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New and Simple Methods for Studying Hemipteran Stylets, Bacteriomes, and Salivary Sheaths in Host Plants

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ABSTRACT Many hemipteran insects are important agricultural pests because they cause direct feeding damage to their host plants and/or transmit plant disease agents including viruses and bacteria. Microscopic and behavioral studies on five hemipteran species from four families (Psyllidae, Aphididae, Cicadellidae, and Aleyrodidae) showed that their exuviae (molten skins) normally had either fully or partially extended stylets in a feeding-like position. In most cases these stylets were still partially embedded in their host plants after ecdysis, which indicated that plant-feeding hemipteran nymphs use their stylets to anchor themselves to host plants during molting. This phenomenon was used here to study the stylet length and ultrastructure in exuviae of various instars, which is normally more difficult in nymphs than in adults because of the fragility and smaller size of nymphs. Additionally, autofluorescence was used for studying the hemipteran salivary sheaths of nymphs and adults in their host plants. This method is based on fixation of free hand sections of plant parts on which hemipteran insects have been feeding, then mounting and examination of these sections with epifluorescence or confocal microscopy. No embedding, microtomy, or staining is necessary for this method that makes it much faster and simpler than other methods. Autofluorescence was also used to study the location and size of bacteriomes/mycetomes (organs containing symbionts) in hemipteran eggs and nymphs. The above methods were applied successfully with the Asian citrus psyllid (Diaphorina citri Kuwayama), melaleuca psyllid (Boreioglycaspis melaleucae Moore), oleander aphid (Aphis nerii Boyer de Fonscolomb), the whitefly (Bemisia tabaci Gennadius), and/or the glassy-winged sharpshooter leafhopper (Homalodisca citri Pennisi Germar).

KEY WORDS hemiptera, molting, stylet, salivary sheath, bacteriome

Plant-feeding hemipteran insects are important pests of agriculture, either directly through their feeding and excretion activities on their host plants (Miles 1999, Kaloshian and Walling 2005), or indirectly as vectors of many important plant pathogens (Nault, 1997, Ammar and Hogenhout 2006, Ammar et al. 2009). Concerning their direct feeding damage, Miles (1999) indicated that aphids can induce ‘toxicoses’ to their host plants, and Kaloshian and Walling (2005) stated that feeding tactics in Hemiptera range from repeated stylet punctures to long-term feeding and ingestion of cell contents. On their roles as vectors, there are hundreds of plant viruses and several bacterial pathogens, most of which are dependent for their transmission and spread in nature mainly on hemipteran vectors (Purcell and Hopkins 1996, Nault 1997). Hemipteran insects have piercing-sucking mouth parts, the most important of which are the labium, two mandibular and two maxillary stylets, with the latter interlocking to form the food and salivary canals between them (Backus 1988, Chapman 2003). During feeding, these insects produce salivary secretions some of which solidify around the stylets and are termed ‘salivary sheaths’ or ‘stylet tracks’ because they show which tissue in the host plant the insect had been probing and/or feeding on (Miles 1999, Bonani et al. 2009). Because feeding and probing on host plants are instrumental for pathogen transmission by hemipteran insects, the stylets and salivary sheath have been subject of several studies to understand the mechanisms involved in the transmission of viruses and bacteria by their vectors (Ng and Falk 2006, Moury et al. 2007, Uze et al. 2010). Hemipteran insects also contain several symbiotic organisms, some of which are housed in an organ called ‘bacteriome’ or ‘mycetome’ found in the eggs, nymphs, and adults (Buchner 1965, Ammar 1985, Baumann 2005). Some of these symbionts are required for host survival, and some reports suggest that they may play a role in the transmission of plant pathogens (Hogenhout et al. 2008).

