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Ultrastructural differentiation of plasma membrane and cell junctions in the hindgut cells is synchronized with key developmental transitions in *Porcellio scaber*

Author names and affiliations:

Urban Bogataj^a, Polona Mrak^a, Jasna Štrus^a, Nada Žnidaršič^a

^aDepartment of Biology, Biotechnical faculty, University of Ljubljana, Večna pot 111, SI-1000 Ljubljana, Slovenia

Corresponding author:

Urban Bogataj

Department of Biology, Biotechnical faculty, University of Ljubljana, Večna pot 111, SI-1000 Ljubljana, Slovenia

e-mail address: urban.bogataj@bf.uni-lj.si

Tel.: +386(0)13203370

Abstract:

Differentiation of transporting epithelial cells during development of animal organisms includes remodelling of apical and basal plasma membranes to increase the available surface for transport and formation of occluding junctions, which maintain a paracellular diffusion barrier. This study provides a detailed ultrastructural analysis of apical and basal plasma membrane remodelling and cell junctions formation in hindgut cells during late embryonic and early postembryonic development of crustacean *Porcellio scaber*. Hindgut cells in late-stage embryos are columnar with flat apical and basal plasma membranes. In early-stage marsupial mancae the hindgut cells begin to acquire their characteristic dome shapes, the first apical membrane folding is evident and the septate junctions expand considerably, all changes probably associated with the onset of active feeding. In postmarsupial mancae the apical labyrinth is further elaborated and the septate junctions expanded. This coincides with the transition to an external environment and food sources. First basal infoldings appear in the anterior chamber of early-stage marsupial mancae, but in the papillate region they are mostly formed in postmarsupial mancae. In molting late-stage marsupial mancae, the plasma membrane acquires a topology characteristic of cuticle-producing arthropod epithelia and the septate junctions are considerably reduced.

Keywords: cell differentiation, cell junctions, development, hindgut epithelium, plasma membrane infoldings

1 Introduction

Differentiation of epithelial cells during development commonly involves the establishment of distinct apical, lateral and basal plasma membrane domains and formation of cell junctions at the appropriate sites of the plasma membrane, resulting in apico-basal polarity of cells and tissues (Rodriguez-Boulán and Nelson, 1989; Laprise and Tepass, 2011; Rodriguez-Boulán and Macara, 2014). The epithelial cells subsequently undergo further differentiation processes which result in specific morphological and functional characteristics of different epithelia. These processes include cell shape modification (Schöck and Perrimon, 2002), reshaping of plasma membrane (Berridge et al. 1976; Louvard et al. 1992; Baumann and Bauer, 2013; Crawley et al., 2014) and reorganization of cell junctions and the cytoskeleton (Tepass and Hartenstein, 1994; Tepass et al., 2001; Schöck and Perrimon, 2002). The hindgut epithelium of *Porcellio scaber* (Crustacea: Isopoda) in its embryonic and postembryonic stages provides an *in situ* model for investigation of invertebrate epithelial cell differentiation in a relevant physiological environment of the surrounding extracellular matrix, cells and tissues. This allows us to study how the selected ultrastructural characteristics of cell differentiation correspond with the gross morphological and physiological changes that the organism undergoes during its development.

Elaborations of apical and basal plasma membranes, such as microvilli and membrane infoldings, are important characteristics of the transporting epithelial cells in general. These modifications greatly increase the available surface area for positioning of membrane transporters and channels and provide enclosed extracellular spaces where ion gradients can build up (Cioffi, 1984; Baumann and Walz, 2012). Cell junctions contribute to the establishment of separate apical and basal plasma membrane domains. They also prevent paracellular diffusion across the epithelial barrier and provide the adhesion between neighbouring epithelial cells as well as between cells and extracellular matrix (Giepmans and van Ijzendoorn, 2009). Morphogenesis of apical microvilli is well explained in the case of intestinal enterocytes of vertebrates (Louvard et al. 1992). In various transporting epithelial cells of arthropods the apical and basal plasma membranes form densely stacked membrane infoldings (Cioffi, 1984) which constitute the apical and basal membrane labyrinths. The data on the formation of such plasma membrane labyrinths is scarce. Formation of cell junctions during embryonic and larval development has been extensively investigated in various epithelia of *Drosophila* (Tepass and Hartenstein, 1994).

The entire alimentary canal of terrestrial isopods is ectodermally derived and consists of cuticle lined foregut and hindgut which are in direct contact with each other. The blind ending tubules of digestive glands, which are connected to the atrium of the stomach, represent the only endodermal part of the digestive system (Vernon et al., 1974; Hassall and Jennings, 1975; Holdich and Mayes, 1975; Bettica et al., 1987; Storch and Štrus, 1989; Štrus et al., 2008). The hindgut epithelium of *P. scaber* is involved in the absorption of digestion products in the anterior part of the hindgut, the anterior chamber, and in transcellular transport of ions and water in the posterior part of the hindgut, the papillate region (Palackal et al., 1984; Hryniewiecka-Szyfter and Storch, 1986; Hames and Hopkin, 1989). The dorsal wall of the anterior hindgut forms a longitudinal fold termed typhlosole and two typhlosole channels (Hames and Hopkin, 1989; Storch and Štrus, 1989). Isodiametric epithelial cells in the anterior chamber have dome-shaped apical parts, which protrude into the hindgut lumen, while dome-shaped basal parts of isodiametric cells in the papillate region protrude into the haemocoel (Bogataj et al., 2018). The outstanding ultrastructural characteristics of the hindgut epithelial cells in terrestrial isopods associated with their transport and barrier functions are

the extremely intensely infolded apical and basal plasma membranes in the form of apical and basal membrane labyrinths and the extensive adherens and septate junctions on the lateral membranes (Vernon et al., 1974; Palackal et al., 1984; Hryniewiecka-Szyfter and Storch, 1986; Storch and Štrus, 1989). In *P. scaber* the apical labyrinth in the papillate region is deeper and exhibits wider extracellular spaces than the labyrinth in the anterior chamber. The infoldings of basal labyrinth with numerous mitochondria are more densely stacked in the papillate region. Hindgut cells are connected laterally with the subapical adherens junctions and extensive septate junctions. Connections to the extracellular matrix comprise hemidesmosome-like junctions between the basal plasma membrane and basal lamina and junctions between the apical plasma membrane and cuticle (Bogataj et al., 2018).

