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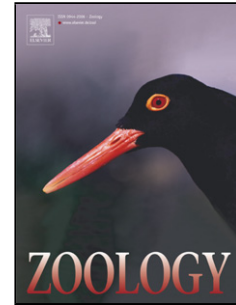


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Fine details of the choanocyte filter apparatus in asconoid calcareous sponges (Porifera: Calcarea) revealed by ruthenium red fixation

Andrey I. Lavrov^{1*}, Fyodor V. Bolshakov¹, Daria B. Tokina², Alexander V. Ereskovsky^{2,3,4}

¹ Pertsov White Sea Biological Station, Biological Faculty, Lomonosov Moscow State University, 129281, Leninskie gory 1-12, Moscow, Russia

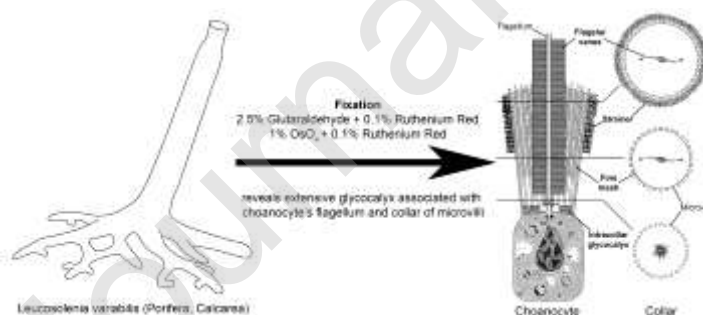
² Institut Méditerranéen de Biodiversité et d'Ecologie marine et continentale (IMBE), Aix Marseille University, CNRS, IRD, Avignon University, Station Marine d'Endoume, Rue de la Batterie des Lions, 13007, Marseille, France

³ Dept. Embryology, Faculty of Biology, Saint-Petersburg State University, Universitetskaya emb. 7/9, 199034, Saint-Petersburg, Russia

⁴ Koltzov Institute of Developmental Biology of Russian Academy of Sciences, Russia, Moscow

* Corresponding author: lavrovai.bio@yandex.ru

Graphical abstract



Highlights

- Application of 0.1% ruthenium red during fixation reveals glycocalyx structures in choanocytes
- Flagella of choanocytes have a pair of flat, symmetrical processes, flagellar vanes
- Microvilli in a collar are sealed in the apical parts by a fine glycocalyx mesh
- The apical parts of collars are connected by a glycocalyx network, strainer

- Glycocalyx structures of choanocytes seem to be involved in pumping and filtration process

Abstract

Sponges (phylum Porifera) are highly specialized filter-feeding metazoans, pumping and filtering water with a network of canals and chambers, the aquiferous system. Most sponges have a leuconoid aquiferous system, characterized by choanocytes organized in small spherical chambers connected with ambient water by a complex net of canals. Such organization requires substantial pressure difference to drive water through an elaborate system of canals, so the choanocytes in leuconoid sponges have several structural features to generate pressure difference. In contrast, it is generally accepted that asconoid and syconoid sponges with long choanocyte tubes or large choanocyte chambers have no similar structures in their choanocytes. The present study is devoted to the detailed ultrastructural analysis of the choanocytes and their filter apparatus in the asconoid calcareous sponge *Leucosolenia variabilis*. The general structure of *L. variabilis* choanocytes is similar to that described for other sponge species. However, the fixation with 0.1% ruthenium red allowed us to reveal for the first time a complex of glycocalyx structures (vanes on the flagella, a fine glycocalyx sealing microvilli in the collar, and a glycocalyx strainer, embedding the apical parts of neighboring collars) in the choanocytes of *L. variabilis*, which are traditionally associated with the pumping and filtration process in leuconoid demosponges. All revealed glycocalyx structures have dimensions and locations similar to those found in the choanocyte chambers of some demosponges. The data suggest that *L. variabilis* utilizes the principles of water pumping and filtration similar to those in demosponges and revealed glycocalyx structures are potentially crucial for these processes. It seems that sponges from distant phylogenetic lineages and with different body plans rely on common principles of choanoderm organization for effective pumping and filtration of water. However, while some adaptation for effective pumping and filtration of water have possibly arisen before the diversification of Porifera, others have appeared independently in different lineages.

Keywords: choanocyte, extracellular matrix, glycocalyx strainer, flagellar vanes, filtration,

1. Introduction

Sponges (phylum Porifera) are highly specialized filter-feeding metazoans. All major metabolic activities of sponges, such as nutrition, respiration, excretion, etc. rely on the pumping and filtering of large volumes of the surrounding water. Thus, the maintenance of highly effective water pumping is of paramount importance for these animals. Sponges continuously change and rearrange their bodies to sustain effective pumping in changing hydrodynamic conditions (Bond, 1992, 1999; Gaino et al., 1995; Ereskovsky, 2010; Lavrov and Kosevich, 2018).

