Mediterranean Marine Science

Vol 15, No 4 (2014)

Vol 15, No 4 (2014) special issue

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doi: 10.12681/mms.819

To cite this article:

CATALÁN, I. A., PÉREZ-MAYOL, S., ÁLVAREZ, I., RUIZ, J., PALMER, M., BALDÓ, F., PELIZ, Á., & MORALES-NIN, B. (2014). Daily otolith growth and ontogenetic geochemical signatures of age-0 anchovy (Engraulis encrasicolus) in the Gulf of Cádiz (SW Spain). *Mediterranean Marine Science*, *15*(4), 781–789. https://doi.org/10.12681/mms.819

Research Article

Mediterranean Marine Science
Indexed in WoS (Web of Science, ISI Thomson) and SCOPUS
The journal is available on line at http://www.medit-mar-sc.net
DOI: http://dx.doi.org/10.12681/mms.819

Daily otolith growth and ontogenetic geochemical signatures of age-0 anchovy (*Engraulis encrasicolus*) in the Gulf of Cádiz (SW Spain)

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Handling Editor: Ioanna Siokou

Received: 1 March 2014; Accepted: 30 October 2014; Published on line: 22 December 2014

Abstract

The European anchovy fishery in the Gulf of Cádiz (ICES Division IXa South) is largely influenced by age-0 individuals. Knowledge of young-of-the-year-growth dynamics is crucial for management, yet data on daily growth are lacking in the area. Linking growth patterns to the environment requires information on habitat occupancy through ontogeny of the fish that reach the fishery, as anchovy use different areas of the Gulf and the Guadalquivir Estuary throughout development. We describe the growth dynamics of age-0 anchovy through otolith microstructure analysis, and couple these data with data on microchemical signals in the otoliths to shed light into habitat use and growth dynamics in the area. Age-0 anchovy captured in September 2011 in the Gulf ranged from 3 to 6 months old for similar sizes, with average growth rates varying twofold. Individual non-linear growth curves showed that maximum otolith growth was positively correlated with the date of spawing. There was no correlation between growth parameters and body length or condition (Fulton K) at capture. The strontium:calcium (Sr/Ca) and magnesium:calcium (Mg/Ca) ratios were significantly lower at the edge of the otolith (approximately the age of capture) than at ages corresponding to larval and early juveniles (<60 days old), but values fell within typical estuarine-dwelling species values. The barium:calcium ratio (Ba/Ca) increased significantly at the edge of the otolith, which possibly resulted from residing in highly productive coastal waters or from ontogenetic effects. The variance in otolith elemental ratios was larger at otolith back-calculated ages around 50 days old, an age that coincides with the presumed closer dependence of estuarine waters. Our data are a first step towards understanding the contribution of the estuarine system to the anchovy fishery in the Gulf. The limitations of the approach and future steps are discussed.

Keywords: *Engraulis encrasicolus*, Gulf of Cádiz, otolith microchemistry, growth, estuarine, Guadalquivir River.

Introduction

The European anchovy *Engraulis encrasicolus* L. is a small pelagic fish of high economic and ecological importance (Palomera *et al.*, 2007; Pikitch *et al.*, 2012).

In the Gulf of Cádiz (SW Spain, ICES subdivision IXa South), anchovy is of paramount importance to the local purse seine fishery (Sobrino *et al.*, 2005; ICES, 2012). This fishery depends highly on the age-0 individuals, and is considered to be heavily regulated by both top-down fishing effects and bottom-up environmental effects (Bellido *et al.*, 2000; Ruiz *et al*., 2009). Within this framework, fishery management requires accurate knowledge of processes regulating production during the first year of life. Growth is an essential element in fishery models and has been established as a critical parameter for the survival of juvenile marine fishes (Houde, 1989). However, the only anchovy growth data in the area come from scales (Rodríguez-Roda, 1977) or length-frequency analyses (Bellido *et al.*, 2000), with a poor resolution in the first phases of life.

