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Validation of daily increments deposition in the otoliths of European anchovy larvae (*Engraulis encrasicolus* L.) reared under different temperature conditions

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ABSTRACT

European anchovy eggs (*Engraulis encrasicolus* L.) were hatched and larvae reared in the laboratory in order to validate the daily increment deposition in otoliths under different temperature condition. Additionally, the effect of temperature on otolith and larval growth and on the fish length and otolith radius relationship was also analysed. On sagittae, one to four increments can be depositioned during the embryonic stage and the first regular increment was formed the day after hatching. Temperature was found to have a significant effect on the increment deposition rate and otolith growth. A daily increment deposition rate was confirmed in larvae reared under conditions of higher temperatures (20.8 and 22.3 °C), while the apparent rate of increment formation of larvae reared at 17.6 °C was clearly lower. Standard length and otolith radius were closely related and this relationship was affected by both temperature and growth rates. The implications of the effect of these variables on otolith growth are further analysed in relation to non-daily pattern of increment deposition found at the lowest temperatures.

1. Introduction

Otolith microstructure analysis is a powerful technique that provides information about the age and growth history in marine fish (Folkvord et al., 2000, 2004; Xie et al., 2005; Baumann et al., 2006). Estimating the age of individual fish larvae using otolith increments has become a widely used tool in early life history ecology (Villanueva and Molí, 1997; Fox et al., 2003). In these stages, information on age structure can be used to clarify the effects of changes in the environment on growth and survival, and can result in an improved understanding of factors affecting recruitment success (Radtke and Fey, 1996; Quiñonez-Velázquez, 1999; Feet et al., 2002). However, otolith microstructure used for growth studies, the daily increment formation and the relationship between body and otolith growth have to be validated (Siegfried and Weinstein, 1989; Moksness, 1992).

The most relevant aspects to achieve a correct interpretation of the otolith readings are (1) to determine the rhythm of the increments deposition (validation experiments), (2) to know the age at first increment formation and (3) to establish those factors which could be responsible for changes on increments deposition rate. Aging larvae using otolith microstructure is based on the assumption that increments are formed at a rate of one per day and that counts of the number of increments are representative of larvae age (Geffen, 1982). But, without proper validation, reconstruction of early life history events using otolith growth increments is dubious (Beamish and Mcfarlane, 1983; Cieri and McCleave, 2001). Daily growth increment deposition has been validated in many fish species living under optimal conditions (Campana and Neilson, 1985; Jones, 1986; Siegfried and Weinstein, 1989; Villanueva and Molí, 1997; Quiñonez-Velázquez, 1999; Morley et al., 2005; Xie et al., 2005). But some works indicate that the daily deposition of otolith increments is not a universal feature of all larvae in all conditions (Geffen, 1982; Campana and Neilson, 1985; Jones, 1986; Jones and Brothers, 1987; Radtke and Fey, 1996). They also show that environmental conditions which affect larval growth rate can significantly alter increment deposition rate.

To obtain reliable age estimates it is also necessary to know the age of first increment formation and the accuracy of the increment count (Campana and Neilson, 1985). The date of first increment formation may vary from hatching to the time of first feeding (Campana and Neilson, 1985). Brothers et al. (1976) reported that daily increments appeared in otoliths of Northern anchovy *Engraulis mordax* (G.) only after completion of yolk-sac absorption.

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In the absence of data on the age of the first increment production to European anchovy *Engraulis encrasicolus* (L.), some workers have assumed that it commenced at the time the jaw was functional (external feeding ability) (Ré, 1987; Palomera et al., 1988; Dulčić, 1997; Somarakis and Nikolioudakis, 2007).

Differences in environmental conditions may result in different accuracy of the age estimates (Stenevik et al., 1996). It has been suggested that ambient temperature is a potential factor responsible for differences in otolith microstructure in some larvae populations (Folkvord et al., 2004). Furthermore, the increment width is often used as a proxy of somatic growth (Ré, 1986; Palomera et al., 1988) assuming a strong relationship between fish length and otolith radius with no environmental influence in this relationship (Allain et al., 2007). However, the increment width (as indirect measurement of growth rate) seems to be affected by several factors such as temperature, food availability or even the somatic growth rate (Stenevik et al., 1996; Fitzhugh et al., 1997; Folkvord et al., 2000, 2004; Fey, 2006).

For anchovy, daily deposition of otolith increments have been validated for *E. mordax* larvae (Brothers et al., 1976) and it has been assumed for *E. encrasicolus* (Ré, 1987; Palomera et al., 1988; Wilhelm et al., 2005). However, for juveniles of *E. encrasicolus* Cermeño et al. (2003) observed certain variability in daily increment formation in laboratory experiments, using oxy-tetracycline hydrochloride as a marker. Furthermore, to our knowledge, whether the relationship between fish length and otolith radius is constant or varies depending on environmental conditions has not been tested for anchovy under controlled experimental conditions.

