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Gross morphology and ultrastructure of the female reproductive system of *Diaphorina citri* (Hemiptera: Liviidae)

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ABSTRACT. The morphological traits of the female reproductive system of *Diaphorina citri* were examined in detail. *Diaphorina citri* has telotrophic ovaries with ovarioles organized as a "bouquet", displaying a rudimentary terminal filament and a syncytial tropharium. The vitellarium carries a single growing oocyte at each maturation cycle, which is connected with the tropharium by a nutritive cord. Morpho-functional changes occur during oocyte development, mainly during mid to late vitellogenesis. Morphological events such as the patency of the follicular cells and the intense traffic of vesicles through para- or intracellular processes, suggest a possible route for endosymbiont invasion of *D. citri* reproductive tissues. Similar events have been demonstrated to be involved in the process of ovariole invasion by endosymbionts in other sternorrhynchans that share reproductive traits with psyllids.

KEY WORDS. Accessory structures; Asian citrus psyllid; oogenesis; ovary.

Insect ovaries are differentiated as panoistic and meroistic (Büning 1994). In the former all of the oogonia differentiate as oocytes, while in the latter part of the germline cells develop into trophocytes. Trophocytes are specialized cells that supply oocytes with macromolecules and organelles during the previtellogenic growth stage (King & Büning 1985, Büning 2006). A typical insect ovariole is subdivided from apex to base into a fine terminal filament, tropharium (trophic chamber), vitellarium and a pedicel (ovariolar stalk) that opens into the calyx of a lateral oviduct (Büning 1994). The number of ovarioles per ovary varies considerably among different taxa, ranging from a single ovariole in aphids (Büning 1985, 1994) to thousands in termite queens and coccids (Büning 1994, Gillot 2005). Auchenorrhyncha may have from one to 15 ovarioles per ovary, whereas members of Heteroptera commonly have seven ovarioles per ovary (Büning 1994, Lalitha *et al*. 1997, Hodin 2009). Plasticity in the number of ovarioles has been documented for aphids and scale insects in Sternorrhyncha, and the ovary of psyllids can contain up to 100 ovarioles (Büning 1994, Hodin 2009). The structure of the reproductive apparatus and the process of oogenesis in Heteroptera (Hemiptera) have been extensively studied (Lalitha et al. 1997, Caperucci & Camargo-Mathias 2006), but information for the remaining suborders is scarce (Buning 1985, Szklarzewicz et al. 2008, 2013). In order to increase the overall knowledge on the reproductive system of sternorrhynchans, we characterized the morphology and ultrastructure of the female reproductive apparatus of *Diaphorina citri* Kuwayama, 1908 during oogenesis.

MATERIAL AND METHODS

Diaphorina citri was maintained at controlled conditions (28 ± 2°C, 60 ± 10% UR, photophase 14 hours) using orange jasmine seedlings, *Murraya exotica* Linnaeus, 1771 (Sapindales: Rutaceae), as a host (Tsai & Liu 2000, Nava *et al*. 2007). Fifth instars and adult females were sampled at different developmental stages for dissection of their reproductive apparatus.

Last instar, newly-emerged, and reproductively active females of *D. citri* (Wenninger & Hall 2007), at the previtellogenic and vitellogenic stages (Dossi & Cônsoli 2010), were dissected in insect saline (3 mM $CaCl_2.2H_2O$, 182 mM KCl, 46 mM NaCl, 10 mM Tris base, pH 7.2) (CSH Protocols 2007). Ovaries were immediately transferred to the proper fixative solution for further processing.

Dissected ovaries were fixed in AFATD solution (75 mL 96% ethanol, 10 mL 40% formaldehyde, 5 mL acetic acid, 10 mL dimethylsulfoxide, 1 g trichloroacetic acid) (Martínez 2002) for two hours at room temperature, hydrated in a decreasing series of ethanol (96, 80, 70, 50 and 30% – five minutes each) and distilled water (five minutes), hydrolyzed in 2.5 N hydrochloric acid (five minutes), stained with Schiff reagent (30 minutes), rinsed in distilled water (five minutes) and counterstained with light green (one minute) (Martínez 2002). Afterwards, samples were rinsed in distilled water (five minutes), absolute ethanol (2x – 10 minutes/each), diaphanized in xylol (2x – 10 minutes/each), and mounted in Entelan® (Merck). Samples were examined with a Zeiss Axiostar Plus light microscope.

