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The osmoregulatory/excretory organs of the glacier-dwelling eutardigrade *Hypsibius klebelsbergi* MIHELČIČ, 1959 (Tardigrada)

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ABSTRACT. – The glacier-dwelling eutardigrade *Hypsibius klebelsbergi* MIHELČIČ, 1959 is exclusively known from cryoconite holes, i.e., water-filled micro-caverns on the glacier surface. This highly specialized environment is characterized by near zero temperatures and an extreme low conductivity of the water in these holes. Especially low conductivity might require powerful organs of osmoregulation in this unique species. Therefore we examined these organs in *H. klebelsbergi* using light and electron microscopy. *H. klebelsbergi* possesses three large Malpighian tubules at the transition of the midgut to the hindgut. Each tubule consists of a three-lobed distal, a thin middle and a short proximal part. The latter opens at the junction of the midgut and rectum. The distal part consists of three cells, which are characterized by a large nucleus, a fair number of mitochondria, a basal labyrinth, interdigitating plasma membranes and an irregular surface. Apical spaces extend in the middle part, which largely lacks nuclei and probably is made of offshoots of the proximal part. Here basal infolding and mitochondria are sparse. Interwoven cell projections and microvilli give this part and the proximal part a vacuolated appearance. The "vacuoles", i.e. extracellular spaces, are confluent with the intestine. In the proximal part up to 12 nuclei are counted. A valve separates the posterior hindgut from the anterior hindgut. In the anterior region and the valve the epithelium is thickened, covered by a thin cuticle and possesses a fair number of mitochondria and basal infolding. Obviously the structure of the osmoregulatory/excretory organ does not reflect the special conditions under which *H. klebelsbergi* lives.

KEYWORDS: Tardigrada, *Hypsibius klebelsbergi*, Malpighian tubules, rectal pads, glaciers, the Alps.

Introduction

Tardigrades are found in a variety of different habitats ranging from bryophyte cushions and lichens in the highest mountains to the sediments of the abyssal bottom. In each case, however, water must be available for active life at least temporarily (for further reading see MARCUS 1929; RAMAZZOTTI & MAUCCI 1983; GREVEN 1980, 2007).

Remarkable habitats for tardigrades are cryoconite holes, i.e. microcaverns on the ice surface in the glacier's ablation zone. The holes are caused by solar radiation on dark particles on the ice surface; they are predominantly water filled and frozen for most of the year (e.g. STEINBÖCK 1936; GRØNGAARD et al. 1999; DASTYCH et al. 2003).

Conductivity of water from these holes is very low ranging from 1.9 to 6.0 µS only (B. SATTLER, *in litt*.: data from the glacier Rotmoosferner, the Ötztal Alps). Three tardigrade species of the genus *Hypsibius* are known only from such holes and are found often in considerable numbers there: *H. klebelsbergi* MIHELČIČ, 1959; *H. janetscheki* RAMAZZOTTI, 1968 and *H. thaleri* DASTYCH, 2004. These animals are heavily pigmented – perhaps a protection against harmful UV-radiation – and are considered as extremely cold stenothermic (for review, descriptions and redescriptions see DASTYCH et al. 2003; DASTYCH 2004a, b; for the supraspecific status of *H. klebelsbergi* within the Hypsibiidae see also KIEHL et al. 2007).

For years the three "glands" at the transition between midgut and hindgut of Eutardigrada were considered as organs of osmoregulation and excretion. Most authors follow the terminology used for Euarthropoda and suggestively call these "glands" Malpighian tubules, whereas WEGLARSKA (1980) suggested the term "nephridia". Shape, position (MARCUS 1929; ENGLISCH 1936; WEGLARSKA 1989) and ultrastructure of these organs are well known (GREVEN 1979, 1982; WEGLARSKA 1980, 1987a, b; MØBJERG & DAHL 1996). Regardless of the structural differences found in the various eutardigrades, the ultrastructure of the distal part of tubules (highly folded plasma membranes, abundance of mitochondria) suggests ion transport.

Also the anterior hindgut of eutardigades shows specialisations compatible with increased transporting functions (DEWEL & DEWEL 1979; GREVEN 1982). They have been occasionally called "rectal pads" (GREVEN 1982), a term, which is just as suggestive as "Malpighian tubules". For the sake of simplicity, these terms will be retained in the following.

