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Association between Temperature and Reproductive Fitness of *Diaphorina citri* Infected with *Candidatus* Liberibacter Asiaticus

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Abstract: *Diaphorina citri* is a serious insect pest of citrus and an insect vector of *Candidatus* Liberibacter asiaticus (CLas) that causes Huanglongbing disease (citrus greening). In this study, we investigated the effect of the CLas pathogen on the life history parameters of *D. citri* at different temperature regimes. Our results demonstrated that the survival rate of first to fifth instar CLas-positive and CLas-negative *D. citri* fluctuate with the change in temperature over the range of 16–35 °C. Meanwhile, the mean developmental time (52.5 d) (d = day(s)) and adult longevity (5.2 d) of the CLas-positive psyllids was longer as compared to CLas-negative psyllids mean developmental time (32.81 d) and adult longevity (3.50 d) at the low- and high-temperature regimes (16 and 35 °C). However, at high temperature regimes, the significant effect of CLas-bacteria on *D. citri* fecundity was higher than the corresponding non-significant effect on their survivorship when compared to non-vectored psyllids. These results indicate a long-term, stable evolutionary relationship among vector-pathogen and climate change.

Keywords: biology; climate change; *Diaphorina citri*; disease epidemiology; *Candidatus* Liberibacter asiaticus; pathogen; reproductive fitness; temperature

Citation: Hussain, M.; Bano, S.; Qasim, M.; Gao, J.; Arthurs, S.P.; Wang, L.; Qiu, D.; Mao, R. Association between Temperature and Reproductive Fitness of *Diaphorina citri* Infected with *Candidatus* Liberibacter Asiaticus. *Agronomy* **2022**, *12*, 815. https://doi.org/10.3390/agronomy12040815

Academic Editors: Wayne B. Hunter, Liliana M. Cano and Jawwad A. Qureshi

Received: 16 December 2021
Accepted: 25 March 2022
Published: 27 March 2022

1. Introduction

Diaphorina citri Kuwayama (Hemiptera: Liviidae) is an economically important pest of citrus [1–3]. *D. citri* is a phloem-feeding insect responsible for direct and indirect damage to citrus fruit quality and tree health. The principal economic importance of *D. citri* reflects its capability to transmit the bacterial infection that causes citrus greening disease, also known as huanglongbing (HLB) [2,3]. HLB affects all citrus varieties and shortens the productive life of trees, limiting fruit production and ultimately causing tree death [4].

HLB disease is caused by *Candidatus* Liberibacter asiaticus (CLas), a gram-negative phloem-limited bacterium vectored by *D. citri* [4,5]. *D. citri* acquires CLas during the insect's nymphal stages while feeding on HLB-infected trees. The intake of infective phloem fluid allows CLas to colonize the digestive tract of *D. citri* and subsequently transmit the disease [2]. HLB symptoms were first detected in China [6] and South Africa [7] in the late 1800s and 1920s, respectively. *D. citri* and HLB have spread worldwide, being reported

in Brazil [8], the USA [9], Paraguay and Argentina [10], Cuba [11], Belize [12], Iran [13], Mexico [12] and Pakistan [13].

The ability of the CLas/*D. citri* pathosystem to colonize new regions is influenced by environmental conditions. For example, the life cycle of *D. citri* is temperature-dependent, with upper and lower thresholds between 0 and 40 °C and an increased mortality rate in range of 35 to 41 °C [14]. Thus, in some regions, *D. citri* completes its life span in 11 days and develops through up to 8 generations (per year), while in cooler climates, *D. citri* may fail to develop [15]. Empirical and theoretical studies also demonstrate that environmental temperature influences the population ecology of *D. citri* through changes in the flushing cycles of its citrus hosts [15,16]. Additionally, temperature affect the CLas pathogen, with growth halted at temperatures above 35 °C or less in susceptible strains [17].

