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Differentiation of Regenerative Cells in the Midgut Epithelium of *Epilachna cf. nylanderi* (Mulsant 1850) (Insecta, Coleoptera, Coccinellidae)

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Differentiation of regenerative cells in the midgut epithelium of *Epilachna cf. nylanderi* (Mulsant 1850) (Insecta, Coleoptera, Coccinellidae), a consumer of the Ni-hyperaccumulator *Berkheya coddii* (Asteracae) from South Africa, has been monitored and described. Adult specimens in various developmental phases were studied with the use of light microscopy and transmission electron microscopy. All degenerated epithelial cells are replaced by newly differentiated cells. They originate from regenerative cells which act as stem cells in the midgut epithelium. Just after pupal-adult transformation, the midgut epithelium of *E. nylanderi* is composed of columnar epithelial cells and isolated regenerative cells distributed among them. The regenerative cells proliferate intensively and form regenerative cell groups. In each regenerative cell group the majority of cells differentiate into new epithelial cells, while some of them still act as stem cells and persist as a reservoir of cells capable for proliferation and differentiation. Because this species is an obligate monophage of plants which accumulate nickel, proliferation and differentiation of midgut stem cells follow degeneration intensively and in a typical manner.

Key words: Midgut epithelium, stem cells, regenerative cells, ultrastructure.

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The stem cell is a cell which divides from time to time to generate a new cell population and daughter cells differentiate further in a precise way, depending on their purposes (SMITH 2001; LI & XIE 2005). During embryogenesis they enable tissues and organs to develop and differentiate. In adult organisms they are responsible for tissue renewal throughout the entire life of an animal, especially when tissues have suffered damage. They are considered as pluripotent cells which can produce several differentiated cell types (FUCHS *et al.* 2004; MOORE & LEMISCHKA 2006).

Regenerative cells of the insect midgut epithelium fulfill the role of stem cells. They can proliferate intensively and differentiate into distinct cell types (HECKER *et al.* 1971; NISHIITSUTSUJI-UWO & YASUHISA 1981; BILLINGSLEY 1990; BILLINGSLEY & LEHANE 1996; SADRUD-DIN *et al.* 1996; HAKIM *et al.* 2001; TETTAMANTI *et al.* 2007; ROST-ROSZKOWSKA *et al.* 2010b). They may transform into columnar cells of epithelial character, either into endocrine or goblet cells, if present in the midgut epithelium. This happens because the midgut epithelial cells, due to a wide range of digestive functions, degenerate in a cyclic or continuous way. Stem cells thus produce all cells which form the midgut epithelium (SPICE & SPENCE 1985; BALDWIN & HAKIM 1991; LOEB & HAKIM 1996; HAKIM *et al.* 2001).

In this study the process of stem cell differentiation in *Epilachna cf. nylanderi* (Insecta, Coleoptera, Coccinellidae) was investigated. Our recent study on the same species demonstrated how the processes of midgut epithelium degeneration fol-

low through apoptosis, necrosis and autophagy (ROST-ROSZKOWSKA *et al.* 2008). Due to the fact that *E. nylanderi* feeds on *Berkheya coddii* Roessl. (Asteracae), one of the Ni-hyperaccumulating plant species growing on ultramafic outcrops in South Africa (PRZYBYŁOWICZ *et al.* 2006), degeneration of the midgut cells has been recognized as one of the strategies used by this beetle species to cope with the excess of nickel that enters the body together with consumed leaves. Because in the midgut epithelium of *E. nylanderi* all processes of degeneration are intensive, we hypothesized that the structure of the midgut must effectively regenerate at a proper rate in order to maintain its regular function.

