Feeding behaviour of *Bactericera cockerelli* (Šulc) (Hemiptera: Psylloidea: Triozidae) changes when infected with *Candidatus Liberibacter* solanacearum

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Abstract
Pathogens which need a vector for their transmission can alter the vectors’ behaviour to favour their spread. We used the electrical penetration graph technique to investigate this hypothesis by using the tomato potato psyllid *Bactericera cockerelli* infected or not with the plant pathogen *Candidatus Liberibacter* solanacearum (CLso) on African boxthorn and tomato. Probing was not affected by the host type but there was a significant effect on probing due to the infection status of the psyllid. More psyllids carried out probing activities in the sieve elements when infected with CLso, and more probing activities were observed from CLso-infected psyllids by comparison to the non-infected groups. Specifically, significant increases in salivation, phloem ingestion and number of probes, before and after reaching the sieve elements, were noticed in the infected groups. Furthermore, time elapsed to reach the sieve elements was significantly shortened by 2 h in the infected group. Remaining probing activities in xylem tissues were not different between all psyllid groups. The observed changes in feeding behaviour by pathogen-infected psyllids may well ensure further spread of the pathogen as greater salivation has the potential to increase transmission, highlighting at the same time the important role that crop and non-crop hosts play in disease epidemiology.

Keywords Insect–plant–pathogen interactions · Probing · African boxthorn · Tomato

Introduction
Plant pathogenic “*Candidatus Liberibacter*” species are phloem-restricted bacteria that depend on psyllids for their transmission (Haapalainen 2014). Psyllids acquire the bacteria during ingestion of phloem sap from infected plants and transmit it during salivation in the sieve elements (Cen et al. 2012; Sandanayaka et al. 2014). This close relationship between the insect, the host plant and the plant pathogen has important effects on the vector, particularly on its behaviour and fitness, reviewed in (Sugio et al. 2015; Tamborinde-guy et al. 2017; Eigenbrode et al. 2018). For instance, the Asian citrus psyllid *Diaphorina citri* Kuwayama and the tomato potato psyllid *Bactericera cockerelli* (Šulc) preferred to land on pathogen-infected plants by comparison to non-infected plants (Wu et al. 2015; Mas et al. 2014) whilst fecundity and nymphal survival decreased significantly in *B. cockerelli* (Nachappa et al. 2012). Feeding changes were studied when pathogenic bacteria were present in the plant (Cen et al. 2012) or in the psyllid (Sandanayaka et al. 2014; Antolinez et al. 2017a, b; Killiny et al. 2017), with results showing an increase or decrease in feeding related activities, i.e. salivation and phloem and xylem ingestion. It is assumed that these changes promote the spread of the pathogen by increasing contact between the host plant, the insect and the plant pathogen.

This idea fits with the “host manipulation hypothesis”, which focused on animal parasite systems and postulates that parasites can alter their host behaviour to promote their
spread (Poulin 2010). This idea is also relevant to plant pathogens that depend on insect vectors for their spread including phloem-restricted plant pathogenic bacteria that need a phloem feeding vector for its transmission and acquisition (Haapalainen 2014). This hypothesis depends on two conditions being met which are that the vector’s altered behaviour benefits the pathogen and that the altered behaviour is controlled by the pathogen (Mauck et al. 2019; Bernardo and Singer 2017). The molecular pathways by which pathogens induce phenotypic changes in the vectors are not fully understood or known for all pathosystems, but work on bacteria and viruses is suggesting that pathogen specific proteins can alter vector and host plant cell morphology, signalling pathways, immune response and cell physiology (Tamborindeguy et al. 2017; Sackton 2018; Mauck et al. 2019; Sugio et al. 2015; Bernardo and Singer 2017). The rise of functional genomic and phenomic technologies has allowed this field of research to expand rapidly in recent times, given their potential for characterizing molecules which are present in the vector (or the host plant) at any point in time of the infection cycle. More studies, however, have been carried out in virus/vector pathosystems than bacteria/vector pathosystems despite these being important vectors of disease.

*Bactericera cockerelli* is a major pest of Solanaceous crops in North and Central America (CABI 2018) and New Zealand (Teulon et al. 2009). This psyllid is the vector of *Candidatus Liberibacter solanacearum* (CLso), a bacterial plant pathogen that causes Zebra chip disease in potato (Secor et al. 2009) and an unnamed disease in tomatoes (Hansen et al. 2008; Sengoda et al. 2013). In February 2017, *B. cockerelli* was detected in Perth, Western Australia (AIQ 2017) and was declared not apt for eradication due to its widespread distribution around Perth. To date, there are no records of the species having dispersed to the eastern States or of the presence of the pathogen CLSo in mainland Australia. Nevertheless, the presence of the insect in Western Australia increases the risk of disease which is known to affect yield and quality of potatoes (Buchman et al. 2011).

In New Zealand, *B. cockerelli* and Zebra chip disease spread to every potato cultivation region (Teulon et al. 2009) soon after its initial detection in the Auckland region in 2006 (Gill 2006). This rapid spread of the pathogen prompted a large-scale research effort designed to understand the biology of the pest and the pathogen so to minimize impacts on the relevant horticultural industries e.g. potato, taewa, tomato, tamarillo, chilli and capsicum. Many aspects were studied which included psyllid host plant surveys (cultivated and non-cultivated plants) (Dohmen-Vereijssen et al. 2016), CLSo survey (Vereijssen et al. 2015), *B. cockerelli* life history on cultivated and non-cultivated plants (Dohmen-Vereijssen et al. 2016), and feeding studies (Sandanayaka et al. 2017). Host plant surveys identified many non-cultivated Solanaceous plants that served as “true hosts”, as eggs, nymphs and adults were detected, including African boxthorn (*Lycium ferocissimum* Miers), Jerusalem cherry (*Solanum pseudocapsicum* L.) and common thorn-apple (*Datura stramonium* L.) (Dohmen-Vereijssen et al. 2016). These hosts are also found in Australia and have the potential to become reservoirs of *B. cockerelli* outside the cropping areas and growing season as shown in the New Zealand field surveys. The pathogen CLso, was also detected in field-collected Jerusalem cherry and common thorn-apple (Vereijssen et al. 2015), but was not found in African boxthorn. There was, however, a successful transmission of CLSo to African boxthorn plants in laboratory conditions using infected psyllids (Dohmen-Vereijssen et al. 2016).

