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 bacteria including *Wolbachia* (wDi). In *D. citri*,

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

Introduction

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

45 intracellular, circulative, propagative manner (Ammar et al. 2016; Inoue et al. 2009; Higgins et al.
46 2022). While *C*Las can also spread via grafting infected citrus budwood, vector-borne dispersal is
47 the predominant and least controllable method causing new citrus infections.

48 Management of the vector requires an understanding of basic epidemiology and vector
49 biology in the field. In the United States, strict testing regimens are followed in Texas and
50 California to maintain quarantine areas for the vector and pathogen. But the state of Florida has
51 nearly succumbed to HLB. Despite the frequency of psyllid testing and a known increase in
52 insecticide resistance by *D. citri* (Chen et al. 2022; Vázquez-García et al. 2013; Naeem et al. 2019;
53 Rao et al. 2014; Pardo et al. 2018; Venkatesan et al. 2022), relatively few papers have been
54 published on population-level dynamics of *C*Las-*D. citri* infections in the field. A few studies have
55 looked at *D. citri*-*C*Las titer and prevalence in different situations, including specific field sites,
56 month to month changes, year to year trends, and differences between countries (Bové et al.
57 1993; Wulff et al. 2020; Capoor et al. 1974; Coy and Stelinski 2015; Hall 2018; Manjunath et al.
58 2008; Razi et al. 2014; Tiwari et al. 2010; Sétamou et al. 2020; Ukuda-Hosokawa et al. 2015;
59 Walter et al. 2012). These studies found high levels of variation in *C*Las titer across both the
60 individual and population level, but the latest of these was conducted in 2017. Genetic studies
61 have also shown that the variation in *C*Las acquisition, transmission and titer levels is stable and
62 heritable, consistent with these phenotypes being regulated by *D. citri* genes and proteins
63 (Ramsey et al. 2015; Mann et al. 2018; Ammar et al. 2018).

64 In addition to *C*Las, *D. citri* hosts multiple symbiotic bacteria which it maintains because
65 they provide various benefits (such as nutritional supplements and defensive polyketides)
66 (Hosseinzadeh et al. 2019b; Nakabachi et al. 2013). *Wolbachia* (wDi) is one such endosymbiont

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

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

81 **Methods**

82 Sample collection

83 Four unique citrus groves in central Florida, near lake Okeechobee, were sampled for
84 adult *Diaphorina citri* during the first week of October, 2018 (see **Figure S1**). Individual *D. citri*
85 were collected into 70% ethanol using mouth aspiration. At least 1200 individual *D. citri* were
86 collected from each field. Samples were then air dried and randomly aliquoted into groups of 100
87 and promptly flash frozen in liquid nitrogen and stored at -80 °C until DNA extractions.

88 DNA extraction and quantification

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

106 Quantitative PCR

107 Quantitative polymerase chain reaction (qPCR) was performed on all 1062 samples using
108 TaqMan hydrolysis probes, testing for the presence and relative titer quantification of the
109 "*Candidatus* Liberibacter asiaticus" (*C*Las) 16S rRNA gene. The 16S rRNA forward primer (5'-

110 TCGAGCGCGTATGCAATACG-3'), reverse primer (5'-GCGTTATCCCGTAGAAAAAGGTAG-3') and
111 probe (5'-FAM-AGACGGGTG/ZEN/AGTAACGCG-3') were used, as previously established (Wenbin
112 Li, 2006). The probe includes a fluorescent dye (5' 6-FAM) and a quencher (ZEN, 3' Iowa Black
113 FQ). Standard thermal conditions were used during the qPCR. Specifically, 95 °C for 10 min (1
114 cycle) and 95 °C for 15 sec and 60 °C for 60 sec (40 cycles total). Each qPCR 384-well plate included
115 a standard curve dilution series (a serial dilution from 10^9 down to 10^3 of the synthetic plasmid
116 pUC57 containing a *C*Las 16S rDNA gene fragment). The resulting y-intercept was 38.303, R^2 =
117 0.964, slope = -3.095, and efficiency = 110.452%. Each qPCR plate also contained positive and
118 negative controls run with two technical replicates each. A subset of samples from the first batch
119 were subjected to additional qPCR using SYBR Green reagents to access wDi presence-absence
120 and estimate titer. The wDi *ftsZ* gene (ftsZ-81) (A. S. Guidolin 2013) forward primer
121 (5'AGCAGCCAGAGAAGCAAGAG3') and reverse primer (5'TACGTCGCACACCTTCAAAA3'), and the
122 *Wingless* (*WgL*) (MyLo L. Thao 2000) forward primer (GCTCTCAAAGATCGGTTTGACGG) and
123 reverse (GCTGCCACGAACGTTACCTTC) primers were run in parallel on the same 386 well plate.
124 All samples on every qPCR plate were run in duplicate (2 technical replicates) then averaged to
125 create the reported final quantitative cycle (Cq) values. The maximum number of qPCR cycles is
126 40, where Cq = 40 means the primers did not create a product that could be amplified so the
127 sample is non-infected or titers are below the limit of detection, while Cq < 40 suggests copies of
128 the target gene are present, and the insect is infected. *C*Las cell counts were calculated from Cq
129 using the standard curve and the QuantStudio6 software. Each qPCR well contained 20 ng of DNA
130 from a single *D. citri.* The QuantStudio6 software associated with the qPCR machine used the
131 standard curve dilution values and resulting best-fit linear equation and efficiency metrics to

132 internally calculate the *C*Las cell equivalent to the number of copies of the *C*Las rDNA sequence
133 in a single *D. citri*. The values were exported from the software as an excel document. *C*Las cell
134 equivalents for technical replicates of each sample were averaged (note, Cq = 40 yielded
135 undetermined values, which were transformed to zeros for calculations of cell equivalents), then
136 all samples from each field were averaged. Field mean *C*Las cell equivalent was found by taking
137 the difference.

