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Validation of daily microincrement deposition in otoliths of juvenile and adult Peruvian anchovy *Engraulis ringens*

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Wild adult specimens of the Peruvian anchovy *Engraulis ringens* were captured and reared to validate the daily periodicity of otolith microincrement formation. The postcapture stress generated spontaneous spawning, making it possible to conduct a rearing trial on larvae first in an artificial nutrient-enriched system (ANES) for 52 days followed by an artificial feeding regime in a culture tank until day 115 post-hatch. Microincrements of the sagittal otoliths of sacrificed juveniles [mean ± s.d. total length (L_T) = 5·13 ± 0·37 cm, range 5–6 cm; c.v. = 7·5%] showed very distinct light and dark zones. The slope of the relationship between the total number of increments after the hatch check and days elapsed after hatching was not significantly different from 1. The transfer from ANES to the artificial feeding regime induced a mark in the sagittal otoliths. The number of microincrements after this induced mark coincided with the number of days elapsed after the transfer date. In parallel experiments, adult *E. ringens* (mean ± s.d. L_T = 14·92 ± 0·55 cm, range 13–16 cm) were exposed to one of two fluorescent marking immersion treatments with either alizarin red S (ARS; 25 mg l^{-1} per 6 h) or oxytetracycline hydrochloride (OTC; 200 mg l^{-1} per 10 h). The microincrements between fluorescent bands were distinct, ranging from 0·89 to 2·75 µm (mean ± s.d. =1·43 ± 0·28 µm; c.v. = 32%) and from 0·71 to 2·89 µm (1·53 ± 0·27 µm; c.v. = 35%) for ARS and OTC, respectively. The relationship between the number of microincrements between marks and the number of elapsed days for ARS and OCT treatments indicated that there was a significant correspondence between the number of increases observed and the number of days. Hence, daily microincrements of otoliths of *E. ringens* are likely to be formed in juveniles and adults under natural conditions.

Key words: alizarin red S; chemical markers; northern Chile; otolith; oxytetracycline hydrochloride; rearing conditions.

INTRODUCTION

Anchovies are small fishes belonging to the Engraulidae family, comprising *c.* 145 species in 17 genera that are distributed in nearly all the world's oceans. The Peruvian anchovy *Engraulis ringens* Jenyns 1842 caught along the coasts of Peru and Chile, accounts for a substantial fraction (>60%) of the world's total anchovy harvest. In areas where anchovies are commercially exploited, age-based stock assessment models have

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been used to advise on total allowable catches (Cubillos *et al.*, 2002; Borja *et al.*, 2008; Martín *et al.*, 2008; Gutiérrez *et al.*, 2012).

Age estimation for anchovy species has been assessed by counting presumed *annuli*, using either scales or otoliths. To date, the information gathered on age structure for anchovy species associated with highly productive systems has shown that some anchovies (*e.g.* genus *Engraulis*) appear to be relatively short-lived, with a maximum life span ranging from 4 to 5 years and a maximum asymptotic total length (L_T) ranging from 16 to 19 cm (Hayasi & Kondo, 1957; Mais, 1981; Vidal-Talamantes, 1991; Waldron, 1992; Basilone, *et al.*, 2000). Recent studies using otolith microstructure analysis have reported fast growth patterns at the daily level for some anchovies and for other small pelagic fishes as well (La Mesa *et al*, 2009; Aldanondo *et al.*, 2011). Furthermore, Yukami *et al.* (2008) recently aged adult fish of the Japanese anchovy *Engraulis japonicus* Temminck & Schlegel 1846 by counting daily increments of otoliths in specimens ranging from 8 to 14 cm L_T. The ages of all specimens were < 365 days (*i.e.* 1 year), and the largest fish aged was 329 days old.

