OOCYTE SURFACE IN FOUR TELEOST FISH SPECIES POSTSPAWNING AND FERTILIZATION

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ABSTRACT

Cytological and cytochemical studies were carried out to investigate the surface characteristics of oocytes of four teleost species from the São Francisco river. The fishes were submitted to hypophysation at the Três Marias Hybrobiology and Fishculture Station, Minas Gerais, Brazil, in January 1996. Postspawning, oocytes of the curimatãs *Prochilodus affinis*, *Prochilodus marggravii* and dourado *Salminus brasiliensis* were surrounded by a thick, three-layered zona pellucida with radial striae. The surface of spawned oocytes of the surubim, *Pseudoplatystoma coruscans*, was comprised of mucous coat located externally to a thin, two-layered and striated zona pellucida. Oocyte activation during fertilization, lead to cortical reaction, formation of a perivitelline space, reduction of the thickness of the zona pellucida and increase in the oocyte diameter in the four species. Following fertilization, many spermatozoa were embedded in the mucous coat of the surubim oocytes. During embryogenesis, this later coating became thicker, diffuse and less viscous while the zona pellucida (chorion) was thinner in all studied species. Cytochemical analyses indicated species-specific differences in the oocyte surface after spawning. It was suggested that the mucous coat of surubim oocytes play a functional role during fertilization. The knowledge of the morphology of the oocyte surface of teleost is important for our understanding of the interactions between their eggs and surrounding environment and may also contribute significantly to phylogenetic studies.

Key words: zona pellucida, oocyte surface, mucous coat, teleost, micropyle.

INTRODUCTION

Surubim *Pseudoplatystoma coruscans*, dourado *Salminus brasiliensis*, curimatã-pioa *Prochilodus affinis* and curimatã-pacu *Prochilodus marggravii* are important species for the commercial fisheries throughout the basin of the São Francisco river, which migrate upriver in the breeding season, and need as such lotic environments to guarantee their successful reproduction (BRITSKI *et al.*, 1988). These species have total spawning, free and demersal eggs, high fecundity and showing no parental care (LAMAS, 1993). Their populations have been declining as a result of the widespread construction of dams and reservoirs in the Brazilian river basins, transforming extensive tracts into lenthic as opposed to lotic environments. The induced reproduction of migratory species is currently in practice at the Três Marias Hydrobiology and

Fishculture Station, in the state of Minas Gerais, in order to produce fry for the repopulation of the areas impacted by the construction of the Três Marias dam (SATO *et al.*, 1996a, b).

Several studies have examined the histology and histochemistry of vitellogenic oocytes of a number of Brazilian teleost species (BAZZOLI & RIZZO, 1990; RIZZO & BAZZOLI, 1991; BAZZOLI, 1992; RIZZO & BAZZOLI, 1993; BAZZOLI & GODINHO, 1994; BAZZOLI *et al.*, 1996). A funnel-shaped micropyle of the *Prochilodus affinis* oocytes was analysed using a scanning electron microscope (RIZZO & BAZZOLI, 1993). A comparative investigation of the micropylar apparatus recorded that the morphology of the micropyle and the micropylar cell differs among freshwater teleost species, although showing similar patterns in some systematic groupings (RICARDO *et al.*, 1996). The morphological study of the final oocyte maturation and fertilization in pacu *Piaractus mesopotamicus* and curimatã *Prochilodus scrofa* showed a fertilization cone sealing the micropyle postfertilization (RIZZO *et al.*, 1997).

The morphological characteristics of the extracellular matrix which cover the oocytes of fishes are of vital importance in the oocyte development (GURAYA, 1986). Determination of the chemical nature of this structure is, likewise, fundamental for the comprehension of its functional role in fertilization and embryogenesis (HART, 1990). In view of these, the present paper analyse the morphology of the oocytes after spawning and fertilization in four freshwater teleost species, examining particularly the cytology and cytochemistry of the oocyte surface.

