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BIOLOGICAL CONTROL

The Enemy is Outside: Releasing the Parasitoid *Tamarixia radiata* (Hymenoptera: Eulophidae) in External Sources of HLB Inocula to Control the Asian Citrus Psyllid *Diaphorina citri* (Hemiptera: Liviidae)

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Abstract

Huanglongbing (HLB), the most destructive citrus disease worldwide, was first recorded in Brazil in 2004, and since then, more than 50 million trees identified with this disease have been eliminated. The disease is managed mainly by controlling the psyllid vector *Diaphorina citri* Kuwayama, 1908 (Hemiptera: Liviidae). Although the presence of the insect in commercial citrus groves is low, HLB infection rates increase in areas bordering the groves. The disease is transmitted by psyllids from host citrus plants in areas outside the managed groves, such as abandoned or organic groves and residential trees, and from orange jasmine plants in urban settings. In order to provide information to support HLB control, this study evaluated the biotic and abiotic variables that affect the dynamics of *D. citri* populations after releases of the parasitoid wasp *Tamarixia radiata* (Waterston, 1922) (Hymenoptera: Eulophidae) in external sources of HLB inocula. The study was divided into two parts. After releasing the parasitoids in non-commercial areas, we determined the following: (a) the variables that significantly affected the number of nymphs collected on shoots in the same non-commercial area; (b) the variables that significantly affected the number of adult psyllids collected in a neighboring commercial citrus area. Our results indicated that the number of nymphs in external areas was affected only by the host plant and rainfall. However, periodic parasitoid releases significantly reduced the number of adult psyllids collected in the commercial area. The results indicate that the release of parasitoids in external sources of inocula has the potential to maximize actions for *D. citri* control, contributing to the reduction of psyllid populations in commercial areas. Consequently, this strategy may help to manage the disease infection without an increase in insecticide use.

Introduction

Since huanglongbing (HLB) or citrus-greening disease was first recorded in Brazil in 2004 (Teixeira *et al* 2005), more than 50 million plants identified with the disease have been eliminated (CDA 2019). The disease is present in almost all citrus-producing regions worldwide (Bové 2006, Hall *et al* 2013). Although HLB was recently detected in Brazil only, the vector, Asian citrus psyllid (ACP) *Diaphorina citri* Kuwayama, 1908 (Hemiptera: Liviidae), has been present in the country since 1938 (Costa Lima 1942). After the disease was detected, several control strategies were applied, including exclusive use of healthy citrus seedlings, elimination of symptomatic plants, and chemical control of the vector. Because of the intensive use of insecticide sprays to control *D. citri*, classical, augmentative, or conservation biological control (BC) could not be used in commercial areas, since the citrus growers rarely use selective insecticides.

Examining the HLB epidemiology, Bergamin-Filho *et al* (2016) found that the primary inocula occur in areas surrounding the groves, where the psyllids can develop and migrate, transmitting the bacterial infection. Previously, Tiwari *et al* (2010) found that large *D. citri* populations migrated from abandoned areas to commercial groves. These areas include abandoned groves (with no psyllid control), organically managed groves, residential backyards with citrus trees, and urban areas with ornamental orange jasmine (*Murraya paniculata*, a psyllid host) (Sétamou & Bartels 2015).

The ectoparasitoid *Tamarixia radiata* Waterston, 1922 (Hymenoptera: Eulophidae) was first recorded in 2004 in Brazil, associated with high rates of natural parasitism on *D. citri* nymphs (Gómez-Torres *et al* 2006). Although *T. radiata* releases have been studied in order to control *D. citri* (Skelley & Hoy 2004, Flores & Ciomperlik 2017), studies focused on releasing the parasitoid in commercial areas indicated a low effectiveness of biological control, due to the intense use of chemical control (Beloti *et al* 2015). An alternative strategy would be to release parasitoids in non-commercial areas, controlling the source of adult psyllids and their influx into commercial areas (Parra *et al* 2016; Milosavljević *et al* 2018).

Given the importance of managing *D. citri* in external sources of HLB inocula to control the spread of citrus-greening disease, the present study was composed of two different and independent studies, both of them focusing on parasitoid releases in non-commercial areas to control the psyllid vector: (1) releasing *T. radiata* in non-commercial areas to investigate if the parasitism affected these *D. citri* populations outside commercial areas; (2) releasing *T. radiata* in non-commercial areas (neighboring commercial area) to investigate if parasitoid releases in external areas affect the dynamics of adult psyllid populations within the commercial grove.

