Zoology 161 (2023) 126129

Contents lists available at ScienceDirect

Zoology

journal homepage: www.elsevier.com/locate/zool

Ontogenetic dynamics of the nudibranch epithelium in *Onchidoris muricata* (O.F. Müller, 1776)

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ARTICLE INFO

Keywords:
Spicule
Sclerocyte
Doridina
Ultrastructure

ABSTRACT

The integumentary system is the set of organs forming the outermost layer of an animal's body. It comprises the epithelium, muscles, and elements of connective and nerve tissue. The integument acts as a physical barrier between the external environment and the internal environment that serves to protect and maintain the body of the animal. The body of nudibranch mollusks undergo significant changes during ontogenesis, with the subepidermal space changing as the mollusk grows. As the extracellular subepidermal matrix is modified, the number of collagen fibers increases, muscles and nerves develop, and calcite spicules appear and grow. Yet, specific knowledge pertaining to the transformation of the epithelium is absent. In the present work, the ontogenetic dynamics of the surface epithelium of nudibranch mollusks are traced for the first time using *Onchidoris muricata* (O. F. Müller, 1776) during the postlarval stages of development. Ontogenetic changes in the epithelium of *O. muricata* were studied using a complex set of morphological methods. According to our data, the degree of modification to the epithelium in ontogenesis depends on individual body parts and is not consistent throughout. First x-cells were recognized as the probable precursors to sclerocytes.

1. Introduction

Doridina is a unique group of nudibranch mollusks whose notum contains subepidermal calcite spicules (Thompson, 1961; Penney et al., 2018; Nikitenko et al., 2021). The notum of mollusks is significantly transformed at the postlarval stages of development (Thompson, 1961; Goddard, 2005; Nikitenko et al., 2021), where spicules and a collagen matrix are developed in the subepidermal space (Nikitenko et al., 2021). However, there is no information on how the epithelium is transformed. This work describes the reorganization of the surface epithelium in *Onchidoris muricata* (O. F. Müller, 1776) during post-larval stages of development.

The development of doridins occurs with the veliger larval stage (Bickell and Chia, 1979; Chia and Koss, 1978; Goddard, 2005; Thompson, 1961). The body of the dorid veliger is protected by a shell, which is lost during metamorphosis (Thompson, 1961; Chia and Koss, 1978; Goddard, 2005). Shell loss may be a signal for the beginning of integument transformation. The integument plays an important role towards survival in metazoans by separating and protecting them from hostile environments. Its function ranges from protection against injury and infection, participation in the regulation of body temperature and water balance, respiratory activity, monitoring of the environment, and production of signals related to behavior (Bereiter-Hahn et al., 2012). The integument in dorids is located directly in contact with the external environment in all post larval stages of development.

At present, the epithelium in adult nudibranchs has only been partially described in a few species of dorids (Skidmore and Rivera, 1982; Thompson, 1983; Wägele, 1997/98). The basis of the

Abbreviations: Ag, alveolar gland; Aj, adherens junction; Asg, apocrine secret gland; Bl, basal lamina; C, cilia; Chs, chitin spindle; Chv, chitin vacuole; Ci, concentric inclusion; Cr, striated roots.; Ct, ctenidia; E, eye; Ecm, extracellular matrix; Eg, epithelial gland; Ep, epithelia; Epr, endoplasmic reticulum; F, foot; Ga, Golgi apparatus; Gl, gland; Gr, secretory granule; Gs, gland secret; Ic, intercellular cell; Icm, intracellular collagen matrix; Inv, invagination; Lv, large vacuole; M, muscle; Mcc, multicellular cell; Mg, mucous gland; Msc, the membrane of sclerocyte; Mv, microvilli; Mvl, microvillar layer; Mvs, the membrane of sclerocyte vacuole; N, nuclei; Nv, nerve; Pr, protrusion of x-cell; R, rhinophore; Rl, rhinophore lamellae; Rp, rhinophore pocket; S, secrete; Sc, sclerocyte; Sg, subepidermal gland; Sj, septate junction; Sp, spicule; Sr, striated roots; T, tubercle; Tf, tonofilament; Tz, transition zone from notum to tubercle; V, vacuole; Vcf, x-cells vacuole with fine content; Vs, vesicle.

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https://doi.org/10.1016/j.zool.2023.126129
Received 3 May 2023; Received in revised form 24 August 2023; Accepted 19 October 2023
Available online 4 November 2023
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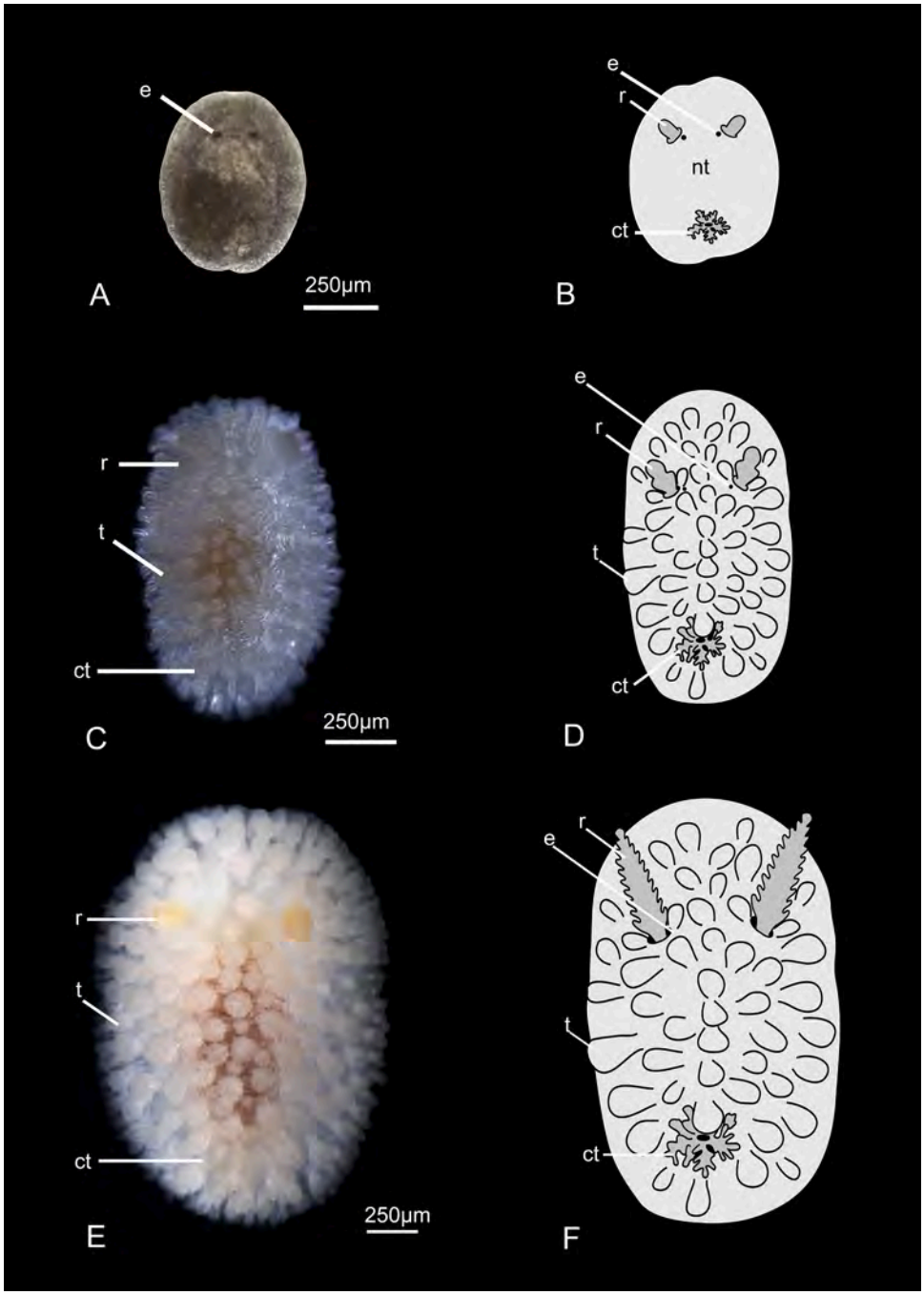

Fig. 1. External morphology of *Onchidoris muricata* body at different sizes. View from the dorsal side. (A, C, E) Intravital photos. (B, D, F) Scheme. (A, B) A newly settled juvenile. (C, D) Immature *O. muricata* with developed tubercle. (E, F) Sexually mature adult specimens with developed rhinophore lamellaes and tubercles. Abbreviation: Ct, ctenidia; E, eye; Nt, notum; R, rhinophore; T, tubercle.

integumentary epithelium is made up of ciliary epithelial and glandular cells, where the ratio of cell types depends on the part of the body. The morphology of the ciliated epithelial cells is however quite universal (Bereiter-Hahn et al., 2012). The nucleus of these cells is displaced basally and the apical part of the cells is connected by adherens and septate contacts. However, the morphology of the glandular cells is extremely diverse and disorganized. The secretions of the glands perform a variety of functions (*i.e.,* protection, locomotory), including playing a vital role in feeding (Davies and Hawkins, 1998).

The early juvenile epithelium is currently only mentioned for two species, *Trinchesia granosa* Schmeckel, 1966 and *Adalaria proxima* (Alder and Hancock, 1854) (Thompson, 1961; Schmekel and Wechsler, 1967). These studies showed there were very large intercellular spaces between the epithelial cells (juveniles) and that the cells are interconnected by desmosomes (Schmekel and Wechsler, 1967). There is however no information regarding the presence of glandular components.

Despite numerous references to the structure of the epithelium, its reorganization in ontogeny has never been studied. Understanding the ontogenetic dynamics of the integument, including the epithelium, will play an important role for future studies into the processes of forming epithelial structures across mollusks.

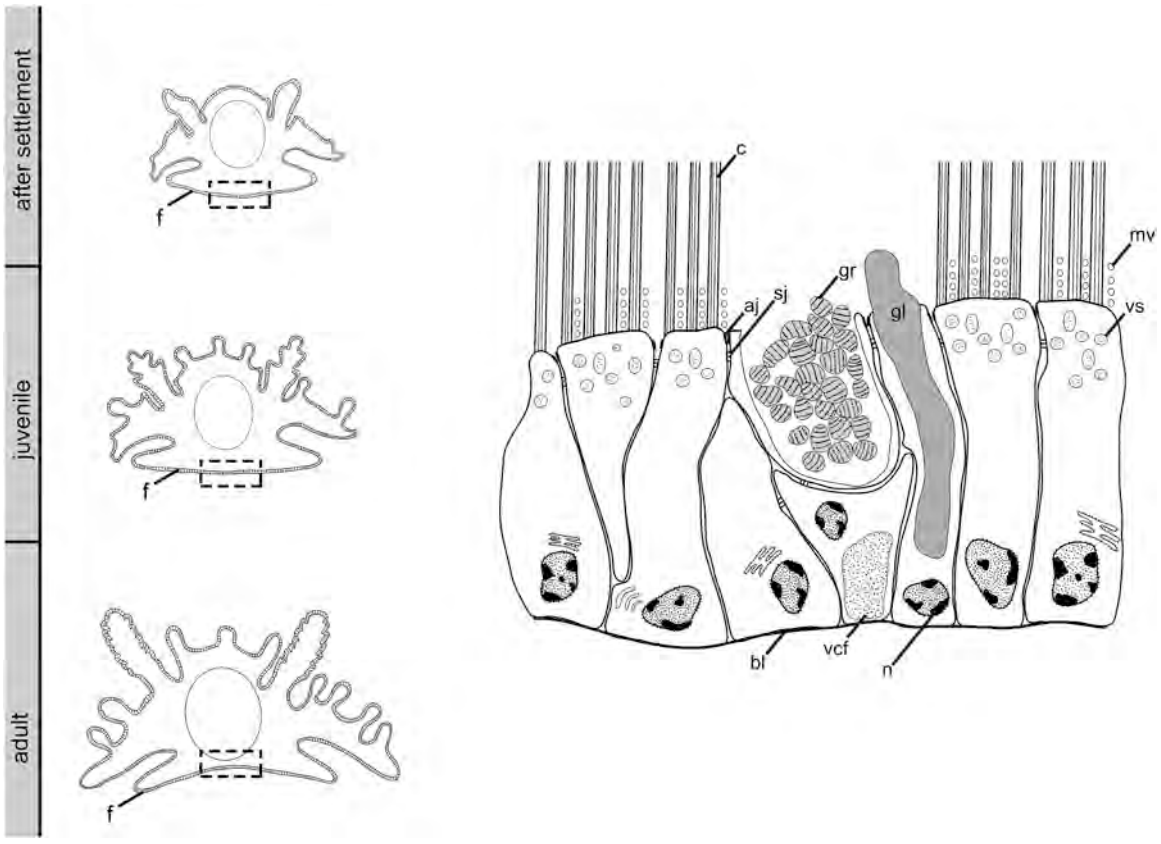

Fig. 2. Scheme of the the epithelial foot structures in *Onchidoris muricata*. Columnar multiciliary epithelium with granular and fine-grained glands. Aj adherens junction; Bl, basal lamina; C, cilia; F, foot; Gl, gland; Gr, secretory granule; Mv, microvilli; N, nuclei; Sj, septate junction; Vcf, x-cells vacuole with fine content; Vs, vesicle.

