Age Validation of the European Anchovy (*Engraulis encrasicolus* (Linnaeus, 1758)) in the Central-Southern Tyrrhenian Sea (West Mediterranean Sea)

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Abstract

The validation of growth of the European Anchovy (*Engraulis encrasicolus* (Linnaeus, 1758) presents several gaps in the Mediterranean Sea, despite its growth has been widely studied using different methods. The uncertainty in estimating the European Anchovy age by otolith interpretation is linked to i) the identification of the first growth ring; ii) the presence of false increments; iii) discrepancies in the applied age scheme (e.g. theoretical birthdate); and iv) the progressive compactness of the last annuli in older specimens. The present study was conducted on specimens caught in Central-southern Tyrrhenian Sea between 2012 and 2016.The analysis of the otolith margin type and the marginal increment analysis elucidated the annuli deposition patterns, with the opaque ring deposited from June to September, and the translucent ring from October to May. No significant differences were found between the von Bertalanffy growth curves calculated by otolith interpretation (back-calculation and direct otolith reading) and the LFDA. The growth pattern inferred from the European Anchovy otoliths was either corroborated or indirectly validated by the agreement between the length-frequency results and the otolith age estimation. These outcomes appear as highly relevant for species, like small pelagic fish, for which the direct validation methods (e.g. mark-recapture, captivity, radiochemical) are particularly difficult to implement.

Keywords

European Anchovy; Age and Growth; Age validation methods; Otoliths; Central-Southern Tyrrhenian Sea.

1. Introduction

The European Anchovy (*Engraulis encrasicolus* (Linnaeus, 1758)) is one the most landed species in Mediterranean basin and thus represents a very important resource for the commercial fisheries (GFCM, 2018). Purse seiners and pelagic trawlers are the fleet accountable for the largest share of total landings, largely dominated by small pelagic fish (mainly European anchovy, with 333,340 tons, and sardine, with 185,700 tons) (GFCM, 2018). In consideration of this species' economic importance, the General Fisheries Commission for the Mediterranean (GFCM) included the European Anchovy as priority species in all the Mediterranean sub-regions (West, Adriatic, Central and East) for which assessments are regularly carried out (GFCM, 2018).

As widely known, age and growth information are crucial data inputs in analytical models for stock assessment (Reeves, 2003). As a result, inaccurate or biased age data may have a great impact on stock assessment outcomes (Eero et al., 2015). Additionally, poor-quality data may often cause inaccurate assessments of the population's level of exploitation, a situation that could eventually lead to the collapse of the stock (Beamish and McFarlane, 1995; Savenkoff et al. 2004; Liao et al. 2013).

According to EU regulation 1004/2017, member states of the European Union are called to gather fisheries data to support scientific assessments of the status of resources. Each member state through the Data Collection Framework (DCF), collects biological information about the exploited species like age and growth. In this regard, a growing amount of work is put into enhancing data quality, especially for the gathering of biological data (such as age and reproduction), in order to get reliable assessment analysis (ICES 2011, 2013). With this purpose, a huge effort to achieve agreement among the readers involved in the species assessment is done under the umbrella of the International Council for the Exploration of the Sea (ICES), performing intercalibration activities (ICES, 2010; 2017; 2018). In this framework, experts have pointed out the first growth ring's identification, the presence of false increments, the employed age scheme's inconsistencies (theoretical birthdates on the 1st of January or the 1st of July), and the annuli compactness in older specimens represent the main sources of disagreement (ICES 2010, 2017).

As a result of the high mortality rate, of anchovies (and generally of small pelagic fish) associated with the capture procedure and handling (stress, scale loss, and wounds) as well as their relative short life span, the most popular direct age validation methods (e.g. mark-recapture, radiochemical dating) (James et al., 1988; ICES, 2020), are frequently affected by a very low feasibility (Politikos et al., 2015; Basilone et al., 2018). Due to these challenges, there are still few studies in the literature about European Anchovy age validation. While Uriarte et al. (2016) used strong year-classes in successive spring surveys to validate the otolith reading, Cermeo et al. (2003) and Aldanondo et al. (2016) used captivity rearing of both juvenile and adult European Anchovy to validate the otolith microstructure (daily increment). Finally, to validate the first annulus formation in the Mediterranean region, Basilone et al. (2020) used semi-direct approaches such as the marginal analysis and the marginal increment analysis.

Indirect (e.g., strong year classes tracking) and semi-direct (e.g., marginal analysis; marginal increment analysis) age validation studies (Campana, 2001; Uriarte et al., 2016; ICES, 2020; Basilone et al., 2020) could be helpful to clarify the European Anchovy growth pattern, thus providing solid data that can hardly be obtained by other ways. Furthermore, the contemporary use and comparison of these age validation techniques in a holistic manner appears to be a reliable method for elucidating growth patterns for both a particular development phase (Basilone et al., 2017) and the complete life cycle of short-lived species (Carbonara et al., 2018).

In this study the otolith age reading the European Anchovy results were compared, in a holistic approach (*sensu* Carbonara et al., 2018), with the growth curves obtained from the back-calculation analysis and from length-frequency distribution analysis (LFDA - Bhattacharya methods) to validate for the first time the species growth in Central-Southern Tyrrhenian Sea.

2. Materials and methods

2.1. Sampling

European Anchovies were collected from commercial fishery catches and landings (DCF; EU Reg. 199/2008) in the period between March 2012 and December 2016 (Tab.Sup.1) in Central-Southern Tyrrhenian Sea (Fig. 1). From each specimens the total length (TL) to the nearest 0.5 cm and the sex were recorded. Moreover, other specimens were obtained from fishery-independent surveys: the Mediterranean International Trawl Survey (MEDITS) from 2012–2016 and from the national trawl survey GRUND (January 2009) in Central-Southern Tyrrhenian Sea. The sampling protocol (Spedicato et al., 2019) used in the MEDITS and GRUND trawl survey (e.g. gear, station position, duration of each station) (Fig. 1) is the same only differing in the sampling period: spring-summer for MEDITS (2012-2016) and winter for GRUND (2009).

The unsexed juvenile specimens were divided into two sexes using the sex ratio value of the first fully sexed class (9 cm).

2.2. Direct age estimation

Age was estimated through the direct observation of winter rings in *saggitta* otoliths extracted from 1855 individuals. Analysis were carried out on the right otolith in order to standardized protocol, while the left one was kept as backup. The translucent rings count was conducted using a stereomicroscope (Leica S9D^{TM}) with reflected light, otoliths were oriented with the distal face up and the proximal surface downward (Fig. 2) and immersed in filtered seawater as clarification medium.

The ageing criteria proposed by Carbonara and Follesa (2019) was utilized (birthdate 1st July; deposition of one annulus, composed by 1 translucent and 1 opaque ring by year). The age estimation process was conducted twice on each otolith by two different readers. The overall accuracy of the readings was evaluated through the coefficient of variation CV% (Chang, 1982), the index of average percent error IAPE (Beamish and Fournier, 1981) and the percentage of agreement A%. Considering that warm months are reported as the main European Anchovy spawning period in the study area (Follesa and Carbonara, 2019 and reference therein) the 1st of July was considered as the species theoretical birthdate. Finally, each fish was given an absolute age (in months) using the procedure suggested by Morales-Nin and Panfili (2002) (Tab.Sup 2). Additionally, the annual deposition pattern of growth rings was investigated through both quantitative (Marginal Analysis, MA) and quantitative approach (Marginal Increment Analysis, MIA). The MA required the annotation of each otolith edge nature in order to follow its monthly evolution. The MIA took into account the average monthly marginal increment. Following the equation proposed by Panfili et al (2002) and Mahé et al. (2021), the Relative Marginal Distance (RMD) was calculated in each otolith as the as the ratio between the farther mark from the edge the Absolute Marginal Distance (AMD), the last completed annulus and the distance separating the two last rings (Di, i-1). Following Campana (2001), in order to avoid the influence of seasonal differences between the age classes on the entire sample, the MIA only considered few age groups (I and II age classes).

2.3. Growth modelling

The age-at-length data obtained from the direct otolith readings were fitted to the standard von Bertalanffy growth curve (VBGC) using R (R Development Core Team 2017; ver. 4.0.5) with the FSA package (version 0.8.25, Ogle et al., 2019) according to the following equation:

$$TL_t = TL_\infty\left(1 - e^{-k(t-t_0}\right)$$

where TL_t is the fish total length at age t, TL_∞ is the species' predicted asymptotic length, k is the growth factor, and t_0 is the theoretical fish's length before birth.

2.4. Back calculation

Using the equipment mentioned above, each otolith was photographed in order to measure the otolith length (OL), otolith radius length (OR), and each true translucent ring distance from the nucleus (R1, R2….Rn). All measurements were taken on the longitudinal axis joining the *sulcus* and the *nucleus* towards the post-rostrum (Fig. 2). According to the ICES recommended criteria, only otoliths with clearly defined annuli were used to register these measurements (ICES, 2010). Additionally, the linear relationships between TL and OR as well as the one between TL and OL were examined. Differences sexes in otoliths morphometric descriptors linear relationships with fish length were investigated with an ANOVA test. The TL at which translucent rings were deposited was back-calculated for each specimen, individually for each sex, using the Campana's formula (1990):

$$TL_i = TL_c + (TL_c - TL_0) * \frac{(R_i - R_c)}{(R_c - R_0)}$$

where TL_i and R_i are the fish's length and otolith's length at age *I*, TL_c and R_c are the fish's length and otolith's length at capture, and TL_0 and R_0 are the fish's length and otolith's length at hatching, respectively (biological intercept) (Campana, 1990). TL_0 and R_0 used are respectively 2.97 mm and 4.07 µm (Aldanondo et al., 2008).

Back-calculated lengths were used to estimate von Bertalanffy (VB) growth curve as described in previous sections.

2.5. Length–frequency distribution analysis.

The length-frequency distribution analysis (LFDA) was conducted on samples collected between 2012 and 2016 through MEDITS survey and in 2009 for the GRUND survey. The Bhattacharya method was applied to the obtained data using the software FISAT II (Gayanilo et al., 2006) in order to discriminate the species normal distribution, considering each mode in the overall size-frequency distribution as a cohort. Only cohorts with values of separation index > 2 were considered, while values <2 indicated a large cohort overlap were considered unacceptable (Gayanilo et al., 2006). The translucent growth increments were assumed to be deposited throughout the winter, when the mode components (cohort) of the GRUND LFD were collected. Because of this, the Bhattacharya approach was used to determine the cohorts' average length. A Kruskal-Wallis non-parametric test was used to compare, the Battacharya analysis outcomes with the mean TL back-calculated from the translucent growth increments discovered during the otolith examination.

