12.53b (8.27-16.79)	7.51c (3.56-11.45)	7.03b (4.29-9.77)	3.27b (2.14-4.39)	2.39c (1.03-3.76)	1.42b (0.95-1.88)	1.14b (0.72-1.56)	0.56c (0.30-0.82)	0.37b (0.24-0.49)	0.30bc (0.13-0.46)
	0.74g Platinum 75SG	0	13.58c (6.07-21.09)	33.39c (16.54-50.23)	33.29b (17.20-49.39)	26.90d (15.37-38.44)	28.10c (17.01-39.19)	16.29c (9.08-23.51)	11.03d (6.18-15.87)	6.09c (2.96-9.23)	3.96c (2.09-5.83)	2.90d (1.48-4.33)	1.52c (0.48-2.57)	0.80c (0.43-1.15)
	Untreated	0	0	0	0	0	0	0	0	0	0	0	0	0
Large 1.34m^3 MCV[a]	0.37g Platinum 75SG	0	0.61ab (0.00-3.14)	0.69a (0.12-1.27)	0.53a (0.00-2.03)	0.39a (0.18-0.60)	0.17a (0.00-2.03)	0.17a (0.00-0.89)	0.16ab (0.04-0.27)	0.11a (0.00-0.58)	0.09a (0.00-0.31)	0.03ab (0.00-0.06)	0.03a (0.00-0.11)	0.05ab (0.00-0.10)
	0.74g Platinum 75SG	0	1.59abc (0.00-4.58)	2.86b (1.00-4.72)	1.90a (0.63-3.17)	2.47bc (0.00-5.70)	0.67a (0.00-2.44)	0.70a (0.01-1.39)	0.39b (0.20-0.59)	0.30a (0.00-0.80)	0.28ab (0.03-0.53)	0.19bc (0.08-0.30)	0.14ab (0.04-0.25)	0.08ab (0.03-0.14)
	p-value	-	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
	X^2	-	28.95	40.27	36.53	43.91	35.18	35.65	40.89	35.23	34.89	41.27	35.08	32.74

a. Mean volume of citrus tree canopy.

Mean separations within columns indicate differences between treatments within weekly samples.
Values sharing the same letter do not differ significantly at $\alpha \leq 0.05$.

Table 3-2. Mean parts per million (ppm) of clothianidin (95% CI) found in citrus leaf tissue during 2016 and 2017 field experiments.

Tree Size	Application rate per tree	Weeks after application												
		0	1	2	3	4	5	6	7	8	9	10	11	12
Small 0.08m^3 MCV[a]	Untreated	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.37g Platinum 75SG	0	1.99b (0.77-3.21)	2.45cd (0.81-4.09)	5.82c (4.07-7.56)	4.50cd (2.85-6.15)	6.29b (5.07-7.52)	4.33b (2.45-6.21)	2.65b (1.57-3.73)	1.84b (1.39-2.29)	1.46b (0.94-1.98)	0.85b (0.52-1.17)	0.55b (0.43-0.66)	0.40b (0.22-0.58)
	0.74g Platinum 75SG	0	3.27b (1.46-5.08)	8.62d (4.27-12.96)	12.12c (6.77-17.47)	13.09d (8.34-17.85)	15.44b (7.32-23.57)	14.02c (7.95-20.09)	11.53c (7.67-15.39)	7.25c (3.63-10.88)	5.01c (3.00-7.02)	5.02c (3.04-6.99)	2.21c (1.05-3.37)	1.45c (0.84-2.05)
Large 1.34m^3 MCV[a]	Untreated	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.37g Platinum 75SG	0	0.26ab (0.00-0.97)	0.39b (0.00-0.82)	0.38b (0.17-0.58)	0.48b (0.10-0.87)	0.21a (0.00-0.78)	0.19a (0.00-0.81)	0.18a (0.00-0.66)	0.14a (0.00-0.81)	0.09a (0.00-0.56)	0.04a (0.00-0.38)	0.03a (0.00-0.22)	0.05a (0.00-0.14)
	0.74g Platinum 75SG	0	0.68ab (0.00-1.42)	1.15bc (0.45-1.85)	1.06b (0.38-1.73)	1.69bc (0.00-3.75)	0.86a (0.37-1.36)	0.74a (0.26-1.23)	0.40a (0.00-0.90)	0.33a (0.00-0.98)	0.30ab (0.00-0.77)	0.29ab (0.00-0.60)	0.17a (0.00-0.36)	0.09a (0.00-0.18)
	p-value	-	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
	X^2	-	29.17	40.86	42.45	43.16	32.66	36.60	35.43	35.16	32.25	32.20	34.93	35.47

a. Mean volume of citrus tree canopy.

Mean separations within columns indicate differences between treatments within weekly samples.

Values sharing the same letter do not differ significantly at $\alpha \leq 0.05$.

Table 3-3. Mean parts per million (ppm) of TZMU (95% CI) found in citrus leaf tissue during 2016 and 2017 field experiments.

Tree Size	Application rate per tree	Weeks after application												
		0	1	2	3	4	5	6	7	8	9	10	11	12
	Untreated	0	0	0	0	0	0	0	0	0	0	0	0	0
Small 0.08m^3 MCV[a]	0.37g Platinum 75SG	0	0.04a (0.02-0.06)	0.05a (0.01-0.08)	0.11b (0.04-0.17)	0.09b (0.05-0.13)	0.05a (0.00-0.09)	0.07ab (0.02-0.12)	0.01a (0.00-0.03)	0.01a (0.00-0.03)	0.01ab (0.00-0.02)	0a	0a	0a
	0.74g Platinum 75SG	0	0.08a (0.02-0.15)	0.44b (0.22-0.65)	0.38c (0.20-0.57)	0.52c (0.32-0.72)	0.40b (0.25-0.54)	0.38b (0.19-0.57)	0.23b (0.10-0.36)	0.15b (0.09-0.22)	0.10b (0.05-0.15)	0.06a (0.00-0.12)	0.02a (0.00-0.04)	0.01a (0.00-0.02)
	Untreated	0	0	0	0	0	0	0	0	0	0	0	0	0
Large 1.34m^3 MCV[a]	0.37g Platinum 75SG	0	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a
	0.74g Platinum 75SG	0	0a	0a	0a	0.01ab (0.00-0.04)	0.01a (0.00-0.01)	0a	0a	0a	0a	0a	0a	0a
	p-value	-	0.0008	< 0.0001	< 0.0001	< 0.0001	0.0005	< 0.0001	0.0014	< 0.0001	0.0030	0.0911	0.0174	0.6178
	X^2	-	21.13	27.40	33.82	39.89	22.11	26.59	19.80	25.37	17.93	9.49	13.73	3.54

a. Mean volume of citrus tree canopy.

Mean separations within columns indicate differences between treatments within weekly samples.

