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Setting up interpretation criteria for ageing juvenile european anchovy otoliths

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SUMMARY: A standardisation of daily age determination in juveniles for this species is proposed by examining a wide range in length of juveniles. Sagitta otoliths and the saggital plane were selected for the analysis and daily increments were read in the postrostrum and antirostrum axes. Certain regions of the otolith show wide, rhythmic double growth patterns. These 2 axes were read with two different objectives (x10 and x20) and using two different interpretation criteria: group band reading (GBR), by which a repetitive cyclic set of growth bands were taken as single daily increments and individual mark reading (IMR), by which each microincrement, regardless of its appearance, constituted a daily count. Hatching dates, relative error and coefficient of variation were applied in order to find the most consistent reading method, and were compared with known growth rates in this and other genera of anchovy. Though the hatching dates produced by the two ageing methods are compatible with the spawning period, we consider the GBR method to be the most reliable ageing procedure because: a) it is the most precise and robust (not being affected by the examination procedure); b) it allows the rhythmic pattern of alternating growth bands to be interpreted as sub-daily microincrements generated in the later phases of larvae and during metamorphosis; and c) it produces high rates of growth compatible with larva growth increments and with other studies for the same genus at similar temperatures.

Keywords: *Engraulis encrasicolus*, otolith, daily increments, sub-daily, juvenile, metamorphosis, growth.

RESUMEN: Establecimiento de un criterio de interpretación para datar otolitos de juveniles de anchoa europea. – Se propone una metodología con el fin de estandarizar la lectura de los incrementos diarios en juveniles de anchoa europea (*Engraulis encrasicolus*) basada en el procesado de un amplio rango de otolitos. Para ello se eligieron los otolitos *saggita* y su plano saggital, que se leyeron por dos ejes y con dos aumentos. Algunas zonas de los otolitos mostraron patrones de crecimiento rítmicos con apariencia de dobles incrementos muy evidentes. Se usaron dos criterios de interpretación: Lecturas por Bandas (GBR), donde cada ciclo repetitivo de grupos de incrementos son leídos como un solo incremento y Lecturas Individuales (IMR) por la que cada incremento, independientemente de su apariencia, constituye un incremento. Se aplicaron Errores Relativos y Coeficientes de Variación buscando el método más consistente y se compararon las tasas de crecimiento con otras anchoas del mismo género así como las fechas de nacimiento. A pesar de que estas fechas resultaron compatibles con la época de puesta por los dos métodos, consideramos que el GBR es el método mas adecuado ya que: a) es el mas preciso y robusto b) permite interpretar el patrón de crecimiento rítmico encontrado, con alternancia de bandas como incrementos sub-diarios generados en las fases tempranas y durante la metamorfosis y c) justifica unas tasas altas de crecimiento compatibles con los incrementos larvarios en consonancia con estudios anteriores en este y otros géneros a similares temperaturas.

Palabras clave: *Engraulis encrasicolus*, otolito, incrementos diarios, subdiarios, juveniles, metamorfosis, crecimiento.

INTRODUCTION

European anchovy (*Engraulis encrasicolus*) is an important species in the Bay of Biscay, from both an ecological and a socio-economic point of view (Uriarte *et al.*, 1996), with average annual catches of around 30000 tonnes for the last fifty years. The main characteristics of this small pelagic species are

a short life span, seasonal migrations, high growth rates, early maturity (at first birthday), a long spawning period and schooling behaviour

Since Pannella's 1971 discovery of otolith daily growth increments (DGI), other works have suggested that the existence of daily microincrements is a universal character among fish (Campana and Neilson 1985; Jones, 1992). DGI formation has been demonstrated for most fish species in which experiments in captivity have been performed (Geffen, 1992). DGI have been applied previously in studies of the genus *Engraulis*, considering larval growth in *E. encrasicolus* (Ré, 1986; Palomera *et al*., 1988; Dulcic *et al*., 1996; García *et al*., 1998), *E. japonicus* (Tsuji and Ayoama, 1984; Takahashi, 2001), *E. mordax* (Methot, 1979), *E. anchoita* (Castello *et al*., 2003), *E. ringens* (Morales-Nin, 1989; Hernández and Castro, 2000) and *E. capensis* (Prosch, 1986; Thomas, 1986); and age determination in *E. mordax* (Methot, 1981; Butler, 1989), *E. capensis* (Waldron 1989, 1992) and *E. encrasicolus* (Allain *et al*., 2003; Cermeño *et al*., 2003). Recently the daily nature of the juvenile and adult European anchovy DGI was demonstrated (Cermeño *et al*., 2003), providing a useful tool to elucidate the recruitment patterns of European anchovy in the Bay of Biscay.