2.6. Statistical Analysis

A Chen test (Chen et al., 1992) was used to look for potential differences in growth between sexes. Additionally, the same test was utilized to compare all the VBG curves derived for this study from direct otolith ageing as well as from LFDA and back-calculation analyses. Moreover, the fitting level of each VBGC (calculated from direct age estimation, back calculation and LFDA) to the observed data was evaluated through the Akaike’s Information Criterion (AIC; Akaike, 1974; Haddor, 2001).

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3. Results

The age estimation process appeared to be characterized by a high level of reproducibility and precision (IAPE =3.86; CV% =4.9; A%=89.7). The observed ages varied between 0.16 and 5.08 years, corresponding to 2 and 61 months respectively. The obtained length-at-age key are presented in Tab.Sup.3. In Table 1 the estimated VBGC parameters for combined sexes and for females and males separately are reported, while in Fig.Sup.1 the obtained VBGC are plotted against observed age. The Chen test did not show any statistical difference in growth curves between males and females ($F_{obs}<F_{crit}$).

The marginal analysis based on the entire sample showed a clear deposition pattern of the annual growth rings, with an overall prevalence of translucent rings throughout the year (52.9% n=801) against opaque ones (47.1% n=714) (Fig. 3a).

According to this result only one translucent ring (mainly found at the otolith’s edge between December and April) followed by one opaque one (mainly found between May and November) appears to be laid down yearly (Fig.3a). This pattern appeared to be further confirmed by the MIA results which revealed a much higher marginal increment during the summer months (June-September) saw, while the winter and early spring (November-May) marginal increments (Fig. 3b).

Otolith morphometric descriptors considered in the present study showed a significant linear relationship with respect to the fish age (Fig. 4a, b). No statistical differences were found in between sexes (ANOVA p>0.05). Likewise, also OL and OR appeared to be linearly correlated (Fig. 4c). Each linear regression equation is presented in Figure 4.

A descriptive summary of growth rings measurement is reported in the table Tab.Sup.4 while the frequency distribution of each growth ring from the nucleus is shown in Fig.Sup.2. The individuals TLs were calculated (Tab.Sup.5). Considering that the back-calculated growth increments represented the winter growth and that the species theoretical birthdate, the corresponding ages assigned to these growth increments were as follows: 1° growth increment 0.5 years, 2° growth increment 1.5 years, 3° growth increment 2.5 years and so on (Carbonara et al., 2018, 2022). The von Bertalanffy growth parameters obtained from the back-calculated length-at-age were as follows: TL_{∞} = 21.512 cm; k = 0.167 years^{-1}; t_0 = -2.739 years (AIC= 11504.5).

The mean length (±sd) for each modal component of the LFD obtained from the length frequency distributions of MEDITS and GRUND surveys (Sup.Fig.3) allowed to calculate VBGF growth parameter TL_{∞} = 20.3579 cm, k = 0.186 year^{-1} and t0 = −2.428 year for sexes combined (AIC= 32.12). In Table 2 mean lengths derived from back-calculation and from the modes observed in the GRUND surveys (winter survey) LFD are reported, no statistical differences were found between mean lengths calculated by the two methods (Kruskal-Wallis p>0.05). The AIC values indicated the VBG curve obtained though the LFD analysis as the most precise in describing the species growth in terms of fitting to the observed data, followed by the direct age estimation (AIC=3430.81) and the back calculated one.

Finally, the Chen test, did not show any statistical difference ($F_{obs} < F_{crit}$) between growth curves obtained from LFDA and both otolith based analysis (back-calculation and direct age reading) (Fig. 5). In Supplementary Figure 4 each VBGC is shown separately together with its length-at-age data (Sup.Fig.4).

4. Discussion

Even though the European Anchovy is one of the species that has been studied the most in the Mediterranean Sea basin (Carbonara and Follesa, 2019), especially in regard to its life cycle features, several aspects of its age estimation still remain unknown. Indeed, based on the growth factors from the analysis of the relevant literature, is it possible to determine an average total length at the first year that ranges from 0.32 cm (Bouaziz and Bennoui, 2004) to 9.81 cm (Bacha et al. 2010) (Tab.Sub.6). It is implausible that ecological variation and genetic differences alone could account for the significant level of variability in the European Anchovy growth patterns described (Carbonara et al., 2018). In general, there are a number of reasons for the variation in age data obtained from reading hard structures (vertebral centra, spines, scales and otoliths). These factors may include the application of multiple sampling methods (commercial fishing or scientific surveys) (Coggins et al. 2013), difference in the sample analyzed and otolith preparation techniques (Smith et al. 2016), and ageing criteria (ICES 2011; 2017; Hüssy et al. 2016; Carbonara and Follesa, 2019). Moreover, the geographical differences of the environmental conditions (ICES, 2017; Carbonara et al. 2018), together to different degrees of fishing pressure (Schindler et al., 2000; Carbonara et al., 2022;) may represent an additional source of difference in ~~ageing data,~~ Furthermore, the method employed to ~~quantify~~ growth could be a source of variation. In general, while the direct age estimation could be distorted by the incorrect interpretation of the annual increments (Uriarte et al. 2016; Carbonara et al., 2018; Basilone et al., 2020), the overlapping of the modes in the LFDA methods (indirect age estimation) could result in an overestimation of growth. Finally, the reader ~~degree~~ of experience ~~(ICES 2010; Carbonara et al., 2019)~~ can be a very important additional source of variability. Indeed, Carbonara et al. (2019), in a medium-short life-span demersal species such as the red mullet (*Mullus barbatus* L. 1758.), pointed out the reader experience as the factor which explained the majority of the variability, even ~~if~~ considering other factors such as the sample geographical origin, the birthd~~ay~~ ~~date~~ (1st of July ~~and~~ 1st of January), and the different identification of the first growth increment.

Age and growth data affected by low accuracy and/or precision levels, potentially may have a significant impact on stock assessment analysis and, as a result, on the implementation of management methods aimed at achieving sustainable exploitation of marine species in general and, particularly, the European Anchovy. The majority of the data-rich stock assessment models in use, particularly the analytical ones require information on the population's age structure. The use of unrealistic and not precise age structured data may lead to unreliable scientific advices (STECF, 2016). The most significant impact of these inaccuracies is related to short-term projections of stock condition and the associated management, leading to improper fisheries management practices and the subsequent collapse of the stocks (Beamish and McFarlane, 1995; Savenkoff et al. 2004; Liao et al. 2013). Therefore, age validation techniques are essential to obtaining highly accurate age and growth data, preventing inaccurate assessments of the health status of the resources. In this regard, approaches, such as indirect (e.g., tracking year class) and/or semi-direct (e.g., margin analysis), appeared as the most applicable and were endorsed to validate small pelagic fish and particularly anchovies age and growth data (ICES, 2020; Basilone et al., 2020).

The current study is one of the first to attempt to validate European Anchovy in Central Tyrrhenian Sea. In our investigation, there were no statistical differences between the growth curves produced by otolith reading (back-calculation and direct age estimation) and the LFDA (Bhattacharya method). According to Campana (2001) and Carbonara et al. (2018), this finding constitutes an indirect validation of the otolith age reading criteria used. Indeed, considering that the modal lengths are assumed to correspond to age classes that can be identified and then compared to individual lengths at age observed in the otolith reading (Morales-Nin and Panfili, 2002), such comparison between discrete length modes and otolith reading data is acknowledged as a solid method to validate the interpretation of annuli and age/growth determination (Campana, 2001).

Additionally, the outcomes of the back-calculation analysis of the translucent growth increment were compared with the mean length of the modes (Bhattacharya's method) obtained from the winter LFD (GRUND 2009). Although being limited to only one sampling occasion, the agreement of the results of these two analyses provided a further indirect validation of the observed age classes (Panfili et al., 2002; Carbonara et al., 2018; ICES, 2020). The VBG curve obtained though the LFD analysis appeared as the most precise in describing the species growth in terms of fitting to the observed data (AIC), followed by the direct age estimation and the back calculated one, however this result might have been strongly influenced by the lower number of age-length data obtained from Battacharya's method in comparison to the other two techniques.

The monthly evolution of the otolith margin (nature of edge) over the course of the year in several areas, including the Bay of Biscay, Galician waters, Gulf of Cadiz, Alboran Sea, and North Adriatic Sea, has been monitored in previous studies using ~~a qualitative method (~~Marginal Analysis~~)~~ (Giraldez and Torres, 2009; Donato and La Mesa, 2009; Millan and Tornero, 2009; Hernandez et al., 2016; Uriarte et al., 2016; Gaamur and Khemiri, 2019). ~~While only~~ the Bay of Biscay (Uriarte et al., 2016) and Tunisian waters (Gaamur and Khemiri, 2019) ~~used a quantitative method (Marginal Increment Analysis)~~ to evaluate the periodicity of growth increment. The pattern revealed in the aforementioned works ~~is~~ consistent with our findings, which indicate that the opaque ~~growth~~ increment is primarily laid down in the summer months (prevalence > 50% of opaque edge) and the translucent ~~growth~~ increment is primarily ~~laid down~~ in the winter months (prevalence > 50% of transparent edge). The prevalence of the opaque margin gradually overwhelms the translucent edge percentage ~~starting~~ in the spring. On the other hand, as autumn approaches, the proportion of opaque margins declines until the transparent margin raises its occurrence. These results~~,~~ ~~seemed to provide a further confirmation of~~ the hypothesis of the deposition of only one annulus per year in the European Anchovy otoliths (Giraldez and Torres, 2009; Donato and La Mesa, 2009; Millan and Tornero, 2009; Hernandez et al., 2016; Uriarte et al., 2016; Gaamur and Khemiri, 2019). However, due to the extraordinary thin morphology of European Anchovy otoliths, which renders them particularly prone to become transparent (especially on the edge) after a short time when immersed in a clearing solution (ICES, 2020), this type of analysis is not always simple to perform and, consequently, the percentage of the opaque margin may be underestimated (Giraldez and Torres, 2009; Hernandez et al., 2016; ICES, 2020). In addition, it should be also considered that this general pattern often exhibits significant inter-annual variability, sometimes due to changes in the habitat conditions (e.g. chlorophyll content, temperature) (Basilone et al., 2004; Giraldez and Torres, 2009), ~~and this~~ may provide a relatively large amount of the variability in European Anchovy growth observed between areas, as well as within the same area.

