SHARPSHOOTER FEEDING BEHAVIOR IN RELATION TO TRANSMISSION OF THE PIERCE'S DISEASE BACTERIUM

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ABSTRACT
Progress this year consisted of completing past projects as well as building infrastructure for future research. Backus's new lab in Parlier was renovated, upgraded and equipped with state-of-the-art facilities for electrical penetration graph (EPG) monitoring of insect feeding and histology of plant and insect tissues. Extensive colonies of glassy-winged, smoke tree, green, and red-headed sharpshooters were established in Fresno and Parlier (with R. Groves, ARS Parlier). New personnel were hired; data was intensively analyzed and grant proposals written. Much effort was also expended in developing new protocols and preliminary findings for feeding waveform correlations with bacterial expulsion and muscle contraction, as well as AC and DC waveforms for several species in colony. Stylet activities and salivary sheath-cell type correlations for the major GWSS waveforms were completed (Objective 1), as was all of the plant histology for the GWSS inoculation test (Objective 2). Results to date support a modified version of last year's hypothesis for the mechanism of *Xf* inoculation to grape. *Xf* bacteria may exit the stylets during brief stylet activities represented by the B1spikelet burst, B1-like portions of N and/or C, probably within seconds of the first puncture of any penetrated cell, both along the pathway to and within xylem. Proper placement of the bacteria appears to be crucial; placement in xylem leads to growth of the bacteria sufficient for detection by less sensitive methods such as culturing. Otherwise, when more sensitive detection methods such as immunocytochemistry of the tissues immediately surrounding the salivary sheath are used, they can detect *Xf* in non-xylem tissues. Three papers from this research are in preparation for submission in late 2004 – early 2005. This work will help solve the PD/GWSS problem by identifying the mechanism of *Xf* inoculation and crucial aspects of inoculation efficiency, and eventually aid host plant resistance through the development of the Stylet Penetration Index.

INTRODUCTION
Almost nothing was known, until this work, about the stylet penetration behaviors of the glassy-winged sharpshooter (GWSS), and how they interact with populations of *Xylella fastidiosa* (*Xf*) to facilitate transmission to grapevine. This project is combining the three most successful methods of studying leafhopper feeding (i.e. histology of fed-upon plant tissues, videotaping of feeding on transparent diets, and electrical penetration graph [EPG] monitoring) to identify most details of feeding.

OBJECTIVES
1. Identify and quantify all feeding behaviors of GWSS on grapevine, and correlate them with location of mouthparts (stylets) in the plant and presence/ population size of *Xf* in the foregut.
2. Identify the role of specific stylet activities in *Xf* transmission, including both the mechanisms of acquisition and inoculation, and their efficiency. This project's emphasis is on inoculation.
3. Begin to develop a simple, rapid method to assess feeding, or detect the likelihood of *X. fastidiosa* transmission (an "inoculation-behavior detection method"), for future studies.

RESULTS
During the first six months of this reporting period (Nov. 2003 – April 2004), Backus's new lab at USDA-ARS in Parlier was closed due to extensive renovation construction underway. Notwithstanding this delay, we made significant progress on several sharpshooter research fronts during this time. We hired new personnel (a post-doc and a second technician), purchased many supplies and pieces of equipment (including a new confocal microscope), and trained in the use of the equipment. Also, we received CDFA importation permits and permission for a GWSS maintenance colony to be established in Fresno Co., at a site on the campus of CSU-Fresno. A trailer was rented, retrofitted for quarantine infrastructure, and inspected by officers of the Fresno Co. Agricultural Commissioner's office. Insect maintenance and research rooms were built and outfitted with lighted shelves, cages, growth chambers, and research equipment. Also, a contract was arranged by Groves and Civerolo with Morgan to supply greenhouse-reared GWSS on a monthly basis. Acquisition of insects began in

September 2004. The new USDA-ARS/CSU-Fresno Insect Maintenance and Research facility went into full operation in October 2004. Also during this time we established colonies in the greenhouse in Parlier of the following species: smoke tree sharpshooter, *H. liturata* (STSS), as well as (with Groves) red-headed sharpshooter, *Xyphon fulgida* (RHSS), green sharpshooter, *Draeculacephala minerva* (GSS) and three-cornered alfalfa hopper, *Spissistilus festinus* (3CAH) (collected locally). Preliminary studies of the feeding behavior and EPG waveforms of all of these species are underway.

