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Morphological and Histochemical Characterization of Gill Filaments of the Brazilian Endemic Bivalve *Diplodon expansus* (Küster, 1856) (Mollusca, Bivalvia, Hyriidae)

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Abstract: This study presents the morphological description and histochemical characterization of gill filaments of the Brazilian endemic bivalve *Diplodon expansus*, aiming to broaden the morphological knowledge of this species and establish the structure of the gills that will serve as control in histopathological studies applied to biomonitoring. The gill filaments are divided into three zones: frontal, intermediate, and abfrontal. In the center of the filament, haemocytes circulate through the haemolymph vessel, which is internally lined by endothelium. The frontal surface of the filament is covered with cilia, the lateral surface exhibits aquifer ducts, and the abfrontal surface presents ciliated and nonciliated cells. The epithelium of the filaments is composed of ciliated cells, nonciliated absorptive cells, and mucocytes. The support of the filaments is made by two specialized structures called skeletal rod and skeletal loop. Based on the obtained information, the gill filaments of the studied species present some peculiar characteristics that are not yet reported in detail in the literature such as the simultaneous presence of skeletal rod and skeletal loop. On the other hand, the general constitution of the filament is similar to that described for both marine and limnic bivalves and seems to be suitable for ecotoxicological studies.

Key words: mussel, ctenidia, ultramorphology, scanning electron microscopy, histology, histochemistry

Introduction

The interest in studying bivalve mollusks has increased considerably due to their population decline in several regions of the world and also due to their potential use as bioindicators in different impacted ecosystems (Colville & Lim, 2003).

The information available in the literature reveals the ability of these mollusks in accumulating pollutants of several chemical natures in their tissues (Alyakrinskaya, 2003), which can lead to the development of histopathologies as observed by Gregory et al. (2002) for the marine bivalve *Perna perna* exposed to mercury. Thus, the morphology of target organs such as gills of estuarine, marine, and limnic bivalves has been studied in order to be used as indicators of environmental pollution, especially in regions under the influence of different pollutant sources (Gregory et al., 1996; Lemaire-Gony & Boudou, 1997; Gregory & George, 2000; David & Fontanetti, 2005; David et al., 2008).

However, most of the ecotoxicological studies in freshwater bodies use exotic species such as *Corbicula fluminea* (Villar et al., 1999; Peltier et al., 2008), *Dreissena polymorpha* (Zupan & Kalafatic, 2003; Mantecca et al., 2006), and *Limnoperna fortunei* (Vilela et al., 2006), which preclude the performance of more accurate toxicological assessments under an ecological point of view. Besides, the responses of native Brazilian malacofauna exposed to potential toxic substances commonly found in freshwater bodies are little known.

The limnic malacofauna in Brazil has about 155 species of bivalve mollusks, which belongs mainly to four families: Hyriidae, Mycetopodidae, Sphaeridae, and Corbiculidae. The first two have a wide geographical distribution, occurring in very varied habitats, such as lakes, marginal lagoons, and reservoirs in most of the hydrographic basins of the South American continent, while the others are of a more restricted occurrence (Avelar, 1999).

The Brazilian endemic species *Diplodon expansus* (Küster, 1856), popularly known as freshwater mussel, belongs to the Hyriidae family (Mansur & Santos, 2008), whose distribution is restricted to South America and Australia (Avelar & Cunha, 2009). Individuals of this species generally occur in rivers that drain into the Atlantic, in the states of São Paulo and Rio de Janeiro, or into the upper Paraná River, as the Tietê River (Mansur & Santos, 2008). The species *D. expansus* has been studied, so far, on reproductive (Curial & Lange, 1974*a*, 1974*b*, 1975) and ecological aspects, such as density and biomass of some populations and their role in the decomposition process in some ecosystems (Henry & Simão, 1984; Henry & Filoso, 1985, 1987).

To contribute to morphological information of the Brazilian species *D. expansus*, for conservation purposes and for its use as model in ecotoxicological studies in impacted limnic environments, this study aimed to characterize its gills, organs that are in direct contact with environ-

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mental contaminants such as metals and agrochemical residues.

Material and Methods

Collection and Acclimation

Specimens of *D. expansus* (average weight ± SD = 13.42 g ± 6.64; mean length ± SD = 4.28 cm ± 0.81) were collected in April 2010 in Ribeirão Claro stream, municipality of Rio Claro (S 22°24′33.1″; O 47°32′25.1″), São Paulo, Brazil. A collection site that does not suffer direct influence of industrial and agriculture practices was chosen and, therefore, can be considered a little impacted region.

In the laboratory, the animals were acclimated for 3 days in aquariums with a 30 L capacity containing artesian well water, temperature ±25°C, constant aeration and light/dark cycle of 12 h. This procedure was adopted to minimize the influence of the stress of collection and transport of the animals.