The Asian citrus psyllid Diaphorina citri Kuwayama (Hemiptera, Psyllidae) is the main vector of Candidatus Liberibacter asiaticus, a bacterium that has been strongly associated with huanglongbing, or citrus greening, one of the most devastating citrus diseases

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worldwide (Gottwald 2010). In the course of studying the stylets and salivary sheaths of *D. citri*, we developed some new and simple techniques that we tested also on four other hemipteran species: the meleuca psyllid (*Boreiglycaspis melaleucae* Moore, Psyllidae), oleander aphid (*Aphis nerii* Boyer de Fonscolomb, Aphididae), the whitely *Bemisia tabaci* (Gennadius) biotype B (Aleyrodidae), and the glassy-winged sharpshooter leafhopper (*Homalodisca vitripennis* Germar, Cicadellidae). *B. melaleucae* has been used recently as a biological control agent of an invasive tree (*Melaleuca quinquenervia* (Cav.), Myrtaceae) in Florida and California (Pratt and Arakelian 2011). *A. nerii* is a pest of several plant families including Apocynaceae, Euphorbiaceae, and Rutaceae (Blackman and Eastop 1984). *B. tabaci* is a major pest as vector of several plant virus diseases worldwide (Nault 1997, Freeman et al. 2001). *H. vitripennis* is a vector of the xylem inhabiting bacterium, *Xylella fastidiosa*, which causes several diseases of fruit crops including Pierce’s disease of grapevine (Daugherty and Almeida 2009).

The methods we describe here include the use of exuviae (molted skins) to study the ultrastructure of stylets and other mouth parts in various nymphal instars, a simpler method for studying the salivary sheaths in host plants, and the use of autofluorescence to localize the bacteriome (mycetome) in hemipteran eggs and nymphs. Additionally, we provide evidence from the five species studied that hemipteran nymphs use their stylets to anchor themselves to their host plants during ecdysis, which has been suggested by some investigators (Zhao et al. 2010) but with little or no supporting data that we could find in the available literature (e.g., Chapman 2003).

**Materials and Methods**

**Insects and Host Plants.** We used the following five hemipteran species from four different families: 1) Nymphs, exuviae, and adults of *D. citri* (Psyllidae) were taken from our laboratory colony that has been maintained for several generations on young healthy citrus plants (*Citrus macrophylla* Westerm.) in the greenhouse. Individuals from the colony were assayed with polymerase chain reaction (PCR) tests every 3 mo to ensure that the colony remained free of *Ca. Liberibacter asiaticus* associated with hulangong disease. 2) Nymphs and adults of *B. melaleucae* (Psyllidae), on young shoots and leaves of naturally growing meleuca trees (*M. quinquenervia*) were kindly provided by Matthew Hentz (USDA–ARS, Fort Pierce, FL). 3) Nymphs and adults of *A. nerii*, (Aphididae) were collected from milkweed plants (*Asclepias* sp.) growing outside the USDA–ARS building in Fort Pierce, FL. 4) Nymphs and adults of *B. tabaci* biotype B (Aleyrodidae) were kindly provided by Dr. C. L. McKenzie and J. Prokop (USDA–ARS) from colonies reared on tomato, tobacco, or cotton plants. 5) Nymphs and adults of *H. vitripennis* (Cicadellidae), reared on okra plants, were provided courtesy of Dr. Wayne Hunter and Maria Gonzalez (USAD–ARS).

**Observation and Photomicrography of Molting Nymphs and Exuviae.** Feeding and molting behavior of the five hemipteran species studied were observed and photographed (Fig. 1) using a stereomicroscope (*Leica DFC 320*) fitted with a *Leica* camera, or using another stereomicroscope (*Leica M60*) fitted with a video camera (*Leica DFC290 HD*) (Leica, Switzerland). For these observations, *D. citri* nymphs were fed in groups (5–10/group) on small pieces of fresh terminal young shoots (8–10 cm long) of sweet orange (*Citrus sinensis* L.) Osbeck variety Ridge Pineapple or of *C. macrophylla*. The cut end of each shoot piece was placed in a small (0.3–0.5 ml) microfuge tube filled with water to keep it fresh for 3–7 d. Each shoot was then placed in a 50-ml polypropylene tube (Fisher, Pittsburgh, PA) as described earlier (Ammar and Hall 2011) or in a petri dish for easier observation under the stereomicroscope. The rearing tubes or petri-dishes were placed on the bench top in the laboratory (at 23.7 ± 1°C) with 14 h light per day. The other four species studied were similarly caged and observed using their respective host plants mentioned above. Identification of various nymphal instars of *D. citri* followed the drawings by Catling (1970). No attempt was made to identify the nymphal instars of other studies species but mainly older nymphs (third–fifth instars) were examined.

