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Author(s): Stefanía Cohen, Gladys Petcoff, Roberto O. Freijo, Enrique L. Portiansky, Claudio G. Barbeito, Gustavo J. Macchi and Alcira O. Díaz
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Histochemical Characterization of Oocytes in the Pink Cuskeel (*Genypterus blacodes*)

Stefanía Cohen[1,2], Gladys Petcoff[3], Roberto O. Freijo[3], Enrique L. Portiansky[2,4], Claudio G. Barbeito[2,5], Gustavo J. Macchi[1,2,6], and Alcira O. Díaz[1]*

[1]*Instituto de Investigaciones Marinas y Costeras (IIMyC), FCEyN, CONICET- Universidad Nacional de Mar del Plata. Funes 3250. (7600) Mar del Plata, Argentina*
[2]*Consejo Nacional de Investigaciones Científicas y Técnicas (Conicet), Argentina*
[3]*Departamento de Biología. Facultad de Ciencias Exactas y Naturales. Universidad Nacional de Mar del Plata*
[4]*Departamento de Histología y Embriología, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata (UNLP), 60 y 118, (1900) La Plata, Argentina*
[5]*Instituto de Patología, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata (UNLP), 60 y 118, (1900) La Plata, Buenos Aires, Argentina*
[6]*Instituto Nacional de Investigación y Desarrollo Pesquero (INIDEP), Paseo Victoria Ocampo 1, Mar del Plata, General Pueyrredón (7600), Buenos Aires, Argentina*

In the present study we histochemically and lectinhistochemically characterized the growing oocytes of the pink cuskeel (*Genypterus blacodes*). We used histochemical methods for the localization and characterization of glycoconjugates (GCs) and lectin histochemical techniques for the identification of specific sugar residues. We analyzed presence and distribution of GCs in the different structures of the growing follicles (cortical alveoli, globules, yolk granules and zona radiata). During the initial stage of vitellogenesis, the oocytes presented small yolk granules composed of GCs that gradually increased during exogenous vitellogenesis. These GCs contained moderate quantities of α-D-mannose, D-glucose, N-acetylglucosamine and N-acetyl-neuraminic acid. The cortical alveoli contained both neutral and carboxylated GCs, and lectin techniques detected N-acetylgalactosamine, galactose and L-fucose. The zona radiata showed a strong positive reaction to PAS and it reacted weakly with more specific techniques, such as KOH/PA*S and PA/Bh/KOH/PAS. This structure showed GCs with oxidizable vicinal diols, O-acyl sugars and sialic acid residues with different substitution types and presented N-acetylgalactosamine and L-fucose specific residues. The oocytes follicular envelope evidenced neutral and acidic non-sulfated GCs and high concentrations of α-D-mannose, D-glucose, galactose and N-acetylgalactosamine. The intergranular cytoplasmic GCs were mainly rich in α-D-mannose, D-glucose, N-acetylgalactosamine, N-acetylglucosamine and N-acetyl-neuraminic acid. These results enhance the comprehension of the structure and functionality of the pink cuskeel ovarian follicles, and provide a useful tool for the study of this tissue in other teleost species.

Key words: *Genypterus blacodes*, Oocytes, Histochemistry, Glycoconjugates, Lectins

INTRODUCTION

The pink cuskeel *Genypterus blacodes* (Schneider, 1801) (Pisces, Ophidiidae) is an economically important resource captured through commercial fishing in the Southwest Atlantic. It is one of the two species of the genus *Genypterus* traditionally exploited in the Argentine Sea, and it distributes both in the Pacific and the Atlantic seawaters of South America. On the Pacific coast, it appears south of 29° 55′ S (Coquimbo), while in the Southwest Atlantic, *G. blacodes* inhabits from 34° S to 55° S. The reproductive activity of this species occurs in Patagonian coastal waters south of 42° S (Province of Chubut, Argentina), and the spawning area goes from 41° S to 45° S, mainly during summer (Cousseau and Perrotta, 2000).

