# Behavioral Responses of Male Diaphorina citri (Hemiptera: Liviidae) to Mating Communication Signals from Vibration Traps in Citrus (Sapindales: Rutaceae) Trees

# Behavioral responses of male *Diaphorina citri* (Hemiptera: Liviidae) to mating communication signals from vibration traps in citrus (Sapindales: Rutaceae) trees

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**Abstract**

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), vectors the bacterium causing citrus greening disease, which has devastated citrus production worldwide wherever it has been introduced. To help monitor and target *D. citri* populations in commercial groves, thereby facilitating more effective management of citrus greening disease, a prototype device has been developed that mimics *D. citri* female vibrational communication signals, attracting males to a trap. For this report, effects of the device on male *D. citri* searching behavior were assessed to consider potential improvements in field applications. Forty-five percent of the males that searched towards the female signal mimic reached the source. In addition, the mean latencies before the initiation of calling and searching responses by males that reached the source were significantly lower than for those that missed, which suggests that trapping efficiency is strongly influenced by variability in male responsiveness to searching cues. Consequently, it is likely that the trapping efficiency of vibration traps could be increased further if they were modified to make use of additional cues strongly attractive to males, such as citrus flush olfactory and visual cues.

Key Words: Asian citrus psyllid; huanglongbing; mate seeking; citrus flush; multimodal orientation

**Resumen**

El sílido asiático de los cítricos *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) es un vector de la bacteria causante de la enfermedad del enverdecimiento de los cítricos, que ha devastado la producción de cítricos en todo el mundo dondequiera que este ha sido introducido. Para ayudar a monitorear y enfocarse a las poblaciones de *D. citri* en los bosques comerciales, facilitando así un manejo más efectivo de la enfermedad del enverdecimiento de los cítricos, se ha desarrollado un aparato prototipo que imita las señales vibracionales de comunicación de las hembras de *D. citri*, que atraen a machos a las trampas. Para este informe, se evaluaron los efectos del aparato sobre el comportamiento de búsqueda de los machos de *D. citri* para considerar posibles mejoras en las aplicaciones de campo. El 45% de los machos que buscaban hacia la señal que imitaba la hembra llegaron a la fuente. Además, el promedio de la latencia antes de la iniciación de la llamada y las respuestas de búsqueda por los machos que llegaron a la fuente fueron significativamente más bajos que para los que se fallaron, lo que sugiere que la eficiencia de captura está fuertemente influenciada por la variabilidad en la capacidad de respuesta de los machos a buscar señales. En consecuencia, es probable que la eficacia de captura de trampas de vibración podría aumentar aún más si se modificaron para hacer uso de señales adicionales fuertemente atractivas para los machos, tales como señales olfativas y visuales de los brotes de nuevas hojas en cítricos.

Palabras Clave: sílido asiático de los cítrico; huanglongbing; búsqueda de pareja; brote de nuevas hojas en cítricos; orientación multimodal

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is an important pest of citrus trees around the world (Hall et al. 2013). It vectors bacteria causing citrus greening disease, huanglongbing, which has spread to 80% of Florida commercial citrus groves (Singerman & Useche 2016) since its initial detection in 2005 in Florida City (Halbert 2005). Recent estimates suggest that huanglongbing accounted for over $1.71 billion of lost revenue in the state of Florida alone between 2006 and 2011 (Hodges & Spreen 2012), with the cost growing every year through 2016 (Monzo & Stansly 2017). Optimization of *D. citri* monitoring tools is needed for improved targeting of this invasive insect vector to enable reduction of huanglongbing incidence in citrus groves (Monzo et al. 2015).

One potential approach for early detection of small *D. citri* populations is to take advantage of their vibrational communication and mate-seeking behaviors (Wenninger et al. 2009). Males initiate courtship by producing a vibrational call. If a receptive female is nearby, she quickly produces a duetting reply. The male then searches toward her, stopping and calling periodically at bifurcations or other transition points to verify the direction of her replies (Lujo et al. 2016). Rohde et al. (2013) and Mankin et al. (2013) found that males search also in response to synthetically generated reply mimics that contain multiple harmonics of the 170 to 250-Hz wingbeat frequency (Wenninger et al. 2009; Mullen et al. 2016). It was hypothesized that such behavior could be co-opted to capture *D. citri* for both sampling and population reduc-

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tion efforts (Mankin et al. 2016). For this study, we monitored behaviors of males responding to a prototype device that attracts them to a piezoelectric buzzer (target) operated by a microcontroller platform. A goal was to identify potential methods to improve trapping efficiency.

