J. Limnol., 2013; 72(s1): 54-61
DOI: 10.4081/jlimnol.2013.s1.e7

The fine structure of the midgut epithelium in *Xerobiotus pseudohufelandi* (Iharos, 1966) (Tardigrada, Eutardigrada, Macrobiotidae)

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ABSTRACT

The aims of our studies were to describe the ultrastructure of the midgut epithelial cells of the eutardigrade Xerobiotus pseudohufelandi *and to determine if there are any differences in the ultrastructure of midgut epithelial cells between males and females. The analysis was performed with the use of the light and transmission electron microscopes. In* X. pseudohufelandi *the midgut epithelium is composed of digestive cells, but in the anterior portion of the midgut a group of cells with different ultrastructure has been observed. Histochemical staining showed the accumulation of reserve material in the cytoplasm of digestive cells. We suggest that some of them fulfil the role of regenerative cells (crescent-like cells, midgut stem cells), whereas others are differentiating cells which form new digestive cells. No differences in the ultrastructure of the midgut epithelium between males and females were distinguished except in the amount of multivesicular bodies.*

Key words: Tardigrada, midgut epithelium, multivesicular bodies.

INTRODUCTION

Tardigrades are small invertebrates, which are widespread in terrestrial, freshwater and marine environments (Dewel *et al.*, 1993; Kinchin, 1994; Nelson *et al.*, 2010). Some species are able to survive under various and also extreme environments that are lethal to most organisms (Rebecchi *et al*., 2009a, 2009b; Nelson *et al.,* 2010). It has been established that encystation and cryptobiosis in tardigrades are adaptive responses to changes in the external environment (Węglarska, 1957; Szymańska, 1995; Wright, 2001; Guidetti *et al.*, 2006, 2011; Rebecchi *et al.*, 2007, 2009b, 2009c; Wełnicz *et al.,* 2011). However, other potential mechanisms which might be involved in the maintenance of homeostasis in invertebrates, such as apoptosis, self-regeneration of tissues and organs, accumulation of spherites, synthesis of methallothioneins or antioxidants (Parthasarathy and Palli, 2007; Park and Takeda, 2008; Hakim *et al.*, 2010; Rost-Roszkowska *et al.,* 2010a, 2010b, 2012; Chajec *et al.,* 2012), have been studied only in some tardigrade species (Rebecchi *et al.*, 2009a; Rizzo *et al.*, 2010; Bonifacio *et al.*, 2012). In many invertebrates the regenerative properties of the midgut epithelium are also considered as adaptive mechanisms for coping with external stress factors (Wilczek, 2005; Tettamanti *et al.*, 2007; Park and Takeda, 2008; Malagoli *et al.*, 2010; Rost-Roszkowska *et al.*, 2011b; Franzetti *et al.*, 2012).

Given that the tardigrade *Xerobiotus pseudohufelandi* (Iharos, 1966) inhabits dry terrestrial environments and is able to survive long periods of drought in an anhydrobiotic state (own observations), it is a suitable organism to study mechanisms for coping with external stress, including the regenerative properties of the midgut epithelium. The aims of our studies were: i) to describe the ultrastructure of the midgut epithelial cells of *X. pseudohufelandi*; ii) to determine if there are any differences in the ultrastructure of midgut epithelial cells between males and females; iii) to distinguish stem cells among the midgut cells.

METHODS

Xerobiotus pseudohufelandi (Eutardigrada, Macrobiotidae) was extracted using standard methods (Dastych, 1980) from sandy soil samples collected from a pine forest near the Collegium Physicum, the Morasko University Campus, Poznań, Wielkopolska Voivodeship, Poland (52°28′04.70″N; 16° 55′45.47″E, 92 m asl).