2. Materials and methods

The object of study in this work was the dorid mollusk *Onchidoris muricata* widespread throughout the White Sea. The composition of spicules, morphology, and ultrastructure of spicules have been studied in detail using *O. muricata* as an example. That is why it was chosen to study the structure and ontogenetic dynamics of the epithelium. However, similar studies need to be carried out on other representative dorids and other mollusks bearing subepidermal spicules. Specimens of *Onchidoris muricata* (Fig. 1) were collected between 12 and 15 m depth in the White Sea near the Pertsov White Sea Biological Station, Moscow State University (Kandalaksha Bay; 66°340 N, 33°080E) by SCUBA diving. The smallest, just-settled juveniles (100–1000 μm length) were sampled in meiobenthos on a large, well-washed *Modiolus modiolus* (Linnaeus, 1758) shell from 20 to 22 m depth. A total of 30 specimens of *O. muricata* from 100 μm to 12 mm in length were studied using light (n = 25), scanning- (n = 5) and transmission electron microscopy (n = 15), and confocal laser scanning microscopy (n = 5). These samples were previously studied in the framework of research by Lisova and Vortsepneva, 2022 and Nikitenko et al. 2021.

2.1. Microscopy

The features of the epithelium in *O. muricata* were studied using microscopical investigations of semi-thin and ultrathin sections. Specimens were relaxed using a magnesium chloride ($MgCl_2$) solution (5.08 g in 100 mL dH2O) isotonic to seawater for 2 h at 4 °C. Specimens were then fixed for electron microscopy and histology using 2.5% glutaraldehyde in a modified cacodylate buffer (Lavrov and Ereskovsky, 2022) for 1 h at room temperature. Afterwards, the initial solution was replaced with new, and the fixation continued for an additional 2 h. Specimens were then washed using the same buffer 3 times every 15 min. Specimens were postfixed with 1% osmium tetroxide in 0.1 M modified cacodylate buffer for 2 h. This was followed by decalcification using a 5% chelator (ethylenediaminetetraacetate, EDTA) kept at pH= 7 and at room temperature for 1 h. Decalcified specimens were washed with distilled water every 20 min for 3 h. Once rinsed, they were dehydrated in an ascending ethanol series (10%, 30%, 50%, 70%, 85%, 96%), followed by a mixture of ethanol and acetone (ratios 3:1, 1:1, 1:3). At each stage, the solution was changed twice every 20 min. Once dehydrated, the specimens were embedded in Spurr's epoxy resin. Resin impregnation was carried out through a mixture of acetone: resin (in ratios 3:1, 1:1, 1:3), each stage lasted a day.

Semi-thin- and ultrathin sections were obtained using a Leica EM UC6 and UC7 (Leica Microsystems, Witzlar, Germany) ultratomes with a diamond knife (Histo Jumbo, Diatome, Biel, Switzerland, and PELCO 12785SU, Ted Pella, USA). Semi-thin sections were stained with 1% Methylene blue (1% toluidine blue, 1% methylene blue, and 1% sodium tetraborate) for 30–60 s, then photographed using a Nikon Eclipse-200 microscope (Nikon, Japan) and an Olympus slide scanner (Olympus Medical Systems Corp., Japan). For transmission electron microscopy (TEM), all ultrathin sections were stained with 1% uranyl acetate for 45 min at 37 °C, followed by 0.4% lead citrate for 7–10 min at room temperature. Ultrathin sections were examined using transmission electron microscopes JEOL JEM-1011, JEOL JEM-100B, and JEOL JEM-1400 Flash (JEOL, Japan).

2.2. Scanning electron microscopy (SEM)

The internal structures of rhinophores and structures of notum glands were studied using scanning electron microscopy (SEM). Specimens were fixed in 2.5% glutaraldehyde, then dehydrated in an ethanol

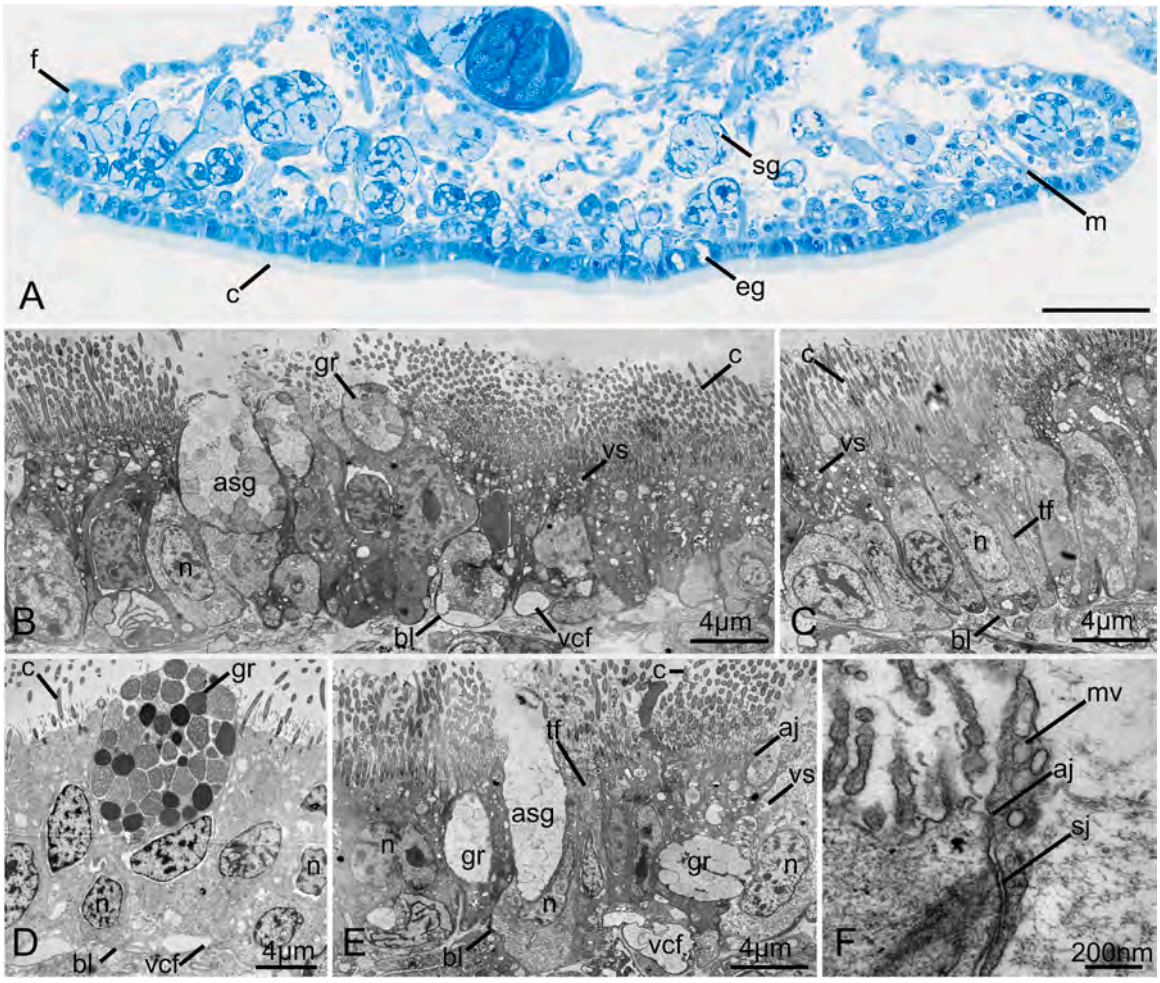

Fig. 3. Morphological features of the foot epithelium *in Onchidoris muricata.* (A) Light microscopy; (B – F) TEM. (A) Columnar multiciliary epithelium with granular and fine-grained glands. (B, D, E) Columnar multiciliary epithelium with granular and fine-grained glands. Aj, adherens junction; Asg, apocrine secretory gland; Bl, basal lamina; C, cilia; Eg, epithelial gland; F, foot; Gr, secretory granule; M, muscle; Mv, microvilli; N, nuclei; Sg, subepidermal gland; Sj, separated junction; Tf, tonofilament; Vcf, x-cells vacuole with fine content; Vs, vesicle.

series (10%, 30%, 50%, 70%, 96%). During each stage, the solution was changed three times, every 20 min. Fixations were carried out similarly in a mixture of alcohol and acetone with ratios of 3:1, 1:1, and 1:3, respectively. Then the specimens were transferred to pure acetone and critical point dried.

Dried specimens were divided into body fragments with tweezers and dissecting needles. The internal structure of the body and spicules were studied on pieces of body parts using scanning electron microscopes CamScan S2 (Cambridge Instrument Scientific Company, Great Britain) and JEOL JSM7000 and JEOL JSM-6380LA (Jeol, Tokyo, Japan).

2.3. Confocal laser scanning microscopy (CLSM)

The features of the rhinophore epithelium were studied using cLSM Nikon A1R-A1 confocal microscope (Nikon Corporation, Japan). Specimens of *O. muricata* were fixed in 4% paraformaldehyde solution (PFA) in 0.1 M phosphate buffer for 4 h at room temperature after being relaxed in $MgCl_2$ (5.08 g in 100 mL dH2O). Following fixation, specimens were washed with 0.1 M PBS 3–5 times over 30 min. The nucleus was stained with Propidium Iodide solution dye (PI, 5 μg/mL in PBS, Sigma), muscles were treated with Phalloidin fluorescein (FITC, F432, Sigma-Aldrich) and Phalloidin (TRITC, P1951, Sigma-Aldrich), and the unpolymerized polysaccharides were stained using Calcofluor-White (Fluorescent Brightener 28, Sigma F3543). Samples were placed in a solution of glycerol with an ascending concentration (10%, 30%, 50%, 70%).

2.4. Three-dimensional reconstruction (3D reconstruction)

Visualization of subepidermal glands in the tubercle were carried out using 3D reconstructions. All reconstructions followed that of Ruthensteiner and Hess (Ruthensteiner, Heß 2008). Digitized image stacks were converted to 8-bit format, aligned using AMIRA 5.2.2 (Amira Visaging GmbH, Germany), and reconstructed using Imaris 7.0.0 (BitplaneAG, Zurich, Switzerland).

2.5. Terminology

The features of the molluscan epithelium have been described numerous times, often with varying terminologies. Below we discuss the existing terms and ones used throughout this work.

Cuticle. The term "cuticle" has been controversial for mollusks and often confusing (see Bereiter-Hahn et al., 2012). However, we consider this term appropriate, and refer to the outer microvillous layer as the cuticle, similar to earlier works by Graham (1938); Harris (1973); Permadani and Retnoaji (2018); Schwalbach and Lickfeld (1962); and Checa, Vendrasco and Salas (2017).

Support cells. The use of this term occurs both for ordinary epithelial cells (Wondrak, 1981) and for cells with a large vacuole (Thompson,

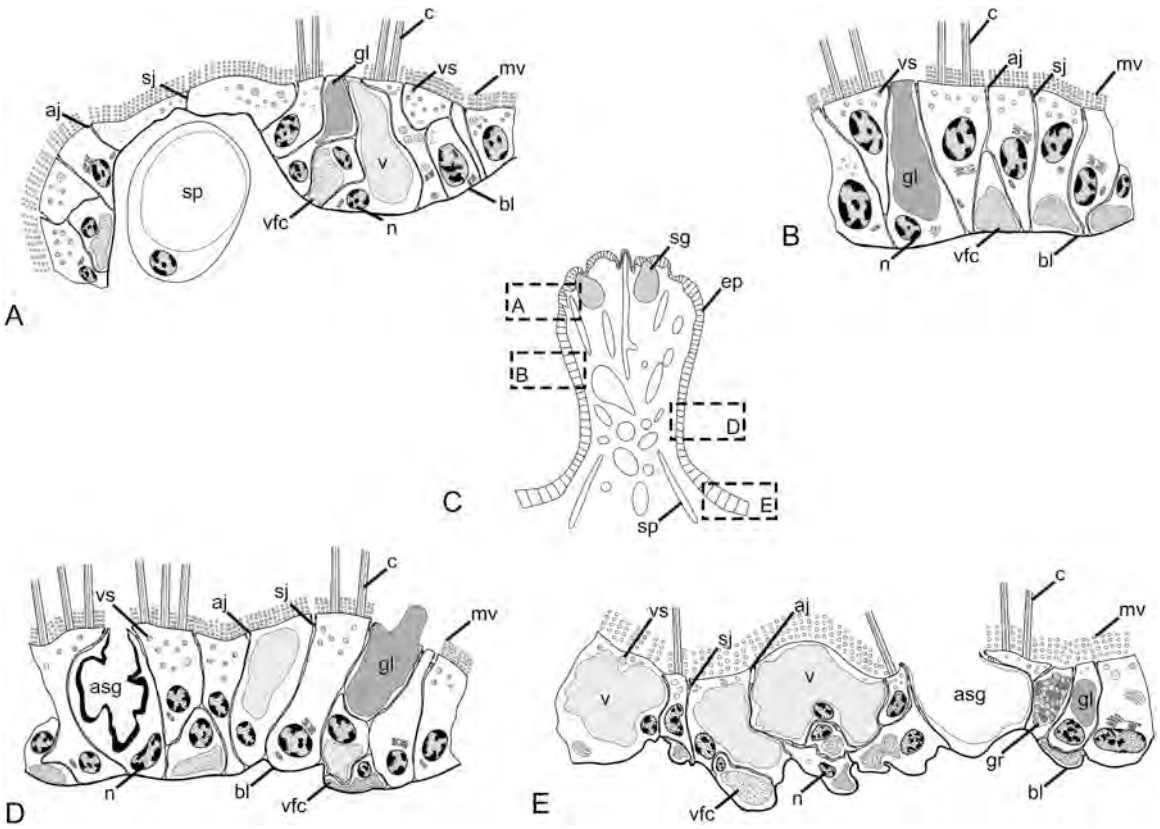

Fig. 4. Scheme of the tubercle epithelium in *Onchidoris muricata*. (A) Flattening of the epithelium next to the spicule. (B, D) Tubercles epithelium. (C) Scheme of the tubercle. (E) Intermediate part of the tubercle epithelium to the epithelium of the notum, a large number of glandular vacuolated cells. Aj, adherens junction; Asg, apocrine secretory gland; Bl, basal lamina; C, cilia; Ep, epithelia; Gl, gland; Gr, secretory granule; Mv, microvilli; N, nuclei; Sg, subepidermal gland; Sj, separated junction; Sp, spicule; Vcf, x-cells vacuole with fine content; Vs, vesicle.