## Materials and Methods

### BIOASSAY ARENA

Tests were conducted on approximately 30-cm-tall *Citrus macrophylla* (Sapindales: Rutaceae) trees taken from the greenhouses at the USDA-ARS, Center for Medical, Agricultural, and Veterinary Entomology, Gainesville, Florida, USA. Virgin adult males, 3 to 10-d old, were obtained from a colony reared using procedures described in Paris et al. (2013). Experiments were conducted in a sound- and vibration-shielded anechoic chamber equipped with a video system enabling viewing and recording of male psyllid movement on the tree (Zagvazdina et al. 2015). Each male was tested only once. To obtain independent, real-time verification of vibrational signals, an accelerometer sensor was attached to the base of the tree (Fig. 1). The accelerometer signals were recorded and analyzed as described in Zagvazdina et al. (2015) and Mankin et al. (2016).

### PROTOTYPE DEVICE DESCRIPTION AND OPERATION

Synthetic mimics of female vibrational signals attractive to male *D. citri* were generated using a low-cost microcontroller platform (Arduino Uno, Arduino Inc., Ivrea, Italy) (Mankin et al. 2013, 2016). In addition to the microcontroller, the platform contained a circuit board that received input from an electret microphone (Model WM-63GNT, Panasonic Corp., Newark, New Jersey, USA) clamped near the base of the tree. The microcontroller processed the incoming signals and, upon detecting a male call, transmitted output signals to a piezoelectric buzzer (9S3174, Taiyo Yuden, Tokyo, Japan) to produce a reply mimic automatically. The microcontroller also had a manual trigger. The buzzer was clamped to a stem near the middle of the tree (Fig. 1). The reply mimics have approximately equal behavioral effects as



**Fig. 1.** Positions of male, accelerometer, and device microphone and buzzer on tree.

replies recorded from females when played back to the male at similar amplitudes (Mankin et al. 2013).

The microcontroller was programmed to collect signals from the microphone at an 8-kHz sampling rate. A Fast Hartley Transform (FHT) algorithm (Bracewell 1984) calculated a 128-point spectrum every 100 ms from 256 consecutive time samples. Given that male calls typically range from 140 to 700 ms in duration, a series of 6 consecutive 100-ms spectra provides an approximate spectrogram of each male call detected. Whenever the spectrum amplitude exceeded a user-set threshold, a microcontroller algorithm was initiated to assess the last 6 spectra before the amplitude decreased below threshold. The algorithm matched the 6 spectra with the last 6 spectra of a spectrogram template that had been optimized to distinguish male calls from background noise (Mankin et al. 2016). If the normalized difference between the signal and template spectrogram was lower than a user-set threshold, the signal was classified as a male call and the microcontroller immediately transmitted a mimic of a female reply signal to the buzzer. Figure 2 displays spectrogram examples of a male call followed by a female reply mimic detected simultaneously by the accelerometer and the microcontroller platform microphone. The observed differences between accelerometer and microcontroller spectrograms reflect the higher noise levels and lower sampling rate of signals collected by the inexpensive microcontroller device compared with the accelerometer recording system.

### MONITORING OF MALE SEARCHING BEHAVIOR AND DEVICE RESPONSES

Thirty-three different males were tested during the (10 AM to 3 PM) period of greatest mating activity (Wenninger & Hall 2007). The handler placed the male onto a tree leaf using the walker tool described in Pregmon et al. (2016). The leaf was on a stem that branched from the tree at a "fork in the road" where the male could decide to move toward the buzzer target or take another direction (Fig. 1). After placement, the handler allowed the psyllid to settle to 3 m before starting the timer. If the psyllid jumped or flew away, the handler attempted to find it and return it to the tree. If the insect escaped from view or would not stay on the plant, the test was discarded.