Further evidence of fast growth comes from two recent studies using otolith microstructure analysis in European anchovy *Engraulis encrasicolus* (L. 1758) from the Gulf of Biscay (Aldanondo *et al.*, 2011) and from the Adriatic Sea (La Mesa *et al.*, 2009; Durovic *et al.*, 2012). These studies demonstrated that juveniles can attain L_T of 6–8 cm in *c.* 70–90 days. In the Gulf of Biscay study by Aldanondo *et al.* (2011), the largest fish aged (*c.* 14 cm standard length, L_S) was < 1 year old. Furthermore, a recent study of the Brazilian herring *Opisthonema oglinum* (LeSueur 1818) raised new questions about the longevity of small pelagic fishes (Lessa *et al.*, 2008). In this species, the life span historically has been estimated to range between 4 and 8 years, using readings of opaque and translucent bands in whole otoliths, but Lessa *et al.* (2008) used daily microincrements to show that the highest proportion of growth occurred during the first year of life, reaching a maximum age of 14 months. Similar cases of overestimation of age resolved using daily microincrements have been reported for other clupeoids such as *Sardinella aurita* Valenciennes 1847 (Boeley *et al.*, 1982) and *Harengula jaguana* Poey 1865 (Pierce *et al.*, 2001). These findings led those authors to infer that in fast-growing species, the frequency of formation of opaque and translucent zones would have a periodicity of only months, due to causes as yet unknown.

The new evidence of faster growth for anchovies relies on the assumption that periodic microincrements represent absolute age at a daily scale. To date, this assumption has been investigated in larvae and juveniles in anchovies and closely related species (Geffen, 1982; McGurk, 1984; Fives *et al.*, 1986; Hayashi *et al.*, 1989; Aldanondo *et al.*, 2008; Lessa *et al.*, 2008). Furthermore, two recent studies have demonstrated that when *E. japonicus* (Namiki *et al.*, 2010) and *E. encrasicolus* (Cermeño *et al.*, 2003) adults were kept under rearing conditions for short periods, they formed very distinctive otolith microincrements at the otolith edges. These studies demonstrated that these microincrements were deposited on a daily basis for these species.

The aim of this study was to validate the periodicity of formation of otolith microincrements in juvenile and adult *E. ringens* in captivity. Such information could be very useful in interpreting the age of adult fish collected in the wild and to test the rapid growth hypothesis in another anchovy species.

TABLE I. Summary information for two immersion experiments used to validate the periodicity of formation of microincrements on the sagittal otoliths of mature *Engraulis ringens*

Marker (mg l^{-1})	N_1	$Date_1$	N_2	$Date_2$	Time (h)	Elapsed days	Number of increment marks between immersion dates: Mean ± S.D.	Range	C.V.
ARS (25)	50	08-10-11	41	16-11-11	6	38	37·82 ± 1·02	36–41	2·64
OTC (200)	50	08-10-11	39	24-11-11	10	46	45·61 ± 1·31	42–49	2·83

N_1, initial sample size; N_2, sample size at the second immersion; $Date_1$, date of first immersion; $Date_2$, date of second immersion; ARS, alizarin red S; OTC, oxytetracycline hydrochloride; C.V., coefficient of variation.

MATERIALS AND METHODS

COLLECTION AND VALIDATION PROCEDURES FOR ADULT FISH

In total, 513 adult *E. ringens* were captured aboard a commercial fishing vessel on 31 July and 1 August 2011 using a purse seine in northern Chile, at *c.* 20° 19′ S; 70° 26′ W. The collection of fish began at the end of the closure of the purse seine, where a 2500 W bulb was installed on the ship. The bright light attracted the fish, which crowded at the surface; a 20 l bucket attached to a polyamide line was lowered to collect only five to 10 individuals to avoid laceration or loss of scales. The same procedure was repeated as many times as necessary to obtain *c.* three groups of 250 fish, which were then placed in three 800 l tanks. The fish were then transported to land to be reared under natural conditions of photoperiod (20° S) and temperature in three cylindrical black tanks with a volume of *c.* 1800 l. The water was pumped from the sea and filtered before entering the tanks. The water circulation flow rate was *c.* 22 l min^{-1}, which generated optimal conditions of oxygen saturation, with values fluctuating between 8·4 and 8·5 mg l^{-1}. Each tank had a ventilation system provided by a Sweetwater blower model S-63 (www.sweetwater.com). The air was released into the water through a diffuser stone. The temperature and pH, which ranged from 13 to 18° C and from 6·8 to 7·2, were monitored daily during the rearing experiments, respectively. There was a high rate of mortality during the first week of the acclimatization period, as most of the fish suffered scale loss and damage to their heads from beating against the tank walls during the transportation and acclimatization period. After 2 weeks of acclimatization, *c.* 150 fish started feeding and their condition improved, and no major episodes of mortality were observed thereafter. The fish were fed twice-a-day with fractionated pellets with low lipid content (5812 Biomarine 2 mm; www.biomar.com) for marine fishes.

