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Structure of the midgut epithelium in four diplopod species: histology, histochemistry and ultrastructure

Magdalena Maria Rost-Roszkowska[1], Jitka Vilimová[2], Karel Tajovský[3], Vladimir Šustr[3], Anna Ostróżka[1], Florentyna Kaszuba[1]

1 *University of Silesia in Katowice, Faculty of Natural Sciences, Institute of Biology, Biotechnology and Environmental Protection, Bankowa 9, 40-007 Katowice, Poland*

2 *Charles University, Faculty of Science, Department of Zoology, Vinicna 7, 128 44 Prague 2, Czech Republic*

3 *Institute of Soil Biology, Biology Centre of the Czech Academy of Sciences, Na Sádkách 7, 370 05 České Budějovice, Czech Republic [vilim@natur.cuni.cz, tajov@upb.cas.cz, sustr@upb.cas.cz]*

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Corresponding author: Magdalena Maria Rost-Roszkowska (magdalena.rost-roszkowska@us.edu.pl)

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Abstract

The middle region of the digestive system of millipedes, the midgut, is responsible for all processes connected with digestion, but also takes part in homeostasis maintenance thanks to the ability to activate many mechanisms which neutralize changes occurring at different levels of the animal's body. Numerous millipede species are treated as bioindicators of the natural environment and they are exposed to different stressors which originate from external environment. To obtain all data on the functioning of midgut of millipedes as the barrier against stressors, it is necessary to have a precise and general description of the midgut epithelium. Members from four millipede orders were selected for the studies: *Polydesmus angustus* (Polydesmida), *Epibolus pulchripes* (Spirobolida), *Unciger transsilvanicus* (Julida) and *Glomeris tetrasticha* (Glomerida). The structure and ultrastructure of their midgut epithelial cells (the digestive, secretory and regenerative cells) were documented using transmission electron microscopy and histochemical methods. The obtained results have been compared and discussed to previous ones, to present the general and structural organization of the midgut in Diplopoda. Our studies revealed that the ultrastructure of all cells which form the midgut epithelium in millipedes is general for all the species studied up to now and it resembles the cell ultrastructure observed in Chilopoda and Hexapoda, including the digestive, secretory and stem cells.

Key words

Diplopoda, digestive system, ultrastructure, midgut stem cells, reserve material.

1. Introduction

Millipedes, living as detritivores in various terrestrial environments, are characterized as "litter transformers" (Lavelle and Spain 2001; Loranger-Merciris et al. 2008). Living in the upper layers of soil, decaying bark of trees, litter and bedding, they take part in the fragmentation of plant litter, have effects on soil micro-

organisms and element cycling, increase nitrogen mineralization and influence soil aeration (Cárcamo et al. 2000; Snyder et al. 2009).

The digestive system has been well described in many species belonging to different groups of myriapods, mainly millipedes and centipedes. However, there are still gaps in knowledge related to this topic, e.g., participation of the intestine in detoxification processes or its adaptation to various types of food. The millipede digestive system that is responsible for secretion, absorption, synthesis and accumulation of reserve materials, is a straight tube that is differentiated into three distinct regions: the fore-, mid- and hindgut. However, its middle region – the midgut - also takes part in homeostasis maintenance thanks to the ability to activate many mechanisms which neutralize changes occurring at different levels of the animal's body (Köhler and Alberti 1992, Rost-Roszkowska et al. 2019) as has been reported for other arthropods such as Crustacea, Insecta or Arachnida (Malagoli et al. 2010, Wilczek et al. 2014, Włodarczyk et al. 2019). The midgut is lined with epithelium that lies on the basal lamina and is surrounded by visceral muscles and hepatic cells. Three types of cell have been described as forming the midgut epithelium: the digestive, secretory and regenerative cells. Their structure and ultrastructure have been studied in numerous millipede species belonging to e.g., Spirobolida or Spirostreptida (e.g. Nunez and Crawford 1977; Seifert and Rosenberg 1977; Fontanetti and Camargo-Mathias 1997; Fantazzini et al. 1998, 2002; Camargo-Mathias et al. 2004, 2011; Deshmukh and Deshmukh 2011; Fontanetti et al. 2001, 2015; Moreira-de-Sousa and Fontanetti 2012; Sosinka et al. 2014; Rost-Roszkowska et al. 2018; Šustr et al. 2020).

However, to obtain a precise and general description of the midgut epithelium of millipedes, further studies are required. Therefore, we selected for our studies the following species, which represent four millipede orders: *Polydesmus angustus* (Latreille, 1802) (Polydesmida), *Epibolus pulchripes* (Gerstäcker, 1873) (Spirobolida), *Unciger transsilvanicus* (Verhoeff, 1899) (Julida) and *Glomeris tetrasticha* (Latreille, 1802) (Glomerida). Additionally, they origin from different biogeographical regions and differ in their diets. They are easy to collect and to maintain in laboratory conditions. *P. angustus* is a middle-size West-European polydesmid species spreading to synanthropic habitats in Central Europe, such as gardens and greenhouses, living in leaf litter, in compost, and under rotten wood. *U. transsilvanicus* is a litter-dwelling species distributed in Central and Eastern Europe and the Balkan Peninsula, inhabiting mainly soils of forest habitats. *G. tetrasticha* is a Central European species preferably inhabiting moist forest and also grassland habitats, living in moist leaf litter and under fallen trees (Kocourek et al. 2017). *E. pulchripes* is a larger tropical species often naturally living in grasslands, drier coastal forests, but also in agricultural land in Kenya and Tanzania. It is a dietary specialist feeding on rotting wood and dead leaves (Sigling 2010). *E. pulchripes* is relatively often kept as a terrarium animal in Europe. The main and primary aim of this study was to conduct a comparative ultrastructural study on the midgut of various diplopods which originate from different biogeographical regions, differ in their diets, and have not been examined yet. However, the detailed goals were also to describe the chemical character of the reserve material and the presence of spherites characteristic for the millipede midgut, as well as to state whether regenerative cells can be identified as the midgut stem cells. Finally, we made a general description of digestive, secretory and regenerative cells in the millipede midgut.

2. Material and methods

2.1. Material

P. angustus, *U. transsilvanicus* and *G. tetrasticha* were collected in the Czech Republic, Central Bohemia, Prague, botanical garden of Charles University. They were obtained from natural, unpolluted environments and were reared in 20 × 15 × 6 cm glass boxes with humus substrate corresponding to the Central European deciduous mixed forest with a relative humidity of about 70% and a temperature of 22 °C. Individuals of each species were kept separately, but all of them had fragments of wet decaying bark of trees covered with algae and lichens together with a mixture of leaf litter as the source of food. They were fed *ad libitum*.

Adult specimens of *E. pulchripes* were obtained commercially from pet shops. The specimens were in good condition, actively moving, taking in food and burrowing in the organic substrate in the breeding containers. They were reared in 60 × 30 × 40 cm glass boxes with a relative humidity of about 70% and at room temperature. *E. pulchripes* were fed with fresh fruits (e.g., apple, pear, banana) and vegetables (e.g., tomato, champignon, cucumber). Pieces of cuttlebone (*Sepia officinalis*) were provided as a source of calcium. They were also fed *ad libitum*. All animals were prepared for sectioning immediately after acclimatization to laboratory conditions for 2 weeks.

