

**Abstract**—Inaccuracy in the aging of postovulatory follicles (POFs) and in estimating the effect of temperature on the resorption rate of POFs may introduce bias in the determination of the daily spawning age classes with the daily egg production method (DEPM). To explore the above two bias problems with field-collected European pilchard (*Sardina pilchardus*, known regionally as the Iberian sardine), a method was developed in which the time elapsed from spawning (POF age) was estimated from the size of POFs (i.e., from the cross-sectional area in histological sections). The potential effect of the preservative type and embedding material on POF size and the effect of ambient water temperature on POF resorption rate are taken into account with this method. A highly significant log-linear relationship was found between POF area and age; POF area shrank by approximately 50% per day. POFs were also shown to shrink faster at higher temperatures (approximately 3% per degree), but this temperature effect is unlikely to be an important source of bias in the assignment of females to daily spawning classes. The embedding material was also shown to influence the size of POFs, the latter being significantly larger in resin than in paraffin sections. In conclusion, the size of POFs provides an indirect, reliable estimation of the time elapsed from spawning and may thus be used to test both the validity of POF staging criteria for identifying daily classes of spawners and the effect of other factors (such as temperature and laboratory processing) in applications of the DEPM to *S. pilchardus* and other fish species.

## Degeneration of postovulatory follicles in the Iberian sardine *Sardina pilchardus*: structural changes and factors affecting resorption

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The postovulatory follicle (POF) consists of the follicular layers that remain in the ovary of fish after the release of the ovum during spawning (Saidapur, 1982). Initially, the POF is a distinct structure, but it rapidly deteriorates and becomes undetectable within a few days (Hunter and Goldberg, 1980). The study of POF degeneration is important in fishery studies because it permits the assignment of spawning females to daily classes to provide estimates of the daily fraction of spawning fish in the population (POF method, Hunter and Goldberg, 1980). The most common application of the POF method is in the daily egg production method (DEPM; Parker, 1980), where spawning fraction, together with other adult parameters, are used to estimate daily specific fecundity (Picquelle and Stauffer, 1985) for the fisheries-independent estimation of spawning biomass. A prerequisite for such applications is the existence of an accurate aging key that describes the time course of POF degeneration (Hunter and Macewicz, 1985).

In most DEPMS, the degeneration of POFs is described by a small number of histomorphological stages (see "Materials and methods" section) that are usually assumed to correspond to distinct daily classes (see review by Stratoudakis et al., 2006). How-

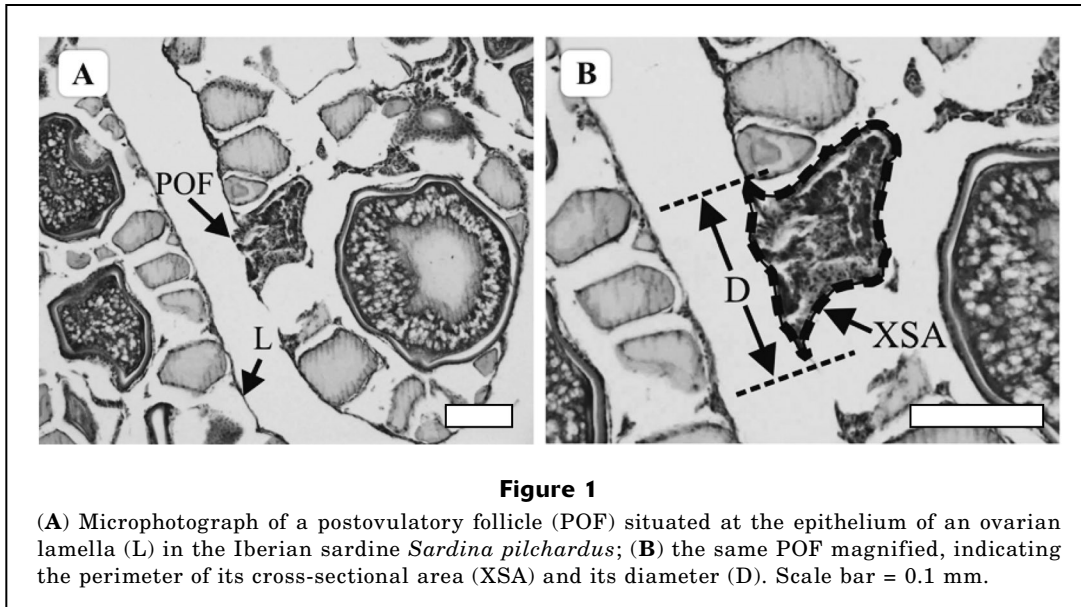
ever, because the process of POF degeneration is continuous and DEPM samples are usually obtained opportunistically throughout the day, the direct assignment of POF stages to daily classes of spawning fish can be imprecise. Also, morphological stages are often attributed to daily classes without prior validation and thus can lead to biased estimates of the spawning fraction. Validation is best performed in the laboratory by sacrificing female spawning fish at known time intervals after ovulation (e.g., Hunter and Goldberg, 1980; Pérez et al., 1992). Alternatively, in fish with daily spawning synchronicity, such as with the Iberian sardine *Sardina pilchardus* (also known as the European pilchard, FAO, 1985) (Bernal et al., 2001; Zwolinski et al., 2001; Ganias et al., 2003), validation can be performed indirectly through the examination of field samples collected at different hours of the day (Goldberg et al., 1984).