Nowadays, the analysis of otolith microstructure enables the detection of daily increments (Panella, 1971) and the reconstruction of individual growth trajectories through life. Understanding (and projecting) juvenile growth, however, requires knowledge of the specific extrinsic (e.g. food, temperature) and intrinsic (e.g. metabolism) effects during development. In highly mobile fish that use estuarine and oceanic environments throughout life, it is key to resolve how and when different developmental stages use identified components of the system. Anchovy are often associated with river plumes and estuarine systems, which can be used by their juvenile and adult forms (García & Palomera, 1996; Drake *et al.*, 2007; Palomera *et al.*, 2007; Borja *et al.*, 2008). In the Gulf of Cádiz, anchovy reproduce from April to August in coastal areas close to the Guadalquivir Estuary, preferably over the 100-m isobaths (Fig. 1), where eggs are found in large concentrations (Rubín *et al.*, 1997; Bellido *et al.*, 2000; Baldó *et al.*, 2006; Catalán *et al.*, 2006a; Ruiz *et al.*, 2006). Early larvae are primarily found in the Gulf close to the estuary (Baldó *et al.*, 2006; Ruiz *et al.*, 2006), and late larvae and juveniles are captured in large amounts in the first 32 km upstream of the river mouth (Baldó & Drake, 2002; Sobrino *et al.*, 2005; Drake *et al.*, 2007; Fernández-Delgado *et al.*, 2007). The role of the Guadalquivir estuary as a nursery ground for anchovy has been postulated for some time (see references above), but whether all or part of the fished stock is linked to the estuary, and at which life stages, remains unknown and is crucial for management.

The analysis of movement patterns through otolith microchemistry has become a widely used technique to explore the association of fishes with particular water properties (Campana, 1999; Elsdon *et al.,* 2008; Phillis *et al.*, 2011). The presence of chemical tracers in the otoliths can be correlated with fish age derived from microstructure analysis. Thus, under some premises (reviewed in Elsdon *et al*., 2008), it is possible to analyze fish movement between water masses through age. The trace elements accumulated in the otoliths come from water composition and ingested food (Limburg, 1995; Milton & Chenery, 2001; Ranaldi & Gagnon, 2010), with strontium (Sr), barium (Ba) and Magnesium (Mg) being the most-studied elements (Elsdon & Gillanders, 2005; Hamer *et al.*, 2006; Elsdon *et al*., 2008; Tabouret *et al.*, 2010).

The works using microchemistry to analyze habitat use by European anchovy are scarce, and controlled experiments have proven difficult. In Atlantic zones, high Sr/Ca ratios have been associated with European anchovy juveniles using oceanic waters to escape from predators (Aldanondo *et al.*, 2010). In the Mediterranean, between-year fluctuations in spawning areas have been suggested for European anchovy through the use of otolith microchemistry, and the technique was highlighted as promising for this species (Guidetti *et al.*, 2013). The objective of this work is to i) describe the growth dynamics of age-0 anchovy through daily growth increment analysis and ii) couple the age data to microchemical signals in the otoliths in order to advance in the knowledge of how European anchovy use different habitats in the Gulf of Cádiz.

Methods

Collection of specimens and otolith pre-processing

Age-0 anchovy (N = 100) were obtained from a commercial purse seine operating over the shelf close to Cádiz (area 1 in Fig. 1) on 29 September 2011. Fish total length (TL, mm), standard length (SL, mm), and weight (W, g) were measured in fresh individuals, which were then frozen and sent to the laboratory for analysis. Their sagittal otoliths were removed using clean methods as in Morales-Nin *et al.* (2014) and stored in acid-cleaned, plastic vials until further handling. The right otoliths were individually glued onto glass microscopic slides with a thermoplastic resin (Crystalbond^{TM}) for age determination; the left otoliths were similarly mounted with distal side up and used for Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICPMS) analysis.

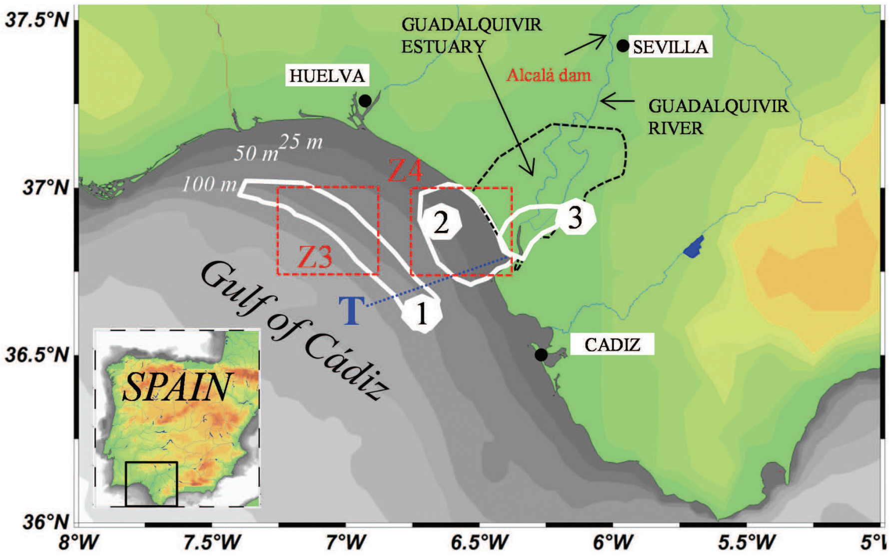

Fig. 1: Area of study. White polygons embrace the main spawning/fishing grounds and collection site (1), areas of high larval presence (2) and areas of high presence of late larvae and juveniles (3). Derived from Bellido *et al.* (2000) and Ruiz *et al.* (2006). T is the transect for which 2011 surface temperature and salinity time-series were derived. Areas Z3 and Z4 were used for surface Chl-a temporal analysis.

