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Light and electron microscopy studies of the midgut and salivary glands of second and third instars of the horse stomach bot, *Gasterophilus intestinalis*

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Abstract. A morphological study of the midgut and salivary glands of second and third instars of *Gasterophilus intestinalis* (De Geer) (Diptera: Oestridae) was conducted by light, scanning and transmission electron microscopy. The midgut is anteriorly delimited by a proventriculus, without caeca, and is composed of posterior foregut and anterior midgut tissue from which a double-layered peritrophic matrix is produced. The midgut can be divided into anterior, median and posterior regions on the basis of the structural and physiological variations of the columnar cells which occur along its length. Two other types of cell were identified: regenerative cells scattered throughout the columnar cells, and, more rarely, endocrine cells of two structural types (closed and open). Different secretion mechanisms (merocrine, apocrine and microapocrine) occur along the midgut epithelium. Abundant microorganisms are observed in the endoperitrophic space of the anterior midgut. The origin and nature of these microorganisms remain unknown. No structural differences are observed between the second and third instar midguts. The salivary glands of *G. intestinalis* second and third instars consist of a pair of elongated tubular structures connected to efferent ducts which unite to form a single deferent duct linked dorsally to the pharynx. Several intermediate cells, without cuticle, make the junction with the salivary gland epithelium layer. Cytological characteristics of the gland epithelial cells demonstrate high cellular activity and some structural variations are noticed between the two larval stages.

Key words. *Gasterophilus intestinalis*, equids, larvae, microorganisms, midgut, peritrophic matrix, salivary glands.

Introduction

Larvae of the horse botfly, *Gasterophilus intestinalis*, cause gastrointestinal myiasis in equids throughout the world (Zumpt, 1965). Adult botflies deposit their eggs on the host's hair and parasitic migration begins when eggs are introduced in the oral cavity of the horse. The first-stage larvae (L1) hatch and undergo a first moult; second-stage larvae (L2) enter the gastrointestinal tract, migrate to the host stomach and undergo another moult. Third-stage larvae (L3) remain attached for about 8–10 months to the mucosa of the non-glandular portion of the host stomach (Cogley & Cogley, 1999). As adult flies do not feed, their nutritional requirements for basic metabolism, dispersal and reproduction must be ingested during the obligatory parasitic larval stage (Hall & Wall, 1995). The digestive system responsible for all steps of food processing in the larvae is comprised of the alimentary canal (gut) and the salivary glands.

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Scanning electron microscopy has been used to examine the pathology associated with the different instars of *Gasterophilus* spp. (Shefstad, 1978; Cogley, 1989) and to analyse the morphological and sensorial properties necessary to enable the larvae to complete their lifecycle inside their host (Principato & Tosti, 1988; Cogley, 1999; Leite & Scott, 1999; Leite *et al*., 1999; Colwell *et al*., 2007). However, a description of the structure of the digestive system of *Gasterophilus* species is lacking.

This paper describes the structural details of the midgut and salivary gland epithelium of *G. intestinalis* L2 and L3 using light, scanning and transmission electron microscopy. Morphological studies on the midgut and salivary glands may be useful to further investigate the larval physiology and the host–parasite interaction.

Materials and methods

Larval collection

Second and third instars of *G. intestinalis* were recovered at necropsy from the stomach of three horses originating from Delémont (47°21′ N, 07°20′ E), in the District of the Swiss Jura, Switzerland. All larvae collected were identified on the basis of morphological keys (Zumpt, 1965).

Specimen preparation for light and transmission electron microscopy

Eight L2 and seven L3 were killed in fixative (2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer at pH 7.4) (Karnowsky, 1965) and partially dissected. The gut and salivary glands were left in their original positions in the larvae. The specimens remained overnight in a fresh fixative solution at 4 °C. Specimens were washed in 0.1 M cacodylate buffer (pH 7.4) and post-fixation was performed using 1% osmium tetroxide in the same buffer for 60 min at room temperature. After three washes in 0.1 M cacodylate buffer (pH 7.4), the specimens were dehydrated in ascending acetone series (30–100%) and embedded in Spurr's resin (Polysciences, Inc., Warrington, PA, U.S.A.). Polymerization occurred for 24 h at 60 °C. The specimens were cut with a diamond knife (Diatome AG, Biel, Switzerland) using an Ultracut S microtome (Reichert GmbH, Vienna, Austria).

Histological sections

Serial semi-thin sections (500 nm) were collected every 10 µm from the proventricle to the hindgut of the eight L2 and the seven L3, placed on albumined slides and stained with toluidine blue (Fluka). Observations were made on an Olympus BX50 (Olympus Optical Co., Geneva, Switzerland). Images were captured with a CC-12 digital camera (Olympus Optical Co.) and treated using analySIS 3.2 image analytic software (Gloor Instruments AG, Uster, Switzerland).

Ultra-thin sections (60–100 nm) were mounted on Formvar-carbonated copper grids, contrasted with uranyl acetate and Reynold's lead citrate. Observations were made on a Philips CM 100 transmission electron microscope (Philips Electron Optics BV, Eindhoven, the Netherlands) at 60 kV. Microphotographs were taken on 35-mm film (Copyline HDU 1p; Agfa Graphics Switzerland AG, Dübendorf, Switzerland) and scanned on an Epson 1640 scanner (Epson America Inc., Long Beach, CA, U.S.A.).

Specimen preparation for scanning electron microscopy

Fixation of the larvae for scanning electron microscopy (SEM) was carried out as above (Karnowsky, 1965). Specimens were dehydrated in ascending acetone series (up to 100%) and desiccated by critical point drying, using carbon dioxide (Baltec CPD 030; Oerlikon Balzers AG, Balzers, Liechtenstein). Dried specimens were mounted on aluminium stubs with Leit-tabs (Plano GmbH, Wetzlar, Germany) and coated with gold (23 nm) in a Sputter Baltec SCD 005 (Oerlikon Balzers AG). Observations and images were made on a scanning electron microscope (PHILIPS XL 30; Philips Electron Optics BV) at 10 kV.

Results

The general features of the digestive tracts of both L2 and L3 *G. intestinalis* are similar to those of other Diptera in that they are comprised of three main regions, namely, the foregut, midgut and hindgut (Fig. 1). The proventriculus, without caeca, represents the junction between the foregut and the anterior midgut (Figs 2A, 3), and the posterior midgut is delimited by the Malpighian tubules.

Proventriculus, peritrophic matrix and microorganisms

The oesophagus passes through the cephalic ganglia and enters the proventriculus (Figs 1, 2A, 3A). Oesophageal epithelial cells have a thin cuticle on their apical surface (Fig. 3C) and join the proventricular cells at the posterior end of the proventriculus, forming an oesophageal invagination (Fig. 3A). The thin cuticle is also present on the proventricular cells. A stretched basal lamina separates the basal surfaces of the proventricular cells and the oesophageal cells (Fig. 3C, D). The junction between the two gut regions is located where the proventricular cells curve around the bulb-like structure and meet the anterior midgut, or columnar, cells (Fig. 2B). In both larval stages, the peritrophic matrix is synthesized where the apical surfaces of the proventricular cells and the columnar cells meet. First represented as a fibrous substance (Fig. 3B), a double-layered peritrophic matrix is formed as it passes through the proventriculus (Fig. 3E). The peritrophic matrix is observed along the entire midgut until it reaches the hindgut (Fig. 9A).

