

# Effects of pymetrozine, an antifeedant of Hemiptera, on Asian citrus psyllid, *Diaphorina citri*, feeding behavior, survival and transmission of *Candidatus Liberibacter asiaticus*

Dhana Raj Boina,<sup>a\*</sup> Youngnam Youn,<sup>b</sup> Svetlana Folimonova<sup>c</sup> and Lukasz L Stelinski<sup>a</sup>

## Abstract

**BACKGROUND:** Vector-borne plant disease management can be enhanced by deployment of antifeedants in addition to the use of broad-spectrum neurotoxic insecticides. The effects of pymetrozine on Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama, feeding behaviour, survival and transmission of *Candidatus Liberibacter asiaticus* (Las), the presumed causal pathogen of huanglongbing, were investigated.

**RESULTS:** Pymetrozine applied at 52 and 104  $\mu\text{g mL}^{-1}$  to citrus plants [Swingle citrumelo (*X Citroncirus webberi* Ingram and Moore)] modified the feeding behavior of ACP and increased the amount of time spent performing non-penetration behaviors while decreasing the time spent performing ingestion behaviors compared with the controls 1 day after treatment. However, the antifeedant effect of pymetrozine subsided 5 days after application. Pymetrozine reduced the survival of both adults and nymphs on treated plants compared with the control. However, it had a greater impact on survival of nymphs than on survival of adults. Pymetrozine applied at 52 and 104  $\mu\text{g mL}^{-1}$  on Las-infected 'Valencia' sweet orange plants [*Citrus sinensis* L. (Osbeck)] reduced acquisition (12 and 21% respectively) and transmission (11 and 18% respectively) of Las by feeding ACP adults compared with the controls; however, these reductions were not statistically significant.

**CONCLUSIONS:** Pymetrozine exhibited moderate antifeedant effects by modifying the feeding behavior of ACP adults with short residual activity. The impact of pymetrozine on survival of nymphs was greater than on adults at the higher concentrations tested. Pymetrozine also reduced the acquisition and transmission of Las by feeding ACP adults up to 21 and 18%, respectively, compared with untreated controls.

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**Keywords:** pymetrozine; antifeedant activity; citrus greening; huanglongbing; Asian citrus psyllid; *Candidatus Liberibacter asiaticus* transmission

## 1 INTRODUCTION

The Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), is an introduced insect into the United States and was first discovered in Florida State in 1998.<sup>1</sup> ACP transmits two gram-negative phloem-restricted bacteria, *Candidatus Liberibacter asiaticus* (Las) and *Candidatus Liberibacter americanus* (Lam). These bacteria are thought to be the causal agents of huanglongbing (HLB) in citrus,<sup>2–5</sup> as Koch's postulates are not fulfilled given that pure cultures of these bacteria have not been established.<sup>4</sup> HLB is a destructive disease of citrus crops, characterized by symptoms such as leaf mottling, asymmetric chlorosis and yellowing of veins and midribs, which resemble nutritional deficiency. HLB causes both qualitative and quantitative yield loss. Diseased trees produce fewer fruit that are small, lopsided and sometimes remain green in color.<sup>4</sup> The juice from diseased tree fruit is bitter tasting, impacting upon both fresh and processed

fruit markets. The economic importance of ACP as a pest in the United States has increased with the detection of HLB in Florida in 2005.<sup>6</sup> Given the ACP short range<sup>7</sup> and possible long-range dispersal capabilities, HLB has spread to most of the citrus-growing

\* Correspondence to: Dhana Raj Boina, Entomology and Nematology Department, Citrus Research and Education Center, University of Florida, Experiment Station Road, Lake Alfred, FL 33850, USA. E-mail: drboina@vt.edu

a Entomology and Nematology Department, Citrus Research and Education Center, University of Florida, Lake Alfred, FL, USA

b Department of Entomology, Chungnam National University, Daejeon, Republic of Korea

c Plant Pathology Department, Citrus Research and Education Center, University of Florida, Lake Alfred, FL, USA

regions (34 counties)<sup>8</sup> within 4 years of HLB's detection in Florida. The profitability and sustainability of the \$US 9.1 billion Florida citrus industry with an annual farmgate value of \$1.5 billion<sup>9</sup> are threatened if the spread of HLB by its vector is not controlled.

Las, the presumed causal pathogen of HLB, is transmitted from tree to tree by ACP in a circulative (persistent) and propagative manner.<sup>10</sup> Both nymphs (fifth instars) and adults are capable of transmitting the bacterium.<sup>10–12</sup> Adult ACP can acquire Las from HLB-infected trees within a 30 min acquisition access period,<sup>13</sup> followed by a latent period of 7–25 days. A continuous inoculation access period of 5–7 h is required for Las-infected ACP to inoculate trees with the pathogen.<sup>12</sup> The long period required for pathogen transmission provides a window of opportunity for interrupting the successful acquisition and/or transmission of bacteria. Although broad-spectrum insecticides effectively reduce populations of ACP, the incidence of HLB continues to increase even under low population densities.<sup>14</sup> Therefore, preventing feeding of the vector with antifeedants may be a reasonable approach as part of an effective integrated pest management program.<sup>15</sup>

Pymetrozine is designated as a reduced-risk chemical by the USEPA<sup>16</sup> that selectively inhibits the feeding of plant-sap-sucking Hemipteran and Homopteran insects including aphids, whiteflies and planthoppers.<sup>17,18</sup> Pymetrozine irreversibly inhibits aphid feeding by blocking stylet penetration into the plant tissue<sup>19</sup> and causes death by starvation.<sup>20</sup> Pymetrozine is both systemic and translaminar, making it highly mobile within plants,<sup>21,22</sup> and has been shown not to affect natural enemies.<sup>23</sup> Pymetrozine reduced whitefly populations in sweet pepper and cotton<sup>24,25</sup> and induced movement of Colorado potato beetle, *Leptinotarsa decemlineata* Say, larvae away from treated plots, suggesting a possible repellent effect.<sup>26</sup> Pymetrozine also significantly reduced acquisition and/or transmission of various plant viruses such as *cauliflower mosaic virus*,<sup>27</sup> *potato virus Y* and *potato leaf roll virus*<sup>28,29</sup> by aphids and *tomato yellow leaf curl virus* by whiteflies.<sup>30</sup> However, it did not prevent transmission of the bacterial pathogen *Xylella fastidiosa* Wells *et al.* by glassy-winger sharpshooter, *Homalodisca vitripennis* (Germer).<sup>14</sup> Based on the above studies, pymetrozine is a good candidate for testing as an antifeedant against ACP. The objectives of the present study were to evaluate the effect of pymetrozine on ACP (1) feeding site selection, (2) feeding behavior, (3) nymph and adult survival and (4) acquisition and transmission of the Las bacterium.

