Insect science

Insect Science (2020) 27, 58–68, DOI 10.1111/1744-7917.12599

ORIGINAL ARTICLE

Effects of "*Candidatus* Liberibacter solanacearum" (haplotype B) on *Bactericera cockerelli* fitness and vitellogenesis

Angélica Albuquerque Tomilhero Frias[1,2,*], Freddy Ibanez[1,*], Azucena Mendoza[1], William Mario de Carvalho Nunes[2] and Cecilia Tamborindeguy[1]

[1]*Department of Entomology, Texas A&M University, 412 Heep Center, College Station, Texas, USA and* [2]*UEM—Depto. de Agronomia, Núcleo de Pesquisa em Biotecnologia Aplicada, Maringá, Brazil*

Abstract "*Candidatus* Liberibacter solanacearum" (Lso) are phloem-restricted and unculturable Gram-negative bacteria. Presently five haplotypes have been identified worldwide; but only haplotypes A and B are associated with the vector *Bactericera cockerelli* (Šulc.) in the Americas. Previous studies showed that Lso-infection reduces *B. cockerelli* reproductive output and that Lso haplotype B is more pathogenic than Lso haplotype A. To understand the interaction of Lso haplotype B and *B. cockerelli*, the fitness of Lso-free and Lso B-infected insects, and the expression of vitellogenin (*BcVg1*-like), a gene involved directly in the insect reproduction were analyzed. Statistical differences in the number of eggs oviposited, and the total number of progeny nymphs and adults were found among crosses of insects with or without Lso. Significant differences in sex proportions were found between Lso B-infected and Lso-free crosses: a higher proportion of F_1 adult females were obtained from Lso B-infected mothers. A significant reduction of *BcVg1-like* was observed in crosses performed with Lso B-infected females compared to the Lso-free insects. In female cohorts of different age, a significant reduction of *BcVg1-like* expression was measured in 7-d-old Lso B-infected females (virgin and mated) compared with 7-d-old Lso-free females (virgin and mated), respectively. The reduction of *BcVg1-like* transcript was associated with a lower number of developing oocytes observed in female's reproductive systems. Overall, this study represents the first step to understand the interaction of Lso B with *B. cockerelli*, highlighting the effect of Lso B infection on egg production, *BcVg1-like* expression, and oocyte development.

Key words feminization; insect–pathogen interaction; oviposition; psyllids; reproduction; vitellogenesis

Introduction

Many plant pathogenic bacteria depend on insect vectors to spread between hosts (Weintraub & Beanland, 2006; Orlovskis *et al.*, 2015; Perilla-Henao & Casteel, 2016; Tamborindeguy *et al.*, 2017). The association between the microorganism and the insect can result in numerous symbiotic interactions that can affect the insect fitness; these associations have been well described in the literature as mutualism, parasitism, and commensalism (Leung & Poulin, 2008; Su *et al.*, 2013; Solomon *et al.*, 2015).

In the last 30 years, emergent diseases caused by plant pathogenic bacteria associated with insects had caused significant economic losses worldwide (Nadarasah & Stavrinides, 2011). One of these associations involves psyllid species with the plant pathogenic bacteria "*Candidatus* Liberibacter spp." Psyllids are phloem-feeding

Correspondence: Cecilia Tamborindeguy, Department of Entomology, 412 Heep Center, Texas A&M University, College Station, TX 77843, USA. Email: ctamborindeguy@tamu.edu

[*]These authors contributed equally to this work and both should be considered first authors.

hemipterans that transmit these pathogenic bacteria threatening the production of several crops (Aubert, 1992; Bové, 2006; Manjunath *et al.*, 2008).

Zebra chip has recently become an economically important disease in potato crops, documented in several countries of the Americas and also in New Zealand (Liefting *et al.*, 2008; Secor *et al.*, 2009; Teulon *et al.*, 2009; Crosslin *et al.*, 2010; Munyaneza *et al.*, 2010; Munyaneza, 2012). The causative agent of zebra chip is "*Candidatus* Liberibacter solanacearum" (Lso) a phloem-restricted and unculturable Gram-negative bacterium transmitted by *Bactericera cockerelli* (Šulc.) (Hemiptera: Triozidae), also known as the potato psyllid (Munyaneza *et al.*, 2007; Hansen *et al.*, 2008; Liefting *et al.*, 2008; Secor *et al.*, 2009). Worldwide, five different Lso haplotypes have been described: Lso A, Lso B, Lso C, Lso D, and Lso E (Nelson *et al.*, 2011, 2013; Teresani *et al.*, 2014). Of those, only Lso haplotypes A and B are associated with *B. cockerelli* and solanaceous crops in the Americas (Nelson *et al.*, 2011), Australia, and New Zealand, whereas the haplotypes C, D, and E are associated with *Trioza apicalis* and *Bactericera trigonica* in the old world (Munyaneza *et al.*, 2010; Teresani *et al.*, 2014; NPPO, 2017; Thomas *et al.*, 2018).

Despite the economic importance of Lso, few studies focusing on the fitness of Lso-infected *B. cockerelli* have been performed. Previous studies showed that Lso-infected females are less fecund on tomato plants compared to uninfected females (Nachappa *et al.*, 2012, 2014; Yao *et al.*, 2016). Similarly, other studies performed in *Drosophila melanogaster*, *Anopheles gambiae*, *Euoniticellus intermedius*, and *Teleogryllus oceanicus* showed that exposure to pathogenic bacteria results in a reduction of the number of eggs oviposited (Ahmed & Hurd, 2006; Reaney & Knell, 2010; McNamara *et al.*, 2014; Nystrand & Dowling, 2014). However, the molecular mechanisms that might affect the egg production in Lso-infected psyllids are still undetermined.

The reproductive success of oviparous species depends on egg production; insects are not an exception. However, egg production is energetically demanding and can be influenced by external or internal factors. During oocyte development, yolk proteins (including vitellogenins), lipids, maternal RNAs, ribosomes, and organelles provide nutrients and/or patterning information for the future zygote (Chen *et al.*, 1997; Arukwe & Goksøyr, 2003). In most insect species, including in members of the Hemiptera order such as *B. cockerelli*, *Nilaparvata lugens*, *Lethocerus deyrollei* and *Riptortus clavatus* (Shinoda *et al.*, 1996; Nagaba *et al.*, 2011; Ibanez *et al.*, 2017), juvenile hormone initiates the vitellogenic process and egg development (Tufail & Takeda, 2008).

The synthesis of vitellogenins (Vgs), which is essential for egg production, occurs in most insects in the fat body. After synthesis, Vgs are secreted into the hemolymph and they accumulate as vitellin proteins in the developing oocytes by a receptor-mediated pathway (Raikhel, 1992). In *B. cockerelli*, *BcVg1-like* is highly expressed in females after mating and following exogenous application of juvenile hormone III (Ibanez *et al.*, 2017).