The present study has four objectives: (1) to determine the age of the first increment formation for European anchovy larvae, (2) to validate the daily frequency of growth increments in sagittae otoliths, (3) to asses the effect of temperature on increment deposition and (4) to study the effect of temperature and somatic growth on fish length and otolith radius relationship.

2. Materials and methods

2.1. Laboratory rearing experiment

Early anchovy juveniles were captured during September 2005 with commercial purse seiners in the southern Bay of Biscay. Anchovies were maintained in captivity in black cylindrical tanks with a volume of 1300 l at facilities of the Aquarium of San Sebastián. The anchovies matured and spawned from July to November 2006. Spawning was induced through control of temperature and photoperiod.

Fertilized eggs were selected and incubated into four black cylindroconical 200 l tanks (44–125 ind l^{-1}), with different temperatures representative of the conditions found in the Bay of Biscay during the spawning period. The larval incubations were carried out at 17.6 ± 1.6; 19.3 ± 1.1; 20.8 ± 0.9 and 22.3 ± 0.8 °C (±S.D.) temperatures, which were monitored daily. The salinity remained stable at around 35.5, whilst oxygen was kept at saturation levels. Tanks were filled with UV-sterilised filtered (1 μm) seawater and temperature conditions were controlled by the combined function of an air-conditioning device and flow-through water cooling (Aqua-Medic, Titan 500). The whole experiment was performed in a controlled temperature room at 18 °C. Eggs were not treated with any antibiotic solution. As it had been previously reported for anchovy eggs in the Bay of Biscay (Motos, 1994; Ibaibarriaga et al., 2007) all egg hatched within 72 h from spawning. The larvae were fed to excess with rotifers *Brachionus rotundiformis* (small strain) from the second day onwards after hatching and high-DHA Superselco enrhiched instar I and II *Artemia franciscana nauplii* and *metanauplii* I and II (Artemia Systems Inc., Belgium) from 10 to 43 days onwards after hatching depending on the size reached by the larvae at each experimental temperature. Additionally, inoculations of algae (e.g. *Nannocloropsis* sp. and *Tetraselmis* sp.) were given, on a daily basis. The light cycle was 16:8 h that approximated the natural light:dark cycle (a nocturnal blue light was left overnight). Eight larvae were sampled daily from each tank from 0 to 8 days after hatch, at 2-day intervals from 9 to 15 days after hatch and at 4 days interval from 16 days after hatch. Three up to eight sampled larvae were preserved in a 95% ethanol solution buffered with Tris-aminomethane and used for otolith analysis. After preservation larvae were measured for standard length (SL) to the nearest 0.1 mm. No correction for ethanol preservation was undertaken (Theilacker, 1980). The other five larvae were measured alive to the nearest 0.1 mm and used for somatic growth analysis.

2.2. Otolith extraction, preparation and analysis

The larvae were placed on a glass slide with a drop of water. The otoliths were extracted from the saccullar chamber under a binocular dissecting microscope. Even when two pairs of otoliths (sagittae and lapillus) were obtained, only the sagittae were used in this study because the microstructures of the lapillus otolith were not as clear as those observed for sagittae. Once the otoliths were extracted, the glass slides with the otoliths were dried carefully with absorbent paper, and the otoliths were covered with a thin layer of nail varnish.

A total of 299 pairs of otoliths were extracted from the larvae. The microstructure analyses were carried out using a light microscope applied to an image-analyser (Visilog, TNPC Software, v.3.2, Ifremer, France), at 400–1000× magnification. Oil immersion was required for the higher magnification.

Palomera et al. (1988) described the presence of a more prominent dark increment near the nucleus which was assumed as the first feeding check. Since the presence of increments prior to the first feeding cannot be discarded, every increment was considered for the age estimation, following always the criterion applied by Palomera et al. (1988) for the discrimination of daily and sub-daily increments. The total number of increments was counted and the distance between consecutive increments was measured. Each otolith was read by two people without knowledge of the specifics of the sample; the result was recorded only if an agreement was reached. Finally, only 163 otoliths were selected. The others were discarded because they were unclear or unsuitable for analysis or because an agreement was not reached.

For comparison purposes, SL and OR data of field larvae sampled from 2000 to 2005 were employed. These larvae were captured in the same area where anchovy juveniles were captured. Surface temperature data, corresponding to the capture day, were split into different thermal groups as an approximation to check the temperature effect.

3. Results

At hatching, the sagittae of each larvae consisted of a lenticular core with a mean ± S.D. radius of 4.07 ± 0.66 μm. In the sagittae of recently hatched larvae some increments were visible in the core (Fig. 1(a)) and a clear overestimation of age was found for those larvae younger than 3 days (Fig. 2). Concentric increments consisting of an adjacent dark and light zone were laid down around the core (Fig. 1(b)) as described to occur daily in other fishes (Pannella, 1971; Morales-Nin, 2000).

