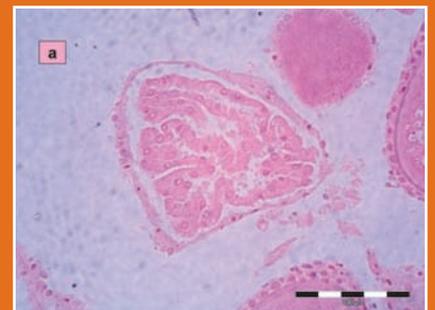# 17(8)

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Revision of criteria for the classification of postovulatory follicles degeneration, for the Bay of Biscay anchovy (*Engraulis encrasicolus L.*)

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Abstract

This study proposes a detailed histological key for the examination of the postovulatory follicle's (POF's) degeneration for the Bay of Biscay anchovy (*Engraulis encrasicolus*). Such key was established uniquely on the basis of histological features not regarding to the time of capture of the samples. The objective of this procedure is to overcome the difficulties associated to the estimation of the Spawning Frequency on the basis of the determination of POF's degeneration stages that have been traditionally divided into three great stages corresponding to daily spawning cohorts. The 7 stages presently proposed suppose a better differentiation and resolution of the different phases of POF's degeneration. This fact allows separating the staging of POF's from that of ageing. In that way, samples obtained at any time throughout the day can be used and not only those sampled during a discrete period of the day.

Key words: Bay of Biscay anchovy, ovary, postovulatory follicle, histological feature, spawning frequency

Introduction

Hunter and Goldberg (1980) working with northern anchovy (*Engraulis mordax*) noticed that the daily spawning rate of populations could be estimated using the incidence of females with the ovaries showing the postovulatory follicles (POFs) remaining after ovulation. The synchronous daily spawning cycle of most small pelagic species allows for the identification of daily cohorts of POFs in the ovary; these have facilitated the estimation of spawning frequency, or the fraction of females spawning per day (Dickerson *et al*., 1992; Yamada *et al*., 1998). This finding has led to the development of the Daily Egg Production Method (DEPM), for the assessment of biomass of fish species with indeterminate fecundity (Parker, 1980; Lasker, 1985; Hunter and Lo, 1997). In order to implement this method, a validated correspondence between degeneration of POFs and daily spawning cohorts is required for the estimation of the spawning frequency. Once the objective classification criteria of ovary's maturation and POFs degeneration is established, it is possible to perform a classification of ovaries, assigning them to the precedent days of spawning as shown by Hunter and Macewicz (1985). For the Bay of Biscay anchovy (*Engraulis encrasicolus*), the POFs classification was established by Motos (1994, 1996), adopting the criteria described for the northern anchovy by Hunter and Macewicz (1985), according to his own experience and to the previous studies undertaken by Santiago and Sanz (1992) and Sanz *et al*. (1992).

Variability in POF deterioration degrees is dependent upon temperature (Fitzhugh and Hettler, 1995) and fixation procedures (Hunter and Macewicz, 1985). Current procedures of ovary examination, for the determination of spawning frequency, combine the process of evaluating the deterioration of POFs (staging), with that of allocating them to daily cohorts (ageing). This implies grouping different POFs states into 24 h periods. For species with defined daily spawning periodicity this procedure is suitable when the adult sampling is reduced to a discrete period of the day. In this way, a 24 h elapsed time between consecutive POF cohorts, should assure sufficiently distinct daily POFs groups (Ganias *et al*., 2003b). However, adult sampling for the Bay of Biscay anchovy DEPM surveys often takes place throughout the day (Motos, 1996). In this case, the distinction between different POF groups and subsequent allocation to daily spawning cohorts becomes difficult. Moreover, when using the criteria of daily POF stages, only the final cohort allocation is retained but, not properly, the state of degeneration of the follicle; this complicates any revision of the ageing procedures of POFs. In these cases, objective classification criteria for the degeneration of POFs independent of the age of the follicle are convenient. To this purpose, definition of POF stages of duration shorter than 24 h would facilitate a sufficiently discrete POF degeneration state for each stage and should minimize the risk of incorrect classification. In the DEPM, ageing of eggs relies in the previous staging of

the eggs; ageing is achieved subsequently according to the stage assigned, incubation temperature, sampling time and daily peak spawning time (Lo, 1985). A similar procedure, separating POFs staging from ageing, could be devised to allocate anchovy specimens to daily spawning cohorts (ICES, 2004; Stratoudakis *et al.*, 2006).

