Oogenesis in the bluefin tuna, *Thunnus thynnus* L.: A histological and histochemical study

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Summary. Histology and histochemistry are useful tools to study reproductive mechanisms in fish and they have been applied in this study. In the bluefin tuna, *Thunnus thynnus* L., oocyte development can be divided into 4 principal phases based on the morphological features of developing oocytes and follicles. The primary growth phase includes oogonia and basophilic or previtellogenic oocytes classified as chromatin-nucleolus and perinucleolus stages. The secondary growth phase is represented by vitellogenic oocytes at early (lipid globule and yolk granule 1), mid (yolk granule 2) and late (yolk granule 3) vitellogenesis stages. The maturation phase involves postvitellogenic oocytes undergoing maturation process. During the spawning period, both postovulatory follicles, which indicate spawning, and atretic follicles can be distinguished in the ovary.

Carbohydrates, lipids, proteins and specially those rich in tyrosine, tryptophan, cystine, arginine, lysine and cysteine, as well phospholipids and/or glycolipids and neutral glycoproteins were detected in yolk granules. Moreover, affinity for different lectins (ConA, WGA, DBA and UEA) was detected in vitellogenic oocytes (yolk granules, cortical alveoli, follicular layer and zona radiata), indicating the presence of glycoconjugates with different sugar residues (Mannose- Man- and/or Glucose -Glc-; N-acetyl-D-glucosamine- GlcNAc- and/or sialic acid- NANA-; N-acetyl-D-galactosamine- GalNAc-; L-Fucose -Fuc-). Histochemical techniques also demonstrated the presence of neutral lipids in globules (vacuoles in paraffin sections) and neutral and carboxylated mucosubstances in cortical alveoli. By using anti-vitellogenin (VTG) serum, immunohisto-chemical positive results were demonstrated in yolk granules, granular cytoplasm and follicular cells of vitellogenic oocytes. Calcium was also detected in yolk granules and weakly in follicular envelope.

In females, the gonadosomatic index (GSI) increased progressively from May, during early vitellogenesis, until June during mid and late vitellogenesis, where the highest values were reached. Subsequently, throughout the maturation-spawning phases (July), GSI decreased progressively reaching the minimal values during recovering-resting period (October).

Key words: Histology, Carbohydrates, Lipids, Proteins, Glycoconjugates, Vitellogenin, Calcium, Ooogenesis, *Thunnus thynnus*

Introduction

During the reproductive cycle of teleost fish, four principal phases can be distinguished: 1) primary growth phase or previtellogenesis (oogonia and basophilic previtellogenic oocytes); 2) vitellogenesis phase (yolk synthesis); 3) maturation phase (migration of the nucleus and its breakdown); and 4) spawning phase (postovulatory follicles and atretic oocytes). During oogenesis, which involves the complex transformations carried out by the oogonia up until the completion of the second meiotic division and their conversion into ovules (Grau et al., 1996), important cytoplasmic and nuclear changes occur (Wallace, 1978; Selman and Wallace, 1982, 1983, 1989, Selman et al., 1988, 1993; Mayer et al., 1988; González de Canales et al., 1992; Grau, 1992) due, essentially, to the synthesis of yolk. During secondary growth phase or vitellogenesis, oestrogens stimulate the synthesis of hepatic proteins (vitellogenin - VTG-), which are incorporated into oocytes (Selman and Wallace, 1982) and the yolk is synthesized.

The chemical composition of the yolk and the distribution of organic reserves during the reproductive cycle of teleosts have been studied by several authors (Khoo, 1979; Gutiérrez et al., 1985; Mayer et al., 1988; González de Canales et al., 1992; Sarasquete et al., 1993a-c; Grau et al., 1996; Lahnsteiner and Patzner, 1999). VTG analysis and immunohistochemical results were also studied during the reproductive cycle of some fish species (Hamazaki et al., 1985; Sarasquete et al.,

1993b; Murata et al., 1994; Fujita et al., 1998; Susca et al., 2001).

Tuna is one of the most important species in the fishery industry. Characteristics that contribute to their high market price include their large size, reaching more than 3 m in total length, their body weight, often up to 500 Kg, and their unique taste. Tuna are subject to intense fishing pressure which has raised concerns for stock survival. Their decreased abundance and high commercial value make them potential candidates for aquaculture. However, fundamental knowledge of their reproductive biology is very limited (Mourente et al., 2001; Susca et al., 2001).

The bluefin, *Thunnus thynnus* L. is the largest tuna and is distributed worldwide. The two main reproductive areas include: 1) the Mediterranean Sea within two differentiated zones: around Baleares islands and in the South of the Tirrene Sea, between Sicilia and Sardinia. In this area spawning occurs in June and July; 2) the second area is the Gulf of Mexico and South of Florida, where spawning occurs in April and May. *Thunnus thynnus* thynnus is the Atlantic tuna, and *Thunnus thynnus* orientalis is the Pacific tuna subspecies, respectively. For spawning, African and European Atlantic tuna specimens migrate to the Mediterranean Sea (Davila, 1985). For Eastern Atlantic bluefin *T. thynnus*, the spawn occurs in the Mediterranean Sea, between May and July (Rodríguez-Roda, 1964, 1967; Cardenas et al., 2001). Recently, Susca et al. (2001) suggested that *T. thynnus* from the central Mediterranean spawn after the middle of June. For the Western Atlantic specimens, Baglin (1982) indicated heaviest spawning in May.

Some reproductive aspects of the bluefin, *T. thynnus* specimens captured in Almadraba of Barbate (Cádiz, SW-Spain) were reported (Rodríguez-Roda, 1957, 1964, 1967). According to this author, first maturity (3-year-old specimens) occurs at 98 cm for females and 105 cm for males. Fecundity is between 5 and 30 million eggs/female (5- and 12-year-old specimens) and tuna eggs have a diameter of 0.85 and 1.26 mm. Hirota and Morita (1976) provided a brief histological description of bluefin gonads from males and females captured in July. The plurimodal frequency distribution of the yellowfin, *Thunnus albacores* oocytes and intra-ovarian ova diameters strongly suggest the spawning of several batches of eggs in the same spawning season (Albaret, 1977). The frequency of ovaries containing postovulatory follicles has been used to estimate spawning frequency in yellowfin (Schaefer, 1996). An interesting study of the sexual maturity of bluefin tuna females in relation to purification, partial characterization of vitellogenin and hormonal plasma levels was studied by Susca et al. (2001). Recently, Mourente et al. (2001) studied the content of lipids and fatty acids composition in different tissues during the reproductive cycle of bluefin tuna females.