After acclimatization, they were exposed to one of two immersion treatments with either alizarin red S (ARS) or oxytetracycline hydrochloride (OTC). The solutions were prepared with distilled water using a magnetic stirrer to ensure effective dilution. The solutions were then filtered to remove residual stain that could potentially obstruct the gills, and they were mixed into the rearing tanks while maintaining neutral pH and suitable oxygenation. A second immersion was performed for each treatment using similar procedures to those of the first immersion (Table I). For both treatments, fish were reared for *c.* 1 month after the second immersion, when the survivors were sacrificed for otolith extraction. Fish that died at least 3 days after the second immersion were also used for validation.

OTOLITH PREPARATION AND ANALYSIS OF CHEMICAL MARKERS

Engraulis ringens sampled during and after the experiments were measured for total length (L_T) and standard length (L_S), ±0·01 cm, sexed and weighed (±0·01 g) for total body mass (M_T), eviscerated body mass (M_{EB}) and gonad mass (M_G). The M_{EB} was used rather than M_T to remove

the effect of stomach and M_G in the analyses of the gonado-somatic index (I_G) calculated as $I_G = 100\, M_G\, M_{EB}{}^{-1}$.

Sagittal otoliths were extracted and stored in polyethylene microtubes. Otoliths were embedded in epoxy resin on glass slides and polished manually with sandpaper of grain size 800–1000, using the slide–glass-embed method (SGEM) (Plaza *et al.*, 2005). As the sagittae of adults become slightly concave, the polishing procedures aimed to obtain a high-quality resolution at the otolith periphery where the chemical markers were expected to be deposited. Examination of microincrements at the otolith periphery was performed with a light microscope (Leica; www.leica-microsystems.com) at magnifications of x400 and x1000 and using image analysis software (Leica LAS EZ). The same otolith areas were also observed under ultraviolet (UV) light to identify and perform two counts of the microincrements deposited between the first and second immersions for both the ARS and OTC markers.

VALIDATION PROCEDURES FOR JUVENILE FISH

As a result of postcapture stress, the *E. ringens* spawned spontaneously during the first night in the rearing tanks. Floating and fertilized eggs were retained in manifolds, which consisted of an 80 l container filled on the inside with plankton mesh collectors (300 microns). After spawning, the eggs retained in the collectors were gathered and transferred for incubation. After hatching, the larvae were transferred to an artificial nutrient-enriched system (ANES) that was previously prepared to reproduce the natural environment using nitrate and phosphate enrichment. The larvae were left in this system without additional management apart from the addition of raw pre-filtered water at a low flow rate of 15 l min^{-1} to compensate for evaporation. To enhance natural phytoplankton and zooplankton growth, microalgae were added daily to a ratio of 100 g day^{-1}. At day 52 post-hatching, on 24 September 2011, a total of 258 juveniles that survived in the artificial pond were transferred to a rearing tank, where they were reared and fed twice-a-day with fractionated pellets (5812 Biomarine 2 mm) for marine fishes.

To validate the daily periodicity of formation of otolith microincrements, 10 individuals were sacrificed weekly and were supplemented by juveniles that had died of natural causes during the interval between sampling. The L_T of each specimen was measured (±0·1 mm), and the sagittal otoliths were extracted and stored for analysis. The preparation of otoliths for examination of microincrements followed the same procedures as that for adults, except that the juvenile otoliths were double polished until the microincrements were distinct from the primordia to the otolith edge. After obtaining a thin section, microincrements were counted from a prominent concentric check surrounding the primordium, using a light microscope at magnifications of x400 and x1000 using the LAS EZ system. This mark was assumed to be the hatch check (hc) following the criteria reported by Hernández & Castro (2000).

The relationship between the days after hatching and the number of microincrements formed to the sampling date was determined using a linear regression model, where the independent variable corresponded to the observed age, which was measured without error. Prior to the analysis, three microincrements were added to the total number of microincrements because this species forms the first microincrements 3 days after hatching (Hernández & Castro, 2000). A *t*-test was used to check whether the slope was significantly different from 1.