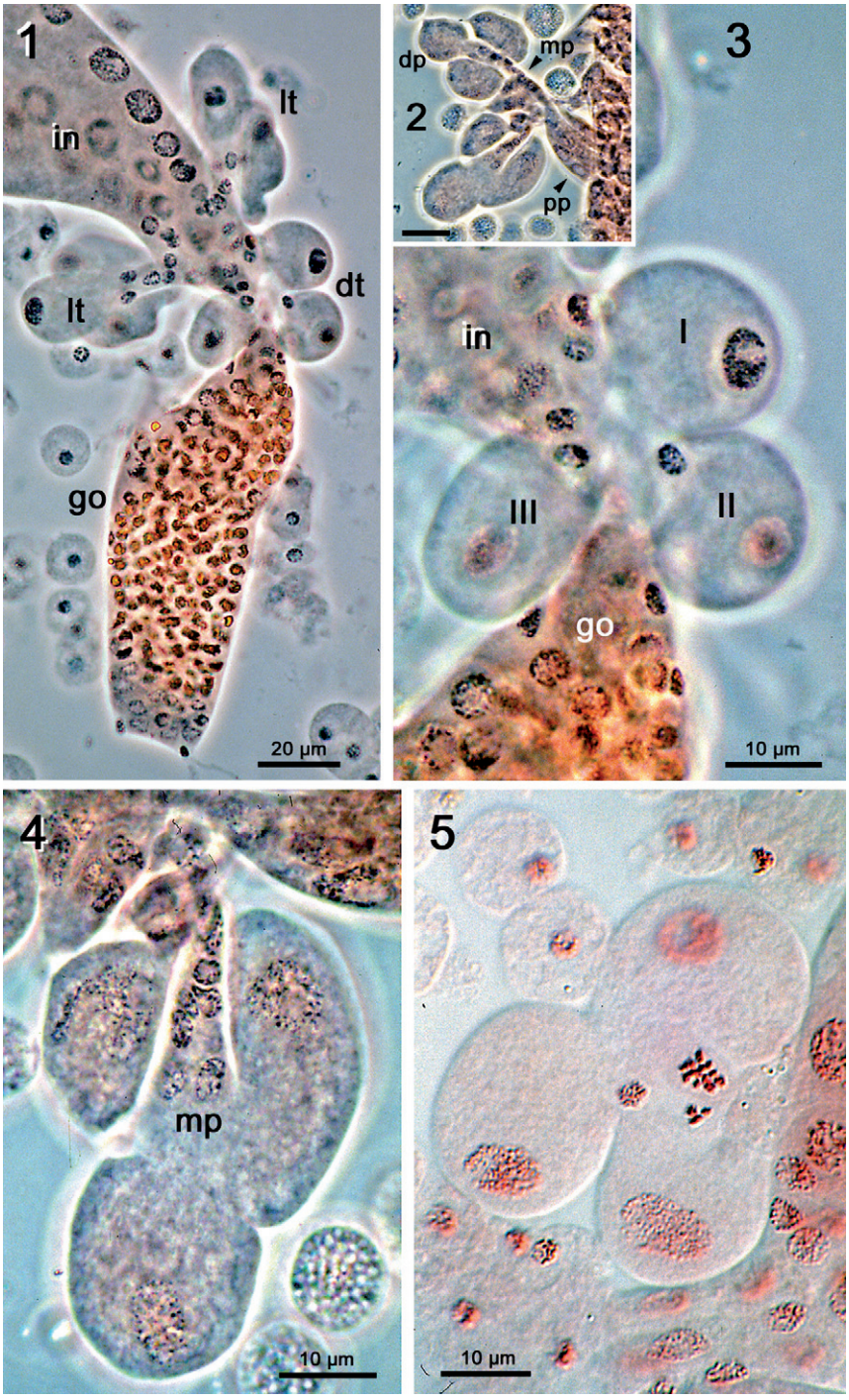
The special environment, in which *H. klebelsbergi* lives, surely requires properly functioning osmoregulatory/excretory organs. Hence, in the present article we take a closer look on these organs of this unique species using light and electron microscopical techniques. In addition, we briefly compare our findings with those obtained from other Eutardigrada, and summarize some functional considerations.

