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Gross and histological observations of ovarian development in twaite shad, *Alosa fallax fallax*, from the Rivers Mira and Guadiana (Portugal)*

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SUMMARY: In order to describe the stages of oogenesis of twaite shad, *Alosa fallax fallax*, 265 females were collected between March and June 1997, February and June 1998 and January and April 1999 in the Rivers Mira and Guadiana. From the histological study of ovaries a total of eight developmental stages were delineated. Gross examination of paired ovary revealed that they could be placed into one of seven maturity stages according to their stage of development. Two stages of atresia, *alfa* and *beta*, were identified. Upon cessation of spawning, the ovaries still contained some oocytes at various stages of development but with a greater number of atretic oocytes. The simultaneous occurrence of oocytes at different stages of development in the ovary indicates asynchronous oocyte development. Oocyte size frequency distributions do not show a gap in size between cortical alveoli and vitellogenic oocytes during the spawning season. This may represent the ability of twaite shad to push oocytes through vitellogenesis from a previtellogenic condition during the spawning period. This has important implications for twaite shad fecundity, because fish with this type of oocyte development depend on estimates of batch fecundity and spawning frequency to determine potential annual fecundity.

Key words: oogenesis, oocyte dynamics, *Alosa fallax fallax,* Portugal.

RESUMEN: OBSERVACIONES DE VISU E HISTOLÓGICAS SOBRE EL DESARROLLO DEL OVARIO DE LA SABOGA, *ALOSA FALLAX FALLAX*, DE LOS RÍOS MIRA Y GUADIANA (PORTUGAL). – Con objeto de describir las etapas de la oogénesis de la saboga, *Alosa fallax fallax*, se recolectaron 265 hembras entre marzo y junio de 1997, febrero y junio de 1998 y enero y abril de 1999 en los ríos Mira y Guadiana. A partir del estudio histológico de los ovarios se definieron ocho estadíos de desarrollo. La observación de visu del ovario par reveló que, dependiendo de su estado de desarrollo, podría ubicarse en uno de los siete estadíos de madurez . Se identificaron dos estadíos de atresia: alfa y beta. Hasta que la freza finaliza, los ovarios tenían todavía algunos oocitos en diferentes estadíos de madurez, aunque presentado un gran número de oocitos atrésicos. La aparición simultánea de oocitos en diferentes estadíos de madurez en el ovario indica que su desarrollo es asincrónico. Las distribuciones de frecuencias de talla de los oocitos no mostró separación por tamaño entre los oocitos con alveolos corticales y los vitelogénicos durante la estación de puesta. Esto podría representar la capacidad de la saboga para forzar el paso de los oocitos en estado previtelogénico a vitelogénico durante la fase de puesta. Este hecho tiene implicaciones importantes para la fecundidad de la saboga, porque la determinación de la fecundidad potencial anual en un pez con este tipo de desarrollo oocitario dependerá de la estima de la fecundidad en cada tanda de puesta y de la frecuencia de las mismas.

Palabras claves: oogénesis, dinámica oocitaria, *Alosa fallax fallax*, Portugal.

INTRODUCTION

The twaite shad, *Alosa fallax fallax*, is an anadromous species that inhabits the North-East Atlantic Ocean and adjacent waters, from the Christiana Fjord to the Iberian Peninsula and the coast of Morocco (Quignard and Douchement, 1991). Anadromous species are highly vulnerable and particularly threatened by anthropogenic activities. Therefore, twaite shad is rare or even extinct in Europe, despite the existing legal protection for

