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2 2 **Age Validation of the European Anchovy (*Engraulis encrasicolus* (Linnaeus, 1758))**
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4 3 **in the Central-Southern Tyrrhenian Sea (West Mediterranean Sea)**
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11 6 Pierluigi Carbonara¹, Andrea Bellodi^{2,3,✉}, Andrea Massaro⁴, Gualtiero Basilone⁵, Loredana
12 7 Casciaro¹, Michele Palmisano¹, Isabella Bitetto^{1,2}, Maria Cristina Follesa^{2,3}
13

14 8
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16
17 10 ¹COISPA - Tecnologia & Ricerca - Via dei Trulli 18/20, 70126 Bari, Italy.

18 11 ²Dipartimento di Scienze della Vita e dell'Ambiente - Università di Cagliari – Via T. Fiorelli 1, 09126
19 12 Cagliari, Italy.

20
21 13 ³CoNISMa Consorzio Nazionale Interuniversitario per le Scienze Mare, Piazzale Flaminio 9, 00196
22 14 Rome, Italy.

23 15 ⁴Aplysia, Ricerche Applicate all'Ecologia e alla Biologia Marina - Via Menichetti 35, 57128 Livorno,
24 16 Italy

25
26 17 ⁵Istituto per lo studio degli impatti Antropici e Sostenibilità in ambiente marino (IAS) - Consiglio
27 18 Nazionale delle Ricerche (CNR), SS Capo Granitola, Campobello di Mazara, TP, Italy
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✉Corresponding Author:

Andrea Bellodi; e-mail: abellodi@unica.it

Dipartimento di Scienze della Vita e dell'Ambiente - Università di Cagliari – Via T. Fiorelli 1, 09126
Cagliari.

Italy tel: +390706758042

46 **Abstract**

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

34 European Anchovy; Age and Growth; Age validation methods; Otoliths; Central-Southern Tyrrhenian
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67 **1. Introduction**

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

76 As widely known, age and growth information are crucial data inputs in analytical models for stock
77 assessment (Reeves, 2003). As a result, inaccurate or biased age data may have a great impact on
78 stock assessment outcomes (Eero et al., 2015). Additionally, poor-quality data may often cause
79 inaccurate assessments of the population's level of exploitation, a situation that could eventually
80 lead to the collapse of the stock (Beamish and McFarlane, 1995; Savenkoff et al. 2004; Liao et al.
81 2013).

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

94 As a result of the high mortality rate, of anchovies (and generally of small pelagic fish) associated
95 with the capture procedure and handling (stress, scale loss, and wounds) as well as their relative
96 short life span, the most popular direct age validation methods (e.g. mark-recapture, radiochemical
97 dating) (James et al., 1988; ICES, 2020), are frequently affected by a very low feasibility (Politikos et
98 al., 2015; Basilone et al., 2018). Due to these challenges, there are still few studies in the literature

99 about European Anchovy age validation. While Uriarte et al. (2016) used strong year-classes in
100 successive spring surveys to validate the otolith reading, Cermeo et al. (2003) and Aldanondo et al.
101 (2016) used captivity rearing of both juvenile and adult European Anchovy to validate the otolith
102 microstructure (daily increment). Finally, to validate the first annulus formation in the
103 Mediterranean region, Basilone et al. (2020) used semi-direct approaches such as the marginal
104 analysis and the marginal increment analysis.

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

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

116 2. Materials and methods

117 2.1. Sampling

118 European Anchovies were collected from commercial fishery catches and landings (DCF; EU Reg.
119 199/2008) in the period between March 2012 and December 2016 (Tab.Sup.1) in Central-Southern
120 Tyrrhenian Sea (Fig. 1). From each specimens the total length (TL) to the nearest 0.5 cm and the sex
121 were recorded. Moreover, other specimens were obtained from fishery-independent surveys: the
122 Mediterranean International Trawl Survey (MEDITS) from 2012–2016 and from the national trawl
123 survey GRUND (January 2009) in Central-Southern Tyrrhenian Sea. The sampling protocol (Spedicato
124 et al., 2019) used in the MEDITS and GRUND trawl survey (e.g. gear, station position, duration of
125 each station) (Fig. 1) is the same only differing in the sampling period: spring-summer for MEDITS
126 (2012-2016) and winter for GRUND (2009).

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

129 2.2. Direct age estimation

130 Age was estimated through the direct observation of winter rings in *saggitta* otoliths extracted from
1 1855 individuals. Analysis were carried out on the right otolith in order to standardized protocol,
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3 4 while the left one was kept as backup. The translucent rings count was conducted using a
4 5
5 6 stereomicroscope (Leica S9D™) with reflected light, otoliths were oriented with the distal face up
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7 8 and the proximal surface downward (Fig. 2) and immersed in filtered seawater as clarification
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9 10 medium.

11 The ageing criteria proposed by Carbonara and Follesa (2019) was utilized (birthdate 1st July;
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13 14 deposition of one annulus, composed by 1 translucent and 1 opaque ring by year). The age
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15 16 estimation process was conducted twice on each otolith by two different readers. The overall
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17 18 accuracy of the readings was evaluated through the coefficient of variation CV% (Chang, 1982), the
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19 20 index of average percent error IAPE (Beamish and Fournier, 1981) and the percentage of agreement
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21 22 A%. Considering that warm months are reported as the main European Anchovy spawning period in
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23 24 the study area (Follesa and Carbonara, 2019 and reference therein) the 1st of July was considered
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25 26 as the species theoretical birthdate. Finally, each fish was given an absolute age (in months) using
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27 28 the procedure suggested by Morales-Nin and Panfili (2002) (Tab.Sup 2). Additionally, the annual
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29 30 deposition pattern of growth rings was investigated through both quantitative (Marginal Analysis,
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31 32 MA) and quantitative approach (Marginal Increment Analysis, MIA). The MA required the
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33 34 annotation of each otolith edge nature in order to follow its monthly evolution. The MIA took into
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35 36 account the average monthly marginal increment. Following the equation proposed by Panfili et al
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37 38 (2002) and Mahé et al. (2021), the Relative Marginal Distance (RMD) was calculated in each otolith
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39 40 as the as the ratio between the farther mark from the edge the Absolute Marginal Distance (AMD),
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41 42 the last completed annulus and the distance separating the two last rings (D_i , $i-1$). Following
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43 44 Campana (2001), in order to avoid the influence of seasonal differences between the age classes on
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45 46 the entire sample, the MIA only considered few age groups (I and II age classes).

46 2.3. Growth modelling

47 The age-at-length data obtained from the direct otolith readings were fitted to the standard von
48 49
49 50 Bertalanffy growth curve (VBGC) using R (R Development Core Team 2017; ver. 4.0.5) with the FSA
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51 52 package (version 0.8.25, Ogle et al., 2019) according to the following equation:

$$52 TL_t = TL_{\infty}(1 - e^{-k(t-t_0)})$$

53 where TL_t is the fish total length at age t , TL_{∞} is the species' predicted asymptotic length, k is the
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55 56 growth factor, and t_0 is the theoretical fish's length before birth.

56 2.4. Back calculation

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

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

171 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
172 otolith's length at capture, and TL_0 and R_0 are the fish's length and otolith's length at hatching,
173 respectively (biological intercept) (Campana, 1990). TL_0 and R_0 used are respectively 2.97 mm and
174 4.07 μm (Aldanondo et al., 2008).

