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Oogenesis in the amberjack *Seriola dumerili* Risso, 1810. An histological, histochemical and ultrastructural study of oocyte development*

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SUMMARY: Macroscopic and microscopic characteristics of the maturity stages of the ovary of the amberjack *Seriola dumerili*, Risso are reported. Oogonia, oocytes at different stages of development, follicular and thecal cells, and postovulatory follicles are described following a light and transmission electron microscope study. Histological observations of oocyte development show that the oocyte diameter increases about 100-fold during oogenesis, the nucleus-to-cytoplasm-ratio ranging from 72% (in oogonia) to 18% (in mature oocytes just before the breakdown of the germinal vesicle). Histochemical and ultrastructural studies show that three types of inclusions are formed during vitellogenesis. Lipid globules (exclusively composed of neutral lipids) appear first (at the end of the perinucleolar stage) followed by cortical alveoli (exclusively composed of carbohydrates). Yolk granules, which contain glycoproteins, glycogen and lipids appear last. The location and movements of these inclusions within the oocyte cytoplasm during oogenesis are described. The vitelline envelope or zona radiata (ZR) is first seen at the lipid globule stage I as a discontinous layer. This structure, mainly proteinaceous in nature, increases in thickness as oogenesis proceeds and peaks (33 µm) during the maturation phase.

Key words: Ovary, histology, histochemistry, ultrastructure, reproduction, fishes.

RESUMEN: ESTUDIO HISTOLÓGICO, HISTOQUÍMICO Y ULTRAESTRUCTURAL DEL DESARROLLO OVOCITARIO EN EL PEZ LIMÓN.– *Seriola dumerili* Risso, 1810. Se describen las características macroscópicas y microscópicas de los estados de maduración ovárica del pez limón, *Seriola dumerili* Risso, 1810. Las ovogonias, los ovocitos en diferentes estados de desarrollo, las células foliculares y tecales, y los folículos postovulatorios son descritos mediante su observación al microscopio óptico y al microscopio electrónico de transmisión. El diámetro ovocitario aumenta aproximadamente 100 veces durante la ovogénesis, pasando la relación núcleo-citoplasma del 72% (en las ovogonias) al 18% (en los ovocitos maduros anteriores a la desaparición de la vesícula germinal). Durante la vitelogénesis se observan tres tipos de inclusiones: primero aparecen los glóbulos lipídicos (compuestos exclusivamente de lípidos neutros) al final del estadío perinucleolar, seguidos de los alveolos corticales (compuestos de carbohidratos). Finalmente aparecen los gránulos vitelinos, que contienen glicoproteinas, glucógeno y lípidos. La envuelta vitelina o zona radiata (ZR) es observada por primera vez en el estadío de glóbulos lipídicos I como una capa discontínua. Esta estructura, principalmente proteica, aumenta de grosor a medida que la ovogénesis avanza, alcanzando su máximo tamaño (33 µm) durante la fase de maduración.

Palabras clave: Histología, histoquímica, ultraestructura, reproducción, ovario, peces.

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INTRODUCTION

The amberjack, *Seriola dumerili*, Risso (Carangidae) is a Perciform fish occurring in tropical and temperate waters. In Europe, this species has been recorded in the Mediterranean and along the Atlantic coast of the Iberian Peninsula as far north as the Gulf of Vizcaya. The amberjack is cultured in Japan (Masuma *et al.*, 1990) and in the Mediterranean area (Benovic, 1980; Giovanardi *et al.*, 1984; Navarro and Belmonte, 1987; Lazzari and Barbera, 1989; Boix *et al.*, 1993). It exhibits very high growth rates in intensive culture, reaching a weight of approximately 1-1.2 kg after nine months in seacages around the Balearic Islands (Pou, unpublished data). However, the future of the intensive culture of this species in the Mediterranean can be seriously compromised by two unsolved problems: pathologies (Crespo *et al.*, 1990, 1992, 1994; Grau and Crespo, 1991) and reproduction. Females do not spontaneously spawn in captivity and the amount of sperm produced by males is lower in captivity than in the wild (Pou, personal communication). Moreover, in contrast to what is described for the Japanese amberjack (Masuma *et al.*, 1990; Tachihara *et al.*, 1993), the development of larvae cannot be successfully achieved in Mediterranean mariculture facilities. Hence, the intensive culture of this species must rely on the capture of juveniles from the wild. It is, therefore, of prime interest to know the reproductive cycle of the Mediterranean amberjack, as a previous step to succeed in the reproduction of this species in captivity. Probably the best method for determining the reproductive cycle of a species is to study the seasonal developmental changes in the gonads, both macroscopically and histologically. But for accomplishing the description of the histological changes of the ovary, it is necessary first to undertake the study of oocyte development. Taking into account that the reports on oogenesis in *S.dumerili* are fragmentary (Micale *et al.*, 1993), the aim of the present investigation was, firstly, to carry out an histological, histochemical and ultrastructural study of oocyte development and, secondly, to make a brief histological and macroscopic classification of the maturity stages of the ovary in this species.

The terminology used in the literature to describe oocyte development in fish is somewhat confusing. According to Barr (1968), four main growth phases are described in marine teleost species: (1) oogonial proliferation by mitotic division; (2) oogenesis, i.e. the meiotic transformation of oogonia into primary oocytes; (3) oocyte growth, and (4) maturation. However, the term oogenesis is somewhat controversial since several authors (Tokarz, 1978; Wallace and Selman, 1981; De Vlaming, 1983) refer to it as exclusively the meiotic transformation of oogonia into primary oocytes, while other authors use this term to describe the whole of the transformations taking place from the oogonial stage to oocyte maturation (Raven, 1961; Lahaye, 1972; González de Canales *et al.*, 1992). Moreover, some reports only deal with oocyte growth without mentioning oogonial development (Wallace and Selman, 1981; Selman and Wallace, 1986; Iwamatsu *et al.*, 1988; Deniel *et al.*, 1989, Selman and Wallace, 1989). In the present study, we refer to oogenesis as the whole meiotic process accomplished by the female germ cells, that is to say, the complex transformations carried out by the oogonia up until the completion of the second meiotic division and their conversion into ovules (Foucher and Beamish, 1977; Schuetz, 1985). Some of these meiotic transformations occur outside of the ovary, but we only describe the stages of oogenesis which take place within the ovary, i.e. primary growth phase, secondary growth phase and maturation. Phases of oogenesis after the expulsion of secondary oocytes (ovulation) are not reported in the present work, although cellular elements within the ovary during the spawning period, such as postovulatory follicles and atretic oocytes, are described.

MATERIAL AND METHODS

Female amberjack (mature and developing immature) were captured from the wild in Port d'Andratx and Port de Pollença (Majorca, Spain). Fish were captured with nets (months of May, June and July) or trawls (November, January and March). The ovaries were macroscopically examined for maturity staging (Table 1). Portions of tissue were then taken from the central part of the left gonad and fixed in Baker's formal-calcium in order to preserve lipid components (Bankroft and Stevens, 1982). Tissues were embedded in either hydroxyethyl methacrylate or paraffin and, in the case of mature ovaries, in celoidin-paraffin (Pearse, 1985). Methacrylate and paraffin embedded samples were sectioned at 0.5-2 μm and 4-6 μm respectively. Sections were stained with Toluidine blue, with Mayer's haematoxylin and eosin (H/E) (Luna, 1968) and with Mayer's haematoxylin and V.O.F. (H/VOF)(a Trichromic dye composed by light green, Orange G and acid Fuchsine; Gutiérrez, 1967) for general histology.

TABLE 1.– Macroscopic and histological characteristics of the maturity stages of the ovary of *Seriola dumerili*.