Values sharing the same letter do not differ significantly at $\alpha \leq 0.05$.

Table 3-4. Mean parts per million (ppm) of TZNG (95% CI) found in citrus leaf tissue during 2016 and 2017 field experiments.

Tree Size	Application rate per tree	Weeks after application												
		0	1	2	3	4	5	6	7	8	9	10	11	12
Small 0.08m^3 MCV[a]	Untreated	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.37g Platinum 75SG	0	0.17ab (0.07-0.27)	0.25bc (0.13-0.37)	0.68c (0.50-0.87)	0.66c (0.51-0.81)	0.65b (0.46-0.83)	0.87c (0.53-1.21)	0.69c (0.57-0.80)	0.59b (0.45-0.74)	0.57b (0.42-0.72)	0.39b (0.32-0.47)	0.33b (0.24-0.41)	0.22b (0.15-0.29)
	0.74g Platinum 75SG	0	0.24b (0.12-0.36)	0.41c (0.28-0.55)	0.77c (0.60-0.95)	0.70c (0.51-0.89)	1.39c (0.92-1.86)	1.36c (0.87-1.86)	1.27c (0.78-1.75)	1.19b (0.66-1.73)	0.81b (0.40-1.23)	0.89c (0.68-1.10)	0.66b (0.41-0.90)	0.52c (0.42-0.63)
Large 1.34m^3 MCV[a]	Untreated	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.37g Platinum 75SG	0	0.02ab (0.00-0.08)	0.08ab (0.00-0.18)	0.12b (0.02-0.23)	0.15ab (0.00-0.30)	0.04a (0.00-0.13)	0.09b (0.05-0.13)	0.06ab (0.01-0.11)	0.04a (0.00-0.12)	0.02a (0.00-0.10)	0.01a (0.00-0.05)	0.01a (0.00-0.04)	0.01a (0.00-0.03)
	0.74g Platinum 75SG	0	0.06ab (0.00-0.13)	0.12ab (0.01-0.22)	0.20b (0.06-0.35)	0.33bc (0.10-0.56)	0.16a (0.05-0.28)	0.19b (0.08-0.30)	0.13b (0.07-0.19)	0.12a (0.04-0.20)	0.11a (0.03-0.19)	0.09a (0.04-0.14)	0.04a (0.00-0.10)	0.03a (0.00-0.06)
	p-value	-	0.0004	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
	X^2	-	22.64	30.73	41.08	37.89	38.01	40.59	40.03	34.70	35.01	37.49	34.14	34.95

a. Mean volume of citrus tree canopy.

Mean separations within columns indicate differences between treatments within weekly samples.

Values sharing the same letter do not differ significantly at $\alpha \leq 0.05$.

Table 3-5. Response of laboratory susceptible and field collected *Diaphorina citri* to thiamethoxam (ppm) administered by ingestion and contact.

Strain	Assay Method	N[a]	Slope + SE	X^2	LC_{50}[b]	95% CL	LC_{90}[b]	95% CL	LC_{99}[b]	95% CL	RR_{50}[c]
Vero Beach	Ingestion	356	0.35 + 0.04	84.53	0.20	(0.10 – 0.34)	7.62	(4.10 – 17.73)	147.91	(52.34 – 693.09)	1.82
	Contact	351	0.69 + 0.11	41.78	0.01	(0.01 - 0.02)	0.07	(0.05 – 0.14)	0.33	(0.16 – 1.13)	1.00
Lab Susceptible	Ingestion	404	0.34 + 0.04	73.58	0.11	(0.05 – 0.21)	4.94	(2.63 – 11.75)	106.45	(36.17 – 555.23)	-
	Contact	405	0.75 + 0.12	38.69	0.01	(0.01 – 0.02)	0.05	(0.04 – 0.11)	0.23	(0.12 – 0.78)	-

a. Number of adult *Diaphorina citri* tested.
b. Parts per million (ppm) of active ingredient.
c. Ratio of Vero Beach LC_{50} divided by Lab Susceptible LC_{50}.

Table 3-6. Nonparametric Spearman correlation between mean number of adult or nymph *Diaphorina citri* per terminal and chemical titer (ppm) during 2016 and 2017 field seasons.

Variable	by Analyte	Spearman ρ	p-value
Mean number of *D. citri* adults per terminal (n = 10 terminals)	thiamethoxam	-0.6440	<0.0001
	clothianidin	-0.6320	<0.0001
	TZMU	-0.5429	<0.0001
	TZNG	-0.6117	<0.0001
Mean number of *D. citri* nymphs per terminal (n = 10 terminals)	thiamethoxam	-0.7010	<0.0001
	clothianidin	-0.6913	<0.0001
	TZMU	-0.6051	<0.0001
	TZNG	-0.6655	<0.0001

Table 3-7. Probability of encountering a *Diaphorina citri* adult on young citrus trees based on thiamethoxam titer (ppm) in leaf tissue.

Probability	Concentration (ppm)	95% Fiducial Limits		Probability	Concentration (ppm)	95% Fiducial Limits	
0.01	64.62813	34.40416	147.16423	0.55	0.24763	0.18915	0.31486
0.02	34.81426	19.88874	72.07353	0.60	0.18534	0.13803	0.23892
0.03	23.51246	14.03654	45.85806	0.65	0.13737	0.09920	0.18047
0.04	17.50123	10.79389	32.65360	0.70	0.10019	0.06976	0.13481
0.05	13.76460	8.71368	24.78236	0.75	0.07127	0.04754	0.09874
0.06	11.21976	7.25962	19.60355	0.80	0.04877	0.03093	0.07002
0.07	9.37867	6.18388	15.96622	0.85	0.03135	0.01868	0.04705
0.08	7.98808	5.35517	13.28972	0.90	0.01797	0.00988	0.02861
0.09	6.90326	4.69711	11.25013	0.91	0.01571	0.00846	0.02538
0.10	6.03539	4.16204	9.65293	0.92	0.01358	0.00716	0.02229
0.15	3.46039	2.51446	5.13735	0.93	0.01157	0.00595	0.01932
0.20	2.22395	1.67583	3.12827	0.94	0.00967	0.00484	0.01648
0.25	1.52197	1.17661	2.05542	0.95	0.00788	0.00382	0.01374
0.30	1.08264	0.85119	1.41831	0.96	0.00620	0.00290	0.01111
0.35	0.78960	0.62631	1.01252	0.97	0.00461	0.00206	0.00855
0.40	0.58525	0.46475	0.74073	0.98	0.00312	0.00131	0.00604
0.45	0.43802	0.34567	0.55145	0.99	0.00168	0.0006408	0.00350
0.50	0.32934	0.25652	0.41537				

Table 3-8. Probability of encountering a *Diaphorina citri* nymph on young citrus trees based on thiamethoxam titer (ppm) in leaf tissue.

