

Gamete interactions in teleost fish: the egg envelope. Basic studies and perspectives as environmental biomonitor

Nibia Berois, María J. Arezo, Nicolás G. Papa

Sección Biología Celular, Instituto de Biología. Facultad de Ciencias. Universidad de la República. Montevideo, República Oriental del Uruguay.

ABSTRACT

The current knowledge about teleost fish egg envelope is summarized. The paper analyzes the organization and deposition process of the protein composition and genes involved in the synthesis of teleost fish egg envelopes and their role in gamete interaction during fertilization. Pelagic and demersal species that our research group is working with are especially considered. The vertebrate ZP family of proteins, the evolution and relationship among the different genes and their expression are taken into account. We consider fish envelope as a possible biomonitor for ecological contaminants. The biotechnological applications for aquaculture and genomic and post-genomic approaches are auspicious.

Key words: fish egg envelope, ZP genes.

INTRODUCTION

Teleost fertilization exhibits special distinct characteristics from those of other vertebrates and even other fish groups. The oocyte is covered by the vitelline envelope (eggshell, zona pellucida, zona radiata or chorion) as in all vertebrate species, but sperm interaction only occurs at the micropyle level. This special teleostean oocyte structure, located at the animal pole, serves as the only entry site for the male gamete and is formed during the egg envelope deposition. It shows morphological differences among species with taxonomic value (Guraya, 1986). In general, it is a funnel-shaped channel whose inner diameter is similar to the sperm head of the species (Hart, 1990). As teleost sperm lacks acrosome, during fertilization there is no acrosome reaction (Guraya, 1986; Hart, 1990). The sperm fuses to the oocyte cell membrane underlying the micropyle and special structures there, such as microvilli or folds, have been reported in some fish (Brummett and Dumont, 1979; Dumont and Brummett, 1980; Hart and Donovan, 1983). In the surface of both gametes lies the key to species-specificity. Consequently, the ultrastructure of egg envelope, micropyle and sperm head are features that considered in phylogenetic analyses and pre-zygotic isolation among closely related species.

In contrast to mammals, where the zona pellucida is involved in sperm binding, in teleosts a single sperm reaches the egg membrane through the micropyle. After the egg is activated by the sperm, the micropyle closes, preventing polyspermy. The vitelline envelope possesses many other functions, such as protection of the growing oocyte and the developing embryo and uptake of nutrients and other molecules during oogenesis, guidance of the sperm to the micropyle (Dumont and Brummet, 1980), as well as possessing bactericidal and fungicidal properties (Kudo and Inoue, 1989; Kudo, 2000).

According to the behavior after ovulation, fish generate two kinds of eggs: a) pelagic highly hydrated eggs that float in seawater; b) demersal non-buoyant eggs, generally in freshwater, which attach to plants or substrate. The oocyte envelope structure is related to environmental conditions. Generally, fish that spawn pelagic eggs are non-adhesive and smooth, with poorly ornamented envelopes, whereas those that place their eggs over plants or on the bottom have sticky and ornamented eggs (Rizzo et al., 2002).

The oocyte envelope appears to be a sensitive biomarker to adverse pollutants. Changes in ZP synthesis and organization of egg envelopes have been reported in both kind of oocyte envelope (pelagic and demersal) as a consequence of aquatic contaminants (especially xenoestrogens) (Arukwe et al., 1997; Arukwe and Goksøyr, 2003).

Framework and objective of the present paper

Our research group is interested in different aspects of reproductive biology of teleost fish both in fishing resources and laboratory models. Among the former is the whitemouth croaker (*Micropogonias furnieri*) (Perciformes; Sciaenidae), a migratory euryhaline teleost fish living in the Atlantic Ocean from Northern Venezuela (20°N) to the Gulf of St. Mathias (41°S) (FAO, 1974; Chao, 1978). In the Rio de la Plata, this species is the most abundant sciaenid and constitutes an important resource for the Uruguayan and Argentinean fisheries (Ehrhardt et al., 1977; Arena, 1990). Histological and cellular studies show that the population inhabiting the Rio de la Plata is a multiple spawner that reproduces on the Uruguayan coast between October and February (Berois et al., 2004).

