FACTORS AFFECTING ACQUISITION OF CANDIDATUS LIBERIBACTER ASIATICUS BY THE ASIAN CITRUS PSYLLID, Diaphorina citri

By

ALICIA J. KELLEY

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To Dad
who helped me catch my first Monarch
ACKNOWLEDGMENTS

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<td>Acquisition access period</td>
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FACTORS AFFECTING ACQUISITION OF *Candidatus Liberibacter asiaticus* BY THE ASIAN CITRUS PSYLLID, *Diaphorina citri*

By

Alicia J. Kelley

December 2016

Chair: Kirsten Pelz-Stelinski
Major: Entomology and Nematology

The Asian citrus psyllid, *Diaphorina citri*, is the most detrimental insect pest of citrus crops due to its role as a vector of *Candidatus* Liberibacter asiaticus (Las), the pathogen responsible for huanglongbing (HLB). Las is a gram-negative, phloem-limited alphaproteobacteria, which causes starch buildup in leaf phloem tissue, disrupting phloem transport (Koh et al. 2012). Trees experience reduced fruit production and poor fruit quality until it eventually dies in 7-10 years.

Several factors influence the successful transmission of Las. Higher acquisition rates occur when the amount of time *D. citri* feed on infected plant material, or acquisition access period (AAP), increases (Pelz-Stelinski et al. 2010, Ammar et al. 2016). In addition, *D. citri* acquire Las more frequently, and are more likely to inoculate susceptible plants, when they acquire Las as nymphs (Inoue et al. 2009, Pelz-Stelinski et al. 2010, Ammar et al. 2016). Thus, successful Las acquisition by nymphs is critical to the transmission cycle. The first objective of this study was to quantify two pathways of nymph acquisition: transovarial transmission and acquisition of Las via feeding at the oviposition site. One Las-infected female was exposed to a citrus seedling and allowed to oviposit. Transovarial transmission was tested by transferring her eggs to a new, uninfected citrus seedling. Oviposition site acquisition was tested by removing the
female’s eggs and re-infesting the flush with nymphs from a healthy psyllid colony. The third treatment allowed the female’s eggs to remain at the oviposition site to test for the combined acquisition rate in nymphs from these two pathways. My results showed that *D. citri* which acquired Las from the oviposition site were more likely to acquire Las than from transovarial transmission. This suggests that transovarial transmission allows Las to spread in low amounts even when infected plant hosts are not available, and that inoculation of the oviposition site provides a source of Las to developing nymphs via the plant phloem.

The second objective evaluated acquisition rates in *D. citri* following thermal treatment for Las management. Current HLB control strategies rely on regular insecticide treatments and replanting infected trees. A new technique, “thermotherapy,” utilizes pressurized steam to reduce Las titers in infected trees and extend the life of the tree. I investigated the effects of thermotherapy on Las titer in infected trees and on *D. citri* acquisition. Thermotherapy was applied to Las-positive sweet orange (*Citrus sinensis*) by enclosing trees in a canvas tent and heating the interior to approximately 55ºC for 30 seconds. Two additional groups of untreated Las-positive and Las-negative trees were included in this study, for a total of three treatments. Psyllid acquisition was compared between treatment groups at 2-3 month intervals using two methods: 1) bagging uninfected adult *D. citri* on branches for 9 days; 2) enclosing branches infested with *D. citri* nymphs and collecting psyllids following adult emergence. Las titers rose significantly over time in all three treatment groups. While untreated Las-negative trees maintained low Las titers and psyllid acquisition rates, Las titers in plants and psyllid acquisition rates did not differ between treated and untreated Las-positive trees. The current results suggest that further development of field application methods is needed to determine the utility of thermotherapy as a tool for HLB management.
CHAPTER 1
LITERATURE REVIEW

Huanglongbing

Causal Agent

*Candidatus* Liberibacter asiaticus (Las), a gram-negative alphaproteobacteria, is the putative causal agent of Huanglongbing (HLB), or citrus greening disease (Bové 2006). This disease is responsible for severe damage to citrus groves worldwide (Gottwald 2010). Las is one of three gram-negative alphaproteobacteria known to cause HLB, or citrus greening. The two other bacteria associated with the disease are *Candidatus* Liberibacter americanus (Lam) and *Candidatus* Liberibacter africanus (Laf). Lam is primarily found in Brazil, and more recently in China, while Laf is found primarily in South Africa; however, Las is the most prevalent bacteria in all regions in which HLB is established (Bové 2006, Gottwald 2010). Thus far, Las is the only HLB-causing agent that has reached Florida. Las was first discovered in Florida in 2005, and has since spread across the major citrus growing regions of Texas and California (Halbert 2005, Kumagai et al. 2013, da Graça et al. 2015).

Disease Symptoms

Las clogs the phloem tissues and causes starch build-up in the leaves (Bové 2006, Koh et al. 2012). Visual diagnostics of HLB include yellow mottling and curling of leaves, stunted growth, twig dieback, deformed and unpalatable fruit, and ultimately, tree death (Capoor et al. 1974). A tree can remain asymptomatic for 1-2 years before showing any of these symptoms (Gottwald 2010).

Disease Management

There is currently no cure for HLB. Management efforts emphasize minimizing the number of Las-infected trees to prevent the disease from spreading. This includes removing
infected trees upon diagnosis and cultivating certified disease-free nursery stock (Brlansky and Rogers 2007, Qureshi and Stansly 2010, Hall and Gottwald 2011). There is also considerable interest in the use of nutritional foliar sprays to increase the production life of infected trees (Hall and Gottwald 2011). Recent work has shown nutritional sprays to be efficacious when combined with vector control (Stansly et al. 2014). However, these results conflict with other studies which suggest nutritional sprays do not significantly effect yield (Gottwald et al. 2012).

Antibiotic treatments have merited considerable research as a potential option of alleviating Las. The most common antibiotics in agricultural use today are streptomycin and oxytetracycline (McManus et al. 2002). These antibiotics disrupt protein synthesis in bacteria by binding to its ribosomes. Streptomycin has been shown to suppress Las titers effectively and cause less phytotoxic side effects when used in combination with penicillin (Zhang et al. 2011). Oxytetracycline causes severe phytoxicity in citrus in graft-based evaluations (Zhang et al. 2014). Trunk injections of tetracycline lower Las titers temporarily, but repeated treatments eventually lead to plant damage (Zhang et al. 2015). Several other compounds, such as Ampicillin, Penicillin, and Rifamycins, also show efficacy against Las (Zhang et al. 2014, 2015); in addition, Ampicillin is readily absorbed by the tree via foliar applications and causes no negative effects on the tree (Zhang et al. 2011, Yang et al. 2015). However, these treatments have not been approved for commercial use. Thus far, only three products have been used in Florida citrus after a crisis declaration in March 2016. Under the emergency exemptions of FIFRA, three antibiotic foliar sprays were approved: streptomycin sulfate (FireWall 50WP, AgroSource Inc.), oxytetracycline hydrochloride (FireLine 17WP, AgroSource Inc.), and oxytetracycline calcium complex (Mycoshield, Nufarm Americas Inc.).
**Asian Citrus Psyllid, *Diaphorina citri***

The Asian citrus psyllid (Hemiptera: Leviidae), *Diaphorina citri* Kuwayama, is the primary insect vector of *Candidatus Liberibacter asiaticus* (Las) (Hall et al. 2013). It feeds on the phloem tissue of citrus, where acquires Las, a phloem-limited bacteria (Grafton-Cardwell et al. 2013, Hall et al. 2013). Once acquired, Las replicates in the psyllid, invading cells in the midgut, Malpighian tubules, muscles, and fat tissues near the reproductive organs (Ammar et al. 2011). It then migrates through the hemolymph to the psyllid’s salivary glands (Xu et al. 1988, Ammar et al. 2011).

Researching the transmission pathway of Las between psyllid vector and plant host is difficult due to the limitations of PCR detection. Since the bacteria is unculturable, it must be present in the insect/plant tissue above a certain threshold before PCR can detect it. Thus, interactions beneath this threshold are difficult to study. We know Las replicates faster in *D. citri* when it is acquired during the nymphal stages, and psyllids which acquire as nymphs are more likely to transmit the pathogen as adults (Inoue et al. 2009, Pelz-Stelinski et al. 2010, Ammar et al. 2016). In addition, when *D. citri* feed on infected plant material as adults, acquisition access periods (AAPs) are positively correlated with the number of Las-positive psyllids (Pelz-Stelinski et al. 2010, Ammar et al. 2016).