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

2.5. Length–frequency distribution analysis.

177 The length-frequency distribution analysis (LFDA) was conducted on samples collected between
178 2012 and 2016 through MEDITS survey and in 2009 for the GRUND survey. The Bhattacharya
179 method was applied to the obtained data using the software FISAT II (Gayanilo et al., 2006) in order
180 to discriminate the species normal distribution, considering each mode in the overall size-frequency
181 distribution as a cohort. Only cohorts with values of separation index > 2 were considered, while
182 values < 2 indicated a large cohort overlap were considered unacceptable (Gayanilo et al., 2006).
183 The translucent growth increments were assumed to be deposited throughout the winter, when the
184 mode components (cohort) of the GRUND LFD were collected. Because of this, the Bhattacharya
185 approach was used to determine the cohorts' average length. A Kruskal-Wallis non-parametric test
186 was used to compare, the Battacharya analysis outcomes with the mean TL back-calculated from
187 the translucent growth increments discovered during the otolith examination.

2.6. Statistical Analysis

188 A Chen test (Chen et al., 1992) was used to look for potential differences in growth between sexes.
189 Additionally, the same test was utilized to compare all the VBG curves derived for this study from
190 direct otolith ageing as well as from LFDA and back-calculation analyses. Moreover, the fitting level

194 of each VBGC (calculated from direct age estimation, back calculation and LFDA) to the observed
195 data was evaluated through the Akaike's Information Criterion (AIC; Akaike, 1974; Haddor, 2001).

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3. Results

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

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

208 According to this result only one translucent ring (mainly found at the otolith's edge between
209 December and April) followed by one opaque one (mainly found between May and November)
210 appears to be laid down yearly (Fig.3a). This pattern appeared to be further confirmed by the MIA
211 results which revealed a much higher marginal increment during the summer months (June-
212 September) saw, while the winter and early spring (November-May) marginal increments (Fig. 3b).
213 Otolith morphometric descriptors considered in the present study showed a significant linear
214 relationship with respect to the fish age (Fig. 4a, b). No statistical differences were found in between
215 sexes (ANOVA $p > 0.05$). Likewise, also OL and OR appeared to be linearly correlated (Fig. 4c). Each
216 linear regression equation is presented in Figure 4.

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

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225 The mean length (\pm sd) for each modal component of the LFD obtained from the length frequency
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226 distributions of MEDITS and GRUND surveys (Sup.Fig.3) allowed to calculate VBGF growth
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227 parameter $TL_{\infty} = 20.3579$ cm, $k = 0.186 \text{ year}^{-1}$ and $t_0 = -2.428$ year for sexes combined (AIC= 32.12).
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228 In Table 2 mean lengths derived from back-calculation and from the modes observed in the GRUND
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229 surveys (winter survey) LFD are reported, no statistical differences were found between mean
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230 lengths calculated by the two methods (Kruskal-Wallis $p > 0.05$). The AIC values indicated the VBG
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231 curve obtained through the LFD analysis as the most precise in describing the species growth in terms
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232 of fitting to the observed data, followed by the direct age estimation (AIC=3430.81) and the back
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233 calculated one.
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234 Finally, the Chen test, did not show any statistical difference ($F_{\text{obs}} < F_{\text{crit}}$) between growth curves
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235 obtained from LFDA and both otolith based analysis (back-calculation and direct age reading) (Fig.
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236 5). In Supplementary Figure 4 each VBGC is shown separately together with its length-at-age data
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237 (Sup.Fig.4).
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25 4. Discussion

258 Even though the European Anchovy is one of the species that has been studied the most in the
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239 Mediterranean Sea basin (Carbonara and Follesa, 2019), especially in regard to its life cycle features,
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240 several aspects of its age estimation still remain unknown. Indeed, based on the growth factors from
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241 the analysis of the relevant literature, is it possible to determine an average total length at the first
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242 year that ranges from 0.32 cm (Bouaziz and Bennoui, 2004) to 9.81 cm (Bacha et al. 2010)
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243 (Tab.Sub.6). It is implausible that ecological variation and genetic differences alone could account
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244 for the significant level of variability in the European Anchovy growth patterns described (Carbonara
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245 et al., 2018). In general, there are a number of reasons for the variation in age data obtained from
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246 reading hard structures (vertebral centra, spines, scales and otoliths). These factors may include the
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247 application of multiple sampling methods (commercial fishing or scientific surveys) (Coggins et al.
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248 2013), difference in the sample analyzed and otolith preparation techniques (Smith et al. 2016), and
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249 ageing criteria (ICES 2011; 2017; Hüsey et al. 2016; Carbonara and Follesa, 2019). Moreover, the
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250 geographical differences of the environmental conditions (ICES, 2017; Carbonara et al. 2018),
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251 together to different degrees of fishing pressure (Schindler et al., 2000; Carbonara et al., 2022;) may
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252 represent an additional source of difference in ageing data. Furthermore, the method employed to
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253 quantify growth could be a source of variation. In general, while the direct age estimation could be
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254 distorted by the incorrect interpretation of the annual increments (Uriarte et al. 2016; Carbonara et
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255 al., 2018; Basilone et al., 2020), the overlapping of the modes in the LFDA methods (indirect age
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257 estimation) could result in an overestimation of growth. Finally, the reader degree of experience
258 (ICES 2010; Carbonara et al., 2019) can be a very important additional source of variability. Indeed,
259 Carbonara et al. (2019), in a medium-short life-span demersal species such as the red mullet (*Mullus*
260 *barbatus* L. 1758.), pointed out the reader experience as the factor which explained the majority of
261 the variability, even if considering other factors such as the sample geographical origin, the birthday
262 date (1st of July and 1st of January), and the different identification of the first growth increment.

263 Age and growth data affected by low accuracy and/or precision levels, potentially may have a
264 significant impact on stock assessment analysis and, as a result, on the implementation of
265 management methods aimed at achieving sustainable exploitation of marine species in general and,
266 particularly, the European Anchovy. The majority of the data-rich stock assessment models in use,
267 particularly the analytical ones require information on the population's age structure. The use of
268 unrealistic and not precise age structured data may lead to unreliable scientific advices (STECF,
269 2016). The most significant impact of these inaccuracies is related to short-term projections of stock
270 condition and the associated management, leading to improper fisheries management practices
271 and the subsequent collapse of the stocks (Beamish and McFarlane, 1995; Savenkoff et al., 2004;
272 Liao et al., 2013). Therefore, age validation techniques are essential to obtaining highly accurate age
273 and growth data, preventing inaccurate assessments of the health status of the resources. In this
274 regard, approaches, such as indirect (e.g., tracking year class) and/or semi-direct (e.g., margin
275 analysis), appeared as the most applicable and were endorsed to validate small pelagic fish and
276 particularly anchovies age and growth data (ICES, 2020; Basilone et al., 2020).

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

286 Additionally, the outcomes of the back-calculation analysis of the translucent growth increment
287 were compared with the mean length of the modes (Bhattacharya's method) obtained from the
288 winter LFD (GRUND 2009). Although being limited to only one sampling occasion, the agreement of

289 the results of these two analyses provided a further indirect validation of the observed age classes
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290 (Panfili et al., 2002; Carbonara et al., 2018; ICES, 2020). The VBG curve obtained through the LFD
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291 analysis appeared as the most precise in describing the species growth in terms of fitting to the
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292 observed data (AIC), followed by the direct age estimation and the back calculated one, however
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293 this result might have been strongly influenced by the lower number of age-length data obtained
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294 from Battacharya's method in comparison to the other two techniques.

