Transmission Parameters for *Candidatus* Liberibacter asiaticus by Asian Citrus Psyllid (Hemiptera: Psyllidae)

K. S. PELZ-STELINSKI,[1,2] R. H. BRLANSKY,[3] T. A. EBERT,[1] AND M. E. ROGERS[1]

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ABSTRACT The purpose of this investigation was to evaluate acquisition and inoculation (together, transmission) efficiency of *Candidatus* Liberibacter asiaticus (Las), the pathogen associated with citrus huanglongbing (HLB) by the Asian citrus psyllid, *Diaphorina citri* (Kuwayama) (Hemiptera: Psyllidae). In laboratory studies, nymphs reared on Las infected plants were more likely to acquire the bacterium than adults. Acquisition by nymphs ranged from 60 to 100%, whereas acquisition by adults only reached 40% after 5 wk of feeding on Las-infected plants. Similar rates of pathogen acquisition by psyllids after nymphal and adult feeding were observed in the field. Transmission of Las from parent to offspring (transovarial) occurred at a rate of 2–6%. One year after psyllid inoculations, successful transmission by individual *D. citri* ranged from 4 to 10%, whereas groups of 100 or more *D. citri* transmitted the pathogen at a rate of ≈88%. In addition, the proportion of Las-positive adult psyllids, determined using quantitative real-time polymerase chain reaction, decreased over time when held on healthy plants. Due to the low rate of pathogen acquisition and long time period required for successful inoculation by adult *D. citri*, experiments designed to determine the latent period required for replication and successful inoculation of Las by *D. citri* did not result in Las-infected plants after >1 yr of incubation after inoculation. Collectively, these results indicate that adult *D. citri* which acquire the HLB pathogen as adults are poor vectors of the pathogen compared with adults that acquired the pathogen as nymphs.

KEY WORDS citrus greening disease, huanglongbing, acquisition, inoculation, vector

Huanglongbing (HLB), or citrus greening disease, is the most economically important disease affecting citrus production worldwide (Halbert and Manjunath 2004, Teixeira et al. 2005, Bové 2006, Wang et al. 2006, Batool et al. 2007, Manjunath et al. 2008). Historically endemic throughout Asia and Africa, HLB recently has spread to South America and some citrus-producing regions of North America, including Florida (Bové 2006). The pathogen associated with citrus greening disease is a vector-borne α-proteobacterium (Jagoueix et al. 1994) that to date has not been maintained in sustained pure culture (Davis et al. 2008); however, limited success was reported recently (for to five single-colony transfers) (Sechler et al. 2009). Three distinct etiologic agents of HLB have been implicated based on their 16S rRNA gene sequence: *Candidatus* Liberibacter asiaticus (Asia, North America, and Brazil), *Ca.* Liberibacter americanus (Brazil), and *Ca.* Liberibacter africanus (Africa) (Garnier et al. 1984, Jagoueix et al. 1996, Sagaram et al. 2009). *Ca.* L. asiaticus (Las) was discovered in Florida in August 2005 (Halbert 2005) and subsequently has infected an estimated 1.6% of orange trees in Florida as of 2008 (Morris et al. 2009a,b). The bacterium systemically infects citrus trees and reduces the quantity and quality of fruit produced. Characteristic symptoms of this disease include mottled leaves, corked veins, yellowed shoots, twig dieback, and ultimately tree death. Recommended management tactics for HLB in Florida primarily rely on controlling *D. citri* populations and removing Las-infected trees that are a source of inoculum for acquisition by healthy psyllids (Brlansky et al. 2007).

There are two reported vectors of citrus greening disease: the Asian citrus psyllid, *Diaphorina citri* (Kuwayama) (Hemiptera: Sternorrhyncha: Psyllidae), and the African psyllid *Trioza erytreae* (del Guercio) (Hemiptera: Sternorrhyncha: Triozidae). *D. citri* is responsible for transmission of Las in North America, Brazil, and Asia and *Ca.* L americanus in Brazil, whereas *T. erytreae* transmits *Ca.* L. africanus in the Middle East, Reunion, and Africa (Halbert and Manjunath 2004). *D. citri* can acquire Las from infected plants as nymphs or adults (Capoor et al. 1974, Xu et al. 1988). If the pathogen is acquired at the nymphal stage, adults are able to transmit immediately after emergence (Capoor et al. 1974, Xu et al. 1988, Inoue et al. 2009).

In past studies with *D. citri* and *T. erytreae*, acquisition of the HLB pathogen was reported to require a minimum of 15 min to 24 h, and the psyllid access time

[1] Department of Entomology and Nematology, Citrus Research and Education Center, University of Florida, Lake Alfred, FL 33850.
[2] Corresponding author, e-mail: pelzstelinski@ufl.edu.
[3] Department of Plant Pathology, Citrus Research and Education Center, University of Florida, Lake Alfred, FL 33850.

for pathogen inoculation of healthy plants ranged from 15 min to 7 h (Capoor et al. 1974, Buitendag and von Broembsen 1993). Generally, previous studies of Las acquisition and inoculation have been conducted by confining psyllids on host plants for feeding and confirming Las presence based on visual observations of symptomatic tissues. More recently, conventional and quantitative real-time PCR (qPCR) have been used to confirm presence, multiplication, and transmission of Las by *D. citri* at different developmental stages (Hung et al. 2004, Inoue et al. 2009); however, reports of pathogen transmission efficiencies are inconsistent and the rates of pathogen acquisition and inoculation by psyllids over variable times have not been quantified. In addition, reports of the latent period of Las in *D. citri* before successful inoculation range from 1 to 25 d postacquisition (Xu et al. 1988, and Roistacher 1991). These differences may be due to variation in the assays used for Las detection or possibly to differences inherent among the populations of *D. citri* evaluated. The nature of the relationship between the purported HLB pathogen and *D. citri* has been defined using populations from regions including India, China, Taiwan, and Japan; consequently, genetic variation among these groups may underlie differences in vector efficiency. To date, no studies have been conducted to evaluate the vector efficiency of *D. citri* in Florida.