Another source of potential bias in the POF method is the duration of follicular degeneration, which may be temperature-dependent (Hunter and Macewicz, 1985), because the metabolic rates of poikilotherms, like fish, may be directly affected by ambient temperature. As a result, POF degeneration may be faster at higher temperatures and may lead to biased

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estimates of the spawning fraction and biomass when spatial variation in ambient water temperature is large within a survey area. This dependence can be assessed under laboratory conditions through inspection of specimens spawning under different temperature regimes (Fitzhugh and Hettler, 1995). Alternatively, the effect of temperature on POF degeneration can be studied in the wild by the examination of individuals caught at variable environmental conditions (Ganias et al., 2003; Roumillat and Brouwer, 2004).

Because of the above unresolved issues of the POF method (that can be species specific), the spawning fraction remains the most poorly estimated DEPM parameter (Hunter and Lo, 1997; Stratoudakis et al., 2006). The main objective of our study was to devise a method whereby one can test indirectly for potential sources of bias in the attribution of stage and age to the POFs of the field-collected sardine *S. pilchardus*. We used the size of POFs (cross-sectional area and diameter) as an index of POF age and, together with other histomorphological characteristics (follicle shape, state of the granulosa layer), we refined existing criteria for determining the stage and age of POFs in sardine. Then, we modeled the size of POFs as a function of POF age, ambient temperature, type of preservative, and type of embedding material. The analysis allowed us to test whether our staging criteria were valid and to examine whether temperature and laboratory processing introduced bias in the process.

## Materials and methods

Adult sardines were collected off Portugal and the Gulf of Cadiz in 1997, 1999, and 2005 within the framework of DEPM surveys for the estimation of the spawning biomass of the Atlanto-Iberian sardine stock (ICES,

2004). Sampling was conducted during peak spawning months for sardine (January–March), either on board the RV *Noruega* or from the commercial purse-seine fleet. During the surveys, sea surface temperature (SST) was recorded on an extensive grid representing hydrographic casts that covered the whole sampling area during the ichthyoplankton surveys (ICES, 2004).

Fish gonads were immediately removed after capture and placed in jars either with AFA (65 parts by volume of 50% alcohol, 32 parts formalin, and 13 parts glacial acetic acid) solution [1997, 1999] or with 4% formalin [2005]. In the laboratory, ovaries were embedded either in paraffin (1997, 2005) or in resin (1999), and histological sections (paraffin: 5  $\mu\text{m}$ ; resin: 3  $\mu\text{m}$ ) were stained with hematoxylin and eosin. Histological slides were examined and scored for maturity, atresia, and presence of POFs. When POFs were detected, slides were scanned in detail to locate the largest follicle situated along the epithelium of the lamellae. These POFs were photographed with a digital camera attached to the microscope at various magnifications so that the whole follicle would fit into the microphotograph (Fig. 1A). The cross-sectional area of the whole POF (all samples) and the distance between the two extremes of the follicle on the lamella (POF diameter, paraffin samples) were measured with UTHSCSA image analysis software (Univ. Texas Health Science Center, San Antonio, Texas) (Fig. 1B). Differences in the dimensional (size, shape) and fine histological (deterioration of the granulosa layer) characteristics were used, together with existing descriptions for *Sardina* populations (Atlantic: Pérez et al., 1992; Mediterranean: Ganias et al., 2003), to describe the pattern of POF resorption.

The evolution in the shape of POFs was assessed through the allometric relationship between diameter ( $D$ ) and cross-sectional area ( $XSA$ ):

$$XSA = a \times D^b, \quad (1)$$

where  $a$  and  $b$  are parameters.