In terrestrial isopods the embryonic and early postembryonic development takes place in the osmotically regulated environment of the female brood pouch (marsupium) (Surbida and Wright, 2001). Embryos are enclosed in two embryonic envelopes (Wolff, 2009; Milatović et al., 2010; Mrak et al., 2012). The embryonic development of *P. scaber* lasts approximately 25 days. The early-stage embryos contain large amounts of yolk and have no visible limb buds. The mid-stage embryos are dorsally bent and are characterised by the appearance of limb buds and by the gradual inclusion of yolk into the digestive glands. After the embryos hatch from the outer egg envelope – chorion – and become ventrally bent, they are termed late-stage embryos. Milatović et al. (2010) divided the marsupial development of *P. scaber* into 20 consecutive stages designated S1 – S20. Stages S1 – S5 comprise the early-stage embryos, stages S6 – S15 comprise the mid-stage embryos, stages S16 – S19 comprise the late-stage embryos and stage S20 designate the marsupial manca. After the release from the inner egg envelope – vitelline membrane – the marsupial mancae develop within the marsupium for up to 10 additional days. The term manca denotes all postembryonic stages that resemble the adult form but lack the seventh pair of pereopods. Mark et al. (2012) defined 3 consecutive stages of marsupial mancae: early-stage marsupial manca, mid-stage marsupial manca and late-stage marsupial manca. The stages differ in size, pigmentation, motility and amount of yolk in the digestive glands. After the release from the marsupium the postmarsupial mancae continue to develop in the external environment for up to 20 days before the juvenile stage is reached (Tomescu and Craciun, 1987).

Hatching from the embryonic envelopes and release from the marsupium into the external environment exert additional physiological demands onto the developing tissues and organs including the hindgut epithelium. The hindgut development in *P. scaber* begins in mid-stage embryos S6 with proctodeum invagination. In mid-stage embryos S6 – S14 the proctodeum gradually elongates anteriorly towards the stomodeum. The proctodeum and stomodeum fuse in mid-stage embryos S15 just prior to dorsoventral rotation of the embryo within the vitelline membrane and afterwards the ventrally bent late-stage embryos S16 exhibit a continuous digestive tract. In late-stage embryos S18 the subdivision of hindgut into the anterior chamber and the papillate region becomes apparent and in marsupial mancae this division is already completely established (Štrus et al., 2008; Wolff, 2009; Milatović et al., 2010). To date, differentiation of the hindgut has not been analysed at the ultrastructural level.

The focus of this study is the *in situ* analysis of apical and basal plasma membrane ultrastructural differentiation and formation of cell junctions in the hindgut epithelium during the embryonic and postembryonic development of *P. scaber*. We evaluate the major ultrastructural changes in plasma membrane shape and formation of cell junctions with respect to the crucial steps in embryonic and postembryonic development.

2 Material and methods

2.1 Specimens of *Porcellio scaber*

Specimens of *P. scaber* were maintained in a laboratory culture in a glass terrarium with ground cover of soil and leaf litter. Animals were bred at 25 °C, in high humidity and in a 12h light/12h dark cycle. Embryos and marsupial mancae were isolated from marsupia of gravid females. The developmental stages of embryos were identified according to Milatović et al. (2010) and marsupial mancae were classified into three consecutive stages according to Mrak et al. (2012). To obtain postmarsupial mancae, gravid females were held in plastic petri dishes with moist filter paper and dry leaf until the mancae were released from the marsupium and they were sampled 3 days and 2 weeks after the release (2 different stages). The developmental stages included in this study are: late-stage embryo S16 (first stage with complete alimentary canal), late-stage embryo S18 (stage when the first hindgut cuticle is deposited), early-stage marsupial manca, mid-stage marsupial manca (first two stages capable of ingesting food material after hatching from the vitelline membrane), late-stage marsupial manca (stage when the hindgut cuticle is renewed for the first time) and 2 stages of postmarsupial manca (stages associated with the transition to the external environment).

2.2 Sample preparation for light and transmission electron microscopy and imaging

The histological structure of hindgut epithelium and the ultrastructure of apical and basal plasma membrane labyrinths and cell junctions in hindgut epithelial cells were investigated in at least 3 specimens of each developmental stage. In all specimens the anterior and posterior region of the hindgut were inspected. Specimens were chemically fixed in aldehydes. All analysed specimens and preparation procedures are shown in detail in the Supplementary Information (SI), Table 1. Embryonic envelopes of late-stage embryos were perforated by a thin needle prior to fixation. After the fixation specimens were rinsed in the same buffer that was used in fixation, post-fixed in 1% OsO₄ and rinsed again in buffer. Before the embedding specimens were dehydrated in a series of ethanol solutions of increasing concentrations and transferred to absolute acetone. After the dehydration, samples were infiltrated and embedded in epoxy resin Agar 100. The exoskeletal cuticle of mancae was perforated by a thin needle before the embedding for better resin infiltration. The resin was thermally polymerised at 60 °C for 48 h in flat embedding molds. Semithin and ultrathin sections were cut with glass and diamond knives on a Reichert Ultracut S ultramicrotome (Leica). Serial semithin sections were prepared in the anterior-posterior axis of the embryo/manca and at the definite anterior and posterior regions the ultrathin sections were made. Semithin sections were stained with Azure II – Methylene Blue and inspected with AxioImager Z.1 light microscope (Zeiss). Light micrographs were acquired with a HRc AxioCam camera using Axiovision software. Ultrathin sections were contrasted with 1% uranyl acetate and 0.5% lead citrate and examined with a Phillips CM100 transmission electron microscope. Electron micrographs were acquired with Bioscan 792 and Orius SC200 (Gatan) cameras using Digital Micrograph software.