Material and Methods

In the first part of this study, we monitored *D. citri* populations at 9 sites in non-commercial areas in the citrus belt of the state of São Paulo, to investigate the variables that may affect nymph dynamics including parasitism by *T. radiata*, by releasing parasitoids biweekly in these areas and observing the presence of eggs and larvae of *T. radiata* in *D. citri* nymphs (the "Releasing the parasitoid *T. radiata* to control *D. citri* populations in non-commercial areas" section). In the second part, we evaluated whether releasing *T. radiata* in a non-commercial area located in the municipality of Itapetininga (Rechã district), São Paulo, could affect the number of adult psyllids in a commercial grove (the "Releasing the parasitoid *T. radiata* to control *D. citri* populations inside commercial areas" section). The two studies are linked and complemented each other, since the first one studied the psyllid in its proliferation zone (non-commercial areas) and the second studied the adult vector psyllids that disperse to commercial areas from external sources of HLB inocula, carrying the greening disease.

Releasing the parasitoid ***T. radiata*** *to control* ***D. citri*** *populations in non-commercial areas*

For this experiment, nine non-commercial areas located in municipalities that compose the citrus belt of the state of São Paulo were chosen (Fig 1). These were divided into three groups: group (1) 3 areas with *Citrus* plants as hosts and with chemical control, in the municipalities of Leme (2.5 ha), Matão (2.5 ha), and Monte Azul Paulista (2.0 ha); group (2) 3 areas with citrus plants as hosts and without chemical control, in the municipalities of Anhembi (2.5 ha), Getulina (2.5 ha), and Itapetininga (2.5 ha), for both citrus areas the plants was 7 to 10 years old; and group (3) 3 areas with orange jasmine (*Murraya paniculata*) as host and without chemical control, in the municipalities of Cajobi (2.5 ha), Pirajuí (1.0 ha), and Rincão (1.5 ha) with unknown age but adult and vigorous plants.

Nymph population was monitored, biweekly, in each area, from February 2012 to January 2013. The first sampling (before start the parasitoid's releasing) in the areas indicated that natural parasitism was present only in Rincão (22.2%), Itapetininga (3.5 %), and Pirajuí (0.96%). During the same period, releases were conducted at a rate of 400 parasitoids/ha at two different points, approximately at the center of the study areas, as part of a biological control program targeting *D. citri*. The individuals of *T. radiata* were reared according to Parra *et al* (2016). *Tamarixia radiata* adults (~ 24 h after emergence) were transported to the field in 2.5-cm glass tubes with a honey droplet for insect nourishment, packed in Styrofoam boxes.

In each area, 60 branches (≅ 10 cm) were collected at the upper third of the plant biweekly during the sampling period, from different trees selected randomly in different parts of the area in order to obtain a representative sample. The branches were individually bagged, placed in a Styrofoam box, and taken to the Laboratory of Insect Biology of the Entomology and Acarology Department, Luiz de Queiroz College of Agriculture, University of São Paulo (USP-ESALQ). At the laboratory, the branches were inspected for *D. citri* nymphs and *T. radiata* under a stereoscopic microscope.