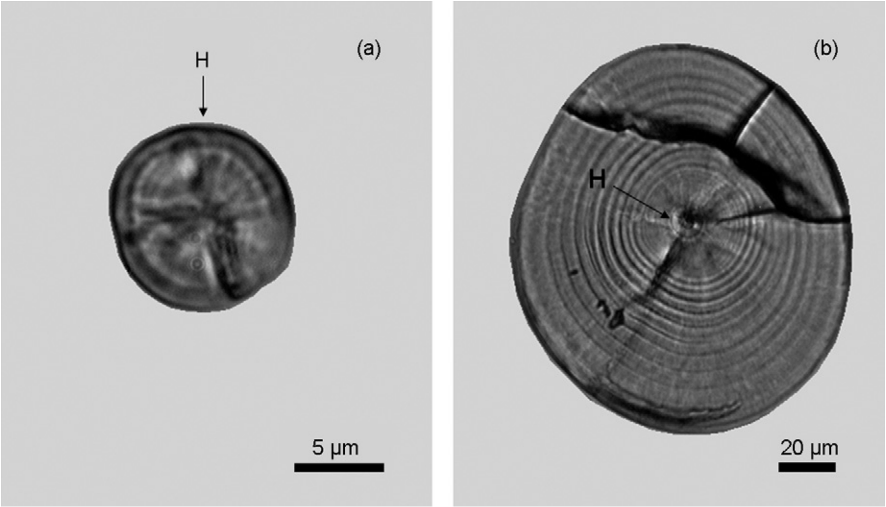

Fig. 1. Sagittae otoliths from (a) 0 day old and (b) 29 days old anchovy larvae, of 2.46 mm and 9.3 mm SL, respectively. H = hatch check.

3.1. Validation of daily increment deposition

The relationship between the number of increments (I) and actual age (D) was high and significant, and was expressed as a linear function for the whole experiment, $I = 0.752 + 0.864D$ ($r^2 = 0.94$; $^{**}P < 0.01$; $n = 163$). The slope of the regression line was significantly different from one (t-test, $^{**}P < 0.01$) and the intercept was significantly higher than zero (t-test, $^{**}P < 0.01$) which indicates that the first increment formation was prior to the hatch as was previously noticed from examination of the otoliths of recently hatched larvae. This pattern was repeated for all the temperatures. The relationship between the number of otolith increments and actual age, in days, for each experimental temperature is shown in Fig. 2. The average deposition rates for each thermal group were also significantly different from one increment per day (t-test, $^{**}P < 0.01$). Likewise, the intercept of the regression equation differed significantly from zero at a temperature of 17.6, 20.8 and 22.3 °C (t-test, $^{*}P < 0.05$), whereas at 19.3 °C did not differ (t-test, $^{*}P > 0.05$). Residuals of the increments at age regression showed positive values of increments for the initial 0–5 days. Negative values were observed for older larvae, suggesting an underestimation of the age.

Taking into account the overestimation found in larvae younger than 3 days for all the experimental condition, the same analysis was performed only for larvae older than 2 days. In this case, the slope of the common regression line ($I = 090D - 0.03$; $r^2 = 0.94$; $^{**}P < 0.01$; $n = 127$) was also significantly different from one (t-test, $^{**}P < 0.01$) but the intercept was not significantly different from zero (t-test, $^{*}P > 0.05$) which indicates that the first increment forma-

