



Acquisition of *Candidatus Liberibacter asiaticus* by psyllid species transiently landing and feeding on *Citrus sinensis*

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Abstract

'*Candidatus Liberibacter asiaticus*' (CLAs), the prevalent agent associated with Huanglongbing (HLB), poses a severe threat to global citrus production. This study aimed to investigate the occurrence of psyllid species (other than *Diaphorina citri*) landing on citrus trees, evaluate their survival on citrus over optimal acquisition access periods (AAP), assess their ability to acquire CLAs, and infer their potential role in pathogen transmission. Field surveys were conducted in *Citrus sinensis* plants located in backyards and poorly managed groves in two locations of São Paulo State, Brazil. Psyllids from fifteen genera were identified, with *Isogonoceraia divergipennis* and *Heteropsylla cubana* being among the most constant or frequent species, showing greater abundance particularly in late winter and early spring. Controlled acquisition assays demonstrated that *I. divergipennis*, *H. cubana*, and *Triozoida limbata* could acquire CLAs following a 72-h AAP, post-acquisition latency of 14 days and a subsequent 72-h post-latency period, with acquisition rates of 8.1%, 3.2%, and 2.4%, respectively, compared to 85.7% in *D. citri*. Notably, CLAs was detected in the 'head-mesothorax' region only in *T. limbata* and *D. citri*, suggesting potential bacterial presence in the salivary glands and possible transmission competence. CLAs titers were consistently higher in the 'metathorax-abdomen' than in the 'head-mesothorax'. While the data indicate occasional CLAs acquisition by psyllids other than *D. citri*, there is currently no evidence supporting their role as natural vectors. Nonetheless, these species may serve as valuable models for elucidating transmission barriers in non-competent psyllids and highlight the need for further investigation into their epidemiological relevance.

Keywords Huanglongbing · Psyllid dispersal · Psyllid food plants · Vector competence

Introduction

Huanglongbing (HLB) has been of major concern for citrus production, affecting key producing regions such as Florida (Singer et al. 2020), China (Zhou 2020), and Brazil (Bassanezi et al. 2020). Despite substantial research efforts and various management strategies, HLB continues to threaten the sustainability of citrus farming worldwide. The presumptive prevalent causal agent in the Americas, '*Candidatus Liberibacter asiaticus*' (CLAs), is a phloem-restricted bacterium transmitted by the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), with

which it has an intimate relationship, as the bacterium persists, circulates, and propagates within the vector.

The transmission of CLAs by *D. citri* exhibits characteristics that significantly complicate HLB management, both in terms of plant-pathogen interactions and vector dynamics. Key challenges include the short pathogen acquisition and inoculation periods by the vector, the relatively short latency period for bacterial establishment within the inoculated plant allowing new acquisition cycles, and the prolonged incubation period before symptom development (Capoor et al. 1974; Xu et al. 1988; Pelz-Stelinski et al. 2010; Canale et al. 2020). The asymptomatic phase of infection (before the end of the incubation period) further complicates control efforts, since the infected citrus trees can serve as inoculum sources before the growers are able to detect and eradicate them (Coletta-Filho et al. 2014). Additionally, *D. citri* dispersal behavior, influenced by some environmental and biotic factors (Hall and Hentz 2011; Lewis-Rosenblum et al. 2015; Lopes et al. 2017; Tomaseto et al. 2018), aligns with

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periods of heightened CLas rates in psyllids, increasing the likelihood of pathogen spread (Martini et al. 2015; Ukuda-Hosokawa et al. 2015).

D. citri dispersal shows seasonal patterns, with increased activity typically beginning in mid to late winter and peaking in spring, as observed in regions such as Brazil, the United States (Florida and California), and Mexico, following environmental conditions (e.g. temperature gradients and rainfall) that precede and promote population increases, and triggered by the onset of new citrus flushing (Hall and Albrigo 2007; Hall et al. 2008; Hall and Hentz 2011; Lewis-Rosenblum et al. 2015; Zorzenon et al. 2021; Alonso-Hernández et al. 2024; Ponvert et al. 2025). In contrast, population peaks of other psyllid species, such as *Isogonoceraia divergipennis* White & Hodkinson and *Russelliana solanicola* Tuthill, have been recorded from late spring to early summer in Brazil, particularly on vegetable crops that are not recognized as their natural hosts (Kuhn et al. 2023).

Evidence of feeding, reproduction, and survival are important factors for analyzing the host relationship of psyllids. In practical terms, a host plant supports breeding and immature survival, a food plant allows feeding but not reproduction, and a casual plant is one where psyllids may land but cannot sustain feeding (Burckhardt et al. 2014). In citrus orchards, beyond the known psyllid vectors of HLB-associated bacteria, *D. citri* and *T. erytrae*, six other psyllid species have been reported to use citrus as a food plant: *Mesohomotoma lutheri* Enderlein, *Cacopsylla (Psylla) citricola* Yang & Li, *Cacopsylla (Psylla) citrisuga* Yang & Li, *Psylla murrayi* Mather, *Trioza citroimpura* Yang & Li, and *Trioza litseae* Bordage (Halbert and Manjunath 2004). In South Texas, adults of several psyllid species, including *Leuronota maculata* Crawford, *Trioza diospyri* Ashmead, *Heteropsylla texana* Crawford, and *H. mimosae* Crawford, are commonly found in citrus groves, possibly using citrus as a casual plant (Thomas 2011), as its host status has not been confirmed.

During dispersal, psyllids can reach non-host plants and may engage in incidental feeding (Cooper et al. 2019). However, psyllids are generally known for their highly specific host-plant relationships, with host switching typically occurring at local scales between closely related plants (Ouvrard et al. 2015). Nonetheless, some reports indicate that species such as *D. citri*, *B. cockerelli*, and *B. maculipennis* can feed on non-hosts, but primarily on xylem and usually for short periods (Borges et al. 2017; George et al. 2020). This behavior may serve as a strategy for survival and/or to facilitate long-distance dispersal (Hodkinson 2009; Tiwari et al. 2010).

Considering that dispersing psyllids eventually feed on non-host (food plants), including citrus, they might be able to acquire ‘*Candidatus Liberibacter*’ if the plants are

infected, raising concerns about their possible role in disease epidemiology.

This gap in knowledge underscores the urgency of identifying potential vectors among dispersing psyllid species eventually collected from citrus plants. To address this challenge, the objectives of the present study are to: (i) investigate the occurrence of migrant psyllids, other than *D. citri*, on citrus groves and backyard plants, (ii) assess their survival on citrus plants over optimum CLas acquisition access periods (iii) evaluate their CLas acquisition ability, and (iv) infer their potential role on CLas transmission, in case bacterial acquisition occurs.

Materials and methods

Psyllid collections on citrus plants

The psyllid sampling was conducted using two complementary methods: a sweep net (40 cm in diameter and 1 m in length) and a portable suction device (Stihl BG86 C/E). Sampling was conducted on *Citrus sinensis* (L.) Osbeck trees in backyards in the municipality of Botucatu, where 14 plants were sampled, and in poorly managed groves in Casa Branca, where five plants were sampled in each of eight groves, in São Paulo State, Brazil (Suppl. Table 1). The seasonal analysis aimed to evaluate the fluctuation of psyllid species across different sampling dates. For this purpose, the mean number of individuals per sampled plant was calculated for each collection date. This mean was obtained by dividing the total number of psyllids collected by the number of plants sampled at each specific site. In the context of “trees in backyards”, absolute values per plant were considered, whereas in “poorly managed groves”, the mean was estimated based on five plants sampled per site.