1983) or tonofilaments (Haszprunar, 1996). We use the term supporting cells for cells with well-developed tonofilaments.

Ellipsoidal inclusions. The problem of terminology for structures in the epithelium of aeolid nudibranchs was previously discussed by Martin et al., (2009). For instance, the following terms are synonymous, "vacuoles", "special vacuoles", "balloon-like vesicles", "highly vacuolated" with "fibrillar inclusions", "partitions", "ellipsoid structures", "ellipsoidal shaped bodies", and "ellipsoidal inclusions" (Bonar and Hadfield, 1974; Edmunds, 1966; Porter and Rivera, 1980; Schmekel and Wechsler, 1967). Later, representatives from Cladobranchia showed that the basic structure of these granules, referred to as spindles, is chitin. In TEM they appear either as a 'stick' capped at both ends, a crescent shape, or, as an oval filamentous disk. In SEM, they appear as biconcave disks about 5 μm diameter with thick rims and a meshwork of filaments in the cavities (Martin et al., 2007a,2007b). We used vacuoles with amorphous content for these structures.

Vacuolated cells. These correspond to the terms macrovacuolated or monovacuolated cells (Àvila, Durfort, 1996), cuboidal to columnar cells that have a characteristic large vacuole (Wägele and Cervera, 2001), or, large vacuole cells (Skidmore and Rivera, 1982). They describe a cell with a large, electron-transparent vacuole that occupies a larger volume of the cell. There are also the term vacuolated cells, which describe cells with spindles (see above) (Wägele, 1997/98).

Transitional zone of notum. This is the place at the base of the tubercle where the notum passes into it.

3. Results

The external morphology of the body of *Onchidoris muricata* changes after metamorphosis. The size of the body, the degree of development of the rhinophore, and the structure of the integument change (Fig. 1). Juveniles (up to 500 μm) have both smooth bodies and smooth rhinophores without lamellaes, which are located at the anterior end of the body (Fig. 1A-B). The rhinophores acquire up to two lamellaes only when the body size reaches 2 mm. Tubercles form on the dorsal surface of the notum after metamorphosis and in early stage of postlarval development (Fig. 1C, D). *Onchidoris muricata* reaches 10–12 mm in length as an adult, having rhinophores with 7–10 lamellaes and tubercles well-developed (Fig. 1E, F). In this work, we consider the tubercle epithelium separately from the notum, despite the fact that they are its continuation.

The epithelium of the foot (Figs. 2–3) and tubercles (Figs. 4–6) in *O. muricata* were nearly identical the same at all stages of life. However, the epithelium of the rhinophores (Figs. 7–10) and the notum between the tubercles (Figs. 11–14) undergo significant ontogenetic transformations.

3.1. Foot epithelium

The foot epithelium is represented by three types of cells: 1) ciliary columnar, 2) glandular, and 3) x-cells. (Figs. 2; 3A-E). The predominant cell type is ciliary columnar, while the glandular cells are less common. These cells are all connected by adherens and septate junctions (Figs. 2; 3 F).

The nucleus of the ciliated columnar cells is located in the basal part of the cell. The apical part is occupied by numerous vesicles (Fig. 3B, C). The cilia have a typical 9 * 2 + 2 structure. Glandular cells of the foot are wider than the ciliated ones and the nucleus is located basally or basolaterally on the side of the vacuole.

There are two types of glandular cells found in the foot (Fig. 3B, D,

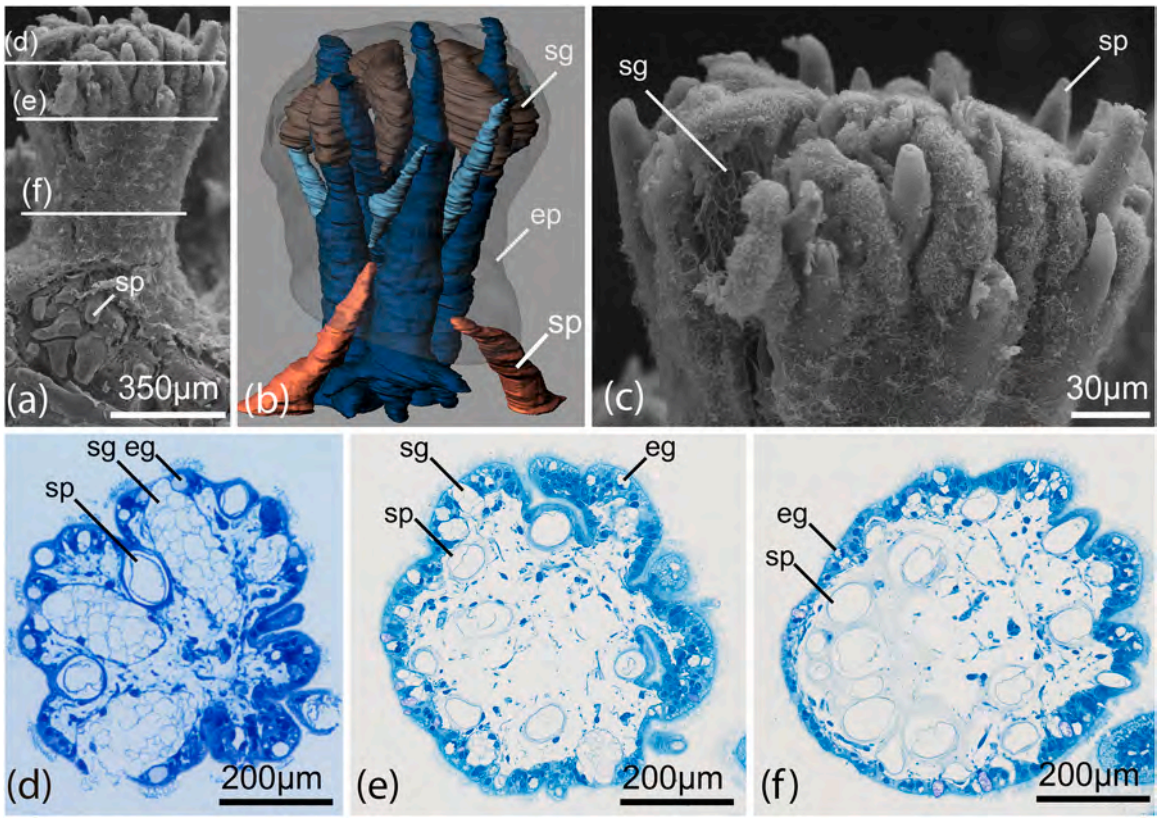

Fig. 5. Morphology of the tubercle integument in *Onchidoris muricata*. (A) External morphology of tubercle (SEM). (B) Tubercle with subepidermal glands and subepidermal spicules (3D reconstruction). (C) Structure of subepidermal gland (SEM). (D) Transverse section through the top of a tubercle with subepidermal glands and spicules. (E) Transverse section through the middle part of a tubercle. (F) Transverse section through the bottom of a tubercle. Acid glands are purple. Eg, epithelial gland; Ep, epithelia; Nt, notum; Sg, subepidermal gland; Sp, spicule; T, tubercle.

E), vacuolized and granular glands. The secretion of the granular glands has the appearance of striated (Fig. 3B) or heterogeneous granules (Fig. 3D). The granules may have varying degrees of osmiophilicity within the same gland (Fig. 3B, E). All glands have an apocrine secretion type (Fig. 3B, E).

The third type of cell is the x-cell. X-cells are always located in the basal part of the epithelium and never come into contact with the external environment. The cells remain covered by other cells of the epithelium (Fig. 3B). A distinctive feature of such cells is the presence of a large vacuole, which occupies almost the entire volume of the cell and is filled with fine-grained contents (Fig. 3B, D, E). The foot also has many large subepidermal glands (Figs. 3A; 15 F) that are weakly acidic (Fig. 15E, F).

3.2. Tubercle epithelium

The morphology of the tubercle epithelium is the same in all sizes of *O. muricata* (Figs. 4–6). The epithelium of the tubercles consists of three cell types: 1) columnar, 2) glandular, and 3) x- cells. Cells are connected by adherens and septate junctions (Fig. 6B, H, I).

The columnar cells cover the main surface of the tubercles. Most of the tubercles epithelial cells bear only microvilli in contrast to the epithelium of the ciliary foot. The ciliary cells of the tubercles are located near the glandular cells (Fig. 6D, E). We distinguished three types of glandular cells: 1) vacuolized (Figs. 6A, C, E, H),) vacuolized with osmiophilic plates (Figs. 6C, E), and) granular (Fig. 15C). Vacuolized glandular cells are distinguished by a large vacuole occupying a larger cell volume. The nucleus of such cells is located basally (Fig. 6A, D, H) and the secretion is electron-clear (Fig. 6A, D) or fine-grained (Fig. 6H, I). The chemical nature is basic or neutral. Vacuolized cells with osmiophilic plates can be distinguished by the presence of an electron-dense vacuole lining (Fig. 6C, E). The vacuole of these glands is also electron transparent. The content is similarly neutral or basic (Fig. 15D-H). Granular cells have granular acid content (Fig. 15C). These cells are associated with the subepidermal glands of the tubercles and are located laterally (Fig. 15C). Subepidermal glands have an alveolar-like structures and are localized at the top of the tubercle (Fig. 5B-E). The secretions of these glands are neutral or basic unlike the subepidermal glands of the foot (Fig. 15C). .

The glandular cells with large vacuoles cover the transition zone between the notum and the tubercles (Fig. 4E; 6D; 15D). The nucleus of these cells is displaced laterally from the vacuole and occupies a basal position. Nearly the entire volume of cells is occupied by vacuoles. Contents of the vacuoles are electron-transparent (Fig. 5D) and acidic (Fig. 15D).

X-cells are less common than in the epithelium of the foot. However, they are similarly located in the basal part of the epithelium (Fig. 6C, E). These cells have a vacuole filled with fine-grained contents and have no connection with the external environment.

3.3. Rhinophore epithelium

The epithelium of the rhinophores has ontogenetic variability. The diversity of epithelial glands and vacuoles with amorphous contents in the apical part of the cells increases (Fig. 7). The epithelium is represented by three types of cells: 1) elongated cilia-bearing epithelial cells, 2) solitary glandular, and 3) x-cells (Figs. 7–9).

The prismatic cells in the rhinophore apex bear microvilli and cilia (Figs. 8B; 9B). Cells are connected by adherens and septate junctions (Fig. 8C). A unique feature of the rhinophore epithelial cells is the presence of numerous vacuoles with amorphous content in the apical part of the cell (Figs. 7; 8B, D, F; 9B, E, H, I; 10). The amorphous contents