2.2. Methods

Adult specimens of millipedes (five males and five females of *P angustus*, *U. transsilvanicus* and *G. tetrasticha*, and three males and three females of *E. pulchripes*) were anesthetized with chloroform and dissected. Their midguts were isolated and immediately fixed with 2.5% glutaraldehyde in 0.1 M PBS (sodium phosphate buffer) at pH 7.4, 4 °C for 2 h. After washing in PBS and postfixing in 2% osmium tetroxide in 0.1 M PBS (4 °C, 1.5 h), the material was dehydrated in a graded concentration series of ethanol (50, 70, 90, 95 and four times 100%, each for 15 min) and acetone (15 min). Then the material was embedded in epoxy resin (Epoxy Embedding Medium Kit; Sigma). Semi- and ultra-thin sections were prepared using a Leica Ultracut UCT25 ultramicrotome. Some of the semithin sections (0.8 μm thick) were stained with 1% methylene blue in 0.5% borax and observed using

an Olympus BX60 light microscope. Ultra-thin sections (70 nm) were stained with uranyl acetate and lead citrate and examined using a Hitachi H500 transmission electron microscope.

The other semi-thin sections that were not stained with 1% methylene blue in 0.5% borax were used for the histochemical analyses: PAS method (detection of glycogen and polysaccharides), Bonhag method (detection of proteins), Sudan Black B staining (detection of lipids) (Litwin 1985). All slides were analyzed using an Olympus BX60 light microscope.

3. Results

We did not observe any differences between females and males in the structure or ultrastructure of the midgut epithelial cells. Therefore, the following description considers both sexes. The midgut of four millipede species is lined with the pseudostratified epithelium which rests on the basal lamina (Figs 1A–G). It is surrounded contraluminally by circular and longitudinal muscles as well as hepatic cells. Three types of epithelial cells were distinguished in the midgut epithelium: the digestive cells, regenerative cells and secretory cells.

3.1. Ultrastructure of the digestive cells

The cytoplasm of digestive cells shows distinct regionalization in organelle distribution, so cytoplasmic regions could be distinguished: the apical, perinuclear and basal.

In *Polydesmus angustus*, *Epibolus pulchripes*, *Unciger transsilvanicus* and *Glomeris tetrasticha* the apical cytoplasm of digestive cells (Figs 2A–F) is rich in mitochondria, cisterns of the rough endoplasmic reticulum, and spheres of the reserve material. In *G. tetrasticha* glycogen granules were also detected (Fig. 2F). The apical cell membrane forms microvilli which protrude into the midgut lumen. The distinct cortical layer of the cytoplasm has been distinguished just beneath the apical cell membrane. The cortical layer is poor in organelles; only a few mitochondria could be observed. However, it has roots of bundled microfilaments entering the cytoplasm of the microvilli (Figs 2A–F). In *E. pulchripes* and *U. transsilvanicus* endosomes which suggest endocytosis were observed – the apical cell membrane forms invaginations covered by an electron dense layer (Figs 2A–C, 2E). Numerous spherites which are membranous structures with concentric layers of electron-dense material inside were visible in the apical cytoplasm just beneath the cortical layer. Spheres with different electron densities were also observed in this part of the cytoplasm (Figs 2A, 2C). Numerous spherites were also detected in the apical cytoplasm of *U. transsilvanicus* (Fig. 2E) and *P. angustus* digestive cells (Fig. 2D).

In the perinuclear cytoplasm of all millipedes examined here, numerous cisterns of the rough endoplasmic reticulum and electron-dense spheres surround the oval nucleus (Figs 3A–C). In *E. pulchripes* numerous spherites occur (Fig. 3B). In *U. transsilvanicus* perinuclear cytoplasm is also rich in Golgi complexes, single mitochondria and electron-dense structures which are probably residual bodies (Fig. 3C).

The basal cytoplasm is devoid of spheres in *P. angustus*, while in *E. pulchripes* it has spherites and a few spheres with the reserve material (Fig. 4A). In *U. transsilvanicus* single spheres of reserve material with medium electron density were detected (Fig. 4B). The basal cell membrane having contact with the basal lamina, forms numerous folds which are accompanied by the accumulation of mitochondria and cisterns of the rough endoplasmic reticulum (Figs 4A–B). In *G. tetrasticha* vacuoles could be observed.

Smooth septate junctions were observed between adjacent digestive cells in their apical region (Figs 2 D–F, 5F–G), while the septate junctions and gap junctions occur in the perinuclear and basal regions. Numerous autophagic structures were observed (autophagosomes, autolysosomes, residual bodies) in the cytoplasm of the digestive cells in all of the four millipede species examined here (Figs 2 E–F, 3A, 3C). The holocrine secretion (Fig. 2D) and the microapocrine secretion (not shown) were detected in *P. angustus*, while in *G. tetrasticha* and *U. transsilvanicus* we managed to observe only the microapocrine secretion, where only small evaginations of the microvillar ends appeared (Fig. 2F). We presented types of secretion in *E. pulchripes* in our previous paper (Šustr et al. 2020)

3.2. Histochemistry

In *P. angustus* the histochemical methods showed the presence of polysaccharides (positive PAS staining; Fig. 1B), small amounts of proteins (positive Bonhag staining; Fig. 1C) and lipids (positive Sudan B staining; Fig. 1D). In *E. pulchripes* polysaccharides (Fig. 1E) and lipids (Fig. 1G) were gathered as the reserve material in the cytoplasm of digestive cells, while proteins were not detected (Fig. 1F). In *U. transsilvanicus* and *G. tetrasticha* lipids, polysaccharides and proteins were accumulated (not shown).

3.3. Regenerative cells

In three millipede species (*P. angustus*, *E. pulchripes*, *U. transsilvanicus*) regenerative cells resting on the basal lamina are isolated from each other by the basal regions of the digestive cells along the entire length of the midgut (Figs 4A–B, 5A–B). They do not reach the midgut lumen (Fig. 1A). Their nucleus possesses electron-dense patches of heterochromatin, and the entire cytoplasm is rich in mitochondria (Figs 4A–B, 5B) and some cisterns of the rough endoplasmic reticulum. Unlike the species mentioned above, in *G. tetrasticha* the regenerative cells form regenerative nests (regenerative groups) (Figs 5C–E). The central part of the regenera-