The freshwater bivalve mollusks of the Hyriidae family, to which the species *D. expansus* belongs, present larvae called glochidium that remain sheltered in the demibranch until they reach maturity (Mansur, 1999). In this study, only animals that did not present marsupial gills sheltering larvae were used.

After acclimation, the mollusks were anesthetized by heat shock and a temperature −18°C; small fragments of their gills were removed and fixed in different solutions.

Histology and Histochemistry

The gill fragments were fixed in aqueous Bouin solution for 24 h and then submitted to phosphate buffer solution 0.1 M pH 7.4. To remove picric acid residues, the material was washed three times with the same buffer solution and then dehydrated in ethanol 70%, 80%, 90%, and 95% for 20 min during each bath and embedded in resin for 24 h at 4°C. Subsequently, the material was transferred to plastic molds containing Leica resin (Leica Microsystems, Wetzlar, Germany) for inclusion. After resin polymerization, the blocks were sectioned with 5 μm thick, using Leica RM2245 microtome with glass knives; the sections were hydrated in histological bath and collected on microscope slides.

For the histological analysis, the sections were stained with Harris haematoxylin for 10 min and washed in running water for 5 min for the reaction; then they were stained with aqueous eosin for 5 min and washed in water. After drying, the slides were mounted in Canada balsam.

The histochemical tests were applied to detect the presence of the following compounds: total proteins—bromophenol blue (Pearse, 1985), polysaccharides—simultaneous technique with PAS and Alcian blue (Junqueira & Junqueira, 1983), calcium—von Kossa (Junqueira & Junqueira, 1983), collagen—Mallory trichromic (Junqueira & Junqueira, 1983), and picrosirius (Junqueira & Junqueira, 1983). This last technique was performed with some modifications described as follows: the sections were previously placed in an incubator for 15 min at 60°C. Subsequently, the sections were submitted to the picrosirius solution at 60°C for 60 min and then washed in distilled water in three baths. The slides were mounted with Canada balsam, dried in an incubator, and observed under light microscope. The photographic records and the measurements of the structures of the gill filaments were obtained using a Leica photomicroscope and QWin Leica software.

Ultramorphology

Fragments of the gills were fixed in Karnovsky solution (Karnovsky, 1965) for 2 h and dehydrated in a series of increasing concentrations of acetone. Subsequently, the material was taken to the critical point (Balzer CPD 030, Bal Tec AG, Fürstentum, Liechtenstein), fixed in a metal holder, and covered with gold using a Sputtering Balzer SCD 050. The material was analyzed and photographed using a Philips scanning electron microscope (Philips, Guildford, Surrey, UK), operated at 12 kV.

Results

Gills of *D. expansus* present an ample surface and are easily observed in the mantle cavity. In each animal there are two gills or ctenidia, one located on the right of the visceral mass and the other on the left (Fig. 1A). Each gill is formed by two demibranchs, the outer and the inner, arranged in a V shape. Each demibranch is constituted by two lamellae, which consist of parallel ciliated filaments (Fig. 1B). In the studied species, the gill filaments of the inner and outer demibranchs present similar morphology.

The frontal surface of the gill filament is completely covered by cilia (Figs. 2A, 2B), called frontal, latero-frontal, and lateral cilia (Fig. 2C). The frontal cilia have an approximate length of 4 μm and are partially covered by latero-frontal cilia. Arranged on each side of the filament, right below the frontal cilia, the latero-frontal cilia are longer (15 μm) (Fig. 2B). The lateral cilia are slightly longer (20 μm) and numerous.

The lateral surface does not have cilia, but aquifer ducts (arrows in Figs. 2C, 2D) that occur at regular intervals can be observed. Ciliated and nonciliated cells are found on the abfrontal surface of the gill filaments (Fig. 2F). Mucus spheres were also observed (Fig. 2E).

Between the two lamellae that compose the demibranch, it is possible to observe the interlamellar space that contains the interlamellar junctions (Fig. 3A) and haemolymph vessels lined internally by endothelium, where the haemocytes pass through (Figs. 3D, 4D).

The filaments are united to each other by interfilamentar junctions present preferentially in the regions where the interlamellar junctions occur (Fig. 3A) and where two or more filaments share the same haemolymph vessel (Fig. 3C). The communication between the external environment and the interlamellar space is done by occasional pores called ostia (* in Fig. 3B), located between the filaments that compose the lamella.