Probability	Concentration (ppm)	95% Fiducial Limits		Probability	Concentration (ppm)	95% Fiducial Limits	
0.01	19.05254	12.16790	34.07621	0.55	0.59899	0.49336	0.72459
0.02	12.96913	8.64334	21.89294	0.60	0.50024	0.40916	0.60505
0.03	10.16081	6.95350	16.54363	0.65	0.41525	0.33608	0.50386
0.04	8.45671	5.90171	13.40459	0.70	0.34126	0.27223	0.41687
0.05	7.28366	5.16330	11.29901	0.75	0.27613	0.21615	0.34088
0.06	6.41433	4.60705	9.77162	0.80	0.21812	0.16664	0.27335
0.07	5.73789	4.16809	8.60486	0.85	0.16570	0.12263	0.21204
0.08	5.19299	3.81004	7.68013	0.90	0.11725	0.08306	0.15463
0.09	4.74248	3.51067	6.92674	0.91	0.10786	0.07556	0.14335
0.10	4.36242	3.25549	6.29960	0.92	0.09850	0.06817	0.13204
0.15	3.08692	2.37788	4.25992	0.93	0.08914	0.06086	0.12066
0.20	2.34503	1.84788	3.12933	0.94	0.07974	0.05361	0.10913
0.25	1.85240	1.48480	2.40760	0.95	0.07023	0.04638	0.09735
0.30	1.49887	1.21695	1.90723	0.96	0.06048	0.03911	0.08514
0.35	1.23180	1.00945	1.54090	0.97	0.05034	0.03170	0.07224
0.40	1.02251	0.84303	1.26208	0.98	0.03944	0.02396	0.05809
0.45	0.85394	0.70607	1.04352	0.99	0.02685	0.01540	0.04125
0.50	0.71519	0.59118	0.86814				

CHAPTER 4
NEONICOTINOID-INDUCED MORTALITY OF *DIAPHORINA CITRI* (HEMIPTERA: LIVIIDAE) IS AFFECTED BY ROUTE OF EXPOSURE

The use of neonicotinoids in citrus has increased substantially to help manage the Asian citrus psyllid, *Diaphorina citri* Kuwayama, 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 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.

Used with permission from: Langdon, K. W., M. E. Rogers. 2017. Neonicotinoid-induced mortality of *Diaphorina citri* (Hemiptera: Liviidae) is affected by route of exposure. J. Econ. Entomol. 110: 2229-2234.

Justification

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, 2010b; Grafton-Cardwell et al. 2013). *Diaphorina citri* serves as the vector of the bacterium, *Candidatus* Liberibacter asiaticus (*C*Las), the presumed causal agent of huanglongbing (HLB), or citrus greening disease. *Candidatus* Liberibacter asiaticus 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). *Diaphorina 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 *C*Las 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 three to five years 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 six-week 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 weeks 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 one to three percent 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 *C*Las-infected citrus tissue with sublethal imidacloprid concentrations which do not inhibit feeding, acquisition and/or inoculation of *C*Las is possible. In Florida, roughly 80-100% of all *D. citri* individuals are *C*Las 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 cultures (Tiwari et al. 2011a, 2013, IRAC 2009, 2011, 2014, Kanga et al. 2016, Coy 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 (Coy et al. 2016, IRAC 2011, Tiwari 2011a, 2013). 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 *C*Las and citrus treated with soil-applied systemic neonicotinoid insecticides.

Materials and Methods

Lab Culture

The laboratory susceptible (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. The LS strain was maintained *C*Las-free, confirmed by routine quantitative real time (qPCR) testing as described in Pelz-Stelinski et al. (2010), 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. *Diaphorina 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. *Diaphorina 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 nine days, 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. Hunt Valley, MD). 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, Walnut Creek, CA). 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 two 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 72h. 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 hours 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 hours 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 24h 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 24h. 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 hours 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 4-1). 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 4-1. 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 9-fold 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 4-2). 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 percent 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).

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, Kanga et al. 2016, Coy 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 9 to 11-fold higher, depending on active ingredient, was required to kill 50 percent 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 parts per billion (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 laboratory susceptible 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 vs 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 one hour on citrus tissue assumed to contain lethal levels of imidacloprid. While one hour of ingestion (E2) is sufficient for *C*Las acquisition to occur (Bonani et al. 2010), Serikawa et al. (2012) explained that subsequent inoculation of nearby uninfected citrus plants following *C*Las acquisition was not likely due to lethal effects of imidacloprid. While lethal levels of imidacloprid may prevent successful *C*Las transmission, sublethal levels that do not deter feeding may allow successful *C*Las 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; Kanga et al. 2016; Coy 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.

Table 4-1. Response of laboratory susceptible *Diaphorina citri* strain to three neonicotinoid insecticides by ingestion and contact routes of exposure.

Insecticide	Assay Method	Strain	N[a]	Slope + SE	LC_{50}[b]	95% CL	LC_{90}	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 72h.

Table 4-2. Response of laboratory and field collected *Diaphorina citri* to imidacloprid by ingestion and contact routes of exposure in 2016.

Method	Population	N[a]	Slope + SE	LC_{50}[bc]	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 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 72h.

CHAPTER 5
EVALUATING THE EFFECT OF IMIDACLOPRID ADMINISTERED IN ARTIFICIAL DIET ON FEEDING BEHAVIOR OF *DIAPHORINA CITRI* (HEMIPTERA: LIVIIDAE) USING ELECTROPENETROGRAPHY

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) is the vector of Huanglongbing (HLB), a global disease of citrus. Following the discovery of HLB in Florida citrus, management strategies were developed using soil-applied neonicotinoids to protect young trees. Despite the implementation of intense management programs, infection continued to spread among even the most intensively managed groves. In the present study, we used electopenetrography (EPG) to test three sublethal imidacloprid doses administered in artificial diet to approximate the dosage required to reduce feeding activity and prevent salivation / ingestion activity altogether. We failed to detect a significant effect of 0.55 ppm imidacloprid on probing behavior, pathway (C), or salivation/ingestion (E1E2) activity when compared to the untreated control. Conversely, we observed a significant reduction in the number of probes and the number of C with both 5.5 and 55 ppm imidacloprid. Furthermore, we detected a significant reduction in the number of E1E2 events at both 5.5 ppm and 55 ppm imidacloprid (57 and 54 percent, respectively) compared to the untreated control, and a reduction in number of sustained (>600 sec) E1E2 (NumLngE1E2) at 55 ppm. Reductions in feeding activity were apparent at dosages of at least 5.5 ppm, which likely helps reduce HLB spread. However, we were unable to prevent E1E2 with dosages of up to 55 ppm, indicating that titers of 55 ppm imidacloprid following application to the soil may not be completely effective in preventing *C*Las inoculaton, and thus spread of HLB in Florida citrus.

