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REGULAR ARTICLE

Histological differentiation of mucus cell subtypes suggests functional compartmentation in the eel esophagus

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Abstract

We investigated the morphological and histological changes in eel esophagus during the course of freshwater (FW) to seawater (SW) transfer and identified multiple types of mucus cells from tissues that were fixed using Carnoy's solution to retain the mucus structure. The FW esophageal epithelium is stratified and composed of superficial cells, mucus cells, club cells (exocrine cells with a large vacuole), and basal cells. Two types of periodic acid–Schiff (PAS)-positive mucus cells were identified, and they can be further distinguished by the periodic acid-thionin Schiff/KOH/PAS (PAT) method, indicating that C7/9- and C8-sialic acids were produced. Isolectin B4-positive mucus cells were found among the C8-sialic acid-producing cells and located at the tips of the villi at mid-posterior regions of the FW esophagus. The two different muci were immiscible and may form separate layers to protect the tissues from the high osmolality of imbibed SW during early SW acclimation. The densities of club cells and isolectin B4-positive cells decreased after SW acclimation, and cuboidal/columnar epithelial cells subsequently developed for active Na^+ and Cl^- absorption. Cuboidal/columnar epithelial cells proliferated in scattered array rather than at the bases of the villi, thereby retaining the characteristic of the stratified epithelium. Prominent leukocyte invasion was found at the base of the stratified epithelium at early SW transfer, indicating that the immune system was also activated in response to antigen exposure from imbibed SW. The mucus composition in FW is more complicated than that in SW, fueling further studies for their functions to form unstirred layers as osmoregulatory barriers.

Keywords Mucus layers · osmoregulation · stratified epithelium · sialic acid · desalination · leukocyte invasion · immune response · unstirred layers

Introduction

Seawater (SW) eel esophagus is an osmoregulatory organ responsible for desalinating imbibed SW with a unique transporting system that actively absorbs the Na^+ and Cl^- with minimal water loss (Ando et al. 2003; Nobata et al. 2013). The eel esophagus is morphologically modified in SW with a prominent simple columnar epithelium and developed capillary system to accommodate the active transport of ions, in contrast to a stratified epithelium that blocks water entry in the freshwater (FW) environment (Yamamoto and Hirano 1978). The molecular mechanism for the dual transport of Na^+ and Cl^- in eel esophagus was shown by a combination of physiological and transcriptomic approaches, and key transporters including chloride channel 2, carbonic anhydrase II, anion exchanger 2, and sodium hydrogen exchanger 3 were identified to be involved in the desalination by the columnar epithelium (Takei et al. 2017).

After the direct transfer from FW to SW, eels drink SW immediately (Kirsch and Mayer-Gostan 1973), but the esophageal desalination of SW begins 3–4 days post-transfer as the SW-type columnar epithelium is developed (Yamamoto and Hirano 1978). We previously showed that the number of esophageal mucus cells increased rapidly after FW to SW transfer, and found a Na-binding substance from the mucus that may moderate the effective osmotic potential of Na^+ and alleviate the steep osmotic gradient caused by the seawater

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imbibition (Wong et al. 2017). These results strongly suggest that mucus production is crucial to the protection of tissues from sudden osmotic challenge. Previous electrophysiological studies showed that the esophageal mucus was separated into layers where the negatively charged mucus contributed to a higher Cl^- gradient in the anterior part of the SW esophagus (Simmoneaux et al. 1987). Moreover, the ultrastructural analysis of the esophageal mucus shown by scanning electron microscopy showed that different types of mucus were secreted in FW and SW environments (Humbert et al. 1984). In the intestine, mucus layer segregation was also suggested by X-ray microanalysis results where the mucus near the luminal layer has an increasing concentration of Cl, S, and Ca compared with that closer to the epithelium (Humbert et al. 1984). While many studies suggested an osmoregulatory role of mucus in fish as water barriers and microclimate layers, the evidence was described as "circumstantial" due to some limitations including (1) poor or failed fixation of mucus; (2) questionable presence of unstirred mucus layers; or (3) indirect and stressful experimental approaches (Shephard 1994).

Histological investigation of mucus characteristics requires specific fixation since common fixatives such as phosphate-buffered paraformaldehyde (PFA) and Bouin's solution contain cross-linking agents that collapse the mucus structure into a thin layer. Successful fixation of mucus was established in the early 1990s using Carnoy's solution to maintain the integrity of the mucus layers (Ota and Katsuyama 1992), and it was demonstrated that the mucus in the human colon is divided into two layers by the secretions from two types of goblet cells (Matsuo et al. 1997). With the establishment of fixation methods, the outer layer of mucus was found to host the microbial community of the large intestine while the inner layer is free of microbes (Johansson et al. 2011; Li et al. 2015). Different layers of mucus provide the opportunity to subdivide the functions such as lubrication, protection from dehydration and/or pathogen, and salinity challenges. For example, galectin in mucus is involved in the immune function in conger eel intestinal lumen (Nakamura et al. 2007). Two lactose-binding lectins expressed by skin club cells (secretory cells in fish epidermis) in Japanese eel can agglutinate bacteria to suppress their growth, indicating that the lectins in mucus are involved in host defense (Suzuki and Kaneko 1986; Tasumi et al. 2002). It is undoubtedly that mucus is the first barrier and defense in fish against environmental challenges. While most previous researches focused on the immunological role of mucus (Reverter et al. 2018), other functions of mucus were scarcely explored in fish. To establish some basic knowledge on the mucus cells and types in eel esophagus, we reexamined the morphology and histology of eel esophagus with various histological markers to study mucus variations under different salinity challenges.

Materials and methods

Animals

Juvenile Japanese eels (*Anguilla japonica*, ca. 200 g) were obtained from a local eel farm and kept in a recirculating aquarium system in the Atmosphere and Ocean Research Institute, The University of Tokyo. The water was maintained at 18 °C, and the eels were exposed to a 14-h/10-h light/dark cycle throughout the experiment. The eels were not fed during the entire course of experiment.

Salinity transfer, tissue sampling, processing, and histology

Eels were transferred from FW (nominally 0‰) to SW (35‰) for 3 h, 12 h, 1 day, 3 days, and 7 days, and FW fish were used as control (Wong and Takei 2012; Wong et al. 2016). Eels were anesthetized in 0.1% ethyl 3-aminobenzoate methanesulfonate (Sigma-Aldrich Chemicals, St. Louis, MO, USA) neutralized with $NaHCO_3$. The eels were sacrificed by decapitation, and the esophagi were dissected out without puncturing the wall. Esophagi were further divided into anterior, middle, and posterior parts of equal length by section with sharp razor blades. The esophageal parts were fixed in Carnoy's solution overnight at 4 °C. The tissues were then transferred to 100% ethanol and stored at 4 °C until further processing. To compare the effects of fixatives, the esophagus from the same individual was divided into two parts and fixed in Carnoy's solution or 4% PFA in 0.1 M phosphate buffer at pH 7.4. Fixed tissues were dehydrated in serial alcohol/xylene solutions and embedded in Paraplast wax (Leica Biosystems, Nussloch, Germany). Cross sections (4 μm) were prepared and mounted on MAS-coated microscope slides (Matsunami, Osaka, Japan). The tissue sections were deparaffinized by xylene and rehydrated to deionized water by serial alcohol solutions.

For hematoxylin and eosin (HE) staining, the hydrated sections were incubated in Mayer's hematoxylin for 2 min, washed in running tap water for 15 min, and subsequently stained with eosin for 2 min. For periodic acid–Schiff (PAS) staining, the sections were incubated in 1% orthoperiodic acid for 10 min followed by Schiff's reagent (Nacalai Tesque, Kyoto, Japan) for 10 min. After washing in sulfurous acid solution three times, the stained sections were counterstained with hematoxylin. For alcian blue staining, sections were incubated in 3% acetic acid (in pH 2.5) for 3 min, and then in alcian blue (pH 2.5) for 30 min, respectively. After washing in 3% acetic acid (pH 2.5) 2 times, the sections were counterstained with nuclear fast red (Muto Pure Chemicals, Tokyo, Japan).