Histological analysis. Dissected ovaries were fixed in PFA-PBTw (5% paraformaldehyde, 1% Tween20 in 0.1% phosphate buffer solution (w/v), pH 7.2) for 24 hours, rinsed in the same buffer (3x – 10 minutes) and dehydrated in a graded series of ethanol (1x – 30, 50, 70 and 90%; 3x – 100% – 10 minutes/each). Samples were then embedded in ethanol:historesin (Leica Historesin) solution (1:1) for 24 hours, followed by 24 hours at 4°C in pure historesin, and polymerized at room temperature for 48 hours. Semithin sections (1-2 µm) were stained in 1% toluidine blue + 1% sodium borate solution or Azan Heidenhain-Mallory (0.5% aniline blue, 1% orange G, 3% acid fuchsin, 1% phosphotungstic acid, w/v) (Behmer *et al*. 2003) for one minute. After staining, sections were quickly rinsed in distilled water, and hot plate dried at 45°C for 20 minutes followed by mounting with Entelan® (Merck) and examination with a Zeiss Axioskop 2 light microscope.

Fluorescence microscopy. For fluorescence microscopy, the dissected ovaries were fixed in PFA-PBTw solution and embedded in historesin as described before. Semithin sections (1 µm) were stained with 1.5 µg DAPI (4', 6'-diamidine-2-phenylindole) in mounting medium Vectashield® (DAPI, Sigma Chemical Co.) for 15 minutes to detect DNA, followed by 15 minutes incubation in rhodamine and phalloidin (Sigma Chemical Co.) for the detection of the actin filaments. Samples were observed under an Olympus BX51 epifluorescence microscope.

Transmission electron microscopy (TEM). Ovaries fixed in Karnovski fixative (3% glutaraldehyde, 3% paraformaldehyde, in 50 mM cacodylate buffer, 5 mM $CaCl_2$, pH 7.2) for 24 h, were rinsed in the same buffer (3x – 10 minutes), post-fixed in 1% osmium tetroxide (OsO_4) in 50 mM cacodylate buffer (60 minutes) and counterstained *in bloco* with 0.5% uranyl acetate for 12 hours. Samples were then dehydrated in a graded series of acetone, followed by embedding (EMBed 812, Electron Microscopy Sciences) (24 hours) and polymerization at 60°C for 24 hours. Ultrathin sections (50-70 nm) were mounted on copper grids, stained in 3% uranyl acetate followed by 1% lead citrate, and analyzed with a Zeiss EM900 transmission electron microscope.

Scanning electron microscopy (SEM). Ovaries fixed as described for TEM were dehydrated in graded series of acetone and critical point dried (CPD-030 Balzers, BAL-TEC). Afterwards, ovaries were mounted on stubs and gold sputter-coated (SCD-050 Sputter Coater, BAL-TEC). Image acquisition and analysis were carried out with a Zeiss LEO 435 VP scanning electron microscope.

RESULTS

Diaphorina citri has meroistic telotrophic ovaries formed by nearly 50 ovarioles arranged in a "bouquet" (Figs 1, 2, and 4). Ovaries are located ventro-laterally in the median region of the abdomen, just below the bacteriome, an organ that harbors the symbiotic associated bacteria (not shown) (for review, see Baumann 2005). They are surrounded by fat body tissue and a dense network of tracheae in both immature and adult stages. In newly-emerged adults, ovaries are small and all oocytes are at the previtellogenic stage (Fig. 2). But in mated females, they are fully developed and have oocytes at all stages of maturation (Figs 3 and 4).

The pedicel of the ovarioles opens into the apical bulb of their respective lateral oviducts (Figs 1, 3, and 4), which will merge forming the common oviduct (Fig. 1). The oviducts are formed by a single epithelium of columnar cells underlying a cuticular intima, which is surrounded by a thick basal lamina and muscle bundles (Fig. 22).

Three accessory structures are laterally connected to the common oviduct: a paired accessory gland, a spermatheca and a colleterial gland (Fig. 1).