The study of gonad development in teleost fish by means of histological tools, leads to the acquisition of basic knowledge of their reproductive biology and has practical applications for the management of their reproductive cycle in captivity. Moreover, cytochemical/immunohistochemical approaches can indicate the synthesis and mobilization of macromolecules between several reproductive organs, i.e. gonads, liver and blood. The present study investigates the histological development of bluefin tuna oocytes, as well as the presence and distribution of carbohydrates, proteins, glycoconjugates, lipids, calcium and VTG in bluefin tuna, *Thunnus thynnus* ovaries, during different phases of the reproductive cycle, in both the Eastern Atlantic and Mediterranean coasts (Barbate-Cádiz and Murcia, Spain).

Materials and methods

Gonads and liver of adult bluefin tuna, *Thunnus thynnus* L. (150-200 Kg) caught during May (n=18), June (n=12) and October (n=9) in Almadraba of Barbate (Cádiz, SW Spain) and in July (n=5) in Mediterranean coasts (Murcia, Spain) were fixed in buffered-formaldehyde 0.1 M pH 7.2 for 24 hours. They were then dehydrated through graded alcohols, cleared in xylene and embedded in paraffin and glycol-metacrylate resin (Historesin embedding kit, Leica, Germany). Serial sections were cut in coronal and sagittal planes and mounted on gelatinized slides. To identify lipid inclusions, some samples were preserved in 70% ethanol, treated with 1% osmium tetroxide 2.5%-potassium dicromate 2.5% during 8 h, washed in running water, dehydrated and embedded in paraffin according to Luna (1968). Haematoxylin-eosin, Haematoxylin-VOF'Gutiérrez (light green-orange G-acid fuchsin) and Toluidine Blue morphological techniques were performed according to Gutiérrez (1990).

The cytochemical techniques for carbohydrates were: periodic acid-Schiff (PAS), diastase-PAS and Alcian Blue pH 0.5, 1 and 2.5. Controls included acetylation, saponification, chlorhydric hydrolysis, neuraminidase-type V from *Clostridium perfringens* and bacterial hyaluronidase from *Streptomices hyalurolyticus*. Glycoproteins (lectins), general proteins (Bromophenol Blue), proteins rich in lysine (Ninhydrin-Schiff), tyrosine (Hg-sulphate-sulfuric acid-sodium nitrate), trytophan (p-dimethylaminobenzaldehyde) and proteins rich in arginine (1,2 napthoquinone-4-sulphonic acid salt sodium) were studied. -SH (cysteine) and -S-S- (cystine) groups were evidenced by means of Ferric ferricyanide-Fe III and Tioglycollate-reduction methods. Histochemical reactions for lipids (Sudan Black B - SBB- and Oil Red O -ORO- for general and neutral lipids; Nile Blue and Luxol Blue for phospholipids and Chloramine-T-performic acid-dinitrophenylhydrazine-PAS for glycolipids) were carried out. Lipid techniques were applied to sections directly processed in a cryostat (Cryocut E) and to paraffin sections. Cold acetone was used to remove neutral lipids; chloroform/methanol (1/1) removed all lipids, and phospholipids were extracted

with pyridin. Calcium was studied with the Alizarin Red method. All techniques and reactions used in this study are described by Martoja and Martoja-Pierson (1970), Pearse (1985) and Bancroft and Stevens (1990).

For the analysis of glycoconjugates, sections were treated with 0.3% hydrogen peroxide for 10 minutes (to inhibit endogenous peroxidase) in Tris buffered saline (TBS) at pH 7.2. The sections were incubated for 30 minutes at room temperature in the presence of the following horseradish peroxidase-conjugated lectins (HRP-lectin conjugated) dissolved in TBS (20 µm/ml): ConA (Mannose- Man- and/or Glucose- Glc-), WGA (N-acetyl-D-glucosamine -GlcNAc- and/or sialic acid-NANA-), DBA (N-acetyl-D-galactosamine -GalNAc-) and UEA-I (L-Fucose -Fuc-). After three washes in TBS, peroxidase activity was visualized with TBS containing 0.05% 3,3'diaminobenzidine tetrahydrochloride and 0.015% hydrogen peroxide. Sections were washed in running tap water for 10 minutes, dehydrated, cleared and mounted in Eukitt. Controls were: omission of the respective lectin; substitution of lectin-HRP conjugates by TBS and treatments with different enzymes (Neuraminidase Type V -0.9 U/mg Prot-; ß-Galactosidase Grade VI -400 U/mg Prot-; α-Mannosidase Type III -20 U/mg Prot -and ß-N-Acetylglucosamine -0.25 U/mg Prot-: L-Fucosidase -20U/mg Prot). Lectins and enzymes were purchased from Sigma Chemical Co. St Louis, MO, USA.

Immunocytochemical staining was performed in gonads and liver paraffin sections using a streptavidin-biotin-peroxidase complex method. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide in Coons buffer (0.01M Veronal, 0.15M NaCl) with 0.1% Triton X-100 (CBT) for 30 minutes at room temperature. Before immunostaining, sections were transferred for 5 minutes to CBT and saturated with 0.5% casein for 30 min. Sections were incubated overnight in a moist chamber at room temperature with a rabbit polyclonal antibody raised against seabass vitellogenin (Mañanos et al., 1994) diluted 1:1000 in CBT with 0.5% casein. After several washes in CBT, sections were incubated for 1 hour at room temperature with biotinylated anti rabbit-IgG diluted 1:1000 in CBT-0.5% casein. After washing in CBT, sections were incubated for 1 hour at room temperature with streptavidin-peroxidase complex diluted 1:1000 in CBT. Finally, sections were washed with CBT followed by Tris-HCl (0.05M, pH 7.4) and peroxidase activity was observed in Tris-HCl 0.05 M, pH 7.6 containing 0.04% 4-chloro-1-naphtol and 0.005% hydrogen peroxide). Some sections were incubated with normal fish serum instead of the primary antibody.