Maturity stage	Macroscopic description	Histological description
I. Immature	Translucent small thread-like ovaries adhering to swimbladder wall; reddish-pink in colour.	Wide ovarian cavity; spaced ovigerous lamellae containing oogonia (nest of 4-5 cells) and primary oocytes at both chromatin-nucleolar (numerous) and perinucleolar (less numerous) stages.
II. Developing immature (or developing virgin)	Ovaries less than 1/3 length of visceral cavity (less than 1/2 length during reproductive season; white-yellow in colour; poorly vascularized; smooth appearance in section.	Smaller ovarian cavity; more developed lamellae with oogonia, primary oocytes (especially at perinucleolar stage: 30% during reproductive season) and oocytes at lipid globule stage I (LGSI): 30% during reproductive season.
III. Early developing	Ovaries 1/3 Length of visceral cavity; pink orange in colour; slightly granular; poorly vascularized.	Ovigerous lamellae fill ovarian cavity; very few oogonia; primary oocytes numerous (45% at perinucleolar stage); oocytes at LGSI also numerous (30%).
IV. Late developing	Ovaries 1/2 to 2/3 of visceral cavity. Slightly granular ovaries with opaque oocytes; orange in colour; external vascularization visible.	Oocytes at all stages of development (either primary or secondary). Very few oogonia (or not any). Follicular bodies not seen.
V. Running	Granular ovaries 2/3 to full length of visceral cavity; orange-yellow in colour; well vascularized; translucent and opaque oocytes visible; translucent eggs within ovarian cavity.	Oocytes at all stages of development (mature oocytes and postovulatory follicles numerous) Ovarian cavity increases in size as spawning proceeds (as well as the number of oogonia and atretic oocytes) ovulated eggs in ovarian cavity.
VI. Spent	Ovary flaccid; 2/3 length of visceral cavity; pink-orange in colour; rough appearance in section; no oocytes within the ovarian stroma; translucent oocytes, irregular in shape, in ovarian cavity; very thick ovarian wall; vascularization visible.	Wide central ovarian cavity, lamellae irregular in shape with atretic oocytes and postovulatory follicles. Oocytes at perinucleolar stage and at LGSI numerous. Not any vitellogenic oocyte.
VII. Recovering	Ovaries 1/3 length of visceral cavity; white-yellow in colour (slightly reddish); very thick ovarian wall, rough appearance in section; poorly vascularized; oocytes not obvious.	Wide ovarian cavity; lamellae irregular in shape. Primary oocytes numerous; oogonia increase in number (25%); oocytes at LGSI less numerous; postovulatory follicles not seen; some atretic oocytes present.
VIII. Resting	Ovaries less than 1/3 of visceral cavity; white in colour; very thick ovarian wall; smooth appearance in section; poorly vascularized.	Primary oocytes numerous as well as oogonia (20%). Atretic oocytes at LGSI are seen; very few oocytes at LGSI.

Histochemical reactions for lipids, carbohydrates and proteins were carried out. Lipids were detected in fixed non-embedded tissue sectioned at 7-8 μm with a Cryo-mycrotome and stained with oil red 0 and sudan black B for general lipids, with Nile Blue-sulphuric acid for phospholipids, and with aqueous Nile Blue for neutral and acidic lipids. Carbohydrate reactions for the detection of glycogen (Diastase-PAS), neutral mucosubstances (PAS), carboxilated mucosubstances (Alcian Blue, pH 2.5), sulphated mucosubstances (Alcian Blue, pH 1 and pH 0.5), glycoproteins (Concanavalin A) and their respective controls were performed. Histochemical reactions for proteins in general (Hg-Bromophenol Blue), and for those proteins rich in lysine (Ninhydrin-ninhydrin-Schiff), cystine (Thioglicolate-potassium-ferrycianide (Fe III)), arginine (1,2 Naphtoquinone-4-sulphonic acid, sodium salt), tyrosine (Hg sulphate-sulphuric acid-sodium nitrite), cysteine (Ferric ferrycianide (Fe III)) and tryptophan (p-dimethylaminobenzaldehyde) and their controls were also accomplished. References of these histochemistry methods are quoted in the monographs of Martoja and Martoja-Pierson (1970), Culling *et al.* (1985) and Pearse (1985).

Samples for the transmission electron microscope (TEM) study were fixed in 4% glutaraldehyde in 0.1 M Na cacodylate buffer (pH=7.3), post-fixed in 2% osmium tetroxide, dehydrated through an ethanol series, stained "en bloc" with uranyl acetate and embedded in araldite (Durcupan, Fluka). Ultrathin sections were stained on the grids with lead citrate and studied in a Hitachi H-7000 TEM.

Oocyte size was obtained, under the light microscope, by measuring the maximum and minimum diameters (Foucher and Beamish, 1980; Hay *et al.*, 1987). Only those oocytes which had been sectioned through the nucleus were measured.

The terminology used in the histological description of oocyte development of *Seriola dumerili* was a modification of that employed by Mayer *et al.* (1988) and Iwamatsu *et al.* (1988).

RESULTS

MATURITY STAGING OF THE OVARY

Eight different developmental stages were described after gross examination of the amberjack ovary (Table 1). The macroscopic classification is a modification of that reported by Holden and Raitt (1975). The histological description of each maturity stage is shown in Table 1. Immature ovaries (Stage I) were observed in each month sampled. We observed developing immature ovaries (Stage II) during May and June, early developing ovaries (stage III) in January, and late developing ovaries (Stage IV) in may. Running ovaries (Stage V), with orange-yellow and granular appearance, were observed during May and June, spent ovaries (Stage VI) at the beginning of July, recovering ovaries (Stage VII) at the end of July, and resting ovaries (Stage VIII) in November.

Macroscopic evaluation of the stage of ovarian maturity generally corresponded to that based on histological observations. However, during the reproductive season (May-June), some samples which had been classified as developing according to macroscopic features exhibited some postovulatory follicles under the light microscope, indicating that spawning had already started.

During the spawning season we distinguished the presence of postovulatory follicles and discrete batches of mature oocytes, together with an heterogeneous population of secondary developing oocytes. A large population of oocytes remained in the primary growth phase. These observations indicate that *Seriola dumerili* is a group-synchronous spawner, that is, at least two populations of oocytes can be distinguished at some time. In the amberjack, recruitment of the succesive clutches occurs from the large heterogeneous population of smaller oocytes at various stages of secondary growth.

HISTOLOGICAL AND ULTRASTRUCTURAL STUDY OF OOCYTE DEVELOPMENT

In the amberjack, oocyte development can be grossly divided into three phases based on the morphological features of developing oocytes and follicles. The primary growth phase includes oogonial and basophilic oocyte (chromatin-nucleolar and perinucleolar) stages. The secondary growth phase includes oocytes in different developmental stages: lipid globule stage, cortical alveolus stage and yolk granule stages I, II and III. The maturation phase involves oocytes undergoing maturation. During the spawning period, postovulatory follicles (the presence of which is indicative that spawning has already begun) and atretic oocytes can be distinguished in the ovary.

Primary growth phase

Oogonia: Oogonia are small rounded cells (9-13 µm diameter) with a pale voluminous spherical nucleus (nucleus-to-cytoplasm ratio or N/C= 72%) and a prominent nucleolus (Fig. 1a). No perinuclear chromatin was observed in oogonia, which makes them easy to distinguish from cells of the granulosa (i.e. follicle cells). Oogonia usually form clusters or nests in the vicinity of either vitellogenic oocytes or postovulatory follicles. The TEM study revealed the presence of electron-dense, amorphous material in the cytoplasm ("nuage" material), close to the nuclear envelope (Fig. 1b), and electron-dense patches, connecting mitochondria ("cement material", Selman & Wallace, 1981).

Chromatin-nucleolar stage: Primary oocytes (16-32 µm diameter) exhibited a strongly basophilic cytoplasm and a prominent nucleus (N/C=53%) (Fig. 1c) with several small nucleoli. With further development, the idiosome (i.e. non-basophilic component of Balbiani's vitelline body) became apparent in a juxtanuclear position: By the transition to the perinuclear stage, the idiosome became more conspicuous (Fig. 1d) or remained as a difuse structure in the yuxtanuclear position; the more basophilic pallial substance can be seen in its vicinity (Fig. 1d). "Nuage" material observed throughout the previous stage disappears and cement material was still very apparent (Fig. 1e). A single layer of flattened follicle cells enveloped the oocyte. A basal lamina covered the external aspect of the follicular layer. No thecal cells were distinguishable. Indentations of the oocyte plasma membrane were not seen during this stage.

