Morphology and ultrastructure of the esophagus during the ontogeny of the spider crab *Maja brachydactyla* (Decapoda, Brachyura, Majidae).

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Short title: *Maja brachydactyla* esophagus morphology and ontogeny

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Abstract.

The esophagus of the eucrustaceans is known as a short tube that connects the mouth with the stomach but has generally received little attention by the carcinologists, especially during the larval stages. By this reason, the present study is focused on the morphology and ultrastructure of the esophagus in the brachyuran *Maja brachydactyla* Balss, 1922 during the larval development and adult stage. The esophagus shows internally four longitudinal folds. The simple columnar epithelium is covered by a thick cuticle. The epithelial cells of the adults are heavily interdigitated and show abundant apical mitochondria and bundles of filamentous structures. The cuticle surface has microspines and mutually exclusive pores. Three muscle types surrounded by the connective tissue are reported: circular muscles forming a broad continuous band, longitudinal muscle bundles adjacent to the circular muscles, and dilator muscles crossing the connective tissue vertically toward the epithelium. The connective tissue has rosette glands. The esophagus of the larvae have epithelial cells with big vesicles but poorly developed interdigitations and filamentous structures, the cuticle is formed by a procuticle without differentiated exocuticle and endocuticle, the connective layer is thin and the rosette glands are absent. The observed features can be explained by his role in the swallowing of the food.

Keywords: Eucrustacea sensu Walossek 1999; larval development; epithelium; microspines; rosette glands

1. Introduction

The comprehension of the digestive process is fundamental to the understanding of the nutrition of decapods (Ceccaldi, 1989; Vogt, 1996) and it is a crucial step for the production of aquaculture species (Zambonino-Infante et al., 2008). The foregut of decapods derives from the embryonary ectoderm and it is lined by a chitinous cuticle. It is constituted by two organs: the esophagus and the stomach (Ceccaldi, 1989; Felgenhauer, 1992; Icely and Nott, 1992; McLaughlin, 1983). The study of the foregut of the decapods has been overshadowed by the stomach (Felgenhauer, 1992; Icely and Nott, 1992) and comparatively little attention has been realized on the esophagus. Henry Milne-Edwards (1834a; b) published one of the earliest descriptions using crabs of the genus *Maja* (presumably *M. brachydactyla*) and other species as models. The first sentence that describes the esophagus indicated that "*l'oesophage ne présenterien de remarquable*" (the esophagus does not show anything remarkable). This impression was maintained during the next decades, being described as a simple, short, vertical tube that connects the mouth with the stomach (Ceccaldi, 1989; Felgenhauer, 1992).

However, in fact the esophagus comprises different structures: longitudinal folds (Felgenhauer, 1992; McLaughlin, 1983); internal cuticle surfaces covered by microspines or setae (Elzinga, 1998; Elzinga and Hopkins, 1994; 1995; McLaughlin, 1983) and pierced by pore-like structures (Robertson and Laverack, 1979); epithelium surrounded by a connective tissue with rosette or "tegumental" glands (Barker and Gibson, 1977; 1978; Erri Babu et al., 1979; Trinadha Babu et al., 1989) and strong circular and longitudinal muscles (Barker and Gibson, 1977; 1978; Erri Babu et al., 1979; Felgenhauer, 1992; McLaughlin, 1983; Trinadha Babu et al., 1989). However, few publications have focused on this organ (Altner et al., 1986; Erri Babu et al., 1979; Robertson and Laverack, 1979; Spirito, 1975), and the study of the esophagus during

62 the larval stages has been largely neglected in studies focused on the foregut or on the
63 general digestive anatomy (Johnston and Ritar, 2002; Minagawa and Takashima, 1994;
64 Schlegel, 1911; Tziouveli et al., 2011).

65 We consider the esophagus an important organ involved in the swallowing of the
66 food pieces provided by the mouth appendages, with the knowledge of their
67 morphology being important for understanding this role. The main objective of this
68 study is a detailed description of the esophagus of a representative Decapoda species
69 from hatching to the first juvenile stage, as well during the adult stage employing
70 morphological, histological and ultrastructural approaches. The species selected for this
71 study was the spider crab *Maja brachydactyla* Balss, 1922. It is a commercial
72 brachyuran species with fisheries located on the NW of the Iberian Peninsula, Denmark,
73 France, Ireland, Portugal, and United Kingdom (FAO, 2012). In *M. brachydactyla*, the
74 larval development has two zoeal stages (zoea I and zoea II) and one megalopa stage
75 (Guerao et al., 2008). Previous studies focused on the feeding and digestive system of
76 *M. brachydactyla* during their larval development, in which the mouthparts (Guerao et
77 al., 2008), the general morphology of the stomach (Castejón et al., 2015), and the
78 ontogeny of the digestive enzymatic capacity were described (Andrés et al., 2010), as
79 well as the tolerance to starvation and refeeding on the success of the larval
80 development (Guerao et al., 2012; Rotllant et al., 2010).

81

2. Material and methods

2.1 Adult and larval culture system

The adult specimens of *M. brachydactyla* were captured along the coasts of the Northwest Iberian Peninsula and Ireland (CADEMAR & LONXANET), and transported to the Institut de Recerca i Tecnologia Agroalimentàries (IRTA, Sant Carles de la Ràpita, Tarragona, Spain). The adult specimens were euthanatized and dissected to obtain the foregut, or employed for reproductive purposes, maintaining a sex ratio of six females and one male per tank. In this last case, the specimens were maintained in 2,000 L cylindrical tanks with a renewal rate of 3.5 m^3 h^{-1}. The environmental parameters were: 18 ± 1 °C, 35 ± 1 psu, and photoperiod 12 h light: 12 h dark provided by fluorescent tubes at 25 lux. The feeding consisted on fresh and frozen mussels (*Mytilus* sp. Linnaeus, 1758). Under these conditions continuous larval hatches can be obtained yearly during 5-6 months (Simeó et al., 2015). The larvae ca. 12 hours after hatch were recovered from the broodstock tanks by the water drainage into 35 L PVC baskets. Then, the larvae were put directly in 600 mL glass beakers placed inside 360 L tanks (96 x 96 x 40 cm) used as incubation chambers. The environmental parameters were: 21 ± 1 °C, 35 ± 1 psu, and photoperiod 12 h light: 12 h dark provided by white LED lights at 1,000 lux. The feeding consisted of fresh *Artemia* sp. Kellogg, 1906 nauplii (INVE Aquaculture Nutrition, Salt Lake UT, USA). Daily, the living specimens were carefully pipetted to glass beakers with clean water and fresh food. The specimens were sampled at each larval stage: zoea I ca. 15 hours after hatching, zoea II at 3-4 days after hatching (dah), megalopa at 6-7 dah, and first juvenile at 12-13 dah, and fixed according to the required procedure (see next sections).

106 *2.2 Optical microscopy study*

107 In the present study the stages of zoea I, zoea II, megalopa and first juvenile will
108 be considered together as "immature stages". For the gross morphology observations,
109 entire specimens of immature stages were fixed in formaldehyde 4 %. Then, these
110 specimens were dissected and the foregut extracted and cleaned by their immersion in a
111 solution of 10 % KOH at 80 °C during 15-20 min. The cleaned foreguts were mounted
112 in microscope slides for their observation without staining. In the case of the adults, a
113 fresh foregut was obtained and the pictures were taken with a digital camera. The
114 esophagus of three specimens of each immature stage were measured; by contrast, the
115 length of the adult esophagus is an approximation because it is strongly attached to the
116 mouth opening and can break when extracted.

117 To study the tissue organization of the esophagus in immature stages the
118 specimens were fixed as a whole; in the case of the adults the fresh foreguts were
119 extracted and the esophagus sectioned with a scalpel into longitudinal and transversal
120 sections before fixation. Thereafter, Davidson's fixative (ethanol absolute: seawater:
121 formaldehyde 37 %: glycine: glacial acetic acid in proportion 3: 3: 2: 1: 1) was
122 employed as fixative during 24 h. An automatic tissue processor was used for the
123 dehydration and embedding in paraffin, then a paraffin processor was used to prepare
124 the paraffin blocks (AP208, Myr, Spain). A microtome (Leica RM2155, Wetzlar,
125 Germany) was employed to cut 2 µm sections. The staining techniques used were: 1)
126 Hematoxylin and Eosin (H-E) to show the general morphology of the tissue; 2) Periodic
127 Acid–Schiff (PAS) to reveal substances with affinity to neutral polysaccharides and
128 mucopolysaccharides; and 3) Mallory's trichrome stain (Acid Fuchsine, Orange G and
129 Aniline Blue stains) to visualize the structure of the muscular and connective tissues.

The observations were realized under an optical microscope (Leica LB30T 111/97, Wetzlar, Germany) connected to a camera (Olympus DP70 1.45 Mpx) and an image analyzing system (DP Controller 2.1.1.83 and DP Manager 2.1.1.163; Olympus).

2.3 Transmission and scanning electron microscopy study

Zoea I specimens and pieces of the esophagus of the adults were fixed with 2 % paraformaldehyde - 2.5 % glutaraldehyde in cacodylate buffer (0.1 mol L^{-1} pH 7.4) in total darkness at 4 °C for 12 h. Then, the samples were rinsed twice with cacodylate buffer and post-fixed in 1 % osmium tetroxide solution in cacodylate buffer. After the post-fixation the samples were dehydrated in a graded series of acetone. For the transmission electron microscopy post-fixed samples were embedded in Spurr's resin and cut into semi-thin (0.5 µm) and ultrathin (50-70 nm) sections with an ultramicrotome (Leica UCT, Wetzlar, Germany). Before observation, grids were counterstained with uranyl acetate and lead citrate. The observations were realized in a JEOL EM-1010 electron microscope at 80 kV equipped with an image analysis system (AnalySIS, SIS, Münster, Germany), a single zoea I specimen and two adult specimens were observed. For scanning electron microscopy, post-fixed samples were critical-point-dried, mounted on SEM stubs with self-adhesive stickers and coated with carbon. Observations were made with a JEOL JSM-7001F scanning electron microscope. The post-fixative treatment and TEM and SEM observations were realized at CCiTUB (Hospital Clinic, University of Barcelona, Spain).