2.3 Measurements of cell size and apical labyrinth depth

Measurements of cell size and apical labyrinth depth were carried out with ImageJ/Fiji software for processing and analysis of digital micrographs. Cell height and cell width were measured on 3 individual animals of each analysed developmental stage. In each animal, 4 cells in the anterior chamber and 4 cells in the papillate region were measured on electron micrographs with the straight line tool in Fiji (SI Fig. 1A). Only cells sectioned across the cell

nucleus were measured. Results are presented graphically with a scatter plot depicting mean values of cell height against mean values of cell width for each developmental stage. Apical labyrinth depth was measured on 3 early-stage marsupial mancae, 3 mid-stage marsupial mancae, 3 postmarsupial mancae 3 days after the release from the marsupium and 3 postmarsupial mancae 2 weeks after the release from the marsupium. In each animal apical labyrinth depth was measured on 4 cells in the anterior chamber and 4 cells in the papillate region. For measurements of apical labyrinth depth, the edges of membrane labyrinths were outlined with a segmented line tool and overlays of a grid with 0.5 μm spacing were made on electron micrographs in Fiji. Apical labyrinth depth was measured with a straight line tool perpendicular to the apical surface (SI Fig. 1B). Measurements were made at every intersection of grid lines with the outline of labyrinth edge, and mean depth for every cell was calculated. Results are presented graphically with a scatter plot depicting mean values of apical labyrinth depth in the papillate region against mean values of the apical labyrinth depth in the anterior chamber for 4 measured stages of mancae.

3 Results

3.1 Cell shape remodelling

Epithelial cells in both hindgut regions of late-stage embryos (S16 and S18) are columnar with the cell nuclei elongated in an apico-basal direction (Figs. 1A, B). In early-stage marsupial mancae, the cells become isodiametric with round cell nuclei. The apical cell parts protrude into the hindgut lumen in the anterior chamber (Fig. 1C) and the basal parts protrude towards the haemocoel in the papillate region (Fig. 1D). During the subsequent development of marsupial and postmarsupial mancae the cell shape remodelling, which approaches the characteristic shape in adults, gradually becomes more pronounced. In postmarsupial mancae cells in the anterior chamber acquire dome-shaped apical parts and cells in the papillate region acquire dome-shaped basal parts (Figs. 1E, F).

Fig. 1. Shape of hindgut epithelial cells in different developmental stages. A. Late-stage embryo S16. B. Late-stage embryo S18. C. Early-stage marsupial manca – anterior chamber. D. Early-stage marsupial manca – papillate region. E. Postmarsupial manca – anterior chamber. F. Postmarsupial manca – papillate region. Hindgut epithelium (HE), hindgut lumen (HL), digestive glands (DG), haemocoel (HC).

In both hindgut regions of analysed late-stage embryos, the cell height is greater than the cell width. In analysed marsupial mancae the hindgut cells in the anterior chamber exhibit approximately equal cell height and cell width, while the cells in the papillate region are still slightly higher than they are wide. Hindgut cells in the papillate region of analysed postmarsupial mancae exhibit approximately equal cell height and width, while cells in the anterior chamber are slightly distended in width (Figs. 2A, B).

Fig. 2. Cell height and cell width in the anterior chamber (A) and in the papillate region (B). Scatter plots depict the mean values of cell height against the mean values of cell width in the two hindgut regions of late-stage embryos S16 (S16), late-stage embryos S18 (S18), early-stage marsupial mancae (EMM), mid-stage marsupial mancae (MMM), late-stage marsupial mancae (LMM), postmarsupial mancae 3 days after release from marsupium (PMM1) and postmarsupial mancae 2 weeks after release from the marsupium (PMM2). The central line is the line of equality. Points lying on this line indicate equal height and width of hindgut cells. Points lying above the line indicate that cell height is greater than cell width. Points lying below the line indicate that cell width is greater than cell height.

3.2 Formation of apical plasma membrane infoldings and junctions with the cuticle

In late-stage embryos S16, the apical plasma membrane of cells along the entire hindgut is almost flat and forms only small bulges with electron-dense tips (Fig. 3A). A similar shape of the apical plasma membrane is present in late-stage embryos S18 (Fig. 3B), when the first cuticle is formed beneath the precuticular matrix. The apical membrane folding is first apparent in hindgut cells of early-stage and mid-stage marsupial mancae. The apical plasma membrane in both hindgut regions forms apical folds, which enclose irregularly arranged cytoplasmic strands separated by distended extracellular spaces (Figs. 3C, D). Abundant mitochondria are evident within the cytoplasmic strands in the anterior chamber and are concentrated in the cytoplasm just beneath the folds in the papillate region.

In both hindgut regions of late-stage marsupial mancae, the apical plasma membrane is shaped into bulges with electron-dense tips similar to the membrane in late-stage embryos S18, a stage of the first cuticle formation. In late-stage marsupial mancae a new cuticle is synthesised beneath the old detached cuticle (Fig. 3E) and in the narrow space between the apical plasma membrane and the thin new cuticle accumulations of electron-dense material are visible above the electron-dense tips of the membrane bulges (Fig. 3F).

Fig. 3. Apical plasma membrane shape in hindgut cells of late-stage embryos and marsupial mancae. A, B. Apical plasma membrane in hindgut cells of late-stage embryos. In late-stage embryos S16 (A) and late-stage embryos S18 (B) apical plasma membrane forms only small

bulges with electron-dense tips (white arrowheads). In both stages the apical epithelial surface is lined by a loose precuticular matrix (PM). In late-stage embryos S18 the first cuticle (CUT) is visible as a thin electron-dense lamina above the apical plasma membrane. The inset in A depicts the boxed area at higher magnification. C, D. Apical plasma membrane in hindgut cells of early-stage marsupial mancae. In anterior chamber (C) and papillate region (D) the apical plasma membrane forms apical folds (AF) with irregular arrangement. E, F. Apical plasma membrane in hindgut cells of molting late-stage marsupial mancae. Low magnification electron micrograph (E) depicting the old cuticle (OC) detached from the apical plasma membrane and a thin new cuticle (NC) present beneath. High magnification electron micrograph (F) of the boxed area in figure E providing a detailed view of the apical plasma membrane which forms bulges with electron-dense tips (white arrows). Cuticle (CUT), mitochondria (M).