The presence of microorganisms was noted in the lumen of the anterior midgut of each larva examined, posterior to the

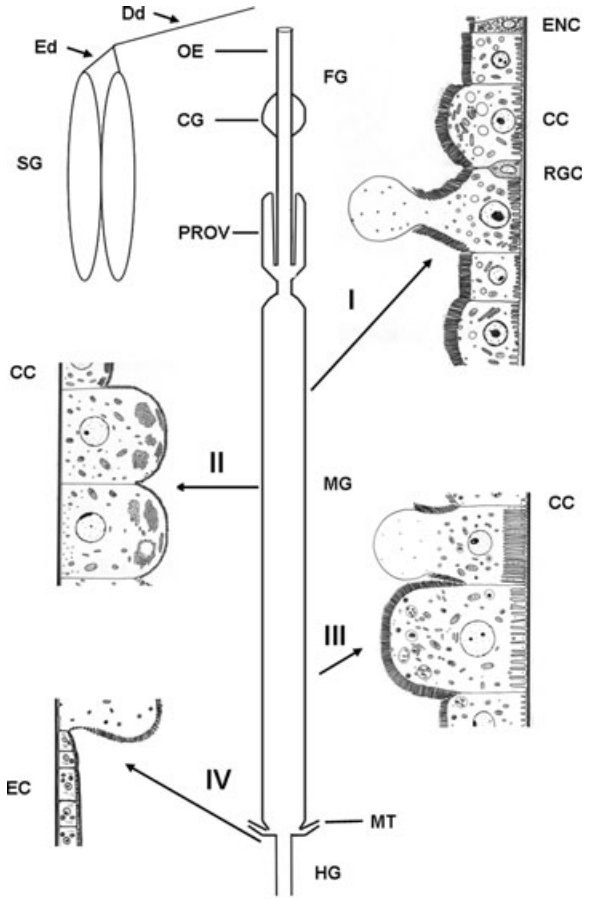

Fig. 1. Schematic drawing of the digestive system of *Gasterophilus intestinalis* L2 and L3 adapted from McFarlane (1985). Proportions are not respected. The three gut regions are represented: foregut (FG), midgut (MG) and hindgut (HG). The oesophagus (OE) passes through the cephalic ganglia (CG) and enters the proventriculus (PROV) (Figs 2–4). The midgut is separated in three different regions: (I) anterior midgut (Fig. 5); (II) median midgut (Fig. 6) and (III) posterior midgut (Fig. 7). The posterior end of the midgut is delimited by the Malpighian tubules (MT), after which the hindgut (IV) (Fig. 9) continues to the rectum. Columnar cells (CC), endocrine cells (ENC) and regenerative cells (RGC) compose the midgut epithelium (here represented in the anterior midgut only). The FG and HG have an epithelium composed of epithelial cells lined by a cuticle (EC). SG, salivary glands; Ed, efferent ducts; Dd, deferent ducts.

proventriculus where the peritrophic matrix is highly abundant (Fig. 4B). These microorganisms were observed especially in the endoperitrophic space of the anterior midgut, but were occasionally also present up to the hindgut (Fig. 4C, D).

Midgut

The proventricular epithelium is continuous with the midgut epithelium and is formed predominantly by columnar cells (also called 'digestive cells'). The histological aspect of the columnar cells varies along the midgut, delimiting three distinct regions: the anterior (Fig. 5), the median (Fig. 6) and the posterior (Fig. 7) midgut. Regenerative cells are found singly, scattered along the entire midgut (Figs 8A, 6A). These undifferentiated cells are typically characterized by a large nucleus and small peripheral cytoplasm and contain few differentiated organelles. Occasional endocrine cells of two types are observed along the midgut: the 'closed-type' (Fig. 8B), which does not extend through the epithelial layer and the 'open-type' (Fig. 8C), which is in contact with midgut lumen. At their basal end these cells show electron-dense vesicles surrounded by a bright halo called 'haloed vesicles' (Fig. 8D).

The columnar cells of the anterior midgut bear long microvilli, around 3.7 µm in length, which extend into the lumen (Fig. 5B, C). The basal plasma membrane is deeply enfolded to form a basal labyrinth associated with vesicles and mitochondria (Fig. 5B, E). The nucleus is centrally located in a vast cytoplasmic area (Fig. 5A, B). Rough reticulum endoplasmic is highly abundant, in particular at the cell apex (Fig. 5D) and near the nucleus. Polymorphous mitochondria, present in the whole cytoplasm, are more concentrated in the basal and apical parts of the cell. Many secretory vesicles and large vacuoles are visible near the base of the microvilli (Fig. 5D). Other small secretory structures are present within or at the apex of the microvilli (Fig. 5C) and remain in clusters, close to the peritrophic matrix.

Some digestive cells of the anterior midgut show an apical protuberance, called a 'protuberant bud' or 'bleb', extending into the lumen (Fig. 5A). These large cytoplasmic projections are devoid of microvilli and contain no organelles. Similar protuberant buds were observed in epithelium of the posterior midgut digestive cells (not shown).

The median midgut epithelium is characterized by columnar cells with short microvilli, around 1.4 µm in length, that extend into the lumen (Fig. 6A, B) and the absence of a basal labyrinth (Fig. 6C). The basal cytoplasm contains numerous polymorphous mitochondria. Some mitochondria seem to be swollen; they appear oval or rounded (Fig. 6C). The prominent nucleolus typical of the nucleus of midgut digestive cells was observed (Fig. 6A).

In the posterior midgut, the structure of the columnar cells resembles that of cells of the anterior midgut. At their apical region, the cells bear long microvilli, around 5 µm in length (Fig. 7A–C) and the basal plasma membrane folds inward to form a basal labyrinth (Fig. 7A, E), associated with mitochondria and microtubules. Small secretory vesicles are released in the lumen from the cell apex or microvilli (Fig. 7C). Numerous swollen mitochondria, Golgi bodies, multi-vesicular bodies and large autolytic vacuoles containing granular and lamellar material are observed in the cytoplasm (Fig. 7A, B, D). A higher concentration of glycogen is noticed in the posterior midgut (Fig. 7B, D).

The larval midgut is posteriorly delimited by the insertion of two Malpighian tubules, which each diverge into two to give a total of four long Malpighian tubules. Only the first region of the hindgut was investigated; this is comprised of epithelial cells with a large nucleus (Fig. 9A, B). A cuticular layer lines the lumen region where fragments of peritrophic matrix and occasionally some microorganisms are present.

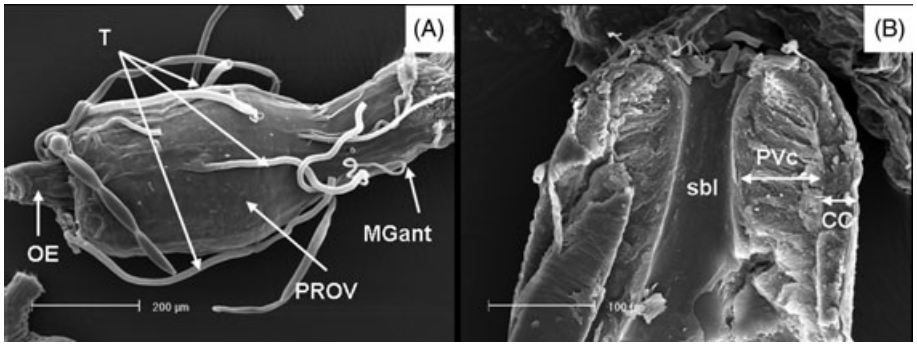

Fig. 2. Scanning electron micrographs of the proventriculus of *Gasterophilus intestinalis* L3. (A) The oesophagus (OE) enters the proventriculus (PROV) and the anterior midgut (MGant) begins at the posterior end of the PROV. Numerous tracheae (T) insert into the PROV. Scale bar = 200 µm. (B) Transverse section of the proventriculus showing the junction between the proventricular cells (PVc) and the columnar cells (CC). The oesophagus has been removed to show the underlying stretched basal lamina (sbl). Scale bar = 100 µm.

Salivary glands

The salivary system of *G. intestinalis* L2 and L3 consists of pairs of long tubules which lie ventrally in the anterior body cavity. A single deferent duct, inserted dorsally to the cephalopharingeal skeleton, separates into two efferent ducts which are connected to the tubular glands. The glands are bathed in haemolymph and are invaded by numerous tracheae (Fig. 10).

The epithelial cells apices of the ducts are lined with cuticle and the basement membrane forms some infoldings (Fig. 11A). These cells contain a small nucleus (about 5 µm in diameter), widely dispersed mitochondria, free ribosomes and microtubules. At the junction between the ductal and the salivary gland epithelial cells, another type of cell, known as the 'intermediate' cell, is present (Fig. 11B). These cells are not lined by a cuticle and do not bear microvilli at the apical region. They show a highly enfolded lateral plasma membrane, a small nucleus and mitochondria.

