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# Ultrastructure and development of the new stylets inside pre-molting first instar nymphs of the Asian citrus psyllid *Diaphorina citri* (Hemiptera: Liviidae)

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The Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is an economically important citrus pest mainly because it vectors the bacteria associated with huanglongbing (HLB, citrus greening), currently the most devastating citrus disease worldwide. The piercing sucking mouthparts of the psyllids play an important role in acquisition and transmission of this phloem limited bacteria, and late instar nymphs of ACP are more efficient transmitters than adults (Hall et al. 2013). Ultrastructure of the mouthparts in ACP nymphs and adults has been described earlier (Garzo et al. 2012; Ammar & Hall 2013; Ammar et al. 2013). However, the development and morphogenesis of new stylets in the psyllids and other Hemiptera has been described only by light microscopy (Weber 1929; Heriot 1934). In the present work, we studied the ultrastructure and development of new stylets inside the pre-molting first instar nymph of ACP using thin-sectioning transmission electron microscopy.

Healthy (non-HLB infected) nymphs of ACP used were from our laboratory colony that has been maintained on young healthy citrus (*Citrus macrophylla* Wester) or orange jasmine (*Murraya exotica* L.) plants in the greenhouse. First instar nymphs of ACP (whole body) were fixed in 3% glutaraldehyde in phosphate buffer, pH 7.4. Specimens were fixed overnight at 4 °C, postfixed in 1% osmium tetroxide in the same buffer for 1 h, washed in buffer, dehydrated in ethanol and propylene oxide, and embedded in Spurr's resin. Ultrathin sections (transverse or tangential in relation to the insect body) were stained with uranyl acetate and lead citrate and examined at 25 Kv using a scanning-transmission electron microscope (Hitachi S-4800, Hitachi, Pleasanton, California) in the TEM mode at magnifications up to 20,000X.

One of the four 1st instar nymphs sectioned and examined turned out to be in a pre-molting phase. This was evidenced by having 2 layers of external cuticle around the whole body: the old (stretched) cuticle and an underlying new cuticle layer secreted by the epidermal cells (Fig. 1A). The new cuticle was folded/wrinkled in several places and an exuvial space was located between the old and new cuticle layers. In cross sections of this and other 1st instar nymphs sectioned and examined, the old (functional) stylet bundle consisted of 2 external mandibular stylets (each with 2 dendrites in its central canal) and 2 maxillary stylets that were interlocked forming 2 canals between them, a wider food canal and a narrower salivary canal (Fig. 1B). In the cephalic region of the pre-molting 1st instar nymph, under the epidermis, 2 oval-shaped/oblong masses of cuboidal hypodermal cells with large nuclei were found (Figs. 1C and 1D). On both sides of each mass, long extensions of these hypodermal cells, sometimes with nuclei, ended with either 2 sections of a mandibular stylet or 2 sections of a

maxillary stylet (Figs. 1C and 1D). Thus, each mass of hypodermal cells apparently produces one mandibular and one maxillary stylets, each of them coiled around this mass during the stylet's development/morphogenesis. The occurrence of 2 sections of a mandibular or a maxillary stylet on each side of the hypodermal cell mass suggests that the 'coil' of each developing stylet around this mass has 2 'turns' inside the cephalic region of first instar nymphs. The above findings and interpretations are consistent with an earlier light microscopy study on other Hemiptera (aphids and scale insects) in which Heriot (1934) described the process of developing stylets as follows: "The retort-shaped organs at the bases of the stylets are masses of hypodermal cells constituting deep invaginations of the integument. Within these invaginations new stylets are built up in each successive instar. The 4 stylets are separately coiled in the cephalic region, and at a definite stage during each ecdysis the new stylets pass down to take the place of the old (stylets) which are discarded at the molt". To our knowledge, this is the first ultrastructural study that confirms and expands that account by Heriot (1934).

