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Histochemical and scanning electron microscopic approaches to gills in juveniles of *Odontesthes argentinensis* (Actinopterygii, Atherinopsidae)

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Abstract: Juveniles of *Odontesthes argentinensis* were collected from Mar Chiquita coastal lagoon, Argentina. The morphology of the gills was analyzed by scanning electron microscopy. The surface of the filaments and the pharyngeal region of the gill arch were covered by a mosaic of polygonal epithelial cells with apical concentric microridges. The apical crypts of mitochondria-rich cells were mainly found in the trailing edge of the filament epithelium and in the interlamellar surfaces. Glycoconjugates (GCs) elaborated by the secretory cells in the epithelium covering the gill filaments and the pharyngeal region of *O. argentinensis* were studied by means of a series of carbohydrate histochemical methods. Mucous cells among the lining epithelium of the pharynx showed a histochemical profile similar to that of mucous cells of filaments and secondary lamellae. Mucous cells showed the presence of neutral, sulphated, carboxylated and sialylated GCs. Glycoconjugates secreted on the surface of the gills could be associated with different functions such as lubrication, ionic regulation and inhibition of pathogen proliferation.

Key Words: gills, histochemistry, SEM, glycoconjugates

Introduction

The teleostean fish gills are the site of respiratory gas exchange, osmoregulation, acid-base balance, metabolic nitrogen excretion and regulation of blood levels of circulating hormones (Evans *et al.*, 2005; Srivastava *et al.*, 2012). Moreover, the gill dimensions and organization of gill filaments and rakers reflect the feeding habits of the fish (Zayed and Mohamed, 2004). The morphology and distribution of the different cell types of the gill epithelium of teleosts have been intensively investigated in order to understand and recognize the integration of several of their functions (Wilson and Laurent, 2002; Díaz *et*

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al., 2010; Monteiro *et al*., 2010).

In addition, the gills represent an appropriate model for the study of potential environmental effects on the organism and they may be indirectly used as indicators of the degree of environmental contamination. In this connection, the gill morphological alterations would reflect the health and physiological state of fishes (Hossler *et al.*, 1985; Machado, 1999; Monteiro *et al*., 2010).

The silverside *Odontesthes argentinensis* (Valenciennes, 1835) is an euryhaline, estuarine-dependent-marine fish species (González Castro *et al.*, 2009). Its distribution in the Argentine coast ranges from approximately 36ºS to 44ºS (Cousseau and Perrotta, 2004). Previous works have been done for this species with reference to its abundance, distribution, relationships with environmental factors and reproductive ecology (González Castro *et al.*, 2009). To our knowledge, there are neither histological nor histochemical studies on gills of *O. argentinensis* at juvenile stages. Histology and histochemistry are valuable tools for the identification and functional characterization of different cell types and the detection of environmentally caused alterations.

In this study, we describe the distribution and characteristics of glycoconjugates (GCs), the surface ultrastructural features, and the correlation to the GCs' possible function in gills of *O. argentinensis* juveniles from the Mar Chiquita coastal lagoon. For those purposes we utilized histochemical specific techniques for acid and neutral GCs as well as classical methods for electron microscopic studies. This is part of a series of studies on GCs and gill ultrastructural analyses of several species that are being carried out in our laboratory (Díaz *et al.*, 2005a, b, 2008, 2009, 2010).

Material and methods

Specimens of *Odontesthes argentinensis* (Valenciennes, 1935) were collected from the Mar Chiquita coastal lagoon. The sampling area was located near the lagoon's mouth with mixo-eurihaline waters and great marine water influence. Once collected, specimens were immediately transported to the laboratory in water-filled containers. Fish were sacrificed by cervical dislocation. Juvenile samples of *O. argentinensis* (9-11 cm total length range; 11-14 g total weight range; n= 5) were selected and their gill arches immediately removed. Fish were collected under permits issued by local and national authorities and all procedures were conducted in accordance with national animal care regulations.

