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The duct connecting Malpighian tubules and gut: an ultrastructural and comparative analysis in various Ephemeroptera nymphs (Pterygota)

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Abstract Malpighian tubules proper are connected to the gut by ducts called trunks, the organization of which is described at ultrastructural level in the nymphs of various mayfly species, namely *Ecdyonurus venosus* (Heptageniidae), *Ephemerella ignita* (Ephemerellidae), *Choroterpes picteti* (Leptophlebiidae), and *Caenis luctuosa* (Caenidae). Trunks are luminal tubes varying in arrangement, number, shape, and length. The main cell type of the trunk wall is represented by cells that are bordered by a thin cuticle along their luminal side (type-A cells). Whereas these cells are exclusive in the long trunks (such as those of *C. picteti* and *C. luctuosa*), in the shorter ones (such as those of *E. venosus* and *Ephemerella ignita*) cells with a microvillous luminal border (type-B cells) are also encountered. These cells are located close to the entrance of the collecting duct of the Malpighian tubules proper, and their long microvilli form a dense filamentous mesh filling up the lumen. Both cell types exhibit fine structural features that are characteristics of ion-transporting epithelia. Ultrastructural investigations show that trunks are not a simple conducting system but are involved in the regulation of the ionic composition of the primary urine for producing a fluid hypo-osmotic with respect to the hemolymph.

A. Introduction

Ephemeroptera maintained many plesiomorphic pterygote and even hexapod features. Investigations on the functional morphology of Malpighian tubules of mayfly nymphs can give insight into the adaptation of these insects to the aquatic environment. Indeed, aquatic insects are usually faced with the problem of loss of salts to the environment and dilution of the body fluids due to a continual diffusion of water through the body surface

(Kapoor 1994). A case in point is the chloride cells of mayflies which are specialized elements for the absorption of inorganic ions from very dilute solutions (Wichard et al. 1972; Komnick 1977; Filshie and Campbell 1984).

Some preliminary morphological aspects of Malpighian tubules in the nymphs of several mayfly species have been reported by Grandi (1950), and the comparative anatomy and arrangement of Malpighian tubules have been intensely studied by Landa (1969), Landa et al. (1980), and Landa and Soldán (1985). In this last paper, Landa and Soldán showed that the external shape of the tubules (degree of coiling of the distal portion), together with their arrangement when entering the gut (connection to the gut with or without intermediate trunks) can be considered important traits to outline phylogenetic diagrams. Ultrastructural data on the Malpighian tubules of mayflies have been presented by Nicholls (1983) for *Ecdyonurus dispar* (Curtis, 1834) and by Gaino and Rebora (2000) for *Baetis rhodani* (Pictet, 1843/45). These studies disclosed regional specialization of the cells in each tract.