Each test lasted a maximum of 1 h after manual triggering of an initial female-reply mimic. Insect ages and the types and timings of different behaviors were noted, including calls, jumps, and crawls to the buzzer target. In addition, times were noted when the device automatically responded within 30 s after a male call. A new reply mimic was triggered after every 30-s period without activity to consider whether periodic broadcasts of female replies would induce quiescent males to begin searching. If the male contacted the target, the test ended.

### STATISTICAL ANALYSES

The latencies of (A) call and (B) search initiations by males that reached or missed the target were compared using Cox proportional hazards analysis (Cox 1972). The regression model (PHREG procedure, SAS Institute Inc. 2012) was

$$minute * status = group. \qquad (Eq. 1)$$

In Eq. 1, *minute* measured the time (in m) from the beginning of the test until (A) first call or (B) first movement towards the target, and *status* was a censoring variable. The data were censored if the test ended before calls or searches occurred. The *group* term was a regression coefficient determining the ratio of the hazard function for males that contacted the target against the hazard function for males with no
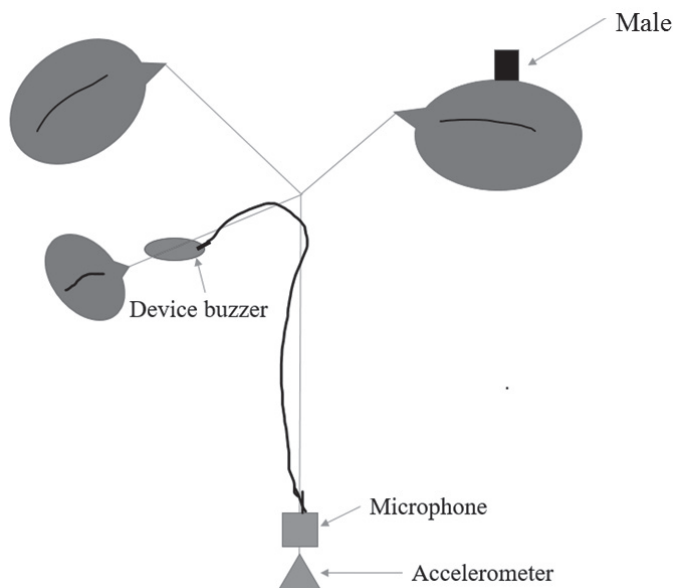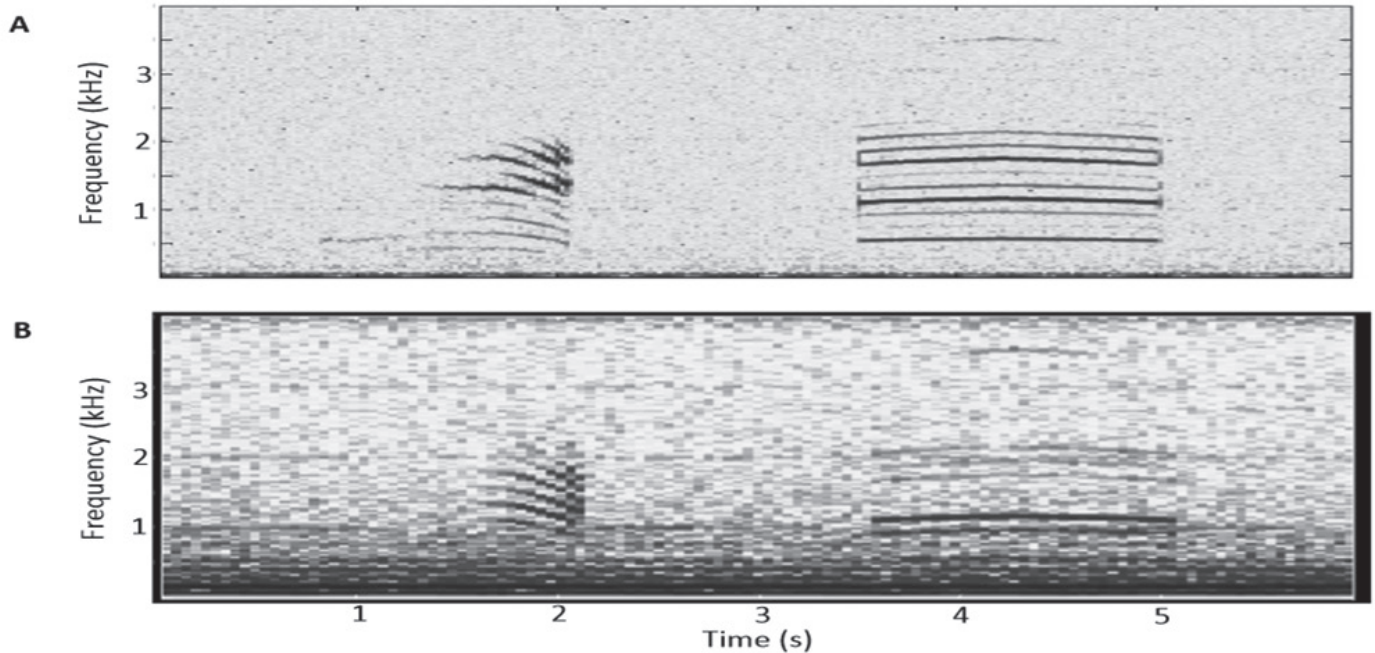
**Fig. 2.** Example spectrograms of a male call followed by buzzer response detected simultaneously by (A) accelerometer, and (B) contact microphone on the microcontroller platform. Darker color indicates greater energy at specified time and frequency.