The second gill arch (BaII) of each fish was isolated for scanning electron microscopy (SEM) study. All the isolated arches were fixed in a 3% glutaraldehyde solution buffered with 0.1M sodium cacodylate and routinely processed for SEM. The dehydration was gradually done in an increasing degree from alcohol to absolute

alcohol. The material was dried with hexamethyldisilazane (HMDS), mounted on aluminium stubs and metalized with gold/palladium for its SEM observation. Observations and photographs were done under a SEM JEOL JSM 6460-LV of the Laboratory for Electron Microscopy of the National University of Mar del Plata. Selection for the SEM analysis of the BaII representing the other gill-arches of the fish was performed according to previously established methodologies in other researches (Hossler, 1980; Eiras-Stofella and Fank-de-Carvalho, 2002).

The gills were fixed by immersion in 10% buffered formalin for light microscopic studies. Samples were routinely processed and embedded in paraffin wax. Four micrometer-thick histological sections were stained with hematoxylin and eosin (H–E) stain and Masson trichrome stain for morphology, and were also subjected to histochemical procedures for the identification of GCs (Tab. 1).

Tab. 1: Histochemical procedures used.

Procedures	Interpretation of staining reactions	References
PAS	GCs with oxidizable vicinal diols and glycogen	McManus (1948)
Acetylation/ PAS	GCs with oxidizable vicinal diols and glycogen	Lillie and Fullmer (1976)
Acetylation/ KOH/ PAS	GCs with oxidizable vicinal diols and glycogen	Culling *et al.* (1976)
α-amylase/ PAS	GCs with oxidizable vicinal diols	Pearse (1985)
PA/P/S	GCs with sialic acid residues without O-acyl substitution with O-acyl substitution at ^{7}C	Reid and Park (1990)
PA/Bh/KOH/PAS	Sialic acid residues with O-acyl substitution at ^{7}C, ^{8}C or ^{9}C and O-acyl sugars	Reid *et al.* (1973)
KOH/PA*/Bh/PAS	GCs with oxidizable vicinal diols and with O-acyl sugars	Volz *et al.* (1987)
PA/Bh/KOH/PA*/Bh/PAS	GCs with O-acyl sugars	Reid and Park (1990)
AB pH 2.5	GCs with carboxyl groups and with O-sulphate esters	Lev and Spicer (1964)
AB pH 1.0	GCs with O-sulphate esters	Lev and Spicer (1964)
AB pH 0.5	Highly sulphated GCs	Lev and Spicer (1964)
AB pH 2.5/PAS	Same as in 9 and 1	Mowry (1963)
AB pH 1.0/PAS	Same as in 10 and 1	Mowry (1963)

AB, Alcian blue; Bh, borohydride; PA, periodic acid; PA*, selective periodic acid oxidation; PA/P/S, periodic acid oxidation-phenylhydrazine-Schiff; PAS, periodic acid Schiff reagent.

Results

Gill filaments

Two epithelial types were clearly recognized in the gills of *Odontesthes argentinensis*: filament epithelium and lamellar epithelium. Filaments were lined up by a stratified epithelium 4 -10 cell layers thick. In addition, mitochondria-rich cells (chloride cells, ionocytes) and mucous cells were spread among the epithelial cells. Branchial lamellae consist of a capillary core covered by a thin epithelium with few mucous cells (Fig. 1A).

The gill filaments of *O. argentinensis* became thinner from the medial to the apical region of the filaments. At the tip of the gill filaments, the secondary lamellae were small and triangular in shape, whereas they were larger and rectangular in the medial and proximal regions (Fig. 2A, B).

The epithelium that lines the gill filaments presented dim folds that gave a softly undulating surface appearance at SEM. The epithelial surface cells were polygonal, with a well-defined contour. In general, the epithelial cell apical surfaces were characterized by the presence of a series of microridges, which appeared smooth, extensive, often unbranched and orderly arranged. In the main, the microridges were almost parallel to each other and formed typical regular concentric patterns. The borders between adjacent epithelial cells were delimited by a well-defined, continuous double row of microridges, narrowly approaching each other. Microridges tended to be shorter in the transition zone that lied between the gill filaments and the secondary lamellae. The microridges were often interconnected with fine transverse connections, the so-called microbridges.