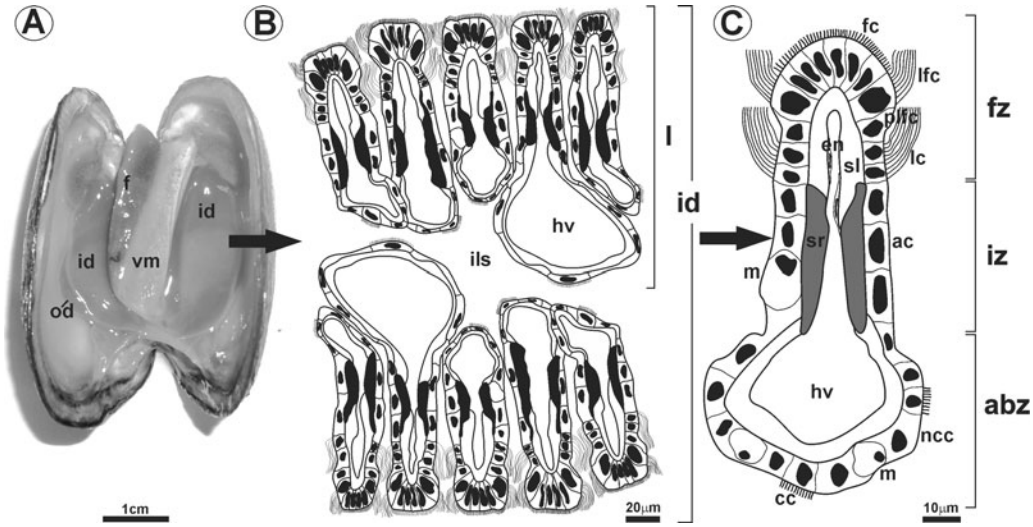

Figure 1. (**A**) Gills, (**B**) demibranch, and (**C**) gill filament of the bivalve *D. expansus* (A). vm = visceral mass; f = foot; od = outer demibranch; id = inner demibranch; ils = interlamellar space; hv = haemolymph vessel; l = lamella; fz = frontal zone; iz = intermediate zone; abz = abfrontal zone; fc = frontal cell; lfc = latero-frontal cell; plfc = post-lateral frontal cell; lc = lateral cell; ac = absorptive cell; ncc = nonciliated cell; cc = ciliated cell; m = mucocyte; en = endothelial cell; sl = skeletal loop; sr = skeletal rod.