contact. A value of zero for the regression coefficient indicated that the hazard functions were not statistically different.

Nonparametric analysis of variance (NPAR1WAY, SAS Institute Inc. 2012) was performed to compare relative

$$Search\ Rate = 1\text{-}\ t\ /\ TD, \quad\quad\quad (Eq.\ 2)$$

for males that reached or missed the target, where $t$ was the time (m) until search initiation, and $TD$ was the 60 m test duration. If movement did not occur, $Search\ Rate = 0$. The mean ages and mean rates of calls produced by males who reached or missed the target were compared using a Student's $t$-test (2-sample assuming unequal variance).

## Results

Twenty-five of 33 males in the bioassay called at least once during the 60-m tests (Fig. 3). Eleven males searched towards the target, 5 of which contacted it. Two males initiated calling behavior before any female mimic signals by the microcontroller platform device, and 23 others began calling when 1 or more signals were triggered manually. Within 30 s of an automatic or manual trigger of the device buzzer, crawling males frequently reversed direction or stopped briefly at bifurcations or other transition points. Males were not observed tapping antennae or legs across transition points to identify the sides from where mimics first appeared, behaviors which had been seen with another hemipteran known to use time delays as directional cues, *Nezara viridula* (L.) (Hemiptera: Pentatomidae) (Čokl et al. 1999). Males that reached the target called at a mean rate of 2.86 calls per m ± 8.42 Standard Error (SEM). Males that missed the target called at a mean rate of 0.307 ± 0.017 calls per m. However, due to the high variation in calling rates of successful males, the difference in mean calling rates was not statistically significant (df = 4, $t$ = -1.31, $P$ = 0.13).

In the 20 tests where a calling male missed the target, female reply mimics were triggered manually a mean of 6.41 times (0.107 triggers per m), and males responded to 43% of the signals within 30 s. In the 5 tests where the male reached the target, female reply mimics were

triggered manually a mean of 7.6 times (0.127 triggers per m), and the males responded to 42% of the signals within 30 s. The differences in the mean rates of manually triggered reply mimics and male callbacks were not statistically significant. The microcontroller triggered a female reply mimic automatically to 33% of male calls. Possible reasons for a low percentage of automated identifications of the male calls in this initial test of the microcontroller algorithm are reviewed in the discussion.