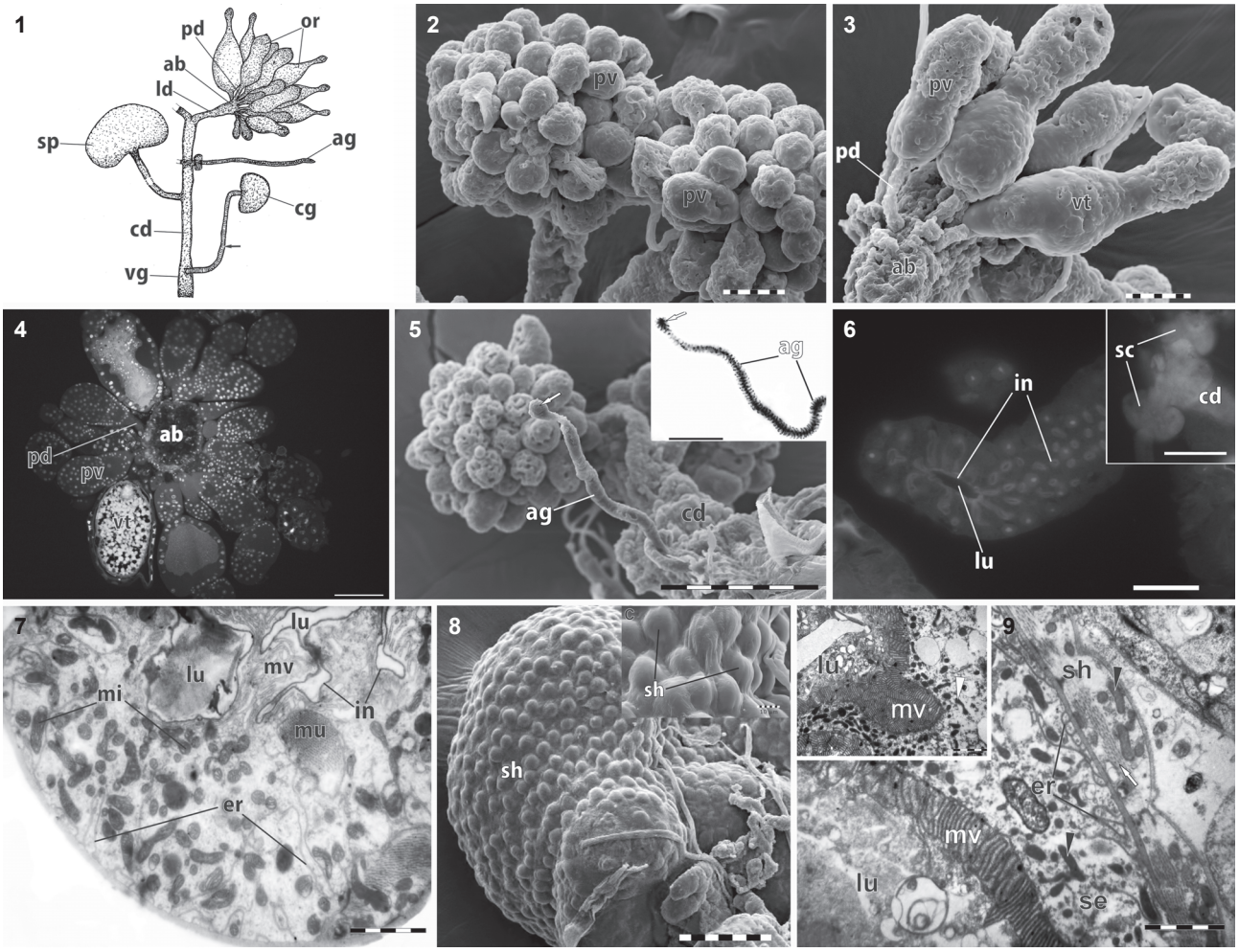
A pair of tubular accessory glands is placed on opposite sides of the upper part of the common oviduct, near to the lateral oviducts. Glands are slightly dilated at their distal region, but bear a small sac-like structure at their base (Figs 1, 5, and 6). These glands are composed of large secretory cells, which contain a nucleus at the base of the cell and a conspicuous nucleolus. Mitochondria are densely distributed, mainly in the distal region of the cell, which has long microvilli (Fig. 7).

The sacciform reservoir of the spermatheca is connected to the common oviduct at a more distal position than the accessory glands (Figs 1 and 8). Both are formed by a single layer of epithelial cells, but only the cells of the reservoir have secretory activity. Their cytoplasm is filled with endoplasmic reticulum, mitochondria, and microvilli facing the gland lumen. These cells are surrounded by a basal lamina and a spermathecal sheath, a single layer of dome-shaped cells filled with muscle fibers and mitochondria (Fig. 9).