For the sweep net method, each sample consisted of 15 sweeps on the middle and upper branches of the citrus trees. In contrast, the suction device samples were collected on the peripheral regions of middle and lower thirds of the tree canopies: each sample consisted of 1.5 min suction period per plant. Sampling was performed biweekly, from August 2017 to March 2020. Collected insects were preserved in absolute ethanol, taxonomically identified, and subsequently quantified. Psyllids collected from citrus plants, including *D. citri*, *I. divergipennis*, *H. cubana*, and *T. limbata*, were individually subjected to real-time polymerase chain reaction (qPCR) analysis for CLas detection.

Psyllid abundance was analyzed by calculating constancy and frequency. Constancy was determined as the proportion of collections in which a given species was recorded relative to the total number of collections performed. Frequency was calculated as the proportion of individuals of a given species

relative to the total number of individuals collected across all species.

Psyllid rearing

In the greenhouse, four psyllid species collected on citrus were reared on their natural host plants: *I. divergipennis* on *Poincianella pluviosa* (DC) LP Queiroz (Fabaceae), *H. cubana* on *Leucaena leucocephala* (Lam.) de Wit (Fabaceae), *Triozoida limbata* Enderlein on *Psidium guajava* L. (Myrtaceae), and *D. citri* on *Murraya paniculata* (L.) Jack (Rutaceae). Adult insects, approximately one-week post-emergence, were used in acquisition experiments.

The colonies were established in 2020 using plants grown from seeds in the case of *P. pluviosa* and *L. leucocephala*, and from cuttings in the case of *P. guajava*. Rearing of *I. divergipennis*, *H. cubana*, and *T. limbata* was conducted in voile cages (50 cm x 80 cm x 100 cm) placed in a greenhouse (26.4±3 °C and an average relative humidity of 54.3%) equipped with a pad-fan cooling system and daily irrigation. *D. citri* was reared as part of a permanent colony in a climatized room at 26±2 °C, an average relative humidity of 60%, and a 16:8 h (L: D) photoperiod.

Each week, 300–500 adult psyllids (males and females) of *I. divergipennis*, *H. cubana*, and *T. limbata* were introduced into their respective cages to start an oviposition cycle. Each cage contained 3–4 plants approximately 60–80 cm tall, with young shoots in early developmental stages to ensure oviposition. After a one-week oviposition period, the adults were removed from the cages to allow development of immature stages of known age.

To ensure reliability of the results, the CLas infection status of the lab-reared psyllids was frequently assessed by qPCR. Genetic consistency of the population was maintained as no new introductions occurred after colony establishment.

Source plants of '*Candidatus liberibacter asiaticus*'

CLas-infected *C. sinensis* plants (source plants) were obtained by grafting with infected buds from the Sylvio Moreira Citrus Center (IAC). The source plants were maintained in a greenhouse under weekly fertilization and daily irrigation. Regular pruning was performed to promote the development of young shoots, which were used for bacterial acquisition experiments. Control plants (non-infected) were maintained under the same conditions as the bacterial source plants.

'*Candidatus liberibacter asiaticus*' detection in plants and insects

CLas presence in source plants was confirmed by qPCR on leaf samples both before and after the acquisition access periods (AAP). Moreover, qPCR was performed to evaluate the presence of CLas in the natural host plants used during the latency period (LP). Additionally, qPCR was performed on insects collected from citrus trees and on those investigated after the post-latency period in the acquisition ability experiments. Detection by qPCR was also performed on dissected individuals, targeting the head and abdomen independently, in the transmission inference experiment. Before qPCR total DNA was extracted from psyllids (three individuals per sample) and plants (from ~100 mg of primary leaf veins) using a modified CTAB-based protocol (Murray and Thompson 1980). DNA concentration and purity were assessed using an Epoch spectrophotometer (BioTek Instruments®, Inc., Winooski, VT, USA). For molecular qPCR analyses, 100 ng/μL of DNA was used from both psyllid and plant samples.

qPCR conditions

qPCR for the detection of CLas was conducted following the protocol described by Lin et al. (2010), with the primer set Las-I-F/Las-I-R (5'-CGATTGGTGTCTTGTAGC G-3'/5'ACAATAGAAGGATCAAGCATCT-3'), targeting the Elongation Factor Ts (EF-Ts) gene (single copy on the CLas genome), along with the probe Las-P' (5'-AATCACC GAAGGAGAAGCCAGCATTACA-3'). qPCR detection of CLas was conducted using the 5x Hot FIREPol Probe qPCR Mix Plus (ROX) (IDT Inc., Coralville, IA, USA) with 2 μl of the DNA template, in an ABI PRISM 7500 Fast Sequence Detection System (Applied Biosystems, USA). Cycling conditions were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 58 °C for 45 s. Reactions were performed in duplicate, including technical (ultrapure water) and biological negative controls (insect and plant), as well as a positive control (previously confirmed CLas-infected). Samples were considered positive when contained 2.1×10^4 (Ct≤35) gene copies (2μL/reaction), as determined from the qPCR standard curve (Supplementary material "qPCR standard curve and quantification").

Analysis of psyllid survival on '*Candidatus liberibacter asiaticus*'-source plants, during latency, and pos-latency periods

An initial survival test was conducted over increasing acquisition access periods (AAPs) of 48, 72, and 96 h, by caging groups of 100 psyllid adults of each species (*I.*

divergipennis, *H. cubana*, and *D. citri*) on branches with shoots in the development stages of v1–v4 of CLas-source plants, aiming to establish the optimal AAP duration that minimizes psyllid mortality while ensuring successful bacterial acquisition.

Following the determination of the optimal AAP duration, the psyllid species (*I. divergipennis*, *H. cubana*, and *T. limbata*) survival (including *D. citri* as a control) was assessed by calculating the proportion (relative to the initial number of psyllids in each cage) of individuals that remained alive at the end of each experimental phase: Acquisition access period (AAP–72 h), latency period (LP–14 days), and post-latency period (PLP–72 h).

To determine the optimal AAP duration, Welch's test was applied using a one-way analysis of variance (ANOVA). This approach accounted for data normality but did not assume homogeneity of variances. The number of replicates (*n*) varied across AAPs, with 50 individuals per species for each experimental condition. The response variable was survival rate, while the factors evaluated were psyllid species and AAP. Post-hoc pairwise *t*-tests with Bonferroni correction were performed to identify significant differences among AAP groups, both within and between treatments. The same analytical approach was used to explore potential interactions between the different experimental phases (AAP, LP, and PLP) and psyllid species.

Assessment of '*Candidatus liberibacter asiaticus*' acquisition ability

Psyllids from three species (*I. divergipennis*, *H. cubana*, and *T. limbata*) were assessed with respect to their ability

to acquire CLAs on *C. sinensis*, using *D. citri* as control species. *I. divergipennis* was selected due to its high constancy in field collections on citrus plants, whereas *H. cubana* was chosen as one of the most frequently collected species. *T. limbata*, although recorded only once on citrus, was included because it belongs to the family Triozidae, which has several known vectors of '*Candidatus Liberibacter* spp.', and because its host plant is guava (*Psidium guajava*), a cultivated fruit species of potential epidemiological relevance. The individuals tested were adults approximately one-week post-emergence, with females and males pooled.

The AAP duration used to analyze the acquisition ability was the optimal determined in this study (72 h, Fig. 3). For each CLas-infected source plant, one cage was assigned per psyllid species, along with an additional cage containing *D. citri* individuals, which served as a control psyllid species. Each cage housed 100 psyllids of a given species, confined in voile cages placed on branches of *C. sinensis*. The psyllids were positioned on branches with shoots at developmental stages v1–v4 (Fig. 1), as suggested by Lopes and Cifuentes-Arenas et al. (2021).