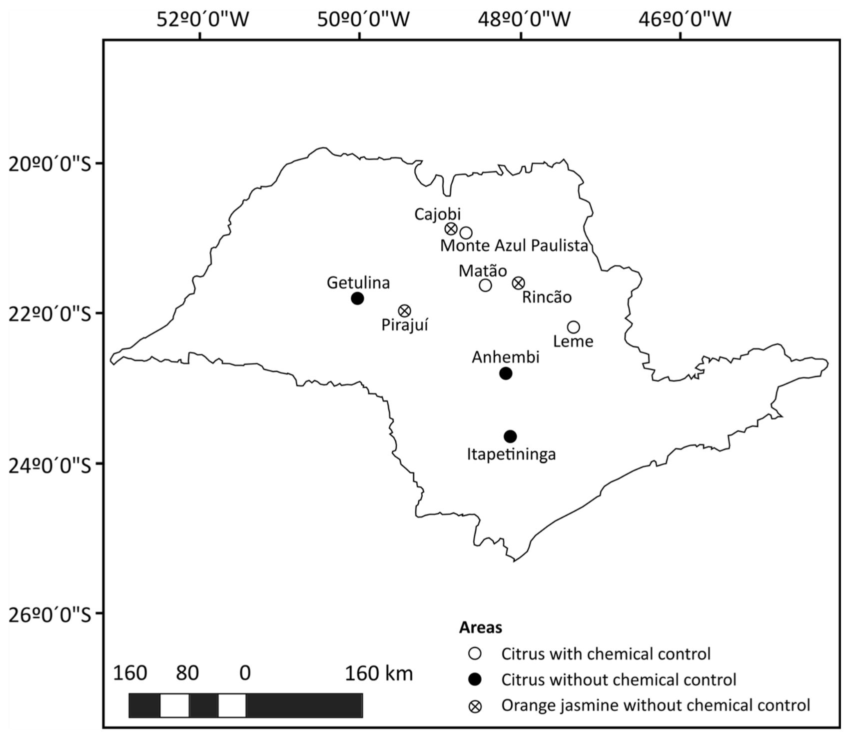

Fig 1 Geographic locations and characteristics of the nine sites chosen for the first part of our study.

Each nymph was examined for the parasitoid, i.e., for the presence of *T. radiata* eggs, larvae or pupae attached, or mummified nymphs with an exit hole. The numbers of non-parasitized and parasitized nymphs were recorded. On each sampling date, air temperatures for each study site were obtained from the closest weather station. We also obtained data for monthly rainfall. Each study site was located within 5 km from the nearest meteorological station.

We analyzed whether *D. citri* populations were affected by the parasitism rate and/or other factors (temperature, monthly rainfall, host plant, and month) by using a generalized linear model (GLM) with a negative binomial distribution since over dispersion was observed in our count data (O'Hara & Kotze 2010). The dependent variable was the nymphs recorded at each site for each sample date. Host plant (citrus, orange jasmine), temperature, rainfall, parasitism rate, and sample month were used as explanatory variables. We studied a wide range of variables, not only the parasitism rate, in order to determine the biotic and abiotic factors that may affect psyllid populations, providing more information for use in management programs. We calculated *P* values of the selected model using the likelihood ratio test. Non-significant explanatory variables ($P > 0.15$) were removed to obtain the minimal adequate model, following the methodology used by Milosavljević *et al* (2018). Model selection with information criteria (Akaike's information criterion [AIC]) was performed to identify the best model (lowest AIC value). All statistical analyses were performed in R® software (Development Core Team R 2018).

*Releasing the parasitoid **T. radiata** to control **D. citri** populations inside commercial areas*

The study was carried out in a commercial grove (Itapetininga; 23°35′30″S, 48°03′11″W). Graminha Farm is a commercial citrus grower with a total area of 2700 ha. The farm has a strict pest-sampling program, with biweekly visual inspection of 1% of the citrus trees for psyllid nymphs. Adult psyllids were monitored with yellow sticky traps located every 150 m along the entire farm border, and other sticky traps within the groves, totaling to 735 traps. The farm uses chemical-control sprays for mature trees and soil-drench applications for new trees (bifentrin and dimethoate). However, the psyllids continue to be present because the insects move in from non-commercial areas outside the farm where no control is practiced. *D. citri* can disperse as far as 1.6 or 2.2 km from the breeding site (Ferreira 2014, Lewis-Rosenblum *et al* 2015).

The farm is bordered by the Rechã district that has approximately 4500 residents, and with citrus trees in residential backyards, all of which can serve as hosts for the psyllid (considered as pest inocula). The district has approximately 200 host plants including all the types mentioned. This experiment was carried out in way to represent the real conditions of the infestation occurring in commercial areas once, as described by Bergamin-Filho *et al* (2016), psyllid movement onto that areas still occur despite of intensive insecticide sprays.

We obtained, from the farm, the number of adult psyllids per yellow sticky trap biweekly from August 2014 to August

2015. At the beginning of the study, we also started biweekly releases of *T. radiata* at 8 different points, inside the Rechã district, approximately 200 m from each other, at a rate of 400 parasitoids/point, and continued the releases for the entire sampling period. Each parasitoid-release location was located approximately 2 km from the farm border. Temperature and monthly rainfall were obtained from automatic meteorological station located inside the farm.