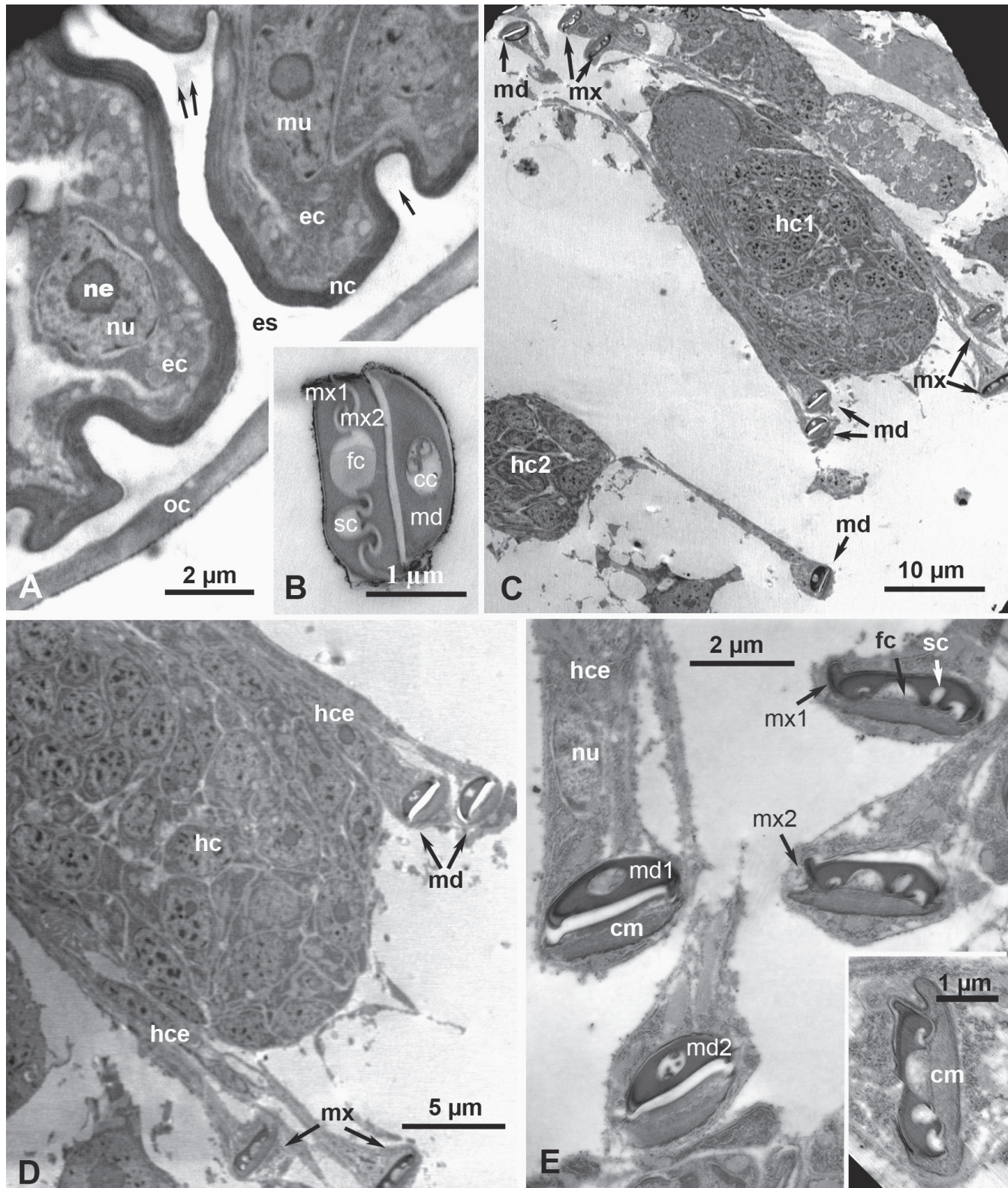
Higher magnifications of cross sections of the developing mandibular and maxillary stylets at the end of the hypodermal cell extensions forming them are shown in Fig. 1E. The mandibular stylet has the usual central canal enclosing 2 dendrites, and the maxillary stylet has grooves representing parts of the food and salivary canals as well as the cuticular ridges that interlock with the opposing maxillary stylet. Additionally, a new structure seemingly composed of lighter (more electron lucent) cuticle was found on the ventral side of each developing mandibular or maxillary stylet usually taking the opposite (mirror) shape of its companion stylet, which suggests that this structure may work as a mold during the secretion of new stylets by the hypodermal cells. This structure has not been reported earlier in previous studies of developing stylets in psyllids or other Hemiptera (Weber 1929; Heriot 1934), probably because the resolution of the light microscope was too low to discern it. Chapman (2013) indicated that insect cuticles are initially formed as rather soft structures, but become hardened in a process called sclerotization, i.e. the cross linking of cuticular proteins. Thus, the above structure associated with the developing stylets may be composed of softer cuticle that works as a mold, then is probably dissolved/ discarded during later stages of ecdysis.