Subsequently, surviving individuals at the end of the AAP were transferred to voile cages placed on branches of their natural host plants (grown from seeds and aged 1–1.5 years) for a LP of 14 days. After the LP, surviving individuals were transferred to healthy citrus seedlings (CLas negative, *C. sinensis* var. Pineapple) at developmental stages v1–v2 for a 72 h PLP (Fig. 1).

To evaluate acquisition ability, psyllids were tested by qPCR at the end of the PLP following the protocol of Lin et al. (2010), with priority given to individuals that were alive at the conclusion of the PLP.

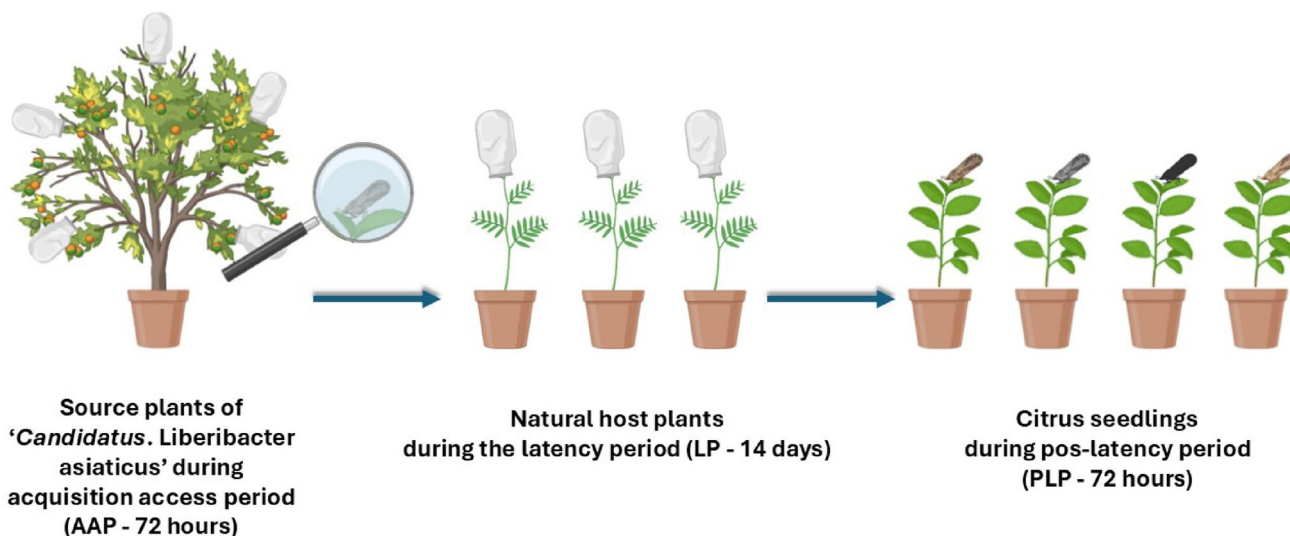


Fig. 1 Experimental workflow for assessing the ability of *Isogonoceraia divergipennis*, *Heteropsylla cubana*, *Triozoida limbata*, and *Diaphorina citri* (control species) to acquire '*Candidatus Liberibacter asiaticus*'. The figure illustrates the three main stages of the experi-

ment: exposure of psyllids to CLas-infected source plants, maintenance during the latency period on their natural host plants, and transfer to healthy citrus seedlings for the post-latency evaluation

To statistically analyze the acquisition ability of CLAs and the effect of psyllid sex (female and male) in four different psyllid species (*I. divergipennis*, *H. cubana*, *T. limbata*, and *D. citri*), a generalized linear mixed-effects model (GLMM) was employed (Supplementary material “Statistical analysis”).

Inference of transmission ability

To assess the potential for CLAs transmission by the different psyllid species, molecular analyses were performed on dissected individuals. Each insect was divided into ‘head-mesothorax’ and ‘metathorax-abdomen’ sections to evaluate the presence of the bacterium in the salivary glands and in organs within the ‘metathorax-abdomen’, including the midgut, hindgut, Malpighian tubules, etc. This approach aimed to infer a potential association between bacterial localization and transmission capability. The ‘head’ was dissected at the mesothoracic region to isolate the salivary glands, based on their anatomical location as described for *D. citri* (Ammar et al. 2017).

The analyzed samples consisted of live individuals collected after an PLP. A total of 19 samples of *H. cubana*, 18 of *I. divergipennis*, 19 of *T. limbata*, and 24 samples of *D. citri* (control) were examined to determine the prevalence and titer of CLAs. Each sample comprised a pool of three insects, and was analyzed by qPCR.

To investigate the relationships between various factors, we addressed two key questions: (i) Is there an association between the copy number of the gene from the ‘head-mesothorax’ and ‘metathorax-abdomen’ regions overall? (ii) Do copy numbers from these two body regions differ across species and sex? To answer the first question, Spearman’s rank correlation was used to assess the relationship between the copy numbers from the two body regions. For the second question, a negative binomial generalized linear model (GLM) using the MASS package was employed to

examine the association between sex (female and male) and the copy number of the ET-s gene in the ‘head-mesothorax’ and ‘metathorax-abdomen’, accounting for potential species-specific effects.

Results

Psyllids collected from citrus trees

A total of 1681 psyllid specimens belonging to four different families: Aphalaridae (*Blastopsylla occidentalis* Taylor, *G. brimblecombei*, *Leurolophus* sp., and *Pseudophacopteron* sp.), Calophyidae (*Calophya* sp., and *Mastigimas* sp.), Psyllidae (*D. citri*, *Eucerosylla* sp., *H. cubana*, *I. divergipennis*, *Mitrapsylla* spp., and *Platycorypha* sp.), and Triozidae (*Calinda* sp., *Trioza* sp., and *T. limbata*), were collected with sweep net and the portable suction device on sweet orange (*C. sinensis*) trees sampled in the municipalities of Botucatu (SP) and Casa Branca (SP). The numbers of insects collected by each method at each location are provided in Suppl. Table 2.

The frequency and constancy rates varied with species (Suppl. Table 2). Among the most frequently collected species (i.e., proportion of individuals relative to the total across all species) were *H. cubana* and *D. citri*, with average frequency of 8.6%, and 17.8% respectively. In terms of constancy, *D. citri* was present in all samplings, followed by *I. divergipennis*, which was recorded in 24.7% of the samples, and *Mitrapsylla* spp., in 12.8% (Suppl. Table 1). The remaining species exhibited frequencies below 1% and constancies under 10% (Suppl. Table 1), all showing seasonal fluctuations (Fig. 2).

Temporal fluctuations in psyllid populations revealed that *D. citri* exhibited a consistent seasonal pattern across both sampling contexts, “trees in backyards” and “poorly managed groves”, characterized by a pronounced increase

Table 1 ‘*Candidatus liberibacter asiaticus*’ detection in ‘head-mesothorax’ and ‘metathorax-abdomen’ samples (pools of three individuals) across psyllid species, stratified by sex

Psyllid species	Sex	qPCR-positive proportion		Average gene copy number	
		Head-mesothorax	Metathorax-abdomen	Head-mesothorax*	Metathorax-abdomen*
<i>Diaphorina citri</i> Kuwayama	Female	8/11	9/11	5.85×10^6 a	3.51×10^7 a
	Male	8/13	8/13	5.83×10^6 b	3.49×10^7 b
<i>Heteropsylla cubana</i> Crawford	Female	0/9	1/9	-	2.80×10^4 a
	Male	0/10	2/10	-	5.44×10^4 a
<i>Isogonoceraia divergipennis</i> White & Hodkinson	Female	0/9	2/9	-	2.86×10^5 a
	Male	0/9	2/9	-	3.88×10^5 a
<i>Trioza limbata</i> Enderlein	Female	0/9	1/9	-	1.49×10^5 a
	Male	2/10	5/10	4.18×10^5	2.69×10^5 a