The evolutionary relationship between insect vector–pathogen and temperature could be evaluated by their mutualistic interactions. For example, coevolution may drive changes in insect vector's reproductive fitness [18]. The production of a high number of offspring in response to pathogen infection may increase the potential for pathogen transmission. In general, the influence of bacterial pathogens has been evaluated as a function of fecundity and survival of their host. Extended maximum host life expectancies may also benefit a pathogen by improving the transmission ability of the vector [19]. High survival rates will similarly improve the chance for disease transmission [20,21].

Currently, there are limited studies investigating the role of CLas on the life history parameters of *D. citri* in relation to temperature [15,16]. While some transmission and fitness parameters between *Candidatus* and their vectors were explored previously [18–21], the association between the *D. citri*/CLas system and temperature is unknown.

In this study, we determine the interaction of CLas and temperature on the life history parameters of *D. citri*. Specifically, we explored further into impact of CLas and temperature on: (1) survivorship (egg to adult), (2) mean developmental periods of adult, and (3) reproductive fitness (fecundity).

2. Materials and Methods

2.1. Plant Colonies

Sour oranges (*Citrus aurantium* L.) were cultivated individually in plastic pots (12 × 18 cm) containing an equal mixture of soil, organic compost and vermiculite. Plants were maintained in a greenhouse until they reach 20 cm, when they were pruned to form the branchlets (10 cm) used in this study. CLas-positive were inoculated using CLas-positive *D. citri*. CLas-infected citrus plants were confirmed with quantitative PCR (qPCR) performed on DNA extracted from midribs of citrus leaves. CLas-infected citrus plants used in this experiment had a less than 28 cycle threshold value (cT). Immediately after the emergence of nymphs, *D. citri* were tested to the confirmed healthy or CLas-positive sour orange seedlings under 27 °C, 65% RH and a 14 h light: 10 h dark photoperiod. *D. citri* populations were reared over 4 generations, and 40 adults were tested each month for CLas infection through qPCR as described below.

2.2. Insect Colonies

The *D. citri* colony was reared in mesh cages (45 × 45 × 50 cm) on sour orange. The original D. citri population used in bioassays was obtained from a culture continuously maintained at the Institute of Zoology, Guangdong Academy of Sciences (Guangdong, China). A CLas-negative *D. citri* colony was kept in a greenhouse without pesticides on sour orange (*Citrus aurantium* L.) plants. To verify that this culture remained CLas-free, random subsamples of *D. citri* and plants were assayed monthly using a quantitative real-time polymerase chain reaction (qPCR), as described below. CLas-positive *D. citri* were obtained from CLas-infected *C. aurantium* plants in a secure quarantine facility at the Guangdong Academy of Sciences' Institute of Zoology. To obtain CLas-infected individuals for bioassays (F1 generation), CLas-negative psyllids (F1 generation) were placed to infected plants immediately prior to assays. Briefly, 20 groups of 25–30 newly emerged (2–5 d old)

psyllids (adult) reared on CLas-infected *C. aurantium* plants for nymphal acquisition of CLas were shifted to a CLas-free plant to assess the effect of CLas on *D. citri* survival. A second group of newly emerged psyllids (adults) reared on CLas-free plant was also shifted to citrus plants as a negative control. Psyllids were confined on sour orange within insect-proof mesh cages (45 × 45 × 50 cm). Psyllids were counted on daily basis (until all psyllids were dead). Psyllids were separated according to sex ratio (stored in 70–80% ethanol at −70 °C for DNA extraction). Based on qPCR detection, monthly sampling of the infected culture conducted along with the current study found that 30 to 70% of psyllids were infected with CLas. For the Las-infected psyllid treatment, only individuals that tested positive for Las in qPCR assays were included in subsequent survival analysis.