Without a better understanding of how Las utilizes its vector to proliferate its spread, we cannot develop targeted control methods to disrupt that pathway. Current pest management practices focus on removing infected trees and pesticide sprays during peak flushing seasons (Rogers and Stansly 2006). However, these methods have been unsuccessful, and *D. citri* is now found in all the major citrus-growing regions of the United States.
**Geographical Range**

Kuwayama (1908) first described *D. citri* in Taiwan, though there is evidence that *D. citri* originated in India (Beattie et al. 2006, Grafton-Cardwell et al. 2013). Reports of *D. citri* in the Western Hemisphere began in the 1940s when they were discovered in Brazil (Halbert and Núñez 2004). *D. citri* was first discovered in the United States when it arrived in Florida in 1998 (Halbert 1998). It has since spread across the southern United States into Alabama, Arizona, California, Georgia, Louisiana, Mississippi, Texas, and South Carolina (Hall et al. 2013).

**Description**

**Eggs.** Females oviposit eggs in clusters on newly-unfurled flush. Eggs are teardrop-shaped and yellow to orange in color, and will hatch in 2-4 days depending on temperature (Chavan and Summanwar 1993, Liu and Tsai 2000). A female lays an average of 500-800 eggs, depending on temperature and her access to males (Grafton-Cardwell et al. 2013, Hall et al. 2013).

**Nymphs.** Nymphs of *D. citri* undergo 5 instars after hatching, which take approximately 11-15 days to complete (Chavan and Summanwar 1993, Grafton-Cardwell et al. 2013). Nymphs are wingless and dorso-ventrally flattened. They are yellow in color, but variations in melanization cause some to appear brown.

**Adults.** *D. citri* adults are 2-3mm in length (Hall et al. 2013). Their exoskeletons are tan in color, with dark brown mottling and a whitish, waxy coating. Their abdomen color has three main color morphisms: yellow-orange, green-blue, and grey-brown (Hall et al. 2013). Abdominal color is thought to be associated with the maturity of reproductive organs (Wenninger and Hall 2008, Wenninger et al. 2009). Adult lifespan varies with temperature, but on average they live approximately 30 to 50 days; however, *D. citri* has been reported to survive as long as 188 days at 27ºC (Liu and Tsai 2000, Hall et al. 2013).
Host Plants

*Diaphorina citri* feeds on plants in the family Rutaceae, subfamily Aurantioidea (Halbert and Manjunath 2004, Hall et al. 2013). Several species in the *Citrus* genus are suitable hosts for *D. citri*, including *Citrus macrophylla* Wester, *Citrus sinensis* (L.) Osbeck, *Citrus reticulata* Blanco, and *Citrus paradisi* (Macfad.). These species are also highly susceptible to HLB (Halbert and Núñez 2004). Another common host plant for *D. citri* is orange jasmine, *Murraya paniculata* (L.). *D. citri* can transmit Las to *M. paniculata*, but the plants rarely develop symptoms (Damsteegt et al. 2008).

Transmission

Transmission of Las by *D. citri* occurs through persistent, propogative transmission. Successful transmission of Las requires an acquisition access period (AAP), during which *D. citri* feeds on infected plant material; a latency period, in which the pathogen replicates in the vector and migrates through the hemolymph to the insect’s salivary glands; and an inoculation access period (IAP), when the psyllid feeds on a healthy plant and inoculates it with the bacteria. An additional latency period occurs after inoculation, during which the bacteria replicates in the plant until it reaches the threshold necessary for psyllid acquisition.

All these stages have been researched thoroughly with inconsistent results. Early studies show *D. citri* need as little as 15 minutes to acquire Las, though these studies relied on visual symptoms of HLB infection (Capoor et al. 1974, Grafton-Cardwell et al. 2013). Studies utilizing PCR detection of Las have reported a directly proportional relationship between AAP and Las detection in the psyllid; however, acquisition rates in adults were extremely low, ranging from 0-20% when the AAP was <4 weeks (Pelz-Stelinski et al. 2010, Ammar et al. 2016). Las titers decrease in adult *D. citri* over time if psyllids are maintained on clean plants (Pelz-Stelinski et al. 2010). The ability of *D. citri* to inoculate new plants also shows significant variation between
studies. Earlier reports showed 80% inoculation efficiency using single psyllids (Xu et al. 1988), whereas more recent studies report 20% efficiency using 5-6 psyllids (Ammar et al. 2016). In both studies, *D. citri* which successfully inoculated new citrus were exposed to Las during their nymphal stages. Psyllids which acquire Las as adults inoculate at a very low or undetectable rate (Inoue et al. 2009, Ammar et al. 2016).

The most consistent result from transmission research is that nymphs are far more likely to acquire Las and to inoculate subsequent plants as adults (Inoue et al. 2009, Pelz-Stelinski et al. 2010, Ammar et al. 2016). Additionally, Las replicates faster in nymphs than adults (Ammar et al. 2016). The underlying mechanism of the enhanced replication and propagation of Las in *D. citri* nymphs is unknown. However, it is evident that nymphs occupy a crucial role in the Las life cycle.

*Diaphorina citri* also transmit Las through horizontal and vertical transmission. A low rate of both transovarial transmission (3.6%) and sexual transmission from male to female (<4%) was shown in previous studies (Pelz-Stelinski et al. 2010, Mann, Pelz-Stelinski, et al. 2011). These data strongly suggest that Las can persist in *D. citri* without infected host plants, which allow Las an alternative transmission mechanism to enhance its dispersal.

Transmission is also enhanced by the bacteria. Las manipulates the behavior and biology of its vector and host plant to facilitate its spread. Volatiles released from citrus leaves are attractive to *D. citri* (Mann et al. 2011), but citrus infected with Las is significantly more attractive (Mann et al. 2012). Las further manipulates *D. citri* after the insect is infected. Infected *D. citri* disperse to uninfected plants at higher rates than *D. citri* which were not exposed to Las (Mann et al. 2012, Martini et al. 2015).

**Integrated Pest Management**

**Cultural control.** Minimizing the incidence and movement of infected plant material is
the primary goal of cultural control practices today. The first line of defense is preventing the dispersal of HLB and *D. citri*. In states such as California where *D. citri* and HLB are not fully established, quarantine zones are established to limit the movement of *Citrus* fruit or leaves out of these areas (Kumagai et al. 2013). No within-state quarantines exist in Florida, but Florida is currently under a state-wide quarantine to prevent infected citrus from leaving the state (CFR 301.75 Subpart Citrus Canker). Aggressive monitoring for *D. citri* and HLB is critical to the efficacy of quarantine zones (Bové 2006, Gottwald 2010). However, due to its long asymptomatic latency period and limitations of early PCR detections, HLB has likely spread to new areas long before it is discovered.

Growers are encouraged to remove trees promptly after it is confirmed to be HLB-positive. Preferably, all the trees in the surrounding area should also be removed, because they are likely to be infected despite being asymptomatic (Gottwald 2010). Citrus nurseries certified under the Citrus Budwood Protection Program supply disease-free stock for replantings.

**Biological control.** Several studies have explored biological control agents. The entomopathogenic fungi, *Isaria fumosorosea*, causes mortality of *D. citri* when humidity is over 80% (Hunter et al. 2011, Hall et al. 2012, Lezama-Gutiérrez et al. 2012, Stauderman et al. 2012). The potential of natural predators has also been examined. Michaud (2004) identified coccinellid beetles (*Harmonia axyridis*) as the most abundant and important naturally-occurring predator of *D. citri* in Florida. *Tamarixia radiata*, a parasitoid wasp which preferentially attacks *D. citri*, has also been investigated. *T. radiata* was imported from Taiwan and Vietnam in 1999, and the parasitoid has established throughout the US citrus regions (Hoy and Nguyen 2001). Large breeding programs and subsequent releases have been conducted with *T. radiata* (Skelley and Hoy 2004), and it is currently being used in organic citrus groves, as well as abandoned groves,
as biological control.

**Chemical control.** *D. citri* management in citrus groves relies on the use of systemic insecticides, such as imidacloprid, thiamethoxam and clothianidin (Dewdney et al. 2016). Soil drenches and are-wide sprays are recommended for young and mature trees, respectively (Rogers and Shawer 2007, Qureshi et al. 2009). Heavy use of chemical sprays has led to the development of insecticide-resistant *D. citri* populations (Tiwari et al. 2011, Kanga et al. 2016). Insecticide resistance has declined in recent years, possibly due to the implementation of insecticide rotation in area-wide sprays; however, monitoring and resistance management remain crucial for effective *D. citri* management (Coy et al. 2016).