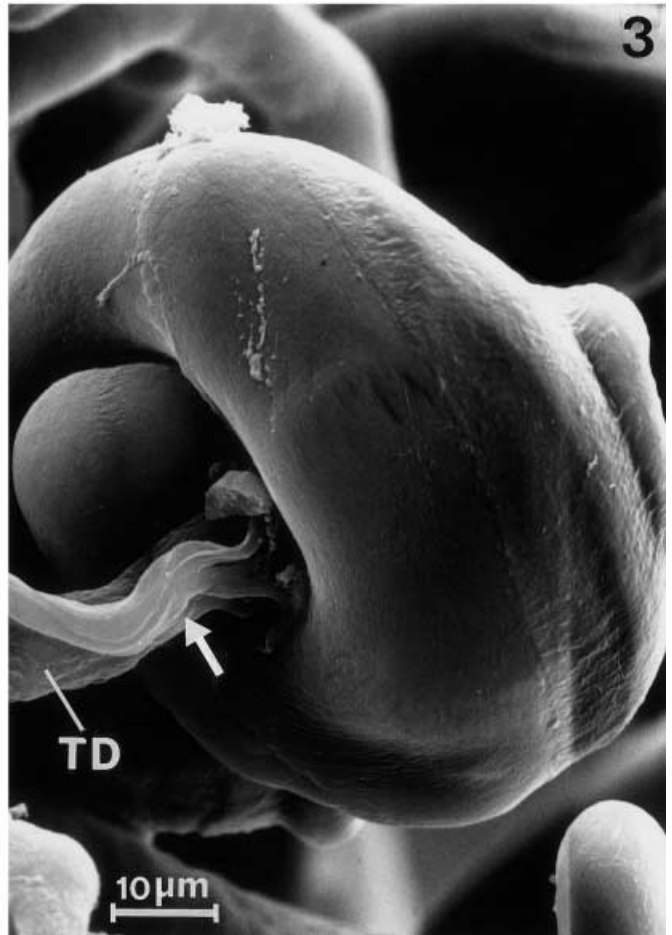
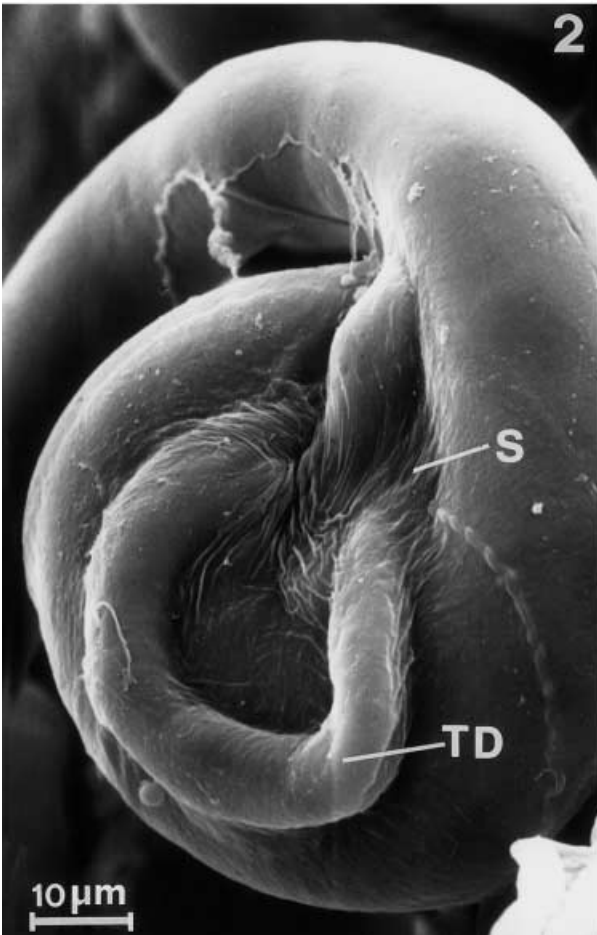
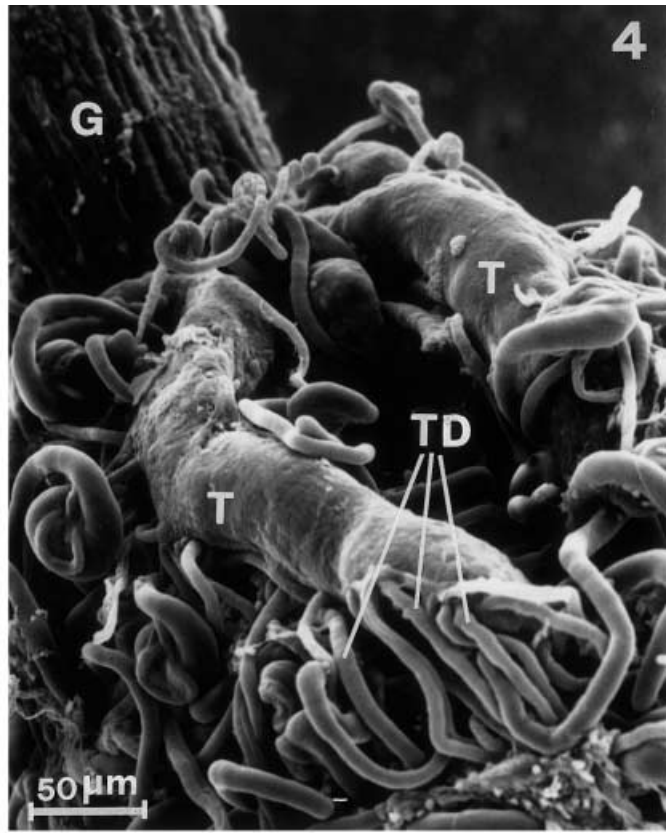
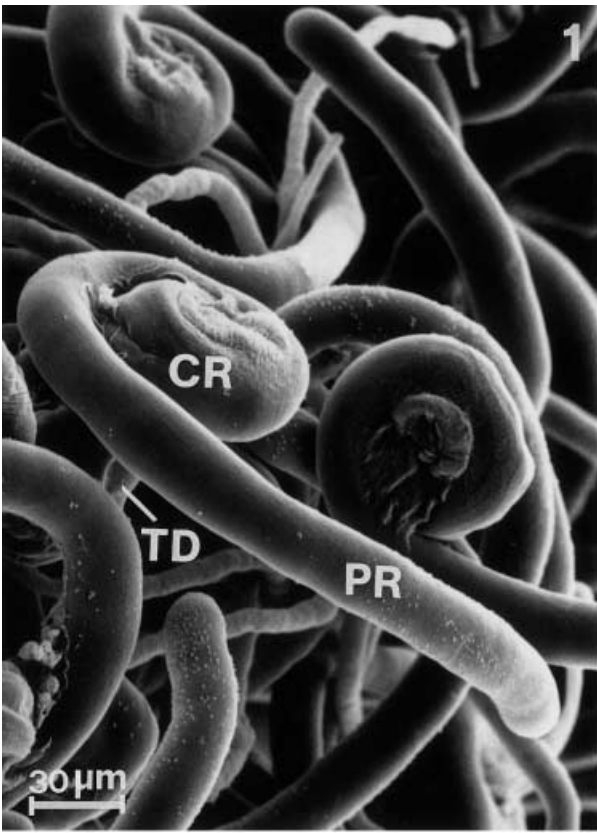
These data indicate two main models, direct connection of each tubule to the gut (“the simple model”) and connection through interposition of trunks of different shape, length, and number (“the complex model”). In the trunks of *E. dispar*, Nicholls (1983) observed two very distinct cell types, thus suggesting that trunks were not simple conducting ducts.