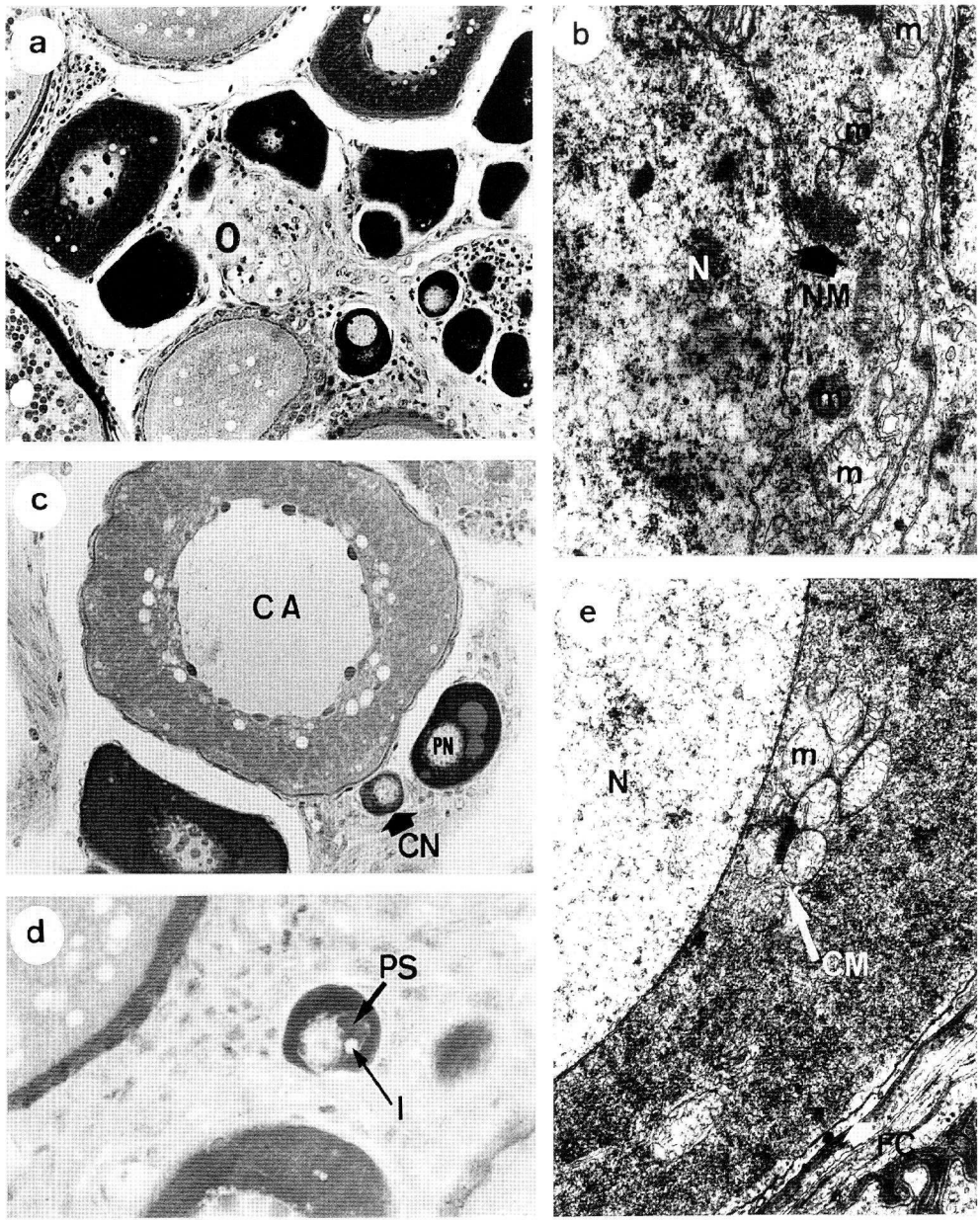

FIG. 1.– Oogonia and chromatin-nucleolar stage of oocytes of *Seriola dumerili*.**(a)** Oogonia. (260 X; methacrylate thin section LM, toluidine blue) Note the nest of oogonia (0) in the stroma of the ovigerous fold. **(b)** Oogonia. (16850 X, TEM) The presence of nuage material (NM) close to the nuclear envelope is observed. **(c)** Chromatin-nucleolar stage. (340 X; methacrylate thin section LM, H/E). A chromatin-nucleolar oocyte (CN) is seen between an early cortical alveolus stage oocyte (CA) and a perinucleolar oocyte (PN). **(d)** Late chromatin-nucleolar stage (540 X; methacrylate thin section LM, H/E) One nucleolus is seen within the nucleus, adjacent to the nuclear envelope. Note the presence of the idiosome (I) and the pallial substance (PS). **(e)** Chromatin-nucleolar stage (13600 X, TEM). Cement material (CM) is found between mitochondria in the cytoplasm. A very thin layer of follicle cells (FC) envelops the oocyte. m, mitochondria; N, nucleus.

Perinucleolar stage: Oocytes (28-84 μm diameter) exhibited a central nucleus (N/C=48%) which contained several round large peripheral nucleoli. At the early perinuclear stage, the idiosome disintegrated and disappeared. Pallial substance gradually increased in size, invading most of the cytoplasm, which gradually lost its basophilia (Fig. 1c, Fig. 2a). The electron-lucent pallial substance appeared, with TEM, to be surrounded by organelles, cement material and filamentous structures (Fig. 2b). Microvilli of the plasma membrane were seen on the oocyte surface facing the junctions of follicle cells connected by desmosomes. Oocytes were covered by three layers: (1) follicular cells, 2) a basement lamina and (3) thecal cells.