MATERIAL AND METHODS

Recently-laid oocytes and fertilized eggs were collected in January 1996 at the Três Marias Hydrobiology and Fishculture Station, in the state of Minas Gerais. The reproduction of the fishes (Table I) were induced artificially by hypophysation (IHERING, 1937), using a crude carp pituitary extract. The amounts injected (one injection for males and two for females), intervals between doses, and the procedures for the egg stripping and fertilization, followed the methods described by WOYNAROVICH & HORVÁTH (1980).

Samples of oocytes and fertilized eggs were fixed in Bouin's fluid and embedded in glycol methacrylate plastic resin. Sections of 3-5μm thickness were stained with toluidine blue/basic fuchsin for cytological examination.

In order to study the chemical nature of the oocyte surface, 5μm thick sections were submitted to the classical cytochemical techniques for the detection of proteins and carbohydrates, following PEARSE (1985):

- periodic acid-Schiff (PAS) for the detection of carbohydrates with 1:2 glycol groups;
- salivary amylase (1h at 37°C) for the extraction of glycogen, followed by PAS;
- alcian blue (AB) 8GX-Sigma at pH 2.5 for the detection of carboxylated acid and sulphated glycoconjugates;
- AB at pH 0.5 for sulphated acid glycoconjugates;
- combined method of AB pH 2.5 followed by PAS for acidic and neutral glycoconjugates;
- ninhydrin-Schiff for the detection of proteins with NH_2 radicals;
- mercuric bromophenol blue for the detection of total proteins;
- combined method of alcian yellow (AY) at pH 2.5 followed by AB at pH 0.5 for the identification of sulphated and carboxylated acid groups (RAVETTO, 1964);
- hydrolysis with hydrochloric acid HCl at 0.1N (8h at 60°C) for the extraction of sialic acid, followed by PAS and AB 2.5 (QUINTARELLI *et al.*, 1961).

Table I: Species submitted to hypophysation and fertilization rate.

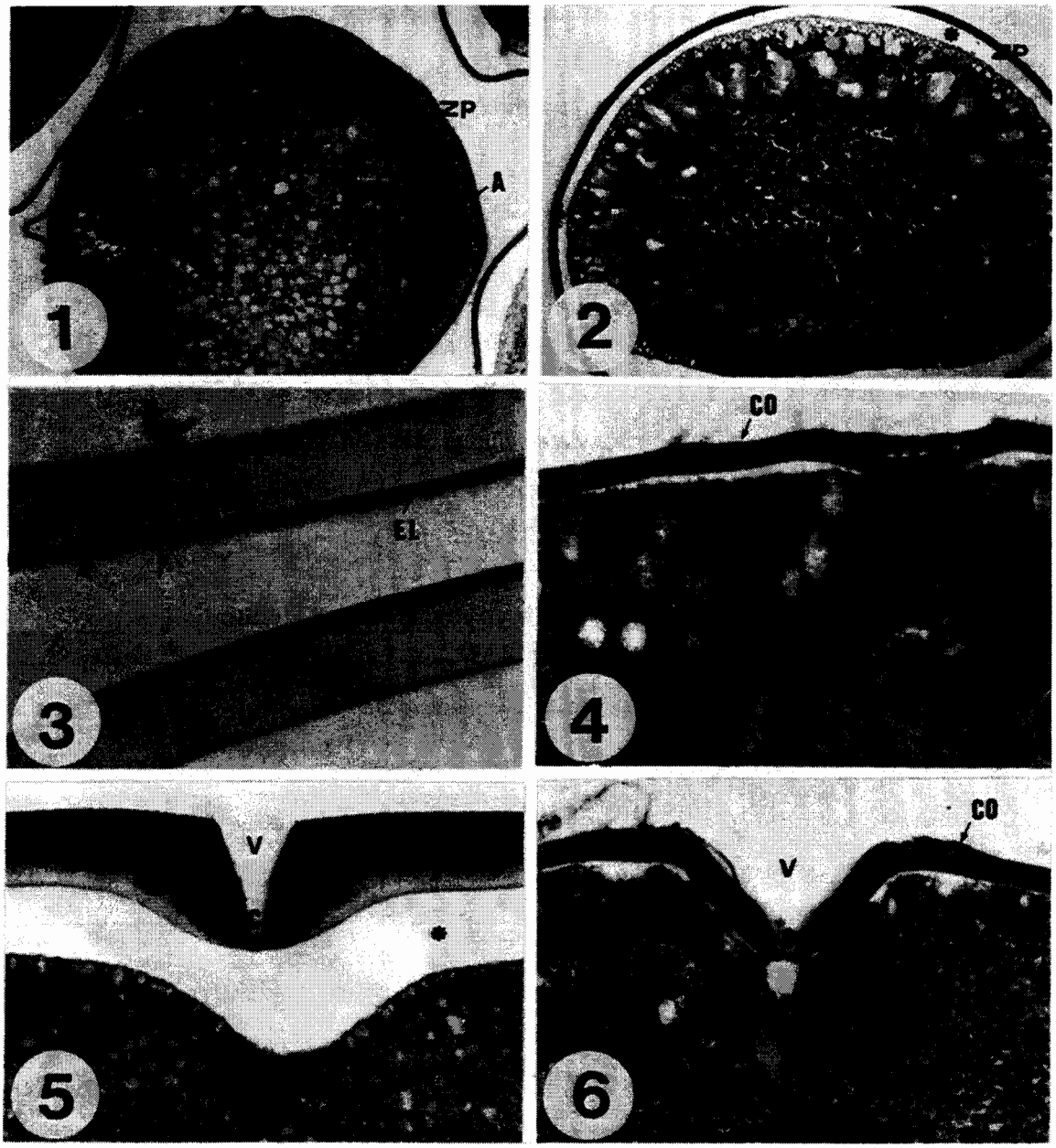
Species	Order/Family	SL (cm)	W (Kg)	FR (%)
P. affinis Reinhardt, 1874	Characiformes/Prochilodontidae	37	0.61	83.0
P. marggravii (Walbaum, 1792)	Characiformes/Prochilodontidae	51	1.90	81.2
S. brasiliensis (Cuvier, 1817)	Characiformes/Characidae	80	5.43	48.5
P. coruscans (Agassiz, 1829)	Siluriformes/Pimelodidae	113	11.90	92.8