The aim of this paper is to compare at ultrastructural level the organization of the trunk in species belonging to different mayfly families, in order to investigate the relationship between external shape and fine structure. The terminology used by Landa and Soldán (1985) was utilized in this study.

B. Materials and methods

Mature nymphs of *Ecdyonurus venosus* (Fabricius, 1775) (Heptageniidae), *Ephemerella ignita* (Poda, 1761) (Ephemerellidae), *Choroterpes picteti* (Eaton, 1871) (Leptophlebiidae), and *Caenis luctuosa* (Burmeister, 1839) (Caenidae) were collected in the

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Figs. 1-4

Tescio stream (Bastia Umbra, Perugia, Italy) during spring and summer 1998 and 1999.

For TEM analysis, the excretory system was dissected in cacodylate buffer and fixed in Karnovsky's medium (Karnovsky 1965) in cacodylate buffer, pH 7.2, repeatedly rinsed in the same buffer, and postfixed for 1 h in osmium tetroxide (1% in cacodylate buffer). It was then rinsed in cacodylate buffer, dehydrated in a graded ethanol series, and embedded in Epon-Araldite mixture resin. Thin sections, cut on a Reichert ultramicrotome, were collected on formvar-coated copper grids, stained with uranyl acetate and lead citrate, and observed with a Philips EM 400 TEM and a Philips EM 208 TEM.

For SEM analysis, the material was fixed following the same procedures utilized for TEM investigation, and then dehydrated in a graded ethanol series, critical-point dried using a CO₂ Pabisch CPD apparatus, mounted on stubs with silver conducting paint, coated with gold palladium in a Balzers Union Evaporator, and observed with a Philips EM 515 SEM.

C. Results

In the nymph of *E. venosus* (Heptageniidae) each Malpighian tubule is constituted of a distal primary region leading to an adjoining coiled region, with the shape of a discoidal plate. The discoidal plate leads to a thin collecting duct (Fig. 1). The discoidal plate has two different faces, one showing a thin raised C-shaped tubular structure (Fig. 2) and the other containing the single emerging thin duct usually associated with a trachea (Fig. 3). Such an organization is the result of an abrupt narrowing of the primary tubule at the end of the coil from which the thin collecting duct departs. The collecting duct is kept adherent to the plate by a fibrous lamina (Fig. 2) and then emerges in the middle from the opposite face of the discoidal plate (Fig. 3). This thin protruding duct empties into the common trunk which is a luminal tube entering the gut (Fig. 4). Numerous thin ducts merge into the common trunk at various levels even though they tend to be more concentrated in its distal part (Fig. 4). Numerous (6–8) trunks are located laterally around the gut. In the specimens we examined they are usually located in segments VII–VIII of the abdomen.

The main cell type of the trunk wall is represented by two cell types: type-A and type-B cells (Figs. 5, 6, 7, 8). Type-A cells are delimited by an apical cuticular border. Type-B cells show long apical microvilli. Type-A cells are prevalent whereas type-B cells are typically located at the junction between each Malpighian tubule and the trunk.

Type-B cells are elongated and show deep infoldings along their basal region (Fig. 5). Their numerous and long microvilli (Fig. 6) fill up the lumen, forming a dense filamentous mesh (Figs. 5, 7). The remaining trunk wall is lined by type-A cells. They are larger than the type-B cells and show apical plasma membrane with marked infoldings (Fig. 7) surmounted by a cuticular border (Figs. 7, 8). Comparison of the apical border of these cells (Figs. 7, 8) reveals that the cuticular border gets thicker towards the connection with the gut, where it acquires a pleated configuration (Fig. 8). In addition, deep canaliculi are interposed between groups of cells (Fig. 7). Their cytoplasm contains a dense population of mitochondria, mainly concentrated along the cell basal region (Fig. 8). The microvilli of the type-B cells may reach the cuticular border of the type-A cells (Figs. 5, 7).

The general external shape of both Malpighian tubules proper and trunks of *E. venosus* may be regarded as the ground pattern of the excretory system of the other species here described. *Ephemerella ignita* (Ephemeroptera) shows eight very short trunks in the form of bud-shaped structures, with similar length and width. Malpighian tubules proper enter the trunk very close to one another. The organization of the trunks consists of type-A cells delimiting the main portion of the trunk wall (Fig. 9), and elongated cells with microvilli (type-B cells) extending into the lumen (Fig. 9). Deep infoldings are present along the basal membrane of the two cell types (Fig. 9).