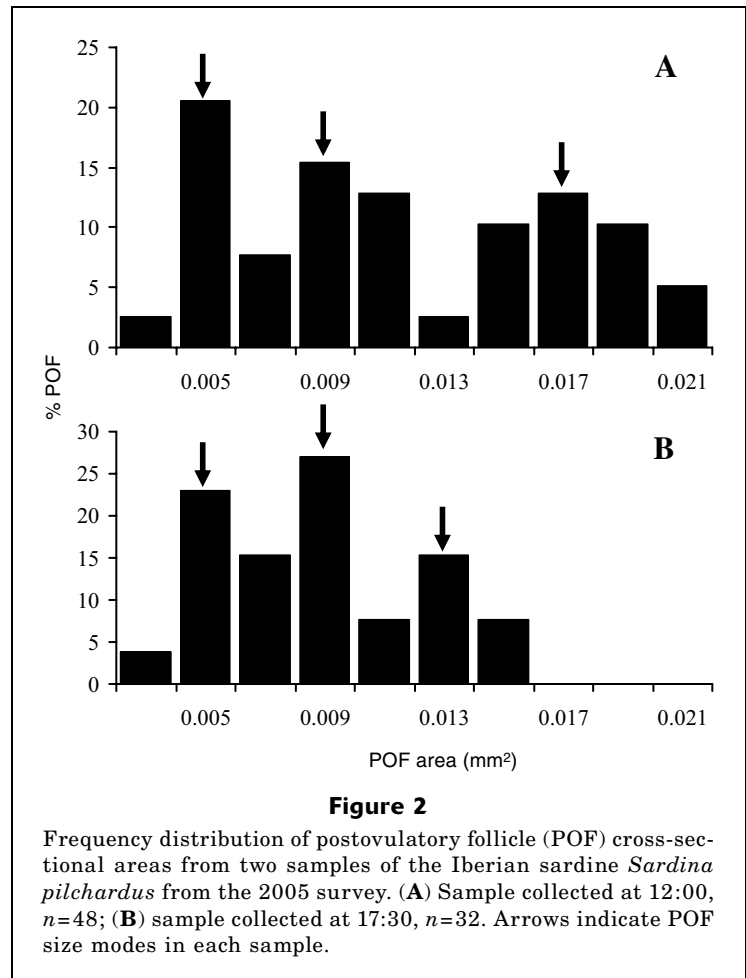
In case of isometry, (i.e.,  $b=2$ ), shrinkage is supposed to occur evenly in all dimensions, and the shape of the regressing POFs does not undergo significant changes. In any other case ( $b>2$ , or  $b<2$ ), the shape is altered along resorption, and thus its intermediate phases may be used in the staging of POFs.

Existing histological criteria for the staging of sardine POFs were refined and based on two hauls from the 2005 survey that contained more than 90 histological specimens each. Given that individuals in each sample were caught at the same time and temperature, POFs in each daily cohort should have been at the same stage and have had the same age, maximizing the morphological contrast among daily classes. Furthermore, one of these hauls was performed in the evening, just before the average daily spawning hour for the Iberian sardine (20:00; Zwolinski et al., 2001; ICES, 2004) and thus provided information on the final histological state of each daily class of POFs.

After refining the staging criteria and classifying the POFs from all surveys into daily classes, we used the time of capture and daily spawning hour to estimate the exact age of POFs, i.e., the time in hours elapsed between spawning and sampling. The effect of POF age and other factors on POF cross-sectional area were tested with a generalized linear model (GLM) with an overdispersed Poisson distribution and a logarithmic link function. Apart from POF age, the effect of temperature, sampling year, and the one-way interaction of year with age were considered in the model. The significance of the relationship between POF age and size indicated whether our staging and aging criteria were accurate. Furthermore, because the laboratory processing of the ovaries differed from year to year, the year effect and its interaction with age was used to test the effect of preservation medium (AFA, formalin) and embedding material (paraffin, resin) on the size of POFs. Residual inspection plots revealed the adequacy of the fitted model.