RESULTS

PERIODICITY OF FORMATION OF MICROINCREMENTS IN ADULT *ENGRAULIS RINGENS*

In both the ARS and OCT experiments, most of the fish (>80%) survived until 1 week after the second marking, and some fish survived for *c.* two more months until being sacrificed. The sacrificed specimens ranged from 13 to 16 cm L_T (mean ± S.D. = 14·92 ± 0·55 cm) and from 13 to 25 g in M_T (mean ± S.D. = 18·68 ± 3·55 g), with similar measurements for males and females, respectively. Gonad development was

TABLE II. General statistical summary of the characteristics of adult *Engraulis ringens* treated with alizarin red S (ARS) and oxytetracycline hydrochloride, (OTC) sacrificed on 5 and 21 January 2012

		ARS			OTC		
Sex	Variable	Mean ± S.D.	Range	C.V.	Mean ± S.D.	Range	C.V.
Females $n=27$	L_T (cm)	15·48 ± 0·58	15–16	3·75	14·61 ± 0·55	14–16	3·80
	L_S (cm)	12·37 ± 0·49	12–14	3·94	12·36 ± 0·43	12–13	3·44
	M_T (g)	21·43 ± 2·32	19–27	10·81	18·89 ± 3·92	13–25	20·73
	M_{EB} (g)	18·35 ± 2·10	15–24	11·46	16·53 ± 3·19	11–22	19·28
	M_G (g)	0·71 ± 0·32	0–1	45·39	0·46 ± 0·50	0–1	53·96
	I_G	3·94 ± 1·87	1–7	47·50	2·69 ± 1·38	0–5	51·35
Males $n=25$	L_T (cm)	14·71 ± 0·31	14–15	2·08	15·00 ± 0·81	14–16	5·37
	L_S (cm)	11·64 ± 2·74	0–14	23·52	12·50 ± 0·65	11–13	5·22
	M_T (g)	20·12 ± 4·71	2–27	23·39	19·35 ± 2·40	15–23	12·40
	M_{EB} (g)	17·27 ± 4·06	2–24	23·49	16·74 ± 2·01	13–19	11·98
	M_G (g)	0·72 ± 0·37	0–1	51·49	0·35 ± 0·22	0–1	62·84
	I_G	4·10 ± 2·19	1–7	53·32	2·07 ± 1·18	0–4	57·08

L_T, total length; L_S, standard length; M_T, total mass; M_{EB}, eviscerated body mass; M_G, gonadal mass; I_G, gonado-somatic index; C.V., coefficient of variation.

somewhat more variable with C.V. of I_G >50% (Table II). Specimens that died during the experiments showed similar characteristics to those of the sacrificed specimens.

Markings made with ARS and OCT produced fluorescent bands in 55 of 62 samples analysed (95% effective). Moreover, marking events could be detected under bright field and appeared as concentric interruptions in the sequence of microincrements [Fig. 1(a)]. The occurrence of microincrements was detected in the markings made in the spring [Fig. 1(b), (c), (d)]. The relationship between the number of microincrements between marks and the number of elapsed days is summarized in Table I. χ^2-tests for the ARS and OTC treatments demonstrated that there was a correspondence between the number of microincrements observed and the calculated number of days (ARS: $\chi^2 = 42{\cdot}95$, d.f. $= 40$ $P < 0{\cdot}05$; OTC: $\chi^2 = 38{\cdot}26$, d.f. $= 38$; $P < 0{\cdot}05$). The increment width between marks ranged from 0·89 to 2·75 μm (mean ± S.D. $= 1{\cdot}43 \pm 0{\cdot}28$ μm; C.V. $= 32\%$) and from 0·71 to 2·89 μm (mean ± S.D. $= 1{\cdot}53 \pm 0{\cdot}27$ μm; C.V. $= 35\%$) for ARS and OCT experiments, respectively.

PERIODICITY OF FORMATION OF MICROINCREMENTS IN JUVENILES

A total of 285 juveniles survived in the artificial pond system (APS) until day 51 (24 September 2012), when they were placed in the rearing tank and fed with an artificial diet. A characteristic feature observed in the otoliths of sacrificed juveniles was the presence of three growth zones [Fig. 2(a)]: (1) in the first zone (A), the microincrements were very distinct; (2) in the second zone (B), the identification of microincrements became less distinct because of the existence of double increments and other perturbations in the otolith growth and (3) in the third zone (C), there was a sharp shift to narrower but more homogenous microincrements. Zone C was absent in juveniles that