2.3. Estimation of Life-History Parameters

For the life table study, we used newly emerged *D. citri* adults, reared (Figure 1) at different temperature regimes (16, 20, 27 and 35 °C) for four continuous generations in growth chambers. At each temperature, *D. citri* cultures were maintained on both confirmed healthy sour orange plants (CLas-negative) and CLas-positive). No insecticides were used. The growth chamber conditions were 65 ± 5% (means ± SE) relative humidity and a 14 h light:10 h dark photoperiod. The growth chamber (LHR-800A-GSI-E3; Tai Hong Jun, Shaoguan, China) was calibrated to maintain relative humidity within the stated range.

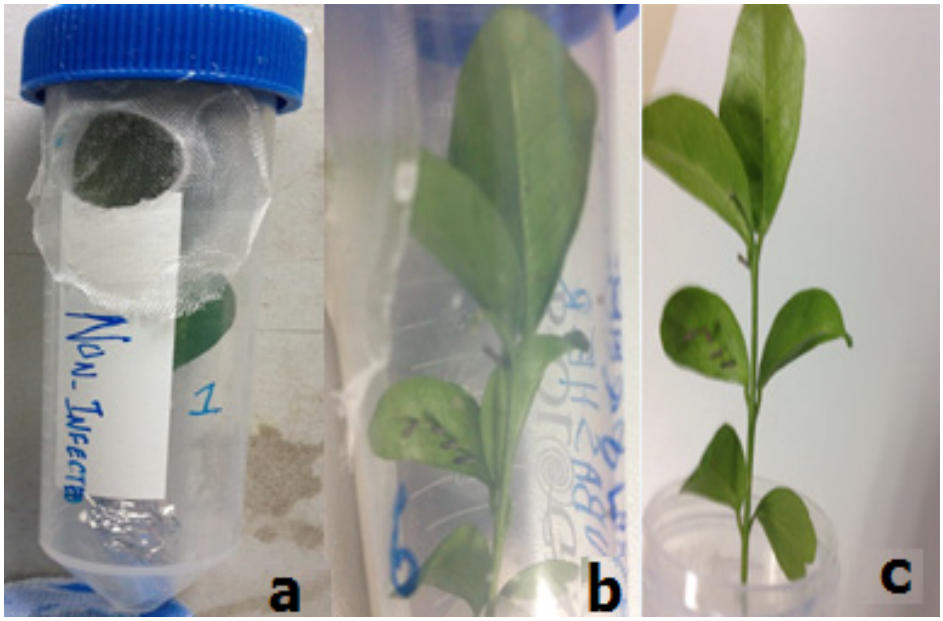

Figure 1. Rearing tubes for life table study of psyllids: (**a**) Showing hole in plastic tube covered with mesh sheath for ventilation; (**b**) Psyllids inside rearing tubes with sour orange branchlets; and (**c**) Psyllids with branchlets.

When *D. citri* reached the fourth nymphal stage, the sour orange branchlets were cut and transferred to plastic rearing tubes (3–4 cm diameter, 8.5 cm high and with a 3-cm diameter ventilation hole covered with fine mesh) until adult emergence. After mating, eggs were collected and kept in growth chambers at each respective temperature on sour orange branchlets under the same experimental temperature condition. After 48 h, 1 egg per leaf (remaining eggs removed) was monitored through development to adults. This process was repeated over eight generations for all temperature conditions. In total, 7 replicate rearing tubes were used for each temperature and CLas status treatment.

2.4. Extraction of Genomic DNA and qPCR

A fresh leaf of sour orange (20 microgram) and 40 *D. citri* were sampled to extract DNA for the detection of CLas (Figure S1) by PCR using CLas specific primers [21]. Genomic DNA