In postmarsupial mancae three days after the release from the marsupium, the apical plasma membrane forms a membrane labyrinth in both hindgut regions. In the anterior chamber, cytoplasmic strands are irregularly arranged and separated by distended extracellular spaces, as in early-stage and mid-stage marsupial mancae (Fig. 4A). The apical infoldings however, are considerably deeper and more numerous mitochondria are evident throughout the cytoplasmic strands. Both elongated and circular section profiles of mitochondria were observed. In the papillate region, cytoplasmic strands and extracellular spaces are densely stacked and parallel (Fig. 4B) and the apical infoldings are deeper than in early-stage and mid-stage marsupial mancae. The cytoplasmic strands contain numerous mitochondria which are concentrated at the labyrinth base and display mostly circular section profiles.

In postmarsupial mancae two weeks after the release from the marsupium, the apical plasma membrane in the anterior chamber forms an apical labyrinth of relatively straight cytoplasmic strands and extracellular spaces. The cytoplasmic strands are densely stacked, have parallel arrangement and are aligned in the apico-basal direction (Fig. 4C). These strands have approximately the same width as the extracellular spaces, but they are broader where they contain the numerous mitochondria. Mostly elongated section profiles of mitochondria were observed throughout the cytoplasmic strands from their bases to the apices. The apical plasma membrane in the papillate region forms an apical labyrinth of densely stacked and convoluted cytoplasmic strands and extracellular spaces (Fig. 4D). The cytoplasmic strands contain numerous mitochondria, which are concentrated near their bases and are only rarely present nearer to the cell apex. Mostly circular section profiles of mitochondria were observed within the cytoplasmic strands.

Fig. 4. Apical membrane labyrinth in hindgut cells of postmarsupial mancae. A, B. Apical membrane labyrinth in hindgut cells of postmarsupial mancae 3 days after the release from the marsupium. In the anterior chamber (A) apical plasma membrane forms apical infoldings with an irregular arrangement. In the papillate region (B) the apical infoldings have a more regular parallel arrangement. C, D. Apical membrane labyrinth in hindgut cells of postmarsupial mancae 2 weeks after the release from the marsupium. In the anterior chamber (C) apical infoldings have a regular parallel arrangement. In the papillate region (D) apical infoldings are highly convoluted. The inset in D depicts the boxed area at higher magnification where extracellular spaces (black arrows) and cytoplasmic strands (white arrows) are visible. Apical labyrinth (AL), cuticle (CUT), mitochondria (M).

Measurements of the apical labyrinth depth indicate that the apical labyrinth in both hindgut regions is deeper in postmarsupial mancae than in the early-stage and mid-stage marsupial mancae. In postmarsupial mancae three days after the release from the marsupium the apical labyrinth is of approximately equal depth in both hindgut regions, while in the postmarsupial mancae two weeks after the release from the marsupium it becomes deeper in the papillate region (Fig. 5).

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Fig. 5. Apical labyrinth depth in hindgut cells of marsupial and postmarsupial mancae. A scatter plot depicts the mean values of apical labyrinth depth in the papillate region against the mean values of apical labyrinth depth in the anterior chamber of early-stage marsupial mancae (EMM), mid-stage marsupial mancae (MMM), postmarsupial mancae 3 days after release from marsupium (PMM1) and postmarsupial mancae 2 weeks after release from the marsupium (PMM2). The central line is the line of equality. Points lying on this line indicate equal depth of apical labyrinth in both hindgut regions. Points lying above the line indicate that the apical labyrinth is deeper in the papillate region. Points lying below the line indicate that the apical labyrinth is deeper in the anterior chamber.

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In all stages of marsupial and postmarsupial mancae, junctions between the apical plasma membrane and cuticle are present. These junctions are visible as electron-dense plaques at the apices of cytoplasmic strands and are structurally the same in both hindgut regions. Electron-dense material is often observed extending from the electron-dense plaques into the procuticle. This material is less electron-dense and tuft-like in early-stage and mid-stage marsupial mancae (Figs. 6A, B), while the connections are more distinct and assume the form of fibrils in postmarsupial mancae (Fig. 6C). The fibrils are more abundant at the cell periphery and in molting specimens of late-stage marsupial mancae electron-dense fibrils are sometimes visible in the exuvial space between the old detached cuticle and the apical plasma membrane (Fig. 6D).

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Fig. 6. Ultrastructure of junctions between apical plasma membrane and cuticle. A, B. Junctions with cuticle in hindgut cells from the anterior chamber of early-stage marsupial mancae. Junctions are visible as electron-dense plaques (arrowheads in A) at the tips of apical cytoplasmic strands. At higher magnifications tuft-like material (arrows in B) extending from the electron-dense plaques into the procuticle is visible. C. In the anterior chamber of postmarsupial mancae the electron-dense material extending from the electron-dense plaques (arrowheads) into the procuticle is in the form of fibrils (arrow). D. In the papillate region of molting late-stage marsupial mancae electron-dense fibrils (arrows) are visible between the apical plasma membrane and the detached partly degraded cuticle (CUT). Cuticle (CUT).

