Comparative Behavioral and Protein Study of Salivary Secretions in *Homalodisca* spp. Sharpshooters (Hemiptera: Cicadellidae: Cicadellinae)

HASAN ALHADDAD, THOMAS A. COUDRON, ELAINE A. BACKUS, AND FRED SCHREIBER

Ann. Entomol. Soc. Am. 104(3): 543–552 (2011); DOI: 10.1603/AN10137

ABSTRACT A novel brush-induced method to physically stimulate salivation was applied to the glassy-winged and smoke tree sharpshooters (Hemiptera: Cicadellidae: Cicadellinae). This technique enabled the direct observation of salivary secretion processes, solidification of saliva, and collection of salivary secretions. For both species, brush-induced saliva was first secreted in liquid form, a portion of which gradually solidified to form the salivary sheath for both sharpshooter species. Proteins of similar molecular weight were obtained from brush-induced saliva extracts from both sharpshooters. Extracts from dried sheaths collected from Parafilm membranes over artificial diet had a different protein profile from brush-induced saliva extracts. The latter contained fewer proteins than extracts of the liquid content of salivary glands. Two proteins appeared in all three of the extracts from hemolymph, salivary glands, and brush-induced saliva, one of which also appeared in dried sheath extracts. Our findings support previous research by others that there is a limited flow of protein from hemolymph to salivary glands and brush-induced saliva. There is also some protein modification associated with saliva solidification. The quantity and composition of proteins suggest the brush-induced saliva collection method has merit for future biochemical analyses of saliva. The implications of this work could potentially include illuminating the mechanism of inoculation by the Pierce’s disease bacterium, *Xylella fastidiosa*.

KEY WORDS glassy-winged sharpshooter, *Homalodisca vitripennis*, *Homalodisca liturata*, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Auchenorrhyncha

Most species of hemipterans are unique in the Animal Kingdom in that they produce two distinctly different types of saliva. One type solidifies (gels) after secretion, forming a protective salivary sheath around the plant-penetrating stylets; hence, it is termed sheath or gelling saliva. The second type remains liquid after secretion and is termed watery or aqueous saliva (Miles 1999). Aphids have been shown to secrete sheath and aqueous saliva separately, during different phases of feeding. Sheath saliva (sometimes mixed with watery saliva) is secreted during pathway phase and sheath formation as the stylets are seeking a phloem sieve element, whereas watery saliva alone is secreted during intracellular punctures of cells along the pathway, as well as in phloem (Tjallingii 2006).

The behaviors involved in salivary secretion, as well as biochemical composition of hemipteran saliva, are complex and vary greatly from species to species. Sheath and watery saliva have been most successfully collected and separated for biochemical analysis from several species of aphids. This is because it is possible to easily rear these parthenogenic insects. Aphids are gregarious and will accept severe confinement. This allows thousands of aphids to be caged on small amounts of highly accepted Parafilm-encapsulated liquid artificial diets, into which they will readily salivate (Miles and Harawign 1991, Miles 1999). The sheath saliva adheres to the Parafilm (which subsequently can be peeled off), whereas the aqueous saliva remains in the liquid diet. Numerous studies on the biochemistry, proteomics, and genomics of aphid saliva have been performed using this technique (Tjallingii 2006, Harmel et al. 2008, Mutti et al. 2008, Carolan et al. 2009). However, a limitation of the diet-collection protocol is a reduced amount of sheath saliva (compared with the amount of total saliva secreted in a plant) and a bias in favor of watery saliva content (Miles and Harawign 1991), because watery salivation...
and ingestion are performed more than sheath salivation during diet feeding.

Many fewer studies have been made of the saliva of nonaphid hemipterans, such as heteropterans (Lau-
rema et al. 1985, Taylor and Miles 1994, Agusti and Cohen 2000) or leaffoppers (Hattori et al. 2005), because it is much more difficult to 1) rear large numbers of these nonparthenogenic insects; 2) house large numbers of these usually less gregarious insects inside confined feeding chambers and induce them to feed; and 3) identify suitable artificial diets, which can require years of work to develop. These insects also ingest more than salivate when feeding on diet (E.A.B., unpublished data), making it particularly difficult to collect significant quantities of saliva from fewer insects. No studies before the present have collected and electrophoresed the saliva of sharpshooter leaffoppers.

The glassy-winged sharpshooter, Homalodisca vitripennis (Germar) (Auchenorrhyncha: Cicadellidae: Cicadellinae) is a polyphagous leaffopper native to the southeastern United States and northeastern Mexico (Hoddle 2004). It preferentially ingests from the xylem (Redak et al. 2004) of at least 72 plant species in 37 families (Hoddle et al. 2003). Xylem ingestion, combined with the salivary sheath feeding strategy used by sharpshooters (Miles 1972, Backus et al. 2005), makes them uniquely adapted to transmit X. fastidiosa (Purcell 1982). A xylem-limited bacterium that survives only in xylem vessels or the alimentary canal of vectors, X. fastidiosa causes both physical (Redak et al. 2004) and chemical (Newman et al. 2004) injury to plants. Infection results in a variety of scorch-like diseases in several crop plants, including the economically important Pierce’s disease (PD) in grapes (Vitis spp.) (Hopkins and Purcell 2002). Yet, despite the economic importance of PD, the exact mechanism of bacterial inoculation by vectors is unknown.