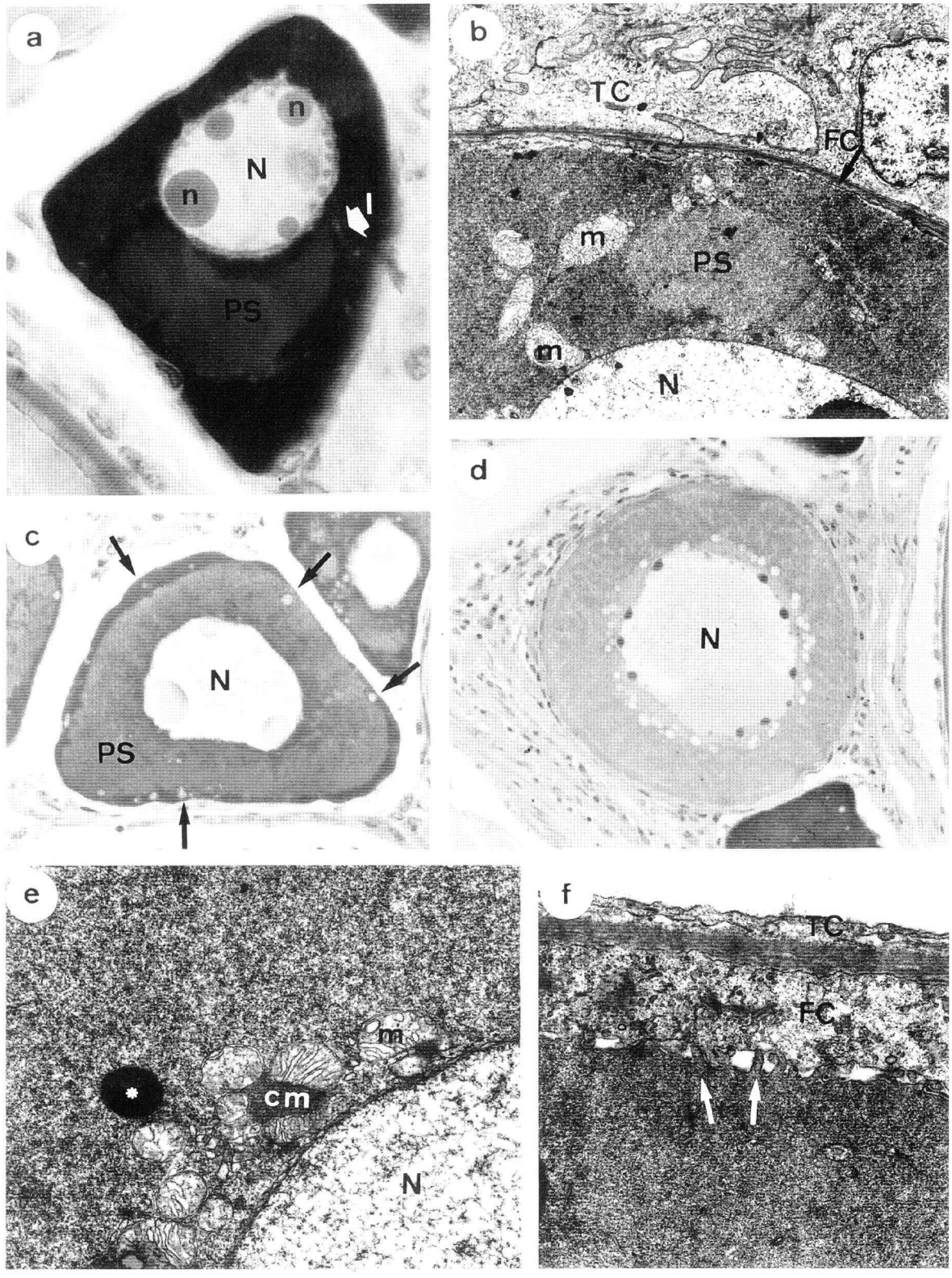

FIG. 2.– Perinucleolar and lipid globule stages of oocytes of *Seriola dumerili*.**(a)** Perinucleolar stage. (1350 X; methacrylate thin section LM, toluidine blue). Note prominent nucleoli (n) in the nucleus (N). The pallial substance (PS) and remnants of an idiosome (I) are visible in the oocyte cytoplasm. **(b)** Perinucleolar stage. (5950 X, TEM) Follicle cells (FC) are seen surrounding the oocyte. **(c)** Lipid globule stage (540 X; methacrylate thin section LM, H/E). Lipid globules (arrows) are seen in the cortical cytoplasm. **(d)** Lipid globule stage (340 X; methacrylate thin section LM, H/E). Lipid globules move centripetally as this stage proceeds and form a perinuclear layer. **(e)** Lipid globule stage. (13600 X, TEM). Lipid globules (asterisc) are devoid of a membrane. Remnants of cement material are seen between mitochondria (m). **(f)** Lipid globule stage. (15300 X, TEM) The microvilli formed by the oocyte plasma membrane are visible (arrow). Follicle cells (FC) and thecal cells (TC) envelop the oocyte. N, nucleus; m, mitochondria.

Secondary Growth Phase

Lipid globule stage: Oocytes in this stage were characterized by the appearance of small globules in the cortical zone of the cytoplasm (Fig. 2c). These globules increased in size and number throughout this stage as they moved from the outer cortex to the vicinity of the nucleus (Fig. 2d). Histochemical reactions carried out in fixed, non embedded tissues revealed that these globules contained exclusively lipids and they were, in fact, oil droplets (Fig. 7a). During the histological procedures, the content of these globules was dissolved and, therefore, they appeared as empty vacuoles on paraffin and methacrylate sections (Fig. 2c and 2d). Their diameter ranged from 1 to 6 μm. The cytoplasm exhibited several changes: it became granular and less basophilic (Fig. 2d) as the pallial substance disintegrated and disappeared (Fig. 2c). The nuclear envelope became indented. At the end of this stage a discontinous layer (the vitelline membrane or zona radiata) appeared around the oocyte. Oocyte size was 53-130 μm (N/C = 51%). Under the TEM, the Balbiani's vitelline body was first seen adjacent to the nucleus. It progressively increased in size and then disintegrated. Cement material disappeared at the end of this stage. Multivesicular bodies occured in the cytoplasm of both oocytes and follicle cells (like in figure 3b). Lipid globules appeared at TEM strongly osmiophilic, in accordance with their lipid content. They were devoid of external membranes and were mainly found adjacent to clusters of cytoplasmic organelles (Fig. 2e). The oocyte plasma membrane exhibited microvilli on the oocyte surface facing microvilli (Fig. 2f) projecting from the surface of follicle cells (which measure 1mm in width). At the end of the lipid globule stage, the vitelline envelope appeared between these projections.

Cortical alveolus stage: In the cortical alveolus stage oocytes measured 101-178 μm (NC= 50%) and were characterized by the appearance of a second layer of globules in the cortical zone of the cytoplasm (Fig. 1c, Fig. 3a). PAS staining revealed that these globules were strongly PAS+ cortical alveoli (6-12 μm diameter; Fig. 7a). The cytoplasm became more acidophilic and the vitelline envelope, also strongly acidophilic and exhibiting a high affinity for eosin and light green dyes, appeared continuous at the periphery of the oocyte (Fig. 3a). TEM observations revealed the presence of multivesicular bodies in oocytes (Fig. 3b) as well as in follicular (1.2 μm thick) and thecal cells (not shown in figures). Electron-lucent vesicles, which exhibited a granular content, appeared in the cortical cytoplasm. The vitelline envelope (Fig. 3b) increased in thickness (0.2-0.8 μm) and, at the end of the stage, a new layer (the electron-dense Z2 layer; Fig. 3c) formed by deposition of granular material. Small electron-lucent vesicles were observed in the cortical cytoplasm.

Yolk granule stage I: Oocytes in the yolk granule stage I measured 170-275 μm (N/C = 40%). This stage is characterized by the appearance of acidophilic small granules (1-3 μm) in the cortical cytoplasm of the oocyte (Fig. 3d). These granules, which had a high affinity for eosin and orange G dye, appeared within the peripheral layer of oil droplets and formed columns with a maximum length of 6-18 μm (up to 6 granules). Lipid globules, which measured 6-16 μm, occupied the greater part of the cytoplasm with the exception of the perinuclear region. The vitelline envelope, as well as the follicular layer, increased in thickness. The nuclear envelope appeared indented. TEM observations revealed that yolk granules, which were surrounded by a membrane and displayed an electron-dense central zone, fused with each other. Within the vitelline envelope, a new layer (Z3 or zona radiata interna ZRI; Fig. 3e) was formed by deposition of electron-dense fibrilar material. The follicle cells increased in size (3-4.4 μm) and modified cells appeared in both the theca and the granulosa (Fig. 3e), exhibiting a well developed smooth endoplasmic reticulum with dilated cisternae, abundant mitochondria, Golgi complexes, and secretory granules.

Yolk granule stage II: Oocytes in this stage measured 178-298 μm (N/C=33%). Yolk granules increased in number and size (4-6 μm). They formed columns of 10-12 granules each and occupied the central part of the oocyte reaching, at the end of the stage, the inner layer of oil droplets (6-22 μm in size) (Fig. 4a). Within the vitelline envelope (5μm thick), the ZRE (Zona radiata externa) appeared more eosinophilic than the ZRI (Zona radiata interna). The follicular envelope reached its greatest thickness (8 μm) and the nucleus was very convoluted. The TEM study revealed the appearance of annulate lamellae (Fig. 4b). The deposition of new fibrilar material was observed in the Z3 layer and the vitelline envelope pores became apparent (Fig. 4c). The ZR was formed by 3 layers: Z1, Z2 and Z3. The electron-luscent Z1 and the electron-dense Z2 were both granular and form the ZRE, while the electron-dense Z3 (or ZRI) was a multilamellar structure composed of 13-14 sheets. The micropylar cell was observed in the follicular envelope. At the end of this stage a perioocyte space appeared (Fig. 4c).