In the pre-molting 1st instar nymph examined, other parts in which double cuticle layers (old and new) were found are the esophagus (Fig. 2A), the hindgut (not shown here) and major trachea (Fig. 2B). As expected, the midgut and filter chamber (shown in detail in Fig. 2C) were not lined with cuticle. Additionally, 2 morphologically different types of bacteria (electron dense and electron lucent) were found in the bac-

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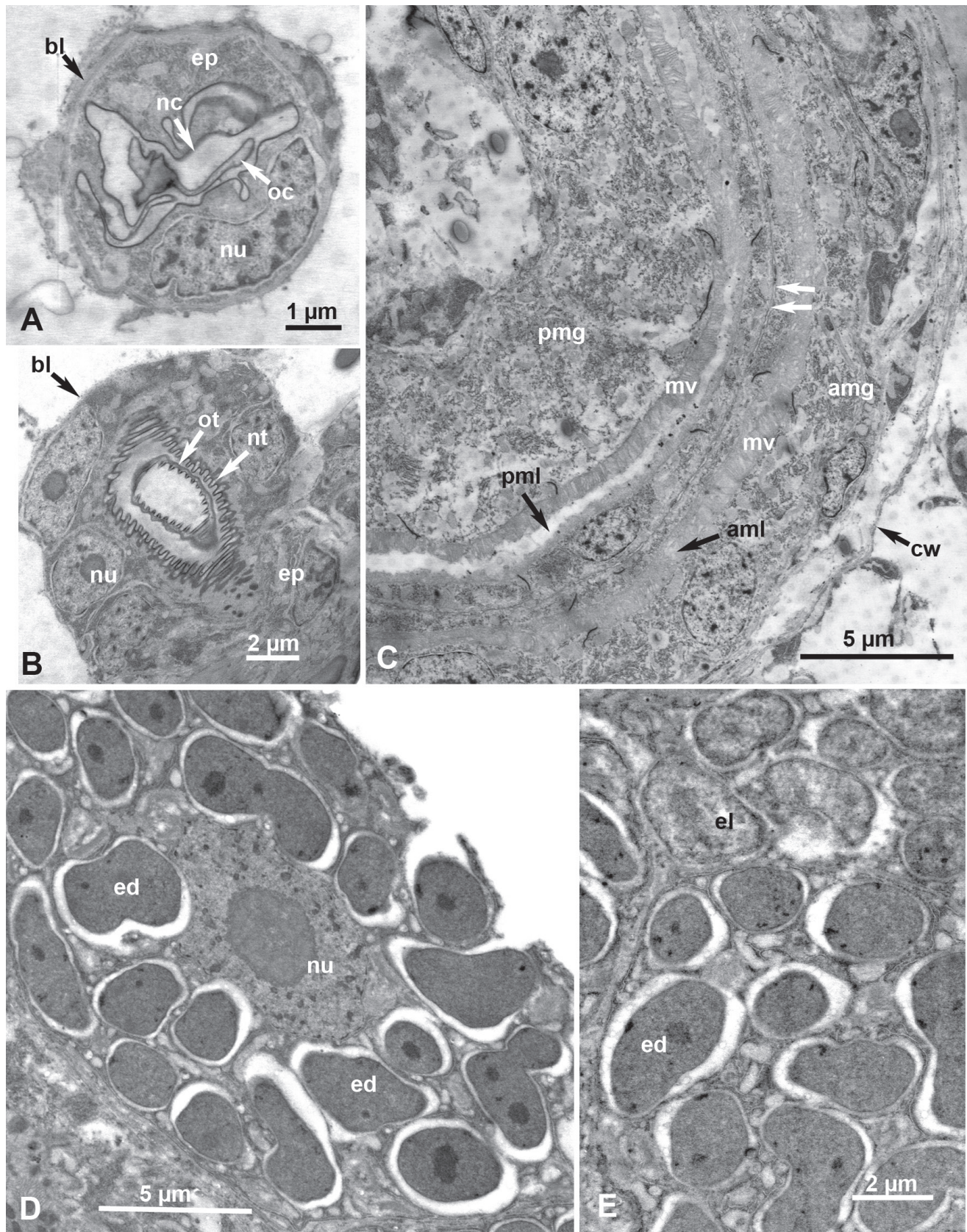
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**Fig. 1.** Transmission electron micrographs of thin sections in the cuticle and new and old stylets of a pre-molting 1st instar nymph of *D. citri*. **A.** Old (oc) and new (nc) layers of the cuticle, with minor (arrow) and major (double arrows) folds in the new cuticle; note the exuvial space (es) between the new and old cuticle, and the epidermal cells (ec) with large nuclei (nu) and nucleoli (ne). **B.** The old (functional) stylets; note the larger food canal (fc) and a narrower salivary canal (sc) between the 2 interlocked maxillary stylets (mx1, mx2), and a mandibular stylet (md) with 2 dendrites in its central canal (cc); the other mandibular stylet has sprung out (during processing) and is not included in this section. **C & D.** Two masses of hypodermal cells (hc1, hc2) with large nuclei, and hypodermal cell extensions (hce) that end with developing mandibular (md) and maxillary (mx) stylets on each side. Note that around hc1, 2 sections of each of the (coiled) mandibular and maxillary stylets are shown, but around hc2 only one section of the mandibular stylet can be seen (the rest of the stylet sections are outside the frame of this image). **E.** Higher magnification of 2 sections in a developing (coiled) mandibular stylet (md1, md2) and a developing (coiled) maxillary stylet (mx1, mx2); fc, part of the food canal; hce, hypodermal cell extension with a large nucleus (nu); sc, part of the salivary canal; inset shows details of the cuticular molding structure (cm).