Material and Methods

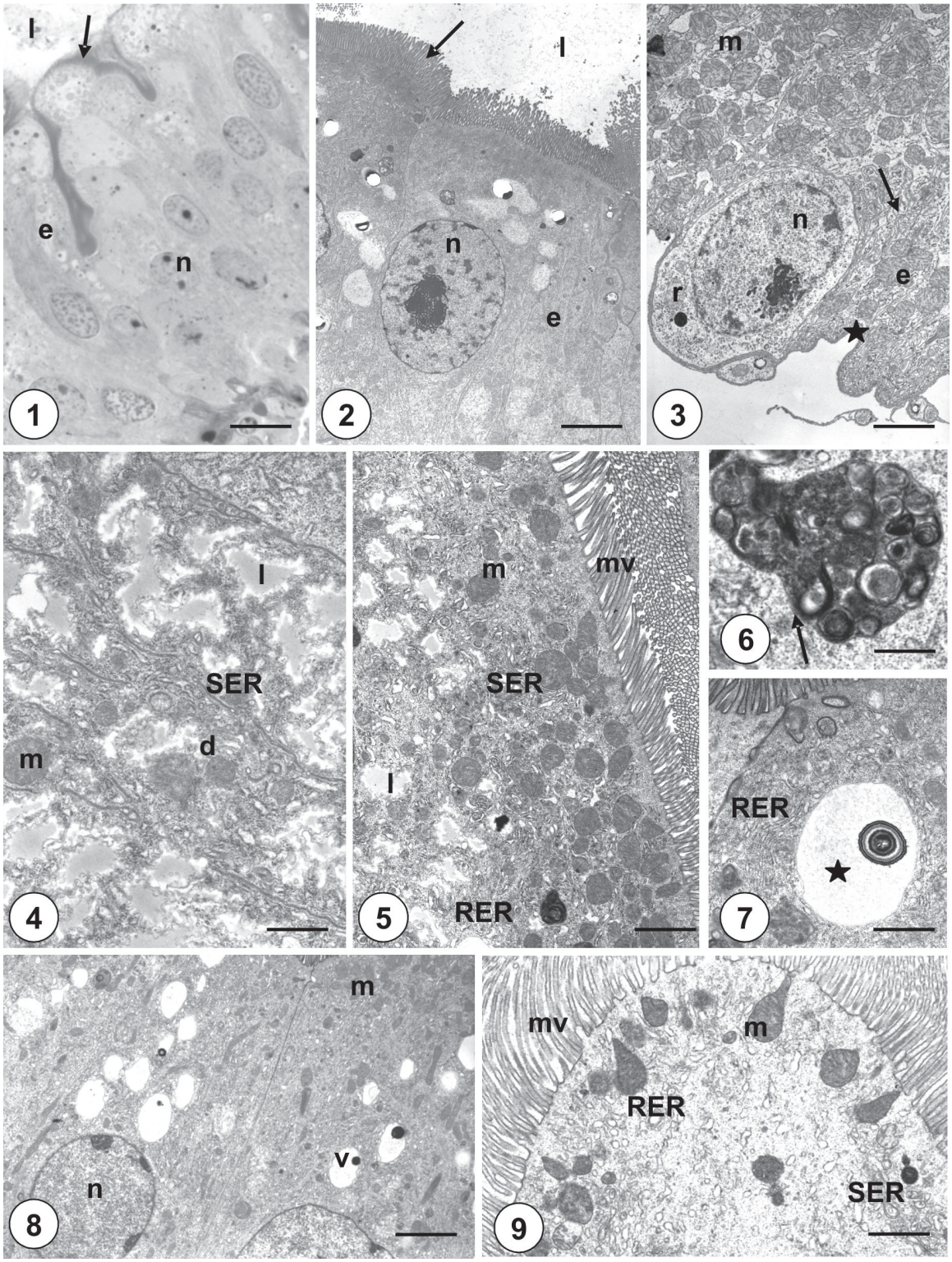
Adult specimens of *Epilachna cf. nylanderi* (Coleoptera, Coccinellidae, Epilachninae) were collected in Groenvaly, Mpumalanga Province, South Africa. The species is multivoltine and can breed continuously in optimal conditions. It·kg^{-1} (MESJASZ-PRZYBYŁOWICZ *et al.* 2004a, 2004b). All collected insects, both just after pupal-adult transformation (before feeding), and older ones which feed with *B. coddii* leaves, were taken alive and kept in small groups on fresh *B. coddii* leaves in plastic containers until used for microscopy preparations.

Isolated midguts (anterior and posterior parts) of about 40 specimens of *E. nylanderi* were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) and postfixed in 2% OsO_4 in 0.1M phosphate buffer with saccharose (1.5 h, temAfter dehydration in alcohols (50%, 70%, 90%, 95%, 100% for 15 min each) and acetone (15 min) the material was embedded in Epon 812. Semi- and ultra-thin sections were cut on a Leica Ultracut UCT25 ultramicrotome. Semi-thin sections were stained with 1% methylene blue in 0.5% borax and observed with an Olympus BX60 light microscope. Ultra-thin sections were stained with uranyl acetate and lead citrate. They were examined with a Hitachi H500 transmission electron microscope at 75 kV.