Considering laboratory models, the research group has focused on annual fish. Although zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) are the most often used teleost models in developmental biology, species of annual fish (Cyprinodontiformes; Aplocheiloidei) are excellent for comparative analyses. They have a short lifespan and are exposed to an extremely variable environment. They

Corresponding author: Nibia Berois, Sección Biología Celular, Facultad de Ciencias, Iguá 4225 (CP 11400), Montevideo, Uruguay. E-mail: berois@fcien.edu.uy Tel. 598-25258618 ext. 145 Fax 598-25258617.

inhabit temporary ponds from Africa and South America that undergo drying during summer. This condition leads to the death of the entire young and adult populations. The survival of the species is entirely dependent on embryos that remain buried in the bottom mud and hatch in the next rainy season (Wourms, 1964, 1967). In contrast to other teleost fish, annual exhibit a unique developmental pattern (Myers, 1952). Epiboly is temporally and spatially detached from organogenesis and embryos undergo one or more reversible arrest (diapauses) at three different stages: diapause I at dispersed stage during epiboly; diapause II at middle somite stage and diapause III at pre-hatching (Wourms, 1972 a,b,c) (Arezo et al., 2005).These developmental adaptations are closely related to the life cycle.

As a tribute to the scientific contribution of Dr. Claudio Barros, the aim of the present paper is to review the information about fish egg envelopes referring to pelagic and demersal oocytes, mainly from the research accomplished with or since his collaboration. Dr. Barros enjoyed teaching and encouraged his students to face the challenges that appeared with each step of their work as a new opportunity to advance and grow. His words and witty remarks will always be with us.

ORGANIZATION AND DEPOSITING PROCESS OF TELEOST EGG ENVELOPE

The fish oocyte develops within the ovarian follicle, which is a structure organized in the early stages of oogenesis before uptake of the yolk. It is formed by the oocyte surrounded by the granulose or follicular cells, the theca cells and a basement lamina as the outer limit of the follicle (Wallace and Selman, 1981). Teleost oogenesis can be divided into five stages: pre-vitellogenic, lipid-yolk, protein-yolk, fully-grown and mature oocyte (Berois et al., 2004). The formation of the envelope, through depositing of successive layers, occurs during the lipid-yolk to fully-grown stages, at the same time as vitellogenesis. During this time the oocyte cell membrane projects microvilli toward the granulose cell of the follicle. Other microvilli from these cells are observed afterwards. The synthesis of the egg envelope starts from the base of the oocyte microvilli and reaches the maximal width and complexity at the fully-grown stage when it acquires a trilaminar structure (Wallace and Selman, 1981; Berois et al., 2007).

At the ultrastructural level (transmission electron microscopy, TEM) the oocyte envelope of the fully grown oocyte (Fig 1 A) shows an outermost fine granular layer, a middle and homogeneous high electron dense layer and the innermost and wider layer, known as zona radiata interna, with a helicoidal-fibrilar aspect (Fig. 1 C). There are numerous channels cross the entire width of the chorion in a regular pattern, each occupied by oocyte and follicle cell microvilli in close apposition (Fig. 1D,E) (Dumont and Brummett, 1980; Hart and Donovan, 1983; Guraya, 1986; Cotelli et al., 1988; Arezo et al., 2007; Berois et al., 2007; Modig et al., 2008). In mature and ovulated pelagic oocytes (Fig. 2 A), the egg envelope became thinner and loses its trilaminar and radial striations, showing parallel bands of different electron density (Fig 2 B). Channels and microvilli are no longer identifiable (Berois et al., 2007; Modig et al., 2007). After fertilization, a perivitelline space is formed and the egg envelope hardens and becomes the fertilization envelope (Guraya, 1986; Oppen-Berntsen et al., 1990).

Comparing the oocyte envelope surface of two fish species, the whitemouth croaker, *Micropogonias furnieri* (pelagic spawner) and the *Austrolebias charrua* (demersal spawner), we can identify by scanning electron microscopy (SEM) that: a) the whitemouth croaker oocyte envelope is smooth, with a regular pattern of empty pores that represent the external openings of oocyte envelope channels. The surface pattern is consistent with the condition of neutrally buoyant pelagic eggs described for the species (Fig. 3 A) (Isaac-Nahum, 1988; Berois et al., 2007); b) the *Austrolebias* egg envelope shows a rough and sticky surface ornamented by hair-like cone-shaped filaments in accordance to its demersal condition (Fig. 3 B) (Arezo et al., 2007).