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

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

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10 **Ethics Statement**

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13 502 Ethical review and approval was not required for the animal study because the vertebrate animals
14
15 503 we worked with for this study were all dead before research began.
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26 **TABLES**

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31 509 Table 1. Anchovy’s von Bertalanffy growth parameters obtained through otolith’s direct age
32 510 estimation.
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	TL_∞(cm)	k (years⁻¹)	t₀ (years)
Combined sexes	18.34	0.29	-1.63
Females	18.81	0.29	-1.53
Males	16.51	0.37	-1.38

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43 513 Table 2. Mean lengths (±sd) of each age class of the European Anchovy calculated by back
44 514 calculation and from the modes observed in the GRUND surveys LFD
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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

51 **FIGURES CAPTIONS**

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

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

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

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

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

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

Pierluigi Carbonara¹, Andrea Bellodi^{2,3,✉}, Andrea Massaro⁴, Gualtiero Basilone⁵, Loredana
Casciaro¹, Michele Palmisano Michele¹, Isabella Bitetto^{1,2}, Maria Cristina Follesa^{2,3}

¹COISPA - Tecnologia & Ricerca - Via dei Trulli 18/20, 70126 Bari, Italy.

²Dipartimento di Scienze della Vita e dell'Ambiente - Università di Cagliari – Via T. Fiorelli 1, 09126 Cagliari, Italy.

³CoNISMa Consorzio Nazionale Interuniversitario per le Scienze Mare, Piazzale Flaminio 9, 00196 Rome, Italy.

⁴Aplysia, Ricerche Applicate all'Ecologia e alla Biologia Marina - Via Menichetti 35, 57128 Livorno, Italy

⁵Istituto per lo studio degli impatti Antropici e Sostenibilità in ambiente marino (IAS) - Consiglio Nazionale delle Ricerche (CNR), SS Capo Granitola, Campobello di Mazara, TP, Italy

✉Corresponding Author:

Andrea Bellodi; e-mail: abellodi@unica.it

Dipartimento di Scienze della Vita e dell'Ambiente - Università di Cagliari – Via T. Fiorelli 1, 09126 Cagliari.

Italy tel: +390706758042

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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, nNo 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, age 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 ~~an~~ 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 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, 2009; 2016; 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 ~~the~~ first growth ring's identification, the

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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 Anchovy 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).

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136 In this study ~~for the first time, the otolith age reading the European Anchovy results were compared,~~
137 ~~in a~~ holistic approach (*sensu* Carbonara et al., 2018), with the growth curves obtained from the
138 ~~back-calculation analysis and from length-frequency distribution analysis (LFDA - Bhattacharya~~
139 ~~methods) fishery-dependent and independent data are used to conduct length-frequency~~
140 ~~distribution analysis (LFDA - Bhattacharya methods), back-calculation, marginal analysis, and the~~
141 ~~results were combined to develop a holistic approach sensu Carbonara et al. (2018) to validate for~~
142 ~~the first time the species growth for the first time, the otolith age reading of the European Anchovy~~
143 in Central-Southern Tyrrhenian Sea (GFCM Geographical Sub-Area₂ (GSA) 10 (Central-Southern
144 Tyrrhenian Sea).

145 **2. Materials and methods**

146 **2.1. Sampling**

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

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

159 **2.2. Direct age estimation**

160 Age was estimated through the direct observation of winter rings in *saggitta* otoliths extracted from
161 1855 individuals. ~~Although both left and right otolith were collected the subsequent a~~ analysis were
162 carried out ~~preferably~~ on the right one otolith in order to standardized protocol, while the left one
163 was kept as backup. The ~~winter translucent~~ rings count was conducted using a stereomicroscope
164 (Leica S9D™) with reflected light, otoliths were oriented with the distal face up and the proximal
165 surface downward (Fig. 2) ~~and immersed in-~~ F filtered seawater ~~was employed~~ as clarification
166 medium.

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8 Given their intrinsic low thickness anchovy's otoliths do not require to be risen before age readings.
9 One opaque zone followed by a completed transparent translucent zone was recognized as an
10 annual increment (annulus). Therefore, the age was assigned counting the transparent zones
11 (winter rings). In this regard, in order to improve the ageing process solidness and reproducibility,
12 †The ageing criteria proposed by Carbonara and Follesa (2019) was utilized (birthdate 1st July;
13 deposition of one annulus, composed by 1 translucent and 1 opaque ring by year). The age
14 estimation process was conducted twice on each otolith by two different readers. The overall
15 accuracy of the readings was evaluated through the coefficient of variation CV% (Chang, 1982), the
16 index of average percent error IAPE (Beamish and Fournier, 1981) and the percentage of agreement
17 A%. Considering that warm months are reported as the main European Anchovy anchovy-spawning
18 period in the study area (Follesa and Carbonara, 2019 and reference therein) the 1st of July was
19 considered as the species theoretical birthdate. Finally, each fish was given an absolute age (in
20 months) using the procedure suggested by Morales-Nin and Panfili (2002) (Suppl. Mat.
21 Tab.SupleTab. 12). Additionally, the annual deposition pattern of growth rings was investigated
22 through both quantitative (Marginal Analysis, MA) and quantitative approach (Marginal Increment
23 Analysis, MIA). The MA required the annotation of each otolith edge nature in order to follow its
24 monthly evolutionthe nature of the otolith edge (opaque or transparent) was noted. The MIA took
25 into account the average monthly marginal increment. Following the equation proposed by Panfili
26 et al (2002) and Mahé et al. (2021), the Relative Marginal Distance (RMD) was calculated in each
27 otolith as the as the ratio between the farther mark from the edge the Absolute Marginal Distance
28 (AMD), the last completed annulus and the distance separating the two last marksrings (Di, i-1).
29 Following Campana (2001), in order to avoid the influence of seasonal differences between the age
30 classes on the entire sample, the MIA only considered few age groups (I and II age classes)-.

2.3. Growth modelling

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

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

35 where TL_t is the fish's total length at age t , TL_{∞} is the species' predicted asymptotic length, k is the
36 growth factor, and t_0 is the theoretical fish's length before birth. A Chen test (Chen et al., 1992) was
37 used to look for potential differences in growth between sexes. Additionally, the same test was
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198 ~~utilized to compare all of the VBC curves derived for this study from direct otolith ageing as well as~~
200 ~~from LFDA and back-calculation analyses (see following paragraphs).~~

201 2.4. Back Calculation

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

$$213 \quad TL_i = TL_c + (TL_c - TL_0) * \frac{(R_i - R_c)}{(R_c - R_0)}$$

214 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
215 and otolith's length at capture, and TL_0 and R_0 are the fish's length and otolith's length at hatching,
216 respectively (biological intercept) (Campana, 1990). TL_0 and R_0 used are respectively 2.97 mm and
217 4.07 μ m (Aldanondo et al., 2008).

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

220 2.5. Length–frequency distribution analysis.

221 The length-frequency distribution analysis (LFDA) was conducted on samples collected between
222 2012 and 2016 through MEDITS survey and in 2009 for the GRUND survey. The Bhattacharya
223 method was applied to the obtained data through-using the software FISAT II (Gayanilo et al., 2006)
224 in order to discriminate the species normal distribution, considering each mode in the overall size-
225 frequency distribution as a cohort. Only cohorts with values of separation index > 2 were
226 considered, while values <2 indicated a large cohort overlap were considered unacceptable
227 (Gayanilo et al., 2006). The translucent growth increments were assumed to be deposited
228 throughout the winter, when the mode components (cohort) of the GRUND LFD were collected.
229 Because of this, the Bhattacharya approach was used to determine the cohorts' average length. A
230 Kruskal-Wallis non-parametric test was used to compare, the Battacharya analysis outcomes with

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231 the mean TL back-calculated from the translucent growth increments discovered during the otolith
232 examination.

