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## Ecology and Behavior

# Disrupting Mating Behavior of *Diaphorina citri* (Liviidae)

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## Abstract

Severe economic damage from citrus greening disease, caused by 'Candidatus Liberibacter asiaticus' bacteria, has stimulated development of methods to reduce mating and reproduction in populations of its insect vector, *Diaphorina citri* (Hemiptera: Liviidae). Male *D. citri* find mating partners by walking on host plants, intermittently producing vibrational calls that stimulate duetting replies by receptive females. The replies provide orientational feedback, assisting the search process. To test a hypothesis that *D. citri* mating can be disrupted using vibrational signals that compete with and/or mask female replies, courtship bioassays were conducted in citrus trees with or without interference from female reply mimics produced by a vibrating buzzer. Statistically significant reductions occurred in the rates and proportions of mating when the buzzer produced reply mimics within 0.4 s after male courtship calls compared with undisturbed controls. Observations of courtship behaviors in the two bioassays revealed activity patterns that likely contributed to the reductions. In both disruption and control tests, males reciprocated frequently between structural bifurcations and other transition points where signal amplitudes changed. Males in the disruption bioassay had to select among vibrational signals combined from the buzzer and the female at each transition point. They often turned towards the buzzer instead of the female. There was a statistically significant reduction in the proportion of males mating if they contacted the buzzer, possibly due to its higher vibration amplitude and duration in comparison with female replies. Potential applications of *D. citri* mating disruption technology in citrus groves are discussed.

**Key words:** vibration, communication, huanglongbing, competitive disruption, noncompetitive disruption

The Florida citrus industry is under threat from huanglongbing disease caused by the bacterium, 'Candidatus Liberibacter asiaticus', vectored by the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) (Hall et al. 2013). The disease kills infected trees within a few years while causing them to produce premature, unmarketable fruit. The severity of economic damage caused by this disease has encouraged efforts to develop greening-resistant citrus varieties (Dutt et al. 2015) as well as to manage and suppress *D. citri* populations (Boina and Bloomquist 2015, Grafton-Cardwell 2015, Monzo et al. 2015), while avoiding development of insecticide resistance (Coy et al. 2016, Naeem et al. 2016).

Asian citrus psyllids (Wenninger et al. 2009), like many species in the Psylloidea (e.g., Lubanga et al. 2014, Liao and Yang 2015) use vibrational communication to locate mating partners. Males pause and buzz their wings at frequencies of 175–250 Hz for durations of 0.1–0.7 s at intervals of 8 ± 0.4 s (mean ± standard error [SE]) (Wenninger et al. 2009) while searching for mates on citrus foliage, twigs, and

branches. Their legs transmit the buzzes as substrate vibrations to receptive females within detection range who respond with reply buzzes (0.3–0.8 s in duration) within 0.3–1.2 s (Wenninger et al. 2009). The resulting duet of call-and-reply buzzes helps direct the male towards the female. We hypothesized that a synthetic mimic of the female reply could disrupt mating, as has been accomplished with several other hemipterans that communicate using vibrational signals (Čokl and Millar 2009, Mazzoni et al. 2009, Eriksson et al. 2012, Mankin 2012, Korinšek et al. 2016, Polajnar et al. 2016). Partly because *D. citri* mates multiple times for high reproductive output (Wenninger and Hall 2008), mating disruption technology has potential to reduce populations sufficiently to enable decreases in frequencies of pesticide applications.

In previous studies (Mankin et al. 2013, 2016; Rohde et al. 2013), synthetic female reply mimics containing multiple harmonics of 200-Hz played for 1.495-s durations were found to elicit *D. citri* duetting behaviors at rates similar to those of live signals or recordings produced by conspecifics. A low-cost electronic system that can

detect male calls and respond with synthetic replies was described in Mankin et al. (2013, 2016). For this report, the electronic system was operated in bioassays to determine whether differences could be observed in the courtship of duetting pairs in the presence or absence of interference from female reply mimics.

