WORKSHOP 3 ON AGE ESTIMATION OF EUROPEAN ANCHOVY (*ENGRAULIS ENCRASICOLUS*) (WKARA3; outputs from 2021 meeting)

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Contents

i Executive summary

The Working Group on Biological Parameters (WGBIOP) approved the realization of a new workshop on age reading of European anchovy to discuss the results of the exchange conducted in 2018 and the development of validation studies in this species. Particularly, the workshop previously scheduled for 2020 has been proposed for 2021 (ICES, 2020). The third Workshop on Age Reading of European Anchovy (WKARA3), chaired by Gualtiero Basilone (Italy) and held from 23 to 25 November 2021, was an online meeting due to the persistence of the pandemic situation. Six countries took part in this workshop (Spain, Italy, Croatia, Greece, Portugal and France), with a total of 26 participants from 12 laboratories [(IEO (Santander, Palma de Mallorca, Cádiz and Malaga), AZTI, CNR-IAS, CNR-IRBIM, CIBM, COISPA, UNIPARTENOPE, UNICA, IOF, HCMR, IPMA, and Ifremer]. Fourteen of the participants in this workshop are readers for the assessment of anchovy. In total 16 areas/stocks were considered for evaluation, particularly 4 from the Atlantic Ocean and 12 from the Mediterranean Sea.

The workshop was focused on the review of information on anchovy age estimations, otolith exchanges, workshops and validation studies done the information on age determination; on analysis and discussion of the results of the previous exchange (2018); on the review of the validation methods existing for the target species (*Engraulis encrasicolus*); and update the age reading protocol.

This workshop was preceded by an otolith exchange (2018), as well as a Workshop on Age estimation of Small Pelagic (WKGESP 2019) in Italy supported by the Italian national fishery data collection plan (Basilone *et al.*, 2019). The main conclusions achieved by both meetings have been presented during the WKARA3.

The otolith exchange programme was focused on two areas (Bay of Biscay and Strait of Sicily) and produced comparable results in both study places. Particularly, the overall agreement between readers and areas is lower than in the previous exchange held in 2014. Moreover, in both areas results from the stock readers group are better than the other groups of readers (including advanced and expert groups) Finally, the growth pattern is commonly identified in both Bay of Biscay and the Strait of Sicily.

The main results of the WKGESP were a new otoliths reference collection (on a national basis) and standardizing age assignments to improve the coherence of age estimation among the participant laboratories. Finally, the WKAGESP group adopted 1 January as the conventional birthdate for anchovy, since the main discrepancy between labs was represented by the adoption of 1 January or 1 July as the species birthdate.

During the second day of the workshop, a new otolith exchange program was set up to integrate and complete the old database. The proposal suggests sharing about 200 otoliths, stratified by area (i.e. the Atlantic Ocean and the Mediterranean Sea) as well as for embedding medium (i.e. water or resin).

As recommended by WKARA2 (ICES, 2017) as well as the last exchange program, age validation studies should be conducted for all anchovy stocks, especially those that are assessed analytically, since the validation of the annual deposition of seasonal zones and the checks in the otolith allow to improve the aging precision. For such reasons, some validation studies have been conducted on anchovy stocks in both Atlantic and Mediterranean waters. Particularly, an age validation study was conducted in the Gulf of Cádiz with the objectives to validate the periodicity of the otolith increment formation through the otolith marginal increment (MIA) and the otolith edge analysis (EA), and to evaluate the temporal link between the reproductive cycle and the somatic conditions with the otolith edge formation and the patterns of monthly increments. For