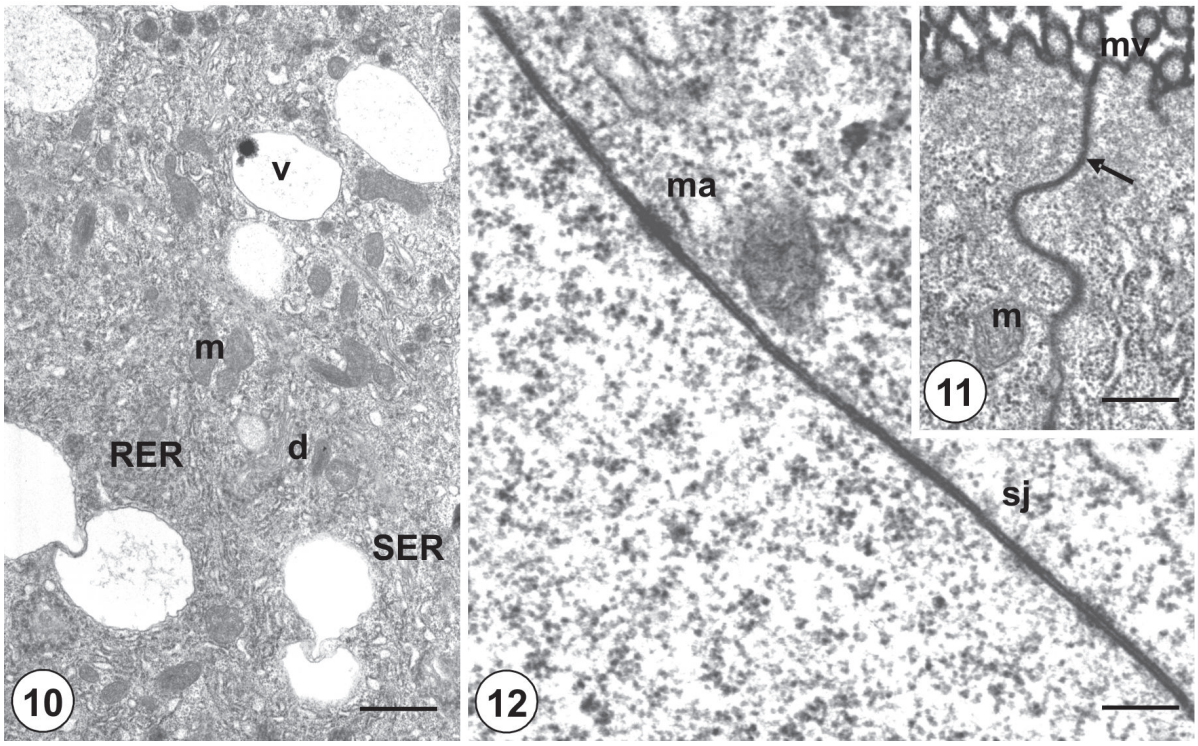
Results

Midgut epithelium of young specimens (just after pupal-adult transformation) of *Epilachna nylanderi* (Fig. 1) is composed of columnar cells of epithelial character (Fig. 2) and regenerative cells singly distributed among epithelial ones (Fig. 3). Midgut cells are located on the non-cellular basal lamina. The peritrophic membrane at this stage is absent. Initially, regionalization in organelle distribution in the cytoplasm of epithelial cells is not observed. The entire cytoplasm is rich in cisterns of smooth endoplasmic reticulum, Golgi apparatus and lipid droplets accumulated at the end of the pupal stage (Fig. 4). Mitochondria begin to aggregate between basal membrane folds (Fig. 3) and in the apical cytoplasm (Fig. 5). Individual mitochondria of the apical region commence transformation. They loose their cristae and start to accumulate lipid droplets (not shown). Mitochondria are surrounded simultaneously by endoplasmic reticulum membranes and autophagosomes are formed (Fig. 6). The peritrophic membrane appears gradually. Together with specimens aging and feeding, when organelle regionalization is distinct, multivesicular bodies and structures resembling "urospherites" of primitive insects occur (Fig. 7). The number of lipid droplets decreases and eventually they completely disappear (Fig. 8). Nuclei of epithelial cells are shifted towards the apical cytoplasm. Cisterns of rough endoplasmic reticulum, seen throughout the entire cytoplasm, demonstrate a typical membranous shape, but they also form circular structures. Near the apical membrane numerous mitochondria form a parallel layer (Fig. 9). Mitochondria do not undergo transformation in the mode described above, but some of them loose their cristae and disintegrate. The number of multivesicular bodies increases (not shown). Afterwards they disintegrate and small liberated vesicles move towards the apical membrane. Cisterns of smooth endoplasmic reticulum and Golgi apparatus of the perinuclear region are accumulated and vacuoles with electron lucent content or recently formed electron dense material appear (Fig. 10). In some epithelial cells vacuoles are formed only in the basal cytoplasm. Then single lamellar bodies appear in the perinuclear region (not shown). Intercellular junctions as smooth septate junctions (continuous junction) (Fig. 11), septate junctions, gap junctions and spot desmosomes (Fig. 12) join neighboring epithelial cells.