However, one of the main problems found by authors dealing with DGI is the presence of sub-daily increments, which were noted by Tsuji and Aoyama (1984) in larvae of *Engraulis japonicus* and in premetamorphic *Engraulis encrasicolus* by Palomera *et al.* (1988). These sub-daily increments have not been described in any of the recent studies of post-larval and juvenile anchovy. Moreover, the methodology for differentiating between daily and sub-daily increments in otoliths of juveniles has not been standardised or described clearly in any paper. The general rule for distinguishing them is to bring the preparation into a focus in which all growth structures can be clearly read, and then vary the focus slightly so that the sub-daily increments disappear (Morales-Nin, 1992).

In a preliminary work with otoliths of juvenile European anchovy, we obtained back-calculated hatching dates incompatible with the known spawning period of this population, based on DGI counts at 400 magnifications, and adopting a 1:1 correspondence between increments and age in days. As the daily periodicity of the increments has been documented (Cermeño *et al*., 2003), there was an overestimation of the age, suggesting the presence of sub-daily increments. This led us to search for plausible age interpretation methods capable of providing sound age estimation by identifying rhythmic patterns of growth.

The first aim of this study was to standardise juvenile European anchovy otolith examination by selecting the pair of otoliths to be used, the optimal magnification and the plane of section. The second aim was to propose and describe repeatable and objective methods accounting for the interpretation of the likely sub-daily increments in order to avoid over-ageing and to enable the application of this powerful tool in future studies of the recruitment process in European anchovy. To this end a description of zones in the sagittal otoliths of anchovy juveniles according to different observed patterns of increment deposition was first made. Next, because so far all attempts to obtain a direct validation of ageing procedures for the early stages, rearing larval to juvenile, have failed for this anchovy, we propose two alternative DGI counting methods and compare them in terms of sensitivity to the examination procedure, precision in age determination, hatch-date back calculation and growth curves in order to discuss their precision, accuracy and reliability in ageing this species.

MATERIALS AND METHODS.

Sampling

Juvenile European anchovy otoliths were obtained from fishes captured in September 1999 (Survey JUVESU99, Uriarte *et al*., 2001). In order to get more contrast from the length distribution, juveniles were added from samples obtained in the port by AZTI in the autumn of 1997. In total the length range of the anchovy juveniles analysed was 3.2 to 15.6 cm.

Selection of the otolith

Anchovies were measured (total length L_T) to the nearest 0.1 mm. The three pairs of otoliths *lapillus, sagitta*, and *asteriscus* were removed using the up through the gills method (Secor *et al*., 1992) and examined. The otoliths were prepared following the methodology of Cermeño *et al*. (2003).

The best observations were obtained in the sagitta and hence these were retained for further studies choosing the sagittal plane as the clearest and easiest to follow along the perimeter.

One reader, ignoring date of capture and length, read each otolith straight from the monitor, using an Olympus microscope coupled to a high-resolution black and white video camera connected to a computer equipped with the TNPC image processing software (Visilog software platform, Noesis, France).

The DGI were observed using two objectives (x10 and x20) along three radii were measured from the otolith core (core-postrostrum, core-antirostrum and core-dorsal edge), although the last one was discarded due to the highly compressed increments towards the edge.

Age reading methodology

Previous methods for differentiating daily from sub-daily rings involve bringing the preparation into a focus in which all growth structures can be clearly read and then varying the focus slightly to remove sub-daily increments (Morales-Nin, 1992). This method did not apply to the juveniles of European anchovy due to the presence of wide, rhythmic double growth patterns in the central zone of the otoliths (whiter and darker bands) and groups of multiple increments.

Indices of precision

Precision was estimated by the coefficient of variation (CV) of increment counting between two readings (Chang, 1982) and also by the Beamish and Fournier equation (Hoenig *et al*., 1995) called relative error (*RE*).

$$RE = 100\frac{1}{N}\sum_{j=1}^{N}\left[\frac{1}{R}\sum_{t=1}^{R}\frac{\left|X_{ij}-X_{j}\right|}{X_{j}}\right]$$

where N = total of fishes read, R= number of times each fish is aged, Xij = microincrements read and Xj = the average calculated for each fish.

Paired *t*-tests were applied to evaluate the significance of the different age determinations (over the same otoliths), produced from the different examination (axes, magnifications) and interpretation criteria considered above.