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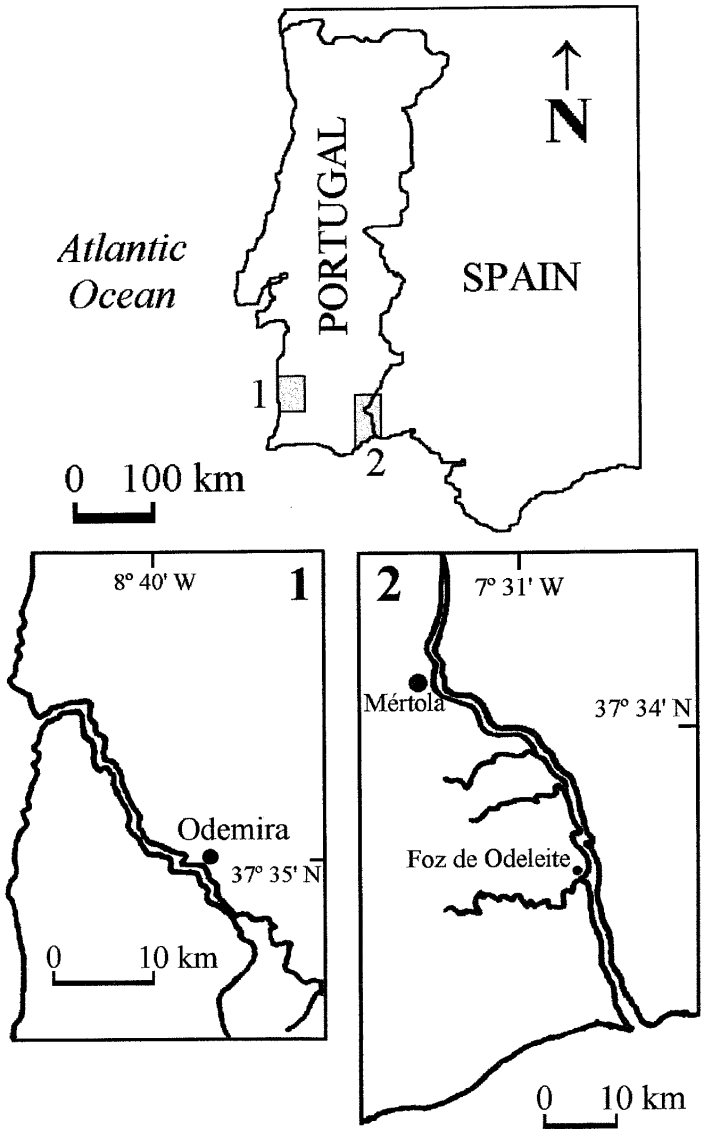

FIG. 1. – Map of Portugal, showing the areas in which the samples were collected: 1, River Mira; 2, River Guadiana.

migratory species (Lelek, 1980). This species still migrates into the River Mira and the River Guadiana, Portugal, to spawn. In these rivers, spawning migration is triggered by favourable environmental conditions, such as the increase in water temperature, and starts between March and April, when adult twaite shad congregate in the sea near the mouth of the river. Subsequently, they enter with the rising tide, migrating to the spawning areas in the upper estuary to spawn. The spawning season can last until June (Pina, 2000).

Several studies have been undertaken on *Alosa* spp., mainly regarding age and growth (Walburg, 1960; Mennesson-Boisneau *et al.*, 1986), migration patterns (Rameye *et al.*, 1976; Loesch, 1987; Quinn And Adams, 1996), reproduction (Hoestlandt, 1958; Walburg, 1960; Nichols and Massmann, 1963; Loesch and Lund, 1977; Leggett and Carscadden, 1978; Loesch, 1987; Manyukas, 1989; Jessop, 1993; Ross *et al.*, 1993; Mylonas *et al.*, 1995; Pina, 2000, Olney *et al.*, 2001), and recruitment (Johnson and Loesch, 1983; Esteves, 1999). However, published information on the reproductive biology of *A. fallax* is restricted to the accounts of Manyukas (1989), Quignard and Douchement (1991) and Pina (2000), which provide no detailed description of the histological development of the gonads. In Portugal, aspects of the feeding ecology and threats to survival (Assis, 1990; Assis *et al.*, 1992), population genetics (Alexandrino *et al.*, 1993, 1996), reproduction (Pina, 2000) and recruitment (Esteves, 1999) of twaite shad have been studied. This study was therefore undertaken as part of a project studying the recruitment, reproduction, age and growth, and

genetics of shad populations in Portuguese rivers, and constitutes the first description of the oogenesis of twaite shad. The outcomes of this project will be used to develop conservation and management programmes that endeavour to halt further declines and to improve twaite shad conservation status.

As a basis for further studies, the present investigation provides a detailed description of the different stages of oocyte development.

MATERIAL AND METHODS

Sampling was carried out between March and June 1997, February and June 1998 and January and April 1999 over the spawning areas of twaite shad in the upper estuaries of the Rivers Mira, South-west Portugal (Odemira) and Guadiana, South-east Portugal (Foz de Odeleite and Mértola) (Fig. 1). Fishing was carried out using trammel nets with an outer mesh size of 12.5 cm and an inner mesh size of 6.0 cm. A total of 265 females (106 in River Mira and 159 in River Guadiana) were measured (total length, to the nearest 0.1 cm) and weighed (wet weight, to the nearest 0.1 g), and the ovaries were removed and weighed (to the nearest 0.1 g). The gonads were staged macroscopically according to a key developed in this study, as follows: I - Immature/Resting; II - Early development; III - Maturing; IV - Ripe; V- Spawning; VI - Partially spent; VII - Spent (Table 1). Samples of gonads were fixed in San Felice solution (160 ml of 1% aqueous solution of chromic acid, 80 ml of 4% buffered formalin, 10 ml of acetic acid). After fixation (36h), samples were dehydrated in ethanol, embedded in paraffin and sectioned at 6-8 µm. Ovaries were stained with Masson's Trichrome (Martoja and Martoja-Pierson, 1967) and Toluidine blue (Bancroft and Stevens, 1990). After staining, the sections were covered with DePex mounting medium for microscopy (BDH Chemicals Ltd., Pools, England). Microscopic developmental stages of oocytes were categorised according to Janssen e*t al.* (1995). Histological classification of the atretic oocytes and postovullatory follicles followed Hunter and Macewicz (1985) and Hunter *et al.* (1986). An oocyte frequency distribution (class interval: 50 µm) was produced for each maturity stage using counts of oocytes on 6 ovaries for each maturity stage. The maximum diameter of the oocytes was measured to the nearest 0.01 µm, using image analysis software (Image Pro Plus 3.0).

