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Ultrastructural and Cytochemical Aspects of Metamorphosis in the Midgut of *Apis mellifera* L. (Hymenoptera: Apidae: Apinae)

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ABSTRACT—The midgut of *Apis mellifera* is remodeled during metamorphosis. The epithelium and, to a lesser extent, the muscular sheath degenerate between the end of the last larval instar and the onset of pupation (prepupa). The larval epithelium is shed to the midgut lumen and digested, while a new epithelium is reconstructed from larval regenerative cells. During pupation, some reorganization still occurs, mainly in brown-eyed pupae. In pharate adult, the midgut wall shows the characteristics of adult, although some cells have pycnotic nuclei. The localization of alkaline and acid phosphatases showed that these enzymes were not involved in the reabsorption of the midgut wall.

Key words: midgut metamorphosis, pupa, ultrastructure, cell death, phosphatase

INTRODUCTION

The larvae and imagos of holometabolous insects differ in body anatomy and physiology. These two forms, use to be considered as ecomorphic phases, or adaptations to the different environments explored by the species during the immature and mature life (Wille, 1983).

The differences in morphology and physiology result from the expression of different sets of genes in each phase, with this expression being regulated by environmental conditions and hormones (Martin *et al.*, 1969; Tata, 1994). During pupation, there is a shift in gene expression, with larval genes inactivated and adult genes being activated. This shift, known as metamorphosis results in the degeneration and reabsorption of larval organs and in differentiation of adult organs.

In *A. mellifera* the larval digestive tract consists of a foregut with a stomodeal valve, a midgut, also undifferentiated, and a hindgut composed of an ileum and a rectum (Snodgrass, 1956). In contrast, the adult digestive tract, consists of a foregut with a pharinx, esophagus, crop and proventriculus, a midgut with a distint peri-stomodeal region and a hindgut subdivided into the pylorus, ileum and rectum (Snodgrass, 1956; Cruz-Landim, 1985).

The remodeling of the bee digestive tract during metamorphosis has been studied by Dobrovsky (1951) and Cruz-Landim and Mello (1970) using light microscopy. Grecorc and Bowen (1997) subsequently examined programmed cell death in the midgut of late larvae . The lack of information on cell morphology during metamorphosis and on the organelles involved lead us to examine these aspects in *A.mellifera*.

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MATERIAL AND METHODS

The fifth 5th instar larvae, pre-pupae and pupae collected from Africanized *A. mellifera* colonies, were used. The bees were collected form the apiary maintained by the, Departamento de Biologia, Instituto de Biociências, UNESP. The fifth instar larvae were studied before and after defecation, the pre-pupae were separated into early and late phases and the pupae were classified according to eye color (white, pink, red and brown) and pharate adult. The eye pigmentation and body darkning degree was used as an indicator of the extent of development.

The midguts of three of each class of individuals were excised and prepared for light and transmission electron microscopy. For light microscopy, the tissues were fixed in 10% neutral formaldehyde for 4 hr, dehydrated in ethanol and embedded in JB4 historesin (Polysciences, Inc., Warrington, PA 1976, USA) according to the manufacturer's instructions. The specimens for transmission electron microscopy (TEM) were fixed in Karnovisky solution prepared with 0.1 M cacodylate buffer, pH 7.4, and post-fixed in 1% osmium tetroxide in the same buffer after dehydration in acetone, the tissues were embedded in Epon-Araldite and the pieces were stained with 2% uranyl acetate in ethanol and dehydration. Thin sections were stained with lead citrate before examination.

Some midgut samples, were also prepared for the detection of acid phosphatase activity and, fixed for 1 hr in 2.5% glutaraldehyde in 0,1 M sodium cacodylate buffer, pH 7.2, containing 5% sucrose, during 1 hr. After fixation and washing in 0.1 M, acetate buffer, pH

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4.8, the tissues were incubated with the substrate p-nitrophenylphosphate (p-NPP) during 45 min, at 37°C according Ryder and Bowen (1975). Pos-fixation was done in 1% osmium tetroxide in 0.1M phosphate buffer, pH 7.4. The same procedure was used for alkaline phosphatase, except that incubation was done in Tris buffer, pH 8.8. Control tissues were incubated without substrate. The grids were examined either stained or unstained.

RESULTS

Light microscopy

The midgut epithelium of last instar larva consisted of an apparently uniform population of digestive cells which were flat to prismatic, depending upon the anatomical region and the food load in the lumen (Fig. 1). Interspersed with the digestive cells were nests of regenerative cells, some of which were undergoing division (Fig. 2). The digestive cells had a well-developed brush border and a large, central nucleus. Although the regenerative cells were in contrast with the epithelium basal lamina, their apices did not reach the lumen (Figs. 1 and 2).