Age estimation

Otoliths were processed for age and growth determination following the otolith core to post-rostrum axis and using standard polishing methods. All readings were conducted on composite images from light microscopy (x1000 magnification for the central part, x100 magnification for the rest) by two experienced readers using the Age & Shape 2.1.1 (Infaimon©, 2007) software, and only agreed readings were accepted. The Double Band reading criterion described by Cermeño *et al.* (2008) was applied, and one day was attributed to each increment and double band (Cermeño *et al.*, 2003; Aldanondo *et al.*, 2008). The spawning date was calculated as the date of hatching (estimated from the number of increments and the date of capture) minus 2.5 days, corresponding to the mean embryonic period at the water temperature registered in the area (Bernal *et al.*, 2012). It was possible to determine the daily growth increments for 56 individuals. For convenience, the life-stages referred to in this work are defined as larvae (incapable of large horizontal displacements), late-larvae (pre-metamorphic, capable of important horizontal swimming), juveniles (post-metamorphic and immature) and age-0 (recruited to the fishery; they may include adults).

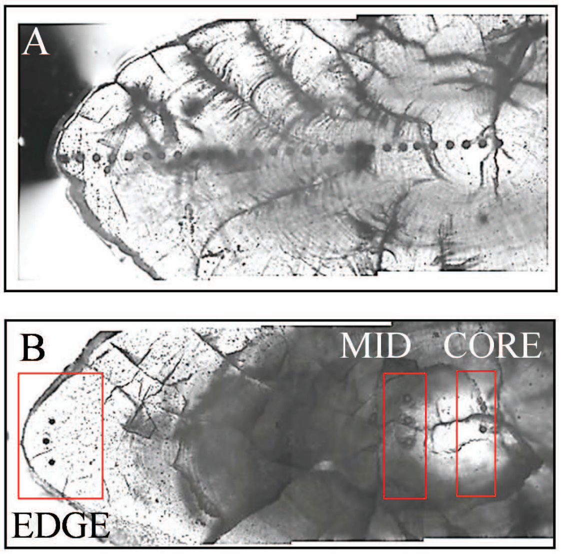

Fig. 2: Micrographs showing laser ablation spots on anchovy otoliths. A: transect performed on a subsample of individuals. B: laser ablation triplets corresponding approximately to the larval period (CORE), late-larvae/early juveniles (MID) and age-0 recruit (EDGE) performed on most samples. Diameter of the spots is 25 µm.

Otolith chemical analyses

The aged otoliths were prepared for LA-ICPMS analyses following Morales-Nin *et al.* (2014). Otolith chemical quantifications for Mg^{24}, Ca^{43}, Ca^{44}, Sr^{88} and Ba^{138} were determined using a Nd:YAG UP-213 laser ablation system (NewWave Research) coupled to an ElementXR plasma mass spectrometer (Thermo-Finnigan). Two different analytical methods were used. First of all, primordium to posterior axis transects (hereafter transects) were performed on a sub-sample (13 otoliths) to inspect fine-scale variations in microchemical signal associated with otolith growth. Then, individual spot samples were taken in triplicate, and orthogonal to the reading axis (henceforth triplets), from 43 otoliths on the core and edge (posterior) areas, as proxies to the natal and sampling period signatures, respectively (Fig. 2). Three additional spot samples were collected at approximately 300 µm from the primordium (posterior direction) to sample an otolith area corresponding to the estuary signature (juveniles approximately two months old, [Drake *et al.*, 2002]). Laser spots were 25 µm in diameter centre-to-centre separated by 45 µm in the transects and by 75 µm in the triplets. Laser conditions were set to a frequency of 10 Hz and 60% energy. The typical Certified Reference Materials (CRMs) were used (Yoshinaga *et al.*, 2000; Sturgeon *et al.*, 2005), and sampled using the same instrument conditions at the beginning and end of each working session and after every 20 LA-spots. LA-ICPMS output data were processed using Glitter software (GEMOC, Macquarie University) to obtain element concentrations ($\mu g_{Me}/g_{otolith}$) based on natural isotope ratios and using Ca^{43} as an internal standard. The analyzed CRMs were used to calibrate element concentrations. The election of one specific CRM was determined following Geffen *et al.* (2013). To reduce bias, the detection of outliers on the CRMs and the election of the concrete CRM was conducted through an ad-hoc script from R software (http://www.r-project.org/) (last accessed February 5, 2014).