3.3 Formation of the basal membrane labyrinth and hemidesmosome-like junctions with basal lamina

In late-stage embryos (S16 and S18) the hindgut cells along the entire hindgut exhibit flat basal plasma membranes; and the hemidesmosome-like junctions are visible as electron-dense plaques at the cytoplasmic side of the basal plasma membrane (Fig. 7A). In early-stage and mid-stage marsupial mancae the first individual infoldings of the basal plasma membrane

begin to form in the anterior chamber (Fig. 7B). The basal plasma membrane in the papillate region is flat with large mitochondria frequently present in the basal cytoplasm, close to the membrane. Basal infoldings are occasionally present only in the lateral regions of basal plasma membrane. In late-stage marsupial mancae the basal plasma membrane of hindgut cells in both hindgut regions forms infrequent basal infoldings (Fig. 7C). The basal infoldings in the papillate region are in close contact with some of the mitochondria in the basal cytoplasm. The infoldings are more common and deeper towards the cell periphery, while in the central region beneath the cell nucleus the basal plasma membrane is often completely flat. In early-stage, mid-stage and late-stage marsupial mancae, many hemidesmosome-like junctions (Fig. 7D) are present along the entire basal plasma membrane, but are more concentrated at the cell periphery. The hemidesmosome-like junctions often form complexes with similar electron-dense structures in muscle cells at the opposite side of the basal lamina (Fig. 7E). The structure and distribution of hemidesmosome-like junctions is similar in both hindgut regions.

A well-established basal membrane labyrinth is first present in the hindgut cells of postmarsupial mancae. In both stages of postmarsupial mancae the basal plasma membrane of hindgut cells in the anterior chamber forms a basal membrane labyrinth of sparse narrow membrane infoldings (Fig. 7F). The basal infoldings are shallow or sometimes even absent in the central region (beneath the cell nucleus) and deeper towards the cell periphery. In the papillate region the basal plasma membrane forms a basal membrane labyrinth of narrow basal infoldings, which are in close contact with the abundant mitochondria in the basal cytoplasm (Fig. 7G). Basal infoldings of uniform depth are present along the entire basal surface of epithelial cells. In both stages of postmarsupial mancae the abundance and the distribution of hemidesmosome-like junctions are similar to those in marsupial mancae.

Fig. 7. Basal plasma membrane shape and hemidesmosome-like junctions in the hindgut cells of late-stage embryos and mancae. A. Flat basal plasma membrane in hindgut cells from the anterior hindgut of late-stage embryo S16. At the cytoplasmic side of basal plasma membrane,

hemidesmosome-like junctions are visible (arrowheads). B. Basal plasma membrane in the anterior chamber of early-stage marsupial mancae exhibits individual basal infoldings (arrow). C – E. Basal plasma membrane and hemidesmosome-like junctions in the hindgut cells of late-stage marsupial mancae. C. Infrequent basal infoldings in hindgut cells in the anterior chamber (arrows). D. Numerous hemidesmosome-like junctions (arrowheads) in hindgut cell from the papillate region. E. Hemidesmosome-like junction (white arrowhead) in hindgut cell (EC) from the anterior chamber and a similar electron-dense structure (white arrow) in muscle cell (MC). F, G. Basal labyrinth in hindgut cells of postmarsupial mancae. In the anterior chamber (F) narrow basal infoldings (arrows) form a basal labyrinth. In the papillate region (G) infoldings of basal labyrinth (arrows) are associated with numerous mitochondria (M) in the basal cytoplasm. Basal lamina (BL), hindgut epithelial cell (EC), muscle cell (MC), mitochondria (M).

3.4 Interdigitations of lateral plasma membrane and differentiation of adherens and septate junctions

In late-stage embryos (S16 and S18) the lateral plasma membranes are relatively straight and are not interdigitated between neighbouring cells (Figs. 8A, B). In all three stages of marsupial mancae however, the lateral plasma membranes are already interdigitated between neighbouring hindgut cells. The section profiles of lateral plasma membranes are often very convoluted, and this is particularly pronounced in the anterior chamber. The lateral plasma membranes in the anterior chamber are more deeply interdigitated in the basal part (Figs. 8C, D) while in the papillate region they are more deeply interdigitated in the apical part (Figs. 8E, F). In postmarsupial mancae, the lateral plasma membranes in the anterior chamber are even more intensely interdigitated, section profiles are convoluted and the area of lateral interdigitations occupies a large proportion of the cell section profile. As in marsupial mancae the lateral plasma membranes are more deeply interdigitated in the basal part. In the papillate region lateral plasma membranes are also highly interdigitated and the section profiles are convoluted. The area of lateral interdigitations is confined more apically than in the anterior chamber, since the basal epithelial surface ingresses between the dome-shaped basal parts of epithelial cells. In the lateral cytoplasm close to the lateral interdigitations abundant microtubules are observed in both hindgut regions (Fig. 8G).

Fig. 8. Shape of lateral plasma membranes in the hindgut cells of late-stage embryos and manca. A, B Lateral plasma membranes in late-stage embryos S18. Lateral plasma membranes (white arrows) between two neighbouring hindgut epithelial cells (EC 1, EC 2) are straight both in the subapical region (A) and in the more basal parts (B). In the subapical

region adherens junction (black arrow) is visible. C – F. Lateral plasma membranes in hindgut cells of early-stage marsupial mancae. In the anterior chamber, lateral plasma membranes in the subapical region (C – white arrow) are only slightly curved. In basal parts lateral plasma membranes form deep convoluted interdigitations (D – white arrows). In the papillate region lateral plasma membranes form convoluted interdigitations in the subapical region (E – black arrows) and straighten basally (F – black arrow). G. Microtubules (MT) in the vicinity of lateral plasma membrane (white arrow) in hindgut cell of postmarsupial manca 2 weeks after the release from the marsupium. Cuticle (CUT), basal lamina (BL), neighbouring epithelial cells (EC 1, EC 2).

Prominent adherens junctions are present in the hindgut cells of all the developmental stages analysed and are located in the subapical region. Ultrastructurally, these junctions are visible as two electron-dense plaques at the cytoplasmic sides of two neighbouring lateral plasma membranes with material of intermediate electron density in the intercellular space (Figs. 9A, B, C). Actin filaments, visible as fibrillar electron-dense material, attach to the electron-dense plaques of adherens junctions.

Fig. 9. Ultrastructure of adherens junctions in hindgut cells of late-stage embryos and mancae. A. Adherens junction in hindgut cells of late-stage embryo S18. B. Adherens junction in hindgut cells of early-stage marsupial manca. C. Adherens junction in hindgut cells of postmarsupial manca 2 weeks after the release from the marsupium. Electron-dense plaques at the cytoplasmic side of lateral plasma membrane (arrows).