3. Results

The mouth of *M. brachydactyla* open ventrally, while the stomach is positioned dorsally (Fig. 1C). The esophagus is a short, almost vertical tube that connects the mouth with the ventral floor of the cardiac stomach (Fig. 1A-C). The esophagus length has been measured during development: 233 ± 5 µm in zoea I, 265 ± 20 µm in zoea II, 265 ± 17 µm in megalopa, 297 ± 24 µm in juvenile 1, and between 15 and 25 mm in the adult (Fig. 1A-C). The cross-section is quadrate; however the lumen has an X- or H-like shape due to the presence of four main internal longitudinal folds or evaginations located on the lateral walls (Fig. 2A-B; 3A, C, E).

The basic structure of the esophagus is similar in all the life stages, but it is more complex in the adults than in the immature stages (Fig. 2-3). The basic structure comprises an epithelium covered by a cuticle. Below the basal lamina a layer of connective tissue is present: in the adults the connective layer is wide and contain groups of rosette glands (Fig. 2A-D; 8A), while in the immature stages it is very thin and glands were not observed (Fig. 3B-C). Three orientations of striated muscle fibers have been observed: circular, longitudinal and dilator muscles (Fig. 2A-D; 3A-D). The circular muscles form a continuous band that wrap the connective tissue and the epithelium along the longitudinal axis of the esophagus (Fig. 2A-C). In the adults, this muscle layer is highly developed and associated with blood vessels (Fig. 2B) and constitutes the most prominent muscle layer of the immature stages (Fig. 3A-C). The bundles of longitudinal muscles of the adults are located in the connective layer, adjacently to the circular muscle band (Fig. 2A-B), but in the immature stages the longitudinal muscles were not identified. The dilator muscles of the adults are identified crossing vertically the connective tissue to reach perpendicularly the epithelium (Fig.

175 2A-B, D), while in the immature stages they connect the epithelium of the esophagus
176 with the epithelial cells of the tegument located behind the mandibles (Fig. 3A-D).

177 *Epithelial cells*. The epithelium of the esophagus is composed of a single cell
178 type and is covered by a cuticle. The morphology of the epithelium differs between
179 adults and immature stages. The adults have a simple columnar epithelium, measuring
180 around 47 ± 19 μm in height (Fig. 2E). In the immature stages, the invaginations have a
181 simple squamous epithelium (3-5 μm in height), while the evaginations have a stratified
182 epithelium composed by basal irregular cells and distal short columnar (25-30 μm in
183 height) cells (Fig. 3E; 5B-C).

184 The adult epithelial cells have a marked polarity (Fig. 4A, B, D); whereas in
185 zoea I no polarity has been observed (Fig. 3E; 5A-B). The apical cell membrane
186 contains a structure denominated by us as an "apical complex": these are irregular
187 electron-dense infolds intracellularly connected to bundles of filamentous structures
188 (Fig. 4F). In the adults, the lateral cell membranes are heavily interdigitated; the number
189 of interdigitations increases toward the cell basis (Fig. 4B, D). By contrast, in the zoeae
190 I the lateral cell membranes are smoothly undulated and lateral interdigitations are
191 marginal (Fig. 5A-C). The cells are joined by cell-to-cell junctions, but their extension
192 is not yet well defined (Fig. 4F; Suppl. Mat. 1). The basal membrane of the adults is
193 heavily infolded (Fig. 4D); while in the zoeae I the basal membrane is generally smooth
194 (Fig. 5B-C).

195 Neither adults nor immature stages showed PAS positive granules in their
196 cytoplasm (Suppl. Mat. 3). In the adults, the cytoplasm is generally lucent, containing
197 sparse ribosomes, small lucent vesicles and multivesicular bodies (Fig. 4A-F). In the
198 immature stages the majority of the cytoplasm can be occupied by a giant vesicle
199 without affinity to the staining techniques used (Fig. 3A-C). By electron microscopy

200 this vesicle is lined by a single membrane and contains aggregations of electron-dense
201 matter (Fig. 3E; 5A-B; Suppl. Mat. 1-2). In the adults the cell nucleus is located medial
202 to basally (Fig. 2E; 4A, D); while in immature stages the cell nucleus can be located
203 from the cell basis to the cell apex (Fig. 3E; 5A-B).

204 The mitochondria concentrate at the cell apex of the adult cells, but in the zoeae
205 I they do not show such a distribution pattern (Fig. 4A, B, F; 5A-B; Suppl. Mat. 1-2).
206 The rough endoplasmic reticulum is composed by thin, short and dispersed cisternae
207 (Fig. 4D-E; 5D). Golgi bodies are scarce (Fig. 4E). One of the most important features
208 of the epithelial cells is the presence of bundles of filamentous structures (Fig. 4C),
209 whose identity is unclear. The bundles of filamentous structures are more prominent in
210 the adults (Fig. 4B-D, F) than in the zoeae I (Fig. 5D; Suppl. Mat. 1). In the adults they
211 cross the cell from the basis (Fig. 4D) to the apex (Fig. 4B, F). The apical extreme of
212 the filamentous structures are attached to the "apical complex" of the apical membrane
213 (Fig. 4F).

214 *Cuticle organization*. The epithelial cells are apically covered by a cuticle layer.
215 In the adults the cuticle thickness is 86 ± 18 µm. The adult cuticle shows the typical
216 layers of the arthropod cuticle: epicuticle, exocuticle and endocuticle (Fig. 2D-E; Suppl.
217 Mat. 3). The epicuticle is the outmost cuticle layer, and represents ca. 10 ± 1 % of the
218 cuticle thickness. It is strongly stained by Orange G and Eosin, and it can be subdivided
219 into three sub-layers: 1) the outermost sub-layer is a thin refractory coat; 2) the next
220 sub-layer shows affinity to Eosin and acquires a yellow color with Orange G, it is
221 composed by fibers without a defined orientation; and 3) the most basal epicuticle sub-
222 layer shows affinity to Hematoxylin and acquires an orange color with Orange G, and is
223 composed of fibers with a vertical orientation that protrudes toward the underlying
224 exocuticle (Fig. 2D-F; 6A-B). The next layers are the exocuticle and the endocuticle.

Both layers differentiate from the epicuticle due to their lamellar structure and their homogeneous staining affinity to Hematoxylin and Aniline Blue (Fig. 2D-E). PAS staining is slightly stronger in the exocuticle than in the endocuticle (Suppl. Mat. 3). The exocuticle represents ca. 30-40 % of the cuticle thickness and it is composed of wide lamellae (5.8 ± 1.1 µm), while the endocuticle represents ca. 50-55 % of the cuticle thickness and it is composed by thin lamellae (2.5 ± 0.6 µm) (Fig. 6B-C). Concomitantly with the proximity to the cell basis the lamellae of the endocuticle gradually lose definition and become extensively wider (Fig. 6D).

The cuticle of the immature stages is much thinner and the typical layers of the arthropod cuticle cannot be identified by optical microscopy (Fig. 3B-C). The cuticle of the zoeal stage is around 570 ± 70 nm, it has an epicuticle that represents ca. 20 ± 5 % of the cuticle thickness and a lamellate procuticle (Fig. 7B; Suppl. Mat. 1-2).

The cuticle surface shows two types of structures: microspines and pores. The microspines appear in all the life stages. The microspines are projected from the epicuticle (Fig. 7B) as hair-like structures difficult to observe by optical microscopy (Fig. 7A). The microspines of the adults form groups of one to three microspines attached to each other, occasionally some groups include more than three microspines (Fig. 7C-D), with the density of groups of microspines calculated to be ca. 0.7 per 10 μm^2. Each microspine is approximately 4 ± 1 µm in length (Fig. 7C). The distribution pattern of the microspines is unclear; we observed areas rich in microspines and areas where the microspines are absent. In the zoeae I, the microspines are shorter (540 ± 180 nm in ZI) and each microspine is individually separated from each other (Suppl. Mat. 4).

The pores have been observed on the adult cuticle but not in the zoeae I. The pores are aggregated in "pore areas", they are microspine free areas with a circular to

250 elliptical shape, slightly elevated in comparison to the surrounding cuticle and pierced
251 by pores (Fig.7F). Two types of pores have been identified. The "large pores" are 2 µm
252 in diameter or more, the cross section is circular and are surrounded by a smooth
253 elevation of the cuticle (Fig. 7G). The "small pores" have around 1 µm in diameter or
254 less, the cross section is horseshoe-like and are surrounded by an abrupt elevation of the
255 cuticle (Fig. 7H).

256 *Rosette glands*. In the adults, the connective tissue of the esophagus contains
257 rosette glands (Fig. 2D; 7E; 8A). The rosette glands are absent from the esophagus of
258 the immature stages, but appear below the tegument of the mouthparts and the anterior
259 distal portion of the thoracic ganglionic mass, nevertheless they seem more associated
260 with the mouth and mouthparts than with the esophagus itself (Fig. 1C). The rosette
261 glands are clusters of secretory cells aggregated into acinar like structures with a
262 diameter of 75-100 µm. The secretory cells surround a central tube that channel the
263 secretions outside the glands (Fig. 8A). The cuticle is pierced by tube-like structures
264 located above some gland agglomerations, showing a possible secretion pathway of the
265 products released by the secretory cells (Fig. 7E). There is only a single type of
266 secretory cell in the rosette glands. These cells have a pyramidal shape and measure ca.
267 35 µm in height (Fig. 8A). The cytoplasm is filled by vesicles of 865 ±118 nm in
268 diameter, however many vesicles are fused into larger structures (Fig. 8B-C). The
269 vesicles contain a dense fibrillar-like matrix with a variable electron-density (Fig.8B-E).
270 Some secretory cells contain a cytoplasm occupied by vesicles that are similar in
271 appearance and size to a "vacuole" (Fig. 8A). The nucleus is located basally (Fig.8A).
272 Mitochondria are usually located around the Golgi bodies (Fig. 8B). Golgi bodies are
273 abundant, large and highly developed (Fig. 8B). The rough endoplasmic reticulum
274 forms a thin layer located adjacent to the nucleus and the basal membrane (Fig. 8D-E).

