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Histological and histochemical study of female germ cell development in the dusky grouper *Epinephelus marginatus* (Lowe, 1834)

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SUMMARY

The developmental stages of female germ cells were analysed in a wild population of the protogynous teleost *Epinephelus marginatus* (Lowe, 1834). 321 wild dusky grouper females were collected in the South Mediterranean Sea during the spawning season and their ovaries analysed using histological and histochemical techniques. Oocyte morphology, nucleus-cytoplasm *ratio* (N/C) range, location and movements of cytoplasmic inclusions during primary growth, vitellogenesis and final oocyte maturation were described. The distribution of proteins, lipids and carbohydrates through oocyte development was also investigated in 50 females. Lipid vesicles appeared firstly in the mid ooplasm of oocytes larger than 130μm, at the beginning of the secondary growth phase. Immediately afterwards, small carbohydrate granules (PAS and Alcian blue positive) appeared before the occurrence of the first yolk granules. Tyrosine-enriched proteins were especially evidenced in the *zona radiata interna* of late vitellogenic oocytes. Specific lectin binding patterns reflected characteristic differences in the content and distribution of specific sugar moieties expressed in the oocytes during vitellogenesis and final maturation. At the end of vitellogenesis and during final maturation, follicular cells, *zona radiata*, and cortical alveoli were characterised by a strong increase of specific binding for WGA.

INTRODUCTION

The study of gonad development in teleost fish leads to the acquisition of basic knowledge of their reproductive biology and has practical applications for the management of their reproductive cycle in captivity. The histological approach permits a precise definition of the process of development and final maturation of the oocytes and allows the identification of the essential morphological parameters that can be used to monitor the sequence and the timing of the ovarian development.

The dusky grouper *Epinephelus marginatus* (family: Serranidae; subfamily: Epinephelinae) is a common, wide-ranging species occurring on both sides of the Atlantic Ocean, throughout the Mediterranean Sea and around the southern tip of Africa (Heemstra, 1991). The dusky grouper is of interest because of its considerable commercial value and because of emerging concern over its over-exploitation (Annex 3 to Berne Convention, 1996, *in* Relini, 1999). Artificial propagation programs are being planned for

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restocking purpose (FAO, 1997) and further knowledge on its reproductive cycle is needed to set up controlled reproduction techniques. *E. marginatus* is a monandric protogynous hermaphrodite, incompletely metagonous, reaching its first maturity late (Marino *et al*., 2001). In captive broodstock, dusky grouper females fail to complete vitellogenesis and final oocyte maturation, and spawning does not spontaneously occur (Glamuzina *et al*., 1998; Marino *et al.,* 2000). Till now few data on the ovarian morphology of wild dusky grouper have been published and they are more related to reproductive patterns than to ovarian development and oocyte morphology and histochemistry. Bruslé and Bruslé (1975) reported a synchronous development of the ovary. Maturation in shifts, through the recruitment of "waves" of previtellogenic oocytes, was described by Bouain and Siau (1983) on the basis of cytoplasm basophilia. A group synchronous development of the ovary was recently determined in the dusky grouper by Massari *et al.* (1999) on the basis of the distribution frequency of vitellogenic oocytes. Multiple spawning was also identified by the same authors in relation to the presence of post-ovulatory follicles in maturing ovaries.

This study describes the developmental stages of the female germ cells through oocyte growth and final maturation. The cytochemical features of the female germinal line were analyzed and the occurrence and distribution of lipid vesicles, yolk granules and cortical alveoli within the developmental oocytes were described by means of routine histochemical techniques. Lectin histochemistry was also applied to study occurrence and distribution of few sugar moieties within oocytes in different developmental stages. Lectins are sugar-binding proteins of non-immune origin that precipitate glycoconjugates having saccharides of appropriate complementarity (Goldstein *et al.*, 1980). In particular, we analysed the spatial distribution of glycoconjugates, which are known to be involved in the binding of hormones, in transport of metabolites and ions across the plasmalemma and sperm-egg interaction (Macek and Shur, 1988; Miller and Ax, 1990). The identification of specific lectin binding patterns in dusky grouper oocytes provides further basic information on the development and maturation process of oocytes in fish and could be utilised to assess the quality of oocyte in captive broodstock.

MATERIALS AND METHODS

Sampling and preservation

Wild dusky grouper females were captured off the Pelagie Islands (Mediterranean Sea, 35°-36° N and 10°-14° E) by professional hook-and-line or by divers. The ovaries of 321 specimens were dissected immediately after capture, sexed and macroscopically analysed for the stage of maturity as described in Marino *et al.* (2001). Apical, central and caudal portions of gonads were fixed in Bouin's solution and/or in 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.2 for histological and histochemical analyses. Some samples were also pre-fixed in 10% buffered formalin and cryostatic sectioned for lipid detection.

Histology

Gonads were embedded in paraffin or glycol methacrylate resin (Kulzer 7100). 4-5µm paraffin embedded sections were stained by haematoxylin-eosin and Mann Dominici staining, 1-2µm resin embedded sections were stained by toluidine blue and Lee's methylene blue-basic fuchsin (Beccari and Mazzi, 1972; Bennet *et al*., 1976). To aid paraffin section adhesion, microscope slides were coated with a thin film of poly-L-lysine solution (1:100, Sigma Chemical Co., USA). The developmental stages of oocytes were studied following the scale described by Mayer *et al.* (1988) adapted to a five phase classification (primary growth phase, early, mid and late vitellogenesis, final maturation) usually utilized for Epinephelinae (Abu-Hakima, 1987; Sadovy and Colin, 1995). Oocytes were classified according to their morphology, affinity for dyes and the presence and position of lipid droplets, yolk vesicles and granules. Postovulatory follicles and atretic follicles were classified on the basis of their histological appearance, following Hunter and Macewicz (1985).