of bacterial strains from citrus was extracted with QIAprep® Spin Miniprep Kit as described previously [21]. PCR (BIO –RAD: Professional Typical Gradient Thermocycler, Singapore), was used to detect the CLas pathogen from leaves and *D. citri*. CLas titer was quantified using qPCR and specific primers; a forward primer (5′-TGAATTCTTCGAGGTTGGTGAGC-3′) and a reverse primer (5′-AGAATTCGACTTAATCCCCACCT-3′) designed on GenBank by using a sequence (M94319) of the CLas pathogen were used for PCR amplification in a 25-µL reaction volume. The qPCR was performed using the Taq polymerase, and Taq master mix (Qiagen, China), qPCR master mix and *D. citri* DNA template (1 µL) were utilized in a 20 µL qPCR reaction volume using fast real-time PCR Biosystems. qPCR conditions were set to 3 min (at 95 °C) and 40 cycles (15 s at 95 °C; 30 s at 60 °C). Duplicate reactions were carried out for each sample in a BIO -RAD T100 CFX Connect Real-Time PCR System (CFX Connect™ Optics module Singapore), and each set of amplifications included positive controls for CLas and CLas negative control sequences. Samples were considered CLas positive when product was amplified (within the range of 36 amplification cycles during reaction).

2.5. Statistical Analysis

The data were statistically analyzed with SPSS software using analysis of variance (ANOVA) and a Tukey's HSD test ($p < 0.05$) to compare the means. Life history data of each individual (mean development periods and fecundity) were analyzed based on the age-stage, two-sex life table theory and the computer software TWOSEX-MSChart [22,23]. Results were considered significantly different at $p < 0.05$.

3. Results

3.1. Effect of CLas on D. citri Development

The developmental periods for immature stages, as well as the adult longevity, fecundity and preoviposition periods, for CLas-positive and CLas-negative psyllids were summarized (Figure 2). Our results indicated that CLas-positive psyllids have some positive effects at high temperature regimes (35 °C) given the higher egg survivorship in CLas-infected samples at 35 °C (91.3%) compared with CLas-non-infected samples (83.3%) (Tables 1 and 2). The survival rate of immature *D. citri* (Tables 1 and 2) fluctuated with temperature over the range of 16–35 °C for both populations (CLas-positive and CLas-negative psyllids).