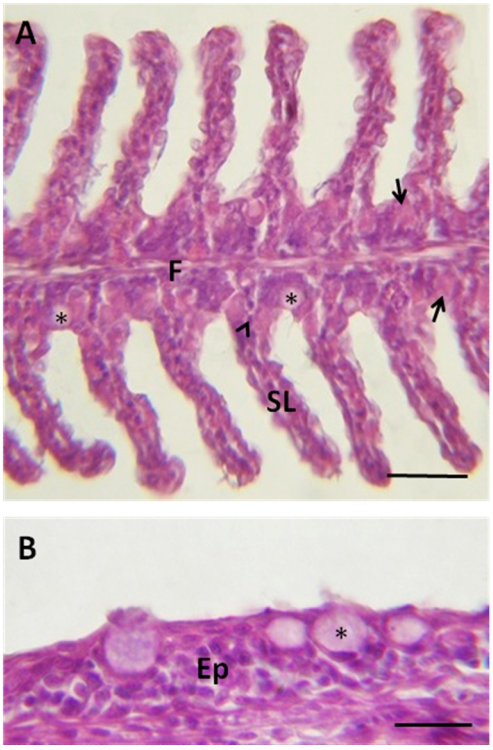

Fig. 1: Histological characteristics of the gills of *Odontesthes argentinensis*, H–E. (A) Sections of gills, Scale bar: 35 µm. (B) Section of pharyngeal cavity. Scale bar: 30 µm. Ep, epithelial cells; F, gill filament; SL, secondary lamellae; asterisk, mucous cells; arrow, chloride cell; arrowhead, capillary core.

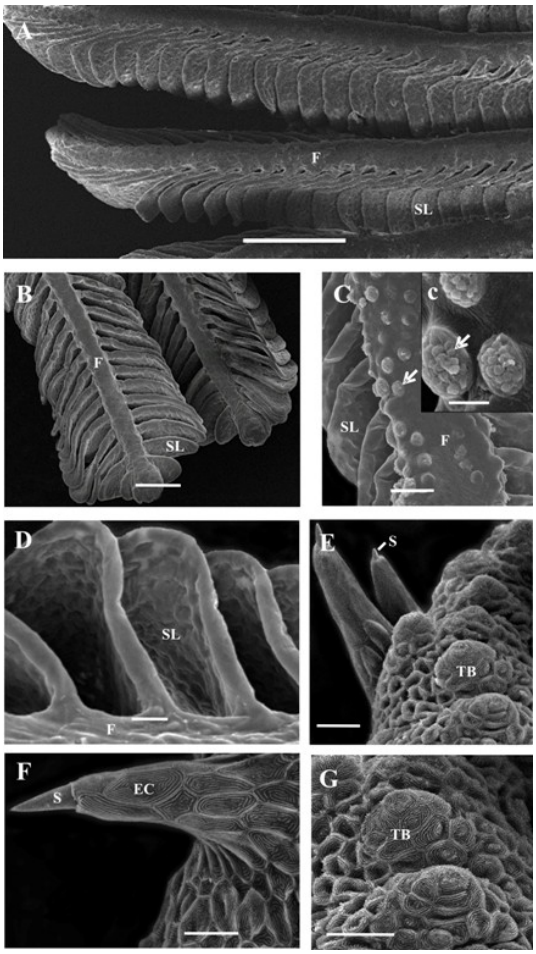

Fig. 2: Surface ultrastructure of the gills of *Odontesthes argentinensis.* (A-D) Structure of the filaments and respiratory lamellae, scale bars: (A) 100 µm; (B) 50 µm; (C) 20 µm, (c) 5 µm; (D) 10 µm. (E-F) Surface of the pharyngeal region studded with villiform spines, scale bars: (E) 15 µm; (F) 10 µm. (G) Detail of taste buds type I, scale bar: 15 µm. EC, epithelial cell; F, gill filament; arrow, mucous cell; S, spines; SL, secondary lamellae; TB, taste bud.