In the midgut of larva beginning with the last instar, the nucleus of most of the digestive cells showed condensed chromatin in the center and a clear halo between this and the nuclear envelope (Fig. 1). By the end of this instar, the larva defecated emptying the midgut, the lumen of which collapses. The digestive cells showed a vacuolated cytoplasm, and granules, which stained like nuclei, were observed in the collapsed lumen (Fig. 3).

The prepupae midgut was in the process of epithelium replacement. The original larval, epithelium was seen in the lumen, while the new epithelium was visible beyond the lumen (Fig. 4).

White-eyed pupae already had a reconstituted epithelial wall, in which digestive and regenerative cells were distinguishable(Fig. 5). In some digestive cells, the nuclei were located in an apical expansion of the cytoplasm and had very condensed chromatin.

In pharate adult the midgut morphology was very similar to that of adults, although some cells had very pycnotic nuclei (Fig. 6).

Ultrastructure

During replacement of the larval midgut epithelium by the new adult epithelium, the larval digestive cells degenerated and were released to the organ lumen. Ultrastructurally, the degenerating cells were extensively vacuolated, mainly in the apical region (Fig. 7). Although the alterations affected the entire cell they were, more intense at the top. In last instar larvae, the apical cytoplasm of digestive cells was very vacuolated and the microvilli had almost disappeared. The mitochondria were swollen but the nucleus was intact and portions of rough endoplasmic reticulum were observed alongside the nucleus surface. At the beginning of the prepupal stage, the midgut cells had an expanded apical cytoplasm with bubbles, deprived of organelles and filled with fine, granular material of median electron density; some vacuoles were also present (Fig. 8). The apparently normal nucleus, tends to be located more apically, sometimes in the bubble. The lumen of the midgut was full of cellular debris and highly electron-dense granules (Fig. 8).

The prepupal epithelium collapsed and the basal lamina appeared thick and very folded. Some intact cells were observed along side the vacuolated cells (Fig. 9). The undamaged cells were regenerative cells. The muscle layer showed disruption of the myofibril and degeneration (Fig. 10). Cells with a clear cytoplasm and large nuclei in close apposition to the impaired muscle cells were probably myoblasts.

In white-eyed pupae, the muscle and epithelial layers of the midgut wall regenerated at the same time. The digestive cells were very slender and contained small vacuoles, while the regenerating cells were broader, with large nuclei, very disperse chromatin, and a cytoplasm poor in organelles (Fig. 11).

As the pupal stage advanced, the appearance of the epithelial cells changed. In pink-eyed-pupae, the cells were flatter, with numerous, well-defined microvilli at the luminal surface. In the apical zone, the cytoplasm contained many free ribosomes and short fragments of rough endoplasmic reticulum, as well as large deposits of lipids (Fig. 12).

In some regions of the epithelium in brown-eyed pupae, there was shedding of some digestive cells (Fig. 13). These cells were round, vacuolated and had few microvilli. Regenerative cells appeared below those cells. The epithelium in this phase was generally lower, vacuolated, and had microvilli with apical bulbous expansions (Figs. 13 and 14). Electrondense structures, some containing crystaline material, were present in the region of the lipid deposits (Fig. 15). Some globular structures seen in the lumen probably resulted from the elimination of dead cells (Fig. 16).