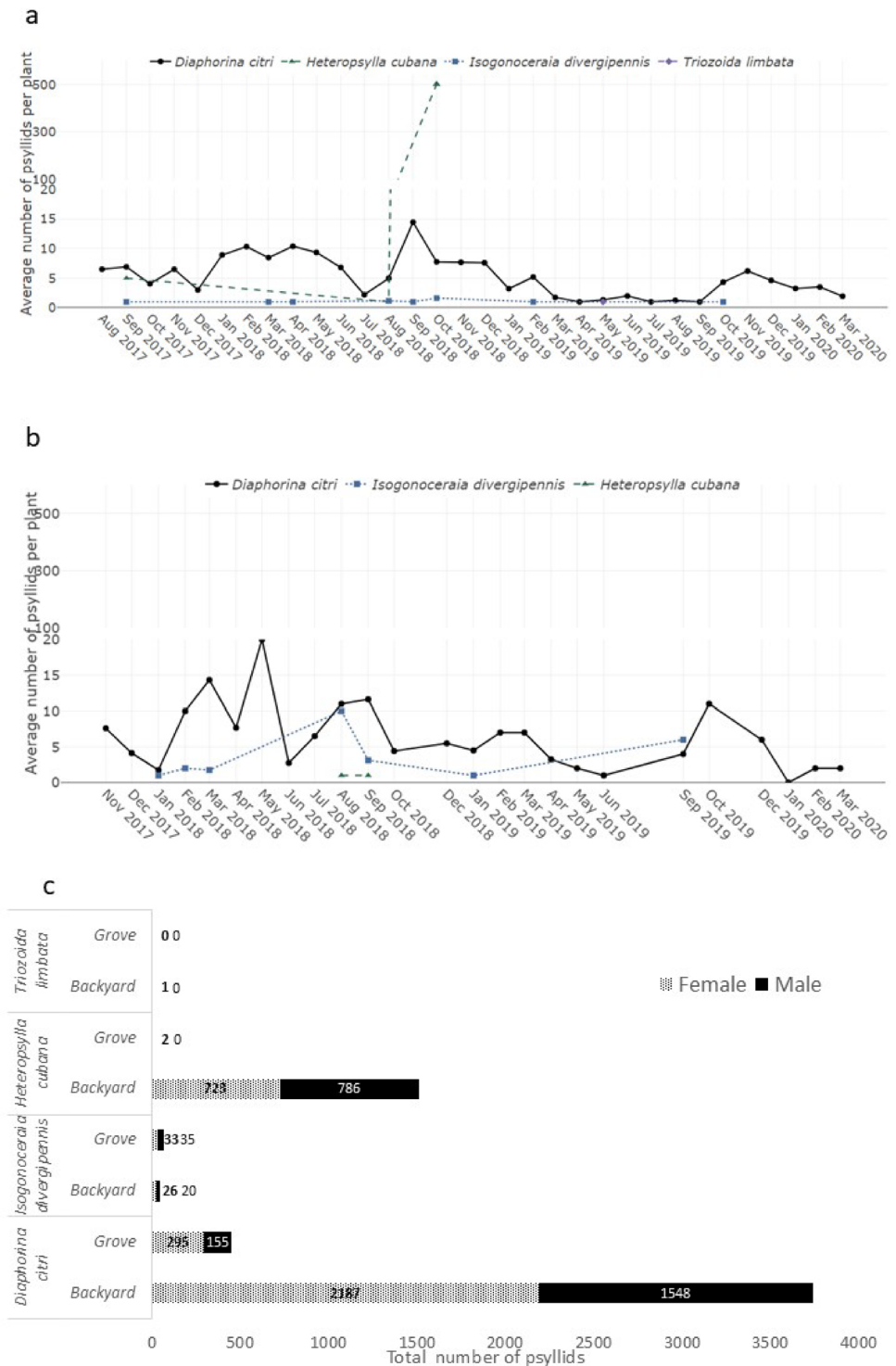
* Comparisons between females and males of the same species were considered significant at $p < 0.05$. Different letters (a, b) indicate statistically significant differences between groups (female and male), whereas identical letters denote no significant difference

during spring. Notably, in 2018, population growth began earlier, towards the end of winter, and the mean abundance of *D. citri* remained higher from January through June in both environments (Fig. 2).

H. cubana was detected exclusively during spring within the “trees in backyards” context across two consecutive years. In 2017, only a few individuals were collected during

the first week of spring, whereas in 2018 a clear outbreak occurred at the onset of the season. No additional captures of this species on citrus were recorded in subsequent years. Conversely, within “poorly managed groves,” a few individuals per plant were observed only at the end of winter 2018 (Fig. 2). Although citrus is not a documented host for *H. cubana*, the transient outbreak likely reflects dispersal

Fig. 2 Seasonal fluctuation and total abundance of four psyllid species collected per plant. Psyllids collected from *Citrus sinensis* plants between August 2017 and March 2020 in backyard plants in Botucatu, SP, Brazil (a), and poorly managed groves in Casa Branca, SP, Brazil (b), using a combination of sweep net and suction device sampling. c Total number of psyllids collected, categorized by sex and sampling context



activity during early spring, suggesting opportunistic use of citrus as a transient resource during this critical period of the year.

Another species not typically associated with citrus, *I. divergipennis*, displayed a similar temporal pattern, with populations increasing in late winter and early spring, approximately 2–4 weeks before the seasonal peak of *D. citri* in “poorly managed groves.” In contrast, in the “trees in backyards” context, *I. divergipennis* population peaks were less pronounced, although individuals were predominantly collected during spring (Fig. 2).

Finally, *T. limbata* was recorded only once on citrus, in May 2019 (Fig. 2). Despite this isolated occurrence, its significance lies in demonstrating that *T. limbata* may also engage in dispersal movements leading to incidental contact with citrus plants, underscoring the dynamic nature of psyllid-plant interactions during seasonal transitions.

Individuals of psyllid species collected (other than *D. citri*) were assayed by qPCR and tested negative for CLAs. Among the analyzed samples, species with higher frequency or constancy, such as *I. divergipennis* and *H. cubana*, were tested in more than 50 composite samples. Specifically, *I. divergipennis* was represented by 57 samples (23 from backyard plants and 34 from poorly managed groves), whereas *H. cubana* was represented by 51 samples (50 from backyard plants and one from poorly managed grove). In contrast, *D. citri* showed a CLAs detection rate of 61.5% among the 384 specimens analyzed, with gene copies number ranging from 1.6×10^8 to 2.5×10^4 .

Survival analysis: optimal AAP

Psyllids (*I. divergipennis* and *H. cubana*) that do not use citrus plants as hosts demonstrated the ability to survive for

at least 96 h on CLAs-infected citrus plants. Across all AAPs analyzed (48, 72 and 96 h), *D. citri* exhibited higher survival rates compared to the other two species, *I. divergipennis* and *H. cubana* (Fig. 3). Oviposition by *D. citri* and *H. cubana* was observed on the shoots where adults were confined during the different AAPs. Some shoots containing eggs of both species were kept for several days, but only the development of immature *D. citri* was observed; no development of *H. cubana* occurred.

The Welch's analysis of variance revealed a statistically significant difference in the survival of all species in relation to the AAPs ($F=26.25$, $p=9.373e-12$). When comparing *H. cubana* across the different AAPs, a statistically significant difference was observed between 48 and 96 h, but not between 48 and 72 h. For *I. divergipennis*, a significant difference was found between 48 h and the other AAPs (72 h: $p=0.0021$; 96 h: $p=2.7e-05$), but no difference was observed between 72 and 96 h. For *D. citri*, a statistical difference was observed between 48 and 96 h, but not between 48 and 72 h.

