Grove-level analysis of titer and prevalence of "*Candidatus* Liberibacter asiaticus" and *Wolbachia* in *Diaphorina citri,* vector of citrus Huanglongbing

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Abstract

Huanglongbing (HLB, or citrus greening disease) affects all citrus varieties world-wide. In the USA, Asia, and South America the causal agent is "*Candidatus* Liberibacter asiaticus" (*C*Las), a phloem-limited, uncultured, alphaproteobacterium. The hemipteran insect vector, *Diaphorina citri* (Asian citrus psyllid) acquires and transmits *C*Las in a circulative, propagative manner. In addition to *C*Las, *D. citri* hosts multiple symbiotic bacterial species including *Wolbachia* (wDi). In

23 *D. citri*, wDi has been sequenced and studied but specific roles in *D. citri* biology are unknown.
24 Using well established quantitative PCR methods we measured *C*Las titer in *D. citri* collected
25 from four groves in central Florida with distinct HLB management strategies and tested
26 whether *C*Las and wDi titer were correlated in a sub-set of these insects. Grove site had the
27 largest effect on *C*Las titer. Sex had no effect on *C*Las titer, while higher wDi titer was correlated
28 with non-infected insects. Our results suggest that more directed follow-up research is
29 necessary and important to clarify whether field management tactics influence *C*Las titer in *D.*
30 *citri* and to better understand gene-by-environment interactions among *D. citri*, wDi and *C*Las.
31 Now that millions of trees in Florida have been treated with injectable formulations of
32 oxytetracycline, which is likely to decrease bacterial populations in *D. citri*, this study may
33 represent the last biologically meaningful snapshot of grove-level vector-pathogen ecology in
34 the state during the HLB epidemic.

35 **Introduction**

36 Huanglongbing (HLB, also known as citrus greening disease) affects all citrus varieties
37 world-wide, a total of 61 countries as of December 2022) (Ghosh et al. 2023) and after decades
38 of targeted research for solutions and over $1 billion US dollars spent in research, HLB remains
39 the most devastating disease of citrus with no cure. It is caused by an unculturable,
40 intracellular, phloem-limited bacteria in the genus "*Candidatus* Liberibacter". The predominant
41 species causing HLB is "*Ca.* L. asiaticus" (*C*Las). Two other species, "*Ca.* L. africanus" and "*Ca.* L.
42 americanus" have also been described in multiple African countries and South-Central
43 American countries, respectively. *C*Las is spread from tree to tree by the insect vector
44 *Diaphorina citri* (Asian citrus psyllid), a hemipteran in the family Psyllidae which feeds

45 exclusively on the phloem of citrus trees as well as species of *Murraya*. *C*Las is transmitted by *D.*
46 *citri* in an intracellular, circulative, propagative manner (Ammar et al. 2016; Inoue et al. 2009;
47 Higgins et al. 2022). While *C*Las can also spread via grafting infected citrus budwood, vector-
48 borne dispersal is the predominant and least controllable method causing new citrus infections.
49 Lastly, *C*Las can infect periwinkle via dodder, but periwinkle is not a host for *D. citri* (Garnier and
50 Bove 1983).

51 Management of the vector requires an understanding of basic epidemiology and vector
52 biology in the field. In the United States, strict testing regimens are followed in Texas and
53 California to maintain quarantine areas for the vector and pathogen. But HLB is now endemic in
54 Florida. Despite the frequency of psyllid testing and a known increase in insecticide resistance
55 by *D. citri* (Chen et al. 2022; Vázquez-García et al. 2013; Naeem et al. 2019; Rao et al. 2014;
56 Pardo et al. 2018; Venkatesan et al. 2022), relatively few papers have been published on
57 population-level dynamics of *C*Las-*D. citri* infections in the field. A few studies have looked at *D.*
58 *citri*-*C*Las titer and prevalence in different situations, including specific field sites, month to
59 month changes, year to year trends, and differences between countries (Bové et al. 1993; Wulff
60 et al. 2020; Capoor et al. 1974; Coy and Stelinski 2015; Hall 2018; Manjunath et al. 2008; Razi et
61 al. 2014; Tiwari et al. 2010; Sétamou et al. 2020; Ukuda-Hosokawa et al. 2015; Walter et al.
62 2012). These studies reported high levels of variation in *C*Las titer across both the individual and
63 population level. Genetic studies have also shown that psyllid populations vary in their *C*Las
64 acquisition, transmission and titer phenotypes and that this variation is stable and heritable
65 over many generations, consistent with these phenotypes being regulated by *D. citri* genes and
66 proteins (Ramsey et al. 2015; Mann et al. 2018; Ammar et al. 2018).