Because X. fastidiosa colonizes the foreguts of vec-
tors, foregut anatomy and expulsion of fluids during specific probing behaviors must be critical compo-
nents of the mechanism of transmission (i.e., acqui-
sition-retention-inoculation) of X. fastidiosa (Almeida and Purcell 2006, Chatterjee et al. 2008). The recently published salivation-egestion hypothesis for inocula-
tion of X. fastidiosa (Backus et al. 2009) implicates sharpshooter saliva (secreted into the plant and then taken up into the anterior foregut) as the critical carrier of bacteria subsequently egested from their foregut colonization sites. However, almost nothing is known about sharpshooter saliva.

Accordingly, the objectives of our work were to 1) observe and document the process of salivation by sharpshooters; 2) develop a new method to collect analyzable amounts of sharpshooter salivary secret-
tions; 3) profile the protein content of the salivary secretions of glassy-winged sharpshooter, as a first step toward future investigations of saliva in the transmis-
sion of X. fastidiosa; and 4) compare salivation behav-
ior and protein profile of a closely related, congeneric, native California species Homalodisca litturata Ball. H. litturata is a less efficient vector of X. fastidiosa (Sis-
terson et al. 2007).

Materials and Methods

Insects. H. vitripennis was collected from ornamental plants along roadsides in Bakersfield, Kern County, CA, during summer 2008 and 2009. The collected in-
sects were transported under permit to the joint USDA-ARS/California State University–Fresno glassy-
winged sharpshooter quarantine rearing facility in Fresno, CA, where they were maintained in cages on cowpea, Vigna unguiculata ssp. dekindtiana (Harms), under artificial lights, a photoperiod of 16:8 (L:D) h, and 24–30°C. For most of this study, only ≈100–200 glassy-winged sharpshooters were available for use at any time.

H. litturata was obtained from established colonies initially collected under permit from jojoba, Simmond-
sia chinensis (Link) C. K. Schneider, plants at Agricult-
ural Operations, University of California–Riverside, CA. The insects were caged on cowpea; sunflower, Helianthus annuus Nutt.; and sorghum, Sorgum bicolor ssp. bicolor Moench in a greenhouse at the US-
DA–ARS San Joaquin Valley Agricultural Science Center, Parlier, CA, at the same temperature and pho-
toperiod as given above. All rearing plants were grown in a different greenhouse at the latter ARS facility and transported to cages as needed.

Collection and Extraction of Saliva From Parafilm Membranes. For one method, five glassy-winged sharpshooters were placed in a cage with a small petri dish filled with distilled water and covered with unstretched Parafilm and allowed to probe for 24 h to produce salivary sheath material while being held under artificial light at 24–30°C. The Parafilm was then removed and air-dried for 2 h at room temperature. Technically, deposits of sheath saliva on the surface of a plant or Parafilm sachet are termed flanges, whereas those inside a plant or sachet are termed sheaths. For ease of discussion, hereafter all deposits of sheath saliva will be termed sheaths. The collected sheaths were placed in 50 μl of Laemmli denaturing sample buffer (catalog no. 161-0737 with added 50 μM mercaptoethanol, Bio-Rad Laboratories, Hercules, CA) and incubated in a heating block at 98°C for 8 min.

For another method, caged glassy-winged sharpshooter were provided with two-chamber slides containing an artificial diet and covered with stretched Parafilm (Habibi et al. 1993). The diet consisted of 0.7 mM L-glutamine, 0.1 mM L-asparagine, and 1 mM sodium citrate (pH 6.4) (Killiny and Almeida 2009). Five feeding chambers, each containing five or six glassy-winged sharpshooter, were used. The feeding cham-
bers were placed under artificial light and held at 24–30°C. Each Parafilm sheet was rinsed with distilled water and then soaked in 0.5 ml of denaturing sample buffer over a boiling water bath for 5 min on each side. Artificial diet from the five feeding chambers was collected and combined. Salivary proteins in the combined diets were precipitated using ice-chilled 20% trichloroacetic acid. The solution was vortexed, incu-
Salivary droplets that accumulated at the tip of the labium after brush stimulation were rapidly absorbed, one at a time, onto each triangle of filter paper. The saliva was collected before there was any indication of solidification: no solid sheath saliva or precipitate was ever observed at any time during collection. Once 20–25 continuous droplets (5–10 min of collection) were absorbed by the tip of the paper, 0.5 mm of the filter paper’s tip was cut under the stereomicroscope and placed, while still wet, into 50 μl of a 1:100 dilution of a protein extraction buffer (0.5 mM sodium acetate, 1 M NaCl, 0.01 M mercaptoethanol, and Sigma protease inhibitor [product no. P1860, Sigma, St. Louis, MO]). This process, termed “milking,” was repeated 20–25 times for each insect to ensure detectable amounts of protein.

The filter paper extracts for each insect (20 insects per trial) were combined and mixed in a 1:1 ratio with the denaturing sample buffer and then incubated in a heating block at 98°C for 5 min. Washed filter papers were extracted with denaturing sample buffer to test for residual protein. Extracts of brush-induced saliva were analyzed for both glassy-winged sharpshooter and H. liturata. All other saliva or gland extracts were analyzed only for glassy-winged sharpshooter.