PROTEIN COMPOSITION

Knowledge about the biochemistry, development and functions of the vertebrate vitelline envelope has expanded as a consequence of the advance in molecular approaches. Alignments of teleost chorion proteins with vitelline envelope proteins from other vertebrate species or zona pellucida proteins (ZPs) indicate that these molecules have been highly conserved during evolution. They show high homology with vitelline envelope proteins of amphibians, birds and mammals, except for the sperm-recognition sequence, which has only been found in mammals. This general similarity suggests that they form a unique group of vertebrate proteins, which function as structural components of the vitelline envelope and are highly conserved (Hyllner et al., 2001; Listcher and Wassarman, 2007)

Most ZP proteins have an N-terminal signal peptide, a conserved ZP domain, a furin cleavage site and a hydrophobic C-terminal. This C-terminal is formed by a transmembrane domain (TMD) and a short cytoplasmic tail. The ZP domain contains about 260 amino acids with 10–12 cystein residues. It is also a common feature in many extracellular matrix proteins (Bork and Sander, 1992).

In spite of the ambiguous nomenclature for egg envelope proteins (choriogenin proteins, ChP, zona pellucida proteins, ZP, zona radiata proteins, ZRP, or vitelline envelope proteins, VEP) four groups of ZPs, all containing the conserved ZP domain signature, have been identified in vertebrates: ZPA, ZPB, ZPC and ZPX. While ZPB and ZPC were found in all vertebrates, ZPX was identified in frogs, chicken and fish and ZPA has not been found so far in fish (Spargo and Hope, 2003).

Comparison of the envelope protein profile among teleost species shows that most share a similar pattern of protein and glycoprotein bands, regardless of their habitats (freshwater or seawater). Major oocyte envelope constituents have molecular masses ranging between 40 and 130 kDa (Hamazaki et al., 1987a; Cotelli et al., 1988; Begovac and Wallace, 1989; Brivio et al., 1991; Oppen-Berntsen et al., 1990; Hyllner et al., 1991; Scapigliati et al., 1994; Bonsignorio et al., 1996; Berois et al., 2007). The Austrolebias egg envelope protein profile analyzed by SDS-PAGE shows seven bands in mature oocytes: four major bands with molecular weights of about 45, 97, 120 and 160 kDa. Bands of 97 and 120 kDa are glycoproteins. More detailed analyzes are currently in progress. The whitemouth croaker protein profile reveals five bands for fully grown oocytes: two major bands with molecular weights of about 47 and 57 kDa, and three minor bands of about 67, 110 and 130 kDa. Bands of 47, 57 and 130 kDa are glycoproteins. In mature ovulated oocytes these



Figure 1: Fully grown oocyte of the whitemouth croacker and ultrastructural details of its chorion. A) Light microscopy of the oocyte (square); histological section. B) High magnification of the chorion showing its striation. C) Ultrastructural aspect of the envelope at this stage. D) Detail of the outer zone showing the apposition between both kinds of microvilli inside the channels. E) Pattern of channels crossing the egg envelope (cross section).

N: nucleus; LY: lipid yolk; PY: protein yolk; C: chorion; F: follicular cell; O: oocyte cytoplasm; 1,2 and 3: chorion zones; M: channels with microvilli; FM: follicular microvillus; OM: oocyte microvillus. A, B: PAS-hematoxyline stain; C, D, E: TEM microscopy.



Figure 2: Mature ovulated oocyte of the whitemouth croacker and ultrastructural details of its chorion. A) Light microscopy of the oocyte. B) Ultrastructural aspect (TEM) of the envelope at this stage showing parallel bands of different electron density. N: nucleus; LD: lipid drop; C: chorion; O: oocyte cytoplasm.



