SPECIAL ISSUE

CSIRO PUBLISHING

Marine and Freshwater Research, 2016, **67**, 943–950
http://dx.doi.org/10.1071/MF15083

Validation of the first annual increment deposition in the otoliths of European anchovy in the Bay of Biscay based on otolith microstructure analysis

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Abstract. In order to validate the first annual increment deposition in European anchovy otoliths, early juveniles were captured in October 2012 in the southern Bay of Biscay. These individuals were maintained under a continuous feeding regimen in a sea cage over a period of 6 months. From October 2012 to January 2013, lengths increased slightly or remained stable at around 9.8 cm. After this period, standard length increased significantly up to a mean value of 12.0 cm in April 2013. Likewise, the age of anchovies was estimated based on otolith microstructure analysis. The estimated age varied from 96 days (for individuals sampled in October 2012) to 293 days (for anchovies sampled in April 2013). A daily increment deposition rate was confirmed in otoliths of individuals maintained in the sea cage during the winter. The general otolith daily growth pattern showed that increment widths increased rapidly and were broadest between 51 and 56 days, with a mean of 19.1 µm. Thereafter, the widths decreased steadily to 1.5 µm and remained almost constant until the end of the experiment. The present study also revealed that the first translucent band formation started in autumn and was completed by spring.

Additional keywords: age, daily increment deposition, *Engraulis encrasicolus*.

Received 28 February 2015, accepted 14 September 2015, published online 6 November 2015

Introduction

Otoliths contain information about the age and growth history of marine fish, at both a daily and annual level. On the annual level, otolith macrostructure analysis enables determination of fish age, which is fundamental to an understanding of population dynamics. It is largely assumed that the annual growth increment, known as an annulus, is laid down at regular intervals following a seasonal cycle (Beckman and Wilson 1995; Pilling *et al.* 2007; Hüssy *et al.* 2010). The annulus is composed of an opaque and translucent band, which differ optically under transmitted light (Neat *et al.* 2008). In temperate species, the general interpretation is that formation of the opaque band is related to a fast growth period during spring and summer, whereas the translucent band or 'winter ring' is generally associated with a period of slow growth rate in autumn and winter (Pannella 1974; Victor and Brothers 1982).

The alternation of opaque and translucent bands in otoliths has been used to estimate fish age (Campana 2001). In the case of the European anchovy in the Bay of Biscay, age determination has traditionally been conducted by interpretation of these structures (ICES 2009) based on an unpublished validation study (A. Uriarte, pers. comm.). In this area, the anchovy has a continuous spawning season between March and August (Motos 1996; Bellier *et al.* 2007) and larvae develop during spring and summer (Cotano *et al.* 2008). Juveniles are regularly observed in autumn (Aldanondo *et al.* 2010; Boyra *et al.* 2013) and, after overcoming the winter period, they are finally recruited to the adult population the following spring. Taking the timing of recruitment into account, in spring 1-year-old individual otolith shows a complete opaque and translucent band and the start of the second opaque band (ICES 2009).

Nevertheless, the factors regulating the formation of the opaque and translucent bands are unclear and substantial variations in the timing of translucent band formation at individual, population and species levels have been reported (Beckman and Wilson 1995). Therefore, to obtain reliable age estimates, it is necessary to validate the periodicity and timing of annual increment deposition in otoliths (Campana 2001).

Daily increment analysis has been used to validate annual increment formation for many fish species, particularly for the first annulus (e.g. Brothers *et al.* 1976; Victor and Brothers 1982; Wright *et al.* 2002; Sequeira *et al.* 2009; Hüssy *et al.* 2010). For European anchovy, daily increment deposition has been validated for larvae (Aldanondo *et al.* 2008) and early juveniles (Cermeño *et al.* 2003). However, certain variability in daily increment deposition was observed for individuals maintained in captivity for long periods (over a period of 2 years), suggesting an underestimation of the age in adults (Cermeño *et al.* 2003).