The present study proposes a set of POF's degeneration stages for estimating the post spawning condition of anchovy ovaries, solely based upon their histological features, independently of their age.

Materials and methods

Bay of Biscay adult anchovies have been caught every year (since 1987), during May-June i.e. the peak of the spawning period, during the DEPM surveys. After histological processing (Hunter and Macewicz, 1985; Motos, 1994), slides of the ovaries of the females are obtained and interpreted, for the estimation of the spawning frequency.

For this study, ovaries from the field samples were randomly selected and examined in detail. When a degree of POF degeneration was considered to be morphologically identifiable, a stage was proposed; it was micro photographed and a brief description of the histological features was established. There are proposed 7 stages for the degeneration of POFs. The classification key of POFs stages is defined on the

basis of objective degeneration criteria described previously in the literature (Hunter and Goldberg, 1980; Goldberg *et al.*, 1984; Hunter and Macewicz, 1985; Fitzhugh and Hettler, 1995) and taking into account our experience. The reduction in size of the POFs stages (Table 1) was estimated upon the examination of several ovary slides, corresponding to surveys in years 1995, 1998 and 2003; those were randomly selected and micro-photographed. The area of the POFs per stage found in each microphotograph was measured and the average area for every stage was calculated.

A total of 11948 ovaries preserved during 12 DEPM surveys (1990-1992, 1994-1995, 1997-1998 and 2001-2005) were selected to apply the new procedures. The mean sea surface temperature during the surveys ranged from 13.7 to 17.7ºC. Since DEPM sampling strategy in Bay of Biscay is rather continuous throughout the day (Motos, 1996), the data were grouped by sampling time to establish the 24 h cycle for POFs degeneration.

Results

POF's degeneration key

POF's degeneration process has been divided into 7 stages (see below and Table 2), together with a Stage 0, when no POFs are found in the ovary.

Table 1. Summary average size (area estimates in mm2), for each postovulatory follicle stages; n refers to the total number of Postovulatory follicles per stages examined whilst relative size refers to the average area of the different postovulatory follicles, relative to the size for Stage I; SD=standard deviation; CV=coefficient of variation.

	Stage I	**Stage II**	**Stage III**	**Stage IV**	**Stage V**	**Stage VI**	Stage VII
n	161	149	144	140	112	103	70
Average (mm²)	39.1	41.7	41.9	35.8	28.5	22.6	10.9
SD	21.6	22.8	20.9	19.0	14.6	13.3	5.7
CV %	55	55	50	53	51	59	53
Max (mm²)	118.8	174.8	151.1	106.9	64.0	59.3	24.1
Min (mm²)	7.7	9.0	9.2	7.0	4.2	3.7	1.7
Relative Size %		100	100	86	68	54	26

Table 2. Proposed identification criteria: New codes and criteria for the identification of the post-spawning stages of the anchovy ovaries. POF: Postovulatory follicles.