RESULTS

Developmental stages of the oocytes

Oogonia (Fig. 2A)
Cell diameter: 50 µm; nuclear diameter: 30 µm

Oogonia were very small, round cells occurring singly or in groups with a relatively narrow zone of clear cytoplasm and a single, prominent nucleolus in the nucleus.

Chromatin nucleolus stage (Fig. 2A)
Cell diameter: 120 µm; nuclear diameter: 61 µm

These newly formed oocytes appeared as small rounded cells and the cytoplasm was very thin and faintly basophilic

Early perinucleolus stage (Fig. 2A)
Cell diameter: 180 µm; nuclear diameter: 75 µm

From this stage onwards, the oocytes were found as single cells, gradually migrating towards the centre of the lamellae. The cytoplasm was homogeneous and strongly basophilic. Numerous, relatively large, basophilic nucleoli appeared at the periphery of the nucleus, indicating increasing nuclear activity.

Late perinucleolus stage (Fig. 2A)
Cell diameter: 200 µm; nuclear diameter: 90 µm

The chromatin material was dispersed throughout the nucleus, causing the nucleoplasm to appear granular. Numerous small round nucleoli were found in the periphery of the nucleus, quite close to the nuclear membrane. The cytoplasm was divided into two concentric zones: the inner dense and deeply basophilic and the outer less dense and only slightly basophilic. A flattened follicular layer surrounding the oocytes could be distinguished at the end of this stage, which is the last one of the primary growth phase.

Cortical alveoli stage (Fig. 2B)
Cell diameter: 380 µm; nuclear diameter: 140 µm.

During this growth phase, the cytoplasm had lost some of its basophilic property, and a narrow zone of small cortical alveoli (Ø 25 µm) was formed in the periphery of the cytoplasm. Several vacuoles

