SEASONAL TRANSMISSION OF *XYLELLA FASTIDIOSA* BY GLASSY-WINGED SHARPSHOOTER FROM GRAPEVINES: SHARPSHOOTER PREFERENCE FOR INFECTED GRAPEVINE TISSUE

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ABSTRACT
This study is part of our larger project aimed at understanding the feeding biology of the glassy-winged sharpshooter (GWSS) as it relates to acquisition and transmission of *Xylella fastidiosa (Xf)*. Over the course of this two year project we have determined that GWSS males and females choose to feed on young leaf, petiole, and stem tissue compared to the same tissues on older parts of the grapevine cane, regardless of the time of year. However, they will feed on old stem tissue, which logically should result in more rapid chronic infection than feeding on young tissue. GWSS adults frequently change position between various tissues through the day, which may contribute to the apparent effectiveness in spreading *Xf*. We have determined that GWSS adults do not feed on cordon tissue, regardless of the time of year. In winter studies, we found that GWSS prefer to feed on grapevine tissue that is infected with *Xf* over tissue that is not infected. This has tremendous implication for bacterial acquisition during the dormant periods of the year, and since GWSS adults retain *Xf* for life, this represents another interesting feature of this invasive vector that may contribute to Pierce's disease (PD) spread. In fall studies when vines were in full flush, the preference for infected tissue was not present. Both GWSS and the closely related smoketree sharpshooter (STSS) fed equally on infected and non-infected grapevine tissue. This work shows yet another aspect of GWSS and STSS biology that is important to the spread of *Xf*.

LAYPERSON SUMMARY
The detailed experiments that have been conducted in this project have tremendous implication for the movement of *Xylella fastidiosa* (*Xf)* by the glassy-winged sharpshooter (GWSS). We have learned that GWSS showed a strong preference for grapevine canes from infected vines in the winter months. This aspect of GWSS biology is interesting and contributes to its status as a vector of *Xf* in grapevines. If we can determine the cause of this preference, we may be able to design methods to reduce it. Studies in the fall months did not reveal a preference for infected or non-infected grapevine tissue. We found that GWSS and smoketree sharpshooter (STSS) move readily between infected and non-infected tissue, again a behavior that would contribute to *Xf* in the field. Studying these detailed behaviors contributes to our understanding of the epidemiology of Pierce's disease vectored by GWSS and STSS.

INTRODUCTION
Pierce's disease (PD), a disease of grapes caused by the bacteria, *Xylella fastidiosa* (*Xf)* Wells et al., was described in California in the 1880s during an epidemic in Orange County (Pierce 1882). A second epidemic occurred in Tulare County in the 1930s (Hewitt et al. 1949), and until the mid-1990s, it was considered only a minor problem in vineyards close to riparian areas. In the early 1990s a new vector, GWSS, was introduced into the state (Sorenson and Gill 1996), and became associated with a devastating epidemic of PD in the Temecula Valley. Since 1994, at least 1,500 acres of vineyards have been lost to the disease in California; in the Temecula Valley alone, losses have been estimated at $13 million (Wine Institute 2002).

The glassy-winged sharpshooter (GWSS) has different feeding and dispersal capabilities than native insect sharpshooter vectors and these attributes are thought to have contributed to the increased number of PD-infected grapevines in California (Almeida et al. 2005a, Blua et al. 1999, Redak et al. 2004). Like other insect-borne plant pathogen systems, there are two potential types of pathogen spread: primary or secondary spread. Primary spread occurs when the pathogen is obtained by the vector from sources outside the crop and transported and inoculated into the crop. Secondary spread occurs when the vector acquires the pathogen from infected vines in the vineyard, and subsequently inoculates healthy vines within the same vineyard (i.e. vine to vine spread). It is thought that *Xf* spread with native California vectors was the result of primary spread, but that rapid spread by GWSS may be the consequence of primary and secondary spread (Almeida et al. 2005a, Hill 2006). GWSS landing and feeding behavior and tissue feeding capacity combine with grapevine phenology, and within-vine *Xf* distribution and phenology to make vine to vine spread possible. Our overall goal is to provide information on these various components to enhance our understanding of vine to vine spread so that strategies can be defined to reduce widespread epidemics in other regions.