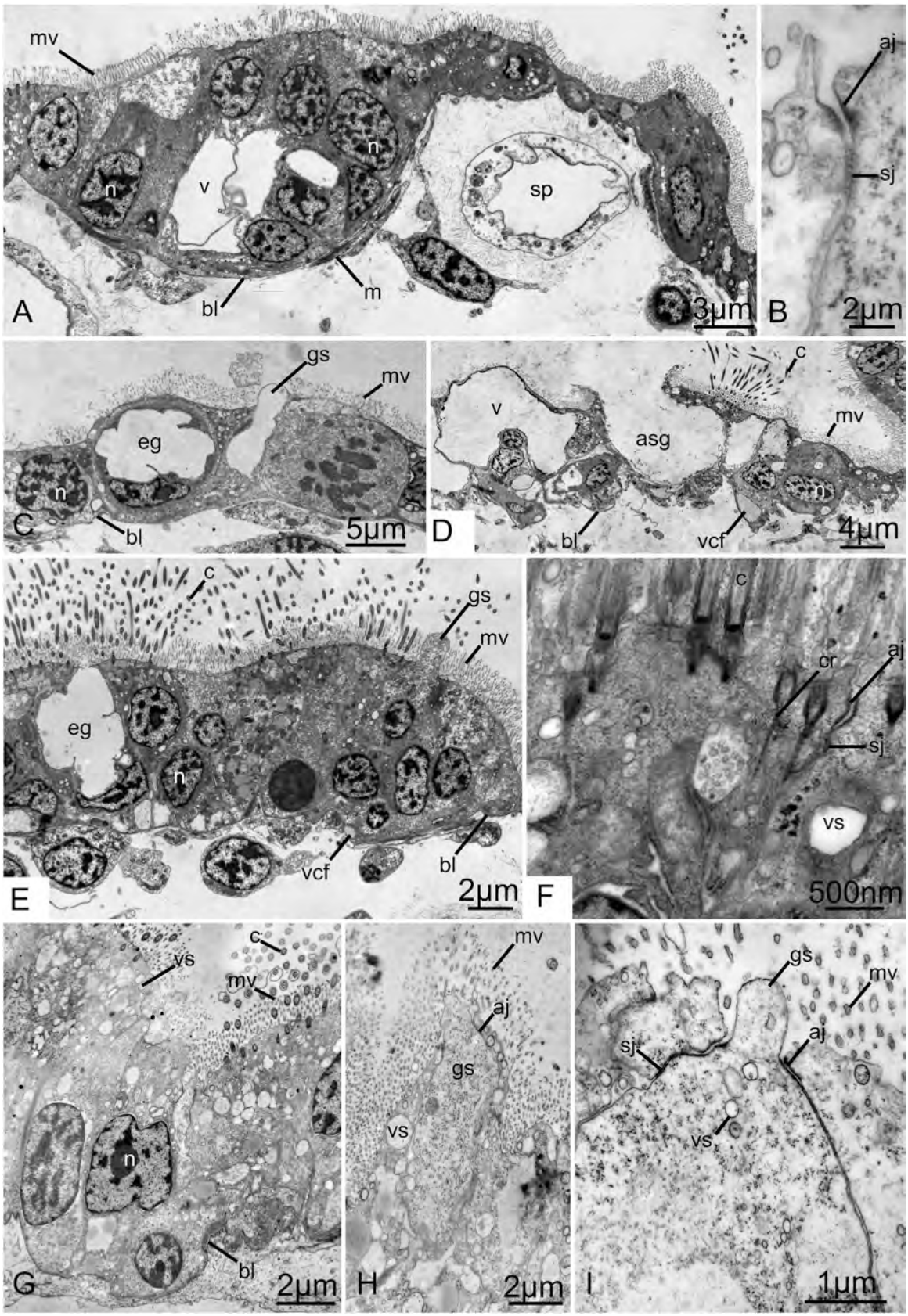

Fig. 6. Morphology of the tubercle integument in *Onchidoris muricata* (TEM). (A) Apical part of tubercles with flattened epithelium above the spicule. (B) Adherens and separated cells in contact. (C) Glands with osmiophilic lining and homogeneous secretions. (D) Glands with large vacuoles and apocrine type of secretion. (E) Surrounding glandular cells with multiciliary cells. (F) Striated roots of multiciliary cells. (G) Tubercle columnar epithelium. (H) Fine-grained secretion of the gland. (I) Extraction of the secretory substance. Aj, adherens junction; Asg, apocrine secretory gland; Bl, basal lamina; C, cilia; Cr, striated roots; Eg, epithelial gland; Gs, secretory gland; M, muscle; Mv, microvilli; N, nuclei; Sj, separated junction; Sp, spicule; V, vacuole; Vcf, x-cells vacuole with fine content; Vs, vesicle.

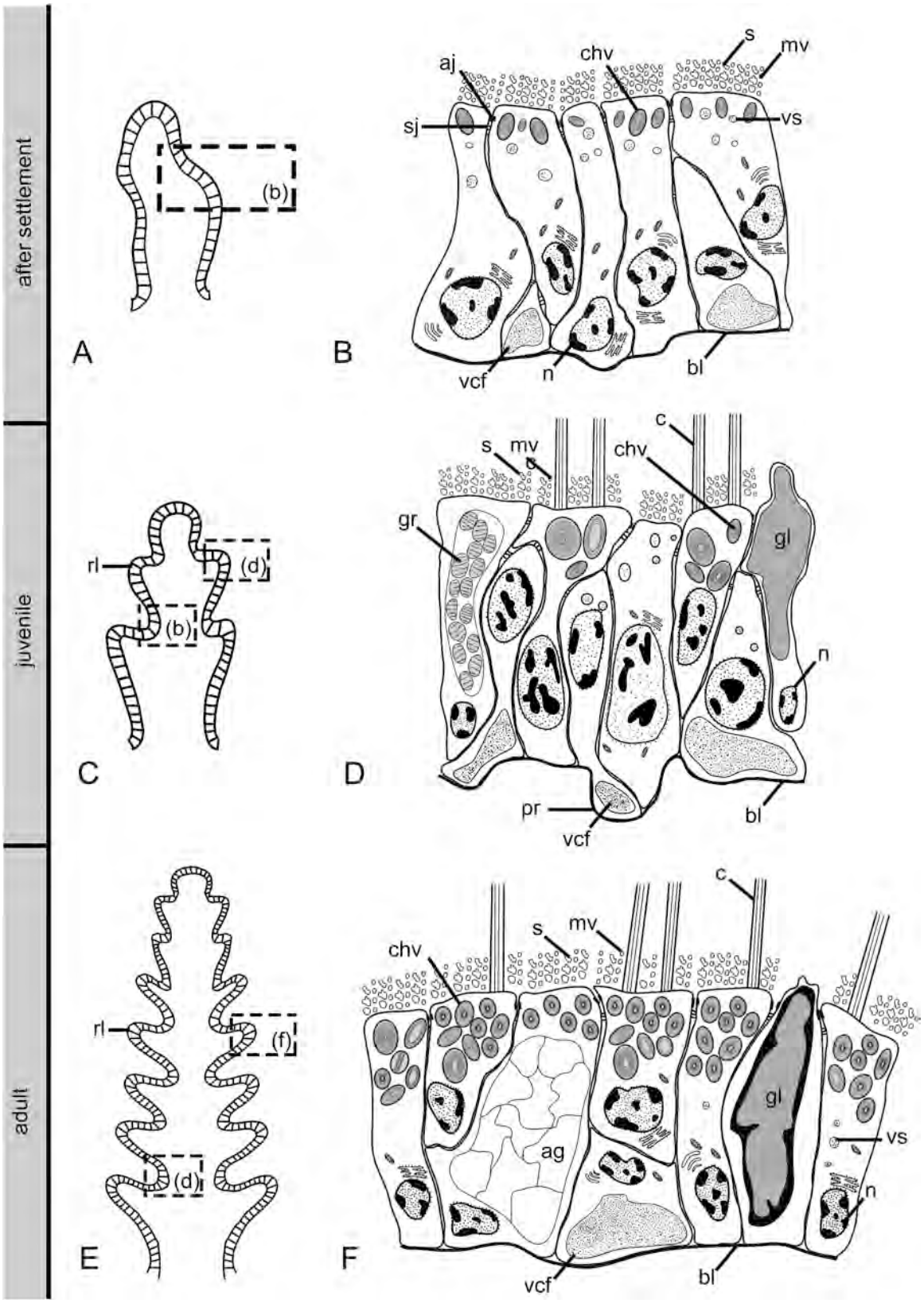

Fig. 7. Scheme of the rhinophore epithelium in *Onchidoris muricata*. (A) Rhinophore in individuals after settling. (B) Columnar epithelium with an underdeveloped layer of vacuoles with amorphous content. (C) A juvenile rhinophore with lamellaes. (D) Columnar epithelium with the glandular component. (E) Lamellaed rhinophore of an adult. (F) Adult rhinophore epithelium. Ag, alveolar gland; Aj, adherens junction; Bl, basal lamina; C, cilia; Chv, Chitin vacuole; Gl, gland; Gr, granule; Mv, microvilli; N, nuclei; Pr, protrusion of x-cell; Rl, rhinophore lamellae; S, secretory; Vcf, x-cells vacuole with fine content; Vs, vesicle.

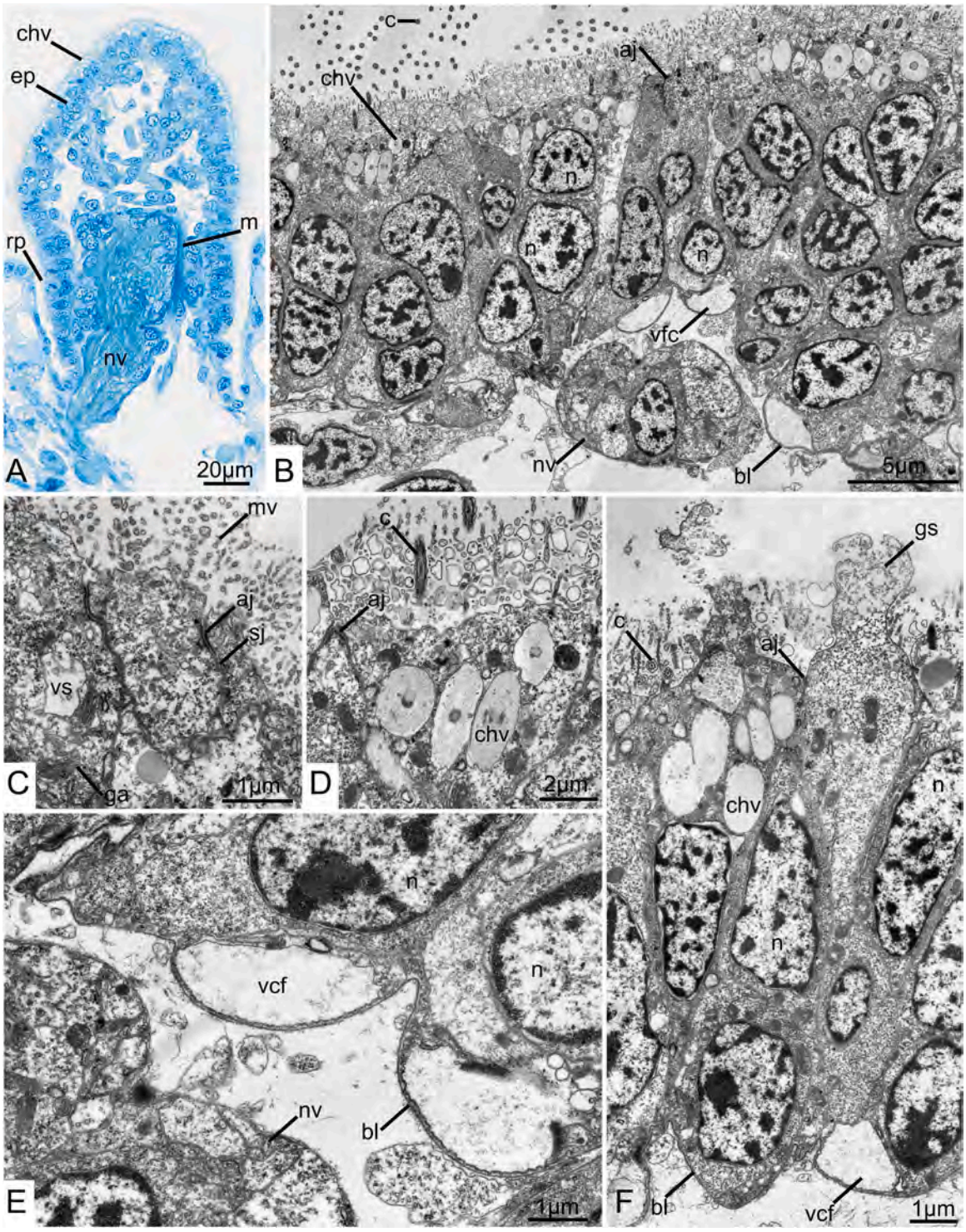

Fig. 8. Morphology of the juvenile rhinophore without leaves epithelium in *Onchidoris muricata*. (A) Light microscopy; (B – F) TEM. (A) Longitudinal section of the rhinophore. (B) Columnar epithelium. (C) Cell contacts. (D) Vacuoles with amorphous content. (E) Vacuoles with finely granular secretion in the basal part of the epithelium. (F) Glandular cell. Aj, adherens junction; Bl, basal lamina; C, cilia; Chv, chitin vacuole; Ep, epithelia; Ga, Golgi apparatus; Gs, secretory gland; M, muscle; N, nuclei; Nv, nerve; Rp, rhinophore pocket; Sj, separated junction; Vcf, x-cells vacuole with fine content; Vs, vesicle.

are unpolymerized polysaccharides (presumably chitin), which is confirmed by staining with calcofluor white (Fig. 10A). In early juveniles with smooth rhinophores, these vacuoles are loosely arranged in a single row (Figs. 7A, B; 8 A, B, D). The number of vacuoles increases when lamellaes of the rhinophore appear, forming up to 2–3 rows. The number of rhinophore lamellaes reaches 10–12 in adult mollusks, and the vacuoles are arranged in 4 rows (Fig. 9B, E, I). The location of vacuoles in the epithelium of molluscan rhinophores, regardless of size, showed zonality – in the depths of the rhinophore lamellaes, their number is less than on the surface of the distal end of the leaves (Figs. 7; 9B, C). The contents of the vacuoles may appear as tightly woven concentric fibers occupying the entire volume (Fig. 10F), being only in the periphery (Fig. 10E), or, appearing as spindles in the central part of the vacuole (Fig. 10G). In addition to the fibrous parts, osmiophilic areas

