FEEDING BEHAVIORS OF THE GLASSY-WINGED SHARPSHOOTER THAT CONTROL INOCULATION OF *XYLELLA FASTIDIOSA*

Project Leader:
Elaine Backus
USDA, ARS, PWA
SJVASC
Parlier, CA 93648
elaine.backus@ars.usda.gov

Primary Researchers:
P.H. Joost
formerly Dept. of Entomol.
University of California
Riverside, CA 92521

S. Dugravot
formerly Dept. of Entomol.
University of California
Riverside, CA 92521

B. Reardon
formerly Dept. of Entomol.
University of California
Riverside, CA 92521

Holly Shugart
USDA, ARS, PWA
SJVASC
Parlier, CA 93648

Cooperators:
JC Chen
USDA, ARS, PWA
SJVASC
Parlier, CA 93648
jianchi.chen@ars.usda.gov

G. Walker
Dept. of Entomology
University of California
Riverside, CA 92521

T. Miller
Dept. of Entomology
University of California
Riverside, CA 92521
thomas.miller@ucr.edu

David Morgan
Calif. Dept. Food & Agric.
Mt. Rubidoux Field Station
Riverside, CA 92521
dmorgan@jps.net

W. Holmes
Dept. of Biology
California State University
Fresno, CA 93710

F. Schreiber
Dept. of Biology
California State University
Fresno, CA 93710
freds@csufresno.edu

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ABSTRACT
In the final eight months of this grant, we completed remaining studies and emphasized writing results for several publications. Overall, all objectives of this grant were successfully completed. The work identified the electrical penetration graph (EPG) waveforms responsible for both ingestion and egestion by the glassy-winged sharpshooter (GWSS), and provided evidence that the mechanism of inoculation of *Xylella fastidiosa* (*Xf*) is a combination of egestion and salivation. Objective 1 successfully showed that ingestion is represented by the EPG waveform C, which nearly always occurs in some type of xylem cell on susceptible grape. Earliest events of waveform C (usually short in duration) often occur in non-functional, primary xylem, whereas later events (longer in duration) always occur in lignified, presumably functional, secondary xylem. GWSS adults virtually never ingest from non-xylem cells while on susceptible grape. Objective 2 showed that the B1s waveform is correlated with muscular fluttering of the precibarial valve, and that the B1w waveform represents salivation. These waveforms occur throughout the pathway phase of feeding, thus occur in all cell types that are penetrated. During Objective 3 work, several experiments with both GWSS and smoke tree sharpshooter also identified for the first time the sharpshooter X-wave, a waveform family that definitively represents xylem penetration by the stylets. The X-wave incorporates the waveforms B1w, B1s, proto-C, and C. X-ray images of GWSS feeding taken at the Argonne National Lab are also discussed. Taken together, our findings support that the B1s and proto-C of the X-wave represent egestion (expulsion) of fluids from the precibarium into xylem, and likely represent the instant that *Xf* cells are inoculated.

INTRODUCTION
The behaviors comprising within-plant feeding (a.k.a. stylet penetration) of hemipteran vectors are intricate and complex, and vary enormously among species. Yet, a deep understanding of stylet penetration is particularly important for sharpshooter vectors because behavior plays a crucial role in transmission of non-circulatively transmitted pathogens like *Xf*. Thanks to EPG monitoring, sharpshooter stylet penetration can now be observed in detail, in real-time. Two stylet penetration behaviors emphasized in this project likely control *Xf* inoculation. They are uptake of plant fluids into the gut (ingestion) and expulsion of bacteria-laden fluids (egestion or extravasation).

OBJECTIVES
1. Characterize ingestion behavior, especially to: (a) identify in which cell types various durations of ingestion (C) are occurring, and (b) how to recognize that by EPG alone.
2. Characterize extravasation (now termed egestion) behavior, especially to: (a) correlate the B1 waveform with fluid flow in and out of the stylets, and (b) determine in which plant cells this behavior occurs.

3. Characterize behavior-*Xf* interactions that permit inoculation, especially to (a) identify the behaviors (i.e. ingestion, egestion or both) during which bacteria are expelled, and (b) whether bacterial expulsion is into xylem, or any plant cell type penetrated, or both.