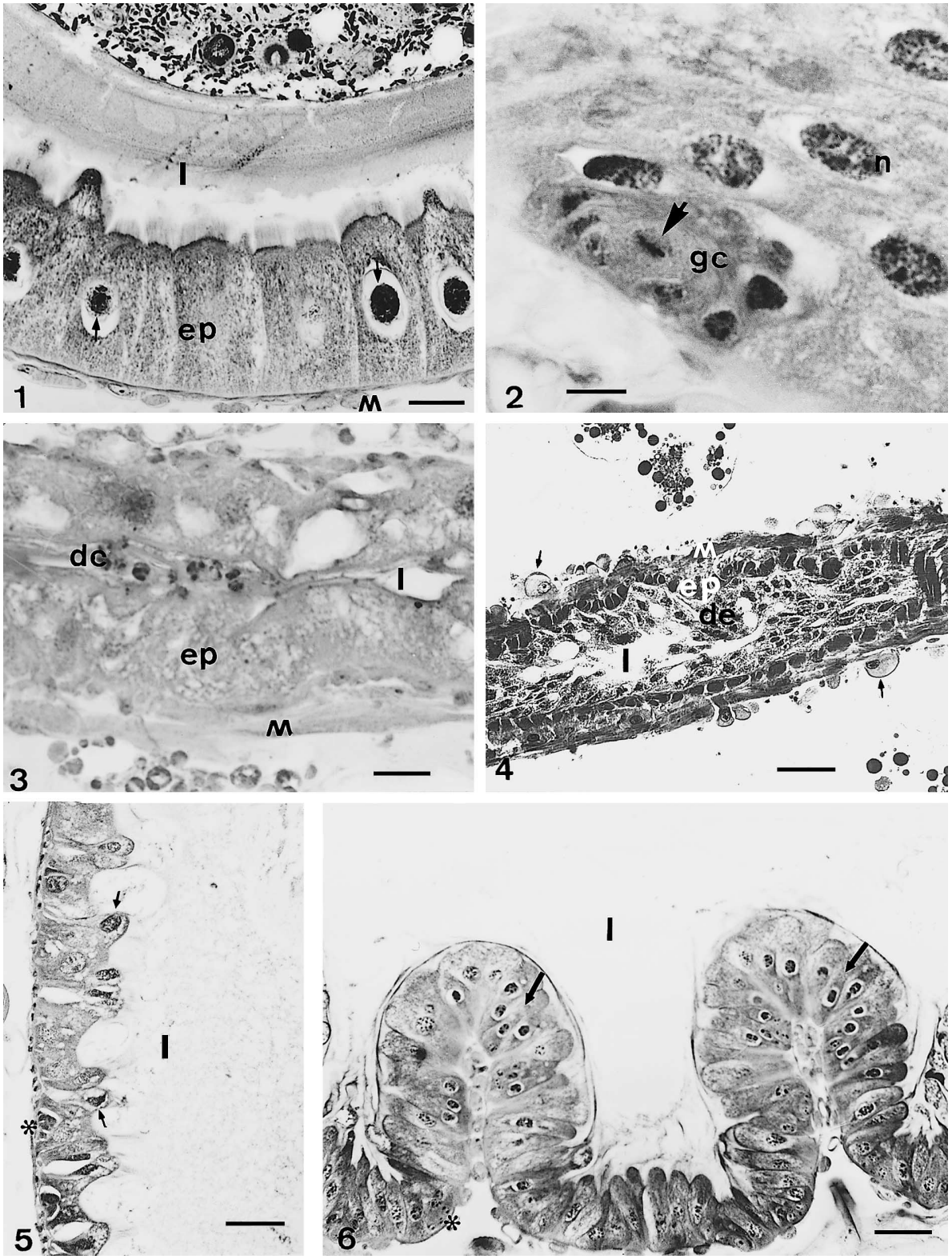
The midgut epithelium of pupae with brown body was fully developed and very similar to that of adults (Fig. 17).

Acid and Alkaline Phosphatase

Acid phosphatase was more widespread in the cells than akaline phosphatase. The most conspiscuous localization of acid phosphatase activity, was in microvilli (Fig. 18). A positive reaction was also observed in the fine granulation dispersed in the cytoplasm as well as in the cell debris in the midgut lumen (Figs. 19 and 20). There was a weak reaction around the mitochondria (Fig. 20) and in the nuclear chromatin (Fig. 21).