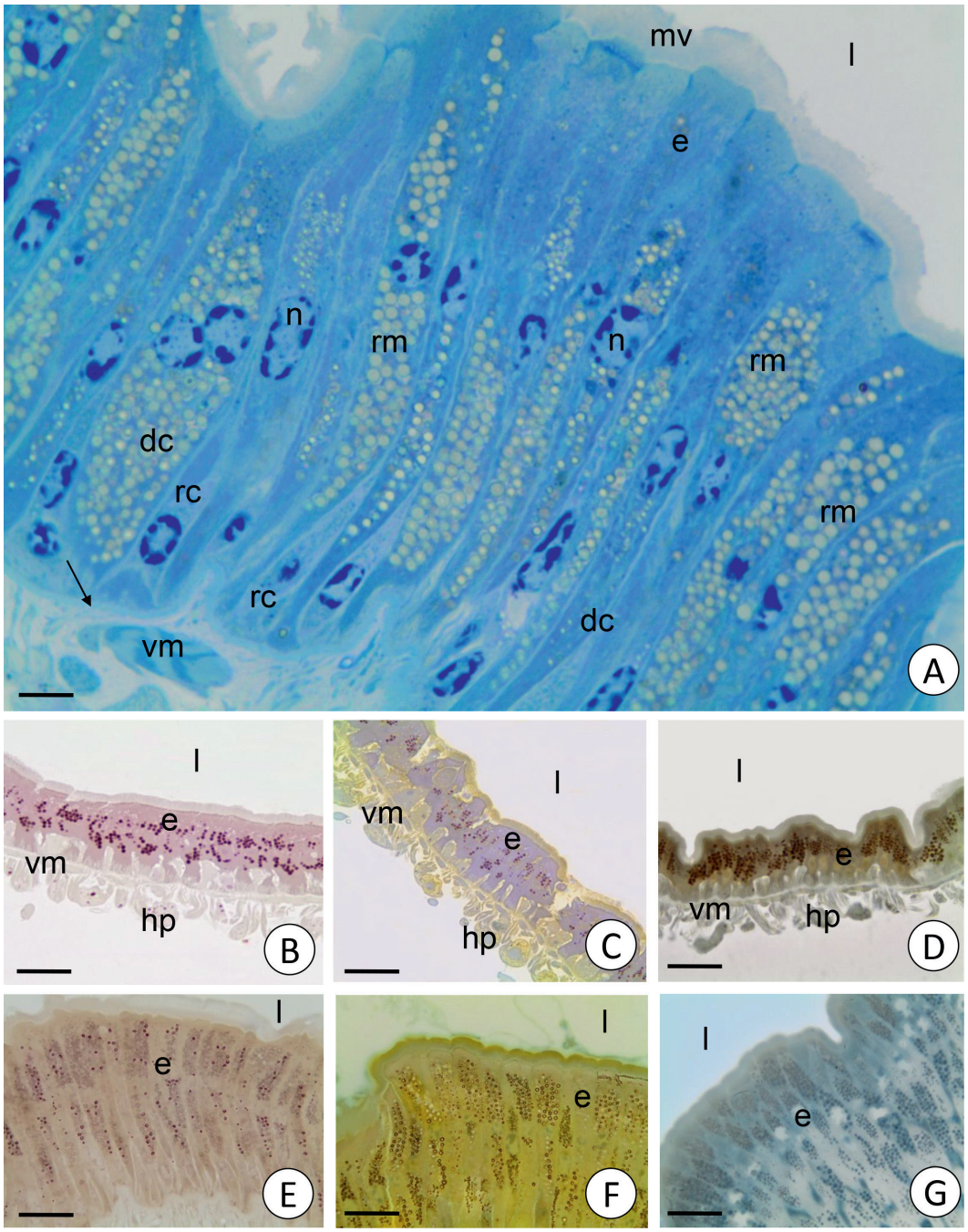

Figure 1. A. *E. pulchripes*. A pseudostratified epithelium (e) of the midgut resting on the basal lamina (arrow). Digestive cells (dc), regenerative cells (rc), midgut lumen (l), visceral muscles (vm), microvilli (mv), nuclei (n), reserve material (rm). Light microscopy. Scale bar = 5 µm. **B–G.** Histochemical staining of the midgut epithelium of millipedes. Midgut epithelium (e), midgut lumen (l), visceral muscles (vm), hepatic cells (hp). **B.** *P. angustus*. PAS. Light microscopy. Scale bar = 16 µm. **C.** *P. angustus*. Bonhag method. Light microscopy. Scale bar = 16 µm. **D.** *P. angustus*. Sudan Black B. Light microscopy. Scale bar = 18 µm. **E.** *E. pulchripes*. PAS. Light microscopy. Scale bar = 18 µm. **F.** *E. pulchripes*. Bonhag method. Light microscopy. Scale bar = 18 µm. **G.** *E. pulchripes*. Sudan Black B. Light microscopy. Scale bar = 12 µm.

tive nest is formed by regenerative cells with the cytoplasm poor in organelles (Fig. 5C, 5E). Mitotic divisions were observed (Fig. 5D). The cells of the regenerative nests located closest to the intestinal lumen start to differentiate into digestive cells (Fig. 5F). The process of differentiation is similar in all species investigated here. The cells elongate and protrude between the major digestive cells (Figs 1A, 2C, 5F). A distinct arrangement of the organelles in the cytoplasm is observed: the mitochondria accumulate distally from the nucleus, which elongates. The apical cell membrane forms microvilli extending into the extracellular space. Smooth septate junctions among the differentiating and adjacent digestive cells are formed (Figs 2C, 5F). Eventually, the differentiating cell reaches the midgut epithelium and new digestive cells are formed.