The tubular salivary glands are comprised of closely packed epithelial cells. The cells are tightly linked to adjacent cells by deeply folded septate junctions (Fig. 12B). Numerous tracheal insertions are found at the basement of the cells and in the intercellular space (Fig. 12A, C). Irregular microvillar-like projections (microvilli linked with apical lamellar membrane) extend into the lumen (Fig. 12D). The basement membrane does not form a basal labyrinth (Fig. 12A). The cytoplasm presents an abundant rough endoplasmic reticulum, and free ribosomes, lipidic droplets, Golgi complexes, multi-vesicular bodies, residual bodies and polymorphous mitochondria, generally swollen, are widespread throughout the cytoplasm (Fig. 12A–C).

Structural differences observed between L2 and L3 salivary glands

The dense vesicles produced by the Golgi apparatus in L2 are less common or more difficult to observe in L3. Numerous lysosomes (residual bodies) appear in L3.

The swollen mitochondria present in L2 become more abundant and bigger in L3. The swollen mitochondria are seen to be closely associated and to form a type of vacuolization, and the matrix seems to be empty.

Discussion

The nutritional phase of *G. intestinalis* larvae is crucial for the accumulation of the energy resources necessary for the survival of adult flies, which complete their lifecycle without feeding. The gut consists of three different regions involved in digestion, absorption and elimination procedures: the foregut, midgut and hindgut. The salivary glands, which are connected to the foregut, usually have a limited or no role in digestion (Terra & Ferreira, 2005). The gut epithelium in *G. intestinalis* L2 and L3 is similar to those described in most insects and is composed of a simple epithelium resting on a basal lamina surrounded by connective tissue and muscle bundles, the contraction of which causes peristalsis that propels food along the gut (Terra & Ferreira, 2005).

The midgut is considered the most important region of the digestive system and is responsible for digestion and absorption (Dow, 1986). The caeca generally present in Diptera larvae are absent in several botfly larvae, such as *Dermatobia hominis* (L. Jr) (Diptera: Oestridae) (Evangelista & Leite, 2003), *Hypoderma bovis* (L.) (Diptera: Oestridae) (Boulard, 1969) or *G. intestinalis* (Keilin, 1944). Unlike the study by Evangelista & Leite (2003), which described no regional and cellular differentiation in the midgut of *D. hominis* L3, this current ultrastructural study demonstrates that the midgut of *G. intestinalis* L2 and L3 is divided into three distinct regions termed the 'anterior', 'median' and 'posterior' midgut, as previously described for *Lucilia* spp. (Robineau-Desvoidy) (Diptera: Calliphoridae) larvae (Hobson, 1931), and three different types of cells were observed. No histological differences were observed in the midgut between the two larval stages and only the number of foldings of the midgut increase with larval growth. Therefore, comments concerning the midgut refer to both L2 and L3.

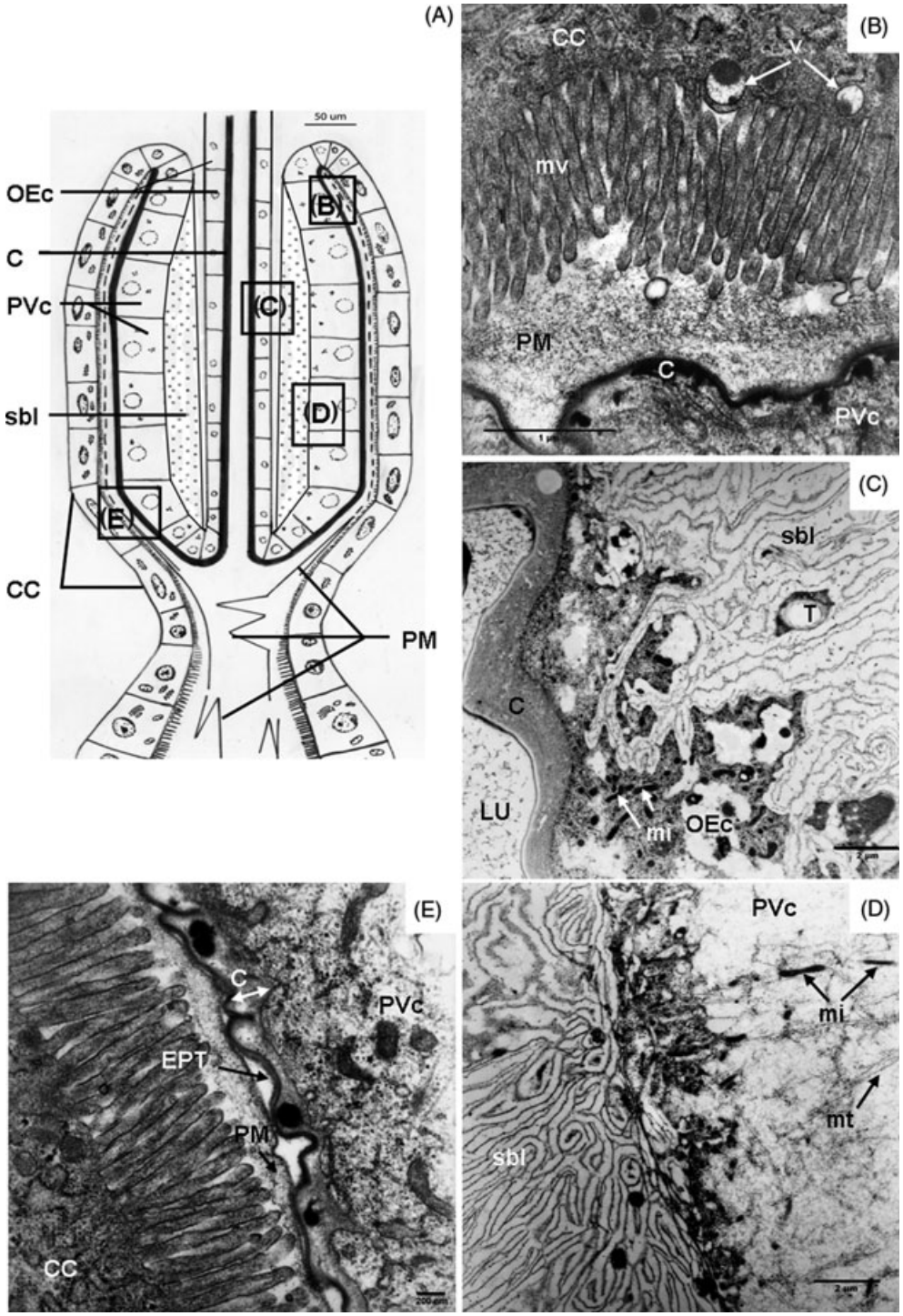

Fig. 3. (A) Schematic drawing of the proventriculus of *Gasterophilus intestinalis* L2 and L3 adapted from Spenve (1991). OEc, oesophageal cells; C, cuticle; PVc, proventricular cells; sbl, stretched basal lamina; CC, columnar cells; PM, peritrophic matrix. (B–E) Ultrastructural details of the regions outlined in (A) in L3. (B) Apical region of CC and PVc at the top of the proventricular bulb-like structure, where the synthesis of the PM begins. v, vesicle; mv, microvilli; scale bar = 1 μm. (C) OEc lined at the apical region by a cuticle (C); the basal surface is surrounded by sbl. T, tracheae; mi, mitochondria; LU, lumen; scale bar = 2 μm. (D) Basal region of PVc, surrounded by the thick layer of sbl. Mt, microtubules; scale bar = 2 μm. (E) Apical region of CC and PVc at the base of the proventriculus, showing the double-layered PM. EPT, ectoperitrophic space; scale bar = 200 nm.