For the Periodic acid–thionin Schiff/KOH/PAS (PAT) method to stain different mucus layers, the hydrated sections were incubated in 1% orthoperiodic acid for 15 min followed

by four washes of deionized water. They were then incubated in thionin Schiff reagent for 30 min followed by washing in running tap water for 10 min. The sections were then rinsed in 70% ethanol for 30 s and treated with 0.5% potassium hydroxide in 70% ethanol for 5 min followed by gentle washing in running tap water. The sections were incubated with 1% orthoperiodic acid for 10 min followed by four rinses in deionized water. They were subsequently incubated in Schiff's reagent for 15 min followed by three changes of deionized water. The sections were next incubated in 0.3% sodium borate for 15 s. Finally, the sections were dehydrated in graded ethanol, cleared in xylene, and mounted with resin.

For Giemsa staining, the hydrated sections were incubated in Giemsa staining solution (1:10 dilution with distilled water, Wako, Osaka, Japan) for 20 min and washed in glacial acetic acid solution (2 drops of glacial acetic acid in 50 mL distilled water) until the color turned into pink. Stained sections were dehydrated with acetone, cleared in xylene, and then mounted with resin.

For detection of α-N-acetylgalactosamine and α-D-galactose, the sections were pre-treated with 0.6% H_2O_2 in methanol for 10 min to inactivate endogenous peroxidase and then incubated in phosphate-buffered saline (PBS) containing 1 μg/mL isolectin B4 (Vector Laboratories, Burlingame, CA, USA) for 60 min. After washing with PBS (5 min × 3 times), immunoreactive signals were developed using a Vectastain ABC Kit (Vector Laboratories, CA, USA) and 3,3′-diaminobenzidine (DAB) as the color reagent according to the manufacturer's protocols. Sections were counterstained with hematoxylin after DAB color development.

For immunohistochemistry, the deparaffinized and rehydrated sections were treated with 0.2% H_2O_2 in methanol for 30 min to inactivate endogenous peroxidase activity and then non-specific sites were blocked by 2% normal horse serum (NHS) in PBS (pH 7.4) for 60 min. Monoclonal proliferating cell nuclear antigen (PCNA) antibody (1:10,000 dilution: NB500-106, Novus Biologicals, CO, USA) and monoclonal cytokeratin AE1/AE3 (Ready-to-use: IS05330-2J, DAKO, Glostrup, Denmark) were incubated with the sections at 4 °C for 16–18 h in a moist chamber saturated with water vapor. Immunoreactive signals were developed using a Vectastain ABC Elite kit (Vector Laboratories) and DAB as described above. Sections were counterstained with hematoxylin after DAB color development.

Sections were viewed using a BX-63 microscope (Olympus, Tokyo, Japan) equipped with a CMOS camera (AdvanCam-U3; AdvanVision, Tokyo, Japan).

Esophageal morphometry

To quantify morphological changes in the eel esophageal epithelium during SW transfer, we counted the number of different cell types (PAS-positive cells, isolectin B4–positive cells, club cells, and PCNA-positive cells) at intervals following FW to SW transfer. Briefly, stained esophageal sections were imaged with a 4× objective lens. Two random areas of epithelial layers (average 50–60 μm^2) from anterior, middle, and posterior esophagus (FW and SW 3 h, 12 h, 1 day, 3 days, and 7 days; $n = 3$ in each group) were measured by ImageJ software v1.50i (National Institutes of Health, Bethesda, MD, USA). Numbers of PAS-positive cells, isolectin B4–positive cells, club cells, and PCNA-positive cells within the region of interest were counted. The cell density was calculated by normalizing the cell number by area (cells/mm^2). For measuring the thickness of the epithelial layer, 10 random epithelia/images were selected and average thickness of the esophageal epithelium was calculated (μm).

Results

General morphology and histology of eel esophagus in FW and SW

The esophagus of FW eels was composed entirely of stratified epithelium with squamous superficial cells forming the surface layer, mucus cells of goblet shape with large secretory granules and small nucleus underneath, club cells with eosinophilic cytoplasm and central vacuoles, and basal cells and leukocytes at the basolateral margin (Fig. 1a, c, e). Taste bud structures were occasionally observed. Superficial cells formed openings for the mucus cells. Undifferentiated basal cells are small in size with a small cytoplasm. In SW eels, the esophageal epithelium remained stratified in the anterior region (Fig. 1b), while thin cuboidal and columnar epithelia were developed in the middle (Fig. 1d) and posterior (Fig. 1f) regions, respectively, along with increased vascularization in proximity. Mucus cells are restricted to basal regions of the villi.

Carnoy's solution vs. PFA fixation

With identical staining procedure, the esophageal epithelial morphology and histology were compared between tissues fixed in Carnoy's solution and PFA (Fig. 2). Carnoy's solution is a protein-coagulating fixative that generally preserves water-soluble substances better than protein-denaturing fixatives such as PFA. In both PFA- and Carnoy's solution–fixed tissues, the secretory granules of mucus cells were transparent in HE (Fig. 2a, b), but were strongly stained purple and blue in PAS and alcian blue, respectively (Fig. 2c–f). In Carnoy's solution–fixed tissues, the mucus cells can be clearly subdivided into two distinct populations with "hollow" and "solid" granules in both PAS and alcian blue stainings (Fig. 2c, e), while these subpopulations were ambiguous