Material and Methods

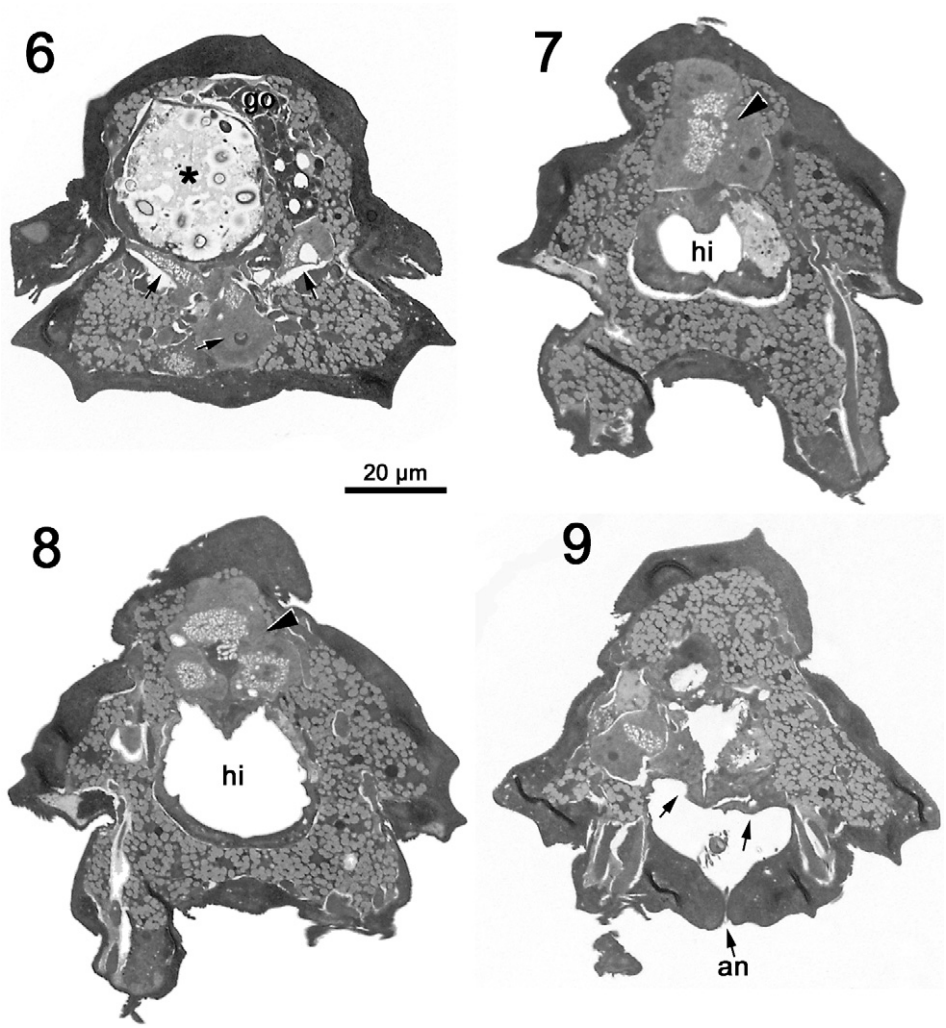
Specimens of *H. klebelsbergi* were collected at 29.8.2005 from the Langtalferner glacier in the Ötztal Alps (Austria) by the method described in DASTYCH et al. (2003). The material has been brought alive to Hamburg and partly used for observations in *LM*. Here they were kept alive in the refrigerator at 0.2-1.2 °C, with water and cryoconite substrate from the collecting site, filled up occasionally with distilled water. For light microscopical investigation animals were squashed and stained with aceto-lactic orcein, the corresponding photomicrographs were taken with ZEISS 'Photomikroscop III'.

For transmission electron microscopy tardigrades were fixed in KARNOVSKY´S fluid (KARNOVSKY 1965) using either 0.1 mol/l phosphate buffer or cacodylate buffer (pH 7.2). Additionally, some tardigrades were fixed in 2.5 % glutaraldehyde in 0.1 mol/l SÖRENSEN buffer after 2 h treatment with 0.05 mol/l tris-buffer plus 0.005 mol/l lanthanum chloride. Specimens were postfixed in 1% osmiumteroxide in the corresponding buffer and embedded in SPURR'S medium (SPURR 1969). The Malpighian tubules are difficult to fix properly and different fixation solutions gave very heterogeneous results from cell to cell and from time to time. Ultrathin sections were cut with diamond knifes, stained with lead citrate (REYNOLDS 1963) and viewed in a ZEISS EM 9-S2 electron microscope.

Abbreviations used are: ***an***- anus; ***cc***- coelomocytes; ***dt***- dorsal tubule; ***dp***- distal part of the tubule; ***go***- gonad; ***hi***- hindgut; ***in***- intestine; ***LM***- light microscope; ***lt***- lateral tubules;



Figs 1-5. *Hypsibius klebelsbergi* MIHELČIČ, squash preparations of the Malpighian tubules stained with aceto-lactic orcein: **1**, dorsal (*dt*) and lateral tubules (*lt*); intestine (*in*), gonad (*go*); **2**, distal (*dp*), middle (*mp*) and proximal (*pp*) portion of a tubule; **3**, magnification of Fig. 1, dorsal tubule; there is no marked difference in size between the three lobules (I-III); **4**, note numerous nuclei of the middle portion (*mp*); **5**, mitosis in a Malpighian tubule (not assigned to a certain cell type).