In the newly emerged adults (just after pupal-adult transformaton) single regenerative cells are seen between the epithelial ones (Fig. 3). Some mitochondria, free ribosomes and cisterns of smooth endoplasmic reticulum are observed in their cytoplasm. The cells begin to divide (Fig. 13), and new groups of regenerative cells (regenerative nests) are formed (Fig. 14). Each regenerative group is composed of about 6-8 regenerative cells. No distinct intercellular junctions are present between regenerative cells. Some regenerative cells in each regenerative cell group proceed to differentiate into epithelial cells (Fig. 15). The number of organelles, such as cisterns of rough endoplasmic re-



Figs 1-9. Fig. 1. Midgut epithelium of *E. nylanderi* composed of columnar epithelial cells (e). Midgut lumen (l), microvilli (arrow), nucleus of epithelial cell (n). LM. Bar = 11 μm. Fig. 2. Midgut epithelium of *E. nylanderi* composed of columnar epithelial cells (e). Midgut lumen (l), microvilli (arrow), nucleus of epithelial cell (n).TEM. Bar = 3.7 μm. Fig. 3. A single regenerative cell (r) distributed among epithelial cells (e). Basal lamina (asterisk), mitochondria (m), nucleus of the regenerative cell (n), folds of basal membrane (arrow). TEM. Bar = 2 μm. Fig. 4. The cytoplasm of epithelial cells rich in cisterns of smooth endoplasmic reticulum (SER), Golgi apparatus (d) and lipid droplets (l). Mitochondria (m). TEM. Bar = 0.62 μm. Fig. 5. Mitochondria (m) accumulated in the apical cytoplasm just beneath the apical membrane. Lipid droplets (l), cisterns of smooth (SER) and rough (RER) endoplasmic reticulum, microvilli (mv). TEM. Bar = 1.4 μm. Fig. 6. Autophagosomes (arrow) gradually formed in epithelial cytoplasm. TEM. Bar = 0.64μm. Fig. 7. Appearance of organelles regionalization structures with crystalline content (asterisk). Cisterns of RER (RER). TEM. Bar = 1.25 μm. Fig. 8. Complete disappearance of lipid droplets and appearance of vacuoles (v) with electron dense content. Visible mitochondria of the apical cytoplasm (m) and nucleus (n). TEM. Bar = 2.8 μm. Fig. 9. Mitochondria (m) of the apical cytoplasm form a layer, which lies parallel to the apical membrane. Cisterns of RER (RER) and SER (SER), microvilli (mv). TEM. Bar = 1.02 μm.