The 72-h AAP was selected for the subsequent experiments due to the lack of statistical difference in survival in relation to the 48-h AAP for *H. cubana* and *D. citri*, as well as for allowing a longer exposure time of the psyllids to the CLAs-infected plant.

Survival analysis: acquisition ability

The linear model, which evaluated survival across the sequential access periods to plants (AAP, LP, and PLP), revealed a significant overall effect ($F(11, 137)=9.79$, $p=6.278e-13$). The psyllids *H. cubana* ($t=-3.1$, $p=0.00218$), *I. divergipennis* ($t=-4.043$, $p=8.72e-05$), and *T. limbata* ($t=-2.327$, $p=0.02145$) presented significantly lower overall

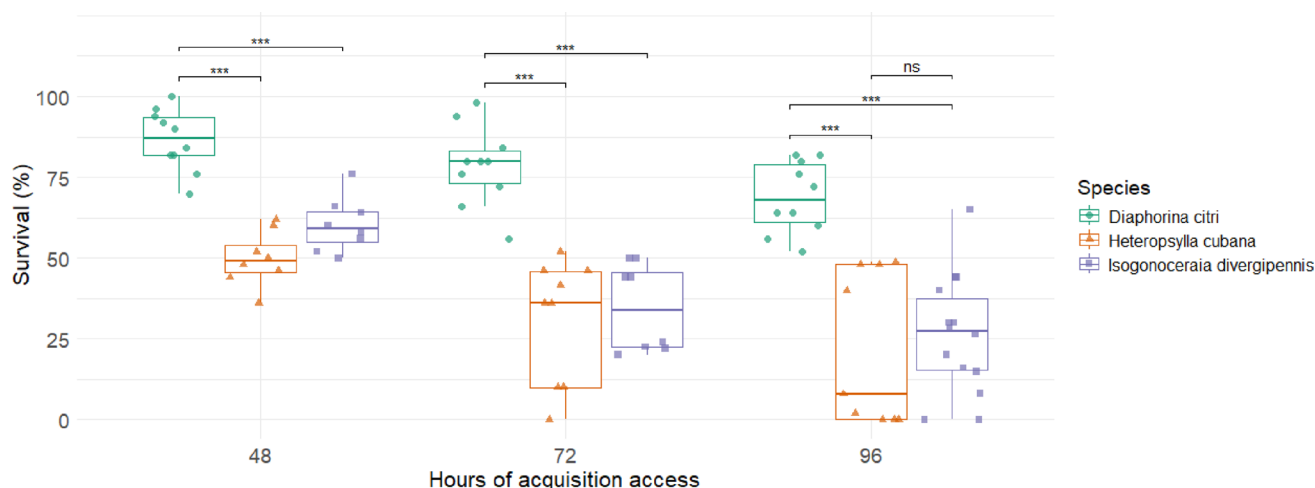


Fig. 3 Survival rates of *Diaphorina citri*, *Heteropsylla cubana*, and *Isogonoceraia divergipennis* over different acquisition access periods (48, 72, and 96 h) of ‘*Candidatus Liberibacter asiaticus*’ on citrus

plants. ns: no significant difference detected ($p \geq 0.05$), *** significant difference detected ($p < 0.05$)

survival rates, with estimated reductions of 24.6%, 34.1% and 26.33%, respectively, compared to *D. citri*.

Comparisons of the survival rate of the different psyllid species between the different periods only showed a statistical difference for *I. divergipennis* whose survival increased by 24.9% ($p=0.039$) during the LP. Although some interactions were not statistically significant, as in the case of *H. cubana* ($p=0.075$) and *T. limbata* ($p=0.057$) during the PLP, the proximity of the values to the significance threshold suggests a marked reduction in survival, greater than 20% and 28%, respectively.

Post-hoc pairwise comparisons using t-tests with unpooled standard deviations between species in each evaluation period revealed a significantly lower survival of *I. divergipennis* relative to *D. citri* ($p=0.0001$) during the AAP. During the PLP on healthy citrus seedlings, the survival rates of all three species (*T. limbata*: $35.9\pm 3.3\%$, *H. cubana*: $39.2\pm 7.75\%$, *I. divergipennis*: $43.7\pm 8.7\%$) were significantly lower than that of *D. citri* ($84\pm 2.6\%$) while in the PL no statistically significant differences were detected between species (Fig. 4).

CLas acquisition ability

Notably, all three psyllid species evaluated were able to acquire CLAs, with at least one qPCR-positive sample (Fig. 5). Across all three seasons, *D. citri* exhibited a high frequency of CLAs-positive samples, with 90.2% of females and 80.8% of males testing positive. Detection rates were highest in autumn, followed by winter and spring. The average gene copy numbers for *D. citri* were 8.8×10^6 and 2.3×10^7 for males and females in autumn, 2×10^5 and 1.4×10^6 in spring, and 4.6×10^6 and 3.3×10^7 in winter, respectively (Fig. 5).

Following *D. citri*, *I. divergipennis* showed the highest acquisition frequency among the other species, with seven positive samples detected during autumn and spring. These represented 9.8% of females and 5.7% of males across both seasons. In autumn, all positive samples were from females, with a mean gene copy number of 5.9×10^5 . In spring, both males and females tested positive, with males exhibiting a significantly higher mean gene copy number (8.9×10^8) compared to females (9.5×10^6), suggesting potential seasonal variation in infection dynamics.

The psyllid *H. cubana* had a lower acquisition frequency of 1.07%, with one positive sample identified in each season. In autumn and winter, the positive samples were from males, with a mean gene copy number of 3.8×10^5 , while the spring sample, also from a male, had a slightly lower gene copy number of 1.1×10^5 . Similarly, *T. limbata* exhibited a low frequency of 2.44% qPCR positives, with a single positive male sample showing 2.3×10^5 gene copies.

Statistical analysis indicated that sex did not significantly influence the odds of bacterial acquisition ($\beta = -0.1663$, $SE=0.4333$, $z=-0.384$, $p=0.701$; $OR=0.85$, $CI: 0.36-1.98$), suggesting that males had a slightly reduced likelihood (15% lower odds) of acquiring CLAs, although this effect was not statistically significant. In contrast, psyllid species had a significant impact on acquisition probability. Compared to the reference species *D. citri*, the other species demonstrated lower predicted acquisition ability: *I. divergipennis* ($\beta = -4.4040$, $SE=0.5496$, $z=-8.013$, $p<0.001$), *H. cubana* ($\beta = -5.1974$, $SE=0.6649$, $z=-7.817$, $p<0.001$), and *T. limbata* ($\beta = -5.6446$, $SE=1.0960$, $z=-5.150$, $p<0.001$). Despite these low probabilities, the detection of positive qPCR cases confirms that all three species can acquire the pathogen (Fig. 6).