We have conducted experiments in the fall, winter, and summer in which we made hourly observations on the location of individual GWSS adults given access to mature tissue and young tissue on the same cane. Both males and females preferred young tissues (particularly the stems) to mature tissues on Cabernet Sauvignon and Chardonnay grapevines throughout the year. However, GWSS spent a substantial amount of time feeding on old stem tissue (7.5%, 11%, 15% in fall, winter, and spring trials, respectively) (Perring et al. 2008), where *Xf* could potentially be transmitted leading to chronic infection. A significant finding is that GWSS moved frequently throughout the days of our studies, changing position in 35%, 14%, and 21% of the observations in the fall, winter and spring, respectively. This has serious consequence for moving *Xf* around the vineyard at various times of the year. Further characterization of GWSS feeding behavior was conducted in no-choice studies. We learned that at no time of the year, were individuals able to feed on the cordon tissue. While others have observed GWSS feeding in this tissue (Almeida et al. 2005b), we were not able to demonstrate it in our trials on mature vines. Aside from cordons, GWSS were able to feed on old and young stems, petioles, and leaves. However, the amount of feeding varied with the season. In the winter and summer, GWSS utilized old stems and young stems, while during the fall they were not able to feed on old stems. In addition, the young stems became hardened and woody, and survival and feeding on the young stems at this time of the year were reduced. Our goal is to integrate the information from these past studies with present and future research on infected grapevines at different times of the year. Through this work, we will understand the interaction between feeding behavior on specific grapevine tissues that contribute to the spread of *Xf* from infected to healthy vines.

OBJECTIVES:

1. Document GWSS feeding preference, through the growing season, on established Cabernet Sauvignon and Chardonnay grapevines that either are healthy or have been infected with *Xf* for two, three, or four years.
2. Evaluate the acquisition by GWSS, through the growing season, from established Cabernet Sauvignon and Chardonnay grapevines that either are healthy or have been infected with *Xf* for two, three, or four years and determine the subsequent transmission from these acquisitions.
3. Determine the relationship between *Xf* inoculation by GWSS at different times of the year and the development of the vine as a source for further acquisition by GWSS.

We were forced to modify the original objectives due to the fact that suspected infections of our grapevines were not present. At the time we started in July 2007, selected vines in our field cages had been needle-inoculated in May 2003, May 2004, and May 2005 by cooperator Groves. An evaluation of all the vines on August 28, 2007 showed almost no infection with *Xf*. It is unclear why the infections did not become systemic, but the fact that we had no multi-year infections dictated a revision of our original plans. We re-inoculated the set of vines that had been inoculated in 2003 by scraping the bark on the cordons to expose green tissue for needle inoculation. This procedure was done on November 5, 2007 and September 8, 2008 and has yielded severe infections for us to use. While waiting for infections, we proceeded with experiments to document GWSS feeding biology through the season in choice and no-choice studies. Below we summarize these studies, the data of which are presented in Perring et al. (2008).

RESULTS AND DISCUSSION

Choice and No-choice Studies

Choice studies were conducted in the fall 2007 (August 29, and September 11, 2007), winter 2008 (January 16, and February 6, 2008) and summer 2008 (July 1, 2008). For this research, we placed GWSS adults individually in observation cages fabricated from acetate cylinders (25cm x 17cm diameter) with organdy sleeves attached to the ends. The cage was placed over the base of a Cabernet Sauvignon or Chardonnay grapevine cane with the cane terminal looped back into the cage. The ends of the observation cage were sealed giving a single GWSS in each cage access to old and young stems, petioles, and leaves inside the cage. We made hourly observations during daylight hours over three consecutive days to determine the location of each GWSS. When given a choice, GWSS males and females chose to feed on young leaf, petiole, and stem tissue compared to the same tissues on older parts of the cane. However, there was substantial time spent feeding on old stem tissue, a phenomenon that would result in more rapid chronic infection than feeding on young tissue. We also learned that

throughout the day, GWSS adults change position frequently between the various tissues, a characteristic that would support the rapid spread of *Xf* that has been associated with GWSS.

