SEASONAL AND LONG-RANGE MOVEMENT OF ASIAN CITRUS PSYLLID, *DIAPHORINA CITRI*

By

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To Dr. Ralph Ibe

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LIST OF ABBREVIATIONS

AAP	Acquisition access period
ACP	Asian citrus psyllid
Cq	Cycle Quantification
ELISA	Enzyme-linked immunosorbent assay
HLB	Huanglongbing
IAP	Inoculation access period
MRR	Mark-release-recapture
OD	Optical Density
PCR	Polymerase chain reaction

Abstract of Thesis Presented to the Graduate School
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SEASONAL AND LONG-RANGE MOVEMENT OF ASIAN CITRUS PSYLLID,
DIAPHORINA CITRI

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Asian citrus psyllid (ACP), *Diaphorina* citri Kuwayama, is a vector of the pathogen that is the presumed causal agent of huanglongbing (HLB), or citrus greening disease. While previous studies have shown that *D. citri* disperse from unmanaged into managed groves, it is not yet clear how seasonality and vector infection status may affect these movement patterns. Although it has been speculated that *D. citri* have the capacity for long-range movement, there has been a lack of supporting data. A novel *in situ* marking technique was used to track the movement of *D. citri* under normal field conditions.

The current results are consistent with previous research indicating that *D. citri* disperse from unmanaged into managed groves. In Florida, this movement was found to be greatest during the spring and summer months, decreasing significantly during the colder months (Sept.-March). *Candidatus* Liberibacter asiaticus (LAS) was found to be present in both citrus trees and *D. citri* adults in unmanaged Florida citrus groves at rates that were comparable to those found in managed groves. Las-infected *D. citri* adults dispersed at least 400 m over four days; from inner rows of unmanaged grove plots to inner rows of managed groves. These unmanaged citrus groves acted as

reservoirs of the bacterium that causes huanglongbing, its vector, and also served as sources of *D. citri* infestation and potential Las infection for nearby commercial groves. *D. citri* traveled at least 2000 m within 12 d regardless of vector infection status. These results directly confirm that the approximately 57,650 ha of abandoned citrus groves in Florida (USDA 2009) are negatively impacting commercial citrus production in the state.

CHAPTER 1
LITERATURE REVIEW

Asian citrus psyllid (ACP), *Diaphorina* citri Kuwayama, is an efficient vector of the pathogen that is the presumed causal agent of huanglongbing (HLB), or citrus greening disease. *D. citri* is an important pest of citrus. Described as early as 1907 in Shinchiku Taiwan, *D. citri* is currently a widely distributed species found in citrus producing regions throughout Asia, the Indian subcontinent, the Middle East, South and Central America, and, more recently, the United States (Halbert and Manjunath 2004). Considered a major pest of citrus in Asia since the mid-1930s, *D. citri* was first detected in south Florida in 1998 (Knapp et al. 2006). In 2002 it was predicted that Florida weather, host plant availability, and high fecundity would allow *D. citri* to spread throughout Florida's citrus producing areas by 2005 (Tsai et al. 2002). Currently, *D. citri* is established in at least 23 counties (Gottwald et al. 2007), which include all of the areas within the state where citrus is commercially produced (Michaud 2004). In the continental US, *D. citri* is now also present in Texas, Louisiana, California, and several other citrus producing states (Qureshi and Stansly 2009, USDA 2010).

Insect Description and Life Cycle

Oogenesis and Oviposition

Asian citrus psyllid females will only lay eggs on unfurled new flush (Rogers et al. 2008), with the first leaf having the highest egg density (Yang et al. 2006). Eggs are approximately 0.3 mm long, orange or bright yellow, and will hatch within 4-8 d (Tsai and Liu 2000). The maximum number of eggs that a single female can lay in a lifetime is temperature dependent. Under ideal conditions, 20-28°C, as many as 300-750 eggs may be deposited, over a lifespan of 30-50 d. The average female lifespan and

fecundity is reduced when temperatures are above 28°C (Tsai and Liu 2000). There is evidence that the lifespan of females is shortened if their “quota” of eggs is laid soon after eclosion, in contrast to the lifespan of females taking longer periods for egg laying (Yang et al. 2006). Nymphs feed on new leaf flush, so egg maturation and oogenesis coincides with the occurrence of this resource. Once the new flush is no longer suitable for egg laying, adult females will either remain on the host plants, feeding on mature leaves until the next new flush, or will go elsewhere to find a suitable host on which to oviposit (Rogers et al. 2010).

Nymphs

Under ideal temperature conditions, *D. citri* will complete all 5 instars within 11-15 d, which will range in size from 0.25 to 1.7 mm in length (Tsai and Liu 2000). Nymphs are somewhat sedentary, but there is evidence that aggregations of first instar nymphs are found on neighboring buds, and that there is a wider dispersal during the final instar, though they remain on immature leaves (Yang et al. 2006). Nymphs may be orange or green with large wing pads and possess circumanal glands which secrete a waxy substance in addition to honeydew excreted from the anus (Tsai and Liu 2000).

Adults

Adult *D. citri* are 3-4 mm in length. Wings have a distinctive pattern, characterized by dark bars across the tops and bottoms, resulting in an X-shaped pattern in the lateral view. When at rest or feeding *D. citri* alight on leaves at a 45º angle to the substrate (Halbert and Manjunath 2004). Adult *D. citri* may appear dusty, as they are covered with a whitish, waxy secretion (Knapp et al. 2006). Psyllids can be quite active at certain temperatures, jumping and taking short flights when disturbed. However, wind has been thought to be the main agent of dispersal, as psyllids are not thought to be strong flyers

(Yang et al. 2006). Adults are often found on south-facing foliage and other warm sites within the canopy (Yang et al. 2006). Reproductive maturity is reached within 2-3 d, and oviposition begins 1-2 d after mating (Wenninger and Hall 2008). Recent behavioral studies give evidence that a volatile sex-attractant pheromone is produced by female *D. citri* (Wenninger et al. 2008). In Florida, the lifespan of *D. citri* ranges between 15 and 47 d, which allows for the possibility of up to 30 overlapping generations per year (Knapp et al. 2006). Outbreaks of *D. citri* can occur anytime during the year, depending on the availability of new flush and favorable environmental factors (Hall et al. 2008).

Host Plants

Potential hosts for *D. citri* and *Ca*. Las include most citrus types and some close relatives, including at least two species of *Murraya*; the ornamental orange jasmine, *Murraya paniculata* (Knapp et al. 2006) and *M. exotica* (Damsteegt et al. 2010). Other plants in the Rutaceae family such as curry leaf tree, *Bergera koenigii*, may be suitable hosts for *D. citri* but not *Ca*. Las (Damsteegt et al. 2010). In the absence of new citrus flush, the presence of untreated *D. citri* host plants (especially *M. paniculata,* which has continual flushes) may allow for the preservation of high psyllid populations (Hall 2008).

Damage

When occurring in high populations, psyllids can cause substantial direct damage to new plant growth from oviposition, feeding, and honeydew production (Michaud 2004). However, *D. citri* has been called the most important insect pest of Florida citrus because of its role as a vector of the presumed causal agent of huanglongbing (HLB) (Rogers et al. 2008).

Management

Biological Control

There are quite a few insect predators of *D. citri*, including predators belonging to the families Anthocoridae, Blattellidae, Syrphidae, Chrysopidae, Miridae, Coccinellidae, and Formicidae (Qureshi and Stansly 2009). *Tamarixia radiata* (Hymenoptera: Eulophidae), an ectoparasitoid of *D. citri*, was imported from Taiwan and south Vietnam, and intentionally released in Florida to help regulate the *D. citri* populations (Michaud 2002). The parasitism rate in Florida on *D. citri* has been documented as seasonally variable, with an average of <20% during the spring and summer months, increasing up to 56% in November (Qureshi et al. 2009).

Chemical Control

Currently, intense use of insecticides is the most effective management strategy for *D. citri*. Most effective are soil-applied systemic insecticides such as imidacloprid or thiamethoxam (Childers and Rogers 2005), along with foliar applications of broad-spectrum insecticides (Rogers et al. 2010). Aldicarb (Temik, Bayer CropScience, Research Triangle PK, NC), which was widely used in the past two years, has been recently discontinued due to a restriction imposed by the U.S. Environmental Protection Agency. With these methods there are concerns about the development of insecticide resistance, and also of the potential harmful effects on beneficial insects.

Systemic insecticides

Imidacloprid and Thiamethoxam. The neonicotinoid insecticides imidacloprid and thiamethoxam may be applied as foliar applications or soil-drenches. Seasonal use restrictions on these insecticides limit the feasibility of drenching in solid blocks to young

trees of ≈1.8 m in height, with tree size determining the number of possible applications per year.

Broad-spectrum insecticides

Foliar applications of broad-spectrum insecticides (organophosphates and pyrethroids) have proven effective in reducing *D. citri* populations, especially when applied to trees prior to flushing and corresponding oviposition. Targeting adult *D. citri* in their overwintering stage should greatly reduce populations during the spring flushes, reducing the need for insecticide applications that would negatively impact pollinators during bloom. *D. citri* populations should be monitored, and additional applications should be made when necessary, as well as prior to or just after hedging and topping before the development of new flush (Rogers et al. 2010).

Huanglongbing

Huanglongbing is one of the most destructive and economically important diseases of citrus throughout the world (Halbert and Manjunath 2004, Manjunath et al. 2008). HLB was first detected in Florida in 2005 (Halbert 2005). The HLB infection rate in Florida is estimated to be 1.6%, with higher infection rates in the southern and eastern parts of the state (Morris et al. 2009). Infected trees show symptoms of fruit drop, off-season bloom, and twig dieback. Fruit from infected trees is often small, misshapen, and bitter tasting (Halbert and Manjunath 2004). Once infected, mature trees die back and become unproductive, often dying within five to eight years (Knapp et al. 2006). Young trees may die within one to two years and, thus, may not survive long enough to become productive (Brlansky et al. 2008). It has been projected that at the current rate of disease spread, all Florida citrus plantings will be infected within 6-12 years (Stover et al. 2008).

Presumed Causal Agent

HLB may be caused by any one of three bacteria in the genus *Candidatus* Liberibacter; *Ca.* L asiaticus (Las), *Ca*. L. africanus (Laf), and *Ca.* L americanus (Lam). These bacteria may be transmitted by two psyllid species; *D. citri* and the African citrus psyllid, *Trioza erytreae* (del Guercio) (Manjunath et al. 2008). In Florida, HLB is believed to be caused by the more heat tolerant *Candidatus* Liberibacter asiaticus (Las), transmitted by *D. citri*. This fastidious gram-negative, phloem limited bacterium can be transmitted to host plants through feeding (Bové 2006).

Disease Transmission

There are conflicting reports about the time required for successful transmission of *Ca.* Las by *D. citri*. Initial research, that based confirmation of transmission on visual symptoms, reported a minimum required feeding time of 15 to 30 min for successful pathogen acquisition, a latency period of 8-12 days within the insect, and a feeding period of 15 min to 1 h required for inoculation. These initial results also suggested that *D. citri* retain the ability to infect throughout their lifespan, and demonstrated a lack of vertical (transovarial) transmission (Capoor et al. 1974). More recent research has utilized quantitative real-time PCR (qPCR) to confirm the presence of Las in both *D. citri* and plant tissue (Hung et al. 2004, Inoue et al. 2009, Pelz-Stelinski et al. 2010). While there continue to be discrepancies between transmission results, it has been suggested that these might be attributable to inherent differences in the tested populations of *D. citri*, or to variations in Las-detection assays (Pelz-Stelinski et al. 2010). Current research from *D. citri* populations in Florida shows a positive correlation between acquisition of the pathogen and feeding time, no significant difference in successfully inoculated plants after being fed on by infected *D. citri* for periods between 1 and 24 d,

and evidence of a low rate (3.6%) of vertical transmission (Pelz-Stelinski et al. 2010). Acquisition of the greening pathogen is possible beginning in the fourth or fifth instar (Inoue et al. 2009).

Disease Management

Currently, there is no cure for HLB. Management programs for HLB include using disease-free planting materials, removal of infected trees and aggressive management of *D. citri* (Halbert and Manjunath 2004). In addition, maintaining productivity of infected trees by application of supplemental micronutrients is being investigated (Spann et al. 2010).

Monitoring insect movement

The development of insect marking techniques has made it possible to track the movement of insects in their natural habitat. In mark-release-recapture (MRR) studies, markers are applied to insects that have been collected from the field or from laboratory colonies. Marked insects are then released into the field where they may eventually be recaptured on traps or in collecting devices and distinguished from the rest of the population. Mark-recapture studies use markers that can be applied to insects directly in the field (Hagler and Jackson 2001). Markers should be chosen based on ease of use, cost-effectiveness, and environmental safety, and should not adversely affect the insects' biology (Hagler and Jackson 2001). Marks may be applied to permit individual insects to be identified from within a population, or to groups of insects to allow for their identification within a larger population. Current insect marking techniques include the application of tags, paint, ink, dust, dye, and trace-elements, as well as creating marks through mutilation, pollen marking, genetic marking, and immunomarking (Hagler and Jackson 2001).

