Interim Progress Report for CDFA Agreement Number 14-0272-SA

SUBSTRATE-BORNE VIBRATIONAL SIGNALS IN INTRASPECIFIC COMMUNICATION OF GWSS

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Introduction

Epidemiological models suggest that vector transmission efficiency, vector population density, and the number of plants visited per vector per unit time are key factors affecting rates of pathogen spread (Jeger et al. 1998). Measures to reduce glassy-winged sharpshooter (GWSS) (*Homalodisca vitripennis*) population density in California include an area-wide insecticide application program and release of natural enemies. Despite such efforts, geographic distribution and population densities of GWSS continues to expand. Chemical control of GWSS in urban areas, organic farms, and crops under integrated pest management programs is problematic because insecticides are ineffective, not used, or incompatible with existing practices, respectively. The near-zero tolerance for GWSS in vineyards, particularly in areas where Pierce's disease is endemic, poses a constant challenge for growers and agencies involved in the area-wide program. Thus, long-term suppression of GWSS populations will rely heavily on novel methods.

Vibrational communication is a widespread form of communication in invertebrate and vertebrate animals including fish, amphibians, reptiles, birds, and mammals (Cocroft et al. 2014). Arthropods emit vibratory signals in connection with aggression, distress, calling, courtship, rivalry, searching, and other behaviors associated with finding conspecifics and avoiding predation (Čokl and Virant-Doberlet 2003). In leafhoppers, mate recognition and localization are mediated exclusively via substrate-borne vibrational signals transmitted through the plant. Vibrational signals in leafhoppers are low-frequency bending (or flexural) waves produced by the abdomen. Signals are transmitted through the legs to the substrate and travel at a speed of about 100 m/s. Signals are detected by the receiver presumably by subgenual and joint chordotonal organs located in the legs (Čokl and Virant-Doberlet 2003).

Exploitation of attractive vibrational signals for trapping leafhoppers or disrupting mating, as well as excluding pests via emission of repellent signals have been considered, but not yet implemented in commercial agricultural landscapes (Polajnar et al. 2014). In Florida, an experimental prototype of a microcontroller-buzzer system attracted the Asian citrus psyllid, *Diaphorina citri*, to branches of citrus trees by playback of insect vibrational signals (Mankin et al. 2013). Recently, small-scale field studies on mating disruption of leafhoppers via playback of vibrational signals through grapevines have demonstrated promising results. Specifically, electromagnetic shakers attached to wires used in vineyard trellis successfully disrupted mating of *Scaphoideus titanus*, vector of a phytoplasma that causes the grapevine disease Flavescence dorée in Europe (Eriksson et al. 2012). Exploitation of disruptive, attractive, and/or repellent signals for suppressing GWSS populations could prove to be a useful tool. However, existing knowledge on GWSS vibrational communication is insufficient to implement a management program for this pest in California.

Objective

To identify and describe substrate-borne signals associated with intraspecific communication of GWSS in the context of mating behavior.

- The experiments are complete for the main objective of this proposal. Description of the methods remains below. GWSS vibrational signals were analyzed for structural and spectral properties. New findings, while still being statistically analyzed, are described in the summary section along with some of the previous findings.

- Manuscript preparation is underway with expected submission date to the journal circa June 2016.

- Success of this project marks the beginning of experiments designed to disrupt mating of GWSS. A brief description of preliminary methods is included in the future directions section below.

Description of methods conducted to accomplish the objective

Test insects and plants. This research project is being conducted at the USDA-ARS SJVASC in Parlier, California. Discovery in the GWSS behavioral analyses are bioassay-driven and focus on the identification of signals that initiate natural responses to conspecifics. Test plants used in the bioassays reported here were okra (*Abelmoschus esculentus* (L.) Moench) and grapevine (*Vitis vinifera* L.). All plants were kept in an insect-free greenhouse located at the SJVASC until used in the experiments. Insects used in the experiments were obtained from colonies maintained year-round in a containment facility located at the SJVASC. Colonies were established using GWSS originated from Bakersfield, CA and maintained in cages (Bug Dorm-2®, BioQuip Products, Rancho Dominguez, CA) using four plant species: cowpea (*Vigna unguiculata* L. Walp. cv. 'Blackeye', Vermont Bean Seed Co., Randolph, WI, USA), basil (*Ocimum basilicum* L. cv. 'Genovese' Ferry-Morse Seed Co., Fulton, KY, USA), okra, and sunflower (*Helianthus annuus* L. cv. 'American Giant Hybrid', Ferry-Morse Seed Co., Fulton, KY, USA). Late-instar (4^{th} and 5^{th}) GWSS nymphs obtained from colonies were separated by gender in cages to generate virgin adult individuals. After molting to the adult stage, a female was transferred individually to a mesh-screen tube cage (10 cm diameter × 40 cm height) containing a cowpea plant. Reproductive maturity in about 150 individually caged females was determined by oviposition of non-fertilized eggs. Virginity of females was confirmed by checking eggs daily for signs of embryo development or nymph emergence. Male insects used in the experiments described below were of the same age as reproductively active females. After female reproductive maturity and virginity was confirmed, test insects were used in the vibrometry experiments described below.