To conclude, although direct age validation methods (marking and re-marking, chemical marking, and aquaculture) could not be used on this species (ICES, 2020), both indirect (LFDA) and semi-direct methods (marginal analysis) were used in this study, taking into account the recommendations of the ICES Working Group on Age Validation of Small Pelagic Fishes (WKVALPEL~~,~~ ICES 2020). ~~The marginal analysis has confirmed that the transparent growth increment is laid down on the otolith during the winter months, supporting the fact that only one annulus is deposited every year. Given that the back-calculation is based on the winter growth increment (translucent), the comparison of the back-calculated mean lengths from the translucent rings and the LFD modes from a winter survey (GRUND) allowed to validate the age readings obtained in this study, as previously reported by Carbonara et al. (2018) for *M. barbatus*.~~ ~~Furthermore,~~ the ICES ~~working group~~ WKVALPEL endorsed and recommended the contemporary use of several highly feasible age corroboration/validation methods (e.g., length and modal frequency analysis, marginal increment analysis, edge nature and microstructure readings), stating that if these methods produce consistent results, the specific ageing criteria could be supported (ICES, 2020). ~~In this regard,~~ the findings from this study represent a valuable contribution to understand the European Anchovy growth pattern in the Central-Southern Tyrrhenian Sea, also allowing to obtain solid and verified age data crucial for an accurate stock status diagnosis, thus favoring the implementation of appropriate management measures with the aim of assuring the sustainability of *E. encrasicolus* populations in the region.

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Ethics Statement

Ethical review and approval was not required for the animal study because the vertebrate animals we worked with for this study were all dead before research began.

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TABLES

Table 1. Anchovy's von Bertalanffy growth parameters obtained through otolith's direct age estimation.

	TL_∞(cm)	k (years^{-1})	t_0 (years)
Combined sexes	18.34	0.29	-1.63
Females	18.81	0.29	-1.53
Males	16.51	0.37	-1.38

Table 2. Mean lengths (±sd) of each age class of the European Anchovy calculated by back calculation and from the modes observed in the GRUND surveys LFD

Age	Back-calculation			GRUND - Survey			p-value
	Total Length (mm)	sd	N° Specimens	Total Length (mm)	sd	N° Specimens	
0.5	89.97	7.22	779	89.58	6.18	195	0.49
1.5	109.14	7.74	520	109.87	7.4	1335	0.06
2.5	125.56	8.54	275	124.95	5.67	425	0.08
3.5	139.76	8.32	81	139.88	10.37	7	0.79

FIGURES CAPTIONS

Figure 1. Study area representing the GFCM GSA (Geographic Sub-Area) 10. Black dots represent the sampling sites in which European Anchovies were caught while black circles are the principal fisheries landing ports sampled in the area.

Figure 1. Definition of the measurement taken on a European Anchovy otolith, where OL is the otolith length; OR represents the otolith radius length; C indicates the Core and R1, R2 and R3 are respectively the distance from the nucleus of the 1st, 2nd and 3rd winter ring.

Figure 3. Monthly deposition percentage of translucent and opaque growth rings (a) and mean monthly marginal increment (MIA) (b) in which numbers indicate sample size, the bars represent the standard error of the mean and the dotted line is the polynomial regression of the means for the European Anchovy In Central-Southern Tyrrhenian Sea.

Figure 4. Linear regression between: fish total length and otolith length (a); fish total length and otolith radius (b); otolith length and otolith radius (c). The equation, R2 and number of specimens are also reported

Figure 5. European Anchovy von Bertalanffy growth curves obtained from direct otolith readings (blue line); back-calculation (red line) and Length frequency distribution (orange line).

Age Validation of the European Anchovy (*Engraulis encrasicolus* (Linnaeus, 1758)) in the Central-Southern Tyrrhenian Sea (West Mediterranean Sea)

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Abstract

The validation of growth of the European Anchovy (*Engraulis encrasicolus* (Linnaeus, 1758) presents several gaps in the Mediterranean Sea, despite its ~~the species'~~ growth has been widely studied using different methods. The uncertainty in estimating the ~~anchovy~~ European Anchovy age by otolith ~~readings~~ interpretation is linked to i) the identification of the first growth ring~~'s identification~~; ii) the presence of false increments; iii) discrepancies in the applied age scheme (e.g. theoretical birthdate); and iv) the progressive ~~overlapping~~ compactness of the last annuli in older specimens. The present study was conducted on specimens caught in Central-southern Tyrrhenian Sea between 2012 and 2016.The analysis of the otolith margin type and the marginal increment analysis elucidated the annuli deposition patterns, with the opaque ring deposited from June to September, and the translucent ring from October to May. ~~The modal components of the length–frequency distribution analysis (LFDA), identified in winter survey (Bhattacharya methods), did not show significant differences from the length back-calculated from the winter (translucent) rings. Moreover, n~~No significant differences were found between the von Bertalanffy growth curves calculated by otolith ~~reading~~ interpretation (back-calculation and direct otolith reading) and the LFDA. The growth pattern inferred from the European Anchovy otoliths was either corroborated or indirectly validated by the agreement between the length-frequency results and the otolith age estimation. These outcomes appear as highly relevant for species, like small pelagic fish, for which the direct validation methods (e.g. mark-recapture, captivity, radiochemical) are particularly difficult to implement.~~ The findings of the current study were compared with those from previous Mediterranean investigations.~~

Keywords

European Anchovy; Age and Growth; Age validation methods; Otoliths; Central-Southern Tyrrhenian Sea.

1. Introduction

The European Anchovy (*Engraulis encrasicolus* (Linnaeus, 1758)) is one the most landed species in Mediterranean basin and thus represents a very important resource for the commercial fisheries (GFCM, 2018). Purse seiners and pelagic trawlers are the fleet accountable for the largest share of total landings, largely dominated by small pelagic fish (mainly European anchovy, with 333,340 tons, and sardine, with 185,700 tons) (GFCM, 2018). In consideration of this species' economic importance,~~ of~~ the General Fisheries Commission for the Mediterranean (GFCM) included the European Anchovy~~anchovy~~ as priority species in all the Mediterranean sub-regions (West, Adriatic, Central and East) for which assessments are regularly carried out (GFCM, 2018).

As widely known, a~~A~~ge and growth information are ~~two of the most ~~crucial data inputs in analytical models for stock assessment (Reeves, 2003). As a result, inaccurate or biased age data may have a~~n~~ great impact on stock assessment outcomes (Eero et al., 2015). Additionally, poor-quality data may often cause inaccurate assessments of the population's level of exploitation~~ have occasionally been caused by poor-quality data~~, ~~with the severe outcome in certain cases being ~~a situation that could eventually lead to the collapse of the stock (Beamish and McFarlane, 1995; Savenkoff et al. 2004; Liao et al. 2013).

According to EU regulation 1004/2017, member states of the European Union are called to gather fisheries data~~ on~~ ~~fisheries ~~to support scientific assessments of the status of ~~of fisheries ~~resources ~~and the Common Fisheries Policy (CFP)~~. Each member state ~~in this context collects fisheries data~~ through the Data Collection Framework (DCF), ~~including ~~collects biological information about the exploited species like age and growth. In this regard, a growing amount of work is put into enhancing data quality, especially for the gathering of biological data (such as age and reproduction), in order to get reliable~~as it is vital for the value and accuracy of stock~~ assessment analysis (ICES 2011, 2013). With this purpose, a huge effort to achieve agreement among the readers involved in the species assessment is done under the umbrella of the International Council for the Exploration of the Sea (ICES), performing intercalibration activities (ICES, 20~~09~~10; 201~~6~~7; 2018).~~Consequently, numerous otolith exchange exercises, workshops, and discussions regarding the most significant species exploited in the waters of the European Union (ICES 2018) were conducted, including, among the other species, the European anchovy. Under the umbrella of the International Council for the Exploration of the Sea (ICES) in 2009 (ICES, 2009) and 2018 (ICES, 2018) two otolith exchanges were carried out, moreover, two workshops on otolith reading were held in 2009 and 2016 (ICES, 2009; 2016).~~ In this framework, experts have pointed out~~T~~ the first growth ring's identification, the presence of false increments, the employed age scheme's inconsistencies (theoretical birthdates on the 1st of January or the 1st of July), and the annuli ~~overlapping~~ compactness in older specimens represent the main sources of disagreement ~~that have been identified during these ageing workshops~~ (ICES ~~2009~~2010, ~~2016~~2017).

~~Considering the age groups calculated by the von Bertalanffy growth function published for the Mediterranean (Suppl. Mat. Table 1 sex combined) the length, for example at age 1, fish total length ranges from 4.56 cm (Bouaziz and Bennoui, 2004) to 12.87 cm (Bacha et al. 2010). Such variability appears to be hard to explain by only taking in consideration geographical/genetic difference (Carbonara et al., 2018). Additional factors, such as the ageing procedures used (for example, length frequency distribution analysis or otolith reading), and age assignment criteria, have been identified as reasons that could significantly contribute to determining high variability in growth studies (Carbonara et al., 2019), this could be the case also of the European Anchovy in the Mediterranean basin (ICES, 2010; ICES, 2017).~~

As a result of the high mortality rate, of anchovies (and generally of small pelagic fish) associated with the capture procedure and handling (stress, scale loss, and wounds) as well as their relative short life span, the most popular direct age validation methods (e.g. mark-recapture, radiochemical dating) ~~for the anchovy and generally for small pelagic fish~~ (James et al., 1988; ICES, 2020), are frequently affected by a very low feasibility (Politikos et al., 2015; Basilone et al., 2018). Due to these challenges, there are still few studies in the literature about European Anchovy age validation. While Uriarte et al. (2016) used strong year-classes in successive spring surveys to validate the otolith reading, Cermeo et al. (2003) and Aldanondo et al. (2016) used captivity rearing of both juvenile and adult European Anchovy to validate the otolith microstructure (daily increment). Finally, to validate the first annulus formation in the Mediterranean region, Basilone et al. (2020) used semi-direct approaches such as the marginal analysis and the marginal increment analysis.

Indirect (e.g., strong ~~mode~~ year classes tracking) and semi-direct (e.g., marginal analysis; marginal increment analysis) age validation studies (Campana, 2001; Uriarte et al., 2016; ICES, 2020; Basilone et al., 2020) could be helpful to clarify the European A~~the a~~nchovy growth pattern, thus providing solid data that can hardly be obtained ~~in~~ by other ways ~~considering the low feasibility of direct age validation methods when applied to most of the small pelagic species~~. Furthermore, the contemporary use and comparison of these age validation techniques in a holistic manner appears to be a reliable method for elucidating growth patterns for both a particular development phase (Basilone et al., 2017) and the complete life cycle of short-lived species (Carbonara et al., 2018).