Oocyte size and frequency were determined on histological slides using a Quantimet (Leica, Cambridge, UK) image analyser, by measuring the maximun and minimum oocyte-diameters. Gonadosomatic index was calculated according to: Gonad weight (Kg)/(Total weight-Gonadal weight) x 100

Results

Histology

In the bluefin tuna, *Thunnus thynnus* L, oocyte development could be divided into 4 phases based on the morphological features of developing oocytes and follicles. The primary growth phase or previtellogenesis included oogonia and previtellogenic oocytes. Two types of these basophilic oocytes, at chromatin nucleolus and perinucleolus stages, could be differentiated. The secondary growth phase included oocytes at early, mid and late vitellogenesis stages. The maturation phase involved postvitellogenic oocytes undergoing maturation. During the spawning period both postovulatory follicles, indicative that spawning has already begun, and atretic oocytes could be distinguished in the ovary.

Some type of oocytes during different stages or phases of the bluefin oogenesis, as well some histochemical results are shown in Figs.1 to 5.

Oogonia were small rounded cells (8-14 µm diameter) with a pale, voluminous and spherical nucleus and a prominent nucleolus (Fig. 1A,B). The cytoplasm presented very weak basophilia. No perinuclear chromatin was observed in oogonia, which made them easy to distinguish from cells of the follicular layer (granulosa cells). Oogonia appeared isolated or forming clusters or nests in the vicinity of either vitellogenic oocytes or post-ovulatory follicles. Oogonia were present throughout the annual cycle, but more easily observed in recovering or resting ovaries.

Primary growth phase or previtellogenic oocytes

These basophilic oocytes (Figs. 1A,B, 2A,B, 3A,B) measured 20 and 100 µm in diameter. They were present in all ovaries analysed and especially in May (72%) and October (90%).

During the chromatin-nucleolus stage (containing filliform and basophilic structures related with condensated chromosomes), oocytes (20-40 µm) showed a strongly basophilic cytoplasm. The nucleus was large with strands of chromatin and a single big nucleolus. The Balbiani's vitelline body appeared at this stage and it comprised 2 parts: the idiosome (non-basophilic component of Balbiani's vitelline body) and the pallial substance (basophilic component). With further development, the idiosome became apparent in a juxtanuclear position. This stage was relatively brief and it was rarely found, especially in maturating ovaries. By the transition to the perinucleolus stage, the idiosome became more conspicuous or remained as a diffuse structure in the juxtanuclear position; the more basophilic pallial substance could be seen in its vicinity.

Oocytes at the perinucleolus stage (various nucleoli close to nuclear membrane) measured 45 and 100 µm in diameter and they had a homogeneous slightly basophilic cytoplasm. The nucleus was central and
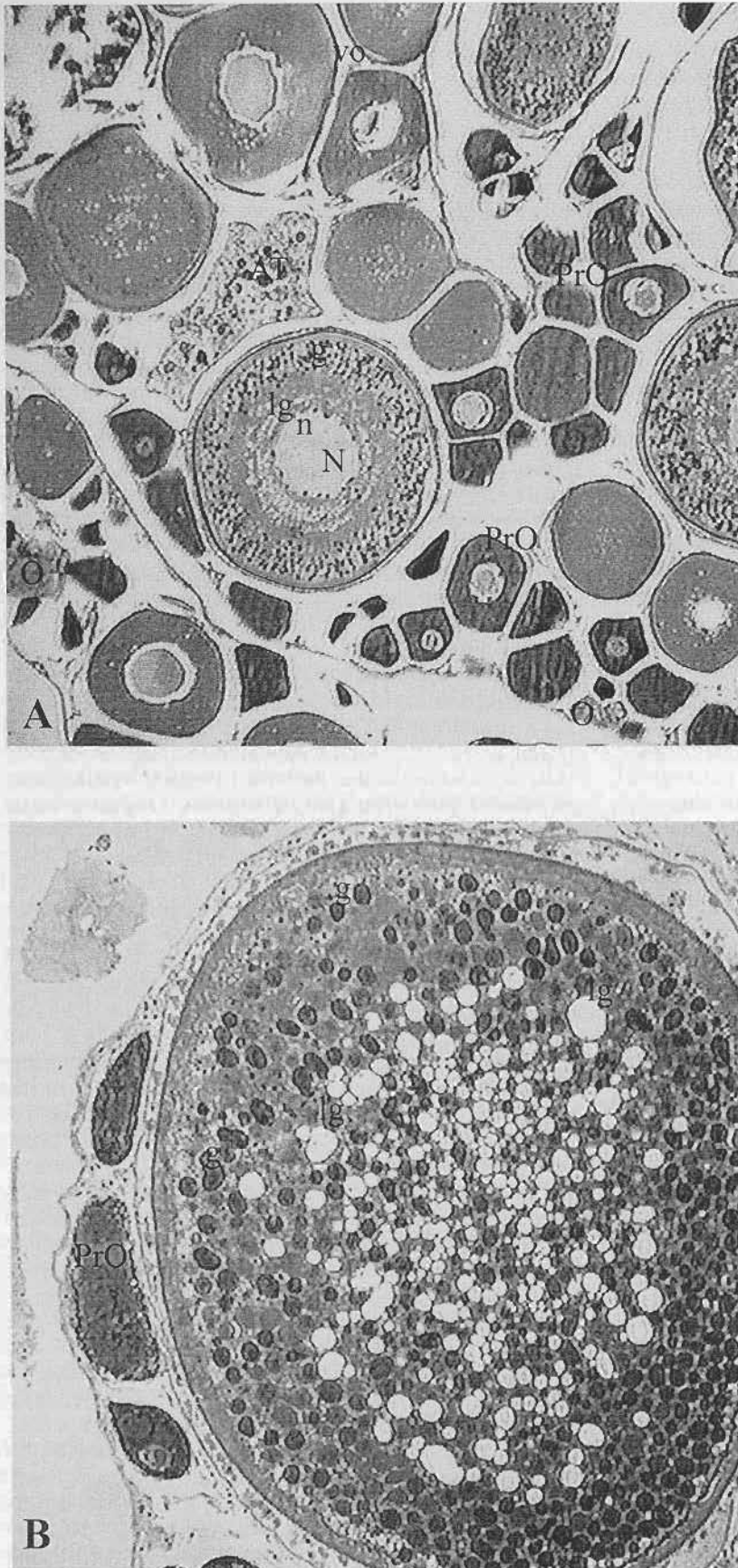
Fig. 1. A. Ovarian section during vitellogenesis phases (June, Barbate-Cádiz, Spain), showing oogonia, previtellogenic and vitellogenic oocytes. Haematoxylin-eosin. x 40. **B.** Semithin ovarian section showing an oocyte during late vitellogenic phase. Some previtellogenic oocytes are also observed. Toluidine Blue. x 250. AT: atretic oocyte; f: follicular layer; g: yolk granules (glycolipoproteins); lg: lipid globules (neutral lipids dissolved -vacuoles-); N: nucleus; n: nucleolus; O: oogonia; PrO: previtellogenic oocyte; r: zona radiata; VO: vitellogenic oocytes.