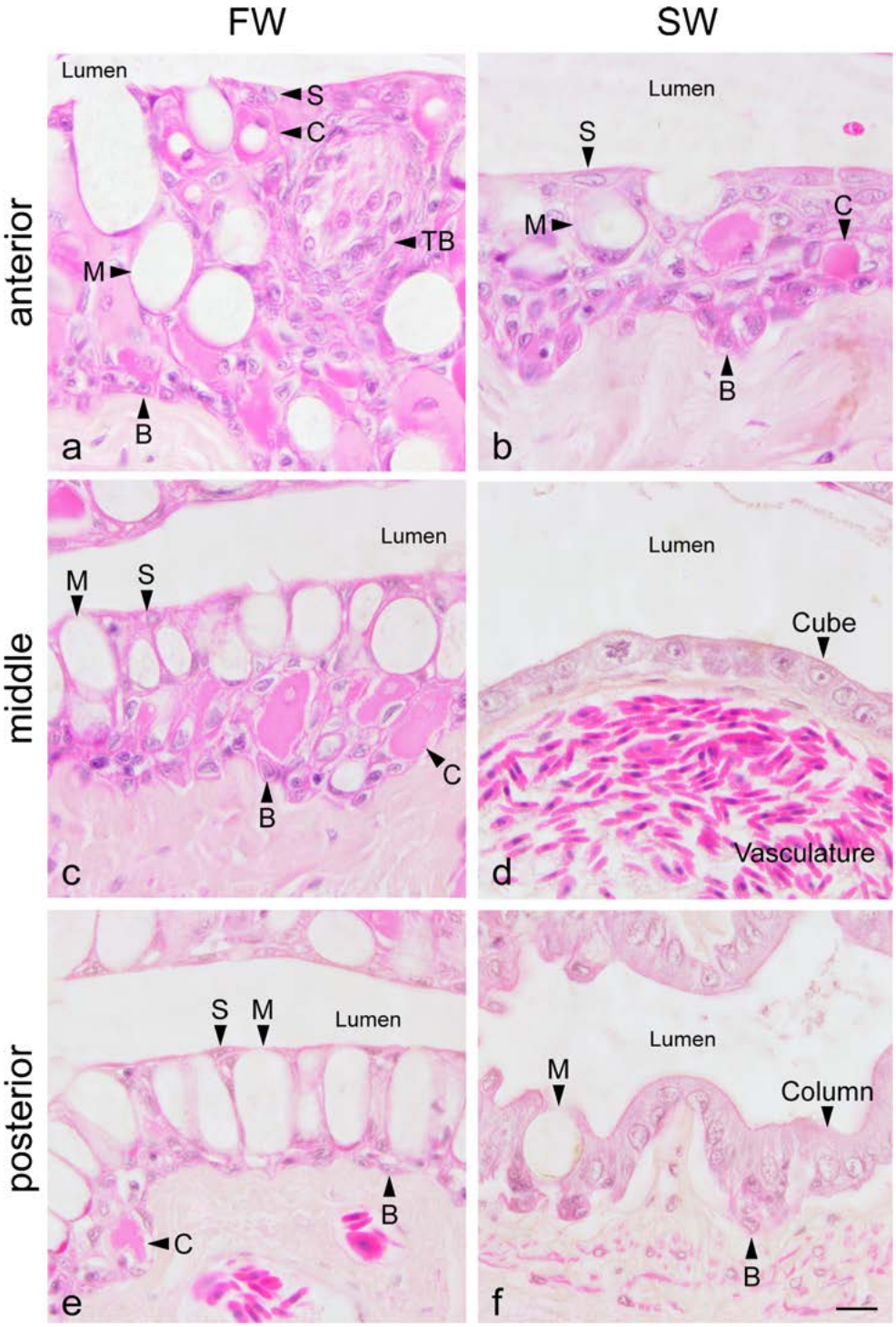


Fig. 1 General morphology of esophageal epithelia among anterior (**a**, **b**), middle (**c**, **d**), and posterior regions (**e**, **f**) in FW and SW eels. Sections were stained with HE. Scale bar = 10 μm. S = superficial cell; M = mucus cell; B = basal cell; C = club cell; TB = taste bud; Cube = cuboidal cell; Column = columnar cell

in PFA-fixed tissues (Fig. 2d, f). Club cells are more eosinophilic in Carnoy's solution–fixed esophagus but less conspicuous in PFA-fixed tissue (Fig. 2a, b). Mucus in the lumen appeared as mesh-like and thread-like structures in Carnoy's solution–fixed tissues (Fig. 2c) while it was collapsed into thin layer lying on the superficial cells in PFA-fixed tissues (Supplementary Fig. 1).

Biochemical properties of mucus and mucus cells

We used serial consecutive sections to study the chemical properties of mucus and mucus cells in Carnoy's solution-fixed tissues. The "hollow" and "solid" mucus in PAS and alcian blue stainings corresponded to each other (Fig. 3a, b, Supplementary Figs. 2 and 3). Using PAT staining that can distinguish mucus

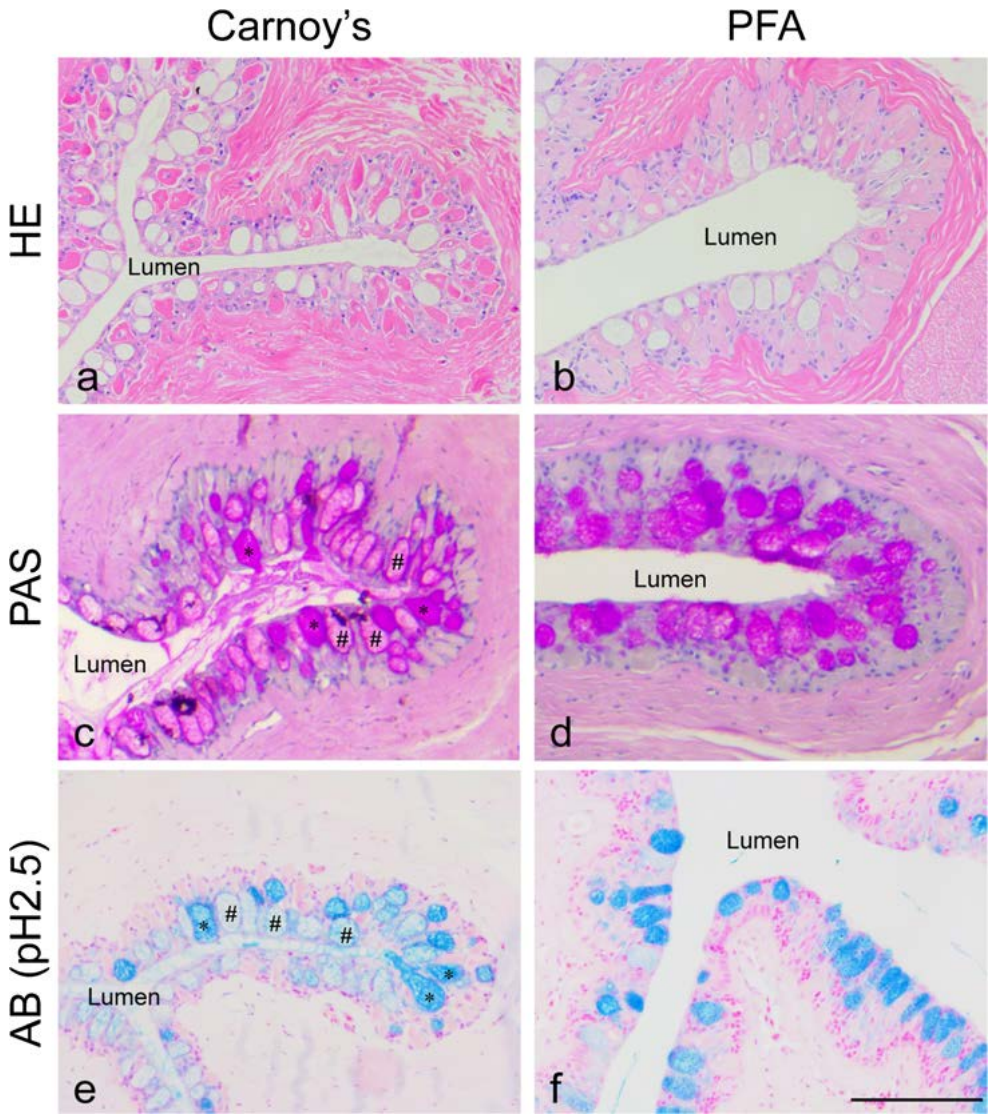


Fig. 2 Comparison between esophageal epithelia fixed by Carnoy's solution and PFA. Sections were stained with HE (**a**, **b**), PAS (**c**, **d**), and alcian blue (AB) pH 2.5 (**e**, **f**). Thread-like mucus can be found in the lumen of Carnoy's solution–fixed esophagus. Scale bar = 100 μm. * indicates "solid" mucus cell, and # indicates "hollow" mucus cell

layers in human colon (Matsuo et al. 1997), we observed that the "solid" mucus cells were stained blue while the "hollow" mucus cells were stained purple (Fig. 3c, Supplementary Fig. 4). Besides the stained mucus in cells, the mucus in the lumen was also stained blue and purple, and corresponded to "solid" and "hollow" mucus as in PAS and alcian blue. On the other hand, isolectin B4 stained the "hollow" mucus and mucus cells but not the "solid" mucus and mucus cells (Fig. 3d). While the isolectin B4-positive mucus corresponded to purple mucus in PAT staining, not all the purple mucus cells are isolectin B4-positive (Fig. 3e and f, Supplementary Fig. 5).