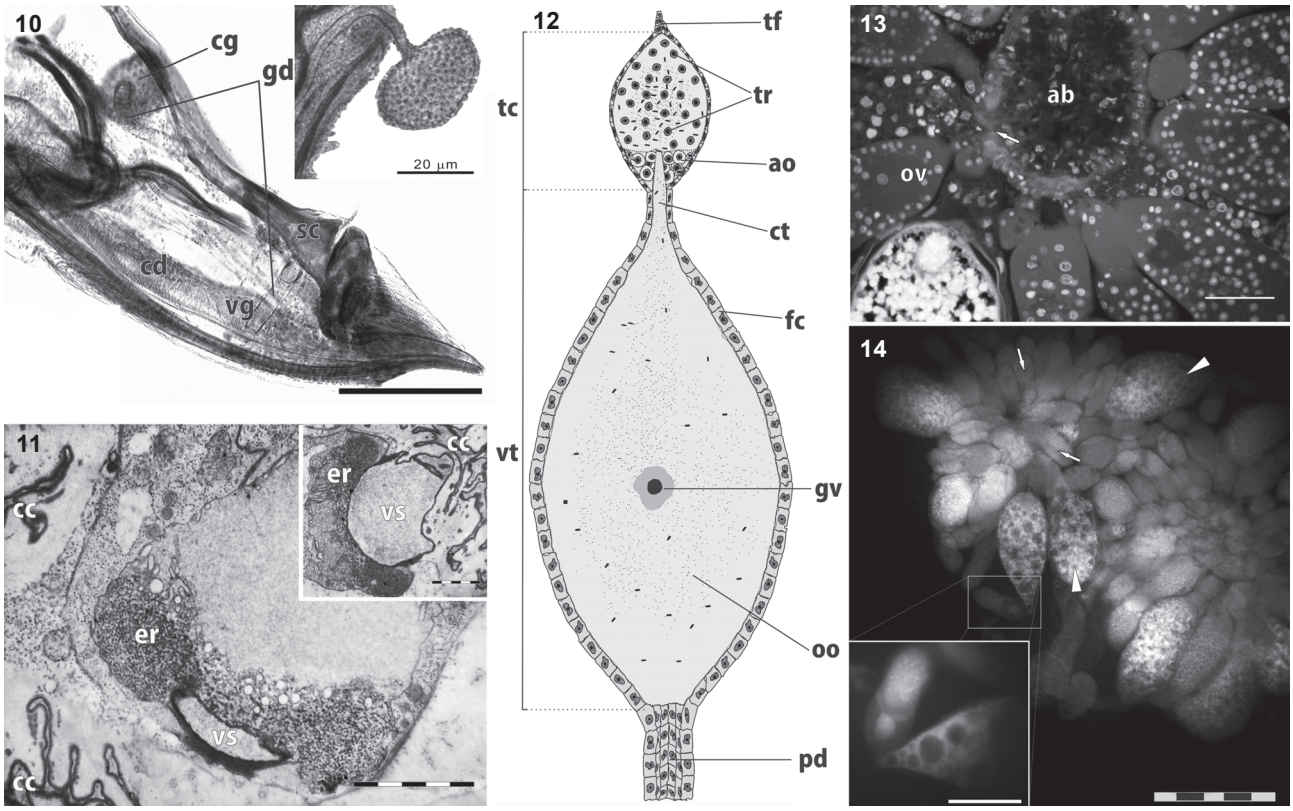
The colleterial gland has a sac-like shape and is located at the base of the ovipositor sclerites and above the accessory glands (Fig. 10). It is connected to the vagina by a long trachea-shaped duct made up of cuticular rings inserted into a single-layer epithelium. The colleterial gland has a single layer of secretory cells characterized by the presence of endoplasmic reticulum and vesicles that temporarily stores secreted molecules. The vesicle contents are unloaded into a network of collection channels that opens in the glandular lumen (Fig. 11).

Four distinct regions were identified in the ovarioles of *D. citri* (Fig. 12): I) a rudimentary terminal filament, II) an ovoid trophic chamber (Figs 15 and 16) that communicates with the growing oocyte into the III) vitellarium (Figs 15 and 16) by means of a cytoplasmic projection, the nutritive cord (Figs 16 and 20). At the base of the vitellarium, there is a IV) ovariolar stalk (pedicel) of a tubular aspect (Figs 3, 12, and 13) that allows the passage of the egg downwards to the calyx of the adjacent lateral oviduct (Fig. 22) after the completion of egg development. Development of the *D. citri* ovarioles is meta-

chronic, with those at the margins maturing before those located internally (Fig. 14). The vitellarium has only one oocyte developing per maturation cycle, but as soon as the basal oocyte choriogenesis has been completed, a new oocyte at a very early previtellogenic stage can be seen at the base of the trophic chamber (Fig. 14, inset).