**Scanning Electron Microscopy (SEM) of Exuviae and Stylets.** Exuviae from the five studied hemipteran species (normally still attached to the plant parts they were feeding on during molting) were pulled out gently from these plant parts using fine forceps (Fon-tax no. 5; Electron Microscopy Sciences, Washington, PA). Two methods were used to prepare these exuviae and their stylets for SEM. The first method involved dehydration in 70% ethanol, 100% ethanol (twice) then air drying before mounting, and the second method was direct mounting without prior dehydration. For both methods, the exuviae were mounted on black conductive double-sided adhesive discs (9–12 mm diameter) placed on aluminum stubs (SPI Supplies, West Chester, PA). Using a stereomicroscope, exuviae were mounted with their ventral or lateral sides up to expose the stylets. In some cases, the stylets were gently handled by the forceps or a fine needle to expose them further for SEM. Mounted specimens were then sputter coated with Gold-Palladium for 100–30,000× (Fig. 2).

**Preparing Plant Sections to Study Salivary Sheaths.** Nymphs or adults of the five studied species were caged for 3–7 d on fresh pieces of their respective host plants in petri-dishes or polypropylene tubes as mentioned above (5–10 insects/dish or tube). To immobilize these insects while they were feeding, a piece of filter paper soaked in chloroform was placed inside each dish or tube for 5–10 min in a fume hood. After removing the filter paper piece, the leaf or young
shoot on which the insects had been feeding was observed under a stereomicroscope. The feeding insect (now anesthetized in its feeding position) was removed by holding the wings and gently pulling it out with fine forceps. A clean razor blade was then used to cut a small piece (≈2–3 mm long) of the plant tissue around the feeding position of the removed insect, normally including the midrib or other veins with the surrounding tissue. This plant piece was immediately transferred to a drop of phosphate buffered saline (PBS, pH 7.4; Polysciences) on a clean glass slide, and sectioned by hand using a sharp razor blade to the thinnest possible sections under a stereomicroscope (at 20× or higher). These sections were quickly transferred using fine forceps to small (0.2–0.3 ml) microfuge tubes with 4% paraformaldehyde in PBS. Sections remained in this fixative at least for 1 h (but can be kept there for several hrs or days if necessary), before they were washed three times with PBS-T (PBS with 0.1% Triton × 100). They were then either stained (or not stained) for 5 min with the nuclear fluorescent stain propidium iodide and, if stained, washed again in PBS-T three times. The samples were then mounted on microscope slides using Fluoro-Gel (antifade mounting medium; Electron Microscopy Sciences, Hatfield, PA).

Mounted sections were kept in the dark at 4°C until examination (within 1–4 d) with ultraviolet (UV) light using an epifluorescence inverted microscope (Olympus IX70, with 4, 10, or 20× objectives) fitted with a camera system. Specimens were examined using no filter cubes, thus mainly autofluorescence in these sections was observed (Fig. 3A–G). Transmitted light was often used at the same time with autofluorescence to show the cell boundaries in the plant sections examined. Some plant sections were also examined with a confocal laser scanning microscope (Zeiss LSM 510, 10 or 20× objectives, He/Ne laser) with excitation wavelength of 543, sometimes simultaneously with DIC (differential interference contrast) (Fig. 3H,I). Identification of the phloem, xylem, and other plant tissues was helped by published works including those by Backus et al. (2009) and Bonani et al. (2010).

Epifluorescence Microscopy of Bacteriomes in Eggs and Nymphs. Eggs and nymphs from D. citri and B. tabaci were fixed in 4% paraformaldehyde in PBS by placing them in small microfuge tubes or in glass cavity
dishes (each with a concave cavity ≈20 mm wide and 6 mm deep). They remained in this fixative for 1 h to overnight, followed by washing three times with PBS-T. They were then mounted on glass slides and examined with epifluorescence microscopy as mentioned above for plant sections.