**Dissection and Extraction of Salivary Glands.** Adults of *H. vitripennis* and *H. liturata* were immobilized in wax as described above. The head was immersed in a droplet of dissection buffer (50 mM sodium acetate buffer, pH 5.0) and carefully lifted into a prognathous position (causing the soft, cervical cuticle to tear slightly) to expose the salivary glands lying underneath the ventral surface. Using sterile fine forceps, the glands were removed and placed in protein extraction buffer (50 glands/50 μl of buffer). Glands were from 650 insects that had not been brush-induced to salivate (hereafter termed “unmilked gland extract”) and from 350 insects that had been brush-induced previously for ~2 h before dissection (hereafter termed “milked gland extract”). Due to the large number of dissections, and the small size of sharpshooter accessory glands, principal and accessory salivary glands were pooled in batches of 50 gland pairs. Each batch was centrifuged at 25,000 rpm for 15 min, the supernatant saved, the pellet resuspended, the suspension centrifuged a second time at 25,000 rpm for 25 min. Tissue rupturing or homogenization was avoided, to minimize extraction of structural proteins. The supernatants were then combined and mixed in 1:1 ratio with denaturing sample buffer and incubated on a heating block at 98°C for 5 min in preparation for protein analysis; the pellet was discarded.

**Collection and Extraction of Hemolymph.** Adults of *H. vitripennis* and *H. liturata* were immobilized as described above; one leg was removed per insect, and the droplets of hemolymph that formed at the point of severance were collected using a pipette and placed in 50 μl of protein extraction buffer. The hemolymph samples from 150 insects were pooled and mixed in 1:1 ratio with denaturing sample buffer and then incubated on a heating block at 98°C for 5 min in preparation for electrophoresis.
Protein Composition. Protein content in the heat-treated protein samples was quantified using the Bradford method (catalog no. 500-0201, Bio-Rad Laboratories) with bovine serum albumen as a standard. After quantification, the proteins in each sample were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis using precast 8–16% gradient Tris-HCl polyacrylamide gel (catalog no. 345-0037, Bio-Rad Laboratories). A molecular weight standard was run with the samples (catalog no. 161-0374, Bio-Rad Laboratories). The gels were stained with Bio-Safe Coomassie stain (catalog no. 161-0786, Bio-Rad Laboratories) or with silver stain (catalog no. 161-0443, Bio-Rad Laboratories). A relative mobility value versus molecular weight standard curve was constructed and used to estimate the molecular mass of the insect proteins to the nearest kilodalton.

Results

Probing of Parafilm Membranes. The few salivary sheaths on the unstretched Parafilm membrane over the petri dish containing distilled water were very short, indicating only brief probes into the water. In contrast, stylet probing was observed directly through the chamber slide glass with the second method. Successful ingestion of diet was determined by observing a reduction of diet volume as well as the number and length of salivary sheaths. Each of the stretched Parafilm membranes from the five feeding chambers contained >100 salivary sheaths.

Salivation Behavior. The salivation behavior of glassy-winged sharpshooter and the *H. liturata* was virtually identical, except that the *H. liturata* has much longer hairs on the tip of its labium, which caused saliva to disperse into smaller droplets than shown in Figs. 2 and 3 for glassy-winged sharpshooter (*H. liturata* images not shown; see Alhaddad 2008). Brushing the clypeus, anteclypeus, and labrum of immobilized sharpshooters stimulated salivation and allowed real-time visualization of the salivation process. This included movement of the mouthparts and the progressive formation of the salivary sheath, when saliva was allowed to air-dry. The first response to brushing stimulus was the appearance of a droplet of saliva on the labium (Fig. 2a). Subsequently, the maxillary stylets gradually extended out of the labium, producing more saliva at the tip of the labium (Fig. 2b and c). As the saliva accumulated at the tip of the labium, the maxillary stylets extended further beyond the saliva bubble and secreted a small droplet of saliva that slid down the stylets and accumulated at the tip of the labium (Fig. 2d–g). With fully extended stylets, the insect continued to produce salivary droplets for as long as 45 min with continuous brushing of the clypeus, anteclypeus, and labrum.

Fig. 2. Glassy-winged sharpshooter salivation behavior. (a) Brushed-induced salivary secretion produced at the tip of the labium. (b) More salivary secretions at the tip of the labium and maxillary stylets gradual extension. (c) Maxillary stylets protracted out of the salivary bubble. (d–g) Maxillary stylets fully protracted out of the salivary bubble producing small salivary droplets (blue arrow) that accumulate at the tip of the labium. Red arrow, saliva changes color at the tip of the labium. Images were captured at different time intervals during salivation. Scale bar = 200 μm.

Fig. 3. Gradual solidification of the glassy-winged sharpshooter salivary sheath. (a) Brushed-induced salivary secretion in a semisolid state. (b) Solidification begins away from the tip of the labium (red arrow). (c and d) Solidification progressively continues. (e) Salivary sheath fully solid. Images were taken at different time intervals during the formation of the salivary sheath. Scale bar = 200 μm.
The salivary secretions were produced in a liquid form. Saliva at the tip of the stylets gradually accumulated, forming a white, semigelatinous substance at the tip of the labium, which progressively enlarged outward as the insect produced more saliva (Fig. 2d, red arrow). Once a substantial quantity of white substance was formed, the maxillary stylets moved in a reciprocating manner, with one fully protracted and the other slightly retracted.