Figures 1-9. *Diaphorina citri*. (1-4) Gross morphology of the female reproductive system: (1) schematic diagram showing the ovary and its ovarioles (or) arranged in a bouquet, inserted by its pedicel (pd) in the apical bulb (ab) at the tip of the corresponding lateral oviduct (ld) which converges with the common oviduct (cd). A pair of accessory glands (ag) a spermatheca (sp) and a colleterial gland (cg) are observed. The colleterial gland (cg) is attached to the common oviduct by a long duct (setae). (2) Previtellogenic (pv) ovarioles of immature ovaries. (3-4) Vitellogenic (vt) and previtellogenic (pv) ovarioles of a mature ovary. (2-3) Scanning electron microscopy; (4) cross-section of ovary at the apical bulb region stained with DAPI (4'-6-diamidino-2-phenylindole), epifluorescence microscopy. (5-7) Accessory gland of *Diaphorina citri*: (5) view of the rounded tip (setae) of the accessory gland (ag) and the intricate network of channels (inset) as seen by whole-mount preparation. (6) Gland lumen (lu) and the intricate network of folded channels underlying a cuticular intima (in) in a tangential section. Whole-mount view (inset) of the sac-like structures (sc) at the base of the gland, placed in apposition to the common oviduct (cd). (7) Cytoplasm filled with mitochondria (mi), endoplasmic reticulum (er), muscle fibers (mu), and microvilli (mv) underlying a cuticular intima (in) at the lumen (lu) interface in a cross section. (5) scanning electron microscopy; (5-inset and 6) autofluorescence as seen by epifluorescence microscopy; (7) transmission electron microscopy. (8-9) Spermatheca: (8) Surface view of the spermathecal sheath (sh) represented in a section in (9) as a layer of cells (sh) containing bundles of muscle fibrils (setae) and mitochondria (arrowhead). Spermathecal sheath is covering a secretory epithelium (se) (er, endoplasmic reticulum; mv, folded network of microvilli; lu, lumen). (8) scanning electron microscopy; (9) transmission electron microscopy. Scale bars: 2 = 30 µm; 3 = 20 µm; 4 = 100 µm; 5 = 100 µm, inset = 50 µm; 6 = 20 µm, inset = 20 µm; 7 = 2 µm; 8 = 100 µm, inset = 10 µm; 9 = 2 µm, inset = 2 µm.



Figures 10-14. *Diaphorina citri*. (10-11) Colleterial gland: (10) general view of the colleterial gland (cg) placed at the distal region of the ovipositor sclerites (sc) and connected through its long and trachea-like duct (gd) to the vagina (vg) (cd, common oviduct); (11) secretory cell filled with endoplasmic reticulum (er) unloading its vesicle (vs) contents (see inset) in an intricate network of cuticular collector channels (cc). (10) bright field microscopy; (11) transmission electron microscopy. (12-14) Ovariole: (12) Schematic view of an ovariole: terminal filament (tf); trophic chamber (tc) containing trophocyte nuclei (tr) inserted into the syncytial tropharium and arrested oocytes (ao); vitellarium (vt) with follicular cells (fc) covering the growing oocyte (oo) that presents a conspicuous germinal vesicle (gv) and communicates with the tropharium through the trophic chord (ct). The pedicel (pd) is placed at the base of the ovariole; (13) Pedicel is connected (setae) to the apical bulb (ab); (14) Metachronic development of ovarioles, with well-developed maturing oocytes located at the periphery of the ovaries (arrowhead), while poor-developed maturing oocytes are internally located (setae). (13-14) epifluorescence microscopy. Scale bars: 10 = 20 µm, inset = 20 µm; 11 = 2 µm, inset = 2 µm; 13 = 50 µm, 14 = 300 µm, inset = 50 µm.

During the previtellogenic stage the follicular cells are columnar or cuboidal, changing their shape during ongoing maturation, becoming rectangular during vitellogenesis and elongated during the late choriogenic stage (Figs 17-18 and 21). The increase in the number of cytoplasmic organelles in the follicular cells and the accumulation of yolk was noticeable once vitellogenin uptake started through a paracellular route. Additionally, the large number of vesicles within the cell cytoplasm is also suggestive of the acquisition and transport of nutrients by pinocytosis (Fig. 18).