Table 1. Sea cage sampling details and the number of individuals, mean standard length (SL), mean weight (W) and relative condition factor (K_n) for anchovies in each sampling

Sampling	Days	Date	Number of individuals	SL (cm)	W (g)	K_n
1	0	5 October 2012	50	9.3	4.3	1.11
2	25	30 October 2012	30	9.8	4.7	0.96
3	55	29 November 2012	30	9.9	5.2	1.01
4	68	12 December 2012	30	10.2	5.5	1.00
5	97	10 January 2013	30	10.1	5.6	0.98
6	111	24 January 2013	27	10.1	5.6	0.98
7	137	19 February 2013	30	10.8	7.4	1.05
8	189	12 April 2013	50	12.0	10.7	1.04

Several authors have reported that laboratory conditions often affect the growth of larval, juvenile and adult fish, and therefore the formation of increments in the otoliths (e.g. Geffen 1982; Folkvord *et al.* 2000; Feet *et al.* 2002; Aldanondo *et al.* 2008; Namiki *et al.* 2010). For validation studies, Geffen (1992) stated that the fish should be reared under conditions that contribute to good growth and natural behaviour, thus avoiding as much as possible the effect of captivity. Large outdoor enclosures, where photoperiod and temperature cycles reflect natural conditions, seem to ensure good fish growth and development, and are considered a suitable solution to overcome possible rearing-related artefacts (Geffen 1992; Folkvord *et al.* 1996, 1997; Feet *et al.* 2002). This approach enables an enclosed and well-defined population to be followed over time under seminatural conditions.

The main objectives of the present study were to: (1) assess temporal variations in juvenile length and weight during the winter under a continuous feeding regimen; (2) validate daily increment deposition in otoliths of the anchovy during the first slow growth period in the Bay of Biscay; and (3) validate the first annual increment deposition in otoliths of the anchovy, as well as to determine the timing of the formation of the first translucent band.

Material and methods

Rearing experiment

Early anchovy juveniles were captured by purse seine in October 2012 in the southern Bay of Biscay. These individuals, weighing ~1000 kg in total weight, were transferred to a cylindrical sea culture cage (16-m diameter, 7-m net depth, 4-mm mesh size) located in Mutriku (Gipuzkoa, Spain; 43°18′N, 02°22′W). Anchovies were fed by hand once daily with a commercial diet (Microbaq; Dibaq Acuicultura, Segovia, Spain) at 4% body weight (Tudela and Palomera 1995) and maintained in captivity until the formation of the first translucent band in otoliths was complete (April 2013). Juveniles were sampled at approximate 27-day intervals, weighed (W; to the nearest 0.1 g) and measured for standard length (SL; to the nearest 1 mm; Table 1).

Otolith analysis

In the laboratory, both sagittal otoliths were removed from each individual. To establish the time of first translucent band deposition, otolith pairs were placed in black containers with the sulcus facing down and examined in water under a dissecting microscope at 10× magnification with reflected light. Translucent band formation was determined according to criteria established by the ICES (2009).

For otolith microstructure analysis, five individuals for each sampling were processed to obtain a representative sample of the whole experiment. For each sample, the right otolith was processed following the methods described by Aldanondo *et al.* (2010). Otoliths were analysed under a light microscope coupled to an image analyser (TNPC Software, version 3.2; Visilog, Ifremer, France). The central part of otoliths was evaluated at a magnification of 1000× in immersion oil, whereas the outer opaque band was analysed at a magnification of 100×. For translucent band analysis, samples were analysed at a magnification of 630×. Composite image files were constructed to enable the reader to scroll across the complete otolith image during analysis.

All increments were counted starting at hatch increment (Aldanondo *et al.* 2008) and the distance between increments was measured along the same axis from the core to the edge of the otolith on the post-rostrum side. In addition, the distance from the core to the beginning and the end of the first translucent band was measured along the same axis. For daily increment interpretation, the group band reading method suggested by Cermeño *et al.* (2008) was used. Each otolith was read independently by two readers (N. Aldanondo and U. Cotano) and any otolith with a difference between readers >5% was rejected. Of 42 otoliths read, 40 were used in the analysis. Because daily increment deposition has been validated for European anchovy larvae (Aldanondo *et al.* 2008) and juveniles (Cermeño *et al.* 2003), the increment number was considered a proxy for age in days.