4. Discussion

The esophagus of *M. brachydactyla* is a short vertical tube that connects the mouth with the stomach as has been described in other decapods (Ceccaldi, 1989; Felgenhauer, 1992; Icely and Nott, 1992), including larval stages such as the zoeae of the brachyuran crabs *Maja* sp. (Schlegel, 1911), *Ranina ranina* (Minagawa and Takashima, 1994), and *Scylla olivacea* (Jantrarotai and Sawanyatiputi, 2005), the zoeae of the caridean shrimp *Lysmata amboinensis* (Tziouveli et al., 2011) and the phyllosomata of the achelatan rock lobster *Panulirus ornatus* (Johnston et al., 2008).

The esophagus is the organ responsible for the transport of the ingested food from the mouth opening to the stomach. The passage of the food into the stomach requires the dilation of the esophageal walls. For this reason, when relaxed the esophagus of *M. brachydactyla* shows four internal longitudinal folds from hatching to the adult stage, which provide capacity for elastic expansion of the esophagus and passage of food to the stomach. Similar infoldings have been described in brachyurans (Erri Babu et al., 1982; Minagawa and Takashima, 1994; Trinadha Babu et al., 1989), astacideans (Factor, 1981; Loya-Javellana et al., 1994; Yonge, 1924), penaeids (Dall, 1967), achelatans (Johnston and Alexander, 1999) and carideans (Patwardhan, 1935; Pillai, 1960; Sousa and Petriella, 2006).

To realize the peristaltic movements required to swallow the food requires a powerful set of muscles. The adult esophagus of *M. brachydactyla* shares with other brachyuran crabs such as *Menippe rumphii* (Erri Babu et al., 1982), *Portunus sanguinolentus* (Trinadha Babu et al., 1989), *Scylla serrata* (Barker and Gibson, 1978), and *Spiralothelphusa hydrodroma* (referred as *Parathelphusa hydrodromus*) (Reddy, 1937), caridean shrimps as *Caridina laevis* (Pillai, 1960), and astacideans such as the Norway lobster *Nephrops norvegicus* (Yonge, 1924) and the European lobster *Homarus*

gammarus (Barker and Gibson, 1977), the presence of a wide band of circular muscles associated with longitudinal muscles. Another set of muscles observed in *M. brachydactyla* receive names such as "dilator muscles" (Erri Babu et al., 1979; Erri Babu et al., 1982; Pillai, 1960; Reddy, 1937; Yonge, 1924), "extrinsic muscles" (Schmitz and Scherrey, 1983) and "radial muscles" (Barker and Gibson, 1977; 1978). Their function has been associated with the expansive efforts required to swallow the food in amphipods (Schmitz and Scherrey, 1983) and the present study suggests the same function for *M. brachydactyla*: the contraction of the "dilator muscles" could press the epithelium toward the connective tissue facilitating the expansion of the lumen. In the case of the larval stages of *M. brachydactyla*, we observed circular and dilator muscles but longitudinal muscles were not identified. Similarly, Schlegel (1911) in the zoea I of the same genus described circular muscle fibers (as "constrictor muscles") and similar "dilator muscles". The circular muscles also were described in the esophagus of *R. ranina* zoeae (Minagawa and Takashima, 1994). The role of the muscles of the larvae must be similar to their adult counterpart.

Epithelial cells. The simple columnar epithelium of the adult esophagus of *M. brachydactyla* is covered by a cuticle, as has been reported in other brachyurans: e.g. *M. rumphii* (Erri Babu et al., 1982), *P. sanguinolentus* (Trinadha Babu et al., 1989), *S. serrata* (Barker and Gibson, 1978) and *S. hydrodroma* (Reddy, 1937). The esophageal epithelium is also described as columnar in astacideans (Barker and Gibson, 1977; Yonge, 1924) and carideans (Pillai, 1960). By contrast, in the brachyuran crab *Pseudocarcinus gigas* the epithelium is cuboidal (Heeren and Mitchell, 1997). This information suggests that the epithelium of the esophagus is generally conserved among the decapods during the adulthood. The epithelial cells of the larvae differs from their adult counterpart, varying from plane to short columnar cells. Schlegel (1911) in his

study of the first zoeal stage of a *Maja* species, described an esophageal epithelium composed of big cells with basal nuclei and covered by a cuticle. In other Decapoda larvae the epithelium is considered short columnar, as in *R. ranina* zoeae (Minagawa and Takashima, 1994) and *P. ornatus* phyllosomata (Johnston et al., 2008), or cuboidal as in *L. amboinensis* zoeae (Tziouveli et al., 2011). The current data are not enough to evaluate if these cell morphologies are related with a functional role, the phylogeny, or a consequence of the molt cycle.

The esophageal epithelium must be able to support the expansive and contractive efforts required for the swallowing of the food. In this sense, one of the most distinctive characteristics of the epithelial cells of *M. brachydactyla* are their richness in interdigitations and bundles of filamentous structures, coinciding with the esophageal epithelial cells of the cirolanid isopod *Natatolana obtusata* (Storch et al., 2002). The filamentous structures have been reported as microtubules in the esophagus and/or hindgut tract of diverse malacostracans, including the brachyuran *Metacarcinus magister* (Mykles, 1979), and astacideans such as the lobsters *Homarus americanus* and *Homarus gammarus* (Mykles, 1979), and the crayfish *Procambarus clarkii* (Komuro and Yamamoto, 1968), as well diverse isopod species (Holdich and Mayes, 1975; Vernon et al., 1974; Witkus et al., 1969). Some authors suggested that the microtubules could help to maintain the cell structure through the support of the expansive and contractive efforts realized by these organs (Komuro and Yamamoto, 1968; Mykles, 1979; Witkus et al., 1969). Similarly, the interdigitations could help to avoid the tearing of the epithelium through the expansive waves. By contrast, the epithelial cells of the larvae are very different from their adult counterpart. Many larval epithelial cells show highly developed vesicles (feature not observed in the adults), while the bundles of filamentous structures and interdigitations are poorly developed. These large vesicles

have not been reported previously and their role is unknown, but tentatively could be considered as having a structural role, maybe maintaining the cell shape.

Cuticle organization. The cuticle surface is rich in small hair-like structures named "microspines" by Elzinga and Hopkins (1994; 1995). These microspines are cuticle specializations observed in ectoderm derivatives such as the foregut and hindgut (Elzinga, 1998; Elzinga and Hopkins, 1994; 1995). They have been reported in the esophagus of the Malacostraca as small aggregations or rows projected toward the stomach (De Jong and Casanova, 1997; Elzinga, 1998; Friesen et al., 1986; Icely and Nott, 1984; Johnston et al., 2004; Storch et al., 2002). Few studies mentioned the presence of microspines in the esophagus of the larval stages. Johnston et al. (2008) mentioned "short spines" in the esophageal lumen of *P. ornatus* phyllosomata. By contrast, in *L. amboinensis* the larval esophagus project dense and thick setae, a feature not observed in *M. brachydactyla* (Tziouveli et al., 2011). The role of the esophageal microspines is unknown. In the hindgut, they have been associated with the grasping of the peritrophic membrane to avoid their backward movement due to anti-peristaltic waves (Felder and Felgenhauer, 1993; Hopkin and Nott, 1980). Other authors suggested a role such as a supporting surface for symbiotic microorganisms (Elzinga, 1998; Harris, 1993). In the case of the esophagus none of these hypotheses looks probable, since no peritrophic membrane or microorganism have been observed. Perhaps the microspines could help to grasp the food for their ingestion.

Other cuticle specializations are the areas devoid from microspines but pierced by "small" (ca. 1 µm diameter) and "large" (ca. 2 µm diameter) pores. In *S. serrata* the cuticle is also pierced by tubes ca. 3 µm in width (Barker and Gibson, 1978). Small (1-3 µm diameter) and large (5-8 µm diameter) pores have been identified in the esophagus of *H. gammarus*, but differs from our study since they are restricted to the esophagus -

stomach junction and are associated with long filaments (Robertson and Laverack, 1979). The "pore areas" can appear at the distal side of cuticle tubes located over the rosette glands, which make it is possible that these pores constitute a release pathway for the gland secretions. An alternative hypothesis suggests that the pores and channels could be "sensors" of the rosette glands, but this hypothesis require confirmation of the presence of nervous structures, i.e. the axons observed in the connective that surrounds rosette glands in carideans such as the common prawn *Palaemon serratus* (Alexander, 1989) and the daggerblade grass shrimp *Palaemonetes pugio* (Doughtie and Rao, 1982).

Rosette glands. The rosette glands received numerous names based on their location ("tegumental", "esophageal", or "intestinal glands"), shape ("rosette glands") or hypothetical function ("salivary glands", or "cement glands"), but has not yet been an agreement in their denomination (Gorvett, 1946; Reddy, 1937; Trinadha Babu et al., 1989; Yonge, 1924). The rosette glands have been described in the adult esophagus of diverse brachyurans (Barker and Gibson, 1978; Erri Babu et al., 1979; Erri Babu et al., 1982; Heeren and Mitchell, 1997; Reddy, 1937; Trinadha Babu et al., 1989), astacideans (Barker and Gibson, 1977; Yonge, 1924; 1932), achelatans (Johnston and Alexander, 1999), carideans (Pillai, 1960) and penaeids (Dall, 1967; Sousa and Petriella, 2006). Although the rosette glands appear to be absent in the esophagus of the immature specimens, they appear near to the mouth opening and mouthparts, coinciding with observations realized in the zoeal stages of *S. olivacea* (Jantrarotai and Sawanyatiputi, 2005) and the pre-zoeal stage of the anomuran *Porcellana platycheles* (Williams, 1944). The rosette glands of *M. brachydactyla* are very active: the cytoplasm of the secretory cells is filled by vesicles and the Golgi bodies are highly developed. Moreover, the secretory cells can be stained by Alcian blue and PAS coinciding to previous studies realized in other brachyurans (Barker and Gibson, 1978; Erri Babu et

al., 1982; Trinadha Babu et al., 1989). These stain affinities reveal a possible composition based on acid mucopolysaccharides (including sulphated mucopolysaccharides, sulphated sialomucins and hyaluronic acid), as well neutral mucopolysaccharides (Erri Babu et al., 1979; Trinadha Babu et al., 1989). Other studies discarded the presence of glycogen (Erri Babu et al., 1979), lipids and phospholipids (Trinadha Babu et al., 1989). Yonge (1932) mentioned numerous possible roles for the rosette glands: "salivary glands" that help the passage and digestion of the food, "slime glands" with no mentioned role, "secretory organs" with a midgut gland-like role, or "cement glands" involved on the oviposition. Considering all the mentioned roles and their location, a probable function for the esophageal rosette glands in *M. brachydactyla* could be comparable to the "salivary glands": the acid compounds could help in digestive processes, while the mucous nature is useful to entangle and lubricate the lumen surface allowing the passage of the food (Barker and Gibson, 1977; Erri Babu et al., 1979; Hunt et al., 1992; Shyamasundari and Hanumantha Rao, 1977; 1978; Yonge, 1924).