No-choice studies were conducted in the winter 2008 (February 26, March 4), summer 2008 (July 15), and fall 2008 (September 19). Individual GWSS were caged on selected grapevine tissue in 50 ml polypropylene centrifuge tubes by one of two methods. The first method, modified from Andersen et al. (1992), was for use on cordons, stems, and petioles. The cages were made by melting a transverse hole in the side of the tube using hot metal cylinders of diameters similar to the grape tissues. The tube was pressed onto the plant tissue, so the GWSS had access to about 2.5 cm length of the plant through the hole. The cage was affixed and sealed to the tissue by wrapping the tube and tissue with ca. 2 cm wide strips of Parafilm. The screw cap was tightened, and the cage rested vertically so that excreta collected in the bottom of the tube. The second cage design was for use on leaf tissue. The mouth of an intact 50 ml tube was pressed to the abaxial leaf surface with a piece of coiled spring steel in a clothes-pin like fashion (Blua and Perring 1992). One end of the spring held the 50 ml tube. The other end of the spring had a plastic ring on which was glued a foam pad 1 cm thick by 3 cm in diameter which gently held the leaf against the polypropylene tube, giving the insect access to leaf tissue of ca. 5.7 cm^2. This cage, too, was oriented vertically, so excreta drained to the bottom of the cage. Each cage type was loosely covered with aluminum foil in order to shade it from direct sunlight. The day before the start of each test, GWSS adults were collected from citrus at Agricultural Operations, UCR, and placed in a cage with a potted rough lemon plant. The following morning, adults were isolated and sexed and then placed individually into the tube cages. Cages were inspected daily and the presence of excreta noted; cages with dead GWSS were removed, and the amount of excreta was weighed. The sharpshooters were allowed to feed for four days.

In these studies, we found that GWSS adults were not able to feed on cordon tissue, regardless of the time of year. They were able to feed on old and young grapevine tissue throughout the year, but the relative amount of feeding on this tissue varied with the season.

GWSS preference for infected/non-infected grapevine tissue

We selected canes from putative infected and non-infected Cabernet Sauvignon and Chardonnay grapevines to study GWSS choice for infected or non-infected tissue. Trials were conducted with GWSS on February 19-21 and February 25-27 2009. Because of the time of year, there were no leaves or petioles on the canes. All tissue had a brown hardened outward appearance, but we confirmed that the internal tissue was green, so GWSS would be able to feed. We placed GWSS adults individually in observation cages, which were placed over a section of cane from an infected vine and a section of cane from a non-infected vine (**Figure 1**). The infected cane was marked with a small wire label. All sharpshooters were placed on the cage, so they were forced to make a choice to find a feeding host. The ends of the observation cage were sealed giving a single GWSS in each cage access to infected or non-infected cane tissue. Twenty cages were used for each trial. We made hourly observations from 8am to 5pm over three consecutive days to document the cane (infected or non-infected) on which the GWSS fed.

Figure 1. Acetate cage uses to evaluate GWSS feeding preference for infected (marked with yellow wire label (in circle) and non-infected grapevine tissue. Notice GWSS feeding in center of infected cane (arrow).

At the conclusion of the studies, we conducted a variety of procedures to verify the infection status of the cane tissue to which the GWSS were exposed. First each section of both canes that were inside the acetate cages was removed from the vine and a small section (0.5 in) was macerated and subjected to ELISA immediately after the trial was concluded. Second, the cane sections were marked and planted into pots. Following growth of these cuttings, we conducted ELISA and culturing to determine the infection status of the section of cane to which GWSS was exposed. Third, when we pruned the vines, we selected six canes and planted an approximately 14 inch section from each cane into pots to grow in the greenhouse. After they pushed leaves, we assayed these plants by ELISA. Finally, each vine was visually assessed in the fall for symptoms of *Xf* infection. Symptomatic canes were sampled and subjected to ELISA.