## 2 MATERIALS AND METHODS

### 2.1 Insects and insecticides

Insects were obtained from a greenhouse colony maintained at the Citrus Research and Education Center, Lake Alfred, FL.<sup>31</sup> Pymetrozine 500 g kg<sup>-1</sup> WG (Fulfill 50WG) was provided by Syngenta Crop Protection Inc. (Greensboro, NC), while technical-grade pymetrozine was purchased from Chem Service (Westchester, PA). As pymetrozine has not been labeled for ACP control in citrus, it was tested at 6.5, 13, 26, 52 and 104 µg mL<sup>-1</sup>, equivalent to 0.12, 0.25, 0.5, 1 and 2× the label rate for use against aphid species in fruit crops such as pecan (57 g AI acre<sup>-1</sup>) at a spray volume of 946 L acre<sup>-1</sup>.

### 2.2 Feeding choice tests

The objective of this experiment was to determine whether pymetrozine affects feeding site selection of ACP adults. The pymetrozine was tested at 52 µg mL<sup>-1</sup>. Potted citrus plants

[Swingle citrumelo (*X Citroncirus webberi* Ingram and Moore), 20–30 cm in height] were sprayed with a handheld bottle atomizer (The Bottle Crew, West Bloomfield, MI) until runoff, with either an aqueous dispersion of pymetrozine WG (52 µg AI mL<sup>-1</sup>) + 0.01% Triton X-100 or tap water + 0.01% Triton X-100 (control). Plants were allowed to air dry under shade for 1–2 h. Air-dried plants were transferred into Plexiglas cages (40 × 40 × 40 cm) with sleeves. Each cage (five cages for each gender) contained one treated and one control plant at opposite corners separated by 40 cm. Approximately 150 ACP adults of the same gender but of mixed age were released into each cage. The numbers of adults present on each plant, irrespective of their location and duration of feeding on the plant, were recorded 1, 24, 48 and 72 h after release. The experiment was repeated twice on different dates.

### 2.3 Electrical penetration graph (EPG) recordings

The objective of this experiment was to determine whether pymetrozine applied topically to ACP or by spray to citrus plants affects ACP feeding behavior. ACP adults (1–5 days old) of mixed gender (ten per treatment) were treated topically with a 0.2 µL droplet of technical-grade pymetrozine dissolved in acetone at 0, 100 or 1000 µg mL<sup>-1</sup>. Ten minutes after treatment, ACP were tethered to a gold wire (18.5 µm diameter) (EPG Systems, Wageningen, The Netherlands) connected to their dorsum with conducting silver glue (Ladd Research Industries, Burlington, VT). Normal-looking (active) ACP were then placed on the upper surface (adaxial) of first or second completely expanded uppermost trifoliate of an untreated potted citrus plant [Swingle citrumelo (*X Citroncirus webberi*), 15–20 cm in height] and connected to the probes using 2–3 cm copper wire (gold wires connected to copper wire using conducting silver glue) of a DC amplifier with an input resistance of 1 GΩ (GIGA-8 DC EPG Amplifier; EPG Systems, Wageningen, The Netherlands). The ground electrode was placed into the soil near the plant stem. Feeding behavior of treated and control ACP was monitored by voltage fluctuations which were amplified 50× using an eight-channel DC amplifier (GIGA-8 DC EPG Amplifier) and displayed and recorded onto a computer hard disk through an A/D conversion (Model DI170-UL; DATAQ Instruments, Inc., Akron, OH) for further analysis.<sup>32</sup> The entire experimental set-up was housed within a Faraday cage. At each concentration, recordings were made on at least 5 ACP ( $n = \geq 5$ ). EPG recordings typically lasted for at least 7 h. Similar recordings were taken with untreated ACP on pymetrozine-treated (52 and 104 µg mL<sup>-1</sup>) and control citrus plants ( $n = 6$ ) 1 and 5 days after application for at least 6 h. The determination of penetration and non-penetration behavior activities was based on EPG waveforms described for aphid<sup>32</sup> and ACP<sup>33</sup>, aided by direct visual observations of ACP activities on the leaf.

### 2.4 Antifeedant effect of pymetrozine, measured by honeydew excretion

The objective of these experiments was to determine whether pymetrozine inhibits feeding of ACP adults by quantifying honeydew excretion of ACP exposed to leaves treated either by leaf dip (contact) or systemically. Systemic application was similar to that described previously,<sup>31</sup> with a few modifications. In brief, petioles of freshly collected citrus leaves were immersed in aqueous pymetrozine WG dispersions at various concentrations (6.5, 13, 26, 52 and 104 µg AI mL<sup>-1</sup>) or tap water (control) in 30 mL glass vials for 48 h. After 48 h, 60 mm diameter leaf discs were cut from soaked leaves. For the leaf-dip method, 60 mm diameter leaf

discs were excised from freshly collected citrus leaves and dipped in aqueous solutions of pymetrozine described above or tap water (control) for 30 s. Treated leaf discs (systemic or contact) were placed individually on 1.5% agar beds in 60 mm plastic disposable petri dishes. Thereafter, ten adult ACP of mixed age and sex were released into each dish, which were sealed with lids lined with 60 mm Whatman filter paper (Whatman International Ltd, Kent, UK). Petri dishes were placed upside down to collect honeydew droplets onto the filter paper. Dishes were maintained at  $25 \pm 1^\circ\text{C}$ ,  $50 \pm 5\%$  RH and 14:10 h light:dark photoperiod in an insectary for 24 h. Filter papers were collected and replaced at 12 h intervals. The adult mortality in these experiments was very low (5% at the highest concentration); therefore, data from both healthy and dead/dying ACP were included in the analysis. Each concentration was replicated 3 times, and the entire experiment was repeated twice on different dates. Collected filter papers were subjected to a ninhydrin (Sigma-Aldrich, St Louis, MO) test to facilitate counting of honeydew droplets.<sup>31,34</sup>

## 2.5 Effect on nymph and adult survival

The objective of these experiments was to determine the effect of pymetrozine on nymph and adult survival. For nymphs, potted citrus plants [Swingle citrumelo (*X Citroncirus webberi*), ~10 cm in height] with new vegetative growth were placed in a Plexiglass cage with sleeves (40 × 40 × 40 cm). Thereafter, 200 mated females were released for egg laying for 24 h. These adults were subsequently removed, and plants with eggs were maintained for 5 days for hatching. The number of live first instars was counted, and unhatched eggs were removed gently with a camel hair brush. Plants with first instars were sprayed gently with a handheld atomizer until runoff with various concentrations of pymetrozine (as WG dispersed in water) + 0.01% Triton X-100. For each concentration, five plants were sprayed, including five controls treated with tap water + 0.01% Triton X-100. Plants were allowed to air dry under shade for 3 h. Plants with nymphs were covered with perforated 1 L plastic cups. Plants were placed on a bench top in an insectary at  $25 \pm 1^\circ\text{C}$  and  $50 \pm 5\%$  RH and a 14:10 h light:dark photoperiod. Nymph development was monitored to determine adult emergence.