The role of the esophagus can explain their histological and ultrastructural characteristics: the internal folds allow the expansion of the lumen, the epithelial cells have numerous interdigitations and filamentous structures to presumably avoid its tearing, the epithelium is covered by a cuticle that protects the epithelial cells and it is covered by microspines that could help to grasp the food, while the connective tissue contains highly developed muscles to realize the peristaltic movements required for these efforts. The rosette glands found in the connective tissue could act as "salivary glands". The esophagus of the larval stages has numerous differences from their adult counterpart: the epithelial cells can contain big vesicles, and the connective tissue and cuticle are thinner than in the adults, lacking rosette glands and blood vessels. The esophagus of the adults is much wider than in the larvae, with the distance between tissues being greatly increased. Consequently, the esophagus of the adult requires additional structures for their maintenance (such as extended connective tissue, blood vessels and glands) and a more complex organization.

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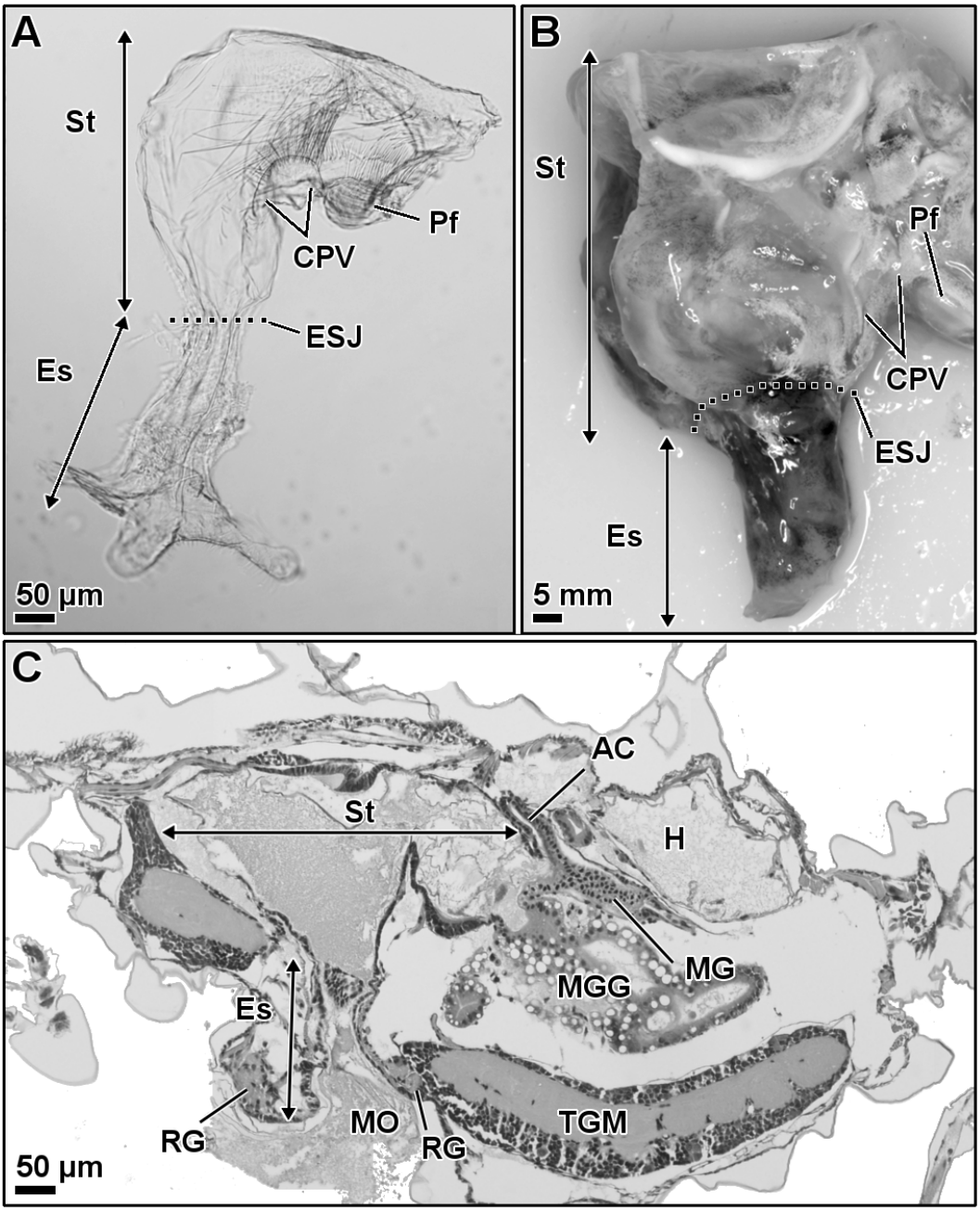

440

441 **Figure 1**. *Maja brachydactyla*. Esophagus, gross morphology and location. Zoea II, optical microscope
442 (A). Adult, digital camera (B). Megalopa, H-E, optical microscope (C). Abbreviations: AC, anterior
443 caeca; CPV, cardio-pyloric valve; Es, esophagus; ESJ, esophagus - stomach junction; H, heart; Pf, pyloric
444 filter; MG, midgut tract; MGG, midgut gland (hepatopancreas); MO, mouth opening; RG, rosette glands;
445 St, stomach; TGM, thoracic ganglionic mass.

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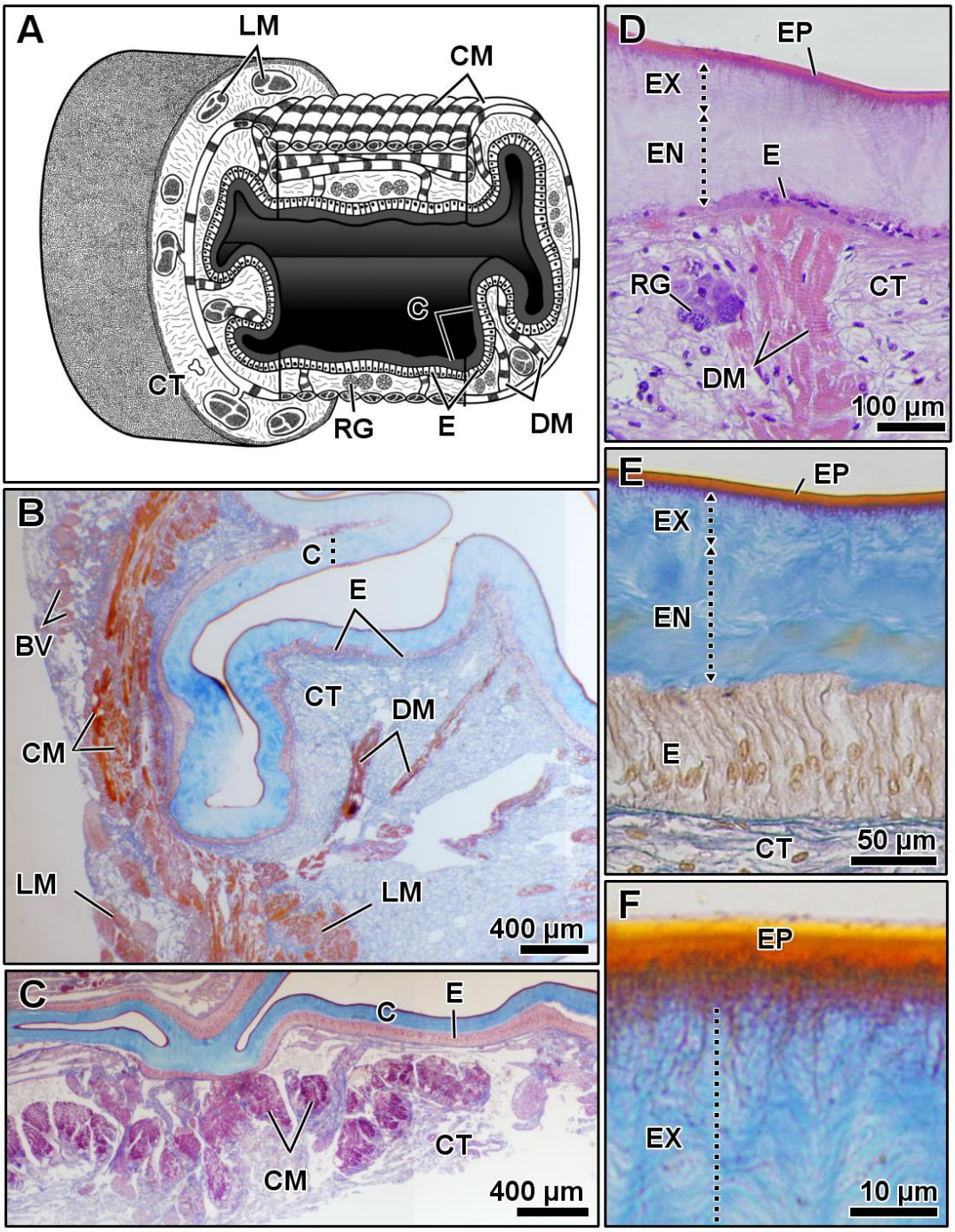
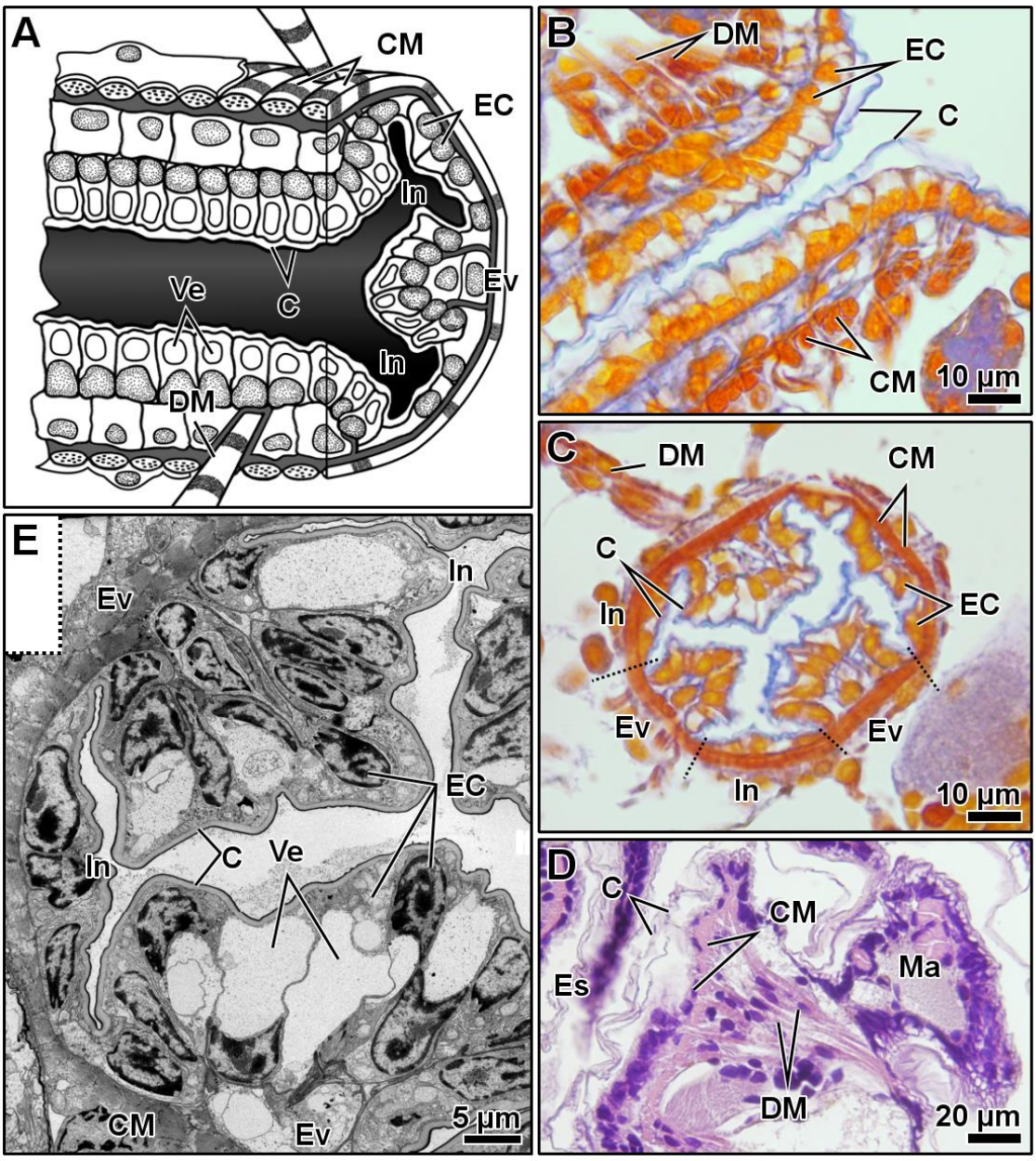


