Fine structures of the mantle tissue in the pinkish-brown salp *Pegea confoederata* (Tunicata: Thaliacea)

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**Abstract:** *Pegea confoederata* is a salp with a pinkish-brown body. The color was retained in the mantle tissue, while the tunic was transparent. We examined the fine structures of the mantle tissue to clarify the cytological basis of the body coloration. Apical cytoplasmic bulges of the epidermal cells were associated with dense tunic fibers, suggesting an involvement in the secretion of the tunic. Light microscopy analysis of the mantle revealed pigment cells that are dendroid-shaped hemocytes filled with brown granules. Five types of hemocytes were recognized in the hemocoel, based on their ultrastructure, and the pigment cells in the present species were hemocytes classified as 'storage cell'. Additionally, some hemocytes were seen to have migrated into the tunic through the epidermis, and were supposed to be presumptive tunic cells.

**Key words:** Pelagic tunicate, Body color, Electron microscopy, Hemocyte types, Tunic, *Pegea*

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**Introduction**

The subphylum Tunicata (Urochordata) is the closest sister group of Vertebrata among the extant metazoans. Tunicates are characterized by a cellulosic tissue that is secreted from the epidermis, called the tunic in Ascidiae and Thaliacea (Belton et al. 1989, Van Daele et al. 1992, Hirose et al. 1999) and the house in Appendicularia (Kimura et al. 2001). Additionally, the presence of an intracellular, cellulosic matrix was suggested in the tail epidermis in appendicularians (Hirose et al. 2011, Nakashima et al. 2011). Salps (Salpida: Thaliacea) usually have a transparent body—a trait also seen in many other gelatinous zooplankton—that probably helps them avoid visual detection by predators. Accordingly, the tunic of salps is expected to have a high transparency and a low reflectance in seawater (e.g., Hirose et al. 2015, Kakiuchida et al. 2017).

In contrast to other salps, *Pegea confoederata* (Forskål, 1775) often has a unique pinkish-brown body coloration. This conspicuous color in the epipelagic layer suggests that a reduction in visibility is not crucial for the survival of this species. In *P. confoederata*, the pinkish-brown color is retained in the mantle, which is comprised of the epidermis, mesenchymal space (hemocoel), and peribranchial epithelium, while the tunic is transparent and rarely reflective (Sakai et al. 2018). Sessile tunicates (ascidians) often have colorful bodies that are comprised of hemocytes (e.g., Burighel et al. 1983, Hirose et al. 1998) or tunic cells, or both (e.g., Hirose 1992, Turon et al. 2005). Salps are also known to have several types of hemocytes (e.g., Péres 1943). Cima et al. (2014) described five types of hemocytes in the transparent salp *Thalia democratica* (Forskål, 1775), based on the ultrastructures, and characterized some functions of each hemocyte type by means of histochemistry and immunohistochemistry. In the present study, we examined the ultrastructure of the mantle of *P. confoederata* to clarify the cytological basis of its pinkish-brown color.

**Materials and Methods**

Floating chains of the salp *P. confoederata* were collected from the ocean surface in Suruga Bay, Japan, using a scoop net. Collections took place on November 5th, 2016, during research cruise of the R/V Hokuto of Tokai University. Solitary zooids that were separated from the ag-
aggregate zooids were fixed in 2.5% glutaraldehyde-seawater onboard and stored at 4°C for microscopy analysis (Fig. 1A). Some pieces of mantle tissue covered with tunic (ca. 5×5 mm) were cut from the middle regions of the bodies of the fixed salps using razor blades. For whole-mount observation, the tissue pieces were mounted in water on glass slides, and observed under a light microscope. For histological and electron microscopic observations, the tissue pieces were rinsed with 0.45 M sucrose and 0.1 M cacodylate buffer (pH 7.5) and post-fixed with 1% osmium tetroxide in a 0.1 M cacodylate buffer (pH 7.5) at 4°C for 1.5 h. The tissue pieces were then dehydrated through an ethanol gradient, cleared with n-butyl glycidyl ether, and embedded in an epoxy resin (Epon 812, TAAB Laboratories). Cross sections of the tunic-mantle tissues were prepared with a diamond knife using an ultramicrotome (EM UC6, Leica). Thick sections were stained with toluidine blue for histological observation. Thin sections were stained with uranyl acetate and lead citrate, and were examined using a transmission electron microscope (TEM) (JEM1011, JEOL) at 80 kV. For morphological classification of the hemocytes, we referred to the terminology for the hemocyte types of the salp *T. democratica* described by Cima et al. (2014).