In *Ch. picteti* (Leptophlebiidae) there are six trunks symmetrically arranged on both sides of the gut. They have different lengths and some of them are very long and may reach segments II–III of the abdomen. Malpighian tubules proper enter at the distal portion of the trunks where these last are slightly enlarged. On serial TEM sections only type-A cells were recognized. Groups of cells are separated by long canaliculi that deeply penetrate inwards (Fig. 10). Highly convoluted junctions are present between cells (Fig. 10). The apical border of the cells is thrown into protrusions towards the lumen (Fig. 11). These protrusions show numerous apical infoldings of the plasma membrane (Fig. 11). No specialization is present at the junction with the cells of the thin duct leading to the Malpighian tubule proper (Figs. 12, 13).

In *C. luctuosa* (Caenidae) only two very long trunks are inserted in the lateral position in the gut. They can

◀ **Figs. 1–4** *Ecdyonurus venosus* (Ephemeroptera) nymph. Malpighian tubules, SEM

Fig. 1 Distal primary region (PR) with adjacent coiled region (CR). TD thin collecting duct

Fig. 2 Coiled region with the thin collecting duct (TD). S Fibrous lamina

Fig. 3 Coiled region with the thin collecting duct (TD). Arrow Trachea

Fig. 4 Thin collecting ducts (TD) from different Malpighian tubules entering common trunks (T). G Gut