Protein marking

Recently developed techniques allow for inexpensive applications of crude food proteins as insect markers which can be detected by enzyme-linked immunosorbent assays (ELISA) (Jones et al. 2006). Protein marking techniques using milk, egg, and soy proteins are an effective way to mark insects *in situ* (Jones et al. 2006, Boina et al. 2009). Markers can be applied in the field using standard spray equipment. Insects can be marked by direct contact during marker application or through contact with dried marker residue on leaves. Insects can then be captured with traps and subjected to an ELISA to see if they have acquired a particular marker protein (Boina et al. 2009). Knowing the marker status and trap location of an insect allows for the creation of an unambiguous map of insect movement in the field.

Justification

As *D. citri* has become quickly established in Florida (and now other citrus producing states in the US), it is obvious that there is the capacity for substantial dispersal. In 2010 the total estimated impact of the citrus industry on the economy of Florida was approximately $9 billion, accounting for over 76,000 jobs in the state. In many counties within central and south-central Florida, the citrus industry is the biggest driver of the economy (Spreen et al. 2006). Understanding movement patterns and dispersal behaviors of *D. citri* will be essential in creating optimal pest control strategies with the hope of curbing the spread of HLB, and protecting citrus production in Florida.

Goal and Hypotheses

The overall goal of this research was to investigate the seasonal and long range movement of *D. citri*. Within that framework, I focused on quantifying movement of *D. citri* from unmanaged groves into nearby commercial citriculture, as well as ascertaining

longer range movement capabilities of *D. citri*, and identifying whether or not those psyllids moving were infected with Las. I hypothesized that unmanaged groves act as breeding grounds or places of refuge for *D. citri*, which can then infest nearby managed groves that practice recommended psyllid control measures.

Specific Objectives

These studies were undertaken in an attempt to determine whether dispersing psyllids carry the HLB pathogen, as well as to investigate the long range movement capabilities of *D. citri*, and to quantify the monthly movement of adult *D. citri* from unmanaged into managed groves.

CHAPTER 2
INCIDENCE OF *CANDIDATUS* LIBERIBACTER ASIATICUS INFECTION IN UNMANAGED CITRUS OCCURRING IN PROXIMITY TO COMMERCIALLY MANAGED GROVES

Huanglongbing (HLB) is one of the most destructive and economically important diseases of citrus throughout the world (Halbert and Manjunath 2004, Manjunath et al. 2008). HLB disease is presumably caused by any of three species of fastidious phloem-inhabiting gram negative bacteria: *Candidatus* Liberibacter asiaticus (Las), *Ca*. L. americanus (Lam), or *Ca*. L. africanus (Laf) (Garnier et al. 1984, Jagoueix et al. 1996). The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), vectors Las in Asia and the Americas and Lam in Brazil, and the South African citrus psyllid, *Trioza erytreae* (Del Guercio), (Hemiptera: Psyllidae) vectors Laf in Africa.

HLB causes yield reduction, fruit drop, reduced fruit quality and, ultimately, tree death. Currently, there is no cure for HLB. Management programs for HLB include using disease-free planting materials, removal of infected trees and aggressive management of *D. citri* (Halbert and Manjunath 2004). The HLB infection rate in Florida is estimated to be 1.6%, with higher infection rates in the southern and eastern parts of the state (Morris et al. 2009). It has been projected that at the current rate of disease spread, all Florida citrus plantings will be infected within 6-12 years (Stover et al. 2008).

HLB spread within a citrus grove has been attributed to the movement of *D. citri* from infected trees to healthy trees (Gottwald et al. 1991a and b). Within a grove, the rate and range of HLB spread is directly dependent on the dispersal range of *D. citri*. Likewise, the spread of HLB from one grove to another is likely to be dependent on the dispersal range of *D. citri* (Halbert and Manjunath 2004). Movement of *D. citri* between unmanaged and managed groves has been found to be bidirectional; however, the

movement is biased, with more *D. citri* moving from unmanaged groves into managed groves (Boina et al. 2009). *D. citri* adults are able to disperse between unmanaged and managed groves separated by a distance of 60-100 m within 48 hours (Boina et al. 2009). However, the dispersal range of Las-infected *D. citri* between groves was previously unknown.

Unmanaged groves are not treated with insecticides or managed to limit the presence and spread of Las, which allows these groves to potentially serve as a continual source of *D. citri* infestation and HLB infection. Several agencies and scientific panels have assessed the risk created by unmanaged citrus located in the vicinity of commercial groves. These agencies have suggested that unmanaged groves may provide a continuous source of *D. citri* and Las, and have recommended possible removal and destruction of unmanaged citrus (DOACS 2006, USDA 2007, USDA 2009). One of the goals of these agencies has been to identify unmanaged citrus groves across the state. The area of unmanaged citrus in Florida increased 6.5% from 2008 to 2009, totaling approximately 57,650 ha (USDA 2008, Morris et al. 2009, USDA 2009). One of the major contributing factors for the increasing acreage of abandoned citrus is the escalating spread of this infection (Morris et al. 2009). Despite the increasing prevalence of unmanaged citrus, the incidence of Las infection in *D. citri* populations and citrus trees in these potential reservoirs has not been previously investigated. We predicted that Las infection in unmanaged citrus should be low due to tree decline. New leaf growth (flush), which is required for *D. citri* oviposition (Halbert and Manjunath 2004), is significantly reduced at these sites, which should result in little or no acquisition or spread of Las by developing *D. citri*. The objectives of this study were to

determine whether unmanaged citrus groves in Florida represent a significant source of Las inoculum, and to quantify the movement of Las-infected *D. citri* from unmanaged into managed grove plots.

Materials and Methods

To determine the potential impact of unmanaged groves on commercial citrus, we investigated seven pairs of unmanaged and managed groves in central Florida (Table 2-1). Each pair of unmanaged and managed groves was separated by approximately 100 m. Managed groves typically received 6-8 insecticide sprays per year, with routine mowing and other disease management programs. Unmanaged groves were completely devoid of irrigation, fertilization and pest management programs for at least 3 years.

DNA Extraction. Leaves were collected from each pair of unmanaged and managed groves during August 2009. Each grove was divided into four transects and 25 trees from each transect were selected based on visual HLB symptoms. Five leaves were selected from each tree (based on visible HLB symptoms), placed into airtight bags, and transported in a cooler to the laboratory. Petiole and midrib tissues from the leaves of 5 trees were combined, and subsequently 100 mg subsamples were ground under liquid nitrogen for DNA extraction. Petiole and midrib tissue was chosen because it contains substantial amounts of phloem, and Las is phloem limited. Total DNA was extracted from plant tissue using the DNeasy® Plant Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Samples were eluted in 50 µL buffer AE (Qiagen) and stored in sterile 1.5 mL microcentrifuge vials at -20°C for use in quantitative real-time polymerase chain reaction (qPCR) assays.

Approximately 100 *D. citri* adults were collected monthly from each grove described above during June, July, and August, 2009. Each grove was divided into four transects, and approximately 25 *D. citri* adults were collected from each transect using an aspirator. Adults were transported in a cooler to the laboratory, where they were stored individually within sterile 1.5 mL microcentrifuge tubes containing 80% ethanol at -20°C until DNA was extracted. Adults were pooled in groups of 10 for each grove and collection date, resulting in 10 samples for each site per collection date. Pooled adults were homogenized in a buffer solution (Qiagen, Valencia, CA, USA) using a sterile pestle and lysed overnight at 56°C in a hybridization oven (Model 136400, Boekel Scientific, Feasterville, PA) prior to extraction of total DNA. DNA was extracted from each batch using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's protocol, with modifications for the extraction of bacterial DNA from arthropods. Samples were eluted in 35 µL buffer AE and stored in a sterile 1.5 mL microcentrifuge tubes at -20°C for use in qPCR assays.

qPCR Assays. qPCR assays of *D. citri* and plant samples were performed in an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using a multiplex TaqMan® qPCR assay developed for detection of *Ca.* Las (Li et al. 2006). qPCR was chosen because of the inconsistent detection of Las in *D. citri* and plant tissues by conventional PCR and other methods (Halbert and Manjunath 2004, Li et al. 2006). Amplification of *D. citri* samples, conducted in duplicate, contained the following: 1 µL template DNA, 12.5 µL TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 235 nM each of target primers (LasF, 5' - TCGAGCGCGTATGCAATACG -3'; LasR, 5' –GCGTTATCCCGTAGAAAAAGGTAG -3')

(GenBank accession number L22532) (Li et al. 2006), internal control primers specific to the *wingless* (*wg*) gene (GenBank accession number AF231365) (WgF, 5'-GCTCTCAAAGATCGGTTTGACGG -3'; WgR, 5'-GCTGCCACGAACGTTACCTTC -3') (Thao et al. 2000) and 118 nM of each probe (WGp, JOE-5'TTACTGACCATCACTCTGGACGC3'-BHQ2) (Duan et al. 2009); HLBp, FAM-5'AGACGGGTGAGTAACGCG-BHQ1) (Li et al. 2006) (Integrated DNA Technologies, Inc., Coralville, IA). Similarly, qPCR amplification of plant samples, conducted in duplicate, contained the following: 1 µL template DNA, 12.5 µL TaqMan® Universal PCR Master, 218 nM each of target primers (LasF and LasR), internal control primers specific to the plant cytochrome oxidase (COX) gene (GenBank accession number CX297817) (CoxF, 5'-GTATGCCACGTCGCATTCCAGA -3' and CoxR, 5' – GCCAAAACTGCTAAGGGCATT -3') (Li et al. 2006), and 136 nM of each probe HLBp and COXp (JOE5'-ATCCAGATGCTTACGCTGG-3'BHQ2) (Li et al. 2006) (Integrated DNA Technologies, Inc., Coralville, IA). DNA amplifications were conducted in 96-well MicroAmp® reaction plates (Applied Biosystems, Foster City, CA). qPCR reactions consisted of 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Each 96-well plate containing *D. citri* samples included a no template control, a positive control (Las DNA in DNA extractions from *D. citri*) and a negative control (no Las DNA in DNA extractions from *D. citri*). Likewise, plates containing plant samples included a no template control, a positive control (Las DNA in DNA extractions from plant) and a negative control (no Las DNA in DNA extractions from plant). Samples were considered positive for *wg* gene, Las, or COX gene if the cycle

quantification (C_q) value determined by the ABI 7500 Real-Time software (Version 1.4, Applied Biosystems) was 35 or less.

Movement of Las-infected *D. citri* adults from unmanaged to managed grove plots. A field study was conducted to quantify the movement of Las-infected *D. citri* from unmanaged into managed grove plots. Unmanaged and managed grove plots were selected from a site that was also used for plant tissue and *D. citri* sampling. Unmanaged and managed grove plots were separated by a distance of approximately 100 m, and each consisted of ≈ 200 Valencia orange trees on 10 x 11 m spacing. Each plot was replicated four times with 40 m spacing between replicates (Fig. 2-1). The study was conducted three times, corresponding with the June, July and August collections of *D. citri*.

Protein Marking. *D. citri* adults in unmanaged plots were marked with protein *in situ* following the protein marking technique described by Boina et al. (2009). The two proteins used in this study were bovine casein (All Natural Whole Milk, Publix Super Markets, Lakeland, FL) and chicken egg albumin (All Whites, Papetti Foods, Elizabeth, NJ). Each protein was sprayed onto a specified row of trees in each unmanaged plot. The two rows defined for protein marking were edge and inner rows. Edge row was the first row of trees facing managed grove and the inner row was the row of trees that was approximately in the center of the grove (150 m from both ends). The edge row of each unmanaged plot was sprayed with a 20% dilution of whole milk in water, with Silwet L-77 (Helena Chemicals, Collierville, TN) added at the rate of 2,000 ppm. The inner row of each unmanaged plot was sprayed with a 10% dilution of egg white in water, with Silwet L-77 added at the rate of 2,000 ppm. These assignments were chosen at

random. The protein solutions were applied using a hand gun sprayer (model 5275016; Fimco Industries, North Sioux City, SD) at ≈ 250 psi (Boina et al. 2009). The spray was applied until there was visible leaf runoff. After the protein markers were applied, four Pherocon AM yellow sticky traps (Trécé, Adair, OK) were placed in both the edge and inner rows of each unmanaged and managed plot (Fig. 2-1). All traps were evenly spaced throughout their respective row and positioned in the tree canopy at a height of ≈ 2 m.