In this study, the effect of Lso B in *B. cockerelli* was evaluated by analyzing life table parameters of crosses between Lso B-infected and/or Lso-free females and males, and by determining the level of expression of *BcVg1-like* in females. Our objective was to start unraveling the cause(s) of the reduced reproductive output previously reported in Lso B-infected *B. cockerelli* females.

Materials and methods

Plant material

Tomato (*Solanum lycopersicum* cv. Moneymaker) plants were cultivated in a room (23 ± 3 °C) in plastic pots filled with Metro-Mix 900 (Sungro, Horticulture Distribution, Inc., WA, USA) and grown with a photoperiod of 16 : 8 h (light : dark). The plants were watered every 2 d.

Insect source

The Lso-free *B. cockerelli* colony used in this study was obtained in 2013 from Dr. Don Henne, AgriLife Research Weslaco, TX, USA. The colony was maintained on tomato plants in 35.5 cm × 35.5 cm × 61 cm insect cages (BioQuip, Rancho Dominguez, CA, USA) at room temperature and photoperiod of 16 : 8 h (light : dark). Insects from Lso-free colony were transferred to tomato plants infected with Lso haplotype B in order to create laboratory psyllid colonies harboring Lso haplotype B. Insects and plants were tested regularly using the SSR1 primers (Lin *et al.*, 2012) as was previously reported (Yao *et al.*, 2016) to verify the presence and the haplotype of Lso.

In order to obtain cohorts of insects of similar age, fourth to fifth instar nymphs from the Lso-free and Lso B-infected colonies were transferred to 4-week-old uninfected tomato plants separately. On the day adults emerged, they were sexed under a dissection microscope (Leica EZ4W, I. Miller Precision Optical Instruments, Inc., PA, USA), and females and males of each colony were kept separated as same age cohorts in different tomato plants until required.

Insect crosses

To perform the individual crosses, one couple of 3-d-old insects (a male and a female) was placed in a 1.7 mL Eppendorf tube for copulation during 4–5 h or until they mated. Different crosses were performed using females and males, those included: Lso-free × Lso-free; Lso-free × Lso B-infected; Lso B-infected × Lso-free; and Lso B-infected × Lso B-infected insects. In each cross the first insect refers to the female and the second to the male. For each cross type, at least 7 couples were allowed to mate. After mating the males were discarded and the females were grouped according to the cross type on 4-week-old tomato plants and allowed to oviposit for 4 d. On the fourth day after mating, females (mothers) were collected, flash frozen in liquid nitrogen and stored at −80 °C for gene expression analyses. The plants and progeny were kept for life history analyses. The experiment was repeated three times.

Life history analyses

To understand the effects of Lso B in *B. cockerelli* insects, we compared several life history traits using the three biological replicates from the "crosses experiment," consisting of 7 mated females per replicate. Traits compared included average number of eggs per female, hatching percentage, average number of nymphs per female, average number of adults per female, percentage of nymphal survival, and sex ratio. Once F_1 adults emerged, the presence of Lso B was assessed by PCR.

Insect adult cohorts

Cohorts of 1-, 3- and 7-d-old virgin females were obtained as previously described. To obtain 7-d-old mated females, groups of ten 1-d-old females from the Lso-free and Lso B-infected colonies were kept on different 4-week-old uninfected tomato plants with ten 1-d-old males from the Lso-free colony, until they reached 7 d old. At day 7, the males were discarded and the females were collected for follow-up analyses such as dissection of reproductive systems and *BcVg1-like* gene expression. Each 10-female group was considered a biological replicate; the experiment was repeated three times.

DNA extraction and Lso-detection

DNA extraction was performed on single F_1 progeny psyllids from each cross following a procedure previously described (Nachappa *et al.*, 2011). The presence of Lso in insects was determined by PCR reaction using the published LsoF/OI2 primers (Li *et al.*, 2009). Each PCR reaction consisted of 50 ng of genomic DNA, 5 μL of Green Master Mix (Promega, Madison, WI, USA), 3.5 μL of water, and 10 μmol/L each Lso primer; the PCR conditions were the following: 94 °C for 5 min, 40 cycles of 95 °C for 30 s; 60 °C for 30 s and 72 °C for 80 s; and a final step of 72 °C for 7 min. The amplicons were analyzed through electrophoresis on 1% agarose gel containing ethidium bromide and the image of each gel was captured using a gel-documentation system (Fotodyne Incorporated, Hartland, WI, USA).

Gene expression analyses

Total RNA was extracted from pools of seven 7-d-old mated females (mothers from the crosses) and from pools of 10 insects per biological replicate (1-, 3-, and 7-d-old virgin and 7-d-old mated females). Insects were homogenized during 1 min on ice with a plastic pestle using 500 μL of TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Genomic DNA was removed by DNase treatment using Turbo DNase (Thermo Fisher Scientific). Quantity and purity of the total RNA samples were determined using an Infinite® 200 PRO NanoQuant (Tecan, Männedorf, Switzerland) and RNA integrity was visualized by electrophoresis in 1.2% agarose gels stained with ethidium bromide.

For females from the life stages cohort experiment, cDNA was synthesized using 300 ng of total RNA, anchored-Oligo (dT) primers and Verso cDNA Synthesis kit (Thermo Fisher Scientific) following the manufacturer's instructions. The gene expression analysis was performed using PowerUp SYBR Green Master Mix according to manufacturer's instructions. Each reaction contained 5 ng of cDNA, 250 nmol/L of each gene specific primer and 1× of SYBR Green Master Mix; the volume was adjusted with nuclease-free water to 10 μL. The real-time PCR program was 95 °C for 2 min followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s. Real-time PCR assays were performed using an Applied Biosystems QuantStudio™ 6 Flex Real-Time PCR System (Thermo Fisher Scientific). *BcVg1-like* gene expression was evaluated using the primers described previously (Ibanez *et al.*, 2017), with *elongation factor-1α* and *ribosomal protein subunit 18* genes as references (Ibanez & Tamborindeguy, 2016).

Gene expression analyses from the mothers from the cross experiments were performed using SensiFAST

SYBR Hi-ROX One-step Kit (Bioline, London, UK) according to manufacturer's instructions. Each reaction contained 50 ng of total RNA, 250 nmol/L of each gene specific primer (*BcVg1-like* and the reference genes *elongation factor-1α* and *ribosomal protein subunit 18*) and 1× of SensiFAST SYBR Hi-ROX One-step Mix; the volume was adjusted with nuclease-free water to 10 μL. The real-time PCR program was 45 °C for 10 min followed 95 °C for 2 min then 40 cycles at 95 °C for 5 s and 60 °C for 30 s. Real-time PCR assays were performed using an Applied Biosystems QuantStudio™ 6 Flex Real-Time PCR System (Thermo Fisher Scientific).