In this study ~~for the first time,~~ the otolith age reading the European Anchovy results were compared, in a~~n~~ holistic approach (*sensu* Carbonara et al., 2018), with the growth curves obtained from the back-calculation analysis and from length-frequency distribution analysis (LFDA - Bhattacharya methods) ~~fishery-dependent and independent data are used to conduct length-frequency distribution analysis (LFDA - Bhattacharya methods), back-calculation, marginal analysis, and the results were combined to develop a holistic approach *sensu* Carbonara et al. (2018)~~ to validate for the first time the species growth ~~for the first time,the otolith age reading of the European Anchovy~~ in Central-Southern Tyrrhenian Sea ~~(GFCM Geographical Sub-Area, (GSA) 10 (Central-Southern Tyrrhenian Sea)~~.

2. Materials and methods

2.1. Sampling

European Anchovies were collected from commercial fishery ~~monitoring~~catches and landings (DCF; EU Reg. 199/2008) ~~carried out~~ in the period between March 2012 and December 2016 (Tab.~~X~~Sup.1) in Central-Southern Tyrrhenian Sea ~~(FAO-GFCM Geographical Sub-Area (GSA) 10)~~ (Fig. 1). From each specimens the total length (TL) to the nearest 0.5 cm and the sex ~~(Follesa and Carbonara, 2019; Follesa et al., 2019)~~ were recorded. ~~Additional~~ Moreover, other ~~samples~~specimens were obtained from the Mediterranean International Trawl Survey (MEDITS) 2012–2016 and from the national trawl survey GRUND (January 2009) in Central-Southern Tyrrhenian Sea. The sampling protocol (Spedicato et al., 2019) used in the MEDITS and GRUND trawl survey ~~was the same~~ (e.g. gear, station position, duration of each station) (Fig. 1) ~~and the only~~is the same only differing in ~~difference was~~ the sampling period: spring-summer for MEDITS (2012-2016) and winter for GRUND (2009).

The unsexed juvenile specimens were divided into two sexes using the sex ratio value of the first fully sexed class (9 cm).

2.2. Direct age estimation

Age was estimated through the direct observation of winter rings in *saggitta* otoliths extracted from 1855- individuals. ~~Although both left and right otolith were collected the subsequent a~~Analysis were carried out ~~preferably~~ on the right ~~one~~ otolith in order to standardized protocol, while the left one was kept as backup. The ~~winter~~ translucent rings count was conducted using a stereomicroscope (Leica S9D^{TM}) with reflected light, otoliths were oriented with the distal face up and the proximal surface downward (Fig. 2) and immersed in~~. F~~ filtered seawater ~~was employed~~ as clarification medium.

~~Given their intrinsic low thickness anchovy's otoliths do not require to be risen before age readings. One opaque zone followed by a completed transparent translucent zone was recognized as an annual increment (annulus). Therefore, the age was assigned counting the transparent zones (winter rings). In this regard, in order to improve the ageing process solidness and reproducibility, t~~The ageing criteria proposed by Carbonara and Follesa (2019) was utilized (birthdate 1st July; deposition of one annulus, composed by 1 translucent and 1 opaque ring by year). The age estimation process was conducted twice on each otolith by two different readers. The overall accuracy of the readings was evaluated through the coefficient of variation CV% (Chang, 1982), the index of average percent error IAPE (Beamish and Fournier, 1981) and the percentage of agreement A%. Considering that warm months are reported as the main European Anchovy ~~anchovy~~ spawning period in the study area (Follesa and Carbonara, 2019 and reference therein) the 1st of July was considered as the species theoretical birthdate. Finally, each fish was given an absolute age (in months) using the procedure suggested by Morales-Nin and Panfili (2002) (~~Suppl. Mat.~~ Tab.Sup~~leTab.~~ ~~1~~2). Additionally, the annual deposition pattern of growth rings was investigated through both quantitative (Marginal Analysis, MA) and quantitative approach (Marginal Increment Analysis, MIA). The MA required the annotation of each otolith edge nature in order to follow its monthly evolution~~the nature of the otolith edge (opaque or transparent) was noted~~. The MIA took into account the average monthly marginal increment. Following the equation proposed by Panfili et al (2002) and Mahé et al. (2021), the Relative Marginal Distance (RMD) was calculated in each otolith as the as the ratio between the farther mark from the edge the Absolute Marginal Distance (AMD), the last completed annulus and the distance separating the two last ~~marks~~rings (Di, i-1). Following Campana (2001), in order to avoid the influence of seasonal differences between the age classes on the entire sample, the MIA only considered few age groups (I and II age classes)~~.~~.

2.3. Growth modelling

The ~~obtained~~ age-at-length data obtained from the direct otolith readings were ~~used~~ fitted to the standard von Bertalanffy growth curve (VBGC) ~~through~~ using R (R Development Core Team 2017; ver. 4.0.5) ~~utilizing~~ with the FSA package (version 0.8.25, Ogle et al., 2019) according to the following equation~~: to calculate the standard von Bertalanffy growth curve (VBGC)~~:

$$TL_t = TL_\infty\left(1 - e^{-k(t-t_0)}\right)$$

where TL_t is the fish~~'s~~ total length at age t, TL_∞ is the species~~'~~' predicted asymptotic length, k is the growth factor, and t_0 is the theoretical fish~~'~~'s length before birth. ~~A Chen test (Chen et al., 1992) was used to look for potential differences in growth between sexes. Additionally, the same test was~~

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~~utilized to compare all of the VBG curves derived for this study from direct otolith ageing as well as from LFDA and back-calculation analyses (see following paragraphs).~~

2.4. Back c~~C~~alculation

Using the equipment mentioned above, each otolith was photographed in order to ~~record~~ measure the otolith length (OL), otolith radius length (OR), and each true ~~winter~~ translucent ring distance from the nucleus (R1, R2….Rn). ~~Differences sexes in otoliths morphometric descriptors linear relationships with fish length were investigated with an ANOVA test.~~ All measurements were taken on the longitudinal axis joining the *sulcus* and the nucleus towards the post-rostrum (Fig. 2). According to the ICES recommended criteria, only otoliths with clearly defined annuli were used to register these measurements (ICES, 2010). Additionally, the linear relationships between TL and OR as well as the one between TL and OL were examined. Differences sexes in otoliths morphometric descriptors linear relationships with fish length were investigated with an ANOVA test.- The TL at which ~~transparent~~ translucent rings were deposited was back-calculated for each specimen, individually for each sex, using the Campana's formula (1990):

$$TL_i = TL_c + (TL_c - TL_0) * \frac{(R_i - R_c)}{(R_c - R_0)}$$

where TL_i and R_i are the fish~~'~~'s length and otolith~~'~~'s length at age *I*, TL_c and R_c are the fish~~'~~'s length and otolith~~'~~'s length at capture, and TL_0 and R_0 are the fish~~'~~'s length and otolith~~'~~'s length at hatching, respectively (biological intercept) (Campana, 1990). TL_0 and R_0 used are respectively 2.97 mm and 4.07 µm (Aldanondo et al., 2008).

Back-calculated lengths were used to estimate von Bertalanffy (VB) growth curve as described in previous sections.

2.5. Length–frequency distribution analysis.

The length-frequency distribution analysis (LFDA) was conducted on samples collected between 2012 and 2016 through MEDITS survey and in 2009 for the GRUND survey. The Bhattacharya method was applied to the obtained data ~~through~~ using the software FISAT II (Gayanilo et al., 2006) in order to discriminate the species normal distribution, considering each mode in the overall size-frequency distribution as a cohort. Only cohorts with values of separation index > 2 were considered, while values <2 indicated a large cohort overlap were considered unacceptable (Gayanilo et al., 2006). The translucent growth increments were assumed to be deposited throughout the winter, when the mode components (cohort) of the GRUND LFD were collected. Because of this, the Bhattacharya approach was used to determine the cohorts~~'~~' average length. A Kruskal-Wallis non-parametric test was used to compare, the Battacharya analysis outcomes with the mean TL back-calculated from the translucent growth increments discovered during the otolith examination.

2.6. Statistical Analysis

A Chen test (Chen et al., 1992) was used to look for potential differences in growth between sexes. Additionally, the same test was utilized to compare all ~~of~~ the VBG curves derived for this study from direct otolith ageing as well as from LFDA and back-calculation analyses ~~(see following paragraphs).~~ ~~The VBGC obtained in this study (otolith reading, LFDA) were statistically compared using the Chen test (Chen et al., 1992)~~. Moreover, the fitting level of each VBGC (calculated from direct age estimation, back calculation and LFDA) to the observed data was evaluated through the Akaike's Information Criterion (AIC; Akaike, 1974; Haddor, 2001).

~~The transparent growth increments were deposited throughout the winter, when the mode components (cohort) of the GRUND LFD were collected. Because of this, the Bhattacharya approach was used to determine the cohorts'' average length. Using the Kruskal-Wallis non-parametric test, the Battacharya analysis outcomes were compared to the mean TL that has been back calculated from the transparent translucent growth increments discovered during the otolith examination.~~

3. Results

~~The direct age estimation process was conducted on 2451 otoliths (1001 females, 854 males and 596 undetermined juveniles) belonging to fish with TLs ranging from 6 up to 16.5 cm caught during the entire year period.~~ The age estimation process appeared to be characterized by a high level of reproducibility and precision (IAPE =3.86; CV% =4.9; A%=89.7). The observed ages varied between 0.16 and 5.08 years, corresponding to 2 and 61 months respectively.The obtained length-at-age key are presented in Tab.Sup.3. In ~~table~~ Table ~~2~~ 1 the estimated VBGC parameters for combined sexes and for females and males separately are reported, while in Fig.Sup.~~Fig.~~1 ~~3~~ the obtained VBGC are plotted against observed age. The Chen test did not show any statistical difference in growth curves between males and females ($F_{obs}<F_{crit}$).

The marginal analysis ~~carried out~~based on the entire sample showed a clear deposition pattern of the annual growth rings, with an overall prevalence of ~~transparent~~ translucent rings throughout the year (52.9% n=801) against opaque ones (47.1% n=714) (Fig. ~~4~~3a).

According to this result only one ~~transparent~~ translucent ring (mainly found at the otolith's edge between December and April) followed by one opaque one (mainly found between May and November) appears to be laid down yearly (Fig.~~54~~3a). This pattern appeared to be further confirmed by the MIA results which revealed a much higher marginal increment during the summer months

(June-September) saw, while the winter and early spring (November-May) ~~had significantly smaller (p<0.05)~~ marginal increments (Fig. 3b).

~~Both the o~~Otolith morphometric descriptors considered in the present study showed a significant linear relationship with respect to the fish age (Fig. ~~5a~~4a, b). ~~while no~~ No statistical differences were found in ~~the linear regressions~~ between sexes (ANOVA p>0.05). ~~Similarly~~Likewise, also OL and OR appeared to be linearly correlated (Fig. ~~5c~~4c). Each linear regression equation is ~~reported~~ presented in Figure ~~5~~4.