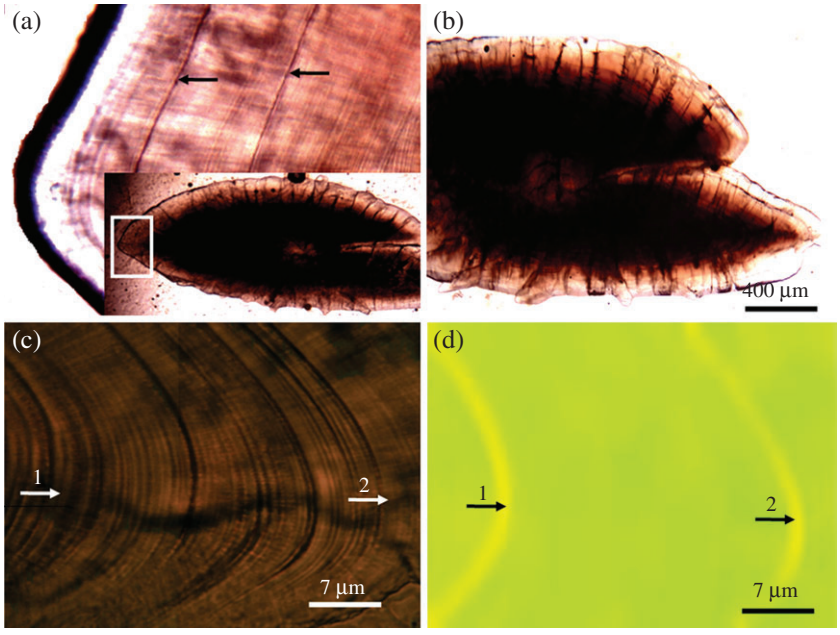

FIG. 1. (a) Sagittal otolith from a 15·42 cm total length (L_T) specimen of *Engraulis ringens*, illustrating marks generated by immersion in alizarin red S (ARS) treatments (➞) that were visible using standard optical microscopy. (b) Correspondence between the first (1) and second (2) ARS immersions on 8 and 16 October 2011 viewed under light (c) microscopy (d) and epifluorescence.

died during transportation from the ANES to the rearing tank, which indicated that this transition occurred when the juveniles were moved from the ANES to the rearing tank.

VALIDATION USING AN OTOLITH TRANSITION MARK AND JUVENILES OF KNOWN AGE

The relationship between the number of microincrements after the transition mark and the number of days elapsed after 24 September was evaluated using otolith sections with highly resolved microincrements obtained from 40 individuals. This linear relationship was highly significant ($y = 0{\cdot}99x + 0{\cdot}37$; $r^2 = 0{\cdot}98$, $F_{1,93} = 16\,997{\cdot}8$, $P < 0{\cdot}001$), and the slope ($t_{cal} = 1{\cdot}21 < t_{obs} = 1{\cdot}93$, $P < 0{\cdot}05$) did not differ significantly from 1.

Because of the occurrence of double rings in zone B, two independent samples were used to determine the relationship between the total number of microincrements and the number of days elapsed between hatching and the sacrifice date. The first sample encompassed juveniles sacrificed weekly between 4 October and 15 November 2011, which ranged from 4·4 to 5·6 cm L_T (mean ± S.D. = 5·13 ± 0·37 cm; C.V. = 7·5%) and from 0·32 to 0·73 g (mean ± S.D. = 0·43 ± 0·14 g; C.V. = 33·5%). After the extraction process, a total of 28 otoliths were available for analysis. The second sample encompassed 61 juveniles sacrificed between 7 October and 27 November 2011 and was supplemented by juveniles that died during the rearing period.