Three biological replicates were used and each RT-qPCR reaction was performed in duplicate with a negative control in each run. The threshold cycle (Ct) values and the primer specificity were monitored with melting curve analysis using QuantStudio™ software V1.3 (Thermo Fisher Scientific). The relative expression of *BcVg1-like* was estimated with the delta delta CT method (Schmittgen & Livak, 2008), using *elongation factor-1α* and *ribosomal protein subunit 18* as reference genes (Ibanez & Tamborindeguy, 2016).

Number of developing oocytes

To determine the effect of Lso B-infection on the number of developing oocytes, 1-d-old Lso-free and Lso B-infected females were crossed with Lso-free males. On the seventh day following mating, the females were anaesthetized on ice for 15 min, and their reproductive organs were dissected using cold 1× phosphate-buffered saline (1× PBS) in a dissection slide as previously described in (Ibanez *et al.*, 2014). The dissected samples were transferred to a new 1.7 mL microcentrifuge tube previously filled with 200 μL of 3.8% formaldehyde/1× PBS buffer (fixation buffer) for 1 h. The fixation buffer was removed by washing the reproductive organs twice for 15 min using 1× PBS. Then, the samples were mounted using 50 μL of Vectashield mounting medium with DAPI (Vector laboratories Inc., Burlingame, CA, USA). The images of at least 20 female reproductive systems per cross category were obtained using an Axioimager A1 microscope (Carl Zeiss microimaging, NY, USA) and visualized with AxioVision SE64 Rel. 4.9.1 software (Carl Zeiss).

Statistical analyses

The effect of Lso B-infection on *B. cockerelli* life history analyses, such as number of eggs and nymphs, nymphal survival, number of adults (F_1 progeny), and number of developing oocytes were analyzed using one-way ANOVA with Tukey's *post hoc* test. Sex ratio

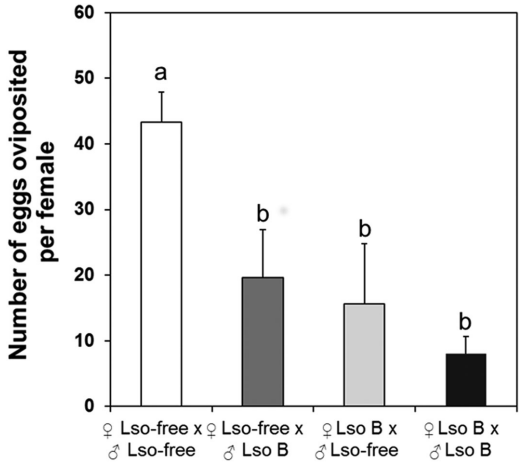

Fig. 1 Average number of eggs oviposited per female from crosses performed with Lso-free and Lso B-infected *B. cockerelli* on tomato leaves in a 4-d period. A significant reduction in oviposition was observed in the crosses in which at least one of the adults (female or male) was infected with Lso haplotype B. Data represent means ± SD of three independent experiments. Different letters indicate statistical differences between treatments using one-way ANOVA with Tukey's *post hoc* test ($P < 0.05$).

statistical analysis was performed using Pairwise comparisons *t*-tests. Gene expression was analyzed using one-way ANOVA with Tukey's *post hoc* test. All analyses were conducted using RStudio environment (RStudio, 2015).

Results

Oviposition

The number of eggs oviposited on leaves of 4-week-old tomato plants by groups of seven mated females from different crosses (Lso-free × Lso-free; Lso-free × Lso B-infected; Lso B-infected × Lso-free; and Lso B-infected × Lso B-infected females and males) were counted after a 4-d-oviposition period (Fig. 1). A significant reduction in the number of eggs oviposited by crosses performed with Lso B-infected insects (females or males) compared to the control Lso-free × Lso-free ($P < 0.05$) was observed. No statistically significant differences were determined among the crosses performed with Lso B-infected insects ($P > 0.05$).

Egg hatching percentage

The percentage of egg hatching was analyzed for each group of samples, no statistically significant differences

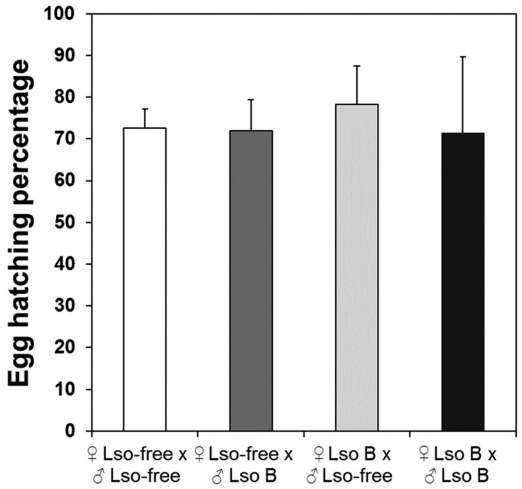

Fig. 2 Influence of Lso B infection in egg hatching percentage in *B. cockerelli*. No statistically significant differences in the percentage of egg hatching among the crosses performed with Lso-free and Lso B-infected insects were observed ($P > 0.05$). Data represent means ± SD of three independent experiments.

were found among the crosses performed with Lso-free and Lso B-infected insects ($P > 0.05$) (Fig. 2).

Number of nymphs, adults, and nymph survival

The number of nymphs on tomato leaves from the different crosses were counted daily during a period of three weeks (Fig. 3A). There were significant differences in the number of nymphs per female produced by the Lso-free × Lso-free cross (31 nymphs) compared with those from crosses with Lso B-infected insects ($P < 0.05$). In detail, there were fewer nymphs in the Lso-free × Lso B-infected (8 nymphs), Lso B-infected × Lso-free (14 nymphs), and Lso B-infected × Lso B-infected (7 nymphs) crosses. No statistically significant differences were identified among the crosses performed with Lso B-infected insects ($P > 0.05$). Fewer adults were also produced in crosses involving Lso B-infected insects compared to the Lso-free × Lso-free cross (Fig. 3B). However, no statistically significant differences ($P > 0.05$) in nymphal survival were observed among the crosses (Fig. 3C).

Each F_1 adult was tested for Lso infection. Lso B was only detected in the progeny of females from the Lso B-infected colony (Fig. S1).