Justification

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), was first detected in Florida in 1998 (Halbert and Manjunath 2004) and is known to transmit the phloem-limited proteobacterium, *Candidatus* Liberibacter asiaticus (*C*Las), the presumed cause of citrus greening disease, or Huanglongbing (HLB) (Halbert and Manjunath 2004, Bové 2006, Grafton-Cardwell et al. 2013). Huanglongbing was discovered in Florida in 2005 (Halbert 2005) and has since caused a significant decline in the state's citrus production (Hodges and Spreen 2015). Upon inoculation of *C*Las into plant phloem, the bacteria moves downward into the roots where the root system is severely compromised. In turn, the canopy is starved of vital nutrients resulting in dead limbs and leaf drop, followed by a reduction in fruit quality and yield, with eventual tree death (Halbert and Manjunath 2004, Bové 2006, Grafton-Cardwell et al. 2013). Following the discovery of HLB in Florida citrus, management strategies were quickly developed and focused on tree health and vector management to aid in reducing the spread of the serious disease (Rogers 2008). Despite the implementation of intense management programs, virtually all *D. citri* are currently infected with *C*Las, and tree infection continues to spread among even the most intensively managed groves (Rogers 2013, Coy and Stelinski 2015). As a result, we must evaluate current vector management practices to elucidate why spread of the pathogen continues in order to develop and deliver improved management tactics to growers.

Diaphorina citri are characterized as insects with high fecundity and rapid development, undergoing completion of the egg to adult life cycle in as little as 15 days during periods of optimal environmental conditions (Liu and Tsai 2000, Grafton-Cardwell et al. 2013). Adult *D. citri* are attracted to volatiles emitted by newly formed flush shoots where they lay up to 800 eggs per female (Patt and Setamou 2010). If egg lay occurs on HLB-infected host tissue, newly hatched nymphs feed on phloem sap and acquire *C*Las (Pelz-Stelinski et al. 2010). Acquisition

efficiency is increased for nymphs developing on infected host tissue compared with *D. citri* acquiring the pathogen in the adult stage (Pelz-Stelinski et al. 2010). Nonetheless, dispersal of infected adults from a point source of inoculum results in the spread of the pathogen within and among groves.

Much of HLB vector management has maintained focus on young tree programs (Rogers 2008, Rogers 2013). The key objective of the young tree management program is to maintain HLB-free trees until trees reach fruit-bearing age. Young trees flush asynchronously and frequently relative to mature trees in Florida (Hall and Albrigo 2007, Rogers 2012). Because adult *D. citri* seek young flush for egg lay or feeding, young trees are presumably at greatest risk of acquiring *C*Las (Stansly and Rogers 2006). Vector management programs in young trees advise an approximate three week alternation between soil-applied neonicotinoids and non-neonicotinoid foliar sprays aimed to maintain *D. citri* populations at low levels in young tree groves (Rogers 2012, Rogers et al. 2015). Neonicotinoids are a unique group of systemic insecticides that when applied to the soil, are absorbed by the roots, and transported through xylem vascular bundles to the foliage (Elbert et al. 2008). According to the Insecticide Resistance Action Committee (IRAC) neonicotinoids are within the insecticide sub-group 4A, and bind to the insect nicotinic acetylcholine receptor (nAChR) resulting in hyper-excitation, paralysis, and eventual death (IRAC 2017). Three neonicotinoid insecticides are currently labeled for use in non-bearing citrus in Florida: 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). A number of studies have investigated the residual activity of neonicotinoids applied to the soil and reported between six and eleven weeks control (Qureshi

and Stansly 2007, Qureshi and Stansly 2009, Ichinose et al. 2010, Setamou et al. 2010, Byrne et al. 2012, Rogers 2012). While influential factors that affect neonicotinoid expression levels in leaf tissue are likely related to the environment (e.g. soil type, application volume, irrigation / rainfall, tree age and size, and climatic and / or weather conditions), uneven insecticide distribution within a plant and / or over time is likely to result in areas of sublethal concentrations within leaf tissue at any time following application to the soil (Boina et al. 2009, Rogers 2012).

Electropenetrography (EPG) is a highly effective method used to study and quantify specific feeding behaviors of piercing-sucking hemipterans (Prado and Tjallingii 1994, Joost et al. 2006, Bonani et al. 2010, Butler et al. 2012, Jacobson and Kennedy 2014) and rasping-sucking Thysanoptera (Harrewijn et al. 1996, Groves et al. 2001, Kindt et al. 2003, Joost and Riley 2005). Each feeding behavior is identified through a behavior-specific waveform captured by an EPG monitor. Bonani et al. (2010) correlated repetitive waveforms for *D. citri* with six specific feeding behaviors including non-probing (NP), pathway (C), xylem ingestion (G), phloem contact (D), phloem salivation (E1), and phloem ingestion (E2). Occurrence, frequency, and duration of specific waveforms can be used to study insect feeding behavior in response to various stimuli. For example, *Diaphorina citri* phloem feeding activities E1 and E2 have been significantly reduced through the use of soil-applied imidacloprid in citrus, however, neither salivaton nor ingestion has been prevented to date (Serikawa et al. 2012, Miranda et al. 2016). Understanding the response of particular feeding behaviors, such as salivation or ingestion, can have major implications in pathogen transmission. Coy and Stelinski (2015) speculated that between 80 and 100 percent of *D. citri* in Florida are infected with *C*Las. Because not all groves are adequately managed for the vector, particularly mature groves and abandoned groves,

preventing the inoculation component of the transmission cycle is key to averting the spread of the deadly disease.

Pathogen transmission is largely a two component phenomenon, 1. Acquisition, and 2. Inoculation. A number of EPG studies reported a focus of feeding behaviors associated with phloem ingestion (E2) activity as related to *C*Las acquisition (Bonani et al. 2010, Miranda et al. 2016, Serikawa et al. 2012, Luo et al. 2015). Bonani et al. (2010) determined that *D. citri* were able to acquire *C*Las when ingestion behavior (E2) was sustained for one hour, albeit acquisition success was low (ca. 6 percent). In contrast, Luo et al. (2015) demonstrated nearly 96 percent successful *C*Las acquisition by adult *D. citri* with a phloem ingestion (E2) period of as little as two minutes. Moreover, Serikawa et al. (2012) found that *D. citri* were able to undergo phloem ingestion (E2) for more than one hour on citrus tissue containing assumed lethal levels of imidacloprid, yet Miranda et al. (2016) determined that both thiamethoxam and imidacloprid disrupted probing behaviors related to phloem ingestion. Each of the aforementioned studies and resultant conclusions maintained focus on the acquisition / ingestion component of the transmission cycle. While a reduction in acquisition (and subsequent inoculation) of *C*Las is likely to reduce the spread of HLB and could be helpful to the industry, given that citrus is a perennial crop where cumulative effects of disease spread are compounded annually, a simple "reduction" in the spread of *C*Las may no longer be economically acceptable to a grower. Moreover, many groves have become abandoned over recent years throughout Florida, and that space serves as an unmanaged source of inoculum to neighboring groves that are intensively managed and still in production. Consequently, perhaps the neonicotinoid dose required to deter and / or prevent salivation into the phloem as related to inoculation is more critical today than the

neonicotinoid dose required to reduce or deter ingestion activity (bacterial acquisition) as studied in the past.