## Materials and Methods

### Insects and Observation Arena
*Diaphorina citri* were reared in a greenhouse at the United States Department of Agriculture, Agricultural Research Service, Center for Medical, Agricultural, and Veterinary Entomology (CMAVE), Gainesville, FL under procedures described in Paris et al. (2013). Nymphs were isolated onto separate young *Citrus macrophylla* and kept enclosed in clear containers until testing. Pairs of previously unmated, 4–15 d males and females were tested during the typical mating hours of 10 a.m.–3 p.m. (Wenninger and Hall 2007). After testing, they were released into the CMAVE greenhouse colony.

For each test, a 30-cm-height tree was chosen randomly from a set of four young *C. macrophylla* Wester plants kept separately in the CMAVE greenhouse. After each day of testing, the trees were returned to the greenhouse.

Testing was conducted in an anechoic chamber at the CMAVE laboratory supplied with video and audio monitoring and recording instruments (Mankin et al. 2013, Zagvazdina et al. 2015). Each tree had multiple branches, serving as an arena providing three or more different search paths. To avoid disturbance, the bioassayer observed the test from outside the chamber using a monitor connected to the video camera and recorder system. An accelerometer attached with clips near the base of the tree detected vibrational signals that the bioassayer monitored with headphones from a digital audio recorder. Raven Pro 1.5 (Charif et al. 2008) was used to display the signals and conduct spectral and temporal pattern analyses on a desktop computer.

### Female Reply Mimics
Before each test, a contact microphone and a piezoelectric buzzer were attached with small binder clips a few cm above the accelerometer near the base of the tree. The microphone supplied substrate vibration signals to a microcontroller platform (Mankin et al. 2013, 2016) which distinguished male calls from background noise using a signal processing algorithm, generating and transmitting a female reply mimic to the buzzer automatically whenever it detected a male call. The time between the end of the male call and the initiation of the reply mimic, 0.1–0.4 s, was governed by the speed of the signal acquisition and processing algorithms, and by the response time of the bioassayer when the microcontroller failed to recognize the call (Mankin et al. 2016). In this study, the resistance in the piezoelectric buzzer circuit was set at 10 kΩ, which resulted in mimic signal amplitudes approximately onefold to fivefold greater than female signal amplitudes, as observed in examples in the Results section.

### Mating Disruption and Control Bioassay Procedures
Using a walker device described in Pregmon et al. (2016), the male and female were placed on outer leaves of two different upward pointing branches. Each of the 32 disruption and 30 control tests began when the male made its initial call and ended after 1 h or when mating began, whichever occurred first. If the male did not initiate calling within 5 min after placement on the tree, the buzzer was triggered manually, which usually stimulated the male to initiate calling within about 30 s.

For each test, the bioassayer noted the tree identity and insect locations, the insects' ages, whether or not mating occurred or the male contacted the buzzer, and the times for the beginning of the test, the first male call, the first female reply, the first buzzer contact, and the beginning of mating. In addition, notes were made for subsequent analysis of the patterns and timing of duetting and searching behaviors during periods when the males were within view of the video camera, including the durations between male calls during first the 5 min after an initial call.

Whenever the male called in a mating disruption test, the buzzer signal was triggered automatically by the microcontroller (or manually by the bioassayer monitoring the test if the microcontroller failed to recognize the call). This allowed the male to select its direction towards the female or the synthetic reply. In control bioassays, the synthetic reply was not triggered after a male call.

### Statistical Analyses
A Cox proportional hazards model (Cox 1972) was fitted to the hazard functions for mating disruption and control bioassays. The regression model (PHREG procedure, SAS Institute Inc. 2012) was

$$\text{minute} * \text{status} = \text{bioassay}, \qquad (1)$$

where minute measured the time in minutes from the beginning of the test until mating, status was a censoring variable (the data were censored if the test ended before mating occurred), and bioassay determined the regression coefficient for the ratio of the disruption and control hazard functions.

Nonparametric analysis of variance (NPAR1WAY, SAS Institute Inc. 2012) was performed to compare the relative rate of mating per test, Mating rate, in disruption and control bioassays,

$$\text{Mating rate} = 1 - t/\text{TD}, \qquad (2)$$

where $t$ was the time until mating initiation (in minutes), and TD was the test duration, 60 min. If mating did not occur, $t$ was set equal to TD.