Sex ratio percentages

Figure 4 shows the sex ratio of the progeny produced by Lso-free and Lso B-infected females. A significant

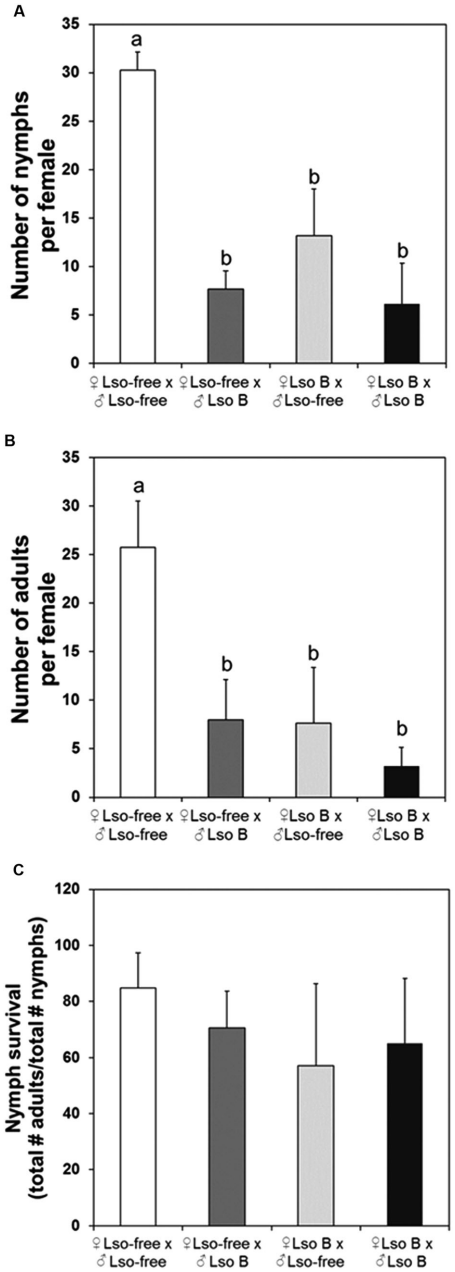

Fig. 3 Influence of Lso B infection in number of nymphs, and adults and in the percentage of nymphal survival in *B. cockerelli*. (A) A reduction in the number of nymphs was observed in the crosses performed with Lso B-infected insects compared to the cross Lso-free × Lso-free. (B) Fewer adults were obtained in the crosses performed with Lso B-infected insects compared to the cross Lso-free × Lso-free. (C) No statistically significant differences in the percentage of nymphal survival were found among the crosses. Data represent means ± SD of three independent experiments. Different letters indicate statistical differences between treatments using one-way ANOVA with Tukey's *post hoc* test.

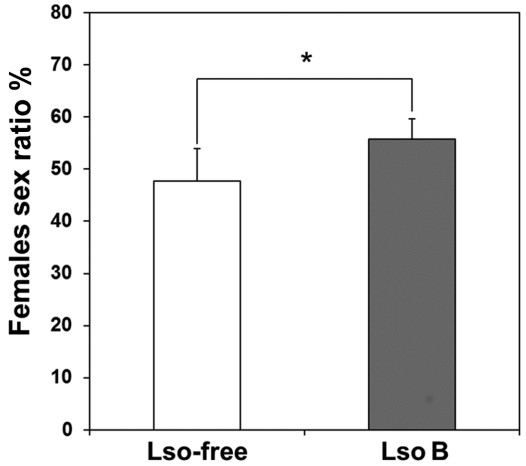

Fig. 4 Sex ratio in progeny of Lso-free and Lso B-infected *B. cockerelli* females. Significant increase in the proportion of females among the progeny of Lso B-infected females was observed. Data represent means ± SD of independent experiments. Asterisks (*) show statistical differences using pairwise *t*-test ($P < 0.05$).

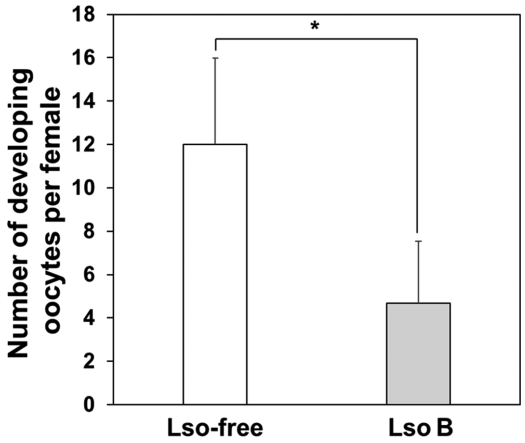

Fig. 5 Average number of developing oocytes present in 7-d-old Lso-free and Lso B-infected mated females. A significant reduction in the number of developing oocytes in the dissected reproductive systems of Lso B-infected mated females was observed. Data represent means ± 20 reproductive female organ samples. Asterisk (*) indicates statistical differences between treatments using unpaired *t*-test ($P < 0.05$).

increase in the percentage of females was observed in the progeny of Lso B-infected females (55.8%) compared to Lso-free females (47.8%) as shown by the pairwise *t*-test ($P < 0.05$).

Developing mature oocytes

To evaluate a possible behavioral change in oviposition by Lso B-infected females such as egg retention, we dissected and counted the number of developing oocytes present on female ovaries in 7-d-old Lso-free and Lso B-infected mated females (in both cases females were crossed with Lso-free males) (Figs. 5 and 6). A significant reduction in number of developing oocytes was observed in the reproductive organs of the Lso B-infected females compared with Lso-free females, excluding a possible behavioral change of egg retention.

Expression of BcVg1-like in 7-d-old mated females (mothers from crosses) and life stages

The relative expression of the *BcVg1-like* transcript was determined in the mothers from the different crosses (those females were 7 d old) and also from Lso-free and Lso B-infected adult females of different ages (1-, 3-, and 7-d-old virgin females and 7-d-old mated females, for the latter Lso-free males were used) (Fig. 7). The expression of *BcVg1-like* transcript was higher in the 7-d-old mothers from the Lso-free × Lso-free cross compared

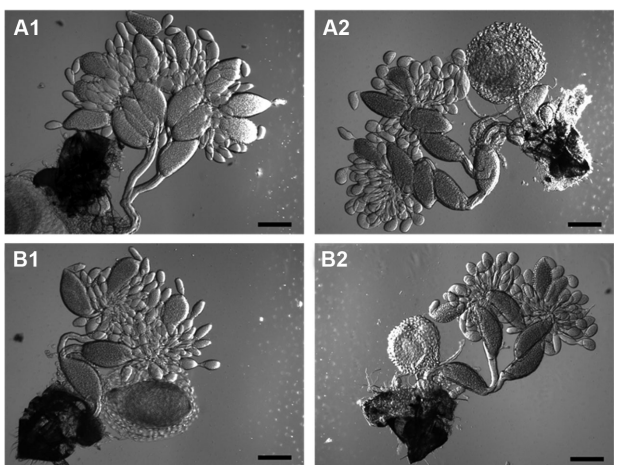

Fig. 6 Representative images of female reproductive systems from 7-d-old Lso-free and Lso B-infected mated females. The dissected ovaries showed a reduction in the number of developing oocytes in Lso B-infected females. A1 and A2 are representative images from Lso-free mated females; B1 and B2 correspond to Lso B-infected mated females. Scale bar is equal to 200 μm.