When brushing was stopped, saliva secretion immediately ceased. The entire salivary bubble at the tip of the labium (Fig. 2g) gradually transformed into an opaque, semisolid gel (Fig. 3a). During this gradual gelling process, the paired maxillary stylets vigorously moved in opposite directions, simultaneously drawing some liquid saliva from the center of the gelled substance, forming a hollow tube of semisolid material (data not shown). The liquid saliva appeared to lubricate the lumen of the salivary sheath as well as the labium, and facilitated movement of the maxillary stylets, inside the lumen. Although the first gelling and partial solidification was seen at the tip of the labium (Fig. 2d, red arrow), the fully solid sheath was first observed most distal to the tip of the labium (Fig. 3b, red arrow). The gel-like substance eventually hardened into the solid salivary sheath in <5 min (Fig. 3e). The core of the salivary sheath remained transparent, either hollow or liquid-filled. Thus, the salivary secretions were secreted in a liquid form and then were manipulated by the stylets such that a portion gradually solidified to form the external salivary sheath.

During salivation, some salivary secretions were seen to pool on a small region on the upper surface of the labium (in the labial groove; Fig. 3, blue arrows). This saliva solidified gradually in a similar manner as explained above. However, with additional liquid saliva secretion, a small amount of solidified saliva was observed to return to its liquid state. This phenomenon was observed repeatedly. Thus, newly secreted liquid saliva could solubilize the gelled salivary sheath.

**Salivary Protein Profiles.** No protein was detected in the distilled water fed upon through the Parafilm membrane (data not shown), probably due to sparse sharpshooter feeding and the dilution of the saliva. Only two bands were detected in the Coomassie-stained artificial diet extract fed upon through the Parafilm membrane; a major band of estimated molecular mass 99 kDa and a minor band of 154 kDa (Fig. 4). The sheath extract from the Parafilm membrane over diet had only two faint bands, with molecular mass 99 and 87 kDa (Fig. 4). Silver staining of the same Coomassie blue-stained gel confirmed the presence of few bands in both samples (Fig. 4a).

The extracted proteins from the unmilked versus milked glassy-winged sharpshooter salivary glands differed in relative protein composition and intensity of individual bands (Fig. 5). A Coomassie blue-stained protein separation of unmilked gland extract contained more bands than the milked gland extract (16 versus 11; Fig. 5, lanes 2 and 3). Nine protein bands with molecular mass 138, 124, 114, 67, 56, 42, 32, 29, and 15 kDa were distinctly present in the unmilked gland extract that were not as prominent in the milked. Conversely, three bands with molecular mass 99, 51, and 46 kDa were present in the milked gland extract, but not the unmilked gland extract (Fig. 5, lanes 2 and 3). Seven protein bands were the same for both types of gland extract, although the relative percentage composition was not the same for these bands in both samples. Six bands with molecular mass 99, 51, 46, 36, 14, and 12 kDa from the milked gland extract constituted a higher percentage composition of total protein than the respective bands in the unmilked gland extract (Fig. 5, lanes 2 and 3).

For comparison, Fig. 6 also shows that the brush-induced saliva extract of glassy-winged sharpshooter
contained many fewer proteins that stained with Coomassie blue and proteins of lower molecular weight than did the unmilked gland extracts (Fig. 6, lanes 4 and 5). The estimated molecular mass of the brush-induced salivary proteins were 56, 51, 46, and 22 kDa (Fig. 6, lanes 4 and 5). All of these bands except the 46-kDa protein band were found in the protein profile from the unmilked gland extract. Silver stain of the brush-induced saliva extract (Fig. 7, lane 2) visualized more proteins than did Coomassie blue (Fig. 6, lane 5). Four protein bands were stained with Coomassie blue, compared with 15 bands with silver stain. The 22-, 19-, 15-,

Fig. 6. Electrophoretic analysis of the glassy-winged sharpshooter salivary gland extract and brush-induced secreted saliva extract stained with Coomassie blue. (a) Lane 1, molecular marker; lane 2, protein extraction cocktail (negative control); lane 3, 30 µg of unmilked salivary gland extract; and lanes 4 (shown in gel but not diagrammed) and 5, 2.5 and 5 µg of secreted saliva extract (collected with cellulose filter paper), respectively. (b) A computerized representation of the bands presented in a. (c) Estimated molecular mass of the bands shown in b. Asterisk (*) and highlighted numbers in lanes 3 and 5, bands in high quantities. The bottom two bands in lanes 2, 4, and 5 are the protease inhibitor (7 kDa) and the sample dye.

Fig. 7. Electrophoretic analysis of glassy-winged sharpshooter brush-induced secreted saliva extract stained with silver stain. (a) Image of gel stained with silver stain. Lane 1, molecular marker; lane 2, 2.5 µg of secreted saliva extract; lane 3, 2.5 µg of hemolymph; and lane 4, dried sheath extract (unquantified). (b) Computerized representation of the bands shown in a. (c) Estimated molecular mass of the bands shown in b. The highlighted numbers are for the bands in high quantities.
and 9-kDa bands appeared to be at significantly higher concentrations when silver stained compared with the other protein bands.

Two major bands in the hemolymph extract, at 99 and 21 kDa, aligned with protein bands in the brush-induced saliva extract (Fig. 7, lanes 2 and 3). One major band at 99 kDa and one minor band at 73 kDa in the sheath extract from the Parafilm membrane over distilled water aligned with protein bands in the brush-induced saliva extract (Fig. 7, lanes 2 and 4). However, a prominent triplet of silver-stained bands of low molecular weight at 10, 11 and 12 kDa was unique to the sheath extract from the Parafilm membrane over distilled water (Fig. 7, lane 4). By comparison, higher molecular mass bands at 73 and 99 kDa had a lower relative composition than the triplet bands.