A contingency test was performed in the disruption bioassay to compare the proportions of pairs successfully mating with or without having contacted the buzzer (JMP, SAS Institute Inc. 2015). Contingency tests also were performed to compare proportions of matings in the two bioassays, as well to compare the proportions of 'orientation success,' i.e., searches in which the male successfully found and mated with the female or contacted the buzzer. For bioassays where the male could be observed on the monitor during the first 5 min after the initial male call, a Welch's unequal-variance *t*-test was conducted to compare mean times between male calls in disruption and control tests.

## Results

Playback of synthetic female replies upon detection of male vibrational courtship calls resulted in a statistically significant difference in the proportion of *D. citri* mating in disruption tests, 0.125, compared with undisturbed control tests, 0.567 (Fig. 1, Table 1A). The Cox proportional hazards analysis indicated that the regression parameter for *bioassay* (equation 1) was significantly different in the survival curves for disruption and control, with Wald $\chi^2 = 5.28$ (df = 1, $P = 0.02$). This difference resulted in a significantly reduced relative rate of mating (equation 2) in disruption tests, 0.07 matings/ h, compared with control tests, 0.42 matings/h (Table 1B). In addition, contingency analysis (SAS Institute Inc. 2015) revealed a statistically significant reduction in the proportion of males mating if they had contacted the buzzer in the disruption bioassay (likelihood ratio $\chi^2 = 4.097$, df = 1, $P = 0.04$).
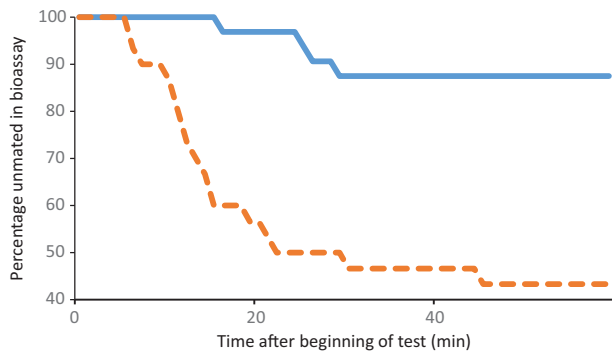
**Fig. 1.** Percentages of psyllids remaining unmated in disruption (solid line) and control (dashed line) bioassays during the 1-h test period.

**Table 1.** Differences between control and disruption bioassays for A) proportions of mating pairs, B) mean relative rates of mating initiation (equation 2), and C) mean time between male calls during first 5 min after initial call

| Measurement | Control | Disruption | Test value | P |
|---|---|---|---|---|
| A) Proportion mating | 0.567 (30) | 0.125 (32) | $13.485^a$ | 0.0002* |
| B) Relative mating rate | 0.42 (30) | 0.07 (32) | $15.415^b$ | <0.001* |
| C) Time between calls (s) | 9.0 (6) | 10.1 (14) | $0.551^c$ | 0.590 |

The numbers of observations in the measurements are listed in parenthesis.

*Statistically significant differences ($P < 0.05$) between control and disruption bioassays are indicated by *.

$^a$ Pearson $\chi^2$, df = 1, null hypothesis: proportions of mating are independent of bioassay.

$^b$ Mann–Whitney U, df = 1, Null hypothesis: relative mating initiation rates are independent of bioassay.

$^c$ Welch's $t$ (two-sample assuming unequal variances and normal distributions), df = 15, null hypothesis: mean times between calls are independent of bioassay. Normality was tested using Shapiro–Wilk W test (SAS Institute Inc. 2015), which indicated that times between calls were distributed normally in disruption (W = 0.897, P = 0.10) and control (W = 0.863, P = 0.20).