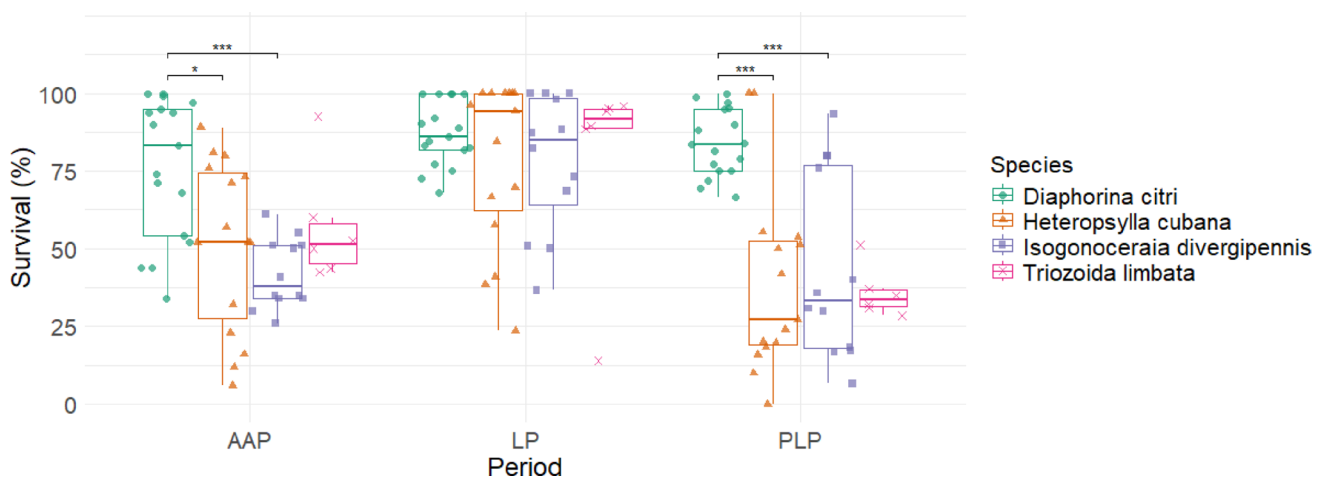


Fig. 4 Survival rates of *Diaphorina citri*, *Heteropsylla cubana*, *Isogonoceraia divergipennis*, and *Triozoida limbata* across the following access periods to plants: acquisition access period (AAP) of 72 h on citrus plants infected with ‘*Candidatus Liberibacter asiaticus*’,

latency period (LP) of 14 days on their natural host plants, and post-latency period (PLP) of 3 days of exposure to healthy citrus plants. In comparisons between species within each period, $p<0.05$ (***) and $p<0.1$ (*) indicate statistically significant differences

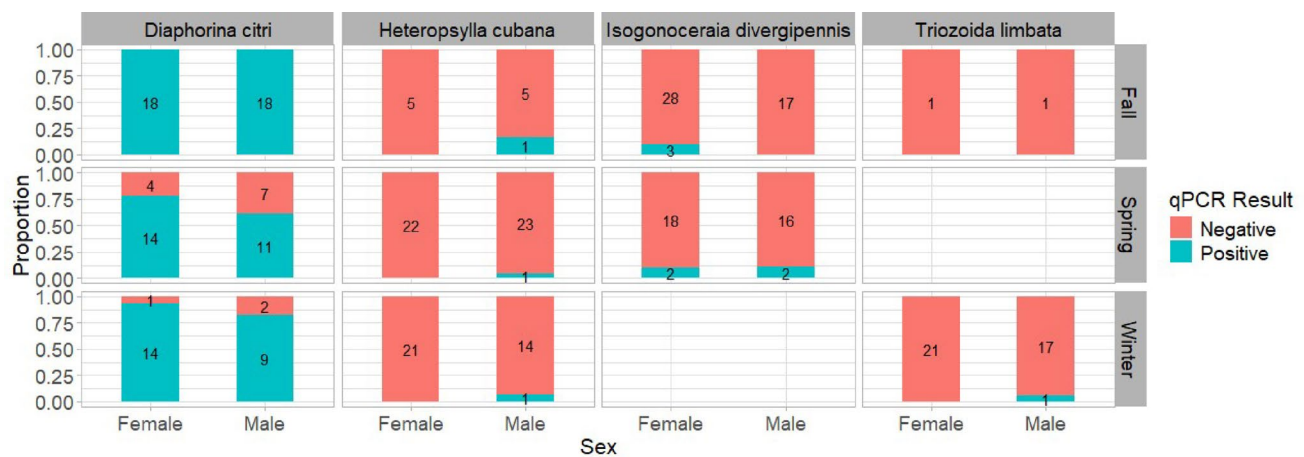


Fig. 5 Proportion of qPCR-positive samples for ‘*Candidatus Liberibacter asiaticus*’ by sex, season and psyllid species. Numbers within the bars represent the number of samples analyzed, each consisting of three individuals

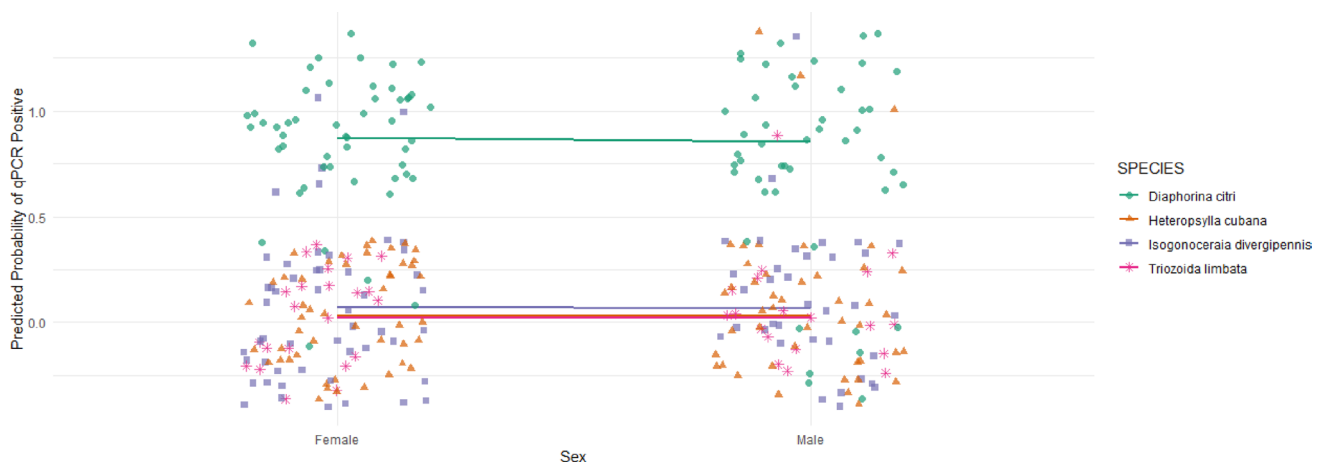


Fig. 6 Predicted probability of ‘*Candidatus Liberibacter asiaticus*’ acquisition by psyllid species and sex, based on a Generalized Linear Mixed Model

When comparing odds among non-vector species, *I. divergipennis* had the highest probability of acquisition at 1.22% (OR=0.0122), followed by *H. cubana* at 0.55% (OR=0.0055), and *T. limbata* with the lowest probability of 0.35% (OR=0.0035). All confidence intervals excluded 1, confirming that these differences are statistically significant (Supplementary material “Statistical analysis” - Table 1).

Inferences on the potential transmission of ‘*Candidatus Liberibacter asiaticus*’

Distinct CLAs detection patterns (by qPCR) were observed between the ‘head-mesothorax’ and ‘metathorax-abdomen’ across psyllid species (*I. divergipennis*, *H. cubana*, *T. limbata*, and *D. citri*). In *D. citri*, CLAs was present in 66.6% of ‘head-mesothorax’ samples and 70.83% of ‘metathorax-abdomen’, while in *T. limbata*, detection rates were lower, at 10.5% and 31.6% for the respective regions, whereas *I.*

divergipennis and *H. cubana* showed 22.2% and 15.8%, respectively, only in the ‘metathorax-abdomen’ (Table 1).

Spearman’s correlation analysis identified a significant positive correlation between EF-Ts gene copy numbers in these regions ($\rho=0.609$, $p=0.007$). This positive correlation suggests that an increase in the EF-Ts gene copy number in the ‘metathorax-abdomen’ is associated with a corresponding increase in the ‘head-mesothorax’ region, reflecting a potential systemic distribution of CLAs across the sampled tissues.