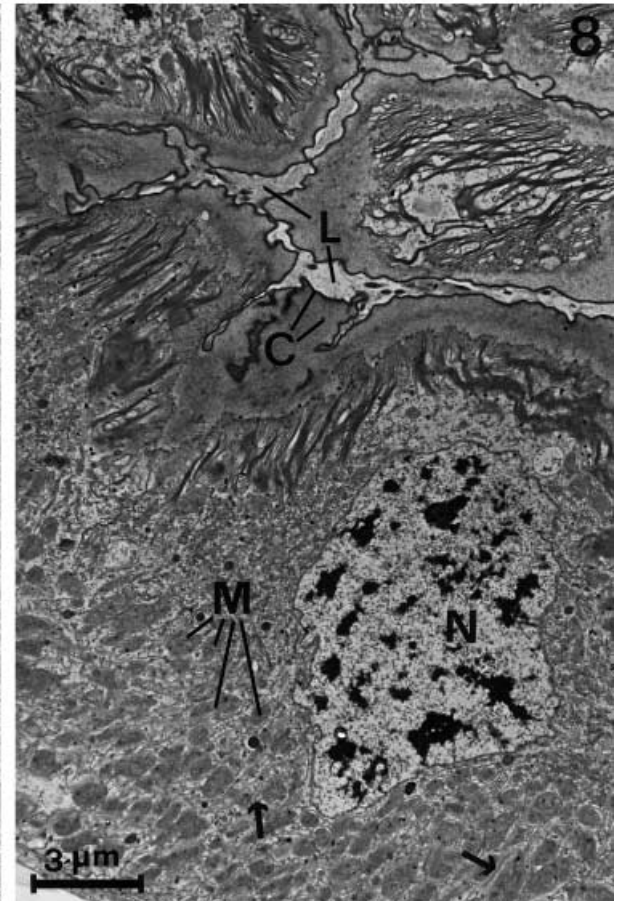
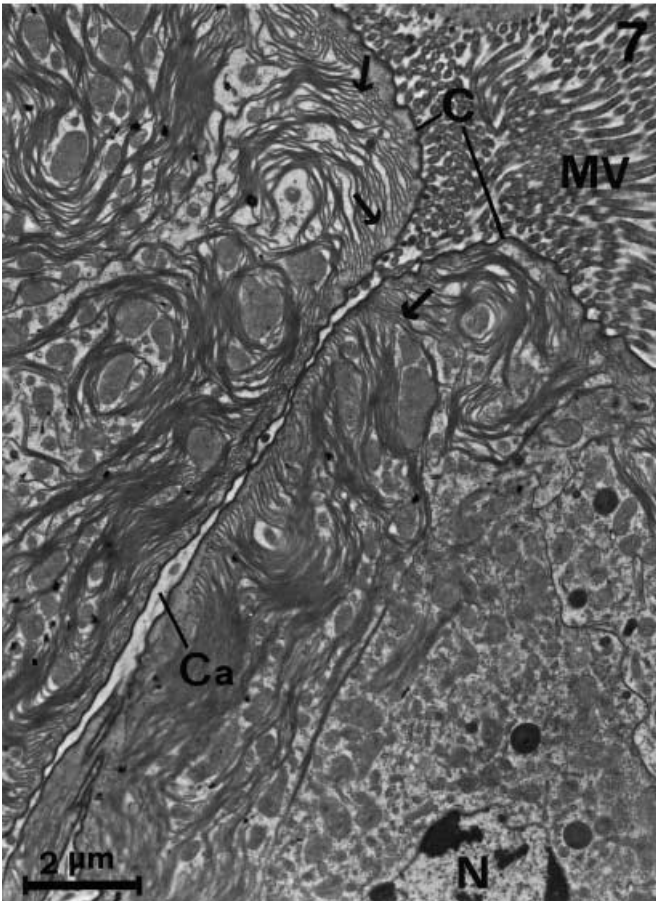
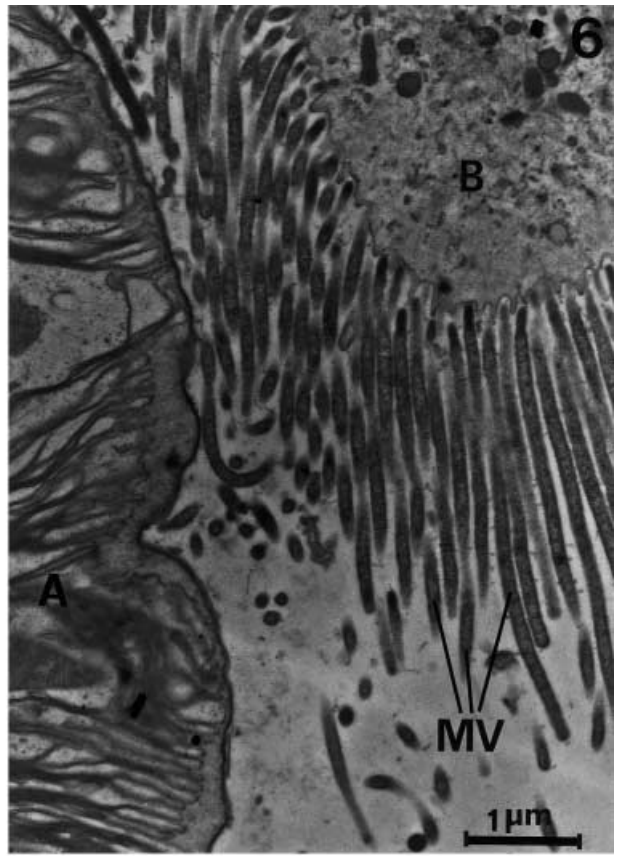
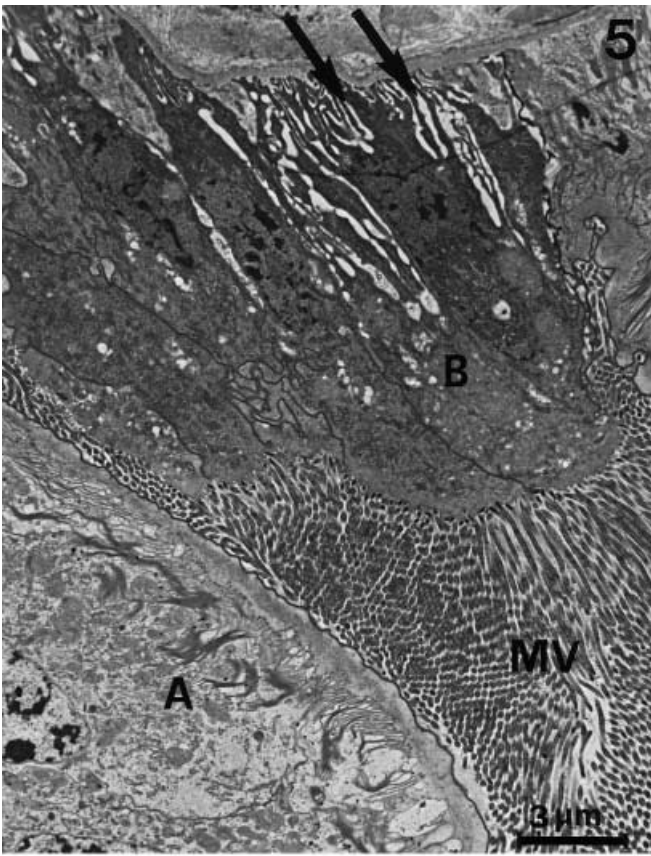
Figs. 5–8 *E. venosus* (Ephemeroptera) nymph. Wall of the trunk with type-A (A) and type-B cells (B), TEM ▶

Fig. 5 Type-B cells with deep basal infoldings (arrows) and numerous microvilli (MV)