Descriptive and quantitative morphological changes in various regions of esophagus during FW to SW acclimation

We summarized the qualitative and quantitative changes of various mucus cell types in Figs. 5, 6, 7, and 8 with a morphometric approach. During early SW transfer (SW 3 h–SW 12 h), the epithelial structure remained intact despite the sudden exposure to hyperosmotic media. By the late SW acclimation, the mid-posterior regions developed the columnar epithelium as described above. Unlike the intestinal epithelium, no prominent brush border was observed in the columnar/cuboidal epithelium. The luminal surface area was enlarged by increased folding and thinning of the epithelial layer (Fig. 4a). We identified various types of leukocytes at the basolateral side of the epithelium in large numbers from SW 3 to 12 h (Fig. 5a–f). From morphological identification, these leukocytes consisted of mostly macrophages with irregular shapes of nuclei and lymphocytes with round nuclei, with few granulocytes that possess horseshoe-shaped nuclei or eosinophilic granules (Fig. 5b, f). Cytokeratin is a histological marker that indicates whether the cells are of epithelial origin. As the leukocytes were not cytokeratin-positive (Fig. 5c, d), it supports the leukocyte-invaded epithelial layer during early

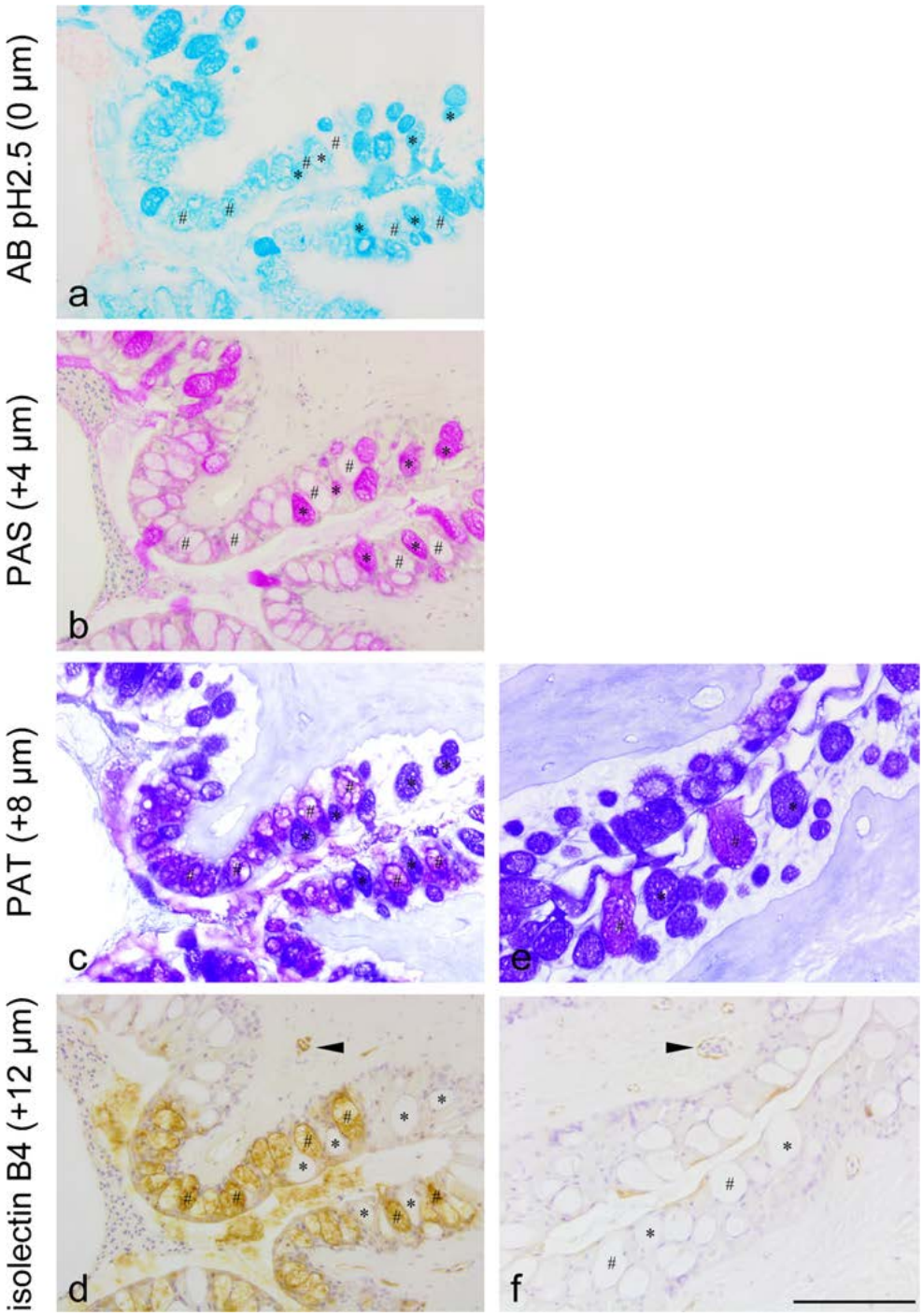
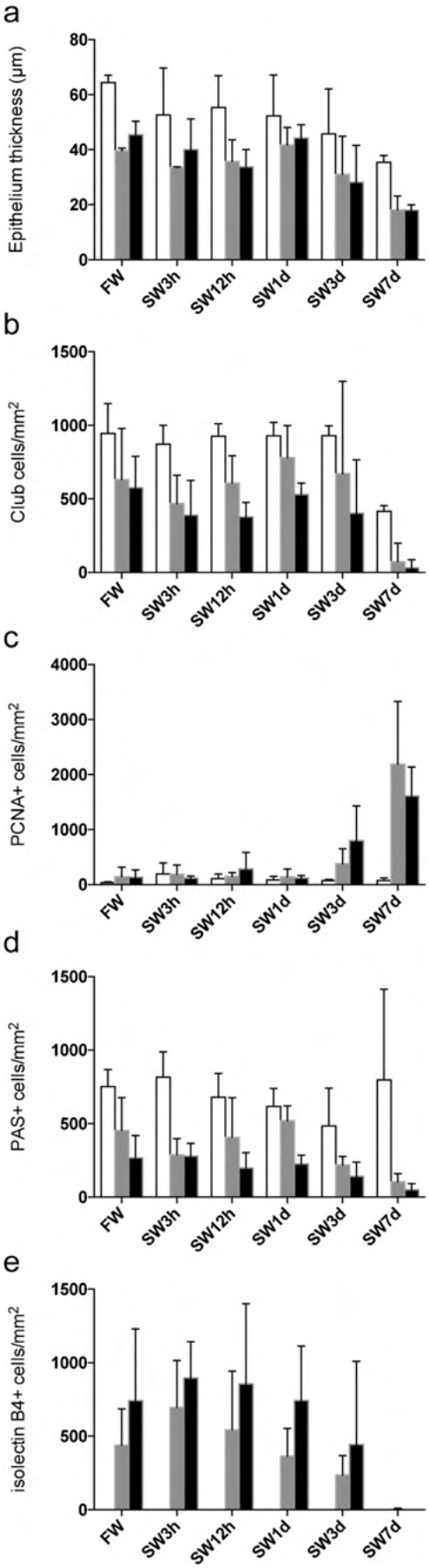


Fig. 3 Mucus and mucus cell characteristics of the esophageal epithelium. Consecutive sections (4 μm) were stained with alcian blue (AB) pH 2.5 (**a**), PAS (**b**), PAT (**c**), and isolectin B4 (**d**). * indicates "solid" mucus cell, and # indicates "hollow" mucus cell. Isolectin-positive mucus is purple mucus in PAT and "hollow" mucus in PAS and AB. In **e** and **f**, another area of consecutive sections are shown to indicate that not all isolectin B4-positive mucus are purple mucus in PAT. Isolectin B4 stains endothelium of blood vessel (arrowheads). Scale bar = 100 μm

SW transfer. The invasion of leukocytes was attenuated after SW 1 d and was followed by nuclear fragmentation of leukocytes (Fig. 5g), which suggested that the inflammatory response ceased after SW 1 day.