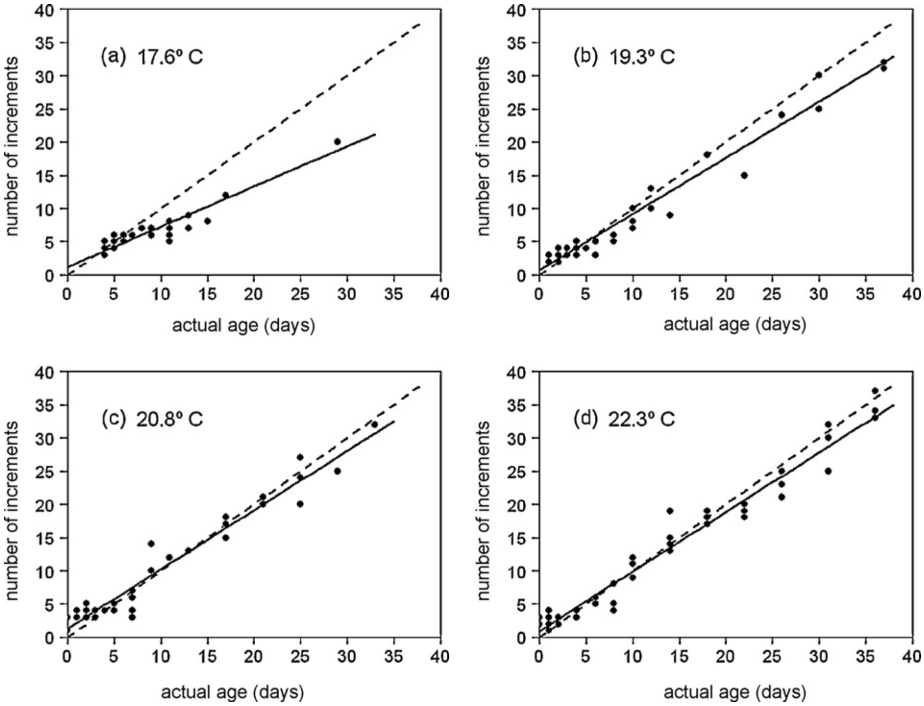

Fig. 2. Relationship between the observed number of increments and the actual age of the European anchovy larvae reared at different temperatures. (a) $y = 0.61 + 1.22\text{age}$, $r^2 = 0.92$; $^{**}P < 0.01$; $n = 27$ at 17.6 °C; (b) $y = 0.73 + 0.85\text{age}$, $r^2 = 0.96$; $^{**}P < 0.01$; $n = 42$ at 19.3 °C; (c) $y = 1.32 + 0.89\text{age}$, $r^2 = 0.96$; $^{**}P < 0.01$; $n = 45$ at 20.8 °C; (d) $y = 0.96 + 0.89\text{age}$, $r^2 = 0.97$; $^{**}P < 0.01$; $n = 49$ at 22.3 °C. Dashed lines represent the 1:1 line.

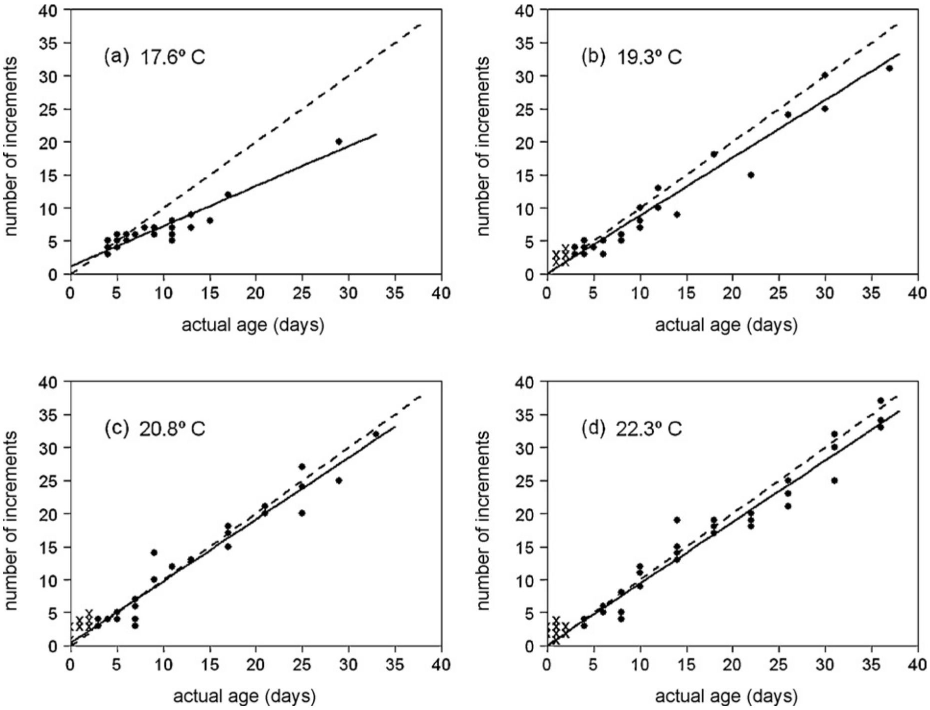

Fig. 3. Relationship between the observed number of increments and the actual age of the European anchovy larvae older than 2 days reared at different temperatures. (a) $y = 1.22 + 0.61\text{age}$, $r^2 = 0.92$; $^{**}P < 0.01$; $n = 27$ at 17.6 °C; (b) $y = 0.14 + 0.87\text{age}$, $r^2 = 0.96$; $^{**}P < 0.01$; $n = 32$ at 19.3 °C; (c) $y = 0.46 + 0.93\text{age}$, $r^2 = 0.96$; $P > 0.05$; $n = 33$ at 20.8 °C; (d) $y = 0.12 + 0.93\text{age}$, $r^2 = 0.96$; $P > 0.05$; $n = 35$ at 22.3 °C. Dashed lines represent the 1:1 line. The crosses represent the youngest larvae no included in the analysis.

tion was at hatch. The relationship between the number of otolith increments and age for larvae older than 2 days and for each experimental temperature is shown in Fig. 3. The average deposition rates for each thermal group were still lower than one increment per day for 17.6 and 19.3 °C (*t*-test, $^{**}P < 0.01$), but were not significantly different from one at 20.8 and 22.3 °C (*t*-test, $^{*}P > 0.05$). For larvae older than 2 days the intercept was significantly different from zero only at 17.6 °C (*t*-test, $^{**}P < 0.01$).