For adults, citrus plants of similar height without new vegetative growth (immediately after pruning) were sprayed with various concentrations of pymetrozine as described above. Sprayed plants were allowed to air dry for 3 h under shade, and ten 2–3-day-old adult ACP of mixed gender were released onto each plant, including controls. Plants were maintained in an insectary at the environmental conditions described above and were counted to determine mortality at 1, 3, 5, 8 and 15 days after treatment.

## 2.6 Effect of pymetrozine on acquisition and transmission of Las bacterium

The objective of these experiments was to determine whether pymetrozine disrupts acquisition or transmission of the Las bacterium by ACP. For acquisition experiments, citrus plants ['Valencia' sweet orange, *Citrus sinensis* (L.) Osbeck, 120–150 cm in height] infected with Las were used (6–8 months after graft inoculation). Inoculation and confirmation of Las presence within these plants was conducted as per standard protocols.<sup>35,36</sup> In brief, eight-month-old seedlings were used for inoculation with Las-infected budwood. Plants were graft inoculated with three pieces of budwood from PCR-positive Las source trees propagated in the greenhouse. Inoculated plants were maintained in a

USDA-APHIS-approved secure greenhouse at  $26\text{--}32^\circ\text{C}$ , and the presence of Las in the plants was confirmed by quantitative real-time polymerase chain reaction (qRT-PCR), as described in Tatineni *et al.*,<sup>35</sup> Folimonova *et al.*<sup>36</sup> and Li *et al.*<sup>37</sup> Four randomly selected branches were sprayed with pymetrozine WG dispersion at either 52 or 104  $\mu\text{g AI mL}^{-1}$  + 0.01% Triton X-100. Similarly, Las-positive and Las-negative control plants of the same variety were sprayed with tap water + 0.01% Triton X-100 only. Sprayed plants were allowed to air dry under shade for 3 h and placed in an insect-proof room maintained under  $25 \pm 1^\circ\text{C}$ ,  $50 \pm 5\%$  RH and 14:10 h light:dark photoperiod. Thereafter, 20 newly emerged Las-negative ACP adults of mixed gender were caged on each branch in 1 L perforated plastic cups for a 7 day acquisition access period (AAP). Following the 7 day AAP, all live adults from each treatment were collected and transferred onto 3–4-month-old untreated citrus plants [Swingle citrumelo (*X Citroncirus webberi*)] within Plexiglass cages with sleeves for 25 days. The experiment was repeated twice on separate dates. All live ACP were freeze killed and transferred, in addition to dead ones (which were collected within 12 h of mortality), individually into sterile 1.5 mL centrifuge tubes containing 80% ethanol and stored at  $-20^\circ\text{C}$  until DNA extraction.

For transmission experiments, 2–3-month-old citrus plants [Swingle citrumelo (*X Citroncirus webberi*), 15–20 cm in height] and adult ACP (10–15 days old) that emerged from nymphs reared on Las-infected plants, resulting in ~60% infection, were used. Ten ACP adults were released onto Las-negative plants treated with pymetrozine at either 52 or 104  $\mu\text{g mL}^{-1}$  + 0.01% Triton X-100 or tap water + 0.01% Triton X-100 only (positive control) and allowed to feed for a 7 day inoculation access period (IAP). All plants were maintained in Plexiglass cages as described above. Similarly, ACP adults emerging from nymphs reared on Las-negative plants were released onto Las-negative plants treated with tap water + 0.01% Triton X-100 only (negative control). Following the 7 day IAP, all living ACP were collected, freeze killed and placed individually into sterile 1.5 mL centrifuge tubes containing 80% ethanol and stored at  $-20^\circ\text{C}$  until DNA extraction. Also, all dead ACP were collected daily and stored in alcohol throughout the experiment. Each treatment was replicated 10 times (ten plants), and the entire experiment was repeated twice. All plants from the transmission experiments were maintained in Plexiglass cages under the conditions described above for 6 months to allow for disease development. The variation in the percentage of Las-positive ACP collected from treatment and positive control plants was 30–80%. Only plants on which an equal number of confirmed Las-positive ACP were observed feeding were retained for disease development. A total of 88 plants from treatment and positive control had this correlation, and 80 plants were retained for disease development.

## 2.7 Total DNA extractions and qRT-PCR

Total DNA from individual ACP samples (both acquisition and transmission experiments) was extracted following standard protocols.<sup>37</sup> In brief, DNA from individual ACP was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions and modified to suit extraction of DNA from insects. PCR-grade DNA was eluted in a final volume of 35  $\mu\text{L}$  AE buffer and stored in sterile 1.5 mL centrifuge tubes at  $-20^\circ\text{C}$  until use in RT-PCR. Similarly, total DNA from individual plants was extracted from 100 mg of plant tissue obtained from midribs and petioles of a representative sample (five leaves) for both treatment and controls (positive and negative) following



standard protocols<sup>37</sup> using DNeasy Plant Kit (Qiagen, Valencia, CA) as per the manufacturer's instructions. PCR-grade DNA was eluted in a final volume of 50 µL AE buffer and stored at −20 °C in sterile 1.5 mL centrifuge tubes until use in RT-PCR.