Hatching dates

Hatch dates were calculated using the mouth opening check as the first day of the anchovy (Campana and Jones, 1992) and the hatching date was deduced by subtraction of the age estimated from the date of sampling.

The spawning of anchovy in the Bay of Biscay lasts from the end of March to the beginning of August, with a maximum peak between May and June (Sanz and Uriarte 1989, Uriarte *et al.*, 1996). Thus, any ageing method leading to hatch dates outside the known spawning period could be discarded.

General growth pattern

In order to understand the biological meaning of juvenile back-calculated growth rates during their post-larval stages by the two methodologies explored in this study, these rates were compared with their likely larval growth rates, so that the hypothesis of post-larval growth rates higher than or equal to that of larval phases (Campana, 1992) could be tested. To this end, since we did not have larvae sampling in 1999, a total of 164 otoliths of larvae collected in the south of the Bay of Biscay in June 2000 and 114 in July 2003 were also analysed. Otoliths were extracted and prepared as described in Cotano and Álvarez (2003) for mackerel larvae. The criteria described by Palomera *et al*., (1988) for otolith readings and distinction between daily and sub-daily rings were employed. Larval standard length (Ls) was measured before the extraction of the otoliths to the nearest 0.1 mm.

After comparing the widths of otolith increments of larvae and juveniles of a certain age, only the 2003 larvae were used. This allowed errors to be minimised, as they had the most similar growth rates to the larval period of the juveniles analysed in this paper (as seen in the close to core portion of their otoliths)

RESULTS

Description of the sagitta otoliths

The sagittal otoliths had a central core surrounded by thin increments that start formation after hatching (Palomera, 1989). However, this area had poor contrast, so we measured a radius of 14 μm in which we assumed that 9 DGI were laid down based on previous larvae otolith examinations (this would imply a maximum error of 2 DGI in that region). From this central area the sagittal otolith showed different

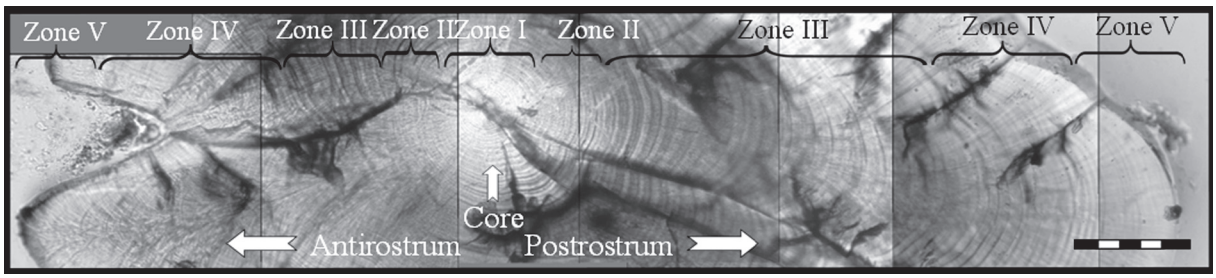

FIG. 1. – Saggital plane in a right saggital otolith showing the difference zones and aspects of the increments (x10) (Bar 50 μm).

growth patterns depending on both the otolith area and the otolith growth, with differences in width, appearance and readability of the increments (Fig. 1). These differences in the postrostrum and antirostrum, resulting in five zones, are described in Table 1. Typically increment widths increased from the core up to a distance from it along the post-rostrum axis of about 200-800 μm, where widths of about 6-13 μm were found (zone III), decreasing steady and gradually towards the edge of the otolith. In zone III, where greater widths were found, a difficult pattern of increments of rhythmic, alternating growth increments appeared (often clear and dark or thick and thin alternating growth increments (Fig. 1 and 2), which made it difficult to interpret them. At first, these group increments were clear even in poorly focused samples, so they could be interpreted as two daily increments or alternatively as sub-daily structures.