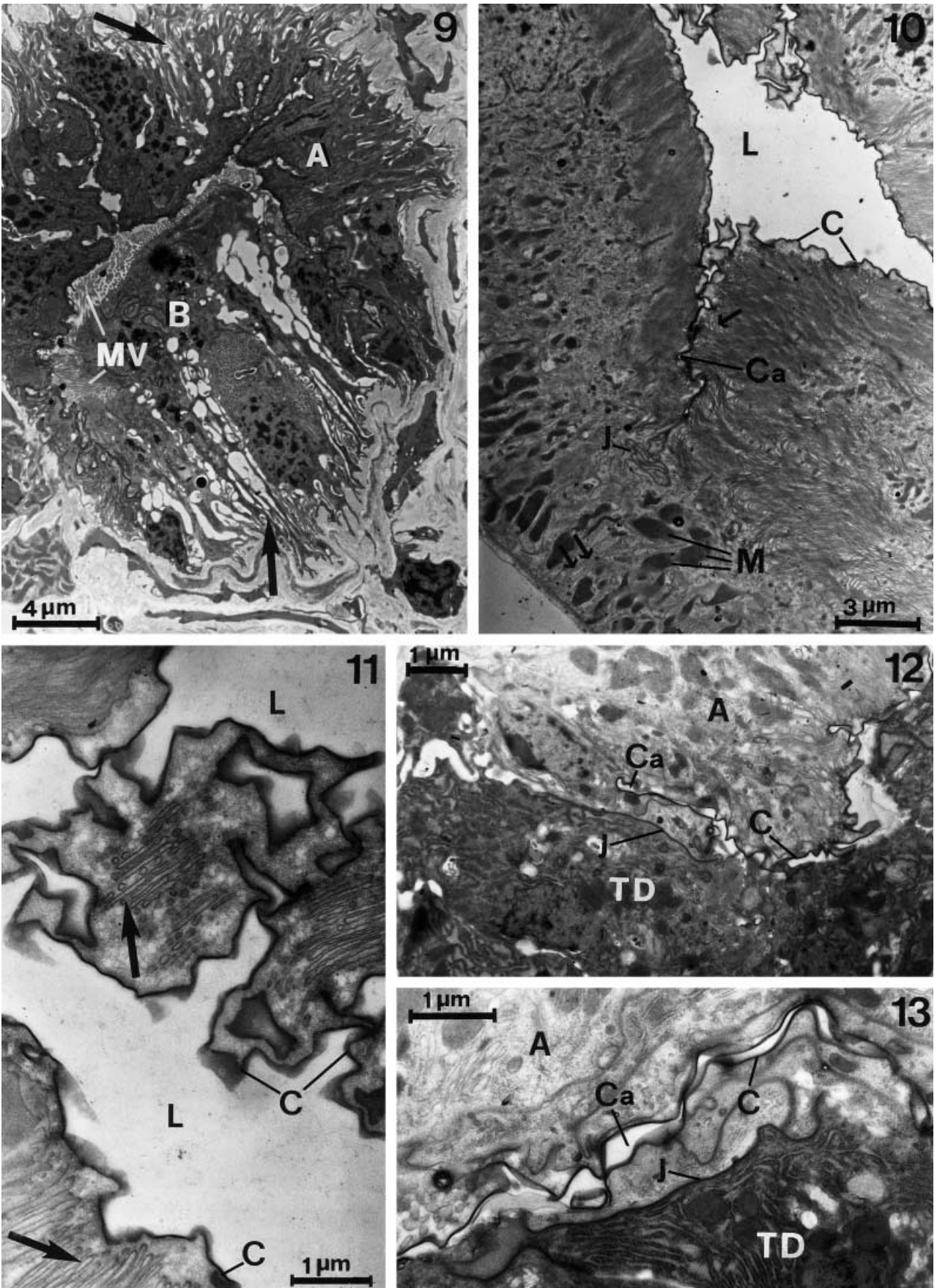
Fig. 6 Long microvilli (MV) of the type-B cells

Fig. 7 Apical infolds (arrows) of type-A cells. Microvilli (MV) of the type-B cells extending towards the cuticular-bordered (C) type-A cells. Ca canaliculus, N nucleus

Fig. 8 Type-A cells with thick cuticle (C) and a dense population of mitochondria (M) mainly located along the basal infolds (arrows). L Lumen of the trunk, N nucleus



Figs. 5-8



Figs. 9-13

reach segments II–III of the abdomen and on occasion even the thorax. They differ from those of *Ch. picteti* because of the narrowing of their distal part. Indeed, each trunk consists of two regions, a distal one where all the Malpighian tubules proper enter (region I) followed by the region that enters the gut (region II; Fig. 14). Ultra-thin sections reveal that there is a marked change in wall thickness and in cell types between the two regions (Fig. 15). The thin wall of region I is very similar to that of the thin duct of the Malpighian tubule proper (Fig. 15) and consists of cells with occasional short, irregular, thin protrusions at their apical border (Fig. 16). The thick wall of region II is composed of cells characterized by numerous infoldings of both their basal and apical plasma membranes (Figs. 15, 17). Their luminal side is delimited by a thin cuticle (Figs. 16, 17). In cross-section these cells are closely apposed to border a narrow lumen (Fig. 17). Groups of cells are separated by narrow canaliculi that extend towards the basal lamina (Fig. 17). These cells show large nuclei and numerous mitochondria mainly located in the basal infoldings (Fig. 17). The cell apical region is considerably pleated and covered with cuticle (Fig. 18). On occasion, mitochondria appear close to cell junctions (Fig. 18).