In all the developmental stages that were inspected, the septate junctions were observed basally to the adherens junctions. In the entire hindgut of late-stage embryos S16 only short, straight and often interrupted strings of electron-dense septa are present in the intercellular space between the two neighbouring lateral plasma membranes (Fig. 10A). In early-stage and mid-stage marsupial mancae, the strings of electron-dense septa are longer when compared to embryos. Septate junctions in the anterior chamber of marsupial mancae are straight or slightly curved (Fig. 10B) and shorter than the convoluted septate junctions in the papillate region (Fig. 10C). In late-stage marsupial mancae the septate junctions in both hindgut regions are short, approximately straight and in some cases interrupted (Fig. 10D).

In postmarsupial mancae three days after the release from the marsupium, septate junctions are similar to those in early-stage and mid-stage marsupial mancae. In postmarsupial mancae two weeks after the release from the marsupium, extensive septate junctions are present in both hindgut regions. The strings of electron-dense septa in the papillate region are longer and more convoluted than in the anterior chamber (Figs. 10E, F).

Fig. 10. Ultrastructure of septate junctions in hindgut cells of late-stage embryos and mancae. A. Septate junctions (SJ) in hindgut cells from the anterior hindgut of late-stage embryo S16 visible as a short string of electron-dense septa. B, C. Septate junctions (SJ) in hindgut cells of mid-stage marsupial manca visible as longer strings of electron-dense septa. In the anterior chamber (B) strings of electron-dense septa are less convoluted than in the papillate region (C). D. The short septate junctions (SJ) in hindgut cells of late-stage marsupial manca. E, F. Septate junctions (SJ) in hindgut cells of postmarsupial manca 2 weeks after the release from the marsupium. In the anterior chamber (E) strings of electron-dense septa are shorter and less convoluted than in the papillate region (F). Adherens junction (AJ).

4 Discussion

4.1 Common ultrastructural features of apical and basal plasma membrane folding in hindgut cells of *P. scaber* and other epithelial cells of arthropods during development

In epithelia the apical plasma membrane is often expanded in surface area by shaping into microvilli and in cuticle-covered ectodermal epithelia of arthropods into apical infoldings (Cioffi, 1984; Lehane, 1998; Noble-Nesbitt, 1998). In absorptive epithelial cells in the midguts of insects and crustaceans the apical plasma membrane is shaped into microvilli of uniform size and shape which are very regularly arranged into a brush border (Martoja and Ballan-Dufrançais, 1984; Icely and Nott, 1992; Billingsley and Lehane, 1996; Lehane, 1998; Ceccaldi, 2006; Shanbhag and Tripathi, 2009). In the hindgut cells in the anterior chamber of adult *P. scaber* the apical plasma membrane is intensely infolded (Bogataj et al., 2018). These cells assist in the absorption of digestive products (Hryniewiecka-Szyfter and Storch, 1986), which is primarily performed by the digestive gland cells of endodermal origin (Hames and Hopkin, 1989). Hindgut cells with absorptive function that also exhibit apical infoldings are known to form the proctodeal fermentation chambers of certain insects (Bignell, 1980; Martoja and Ballan-Dufrançais, 1984). The hindgut cells in the papillate region of adult *P. scaber* with their intensely infolded apical and basal plasma membranes associated with numerous mitochondria (Bogataj et al., 2018) have a typical ultrastructure of ion and water transporting epithelial cells of arthropods present in hindguts of insects and decapod crustaceans (Martoja and Ballan-Dufrançais, 1984; Icely and Nott, 1992; Noble-Nesbitt, 1998; Ceccaldi, 2006), salivary glands of insects (Martoja and Ballan-Dufrançais, 1984) and crustacean gills (Taylor and Taylor, 1992; Freire et al., 2008). The apical and basal infoldings increase the available surface area and provide limited extracellular spaces where the ion gradients can build up (Cioffi, 1984). The associated mitochondria provide the ATP for ion pumps located on the membranes (Weihrauch and O'Donnell, 2015).

Our observations revealed that the formation of apical labyrinth in *P. scaber* hindgut begins in early-stage marsupial mancae with the formation of the first apical membrane folds that are arranged irregularly. These folds become deeper and more numerous during the subsequent development and gradually acquire a regular arrangement present in postmarsupial mancae. The organization of apical plasma membrane in hindgut cells of postmarsupial mancae two weeks after the release from the marsupium closely resembles that in the adults (SI Figs. 2A, B), but the apical labyrinth in adult animals is considerably deeper (Bogataj et al., 2018). The entire process of apical labyrinth formation is interrupted due to molt-related changes in late-stage marsupial mancae. The sequential stages of the apical membrane remodelling into deeper, more numerous and regularly positioned membrane folds in crustacean hindgut cells

described here, to some extent, resemble the steps in apical microvilli formation in the midgut of insects. Generally, the microvilli also gradually increase in number and length and acquire their parallel arrangement perpendicular to the apical surface during development (van der Starre-van der Molen and de Priester, 1972; Martoja and Ballan-Dufrançais, 1984; Hakim et al. 1988). However, in the midgut of certain insects a different mode of microvillar surface formation has been described. Authors of these studies report that the microvilli are pre-formed on the membranes limiting the dilated intercellular spaces between differentiating cells and elaborated microvilli are subsequently exposed to the apical surface (Kadiri and Louvet, 1982; Martoja and Ballan-Dufrançais, 1984; Rost-Roszkowska and Klag, 2011).