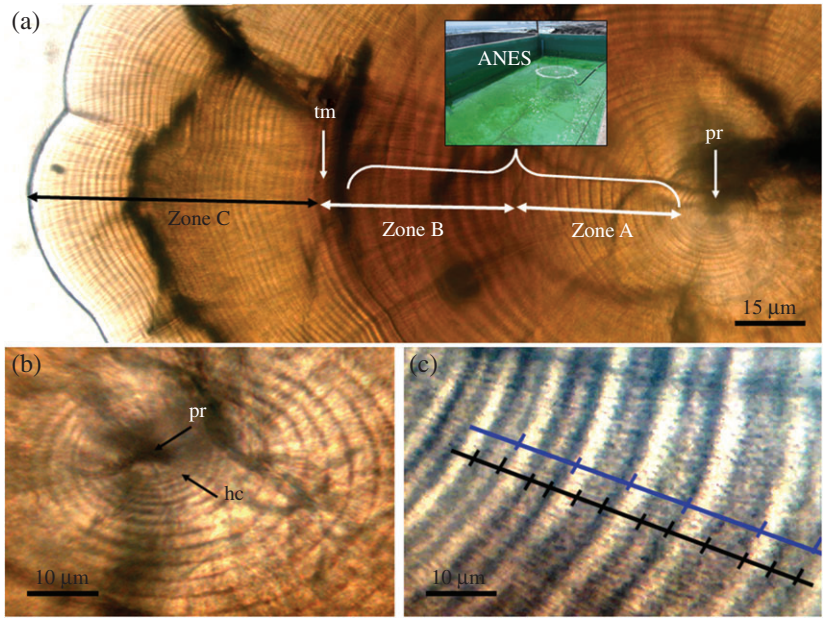

FIG. 2. (a) Sagittal otolith from a 4·54 cm total length (L_T) juvenile *Engraulis ringens* sacrificed on 10 October 2011, showing the three characteristic zones from juveniles that were reared in an artificial nutrient-enriched system (ANES) (zone A and B) and then transferred to culture tanks. (b) Enhanced image near the primordium (pr) and hatch check (hc). (c) Illustration of the two criteria used to identify primary microincrements in the zone of variation (zone B); ▬, band reading criterion; ▬, individual reading criterion; tm: transition mark.

The L_T of juveniles analysed ranged from 3·62 to 5·91 cm (mean ± S.D. = 4·97 ± 0·44 cm; C.V. = 8·92%) and M ranged from 0·19 to 3·8 g (mean ± S.D. = 0·46 ± 0·45 g; C.V. = 79·6%). In both samples, the microincrements were quantified from the hc [Fig. 2(b)] to the otolith edge using two reading criteria. In the first criterion, double microincrements in zone B were counted as a single unit [Fig. 2(c); band reading criterion; BRC]; in the second criterion, each microincrement comprising a double structure was quantified separately [Fig. 2(c); individual reading criterion; IRC]. Non-concentric faint microincrements and disturbances were not quantified. In both samples, irrespective of the type of reader, the slope was significantly different from 1 when the IRC was used, unlike the BRC, where the hypothesis was rejected (Table III). The accuracy of readings for the first sample, determined through average per cent error (E%), fluctuated between 6 and 20% and showed greater variability when the BRC was used. The overall E% was 11 and 6% for the BRC and the IRC, respectively. For the second sample, the accuracy of the readings fluctuated between 3 and 28% with an overall relative error of 17 and 3·7% for the BRC and the IRC, respectively.

DISCUSSION

Three distinct results were associated with this study of validation of the periodicity of formation of microincrements in *E. ringens*: (1) the occurrence of spontaneous

TABLE III. Summary of *t*-statistics testing the correspondence of the number days elapsed after enclosure to the number of increments formed until the date of sacrifice of juvenile *Engraulis ringens* kept under experimental conditions

Criteria	Regression	$b \pm$ S.E.	d.f.	t	t_{obs}	r^2
BRC	Reader A	$0{\cdot}88 \pm 0{\cdot}01$	26	9·34	2·06	0·96
	Reader B	$0{\cdot}90 \pm 0{\cdot}01$	26	7·16	2·06	0·95
IRC	Reader A	$1{\cdot}01 \pm 0{\cdot}01$	26	0·50	2·06	0·96
	Reader B	$1{\cdot}03 \pm 0{\cdot}01$	26	2·02	2·06	0·96

b, slope of regression (all *b* coefficients were statistically significant; $P < 0{\cdot}05$); *t*, calculated *t*-test: t_{obs}, observed *t*-test; BRC, band reading criterion; IRC, individual reading criterion.

spawning, which supplied newly hatched larvae for rearing, (2) the occurrence of three microstructural zones in the sagittal otoliths of the reared juveniles and (3) the confirmation of daily periodicity of the microincrements in juvenile and adult *E. ringens*.

The postcapture stress generated spontaneous spawning in females and males, a process that was used to rear a generation of captive specimens. Unlike some other teleosts, which must be hormonally induced to spawn (DiMaggio *et al.*, 2010; Sink *et al.*, 2010), some species can spawn naturally under rearing conditions (Otterlei *et al.*, 2002; Kawakami *et al.*, 2011). More specifically, studies of some engraulids have documented natural spawning and the subsequent production of larvae and juveniles from mature specimens that were captured and subsequently transferred to culture conditions (Aldanondo *et al.*, 2008; Masuda, 2011; Ohata *et al.*, 2011). To date, the spontaneous spawning of fishes after capture has not been documented for small pelagic fishes, but it appears reasonable to infer that spawning occurred because of the combination of the stress generated by sample collection and the fact that the experiments coincided with the period of reproductive activity of the species in the study area (Claramunt *et al.*, 2012).