|	Codes	Histological features
Post-spawning	0	Ovaries with no post-spawning signs
	I	New POFs
	II	First signs of POFs degeneration. First vacuoles
	III	Small vacuoles affecting ≤50% of granulose cells
	IV	Massive incidence of medium vacuoles
	V	Reduction/absence of lumen. Cell walls rupture
	VI	Very reduced POF. Few vacuoles and few pycnotic nuclei.
	VII	Tissue remains. No cells. Some pycnotic nucleus.
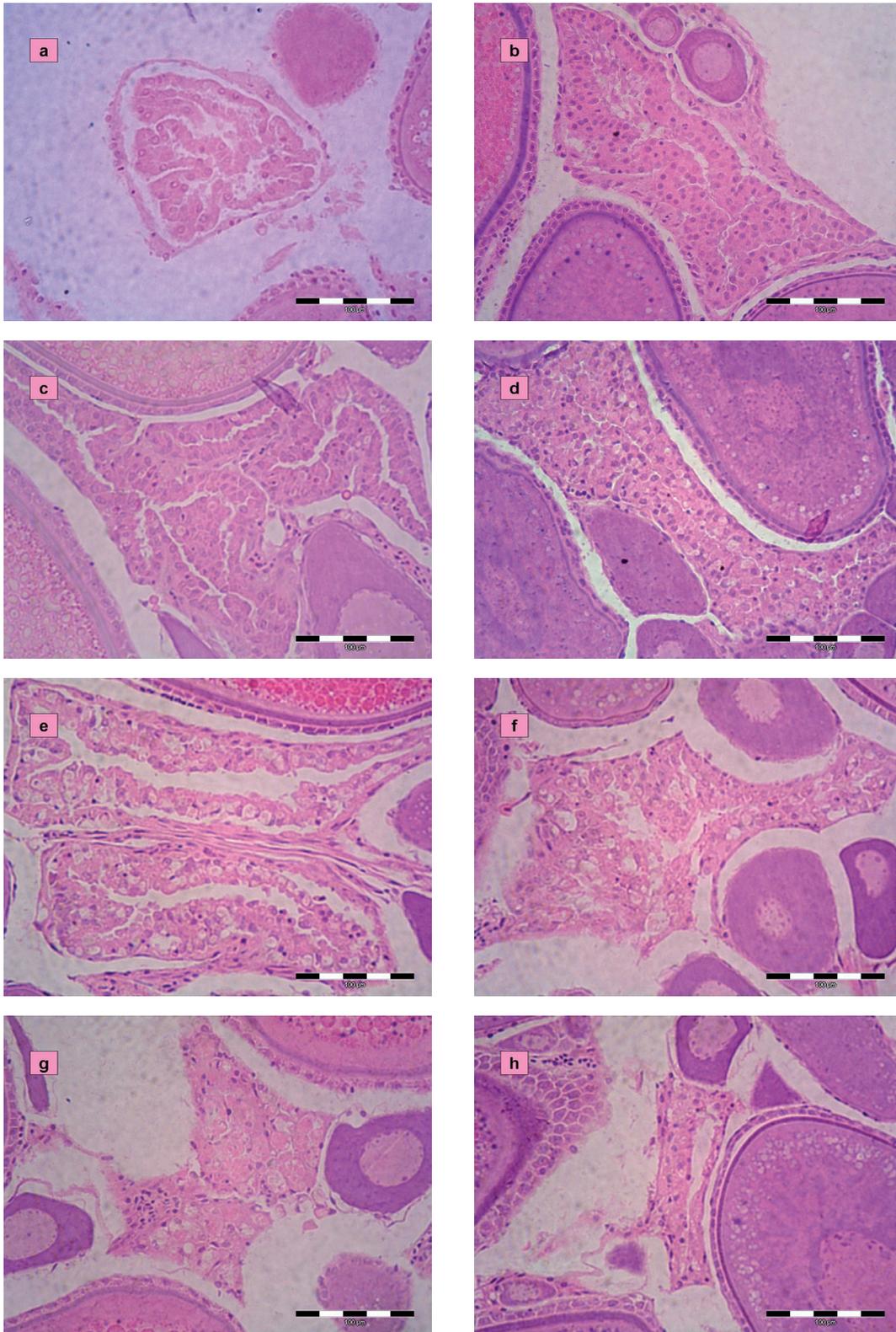
Figure1(a-h). Postovulatory follicle states of anchovy *Engraulis encrasicolus*. Microphotographs of histological sections of anchovy female gonads: a postovulatory follicle Stage I (at ovulation), b postovulatory follicle Stage I (after spawning), c postovulatory follicle Stage II, d postovulatory follicle Stage III, e postovulatory follicle Stage IV, f postovulatory follicle Stage V, g postovulatory follicle Stage VI, h postovulatory follicle Stage VII. Scale bars: 100 mm

