Research Article

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Effects of methoprene, a juvenile hormone analog, on survival of various developmental stages, adult emergence, reproduction and behavior of Asian citrus psyllid, *Diaphorina citri* Kuwayama

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Abstract

BACKGROUND: The Asian citrus citrus psyllid, *Diaphorina citri* Kuwayama, transmits a bacterium that causes huanglongbing in citrus. Frequent and repeated use of neurotoxic insecticides against *D. citri* has resulted in the development of insecticide resistance. We evaluated the effects of the juvenile hormone analog methoprene on egg hatch, nymphal development, adult emergence, reproduction and behavior of *D. citri*.

RESULTS: Methoprene significantly reduced the viability of eggs that were between 0 and 4 days old. Egg hatch of 0–48-h-old and 49–96-h-old eggs was 8 and 9%, respectively, when treated with 320 μg mL^{-1} of methoprene. Methoprene caused significant mortality of first-, third- and fifth-instar *D. citri* nymphs and reduced adult emergence as compared with controls. Methoprene caused less than 5% adult emergence when first- and third-instar stages were treated, respectively, and less than 40% adult emergence when fifth instars were treated. Reduced fertility of females was observed when they emerged from methoprene-treated fifth instars.

CONCLUSION: Methoprene was effective in reducing egg hatch, suppressing nymphal development and decreasing adult emergence of *D. citri* under laboratory conditions. Treatment of fifth instars reduced the fertility of females. Methoprene might be a possible tool for integrated management of *D. citri*.

Keywords: insect growth regulator; juvenile hormone analog; Asian citrus psyllid; citrus greening; huanglongbing

1 INTRODUCTION

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is a serious pest of citrus crops worldwide. It transmits a phloem-restricted, gram-negative bacterium, *Candidatus* Liberibacter asiaticus (Las), that causes huanglongbing (HLB), also referred to as citrus greening.[1–3] HLB is a devastating disease that causes heavy leaf drop, dieback of stems, premature fruit drop, out-of-season flushing and blossoming and eventual tree death within 5–10 years of initial infection.[1] The fruits produced on infected trees are small and distorted and have a bitter taste.[4,5] In the United States, *D. citri* was initially reported in June 1998 in Florida, and since then it has been reported in Alabama, Arizona, California, Georgia, Louisiana, Mississippi and Texas.[6] HLB was reported initially in southern Florida in August 2005 and has subsequently spread to all citrus-growing regions of the state. In the past 5 years, HLB has caused approximately $US 3.63 billion in revenue loss and 6611 lost jobs in agriculture and related sectors in Florida.[7]

Adult *D. citri* are small (2.7–3.3 mm in length) with mottled brown wings. There are three adult morphs which differ in abdominal color (gray/brown, blue/green and orange/yellow)[8]. *D. citri* females are very prolific and can lay approximately 800 eggs in their lifetime on young foliar growth. Upon hatching, the nymphs undergo five nymphal instars to emerge as adults. The life cycle duration ranges from 15 to 47 days, depending upon temperature regime. The nymphs and adults feed on plant sap and excrete honeydew rich in sugars that leads to growth of sooty mold on leaves, which can affect photosynthesis. The pathogen can be acquired by second-, third-, fourth- and fifth-instar nymphs, as well as adults, during feeding on infected trees.[9] However, the pathogen can only be transmitted by fourth- and fifth-instar nymphs and adult *D. citri*.[1,6,10,11] Infected adults play a major role in the spread of the pathogen because of their dispersal capabilities. Once infected, adults remain infective throughout their lives.[9,11] The pathogen multiplies within *D. citri* and has been reported in hemolymph and salivary glands of adults.[9,11–13] Acquisition of Las can also

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occur transovarially from infected females to eggs, but at a low frequency.[14]

Currently, the spread of HLB is managed by removing infected trees, applying fertilizers to maintain tree health and suppressing populations of *D. citri*.[7] Multiple applications of a limited number of insecticides of various modes of action are made to reduce *D. citri* populations during each growing season. Repeated use of limited available insecticides has resulted in the development of insecticide resistance in some *D. citri* populations.[15] There is an immediate need for additional insecticides with novel modes of action for incorporation into integrated management programs for *D. citri*. These additional insecticides would not only serve as possible new tools for *D. citri* management, they could also improve existing rotation with conventional insecticides to impede evolution of insecticide resistance.

Insect growth regulators (IGRs) are reduced-risk insecticides with various modes of action that differ from neurotoxic insecticides. IGRs cause hormonal and biochemical imbalances during the development of the target insect, resulting in various morphological abnormalities and reduced development of immature stages, which leads to reduced emergence of adults. IGRs are less harmful to beneficial insects and humans than neurotoxic insecticides.[16] They have been successfully used to control insect pests of medical and veterinary importance.[16] Juvenile hormone agonists, ecdysteriod agonists and chitin synthesis inhibitors are three commonly used insect growth regulators.[17,18] Reduced egg hatch, suppressed nymphal development and decreased adult emergence were observed when various developmental stages of *D. citri* were treated with pyriproxfen [4-phenoxyphenyl (*RS*)-2-(2-pyridyloxy)propyl ether – a juvenile hormone mimic], buprofezin (2-*tert*-butylimino-3-isopropyl-5-phenyl-3,4,5,6-tetra-hydro-2-thiadiazine-4-one – a chitin synthetase inhibitor) and diflubenzuron [1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl) urea – a chitin synthetase inhibitor].[5,6]

Methoprene [1-methylethyl (*E*,*E*)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate] is a juvenile hormone analog that has been widely used for insect pest management. It has been used as a mosquito larvacide, as a grain protectant against stored insect pests and for control of a wide range of pests, including Coleoptera, Diptera, Hemiptera and Siphonoptera.[16] Methoprene has been shown to reduce adult emergence of several economically important insect pests such as *Rhyzopertha dominica*, *Tribolium castaneum*, *Triboleum confusum*, *Aedes aegypti* and *Cimex lectularius*.[17–21] External application of methoprene to eggs during blastokinesis is lethal to embryo development, and application during metamorphosis leads to faulty development of immature stages. Application to the adult stage can cause sterility.[16,17] Because of its low mammalian toxicity, selectivity and unique mode of action, methoprene is a potential candidate for integration into management programs for *D. citri*. The objectives of this study were to quantify the effects of methoprene on: (1) egg hatch, nymphal development and adult emergence; (2) fecundity and fertility of treated females or females emerging from treated fifth instars; (3) feeding behavior of adults on treated leaves; (4) settling behavior of adults on treated plants.