We analyzed whether the number of psyllids per sampling date (dependent variable) was affected by the cumulative number of parasitoid releases since the beginning of the study and/or by abiotic factors (temperature, month, and rainfall) (explanatory variables), using generalized linear models (GLM). We calculated *P* values of the selected model using the likelihood ratio test. Non-significant explanatory variables ($P > 0.15$) were removed to obtain the minimal adequate model, following the methodology used by Milosavljević *et al* (2018). Model selection with information criteria (Akaike's information criterion) was performed to identify the best model (lowest AIC value). All statistical analyses were performed in R software (Development Core Team R 2018).

Results

Effects of the parasitism rate on ***D. citri*** *nymphs in non-commercial areas*

In Leme, Matão, and Monte Azul Paulista (Group 1, regular insecticide use), *D. citri* nymphs and the parasitoid were technically absent. Therefore, we did not use these three areas in our analysis. In the municipalities that composed groups 2 and 3, the incidence of nymphs per branch ranged from 1.92 to 2.8 and 0.88 to 2.57, respectively, throughout the study.

The number of *D. citri* nymphs was not significantly affected by month, *T. radiata* parasitism (Fig 2), temperatures, or interactions among these variables ($P > 0.15$). Nymphs were significantly affected only by the host plant (estimate = – 0.24026, SE = 0.13503, $\chi^2 = 2.9089$, $Df = 1$, $P = 0.088$) and rainfall (estimate = – 0.03161, SE = 0.02078, $\chi^2 = 2.2295$, $Df = 1$, $P = 0.135$). Regarding the host plant, the mean number of psyllids per sampling was equal to 117.06 and 93.58 in citrus and orange jasmine, respectively (Fig 3).

Effect of the number of parasitoid releases in an external area on the number of adult Asian citrus psyllids collected inside a commercial area

In the second part of our study, the cumulative number of parasitoid releases (estimate – 0.08874, SE = 0.0460310, $\chi^2 = 90783$, $Df = 1$, $P = 0.0026$) was the only variable found to be significant (Fig 4). The climate variables (temperature and rainfall) and the month did not significantly affect the *D. citri* population.

Discussion

Effects of the parasitism rate on ***D. citri*** *nymphs in non-commercial areas*

Between 2005 and 2010, the use of agrochemicals in Brazilian citriculture increased more than 600% (Neves *et al* 2011). Beloti *et al* (2015) found that more than half of the 25 insecticides recommended for citrus pests were classified as harmful (IOBC class 4), and only 20% were considered harmless to *T. radiata*. This information could explain our results for the commercial groves with insecticide use, where no parasitism was recorded. This observation demonstrates the importance of environments without any control for *D. citri* reproduction and dispersal, mainly to commercial groves (Bergamin-Filho *et al* 2016).

Rainfall was negatively correlated with the number of psyllid nymphs. Heavy rainfall reduces populations by washing 1st- and 2nd-instar nymphs from the trees. Only adults manage to survive because they hide on the lower surfaces of the leaves and twigs (Aubert 1987, Ahmed *et al* 2004, Teck *et al* 2011a). Hall *et al* (2008) also observed that high levels of monthly rainfall, above 150 mm, drastically reduced the density of nymphs, due to a flushing effect that leaves nymphs exposed to the rain impact. However, in our study, the temperature did not significantly affect the number of nymphs. Published sources report divergent effects of climate variables on the dynamics of *D. citri* nymphs. Chong *et al* (2010) found no correlation of natural factors (wind, rainfall, relative humidity, and temperature) with psyllid populations. Similarly, Michaud (2004) found no correlation between rainfall and psyllid populations. However, according to Tsai *et al* (2002), weather factors should in fact directly affect flushing, this will affect the *D. citri* populations.

Host plant also significantly affected the population of psyllid nymphs. A larger number of insects were associated with citrus plants than with orange jasmine. Although orange jasmine was reported as the most suitable host for psyllid development (Aubert 1987, Nava *et al* 2007; Teck *et al* 2011b), Stockton *et al* (2017) observed that nymphs were larger in size and developed more rapidly on citrus than on orange jasmine. Other studies found no significant difference in biological traits (oviposition, development time, longevity, viability) between psyllids on citrus and orange jasmine (Alves *et al* 2014, 2018). However, all these studies were conducted under laboratory conditions, whereas we evaluated the number of nymphs in the field.