**Justification**

The interaction of Las with its plant host and insect vector is not currently well-understood. It is necessary to deepen our understanding of the transmission process in order to improve our pest control programs. This research further characterizes how Las uses *D. citri* to reach new citrus hosts, and offer more insight into which factors contribute to its successful spread. I explore how small amounts of Las can be transmitted to developing nymphs, because of their critical role in the replication of Las and inoculation of new plants. Part of the difficulty in studying these interactions is due to the inability to detect low titers of Las with conventional PCR techniques. With the recent advances in detection methods, we now have access to an extremely sensitive nested-qPCR method developed by Coy et al. (2014). Using this method to investigate interactions of Las with *D. citri* that were previously undetectable. This will provide insight into how nymphs are able to acquire and spread Las quickly in previously healthy agro-ecosystems.

I also examine adult and nymph acquisition under field conditions. This project aimed to study the efficacy of treating diseased trees with heat (referred to as “thermotherapy”), and to
determine how a change in Las titer would affect the acquisition rate in *D. citri* which feed upon that plant material. While research suggests thermotherapy decreases Las titers in infected trees (Hoffman et al. 2012, Ehsani et al. 2013), it is not known if *D. citri* can still acquire small amounts of the bacteria which were not eliminated.

**Goal and Hypotheses**

The goal of this research was to identify which inoculum sources contribute more effectively to acquisition of Las by *D. citri* nymphs. I hypothesized that nymphs initially acquire Las at the oviposition and maternal feeding site on young flush, as well as a small amount of Las from transovarial transmission. I also hypothesized that nymphs are more likely to acquire Las through feeding at the oviposition site than transovarial exposure.

If thermotherapy successfully decreases Las titer in the treated plants, I hypothesized that psyllid acquisition from those trees will also decrease. I postulated that as the pathogen slowly reestablishes its population in the tree, acquisition of Las by *D. citri* will rise at a similar rate.

**Specific Objectives**

The first objective of this research was to determine whether an infected female can inoculate flush where she oviposits, thus providing an immediate source of Las to her offspring. I aimed to differentiate this mode of transmission from transovarial transmission to determine which pathway results in a higher rate of nymph acquisition. My second objective was study how thermotherapy affects disease transmission.
CHAPTER 2
CONTRIBUTION OF MATERNAL INOCULATION OF LAS AT OVIPOSITION SITE AND TRANSOVARIAL TRANSMISSION TO NYMPH ACQUISITION OF LAS

Huanglongbing (HLB) can spread rapidly through a healthy grove. After initial inoculation with Las, a tree may remain symptomatic for 1-2 years before it is detected (Bové 2006, Gottwald 2010). During this time, the tree can serve as a source of acquisition long before Las reaches a detectable level (Gottwald 2010). Research has shown that D. citri can transmit Las without a source of infected plant material, such as via sexual transmission from males to females through seminal fluids (<4%) (Mann et al. 2011) and transovarial transmission (2-6%) (Pelz-Stelinski et al. 2010). The low rate of Las transmission via horizontal or transovarial transmission suggests these pathways are not the primary source of Las for D. citri, but provide an alternative mechanism for Las dissemination in the absence of infected plants.

Multiple studies indicate the transmission efficiency of D. citri is much higher when Las is acquired from infected plants as nymphs (Inoue et al. 2009, Pelz-Stelinski et al. 2010, Ammar et al. 2016). This suggests that nymphal acquisition is important to the lifecycle of Las. In addition, infected psyllids can transmit Las rapidly during this asymptomatic latency period by inoculating the flush where females oviposit, thus providing an immediate source of Las to developing nymphs (Lee et al. 2015). However, due to the limitations of PCR detection, studying the pathway of Las between a single female psyllid and her offspring is difficult. It is not known how Las titer in infected adults influences nymphal acquisition, nor how transovarial transmission compares to feeding on small amounts of Las in the flush. This study utilizes a more sensitive Las detection technique (Coy et al. 2014) to investigate the efficiency of Las acquisition associated with inoculation of oviposition sites and compare this to transovarial transmission.
Materials and Methods

Psyllid and plant materials. Las-exposed females were collected from 1) a laboratory colony in which *D. citri* are reared on Las-positive *Citrus sinensis* (L.) Osbeck, *Swingle citrumelo*, and *Citrus macrophylla*, maintained at 27°C and 50-60% RH on an 18:6h L:D photoperiod; 2) Las-infected *C. sinensis* trees in a research grove located in Lake Alfred, FL (28.130996, -81.71710). Females were of mixed age and preferentially selected based on visual assessment of gravidity (females with enlarged, bright orange abdomen). These selection criteria were recommended to improve the success rate of oviposition and were based on unpublished data.

Healthy male *D. citri* (unexposed to Las) were collected from an uninfected (Las-free) colony maintained on *Citrus sinensis* (L.) Osbeck, *Swingle citrumelo, Murraya paniculata* (L.) Jack, and *Citrus macrophylla* at 2 ±2°C and 50-60% RH on an 18:6h L:D photoperiod. *Citrus macrophylla* seedlings, grown from seed in an insect-proof greenhouse, were used in all assays. Seedlings were 5-7 inches tall with approximately 6-8 leaves. Plants with new, actively sprouting foliage (flush) were selected for all assays.

Oviposition bioassay. A single infected female was confined with 3-5 uninfected males onto a healthy seedling. A fine mesh bag, held erect with a metal rod inserted in the soil, was used to enclose *D. citri* on plants. Females were allowed a 5- to 7-day AAP to lay eggs. After the AAP, any replicates in which the female was not recovered, or in which the female did not oviposit, were discarded. Females were collected and stored in 80% ethanol at -80 °C for DNA collection. Plants and eggs were subjected to one of three acquisition treatments: 1) oviposition site acquisition (OA), 2) transovarial acquisition (TO), and 3) oviposition site and transovarial acquisition (OVTR). Nymph acquisition from the oviposition site (OA) was evaluated by removing eggs from the flush and replacing with 10-15 2nd-3rd instar nymphs. Transovarial
transmission (TO) was evaluated by removing eggs from the plant immediately after oviposition. The flush and eggs were sterilized to prevent fungal or bacterial contamination prior to egg hatch. Eggs were sterilized with the following procedure: 5 s agitation in 10% bleach; 5 s rinse in DI water; 5 s agitation in 80% ethanol; 10 s rinse in DI water. The excised flush was then placed in a petri dish lined with moistened filter paper to provide humidity. The dishes were kept in a Percival Incubator (Fisher Scientific, Waltham, MA) at 28ºC and 80% RH. They were checked for nymph emergence daily. Flush with newly hatched nymphs was placed onto flush of a new, uninfected C. macrophylla seedling. The excised flush was held in place with a small amount of Elmer’s glue at the cut end of the flush. A plastic cup with holes for airflow was overturned over the seedling to provide a lid, and the pot was returned to the incubator. The seedling was checked daily until all nymphs had crawled onto the new plant. The old flush was removed, and a screen mesh was secured over the pot as described previously.

The third treatment evaluated Las transmission from the oviposition site and transovarial transmission (OVTR). Eggs laid by infected females were allowed to hatch and develop on the flush where they were deposited.

Seedlings were enclosed in insect-proof mesh sleeves following oviposition treatments and held in an insectary at 26-29ºC and 60-80% RH with an 18:6h L:D photoperiod. The nymphs were reared to adults, then collected in 80% ethanol and held at -80ºC for subsequent nucleic acid preparation. Replicates replicate with fewer than five adults were not included in subsequent analyses.

**DNA isolation from psyllid samples.** All D. citri (females and offspring) were processed individually. DNA was isolated using a DNeasy Blood & Tissue kit (Qiagen,
Valencia, CA) for preparation of nucleic acids, with modifications as described in previous studies (Pelz-Stelinski et al. 2010, Pelz-Stelinski and Killiny 2016).

**Pre-amplification of Las for nested-qPCR.** Psyllid offspring were tested for Las using nested-qPCR method developed by Coy et al. (2014). Nested-qPCR tests were conducted in triplicate for each sample. Samples were amplified with conventional PCR using 0.5 µM each of external Las primers (Ex-LasF: 5’-TGACGTTGGAAGATGTTTGTAGC-3’; Ex-LasR: 5’-ACGCAGGCTCATCTCTCTCC-3’), 0.2 µM dNTPs, 0.03U/µL TaKaRa Taq™ DNA Polymerase (TaKaRa Bio USA, Inc., Mountain View, CA) in 2 µL Mg+ Plus 10x Buffer, and 1 µL of template DNA, in a total reaction volume of 20 µL. Amplifications conditions consisted of two cycles at 94 °C for 90 s, 62 °C for 45 s, 72 °C for 20 s, 34 cycles at 94 °C for 30 s, 62 °C for 45 s, 72 °C for 20 s, and a final extension of 72 °C for 3 min. The PCR products were diluted to 1:100 in 0.5 mL microcentrifuge tubes with Nanopure water for use in subsequent quantitative real-time polymerase chain reactions (qPCR).