Stage I: New POFs, showing no signs of degeneration. Immediately following ovulation (Figure 1/a) the follicle cell layers appearing cord-like, forming loose folds or loops. The granulosa cells, which have been stretched extensively during hydration, appear elongated and extremely narrow. The lumen contains eosinophilic granules, of uncertain origin. The underlying theca layer is thin, not very noticeable and is not adhered to the granulosa.

After spawning (Figure 1/b), the fully collapsed postovulatory follicle is a much more tightly folded structure; it is relatively large, irregular in shape, with an irregular lumen. The granulosa cells are characteristically columnar or cubical and in some cases, have slight hypertrophied. These cells are arranged orderly along the edge of the lumen, with their cell walls evident and possessing prominent nuclei. The nucleus of the granulosa cells may be located at either the apex, or the base of the cell. The thecal cell layer is more clearly defined surrounding the granulosa, but separated from it.

Stage II (Figure 1/c): The recently spawned follicle undergoes the early signs of deterioration. The postovulatory follicle is large in size, but presents a still tightly folded structure. The lumen is easily visible and contains granular material. The cells of the granulose layer conserve the orderly alignment characteristics, although not as arranged as in Stage I. The critical feature to distinguish this stage from the previous one is the occurrence of some small vacuoles affecting to the granulose cells cytoplasm; however, the majority of the cell walls remain intact. The granulose cells nuclei are prominent, even tough a few of them could become pycnotic. The underlying thecal layer conserves its integrity, separated from the granulosa; it is associated often with blood capillaries.

Stage III (Figure 1/d): The postovulatory follicle is more shrunken. Conversely, preliminary measures of the average area (Table 1) of POFs provide an indication of a slight increase in size of Stage III POFs. This is due probably to modifications in the granulose cells morphology, changing from a columnar shape in Stage I to a more expanded cubical or spherical form in this stage. The lumen is easily visible and it may contain some eosinophilic granules. The vacuoles occurrence is once again helpful in defining this stage, since it is observed now a great number of small vacuoles (affecting approximately one half of the granulosa cells). Some breakdown of the cells walls may occur. At this point, many of the granulosa cells nuclei become pycnotic. Some pattern in arrangement of cells can still be identified. The thecal layer is observable easily adhered to the granulose; the nuclei of their cells could also be pycnotic.

Stage IV (Figure 1/e): The degeneration is pronounced within this stage. The POFs show, for the first time, a clear decrease in size reaching on average 85 % of the new POFs. The regressing follicle has few folds; as such as it acquires, a more regular form. The lumen is reduced greatly and eosinophilic granules are no longer observed within it. The vacuoles have a wide incidence within this stage, as they increase in number (affecting almost all the granulose cells) and in size (occupying the complete cell cytoplasm and producing the breakdown of many of the walls). This fact, together with the pycnosis in most of the nuclei cells, infers that no arrangement pattern of the cells can be identified. The theca layer becomes thinner and adheres more closely to the granulosa.

Stage V (Figure 1/f): As the degeneration advances, the folds observed previously disappear completely. The postovulatory follicle becomes a compact structure of medium size (65% of the original size), which prevents often the observation of the lumen. The vacuoles are still of high incidence (but less than in the previous Stage (IV)); their great growth in size produces the tear in the remaining cell walls. All of the granulose cells nuclei have become pycnotic. The theca layer is present, although its cells are less distinct.