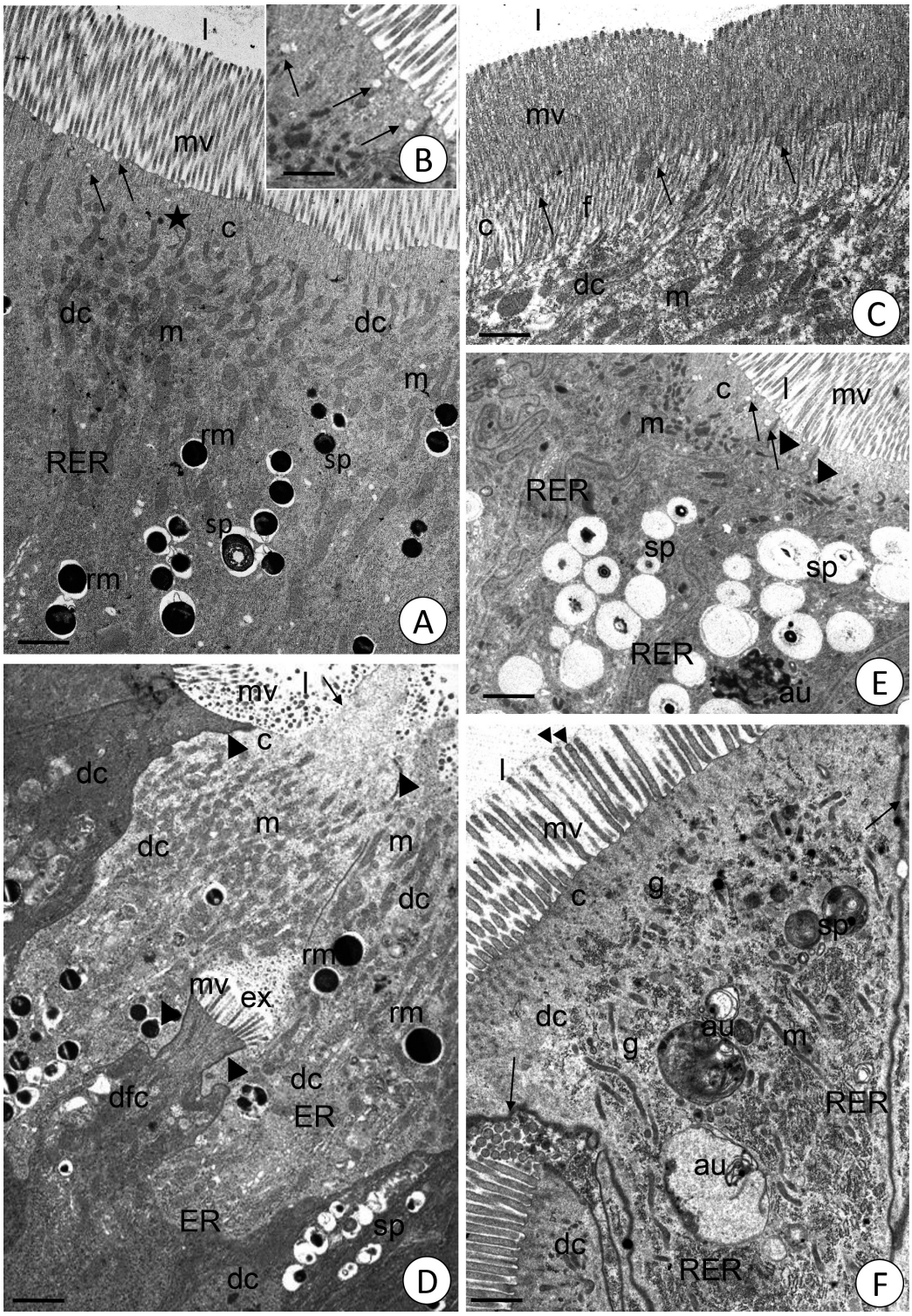

Figure 2. Apical cytoplasm of several digestive cells (dc) in millipede midgut. **A.** *E. pulchripes*. Microvilli (mv), midgut lumen (l), cortical layer (c), cisterns of the rough endoplasmic reticulum (RER), filaments in cortical layer (star), mitochondria (m), spheres of reserve material (rm), spherites (sp), endosomes (arrows). Longitudinal section. TEM. Scale bar = 3.5 µm. **B.** *U. transsilvanicus*. Higher magnification of Fig. E. Endosomes (arrows). TEM. Scale bar = 0.8 µm. **C.** *E. pulchripes*. Cortical layer (c) in the digestive cells within apical cytoplasm with distinct roots of filaments (f). Midgut lumen (l), microvilli (mv), mitochondria (m), endosomes (arrows). Longitudinal section. TEM. Scale bar = 1.8 µm. **D.** *P. angustus*. Microvilli (mv), midgut lumen (l), cortical layer (c), smooth septate junctions (arrowheads), cisterns of the endoplasmic reticulum (ER), mitochondria (m), spheres of reserve material (rm), spherites (sp), differentiating cell (dfc) with microvilli (mv) protruding the extracellular space (star), holocrine secretion (arrow), digestive cells (dc). Longitudinal section. TEM. Scale bar = 1.2 µm. **E.** *U. transsilvanicus*. Microvilli (mv), midgut lumen (l), cortical layer (c), endosomes (arrows), smooth septate junctions (arrowheads), mitochondria (m), spherites (sp), autophagosomes (au), cisterns of the rough endoplasmic reticulum (RER). Transverse section. TEM. Scale bar = 1.7 µm. **F.** *G. tetrasticha*. Microvilli (mv), midgut lumen (l), cortical layer (c), smooth septate junctions (arrows), accumulation of glycogen granules (g), mitochondria (m), spherites (sp), cisterns of the rough endoplasmic reticulum (RER), microapocrine secretion (arrowheads), digestive cells (dc). Longitudinal section. TEM. Scale bar = 1.3 µm.

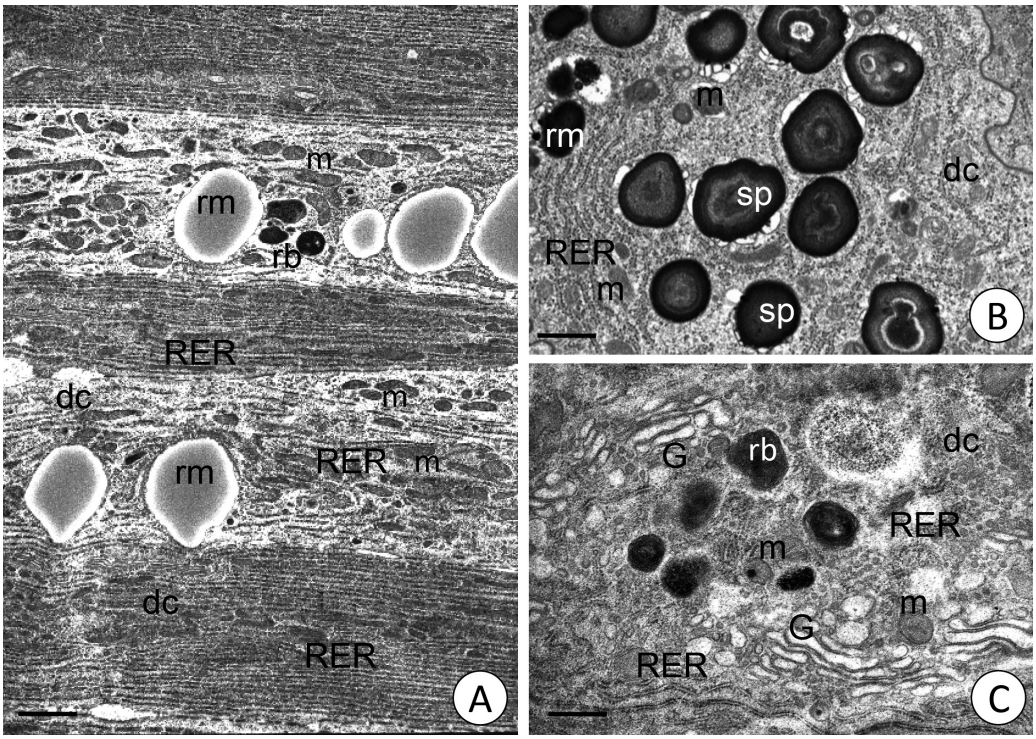

Figure 3. Perinuclear cytoplasm of digestive cells (dc) in millipedes. **A.** *U. transsilvanicus*. Cisterns of the rough endoplasmic reticulum (RER), electron-dense residual bodies (rb), mitochondria (m), reserve material (rm). Transverse section. TEM. Scale bar = 0.5 µm. **B.** *E. pulchripes*. Cisterns of the rough endoplasmic reticulum (RER), mitochondria (m), spherites (sp), reserve material (rm). Transverse section. TEM. Scale bar = 1.5 µm. **C.** *U. transsilvanicus*. Cisterns of the rough endoplasmic reticulum (RER) and Golgi complexes stacks (G), mitochondria (m) and electron-dense residual bodies (rb). TEM. Transverse section. Scale bar = 0.3 µm.

3.4. Secretory cells

In all species examined, the secretory cells resting on the non-cellular basal lamina are scarce and stand contraluminally, isolated between the basal regions of digestive cells. However, no intercellular junctions between the secretory and digestive cells were detected. They do not contact the midgut lumen; therefore, they are of the closed type (Fig. 6A). The oval nucleus is located in the central part of the cytoplasm, which is rich in small electron-dense granules (Figs 6A–B). Some mitochondria and cisterns of the rough endoplasmic reticulum were present.