The protein profiles of brush-induced saliva extracts from glassy-winged sharpshooter and *H. liturata* were very similar, but not identical. Glassy-winged sharpshooter saliva extract produced 13 protein bands (Fig. 8, lane 1), whereas that from *H. liturata* produced 11, of slightly different estimated molecular mass (Fig. 8, lane 3). Ten protein bands aligned between the two species; thus, glassy-winged sharpshooter produced three protein bands not produced by *H. liturata* (molecular mass of 154, 67 and 36; Fig. 8), whereas *H. liturata* produced only one band (114 kDa) not found on the glassy-winged sharpshooter gel (Fig. 8). The 22-, 19-, and 9-kDa bands were at high concentrations in both of the glassy-winged sharpshooter and *H. liturata* brush-induced saliva samples.

Comparing molecular weight profiles among all the treatments for both species (Fig. 9) shows large differences that form a pattern. First, extracts from both unmilked glands and brush-induced saliva have many more protein bands than milked gland extracts, Parafilm-collected dried sheaths, diet water, or hemolymph. Second, unmilked gland extract and brush-induced saliva extract (Fig. 7, lanes 2 and 3). One major band at 99 kDa and one minor band at 73 kDa in the sheath extract from the Parafilm membrane over distilled water aligned with protein bands in the brush-induced saliva extract (Fig. 7, lanes 2 and 4). However, a prominent triplet of silver-stained bands of low molecular weight at 10, 11 and 12 kDa was unique to the sheath extract from the Parafilm membrane over distilled water (Fig. 7, lane 4). By comparison, higher molecular mass bands at 73 and 99 kDa had a lower relative composition than the triplet bands.

The protein profiles of brush-induced saliva extracts from glassy-winged sharpshooter and *H. liturata* were very similar, but not identical. Glassy-winged sharpshooter saliva extract produced 13 protein bands (Fig. 8, lane 1), whereas that from *H. liturata* produced 11, of slightly different estimated molecular mass (Fig. 8, lane 3). Ten protein bands aligned between the two species; thus, glassy-winged sharpshooter produced three protein bands not produced by *H. liturata* (molecular mass of 154, 67 and 36; Fig. 8), whereas *H. liturata* produced only one band (114 kDa) not found on the glassy-winged sharpshooter gel (Fig. 8). The 22-, 19-, and 9-kDa bands were at high concentrations in both of the glassy-winged sharpshooter and *H. liturata* brush-induced saliva samples.

Comparing molecular weight profiles among all the treatments for both species (Fig. 9) shows large differences that form a pattern. First, extracts from both unmilked glands and brush-induced saliva have many more protein bands than milked gland extracts, Parafilm-collected dried sheaths, diet water, or hemolymph. Second, unmilked gland extract and brush-induced saliva extract (Fig. 7, lanes 2 and 3). One major band at 99 kDa and one minor band at 73 kDa in the sheath extract from the Parafilm membrane over distilled water aligned with protein bands in the brush-induced saliva extract (Fig. 7, lanes 2 and 4). However, a prominent triplet of silver-stained bands of low molecular weight at 10, 11 and 12 kDa was unique to the sheath extract from the Parafilm membrane over distilled water (Fig. 7, lane 4). By comparison, higher molecular mass bands at 73 and 99 kDa had a lower relative composition than the triplet bands.

The protein profiles of brush-induced saliva extracts from glassy-winged sharpshooter and *H. liturata* were very similar, but not identical. Glassy-winged sharpshooter saliva extract produced 13 protein bands (Fig. 8, lane 1), whereas that from *H. liturata* produced 11, of slightly different estimated molecular mass (Fig. 8, lane 3). Ten protein bands aligned between the two species; thus, glassy-winged sharpshooter produced three protein bands not produced by *H. liturata* (molecular mass of 154, 67 and 36; Fig. 8), whereas *H. liturata* produced only one band (114 kDa) not found on the glassy-winged sharpshooter gel (Fig. 8). The 22-, 19-, and 9-kDa bands were at high concentrations in both of the glassy-winged sharpshooter and *H. liturata* brush-induced saliva samples.

Comparing molecular weight profiles among all the treatments for both species (Fig. 9) shows large differences that form a pattern. First, extracts from both unmilked glands and brush-induced saliva have many more protein bands than milked gland extracts, Parafilm-collected dried sheaths, diet water, or hemolymph. Second, unmilked gland extract and brush-induced saliva extract (Fig. 7, lanes 2 and 3). One major band at 99 kDa and one minor band at 73 kDa in the sheath extract from the Parafilm membrane over distilled water aligned with protein bands in the brush-induced saliva extract (Fig. 7, lanes 2 and 4). However, a prominent triplet of silver-stained bands of low molecular weight at 10, 11 and 12 kDa was unique to the sheath extract from the Parafilm membrane over distilled water (Fig. 7, lane 4). By comparison, higher molecular mass bands at 73 and 99 kDa had a lower relative composition than the triplet bands.
induced saliva bear a remarkable similarity in protein profile. Unmilked gland extract had eight more bands (molecular mass of 138, 114, 103, 80, 73, 44, 32, and 15 kDa) than brush-induced saliva, whereas the latter had only one more band (21 kDa) more than the former. Milked gland extract lacked many of these proteins, but it gained only one (46 kDa).