~~Appling the Campana's formula (Campana, 1990)~~ A descriptive summary of growth rings measurement is reported in the table Tab.Sup.~~3~~4 while the frequency distribution of each growth ring from the nucleus is shown in Fig.Sup.2. The individuals TLs ~~corresponding to each detected true winter ring~~ were calculated (Tab.Sup.~~ 3~~45). Considering that the back-calculated growth increments represented the winter growth and that the species theoretical birthdate, ~~in consideration also of the criteria used for otolith direct age estimation, was set at the 1st of July,~~ the corresponding ages assigned to these growth increments were as follows: 1° growth increment 0.5 years, 2° growth increment 1.5 years, 3° growth increment 2.5 years and so on (Carbonara et al., 2018,~~;~~ 2022). The von Bertalanffy growth parameters obtained from the back-calculated ~~age~~ length--at--age ~~length data~~ were as follows: $TL_\infty = 21.512$ cm; $k = 0.167$ years^{-1}; $t_0 = -2.739$ years (AIC= 11504.5).

~~The Bhattacharya method was used in order to isolate the normal components of each length frequency distributions obtained through MEDITS and in GRUND survey (Sup.Fig. 62), thus providing t~~The mean length (±sd) for each modal component of the LFD obtained from the length frequency distributions of MEDITS and GRUND surveys (Sup.Fig.~~2~~3) allowed to calculate~~. Subsequently, following the same criteria used for the direct age estimation, a putative age was assigned each mode, and the obtained length-at-age data used to calculate~~ VBGF growth parameter $TL_\infty = 20.3579$ cm, $k = 0.186$ year^{-1} and $t0 = -2.428$ year for sexes combined (AIC= 32.12). In Table ~~4~~ 2 mean lengths derived from ~~back~~ back-calculation and from the modes observed in the GRUND surveys (winter survey) LFD are reported, no statistical differences were found between mean lengths calculated by the two methods (Kruskal-Wallis p>0.05). The AIC values indicated the VBG curve obtained though the LFD analysis as the most precise in describing the species growth in terms of fitting to the observed data, followed by the direct age estimation (AIC=3430.81) and the back calculated one.

Finally, the Chen test, did not show any statistical difference ($F_{obs} < F_{crit}$) between growth curves ~~computed in order to detect possible discrepancies between growth curves~~ obtained from LFDA and both otolith ~~driven~~ based analysis (back-calculation and direct age reading) (Fig. ~~7~~5~~), did not show~~

~~any statistical differences in every possible match (F_{obs} < F_{crit}).~~). In Supplementary Figure 4 each VBGC is shown separately together with its length-at-age data (Sup.Fig.4).

4. Discussion

Even though the European Anchovy is one of the species that has been studied the most in the Mediterranean ~~setting~~ Sea basin (Carbonara and Follesa, 2019), especially in regard to its life cycle features, several aspects of its age estimation still remain ~~debatable~~unknown. Indeed, based on the growth factors from the analysis of the relevant literature, is it possible to determine an average total length at the first year that ranges from 0.32 cm (Bouaziz and Bennoui, 2004) to 9.81 cm (Bacha et al. 2010) (~~Supp. Mat. Table~~Tab.~~s~~Sub. ~~5~~6~~1~~). It is implausible that ecological variation and genetic differences alone could account for the significant level of variability in the European ~~anchovy~~ Anchovy growth patterns described (Carbonara et al., 2018). In general, there are a number of reasons for the variation in age data obtained from reading hard structures (vertebral centra, spines, scales and otoliths). These factors may include the application of multiple sampling methods (commercial fishing or scientific surveys) (Coggins et al. 2013), difference in the sample analyzed and otolith preparation techniques (Smith et al. 2016), and ageing criteria (ICES 2011; 2017; Hüssy et al. 2016; Carbonara and Follesa, 2019). Moreover, the geographical differences of ~~the habitats colonized by different populations~~the environmental conditions (ICES, 2017; Carbonara et al. 2018), together to different degrees of fishing pressure (Schindler et al., 2000; Carbonara et al., 2022;) may represent an additional source of difference in ageing data. Furthermore, the method employed to quantify growth could be a source of variation. In general, while the direct age estimation could be distorted by the incorrect interpretation of the annual increments (Uriarte et al. 2016; Carbonara et al., 2018; Basilone et al., 2020), the overlapping of the modes in the LFDA methods (indirect age estimation) could result in an overestimation of growth. Finally, the reader degree of experience (ICES 2010; Carbonara et al., 2019) can be a very important additional source of variability. Indeed, Carbonara et al. (2019), in a medium-short life-span demersal species such as the red mullet (*Mullus*~~.~~ *barbatus* L. 1758.), pointed out the reader experience as the factor which explained the majority of the variability, even if considering other factors such as the sample geographical origin, the birthday date (1st of July and 1st of January), and the different identification of the first growth increment.~~found that the reader experience was the parameter that explained most of the variability, considering geographic origin, birthday date (1st of July and 1st of January), the different identification of the first growth increment.~~

Age and growth data affected by low accuracy and/or precision levels, potentially may have a significant impact on stock assessment analysis and, as a result, on the implementation of management methods aimed at achieving sustainable exploitation of marine species in general and, particularly, the European Anchovy. The majority of the data-rich stock assessment models in use, particularly the analytical ones ~~like statistical catch-at-age (e.g., Stock Synthesis [Methot and Wetzel 2013]), Age Structured Assessment Program (Legault and Restrepo 1999), or virtual population analysis (e.g., Extended Survivor Analysis [Shepherd 1999], ADAPT VPA [Gavaris 1988]),~~ require information on the population's age structure. The use of unrealistic and not precise age structured data~~The transformation of the LFD of catches into an age structure, based on the growth pattern analyzed through direct and/or indirect age estimations, is one of the basic steps in running these kinds of stock-assessment models. This process it is usually carried out by means of age slicing algorithms using growth parameters from the von Bertalanffy growth function (VBGF) or age-length keys (ALK). Consequently, incorrect growth parameters or ALK, used to convert size distribution into age structure,~~ may lead to unreliable scientific advices (STECF, 2016). ~~In this regard, uncertain ageing data can lead to two possible outcomes: in the case of an age overestimation, the stock assessment will give an incorrect picture with a population made up of older individuals and, consequently, affected by lower fishing mortality; in the alternative case, fish would be considered younger resulting in an overestimation of fishing mortality (Campana 2001). Furthermore, also the calculation of natural mortality and maturity-at-age data can be impacted by biases in age and growth data, which can therefore have an impact on estimates of recruitment strength and spawning stock biomass. According to Punt et al. (2008), Eero et al. (2015), and Hüssy et al. (2016), t~~The most significant impact of these inaccuracies is related to short-term projections of stock condition and the associated management, ~~measures. Inconsistencies in age estimation in some cases may have contributed to larger errors in population assessments, which in turn led~~leading to improper fisheries management practices and the subsequent collapse of the stocks (Beamish and McFarlane, 1995; Savenkoff et al. 2004; Liao et al. 2013). Therefore, age validation techniques are essential to obtaining highly accurate age and growth data, preventing inaccurate assessments of the health status ~~condition~~ of the resources. ~~Due to this, all age validation approaches were assessed in the context of an International Council for the Exploration of the Sea (ICES) workshop for their level of relevance for the small pelagic species, including European anchovy (ICES, 2020). The findings of this workshop highlighted that direct approaches, despite frequently being regarded as the most consistent validation method (Campana, 2001), demonstrated a low degree of~~

~~application, generally for small pelagic and particularly for anchovies (ICES, 2020). As a result, it could be helpful to~~ In this regard, ~~employ additional~~ approaches, such as indirect (e.g., tracking year class) and/or semi-direct (e.g., margin analysis), appeared as the most applicable and were endorsed to ~~get~~ validate~~d~~ small pelagic fish and particularly anchovies age and growth data (ICES, 2020; Basilone et al., 2020).

The current study is one of the first to attempt to validate European ~~anchovy~~ Anchovy in the Mediterranean basin. ~~Two factors are determinants in validation studies: the periodicity time scale of the growth increments deposition (precision) and the presence of a consistently interpretable pattern of increments in the hard structure (Campana, 2001; Panfili et al., 2002; Carbonara and Follesa 2019). However, both of these issues have received scant attention in earlier studies on *E. encrasicolus* in the Mediterranean basin (ICES, 2020; Basilone et al., 2020). The indexes used in the present paper to evaluate age readings precision indicated the direct age estimation on European Anchovy otoliths as a highly accurate and reliable process.~~

In our investigation, there were no statistical differences between the growth curves produced by otolith reading (back-calculation and direct age estimation) and the LFDA (Bhattacharya method). According to Campana (2001) and Carbonara et al. (2018), this finding ~~constituted~~ constitutes an indirect validation of the otolith age reading criteria used. Indeed, considering that the modal lengths are assumed to correspond to age classes that can be identified and then compared to individual lengths at age observed in the otolith reading (Morales-Nin and Panfili, 2002), such comparison between discrete length modes and otolith reading data is acknowledged as a solid method to validate the interpretation of annuli and age/growth determination (Campana, 2001).

Additionally, the outcomes of the back-calculation analysis of the ~~winter~~ translucent growth increment ~~(transparent)~~ were compared with the mean length of the modes (Bhattacharya's method) obtained from the winter LFD (GRUND 2009)~~ used in this analysis because the winter period represents the period of deposition of transparent growth increment~~. Although being limited to only one sampling occasion, the agreement of the results of these two analyses provided a further indirect validation of the~~ detected~~ observed age ~~groups~~ classes (Panfili et al., 2002; Carbonara et al., 2018; ICES, 2020). The VBG curve obtained though the LFD analysis appeared as the most precise in describing the species growth in terms of fitting to the observed data (AIC), followed by the direct age estimation and the back calculated one, however this result might have been strongly influenced by the lower number of age-length data obtained f~~or~~rom Battacharya's method in comparison to the other two techniques.