In addition to significant deviations from daily increment deposition, significant differences were observed between the slopes of the observed number of increments versus actual age regressions at different temperatures. The slopes at 19.3, 20.8 and 22.3 °C temperatures did not differ statistically (ANCOVA, $^{*}P > 0.05$); however, the slope at 17.6 °C was significantly different to the others (ANCOVA, $^{*}P < 0.05$).

The accuracy of the age estimates of larvae reared at 17.6 °C using increment deposition was extremely low. The age of larvae was underestimated by around 40%. Age estimates for larvae reared under conditions of higher temperatures were more accurate with age underestimations below 15%. The highest accuracy was found at temperatures of 20.8 and 22.3 °C.

The same results were found when only larvae older than 2 days were considered for analysis with a significant lower slope of the observed number of increments versus actual age regression for the 17.6 °C thermal group. Accuracy increased when larvae older than 2 days were considered for the three warmest thermal groups with underestimation values between 7 and 13%.

3.2. *Fish standard length and otolith radius relationship*

The relationship between OR and SL was high and significant, and was expressed as an exponential function for the whole experiment, $\text{OR} = 3.561e^{0.22\text{SL}}$, ($r^2 = 0.92$; $^{**}P < 0.01$; $n = 163$). Also an exponential relationship was found for field larvae ($\text{OR} = 3.19e^{0.18\text{SL}}$, $r^2 = 0.92$; $^{**}P < 0.01$; $n = 753$), although they showed significantly lower OR at length than those reared in laboratory (ANCOVA, $^{***}P < 0.001$).

However, significant differences in the slope of the OR versus SL regressions were found depending on temperature (Fig. 4(a) and Table 1). The slopes at 20.8 and 22.3 °C temperatures did not differ statistically (ANCOVA, $^{*}P > 0.05$); however, the slopes of the rest of the thermal groups were significantly different (ANCOVA, $^{**}P < 0.01$). For specimens of the same SL, larvae reared at higher temperatures exhibited larger OR.

Similar results were observed on field larvae caught in summer at different temperature ranges. The relationship between OR and SL was analyzed for these larvae (Fig. 4(b) and Table 1) and significant differences were also observed between them (ANCOVA, $^{**}P < 0.01$). However, the slopes for 20–21 °C and >21 °C temperature ranges did not differ statistically (ANCOVA, $^{*}P > 0.05$).

3.3. *Somatic growth and otolith growth*

The mean (±S.D.) length at hatching of reared larvae was 2.97 (±0.24) mm. There were significant differences between temperatures. At hatching, the mean SL was significantly smaller at 19.3 °C than at 20.8 and 22.3 °C (*t*-test, $^{*}P < 0.05$), but there were no signif-

Table 1
Estimated coefficients of the OR versus SL ($\text{OR} = a_i e^{b_i \text{SL}}$) exponential equation

	Temperature (°C)	a	b	r^2	P	n
Reared larvae	17.6	4.48	0.16	0.77	<0.001	27
	19.3	3.97	0.20	0.91	<0.001	42
	20.8	3.13	0.25	0.88	<0.001	45
	22.3	3.47	0.23	0.96	<0.001	49
Field larvae	<19	3.08	0.18	0.90	<0.001	55
	19–20	2.93	0.18	0.95	<0.001	263
	20–21	4.05	0.16	0.90	<0.001	117
	21–22	3.23	0.18	0.91	<0.001	278
	>22	3.62	0.17	0.94	<0.001	40