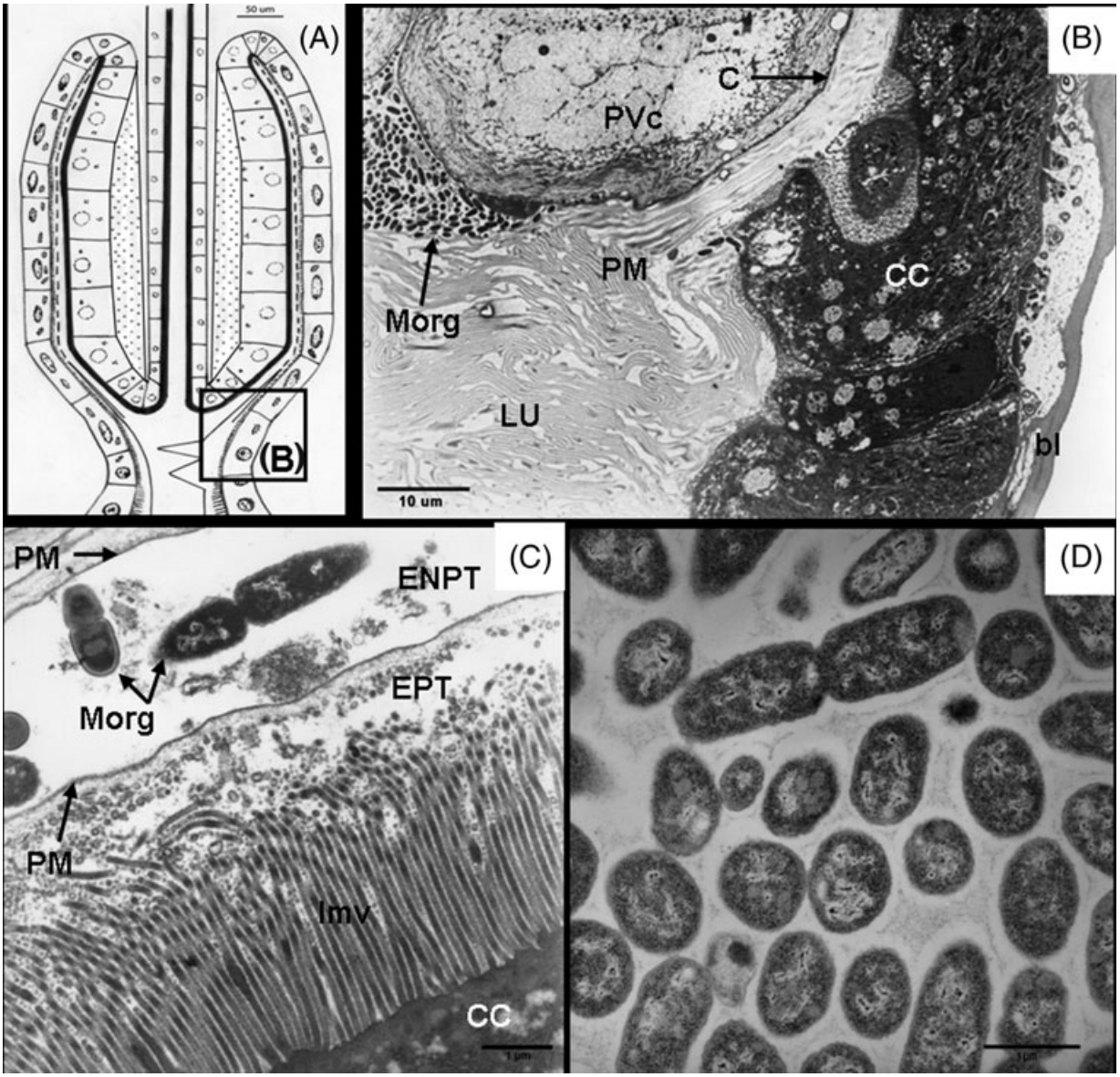

Fig. 4. (A) Schematic drawing of the proventriculus of *Gasterophilus intestinalis* L2 and L3 adapted from Spenve (1991). (B) Transmission electron microscopy (TEM) of the section outlined in (A) showing a transverse section of the posterior proventriculus of *G. intestinalis* L2. PVc, proventricular cells; C, cuticle; CC, columnar cells; PM, peritrophic matrix; LU, lumen; Morg, microorganisms; bl, basal lamina; scale bar = 10 μm. (C) TEM of the apical section of a CC showing Morg in the endoperitrophic space (ENPT) of the anterior midgut of *G. intestinalis* L2. EPT, ectoperitrophic space; lmv, long microvilli; scale bar = 1 μm. (D) TEM of Morg in the ENPT of *G. intestinalis* L2. Scale bar = 1 μm.

The peritrophic matrix compartmentalizes the midgut lumen in two compartments: the ectoperitrophic space (between the epithelium and the peritrophic matrix), and the endoperitrophic space (between the peritrophic matrix and the lumen). This semi-permeable matrix, which lines the gut in most insects, is thought to be associated with many digestive processes and also has a protective function (Tellam *et al*., 1999). A peritrophic matrix, produced by the specialized epithelial cells of the proventriculus, has been identified in *G. intestinalis* L2 and L3. Further investigations to characterize the function and composition of the peritrophic matrix in these larvae will help to elucidate the digestion processes and may allow for the isolation of molecules that could serve as pest control agents, as demonstrated for *Lucilia cuprina* (Wiedemann) (East & Eisemann, 1993; East *et al*., 1993; Tellam *et al*., 2000). An intriguing feature of this study concerned the presence of microorganisms in each larva examined. They were generally abundant at the junction between the proventriculus and the anterior midgut. Usually they were localized in the endoperitrophic space, although several microorganisms were observed in the ectoperitrophic space and between the microvilli (not shown). If the presence of microorganisms