The vibrometer. Experiments were conducted in a transparent experimental arena (60-cm length × 60-cm width × 80-cm height) made of 1-cm thick acrylic walls. The arena was centered inside a chamber formed by 86-cm × 86-cm × 98-cm high black fabric and sound isolating walls. The arena and chamber were placed on an active vibration isolation table (Model 20-561, Technical Manufacturing Corporation, Peabody, MA) to reduce external noise. LED lights were affixed to the top of the chamber. Insect behaviors were monitored via video surveillance and recorded to a computer. Vibrational signals produced by individuals were recorded, digitized, and measured using a laser Doppler vibrometer (NLV-2500, Polytec, Inc., Irvine, CA) and associated softwares (Raven Pro 1.5, The Cornell Lab of Ornithology, Ithaca, NY and Adobe Audition® C26, Adobe Systems, Inc., San Jose, CA). Recorded vibrational signals were digitized with 44.1 kHz sample rate and 32 bits resolution and analyzed using FFT type Hann with a window size of 2560 samples and overlap of 80% to characterize and determine key spectral and temporal features of signals such as frequency span, dominant frequency, intensity, and pulse repetition rate. Recorded signals were used to perform playback experiments conducted with an electrodynamic mini-shaker (Type 4810, Brüel & Kjær, Inc., Norcross, GA) driven by a computer, where individuals were stimulated to produce natural behavioral responses to selected signals transmitted on host plants.

Bioassay methodology. Bioassays were conducted between 0800 and 1900 h at 25 ± 0.5°C. Before testing, insects were allowed 15 min to acclimatize to ambient conditions in 130-ml plastic vials placed within the chamber housing the plant. After the acclimatization period, insects introduced into the acrylic arena had free movement to and from a host plant. A series of laboratory studies were conducted to identify and describe GWSS vibrational signals. First, virgin males (n = 21) and females (n = 25) were placed on host plants individually to identify common and unique signals produced by each gender. Second, males and females were paired (n = 32) on host plants to identify signals used in advertisement and species recognition, male-female duetting that result [or not] in oriented movement of one individual to another, and courtship. Third, groups of individuals (two males and one female) (n = 30) were placed on plants to identify potential rivalry or distress signals. Trials consisted of 90-min observations. In cases where mating occurred during the trials, the couple was immediately transferred to a tube cage containing a cowpea plant and kept until copulation ended. After copulation, the male was removed and preserved in alcohol and the female was kept individually on a plant until fertility was confirmed by deposition of fertilized eggs. All four conditions described above (insects alone and in groups) were tested on the same day. The order in which the four conditions were tested was randomized within each day.

Data analysis. The data set is complete. Data from vibrometry were analyzed to characterize the spectral and temporal features of signals such as frequency span, dominant frequency, intensity, and pulse repetition time. Statistical comparison of signals produced by individuals tested under different conditions is underway.

Summary of accomplishments and results

Signal analysis

Several call types were identified for GWSS, with male and female calls being unique to each sex. Females possessed two calls that were identifiable and analyzed. Conversely, male calls had one main call but with two additional components that may or may not be present in some calls. Furthermore, the main male call could be broken up into two sections (MCs2 and MCs3) resulting in some male calls having four sections (Fig 1). The main female call (Fig 2) and main male call were used by the animals when they were alone on the plant as well as in duets during the first stages of communication. There were no significant differences in the main call duration or dominant frequency when signaling alone or in a duet or when compared within or between sexes (Fig 3). However, the second type of call emitted by females (PCS), which was emitted just before mating, was significantly shorter and had a lower dominant frequency. The addition of a male wing flip behavior (MCcrs) created a significantly longer signal but did not change the overall dominant frequency. Another male signal, called quivering (MCs1), varied greatly in length and was significantly longer than other signal components (duration 20.4 ±13.2s). Dominant frequency of MCs1 was lower than the other calls (Fig 3B). The main female and male calls increased in frequency during the duration of the call. The increased frequency range for females was smaller than that of the males (female: 8.9 ± 6.7, male: 25.5 ± 12.6).