D. Discussion

Nicholls (1983), in his ultrastructural investigation on the Malpighian tubules of *E. dispar*, described the trunks as complex structures and hypothesized a possible involvement of the cells forming its wall in ion resorption. The present ultrastructural comparative investigation showed a correlation between the length of the trunk and the cellular component of the wall: two cell types (cuticular-bordered and microvillous cells) are associated with the short trunk (*E. venosus* and *Ephemerella ignita*) and a sole cell type (cuticular-bordered) is associated with the long trunk (*Ch. picteti* and *C. luctuosa*). The ultrastructural characteristics of both cell types, mainly consisting of an extensive membrane elaboration and a dense population of mitochondria, offer the basis for a hypothesis on their osmoregulatory function. Indeed,

these features are essentially similar to those of the resorption epithelia of a number of insects (Wessing and Eichelberg 1973; Wichard and Komnick 1973; Da Cruz-Landim 1994). This role is particularly relevant in aquatic larvae that need to take ions up from the primary urine in order to achieve a filtrate hypo-osmotic with respect to the hemolymph (Bradley 1985).

In particular, the morphology of the cells delimited by cuticle is very close to that shown by the cells lining the lumen of the insect hindgut (Wall and Oschman 1975; Green 1979; Martoja and Ballan-Dufrançais 1982), the surface specialization of which is essential for the diffusion of ions and water. A case in point is the rectum of damselfly larvae belonging to *Coenagrion puella* (Linné, 1758) and *Coenagrion* sp., whose cells constitute an ion-transporting epithelium (Wichard and Komnick 1974) with an ultrastructural organization very similar to that of the cuticular-bordered cells described by us in this paper.

In the long trunk, urine moves towards the gut along an extended absorbing tract where the useful electrolytes can be taken up. This uptake is greatly enhanced by the extracellular deep canaliculi interposed between groups of cuticular-bordered cells. A similar function could be attributed to the microvillous cells, which coexist with the previous ones in the short trunks, since their luminal surface is remarkably enlarged by the long microvilli. Moreover, microvilli extending to fill the lumen form a mesh of filaments that might be able to slow down the flow of the primary urine. This notion is supported by the fact that this cell type is restricted to the short trunks. Therefore, the superficial specialization of the type-B cells seems to accomplish a double function: physiological (absorption) and mechanical (fluid flow slowdown).

Considering the possible origin of the two cell types that constitute the trunks, it appears evident the morphological link between hindgut cells described in various order of insects and type-A cells of the trunk, especially owing to the occurrence in both cases of a luminal cuticular border. This feature is consistent with an ectodermal origin. In contrast, type-B cells lack cuticle and the presence of microvilli may represent a link between these cells and those lining the midgut, even though microvilli are not arranged to form the brush border that is typical

◀ **Figs. 9–13** *Ephemerella ignita* (Fig. 9) and *Choroterpes picteti* (Figs. 10, 11, 12, 13) (Ephemeroptera) nymph. Trunk, TEM

Fig. 9 Bud-shaped trunk with type-A (A) and microvillous (MV) type-B (B) cells. Note the basal infoldings (arrows)

Fig. 10 Long trunk constituted of type-A cells with apical cuticular border (C). Note the apical (arrow) and basal (double arrow) infoldings. J Junctions, Ca canaliculus, L lumen, M mitochondria

Fig. 11 Apical border of type-A cells extending towards the lumen (L). Note the apical infoldings of the cellular membrane (arrows). C Cuticle

Fig. 12 Connection between thin collecting duct (TD) cells and type-A cells (A). C Cuticle, Ca canaliculus, J junction

Fig. 13 Detail of the junction (J) between thin collecting duct (TD) cells and type-A cells (A). C Cuticle, Ca canaliculus

Figs. 14–18 *Caenis luctuosa* (Ephemeroptera) nymph. Long trunk. Schematic drawing (Fig. 14) and TEM (Figs. 15, 16, 17, 18) ▶