## Results

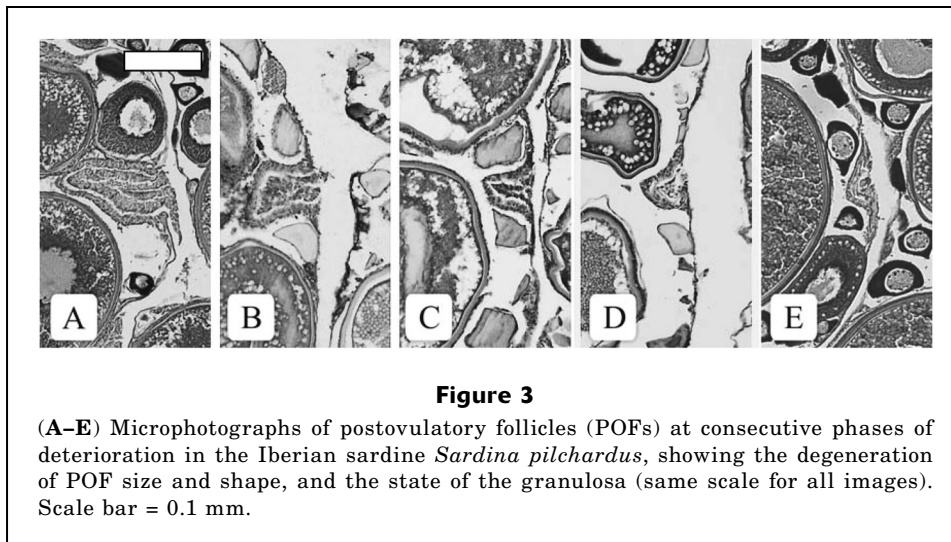
A total of 249 ovaries with POFs were detected and used in our analysis (1997: 65, 1999: 104, 2005: 80). The two hauls from the 2005 survey that were used for refining POF staging criteria contained many females with POFs at various stages and sizes, facilitating the distinction between successive daily classes of spawners. The frequency distribution of POF cross-sectional areas in the two hauls displayed three size modes, which were considered to correspond to different age classes (Fig. 2).



**Figure 2**  
Frequency distribution of postovulatory follicle (POF) cross-sectional areas from two samples of the Iberian sardine *Sardina pilchardus* from the 2005 survey. (A) Sample collected at 12:00,  $n=48$ ; (B) sample collected at 17:30,  $n=32$ . Arrows indicate POF size modes in each sample.

The most advanced size mode in the sample hauled at 12:00 (Fig. 2A) consisted of larger POFs compared to the POFs in the sample hauled at 17:30 (Fig. 2B). Re-examination and comparison of POF size modes in each sample showed that, apart from size, they differed in shape and in the fine histological characteristics of the follicular layers (Fig. 3). In particular, large POFs had an irregular shape and contained a large, convoluted, and thick granulosa layer (Fig. 3A). In the intermediate size mode, the shape of the follicle changed to semirectangular and the granulosa layer tended to lose its convoluted appearance and to form a single layer (Fig. 3B). Finally, in the smaller size mode, all POFs displayed a triangular shape (Fig. 3, C–E). However, more detailed examination showed that these triangular POFs could be further separated into 1) a group of slightly larger POFs with a thin layer of the granulosa (Fig. 3C), and 2) a group of very small POFs that contained only granulosa remnants in the form of residual vacuoles (Fig. 3, D and E).

POF diameter increased significantly with POF cross-sectional area (Fig. 4) and the relationship was not significantly different between the paraffin samples from 1997 and 2005 ( $P>0.05$ ). The allometric coefficient  $b$  of Equation 1 differed significantly from the square



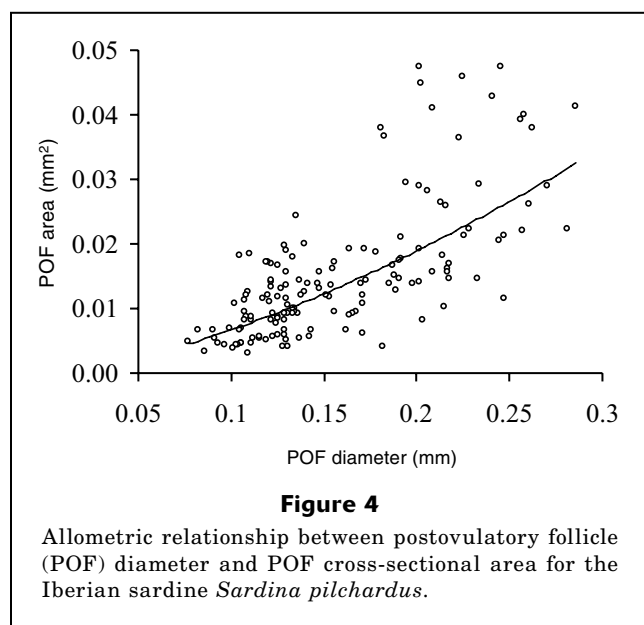
( $P < 0.001$ ), indicating that POF resorption in the Iberian sardine is not isometric, i.e., the shape of POFs changes throughout degeneration. More specifically,  $b$  was estimated to be 1.5 (standard error=0.13), indicating that the diameter of POFs along the lamellar epithelium diminishes at a lower rate than that for the overall POF area. This allometric pattern of POF resorption confirms the shape differences along POF degeneration described above and shown in Figure 3.

The differences in the dimensional characteristics and the morphological state of the granulosa (Table 1) were used to assign POFs from all surveys into four daily classes. The reliability of these aging criteria was confirmed by a very good relationship between POF age and POF size in all years of the study (Table 2; Fig. 5A). The rate of resorption, i.e., the relationship of

the slope of the POF cross-sectional area to POF age was not found to differ significantly between the three years, indicating that estimates of resorption rate are not biased either by the preservation medium (AFA or formalin) or by the embedding material (paraffin or resin). In all study years, POFs were shown to shrink exponentially with time in a way that their cross-sectional area decreased daily by almost 50% (Fig. 5A).