Behavioral Analysis

When placed alone on plants, males were less likely to signal than females, with 19% of the animals signaling (n = 21) compared with 79% of females (n = 25) ($G = 15.61$, $P < 0.001$). The latency to begin calling was longer for males ($F = 7.715$, $P = 0.012$) than females (Fig 4A). Within females, there was large variation in the latency to begin calling as well as the calling rate. Calling rate varied from 0 to 143 calls during a 45-min recording. When normalized on a log scale, females showed a shorter latency to calling when they had a faster calling rate ($F = 6.45$, $P = 0.023$) (Fig 4B).

Recordings of a virgin male and female placed together on the plant revealed a complex series of behaviors linked to vibrational signals that lead to mating, or not. Male-female GWSS communication can be divided into three stages: 1) duet, 2) courtship and location, and 3) precopula. In Stage 1, females initiated the duet in 15 of 21 cases. After the initial duet lead by the female, the behavior was reverted with the male leading duets. During the location phase (Stage 2), the female remained on the same position on the plant as the male searched and approached her. During Stage 2, the male added another component to the beginning of the signal, quivering (MCs1), which was alternated with MCcrs and MC. Males searched for the female on the plant by alternating a walking behavior and short stops to emit additional signals; likely to maintain communication with the female. The length of the duet was variable from as few as two calls each to over 10 calls each and does not appear to be correlated with the final outcome of mating. Mating success occurred in 55% of the animals (n = 20). In many cases, females were not receptive for mating despite long duets with males. To avoid copulation, females lifted the posterior part of the body and stretched the hind legs to keep males away. Unsuccessful courtship interactions only entered the second stage of communication in 55% of the cases (n = 5/9), while successful courtship entered the second stage 100% of the cases (n = 12/12). When mating was successful, the couple remained in copula for more than 6 hours. All mating pairs produced viable progeny.

GWSS male-male rivalry signals were recorded during establishment of dominance and subordination between males competing for mates. Different rivalry signals are currently being characterized (see one example in Figure 5). These rivalry calls had a much lower range of frequency increase (male-female male call: 25.5 ± 12.6, male-male male call: 9.8 ± 7.05). Preliminary data show that visual signals, physical contact, and specific vibrational signals may be used by GWSS to establish male hierarchy and thus, preferential access to mates. In relatively more aggressive situations, a male bended his body by lowering the posterior part of the abdomen forming an arc. Male rivalry signals were observed after Stages 1 and 2 of male-female communication. In cases where male rivalry was observed, mating occurred in only 4 of 12 trials. However, GWSS females can also be “choosy”, which suggests that both intrasexual (male-male combat) and intersexual (mate choice) selection may occur in GWSS. While GWSS male rivalry appears to negatively impact courtship behaviors, it is not known if the overall reproductive success of a male can be artificially affected by playback of rivalry signals.

Future Directions

In conclusion, the project is providing a detailed description of vibrational communication signals that are key for understanding fundamental behaviors of GWSS for mating success. Our work has shown that 1) GWSS uses substrate-borne vibrational signals in intra- and inter-gender communication and 2) specific signals are required for GWSS to achieve mating. Although the role of some signals reported here could be inferred from observations, the role and relevance of individual signals to insect behaviors can be ultimately determined only when insects are stimulated via playback of select signals. Construction and testing of an arena (Fig 6) to conduct playback experiments and mating disruption trials is underway. So far, playback of GWSS calls through a wire similar to the ones used in vineyard trellis matches the frequency spectra of natural calls. In the upcoming season, pending availability of research funds, field trials are planned to validate results that will be obtained with the arena and playback methods. The ability to establish a communication channel and elicit GWSS response to select signals represents an important step towards the next goal, which is to determine the role of specific signals in GWSS communication and identify signals capable of influencing GWSS behavior for applicative purposes (e.g., disruption of mating communication, attraction).