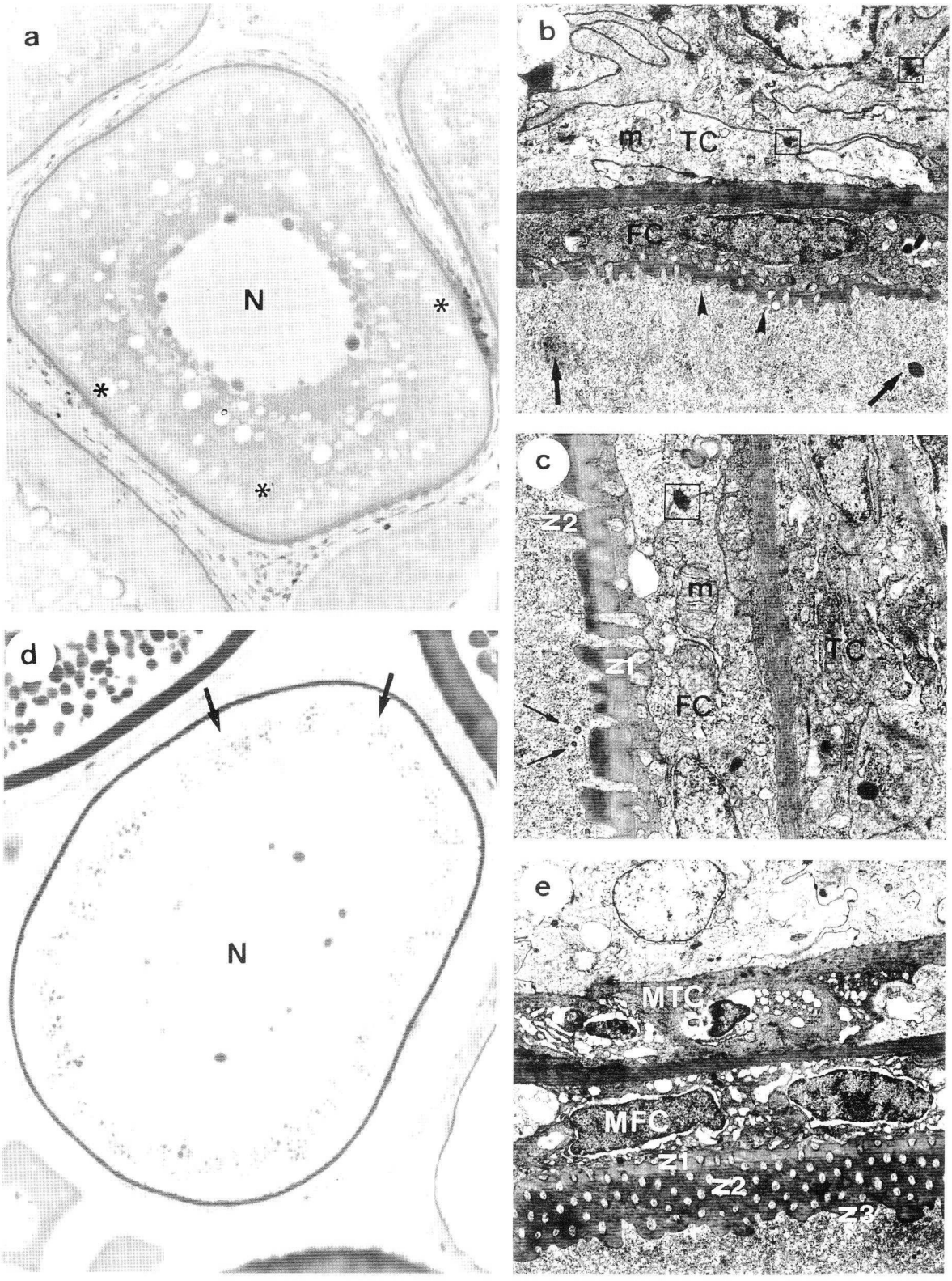

FIG. 3.– Cortical alveolus stage and yolk granule stage I of oocytes of *Seriola dumerili*.**(a)** Cortical alveolus stage (345 X; methacrylate thin section LM, H/E). Note the presence of a second layer of lipid vacuoles (asterisc) at the cortical cytoplasm. **(b)** Cortical alveolus stage (11900 X, TEM). Multivesicular bodies are seen in the cytoplasm and the vitelline envelope is clearly visible (arrowheads). **(c)** Cortical alveolus stage (13600 X, TEM). The electron-lucent Z1 and the electron-dense Z2 layers of the vitelline envelope are observed at the end of this stage. Small vesicles appear (arrow) in the outer cortex of the oocyte. **(d)** Yolk granule stage I (345X; methacrylate thin section LM, H/VOF). Note the presence of small granules in the cortical cytoplasm (arrow). **(e)** Yolk granule stage I (5950 X, TEM). A third layer (Z3) appears in the vitelline envelope. Modified thecal cells (MTC) and modified follicle cells (MFC) are observed. N, nucleus; m, Mitochondria; ⊐, desmosome.

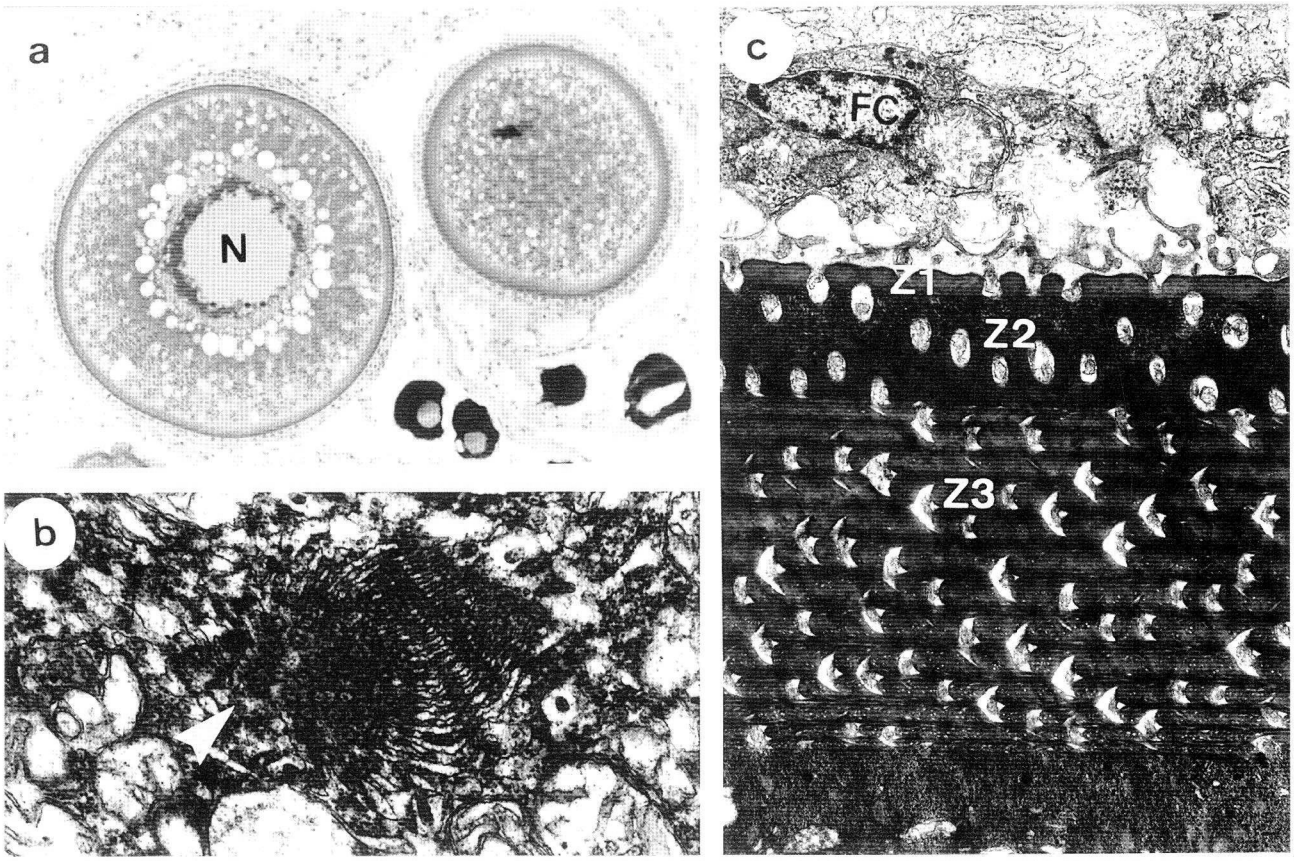

FIG. 4.– Yolk granule stage II of oocytes of *Seriola dumerili*. Note, in **a** (135 X; methacrylate thin section LM, toluidine blue) yolk granules reaching the inner layer of lipid globules. **(b;** 25500 X, TEM) Annulate lamellae (arrow) are seen in the oocyte cytoplasm at this stage. **(c;** 8500X, TEM) The vitelline envelope is formed by three layers (Z1, Z2 and Z3). N, nucleus; FC, follicle cell.