Many studies have investigated the effects that plant pathogens have on their vectors; particularly on the traits that favour pathogen spread such as host choice (Mauck et al. 2010), dispersal and sexual behaviours (Martini et al. 2015), production of wing forms (Shi et al. 2016), life history traits (Mann et al. 2008) and feeding (Lei et al. 2016), reviewed in (Fereres and Moreno 2009; Bosque-Pérez and Eigenbrode 2011; Sugio et al. 2015; Han et al. 2015; Tamborindeguy et al. 2017; Eigenbrode et al. 2018). The electrical penetration graph (EPG) technique was developed to understand Hemipteran feeding by measuring small changes in electrical resistance associated to specific probing activities of the stylet tip position in plant tissue (McLean and Kinsey 1964; Tjallingii 1978; Walker 2000). This technique is increasingly being used for understanding the effects that plant pathogens have on their vectors’ feeding behaviour as it allows for real-time observations of the stylet tip position in plant tissue helping researchers to make inferences about specific feeding changes, and pathogen movements from the plant to the insect and vice versa. This is the case for many psyllids that have become important pests in recent times due to their ability to transmit pathogenic *Candidatus* Liberibacter species in Rutaceae (Halbert and Manjunath 2004), Solanaceae (Butler and Trumble 2012), Apiaceae (Alfaro-Fernández et al. 2012), and Rosaceae (Thébaud et al. 2009).

Our study aimed at investigating the feeding behaviour of infected and non-infected *B. cockerelli*, by testing the hypothesis that insect feeding behaviour is altered by the presence of a pathogen. This idea has been tested on *B. cockerelli* feeding on tomato (Sandanayaka et al. 2014), *B. trigonica* feeding on carrots (Antolínez et al. 2017a, b) and *D. citri* feeding on *Citrus* sp. (Killiny et al. 2017). This idea, however, has not been tested for *B. cockerelli* feeding on the perennial non-crop host African boxthorn. Yet, this host may be playing an important role in the epidemiology of the disease in New Zealand. Potato crops in New Zealand, which are frequently infected with CLSo, are in some areas surrounded by African boxthorn plants which are used as wind protective hedges or occur in rived beds and sea dunes. CLSo has not been detected in field African boxthorn
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plants but has been successfully transmitted in laboratory conditions. In order to understand better the behaviour of infected psyllids when exposed to this host and to validate laboratory experiments of CLso transmission to African boxthorn plants, we used the direct current electrical penetration graph (DC-EPG) technique to investigate changes in probing behaviour from CLso-infected and non-infected *B. cockerelli* on two host plant species; the weed African boxthorn (also widely present in Australia) and the crop plant tomato (*S. lycopersicum* L.). We used tomato plants alongside African boxthorn plants as control plants given the availability of published EPG results on this host. A better understanding of *B. cockerelli* and CLso interactions with their crop and non-crop hosts will improve pest and disease management strategies in New Zealand and biosecurity readiness in Australia.

**Material and methods**

**Insects and plants**

The CLso-free *B. cockerelli* colony was established from the adults and nymphs originally collected from greenhouse tomatoes in Auckland and were reared on CLso-free capsicum plants (*Capsicum annuum* L. cv. “Giant Bell”). Adults and nymphs of CLso-infected TPP were originally collected from CLso-infected potato plants in 2009 in Pukekohe, Auckland, and reared on CLso-symptomatic tomato plants (*S. lycopersicum* L. cv. “Moneymaker”). Uninfected (CLso-free) and CLso-infected colonies of *B. cockerelli* were maintained in separate glasshouses at Plant & Food Research (PFR) Mt Albert, Auckland, New Zealand. Both colonies were maintained in steel frame cages (50×50×50 cm) covered with fine netting, containing 4 plants. The plant material provided to the CLso-free colony was tested for CLso before being used in the colonies, any positive or inconsistent reading resulted in the plants being discarded. The colonies were maintained at 25 °C, 16:8 L:D, 40–50% RH. Ten randomly selected *B. cockerelli* per cage (4 cages for each colony type) were tested for CLso every 5–10 weeks, first following the multiplex PCR protocol by Liefting et al. (2008) and later using the DNA extraction and qPCR protocols developed by Beard and Scott (2013). The non-infected colonies have tested 100% negative since 2006, and the infected colonies have tested 100% positive for CLso since 2010. All insects used in the EPG recordings were young females which had recently moulted to the adult stage (we selected yellow coloured psyllids indicating their recent moult to adulthood), and consequently unlikely to be gravid and oviposited.

Test plants, one-month old tomato cv. “Moneymaker” and four months old African boxthorn, were grown in glasshouse conditions at 24 ± 1 °C and L15:D9 photoperiod and used for EPG recordings. Tomato cv. “Moneymaker” was obtained as seed stock (Yates) and was chosen for its commercial use in New Zealand and Australia, whereas African boxthorn seeds were collected from the field in 2015 in New Zealand. Tomato seeds were placed in a germinating tray containing potting mix and the soil was watered every 2–3 days. African boxthorn seeds were soaked in water for 24 h before being placed in potting mix. The germinating and seedling growth periods were longer for African boxthorn than for tomato.