2 MATERIALS AND METHODS

2.1 Insect culture and insecticides

Diaphorina citri were collected from a laboratory colony maintained in a greenhouse at the Citrus Research and Education Center, University of Florida. The culture was established in 2000 from a field population collected in Polk County, Florida (28.0′ N, 81.9′ W), prior to discovery of HLB in Florida. The colony is continuously maintained on sweet orange [*Citrus sinensis* (L.) Osbeck] plants at 27–28 °C, 60–65% RH and a 14:10 h (L:D) photoperiod. Analytical-grade methoprene (purity 99.2%), pyriproxyfen (purity 99.0) and imidacloprid (purity 99.9) were procured from Sigma-Aldrich (St Louis, MO). The insecticides were diluted in tap water for spray and dip treatments and in acetone for topical treatments. Pyriproxyfen and imidacloprid were used as positive controls. The concentrations of positive controls were selected on the basis of effective concentrations recorded against *D. citri* in previous studies.[6,22]

2.2 Effect of methoprene on egg hatch

The objective of this experiment was to determine the effect of methoprene on egg hatch. Forty-five potted 'Swingle citrumelo' plants (*Citrus paradisi* MacFaden × *Poncirus trifoliata* L. Raf.), 2–3 months old and with new flush as defined by Hall and Albrigo,[23] were placed into four fine-mesh screen cages (61 × 61 × 91 cm; Bioquip, Ranch Dominguez, CA). Within each cage, approximately 400 *D. citri* adults of mixed gender were released for mating and oviposition for 48 h at 25 ± 2 °C and 50 ± 5% RH and a 14:10 h (L:D) photoperiod. After 48 h, the adults were removed from the plants. After 48 h or 72 h, leaf flush with eggs was excised to obtain 0–48-h-old and 49–96-h-old eggs. Only one flush per plant was removed. The excised flush was placed individually into 1.5 mL microcentrifuge tubes (Fisher Scientific, Pittsburg, PA) containing a 2% agar–water solution. Eggs per individual flush were counted under the stereomicroscope. The flush samples were then dipped in solutions of methoprene at various concentrations: 10, 20, 40, 80, 160 and 320 μg mL^{-1} prepared in tap water. Pyriproxyfen (128 μg mL^{-1}) and imidacloprid (100 μg mL^{-1}) were both included as positive controls. Water alone served as the negative control. The flush was dipped for 30 s and allowed to air dry. The entire experiment was repeated twice, and each treatment, including the controls, was replicated 12–18 times. The number of eggs per flush ranged between 7 and 95. Treated flush samples were placed in a rearing chamber maintained at 25 ± 2 °C and 50 ± 5% RH and a 14:10 h (L:D) photoperiod. After 5 days, the numbers of hatched and unhatched eggs were counted using a stereomicroscope.

In the second experiment, contact toxicity of methoprene to eggs was assessed by allowing *D. citri* to oviposit on plants that were previously treated with methoprene. Potted 'Swingle citrumelo' plants with new flush were sprayed until run-off with a handheld atomizer using various concentrations of methoprene (20, 40, 80, 160 and 320 μg mL^{-1}), as well as pyriproxyfen (128 μg mL^{-1}) or imidacloprid (100 μg mL^{-1}), diluted in tap water. Water alone served as the control treatment. The foliage was air dried. Each plant was infested with 20 *D. citri* adults of mixed gender and individually covered within mesh screens. After 48 h, *D. citri* were removed from the plants. After 96 h, one flush with eggs was removed from each plant and placed into a 1.5 mL microcentrifuge tube containing a 2% agar–water solution. Eggs were then counted using a stereomicroscope, and the flush was placed within a rearing chamber maintained at 25 ± 2 °C and 50 ± 5% RH and a 14:10 h (L:D) photoperiod to allow for egg hatch. The experiment was replicated twice over time, with 11–12 replicates per treatment and with 7–98 eggs per flush sample. After 5 days of treatment, the numbers of hatched and unhatched eggs were counted.

2.3 Effect of methoprene on development of nymphs and adults

The objective of this experiment was to quantify the development of *D. citri* nymphs following treatment with methoprene during various instars. Adult *D. citri* of mixed gender were released into a cage with potted 'Swingle citumelo' plants as described above. Females were allowed to lay eggs on the plants for 5 days, and then all adults were aspirated from the cage. A total of six cages were infested with *D. citri*, and subsequently first-, third- or fifth-instar *D. citri* nymphs were obtained from two cages for each instar. The numbers of each nymphal instar were counted under a stereomicroscope, and the plants were sprayed with various concentrations of methoprene diluted in tap water (16, 32, 64, 128 and 256 μg mL^{-1}), as well as a set sprayed with pyriproxyfen (128 μg mL^{-1}), imidacloprid (100 μg mL^{-1}) or water alone as a negative control treatment. Plants were sprayed until run-off using a handheld atomizer (Chemical Guys MFG. Co., Gardena, CA) and were left to air dry. Each plant was covered with an individual screen sleeve. The experiments were replicated twice over time, with each treatment replicated 7–11 times and each plant receiving 7–104 nymphs. To determine acute toxicity, the number of nymphs surviving to the subsequent instar was recorded after 72 h, and adult emergence was quantified for each treatment.