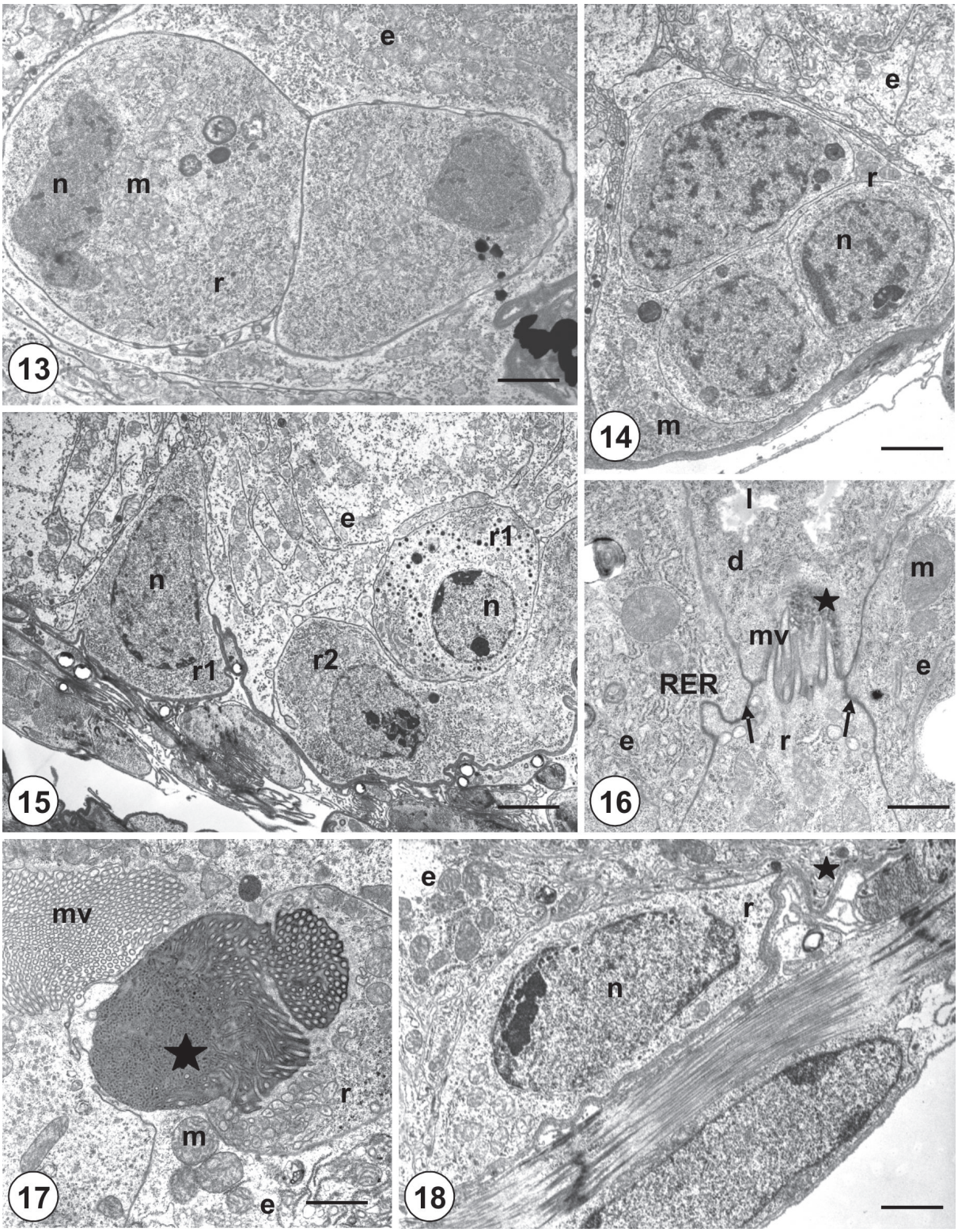
Figs 10-12. Fig. 10. Increase in the number of vacuoles (v). Cisterns of RER (RER) and SER (SER), Golgi apparatus (d), mitochondria (m). TEM. Bar = 1.125 μm. Fig. 11. Intercellular junction of smooth septate junction (arrow) observed between apical regions of epithelial cells. Mitochondria (m), microvilli (mv). TEM. Bar = 0.38 μm. Fig. 12. Spot desmosome (ma) and septate junction (sj) between epithelial cells in their perinuclear regions. TEM. Bar = 0.25 μm.

ticulum, Golgi apparatus and electron dense bodies in their cytoplasm, increases gradually. Intercellular junctions appear between differentiating new cells and adjacent epithelial cells (Fig. 16), and the first septate and gap junctions are formed. These cells are elongated and gradually separate degenerating epithelial cells from the basal lamina. Extracellular vacuoles are formed between differentiating cells and degenerating ones (Fig. 16). The apical membrane of the differentiating cell forms small microvilli (Fig. 16). Together with the increasing extracellular vacuole, its vacuolar space becomes electron dense (Fig. 17). The number of electron dense bodies in the differentiating cell decreases. Regionalization in organelle distribution in their cytoplasm appears when the apical membrane reaches the midgut lumen.