**Results**

**Gross Morphology**

The body of *P. confoederata* is almost transparent or pinkish-brown in fresh specimens, and transparent, light brown in fixed specimens (Fig 1A), except for the remnant of the placenta and gut nucleus (see ‘rp’ and ‘gn’, respectively, in Fig. 1A). The body is entirely covered by a transparent, gelatinous tunic (facing arrows in Fig. 1A). The mantle appears light brown in color.

In the histological sections, toluidine blue rarely stained
Pigmentary cells in a pinkish-brown salp

the tunic matrix (Fig. 1B–D), while the outermost layer of the tunic, i.e., the cuticle, was homogeneously stained. The mantle, i.e., the tissue layer beneath the tunic matrix, is comprised of two epithelial layers (the epidermis and peribranchial epithelium) and the hemocoel (or connective tissue) between them (Fig. 1D). Various types of hemocytes were found in the hemocoel, and the tunic cells were found to be sparsely distributed within the tunic matrix.

We found several types of hemocytes, including brown-pigmented cells, in the whole mount sections of the mantle (Fig. 1E–I). These hemocytes were characterized by ameboid cell shape (Fig. 1E), some pseudopodia and roundish vacuoles (Fig. 1F), granules that were approximately 2–5 µm in diameter (Fig. 1G) or refractile granules that were about 1 µm in diameter (Fig. 1H). Pigment cells were seen to be dendroid and filled with brown granules that were less than 1 µm in diameter (Fig. 1I).

Fine structures

Epithelia

The peribranchial epithelium is a simple squamous epithelium between the hemocoel and peribranchial cavity (Fig. 2A). The cytoplasm contained mitochondria and endoplasmic reticulum (ER) but no granular inclusions. The epidermis is a simple cuboidal epithelium between the tunic and hemocoel (Fig. 2B). The epidermal cells contained...
mitochondria and rough ER but no granular inclusions. In Figure 1D, a cytoplasmic bulge is seen protruding toward the tunic, resulting in a serrated appearance of the epidermis. Bulges are filled with rough ER, and dense tunic fibers are closely associated with the apical membrane of the bulge (Fig. 2C).

Muscle

The muscle band is located in the hemocoel, and consists of two parts: peripheral myofibrils and the core that contains the nucleus and mitochondria (Fig. 2D). Muscle fibers are multinucleated. It can be seen in Fig. 2E that the core and peripheral parts are not clearly separated, and the nucleus and myofibrils are occasionally contiguous. The nucleus usually has a large nucleolus.
Hemocytes

We recognized five hemocyte types based on their ultrastructural features. Hyaline amoebocytes are often fusiform and less differentiated than the other cell types (Fig. 3A). Their cytoplasm contains mitochondria, ER and small membrane-bounded vesicles (arrowheads in Fig. 3A). Amoebocytes with large vacuoles are roundish and their cytoplasm is filled with large vacuoles (up to 1.2 µm in diameter) (Fig. 3B). These vacuoles contain moderately electron-dense, fibrous materials (Fig. 3C). The granules vary in size and are approximately 2.5 µm in diameter. The type-2 cells are often fusiform and contain round granules (approximately 1 µm in diameter) that are homogeneously and strongly electron-dense (Fig. 3D). Storage cells are irregularly shaped and contain round vesicles of approximately 0.5 µm in diameter (Fig. 3E). These features are consistent with those of the brown pigment cell (Fig. 1I). Within the round vesicles, electron-dense materials form organized structures as a central core and a surrounding shell (Fig. 3E inset). Figure 3F shows an amoebocyte with large vacuoles that is located in the epidermis, suggesting that hemocytes migrate from the hemocoel into the tunic matrix via the epidermis.