Yolk granule stage III: Yolk granules (6-18 μm) increased in number and reached the inner layer of lipid droplets (14-26 μm) (Fig. 5a), which moved towards the innermost cytoplasmic region where they initiated coalescence. The perinuclear space disappeared. The vitelline envelope increased in thickness (8-29 μm) and became striated (Zona radiata). The micropyle was observed for the first time under the light microscope. Intercellular spaces between follicle cells enlarged. Oocytes measured 231-454 μm (NC=27%). The TEM revealed that the perioocytary space enlarged and the vitelline envelope became more compact (Fig. 5b). Oocyte and follicle cell processes (measuring 0.09 and 0.2 μm respectively) were seen through the vitelline envelope pores.

Maturation Phase

Oocyte maturation was characterized by the migration of the nucleus (or germinal vesicle) towards the animal pole (Fig. 5c) and its subsequent breakdown (N/C before nucleus breakdown = 18%). As the germinal vesicle migrated, yolk granules and lipid globules started to coalesce and the formation of a single oil drop was observed (Fig. 5c). Oocytes rapidly increased in volume due to hydration (221% increase in cell volume) and appeared translucent. The vitelline envelope (now termed the chorion) exhibited a more compact aspect (26-33 μm). The follicle decreased in thickness due to the rapid increase in oocyte volume. A large perioocyte space was observed and the micropyle was apparent. Completely mature oocytes now appeared homogeneous and measured about 930 μm. They were ovulated as secondary oocytes after the first polar body was released. Fresh ripe eggs, which measured 1031-1163 μm prior to fixation, appeared translucent and homogeneous and contained a single oil globule (281 μm diameter). The TEM revealed the appearance of an electron-dense sheet within the Z1 layer (Fig. 5d) resulting from the compression of the chorion. Chorionic pores become narrower and intercellular spaces between follicle cells enlarged. Neither multivesicular bodies nor fibrilar structures were found within the cytoplasm. Cortical alveoli were observed without coalescing.

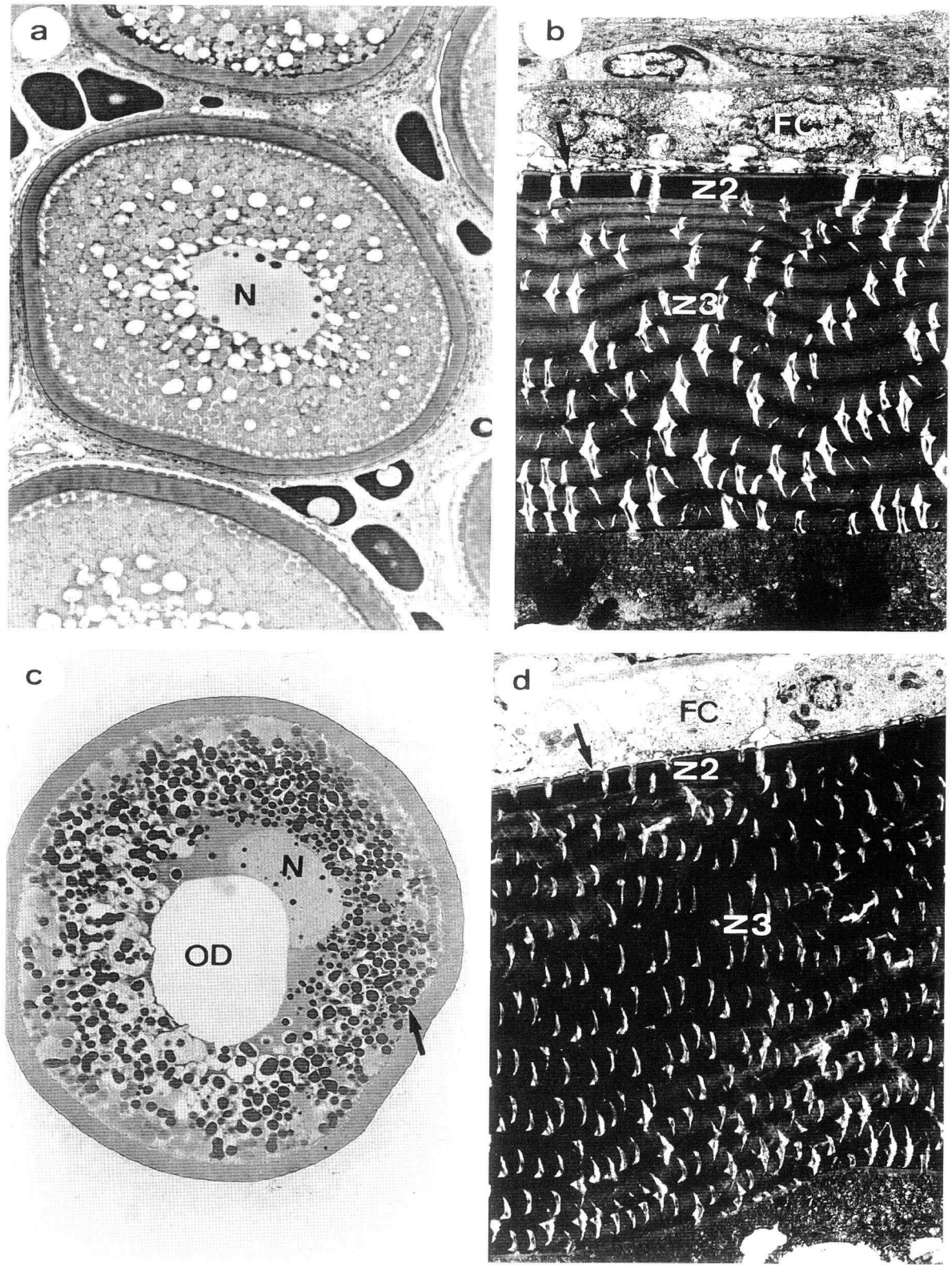

FIG. 5.– Yolk granule stage III and maturation phase of oocytes of *Seriola dumerili*. **(a)** Yolk granule stage III (135 X, methacrylate thin section LM, H/E). Yolk granules fill the oocyte cytoplasm. Note the disappearance of the perinuclear space. **(b)** Yolk granule stage III. (3400 X, TEM) The vitelline envelope becomes more compact and the perioocyte space larger. An electron-dense thin layer appears at the outer Z1 layer (arrow). **(c)** Maturation Phase. Migratory nucleus stage (100 X, methacrylate thin section LM, H/E) Note the germinal vesicle migrating towards the animal pole and the coalescence of the yolk granules and lipid droplets (→). A single oil drop is forming (OD). **(d)** Maturation phase (3400 X, TEM). The vitelline envelope appears more compact and the new-formed electron-dense layer within the Z1 (arrow) more visible. N, nucleus; TC, thecal cells; FC, follicle cells.

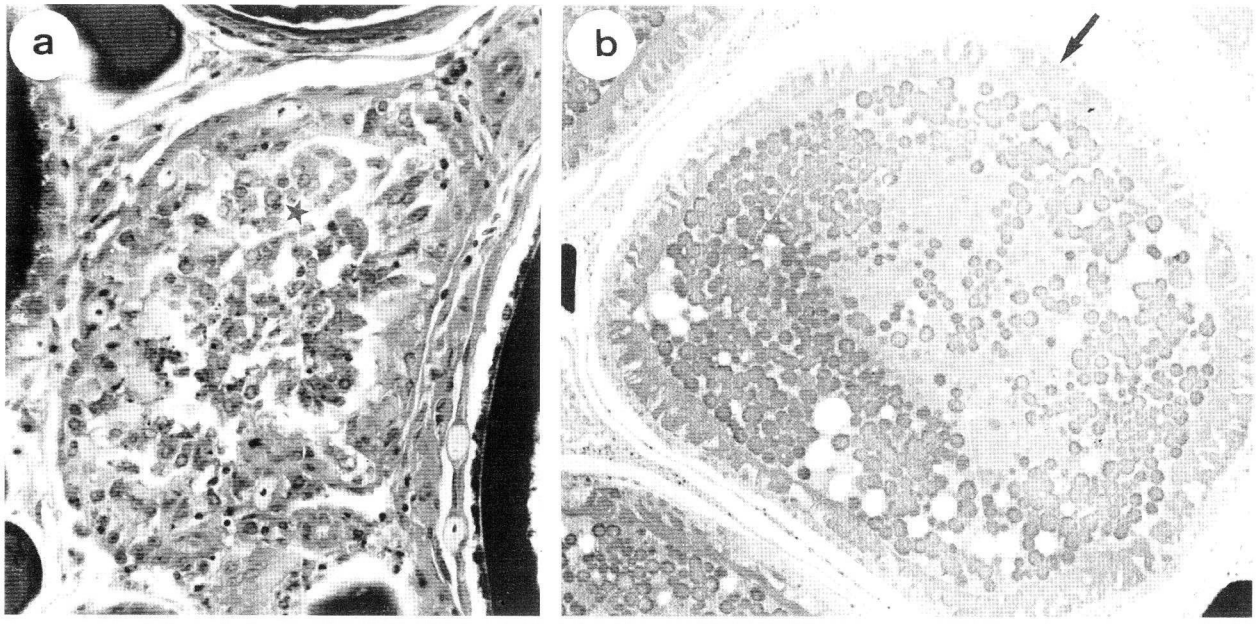

FIG. 6.– Postovulatory follicle and atretic oocyte of *Seriola dumerili*. **(a)** Postovulatory follicle (340 X, methacrylate thin section LM, toluidine blue). The presence of blood remnants (star) within the follicular cavity is observed. **(b)** Atretic oocyte (130 X; paraffin section LM, H/E). Note the fragmentation of the vitelline envelope (arrow).