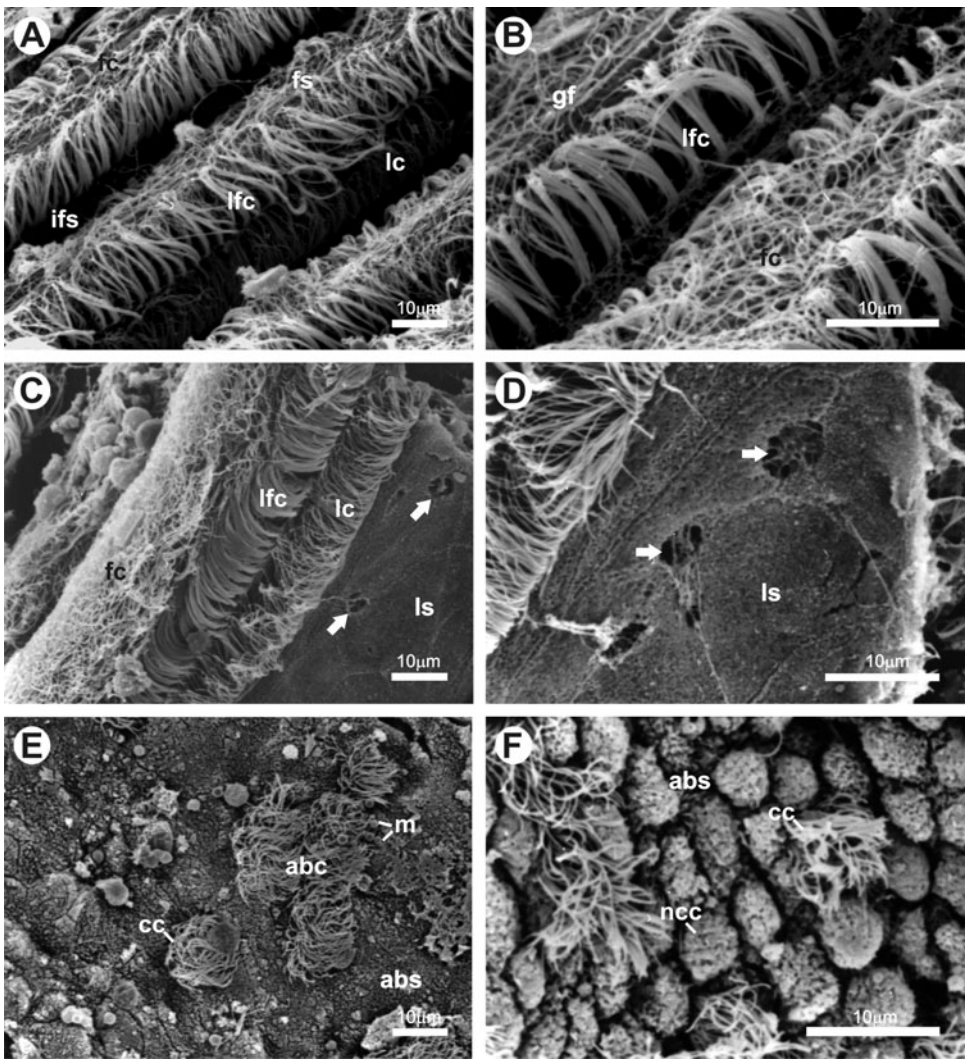

Figure 2. Scanning electron micrographs of the gills of the bivalve *D. expansus*. (**A**, **B**) Frontal surface, (**C**, **D**) lateral surface, and (**E**, **F**) abfrontal surface of the gill filaments. gf = gill filament; ifs = interfilamentar space; fs = frontal surface; ls = lateral surface; abs = abfrontal surface; fc = frontal cilia; lfc = latero-frontal cilia; lc = lateral cilia; abc = abfrontal cilia; cc = ciliated cells; ncc = nonciliated cells; m = mucus; arrows in **C** and **D** = aquifer ducts.