4. Discussion

4.1. General ultrastructure and functions of the digestive cells

The midgut epithelium of millipedes has been described as pseudostratified, e.g. in *Floridobolus penneri* (Spirobolida), *Narceus gordanus* (Spirobolida), *Telodeinopus aoutii* (Spirostreptida), *Rhinocricus padbergi* (Spirobolida), *Archispirostreptus gigas* (Spirostreptida) and *Julus scandinavius* (Julida) (Bowen 1968; Camargo-Mathias et al. 2004; Sosinka et al. 2014; Rost-Roszkowska et al. 2018) and in *P. angustus* (Polydesmida), *U. transsilvanicus* (Julida), *E. pulchripes* (Spirobolida), and *G. tetrasticha* (Glomerida). However, in the majority of millipedes the simple prismatic or columnar epithelium lines the midgut (see Fontanetti et al. 2015). The similar type of epithelium in centipedes has been described previously (Kaufman 1961a, 1961b, 1962, Chajec et al. 2012, 2014). The principal cells which form the midgut epithelium are the digestive cells, the cytoplasm of which shows distinct regionalization in organelle distribution; thus the apical (with the cortical layer), perinuclear and basal regions could be distinguished (Nunez and Crawford 1977; Camargo-Mathias et al. 2004; Fonanetti et al. 2006, 2015; Sosinka et al. 2014; Moreira-de-Sousa et al. 2017; Rost-Roszkowska et al. 2018). The apical cytoplasm is characterized by microvilli formed by the apical cell membrane and internal actin filaments. The filaments enter the cytoplasm of the cell from the microvilli, forming the cortical layer. The presence of microvilli and numerous granules accumulated near the apical cell membrane suggests the secretory and absorptive functions of digestive cells. Different types of secretion have been described: apocrine, microapocrine, merocrine and holocrine secretion (Fontanetti & Camargo-Mathias 1997; Fontanetti et al. 2001; Camargo-Mathias et al. 2004; Sosinka et al. 2014; Rost-Roszkowska et al. 2018; Šustr et al. 2020). In *E. pulchripes* merocrine secretion occurred: small vesicles released their electron-lucent content into the midgut lumen (Šustr et al. 2020). Apocrine, microapocrine, and merocrine types of secretion were

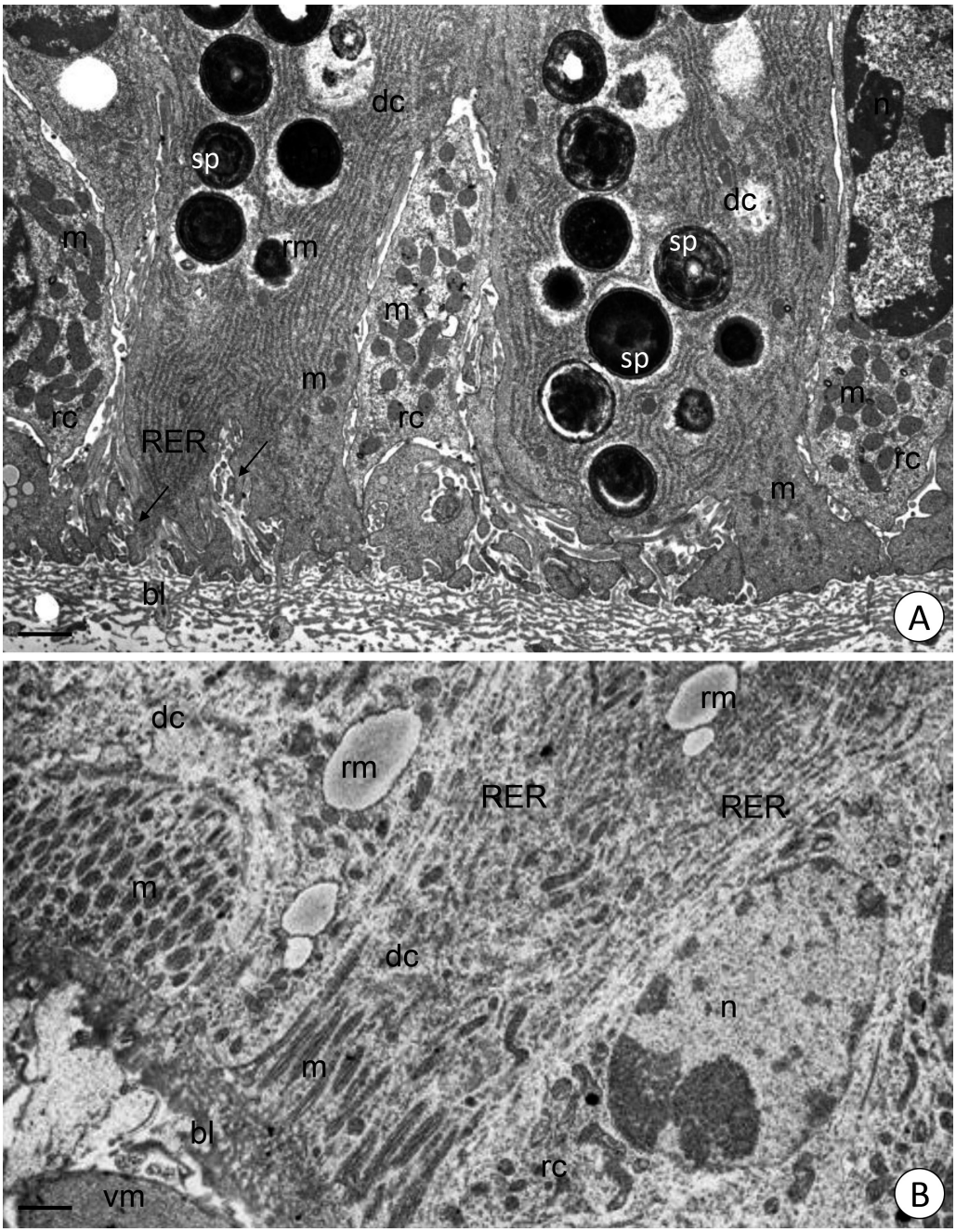

Figure 4. Basal cytoplasm of several digestive cells (dc) in millipedes. **A.** *E. pulchripes*. The cytoplasm is rich in mitochondria (m) and cisterns of the rough endoplasmic reticulum (RER) in the neighborhood of basal cell membrane folds (arrows). Basal lamina (bl), spherites (sp), spheres with reserve material (rm), fragments of regenerative cells (rc). Transverse section. TEM. Scale bar = 1.4 µm. **B.** *U. transsilvanicus*. Regenerative cells (rc), basal lamina (bl), cisterns of the rough endoplasmic reticulum (RER), nucleus (n), visceral muscles (vm), reserve material (rm), digestive cells (dc), mitochondria (m). Transverse section. TEM. Scale bar = 0.6 µm.

detected in Spirostreptida (Fontanetti and Camargo-Mathias 1997; Sosinka et al. 2014; Rost-Roszkowska et al. 2018a, Šustr et al. 2020), apocrine and merocrine secretion in Spirobolida (Fantazzini et al. 2002; Šustr et al. 2020), microapocrine or apocrine secretion in Julida (Hefner 1929; Rost-Roszkowska et al. 2018a), and merocrine and apocrine secretion in Polyxenida. The results of our research confirm the types of secretion for Julida and Spirobolida and indicate the participation of microapocrine and holocrine secretion in Polydesmida and the microapocrine secretion of Glomerida.