Examples of similarities and differences among tests in the two bioassays are seen in Figs. 2 and 3. A 60-s interval of a mating disruption test (Fig. 2) shows a series of five calls, each followed by a duetting reply mimic within $0.38 \pm 0.11$ s. The male in this example failed to locate either the female or the buzzer during the 1 h disruption test. For comparison, a 60-s interval is shown from a control test wherein mating occurred (Fig. 3). The female replied within $0.56 \pm 0.08$ s to all but one of the male calls in this interval. There was a relatively constant, 4–10 s interval between male calls during the 19 min this male required to find the female. The female reply was larger in amplitude than the male call in this example. However, the relative amplitudes of the detected signals depended on the substrates from which they were calling and their distances from the accelerometer; consequently, there was considerable variation in relative amplitudes of male and female signals in different tests. Notable similarities among the male and female signals in Figs. 2 and 3 include the occurrence of strong harmonics between 1 and 2.4 kHz in the two spectrograms. This similarity was observed as well in numerous other disruption and control tests, including Rohde et al. (2013).

The reply mimics often overlapped or completely masked the female replies in disruption tests. An example (Fig. 4) shows a female reply beginning approximately 1.35 s after the beginning of the recording, 0.15 s before the end of a reply mimic, and ending 0.22 s after the mimic ended. Despite the higher amplitude of the mimic, the female signal is visible in the spectrogram due to slight differences between the mimic harmonics and the female harmonics. In this example the female had a dominant harmonic at 1.2 kHz. This recording was obtained from a disruption test where no mating occurred and the male contacted the buzzer. In six of the disruption tests (19%), it was observed that the female ceased replying to the male after the buzzer had produced 1–3 reply mimics.