Figure 3: Animal pole of oocytes belonging to the fish species, whitemouth croaker and annual fish Austrolebias (SEM). Note the differences of the chorion surfaces: smooth in the case of pelagic egg (A) and highly ornamented with filaments in the demersal egg (B). Squares show high magnifications of the micropyles in both species. Arrow: micropyle at low magnification; Mi: micropyle:

glycoproteins are the only proteins that remain in the oocyte surface suggesting that the morphological changes observed following maturation might involve changes in the oocyte envelope protein composition. Following 2-DE electrophoresis 14 major polypeptides and some minor spots were detected. MALDI-TOF-MS analyses have shown no matching entries in the database (Berois et al., 2007). One limitation is the lack of genome sequencing or expressed sequence tag (EST) projects for this species. Proteomic studies currently underway could play an important role in evaluating changes in the expression levels of whitemouth croaker oocyte ZP proteins in response to environmental factors and determining whether an accurate and sensitive biomarker can be selected.

ZPS SITES OF SYNTHESIS. EGG ENVELOPE GENES

Regarding ZP synthesis, the earlier ultrastructural data on medaka gave support to the idea that this envelope was secreted by the oocyte itself (Tesoriero 1977, 1978). In the 1980s data was collected about the presence of an immunoreactive substance with antibodies against chorion proteins in the liver and blood of spawning medaka females (Hamazaki et al., 1985, 1987, 1989). Similar results were found for rainbow trout (Hyllner and Haux, 1992). A later study that characterized the three vitelline envelope proteins (VEP) cDNA of this species showed that VEP mRNAs were transcribed in the liver under estrogen regulation in both sexes (Hyllner et al., 2001). There was assumed a general agreement that the main proteins of the teleost chorion (specially zone 3) were synthesized in the hepatocyte under regulation of estradiol-17 β (Hyllner et al., 1991; Oppen-Berntsen et al., 1992 a and b; Hyllner and Haux, 1992; Hyllner et al., 1994; Hyllner et al., 2001). In contrast to these findings, the synthesis of chorion proteins in pipefish (Begovac and Wallace, 1989), in the carp (Chang et al., 1996, 1997) and in zebrafish (Wang and Gong, 1999; Mold et al., 2001) was reported in the ovary. Therefore the sites of ZP synthesis differ among species, but the complexity is even greater because this condition affects different ZPs in the same fish. Examples are the dual expression of rainbow trout ZPC and gilthead sea bream ZPBa and ZPX in both liver and ovary (Modig et al., 2006). Liver expression appears to be regulated by estrogen

while ovarian expression can either be under estrogenic control or independent of it (e.g. zebrafish, Liu et al., 2006).

Analyses of teleost fish ZP gene sequences have demonstrated that this group of vertebrates includes two classes of genes that encode ZP proteins further distinguished by their expression in the liver, in the ovary or both, depending on the species. The ancestral condition for vertebrates is ovary expression (Conner and Hughes, 2003). It has been hypothesized that teleostean fish experience a duplication event followed by a switch to hepatic expression of one of the paralogue genes and that the acquisition of dual sites of synthesis is the result of an ancient polyploidization event plus additional species-specific gene amplifications (Conner and Hughes, 2003). Probably the first step in ZP gene evolution was a gene duplication that might have originated both the ancestral ZPC gene and the precursor of the ZPA, ZPB and ZPX subfamilies. Because no ZPA gene has been found in fish, it has been assumed that it has been lost, probably through deletion events (Spargo and Hope, 2003). On the other hand the ZPX genes are present in fish, amphibians and birds, but not in mammals. It has been suggested that they have been lost through evolutionary mutations (Smith et al., 2005).

In the context of a comparative research project we are characterizing the expression of ZP genes in the whitemouth croaker and in annual fish by means of RT-PCR. With respect to the first species, we have isolated a first cDNA, (czp10 560 bp) expressed in the liver. The deduced amino acid sequence presented 81 % of identity with choriogenin L from Sparus aurata, another Perciforme (D'Alessandro et al., 2007). In annual fish (*A. charrua*) two cDNA, *achzpL* and *achzpH* (160 bp and 670 bp respectively) have been identified, both expressed in the liver. The deduced amino acid sequence in both fragments showed identity values between 65 to 80 % with ZPs from species belonging to diverse orders (*Fundulus heteroclitus, Oryzias latipes, Sparus aurata* and *Danio rerio*) (Ms in preparation)

BIOMONITOR VALUE

Teleosts are increasingly important indicators of environmental health. Considerable information exists about the impact

that pollutants may cause on fish reproduction: on sex differentiation, gonad morphology and rates of gametogenesis and sex phenotypes (reviewed by Devlin and Nagahama, 2002; Arukwe and GoksØyr, 2003).