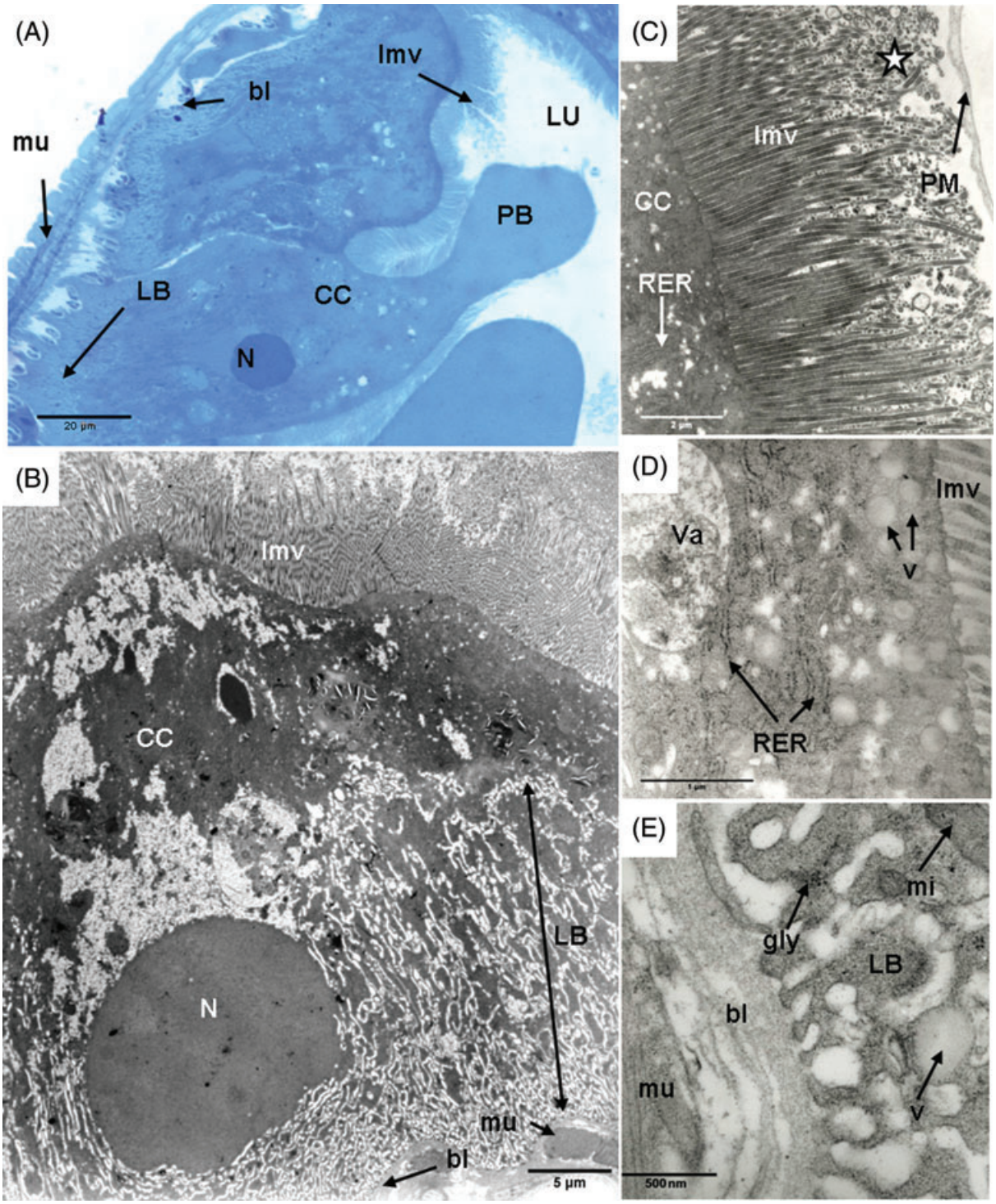

Fig. 5. (A–E) Ultrastructural detail of *Gasterophilus intestinalis* L3 anterior midgut epithelium. (A) Light micrograph showing a well-developed apical protuberant bud (PB) projecting into the midgut lumen (LU). CC, columnar cell; Nu, nucleus; LB, basal labyrinth; lmv, long microvilli; bl, basal lamina; mu, muscle; scale bar = 20 µm. (B) CC showing the deeply enfolded basal membrane forming the LB. Scale bar = 5 µm. (C) Apical region of a CC showing pinching off vesicles (star). RER, rough endoplasmic reticulum; PM, peritrophic matrix; scale bar = 2 µm. (D) Details of a CC apical cytoplasm showing numerous small vesicles (v), large vacuole (Va) and RER. Scale bar = 1 µm. (E) Details of the basal region of a columnar cell representing the channels of the LB. mi, mitochondria; gly, glycogen; scale bar = 500 nm.

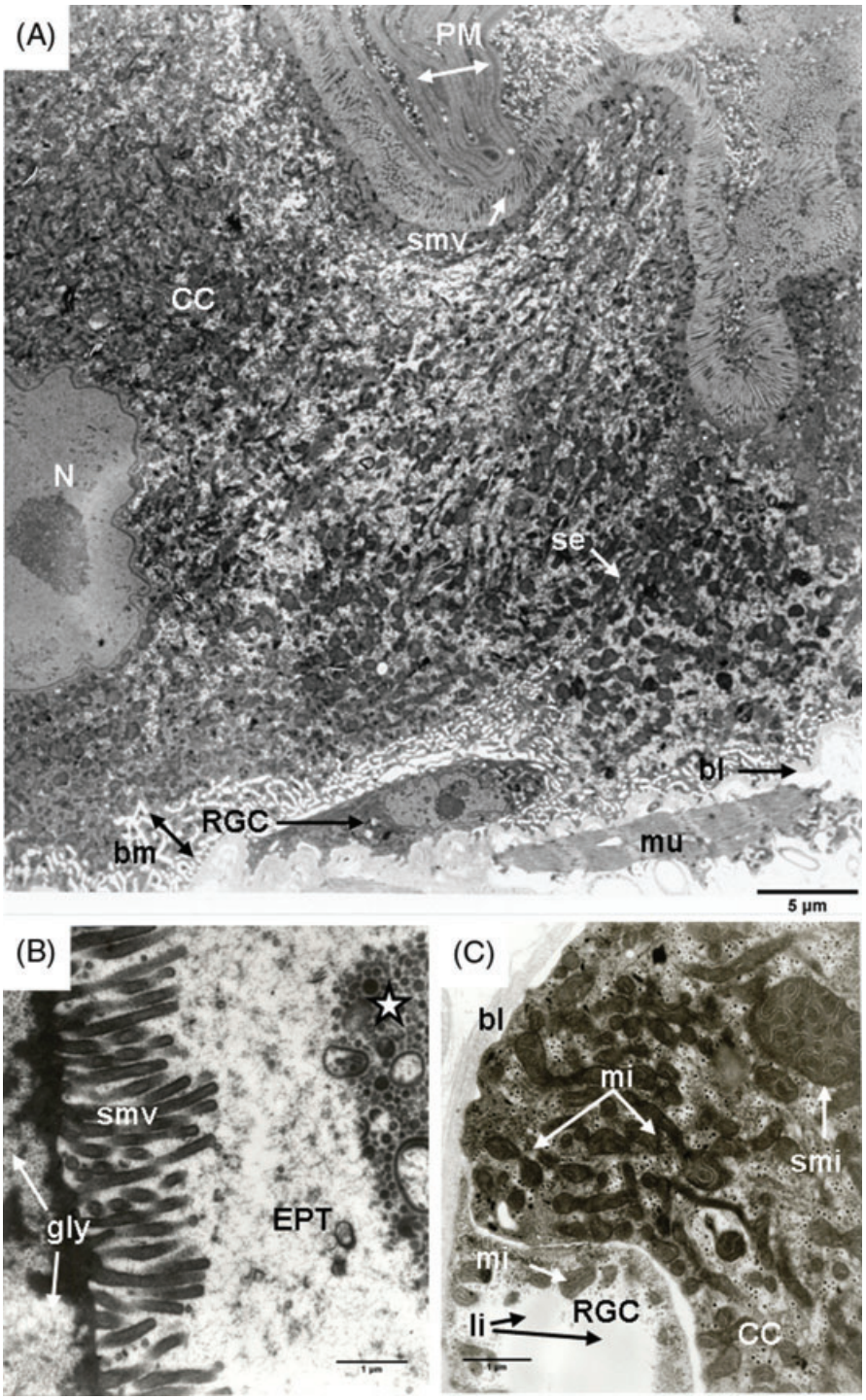

Fig. 6. (A–C) Ultrastructural detail of *Gasterophilus intestinalis* L3 median midgut epithelium. (A) View of a columnar cell (CC) showing a few infoldings of the basal membrane (bm) and short microvilli (smv). A regenerative cell (RGC) is present along the basal lamina (bl) at the base of the CC. N, nucleus; PM, peritrophic matrix; se, septate junction; mu, muscle; scale bar = 5 µm. (B) Detail of the smv and glycogen (gly) at the apical part of the CC. Secretory or digestive material (star) is highly abundant in the ectoperitrophic space (EPT). Scale bar = 1 µm. (C) Basal cytoplasm of a CC showing numerous mitochondria (mi) and swollen mitochondria (smi). The RGC contains lipidic droplets (li). Scale bar = 1 µm.