to the expression in the females from the Lso B-infected × Lso-free and Lso B-infected × Lso B-infected crosses. While no statistically significant differences were found in the *BcVg1-like* expression level between Lso-free females mated with Lso-free or Lso B-infected males, the relative expression of the transcript was reduced

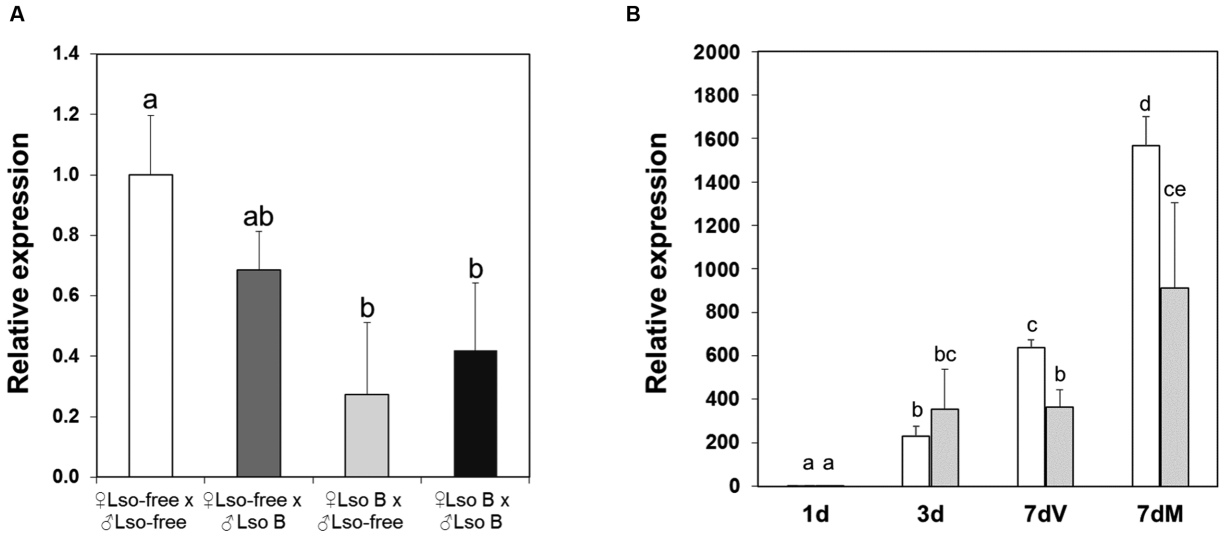

Fig. 7 Expression analyses of *BcVg1-like* transcript in mothers from the crosses and among adult females of different ages. Relative *BcVg1-like* transcript expression was determined by RT-qPCR and normalized to the expression value of *RpS18* and *Ef-1a* transcripts. (A) The expression of *BcVg1-like* was reduced in Lso B-infected females compared to Lso-free females mated with Lso-free males; however, no statistical differences were observed between females from the Lso-free × Lso B-infected cross and any of the other females. (B) Overall, *BcVg1-like* relative expression was reduced in 7-d-old Lso B-infected females (virgin and mated; V and M in the figure, respectively) compared with 7-d-old Lso-free females (virgin and mated). White bars indicate the cross performed with Lso-free insects, while gray bars indicate the cross performed with Lso B-infected insects. Each bar represents the means ± standard deviation (SD) of three independent experiments. Different letters indicate statistical differences among life stages using one-way ANOVA with Tukey's *post hoc* test ($P < 0.05$).

to 0.69 in the cross using the Lso B-infected male (Fig. 7A).

Overall, relative expression analyses of *BcVg1-like* among adult females of different ages from the Lso-free and Lso B-infected colonies revealed that the transcript was expressed in all samples and that its expression increased as females aged and following mating (Fig. 7B). In Lso-free females, *BcVg1-like* expression increased from 1-d-old virgin to 7-d-old virgin females, and it was further upregulated after mating. However, in Lso B-infected females, no differences in *BcVg1-like* expression were found between 3- and 7-d-old virgin females, or between 7-d-old mated and 3-d-old virgin females even if on average the expression in 7-d-old mated females was three times higher than in 3- and 7-d-old virgin females. *BcVg1-like* expression was significantly higher in 7-d-old virgin and mated Lso-free females compared to 7-d-old virgin and mated Lso-infected females, respectively.

Discussion

The effect of *Liberibacter* infection on psyllid fitness is not well understood. While the fitness analyses performed in *Diaphorina citri* revealed that infection by "*Candidatus* Liberibacter asiaticus" (CLas) resulted in increased fecundity, reduced nymphal development time, and increased female longevity of the insects harboring CLas (Pelz-Stelinski & Killiny, 2016; Ren *et al.*, 2016), the interaction of *B. cockerelli* with Lso resulted in reduced oviposition (Nachappa *et al.*, 2012, 2014). Also, previously, it was shown that the effects of Lso infection in *B. cockerelli* can differ depending on the Lso haplotype. The infection with each Lso haplotype resulted in the decrease of *B. cockerelli* fecundity, but only the infection with Lso B reduced the nymphal survival (Yao *et al.*, 2016). On potato and silver leaf nightshade, Lso-infected *B. cockerelli* nymphs developed faster than uninfected psyllids, but their mortality was higher (Thinakaran *et al.*, 2015). Also in tomato, Lso-infection resulted in lower nymphal survival (Nachappa *et al.*, 2012, 2014; Yao *et al.*, 2016). However, the effect of Lso infection on psyllid sex ratio and the effect of male infection on female oviposition, and nymphal development have not been investigated.

The present study confirms previous findings (Yao *et al.*, 2016) that crosses between Lso B-infected insects produce fewer eggs than crosses between Lso-free psyllids. This result might support the observed trade-off

between immune defense and reproduction, both energetically demanding processes (Schwenke *et al.*, 2016). However, in this study no differences in nymphal survival between those crosses were determined; one reason for this difference might be related to the difference in oviposition period between the two studies.

In addition, the present study revealed that the infectious status of the male can affect the oviposition of Lso-free females but not the percentage of nymphal survival. We had previously shown that mating induces the expression of the *BcVg1-like* transcript (Ibanez *et al.*, 2017). Here, we found a 30% reduction on the level of the *BcVg1-like* transcript when Lso-free females mated with Lso B-infected males compared to the females that mated with Lso-free males. However, this difference was not statistically significant. Seminal fluid proteins transferred to females during mating affect the physiology and behavior of the female (Avila *et al.*, 2011; Sirot *et al.*, 2015); changes in seminal fluid proteins between Lso-free and Lso B-infected males could lead to the identification of key proteins involved in psyllid reproduction.