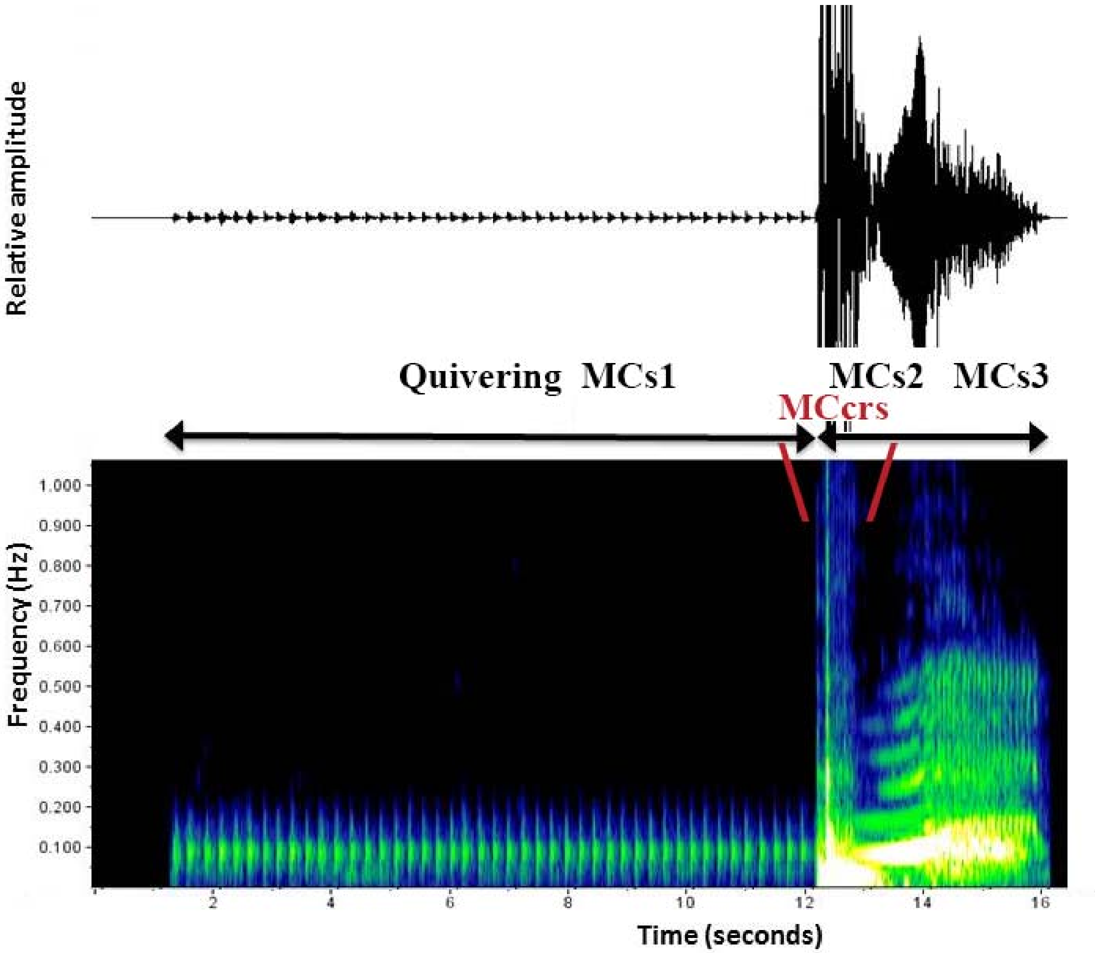

Figure 1. Oscillogram (above) and spectrogram (below) of a GWSS male call (MC2). Three different sections of MC2 are indicated by MCs1, MCs2, and MCs3.