233 2.6. Statistical Analysis

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

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

246 3. Results

247 The direct age estimation process was conducted on 2451 otoliths (1001 females, 854 males and
248 596 undetermined juveniles) belonging to fish with TLs ranging from 6 up to 16.5 cm caught during
249 the entire year period. The age estimation process appeared to be characterized by a high level of
250 reproducibility and precision (IAPE =3.86; CV%=4.9; A%=89.7). The observed ages varied between
251 0.16 and 5.08 years, corresponding to 2 and 61 months respectively. The obtained length-at-age key
252 are presented in Tab.Sup.3. In table-Table 2-1 the estimated VBG parameters for combined sexes
253 and for females and males separately are reported, while in Fig.Sup.Fig-1-3 the obtained VBGC are
254 plotted against observed age. The Chen test did not show any statistical difference in growth curves
255 between males and females ($F_{obs} < F_{crit}$).

256 The marginal analysis carried out based on the entire sample showed a clear deposition pattern of
257 the annual growth rings, with an overall prevalence of transparent-translucent rings throughout the
258 year (52.9% n=801) against opaque ones (47.1% n=714) (Fig. 43a).

259 According to this result only one transparent-translucent ring (mainly found at the otolith's edge
260 between December and April) followed by one opaque one (mainly found between May and
261 November) appears to be laid down yearly (Fig. 543a). This pattern appeared to be further confirmed
262 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 otolith morphometric descriptors considered in the present study showed a significant linear relationship with respect to the fish age (Fig. 5a4a, b), while 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. 5c4c). Each linear regression equation is reported presented in Figure 54.

Applying the Campana's formula (Campana, 1990) a descriptive summary of growth rings measurement is reported in the table Tab.Sup.34 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.345). 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^o growth increment 0.5 years, 2^o growth increment 1.5 years, 3^o 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 the mean length (\pm sd) for each modal component of the LFD obtained from the length frequency distributions of MEDITS and GRUND surveys (Sup.Fig.23) 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 $t_0 = -2.428$ year for sexes combined (AIC= 32.12). In Table 42 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 through 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. 75), did not show

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

10 **4. Discussion**

11 Even though the European Anchovy is one of the species that has been studied the most in the
12 Mediterranean ~~setting~~ Sea basin (Carbonara and Follesa, 2019), especially in regard to its life cycle
13 features, several aspects of its age estimation still remain ~~debatable~~ unknown. Indeed, based on the
14 growth factors from the analysis of the relevant literature, is it possible to determine an average
15 total length at the first year that ranges from 0.32 cm (Bouaziz and Bennoui, 2004) to 9.81 cm (Bacha
16 et al. 2010) (~~Supp. Mat. TableTab.sSub. 564~~). It is implausible that ecological variation and genetic
17 differences alone could account for the significant level of variability in the European ~~anchovy~~
18 Anchovy growth patterns described (Carbonara et al., 2018). In general, there are a number of
19 reasons for the variation in age data obtained from reading hard structures (vertebral centra, spines,
20 scales and otoliths). These factors may include the application of multiple sampling methods
21 (commercial fishing or scientific surveys) (Coggin et al. 2013), difference in the sample analyzed
22 and otolith preparation techniques (Smith et al. 2016), and ageing criteria (ICES 2011; 2017; Hüsey
23 et al. 2016; Carbonara and Follesa, 2019). Moreover, the geographical differences of ~~the habitats~~
24 colonized by different populations the environmental conditions (ICES, 2017; Carbonara et al. 2018),
25 together to different degrees of fishing pressure (Schindler et al., 2000; Carbonara et al., 2022;) may
26 represent an additional source of difference in ageing data. Furthermore, the method employed to
27 quantify growth could be a source of variation. In general, while the direct age estimation could be
28 distorted by the incorrect interpretation of the annual increments (Uriarte et al. 2016; Carbonara et
29 al., 2018; Basilone et al., 2020), the overlapping of the modes in the LFDA methods (indirect age
30 estimation) could result in an overestimation of growth. Finally, ~~the reader~~ degree of experience
31 (ICES 2010; Carbonara et al., 2019) can be a very important additional source of variability. Indeed,
32 Carbonara et al. (2019), in a medium-short life-span demersal species such as the red mullet (*Mullus-*
33 *barbatus* L. 1758.), pointed out the reader experience as the factor which explained the majority of
34 the variability, even if considering other factors such as the sample geographical origin, the birthday
35 date (1st of July and 1st of January), and the different identification of the first growth
36 increment. ~~found that the reader experience was the parameter that explained most of the~~
37 ~~variability, considering geographic origin, birthday date (1st of July and 1st of January), the different~~
38 ~~identification of the first growth increment.~~

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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 Hüseyin et al. (2016), †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

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~~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-validated~~ 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 through 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.

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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 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

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8 annulus is deposited every year. Given that the back-calculation is based on the winter growth
9 increment (~~transparent~~translucent), the comparison of the back-calculated mean lengths from the
10 winter-translucent rings and the LFD modes from a winter survey (GRUND) allowed to validate the
11 age readings obtained in this study, as previously reported by Carbonara et al. (2018) for *M. ~~ullus~~*
12 *barbatus*. ~~1758~~ Furthermore, the ICES working group WKVALPEL endorsed and recommended
13 the contemporary use of several highly feasible age corroboration/validation methods (e.g., length
14 and modal frequency analysis, marginal increment analysis, edge nature and microstructure
15 readings), stating that if these methods produce consistent results, the specific ageing criteria could
16 be supported (ICES, 2020). In this regard, the findings from this study represent a valuable
17 contribution to understand the European Anchovy growth pattern in the Central-Southern
18 Tyrrhenian Sea, also allowing to obtain solid and verified age data ~~indispensable-crucial~~ for an
19 accurate stock status diagnosis, thus favoring the implementation of appropriate management
20 measures with the aim of ~~assuring the sustainability preventing the collapse~~ of *E. encrasicolus*
21 populations ~~in the region~~.

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8 **Credit authorship contribution statement**

9 ~~PC: Conceptualization, Methodology, Writing — original draft, Software, Investigation, Review,~~
10 ~~Supervision, project administrator. AB: Methodology, Writing — original draft, Writing — review &~~
11 ~~editing, Software, Investigation; AM: Formal analysis, Investigation, Review. GB: Formal analysis,~~
12 ~~Investigation, Review. LC: Formal analysis, Investigation, Software; MP: Formal analysis,~~
13 ~~Investigation Software. IB: Formal analysis, Investigation, Software. MCF: Conceptualization,~~
14 ~~Supervision, Review.~~

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21 **Fundings**

22
23 The samples from commercial fishery (landing and discard) are collected under the Data Collection
24 Framework supported by the Italian Ministry of Agriculture, Food and Forestry Policy (MiPAAF) and
25 by the European Commission (EU Reg. 199/2008).

26
27
28 **Ethics Statement**

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

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36 **TABLES**

37 ~~Table 1. The employed scheme for the assignation of anchovy absolute age. N represent the~~
38 ~~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
$N + \lfloor 12 - (\text{month of birth} - \text{capture month}) / 12 \rfloor$						$N + \lfloor (\text{month of birth} - \text{capture month}) / 12 \rfloor$					

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47 Table 21. Anchovy's von Bertalanffy growth parameters obtained through otolith's direct age
48 estimation.

	<u>IL_∞(cm)</u>	<u>k (years⁻¹)</u>	<u>t₀ (years)</u>
Combined sexes	18.33834	0.28829	-1.63±
Females	18.80581	0.28529	-1.53±
Males	16.510	0.37±	-1.37638

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.