Histochemical procedures

Histochemical analyses were performed on 50 specimens. Staining such as 1% Coomassie Brilliant blue (G250, in ethanol 50%) for proteins in general, Morel Sisley for tyrosine-, ninhydrin-Schiff for lysine- and Adams for tryptophan-enriched proteins were used. The lipid droplet content was studied in cryostatic sections with

Sudan Black B for the detection of general lipids, while phospholipids, neutral and acid lipids were studied with Nile blue sulphuric acid and aqueous Nile blue, respectively. Periodic acid Schiff (PAS) for neutral muco-substances and Alcian blue for carboxylated and sulphated muco-substances (pH 2.5 and 0.5, respectively) were utilized for the study of cortical alveoli on paraffin embedded sections. All the histochemical methods were performed according to routine techniques (Beccari and Mazzi, 1972; Barka and Anderson, 1963). The identification of sugar residues (Table I) in the oligosaccharide chains of the mucosubstances was accomplished using specific HRP- and/or fluorescein-conjugated lectins (Sigma Chemical Co., USA) according to Skutelsky *et al.* (1994). When HRP-conjugated lectins were used, endogenous peroxidase was always blocked with 4‰ H_2O_2 in saline phosphate buffer, for 30 min. Moreover, endogenous peroxidase activity was checked by incubating untreated sections with DAB solution. The respective peroxidase conjugated lectins were omitted and/or their hapten sugars were added in control sections. Lectin staining was partially or completely inhibited when the corresponding hapten was added to the standard lectin solution. No endogenous peroxidase was observed after incubation with DAB-H_2O_2 solution.

Oocyte measurement

At least 30 oocytes from each stage were measured in apical, central and caudal sections along the median axis of the oocyte parallel to the horizontal micrometer gradation (De Martini and Fountain, 1981). The study was carried out using image-processing and analysis software (Global Lab Image).

RESULTS

The ovaries were two unequal lobes fused along the medial-caudal portion, surrounded by a thick tunica albuginea. In each lobe, lamellae of connective tissue were projecting into the central lumen. Each lumen fused posteriorly into a common cavity, gradually reducing in size to form a small post-ovarian sinus. The ovarian wall contained smooth muscle layers and connective tissue with blood and lymphatic vessels and nerve fibres.

Six female developmental stages histologically described by Massari *et* al. (1999) are summarized in Table II. Pockets of testicular tissue, constituted by small cysts of male germ cells, were always present amongst the female tissue, following the "Epinephelus type" organization (Smith, 1965).

The histological and histochemical aspects of oocytes through primary growth phase, early-, mid- and late vitellogenesis and final maturation are given below.

Table I

Lectins used and their binding specificities according to Spicer and Schulte (1992) and Skutelsky *et al.* (1994)

Lectin: latin name (common name) and acronym	**Concentration ($\mu g/ml^{-1}$)**	**Binding specificities**
Canavalia ensiformis (jack bean) Con-A	50	a.D-glucose, terminal, internal a-D-mannose
Triticum vulgaris (wheat germ) WGA	50	ß-1-4-N-acetyl-D-glucosamine, sialic acid
Dolichos biflorus (horse gram) DBA	10	a-N-acetyl-D-galactosamine
Glicine max (soybean) SBA	10	a-N-acetyl-D-galactosamine, a-D-galactose
Bauhinia purpurea (camels foot tree) BPA	50	ß-galactose-1-3- N-acetyl-galactosamine
Griffonia simplicifolia GS-I	50	terminal a-D-galactose
Griffonia simplicifolia GS-II	50	a,ß-N-acetylglucosamine, glycogen
Maclura pomifera (hedge apple tree) MPA	50	terminal-ß-galactose-1,3-N-acetyl-galactosamine, galactose-1,6-N-acetyl-a-galactosammine
Ulex europaeus (gorse seed) UEA-I	10	a-L-fucose
Arachis hypogaea (peanut) PNA.	20	terminal ß-D-galactose-1-3-N-acetyl-galactosamine

Table II
Ovary maturity stages in dusky grouper females, according to Massari *et al.* (1999)

Maturity stage	Histological description
J. Immature	Primary germ cells (PGC), oogonia and small previtellogenic oocytes (PVG) filling the short ovigerous folds
F1. Resting	PGC, oogonia and PVG in both CNS and PNS stages especially present at the periphery of the elongated ovarian folds.
F2. Developing	Ovigerous folds filling ovarian cavity; numerous late PVG near one batch of LV oocytes.
F3. Maturing	Groups of coetaneous oocytes from PVG to vitellogenic stages (LV, YI, YII and YIII).
F4. Mature	One group of hydrated oocytes amongst batches of vitellogenic oocytes in different stage.
F5. Partially running	Ovigerous folds partially empty. Post-ovulatory follicles (POF) together with new batches of oocytes in all stages of vitellogenesis included YIII oocytes.
F6. Post-spawning	Empty ovigerous folds with few residual yolked oocytes and numerous atretic vitellogenic follicles.

Histology

Primordial germ cells (PGC) were oval unstained cells. They were relatively large (12-24μm in diameter) and usually arranged in clusters of a few cells (Fig. 1a) both along the edge and the axis of the ovigerous folds. The diameter of the nucleus ranged between 9 and 15μm. The nucleus/cytoplasm *ratio* (N/C) was about 0.7. These cells were present both in the female and male phase of the gonad.

Oogonia (O) developed from PGC and their cytoplasm was stained with haematoxylin. These cells were 15-24μm in size and contained a pale large nucleus (9-13μm). The N/C was about 0.6 (Fig. 1a). One nucleolus was usually present within the chromatin network. Oogonia, isolated or in clusters, were present during the entire annual cycle even though they could be more easily observed along the edge of the ovarian folds, in immature and developing ovaries.

Primary growth phase

Chromatin nucleolus stage (CNS) oocytes (25-40μm) with a narrow cytoplasm ring were deeply stained with haematoxylin. The nucleus was large (15-23μm) with strands of chromatin and a single large nucleolus (5-8μm). N/C was 0.59. This stage was relatively rapid and was rarely found, especially in maturing, mature and running ovaries.

Perinucleolus stage (PNS)

At the beginning of this stage, oocyte diameter ranged between 40-60μm and the N/C was about 0.58. Cytoplasm was homogeneous and strongly basophilic. The large nucleolus was still evident, but small nucleoli were detectable close to the nuclear membrane. Later PNS oocytes increased in size, reaching 80-110μm in diameter while the cytoplasm became less homogeneous and gradually decreased its affinity to haematoxilin. The nucleus reached 45-65μm (N/C 0.57) with more numerous small peripheral nucleoli (Fig. 1b). Basophilic material (Balbiani's body) appeared close to the nucleus in early PNS and moved to the periphery, gradually losing its affinity for haematoxylin in late PNS oocytes (Fig. 1c). A single layer of cells surrounding the oocyte became visible at this stage.