447

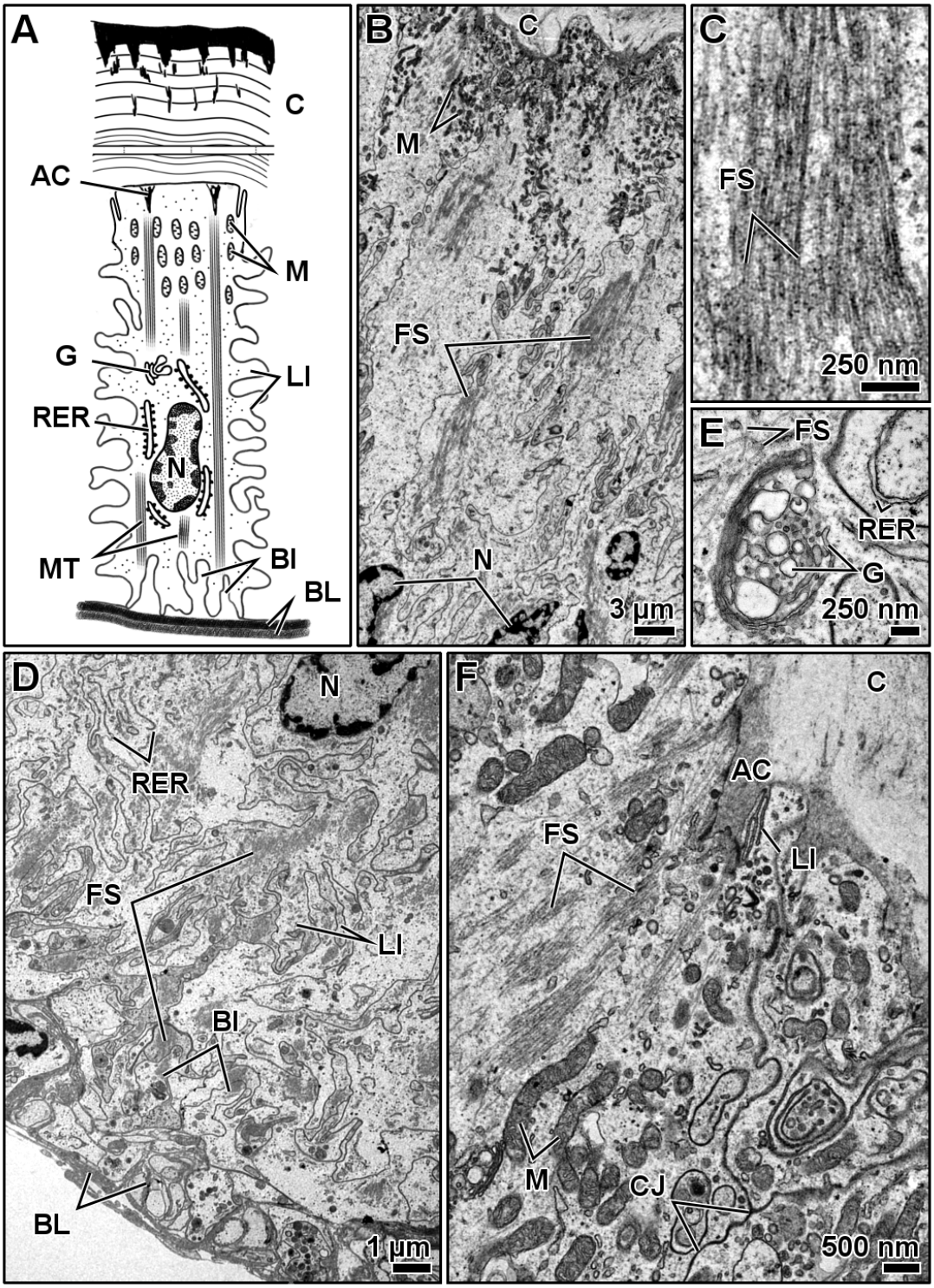
448 **Figure 2**. *Maja brachydactyla*. Adult. Esophagus, histological organization. Optical microscopy. General
449 diagram (A). General view, Mallory's trichrome (B-C): transversal (B) and longitudinal sections (C).
450 Epithelium and connective tissue, H-E (D). Close view of the epithelium, Mallory's trichrome (E).
451 Exocuticle and epicuticle, Mallory's trichrome (F). Abbreviations: BV, blood vessels; C, cuticle; CT,
452 connective tissue; CM, circular muscles; DM, dilator muscles; EN, endocuticle; EP, epicuticle; EX,
453 exocuticle; RG, rosette glands; LM, longitudinal muscles.



454

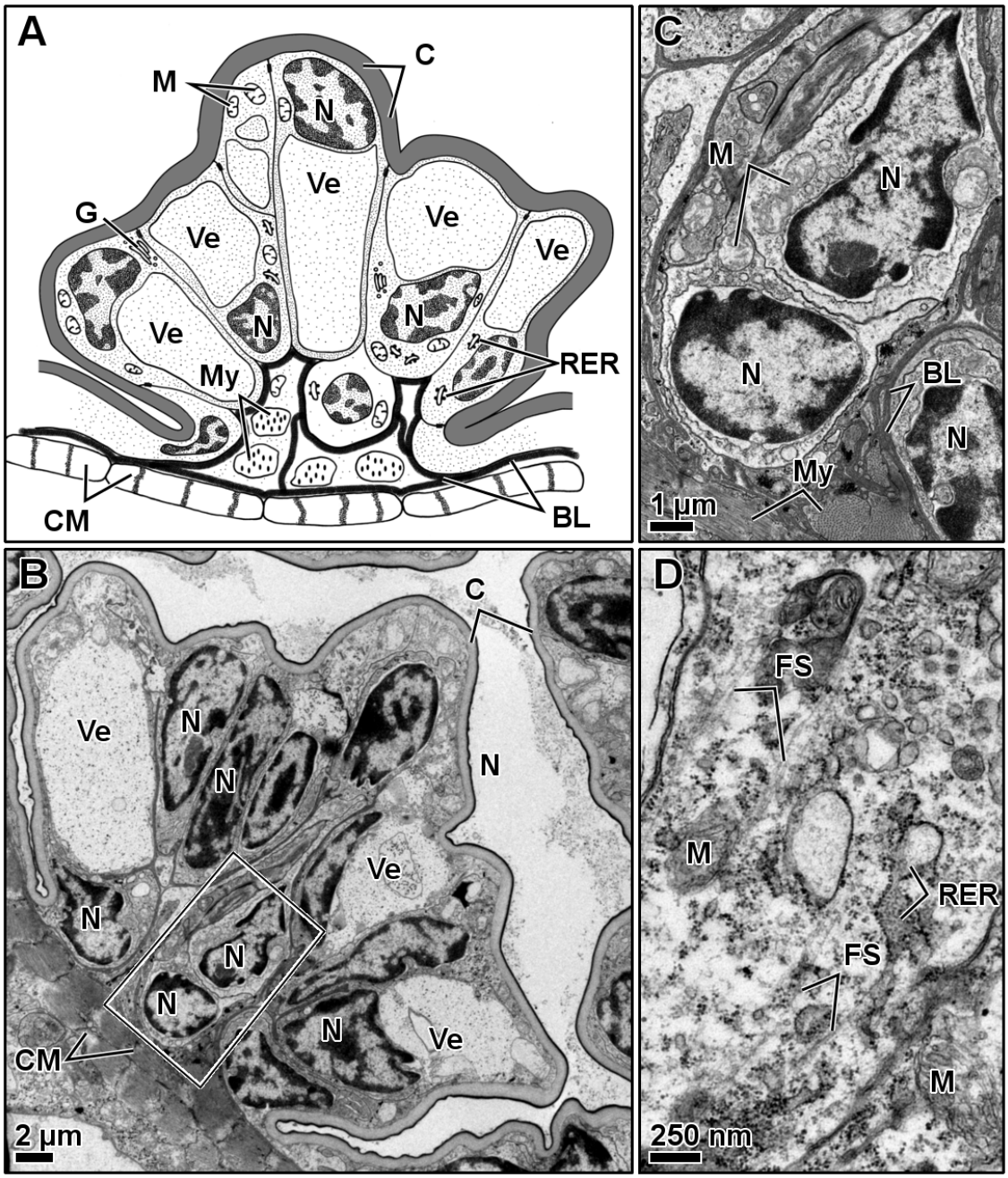
455 **Figure 3**. *Maja brachydactyla*. Larvae. Esophagus, histological organization. Optical microscopy.
456 General diagram (A). Megalopa, Mallory's trichrome (B-C): longitudinal (B) and transversal sections (C).
457 Megalopa, close view of the dilator muscles, HE (D). Zoea I, transversal section, TEM. Abbreviations:
458 C, cuticle; CM, circular muscles; DM, dilator muscles; EC, epithelial cells; Es, esophagus; Ev,
459 evaginations; If, infold; In, invaginations; Ma, mandible; Ve, vesicles.