67 In addition to *C*Las, *D. citri* hosts multiple symbiotic bacteria which it maintains because
68 they provide various benefits (such as nutritional supplements and defensive polyketides)
69 (Hosseinzadeh et al. 2019b; Nakabachi et al. 2013). *Wolbachia* (wDi) is one such endosymbiont
70 found in *D. citri*, as well as over 50% of all insects. At least two genetically distinct populations
71 of wDi have been shown to infect *D. citri* in Florida (Chu et al. 2019). Understanding how wDi
72 interacts with both *D. citri* and *C*Las may be important to the future management of psyllid
73 population (Chu et al. 2019; Jain et al. 2017). In *D. citri*, wDi has been sequenced (Neupane et
74 al. 2022; Saha et al. 2012; Shippy et al. 2019) and studied but its specific role in *D. citri* is still
75 unclear. Studies suggest wDi is 100% present in *D. citri* individuals (Hosseinzadeh et al. 2019b;
76 Guidolin and Cônsoli 2013; Hoffmann et al. 2014) and is found infecting many organs
77 (Hosseinzadeh et al. 2019b; Ren et al. 2018) of *D. citri*. The bacteriome, the endosymbiotic
78 organ where the other psyllid endosymbiont bacteria reside, maintains lower titers as
79 compared to other organs (Hosseinzadeh et al. 2019b).

80 The goal of this study was to compare *C*Las titer of psyllids collected from four groves in
81 Florida in 2018 with high disease pressure to better understand the natural variation that exists
82 in *C*Las titer across different populations of *D. citri* and to test whether factors such as sex or
83 wDi titer influence *C*Las titer.

84 **Methods**

85 Sample collection

86 Four citrus groves in central Florida, near lake Okeechobee, were sampled for adult
87 *Diaphorina citri* during the first week of October, 2018 (see **Figure S1**). Individual *D. citri* were
88 collected into 70% ethanol using mouth aspiration. At least 1200 individual *D. citri* were

89 collected from each field. Samples were air dried and randomly aliquoted into groups of 100,
90 promptly flash frozen in liquid nitrogen and stored at -80 ℃ until DNA extractions.

91 DNA extraction and quantification

92 DNA extractions were performed in three batches on a total of 1062 samples, randomly
93 selected from each field site in varying amounts, using the Qiagen DNeasy Blood and Tissue 96
94 extraction kit. This kit allowed 96 samples to be extracted simultaneously with a filter-based
95 method. The methods advertised with the kit were followed with minor adjustments as follows.
96 First, individual *D. citri* were sexed under a dissecting scope over ice, then moved via paint
97 brush to the 96-well racks of sample tubes supplied by the Qiagen kit. Three, 3.2mm steel
98 beads were added to each tube and the samples were capped using the rubber tube caps
99 provided, throughout the first and last steps of the protocol. Samples were disrupted for 2
100 minutes at 1000 rpm using a mechanical homogenization with a SPEX Sample Prep MiniG 1600
101 (Thermo Fisher). The samples were kept on ice before and after sample disruption. All
102 centrifugation steps were performed at the maximum 4000 rpm (RCF of 3434 x g) available
103 when using the Hermle Z466 3L swing-out rotor with square microplate buckets and inserts to
104 accommodate the stacked column and flow-through plates. Samples were lysed for 45 min at
105 56 ℃ in a water bath and mixed once midway. Following lysis, 4 ul RNase A per sample was
106 added to purify the genomic DNA, at which point, instead of tube caps, plastic PCR plate covers
107 were used to cover the 96-well racks. Samples were eluted once using 50 ul AE buffer then
108 stored at -20 ℃ until DNA quantification and purity checks, which were performed using a
109 Nanodrop 2000.