Fig. 2. A. Postvitellogenic oocyte starting the maturation phase (July, Murcia, Spain). Migration of the nucleus and coalescence of lipid globules are observed. Haematoxylin-eosin. x 100. **B.** Ovarian histological section during spawning phase (July, Murcia, Spain) showing postovulatory follicles and some previtellogenic (most at perinucleolus stage) and residual vitellogenic oocyte. Haematoxylin-eosin. x 40. f: follicular layer; g: yolk granules; lg: lipid globules; N: nucleus; PrO: previtellogenic oocyte; PF: postovulatory follicles; r: zona radiata; VO: vitellogenic oocytes.
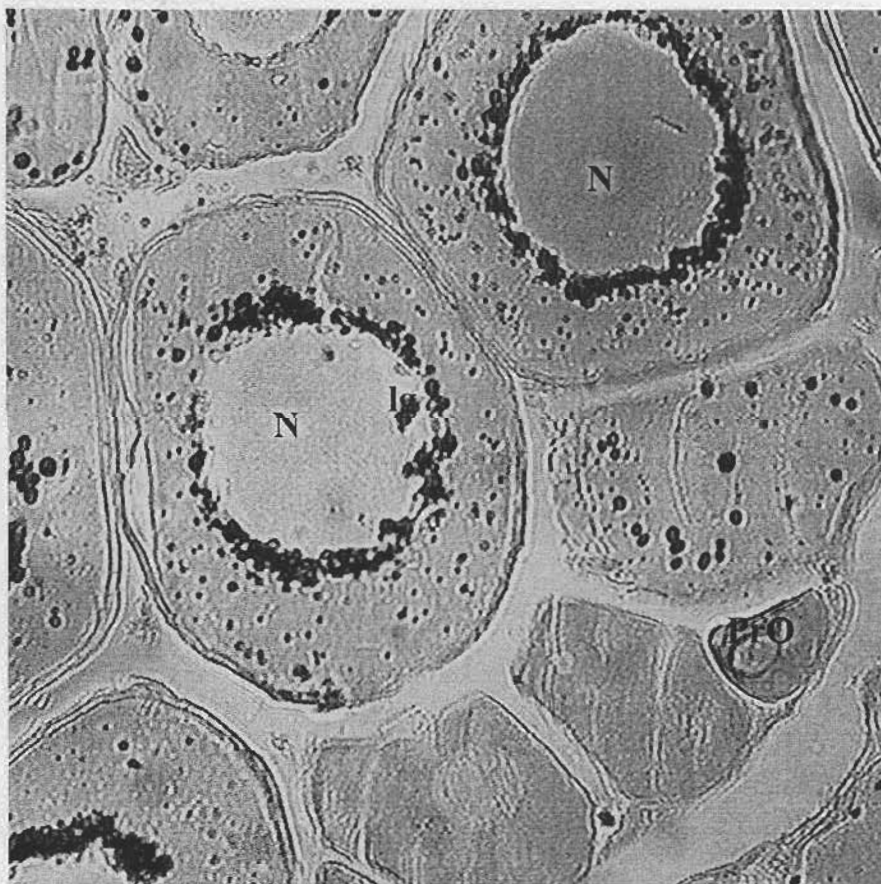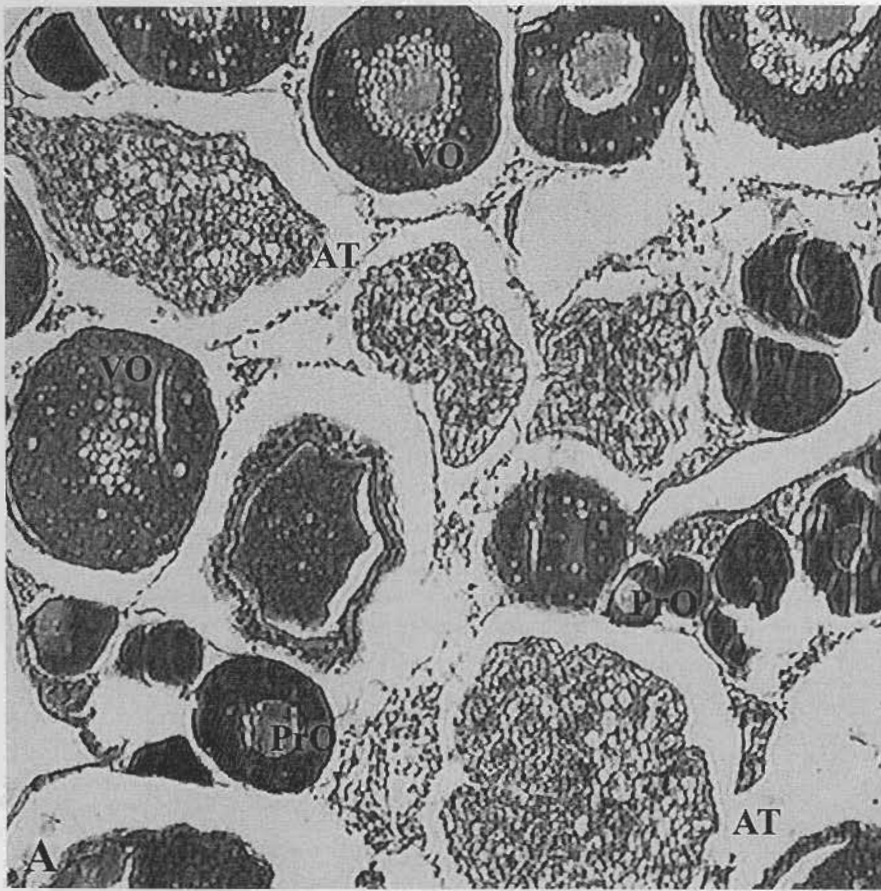
Fig. 3. A. Histological ovarian section showing numerous vitellogenic atretic oocytes, as well as previtellogenic and vitellogenic oocytes (July, Murcia Spain). Haematoxylin-V.O.F´Gutiérrez. x 40. **B.** Oocytes at early and mid vitellogenic phases (June, Barbate-Cádiz, Spain) Active lipidogenesis (numerous neutral lipid globules) around nucleus is observed. Osmium tetroxide-sodium dichromate treatment, before paraffin embedding process. x 100. AT: atretic oocytes; lg: lipid globules; N: nucleus; PrO: previtellogenic oocytes; VO: vitellogenic oocytes.

contained several large, round polychromatophilic (affinity to Haematoxylin, eosin and acid fuchsin dyes) peripheral nucleoli adhering to the nuclear membrane. The non-basophilic component of Balbiani´s body or idiosome disintegrated and disappeared. The basophilic pallial substance gradually increased in size, invading most of the cytoplasm, which progressively lost its basophilia (less affinity to Haematoxylin dye). A single layer of cells surrounding the oocytes became visible at this stage.