Compounding the lack of clear information regarding Las transmission, there are inconclusive reports of vertical (transovarial) passage of the bacterium by psyllids. Studies suggest that *T. erytreae* can pass *Ca.* L. africanus transovarially, but that transovarial passage of Las by *D. citri* does not occur (van den Berg et al. 1992 and Xu et al.1988). In addition, transovarial transmission of the pathogen associated with Zebra Chip disease, *Ca.* L. psyllaurous/solanacearum (Hansen et al. 2008; Liefting et al., 2008, 2009) by its psyllid vector *Bactericera cockerelli* (Sulc) has been reported previously (Hansen et al. 2008). Previous investigations of transovarial transmission of Las have been hampered by detection methods with little sensitivity to low bacterial titers; therefore, we conducted experiments to assess the prevalence of transovarial transmission in *D. citri* using qPCR for detection of Las. In addition, we conducted a series of experiments to quantify the acquisition and inoculation of Las by *D. citri* in Florida. Specifically, we examined the efficiency of Las acquisition and inoculation by *D. citri* over time, latency of the pathogen before psyllid inoculation, and retention of the pathogen by *D. citri* postacquisition.

Materials and Methods

Maintenance of Pathogen and Host Plants. In planta cultures of Las were maintained by graft-inoculation of healthy 'Pineapple' sweet orange plants [*Citrus sinensis* (L.) (Rutaceae)] with Las-infected budwood collected from citrus groves in Immokalee, FL (Collier Co.). Healthy (Las-free) host plants used in the following experiments were cultivated from seed (*C. sinensis* Pineapple) or obtained as potted seedlings [*Bergera koeniggii* (L.) Sprengel] (Logee's Greenhouse, Danielson, CT). With the exception of transovarial transmission, all experiments described below were conducted with infected of healthy *C. sinensis* Pineapple. Before using plants in psyllid transmission experiments, multiplex qPCR assays by using primers and probes described by Li et al. (2006) were conducted to determine the presence of Las.

Psyllids. *D. citri* were obtained from a culture collected in 2005 from Polk Co., FL (28° 00′ 39.91″ N, 81° 54′ 01.96″ W) before the discovery of HLB in the state, and maintained on healthy sweet orange plants in an insect-proof greenhouse at the Citrus Research and Education Center (University of Florida, Lake Alfred, FL). Psyllids from this colony were sampled monthly to confirm that insects remained free of Las. Psyllids harboring the bacterium were obtained by rearing *D. citri* from the culture on infected citrus plants maintained in a secure quarantine facility. Routine sampling indicated that 70–100% of *D. citri* obtained from this colony were positive for Las when groups of 10–20 psyllids were individually tested by qPCR. Both psyllid colonies were maintained at 25°C, 60–80% RH, and a photoperiod of 16:8 (L:D) h.

Acquisition of Las by *D. citri* in the Laboratory. The ability of *D. citri* nymphs and adults to acquire Las by feeding on infected citrus was evaluated in two experiments. Adult acquisition was evaluated by confining groups of 50 adult *D. citri* in mesh enclosures on psyllid-free branches of Las-infected citrus plants for acquisition access periods (AAPs) ranging from 1 to 52 d at 25°C and 80% RH. Although the extent of Las multiplication in *D. citri* is unclear, surviving adults were transferred with an aspirator to healthy citrus seedlings after each AAP for a 1-wk latent period during which multiplication of Las could occur. At the end of each acquisition experiment, *D. citri* were collected and preserved in 80% ethanol at −20°C for subsequent detection of Las by qPCR. AAPs were pooled for analysis into six groups representing the number of days psyllids spent feeding on infected plants:1–6, 7–13, 14–20, 21–27, 28–34, and ≥35 d. Each AAP group was replicated three times (n = 20–120 psyllids per AAP).

Acquisition of Las by *D. citri* nymphs was evaluated by placing excised flush containing eggs on mature leaves of infected citrus plants. Before placement, excess flush was carefully removed from around the eggs under a stereomicroscope by using forceps and a camel's-hair brush. Branches containing nymphs were enclosed in mesh bags to prevent dispersal of nymphs during the AAP. Upon emergence after the 11–15-d nymphal developmental period, adults arising from eight replicate groups containing 20–105 psyllids were collected and preserved for Las detection.

Acquisition of Las in the Field. The rate of Las acquisition by *D. citri* under field conditions was assessed by caging healthy psyllids from our laboratory colony on HLB symptomatic sweet orange trees in five Florida citrus groves located in Avon Park (27° 43′ 47.66″ N, 81° 41′ 43.63″ W), Lake Placid (27° 21′ 51.57″ N, 81° 19′ 39.66″ W), and Lake Henry (27° 44′ 59.44″

N, 81° 43′ 37.5″ W). Groups of newly emerged *D. citri* adults were enclosed in mesh bags on branches for acquisition access. Before acquisition experiments, the presence of the bacterium in individual branches was confirmed by qPCR. Adults were confined for AAPs of 10–51 d. AAPs were pooled for analysis into six groups representing the number of days psyllids spent feeding on infected plants:1–13, 14–27, 28–34, 35–42, and ≥42 d. Each pooled acquisition period was replicated at least five times, with at least 100 *D. citri* per replicate. At the end of each AAP, *D. citri* adults were collected and preserved for subsequent assessment of Las acquisition by qPCR.