**Experimental procedures used in EPG assays**

All EPG recordings were conducted at PFR, Mt Albert, New Zealand using concurrently a Giga-4 and Giga-8 DC-EPG monitor (EPG Systems, Wageningen, Netherlands). The Giga-4 monitor was connected to African boxthorn plants and the Giga-8 monitor to tomato plants, to be able to test plants and insects at the same time. The signals from Giga-4 EPG monitor were digitized using the analogue-to-digital device DATAQ Di-720 card (Dataq® Instruments, Akron, OH, USA) and data were acquired and stored using WinDaq® Pro+ software (Dataq® Instruments, Akron, OH, USA). The data from Giga-8 DC-EPG monitor were acquired and stored using Stylet + d for Windows 7 (EPG Systems, Wageningen, Netherlands). Faraday cages were used in all cases to reduce electrical interference and the experiments took place in a climate-controlled room at 21 °C ± 1, under fluorescent lights with 16 h photoperiod (6:00-am to 10:00-pm).

*Bactericera cockerelli* females collected from colony cages were starved for about 4 h and then anaesthetized with CO₂ for ca. five seconds. Psyllids were then immobilized using fine forceps by carefully seizing the back of their wings to allow the attachment of a gold wire (3 cm long and 18.0 µm diameter) using a water-based silver conductive glue (EPG Systems, Wageningen, The Netherlands) applied on the psyllids’ pronotum using a micropin. Once wired psyllids were left for another 20–30 min of recovery period to reduce any potential tethering effect (Lei et al. 1997). Each wired psyllid was placed on the abaxial side of leaves which had been secured to a plastic stand to expose this side and left to feed on the test plants overnight (the EPG recordings were based on 12 h recording periods between 6:00-pm and 6:00-am next day with 16 h photoperiod (6:00-am to 10:00-pm). The leaves chosen for EPG were fully expanded young and mature leaves for tomato and African boxthorn, respectively. Older leaves of African boxthorn were chosen as they did not break off from the plant when exposing the underside of the leaves (younger leaves tended to break off the plant).

We tested four psyllid/host plant combinations; infected and uninfected psyllids on tomatoes and African boxthorn. The total number of psyllids tested for the African boxthorn...
EPG assays was 48 (24 infected and 24 non-infected psyllids) and 64 psyllids for the tomato EPG assays (32 infected and 32 non-infected psyllids). Any recordings which were disturbed by psyllids trying to escape from the wire attachment were not included in the analyses. The final number of psyllids that produced EPG recordings suitable for analyses was 18 and 19 infected and, 17 and 18 non-infected psyllids tested on tomato and African boxthorn for each infection group, respectively.

All EPG recordings were set at 100 × gain and voltage was adjusted to 1 V approximately during the initial phases of probing. Once the recordings had taken place, waveform interpretation and measurements were performed using WinDaq Waveform Browser (Dataq® Instruments, Akron, OH, USA) and Stylet + a (EPG Systems, Wageningen, The Netherlands) for the Giga-4 and Giga-8 DC monitors, respectively. The program Stylet + a, developed for aphids does not have the D waveform. Nevertheless, we used this program and substituted the waveform E1e for D. We did not individually analyse waveforms A, B (initial waveforms related to the establishment of electrical contact between the stylet and plant tissues shortly after penetration) (Cen et al. 2012) and Z (waveform related to walking activities) (Youn et al. 2011). Therefore our C waveform analyses included waveforms A and B and our NP waveform analyses included waveform Z. The term salivation is used throughout the manuscript and refers to salivation in phloem tissue (E1) followed or not followed by phloem ingestion (E2), the latter referred to as single salivation (single E1). Also, the full 12 h of recording was counted from beginning to end, which included the artificially terminated waveforms at the end of the recording. Waveforms measured were: NP (non-probing), C (pathway phase through epidermis and parenchyma), D (first contact with sieve elements), E1 (salivation in sieve elements), E2 (ingestion from phloem tissue) and G (ingestion from xylem tissue).

Selected sequential and non-sequential variables were calculated using “Sarria” Excel workbook (Sarria et al. 2009) and were used for the comparative analysis of feeding differences between hosts and between CLso-infected and non-infected psyllids. We analysed the proportion of individual psyllids that produced each waveform type (waveforms D, E1, single E1, E2, sustained E2 and G; with single E1 being E1 not followed by E2 and sustained E2 being E2 > 10 min), and EPG variables as determined by Sarria et al. (2009). These were (i) the time spent (as percentage of total probing) in each waveform type (waveforms C, D, E1, E2, sustained E2, and G), (ii) the mean number of waveform events produced per psyllid (in waveforms NP, C, D, E1, E2 and G), and (iii) the mean duration of waveform events produced per psyllid (in waveforms NP, C, D, E1, E2 and G). Along the non-sequential variables, we calculated the sequential variables (iv) the number of probes before and after the 1st E1, (v) the time to 1st probe from start of EPG, (vi) the time from 1st probe to 1st E1, (vii) the time from 1st probe to 1st E2, and (viii) the time from 1st probe to 1st sustained E2. Probe is defined in Sarria et al. (2009); a probe = number of waveforms between non-probing events. Waveform terminology and putative stylet tip position in plant tissue follow (Bonani et al. 2010). The choice of EPG variables was based on previous studies that tested the effects of multiple hosts and CLso infection status on feeding behaviour of its psyllid vector (Sandanayaka et al. 2013, 2014, 2017).

Statistical analyses

All sequential and non-sequential variables were initially plotted using boxplots with groups (Plant: African boxthorn and tomato; Infection status of psyllid: Yes/No) to illustrate any potential treatment differences, complementing the formal analyses to follow (boxplots were plotted using R). These variables were then formally analysed using analysis of variance (ANOVA) for continuous variables and non-parametric Kruskal–Wallis ANOVA for discrete/count data.