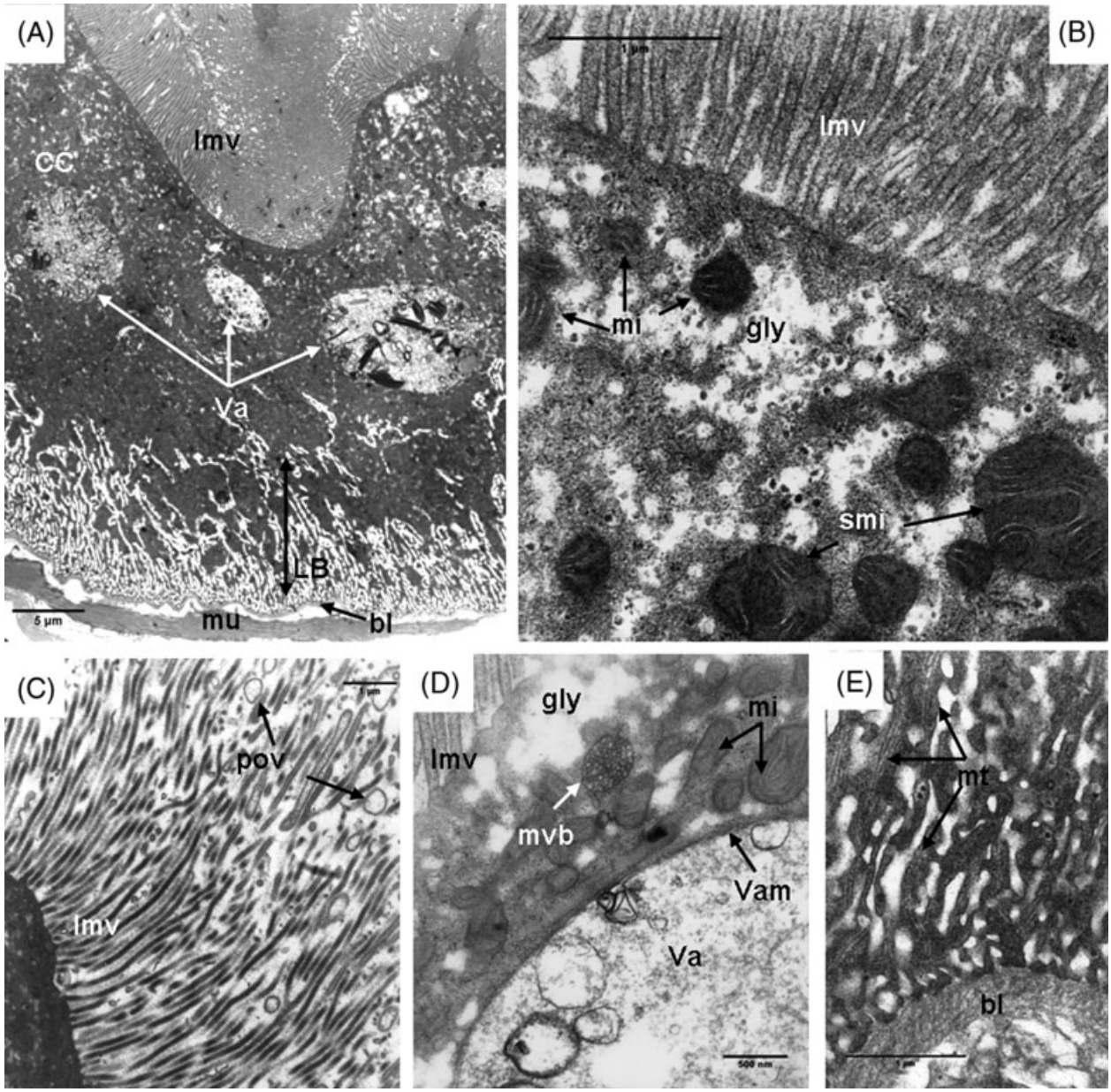

Fig. 7. (A–E) Ultrastructural detail of *Gasterophilus intestinalis* L3 posterior midgut epithelium. (A) View of a columnar cell (CC) with the folded plasma membrane forming the basal labyrinth (LB). Long microvilli (lmv) extend into midgut lumen. Cytoplasm contains large autolytic vacuoles (Va). bl, basal lamina; mu, muscle; scale bar = 5 µm. (B) Detail of the apical cytoplasm showing mitochondria (mi), swollen mitochondria (smi) and glycogen (gly). Scale bar = 1 µm. (C) Detail of pinching off vesicles (pov). Scale bar = 1 µm. (D) Detail of a Va containing granular and lamellar material and surrounded by a vacuolar membrane (Vam). mvb, multi-vesicular body; scale bar = 500 nm. (E) Basal region of a CC showing the channels of the LB. mt, microtubules; scale bar = 1 µm.

in the intestinal tract of insects is common and widespread, little is known about their existence in the myiasis-causing species. They may be symbiotic or fortuitous contaminants that gain access from the external environment (Douglas & Beard, 1996). Symbionts are rarely associated with digestion, but are thought to provide nutrient factors such as essential amino acids and B vitamins, or to prevent the colonization of the gut by other species (Dillon & Dillon, 2004). Therefore, further studies are required to identify and understand the origin and function of these microorganisms.

The distinction between the different midgut regions was based upon morphological comparison of the columnar cells. The anterior and posterior segments demonstrate morphological similarities. The columnar cells constituting the epithelium of the anterior and posterior midgut bear long microvilli at the apical plasma membrane and thus the available membrane

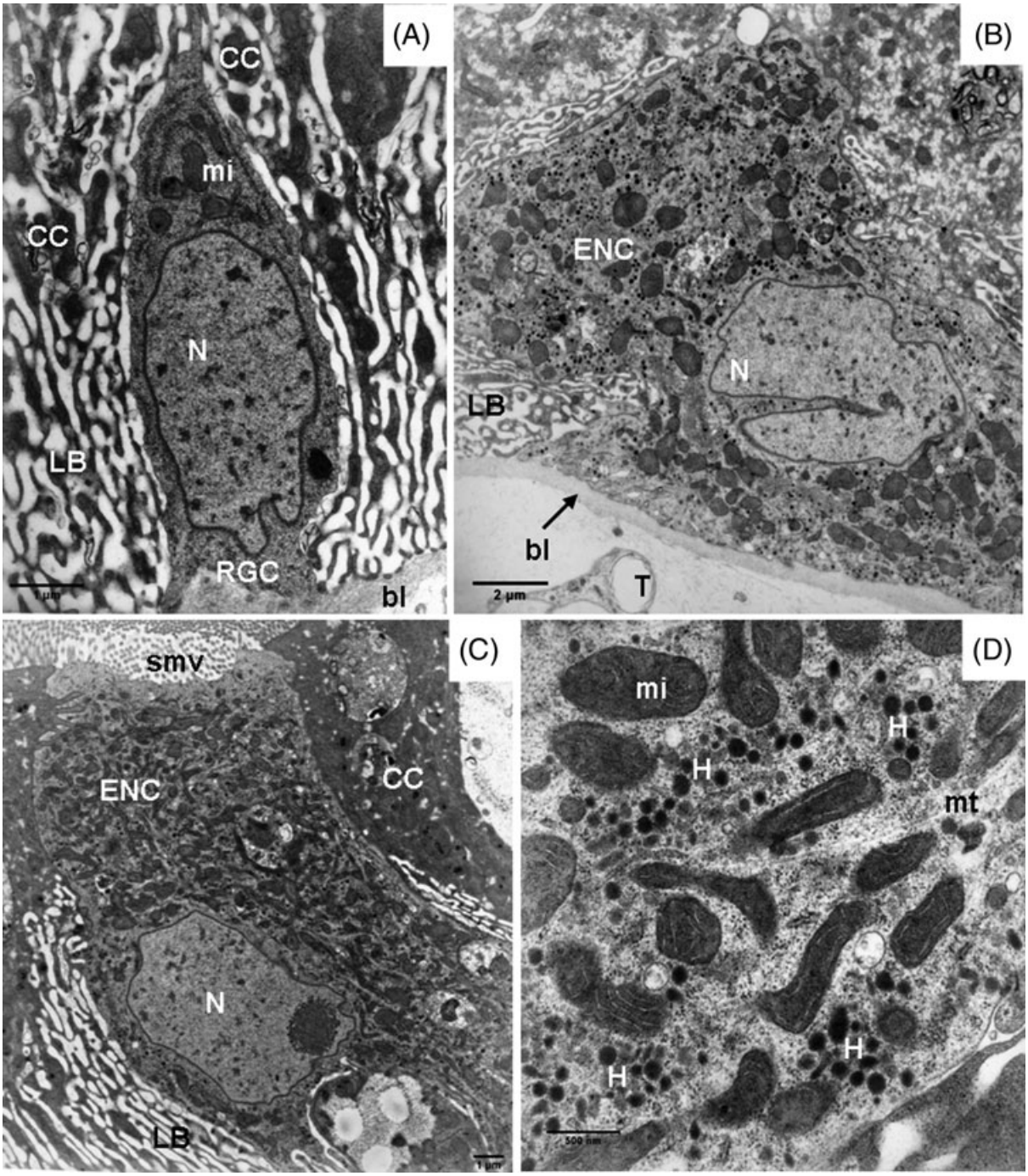

Fig. 8. (A–D) Ultrastructural view of the regenerative and endocrine cells in *Gasterophilus intestinalis* L3. (A) Regenerative cell (RGC) in an undifferentiated state located between the bases of two columnar cells (CC). N, nucleus; mi, mitochondria; LB, basal labyrinth; bl, basal lamina; scale bar = 1 µm. (B) Endocrine cell (ENC) in a 'closed' shape (not extending to the midgut lumen). T, tracheae; scale bar = 2 µm. (C) ENC in an 'open' shape (in contact with the midgut lumen). smv, short microvilli; scale bar = 1 µm. (D) Detail of the haloed vesicles (H) characteristic of endocrine cells. mt, microtubules; scale bar = 500 nm.

for secretion or absorption is enlarged. The basal plasma membrane has numerous infoldings, which form a complex labyrinth of channels associated with numerous mitochondria and microtubules, sometimes stretching into the apical third of the cell. These membrane infoldings are generally related to the active transport of water and ions (Martoja & Ballan-Dufrançais, 1984; Terra *et al*., 1988) and may be involved in midgut fluxes important for the translocation of enzymes and