We only used the elements that were not under the detection limit (DL) for at least 80% of the spots, and only the standards showing no significant differences between sessions (ANOVA). In the case of Ba^{138}, for the few values that were below the DL we used DL as the value. Both for transects and the triplets, the elemental ratios (molar ratios) used for comparisons were Sr/Ca, Mg/Ca and Ba/Ca. After the LA-ICPMS analyses, calibrated digital images from the slides were used to measure the otolith diameter (anterio-posterior axis) and the spot distances to the otolith primordium using ImageJ free software (Schneider *et al.*, 2012).

Statistical analyses

Individual otolith growth trajectories were explored and related to microchemical and environmental variation. We fitted a Gompertz growth equation, previously used in juvenile anchovy (Aldanondo *et al.*, 2008), through a non-linear Mixed Effects Model fitted by maximum likelihood, where individuals were considered as a random factor and an autocorrelation AR1 structure was included to account for repeated measurements (Crawley, 2012).

The Gompertz equation had the form $D\,(\mathrm{t}) = \alpha e^{-e^{-b(\mathrm{age}-\mathrm{c})}}$

where *D* is the distance from the otolith core to a given daily increment (µm), *a* is the asymptotic otolith growth (µm), *b* is the maximum otolith growth rate (d^{-1}) and *c* is the age (d) at which maximum otolith growth is attained. Further, for an average estimate of somatic growth, anchovy length at capture was divided by the total number of daily increments. We assumed a linear relationship between otolith and somatic growth for the juvenile period (Aldanondo *et al*., 2011) due to the small length-range of the samples.

The procedure to assign an age (and a variability in age-integration) to each spot involved *i)* measuring the distances from the otolith core to the center and borders of each spot, and *ii)* transforming these distances into ages through the individual Gompertz curves, fitted to the other otolith pair (the same otolith had to be used just in five cases). Variation in the chemical signal of the transects was inspected visually in search for fine-scale trends and peaks. For the triplets, an average was obtained for each elemental ratio by triplet as the variability between otoliths, for a given otolith region, was lower than between regions (inspected through non-metric Multi-dimensional scaling).

Differences in averaged elemental ratios between core, mid-otolith and edge were tested through ANOVA or Kruskall-Wallis tests followed by appropriate post-hoc tests, after testing for homoscedasticity. The relationship between key individual growth parameters (*b* and *c*) and condition factor at capture (Fulton's K, measured as $100W/L^3$, where L is TL) on the multivariate chemical signal at early (core and mid-otolith) and edge triplets was tested through separate Redundancy Analysis (RDA, Borcard *et al*., 2011). Parameter *a*, spawning date, length and weight were not included in these analyses as they were highly correlated to other growth parameters or to K. All analyses were performed in R.

Environmental data

The near-surface (5 m) salinity (SS), temperature (ST, °C) and chlorophyll a (Chl-a, mg m^{-3}) of the Gulf of Cádiz were used to interpret the results of otolith microchemistry and growth. A shelf across-shore transect (transect T in Fig. 1) of hydrodynamic model outputs (available from April to December 2011) extending from the river mouth to the main spawning area was used to characterize the variability of ST and SS. The model is a fully operational product operated by Puertos del Estado in the frame of MyOcean consortium (MyOcean IBI-MFC Ocean Forecast [NEATL36-NEMO], http://www.myocean.eu.org [accessed February 5, 2014]). Furthermore, the 2011 series of eight day-averaged surface Chl-a was obtained from NASA GES DISC (MODIS AQUA, 4 km resolution) and averaged over each of two zones corresponding to the two main productive zones in the area (Zones 3 and 4 in Fig.1, in accordance with the nomenclature in Navarro & Ruiz [2006]). These two zones are considered to be representative of spawning areas (Zone 3) and high larval/recruit presence (Zone 4, see Fig. 1). The latitudinal range for both zones was 36°45'0.00" North to 37°0'0.00" North. Longitude was 7°15'0.00" West to 6°52'30.00" West (Zone 3) and 6°45'00.00" W to the shoreline (Zone 4). To characterize the estuarine environment, monthly measurements of T and S (measured with an ATAGO S/Mill Refractometer) were made 8, 20 and 32 km upstream from the river mouth to cover the main river section used by late-larval and juvenile anchovy (Sobrino *et al.*, 2005; Drake *et al.*, 2007). An average monthly T and S value was derived for each site as described in Drake *et al*. (2007).