N° Growth increments	N° specimens	Growth increments				
		1°	2°	3°	4°	5°
4	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. 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	89.96897	7.2239	779	89.58	6.18	195	0.490313
1.5	109.144	7.744	520	109.874	7.4	1335	0.060961
2.5	125.563	8.53554	275	124.95	5.67	425	0.080056
3.5	139.760	8.31932	81	139.88	10.37	7	0.79023

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.

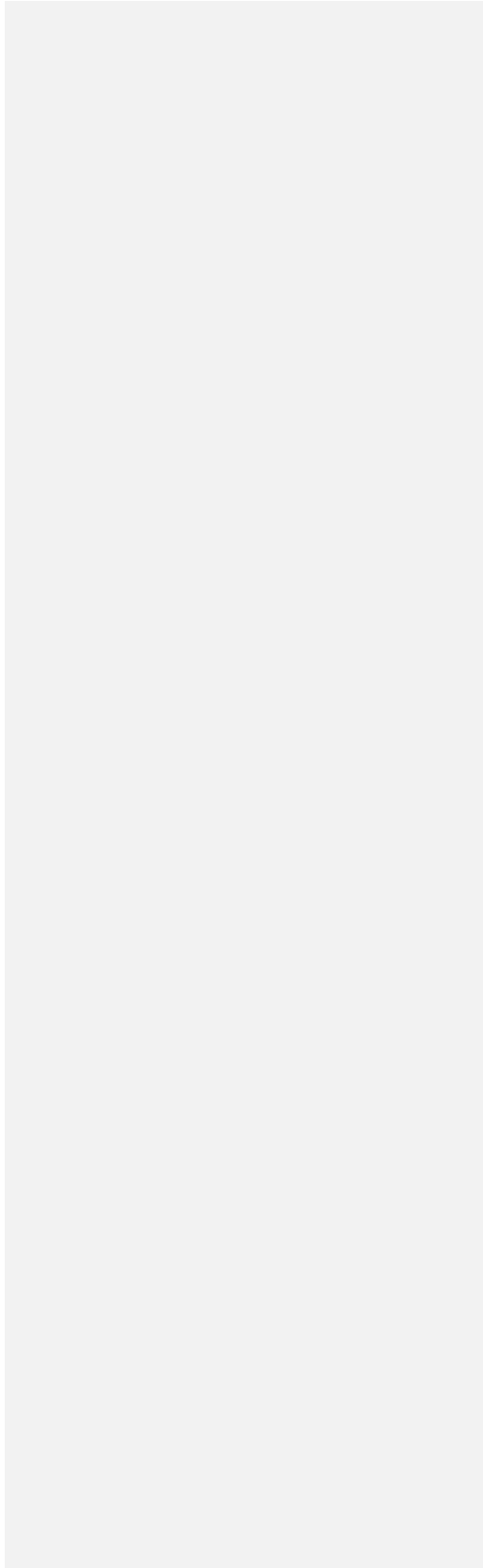
Figure 4.3. Annual-Monthly deposition pattern-percentage of transparent-translucent and opaque growth rings (a) and mean monthly marginal increment (MIA) (b) in which numbers indicate sample size, the bars represent the standard error of the mean and the dotted line is the polynomial regression of the means for the European Anchovy in Central-Southern Tyrrhenian Sea.

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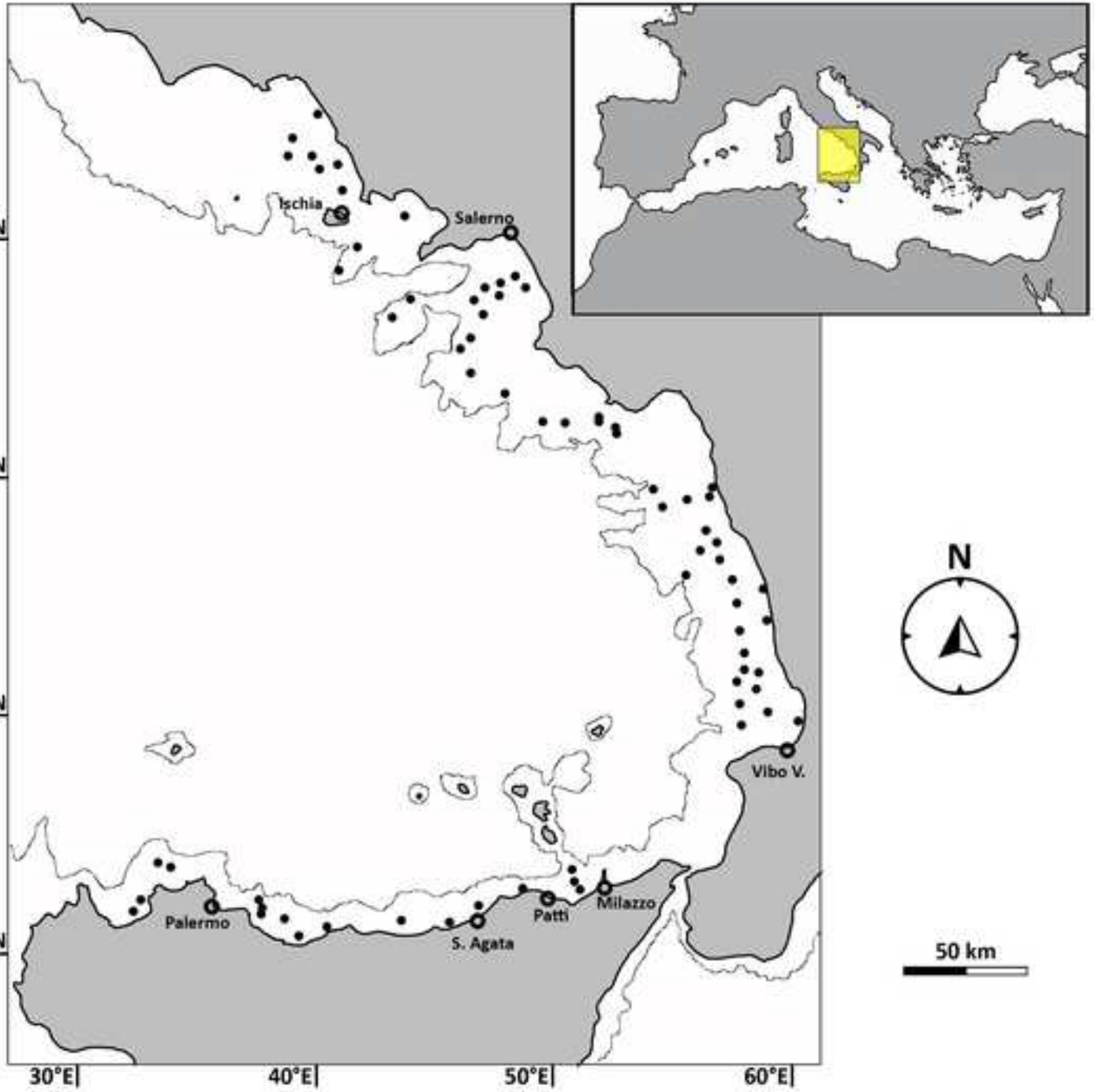
Figure 5-4. Linear regression between: fish total length and otolith length (a); fish total length and otolith radius (b); otolith length and otolith radius (c). The equation, R² and number of specimens are also reported