ELISA. Traps were removed four days after application of protein sprays. *D. citri* adults were removed individually from the traps using clean, disposable wooden toothpicks (Hearthmark, LLC, Muncie, IN) and placed into 1 mL protein extraction buffer (Tris-buffered saline, pH 8.0 and 0.5 g/liter tetrasodium ethylenediamine tetraacetic acid [EDTA]) (Sigma-Aldrich, St. Louis, MO) for 3-5 min in separate 1.5 mL centrifuge tubes. Adults were then removed from the buffer using clean wooden toothpicks and placed into new 1.5 mL centrifuge tubes containing 1 mL of 80% ethyl alcohol, and stored at -20°C for later analysis by qPCR. Trap location was recorded for each *D. citri* captured. Extracts from field collected *D. citri* were analyzed for both milk and egg proteins by using an indirect enzyme linked immunosorbent assay (ELISA) as described by Boina et al. (2009). Individual *D. citri* collected from traps in the managed plots that were positive for either protein were subjected to qPCR as described earlier for detection of Las. Uninfected *D. citri* adults, with no exposure to a protein marker, that served as controls for both assays were collected from a greenhouse culture maintained in Lake Alfred, FL and described in Wenninger et al. (2008).

Statistical Analyses. The incidence of Las-infected plant samples in unmanaged and managed groves was compared using a paired *t*-test ($P < 0.05$) (PROC TTEST, SAS INSTITUTE 2005). The mean number of Las-infected *D. citri* samples occurring in unmanaged and managed groves was compared using two-way analysis of variance (ANOVA) followed by Fisher's protected LSD mean separation test (PROC GLM; SAS Institute 2005). Grove type (managed and unmanaged) and month served as main effects.

Two-way ANOVA followed by Fisher's protected LSD mean separation tests, were used to compare the total number of protein-marked *D. citri* adults and the number of Las-infected *D. citri* adults moving from either row of unmanaged into managed plots. For each analysis, movement type (four levels) and month (three levels) served as main effects. Four possible outcomes of *D. citri* movement were included in the analysis: inner row of the unmanaged plots to the inner row of managed plots (1), inner row of the unmanaged plots to edge row of the managed plots (2), edge row of the unmanaged plots to the inner row of the managed plots (3) and edge row of the unmanaged plots to the edge row of the managed plots (4).

Results

Two hundred and eighty plant tissue samples collected from 1,400 trees belonging to seven pairs of unmanaged and managed groves were analyzed by qPCR. The mean Las infection rate in plant samples was numerically greater in unmanaged (3.57%) than managed groves (1.43%) (Fig. 2-2a); however, the difference was not statistically significant ($t = -0.93$, df = 12, $P = 0.3691$).

Four hundred and twenty *D. citri* samples comprising 4,200 total adults collected from 7 pairs of unmanaged and managed groves were analyzed by qPCR. The mean

Las infection rate in *D. citri* samples was numerically greater in unmanaged (1.90%) than managed (1.19%) groves (Fig. 2-2b); however, the difference was not statistically significant (F = 0.38; df = 1, 36; P = 0.5441). The mean (± SEM) number of Las-infected samples found in unmanaged and managed groves was higher during the month of August (1.00 ± 0.53, 0.29 ± 0.28, respectively) than June (0.14 ± 0.14, 0.14 ± 0.13) and July (0.00 ± 0.00, 0.29 ± 0.28); although month was not a significant main effect (F = 2.04; df = 2, 36; P = 0.1446). Las-infected *D. citri* adults were never collected from the same grove on more than one occasion, with the exception of one managed grove, where Las-infected *D. citri* adults were found during two consecutive sampling periods.

A total of 204, 281 and 29 *D. citri* adults were marked in the unmanaged plots and re-captured in the managed plots over a four-day period during June, July and August, respectively. Overall, the mean number of *D. citri* adults dispersing during June or July was significantly greater than during August (F = 5.02; df = 2, 9; P = 0.0344) (Fig. 2-3). The majority of recaptured *D. citri* moved from the inner rows of unmanaged plots to the edge rows of managed plots (Fig. 2-4). This occurred during each month. Las-infected *D. citri* adults comprised 2.9% percent of the total *D. citri* dispersing from unmanaged into managed plots. Most Las-infected *D. citri* moved from the inner row of the unmanaged plots to the inner row of the managed plots, followed by those moving from the inner row of unmanaged plots to the edge row of managed plots (Fig. 2-4).

Discussion

Candidatus Liberibacter asiaticus is present in both citrus trees and *D. citri* adults in unmanaged Florida citrus groves at rates that are comparable to those found in managed groves. Although trees in the unmanaged groves used in our study were in a

state of severe decline compared with those in the managed groves, they harbored comparable levels of Las infection. While fewer psyllids were found in unmanaged groves, our results indicate that the percentage of collected samples with *Ca*. Las inoculum tended to be slightly greater in unmanaged than managed groves. Las-infected *D. citri* adults dispersed at least 400 m over four days; from inner rows of unmanaged grove plots to inner rows of the managed groves. These results confirm that unmanaged citrus groves act as reservoirs of the bacterium that causes huanglongbing, its vector, and also serve as sources of *D. citri* infestation and potential Las infection for nearby commercial groves.

Results from the present study should assist regulatory agencies in deciding the fate of unmanaged citrus. Unmanaged citrus has not been considered a significant reservoir of HLB, given the limited growth of leaf flush on unmanaged trees. Despite limited habitat for egg laying and feeding, *D. citri* in these sites harbored Las inoculum levels comparable to those found in managed sites. Unmanaged groves harboring *D. citri* populations will likely require removal or control for successful area wide management of HLB in Florida. Management programs conducted on an area wide basis may also reduce short range movement of Las-infected *D. citri* and thus reduce the spread of the HLB pathogen.

Commercially managed citrus groves receive 6-8 insecticide applications per year (Srinivasan et al. 2008) and other routine maintenance, such as mowing and disease management sprays. Therefore, the biased movement of *D. citri* (Boina et al. 2009) from unmanaged into managed groves is likely due to the presence of more citrus flush in managed groves, which serve as oviposition sites and food for developing nymphs.

The short range movement of *T. erytreae* is also influenced by the availability of new flush (Catling 1969, and Samways and Manicom 1983). Immigrating Las-infected *D. citri* adults that settle and feed on new flush in managed groves could result in rapid multiplication of Las within healthy flushing trees. Multiplication of Las is greater on the distal end of citrus branches, especially where new growth occurs regularly (Teixeira et al. 2008).

The dispersal capabilities of *D. citri* have not been experimentally quantified; however, it has been speculated that *D. citri* can disperse from 90-145 km (Gottwald et al. 2007) up to 470 km (Sakamaki 2005). *D. citri* was found to disperse up to 100 m within 3 d between unmanaged and managed groves in our previous investigation (Boina et al. 2009). The above studies on the dispersal capabilities of *D. citri* have not distinguished between the movement of Las-infected and uninfected *D. citri*. At this point, it is unknown whether the presence of Las may alter the dispersal behavior of *D. citri* or whether presence of Las in host plants may alter the dispersal of *D. citri*. However, the psyllid, *Cacopsylla picta*, which vectors *Ca*. Phytoplasma mali in apple, is known to be attracted by the odor of phytoplasma-infected apple plants (Mayer et al. 2008). Determining the dispersal range capabilities of *D. citri*, specifically of Las-infected *D. citri*, is critical to thoroughly understand the potential impacts of unmanaged citrus on commercial citrus production. In addition, determining the effect of HLB infection on the dispersal behavior of uninfected and Las-infected *D. citri* should improve the understanding of this arthropod-pathogen interaction. Also, this information should help establish effective area wide management programs, determine useful

quarantine boundaries and develop guidelines for management or removal of unmanaged citrus groves.

Table 2-1. Location of unmanaged and managed citrus grove pairs sampled in central Florida for *Candidatus* Liberibacter asiaticus infection in *Diaphorina citri* and citrus trees.

Grove	City, County	Latitude	Longitude
1	Lake Alfred, Polk	N 28˚06.613′	W 81˚43.788′
2	Winter Garden, Orange	N 28˚28.451′	W 81˚38.498′
3	Lake Alfred, Polk	N 28˚05.656′	W 81˚43.350′
4	Lake Alfred, Polk	N 28˚06.978′	W 81˚43.868′
5	Kissimmee, Osceola	N 28˚07.402′	W 81˚42.952′
6	Groveland, Lake	N 28˚06.883′	W 81˚42.823′
7	Lake Alfred, Polk	N 28˚06.784′	W 81˚42.584′

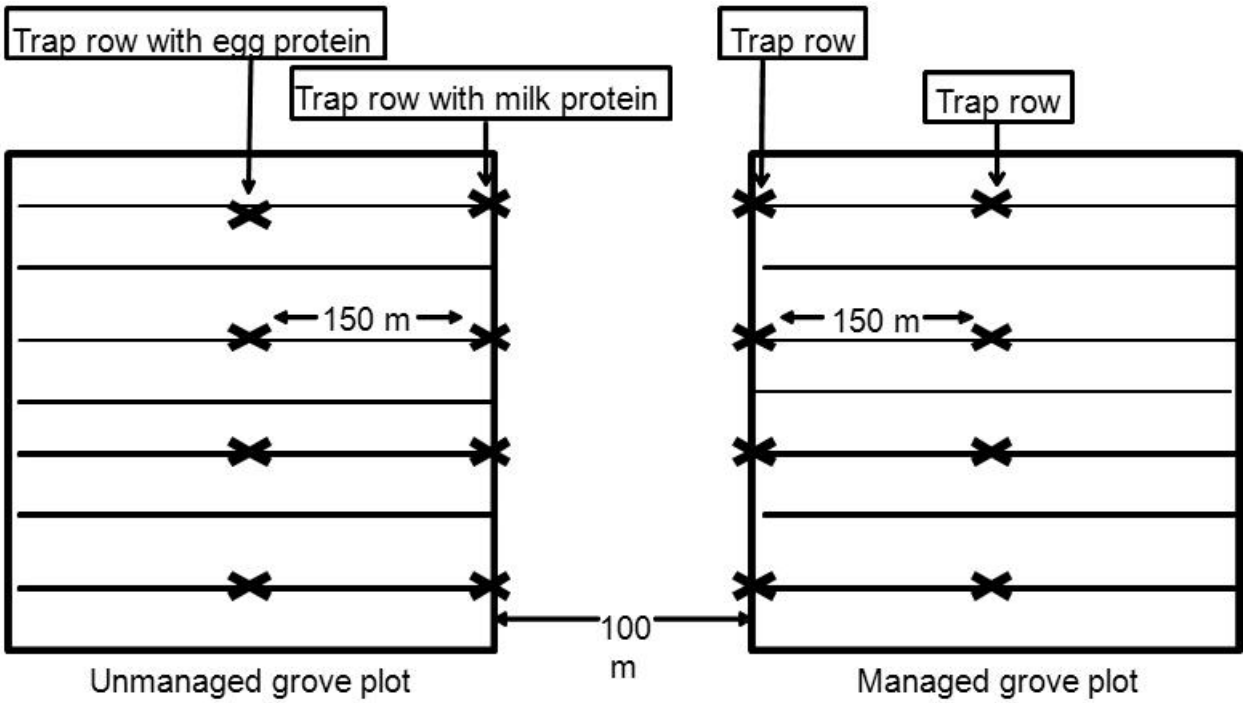

Figure 2-1. Schematic layout of a plot used to quantify the movement of Las-infected *Diaphorina citri* from an unmanaged into managed groves. In total, there were 4 sets of such plots, each plot set serving as a replicate.