Light and transmission electron microscope analysis

We examined midguts of 42 adult specimens (males and females). Specimens were fixed with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4), for 2 h at 4°C. The material was then post-fixed in 2% osmium tetroxide in a 0.1 M phosphate buffer for 1.5 h at 4°C, dehydrated in increasing concentrations of ethanol (50, 70, 90, 95 and 100%, each for 15 min), transferred to acetone for 15 min, and finally embedded in epoxy resin (Epoxy Embedding Medium Kit; Sigma Aldrich Inc., St Louis, MO, USA). Semi- (800 nm thick) and ultra-thin (70 nm) sections were cut on a Leica Ultracut UCT25 ultramicro-

tome (Leica, Wetzlar, Germany). Semi-thin sections were stained with 1% methylene blue in 0.5% borax and observed with an Olympus BX60 light microscope (Olympus Corporation, Tokyo, Japan). Ultra-thin sections were stained with uranyl acetate and lead citrate and examined with a Hitachi H500 transmission electron microscope at 75 kV (Hitachi Ltd., Tokyo, Japan).

Histochemistry

To detect lipids, semi-thin sections were stained with Sudan Black B at room temperature for 20 min.

Glycogen and polysaccharides were detected using the Periodic acid-Schiff (PAS) method. To remove osmium, semi-thin sections were treated with 2% solution of periodic acid (HIO_4) for 10 min at room temperature and then they were stained with Schiff's reagent for 24 h at 37°C.

To detect protein, first semi-thin sections were treated with a 2% solution of periodic acid for 10 min at room temperature to remove osmium, then they were stained with bromophenol blue (BPB) for 24 h at 37°C.

RESULTS

No differences between females and males of *X. pseudohufelandi* were observed in the ultrastructure of the midgut epithelium with the exception in the amount of multivesicular bodies (MVBs). Therefore, the results presented here refer to both females and males.

The midgut epithelium of *X. pseudohufelandi* (Fig. 1a and 1b) is formed by flat or cubic digestive cells. Their nuclei are located in the central region of the cytoplasm (Fig. 1c). In some cases the apical cell region forms a large evagination into the midgut lumen, making the epithelial surface folded. In these digestive cells the nucleus is present in this evagination of the apical cytoplasm (Figs. 1c and 3d).

The cytoplasm of the digestive cells does not show any regionalization in organelle distribution, which is most likely caused by the flat or cubic shape of cells. The basal cell membrane invaginates slightly into the cytoplasm forming membranous folds. Numerous cisterns of the rough endoplasmic reticulum (RER) are present (Fig. 1d) in their neighbourhood. Near the apical membrane, which forms microvilli that enter the midgut lumen, numerous small, coated vesicles are visible (Fig. 1b, 1d, 1e). They are formed due to endocytosis, when coated pits appear (Fig. 1e and 1f). Between the midgut and foregut or hindgut the number of microvilli is low (Fig. 1g). The entire cytoplasm of the digestive cell is rich in cisterns of RER and smooth endoplasmic reticulum (SER), mitochondria and free ribosomes (Fig. 2a). Golgi complexes have not been observed in the cytoplasm of digestive cells.

In many individuals several electron-lucent and electron-dense spheres of reserve material occur in the cytoplasm of digestive cells (Fig. 2b). Histochemical analyses showed that these are mainly lipids, whereas proteins and saccharides are rare (Fig. 3a, 3b, 3c). No differences in the amount of reserve material in the cytoplasm of digestive cells are found between females and males. In some specimens, which have the reserve material, large MVBs also appear (Fig. 2d and 2f). Intensive endocytosis (Fig. 2d and 2e), numerous small coated vesicles that originate from endocytosis (Fig. 2c and 2e), and cisterns of RER are present in the neighbourhood of MVBs. The process of vesicle fusion with MVBs has been recorded (Fig. 2g and 2h). Many more MVBs are present in the midgut epithelium of females than that of males (Fig. 2d, 2f, 2j).