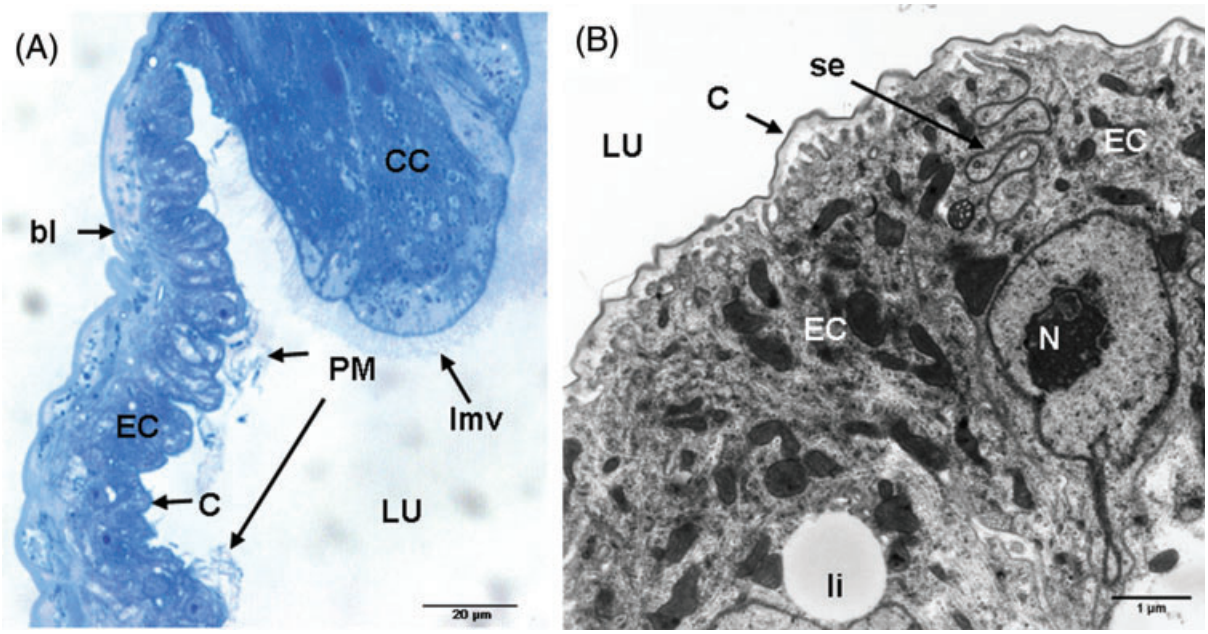

Fig. 9. (A) Light micrograph of the transition between the posterior midgut columnar cells (CC) and hindgut epithelial cells (EC) of *Gasterophilus intestinalis* L3. EC are lined by a cuticle (C). Fractions of peritrophic matrix (PM) are present in hindgut lumen (LU). bl, basal lamina; lmv, long microvilli; scale bar = 20 µm. (B) Transmission electron micrograph of the apical part of the hindgut epithelial cells (EC) of *G. intestinalis* L3. N, nucleus; li, lipidic droplet; se, septate junction; scale bar = 1 µm.

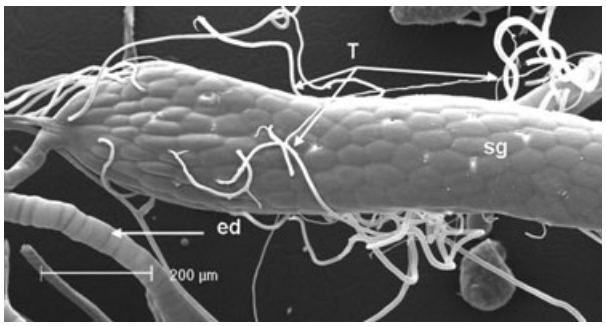

Fig. 10. Scanning electron micrograph of *Gasterophilus intestinalis* L2 salivary gland (sg). The gland is connected to an efferent duct (ed) and numerous tracheae (T) insert into the gland. Scale bar = 200 µm.

products of digestion (Billingsley & Lehane, 1996; Terra & Ferreira, 2005).

The columnar cells of the anterior and posterior midgut regions in both larval stages exhibit intense secretory activity and their cytoplasm contains a high concentration of organelles related to secretion, such as rough endoplasmic reticulum, Golgi complexes and secretory vesicles (Rothman & Orci, 1992). The continuous synthesis and secretion of digestive enzymes have been described in continuous feeders (e.g. Diptera larvae and Lepidoptera) (Baker *et al*., 1984). Three secretory mechanisms were observed in these two midgut regions (Terra & Ferreira, 2005): (a) apocrine secretions; (b) microapocrine secretions, and (c) merocrine secretions, or exocytosis. Apocrine secretions involve the loss of part of the apical cytoplasm and, indeed, apical extrusions, the so-called protuberant buds or blebs, projecting into the lumen were frequently noted (Fig. 5A); according to some authors these blebs can be associated with normal renewal processes in healthy cells (Anderson & Harvey, 1966; Baker *et al*., 1984). Apocrine secretions are referred to as microapocrine secretions when the loss of cytoplasm is small. Numerous pinched-off vesicles were observed at the top of the long microvilli and it has been suggested that the content of these vesicles may be incorporated into the peritrophic matrix (Jordao *et al*., 1999). Merocrine secretions, or exocytosis, consist of the fusion of the secretory vesicles with the apical cell membrane without cytoplasm loss; content is emptied in lumen (not shown).

The columnar cells of the anterior midgut present some large vacuoles, whereas the posterior midgut cells appear to be more vacuolated and show large autolytic vacuoles containing various lamellar and granular residues. The presence of numerous autolytic vacuoles and multi-vesicular bodies can be associated with autolytic activity (Anton-Erxleben *et al*., 1983).

The morphology of the columnar cells of the median midgut differs from those of the two other regions. The surface of basal membrane exposed to haemolymph is not increased by a basal labyrinth and only short microvilli are visible at the apical region of the cells. Microapocrine secretion consisting of the release of budding secretory vesicles was the only secretion mechanism noticed in these columnar cells. Blebs were never observed in this region. These cells seem to contain a much higher concentration of mitochondria compared with the other columnar cells. Even if the anterior and posterior midgut regions carry out the dual functions of absorption and

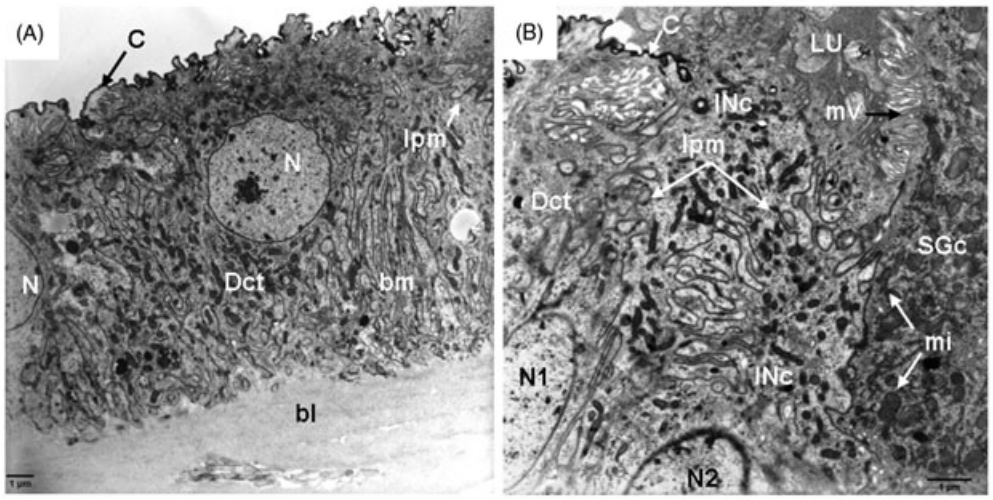

Fig. 11. (A) Transmission electron microscopy (TEM) of salivary gland efferent duct cells (Dct) of *Gasterophilus intestinalis* L2 showing the cuticle (C) that lines the cells at the apical region, the infoldings of the basement membrane (bm) and the lateral plasma membrane (lpm). bl, basal lamina; N, nucleus; scale bar = 1 µm. (B) TEM of the intermediate cells (INc) at the junction of the Dct and the glandular cells (SGc) of *G. intestinalis* L2. The nuclei of the INc are indicated as N1 and N2. mv, microvilli; LU, lumen; mi, mitochondria; scale bar = 1 µm.

secretion, it is difficult to attribute a definitive function to the median midgut.