Condition factor

The length–weight relationship for anchovies in the experiment was established using the simple allometric equation

$$W' = a\mathrm{SL}^b$$

where W′ is predicted length-specific mean weight for the sample (g), a is a constant of proportionality, and b is the allometric factor. Because Fulton's condition factor was significantly correlated with body length, relative condition

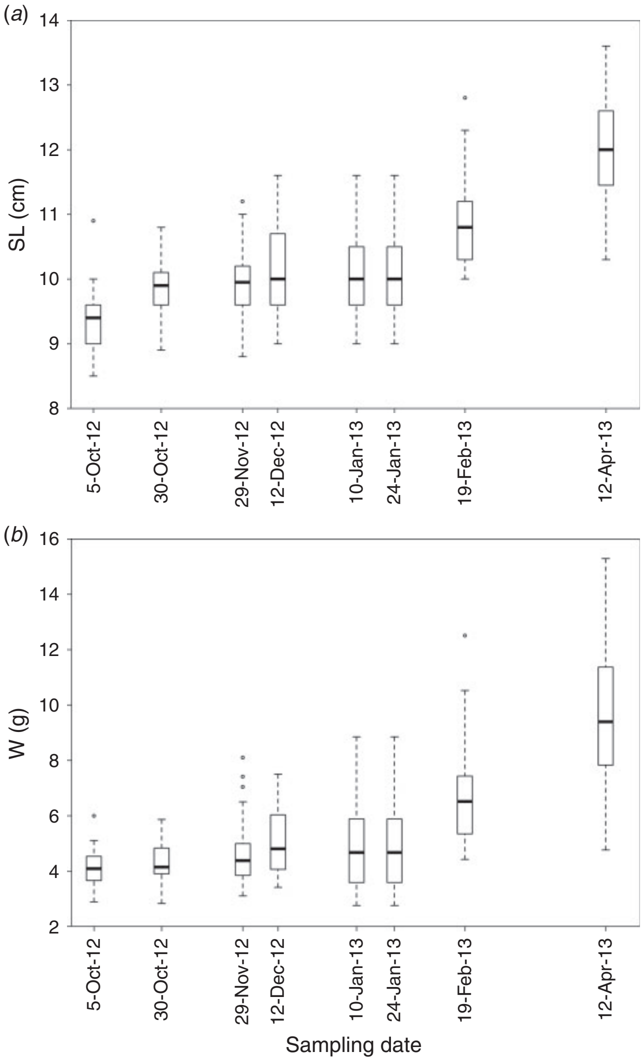

Fig. 1. Temporal variations in juvenile (*a*) standard length (SL) and (*b*) weight (W) in the sea cage. Median values are indicated by horizontal lines within the boxes, which show the interquartile range; whiskers indicate minimum and maximum values of the calculated non-outlier values, with outliers indicated by the open circles.

factor (K_n; Le Cren 1951) was used to compare body weight as follows:

$$K_n = \frac{W}{W'} \qquad (1)$$

Results

Length and weight temporal variation

There was a significant increase in length during the initial 3 weeks of the experiment ($P < 0.001$, Kruskal–Wallis test; Fig. 1*a*). From the end of October 2012 until January 2013, anchovy length increased slightly or remained stable at around a mean ± s.d. value of 9.8 ± 0.6 cm. After this period, SL increased significantly up to a mean value of 12.0 ± 0.8 cm in April 2013 ($P < 0.001$, Kruskal–Wallis test).

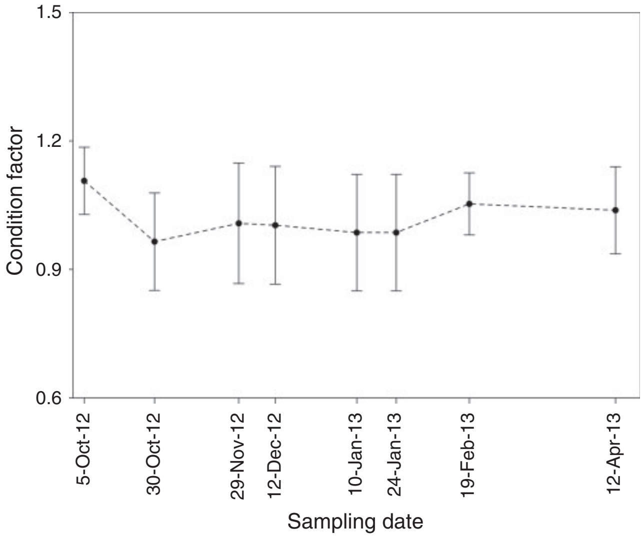

Fig. 2. Temporal variations in the relative condition factor of anchovies maintained in the sea cage. Data are the mean ± s.d.