Various mucous cells among the epithelial cells primary lamellae were observed (Fig. 2C, c). Their surface appeared covered by polygonally or round-shaped secretion globules limited by a smooth edge membrane. The apical crypts of mitochondria-rich cells, which contained apical extensions, were found in the trailing edge of the filament epithelium, in the interlamellar surfaces and around the bases of the respiratory lamellae. The secondary lamellae were mostly wrinkled with a much undulated surface. Their epithelial cells showed soft although well-defined borders (Fig. 2D). No mitochondria-rich cells and few mucous cells were present among the epithelial cells in the secondary lamellae.

Pharyngeal region

The epithelium that lined the gills arches and gills rakers of *O. argentinensis* was stratified, with high cuboidal cells in the basal layer, cuboidal cells in the intermediate layers and more flattened cells in the superficial layers. A great many large mucous cells with a foamy appearing cytoplasm were visible in between the epithelial layers (Fig. 1B).

As evidenced by SEM, the surface of the gill arches and rakers showed a large number of conspicuous, irregularly dispersed epithelial projections separated by shallow wavy depressions (Fig. 2E- G). It was also covered by a mosaic of irregular polygonal epithelial cells of varied dimensions with long apical concentric microridges (Fig. 2F). This arrangement is similar to that of the epithelium lining the gills. The epithelium surface was studded with villiform spines (Fig. 2E, F). They were

elongated with sharp pointed ends. The spines were similar in dimensions and morphology, and they projected considerably from the epithelium surface that lined the rakers.

Several mucous cell apertures and prominent taste buds were identified on the surface of both the epithelium of the gill arch and the rakers. The mucous cell apertures were frequently limited by three or four epithelial cells forming pore-like structures. These pores were wide and often full of mucous goblets.

The taste buds, both individually or in groups, were found at intervals at the apical ends of the epithelial protuberances, and projected well above the general surface of the epithelium (Fig. 2E, G). Type I taste buds were located on conical epithelial heaps. At the peak of every elevation, closely packed microvilli representing taste hairs projected through a rounded taste pore.

Histochemical characterization

Although the number of mucous cells was fewer in the secondary lamellae, no histochemical differences were detected between the mucous cells of gill filaments and secondary lamellae. Mucous cells with periodic acid Schiff (PAS) presented a weak positive reaction (Fig. 3A); the coloration disappeared after acetylation and recovered after saponification. Sections exposed to α-amylase, to determine periodate reactive vicinal diols and exclude the presence of glycogen, were positive for the PAS reaction after the same treatment. Mucous cells reacted weakly to the PA/P/S technique (Fig. 3B). The PA/Bh/KOH/PAS method gave a slight to moderate reaction that indicated sialic acid residues with O-acyl substitution at C7, C8 or C9 and O-acyl sugars. Neutral GCs with oxidizable vicinal diols and with O-acyl sugars were revealed by using the KOH/PA*/Bh/PAS method. The moderate reaction with the PA/Bh/KOH/PA*/Bh/PAS indicated the presence of GCs with O-acyl sugars (Fig. 3C). Therefore, a procedure sequence using AB at different pH´s showed the presence of strong and weak sulphated GCs. A positive reaction with AB/PAS sequence demonstrated the presence of neutral and acid GCs (Fig. 3D).

Mucous cells among the lining epithelium of the pharynx showed a histochemical profile similar to that of mucous cells of filaments and secondary lamellae. However, the pharyngeal mucous cells showed a stronger reaction with AB pH 1.0 technique, indicator of pharyngeal secretions with a greater presence of sulphated GCs (Fig. 4A). Neither PA/P/S nor KOH/PA/Bh/PAS positive mucous cells were observed (Fig. 4B, D). It was clear a marked reaction of the glycocalyx lining epithelium of the pharynx with all the techniques tried, which demonstrated the presence of the different GCs types (Fig. 4A-D). We identified GCs with oxidizable vicinal diols, GCs with carboxyl groups, GCs with O-acyl sugars, GCs with sialic