138 **Statistical Methods**

139 A linear mixed-effects model (LMM) was used to analyze the *C*Las Cq data collected on
140 the individual insects. The model contained fixed effects of sex and grove and a random effect of
141 DNA extraction batch. Fixed effects were tested using F tests, and post-hoc pairwise comparisons
142 were performed using Tukey's HSD. Model assumptions of normality and homogeneous variance
143 were assessed visually using the model residuals. *P*-values < 0.05 were considered statistically
144 significant. The mean absolute titer of 918 individuals was calculated from a standard curve, then
145 the mean absolute titer was calculated for each field site. For each pairwise field comparison, the
146 difference in absolute mean was taken by subtracting the second from the first (**Table 1**). A subset
147 of the samples from batch number one, representing all four fields, was additionally tested for
148 the dual effect of wDi (Cq values normalized by *Wingless* Cq) and grove on *C*Las Cq. R packages
149 (R Core Team 2022) were required for the analysis of both *C*Las and wDi, including *ggplot2*
150 (Wickham 2-16)*, ggpubr* (Kassambara 2020), *emmeans* (Lenth 2023), and *lmertest* (Kuznetsova
151 et al. 2017). To test for interactions between *C*Las and wDi in the insects, wDi Cq and *C*Las Cq
152 were converted into binary values as follows: for wDi, a value of 0 was assigned for Cq values
153 below the median, and a value of 1 was assigned for values above median and for *C*Las, a value

154 of 0 was assigned for Cq values less than 40, and a value of 1 for undermined Cq values or Cq=40).
155 The association between wDi and *C*Las titer was assessed using a Wilcoxon test, which clarified
156 whether wDi Cq above and below the median was different in CLas (+) and (-) insects, and a Chi-
157 squared test in R to test whether the wDi Cq distribution around the median differs from the
158 distributions of *C*Las Cq below or at Cq=40.

159 **Results**

160 1. Each grove had a distinct management strategy.

161 Groves were assigned names corresponding to their town of origin to protect the identity
162 of the participating growers. Psyllid infestations were found by scouting for flush points with
163 nymph honeydew deposits. A total of 450 adult *D. citri* were used from the Port St. Lucie grove.
164 This grove was one of two organic groves we sampled and did not utilize any pesticides,
165 herbicides or other chemical inputs at the time of sampling. It maintained a high *D. citri*
166 population as well as the highest observed levels of in-field entomopathogenic fungi (Higgins et
167 al. 2022) and other insects (including ladybeetles, ants, caterpillars and wasps). Multiple citrus
168 species as well as *Murraya spp.* grew within the grove. The majority of *D. citri* samples were
169 taken from *Citrus sinensis* (Valencia orange). Trees ranged in age from recently re-planted to well
170 established, bearing trees. We used a total of 164 *D. citri* adults from *C. sinensis* in the second
171 grove site, referred to as Pioneer. This grove was also organically managed and under a regimen
172 of periodic nitrogen, phosphorous and potassium (NPK) supplementation. Trees were mature
173 and bearing and well-spaced out. A total of 176 samples were used from an experimental plot
174 within a third grove, Whidden Corner. This grove was composed of mostly small, juvenile,
175 recently planted *C. sinensis* trees undergoing heavy pesticide treatment for *D. citri* control, with

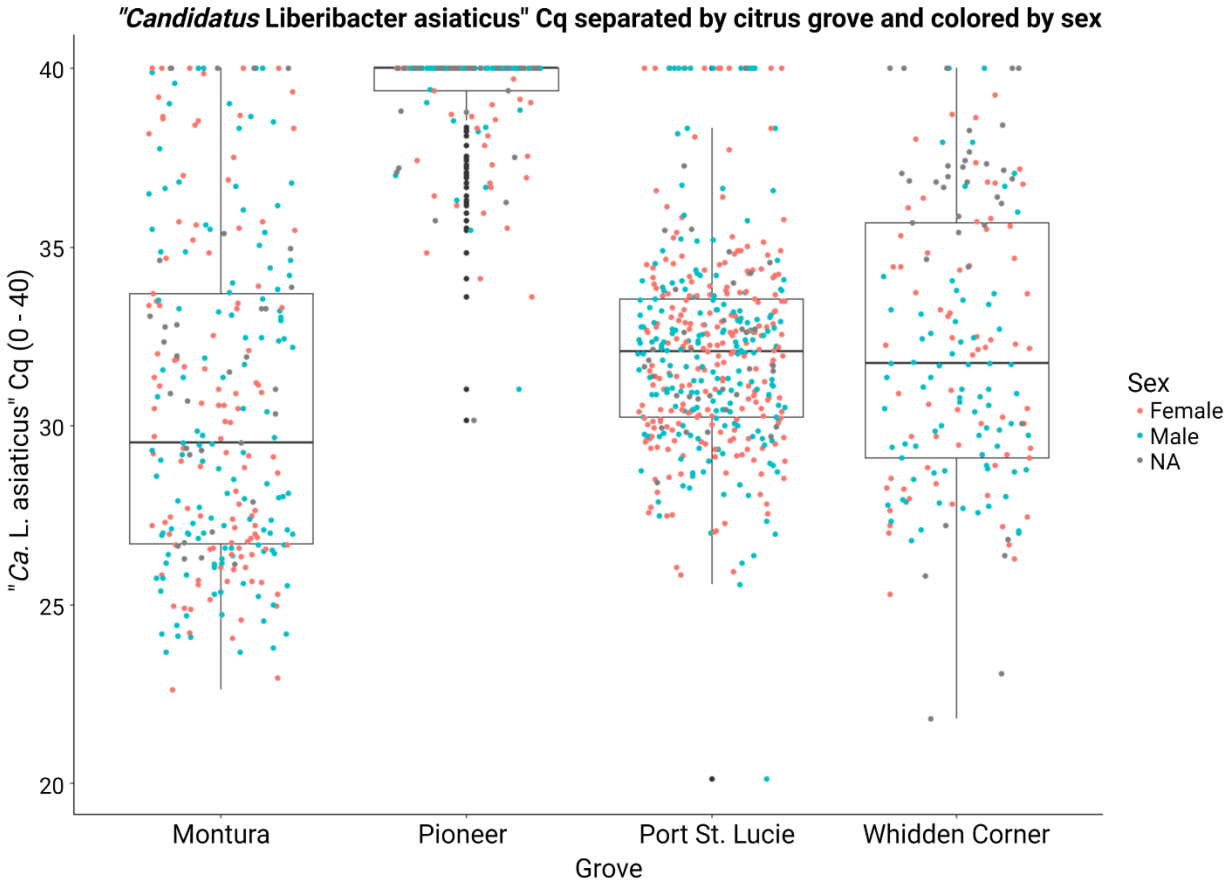
applications occurring every three weeks. A total of 272 adult *D. citri* were used from a fourth citrus grove, referred to as Montura. These insects were sampled from *C. sinensis,* and this grove also experienced insecticide sprays every three weeks. The trees were bearing, tall, and thick with leaves. In all groves, rootstock variety was unknown, and the majority of *D. citri* were collected from flush.