**Fig. 2.** Transmission electron micrographs of thin sections in other organs of 1st instar nymphs of *D. citri*. **A & B.** The esophagus (A) and tracheal cells (B) of a pre-molting nymph; bl, basal lamina; ep, epithelial cells; nc, new cuticle; nt, new taenidia; nu, nucleus; oc, old cuticle, ot, old taenidia. **C.** Part of the filter chamber showing the chamber wall (cw), anterior midgut (amg) and posterior midgut (pmg); double arrows indicate closely apposed basal lamina of the anterior and posterior midgut; mv, microvilli; pml, posterior midgut lumen; aml, anterior midgut lumen. **D.** Bacteriocyte cell in the bacteriome with a large nucleus (nu) and various shaped electron-dense bacterial cells (ed). **E.** Another part of the bacteriome with 2 types of bacterial cells: electron-dense (ed) and electron-lucent (el).

teriome cells (bacteriocytes) of the 1st instar nymphs sectioned and examined (Figs. 2D and 2E), which is consistent with previous reports from adults of other psyllids (Baumann 2005).

## Summary

The ultrastructure and development of new stylets was studied in pre-molting first instar nymph of *Diaphorina citri* Kuwayama (Hemiptera: Liviidae). Two oval-shaped masses of cuboidal hypodermal cells, located in the cephalic region, had long extensions that ended with developing pairs of mandibular and maxillary stylets, apparently coiled around these masses. A new structure, probably composed of softer cuticle, was found on the ventral side of each developing stylet suggesting that this structure may work as a mold during formation of the new stylets. Other organs of 1st instar nymphs, including the filter chamber and bacteriome, are also ultrastructurally described.

Key Words: bacteriome, citrus greening disease, filter chamber, huanglongbing, mandibular stylets, maxillary stylets

## Sumario

Se estudiaron la ultraestructura y el desarrollo de los nuevos estiletes en la pre-muda del primer instar de la ninfa de *Diaphorina citri* Kuwayama (Hemiptera: Liviidae). Dos masas de células cúbicas hipodermales de forma ovalada, ubicadas en la región cefálica, tenían extensiones largas que termine en el desarrollo de pares de estiletes mandibulares y maxilares, al parecer, enrollados alrededor de estas masas. Una nueva estructura, probablemente compuesta de cutícula

más suave, se encuentro en el lado ventral de cada estilete en desarrollo lo que sugiere que esta estructura puede funcionar como un molde durante la formación de los nuevos estiletes. Otros órganos de las ninfas del primer estadio, incluyendo la cámara de filtración y bacteriome, también se describen ultraestructuralmente.

Palabras Clave: bacteriome, enverdecimiento de los cítricos, cámara de filtración, Huanglongbing, estiletes mandibulares, estiletes maxilares

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