The monthly evolution of the otolith margin (nature of edge) over the course of the year in several areas, including the Bay of Biscay, Galician waters, Gulf of Cadiz, Alboran Sea, and North Adriatic Sea, ~~was~~ has been monitored in previous studies using a qualitative method (Marginal Analysis) (Giraldez and Torres, 2009; Donato and La Mesa, 2009; Millan and Tornero, 2009; Hernandez et al., 2016; Uriarte et al., 2016; Gaamur and Khemiri, 2019). While only the Bay of Biscay (Uriarte et al., 2016) and Tunisian waters (Gaamur and Khemiri, 2019) used a quantitative method (Marginal Increment Analysis) to evaluate the periodicity of growth increment. The pattern ~~mentioned~~revealed in the aforementioned works is consistent with our findings, which indicate that the opaque growth increment is primarily laid down in the summer months (prevalence > 50% of opaque edge) and the ~~transparent~~ translucent growth increment is primarily laid down in the winter months (prevalence > 50% of transparent edge). The prevalence of the opaque margin gradually overwhelms the ~~transparent~~ translucent edge percentage starting in the spring. On the other hand, as autumn approaches, the proportion of opaque margins declines until the transparent margin raises its occurrence. These results, seemed to provide a further confirmation of the hypothesis of the deposition of only one annulus per year in the European Anchovy otoliths (Giraldez and Torres, 2009; Donato and La Mesa, 2009; Millan and Tornero, 2009; Hernandez et al., 2016; Uriarte et al., 2016; Gaamur and Khemiri, 2019). However, due to the extraordinary thin morphology of ~~anchovy~~ European Anchovy otoliths, which renders them particularly prone to become transparent (especially on the edge) after a short time when immersed in a clearing solution (ICES, 2020), this type of analysis is not always simple to perform and, consequently, the percentage of the opaque margin may be ~~higher than it is reported~~underestimated (Giraldez and Torres, 2009; Hernandez et al., 2016; ICES, 2020). In addition, it should be also considered that this general pattern often exhibits significant inter-annual variability, sometimes due to changes in the habitat conditions (e.g. chlorophyll content, temperature) (Basilone et al., 2004; Giraldez and Torres, 2009), and this may provide a relatively large amount of the variability in European ~~a~~Anchovy growth observed between areas, as well as within the same area.

~~Although~~ To conclude, although direct age validation methods (marking and re-marking, chemical marking, and aquaculture) could not be used on this species (ICES, 2020), both indirect (LFDA) and semi-direct methods (marginal analysis) were used in this study, taking into account the recommendations of the ICES Working Group on Age Validation of Small Pelagic Fishes (WKVALPEL, ICES 2020). The marginal analysis has confirmed that the transparent growth increment is laid down on the otolith during the winter months, ~~in addition to proving~~supporting the fact that only one annulus is deposited every year. Given that the back-calculation is based on the winter growth increment (~~transparent~~translucent), the comparison of the back-calculated mean lengths from the ~~winter~~ translucent rings and the LFD modes from a winter survey (GRUND) allowed to validate the age readings obtained in this study, as previously reported by Carbonara et al. (2018) for *M.~~ullus~~ barbatus*. ~~L. 1758.~~ Furthermore, the ICES working group WKVALPEL endorsed and recommended the contemporary use of several highly feasible age corroboration/validation methods (e.g., length and modal frequency analysis, marginal increment analysis, edge nature and microstructure readings), stating that if these methods produce consistent results, the specific ageing criteria could be supported (ICES, 2020). In this regard, the findings from this study represent a valuable contribution to understand the European Anchovy growth pattern in the Central-Southern Tyrrhenian Sea, also allowing to obtain solid and verified age data ~~indispensable~~ crucial for an accurate stock status diagnosis, thus favoring the implementation of appropriate management measures with the aim of assuring the sustainability ~~preventing the collapse~~ of *E. encrasicolus* populations in the region.

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~~Credit authorship contribution statement~~

~~**PC:** Conceptualization, Methodology, Writing – original draft, Software, Investigation, Review, Supervision, project administrator. **AB:** Methodology, Writing – original draft, Writing - review & editing, Software, Investigation; **AM:** Formal analysis, Investigation, Review. **GB:** Formal analysis, Investigation, Review. **LC:** Formal analysis, Investigation, Software; **MP:** Formal analysis, Investigation Software. **IB:** Formal analysis, Investigation, Software. **MCF:** Conceptualization, Supervision, Review.~~

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Ethics Statement

Ethical review and approval was not required for the animal study because the vertebrate animals we worked with for this study were all dead before research began.

TABLES

~~Table 1. The employed scheme for the assignation of anchovy absolute age. N represent the number of true winter (transparent) rings~~

~~Capture Month~~											
~~Jan~~	~~Feb~~	~~Mar~~	~~Apr~~	~~May~~	~~Jun~~	~~Jul~~	~~Aug~~	~~Sep~~	~~Oct~~	~~Nov~~	~~Dec~~
~~1~~	~~2~~	~~3~~	~~4~~	~~5~~	~~6~~	~~7~~	~~8~~	~~9~~	~~10~~	~~11~~	~~12~~
~~N + [12-(month of birth-capture month)/12]~~						~~N + [(month of birth-capture month)/12]~~					

Table ~~2~~1. Anchovy's von Bertalanffy growth parameters obtained through otolith's direct age estimation.

	TL_{∞}(cm)	k (years^{-1})	t_0 (years)
Combined sexes	18.~~338~~34	0.~~288~~29	-1.63~~1~~
Females	18.~~805~~81	0.~~285~~29	-1.53~~1~~
Males	16.51~~0~~	0.37~~1~~	-1.~~376~~38

~~Table 3. Mean back-calculated length for each growth increment for European Anchovy collected in the Central-Southern Tyrrhenian Sea. SD = standard deviation; CV = coefficient of variation.~~

		~~Growth increments~~				
~~N° Growth increments~~	~~N° specimens~~	~~1°~~	~~2°~~	~~3°~~	~~4°~~	~~5°~~
~~1~~	~~259~~	~~87.563~~	-	-	-	-
~~2~~	~~245~~	~~89.970~~	~~108.041~~	-	-	-
~~3~~	~~194~~	~~91.303~~	~~109.286~~	~~124.621~~	-	-
~~4~~	~~67~~	~~94.053~~	~~111.782~~	~~127.637~~	~~139.668~~	-
~~5~~	~~14~~	~~96.382~~	~~113.873~~	~~128.689~~	~~140.201~~	~~149,982~~
~~Tot. Number~~	~~779~~	~~779~~	~~520~~	~~275~~	~~81~~	~~14~~
~~mean (mm)~~		~~89.968~~	~~109.144~~	~~125.563~~	~~139.760~~	~~149.982~~
~~mean increment (mm)~~		~~85.746~~	~~19.176~~	~~16.419~~	~~14.197~~	~~10.222~~
~~sd~~		~~7.223~~	~~7.744~~	~~8.535~~	~~8.319~~	~~8.521~~
~~CV~~		~~8.029~~	~~7.095~~	~~6.797~~	~~5.952~~	~~5.681~~

Table ~~4~~2. Mean lengths (±sd) of each age class of the European Anchovy calculated by back calculation and from the modes observed in the GRUND surveys LFD

	Back-~~Calculation~~calculation			GRUND - Survey			
Age	**Total Length (mm)**	**sd**	**N° Specimens**	**Total Length (mm)**	**sd**	**N° Specimens**	**p-value**
0.5	89.~~968~~97	7.22~~39~~	779	89.58	6.18	195	0.49~~0313~~
1.5	109.14~~4~~	7.74~~4~~	520	109.87~~1~~	7.4	1335	0.06~~0961~~
2.5	125.56~~3~~	8.~~535~~54	275	124.95	5.67	425	0.08~~0056~~
3.5	139.76~~0~~	8.~~319~~32	81	139.88	10.37	7	0.79~~023~~

FIGURES CAPTIONS

Figure 1~~–~~. Study area representing the GFCM GSA (Geographic Sub-Area) 10. Black dots represent the sampling sites in which European Anchovies were caught while black circles are the principal fisheries landing ports sampled in the area.

Figure 1. Definition of the measurement taken on a European Anchovy otolith, where OL is the otolith length; OR represents the otolith radius length; C indicates the Core and R1, R2 and R3 are respectively the distance from the nucleus of the 1st, 2nd and 3rd winter ring.

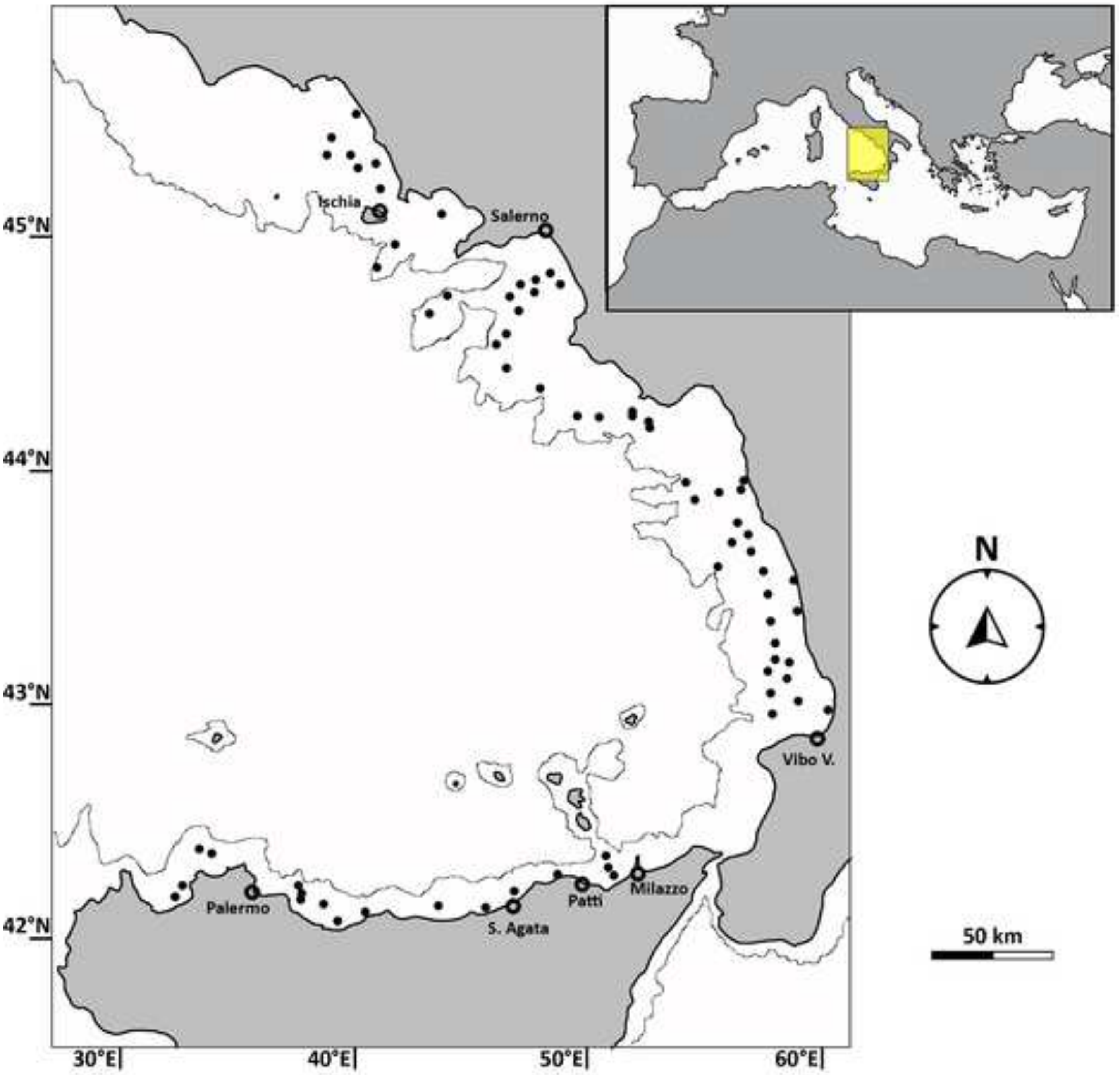
~~Figure 3. von Bertalanffy growth curve (solid line) for the European Anchovy for female (a) male (b) and combined sexes(c) in GSA10 estimated from age-at length data (blue dots) obtained from direct otoliths age estimation. The dotted black line represents the asymptotic length.~~