RESULTS

Insect Availability

This year, we were able to resume use of lab-reared insects very kindly provided by D. Morgan, CDFA.

Objective 1 – Correlation of ingestion with EPG Waveforms

Study a: Ingestion-waveform correlations and cell types in which ingestion occurs

Prior to this grant, we correlated the C waveform (Figure 1b) with ingestion via observation of particle movement in artificial diets (Joost et al. 2005). Last year's electromyographic (EMG) study of cibarial muscle potentials by former post-doc S. Dugravot showed that C waveform was also correlated with both cibarial dilator activity and excretory droplet production. Two to four cibarial pumps produced a single droplet. This year, additional analysis of the data showed that the fine structure of the C waveform is correlated with directional flow of fluid through the foregut. Thus, whether fluid is flowing into (rise portion of each plateau) or out of (falling portion) the cibarium can be judged by voltage changes alone. A paper of these findings is *in press* (Dugravot et al. 2008).

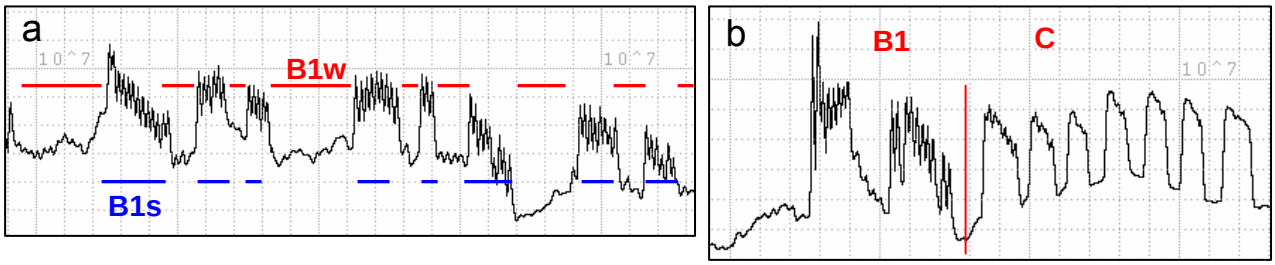

Figure 1. Examples of the main EPG waveforms studied in this project, in the order in which they occur during stylet penetration. **a.** The B1 waveform, composed of B1w (wave) and B1s (spikelets). This waveform occurs frequently throughout pathway and interruption phases. **b.** The C waveform, following a length of B1. C waveform is the landmark waveform for ingestion phase.

Last year we also performed a study at the Argonne National Lab. EPG waveforms were recorded from wired GWSS that were subjected to high-energy X-ray imaging during feeding. This allowed the cibarial muscle movements to be viewed via movements of two sets of tracheae that apparently aerate different functional groups of the cibarial muscles. Video images and waveforms were further analyzed during this year. Results to date confirm and expand upon the EMG study. The two sets of muscle groups move at different times during feeding. Major, coordinated convulsions of both muscle groups are correlated with the rise phase of the C waveform, and therefore with the muscle potentials and cibarial pumping previously recorded. However, very small contractions of the major cibarial muscle group were correlated with a new waveform, proto-C. We believe these small, C-like plateaus represent test pulls of the cibarial pump. In addition, atypical C waveform shapes, hypothesized to correlate with ingestion from non-xylem cells, were actually caused by unusual cibarial muscle contractions. In fact, C waveform fine structure was entirely correlated with cibarial muscle movements alone.

Last year, we reported results of a third project to determine which ingestion events were performed in xylem, and whether xylem ingestion can be identified by waveform appearance alone. Further analysis this year found that waveform C always occurred in xylem, but any of several xylem cell types. Very early, especially short-duration, C events occurred in primary proto-xylem cells or small, unlignified secondary xylem cells. In contrast, later, longer-duration, C events occurred in large, lignified secondary xylem cells. The thesis for this work was completed this year (Holmes 2007) and a manuscript is in preparation.

Study b: Recognizing ingestion from waveforms alone

Results from Study a, altogether, support that waveform C represents ingestion (i.e. cibarial pumping), but its fine structure is not correlated with ingestion tissue type, as we hypothesized. Instead, waveform C predominantly occurs in xylem, and the type of xylem cell is correlated with the C event's order and/or event duration.