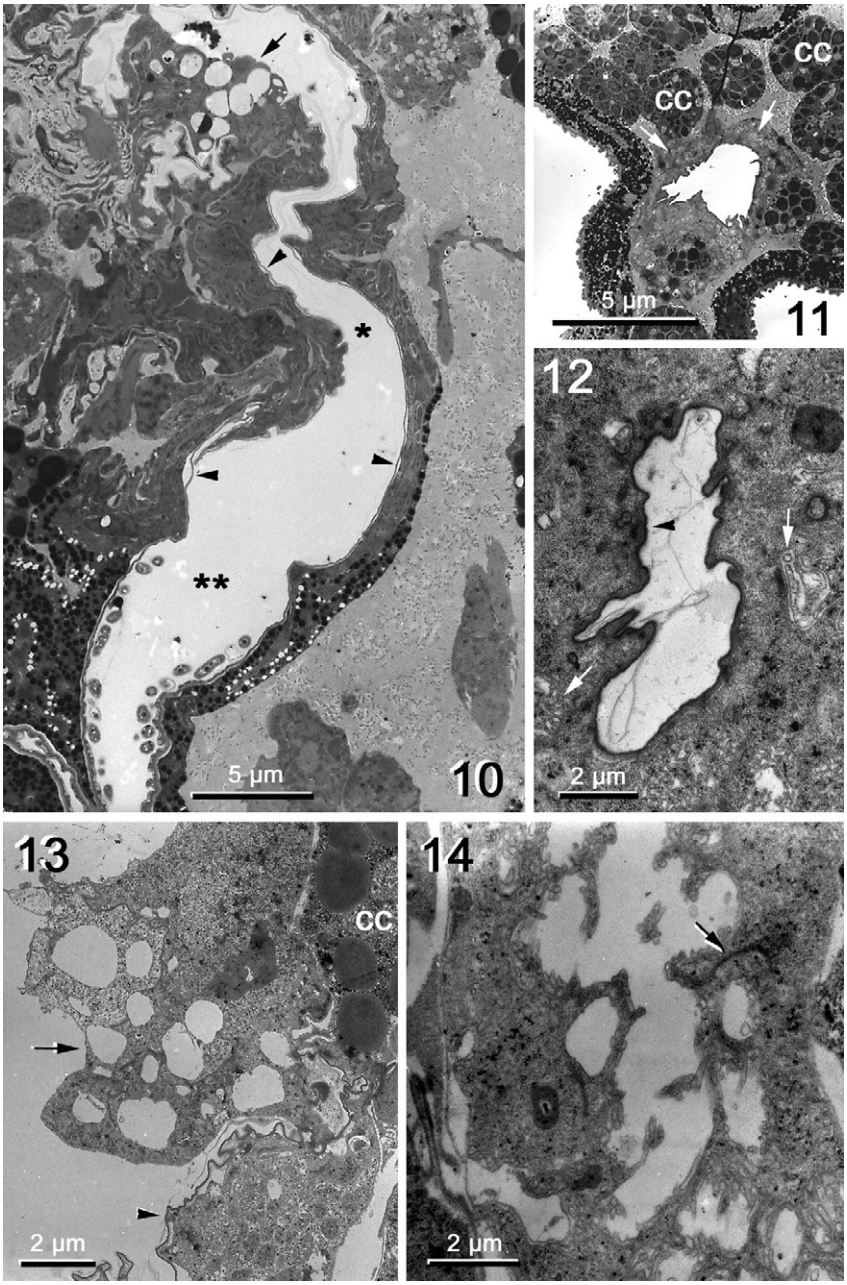


Figs 6-9. *Hypsibius klebelsbergi* MIHELČIČ, serial semithin cross sections from cranial to caudal, stained with toluidine-blue borax: **6**, intestine (asterisk) filled with unidentified material, gonad (*go*) and lateral Malpighian tubules (arrows); **7**, the gonad is no longer seen; dorsal Malphigian tubule (arrowhead); hindgut (*hi*); **8**, the three-lobed dorsal Malpighian tubule (arrowhead); hindgut lumen (*hi*); **9**, the hindgut possesses a prominent valve with thickened epithelium (arrows); anus (*an*).

ly- lysosome-like body; ***mi***- mitochondrion; ***mp***- middle part of the tubule; ***nu***- nucleus; ***pp***- proximal part of the tubule; ***rer***- rough endoplasmatic reticulum.