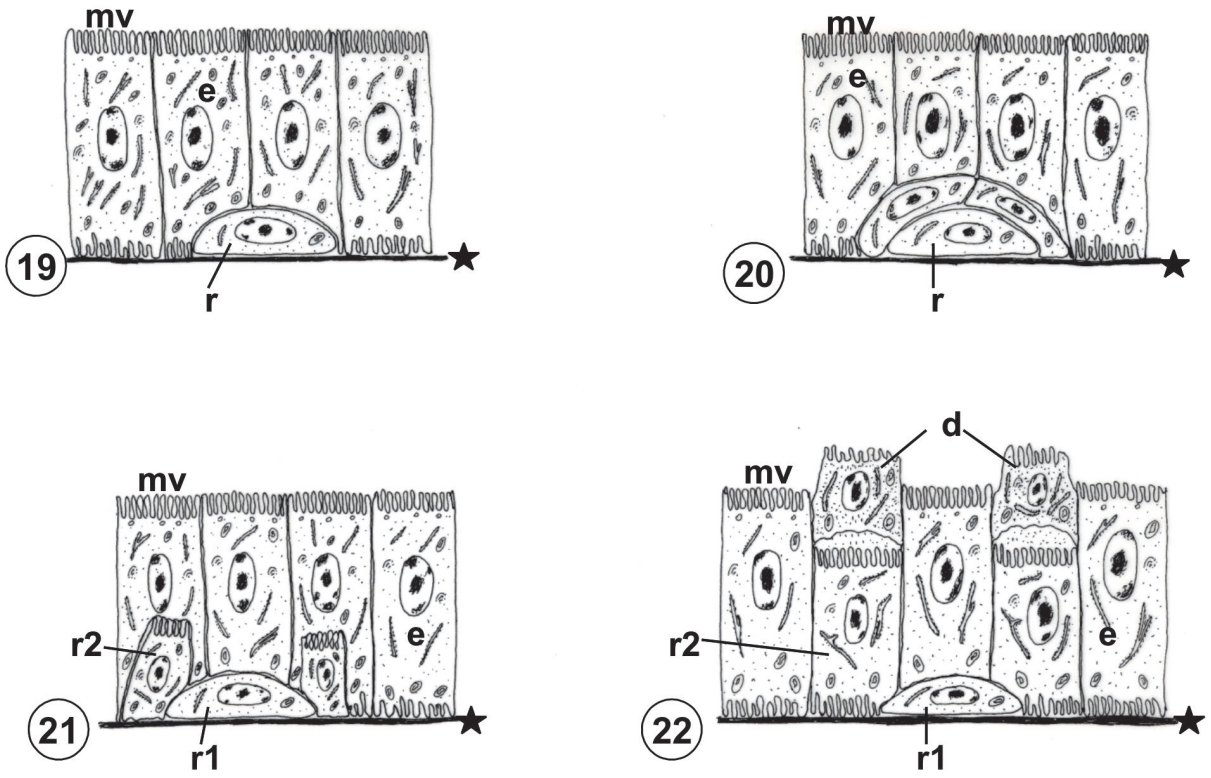
In each regenerative group most regenerative cells differentiate into epithelial ones, while only one of them continues to maintain the functions of the midgut stem cell. It achieves a spindle-like shape and its cytoplasm is poor in organelles (Fig. 18). Singly distributed midgut stem cells proliferate intensively forming succeeding regenerative cell groups. Distinct synchrony of their proliferation and differentiation between anterior and posterior midgut regions is not observed. It only proceeds more intensively in the posterior midgut. The described processes are diagrammatically presented in Figs 19-22.