Secondary growth phase or vitellogenic oocytes

Early vitellogenic oocytes (Lipid globule stage) measured 162±49 µm in diameter. They showed a granular, lightly basophilic cytoplasm and contained small lipid globules scattered in the mid portion of the ooplasm and, later on, also around nucleus. These lipid globules increased in number and size during the oogenesis. Because of the histological procedure, the lipid of these globules was dissolved (Figs. 1A,B, 2A, 3A) and appeared as empty vacuoles. However, neutral lipid (globules) were preserved in those samples treated, previous to the paraffin embedding process, with osmium tetroxide-potassium dichromate (Figs. 3B, 4A, 5A), as well as in those unfixed samples processed directly in cryostat (data not shown). At the end of this stage, some very small and acidophilic yolk granules (yolk granule stage 1) appeared in the cortical portion of the cytoplasm

The cytoplasm exhibited several changes: more granular, weakly acidophilic and the pallial substance disintegrated and disappeared. The nuclear envelope became indented and oocytes were surrounded by a thin double layer of follicular cells (granulosa and theca) and a thin zona radiata (1-2 µm).

Mid vitellogenesis (Yolk granule stage 2): oocytes measured 300±44 µm and contained numerous lipid globules (vacuoles) which occupied the greater part of the strongly acidophilic cytoplasm with the exception of the perinuclear region. Numerous acidophilic yolk granules were detected in the cortical cytoplasm. The follicular envelope reached its greatest thickness and it was organized in two well differentiated layers: the granulosa and a thin theca externa cellular layer. Zona radiata showed higher affinity to eosin and started to exhibit a bipartite structure (8 µm) constituted by a thinner external zone and an internal and less acidophilic zone. Some peripheral cortical alveoli were still detected at this stage.

During late vitellogenesis (yolk granule stage 3), oocytes measured 449±79.04 µm. Ooplasm was completely filled by lipid globules (around 25 µm in diameter) and acidophilic yolk granules which reach their largest size (10 µm) and they move towards the innermost cytoplasmic region. The perinuclear space disappeared and the vitelline envelope increased in thickness and becames striated. Cortical alveoli were evident in the periphery of the oocytes. Granulosa cells were cuboidal and the zona radiata was around 20 µm wide (Figs. 1B, 2A, 4A,B, 5B). Vitellogenic oocytes were abundant in May (25%), June (35%) and July (15%).

Postvitellogenic or maturating oocytes

Postvitellogenic oocytes measured 586±99 µm in diameter. They were only observed in those specimens captured in July (Murcia), representing 28% of total oocytes. The nucleus (germinal vesicle) was still in a central position and showed numerous peripheral nucleoli. Lipid vacuoles started to fuse becoming larger. The process of lipid coalescence resulted in the formation of a continuous mass of lipid starting from the perinuclear zone. Large yolk granules moved towards the innermost cytoplasmic region where they initiated coalescence. Subsequently, these postvitellogenic oocytes were characterized by the migration of the nucleus or germinal vesicle towards the animal pole and yolk granules and lipid globules started to coalesce (Fig. 2A) and they were fused in large amorphous plaques. After the germinal vesicle breakdown was completed, the confluence in a single mass of yolk granules, lipid droplets and glycoproteic material (cortical alveoli) gave a translucent aspect to the ooplasm. In this stage, oocytes rapidly increased in volume due to hydration (>>600 µm diameter) and assumed an irregular shape. Follicular layers stretched, became flattened and decreased in thickness. The vitelline envelope (or chorion) showed a more compact appearance (35-40 µm). Some cortical alveoli were still detected in these ovaries.

Spawning Phase. Postovulatory follicles

During the spawning phase, follicular cells collapsed and constituted empty follicular envelopes characterized by a large irregular lumen formerly occupied by the oocyte (Fig. 2B). At the beginning, follicular cells, which were folded in loops with hypertrophic granulosa cells, cuboidal in shape and with a large nucleus, were evident. Theca cells were closely adherent to the granulosa cells and contained numerous blood capillaries. Later, this structure became less convoluted and irregular with a small lumen and started to be reabsorbed. The nucleus of granulosa cells became pyknotic. Postovulatory follicles remained for a short time in the ovary and at the end it was very difficult to distinguish these structures from atretic oocytes. Postovulatory follicles were always present in running ovaries, together with new batches of growing oocytes. These structures, related to the spawning-phase were only observed in specimens captured in July (Murcia), representing 25% of total oocytes.