Secretory granules, numerous cisterns of the rough endoplasmic reticulum, and autolysosomes connected with digestion indicate the involvement of digestive cells in extra- and intra-cellular digestion in myriapods (Chajec et al. 2012, 2014, Sosinka et al. 2014, Rost-Roszkowska et al. 2018a, Šustr et al. 2020) or in other arthropods (Azevedo et al. 2009; Caccia et al. 2019). The nucleus is accompanied by cisterns of the rough and smooth endoplasmic reticulum or even Golgi complexes, thus suggesting the intensive processes of synthesis. The substances produced may either be accumulated in digestive

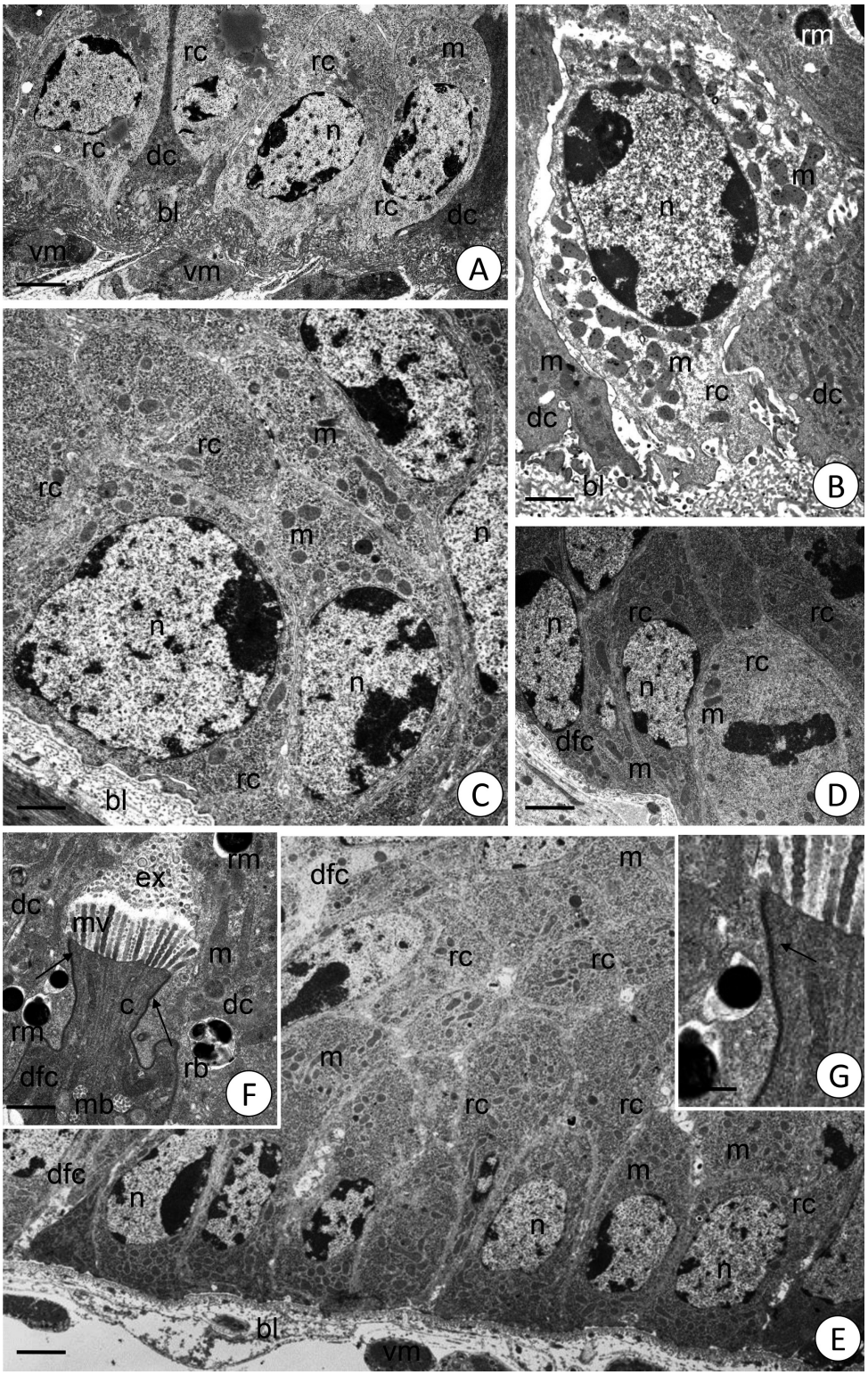

Figure 5. Midgut epithelia in various millipedes. **A.** *P. angustus*, transverse section. Regenerative cells (rc) among basal regions of digestive cells (dc). Nucleus (n), clusters of mitochondria (m), basal lamina (bl), visceral muscles (vm). TEM. Scale bar = 1.8 µm. **B.** *E. pulchripes*, longitudinal section. Regenerative cells (rc) among basal regions of digestive cells (dc). Nucleus (n), basal lamina (bl), clusters of mitochondria (m), reserve material (rm). TEM. Scale bar = 1 µm. **C.** *G. tetrasticha*, longitudinal section. Nest of regenerative cells (rc) in the midgut epithelium, note the cytoplasm poorly supplied with organelles. Basal lamina (bl), nucleus (n), clusters of mitochondria (m). TEM. Scale bar = 0.8 µm. **D.** *G. tetrasticha*, transverse section. Detail of a regenerative nest with dividing regenerative cells (rc) and differentiating cells (dfc), nucleus (n), mitochondria (m). TEM. Scale bar = 1.6 µm. **E.** *G. tetrasticha*. Nest of regenerative cells (rc) in the midgut epithelium displaying cytoplasm with numerous mitochondria (m). Basal lamina (bl), visceral muscles (vm), nucleus (n), differentiating cells (dfc). Transverse section. TEM. Scale bar = 1.6 µm. **F.** *P. angustus*, transverse section. A differentiating cell (dfc) with microvilli (mv) entering the extracellular space (ex). Multivesicular bodies (mb), mitochondria (m), filaments in the cortical layer (c), electron dense spheres of the reserve material (rm), residual bodies (rb), smooth septate junctions (arrows). TEM. Scale bar = 0.8 µm. **G.** Higher magnification of Fig. F. Smooth septate junction (arrow). TEM. Scale bar = 0.3 µm.