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

There was a significant difference in *C*Las Cq among grove sites after controlling for the effects of sex and DNA extraction batch ($F = 212.408$, $df = 3$, $P < 0.001$). The effect of DNA extraction batch was included in the model to rule out a potential bias, and we found no between-batch variability (var < 0.001) and low, or no standard deviation (SD < 0.001), suggesting high accuracy of technical replications of DNA extraction.

The four populations of *D. citri* fell into three broad categories based on *C*Las titer and secondarily, infection rates. After accounting for effects of batch and sex, Pioneer represents the "low titer" population and has the lowest overall titer (bacterial load per individual *D. citri*) with mean Cq = 39.0 and standard error (SE) = 0.32. Montura has the highest titer (mean Cq = 30.4, SE = 0.28) and represents the "high titer" population, while Port St. Lucie (mean Cq = 32.1, SE = 0.30) and Whidden Corner (mean Cq = 31.6, SE = 0.30) report similar bacterial titers and make up the third population of "medium titer" individuals. When absolute titer of *C*Las is calculated from Cq and a standard curve, the three groups are still present. Means were calculated using only samples with Cq<40, and Montura had the highest titer (29,630 *C*Las cell equivalents), Pioneer the lowest titer (41 *C*Las cell equivalents), while Whidden Corner and Port St. Lucie fell in the middle (8,912 and 11,270 *C*Las cell equivalents, respectively).

198 The percent infection rate is calculated using the proportion of insects with Cq values
199 lower than 40, as Cq = 40 are considered non-infected with *C*Las. While the three groups above
200 are determined by *C*Las titer, their infection rates do not necessarily match the trend. The "low
201 titer" population represented by Pioneer did also have the lowest *C*Las infection rate (26.82 %).
202 However, the "medium titer" populations represented by Port St. Lucie (97.11 %) and Whidden
203 Corner (96.02 %) recorded the highest percent infection rates. Finally, the "high titer" population
204 represented by Montura maintained the second-lowest percent infection rate (94.85 %). The
205 three populations as determined by mean Cq can be seen in **Figure 1** and pair-wise results
206 between grove populations (**Table 1**) additionally confirm visual differences between groves.
207



208

209 Figure 1. Box plot of *C*Las Cq distribution by citrus grove collection site and colored by sex.

210 Box plot center lines are the median, box top and bottom edges are the first and third quartile

211 respectively, and outliers are points that extend past the tails. NA samples (grey) are those that

212 have a *C*Las Cq value but no recorded sex. M = male (blue), F = female (red).

213 **Table 1:** Statistical results from each pair-wise field comparison using both mean *C*Las Cq and

214 absolute titer of *C*Las (genome copies of *C*Las in 20 ng of *D. citri* DNA).

	***C*Las cell equivalent**	**Quantification Cycle (Cq) of *C*Las[3]**				
Field site	**Mean difference[1]**	**Mean difference[2]**	**SE**	**t.ratio**	**df**	***P***
Montura vs. Pioneer	29,588	-8.870	0.371	-23.910	910	< 0.001
Montura vs. Port St. Lucie	18,360	-1.711	0.285	-5.995	763	< 0.001
Montura vs. Whidden Corner	20,717	-1.139	0.369	-3.086	850	0.011
Pioneer vs. Port St. Lucie	-11,228	7.159	0.360	19.906	476	< 0.001
Pioneer vs. Whidden Corner	-8,870	7.731	0.411	18.806	924	< 0.001
Port St. Lucie vs. Whidden Corner	2,357	0.573	0.362	1.580	305	0.391