A second experiment to determine the rate of acquisition by nymphs was conducted by similarly caging adult psyllids on infected citrus trees. The wings of adults were notched to distinguish this generation of adults from their progeny. In addition, because a minimum of 15 d is required for the production of adult progeny after oviposition (Mead 1977, Chavan and Summanwar 1993), the original *D. citri* adults were removed before this period to preclude interaction between progenitors and offspring (F_1 generation). F_1 adults were collected 14–37 d after oviposition and preserved as described above before testing to determine the level of Las acquired by *D. citri* during nymphal development. Acquisition access periods were calculated from the day original *D. citri* adults were placed on plant (day 0), because this day was the earliest possible date on which oviposition could occur.

Retention of Las by *D. citri*. Retention of Las was assessed in *D. citri* reared on Las-infected sweet orange trees. Las-infected branches containing psyllid eggs were enclosed in mesh sleeves until 3 d after the appearance of the first adult psyllid. At that time, *D. citri* adults and nymphs were aspirated and then transferred individually to healthy sweet orange seedlings where they were enclosed under 1-liter clear plastic deli cups perforated for ventilation. *D. citri* and plants were held at 25°C and a photoperiod of 16:8 (L:D) h. Adults psyllids (n = 10–40 per date) were collected every 3–4 d and tested for the presence of Las by using qPCR as described below. In addition, the status of infected and healthy plants was confirmed with qPCR before and after retention tests.

Inoculation of Citrus With Las by *D. citri*. Two experiments were conducted to assess the success of Las transmission over time. Individual, putatively infective adult psyllids (reared on Las-infected citrus plants) were each caged on a single 6–9-mo-old healthy sweet orange seedlings (Pineapple) ≈20 cm high and allowed to feed for 1-, 4-, 7-, 15-, or 24-d inoculation access periods (IAPs). The number of single-psyllid inoculations conducted for each IAP ranged from 40 to 60. Psyllids were caged on plants within Plexiglas tubes vented with mesh to permit air circulation such that the entire plant was accessible for feeding. After each IAP, psyllids were removed and tested for Las with qPCR (n = 214), and plants were held for 3 mo in an insect-proof greenhouse to allow time for pathogen replication and symptom development before testing for the presence of Las. Citrus seedlings that tested negative after 3 mo were retested bimonthly for 1 yr after inoculation experiments. Only plants exposed to psyllids harboring Las were included in subsequent tests for successful inoculation.

In a second experiment, transmission of Las to healthy citrus plants by groups of psyllids was assessed by allowing Las-positive *D. citri* to feed for an IAP of 30 d. A group of 200 *D. citri* obtained from our Las-positive colony was caged with test plants (n = 15) for the course of the experiment. Live psyllids remaining on the plant at the end of this period were collected to confirm the presence of Las. Test plants were retained as above and evaluated bimonthly beginning 3 mo after the IAP.

Transovarial Transmission. The objective of this experiment was to determine the potential for and frequency of vertical transmission of Las from adult *D. citri* females to progeny. One female and two male psyllids reared from eggs on infected citrus plants were caged on flushing, Las-negative sweet orange seedlings for oviposition. Adults were removed after one week or upon the appearance of eggs and tested as described previously to confirm the presence of Las by using qPCR. Introduction of bacteria at the oviposition site may occur as a result of female feeding. To prevent subsequent acquisition of the pathogen by offspring, eggs were transferred to *B. koenigii.* Although the latter is known to be an alternate host of *D. citri* (Halbert and Manjunath 2004), it is not a host for Las (Damsteegt et al. 2010). Egg transfers were conducted by placing egg containing flush on new plants after carefully removing excess plant tissue from around the eggs with forceps and a camel's-hair brush under a stereomicroscope. Eggs and any attached leaf tissue remaining were rinsed with 10% bleach followed by sterile water to eliminate potential surface contamination. All plants used in transovarial transmission experiments were confirmed to be negative for Las with qPCR before oviposition and after offspring were collected.

Replication of maternally derived Las may occur as progeny progress through life stages, such that bacterial titers undetectable in eggs may be discernible in later stages. Therefore, we evaluated the extent of transovarial passage on three distinct *D. citri* developmental stages: eggs, nymphs, and newly eclosed adult offspring. Allowing eggs to develop increases the likelihood the bacteria will multiply to detectable levels within the psyllid. Due to the low concentration of DNA recovered (Hung et al. 2004), eggs and nymphs arising from individual infected females were pooled (20–50 and 20–30 per pool, respectively) for testing. We chose the lower boundary for egg pools based on a previous study by Hung et al. (2004) that determined that DNA extracts from pools of 10 or fewer *D. citri* eggs did not contain sufficient material for spectrophotometric detection of DNA at optical $density_{260}$. DNA was extracted from pooled egg and nymphal samples, individual *D. citri* adult offspring, and progenitor female *D. citri* and then tested for the presence of Las. The experiment was replicated until a mini-

mum of 40 samples per developmental stage produced by Las-positive females were available for analysis.

Latent Period of Las in *D. citri*. The time (latent period) between acquisition and subsequent inoculation of citrus with Las by adult *D. citri* was investigated. In total, 30 adult psyllids ($n = 3$) were confined on Las-infected sweet orange plants for 7 d AAPs. After each AAP, single psyllids were transferred to separate healthy sweet orange seedlings. Serial transfers of the individual psyllids to a new, healthy sweet orange seedling were then made every 7 d for ≈1 mo to estimate the number of days until pathogen inoculation occurred. At the end of the experiment, psyllids were collected and immediately stored in 80% ethanol at −20°C for subsequent testing for the presence of Las by using qPCR. All plant material was held for 3 mo and then tested bimonthly for an 18-mo period to assess successful inoculations of plants with Las.