The relationship of the intercept of the POF cross-sectional area on POF age in the GLM was similar for the two preservation mediums (no significant difference between 1997 and 2005), but differed significantly between the two embedding materials (significant difference between 1999 and the other two years) (Table 2). POF area at any given time was significantly higher for resin (Fig. 5A), indicating that processing in paraffin wax leads to a higher shrinkage of all cellular structures in the gonad. This higher rate of shrinkage was also evident by differences in the histological appearance of POFs between the two embedding materials, especially at earlier phases of degeneration (Fig. 6). The structure of POFs in resin was more compact and the cellular organization of the granulosa was clearly visible (Fig. 6A). On the other hand, in paraffin sections, the cells of the granulosa layer were hardly detectable and the follicular folds were usually detached from the surrounding theca (Fig. 6B).

During the entire survey period, sea surface temperature ranged between 11.6° and 19.3°C, and there were marked interannual differences (2005 being the coldest [mean SST: 14°C ±2.1] and 1997 being the warmest year [mean SST: 16.2°C ±2.2°]). The fitted GLM showed that ambient temperature had a significant effect on the rate of POF degradation (Table 2; Fig. 5B). However, this effect appeared to be limited because an increase of 1°C in ambient temperature accelerated the rate of POF resorption by only 3% (compared to the reduction of POF area by almost 50% per day since spawning; Table 2). This finding indicates that the maximum dif-



**Table 1**

Summary of dimensional (shape, cross-sectional area) and fine histological (state of the granulosa layer) characteristics of different daily classes of postovulatory follicles (POFs) in the two embedding materials (P=paraffin; R=resin) for the Iberian sardine *Sardina pilchardus*.

Daily POF class	Shape	State of granulosa	Cross-sectional area (mm <sup>2</sup> )
<1	Irregular	Thick and looped	0.0164 ± 0.0004 (P)
			0.0355 ± 0.0001 (R)
1–2	Rectangular	One well-formulated layer	0.0093 ± 0.0002 (P)
			0.0176 ± 0.0005 (R)
2–3	Triangular	A thin receding layer	0.0059 ± 0.0002 (P)
			0.0126 ± 0.0003 (R)
>3	Triangular	Resorption almost completed, some residual vacuoles	0.0042 ± 0.0002 (P)
			0.0072 ± 0.0004 (R)

ference in POF duration for the Iberian sardine across the study years could never exceed 0.5 days, thus reducing the concerns in relation to the potential bias introduced by varying ambient temperatures in the estimation of the spawning fraction.

## Discussion

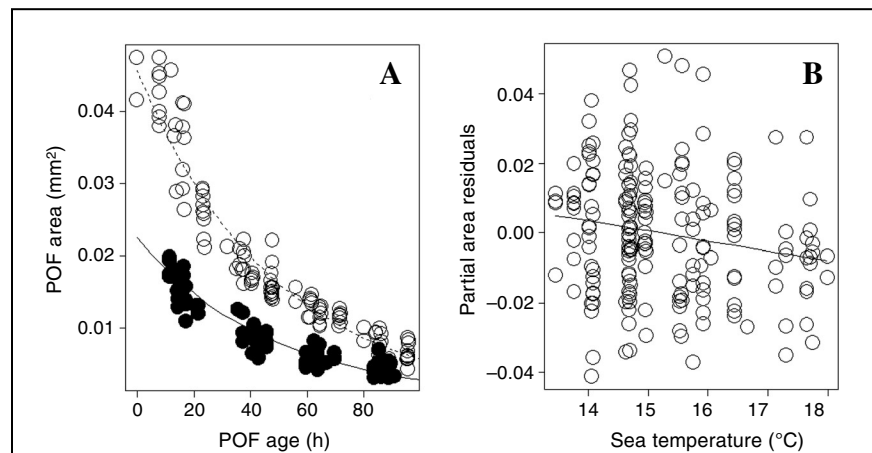
The incorrect attribution of age to POFs and the effect of temperature on POF resorption rate constitute major sources of potential bias in the determination of daily spawning classes in routine applications of the DEPM (Stratoudakis et al., 2006). Our method was devised as an indirect way to test the above issues together with the effect of laboratory processing, i.e., type of preservative and embedding material. The method is based on the assumption that in species with diel spawning synchronicity, such as in *S. pilchardus*, daily classes of spawning fish have, at any given time, POFs of similar size. The first task in our analysis was to refine existing staging criteria for the POFs of *Sardina pilchardus*. This task was mainly attempted through analyzing the frequency distribution of POF cross-sectional areas in females caught simultaneously and by inspecting POFs in each size-age mode for differences in the cytomorphological characteristics.