For the number of psyllids that produced each waveform type, a logistic regression was fitted, and odds ratio was calculated from the regression parameters. From these odds ratio, the probability of the number of psyllids that produced each waveform type for each of the treatment combinations were calculated using the odds ratio via the formula

$$p = \left( \frac{e^z}{1 + e^z} \right)$$

where $p$ = probability and $z$ is the relevant odd ratios for each treatment combination.

For ANOVA analyses, Plant (with two levels: African boxthorn and tomato) and Infection status of psyllid (with two levels: Yes/No) was modelled as the Treatment structure (Plant × Infection). There was no block structure. All residual values were examined graphically to ensure normality and homogeneity of variances. Observations with standardized residuals greater than 3.0 were excluded from analyses. Fisher’s protected LSD test ($p < 0.05$) was used to separate means where $F$ tests were significant. We report Fisher’s protected LSDs and H-statistics for all cases (that is whether they were significant or not) so that readers can conduct their own pair-wise comparisons. To confirm/check the $F$-probabilities (because of the relatively small number of residual degrees of freedom in the ANOVA), a permutation test with 5000 iterations were also performed. Where appropriate, non-sequential and sequential parameters were log-transformed to homogenize variances and normalize the data. For Kruskal–Wallis ANOVA analyses, Plant*Infection was modelled as the Group factor and ranks were calculated. The differences between the ranks for the four groups were then compared using the test statistic H. All analyses were
performed in GenStat 18th Edition (VSN International n.d.). Figures were created using R.

**Results**

**EPG waveforms**

Females of *B. cockerelli* showed six different EPG waveforms on both host plant species and were identical to waveforms previously described from this psyllid (Pearson et al. 2010, 2014; Sandanayaka et al. 2011). Waveforms observed in this study comprised of waveforms NP (non-probing), C (pathway phase through epidermis and parenchyma), D (first contact with sieve elements), E1 (salivation in sieve elements), E2 (ingestion from phloem tissue) and G (ingestion from xylem tissue). Hereafter, activities in the sieve elements refer to activities carried out in D, E1 (including single E1 where appropriate) and E2 (including sustained E2 where appropriate).

**Number of psyllids displaying a particular waveform**

The number of psyllids that produced a waveform was not affected by host plant type, but there was an effect due to psyllid CLso infection status with more psyllids carrying out sieve element activities when infected with CLso by comparison to the non-infected group (Table 1). The number of psyllids that carried out xylem tissue activities was the same for all groups (21 psyllids) but more psyllids carried out xylem activities by comparison to sieve element activities (Table 1). All psyllids carried out non-probing and pathway phase activities (data not shown).

Analyses between the four psyllid infection/host plant groups showed a greater number of psyllids carrying out sieve element activities when infected with CLso tested on African boxthorn followed by tomato (Table 1).

**Time spent in each waveform type (%)**

Comparative analyses of percentage time spent in each waveform type revealed there were no effects of host plant species for any of the waveforms, but there were significant effects of psyllid CLso infection status on time spent in the sieve elements. For instance, time spent in E1 increased 13 times approximately by comparison to the non-infected group (Table 2).

Further analyses between the four psyllid infection/host plant groups revealed that infected psyllids feeding on African boxthorn spent more time in the sieve elements by comparison to any psyllid infection/host plant group combination. For this group, time spent in D, E1 and E2 increased 13, 100 and 3 times approximately for each waveform type, respectively, by comparison to the non-infected group also feeding on the same host plant (Table 2). Similar results were observed for the same group tested on tomato. Boxplots show the distribution of the data used for calculating percentage time spent in each waveform type for each psyllid infection/host plant group (Fig. 1).

**Table 1** Proportion and probability (in parentheses) of *Candidatus Liberibacter solanacearum*-infected and non-infected *Bactericera cockerelli* producing a waveform during electrical penetration graph assays on leaves of either tomato or African boxthorn

| EPG experimental conditions | D | E1 | Single E1 | E2 | Sustained E2 | G |
|----------------------------|---|----|-----------|----|--------------|---|
| Host plant                 |   |    |           |    |              |   |
| Tomato (n = 35)            | 17/35 | 16/35 | 16/35 | 13/35 | 7/35 | 21/35 |
| African boxthorn (n = 37)  | 17/37 | 16/37 | 15/37 | 13/37 | 7/37 | 21/37 |
| F test probability         | 0.972 | 0.994 | 0.718 | 0.997 | 0.994 | 0.794 |
| Psyllid CLso infection status |   |    |           |    |              |   |
| TPP− (n = 35)              | 9/35 | 8/35 | 7/35 | 6/35 | 1/35 | 21/35 |
| TPP+ (n = 37)              | 25/37 | 24/37 | 24/37 | 20/37 | 13/37 | 21/37 |
| F test probability         | 0.001 | 0.002 | 0.001 | 0.002 | 0.001 | 0.756 |
| Interaction                | 0.146 | 0.303 | 0.150 | 0.244 | 0.261 | 0.023 |
| Host plant and psyllid CLso infection status |   |    |           |    |              |   |
| Tomato/TPP− (n = 17)       | 6/17 (0.353) | 5/17 (0.295) | 5/17 (0.295) | 4/17 (0.235) | 1/17 (0.058) | 8/17 (0.470) |
| Tomato/TPP+ (n = 18)       | 11/18 (0.611) | 11/18 (0.611) | 11/18 (0.611) | 9/18 (0.500) | 6/18 (0.333) | 13/18 (0.722) |
| African boxthorn/TPP− (n = 18) | 3/18 (0.167) | 3/18 (0.167) | 2/18 (0.111) | 2/18 (0.111) | 0/18 (0.000) | 13/18 (0.722) |
| African boxthorn/TPP+ (n = 19) | 14/19 (0.737) | 13/19 (0.684) | 13/19 (0.684) | 11/19 (0.579) | 7/19 (0.368) | 8/19 (0.421) |