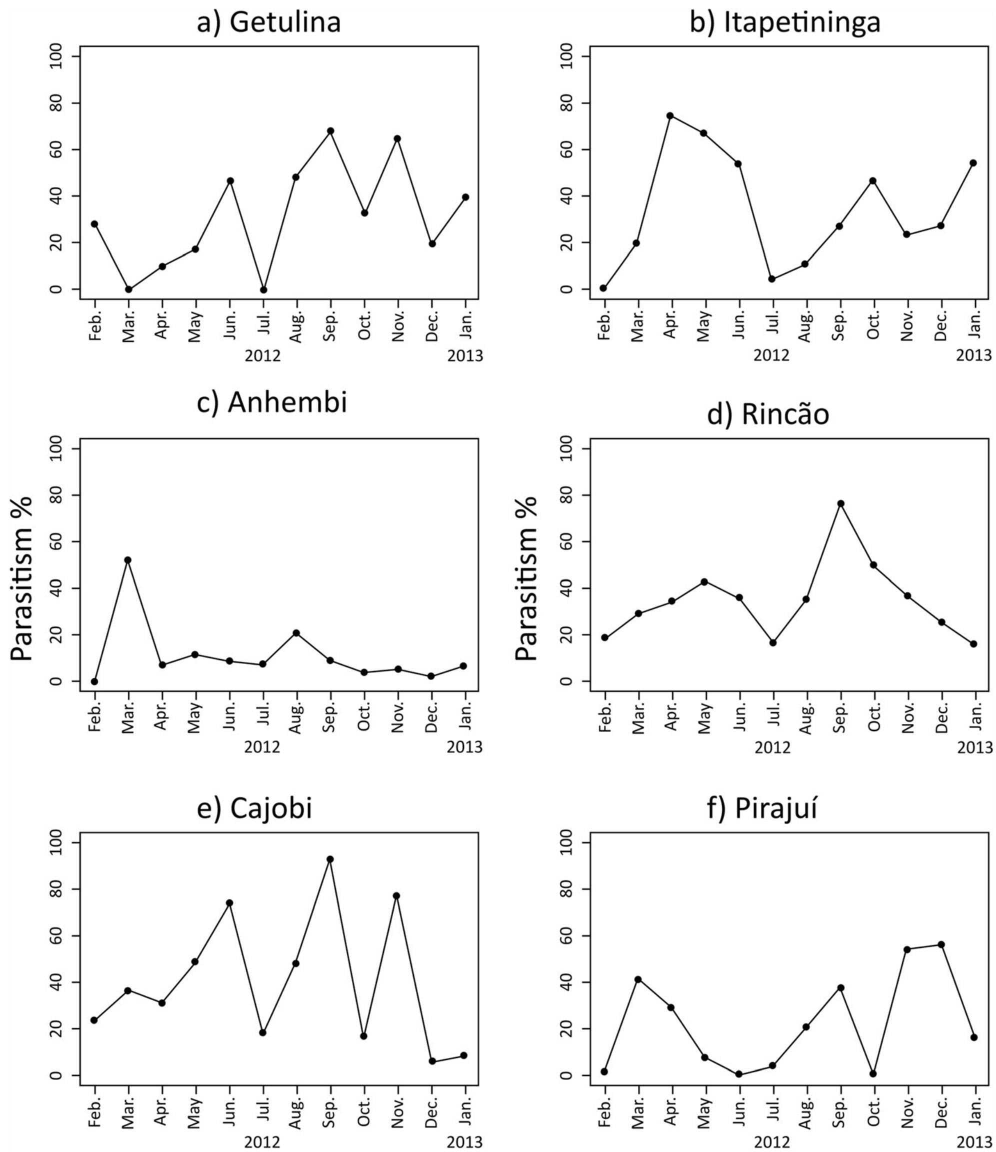

Fig 2 Monthly percentage of parasitism (%) for *Tamarixia radiata* attacking *Diaphorina citri* nymphs at each sampling site.

Surprisingly, the parasitism rate did not significantly affect the nymph population. Milosavljević *et al* (2018) found that only large nymphs (4th and 5th instars) are susceptible to the parasitoid. Gómez-Torres *et al* (2012) found the same under laboratory conditions. In our study, we did not separate nymphs by instar and the effects of the parasitoid on the nymph population were probably masked. Another potential reason was the size of the sampled area. Our study was conducted in the field, and therefore, we could not precisely determine the spatial dynamics of the parasitoid after its release, nor the variables that affected its spatial distribution. Additionally, recruitment of adult psyllids from other areas to the resident population may affect the dynamics of nymphs on shoots. For future studies, we recommend sampling nymphs over a larger area in order to cover more possible sites with psyllids to where the parasitoids can disperse.