2.4 Effect of methoprene on reproduction

The objective of this experiment was to determine the reproductive potential of *D. citri* adults obtained from nymphs, as well as adults, topically treated with methoprene. Fecundity and viability of eggs were measured to ascertain the effects on reproductive potential of *D. citri*. Ten-day-old adults obtained from treated fifth-instar nymphs, described in the previous experiment, were collected and sexed for each concentration of methoprene tested (0, 32, 64, 128 and 256 μg mL^{-1}). Two pairs of *D. citri* (male:female in 1:1) were released onto untreated 'Swingle citrumelo' plants with new flush. The plants were placed in a rearing chamber maintained at 25 ± 2 °C and 50 ± 5% RH and a 14:10 h (L:D) photoperiod. Adults were allowed to lay eggs for 72 h. After 72 h, the total number of eggs per plant was counted using a stereomicroscope. The flush was excised, inserted into 1.5 mL microcentrifuge tubes filled with agar and placed into a rearing chamber for 5 days, after which the hatched and unhatched eggs were counted. There were 6–9 replicates per treatment.

In a second experiment, the effects of methoprene on fertility of *D. citri* were evaluated following topical treatment of adults with various concentrations (128 and 256 μg mL^{-1}) diluted in acetone or a negative control treatment of acetone alone. A total of 0.2 μL of the treatment solution was applied to the abdomen of adults using a 10 μL Hamilton syringe (Hamilton Co., Reno, NV). After treatment, three pairs of adults of the same age were placed onto untreated 'Swingle citrumelo' plants. Treatments were replicated 3 times, and the entire experiment was repeated twice over time. *D. citri* adults were removed from plants after 2, 7 and 14 days. The number of eggs per plant was counted 2, 7 and 14 days after treatment. The numbers of eggs that hatched and first-instar nymphs were counted at 6, 11 and 18 days after treatment.

2.5 Effect of methoprene on adult feeding

The objective of this experiment was to determine the effect of methoprene on feeding behavior of *D. citri* on treated plant surfaces. Surfaces treated with various concentrations of insecticides were exposed to *D. citri*, and honeydew excretions were quantified to assess the amount of feeding using a ninhydrin test.[24] Fresh citrus leaves collected from insecticide-free Valencia orange trees were cut into discs 34 mm in diameter. The leaf discs were dipped in solutions of methoprene (20, 40, 80, 160 and 320 μg mL^{-1}), pyriproxyfen (128 μg mL^{-1}) or water alone for 30 s and then air dried in a fume hood. The treated leaf discs were then placed on 35 mm × 10 mm petri dishes (Fisher Scientific) upon a bed of agar to maintain moisture for the leaf disc. The lids of the petri dishes were lined with Whatman filter paper (Whatman International Ltd, Kent, UK). Five adult *D. citri* of mixed gender were placed into each dish. The lids were sealed with parafilm (Beemis Flexible Package, Neenah, WI) and held upside down in an incubator (Thermo Scientific, Waltham, MA) set at 25 ± 2 °C and 50 ± 5% RH with a 14:10 h (L:D) photoperiod. Filter papers were removed after 48 h, replaced and then again collected 72 h post-treatment. Collected filter papers were subjected to the ninhydrin test[24] to quantify honeydew droplet production as an indirect measure of feeding. There were at least six replicates (individual petri dish with treated disc) per treatment.

2.6 Settling behavior

The objective of this experiment was to determine whether methoprene affects *D. citri* settling on plants. 'Swingle citrumelo' plants of the same age (12–14 weeks old) and same height and vigor were sprayed until run-off with various concentrations of methoprene (20, 40, 80, 160 and 320 μg mL^{-1}) diluted in water or water alone as a negative control. Plants were allowed to air dry and were randomly placed into Plexiglas cages (40 cm × 40 cm × 40 cm). All six treatments were randomly arranged within each cage as a choice test. There were six cages set up for choice tests with each treatment. Fifty *D. citri* adults of mixed gender were released into the center of each cage. The cages were placed in the rearing chamber maintained at 25 ± 2 °C and 50 ± 5% RH and at a 14:10 h (L:D) photocycle. The number of adults settling on plants treated with various concentrations of methoprene and the control were recorded after 24, 48 and 72 h.

2.7 Statistical analyses

Percentage data from the experiments were tested to ensure that the assumptions of homogeneity of variance and normality were met before data were analyzed. Percentage egg hatch and survival of first, third, and fifth instars, as well as percentage adult emergence, were subjected to separate one-way analysis of variance (ANOVA) and Fisher's protected LSD means separation tests. Concentrations causing 50% egg inhibition (LC_{50}) and 50% first-instar nymphal mortality (LC_{50}) and the corresponding 95% confidence intervals (CIs), at both ages of eggs, were determined by subjecting the data to probit analysis. The numbers of eggs laid per plant by the females that developed from treated fifth instars with various concentrations of methoprene and the percentage egg hatch for these treatments were subjected to separate one-way ANOVA and Fisher's protected LSD tests. The data for the number of eggs laid per female and the percentage egg hatch for topically treated adults at 2, 7 and 14 days were analyzed by conducting separate one-way ANOVAs and Fisher's protected LSD tests. The differences in feeding of *D. citri* across different treatments at the two different time intervals were determined by conducting separate one-way ANOVAs and Fisher's protected LSD tests for each time interval. The differences in the number of *D. citri* adults settling on plants treated with various concentrations of methoprene or the control at 24, 48 or 72 h after release were determined by one-way ANOVAs and Fisher's protected LSD tests for each time interval. Proc GLM was

used to conduct one-way ANOVAs and Fisher's protected LSD tests, and Proc Probit was used for probit analysis with SAS software (SAS Institute, Cary, NC).[25]