SL- standard body length of the female , W- body weight of the female, FR- egg fertility rate at 18h postspawning

For measurements of the oocyte diameter, the thickness of the zona pellucida and mucous coat at stripping, were selected 10 major oocytes of five slides for species. The micropylar apparatus were measured in three to five oocytes. The values were obtained by using a micrometer ocular connected to a Zeiss microscope.

RESULTS AND DISCUSSION

Cytological features of the oocytes at stripping (Fig. 1) are similar to vitellogenic oocytes at final stage of ovarian development, including acidophilic yolk globules, slightly basophilic cortical alveoli at the peripheral ooplasm and layered zona pellucida with radial striae (BAZZOLI & RIZZO, 1990; BAZZOLI, 1992; BAZZOLI & GODINHO, 1994). One of the events of the final oocyte maturation is the migration of the nucleus or germinative vesicle toward the micropyle where it breakdown allowing for fusion of the genetic material at fertilization



Figures 1 to 6: 1 - Recently-laid oocyte of the dourado, *Salminus brasiliensis.* x 68; **2** - Oocyte of curimatã-pacu, *Prochilodos marggravii* at the initial stage of hidration and concentration of basophilic material. x 45; **3** – Zona pellucida with radial strie at the oocyte surface of curimatã-pacu, *Prochilodos marggravii.* x 1040; **4** – Mucous coat on the zona pellucida at the oocyte surface of surubim, *Pseudoplatystoma coruscans.* x 1000; **5** – Funnel-shaped micropyle separated of the plasma membrane by the perivitelline space in formation on oocyte of curimatã-pioa, *Prochilodos affinis.* x 400; **6** – Micropyle of the surubim, *Pseudoplatystoma coruscans* constituted by a large deep vestibule and a short micropylar canal. Provable site of sperm incorporation at the micropyle base. x 1320.

Stain with toluidine blue/basic fuchsin (figs. 1,2,4,5,6) and PAS (fig. 3). Zona pellucida (ZP); yolk globules (YG); cortical alveoli (A); perivitelline space (*); concentration of basophilic material (★); internal layer of the zona pellucida (IL); middle/external layers of the zona pellucida (EL); mucous coat (CO); vestibule (V); mycropyle canal (C); perihperal ooplasm (OP).