Results

Molting Mechanism and the Role of Stylets. Long and frequent observations with stereomicroscopy as well as several video recordings (30–90 min each) showed that various instars of *D. citri* nymphs may continue feeding/probing for several hours in the same site on young citrus leaves or young terminal shoots, but they can quickly move away from a feeding site when they are disturbed. During molting, *D. citri* nymphs usually stay flat in their normal feeding position on the plant parts they are feeding on (Fig. 1A,B). The first externally visible sign of molting is peristaltic/pumping movement of the nymph during which the thorax expands greatly in size, which leads to a median v-shaped split in the head and thoracic terga (Fig. 1A). Peristaltic movement of both the tho-
and abdomen continues until the molting nymph frees itself from its exuvia (Fig. 1B), leaving the latter in place sometimes with the white anal excretion material still attached to it (Fig. 1C). In the great majority of cases examined from *D. citri* of various instars (*N* >100), the exuviae had fully extended stylets (Figs. 1C,D and 2A,B), usually still attached at least partially to the plant tissue on which they were feeding (Fig. 1C,D). Among most of *D. citri* exuviae examined, a confocal micrograph of extended stylets in *D. citri* exuvia with part of the ‘salivary flange’ (top of the salivary sheath) was still attached to the stylet bundle a short distance (usually <100 μm) from the distal end (Fig. 2B inset).

Fully or partially extended stylets, mostly still attached to the plant host, were also consistently observed in exuviae from the other four hemipteran species examined (*N* = 20–50 exuviae/species). These are: *B. melaleucae* (Fig. 2D), *A. nerii* (Figs. 1E and 2G,H), *B. tabaci* (Figs. 1G and 2E), and *H. vitripennis*.

**Fig. 3.** (A–G) Salivary sheaths (unlabeled arrows) of four hemipteran species in free hand sections of their respective host plants: *D. citri* on citrus leaves (A–C), *A. nerii* on milkweed plant (D), *B. tabaci* on cotton leaves (E), and *H. vitripennis* on okra (F,G). All sections were stained with propidium iodide except that in (C), and all were observed with epifluorescence microscopy, sometimes in addition to transmitted light to show cell boundaries (B,D,E,G). (H,I) Salivary sheaths (unlabeled arrows) of *D. citri* stained with propidium iodide and observed by confocal microscopy with DIC. (J–N) Localization of bacteriomes (unlabeled arrows, blue/green fluorescence) in eggs of *B. tabaci* (J,K), and in nymphs of *B. tabaci* (L) and *D. citri* (M,N). Eggs and nymphs were observed with epifluorescence microscopy in addition to transmitted light (except in M where only fluorescence was used). Abbreviations: cp, cortical parenchyma; fl, fibrous layer around the phloem; he, hatched egg shell; lt, leaf tissue; mt, median tube inside the salivary sheath; ph, phloem; sf, salivary flange; st, stylets; xy, xylem.
(Figs. 1F and 2I,J). In none of these four species, however, did the exuviae stylets contain part of the salivary flange as we observed with D. citri. In addition, with the exuviae of H. vitripennis the stylets were usually only partially extended (Figs. 1F and 2I), whereas in exuviae of the other four species most of the stylets examined appeared to be fully extended.

In all five species examined most of the exuviae were still attached to the host plant after eclosion, and several stayed in the middle of the colony for several hours or days (Fig. 1H, I). In a stereomicroscopy video recording we observed a small colony of aphids (20–30 nymphs and adults of A. nerii and several exuviae with their stylets attached to the plant) on a small piece of a milkweed terminal shoot (Supp. Video S1 [online only]). These live aphids were moving actively knocking and walking on/trampling the attached exuviae without removing any of them from the plant for a period of 66 min (the full length of the recording). This indicates that the attachment of the exuviae stylets to the host plant is strong enough to withstand such a harsh treatment for considerable time.