Stage VI (Figure 1/g): The average size for this stage is approximately one-half of the new POFs; their incidence is low due probably to the reduction in size caused by the resorption process and to the growth of the larger oocytes within the ovary (Hunter and Macewicz, 1985).The cell walls are mostly absent in the remaining granulosa layer tissue; likewise, few vacuoles or pycnotic nuclei may be seen. The theca might be still present, but is often indistinct; it becomes incorporated into the ovarian connective tissue stroma. Classification beyond Stage VI becomes difficult, because there are not arguments for differentiation of the follicles from old atretic stages. However, for the Bay of Biscay anchovy, atresia incidence during the DEPM cruise time is at minima (Motos, 1996) and degenerated structures are common within the ovary. As such, there is a higher likelihood to be one of the oldest postovulatory follicles, than the latest stages of atresia.

Stage VII (Figure 1/h)**:** POFs considered as Stage VII are all the polyhedron/shaped tissue remains that can be found occupying space among the oocytes. They might still present some pycnotic nuclei, but no cellular differentiation can be observed. The theca layer is not visible. The size of those POFs is small reaching approximately one-fourth of the size of the new POFs. They are usually scarce and difficult to be found.

Application of the key to field samples

The incidence of the different POF's stages in the survey samples throughout the day is shown in Figure 2. Stages I to IV show marked minima during some daytime intervals. Conversely, advanced degeneration stages of POF (Stages V, VI and VII) show significant incidence throughout the day. Gonads with no traces of POF's (Stage 0) have marked incidence from the early afternoon until night while they are scarcely found during the early morning

Discussion

Since the development of the postovulatory follicle method (Hunter and Goldberg, 1980), the POF degeneration process has been studied widely in various multiple spawning fishes. The present proposal for the classification of POFs degeneration based upon 7 stages is so far the most detailed key proposed. During the process of description and establishment of the
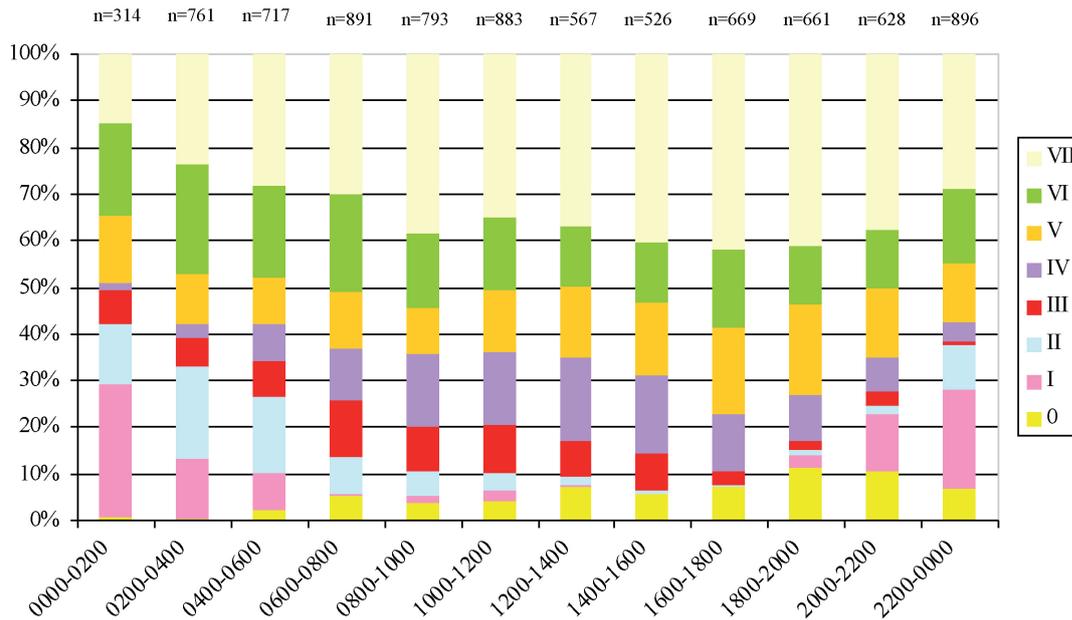
Figure 2. Postovulatory follicles incidence-time analysis for the anchovy females corresponding to years 1990-1992, 1994,-1995, 1997-1998, 2001-2005 (% incidence), through 2 hourly daytime intervals