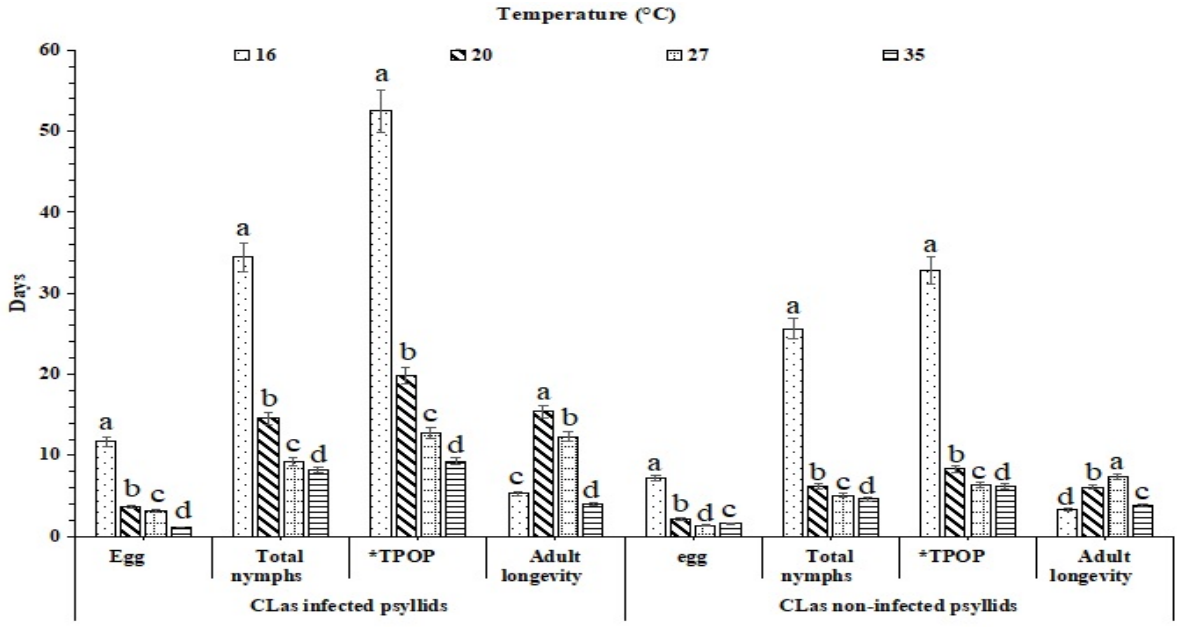

Figure 2. Mean developmental periods of CLas-positive and CLas-negative *D. citri* at different temperature regimes. Different letters show differences according to Tukeys test. * TPOP; total preoviposition period. Standard error is shown by error bars.

Table 1. Survivorship (%) of CLas infected *D. citri* immature stage at four temperatures.

Temperature (°C)	Egg	N1	N2	N3	N4	N5	Egg to Adult
16	86.9 ^{b}	83.3 ^{b}	93.2 ^{a}	98.6 ^{a}	97.7 ^{a}	95.0 ^{a}	65.4 ^{b}
20	89.6 ^{b}	86.0 ^{b}	95.0 ^{a}	99.4 ^{a}	98.7 ^{a}	94.1 ^{a}	70.7 ^{a}
27	95.9 ^{a}	93.2 ^{a}	94.1 ^{a}	99.1 ^{a}	94.1 ^{ab}	87.9 ^{b}	73.4 ^{a}
35	91.3 ^{a}	91.4 ^{a}	92.3 ^{ab}	94.1 ^{ab}	93.3 ^{ab}	86.9 ^{b}	69.8 ^{ab}
F	14.0	2.5	2.1	1.2	3.4	1.6	17.6
df	4.21	4.21	4.21	4.21	4.21	4.21	4.21
p	<0.001	0.03	0.05	0.59	0.07	0.52	<0.001

Mean values followed by the same letter in the column do not differ by Tukey test ($p \leq 0.05$). N1: first instar nymph; N2: second instar nymph; N3: third instar nymph; N4: fourth Instar nymph; N5: fifth instar nymph.

Table 2. Survivorship (%) of CLas-non-infected *D. citri* immature stage of at four temperatures.

Temperature (°C)	Egg	N1	N2	N3	N4	N5	Egg to Adult
16	81.5 ^{b}	80.5 ^{ab}	90.5 ^{ab}	89.7 ^{a}	89.7 ^{a}	88.8 ^{a}	60.5 ^{a}
20	82.4 ^{b}	83.3 ^{ab}	93.2 ^{a}	85.1 ^{a}	81.5 ^{b}	82.4 ^{ab}	59.8 ^{ba}
27	93. ^{a}	89.9 ^{a}	92.3 ^{a}	82.4 ^{ab}	82.4 ^{b}	85.1 ^{a}	64.2 ^{a}
35	83.3 ^{b}	82.4 ^{ab}	88.7 ^{b}	84.2 ^{ab}	83.3 ^{b}	84.2 ^{ab}	52.41 ^{b}
F	11.3	2.1	2.5	0.7	2.3	2.5	15.1
df	4.21	4.21	4.21	4.21	4.21	4.21	4.21
p	<0.001	0.03	0.06	0.52	0.05	0.25	<0.001

Mean values followed by the same letter in the column do not differ by Tukey test ($p \leq 0.05$). N1: first instar nymph; N2: second instar nymph; N3: third instar nymph; N4: fourth Instar nymph; N5: fifth instar nymph.

The survivorship of the *D. citri* CLas-positive nymphs was not negatively affected compared with CLas-negative psyllids population (Table 3). However, the mean development periods of CLas-positive psyllids were slower than CLas-negative psyllids population (Figure 2).

Table 3. Interaction effects of temperature and CLas infection on *Diaphorina citri*.