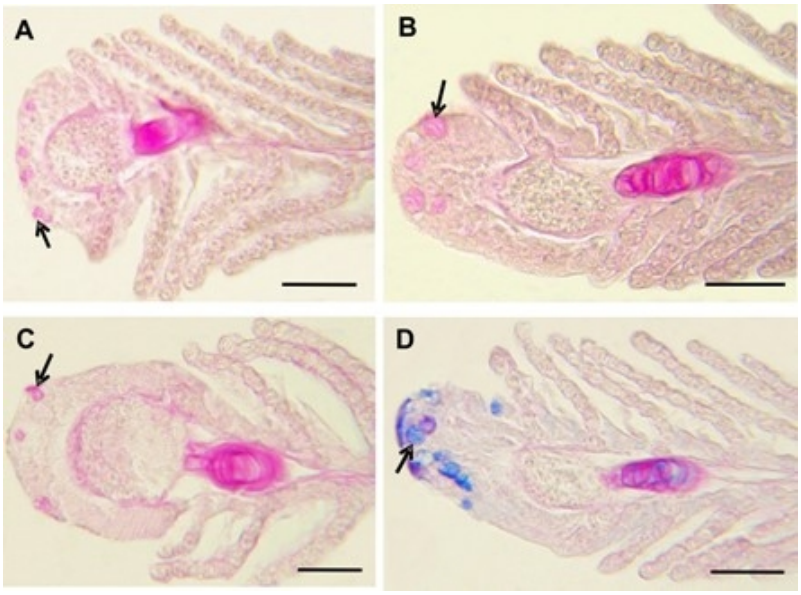

Fig. 3: Sections of *Odontesthes argentinensis* gills showing reactions to GCs in the mucous cells from gill filaments (arrow). (A) PAS reaction. (B) PA/P/S reaction. (C) PA/Bh/KOH/PA*/Bh/PAS. (D) AB pH 2.5/PAS. Scale bars: 35 µm.

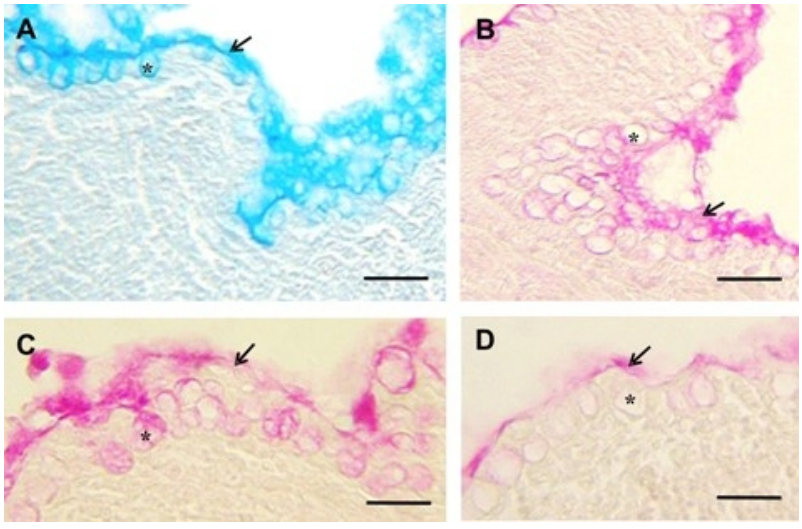

Fig. 4: Sections of *Odontesthes argentinensis* pharyngeal cavity showing reactions to GCs in the mucous cells (*) and the glycocalyx (arrow). (A) AB pH 1.0. (B) KOH/PA/Bh/PAS reaction. (C) PAS. (D) PA-P-S. Scale bars: 30 µm.

acid residues and GCs with O-sulphate esters. Instead, the glycocalyx of the gill epithelium only gave a slight labeling to PAS and KOH/PA*/Bh/PAS.