Results

Anchovy length ranged from 84 to 104 mm SL (average = 94.0 ± 4.5 [SD] mm). The age (from spawning) at capture ranged from 103 to 183 days (average = 132 ± 20 days). The range of linear somatic growth was from 0.5 to 1 mm day^{-1} (average = 0.7 ± 0.11 mm day^{-1}). The estimated spawning dates were centred on May 21 (mean calendar day = 141, SD = 20) and ranged from March 30 (day 89) to June 18 (day 169). Therefore, our samples were spawned at the beginning of the period of strong surface warming and coinciding with an extended influence of riverine signal relatively far offshore (Fig. 3, A-C). Riverine influence (here defined as waters with SS <35) was detectable between 5 and 25 km offshore during the spawning months (Fig. 3A), and was later confined to the nearshore coastal zone. In the estuary, salinity increased during the spawning period but it was clearly below the values of the Gulf (Fig. 3 D).

The otolith population averaged parameter values of the Gompertz fit were *a* = 1805.12 (33.63, SE), *b* = 0.028 (0.001, SE) and *c* = 69.02 (1.70, SE). Between-fish variability (random effect, standard deviation) for the different parameters was a = 214.31, b = 0.0068, c = 11.04. The individual growth curves showed that otolith maximum growth, parameter *b*, was positively correlated with the calendar spawning date (r_p = 0.72, df = 41, p<0.001). Thus, individuals that were spawned in June grew faster than those spawned in April. Parameter *c* was negatively correlated with spawning date (r_p = -0.41, df = 41, p<0.01); the maximum otolith growth of April-spawned anchovy (observed at ca. 93 days old) was attained at ages ca. 56% older than in anchovy spawned in June (maximum growth observed at approximately 53 days old). Both early-spawned and late-spawned anchovy attained similar lengths and body condition at the date of capture, as neither length nor condition were significantly correlated with the back-calculated spawning date (SL: r_p = -0.07, df = 41, p = 0.63; Fulton K: r_p = 0.12, df = 41, p = 0.44). Parameter *a* was not analyzed as it was considered unreliable for the purposes of this work.

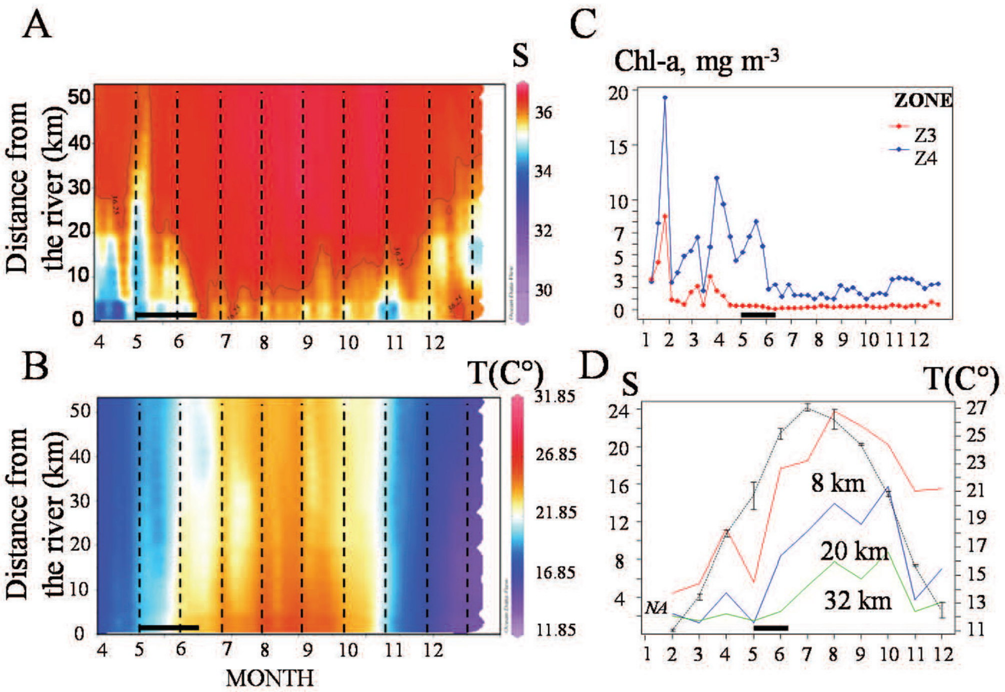

Fig. 3: A and B, daily modelled sea surface (-5 m) salinity and temperature from April-December 2011 in the Gulf transect T from Fig. 1. Data are from the operational model of Puertos del Estado. C, observed eight-days averaged values of surface Chl-a (MODIS-AQUA 4 km) in the Gulf, in each of the two zones in Fig. 1. D, estuarine conditions during February-December 2011, where solid lines are salinity at three estuarine stations located at 8, 20 and 32 km from the river mouth. In D, temperature (dashed) is shown as mean ± SD due to low spatial variability. The horizontal black bars shows the estimated (mean ± SD) spawning dates.