Preparation of Nucleic Acids From Sample Tissues. Individual adult psyllids, pooled nymphs, or pooled eggs collected from experiments were homogenized in a buffer solution (QIAGEN, Valencia, CA) by using a sterile mortar and lysed overnight at 56°C in a hybridization oven (model 136400, Boekel Scientific, Feasterville, PA). Subsequent extraction of DNA from Las-positive psyllids and healthy controls was conducted according to the manufacturer's instructions using the DNeasy Blood and Tissue kit (QIAGEN) with a protocol modified for detection of bacterial DNA in arthropods (QIAGEN, Anonymous 2008). Whole-psyllid DNA was eluted in a final volume of 35 μl.

Leaf samples collected from citrus plants were stored in plastic bags at 4°C and used for DNA extraction within 24 h. Composites of leaf petiole and midvein tissue from four leaves were subsampled (100 mg) for preparations of total DNA from plant samples. Tissue samples were ground in a bead mill (TissueLyzer II, QIAGEN) under liquid nitrogen for 1 min at 30 Hz/s in 1.5-ml microcentrifuge tubes containing a 5-mm stainless steel bead. Plant DNA was then extracted from the ground samples using the DNeasy Plant kit (QIAGEN) according to the manufacturer's protocol and eluted in a final volume of 50 μl.

Real-Time PCR Assays. A multiplex *Taq*Man qPCR assay was used to determine the presence of Las in insect and plant tissues. Duplicate amplifications of Las-specific DNA from psyllid and plant extracts were conducted in conjunction with probe-primer sets targeting insect wingless (Wg)- or citrus cytochrome oxidase (Cox) gene regions, respectively (Thao et al. 2000, Li et al. 2006), as internal controls for DNA extractions. Each PCR assay included positive and negative controls for target and internal control sequences. PCR amplifications were carried out in an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). Psyllid reactions (26 μl) contained 12.5 μl of *Taq*Man Universal PCR mastermix (Applied Biosystems), 235 nM each of internal control primers (WgF, 5′-GCTCTCAAAGATCGGTTTGACGG-3′; WgR, 5′-GCTGCCACGAACGTTACCTTC-3′) and Las primers (LasF, 5′-TCGAGCGCGTATGCAATACG-3′; LasR, 5′-GCGTTATCCCGTAGAAAAAGGTAG-3′), 118 nM each of Wg hydrolysis probe (TTACTGACCATCACTCTGGACGC) and Las hydrolysis probe (AGACGGGTGAGTAACGCG), and 1 μl of template DNA (Li et al. 2006). Plant reactions (27 μl) were conducted similarly, but contained 270 nM each of internal control primers (CoxF, 5′-GTATGCCACGTCGCATTCCAGA-3′; CoxR, 5′-GCCAAAACTGCTAAGGGCATTC-3′), 216 nM Las primers, and 135 nM each of Cox hydrolysis probe (ATCCAGATGCTTACGCTGG) and Las hydrolysis probe (Li et al. 2006). The default ABI 7500 Real-Time amplification protocol used consisted of an initial denaturation step of 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Reactions were considered positive for either target sequence if the cycle quantification (Cq) value, determined by the ABI 7500 Real-Time software (version 1.4, Applied Biosystems), was ≤35.

Statistical Methods. Acquisition of Las by *D. citri* in laboratory and field assays was evaluated with analysis of variance (ANOVA) followed by means separation by using Fisher protected least significant difference (LSD) test (PROC GLM, SAS Institute 2005). In addition, linear regression and Pearson's correlation coefficient were used to assess the relationship between Las infection of *D. citri* and time for acquisition and pathogen retention studies. Differences among transovarial transmission rates at different life stages were compared using the Kruskal–Wallis chi-square test (PROC npar1way, SAS Institute 2005).

Results

Acquisition of Las by *D. citri* in the Laboratory. Acquisition of Las by *D. citri* adults and nymphs was positively correlated with confinement time on infected plants (b = 9.6; Pearson's correlation coefficient, $r = 0.79$, $F = 56.5$, $P < 0.0001$). Acquisition of the pathogen by adults *D. citri* was significantly lower when psyllids fed on Las-infected plants for AAPs <7 d compared with longer AAPs (ANOVA: $F = 9.16$; df = 6, 36; $P = 0.0001$). After ≥35 d of confinement on infected citrus, significantly more (39%) adult *D. citri* acquired Las than for any other AAP (Fig. 1). In addition, the level of Las infection in *D. citri* was significantly higher after a 28 d AAP than for AAPs of ≤20 d. Psyllids reared from eggs to adults on Las-infected citrus accounted for the greatest percentage of pathogen acquisition. More than 60% of newly emerged adult *D. citri* tested positive for Las after acquisition as nymphs, a significantly greater number than obtained from AAPs <35 d (Fig. 1).

Acquisition of Las in the Field. Acquisition of Las by adult *D. citri* was positively correlated with time (b = 0.02; Pearson's correlation coefficient, $r = 0.71$, $F = 10.1$, $P = 0.01$) (Fig. 2). Acquisition rates remained <15% until adults fed on infected citrus for >40 d and increased thereafter to as much as 100%. An exception to this occurred with psyllids held on plants for 28 d, which exhibited an acquisition rate of 80%. Las acqui-