Discussion

Gill filaments

As in other fish species, the surface of the stratified epithelium of the primary lamellae has shown to be made up of polygonal cells (Zayed and Mohamed, 2004; Díaz *et al.*, 2009; Srivastava *et al.*, 2012). The present study showed the presence of extensive microridges, which formed representative patterns on the surface of the epithelial cells of primary lamellae. Like several other fish, *Odontesthes argentinensis* showed a reduction in microridges number and size at the region close to the secondary lamellae, thus indicating a transition among the different cell surface configurations (Eiras-Stofella *et al.*, 2001; Zayed and Mohamed, 2004). The teleostean secondary lamellae have been frequently mentioned as wrinkled structures covered by squamous epithelial cells with few or no microridges (Díaz *et al.*, 2009; Kumari *et al.*, 2012). Comparable features were found in *O. argentinensis*. According to Arellano *et al*. (2004) differences in the topography of lamellar and interlamellar pavement cells have either phylogenetic or physiological bases.

The existence of mucous cells in the gill lamellae is a common feature of teleosts; however, their number and distribution may vary among species (Díaz *et al.*, 2005a, 2009, 2010). Like other fish species, *O. argentinensis* has shown a close relationship between the presence of mucous cells and the concentration of microridges at various regions of the branchial arches (Eiras-Stofella *et al.*, 2001; Kumari *et al.*, 2012). Thus, numerous mucous cells and abundant microridges have been observed in the primary lamellae and in the pharyngeal region, while secondary lamellae presented few mucous cells and scarce and short microridges. No doubt, these morphological characteristics are associated to assist the fish to utilize the maximum surface area of the secondary lamellae for efficient respiration (Kumari *et al.*, 2012).

Mitochondria-rich cells (MRC) are specialized ionocytes, and the main site responsible for the active transport of ions in gills. Several studies describing the characteristics of MRC in the gill epithelium of teleosts have shown a wide interspecific diversity as far as distribution, subtypes, morphology and number are concerned (Wilson and Laurent, 2002; Chun-Nian *et al*., 2004; Evans *et al.*, 2005; Hwang and Lee, 2007; Monteiro *et al*., 2010). The presence of typical crypts has been described either as a marine teleost MRC characteristic or as a structural change produced in this cell type when euryhaline species shift from fresh to salt waters (Carmona *et al.*, 2004). Apart from the presence of crypts in the seawater fish MRC

deep pits with few or no visible apical extensions have been described. On the other hand, fish inhabiting freshwater environments possess MRC with wider apical surfaces, less evident crypts and no pits (Eiras-Stofella *et al.*, 2001; Díaz *et al.*, 2009). These observations agree with the results of our study which revealed MRC with clear deep crypts.

Pharyngeal region

In most teleosts the pharyngeal region of the gill arches possesses similar structural and morphological organization along each arch, and only minor variants between the external and internal sides (Eiras-Stofella *et al.*, 2001). *O. argentinensis* juveniles showed a similar morphology at both sides of the BaII. The pharyngeal region inherently possessed short rakers with spines at both sides all along the gill arches. Various authors have determined that the structure and shape of the pharyngeal region of the teleostean gill arches are indicative of the particular feeding habits of fish (Eiras-Stofella *et al.*, 2001; Cousseau, 2010). The short and slightly sculptured gill rakers of *O. argentinensis* would demonstrate that this species should not be a typical filtering species. The rakers of ilyophagous species suggest important differential features like marked development, length and particular sculpture (Eiras-Stofella *et al.,* 2001). Ojha *et al*. (1987) have also reported gill rakers with secondary and tertiary structures for increasing the filtering mechanism efficiency in plankton feeders.

Taste buds are greatly developed in fishes and are important for feeding, orientation and social behavior. In most fishes, they are not only dispersed in the oropharyngeal cavity of the mouth, but also on the basal parts of the gill arches and in the skin (Fishelson *et al.,* 2004; Díaz *et al.,* 2009; Xiong *et al.,* 2011). The morphology of the *O. argentinensis* pharyngeal taste buds showed that they were situated in the apex of elevated papillae projecting from the surface. Their gross structure closely relates to the type I taste buds. In most fishes, three types of taste buds have been described: types I and II protrude on papillae above the surrounding epithelium, whereas type III taste buds remain level with it (Boudriot and Reutter, 2001; Fishelson and Delarea, 2004; Xiong *et al.,* 2011). Moreover, Fishelson *et al.,* 2004 proposed that type I and type II taste buds are generally mechanoreceptors while type III taste buds are basically chemoreceptors. It is evident that gills with this conformation will take part in a mechanical type way of food selection rather than in a sensory way. The presence of only type I taste buds and numerous mucous cells in this species upholds this hypothesis. Therefore, a series of taste buds that transmit information about food would not be needed, and the morphology of gill-rakers would work as a prevention barrier to the entering of large and non-desirable organisms (Eiras-Stofella *et al.*,

2001; Díaz *et al.*, 2009).