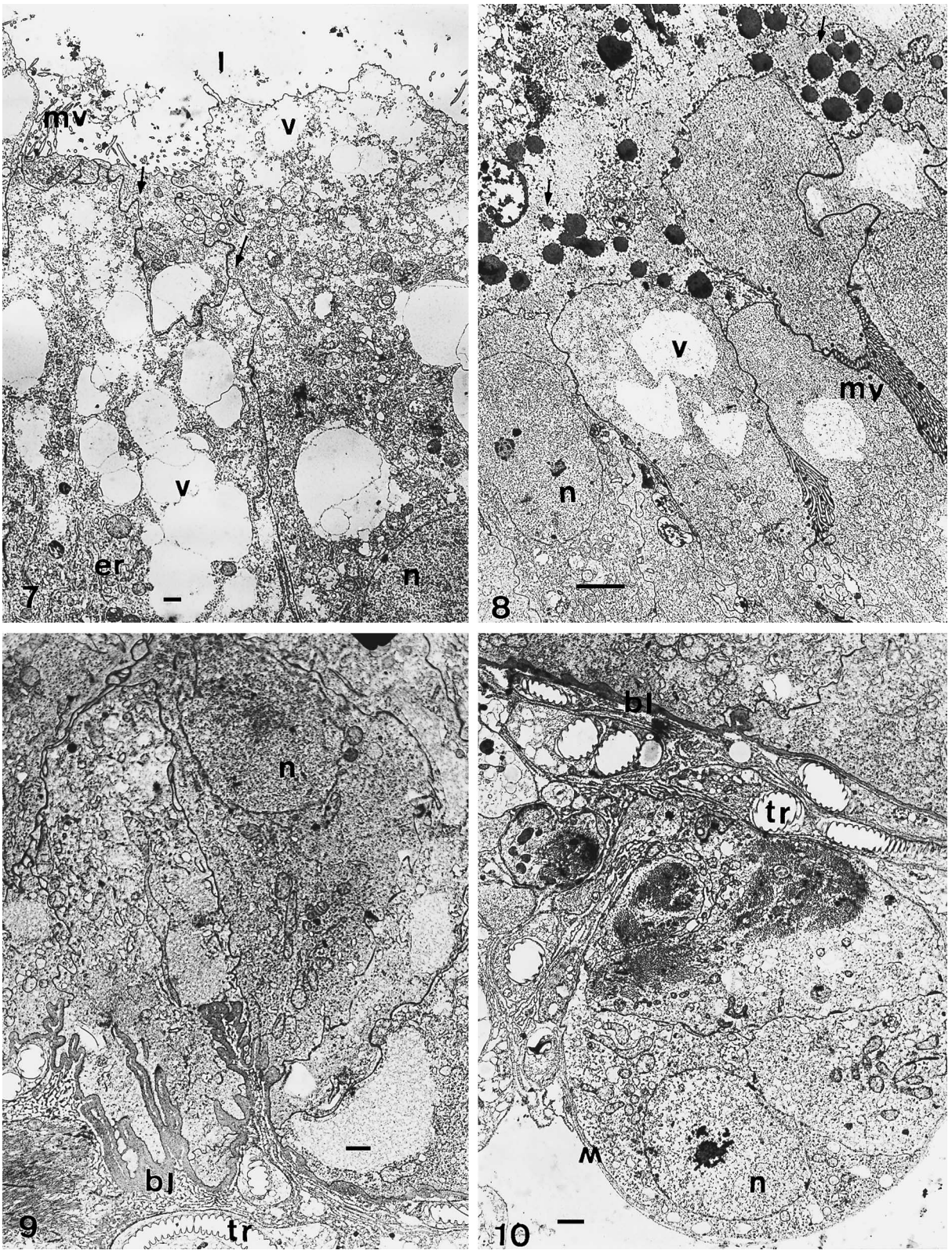
While the positive reaction for acid phosphatase in the microvilli appeared as small dots over the microvillar surface, alkaline phosphatase was detected in discrete regions along the length of the microvilli (Figs. 24 and 25). The location of alkaline phosphatase was always more conspiscuous and clearer than that of acid phosphatase. The basal plasma membrane invaginations of the digestive cells were filled with lead, indicative of the presence of enzyme (Fig. 22). The regenerative cells contained only a few positive dots over the mitochondria and nuclear envelop (Fig. 23).

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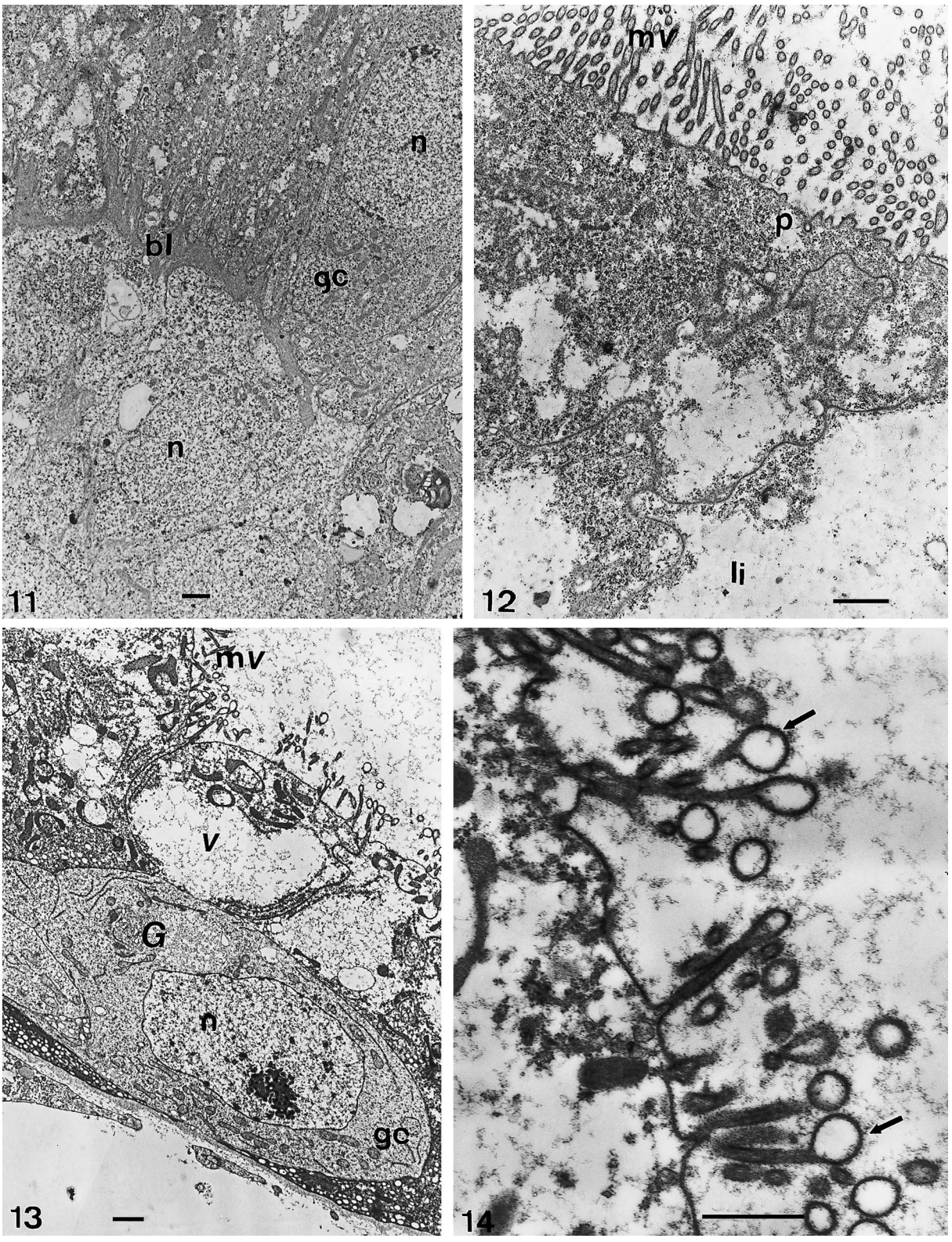
Figs. 1–6. Light microscopy of the metamorphosing *A. mellifera* midgut. 1. Cross section of the last larval instar midgut showing pycnotic nuclei (arrows) in the epithelial cells (ep) Bar=25 μm. 2. Group of regenerative cells (gc) from last larval instar, showing cell division (arrow) Bar=10 μm. 3. Midgut wall from early prepupa showing the midgut lumen (l) with cell debris; degenerating cells in the lumen (dc) and the vacuolated epithelium. 4. Midgut wall of late prepupae showing the larval degenerating epithelium (de) in the lumen and a new epithelium (ep) forming below. Allows point to myblasts, Bar=35 μm. 5. Epithelial wall of white-eyed pupa showing nests of regenerative cells (asterisc) and some digestive cells with pycnotic nuclei (arrows) in bulbous tips Bar=20 μm. 6. Epithelial wall of the midgut of a pharate adult showing nests of regenerative cells (asterisc) and digestive cells with pycnotic nuclei (arrows) Bar=35 μm. M=muscle; l=lumen; n=nucleus.