The aquiferous system is a central anatomical and functional structure in a sponge body, responsible for water pumping and filtering. The principal cell type of the aquiferous system is the choanocyte, a cell that bears a single flagellum, surrounded by a collar of microvilli (Ereskovsky and Lavrov, 2021). The choanocytes generate a water flow by the constant beating of their flagella and retain food particles from water by their collars. Despite the general simplicity of the body organization in sponges, there are five different types of aquiferous systems. The majority of sponges have a leuconoid aquiferous system. Such an aquiferous system is characterized by choanocytes organized in small spherical chambers connected with ambient water by an elaborated net of inhalant and exhalant canals (Leys and Hill, 2012; Ereskovsky and Lavrov, 2021). As much pressure is required to drive water through a long canal system, the choanocyte chambers in leuconoid sponges are subdivided into sealed low- and high-pressure zones (Weissenfels, 1992; Asadzadeh et al., 2019). Maintenance of the pressure difference between these zones relies on several structural features, which were found in the choanocyte chambers of demosponges and hexactinellids: 1) vaned flagella, 2) microvilli in a collar sealed by a glycocalyx mesh across their apical parts, and 3) glycocalyx strainer (in demosponges) or secondary reticulum (in hexactinellids) uniting apical parts of neighbor collars (Afzelius, 1961; Fjordingstad, 1961; Brill, 1973; Mackie and Singla, 1983; Mehl and Reiswig, 1991; Reiswig and Mehl, 1991; Saller, 1989, 1991; Weissenfels, 1992; Leys, 1999; Leys et al., 2007, 2011; Mah et al., 2014).

In contrast, asconoid and syconoid sponges possess simpler aquiferous system organization with relatively short canal systems and numerous choanocytes lining large cavities (Leys and Hill, 2012; Ereskovsky and Lavrov, 2021). It is thought that they do not need high pressure difference to pump water through their aquiferous system and, consequently, do not have the low- and high-pressure zones and corresponding structural features in their choanocyte cavities (Leys and Eerkes-Medrano, 2006; Pozdnyakov and Karpov, 2013; Asadzadeh et al., 2019, 2020).

The present study is devoted to the detailed ultrastructural study of the choanocytes and their filter apparatus in the asconoid calcareous sponge *Leucosolenia variabilis* using the ruthenium red fixation technique. Here we, for the first time, show the occurrence of a complex of glycocalyx structures (vanes on the flagella, a fine glycocalyx sealing microvilli in the collar, and a glycocalyx strainer, embedding apical parts of neighboring collars), which are traditionally associated with the filtration process in demosponges, in the choanocytes of the calcareous sponge *L. variabilis*.

2. Materials and methods

Specimens of the asconoid calcareous sponge *Leucosolenia variabilis* Haeckel, 1870 (Porifera, Calcarea) (Fig. 1A) were collected in the Kandalaksha Bay, White Sea, in June-August 2019 and July-September 2020 in the environs of the Pertsov White Sea Biological Station of Lomonosov Moscow State University (66°34' N, 33°08' E). The sponges were manually collected in the upper subtidal zone (0-2 m) at low-tide with the brown algal substrate (*Ascophyllum* sp., *Fucus* sp.) and maintained in a 70 L laboratory aquarium with biological filters and natural seawater at a temperature of 6-10°C for no longer than 3 days.

For conventional histological and ultrastructural studies, sponge tissues were fixed with 2.5% glutaraldehyde (Electron Microscopy Science) in modified 0.1M Na-Cacodylate buffer (0.1M Na-Cacodylate, 85.55 mM NaCl, 5 mM CaCl₂, 5 mM MgCl₂; pH 7.0-7.5) for 2 hr at 4°C (half of the fixative was changed by a fresh one after 30 min) and post-fixed with 1% OsO₄ (Electron Microscopy Science) in the same buffer for 1 hr at room temperature (RT). Between the fixation and post-fixation, specimens were rinsed three times with modified 0.1M Na-Cacodylate buffer for 30 min each time at RT with constant orbital shaking at 70 rpm (Supplementary File 1).

For studies of the fine extracellular matrix associated with choanocytes, sponge tissues were fixed with 2.5% glutaraldehyde supplemented with 0.1% ruthenium red (Sigma-Aldrich) in modified 0.1M Na-Cacodylate buffer for 2 hr at 4°C and post-fixed with 1% OsO₄ supplemented with 0.1% ruthenium red in the same buffer for 3 hr at room temperature (RT) (Lavrov and Ereskovsky, 2021; Luft, 1971a). Between fixation and post-fixation, specimens were rinsed three times with modified 0.1M Na-Cacodylate buffer for 30 min each time at RT with constant orbital shaking at 70 rpm (Supplementary File 1).