During SW transfer, the density of club cells decreased and these cells were nearly absent in the posterior region at SW 7 days (Fig. 4b). Club cells remained relatively abundant in the anterior esophagus after SW 7 days; however, the density was decreased to half of that in the FW epithelium. Cell proliferation activity was monitored using PCNA immunohistochemistry, and positive signals are present in mitotic cells with nuclear staining. PCNA-positive cells were found among the stem cells at the basement membrane, but their density was low in epithelia of FW-acclimated eels. Intriguingly, the density of PCNA-positive cells did not increase until SW 7 days (Fig. 4c), and then they are located among the columnar/cuboidal epithelial cells in scattered array (Fig. 6). "Solid" PAS mucus cell density was higher in the anterior region



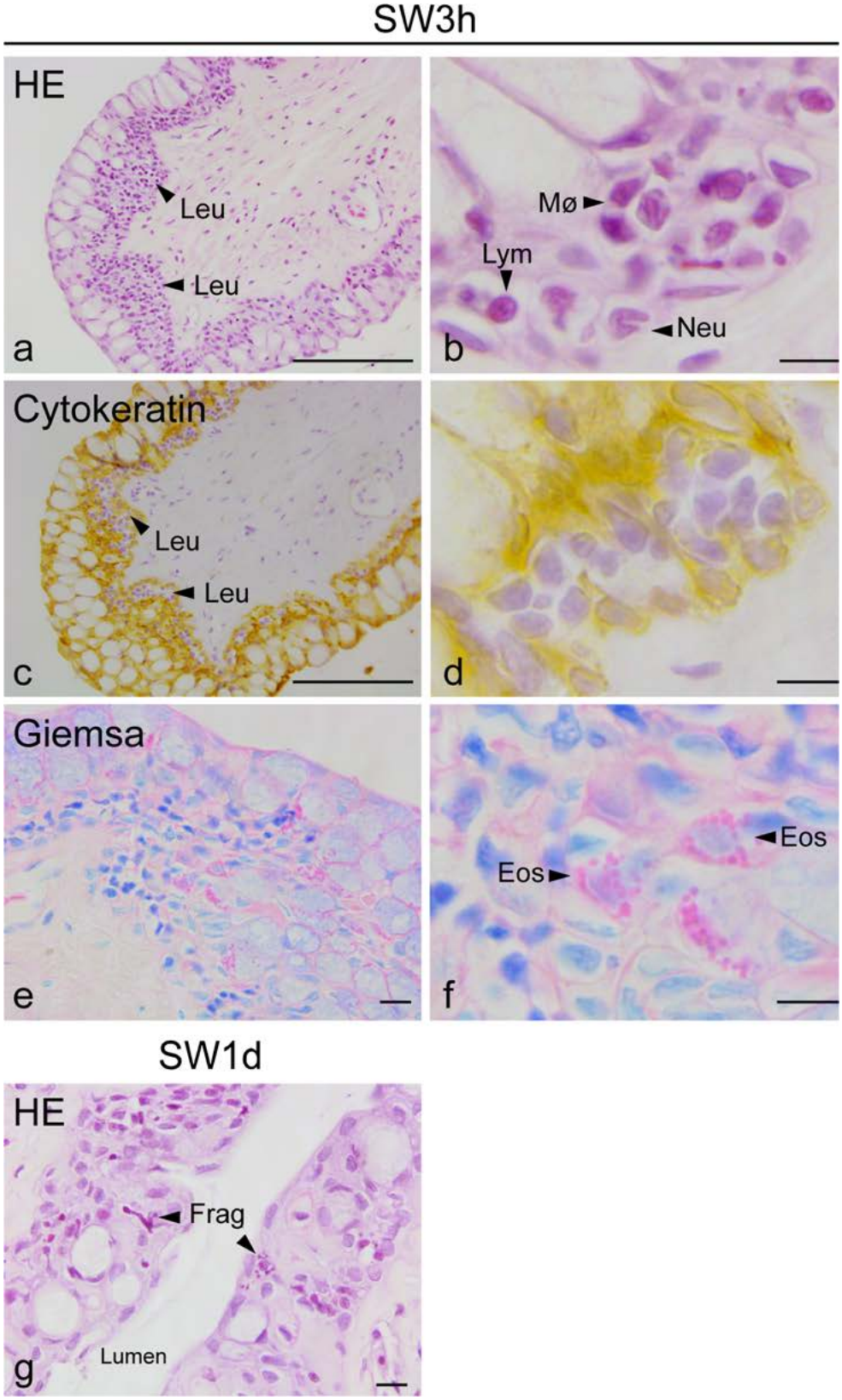
◀ **Fig. 4** Morphometry of esophageal epithelia during the time course FW to SW transfer. Epithelial thickness (**a**), club cell density (**b**), PCNA-positive cell density (**c**), PAS-positive cell density (**d**), and isolectin B4–positive cell density (**e**) were measured in anterior (white), middle (grey), and posterior (black) regions at various time points ($N = 3$)

and decreased towards the posterior region (Fig. 4d). The PAS cells were mainly found at the bases of the villi after SW acclimation (Fig. 7). Isolectin B4-positive cells were found exclusively in middle and posterior regions, and they were located close to the tip of the villi (Fig. 8). Their density decreased from SW 1 day and were rarely seen after SW 7 days (Fig. 4e). From PAT staining, SW acclimation generally decreased the proportion of blue-stained mucus, resulting in an increased proportion of purple-stained mucus. Morphometric analysis was not performed in PAT-stained sections, because the blue staining tends to hide the purple staining, which may lead to ambiguous measurement of cell types.

Discussion

The use of Carnoy's solution as the fixative to observe mucus was a breakthrough in histological technique (Ermund et al. 2013). So far, this fixation process has not been applied in fish studies and we first investigated whether this fixation process can lead to new observations in the eel esophagus, which undergo dramatic anatomical, histological, and physiological modification during transfer from FW to SW. We summarized the time-course changes in cell types, density, and morphology of the eel esophagus in Fig. 9, showing that the eel esophageal epithelium displayed complex and regional specialization for its multifunctional nature, rather than a simple conduit conveying foods and water to the stomach. Studies using a single, simple model of the eel esophagus may underestimate the differential roles played by various regions, which integrate coherently to perform osmoregulatory and immune functions.

With Carnoy's solution–fixed tissues, two subpopulations of mucus cells were identified in the eel esophagus. They have distinctive "solid" and "hollow" characteristics after PAS and alcian blue stainings, which were masked when tissues were fixed by PFA. To further confirm that different types of mucus are produced by these subpopulations, we performed PAT staining and found that "solid" and "hollow" mucus cells are stained blue and purple, respectively, suggesting that the "solid" cell mucus contains C7/C9 sialic acids while "hollow" cell mucus contains C8 sialic acids (Ramey et al. 2005). Isolectin B4 is usually used as a marker for endothelial cells of blood vessels, and it readily stained the endothelium of eel blood vessels (Fig. 3d, f, arrowheads). To our surprise, the isolectin B4 stained some of the "hollow" mucus cells on the villi except those at the basal region, indicating that the isolectin



B4–positive molecules were secreted along with C8-sialic acid mucus. This also indicated that the C8-sialic acid mucus cells can be subdivided into at least two groups based on isolectin B4 reactivity. Isolectin B4 is specific to α-galactose residues that are involved in the immune response by adhering to the micro-organism surface (Baos et al. 2012). This

◀ **Fig. 5** Representative esophageal epithelia showing leukocyte invasion after SW 3 h (**a**–**f**) and nuclear fragmentation after SW 1 day (**g**). **a**, **b** HE staining shows invasion of leukocytes in the epithelial layer. Cells having a round, horseshoe-shaped, and irregular shape nuclei are identified as lymphocytes, neutrophils, and macrophages, respectively. **c**, **d** Cytokeratin immunohistochemistry indicates that invaded cells are not of epithelial origin, supporting that they are invaded leukocytes, but not proliferated resident epithelial cells. **e**, **f** Giemsa staining shows eosinophils having pink granules. Some granules are secreted and scattered within the epithelial layers. Leu = leukocyte; Mø = macrophage; Lym = lymphocyte; Neu = neutrophil; Eos = eosinophil; Frag = nuclear fragmentation. Scale bars = 100 μm in **a**, **c**, 10 μm in **b**, **d**–**g**

suggests that the isolectin B4-positive mucus could be related to immune responses, but its absence in the anterior esophagus discounted that possibility since the anterior region experiences first contact with external agents including foods and water. Moreover, the isolectin B4–positive mucus decreased after SW acclimation, where the esophagus is constantly exposed to pathogens via copious drinking. In contrast, the isolectin B4-positive mucus could have an osmoregulatory role since it was abundant in FW and increased during early SW transfer, but was not produced in the SW-type esophagus that is actively engaged in desalination (Takei et al. 2017). An increase in leukocyte migration towards the epithelium during early SW acclimation suggested that acute immune responses occur against foreign antigens in the imbibed SW (Fig. 5a–f). Nuclear degranulation and degrading leukocytes were observed in the lumen at SW 1 day, indicating that the leukocytes actively degraded the foreign antigens from SW (Fig. 5g). The esophagus faces both pathogen and osmotic challenges when eel initiates copious drinking of SW, and the secretion pattern of isolectin B4-positive mucus matched with the Na-binding role suggested in a previous study (Wong et al. 2017).