Juvenile weight distribution showed a similar trend to that observed for length (Fig. 1*b*). In this case, individual weight was less variable from the beginning of the experiment until January 2013 (mean ± s.d. weight = 4.6 ± 1.2 g). Thereafter, weight increased significantly up to a mean value of 10.7 g at the termination of the experiment ($P < 0.001$, Kruskal–Wallis test).

The relationship between length and weight was significant, and was given by the equation:

$$W' = 0.0013SL^{3.56}$$

where $n = 261$; $R^2 = 0.89$; and $P < 0.001$. There were significant differences in the condition of anchovies among samplings ($P < 0.05$, Kruskal–Wallis test; Fig. 2). Individuals sampled in February and April 2013 had the highest mean values (1.04 ± 0.09), indicating that these anchovies were better conditioned than those from the previous samplings. No significant differences were found among individuals sampled between the end of October 2012 and January 2013 (0.98 ± 0.13; $P > 0.05$, Kruskal–Wallis test).

Age estimation and increment deposition rate

The age (in days) of anchovy juveniles was estimated based on otolith microstructure analysis. The estimated age varied from 96 days for individuals sampled in October 2012 to 293 days for anchovies sampled in April 2013 (Fig. 3). Otolith-derived hatch date distributions indicated that these anchovies originated from late spring and early summer (between 12 June and 16 July), with a mean hatch date of 1 July (±10.2 days).

The relationship between the number of increments (I) and days in captivity (D) was significant, and was given by the following equation

$$I = 98.04 + 0.96D$$

where $n = 40$; $R^2 = 0.97$; and $P < 0.001$ (Fig. 3). The estimated number of increments was compared with the number of days in

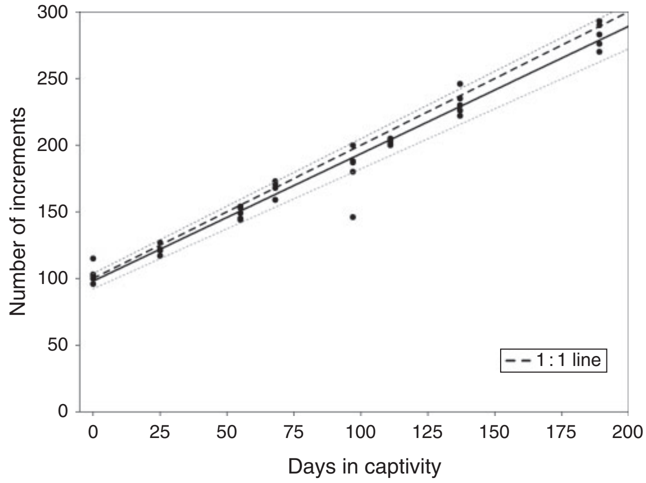

Fig. 3. Relationship between the observed number of increments and days in captivity of European anchovy reared in the sea cage. The grey dashed lines represent 95% confidence intervals; the black dashed line indicates the line of 1 : 1 agreement. The solid line indicates the regression line between the observed number of increments and days in captivity.

captivity using linear regression. To this end, and based on estimated mean age, it was assumed that juveniles were, on average, 100 days old at the beginning of the experiment. The regression analysis showed that the slope approached 1 (95% confidence interval 0.89–1.01) and therefore the number of increments was in accordance with a daily increment deposition rate in otoliths of anchovies maintained in the sea cage.

Somatic and otolith growth

The length at age data were described by linear regression analysis (Fig. 4*a*). The estimated mean somatic growth rate for anchovies during the experiment was 0.16 mm day^{-1}. The otolith growth pattern was similar to that observed for somatic growth. A linear regression line was fit for otolith radius as a function of age and the estimated mean otolith growth rate was 1.8 μm day^{-1} (Fig. 4*b*).

A linear relationship was found between otolith radius and length (ln transformed), indicating that otolith radius was a suitable proxy for anchovy size in the present study (otolith radius = 986.6 ln(SL) – 1006.8; $n = 40$, $R^2 = 0.69$, $P < 0.001$; Fig. 5).