Postovulatory follicles

Empty follicular envelopes were observed within the ovary after ovulation. These envelopes appeared as flat folded structures and might contain blood remnants (Fig. 6a) They are termed postovulatory follicles or corpora lutea. These structures degenerated and were finally observed as a fibrous mass or corpora albicans. The presence of postovulatory follicles indicated that the spawning period had already initiated.

Atretic oocytes

Those oocytes at the end of the tertiary granule stage can, according to Wasserman and Smith (1978), either undergo maturation in response to the appropiate hormonal stimulus, or become atretic. All the ovaries sampled during the spawning season exhibited some atretic oocytes. The number of atretic oocytes increased during the spawning period. At the onset of atresia, the vitelline envelope of the oocyte began fragmentation. Follicle cells hypertrophy, invaded the oocyte through the ruptured ZR and resorbed the oocyte cytoplasmic content (Fig. 6b). The resulting structure, which consists of follicle cells, appeared compact and well vascularized and was termed the corpus atreticum. Eosinophilic granules were observed within the cytoplasm of follicle cells which, in turn, underwent degeneration. The corpus atreticum turned into a fibrous mass within the ovarian stroma (corpus albicans).

HISTOCHEMISTRY OF OOCYTE DEVELOPMENT

The oocyte cytoplasm contained a light quantity of glycoproteins, neutral mucosubstances and glycogen, the amount of which did not vary throughout development. Sulphated mucosubstances were not found at any stage. Carboxylated mucosubstances increased in the cytoplasm during oocyte development, the amount of which varied from a light to a moderate quantity. The oocyte cytoplasm was moderately rich in proteins. Proteins rich in basic aminoacids (Lys-Arg) increased as vitellogenesis proceeded. The lipid globules (oil droplets) that characterized the onset of vitellogenesis contained neutral lipids exclusively (Fig. 7a). Yolk granules mainly contained proteins and a moderate quantity of lipids (neutral and acid lipids and phospholipids) and carbohydrates (glycogen and glycoproteins). Cortical alveoli (Fig. 7b) contained exclusively carbohydrates (neutral and carboxylated mucosubstances). The ZR was mainly proteinaceous in nature and did not contain lipids. The ZRE gave a stronger PAS-positive reaction (glycoproteins) than the ZRI (Fig. 7b). The ZRI contained a small quantity of proteins rich in cystine (Fig. 7c).

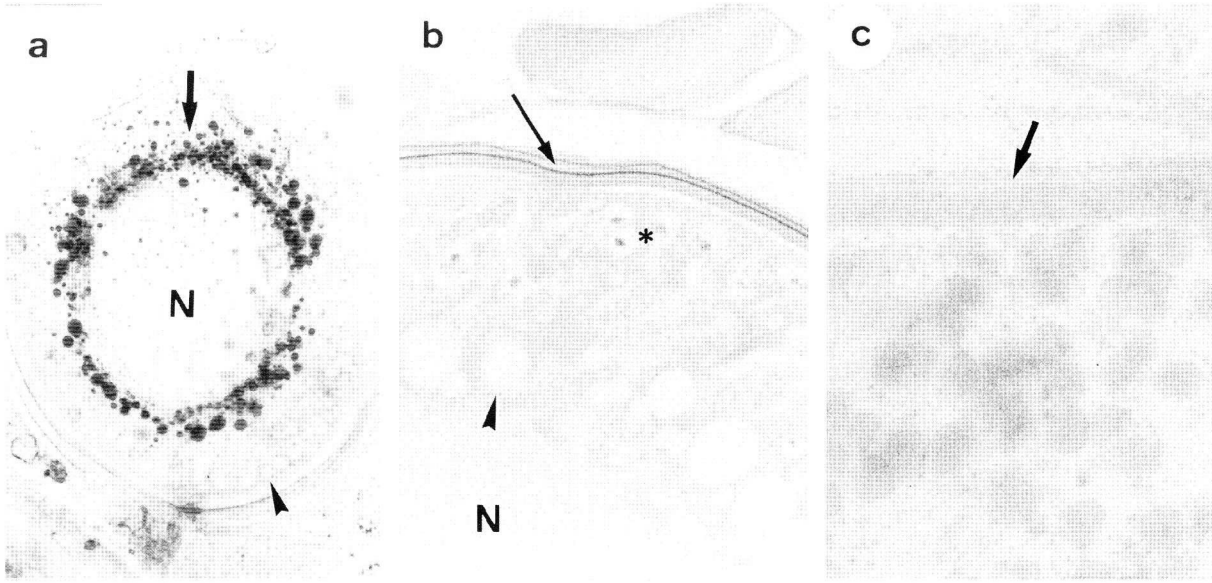

FIG. 7.– Histochemical reactions during oocyte development of *Seriola dumerili*. **(a;** 270x, fixed non-embebed section LM, oil red 0) Oil droplets (arrows) are positive to lipids, whilst cortical alveoli are negative (arrowheads). Note in **(b)** (540 X, paraffin section LM, PAS) a stronger PAS positive reaction of the ZRE (arrow) as well as the yolk vesicles (asterisc). Oil droplets are negative to PAS. **(c;** 540 X, paraffin section LM, Thioglicolate-potassium ferricyanide (Fe III)) Proteins rich in cystine are demostrated in the ZRI while the ZRE (arrow) does not exhibit a positive reaction.

DISCUSSION

During the oogenesis of teleost species, the oocyte primary growth phase (Wallace and Selman, 1981), also termed previtellogenesis (Raven, 1961; González De Canales *et al.*, 1992), includes oogonia and basophilic oocytes. It is a gonadotropin independent phase (Khoo, 1979) where important nuclear transformations occur (Barr, 1968; Tokarz, 1978). According to Selman and Wallace (1989), the oocyte follicle is fully developed at the end of the primary growth phase. In the amberjack, the yolk nucleus or Balbiani's body (Raven, 1961) also termed the Balbiani's vitelline body (Beams and Kessel, 1973) appears at the chromatin-nucleolar stage and exhibits similar features to those described for other teleost species (Beams and Kessel, 1973; Wallace and Selman, 1981; Iwamatsu *et al.*, 1988; Mayer *et al.*, 1988; Coello and Grimm, 1990). The Balbiani's vitelline body comprises 2 parts (Beams and Kessel, 1973), the idiosome, also called the yolk nucleus (Coello and Grimm, 1990) and the pallial substance which, in *S. dumerili*, disintegrates during the lipid globule stage. In other fish species the presence of these structures within previtellogenic oocytes is not mentioned (Bengen *et al.*, 1991; González De Canales *et al.*, 1992). Although their existence is well documented in the literature, their function is not understood. Guraya (1979, 1986) suggested that the yolk nucleus might function as a centre for the formation of various organelles within the oocyte during the primary growth phase. Balbiani's bodies in *S. dumerili*, as well as in other teleost species (Selman and Wallace, 1989), are not homogeneous structures: they are composed of amorphous electron-dense material and various cytoplasmic organelles. The electron-dense material is found either freely within the cytoplasm (nuage material) or bound to mitochondria (cement material) and has been shown to consist of RNA (Selman and Wallace, 1989) and proteins (Kessel, 1983). Its function might be related to the stocking of long-lived RNA (Kessel, 1983). According to Toury *et al.* (1977), cement material contains not only a great variety of RNA but most of the proteins which will be included in mitochondria. Both nuage and cement material have been described in oogonia of the golden grey mullet *Mugil auratus* (Bruslé and Bruslé, 1978) and are considered as germ cell markers (Hogan, 1978).