Smooth septate junctions and septate junctions are distinguished between adjacent digestive cells (Fig. 4a). A group of cells with cytoplasm that differs from that of the digestive cells is present between the foregut and the midgut (Fig. 4b, 4c, 4d). These cells (herein called crescent-like cells) form a kind of *epithelial ring* that surrounds the epithelium of the foregut (Fig. 4b). The cytoplasm of cells in the *epithelial ring* contains many mitochondria and some cisterns of RER, but it is completely devoid of the reserve material (Fig. 4c). Between the crescent-like cells and the digestive cells of the midgut are young differentiating digestive cells (Fig. 4c and 4d). Together with numerous cisterns of RER and mitochondria, small amounts of the reserve material are also visible in their cytoplasm (Fig. 4c).

No mitotic divisions in the midgut epithelium or in the crescent-like cells have been described.

DISCUSSION

Up to now two kinds of cells have been described in the midgut epithelium of tardigrades: digestive cells that form the epithelium and crescent-like cells situated between the foregut and the midgut (Bertolani, 1970; Dewel and Clark, 1973; Greven, 1976; Kristensen, 1976; Pirch and Greven, 1994; Ząbczyk, 2000; Avdonina *et al.*, 2007; Rost-Roszkowska and Poprawa, 2008; Rost-Roszkowska *et al.*, 2011a).

Fig. 4d shows a schematic representation of the different kinds of cells observed in the midgut of *X. pseudohufelandi.* From the beginning of the midgut these cells are: midgut regenerative cells, midgut differentiating cells, and finally digestive cells. Therefore at the anterior end of the midgut epithelium of *X. pseudohufelandi* a group of cells forms a structure that we refer to as *the epithelial ring*. Cells of the *epithelial ring* correspond to crescent-like cells (midgut regenerative cells) described in some tardigrade species (Bertolani, 1970; Dewel and Clark, 1973; Greven, 1976; Ząbczyk, 2000; Rost-Roszkowska *et al.*, 2011a). However, we did not observe their proliferation, a characteristic feature of midgut regenerative cells of invertebrates (Hakim *et al*., 2010; Nardi *et al.*, 2010). Still, it is probable that these cells may divide at specific periods of the life cycle (*e.g.* during moulting) as has been described or sug-