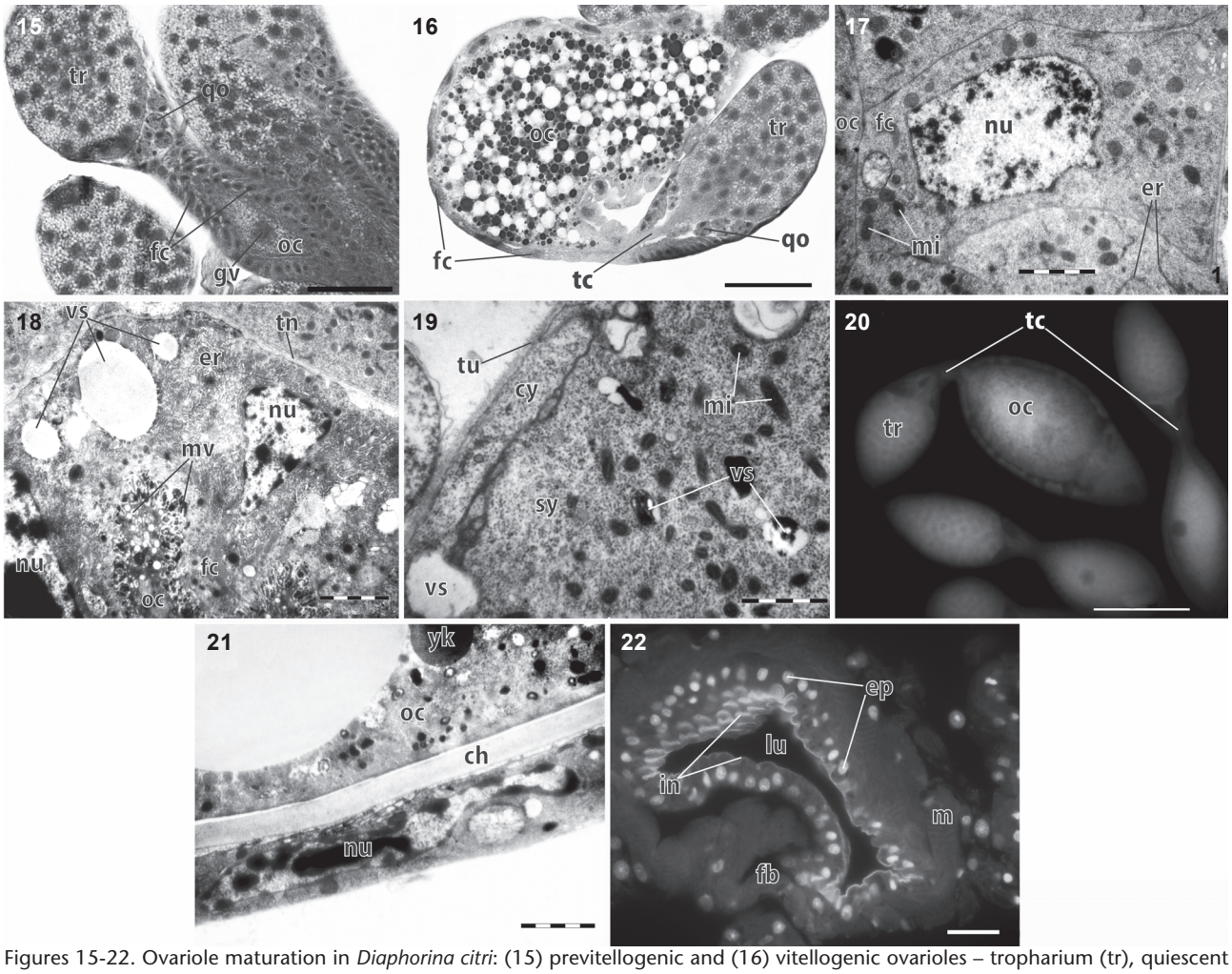
The trophic chamber of *D. citri* is surrounded by a single layer of flattened somatic cells which is covered by a tunica propria. The thickness of this layer of cells is variable along the periphery of the tropharium as the cells become very elongated and thin on the edges away from the nucleus (Fig. 19).