In addition to major infrastructure improvements in the first 6 months, we also analyzed past data, and Joost performed extensive preliminary tests to develop new protocols in electromyography and real-time imaging of sharpshooter muscles controlling feeding. We also wrote papers, and reviewed and wrote grant proposals. Among these were revisions of the Almeida & Backus paper on blue-green sharpshooter waveforms, now in print [1] and a newly funded UC PD proposal to continue research on mechanisms of *Xf* transmission and details of ingestion behavior. Once we had moved back into the lab and set up, progress resumed on existing objectives during the last four months of the reporting period (July – October 2004).

Objective 1 - Waveform Correlations

Experiment 1: AC-DC Correlation Monitor

Significant progress was made this year in the continuing development of this technology. Bennett built two new prototype monitors, the last of which included design suggestions developed by Backus in consultation with W. F. Tjallingii, Wageningen Agricultural University, The Netherlands. These prototypes for the first time succeeded in achieving waveform fidelity with the original, separate AC and DC waveforms, a goal sought for the last two years of work developing these instruments [2].

Experiment 2: Salivary Sheath-Cell Type Correlation

Backus analyzed histological images produced last year by Habibi from recordings made by Yan (see methods and preliminary findings in [2, 3]). Preliminary findings and waveform appearances are the same as those pictured in the 2002 and 2003 progress reports [2, 3], but waveform names are as in [3]. Results show that early pathway activities, especially A1, occur in the shallow epidermal/parenchyma tissues, A2 and continuous B1 usually occur in the parenchyma peripheral to the vascular bundle (although the sample size of tissues collected for B1 is very small). B2 usually occurs in the parenchyma or phloem, and is often associated with a large deposit of sheath saliva sometimes at a branching point in the sheath. The number of B2 events is also correlated with the number of sheath branches. Short, early C and N events can occur variably, in parenchyma, phloem or xylem; however, longer later C and N events are almost always in mature xylem cells. It is still uncertain whether B1 or C may represent the first penetration of a xylem cell. Correlations were completed and a manuscript is *in prep* for submission in late November [4]. Appendix Table A further summarizes the plant tissue/cell correlations known at the end of the reporting period (late Sept. 2003).

Experiment 3: Stylet Activities Correlation

Joost analyzed the videomicrography data collected by Yan of the stylet activities in artificial diet (see methods and preliminary findings in [2, 3], as well as a schematic of the equipment in the Backus et al. 2004 poster). Stylets could clearly be seen performing stereotypical behaviors during three waveform types frequently seen on grape, i.e. A1, A2 and B1. Results are summarized in Figures 1 – 4 below, Table A and in the Backus et al. 2004 poster. They reveal for the first time that A1 represents the primary formation of the salivary sheath (Figures 1, 2), B1 represents stylet tip fluttering (Figures 1, 3), and B2 represents stylet sawing through the hardened sheath (and, we speculate, perhaps also through tough plant material) (Figures 1, 4). It is particularly interesting that the B1 spikelet burst is dispersed intermittently throughout other pathway waveforms, e.g. between peaks of A1 (Figure 2), as well as in continuous durations by itself (Figure 3). This dispersion, plus last year's Experiment 4 finding [3] that B1 was the only pathway waveform associated with *Xf* inoculation, suggest that the spikelet bursts might represent precibarial valve movement, an important component of a hypothesized inoculation behavior [4]. A manuscript describing these results is *in prep* for submission in late November [5].

Objective 2 - Inoculation Behavior:

Experiment 4: EPG Waveforms Associated with Inoculation

Habibi completed sectioning and photomicrography of the remaining grape tissues probed by EPG-recorded GWSS, i.e. those during the short probe treatment (see the 2003 progress report [3] for methods and preliminary findings). Results from each of the three bacterial detection methods used (Table 1) continue to support that immunocytochemistry may be the most sensitive detection method; 56% of probes showed positive detection of *Xf* near the salivary sheath, while 45% were positive with PCR, and only 10% with culturing. These findings continue to support the interpretations discussed in the 2003 progress report [3]. Unlike PCR, immunocytochemistry results suggest that detectable bacteria are inoculated more often during long than short probes (Table 1). However, it will be important to determine how many insects were actually inoculative before we can state that conclusively. We have begun to dissect the fixed, dried heads of the recorded sharpshooters for scanning electron microscopy, to determine how many of them contained *Xf* and in exactly which areas in the precibarium/cibarium. This information will be

Table 1: Number of EPG-GWSS-probed grape samples that was positive for *Xf* near the probe out of the total number tested, for each of the three bacterial detection methods.