RT-PCR assays of insect and plant samples were performed in an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA) using a TaqMan assay developed for the detection of Las.<sup>37</sup> The total volume of the reaction mixture was 26 µL, consisting of 1 µL of template DNA (plant or insect), 13 µL of TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 3 µL (231 nM) of Las 16S rDNA-specific primers (LasF: 5'-TCGAGCGCGTATGCAATACG-3'; LasR: 5'-GCGTTATCCCGTAGAAAAAGGTAG-3'), for amplifying a portion of 16S rDNA, and 3 µL (115 nM) of Las-specific probe (FAM 5'-AGACGGGTGAGTAACGCG-3' BHQ1). In addition, the insect PCR reaction mixture included 3 µL (231 nM) of wingless (*wg*) gene-specific primers (*wgF*: 5'-CTCTCAAAGATCGGTTGACGG-3'; *wgR*: 5'-GCTGCCACGAACGTTACCTTC-3') and 3 µL (115 nM) of *wg* probe (*wgP*: JOE 5'-TTACTGACCATCACTCTGGACGC-3' BHQ2) as an internal control, while the plant PCR reaction mixture included 3 µL (231 nM) of cytochrome oxidase (*cox*) gene-specific primers (*coxF*: 5'-GTATGCCACGTGCGATTCAG-3'; *coxR*: 5'-GCCAAAAGTCTAAGGGCATTG-3') and 3 µL (115 nM) of COX probe (*coxP*: JOE 5'-ATCCAGATGCTTACGCTGG-3' BHQ2) as an internal control (Integrated DNA Technologies, Inc., Coralville, IA).

For each DNA sample, PCR reactions were conducted in duplicate in 96-well MicroAmp<sup>®</sup> reaction plates (Applied Biosystems, Foster City, CA) containing three controls: no template control (DNA volume replaced with buffer), a positive control (DNA from ACP or plant with Las DNA) and a negative control (DNA from ACP or plant without Las DNA). Target DNA was amplified under PCR conditions of 95 °C for 10 min, then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The percentage of Las-infected ACP was determined in treatments as well as in controls (positive and negative) for acquisition and transmission experiments. The Ct values used to differentiate between Las-positive and Las-negative ACP in acquisition and transmission experiments were 16.66 and 25.8 respectively. Similarly, the percentage of Las-infected plants was determined for treatments and controls in the transmission experiment. The Ct value used to differentiate between Las-positive and Las-negative citrus plants in the transmission experiment was 24.71.

## 2.8 Statistical analyses

The percentage data were arcsine square root transformed before analysis to meet assumptions of normality and homogeneity of variances. Significant differences in numbers of ACP adults feeding on treatment and control plants in feeding site selection experiments were determined by  $\chi^2$  analysis (PROC FREQ<sup>38</sup>). Significant differences in the duration of time (expressed as percentage) ACP spent performing various feeding behaviors (non-penetration, pathway and ingestion) between treatments (treated ACP and treated plants) and controls for EPG recordings were determined by *t*-tests (PROC TTEST<sup>38</sup>). Significant differences in the number of honeydew droplets excreted between treatments were determined by two-way analysis of variance (ANOVA) (concentration and method of treatment as factors) (PROC GLM<sup>38</sup>). Similarly, significant differences in adult and nymph survival between treatments were determined by two-way ANOVA (concentration and time as factors) and one-way ANOVA respectively. Least significant difference (LSD) tests were used for mean separation. Significant differences in the percentage

of ACP acquiring Las between the pymetrozine treatment and positive control were determined by *t*-tests. Similarly, significant differences in the percentage of plants infected by Las between treatment and positive control plants were determined by *t*-tests. All mean separation tests were performed at  $\alpha = 0.05$ .

## 3 RESULTS

### 3.1 Feeding choice tests

Significantly fewer female ACP adults were present on pymetrozine-treated plants than on control plants 24 and 48 h after release during both experiments (Table 1) ( $\chi^2 = 4.72$ –42.84; df = 1;  $P = <0.0001$ –0.02). In no other case did pymetrozine treatment significantly affect host plant acceptance of ACP compared with the control (Table 1) ( $\chi^2 = 0.007$ –2.79; df = 1;  $P = 0.09$ –0.93).

### 3.2 EPG recordings

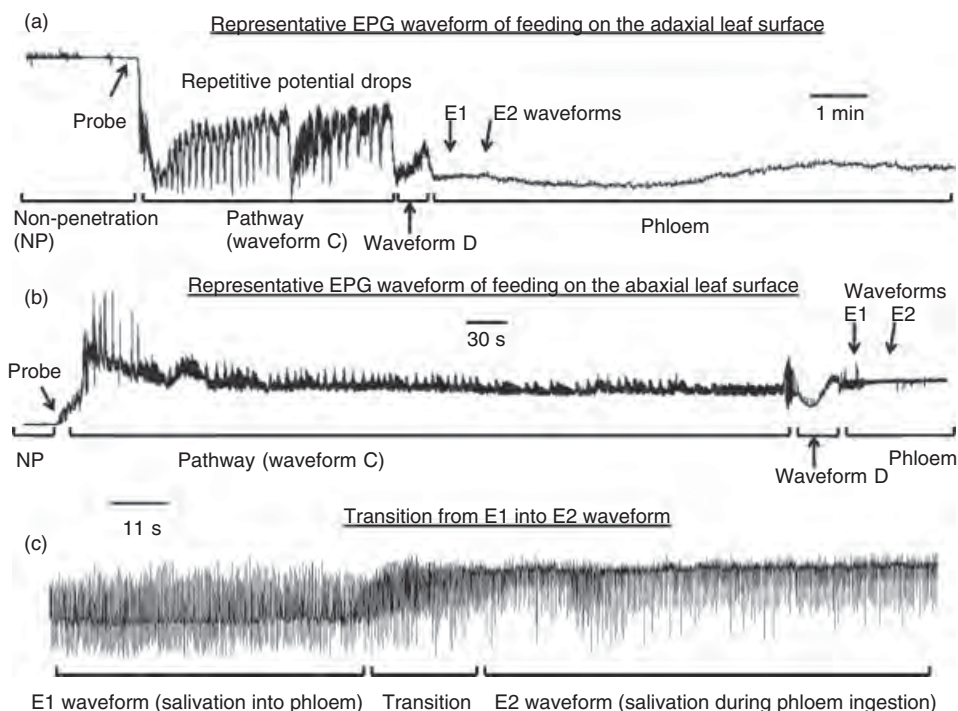
Representative EPG recordings of ACP feeding on citrus plants are presented in Figs 1 and 2. Among the waveforms recorded, waveform C (stylet pathway activity through epidermis and parenchyma) (Figs 1a and b), waveform E1 (salivation into phloem, which is typical for phloem-feeding insects) (Figs 1a and b), waveform E2 (salivation during phloem ingestion) (Figs 1a and b) and waveform G (probably feeding from xylem) (Fig. 2a) were observed, as well as waveform D (stylet first contact with phloem and rupture of phloem cell wall), which is typically observed in ACP (Figs 1a and b). However, both negative and positive in voltage waveforms were recorded (Figs 1a and b), depending on the leaf surface on which ACP fed, indicating intracellular and intercellular stylet probing during pathway activity through epidermis and parenchyma cells respectively. The intracellular