The trophocytes become devoid of plasmic membrane before the previtellogenic stage, originating a syncytial tropharium. There is an accumulation of electron-dense vesicles and multivesicular bodies, mainly in the central region of the trophic chamber. The previtellogenic oocyte accumulates the cytoplasmic components produced in the tropharium, transferred through the nutritive cord (Fig. 20), in which we did not observed microfilaments.

There are about 10 quiescent oocytes (i.e. oocytic cells) located at the base of the trophic chamber in each ovariole, but only one matures at a time (Fig. 15). During the previtellogenesis, the oocyte becomes enlarged due to the incorporation of cytoplasmic components (e.g., organelles, proteins and RNA) produced in the tropharium. The mid-previtellogenic stage is marked by the moderate secretory ac-

tivity of the follicular cells, characterized by the presence of small vesicles between the plasmatic membrane and the cytoplasm of the oocyte. The follicular cells reach their maximal activity during the mid to late vitellogenesis. During this stage of oocyte development, their secretory activity is characterized by the abundance of vesicles of different sizes, probably due to the processing of macromolecules (mainly proteins and lipids) from hemolymph. Glycogen deposits were detected by transmission electron microscopy at the perivitelline space, suggesting that such elements are thus added to the oocyte. The oocyte reaches its maximum size at the end of the vitellogenic stage, when the surrounding follicular cells become elongated and thin.

During late choriogenesis, the chorion (Fig. 21) is observed as a result of the accumulation of precursors secreted by the follicular cells into the perivitelline space.



Figures 15-22. Ovariole maturation in *Diaphorina citri*: (15) previtellogenic and (16) vitellogenic ovarioles – tropharium (tr), quiescent oocytes (qo), nutritive cord (tc), growing oocyte (oc), germinal vesicle (gv) and follicle cells (fc). (17-18) follicle cells from previtellogenic (17) and vitellogenic (18) ovarioles. The distinct metabolic condition of the cells in each stage is revealed by the difference in the abundance of organelles, such as mitochondria (mi), endoplasmic reticulum (er) and secretory vesicles (vs) at the interface (mv) between follicular cells (fc) and oocyte (oc) during the accumulation of yolk. Note the presence of the tunica propria (tn) at the distal region of the follicular cell. (19) partial view of tropharium showing the thin, elongated epithelial cells (tu, tunica propria, vs, vesicle, mi, mitochondria, sy, syncytium). (20) ovarioles at different stages of maturation showing the nutritive cord (tc) (oc, oocyte, tr, tropharium). (21) mature oocyte revealing the elongated format of the follicle cell (nu, nucleus, ch, chorion, oc, oocyte cytoplasm, yk, yolk granules). (22) cross-section of the common oviduct showing the epithelium (ep) underlying a folded cuticular intima (in) (lu, lumen, fb, dense fibrous layer, m, muscle bundles), (15-16), bright field microscopy; (17-19 and 21) transmission electron microscopy; (20 and 22) epifluorescence microscopy. Scale bars: 15 = 30 µm; 16, 20 = 50 µm; 17, 18, 21 = 2 µm; 19 = 1 µm; 22 = 20 µm.

DISCUSSION

The meroistic telotrophic ovaries of *D. citri* are common to other Sternorrhyncha (Büning 1994, Michalik *et al*. 2013). The mature ovaries of this species, in which the ovarioles lack a functional terminal filament, are similar to those of some Aphidomorpha and Aleyrodomorpha (Büning 1985, Szklarzewicz & Moskal 2001). However, the types and localization of the accessory structures are variable from previously studied species (Lococo & Hubner 1980, Stacconi & Romani 2011, Sturm 2012, Ma *et al*. 2013). The occurrence and location of accessory structures may differ among species as they are highly specialized secretory organs involved in the synthesis of molecules related to several aspects of reproduction (sperm storage and nourishment, egg laying, among others). The synthesis and release of their contents are often linked to time-dependent physiological events during the reproductive stage (Sturm & Pohlhammer 2000, Gillott 2002, Klowden 2007). Although the accessory structures are frequently linked to the basal region of the common oviduct (Büning 1994), they are placed in different regions of the common oviduct in *D. citri*. One exception is the duct of the colleterial gland, which is linked to the distal part of the vagina.

The number of ovarioles of *D. citri* is much greater than in aphids (1-11) (Couchman & King 1979, Büning 1985) and aleyrodids (5-15) (Szklarzewicz & Moskal 2001), indicating that this trait is highly variable among Sternorrhyncha. The metachronic development observed in the ovarioles of *D. citri* has also been described for aphids, aleyrodids, scale insects (Couchman & King 1979, Büning 1985, 1994), and some Diptera and Psocoptera (Büning 1994).