460



461

462 **Figure 4**. *Maja brachydactyla*. Adult. Esophagus. Epithelial cells. TEM. General diagram (A). Apex of
463 the epithelial cell (B). Close view of the filamentous structures (C). Basis of the epithelial cell (D). Close
464 view of a Golgi body (E). Cell apex showing the cell-to-cell junctions and the "apical complex" (F).
465 Abbreviations: AC, apical complex; AI, apical infolds; BI, basal infolds; BL, basal lamina; C, cuticle; CJ,
466 cell-to-cell junctions; FS, filamentous structures; G, Golgi body; LI, lateral interdigitations; M,
467 mitochondria; N, nucleus; RER, rough endoplasmic reticulum.



468

469 **Figure 5**. *Maja brachydactyla*. Zoea I. Esophagus. Epithelial fold. TEM. General diagram (A). General
470 view, the rectangle marks the epithelial cells located on the fold center (B). Detailed view of the rectangle
471 marked in B (C). Close view of the filamentous structures (D). Abbreviations: BL, basal lamina; C,
472 cuticle; CM, circular muscles; EC, epithelial cell; FS, filamentous structures; G; Golgi body; M,
473 mitochondria; My, myofibrils; N, nucleus; RER, rough endoplasmic reticulum; Ve, vesicles.

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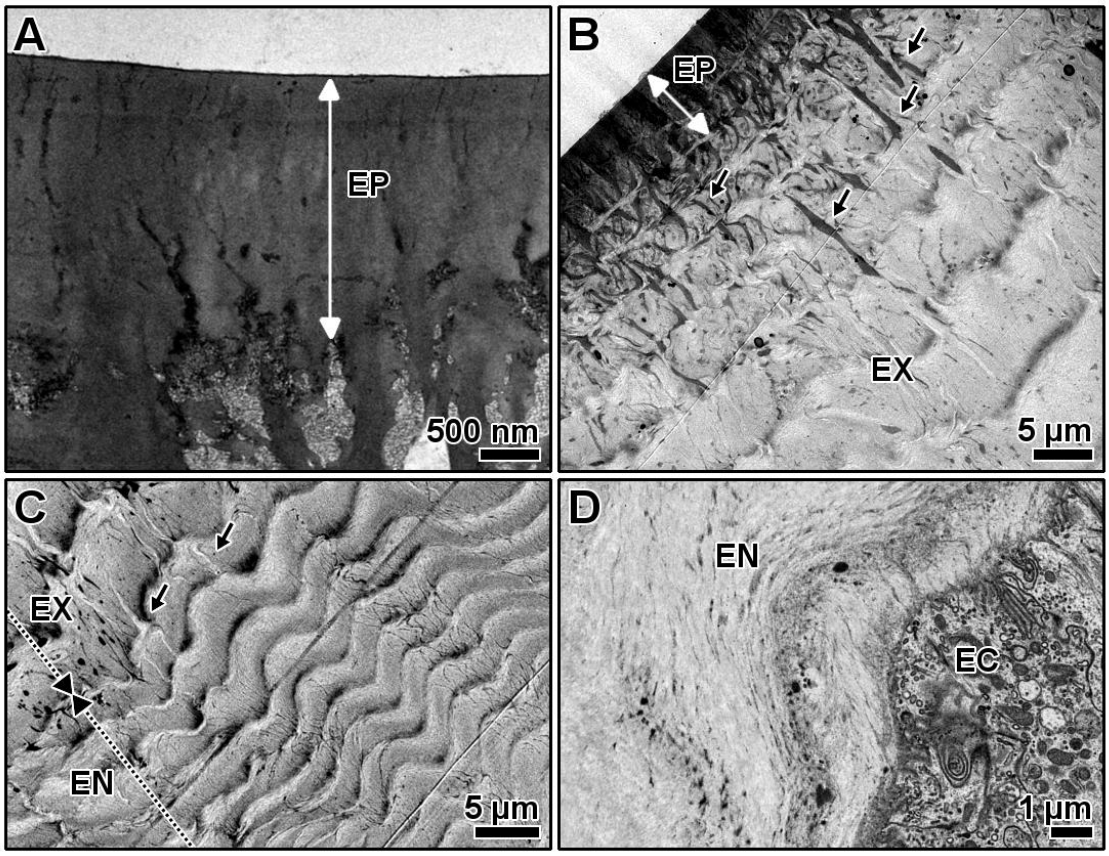


476

477 **Figure 6**. *Maja brachydactyla*. Adult. Esophagus. Cuticle. TEM. Epicuticle (A). Epicuticle, exocuticle
478 and protrusions of epicuticle crossing the exocuticle (arrows) (B). Transition from the exocuticle to the
479 endocuticle, pore canals (arrows) are showed (C). Basal endocuticle and apex of the epithelial cell (D).
480 Abbreviations: EC; epithelial cell; EN, endocuticle; EP, epicuticle; EX, exocuticle.

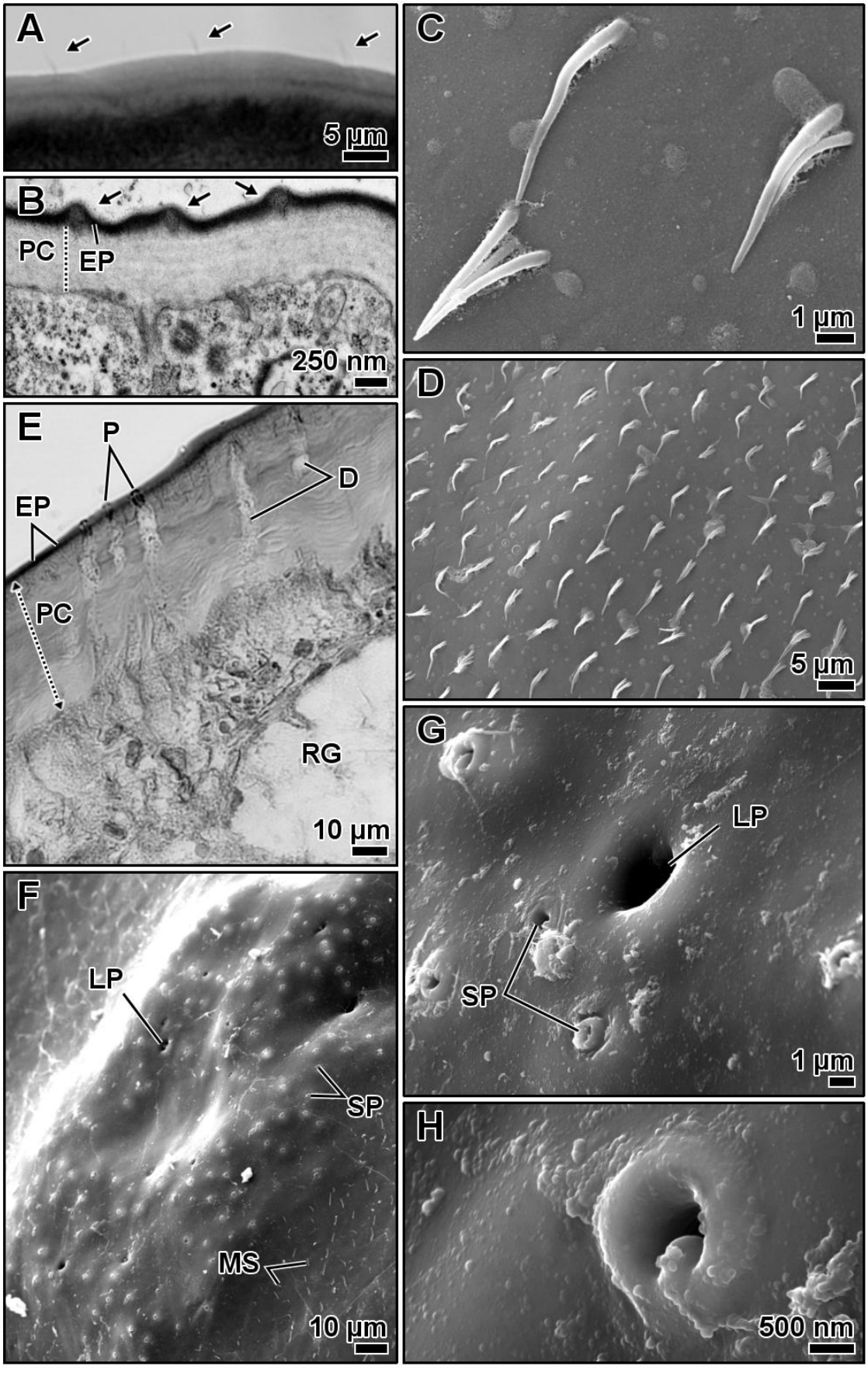
481

482

483

484 **Figure 7** (next page). *Maja brachydactyla*. Esophagus, superficial structures of the cuticle. Adult,
485 microspines (arrows), Mallory's trichrome (A). Zoea I, microspines protruding from the cuticle, TEM (B).
486 Adult, microspines, SEM (C-D): close view (C) and field of microspines (D). Rosette glands associated
487 with duct-like structures and pores, Mallory's trichrome (E). "Pore area" with elongated shape, SEM (F).
488 Close view of the cuticle pores, SEM (G-H): "large pore" surrounded by "small pores" (G) and "small
489 pore" (H). Abbreviations: D, duct-like structure; EC, epithelial cells; EN, endocuticle; EP, epicuticle; EX,
490 exocuticle; LP, "large pore"; MS, microspines; P, pores; PC, procuticle; RG, rosette glands; SP, "small
491 pore".