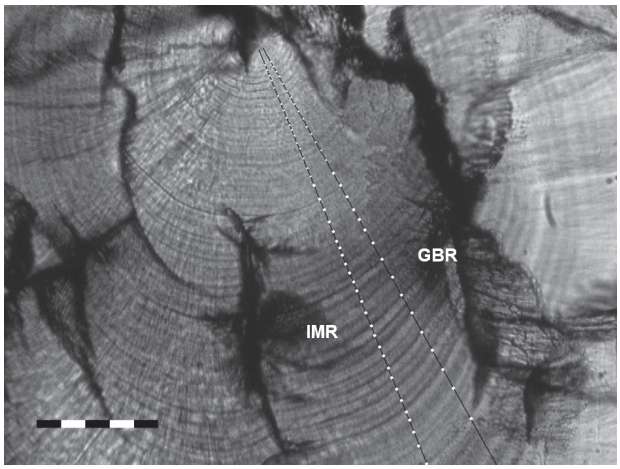

FIG. 2. – Saggital section of an anchovy otolith, showing the problems and reading methods for discriminating daily from sub-daily increments. Core showing the special rhythmic pattern of growth consistent in alternate white and dark bands (Zone I, II and III). GBR represents the growth band reading method, while IMR represents the individual mark reading method (Bar 100 μm).

We therefore tried two interpretation methods. In the first one, named group band reading (GBR), the reader counted as one every repetitive cyclic set of growth bands or groups of microincrements (usually 2 but occasionally more), assuming that they were sub-daily marks. The second one, named individual mark reading (IMR), took into consideration every clear increment regardless of its appearance (Fig. 2).

Double blind readings by each method (GBR and IMR) were done to assess precision, and if the dif-

TABLE 1. – Characteristics of the five zones in the sagittal otolith of European anchovy.

	Distance to core	Width of increments GBR	IMR	Description
POSTROSTRUM				
Zone I	0 to 22 μm	0.9 to 2 μm	0.9 to 2 μm	Typical larval core, rthymic and regular increments
Zone II	22 to 80 μm	3 to 7 μm	3 to 6 μm	First subdaily increments appear, easy to discriminate
Zone III	80 to 700 μm	7 to 17 μm	6 to 13 μm	Pattern of growth shows clear bands of alternant translucency. These structures do not dissapear in any case.
Zone IV	700 to 1200 μm	9 to 13 μm	9 to 13 μm	Organic matter desposited. Increments become regular, decreasing towards the edge. Sub-daily increments are less evident.
Zone V	1200 to edge	5 to 9 μm	5 to 9 μm	Continously decreasing.
ANTIROSTRUM				
Zone I	0 to 22 μm	0.9 to 2 μm	0.9 to 2 μm	Typical larval core, rthymic and regular increments
Zone II	22 to 60 μm	2 to 8 μm	2 to 8 μm	First subdaily increments appear, easly to discriminate
Zone III	60 to 400 μm	8 to 15 μm	6 to 11 μm	Pattern of growth shows clear bands of alternant translucency. These structures do not dissapear in any case.
Zone IV	400 to 900 μm	6 to 9 μm	6 to 9 μm	Organic matter desposited. Increments become regular, decreasing towards the edge. Sub-daily increments are less evidents.
Zone V	900 to edge	5 μm	5 μm	Continously decreasing.

TABLE 2. – Comparision of the amplification effects on the different age reading methods for the two sagittal axes, checked by paired *t* test (two tailed) on individual otolith age determinations.

	Group Band Method Paired t-test (10 vs 20)			Individual Mark Method Paired t-test (10 vs 20)		
	Nº Otoliths	Mean difference (20-10)	P(Dif=0)	Nº Otoliths	Mean difference (20-10)	P(Dif=0)
Postrostrum	21	-3.6	**0.0709**	21	-6.3	0.0390
Antirostrum	15	0.4	**0.7815**	15	-8.5	0.0012

TABLE 3. – Average daily increments and precision obtained for the two age reading procedures (GBR and IMR) and for the otolith axes used for the examination and comparison of the different age reading results by paired *t* test (two tailed) on individual otolith's age determination.

POSTROSTRUM Method	No.of otoliths	Mean age	CV %	RE %
GBR	25	85.3	2.41	1.85
IMR	19	101.1	2.65	1.89
ANTIROSTRUM	No.of otoliths	Mean age	CV %	RE %
GBR	17	79.6	3.65	2.65
IMR	14	86.0	3.89	2.78

Comparison between otolith Axes by methods	Average difference in age (Post-Anti)	P(Dif=0)	No.of otoliths
GBR	2.3	**0.3494**	17
IMR	9.1	**0.0628**	14

Comparison between methods by axes		
Average difference in age (GBR-IMR)	-16.4	-11.07
P(Dif=0)	6.96E-10	5.88E-06
No.of otoliths	19	14
Increase of IMR over GBR	19%	14%

ference were more than 7%, a third reading was performed; the otolith was discarded if the difference between the new ageing and the closest among the previous readings was still higher than 7%. Otherwise, the two closest readings were retained. Individual DGI were measured for each method (making use of the TNPC image processing software) and they were compared along the postrostrum axis radius.