Results

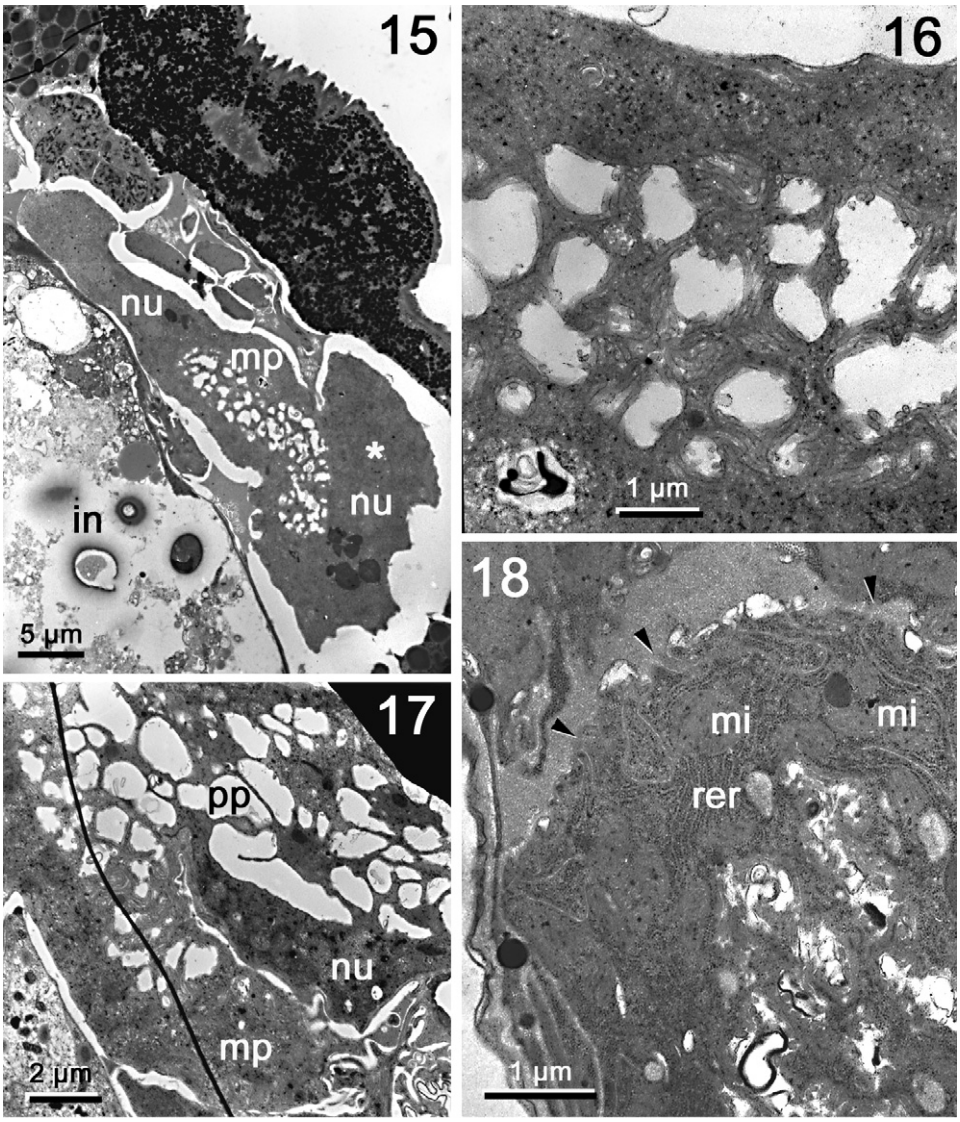
Squash preparations stained with aceto-lactic orcein of *H. klebelsbergi* show three Malpighian tubules of considerable, but similar size, which empty into the digestive tract at the transition zone of the midgut and hindgut (Figs 1-3). The tubules are suspended from ligaments and muscle cells attached to the midgut, hindgut, and the body wall. Each tubule consists of 1) a three-lobed distal part, 2) a slender middle segment, and



Figs 10-14. *Hypsibius klebelsbergi* MIHELČIČ, ultrathin sections of the hindgut and Malpighian tubules: **10**, low power micrograph of the anterior (asterisk) and posterior (two asterisks) hindgut. The thickened epithelium in the anterior hindgut is very electron dense; note the cuticular covering (arrowheads) and the vacuolated hindgut epithelium (arrow); **11**, cross section through the thickened portion of the anterior hindgut; mitochondria (arrows); coelomocytes (*cc*); **12**, anterior hindgut with the thin cuticular covering (arrowhead) and membranous infolding (arrow); **13**, transition from the mitochondria-rich hindgut cells covered with a cuticle (arrowhead) to a vacuolated proximal cell of a Malpighian tubule (arrow); coelomocyte (*cc*); **14**, middle part of the tubule with intercellular channels and junctional complex (arrow).

3) a slightly thicker proximal part (Figs 1, 2). The three lobes represent three large cells having a prominent nucleus each (Figs 1, 3). Occasionally, mitoses were seen, but were not assigned to a certain cell type (Fig. 5; see also BERTOLANI 1970). The slender middle part lacks nuclei; in the somewhat broader proximal part we counted seven to 12 nuclei.