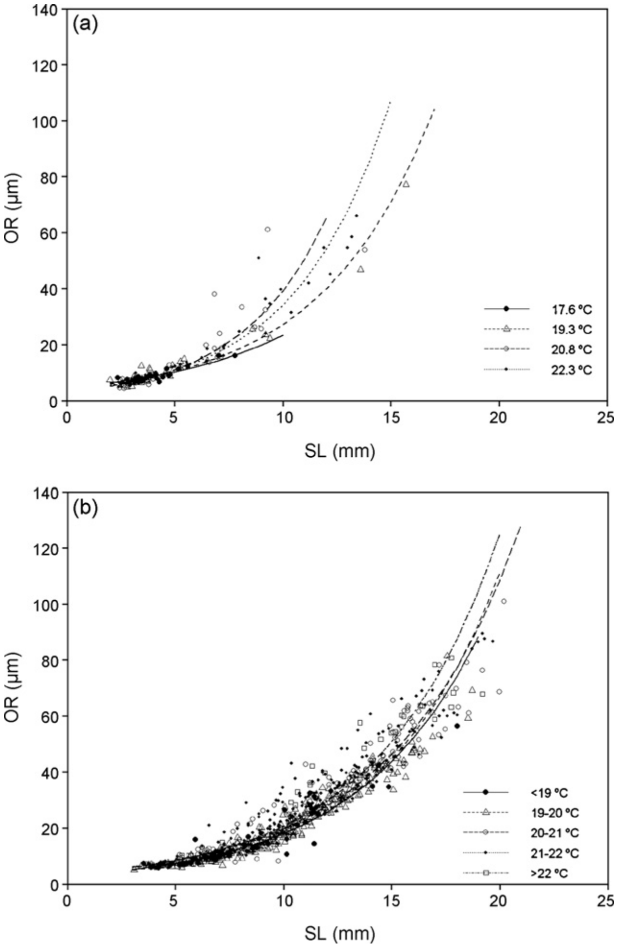

Fig. 4. Relationship between OR and SL of European anchovy larvae at different temperatures. (a) Reared larvae at the four experimental conditions. (b) Field larvae in summer at different temperature ranges.

icant differences between larvae reared at 20.8 and 22.3 °C (*t*-test, *$P > 0.05$).

Larval growth was clearly temperature dependent (Fig. 5(a)) and significant differences were observed in growth rates at different temperatures (ANCOVA, *$P < 0.05$). Maximum growth rates were found for larvae reared at 20.8 °C with $0.33\ \mathrm{mm\ day^{-1}}$ and minimum values of $0.15\ \mathrm{mm\ day^{-1}}$ were observed for the 17.6 °C group. There was no significant differences in size at age between larvae reared at 20.8 and 22.3 °C (ANCOVA, *$P > 0.05$).

The overall regression of OR at age was highly significant and was given by the equation: $R = 5.45e^{0.062\mathrm{days}}$ ($r^2 = 0.89$; **$P < 0.01$; $n = 163$). However, a temperature dependent otolith growth was observed in the experiment (Fig. 5(b)). The group reared at 17.6 °C had significantly lower growth than the other groups reared at 19.3, 20.8 and 22.3 °C temperatures (ANCOVA, *$P < 0.05$).

The otolith daily growth pattern revealed an increasing average increment width in the 19.3, 20.8 and 22.3 °C groups (Fig. 5(c)). On the contrary, the average increment widths in the 17.6 °C group remained constant and below 1 µm and were significantly lower than for the other thermal groups.

4. Discussion

Previous studies have validated successfully daily rhythm deposition for several engraulidae (Brothers et al., 1976; Struhsaker and Uchiyama, 1976; Fives and Warlen, 1986; Waldron et al., 1989; Ekau, 1997; Castello and Castello, 2003). For *E. encrasicolus*, Cermeño et al. (2003) validated the daily deposition of increments of juveniles and adults maintained for short periods in an aquarium. But to our knowledge this is the first work to validate daily increment formation in European anchovy larvae.

4.1. *Age of first increment deposition*

The slope of the increment formation was less than one increment per day, whilst the intercept value differed from zero. This could be due to the presence of embryonic increments and to the rearing conditions, mainly temperature. The age at first increment deposition is species specific (Jones, 1986; Høie et al., 1999; Morley et al., 2005). Usually, initial increment formation on otoliths is associated with hatching, first feeding, or the start of activity (Morales-Nin, 2000; Joh et al., 2005). However, some species can start forming increments before hatching (Quiñonez-Velázquez, 1999). For *E. mordax*, Brothers et al. (1976) observed that larvae showed increments only after completion of yolk-sac absorption by the fifth day after hatching. For Japanese anchovy *Engraulis japonicus* (S.), Aoki and Miyashita (2000) also reported that daily increment formation began at the start of external feeding. Due to lack of data for *E. encrasicolus*, Palomera et al. (1988) assumed that, for this species, initial increment formation begin at first feeding (2 days of age), and then, the frequency of increments deposition is daily.

The present study, carried out in a laboratory from larvae of known ages, establishes that the first of the regular increment is formed only on the day after hatching. This disagrees with previous assumptions (Ré, 1987; Palomera et al., 1988; Dulčić, 1997), although some of these studies also observed some thin increments before the established first increment in yolk-sac larvae. The development of otolith starts before hatching, and one to four increments can be deposited during the embryonic stage. These increments are more visible in larvae younger than 5 days.

In some otoliths examined, at $4.07 \pm 0.66\ \mu\mathrm{m}$ a well-defined first increment laid down around the nucleus that coincide with a hatch check is observed. Outside this increment, two to four increments were bounded by another well-defined dark increment which could be the yolk-sac increment. Regner (1985) reported that at 21 °C yolk-sac absorption occurs 3 days after hatching for European anchovy. It seems likely that the second well-defined growth increment is associated with the switch from subsistence on endogenous yolk supplies to exogenous feeding.