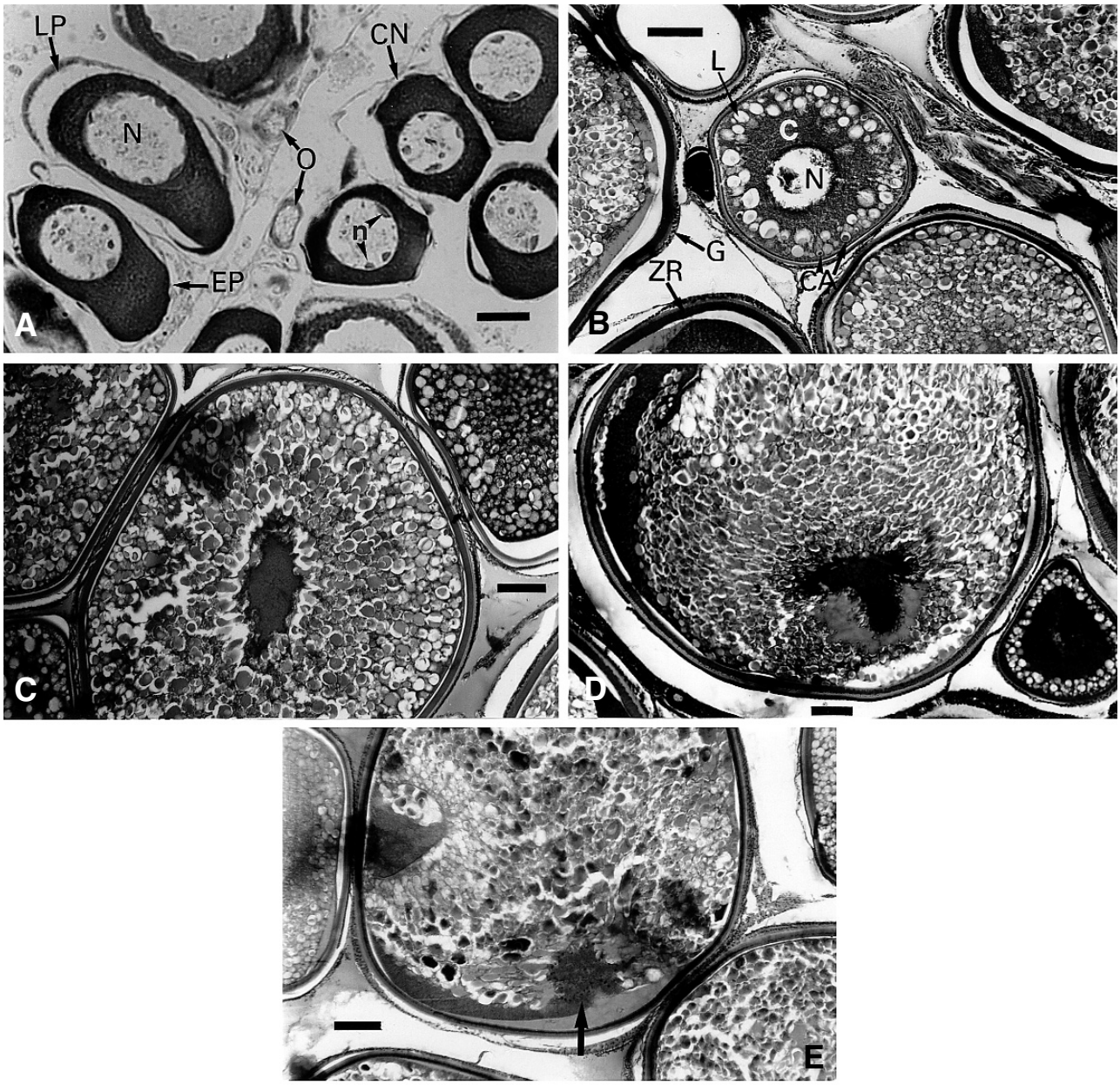

FIG. 2. – Histological appearance of oocytes of the twaite shad. **A**, primary growth phase (O, oogonia; CN, chromatin nucleolus; EP, early perinucleolus; LP, late perinucleolus; N, nucleus; n, nucleoli) (Bar = 50 µm). **B**, cortical alveoli stage (CA, cortical alveoli; N, nucleus; C, cytoplasm; G, granulosa; ZR, zona radiata; L, lipids) (Bar = 110 µm). **C**, vitellogenesis (Bar = 60 µm). **D**, germinal vesicle migration (Bar = 75 µm). **E**, Germinal vesicle breakdown (Bar = 105 µm).

appeared in the basophilic layer of the cytoplasm around the nucleus. These vacuoles increased in number and volume and after a while formed a spongy-looking zone in the inner part of the cytoplasm. The nucleus contained numerous nucleoli, which were arranged in a ring close to the nuclear membrane. The zona radiata and the theca had become visible.

Vitellogenesis (Fig. 2C)
Cell diameter: 550 µm; nuclear diameter: 170 µm.

Exogenous vitellogenesis started with the deposition of protein yolk granules at the periphery of the oocyte. These have multiplied increased in number and displaced most of cortical alveoli. The lipid droplets enlarged and occurred scattered between the yolk granules. The cytoplasm became less basophilic as vitellogenesis proceeded. Both the zona radiata, which assumed a fine, striated appearance, and the follicle epithelium were more prominent. Oocytes can remain in this stage until they either resume meiosis in response to appro-

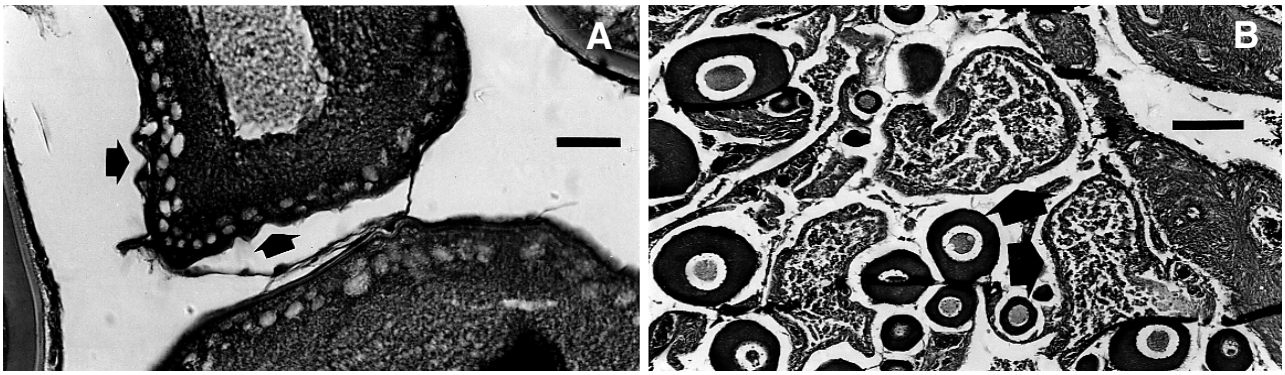

FIG. 3. – Histological appearance of **A**, *alfa* (arrows indicate wrinkled zona radiata) (Bar = 50 µm) and **B**, *beta* atresia (arrows) oocytes of the twaite shad. (Bar = 130 µm).