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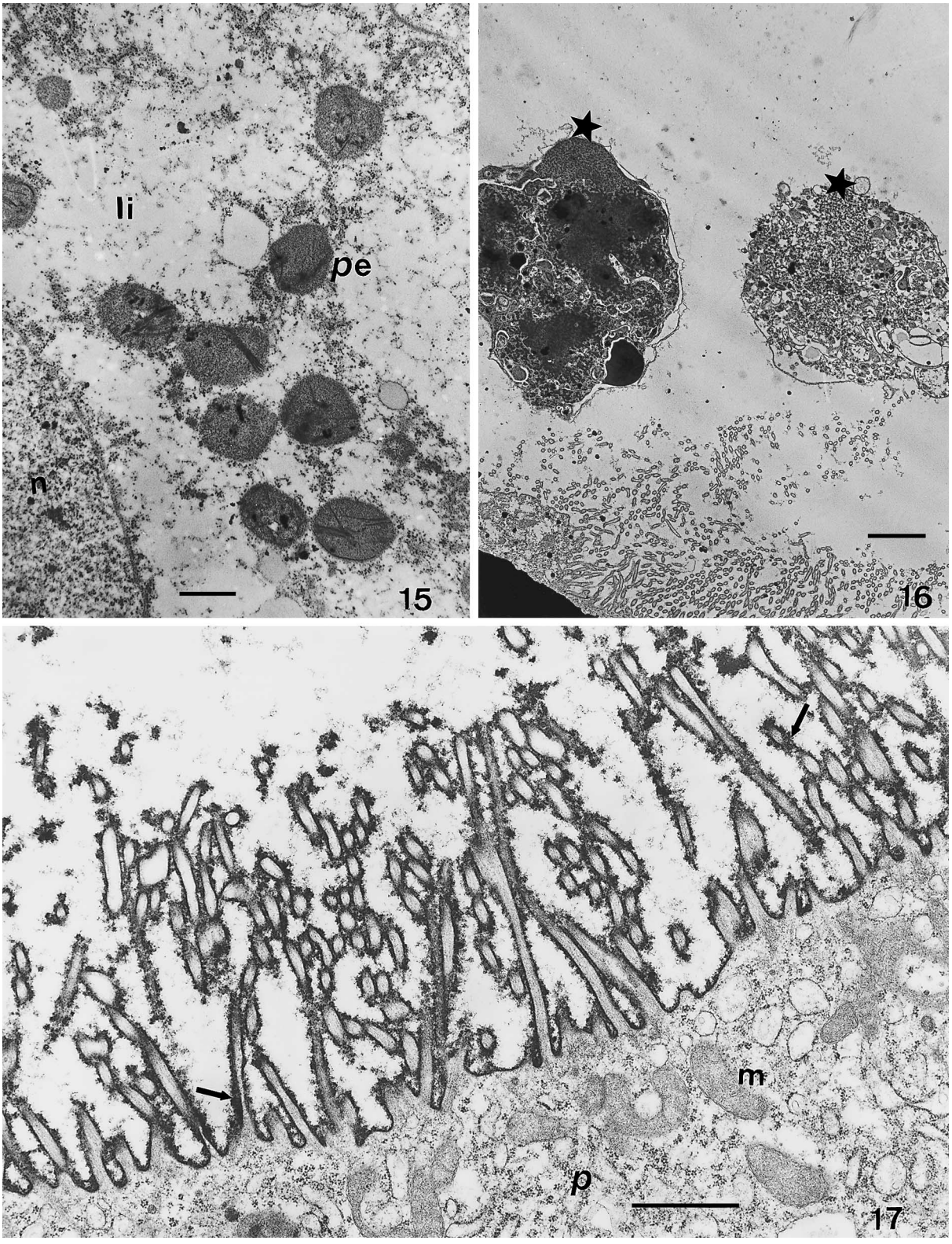
Figs. 7–10. Transmission electron microscopy of the midgut wall of *A. mellifera* prepupae. 7. Disorganized digestive cells showing loss of microvilli and extensively vacuolated cytoplasm (v) The arrows point to very simuous cell contacts. Note the normal features of the nucleus (n) and basal portion of the cells. 8. Digestive cells showing bulbous apices, apical nucleus, and electrondense granules released into the midgut lumen (arrows). 9. Basal region of the midgut epithelium showing the folds of the basal lamina (bl). Note damaged and intact cells side by side. 10. Cross section of a muscule fiber (M) in the process of reorganization. tr=tracheoles; n=nuclei; er=rough endoplasmic reticulum; v=vacuole; l=lumen; mv=microvilli. All scales=1 μm