~~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.~~

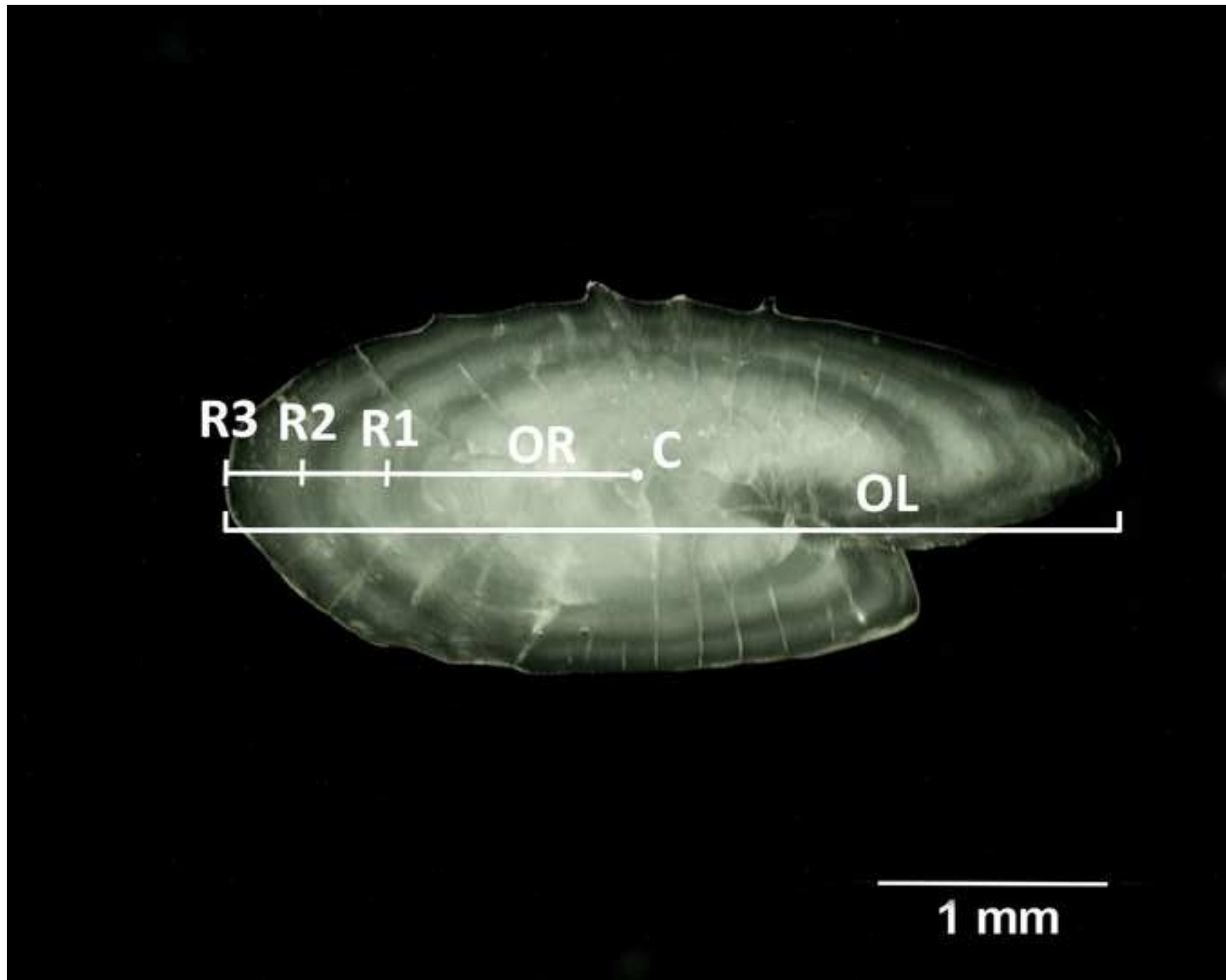
Figure 75. European anchovy von Bertalanffy growth curves obtained from direct otolith readings (blue line); back-calculation (red line) and Length frequency distribution (orange line)