110 Quantitative PCR

111 Quantitative polymerase chain reaction (qPCR) was performed on all 1062 samples
112 using TaqMan hydrolysis probes, testing for the presence and relative titer quantification of the
113 "*Candidatus* Liberibacter asiaticus" (*C*Las) 16S rRNA gene. The 16S rRNA forward primer (5′-
114 TCGAGCGCGTATGCAATACG-3′), reverse primer (5′-GCGTTATCCCGTAGAAAAAGGTAG-3′) and
115 probe (5′-FAM-AGACGGGTG/ZEN/AGTAACGCG-3′) were used, as previously established
116 (Wenbin Li, 2006). The probe includes a fluorescent dye (5′ 6-FAM) and a quencher (ZEN, 3′
117 Iowa Black FQ). Standard thermal conditions were used during the qPCR. Specifically, 95 °C for
118 10 min (1 cycle) and 95 °C for 15 sec and 60 °C for 60 sec (40 cycles total). Each qPCR 384-well
119 plate included a standard curve dilution series (a serial dilution from 10^9 down to 10^3 of the
120 synthetic plasmid pUC57 containing a *C*Las 16S rDNA gene fragment). The resulting y-intercept
121 was 38.303, R^2 = 0.964, slope = -3.095, and efficiency = 110.452%. Each qPCR plate also
122 contained positive and negative controls run with two technical replicates each. A subset of
123 samples from the first batch were subjected to additional qPCR using SYBR Green reagents to
124 access wDi presence-absence and estimate titer. The wDi *ftsZ* gene (ftsZ-81) (A. S. Guidolin
125 2013) forward primer (5'AGCAGCCAGAGAAGCAAGAG3') and reverse primer
126 (5'TACGTCGCACACCTTCAAAA3'), and the *Wingless* (*WgL*) (MyLo L. Thao 2000) forward primer
127 (GCTCTCAAAGATCGGTTTGACGG) and reverse (GCTGCCACGAACGTTACCTTC) primers were run
128 in parallel on the same 386 well plate. All samples on every qPCR plate were run in duplicate (2
129 technical replicates) then averaged to create the reported final quantitative cycle (Cq) values.
130 The maximum number of qPCR cycles is 40, where Cq = 40 means the primers did not create a
131 product that could be amplified so the sample is non-infected or titers are below the limit of

132 detection, while Cq < 40 suggests copies of the target gene are present, and the insect is
133 infected. *C*Las cell counts were calculated from Cq using the standard curve and the
134 QuantStudio6 software. Each qPCR well contained 20 ng of DNA from a single *D. citri.* The
135 QuantStudio6 software associated with the qPCR machine used the standard curve dilution
136 values and resulting best-fit linear equation and efficiency metrics to internally calculate the
137 *C*Las cell equivalent to the number of copies of the *C*Las rDNA sequence in a single *D. citri*. The
138 values were exported from the software as an excel document. *C*Las cell equivalents for
139 technical replicates of each sample were averaged (note, Cq = 40 yielded undetermined values,
140 which were transformed to zeros for calculations of cell equivalents), then all samples from
141 each field were averaged. Field mean *C*Las cell equivalent was found by taking the difference.

142 **Statistical Methods**

143 A linear mixed-effects model (LMM) was used to analyze the *C*Las Cq data collected on
144 the individual insects... Two models were compared to determine whether grove makes a
145 significant difference as a random effect. Model 1 included both DNA extraction batch and
146 grove as random effects, and sex as a fixed effect. Model 2 was reduced, containing only batch
147 as a random effect and sex as a fixed effect, with grove absent. An ANOVA was used to test the
148 difference between the two models. Model assumptions of normality and homogeneous
149 variance were assessed visually using the model residuals. *P*-values < 0.05 were considered
150 statistically significant. The mean absolute titer of 918 individuals was calculated from a
151 standard curve, then the mean absolute titer was calculated for each field site. For each
152 pairwise field comparison, non-zero titers were used to calculate field-specific means, the
153 means subtracted to find the difference and the absolute value was reported to highlight the