Atretic oocytes

Most ovaries sampled showed atretic oocytes (Figs. 1A, 3A). At the onset of atresia, the vitelline envelope began fragmentation; the nucleus of follicle cells became to be more irregular in shape and more basophilic and
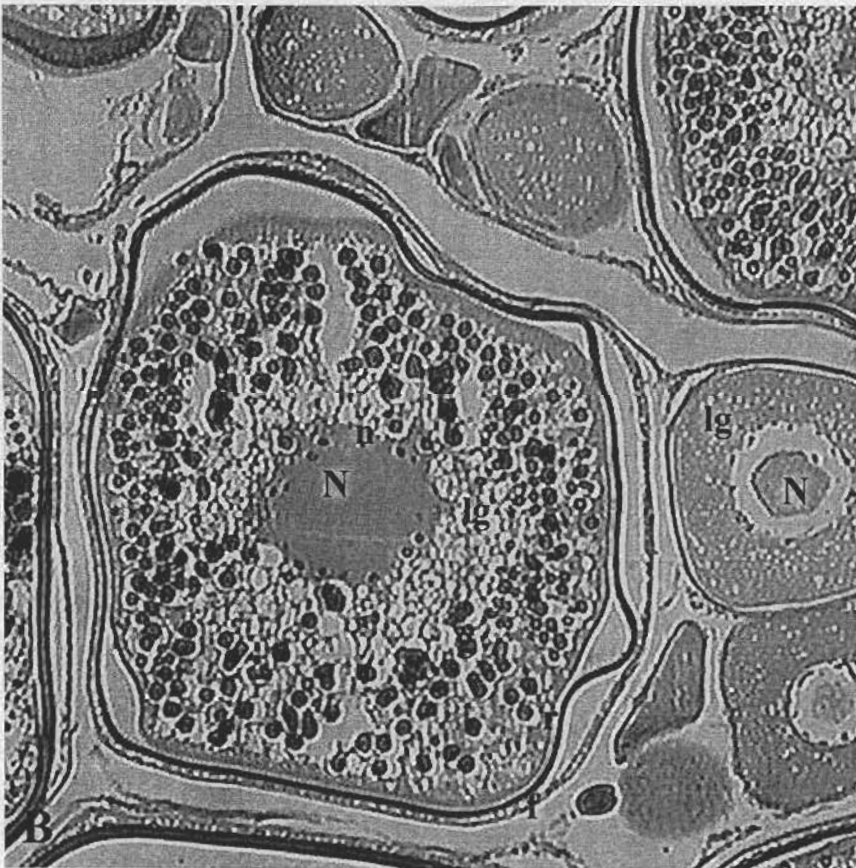
Fig. 4. A. Lipid globules showing strong affinity by osmium tetroxide (similar to Fig. 3B). x 250. **B.** Oocytes during early and late vitellogenic phases showing the distribution of proteins in zona radiata and follicular envelope. Bromophenol Blue. x 100. lg: lipid globules; N: nucleus; n: nucleoli; f: follicular layer; r: zona radiata: VO: vitellogenic oocytes.

Fig. 5. A. Ovarian section showing a vitellogenic oocyte with abundant lipid globules (osmium tetroxide affinity) and yolk granules, zona radiata and follicular layer containing abundant proteins (affinity to Bromophenol Blue). Treatment with Osmium tetroxide-potassium dichromate previous to paraffin embedding process and posterior staining with Bromophenol Blue technique. x 250. **B.** Histological section of an ovary during vitellogenic phase stained with anti seabass VTG serum. Positive immunostaining within granular cytoplasm, yolk granules and in follicular layer (granulose cells). Streptavidin-biotin-peroxidase method. x 250. f: follicular layer; g: yolk granules; lg: lipid globules; N: nucleus; n: nucleoli.

disintegrated discharging its content into the cytoplasm. Afterwards, the zona radiata became fragmented, it lost striation and formed irregular structures enveloped, while the degenerating yolk and numerous blood capillaries were visible. Finally, yolk granules lost their structural integrity and were phagocytosed by the hypertrophied granulosa cells invading the degenerated oocytes (Figs. 1A, 3A).

Different patterns of atretic oocytes, specially those related to degenerating vitellogenic and/or maturating oocytes were observed throughout the spawning season, during May (10%), June (15%) and July (35%). Moreover, a very small number of atretic unyolked oocytes were observed in some studied samples (data not shown).

In the bluefin tuna females studied, the gonadosomatic index (GSI) increased progressively during May (0.143-0.309) coinciding with the early vitellogenesis phase. The maximal values were reached in June (0.446-1.226) during mid and late vitellogenesis and, subsequently decreased throughout the maturation-spawning phases (July), showing values ranging between 0.169 and 0.596. During the recovering-resting period (October), GSI varied between 0.071 and 0.100.

Histochemistry

Histochemical results are summarized in Table 1. Yolk granules showed orange G (Haematoxylin-V.O.F´Gutiérrez) or eosin affinities (Haematoxylin-eosin), and lipid globules appeared unstained with these morphological dyes. The lipids (globules) of the vitellogenic oocytes were dissolved (vacuoles) during the paraffin and resin embedding procedures (Figs. 1A,1B, 2A,B) but preserved with 1% osmium tetroxide and 2.5% potassium dichromate treatment before histological embedding processes (Figs. 3B, 4A). Moreover, in unfixed fresh samples processed in a cryostat, these globules (vacuoles) reacted with Oil Red O (ORO) and with Black Sudan B (BSB) indicating the presence of neutral lipids exclusively. However, yolk granules observed in the vitellogenic oocytes of *Thunnus thynnus* did not contain neutral lipids. They showed a moderate sudanophilia in paraffin sections incubated with pyridine and then stained with SBB. These results, as well as their tinctorial affinity to Nile Blue, Luxol Blue and Chloramine T-performic acid-dinitrophenylhydrazyne-PAS, indicate the absence, in yolk granules, of neutral lipids but the presence of phospholipids and glycolipids.

Yolk granules showed weak staining with PAS and diastase-PAS reactions (presence of neutral glycoproteins/glycolipids and absence of glycogen). They were strongly stained with protein techniques (Figs. 4B, 5A) and unstained with Alcian Blue (pH 0.5, 1 and 2.5). Glycoconjugates containing different sugar residues were detected in vitellogenic oocytes. DBA lectin (α–GalNAc) was positive within yolk-granules and cortical alveoli of the vitellogenic oocytes of bluefin tuna specimens. Other lectins, such as Con A (α-Man, α-Glc, α-GlcNAc), UEA (α-Fucose) and WGA (ß-GlcNAc, sialic acid) were weakly positive or negative.

Table 1. Histochemical results in the vitellogenic oocytes of the bluefin tuna, *Thunnus thynnus* L.