We also observed that the parasitism rate fluctuated substantially from month to month across the study cities, with 1–3 peaks per year, but parasitism peaks were not concentrated in preferred months as reported in previous studies (Kistner *et al* 2016, Milosavljević *et al* 2018). Our results can be explained by the mild temperatures during most of the year in the tropical and subtropical climates of the study sites. For instance, the coldest municipality sampled in this study, Itapetininga, had winter mean temperatures from 15.03 to 17.01°C. According to Gómez-Torres *et al* (2012), the optimal temperature for parasitism is 26.3°C, but parasitism rates around 25% can be observed at 15°C. In a study in Taiwan, Chu & Chien (1991) also did not find that parasitism peaks were concentrated in certain months. Therefore, considering the mild year-round temperatures in São Paulo, high parasitism rates can be observed in different seasons, even

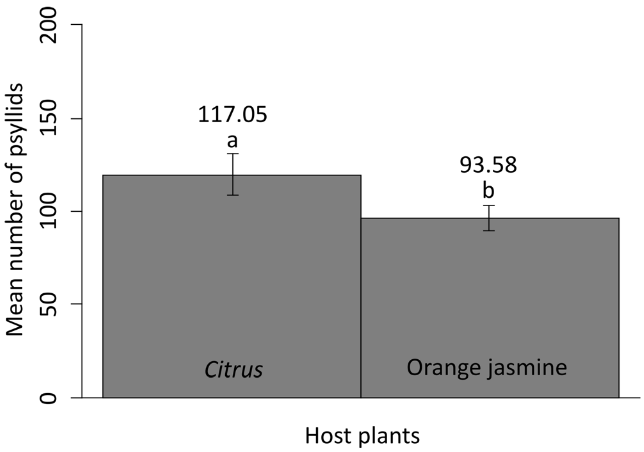

Fig 3 Mean number of nymphs of *Diaphorina citri* (± SE) captured per sample on each host plant. Different letters above bars within a life stage indicate significant differences between host plants (α = 0.05).

during the winter (June to September in the southern hemisphere). The only exception was Anhembi, which had parasitism rates around 5% during most of the year, with only one accentuated peak in March and another smaller one in August. According to Santos (2013), low levels of parasitism in this region can be associated with insecticide drift from nearby commercial groves that affects the development of young parasitoids and the oviposition rate in females.

Effect of the number of parasitoid releases in an external area on the number of adult Asian citrus psyllids collected in a neighboring commercial area

The highest number of psyllids was collected in October 2014 and the lowest in April 2015. The primary factor affecting the number of adult psyllids captured was the number of parasitoid releases, which was negatively correlated with the response variable. That is, the number of psyllids showed a tendency to decrease as the number of parasitoid releases increased, indicating that biological control by releasing parasitoids in external areas achieved its main goal. Our study complements that of Hall & Rohrig (2015), who suggested that higher release rates are more effective in managing psyllids. Here, we found that the number of releases affected the success of biological control by *T. radiata*, but not the release rate as previously reported by Hall & Rohrig (2015).

This is the first report describing the release of the parasitoid *T. radiata* in areas outside commercial groves and significantly affecting the number of adult psyllids within the groves. Recently, Milosavljević *et al* (2018) did not find any effect of *T. radiata* activity on population trends of *D. citri* adults in non-commercial areas. They associated this result with the continuous recruitment of new psyllids to the resident population and with compensatory survivorship of large nymphs when competition is reduced by parasitism. However, in their study, the authors tested if the natural parasitism by *T. radiata* in non-commercial urban sites affects the number of adults in those same areas. Our study used a different approach, since we studied adult psyllids in a commercial area, where the level of chemical control was sufficiently high to prevent the presence of eggs and nymphs and consequently of parasitic wasps.