**Quantitative PCR.** The diluted PCR products from the nested-qPCR protocol described above were tested for Las via qPCR. ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) was used for qPCR assays. A 25 µL reaction was prepared containing 0.2 µM each of Las primers (LasF: 5’-TCGAGCGCTATGCGAATAC-3’; LasR: 5’-GCGTTATCCCGTAGAAAAGGTAG-3’) and probe (5’/-56-FAM/AGACGGGTAGTAACGCG/3BHQ_1/-3’) in PerfeCTa qPCR ToughMix, Low ROX (Quanta Biosciences, Beverly, MA). Reactions contained 1 µL DNA with 24 µL of master mix. Each plate included a non-template control (Thermolyne Nanopure Infinity Ultrapure Water System D8981), a known Las-positive sample, and a known Las-negative sample. All qPCR reactions were performed in duplicates. The qPCR routine was set to: 50 °C for 2 min, 95 °C for
10 min, 40 cycles at 95 °C for 15 sec, and 60 °C for 1 min. Psyllid offspring were considered positive if at least one of the three nested-qPCR tests produced a Ct<19.

**Las quantification in female *D. citri.*** Detection and quantification of Las in maternal psyllids was conducted using a plasmid-based standard curve for Las and the psyllid wingless gene (Wg). Plasmids were created for both genes using the pGEM®-T Easy Vector System (Promega Corporation, Madison, WI). The Las plasmid was ligated with the Las PCR product, using the Las primers described for qPCR. The Wg plasmid was ligated with Wg PCR product (WgF: 5’- GCTCTCAAAGATCGGTTTGACGG-3’; WgR: 5’-GCTGCCACGAACGTTACCTTC-3’) using the same procedure. Ligated plasmids were extracted from high-efficiency competent cells using the Qiagen DNeasy Blood and Tissue kit. Extracted plasmid DNA was cut with PstI (New England Biolabs, Ipswich, MA) and quantified with a NanoDrop 2000 (Thermo Scientific, Wilmington, DE) and diluted to a starting concentration of 0.05 ng/µL. Five ten-fold serial dilutions were made to create a six-point standard curve. The curve for Las and Wg was included on each qPCR plate. qPCR reactions remained the same as described previously, except for the addition of 0.2 µM each of Wg primers and probe (WgP: 5’/-56-JOEN/TTACTGACCATCATCCTGGACGC/3BHQ-2/-3’). Plates were rerun if the R² of either of the standard curves was less than 0.98.

The amount of Las DNA and Wg DNA in each female psyllid (ng/µl) was determined using the ABI 7500 Real-Time PCR System software. Las and Wg quantities were converted to copy numbers per microliter using the DNA quantification equation described in previous publications (Whelan et al. 2003, Dossi et al. 2014). Las and Wg copy numbers were averaged between duplicate qPCR reactions for each sample. The average Las copy number divided by the average Wg copy number was used to calculate the Las titer per psyllid. The average Las/Wg
copy number per sample was transformed as: \( \log_{10}(x+1) \), where \( x \) was the average Las/Wg copy number. This normalized the data set and eliminated errors from zeros.

Female psyllids were ranked into three categories based on their Las titer: 1) \( \text{Ct}<25 \) and \( \log(\text{Las/Wg}) < -1 \) (high); \( \text{Ct} \) between 25 and 35, and \( \log(\text{Las/Wg}) \) between -1 and -5 (medium); \( \text{Ct}>35 \) and a \( \log(\text{Las/Wg}) > -5 \), or any female which defaulted to zero due to an undetermined Ct (low) (Fig. 2-1).

**Statistical analysis.** ANOVA with post-hoc multiple comparisons (Tukey’s HSD test) was used to compare the average percent of Las-positive psyllids between the three treatments.

The mean acquisition rate in nymphs was compared to the maternal inoculation source to determine if the Las titer in the female psyllid correlated with the successful acquisition in psyllids nymphs. High-, medium-, and low-ranked females were compared within the OVTR replicates via an ANOVA with post-hoc multiple comparisons (Tukey’s HSD test). The other two treatments were not subjected to this comparison due to inadequate replication of females in each rank category.

**Results**

Las-exposed females produced an average of 53.8%, 18.9%, and 70.0% Las-positive offspring in OA, TO, and OVTR treatments, respectively (Fig. 2-2). TO offspring acquired Las less frequently than OA offspring (\( F_{2,23}=7.191, P=0.046 \)) and OVTR offspring (\( F_{2,23}=7.191, P=0.003 \)).

Females in the OVTR treatment group with low Las titers had significantly fewer Las-positive offspring as compared with females that had high (\( F_{2,6}=16.47, P=0.004 \)) and medium (\( F_{2,6}=16.47, P=0.007 \)) Las titers. Female Las titer did not influence transmission in OA and TO treatments (Table 2-1).
Discussion

This is the first study to use nested-qPCR to study transmission interactions between *D. citri* and small amounts of Las. This work confirms that *D. citri* can transmit Las vertically, and the nested-qPCR reveals a higher rate of transovarial transmission than previously reported by Pelz-Stelinski et al. (2010). However, *D. citri* that fed on plants exposed to the infected female acquired Las more frequently. The combination of transovarial transmission and acquisition from the oviposition site (OVTR) did not yield more Las-positive offspring than only feeding on the oviposition site (OA). These data further suggests that transmission through infected plant material is the primary pathway of Las between vector and host.

These results highlight the capacity of individual females to transmit Las to her offspring within 5-7 days. She accomplishes this through transovarial transmission and by immediately inoculating her oviposition site with Las. In a field setting, maternal transmission may be enhanced by the presence of multiple psyllids on the plant flush. Lee et al. (2015) conducted a simulation of the spread of Las under field-like conditions which confirmed that young flush became Las-positive within 15 days of exposure to Las-positive *D. citri*. The ability of psyllids to transmit Las through plant flush is critical when considering how Las manipulates the behavior and biology of its vector. Psyllids infected with Las are more likely to spread to healthy host plants than *D. citri* with undetectable Las titers (Martini et al. 2015). Infected psyllids also experience increased oviposition and population growth rates (Pelz-Stelinski and Killiny 2016). This suggests that infected host plants are not only unnecessary for transmission, but that Las promotes *D. citri* to disperse and reproduce on healthy plants. My study compliments these previous works by confirming the ability of *D. citri* to spread the bacteria to developing nymphs through healthy plant flush. In these experiments, transmission occurred when Las was still undetectable by standard qPCR or visual assessments. This further supports that *D. citri*
transmitted Las throughout Florida and the southern United States several years before the
disease was diagnosed (Halbert 2005, Manjunath et al. 2008).

Only the OVTR treatment produced enough females in each ranking to quantify the
effect of maternal Las infections on transmission. Results from the OVTR assay indicate an
affect of maternal Las titer on nymph acquisition; however, the data strongly suggest that Las
transmission is able to occur regardless of Las titer in the female. Conducting replicates with
females from healthy colonies would confirm that Las is constantly present in \textit{D. citri} in very
low amounts. Coy et al. (2014) reported 98\% of psyllids to test positive for Las using the nested-
qPCR protocol, compared to 43\% of psyllids testing positive with the standard qPCR protocol
used to detect Las. This study further suggests the presence of Las in \textit{D. citri} has been vastly
underestimated. Understanding this baseline infectivity would allow further comparison between
psyllids and plants and their contribution of Las to the transmission cycle. Future research on Las
transmission will test the ability of psyllids with low Las titers to inoculate healthy citrus. If
plants develop HLB following exposure to \textit{D. citri} with low or undetectable levels of Las, this
would be strong evidence that the baseline titers of Las in \textit{D. citri} are sufficient to sustain Las
until it is inoculated into a suitable plant host.