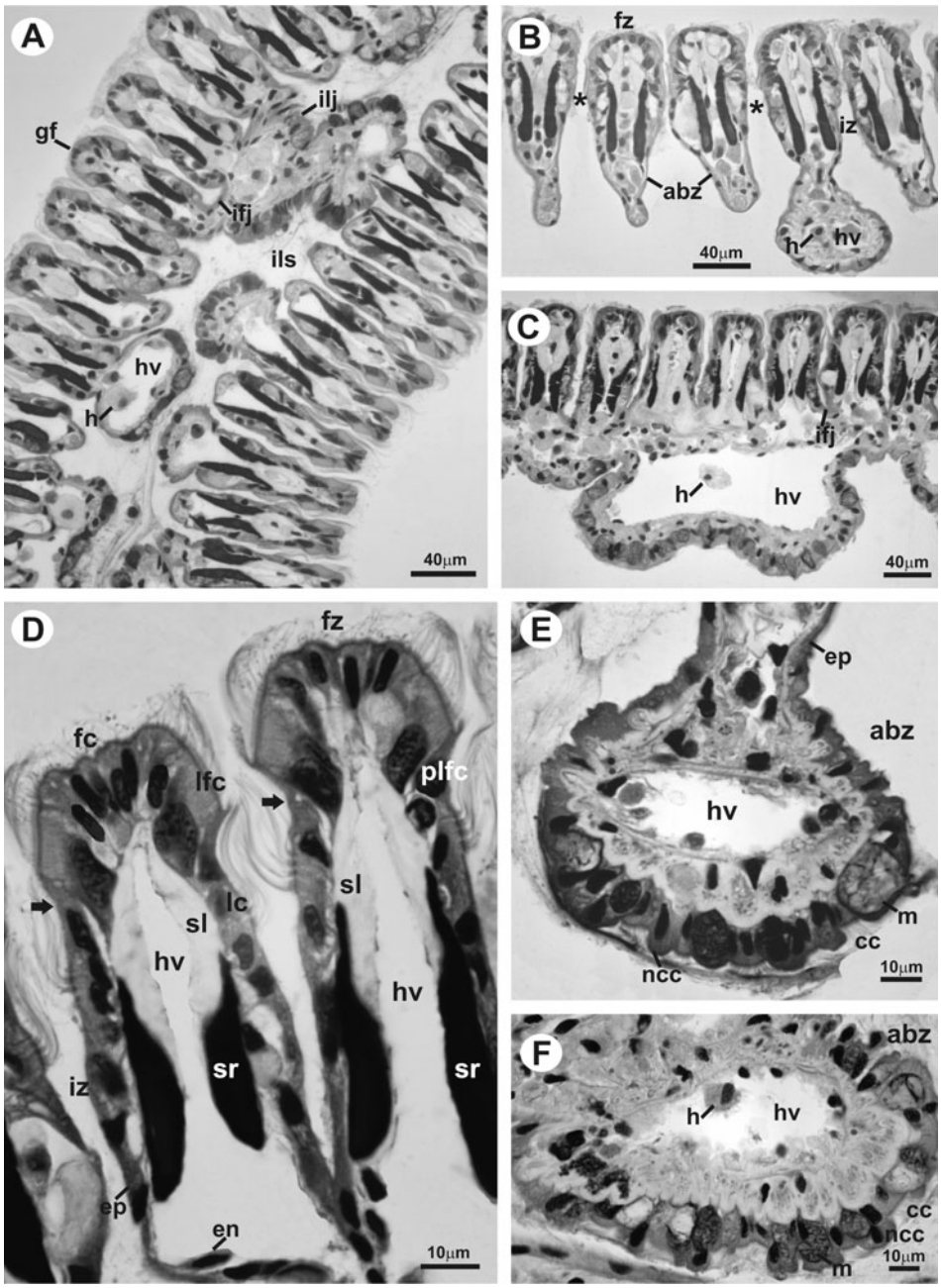

Figure 3. Gill filaments of *D. expansus* stained with Harris haematoxylin and eosin. gf = gill filaments; ils = interlamellar space; ifj = interfilamentar junction; ilj = interlamellar junction; hv = haemolymph vessel; h = haemocyte; sr = skeletal rod; sl = skeletal loop; ep = epithelium; en = endothelium; fz = frontal zone; iz = intermediate zone; abz = abfrontal zone; fc = frontal cells; lfc = laterofrontal cells; plfc = post-lateral frontal cell; lc = lateral cells; cc = ciliated cell; ncc = nonciliated cell; m = mucocyte; * = ostia; arrows= narrowing of the gill filament.

Each filament is divided into three zones: frontal, intermediate, and abfrontal (Figs. 1C, 3B). In the frontal zone, ciliated and nonciliated epithelial cells are present, which exhibit different morphologies depending on their position in the gill filament (Fig. 3D). The apical region of the frontal zone is composed of columnar cells with preferably ovoid nuclei and short cilia, called frontal cells. Adjacent to these cells, there are large latero-frontal cells that have ovoid nucleus and cilia. Below these cells, where the narrowing of the gill filament occurs, nonciliated cells, called absorptive postlateral-frontal cells, were observed. Next, cuboidal cells with round nucleus and long cilia were observed and named lateral cells (Fig. 3D).

The intermediate zone is composed of nonciliated absorptive cells, whose nuclei are flattened, gradually approaching the morphology of squamous cells toward the abfrontal zone (Fig. 3D). Eventual mucocytes, rich in acid polysaccharides (Fig. 4E), were also observed in this zone.

The abfrontal zone is composed of a simple layer of different epithelial cell types that join to each other delimiting the haemolymph vessel in the interlamellar space (Figs. 3E, 3F). The cells that compose this zone are ciliated cells, nonciliated absorptive cells, and mucocytes, which are supported by a thick basal membrane rich in neutral polysaccharides (Fig. 4F). Mucocytes occur in higher or lower number depending on the gill filament.

Beneath the epithelium, in the intermediate zone, highly basophilic paired structures are found, one on each side of the filament (Fig. 3D), called skeletal rods. These structures are rich in collagen (Figs. 4A, 4B), calcium (Fig. 4C), and