the Strait of Sicily, the results of published research were presented (Basilone *et al*., 2020). A statistical modelling approach has been here adopted to assess the coherence of two well-known methods, namely Edge Analysis and Marginal Increment Analysis, to validate the first annulus formation in European anchovy. Both methodologies in two different yearly cycles converged toward the same result, thus confirming the annulus identification for the first-year class. In addition, the completion dates of the checks and the first annulus were computed to gain a better insight into otolith growth dynamic. The obtained results corroborated temporally coherent patterns providing a better insight into the otolith growth dynamic as well as a more robust validation of the first annulus formation in the European anchovy. Finally, a preliminary validation on anchovy stock inhabiting the South Tyrrhenian Sea was presented during the WKARA3, according to the experience the researcher already obtained by validation on *Mullus barbatus* (Carbonara *et al*., 2018). The work done assigning the age according to the criteria by Carbonara and Follesa (2019), then the growth parameter of the von Bertalanffy curve (VBGC) was estimated for males and females, later VBGC parameters for each sex were compared to identify a possible sexual dimorphism.

On the last day, the workshop proceeded with a detailed and joint discussion on the growth patterns shown by otoliths from the different areas, proposed by attendees to the meeting. This common examination and discussion of otoliths by areas allowed finding out the major reasons for discrepancies in age determination among readers and a better understanding of the pattern of otolith growth increments by areas to improve the guidelines for their interpretation (ToR b). Such an exercise served to enhance the collections of agreed-aged otoliths (ToR d), as well as to highlight the importance of a new exchange program.

The discussions on examples among the set of otoliths which generated the discrepancies in the age determination led to conclude that mainly source of disagreement is linked to a divergent otolith interpretation. Particularly, different interpretations of the marks, growth bands and edges through their conformity with the expected growth pattern of the anchovies, seasonal formation of the otolith by age and most common checks.

The importance of technical advice on storage was also highlighted. In fact, before embedding them in resin or before placing them in cuvettes, readers should be ensured that the otolith is completely dry. Moreover, this discussion strongly encouraged each lab area to conduct validation studies by different techniques as reported in Basilone *et al*. (2020).

In conclusion of the former statements, a new exchange exercise has been planned for after 2022, also suggesting two new chairs who will join the coordination with Gualtiero Basilone. Similarly, to improve the coherence of the age estimates among Atlantic and Mediterranean areas and corroborate the kindly suggested studies of validation, the group recommend a new WKARA in 2023, which will be chaired by new coordinators together with the former one (G. Basilone).

ii Expert group information

Expert group name	Workshop 3 on age estimation of European anchovy (*Engraulis encrasicolus*) (WKARA3)
Expert group cycle	Annual
Year cycle started	2020
Reporting year in cycle	1/1
Chair	Gualtiero Basilone, Italy
Meeting venue(s) and dates	23–25 November 2021, online meeting, (26 participants)

1 Introduction

WKARA3 – Workshop 3 on age estimation of European anchovy (*Engraulis encrasicolus*)

1.1 Terms of reference

A **Workshop on Age estimation of European anchovy** (***Engraulis encrasicolus)*** (WKARA3) chaired by Gualtiero Basilone, Italy, has been held online, 23–25 November 2021, following the subsequent Terms of Reference (ToRs):

a) Review information on anchovy age determination, otolith exchanges, workshops and validation work done so far;
b) Analyse growth increment patterns in anchovy otoliths and to improve (if necessary) the guidelines for their interpretation;
c) Analyse the results of the exchanges conducted in 2018 and the potential source of discrepancies, in light of ToRs a) and b);
d) Increase existing reference collections of agreed aged otoliths by stocks and areas.
e) Address the generic ToRs adopted for workshops on age calibration (see 'WGBIOP Guidelines for Workshops on Age Calibration').

1.2 Participants

A list of all participants to this workshop is shown in Table 1.1. and Annex 1.

A total of 26 readers participated in the present Workshop, 9 from Spain [7 from IEO (laboratories of Santander, Palma de Mallorca, Cádiz and Malaga) and 2 from AZTI (Basque Country)], 11 from Italy (3 from CNR-IAS, 2 from CNR-IRBIM, 3 from COISPA, 1 from UNIPARTENOPE, 1 from UNICA, 1 from CIBM), 2 from Greece (HCMR), 1 from Croatia (IOF), 2 from Portugal (IPMA), 1 from France (Ifremer). All the participants were connected by the Microsoft Teams platform.