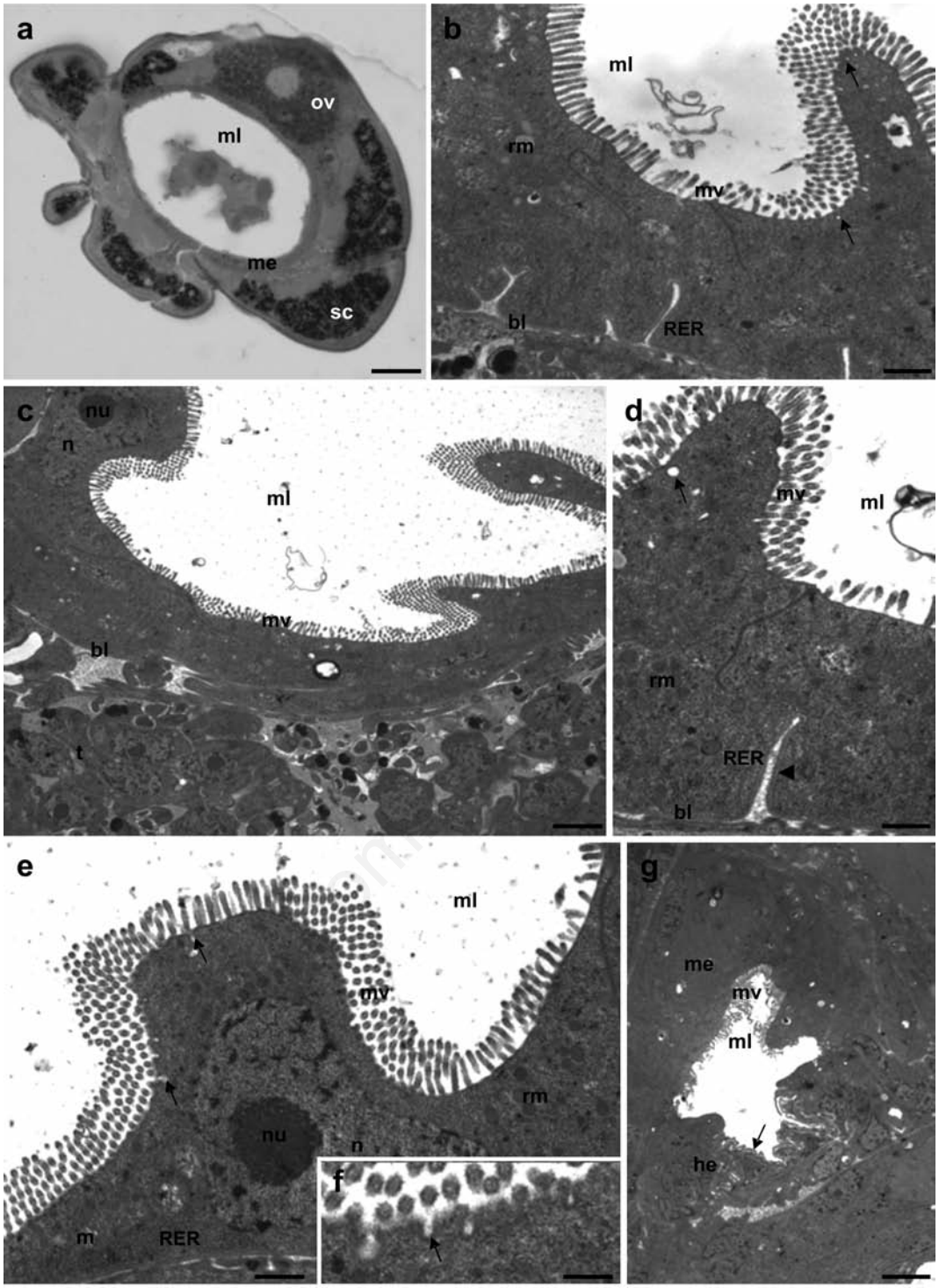

Fig. 1. Midgut epithelium of *Xerobiotus pseudohufelandi*. a, image obtained with light microscopy (LM); b-g, image obtained with transmission electron microscopy (TEM). a) Cross section through the body of a tardigrade; b) a fragment of the midgut epithelium showing cisterns of rough endoplasmic reticulum, reserve material and small vesicles (arrows); c) digestive cells of the midgut epithelium with distinct basal lamina and testis; d) basal membrane of digestive cells with membranous folds (arrowhead); e) apical membrane of digestive cells showing microvilli (mv) and endocytosis (arrow); f) higher magnification of the Fig. 1e showing endocytosis (arrow); g) microvilli between midgut (me) and hindgut (he); arrow indicates the cuticle of hindgut. bl=basal lamina; m=mitochondria; me=midgut epithelium; ml=midgut lumen; mv=microvilli; n=nucleus; nu=nucleolus; o=ovary; RER=rough endoplasmic reticulum; rm=reserve material; sc=storage cells; t=testis. Scale bars of a)=8.2 μm; b)=0.7 μm; c)=1.7 μm; d, e)=bar=0.6 μm; f)=0.2 μm; g)=2.3 μm.