The general otolith daily growth pattern showed that increment widths increased rapidly and were broadest between 51 and 56 days, with a mean (± s.d.) of 19.1 ± 3.7 μm. Thereafter, the widths decreased steadily to around 1.5 μm on Day 173 and remained almost constant at approximately this value until the end of the experiment (Fig. 6).

Deposition of the first translucent band

Deposition of the first translucent band was observed in otoliths of anchovies maintained in the sea cage. At the end of the experiment, anchovy otoliths showed a complete opaque and translucent band and the start of the second opaque band. Estimated mean hatch date in relation to otolith daily growth pattern indicated that the formation of the first translucent band started

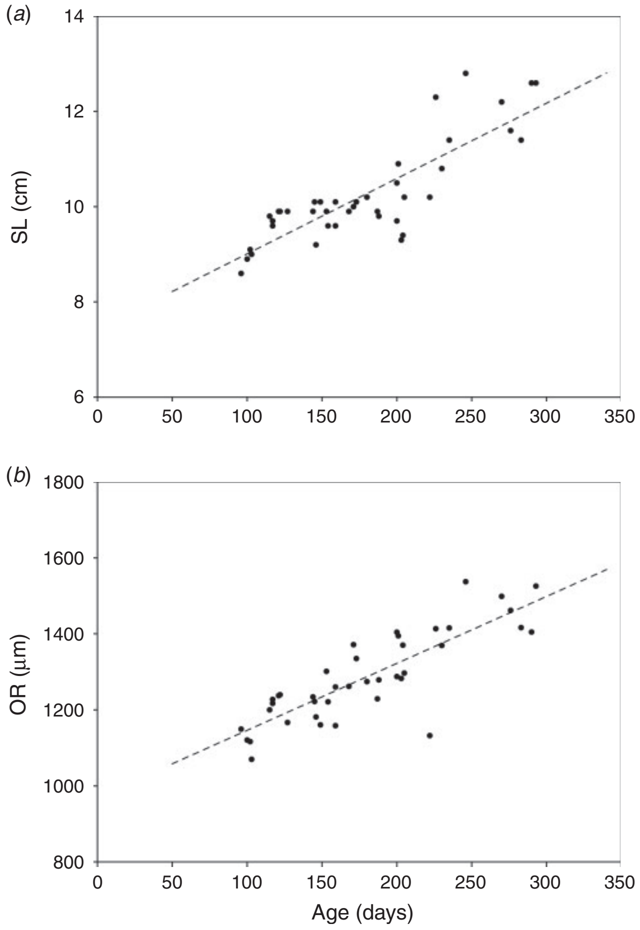

Fig. 4. (*a*) General somatic growth pattern of anchovies: standard length (SL) = 7.4 + 0.016days ($n = 40$, $R^2 = 0.71$, $P < 0.001$). (*b*) Otolith radius (OR) at age for individuals reared in the sea cage: OR = 970.1 + 1.76days ($n = 40$, $R^2 = 0.70$, $P < 0.001$).

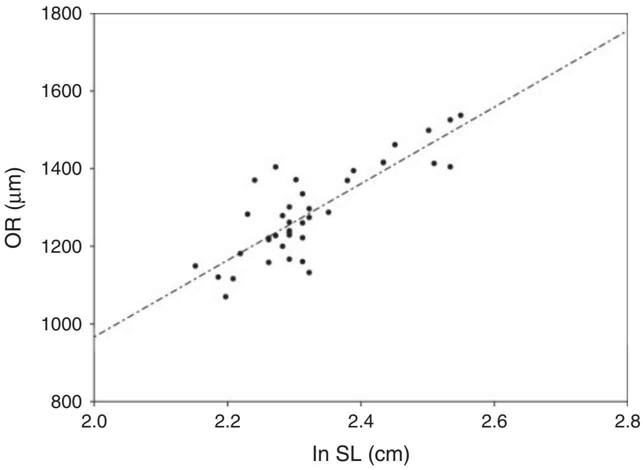

Fig. 5. Regression of otolith radius (OR) plotted against ln-transformed standard length (SL) for anchovy juveniles maintained in the sea cage.