Males that reached the target had significantly shorter latencies for call initiation than those that missed, as the regression coefficient for *group* (Eq. 1) was significantly different in the survival curves for call initiation (Fig. 4A), with Wald $\chi^2$ = 6.58 (df = 1, $P$ = 0.01). Similarly, the regression coefficient for *group* was significantly different in the survival curves for search initiation with and without successful contacts (Fig. 4B), with Wald $\chi^2$ = 4.73 (df = 1, $P$ = 0.03). The shorter latencies to search initiation by males that reached the target resulted in a signifi-
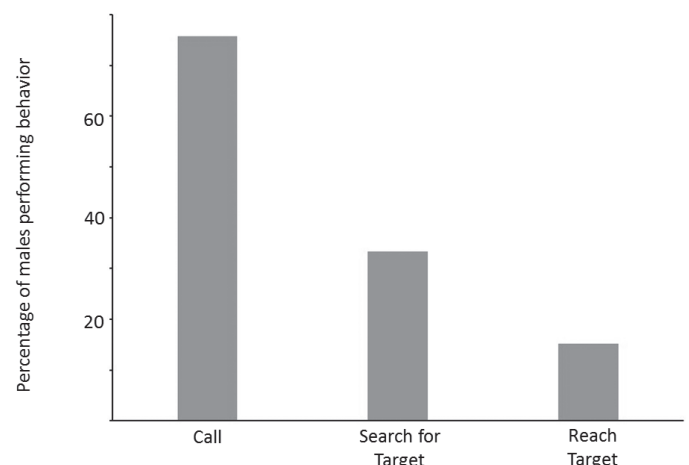


**Fig. 3.** Percentages of tested males calling, searching for target, and contacting target in response to female reply mimics.
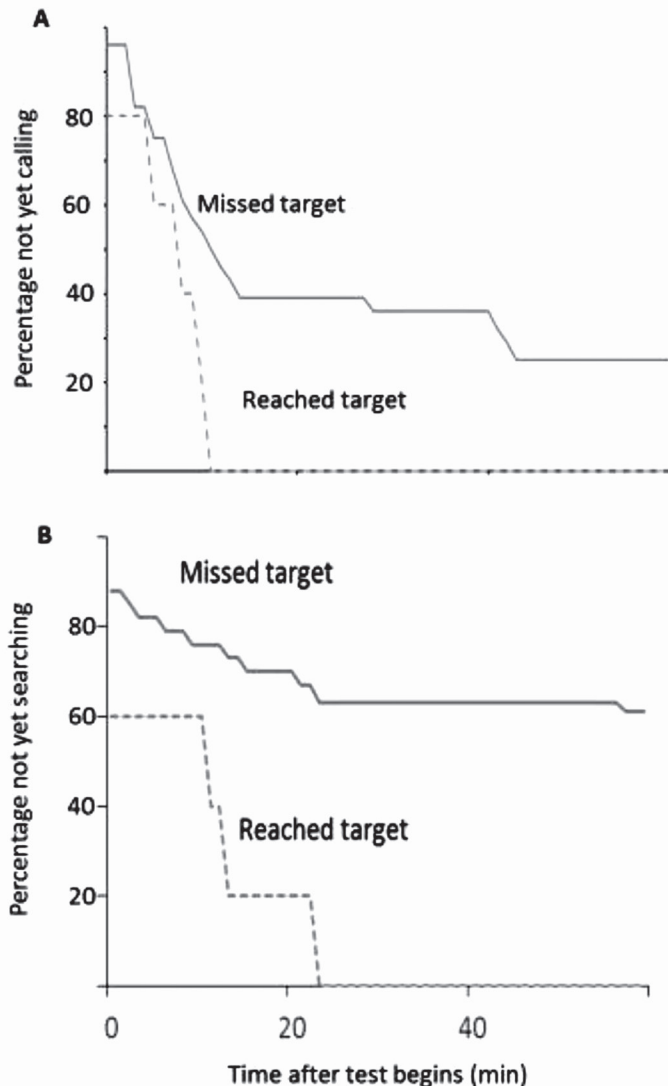
**Fig. 4.** Percentages of tested males missing target (solid line) and contacting target (dashed line) that (A) had not called or (B) had not reached target within specified time after test beginning.

cantly higher *Search Rate* (Eq. 2), 0.83 per h, compared with 0.38 per h for males that missed the target (Mann-Whitney U test, $F = 4.90$, df = 1, $P = 0.034$).