	GLOBULES OR VACUOLES	YOLK GRANULES	CORTICAL ALVEOLI	FOLLICULAR ENVELOPE	ZONA RADIATA
PAS	0	±	1	+	1
Diastase-PAS	0	±	1	+	1
KOH-PAS	0	±	2	+	1
AA 2.5	0	0	±	±	0
AA 1	0	0	0	0	0
AA 0.5	0	0	0	0	0
Proteins	0	3	0	2	3
Lysine	0	±	0	±	±
Arginine	0	±	0	±	±
Tyrosine	0	3	0	2	2
Tryptophan	0	2-3	0	2	1
Cysteine	0	±	0	±	±
Cystine	0	1	0	1	2
Lipids (phospholipids and/or glycolipids)	0(V)	2	0	1	1
Neutral lipids	3(*/**)	0	0	0	0
Vitellogenin	0	1	0	1	0
Calcium	0	1-2	0	±	0
DBA	0	2	1	0	1
Con A	0	±	0	±	±
UEA	0	±	0	±	±
WGA	0	0	1-2	±	0

Intensity of reaction: 0: negative, ±: very weak, 1: weak, 2: moderate, 3: strong. Neutral lipids: *, Oil Red O/ Sudan Black B techniques in cryostat sections and **, Osmium tetroxide -potassium dichromate previous to paraffin embedding procces; V: vacuoles (neutral lipids dissolved in paraffin sections).

The calcium technique was strongly positive within yolk-granules and weakly in the follicular envelope. Granular cytoplasm of the vitellogenic oocytes also contained glycogen, lipids, proteins, glyconjugates (containing Mannose -Man- and/or Glucose -Glc-; N-acetyl-D-galactosamine-GalNAc-; L-Fucose -Fuc- sugar residues and N-acetyl-D-glucosamine -GlcNAc- and/or sialic acid -NANA-), vitellogenin (VTG) and calcium (Table 1).

Cortical alveoli were composed by carboxylated and neutral glycoproteins, especially rich in N-acetyl-D-glucosamine, sialic acid and N-acetyl-D-galactosamine. No affinity was detected for Con A and UEA lectins (Table 1).

In vitellogenic oocytes, the zona radiata presented strong acidophilia/eosinophilia (Haematoxylin-eosin/VOF) and was mainly proteinaceous in nature (Figs. 4B, 5A). The zona radiata was formed by an internal part with affinity to acid fuchsine and another external portion with tinctorial affinity to light green of the polychrome V.O.F. ´Gutiérrez. The external portion gave a stronger PAS-positivity reaction than the internal part of the zona radiata, which contained abundant disulphide groups (cystine), as well as glycoconjugates with Man and/or Glc sugar residues (Table 1).

Interestingly, yolk granules, granular cytoplasm and follicular layer showed positive anti-seabass VTG immunostaining (Fig. 5B). The liver also showed positive immunostaining angainst anti-VTG, especially in the vascular system (biliar ducts and sinusoids) and a weak staining was also observed, during April and May, within the cytoplasm of hepatocytes from females. Negative results were observed in the liver and gonad of males (data not shown).

Discussion

During the reproductive cycle of the bluefin tuna, *Thunnus thynnus* females, as in other fish species (Wallace, 1978; Selman and Wallace, 1982, 1983, 1989; Mayer et al., 1988; Selman et al., 1988, 1993; Grau, 1992) 4 phases can be observed: 1) primary growth phase including oogonia and basophilic previtellogenic oocytes at chromatin nucleolus and perinucleolus stages; 2) vitellogenesis phase (early, mid and late) related to yolk synthesis; 3) maturation phase (migration of nucleus and its breakdown); and 4) spawning phase characterized by the presence of atretic oocytes and especially for containing numerous postovulatory follicles. In bluefin oogenesis, the vitellogenesis phase (early, mid and/or late) was especially observed in ovaries from specimens captured during May and June (Barbate-Cádiz, Spain) and the maturation and spawning phases occur during July in Mediterranean Sea (Murcia, Spain). Similar results were observed during spermatogenesis of this species. The maturating/spermiation phases take place during July and parallely, GSI decreases during this time in both males (Cardenas et al., 2001) and females. We have no available data from females captured in August. Recovering and resting ovaries were observed in females captured in October, which contained oogonia and previtellogenic oocytes exclusively, as well the lowest GSI values. In bluefin specimens (3 full years-old) captured in July (Bay of Komame, Japan), Hirota and Morita (1976) observed oocytes at perinucleolus, oil droplet and yolk vesicle stages, as well some atretic oocytes. Another later stage of oogenesis was not observed, suggesting that the ovary of these specimens might not be developed to the further maturing condition. In males, the lobules of the testis were filled with spermatozoa, while some of them consisted of a few cysts including spermatids and spermatozoa.

Oocytes at chromatin nucleolus stage were not commonly found in bluefin tuna ovaries, suggesting that the transformation of oogonia into these previtellogenic oocytes and then in perinucleolus stage oocytes was a very fast process in *T. thynnus* ovaries. Most previtellogenic oocytes were in the perinucleolus stage. The yolk nucleus or Balbiani's vitelline body comprises 2 parts: the idiosome (non-basophilic component) and the more basophilic part or pallial substance (Beams and Kessel, 1973; Coello and Grimm, 1990). The Balbiani's body observed in previtellogenic oocytes at chromatin nucleolus stage might function as a center for the formation of various organelles within the oocyte, during the primary growth phase. Balbiani's bodies are not homogeneous structures; they are composed of electron-dense material (nuage material) or bound to mitochondria (cement material) and have been shown to consist of RNA and proteins (Kessel, 1983; Selman and Wallace, 1989; Grau et al., 1996). The pallial substance disintegrates during the early vitellogenesis phase (lipid globule stage), such as was indicated for other fish species (Guraya, 1979; Grau et al., 1996)

During the vitellogenesis phase of the *T. thynnus*, three types of inclusions: lipid globules, yolk granules and cortical alveoli were usually and progressively synthesized within the cytoplasm, although the sequence of their appearance varies in different fish species (Groman, 1982; Mayer et al., 1988; Selman and Wallace, 1989; González de Canales et al., 1992; Sarasquete et al., 1993a; Grau et al., 1996; Merson et al., 2000). In the bluefin ovaries, as in seabass (Mayer et al., 1988), lipid globules appear first, followed by yolk granules and cortical alveoli, respectively.

In bluefin ovaries, lipid globules present in the cytoplasm during early vitellogenesis contain only neutral lipids, which are dissolved (vacuoles) during paraffin and resin embedding procedures. This fact indicates that they could represent free unsaturated lipids (tryacylglicerides, wax esters and/or cholesterol) described for other fish species (Mayer et al., 1988; Rosety et al., 1992; Grau et al., 1996; Muñoz-Cueto et al., 1996). However, neutral lipids are preserved and stained in unfixed cryostatic sections and they show affinity to Sudan Black B and Oil Red O, as well in paraffin sections previously treated with osmium

tetroxide/ potassium-dicromate (osmium tetroxide affinity) according to Luna (1968). Lipid globules (neutral lipids exclusively) are not present in oocytes of some species, while phospholipids and neutral lipids may or may not be observed in the yolk granules of different teleosts (Khoo, 1979; Mayer et al., 1988; González de Canales et al., 1992; Sarasquete et al., 1993a; Muñoz-Cueto et al., 1996; Lahnsteiner and Patzner, 1999).