In conclusion, this study highlights the difficulty of controlling the spread of Las.
Alternative transmission pathways, such as horizontal and vertical transmission, allow \textit{D. citri} to
transmit Las to new hosts even when infected plant sources are scarce. This explains the rapid
spread of HLB throughout Florida. These data suggest that flushing sites are crucial components
of the transmission cycle, and underscore the importance of psyllid management during flushing
seasons.
Figure 2-1. Ct value of maternal psyllid versus the log transformation of Las copy number per Wg copy number for the same female. Red circle indicates females with a Ct<25 and log(Las/Wg) < -1 (high). Green dotted circle indicates females with Ct between 25 and 35, and log(Las/Wg) between -1 and -5 (medium). Purple dashed circle indicates females with Ct>35 and a log(Las/Wg) > -5, or any female which defaulted to zero due to an undetermined Ct (placed at 40) (low).
Figure 2-2. Mean (±SE) proportion of Las-positive offspring for all females in each treatment. Treatments with difference letters are significantly different according to Tukey’s Honestly Significant Difference (HSD) test (α=0.05). Transmission treatments: oviposition site acquisition (OA); transovarial acquisition (TO); oviposition site and transovarial acquisition (OVTR).
Table 2-1. Mean Las-positive offspring per female (%) in high, medium, and low rankings for each treatment. Letters indicate significantly different means (within OVTR only).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Maternal Las titer rank</th>
<th>n</th>
<th>Mean Las-pos offspring</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>OA</td>
<td>High</td>
<td>4</td>
<td>56.0%</td>
<td>17.1%</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>4</td>
<td>50.0%</td>
<td>14.0%</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>1</td>
<td>60.0%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>1</td>
<td>28.6%</td>
<td></td>
</tr>
<tr>
<td>TO</td>
<td>Medium</td>
<td>1</td>
<td>13.3%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>6</td>
<td>18.3%</td>
<td>9.2%</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>3</td>
<td>93.3% a</td>
<td>6.7%</td>
</tr>
<tr>
<td>OVTR</td>
<td>Medium</td>
<td>4</td>
<td>80.0% a</td>
<td>9.8%</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>2</td>
<td>15.0% b</td>
<td>5.0%</td>
</tr>
</tbody>
</table>
CHAPTER 3
EFFECT OF THERMOTHERAPY ON THE ACQUISITION OF \textit{CANDIDATUS LIBERIBACTER ASIATICUS} BY THE ASIAN CITRUS PSYLLID, \textit{Diaphorina citri}

\textit{Candidatus} Liberibacter asiaticus (Las) is a gram-negative, phloem-limited alphaproteobacterium which is the putative causal agent of citrus greening disease, or Huanglongbing (HLB) (Bové 2006). This disease is responsible for the most severe damage to citrus groves worldwide. Symptoms of this disease include blotchy, mottled leaves, premature fruit drop, reduced fruit size, and bitter, misshapen fruits. The tree produces less marketable fruit every year until it ultimately becomes unproductive within seven to ten years (Gottwald 2010).

Current management strategies for HLB focus on the propagation of young nursery stock, maintaining tree health, removing inoculum sources (infected trees), and area-wide insecticide sprays to control \textit{D. citri} (Brlansky and Rogers 2007, Rogers and Shawer 2007, Qureshi et al. 2009). Insecticides are an attractive option due to the immediate mortality rate they provide, and the use of these chemicals has the benefit of simultaneously controlling other insect citrus pests such as leaf miners and root weevils. However, the use of insecticides has many negative impacts on the environment, and developing alternative control methods is necessary in order to reduce the use of these chemicals and the development of resistance among insect populations (Gottwald 2010).

While these treatments minimize the dispersal of Las, even a single psyllid can transmit the pathogen. Despite rigorous efforts to reduce psyllid abundance, since its introduction to the United States in 2005, it has spread throughout the citrus-growing regions in Texas and California (French et al. 2001, Kumagai et al. 2013). \textit{D. citri} are highly mobile, especially during spring and summer months, and can transmit the bacteria rapidly throughout a grove even when Las incidence is low (Gottwald 2010, Lewis-Rosenblum et al. 2015). A citrus tree can take 1-2 years before displaying symptoms of HLB. During this asymptomatic phase, the infected citrus
can serve as an inoculum source for *D. citri*. Psyllids can also inoculate the flush upon which they oviposit, thus providing an immediate source of Las for the nymphs which develop there (Lee et al. 2015). In addition, *D. citri* infected with Las disperse more frequently and for longer distances than uninfected psyllids, which further increases the rate of Las transmission to new plant hosts (Martini et al. 2015).

Once a tree is infected, there are few options for growers to treat the bacteria directly to improve the production and lifespan of the tree. Long-term HLB management will rely on the development of resistant citrus varieties. Some resistance has been observed in the *Citrus* genus (Ramadugu et al. 2016), but the use of these lines in resistant crosses is still under investigation. RNA interference (RNAi) is also an appealing option for HLB management. Non-virulent strains of *Citrus tristeza virus* (CTV), another phloem-limited citrus pathogen, can be engineered as a vector to induce the expression of RNAi silencing genes targeting psyllid development (Hajeri et al. 2014). While promising, the research into transgenic and resistant varieties is many years away from commercial application. It will also require replanting susceptible citrus trees, which will be expensive and time-consuming.

The citrus industry is in need of short-term solutions to combat HLB. A crisis declaration in March 2016 permitted the use of antibiotic foliar applications for use in Florida under the Emergency Exceptions provisions of FIFRA. Studies have shown antibiotic treatments to significantly lower Las titers in trees (Zhang et al. 2011, 2013). While these treatments provide temporary relief from HLB, the use of antibiotics raises many public concerns, such as the potential for antibiotic resistance to develop, or the unknown effects on human health.

A new technique referred to as “thermotherapy” has been explored due to the heat sensitivity of Las (Lopes et al. 2009). Under laboratory conditions, Las-infected trees exposed to
temperatures of 40–42°C for 48 hours experienced a reduction in pathogen titers to low or undetectable levels (Hoffman et al. 2012). Multiple studies have shown the efficacy of thermotherapy, including some combination treatments with heat and antibiotics (Ehsani et al. 2013, Al-jumaili and Ehsani 2015, Yang, Powell, Duan, Shatters, Fang, et al. 2016, Yang, Powell, Duan, Shatters, Lin, et al. 2016). However, the most efficient and effective method for applying thermotherapy under field conditions is still undetermined. This study continues the exploration of thermotherapy as a control method for HLB by measuring the titer of Las in leaf tissue over the course of a year. The thermotherapy treatment involved lowering a heat-resistant tarp over an individual tree and using steam to raise the internal temperature of the enclosure for an allotted amount of time.

In addition, there is no research showing how thermotherapy affects the vector efficiency of *D. citri*. The trees could still serve as a source of inoculum if Las is present, even if it is not detectable (Gottwald 2010). *D. citri* can acquire Las in as little as 5 hours after feeding on infected citrus (Halbert and Manjunath 2004). *D. citri* which feed as nymphs are far more likely to acquire Las and subsequently inoculate new trees (Inoue et al. 2009, Pelz-Stelinski et al. 2010, Ammar et al. 2016). Thus, thermotherapy may disrupt the Las available to *D. citri* and inhibit acquisition. Alternatively, heat-stressed trees may be unable to defend itself against re-inoculation from psyllids, allowing Las to multiply rapidly and provide new inoculum to psyllids.

The goal of this study was to investigate the effect of thermotherapy on the transmission of Las between plants and *D. citri*. I hypothesized that reductions in the titer of Las in trees receiving thermal treatment should be associated with a reduction of Las acquisition by *D. citri*. 
Las titers in the leaf tissue of thermotherapy-treated trees were monitored over the course of one year. I also measured the rate of pathogen acquisition in both adult and nymph *D. citri* over time.

**Materials and Methods**

**Field Study**

**Grove site.** Thermotherapy trials were conducted in a research grove located in Lake Alfred, FL (28.130996, -81.71710). Three-year-old sweet orange trees (*Citrus sinensis*) were screened for Las status using qPCR. Twenty-four Las-positive trees (Ct<36) and 10 Las-negative trees (Ct>36 or undetectable) were selected for the experiments. The site was regularly mowed, but no other pest control measures (i.e. pesticides or biological control agents) were conducted during the course of this study. The trees were not harvested for fruit during the study.

A second thermotherapy trial was prepared at a field site in Lake Wales, FL (27.962312, -81.586095) with 22 Las-positive trees (11 untreated positive controls; 11 thermotherapy-treated) and 11 Las-negative trees. However, frequent pesticide applications prevented use of the Lake Wales site for psyllid assays; thus, this site was discontinued from further study. All subsequent methods were conducted at the Lake Alfred site.