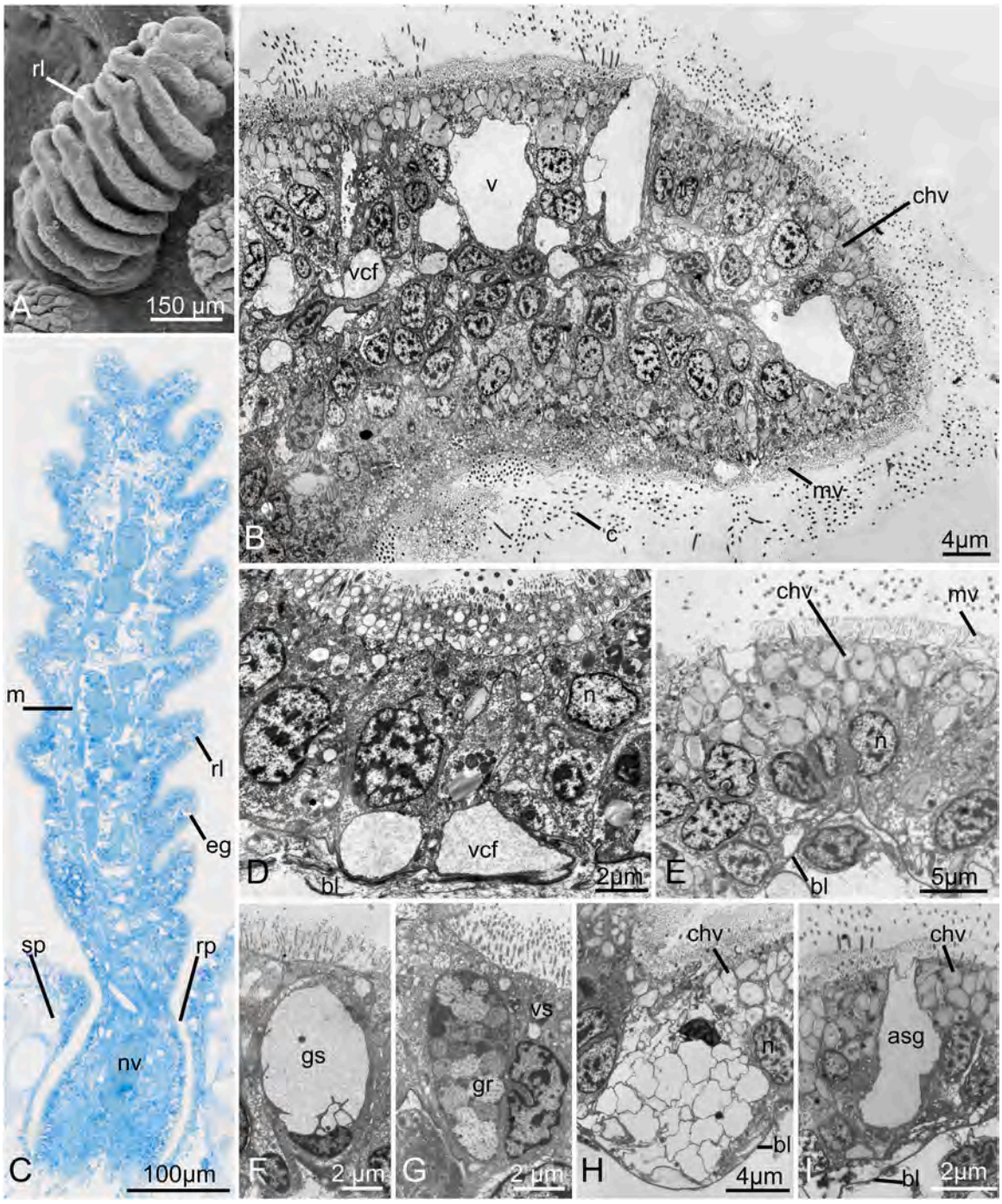

Fig. 9. Morphological features of the rhinophore epithelium with lamellae in *Onchidoris muricata*. (A) SEM. (B, D – I) TEM. (C) Light microscopy. (A) External morphology of rhinophore. (B) Frontal section through the rhinophore lamellae. (C) Longitudinal section through the rhinophore. (D) Epithelium of the ventral side of the inner rhinophore lamellae. (E) Epithelium of the dorsal side of the rhinophore lamellae, distal. (F–I) Diversity of rhinophore epithelial glands. Asg, apocrine secretory gland; Bl, basal lamina; C, cilia; Chv, chitin vacuole; Eg, epithelial gland; Gs, secretory gland; Gr, granule; M, muscle; N, nuclei; Nv, nerve; Rl, rhinophore lamellae; Rp, rhinophore pocket; Sp, spicule; V, vacuole; Vcf, x-cells vacuole with fine content; Vs, vesicle.

were also found in some vacuoles. (Fig. 10C, D).

Juveniles have only one type of vacuolated glandular cell. These cells have a vacuole with homogeneous electron-transparent secretions that occupy nearly the entire volume of the cell (Fig. 8F). Four types of glandular cells occur in adults: 1) vacuolated (Fig. 9B, I), 2) vacuolated with an osmiophilic lining (Fig. 9F), 3) granular (Fig. 9G), and 4) alveolar-like (Fig. 9H). The structure of the vacuolized, vacuolated with osmiophilic lining, and granular gland cells is similar to those of the tubercle. Glandular epithelial cells with an alveolar-like structure are characteristic to only the rhinophores. The nucleus of these cells is displaced basolaterally. The secretory vacuole is divided into cells.

The structure of the x-cells is the same as that in other body parts (Figs. 7; 8B, E, F; 9B, D; 10B). Rhinophore x-cells were noted to form numerous protrusions into the subepidermal space, but remain lined with a basal membrane (Figs. 8B, E; 10B). Such protrusions are characteristic in juveniles, but are less common in adults.

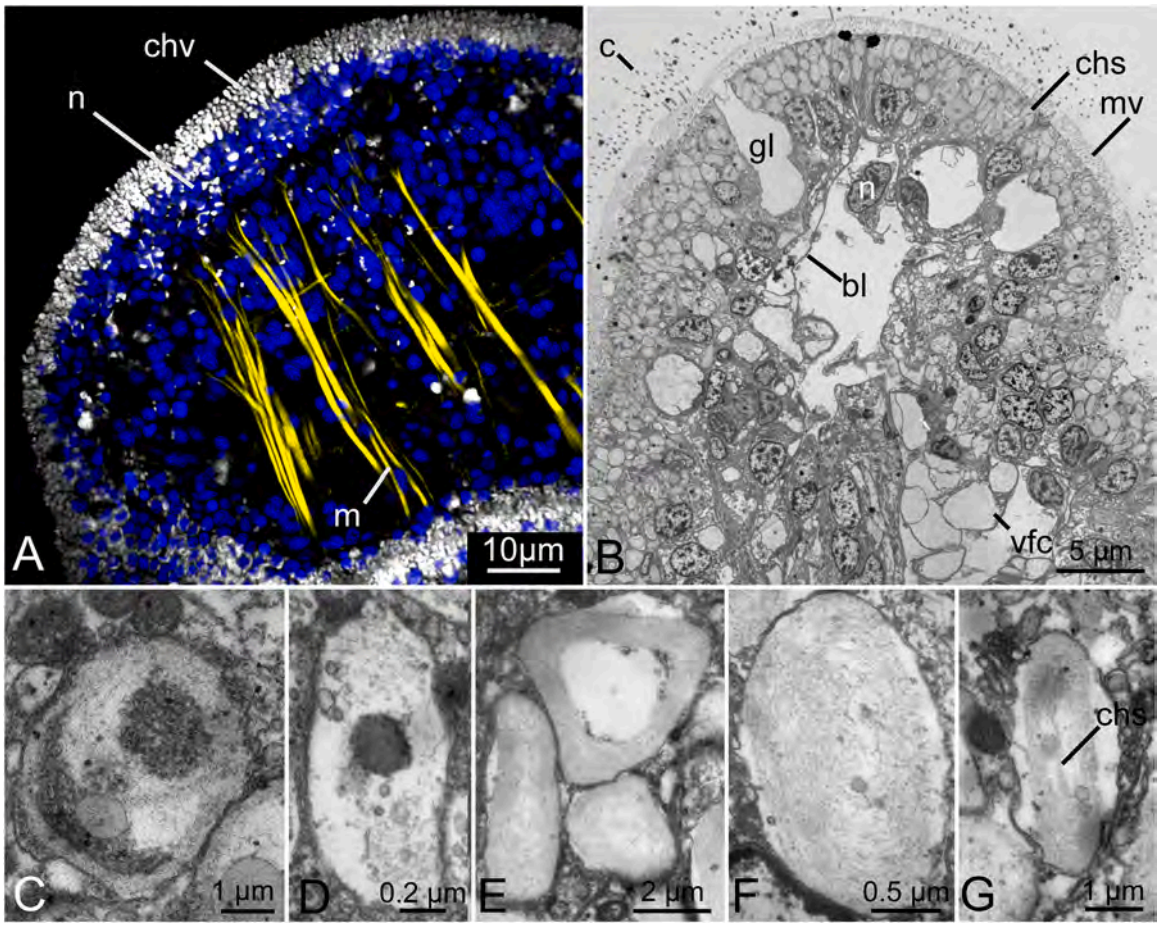

Fig. 10. Features of the epithelium in the apical part of the rhinophore in *Onchidoris muricata*. (A) Apical rhinophore epithelium with numerous chitin granules (cLSM). (B) Cross section through the crease at the apical rhinophore (TEM); Epithelial cells with chitinous granules are interspersed with glands, some cells have vacuoles in their basal part. (C – G) Types of chitin inclusions in rhinophore epithelial cells (TEM). Bl, basal lamina; C, cilia; Chs, chitin spindle; Chv, chitin vacuole; Gl, gland; M, muscle; Mv, microvilli; N, nuclei; Vcf, x-cells vacuole with fine content.

3.4. Notum epithelium

The notum epithelium in *O. muricata* undergoes the greatest changes in ontogenesis, having very different morphology across different stages of postlarval development (Figs. 11–14). We have identified three morphological stages in the development of the notum epithelium – juvenile (Figs. 11A, B; 12), intermediate (Figs. 11C, D; 13), and adult (Figs. 11E, F; 14). The juvenile type of epithelium is characteristic of newly settled juveniles with a body size of about 200 μm. This type of epithelium is represented by loosely arranged cells with a large number of vesicles inside, and large intercellular spaces (Fig. 12). The intermediate type of notum epithelium is typical for juveniles up to 2 mm in body size, and having highly vacuolated cells (Fig. 13). The adult epithelium is found in all individuals up to 12 mm. This type of epithelium is represented by columnar and glandular cells (Fig. 14).

3.4.1. Juvenile notum epithelium type

Four types of cells are found in the epithelium of juveniles: 1) strongly elongated in the latitudinal direction, 2) columnar, 3) glandular, and 4) x- cells.

Strongly elongated cells in the longitudinal direction are the predominant type, being joined by adherens and septate junctions to each other (Fig. 12C, D) but with intercellular spaces between them. These cells are filled with numerous vesicles with a diameter of about 0.1 μm. Numerous microvilli cover the cells in the apical part. These elongated cells are filled with a large number of vesicles (0.6–0.8 μm in cross-section). The content of these vesicles varies, and includes content ranging from fibrous (Fig. 12A, B), to concentric (Fig. 12B, D, E), and even electron-dense inclusions resembling lipid droplets (Fig. 12A, D).

Columnar cells are carried on the surface of the microvilli and the nucleus is located basally. The tonofilaments are well-developed in these cells (Fig. 12D). The glandular component is poorly developed. There are the only vacuolated glandular cells with an electron-transparent secretion (Fig. 12C).

The structure of the x-cells is similar to those from other body parts (Fig. 12A, E). X-cells and basal membranes have protrusions deep into the epithelial space in some areas of the epithelium. The cells with fine-grained contents are localized under these invaginations in the subepidermal space (Fig. 12E). The content of these cells is similar to the contents of the x-cell vacuoles.

3.4.2. Intermediate notum epithelium type

This type of epithelium combines features of the juvenile and adult epithelium (Figs. 11; 13). Three types of cells have been identified: 1) columnar, 2) glandular, and 3) x-cells. The cells are closely adjacent to each other in contrast to the juvenile type. The cells are also joined by adherens and septate junctions (Fig. 13A-D). However, a few intercellular spaces filled with vesicles still occur (Fig. 13C). Columnar epithelial cells in the basal position bear a nucleus. The surface of these cells is covered by microvilli. The apical part of the cell is filled with vesicles, similar to the loose epithelium of juveniles (Fig. 13D). Contents of the apical vesicles are similar to the juvenile epithelium (Fig. 13 F-I).