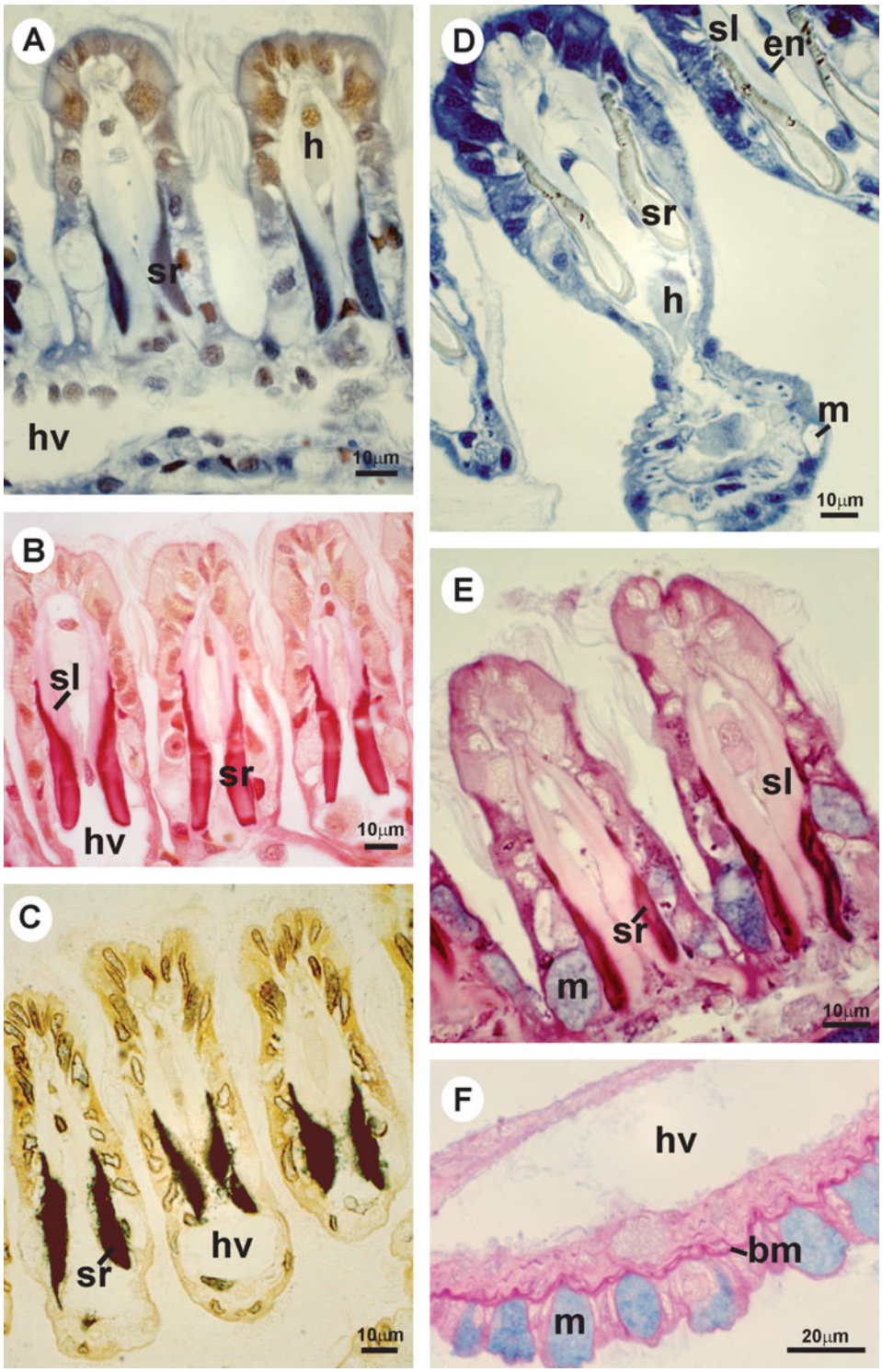

Figure 4. Gill filaments of *D. expansus* submitted to the histochemical techniques of (**A**) Mallory trichromic, (**B**) picrosirius, (**C**) von Kossa, (**D**) bromophenol blue, and (**E**, **F**) simultaneous PAS and Alcian blue. sr = skeletal rod; sl = skeletal loop; h = haemocyte; en = endothelium; hv = haemolymph vessel; m = mucocyte; bm = basal membrane.

neutral polysaccharides (Fig. 4E); however, they do not present protein constitution (Fig. 4D).

The structure of the gills and the haemolymph vessel located in the central region of each filament are also supported by the skeletal loop (Fig. 3D). This support structure is found more internally in relation to the skeletal rod in the intermediate zone and adjacent to the epithelium in the frontal zone of the filament (Fig. 3D). The skeletal loop shows little presence of proteins (Fig. 4D), neutral polysaccharides (Fig. 4E), and collagen, the latter being detected only by the picrosirius technique (Fig. 4B). The results of the histochemical tests are summarized in Table 1.

Discussion

The present study is the first to use histochemical and ultramorphological techniques to analyze the gill filaments of a species of the genus *Diplodon*. Previously, gill filaments

Table 1. Results of the Histochemical Tests Applied in the Gill Filaments of the Bivalve *D. expansus*.

		Histochemical Tests Applied					
		Total Protein	Polysaccharides		Collagen		Calcium
Filament Zones	Structure	Bromophenol Blue	Neutral	Acid	Picrosirius	Mallory Trichromic	Von Kossa
	Skeletal rod	−	+++	−	+++	+++	+++
	Skeletal loop	+	+	−	+	−	−
Frontal	Ciliated cell	+++	++	−	−	−	−
	Postlatero frontal cell	+++	+	−	−	−	−
Intermediate	Absorptive cell	++	++	−	−	−	−
	Mucocyte	−	−	+++	−	−	−
Abfrontal	Ciliated cell	++	++	−	−	−	−
	Nonciliated absorptive	+	++	−	−	−	−
	Mucocyte	−	−	+++	−	−	−
	Basal membrane	−	+++	−	−	−	−

Note: +++, strongly positive; ++, moderately positive; +, weakly positive; −, negative.

of *Diplodon rotundus gratus* were analyzed at light microscopy level, in a study about the functional anatomy of the species (Hebling & Penteado, 1974). However, these authors only illustrated the general morphology of the filaments, without detailing the structure of their cells. Avelar and Cunha (2009) studied the anatomy and functional morphology of *Diplodon rhombeus fontainianus*. The authors detailed the beating dynamics of the cilia present in the different regions of the gill filaments and discussed their function; however, they did not bring information about the morphology of the epithelial cells that compose the filament.