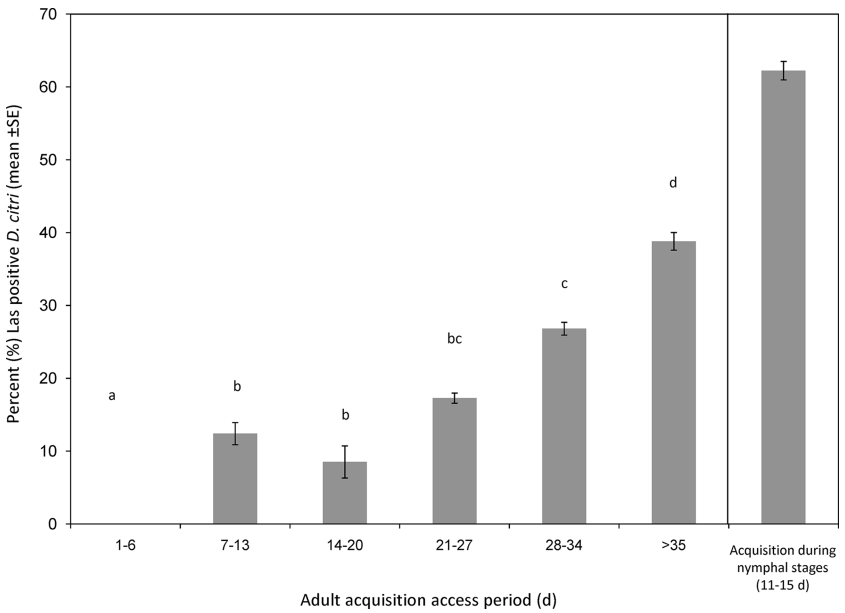

Fig. 1. Percentage of *D. citri* (mean ± SE) that tested positive for Las in laboratory experiments after feeding on infected citrus for AAPs lasting 1–45 d (adults) or for the duration of nymphal development from the egg stage through the fifth instar (11–15 d). Presence of Las was assessed using qPCR after each AAP. AAPs for adults were grouped by week for analysis. Means for adult AAPs with different letters are significantly different ($\alpha = 0.05$; Fisher protected LSD).

sition by *D. citri* was significantly higher when psyllids fed on Las-infected plants for AAPs lasting 28–42 d compared with AAPs of <28d (ANOVA: $F = 21.8$; df = 4, 72; $P < 0.0001$). The highest level of Las acquisition, 80%) occurred when adults were given access to infected plants for >42 d.

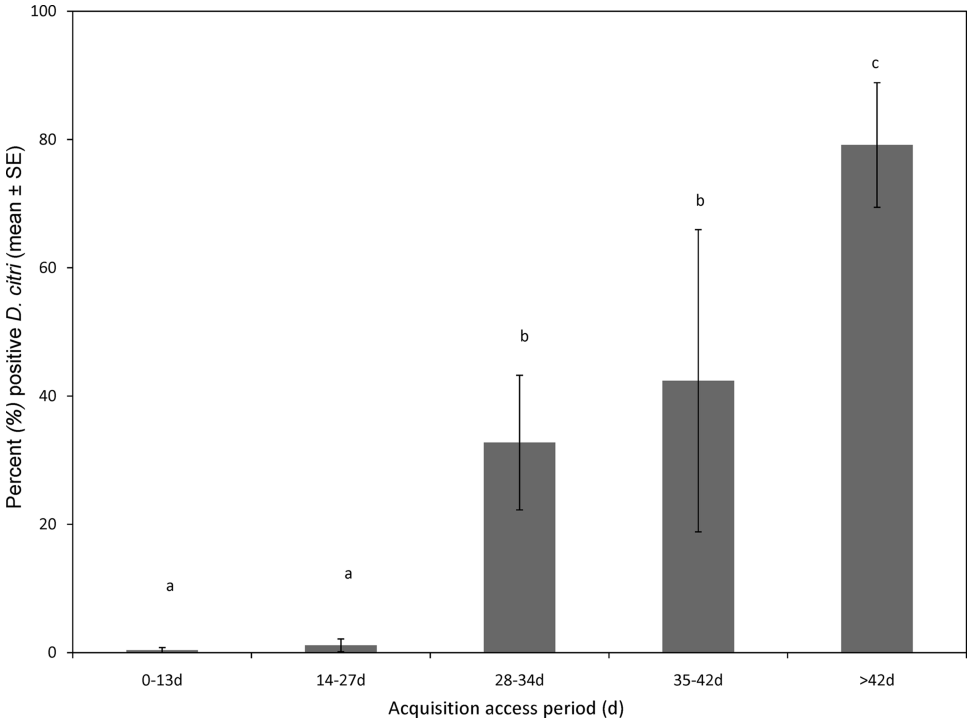

Fig. 2. Percentage of *D. citri* (mean ± SE) that tested positive for Las under field conditions after feeding on infected citrus for AAPs of 1–51 d. Presence of Las was assessed using qPCR after each AAP. AAPs for adults were grouped for analysis. Means for adult AAPs with different letters are significantly different ($\alpha = 0.05$; Fisher protected LSD).