Glandular cells are similar to those in the juveniles. Glandular cells have huge vacuolated vacuoles with electron-transparent contents. The

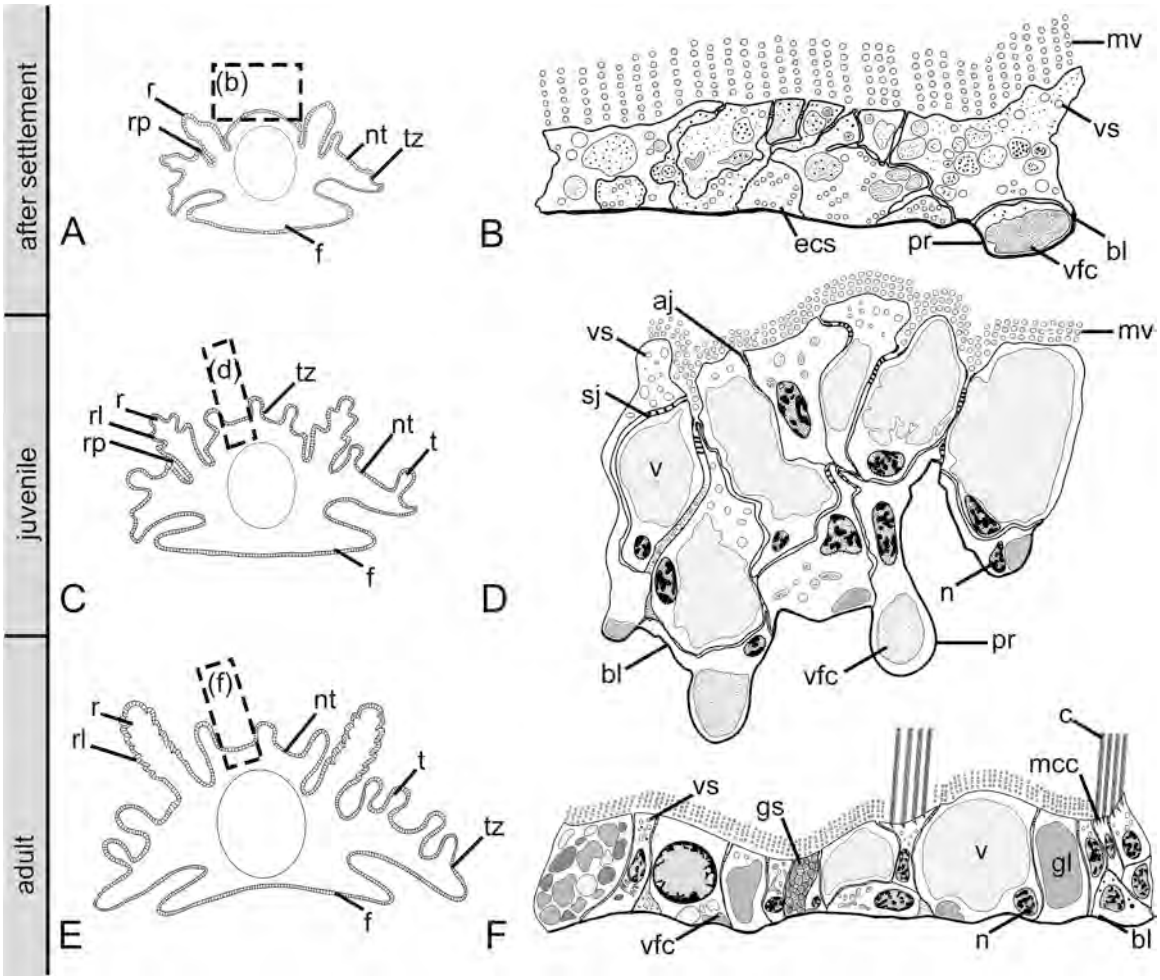

Fig. 11. Notum epithelial types in *Onchidoris muricata*. (A, B) Juvenile type of notum epithelial (C, D) Intermediate type of notum epithelial (E, F) Adult type. Aj, adherens junction; Bl, basal lamina; C, cilia; Ecm, extracellular matrix; F, foot; Gl, gland; Gs, secretory gland; Lv, large vacuole; Mcc, multicellular cell; Mvl, microvillar layer; N, nuclei; Nt, notum; Pr, protrusion of x-cell; R, rhinophore; Rl, rhinophore lamellae; Rp, rhinophore pocket; Sj, septate junction; T, tubercles; Tz, transition zone from notum to tubercle; Vcf, x-cells vacuole with fine content; Vs, vesicle.

content is chemically neutral or basic (Figs. 13A; 15 A). The nuclei of the glandular cells are arranged in several rows and occupy a basal position.

Intermediate epithelium is characterized by a large number of x-cells. X-cells form the largest number of protrusions into the subepidermal space (Fig. 13E). However, such protrusions remain lined with a basal membrane.

3.4.3. *Epithelium of the adult notum*

The epithelium of the adult notum is composed of three cell types: 1) columnar, 2) glandular, and 3) x-cells (Fig. 14). All cells are densely packed together unlike those found in the juveniles and intermediate epithelium. Intercellular spaces filled with small vesicles are absent. The columnar cells are attached by adherens and septate junctions. The nuclei are located basally and microvilli cover the surface of the cells (Fig. 14B, D, E). Some columnar cells bear cilia and are associated with the glandular cells (Fig. 14D).

Glandular cells are the predominant type (Fig. 14). All glands have an apocrine type of secretion. The apical part of the cell is destroyed when the secretion is released (Fig. 14H). Glandular cells carry different secretions and chemical composition (Figs. 14D-H; 15B). There are three types of gland cells 1) vacuolated (Fig. 14H),) vacuolated with an osmiophilic lining (Fig. 14A), and) granular (Fig. 14A, D, E. F). The structure of the glands is similar to the glands of the tubercles. X-cells have a basal vacuole filled with fine granular contents (Fig. 15A) but protrusion into the subepidermal space were not found.

3.5. *Epithelium near the spicules*

Onchidoris muricata contains subepidermal calcite spicules in the notum, tubercles, foot, and rhinophores. Despite their subepidermal position, spicules can approach the surface of the tubercles and at the edge of the notum and protrude, remaining covered with epithelium (Figs. 6A; 15C). The epithelial cells flatten to 0.5 μm at the spicule attachment sites (Fig. 6A). The main organelles of the cell are displaced away from the spicule. Only small vesicles free from inclusions of the cytoplasm are located above it.

4. Discussion

Changes to the structure of the body epithelium during early ontogeny has not previously been shown for nudibranch mollusks. For the first time, we traced the change in the body epithelium from the moment of metamorphosis to adults in the dorid species *O. muricata*. The epithelium is modified to varying degrees depending on the body part according to our results on the morphology and ultrafine structure of the integument across different stages of ontogeny. The greatest changes are those of the epithelium in the notum and rhinophores. There were no significant differences in the epithelium between different life stages in the foot or tubercles. We are only discussing features that differed or that seem of new interest.

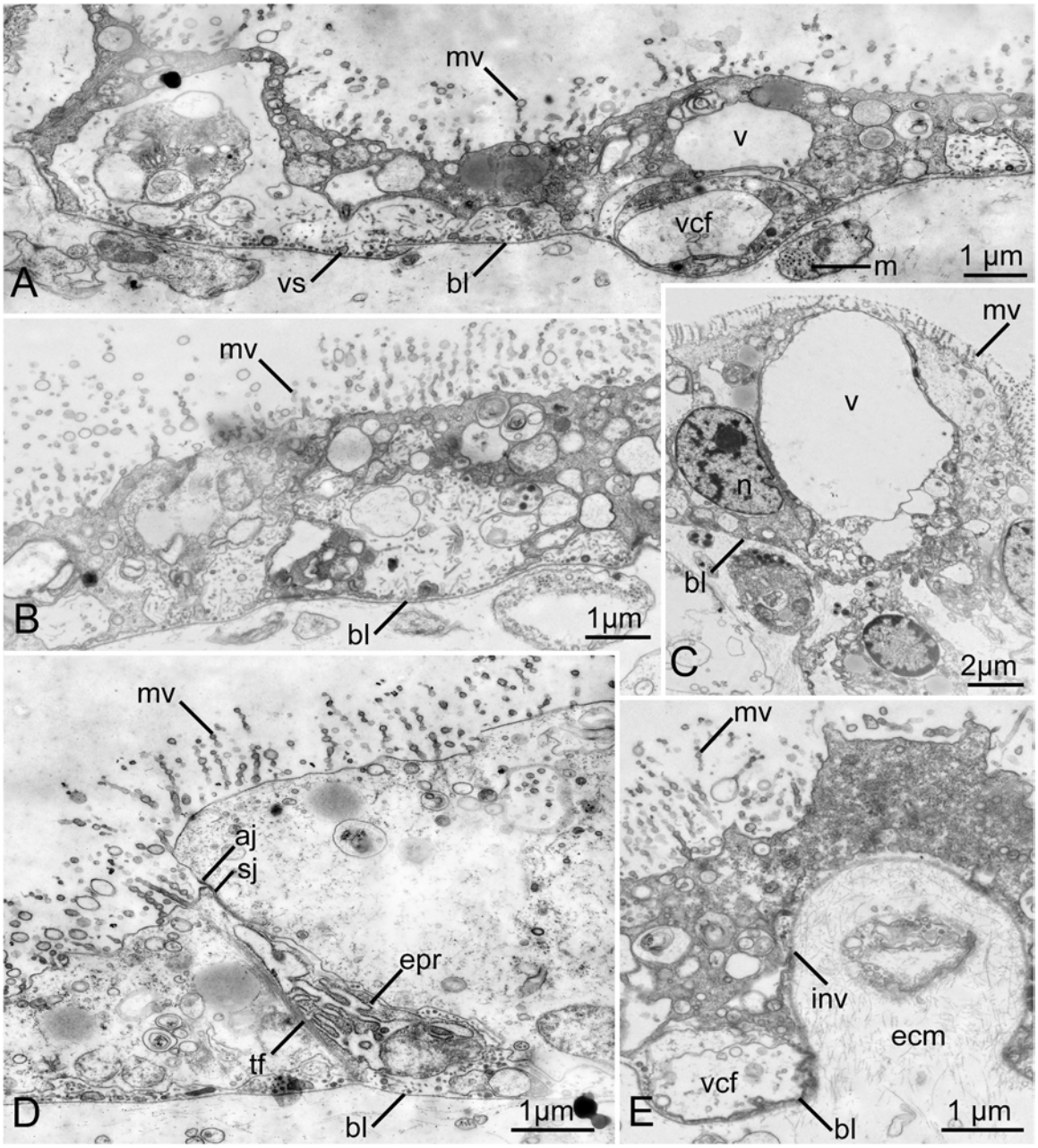

Fig. 12. Morphology of the dorsal part of the juvenile notum epithelium in *Onchidoris muricata* (200 μm in length (TEM)). (A, B) Fragment of loose epithelium with gaps between cells and basal laminae with many inclusions. (C) A gland with a large vacuole and electron-transparent contents. (D) Supporting epithelial cells with a developed endoplasmic reticulum and tonofilaments. (E) Invagination of the epithelium, formation of a compartment with collagen fibers, and a cell with a vacuole. Aj, adherens junction; Bl, basal lamina; Ecm, extracellular matrix; Epr, endoplasmic reticulum; Inv, invagination; M, muscle; Mv, microvilli; N, nuclei; Sj, septate junction; Tf, tonofilament; V, vacuole; Vs, vesicle.

4.1. Notum epithelium

The greatest changes occur in the epithelium of the notum in *O. muricata*. This change is probably associated with the transition from a floating larva with a shell, to a juvenile crawling along the substrate without a shell. The epithelium retains features of the integument located under the protoconch in the larva immediately after metamorphosis (Schmekel and Wechsler, 1967). Large intercellular spaces remain between epithelial cells (Fig. 12). A similar state of the epithelium has been similarly described for postmetamorphic specimens of two other species of nudibranchs; the cladobranchia *Trinchesia granosa* (Schmekel and Wechsler, 1967) and the doridid *Adalaria proxima* (Thompson, 1958). The transformation of the loose (larval) epithelium through the intermediate stage into the adult occurs quite quickly. This is probably due to the need to perform a protective function that belonged to the larval shell and why a large number of glands are formed with a secretory substance that serves for chemical protection. The intermediate epithelium is dominated by vacuolated cells that presumably perform both supportive and protective functions. We suggest, that the supportive function is realized due to turgor inside large vacuoles. The protective function is due to the neutral or main secretions of their contents. Similar cells are also noted in juveniles of *Bathydoris hodgsoni* Eliot, 1907 (Moles et al., 2017) and *Ad. proxima* (Thompson, 1958).