Magnification

The paired *t*-test showed that the magnification (x10 or x20) did not affect the number of DGI counted with the GBR method in either the postrostrum or antirostrum axes (Table 2). A significant difference was found using the IMR method, by which the higher the magnification the more DGI were counted. We decided to use the x10 magnification to gain more perspective of the whole otolith and to reduce the processing work.

Precision

In all cases the indices of precision applied to the readings on the two axes (postrostrum and antirostrum) using different magnifications and the two methods showed a low relative error (less than 5%). These results indicate that readings for a given selected method and axis are internally consistent and precise, being slightly better in the postrostrum axis (Table 3). However the consistency of the reading methods according to the axes of examination was different: while GBR produced consistent results, significant differences (at alpha 10%) were found between axes for IMR (Table 3).

Age readings

IMR gave consistently older ages than GBR: by about 16 days for the postrostrum readings (Table 3) and about 11 days for the antirostrum readings. This represents a relative increase in age of IMR over GBR of c. 19% and 14% respective to these two axes. These differences are mostly already established in the smallest of the juveniles here analysed (Fig. 3): At 40 mm length the methods showed an average difference of about 16 days in the age determinations,

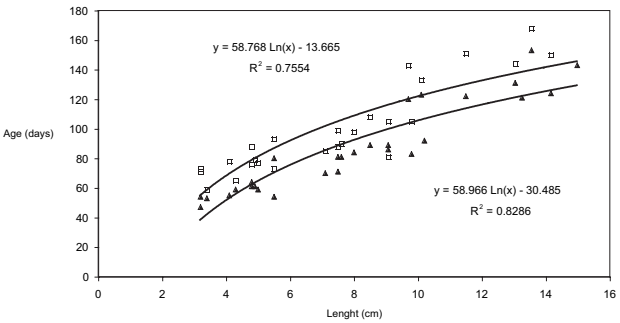

FIG. 3. – Logarithmic growth curve for European anchovy, read in the postrostrum with the two methods. GBR (triangles) n=22 and IMR (squares) n=22

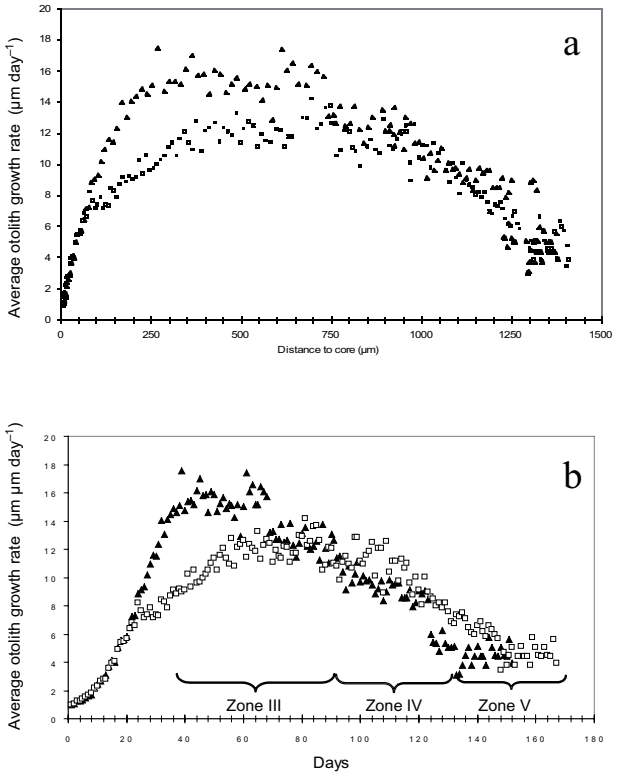

FIG. 4. – a) Average otolith growth rate of juvenile measured in the postrostrum axis from core. Group band method (triangles) and individual mark method (squares) n=22. b) Average otolith growth rate of juvenile measured in the postrostrum axis in days. Group band method (triangles) and individual mark method (squares) n=22.

increasing very slightly afterwards. The differences in age determination by the two methods originate from zones II and III (Fig. 1 and 2), where the presence of multiple increments with the appearance of double bands or rhythmic growth bands made interpretation difficult. The main difference between the methods was induced by interpretation of the micro-increments in these two zones, which were formed well before the time these juveniles were caught and started at an estimated age of 22 days. These two age determination methods therefore produce different growth patterns of the otolith by individual DGI along the postrostrum axis: the greatest average DGI (average increment growth of each otolith in each day) are greater and are laid down earlier by GBR than by IMR (Fig. 4).