**Thermotherapy methods.** Twelve Las-positive trees were randomly selected to receive thermotherapy treatment (THERM). The remaining 12 Las-positive trees (POS) and 10 Las-negative trees (NEG) remained untreated. The thermal treatments were applied per methods described by Al-jumaili and Ehsani (2015). Briefly, the tree canopy was enclosed in a canvas tent, and the interior of the tent was heated with pressurized steam until the temperature reached 55°C. This temperature was maintained for 30 seconds. A second treatment of thermotherapy was applied to the same THERM trees (minus one tree which had died) at 153 days after the first treatment (Table 3-1).
Las acquisition in uninfected adult *D. citri*. Uninfected adult *D. citri* used in acquisition assays were collected from a Las-free colony maintained on *Citrus sinensis* (L.) Osbeck, *Swingle citrumelo*, *Murraya paniculata* (L.) Jack, and *Citrus macrophylla* at 26-29°C and 60-80% RH on an 18:6h L:D photoperiod. Psyllids are tested for Las every 2-3 months to confirm Las-free status.

Adult psyllid acquisition trials were conducted as described by Pelz-Stelinski (2010). Briefly, 25-30 adult *D. citri* from a Las-free colony were enclosed on tree shoots with fine nylon mesh sleeve cages for 9-day acquisition access periods (AAPs). Following the 9-day AAP, a maximum of 10 psyllids were collected and stored in 80% ethanol at -80°C for subsequent Las detection.

This assay was completed once prior to thermal treatments, and every 2-3 months post initial treatment for one year, resulting in a total of 4 collections periods (Table 3-1). The same branch was used for subsequent acquisition assays on the tree, when possible; alternatively, a branch close to the original branch site was used.

Las acquisition in *D. citri* during nymph development. The second psyllid acquisition assay tested the number of Las-positive *D. citri* in the natural population emerging from the trees. Trees were periodically examined for flush infested with *D. citri* nymphs. These branches were bagged, and a maximum of 10 psyllids were collected 2-3 weeks later, or when adults emerged. This assay was conducted every 2-3 months post-treatment for one year, resulting in 4 collection periods (Table 3-1). Tree shoots were chosen according to nymph presence and were not consistent between collection periods.

DNA isolation from psyllid samples. *D. citri* were processed individually. DNA was isolated using the “Blood & Tissue” DNeasy kit (Qiagen, Valencia, CA) for extraction of nucleic
acids, with modifications as described in previous studies (Pelz-Stelinski et al. 2010, Pelz-Stelinski and Killiny 2016).

**Pre-amplification of Las for nested-qPCR.** *D. citri* acquire Las less efficiently during the adult stages, and previous acquisition studies have shown <15% of adults will test positive for Las after an AAP lasting less than 14 days (Pelz-Stelinski et al. 2010, Ammar et al. 2016). Thus, the nested-qPCR protocol designed by Coy et al. (2014) was used to test *D. citri* from adult acquisition assays. *D. citri* collected from nymph acquisition assays were not pre-amplified, and were tested for Las with the qPCR assay described in the next section.

Samples were pre-amplified with conventional PCR using 0.5 µM each of external Las primers (Ex-LasF: 5’-TGACGTTGGAAGATGTTTGTAGC-3’; Ex-LasR: 5’-ACGCAGGCTCATCTCTCTCC-3’), 0.2 µM dNTPs, 0.03U/µL TaKaRa Taq™ DNA Polymerase (TaKaRa Bio USA, Inc., Mountain View, CA) in 2 µL Mg+ Plus 10x Buffer, and 1 µL of template DNA, in a total reaction volume of 20 µL. Thermal cycler routine was set to: 2 cycles at 94 °C for 90 s, 62 °C for 45 s, 72 °C for 20 s, 34 cycles at 94 °C for 30 s, 62 °C for 45 s, 72 °C for 20 s, and a final extension of 72 °C for 3 min. The PCR products were diluted to 1:100 in 0.5 mL microcentrifuge tubes with Nanopure water for use in subsequent quantitative real-time polymerase chain reactions (qPCR).

**Quantitative PCR.** The diluted PCR products from the nested-qPCR protocol described above, or DNA collected from nymph acquisition assays, were tested for Las via qPCR. ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) was used for qPCR assays. A 25 µL reaction was prepared containing 0.2 µM each of Las primers (LasF: 5’-TCGAGCGCGTATGCGAATAC-3’; LasR: 5’-GCGTTATCCCGTAGAAAAAGGTAG-3’) and probe (5’-/56-FAM/AGACGGGTAGTAACGCG/3BHQ_1/-3’) in PerfeCTa qPCR ToughMix,
Low ROX (Quanta Biosciences, Beverly, MA). Reactions contained 1 µL DNA with 24 µL of master mix. Each plate included a non-template control (Thermolyne Nanopure Infinity Ultrapure Water System D8981), a known Las-positive sample, and a known Las-negative sample. All qPCR reactions were performed in duplicates. The qPCR routine was set to: 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min.

**Leaf tissue sampling.** Three leaves were sampled from the tree at the time of each psyllid collection. Leaves were selected from the same branch that psyllids were caged upon. Leaf samples were stored at -80ºC until subsequent DNA processing.

**DNA isolation from leaf tissue.** Midribs were cut from leaf samples and 100mg of chopped plant tissue was subsampled for DNA processing. DNeasy Plant Kits (Qiagen) were used for DNA isolation, with modifications described previously (Li et al. 2006, Pelz-Stelinski et al. 2010, Pelz-Stelinski and Killiny 2016). Isolated plant DNA was quantified to nanograms per microliter with a NanoDrop 2000 (Thermo Scientific, Wilmington, DE).

**Las quantification in plant samples.** Las detection in plant tissue was conducted using the same qPCR reagents and protocol as described previously. To quantify the relative amount of Las in each sample, plant DNA was first diluted to 10 ng/µl based on Nanodrop data. A plasmid-based standard curve for Las was developed using the pGEM®-T Easy Vector System (Promega Corporation, Madison, WI) with the Las PCR product using the qPCR primers. Ligated plasmids were extracted from high-efficiency competent cells using the Qiagen DNeasy Blood and Tissue kit. Extracted plasmid DNA was quantified with a NanoDrop 2000 and diluted to a starting concentration of 0.05 ng/µL. Five ten-fold serial dilutions were made to create a six-point standard curve. This curve was included on each qPCR plate. Plates were rerun if the $R^2$ of the standard curve was less than 0.98.
**Statistical analyses.** Psyllids tested with qPCR were considered positive if Ct<36; psyllids tested with nested-qPCR were considered positive if Ct<19.

The percentage of Las-positive *D. citri* was calculated per replicate, and the average percentage was calculated per treatment group for each collection period. An analysis of variance (ANOVA) with post-hoc multiple comparisons (Tukey’s HSD test at *p*<0.05) was used to compare the mean percentage of Las-positive *D. citri* between the three treatment groups for each collection period.

The amount of Las DNA in plant tissue (g/µl) was determined using the ABI 7500 Real-Time PCR System software. Las copy numbers per microliter were calculated using the DNA quantification equation described in previous publications (Whelan et al. 2003, Dossi et al. 2014). Las copy number was averaged between the two qPCR duplicates run for each sample. The average copy number per sample was transformed as: $\log_{10}(x +1)$, where *x* was the average copy number. This normalized the data set and eliminated errors from zeros.

The transformed Las copy number in leaf tissue was averaged per treatment group in each sampling block. An analysis of covariance (ANCOVA) was used to compare the effect of the treatment group on the Las titer in leaf tissue over time.

Linear regression and Pearson’s correlation co-efficient was conducted to determine if the mean psyllid acquisition correlated with mean Las titer in leaf tissue for each treatment group at each sampling period. All statistical tests were conducted in R Studio with R script (the R Foundation for statistical software R; Vienna, Austria).

**Whole-Tree Thermotherapy**

**Plant materials.** Potted Valencia (30 trees, approx. 36 in tall) were placed in screen cages with Las-exposed *D. citri* obtained until a systemic Las infection developed in the trees (approx. 4 months). *D. citri* were collected from 1) a laboratory colony in which psyllids are
cultured on Las-positive *Citrus sinensis* (L.) Osbeck, *Swingle citrumelo*, and *Citrus macrophylla*, maintained at 26-29°C and 60-80% RH on a 16:8h light schedule; 2) Las-infected *C. sinensis* trees in a research grove located in Lake Alfred, FL (28.130996, -81.71710). Leaves were sampled from each tree and screened with qPCR prior to treatment to establish initial Las infection status.

**Psyllid materials.** Adult *D. citri* used in whole-tree thermotherapy (“WTT”) assays were collected from our Las-free laboratory culture described previously.