The juveniles of *O. muricata* are characterized by active growth and synthesis of calcite subepidermal spicules (Nikitenko et al., 2021; Lisova and Vortsepneva, 2022). The spicules take on the supporting and

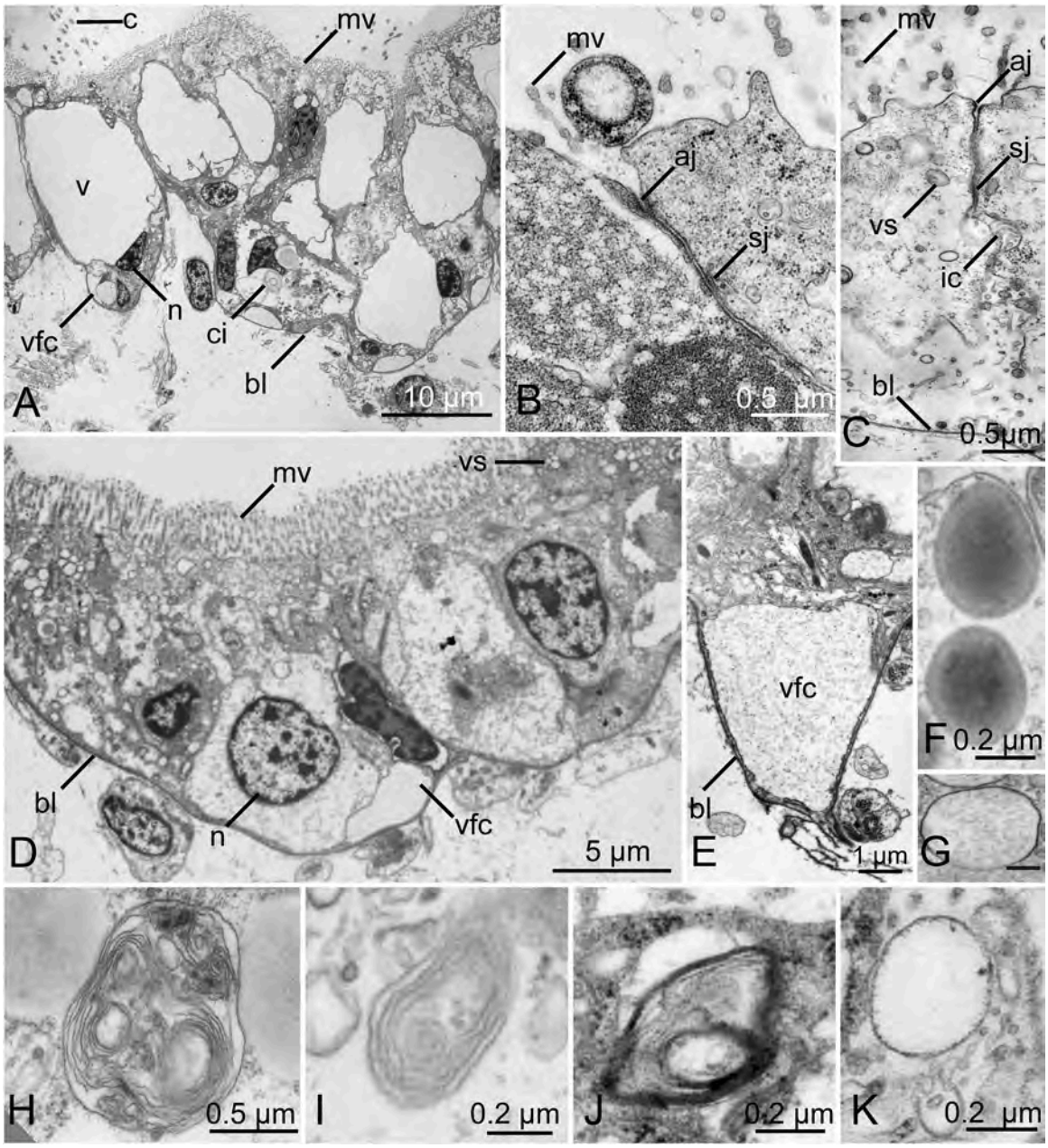

Fig. 13. Morphology of the dorsal part of the epithelium in the notum of *Onchidoris muricata* (up to 2 mm in length (TEM)). (A) Fragment of epithelium with vacuolated cells. (B) Adherens junction between epithelial cells. (C) Connection of cells in the apical part by adhesive and separate contacts in the basal part of the lumen with vesicles between cells. (D) Fragment of epithelial cells with vesicles in the apical part and a structure with fine-grained content in the basolateral part of the cells. (E) Protrusion of X-cell with fine-grained contents deep into the subepidermal space. (F) Electron-dense inclusions. (G) Inclusions with granular content. (H – O) Inclusions with concentric content. (l) Vesicle. Aj, adherence junction; Bl, basal lamina; C, cilia; Ci, concentric inclusion; Ic, intercellular cell; Mv, microvilli; N, nuclei; Sj, septate junction; V, vacuole; Vcf, x-cells vacuole with fine content; Vs, vesicles. Scale bar: G, 0.1 μm.

protective functions (Thompson, 1960) of the epithelium in early juveniles, which correlates with the restructuring of the surface epithelium. The notum epithelium in adults is however dominated by columnar cells without large vacuoles, and glandular cells. Glandular cells have a variety of secretions that can perform, including a protective function.

4.2. Rhinophore epithelium

Ontogenetic changes in the rhinophores are expressed with age as an increase in the number of rhinophore leaves (Lisova and Vortsepneva, 2022). As development proceeds, the diversity of glandular cells increases and specific vacuoles in the apical part of prismatic epithelial cells appear.

Similar vacuoles (with amorphous content) in the epithelium have been described for nudibranch mollusks of both groups (cladobranchia and dorid) (Martin et al., 2007a,2007b). The chitinous nature of the content of these vacuoles has been shown in several species of cladobranchia (Schmekel and Wechsler, 1967; Martin et al., 2007a,2007b). They perform a protective function against the stinging cells of cnidarians, their main food source (Martin et al., 2007a,2007b; Moles et al., 2016; Vorobyeva et al., 2017). Chitin spindles can be found in the epidermis, esophagus, stomach, and the intestine (Schmekel and Wechsler, 1967). Similar vacuoles (with amorphous content were also noted in the epithelium of dorids, specifically in the notum (*Prodoris clavigera* (Thiele, 1912), *Acanthodoris pilosa* (Abildgaard [in Müller], 1789)), tubercles (*Jorunna tomentosa* (Cuvier, 1804)) and in the

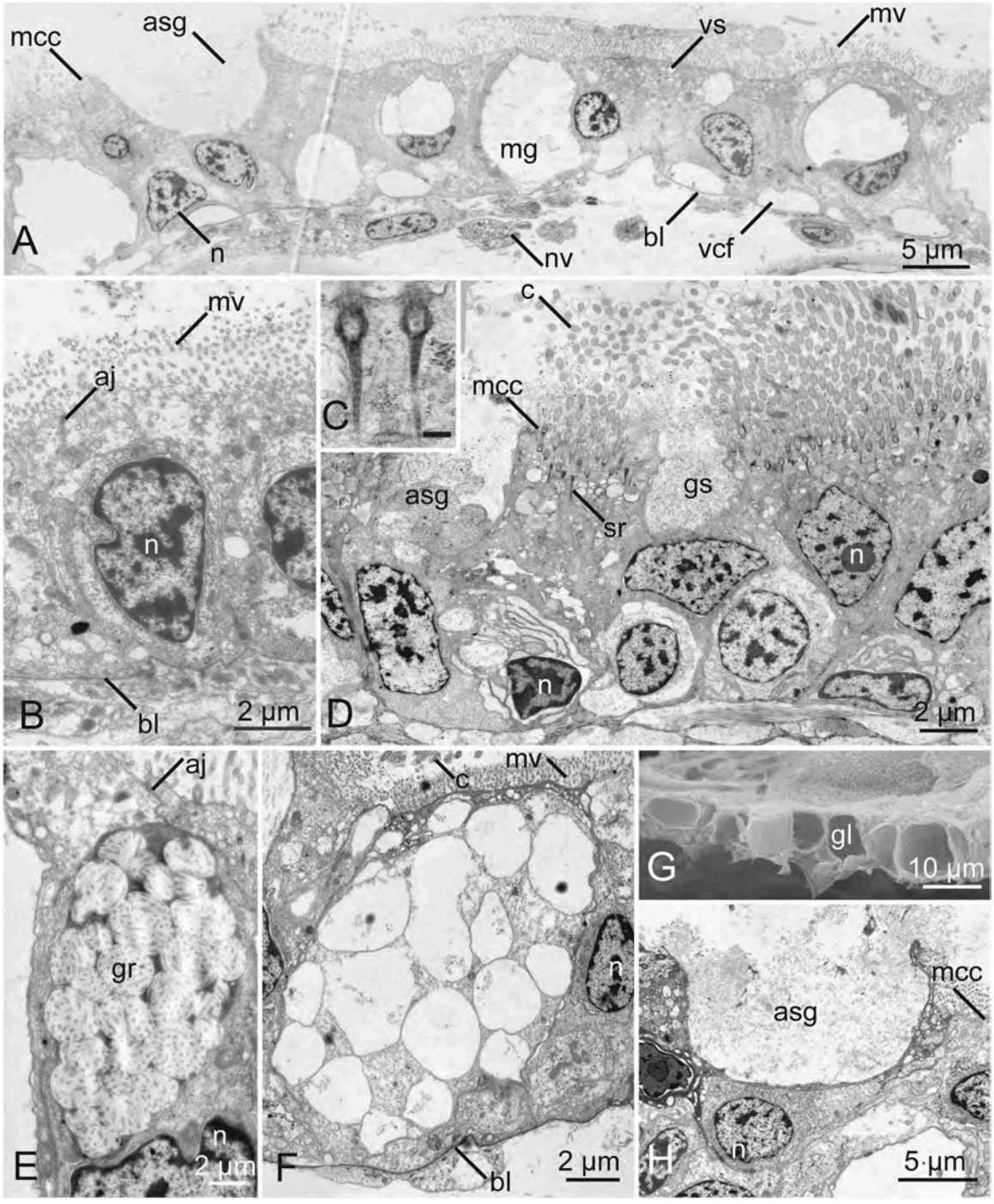

Fig. 14. Morphology of the dorsal part of the adult notum epithelium in *Onchidoris muricata* (2–12 mm (TEM)). (A) Fragment of glandular epithelium. (B) Integumentary epithelial cell. (C) Striated cilia root of a multiciliary cell. (D) Granular gland and gland with homogeneous granular contents surrounded by multiciliary cells. (E) Epithelial gland with granular secretion. (F) Epithelial gland with vesicles. (G) Epithelial gland of the apocrine secretion type. Acg, apocrine secretion gland; Aj, adherence junction; Bl, basal lamina; C, cilia; Gl, gland; Gs, secretory gland; Mcc, multicellular cell; Mg, mucous gland; Mv, microvilli; N, nuclei; Nv, nerve; Sr, striated roots; Vcf, x-cells vacuole with fine content; Vs, vesicle. Scale bar: C, 0.02 µm.

rhinophores (Doridoidea) (Schmekel and Wechsler, 1967; Storch and Welsch, 1972; Wägele, 1997). However, the morphology, ultrastructure, function, and composition of these contents are not described.

For the first time we have shown the presence of vacuoles only in the rhinophores for *O. muricata,* as they were not found in other parts of the body. Despite the fact that the epithelium vacuoles of the rhinophores in *O. muricata* are stained with calcofluorwhite similarly to the chitin spindles of Claobranchia, their ultrastructure is significantly different. Interestingly, the ultrastructure is more reminiscent of the inclusions described in the epithelium of the foot and notum in *Rostanga alisae* Martynov, 2003 (Zhukova et al., 2022). However, these inclusions have been shown to be endosymbiotic single bacteria. This casts doubt on earlier descriptions of the contents belonging to amorphous chitin. Thus, despite numerous descriptions of vacuoles, there is still no reliable chemical nature of their contents.

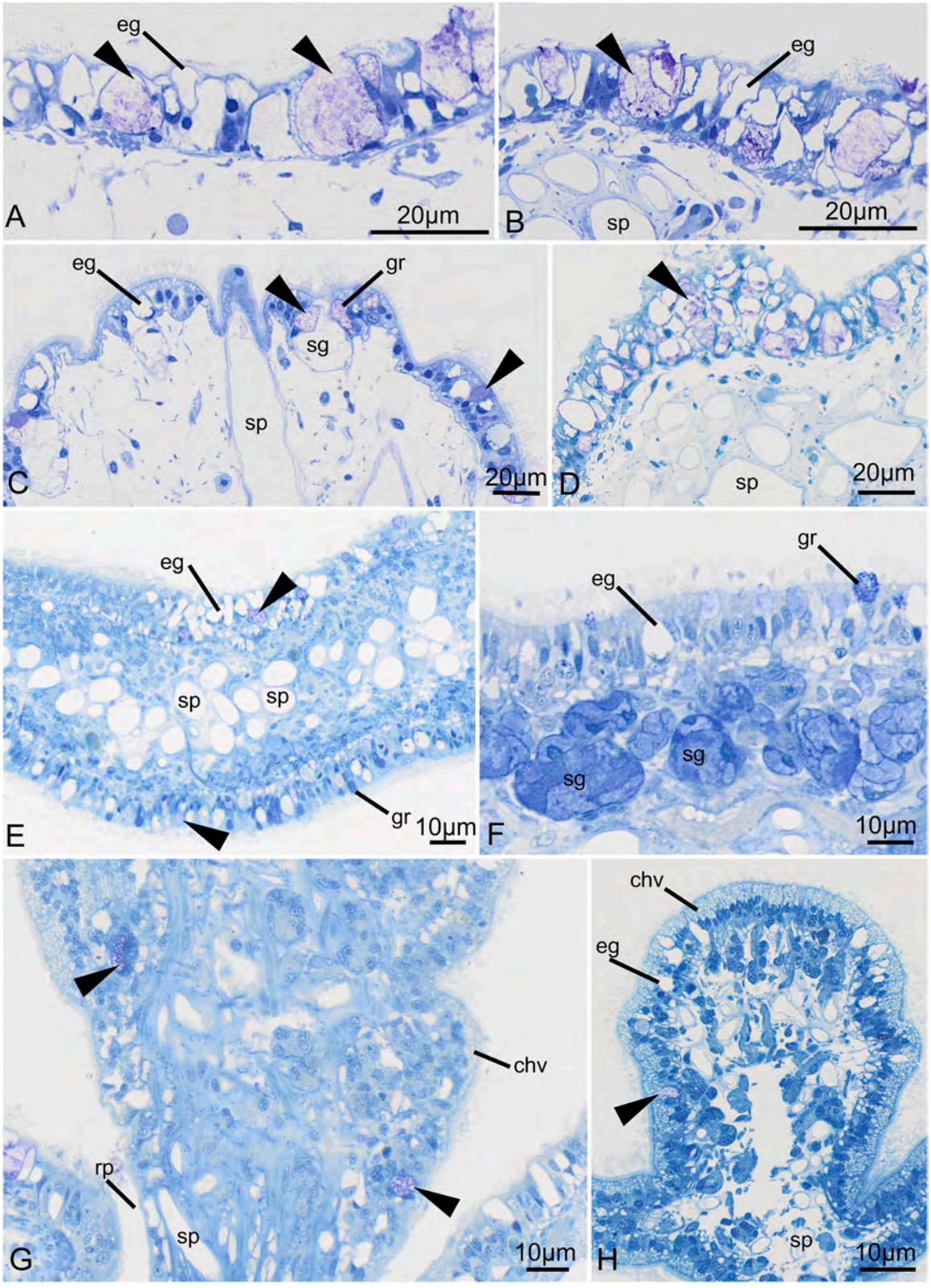

Fig. 15. Histological staining with methylene blue and toluidine blue of the epithelium in *Onchidoris muricata*. Violet - acidic glands (arrows). (A) Intermediate notum epithelium. (B) Adult notum epithelium. (C) Epithelium of the apex of the tubercle. (D) The transition zone between tubercle and notum with pseudostratified epithelium. (E) Longitudinal section of the leg. (F) Transverse section of the anterior leg with subepidermal glands. (G) Longitudinal section through the basal part of an adult rhinophore. (H) Transverse section through the lamellae of an adult rhinophore. Eg, epithelial gland; Chv, chitin vacuole; Gr, granule; Rp, rhinophore pocket; Sg, subepidermal gland; Sp, spicule.