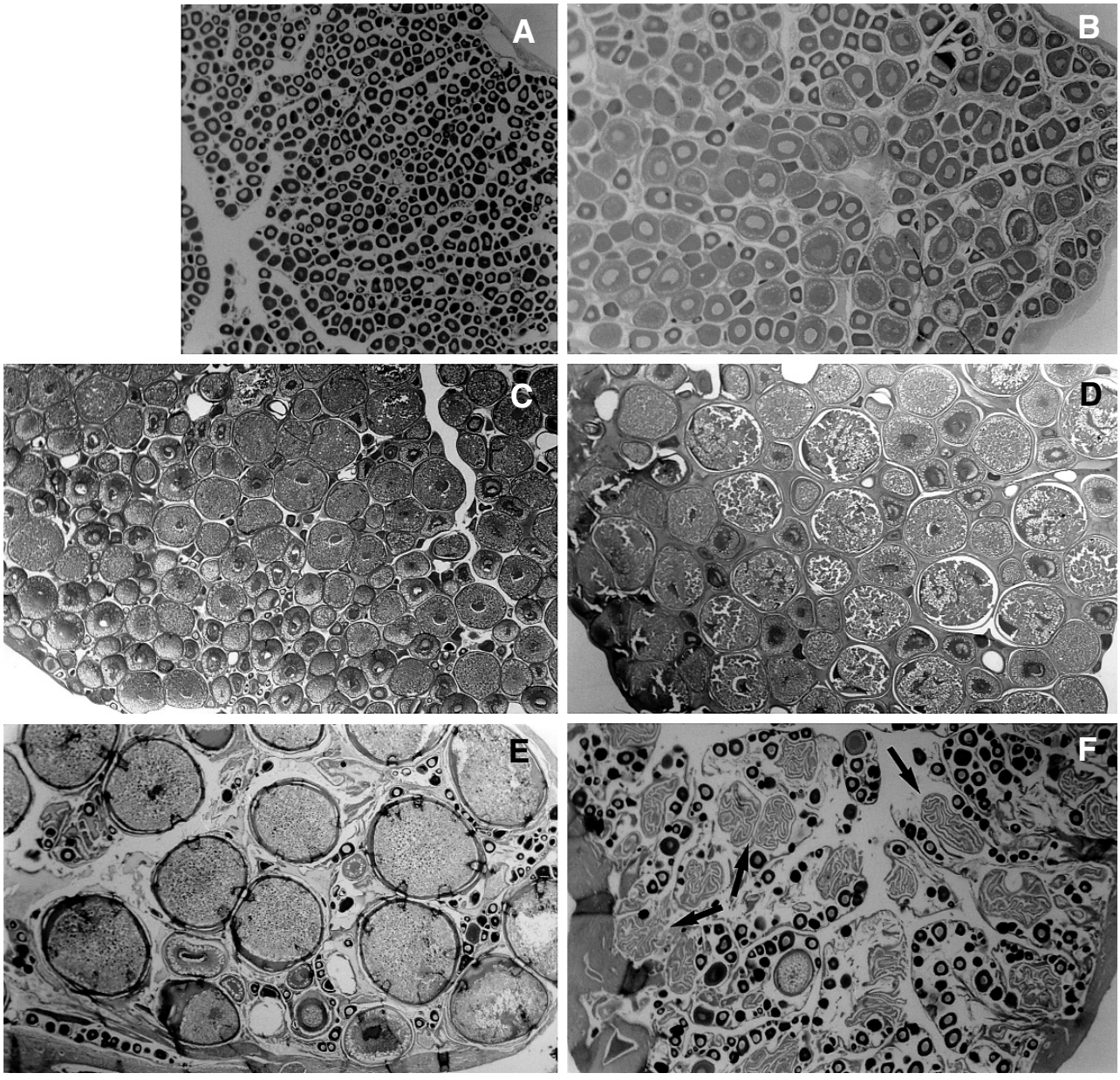

FIG. 4. – Photomicrograph of twaite shad ovaries at various magnifications. **A**, immature/resting; **B**, early development; **C**, maturing; **D**, ripe; **E**, partially spent; **F**, spent (arrows indicate POF's).

priate hormonal stimulus or are resorbed (oocyte atresia)

Germinal vesicle migration (GVM) stage (Fig. 2D)
Cell diameter: 850 µm; nuclear diameter: 180 µm

The nucleus, or germinal vesicle (GV), migrated towards the periphery of the oocyte and contained many nucleoli. The protein yolk granules and lipid droplets started to coalesce and the oocyte rapidly increased in volume due to hydration. The cytoplasm was surrounded by a marked zona radiata.