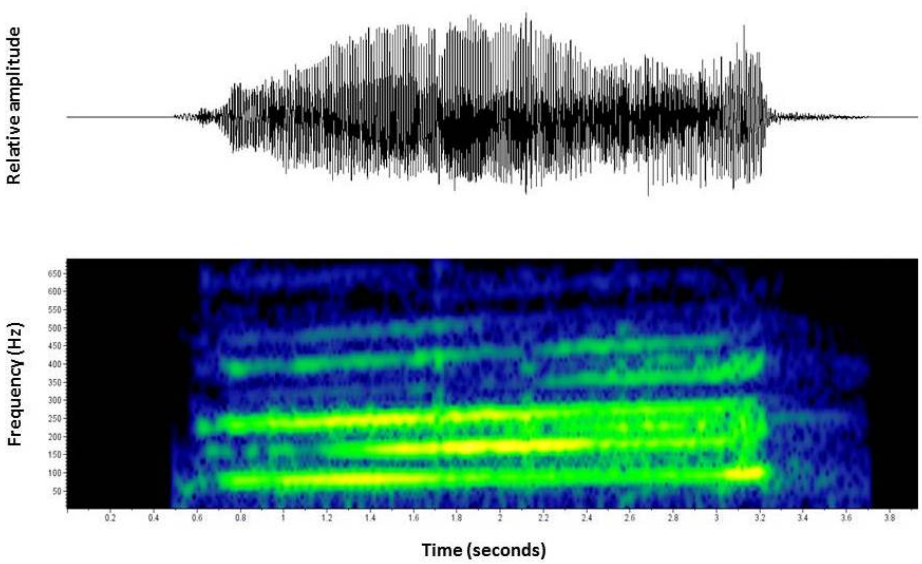

Figure 2. Oscillogram (above) and spectrogram (below) of a GWSS female call (FC).

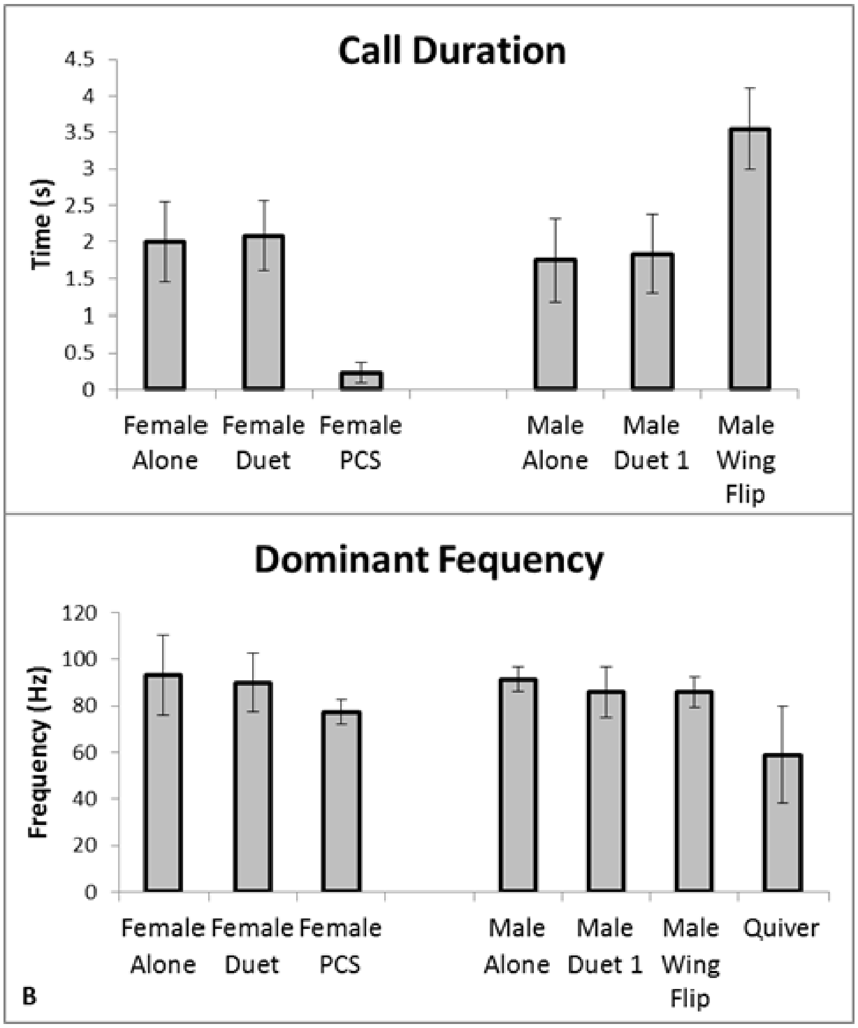

Figure 3. Analysis of duration (A) and dominant frequency (B) of the various calls used in GWSS communication.