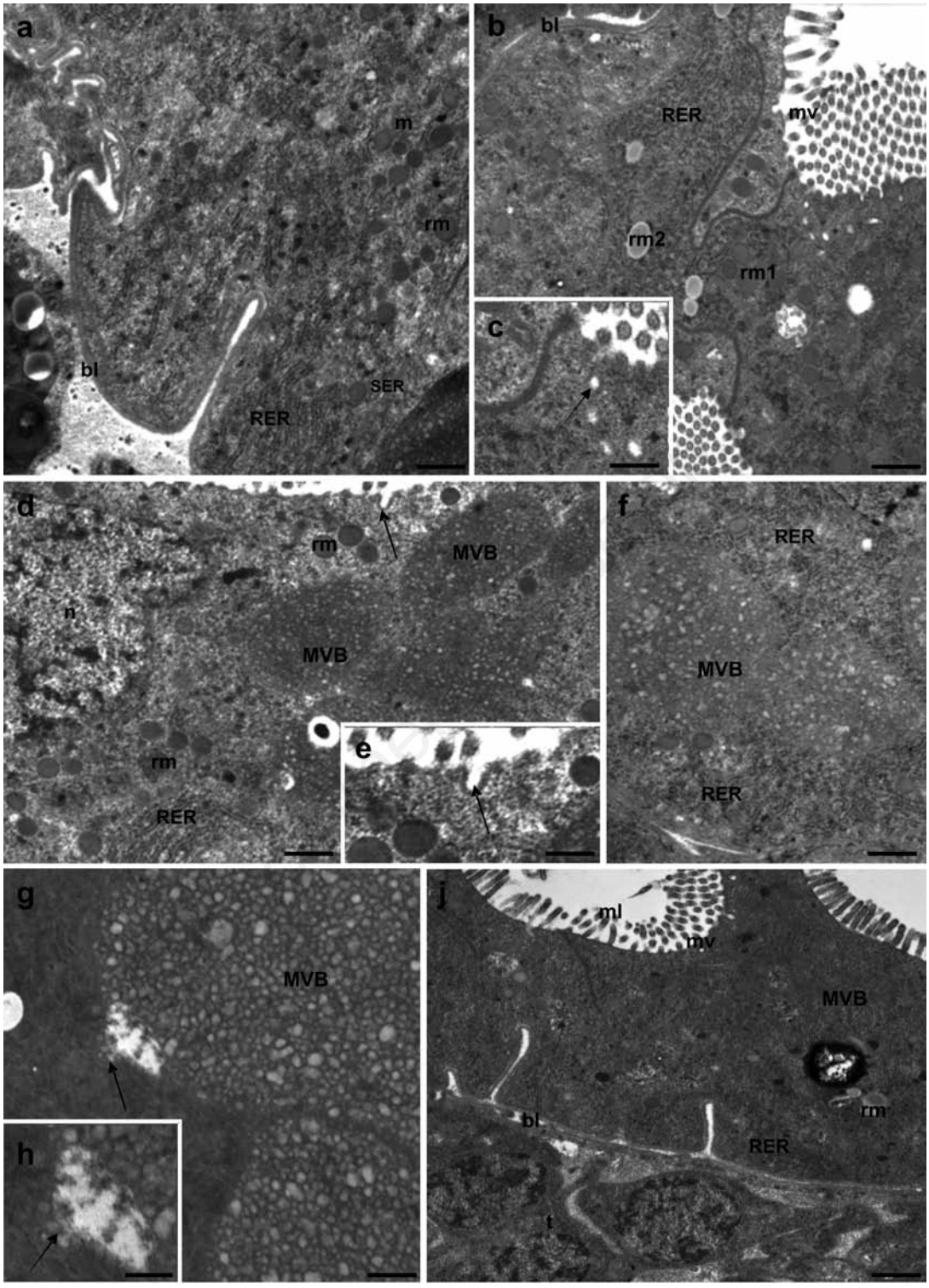

Fig. 2. Digestive cells of *Xerobiotus pseudohufelandi.* a-j, image obtained with transmission electron microscopy (TEM). a) Basal part of the cytoplasm in a digestive cell; b) cytoplasm of digestive cells; c) higher magnification of the Fig. 2b showing endocytotic vesicle (arrow); d) endocytosis (arrow) in the digestive cell of a female; e) higher magnification of the Fig. 2d showing endocytosis (arrow); f) cisterns of rough endoplasmic reticulum (RER) near multivesicular bodies (MVB) in a female; g) fusion of coated vesicle (arrow) with MVB; h) higher magnification of Fig. 2g showing coated vesicle (arrow); j) MVB in the midgut epithelial cells of a male. bl=basal lamina; m=mitochondria; ml=midgut lumen; mv=microvilli; n=nucleus; rm, rm1 and rm2=reserve material; SER=smooth endoplasmic reticulum; t=testis. Scale bars of a, b)=0.5 µm; c)=0.2 µm; d)=0.5 µm; e)=0.3 µm; f)=0.5 µm; g)=0.2 µm; h)=0.1 µm; j)=0.6 µm.