Germinal vesicle breakdown (GVBD) stage (Fig. 2E)
Cell diameter: 950 µm

During this stage, a pronounced and rapid size increase occurred due to both hydration and limited protein uptake and the nuclear membrane disappeared (germinal vesicle breakdown). Most of the oocytes in this stage collapsed during histological processing. Thus, photographs were possible only on an earlier phase of this stage (Fig. 2E).

Post-ovulatory follicles (POF's)

The POF's consisted of residual follicle layers, which remained in the ovaries after ovulation and degenerated during the following period (*c.f.* Fig. 4F).

Atretic follicles (*alfa* and *beta*)

From final spawning onwards, a number of post-vitellogenic oocytes failed to undergo maturation or ovulation and subsequently degenerated and were resorbed, i.e. became atretic. At the onset of atresia (*alfa* atresia), the zona radiata wrinkled and started to break up (Fig. 3A). The follicle granulosa cells proliferated and hypertrophied to form a compact, well-vascularised structure. These active granulosa cells invaded the oocyte through the broken down zona radiata and digested and resorbed the yolk contents by active phagocytosis. The phagocitic granulosa cells in turn also degenerated, leaving behind a lightly staining fibrous mass surrounded by connective tissue elements (*beta* atresia) (Fig. 3B).

The stages of *gamma* and *delta* atresia referred to by Hunter and Macewicz (1985) were not observed. The follicle was completely resorbed during the *beta* stage or a rapid evolution of atresia might have occurred after this stage, leaving no histological characteristics that could be identified.

Maturity stages of the ovary

Gross examination of ovaries revealed that they could be placed into one of seven maturity stages according to its stage of development (Table 1 and Fig. 4). This seven-stage classification is a modification of that given by Macer (1974).

TABLE 1. – Macroscopic characteristics and histological description of the maturity stages of the ovary of *Alosa fallax fallax*.

Maturity stage	Macroscopic description	Histological description
I. Immature/resting	Small thread-like ovary, pinkish in colour, no oocytes visible	Well-spaced ovigerous fold, orientated towards the centre of the ovary, containing both oogonia and chromatin nucleolus and early perinucleolus stage; oogonia generally occur in nests
II. Early development	Ovaries one-third of ventral cavity, yellowish in colour, opaque oocytes visible through the tunica	Oogonia still present, ovigerous folds fill cavity, late perinucleolus and cortical alveoli oocytes present
III. Maturing	Ovaries two-thirds of ventral cavity, yellowish in colour, opaque and translucent oocytes visible, increasing vascularization	Cortical alveoli and yolk granule oocytes predominate; late perinuceolus and vitologenic oocytes can also be observed
IV. Ripe	Ovaries highly vascularized occupy most of ventral cavity, opaque, translucent and dominant hyaline oocytes visible	Oocytes observed in previous stage still present; predominance of GVM stage oocytes
V. Spawning	Ovaries occupying most of ventral cavity, eggs released under slight pressure of the abdomen	Predominance of GVM oocytes and POFs
VI. Partially spent	Ovaries flaccid, deep red in colour, occupying two-third of ventral cavity, with hyaline oocytes visible	Irregular convoluted ovigerous folds, conspicuous spaces in the septa and POFs in the lumen, early perinucleolus and atresia oocytes present
VII. Spent	Ovaries flaccid and fully empty, a few residual oocytes visible	Ovigerous folds reorganizing, containing atretic follicles and perinucleolus oocytes