In contrast, very few proteins were extracted from hemolymph, diet water or Parafilm-collected dried sheaths (Figs. 4 and 9). All proteins extracted from these treatments also were found in unmilked gland extracts and brush-induced saliva. Strikingly, one protein that occurred in all six treatments, the 99-kDa protein, was found in high concentrations in both hemolymph and milked saliva. In opposite manner, the 154-kDa protein was found in all glassy-winged sharpshooter treatments except dried Parafilm-collected sheath and hemolymph. The 154- and 99-kDa proteins may have been highly soluble in water, because they were the only proteins found in the diet water. Both the 99- and 87-kDa proteins were the only ones extractable from the dried sheath on Parafilm.

Discussion

Salivation Behavior. The salivation and feeding behaviors of most phytophagous hemipterans are difficult to study, because they occur beneath the surface of the plant and cannot be easily visualized and analyzed without disturbing the process(es). Sharpshooters seem to be unique among species tested to date in initiating salivation after physical stimulation, allowing direct observation of the solidification process of sheath saliva. Herein, we revealed how the stylets facilitate alternating saliva solidification and liquefaction in the formation of the salivary sheath. Previous methods could not visualize this process as clearly, and analysis of salivary sheath material was limited to what had hardened on parafilm, in plants via histochemical staining (Backus et al. 2005), or by observing salivation into liquid diets (Joost et al. 2005).

The application of chemical stimuli, such as acetic acid, to the external chemoreceptors on the foretarsi or antennae (Miles 1965) or plocarpene based on a protocol used with ticks (Binnington and Schotz 1973), has been reported to stimulate salivation. However, chemical effects on the content and contamination of the salivary secretions are a concern. Presumably, brushing presented no chemical stimulus and the area touched is not the foretarsi or the antenna. Perhaps by brushing the clypeus, anteclypeus, and labrum, the muscles that control the mouth parts were stimulated, because they lie directly underneath those plates. Subsequently, such stimulation induced a reflex behavior of salivation. Furthermore, we hypothesize that natural stimuli might occur from hairs on the plants that might perform a similar function as did the camel’s-hair brush.

The increased opacity of the saliva accumulated at the tip of the labium seemed to represent coagulation, which increased with time but was not seen in all the brush-induced saliva. Coagulation of saliva contradicts claims that saliva collected directly from the mouth parts is intended only for sampling of the surface of a substrate (Miles 1967, 1999). This claim led to many years of concern that stylet collection of saliva was not ideal. Yet, our work suggests that this does not apply to all hemipterans, even if it is true for aphids.

Observations described herein suggest that, at least in the earliest stages of feeding by glassy-winged and Hypera postica, sheath and watery saliva are secreted simultaneously in a liquid form. Some of this saliva then gradually solidifies to form the sheath, and the rest remains liquid, becoming the watery saliva. In addition, our observations suggest that an active chemical process is responsible for the liquid form of the saliva to partially or fully coagulate, resolubilize, or both. During solidification of the salivary sheath, the paired stylets rapidly protracted and retracted in opposition. This type of movement also was observed by Miles (1959), who suggested that such stylet movement in aphids is highly associated with the solidification process. Miles (1959) proposed this might occur by dissolving oxygen to assist oxidization as part of the salivary sheath formation. Collection of saliva using our new method might facilitate testing these hypotheses.

The salivation behavior of the H. liturata seemed to be identical to that of the glassy-winged sharpshooter, especially in their response to the same artificial stimulation to produce salivation. Longer and denser hairs on the labium caused smaller clumps of saliva to form. Nonetheless, a similar pattern of salivation and salivary sheath formation occurred. In both species, sheath saliva gelled out of a secreted liquid but also left a liquid fraction behind in the lumen of the hardend sheath, which could become the watery saliva. This interesting finding is therefore not aberrant, or found only with H. vitripennis, but seems to be at least genus-specific (possibly also specific to all sharpshooters [subfamily Cicadellinae]).

Salivary Protein Composition. The brush stimulation method provided an opportunity to directly analyze salivary protein content of saliva, as well as to compare salivary profiles from partially empty (milked) glands versus full (unmilked) glands. Our gland extraction protocol was intended to capture primarily the liquid in the glands and not structural proteins. Although there were bands of comparable molecular weight in milked and unmilked gland extracts, the relative composition differed greatly. Extensive emptying of the salivary glands caused some proteins (that were present in unmilked gland extract) to disappear from the liquid fraction, others (that were present only in milked extract) to appear, and the remainder (those present in both extracts) to either remain the same or change in relative amount after milking. Overall, the number of protein bands decreased 63% after milking, showing that supplies of certain proteins could become depleted after extensive salivation and require replenishing.

The prominent bands in the milked gland extracts probably represent those proteins that are most rapidly replenished. These proteins probably serve critical roles and have not undergone lysis. For example,
the intensely silver-stained band at 99 kDa was seen in the hemolymph, milked gland extracts, brush-induced saliva extracts, and dried sheath extract, whereas the intensely Coomassie blue-stained band at 46 kDa was seen only in milked gland extracts and brush-induced saliva extracts. Higher molecular mass bands had a high relative composition in unmilked gland and brush-induced saliva extracts but lower in the unmilked gland extract and dried Parafilm-collected sheath extract.