The analysis of the laser transects in a subsample of anchovy otoliths showed that laser spots of estimated age < 20 d integrated between 6 and 14 days; spots of estimated age between 20 and 40 d integrated between 1 and 9 days, and spots estimating ≥ 40 d integrated between 1 and 3 days. The trajectories of the elemental ratios in these transects (not shown) showed high variability between individuals and within transects. Variability ranges in the transects were 0.7 to 2.3 for Sr/Ca, 0.4 to 24.8 for Ba/Ca and 43.7 to 440.1 for Mg/Ca.

The analysis of single elemental ratios in the three otolith regions showed significant decreases in Sr/Ca and Mg/Ca at the otolith edge (approximately 129 days old) with respect to the core (approximately 25 days old) and mid-otolith (approximately 48 days old) (Table 1). The opposite pattern was observed for Ba/Ca ratios, although in this case the mid-otolith data were different from the core and edge data. As core and mid-otolith spots behaved similarly (Table 1), we performed an RDA analysis on the multivariate elemental response to individual growth drivers by making two separate analyses: elemental ratios in early stages including core and mid-otolith (average estimated spots ages = 37 ± 9 days) and edge signals (130 ± 21 d) (these ages do not include age-dependent spot uncertainty in age estimation). There was no significant effect of individual growth parameters or body condition on the multivariate chemical signal in either the core or the edge of the otolith.

Discussion

The current view suggests that adult anchovy spawn in the Gulf, particularly over the 100-m isobath (close to Zone 3, Fig. 1); however, other close-by spawning areas of minor importance have been documented. In general, larvae are found closer to the Guadalquivir River plume than eggs, which implies that either larvae actively swim to a river cue or they exhibit higher survival near the plume (Baldó *et al.*, 2006; Catalán *et al.*, 2006 b; Ruiz *et al.*, 2006). The current view also suggests that late larvae and juveniles travel up the first 32 km of the estuary, particularly during periods of high stability (low discharge) and warm months (July-August, Drake *et al.,* 2007), and then leave the estuary to recruit to the fishery stock. River discharges are primarily regulated by the Alcalá Dam, located 110 km upstream (Fig. 1), which acts as a weir and is particularly relevant during the summer period (Baldó & Drake, 2002). Although the dam can provide a relatively stable flow in summer, large discharges in spring linked to high precipitation can negatively affect anchovy estuary occupancy (Drake *et al.*, 2007; González-Ortegón *et al.*, 2010), with consequences for the stock (Ruiz *et al.*, 2009). On the other hand, age-0 recruitment appears to depend both on wind transport, river discharge and fishing intensity (Ruiz *et al.*, 2009). Despite this general picture, the relative contribution of the estuary to the anchovy ICES

Table 1. Variation between otolith areas for each element ratio (mmol/mol for Sr/Ca and μmol/mol for the rest). The estimated ages of each otolith area (in days from spawning) is shown. For each individual, the three laser spots were averaged in each otolith area and for each element ratio after checking that variability within spots was lower than between individuals. For Sr/Ca and Mg/Ca, tests are ANOVAS on log10-transformed values followed by post-hoc Tukey's HSD test for multiple comparisons. For Ba/Ca, a Kruskall-Wallis test followed by Pair-wise Wilcoxon Rank test was used due to lack of homoscedasticity.(*), medians and inter-quartilic range. (**), p<0.001. Letters that are different in post-hoc tests indicate significant differences between groups.

Ratio	Otolith area	Mean spot age, days ± SD	N	Mean elemental ratio ± SD	TEST	Post-hoc
	Core	25 ± 10.9	32	1.70 ± 0.287		a
Sr/Ca	Middle	49 ± 9.8	32	1.66 ± 0.405	F=54.4**	a
	Edge	130 ± 20.7	31	1.07 ± 0.199		b
	Core	25 ± 10.6	27	0.38 (0.29-0.68)*		a
Ba/Ca	Middle	48 ± 9.1	27	0.69 (0.39-2.46)*	Chi^2= 61.02**	b
	Edge	129 ± 19.3	27	11.76 (8.63-16.93)*		c
	Core	25 ± 10.9	32	131.26 ± 47.561		a
Mg/Ca	Middle	49 ± 9.8	32	147.97± 54.267	F=45.53**	a
	Edge	130 ± 19.3	32	73.19 ± 11.790		b

IXa South stock is not clear as other nursery areas exist nearby (e.g. close to Huelva, see Fig.1), and adults and late juveniles may move extensively within the Gulf.