(NAGAHAMA, 1983; RIZZO *et al.*, 1997). Even though, the nucleus was not evident after eggs stripping, basophilic granular material to accumulate gradually around the micropyle (Fig. 2).

On the surface of the oocytes of the curimatãs and dourado, the zona pellucida was divided into layers (Fig. 3): a thick internal layer and an thin external layer, with a strongly-stained line indicating a medial layer between the two. During oocyte development, deposits of extracellular material between microvilli from both oocyte and follicular cells leading to formation of pores or canals which represent the radial striations of this envelope being thus referenced as zona radiata (GURAYA, 1986; SELMAN & WALLACE, 1989). After stripping and oocyte activation, polymerization of proteins hardens it into a fertilization envelope or chorion which provides protection to the embryo (YAMAGAMI *et al.*, 1992). Our results indicated, for all four species, a progressive reduction in the thickness of the zona pellucida during hydration of the egg and formation of the chorion, while maintaining, however, its layered structure. The disappearment of the outermost layer and formation of a new layer of material released by the cortical alveoli (REDDING & PATINO, 1993) was not observed in the present study.

In the surubim, the oocyte surface was comprised of a thinner zona pellucida showing two layers with radial striae and, on the outside, a fine coating of mucous material (Fig. 4, Table II). A similar gelatinous coating, called sometimes jelly-coat, has also been reported for other siluriformes (IHERING & AZEVEDO, 1936; GODINHO *et al.*, 1978; ESPINACH ROS *et al.*, 1984; RIEHL & APPELBAUM, 1991; ABRAHAM *et al.*, 1993). According to LAALE (1980), this structure, considered a secondary envelope in teleost eggs, undergoes physical and chemical changes after spawning, acquiring adhesive properties which make the attachment of the egg to vegetation or submersed objects as well as to other eggs. Besides, he have also indicated that the stickiness is lost in the majority of species after egg hydration and the hardening of the chorion, which is in agreement with the non-adhesive characteristics of surubim eggs (CARDOSO *et al.*, 1995). Likewise, eggs of *Silurus lithophilus* have also a jelly-coat on the surface which is also non-adhesive (KOBAYAKAWA, 1985 *apud* RIEHL & APPELBAUM, 1991). On the other hand, there is an attachment-disc around the micropyle, the future animal pole of ova in *Clarias gariepinus*, consisting of numerous tiny attaching-filaments embedded in a cementing substance containing acidic mucopolysaccharides (RIEHL & APPELBAUM, 1991).

The micropyle, opening in the zona pellucida which allows for fertilization by giving the sperm access to the plasmatic membrane of the oocyte postspawning, varies in its morphology from species to species (GURAYA, 1986; RICARDO *et al.*, 1996). The micropyle of the curimatãs