A unique finding associated with the rearing experiments was the occurrence of three microstructural zones in the sagittal otoliths of the reared juveniles. A first zone (A) extended from the point of hatching to *c.* the first 25 days; an intermediate zone (B) formed until approximately the 50th day and a third zone (C) formed after the juveniles were transferred to rearing tanks. The identification of microincrements in zone B was more difficult than in the other zones because of the existence of double microincrements, which showed a rhythmic growth patterns. These structures did not have the consistence of sub-daily increments, neither did they resemble the typical discontinuity checks or other growth-band related structures described in otolith microstructure analysis (Campana & Neilson, 1985; Campana, 1992; Wright *et al.*, 2002). Similar double structures, however, have been reported in sagittal otoliths of *E. encrasicolus* (Palomera *et al.*, 1988; Cermeño *et al.*, 2006; Cermeño *et al.*, 2008). Moreover, for this species two interpretation criteria have been suggested to read daily microincrements: group band reading (GBR), where a repetitive cyclic set of growth bands were taken as single daily increments and individual mark reading (IMR), where each microincrement, regardless of its appearance was considered as a daily count (Cermeño *et al.*, 2008). These authors concluded the GBR was the most reliable ageing procedure for this species, although the way of counting daily increments on otoliths of juvenile *E. encrasicolus* is still under controversy because of the absence of a validation

experiment to support interpretation criteria (Arneri *et al.*, 2011). In the case of *E. ringens*, this study demonstrated that the double microincrements corresponded to two daily increments and must be counted individually to obtain reliable quantification of daily age for this species. Conversely, age of juveniles can be underestimated by an average of 12 days when double rings are counted as only one daily increment. In addition, it is important to underline the existence of double microincrements and growth bands that have also been reported in the otoliths of other fishes, presumably associated with the transition from larva to juvenile (Morales-Nin & Aldebert, 1997; Thomas & Panfili, 2000), larval vertical migration and tidal cycles (Rahman & Cowx, 2006), although the definitive causes of their formation are currently unknown. It appears necessary however, to incorporate in future studies other otolith pairs (*e.g.* lapillus and asteriscus) to evaluate whether the existence of these double structures are not merely a structural effect associated with sagittal otoliths.

One feature of this study was the observation of a transition in the sagittal otoliths linked to the transfer from the ANES to the rearing tank, where juveniles were fed with an artificial diet. After this induced mark microincrements were formed daily in this species. To date, transition zones in otoliths, presenting as abrupt changes in the thickness and resolution of the microincrements, have been reported in a variety of species, particularly in demersal fishes during the juvenile period and associated with habitat change from a pelagic to a benthic environment (Victor, 1986; Wellington & Victor, 1989; Sponaugle, 2010; Kohn & Clements, 2011). This finding confirms that fish otoliths can register ontogenetic events and become natural tags for a variety of ecological applications. In the current experiment, otolith microstructures comprising double and irregular rings suddenly changed to very distinctive and homogeneous microincrements when only the feeding regime was varied because the natural temperature and photoperiod variations remained unchanged. To date, it is known that daily increment formation in otoliths of teleosts is controlled by an endogenous circadian rhythm mediated by the influence of photoperiod, where temperature and feeding frequency could mask circadian rhythmicity and trigger the formation of sub-daily increments (Radtke & Dean, 1982; Campana & Neilson, 1985; Wright *et al.*, 1991, 1992) Hence, it is reasonable to infer that the otherwise regular nature of the artificial feeding regime might be associated with the regular pattern of homogenous microincrements. Further research on the physiological process and regulation of otolith accretion is needed in order to reveal the occurrence of double increments and the sudden shift to homogeneous otolith growth patterns and reveal if this pattern occurs in wild juvenile *E. ringens* as well.