## Discussion

The finding that male *D. citri* that successfully reached the device target had significantly lower latencies for initiation of calling and searching behavior than those that missed is of both fundamental and practical interest. The differences in latencies are supportive of a hypothesis that males exhibit different levels of mating drive akin to variation observed in responsiveness of honeybees to waggle dances (von Frisch 1971) and responsiveness of male *Drosophila* to mating cues (Zhang et al. 2016). Additional investigations of variation in mating drive are needed to fully understand such phenomena, however, and the range of species and behavioral contexts where such a hypothesis may be valid.

A practical result of such variable responsiveness is that males with strong engagement in searching activity increase the trapping effective-

ness of the prototype device. Stimuli that might heighten male engagement would include the incorporation of known visual and olfactory attractants into the trap. For example, both males and females are attracted to flush (Catling 1970; Hall & Albrigo 2007; Sétamou et al 2016), light (Paris et al. 2015; Pregmon et al. 2016) and host odors (Patt & Sétamou 2010; Hall et al. 2015; Beloti et al. 2017). Consequently, placement of trapping devices near flush at the outer edges of trees likely would result in higher trapping efficiency. This is particularly important for *D. citri*, which may not be able to determine the direction of a source as effectively as other, larger hemipterans such as *N. viridula* (Čokl et al. 1999) and *Halyomorpha halys* (Stål) (Hemiptera: Liviidae) (Mazzoni et al. 2017), both of which locate mates using directional cues provided by duetting vibrations. The search behaviors of *N. viridula* and *H. halys* males indicated that they were able to use differences in the arrival times of signals at different legs or antennae and legs to sense the signal source direction (Mazzoni et al. 2014, Hager & Kirchner 2014). However, in this report and in Lujo et al. (2016), males *D. citri* often made multiple reversals between bifurcations or other transition points, which suggests they were uncertain about the signal source direction.

It should be noted that others have reported variation in engagement in mating behavior among *D. citri* males, including Wenninger & Hall (2007), who found that male latency to initiate calling significantly decreased and the numbers of calls detected in 10-m bioassays significantly increased with age. Zagvazdina et al. (2015) found that mean responses of *D. citri* males in mate-seeking bioassays decreased when barometric pressure rose or fell more than 1 standard deviation over a 24-h period. Stockton et al. (2017) found that the female-odor attractiveness to male *D. citri* increases after successful mating. In other Hemiptera, Mazzoni et al. (2017) found that male *H. halys* latency to initiate searching activity was lower for males that reached the female song source than those that missed.

The prototype device responded correctly to 33% of the male calls in this study, slightly below the 50% rate observed in Mankin et al. (2016), which raises questions about factors that influence identification of male calls by the microcontroller. Some of the variation in recognition of male calls by the microcontroller may be due to the considerable variation among calls produced by different males (Mankin et al. 2016). The device recognized calls more readily from some males than others because they matched more closely with the male call template developed in the initial study. In addition, the original template derived partly from field-collected signals of males and females in trees larger than used in the laboratory. Previous studies have demonstrated that the size and structure of the substrate affect the acoustic characteristics of detected signals (Mankin et al. 2008). Finally, power-supply noise was present on several frequency bands (see e.g., Fig. 2), which may have interfered with microcontroller identifications of male signals. Because Lujo et al. (2016) observed that reply mimics produced within 0.4 s after a male call elicited higher levels of search activity in male *D. citri* than mimics did after longer intervals, improvements in the trapping device capability to recognize male calls in field environments likely would improve its efficiency. Kuhelj et al. (2015) reported also that delays in duetting replies > 0.4 s reduced the responsiveness of male *Aphrodes makarovi* Zachvatkin leafhoppers.

Overall, the results demonstrated that the microcontroller platform device has capability to respond automatically to male *D. citri* calls and thereby to influence male behavior in the laboratory. In addition, the result that males responded within 30 s to > 40% of manually triggered reply mimics suggests that trap effectiveness is enhanced by periodic broadcasts of female reply mimics whenever males are present but have not initiated calling activity. It has been reported also that male *N. viridula* searched more frequently in the presence than in the absence of female song (Čokl et al. 1999).

## Acknowledgments

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