Oocytes in primary growth phase were observed in the ovary throughout all stages of ovarian development, but were particularly numerous at the beginning and at the end of the reproductive period (Table II).

From now on, cells in primary growth phase will be also indicated as previtellogenic oocytes (PVG).

Early vitellogenesis

Depending on hormonal and environmental factors, a number of PVG underwent early vitellogenesis. These oocytes (*lipid vesicle stage, LV*) (130-

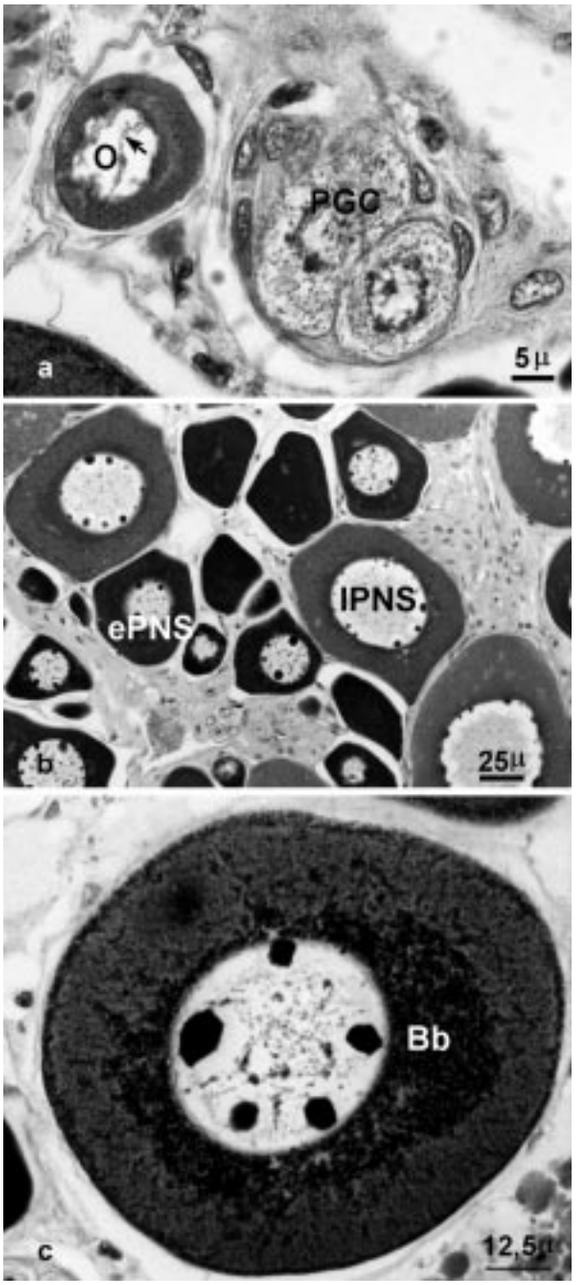

Fig. 1 - (a) Magnification of an *E. marginatus* ovary showing cyst of primary germ cells (PGC) and oogonia (O). Arrow indicates chromatin network. **(b)** Early and late perinucleolus stage oocytes (ePNS and lPNS). **(c)** Magnification of Balbiani 's body (Bb) in a PNS oocyte. **(a)** and **(c)** toluidine blue, **(b)** haematoxylin-eosin.

170µm) showed a granular, lightly basophilic cytoplasm and contained small lipid vesicles (Fig. 2a) scattered in the mid portion of the ooplasm and, later on, also around the nucleus. These vesicles increased in number and size throughout the LV stage, ranging from 3.5 to 9µm in diameter. Because of the histological procedures, the lipid content was dissolved and empty structures were apparent in paraffin embedded tissues. At the end of the stage, many small granules of about 2µm diameter appeared in the cortical portion of the cytoplasm. Numerous flattened nucleoli and lampbrush chromosomes were present in the nucleus. The nucleus was 75-85µm in diameter and showed an irregular edge. At the end of this stage N/C was 0.5. A thin double layer of follicular cells and a very thin *zona radiata* (1-2 µm) surrounded the oocyte surface. LV oocytes characterised developing ovaries, but were always present in maturing, mature and running ones.

Mid vitellogenesis

Primary yolk stage oocytes *(YI)* had a diameter of 170-240µm and a nucleus of 75-110µm (N/C 0.45). Lipid vesicles increased in number and size in the mid portion of the cytoplasm (5.8µm) and around the nucleus (6.41µm) and, from here on, will be indicated as lipid droplets as suggested by Mayer *et al.* (1988). These oocytes were characterised by the presence of numerous small yolk granules (2-3µm) in the middle of cytoplasm that became more acidophilic (Fig. 2b). Follicular cells were organised in two well developed layers, the granulosa cells with a constant thickness of about 4µm, and a thin theca *externa*. *Zona radiata* (ZR) showed higher affinity for eosin and started to exhibit a bipartite structure constituted by *zona radiata externa* (ZRE) and *zona radiata interna* (ZRI). ZRE was thinner (2.8µm vs.1.7µm) but more strongly stained with eosin than ZRI.

Secondary yolk stage oocytes *(YII)* had a diameter of 250-350µm and an irregular nucleus of 95-120µm (N/C of about 0.35). As vitellogenesis proceeded, yolk granules increased in number and in size (10-16 µm), especially in the inner portion of the cytoplasm and became slightly acidophilic. Lipid droplets were distributed in the inner part of the cytoplasm and further enlarged (11-12µm), but smaller ones (7-8µm) were still present in all the ooplasm (Fig. 2c). ZR was prominent (7-8µm wide) and divided almost equally in ZRE and ZRI.