215

216 [1]The mean absolute *C*Las titer, measured as the number of genome copies per 20ng of *D. citri*
217 DNA, of all samples with Cq < 40 per field site, was subtracted from each other to yield this
218 difference in mean absolute *C*Las titer by field. "Montura vs. Pioneer" means the mean *C*Las cell
219 equivalent of the Pioneer psyllids was subtracted from the mean *C*Las cell equivalent of Montura
220 psyllids, in this case, yielding a positive value showing Montura psyllids have a higher bacterial
221 load (total number of *C*Las genome copies) than psyllids from the Pioneer grove site.
222 [2]The difference in adjusted mean Cq value between two field sites. For example, the mean
223 difference of -8.870 shows that Montura has 8.87 Cq values more (meaning higher titer) than
224 Pioneer.
225 [3]The Linear Mixed-effect model transforms the data and accounts for co-variates. DF = degrees
226 of freedom are calculated using the Kenward-Rodger method, SE = Standard Error, Mean
227 difference = estimated relative difference in means, t.ratio = test statistic for testing the pairwise
228 contrasts, *P*-values are adjusted using the Tukey method. *C*Las = "*Candidatus* Liberibacter
229 asiaticus", Cq = quantification cycle in a quantitative polymerase chain reaction (qPCR).
230

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

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

238 The effect of sex on *C*Las Cq was tested and included in our model as *C*Las infection has
239 been shown to affect *D. citri* behavior differently by sex. There was no statistically significant
240 effect of sex on *C*Las Cq after controlling for grove and DNA extraction batch (F = 0.482, df =1, *P*
241 = 0.487). This can also be seen in **Figure 1**, as the blue and red colors show a random distribution
242 of sex by Cq value for each of the four groves.

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

250 4. *Wolbachia* (wDi) has higher titer in uninfected *D. citri.*

251 A subset of *D. citri* samples from the first DNA extraction batch were tested for wDi titer
252 (Total = 176: Port St, Lucie = 46, Pioneer = 42, Montura = 41, Whidden Corner = 47). WDi Cq was

253 compared to *C*Las Cq using a linear regression. The regression showed that there was no
254 correlation between *C*Las and wDi Cq in these *D. citri* from Florida citrus groves (R^2 = 0.0005)
255 **(Figure S2**). Additionally, wDi Cq was normalized against the *D. citri Wingless* gene (**Figure S3).** A
256 linear model assessed the relative effects of both grove and normalized wDi Cq on *C*Las Cq, and
257 also found that wDi was not significantly correlated with *C*Las Cq (F = 0.646, df = 1, *P* = 0.426) and
258 that the source grove maintains the strongest effect on *C*Las titer (F = 22.881, df = 3, *P* < 0.001,
259 **Table S2**).

260 In the regression analysis, we noticed that there was a large number of wDi samples with
261 low Cq values when *C*Las Cq = 40 (*C*Las uninfected samples, **Figure S2)**. We tested whether wDi
262 titer is significantly different depending on the presence or absence of *C*Las (present when Cq <
263 40, absent when Cq = 40), and found that when *C*Las Cq = 40 in a sample, wDi Cq was more likely
264 to be below the median wDi Cq value (Wilcoxon, n=119, *P* = 0.017). A Chi^2 test confirmed that
265 the proportion of wDi with Cq above and below the median differs significantly from expected
266 when compared to the *C*Las Cq distribution based on infection or no infection, X^2(1, n=119)
267 =6.086, *P*=0.013. *C*Las-uninfected *D. citri* tend to have higher wDi titers as compared to *C*Las-
268 infected individuals.

269

270 **Discussion**

271 Natural variation in vector competency plays a role in disease epidemiology. Vector
272 competency is the potential of a vector to transmit a pathogen. In *D. citri*, vector competency is
273 apparently in part regulated by *D. citri* genes and proteins functioning at distinct tissue barriers
274 which *C*Las must overcome prior to inoculation into a new host tree (Ammar et al. 2018; Mann

275 et al. 2018; Kruse et al. 2017, 2018; Hosseinzadeh and Heck 2023). *D. citri* acquires and transmits
276 *C*Las in an intracellular, circulative, propagative manner. Vector competency in our study was
277 measured through infection rate and *C*Las titer for each population of vectors studied. Studies
278 have shown that approximation of *C*Las titer in individual insects can predict vector capacity.
279 There is a strong positive correlation between *C*Las titer in the psyllid and transmission efficiency
280 (Ammar et al. 2018; Hosseinzadeh et al. 2019a). Broadly, our study shows that there are
281 population-level differences in *D. citri* vector competency that are likely maintained through a
282 combination of gene by environment interactions.

283 During circulative propagative transmission, *C*Las must cross the gut epithelial cell layer
284 to enter the body cavity of the psyllid. Next, *C*Las must circulate throughout the body and
285 replicate in tissues, especially the salivary glands. Finally, *C*Las must be inoculated into healthy
286 citrus plants while feeding. The efficiency of the first and second steps can be approximated by
287 using qPCR to detect *C*Las titer in adults that have acquired *C*Las, while the third step can only be
288 measured by careful transmission studies. Also, the development of vector-competent adults
289 requires *C*Las acquisition to occur during the psyllid's nymphal stages (Inoue et al. 2009; Ammar
290 et al. 2016; Igwe et al. 2022). This study approximates the grove-wide vector capacity of four *D.*
291 *citri* populations through measurements of *C*Las titer in individual insects. A key assumption of
292 this study is that the majority of trees in all citrus-growing counties of Florida are infected with
293 *C*Las, and thus, all adult psyllids that are infected with *C*Las likely acquired as nymphs and are
294 competent transmitters. However, our results suggest these competent transmitters may still fall
295 into categories of low to high vector capacity, as determined by mean *C*Las titer of the population.