Clear overestimation of age was found for larvae younger than 5 days. That overestimation is probably due to the presence of embryonic increments, since they are more visible in the smallest otoliths. Owing to otolith enlargement there is a loss of embryonic increment, which is related to otolith thickening. Hence, when larvae younger than 2 days were removed from the analyses, the age estimation became more accurate. These results are supported by similar observations reported by Palomera et al. (1988) for same species larvae.

4.2. *Frequency of growth increments deposition*

Temperature was found to have a significant effect on the increment deposition rate, which agrees with results from other species (Geffen, 1982; Radtke and Fey, 1996; Folkvord et al., 2004). When the increment formation was estimated for each thermal group, lower increment deposition rates were observed at lower temperatures. This temperature dependency affects the accuracy of the age estimates with an underestimation of around 40% for larvae raised at 17.6 °C. Several authors have reported similar discrepancies at low temperatures raised larvae (Geffen, 1982; Feet et al.,

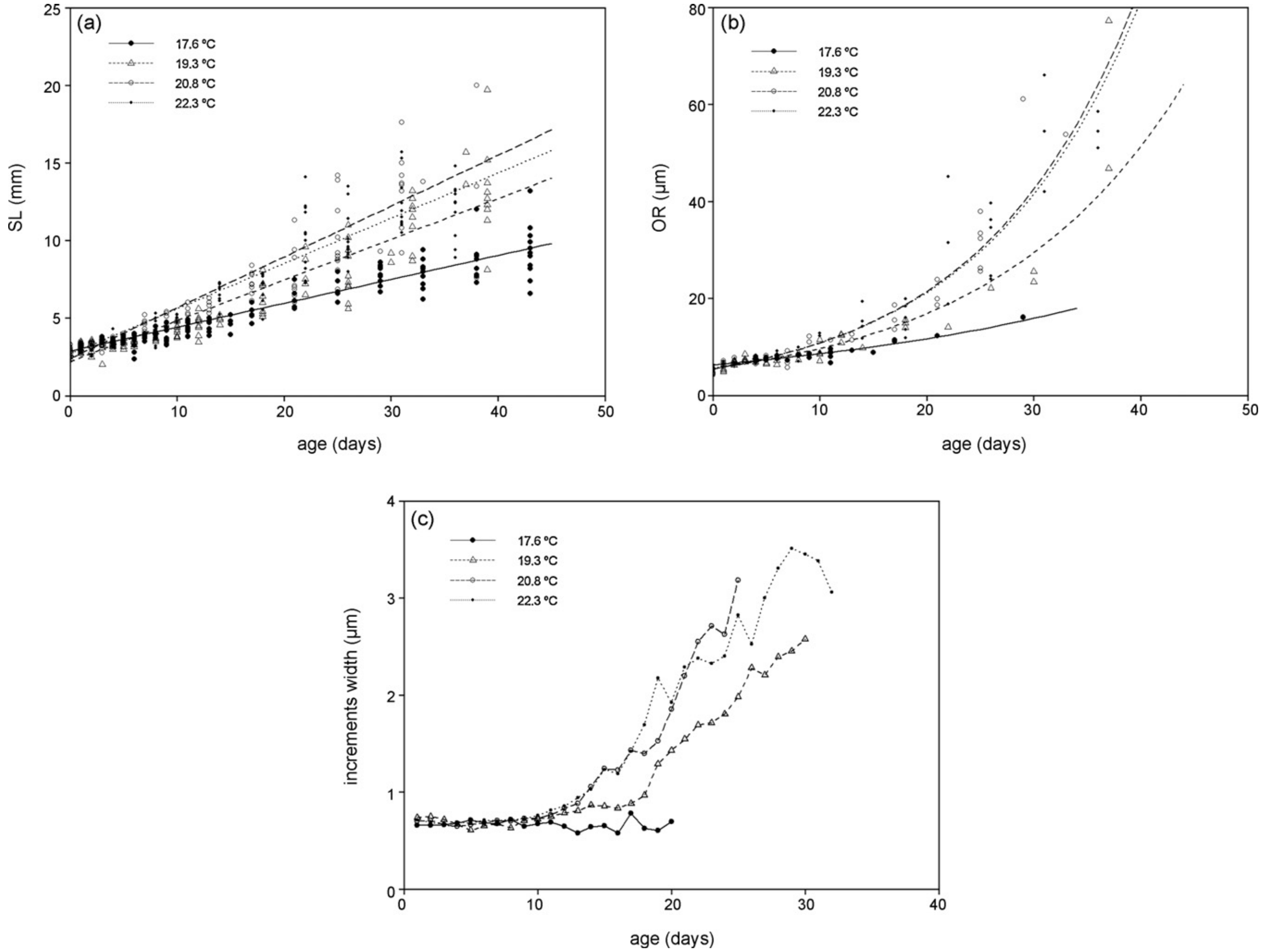

Fig. 5. (a) SL at age of anchovy larvae from the different groups in the experiment. $y = 2.88 + 0.15\text{age}$, at 17.6 °C; $y = 2.21 + 0.26\text{age}$, at 19.3 °C; $y = 2.4 + 0.33\text{age}$, at 20.8 °C; $y = 2.69 + 0.29\text{age}$, at 22.3 °C. (b) OR at age from the different groups. $y = 6.32e^{0.03\text{days}}$, at 17.6 °C; $y = 5.55e^{0.05\text{days}}$, at 19.3 °C; $y = 5.46e^{0.07\text{days}}$, at 20.8 °C; $y = 5.56e^{0.07\text{days}}$, at 22.3 °C. (c) Mean increment widths versus age from the different groups in the experiment.