Discussion

Epidermis and tunic synthesis

The cuboidal, epidermal cells of *P. confoederata* have a large amount of rough ER, which suggests active biosynthesis. These cells have a cytoplasmic bulge where the apical membrane is closely associated with dense tunic fibers, and is, therefore, probably the site for tunic synthesis. In ascidians, the apical membrane of the epidermis has a complex of cellulose synthase, i.e., the terminal complex, and cellulose microfibrils are assembled at this site (Kimura & Itoh 1996, 2004). The epidermal morphology of *P. confoederata* indicates that salps synthesize tunic in the same way as ascidians do.

Muscle

In the cross sections of the muscle band, the mitochondria-surrounded nuclei form a core, and the myofibrils are located in the periphery (Fig. 1H). This organization has also been reported in other salps, such as *lasis zona*ria (Pallas, 1774) and *T. democratica* (Bone & Ryan 1973, Bone 1998). In the present species—*P. confoederata*—the core and periphery are not clearly separated and the nucleus, mitochondria, and myofibrils are occasionally seen overlapping one another.

Hemocyte types and their possible functions

Cima et al. (2014) described five types of hemocytes from the transparent-bodied salp *T. democratica*: 1. undifferentiated cells (lymphocyte-like cells), 2. hyaline amoebocytes, 3. amoebocytes with large vacuoles, 4. granular cells, and 5. storage cells (nephrocytes). Based on TEM observations, we also recognized five types of hemocytes in *P. confoederata*, many of which are morphologically similar to those in *T. democratica*. We, therefore, followed the terminology of Cima et al. (2014) to classify the five different hemocyte types in *P. confoederata*. Although the undifferentiated cells (lymphocyte-like cells) in Cima et al. (2014) were characterized by small hemocytes with an oval nucleus and scanty cytoplasm, we did not find these hemocytes in the present specimens. This cell-type morphologically is consistent with the hemoblasts or lymphocyte-like cells described in various ascidians (e.g., Burighel & Cloney 1997) and are supposed to serve as hematopoietic stem cells, producing other cell types in the hemocoel. It is possible that undifferentiated cells are localized in a particular site within the hemocoel in *P. confoederata*, and that we did not encounter the site during our examinations.

In *T. democratica*, hyaline amoebocytes are characterized by the massive presence of small membrane-bounded vesicles in the cytoplasm (Cima et al. 2014), whereas the vesicles are not abundant in *P. confoederata*. Cima et al. (2014) histochemically demonstrated that both hyaline amoebocytes and amoebocytes with large vacuoles in *T. democratica* contain acid phosphatase, non-specific esterase, and peroxidase that are generally involved in phagocytosis, and are labeled with *Narcissus pseudonarcissus* agglutinin that serves as a specific marker of mammalian macrophages. These hemocytes may also serve as phagocytes in *P. confoederata*, although we did not conduct such histochemical analyses in this study.

The granular cell type-1 in the present species is consistent with the granular cells in *T. democratica*, in that the granules in both hemocytes have heterogeneous electron densities. Cima et al. (2014) demonstrated by means of immunoelectron microscopy that the granules in *T. democratica* contain heparin and histamine, which classifies the granular cell as a mast cell-like hemocyte. The granular cell type-2 was not consistent with any of the five hemocytes described in *T. democratica* (Cima et al. 2014), and the function(s) of this hemocyte is uncertain. In contrast to the transparent body of *T. democratica*, *P. confoederata* is pinkish-brown, and therefore, the refractile granules in granular cell type-2 may possibly contribute to its body color.