The two investigations discussed above used EPG to study feeding behavior in response to imidacloprid exposure (Serikawa et al. 2012, Miranda et al. 2016). These studies each have a single key limitation: imidacloprid dosages in which *D. citri* were exposed are unknown. In both Serikawa et al. (2012) and Miranda et al. (2016), various rates of Admire Pro ranging from 0.25 – 0.35 g plant^{-1} were applied to the soil of varying plant sizes up to 80 cm tall. While the amount of imidacloprid applied to the soil is known, application rate and plant size can each have a significant impact on expression in leaf tissues (Langdon 2017). Moreover, expression in leaf tissue can only be quantified after the EPG monitoring period using analytical methods such as enzyme-linked immunosorbent assay (ELISA) (Castle et al. 2005, Garlapati 2009, Setamou et al. 2010) or liquid chromatography mass spectrometry (LC-MS) (Langdon 2017). One must chemically analyze the leaf tissue following each EPG monitoring period to develop a mean imidacloprid titer across the test leaf, which likely would not accurately emulate the imidacloprid concentration within the phloem due to potential in-leaf concentration gradients as proposed by Boina et al. (2009), as well as potential changes in concentration during the EPG monitoring period. Because phloem feeding activity is of most interest to researchers studying transmission of *C*Las, knowing the concentration of imidacloprid expressed specifically within the phloem sap is paramount to behavioral studies regarding the *C*Las-*D. citri* transmission matrix.

Despite demonstrations of changes in feeding behavior under the influence of imidacloprid, the imidacloprid dosage required to elicit a particular behavioral response remains unknown (Serikawa et al. 2012, Miranda et al. 2016). The ability to study feeding behavior during ingestion of a range of known imidacloprid dosages would allow us to develop an

improved understanding of the effects of sublethal imidacloprid exposure to *D. citri* feeding behavior. Herein, we used electropenetrography to evaluate *D. citri* feeding behavior during exposure to a sucrose-based liquid diet spiked with varying, known concentrations of imidacloprid. The overarching goal of this research was to determine the concentration of imidacloprid in citrus leaf tissue required to reduce feeding activity and the concentration required to prevent salivation / ingestion. Langdon and Rogers (2017) defined [feeding] deterrence as the, "gustatory avoidance of less or non-suitable feeding sources." Ascertaining the imidacloprid concentration required to deter and / or prevent *D. citri* salivation / ingestion in phloem will allow us to refine current vector management programs which will help either maximize the reduction or perhaps prevent the spread of *C*Las in Florida citrus.

Materials and Methods

Electropenetrography Assays

Two electropenetrography experiments were conducted to determine the imidacloprid dosage required to reduce feeding activity and prevent salivation / ingestion feeding behaviors when exposed via ingestion. Three sublethal imidacloprid dosages were administered across two experiments using a combination of Admire Pro 4.6F and a 30% sucrose based artificial diet described in detail within Langdon and Rogers (2017). The first experiment tested 0.55 ppm imidacloprid against an untreated control, and the second experiment tested 5.5 ppm and 55 ppm imidacloprid against an untreated control.

To monitor insect feeding behavior, the sucrose based diet, with or without insecticide, was used to fill a polystyrene petri dish 3.5 cm in diameter by 1 cm deep (Corning Glass Works, Corning NY 14831, part #25050-35). A 26 AWG copper wire was inserted into the diet, with the tag end folded over the outer rim of the petri dish. Parafilm M® (Pechiney Plastic Packaging, Menasha WI 54952) was then stretched over the diet filled petri dish in a manner that prevented

air gaps between the undersurface of stretched Parafilm M® and top concave surface of liquid diet. The equipment and its set-up was described in detail elsewhere (Ebert and Rogers 2016). In brief, two 4-channel AC-DC monitors (Backus & Bennett 2009) custom-built by William H. Bennett (EPG Equipment Co., Otterville, MO) were used in DC mode with 150 mV substrate voltage. Data was acquired through a DI710 AD converter (Akron, OH) using Windaq software at a sampling rate of 100 Hz/channel. *Diaphorina citri* adults were tethered using a 2 cm long by 25.4 µm diameter gold wire (Sigma Cohn Corp., Vernon, NY) attached to the thoracic tergites using silver glue (1:1:1 w:w:w, white glue:water:silver flake [8-10 µm, Inframat Advanced Materials, Manchester CT]). The opposite end of the gold wire was connected to the unit head amp set to an impedance of 10^9 Ω, and the copper wire from the petri dish was connected to the "soil probe" electrode from the monitor.

Test insects were subjected to a starvation period of 30 minutes from the time the insects were removed from the colony until they were placed on the plant. All insects were wired during this period without being chilled or anesthetized with CO_2. Recording began before *D. citri* were placed on the Parafilm M® covered petri dish to ensure that all recordings started in the NP behavior and recordings were made over a 23h period. The insects, diet, and head amp were contained in a Faraday cage to minimize electronic noise. Light was provided by overhead fluorescent lights (24:0 L:D) and room temperature was maintained at 26.6° C. When on a plant, *D. citri* are known to exhibit at least six waveforms: non-probing (NP), pathway (C), phloem contact (D), phloem salivation (E1), phloem ingestion (E2), and xylem ingestion (G) (Bonani et al. 2010). When exposed to artificial diet in this study, three waveforms were identified: non-probing (NP), pathway (C), and salivation / ingestion (E1E2). In the first experiment, 28 adult *D. citri* were monitored in the control treatment and 27 adult *D. citri* were monitored in the 0.55

ppm imidacloprid treatment. In the second experiment, 26 adult *D. citri* were monitored in the control treatment, 27 in the 5.5 ppm imidacloprid treatment, and 31 in the 55 ppm imidacloprid treatment. For each run of the experiment, all treatments were run at least once. Multiple replicates of the same treatment within a single run were evenly split between two monitors. The position in the room for any one treatment was rotated between runs to ensure that any potential room effects were evenly distributed between all treatments.

Insect Culture

A continuous culture of laboratory susceptible (LS) *D. citri* was reared 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. Following establishment in 2005, the LS strain did not receive any exposure to insecticides and routine quantitative real time (qPCR) testing as described in Pelz-Stelinski et al. (2010) was used to confirm the colony was *C*Las-free.