The structure of the ovarioles of *D. citri* is very similar to that of aleyrodids (Büning 1994). The arrangement of the germline cells (cystocytes) in rosettes during the development of the trophic chamber is a relatively common trait in psyllids, aphids, aleyrodids (King & Büning 1985, Büning 1994), and scale insects (Szklarzewicz 1997).

We did not observe bundles of microfilaments into the nutritive cord of *D. citri*, as reported for other psyllids (Büning 1994), but mechanisms based on ionic gradients and on osmotic pressure are also known to be involved in the translocation of cytoplasmic components from the trophic chamber to the growing oocyte (Huebner & Diehl-Jones 1993, Telfer & Woodruff 2002).

The ultrastructural characteristics of the inner epithelial sheath of the trophic chamber of *D. citri* resemble those in aphids (Michalik *et al*. 2013). However, the syncytium of the tropharium of *D. citri* lacks a trophic core, differently from what is observed in aphids and heteropterans (Brough & Dixon 1989, Szklarzewics *et al*. 2000).

In *D. citri*, the activity of the nuclei in the tropharium is evident by the presence of "*nuage*-like", electron-dense agglomerates facing the nuclear envelope (as visualized by transmission electron microscopy), and is an indication of the nucleocytoplasmic transference of substances (Davenport 1976, Huebner & Diehl-Jones 1993). The accumulation of protein-filled vesicles, commonly associated with the Golgi complex and mitochondria in the tropharium, points to the selective uptake of molecules from hemolymph, and synthesis of morphogens (Couchman & King 1979). The *nuage* can be a ribonucleoprotein complex, as it shares similar morphology with different organisms. Nuages are frequently seen as small patches of dense granular material. They can be associated with mitochondrial clusters or lie adjacent to the nuclear envelope. Larger accumulations of dense material can also occur free in the cytoplasm (Eddy 1976). The major function of nuage is to maintain genome stability by repressing the expression of selfish genetic elements via small interference RNA-mediated gene silencing (for review, see Eddy 1976, Kim Lim & Kai 2007, Kim Lim *et al*. 2013). Furthermore, the presence of multivesicular structures in *D. citri*, which are similar to the cytolysomes dispersed in the syncytium, suggests that the tropharium may play a role in molecule processing and organelle resorption. The basal location of the arrested oocytes in the trophic chamber of *D. citri* follows the common pattern described for telotrophic ovaries (Büning 1994, Michalik *et al*. 2013), with only one oocyte developing per reproductive cycle. This reproductive strategy is also observed in *Megoura viciae* Buckton, 1876 (Homoptera: Aphididae) (Brough & Dixon 1989) and *Adelges laricis* Vallot, 1836 (Hemiptera: Adelgidae) (Szklarzewicz *et al*. 2000), and may be an adaptation to space or nutritional limitations, given that other insects have the capacity to produce several eggs per cycle (Lalitha *et al*. 1997, Szklarzewicz *et al*. 2008, Winnick *et al*. 2009).

The follicular cells surrounding the growing oocytes in the telotrophic ovarioles of *D. citri* have an important role during vitellogenesis, as they participate in the transport of molecules from hemolymph, and actively synthesize and incorporate macromolecules in the oocyte (Huebner & Anderson 1972, Raikhel & Dhadialla 1992). The occurrence of pinocytic vesicles in the follicular cells of *D. citri* was observed even during choriogenesis. Nevertheless, the late occurrence of molecule transport has been seldom indicated (e.g., Beams & Kessel 1969, Cruickshank 1972).

The accessory glands of the reproductive apparatus of insects are associated with the secretion of substances that participate in fertilization and oviposition (Wheeler 2003). The release of the electron-lucent secretions from the accessory gland of *D. citri*, may be linked to time-dependent physiological mechanisms, such as egg laying and fixation onto the substrate, as reported elsewhere (Gillott 2002, Klowden 2007, De Santis *et al*. 2008).

We provided here the first overview of the ultramorphology of the female reproductive system of *D. citri*. We believe that this information will be useful in furthering our understanding of the reproductive biology of this psyllid. Our data

will also support future investigations on other reproductive traits of the Asian citrus psyllid and on the vertical transmission of symbiotic bacteria associated with this insect during its reproductive period.

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