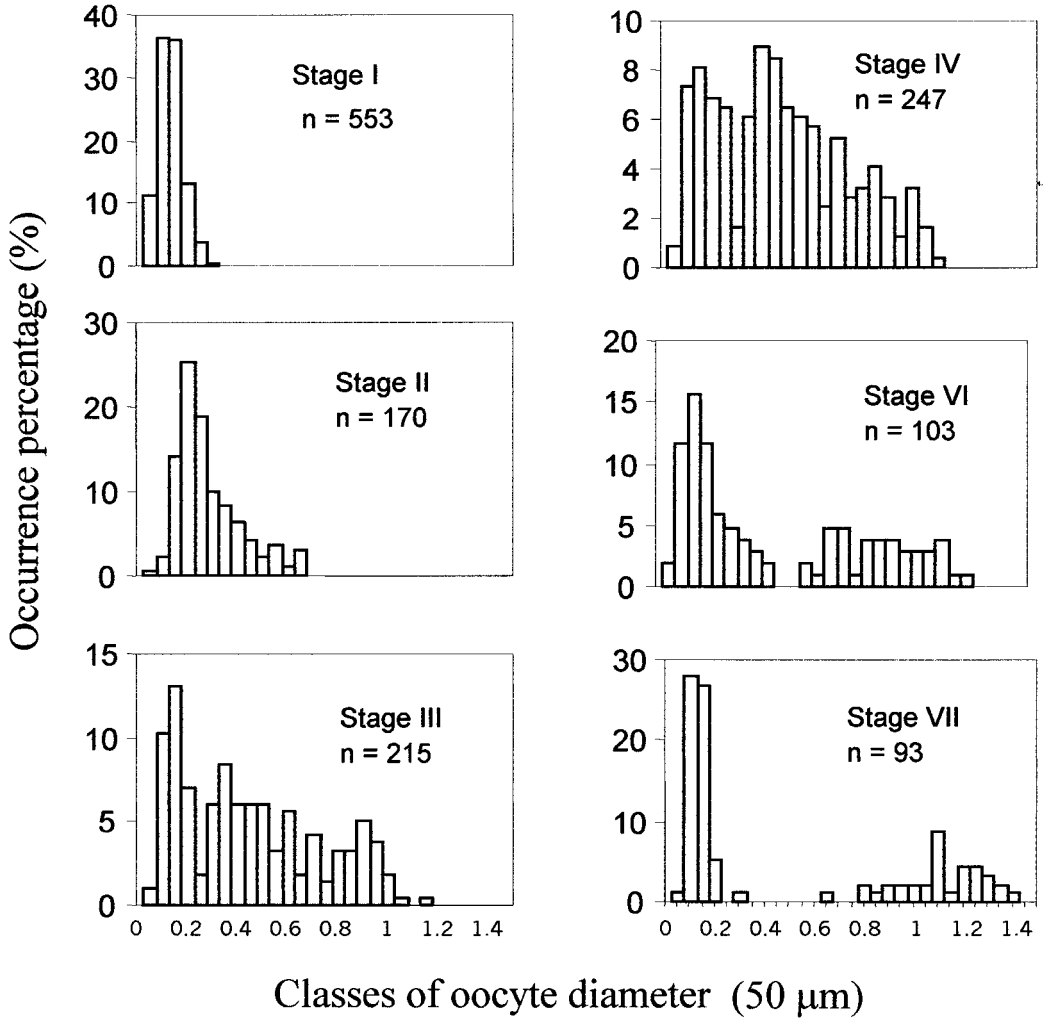

FIG. 5. – Oocyte size frequency distribution for each maturity stage of twaite shad; n, total number of oocytes measured.

Oocyte size frequency distribution

Figure 5 shows the oocyte size frequency distribution in each maturity stage. In maturity stages I, II and III, only one modal class is identified. This distribution is clearly polimodal from this stage onwards, when different cohorts of developing oocytes are identified. In ripe ovaries, oocyte diameter reaches 1.052 mm. Nevertheless, larger oocytes (1.415 mm) can be observed in partially spent and spent ovaries, which were not shed and will be resorbed.

DISCUSSION

The process of oocyte development in *A. fallax fallax* follows the same basic progression as that described for *Alosa sapidissima* (Mylonas *et al.*, 1995; Olney *et al.*, 2001) and other teleost fish (Tyler and Sumpter, 1996).

In this work, oogenesis was divided into 8 stages according to size differences and the occurrence of new structures that are easily recognisable during the different phases of oocyte growth. Hence, oogonia give rise to immature oocytes with multiple nucleoli. The oocyte enlarges as vitellogenic yolk is deposited and its zona radiata thickens. The nucleus migrates to the animal pole prior to the breakdown of its membrane. Hydration precedes ovulation and the appearance of these hyaline oocytes indicates imminent spawning. The follicle collapses after the oocyte has been released, leading to the emergence of the postovulatory follicles.

The origin of the young oocytes that appear at the beginning of each reproductive cycle is a question of great biological interest, since those oocytes represent the initial stage of oogenesis, and therefore the

reserve from which all oocytes will develop. There has been some controversy as to the origin of the new oocytes. Hann (1927), Matthews and Marshall (1956) and Ramos (1983) suggested that these oocytes originate from residual oogonia that remain in the ovary from year to year. Bara (1960) and Arruda (1988) concluded that oogonia were derived from germinal epithelial cells. According to Wheeler (1930) and Yamamoto (1956), at least some oogonia are supplied each year by the transformation of certain cells remaining from the empty follicles of the previous year's spawning. According to Howell (1983), the observation of mitotic activity in oogonia of mature fish suggests that a new stock of oogonia arises during each reproductive cycle. In this study, oocytes in the chromatin nucleolus stage were usually present in small groups but no mitotic divisions were apparent. Furthermore, at all stages, from late immature onwards and throughout the year, a "reserve fund" was present in the ovaries of the twaite shad. This "reserve fund" consisted of oocytes that had grown from the chromatin nucleolus stage up to a size of 0.37 mm, at which point they seem to suspend development for some time and resume growth towards maturation. This dynamics would agree with the conclusion of Foucher and Beamish (1977), indicating an apparent regeneration of the oocytes in the perinucleolus stage at the beginning of each reproductive cycle. On the other hand, the observation of oocytes in the chromatin nucleolus stage near the base of the septa supports an epithelial origin of these cells.