Macroscopically, the ovaries of *G. blacodes* present an elongated shape; they are surrounded by a muscular connective tunic that invaginates into the ovary, forming two lobes. As characteristically seen in teleosts, they are of the cystovarian type. They are hollow organs into which numerous ovigerous lamellae project to the central lumen, and are covered by a single-layered flat epithelium, the epithelium of the ovarian lamellae. These lamellae possess follicles at different developmental stages (Uribe et al., 2012; Macchi and Díaz, 2013).

In order to classify female gonads of *G. blacodes*, Machinandiarena et al. (2003) proposed a macroscopic

* Corresponding author. Tel. : +54-223-4753554;
Fax : +54-223-4753150;
E-mail: adiaz@mdp.edu.ar
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ovary maturation scale based on appearance and size changes. They defined five developmental ovary stages: (1) immature, (2) developing, (3) spawning, (4) post-spawning and (5) resting. Likewise, ovarian tissue maturity diagnostics can be done according to the oocytes developmental stage. A five-stage maturation scale for describing the oocytes of *G. blacodes* has been microscopically determined: 1) previtellogenic stage or primary growth; 2) with cortical alveoli or early maturation; 3) early vitellogenic stage; 4) secondary vitellogenic stage; and 5) hydrated oocytes or final maturation (Machinandiarena et al., 1998).

Many authors have studied the reproductive cycle of diverse fish species, analyzing the oocytes chemical composition taking into account their cytoplasm, cortical alveoli, yolk, zona radiata, and the theca and granulosa layers (Sarasquete et al., 2002; Calabro et al., 2008; Ortiz-Delgado et al., 2008; Martins et al., 2012; Sales et al., 2013). However, few are the studies on glycoconjugate (GC) characterization in cell structures of vitellogenic and mature oocytes (Ortiz-Delgado et al., 2008).

The carbohydrate composition and characterization of the GCs present in the epithelium of the ovarian lamellae of *G. blacodes* during different maturation stages using histochemical and lectinhistochemical methods have been reported in previous studies (Freijo et al., 2009; Díaz et al., 2012). Lectins are a valuable tool for detection and localization of sugar sequences in oligosaccharide chains of GCs (Parillo et al., 2009).

It is suggested that GCs during fish oogenesis may be involved in binding hormones, in bacteria agglutination, in metabolites and ions transport across the plasmalemma, in sperm-egg binding and in polyspermic inhibition (Ortiz-Delgado et al., 2008).

The purpose of this study was to analyze the carbohydrate composition of the oocytes of *G. blacodes*. This study was performed with a battery of seven lectins and carried out through traditional histochemical treatments.

This work is part of a series of researches our group is developing on glycoconjugates of several fish species of high commercial value (Díaz et al., 2005a, b, 2008, 2010; Freijo et al., 2009).

MATERIALS AND METHODS

Sampling

Forty females of *G. blacodes* were collected during two research trawl cruises carried out in San Jorge Gulf (45°S–47°S, 65°W–67°W) during January 1997 and March 1998. The maturation stage of gonads and oocytes was determined. Firstly, ovaries were macroscopically examined on board for maturity staging, and a diagnosis was made according to a five-stage maturity scale: (1) immature, (2) developing, (3) spawning, (4) post-spawning and (5) resting (Machinandiarena et al., 2003). The maturation stage of oocytes of every ovary was then microscopically determined after

Table 1. Histochemical procedures for identifying glycoconjugates (GCs) in the oocytes of *G. blacodes*.