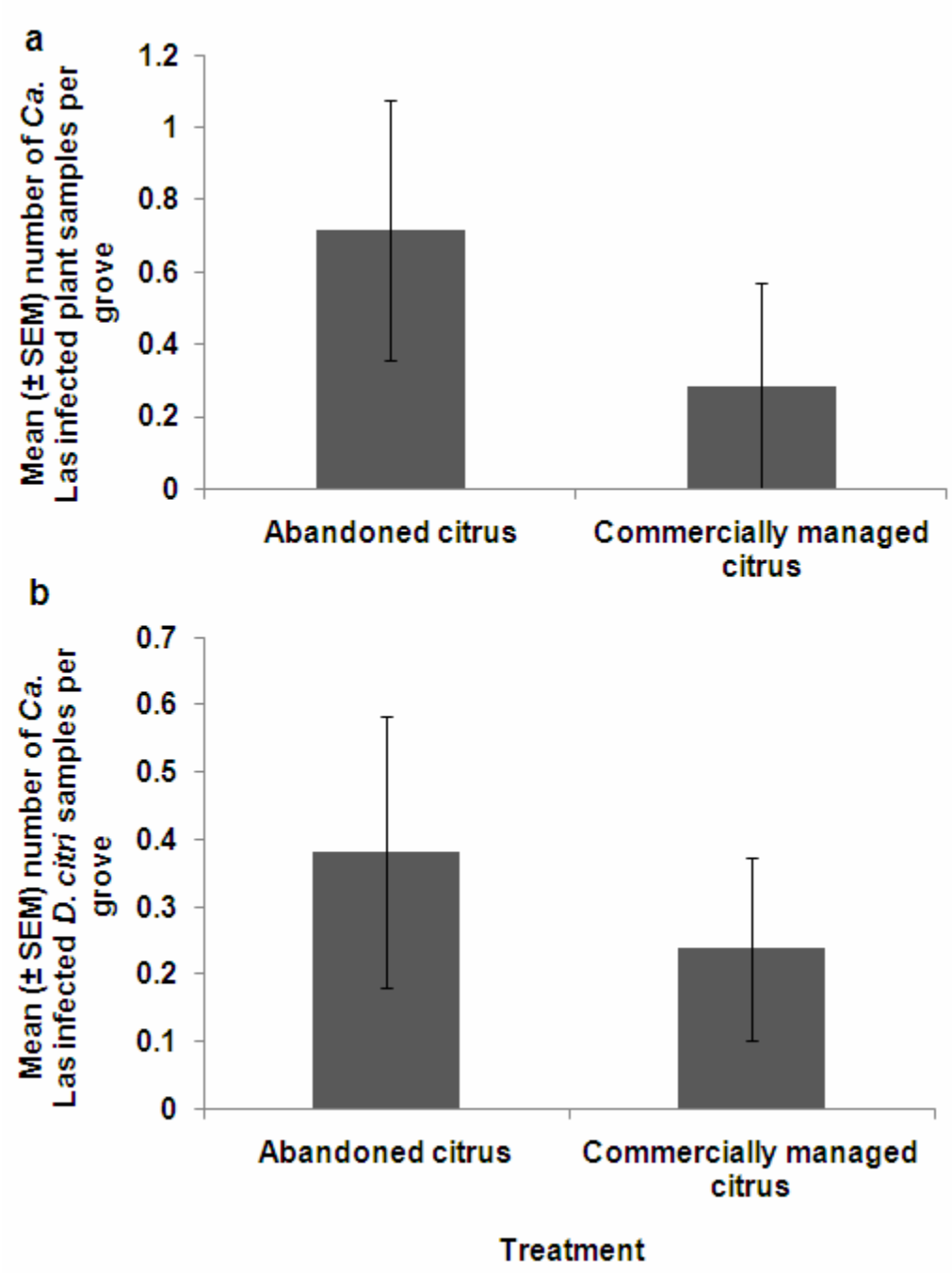

Figure 2-2. Mean (± SEM) number of *Candidatus* Liberibacter asiaticus infected tissue samples from seven pairs of unmanaged and managed citrus groves in central Florida. a) In plant tissue. b) In *Diaphorina citri* tissue. For plant tissue samples, 20 DNA samples were analyzed from each grove and each DNA sample consisted of pooled plant tissues from 5 trees. For *D. citri* samples, 10 DNA samples were analyzed from each grove and each DNA sample consisted of 10 *D. citri* adults.

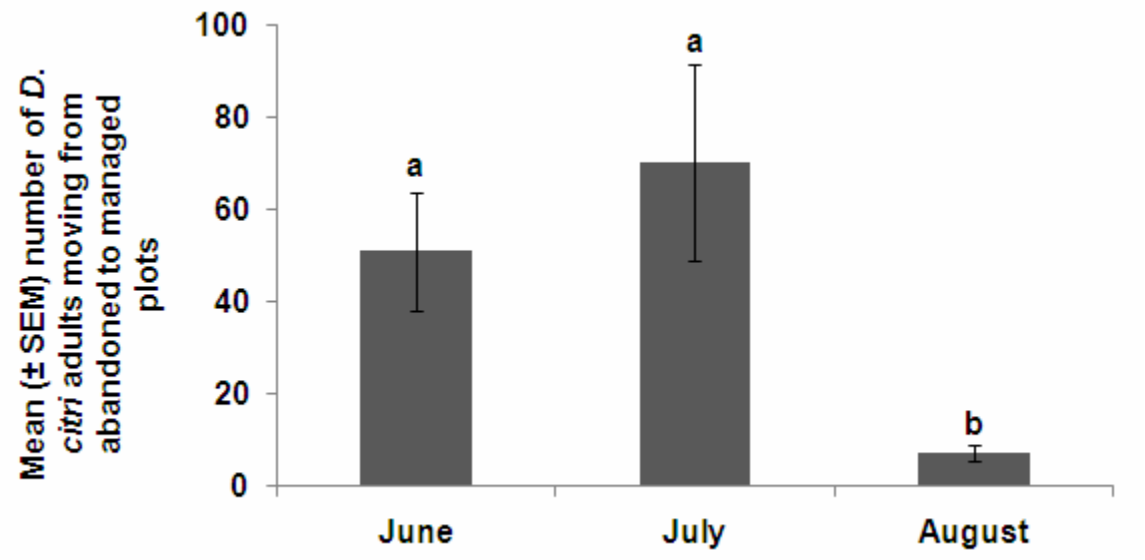

Fig. 2-3. Mean (± SEM) number of *Diaphorina citri* adults dispersing from unmanaged into managed grove plots during June, July and August.

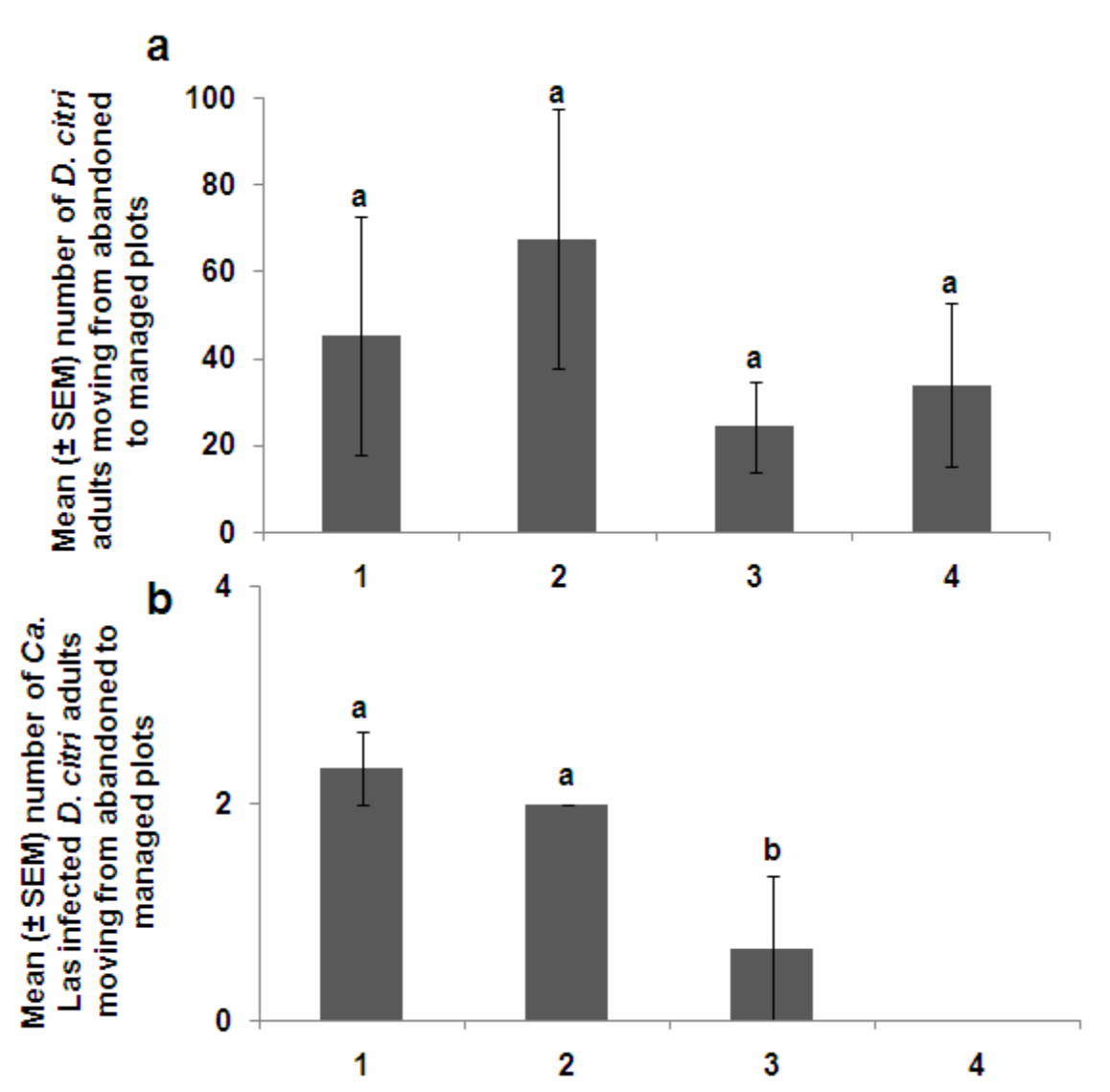

Fig. 2-4. Mean (± SEM) total number of marked *Diaphorina citri* (a) and mean number of *Candidatus* Liberibacter asiaticus-infected *Diaphorina citri* (b) found moving from unmanaged into managed citrus grove plots. 1 refers to the movement of adults from the inner row of the unmanaged to the inner row of the managed plots, 2 refers to the movement of adults from the inner row of the unmanaged to the edge row of the managed plots, 3 refers to the movement of adults from edge row of the unmanaged to the inner row of the managed plots, and 4 refers to the movement of adults from the edge row of the unmanaged to the edge row of the managed plots.

CHAPTER 3
POTENTIAL FOR LONG DISTANCE VECTOR MOVEMENT

Huanglongbing (HLB) is a devastating disease of citrus wherever it is found (Halbert and Manjunath 2004). HLB was first detected in Florida in 2005 (Halbert 2005) and as of February 2009, 33 counties have confirmed cases (DOACS 2009). Infected trees show symptoms of fruit drop, off-season bloom, and twig dieback. Fruit from infected trees is often small, misshapen, and bitter tasting (Halbert and Manjunath 2004). Tree death may eventually occur as a result of HLB infection (Gottwald et al. 2007).

Three species of fastidious, phloem inhabiting, gram negative bacteria are the presumed causal agents of HLB; Candidatus Liberibacter asiaticus (Las), *Ca.* L. americanus (Lam), and *Ca.* L. africanus (Laf). The Asian citrus psyllid, *Diaphorina* citri Kuwayama, (Hemiptera: Psyllidae), is believed to vector Las in Florida, transmitting the disease to host plants through feeding (Bové. 2006). Spread of the disease within citrus groves has been attributed to *D. citri* movement between infected and healthy trees (Gottwald et al. 1991a and b), and the dispersal range of *D. citri* directly affects the rate and range of HLB spread within a grove (Halbert and Manjunath 2004). It is assumed that HLB spread between groves is also dependent on the dispersal capabilities of *D. citri* (Halbert and Manjunath 2004).

It has been postulated that wind-assisted *D. citri* movement in Florida can range from 90-145 km (Gottwald et al. 2007). Sakamaki (2005) suggested that *D. citri* dispersal of up to 470 km throughout the Okinawan islands may have been mediated by lower jet airstreams. However, the actual dispersal capabilities of *D. citri* have not yet been experimentally quantified. Wind is thought to be the main agent of dispersal as

psyllids are not thought to be strong flyers (Yang et al. 2006). It is clear that maximum flight distance of *D. citri* needs to be estimated in order to identify safe isolation and quarantine boundaries as well as to establish area-wide control protocols (Halbert and Manjunath 2004, Hall et al. 2010).

A protein marking technique has been developed that allows for wild populations of *D. citri* to be marked *in situ* (Jones et al. 2006, Boina et al. 2009). Crude food proteins such as milk, egg, and soy can be applied in the field using standard spray equipment. The marker can be acquired either by direct contact during application or through contact with dried marker residue on leaves. Insects can then be captured with traps and subjected to an enzyme-linked immunosorbent assay (ELISA) to see if they have acquired a particular marker protein (Jones et al. 2006, Boina et al. 2009). Knowing the marker status and trap location of an insect allows for the quantification of insect movement in the field. The objective of this study was to experimentally quantify the dispersal capabilities of *D. citri* under normal field conditions in central Florida, identifying influencing factors such as predominant wind direction, flush availability, distance, and grove type (unmanaged vs. managed).

Materials and Methods

Protein Marking. A protein marking solution made up of 10% chicken egg albumin (All Whites, Papetti Foods, Elizabeth, NJ) in water with Silgard (Wilbur-Ellis, San Francisco, CA) added at 2000 ppm was applied to a block of 200 mature sweet orange trees in the central area of a well managed 40 ha grove in Lake Alfred, FL (N 28°07.053', W 81°44.003') using an ATV-mounted handgun sprayer (model 5275016; Fimco Industries, North Sioux City, SD) run at ≈250psi (Boina et al. 2009). Marker spray was applied at the rate of ≈7.5 L/tree.

Trapping. Eight yellow sticky traps (Pherecon AM, Trece, Adair, OK) were placed within the marked area; two each on the northern, eastern, southern, and western borders. Traps were also placed concentrically, radiating away from the marked area at distances of 100, 300, 400, 500, 650, 1000, 1200, and 2000 meters. At each distance one pair of traps was set up in five separate locations, with an attempt made to get good radial coverage (Fig. 3-1). Some traps placed at distances of 500 m and further from the marked area extended beyond the border of the grove in which the marker protein was applied; into nearby managed and unmanaged orange groves. Traps were removed 11 days after application of the marker protein. This experiment was conducted twice, once in June 2010, and again in July 2010.