The formation of basal labyrinth in *P. scaber* is slightly delayed relative to the apical labyrinth and also proceeds gradually. The first individual basal infoldings appear in the anterior chamber cells of early-stage marsupial mancae, when the apical labyrinth in these cells is already developed. During the subsequent development the basal infoldings increase in number and in the papillate region become associated with numerous mitochondria. The basal labyrinth in the anterior chamber of postmarsupial mancae already resembles that in the adult animals (SI Fig. 2C), while the basal labyrinth in the papillate region of adult animals is considerably deeper and exhibits more densely stacked infoldings and more numerous mitochondria (SI Fig. 2D) (Bogataj et al., 2018). Similarly, the formation of apical microvilli also precedes the basal infoldings formation in the midgut cells of insects (Hakim et al., 1988; Baldwin et al., 1996; Rost-Roszkowska et al., 2007) and in the labyrinth cells in the antennal glands of the crayfish *Astacus leptodactylus* (Khodabandeh et al., 2005). The exception among insects is the stick insect *Clitumnus extradentatus*, for which Kadiri and Louvet (1982) reported, that the basal infoldings in the midgut cells form prior to apical microvilli.

4.2 Comparison of ultrastructural differentiation of cell junctions in *P. scaber* hindgut and other arthropod epithelia

In general, a subapical junctional complex in epithelia consists of adherens junctions that provide the adhesion between epithelial cells and occluding junctions which establish a paracellular diffusion barrier. In arthropods the occluding function is provided by the septate junctions (Tepass and Hartenstein, 1994; Izumi and Furuse, 2014; Jonusaite et al. 2016), while in vertebrates this function is provided by the tight junctions (Giepmans and van Ijzendoorn, 2009). However, the tight junctions have also been reported in various tissues of arthropods (Lane and Chandler, 1980; Lane, 1982). The septate junctions in arthropods are located basally to the adherens junctions and contain proteins that are determinants of the basolateral membrane domain (Tepass et al., 2001; Knust and Bossinger, 2002). In arthropods two ultrastructurally distinct types of septate junctions are known. The pleated septate junctions are present in ectodermally derived tissues including the foregut and hindgut and the smooth septate junctions are present in tissues of endodermal origin including the midgut (Lane, 1982).

In our investigation, adherens junctions of uniform ultrastructural appearance were observed in the subapical region of hindgut cells in all analysed developmental stages. This shows that adherens junctions are already fully formed before the late-stage embryo S16 and that they do not change noticeably afterwards. This is in agreement with observations in *Drosophila*, where spot adherens junctions begin to form during cellularization of blastoderm and fully formed belt adhesion junctions are present from gastrulation onwards in all ectodermal epithelia including the hindgut (Tepass and Hartenstein, 1994).

Septate junctions were observed in all investigated developmental stages of *P. scaber*, but the strings of electron-dense septa in the intercellular space were shorter and more frequently interrupted in late-stage embryos than in subsequent stages of early-stage and mid-stage marsupial mancae. We clearly show that the extent of septate junctions on lateral plasma membranes gradually increases during late embryonic and early postembryonic development and the extensive septate junctions in the hindgut of postmarsupial mancae two weeks after the release from the marsupium closely resemble those in the hindgut of adult animals (SI Figs. 2E, F) (Bogataj et al., 2018). A similar sequence was reported in *Drosophila* ectodermal epithelia where the first septa appear at embryonic stage 14 and additional septa are continuously added during subsequent embryonic stages until hatching (Tepass and Hartenstein, 1994).

Studies on insects demonstrated that the septate junction proteins are localized to the lateral plasma membranes before the first septa can be discerned (Lane and Swales, 1982; Tiklova et al., 2010). The occluding function is established subsequently (Paul et al., 2003). Besides that, certain septate junction proteins have important roles in various morphogenetic events, well before the occluding function is established (Hall and Ward, 2016). In this regard further molecular studies are required in *P. scaber* and other arthropod species to elucidate common features and possible differences in the molecular composition and assembly of septate junctions among the arthropods.

The junctions between the apical plasma membrane and the cuticle were observed in *P. scaber* hindgut from the early-stage marsupial mancae onwards. This corresponds with the replacement of hindgut precuticular matrix in embryos with the first hindgut cuticle in early-stage marsupial mancae (Mrak et al., 2015). In postmarsupial mancae and adult animals (Bogataj et al., 2018) these junctions ultrastructurally resemble connections with the cuticle in epidermal tendon cells, which serve as attachments of muscles to exoskeleton (Žnidaršič et al., 2012). In *Drosophila* apical hemiadherens junctions with cuticle were reported only in epidermis and appear in first instar larvae (Tepass and Hartenstein, 1994). Hemidesmosome-like junctions with basal lamina were present in all the developmental stages of *P. scaber* that were analysed. No alterations in their ultrastructure were noted, but they became concentrated in the peripheral regions of basal plasma membrane in marsupial mancae. In comparison to postmarsupial mancae, adult animals display more numerous hemidesmosome-like junctions in the papillate region (Bogataj et al., 2018). In *Drosophila* basal hemiadherens junctions form in the second half of embryogenesis in all ectodermal epithelia together with the first appearance of basal lamina (Tepass and Hartenstein, 1994).

4.3 Remodelling of plasma membrane and septate junctions in *P. scaber* hindgut during de novo formation and renewal of apical extracellular matrix

The apical plasma membrane of hindgut cells in late-stage embryos S16 and S18 and in late-stage marsupial mancae is shaped into bulges with electron-dense tips. In these three developmental stages a new apical extracellular matrix is formed. In late-stage embryo S16 a precuticular matrix is synthesised, in late-stage embryos S18 the first cuticle is formed and in late-stage marsupial mancae the cuticle is renewed during the molt. A similar organization of apical plasma membrane in bulges with electron-dense tips is also present in the epidermal cells of the same developmental stage that produce the exoskeletal cuticle (Mrak et al., 2014). Studies of cuticle formation in ectodermal epithelia of *Drosophila* have shown, that such a membrane architecture is necessary for the correct organization and orientation of chitin-protein fibers within the newly assembled procuticle (Moussian et al., 2006; Moussian et al.,

2007; Uv and Moussian, 2010; Moussian, 2013). Evidently, the already infolded apical plasma membrane in the hindgut cells of early-stage and mid-stage marsupial mancae is remodelled in molting late-stage marsupial mancae and acquires a topology characteristic for cuticle producing arthropod epithelia, which can facilitate the appropriate cuticle formation.