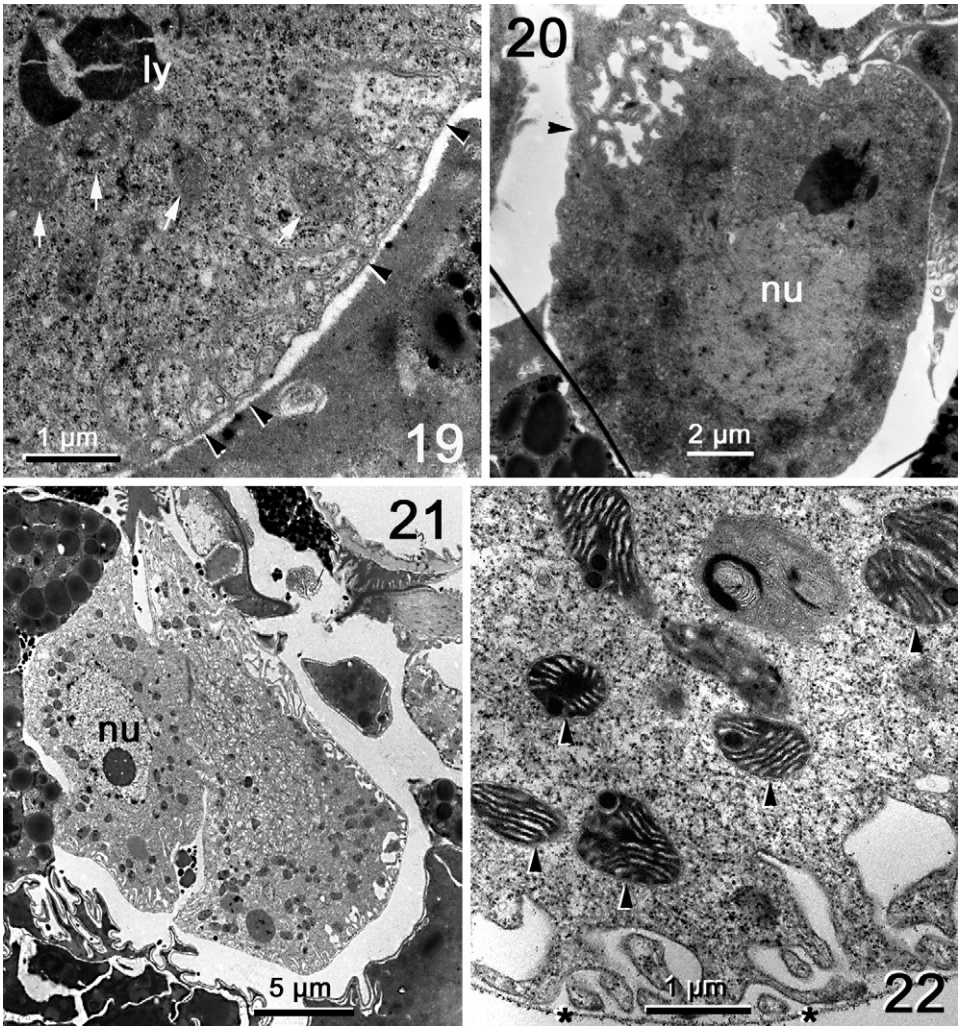
Semithin serial sections from cranial to caudal first show the large intestine (Fig. 6) followed by the hindgut and the large Malpighian tubules, which have a vacuolated



Figs 15-18. *Hypsibius klebelsbergi* MIHELČIČ, ultrathin sections of Malpighian tubules: **15**, overview, middle part (*mp*) with transition to the large proximal cell (asterisk); note the two elongated nuclei (*nu*); **16**, relatively thick section of the middle part showing the densely arranged microvilli; **17**, transition of the middle part (*mp*) to the proximal part (*pp*) near the opening to the gut; intercellular spaces become larger**;** **18**, transition to the stylus; note basal infolding (arrowheads), mitochondria (*mi*) and rough endoplasmic reticulum (*rer*).

appearance (Figs 7-9). The vacuolated parts approach the gut; however, we did not find a duct with a large lumen opening into the gut. The hindgut is divided in an anterior and posterior portion; both are separated by a valve or flap. In the anterior part and the flap region the epithelium appears to be thickened (Fig. 9).

At the ultrastructural level each rounded distal cell is characterized by some basal infoldings of the plasma membrane forming a moderate basal labyrinth (Figs 19, 21). The basal lamina, which surrounds the entire organ, does not follow these infolding (Fig. 22). The apices of the three cells possess finger-shaped, interdigitating projections



Figs 19-22. *Hypsibius klebelsbergi* MIHELČIČ, ultrathin sections of Malpighian tubules: **19**, the large distal cell shows some basal infolding (arrowheads), numerous mitochondria not well preserved (arrows), lysosome-like bodies *(ly)*; **20**, distal cell with large nucleus *(nu)* and the transition to the stylus (arrowhead); **21**, overview with a fixative showing an insufficiently preserved cytoplasm; mitochondria appear well-preserved; **22**, basal portion of a distal cell showing basal finger-like extensions, a thin basal lamella (asterisks) and well-preserved mitochondria (arrowheads); fixation as in Fig. 21.

obscuring a continuous lumen (Figs 18, 20). Between these projections occasionally some membranous whirls are seen. Cells contain some mitochondria generally not properly preserved and difficult to distinguish (Figs 18, 19). A much better preservation of mitochondria was seen in a specimen fixed after lanthanum treatment (Fig. 22). Mitochondria are not very closely associated with the membrane infolding (Figs 18, 19, 22).

Offshoots of the cells of the proximal part probably form the slender middle part. Here, infolding of the basal plasma membrane is scarcely developed, and the number of mitochondria is markedly reduced (Figs 15-17). Towards the interior there are numerous interwoven projections and microvilli-like extensions giving this and the next part a vacuolated appearance (Fig. 16). Adjacent cells are connected by junctional complexes (Fig. 14).