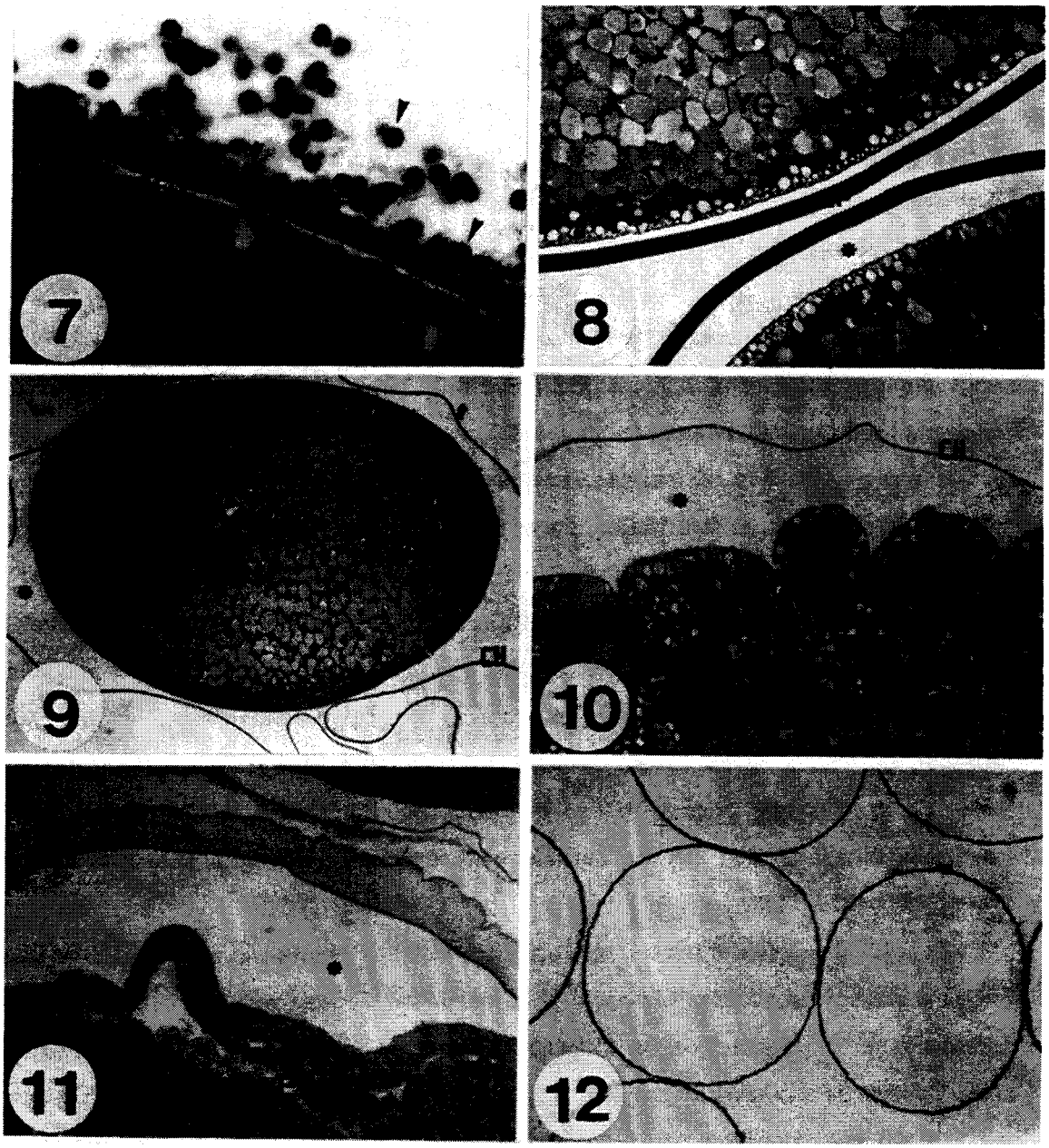
and dourado eggs was funnel-shaped (Fig. 5), comprised of a shallow vestibule and a long micropylar canal, agreeing with a scanning electron microscope study (RIZZO & BAZZOLI, 1993). The micropyle of surubim eggs (Fig. 6), on the other hand, has a large deep vestibule and a short micropylar canal, similar to the Type 1 described in other pimelodids being those of curimatãs and dourado such as Type 2 (RICARDO *et al.*, 1996). .

Table II: Mean values (μm) of the oocyte diameter and structures of the oocyte surface at stripping in four teleost species submitted to hypophysation.

SPECIES	OOCYTE(d)	ZONA PELLUCIDA (t)		MUCOUS COAT (t)	MICROPYLE	
		IL	M/EL		Vestibulo (ed)	Canal (dp)
P. affinis	1086.0 ± 48.6	16.4 ±3.4	3.5 ± 1.6	-	23.3 ± 7.4	25.8 ± 1.0
P. marggravii	1047.0 ± 60.8	14.7 ± 3.1	2.3 ± 1.8	-	29.5 ± 0.7	26.0 ± 1.4
S. brasiliensis	984.0 ± 34.1	12.0 ± 2.7	3.5 ± 2.3	-	25.0 ± 6.9	18.5 ± 0.7
P. coruscans	663.0 ± 22.2	1.3 ± 0.3	0.5 ± 0.1	2.3 ±1.0	26.4 ± 6.0	7.0 ± 1.4

d= diameter; t = thickness; IL= internal layer; M/EL= middle/external layer; ed= external diameter; dp=depth.