Hatching dates and spawning time

To test the compatibility of the methods with the spawning season indirectly, the birth date was back-calculated from the date of capture and the age obtained with each reading method in the postrostrum axis. Though large individuals belonging to the 1997

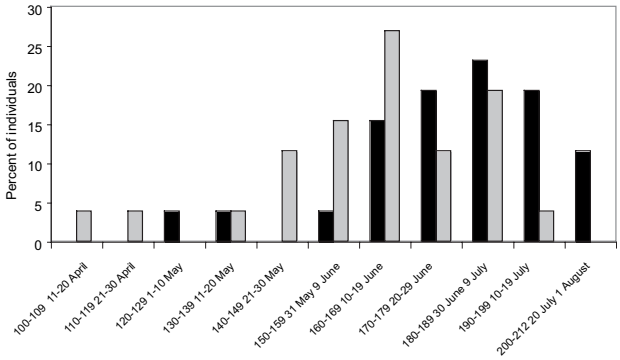

FIG. 5. – Birth date distribution comparing the two methods of reading in the postrostrum using x10 amplification. Solid bar (IMR), spotted bar (GBR). The horizontal axis represents days in Julian calendar days and dates.

cohort were inlcuded, the birthdates calculated by the two methods gave hatching between late April and the end of July, which falls within the spawning season (Fig. 5).

Larval and juvenile growth

Standard length, from the tip of the snout to the tip of the notochord, (L_S) for the 2003 anchovy larvae ranged between 4.7 mm to 20.2 mm for individuals between 2 and 26 days old (using Palomera, 1989 methodology). Length-at-age of these larvae was well described by a linear model, which explained 87.3% of the variability in L_S. The equation relating larvae length and age is described in Figure 6:

$$L_S = 0.6517*age + 2.8199$$

This model predicted an average growth rate of 0.65 mm.day-1 for anchovy younger than 26 days old. According to the model, larvae will reach a L_S of 19.8 mm at that age. In Figure 6 we contrast this information with the growth rates resulting from the two age reading methods for a random set of juveniles smaller than 50 mm (mean total length, L_T, of 41.9 ± 0.75 mm ±S.D.). The two ageing methods gave average ages of 57 and 74 days, respectively. This would imply a mean daily growth rate since hatching of 0.73 mm.day^{-1} by GBR and 0.57 mm.day^{-1} by IMR. Assuming the same larvae growth rates for the larval phase of this set of juveniles as that of the larvae in 2003, we would infer mean daily growth rates from the period of late larvae to the early juvenile stage of 0.71 mm.day^{-1} by GBR and 0.46 mm.day^{-1} by IMR (Fig. 6). This would imply a reduction in growth rates in the post-larval stages by the IMR method.

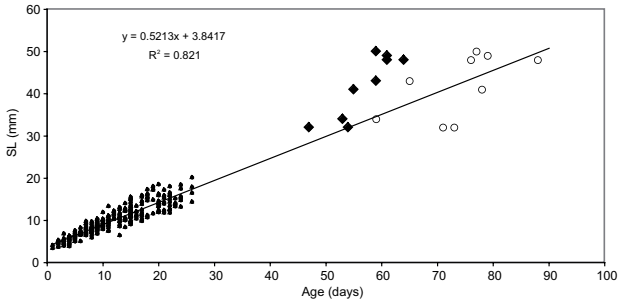

FIG. 6. – Comparison between the linear model of larval growth (solid line) and the length at age of juveniles obtained from postrostrum readings of age based on the IMR method (white circles) and the GBR method (black diamonds). Solid black circles correspond to larval age determinations

DISCUSSION

Otolith/plane/magnification/axis selection

The indices (CV and RE) used show that the lowest internal error in readings was found on the postrostrum axis (Table 2), which also showed wider increments that were easier to discriminate and measure. Increments in the antirostrum axis, despite the more regular growth rate, caused difficulties for ageing older juveniles (e.g. from 1997) due to more compressed increments along a shorter axis, with the risk of underestimating the age.

Ageing method selection and biological interpretation of increments

In terms of methodology, both methods showed an internal consistency in the age determination for a given magnification and axes of examination with a low coefficient of variation and relative errors (CV of about 2-4% and RE of about 1-3%), slightly better for the GBR than the IMR method. However, the two methods were different in their sensitivity to the selected examination methodology (Table 2 and 3): The GBR age determinations were not affected by the selected magnification or by the axes of reading, which made it the most robust method (objective and repeatable). On the other hand, the IMR ageing results were especially sensitive to magnifications and to the axes of reading (although in the latter case at an alpha of 10% [type I error]).