In bluefin tuna vitellogenic oocytes, yolk granules were weakly positive to PAS and diastase-PAS, moderately stained with lipid reaction (paraffin, SBB) and strongly stained with different specific protein techniques (rich in tryptophan, tyrosine, arginine, cysteine and cystine). However, in goldfish and seabass, yolk granules were PAS-negative (Khoo, 1979; Mayer et al., 1988). In *Solea senegalensis* oocytes, yolk granules contain acid glycoproteins (Gutiérrez et al., 1985), which were only detected within granular cytoplasm on bluefin oocytes. In *Signatus rivulatus* (Lahnsteiner and Patzner, 1999), the postvitellogenic oocytes contain several large lipid droplets composed of neutral lipids and glycolipids. In fish, lipid droplets of the eggs are energy sources, and do not influence their buoyancy (Heming and Buddington, 1988; Mommsen and Walsh, 1988).

The presence of glycoproteins, glycolipids and phospholipids in the yolk granules of the bluefin tuna vitellogenic oocytes, such as was pointed out for other species (Gutiérrez et al., 1985; Grau et al., 1996; Sarasquete et al., 1993a; Muñoz-Cueto et al., 1996) could be related to the vitellogenin-molecule (VTG). In different fish species (Dasmahapatra and Medda, 1982; Singh and Singh, 1990; Rosety et al., 1992; Muñoz-Cueto et al., 1996), an increase in liver triglycerides (energy source) and diglycerides (vitellogenin precursor) was observed during the reproductive period (vitellogenesis phase). Haux and Norberg (1985) indicated the importance of phospholipids and triglycerides during vitellogenin -VTG- synthesis, since they are functional components of this molecule.

Yolk granules of the bluefin tuna vitellogenic oocytes were also immunostained with anti- seabass-VTG and they contained calcium. According to Hara and Hirai (1978), the vitellogenin -VTG- is a glycolipophosphoprotein complex which binds calcium and iron; it is synthesized in the liver during oogenesis, released into the blood and transported to the ovaries forming the major constituent of the yolk (Ng and Idler, 1983). In *Sparus aurata* (Sarasquete et al., 1993c) as in bluefin vitellogenic oocytes, VTG was detected in yolk granules, granular cytoplasm and follicular envelope by using seabass anti-VTG serum, such as was pointed out previously by Susca et al. (2001) using a specific bluefin VTG antisera, According to Abraham et al. (1984), the follicular cells are not involved directly in the transfer of exogenous material into the oocytes, suggesting (Susca et al., 2001) that the immunoreactivity displayed by the follicular layer of bluefin tuna oocytes was of uncertain interpretation.

VTG immunostaining was also observed in the hepatic vascular system and weakly in the cytoplasm of hepatocytes of bluefin tuna females. Hepatocytes have previously been identified as the site of exogenous vitellogenesis, while the vascular system has been identified as the transferral route between the liver and ovary (Wallace, 1978). Wahli et al. (1998), by using different monoclonal and polyclonal commercial VTG antibodies observed positive results in the liver of brown trout, *Salmo trutta fario* females, but no immunostaining was detected in the liver of males. In addition to VTG, an apparent precursor of the vitelline envelope (VEP) has been identified immunochemically in some teleost fish (Hamazaki et al., 1985; Murata et al., 1994). In *Diodon holocanthus*, Fujita et al. (1998) showed that yolk granules and the vitelline envelope enclosing developing oocytes were immunostained with anti-VTG and with anti-VEP. It was noted that the appearance of VEP precedes that of VTG in the plasma of rainbow trout (Hyllner et al., 1994).

As in other fish species (Grau et al., 1996), the internal layer of the zone radiata contained neutral glycoproteins, and proteins rich in different aminoacids and especially rich in cystine, which suggests that most of the proteinaceous material of this multilamellar layer is determined by the formation of disulphide bonds (Hagenmaier, 1973; Mayer et al., 1988). The zona radiata of the *Salmo salar* oocytes contains proteins, mucopolysaccharides, phospholipids, cholesterol, DNA and RNA, as well as oxidative enzymes such as peroxidase, uricase, etc. (Hamor and Garside, 1973). Studies in other teleosts (Tesoriero, 1977) have shown that the outer layer of the zona radiata is rich in polysaccharides, whereas the inner layer is rich in proteins. In *Carassius auratus* (Khoo, 1979) and *Dicentrarchus labrax* (Mayer et al., 1988), polysaccharides adhered to the outer layer which might contribute to the adhesion of the eggs. Recently (Lahnsteiner and Patzner, 1999), indicated that the chorion of the *Siganus rivulatus* oocytes consisted exclusively of proteins. The follicular envelope of the bluefin tuna oocytes contain vitellogenin, lipids, calcium and proteins, and the zona radiata is formed by proteins specially; few lipids (phospholipids and/or glycolipids) and neutral glycoproteins were also observed in this layer of the vitellogenic oocytes.

Glycoconjugates are involved in the binding of hormones, in the transport of metabolites and ions across plasmmalema, and in sperm-egg interaction (Miller and Ax, 1990). In mammals, specific sugar residues present in the zona pellucida are the key for species-specificity to the interaction between oocytes and spermatozoa (Skutelsky et al., 1994). Fish egg polysialoglycoprotein was first isolated from eggs of rainbow trout (50% sialic acid). Although its biological function is unknown, Iwasaki and Inoue (1985) showed that this glycoprotein is closely associated with the cortical vesicles of the eggs and undergoes fertilization-induced specific depolymerization. In bluefin tuna oocytes, cortical

alveoli contain N-acetyl-D-glucosamine, sialic acid and N-acetyl-D-galactosamine sugar residues. Glyco-conjugates of the zona radiata contain acetyl-D-galactosamine, α-D mannose and/or glucose and α-L fucose residues. In fish, the autosynthetic origin of the cortical alveoli present in vitellogenic oocytes has been pointed out (Nahagama, 1983) and because their content is released during fertilization (Selman et al., 1988), they cannot be considered as true yolk.

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