A specific feature observed in the midgut columnar cells and salivary gland cells concerns the numerous swollen mitochondria. The ultrastructure of mitochondria is known to vary between tissues, organisms and the physiological status of cells (Zick *et al*., 2009). Swollen mitochondria have often been described as a manifestation of the ageing process, cellular disorder or apoptosis in all kinds of organisms (Anton-Erxleben *et al*., 1983; Charles, 1987; Yasuda *et al*., 2006). These structures may also be a consequence of the microaerobic conditions in which *G. intestinalis* larvae live, in that swollen mitochondria may optimize the production of energy.

Associated with the columnar cells, regenerative cells were found scattered throughout the epithelium, indicating that epithelium may be renewed regularly or under certain conditions. Regenerative cells were found in *D. hominis* larvae (Evangelista & Leite, 2003), but appear to be absent in some dipteran larvae (Terra *et al*., 1988). The third type of cell observed was the endocrine cell. The role played by endocrine cells in the control of midgut events is as yet unclear, but, as in vertebrates, these cells are likely to have a function in the regulation of intestinal activities (Lehane *et al*., 1996).

The tubular form of the salivary glands of *G. intestinalis* L2 and L3 can be considered similar to that of other Diptera, such as *D. hominis* (Evangelista & Leite, 2007) or *H. bovis* (Boulard, 1969). The group of cells which represent the transition between the salivary canal cells and the glandular cells, termed 'intermediate' cells, were also identified in the salivary system of *H. bovis* (Boulard, 1969). These may have only a transitional function between the canal and gland structures as their cytoplasm contains few organelles and a small nucleus.

Unlike the glandular cells, in which the basement membrane shows only a few infoldings, the plasma membrane of the salivary canal cells forms a sort of basal labyrinth, exposing a larger membrane surface to haemolymph that may have an active transport function.

In the glandular cell, the septate junctions that link the adjacent cells tightly to one another show numerous infoldings, allowing the cells to increase in volume when necessary, possibly to store synthesized products. Indeed, these cells demonstrate an intense synthesis activity; the rough endoplasmic reticulum, free ribosomes and Golgi complexes seem to completely fill the apical region of some cells. Additionally, the numerous tracheae insertions, which supply the cells with oxygen, the highly abundant mitochondria (Fig. 12A) and the large nucleus exhibiting several nucleoli (not shown) support the hypothesis of important cellular synthesis and storage processes. The microvillar-like projections associated with the microvilli at the apical portion of the glandular cell may serve to reinforce the structure of the gland or to resist osmotic pressure.

The differences noted between the two larval stages concerned only the glandular cell content. However, these results should be confirmed as they are based on only a few observations. Further investigations are needed to elucidate the contribution of the salivary glands to digestion processes and the impact of salivary enzymes on host tissue during migration and maturation phases.

This work presents a first step towards understanding the functional morphology of the midgut and salivary glands of *G. intestinalis* second and third instars. It is known that in insects the organization of the digestive processes depends on the compartmentalization of digestive enzymes and on midgut fluxes (Terra *et al*., 1996). The localization in each midgut luminal compartment and corresponding tissue of

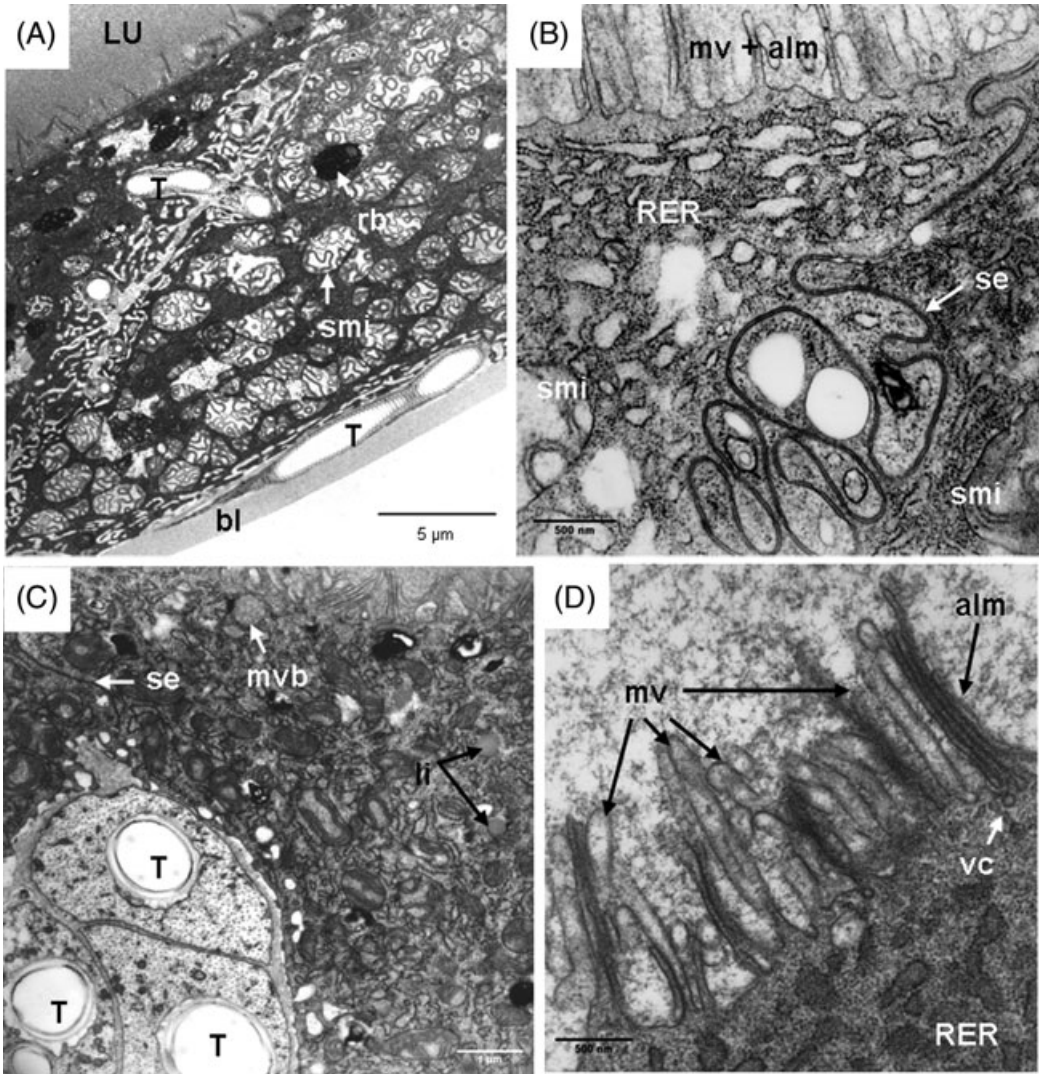

Fig. 12. (A–D) Ultrastructural details of the salivary glandular cells (SGc) of *Gasterophilus intestinalis* L3. (A) View of salivary glandular cells, showing the high concentration of mitochondria and swollen mitochondria (smi). T, tracheae; rb, residual bodies; bl, basal lamina; LU, lumen; scale bar = 5 µm. (B) Apical cytoplasm of SGc showing the abundant rough endoplasmic reticulum (RER) and the folded septate junction (se). Microvilli (mv) linked with apical lamellar membrane (alm) extends into the LU. Scale bar = 500 nm. (C) Detail of the tracheae 'clusters' that penetrate the glandular cells. li, lipidic droplets; scale bar = 1 µm. (D) Detail of the apical projections (mv and alm) of SGc with coated vesicles (vc) at the base of the projections. Scale bar = 500 nm.

various digestive enzymes described in *G. intestinalis* larvae (Tatchell, 1958; El-Ebiarie *et al*., 2005) will contribute useful information to the further study of midgut functions.

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