gested for many arthropods (Rost-Roszkowska *et al.*, 2007; Hakim *et al.*, 2010; Nardi *et al.*, 2010; Chajec *et al*., 2012). Finally, several cells forming the layer of the new/young digestive cells have been observed between crescent-like cells and digestive cells. Because their cytoplasm also possesses small amounts of the reserve material, together with mitochondria and cisterns of RER, we conclude that these cells are differentiating midgut regenerative cells (*i.e.* midgut progenitor cells). Midgut progenitor cells that differentiate into all types of the midgut epithelial cells have been described mainly for arthropods. They are described as cells which show the features of both midgut stem cells and digestive cells (Martins *et al.*, 2005; Cruz *et al.*, 2011; Chajec *et al*., 2012).

The amount of reserve material (*e.g.* lipids, proteins, saccharides) in the tardigrade midgut cells increases to enable the survival of the animal in case of, for example, starvation or any other external stress factor (Greven, 1976; Pirch and Greven, 1994; Rost-Roszkowska and Poprawa, 2008). In *Isohypsibius granulifer granulifer* Thulin, 1928 (Eutardigrada) numerous structures with reserve material accumulate gradually in the cytoplasm of the midgut digestive cells as oogenesis occurs (Rost-Roszkowska *et al.*, 2011a). The presence of reserve material in the cytoplasm of the digestive cells has also been described in the eutardigrade *Dactylobiotus dispar* (Murray, 1907) (Ząbczyk, 2000; Rost-Roszkowska and Poprawa, 2008). In case of *X. pseudohufelandi* differences in the amount of reserve material were not observed between midgut cells of males and females.

Multivesicular bodies are round or oval organelles which possess numerous internal spherical or ellipsoidal vesicles enclosed within a single outer membrane (von Bartheld and Altick, 2011). These organelles may be formed by small vesicles that originate from Golgi complexes or from endocytosis (Martin and Spicer, 1973; Piper and Katzmann, 2007; Büning *et al.*, 2008). In the midgut digestive cells of *X. pseudohufelandi* we observed the fusion of a coated vesicle with a multivesicular body, which might suggest that endocytosis participates in MVB formation. Also, no Golgi complexes were detected in the cytoplasm of digestive cells, which cannot participate in MVB formation, contrary to what is known in other taxa (Martin and Spicer, 1973; Büning *et al.*, 2008). The role of MVBs can be multiple; they may participate in enzyme accumulation (Martin and Spicer, 1973), degradation of membrane proteins (Piper and Katzmann, 2007), growth factor receptor down-regulation (Futter *et al*., 1996), developmental signalling (Lai *et al.*, 2001), and finally in protein sorting, recycling, transport and release (von Bartheld and Altick, 2011). Because MVBs in *X. pseudohufelandi* probably are formed with the participation of coated vesicles that originate from endocytosis, we may suppose that these structures take part in the accumulation of substances (*e.g.* water) that enter the midgut lumen with the food. Further studies are necessary to understand the nature of MVBs and if their presence is widespread among terrestrial tardigrade species.