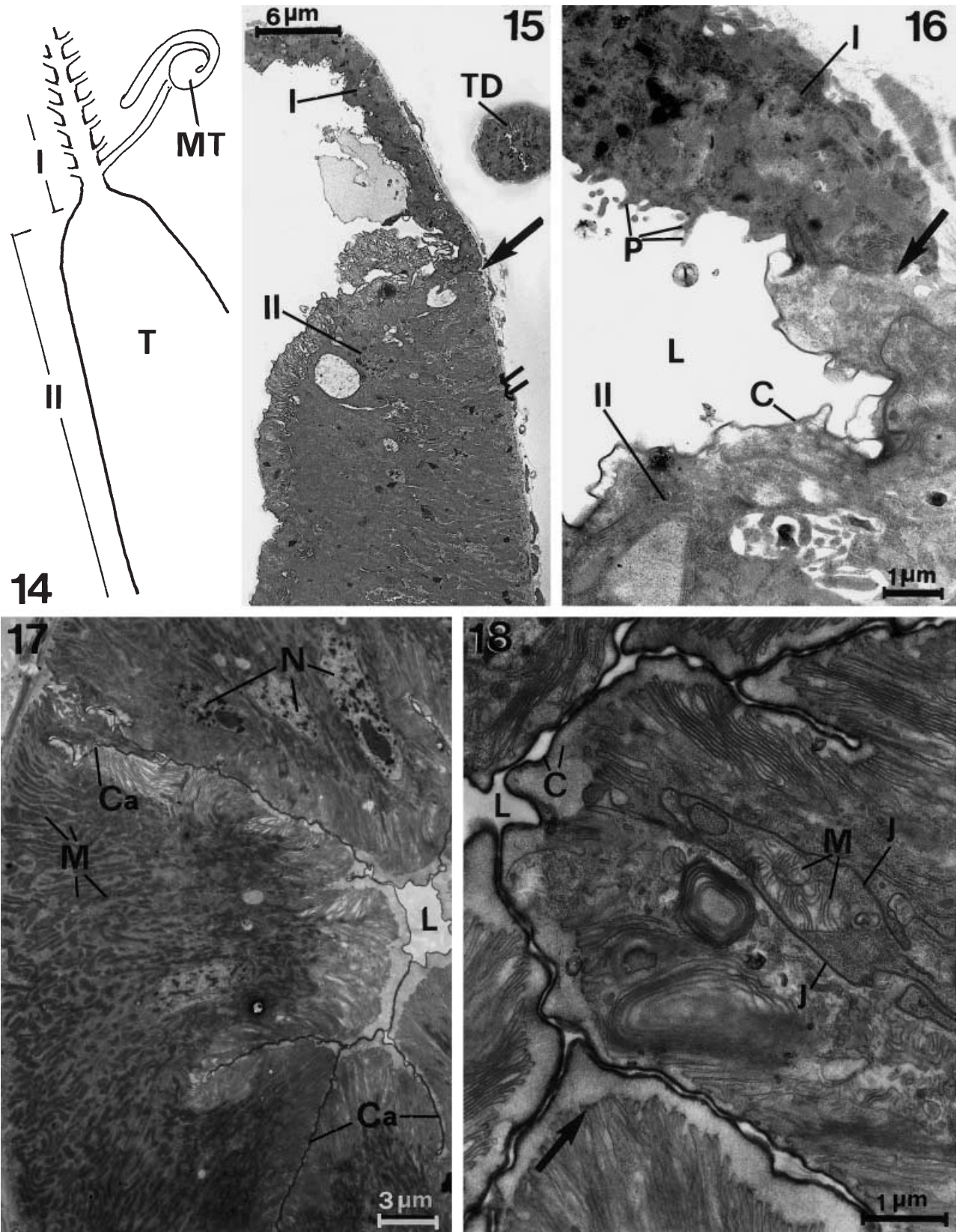
Fig. 14 Distal (I) and proximal (II) regions of the trunk (T). MT Malpighian tubule proper

Fig. 15 Connection (arrow) between regions I and II. Note the infoldings (double arrow) of the apical and basal membrane of region II. TD Cross-section of the thin collecting duct of a Malpighian tubule

Fig. 16 Magnification of the connection (arrow) between regions I and II. C Cuticle, L lumen, P thin protrusions from region I cells

Fig. 17 Region II. Ca canaliculi, L lumen, M mitochondria, N nuclei

Fig. 18 Region II. Infoldings of the cellular membrane (arrow) covered by cuticle (C). J Cell junctions, L lumen, M mitochondria



Figs. 14–18

of the midgut epithelium (Billingsley and Lehane 1996; Gaino et al. 1997). Even though the origin of type-B cells remains uncertain, their structure is consistent with an endodermal origin like that of the midgut epithelium. As a consequence, the short trunks of Ephemeroptera are characterized by the coexistence of two cell types arising from two different sources, which play a similar functional role in the regulation of the hemolymph composition. In the long trunks this function is accomplished by the type-A cells only. Nevertheless, in *C. luctuosa* it is worth stressing that the apical region (region I) of each trunk, which collects all the Malpighian tubules proper, lacks cuticle and the thin walls recall those of the ducts connecting this region to the coiled part of the Malpighian tubule. The region I could be regarded as one of the collecting ducts of the Malpighian tubules proper, specialized to gather them. Grandi (1950) in drawing the excretory system of *Caenis macrura* illustrates that all the discrete Malpighian tubules enter a thin non-apically coiled duct. Therefore, it seems acceptable that in *Caenis* the trunk *sensu strictu* is limited to the region II, which is characterized by a thick epithelium resulting only from absorptive cells delimited by cuticle.

McCafferty (1991) in his review on the phylogenetic classification of the Ephemeroptera, included Baetidae in the newly introduced taxa (suborder Pisciforma, infraorder Imprimata). This classification is in agreement with our observation on the excretory system of *B. rhodani* where straight tubules entering the gut individually represent the simplest model (Gaino and Rebora 2000) when compared with the more complex organization of the Malpighian tubules of the remaining mayfly. Indeed, the coiled distal portion of Malpighian tubules and the acquisition of trunks, along with their progressive reduction in number, seem to be a specialization (Landa and Soldán 1985; McCafferty 1991).

In summary, trunks represent important osmoregulatory organs allowing Ephemeroptera to keep the suitable ionic composition of the hemolymph during their aquatic life cycle. Our data confirm that these structures, together with the morphology of the Malpighian tubules proper, may represent a set of characters useful in the reconstruction of Ephemeroptera phylogeny.

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