A negative binomial GLM was employed to assess the effects of body region and sex on CLAs copy number. Comparisons between the ‘head-mesothorax’ and ‘metathorax-abdomen’ regions were conducted exclusively for *D. citri* and *T. limbata*, as these were the only species with CLAs detection in the ‘head-mesothorax’. In *D. citri*, the model revealed significant effects of both body region and sex ($\beta_0=17.9$, $p<0.001$), with bacterial abundance being highest in the ‘metathorax-abdomen’ compared to the

‘head-mesothorax’ ($\beta_1 = -1.9$, $p = 0.0009$). CLas prevalence was also greater in the ‘metathorax-abdomen’ (70.83%) than in the head-mesothorax (66.6%) across both sexes. Additionally, males exhibited significantly lower CLas copy numbers (not separated by region) than females ($\beta_2 = -2.2$, $p = 0.0001$).

T. limbata showed no significant difference in bacterial copies in the ‘head-mesothorax’ and ‘metathorax-abdomen’ regions ($\beta_1 = 0.44$; $p = 0.49$), and although males had more copies than females, this difference was also not statistically significant ($\beta_2 = 0.59$, $p = 0.48$) (Table 1).

H. cubana and *I. divergipennis* showed significantly lower bacterial loads compared to *D. citri*, with detectable levels restricted to the ‘metathorax-abdomen’. For all species, the negative binomial generalized linear model revealed significant associations between ‘metathorax-abdomen’ copy number of EF-Ts gene and both sex and species. In general terms, males exhibited a significantly lower ‘metathorax-abdomen’ copy number compared to females ($\beta = -1.27$, $SE = 0.49$, $z = -2.58$, $p = 0.009$). Furthermore, significant interspecific differences were observed, with *H. cubana* ($\beta = -5.51$, $SE = 0.81$, $z = -6.8$, $p < 0.001$), *I. divergipennis* ($\beta = -3.9$, $SE = 0.7$, $z = -5.5$, $p < 0.001$), and *T. limbata* ($\beta = -3.9381$, $SE = 0.63$, $z = -6.1$, $p < 0.001$) exhibiting significantly lower ‘metathorax-abdomen’ copy number relative to *D. citri*. In contrast, no statistically significant differences were found among *H. cubana*, *I. divergipennis*, and *T. limbata*.

Analysis of EF-Ts gene copy number revealed sex-specific differences in bacterial load. In the ‘metathorax-abdomen’, *D. citri* males carried significantly fewer bacteria than females ($\beta = -2.03$, $p = 0.004$), whereas *H. cubana* males had slightly higher loads without statistical significance ($\beta = 0.88$, $p = 0.059$); no significant sex effects were observed in *I. divergipennis* ($\beta = 0.30$, $p = 0.726$) or *T. limbata* ($\beta = 0.59$, $p = 0.387$), indicating that sex-related variation is not consistent across species (Table 1). In the ‘head-mesothorax’, males generally exhibited lower bacterial loads than females ($\beta = -2.3$, $p = 0.007$), though interspecific differences were not significant, as *T. limbata* did not differ from *D. citri* ($\beta = -0.85$, $p = 0.54$); however, this outcome is largely driven by *D. citri*, given amplification success in only two *T. limbata* males.

Discussion

This study presents the first record of *H. cubana*, *I. divergipennis*, and *T. limbata* on *C. sinensis* plants in natural conditions, suggesting that citrus may serve as either a food or casual plant for these species.

The relationship between psyllids and their host plants is generally known to be highly specific, but they also present host switching between related plants at local scales (Ouvrard et al. 2015). Despite that, some psyllid genera, such as *Trioza* and *Cacopsylla*, exhibit a broad host range. *Trioza*, for instance, includes 346 species associated with 154 plant genera across 59 families (Ouvrard et al. 2015). However, the psyllids investigated here have Fabales as hosts, as in the case of *I. divergipennis* and *H. cubana*, and Myrtaceae as in the case of *T. limbata*, and are clearly not closely related to Rutaceae. Although some psyllid families have genera (i.e. *Trioza* and *Cacopsylla*) with a large number of host plants, the psyllid species investigated here are restricted to Fabaceae as far as we know.

The 2018 outbreak of *H. cubana*, involving an aggregation of 1509 individuals on a single citrus plant (Suppl. Tables 2 and Fig. 2a), underscores the potential significance of convective air currents in facilitating the wind-assisted movement of psyllids, particularly during periods of passive dispersal. This phenomenon highlights the complex interplay between atmospheric dynamics and insect dispersal, and the role of non-hosts plants serving either as land or food sources. For example, the recurrent presence of *I. divergipennis* on citrus plants (24.7%), albeit at low population densities, suggests that citrus may function as a food plant for this species.

Seasonal patterns of psyllid occurrence on host plants are generally consistent, with a notable tendency for increased dispersal activity during spring (Hall and Hentz 2011). However, this study revealed interspecific variations in the timing of population movements compared to *D. citri*. Specifically, while *D. citri* and *H. cubana* populations exhibited typical increases in early spring, *I. divergipennis* displayed a distinct pattern, with population peaks occurring in late winter and early spring, preceding the *D. citri* peak by approximately 2–4 weeks. These findings highlight species-specific differences in seasonal population dynamics, which may be influenced by variations in environmental cues, host plant phenology, or physiological adaptations.

Ability of feed and survive on non-hosts

The ability of monophagous or oligophagous psyllids to survive and feed on non-host plants is a critical factor in understanding their adaptability and potential ecological impact. Our results demonstrate that *I. divergipennis*, *T. limbata*, and *H. cubana* exhibit varying degrees of survival when forced to feed on *C. sinensis*, which is not their breeding host, as evidenced by the absence of oviposition by *I. divergipennis* and *T. limbata*, and the lack of egg development in *H. cubana* during the AAPs on citrus shoots. Notably, all three species had a lower survival rate compared to *D. citri*

on its host plant, which is expected but suggests a surprising level of resilience in suboptimal feeding conditions.

I. divergipennis exhibited the lowest survival rate ($41.9 \pm 3.24\%$) while feeding on CLas+citrus plants, highlighting its difficulty in utilizing non-host plants as a food source. However, survival increased dramatically to 78.2% when transferred back to its natural host. The inability to access phloem cells, as suggested by Sandanayaka et al. (2019), likely plays a critical role in this reduced survival, as phloem serves as a primary nutrient source for psyllids. In spite of that, the ability of *I. divergipennis*, *H. cubana*, and *T. limbata* to sustain themselves for extended periods (~96 h) on a non-host plants suggests that they may incidentally utilize citrus as a temporary food source.

During the post-latency period on healthy citrus seedlings, the survival rates of all three species (*T. limbata*, *H. cubana*, *I. divergipennis*) were significantly lower than that of *D. citri*. This reinforces the idea that while these species can persist on non-host plants, they are also capable of returning to their host plants and even refeeding on non-host plants. Notably, survival rates exceeding 35% after 20 days of alternating between citrus (3 days at the beginning and end) and host plants (14 days) suggest a level of adaptability that allows them to sustain themselves despite shifts between host and non-host environments.

'*Candidatus Liberibacter asiaticus*' acquisition by psyllids other than *Diaphorina citri*

We found that all psyllid species tested were able to acquire CLas, at rates that differed moderately according to sex and seasons. Overall, *I. divergipennis* showed the highest acquisition rate compared to *T. limbata* and *H. cubana*. The ability to acquire and retain CLas by distinct psyllid species is not unexpected. Although *Candidatus Liberibacter* spp. are thought to have a specific relationship with their insect vectors, in the context of HLB it has demonstrated that CLas can also be transmitted by the African citrus psyllid, *T. erytrae* (Reynaud et al. 2022), which belongs to a distinct psyllid family (Triozidae), whereas *D. citri* has been shown to transmit *Ca. Liberibacter africanus* (CLaf), which has *T. erytrae* as its natural vector in endemic regions of Africa (Lallemand et al. 1986). In addition, CLas has been detected in individuals of *Cacopsylla citrisuga* collected on *Citrus limon* L. in China (Cen et al. 2012), for which there is still no evidence of transmission.