In the lumen, mucus compartmentation was demonstrated by PAT staining, which was first applied to show two distinct mucus layers in the mammalian colon (Matsuo et al. 1997). C8 sialic acid may provide the structural difference underlying the hydrophobicity of the mucus, and explain the immiscible characteristics with C7/9 sialic acid mucus (Jaques et al.

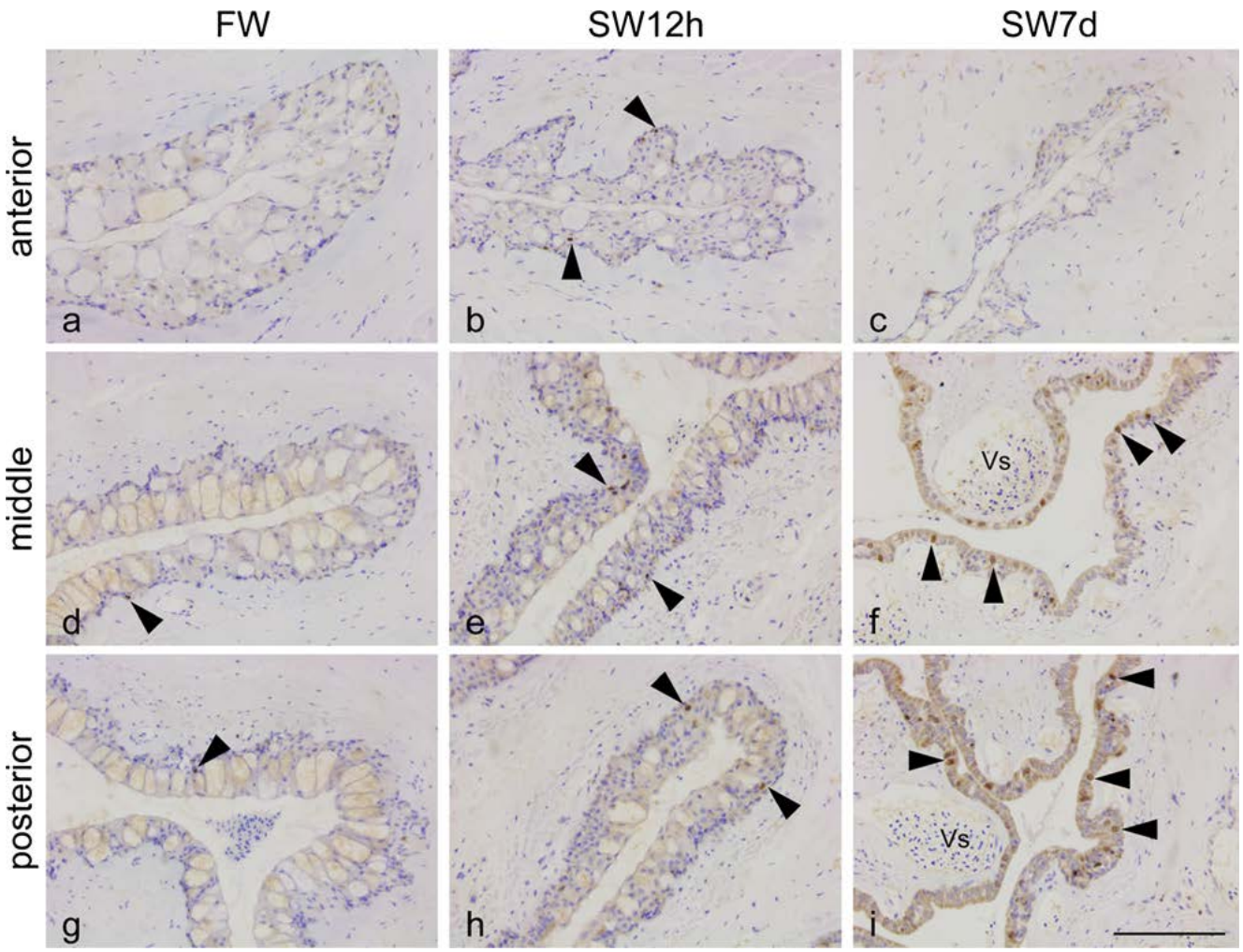


Fig. 6 PCNA immunoreactive staining of esophageal epithelia from anterior (**a**–**c**), middle (**d**–**f**), and posterior (**g**–**i**) regions at representative time points during FW to SW transfer. Vs = vasculature; arrowheads indicate PCNA-positive cells with proliferation activities. Scale bar = 100 μm

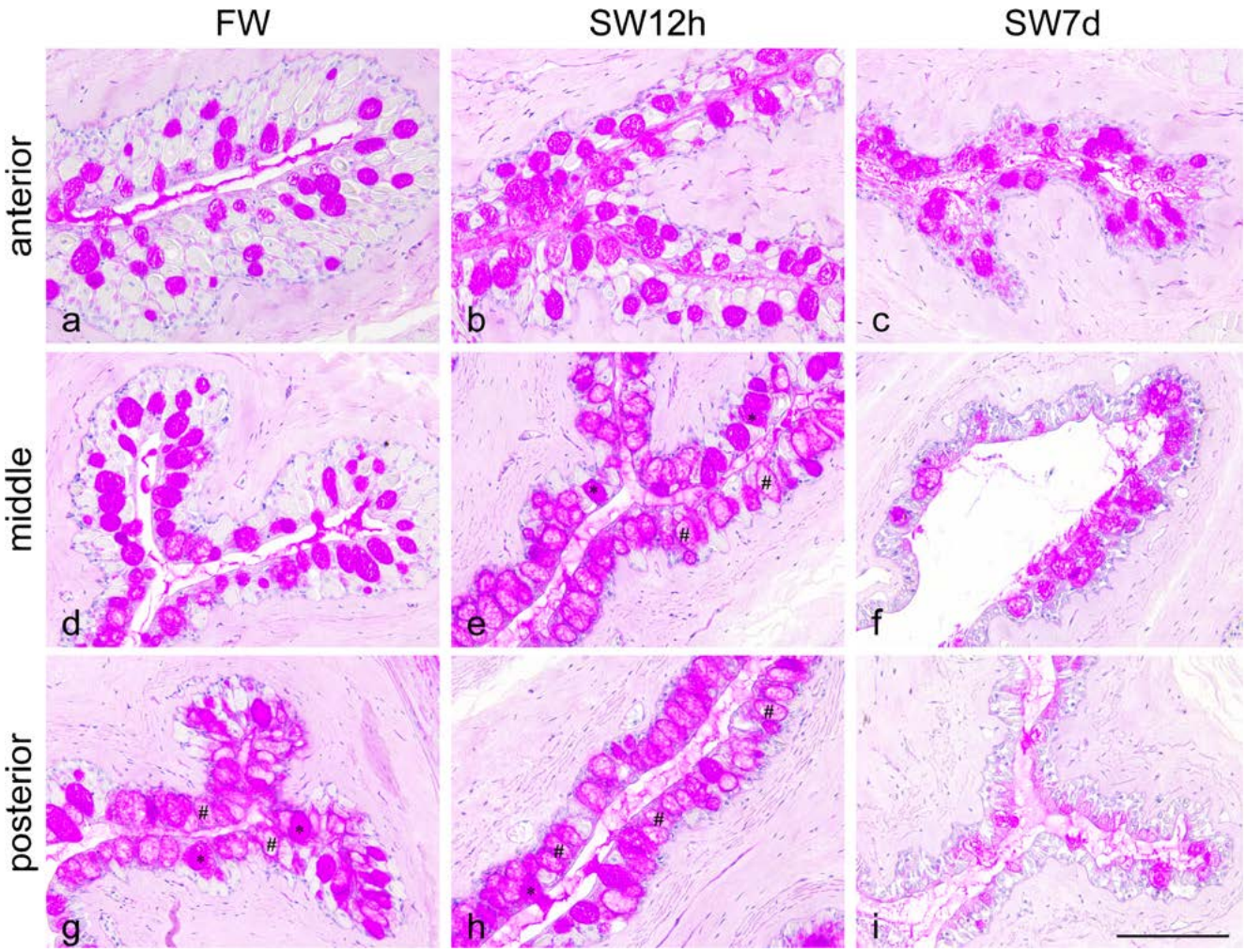


Fig. 7 PAS staining of esophageal epithelia from anterior (**a**–**c**), middle (**d**–**f**), and posterior (**g**–**i**) regions at representative time points during FW to SW transfer. Open arrow heads indicate "hollow" mucus cells (isolectin B4–positive cell in Fig. 8); solid arrow heads indicate "solid" mucus cells (isolectin B4–negative cells in Fig. 8). Scale bar = 100 μm