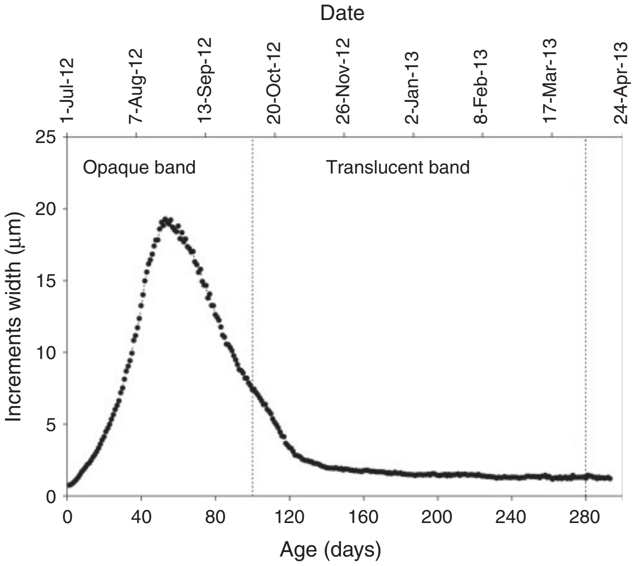

Fig. 6. Average increment widths at age from otoliths of anchovy juveniles. The vertical dashed lines indicate the beginning and end of translucent band formation in the otoliths.

in October 2012 and was complete by April 2013 (Fig. 6). This translucent band was characterised by a decline in increment widths, which were significantly narrower than those in the adjacent opaque band.

Discussion

In the present study, juveniles were successfully maintained in a sea cage under continuous feeding conditions to assess anchovy length and weight temporal variation in the Bay of Biscay. Both length and weight remained almost constant during the autumn and early winter; thereafter they increased significantly until early spring (Fig. 1). The relative condition factor followed a similar trend to that observed for anchovy length and weight (Fig. 2). Unfortunately, no references to temporal variations in length and weight during the winter were found in the literature against which the results of the present study could be compared.

The mean length of individuals maintained in the sea cage until April 2013 falls within the range of 10.5–18.0 cm in length reported for 1-year-old anchovy in the Bay of Biscay (Junquera and Perez-Gándaras 1993). However, these anchovies were noticeably smaller than those caught in the field during the same period (14.9 ± 0.8 cm; I. Martín and I. Rico, unpubl. data). Similarly, the condition of the individuals maintained in the sea cage was lower than that of anchovies caught in the field (1.15 ± 0.08; I. Martín and I. Rico, unpubl. data). These differences could be attributable to the small size range of anchovies analysed in the present study, because only a homogeneous cohort of a juvenile population was sampled in autumn. The mean length of juveniles captured in autumn 2012 was 9.3 ± 0.4 cm, whereas the juvenile population size at that time ranged from 2.7 to 13.7 cm (ICES 2012).

Furthermore, anchovies in the sea cage were not subjected to predation mortality, which could affect individual size distribution in the cage. Several studies have reported a size-dependent vulnerability to predation (i.e. smaller individuals are more susceptible to predators and experience a higher mortality rate than larger individuals; Miller *et al.* 1988; Rice *et al.* 1993; Sogard 1997). Therefore, the absence of predators could also explain the observed differences in length, because the smallest individuals were not removed from the sea cage. In addition, overwinter mortality associated with either starvation or adverse environmental conditions could be an additional size-selective process in the field (Henderson *et al.* 1988; Johnson and Evans 1991; Cargnelli and Gross 1996; Schindler 1999).

Validation of daily increment deposition rate

Daily increment deposition rate has been validated in otoliths of anchovies maintained under a continuous feeding regimen during the winter. For this species, Cermeño *et al.* (2003) previously validated the daily periodicity of increment formation in otoliths of juveniles and adults in laboratory rearing experiments using oxytetracycline hydrochloride as a marker. However, they observed a loss of daily increment deposition in otoliths and suggested that this could be associated with a slow growth rate in the aquarium. The lack of apparent daily increment deposition because of a slow growth rate has also been reported by other authors (Folkvord *et al.* 2000; Fox *et al.* 2003; Aldanondo *et al.* 2008). However, in the present study, the daily periodicity of increment formation was confirmed for winter, when the growth rate decreases markedly (0.16 mm day^{-1}). The observed differences in increment deposition rate between these studies could be associated with the rearing conditions, because in the present study anchovies were maintained under seminatural conditions, in contrast with the other study, where individuals were maintained under aquarium conditions. Therefore, it is expected that the cage conditions contribute to noticeable growth compared with the laboratory conditions. Conversely, as mentioned previously, anchovies in the sea cage at the end of the experiment were smaller than those captured in the field. Therefore, a higher growth rate is expected in the field, and consequently higher age accuracy, as suggested by others (Folkvord *et al.* 2000; Feet *et al.* 2002; Ivarjord *et al.* 2008).