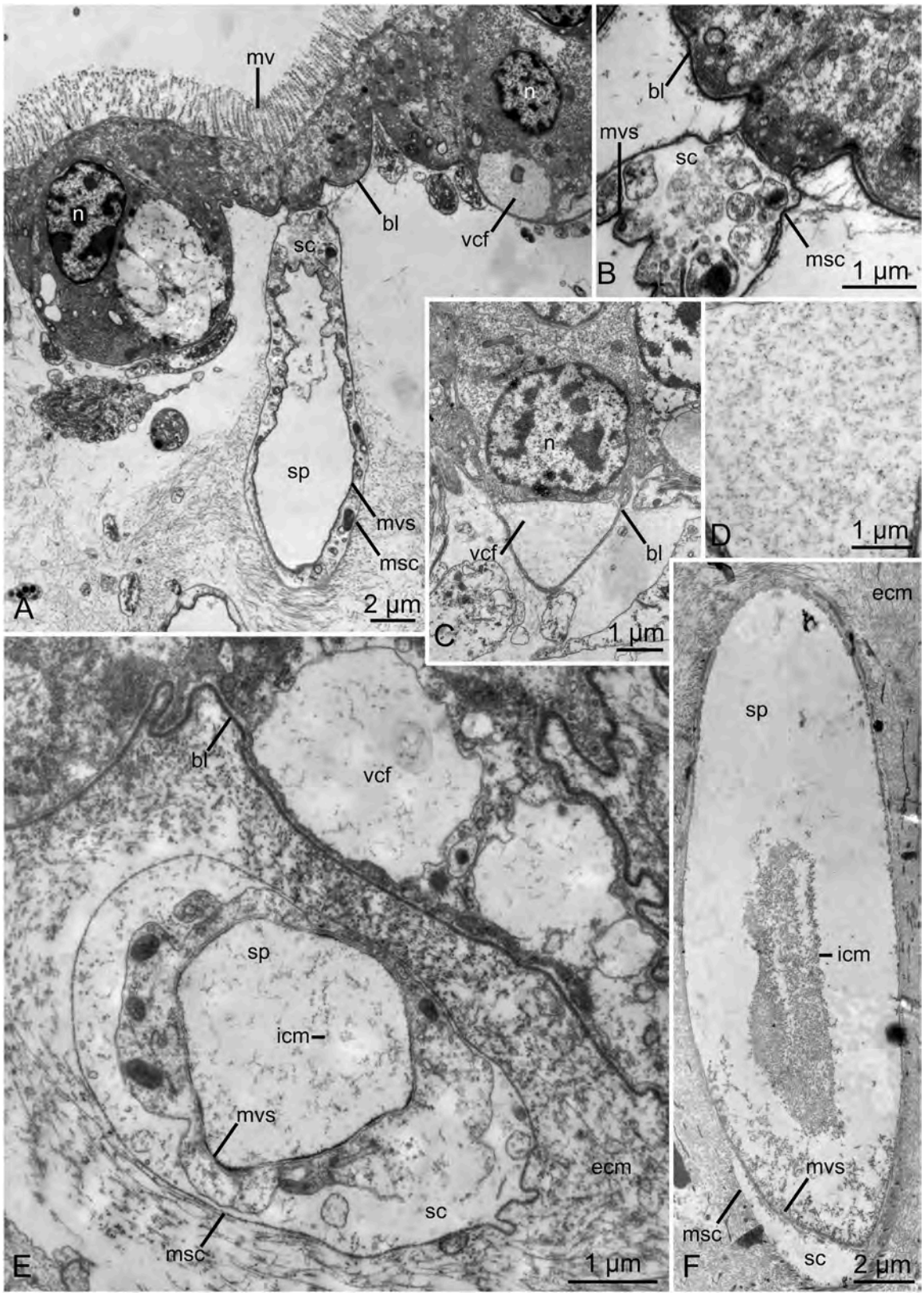

Fig. 16. Comparison of the structure of sclerocytes with cells in the basal part of the epithelium (TEM). (A) Protrusion of a sclerocyte in the subepidermal space in juvenile *Onchidoris muricata*. (B) Communication of the sclerocyte with the epithelium. (C) Protrusion of a cell with a vacuole and fine-grained contents into the subepidermal space. (D) Structure of the fine-grained contents of the vacuole. (E) A juvenile sclerocyte in the subepidermal space next to a cell with a vacuole. (F) *Onchidoris muricata* sclerocyte with intracellular collagen matrix (individual size 3 mm). Bl, basal lamina; Ecm, extracellular matrix; Icm, intracellular collagen matrix; Msc, the membrane of sclerocyte; Mv, microvilli; Mvs, the membrane of sclerocyte vacuole; N, nuclei; Sc, sclerocyte; Sp, spicule; Vcf, x-cells vacuole with fine content.

Table 1
Gland types of the *Onchidoris muricata* body epithelium.

Type of gland / location			Foot	Tubercle	Rhinophore	Notum		
						Juvenile	Inter mediate	Adult
with large vacuole	"empty", electronically transparent content	with osmiophilic lining	+	+	+	-	+	+
		without osmiophilic lining	+	+	+	+	+	+
		alveolar structure	-	-	+	-	-	?
	with fine granular content	+	+	+	-	+	+	+
Granulate	with striped granules		+	-	+	-	-	+
	with heterogeneous granules		+	+	?	-	-	+

Blue - acidic content
Grey - pH not specified

4.3. Epithelium of the foot and tubercles

The epithelium of the foot and tubercles have similar structures in *O. muricata* regardless of size. Our data on the structure of the foot epithelium in *O. muricata* is consistent with previous results in the adults. However, we found an additional type of epithelial gland that has a large vacuole and granular secretions of a heterogeneous structure. These glands are in addition to those glands previously noted with striated granules (Skidmore and Rivera, 1982). Because the secretions of these glands help with locomotion by facilitating gliding over the substrate, it makes sense that they appear in the epithelium of the foot, regardless of the size of the individual.

The epithelium structure of the tubercle does not change with ontogeny. The apical part of the tubercles in *O. muricata* contain a large number of multiciliary cells. These cells perform a sensory function and also prevent overgrowth by invertebrates and bacteria by generating water currents that flow from the anterior to the posterior end (Potts, 1981). The transition zone between the tubercles and the notum is completely lined with numerous glands that contain a large, acidic vacuole (Table 1) that provides additional protection for the integument.

4.4. Variety of glands

In this paper, we described and classify numerous epithelial glands found throughout the body in *O. muricata*. Based on ultrastructural data, epithelial glandular cells can be divided into two types - glands with a large vacuole (I), and granular glands (II). Type I glands contain vacuoles with electron-clear content, fine-grained content, or with osmiophilic linings. Type II glands contain uniform striated granules and heterogeneous granules. Glands may contain acidic, basic, or neutral secretions. The contents of the gland with a large vacuole may have a different pH, while granular glands have an acidic reaction. Glands can perform extremely diverse functions, including protective, facilitating locomotion, or even playing a vital role in feeding (Edmunds, 1968; Martin et al., 2007a,2007b; Moles et al., 2016). It was previously noted that any one cell will usually produce only one type of granule. Not only is there a segregation in the types of granules between cells, but there is also a difference in the amount of granular material present within each cell (Porter and Rivera, 1980).

For the first time, we provide evidence showing that glandular cells in the epithelium are surrounded by multiciliary cells (Fig. 14 A, D). It is likely that these cells perform a sensory function, and are involved in the regulation of the secretory activity of the glands.

4.5. X-cells and spiculogenesis

In the study, we found numerous x-cells in the various parts of the epithelium in *O. muricata* at different stages of postembryonic development (Fig.16). Most often, such cells were found in the epithelium of juveniles from the moment of settling up to 2 mm, less often in individuals from 2 to 5 mm, and even less often in adults. Previously, such formations were noted only in the foot in *O. muricata* (Skidmore and Rivera, 1982). However, no particular importance was attached to these structures. A similar arrangement is also characteristic of pigment granules in the epithelium in *O. bilamellata* (Linnaeus, 1767) (Edmunds, 1968).

X-cells are located in the basal part of the epithelium (Figs. 3B, D, E; 6D, E; 8 E, F; 9B, D; 10B; 12A, E; 13A, D, E; 14A; 16C, D, E) and never interact with the external environment and remain covered with epithelial cells. X-cells carry a vacuole that is filled with fine-grained contents resembling cross-cut collagen fibers. The contents of these vacuoles resemble contents of sclerocytes in the subepidermal space at certain stages of maturation (Nikitenko et al., 2021). Similar cells were found by us in all parts of the body containing spicules (foot, notum, tubercles, rhinophores). In addition, spicules are deposited and formed in juveniles. X cells protrude deep into the subepidermal space (Figs. 13E; 16C). The stages when X cells are found in abundance is also when spicules are forming in juveniles, and that the x cells decrease in abundance (Table1) at the same time that spicule formation slows (Nikitenko et al. 2021).

Since the spicules in other mollusks (Aplacophora (Salvini-Plawen,

1967; Kocot et al., 2017), Polyplacophora (Leise, 1984), Bivalvia (Harper et al., 2006)) are formed by cells of the integumentary epithelium, it is most likely that doridid sclerocytes also are ectodermal. Numerous protrusions of x-cells into the subepidermal space may indirectly confirm the participation of these cells in spiculogenes. We suggest that x-cells are sclerocytes in the early stages of formation in the epithelium. However, to test this hypothesis, further studies using a set of methods are required. Thus, the study of epithelia at different stages of development is also relevant for understanding spiculogenesis.

5. Conclusion

A detailed study of the epithelia in *O. muricata* during postlarval stages of development made it possible for the first time to note the ontogenetic variability in the morphology of epithelia. The degree of modification of the epithelium in ontogeny depends on the part of the body. However, they are most pronounced in that the notum, which in larvae, was protected by the protoconch.

The epithelial glands were classified for dorids. Two fundamental types of gland structure have been identified. There are glands with a large vacuole, and those with numerous granules inside.

Numerous vacuoles are present in the epithelium of the rhinophores and increase in number during ontogeny. It was noted that vacuoles with amorphous contents formed ordered rows, there are fewer vacuoles in the depth of the leaves and more vacuoles at the distal edge. For the first time, a positive calcoflour white color was shown, which may indicate that the content is unpolymerized polysaccharide, could be chitin. However, our first ultrastructural data on vacuoles with amorphous contents of dorids are extremely similar to the ultrastructure of single bacteria. Thus, the chemical nature of the content has not been determined.

In the present work, unique x-cells are described in detail. These cells form protrusions into the subepidermal space and look like sclerocytes in their early stages of formation. Numerous protrusions were found in individuals whose spicules were at the stage of formation. This confirms the above assumption that x-cells belong to sclerocytes.

Thus, questions about the chemical nature of the contents of vacuoles in the epithelium of dorids rhinophores remain open. More x-cell studies are needed to test for their involvement in spiculogenesis.

Funding

This work was supported by the Russian Science Foundation, Grants no. 21-14-00042.

Declaration of Competing Interest

The authors report no conflict of interest.

Data Availability

Data will be made available on request.

Acknowledgements

We are grateful to Dr. B.C. Gonzalez for the invaluable help with the English. We would like to thank our colleagues of the Pertsov White Sea Biological Station and the Zoology Department of Lomonosov Moscow State University. We thank A.A. Semenov, D. Ozerov, G.D. Kolbasova, A. L. Mikhlina, and A.A. Prudkovsky for helping with the sampling. We want to thank the unknown reviewers for their most helpful comments on the typescript and any fruitful discussion. We are very thankful to I.A. Kosevich, A.I. Lavrov, E.N. Temereva, F.V. Bolshakov, and I.A. Ekimova for their assistance with the morphological methods and experimental protocols. Sincere appreciation goes to G. Davidovich, A. Bogdanov, and the Electron Microscopy Laboratory of the Shared Facilities Center of Lomonosov Moscow State University, sponsored by the RF Ministry of Education, Science and Research. The authors would also like to thank S. Metelev, G. Bykov, and I.D. Papanin from the Institute for the Biology of Inland Waters, Russian Academy of Sciences for their help with the electron microscopy. Light microscopy was made possible at the Center of microscopy, WSBS, MSU.

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