In a previous study, it was observed that the expression of *BcVg1-like* was correlated with the number of eggs oviposited, suggesting its important role in psyllid reproduction (Ibanez *et al.*, 2017). We also showed that in *B. cockerelli* juvenile hormone regulated Vg expression and oocyte development. Results from the present study showed that there was a significant reduction in the level of *BcVg1-like* transcript in Lso B-infected mated females (Fig. 7). Thus, these results might support the model whereby the physiological trade-off between reproduction and immunity is mediated by endocrine and metabolic signaling via juvenile hormone and/or other signaling molecules (Schwenke *et al.*, 2016). More research is needed to evaluate the role of juvenile hormone on the immune function of psyllids.

The negative effects of pathogen-infection in insect reproduction have been shown in many insect species (Ahmed & Hurd, 2006; Reaney & Knell, 2010; McNamara *et al.*, 2014; Nystrand & Dowling, 2014). For instance, in *A. gambiae*, the transcription of *Vg*, the concentration of the Vg protein in the hemolymph, and Vitellin accumulation in oocytes were reduced following the infection with *Plasmodium yoelii nigeriensis* when ookinetes invaded the female's mosquito midguts (Ahmed *et al.*, 2001). Also, when *A. gambiae* was challenged with lipopolysaccharides, an immune elicitor, there was a significant decrease in the accumulation of Vitellin in the oocytes and in the number of eggs oviposited (Ahmed *et al.*, 2002). In *D. melanogaster* a reduced fecundity was observed during the acute phase of infection of the Gram-negative pathogen, *Providencia rettgeri* (McKean *et al.*, 2008). However, Vgs have been also associated with other biological functions. For instance, another *B. cockerelli* Vg, *BcVg6-like*, has a putative function involving the transport of lipids or other molecules (Ibanez *et al.*, 2018). Similarly, other Vgs were shown to be involved in immune defense in nonmammalian vertebrates and invertebrates (Zhang *et al.*, 2011). *Hexagrammos otakii* Vg binds lipopolysaccharides from Gram-negative bacteria, lipoteichoic acids from Gram-positive bacteria, peptidoglycans from both Gram-positive and Gram-negative bacteria, β-1,3-glucans from eukaryotic fungi and laminarin from brown algae (Li *et al.*, 2008). Similarly, the *Bombyx mori* Vg protein can bind the Gram-negative bacterium *Escherichia coli* and the Gram-positive bacterium *Bacillus subtilis*, resulting in a strong antibacterial activity (Singh *et al.*, 2013). In *A. gambiae*, Vg was able to interfere with the anti-Plasmodium response (Rono *et al.*, 2010). These findings suggest that Vg might be involved in the defense of hosts against microbes. However, this is a hypothesis that needs to be examined in *B. cockerelli*.

The third finding of this study was the change in the sex proportion between the progeny of Lso B-infected and Lso-free mothers; an increase in the female proportion in the progeny produced by *B. cockerelli* Lso B-infected females was determined (Fig. 4). Other studies had reported the association of insects with bacteria inducing feminization. For example, in whiteflies (*Bemisia tabaci*), *Rickettsia* was found to be associated with a significant female bias in two whitefly genetic lines (Cass *et al.*, 2016). Also, other members of the order *Rickettsiales*, such as *Wolbachia*, a diverse group of intracellular bacteria with different symbiotic relationships, induce diverse phenotypes in their insect hosts including female-bias, parthenogenetic induction, and cytoplasmic incompatibility (Werren *et al.*, 2008). Since in this study only the progeny from Lso B-infected females harbored the pathogen, the female bias observed in this report might be an adaptive strategy of Lso B to increase the fitness of Lso B-infected *B. cockerelli*; however, this interaction needs to be investigated in more detail. While the progeny of Lso B-infected females had a higher female ratio than the Lso-free counterpart, overall there were fewer females in the Lso B-infected progeny. Therefore, the increased proportion of females in the Lso B-infected population did not compensate for the decreased fecundity of the Lso B-infected females.

In conclusion, this study focused in the interaction between Lso B and *B. cockerelli* reproduction; in particular, it determined the effects of the bacteria on the female fecundity at the molecular level (*BcVg1-like* expression). These new findings could pave the way to a better understanding of the interaction between *Liberibacter* pathogens and their psyllid vectors. Further, knowledge

gained in the control of psyllid reproduction could lead us to design and adopt novel strategies of integrated pest management to control the vector population.

Acknowledgments

This work was supported by U.S. Department of Agriculture-National Institute of Food and Agriculture (USDA-NIFA) 2012-67013-19431, Texas A&M University and Texas A&M AgriLife Research (Controlling Exotic and Invasive Insect-Transmitted Pathogens). Angelica Albuquerque Tomilhero Frias was supported by a scholarship from the PDSE/CAPES - Brazilian Federal Agency for Support and Evaluation of Graduate Education within the Ministry of Education of Brazil (Process: PDSE 88881.131726/2016-01). We thank The National Council for Scientific and Technological Development for scholarship granted to William Mario de Carvalho Nunes.

Disclosure

The authors declare no conflicts of interest.

References

Ahmed, A., Baggott, S., Maingon, R. and Hurd, H. (2002) The costs of mounting an immune response are reflected in the reproductive fitness of the mosquito *Anopheles gambiae*. *Oikos*, 97, 371–377.

Ahmed, A., Maingon, R., Romans, P. and Hurd, H. (2001) Effects of malaria infection on vitellogenesis in *Anopheles gambiae* during two gonotrophic cycles. *Insect Molecular Biology*, 10, 347–356.

Ahmed, A.M. and Hurd, H. (2006) Immune stimulation and malaria infection impose reproductive costs in *Anopheles gambiae* via follicular apoptosis. *Microbes and Infection*, 8, 308–315.

Arukwe, A. and Goksøyr, A. (2003) Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. *Comparative Hepatology*, 2, 1.

Aubert, B. (1992) Citrus greening disease, a serious limiting factor for citriculture in Asia and Africa. *Proceedings of the International Society of Citriculture*, 2, 817–820.

Avila, F.W., Sirot, L.K., LaFlamme, B.A., Rubinstein, C.D. and Wolfner, M.F. (2011) Insect seminal fluid proteins: identification and function. *Annual Review of Entomology*, 56, 21–40.

Bové, J.M. (2006) Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. *Journal of Plant Pathology*, 88, 7–37.

Cass, B.N., Himler, A.G., Bondy, E.C., Bergen, J.E., Fung, S.K., Kelly, S.E. *et al*. (2016) Conditional fitness benefits of the *Rickettsia* bacterial symbiont in an insect pest. *Oecologia*, 180, 169–179.

Chen, J.S., Sappington, T.W. and Raikhel, A.S. (1997) Extensive sequence conservation among insect, nematode, and vertebrate vitellogenins reveals ancient common ancestry. *Journal of Molecular Evolution*, 44, 440–451.