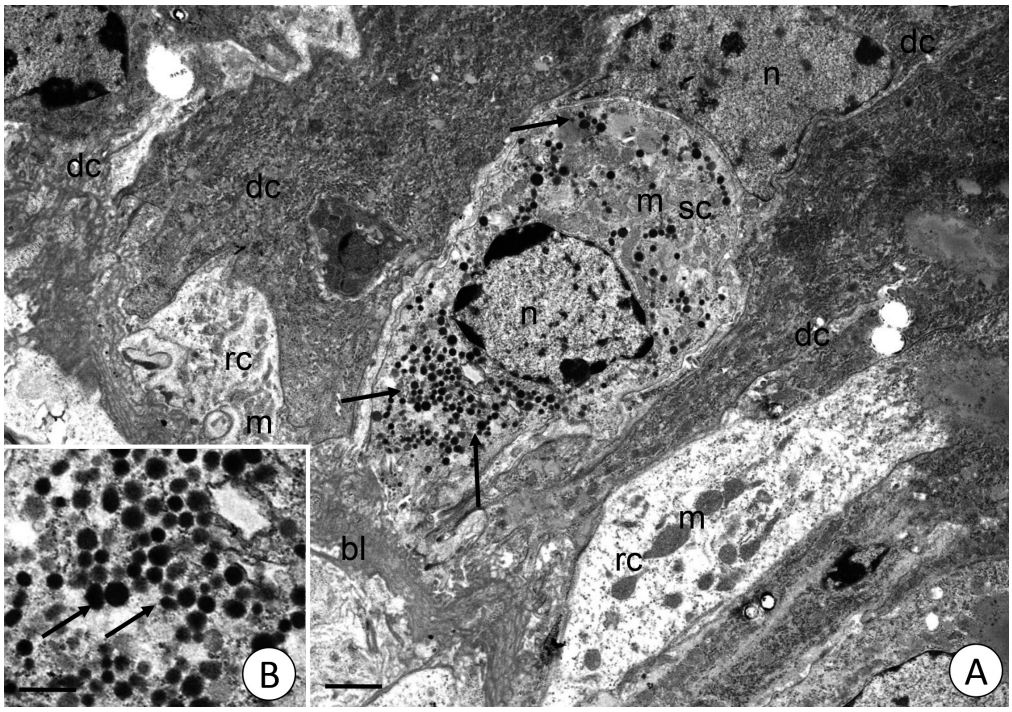

Figure 6. Secretory cell (sc) in the midgut epithelium of *P. angustus.* **A.** Localization of secretory cell (sc) in the epithelium among basal regions of digestive cells (dc). Fragments of regenerative cells (rc), mitochondria (m), nuclei (n), electron dense granules (arrows), basal lamina (bl). Longitudinal section. TEM. Scale bar = 1.2 μm. **B.** A fragment of Fig. A with the electron dense granules (arrows). TEM. Scale bar = 0.3 μm.

cells as reserve material or may be secreted into the midgut lumen. The basal cytoplasm has well-developed infoldings surrounded by mitochondria and cisterns of the rough endoplasmic reticulum. They suggest the active transport of water and ions across the cell membrane, as has been suggested for insects (Billingsley and Lehane 1996; Caccia et al. 2019).

The cytoplasm of the digestive cells of millipedes possesses spheres with reserve material (polysaccharides, glycolipids, proteins, proteoglycans) and/or lipid droplets, and the type of food consumed may have an effect on the chemical character of the material accumulated in the cytoplasm (Hopkin and Read 1992; Fantazzini et al. 2002; Deshmukh and Deshmukh 2011; Sosinka et al. 2014; Fontanetti et al. 2015; Rost-Roszkowska et al. 2018a). Therefore, in typical herbivorous millipedes which feed on material rich in polysaccharides (e.g., fruit, vegetables), polysaccharides, glycolipids, and lipids accumulate; in herbivorous species which feed on plant material rich in proteins, a huge amount of proteins is gathered together with small amounts of lipids and/or polysaccharides. However, in detritivorous species (both herbivorous and saprophagous), mainly lipids, polysaccharides or even reserve material of a mixed type (e.g. glycolipids) accumulates, while proteins are not stored there or their amount is very low (Sosinka et al. 2014; Rost-Roszkowska et al. 2018a). Polysaccharides, glycolipids and lipids have been detected in the digestive cells of detritivorous millipedes: *T. aoutii*, *J. scandinavius*, and *A. gigas*. However, in the midgut epithelium of *P. lagurus*, which feeds on plant material such as algae or lichens, together with above-mentioned reserve material, proteins are also gathered (Sosinka et al. 2014, Rost-Roszkowska et al. 2018a). Algae and lichens are treated as a good source of proteins and polysaccharide (Ullah et al. 2019, Rando and Rene 2020). *P. angustus*, *G. tetrasticha* and *U. transsilvanicus* are litter and humus inhabitants which feed on decomposing litter and decaying rotting wood materials. *E. pulchripes* feeds on leaf litter and rotting wood (Sigling 2010), but it can also be fed with fruit and vegetables (e.g., cucumbers, mushrooms) (own observation), which are a good source of polysaccharides (Ullah et al. 2019). Our study revealed that in *E. pulchripes* only polysaccharides and lipids could be accumulated, while proteins were not observed. However, in our previous paper we suggested the accumulation of a small amount of proteins in the cytoplasm of the midgut epithelium of this species, which must have been connected with mainly detritivorous materials in their diet (Šustr et al. 2020). Adding fresh fruit and vegetables probably caused the accumulation of mainly polysaccharides in *E. pulchripes*. *P. angustus* is a detritivorous millipede and has been observed to occasionally consume remnants of animal origins, probably cadavers of small invertebrates (e.g. springtails, dipteran larvae) (Šustr et al. 2013, own observation). Therefore, we can conclude that small amounts of proteins would probably originate from digested animal material (Rumpold and Schlüter 2013, Van der Spiegel et al. 2013, Rando and Rene 2020). *U. transsilvanicus* and *G. tetrasticha* consume only dead materials of plant origin; they use as food resources decomposing leaf litter and rotting wood (Tajovský et al. 1992; David and Gillon 2002; own observation). It is worth mentioning that the material synthesized in the cytoplasm of digestive cells is

transferred to the hepatic cells which surround the midgut epithelium (Hubert 1988; Godoy and Fontanetti 2010; Nogarol and Fontanetti 2011; Nardi et al. 2016; Rost-Roszkowska et al. 2018b). Monosaccharides and polysaccharides are transported to the hepatic cells, where they are accumulated as glycogen granules, which explains the fact that glycogen has not been detected in the cytoplasm of digestive cells (Hubert 1978; Bozzatto and Fontanetti 2012; Rost-Roszkowska et al. 2018b). The reserve material, mainly lipids and glycolipids, accumulated in the cytoplasm of the digestive cells or even in hepatic cells, could be utilized due to autophagy, the pathway of lipid metabolism through the lysosomal degradative pathway of autophagy (Liu and Czaja 2013). It is enclosed inside autophagosomes and digested in autolysosomes. Thanks to this, the energy which originates from reserve material digestion is supplied (Fontanetti et al. 2015; Rost-Roszkowska et al. 2018b, 2019). Autophagy as a pro-survival process would also protect the digestive cells against the accumulation of toxic substances including heavy metals (e.g., cadmium) (Franzetti et al. 2012, 2016; Włodarczyk et al. 2019). Autophagy has been described as a survival process commonly observed in the midgut epithelium in millipedes. It protects cells against infection by pathogens and enables neutralization of degenerated or disrupted organelles and toxic substances (e.g., heavy metals) or digestion of the reserve material as a source of energy. It may occur in all epithelial cells: digestive, secretory, and regenerative cells. However, a distinct crosstalk between autophagy and apoptosis was observed in the digestive cells, while it was not detected in the secretory and regenerative cells (Rost-Roszkowska et al. 2019).

Spherites, which are membranous structures with concentric layers of electron-dense material inside, have been described in the cytoplasm of digestive cells in millipedes (Hubert 1979; Hopkin and Read 1992; Fantazzini et al. 2002; Fontanetti et al. 2006, 2015; Godoy and Fontanetti 2010; Nogarol and Fontanetti 2010, 2011; Sosinka et al. 2014; Rost-Roszkowska et al. 2018a, 2019; this study) and centipedes (Chajec et al. 2012, 2014). They resemble A and B granules described for insects (Hopkin 1989), where zinc, lead, cadmium and/or copper can be stored. The accumulation of spherites that contain all of the organic and inorganic chemical compounds is connected with feeding on decaying leaves, feces and organic matter mixed with soil (Hubert 1979; Köhler and Alberti 1992; Köhler et al. 1995; Köhler 2002; Nogarol and Fontanetti 2010; Godoy and Fontanetti 2010) or even calcium (Hubert 1988; Hopkin and Read 1992; Fantazzini et al. 2002; Fontanetti et al. 2006; Moreira-de-Sousa et al. 2017). These structures may be utilized in the digestive cells of the millipede midgut inside autophagosomes (Rost-Roszkowska et al. 2019). Therefore, we can conclude that the general structure and ultrastructure of the digestive cells in the millipede midgut epithelium resemble the ultrastructure of the digestive cells in hexapod invertebrates (Billingsley and Lehane 1996; Azevedo et al. 2009; Rost-Roszkowska et al. 2010a,b,c; Caccia et al. 2019) and centipedes (Chilopoda) (Chajec et al. 2012, 2014), showing their role in digestion and detoxification.