It should be noted also that in approximately 10–15% of control tests, the female replied to some but not all male calls (including an instance in Fig. 3). There were 11 (discarded) trials during which either the male or the female did not signal within 20 min after the beginning of the test.
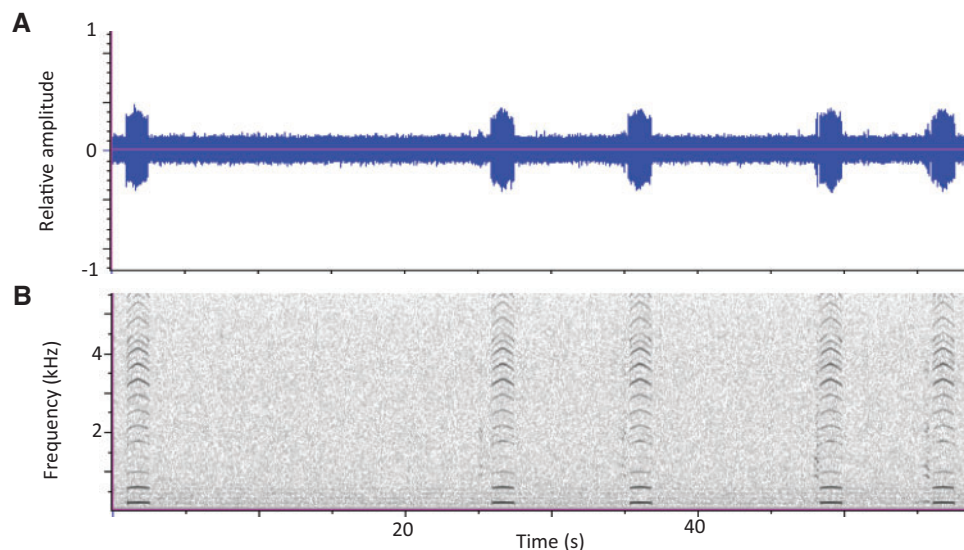
**Fig. 2.** Oscillogram (A) and spectrogram (B) of male calls and female reply mimics collected over a 60 s interval containing the first male call in a mating disruption test. Darker shades in the spectrogram indicate frequencies of higher energy at the specified time. The calls by the male in this test had dominant frequency harmonics of 1.0 or 1.2 kHz.
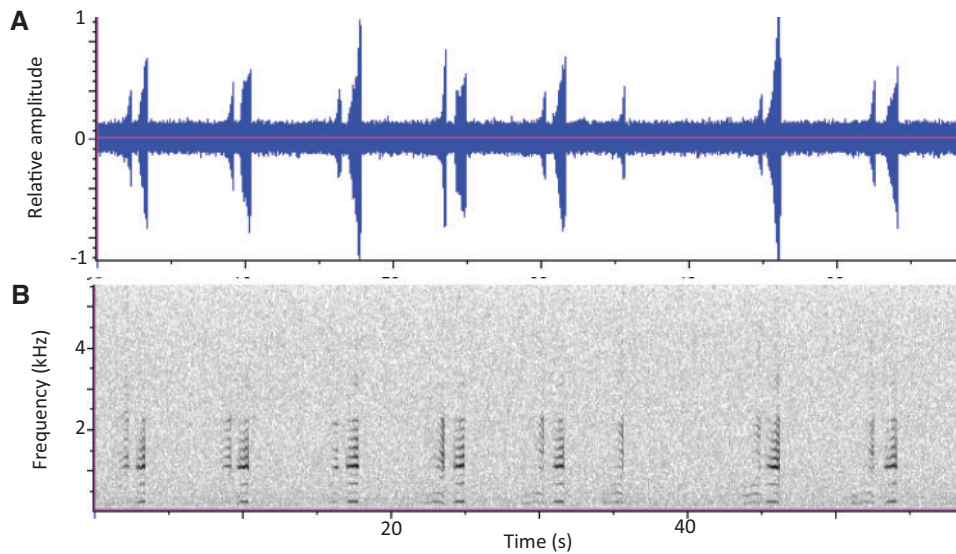
**Fig. 3.** Oscillogram (A) and spectrogram (B) of male calls and female replies collected over a 60 s period of courtship duetting in a control test. Darker shades in the spectrogram indicate frequencies of higher energy at the specified time. Both the male and female varied considerably in call amplitudes and durations, but both had strong harmonics at 1 kHz.
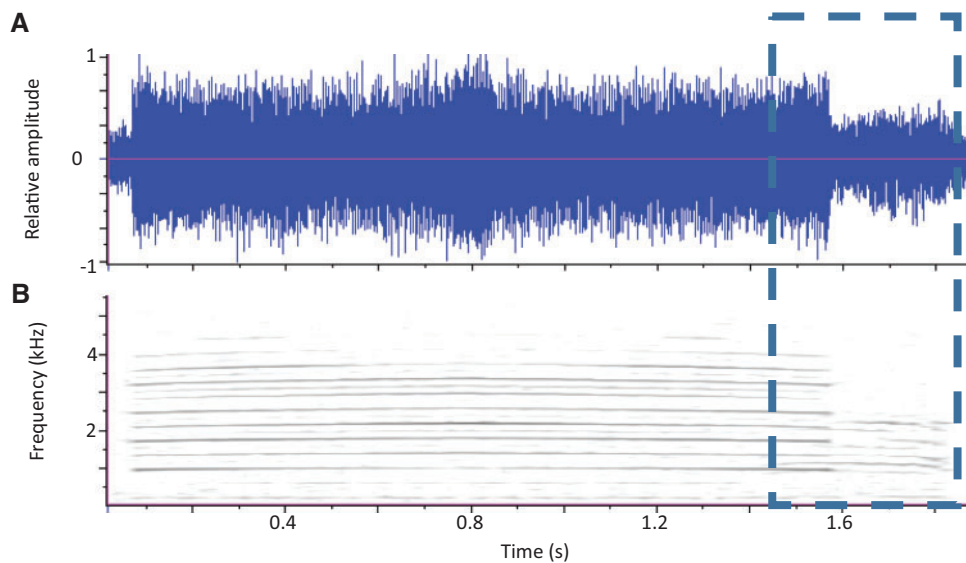


**Fig. 4.** Oscillogram (A) and spectrogram (B) of a synthetic reply mimic that overlaps a female reply shown in dashed box. The mimic has greater amplitude than the female reply, but the reply appears clearly in the spectrogram at its dominant frequency harmonic, 1.2 kHz, as well as at additional harmonics between 1.4 and 2.4 kHz. Darker shades in the spectrogram indicate frequencies of higher energy at the specified time.

Analysis of video recordings of searching behaviors in the disruption and control bioassays revealed activity patterns that likely contributed to the reductions of mating in the disruption tests.