From the various tests, we determined the infection status of all the canes used in the experiments and discarded the cages in which we were unable to make a confident determination. We also discarded cages in which the GWSS died, because this indicated the inability of the insect to successfully feed on either cane. This filtering resulted in nine total cages for the February 19-21 trial (four Cabernet Sauvignon, five Chardonnay, four females and five males) and a total of 11 cages for the February 25-27 trial (four Cabernet Sauvignon, seven Chardonnay, six females, and five males). Because of the small numbers present in each variety and gender, the data are presented as totals for each trial.

Results from both trials showed that GWSS was found more often on the infected vines (**Figure 2**). For the February 19-21 test, GWSS were present on the infected tissue 71% of observed times, while they were on non-infected tissue just 22% of the time. They were found on the cage only 7% of the time. In the second trial (February 25-27), they again were found more often on the infected cane (71%) compared to the non-infected cane (22%) or the cage (7%). We were surprised that the proportions for each of these trials were the same, and have no explanation for this similarity. This is particularly remarkable, given that there was a total of 215 observation times in the first trial and 303 observation times in the second trial (**Table 1**) and the two trials were conducted with different insects on different canes, often from different vines, and at two distinct times.

Also interesting were the movements that sharpshooters made throughout the studies. More GWSS moved to infected canes and stayed for three or more hours than to non-infected canes (**Table 1**). Additionally, there were more sharpshooters that fed on infected canes, left these canes and returned to the infected canes, than those on non-infected canes. Clearly there was something unique about the infected canes that the sharpshooters preferred. It also is apparent that sharpshooters in this study moved about the cages often (17 of a possible 215 observations in trial 1 (8%) and 36 of a possible 303 observations (12%) in trial 2).

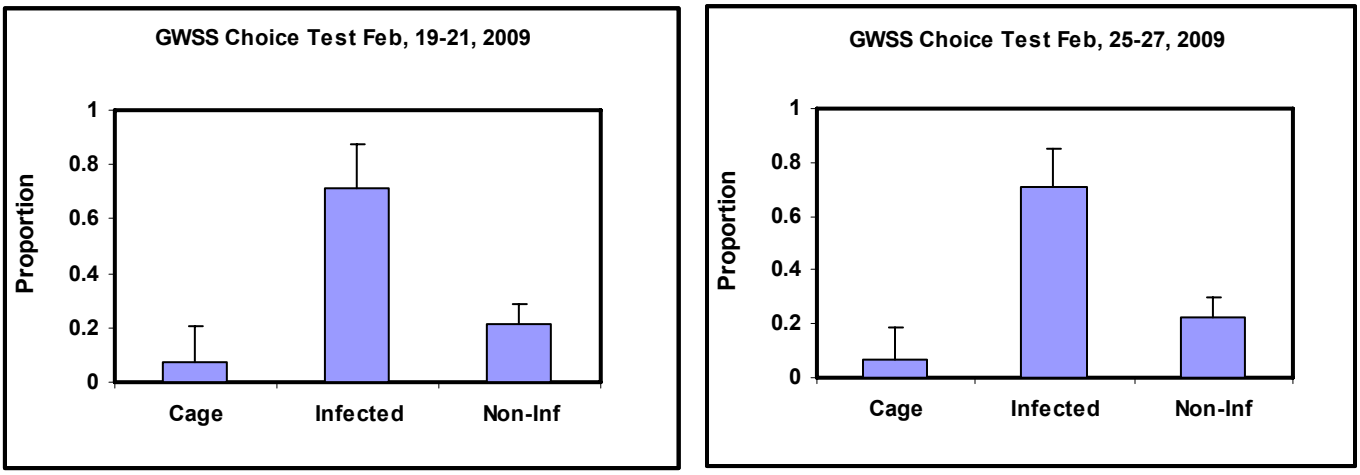

Figure 2. GWSS preference on field-grown Cabernet Sauvignon and Chardonnay grapevines in choice experiments initiated on 19 February (left) and 25 February (right), 2009. Bars represent average proportions of GWSS (± SE) observed on the cage, on the infected canes, and on the non-infected canes.