7 stages of degeneration of the POFs some morphological characteristics have been identified as being critical for this anchovy. Features such as the vacuoles appearance, their development in the granulosa cells and the subsequent breakdown of cell walls, are determinant to describe the process of POF degeneration from the early signs until its latest phases. Of similar relevance are the characteristics related to the state of the cell's nuclei, such as the alignment degree in the granulose and the balance between nuclear prominence and pycnosis, in both the granulosa and the theca layers. Another key aspect considered here is the presence/absence of the lumen, which affects greatly the main appearance, shape and size of the degenerating follicle. In the studies undertaken by Hunter and Goldberg (1980) and latterly, by Hunter and Macewicz (1985) with northern anchovy all these characteristics were reported as key references for assessing the degeneration of the POFs, which was corroborated by other teams working with different species of multiple-spawning fishes (*Sardinops sagax*: Goldberg *et al*., 1984; *Encrasicholina purpurea*: Clarke, 1987; *Sardina pilchardus*: Perez *et al*., 1992; Ganias *et al*., 2003b; *Scomber japonicus*: Dickerson *et al*., 1992; *Brevoortia tiranus*: Fitzhugh and Hettler, 1995; *Merluccius merluccius*: Murua *et al*., 1998; and *Cynoscion nebulosus*: Roumillat and Brouwer, 2004;). Other important characteristics described by all these researchers are the distinction/indistinction of the thecal layer and its position, in relation to the granulosa. However, this aspect is less relevant in the degeneration processes of the follicles of the Bay of Biscay anchovy; this is due probably to the compacted appearance of the gonads of this species, making the observation of this feature often difficult, from the

Stage III onwards. Lastly, minor events such as the migration of lymphocytes in the POF, as described by Hunter and Macewicz (1985) and latterly, by Fitzhugh and Hettler (1995), are not considered in our description or in the studies of the other teams previously cited.

This POFs classification key provides an improved description of the degeneration process and refines the daily POF stages used formerly. There exist in literature previous efforts to better understand the process of degeneration of POFs in multiple spawning fishes during the first hours after spawning. Dickerson *et al*. (1992) working with chub mackerel (*S. japonicus*) divided the first daily class into 3 stages. Roumillat and Brouwer (2004) separated the process of degeneration of Day 0 POFs of spotted seatrout (*C. nebulosus*) into 4 stages. Those works support the convenience of having a largest number of shortly enduring POF stages that would result subsequently, in a more accurate ageing procedure. Moreover, the independence between POFs staging and ageing allows to undertake studies about the role of environmental variables (as sea temperature) inhabited by the fishes on the degeneration of the POFs as reported by Fitzhugh and Hettler (1995).

Conclusions

Given that the POFs degeneration is a continuous biological process some subjectivity in the process of allocation of POFs into stages will always remain. This new proposal supposes an improvement over the daily stages used previously because it allows for separating out the estimation of POF degeneration,

from that of ageing POFs; as such for retaining knowledge of their degeneration stage. A subsequent analysis of the duration of the proposed POF stages, in relation to different variables affecting the samples has been implemented in Alday *et al* (2008). The morphologically defined degeneration stages of POFs could be of interest to other investigators attempting to determine the spawning frequency of small pelagic fishes. This work shows that their application to a 24 h daily cycle of samples can provide firm indication about the duration of the first phases of degeneration of POFs, even in the absence of experimental studies. Such an approach could provide a tool for checking the ageing of POFs, hereby improving the spawning frequency estimation.

Acknowledgements

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