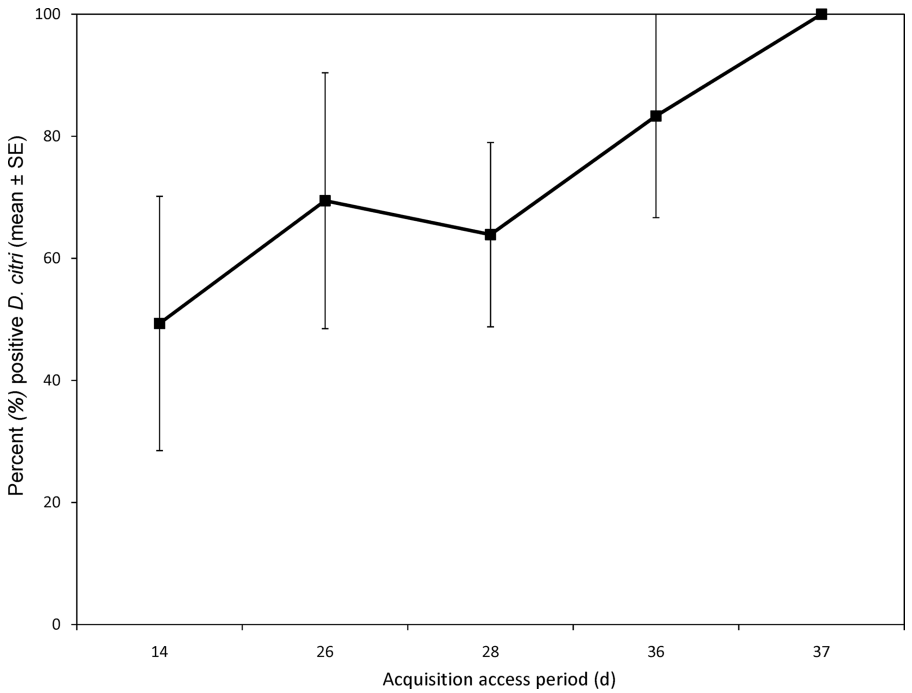

Fig. 3. Percentage of *D. citri* (mean ± SE) reared on infected citrus under field conditions that tested positive for Las (mean ± SE). Adult psyllids were collected from infected plants 14–37 d after oviposition. Presence of Las was assessed using qPCR after each AAP. The solid line indicates the regression line of best fit (y = −0.04 + 0.98).

In contrast, psyllids reared on infected citrus exhibited a mean acquisition rate of 50% at 14 d postemergence (Fig. 3). Because the progenitors of these psyllids were removed after 14 d, before the emergence of adult offspring, adults collected on day 28 could be no >14 d old. The rate of acquisition significantly increased over time such that *D. citri* offspring collected at 37 d were nearly 100% positive for Las (b = 0.02; Pearson's correlation coefficient, $r = 0.93$, $F = 18.0$, $P = 0.02$).

Retention of Las by *D. citri*. After acquisition of Las as nymphs, newly emerged adults transferred to healthy citrus experienced decreased levels of the bacterium over their lifetime. The proportion of Las-positive adult *D. citri* decreased significantly over time ($R^2 = 0.72$; Pearson's correlation coefficient, $r = -0.85$, $F = 31.3$, $P = 0.0001$) when not continuously exposed to Las-infected plants, resulting in a retention rate of <20% after 24 d on healthy plants (Fig. 4).

Inoculation of Citrus With Las by *D. citri*. In total, eight plants tested positive for the presence of Las at 3, 5, 7, 9, and 12 mo after inoculation access. The mean numbers of plants testing positive for Las after IAPs of 1, 4, 7, 15, and 24 d were not significantly different (ANOVA: $F = 0.41$; df = 4, 122; $P = 0.801$). Individual positive adult psyllids successfully inoculated citrus with Las, with a mean efficiency of 5% (Table 1). In a second experiment, groups of 200 newly emerged *D. citri* reared on Las-infected plants ($n = 15$) transmitted the bacterium to healthy sweet orange plants at a rate of 73% after a 30-d IAP. Psyllids sampled from the inoculating population tested 93.9% ($n = 33$) positive for the presence of Las.

Transovarial Transmission. In transovarial transmission tests, three stages of offspring were screened for the presence of transovarially-inherited Las: pooled eggs, third–fifth instars, and newly eclosed adults. Despite being reared on healthy (Las-free) plants, 3.6% of combined psyllid offspring (eggs, nymphs, and adults) derived from Las-infected females tested positive for Las, indicating that transovarial transmission occurred at a low rate in *D. citri* (Table 2). There were no significant differences among life stages in the F_1 generation testing positive for the presence of Las (Kruskal–Wallis, $\chi^2 = 2.56$, df = 2, $P = 0.279$).

Latent Period of Las in *D. citri*. Although positive psyllids were collected from test plants at the end of experiments, transmission of Las was not observed; therefore, we were unable to determine the latent period between acquisition of the pathogen by adult *D. citri* and subsequent inoculation of uninfected host plants.

Discussion

Acquisition of Las was greater for psyllids reared from eggs on Las-infected citrus plants compared with *D. citri* that were caged (and presumably fed) only on infected citrus as adults. The percentage of adult *D. citri* acquiring Las increased with confinement time on infected citrus plants from 0% at 1 wk to 39% at 5 wk.