Factors	df, Residuals	*F* Value	*p* Value
CLas infection	2, 212	14.10	0.0030 *
Temperature	3, 212	8.12	0.0041 *
Fecundity	3, 212	5.22	0.0014 *
CLas infection × temperature	4, 212	1.10	0.1390
CLas infection × fecundity	3, 212	0.58	0.0018 *
Temperature × fecundity	19, 212	10.76	0.0044 *
Las infection × temperature × fecundity	19, 212	0.27	0.5110

* Represent significant factors and interactions at $p < 0.05$.

3.2. Effect of CLas on Adult D. citri Fecundity

CLas-positive *D. citri* fecundity was significantly changed at different temperature regimes (Figure 3). Increased fecundity of CLas-positive psyllids (Figure 3) was observed within the range of 20 to 27 °C. Thus, the effect of CLas-bacteria on *D. citri* fecundity was greater at high temperatures (35 °C) than the non-significant effect on their survival when compared to non-vectored *D. citri*.

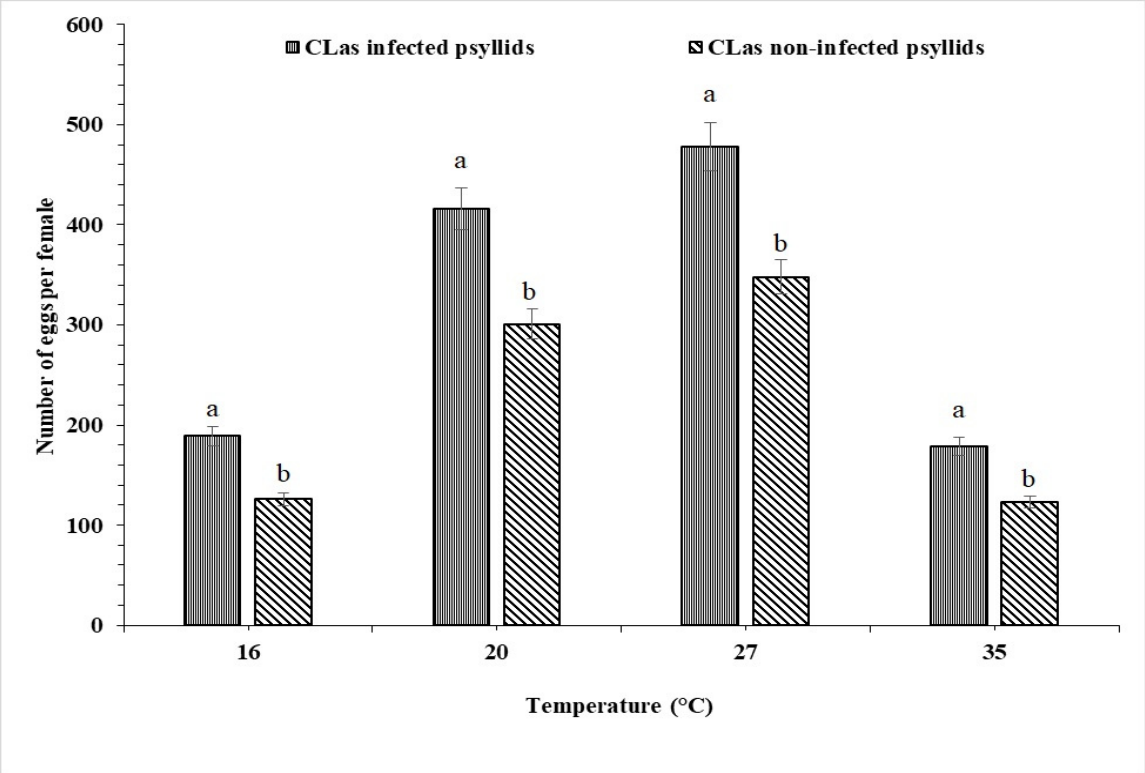

Figure 3. Fecundity of CLas-positive and negative *D. citri* at different temperatures. Different letters show differences according to Tukeys test. Standard error is shown by error bars.