1977). Indeed, our PAT staining clearly showed separation of blue and purple mucus (Fig. 3c). The compartmentation allows functional specification, and our data showed that isolectin B4–positive carbohydrate or glycoprotein was present in the C8-mucus compartment, although the exact roles for it are not yet delineated. The PAT staining differentiates the mucus of C8-sialic acid as purple while that of C7/9-sialic acid as blue, but some purple mucus cells were capsulated by blue mucus (Fig. 3c, e), indicating that the cells can potentially produce both types of mucus, but they are not mixed even before secretion.

The eel esophagus is a stratified epithelium and undergoes dramatic morphological and physiological changes during FW to SW transfer. The entire length of the FW esophagus is stratified, containing mucus cells, club cells, superficial cells, and basal cells. The cellular composition is more comparable to skin rather than to the intestine although it is part of the digestive tract. The SW esophagus retained a similar stratified structure in the anterior portion while the mid-posterior portions developed absorptive cuboidal-columnar epithelia with extensive vascularization. After the peak of mucus production in early SW acclimation (SW 3 h–12 h), the number of mucus cells dramatically decreased in the mid-posterior regions, and the mucus cells were restricted to the basal intervillar regions, suggesting that the SW eel esophagus may secrete less mucus. Coincidently, the isolectin B4–positive mucus cells were more abundant in areas away from the bases of the villi (Fig. 3d), and they ceased production in late SW acclimation, leaving only mucus cells at the bases of the villi. This indicated that the isolectin B4–positive mucus became redundant after the columnar epithelium was developed for the desalination of imbibed SW. FW eels do not drink a significant amount of water, and mucus secretion is important for maintaining the fluidity of the esophagus, while SW eels drink copiously and the imbibed volume could provide sufficient fluidity (Takei et al. 1998), which coincides the decrease in mucus cell numbers. Previous electrophysiological results demonstrated that the anterior esophageal mucus

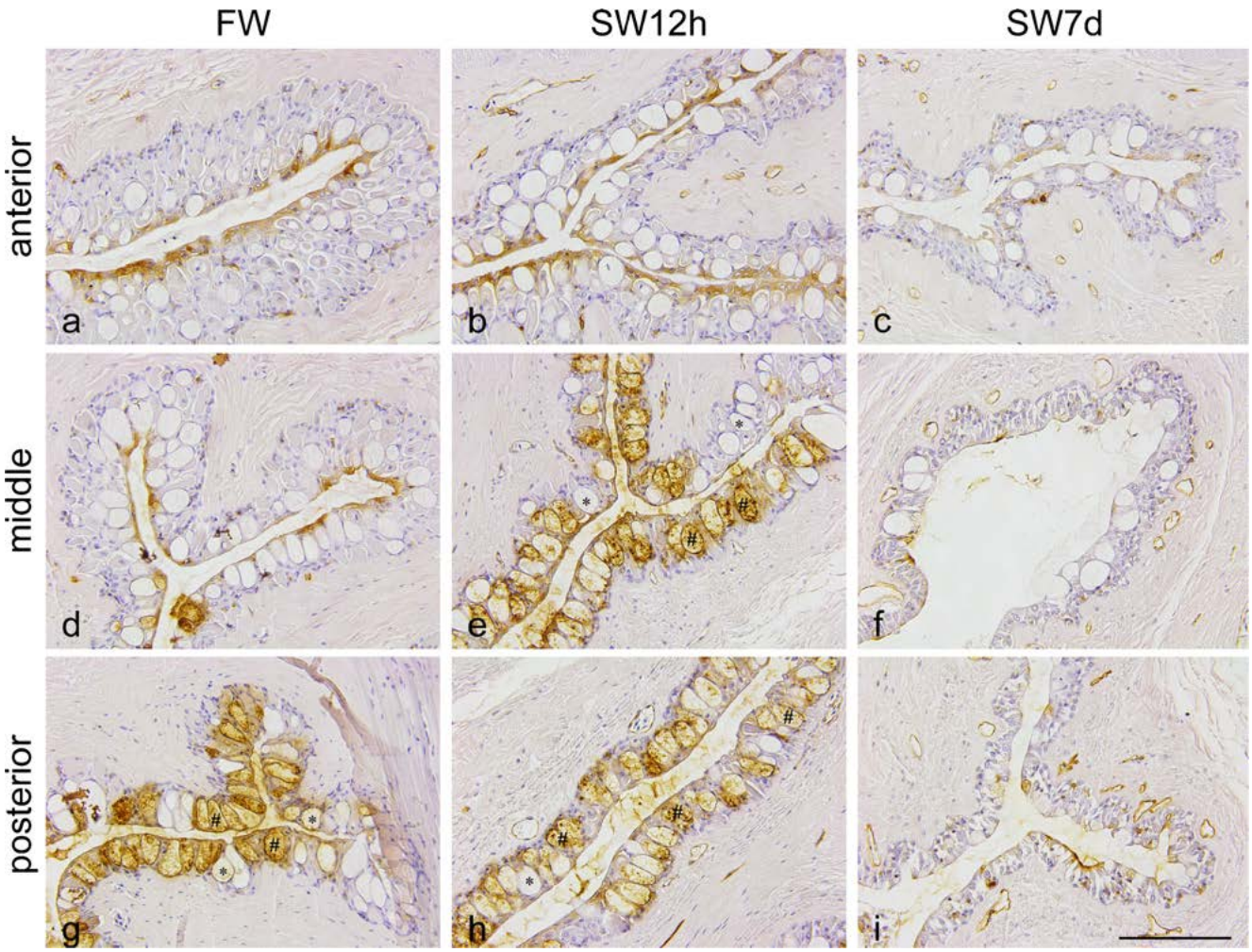
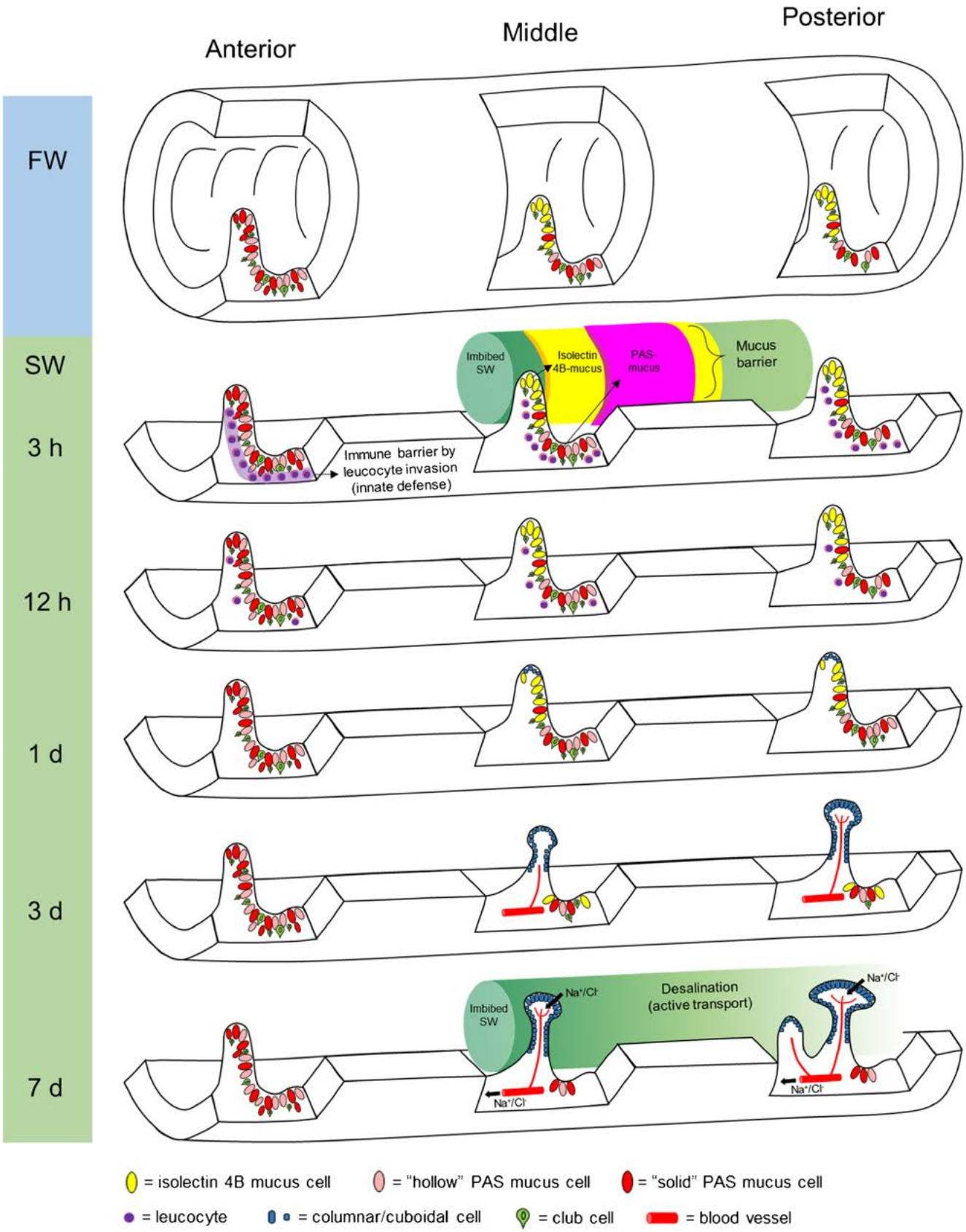