The mollusk bivalve *D. expansus* can be considered a eulamellibranch because there are permanent and developed connections of tissue in the gills, called interfilamentar and interlamellar junctions. These structures arise as a response to the need for structural support of the filaments in order to maintain the proper and constant space for filtration and avoid the passage of larger particles of food between adjacent filaments.

In addition to the connections of tissue, there are two specialized structures that help in the structural support of the gill filaments: skeletal rod and skeletal loop. The skeletal rod is a structure composed of fibrous elements rich in collagen associated with neutral polysaccharides and calcium, which confers to the intermediate zone of the gill filament higher resistance and rigidity. Hebling and Penteado (1974) illustrated the presence of this structure in the gill filaments of *D. rotundus gratus*. For the bivalve *Anodonta woodiana lauta*, the presence of a pair of small chitinous rods was observed, arranged in parallel within each gill filament (Nakao, 1975). However, until now, the morphology, constitution, and occurrence of this structure was not described in detail.

In the bivalve *D. expansus*, each gill filament exhibits internally a skeletal loop, present in the frontal and intermediate zones. This structure is composed of smaller amounts of collagen and neutral polysaccharides when compared to the skeletal rod. The skeletal loop seems to help the skeletal rod in maintaining the structure of the filament, especially in the frontal zone where it is in direct contact with the epithelium.

The structure of the gill filaments of the limnic bivalve *C. fluminea* is supported by a fiber rod that underlines the epithelium and resembles the skeletal loop of *D. expansus* described in this study. This fiber rod is thick in the ciliated region and very thin in the respiratory region (Lemaire-Gony & Boudou, 1997). For the estuarine bivalve *Mytella falcata*, this structure was called connective tissue due to its composition, which includes collagen and neutral polysaccharides (David et al., 2008). On the other hand, this structure on the marine bivalve *P. perna* presented a chitinous constitution (Gregory et al., 2002).

In the present study, the specialized structures of support, skeletal rod and skeletal loop, occur simultaneously giving to the gill filaments great stability in structural terms.

Respiration is the main function attributed to the gills of bivalve mollusks. According to Gómez-Mendikute et al. (2005), gills present two elements to perform the respiratory function: a peripheral ciliated pump that generates a water flow rich in oxygen over and through the demibranchs, and an internal circulatory system that carries the haemolymph rich in oxygen to the heart.

In the limnic bivalve *C. fluminea*, the abfrontal area of the gill filament performs the respiratory function (Lemaire-Gony & Boudou, 1997). On the other hand, Gómez-Mendikute et al. (2005) affirm that, for the marine bivalve *Mytilus galloprovincialis*, the flattened cells present in the intermediate zone of the filament is the place where the gas exchanges and the interaction between the external environment and the haemolymph occur. In *D. expansus*, this interaction would be impaired due to the presence of the rigid and thick skeleton rod between the epithelium and the haemolymph vessel. In this sense, the aquifer ducts, located on the sides of the filament, possibly occur to enhance the contact of circulating water and the haemolymph, facilitating oxygenation.

The gills of bivalves that feed on suspended particles, such as *D. expansus*, are morphologically complex and also play a role in capturing and processing particles present in the water column (Dufour & Beninger, 2001). The food is filtered and separated by the ciliated epithelium and sent to the oral lobes. Later, by the action of the ciliary activity, the food particles reach the mouth opening of the animal (Alyakrinskaya, 2003). The rejected particles are swept to the edge of the lamella and directed toward the tips, which touch the mantle. Subsequently, the particles are transferred to the surface of the mantle and discarded into the environment (Nakao, 1975). Silverman et al. (2000) concluded that the mechanism of processing food particles can be divided into different stages such as contact, capture, transport, selection, and finally ingestion.

Thus, the presence of cilia in the gill filaments and their ordered activity are fundamental to the properly functioning of the feeding of these mollusks. As described for *D. rotundus gratus* (Hebling & Penteado, 1974) and *C. fluminea* (Lemaire-Gony & Boudou, 1997), there are three types of cilia on the outer surface of some epithelial cells of the gill filaments: frontal, latero-frontal, and lateral cilia. In *D. rhombeus fontainianus* (Avelar & Cunha, 2009), the denomination latero-frontal cilia were substituted by eulatero-frontal cilia. In the present study, the terms first applied in the description of the gills of the genus *Diplodon* were used.