**Table 1.** Feeding site selection by ACP adults when presented with pymetrozine-treated (52 µg mL<sup>−1</sup>) or control plants

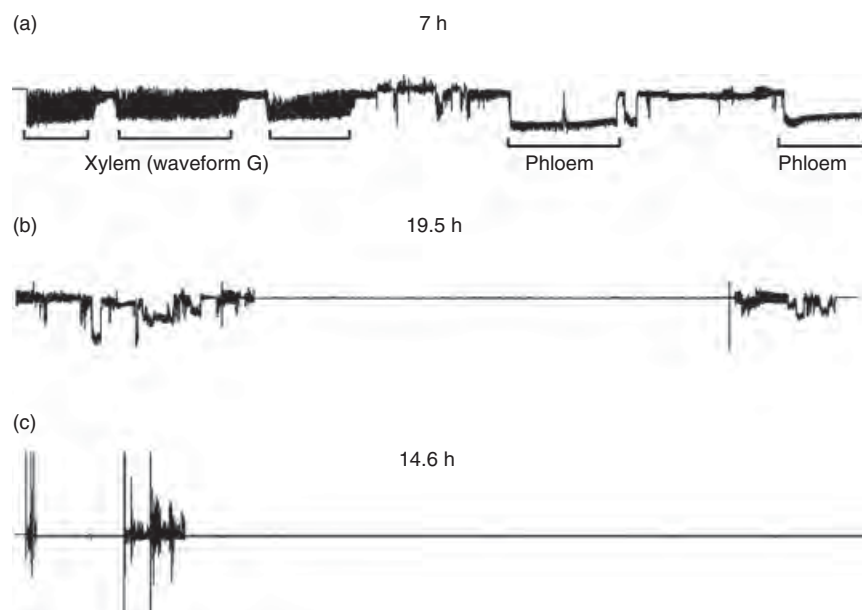
Observation time (h)	ACP gender	Experiment	n	Number of adults on		
				control plants	treated plants <sup>a</sup>	cage walls and pot soil <sup>b</sup>
1	Male	1	150	18	22	110 (0)
		2	150	22	29	99 (0)
1	Female	1	150	26	20	104 (0)
		2	150	24	25	101 (0)
24	Male	1	150	63	57	30 (0)
		2	150	76	61	13 (0)
24	Female	1	150	104	30*	15 (1)
		2	150	73	49*	26 (2)
48	Male	1	150	79	69	2 (0)
		2	150	74	60	15 (1)
48	Female	1	150	110	32*	6 (2)
		2	150	83	55*	10 (2)
72	Male	1	150	54	67	27 (2)
		2	150	60	56	20 (4)
72	Female	1	150	69	70	9 (2)
		2	150	74	55	19 (2)

<sup>a</sup> \* Indicates significant difference from the respective control at a given observation time according to  $\chi^2$  analysis.

<sup>b</sup> The numbers in parentheses indicate the number of dead ACP adults found during the experiment.



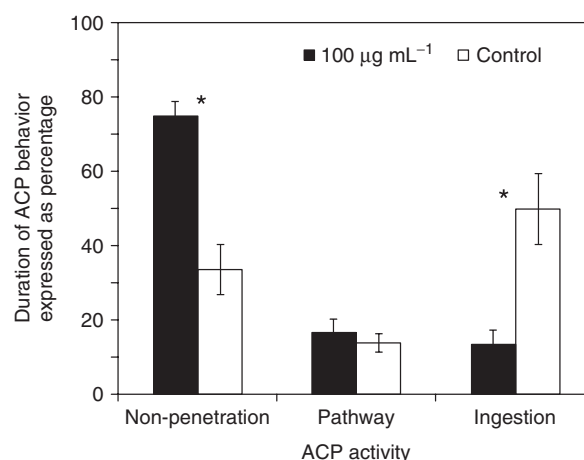
**Figure 1.** Representative EPG recordings showing waveform patterns produced when ACP adults feed on citrus plants. (a) The EPG waveform showing negative voltage reflecting intracellular probing activities. (b) The EPG waveform showing positive voltage reflecting intercellular probing activities. 'Non-penetration' indicates stylets have not penetrated plant tissue. 'Pathway' indicates probing activities in epidermis, mesophyll or parenchyma cells. 'Phloem' indicates sap ingestion from phloem sieve elements with short periods of salivation. The phloem ingestion phase has both E1 (salivation into sieve element) and E2 (salivation during sieve element ingestion) waveforms. (c) Magnified view of transition of E1 waveform into E2 waveform.



**Figure 2.** (a) Representative EPG recordings from ACP adults ( $n = 5$ ) treated topically with 0.2  $\mu\text{L}$  of acetone containing (a) 0, (b) 100 or (c) 1000  $\mu\text{g mL}^{-1}$  of pymetrozine, feeding on untreated citrus plants. 'Xylem' and 'phloem' indicate sap ingestion from xylem and phloem sieve elements respectively.

stylet probing waveforms were mainly observed when ACP were feeding on the adaxial leaf surface (Fig. 1a), whereas ACP feeding from the abaxial leaf surface resulted in intercellular stylet probing waveforms (Fig. 1b). Repetitive potential drops were also observed when ACP were feeding from the adaxial leaf surface (Fig. 1a). The typical EPG waveforms described above were observed from

both control and treated ACP feeding. ACP treated topically with 100  $\mu\text{g mL}^{-1}$  of pymetrozine spent significantly less time performing ingestion behavior activities (waveforms E1, E2 and G) and more time performing non-penetration behavior activities (searching and walking) than control ACP (Figs 2a and b and Fig. 3) ( $t = 4.18$  and  $6.16$ ;  $df = 4$ ;  $P = 0.01$  and  $0.003$ ). ACP



**Figure 3.** Duration (expressed as percentage) that ACP adults spent performing various feeding behaviors after topical treatment with 100 µg mL<sup>-1</sup> of pymetrozine or acetone alone during 7 h feeding period. Each bar represents mean ± SEM ( $n = 5$ ). Bars labeled with an asterisk within a given activity are significantly different from one another according to  $t$ -tests ( $P < 0.05$ ).

treated topically with 1000 µg mL<sup>-1</sup> of pymetrozine did not make any probing attempts, made uncoordinated movements with leg and wing stretching and died within 3 h of treatment (Fig. 2c). Similarly to observations with topical application, ACP spent significantly less time performing ingestion behavior activities and more time performing non-penetration behavior activities on treated plants than on untreated plants at both concentrations (52 and 104 µg mL<sup>-1</sup>) 1 day after treatment ( $t = 2.48$ – $2.90$ ;  $df = 5$ ;  $P = 0.03$ – $0.05$ ) but not 5 days after treatment (Table 2) ( $t = 0.67$ – $1.42$ ;  $df = 5$ ;  $P = 0.21$ – $0.53$ ). However, there was no significant difference in the amount of time spent in stylet pathway activity (waveform C) between treatments and controls after topical application to ACP ( $t = 0.38$ ;  $df = 4$ ;  $P = 0.72$ ) or spray application to plants 1 and 5 days after treatment (Table 2 and Fig. 3) ( $t = 0.13$ – $1.59$ ;  $df = 5$ ;  $P = 0.17$ – $0.90$ ).