Probability of psyllids producing a waveform type was calculated from the regression parameters from a logistic regression

**TPP**− non-infected *B. cockerelli, TPP**+ CLso-infected *B. cockerelli*. Waveforms, D first contact with sieve elements, *E1* salivation in the sieve elements, *single E1* E1 not followed by E2, *E2* ingestion from phloem tissue, *sustained E2* E2 > 10 min, *G* ingestion from xylem tissue
Analyses of the number of waveforms showed there was no effect of host plant species but there was a significant effect of psyllid CLso infection status on the number of non-probing, pathway and sieve element events. In all these tissues, the number of waveforms increased significantly in the infected group. For instance, there were 5, 7, 8.5 times more Ds, E1s and single E1s, 5 times more E2s and 2 times more non-probing and pathway events (Table 3).

Analyses between the four psyllid infection/host plant groups showed a significant increase in the number of the waveforms mentioned above by the infected psyllid group tested on African boxthorn. For instance, there were 5, 7, 8.5 times more Ds, E1s and single E1s, 5 times more E2s and 2 times more non-probing and pathway events (Table 3).

Analyses between the four psyllid infection/host plant groups showed a significant increase in the number of the waveforms mentioned above by the infected psyllid group tested on African boxthorn. For instance, there were 5, 7, 8.5 times more Ds, E1s and single E1s, 5 times more E2s and 2 times more non-probing and pathway events (Table 3).

### Duration of EPG waveforms

There was no effect of plant species on the duration of waveforms, except for the duration of the pathway phase that was shorter on African boxthorn by comparison to tomato, although the difference was marginal ($p = 0.038$) (Table 4). By contrast, there was a significant effect of CLso psyllid infection status on the duration of all sieve element waveform types. For instance, duration of salivation increased 11 times in the infected group whilst ingestion from phloem tissue decreased by 1.5 times in the infected psyllid group (Table 4).

Analyses between the four psyllid infection/host plant groups revealed significantly longer duration of salivation carried out by infected psyllids feeding on African boxthorn. For instance, the duration of salivation increased 71 times in this group when compared to the uninfected group feeding on the same host plant (Table 4). The duration of phloem ingestion decreased by up to 1.4 times in this group, although the difference was marginal ($p = 0.048$).
Fig. 1 Time spent in each waveform type (%) of *Candidatus Liberibacter* solanacearum-infected and non-infected *Bactericera cockerelli* during electrical penetration graph assays on leaves of either tomato or African boxthorn. *T/TPP* Tomato/non-infected *B. cockerelli* (*n* = 17), *T/TPP* + Tomato/CLso infected *B. cockerelli* (*n* = 18), *AB/TPP* - African boxthorn/non-infected *B. cockerelli* (*n* = 18), *AB/TPP* + African boxthorn/CLso-infected *B. cockerelli* (*n* = 19). Waveforms, *C* pathway phase, *D* first contact with sieve elements, *E1* salivation in the sieve elements, *E2* ingestion from phloem tissues, *sustained E2* > 10 min, *G* ingestion from xylem tissues

(Table 4). Boxplots show the distribution of the data used for calculating durations of non-probing and waveform events for each pathogen–host group and EPG waveform type (Fig. 3).
Sequential EPG variables

As in previous non-sequential EPG variables there was no effect of plant species on any of the sequential variables examined here, but there was a significant effect due to psyllid infection status on the number of probes before and after the 1st salivation event. Non-infected psyllids probed on average once, whilst CLso-infected psyllids probed 7 and 6 times before and after reaching the sieve elements, respectively (Table 5). There was as well as significant effect in the time elapsed to reach the sieve elements (variables: time from 1st probe to 1st E1 and 1st E2), that decreased from 10 and 11 h (in the non-infected group) to 8 and 9 h (in the infected group) for each variable, respectively (Table 5).

Further analyses between the four psyllid infection/host plant groups revealed a significantly higher number of probes carried out before and after the 1st salivation event by the infected psyllid group tested on African boxthorn (up to 10 and 7 times more probes for each variable, respectively) (Table 5). However, there were no differences in the remaining sequential variables. All psyllids spent similar time reaching the sieve elements, i.e. 14 to 18 min to start probing from the beginning of EPG recordings, 7 to 11 h to reach the sieve elements (variables: time from 1st probe to 1st E1 and E2) and, 10 to 11 h to initiate prolonged phloem ingestion (Table 5). Boxplots show the distribution of the data used for calculating sequential variables for each pathogen–host group (Fig. 4).