The distinct band at 99 kDa was found in the hemolymph extract, as well as in all other treatments, including dried Parafilm-collected sheath, albeit at varying relative concentrations. Previous reports have suggested that salivary secretions may contain hemolymph proteins (Miles 1967, Miles and Slovik 1970). Consequently, it is probable that the 99-kDa protein associated with salivary material in glassy-winged sharpshooter was originally derived from the hemolymph, passed into the brush-induced saliva, and ultimately solidified unchanged in the sheath. Thus, our results support that at least one protein in the saliva is derived from the hemolymph; the salivary glands filter out these proteins and supplement them with additional proteins before secretion. Furthermore, the 22-, 36-, and 51-kDa proteins were probably derived from the salivary glands and may have been altered or excluded during the solidification process of the sheath. As shown by the 21- and 22-kDa proteins, the brush-induced saliva captured the highest proportion of proteins derived from both the hemolymph and salivary glands of all the saliva-capture methods tested.

The molecular weights of proteins extracted from brush-induced saliva of *H. liturata* seemed similar to those from salivary secretions of glassy-winged sharpshooter. There were differences in relative composition between the two insects. However, proteins with molecular mass of 9, 19, and 22 kDa were in high concentration in the salivary secretions from both insects. This suggests that glassy-winged sharpshooter and *H. liturata* share some of the same salivary proteins as well as salivation behavior. Potentially, this similarity could have been even greater if the two test species had been reared on the same host plants. It has been shown that food source may affect salivary protein composition (Habibi et al. 2001). The fact that these two sharpshooters share similar salivary proteins and are also capable of inoculating similar, foregut-borne plant pathogens such as *X. fastidiosa* suggests by the salivary and midgut digestive enzymes. J. Entomol. Sci. 35: 176–186.

Alhaddad, H. 2006. Patterns of *Xylella fastidiosa* colonization on the precibarium of sharpshooter vectors relative to transmission to plants. Ann. Entomol. Soc. Am. 99: 884–890.

Almida, R.F.P., and A. H. Purcell. 2006. *Xylella fastidiosa* colonization on the precibarium of sharpshooter vectors relative to transmission to plants. Ann. Entomol. Soc. Am. 99: 884–890.

Almida, R.F.P., and A. H. Purcell. 2006. Patterns of *Xylella fastidiosa* colonization on the precibarium of sharpshooter vectors relative to transmission to plants. Ann. Entomol. Soc. Am. 99: 884–890.

Acknowledgments

We thank Holly Shugart (ARS, Parlier) for experimental assistance and Kim Andrews (formerly Kingston) (University of California–Davis; presently Department of Primary Industries, Attwood, Australia) for constructive discussions. We are also grateful to Jose Gutierrez and Cole Pearson (ARS, Parlier) for help with insect and plant maintenance. This research was performed in partial fulfillment of H.A.’s M.S. degree at California State University–Fresno. Funding for this work came from a scholarship to H.A. from the government of Kuwait and in-house USDA–ARS research funds to E.A.B.

References Cited

Agosti, N., and A. C. Cohen. 2000. *Lygus hesperus* and *L. lineolaris* (Hemiptera: Miridae), phytophages, zoophages, or omnivores: evidence of feeding adaptations suggested by the salivary and midgut digestive enzymes. J. Entomol. Sci. 35: 176–186.

Alhaddad, H. 2005. *Xylella fastidiosa*. Department of Biology, California State University–Fresno, Fresno, CA.

Almida, R.F.P., and A. H. Purcell. 2006. *Xylella fastidiosa* colonization on the precibarium of sharpshooter vectors relative to transmission to plants. Ann. Entomol. Soc. Am. 99: 884–890.

Backus, E. A., Habibi, J., Yan, F., and M. Ellersieck. 2005. *Xylella fastidiosa* colonisation on the precibarium of sharpshooter vectors relative to transmission to plants. Ann. Entomol. Soc. Am. 99: 884–890.

References Cited

Agosti, N., and A. C. Cohen. 2000. *Lygus hesperus* and *L. lineolaris* (Hemiptera: Miridae), phytophages, zoophages, or omnivores: evidence of feeding adaptations suggested by the salivary and midgut digestive enzymes. J. Entomol. Sci. 35: 176–186.

Alhaddad, H. 2005. *Xylella fastidiosa*. Department of Biology, California State University–Fresno, Fresno, CA.

Almida, R.F.P., and A. H. Purcell. 2006. Patterns of *Xylella fastidiosa* colonization on the precibarium of sharpshooter vectors relative to transmission to plants. Ann. Entomol. Soc. Am. 99: 884–890.

Backus, E. A., Habibi, J., Yan, F., and M. Ellersieck. 2005. Sterile penetration by adult *Xylemodonas coagulata* on grapes: electrical penetration graph waveform characterization, tissue correlation, and possible implications for transmission of *Xylella fastidiosa*. Ann. Entomol. Soc. Am. 96: 787–813.

Backus, E. A., W. J. Holmes, F. Schreiber, B. J. Beardon, and G. P. Walker. 2009. *Xylemodonas* X wave: correlation of an electrical penetration graph waveform with xylem penetration supports a hypothesized mechanism for *Xylella fastidiosa* inoculation. Ann. Entomol. Soc. Am. 102: 847–867.

Binnington, K. C., and M. Schotz. 1973. Automatic collection of tick saliva. J. Aust. Entomol. Soc. 12: 75–79.