After the post-fixation, all specimens were subjected to spicule dissolution in 5% EDTA (Sigma-Aldrich) solution for 2 h at RT. For light microscopy and transmission electron microscopy (TEM), specimens were dehydrated in an ethanol series at 4°C and embedded in Epon/Araldite epoxy embedding media (Electron Microscopy Science). Semi-thin sections (1 µm) and ultrathin sections (60–80 nm) were cut using Leica UCT6 ultramicrotome (Leica) or

Ultramicrotome PowerTome XL (RMC Boeckeler). The semi-thin sections were stained with 1% toluidine blue – 0.2% methylene blue mixture for 1-1.5 min at 60°C and studied with Carl Zeiss Axioplan 2 microscope (Carl Zeiss) equipped with digital camera AxioCam HRm (Carl Zeiss) and software AxioVision 3.1 (Carl Zeiss). The ultrathin sections were double-stained with 4% aqueous uranyl acetate solution (40-60 min at +37°C) and 0.4% lead citrate solution (25-30 min at RT in the dark) according to Reynolds (1963) (Supplementary File 1). The ultrathin sections were studied using JEM-1400 Flash (Jeol), JEM-1011 (Jeol), and Zeiss-1000 (Carl Zeiss) transmission electron microscopes.

For scanning electron microscopy (SEM), specimens were dehydrated in an ethanol/acetone series, critical point dried with Hitachi Critical Point Dryer HCP-2 (Hitachi) (Supplementary File 1), gold/platinum-coated with Eiko IB-3 (Eiko), mounted onto aluminum stubs, and studied using CamScan S-2 (Cambridge Instruments) and Jeol JSM6380-LA (Jeol) microscopes.

Measurements of the selected choanocytes structures (Table 1) were done on TEM and SEM micrographs. For each measurement, mean, maximum (max), minimum (min), and standard deviation (SD) were calculated. The means are presented in the Results section, while additional calculations are given in Table 1.

For the analysis of the porocyte distribution in the body wall, sponge tissues were fixed with 2.5% glutaraldehyde in modified 0.1M Na-Cacodylate buffer for 2 hr at 4°C (half of the fixative was changed by a fresh one after 30 min). After the fixation, specimens were rinsed three times with modified 0.1M Na-Cacodylate buffer for 30 min each time at RT with constant orbital shaking at 70 rpm and then subjected to spicule dissolution in 5% EDTA (Sigma-Aldrich) solution for 2 h at RT. Finally, specimens were rinsed twice with modified 0.1M Na-Cacodylate buffer and studied using differential interference contrast (DIC) with Leica DM2500 (Leica) microscope equipped with digital camera Leica DFC 420 (Leica) and software Leica LAS Store and Recall v.4.2 (Leica).

3. Results

Leucosolenia variabilis has a typical asconoid body organization (Fig. 1B) with a thin body wall consisting of three layers: the external exopinacoderm, internal choanoderm, and thin mesohyl located in-between them (Fig. 1B, C). The body wall is perforated by numerous ostia formed by the porocytes (Fig. 1C, E).

The choanocytes of *L. variabilis* have a structure typical for calcareous sponges. They are strongly polarized, trapeziform or prismatic cells (8.7 μm in height, 4.7 μm in width) (Table 1),

which bear a distal flagellum surrounded by a collar of microvilli consistently oriented towards the lumen of the choanocyte tube (Figs. 1C, D, F, G; 2A, B). The choanocytes are united laterally, mainly in the middle part of the cells, by specialized intercellular contacts similar to septate junctions but less regular (Fig. 1H). The basal part of the choanocytes has no basal membrane.

The cytoplasm of the choanocyte exhibits a clear basal-apical gradient in the organelle distribution (Fig. 1F). A flagellar kinetosome is located in the most apical position and composed of microtubular triplets. A cone-shaped basal foot is attached by its broad base to the proximal half of the kinetosome at a right angle. A prominent fibrous root projects from the proximal end of the kinetosome deep into the cell and attaches to the apical nuclear surface. The non-flagellar kinetosome (additional centriole) is orthogonal to the flagellar kinetosome but at a different plan from the flagellar kinetosome, lying underneath and lateral to it under the basal foot. An amorphous electron-dense material surrounds the additional centriole (Fig. 1G).

The pyriform nucleus occupies the apical position. The nucleus has a distal beak-like protrusion that consistently occurs below the flagellar kinetosome. The nuclear chromatin is visualized as a combination of granular and fibrous material, with heterochromatin often associated with the inner side of the nuclear membrane. Often choanocytes have one 0.7 – 1 μm nucleolus. Large Golgi dictyosomes are located atop and at one side of the distal beak-like protrusion of the nucleus. Numerous small vesicles with electron-clear content are characteristic of the apical cytoplasm. Some small spherical mitochondria (0.4 – 0.5 μm in diameter) with the lamellar cristae occur both in the apical and middle regions of the cytoplasm. The middle part of the choanocyte cytoplasm contains most of the lysosome-like vesicles and some phagosomes. The basal part of the choanocyte contains large vacuoles (about 1.7 μm in diameter) and phagosomes (1.8 – 2.7 μm in diameter), as well as microvesicles (Fig. 1F).