Flush Abundance. Relative abundance of leaf flush was evaluated on the final day of the experiment in July 2010. A cubic square frame made up of PVC pipe and fittings (15.24 × 15.24 × 15.24 cm, 3.375dm³) was randomly placed into the tree canopy, and the number of flush shoots originating from within the square frame was counted (Hall and Albrigo 2007). At each trap site, the frame was randomly placed in the canopy of each of five adjacent trees twice, and the 10 numerical values were averaged to estimate relative flush abundance.

Wind Direction. Average daily wind direction during the experiment was collected from the Florida Automated Weather Network (FAWN) database with readings from a weathering monitoring station located within the 4 km diameter study area. The degree numbers provided by the database were categorized into eight components (N (338°-22°), NE (23°-67°), E (68°-112°), SE (113°-157°), S (158°-202°), SW (203°-247°), W (248°-292°), NW (293°-337°)). A compass graph was created and superimposed

over a satellite map of the study area and used to determine how many days a trap was downwind from the marked area.

Grove Type. Grove type was separated into three categories; unmanaged (no pesticide applications for at least three years), managed (areas where recommended *D. citri* control protocols are followed), and intensively managed (areas with young trees where insecticides are applied at the maximum allowable rate for *D. citri* control).

Indirect ELISA. Adult *D. citri* were individually removed from traps using disposable wooden toothpicks to minimize the risk of cross-contamination and placed in 1 mL protein extraction buffer (Tris-buffered saline, pH 8.0 + 0.3 g/L sodium ethylenediamine tetraacetic acid [EDTA]) (Sigma-Aldrich, St. Louis, MO) in 1.5 mL microcentrifuge tubes. Psyllids were removed from the extraction buffer after 3-5 minutes and placed into sterile 1.5 mL centrifuge tubes and preserved in 1mL 80% EtOH at -8°C for future use in PCR assays. Eighty µL of extraction buffer was removed from each microcentrifuge tube and placed in a single well of a 96-well microplate (Nunc Polysorp; Fisher, Pittsburgh, PA) to test for the presence of the egg protein mark. On each plate, 8 wells of 80 µL extraction buffer alone, and 8 wells of 80 µL extraction buffer from marker-free greenhouse psyllids served as blanks and negative controls, respectively. Eighty µL of a 0.001% dilution of each protein in TBS-EDTA was added to 3 wells as a positive control. Microplates were covered with aluminum foil and incubated for 2 h at 37°C. After this incubation, each plate was washed 5 times with 300 µL phosphate-buffered solution (PBS; Sigma-Aldric, St. Louis, MO) pH 7.4 + 0.09% Triton X-100 (PBST; Sigma-Aldrich) per well. Washing was accomplished with a microplate washer (Wellwash 4 Mk 2, Thermo Electron Corporation, Vantaa, Finland). After this

wash, 300 µL of blocking solution (StartingBlock, Pierce Biotechnology, Rockford, IL) was added to each well, followed by 1 h incubation at 37°C. Plates were then washed once with 300 µL PBST, and 80 µL of appropriately diluted primary antibody (Rabbit anti-chicken Egg Albumin, Sigma-Aldrich) diluted in StartingBlock with 0.05% Tween-20 (37539; Pierce Biotechnology) and Silwet L-77 (Helena Chemicals, Collierville, TN) added at 1300 ppm was added to each well. Following a 30 min incubation at 37ºC, primary antibodies were discarded and each plate was washed five times with PBST before adding 80 µL of appropriately diluted secondary antibodies (Donkey anti-rabbit IgG (H + L) with a peroxidase conjugate, Pierce Biotechnology, Rockford, IL) in StartingBlock. Following a 2 h incubation at 37ºC, plates were washed three times with 300 µL of PBS-SDS (PBS) + 2.3g/L sodium dodecyl sulfate (Sigma-Aldrich), and twice with 300 µL of PBS. Thereafter, 80 µL of TMB (1-Step Ultra TMB-ELISA, Pierce Biotechnology, Rockford, IL) was added to each well, and the plate was covered with foil and placed on a shaker (IKA* MTS 2/4 Digital Microtiter Plate Shaker, IKA Works, Wilmington, NC) at room temperature for 10 min. Eighty- µL of 2N H2SO4 was added to each well to stop the reaction. Optical density (OD) from each well was read twice at 450 nm with 490 nm as a reference standard on an Emax microplate reader (Molecular Devices, Sunnyvale, CA), and the 2 values for each well were averaged. Error correction was accomplished by subtracting the mean OD value of the blanks from each value of trapped and negative control sample OD values. Any OD values higher than 4 standard deviations above the mean of the control were considered positive for having a protein mark (Jones et al. 2006).

qPCR Assays. qPCR assays of *D. citri* were performed in an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using a multiplex TaqMan qPCR assay developed for detection of *Ca.* Las (Li et al. 2006). qPCR was chosen because of the inconsistent detection of Las in *D. citri* by conventional PCR and other methods (Halbert and Manjunath 2004, Li et al. 2006). Amplification of *D. citri* samples, contained the following: 1 µL template DNA, 12.5 µL TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 235 nM each of target primers (LasF, 5' -TCGAGCGCGTATGCAATACG -3'; LasR, 5' –GCGTTATCCCGTAGAAAAAGGTAG -3') (GenBank accession number L22532) (Li et al. 2006), internal control primers specific to the *wingless* (*wg*) gene (GenBank accession number AF231365) (WgF, 5'-GCTCTCAAAGATCGGTTTGACGG -3'; WgR, 5'-GCTGCCACGAACGTTACCTTC -3') (Thao et al. 2000) and 118 nM of each probe (WGp, JOE-5'TTACTGACCATCACTCTGGACGC3'-BHQ2) (Duan et al. 2009); HLBp, FAM-5'AGACGGGTGAGTAACGCG-BHQ1) (Li et al. 2006) (Integrated DNA Technologies, Inc., Coralville, IA). DNA amplifications were conducted in 96-well MicroAmp® reaction plates (Applied Biosystems, Foster City, CA). qPCR reactions consisted of 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Each 96-well plate containing *D. citri* samples included a no template control, a positive control (Las DNA in DNA extractions from *D. citri*) and a negative control (no Las DNA in DNA extractions from *D. citri*). Samples were considered positive for *wg* gene or Las or COX gene if the cycle quantification (C_q) value determined by the ABI 7500 Real-Time software (Version 1.4, Applied Biosystems) was 35 or less.

Statistical Analyses. An analysis of variance (ANOVA) was used to compare the number of marked *D. citri* trapped with respect to trap distance, number of days trap was downwind of the marked area, trap placement in unmanaged or managed areas of the study site, and flush abundance, when available (PROC GLM; SAS Institute 2005). Main effects that were found to be significant in the ANOVA were then compared using a one-way analysis of variance (ANOVA) followed by Fisher's protected LSD mean separation tests (PROC GLM; SAS Institute 2005). Individual correlations were performed to identify relationships between trap distance and number of marked *D. citri* trapped as well as between number of days a trap was downwind of the marked area and number of marked *D. citri* found on that trap (PROC CORR; SAS institute 2005).

Results

June. A total of 179 adult *D. citri* were trapped, and 19% were marked with the marker protein. Protein marked adult *D. citri* were trapped within the marked area, and at all distances except 1000 m. A total of 10 marked *D. citri* were trapped at a distance of 2000 m. Of the ten psyllids trapped at 2000 m, five tested positive for *Ca.* Las by qPCR. Flush was not considered during the June experiment, and grove type (Fig. 3-2) was the only variable found to be significant (F = 5.45, df = 1, 36; P = 0.0253). The number of days that a trap was in the downwind direction from the marked area was not found to be significant in determining the number of marked psyllids found on that trap (F = 1.25, df = 2, 31; P = 0.7999), nor was the distance of a trap from the marked area (F = 0.96, df = 8, 31; P = 0.4844) (Fig. 3-3).

July. A total of 541 *D. citri* were sampled and 18% were protein marked. Protein marked *D. citri* were trapped at all distances except 1000 m. Two traps located 2000 m from the marked area each contained over 1000 *D. citri* and sub-sampling was

necessary; 50 males and 50 females were selected from each trap for analysis. A total of 73 marked *D. citri* were identified from the sampled population at 2000 m; of these, six tested positive for *Ca.* Las by qPCR. Flush abundance was estimated around each trap and was found to be highly significant (F = 5.31, df = 6, 18; P = 0.0013), showing a positive correlation between abundance of flush and number of marked *D. citri* trapped (Fig. 3-4a). This was also found with distance of the trap from the marked area, with more marked *D. citri* trapped at greater distances (F = 4.92, df = 8, 18; P = 0.0011) (Fig. 3-4b). Wind direction was not found to be a significant factor in *D. citri* movement (F = 0.32, df = 2, 18; P = 0.7300) (Fig. 3-4c). There was a correlation between grove type and the number of marked *D. citri* trapped, with more marked *D. citri* trapped in unmanaged areas of the study site than in managed areas (r^2 = 0.37, P = 0.0172) (Fig. 3-5). As no correlations were found between grove type and flush abundance or between distance and flush abundance, flush abundance was further evaluated and found to have two outliers. When these outliers were removed and the data were reanalyzed, flush abundance was the only significant factor affecting the movement of *D. citri* (r^2 = 0.58, P < .0001) (Fig. 3-6).

Discussion

The results indicate that adult *D. citri* are capable of travelling at least 2000 m within 12 d. Surprisingly, wind direction did not appear to be a major factor affecting psyllid movement, which was influenced more by flush availability and areas of low or no psyllid control (grove type). Flush is a necessary resource for *D. citri* reproduction, and thus it is not surprising that this would affect *D. citri* dispersal. It is not yet completely understood how the presence of *Candidatus* Liberibacter asiaticus might affect the biology of *D. citri*. This study indicates that *D. citri* carriers of *Ca.* Las may be

just as fit as their *Ca.* Las-free cohorts in terms of dispersal abilities. *D. citri* were trapped in both months at all distances except for 1000 m. This result is likely due to the fact that all ten of the traps placed at 1000 m were, due to the constraints of the study area, placed in trees that were either in a state of severe decline or on young trees (< 4 y) in intensively managed areas.

This study expands on the work initiated by Boina et al. (2009) where an *in situ* protein marking technique was shown to be an effective method for tracking the relatively short distance movement (≈300m) of *D. citri* from unmanaged groves into proximal managed groves over the course of three days. The present study has utilized that protein marking technique for the purpose of determining longer range movement capabilities of *D. citri* over an 11 day period. It has been shown that a different psyllid vector of huanglongbing, *Trioza erytreae* (Del Guercio), the African citrus psyllid, has the ability to disperse up to 1.52 km within seven days in the absence of host plants (van den Berg and Deacon 1988). Thus, it was determined that in geographical locations where *T. erytreae* is the primary vector of HLB, groves could only be considered isolated if they were at least 1.5 km away from any other grove. Van den Berg and Deacon's (1988) results support my finding that there is no strong correlation between prevailing wind direction and psyllid dispersal; they postulate that the apparent upwind movement recorded in their study was actually the result of psyllids moving actively during periods when the wind was calm.

There is a need for area-wide management practices to control huanglongbing and its psyllid vector in Florida, and growers are beginning to implement more coordinated measures in an attempt to gain effective control (Hall et al. 2010). The

information presented in this study gives an indication of exactly how far *Ca.* Las-infected *D. citri* are capable of moving under normal field conditions in a relatively short period of time, and should be considered when determining boundaries in area-wide studies. Citrus groves that are within 2 km of any other citrus plantings are at risk for *D. citri* infestation and HLB disease introduction from those areas. Further studies should seek to identify if *D. citri* are capable of movement greater than the 2 km recorded here. Results from this study should also serve as further evidence of the threat of infestation and infection posed by unmanaged groves in proximity to managed citrus as grove type was a significant factor in psyllid movement regardless of flush abundance.

Table 3-1. Number of traps placed in unmanaged, regularly managed, and intensively managed areas within the study site.