3.3. Effect of Temperature and CLas Infection on Diaphorina citri

Our findings indicated that the interactions between CLas-infection, temperature and fecundity had a significant effect on the reproductive fitness of psyllids (Table 3).

4. Discussion

Temperature affects the developmental parameters of insects in a non-linear manner, which can be modelled around high and low threshold parameters [24]. However, in nature, insects are not continuously subject to constant temperatures, with diurnal measurements providing a better understanding of the population dynamics of a specific species [25].

Our results showed the effects of different temperature regimes on the survival rate, longevity and reproduction of *D. citri*. Within the temperature range of 16–35 °C, the survival of immature stages declined as the temperature increased. Although adult longevity of *D. citri* CLas-positive nymphs was numerically similar with CLas-negative psyllids, our results suggest that CLas-infection may confer some benefit at high temperatures. This was reflected in higher egg survivorship in CLas-infected samples compared with CLas-non-infected at 35 °C (Tables 1 and 2). The negative influence of CLas infection on egg survivorship may reflect physiological costs related to the growth of the CLas bacteria inside the developing embryo. However, the development of CLas-positive psyllids was slower than CLas-negative psyllids. This latter observation may reflect reduced host plant suitability resultant from CLas infection. Thus, our results indicated that CLas-positive psyllids mean development period has been significantly impacted at extreme temperature regimes (16 and 35 °C). These results are in line with earlier studies with *D. citri* and the potato psyllid, *Bactericera cockerelli* [26].

This is in line with a study on another psylllid species [27]. Similar to this study, our study results also showed that the longevity of adult CLas-positive *D. citri* (Figure 2) was lower at 16 and 35 °C compared with 20 and 27 °C; however, the fecundity of CLas-positive female *D. citri* (Figure 3) was also higher as compared with CLas-negative psyllids (Figure 3). The higher temperature regime (35 °C) has another effect on the physiology of *D. citri*. When they were kept at an optimum temperature range, their development was ceased (MH personal observation). However, cumulatively, optimum temperature regimes (20–27 °C) have significant effects on the mean development periods of CLas-positive psyllids as compared with CLas-negative psyllids, which indicates a long-term, stable evolutionary relationship among vector-pathogens and temperature. As stated before, this may have been because of reduced plant suitability resultant from plant pathogen (CLas). Thus, our results suggest that *D. citri* CLas-positive populations may increase more rapidly at different temperature regimes than their counterpart, CLas-negative psyllid populations.

D. citri CLas–pathogen–temperature interactions are also complicated and different and may employ significant impacts on epidemiology of the huanglongbing disease, vascular tissues blockage (phloem tissue) and collapse are remarkable features of this disease [28] as well as the population dynamics of the vectors. The effect of viral infection in some Hemipterans such as the *Bemisia tabaci* [29] has been recorded previously, but, to our knowledge, no studies investigated the possible roles of *D. citri* CLas–pathogen–temperature interactions in the invasion process of psyllids to population dynamic growth. Our results show that the most favorable host plant, sour orange, significantly improved the performance of CLas-positive psyllids at different temperature regimes (16, 20, 27 and 35 °C) as compared with CLas-negative psyllids (Tables 1 and 2). When the psyllids were vectored by plant pathogen (CLas) adult longevity increased compared with CLas-negative pysyllids at optimum temperature (20–27 °C) regimes (Figure 2). These results are correlated with Purcell [30] studies among Liviidae family so far revealed the fitness rate of vector infected with pathogen infection, which was increased with temperature under laboratory conditions; for example, hemipterans, such as leafhopper vectors, also show higher fitness rate after acquisition of plant pathogens. For example, *Macrosteles quadrilineatus* Forbes and *Dalbulus maidis* (DeLong & Wolcott) leafhoppers, when vectored to the plant pathogens (Aster yellows phytoplasma and corn stunt spiroplasma, respectively), had longevity higher than CLas-negative populations [31]. These significantly positive effects of a plant pathogen on vector fitness may suggest that the pathogen developed a correlation with the insect vector before secondarily transferring to plants. These findings suggested that CLas-positive psyllids population growth at optimum temperature regimes may be higher between populations that infected with the CLas-pathogen with greater frequency than with those psyllid populations that are less infected at optimum temperature regimes.