296 Other factors may influence *D. citri* vector competency and the variation we observed in
297 our study. Prior work has shown that host switching influences heritable variation in *D. citri* vector
298 competency (Ramsey et al. 2022). While grove-collected sampling makes it difficult to control or
299 understand all variables that may influence *C*Las titer in psyllids, all four *D. citri* populations were
300 sampled from the same citrus variety (*Citrus sinensis*), within the same week in October, 2018,
301 so it represents a snapshot in time of the potential of *D. citri* in these regions to acquire and
302 transmit. Additionally, the role that symbionts like wDi might play in mediating vector-pathogen
303 interactions is unclear, so the effect of wDi on *C*Las titer from these four fields was also tested.
304 Our results show that *D. citri* has higher wDi titer in healthy individuals, while field site has the
305 strongest effect on *C*Las titer, and sex does not have any effect. The field effect could be related
306 to the heritability of *D. citri* genes involved in vector competency, the management regimen, or
307 transient access to and feeding on non-citrus host plants such as *Murraya spp.* in the grove agro-
308 ecosystem. More work is required to fully explore these hypotheses.
309 Abandoned groves have also been shown to maintain high *D. citri* populations that travel
310 frequently between managed and abandoned areas (Sétamou et al. 2022; Boina et al. 2009) with
311 equally high *C*Las titers and infection rates as alive and managed groves (Hall 2018). Prior to 2010,
312 Tiwari et al (Tiwari et al. 2010) studied the *D. citri*-*C*Las infection rate and found 1.9 % in
313 abandoned and 1.2 % in managed grove populations while the mean *C*Las Cq value was 32.52 in
314 abandoned and 25.95 in managed groves. Over 10 years after Tiwari et al collected their data,
315 the infection rates found by our study in the least infected, but still managed, grove are at least
316 1.5x higher incidence while our other three groves have at least 50x higher infection rates. This
317 is undoubtedly due to the vastly increased number of infected trees in Florida over the past ten

318 years. A study by Pacheco et al (De Souza Pacheco et al. 2020) shows that when *D. citri* are raised
319 on *C*Las-infected citrus for four consecutive generations in a greenhouse setting, the percent-
320 infection and the titer in individuals increased with each generation from 42% to 100% infection
321 incidence, and 47.9 to 5,390 *C*Las cells per 100 ng of DNA. These results suggest that interruptions
322 in vector generations (such as through regular insecticide applications or during seasons of low
323 flush) in the field may help keep *C*Las titer and incidence lower in vector populations. The increase
324 in infection incidence seen in Florida groves despite various management innovations over the
325 last decade is a testament to both the effect of continuous populations of *D. citri* replicating on
326 infected citrus and reference to the accumulating impact HLB has wrought on the Florida citrus
327 industry.

328 Vector competency of *D. citri* populations and individuals varies widely in the field as we
329 have seen through this study and others (Capoor et al. 1974; Coy and Stelinski 2015; Hall 2018;
330 Ukuda-Hosokawa et al. 2015; Ammar et al. 2018; Sétamou et al. 2016; Xu et al. 1988), in addition
331 to laboratory-kept colonies (Inoue et al. 2009; Hosseinzadeh et al. 2019b; De Souza Pacheco et
332 al. 2020). In agreement with our results, Chu et al (Chu et al. 2016) show that three wild
333 populations of *D. citri* vary in their *C*Las titer and *C*Las infection incidence, and that like our study,
334 population (grove site) has the strongest effect on all their models. While grove is highly
335 correlated in the current study with *C*Las titer, the base differences may still come down to
336 molecular or genetic differences between the psyllid populations, *C*Las strains, other
337 endosymbionts, and unknown environmental factors. Methods to track genetic changes in *C*Las
338 between populations of *D. citri* show that greenhouse and field *D. citri* maintain diverging strains
339 of *C*Las (Higgins et al. 2022), and that microbiota vary with *D. citri* collection method. Research

340 has shown that the ability of *D. citri* to acquire and transmit *C*Las may be genetically linked
341 (Ammar et al. 2018). Studies that separate acquisition and transmission (Ammar et al. 2016;
342 Ukuda-Hosokawa et al. 2015; Ammar et al. 2018; Xu et al. 1988) agree that these are likely two
343 separate traits, but that they are correlated, such that higher acquisition rates often lead to more
344 efficient transmission (Ammar et al. 2018). The current study used *C*Las titer as a measure of
345 acquisition in adult *D. citri*, (which may also include effects of internal *C*Las replication) but the
346 high numbers of infected individuals (infection rate) also suggests transmission of *C*Las is
347 occurring in the field and that adult psyllids acquired as nymphs from infected trees. Now that
348 direct injection of the antibiotic oxytetracycline is becoming a wide-spread HLB management
349 tactic in Florida, understanding the impact of OTC on *C*Las titer in psyllids will be important to
350 understand if the use of OTC will indirectly lower vector competency in psyllid populations.