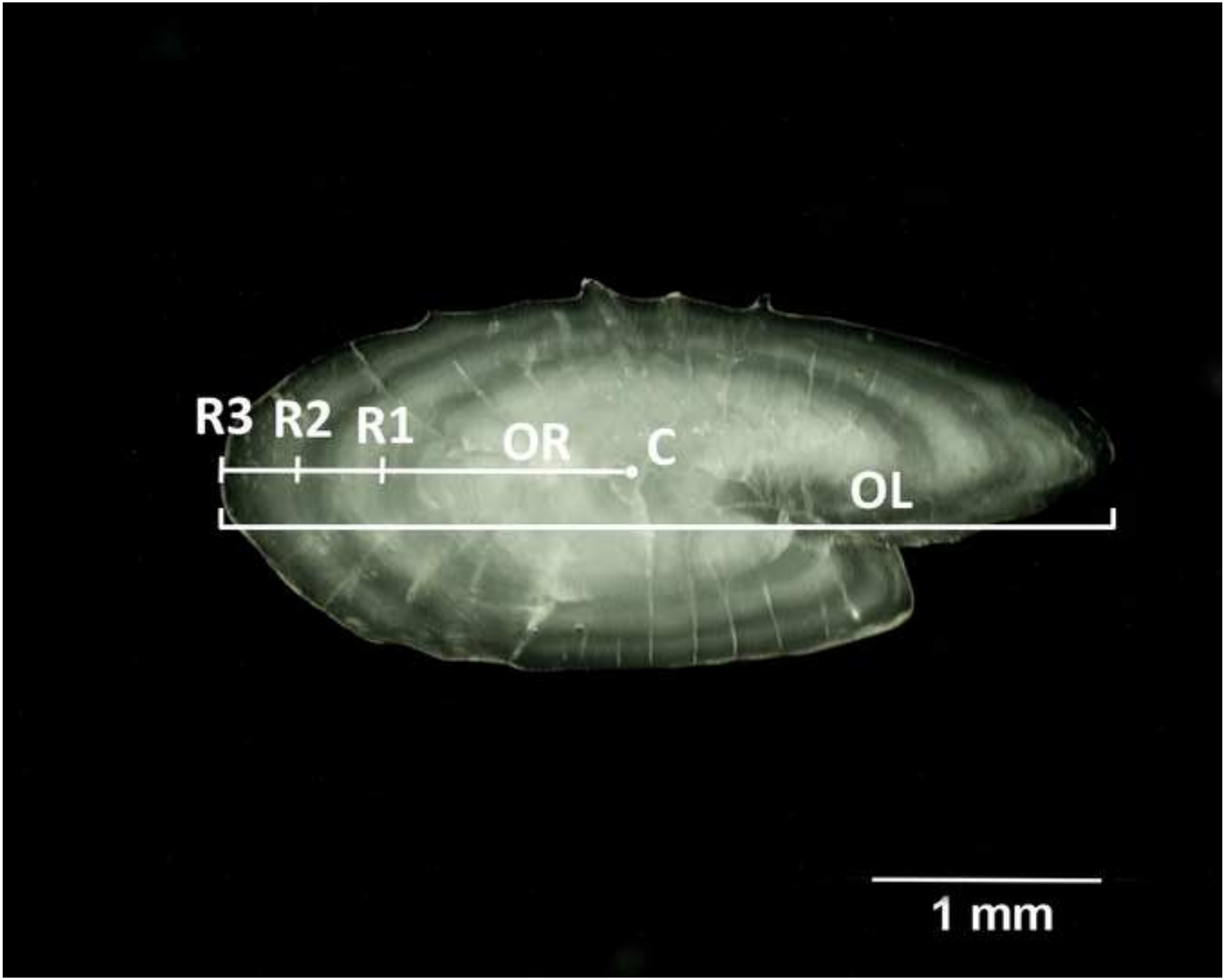
Figure ~~4~~ 3. ~~Annual~~ Monthly deposition ~~pattern~~ percentage of ~~transparent~~ translucent and opaque growth rings (a) and mean monthly marginal increment (MIA) (b) in which numbers indicate sample size, the bars represent the standard error of the mean and the dotted line is the polynomial regression of the means for ~~in~~ the European Anchovy In Central-Southern Tyrrhenian Sea.

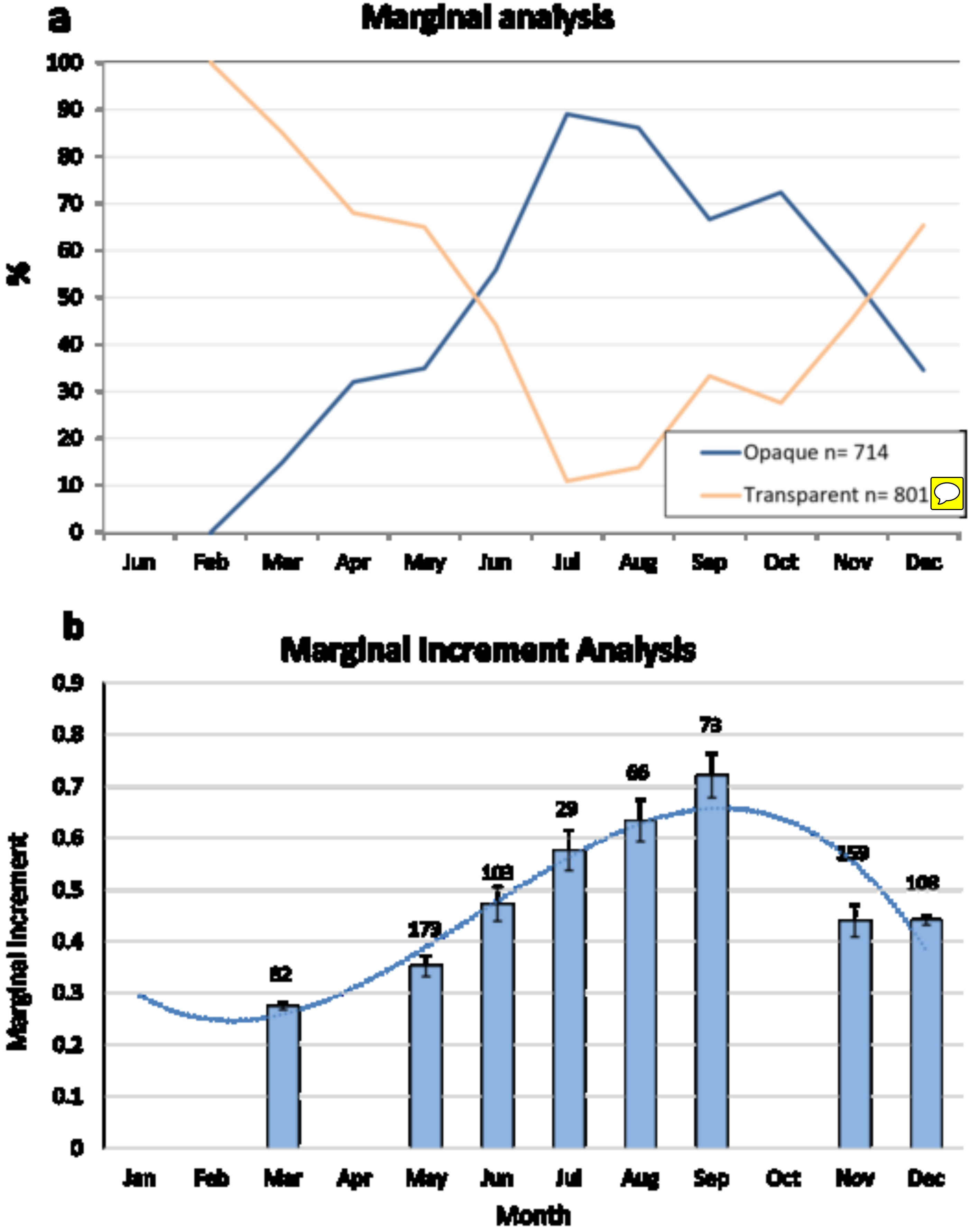
Figure ~~5~~ 4. Linear regression between: fish total length and otolith length (a); fish total length and otolith radius (b); otolith length and otolith radius (c). The equation, R^2 and number of specimens are also reported

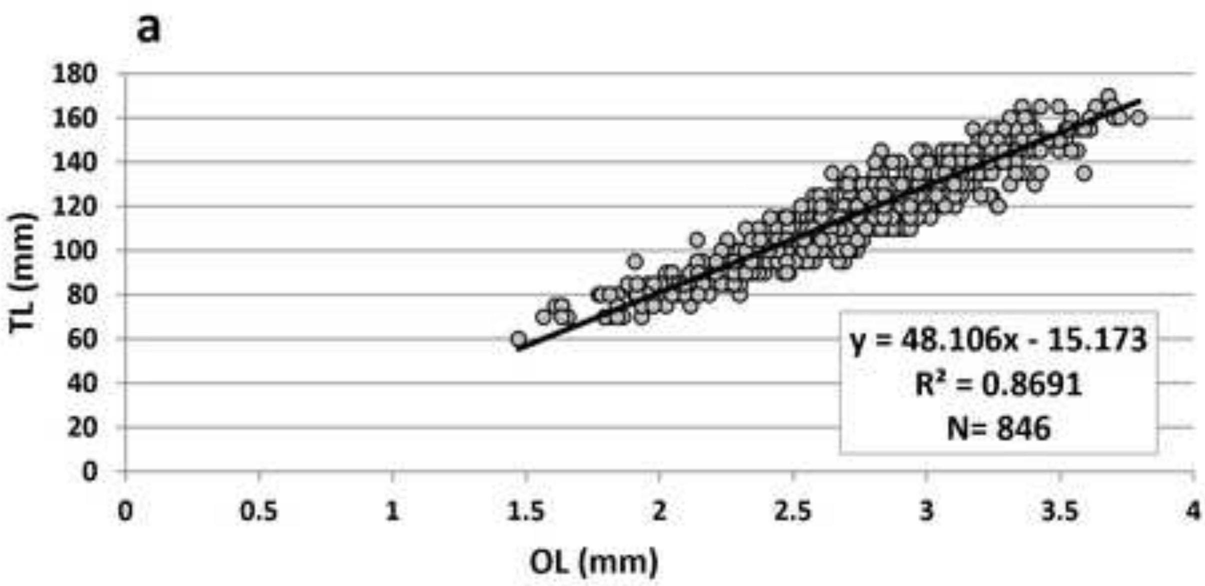
~~Figure 6. Length-frequency distribution of the catches of the European Anchovy recorded during MEDITS surveys 2012 and 2016 in the Central-Southern Tyrrhenian Sea.~~