The brown pigment cells in *P. confoederata* are usually dendroid in shape, and have numerous brown, round granules throughout the cytoplasm (Fig. 1I). Because these characteristics are consistent with the ultrastructural fea-
tures of storage cells in Cima et al. (2014), we conclude that the brown pigment cells seen via light microscopy are storage cells. The dendroid cell shape of pigment cells suggests that the cells adhere and extend pseudopodia on to the inner wall of the mantle epithelia. In ascidian hemocytes, nephrocytes and pigment cells have a large vacuole that contains granules that are often crystalline, geometric, or tubular in form (e.g., Burighel & Cloney 1997). Storage cells in T. democratica have vacuoles of various sizes that contain tubular, crystalline granules, and Cima et al. (2014) classified this cell type as a nephrocyte. Storage cells in P. confoederata have small vesicles that contain electron-dense granules that form an organized structure. The difference in structure of granular inclusions in the vacuoles is probably attributable to the difference of the components in the granules. In botryllid ascidians, the colonies of the differently-colored strains have distinct structures of the pigment cell granules (Burighel et al. 1983), and polychromatic colonies have several types of pigment cells characterized by different forms of granules in the vacuole (Hirose et al. 1998, Cima et al. 2015). Accordingly, the difference in the granular contents of the storage cells is probably the reason for the difference in coloration between P. confoederata (pinkish-brown) and T. democratica (transparent or blueish). Notably, the pigments are included in a number of vacuoles in salps, whereas they are contained in a single, large vacuole in ascidians.

**Tunic cells**

Tunic cells are free mesenchymal cells that are distributed in the tunic matrix and perform various roles that depend on the cell types (Hirose 2009). In salps, tunic cells are usually amoeboid and the cell densities in the tunic are much lower when compared with those in the ascidians and pyrosomas (Hirose et al. 1999). This is consistent with the findings of the present study. On the contrary, phagocytic cells that were alive were found even in the 'barrel’ made from a salp tunic that was processed by the amphipod *Phronima sedentaria* (Forskål, 1775) (Hirose et al. 2005). The tunic cells in salps are probably involved in innate immunity.

We found an amoebocyte with large vacuoles in the epidermis (Fig. 3F). This hemocyte probably migrated from the hemocoel into the tunic by crossing the epidermis. This migration suggests that tunic cells originated from hemocytes. Migration of hyaline amoebocytes into the tunic through the epidermis has also been reported in *T. democratica* (Seeliger 1893, Perès 1943, Cima et al. 2014), suggesting that trans-epidermal migration of amoebocytes is common in salps.

**Conclusion**

The present study showed that *P. confoederata* and *T. democratica* share many morphological features, such as muscle organization and certain types of hemocytes. According to the molecular phylogeny of thaliaceans based on 18S rDNA sequences, these genera are relatively specialized and similar to the ancestral condition inside salps (Govindarajan et al. 2011). As a remarkable difference, *P. confoederata* displays an unique body color. It originates from the pigment cells located in the hemocoel of the mantle. This hemocyte is classified as a storage cell based on the ultrastructural features. The pigment is located in small vacuoles as crystalline contents that are brown in *P. confoederata* but are transparent in *T. democratica*. Considering visual detection by predators, a colored body should be unfavorable for gelatinous planktons that occur in the epipelagic layer. Because the present species is often distributed in the euphotic zone during the daytime, the protection from harmful solar radiation may be a possible function of the color. However, other salp species distributed in similar depths in the daytime usually have transparent body, and ultraviolet radiation permeates well through their transparent tunic (e.g., Hirose et al. 2015, Kakiuchida et al. 2017). Therefore, the biological significance of this conspicuous coloration of *P. confoederata* remains unknown.

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