Using Exuviae to Study the Ultrastructure and Length of Stylets. The mouthparts of exuviae from the five studied species were examined using SEM, either after dehydration or without dehydration. In both cases, the ultrastructure of the stylets and other mouth parts was clearly visible (Fig. 2). In most of the exuviae examined the stylet bundle was compact, in the feeding position, with the two mandibular stylets encasing and covering part of the two interlocking maxillary stylets, and in these cases only the outer surfaces of the mandibular stylets and part of the maxillary stylets could be examined (Figs. 2C–E and 2I–J). The serration/barbing of the distal part of the mandibular stylets was most prominent in H. vitripennis (Fig. 2I), followed by D. melaleucae and D. citri (Fig. 2C,D), A. nerii (Fig. 2H), and finally B. tabaci (Fig. 2E). Slight serration or small ridges were also observed on the terminal sides of the maxillary stylets in D. citri, B. melaleucae, and A. nerii (Fig. 2C,D,H). In some cases, the stylet bundle in the exuviae was not compact at all the way, with the mandibular or maxillary stylets separated from each other either fully (Fig. 2G), or only near the distal end (Fig. 2H), which allowed examination of the inner surfaces of the mandibular or maxillary stylets more freely (Fig. 2H). In other cases, the mandibular or maxillary stylets had apparently slid over each other, thus exposing the distal part of the food and salivary canals formed between the two maxillary stylets (Fig. 2K). Handling of the stylets with fine forceps while mounting the exuviae on the SEM stubs sometimes resulted in separation of the mandibular and maxillary stylets (that allowed studying them more freely from ventral and/or dorsal sides). Occasionally, it also resulted in breaking of the stylet bundle, which allowed examining both the food and salivary canals inside the interlocked maxillary stylets (Fig. 2F).

In addition to studying the stylet ultrastructure in exuviae of various instars, SEM of other mouth parts is possible, including the labrum, labium, and various sensilla on the labial tip (Fig. 2G, I). SEM of exuviae can be used also to study the ultrastructure of other body parts in various nymphal instars, including the antennae, head, thorax, legs, abdomen (Fig. 2A,G), and the anal ring located ventrally near the posterior tip of the abdomen in psyllids (Fig. 2A). Brochosomes, that is, spherical bodies with characteristic sculpturing (Fig. 2J inset), were found on the stylets, labium and other cuticular parts of exuviae from the leafhopper H. vitripennis, but not on the exuviae of other species examined.

To study the length of the stylets in exuviae of various nymphal instars, confocal, or epifluorescence microscopy, rather than SEM, was used and the images analyzed with ‘ImageJ’ computer program (http://rsb.info.nih.gov/ij/). This is because, for confocal and epifluorescence microscopy, exuviae were mounted on glass slides with cover slips that rendered the stylets more flat (Fig. 2B), compared with those in SEM preparations, and thus more easily traceable using a computer mouse. We used exuviae to study the stylet length of various nymphal instars of D. citri (results not shown).

Autofluorescence for Studying the Salivary Sheaths in Plant Hosts. The salivary sheaths of some of the hemipteran species studied, observed in free hand sections in the midrib or other parts of various plant hosts are shown in Fig. 3A–I. The thickness of these free hand sections ranged between 50 and 70 μm, as measured by focusing up and down with confocal laser scanning microscopy (Fig. 3H, I). Epifluorescence microscopy of these sections using UV alone or with transmitted light revealed the salivary sheaths, which were auto-fluorescing in greenish blue color, inside various tissues of the plant host. The route and branching of these sheaths were clear whether propidium iodide was used for staining (Fig. 3A, B, D–G) or not (Fig. 3C). With D. citri (Fig. 3A–C), A. nerii (Fig. 3D), and B. tabaci (Fig. 3E) most of these sheaths appeared to target, branch, and end in the phloem tissue. In most cases, these sheaths took a slightly tenuous course, through the cortical parenchyma or mesophyll layers, before branching in one or several directions within the phloem tissue (Figs. 3A–D). However, with H. vitripennis the salivary sheaths were much wider, and took a more direct path through the cortical parenchyma before ending in xylem vessels (Fig. 3F, G). Most of the xylem vessels also autofluoresced in brighter bluish color (Fig. 3A–C, E–G), and the fibrous layer, or bundle sheath, surrounding the phloem fluoresced in darker blue color (Fig. 3A, B). Using transmitted light along with UV fluorescence imaging allowed the visualization of cell boundaries in various tissues (Fig. 3B, D, E) that were not clear if only fluorescence imaging was used (Fig. 3A, C, F). In some cases, the glistening structure of the salivary sheaths was clear with epifluorescence microscopy (Fig. 3B) but much more so with confocal microscopy and propidium iodide staining (Fig. 3H, I). In the later case, a
narrow median canal inside the salivary sheaths (where the stylets had been during feeding) was also clear especially at higher magnifications (Fig. 3I). However, within the sheaths of *H. vitripennis* this median canal was apparently wide enough to be observed using epifluorescence microscopy especially with transmitted light (Fig. 3G). In some cases, the salivary flange (top of the salivary sheath) was observed at or near the surface of the epidermal layer of a host plant (Fig. 3F–H).