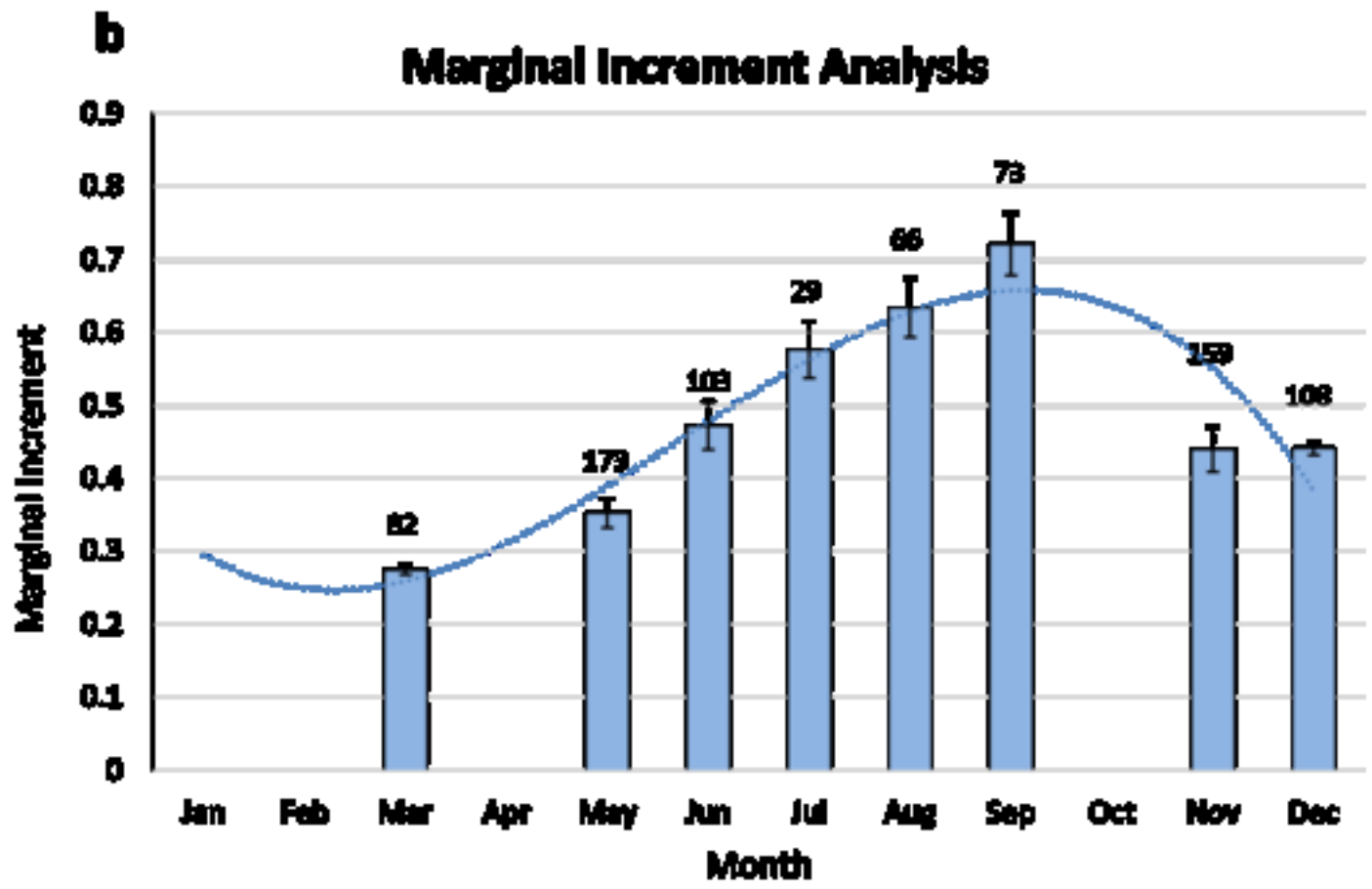
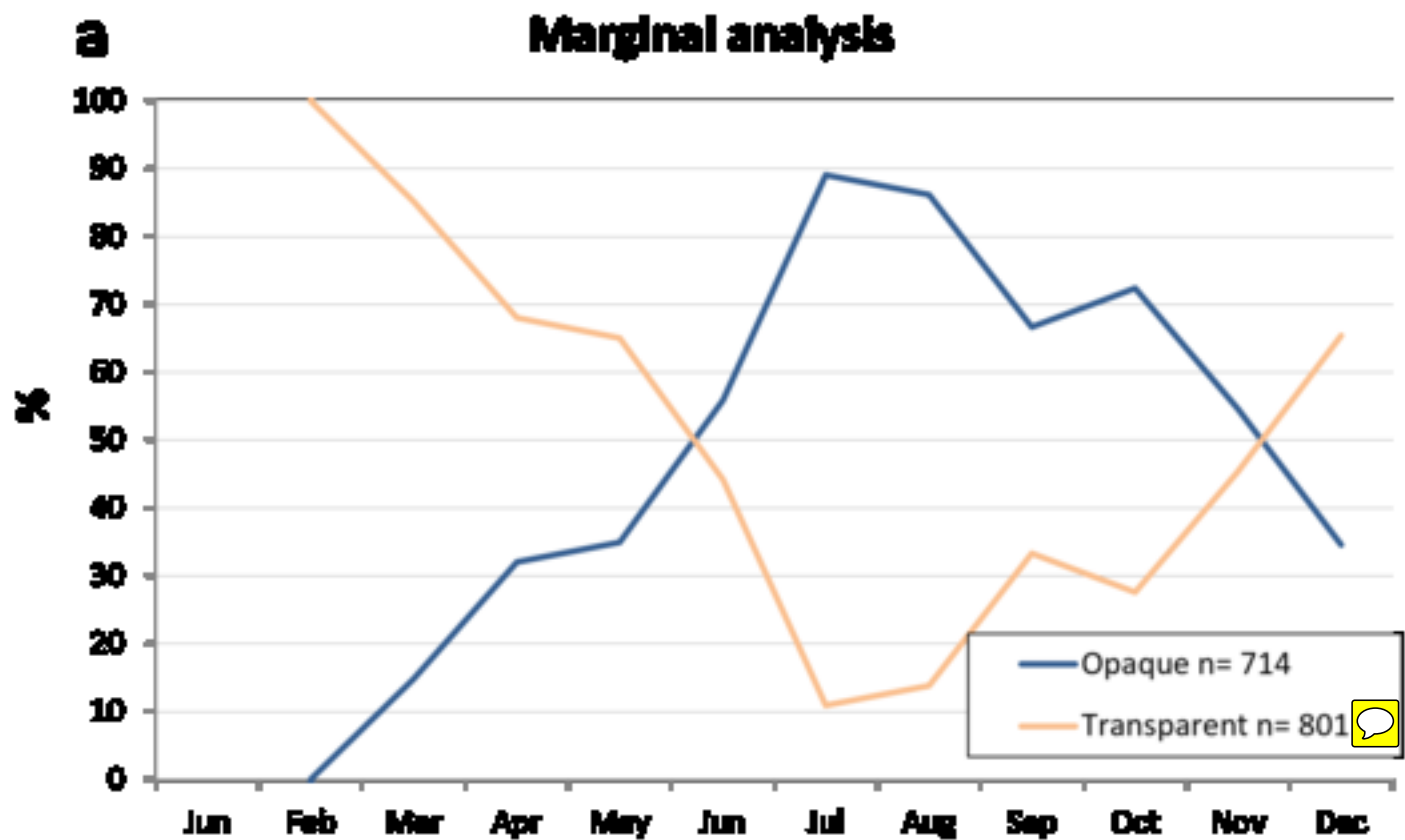
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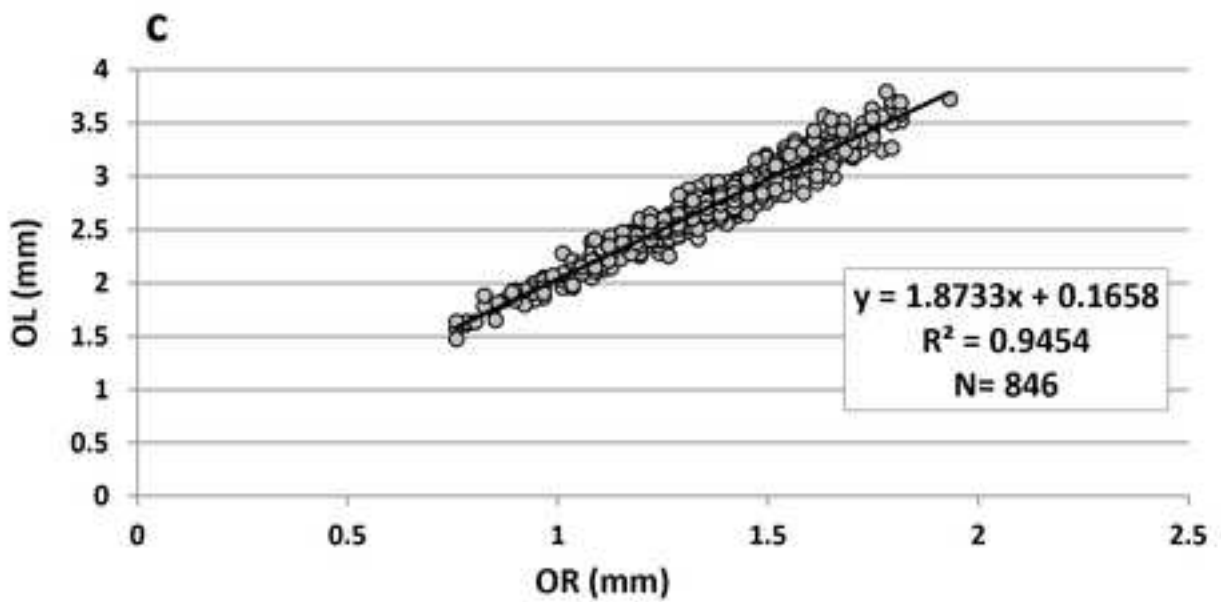
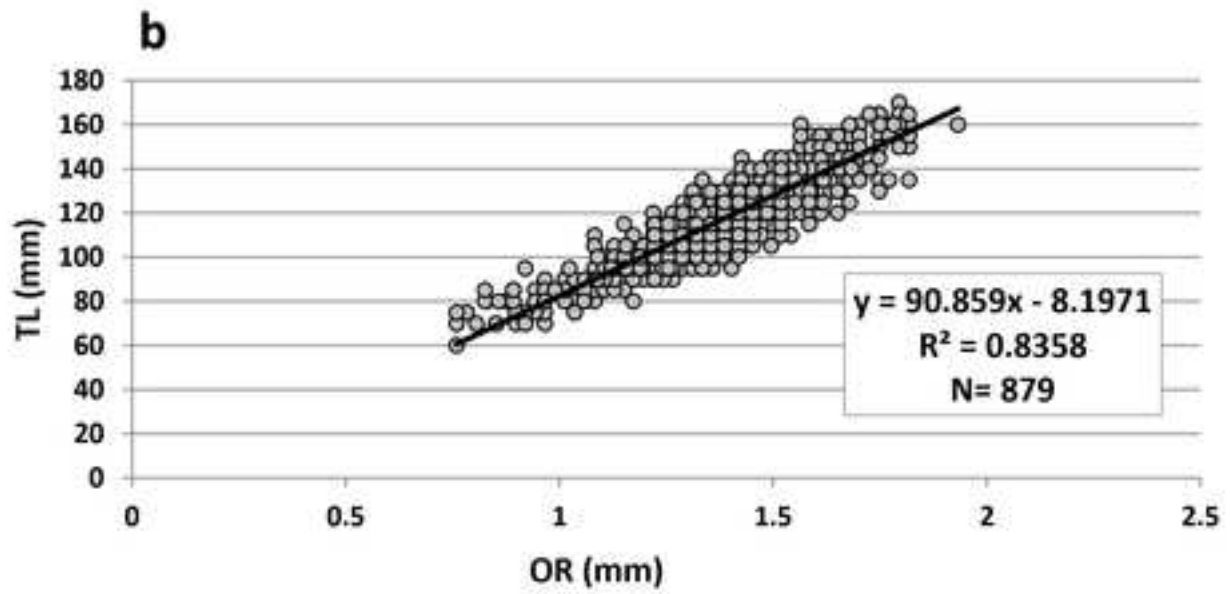
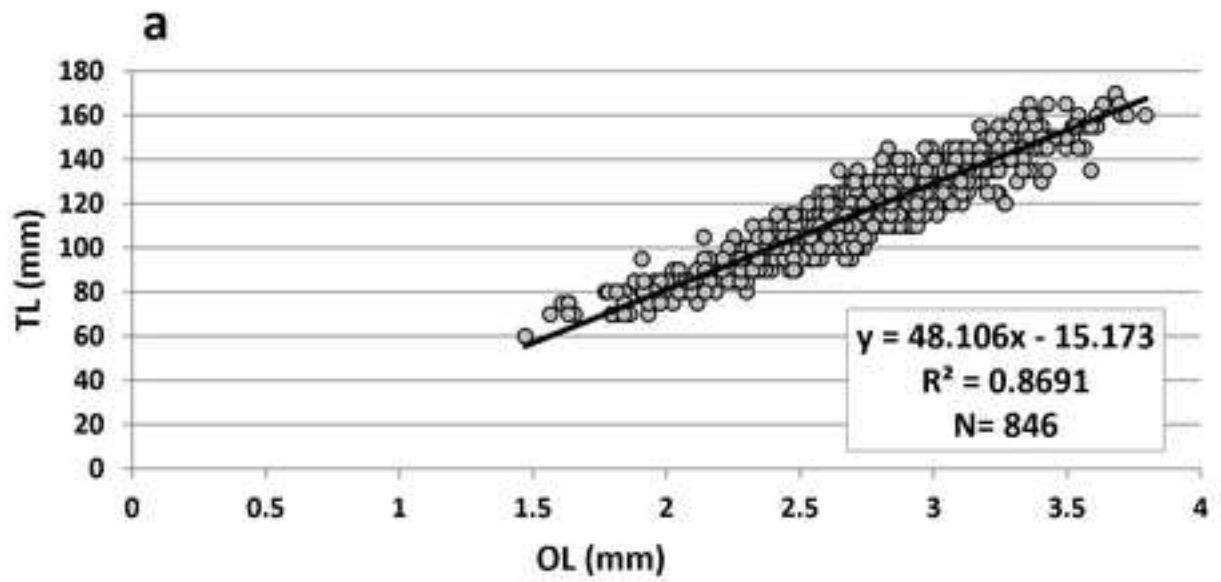