4.2. Secretory cells

Specific secretory cells with the cytoplasm abundant in granules which differ in size and electron density have been detected in the millipede midgut (Camargo-Mathias et al. 2004; Godoy and Fontanetti 2010; Sosinka et al. 2014; Rost-Roszkowska et al. 2018a, 2019). The presence of numerous granules of different electron densities, some mitochondria, cisterns of the endoplasmic reticulum, and glycogen granules suggests that the secretory cells in the millipede midgut resemble the ultrastructure of the closed secretory cells of centipedes (Chajec et al. 2012, 2014) or closed endocrine cells in the insect midgut (Neves et al. 2003; Rocha et al. 2014; Caccia et al. 2019). While two types of secretory cells have been described in insects – a closed type which does not come into contact with the midgut lumen, and open type cells with their apical cell membrane attaching the gut lumen (Neves et al. 2003, Rocha et al. 2014) – only the closed secretory cells have been described in millipedes (see Fontanetti et al. 2015). Secretory cells were described in the midgut epithelium of Spirobolida (Bowen 1986) and Spirostreptida (Nunez and Crawford 1977; Rost-Roszkowska et al. 2018a). While the cells described here have endocrine character in insects (Bonelli et al. 2019), there are no data on their endocrine functions in centipedes (Chajec et al. 2012, 2014) or millipedes (Fontanetti et al. 2015). Their secretory nature is evidenced by the presence of numerous electron-dense granules, as has been described for some millipede species (Camargo-Mathias et al. 2004; Godoy and Fontanetti 2010; Sosinka et al. 2014; Rost-Roszkowska et al. 2018a, 2019). However, further research into the nature of these cells is necessary.

4.3. Regenerative cells as midgut stem cells

The regenerative cells (generative cells) have been described in the midgut epithelium of invertebrates, and the majority of data refer to arthropods. They are dispersed within basal regions of digestive and secretory cells or may occur as one of two types of regenerative cell aggregation: regenerative crypts or regenerative nests (Martins et al. 2006; Hakim et al. 2007, 2010; Nardi et al. 2010; Rost-Roszkowska et al. 2010 a,b,c; Mehrabadi et al. 2012; Franzetti et al. 2016; Bonelli et al. 2019; Bruno et al. 2019; Caccia et al. 2019; Tettamanti et al. 2019). The regenerative cells in myriapods have been described as forming regenerative crypts (Kaufman 1960, 1961) or as cells that are individually located between basal regions of the digestive and secretory cells along the entire length of the midgut (Köhler and Alberti 1992; Fontanetti et al. 2001, 2015; Fantazzini et al. 2002; Camargo-Mathias et al. 2004; de Godoy and Fontanetti 2010; Nogarol and Fontanetti 2011; Chajec et al. 2012, 2014; Sosinka et al. 2014; Fontanetti et al. 2015, Rost-Roszkowska et al. 2018a, 2019). Our research on three millipede species

(*P. angustus*, *E. pulchripes*, *U. transsilvanicus*) confirms the general description of the structure and localization of the regenerative cells in millipedes with regenerative cells singly distributed among digestive and secretory cells. However, in *G. tetrasticha* the regenerative cells occur in clusters and may form regenerative nests. Cells which can divide mitotically occupy the central part of each regenerative nest, while cells which differentiate into digestive cells are located the most externally of the regenerative nests. Such structure of the regenerative nest resembles that characteristic for wingless Hexapoda, e.g. Zygentoma and Archaeognatha (Rost-Roszkowska et al. 2007, 2010 a,b,c), while up to now it has not been described for millipede species. Regenerative cells in the midgut epithelium of Hexapoda species possess cytoplasm poor in organelles, with groups of mitochondria accumulated above the nucleus. It is suggested that they supply energy for cell differentiation when the regionalization in the organelle distribution characteristic for epithelial cells appears gradually. Proliferation and differentiation processes occur in regenerative groups more intensively and continuously than in the case of individually distributed cells (Martins et al. 2006; Rost-Roszkowska et al. 2007, 2010 a,b,c; Caccia et al. 2019). As has been described in arthropods, the regenerative cells will take over specific functions to regenerate/self-repair epithelium damaged by any type of cell death (autophagy, apoptosis and/or necrosis) (Bonelli et al. 2019; Franzetti et al. 2016; Bruno et al. 2019; Caccia et al. 2019; Tettamanti et al. 2019). In the millipede, the regenerative cells have also been described as cells that could replace damaged parts in the midgut epithelium (Godoy and Fontanetti 2010; Souza and Fontanetti 2011; Bozzatto and Fontanetti 2012; Christofoletti et al. 2012; Fontanetti et al. 2015; Rost-Roszkowska et al. 2018a, 2019). Because of the fact that midgut regenerative cells of arthropods, e.g. insects (Martins et al. 2006; Hakim et al. 2007, 2010; Rost-Roszkowska et al. 2007, 2010 a,b,c; Franzetti et al. 2016; Bruno et al. 2019; Caccia et al. 2019; Tettamanti et al. 2019), crustaceans (Sonakowska et al. 2015), and myriapods (Nardi et al. 2010; Chajec et al. 2012, 2014; Sosinka et al. 2014), are able to remain in a quiescent state, divide mitotically or differentiate into other epithelial cells, taking on their characteristic features, they are considered as midgut stem cells. Despite the fact that three types of cells have been described in the midgut epithelium of millipedes – digestive, regenerative and secretory cells – the regenerative cells were found to only differentiate into digestive cells in myriapods. The formation of secretory cells has not been described so far in (Sosinka et al. 2014; Fontanetti et al. 2015; Rost-Roszkowska et al. 2018a). However, it is also suggested that secretory cells might present one of the functional stages of digestive cells' activity (Fontanetti et al. 2015). Therefore, we can assume that in all the millipede species the regenerative cells, regardless of their location in the midgut epithelium, play the role of unipotent stem cells (Sosinka et al. 2014; Rost-Roszkowska et al. 2018a).

5. Conclusions

Our study concerning the ultrastructure of the midgut epithelium in millipedes helped us to elucidate its general structure. Therefore, we can conclude that: (a) the midgut is composed of three types of cells: digestive, secretory and regenerative cells; (b) the ultrastructure of the digestive cells suggests that they are responsible for the synthesis, absorption and accumulation of reserve material; (c) the secretory cells are of the closed type; (d) the regenerative cells are the unipotent midgut stem cells; (e) the differentiation of the digestive cells is the common process observed in the midgut epithelium, while the regeneration of secretory cells has not been detected; (f) the ultrastructure of all cells which form the midgut epithelium in millipedes is general for all the species studied up to now, and it resembles the cell ultrastructure observed in centipedes and Hexapoda.

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