**Thermotherapy methods.** Fifteen Las-positive trees (Ct<36) were randomly selected for treatment with WTT (“Twtt”). Trees were heated in a mobile steaming chamber (6x6x10’) at 40°C (108°F) for 48 hours. After treatment, trees were sprayed with Magna-Bon CS (250ppm) to reduce secondary fungal infection. Fifteen Las-positive trees remained untreated and served as positive controls (“Pwtt”). An additional fifteen trees, which were maintained in an insect-free greenhouse and were never exposed to Las, were used as negative controls (“Nwtt”).

After treatment, all 45 trees were held in screen cages, with 6-8 trees from the same treatment group to a cage. Cages were placed in an environmentally-controlled greenhouse at 26-29°C and 60-80% RH on a 16:8h L:D photoperiod.

**Psyllid acquisition assays.** Four weeks post-treatment, 10-20 adult *D. citri* from the Las-free colony were released into each cage of 6-8 trees and allowed to reproduce. An additional 20-25 adult *D. citri* were released into each cage the following week. Trees were pruned and fertilized every 2-3 weeks to promote flushing and oviposition. Adult psyllids were collected from each cage at 11 weeks post treatment.

**Las in leaf tissue.** Three leaves were collected from each tree prior to thermotherapy treatment and at 6 weeks post treatment, for a total of 2 sampling periods.
DNA isolation from leaf and psyllid tissues. All DNA extractions were processed as described for leaves and psyllids previously.

Las quantification in plant tissue. Because plant Las titers were much lower in WTT trials, diluting the DNA samples yielded many “Undetermined” results. Thus, an alternate method was conducted which allowed the normalization of Las quantities between samples without need for diluting the DNA.

A plasmid-based standard curve was created for the plant cytochrome oxidase gene (Cox), a common housekeeping gene, using the same methods described previously, with the exception that a Cox-specific primer set (CoxF: 5’-GTATGCCACGTCGATTCCAGA-3’; CoxR: 5’-GCCAAAACTGCTAAGGCGATTC-3’) was used. The standard curve for Las and Cox was included on each plate in triplicate. qPCR reactions remained the same, except for the addition of 0.2 µM each of Cox primers and probe (CoxP: 5’/-/56-JOEN/ATCCAGATGCTTACGCTGG/3BHQ-2/-3’).

Las detection in D. citri. Adult D. citri collected from cages were tested for Las via the standard qPCR (non-nested) protocol as described previously.

Statistical analyses. D. citri tested with qPCR were considered positive if Ct<36. The percentage of Las-positive psyllids was calculated per cage (2 cages per treatment group). Statistical tests were not conducted on psyllid acquisition due to low replication.

The amount of Las/Cox DNA in plant tissue (g/µl) was determined using the ABI 7500 Real-Time PCR System software. Las/Cox copy numbers per microliter were calculated using the DNA quantification equation described in previous publications (Whelan et al. 2003, Dossi et al. 2014). Las copy number divided by Cox copy number provided the amount of Las DNA.
relative to the amount of plant DNA. The mean Las/Cox copy number was transformed as: 

$$\log_{10}(x + 1)$$, where \(x\) was the average copy number.

Due to non-normality of data, the transformed Las/Cox copy numbers in leaf tissue were tested with Kruskal-Wallis rank sum tests for each sampling period.

All statistical tests were conducted in RStudio with R script (the R Foundation for statistical software R; Vienna, Austria).

**Results**

**Field Study**

**Adult acquisition.** In 9-day AAP adult acquisition assays, significantly more *D. citri* tested positive for Las on POS trees than NEG trees for A2 (\(F_{2,27}=8.323, P=0.003\)), A3 (\(F_{2,29}=3.194, P=0.047\)), and A4 (\(F_{2,28}=4.617, P=0.014\)). Adult acquisition was also higher on THERM trees than NEG trees for A2 (\(F_{2,27}=8.323, P=0.007\)) (**Fig. 3-1**).

**Nymph acquisition.** *D. citri* collected from POS and THERM trees had significantly higher acquisition than NEG trees for N3 (\(F_{2,15}=6.32\), \(P=0.008\), \(P=0.045\), respectively). No other significant differences were observed in nymph acquisition assays (**Fig. 3-2**).

Psyllid acquisition was not significantly different between THERM and POS for any collection period in adult or nymph acquisition assays.

**Las quantification in leaf tissue.** When all three treatments (NEG, POS, THERM) were tested for an effect on Las titer over time, ANCOVA showed a significant treatment on Las quantity over time (\(F_{2,225}=2.96, P=0.05\)). A subsequent ANCOVA was conducted which compared only POS and THERM treatments. These results showed no significant effect of treatment on Las quantity over time (\(F_{1,154}=0.024, P=0.88\)) (**Fig. 3-3**).

**Psyllid acquisition correlation with Las titer in leaves.** There was a significant increase in adult acquisition as the mean Las titer in leaves increased (\(R^2=0.45\); Pearson’s
correlation co-efficient = 0.67, F=8.279, P=0.0165) (Fig. 3-5). This trend was also observed in nymph acquisition when compared to Las titer in leaves (R²=0.35; Pearson’s correlation co-efficient = 0.59, F=5.315, P=0.044) (Fig. 3-4).

Whole-Tree Thermotherapy

**Las titer in leaves.** The Las/Cox titer in leaves was not significantly different between treatment groups for the pre-treatment sampling period (Chi-squared=3.85; P=0.1462) or the 6-week post-treatment sampling period (Chi-squared=2.33; P=0.3125).

**Psyllid acquisition.** No *D. citri* tested positive for Las in Nwtt or Twtt cages. In the two Pwtt cages, an average of 75% of *D. citri* tested positive for Las (Table 3-2).

**Discussion**

This study represents the first to present data on how field applications of thermotherapy affect psyllid acquisition of Las from *Citrus* trees. The acquisition data are supplemented with measurements of Las in leaf tissue over time, which did not show a reduction in Las titer. In addition, the WTT trial with potted citrus showed psyllid acquisition occurring in the untreated Las-positive trees, while *D. citri* in thermal-treated trees did not test positive. The replication in this trial was low, and further studies are needed to confirm the efficacy of WTT on psyllid acquisition; however, the data indicate that WTT reduces psyllid acquisition. The field trial did not provide the same reduction in psyllid acquisition. This suggests that treating the above-ground portion of the tree at 55ºC for 30 seconds is not adequate to replicate the efficacy of thermotherapy observed in potted trials (Hoffman et al. 2012).

The percentage of *D. citri* acquiring Las from treated trees did not decrease relative to untreated trees in the field study. Psyllid acquisition rates followed similar patterns as leaf titer, in which the mean adult and nymph acquisition rates correlated to the mean Las titer in leaves (Figs. 3-4, 3-5). Thus, psyllid acquisition is directly related to and affected by the Las titer in the
trees. This suggests the trees still serve a source of inoculum for *D. citri* to acquire and spread Las.

The field study required psyllid populations to survive to collect acquisition data, therefore the grove used in this research was not managed for insect pests. It is possible that psyllids were able to re-inoculate the tree faster than the effects of thermotherapy could be observed. However, a healthy tree remains asymptomatic for 6 months to 2 years after initial inoculation (Gottwald 2010), and assessments in this study were every 2-3 months after treatment. Thus, it is unlikely that the efficacy of thermotherapy would be obscured by the amount of Las inoculated by wild psyllid populations. In addition, residual dead bacteria in the tree may result in higher calculations of Las titer (Trivedi et al. 2009). While the effect of dead bacterial fragments was also noted by Hoffman et al. (2012), significant titer reductions were observed 30 days post treatment in their study. This research did not find a significant decrease in Las 119 days after the first treatment, nor 164 days after the second treatment.

There are several challenges to overcome before thermotherapy can succeed on a commercial scale. Currently it is only possible to treat the above-ground portion of the tree in the field, and so the root system remains infected with Las. Because trees in the field are much larger than the potted varieties used in laboratory tests, it is currently not known what optimal temperature and time of exposure is necessary to achieve the same results (Ehsani et al. 2013). Furthermore, it is difficult to control the application of thermotherapy under field conditions. Enclosing single trees under a plastic tarp is an alternative thermotherapy technique which relies on a greenhouse effect to raise internal temperatures of the enclosure. However, this method is weather dependent, and can take 6-7 hours to complete the treatment (Ehsani et al. 2013). Thus, mobile heat treatments such as the one used in this study are under development to allow better
control of heat and time (Al-jumaili and Ehsani 2015). While the ability to customize the conditions of thermotherapy is desirable, this presents further challenges. A manned vehicle has time limitations for efficient operation and cannot be left unsupervised in the field. Additionally, longer treatment times would increase the cost per tree. This complicates the improvement of the heat/time ratio since the treatment time necessary to produce efficacy may be cost prohibitive.