The main cytomorphological changes during the degeneration of POFs in sardine ovaries are the gradual deterioration of the structure of the follicular layers and alterations in the dimensional characteristics (shape and size), accompanied by a decrease in their overall number in the slide. Histo-

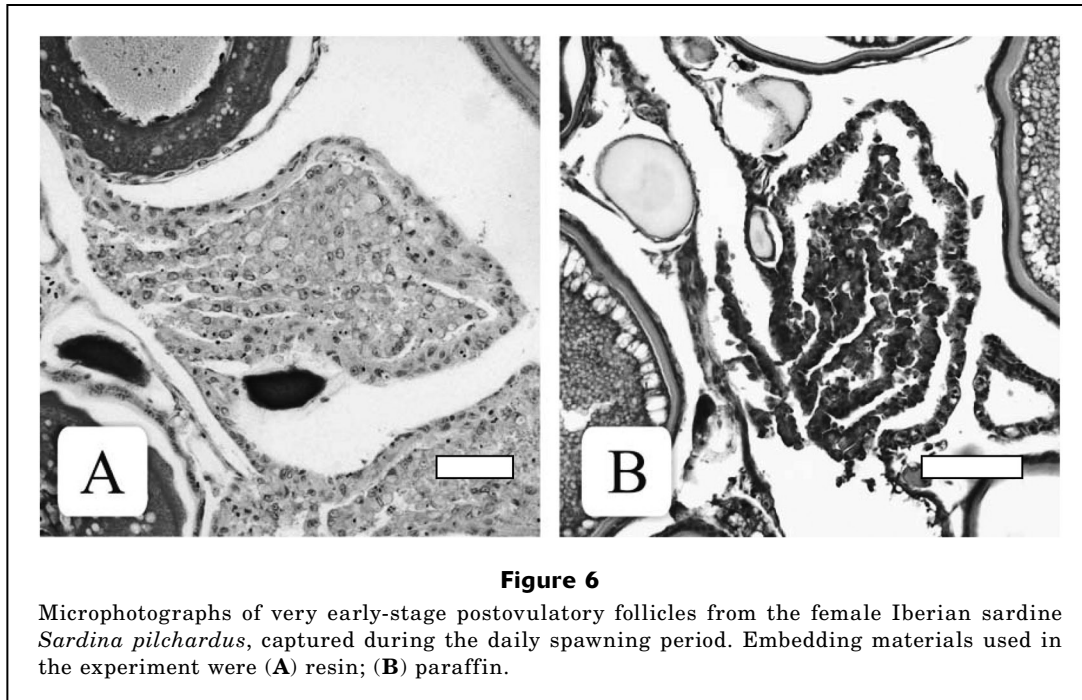
**Table 2**

Summary statistics of the general linear model fitted to the postovulatory follicle cross-sectional area for the sardine *Sardina pilchardus*. All parameter estimates were tabulated at the scale of the linear predictor (the intercept for resin as an increment). SE = standard error.

Variable	Estimate	SE	<i>t</i> -value	<i>P</i>
Age (hour)	-0.021	0.0004	-46.78	<0.001
Temp (°C)	-0.030	0.012	-2.44	0.016
Intercept (paraffin)	-3.344	0.197	-16.98	<0.001
Intercept (resin)	0.710	0.027	25.90	<0.001

**Figure 5**

(A) Degeneration of the postovulatory follicle (POF) cross-sectional area (POF area) with time elapsed from spawning (POF age) for the Iberian sardine *Sardina pilchardus*; ○=resin; ●=paraffin. (B) Effect of ambient temperature on the sardine POF cross-sectional area.