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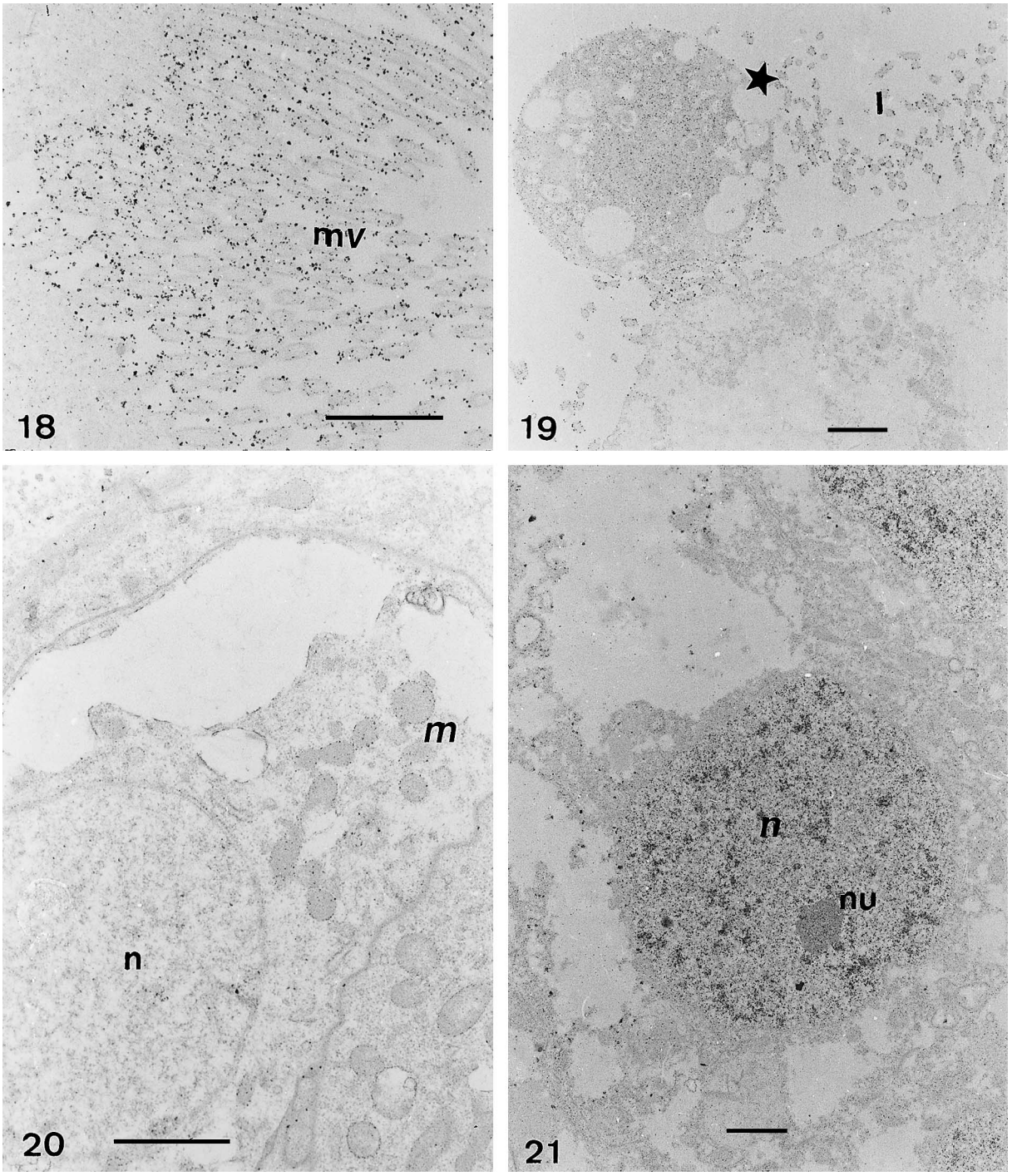
Figs. 11–14. Transmission electron microscopy of the midgut wall of *A. mellifera* pupae. 11. Contact between digestive and regenerative cells (gc) in white-eyed pupa. Note the vacuolation of the digestive cells. 12. Apical region of digestive cells in a pink-eyed pupa showing lipid deposits (li) and polysomes (p). 13. Midgut epithelium from a brown-eyed pupa showing a cell being shed into the lumen and below, the regenerative cells (gc). 14. Magnification of the apex of digestive cells in a brown-eyed pupa showing the microvilli with swollen tips (arrows). v=vacuole; G=Golgi; bl=basal lamina; n=nucleus. All scales=1 μm