Crosslin, J.M., Munyaneza, J.E., Brown, J.K. and Liefting, L.W. (2010) Potato zebra chip disease: a phytopathological tale. *Plant Health Progress*, https://doi.org/10.1094/PHP-2010-0317-01-RV.

Hansen, A., Trumble, J., Stouthamer, R. and Paine, T. (2008) A new huanglongbing species, "*Candidatus* Liberibacter psyllaurous", found to infect tomato and potato, is vectored by the psyllid *Bactericera cockerelli* (Šulc). *Applied and Environmental Microbiology*, 74, 5862–5865.

Ibanez, F., Hancock, J. and Tamborindeguy, C. (2014) Identification and expression analysis of aquaporins in the potato psyllid, *Bactericera cockerelli*. *PLoS ONE*, 9, e111745.

Ibanez, F., Levy, J. and Tamborindeguy, C. (2017) Identification and expression analyses of vitellogenin in *Bactericera cockerelli* (Šulc). *Journal of Insect Physiology*, 98, 205–213.

Ibanez, F. and Tamborindeguy, C. (2016) Selection of reference genes for expression analysis in the potato psyllid, *Bactericera cockerelli*. *Insect Molecular Biology*, 25, 227–238.

Ibanez, F., Tang, X. and Tamborindeguy, C. (2018) *Bactericera cockerelli vitellogenin-6 like*, a vitellogenin without a direct reproductive function? *Insect Molecular Biology*, 27, 166–176.

Leung, T. and Poulin, R. (2008) Parasitism, commensalism, and mutualism: exploring the many shades of symbioses. *Vie Et Milieu*, 58, 107–115.

Li, W., Abad, J.A., French-Monar, R.D., Rascoe, J., Wen, A., Gudmestad, N.C. *et al*. (2009) Multiplex real-time PCR for detection, identification and quantification of "*Candidatus* Liberibacter solanacearum" in potato plants with zebra chip. *Journal of Microbiological Methods*, 78, 59–65.

Li, Z., Zhang, S. and Liu, Q. (2008) Vitellogenin functions as a multivalent pattern recognition receptor with an opsonic activity. *PLoS ONE*, 3, e1940.

Liefting, L., Perez-Egusquiza, Z., Clover, G. and Anderson, J. (2008) A new "*Candidatus* Liberibacter" species in *Solanum tuberosum* in New Zealand. *Plant Disease*, 92, 1474–1474.

Lin, H., Islam, M.S., Bai, Y., Wen, A., Lan, S., Gudmestad, N.C. *et al*. (2012) Genetic diversity of "*Candidatus* Liberibacter solanacearum" strains in the United States and Mexico revealed by simple sequence repeat markers. *European Journal of Plant Pathology*, 132, 297–308.

Manjunath, K., Halbert, S., Ramadugu, C., Webb, S. and Lee, R. (2008) Detection of "*Candidatus* Liberibacter asiaticus" in *Diaphorina citri* and its importance in the management of

citrus huanglongbing in Florida. *Phytopathology*, 98, 387–396.

McKean, K.A., Yourth, C.P., Lazzaro, B.P. and Clark, A.G. (2008) The evolutionary costs of immunological maintenance and deployment. *BMC Evolutionary Biology*, 8, 76.

McNamara, K., Lieshout, E. and Simmons, L. (2014) Females suffer a reduction in the viability of stored sperm following an immune challenge. *Journal of Evolutionary Biology*, 27, 133–140.

Munyaneza, J., Crosslin, J. and Upton, J. (2007) Association of *Bactericera cockerelli* (Homoptera: Psyllidae) with "zebra chip," a new potato disease in southwestern United States and Mexico. *Journal of Economic Entomology*, 100, 656–663.

Munyaneza, J.E. (2012) Zebra chip disease of potato: biology, epidemiology, and management. *American Journal of Potato Research*, 89, 329–350.

Munyaneza, J.E., Fisher, T.W., Sengoda, V.G., Garczynski, S.F., Nissinen, A. and Lemmetty, A. (2010) Association of "*Candidatus* Liberibacter solanacearum" with the psyllid, *Trioza apicalis* (Hemiptera: Triozidae) in Europe. *Journal of Economic Entomology*, 103, 1060–1070.

Nachappa, P., Levy, J., Pierson, E. and Tamborindeguy, C. (2011) Diversity of endosymbionts in the potato psyllid, *Bactericera cockerelli* (Hemiptera: Triozidae), vector of zebra chip disease of potato. *Current Microbiology*, 62, 1510–1520.

Nachappa, P., Levy, J., Pierson, E. and Tamborindeguy, C. (2014) Correlation between "*Candidatus* Liberibacter solanacearum" infection levels and fecundity in its psyllid vector. *Journal of Invertebrate Pathology*, 115, 55–61.

Nachappa, P., Shapiro, A.A. and Tamborindeguy, C. (2012) Effect of "*Candidatus* Liberibacter solanacearum" on fitness of its insect vector, *Bactericera cockerelli* (Hemiptera: Triozidae), on tomato. *Phytopathology*, 102, 41–46.

Nadarasah, G. and Stavrinides, J. (2011) Insects as alternative hosts for phytopathogenic bacteria. *FEMS Microbiology Reviews*, 35, 555–575.

Nagaba, Y., Tufail, M., Inui, H. and Takeda, M. (2011) Hormonal regulation and effects of four environmental pollutants on vitellogenin gene transcription in the giant water bug, *Lethocerus deyrollei* (Hemiptera: Belostomatidae). *Journal of Insect Conservation*, 15, 421–431.

Nelson, W.R., Fisher, T.W. and Munyaneza, J.E. (2011) Haplotypes of "*Candidatus* Liberibacter solanacearum" suggest long-standing separation. *European Journal of Plant Pathology*, 130, 5–12.

Nelson, W.R., Sengoda, V.G., Alfaro-Fernandez, A.O., Font, M.I., Crosslin, J.M. and Munyaneza, J.E. (2013) A new haplotype of "*Candidatus* Liberibacter solanacearum" identified in the Mediterranean region. *European Journal of Plant Pathology*, 135, 633–639.

NPPO (2017) First report of "*Candidatus* Liberibacter solanacearum" on carrots in Israel. *European Plant Protection Organisation*, no. 01–2017, 020.

Nystrand, M. and Dowling, D. (2014) Dose-dependent effects of an immune challenge at both ultimate and proximate levels in *Drosophila melanogaster*. *Journal of Evolutionary Biology*, 27, 876–888.

Orlovskis, Z., Canale, M.C., Thole, V., Pecher, P., Lopes, J.R. and Hogenhout, S.A. (2015) Insect-borne plant pathogenic bacteria: getting a ride goes beyond physical contact. *Current Opinion in Insect Science*, 9, 16–23.