Probing Treatment	PCR	Culture	Immunocyt.
3 short probes	5/10	0/10	3/8
1 long probe	4/10	1/8	6/8

correlated with all other findings to determine how often the inoculation behavior, when performed by bacteria-laden insects, actually results in expulsion of *Xf*. Present findings [3] still implicate waveforms B1, C and N, especially during long probes. All data analysis will be completed and a manuscript submitted in early 2005 [6].

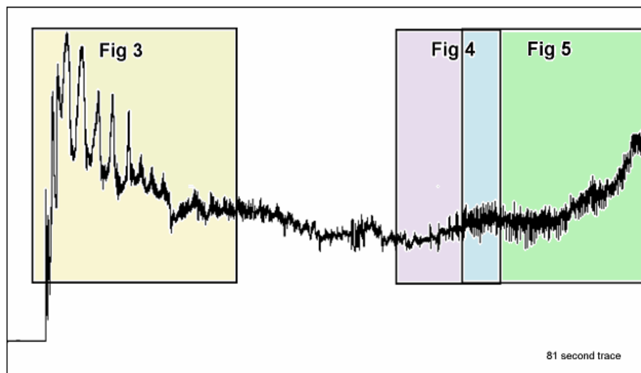

Figure 1. Waveform of GWSS probe in artificial diet compressed 35 times. Box labels indicate where Figures. 3-5 were taken from this trace.

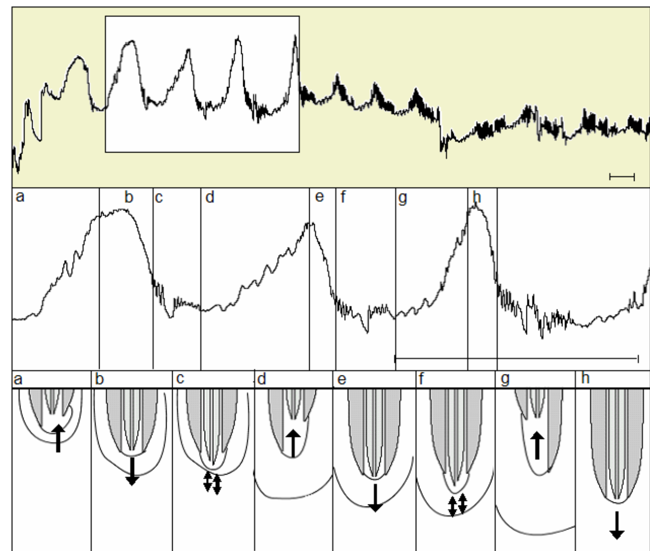

Figure 2. A1 waveforms were correlated with GWSS stylet activities in artificial diet. Top panel trace contains an A1 waveform compressed 5 times. The middle panel is an uncompressed A1 waveform trace that corresponds to the boxed waveform trace in the top panel. Subdivisions, a-h, in middle panel are correlated with stylet activities in the bottom panel with the same subdivision letters. Time marks in the lower right hand corner of the top and middle panel equal one second.

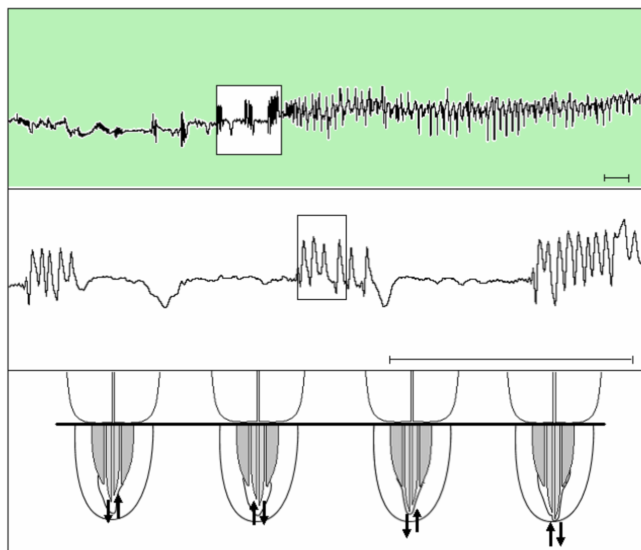

Figure 3. Correlation of B1 waveforms with GWSS stylet activities in artificial diet. Top panel is a waveform trace with B1 compressed 5 times. The middle panel is an uncompressed B1 waveform trace that corresponds to the boxed waveform portion in the top panel. The boxed waveform portion of the middle panel is a B1 spikelet burst and correlates with the stylet activities in the bottom panel. Time marks in the lower right hand corner of the top and middle panel equal one second.