Objective 2 – Correlation of egestion with EPG Waveforms

Study a: Correlate B1 waveform with muscle movements and fluid flow in and out of stylets

A second EMG study was also performed last year, and results were analyzed this year. Muscle potentials were recorded from the precibarial valve muscles, which were hypothesized to control egestion. Results conclusively showed that the valve's muscle potentials occurred only during pathway and were temporally correlated with B1 spikelet bursts (B1s)

(Figure 1a). Muscle potentials also strongly resembled waveform B1s (data not shown) (Backus & Dugravot 2008). Thus, B1s represents voluntary valve fluttering, and this occurs only during pathway phase, not during ingestion. The directionality of fluid flow (described in Objective 1 results, above) further supports that fluid moves up and down within (and possible out of) the precibarium during B1s.

Study b: Determine in which plant cells B1 occurs

A combination of the precibarial EMG study and Holmes sheath histology study, above, show that B1 occurs throughout pathway, in all cell types penetrated by the stylets. Work described below shows that B1 also can occur in xylem cells, just prior to or interrupting ingestion events.

Objective 3 – Characterize behavior-*Xf* interactions that permit inoculation

Study a: Identify the behaviors (ingestion, egestion, or both) during which bacteria are expelled

Last year's report detailed findings by two previous post-docs, P.H. Joost and S. Dugravot, that green fluorescent protein (GFP)-expressing *Xf* were seen embedded in salivary sheaths in artificial diet on which putatively inoculative GWSS fed. Yet, only eight out of a combination of nearly 81 EPG-recorded probes revealed GFP, as confirmed by confocal microscopy. Problems with our insects acquiring the GFP-*Xf* had been the cause. This year, a third post-doc, B. Reardon, attempted this diet-inoculation project again. He found two out of 30 confirmed salivary sheaths with GFP-*Xf* (Figure 2). We suspect that the rarity of GFP-*Xf* in diet was due to lack of acceptance of our diet. Nonetheless, these findings strongly support that *Xf* is inoculated during salivation.

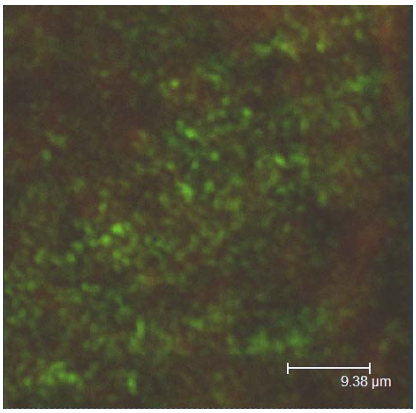

Figure 2. Confocal laser scanning micrograph of green GFP-*Xf* inside the lumen of a GWSS salivary sheath in artificial diet.

During completion of the Holmes (2007) thesis project in the last year, fine-structure analysis of the interruptions (waveform N) between trial ingestion (C) events was performed. We found that each interruption consistently was composed of B1w, B1s, and proto-C waveforms (Figure 3). At the same time, further analysis of the Argonne X-ray data suggested (although the data are not yet fully analyzed) that the smaller of the cibarial muscle groups twitches slightly but rhythmically during B1w, during both pathway and interruption phases. This supports that "micro-ingestion" of a small amount of fluid occurs, probably into only the precibarium, where it can be tasted by the precibarial chemosensilla. In addition, salivary sheath tips were always in a xylem cell during interruption and trial ingestion events. The first "interruption" (actually at the end of pathway, before the first C event) marked the first stylet penetration of the ingestion cell; this is the definition of a specialized EPG waveform family called the "X-wave" that is seen in many vectors species. Thus, we have now discovered the sharpshooter X-wave (Figure 3), and theorize that it is the *Xylella* inoculation behavior.