351 The effect of wDi titer on *C*Las in *D. citri* is still uncertain, and there was no effect of sex
352 on wDi or *C*Las observed in this study. However, in a related psyllid, *Cacopsylla pyri* (pear psyllid)
353 studied by Serbina et al (Serbina et al. 2022), wDi titer was reduced in males. Several lab studies
354 investigated interactions among wDi, *D. citri* and *C*Las. Hosseinzadeh et al. (Hosseinzadeh et al.
355 2019b) found that wDi titer varied between sexes, and *D. citri* had higher titer in males that were
356 infected with *C*Las than uninfected males, though wDi did not significantly differ between *C*Las
357 infected and uninfected populations as a whole. Others found that wDi titer was significantly
358 higher in *C*Las-negative *D. citri*, except when controlling for population effect (Chu et al. 2019;
359 Ren et al. 2018; Chu et al. 2016). Fagen et al (Fagen 2012) showed the opposite trend, that *C*Las
360 has a negative relationship with other *D. citri* obligate endosymbionts, but a positive relationship
361 to wDi regarding titer. Our results, using insects collected from citrus groves instead of

362 greenhouse and chamber-raised populations, contradict some of these previous lab-based
363 results. We saw 100% wDi infection rate among our individuals tested, and the titer of wDi
364 tended to be higher in *C*Las-uninfected *D. citri* individuals. These contradictions underscore the
365 importance to not over-generalize the results from lab studies and the importance of field-based
366 inquiries on vector-microbe interactions.
367

368 **Conclusion**

369 This snapshot assessment of vector populations showed grove-specific differences in *C*Las
370 infection. Specifically, infection rate and titer of *C*Las within individual *D. citri* varied by grove site,
371 exhibiting three patterns of vector competency. However, no conclusions could be drawn on the
372 components of field management that influenced *C*Las titer in *D. citri.* Additionally, while there
373 was no impact of sex on *C*Las titer, wDi titers were higher in *C*Las-uninfected individuals. These
374 results contradict some studies using lab-reared psyllid colonies. Unknown environmental factors
375 may contribute to titer levels in insects in the grove and caution is necessary when interpreting
376 results from lab-reared insects for field-based applications. The data suggest that future studies
377 should prioritize understanding the effect of grove management practices, including the use of
378 non-citrus plants that are hosts of *D. citri* in the grove, on the dynamics of *D. citri* infection by
379 *C*Las. Additionally, understanding the contributions that both environment and psyllid genes –
380 the G x E interaction – have on vector competency will help make all future genetic, genomic and
381 field-based studies of HLB more informed when developing control strategies or other programs
382 aimed blocking *C*Las transmission by *D. citri*.

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391 **Competing Interests**

392 The authors declare that they have no competing interests.

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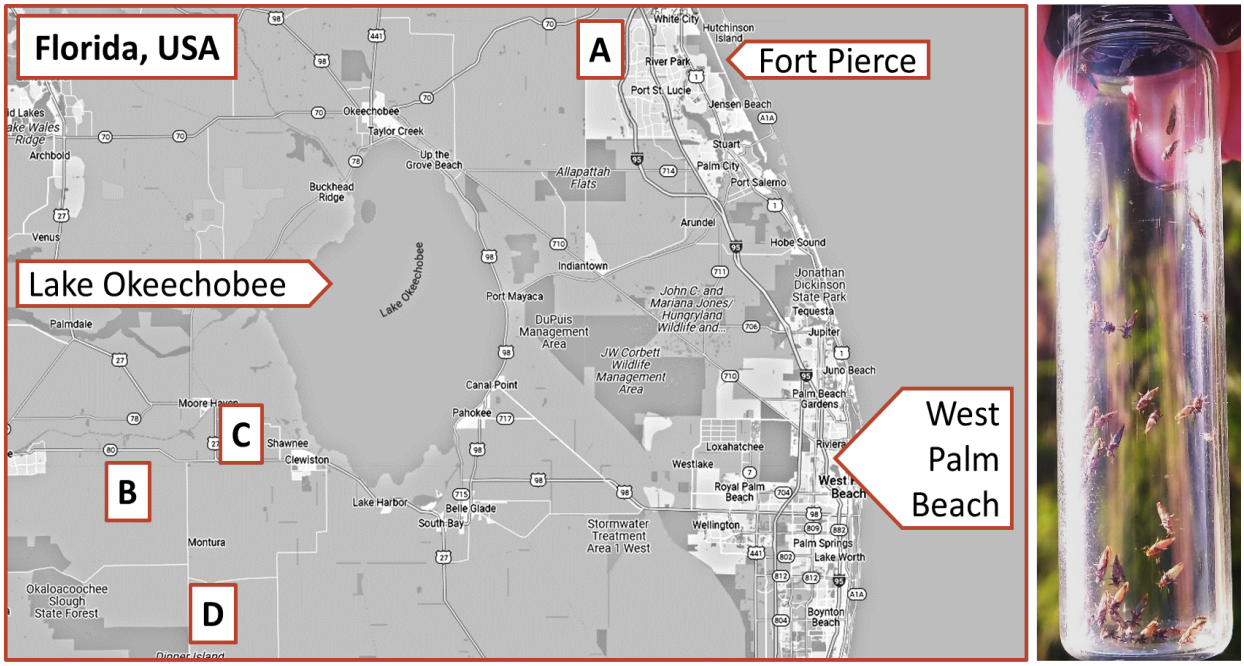

Figure S1: Map of four grove sites in the central Florida region. A) Port St. Lucie, B) Pioneer, C) Whidden Corner, D) Montura.