3 RESULTS

3.1 Effect of methoprene on egg hatch

Methoprene significantly reduced the hatch of 0–48-h-old eggs in a concentration-dependent manner as compared with the control ($F = 42.87$; df = 8, 129; $P = <0.0001$) (Fig. 1). Similarly, methoprene significantly reduced the hatch of 49–96-h-old eggs ($F = 30.73$; df = 8, 114; $P = <0.0001$) (Fig. 1). We observed 74 and 77% hatch for 0–48-h-old and 49–96-h-old eggs, respectively, at the lowest concentration of methoprene tested (10 μg mL^{-1}). Furthermore, 8 and 9% of 0–48-h-old and 49–96-h-old eggs, respectively, hatched following methoprene treatment at the highest rate tested (320 μg mL^{-1}). *D. citri* egg hatch was 23 and 38% following treatment with pyriproxfen (128 μg mL^{-1}) after 0–48 h and 49–96 h of ageing respectively (Fig. 1). The percentage of eggs hatched following treatment with imidacloprid (100 μg mL^{-1}) was 51 and 52% for 0–48-h-old and 49–96-h-old eggs respectively (Fig. 1). Although 0–48-h-old eggs were more sensitive to methoprene treatments than 49–96-h-old eggs, the overlapping 95% CIs for LC_{50} and LC_{90} did not indicate a statistical difference in susceptibility of eggs, based on age (Table 1). In the second experiment, contact toxicity of methoprene resulted in a reduction in egg hatch in a concentration-dependent manner as compared with the water control ($F = 57.16$; df = 6, 76; $P = <0.0001$). The lowest concentration of methoprene tested resulted in a mean percentage egg hatch of 53%, and the highest concentration tested resulted in a mean percentage egg hatch of 7% (Fig. 2). Pyriproxyfen resulted in a mean egg hatch of 22% (Fig. 2). There were no eggs observed on plants sprayed with imidacloprid because all of the adults died before egg laying owing to contact toxicity. There were no significant differences in the LC_{50} and LC_{90} values of methoprene when applied before and after egg laying, based on the overlapping of 95% CIs (Table 1).

3.2 Effect of methoprene on development of nymphs and adults

There was a significant reduction in the survival of first-instar nymphs to the second instar ($F = 35.56$; df = 7, 70; $P = <0.0001$) after treatment with methoprene. Acute mortality of first-instar nymphs was observed in a concentration-dependent manner. The lowest concentration tested caused 46% mortality, and the highest caused 93% mortality of first-instar nymphs (Fig. 3A). Pyriproxyfen and imidacloprid caused 76 and 98% mortality of first instars respectively (Fig. 3A). The LC_{50} and LC_{90} values calculated for methoprene were 27.2 and 371.8 μg mL^{-1} respectively (Table 1). There was reduced development of methoprene-treated first-instar nymphs to adults as compared with the control ($F = 52.47$; df = 7, 49; $P = <0.0001$). There was no emergence of adults from first-instar nymphs treated at the higher concentrations of methoprene tested, and less than 2% survival was recorded at the lower concentrations tested (Fig. 4A). First-instar nymphs treated with pyriproxyfen or imidacloprid did not survive to emerge as adults (Fig. 4A).

Treatment of third instars with various concentrations of methoprene, pyriproxyfen or imidacloprid resulted in a significant reduction in survival of third-instar nymphs ($F = 18.01$; df = 7, 60; $P = <0.0001$). The lowest concentration of methoprene that caused significant mortality of third-instar nymphs (34%) was

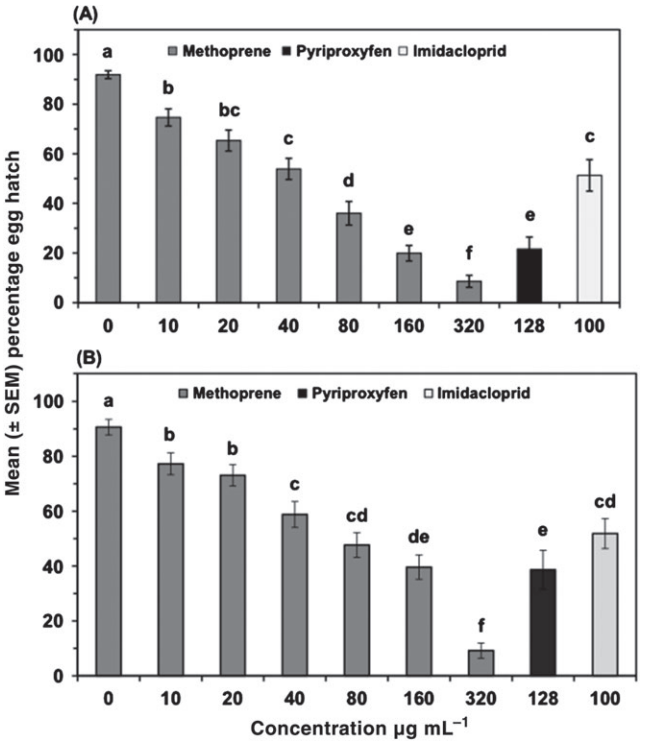

Figure 1. Mean percentage hatch of *Diaphorina citri* eggs of (A) 0–48 h or (B) 49–96 h age prior to treatment with various concentrations of methoprene, pyriproxyfen or imidacloprid. Bars represent treatment means with SEM. Treatment bars not labeled with the same letter are significantly different from one another according to an LSD mean separation test ($P < 0.05$).