CONCLUSIONS

Our study shows that: i) the midgut epithelium of *X. pseudohufelandi* is composed mostly of digestive cells, but

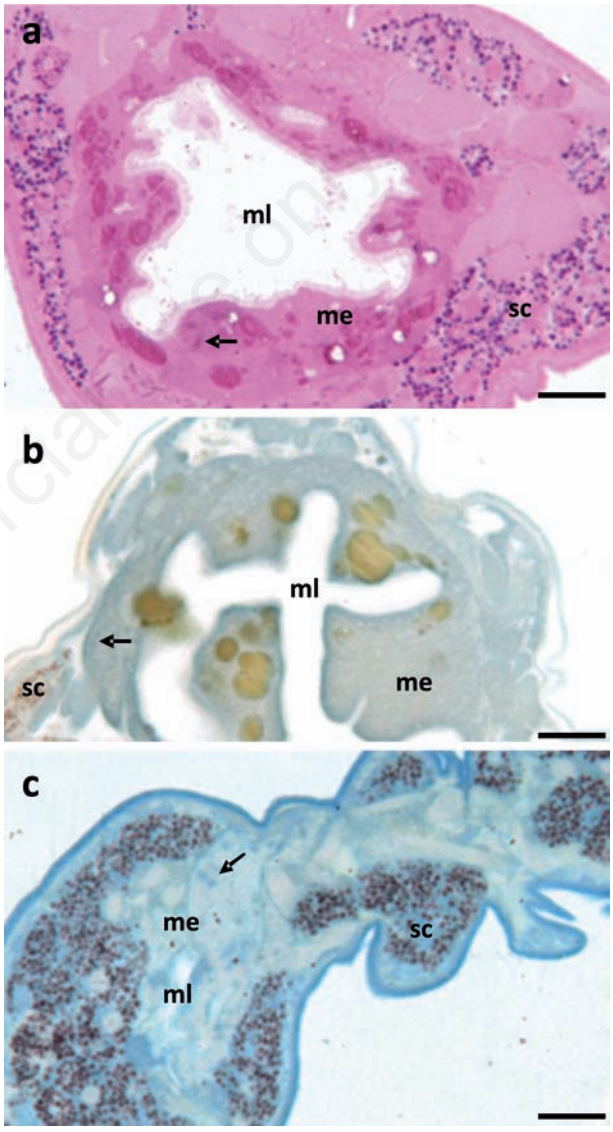

Fig. 3. Histochemical staining of *Xerobiotus pseudohufelandi*. a-c, image obtained with light microscopy (LM). a) Cross section through the body of a tardigrade. Arrow indicates Periodic acid-Schiff (PAS)-positive granules; b) cross section through the body of a tardigrade. Arrow indicates Sudan-positive granules; c) longitudinal section through the body of a tardigrade. Arrow indicates bromophenol blue (BPB)-positive granules. me=midgut epithelium; ml=midgut lumen; sc=storage cells. Scale bars of a)=4.5 μm; b)=3.1 μm; c)=5.1 μm.