Trap Distance	Unmanaged Areas	Regularly Managed Areas	Intensively Managed Areas
Marked Area	0	8	0
100 m	0	10	0
300 m	0	10	0
400 m	0	10	0
500 m	2	8	0
650 m	4	6	0
1000 m	6	2	2
1200 m	4	4	2
2000 m	8	0	2

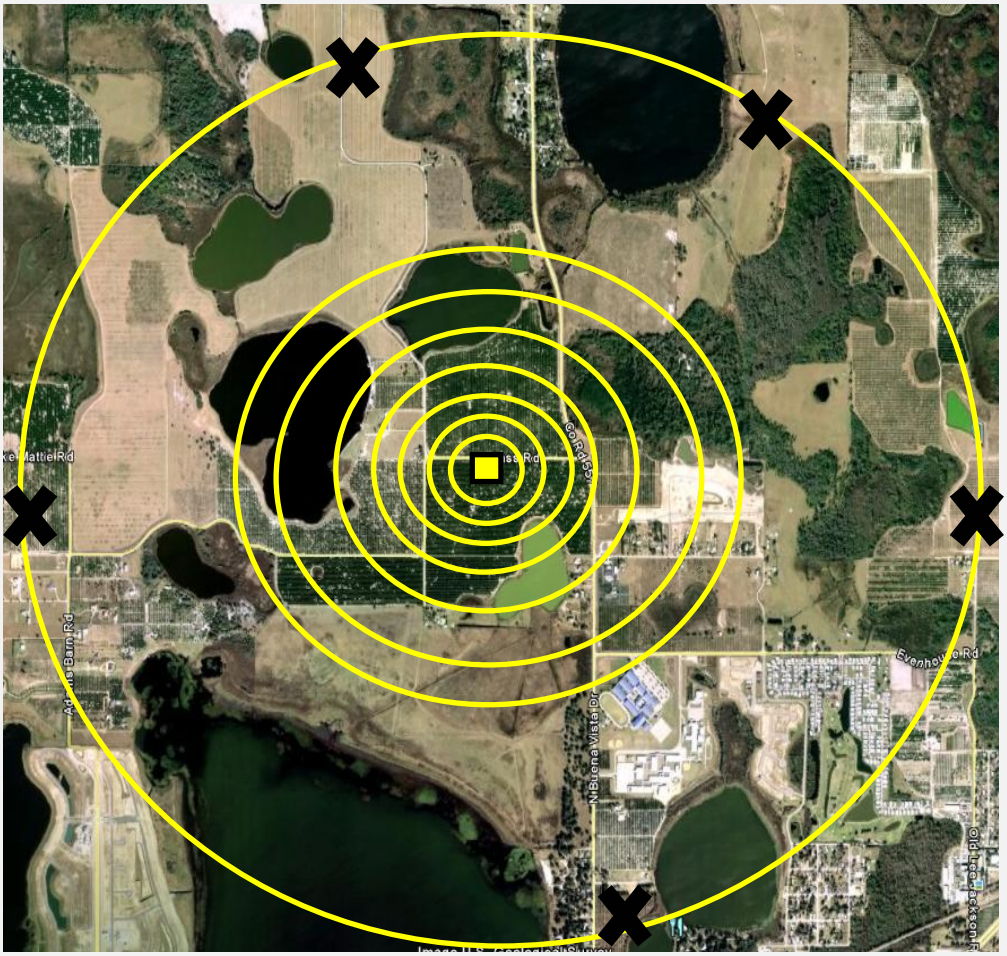

Figure 3-1**.** Satellite view delineating marked area (yellow square) with trap distances (yellow circles). Trap placement at 2 km is also shown (black X's).

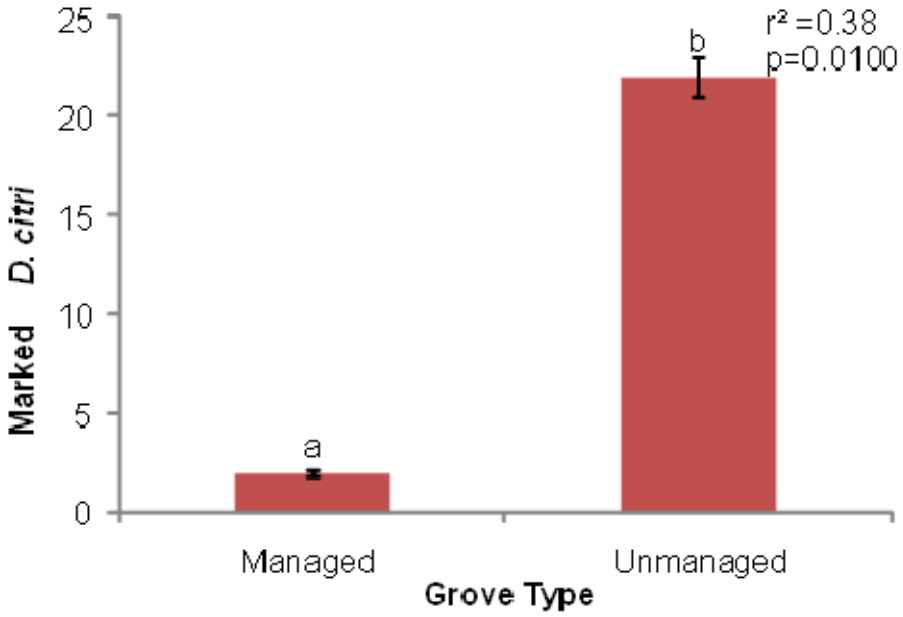

Figure 3-2. Mean (±SEM) number of marked *D. citri* trapped in managed and unmanaged areas during the month of June.

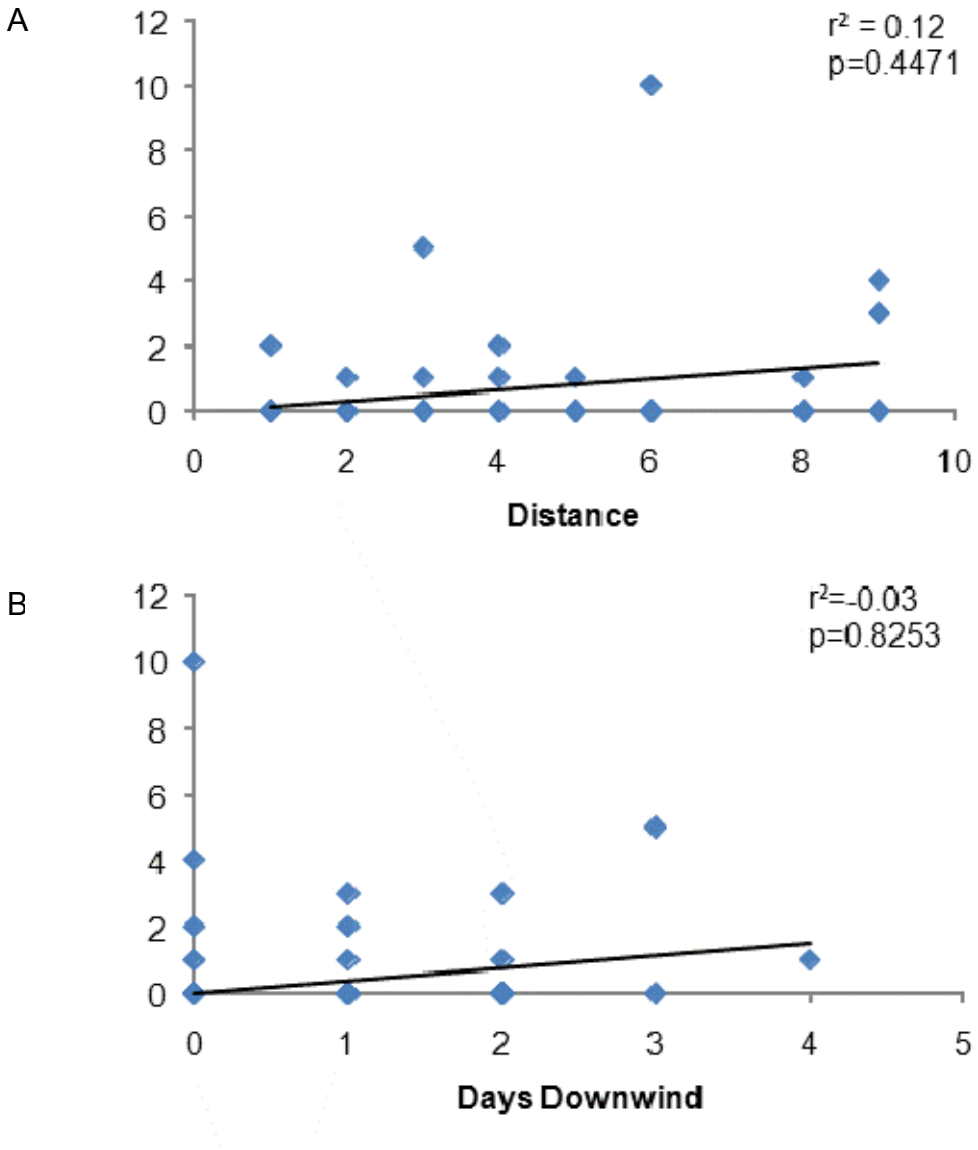

Figure 3-3. Correlations between trap distance (A), days traps were downwind of the marked area (B), and captured marked *D. citri* during June.

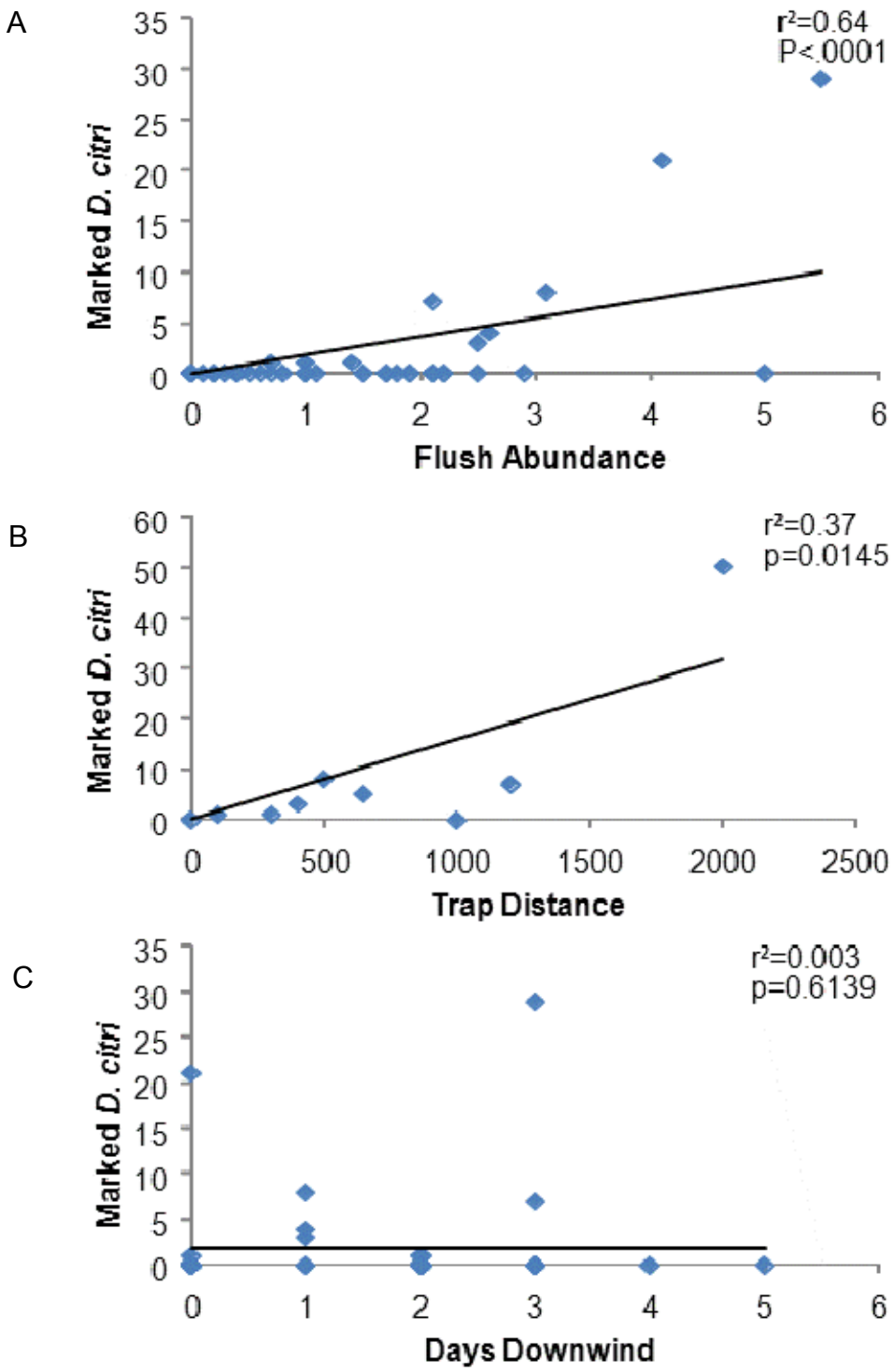

Figure 3-4. Correlations between flush abundance (A), trap distance (B), days traps were downwind of the marked area (C) and captured marked *D. citri* during July.