154 magnitude of the difference the second from the first (**Figure 2**). A subset of the samples from
155 batch number one, representing all four fields, was additionally tested for the dual effect of wDi
156 (Cq values normalized by *Wingless* Cq) and grove on *C*Las Cq. R packages (R Core Team 2022)
157 were required for the analysis of both *C*Las and wDi, including *ggplot2* (Wickham 2-16), *ggpubr*
158 (Kassambara 2020), and *lmertest* (Kuznetsova et al. 2017). To test for interactions between *C*Las
159 and wDi in the insects, wDi Cq and *C*Las Cq were converted into binary values as follows: for
160 wDi, a value of 0 was assigned for Cq values below the median, and a value of 1 was assigned
161 for values above median and for *C*Las, a value of 0 was assigned for Cq values less than 40, and
162 a value of 1 for undermined Cq values or Cq=40). The association between wDi and *C*Las titer
163 was assessed using a Wilcoxon test, which clarified whether wDi Cq above and below the
164 median was different in CLas (+) and (-) insects, and a Chi-squared test in R to test whether the
165 wDi Cq distribution around the median differs from the distributions of *C*Las Cq below or at
166 Cq=40.

167 **Results**

168 1. Each grove had a distinct characteristics, including tree age, type and management
169 strategies.

170 Groves were assigned names corresponding to their town of origin to protect the
171 identity of the participating growers. Psyllid infestations were found by scouting for flush points
172 with nymph honeydew deposits. A total of 450 adult *D. citri* were used from the Port St. Lucie
173 grove. This grove was one of two organically managed groves we sampled from, however, Port
174 St. Lucie did not utilize any pesticides, herbicides or other chemical inputs to our knowledge, at
175 the time of sampling. It maintained a high *D. citri* population as well as the highest observed

176 levels of in-field entomopathogenic fungi (Higgins et al. 2022) and other insects (including
177 ladybeetles, ants, caterpillars and wasps). Multiple citrus species as well as *Murraya spp.* grew
178 within the grove. The majority of *D. citri* samples were taken from *Citrus sinensis* (Valencia
179 orange). Trees ranged in age from recently re-planted to well established, bearing trees. We
180 used a total of 164 *D. citri* adults from *C. sinensis* in the second grove site, referred to as
181 Pioneer. This grove was also organically managed, and while organic pesticides were used, only
182 a regimen of periodic nitrogen, phosphorous and potassium (NPK) supplementation was
183 described in particular during sample collection, and any other treatments utilized are
184 unknown. Trees were mature and bearing and well-spaced out. A total of 176 samples were
185 used from an experimental plot within a third grove, Whidden Corner. This grove was
186 composed of mostly small, juvenile, recently planted *C. sinensis* trees undergoing heavy
187 pesticide treatment for *D. citri* control, with applications occurring every three weeks. A total of
188 272 adult *D. citri* were used from a fourth citrus grove, referred to as Montura. These insects
189 were sampled from *C. sinensis,* and this grove also experienced insecticide sprays every three
190 weeks. The trees were bearing, tall, and thick with leaves. In all groves, rootstock type was
191 unknown, and the majority of *D. citri* were collected from flush.

192 2. Grove had the largest effect on *C*Las Cq.

193 The random effect of grove site significantly outweighed any effect of sex or DNA
194 extraction batch on *C*Las Cq (Chi^2 = 462.01 , df = 2, $P < 0.001$). The effect of DNA extraction
195 batch was included in the model to rule out a potential bias, and we found no between-batch
196 variability (var. = 0.010, SD = 0.010) suggesting high accuracy of technical replications of DNA
197 extraction.

198 The four populations of *D. citri* fell into three broad categories based on *C*Las titer and
199 secondarily, infection rates. After accounting for effects of batch and sex, Pioneer represents
200 the "low titer" population and has the lowest overall titer (bacterial load per individual *D. citri*)
201 with mean Cq = 39.0 and standard error (SE) = 0.32. Montura has the highest titer (mean Cq =
202 30.4, SE = 0.28) and represents the "high titer" population, while Port St. Lucie (mean Cq = 32.1,
203 SE = 0.30) and Whidden Corner (mean Cq = 31.6, SE = 0.30) report similar bacterial titers and
204 make up the third population of "medium titer" individuals. When absolute titer of *C*Las is
205 calculated from Cq and a standard curve, the three groups are still present. Means were
206 calculated using only samples with Cq<40, and Montura had the highest titer (29,630 *C*Las cell
207 equivalents), Pioneer the lowest titer (41 *C*Las cell equivalents), while Whidden Corner and Port
208 St. Lucie fell in the middle (8,912 and 11,270 *C*Las cell equivalents, respectively).
209 The percent infection rate is calculated using the proportion of insects with Cq values
210 lower than 40, as Cq = 40 are considered non-infected with *C*Las. While the three groups above
211 are determined by *C*Las titer, their infection rates do not necessarily match the trend. The "low
212 titer" population represented by Pioneer did also have the lowest *C*Las infection rate (26.82 %).
213 However, the "medium titer" populations represented by Port St. Lucie (97.11 %) and Whidden
214 Corner (96.02 %) recorded the highest percent infection rates. Finally, the "high titer"
215 population represented by Montura maintained the second-lowest percent infection rate
216 (94.85 %). The three populations as determined by mean Cq can be seen in **Figure 1** and pair-
217 wise results between grove populations (**Figure 2**) additionally confirm visual differences
218 between groves.
219