For Japanese anchovy (*Engraulis japonicus* S.), Namiki *et al.* (2010) also reported non-daily increment deposition in otoliths of immature anchovies in winter. The authors argued that low water temperature conditions (below 13–14°C) reduced otolith growth rate, making interpretation of daily increments more difficult. In the present study, anchovies experienced similar temperatures during the winter to those reported for Japanese anchovy (Fig. 7); however, daily periodicity of increment deposition in the present study was not impaired at low temperatures, even though otolith growth noticeably decreased.

The general otolith growth pattern was characterised by a period of continuous increase in increment widths during the first 56 days, which corresponds to the larval and early juvenile stages. After this period, increment widths decreased until the age of 139 days; thereafter, they remained relatively stable until the termination of the experiment (Fig. 6). To our knowledge, the present study is the first to report otolith growth during the late juvenile stage. Conversely, several authors have studied the otolith growth of this species in the Bay of Biscay. A study by Cermeño *et al.* (2008) found a maximum average increment

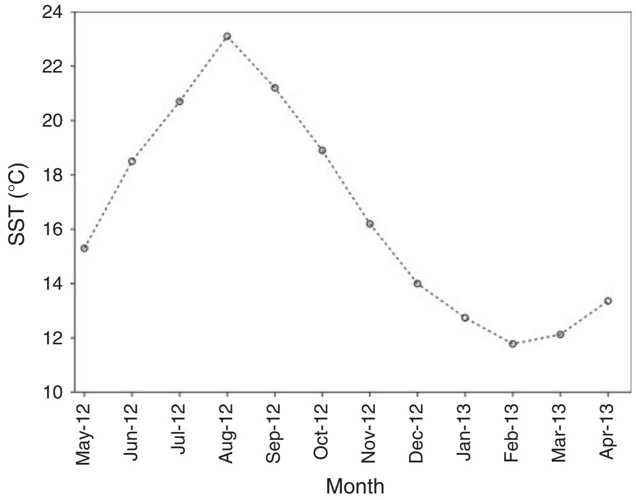

Fig. 7. Monthly mean sea surface temperature (SST) values obtained from data provided by the Aquarium of San Sebastian (43°19′N, 02°W).

width of 18 μm day^{-1} between 40 and 60 days, similar to that measured in the present study. Similarly, Aldanondo *et al.* (2011) described otolith growth of anchovy during the first growth season with a Gompertz model and observed a similar pattern to that observed for juveniles in the sea cage. A similar growth pattern has been reported previously in other pelagic fish species (Cotano and Álvarez 2003; Xie and Watanabe 2005; Baumann *et al.* 2009).

The trend observed suggests that growth rates are primarily related to endogenous factors, although they are also modulated by environmental variables, such as temperature or food availability (Campana and Neilson 1985; Alemany *et al.* 2006; Baumann *et al.* 2006). In this case, the otolith growth pattern seems to reflect the seasonal temperature cycle in the Bay of Biscay (Figs 6, 7). The increase in growth rates was coincident with high water temperatures in summer, whereas the subsequent decline in growth rates occurred during the autumn, the time when sea surface temperature started to decline.

Growth discontinuities or underestimation of narrow increments because of resolution problems with the light microscope have been reported in many studies (e.g. Feet *et al.* 2002; Paul and Horn 2009; Hüssy *et al.* 2010; Namiki *et al.* 2010). However, in the present study, daily increments were well defined along the otolith, although they were narrow and with low contrast during the late juvenile stage. Unclear increments or non-defined daily deposition patterns were not observed. Furthermore, the average increment width during the slow growth period was 1.5 ± 0.2 μm, which is well above the resolution limit of the light microscope (0.3 μm according to Fox *et al.* 2003). Therefore, significant age underestimation is not expected.