2002). Limitations of the light microscope may explain non-daily increment formation in larvae reared at low temperatures. The theoretical resolution limits for standard optical microscopy will be around 0.30 µm (Fox et al., 2003). In our experiment, at 17.6 °C the average increment width for the first 10 days was approximately 0.67 ± 0.11 µm; as the otolith grew, the increments width was the same or fell. These results suggest that the observation of non-daily increment formation is caused by limitation in the resolution of the light microscope, and increments, if present, might only reliably be detected with scanning electronic microscopy (SEM) (Campana et al., 1987). Underestimation of narrow increments due to resolution problems has been previously reported by several authors (Campana et al., 1987; Folkvord et al., 2000). However, the light microscope limitations may not be the sole explanation of the loss of daily increment deposition; since the mechanisms of otolith formation at the highest temperatures might be different from that at lower temperatures (Mosegaard et al., 1988).

4.3. *Fish standard length and otolith radius relationship*

The results obtained in our study suggest an exponential model for the allometric relationship between fish length and OR; however, the presence of an inflexion point in this relationship should be considered. Somarakis and Nikolioudakis (2007) modelled this relationship for the same species in Mediterranean waters and found a marked ontogenetic change in allometric slope at about 6 mm length, which would be related to the development of the caudal fin.

From our experiment, it is also deduced that fish SL and OR relationship is affected by temperature in both laboratory reared and field caught larvae. For the same SL fish growing at lower temperatures have smaller otoliths. Similar results were also obtained by several authors for other species (Barkman and Bengtson, 1987; Mosegaard et al., 1988; Fitzhugh et al., 1997; Otterlei et al., 2002; Folkvord et al., 2004; Fey, 2006).

Somatic growth has often an optimal rate below the maximum temperature tolerance point (Webb, 1978). At higher temperatures, growth rates tend to decline, since respiration rates are progressively higher than anabolic processes (Webb, 1978). Since otoliths are extracellular structures, otolith formation may occur in a different way than cellular growth (Mosegard, 1988). So, otolith growth rates may increase even above the optimal temperature for somatic growth.

On the other hand, a marked difference was observed in fish size and OR relationship between reared and field caught larvae (Fig. 5). Reared larvae had larger otolith at length than field caught larvae. This difference might be related to relatively low growth rates of reared larvae, since as showed previously in many studies (e.g., Secor and Dean, 1989; Hare and Cowen, 1995; Otterlei et al., 2002; Fey, 2006) faster growing fish often have smaller otoliths at length than slower growing fish.

As shown in previous studies, apparent loss of daily increment deposition in otolith was often associated with low growth rates (Geffen, 1982; Folkvord et al., 2000, 2004; Fox et al., 2003). Growth rates for laboratory reared larvae in this experiment are lower than those reported for anchovy larvae in the Bay of Biscay (Cotano et al., 2007). Therefore, to obtain accurate age estimates might be difficult, especially in larvae reared at low temperature. However, the age-accuracy of field caught specimens is expected to be higher, as a consequence of higher growth rates, as suggested by Folkvord et al. (2000).

In conclusion, our results indicate that in *E. encrasicolus* one to four increments can be deposited during the embryonic stage and that the first of the regular increment is formed only on the day after hatching. This needs to be taken into account to avoid overestimation of very early ages, although, for external feeding larvae the otolith thickening may help to avoid the observation of embryonic increments. The daily deposition of increments detectable by optical microscopy is affected by temperature with an important underestimation (40%) at temperatures around 17 °C. As peak spawning of *E. encrasicolus* in the Bay of Biscay occurs at around 16–17 °C (Ibaibarriaga et al., 2007) this needs to be considered because otherwise, the age of a significant proportion of the larvae collected in the field can be gravely underestimated with the consequent overestimation of growth rates. Anyway higher growth rates expected at field should partially improve aging estimates.

Although fish SL and OR are closely related, this relationship varies through anchovy's lifetime. Moreover, the slope of this relationship is affected by temperature. As indicated by Fey (2006), because the general relation between size and otolith width remains strong, otolith width can still be used for average growth back calculation in anchovies. However, it has to be considered average estimates will have lower accuracy than for other species. Furthermore, due to the temperature influence otolith width should not be used for short-term growth estimates (Fey, 2006).

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