Pelz-Stelinski, K.S. and Killiny, N. (2016) Better together: association with "*Candidatus* Liberibacter asiaticus" increases the reproductive fitness of its insect vector, *Diaphorina citri* (Hemiptera: Liviidae). *Annals of the Entomological Society of America*, 109, 371–376.

Perilla-Henao, L.M. and Casteel, C.L. (2016) Vector-borne bacterial plant pathogens: interactions with hemipteran insects and plants. *Frontiers in Plant Science*, 7, https://doi.org/10.3389/fpls.2016.01163.

Raikhel, A.S. (1992) Vitellogenesis in mosquitoes. *Advances in Disease Vector Research*, pp. 1–39. Springer, New York, NY.

Reaney, L.T. and Knell, R.J. (2010) Immune activation but not male quality affects female current reproductive investment in a dung beetle. *Behavioral Ecology*, 21, 1367–1372.

Ren, S.L., Li, Y.H., Zhou, Y.T., Xu, W.M., Cuthbertson, A.G., Guo, Y.J. *et al*. (2016) Effects of "*Candidatus* Liberibacter asiaticus" on the fitness of the vector *Diaphorina citri*. *Journal of Applied Microbiology*, 121, 1718–1726.

Rono, M.K., Whitten, M.M., Oulad-Abdelghani, M., Levashina, E.A. and Marois, E. (2010) The major yolk protein vitellogenin interferes with the anti-plasmodium response in the malaria mosquito *Anopheles gambiae*. *PLoS Biology*, 8, e1000434.

RStudio (2015) *RStudio: Integrated Development for R*. RStudio, Inc., Boston, MA, URL http://www.rstudio.com.

Schmittgen, T.D. and Livak, K.J. (2008) Analyzing real-time PCR data by the comparative C(T) method. *Nature Protocols*, 3, 1101–1108.

Schwenke, R.A., Lazzaro, B.P. and Wolfner, M.F. (2016) Reproduction-Immunity Trade-Offs in Insects. *Annual Review of Entomology*, 61, 239–256.

Secor, G., Rivera, V., Abad, J., Lee, I.M., Clover, G., Liefting, L. *et al*. (2009) Association of "*Candidatus* Liberibacter solanacearum" with zebra chip disease of potato established by graft and psyllid transmission, electron microscopy, and PCR. *Plant Disease*, 93, 574–583.

Shinoda, T., Miura, K., Taylor, D. and Chinzei, Y. (1996) Vitellogenins and vitellins in the bean bug, *Riptortus clavatus* (Hemiptera: Alydidae): purification, immunological identification, and induction by juvenile hormone. *Archives of Insect Biochemistry & Physiology*, 31, 395–412.

Singh, N.K., Pakkianathan, B.C., Kumar, M., Prasad, T., Kannan, M., König, S. *et al*. (2013) Vitellogenin from the silkworm, *Bombyx mori*: an effective anti-bacterial agent. *PLoS ONE*, 8, e73005.

Sirot, L.K., Wong, A., Chapman, T. and Wolfner, M.F. (2015) Sexual conflict and seminal fluid proteins: a dynamic landscape of sexual interactions. *Cold Spring Harbor Perspectives in Biology*, 7, a017533.

Solomon, N.U., James, I.M., Alphonsus, N.O. and Nkiruka, R.U. (2015) A review of host–parasite relationships. *Annual Research & Review in Biology*, 5, 372–384.

Su, Q., Zhou, X. and Zhang, Y. (2013) Symbiont-mediated functions in insect hosts. *Communicative & Integrative Biology*, 6, e23804.

Tamborindeguy, C., Huot, O.B., Ibanez, F. and Levy, J. (2017) The influence of bacteria on multi-trophic interactions among plants, psyllids, and pathogen. *Insect Science*, 24, 961–974.

Teresani, G.R., Bertolini, E., Alfaro-Fernández, A., Martínez, C., Tanaka, F.A.O., Kitajima, E.W. *et al*. (2014) Association of "*Candidatus* Liberibacter solanacearum" with a vegetative disorder of celery in Spain and development of a real-time PCR method for its detection. *Phytopathology*, 104, 804–811.

Teulon, D., Workman, P., Thomas, K. and Nielsen, M. (2009) *Bactericera cockerelli*: incursion, dispersal and current distribution on vegetable crops in New Zealand. *New Zealand Plant Protection*, 62, 136–144.

Thinakaran, J., Yang, X.B., Munyaneza, J.E., Rush, C.M. and Henne, D.C. (2015) Comparative biology and life tables of "*Candidatus* Liberibacter solanacearum"—infected and free *Bactericera cockerelli* (Hemiptera: Triozidae) on potato and silverleaf nightshade. *Annals of the Entomological Society of America*, 108, 459–467.

Thomas, J.E., Geering, A.D.W. and Maynard, G. (2018) Detection of '*Candidatus* Liberibacter solanacearum' in tomato on Norfolk Island, Australia. *Australasian Plant Disease Notes*, 13(1), 7.

Tufail, M. and Takeda, M. (2008) Molecular characteristics of insect vitellogenins. *Journal of Insect Physiology*, 54, 1447–1458.

Weintraub, P.G. and Beanland, L. (2006) Insect vectors of phytoplasmas. *Annual Review of Entomology*, 51, 91–111.

Werren, J.H., Baldo, L. and Clark, M.E. (2008) *Wolbachia*: master manipulators of invertebrate biology. *Nature Reviews Microbiology*, 6, 741–751.

Yao, J., Saenkham, P., Levy, J., Ibanez, F., Noroy, C., Mendoza, A. *et al*. (2016) Interactions "*Candidatus* Liberibacter solanacearum"—*Bactericera cockerelli*: haplotype effect on vector fitness and gene expression analyses. *Frontiers in Cellular and Infection Microbiology*, 6, https://doi.org/10.3389/fcimb.2016.00062.

Zhang, S.C., Wang, S.H., Li, H.Y. and Li, L. (2011) Vitellogenin, a multivalent sensor and an antimicrobial effector. *The International Journal of Biochemistry & Cell Biology*, 43, 303–305.

Manuscript received February 6, 2017
Final version received April 4, 2018
Accepted April 8, 2018

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Evaluation of Lso presence in the progeny of the crosses between Lso-free and Lso B-infected females with Lso B-infected males. Individual insects were used to determine the incidence of Lso in the F^1 progeny for each cross. The presence of Lso was not detected in the F^1 progeny from the cross Lso-free females × Lso B-infected males. While all samples of F^1 progeny from the cross Lso B-infected females × Lso B-infected males showed the amplicon for Lso-detection (highlighted as Lso). The positive control of PCR amplification was performed detecting the psyllid 28S ribosomal RNA.