The presence of two differently stained zones in the cytoplasm of the oocytes in the cortical alveoli stage is related to the development of the Balbiani body, and has been described for a number of species (Bara, 1960; Arruda, 1988; Mayer *et al*., 1988; Coello and Grimm, 1990). According to Guraya (1986) and Coello (1990), it is composed of two parts, the yolk nucleus and the pallial substance. The pallial substance appeared inside the resting oocytes as the more deeply basophilic ring in the inner cytoplasm. The proportion of these oocytes showing Balbiani's body increases with cell size. Similar results were described by Coello and Grimm (1990) in the oocytes of *Scomber scombrus*.

Migration of the germinal vesicle (GV) is an event associated with the onset of final oocyte maturation and was apparent in oocytes larger than 800 µm. Oocytes greater than 950 µm exhibited varying degrees of yolk coalescence, but the GV was not apparent after histological examination. According to Mylonas *et al.* (1995), this suggests the dissolution of the nuclear wall (GV breakdown).

Atresia followed one of the three patterns described by Hunter and Macewicz (1985), and the follicle was completely resorbed during the *beta* stage. Atresia affected only the oocytes that began the second growth phase and the younger oocytes were not affected in any way.

In post-spawning fish, the postovulatory follicles (POF's) can be found embedded in the lamellae. The POFs degenerate rapidly, making it difficult to access the percentage of oocytes spawned, unless samples are collected daily (Hunter and Macewicz, 1985). Also, the elastic nature of ovarian tissue allows the ovary to contract as oocytes are spawned so that remaining oocytes are still closely grouped, making loss of oocytes less apparent. It is also difficult to determine whether the observed atresia represented premature resorption of oocytes that could have been released later or was part of the natural tissue resorption process after cessation of spawning.

According to Bye (1984), in wild populations the only factor that can cancel out or reverse the effect resulting from increased reproductive investment is temperature. Ovulation marks the commitment of considerable metabolic investment and can lead to successful reproduction only if critical external factors (presence of a male and spawning substrate, low level of predation, etc.) are appropriate (Stacey, 1984). Thus, an overproduction of maturing oocytes can be part of a reproductive strategy of *A. fallax fallax* and oocytes can be shed or not, depending on both the abiotic and the biotic conditions. Furthermore, fasting during anadromous migration can induce atresia, suggesting that some feedback mechanism may exist to use resources, particularly yolk, efficiently so that there is a minimum requirement for resorption in migrating females. According to Bagenal (1978) and Guraya (1986), food shortening is one of the main factors determining increasing rates of atresia. Thus, the study of the direct effects of atresia in oocyte production is a major issue to be addressed in future studies, particularly in fecundity estimates.

Evidence that twaite shad are serial spawners releasing discrete batches of eggs over an extended spawning season includes macroscopic and histological indications of recent spawning concurrent with mature vitellogenic oocytes. The development of oocytes in twaite shad is asynchronous because these fish are capable of bringing oocytes from an

immature condition through vitellogenesis during the spawning season. Eggs are recruited from a heterogeneous population of developing oocytes and are subsequently ovulated in several batches during each spawning season. This is supported by the lack of a hiatus in the size frequency distribution of immature versus mature oocytes. Similar results were also reported for twaite shad by Le Clerc (1941) and Hass (1968) and for American shad, *A. sapidissima*, by Mylonas *et al.* (1995) and Olney *et al.* (2001).

More information about the number of batches spawned can be obtained through the process of identifying and ageing the postovulatory follicles. Future studies will have to address this problem, since the delineation of the spawning frequency is essential for accurate fecundity estimates. Further research is also necessary to determine how the reproductive patterns affect the investment of energy and nutrients in the somatic and reproductive tissues of twaite shad.

This study indicates that there are still numerous gaps in our understanding of this complex process in twaite shad. Nevertheless, this study might serve as background information for future studies and may stimulate further research, leading to insights into the events and control mechanisms of egg production among vertebrates. Moreover, a greater understanding of the relation between reproductive strategy and habitat characteristics is needed in order to evaluate exploitation techniques and permissible habitat changes.

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