The flagellum has a length of approximately 25.4 μm and a diameter of 0.39 μm (Table 1). It emerges from the center of the apical cell surface, where a small cone outgrowth (0.19 μm in height, 0.6 μm in diameter at the base) is located (Fig. 1G) (Table 1). The axoneme of the flagellum has a typical eumetazoan structure 9+2 and is covered with a thin wavy layer of the cytoplasm (Fig. 2D, E). At the proximal part near the cell surface, the flagellum is thinner than in the more distal part, forming a transition zone with a diameter of 0.2 μm and height of 0.36 μm (Fig. 1G) (Table 1). The flagellar transition zone has no special structures. The flagellum protrudes about 12 μm above the collar, which is about half of its length (Fig. 1D).

The collar of microvilli surrounds the flagellum and consists of 31 microvilli (Table 1), which arise directly from the apical cell surface (Figs. 1C, D, F, 2B, C, 3A, B, E). The microvilli of the collar are arranged parallel to the apical-basal axis of the cell. The height of the collar is

12.6 μm (Table 1). It slightly expands from its base to the distal part, having a diameter of 4 μm in the proximal part and 4.8 μm in the distal part (Table 1). The distance between neighboring microvilli in the collar is 0.29 μm (Table 1). The diameter of the microvillus is 0.22 μm (Table 1). After the conventional fixation, many collars show a constriction in their lower half, formed by a bend of microvilli of varying degree.

Application of 0.1% ruthenium red during fixation allows us to reveal extensive glycocalyx associated with the flagellum and collar of microvilli in *L. variabilis* (Figs. 1C, D, 2, 3, 4, 5). The proximal part of the flagellum is covered by the loose layer of glycocalyx 0.58 μm thick (Fig. 2D) (Table 1). At the height of 1.44 μm (Table 1), the pair of flat, symmetrical thread-like processes, flagellar vanes, appears at the lateral sides of the flagellum (Figs. 1D, 2B, E, 4). They occupy approximately 2/3 of flagellar length, including the free part of the flagellum outside the collar, above which the vanes rise by 4.6 μm (Figs. 1D, 2B) (Table 1). The most distal part of the flagellum is naked.

The flagellar vanes consist of glycocalyx fibrils oriented perpendicular to the plasmalemma of the flagellum. Their width is 2 μm , the thickness at the base is 0.11 μm (Fig. 2B, E) (Table 1). According to our materials, there is always a gap of 1 μm between the tips of the vanes and the inner side of the collar (Figs. 2A, 3B) (Table 1).

The plasmalemma at the apical choanocyte surface inside the collar is covered by a 0.58 μm -thick glycocalyx (Figs. 2A, C, 3A) (Table 1). This intracollar glycocalyx has a structure similar to the glycocalyx of the proximal part of the flagellum and forms a continuity with it. Around the transitional zone of the flagellum, the intracollar glycocalyx becomes much thinner, forming a 0.44 μm -wide zone almost free of the glycocalyx (Fig. 2C) (Table 1).

Each microvillus of the collar is covered by a loose layer of the glycocalyx with a thickness of about 0.1 μm (Figs. 3B, C, 4) (Table 1). The glycocalyx of the microvillus extends from the base of the microvillus to its apical end. From the height of 3.9 μm (Table 1) and up to the top of a collar, the microvilli are interconnected by a fibrous material forming a fine mesh that join the microvilli into a tube (Figs. 3B-E, 4). After the fixation with the ruthenium red, all microvilli are straight and do not bend, and the collar has no constrictions like after the conventional fixation.

Additionally, to the fine mesh connecting the microvilli in a collar, the collars of all choanocytes in the choanoderm are interconnected by a dense glycocalyx network (Figs. 1C, D, 2A, 3A, B, F, 5). This glycocalyx network begins at the height of 6.6 μm from the collar base and has a thickness of 4.4 μm (Table 1), thus embedding the apical ~1/3 of each collar (Figs. 3A, 4, 5). The distance between neighboring collars is 2.6 μm (Table 1). Due to this network, microvilli and

glycocalyx form a fairly rigid three-dimensional structure, strainer (Weissenfels, 1992), located on one level and extending throughout the entire system of choanocyte tubes of the sponge. The distances between the elements of the strainer are stable.

4. Discussion

The general structure of *Leucosolenia variabilis* choanocytes is similar to that described for a number of other sponge species (Fjerdingstad, 1961; Brill, 1973; Eerkes-Medrano and Leys, 2006; Leys and Eerkes-Medrano, 2006; Gonobobleva and Maldonado, 2009). The comparison of *L. variabilis* and *Sycon* spp. choanocytes shows that they have almost identical structure (Eerkes-Medrano and Leys, 2006; Leys and Eerkes-Medrano, 2006; Pozdnyakov and Karpov, 2013). This concerns both general traits, e.g., cell shape and size, as well as fine structural details, like the organization of the flagellum basal apparatus (Pozdnyakov and Karpov, 2013) and occurrence of septate-like specialized intercellular contacts, described only in calcareous sponges (Green and Bergquist, 1979; Eerkes-Medrano and Leys, 2006; Lavrov et al., 2018). However, *L. variabilis* choanocytes show stable morphology across different regions of the sponge body in numerous specimens collected over prolonged periods, while choanocytes of *Sycon coactum* demonstrate substantially variable morphology even within a single choanocyte chamber. The described variations of *S. coactum* choanocyte morphology concern cell size, occurrence of the neck region, and morphology of the collar (Eerkes-Medrano and Leys, 2006).