As a consequence, it was possible to validate the daily periodicity of microincrements in juvenile otoliths of *E. ringens* using induced marks in the otoliths along with juveniles of known age. These results are consistent with those reported for other teleosts that have confirmed this same condition in larvae and juveniles (Hernaman *et al.*, 2000; Joh *et al.*, 2005, Yamada *et al.*, 2009; Parkinson *et al.*, 2012). Moreover, this finding supports results reported by Cermeño *et al.* (2003) and Namiki *et al.* (2010) who also validated the daily periodicity of microincrements in juvenile otoliths in *E. encrasicolus* and *E. japonicus*. Additionally, the results of this study are also consistent with those of previous studies in other clupeiformes, which have confirmed the daily periodicity of formation of microincrements for both larvae and juveniles such as Californian anchovy *Engraulis mordax* Girard 1854 (Brothers *et al.*, 1976), nehu *Encrasicholina purpurea* (Fowler 1900) (Struhsaker & Uchiyama, 1976), bay anchovy

Anchoa mitchilli (Valenciennes 1848) (Fives et al., 1986), South African anchovy *Engraulis capensis* Gilchrist 1913 (Waldron *et al.*, 1989), South American pilchard *Sardinops sagax* (Jenyns 1842) (Hayashi *et al.*, 1989), Argentine anchovy *Engraulis anchoita* Hubbs & Marini 1935 (Castello & Castello, 2003) and *E. encrasicolus* (Cermeño *et al.*, 2003; Aldanondo *et al.*, 2008).

A distinctive finding from the validation experiments in adult fish was the appearance of clear chemical marks in the otolith, particularly when ARS was used. These marks were visible under light microscopy as sharp checks that fluoresced under UV light. Similar findings have been reported in other validation studies using ARS (Lagardère et al., 2000; Meisfjord *et al.*, 2006; Durham & Wilde, 2008). Therefore, ARS appears to be an effective marker for use in immersion procedures for larvae, juveniles and adults, with similar results to those from studies using more traditional markers such as alizarin complexone, calcite and other tetracycline derivatives (Bashey, 2004; Meisfjord *et al.*, 2006; Crook *et al.*, 2007; Liu *et al.*, 2009). It is important to note, however, that ARS has a very low solubility in water; hence, it is necessary to develop effective dilution and filter procedures to avoid small particles that can adhere to the fish gill and would trigger massive mortality during the immersion process.

A further result derived from experiments of adult fish was the high mortality found during the first week of the acclimatization period. Such a condition appears to be characteristic of small pelagic fishes with similar results reported in other studies, where high mortalities have also been reported at the beginning of experiments (Cermeño *et al.*, 2003; Namiki *et al.*, 2010). Certainly, the shift from a highly mobile pelagic life to rearing conditions generates a high stress for a fish. Survivors, however, become very healthy thereafter, being able to receive artificial food. Good conditions after acclimatization could be favoured by the fact that the temperature under rearing conditions was unregulated (range 13 to 18° C), similar to the sea temperature range in spring in the study area.

The results derived from the rearing experiments with both ARS and OTC indicated a correspondence between the number of microincrements and the number of days between chemical marks. These results showed that the microincrements were produced on a daily basis for fish between 12 and 15·5 cm L_T. These findings are also similar to the results reported by Cermeño *et al.* (2003) for adults of *E. encrasicolus* and more recently reported by Namiki *et al.* (2010) for *E. japonicus.* It is important to underline, however, that validation in this study were carried out in spring, when resolution of microincrements could be expected to be high. Hence, it is necessary to perform further research in winter. A recent study reported that both resolution and periodicity of formation were more variable in individuals of *E. japonicus* that experienced low temperatures (Namiki *et al.*, 2010). Nevertheless, based on these studies and the results of this study, microincrements can be used to produce reliable estimates of ages and birth dates for juveniles and adults in natural environments, where conditions linked to circadian rhythms associated with the formation of one microincrement per day are expected to be even more optimal, and where the main driver (photoperiod) and masking agents (temperature and feeding frequency) (Campana & Neilson, 1985; Wright *et al.*, 1991, 1992) of otolith accretion are expected to be operating as well. Finally, the results of this study are preliminary to further studies of validation of the first annulus, still an unachieved task in this species. Such effort is necessary because uncertainty in age determination of *E. ringens* can affect mortality estimates for stock assessment purposes, particularly in short-lived small pelagic fishes, where

high natural mortality is expected. Hence, validation of the first annulus is highly recommended and feasible because otoliths are normally collected and stored as part of stock assessment procedures.

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