Statistical Analysis

Data analysis used an adaptation of Ebert 2.01 (Ebert and Rogers 2016) that was simplified to deal with a psyllid exhibiting only three waveforms (non-probing (NP), pathway (C), and salivation / ingestion (E2)). There was no clear separation between salivation (E1) and ingestion (E2), therefore all salivation and ingestion behaviors were pooled into one unit: salivation / ingestion (E1E2). Count data were square root transformed, duration data were $\log_e$ transformed, and percentage data were logit transformed prior to analysis. Analyses were performed using Proc Glimmix in SAS 9.4M4 running under SAS® Enterprise Guide 7.13. A detailed description of each measured parameter can be found in Table 5-1.

Results and Discussion

In the present study, we tested a range of three sublethal imidacloprid doses across two experiments to approximate the dosage required to: 1) Reduce feeding activity, and 2) Prevent

salivation / ingestion activity. Electropenetrography has been used to study feeding behavior for a number of insect species on artificial media (Joost et al. 2006, Jin et al. 2012, Trebicki et al. 2012). This was the first formal study to use EPG to monitor the feeding behavior of *D. citri* against an insecticide-spiked liquid diet. During the first experiment, we failed to detect a significant effect of 0.55 ppm (550 ppb) imidacloprid on *D. citri* probing behavior, pathway, or salivation / ingestion activity when compared to the untreated control (Table 5-2). These results indicate that a concentration of 0.55 ppm may not deter *D. citri* feeding activity or prevent E1E2, resulting in a failure to intercept bacterial transmission. Conversely, imidacloprid doses of 5.5 (5500 ppb) and 55 ppm (55,000 ppb) generally influenced a majority of probing and pathway parameters (Table 5-3). A significant reduction in the number of probes (NumPrbs) and the number of pathway events (NmbrC) was observed with both 5.5 and 55 ppm of imidacloprid compared to the untreated control. Similarly, Miranda et al. (2016) found that significantly fewer probing and pathway events occurred on plants treated with imidacloprid compared to untreated plants at 35 days following insecticide application to the soil, although the precise dosage received by the insect was unknown. Furthermore, we failed to detect a reduction in the duration of the first (DurFrstPrb) probe event, a reduction in the percentage of probe events that resulted in pathway (PrcntPrbC), nor a reduction in the percentage of probe events that resulted in E1E2 (PrcntPrbE1E2), which may indicate that *D. citri* adults were unable to detect imidacloprid at concentrations up to 55 ppm. Miranda et al. (2016) determined that *D. citri* were able to detect imidacloprid treated plants only following a short period of ingestion (E2), and went on to conclude that imidacloprid likely acts as a feeding deterrent when applied to the soil. In addition, the total duration of non-probing (TtlDurNP) and mean duration of non-probing (MnDurNP) was significantly longer at 55 ppm imidacloprid compared to the untreated control, and the total

duration of pathway (TtlDurC) was significantly reduced at 5.5 ppm, and further reduced at 55 ppm. Similarly, Butler et al. (2012) reported longer periods of non-probing activity for the potato psyllid, *Bactericera cockerelli* (Sulc) on potato plants treated with imidacloprid.

We detected an effect of imidacloprid on two E1E2 parameters: 1) the number of E1E2 events (NumE1E2), and 2) the number of sustained (>600 sec) E1E2 events (NumLngE1E2) (Table 5-3). A significant reduction in the number of E1E2 events was observed at both 5.5 ppm and 55 ppm of imidacloprid (57 and 54 percent, respectively) compared to the untreated control. In addition, the number of sustained (>600 sec) E1E2 events was significantly reduced (ca. 61 percent) at only 55 ppm of imidacloprid relative to the untreated control. However, we failed to detect a difference between treatments in the total (TtlDurE1E2) or mean (MnDurE1E2) duration of E1E2. These results clearly demonstrate a reduction in feeding activity (ie. salivation / ingestion), which presumably would equate to a reduction in bacterial acquisition from *C*Las-infected leaf material in the field, yet a number of *D. citri* were able to successfully salivate in / ingest imidacloprid-spiked diet at our highest dose of 55 ppm for a period that exceeded 10 minutes. An inoculation access period (IAP) of as little as 15 minutes is known to result in inoculation of *C*Las into uninfected citrus tissue (Capoor et al. 1974, Grafton-Cardwell et al. 2013), therefore, it remains possible that sustained salivation / ingestion activity exhibited in our study may result in inoculation of *C*Las into uninfected tissue. We failed to detect a significant difference between 0, 5.5, and 55 ppm imidacloprid in the percent of E1E2 events that resulted in sustained (>600 sec) E1E2, time to first E1E2 from start of probe (TmFrstE1E2FrmPrbStrt), nor time to first sustained E1E2 from start of probe (TmFrstSusE1E2StrtPrb), indicating that *D. citri* adults that did undergo salivation / ingestion, did not stop feeding due to imidacloprid detection. Nevertheless, in two separate whole plant studies where small potted citrus plants were drenched

with some rate of imidacloprid, a reduction in the number of E1 events was observed (Serikawa et al. 2012, Miranda et al. 2016), yet neither investigation indicated that E1 occurrence was prevented. While insecticide did influence feeding behavior in the present study, our highest imidacloprid dose of 55 ppm did not completely prevent E1E2, therefore inoculation of *C*Las into uninfected leaf material remains possible at 55 ppm imidacloprid.

Despite intensive *D. citri* management programs implemented by growers that utilize frequent soil applications of neonicotinoid insecticides, groves continue to succumb to *C*Las infection. We revealed a reduction in a number of probing activities, an increase in non-probing behaviors (NP), a reduction in pathway behaviors (C), and a reduction in salivation / ingestion behaviors (E1E2) under oral exposure of at least 5.5 ppm imidacloprid-spiked artificial diet using EPG. Reductions in feeding activity observed in the present study confirm findings of previous studies (Serikawa et al. 2012, Miranda et al. 2016), and are likely to elucidate a reduction in the spread of HLB within and among commercial citrus groves, demonstrating some level of value in the use of neonicotinoids applied to the soil. Langdon and Rogers (2017) found that the LC_{90} of imidacloprid following ingestion ranged from 62.19 ppm in the lab population to as much as 522.58 ppm in a potentially resistant field collected population, indicating that 55 ppm is a sublethal imidacloprid dose when administered through ingestion. In addition, they found increased activity when imidacloprid was administered through contact (laboratory susceptible population LC_{90} = 0.13 ppm imidacloprid) than by ingestion (laboratory susceptible population LC_{90} = 62.19 ppm imidacloprid). Nevertheless, while reductions in feeding activity are apparent following ingestion of imidacloprid, because we were unable to prevent salivation / ingestion feeding behavior by oral administration of imidacloprid doses of up to 55 ppm, and because imidacloprid titer following the soil-application of Admire Pro in commercial groves is not

known to exceed 55 ppm (Langdon, 2017), we are certain that soil-applied imidacloprid is not capable of completely preventing *C*Las inoculaton, and thus preventing the spread of HLB in Florida citrus. Future work should investigate imidacloprid residues following foliar application and resulting *D. citri* feeding behaviors at those concentrations in the attempt to find an effective use for imidacloprid that is likely to prevent inoculation of *C*Las into uninfected citrus.