### 3.3 Effect on adult and nymph survival

Two-way ANOVA indicated a highly significant effect of concentration ( $F = 25.48$ ;  $df = 5$ ;  $P < 0.0001$ ) and time (days) ( $F = 28.73$ ;  $df = 4$ ;  $P < 0.0001$ ) on adult survival, but no significant interaction between main effects ( $F = 0.78$ ;  $df = 20$ ;  $P = 0.73$ ). In general, percentage survival of adults on treated plants decreased over time (Fig. 4). One day after treatment, the 104 µg mL<sup>-1</sup> treatment significantly decreased (6%) adult survival compared with the control and 6.5 µg mL<sup>-1</sup> treatment ( $F = 2.41$ ;  $df = 5$ ;  $P = 0.02$ ). Three days after treatment, adult survival decreased by 9 and 13% compared with the control for the 26 and 104 µg mL<sup>-1</sup> treatments respectively, which were significant reductions compared with the control and 6.5 µg mL<sup>-1</sup> treatment (Fig. 4) ( $F = 3.05$ ;  $df = 5$ ;  $P = 0.01$ ). At 5, 8 and 15 days after treatment, adult survival decreased further in the 26 µg mL<sup>-1</sup> (14–23%), 52 µg mL<sup>-1</sup> (15–25%) and 104 µg mL<sup>-1</sup> (16–22%) treatments compared with the control; these were significant reductions compared with the 6.5 µg mL<sup>-1</sup>, 13 µg mL<sup>-1</sup> and control treatments (Fig. 4) ( $F = 7.10$ – $9.36$ ;  $df = 5$ ;  $P < 0.0001$ ). Pymetrozine exhibited greater activity against nymphs compared with adults. Significantly fewer nymphs survived into adults at the 26 µg mL<sup>-1</sup> (13%), 52 µg mL<sup>-1</sup> (35%) and 104 µg mL<sup>-1</sup> (38%) treatments compared with the control, which were significant reductions compared with the 13 µg mL<sup>-1</sup> treatment and control (Fig. 5) ( $F = 24.11$ ;  $df = 4$ ;  $P < 0.0001$ ).

**Table 2.** Mean (± SEM) duration (expressed as percentage) that ACP adults spent performing various feeding behaviors on pymetrozine-treated (52 or 104 µg mL<sup>-1</sup>) or control plants during the EPG recording period<sup>a</sup>

DAT <sup>a</sup>	Concentration (µg mL <sup>-1</sup> )	Non-penetration <sup>b</sup>	Pathway	Ingestion <sup>b,c</sup>
1	0	24.88 (±4.09)	7.17 (±0.70)	68.56 (±6.98)
	52	44.25 (±6.38)*	9.75 (±2.63)	45.36 (±3.93)*
5	0	22.81 (±6.16)	6.65 (±0.68)	70.53 (±5.91)
	52	32.61 (±10.18)	10.55 (±2.24)	57.22 (±10.96)
1	0	27.87 (±4.35)	9.58 (±1.21)	62.54 (±5.23)
	104	49.10 (±6.70)*	10.97 (±3.77)	39.92 (±6.26)*
5	0	19.07 (±3.23)	9.46 (±1.49)	71.45 (±3.66)
	104	16.13 (±5.08)	7.94 (±1.30)	75.92 (±5.06)

<sup>a</sup> Days after treatment.

<sup>b</sup> \* Indicates significant difference from the respective control at a given DAT and observation.

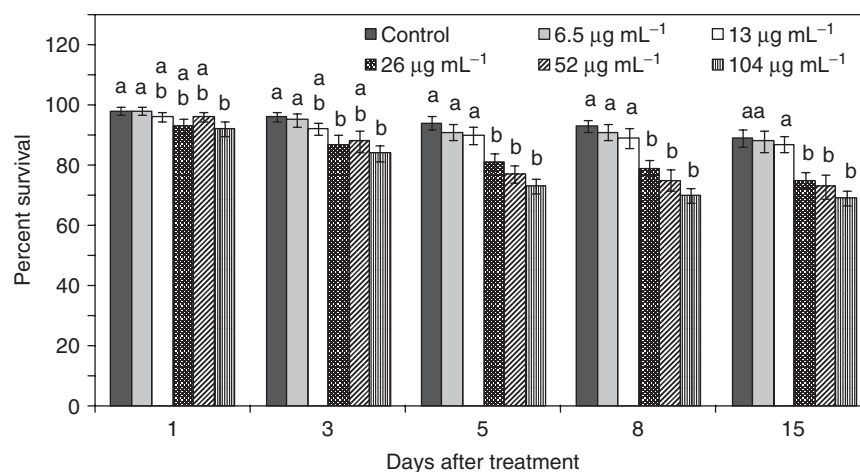
<sup>c</sup> Includes feeding from xylem and phloem.