Figure 1. Box plot of *C*Las Cq distribution by citrus grove collection site and colored by sex. Box plot center lines are the median, box top and bottom edges are the first and third quartile respectively, and outliers are points that extend past the tails. NA samples (grey) are those that have a *C*Las Cq value but no recorded sex. M = male (blue), F = female (red).

Absolute *C*Las Cell Equivalent

	Port St. Lucie	Pioneer	Whidden Corner	Montura
Montura	18,360	29,588	20,717	**29,630**
Whidden Corner	2,357	8,870	**8,912**	
Pioneer	11,288	**41**		
Port St. Lucie	**11,270**			

Quantification Cycle (Cq) of *C*Las

	Port St. Lucie	Pioneer	Whidden Corner	Montura
Montura	1.7	8.6	1.2	**30.4**
Whidden Corner	0.5	7.4	**31.6**	
Pioneer	6.9	**39**		
Port St. Lucie	**32.1**			

Figure 2: Pair-wise field comparisons using both mean *C*Las Cq and titer of *C*Las (genome copies of *C*Las in 20 ng of *D. citri* DNA). Field-specific means were computed using only non-zero absolute titer values, or Cq < 40 values and the absolute values are reported.

3. The sex of *D. citri* and color morph had no effect on *C*Las Cq means.

Sex of each *D. citri* individual was recorded. Of the 929 samples with recorded sex, the overall ratio of males to females was nearly equal (Male: n=454, 48.86%; Female: n=475, 51.13%) as was the ratio of males to females in each grove (Montura: Female=41.54%, n=113, Male=46.32%, n=126; Pioneer: Female=42.68%, n=70, Male=39.02%, n=64; Port St. Lucie: Female=50.00%, n=225, Male=42.22%, n=190; Whidden Corner: Female=38.07%, n=67, Male=42.05%, n=74) (see **Table S1A** for further breakdown of male-female results).

The effect of sex on *C*Las Cq was tested and included in our model as *C*Las infection has been shown to affect *D. citri* behavior differently by sex. There was no effect of sex on *C*Las Cq

241 after controlling for grove and DNA extraction batch (F = 0.489, df =1, *P* = 0.484). This can also
242 be seen in **Figure 1**, as the blue and red colors show a random distribution of sex by Cq value
243 for each of the four groves.

244 In laboratory experiments, the color morph of *D. citri* was shown to be associated with
245 *C*Las titers (Hosseinzadeh et al. 2019a). While it was technically challenging to accurately assign
246 the color morphology to each specimen due to the insects being stored in ethanol prior to data
247 collection, no obvious associations between *C*Las titer and color morph emerged in these
248 insects (**Table S1B**). The lack of association between color morphology and *C*Las infection status
249 has previously been reported in the analysis of other grove-captured, Florida psyllid
250 populations (Hall et al. 2018).

251 4. *Wolbachia* (wDi) has higher titer in non-infected *D. citri.*

252 A subset of *D. citri* samples from the initial DNA extraction were tested for wDi titer. The
253 total number of samples tested was 176 (Port St, Lucie = 46, Pioneer = 42, Montura = 41,
254 Whidden Corner = 47). A linear regression compared wDi Cq to *C*Las Cq , revealing no
255 correlation between *C*Las and wDi Cq among *D. citri* from these Florida citrus groves (R^2 =
256 0.0005) **(Figure S2**). Additionally, wDi Cq was normalized against the *D. citri Wingless* gene
257 (**Figure S3).** A linear model assessed the relative effects of both grove and normalized wDi Cq
258 on *C*Las Cq. The analysis indicated that wDi was not significantly correlated with *C*Las Cq (F =
259 0.646, df = 1, *P* = 0.426). Moreover, the source grove had the most substantial effect on *C*Las
260 titer (F = 22.881, df = 3, *P* < 0.001, **Table S2**).