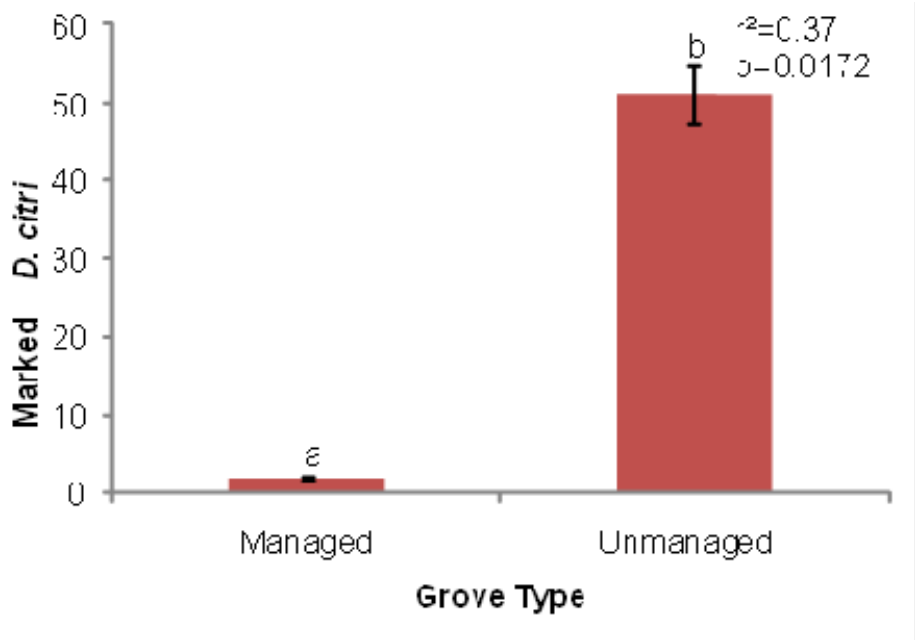

Figure 3-5. Mean (±SEM) number of marked *D. citri* trapped in managed and unmanaged areas during the month of July.

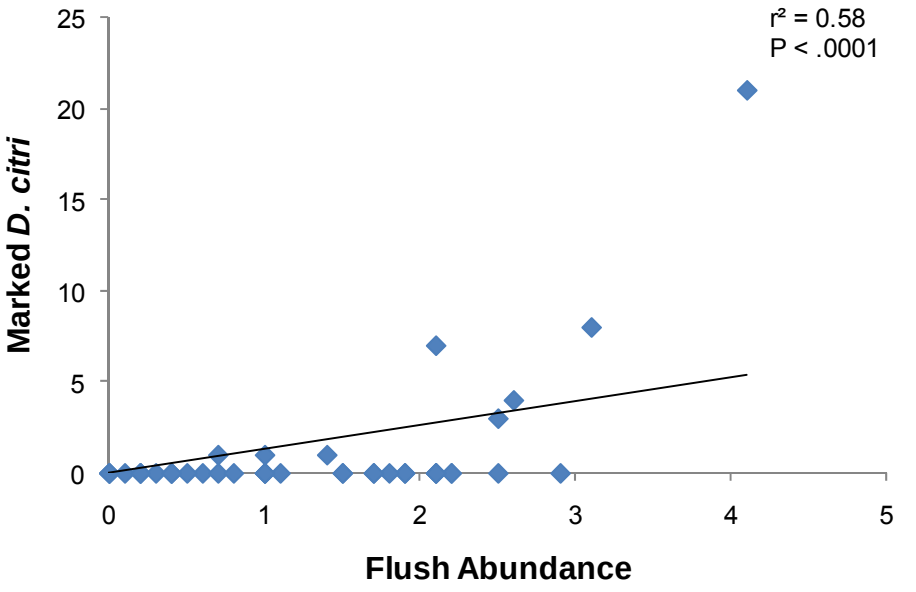

Figure 3-6. Correlation between flush abundance (outliers removed) and captured marked *D. citri* during the month of July.

CHAPTER 4
SEASONAL IMPACT OF UNMANAGED GROVES ON NEARBY COMMERCIAL GROVES; D. CITRI INFESTATION

Mark-recapture studies may be undertaken using markers that can be applied to wild insects directly in the field, allowing their movement to be tracked in their natural habitat (Hagler and Jackson 2001). An inexpensive technique has recently been developed that allows for *in situ* applications of crude food proteins, which can be used to mark insects, and can be detected by enzyme-linked immunosorbent assays (ELISA) (Jones et al. 2006). Milk, egg, and soy proteins can be used as marker proteins and applied in the field using standard spray equipment (Jones et al. 2006, Boina et al. 2009). Insects can acquire a protein mark either by direct contact during marker application or by coming into contact with dried residue. Captured insects can then be subjected to an ELISA to determine if they have acquired a particular protein mark (Boina et al. 2009). The location and marker status of an insect allows for a map of insect movement to be constructed. *D. citri* are capable of dispersing at least 300 meters within 3 days; from unmanaged into nearby managed groves (Boina et al. 2009). There are currently 57,650 ha of abandoned or unmanaged citrus in Florida, and *D. citri* are able to exploit these areas of host plants unchecked (USDA 2008, Morris et al. 2009, USDA 2009). This study was undertaken to identify any seasonal patterns in the movement of *D. citri* from unmanaged into nearby managed citrus groves. Understanding these patterns should benefit growers by providing them with information on timing and location of *D. citri* infestations, allowing for the optimization of psyllid control measures.

Materials and Methods

To quantify the movement of *D. citri* from unmanaged groves into managed groves 4 replicate plots were delineated in citrus groves located in and around Lake Alfred, FL. Each plot included a pair of groves; an unmanaged grove (no pesticide treatments for at least 3 years prior to this study) and a regularly managed commercial grove, which were separated by 50-100 meters. Twenty un-baited yellow sticky traps (Trécé; Adair, OK) were deployed within each replicate. These sticky traps have been shown to attract and capture adult *D. citri* (Hall et al. 2007). Each replicate consisted of 10 rows of 30 mature sweet orange trees (0.4-ha plots). A minimum buffer of 40 m (5 rows) was left untreated between replicates. Traps were placed within the canopy on the facing edge trees of both the unmanaged and managed groves in rows 2, 4, 6, and 8. Traps were also placed 15 trees deep in each grove, again in rows 2, 4, 6, and 8. Four traps were placed centrally between the two groves, in line with the edge traps on opposing sides attached to 1.8 m wooden posts.

Protein Marking. A protein marking technique developed by Jones et al. (2006) and tested and modified for optimal use under Florida weather conditions by Boina et al. (2009) was used to mark unmanaged plots. Within each plot, one row of ten trees (the 15^{th} trees on the interior of the unmanaged grove) was sprayed with 10% chicken egg albumin (All Whites, Papetti Foods, Elizabeth, NJ) in water, with Silwet L-77 (Helena Chemicals, Collierville, TN) added at 2000 ppm. In previous tests (Boina et al. 2009) this solution has been shown to be detectable up to 35 days post application. Ten trees located along the edge of the unmanaged plot (closest to the managed grove) were sprayed with 20% bovine casein (All Natural Whole Milk, Publix Super Markets, Lakeland, FL) in water with Silwet L-77 added at 2000 ppm. This mixture also has the

ability to be detected up to 35 days post application (Boina et al. 2009). The solutions were applied in the field using an ATV mounted handgun sprayer (model 5275016; Fimco Industries, North Sioux City, SD) run at ≈250psi. The spray was applied until there was visible leaf runoff. *D. citri* acquired a protein mark in the marked areas either from direct contact during spraying, or through contact with dried residue on leaves. After spraying the marker proteins, traps were deployed. Traps were collected 4 days post application of the protein spray. This study was conducted monthly from June 2009 through September 2010 to understand the effect of seasonality on the movement of *D. citri*, which is essential to improving the timing of pesticide sprays and effectively managing HLB. Stem tap counts (Hall et al. 2007) were conducted after each field experiment was complete (beginning in September 2009) to estimate *D. citri* populations.

ELISA. In the lab, psyllids were identified, sexed, and individually removed from traps with disposable toothpicks to eliminate the possibility of cross-contamination before being placed into 1.5mL centrifuge tubes. Once all psyllids were removed from the traps, 1mL of protein extraction buffer (Tris-buffered saline, pH 8.0 + 0.3 g/L sodium ethylenediamine tetraacetic acid [EDTA]) (Sigma-Aldrich, St. Louis, MO) was added to each centrifuge tube. Psyllids were removed from the extraction buffer after 3-5 minutes and placed into sterile 1.5 mL centrifuge tubes with 1mL 80% EtOH and stored at -8°C for future use in quantitative real-time polymerase chain reaction (qPCR) assays.

The extraction buffer was then subjected to an indirect enzyme-linked immunosorbent assay (ELISA), according to the protocols described in Boina et al. (2009) and Jones et al. (2006). Eighty μL of extraction buffer was removed from each

centrifuge tube and placed in a single well of a 96-well microplate (Nunc Polysorp; Fisher, Pittsburgh, PA) to test for the presence of egg protein, and an 80μL aliquot from the same centrifuge tube was placed into the corresponding well of a separate 96-well microplate to test for the presence of milk protein. On each microplate, 8 wells of 80μL extraction buffer alone, and 8 wells of 80μL extraction buffer with marker-free greenhouse psyllids served as blanks and negative controls respectively. Eighty μL of a 0.001% dilution of each marker protein in extraction buffer was added to 3 wells on each microplate as positive controls. Microplates were initially incubated for 2 h at 37°C. After this incubation, each plate was washed 5 times with 300μL phosphate-buffered solution (PBS, Sigma-Aldrich, St. Louis, MO), pH 7.4 + 0.09% Triton X-100 (PBST; Sigma-Aldrich) per well. Washing was either conducted by hand, or by using a microplate washer (Wellwash 4 Mk 2; Thermo Electron Corporation, Vantaa, Finland). Following this wash, 300μL of blocking solution (StartingBlock, Pierce Biotechnology, Rockford, IL) for egg, and 10% ethanolamine (Sigma-Aldrich) in PBS for milk, was added to each well. This was followed by 1 h incubation at 37°C, and one wash of 300μL PBST. Eighty μL of appropriately diluted primary antibody was then added to each well. For egg, the primary antibody used was Rabbit anti-chicken Egg Albumin (Sigma-Aldrich) diluted in StartingBlock with 0.05% Tween-20 (37539; Pierce Biotechnology) and Silwet L-77 added at 1300 ppm. The primary antibody used for milk was Sheep anti-Bovine Casein (Biodesign International, Saco, ME) diluted in PBS with 20% bovine serum albumin (HyClone, Logan, UT) and Silwet L-77 added at 1300 ppm. The microplate used to detect milk protein was incubated for 1 h, and the egg for 30 min at 37°C. Primary antibodies were discarded and each well was then washed five times

with PBST before adding 80µL of appropriately diluted secondary antibodies. The egg secondary antibody used was Donkey anti-rabbit IgG (H + L) with a peroxidase conjugate (Pierce Biotechnology, Rockford, IL) in StartingBlock. Donkey anti-sheep IgG with a peroxidase conjugate (Sigma-Aldrich) in StartingBlock was used as the secondary antibody for milk. This was followed by 2 h incubation at 37°C. Plates were washed three times with 300µL of PBS-SDS (PBS + 2.3g/L sodium dodecyl sulfate) (Sigma-Aldrich) and twice with 300µL of PBS. Thereafter, 80µL of 1-Step UltraTMB (ImmunoPure, Ultra TMB substrate kit 34028; Pierce Biotechnology, Rockford, IL) was added to each well, and the microplates were covered with foil and placed on a shaker (IKA* MTS 2/4 Digital Microtiter Plate Shaker; IKA Works, Wilmington, NC) at room temperature. After 10 min at 900 rpm, the plates were removed from the shaker and 80µL of 2N H_2SO_4 was added to each well to stop the reaction.

Optical density (OD) from each well was read twice at 450 nm with 490 nm as a reference standard on an Emax microplate reader (Molecular Devices, Sunnyvale, CA). The two values for each well were averaged. The OD values of the trapped *D. citri*, as well as the wells used as negative control, were corrected for error by subtracting the mean OD value of the blanks from each. Any OD values higher than four standard deviations above the mean of the control were considered positive for having a protein mark (Jones et al. 2006).

Population Estimates. The *D. citri* population within the study area was estimated each month beginning in September 2009. Tap counts (Hall et al. 2007) were performed at the rate of two taps per tree on 40 trees within each managed and unmanaged block: 10 edge row trees, and 10 trees immediately to the interior of the

edge, as well as 10 trees each from the 14th and 15th rows interior (≈150 m from the edge row).

Statistical Analyses. *D. citri* infestation, as measured by tap counts, was analyzed by an analysis of variance (ANOVA) at a significance level of α=0.05, followed by Fisher's protected LSD mean separation test (PROC GLM; SAS Institute 2005). Main effects were 1) month 2) block and 3) location; interior and edge row of both unmanaged and managed grove plots. Total number of *D. citri* captured on traps was compared to the total number of protein marked *D. citri* captured, and the total number of *D. citri* identified through tap counts. Percent of protein marked *D. citri* captured in each of the four possible locations was calculated.