Discussion

Insect midgut epithelium is always formed by two main types of cells: epithelial and regenerative (BILLINGSLEY 1990, BILLINGSLEY & LEHANE 1996; LEITE & EVANGELISTA 2001; SILVA-OLIVARES *et al.* 2003; BATON & RANFORD-CARTWRIGHT 2007; FIALHO *et al.* 2009). Regenerative cells are either distributed as isolated cells among epithelial cells, or form regenerative groups which, depending on the shape, are called regenerative nests or crypts (GARCIA *et al.* 2001; SILVA-OLIVARES *et al.* 2003; ILLA-BOCHACA & MONTUENGA 2006; ROST 2006a, 2006b; WANDERLEY-TEIXEIRA *et al.* 2006; BATON & RANFORD-CARTWRIGHT 2007; ROST-ROSZKOWSKA *et al.* 210a, 210b). When regenerative cells are absent it is suggested that their proliferative functions are taken over by epithelial cells (LAUGA-REYREL 1980; ROST-ROSZKOWSKA & UNDRUL 2008). In *E. nylanderi* regenerative cells are singly distributed among basal parts of epithelial cells, but after intensive proliferation they form regenerative cell groups.



Figs 13-18. Fig. 13. Regenerative cells (r) begin to divide intensively. Nucleus of regenerative cell (n), mitochondria (m), epithelial cell (e). TEM. Bar = 1.3 μm. Fig. 14. Formation of regenerative cell groups (regenerative nests) (r). Nucleus of regenerative cell (n), mitochondria (m). Epithelial cells (e). TEM. Bar = 1.68 μm. Fig. 15. Differentiation of some regenerative cells (r1) in each regenerative cell group into epithelial cells (e). Basal lamina (asterisk), regenerative cell fulfilling the role of a stem cell (r2), nuclei of regenerative cells (n). TEM. Bar = 2.44 μm. Fig. 16. Formation of extracellular vacuole (asterisk) between the degenerating (d) and differentiating (r) cells. Apical membrane of differentiating cell forms small microvilli (mv). Epithelial cell (e), lipid droplets in degenerating cell (l), mitochondria (m), Golgi apparatus (d), cisterns of RER (RER), intercellular junctions formed between apical regions of epithelial cells (arrows). TEM. Bar = 0.9 μm. Fig. 17. According to the increasing of extracellular vacuole (asterisk) its interior becomes electron dense. Epithelial cell (e), differentiating cell (r), microvilli (mv), mitochondria (m). TEM. Bar = 1.35 μm. Fig. 18. The cytoplasm of regenerative cells (r) poor in organelles. They take a spindle-like shape. Basal lamina (asterisk), nucleus of stem cell (n), epithelial cell (e). TEM. Bar = 1.25 μm.



Figs 19-22. Figs 19-22. Diagrammatic representation of regenerative cell differentiation in the midgut epithelium of *E. nylanderi.* Basal lamina (asterisk), microvilli (mv), epithelial cells (e), regenerative cells (r). Fig. 19. Regenerative cells singly distributed among epithelial cells. Fig. 20. Regenerative cells proliferate and regenerative cell groups are formed. Fig. 21. Differentiation of regenerative cells into epithelial cells. Between a degenerating epithelial cell and a differentiating regenerative cell (r2), a small extracellular vacuole is formed. Regenerative cells as stem cells (r1). Fig. 22. Extracellular vacuole extends, differentiating cells (r2) elongate. Regionalization in organelle distribution is not seen before their apical membrane reaches the midgut lumen. Some regenerative cells fulfill the functions of stem cells (r1). Degenerating cells (d).

It is known that midgut epithelial cells degenerate during digestion, and new cells originate from regenerative cells due to their mitotic activity (HECKER *et al.* 1971; SPICE & SPENCE 1985; SADRUD-DIN *et al.* 1996; HAKIM *et al.* 2001; TETTAMANTI *et al.* 2007; ROST-ROSZKOWSKA 2008; ROST-ROSZKOW- SKA *et al.* 2010a, 2010b). Degeneration and regeneration of the midgut epithelium may proceed cyclically or continuously. Cyclic cellular renewal in an insect midgut appears at each molt, when the digestive tract is growing (CHIANG *et al.* 1986; GARCIA *et al.* 2001; ROST 2006b). Continuous processes are observed during the entire life of the animal and depend on various, often stressing, external factors (EVANGELISTA & LEITE 2003; ROST 2006b; BATON & RANFORD-CARTWRIGHT 2007; ROST-ROSZKOWSKA *et al.* 2008). Midgut epithelium is in the first line of defense of the entire organism against different stressors such as harmful or toxic chemical compounds. In such circumstances all processes of degeneration would proceed very intensively. Simultaneously, regeneration of the midgut epithelium must follow the degeneration rates replacing eliminated cells with new ones. In the midgut epithelium of *E. nylanderi* all processes combined with degeneration (apoptosis, necrosis and autophagy) are intensive (ROST-ROSZKOWSKA *et al.* 2008) because this species, as the obligate feeder of the Ni-hyperaccumulating plant e.g. *Berkheya coddii,* is adapted to a diet extremely overloaded with toxic nickel (MIGULA *et al.* 2005). This is connected with many spherites which accumulate the excess of nickel, discussed for *E. nylanderi* by ROST-ROSZKOWSKA *et al.* (2008). Regenerative cells in the midgut of this species must then divide and differentiate intensively.