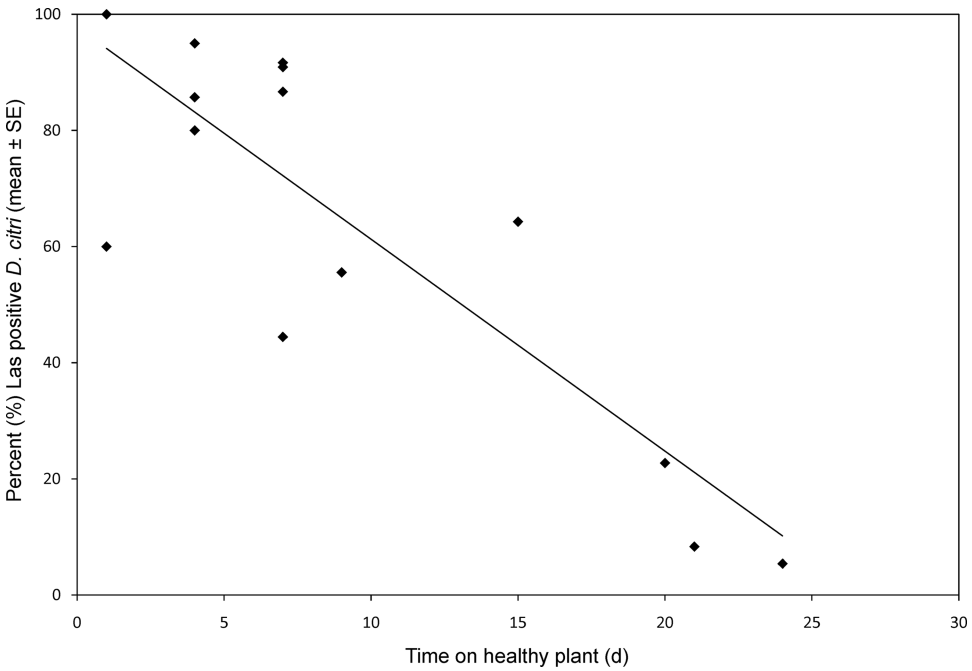

Fig. 4. Retention of Las over time by *D. citri* that were reared as nymphs on infected citrus and then held on healthy (Las-free) citrus for 1–24 d. Presence of Las was assessed using qPCR.

Longer confinement on infected plants may allow time for bacterial titer to increase. In addition, higher acquisition rates of Las over longer times is probably due to the uneven distribution of the bacterium throughout infected plants (Teixeira et al. 2008), because the probability of encountering the bacterium increases with the duration of insect feeding (Almeida et al. 2005). Our results differed from a previous Japanese study in which 88% of *D. citri* adults acquired Las after just 24 h of feeding (Inoue et al. 2009). This comparatively higher level of infection could possibly be an artifact of the small number of psyllids tested in the latter ($n = 25$). In contrast, the large sample population ($n = 478$) evaluated in our study did not produce any positive psyllids, suggesting that differences in vector competence could exist between the Japanese and Florida populations of *D. citri*. Differences in acquisition efficiencies also may be linked to features associated with the physiology of host plants, the pathogen, or both.

Changes in the efficiency of Las inoculation by single adult psyllids did not occur in response to the length of the IAP; however, a higher rate (up to 100%) of successful pathogen inoculation occurred when larger numbers of Las-positive adult psyllids were held on plants for inoculation feeding. Our results showed that a single adult psyllid was able to inoculate the pathogen after a 1-d IAP, but the overall rate of successful inoculation did not exceed 6.3% with single-psyllid inoculation feedings. The probability of successful inoculation may increase if a critical number of bacteria are required for an infection to establish (Almeida et al. 2005). We suspect that the titer of Las required to cause disease is higher than that typically

Table 1. Inoculation of *C. sinensis* with Las by individual adult *D. citri* reared on Las-infected plants

IAP[a]	Positive psyllids[b]/ total psyllids confined	Infected plants (%)[c]
1	16/35	6.3
4	42/58	7.1
7	39/48	7.7
15	27/39	3.7
24	4/61	0.0

[a] Inoculation access period (in days) provided to *D. citri* on test plants.

[b] Confirmation of Las presence in single *D. citri* determined by qPCR after IAPs.

[c] Confirmation of the presence of Las in plants determined by qPCR by 18 mo after the IAP. Percentage of infected plants refers to number of Las-infected plants/total number of plants with positive *D. citri*.

Table 2. Transovarial transmission of Las from female *D. citri* to offspring (F_1 generation)

Developmental stage[a]	n[b]	%[c]
Egg	49	2.0
Nymph	48	6.3
Adult	42	2.4

[a] Samples consisting of pooled (20–50) eggs, pooled (20–30) nymphs, or individual F_1 adults from Las-positive females were tested for the presence of Las by using qPCR.

[b] n is number of pooled (eggs and nymphs) or individual *D. citri* screened by qPCR.

[c] Percentage of *D. citri* positive by qPCR.

inoculated by a single psyllid. The high rate of successful inoculation we observed when plants were exposed to 200 Las-positive psyllids supports this hypothesis, although additional studies with quantitative PCR are needed to determine the minimum titer required for successful inoculation.

Inoculation rates resulting from IAPs by single adult psyllids in this study were generally less efficient than rates previously reported for *D. citri.* Xu et al. (1988) reported 80% of test plants exhibited HLB symptoms after inoculation feeding by single adult psyllids in China. Successful transmissions in this study was determined based on the appearance of HLB symptoms on test plants, in absence of molecular diagnostic tools now available to researchers. In a more recent study using qPCR to detect successful transmission, no inoculation was observed from a population of Japanese psyllids that acquired the pathogen as adults, although groups of three adult *D. citri* newly emerged from nymphs reared on Las-infected plants transmitted at a rate of 66% after a 30-d IAP (Inoue et al. 2009). In contrast, transmission by psyllids in Taiwan less efficient than observed in our study; after inoculation assays; only five of 380 plants exposed to adult psyllids for a 1-mo IAP exhibited HLB symptoms (Huang et al. 1984). Differences in inoculation efficiency among populations of insect vectors of plant viruses have been documented, although there are few examples of population-level differences in the transmission of plant bacterial pathogens (Purcell 1982, Galetto et al. 2009). The mollicute *Spiroplasma citri,* for example, is transmitted with differential efficiency by different populations of beet leafhopper, *Circulifer tenellus* (Baker) (De Almeida et al. 1997). It is possible that genetic differences in the Florida population of *D. citri* produce phenotypes that have a reduced capacity for transmission. Furthermore, the physiological condition of plants may confer resistance to Las inoculated by psyllids.