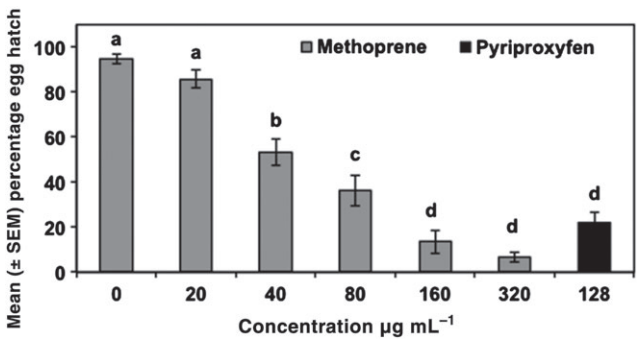

Figure 2. Mean percentage hatch of *Diaphorina citri* eggs laid after treatment of plants with various concentrations of methoprene or pyriproxyfen. Bars represent treatment means with SEM. Treatment bars not labeled with the same letter are significantly different from one another according to an LSD mean separation test ($P < 0.05$).

32 μg mL^{-1}. The highest concentration tested, 256 μg mL^{-1}, caused 77% mortality of third-instar nymphs. Pyriproxyfen and imidacloprid caused 48 and 93% mortality respectively (Fig. 3B). Each treatment tested significantly reduced emergence of adults from treated third-instar nymphs as compared with the control ($F = 23.36$; df = 7, 60; $P = <0.0001$). The greatest reduction in adult emergence occurred with imidacloprid (100 μg mL^{-1}) and methoprene (at 256 and 128 μg mL^{-1} concentrations), followed by pyriproxfen (128 μg mL^{-1}) (Fig. 4B).

Survival of fifth-instar nymphs was significantly reduced after treatment with methoprene, pyriproxyfen or imidacloprid as

Table 1. Toxicity of methoprene to various developmental stages of *Diaphorina citri*

Developmental stage	Age (h)	*n*	LC_{50} (μg mL^{-1}) (95% CI)[a]	LC_{90} (μg mL^{-1}) (95%CI)[b]	Slope (± SE)	χ^2 (df)[c]
Egg[d]	0–48	2773	41.91 (32.71–52.77)	336.16 (227.72–590.13)	1.41 ± 0.10	15.1 (4)
Egg[d]	49–96	2006	56.11 (34.78–91.28)	645.17 (289.62–3711)	1.20 ± 0.17	31.1 (4)
Egg[e]	49–96	2228	56.81 (48.74–66.59)	211.21 (166.08–298.48)	2.24 ± 0.12	10.5 (4)
First-instar nymph		1909	27.19 (13.38-40.8)	371.84 (194.7–1569)	1.12 ± 0.14	11.2 (3)

[a] 95% confidence intervals for LC_{50}.
[b] 95% confidence intervals for LC_{90}.
[c] Chi-square goodness-of-fit test and degrees of freedom.
[d] Leaf-dip bioassay was conducted after egg laying.
[e] Plants underwent different treatments before egg laying.

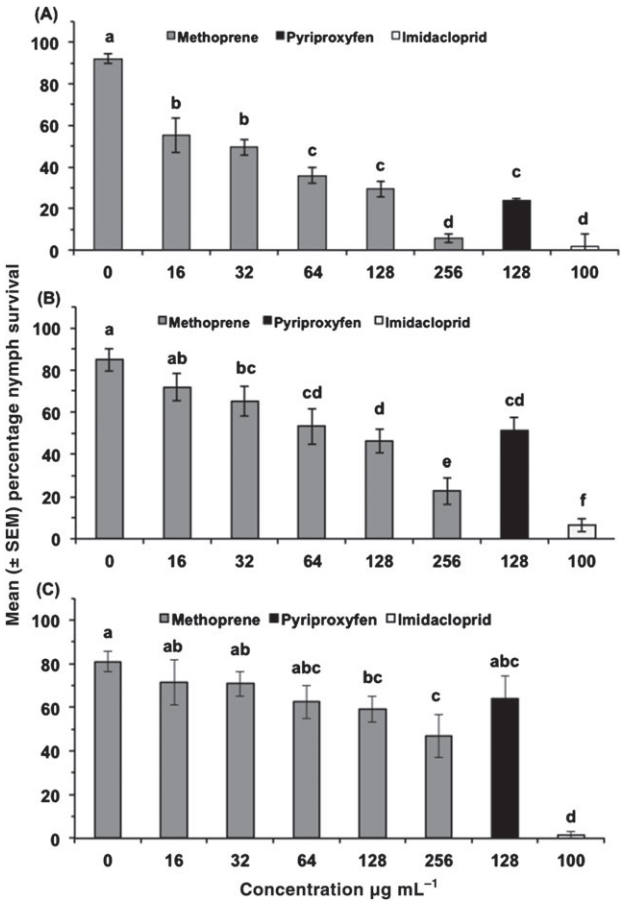

Figure 3. Mean percentage survival of (A) first-, (B) third- and (C) fifth-instar nymphs of *Diaphorina citri* when treated with various concentrations of methoprene, pyriproxyfen or imidacloprid. Bars not labeled with the same letter are significantly different from one another according to an LSD mean separation test ($P < 0.05$).

compared with the controls ($F = 7.83$; df = 7, 68; $P = <0.0001$) (Fig. 3C). The highest reduction in survival occurred with imidacloprid, followed by methoprene at the 256 μg ml^{-1} concentration. Application of methoprene, pyriproxyfen or imidacloprid significantly reduced the emergence of adults ($F = 3.29$; df = 7, 68; $P = 0.0045$) as compared with the controls. The higher concentrations of methoprene tested (256 and 128 μg mL^{-1}), as well as the pyriproxfen and imidacloprid treatments, reduced the emergence of adults from fifth-instar nymphs as compared with the control (Fig. 4C).