Each stem cell has its own extracellular environment, forming the so called stem cell niche. The niche is created by neighboring differentiated cells which allow stem cells to present the unique ability of self-renewal. The signal to the cells must come from the stem cell niche, which induces proliferation and differentiation, or ends these processes (FUCHS *et al.* 2004; LI & XIE 2005; MOORE & LEMISCHKA 2006). The stem cell niche of insect midgut epithelium is composed of basal lamina and the epithelial cells. When the degeneration of

epithelial cells begins, they probably send signals to the regenerative cells, which activate proliferation processes. Midgut epithelial cells of *E. nylanderi* degenerate intensively (ROST-ROSZKOWSKA *et al.* 2008), probably signaling to stem cells that an acceleration of regenerative cell proliferation is needed.

Regenerative cells have only a small amount of cytoplasm poor in organelles (KLAG *et al.* 2002; EVANGELISTA & LEITE 2003; LEVY *et al.* 2004; ROST 2006a, 2006b). In some insect species regenerative cells of the posterior midgut have numerous cisterns of rough endoplasmic reticulum and mitochondria. This is probably associated with more intensive degeneration and, in consequence, midgut epithelium regeneration (ROST-ROSZKOWSKA 2008). A similar phenomenon has been observed in *E. nylanderi*. The appearance of numerous mitochondria, cisterns of rough and smooth endoplasmic reticulum, Golgi apparatus and free ribosomes in the cytoplasm of regenerative cells is a morphological sign that differentiation has started. In *E. nylanderi* the cytoplasm of regenerative cells becomes rich in organelles just after it begins to differentiate, but the distribution of organelles forms a characteristic arrangement after reaching the midgut lumen. The characteristic regionalization in organelle distribution is common for midgut epithelial cells, responsible for digestion, secretion and absorption (EVANGELISTA & LEITE 2003, FIALHO *et al.* 2009). Extracellular vacuoles with electron dense content are formed between recently formed epithelial cells and the degenerated ones. As in embryos of chrysomelid beetles, *Melasoma saliceti* and *Chrysolina pardalina,* such vacuoles probably play a distinct role in the formation of microvilli (CRUZ-LANDIM 1999; ROST 2006b; ROST-ROSZKOWSKA *et al.* 2007).

It is known that regenerative cells are able to proliferate and differentiate. Therefore they might be treated as stem cells which, depending on the kind of cells forming distinct tissues, would differentiate into epithelial or even endocrine or goblet cells (SMITH 2001; FUCHS *et al.* 2004; TETTAMANTI *et al.* 2007). Stem cells which are able to differentiate in several cell types characteristic for a given tissue are called pluripotent (multipotent) (HAKIM *et al.* 2001; FUCHS *et al.* 2004). However, in most organs such abilities of stem cells are limited. Unipotent cells give rise to e.g. only one cell type. In *E. nylanderi* regenerative cells are treated as unipotent ones, because they transform into only one cell type, which represents the midgut epithelial tissue. Some regenerative cells do not differentiate and they remain as stem cells (BALDWIN & HAKIM 1991; LOEB & HAKIM 1996; MARTINS *et al.* 2006; BATON & RANFORD-CARTWRIGHT 2007; ROST-ROSZKOWSKA 2008; ROST-ROSZKOWSKA *et al.* 2010b). Depending on necessity, they will proliferate more or less intensively. In *E. nylanderi* processes of stem cell proliferation and differentiation proceed intensively and probably depend on the amount of consumed nickel which should be rejected as toxic from the organism with degenerated midgut epithelial cells.

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