Procedures[a]	Summarized Protocol	Interpretation of staining reactions	References
1. PAS	Oxidation with periodic acid followed by Schiff's reagent	GCs with oxidizable vicinal diols and glycogen	McManus (1948), Bancroft and Gamble (2008)
2. α-amylase-PAS	Ptyalin incubation at 36°C during 45 min, previous to PAS reaction	GCs with oxidizable vicinal diols	Pearse (1985)
3. Acetylation-PAS	Acetylation with an acetic anhydride-anhydrous pyridine mixture at 58°C during 30 min, followed by PAS reaction	GCs with oxidizable vicinal diols and glycogen	Pearse (1985)
4. Acetylation-KOH-PAS	Acetylation previous to saponification with 0.5% potassium hydroxide in 70% ethanol for 30 min at room temperature, and finally PAS reaction	GCs with oxidizable vicinal diols and glycogen	Pearse (1985)
5. KOH/PA*S	Saponification followed by PA*S	GCs with sialic acid residues	Culling et al. (1976)
6. KOH/PA*/Bh/PAS	Saponification followed by selective oxidation at 4°C during 1 h, reduction with borohydride and finally PAS reaction	GCs with oxidizable vicinal diols and O-acyl sugars	Volz et al. (1987)
7. PA/Bh/KOH/PAS	Two-h oxidation at room temperature with 1% periodic acid, followed by reduction with sodium borohydride (PA-Bh). Then, saponification (KOH) and finally PAS reaction.	Sialic acid residues with O-acyl substitution at ^{7}C, ^{8}C or ^{9}C and O-acyl sugars	Reid et al. (1973)
8. AB pH 2.5	Alcian blue pH 2.5 during 30 min.	GCs with carboxyl groups and O-sulphate esters	Lev and Spicer (1964), Bancroft and Gamble (2008)
9. AB pH 1.0	Alcian blue pH 1.0 during 30 min.	GCs with O-sulphate esters	Lev and Spicer (1964), Bancroft and Gamble (2008)
10. AB pH 0.5	Alcian blue pH 0.5 during 30 min.	Highly sulphated GCs	Lev and Spicer (1964), Bancroft and Gamble (2008)
11. TB pH 5.6	Toluidine blue in acetate buffer pH 5.6 during 15 min.	Acid sulphated and carboxylated GCs	Lison (1953)
12. TB pH 4.2	Toluidine blue in acetate buffer pH 4.2 during 15 min.	Acid sulphated GCs	Lison (1953)

[a] AB, Alcian blue; Bh, borohydride; KOH, potassium hydroxide; PA, periodic acid oxidation; PA*, selective periodic acid oxidation; PAS, periodic acid Schiff reagent; PA*S, periodic acid/Schiff at low temperature and low pH (oxidation with 0.4 mM periodic acid in 1.0 M hydrochloric acid at 4°C); TB, Toluidine blue.

Machinandiarena et al. (1998) as (1) previtellogenic stage or primary growth; 2) with cortical alveoli or early maturation; 3) early vitellogenic stage; 4) secondary vitellogenic stage; and 5) hydrated oocytes or final maturation.

Histological methods

Ovaries from sampled individuals were removed immediately after capture, fixed by immersion in Bouin's fluid or 10% buffered formalin during 24 h for light microscope studies. Samples were routinely processed, embedded in paraffin wax and stored in paraffin blocks for later studies. In this work 4-μm-thick histological sections were cut by microtome (Microtome 1512, Leitz Wetzlar), prepared according to a standard protocol and then stained using hematoxylin and eosin technique (H-E) and Light Green-Orange G-Acid Fuchsin (VOF) stain for morphology. Micrographs were taken with an Olympus microscope, CH 30 (Olympus; www.olympus.com).

Histochemical methods

Sections of tissue were also subjected to histochemical procedures for visualization and identification of GCs, as detailed in Table 1.