In several teleost species the oocyte secondary growth phase or vitellogenesis has been divided into two subphases: endogenous vitellogenesis (Shackley and King, 1977) and exogenous or true

vitellogenesis (Wallace and Selman, 1981). Three types of inclusions are in general formed during vitellogenesis of teleosts, although the sequence of their appearance varies greatly with the species. In most teleosts (Yamamoto *et al.*, 1965; Lahaye, 1972; Selman and Wallace, 1986, 1989; Landry and McQuinn, 1988; Bengen *et al.*, 1991; Sarasquete *et al.*, 1993), cortical alveoli appear first, followed by lipid globules. However, lipid globules have not been reported in a few species (Khoo, 1979; Wallace and Selman, 1981; Landry and McQuinn, 1988), while in the japanese sea bream *Pagrus major*, they have been described to appear last (Matsuyama *et al.*, 1987). In the seabass *Dicentrarchus labrax* (Mayer *et al.*, 1988), lipid globules accumulate first, followed by yolk granules and finally by cortical alveoli. In *S. dumerili*, as well as in other species, such as the striped bass *Morone Saxatilis* (Groman, 1982), the medaka *Oryzias latipes* (Iwamatsu *et al.*, 1988) and the teleost *Blennius pholis* (Shackley and King, 1977) the sequence is: 1, lipid globules, 2, cortical alveoli, and 3, yolk granules. According to Shackley and King (1977), the appearance of lipid globules might indicate the start of endogenous vitellogenesis, in those species which exhibit such inclusions first. In other teleost species (Selman and Wallace, 1989; González De Canales *et al.*, 1992; Sarasquete *et al.*, 1993) cortical alveoli (which appear first) are the markers of the onset of endogenous vitellogenesis. In most teleosts (Wiegand, 1982; Nagahama, 1983; Selman and Wallace, 1989), lipid globules are first found at the vicinity of the nucleus and then move to the periphery of the oocyte.

On the contrary, in *S. dumerili* (present study) and *D. labrax* (Mayer *et al.*, 1988) lipid globules are first seen in the cortical cytoplasm and then migrate to the juxtanuclear region. Our histochemical study shows that, in the amberjack, lipid globules are composed exclusively of neutral lipids. They are dissolved during histological embedding procedures, which suggests they are not bound, as in gilt-head seabream *Sparus aurata* (Sarasquete *et al.*, 1993). Although their origin seems to be endogenous, Mommsen and Walsh (1988) hypothetized that they might be synthetized outside the oocyte and subsequently transported into it by a lipoprotein other than vitellogenin. In *S. dumerili*, as in other teleosts (Nagahama, 1983), lipid globules increase in size and start to fuse with each other before ovulation. The fusion of the lipid globules leads to the formation of a single oil drop which will act as an hydrostatic device in the amberjack, as well as in other fish with pelagic eggs (Groman, 1982). Our histochemical observations show that cortical alveoli, which are formed in *S. dumerili* following the appearance of lipid globules, are exclusively composed of carbohydrates, similarly to that is described for *S. aurata* (Sarasquete *et al.*, 1993). On the contrary, in other teleost species (Khoo, 1979; Gutiérrez *et al.*, 1985; González De Canales *et al.*, 1992), the cortical alveoli also contain proteins. The autosynthetic origin of the cortical alveoli has been pointed out (Nagahama, 1983) and, as their content is released during fertilization (Selman *et al.*, 1988), they cannot be considered as true yolk. The yolk granules of *S. dumerili* oocytes, as well as those of *S. aurata* (Sarasquete *et al.*, 1993) contain glycoproteins, glycogen and lipids. In some species, these granules are PAS-negative (Khoo, 1979, Mayer *et al.*, 1988), whereas in others they contain acid mucosubstances (Aketa, 1954; Yamamoto, 1956; Gutiérrez *et al.*, 1985).

The funtion of carbohydrates and lipids present in the yolk is to supplement the nutritional requirements of the embryo during the first stages of life, when it does not receive external nutritional contributions and its metabolism of carbohydrates and lipids is very intense (Brachet, 1950). The function of phospholipids observed in the yolk granules of the amberjack might be related to the transfer of lipids from the yolk reserves to the embryo, as Fraser *et al.* (1988) hypothesized for the cod *Gadus morhua*. In teleosts, the lipids and proteins which form yolk granules are mostly of exogenous origin (Nagahama, 1983). Yolk precursors (vitellogenin) are synthetized in the female liver during ooogenesis, released into the blood and transported to the ovary (Wallace, 1978; Selman and Wallace, 1983). After proteolysis, vitellogenin splits into lipovitelin and phosvitin, which are both found in yolk granules (Wallace and Begovac, 1985). Multivesicular bodies, which as in *S. dumerili* have been described in the pipefish *Syngnathus scovelli* to be present throughout vitellogenesis and disappear during the maturation phase (Begovac and Wallace, 1989), seem to play an important role in these proteolytic processes (Selman and Wallace, 1989). Multivesicular bodies are considered as a type of lysosome since they contain acid phosphatase and catepsin (Sire and Vernier, 1991). Within the teleost vitellogenic oocyte, the occurrence of typical lysosomes has not been reported, which is not unexpected since lysosomes could digest exogenous proteins and prevent yolk formation (Wallace and Hollinger, 1979).

In *S. dumerili*, as in *O. latipes* (Iwamatsu *et al.*, 1988), the vitelline envelope is first seen at the lipid globule stage I. During the perinucleolar stage the plasma membrane of both oocytes and follicle cells becomes indented. These indentations transform into micro- and macrovilli which cross the vitelline envelope and allow the transfer of information between the oocyte and follicle cells (Selman and Wallace, 1982). At the end of the yolk granule stage I, modified follicular and thecal cells are observed which have also been described in other species (Nagahama *et al.*, 1978; Wallace and Selman, 1980; Iwamatsu and Ohta, 1981; Iwamatsu *et al.*, 1988; Kagawa, 1991; Kagawa *et al.*, 1981) and have been related to the synthesis of hormones inducing maturation (Wallace and Selman, 1980). The vitelline envelope of teleosts is composed of 3 layers: Z1, Z2 and Z3 (Begovac and Wallace, 1989), also termed zona externa, interna and subinterna (Gillis *et al.*, 1990). In *S. dumerili*, during the maturation phase, the compacting of the vitelline envelope layers is apparent as the oocyte microvilli disappear, although remnants of vitelline envelope pores can still be observed. On the contrary, Kagawa (1991) reports the complete disappearance of the vitelline envelope pores in the yellowtail, *Seriola quinqueradiata* at this stage. The Z3 (or ZRI) layer of the amberjack contains proteins rich in cystine, which suggests that most of the proteinaceous material of this multilamellar layer is ordered by the formation of disulphide bonds (Hagenmaier, 1973; Mayer *et al.*, 1988).

Macroscopic maturity stages of the ovary of *S. dumerili* generally corresponded to the microscopic classification, based on oogenesis stages. However, during the reproductive season, the onset of spawning could only be detected microscopically by the presence of postovulatory follicles. The histological and histochemical features of oocytes, and the presence of postovulatory follicles (Hunter and Goldberg, 1980) lead us to state that the spawning season of *S. dumerili* in the Balearic Islands occurs at the end of spring and the beginning of summer, earlier than previously reported for the same species in other Mediterranean areas (Lazzari and Barbera, 1988, 1989). Despite the knowledge of the spawning season in the amberjack, their reproductive cycle has not been described until now. We hope that the present study of oogenesis in the amberjack, which has permitted us to delineate the maturity stages of the ovary in this species (macroscopic and histological, table 1), will contribute to improve the knowledge of their reproductive cycle in the different regions of the mediterranean basin and will be of benefit to basic fisheries research on the amberjack.

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