The efficacy of thermotherapy in combination with other control techniques, such as antibiotic injections, may improve its application in HLB management in the field. Studies have shown that chemo-thermotherapy decreases Las more than thermotherapy treatments in potted trees (Yang et al. 2016). This has not yet been tested under field conditions.

In conclusion, further research is required to optimize thermotherapy application in field conditions. While this study showed no effect of thermotherapy in an unmanaged grove, its efficacy in a managed grove with low psyllid populations has yet to be determined. In addition, thermotherapy may improve the efficacy of chemical treatments in the field, which has the potential to prolong the production life of a tree. This could provide temporary relief to growers until a resistant citrus variety is available.
Table 3-1. Field trial: Dates of thermotherapy treatments, date ranges for psyllid acquisition assay collection periods, and associated abbreviations.

<table>
<thead>
<tr>
<th>Start date</th>
<th>End date</th>
<th>Event</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>5/20/15</td>
<td>6/4/15</td>
<td>Adult acquisition 1</td>
<td>A1</td>
</tr>
<tr>
<td>7/14/15</td>
<td>7/14/15</td>
<td>Thermotherpay treatment 1</td>
<td>TT1</td>
</tr>
<tr>
<td>8/28/2015</td>
<td>8/28/2015</td>
<td>Adult acquisition 2</td>
<td>A2</td>
</tr>
<tr>
<td>8/12/2015</td>
<td>9/18/2015</td>
<td>Nymph acquisition 1</td>
<td>N1</td>
</tr>
<tr>
<td>11/5/2015</td>
<td>11/5/2015</td>
<td>Adult acquisition 3</td>
<td>A3</td>
</tr>
<tr>
<td>11/10/2015</td>
<td>11/24/2015</td>
<td>Nymph acquisition 2</td>
<td>N2</td>
</tr>
<tr>
<td>12/14/2015</td>
<td>12/14/2015</td>
<td>Thermotherpay treatment 2</td>
<td>TT2</td>
</tr>
<tr>
<td>2/1/2016</td>
<td>2/1/2016</td>
<td>Nymph acquisition 3</td>
<td>N3</td>
</tr>
<tr>
<td>3/17/2016</td>
<td>3/17/2016</td>
<td>Adult acquisition 4</td>
<td>A4</td>
</tr>
<tr>
<td>5/26/2016</td>
<td>6/24/2016</td>
<td>Nymph acquisition 4</td>
<td>N4</td>
</tr>
</tbody>
</table>
Table 3-2. Percentage of Las-positive *D. citri* collected from whole-tree thermotherapy trials. Each cage contained 6-8 trees of the same treatment group.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cage number</th>
<th>Percentage of Las-positive <em>D. citri</em></th>
<th>n psyllids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermal treatment</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0%</td>
<td>6</td>
</tr>
<tr>
<td>Positive control</td>
<td>1</td>
<td>60%</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>90%</td>
<td>10</td>
</tr>
<tr>
<td>Negative control</td>
<td>1</td>
<td>0%</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0%</td>
<td>9</td>
</tr>
</tbody>
</table>
Figure 3-1. Mean percent (±SE) of lab-reared adult *D. citri* in each treatment group which tested positive for Las via nested-qPCR following a 9-day AAP on trees. An ANOVA was conducted separately for each sampling period to compare means between treatment groups. Different letters indicate significant differences between treatments within a sampling period (Tukey’s HSD, α=0.05).
Figure 3-2. Mean percent (±SE) of feral adult *D. citri* (bagged onto tree branches during nymphal stages) which tested positive for Las via qPCR. An ANOVA was conducted separately for each sampling period to compare means between treatment groups. Different letters indicate significant differences between treatments within a sampling period (Tukey’s HSD, α=0.05).
Figure 3-3. Mean Las titer (±SE) in trees for each treatment group on the first date leaf samples were collected for each sampling period.
Figure 3-4. Mean nymph acquisition (±SE) compared to mean titer (±SE) of Las in leaf tissue. Means calculated per treatment group (3), per sampling period (4). y = 0.1197x + 0.2898
Figure 3-5. Mean adult acquisition (±SE) compared to mean titer (±SE) of Las in leaf tissue. Means calculated per treatment group (3), per sampling period (4). $y = 0.1138x + 0.1237$
CHAPTER 4
SUMMARY AND CONCLUSIONS

It is critical to have a deeper understanding of how a pathogen interacts with its vector and host to develop methods for disrupting disease spread. This research further illustrates how D. citri are able to acquire Las, and how this complicates HLB management. These data show that a single psyllid can provide an inoculum source for developing nymphs through a combination of vertical and horizontal transmission. In addition, my field study confirms that psyllids acquire Las from trees which test negative for Las with qPCR, which is consistent with previous studies (Lee et al. 2015). Thus, my research presents further evidence that D. citri are efficient vectors which are capable of spreading Las before it is detectable in the plant or the psyllid.

Acquisition during the nymphal stages is crucial to the proliferation of Las. Bacterial replication is enhanced and psyllids are more likely to inoculate new hosts as adults when they acquire Las as nymphs (Inoue et al. 2009, Pelz-Stelinski et al. 2010, Ammar et al. 2016). In this research, nymphs which were exposed to plant tissue inoculated by a single female psyllid were more likely to acquire Las than through transovarial transmission alone. This is consistent with previous research which found transovarial transmission to occur at a much lower rate (2-6%) than acquisition via feeding on infected plant material (>60%) (Pelz-Stelinski et al. 2010). This demonstrates how healthy plants contribute to the transmission cycle prior to symptom development. It also emphasizes the challenge of controlling Las spread. Previous works showed that a single psyllid is able to transmit the bacteria to a new plant host (Xu et al. 1988, Gottwald 2010, Ammar et al. 2013). Here I present research which shows a single psyllid can also transmit Las to nymphs developing on plant flush.
This rapid rate of transmission makes HLB management extremely challenging. Once a tree is inoculated, it becomes a reservoir for Las. The thermotherapy experiment showed that as Las titer in the trees increase, the number of psyllids which acquire Las from the trees also increase. Thus, the inoculated tree contributes more infected psyllids to the population as the disease progresses. In addition, the tree experiences reduced fruit production until it eventually dies (Gottwald 2010). Pesticide sprays can reduce vector populations, but overuse of pesticides disrupt IPM practices and cause outbreaks of secondary pests such as mites (Grafton-Cardwell 2015). Frequent pesticide use can also lead to the development of resistant psyllid populations (Tiwari et al. 2011). Thus, there is a need for potential short-term solutions for growers to minimize the impact of HLB while long-term strategies, such as resistant citrus varieties or transgenic psyllids, are being researched.

My research on thermotherapy sought to determine the efficacy of the heat treatments currently in use to combat HLB. While the data presented here shows no significant effect of the treatment in the field, the potential use of heat to reduce HLB symptoms should not be dismissed. The data I collected on whole-tree thermotherapy is consistent with previous work which demonstrated efficacy (Hoffman et al. 2012), and this research also shows reduced numbers of Las-positive psyllids on treated trees compared to untreated trees. Thus, whole-tree thermotherapy is a viable solution for mitigating HLB in potted citrus. Further research is needed to determine the optimum heat to time ratio needed to reduce bacterial titers in the field. This treatment may also be more effective in combination with other treatments and management practices (Yang, Powell, Duan, Shatters, Lin, et al. 2016).

The research presented here provides additional evidence for how D. citri transmit Las swiftly through healthy citrus groves. Further research is needed to determine how transmission can
be disrupted to prevent the spread of Las. My data suggest these efforts should focus on Las acquisition by nymphs via inoculated plant tissue. I have also shown that the use of thermotherapy in the field requires further development before it can be implemented as an effective component of IPM programs to minimize the impact of HLB.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Alicia Joy Kelley graduated from Purdue University in 2010 with a Bachelor of Science in entomology. In 2011, she joined the team at i2LResearch USA, Inc. and worked as a Study Director for three years, conducting pesticide efficacy studies on urban insect pests. She then moved to Florida to pursue a graduate degree under the guidance of Dr. Kirsten Pelz-Stelinski. Alicia graduated with a Master of Science in Entomology and Nematology in 2016, and then transitioned into the Doctor of Plant Medicine program at the University of Florida.