Alternatively, differences in acquisition and inoculation efficiency among psyllid populations may be due to the host plant used in assays. We selected sweet orange as our source for pathogen acquisition because it is representative of the typical host encountered by *D. citri* in commercial citrus groves in Florida. Certainly, the host plant–pathogen interaction could influence the efficiency of acquisition and inoculation. Differential susceptibility of hosts is commonly associated with generalist pathogens (Tooley and Kyde 2007, Lopes et al. 2009), but there is little information regarding differences associated with narrow host ranges. Although numerous *Citrus* species and their relatives have been reported as hosts for Las (Halbert and Manjunath 2004), pathogen acquisition from these various hosts and subsequent inoculation of citrus plants remains unexplored. Thus, use of varying citrus varieties (e.g., sour orange, sweet orange and rough lemon citrus) for assays may underlie differences in reported transmission efficiency.

In addition to laboratory assays, we conducted acquisition experiments under field conditions which corroborate the correlation between access time (with actual feeding of unknown duration) and pathogen acquisition rate. These studies further confirm the higher rate of pathogen acquisition by psyllids reared on Las-infected trees, compared with psyllids that acquired the pathogen as adults. Variation in the rate of psyllids acquiring Las under field conditions as adults, particularly on day 28 (Fig. 2), may reflect the variable distribution of the pathogen within host trees. Adults caged on branches with high bacterial titers would presumably have a greater likelihood of acquiring the pathogen compared with psyllids held on branches with low titers. Additional studies of Las acquisition by adults by using qPCR are needed to determine the relationship between acquisition and bacterial titer within the plant. The observed high acquisition rates in psyllid offspring caged on infected trees for longer periods probably resulted from continuous acquisition postemergence, bacterial replication, or possibly a combination. Although the occurrence of transovarial transmission could not be completely addressed in the field acquisition experiment, the results of our transovarial transmission study indicate that the acquisition rate in adult offspring was probably augmented by maternal passage of the bacteria directly to offspring.

Retention of Las in adult psyllids that acquired the pathogen as nymphs declined over time, despite reports of bacterial multiplication in previous studies conducted with different populations of *D. citri* or *T. erytrae* and Liberibacter (Moll and Martin 1973, Inoue et al. 2009). Lower rates of inoculation efficiency after increased time postpathogen acquisition have been reported in other plant–pathogen–insect systems, including *Spiroplasma citri* and its insect host beet leafhopper (Purcell 1982, Whitcomb et al. 1973). Here, we used qPCR to assess the level of Las in psyllids in response to time postacquisition, rather than directly assessing the relationship between time postacquisition and inoculation rate, due to the generally low rate of inoculation observed in our previous experiments. Declines in pathogen titer over time may be the result of initial concentrations of Las in the psyllid, host aging, or negative effects of the bacterium on the insect host (e.g., Purcell 1982), even though multiplication may occur. Alternatively, decreases in the number of psyllids carrying Las strongly suggest that the pathogen does not persist in *D. citri.* Although these psyllids may test positive for Las immediately after acquisition, the absence of multiplication in adult *D. citri* would explain our observation of increasingly higher rates of Las-negative psyllids over time. Although multiplication of Las in nymphs was not directly assessed in our studies, our results to not exclude the possibility that replication of Las does occur in *D. citri* nymphs. Additional studies that quantify bacterial titers over time are needed to determine whether Las propagates in nymphs and to confirm our observation that Las are not propagative in *D. citri.*

The latent period of Las before successful inoculation of citrus plants by *D. citri* was not defined in our study, presumably due to the low inoculation effi-

ciency by individual psyllids. Previous reports of the latent period in *D. citri* ranged from 24 h to 25 d (Roistacher 1991, Xu et al. 1988). Although only plants exposed to psyllids that tested positive for Las by qPCR were screened in the latency experiments, we suspect that an insufficient titer of bacteria was inoculated into susceptible plants for the development of HLB. Below a certain threshold pathogen titer, it is possible that plants may be able to resist Las infections. Further studies are needed to elucidate plant resistance mechanisms that may confer protection against Las infection. Under field conditions, longer feeding periods and exposure to multiple infected psyllids would be expected to result in higher pathogen transmission rates.

One of the primary goals of this study was to determine whether *D. citri* can transmit the HLB associated bacterium vertically from parent to offspring. Although Las-positive nymphs have been reported from asymptomatic citrus in Florida, it was not determined whether the plant tissues from which the nymphs were collected were also Las-positive before psyllid infestation (Manjunath et al. 2008). Xu et al. (1988) reported that no transovarial transmission occurs in *D. citri;* albeit, in their study, the infection status of the parental generation was unknown and successful pathogen inoculations were scored on the basis of visual symptoms. If transovarial transmission does not occur, psyllids nymphs could possibly act as sentinels for asymptomatic plants in citrus groves because the bacteria are more readily detected in psyllids than in plant tissue; however, the feasibility of sampling psyllid nymphs remains untested (Manjunath et al. 2008). Field-collected nymphs that test positive for the presence of Las would indicate that their natal host tree also is infected with the bacterium. Our observation of transovarial passage in the current study suggests that a small number of psyllids have the capacity to inoculate Las immediately after emerging as adults on a healthy plant if they were the offspring of a Las-positive female.

Control measures for arthropod-borne plant pathogens are more effective when the nature of the pathogen–vector relationship is understood. The current strategies for managing the spread of HLB rely on vector control and removal of Las-infected trees (Brlansky and Rogers 2007); therefore, a clear understanding of the salient aspects of Las transmission by Florida *D. citri* is essential for constructing and tailoring disease prevention tactics. Collectively, our results indicate that adult *D. citri* that acquire Las through feeding on infected plants are less likely to successfully inoculate the pathogen compared with adults that acquired the pathogen as nymphs. Preventing oviposition and subsequent development of nymphs on Las-infected citrus plants may provide significant disease control if included as part of an integrated pest management strategy. Future studies are needed to elucidate whether there is a genetic basis to the clear variation in transmission biology that occurs among *D. citri* populations. In addition, this is the first study to provide evidence for the existence of transovarial transmission in *D. citri.*

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