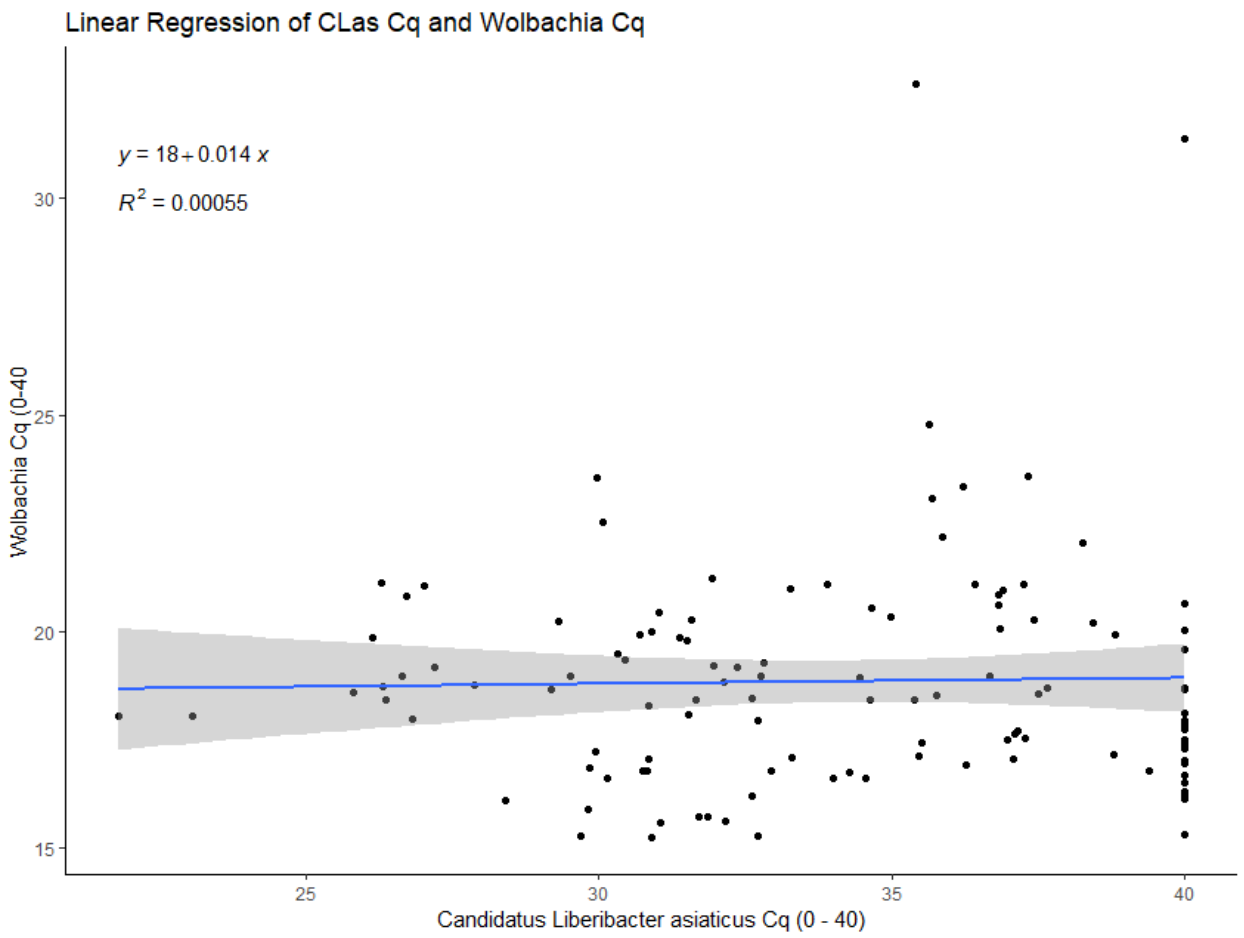

Figure S2: Linear regression of 176 samples with paired *C*Las-*Wolbachia* Cq values, showing no correlation (R^2=0.00055) in *D. citri*. Grey area is 95% confidence, while the blue line is the best fit line which follows the equation stated. Note that many of the *Wolbachia* samples with Cq < 20 Cq have *C*Las Cq = 40 (uninfected).