Carolan, J. C., C. J. Fitzroy, P. D. Ashton, A. E. Douglas, and T. L. Wilkinson. 2009. The secreted salivary proteome of the pea aphid *Acyrthosiphon pisum* characterised by mass spectrometry. Proteomics 9: 2457–2467.

Chatterjee, S., R.F.P. Almida, and S. Lindow. 2008. Living in two worlds: the plant and insect lifestyles of *Xylella fastidiosa*. Annu. Rev. Phytopathol. 46: 243–271.
Habibi, J., E. A. Backus, and T. H. Czapla. 1993. Plant lectins affect survival of the potato leafhopper (Homoptera: Cicadellidae). J. Econ. Entomol. 86: 945–951.

Habibi, J., Backus, E. A., Coudron, T. A., and S. L. Brandt. 2001. Effect of different substances on hemipteran salivary protein profiles. Entomol. Exp. Appl. 98: 369–375.

Hattori, M., H. Konishi, Y. Tamura, K. Konno, and K. Sogawa. 2005. Laccase-type phenoloxidase in salivary glands and watery saliva of the green rice leafhopper, *Nephotettix cincticeps*. J. Insect Physiol. 51: 1359–1365.

Harmel, N., E. Letocart, A. Cherqui, P. Giordanengo, G. Mazzucchelli, F. Guillonneau, E. De Pauw, E. Haubruge, and F. Francis. 2008. Identification of aphid salivary proteins: a proteomic investigation of *Myzus persicae*. Insect Mol. Biol. 17: 165–174.

Hoddle, M. S. 2004. The potential adventive geographic range of glassy-winged sharpshooter, *Homalodisca coagulata* and the grape pathogen *Xylella fastidiosa*: implications for California and other grape growing regions of the world. Crop Prot. 23: 691–699.

Hoddle, M. S., S. V. Triaptsyn, and J. W. Morgan. 2003. Distribution and plant association records for *Homalodisca coagulata* (Hemiptera: Cicadellidae) in Florida. Fla. Entomol. 86: 89–91.

Hopkins, D. L., and A. H. Purcell. 2002. *Xylella fastidiosa*: cause of Pierce’s disease of grapevine and other emergent diseases. Plant Dis. 86: 1056–1066.

Joost, P. H., Backus, E. A., Morgan, D., and F. Yan. 2005. Correlation of stylet activities by the glassy-winged sharpshooter, *Homalodisca coagulata* (Say), with electrical penetration graph (EPG) waveforms. J. Insect Physiol. 52: 327–337.

Killiny, N., and R.P.P. Almeida. 2009. Host structural carbohydrate induces vector transmission of a bacterial plant pathogen. Proc. Natl. Acad. Sci. U.S.A. 106: 22416–22420.

Laurema, S. A. L., Varis, and H. Miettinen. 1985. Studies on enzymes in the salivary glands of *Lygus rugulipennis* (Hemiptera, Miridae). Insect Biochem. 15: 211–224.

Miles, P. W. 1968. Insect secretions in plants. Annu. Rev. Phytopathol. 6: 137–164.

Miles, P. W. 1972. The saliva of Hemiptera. Adv. Insect Physiol. 9: 183–255.

Miles, P. W. 1999. Aphid saliva. Biol. Rev. 74: 41–85.

Miles, P. W., and P. Harrewijn. 1991. Discharge by aphids of soluble secretions into dietary sources. Entomol. Exp. Appl. 59: 123–134.

Miles, P. W., and D. Svolka. 1970. Transport of whole protein molecules from blood to saliva of a plant-bug. Experimentia 26: 611–612.

Muttii, N. S., J. Louis, L. K. Pappan, K. Pappan, K. Begum, M. S. Chen, Y. Park, N. Dittmer, J. Marshall, J. C. Reese, et al. 2008. A protein from the salivary glands of the pea aphid, *Acyrthosiphon pisum*, is essential in feeding on a host plant. Proc. Natl. Acad. Sci. U.S.A. 105: 9965–9969.

Newman, K. L., Almeida, R.P.P., Purcell, A. H., and S. E. Lindow. 2004. Cell-cell signaling controls *Xylella fastidiosa* interaction with both insects and plants. Proc. Natl. Acad. Sci. U.S.A. 101: 1737–1742.

Purcell, A. H. 1982. Insect vector relationships with procaryotic plant pathogens. Annu. Rev. Phytopathol. 20: 397–417.

Redak, R. A., Purcell, A. H., Lopes, J.R.S., Blua, M. J., R. F. Mizell, III, and P. C. Anderson. 2004. The biology of xylem fluid-feeding insect vectors of *Xylella fastidiosa* and their relation to disease epidemiology. Annu. Rev. Entomol. 49: 243–270.

Sisterson, M., R. Groves, K. Daane, and S. Thammiraju. 2007. Assessing the potential for forage crops to serve as *Xylella fastidiosa* primary inoculum sources in the San Joaquin Valley. 2007 Pierce’s Disease Research Symposium, T. Esser (ed.), California Department of Food and Agriculture, San Diego, CA.

Taylor, G. S., and P. M. Miles. 1994. Composition and variability of saliva of coreids in relation to phytotoxicoses and other aspects of the salivary physiology of phytophagous Hemiptera. Entomol. Exp. Appl. 73: 265–277.

Tjallingii, W. F. 2006. Salivary secretions by aphids interacting with proteins of phloem wound responses. J. Exp. Bot. 57: 739–745.

Received 8 September 2010; accepted 31 January 2011.