In order to validate daily increment deposition, it was assumed that juveniles were, on average, 100 days old at the beginning of the experiment, because their actual age was not known. This assumption was based on estimated average age at that moment. It should be noted that individuals caught in the field in October 2012 belonged to a homogeneous cohort and originated from 1 July (±10.2 days). Therefore, the expected deviation is low and it could be assumed that there is negligible ageing error.

Validation of the first annual increment deposition

Based on otolith microstructure analysis, the first annual increment deposition has also been validated in anchovy otoliths in the Bay of Biscay. It is generally recognised that the translucent band is usually formed during winter–spring in the otoliths of temperate species (Beckman and Wilson 1995; Hüssy *et al.* 2010). The findings for the European anchovy are in accordance with this assumption, because the formation of the first translucent band starts in October and is complete by April. A similar deposition pattern has been observed in other species (Woodroffe *et al.* 2003; La Mesa 2007). The formation of the first annulus was also validated for South African anchovy using daily increment counts, but in this case the deposition of the first translucent band occurs in the austral summer (Waldron 1994).

Numerous studies have reported large variations in seasonality of otolith band formation between species (Beckman and Wilson 1995), as well as between populations within the same species (Williams *et al.* 2005; Høie *et al.* 2009). In order to explain these differences, several factors have been postulated, for example somatic growth (Wright *et al.* 2002), reproduction (Morales-Nin *et al.* 1998), photoperiod (Wright *et al.* 1992) and environmental temperature (Beckman and Wilson 1995; Pilling *et al.* 2007; Neat *et al.* 2008).

In this sense, the formation of opaque and translucent bands is expected to be associated with seasonality of somatic growth or general environmental conditions (Wright *et al.* 2002). In the case of the European anchovy, the daily growth pattern of the otolith reveals that the deposition of the opaque band is related to a fast growth period, whereas formation of the translucent band is linked to a slow growth period (Fig. 6). This pattern is consistent with previous descriptions of the translucent band in this species (ICES 2009) and in several other species (Brothers *et al.* 1976; Pannella 1980; Victor and Brothers 1982; Beckman and Wilson 1995; Wright *et al.* 2002). However, in the case of the South African anchovy, whether the formation of the translucent band is associated with a fast or slow growth period is unknown because it was not reported by Waldron (1994).

Seasonal changes in water temperature have also been suggested to promote translucent band formation in otoliths (Pannella 1980; Schramm 1989; Beckman and Wilson 1995). Nevertheless, the relationship between translucent band formation and temperature remains uncertain (Høie and Folkvord 2006; Pilling *et al.* 2007; Neat *et al.* 2008; Hüssy *et al.* 2010). In the case of the European anchovy, the formation of the opaque and translucent bands resembles the seasonal temperature variation in the Bay of Biscay (Fig. 7). Deposition of the opaque band coincides with high seasonal temperatures in summer and early autumn, whereas formation of the translucent band coincides with low temperatures in winter and early spring (Figs 6, 7).

Finally, the possibility that annulus formation could be under endogenous physiological control should be taken into account. Some studies have addressed this issue and reported that many fish form an annulus in the absence of variable water temperature (Schramm 1989; Johnson and Belk 2004; Neat *et al.* 2008).

In conclusion, the results of the present study indicate that daily increment deposition in otoliths of anchovies is not impaired during their first growth season. Therefore, the age and growth of juveniles during the winter can be estimated based on analysis of these structures. This information will enable overwinter mortality estimates to be made in order to determine the factors that control anchovy recruitment strength in the Bay of Biscay. In addition, the results of the present study confirm that the first annulus is composed of an opaque band, which is deposited during the spring and summer, and a translucent band, which is formed during autumn and winter. Consequently, the study validates age determination based on annulus interpretation and shows that this method is a reliable tool for age determination in the anchovy.

Acknowledgements

The authors thank Iñaki Rico for assistance with laboratory work and Victor Valencia for providing temperature data. This work was supported by the research project Jaula (Department of Agriculture, Fisheries and Food of the Basque Country Government). This paper is contribution number 732 from AZTI (Marine Research Division).

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