Reproductive fitness is a very important phenomenon in insect life. Our results also showed that CLas-positive psyllids' fecundity significantly decreased at low and high temperature regimes (35 °C) in comparison to CLas-negative psyllids (Figure 3). Thus, as a re-

sult of the increased fecundity rate in highly infected areas at optimum temperature regimes (20 and 27 °C), the transmission rate increases. Similar studies by Ammar, et al. [32] showed that the plant pathogen (CLas) is persistently transferred and capable of inhabiting a majority of psyllid tissues, effecting the reproductive rate. A close correlation between the plant pathogen (CLas) and its vector has also been revealed in previous studies [33,34], showing the existence of vertical and parallel sexual transmission of CLas bacteria. Plant pathogens could result in plant metabolic or immune costs related to pathogenic bacterial growth which play an important role in plant defense mechanisms against herbivores [35–39]. For example, when leafhoppers were infected with the phytoplasmic X-disease, Flavescence dore'e, it reduced their fitness [18]. Similar studies have shown negative effects of plant pathogen on life history parameters of some psyllid species. Some phytopathogens, such as, *Candidatus* Liberibacter solenacearum and *Candidatus* Phytoplasma mali are linked with apple zebra chip and proliferation pathogenic diseases, respectively. These pathogens significantly reduce the fecundity of *Cacopsylla melaneura* and *Bactericera cockerelli*, psyllid vectors [40]. However, Nachappa, Shapiro and Tamborindeguy [41] showed that the survival rates of adult *B. cockerelli* were not affected by Ca. L. solenacearum, but survival rate of nymphs to the adult stage was significantly decreased when *B. cockerelli* were infected with plant pathogen.

5. Conclusions

In summary, the survivorship (%), mean developmental periods, and reproductive fitness of CLas-positive and CLas-negative *D. citri* were significantly influenced by temperature. An optimum temperature range of 20-27°C appears to be most suitable for the population growth and for rearing of this insect in the laboratory. Our findings suggests that population growth of CLas-positive psyllids at optimum temperature regimes (20–27°C) may be faster in populations infected with the CLas-pathogen more frequently than in populations that are less infected. Therefore, our insights especially on the association between temperature and reproductive fitness of *D. citri* might be used to build an experimental foundation for the construction of a psyllid population model (a better way to quantify the effect of climate change factors such as temperature on this important pest) for better HLB management.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12040815/s1, Figure S1: Electrophoresis gel.

Author Contributions: Conceptualization, M.H.; formal analysis, M.H., S.B., J.G. and M.Q.; funding acquisition, M.H. and R.M.; investigation, M.H., S.B. and M.Q.; methodology, M.H.; project administration, M.H., L.W. and R.M.; software, M.H., J.G. and M.Q.; supervision, M.H. and R.M.: writing—original draft, M.H., S.B. and M.Q.; writing—review and editing, M.H., S.B., M.Q., J.G., R.M., S.P.A., L.W. and D.Q. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the GDAS Special Project of Science and Technology Development (2019GDASYL-0501006, 2021GDASYL-20210103051); Science and Technology Planning Project of Guangdong Province (No. 2017B020202005) and the Research Programs of Guangzhou (202103000065).

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results. S.P.A. works for a commercial company that has no commercial relationship to any products or services mentioned in this manuscript.

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