The age determinations resulting from the two methods were statistically different, resulting in older ages with IMR than with GBR (by about 16 days in the post-rostrum axes). This difference was already present for the smallest of the juveniles analysed (of about 3.5 cm) and remained constant for all other larger sizes (Fig. 3 and Table 2), indicating that the total difference was between the larvae and the juvenile stages, i.e. during metamorphosis.

There is a lack of knowledge about the metamorphic stage in the European anchovy, because only larvae up to c. 20 mm and early juveniles over c. 30 mm have been collected in samples, indicating that during this stage anchovy is not accessible. Metamorphosis should therefore occur between 25 and 45 days after hatching (GBR), or between 25 and 65 days (IMR) (Fig. 6). This is the period of maximum presence of rhythmic double growth bands (zone III, Figs. 1 and 2), which causes the maximum discrepancy between the two methods (Fig. 4). The presence of sub-zones or double increment zones (Fig. 2) in European anchovies, in which a great quantity of protein matrix is probably present (Cermeño *et al*., 2006), make them complicated to interpret, leading to a possible over- or underestimation of the age. At first, these group increments are clear even in poorly focused samples, so they could be interpreted as two daily increments or alternatively as sub-daily structures. Studies of double or sub-daily increments are scarce. In larval otoliths of anchovy, Palomera (1989) described the presence of sub-daily increments that correspond to zone II in juvenile otoliths; in several species Zhang and Runham (1992) found similar structures during fast growing periods; and Morales-Nin and Aldebert (1997) and Tomás and Panfili (2000) relate sub-daily increments to metamorphosis stage and changes in habitats. The interpretation as sub-daily increments provided by the GBR method for the rhythmic double growth band pattern of zone II and III in the otoliths of juvenile anchovy would be in agreement with the interpretation followed by the above authors for those early stages.

Growth studies on *Engraulidae* species including early juvenile individuals are scarce in the literature (e.g. Methot, 1979; Rilling and Houde, 1999; Aoki and Miyashita, 2000; Takahashi *et al*., 2001, Allain *et al*., 2003). Both linear and exponential models, such as that of Gompertz, have been commonly used to describe growth curves in these studies. In these, daily growth rates are predicted to remain constant or increase with age for individuals beyond 40 days old. The results calculated by GBR fit better with the growth obtained for the larval stages, since this method implies a slight increase in daily growth rates for larvae older than 26 days to reach the actual length of the early juve-

niles caught, which is in agreement with sustained or slightly increasing growth rates over these early stages of life, as reported by Campana (1992). On the other hand, the result obtained by IMR does not agree with this idea, because it implies an unexpected reduction in daily growth rates for larvae older than 26 days (Fig. 6). A reduction in daily growth rates from larval to juvenile stage (as IMR predicts) could be considered assuming that the metamorphosis is an energetically costly non-feeding period. Hunter (1976) stated that anchovy was able to withstand starvation for two weeks during metamorphosis. Nevertheless, some of the analysed juveniles show a characteristic otolith structure with thick and thin alternative growth bands in a very constant and rhythmical pattern (see Fig. 2). Assuming the IMR method is correct, the described pattern would imply alternative high and low daily growth rates, which are difficult to explain and have not been described previously in the literature. This type of distinct alternate growth pattern is considered by other authors (Tomas and Panfili, 2000; Hwang and Lee, 2005) as a sufficient indication of sub-daily increments, giving preference to the interpretation made by the GBR method. The interpretation of sub-daily increments for these rhythmic thick and thin alternative bands is further supported by the analysis of the microincrements of zone III of the otolith in the electronic microscopic (Cermeño *et al*., 2006), which confirms these composite wide increments to be single growth units, where a thin hypermineralised layer is laid down before the discontinuity with the next increment, which forms the second thin sub-daily increment of the composite band.