Table 1. Actions taken by GWSS in two trials (February 19-21 and February 25-27, 2009). Sharpshooters were given a choice between infected and non-infected cane tissue over the 3 day period and observations were made hourly during the daylight hours.

Parameter	Trial 1 (Feb 19-21)	Trial 2 (Feb 25-27)
Chose Infected and stayed 3h or more	12	13
Chose Non-Infected and stayed 3h or more	3	3
Chose Inf. for 3h, left, returned for 3h or more	3	4
Chose Non-I for 3h, left, returned for 3h or more	0	0
Moved from Cage to Inf.	7	12
Moved from Cage to Non-I	1	7
Moved from Inf. to Cage	5	5
Moved from Non-I to Cage	1	4
Moved from Inf. to Non-I	1	4
Moved from Non-I to Inf.	2	4
Total number of Times insect moved	17	36
Total number of Observed Times	215	303

A second set of choice experiments was conducted in September, 2009. These studies, which had the same design as those conducted in February, utilized infected canes that were severely diseased. A healthy, asymptomatic cane was paired with each diseased cane and the canes were stripped of all but 1 leaf within the experimental cage. Twenty cages were established on Chardonnay vines on September 17, and into each cage we introduced a single GWSS female. Observations were made hourly from 8am to 6pm for three days. Utilizing the same 20 cages on the same canes, a second trial was initiated on September 20 with 20 female smoketree sharpshooters (STSS). Observations again were made each hour from 8am to 6pm for a period of three days.

Sharpshooter responses from these trials were distinctly different from the studies conducted in February. In the September 17-19 trial, a slightly higher proportion of GWSS were observed on the non-infected cane (56%) than on the infected canes (40%), with just 4% of the observations on the cage (**Figure 3**). Interestingly, similar results were found for the STSS. This species showed a slight preference for the non-infected canes (51%) rather than the infected canes (40%), with 9% of the observations on the cage (**Figure 3**). We will be collecting the canes from this study in an effort to analyze the xylem sap to see if any particular chemical constituents were present in the canes on which sharpshooters predominantly fed.