### Video Analyses of Control Tests

In a majority of control tests, the male moved to the end of the petiole of the leaf on which it had settled initially, paused, and called. Then it moved forward rapidly along a branch, thereafter continuing a pattern of pauses, calls, and then forward or reverse movements. The times between calls were of varying durations, with a range of approximately 3–15 s and a mean of $9.00 \pm 1.38$ s between successive male calls for the first 5 min after the initial male call (Table 1C). Typically the males would pause to call at the beginning or end of a leaf, a bifurcation, or other usually visually identifiable transition point. At such transition points, the male frequently but not always moved towards a duetting reply. The male often travelled multiple times between pairs of previously visited transition points before it eventually traveled towards the duetting reply. Typically, several instances of such reciprocating behavioral patterns occurred, each instance ending somewhat closer to the female. The process continued until the male reached the female or ceased searching. When the male reached the female before the end of the 1 h test, the mean time from the beginning of the test until mating was $15.9 \pm 2.38$ min ($n = 17$), sometimes with more than a hundred reversals of direction among transition points. In this study, all of the males initiated mating if they found the female.

### Video Analyses of Disruption Tests

The males in disruption tests reciprocated multiple times between transition points as in the controls. Now, however, they also had additional choices of moving towards the female or the buzzer, or of reciprocating between them. During the hour of testing, 38% of males in the disruption bioassay eventually contacted the buzzer. The durations between calls at transition points were not significantly different from those in the controls, 10.1 ± 1.38 s (Table 1C).

For the 12.5% of males in the disruption bioassay that mated even with interference from the buzzer, the mean time from the beginning of the test until mating was 24.13 ± 2.38 min (Table 1A). This is a longer duration than in the control, in accordance with the Cox proportional hazards analysis, which determined that the percentages remaining unmated over time in the control was significantly lower than in the disruption bioassay. It should be noted also that, combining the counts of males contacting the buzzer with the counts of males mating with females into a single category of 'orientation success,' the difference between the proportions of orientation in the disruption bioassay and the control, 0.5 versus 0.567 were not significantly different (Pearson $\chi^2 = 0.276$, df = 1, $P = 0.599$), lending support to a hypothesis that the males believed the buzzer signal was produced by a female conspecific.

## Discussion

In this study, females were placed at a typical feeding position near the top of the tree, towards which D. citri have a natural phototactic bias (Sétamou et al. 2011; Anco and Gottwald 2015; Paris et al. 2015); yet, the response to female mimic signals often overcame the combined response to phototactic stimuli and female reply signals. In disruption tests with successful matings, the male often had moved oppositely towards the buzzer earlier in the assay but, as an active, rapidly moving searcher, had moved into visual range of the female during the search process. Consequently, the overall extent of the search ranges of these males likely contributed to their mating success.

Throughout the mating disruption bioassay, males usually moved preferentially towards the louder, longer reply mimic relative to the weaker, shorter female psyllid response. However, signals are more intense at certain transition or bifurcation points than others in tree structures (Michelsen et al. 1982, Mankin et al. 2008), which may contribute to the frequent reciprocations of males between multiple transition points seen in this study. As discussed in Mazzoni et al. (2014), the vibration active space, i.e., the spatial extent over which a signal is detectable to the insect, differs from the spherical airborne puffs or elongated plumes of olfactory active spaces (Mankin et al. 1980, Elkinton et al. 1984) or the hemispherical active spaces of terrestrial acoustics (Schmidt and Balakrishnan 2015). Rather, it is a network of one- or two-dimensional spaces comprising branch, shoot, and leaf tissues. Vibration amplitude cannot lead the male to the female simply through vibrotaxis, although it can provide directional information. Consequently, the male must employ additional searching mechanisms to successfully find a female in the complex environment of a citrus tree, possibly including attraction to female odors (Wenninger et al. 2008, Martini et al. 2014, Moghbeli et al. 2014) or to female visual or tactile cues that have not been studied in detail. Even the full range of vibrational frequency and temporal pattern cues that elicit male searching behavior is not yet fully established, although multiple harmonics of the wing-beat frequency appear to be important (Mankin et al. 2016). A capacity to employ multiple sensory modalities for mate seeking

may have evolved in D. citri to mitigate the effects of interference with one or more sensory modalities in different environments or social contexts. Development of a more comprehensive understanding of the entire searching process may improve the capability to disrupt mating as attempts to use vibration disruption technology expand.