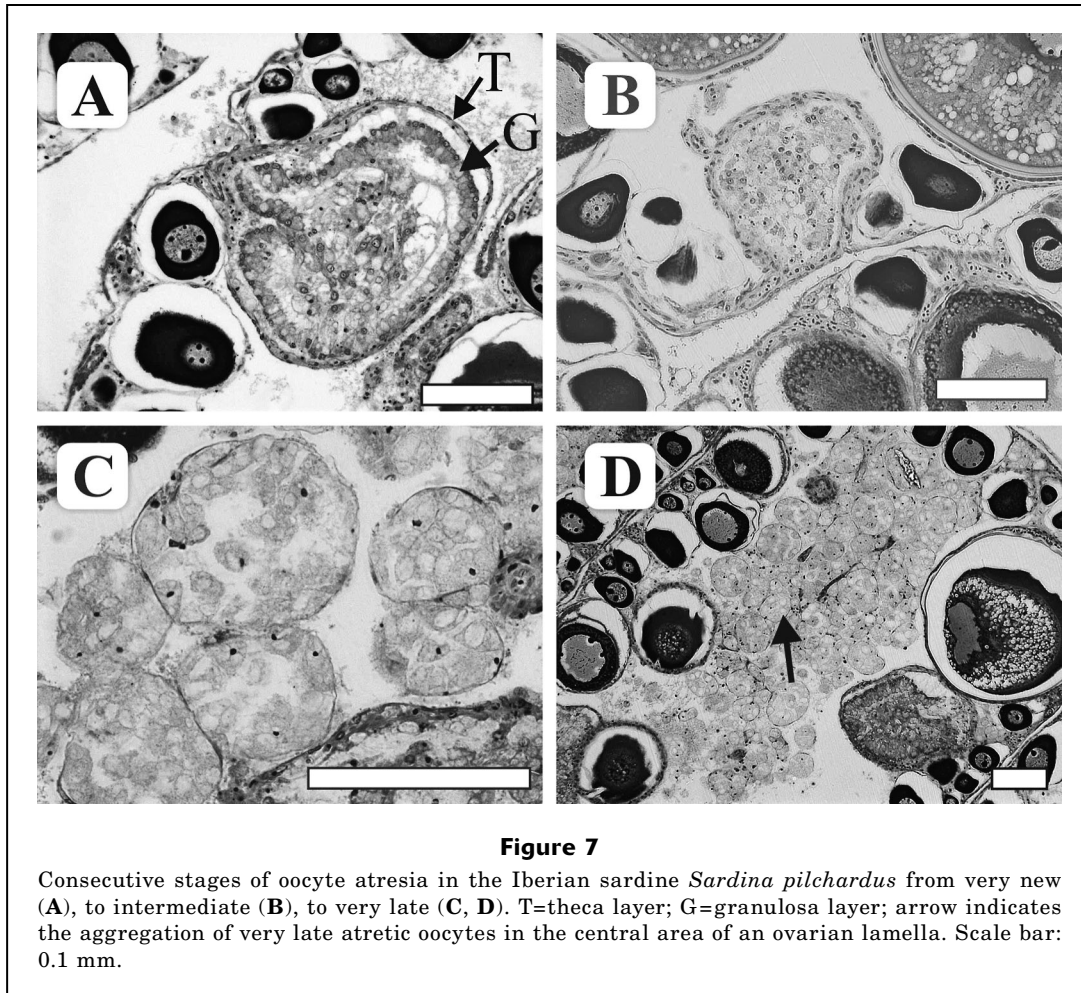


logical changes in the granulosa seem to follow the general pattern of degeneration described for other populations of sardine (Japanese sardine [*Sardinops melanostictus*]: Murayama et al., 1994; Mediterranean sardine [*S. pilchardus*]: Ganias et al., 2003; Pacific sardine [*Sardinops sagax*]: Goldberg et al., 1984; South African sardine [*S. sagax*]: Akkers et al., 1996) and other fish species (see interspecific comparison in Hunter and Macewicz, 1985). The information provided in our study mostly concerns the changes in the dimensional characteristics of POFs and how these may be used in the aging of these POFs.

The evolution in the shape of POFs is allometric because the POF surface along the lamellar epithelium decreases at a lower rate than the overall area of the follicles. As a result, throughout degeneration, POFs passed consecutively from an irregular to a semirectangular and finally a triangular shape, providing a useful additional morphological criterion for determining the stage of the POF. POFs remain for the whole of their “life” on the epithelium of the lamellae, where they occupy approximately the space of an oocyte at the yolk vesicle stage (early POFs) or of a primary oocyte (late POFs). For an indeterminate spawner like sardine, where recruitment of new spawning batches of oocytes occurs continuously and directly from the oogonia, fast resorption is necessary because the aggregation of old POFs would restrict the space available for the development of new oocytes. On the other hand, late atretic pre-ovulatory follicles separate from the epithelium and concentrate medially in the lamellae—a pattern that has also been observed in other fish species such as striped mullet (*Mugil cephalus*) (McDonough et al., 2005). Late atretic follicles remain in fish ovaries for

long periods, which might extend up to the next spawning season (Hunter and Lo, 1997; Miranda et al., 1999). Therefore, their separation from the lamellar epithelium possibly constitutes a mechanism for managing space availability for the newly recruited spawning batches.