261 During the regression analysis, many wDi samples exhibited low Cq values when *C*Las Cq
262 = 40 (indicating non-infected samples, see **Figure S2)**. We investigated if wDi titer differed

263 significantly based on *C*Las presence (defined as Cq < 40) or absence (Cq = 40 Our analysis
264 revealed that when *C*Las is absent, wDi Cq was more likely to be below the median wDi Cq
265 value (Wilcoxon, n=119, P = 0.017). Furthermore, a Chi^2 test confirmed a significant difference
266 in the proportion of wDi samples with Cq above and below the median *C*Las Cq distribution,
267 when that distribution is divided by infection or no infection ($X^2(1, n = 119) = 6.086$, P = 0.013).
268 This exploratory testing appears to show that non-infected *D. citri* tend to have higher wDi
269 titers compared to *C*Las-infected individuals.
270

271 **Discussion**

272 Natural variation in vector competency plays a role in disease epidemiology. Vector
273 competency is the potential of a vector to transmit a pathogen. In *D. citri*, vector competency is
274 in part regulated by *D. citri* genes and proteins functioning at distinct tissue barriers which *C*Las
275 must overcome prior to inoculation into a new host tree (Ammar et al. 2018; Mann et al. 2018;
276 Kruse et al. 2017, 2018; Hosseinzadeh and Heck 2023). The infection of the host tree and citrus
277 variety has also been shown to play a role in modulating vector capacity (Ramsey et al. 2022;
278 Chin et al. 2020; Lombardi et al. 2024; Coletta-Filho et al. 2014). *D. citri* acquires and transmits
279 *C*Las in an intracellular, circulative, propagative manner. Vector competency in our study was
280 measured through infection rate and *C*Las titer for each individual in each population studied.
281 Studies have shown that approximation of *C*Las titer in individual insects can predict vector
282 capacity. There is a strong positive correlation between *C*Las titer in the psyllid and
283 transmission efficiency (Ammar et al. 2018; Hosseinzadeh et al. 2019a). Broadly, our study

284 shows that there are population-level differences in *D. citri* vector competency that are likely
285 maintained through a combination of gene by environment interactions.

286 During circulative propagative transmission, *C*Las must cross the gut epithelial cell layer
287 to enter the body cavity of the psyllid and to be considered "acquired". Next, *C*Las must
288 circulate throughout the body and replicate in tissues, especially the salivary glands, where it
289 has been shown to reach high relative titers. From the salivary glands, transmission is possible
290 and can be inoculated into healthy citrus plants while feeding. The efficiency of the first and
291 second steps can be approximated by using qPCR to detect *C*Las titer in adults that have
292 acquired *C*Las, while the third step can only be measured by careful transmission studies. The
293 development of vector-competent adults requires *C*Las acquisition to occur during the psyllid's
294 nymphal stages (Inoue et al. 2009; Ammar et al. 2016; Igwe et al. 2022). This study
295 approximates the grove-wide vector capacity of four *D. citri* populations through
296 measurements of *C*Las titer in individual adult insects. A key assumption of this study is that the
297 majority of trees in all citrus groves of Florida are infected with *C*Las and thus, all adult psyllids
298 will have fed on *C*Las-infected citrus as nymphs and are competent transmitters as adults.
299 However, even if this assumption did not hold for every individual in our dataset, multiple
300 studies on vector acquisition rates in the lab have recorded <100% acquisition, despite feeding
301 on known infected material (Pelz-Stelinski et al. 2010; De Souza Pacheco et al. 2020). Samples
302 that do test positive for *C*Las are competent transmitters and fall into categories of low to high
303 vector capacity, as determined by their mean *C*Las titer (Ammar et al. 2018).