Both the conventional fixation and the fixation with 0.1% ruthenium red initially demonstrate a principally similar quality of tissue and cell preservation in *L. variabilis*. However, fixation with ruthenium red preserves and reveals extensive non-fibrillar extracellular matrix (glycocalyx), associated with the choanocyte flagellum and the collar of microvilli. The glycocalyx quite likely consists of acid mucopolysaccharides as ruthenium red seems to show affinity to these compounds (Behnke, 1968; Luft, 1971b). Preservation of the glycocalyx leads to better preservation of the flagellum-collar system in general: specimens fixed with ruthenium red have a more accurate and regular organization of the collar. While choanocytes after the conventional fixation usually have a constriction in the lower part of the collar (the so-called “Minchin’s ring”), choanocytes fixed with ruthenium red always have straight collars. Possibly, after the conventional fixation, collars lose their rigidity due to erosion of ECM and form a wrinkle in the lower part (Fig. 1F). Thus, “Minchin’s ring” possibly is not a natural condition (Eerkes-Medrano and Leys, 2006) but an artefact of fixation.

The fixation with 0.1% ruthenium red allows us to reveal for the first time a full complex of glycocalyx structures required for the formation of different pressure zones in the choanocyte tubes of the asconoid sponge *L. variabilis*: 1) varied flagella, 2) microvilli in a collar sealed by a

fine glycocalyx mesh across their apical parts, and 3) glycocalyx strainer, embedding apical parts of neighboring collars (Figs. 4, 5). Additionally, we revealed the intra-collar glycocalyx, which covers the apical surface of the choanocyte inside the collar. All revealed glycocalyx structures generally have locations and dimensions similar to those found in choanocyte chambers of some demosponges (Fjerdningstad, 1961; Brill, 1973; Saller, 1989, 1991; Weissenfels, 1992; Mah et al., 2014).

Results of computational modeling show that the glycocalyx structures are of high importance for effective pumping in demosponges (Asadzadeh et al., 2019). The vanes, which are wide enough to create a minimum gap with the collar, allows to generate maximum pressure; the fine mesh connecting the microvilli in a collar prevents leaking of filtered water through the distal part of the collar; finally, the strainer precludes the recycling of water from the inner parts of the choanocyte chamber (Asadzadeh et al., 2019).

It is generally accepted that these glycocalyx structures are required for pumping only in sponges with the leuconoid aquiferous system as they have an extended and elaborated system of canals, which generate substantial resistance for the water flow (Asadzadeh et al., 2019, 2020). In the case of open-type aquiferous systems of asconoid and syconoid sponges (characterized by rather short canals and voluminous cavities lined by the choanocytes), it is assumed that glycocalyx structures are redundant as there is no need for high pressure differences to pump water (Asadzadeh et al., 2019, 2020). A recent study on another sponge with open-type aquiferous system, the syconoid *S. coactum*, combining computational modeling and experimental approach shows that at least this species can substitute a physical strainer made of glycocalyx by creating a “hydrodynamic gasket” above choanocytes’ collars (Asadzadeh et al., 2020). The “hydrodynamic gasket” is a stagnation zone created by two opposite flows: by the inhalant flow from an ostium and the backflow generated by pressure drop above the ostium. The “hydrodynamic gasket” ensures high effective particle capture, preventing them from bypassing the collars in the absence of a glycocalyx strainer. At the same time, the “hydrodynamic gasket” is inefficient in preventing re-filtering of water, and a large portion of water is recirculated inside the chamber (Asadzadeh et al., 2020). The study by Asadzadeh et al. (2020) confirms that choanocyte chambers without physical sealing elements could be effective pumping units in open aquiferous systems.

Altogether experimental and computational approaches show that sponges can use different adaptations to achieve a highly effective pumping and filtration of water. The effective pumping and filtration can rely on the ultrastructural features of choanocytes like in leuconoid demosponges (Asadzadeh et al., 2019) or on the hydrodynamics inside the choanocyte chamber like in the syconoid calcareous sponge *S. coactum* (Asadzadeh et al., 2020). The nature of the

adaptations seems to depend on the general organization of the aquiferous system but can also be influenced by minor structural characteristics (Asadzadeh et al., 2019, 2020).

Concordantly with this assumption, our data show that choanocyte tubes in *L. variabilis*, another calcareous sponge with an even more open aquiferous system (the asconoid aquiferous system), possess complex glycocalyx structures, which was considered to be necessary for the filtration in demosponges with closed leuconoid aquiferous system. The locations and dimensions of the glycocalyx structures potentially indicate that *L. variabilis* utilizes the principles of water pumping and filtration similar to those in leuconoid demosponges (Weissenfels, 1992; Asadzadeh et al., 2019). Moreover, considering the height of the collar (12.6 μm) and the length of the flagellum (25.4 μm) as well as the waveform of the flagellum in action, it seems that more than half of the flagellum resides inside the collar in *L. variabilis*. This further indicates that the pumping mechanism in *L. variabilis* is similar to that of demosponges (Asadzadeh et al., 2019), and that the “collared” part of the flagellum is the pumping unit.