Table III
Lectin reactivities during vitellogenesis and final maturation

Lectins*		LV	YI	YII	YIII	H
DBA	**FC**	-	±	±	±	-
	ZR	-	-	-	±	+
	YGm		-	+	+	
	CA	±	±	+	++	+
SBA	**FC**	±	±	±	+	-
	ZR	-	-	-	±	+
	YGm		+	±	±	
	CA	+	±	+	++	+
PNA	**FC**	±	-	-	-	-
	ZR	-	-	-	-	-
	YGm		-	-	-	
	CA	-	-	-	±	-
WGA	**FC**	-	±	+	++	+
	ZR	-	±	+	++	±
	YGm		-	-	±	
	CA	±	±	+	++	++
Con-A	**FC**	±	-	-	-	-
	ZR	±	-	-	-	-
	YGm		-	-	-	
	CA	-	-	-	-	-
GS-I	**FC**	-	-	-	-	-
	ZR	±	±	±	+	-
	YGm		+	++	++	
	CA	-	++	++	++	+
GS-II	**FC**	-	-	-	-	-
	ZR	-	-	-	-	-
	YGm		-	-	-	
	CA	-	-	-	-	-
BPA	**FC**	-	±	±	±	-
	ZR	-	-	-	-	+
	YGm		-	-	±	
	CA	-	-	-	±	+
MPA	**FE**	±	-	-	-	-
	ZR	±	±	±	±	±
	YGm		-	-	-	
	CA	-	-	-	±	+
UEA-I	**FC**	-	-	-	-	-
	ZR	-	-	-	-	-
	YGm		-	-	-	
	CA	-	-	-	-	-

Results are summarised as follows: -, negative; ±, weak; +, moderate; ++, strong staining.
LV, lipid vesicle stage; YI,YII and YIII, yolk granules type I, II and III, H, hydrated oocytes, FC, follicular cells; ZR, zona radiata; YGm, yolk granules membrane; CA, cortical alveoli.
*See table 1 for lectin name.

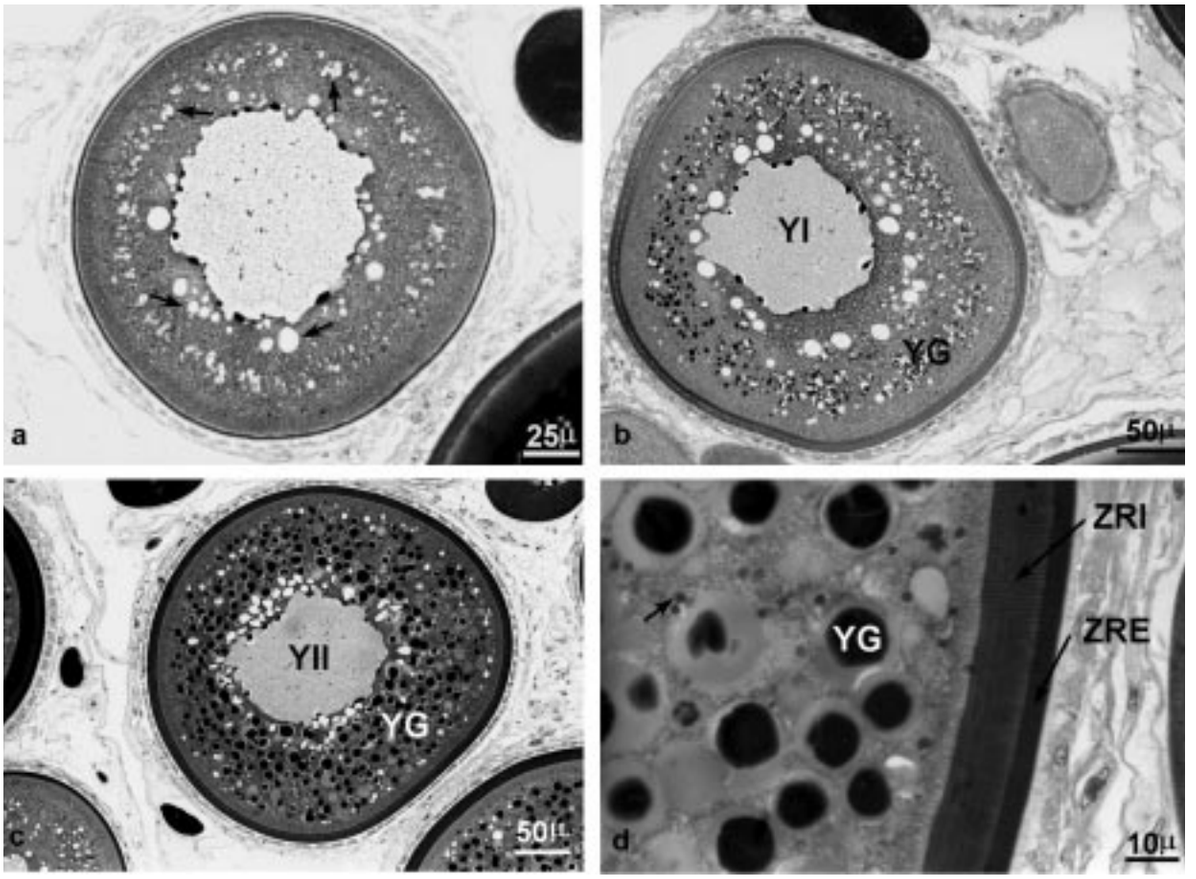

Fig. 2 - Vitellogenic phase of *E. marginatus* ovary. **(a)** Oocyte in LV stage showing lipid vesicles both around the nucleus and in the mid portion of the cytoplasm (arrows). **(b)** and **(c)** Yolk granules (YG) in the ooplasm of type I (YI) and type II (YII) oocytes, respectively**. (d)** Magnification of YIII oocyte showing yolk granules (YG), cortical alveoli (arrow) and zona radiata externa and interna (ZRE and ZRI). **(a)** toluidine blue, **(b)** and **(c)** Lee's method, **(d)** Mann Dominici.

Late vitellogenesis

Tertiary yolk stage oocytes *(YIII)* had a diameter of 350-500µm and nucleus of 110-150µm (N/C 0.3). Ooplasm was completely filled by acidophilic yolk granules that reached their largest size. Mann Dominici staining showed two types of yolk granules; one group, larger in size (16µm), was pale pink and scattered within the cytoplasm, the other group, deep blue, was located both near the nucleus (12µm) and at the periphery (8-9µm). Lipid droplets (12-13µm) were especially localised in the peri-nuclear zone and started to fuse one with each other becoming larger. Cortical alveoli were evident at the periphery of the oocytes. Granulosa cells were cuboidal and ZRE and ZRI were 16-18µm wide in total (Fig. 2d).

Groups of healthy YI, YII and YIII oocytes were always present in maturing, mature and partially running ovaries.