### 3.4 Antifeedant effect as measured by honeydew excretion

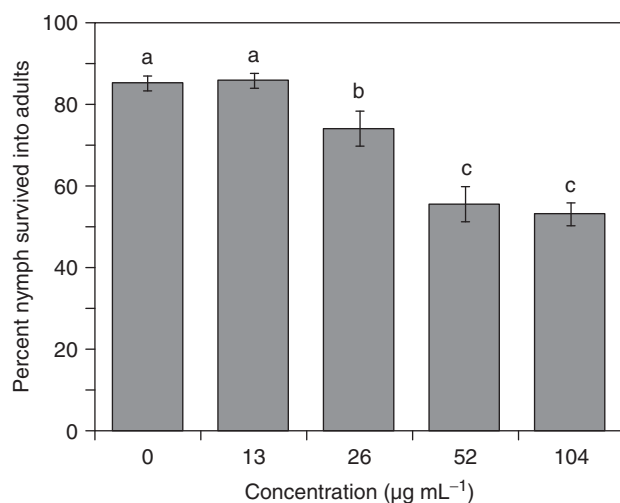
Two-way ANOVA indicated a significant effect of method of treatment ( $F = 4.10$ ;  $df = 1$ ;  $P = 0.04$ ), a highly significant effect of concentration ( $F = 181.36$ ;  $df = 5$ ;  $P < 0.0001$ ) and a significant interaction between main effects ( $F = 2.32$ ;  $df = 5$ ;  $P = 0.04$ ). The number of honeydew droplets excreted decreased as the concentration of pymetrozine was increased for both contact and systemic application experiments. Each concentration significantly decreased honeydew droplet excretion compared with the control, and significant differences existed between any two concentrations being compared within a method of treatment (Fig. 6) ( $F = 48.67$  and  $40.40$ ;  $df = 4$ ;  $P < 0.0001$ ). However, no significant difference existed between methods of treatment at a given concentration except at 104 µg mL<sup>-1</sup> ( $t = 0.37$ – $3.20$ ;  $df = 9$ ;  $P = 0.01$ – $0.72$ ).

### 3.5 Effect of pymetrozine on acquisition and transmission of Las

There were 12 and 21% reductions in acquisition of Las by ACP feeding on Las-infected plants treated with pymetrozine at 52 and



**Figure 4.** Effect of pymetrozine on the survival of ACP adults on citrus plants treated at various concentrations. Each bar represents mean  $\pm$  SEM ( $n = 10$ ). Bars not labeled by the same letter at a given day are significantly different from one another according to LSD ( $P < 0.05$ ).

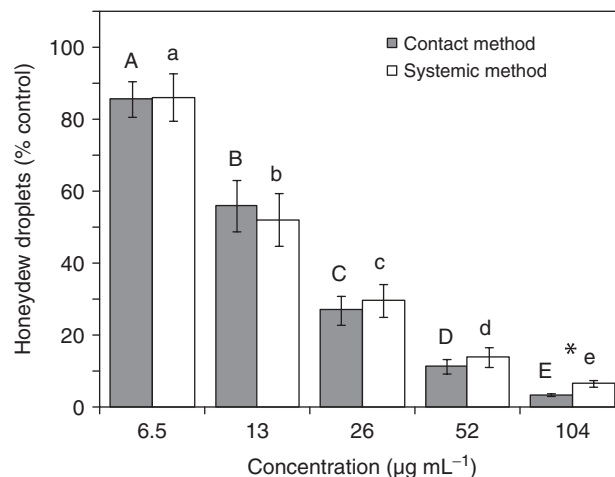


**Figure 5.** Effect of pymetrozine on survival of ACP nymphs into adults on citrus plants treated with pymetrozine at various concentrations. Each bar represents mean  $\pm$  SEM ( $n = 10$ ). Bars not labeled by the same letter are significantly different from one another according to LSD ( $P < 0.05$ ).

104  $\mu\text{g mL}^{-1}$ , respectively, compared with the control (Fig. 7a). Similarly, there were 11 and 18% reductions in transmission of Las and subsequent disease development by Las-infected ACP feeding on plants treated with pymetrozine at 52 and 104  $\mu\text{g mL}^{-1}$ , respectively, compared with the control (Fig. 7b). However, these were not statistically significant reductions in Las acquisition and transmission by ACP compared with positive controls (Figs 7a and b) ( $t = 0.39\text{--}1.00$ ;  $\text{df} = 1\text{--}7$ ;  $P = 0.37\text{--}0.70$ ).

## 4 DISCUSSION

Pymetrozine is the first compound of a new class of insecticides known as the pyridine azomethines. It is mostly active against plant-sap-sucking insect pests of field, fruit and ornamental crops.<sup>39</sup> Although ACP preferentially alighted on control plants rather than on those treated with pymetrozine at 52  $\mu\text{g mL}^{-1}$ , the significant repellent effect exhibited on females was after 24 and 48 h of exposure. Moreover, although pymetrozine is a known

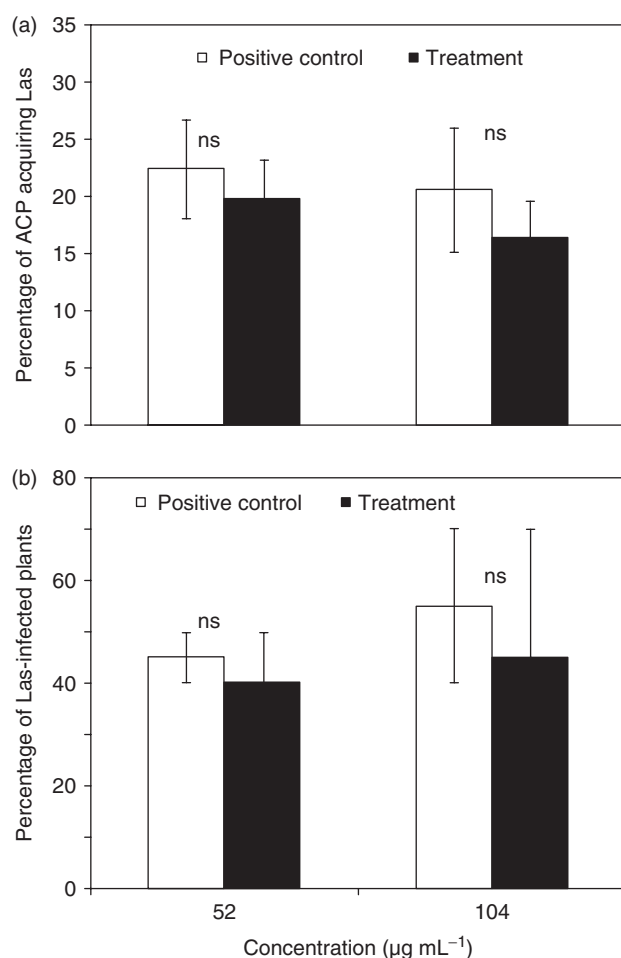


**Figure 6.** Effect of pymetrozine on ACP adult feeding on citrus leaf discs treated by contact or systemic method and confined to petri dishes at various concentrations. Each bar represents mean  $\pm$  SEM ( $n = 10$ ). Bars not labeled by the same upper-case letter are significantly different from one another according to LSD ( $P < 0.05$ ) for the contact method. Bars not labeled by the same lower-case letter are significantly different from one another according to LSD ( $P < 0.05$ ) for the systemic method. Bars labeled by an asterisk at a given concentration are significantly different from one another according to  $t$ -tests ( $P < 0.05$ ).

repellent of certain insects,<sup>26</sup> the present results indicate that it is not a potent repellent for ACP.