Soon after fertilization, the following events were observed progressivelly in oocytes of four species: liberation of glycoconjugates from the cortical vesicles (cortical rection), formation of a perivitelline space due to hydration, increase in the oocyte diameter, and reduction in the thickness of the zona pellucida. In surubim, numerous spermatozoa were embedded in the mucous coat of their eggs immediately after fertilization (Fig. 7), however the number of sperm was reducing following the hydration of the egg. The yolk globules remained intact and no liquefaction processes were observed postspawning (Fig. 8). Fusion of the yolk globules to form a homogeneous mass of vitelline fluid making the eggs transparent and has been related for marine teleosts during preovulatory hydration but this do not occur in several freshwater species (CHAVES, 1989; SELMAN & WALLACE, 1989; BAZZOLI, 1992). The postspawning hydration of freshwater teleost differs from the preovulatory hydration of marine fishes and may to represent a important factor for the functional activity of the glycoconjugates released during cortical reaction. The fertilization cone, observable 1-2 minutes after fertilization in teleosts such as *Cyprinus carpio* (KUDO & SATO, 1985), *Oryzias latipes* (IWAMATSU *et al.*, 1991) and *Piaractus mesopotamicus* (RIZZO *et al.*, 1997), regresses rapidly after its formation and was not observed in this study. Likewise, the elimination of the second polar body was not detected by cytological techniques.



Figures 7 to 12: **7** - Spermatozoa embedded in the mucous coat of the oocytes of surubim, *Pseudoplatystoma coruscans,* immediately following fertilization. x 1440; **8** - Formation of the perivitelline space, intact yolk globules and cortical alveoli in the oocyte of curimatã-pioa, *Prochilodos affinis.* x 192; **9** - Concentration of basophilic material at the animal pole determining the egg polarity of curimatã-pioa. *Prochilodos affinis*, 30 minutes after fertilization and hydration. x 66; **10** - Thin chorion on the egg surface of the dourado, *Salminus brasiliensis* at blastula stage. x 160; **11** - Thick mucous coat and a thin chorion on the egg surface of surubim, *Pseudoplatystoma coruscans* at gastrula stage. x 470; **12** – Positive Alcian Blue pH 2.5 reaction for the mucous coat of the oocytes of surubim, *Pseudoplatystoma coruscans,* at stripping after hypophysation. x 58.

Stain with toluidine blue/basic fuchisn (figs. 7 to 11) and AB pH 2.5 (fig. 12). Spermatozoa (arrowhead); zona pellucida (ZP); perivitelline space (*); cortical alveoli (A); yolk globules (YG); concentration of basophilic material (★); chorion (CH); developing embryo (E); mucous coat (CO).

Several factors contribute to prevent polyspermy in teleost fishes (KOBAYASHI & YAMAMOTO, 1981; HART, 1990). The diameter of the micropylar canal is close to the size of the head of the spermatozoon, there is just a single site at the base of the micropyle where sperm can enter, a fertilization cone is formed which seals the micropyle after the penetration of the fertilizing spermatozoon, and a cortical reaction produces morphofunctional changes in the zona pellucida to form the chorion. According to YAMAGAMI *et al.* (1992), these processes and events occur with or without fertilization, being activated by contact with freshwater which makes the egg turgid and hardens the chorion. After hardening, the chorion acquires bactericidal and fungicidal properties, probably derived from the cortical alveoli, protecting the embryo from physical damage and disease (REDDING & PATIÑO, 1993).

About 30 minutes after fertilization, the eggs reached fully hydration. Histologically, it was possible to observe the polarity of the egg with a concentration of basophilic material at the animal pole where the micropyle was located, and an accumulation of yolk globules at the vegetative pole (Fig. 9). Embryonic development up to the closure of the blastopore was similar in the four species. These results are in according to that described for *P. affinis* and *P. coruscans* (ALVES & MOURA, 1992; CARDOSO *et al.*, 1995). During this period, the embryo of the all species was coated by an ample perivitelline space and a thin chorion or fertilization envelope (Fig. 10). On surubim eggs surface, the mucous coat became thick and poorly viscous (Fig. 11).