Final oocyte maturation (FOM)

Post-vitellogenic oocytes showed an increase of lipid droplets scattered amongst the deeply stained yolk granules. Numerous cortical alveoli, intensely blue with Mann Dominici, were confined at the periphery of the ooplasm. The nucleus (germinal vescicle) was still in a central position and showed small peripheral nucleoli. The process of lipid coalescence resulted in the formation of a continuous mass of lipids starting from the perinuclear zone (Fig. 3a). Subsequently, the germinal vesicle (GV) moved to the animal pole (germinal vescicle

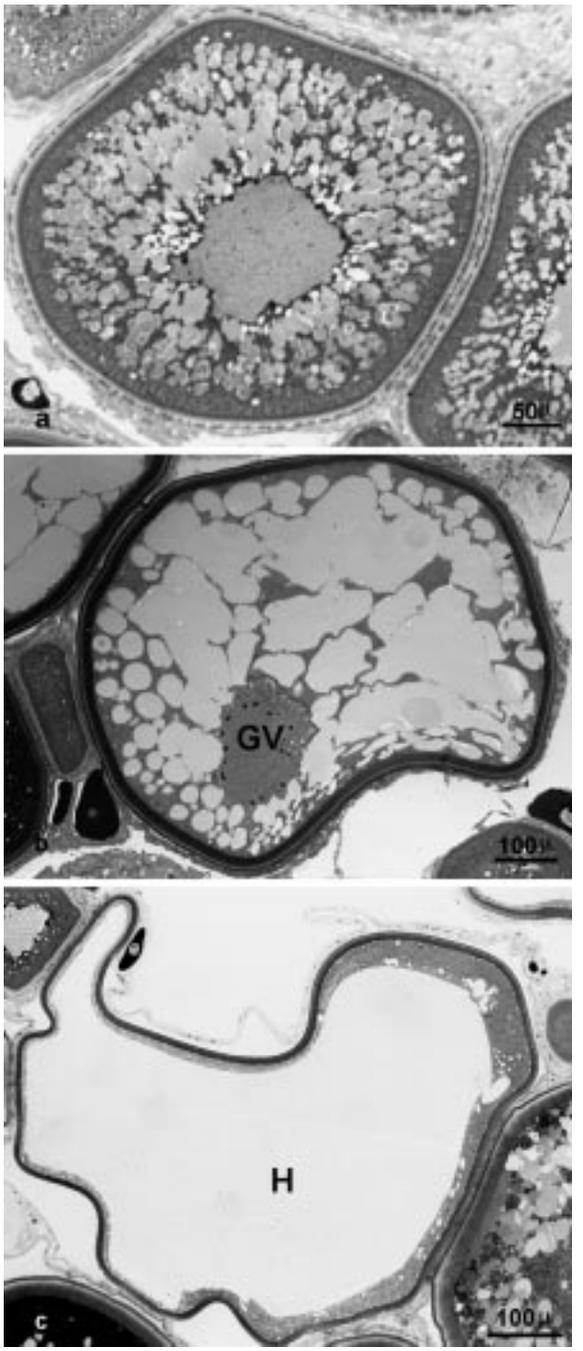

Fig. 3 - Maturation phase of an *E. marginatus* ovary. **(a)** Lipid vesicles and yolk granules start to coalesce. **(b)** Migration of germinal vesicle (GV). **(c)** Translucent hydrated oocyte (H). **(a)**, **(b)**, **(c)** toluidine blue.

migration, GVM, 550-600µm) and yolk granules and lipid droplets were fused in amorphous large plaques (Fig. 3b). After the germinal vesicle breakdown (GVBD, 600-650µm) was completed, the confluence in a single mass of yolk granules, lipid droplets and mucopolysaccharidic material gave a translucent aspect to the ooplasm (Fig. 3c). These *hydrated (H)* oocytes showed several cortical alveoli still detectable at the periphery of the ooplasm.

In this stage, oocytes rapidly increased in volume (550-800 µm diameter) due to hydration. Follicular layers stretched and became flattened with a decrease in thickness. The vitellin envelope showed a more compact appearance. Later these oocytes assumed an irregular shape.

These oocytes were observed in mature females.

Post-ovulatory follicles

After spawning, follicular cells collapsed and constituted a convoluted post-ovulatory follicle (POF) characterised by a large and irregular follicular lumen formerly occupied by the oocyte. At first follicular cells were folded in loops in which hypertrophic granulosa cells, cubic in shape and with a large nucleus, were especially evident. The theca layer was closely adherent to the granulosa layer and contained numerous blood capillaries (Fig. 4a). Thereafter, the structure became less convoluted and irregular with a smaller lumen that started to be reabsorbed. Some of the nuclei of the granulosa cells became pycnotic (Fig. 4b). POFs remained for a short time in the ovary, and in the latest stage it was very difficult to distinguish them from the late atretic structures. POFs were always present in partially running ovaries together with new batches of healthy growing yolked oocytes.

Atretic follicles

Those oocytes that had not been ovulated underwent atresia. In early atretic follicles (early α-stage), the nucleus became more irregular in shape, more basophilic and then underwent disintegration, discharging its contents into the cytoplasm (Fig. 4c). In late α-stage, *zona radiata* became fragmented, lost striations and formed irregular structures enveloped within the degenerating yolk (Fig. 4d). Numerous blood capillaries within the thecal layer were visible. Later (ß-stage), yolk granules lost their structural integrity and were phagocytized by the hypertrophied granulosa cells invading the degenerating oocyte.