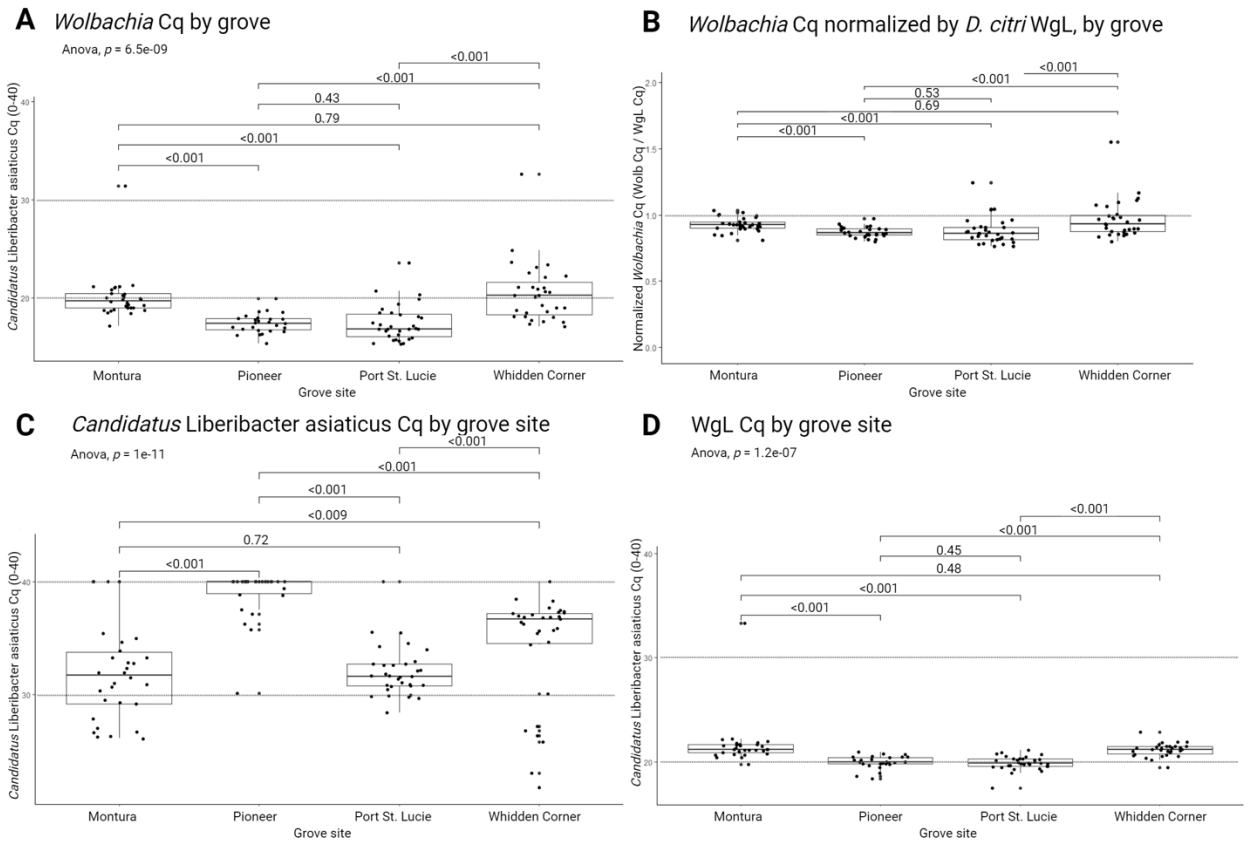

Figure S3: Normalization of *Wolbachia* wDi Cq values using a psyllid gene internal standard. In addition to measuring the Cq of wDi (A), wDi Cq can be normalized by dividing with *Wingless* Cq (B), which is the *D. citri* endogenous control gene. Paired *C*Las Cq values are also available for each sample (C), and a standard curve was used while measuring *C*Las Cq instead of *Wingless.* W*ingless (WgL)* Cq alone can be seen in (D). The goal of running the reference gene is to see consistent Cq values, despite variation in the variable of interest (in this case, testing for wDi Cq. Two 386-well qPCR plates were run (First: Pioneer and Port St. Lucie, Second: Montura and Whidden Corner samples). Statistical results are from pair-wise T-tests.

Table S1: Data for sex and color of individual *Diaphorina citri* from four citrus groves. Pioneer is a consistent outlier due to a higher proportion of *C*Las-uninfected (Cq = 40) samples relative to other groves, yielding a higher mean Cq as well.

A	*Total	Sex	% of Total	% of Sex w/Cq=40	**Mean Cq
Montura	239	Female	47.28	4.42	30.14
		Male	52.71	3.17	29.94
Pioneer	134	Female	52.24	65.71	37.33
		Male	47.76	82.81	37.18
Port St. Lucie	415	Female	54.22	4.89	31.73
		Male	45.78	5.79	31.65
Whidden Corner	141	Female	47.52	0.00	32.01
		Male	52.48	2.70	30.89

B	*Total	Color	% of Total	% of Color w/Cq=40	**Mean Cq
Montura	239	Blue	17.99	4.65	31.75
		Orange	26.36	15.87	29.27
		White	55.65	51.13	29.45
Pioneer	134	Blue	32.09	69.77	37.54
		Orange	47.01	79.37	37.31
		White	20.90	67.86	36.86
Port St. Lucie	415	Blue	20.24	4.76	32.23
		Orange	40.96	6.47	31.51
		White	38.80	4.35	31.60
Whidden Corner	141	Blue	13.48	0.00	31.49
		Orange	45.39	1.56	31.81
		White	41.13	1.72	30.99

*NA samples are excluded from total counts and from all percent and mean calculations.

**mean Cq uses samples with Cq<40, excludes samples with Cq=40.

37 **Table S2**: Statistical results and post-hoc tests from the linear model used to test the effect of

38 grove on *C*Las Cq while controlling for normalized *Wolbachia* Cq.

Pair-wise grove (field) comparisons controlling for effect of *Wolbachia* *lm*(CLas.Cq ~ Field + Norm_Wolb, data=dataset)					
Contrast	**Est**	**SE**	**t.ratio**	**df**	***P***
Montura vs. Pioneer	-7.264	0.996	-7.296	114	<0.0001
Montura vs. Port St. Lucie	-0.278	0.943	-0.295	114	0.9911
Montura vs. Whidden Corner	-2.329	0.943	-2.471	114	0.0701
Pioneer vs. Port St. Lucie	6.986	0.961	7.271	114	<0.0001
Pioneer vs. Whidden Corner	4.935	1.028	4.800	114	<0.0001
Port St. Lucie vs. Whidden Corner	-2.052	0.974	-2.107	114	0.1572

39

40 *The estimate (Est) value of -7.264 (for example) shows that Montura has on average about 7

41 points smaller Cq value than Pioneer, which can be understood as a multi-fold higher mean *C*Las

42 titer.

43

44 **Table S2 Legend**: DF = degrees of freedom, SE = Standard Error, Est = estimate, t.ratio = test

45 statistic for testing the pairwise contrasts, $P < 0.0001$ is adjusted using the Tukey method. *C*Las =

46 "*Candidatus* Liberibacter asiaticus", Norm.Wolb = *Wolbachia* titer normalized by dividing

47 *Wolbachia* Cq by *Wingless* Cq, Cq = quantification cycle in a quantitative polymerase chain

48 reaction (qPCR).

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