In the proximal part, cells and their nuclei are considerably smaller than the large cells of the distal part (Figs 15, 17) and the luminal spaces become somewhat larger (Fig. 17). The region where the proximal part opens into the intestine is characterized by large mostly intercellular spaces. In its immediate proximity the anterior hindgut begins; it is recognizable by the thin cuticular covering consisting largely of the epicuticle (for the cuticle structure of *H. klebelsbergi* see GREVEN et al. 2005) and the relative high number of mitochondria (Fig. 13). Towards the anus the cuticle becomes thicker, resembling the body cuticle (Fig. 10).

The anterior hindgut is clearly thickened forming a kind of pads. The cytoplasm of the epithelium of the anterior hindgut and the valve is rather electron dense. Number of mitochondria as well as basal infolding of the cell vary (Figs 10-13).

Discussion

Malpighian tubules of Eutardigrada are positioned at the junction of the midgut and the hindgut (e.g. MARCUS 1929; ENGLISCH 1936; WEGLARSKA 1989), lack a luminal cuticular covering and the cellular characteristics (numerous mitochondria, enlarged cellular surfaces) of the distal part are consistent with their roles in ion transport. The tubules do not show any structures indicating ultrafiltration, although WEGLARSKA (1980: p. 180/181) stated a "close resemblance to podocytes" (however, see the discussion in GREVEN 1982). Terminology of the various parts or segments of Malpighian tubules varies in the literature, e.g. external or distal lobe or initial segment for the distal part, internal column or stylus for the middle and proximal part, and distal part for the part called herein middle part (see literature cited below). Also shape, size, cell number and the mode to achieve an enlarged apical surface of the cells (long microvilli, irregular projections of the apical plasmalemma) as well as the structure of the opening into the gut vary among species, but commonly these tubules are considered as organs of osmoregulation and excretion. This assumption is based solely on their position and their ultrastructure; some authors emphasize different sensitivity to the osmolarity of saline solutions, but conclusive experimental work is lacking yet (see *Pseudobiotus megalonyx* (THULIN, 1928): GREVEN 1979, 1982; *Macrobiotus richtersi* MURRAY, 1911: WEGLARSKA 1980; *Isohypsibius granulifer* THULIN, 1928: WEGLARSKA 1987a; *Dactylobiotus dispar* (MURRAY, 1907): WEGLARSKA 1987b; *Halobiotus crispae*

KRISTENSEN, 1982: in KRISTENSEN 1982; MØBJERG & DAHL 1996). A second component of the osmoregulatory/excretory system in Eutardigrada that obviously forms a functional unit with the Malpighian tubules is the more or less thickened mitochondria-rich epithelium of the anterior hindgut, which is capable of accumulating chloride (*Milnesium tardigradum* DOYÉRE, 1834: DEWEL & DEWEL 1979; *P. megalonyx*, *Macrobiotus hufelandi* SCHULTZE, 1834: GREVEN 1982).

We do not want to deepen herein the discussion on the phylogenetic significance of the Malpighian tubules of eutardigrades and their similarity or supposed homology with the osmoregulatory/excretory system of various taxa ranging from nematodes and rotifers (WEGLARSKA 1980, 1987) to proturans (MØBJERG & DAHL 1996). We think the Malpighian tubules of Eutardigrada show only a superficial similarity, if any, to the osmoregulatory/excretory system of the nephridia of the onychophorans (e.g. STORCH & RUHBERG 1993; MAYER 2006) or the Malpighian tubules of euarthropods (e.g. ALBERTI 1999; BRADLEY 1998), united with Tardigrada to the Panarthropoda (*sensu* NIELSEN 2001). However, eutardigrades share the positional conformity of the Malpighian tubules-rectal pads complex with Euarthropoda. Only a superficial similarity holds also for the osmoregulatory/excretory organs of Cycloneuralia, namely nematodes (e.g. WRIGHT 1991), which are currently regarded as sister-group of Panarthropoda constituting the common taxon Ecdysozoa (e.g. SCHMIDT-RHAESA et al. 1998; GAREY 2001), and the metanephridia of Annelida (HARRISON & GARDINER 1992) combined with the Panarthropoda to the traditional assemblage Articulata. Malpighian tubules of eutardigrades are structurally highly derived and represent a potential autapomorphy of this taxon, i.e. either eutardigrades have modified an already existing organ they shared with the common ancestor of Euarthropoda, the possible sister group of Tardigrada (e.g. GAREY 2001) – MØBJERG & DAHL (1966) considered the Malpighian tubules of eutardigrades as a plesiomorphic trait – or Malpighian tubules have evolved convergently in euarthropods and eutardigrades (for further discussion and readings see GREVEN 1982).