Each type of cilia plays a fundamental role in the transport and uptake of nutrients in the frontal zone of the gill filament. Frontal cilia transport particulate material to the palps. In *D. rotundus gratus* (Hebling & Penteado, 1974) and *D. rhombeus fontainianus* (Avelar & Cunha, 2009), latero-frontal cilia of a filament alternate with those of the adjacent filament, forming a network that prevents the passage of larger particles into the interior of the demibranch. The gill filaments of *D. expansus* have latero-frontal cilia whose length, disposition, and morphology resemble those of the species of the genus *Diplodon*, previously described. In this sense, the latero-frontal cilia of the gill filaments of the species studied also seem to help in the selective function of the food particles.

In comparison with *D. rotundus gratus* (Hebling & Penteado, 1974) and *D. rhombeus fontainianus* (Avelar & Cunha, 2009), the bivalve *D. expansus* has considerably longer lateral cilia. The lateral cilia are responsible for pumping water into the bivalve shell (David & Fontanetti, 2005) and transporting water through the ostia, creating the food and respiratory flow (Jorgensen, 1976). Thus, the food and respiratory functions may be optimized due to the presence of longer lateral cilia in the species studied.

The abfrontal surface of the gill filaments is not directly involved with feeding process because the capture of food particles occurs on the frontal surface of the epithelium (Dufour & Beninger, 2001). However, some elements related to the processing of alimentary particles, such as cilia and mucus, are present in the abfrontal surface of the gills of *D. expansus*.

The cilia are scarce and the mucocytes rich in acid polysaccharides are more frequent in the abfrontal zone of the gill filament of the species studied, a situation similar to that found in the eulamellibranch bivalve *Spisula solidissima* (Dufour & Beninger, 2001). In *D. expansus*, the cilia found in the abfrontal zone of the filaments do not seem to be related to the creation of water flow because such cilia are short and few in number when compared to the lateral cilia that admittedly perform this function. Possibly these structures appear as vestigial along the evolution of bivalves (Dufour & Beninger, 2001) or play the sensorial function, as previously discussed by Atkins (1936).

According to Beninger et al. (1997), mucocytes occur in the abfrontal zone of eulamellibranch bivalves, such as *D. expansus*, due to the great lubrication needed in this region in order to decrease the friction between water and epithelium because the water pumped through the gill filaments is directed to the abfrontal chambers (present in the interlamellar space) of reduced volume. In limnic bivalves, gills secrete mainly acid mucosubstances, which help in the transport of food particles and in the formation of a protective and lubricant mucous layer (Kale & Patil, 1977). The mucus of acid nature such as that found in the mucocytes of *D. expansus* is highly viscous and considered a good lubricant because it is not easily hydrated or removed from the epithelium (Hunt, 1970; Faillard & Schauer, 1972). Therefore, it is possible that the mucus secreted in the abfrontal zone of *D. expansus* acts as an effective lubricant, considerably reducing the friction between water and epithelium.

In the intermediate zone of the gill filaments of *D. expansus*, mucocytes rich in acid polysaccharides were also found. In the marine bivalve *M. galloprovincialis*, the mucocytes rich in acid polysaccharides occurred in the frontal zone of the filament, while mucocytes rich in neutral polysaccharides were observed in the abfrontal zone (Gómez-Mendikute et al., 2005). In the estuarine bivalve *M. falcata*, mucocytes rich in neutral polysaccharides were often observed in the abfrontal zone of the filament. Such cells were also observed in the frontal zone near the intermediate zone of the filament (David et al., 2008). In animals exposed to pollutants, the authors found that the increase in the number of mucocytes occurred preferentially in the frontal zone of the filament (David & Fontanetti, 2009).

In the gill filament of the marine bivalve *Mya arenaria*, the arrangement of the mucocytes rich in acid polysaccharides occurs similarly to that observed in *D. expansus*; however, such cells are less abundant in the abfrontal zone of the marine bivalve (Beninger et al., 1997). The authors affirm that it is unlikely that the mucous secreted in the intermediate zone be transported toward the frontal zone because the lateral cilia beat in the opposite direction and are separated from the latero-frontal cilia by nonciliated cells. Since residues of mucous are found in the frontal zone of the filaments, the authors pointed out the need to con-

duct further investigations to verify the possible existence of a glandular system integrated into these mucocytes that would make possible the direct secretion of mucus on the frontal zone of the filament.

Conclusions

The information obtained in this study showed that the gills of the Brazilian endemic bivalve *D. expansus* are typical of eulamellibranch bivalves and are suitable for histopathological studies applied on the effects of water pollutants. Important peculiarities never related in detail before showed the morphological and histochemical differences in bivalve species gills such as the constitution of the structure supporting the haemolymph vessel and the simultaneous presence of two specialized structures that play an important role in the structural maintenance of the gills, the skeletal rod and skeletal loop. Ultrastructural studies will be carried out to provide morphological detailing of the structures and cells that compose the gill filaments of the bivalve.

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