The reported mode of action of pymetrozine for aphids is an immediate cessation of feeding by preventing stylet penetration.<sup>19</sup> ACP adults treated topically with 1000  $\mu\text{g mL}^{-1}$  of pymetrozine (0.2  $\mu\text{g AI adult}^{-1}$ ) were unable to probe, made uncoordinated movements with wing and leg stretching and died within 3 h of treatment. Injecting the locust, *Locusta migratoria* L., with pymetrozine caused similar toxic symptoms including increased activity and extension of legs and wings without associated paralysis or knockdown.<sup>40</sup> It is thought that the serotonin (5-HT)-mimicking effects of pymetrozine on 5-HT receptors inhibit feeding for a prolonged period.<sup>40</sup> On the other hand, leg and wing stretching effects could be the result of pymetrozine action on femoral and wing hinge stretch receptor chordotonal organs.<sup>41</sup> In the present study, pymetrozine applied at 100  $\mu\text{g mL}^{-1}$  affected





**Figure 7.** Effect of pymetrozine at 52 and 104 µg mL<sup>-1</sup> on ACP (a) acquisition and (b) transmission of Las. Each bar represents mean ± SEM ( $n = 122$ – $156$  for acquisition experiments,  $n = 20$  for transmission experiments). Bars labeled by 'ns' are not significantly different from one another according to  $t$ -tests ( $P < 0.05$ ). Negative controls had zero Las-infected ACP or plants.

the probing behavior of ACP, which may indicate an effect on stylet penetration. However, this effect was short lived and completely subsided by 5 days after treatment. Pymetrozine has a half-life of ~3 days within tomato plants, and resulting metabolites are biologically inactive or highly unstable.<sup>22</sup> Furthermore, foliar applications of pymetrozine result in uneven distribution within leaf tissues.<sup>22</sup> The above reports are congruent with the present findings indicating a short residual activity of pymetrozine against ACP.

During EPG waveform recordings of both control and treated ACP, both intercellular and intracellular stylet pathway activities were observed. The majority of EPG waveform recordings from ACP feeding on the adaxial leaf surface suggested intracellular stylet pathway activity (negative in voltage). On the other hand, the majority of EPG recordings from ACP feeding on the abaxial leaf surface suggested intercellular stylet pathway activity (positive in voltage). This indicates that ACP can perform both intracellular and intercellular probing activities, and the most prevalent type of pathway activity may depend on the leaf surface (abaxial versus adaxial). Bonani *et al.*<sup>33</sup> reported EPG waveform recordings from female ACP that were only positive in voltage, suggesting intercellular pathway activities. The present EPG recordings

from ACP feeding on the adaxial leaf surface also revealed repetitive potential drop waveforms during stylet pathway activity, indicating puncturing of epidermal and/or parenchyma cells which was not observed in EPG waveform recordings of ACP feeding on the abaxial leaf surface in this study or that of Bonani *et al.*<sup>33</sup> The differences in ACP adults' age and sex (mixed sex versus females), feeding leaf surface (adaxial versus abaxial) and/or citrus variety [Swingle citrumelo (*X Citroncirus webberi*) versus sweet orange] may explain to some extent the observed differences between the present study and that of Bonani *et al.*<sup>33</sup> Although different stylet pathway activities were observed by the present authors, depending on the leaf surface on which ACP were feeding, further histological studies at ACP feeding sites will confirm the present findings.

Treatment of citrus plants with pymetrozine reduced the survival of feeding ACP in a time- and concentration-dependent manner. Survival of ACP continued to decrease on pymetrozine-treated plants up to 15 days after treatment. Survival was impacted most for the first 8 days after treatment, which corroborated the present EPG experiments indicating a short-lived antifeedant effect. Even though the treatment was active for only 5 days, it is possible that death might have occurred and continued up to 15 days owing to starvation. Approximately 50% of adult ACP died within 48 h when they were completely deprived of food.<sup>31</sup> ACP nymphs appeared to be more susceptible to pymetrozine treatment than adults. The limited movement capabilities of nymphs compared with adults may impact upon feeding site selection, rendering immature stages more susceptible to pymetrozine than adults. Furthermore, it is possible that mobile adults are more capable of finding and selecting plant parts with lower concentrations of active ingredients than nymphs. The antifeedant effect of pymetrozine appeared to be greater in the present honeydew quantification experiment than in host choice and EPG experiments. The likely greater exposure to pymetrozine in petri dishes than on treated plants may have accounted for this difference.<sup>21</sup> Furthermore, the observed higher decrease in honeydew excretion at the higher concentrations could be the result of inclusion of dead/dying ACP in the analysis.

Pymetrozine treatment did not prevent acquisition of Las by ACP from infected plants or transmission of Las by bacterialiferous ACP into healthy plants. These results are congruent with those obtained with *H. vitripennis*, where pymetrozine negatively affected feeding behavior but did not prevent the transmission of *X. fastidiosa* by bacterialiferous *H. vitripennis*.<sup>14</sup> However, the present findings are in contrast to those obtained with insect vectors of plant viruses. Pymetrozine is known to reduce transmission of tomato yellow leaf curl virus by viruliferous whiteflies, *Bemisia tabaci* Genn.,<sup>30</sup> and cauliflower mosaic virus transmission by aphids.<sup>27</sup> Given that pymetrozine treatment affected feeding behavior of ACP for only up to 5 days, it is not surprising that transmission of Las by bacterialiferous ACP was not affected.

## 5 CONCLUSIONS

The treatment of citrus foliage with pymetrozine at 52 and 100 µg mL<sup>-1</sup> disrupted feeding by ACP; however, the short residual activity of this treatment did not significantly affect acquisition or transmission of the Las pathogen presumably responsible for HLB. Although the use of this chemical as a stand-alone tool for ACP and HLB management will not be effective, it may be a useful component of an integrated management



program for ACP. Further field-based evaluation of the 52 and 104  $\mu\text{g mL}^{-1}$  rates is necessary to determine whether integration of pymetrozine with the use of broad-spectrum pesticides may have a positive impact on ACP and HLB management. Pymetrozine is known to have less impact on beneficial insects,<sup>23,42</sup> and thus its use may benefit biological control.

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