However, our data also corroborate the idea that open-type aquiferous systems generate somewhat lower pressure resistance for the water flow: the choanocytes of *L. variabilis* show a relatively large gap between the vanes and the collar. As the smaller gap is highly important for the generation of maximum pressure (Asadzadeh et al., 2019, 2020), it seems that while glycocalyx structures in *L. variabilis* are potentially crucial for efficient pumping and filtration, the pressure resistance generated by the asconoid aquiferous system and the degree of the sealing of the pumping units differ from those parameters in leuconoid demosponges.

The reason why *L. variabilis* and *S. coactum*, two calcareous sponges with open-type aquiferous systems, utilize different adaptations for effective pumping and filtration of water could possibly be found in the minor structural characteristics of the choanocytes of these sponges. The dimensions of the collar-flagellum system could be one of such characteristics: the choanocytes of *S. coactum* possess much shorter collar and flagellum (4.8 μm and 15.5 μm , respectively) (Leys and Eerkes-Medrano, 2006; Asadzadeh et al., 2020). The “hydrodynamic gasket” is possibly effective for capture of particles only in systems with relatively short collar and flagellum, while the inclusion of a physical glycocalyx strainer is required for efficient filtering in systems with longer collar and flagellum. However, other parts of the aquiferous system should also be studied in detail as they can influence pumping and filtering, e.g., computational modeling predicts that *S. ciliatum* choanocyte chambers with “hydrodynamic gasket” will be ineffective if they are connected to yet open canal system but with resistive ostia (Asadzadeh et al., 2020).

5. Conclusion

The ultrastructural and computational data collected so far suggest that sponges from distant phylogenetic lineages and with different body plans rely on common principles of choanoderm organization for effective pumping and filtration of water. Studied representatives of demosponges, hexactinellids, and calcareous sponges utilize varied flagella to achieve a pressure required for driving water through the aquiferous system and have collars supplemented with the fine mesh of glycocalyx and the “gasket”, both of which prevent leaking and recirculation of water as well as bypassing of food particles (Afzelius, 1961; Asadzadeh et al., 2020, 2019; Brill, 1973; Fjerdingstad, 1961; Leys, 1999; Leys et al., 2011, 2007; Mackie and Singla, 1983; Mah et al., 2014; Mehl and Reiswig, 1991; Reiswig and Mehl, 1991; Saller, 1991, 1989; Weissenfels, 1992).

The flagellar vanes and fine mesh of glycocalyx connecting microvilli in the collar have similar appearance and structure in all studied sponge species (Afzelius, 1961; Brill, 1973; Fjerdingstad, 1961; Leys et al., 2011, 2007; Mackie and Singla, 1983; Mah et al., 2014; Mehl and Reiswig, 1991; Saller, 1991, 1989; Weissenfels, 1992), implying that they had arisen before the diversification of the sponge lineages. Moreover, as the flagellar vanes of the similar structure are also known in choanoflagellates, it could be hypothesized that they have arisen in the common ancestor of Choanoflagellata and Metazoa (Brunet, King, 2017; Leadbeater, 2014; Mah et al., 2014). On the other hand, the fine mesh of the collar is absent in choanoflagellates (Leadbeater, 2014; Mah et al., 2014) and seems to be a sponge-specific trait.

In contrast to flagellar vanes and fine mesh, the “gasket” has different nature in different sponge species. Leuconoid demosponges (Brill, 1973; Saller, 1991, 1989; Weissenfels, 1992) and the asconoid calcareous sponge *L. variabilis* have a physical “gasket” built of the glycocalyx, the strainer (Weissenfels, 1992); studied hexactinellids also show a physical “gasket” but formed by a syncytial tissue, the secondary reticulum (Leys, 1999; Leys et al., 2011, 2007; Reiswig and Mehl, 1991); finally, the syconoid calcareous sponge *S. coactum* seems to have the “hydrodynamic gasket”, a stagnation zone created by two opposite flows inside the choanocyte chambers. Such a situation suggests that the “gasket” as an adaptation for preventing water recirculation in the choanocyte chambers and bypassing of food particles have arose independently in several sponge lineages.

Pumping and filtering of water are central activities of sponges, defining the lifestyle of these animals. The aquiferous system of sponges is perfectly adapted to fulfill these activities. A comprehensive understanding of the biology of sponges, including their ecology, evolutionary history, and the peculiarity of their anatomy and functional morphology, is possible only through meticulous studies of pumping and filtering activities and structure of the aquiferous system across the phylum Porifera. Our study demonstrates that even long-established methods of

ultrastructural studies allow the generation of reliable and relevant data. Similar detailed studies of the ultrastructure of the aquiferous system in different sponge lineages are essential for further understanding of the evolution of sponge body plans and adaptation for highly effective pumping.

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Conflict of interest

All authors declare no conflict of interest.