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Figs. 15–17. Transmission electron microscopy of the midgut of *A. mellifera* pupae. 15. Electrondense granules, probably peroxisomes (pe) in the cytoplasm of digestive cells from a brown-eyed pupa. 16. Cellular debris (stars), similar to apoptotic bodies, in the midgut lumen of a brown-eyed pupa. 17. Apex of a digestive cell in a brown pupa. Notice the glycocalyx (arrows) around the microvilli. m=mitochondria; li=lipids; p=polysomes; n=nucleus. All scales=1 μm

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Figs. 18–21. Transmission electron microscopy of the acid phosphatase reaction. 18. Positive reaction in the microvilli (mv) of digestive cell membranes from a pupa. 19. Positive reaction in the blebs (star) in the midgut lumen (l) and microvilli of a prepupa. 20. Positive reaction around the mitochondria (m). 21. Positive reaction in the nucleus (n) and cytoplasm. nu=nucleolus. All scales=1 μm

Both enzymes give unexpected positive reactions in the nuclear chromatin and nucleolus of some cells (Fig. 21).

DISCUSSION

During metamorphosis, the larval midgut epithelium is partially destroyed and replaced by a new epithelium that will function during adult life.

After emptying the midgut, the cells of last instar larvae already show signs of degeneration, such as nuclear chromatin condensation. However metamorphosis itself begins in the prepupal stage and involve the shedding of larval epithelium into the lumen and the formation of a new epithelium, mainly by mitosis of the regenerative cells. It was unclear