Lectin histochemistry

Labeling with biotinylated lectins was used to identify specific sugar residues of GCs (Díaz et al., 2008). Briefly, paraffin sections mounted on slides coated with poly-L-lysine (Sigma Diagnostics, St Louis, MO, USA) were deparaffinized with xylene and then incubated in 0.3% H_2O_2 in methanol for 30 min at room temperature in order to block the endogenous peroxidase activity. Later, they were hydrated, washed in a phosphate-buffered saline (PBS) 0.01 M, pH 7.2 solution and incubated for 30 minutes with biotinylated lectins. Seven different specific lectins, purchased at Vector Laboratories, Inc. (Burlingame, CA, USA) were employed (Table 2). Afterwards, sections were incubated with avidin biotin-peroxidase complex (ABC) for 30 min (Vectastain Elite PK 6200 Vector Laboratories Inc, Burlingame, CA, USA). Lectin binding was revealed by incubation with diaminobenzidine tetrahydrochloride (DAB) (DAKO) 0.5 mg/mol in tris buffer 0.1 M, pH 7.2, plus 0.02% H_2O_2. Each lectin was used at a 30 μg/ml dilution in PBS, except for PNA, which was applied at a concentration of 10 μg/ml. All dilutions and thorough washes between steps were carried out using PBS unless otherwise stated. Two types of controls were performed: (i) lectin solution was replaced by PBS and (ii) lectin labeling was performed as described previously after lectin preincubation for 1 h in the presence of the appropriate hapten sugars (0.2 Min PBS), as listed in Table 2, at room temperature.

Evaluation of labeling intensities was based on subjective estimates of the authors through the examination of two sections per sample of all the animals tested.

RESULTS

Histochemical characterization

Oocytes at different developmental stages were distinguished in the analyzed *G. blacodes* ovaries (Fig. 1). The oocytes in primary growth showed ooplasmic basophilia that progressively decreased together with the oocyte growth. In the early primary growth oocytes numerous nucleoli within the large central nuclei reacted to the hematoxylin-eosin technique basic dye (Fig. 1A). The beginning of the yolk deposition was indicative of vitellogenic oocytes. In advanced maturation phases, the oocyte cytoplasm was completely covered by yolk granules and lipid globules.

During the early maturation stage, the observed small yolk granules were composed of GCs that gradually increased during the exogenous vitellogenesis. The yolk granules showed orange G and eosin affinities with the VOF and the hematoxylin-eosin procedures respectively (Fig. 1) They were weakly stained with the PAS technique and not stained with AB techniques (Fig. 2A–C). Lipid globules appeared unstained with both morphological dyes, increased in number and size, and gradually fused and coalesced during the maturation phase (Fig. 1). During the developing phase, cortical alveoli progressively appeared and formed a discrete layer close to the oolema. They showed GCs with carboxyl groups and neutral GCs (Fig. 2E).

Table 2. Lectins used for histochemical characterization of sugar residues of glycoconjugates.

Lectin	Acronym	Specificity a,b	Hapten
GROUP I		Glc/Man	
Canavalia ensiformis agglutinin	Con-A	α-D-Man; α-D-Glc	α-D-Methyl-Man
GROUP II		GlcNAc	
Triticum vulgaris (wheatgerm) agglutinin	WGA	β-D-GlcNAc; NeuNAc	NeuNAc
GROUP III		GalNAc/Gal	
Dolichos biflorus Agglutinin	DBA	α-D-GalNAc	D-GalNAc
Glycine maximus agglutinin	SBA	α-D-GalNAc; β-D-GalNAc	D-GalNAc
Ricinus communis agglutinin-I	RCA-I	β-Gal	Lactose
Arachis hypogaea agglutinin	PNA	β-D-Gal (b1-3) > D-GalNAc	Lactose
GROUP IV		L-Fuc	
Agglutinin-I			
Ulex europaeus	UEA-I	α-L-Fuc	L-Fuc

aGoldstein and Hayes (1978).
bGal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcNAc, N-acetylglucosamine; L-Fuc, L-fucose; Man, mannose; α-D-Methyl-Man, α-D-Methyl-mannose; NeuNAc, N-acetyl-neuraminic acid (sialic acid).