Discussion

Probing behaviour of CLso-infected and non-infected B. cockerelli

A handful of EPG-based studies have recorded psyllid feeding behaviour when pathogenic Candidatus Liberibacter species are present in the plant (Cen et al. 2012; Sandanayaka et al. 2014; George et al. 2018) or in the insect (Sandanayaka et al. 2014; Antolinez et al. 2017a, b; Killiny et al. 2017). When Ca. Liberibacter species or haplotypes are present in a psyllid, EPG-based studies have shown changes in feeding. For instance, the duration of the pathway phase, salivation phase and time to reach the sieve elements decreased in CLso-infected B. cockerelli and B. trigonica (Sandanayaka et al. 2014; Antolinez et al. 2017a, b). A third study carried out on Candidatus Liberibacter asiaticus (CLas)-infected D. citri (Killiny et al. 2017) also showed a decrease in the duration of the pathway and salivation phases as seen in the B. cockerelli and B. trigonica studies. Our study also showed a decrease in the duration of the pathway phase and time to reach the sieve elements but, in contrast with the
afore-mentioned studies; an increase in salivation. In our study, CLso-infected psyllids spent more time on phloem ingestion activities by comparison to non-infected ones, an effect also seen in the Bactericera spp. studies. In addition, two of the above-mentioned studies reported an increase in xylem ingestion by Ca. Liberibacter spp.-infected psyllids (Sandanayaka et al. 2014; Killiny et al. 2017), whereas our study found a decrease in xylem ingestion similar to (Antolínez et al. 2017a, b). Possible explanations for the observed differences could be osmotic stresses (Pompon et al. 2011), host plant health (Cen et al. 2012), host mechanical/chemical stimuli (Valenzuela et al. 2017) and plant age (Luo et al. 2015a, b) to mention a few. Our study did not test specific reasons why the feeding behaviour of infected psyllids was different to non-infected ones and to results from some previous studies. But results from other studies are suggestive of the pathogen being capable of altering important physiological processes in the insect such as the ones observed in (Killiny et al. 2017), where increased Adenosine triphosphate (ATP) levels were observed in CLas-infected D. citri adults. Additional physiological changes have also been observed in CLas-infected D. citri nymphs (Killiny and Jones 2018). These or other physiological changes could have contributed to the observed differences in our study, where the pathogen was able to change the feeding behaviour of its vector to presumably promote its spread through faster access to the sieve elements and an increase in salivation and phloem ingestion.

Despite the small number of studies on pathogen mediated feeding changes (particularly of studies based on persistent circulative bacterial pathogens), a pattern is emerging whereby infected insects spent less time in the pathway phase therefore reaching the sieve elements sooner by comparison to a non-infected group. However, it is difficult to find a common pattern of probing activities in the sieve elements and in xylem tissues with results differing depending on the study. For instance, in our study the CLso-infected group salivated significantly more by comparison to the non-infected group but the reverse was seen in other psyllid studies (Sandanayaka et al. 2014; Antolínez et al. 2017a, b; Killiny et al. 2017). This could be explained by different recording periods (from 5 to 24 h) used in different studies which may have influenced the results as shown in (Sandanayaka et al. 2017), and other factors like the age of the plant and the type of leaf (Luo et al. 2015a, b; Ebert et al. 2018), the pathogen titre in the plant or the insect, pathogen genotype and insect age.

The molecular interactions that mediate feeding changes are not fully characterized or known for many pathosystems but, with the advent of functional genomics more pathways are being discovered that point out the important role that pathogen related proteins have on the vectors’ phenotypic changes (Tamborindeguy et al. 2017; Sackton 2018; Mauck et al. 2019; Sugio et al. 2015; Bernardo and Singer 2017). In absence of ‘omics’ data and based on other pathosystems, we propose that the feeding effects observed in our study could be due to the result of bacteria-vector specific responses, pointing out to a possible manipulation by the pathogen. This inference, however, needs to be studied further using the appropriate technologies.

We are also aware that plant mediated responses have not been excluded from our study given that our infected group became infected whilst feeding from an infected plant during its entire nymph development. Although difficult to separate, studies have shown that leaving insects to feed on an infected host plant for a short time and preferably during their early stages of development, allows sufficient time for the pathogen to replicate in the insect without the host effects (Moreno-Delafuente et al. 2013; Stafford et al. 2011). Further to this, we are also aware that our psyllids had different rearing and test hosts. Application of the same host plant species for rearing and testing insects may exclude host switching effects from the results. However, it is not uncommon to find experiments that include different rearing and test hosts, as seen in other EPG studies on psyllids (Antolínez et al. 2017a, b; George et al. 2018; Butler et al. 2012) and aphids (Simon et al. 2017). In this study, most test insects were not reared on the same plant type as tested. Rearing TPP on African boxthorn was not feasible as this host does not meet the basic criteria used for long term rearing of psyllids because of its poor germination and development under glasshouse conditions. In addition, leaves drop off easily and complete leaf drop can happen randomly (Vereijssen pers. observation). A preliminary EPG study investigating the effects of switching hosts between tomato and capsicum found no effects on feeding behaviour of TPP due to host switching between these two host plants (Sandanayaka unpublished data).

Implication to potential CLso transmission to tomato and African boxthorn

Candidatus Liberibacter spp. inoculation studies suggest that successful transmission is dependent on several factors mainly: the acquisition access period, the post-acquisition period where the pathogen replicates inside the insect, the insect life-stage at time of acquisition, and pathogen titre in the plant (Inoue et al. 2009; Pelz-Stelinski et al. 2010; Lopes et al. 2016; George et al. 2018). Our study did not assess CLso transmission to tomato or African boxthorn. A previous study reported successful transmission of CLso to tomato (Sandanayaka et al. 2014). Our results validate the observed transmission of CLso to African boxthorn in laboratory conditions (Dohmen-Vereijssen et al. 2016).

Although not easy, it is important to reduce psyllid probing to avoid the spread of the pathogen. Some insecticides
have proven relatively effective at controlling/stoppping psyllids from feeding such as cytantriliprole and abamectin sprayed on potatoes (Mustafa et al. 2015a, b), and less effective was soil applied imidacloprid and aldicarb to protect citrus (Serikawa et al. 2012, 2013). Protecting non-crop host plants is more difficult than protecting crops yet they are important in the epidemiology of the disease. A better strategy than spraying insecticides in non-crop hosts, is to eliminate host availability if possible. This becomes important as seen in New Zealand potato crops that had African boxthorn planted along the crops’ boundaries as wind breaks. These crops showed high incidences of Zebra chip which was likely due to the movement of infected psyllids from African boxthorn to potatoes and vice versa (Dohmen-Vereijssen et al. 2016). In New Zealand, efforts to control TPP and the pathogen CLso are following integrated pest management strategies which combine cultural, biological and chemical control methods (Vereijssen et al. 2018).