**Autofluorescence for Studying Bacteriomes.** In fixed intact eggs and/or nymphs of *B. tabaci* and *D. citri*, bacteriomes were clearly visible without staining, by their bluish/greenish autofluorescence, using epifluorescence microscopy (Fig. 3J–N). Those in *B. tabaci* eggs appeared as single spherical bodies either in the anterior, middle, or posterior part of the egg (Fig. 3J,K), whereas those in *B. tabaci* nymphs appeared as two oval/elongated bodies in the lower middle part of the abdomen (Fig. 3L). Bacteriomes in the nymphs of *D. citri*; however, appeared as two lateral oblong bodies connected to a centrally located larger rectangular body in the middle of the abdomen (Fig. 3M,N). Using transmitted light in addition to UV fluorescence imaging (Fig. 3J,K,L,N) helped in the localization of the bacteriome in relation to the whole body of the egg or nymph, as opposed to using UV fluorescence alone (Fig. 3M).

**Discussion**

Some investigators have noted that in hemipteran insects the exuviae often remain within the colony and suggested that, with aphids, this can be either ‘bad housekeeping’ or a defense mechanism against parasitoids (Battaglia et al. 2000; Muratori et al. 2006, 2008). In our study, we found that in the great majority of exuviae examined from five hemipteran species belonging to four different families, the stylets were partially or fully extended and were mostly still attached to the host plant after ecdysis. This suggests that, in hemiptera, the exuviae remain in the colony because the nymphs anchor themselves to the plant host, by inserting their stylets deeply into the plant tissue before ecdysis, as part of the molting mechanism. This was also suggested, but not shown, by Zhao et al. (2010) who studied the stylets of the leafhopper *Psammotettix striatus* (Cicadellidae) and stated that the mandibular stylets have sculpture on their tips that may function in attaching the body to the host plant during molting. In our study, most the exuviae stylets were fully extended except in *H. vitripennis* where the stylets were only partially extended. This is probably because the serration/ridges in the distal part of the mandibular stylets in sharpshooters are much more prominent than those in the other species studied. However, even in the fully extended stylets, most of these were found to be only partially embedded in the plant host, probably because the peristaltic movement by the nymphs to shed the old cuticle may have resulted in withdrawing most of the stylet out, except the distal part which is barbed with serrations and ridges in all the species studied. This may also explain why, in the fully extended exuviae stylets of *D. citri*, part of the salivary flange was found usually near the distal end of the stylet bundle (Fig. 2B). That part of the salivary flange was found only around the stylets of *D. citri* may be because of the more waxy surface on citrus leaves compared with that on plant hosts used for other insects studied, which may make the salivary flange more easy to separate from the leaf surface in citrus than in other hosts.