The strong correlation of age-0 anchovy maximum growth (parameter *b*) with the spawning day was probably a function of temperature (Fig. 3 B), although both feeding and temperature might explain growth rates (Meekan *et al.*, 2003). Relatively high Chl-a in Zone 4 was observed throughout the year (Fig. 3 C), and this area was also the warmest one in summer. The literature suggests that anchovy larvae are primarily found close to Zone 4 (Baldó *et al.*, 2006; Ruiz *et al.*, 2006). Therefore, it is plausible that feeding was not a limiting factor and that the temperature explained the differences in maximum growth. We did not correlate growth parameters with environmental variables because, this being at the core of the work, environmental variables vary largely in space, particularly during habitat transitions.

The usefulness of detecting changes in salinity through the analysis of Sr, Ba and Mg in otoliths has been highlighted in general terms, but data interpretation must account for multiple sources of variation including species-specificity, temperature and growth effects (reviewed in Campana, 1999; Elsdon *et al.*, 2008; Sturrock *et al.*, 2012; Chang & Geffen, 2013). One concern in the use of otolith geotags to track changes in water characteristics is linked to the effect of physiology in ion incorporation (e.g. Kalish, 1989; Campana, 1999; Sturrock *et al.*, 2014). The lack of effect of individual-based information (maximum growth and condition) on multivariate otolith microchemistry in either the early portion of the otolith and at the otolith edge suggests (but not proves) that growth was not significantly affecting the chemical signals in anchovy otoliths. Growth, as a proxy for physiological status of a non-mature fish, was highly correlated with age at capture (and probably with temperature at hatch and degree-days); thus, the specific effect of age was not tested. However, the long term effect of age (e.g. in decreasing Sr/Ca, Campana, 1999 and references cited therein) is contrary to the sign of our results at least for Sr/Ca (Table 1), probably because we are examining young individuals. Effects of maturation, sex and age have been shown in a long-term experiment on adult *Pleuronectes platessa* in the laboratory (Sturrock *et al.*, 2014). In our otolith transects (not shown), high variability in Sr/Ca and Mg/Ca was observed, with few individuals showing little variation through age and others showing strong differences. The fact that the Sr/Ca chronology does not always follow an age-dependent function in juvenile anchovy was already observed by Aldanondo *et al.* (2010), and they argued that it was the environment (populations were sampled in oceanic vs coastal areas differing mainly in salinity and production) and not the ontogeny that explain the differences in Sr/Ca. However, experimental evidence at species level is needed to better understand physiological effects on elemental deposition.

One typically unaddressed problem in microchemistry analyses of otoliths is the uncertainty in the estimates of the age assigned to the spot samples. In the few existing studies of the otolith microchemistry of anchovy (e.g. Aldanondo *et al.*, 2010; Guidetti *et al.*, 2013), the age corresponding to the spots close to the core is not clarified. We here demonstrated, using the transects data, that laser spots close to the core can integrate up to two weeks of life. If we account for the potential loss of resolution due to the time needed to detect ambient changes in the otolith, and potentially on the effect of ablation depth (Hoover & Jones, 2013), it is clear that detecting the habitat transition from the larval (presumably oceanic) period to the coastal juvenile phase may not be straightforward. This observation may be one of the reasons why mean values in the core and mid-otoliths (Table 1) are similar.

We showed consistent differences between the microchemical signal at the otolith edge, presumably corresponding to the environmental conditions at capture, vs. otolith areas corresponding to early stages. Our data on Sr/Ca agree with the usual interpretation, i.e. that the decrease in Sr/Ca through age-0 life history depicts offshore fish (from more saline waters) approaching coastal (fresher) waters (Thorrold *et al.*, 1997; Hamer *et al.*, 2006). However, all our Sr/Ca values fit estuarine-dwelling fish (Secor & Rooker, 2000). This is not surprising as river discharges may provoke anchovy mortality in individuals up to 6 months old (Ruiz *et al.*, 2009), which embraces the whole life-span of the present samples and supports the view of anchovy being in contact with non-oceanic waters during all that period (riverine signals can extend several km offshore, Fig.3). We acknowledge that potential confounding factors, mainly temperature, may affect Sr/Ca. However, extreme changes in temperature are needed to produce the same effect as salinity transitions from marine to estuarine areas (Campana, 1999). Furthermore, the same review suggested that the effect of temperature tends to be relevant in cold-temperature species (<10°C), which is not the case.