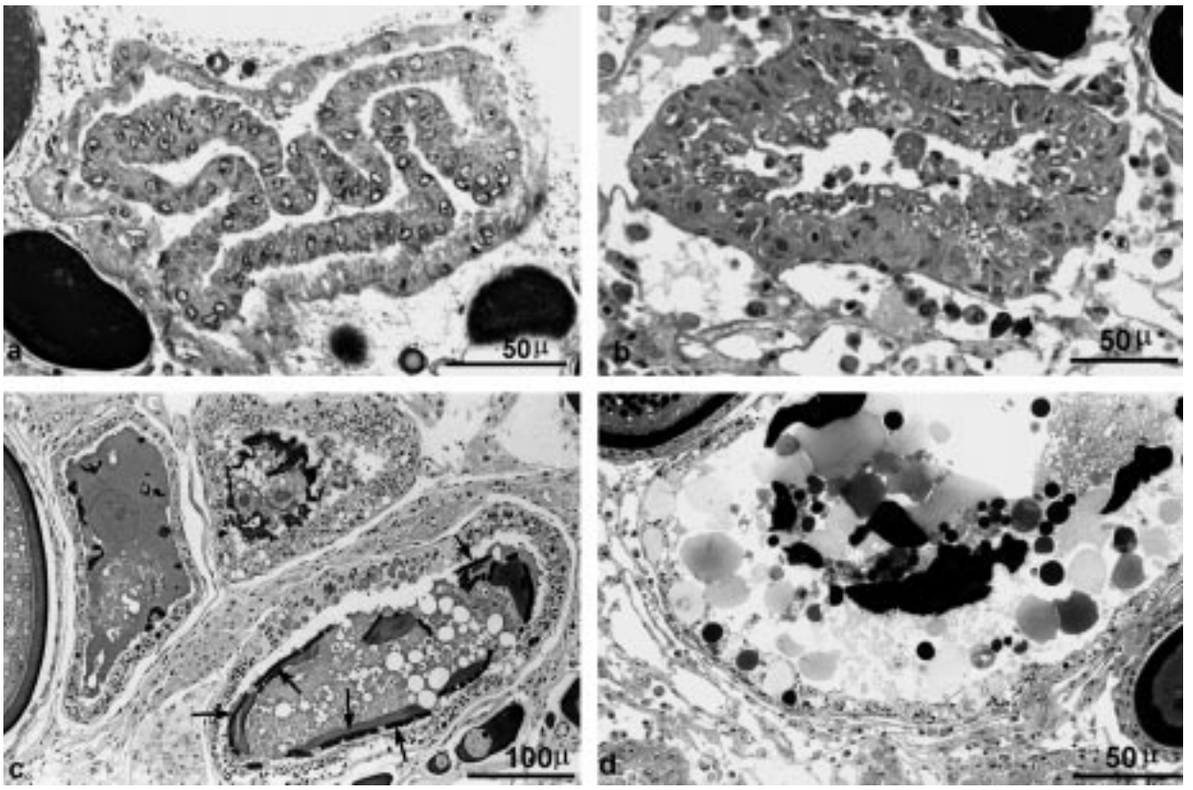

Fig. 4 - Early **(a)** and late **(b)** post-ovulatory follicles (POF) of *E. marginatus*. **(c)** α-stage atretic vitellogenic oocytes showing fragmentation of zona radiata (arrow) and hypertrophy of granulosa cells (*). **(d)** Magnification of late α-stage atretic vitellogenic oocyte. **(a)** and **(d)** toluidine blue, **(b)** haematoxylin-eosin, **(c)** Lee's method.

Different patterns of atresia were observed in dusky grouper ovaries. Early in the spawning season, numerous developing ovaries (about 33.3 %) showed a large number of atretic oocytes in the first batch undergoing vitellogenesis. During the mid spawning season, a small amount of atretic vitellogenic follicles (2-5%) was observed near healthy advanced yolked oocytes. Later, at the end of the reproductive season, strong atresia of vitellogenic follicles was a physiological event indicating the cessation of spawning. Occasionally, atresia of unyolked oocytes, with a process similar to α-stage but without yolk, was observed in resting and developing ovaries.

Histochemistry

Proteins

In previtellogenic oocytes, cytoplasm and nucleoli were positively stained for proteins. Vitellogenic oocytes showed yolk granules, as well as nucleoli and ZR, strongly positive to Coomassie blue (Fig. 5a). Tyrosine-enriched proteins (Fig. 5b) were present in the ZR starting from the LV stage and reaching the highest intensity in the ZRE of YIII oocytes; from YI to YIII oocytes, yolk granules increased in positivity too. Very few lysine-enriched proteins were observed during the proceeding of vitellogenesis in the ooplasm. Tryptophan-enriched proteins were not detectable.

Lipids

In order to verify the lipidic nature of the "lipid vesicles-droplets" observed during the histological study, specific histochemical methods on cryostatic sections were performed. The most intense Sudan Black B reaction was observed in the largest droplets distributed along the mid and inner cortex of YII and YIII oocytes. A faint reaction for lipids was sometimes present in the membrane around the yolk granules of the vitellogenic oocytes.

Carbohydrates

Carbohydrate study was firstly performed by means of Periodic Acid Schiff (PAS) and Alcian blue reactions. No positive material for either staining was ever observed in PGC, oogonia and primary oocytes. At the end of the lipid vesicle stage, a strong PAS positivity was evident in the ZR of LV oocytes (Fig. 5c). In these oocytes, small PAS-positive granules appeared for the first time in the mid portion of the cytoplasm (Fig. 5c). Alcian blue positivity was also observed in these structures at pH 2.5. Weaker positivity was observed in the same granules and in the ZRE of yolky oocytes. At the beginning of the final maturation, numerous cortical alveoli, stained deeply with PAS and slightly with Alcian blue, pH 2.5, were detectable at the periphery of the ooplasm.

Carbohydrates were further studied by means of specific lectins in follicular cells (FC), *zona radiata* (ZR), yolk granule membranes (YGm) and cortical alveoli (CA) during vitellogenesis and final maturation. Results are summarised in Table III.

Follicular cells. Specific binding for GS-I and GS-II was never detectable in the follicular envelope. Very weak affinity for PNA, Con A and MPA was observed in LV oocytes. Weak affinity for DBA, SBA and BPA was present in YI-YIII oocytes. WGA affinity increased from YI to YIII oocytes (Fig. 5d), but was weakly present in the follicular cells in H oocytes. Follicular cells were negative for UEA-I, such as observed in all structures of the oocytes.

Zona radiata. No specific binding for GS-II and PNA was detected in any oocyte development stage. Specificity for Con A was only present in LV oocytes. Weak affinity for DBA and SBA in YII, YIII and H oocytes, and for GS-I and MPA in all stages, were observed. PNA was always negative, while moderate to strong WGA binding was observed in oocytes during vitellogenesis. Affinity for WGA was inhibited in the sections by pre-treating them with sialidase.

Yolk granules membranes. Strong affinity was evidenced in granule membranes of YII and YIII oocytes only for GS-I, even though moderate or weak affinity for DBA, SBA, WGA and BPA were observed in YIII oocytes.

Cortical alveoli. From vitellogenesis to final maturation, WGA, DBA, SBA and GS-I (Fig. 5f) were specifically bound in cortical alveoli showing a strong reaction in YIII oocytes. No or weak affinity was observed for Con-A, MPA, BPA and PNA.