Table 5-1. Description of adult *Diaphorina citri* feeding behavior by EPG model abbreviation.

Behavior	Abbreviation*	Behavior Description
Probing	NumPrbs	total number of probing events
	MnPrbs	mean number of probing events
	DurFrstPrb	Duration (sec) of first probe
	NumNP	Total number of non-probing events
	TtlDurNP	Sum of duration (sec) of all non-probing events
	MnDurNP	Mean duration (sec) of all non-probing events
	DurNpFllwFrstSusE1E2	duration (sec) of non-probing event before first sustained (>600sec) ingestion
Pathway	NmbrC	Number of pathway events
	TtlDurC	Total duration (sec) of pathway events
	MnDurC	Mean duration (sec) of pathway events
	PrcntPrbC	Percent of probe events that result in pathway
Salivation / Ingestion**	NumE1E2	Number of salivation / ingestion events
	NumLngE1E2	Number of long (>600 sec) salivation / ingestion events
	TtlDurE1E2	Total duration (sec) of salivation / ingestion
	MnDurE1E2	Mean duration (sec) of salivation / ingestion
	TmFrstSusE1E2StrtPrb	Time (sec) until first sustained (>600sec) salivation / ingestion from start of probe with the sustained event
	TmFrstE1E2FrmPrbStrt	duration (sec) of first salivation / ingestion event from start of probe
	PrcntPrbE1E2	Percent of probe duration in salivation / ingestion
	PrcntE1E2SusE1E2	Percent of salivation / ingestion duration spent in sustained (>600sec) salivation / ingestion
	TmFrstSusE1E2	Duration (sec) of first sustained salivation / ingestion event

* All variables are by insect. Means are counts, durations, or percentages per insect, where durations are expressed in seconds.

** There is no clear separation between E1 and E2 in the artificial diet. The waveforms blend one into the other, and separating them would introduce considerable error into the measurements.

Table 5-2: LSMeans ± SEM for each behavioral parameter following exposure of adult *Diaphorina citri* to artificial diet with and without 0.55 ppm imidacloprid.

Behavior	Parameter	Control LSMeans ± SE	0.55 ppm LSMeans ± SE	p-value
Probing / non-probing	NumPrbs	7.10 ± 0.48	6.22 ± 0.49	0.2034
	MnPrbs	4.70 ± 0.17	5.02 ± 0.17	0.1843
	DurFrstPrb	3.94 ± 0.17	3.95 ± 0.18	0.9712
	NumNP	7.19 ± 0.47	6.29 ± 0.48	0.1884
	TtlDurNP	11.25 ± 0.06	11.15 ± 0.06	0.2503
	MnDurNP	7.52 ± 0.17	7.54 ± 0.18	0.9321
	DurNpFllwFrstSusE1E2	1008.18 ± 2607.27	6968.35 ± 3057.30	0.1563
Pathway	NmbrC	7.22 ± 0.49	6.30 ± 0.50	0.1901
	TtlDurC	8.20 ± 0.19	8.13 ± 0.20	0.8112
	MnDurC	4.49 ± 0.09	4.52 ± 0.09	0.7777
	PrcntPrbC	1.19 ± 2.30	4.84 ± 2.16	0.2567
Salivation / Ingestion*	NumE1E2	1.06 ± 0.20	0.86 ± 0.21	0.4883
	NumLngE1E2	0.53 ± 0.14	0.48 ± 0.14	0.8102
	TtlDurE1E2	7.81 ± 0.49	7.61 ± 0.49	0.7735
	MnDurE1E2	6.70 ± 0.49	6.85 ± 0.49	0.8294
	TmFrstSusE1E2StrtPrb	5.45 ± 0.32	4.88 ± 0.34	0.2383
	TmFrstE1E2FrmPrbStrt	4.77 ± 0.24	4.71 ± 0.24	0.8415
	PrcntPrbE1E2	-1.19 ± 0.45	-0.59 ± 0.45	0.3547
	PrcntE2SusE1E2	-1.07 ± 0.42	-0.23 ± 0.39	0.1780
	TmFrstSusE1E2	11.07 ± 0.11	10.93 ± 0.12	0.3993

*All variables are by insect. Means are counts, durations, or percentages per insect, where durations are expressed in seconds.

**There is no clear separation between E1 and E2 in the artificial diet. The waveforms blend one into the other, and separating them would introduce considerable error into the measurements.

Table 5-3: LSMeans ± SEM for each behavioral parameter following exposure of adult *Diaphorina citri* to artificial diet with 0, 5.5, or 55 ppm imidacloprid.

Behavior	Parameter	Control LSMeans ± SE*	5.5 ppm LSMeans ± SE*	55 ppm LSMeans ± SE*	p-value
Probing / non-probing	NumPrbs	8.44 ± 0.46 a	6.32 ± 0.45 b	5.31 ± 0.42 b	<.0001
	MnPrbs	4.93 ± 0.17 a	4.81 ± 0.17 ab	4.28 ± 0.16 b	0.013
	DurFrstPrb	3.61 ± 0.16	3.83 ± 0.16	3.49 ± 0.15	0.2901
	NumNP	8.49 ± 0.45 a	6.40 ± 0.44 b	5.42 ± 0.42 b	<.0001
	TtlDurNP	11.17 ± 0.04 b	11.23 ± 0.04 ab	11.33 ± 0.04 a	0.014
	MnDurNP	6.97 ± 0.15 b	7.66 ± 0.15 a	8.09 ± 0.14 a	<.0001
	DurNpFllwFrstSusE1E2	5.59 ± 0.40 b	9.11 ± 0.55 a	10.38 ± 0.45 a	<.0001
Pathway	NmbrC	8.64 ± 0.46 a	6.37 ± 0.45 b	5.39 ± 0.42 b	<.0001
	TtlDurC	8.78 ± 0.16 a	7.81 ± 0.16 b	7.23 ± 0.15 c	<.0001
	MnDurC	4.54 ± 0.09 a	4.26 ± 0.09 ab	4.01 ± 0.09 b	0.0003
	PrcntPrbC	1.94 ± 1.78	3.08 ± 2.04	4.44 ± 1.74	0.6061
Salivation / Ingestion**	NumE1E2	1.72 ± 0.20 a	0.74 ± 0.19 b	0.79 ± 0.18 b	0.0006
	NumLngE1E2	0.75 ± 0.14 a	0.39 ± 0.14 ab	0.29 ± 0.13 b	0.0497
	TtlDurE1E2	7.06 ± 0.44	7.02 ± 0.52	6.27 ± 0.45	0.404
	MnDurE1E2	5.85 ± 0.38	6.58 ± 0.45	5.94 ± 0.39	0.4243
	TmFrstSusE1E2StrtPrb	4.87 ± 0.25	4.95 ± 0.32	4.46 ± 0.31	0.4881
	TmFrstE1E2FrmPrbStrt	4.68 ± 0.22	4.76 ± 0.26	4.48 ± 0.22	0.6951
	PrcntPrbE1E2	-1.94 ± 0.46	-0.83 ± 0.54	-1.21 ± 0.47	0.2756
	PrcntE1E2SusE1E2	-1.01 ± 0.37	0.22 ± 1.16	-0.80 ± 0.82	0.6098
	TmFrstSusE1E2	10.70 ± 0.19	10.87 ± 0.19	10.97 ± 0.18	0.581

* All variables are by insect. Means are counts, durations, or percentages per insect, where durations are in expressed in seconds.