The fish oogenesis process has been shown to be a sensitive biomarker to environmental pollutants and endocrinedisrupting chemicals, which induce noticeable changes in the synthesis of fish oocyte proteins, including envelope proteins and vitellogenin (Vtg) (Arukwe et al., 2000; Arukwe and Goksøyr, 2003). The induction of vitellogenin is mainly used as a biomarker but it has been demonstrated that the expression of ZPs precedes that of Vtg (Arukwe et al., 2000). Furthermore, to use ZP proteins as biomonitor appear to have a higher potential than vitellogenin because, while subtle changes in VTg would not be a threat to the survival of the offspring, small changes in ZP expression or synthesis might cause differences in thickness and strength of the egg envelope. The consequences of these alterations could affect fertilization and polyspermy prevention and protection of the embryo during development (Arukwe et al., 1997; Arukwe and Goksøyr, 2003).

Some parameters related to regulation of ZP synthesis are involved in the selection of these proteins as accurate biomonitors. E_2 appears as the main regulator in most of the studied species (Modig et al., 2007) but it has been shown that other hormones, such as cortisol and androgens or physiological conditions, such as stress, affect estrogendependent regulation of ZP proteins in some species (Berg et al., 2004). Consequently, when proposing these proteins as biomonitors it is necessary to have first elucidated the sites and basal level of ZP expression for the species under research.

In order to provide a baseline to the potential effect of contaminants on an important economic fishery resource of the Rio de la Plata, our group analyzed the detailed structure, depositing dynamic and chemical composition of the oocyte envelope in the whitemouth croaker, *M. furnieri*. The assays performed on samples from three time periods were consistent and analogous in spite of the natural variable conditions of the estuary, which were emphasized under the influence of El Niño (1997– 98). This information provides a useful baseline to assess future detrimental effects of pollutants on the oogenesis of this important renewable fishing resource (Berois et al., 2007). Currently we are working in order to establish the expression pattern of the ZP genes and its regulation in this species.

A comparable approach is being developed for annual fish. The species under research, *A. charrua*, inhabits a Uruguayan zone declared a Biosphere Reserve Area (UNESCO, 1976). Sex differentiation, determination of sexual strategy, and cellular aspects of gametogenesis of this annual fish have been established by our group (Arezo et al., 2007). The growing information about different topics of reproduction of *A. charrua* makes it a useful taxon to monitor contamination effects in a protected area.

CONCLUSIONS AND PERSPECTIVES

Current knowledge about fish egg envelope was summarized in the present paper. The information about organization and depositing at the ultraestructural level, protein composition, the multiple genes involved in the synthesis of this structure and its role in fertilization were analyzed comparatively. Our contribution is focused on increasing basic and applied knowledge about gamete biology in two phylogenetically distant fish species: the perciforme *Micropogonias furnieri* and the cyprinodontiforme *Austrolebias charrua*. In addition, fish chorion could be a feasible biomonitor of the aquatic environment. This condition is more relevant in species that are diminishing by pressure on the habitat or overfishing

Finally, studies of teleost fish reproduction support aquaculture in different areas of concern, one of these being the production of large number of healthy eggs that maintain fish culture at high levels. Endocrine regulation of spawning and new protocol development to rear embryos, fries and juveniles has been the main focus of many applied investigations. Nevertheless, there are many gaps of information about molecular and physiological mechanisms underlying oocyte development and maturation.

As teleosts represent the most diverse vertebrate group, to have access to further genome sequencing data and the application of approaches such as transcriptomic and proteomic in more fish clades will enhance our understanding of fish oocyte as a whole and help in the near future to develop useful biotechnological applications in aquaculture.

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