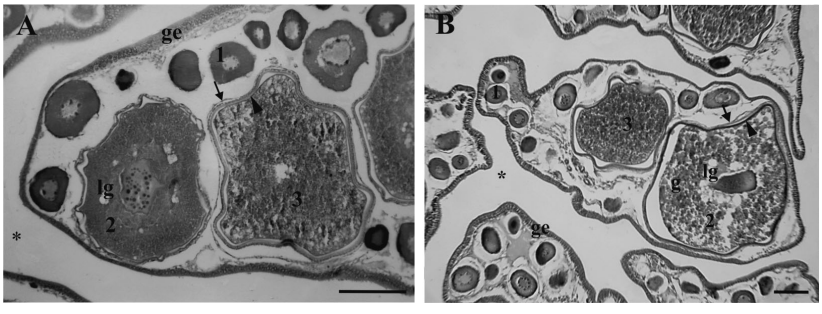

Fig. 1. Histological characterization of the oocytes of *G. blacodes*. **(A)** H-E technique **(B)** VOF technique. Scale bars: 100 μm. g, yolk granules; ge, epithelium of ovarian lamellae; lg, lipid globules; ►, zona radiata; →, follicular envelopment; *, ovarian lumen; 1, previtellogenic oocytes; 2, oocytes at an early maturation stage; 3, oocytes at the secondary vitellogenic stage.

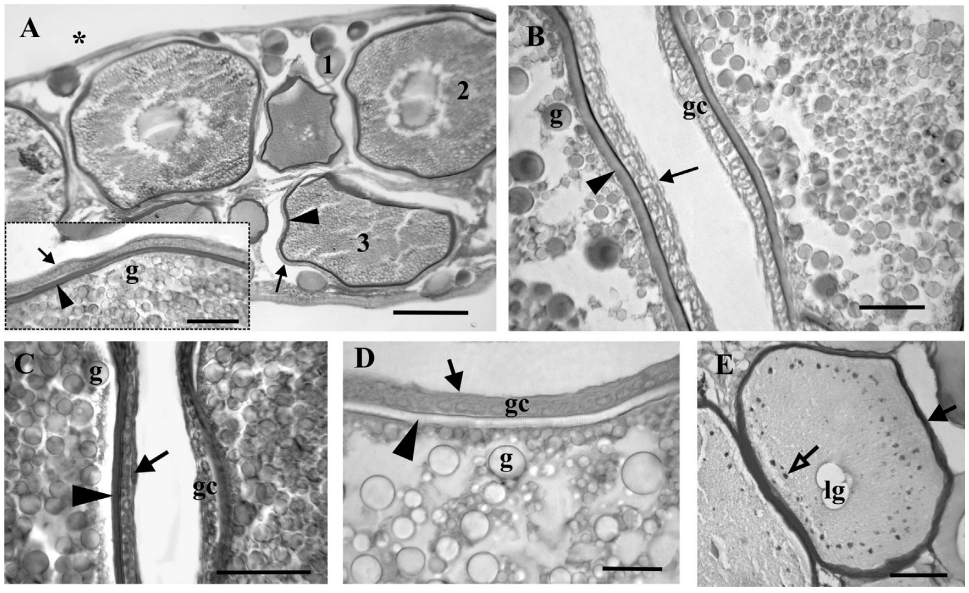

Fig. 2. Histochemical characterization of the oocytes of *G. blacodes*. **(A)** PAS technique. **(B)** PA/Bh/KOH/PAS technique. **(C)** PAS-H technique. **(D)** AB pH 2.5 technique. **(E)** KOH/PA/Bh/PAS technique. Scale bars: **(B)** and **(D)**, 25 μm; **(C)**, **(E)** and detail of **(A)**, 50 μm; **(A)**, 100 μm. g, yolk granules; gc, granulosa cell layer; lg, lipid globules; ►, zona radiata; →, follicular envelopment; ⇾, cortical alveoli; *, ovarian lumen; 1, previtellogenic oocytes; 2, oocytes at an early maturation stage; 3, oocytes at the secondary vitellogenic stage.