In *Odontesthes argentinensis* the microridges on the surface of the epithelial cells, like in the gill arches and the gill rakers of other fish species, are often compactly arranged and organized into elaborate spirals forming intricate patterns (Eiras-Stofella *et al.*, 2001). Many are the physiological roles ascribed to microridges, among them, the ability to augment the surface area and to provide mechanical flexibility and protection (Kumari *et al.*, 2009b). Fishelson (1984) and Mittal *et al.* (2010) have suggested that microridges developed as an adaptation to retain mucous secretions at the surface. It is remarkable the presence of microbridges frequently interconnecting the microridges of *O. argentinensis*. The presence of microbridges would collaborate in protecting the epithelium of the pharyngeal zone providing mechanical strength to the microridges (Mittal *et al*., 2010).

Histochemical characterization

Mucus production in fishes is definitely very common and could take place in all fish species. The functions of fish mucus are various and diverse and include the streamlining for water flow during movement through the water, defense against infections, respiration, ionic and osmotic regulation, lubrication, protection, reproduction and nest building (Shephard, 1994; Yan, 2009; Díaz *et al.*, 2008, 2009).

The gill arches and the gill rakers of *O. argentinensis* showed acidic GCs in the mucous cells. The mucus comprising GCs with O-sulphate esters is more viscous than that containing GCs with sialic acids (Kumari *et al.*, 2009a). The increase of mucus viscosity confers it a gel-type consistency which results in the slipperiness of the mucus. In consequence, the mucus secreted on the surface of the gill arches and the gill rakers of *O. argentinensis* might be associated to the lubrication of the pharyngeal cavity as well as to the food items ingested by the fish. Lubrication could play a central role in protecting the epithelium covering the pharyngeal cavity against mechanical injuries to which it is greatly exposed during the manoeuvring and transport of preys toward the oesophagus. Moreover, lubrication might be considered to assist in smooth transport and in food swallowing (Díaz *et al.*, 2009; Kumari *et al.*, 2009a; Mittal *et al*., 2010). Further, the presence of sulphated GCs, in addition to their lubricating property, may prevent the proliferation of pathogenic micro-organisms on the epithelial surfaces as well, as suggested by Yashpal *et al.* (2007).

The histochemical composition of the mucous secretion in the gills and pharyngeal region of *O. argentinensis* has also revealed the presence of sialic acid and neutral GCs. It has been proposed that GCs with sialic acid residues are associated with protection against bacterial and viral invasion (Díaz *et al.*, 2010). According to Mittal *et al.* (2004) mucus with neutral GCs is

considered to be fairly easy to wash away with the respiratory water current. This fact could facilitate the respiratory process (Díaz, *et al.* 2008). In addition, neutral GCs are associated with the absorption and transport of molecules through the membranes (Sarasquete *et al.*, 2001).

In conclusion, the present study gives a deeper insight into the morphological and functional aspects of the gill arches, gill rakers, gill filaments and secondary lamellae of *O. argentinensis* juveniles. The secretions on the surface of these areas are considered to achieve diverse functions with great specificity, thus they would be involved in feeding activities in gill arches and gill rakers, and in respiration in gill filaments and secondary lamellae. The high functional specificity of glycoconjugates in each of the areas studied could play a significant role in the preservation of the structural and functional integrity, an adaptation for the fish in relation to its habit. Knowledge of the normal glycoprofile of the gills of *O. argentinensis* may constitute a basis for the study of this structure in other teleost species.

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