Another ultrastructural feature in *P. scaber* hindgut cells that is strikingly modified in molting late-stage marsupial mancae are the septate junctions. Septate junctions are considerably shorter in this stage than in the previous manca stages, which exhibit relatively long and convoluted strings of intercellular septa. This ultrastructural change in hindgut of late-stage marsupial mancae is probably associated with the formation of a new cuticle or with a feeding pause in molting specimens, but currently there are no other reports of a similar remodelling of septate junctions in relation to chitinous apical matrix renewal in other arthropods.

Nonetheless, the septate junctions seem to be required for the correct formation of chitinous matrix in arthropods as shown by several genetic studies in *Drosophila*. Mutations in *coracle*, which encodes one of the key components of septate junctions, cause defects in cuticle secretion during embryonic development. In mutant embryos the secreted cuticle is thinned and displays delamination between the epicuticle and the procuticle (Lamb et al., 1998). During the differentiation of the tracheal system, septate junctions are also necessary for the apical secretion of two chitin deacetylases (Vermiform and Serpentine), which are required for the correct organization of luminal chitinous matrix (Wang et al., 2006; Nelson et al., 2010). In addition, the transcription factor Grainy head in *Drosophila* regulates the expression of some of the key enzymes that cross-link the apical chitinous matrix and some of the proteins that are the key components of septate junctions (Narasimha et al., 2008; Boivin and Schmidt-Ott, 2017). To conclude, in this stage of research we cannot comprehensively interpret the functional significance of hindgut septate junctions remodelling in molting specimens, but our results clearly show that septate junctions in the hindgut epithelial cells are highly dynamic structures with the ability to form and disassemble in accordance with functional demands.

4.4 Ultrastructural modifications of hindgut cells during differentiation correspond with crucial transitions in *P. scaber* embryonic and postembryonic development

Two main developmental transitions to which *P. scaber* is subject during its late embryonic and early postembryonic development are the hatching from the inner embryonic envelope (vitelline membrane) and the release from the marsupium into the external environment (Wolff, 2009; Milatović et al., 2010). These transitions are associated with changes in feeding and osmoregulation, that likely demand increased apical and basal surfaces of hindgut cells for transport of digestion products, ions and water and stronger paracellular barrier provided by septate junctions.

The early-stage marsupial manca is the earliest stage in which the hindgut cells begin to acquire their characteristic dome shapes and the first foldings of their apical membranes. Additionally, the septate junctions are evidently more extensive than in late-stage embryos. These characteristics are likely associated with the onset of active feeding and absorption of digestion products in the hindgut. We observed that the hindgut lumen in late-stage embryos was always empty, as the embryos rely on yolk stored in the digestive glands as their nutrient supply. At the end of embryonic development, embryos hatch from the vitelline membrane; thus the early-stage marsupial mancae can begin to ingest contents of marsupial fluid. The yolk is still an important nutrient source as indicated by gradual yolk reduction in the

digestive glands during marsupial manca development (Milatović et al., 2010; Mrak et al. 2012). As observed in the present study the hindgut lumen contains densely stained homogenous content, often with bacteria and stacks of shed egg envelopes. The exceptions were molting late-stage marsupial mancae, which always exhibited an empty gut lumen. Hoese and Janssen (1989) have reported that the marsupial mancae are able to ingest and actively feed on contents of marsupial fluid, including mucous mass of polysaccharide matrix and even bacteria, and Warburg (1994) reported that they often cannibalize their immature or undeveloped siblings.

In postmarsupial mancae the apical labyrinth in hindgut cells is further elaborated, and this is likely associated with the transition to entirely external food sources. Postmarsupial mancae begin to ingest food available in their environment immediately after the release from the marsupium (Tomescu and Craciun, 1987; Brum and Araujo, 2007; Montesanto et al., 2012). In our study, the hindguts of all analysed postmarsupial mancae contained food particles of predominantly partially degraded plant material.

During the development of blowfly *Calliphora* the apical plasma membrane differentiation in secretory epithelial cells in the salivary glands coincides with the eclosion of an imago from a puparium. The secretory epithelial cells in the salivary glands have deeply infolded apical plasma membrane in the form of a branching system of canaliculi (Baumann and Walz, 2012). These apical infoldings begin to form shortly before the eclosion of an imago and gradually elongate during the first 24 h after the eclosion, when the salivary glands begin to perform their function (Baumann and Bauer, 2013).

The basal labyrinth in the papillate region of *P. scaber* is formed relatively late in stages of the late-stage marsupial and postmarsupial mancae, which coincides with the release from the marsupium. The hindgut epithelium in the papillate region is involved in absorption of water and osmoregulation (Palackal et al., 1984; Hames and Hopkin, 1989). Thus the formation of basal labyrinth in the papillate region is probably associated with increased osmoregulatory demand after the release of mancae from the aqueous marsupial fluid to the terrestrial environment. As reported by Surbida and Wright, (2001) the marsupial fluid is osmotically relatively well regulated. Nevertheless, the basal labyrinth in the papillate region of postmarsupial mancae is still considerably less developed than in the papillate region of adult animals, thus further elaboration of basal labyrinth in the papillate region progresses in juvenile animals.

Cell differentiation and tissue morphogenesis are complex and interrelated processes that depend on a network of interactions across multiple scales, including subcellular, cellular, tissue, organ and whole organism levels. Therefore it is advantageous also to study and analyse ultrastructural differentiation of certain cells in the context of the whole organism in its environment during development. This enables us to relate different ultrastructural characteristics of cell differentiation, including cell shape, plasma membrane modifications, cell junctions and extracellular matrix formation and additionally, to relate ultrastructural modifications to developmental transitions at the organism level.

Author contributions

UB and NŽ designed the study. UB reared the animals in a laboratory culture. UB and PM prepared the samples for electron and light microscopy. UB performed imaging with light and transmission electron microscopy. UB performed measurements on digital images and

designed the figures. PM, JŠ and NŽ contributed to the interpretation of data. UB wrote the first draft of the manuscript. PM, JŠ and NŽ contributed to the text and made numerous comments and recommendations. All authors revised the manuscript and read and approved the final version.

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