3.3 Effect of methoprene on reproduction

There were no significant differences between the number of eggs oviposited by female adults that emerged from treated fifth-instar nymphs as compared with the control ($F = 1.36$; df = 4, 32; $P = 0.26$) (Fig. 5). However, egg hatch of treated females differed significantly from the control ($F = 5.03$; df = 4, 26; $P = 0.0039$) (Fig. 6). Only the higher concentrations of methoprene tested (256 and 128 μg mL^{-1}) reduced egg hatch as compared with the control.

Topically applied methoprene significantly reduced the hatch of eggs laid 2 days after treatment ($F = 4.90$; df = 2, 7; $P = 0.046$) for the higher dosages of methoprene tested (Fig. 7). There were no significant differences in egg hatch between treatments and controls for eggs laid at 7 days ($F = 1.15$; df = 2, 11; $P = 0.35$) and 14 days after treatment ($F = 0.07$; df = 2, 14; $P = 0.93$) (Fig. 7).

3.4 Effect of methoprene on adult feeding

There were significant differences in the number of honeydew droplets recorded per filter paper for the methoprene and pyriproxfen treatments, as compared with the controls, after 0–48 h ($F = 4.57$; df = 7, 53; $P = 0.0005$) and 49–72 h ($F = 5.92$; df = 7, 56; $P = 0.0001$) of feeding. The lowest concentrations that significantly reduced *D. citi* adult feeding during the 0–48 and 49–72 h intervals were 40 and 160 μg mL^{-1} respectively (Fig. 8).

3.5 Settling behavior

Settling behavior of *D. citri* did not differ between the methoprene and control treatments 24 h ($F = 0.95$; df = 5, 30; $P = 0.46$), 48 h ($F = 0.96$; df = 5, 30; $P = 0.45$) or 72 h ($F = 0.96$; df = 5, 30; $P = 0.46$) after release.

4 DISCUSSION

Currently, management of HLB in citrus relies heavily on management of *D. citri* populations by frequent application of available, registered insecticides for use in citrus crops. Given the limited modes of action available, the repetitive and frequent use of these insecticides has resulted in the development of insecticide resistance in certain populations of *D. citri* in Florida.[15] It is necessary to prevent or delay insecticide resistance development in *D. citri*, and inclusion of additional insecticide modes of action into an integrated management program is one possible approach. Insect growth regulators are characterized by several modes of action that differ significantly from that of neurotoxic insecticides, and that can be incorporated into the current rotation for *D. citri* management. The objective of this investigation was to evaluate the efficacy of the juvenile hormone analog, methoprene, against *D.*

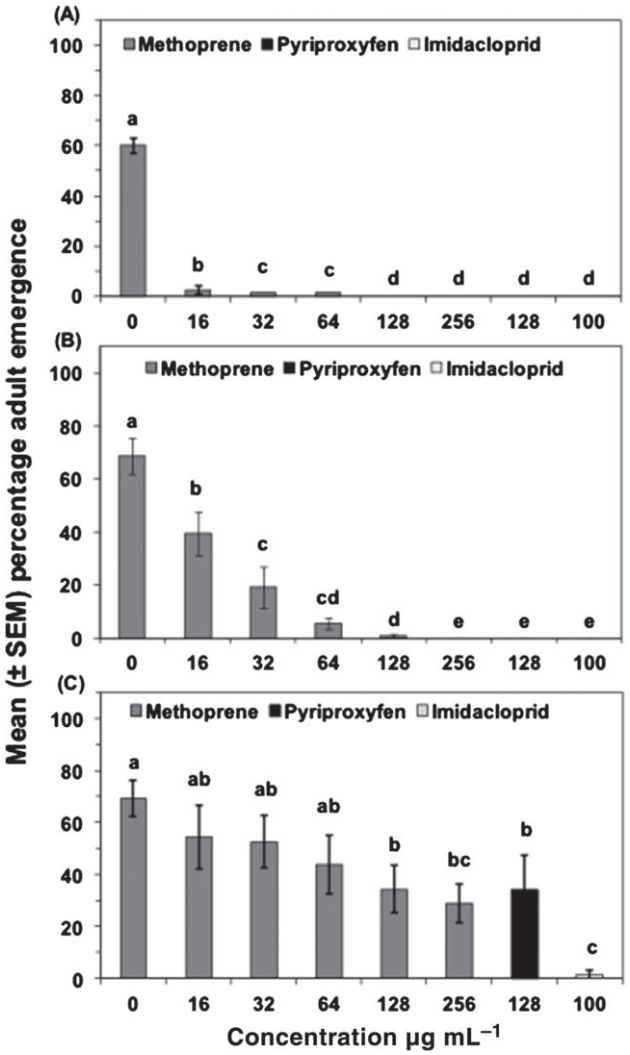

Figure 4. Mean percentage adult emergence from (A) first-, (B) third- and (C) fifth-instar nymphs of *Diaphorina citri* when treated with various concentrations of methoprene, pyriproxyfen or imidacloprid. Bars not labeled with the same letter are significantly different from one another according to an LSD mean separation test ($P < 0.05$).

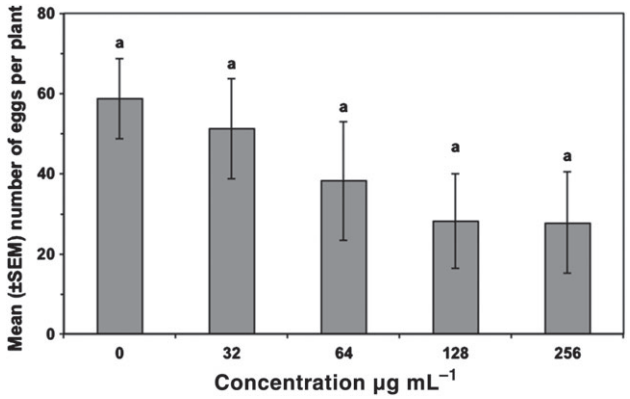

Figure 5. Mean number of eggs oviposited per plant by *Diaphorina citri* females emerging from fifth-instar nymphs treated with various concentrations of methoprene. Bars not labeled with the same letter are significantly different from one another according to an LSD mean separation test ($P < 0.05$).