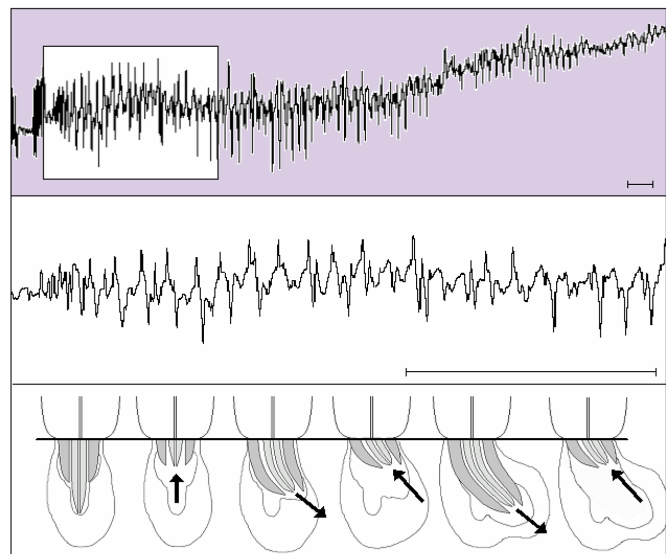

Figure 4. Correlation of B2 waveform with GWSS stylet activities in artificial diet. Top panel is a B2 waveform trace compressed 5 times. The middle panel is an uncompressed B2 waveform trace that corresponds to the boxed portion of the waveform in the top panel. The bottom panel are the stylet activities that were observed at the onset of the B2 waveform and through out the waveform. Time marks in the lower right hand corner of the top and middle panel equal one second.

CONCLUSIONS
These findings will help solve the PD/GWSS problem by:

- Identifying the mechanism of *Xf* inoculation and using EPG to observe it real-time as it occurs,
- Identifying one determinant of inoculation efficiency, i.e. the role(s) of inoculation behavior vs. bacterial presence and/or detachment in the foregut,
- Developing protocols for further tests of transmission biology and efficiency, especially with respect to acquisition.
- Developing a Stylet Penetration Index for testing among host and non-host species or cultivars, diets, etc. for performance of transmission behaviors, ultimately leading to improved host plant resistance.

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Appendix Table A. Current definitions of the AC EPG waveform phases, families and types of GWSS on grape.

Waveform Phase	Waveform Family	Waveform Type	Waveform Characteristics	Proposed Biological Meanings	
				Plant Tissue/Cell	Insect Activity
Pathway	A	A1	Highest amplitude, hump-like waveform at beginning of probe; usually with spike at the top	Parenchyma or mesophyll	Major salivary sheath formation; deep extension/ retraction of stylets; some watery salivation
		A2	Medium amplitude, variable slope; irregular, high frequency with occasional trenches and/or potential drops	Parenchyma or mesophyll	Lengthening and/or hardening of salivary sheath; cell membrane breakage; some watery salivation
	B	B1	Short, single- or multi-peak "spikelet bursts" (20-28 Hz) separated by flatter, wave-like sections	Parenchyma or xylem or pith	Stylet tip fluttering; possible internal muscle/valve movement; involved in inoculation
		B2	Extremely regular, stereotypical pattern of peaks (6 Hz), with distinct phrases	Parenchyma or xylem or pith	Stylet sawing through salivary sheath or tough wood; sheath branching; sheath salivation
Ingestion	C	C (to be subdivided)	Very regular, low rep. rate (3 Hz) with with distinct phrases	Parenchyma or xylem or pith	Trial (short) or sustained (long) ingestion (watery excretory droplets correlated)
Interruption	N	N (to be subdivided)	Irregular, appearing A-like at times, but interrupting continuous C; ave. dur. 16 sec.	Parenchyma or xylem or pith	Sheath or watery salivation in ingestion cell; sheath extension

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