Fig. 8 Isolectin B4-immunoreactive staining of esophageal epithelia from anterior (**a**–**c**), middle (**d**–**f**), and posterior (**g**–**i**) regions at representative time points during FW to SW transfer. Open arrowheads indicate isolectin B4–negative mucus cells ("solid" cells in Fig. 7); solid arrowheads indicate isolectin B4-positive mucus cells ("hollow" cells in Fig. 7). Scale bar = 100 μm

possesses a Cl^- gradient twice as deep as that of the posterior part, while the Na^+ gradients among different regions were constant in SW eels (Simmoneaux et al. 1987). However, few FW esophageal mucus studies were performed previously, perhaps due to the assumption that mucus is more important as the salt barrier in SW (Karlsen et al. 2018). In the esophagus of FW minnow, the mucus layer lowers the Cl^- diffusion gradient, suggesting that an unstirred layer of mucus could be important for the reduction of ion loss (Shephard 1984). Our data suggested that mucus composition and functions change in different salinities, with FW mucus being more complicated than that of SW and deserving further investigation.

In human, Clara cells were renamed as club cells in 2013 (Irwin et al. 2013), but our definition for club cells is based on the morphological similarity to those of skin club cells found in other teleost species (Suzuki and Kaneko 1986). Skin club cells release alarm cues such as chondroitins as warning signals (Reverter et al. 2018) and galectin for immune function (Nakamura et al. 2001). The exact functional roles of skin club cells are still under debate, and so far no studies on esophageal club cells are available. Our data suggests that they could be part of the FW mucus as the distribution pattern is highly similar to that of PAS-positive mucus cells. Desquamated club cells were observed in the mucus layers in the lumen during early SW transfer, suggesting that the mode of secretion could be holocrine in eels.

The SW esophagus desalinates imbibed SW to reduce the osmolality via active absorption of Na^+ and Cl^- at equal molar ratios without water loss (Ando et al. 2003; Takei et al. 2017). However, the time course for morphological changes (Fig. 9) showed that the absorptive epithelium does not develop until 3 days after SW transfer, suggesting that the transporter-dependent desalination was not dominant during early SW transfer. In the mid-posterior regions, columnar epithelia with blood vessel formation indicated increased ion absorption capacities, which accords with the osmoregulatory role for esophagus in long-term SW acclimation. The abundant



◀ **Fig. 9** Schematic diagram summarizing the dynamic changes in various regions of esophageal epithelia in during FW to SW transfer. Relative mucus cell and epithelial cell densities are shown at the tips and bases of villi along the esophagus. In early SW exposure (e.g., SW 3 h), massive leukocyte invasion enhances the innate immune response against antigens from SW. Layers of mucus could be formed by different types of mucus cells located at the tips and bases of villi, providing protection against imbibed SW. In late SW exposure (e.g., SW 7 days), columnar epithelium forms and active transport of Na^+/Cl^- are prominent to desalinate the imbibed SW

PCNA staining indicated a higher turnover rate for the columnar epithelium (Figs. 4c and 6), consistent with active reorganization of the esophageal epithelium during late SW acclimation. However, eels start drinking SW immediately after SW exposure (Nobata et al. 2013), and the imbibed SW is not desalinated by the columnar epithelium. The mucus cells are important for protecting against the osmotic stress caused by the imbibed SW, probably via Na-binding molecules in the mucus (Wong et al. 2017). Combining the features of different localities of isolectin B4-positive mucus cells and PAS-positive mucus cells and apparent compartmentalization, a layer of isolectin-B4 positive mucus could be surrounded by a layer of PAS-mucus in the lumen, thus providing a protection gradient. It is particularly intriguing that the isolectin B4-positive mucus may contain the Na-binding molecules and possibly form the first barrier against the salt challenge during the early exposure to SW.

It is surprising that PCNA staining suggested that cell proliferation in the esophageal epithelium is low, even during the early SW transfer when it was expected that rapid reorganization of the epithelial layer for desalination was necessary. However, the small amount of PCNA staining suggested that the increase in mucus cells during the early SW transfer was a result of increased activity of existing mucus cells rather than increased proliferation of new mucus cells. During late SW acclimation, moderate PCNA staining was observed on the cuboidal-columnar epithelial cells, indicating that they express mitotic activity (Fig. 6). The proliferation of the intestinal villi usually appears at the base of the intervillar region of teleosts (Sanden and Olsvik 2009) or crypt in mammals (Lo et al. 2018), but the scattered PCNA staining on the esophageal simple columnar epithelium of eel suggests that absorptive cells proliferate ubiquitously on the villi. This indicates that the columnar epithelium retains the ubiquitous proliferating pattern as in other stratified epithelia. The proliferation activity in esophagus is relatively low compared with organs such as stomach, intestine, and spleen, which display high PCNA immunoreactivity (Abdo et al. 2014).

In summary, we identified different types of mucus cells and mucus in eel esophagus by Carnoy's solution fixation, which may contribute to functional compartmentation. The isolectin B4–positive mucus and club cells were prominent in FW and during early SW transfer, and could be related to reduction in osmotic stress via Na binding. We also demonstrated that the absorptive columnar epithelium is region-specific to mid-posterior and only develops late in SW transfer. Further studies are required to identify the mucus cell types that may secrete the Na-binding molecules, and the use of salt-free fixatives will be crucial to avoid additional salt that may mask the intrinsic ion-binding properties.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All animal studies were performed according to the Guideline for Care and Use of Animals approved by the Animal Experiment Committee of The University of Tokyo.

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