DISCUSSION

This study provides the first detailed histological and histochemical description of female germ cell development in the protogynous dusky grouper *E. marginatus*. Oocytes were primarily analyzed following the classification usually utilised for Epinephelinae (Abu-Hakima, 1987; Sadovy and Colin, 1995). Nevertheless, in order to give a more detailed cytological description, 8 cellular stages (chromatin nucleolus, early and late perinucleolus, lipid vesicle, yolk granules I, II and III, hydrated) were described following the classification of Mayer *et al.* (1988).

Dusky grouper oogonia entered the period of primary growth phase, gonadotropin-independent, after reaching 25µm diameter. These chromatin nucleolus stage oocytes (CNS) were not commonly found in ovaries, suggesting that the transformation of oogonia into CNS and then perinuleolus stage oocytes (PNS) was a rapid process. Early and late PNS oocytes appeared to be the most characteristic previtellogenic oocytes in the ovary of *E. marginatus* and were predominant in immature, developing and resting ovaries. PNS were also always present during the vitellogenic phases, suggesting a continuous recruitment of oocytes during the reproductive season. At the beginning of the perinucleolus stage, a structure similar to that described as Balbiani's body (Raven, 1961) in other teleosts (Wallace and Selmann, 1981; Iwamatsu *et al.*, 1988; Mayer *et al.*, 1988; Coello and Grimm, 1990) appeared, even though not as strongly basophilic as previously described. It usually disappeared at the end of the PNS stage.

During vitellogenesis, three types of inclusions (lipid globules, cortical alveoli and yolk granules) are usually formed although the sequence of their appearance may vary in the different species (Selman and Wallace, 1986). In several teleosts, such as *Morone saxatilis* (Groman, 1982), *Oryzias latipes* (Iwamatsu *et al*., 1988) and *Seriola dumerilii* (Grau *et al*., 1996) lipid globules, cortical alveoli and yolk granules appear subsequently. In other fish, cortical alveoli appear first, just before the lipid globules (Selman and Wallace, 1986, 1989; Landry and McQuinn, 1988; Bengen *et al*., 1991; Gonzáles De Canales *et al.*, 1992) while in the sea bass lipid droplets appear first, followed by yolk granules and cortical alveoli (Mayer *et al*., 1988).

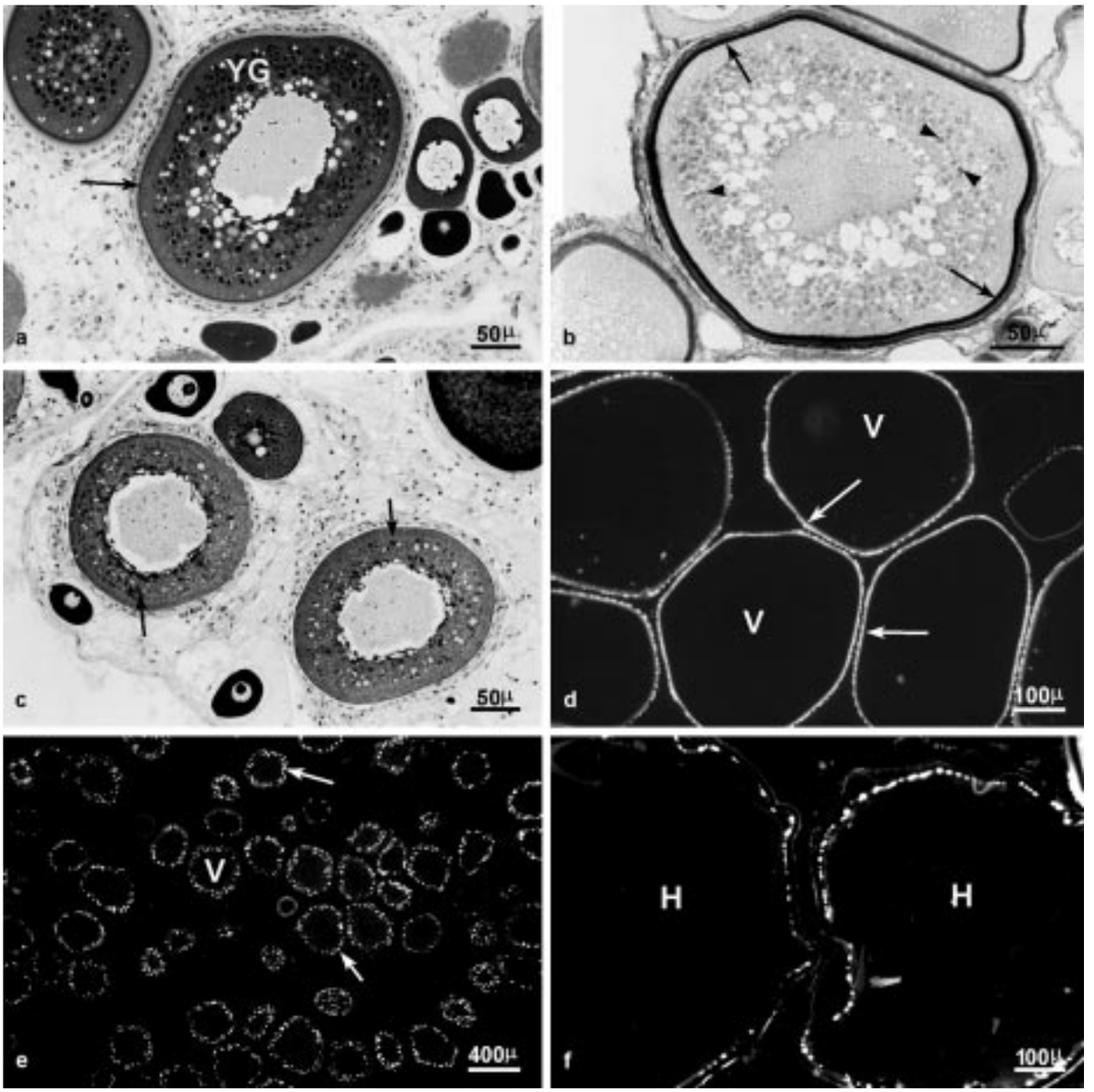

Fig. 5 - (a) Proteins in yolk granules (YG) and zona radiata interna (arrow) of a YII oocyte. **(b)** Tyrosine-enriched proteins in the zona radiata (arrow) and yolk granules (arrowhead) of a vitellogenic oocyte. **(c)** Granules containing neutral mucopolysaccharides (arrows) in the mid cytoplasm of LV oocytes. **(d)** WGA affinity (arrows) in vitellogenic oocytes (V) follicular envelope and zona radiata. **(e)** Strong affinity for SBA in cortical alveoli (arrows) of late vitellogenic (V) follicles. **(f)** Specific binding for GS-I in the cortical alveoli of hydrated oocytes (H). **(a)** Coomassie brilliant blue, **(b)** Morel-Sisley, **(c)** PAS-haematoxylin, **(d)** WGA-FITC, **(e)** SBA-FITC, **(f)** GS-I-FITC.