### Probing behaviour of *B. cockerelli* on tomato and African boxthorn

The present study showed there were no effects of host plant species on the probing behaviour of female adult *B. cockerelli*, indicating that physical and chemical stimuli of tomato or African boxthorn did not affect psyllids’ probing behaviour during the 12 h that psyllids were monitored using the EPG technique. This supports the findings from field surveys carried out in New Zealand where large numbers of all life stages of *B. cockerelli* were found on African boxthorn throughout the year (Vereijssen et al., unpublished data). The presence of African boxthorn in Australia poses a challenge for the management of *B. cockerelli* as African boxthorn is a widespread species in pastures and neglected areas (Noble and Rose 2013; ALA 2018a). Additional *Lycium* species, which are also abundant in Australia, could become potential hosts for *B. cockerelli*, such as the indigenous *L. australiense* which is also widespread (ALA 2018b).

The predominant waveform in *B. cockerelli* was the pathway phase through epidermis and parenchyma tissues with 82% and 81% of the total time spent in this waveform on tomato and African boxthorn, respectively (Table 2).

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### Table 4 Duration (minutes) of waveform events of *Bactericera cockerelli* by host plant and psyllid *Candidatus* Liberibacter solanacearum infection status for each EPG waveform representing different stylus penetration activity. Data are expressed as means. One-way ANOVA (F-statistics) was used for waveforms NP and C, which were log-transformed. The values given are the raw means followed by the log-transformed means in parentheses.

| EPG experimental conditions | NP | C | D | E1 | E2 | G |
|----------------------------|----|---|---|----|----|---|
| Host plant                 |    |   |   |    |    |   |
| Tomato (*n* = 35)          | 19 (3.1) | 86 (3.7) | 21 (38) | 120 (37) | 23 (37) | 19 (36) |
| African boxthorn (*n* = 37)| 10 (2.8) | 59 (3.6) | 17 (35) | 185 (36) | 24 (36) | 32 (36) |
| LSD (5%), (H-statistics)   | 0.5 0.4 | 0.28 | 0.03 | 0.11 | 0.00003 |
| *F* test probability, (Chi-square probability) | 0.666 0.038 | 0.560 | 0.827 | 0.699 | 0.995 |
| Psyllid CLso infection status |    |   |   |    |    |   |
| TPP− (*n* = 35)            | 11 (2.8) | 96 (3.8) | 8 (28) | 26 (27) | 28 (31) | 37 (40) |
| TPP+ (*n* = 37)            | 18 (3.0) | 50 (3.5) | 30 (44) | 274 (45) | 19 (42) | 14 (33) |
| LSD (5%), (H-statistics)   | 0.5 0.4 | 11.23 | 14.20 | 5.13 | 2.10 |
| *F* test probability, (Chi-square probability) | 0.988 <0.001 | <0.001 | <0.001 | 0.008 | 0.131 |
| Host plant and psyllid CLso infection status |    |   |   |    |    |   |
| Tomato/TPP+ (*n* = 17)     | 13 (2.9) | 93 (3.7) | 11 (32) | 48 (30) | 30 (34) | 17 (33) |
| Tomato/TPP− (*n* = 18)     | 25 (3.2) | 80 (3.7) | 30 (43) | 188 (43) | 17 (41) | 20 (40) |
| African boxthorn/TPP− (*n* = 18) | 8 (2.7) | 100 (3.8) | 4 (24) | 5 (24) | 26 (28) | 57 (47) |
| African boxthorn/TPP+ (*n* = 19) | 12 (2.9) | 21 (3.1) | 29 (45) | 355 (48) | 22 (43) | 9 (27) |
| LSD (5%), (H-statistics)   | 0.8 0.6 | 12.46 | 15.54 | 5.13 | 0.003 |
| *F* test probability, (Chi-square probability) | 0.503 0.026 | 0.002 <0.001 | 0.048 | 0.019 |

The LSD given is also in log-transformed mode. Kruskal–Wallis one-way ANOVA was conducted for the remainder waveforms. In these cases, we report the raw mean followed by the mean rank in parentheses, as well as H-statistics and Chi-square probability.

TPP− non-infected *B. cockerelli*, TPP+ CLso-infected *B. cockerelli*. 1Waveforms D and E1 are shown in seconds. Waveforms: NP non-probing, C pathway phase, D first contact with sieve elements, E1 salivation in the sieve elements, E2 ingestion from phloem tissue, G ingestion from xylem tissue.
Meanwhile, percentage time spent ingesting from phloem tissues occurred less frequently with 12–17% on tomato and 8–13% on African boxthorn for each waveform type, respectively (Table 2). This suggests that *B. cockerelli* either (i) preferred feeding from the parenchyma (unlikely as previous EPG studies on various hosts, including tomato characterized this species as being predominantly a phloem feeder Butler et al. 2012; Mustafa et al. 2015a, b; Sandanayaka et al. 2014, 2017), or (ii) found some type of impediment that delayed reaching the sieve elements.