The acquisition of CLas by psyllids not previously identified as vectors can be influenced by physiological and anatomical factors that regulate bacterial retention within the insect. Based on the predicted acquisition rate (odds ratio), *I. divergipennis* exhibited a 1.22% probability of acquiring CLas, while *H. cubana* and *T. limbata* showed lower

probabilities of 0.55% and 0.35%, respectively. Since these probabilities were derived from CLas detection at the end of the PLP, they primarily reflect bacterial retention, which depends on cell-cell interactions within different insect tissues.

In *D. citri*, the formation of *Liberibacter*-containing vacuoles (LCVs) in gut cells facilitates bacterial survival and propagation, whereas *B. trigonica* infected with *Ca. L. solanacearum* lacks such structures and shows no cell death in the midgut cells, unlike the *D. citri*-CLas interaction (Ghanim et al. 2016, 2017). Similarly, no apoptosis was observed in *B. cockerelli* midgut cells, although vesicle-like structures on the basal lamina suggest exocytosis as a route for bacterial passage (Tang and Tamborindeguy 2019; Cicero et al. 2017). These findings highlight species-specific strategies for *Ca. Liberibacter* retention and dissemination, and it is plausible that analogous mechanisms occur in *I. divergipennis*, *H. cubana*, and *T. limbata*, which retain CLas for up to 20 days at low rates. Notably, *I. divergipennis* exhibited bacterial loads exceeding those of *D. citri*, particularly in one male sample during spring (3×10^8), a magnitude far surpassing the transmission threshold ($\sim 10^6$ bacteria per insect in citrus) (Ukuda-Hosokawa et al. 2015). Females of *I. divergipennis* carried bacterial loads comparable to *D. citri* males, ranging from 3.1×10^6 to 1.9×10^5 . Although *I. divergipennis* is not recognized as a vector, its ability to sustain such high bacterial densities suggests an internal environment conducive to CLas persistence, potentially through mechanisms similar to those facilitating bacterial retention in *D. citri* (Ammar et al. 2018). In contrast, CLas detection in *H. cubana* (1.1×10^5 to 3.8×10^5) and *T. limbata* (2.3×10^5) was restricted to males and occurred sporadically across seasons, indicating lower bacterial retention.

In our study, *D. citri* females consistently exhibited higher CLas titers than males across seasons, although the difference in acquisition probability was not statistically significant. Similar sex-related differences were previously reported by Lopes and Cifuentes-Arenas (2021) and Wu et al. (2016), who found higher detection and transmission rates in females (26%) than in males (18%), despite males displaying more frequent phloem salivation events (82% vs. 76%). A positive correlation between bacterial titer and acquisition and inoculation capacity has been demonstrated (Ammar et al. 2018), and in our assays, the highest titers occurred in females during fall and winter, with the lowest concentrations in spring. These seasonal fluctuations are consistent with the observations of Ukuda-Hosokawa et al. (2015), who reported low CLas densities in early spring ($\sim 10^3$), rising in late spring to 10^6 – 10^7 , and remaining constant in autumn ($\sim 10^6$ – 10^7). In psyllid species other than *D. citri*, the prevalence of *Ca. Liberibacter* spp. infection varies across species. Lin et al. (2022) reported sex-based

differences in *Macrophoma gladiata* Kuwayama (Car-sidaridae) populations collected from *Ficus macrocarpa* L. (Moraceae), with males exhibiting a higher proportion of individuals carrying high *Ca. Liberibacter* sp. titers. In contrast, *Cacopsylla oluanpiensis* Yang showed no significant sex-related differences in infection rates of ‘*Ca. Liberibacter europaeus*’ (Fang et al. 2023). Therefore, species-specific variations in insect-plant-bacteria interactions beyond sex may drive differences in *Ca. Liberibacter* spp. acquisition and retention.

Inference of ‘*Candidatus Liberibacter asiaticus*’ transmission by detection in separate regions of the psyllid body

Detecting bacteria in distinct body regions provides crucial insights into their circulation within the host and potential for transmission. Our results revealed a positive correlation between CLas copy numbers in the ‘head-mesothorax’ and ‘metathorax-abdomen’ consistent with the findings of Ukuda-Hosokawa et al. (2015), who observed this correlation across sexes and seasons (spring and autumn). They also reported that bacterial titers in the head were generally lower than those in the abdomen, although the difference was not statistically significant. A similar pattern was observed in our study; however, the difference between ‘head-mesothorax’ and ‘metathorax-abdomen’ titers was statistically significant.

CLas was detected in both *D. citri* and *T. limbata* across the ‘head-mesothorax’ and ‘metathorax-abdomen’ regions, whereas in *H. cubana* and *I. divergipennis*, it was exclusively found in the ‘metathorax-abdomen’. The absence of CLas detection in the ‘head-mesothorax’ of *H. cubana* and *I. divergipennis* raises important questions regarding potential barriers to bacterial circulation. Structural or physiological constraints may impede bacterial movement from the midgut to the hemolymph, preventing colonization of the salivary glands. Alternatively, bacterial replication in these species may be insufficient to reach detectable levels in transmission-relevant tissues.

The midgut and salivary glands of *D. citri* are well-established barriers to CLas transmission, with bacterial titers in the salivary glands remaining notably low (Ammar et al. 2011). The detection of CLas in the ‘head-mesothorax’ of *T. limbata* suggests potential bacterial movement from the ‘metathorax-abdomen’ region and raises the possibility of transmission. However, the mere presence of CLas in this region does not confirm vector competence, as successful transmission depends on multiple factors, including bacterial replication within the host, ability to overcome physiological barriers, and eventual delivery into plant tissues during feeding.

The possible involvement of psyllid species other than *D. citri* in HLB epidemiology is an issue that requires investigation, considering the risk of intermittent pathogen introduction into citrus ecosystems during psyllid dispersal events. While these psyllids may occasionally acquire CLas in the field, there is no yet evidence that they naturally carry (based on the infectivity assays conducted in the present study) and transmit the pathogen to citrus. In case their vector competence is not confirmed in future transmission assays, they still may serve as models for investigating transmission barriers of CLas in the vector.

Field-based research remains indispensable to quantify the epidemiological impact of alternative psyllid species and their role in HLB dynamics under natural conditions. Comprehensive surveillance strategies incorporating non-vector psyllid populations could result in more precise risk assessments and management interventions. Considering that the population peaks of some psyllid species (for instance *I. divergipennis*) are typically observed a few weeks before those of *D. citri*, the analyzes of their seasonal fluctuations could be integrated into disease forecast models for predicting immigration of *D. citri* into citrus orchards in late winter and early spring.

By integrating these research directions, future studies can help to resolve key uncertainties on the involvement of other psyllid species in HLB epidemiology. A refined understanding of their ecological roles, coupled with improved diagnostic and modeling tools, will ultimately contribute to more effective and adaptive disease management strategies.

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Author contributions All authors contributed to the study. M.T.B.C. and J.R.S.L. were responsible for the conceptualization, design, and methodology. M.T.B.C. conducted the field and laboratory work, performed the data analyses and prepared the original draft. J.R.S.L. critically reviewed and provided input on previous versions of the manuscript. All authors read and approved the final version.

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Declarations

Conflict of interest The authors have no competing interests to declare that are relevant to the content of this article.

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