**There is no clear separation between E1 and E2 in the artificial diet. The waveforms blend one into the other, and separating them would introduce considerable error into the measurements.

CHAPTER 6
CONCLUSIONS

The overarching goal of this research was to help refine current vector management programs which will help either maximize the reduction or prevent the spread of *C*Las in Florida citrus. This research quantified the concentration of imidacloprid, thiamethoxam, and clothianidin in citrus leaf material in space and over time following application to the soil. While factors including tree canopy region, leaf section, tree size, and application rate each effected expression of neonicotinoids following application to the soil, observed titers were much lower than expected when used in the field. The target concentration threshold of imidacloprid following application to the soil was 200 to 250 ppb based on the report by Setamou et al. (2010). Since a correlation between *D. citri* abundance and clothianidin or thiamethoxam titer did not exist, 200 to 250 ppb became the assumed efficacy threshold concentration for all neonicotinoids. This research was the first to quantify the lethal concentration of each of the three currently labeled 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, Kanga et al. 2016, Coy et al. 2016). 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. We found that lethal activity from contact exposure to neonicotinoid insecticides occurs at very low concentrations compared with ingestion. Moreover, we were able to use electropenetrography to study feeding behaviors under exposure to precise neonicotinoid dosages. We found that 0.55 ppm imidacloprid did not

reduce in any feeding behavior including probing, pathway, or salivation / ingestion activities, yet 5.5 and 55 ppm imidacloprid caused significant reductions in probing, pathway, and salivation / ingestion behaviors. While the highest mean titer observed for any neonicotinoid in the present composition of studies was likely to have reduced the incidence of *C*Las inoculation in the field, that titer was unlikely to completely intercept inoculation. We observed a number of effects that are of significant concern regarding use of neonicotinoids by soil application in Florida citrus: 1) failure to achieve lethal concentrations in leaf tissue following application to the soil; 2) persistence of neonicotinoid concentrations less than 1 ppm through 12 weeks following application; 3) failure to achieve acceptable *D. citri* control following application to trees 18 months of age (MCV = 1.34m^3); 4) lack of efficient uptake relative to dose applied (e.g. high rate of 0.74g Platinum 75SG in 237 mL water per tree is equivalent to 2370 ppm thiamethoxam applied to the soil, and low rate of 0.37g Platinum 75SG in 237 mL water per tree is equivalent to 1185 ppm thiamethoxam applied to the soil); and 5) higher sensitivity of *D. citri* to neonicotinoids through contact exposure compared to ingestion.

While the potential risk of the spread of HLB is highly important, and within the scope of this research, neonicotinoid resistance following exposure to sublethal dosages is of significant concern. Our results indicated a second event of neonicotinoid resistance in field populations of *D. citri*, which may have been exacerbated by sublethal neonicotinoid expression following application to the soil. Development of resistance to neonicotinoids by *D. citri* has occurred in the field, and therefore, applications of neonicotinoids must be carefully administered such that *D. citri* exposure to sublethal dosages is minimized. To potentially maximize the activity of neonicotinoids and permit the longevity of their use, subsequent work should investigate neonicotinoid residues over time following foliar application. Presumably, foliar application

would result in much higher acute residues following application, with a more rapid residue degradation, more suitable within the scope of insecticide resistance management. The foremost strategy for stewardship and future implementation of neonicotinoids in citrus must be resistance management. Therefore, the current results suggest that foliar use of neonicotinoids may be a superior tactic than their applications to the soil, particularly in trees with canopies larger than $0.08m^3$ to mitigate resistance development and thus preserve efficacy of this mode of action. Given the dynamic nature of susceptibility of *D. citri* to insecticides, we must remain diligent in research efforts with a keen focus on resistance management and be willing to adjust insecticide use patterns to ensure the longevity of each available chemical class.

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BIOGRAPHICAL SKETCH

Kevin William Langdon was born to Mr. and Mrs. Andy William Langdon and was the youngest sibling of three. Kevin was raised between his home in rural eastern Wake County and the family swine and horse farm in southwestern Johnston County. He became passionate about the outdoors through his pursuit of fish and game, with a focus on chasing whitetail deer with his bow and bobwhite quail behind his pair of English pointers, Junior and Bailey. Kevin developed a true affinity for plant agriculture by working on a neighboring tobacco farm throughout his boyhood.

Kevin was graduated from East Wake High School in 2006, where he was an active member of the FFA under Ms. Janet Harris. He attended North Carolina State University where he earned a Bachelor of Science degree in Agricultural and Environmental Technology with a minor in Soil Science in 2010. Kevin worked as a research assistant between 2006 and 2008 in the entomology lab of Dr. George G. Kennedy. In the summer of 2009, Kevin moved to Vero Beach, Florida to fill an internship position with Syngenta Crop Protection working for Dr. Tony Burd in the Insect Control Lab at the Vero Beach Research Center. It was in Vero Beach where Kevin decided to pursue a graduate degree in entomology and where he met his wife, an intern in the Weed Control Lab, Miss Barbara Lee Adams. After a love-filled summer, Kevin returned to Raleigh where he earned a Master of Science degree in entomology at North Carolina State University under the direction of Dr. Mark R. Abney. Upon graduation in August 2012, he took a position back at Syngenta's Vero Beach Research Center as a Research and Development Scientist, where he conducted research in insecticide development and insecticide resistance. Following encouragement from his manager, Dr. Clark Lovelady, in 2014 Kevin decided to return to academia to pursue a Doctor of Philosophy in entomology at the University of Florida under the direction of Dr. Michael E. Rogers, while maintaining full-time employment with

Syngenta. The overarching goal of his research was to provide solutions to citrus growers to help manage the devastating, insect-transmitted citrus disease, huanglongbing. Kevin received his Ph.D. from the University of Florida in the fall of 2017 and continued his career with Syngenta while maintaining his passion for the outdoors.