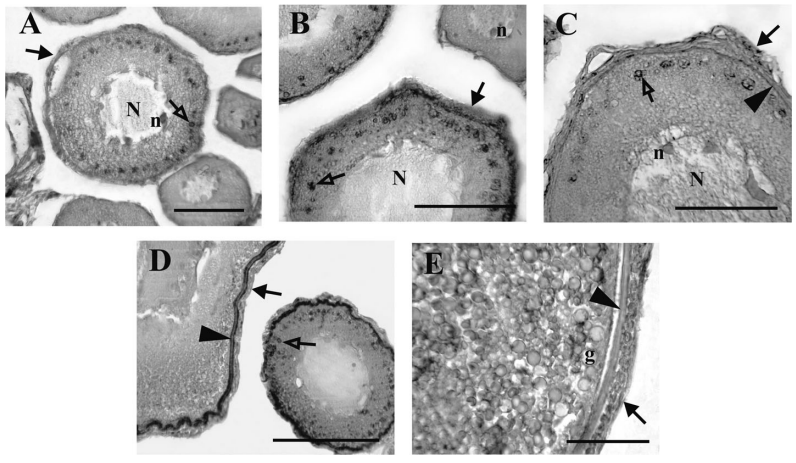

Fig. 3. Lectinhistochemical characteristics of the oocytes of *G. blacodes*. **(A)** Lectin SBA. **(B)** Lectin RCA-1. **(C)** Lectin WGA. **(D)** Lectin PNA. **(E)** Lectin DAB. Scale bars: **(A)** and **(C)**, 25 μm; **(B)**, **(D)** and **(E)**, 50 μm. g, yolk granules; ►, zona radiata; →, follicular envelopment; ⇾, cortical alveoli.

The zona radiata showed a strong PAS-positive reaction; it reacted weakly with more specific techniques, such as KOH/PA*S and PA/Bh/KOH/PAS, displaying the presence of GCs with vicinal oxidizable diol groups, O-acyl sugars and sialic acid in several forms of substitution (Fig. 2A, C, D). The follicular envelope stained with PAS and AB pH 2.5 techniques, which allowed the identification of neutral and acid GCs (Fig. 2A–C). A further source of information was provided by toluidine blue procedures at pH 5.6 that revealed the existence of acid-sulfated and carboxylated GCs.

Lectinhistochemical characterization

Biotinylated lectins were used to identify specific sugar residues of GCs.

Lectin Con-A was found to have affinity to the follicular envelope, intergranular cytoplasm and yolk granules, showing a moderated staining. No labeling was observed in cortical alveoli.

Yolk granules and cortical alveoli labeled strongly with WGA, while the intergranular cytoplasm and the follicular envelope did it moderately (Fig. 3C).

DBA and SBA showed similar distribution patterns. They stained cortical alveoli as well as yolk granules and the follicular envelope. However, a moderate staining was detected in the intergranular cytoplasm with these lectins (Fig. 3A, E).

PNA showed a strong affinity for cortical alveoli and the zona radiata (Fig. 3D). Using RCA-I the reaction was strong in cortical alveoli and the follicular envelope, and weak in the intergranular cytoplasm (Fig. 3B).

Cortical alveoli labeled moderately to strongly with UEA-I, and the intergranular cytoplasm showed a weak affinity for this lectin.

No labeling was detected in control sections.

DISCUSSION

The present study presents the first information on the carbohydrate composition of oocytes of *Genypterus blacodes*.

Sexual development and gonad differentiation in some vertebrates involve changes that take into account both type and distribution of different proteins and GCs (Fröjdman et al., 1992). During development, large quantities of GCs are synthesized. They represent an important component of diverse structures such as cortical alveoli, vitelline envelope and yolk granules of mature oocytes of many marine organisms (Sarasquete et al., 2002; Ortiz-Delgado et al., 2008).