Only the adult stylets have been previously studied for *D. citri* (Garzo et al. 2012), whereas only those of first instar nymphs were studied for the lac insect *Kerria lacca* (Kerr) (Hemiptera, Tachardiidae) (Ahmad et al. 2012). Very few studies, however, have compared the length or ultrastructure of hemipteran nymphs with those of adults, which can be important for studying the feeding behavior and/or pathogen transmission at various stages of development. Leopold et al. (2003) reported that, with the sharpshooter *H. vitripennis*, the number and size of labial sensilla, the length of mandibular and maxillary stylets, and the serration in the mandibular stylets, varied among various instars. Freeman et al. (2000) also reported differences in the stylet length of various instars of the whitely *Bemisia argentifolii*. Obviously, it is more difficult to study the stylets in nymphs than in adults because of the fragility and smaller size of nymphs. Thus, the fact that hemipteran exuviae have fully or partially extended stylets should make it easier to study the morphology and ultrastructure of the stylets, other mouthparts and even other body parts in hemipteran nymphs of various instars. Preparing the exuviae for SEM is much easier than preparing live nymphs because, as we did here, there is no need for fixation, dehydration, or critical point drying of the exuviae. However, manipulation of the stylets in mounted exuviae may help in the separation of different stylets and/or in observing the inner structures of the maxillary food and salivary canals.

The brochosomes, found here on the stylets and labium of the exuviae from the *H. vitripennis* have been reported to cover the outer cuticle in most of the leafhopper species previously studied (Rakitov 1999). These structures are known to be excreted by the Malpighian tubules and then distributed on the cuticle as part of the ‘anointing’ behavior in leafhoppers (Rakitov 1999). Studying the exuviae of leafhoppers may provide more information on the distribution of these interesting structures in nymphs of various species.

To study the hemipteran salivary sheaths of various insects in their host plants, most investigators have previously used microtome cut, stained, 1–12 μm thick sections from fixed and embedded plant material (Young et al. 1995, Brennan et al. 2001, Backus et al. 2009, Bonani et al. 2009). Others have used stained, unsectioned plant parts especially with whiteflies (Freeman et al. 2001). Only a few, however, have used free hand sections with clearing, staining and dehydration (Leopold et al. 2003, Ahmad et al. 2012). In our study, we used autofluorescence to observe the sali-
vary sheaths of hemipteran insects in free hand sections of various host plants after fixation with or without staining. We believe that this method, which does not involve clearing, dehydration, embedding, microtomy, or necessarily staining, is much faster and simpler than most other methods previously used. In addition, the larger thickness of the hand-cut sections that we used (50–70 μm) may have allowed observing the gnarling and branching of the salivary sheaths more readily than would be possible with the thinner sections obtained by microtomy. However, some resolution may be lost with hand sections compared with microtome sections unless confocal microscopy is used for greater resolution. The nuclear fluorescent stain propidium iodide that we used did not make much difference when examining plant sections with epifluorescence, but it improved observation of details of the salivary sheath structure on examination with confocal microscopy. The salivary sheaths of aphid, psyllid, and whitefly species examined here appeared to target and end in the phloem tissues, but the sharp-shooter leafhopper sheaths ended in xylem vessels, which is consistent with previous reports (Brennan et al. 2001, Freeman et al. 2001, Bonani et al. 2009, Backus et al. 2009). However, some aphids may feed on the xylem sap in addition to the phloem to regulate osmotic potential (Spiller et al. 1990, Pompon et al. 2011).

Confocal microscopy was also used here to visualize the bacteriomes (mycetomes) in fixed, intact hemipteran eggs and nymphs without staining. This can be a useful tool for studying the location and size of bacteriomes in various instars of hemipteran insects. In our study, the bacteriome observed in the nymphs of D. citri is fairly similar in shape and location to that reported in the nymphs of another psyllid, Glycaspis brimblecombei Moore, which is known to harbor several endosymbionts (Baumann 2005). Whiteflies are known to have a relatively small, paired, and roundish or oval bacteriome (Buchner 1965, Baumann, 2005), which is consistent with the bacteriome we found in the nymphs of B. tabaci. In eggs of B. tabaci, however, we observed one spherical bacteriome in the anterior, middle, or the posterior end of the egg. The position of the bacteriome in hemipteran eggs depends on their stage of embryonic development (Ammar 1985), which is consistent with blastokinesis, that is, reversion of the embryo inside the egg during development (Chapman 2003).

Finally, it is hoped that the faster and simpler methods described here will be useful to other investigators studying the mouth parts, bacteriomes, salivary sheaths, and feeding behavior of these and other economically important groups of plant feeding hemipteran insects.

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References Cited


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