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Table 1. Dimensions of various structures in the choanocytes of *Leucosolenia variabilis*

Object	Parameter	Min, μm	Mean, μm	Max, μm	SD	n
Choanocyte	Height	7.4	8.7	10	0.80	12
	Width	4	4.7	5.3	0.37	12
	Nucleus diameter	2.5	2.7	2.9	0.14	8
	Nucleus height	3.8	4	4.4	0.22	8
	Intracollar glycoalyx thickness	0.49	0.58	0.7	0.06	10
	Glycoalyx-free zone at the base of flagellum	0.3	0.44	0.54	0.09	9
Flagellum	Length	22	25.4	30.5	2.35	33
	Diameter	0.33	0.39	0.56	0.06	14
	Transition zone height	0.25	0.36	0.4	0.05	9
	Transition zone diameter	0.2	0.2	0.29	0.03	9
	Proximal cone outgrowth height	0.15	0.19	0.23	0.03	14
	Proximal cone outgrowth diameter	0.52	0.6	0.7	0.05	14
	Glycoalyx of the proximal part, thickness	0.34	0.58	0.9	0.28	4
	Flagellar vanes thickness	0.07	0.11	0.22	0.04	12
	Flagellar vanes width	1.2	2.0	3.2	0.62	29
	Distance between apical surface of choanocyte and lower edge of flagellar vanes	1.12	1.44	1.82	0.25	5
	Flagellar vanes height above collar	2.9	4.6	6.3	1.11	15
	Gap between vanes and collar	0.3	1.0	1.9	0.42	15
	Collar	Collar diameter proximal	3.2	4.0	5.3	0.63
Collar diameter distal		3.7	4.8	6.6	0.89	38
Collar height		8.7	12.6	15.3	2.01	41
Number of microvilli		26	31	36	3.22	16
Microvillus diameter		0.11	0.22	0.27	0.04	24
Distance between microvilli		0.2	0.29	0.46	0.07	33
Microvillus glycoalyx thickness		0.08	0.1	0.15	0.02	7
Distance between base of collar and lower edge of fine mesh		3.4	3.9	4.4	0.44	12
Strainer	Distance between collars	0.4	2.6	4.8	2.25	45
	Distance between base of collar and lower edge of strainer glycoalyx	4	6.6	9.5	1.36	40
	Strainer glycoalyx thickness	2.4	4.4	6.3	0.93	40

SD – standart deviation, n – number of measurements

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Figure legends

Figure 1. General organization of *Leucosolenia variabilis* and ultrastructure of its choanocytes. A – general view of *L. variabilis* *in vivo*; B – transversal semithin section of the sponge body showing the asconoid organization (histology, conventional fixation); C – three-layered structure of the body wall (histology, ruthenium red (RR) fixation); D – general view of the choanoderm (SEM, RR fixation); E – distribution of the porocytes (black asterisks) in the body wall (DIC microscopy, conventional fixation); F – general view of the choanocyte (TEM, conventional fixation); G – structure of the apical part of the choanocyte (TEM, conventional fixation); H – specialized intercellular junctions (black arrowheads) between choanocytes (TEM, RR fixation). Ac – accessory centriole, AG – Golgi apparatus, Bb – basal body, Bf – basal foot, C – cormus, Ch – choanocyte, Chd – choanoderm, Co – collar, Exp – exopinacoderm, F – flagellum, Fr—fibrillar root, M – mesohyl, Mc – mesohyl cell, Mt – mitochondria, Mv – microvilli, N – nucleus, Nu – nucleolus, Os – oscular tube, P – porocyte, Ph – phagosome, RER – rough endoplasmic reticulum, Sc – spherulous cell of mesohyl, Sp – hollow from spicule, St – strainer, Tz – transitional zone of axoneme, V – flagellar vanes, Va – vacuole. Scale bars: A – 2mm; B – 100 μ m; C – 20 μ m, Insert – 500 nm; D – 10 μ m; E – 10 μ m; F – 2 μ m; G – 1 μ m; H – 200 nm.

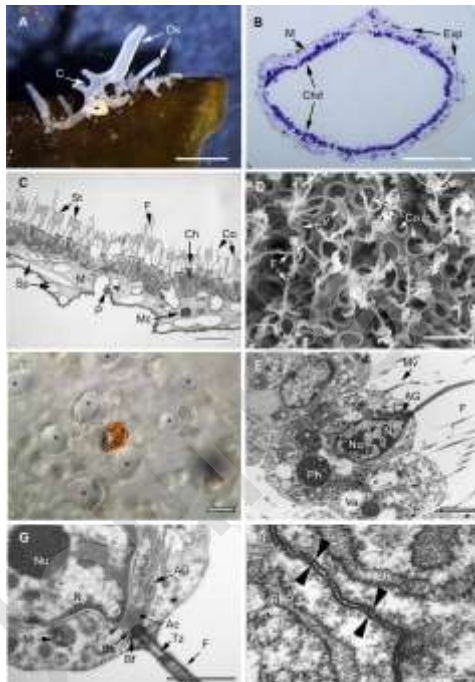


Figure 2. The extracellular matrix associated with the choanocytes' flagella of *Leucosolenia variabilis*. A – extracellular matrix associated with the choanocytes and their flagella and collars (TEM, RR fixation); B – the choanocyte flagella with the paired lateral vanes (SEM, RR fixation); C – the apical part of the choanocyte covered by the intracollar glycocalyx, inset – enlarged view of the intracollar glycocalyx (TEM, RR fixation); D – the proximal part of