492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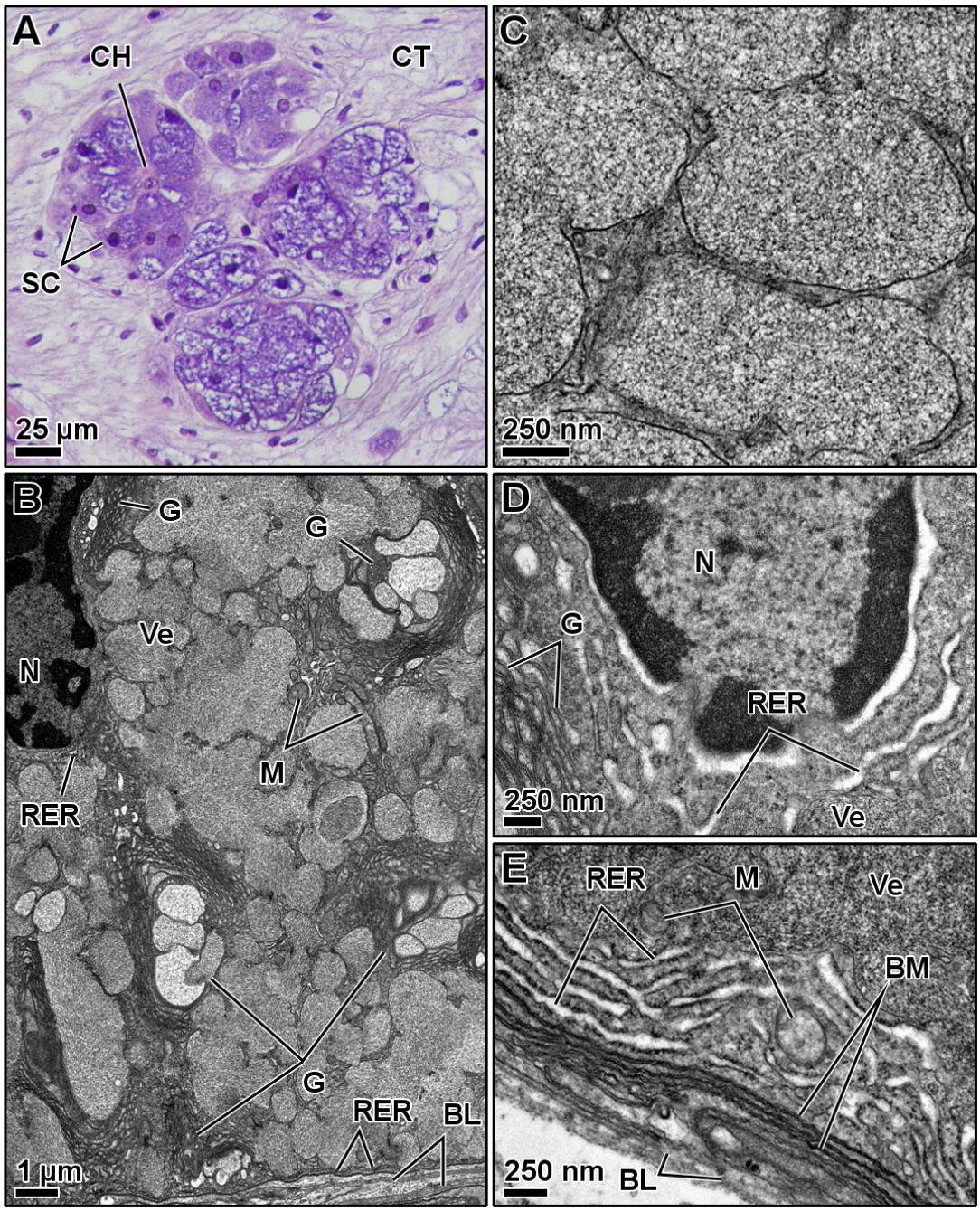
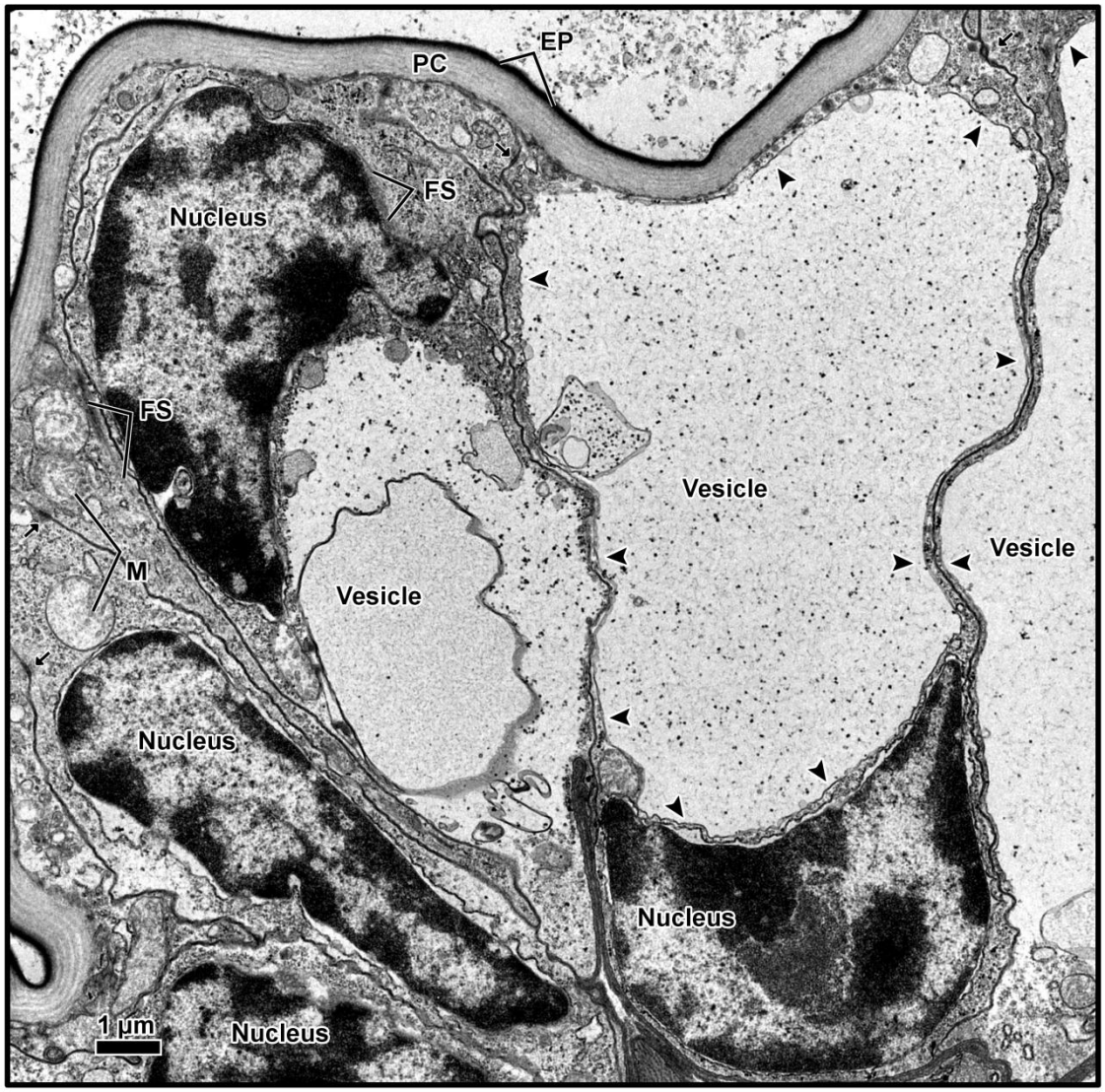