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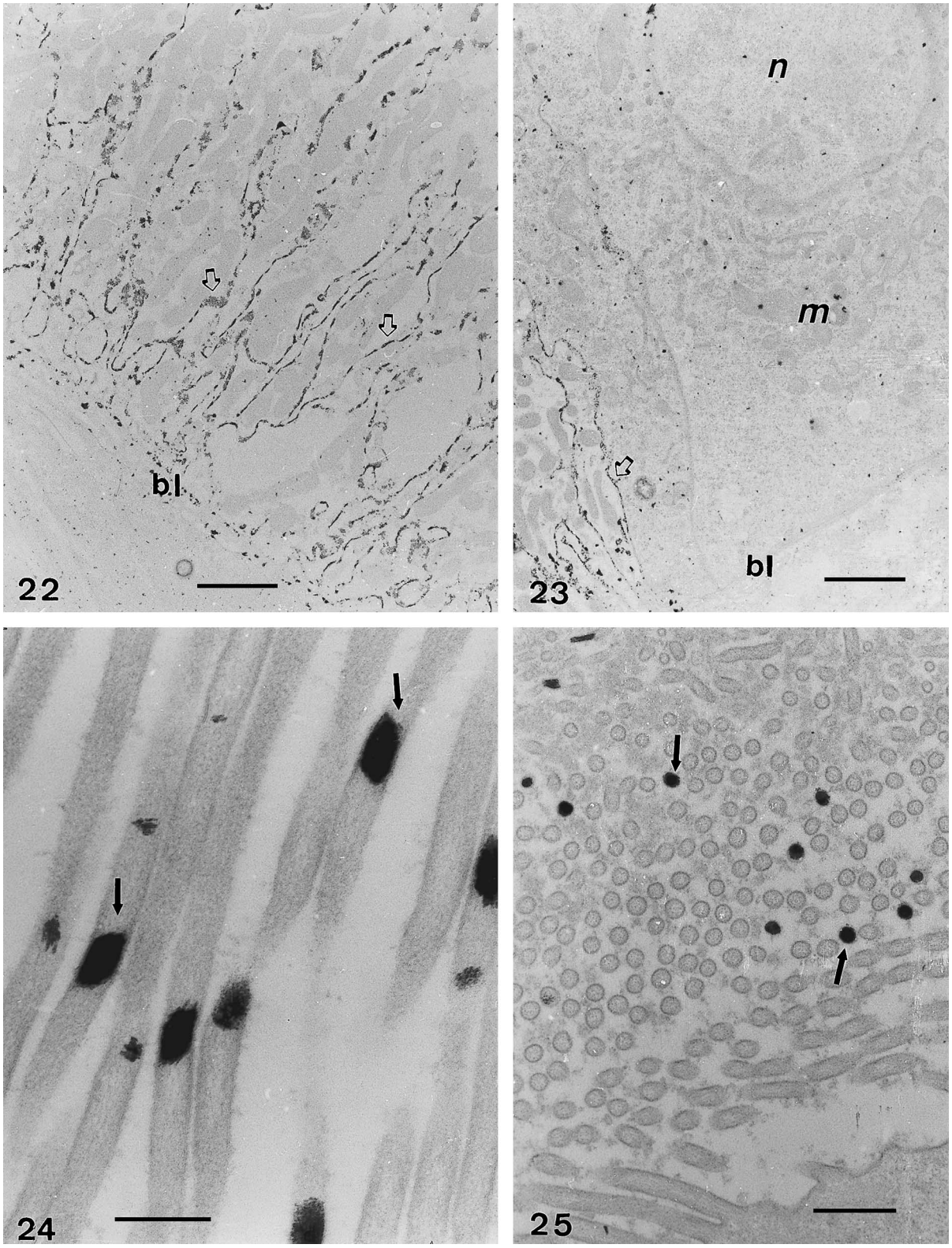


Fig. 22–25. Transmission electron microscopy of the alkaline phosphatase reaction. 22. Positive reaction in the basal lamina (bl) and in the invaginations of the plasma membrane (arrows). 23. Contact between a digestive cell and regenerative cell, showing the lack of basal invaginations in the latter and some deposits of lead in the nuclear envelop and mitochondria (m). 24. Longitudinal section of microvilli showing positive reaction (arrows). 25. Cross-section of microvilli showing the spots (arrows) indicating a positive reaction. This section corresponds to the regions positive in 24. bl=basal lamina; n=nucleus; m=mitochondria. All scales=1 μm

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whether all of the midgut larval cells are eliminated or whether some remains in the adult. The greatest damage is seen in the cell apices, which appeared vacuolated and forming bulbous projections toward the lumen. It is possible that the apices of these cells are casted off and the remaining basal part preserved to participate in the new regenerated epithelium, together with the new cells produced by mitosis of the regenerative cells.

Although light microscopy showed chromatin condensation in the nuclei, typical apoptosis was never observed, probably because the damaged cells are sheded into the lumen. Nevertheless, some structures similar to the apoptotic bodies described by Gimenez and Gilliam (1990), were observed in the lumen and may represent entire cells or parts of them.

Our observations supporte the idea that acid phosphatase was not directly involved in cell reabsorption since the cells had no conspicuous autophagic structures and the acid phosphatase activity was no related to the presence of lysosomes, although structure with a similar morphology were sometimes present.

Positive reactions, for phosphatase, dispersed in the cytoplasm, are indicative of cell death (Jones and Bowen, 1993). Therefore the presence of lead deposits in the cytoplasm and nucleus of digestive cells probably indicated that the cells involved are dying.

The dead cells and cellular debris from metamorphosis are shed into the organ lumen were they are probably digested, since we have detected acid phosphatase in the midgut lumen, of pre-pupae and pupae (unpublished observations).

The lipids present in brown-eyed pupae may represent reserves for cellular differentiation or may indicate cell lipid degeneration.

The alkaline phosphatase was detected only at certain points along the microvilli and in folds of the basal plasma membrane. This locatization suggests that this enzyme is more related to cell absorptive activity, than to cellular digestion.

In conclusion, the larval midgut epithelium and, to some extent, the midgut muscular sheath, degenerate and are replaced between the end of fifth larval instar and the onset of pupation (prepupae). This process is very rapid. During pupation, the midgut epithelium differentiates, and the dead cells are shed into the midgut lumen.

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