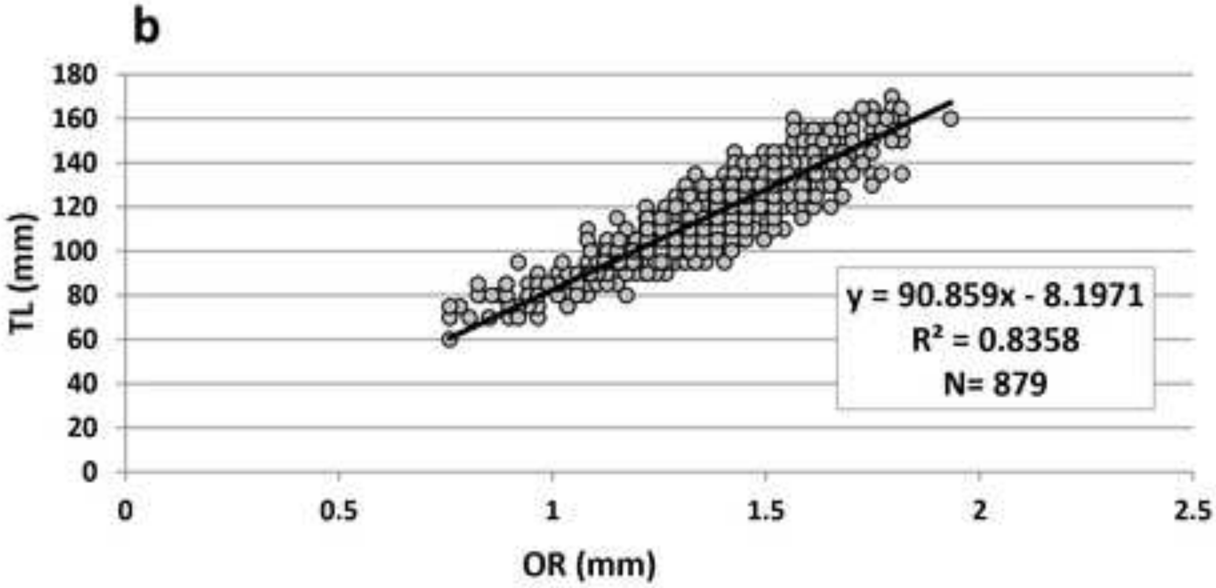
Figure ~~7~~5. European anchovy von Bertalanffy growth curves obtained from direct otolith readings (blue line); back-~~-~~calculation (red line) and Length frequency distribution (orange line)

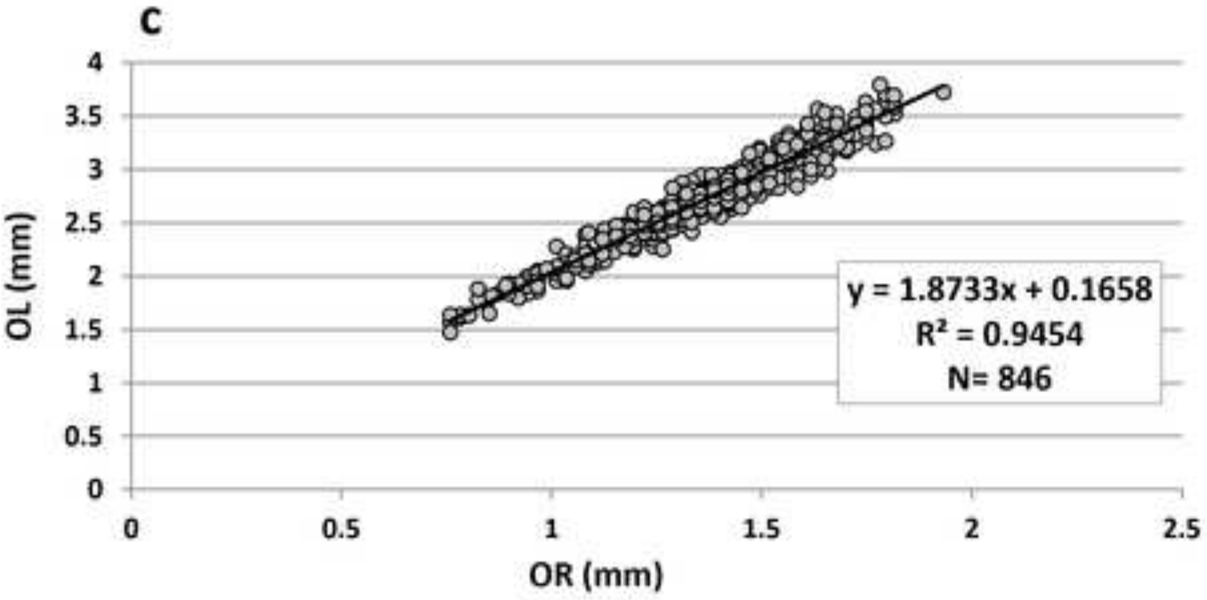

Ischia
Salerno
Vibo V.
Palermo
S. Agata
Patti
Milazzo
45°N
44°N
43°N
42°N
30°E
40°E
50°E
60°E
N
50 km

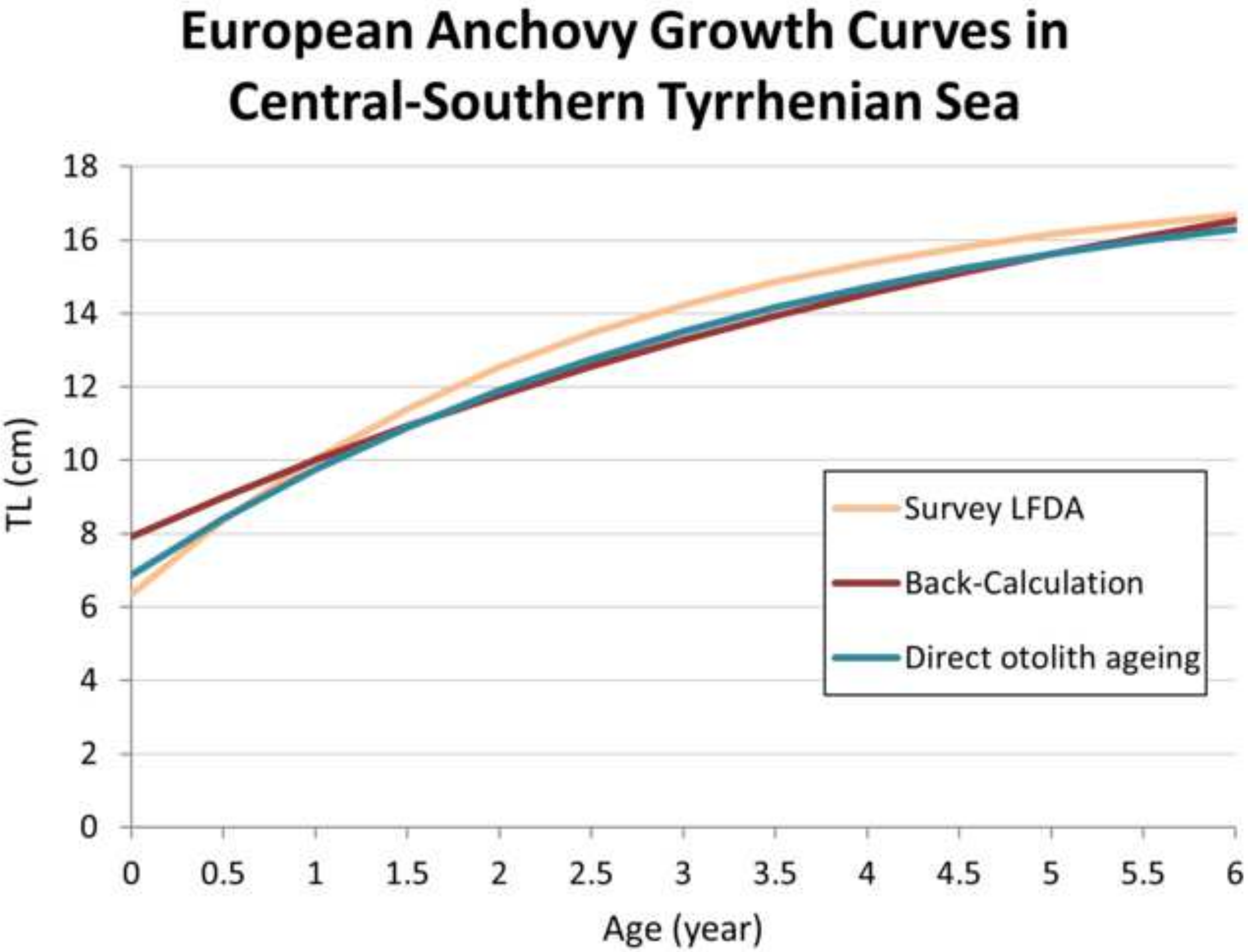

R3
R2
R1
OR
C
OL
1 mm


a
Marginal analysis
%
100
90
80
70
60
50
40
30
20
10
0
Jun
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Opaque n= 714
Transparent n= 801
b
Marginal Increment Analysis
Marginal Increment
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
82
173
103
29
66
73
159
108
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Month


a
TL (mm)
180
160
140
120
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
3
3.5
4
OL (mm)
y = 48.106x - 15.173
R² = 0.8691
N= 846


b
TL (mm)
180
160
140
120
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
OR (mm)
y = 90.859x - 8.1971
R² = 0.8358
N= 879


c
OL (mm)
4
3.5
3
2.5
2
1.5
1
0.5
0
0
0.5
1
1.5
2
2.5
OR (mm)
y = 1.8733x + 0.1658
R² = 0.9454
N= 846


European Anchovy Growth Curves in
Central-Southern Tyrrhenian Sea
TL (cm)
0
2
4
6
8
10
12
14
16
18
Age (year)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
Survey LFDA
Back-Calculation
Direct otolith ageing