304 Many factors may influence *D. citri* vector competency and the variation we observed in
305 our study. Prior work has shown that host switching influences variation in *D. citri* vector

306 competency (Ramsey et al. 2022; Lombardi et al. 2024). While grove-collected sampling makes
307 it difficult to control or understand all variables that may influence *C*Las titer in psyllids, all four
308 *D. citri* populations were sampled from the same citrus variety (*Citrus sinensis*), within the same
309 week in October, 2018, so it represents a snapshot in time of the potential of *D. citri* in these
310 regions to acquire and transmit. Additionally, the role that symbionts like wDi might play in
311 mediating vector-pathogen interactions is unclear, so the effect of wDi on *C*Las titer from these
312 four fields was also tested. Our results show that *D. citri* has higher wDi titer in CLas-negative
313 individuals, while field site has the strongest effect on *C*Las titer, and sex does not have any
314 effect. The field effect could be related to the heritability of *D. citri* genes involved in vector
315 competency, the management regimen, or transient access to and feeding on non-citrus host
316 plants such as *Murraya spp.* in the grove agro-ecosystem. More work is required to fully explore
317 these hypotheses.

318 Abandoned groves have also been shown to maintain high *D. citri* populations that
319 travel frequently between managed and abandoned areas (Sétamou et al. 2022; Boina et al.
320 2009) with equally high *C*Las titers and infection rates as alive and managed groves (Hall 2018).
321 Prior to 2010, Tiwari et al. (Tiwari et al. 2010) studied the *D. citri*-*C*Las infection rate and found
322 1.9 % in abandoned and 1.2 % in managed grove populations while the mean *C*Las Cq value was
323 32.52 in abandoned and 25.95 in managed groves. Over 10 years after Tiwari et al. collected
324 their data, the infection rates found by our study in the least infected, but still managed, grove
325 are at least 1.5x higher incidence while our other three groves have at least 50x higher infection
326 rates. This is undoubtedly due to the vastly increased number of both managed and abandoned
327 infected trees in Florida over the past ten years. A study by De Souza Pacheco et al. (De Souza

328 Pacheco et al. 2020) shows that when *D. citri* are raised on *C*Las-infected citrus for four
329 consecutive generations in a greenhouse setting, the percent-infection and the titer in
330 individuals increased with each generation from 42% to 100% infection incidence, and 47.9 to
331 5,390 *C*Las cells per 100 ng of DNA. These results suggest that interruptions in vector
332 generations (such as through regular insecticide applications or during seasons of low flush) in
333 the field may help keep *C*Las titer and incidence lower in vector populations. The increase in
334 infection incidence seen in Florida groves despite various management innovations over the
335 last decade is a testament to both the effect of continuous populations of *D. citri* replicating on
336 infected citrus and reference to the accumulating impact HLB has wrought on the Florida citrus
337 industry.

338 Vector competency of *D. citri* populations and individuals varies widely in the field as we
339 have seen through this study and others (Capoor et al. 1974; Coy and Stelinski 2015; Hall 2018;
340 Ukuda-Hosokawa et al. 2015; Ammar et al. 2018; Sétamou et al. 2016; Xu et al. 1988), in
341 addition to laboratory-kept colonies (Inoue et al. 2009; Hosseinzadeh et al. 2019b; De Souza
342 Pacheco et al. 2020). In agreement with our results, Chu et al (Chu et al. 2016) show that three
343 wild populations of *D. citri* (including a total of 123 insects collected in 2015) vary in their *C*Las
344 titer and *C*Las infection incidence, and that like our study, population (grove site) has the
345 strongest effect on all their models. Coy and Stelinski collected their samples in 2014 from six
346 locations and showed large vector capacity variation ranging from 35-100%, but they collected
347 less than 300 samples in total (Coy and Stelinski 2015). Hall published results from two studies
348 with multiple time points and similar numbers of samples as the current study, but only from
349 one location, and again showed strong variability (Hall 2018). This study not only adds to the