the flagellum covered with a loose layer of glycocalyx (TEM, RR fixation); E – transversal section of the flagellum with the vanes (TEM, RR fixation). Ch – choanocyte, Co – collar, F – flagellum, Gl – glycocalyx, Mv – microvilli, St – strainer, V – flagellar vanes. Scale bars: A – 10 μm ; B – 3 μm ; C – 2 μm ; D – 1 μm ; E – 1 μm .

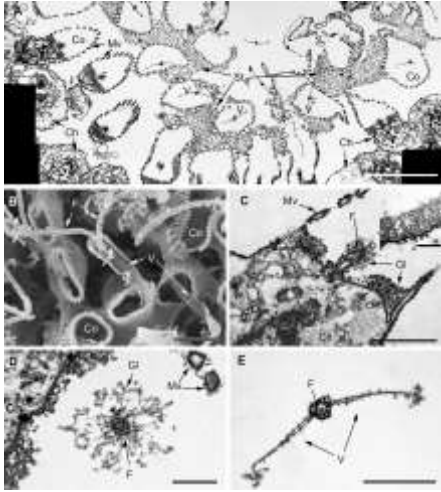


Figure 3. The extracellular matrix associated with the choanocytes' collars of *Leucosolenia variabilis*. A – the choanocytes' collars connected by the glycocalyx of the strainer (TEM, RR fixation); B – the choanocyte collar embedded in glycocalyx of the strainer with microvilli interconnected by the fine mesh of the extracellular matrix (TEM, RR fixation); C, D, E – the microvilli of the choanocyte collar interconnected by the fine mesh of the extracellular matrix, C – longitudinal section (TEM, RR fixation), D – transversal section (TEM, RR fixation), E – SEM (RR fixation); F – the microvilli of the neighboring choanocyte collars interconnected by the glycocalyx of the strainer. Ch – choanocyte, Co – collar, F – flagellum, Fm – fine mesh, Gl – glycocalyx, Mv – microvilli, St – strainer, V – flagellar vanes. Scale bars: A – 5 μm ; B – 2 μm ; C – 1 μm ; D – 1 μm ; E – 600 nm; F – 1 μm .

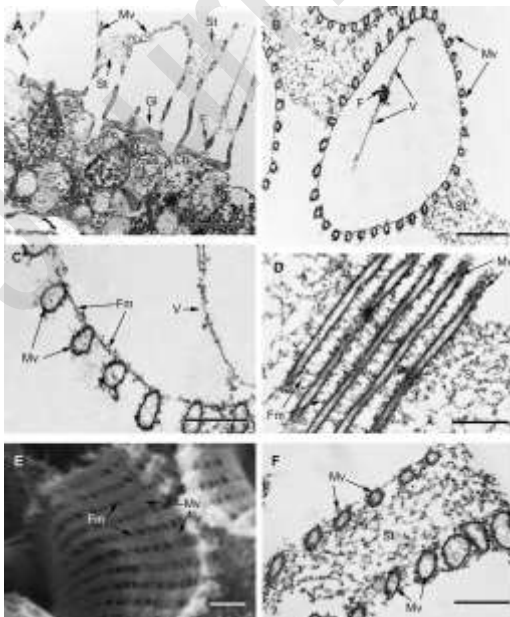


Figure 4. Organization of the choanocyte and glycocalyx associated with its flagellum and collar of microvilli in *Leucosolenia variabilis*. Differences in the organization of glycocalyx along flagellum and collar are shown on the cross-sections. Ac – accessory centriole, AG – Golgi apparatus, Bb – basal body, Bf – basal foot, Co – collar, F – flagellum, Fm – fine mesh, Gl – intra-collar glycocalyx, Mv – microvilli, N – nucleus, Ph – phagosome, St – strainer, Tz – transitional zone of axoneme, V – flagellar vanes.

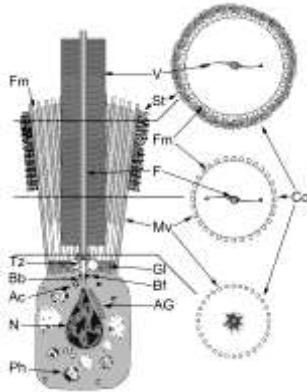


Figure 5. Organization of a body wall and strainer system of choanoderm in the asconoid calcareous sponge *Leucosolenia variabilis*. Arrows show directions of the water flow. Ch – choanocyte, Co – collar, Ex – exopinacocyte, F – flagellum, M – mesohyl, Mc – mesohyl cell, Mv – microvilli, N – nucleus, P – porocyte, Sp – spicule, St – strainer, V – flagellar vanes.