![Fig. 3 Duration (seconds) of waveform events of *Candidatus Liberibacter* solanacearum-infected and non-infected *Bactericera cockerelli* during electrical penetration graph assays on leaves of tomato or African boxthorn. *Tomato/TPP* Tomato/non-infected *B. cockerelli* (*n* = 17), *Tomato/TPP* + *Tomato/CLso-infected* *B. cockerelli* (*n* = 18), *AB/TPP* − *African boxthorn/non-infected B. cockerelli* (*n* = 18), *AB/TPP* + *African boxthorn/CLso-infected B. cockerelli* (*n* = 19). Waveforms, NP non-probing, C pathway phase, D first contact with sieve elements, E1 salivation in the sieve elements, E2 ingestion from phloem tissue, G ingestion from xylem tissue]
Feeding behaviour of *Bactericera cockerelli* (Šulc) (Hemiptera: Psylloidea: Triozidae)…

This line of evidence is supported by the long-time psyl-
lids took to reach the sieve elements on both hosts; psyllids
took 9–10 h to reach the sieve elements and 10 h to initiate
sustained phloem ingestion (Table 5). This contrasts with
previous *B. cockerelli* feeding studies that reported shorter
times for the same variables. For instance, when feeding
on potato, *B. cockerelli* took 2.6 to 4.2 h to 1st phloem
salivation and 3.2 to 5.5 h to 1st phloem ingestion (Butler,
Walker and Trumble 2012; Mustafa et al. 2015a, b) and
when feeding on tomato, *B. cockerelli* took 2.1 h to 1st
phloem salivation and 1.1 to 6.3 h to 1st phloem ingestion
(Sandanayaka et al. 2011, 2014).

It is not clear why reaching the sieve elements was
delayed in our study by comparison to other studies. Given
the longer times reported here (9–10 h to reach the sieve
elements and 10 h to initiate prolonged phloem inges-
tion), we recommend that EPG recordings using *B. cock-
erelli* is carried out for a minimum of 12 h particularly, in
phloem-restricted pathogen transmission and acquisition
experiments, as well as measuring effectiveness of sys-
temic insecticides and assessing phloem-based host plant
resistance.

**Conclusion**

The present study showed there was an effect of psyllid
infection status on probing behaviour. In summary, more
psyllids carried out probing activities in the sieve elements

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**Table 5** Sequential EPG variables of *B. cockerelli* by host plant and by psyllid *Candidatus Liberibacter solanacearum* infection status

| EPG experimental conditions | Number of probes before 1st E1 | Number of probes after 1st E1 | Time to 1st probe from start of EPG recording (min) | Time from 1st probe to 1st E1 (h) | Time from 1st probe to 1st E2 (h) | Time from 1st probe to 1st sustained E2 (h) |
|-----------------------------|-------------------------------|-------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Host plant                  |                               |                               |                                 |                                 |                                 |                                 |
| Tomato (n = 35)             | 3 (36)                        | 3 (37)                        | 15 (2.9)                        | 9 (4.5)                         | 9 (4.5)                         | 10 (4.5)                         |
| African boxthorn (n = 37)   | 5 (37)                        | 4 (36)                        | 16 (3.0)                        | 9 (4.5)                         | 10 (4.5)                        | 10 (4.6)                        |
| LSD (5%), (H-statistics)    | 0.0008                        | 0.02                          | 0.75                            | 0.36                            | 0.31                            | 0.30                            |
| *F* test probability, (Chi-square probability) | 0.975                      | 0.844                         | 0.789                           | 0.795                           | 0.823                           | 0.383                           |
| Psyllid CLso infection status |                               |                               |                                 |                                 |                                 |                                 |
| TPP− (n = 35)               | 1 (27)                        | 1 (31)                        | 14 (2.9)                        | 10 (4.6)                        | 11 (4.6)                        | 11 (4.6)                        |
| TPP+ (n = 37)               | 7 (46)                        | 6 (42)                        | 17 (3.0)                        | 8 (4.5)                         | 9 (4.5)                         | 10 (4.5)                        |
| LSD (5%), (H-statistics)    | 14.59                         | 5.15                          | 0.75                            | 0.36                            | 0.31                            | 0.30                            |
| *F* test probability, (Chi-square probability) | <0.001                      | 0.003                         | 0.653                           | 0.025                           | 0.039                           | 0.271                           |
| Host plant and psyllid CLso infection status |                               |                               |                                 |                                 |                                 |                                 |
| Tomato/TPP− (n = 17)        | 1 (29)                        | 1 (35)                        | 14 (2.9)                        | 9 (4.5)                         | 10 (4.5)                        | 10 (4.5)                        |
| Tomato/TPP+ (n = 18)        | 5 (43)                        | 4 (39)                        | 16 (3.0)                        | 9 (4.5)                         | 9 (4.5)                         | 10 (4.5)                        |
| African boxthorn/TPP− (n = 18) | 1 (25)                       | 0 (27)                        | 15 (2.9)                        | 11 (4.6)                        | 11 (4.6)                        | 11 (4.6)                        |
| African boxthorn/TPP+ (n = 19) | 10 (48)                      | 7 (45)                        | 18 (3.0)                        | 7 (4.4)                         | 8 (4.5)                         | 10 (4.5)                        |
| LSD (5%), (H-statistics)    | 15.38                         | 7.06                          | 1.06                            | 0.51                            | 0.43                            | 0.42                            |
| *F* test probability, (Chi-square probability) | <0.001                      | 0.008                         | 0.934                           | 0.082                           | 0.161                           | 0.411                           |

Data are expressed as means. Kruskal–Wallis one-way ANOVA was calculated for variables: Number of probes to the 1st E1 and number of probes after the 1st E1. In these cases, we report the raw mean followed by the mean rank in parentheses, as well as H-statistics and Chi-square probability. One-way ANOVA (F-statistics) was used for the remainder variables which were log-transformed (the values given are the raw means followed by the log-transformed means in parentheses). LSDs are given in log-scale

TPP− non-infected *B. cockerelli*, TPP+ CLso-infected *B. cockerelli*, E1 salivation in the sieve elements, E2 ingestion from phloem tissue, sustained E2 E2 > 10 min
when infected with CLso and more probing activities were observed from the CLso-infected psyllid group by comparison to the non-infected group. These findings are important for understanding pathogen and disease epidemiology as an increase in salivation and ingestion activities has the potential to intensify pathogen transmission and acquisition rates.
But further studies are needed to elucidate the molecular interactions that induced such changes, considering potential host plant effects.

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