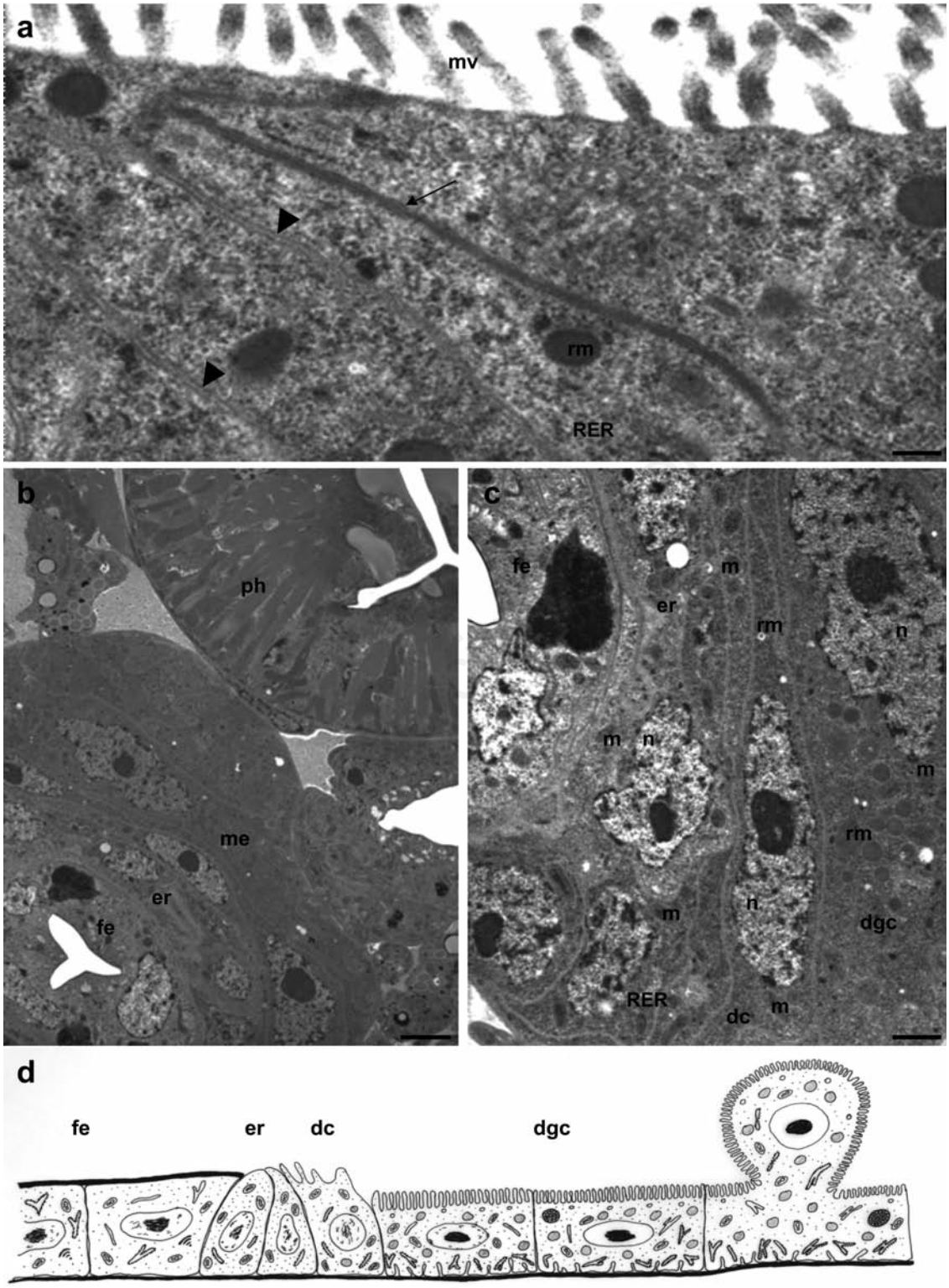

Fig. 4. Digestive system of *Xerobiotus pseudohufelandi.* a-c, image obtained with transmission electron microscopy (TEM). a) Digestive cells connected with smooth septate junction (arrow) and septate junction (arrowhead); b) *epithelial ring* between foregut (fe) and midgut (me); c) young differentiating digestive cell between *epithelial ring* (er) and digestive cell; d) a diagrammatic representation of the midgut epithelium. dc=young differentiating digestive cell; dgc=digestive cell; er=*epithelial ring*; m=mitochondria; me=midgut epithelium; mv=microvilli; n=nucleus; ph=pharynx; RER=rough endoplasmic reticulum; rm=reserve material. Scale bars of a)=0.2 μm; b)=2.0 μm; c)=0.8 μm.

crescent-like cells are also present; ii) no differences in the ultrastructure of the digestive and crescent-like cells have been observed between males and females with the exception of the amount of multivesicular bodies; iii) a large amount of multivesicular bodies occurs in female midgut digestive cells; iv) crescent-like cells placed between the foregut and midgut probably play a role as midgut stem cells, and v) they differentiate into digestive cells.

ACKNOWLEDGMENTS

We would like to express our gratitude to Dr. Łukasz Michalczyk (Jagiellonian University, Poland) for valuable remarks on the manuscript.

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