The synthetic reply mimic was purposely set higher in amplitude and longer in duration than typical signals produced by females because higher amplitude signals had been demonstrated to have greater disruptive effect in other hemipteran species, e.g., Polajnar et al. (2016). A preference for higher-energy calls also may apply to females in hemipteran species (Wood et al. 2016). The long duration of the mimic signal did not appear to reduce its attractiveness to males in previous studies (Mankin et al. 2013). In addition, the synthetic replies usually were triggered before the females initiated their replies. A short, <0.4 s interval between the end of the male call and the beginning of the female reply is known to be important for mate recognition and location in other Hemiptera (Kuhelj et al. 2015). The mimic was longer than a typical female reply, 1.49 s compared with 0.408 s (Wenninger et al. 2009), and often overlapped with the female reply (Fig. 4) or ended after the female reply finished, making it difficult for the male to detect any part of her reply. These effects may have contributed to the reduction of mating rate in the disruption bioassay by a mechanism somewhat analogous to noncompetitive pheromone mating disruption (Miller and Gut 2015). Continuous application of calling signals also would mask signals, but it remains to be tested whether continuous signaling disrupts mating as effectively as reply mimics produced during courtship duets. The difference between disruption by buzzers competing with females or by masking of female signals is akin to the difference between competitive and noncompetitive mating disruption with pheromones (e.g., Miller and Gut 2015), and the understanding of how these two mechanisms combine to result in mating disruption has never been fully resolved.

It was of interest that in multiple tests, the female ceased replying to the male after the buzzer had produced 1–3 reply mimics. In other studies of hemipteran duetting (Mazzoni et al. 2009), it was found that females often ceased calling when two males were signaling in rivalry. Instances were observed in Wenninger et al. (2009) where two female D. citri replied in response to a male, but usually only one female continued to reply after the second and subsequent male calls. Males responded differently in the presence of a potential competitor in that an inactive male often would begin calling within a few seconds after it detected either a male call or a female or mimic reply, as was noted also by Zagvazdina et al. (2015). In other insects that communicate with vibrations (e.g., De Souza et al. 2011), males are known to exchange signals during agonistic encounters, and the male producing the greatest signal amplitude usually forces the weaker male to exit the courtship arena. Because D. citri adults frequently are observed in aggregations (Costa et al. 2010, Sule et al. 2012), it may be worthwhile in future investigations of D. citri courtship to consider effects of conspecific interactions in more detail.

The capability to manipulate and disrupt D. citri courtship signals has considerable potential as a tool for research on the management of this important pest. However, the technology by which disruptive signals could be applied to multiple trees in large citrus groves, as well as the optimal timing of such signals, remains under study. Minishakers were used to activate the top wires of the fences supporting grape vines in recent studies of mating disruption in a grapevine pest, Scaphoideus titanus Ball (Polajnar et al. 2016). However, the details of implementing such an approach in citrus groves are in an earlier stage of development than for vineyards. It

has not been determined whether introduction of wire waveguides within the groves could be accomplished easily without intrusion on management and harvesting operations. Variants of tree-shaking technology used to harvest citrus (Gupta et al. 2015), or direct transmissions of vibration into the ground (Kim and Lee 2000) might avoid some of these concerns but have not yet been tested.

A different approach to mating disruption was used by Saxena and Kumar (1980), who interrupted mating of *Amrasca devastans* (Dist.) and *Nilaparvata lugens* Stal using vibrations induced in host plants by ∼80 dB, 200–300-Hz produced sounds aerially. Unpublished preliminary studies with speaker-produced, 90-dB frequency sweeps confirmed the utility of this approach for *D. citri* in the laboratory, suggesting potential for future use in field environments. In addition, there is potential for use of alarm or stress vibrations as mating disruptors, as was demonstrated by Hofstetter et al. (2014) with *Dendroctonus frontalis* Zimmerman, but *D. citri* has not been observed to produce such signals.

## Acknowledgments

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