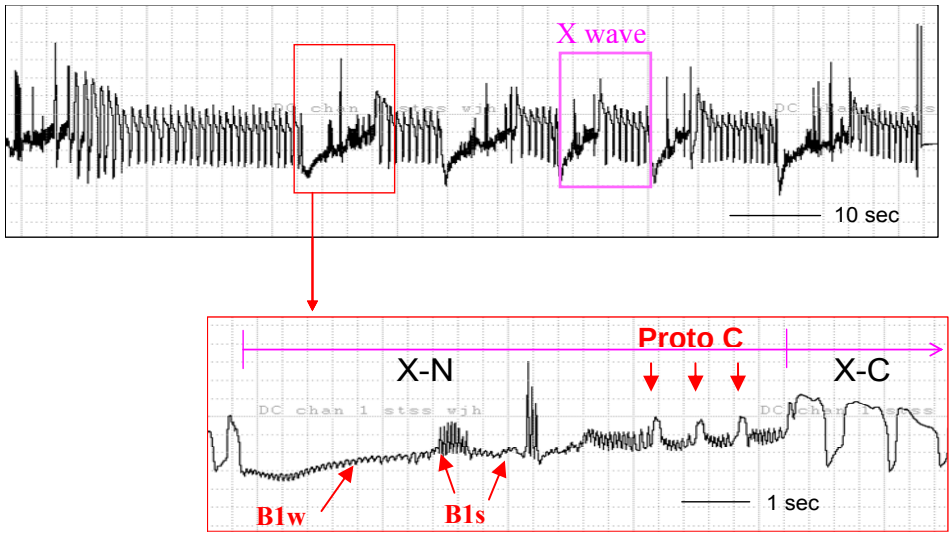

Figure 3. A repetitive series of sharpshooter X-waves (one in pink box). Each includes interruption and trial ingestion events. The red box, expanded below, shows components of the X-wave.

The behaviors of the X-wave apparently function in tasting the fluid in the xylem cell to determine its acceptability, and testing the strength of the mechanical seal of the stylets. B1w represents watery salivation (Joost et al. 2005), perhaps simultaneous with micro-ingestion into the precibarium for the purpose of tasting partially-digested xylem fluid. If so, the insect would be micro-ingesting a mixture of plant fluid and its own saliva. B1s is precibarial valve fluttering, which probably further aids movement of fluids back and forth across the sensilla, during tasting. When tasting is nearly complete, proto C may represent small contractions of the large cibarial muscle group, for "mini-ingestion" into the cibarium. Perhaps when these muscles release, some fluid can be pushed back into the precibarium, then leak past the valve and into the stylets. This would be egestion of a mixture of plant fluid and saliva.

Thus, there is good evidence that the component waveforms of the X-wave represent all behaviors necessary for *Xf* inoculation via a mechanism of combined salivation and egestion. We are in the process of conclusively testing this hypothesis with *Xf* inoculation studies underway.

Study b: Determine into which plant cells bacteria are expelled

In Backus 2007, we describe a plant inoculation experiment using EPG-identified probes that artificially terminated stylet penetration after 3-6 min of ingestion phase (3 to 8 X waves). This work complemented the diet inoculation study described above. PCR evidence from that study showed that a single GWSS probe of this type could inoculate enough *Xf* to kill the test plant (3 months later), in 100% of 36 plants tested. This indirectly supports that *Xf* is inoculated into the xylem during X waves, at the start of ingestion. Thus, duration of ingestion probably does not directly relate to inoculation. This experiment is the first time that GWSS has been shown to exhibit 100% vector efficiency per individual insect, let alone from a single probe. This project will be repeated with histology of salivary sheaths in the coming year, using a new method we have developed to retain the fluorescence of GFP-*Xf* through paraffin sectioning and process for confocal microscopy (see Backus & Labavitch 2007).

CONCLUSIONS

We have succeeded in meeting all objectives of this grant proposal. The accumulated evidence supports that the EPG waveform C represents ingestion from xylem, and the B1s and proto-C waveforms represent egestion of a mixture of plant fluid and saliva. Our findings will help solve the PD/GWSS problem by providing: 1) insights into the mechanism of *Xf* transmission (acquisition and inoculation); 2) a powerful tool in EPG for studies of host plant resistance, including a natural, insect-inoculation bioassay and eventual development of a resistance index for genotype screening (the Stylet Penetration Index); 3) numerous spin-offs from such basic findings, such as information for risk assessment models, with implications for all levels of the *Xylella*-sharpshooter-grape pathosystem, including ecological, epidemiological and management; and 4) knowledge of new potential targets for grape breeding and transgenic resistance.

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