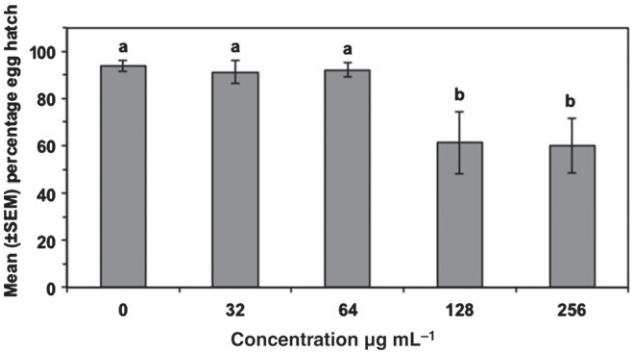

Figure 6. Mean percentage hatch of eggs oviposited per plant by *Diaphorina citri* females emerging from fifth-instar nymphs treated with various concentrations of methoprene. Bars not labeled with the same letter are significantly different from one another according to an LSD mean separation test ($P < 0.05$).

citri. Both direct effects against immature stages and sublethal effects against adults were investigated.

In the present investigation, application of methoprene resulted in acute toxicity to *D. citri* eggs, leading to reduced egg hatch of both younger and older stages tested. Egg hatch inhibition increased in a dose-dependent manner, irrespective of egg age. Older-stage eggs were less susceptible to methoprene than younger-stage eggs, which is congruent with previous observations for pyriproxyfen.[6] Also, eggs of glassy-winged sharpshooter, *Homalodisca vitripennis* (Germar), are similarly more susceptible to pyriproxyfen at younger than at older stages.[26] This trend is also true for *Bemisia tabaci* (Gennadius) and *Trialeurodes vaporariorum* (Westwood).[27] Juvenile hormone analogs inhibit egg hatch by penetrating into the egg and blocking embryogenesis at the blastokinesis stages. Juvenile hormone analogs are more potent if they enter the egg prior to blastokinesis.[27,28] The higher probability of blockage of blastokinesis in younger eggs may have resulted in increased susceptibility of younger than of older eggs. Methoprene exhibited similar age-dependent effects on egg mortality to those observed previously with other juvenile hormone mimics.

Treatment with pyriproxyfen at 128 µg mL^{-1} resulted in similar egg hatch inhibition to methoprene applied at 160 µg mL^{-1}. Imidacloprid applied at 100 µg mL^{-1} resulted in similar egg inhibition to methoprene applications at 40 µg mL^{-1} for younger eggs. In the present investigation, the juvenile hormone analogs methoprene and pyriproxyfen appeared to be more effective than imidacloprid, a commonly used neonicotinoid, in reducing egg survival. Imidacloprid had no effect on egg hatch of *Popillia japonica* Newman eggs in soil treated with concentrations ranging from 0 to 2 µg mL^{-1}.[29] Similarly, the mortality of *Helicoverpa zea* Boddie eggs was not affected by imidacloprid treatment in the field.[30,31] Given that the lipid layer of the insect chorion acts as a barrier to hydrophilic compounds (low octanol–water partitioning coefficient log K_{ow}), it prevents these compounds from reaching the embryo. We hypothesize that in the case of *D. citri* eggs the chorion may have selectively prevented imidacloprid (log $K_{ow} = 0.57$)[30] from interfering with embryogenesis. Methoprene exhibited residual effectiveness in reducing egg hatch in a concentration-dependent manner. Similarly, residues of pyriproxyfen resulted in reduced egg hatch of *D. citri*.[6] In the present study, no oviposition was observed on plants treated with imidacloprid.

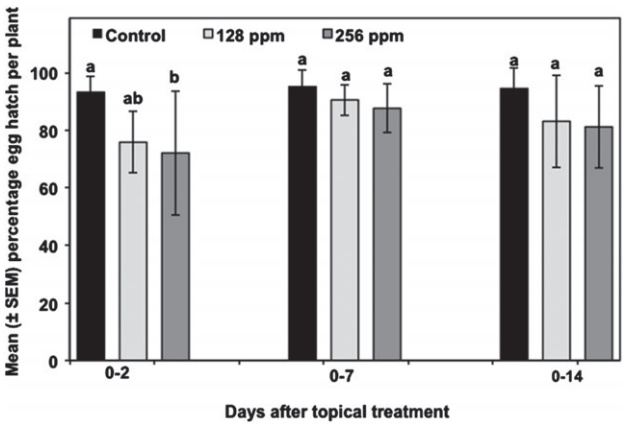

Figure 7. Mean percentage egg hatch per plant for eggs oviposited by *Diaphorina citri* females topically treated with various concentrations of methoprene or control (acetone alone) at three different time intervals. Bars not labeled with the same letter within each time interval are significantly different from one another according to an LSD mean separation test ($P < 0.05$).