Our microchemistry data showed that variability in Sr/Ca and Ba/Ca tended to be highest in the mid-otolith signal (Table 1), suggesting high variability in the environment of ca. 50-day-old anchovy. These results support the prominent role of the estuary for juvenile stages of anchovy (Baldó & Drake, 2002). We acknowledge that care must be taken when interpreting life history movements when high environmental variability occurs at short time scales (e.g. larger within a day than within months) (Elsdon & Gillanders, 2006). Salinity varied strongly on a daily basis in the estuary (up to three-fold, data not shown) compared to the Gulf (1-2 psu). However, given the fact that anchovy perform vertical migrations in the Gulf, and accounting for the fact that spots integrate information over days, we contend that this short-term (within-day) variability would be smoothed in the signal, as observed in other anchovy studies (e.g. Aldanondo *et al*., 2010).

The Mg/Ca ratios paralleled the Sr/Ca trends. Several works indicate that Mg is one of the elements showing the least biological fractionation and the highest sensitivity to ambient concentrations (e.g. Sturrock *et al.*, 2014). Although many works have shown the positive relationship between salinity and both Mg/Ca and Sr/Ca (Thorrold *et al*., 1997; Elsdon *et al*., 2008; Mercier *et al*., 2011), that is not always the case (e.g. Elsdon & Gillanders, 2002), and controlled species-specific studies should be conducted.

Our Ba/Ca values increased from the core to the edge of the otolith by two orders of magnitude, which is not an uncommon observation in other species (e.g. Milton & Chenery, 1998). Laboratory experiments in Chinook salmon *Oncorhynchus tshawytscha* have suggested that Ba/Ca may show complex interactions between temperature, salinity and growth (Miller, 2011). Reis-Santos *et al.* (2013) also accept that temperature and salinity affects the incorporation of Ba in sea bass, but other studies show that Ba/Ca is rather insensitive to temperature and salinity and depends on ambient Ba concentrations (Webb *et al.*, 2012). In general, there is a tendency to attribute a negative correlation between salinity and Ba, and a positive correlation between productivity and Ba. The latter has been attributed to migrations from offshore to more productive coastal areas in diverse taxa including sparids (e.g. Hamer *et al.*, 2006) and samonids (Miller, 2011). In our case, the highest production may be detected in the river plume, which can extend dozens of kilometres offshore. In fact, 9-cm anchovy can actively select hotspots for feeding (Pikitch *et al.*, 2012), thus choosing environments where the incorporation of Ba was particularly high. Nevertheless, experiments with anchovy would be required to fully understand our observations.

One crucial piece of knowledge that would help understanding the ontogenetic vs habitat-effect should come from the analysis of estuarine-collected individuals. Preliminary analyses (I.A. Catalán, unpublished) performed on 20 late-larvae from the estuary in July 2011 showed that the multivariate signal (using the same elements) of the post-larval otolith edges was closer to the corresponding post-larval signal in the present samples than to their edge signal, thus suggesting a habitat-related effect. However, individuals were preserved in ethanol and thus data are not fully comparable.

Overall, our results should be viewed as a relevant step towards the description of age-0 growth dynamics of anchovy in the Gulf of Cadiz, and as a first step towards understanding the interplay between growth dynamics and spatial segregation through life in this area. Despite the patterns of increase/decrease of the elemental concentrations during the first 6 months of life were clear, many unknowns still remain. Literature on clupeiform fish with protracted spawning seasons (herring, anchovy) suggests high variability within and between seasons with respect to natal origin (e.g. Geffen *et al.*, 2011; Guidetti *et al*., 2013). Therefore, future work should be focused on obtaining experimental evidence and/or implementing extended spatial and temporal sampling, including several life-stages.

Acknowledgements

The salary of SPM was funded by the Spanish Ministry as part of the MICINN-PTA (PTA2010-03812) program. We thank Ms. A. Cantarero from the Universidade A Coruña for her help with the LA-ICPMS system and analyses. Analyses and visualizations of surface chlorophyll used in this study were produced with the Giovanni online data system, developed and maintained by the NASA GES DISC. This work was partly supported by the MEDEX (MarinEra, Contract Nº ERAC-CT-2004-515871) and ECOKNOWS (FP7-KBBE, project 244706) projects.

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