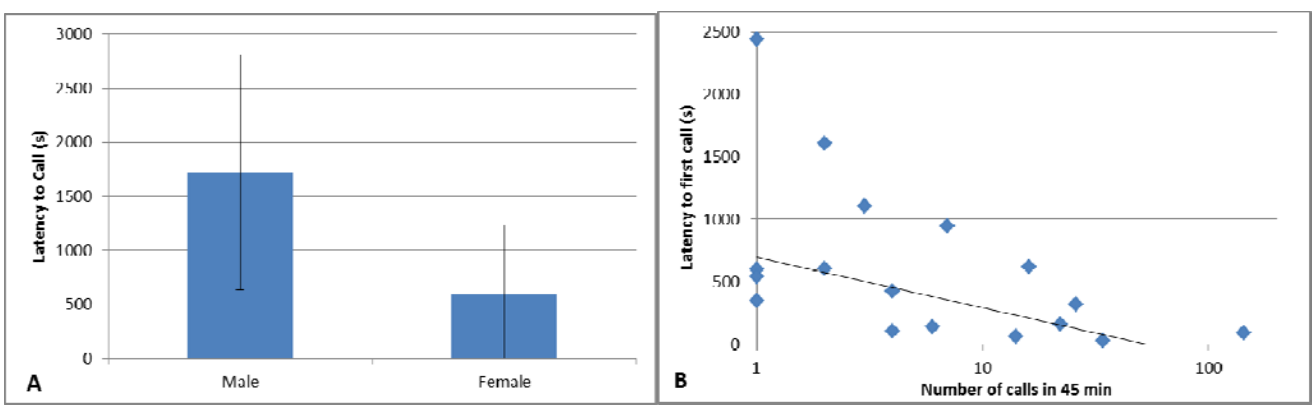

Figure 4. Mean time taken for isolated GWSS individuals to begin calling (i.e., latency to call). A) Average male (n = 4) and female (n = 18) latency to call. B) Female latency to call based on calling rate over a 45 min trial.

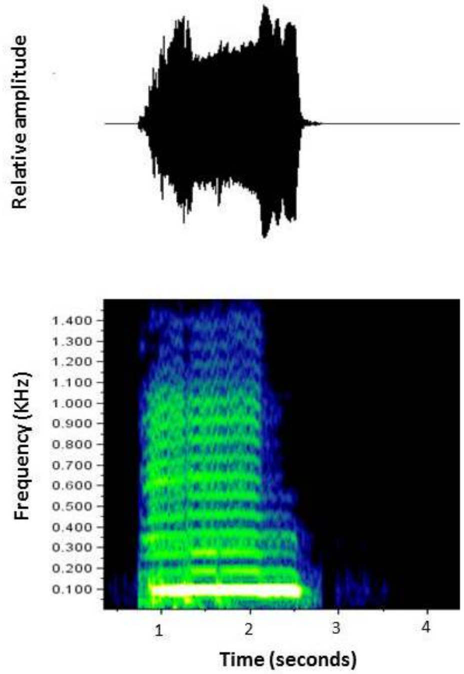

Figure 5. Oscillogram (above) and spectrogram (below) of an aggressive signal of a male GWSS produced during a male-male competition for a female.

Figure 6. Arena designed for studies on the effects of playbacks on GWSS behaviors. The light source is above. Plant(s) are leaned on wires with the shaker attached on the outside/right of the cage. When the door is closed, white sleeves enable access into the cage while a blackout screen isolates animals from the observer. The laser Doppler vibrometer is in the bottom right of the image. Signal intensity and spectral properties can be monitored with the laser shot through the mesh, or through a sleeve for higher resolution. Insects will be released in the cage in pairs (male and female) to determine effectiveness of select signals in disrupting mating or in groups to study aggregation or dispersal behaviors.

Publications produced and pending, and presentations made that relate to the funded project

Nieri, R, Mazzoni, V., Gordon, S.D., and Krugner, R. Substrate-borne signals involved in the mating behavior of the glassy-winged sharpshooter. Manuscript in preparation.

Krugner, R. 2015. GWSS communication: We heard it [Directly] Through the Grapevine. Seminar at the Department of Biology, California State University-Fresno, September 11, 2015.

Krugner, R. 2015. Substrate-borne Vibrational Signals in Intraspecific Communication of the Glassy-winged Sharpshooter. Seminar at the Department of Entomology, UC-Riverside, April 6, 2015.