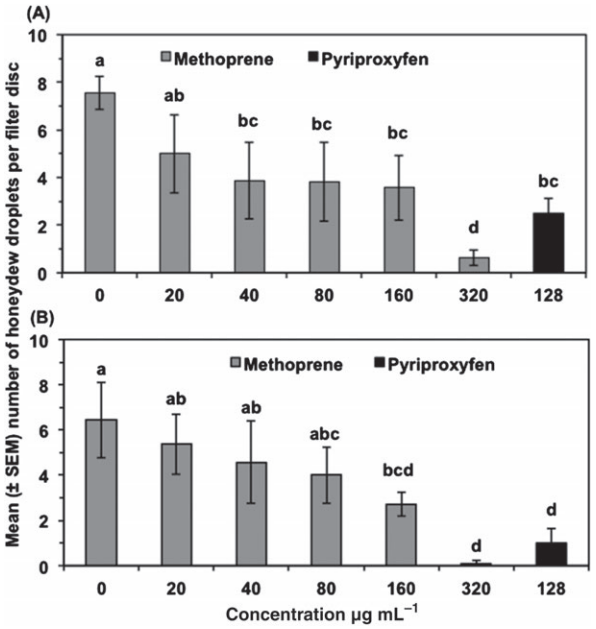

Figure 8. Effect of methoprene or pyriproxfen on feeding by *Diaphorina citri* adults at (A) 0–48 h and (B) 49–72 h after placing five adults on each treated leaf disc. The number of honeydew droplets per filter paper disc was quantified after (A) 48 h and (B) 72 h for each concentration. Bars not labeled with the same letter are significantly different from one another according to an LSD mean separation test ($P < 0.05$).

In the present study, treatment with methoprene resulted in acute mortality of early instars and delayed mortality of late-instar *D. citri* nymphs. Methoprene was most effective when applied to the first-instar nymphs and least effective when applied to fifth instars. Methoprene and pyriproxfen resulted in similar mortalities of first, third and fifth instars at concentrations of 128 µg mL^{-1}. Imidacloprid was highly effective, resulting in 98, 94 and 94% mortality of first, third and fifth instars respectively. For methoprene, the highest concentration tested caused 93, 77 and 53% mortality of first, third and fifth instars respectively. Methoprene treatments were more effective in suppressing adult emergence when applied to earlier than to later instars. Inhibition of adult emergence following treatment with methoprene is well documented among several insect species.[16,17,32,33] The present findings are consistent with earlier studies conducted with pyriproxfen on *D. citri* nymphs.[6] Pyriproxyfen was also more effective in reducing survival and adult emergence when applied to earlier than to later instars of *D. citri*.[6] Similarly, first-instar nymphs of the German cockroach, *Blattella germanica* (L.), were more susceptible than fourth instars when treated with a juvenile hormone analog, fenoxycarb.[34] These results suggest that methoprene treatment would be most effective against *D. citri* by targeting eggs and early instars during applications. Our positive control treatment, imidacloprid, was more effective against all stages of *D. citri* than methoprene or pyriproxyfen.

There was no effect of treating fifth-instar nymphs or females directly with methoprene on subsequent fecundity. However, there were sublethal effects observed with reduced fertility of females that emerged from treated fifth-instar nymphs or females that were treated topically. The percentage egg hatch for females emerging from treated fifth-instar nymphs was reduced in a concentration-dependent manner. Egg hatch was reduced for up to 1 week when females were treated topically with methoprene. These results with methoprene against *D. citri* are similar to those observed with another juvenile hormone analog, pyriproxifen.[6] Juvenile hormone analogs are typically characterized by a window of maximum effectiveness, and in many cases younger stages of immatures exhibit greater sensitivity than older stages, but there is variation among species.[17,28,32] The application of methoprene to two-day-old and six-day-old *Cydia pomonella* (L.) (Lepidoptera: Torticidae) larvae did not result in reduction in fecundity, but application to four-day-old larvae did reduce fecundity.[35] Pyriproxyfen caused abnormalities in the development of embryos of *Acheta domesticus* (L.) (Orthoptera: Gryllidae) only when applied during a limited window of sensitivity, prior to the second embryonic (pronymphal) molt.[36]

The Las pathogen is acquired and transmitted by *D. citri* during feeding. Acquisition by *D. citri* may require approximately 30 min of continuous feeding on infected trees.[6,12] Transmission of the Las pathogen by *D. citri* is thought to require 5–7 h of feeding on healthy plants.[6,10,12,37] Deterring feeding may be one method to decrease the spread of HLB in citrus. At the higher concentrations of methoprene tested (80–320 µg mL^{-1}), it appears that psyllid feeding was reduced. Similar effects have been observed with pyriproxyfen,[6] imidacloprid[17] and cyantraniliprole[18] with *D. citri* under laboratory conditions. In our laboratory investigation, methoprene did not affect the settling behavior of *D. citri* and therefore does not appear to act as a repellent to this insect.

Methoprene was first registered by the US Environmental Protection Agency in 1975 and has proven to be effective against a wide variety of insect pests. It is particularly useful for stored-product pests, as well as pests of livestock and domestic animals, because of its low mammalian toxicity.[38] Methoprene could be a good choice for management of *D. citri* because of the demonstrated effects on nymphal development and egg hatch. As the HLB pathogen is acquired more effectively by nymphs than by adult *D. citri*,[14] methoprene could potentially be used in an insecticide rotation when nymphal populations are high in order to reduce the transmission of pathogen by emerging and mobile adults. Given the effectiveness of methoprene in laboratory assays, further field testing is warranted. Registration of additional and more environmentally friendly insecticides for integration into *D. citri* management is needed.

5 CONCLUSIONS

Methoprene reduced survival of *D. citri* eggs at each age tested and effectively reduced the survival of early-instar nymphs in a concentration-dependent manner. Methoprene significantly reduced adult emergence from treated nymphs. Early instars were more sensitive to methoprene than late instars. Methoprene significantly reduced fertility of females that emerged from treated fifth instars. Treatment of adults with methoprene reduced fertility of females for 1 week. *D. citri* fed significantly less on methoprene-treated surfaces than negative controls in a concentration-dependent manner. There were no effects of methoprene on the settling behavior of *D. citri* on treated plant surfaces. The present investigation suggests that methoprene should effectively reduce *D. citri* populations as part of an integrated program. Unlike broad-spectrum neurotoxins, methoprene is stage specific and will require targeting of early developmental stages. Field efficacy testing is warranted, based on these laboratory results.

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