Cytochemical analysis of the surface components in the recently-laid oocytes (Tables III and IV) demonstrated the presence of neutral glycoproteins in the zona pellucida of the four species similar to other Brazilian teleost (BAZZOLI & RIZZO, 1990; RIZZO & BAZZOLI, 1991; BAZZOLI, 1992). Our results also revealed the presence of carboxylated glycosaminoglycans in the middle and external layers oocytes of the curimatãs. Moreover, the mucous coat of the surubim oocytes, also of an acidic nature, contains sulphated glycosaminoglycans similar to the follicular layer surrounding the vitellogenic oocytes (BAZZOLI, 1992). These results suggest that this coating may be a product of the follicular cells such as the jelly-coat in the oocyte of the *Sillurus glanis* (ABRAHAM *et al.*, 1993). Sialo and sulphomucins in vitellogenic oocytes have been found in the cortical alveoli, the outermost layer of the zona pellucida and the follicular layer of oocytes of some Brazilian species (BAZZOLI, 1992; BAZZOLI & GODINHO, 1994; BAZZOLI *et al.*, 1996). These substances are also found in the secretion of the sperm ducts glands of some blenniids and gobbids, increasing the viscosity of the semen and delaying the

activation of the spermatozoa during fertilization (LAHNSTEINER & PATZNER, 1990; OTA *et al.*, 1996). Furthermore, the glycoproteins in the mucous area on the oocyte surface of *Oryzias latipes,* which have an affinity for spermatozoa, play an important role in guiding the sperm into the micropyle (IWAMATSU *et al.*, 1997). In surubim oocytes, the fact that the mucous coat retains spermatozoa immediately after spawning suggest that this structure may to be essential during the process of fertilization for this species, however this fact need to be still to investigated.

Table III: Chemical nature of the oocyte surface at stripping in four teleost species submitted to hypophysation

SPECIES	ZONA PELLUCIDA		MUCOUS
	internal layer	middle/external layer	COAT
P. affinis	NEG	NEG/CAG	-
P. marggravii	NEG	NEG/CAG	-
S. brasiliensis	NEG	NEG	-
P. coruscans	NEG	NEG	SAG/NEG

NEG = neutral glycoproteins; NEG/CAG = predominance of neutral glycoproteins over carboxilated glycosaminoglycans; SAG/NEG = predominance of sulphated glycosaminoglycans over neutral glycoproteins.

Table IV: Cytochemical reactions of the substances of the oocyte surface in four teleost species submitted to hypophysation

	CYTOCHEMICAL REACTIONS								
SUBSTANCES	PAS	AB2.5	AB0.5	PAS + AB 2.5	AH + PAS	AH+ AB2.5	AY2.5+ AB0.5	BF	NS
NEG	++	-	-	++/-	++	-	-/-	++	++
NEG/CAG	++	+	-	++/+	++	+	+/-	++	++
SAG/NEG	+	++	++	+/++	+	++	++/++	+	+

PAS = Periodic acid-Schiff; AB 2.5 = alcian blue pH 2.5; AB 0.5 = alcian blue pH 0.5 ; PAS+AB 2.5 = PAS followed by AB pH 2.5; HA+PAS = acid hydrolysis followed by PAS; HA+AB 2.5 = acid hydrolysis followed of AB pH 2.5; AY 2.5+AB 0.5 = alcian yellow pH 2.5 followed of AB pH 0.5; BF = mercuric bromofenol blue; NS = ninhidrin-Schiff. NEG = neutral glycoproteins; NEG/CAG = predominance of neutral glycoproteins over carboxilated glycosaminoglycans; SAG/NEG = predominance of sulphated glycosaminoglycans over neutral glycoproteins; ++ strong positive reaction; + positive reaction; - negative reaction.

The results of the present study indicated morphological and chemical variation in the oocyte surface of teleosts which may be related to the different reproductive strategies of the species. In addition, knowledge of the characteristics of the oocytes after spawning and fertilization could

provide an important basis for our understanding of the interactions of the eggs with their surrouding environment, as well as useful parameters for phylogenetic studies of teleost fish.

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