Results

There was no clear pattern to the location of *D. citri* infestation throughout this study. Tap counts were initiated to estimate relative population abundance beginning in September 2009. Significantly more *D. citri* were found in December of 2009, than in any other month. In that same month, significantly more *D. citri* were found in the managed groves when compared to the unmanaged plots (Table 4-1). The greatest number of both trapped, and protein marked *D.* citri occurred in June, July, and August of 2009 (Fig. 4-1). After August 2009, the total number of *D. citri* trapped, and the total number marked decreased dramatically. The number of *D. citri* trapped within the study area was low from Sept. 2009 through Sept. 2010, with the most (32 total, 1 marked) being captured in April 2010 (Fig. 4-1). No marked *D. citri* were trapped in December of 2009 through March 2010. The greatest amount of movement from unmanaged to managed grove plots occurred during June, July, and August of 2009, with a very low percentage of marked *D. citri* trapped within the unmanaged groves (Fig. 4-2). This

trend began to change in October of 2009. During Oct., Nov., and Dec. 2009, there was a marked decrease in the movement between groves. While there was some movement from unmanaged to managed grove plots during this time, most protein-marked *D. citri* were trapped within the unmanaged groves (Fig. 4-2); during the spring and summer of 2010 (April-August), movement between groves resumed and almost all of the marked *D. citri* were captured in the interiors of managed grove plots.

Discussion

Local movement of *D. citri* is greatest during the spring and summer months, and decreases greatly during the colder months (Sept.-March). During the warmer months of this study, *D. citri* were consistently capable of dispersing at least 300 meters within four days; from inner rows of unmanaged grove plots to inner rows of managed groves.

The results indicate that *D. citri* can disperse from unmanaged groves into nearby managed groves within only a few days, corroborating previous findings (Boina et al. 2009). Unlike the previous study, this experiment attempted to identify how this movement pattern changes throughout the year. While it was hypothesized that monthly monitoring of the movement of *D. citri* from unmanaged into managed grove plots would elucidate seasonal trends in locations of infestations and preference for interiors or edge rows of groves, low psyllid populations for 13 out of the 16 months of this study hampered those efforts. The population increase in the spring of 2010 was lower than in 2009 and occurred later in the season. Possible reasons for the extended low populations could be the result of either lower than average winter temperatures in Florida, or an increase in intensity of psyllid control measures practiced by growers in Polk county beginning in July or August of 2009.

It is clear that unmanaged groves can act as reservoirs of the vector and presumed pathogen that causes huanglongbing. The extent of the impact of these unmanaged groves on the commercial citrus industry in Florida is difficult to quantify. However, knowing how devastating HLB can be to citrus production, the presence of these areas of refugia should be of immediate concern. While intensity and coordination of control measures for *D. citri* are an important step in mitigating the damage caused by HLB, the presence of large numbers of unmanaged groves in Florida may undermine these efforts.

This study focused on commercial groves in immediate proximity to unmanaged groves, however, there is evidence that *D. citri* are capable of dispersing much greater distances in a relatively short period of time (Chapter 3). These results suggest that far more commercial groves are at risk of infestation and infection from isolated sources than previously believed. With respect to annual fluctuations of *D. citri* movement, the current results would suggest that *D. citri* exhibit greatest mobility during the spring and summer months. Intensive insecticide use likely prevented demonstration of this annual pattern in the second year of the study. These results lend further support to the currently recommended implementation of large-scale, coordinated insecticide applications for *D. citri* management prior to occurrence of spring flushes. Also, these results indicate that *D. citri* populations should be actively monitored during the spring and summer months to determine if additional coordinated sprays are necessary, following dormant-season insecticide applications, to prevent infiltration of psyllids from unmanaged areas.

Table 4-1. Mean (± SEM) number of *D. citri* per tree as measured by tap counts.

Month	*Unmanaged Edge*	*Unmanaged Interior*	*Managed Edge*	*Managed Interior*	*Overall Mean*
Sept. 09	0.12 ± 0.07ab[1]	0.07 ± 0.07b	0.29 ± 0.05a	0.10 ± 0.04b	0.15 ± 0.04b[2]
Oct. 09	0.01 ± 0.01a	0.0 ± 0.0a	0.08 ± 0.04a	0.08 ± 0.03a	0.04 ± 0.02b
Nov. 09	0.05 ± 0.04a	0.11 ± 0.10a	0.23 ± 0.13a	0.13 ± 0.07a	0.13 ± 0.04b
Dec. 09	0.01 ± 0.01b	0.18 ± 0.13b	0.83 ± 0.21a	1.26 ± 0.33a	0.57 ± 0.16a
Jan. 10	0.04 ± 0.04a	0.03 ± 0.01a	0.15 ± 0.06a	0.11 ± 0.10a	0.08 ± 0.03b
Feb. 10	0.0 ± 0.0a	0.0 ± 0.0 a	0.08 ± 0.04a	0.09 ± 0.05a	0.04 ± 0.02b
Mar. 10	0.0 ± 0.0a	0.0 ± 0.0a	0.03 ± 0.01a	0.04 ± 0.02a	0.02 ± 0.01b
April 10	0.0 ± 0.0b	0.05 ± 0.05ab	0.07 ± 0.04ab	0.17 ± 0.07a	0.07 ± 0.03b
May 10	0.01 0.01a	0.0 ± 0.0a	0.06 ± 0.02a	0.05 ± 0.04a	0.03 ± 0.01b
June 10	0.01 ± 0.01a	0.0 ± 0.0a	0.0 ± 0.0a	0.13 ± 0.09a	0.03 ± 0.03b
July 10	0.0 ± 0.0a	0.0 ± 0.0a	0.01 ± 0.01a	0.0 ± 0.0a	0.0 ± 0.0b
Aug. 10	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.0b	0.15 ± 0.08a	0.04 ± 0.02b
Sept. 10	0.01 ± 0.01a	0.0 ± 0.0a	0.01 ± 0.01a	0.03 ± 0.03a	0.01 ± 0.0b

[1] Means within rows followed by the same letter are not significantly different.

[2] Means within this column followed by the same letter are not significantly different.

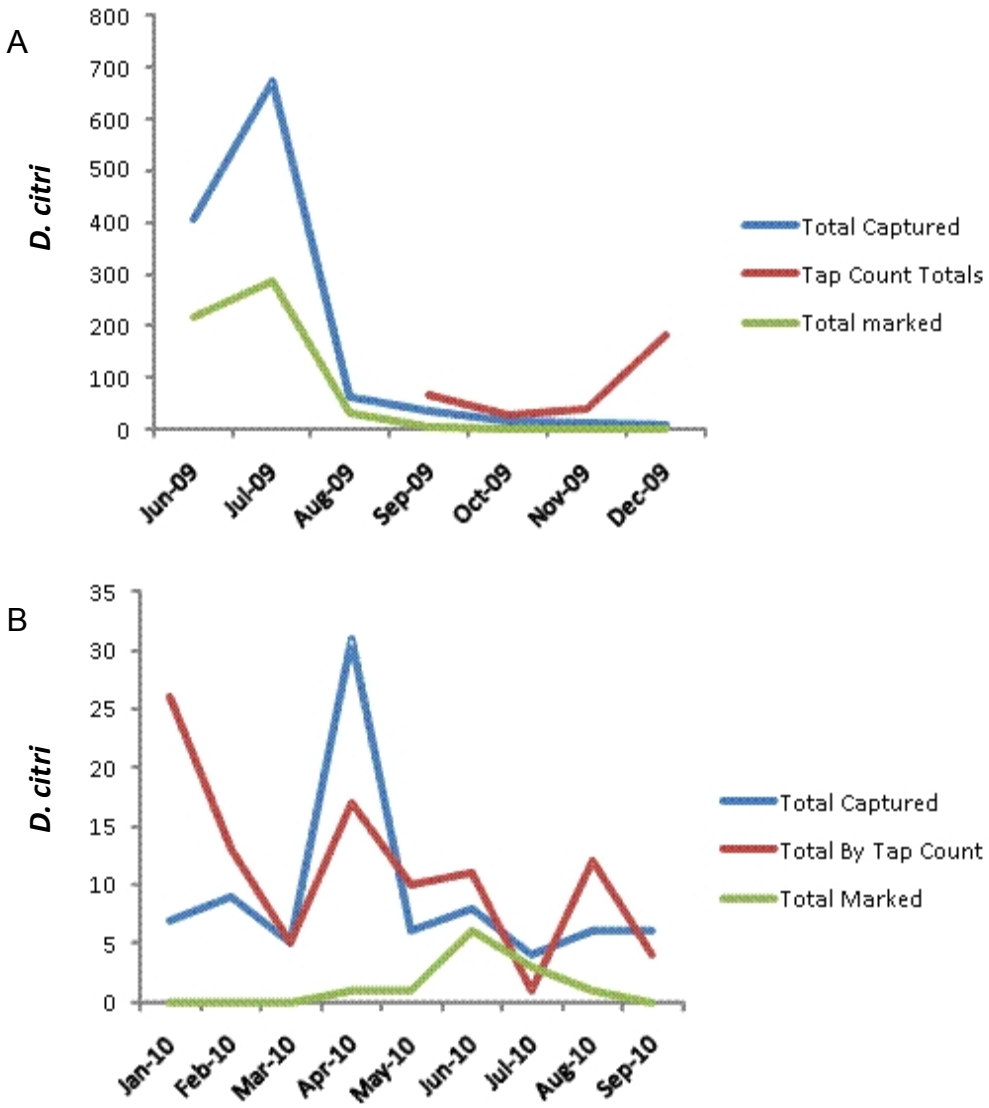

Figure 4-1. Monthly trends in numbers of *D. citri* found on traps and by tap count, as well as total number possessing a protein mark. A) In 2009. B) In 2010.

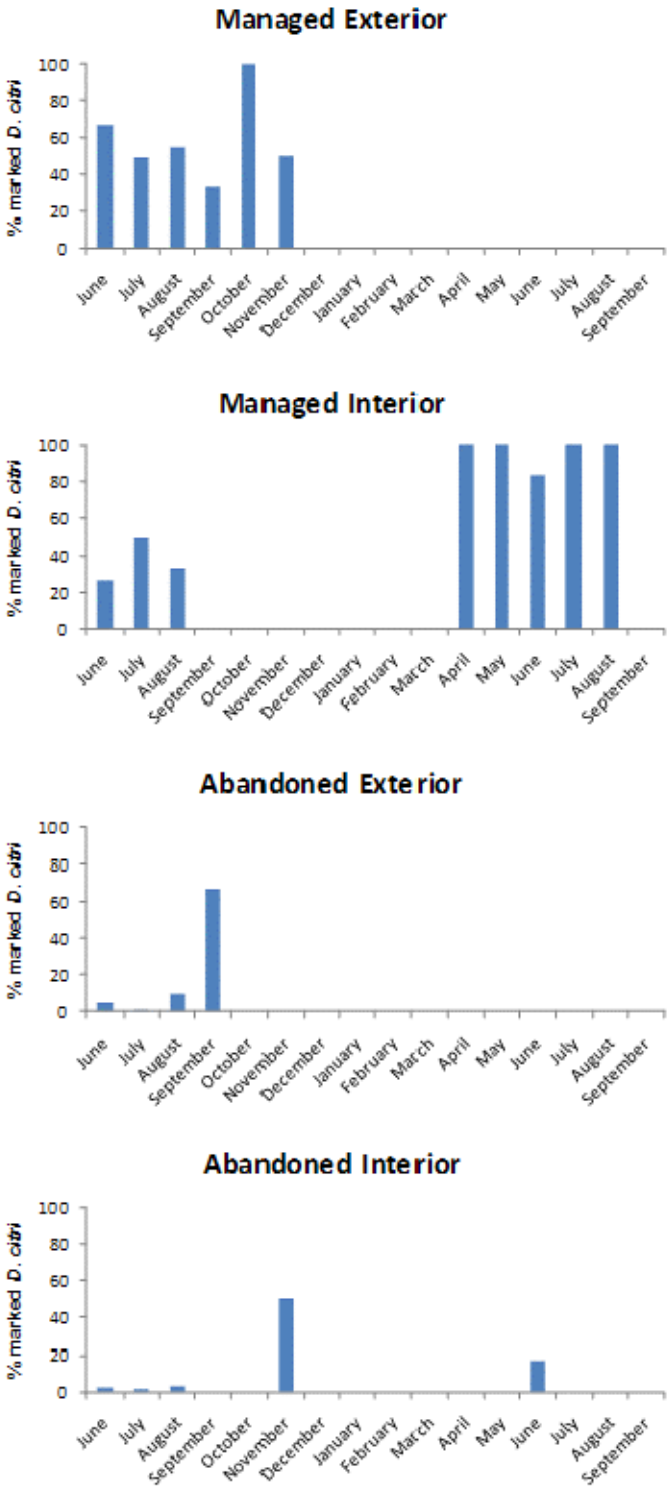

Figure 4-2. Percentage of protein marked *D. citri* found in each of the four studied areas. Monthly proportions are calculated from monthly totals presented in Figure 4-1.

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BIOGRAPHICAL SKETCH

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