The aforementioned morphological phases and the different histological characteristics of the granulosa layer were used, together with follicle size, in the aging of POFs. Four daily classes were identified, implying that full POF resorption in the Iberian sardine exceeded 72 hours. The duration of POF degeneration is variable among fish species, ranging from less than 1 day in the skipjack tuna (*Katsuwonus pelamis*) (Hunter et al., 1986) to more than 7 days in the piau-jejo (*L. taeniatus*) (Santos et al., 2005). However, there are reports of intraspecific variability in the duration of POF resorption, both under laboratory conditions (e.g., Atlantic menhaden, *Brevoortia tyrannus*; Fitzhugh and Hettler, 1995) and in the field (e.g., spotted seatrout [*Cynoscion nebulosus*]: Roumillat and Brouwer, 2004). In some cases the duration of POF resorption is underestimated because late POFs can be confused with late atretic stages (e.g., northern anchovy [*Engraulis mordax*] Hunter and Macewicz, 1980). However, given that surveys for refining the DEPM are undertaken at peak spawning months (Stratoudakis et al., 2006), POFs would so greatly outnumber atretic follicles in fish ovaries that there would be little confusion in distinguishing the two and thus would lead to a very minor bias in the duration of POF resorption. In addition, POFs in *S. pilchardus* are effectively distinguished from all types of atresia, and this distinction has been confirmed by the high degree of consistency in the scoring of post-ovulatory and atretic follicles by different observers. At



all stages, atretic follicles constitute enclosed cellular structures (Fig. 7), whereas POFs always maintain an opening towards the ovarian lumen (Fig. 3). In addition, as previously reported, late atretic oocytes separate from the epithelium of the lamellae (Fig. 7D), whereas POFs remain on the epithelium until full resorption (Fig. 3).

Sardine POFs shrank exponentially with time, reducing to almost half their size daily. Therefore, after the exponential relationship of POF resorption with time is estimated, ages may be estimated by applying the data for the cross-sectional areas of POFs and times of capture to the model. The measurement of POF cross-sectional areas is not very time consuming and could be merged into the routine of histological analysis for DEPM analyses. Finally, the attribution of ages may be finely tuned by inspecting histomorphological characteristics, especially from specimens that lie outside the fitting curve.

Before applying the above aging procedure to fish populations, attention should be drawn to other factors that may affect POF cross-sectional area, such as the laboratory treatment of ovaries and the environmental conditions at sampling, e.g., water tempera-

ture. In our study, the use of different preservation media (AFA solution and formalin) did not appear to affect the size of the POFs. On the other hand, the embedding material significantly affected the follicle's size, for POFs in resin were almost double those in paraffin. Resin is known to maintain cellular organization in tissues slightly affected, whereas paraffin causes significant shrinkage (Casotti, 2001; Dorph-Petersen et al., 2001). Besides size, the morphological characteristics of the follicles also differed between the two materials. Although the whole POF structure remained compact in resin, follicular folds in paraffin had shrunk and were detached from the thecal layer, even in young POFs. These differences, in combination to the thinner slices that are achieved with resin, make resin a much better material for detailed histological observations. However, for accuracy in the staging of POFs, both resin and paraffin seem to provide similar results.

Water temperature may affect the rate of POF resorption in teleosts both in the field (Ganias et al., 2003; Roumillat and Brouwer, 2004) and laboratory studies (Fitzhugh and Hettler, 1995). However, this effect has never been precisely quantified and thus temperature

could never be introduced as an auxiliary factor in the aging of POFs. In the present study, temperature had a significant effect on the rate of POF resorption. This effect appeared to be limited because an increase of 1°C in ambient temperature was estimated to accelerate the rate of POF resorption by almost 3%. Nevertheless, the maximum range of 4–5°C that can be observed during sardine DEPM surveys off the Iberian Peninsula corresponds to a 12–15% maximum difference in the rate of resorption and thus to a maximum of 8 hours lag in the degree of POF degeneration. The results of this analysis imply that in each DEPM survey, temperature differences between the subareas of the survey area are not expected to introduce serious bias in the correct classification of POFs and to subsequently affect estimates of their ages. Finally, given that the late daily classes of POFs are usually excluded from the estimation of spawning fraction, the maximum effect between the most extreme temperatures is even less important (<10%).

Tests, such as described in our study, should be performed at least once for each species or population to assess bias in the criteria used to determine POF stages and ages. Moreover, the test would provide comparative information on POF resorption rates, the impact of embedding material, and the effects of temperature and other environmental parameters on the estimates. In routine DEPM analyses, the measurement of POF cross-sectional areas could increase technical work, but not necessarily the precision in the estimates of the spawning fraction because females are again broken down into spawning nights as they were with the histological staging method. However, in cases where such relationships of POF age on POF size are already available, correspondence of POF sizes to spawning nights would be much more realistic than simple histological staging, which strongly depends on the experience of the observer and the quality of the slides.

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