Krugner, R., and Mazzoni V. 2015. Substrate-borne vibrational signals in intraspecific communication of GWSS. pp. 104-111. In T. Esser and R. Randhawa (eds.), 2015 Pierce's Disease/GWSS Progress Report. California Department of Food and Agriculture, December, Sacramento, CA.

Nieri, R., Mazzoni, V., and Krugner, R. 2015. Substrate-borne signals involved in the mating behavior of the glassy-winged sharpshooter (GWSS). Poster presentation at the 2015 Entomology Congress of the German Society for General and Applied Entomology. March 2-5, 2015, Frankfurt, Germany.

Krugner, R. 2014. Substrate-borne Vibrational Signals in Intraspecific Communication of the Glassy-winged Sharpshooter. Oral presentation at the 2014 CDFA Pierce's disease Research Symposium. December 15-17, 2014, Sacramento, California.

Krugner, R., and Mazzoni V. 2014. Substrate-borne vibrational signals in intraspecific communication of GWSS. pp. 2-5. In T. Esser and R. Randhawa (eds.), Proceedings, 2014 Pierce's Disease Research Symposium. California Department of Food and Agriculture, 15-17 December, Sacramento, CA. Time Printing, Sacramento, CA.

Research relevance statement, indicating how this research will contribute towards finding solutions to Pierce's disease in California

This project encompasses three compounding phases build on research findings of previous phases: 1) Exploratory Phase – identify and describe the substrate-borne signals associated with intraspecific communication of GWSS; 2) Developmental Phase - determine which signals are effective to attract, repel, and/or disrupt mating of GWSS; and 3) Application Phase - technology transfer for implementation of a sustainable management strategy for GWSS. Our work has shown that 1) GWSS uses substrate-borne vibrational signals in intra- and inter-gender communication and 2) specific signals are required for GWSS to achieve mating. Pre-recorded GWSS signals were used to perform preliminary playback experiments, where males and females were artificially stimulated to produce natural responses to signals transmitted to host plants. In these preliminary trials, we were able to establish communication with GWSS males and females using pre-recorded female and male calls, respectively. The ability to establish a communication channel and elicit GWSS response to select signals represents an important step towards our next goal, which is to determine the role of specific signals in GWSS communication and identify signals capable of influencing GWSS behavior for applicative purposes (e.g., disruption of mating communication, attraction).

Layperson summary of project accomplishments

Leafhoppers and sharpshooters communicate via vibrational signals transmitted through the plant. Signals are very low frequency and intensity "sound" waves that could be the key to a novel control method that may be incorporated in an integrated management strategy. A laser-Doppler vibrometer was used to identify and describe signals used by glassy-winged sharpshooter (GWSS) to communicate. GWSS uses signals in intra- and inter-gender communication with specific signals required to achieve mating. Bioassays using paired virgin males and females on plants revealed that GWSS males search for females on plants while females wait for males to approach. Visual signals, physical contact, and specific vibrational signals are used by GWSS to establish male and female hierarchy and preferential access to mates. However, GWSS females also can be "choosy", suggesting that both intrasexual (male-male combat) and intersexual (mate choice) selections may occur. While GWSS rivalry calls negatively impact courtship behaviors, it is not known if the overall reproductive success of individuals can be artificially affected by signal playbacks.

Since what they “say” to each other has a big effect on behaviors, signals may be exploited as an attractant, repellent and/or disruptive signal which could be a useful, non-chemical control method for suppressing GWSS populations. Can these sounds be reproduced to manipulate GWSS behaviors? The answer is: Yes. In the laboratory, mini-shakers and speakers deliver pre-recorded natural sounds or synthetic sounds to plants through a trellis wire thereby artificially stimulating individuals to produce natural responses to playback signals. In preliminary trials, communication (duets, trios, and quartets) with GWSS males and females has been established using pre-recorded calls. The ability to establish a communication channel and elicit GWSS response to select signals represents an important step towards the next goal: identification of signals capable of influencing GWSS behavior for disruption of mating.

Status of funds

Part of funds obtained through an agreement between USDA and CDFA were successfully transferred to Fondazione Edmund Mach to cover Dr. Mazzoni’s travel expenses during the first visit to the laboratory in Parlier (May to July 2015: $3,536). A total of $7,939 is available for travel expenses of the second trip to Parlier planned for March 31 to June 10, 2016. Funds obtained for salary and benefits to support a post-doctoral researcher were exhausted in early March 2016.

Summary and status of intellectual property associated with the project

None

Literature cited

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