The average growth rate from hatching to early juveniles estimated for the European anchovy in this work is 0.56 mm day^{-1} for IMR and 0.73 mm day^{-1} for the GBR. Similar ranges in growth rates for early stages (before and after metamorphosis) are found in other anchovy species: for *E. mordax* larvae Hunter (1976), Methot (1983) and Butler (1989) found a growth rate of 0.36 to 0.55 mm day^{-1} (10-35 mm length and a period of metamorphosis of c. 55 days), while for *E. japonicus* Sigel (1988), Tsuji and Aoyama (1984), Mitani (1990), Takahashi *et al.* (2001), and Takahashi and Watanabe (2004a) found the growth rate to range from 0.49 to 0.73 mm day^{-1} (5-35 mm length and a period of metamorphosis of c. 30 days). Thus, depending on the reading method our growth estimates would be closer to those of one or the other of the engraulidae considered above (the GBR method being out of range with *E. mordax*). Differences in growth should be expected between different species and places, since temperature affects larval metabolism (Houde, 1989). *E. mordax* early stages develop up to early juveniles in cooler waters (generally below 18ºC, Methot [1983]) than *E. japonicus* (generally at or above 18ºC, Aoki and Miyashita (2000), Nakata *et al*., 2000) and than this European anchovy (generally above 18 as well, Uriarte *et al.*, 2001). According to the similar environmental temperature ranges and metamorphosis duration, the European anchovy could probably have a growth pattern more similar to the Japanese anchovy than to the northern anchovy.

Studies with Japanese Anchovy (*E. japonicus*) by Takahashi and Watanabe (2004b) showed similar daily increment widths to the ones found here with the GBR method. They found that the average maximum increment width increased to c. 16 μm, and after this peak gradually decreased as we have shown in *E. encrasicolus* when following the group band method. This method results in a maximum average increment width of c. 15 μm, followed by a slow downward curve with a decrease in the otolith daily growth rates. Using the IMR method, average maximum increments of c. 12 μm are reached and remain stable for a long period (c. 50 days). A study by Allain *et al.* (2003) showed for this anchovy an average maximum increment of c. 9-12 μm, which is similar to that shown by the IMR method. Cermeño *et al*. (2003), during the validation experiment, found average increments of 1.1 μm in the margin of the antirostrum in both elder juveniles and adults otoliths using x40 magnification. It is impossible to compare the two studies because, firstly, a different axis was used due to problems with the oxitetracycline deposition and, secondly, a reduction in growth rates could be expected in the validation study for these stages (end of juveniles and adult stage).

The spawning season occurs from March to the beginning of August, with a peak in May-June (Uriarte *et al*., 1996). Bearing in mind the different reading methods, only previous readings to this study performed using a 1:1 criterion at greater magnification gave birthdays outside the spawning time (December-February) and could therefore be eliminated, thus confirming the presence of a great number of sub-daily increments. However, the two ageing methods (GBR and IMR) back-calculated hatch dates compatible with spawning time. Not enough

contrast was obtained, even using old juveniles from the end of the 1997, because the final error did not increase with length. Even the presence of hatching dates in early April pointed out by the IMR method (Fig. 5) might occasionally occur for this anchovy since spawning is in fact occurring at that time.

Though the hatching data do not allow either of the two age reading methods to be discarded, all other former criteria lead us to consider the GBR method to be the most reliable one for age determination of European juvenile anchovy. First, it is the most precise (lowest CV and RE) and robust (not affected by the examination procedure) of the two. Second, this method responds to the rhythmic pattern of alternating growth bands (clear and dark often present in zone III of these otoliths) to be interpreted as sub-daily microincrements. This growth pattern would generate sub-daily increments in the later phases of larvae stages and during the metamorphosis stage, in agreement with previous studies on this and other genera. Finally, it produces high rates of growth compatible with larva growth increments and with other studies for the same genus at similar temperatures. Moreover the presence of juveniles between 10 and 15 cm in the live bait fisheries at the end of summer has been recorded (Boyra *et al.*, 2007), it would imply a very high growth rate for the European anchovy (Fig. 5).

Juvenile fish otoliths are generally very difficult to interpret and few studies encompass this development stage. This complexity is mainly due to the changes related to metamorphosis and associated life patterns (zone II and III). Moreover, the otoliths adapt to them with shape changes and complex otolith microstructure. In assessing the otolith interpretation, first an alphabet has to be defined and then a series of grammatical rules (i.e. the growth structures and their meaning) (Morales-Nin and Panfili, 2002) to allow the information encoded in the otoliths to be understood.

The adoption of the GBR method seems more suitable. Only a controlled experiment rearing European anchovy from eggs to juveniles could definitively verify whether the GBR interpretations of the otolith growth pattern during the metamorphosis stage is correct. In this contribution we have aimed to establish sound rules for juvenile European anchovy otoliths: we provide a description of the otolith zones according to growth patterns and we propose an examination procedure and an age reading method (GBR) that is robust and repeatable.

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