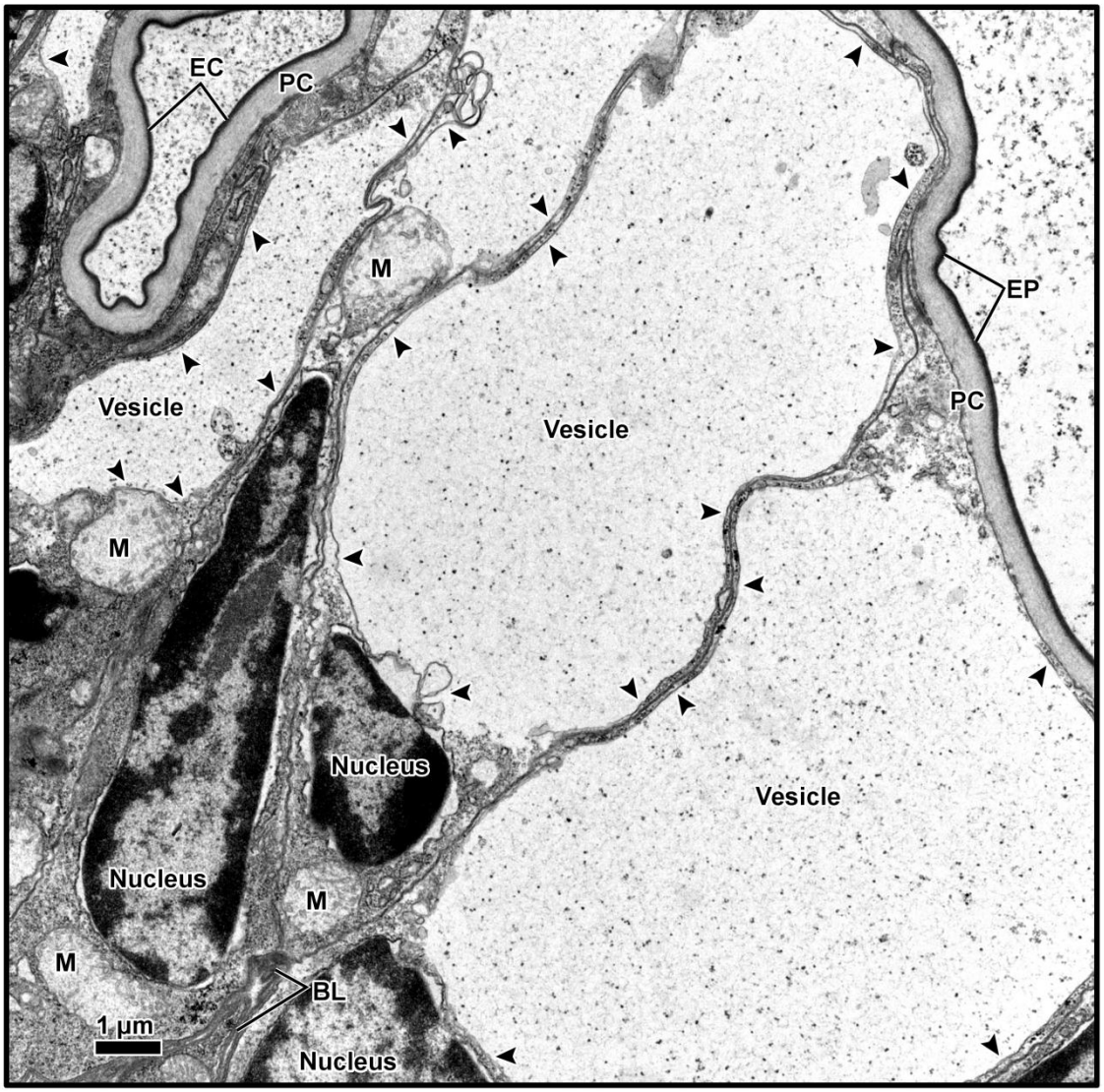
494

495 **Figure 8**. *Maja brachydactyla*. Adult. Esophagus, rosette glands. General view of the rosette glands, H-E
496 (A). General view of the gland cells, TEM (B). Close view of the secretory vesicles (C). Close view of the
497 nucleus and rough endoplasmic reticulum (D). Close view of the basal layer of rough endoplasmic
498 reticulum. Abbreviations: BL, basal lamina; BM, basal membrane; CH, central channel; CT, connective
499 tissue; G, Golgi bodies; N, nucleus; RER, rough endoplasmic reticulum; RG, rosette glands; SC,
500 secretory cells; Ve, vesicles.

501



Supplementary Material 1 (Suppl. Mat 1). *Maja brachydactyla*. Zoea I. Esophagus, epithelial cells. TEM. The cell-to-cell junctions are marked by arrows and the single membrane of the vesicles by arrow-heads. Abbreviations: EC, epicuticle; FS, filamentous structures; M, mitochondria; PC, procuticle.



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508 **Supplementary Material 2 (Suppl. Mat 2)**. *Maja brachydactyla*. Zoea I. Esophagus, epithelial cells.
509 TEM. The single membrane of the vesicles are marked by arrow-heads. Abbreviations: EC, epicuticle; M,
510 mitochondria; PC, procuticle.

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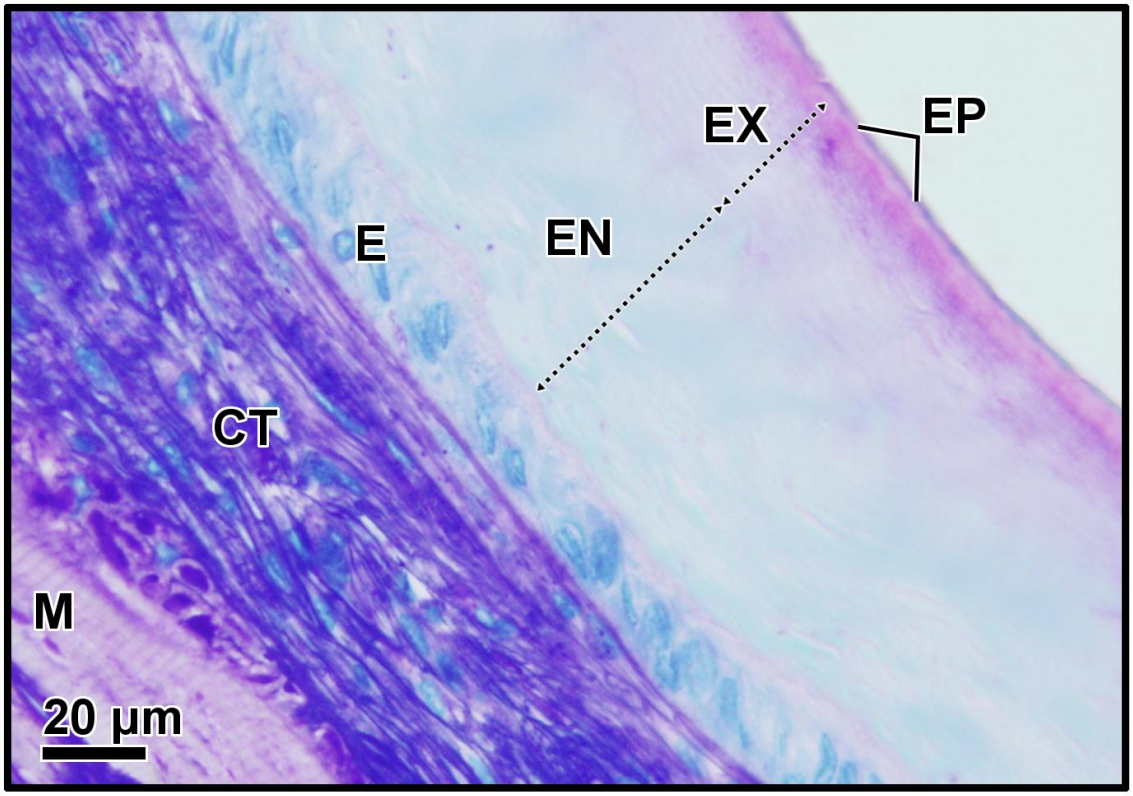
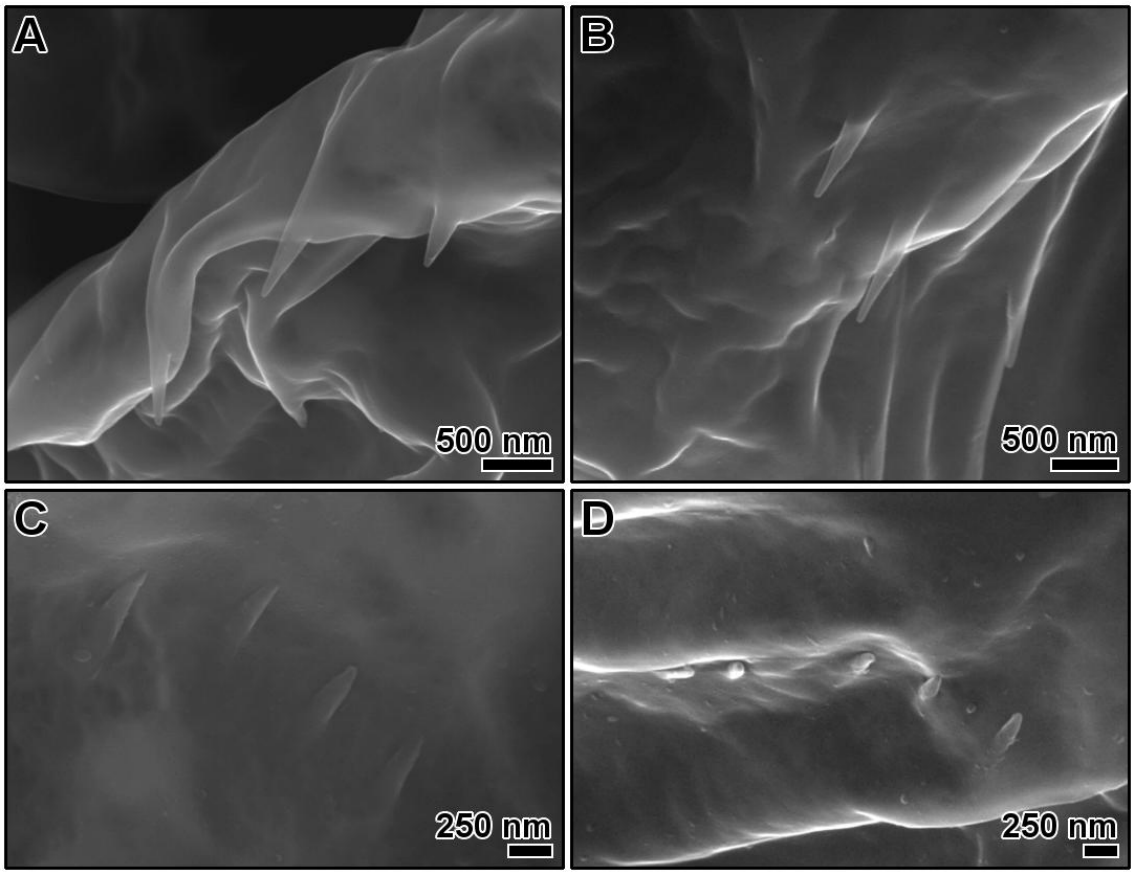


Supplementary Material 3 (Suppl. Mat 3). *Maja brachydactyla*. Adult. Esophagus. Close view of the epithelium, PAS and Methylene Blue. Abbreviations: CT, connective tissue; E, epithelium; EN, endocuticle; EP, epicuticle; EX, exocuticle; M, muscle.



Supplementary Material 4 (Suppl. Mat 4). *Maja brachydactyla*. Zoea I. Esophagus, microspines of the cuticle surface (A-D): three microspines (A), three microspines (B), four microspines (C) and five microspines (D).

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