In *E. marginatus*, cytoplasmic inclusions occurred in oocytes larger than 130µm (LV oocytes). Firstly, lipid droplets containing neutral lipids appeared in the mid portion of the cytoplasm. Small granules, deeply stained with PAS reaction and characterised as polysaccharidic material, were visible at the end of this stage in the mid-outer portion of the cytoplasm. The presence of carboxylic groups was shown up by the positive reaction to Alcian blue, pH 2.5. The same histochemical characteristics were

observed in "cortical alveoli" which appeared at the periphery of cytoplasm in late vitellogenic oocytes. Lectin binding pattern confirmed the presence of neutral and acid sugar residues with an increase of the latter in late vitellogenic and hydrated oocytes.

Vitellogenic oocytes increased in diameter from 130 to 500µm, and the final stage of vitellogenesis was first observed in oocytes larger than 350µm. Yolk granules were PAS negative and contained proteins above all. Specific histochemical staining evidenced the increasing presence of tyrosine-enriched proteins from YI to YIII oocytes, while only small amounts of lysine-enriched proteins were detectable.

The follicular envelope, as a single layer, first appeared at the end of the PNS stage. A double cell layer started to be visible in LV oocytes and granulosa and theca cells were clearly visible from YI oocytes. During early vitellogenesis, these cells began to enter into contact with the oocyte through interweaving of the basal membrane**,** as usually observed in teleosts (Kagawa *et al*., 1981; Wallace and Selman, 1981) and the identifiable *zona radiata* appeared PAS positive. Successively, the *zona radiata* became divided in a ZRE and ZRI. ZRI contained tyrosine-enriched proteins, as evidenced by the Morel Sisley reaction.

Fully grown oocytes accomplished final maturation through several processes involving both nucleus and cytoplasm. The first event was the migration of the nucleus (germinal vesicle migration) to the animal pole, where the micropile was located. Immediately before and during this phase, lipid vesicles started to coalesce in dusky grouper oocytes. The GV appeared to migrate to the periphery together with the fusing lipid droplets as observed in *Dicentrarchus labrax* and *Morone saxatilis* (Alvariño *et al*., 1992; Mylonas *et al*., 1997). GV migration was observed very rarely in the ovaries analysed. The poor frequency of oocytes with GVM in relation to the total number of estimated oocytes undergoing final maturation within the same batch would suggest this phase to be very rapid in the dusky grouper. Germinal vesicle dissolution was followed by yolk globule coalescence. During GV migration and breakdown, oocyte diameter significantly increased as a consequence of oocyte hydration. Presumably GVBD and yolk globule coalescence took place concomitantly, since both phenomena were observed in the same analysed oocytes.

Atresia plays a major role in oocyte growth and recruitment in vertebrates. Few studies are focused on the cause of atresia and its role in regulating the number of oocytes progressing from stage to stage in teleosts. Although the atresia process has been poorly described in groupers, it was routinely observed in *E. tauvina* ovaries throughout the spawning season (Abu-Hakima, 1987). In *E. marginatus*, at the beginning of the reproductive season, some ovaries showed a large number of LV oocytes in α-stage atresia (in agreement with Hunter and Macewicz, 1985). Extensive atresia of LV and YI oocytes was observed in young *Epinephelus aeneus* females before the attainment of first sexual maturity (Hassin *et al*., 1997). Rare atretic vitellogenic oocytes were also observed in maturing ovaries, where the simultaneous presence of many healthy YIII oocytes demonstrated a regular reproductive activity as also reported for other teleosts (Hunter and Macewicz, 1985; Matsuyama *et al*., 1987; Hay and Brett, 1988; Barbieri *et al*., 1994; Lowerre-Barbieri *et al*., 1995). At the end of the reproductive season, the cessation of spawning was indicated by the presence of ovaries in which all vitellogenic oocytes were interested by the atretic process.

The anatomy and histology of teleost ovaries have been described for a lot of species. Few studies have been made to characterise the gonadal mucosubstances of fish other than their affinity for Alcian blue, PAS and other conventional staining. Lectins are carbohydrate-binding proteins that have been found to be powerful and reliable tools to characterise glycoconjugates in tissues (Lis and Sharon, 1986; Spicer and Schulte, 1992; Pajak and Danguy, 1993). Since lectins bind to specific carbohydrate residues (Lis and Sharon, 1986), the lectin binding patterns observed in this study could reflect characteristic differences in the content and distribution of specific sugar moieties expressed in the oocytes during vitellogenesis and final maturation. In our study, *zona radiata* and cortical alveoli, characterised by a higher content of carbohydrate residues compared with the oocyte itself, showed different patterns during oocyte growth. The strongest affinity observed in *zona radiata* and follicular envelope at the end of vitellogenesis was for WGA. Cortical alveoli in YIII oocytes showed strong affinity for DBA, SBA and GS-I, too. Lectins have been used to characterise the *zona pellucida* in mammals with light and electron microscopes (Skutelsky *et al*., 1994). Several authors have suggested that, in mammals, specific sugar residues in the *zona pellucida* are the key for

species specificity of the interaction between spermatozoa and oocytes. The high reactivity for WGA in *zona radiata* and cortical alveoli of dusky grouper postvitellogenic oocytes may reflect a high content of either N-acetyl-glucosamine or sialic acid residues, or both (Monsigny *et al.*, 1980) in these structures. Nevertheless, negative reaction for WGA, after pre-incubation with sialidase, and negative reaction to GS-II, appear to suggest the presence of sialic acid in these structures.

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