Genypterus blacodes oocytes are spherical with cortical alveoli initially small, regularly distributed around the nucleus. Phylogenetic studies show that the cortical alveoli morphology is an important parameter among the fish family, and that the shape and histochemical content similarities suggest that the analyzed species could have a similar polyspermy block (Martins et al., 2012). Barros et al. (2007) have determined that GCs are released to the perivitelline space in order to block polyspermy after fertilization. Thus, the cortical alveoli have a large GCs heterogeneity and react

to different reagents according to their contents (Gomes et al., 2007; Ortiz-Delgado et al., 2008). These structures are considered homologous to the cortical granules present in invertebrates (Schuel, 1985) and some species of vertebrates (Gilkey, 1981). Gallo and Constantini (2012) suggest that the GC pattern is specific to the species. In this work we have observed that the *G. blacodes* cortical alveoli possess neutral GCs just as other teleosts, like *Lophiosilurus alexandri* (Barros et al., 2007), *Heterandria formosa* (Uribe and Grier, 2011) and *Pimelodella vittata* (Sales et al., 2013). Through lectin histochemistry it was possible to detect a great variety of sugar residues in the cortical alveoli that reacted positively with most of the employed lectins. This same diversity was observed in species like *Xiphias gladius* (Ortiz-Delgado et al., 2008).

As the oocyte continues the reproductive cycle, size increases and vitellogenesis with vitelline granules starts. The lipidic globules and yolk granules represent a very important source of energy for eggs, embryos and larvae of many fish species. Since the lipidic drops are scant in some species, the necessary lipids to the embryonic development should somehow be related to proteins coming from the vitelline granules (McMillan, 2007; Quagio-Grassiotto et al., 2011, 2014). In these cases the cortical alveoli are thought to be formed at the end of the primary growth, simultaneously with the lipid deposition (Patiño and Sullivan, 2002; McMillan, 2007).

With the use of histochemical techniques the heterogeneity of *G. blacodes* vitelline granules sugar residues could be observed, evidencing N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, galactose and sialic acid groups. Similar results were observed in *Hoplostethus mediterraneus* and *Coelorhinchus coelorhynchus* (Calabro et al., 2008).

The structure of the zona radiata varies between teleosts and reflects adaptations to different ecological conditions (Fausto et al., 2004). Moreover, the zona radiata is responsible for the interaction of eggs in water and the protection of the embryo against microorganisms (Sales et al., 2013). Riehl and Patzner (1998) describe the teleosts zona radiata usually consisting of a proteic inner layer and an outer layer that mainly contains neutral and acid GCs. In *G. blacodes*, however, this structure presented GCs with oxidizable vicinal diols and substituted sialic acid residues. Diverse authors have observed neutral, carboxylated and/or sulfate GCs in the zona radiata of different teleosts (Rizzo and Bazzoli, 1991; Rizzo et al., 1998; Gomes et al., 2007; Sales et al., 2012; Quagio-Grassiotto et al., 2014).

Among other functions, GCs may be involved in hormone binding, metabolite and ion membrane transport, chorion hardening, binding of sperms to the egg surface, and polyspermy block after genetic fusion (Ortiz-Delgado et al., 2008). On the other hand, the attachment of deposited eggs to substrate is in some species largely mediated by the zona radiata externa. In these cases, the surface GCs become sticky in contact with water permitting eggs to be fixed to the substrate. There are, however, some teleosts where the zona radiata has no participation in the adhesion to substrate, but the female produces a jelly layer around the eggs. (Riehl and Patzner, 1998; Patiño and Sullivan, 2002).

The results of the present study expand our understanding of the morphology and physiology of the *G. blacodes* ovary, thus contributing to the knowledge of the chemical structure of the GCs produced by different ovarian structures. This work offers a useful tool as a basis to the study of this organ in other teleost species.

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