Size and shape of Malphigian tubules in Eutardigrada may roughly reflect the environment the animals live in (MARCUS 1929; ENGLISCH 1936). The authors *(l.c.)* distinguish long tubules, e.g. in the xerophilous *M. tardigradum* and short tubules in freshwater and semiterrestrial species. In *M. richtersi* a highly complex situation has been found (see WEGLARSKA 1980), not fully understood as yet, but indicating a considerable diversity of these organs among eutardigrades. The Malpighian tubules of *H. klebelsbergi* are short, as in *H. crispae,* but relatively large and possess comparable segments: 1) the distal part corresponding to the initial segment of *H. crispae* (see MØBJERG & DAHL 1996), 2) the thin middle part (called distal part by KRISTENSEN 1982 and MØBJERG & DAHL 1996) and 3) the proximal part opening at the junction of mid- and hindgut. In *H. crispae* the mouth to the intestine is characterized by microvilli not found in *H. klebelsbergi* (see a similar organisation in *P. megalonyx*: GREVEN 1979).

Authors, who attempted a functional interpretation of these organs, stressed the absence of morphological signs of ultrafiltration and compared the excretory/osmoregulatory system of eutardigrades with the similarly positioned system of many euarthropods, mainly insects, assuming a similar mode of operation (GREVEN 1979, 1982; DEWEL & DEWEL 1979; DEWEL et al. 1993; MØBJERG & DAHL 1996). In freshwater this

includes 1) formation of the primary urine by secretion from the body cavity *via* the transporting epithelium towards the lumen of the Malpighian tubules, 2) modification of the primary urine along the tubules and, considering the anterior hindgut as significant part of this system (see GREVEN 1982), 3) active resorption of ions in the hindgut to a finally dilute urine, also to compensate for the loss of ions through the cuticle. This kind of urine formation and the need to reabsorb solute rather than fluid in the hindgut appears to be the basic mechanism present in obligatory freshwater eutardigrades. Membranous whirls, precipitations or even discrete concretions in the tubular lumen are considered as indicative of modifying processes (see WEGLARSKA 1980; KRISTENSEN 1982; MØBJERG & DAHL 1996).

Sites of active ion uptake directly from the dilute external medium (we have to assume a hyperosmotic body fluid in *H. klebelsbergi* and other freshwater eutardigrades: see CROWE 1972) have not been demonstrated yet. Thus, obligatory freshwater eutardigrades, normally not exposed to large fluctuations of their environmental salinities, may be suitable objects to start experimental work.

H. crispae is a eutardigarde, which secondarily has colonized marine habitats (KRISTENSEN 1982). This species possesses considerably enlarged Malpighian tubules attributed to the shift to seawater. Active stages exposed to low salinities die, but *pseudosimplex* stages are said to have greater osmoregulatory capacities; the latter statement has been, however, not substantiated (MØBJERG & DAHL 1996). Higher environmental salinities should lead to changes in the rate and direction of ion transport in the Malpighian tubules and the hindgut. Data on the hindgut are not available for *H. crispae*, but "rectal pads" are present in this species, too (see Fig. 19 in KRISTENSEN 1982 and Fig. 4 in MØBJERG & DAHL 1996). Strikingly, an increasing size of the distal part of the Malphigian tubules was mentioned after exposing the animals to high salt concentrations (KRISTENSEN 1982).

DEWEL & DEWEL (1979) have depicted a scenario what might happen when "terrestrial" eutardigrades enter anhydrobiosis. During this process osmolarity of the environment might dramatically increase. Authors speculated that the increasing osmotic pressure of tissue and fluid in the body cavity might slow urine formation and facilitate water uptake from the gut into the gut cells to conserve water during the drying process. Further, an active role of the hindgut was suggested.

Keeping tardigrades in water of higher salinity their body fluid would result in loss of water and keeping them in water of low salinity in loss of solutes. Thus, these losses need to be compensated for by a predominant reabsorption either of fluid or of solutes. Malpighian tubules of insect show microvillous shrinkage and growth in association with changes in fluid transport (for further readings see BRADLEY 1998), but despite the substantial differences in the physiological function, e.g. in surviving of various mosquito larvae in a wide range of salinities, the general ultrastructure of Malpighian tubules and the rectum does not vary in these insects in contrast to the anal papillae, the latter representing the sites of ion uptake from the external medium (e.g. GARRETT & BRADLEY 1984).

Currently, possible dependence of the osmoregulatory/excretory system of eutardigrades (Malpighian tubules, hindgut) on environmental variables is not reflected

in the ultrastructure of this system. However, the results of our study are somewhat puzzling, showing for example a relative low number of mitochondria and basal infoldings in the distal part of the Malpighian tubules of *H. klebelsbergi* – obviously the most active part – whereas in many other species these features appear to be more pronounced (see the figures in the articles on the Malpighian tubules cited above). Also the hindgut cells in *H. klebelsbergi* seem less elaborated compared to those of e.g. the "terrestrial" *M. tardigradum* (see DEWEL & DEWEL 1979) or some obligatory freshwater species (e.g. GREVEN 1982), where mitochondria and basal infolding are abundant. For comparative studies of environmentally induced changes of the osmoregulatory system in eutardigrades and even for interspecific comparisons a defined and standardized experimental design and fixation procedures that give more consistent results are necessary.

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