350 literature on in-field vector variability, but it fills a gap in the literature by studying multiple
351 locations with over 150 samples from each location, in 2018 – the most recent timepoint for a
352 study like this, available to our knowledge in the literature. One study focusing on
353 overwintering survival rates assessed psyllids in northern Florida from 2017-2018, but their
354 measurements of *C*Las titer were based on sticky card captures and did not record any psyllids
355 from commercial groves (Martini et al. 2020). Finally, a recent paper, published in 2023 by
356 Ebert et al., surveyed multiple commercial sites in Florida. However, their samples were
357 collected between 2008 and 2012, (Ebert et al. 2023) making our study the latest published
358 record of commercial psyllid vector capacity variability in Florida and perhaps the last one
359 before the wide-spread adoption of injectable oxytetracycline (OTC) for HLB management in
360 Florida. Now that direct injection of the OTC is becoming a wide-spread HLB management tactic
361 in Florida, understanding the impact of OTC on *C*Las titer in psyllids will be important to
362 understand if the use of OTC will indirectly lower vector competency in psyllid populations.

363 While grove is highly correlated in the current study with *C*Las titer, the base differences
364 may still come down to molecular or genetic differences between the psyllid populations, *C*Las
365 strains, other endosymbionts, and unknown environmental factors. The ability of *D. citri* to
366 acquire and transmit *C*Las may be genetically linked (Ammar et al. 2018) and studies that
367 separate acquisition and transmission (Ammar et al. 2016; Ukuda-Hosokawa et al. 2015;
368 Ammar et al. 2018; Xu et al. 1988) agree that these are likely two separate traits, but that they
369 are correlated, such that higher acquisition rates often lead to more efficient transmission
370 (Ammar et al. 2018). The current study used *C*Las titer as a measure of acquisition in adult *D.*
371 *citri*, (which may also include effects of internal *C*Las replication) but the high numbers of

372 infected individuals (infection rate) also suggests transmission of *C*Las is occurring in the field
373 and that adult psyllids acquired as nymphs from infected trees.

374 The relationship between wDi titer on *C*Las in *D. citri* requires further investigation.
375 However, in a related psyllid, *Cacopsylla pyri* (pear psyllid) studied by Serbina et al (Serbina et
376 al. 2022), wDi titer was reduced in males. Several lab studies investigated interactions among
377 wDi, *D. citri* and *C*Las. Hosseinzadeh et al. (Hosseinzadeh et al. 2019b) found that wDi titer
378 varied between sexes, and *D. citri* had higher titer in males that were infected with *C*Las than
379 non-infected males, though wDi did not significantly differ between *C*Las infected and non-
380 infected populations as a whole. Others found that wDi titer was significantly higher in *C*Las-
381 negative *D. citri*, except when controlling for population effect (Chu et al. 2019; Ren et al. 2018;
382 Chu et al. 2016). Fagen et al (Fagen 2012) showed the opposite trend, that *C*Las has a negative
383 relationship with other *D. citri* obligate endosymbionts, but a positive relationship to wDi
384 regarding titer. Our results, using insects collected from citrus groves instead of greenhouse
385 and chamber-raised populations, contradict some of these previous lab-based results. We saw
386 100% wDi infection rate among our individuals tested, and the titer of wDi tended to be higher
387 in *C*Las non-infected *D. citri* individuals. These contradictions underscore the importance to not
388 over-generalize the results from lab studies and the importance of field-based inquiries on
389 vector-microbe interactions.

390

391 **Conclusion**

392 This snapshot assessment of vector populations showed grove-specific differences in
393 *C*Las infection. Specifically, infection rate and titer of *C*Las within individual *D. citri* varied by

394 grove site, exhibiting three patterns of vector competency. However, no conclusions could be
395 drawn on the components of grove site that influenced *C*Las titer in *D. citri.* Additionally, while
396 there was no impact of sex on *C*Las titer, wDi titers were higher in non-infected individuals.
397 These results contradict some studies using lab-reared psyllid colonies. Unknown
398 environmental factors may contribute to titer levels in insects in the grove and we stress that
399 caution is necessary when applying and interpreting results from lab-reared insects to field-
400 based studies and applications. The data suggest that future studies should prioritize
401 understanding the effect of grove management practices, including the potential benefits of
402 using non-citrus plants that are hosts of *D. citri* or of its natural enemies in the grove.
403 Additionally, understanding the contributions that both environment and psyllid genes – the G
404 x E interaction – have on vector competency will help make all future genetic, genomic and
405 field-based studies of HLB more informed when developing control strategies or other
406 programs aimed at blocking *C*Las transmission by *D. citri*.

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Competing Interests

The authors declare that they have no competing interests.

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