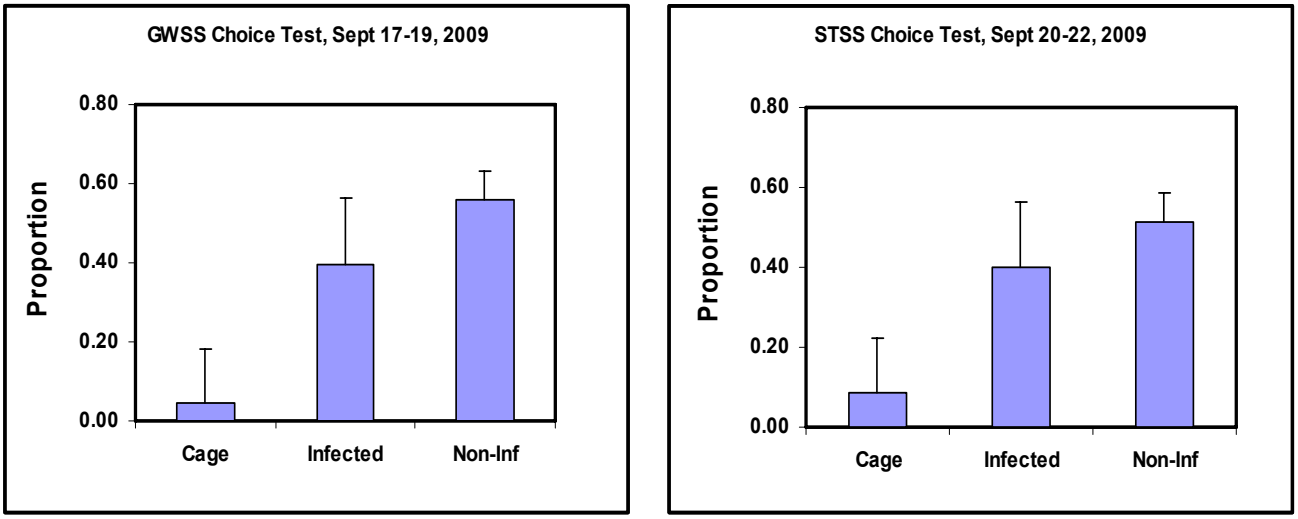

Figure 3. Female GWSS preference (left graph) and STSS preference (right graph) on field-grown Chardonnay grapevines in choice experiments initiated on September 17 (STSS) and September 20 (STSS). Bars represent average proportions of sharpshooters (± SE) observed on the cage, on the infected canes, and on the non-infected canes.

Table 2. Actions chosen by GWSS (September 17-19) and STSS (September 25-27) in choice studies between infected and non-infected cane tissue over the three day period . Observations were made between 8am and 6pm.

Parameter	GWSS (Sept. 17-19)	STSS (Sept. 25-27)
Chose Infected and stayed 3h or more	11	21
Chose Non-Infected and stayed 3h or more	19	25
Chose Inf. for 3h, left, returned for 3h or more	3	9
Chose Non-I for 3h, left, returned for 3h or more	10	5
Moved from Cage to Inf.	15	23
Moved from Cage to Non-I	14	26
Moved from Inf. to Cage	3	17
Moved from Non-I to Cage	10	14
Moved from Inf. to Non-I	12	10
Moved from Non-I to Inf.	8	12
Total number of Time insect moved	62	102
Total number of Observed Times	485	633

Sharpshooters moved slightly more often in this set of experiments than in the February study. In the GWSS trial, insects moved 62 out of a possible 485 observations (13%) and 102 out of 633 observations (16%) (**Table 2**). There were more GWSS that settled and had prolonged feeding (at least 3 hr) on non-infected canes than on infected canes. There was only a slightly higher number of STSS that had prolonged feeding on the non-infected canes than the infected canes.

CONCLUSIONS

In winter choice studies in which GWSS were given access to infected and non-infected grapevine tissue, GWSS were found more often on grapevine tissue that was infected with *Xf* over tissue that was not infected. The reason why this choice was made is unknown, but likely is related to the biochemical components in the various cane tissues (Anderson et al. 1992). Regardless, the fact that GWSS prefers infected tissue has important epidemiological ramifications. Specifically, feeding on infected tissue increases the likelihood of sharpshooters acquiring *Xf*. Movement by these sharpshooters to non-infected tissue, which occurred 1/17 (6%) and 4/36 (11%) times in the two winter trials, could rapidly move the bacteria causing new infections. It is important to remember that the cane tissue was woody (although green inside) and sharpshooters easily fed on this tissue.

In fall experiments, on vines containing green leaves, sharpshooter preference for infected tissue was not apparent. Both GWSS and STSS fed equally on infected and non-infected grapevine tissue. These data suggest that there was nothing in either infected or non-infected tissue that caused sharpshooters to feed preferentially. Both insect species moved readily from infected to non-infected tissue (12/62 = 19% and 10/102 = 10% for GWSS and STSS, respectively). They also moved from non-infected tissue to infected tissue with similar frequency. These results suggest that transmission between infected and healthy vines may be greater at this time of year.

The work reported here is valuable to our understanding of GWSS and STSS feeding behavior that can influence transmission of *Xf*. These studies fill an important data gap in our knowledge of GWSS- and STSS-vectored epidemiology at various times of the year. We plan to continue studies through next year, to confirm the preference of sharpshooters for infected tissue. During this work, we will conduct biochemical assays similar to Andersen et al. (1992) to determine what components are correlated with GWSS and STSS feeding.

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