In our study, adult psyllids were not affected by any climate variable (temperature and rainfall). Aubert (1987) suggested that rainfall dislodges adult psyllids from plants and promotes the growth of entomopathogenic fungi, negatively affecting the number of insects. However, this was not

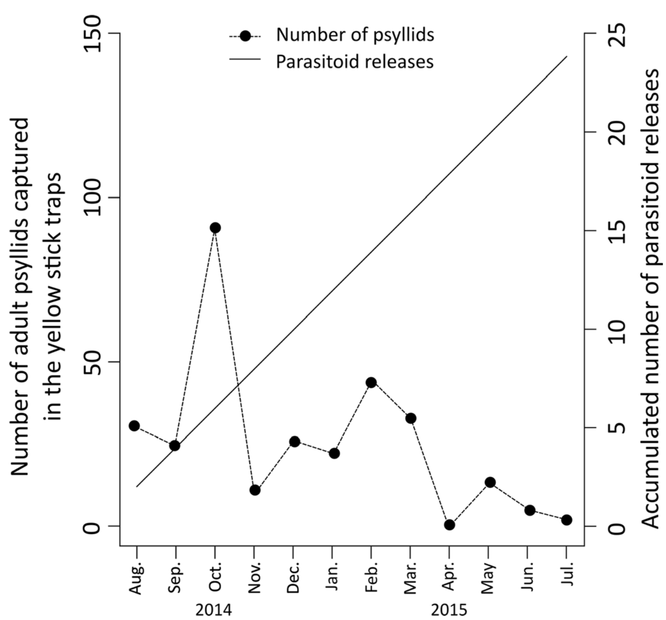

Fig 4 Number of adult psyllids captured per month on yellow sticky traps, and the number of parasitoid releases since the beginning of the study.

confirmed by Milosavljević *et al* (2018), who found that the numbers of adult psyllids were affected by the year, temperature, and month, but not by rainfall. Tomaseto *et al* (2018) suggested that the temperature may indirectly affect the number of adult psyllids by interfering with their flight capacity. By contrast, Martini *et al* (2016) found that adult psyllids were affected by relative humidity, but not by temperature. In general, there is extensive controversy about the abiotic factors that affect the dynamics of *D. citri*, and more studies are needed to clarify and explore the possible abiotic factors affecting adult populations. One suggestion is a long-term study covering a wide range of climate conditions. Our study lasted only 1 year, which may not have been sufficient to indicate trends in the number of psyllids affected by abiotic factors.

In conclusion, this strategy could be termed "External Management," since the focus of this approach aims to manage the vector psyllid outside commercial areas, to reduce the influx of psyllids, and could be included as a strategy in the integrated management of *D. citri*, combined with other measures to increase the parasitoid populations, including introduction, conservation, and multiplication (Parra *et al* 2002).

This study clarified some important points regarding the release of parasitoids into external sources of HLB inocula and helped us to determine possible improvements in our experimental design for application in future studies.

The first part of our study agreed with previous studies that identified external areas as proliferation zones for psyllids. We found that nymph populations were affected only by the host plant and rainfall. For future studies, we recommend that nymphs be separated by size in order to determine the effect of the parasitism rate on psyllid dynamics. We also recommend sampling insects in a larger and more representative area.

In the second part of the study, we found that the number of parasitoid releases in non-commercial areas affected the number of psyllids inside the commercial area. This is the first study of the efficiency of this strategy, although further studies are needed to clarify some unsolved problems. For instance, what is the optimal number of parasitoids to be released and what is the periodicity of release? What are the long-term effects on psyllid populations? What is the spatial distribution of the parasitoid *T. radiata* after release? All these questions should be addressed in future studies in order to provide more information on this management strategy and improve its efficiency.

The strategy proposed in this study (External Management) can be combined with the current measures for HLB management (planting healthy seedlings, eradicating symptomatic plants, and applying insecticides), mainly in the borders to prevent the vector migration discussed here, and can help to reduce HLB disease in citrus crops. Establishing the parasitoid throughout an area over time can gradually reduce the occurrence of HLB and consequently the need for agrochemicals.

As described for HLB pathosystem, External Management could be applied to other pests, even those that are not disease vectors, enhancing the Integrated Pest Management. The release of natural enemies in sites outside commercial areas, where insect pests are capable of reproducing, can contribute to reducing pest migration especially in the early stages of plant growth.

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Author Contributions AJFD and JRPP conceived the project, conducted the field experiments, and wrote the manuscript. AGG and CR performed the statistical analysis. AGG, CR, JMV, and GRA wrote the manuscript and participated in discussing the results, critiquing the scientific aspects, and proofreading the manuscript.

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