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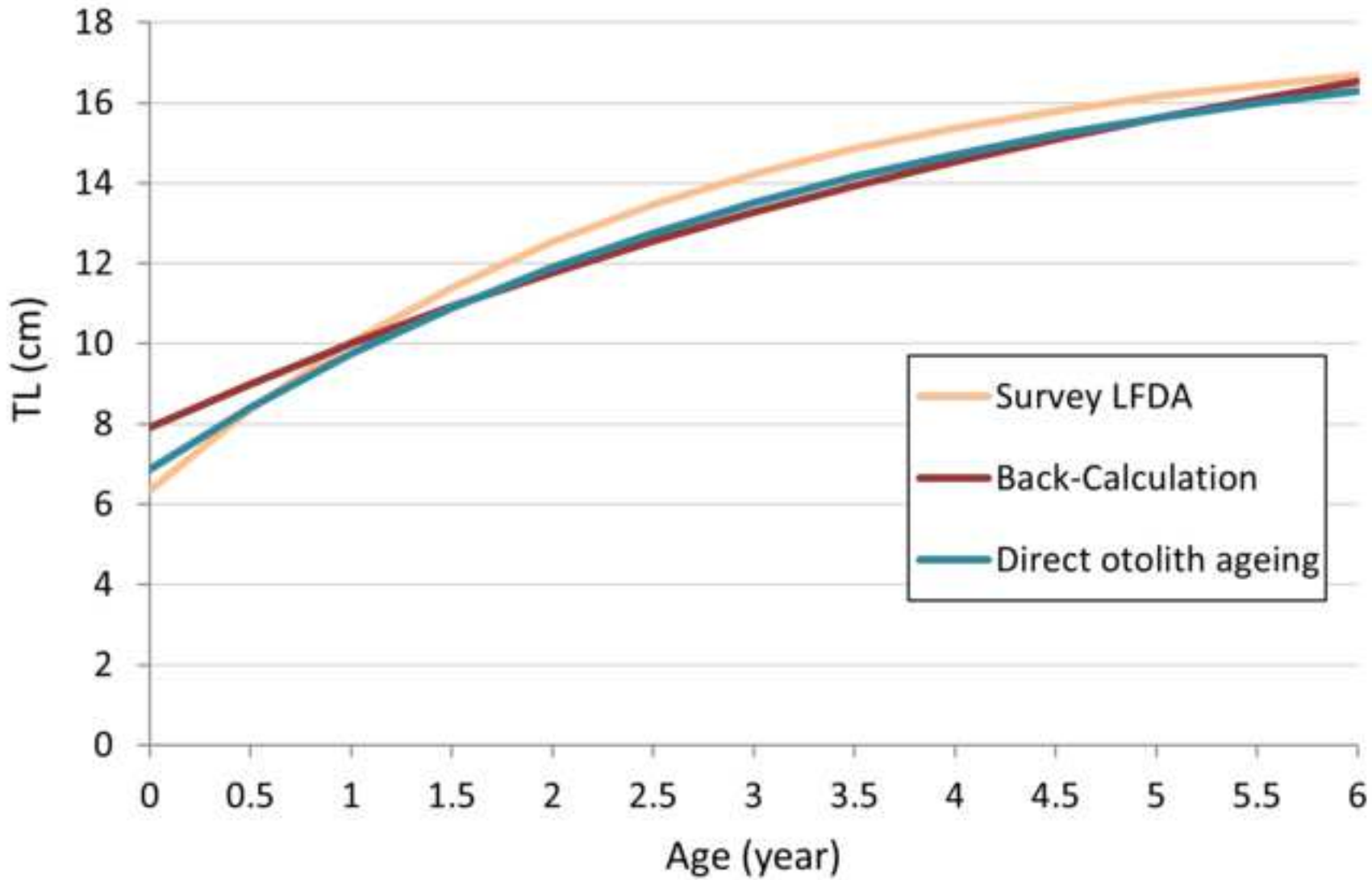
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European Anchovy Growth Curves in Central-Southern Tyrrhenian Sea



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Supplementary Material

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