All the participants are from Europe, most people involved in age reading of anchovy stocks in Mediterranean Sea, while 9 readers are readers of anchovy from Atlantic Ocean.

Table 1.1. shows with a summary of the participants' experience in age estimation and the area where they work on. The level of experience in anchovy reading was considered by number of otoliths (first) and by years of experience in this species (second).

Fifteen of the participants to this workshop are readers for the assessment of anchovy, while only eight readers participated in the otolith exchange held in 2014. Six of them have also participated in the 2009 Exchange and five in the WKARA 2009 (ICES, 2010).

In summary, thirteen participants are experts/coordinators in otolith analyses, while five are new readers (i.e. trainees).

Table 1.1. Summary of WKARA3 participants and reading experience of anchovy otoliths.

Country	Participants in Workshop 2021	Age reading expertise:	Reads for assessment	Does your institute sample Anchovy otoliths? Which stock? Which areas?
Spain-IEO	Carmen Hernández	Coordinator	No	Bay of Biscay anchovy: VIIIc, VIIIb
	Clara Dueñas	Expert	Yes	
	Pedro Torres	Expert	Yes	Anchovy IXa: IXa North
	CRISTINA BULTO ESTEBANEZ			
	Jorge Tornero	Expert		
	Ana Ventero	Expert	Yes	Mediterranean Geographical Subareas GSA01 (Northen Alboran Sea) GSA06 (Northen Spain)
	Magdalena Iglesias	Coordinator	No	
Spain-AZTI	Iñaki Rico	Expert	Yes	Bay of Biscay anchovy: VIIIc,
	Beatriz Beldarrain	Trainee	No	VIIIab
Italy- IRBIM-CNR	Ilaria Costantini	Expert	YES	GSAs 17, 18 (Adriatic Sea -Italian Side)
	Fortunata Donato	Expert	YES	
Italy-IAS-CNR	Gualtiero Basilone	Coordinator	Yes	GSA 9 (north Tyrrhenian and Ligurian Sea), GSA10 (south Tyrrhenian Sea), GSA16 (Strait of Sicily)
	Maurizio Pulizzi	Expert	Yes	
	Rosalia Ferreri	Observer	no	
Italy- CIBM	Andrea Massaro	Intermediate	No	GSA 9 (north Tyrrhenian and Ligurian Sea)
Italy- COISPA	Loredana Casciaro	Intermediate	No	GSA10-19 (Southern TyrrHenian & Western Ionian Sea)
	Michele Palmisano	Trainee	No	
	Pierluigi Carbonara	Expert	Yes	
France-IFREMER	Geoffrey Bled Defruit	Intermediate	Yes	GSA07 (Gulf of Lion)
Greece - HCMR	Konstantinos Markakis	Trainee	YES	GSA 22 (North Aegean Sea) GSA 20 (Eestern Ionian Sea)
	Amalia Mina	Trainee	NO	
Croatia - IOF	Denis Gašparević	Expert	Yes	GSA 17 (Eastern Adriatic Sea)
Portugal (IPMA)	Raquel Milhazes	Expert	YES	Western and Southern Iberia (9a.CN, 9a.CS, 9a.S)
	Dina Silva	Trainee		
Italy-UNIPARENOPE	Ciorciaro Domenico	Trainee	No	GSA10 (Tyrrhenian Sea)
Italy -UNICA	Andrea Bellodi	Intermediate	yes	GSA 11 (Sardinian Sea)

1.3 Work conducted during the workshop

The work conducted during the meeting is detailed in the updated agenda, attached in the Annex 2.

The workshop focused on the ToRs a, b, c, and d.

During the first day of the meeting, the participants showed the available information on anchovy age validations, otolith growth patterns and age estimation procedures applied in each laboratory and study area (ToRs a and b).

In 2019, a national workshop on age estimation of European anchovy (*Engraulis encrasicolus*) (WKAGESP, Basilone *et al.*, 2019) involved the seven Italian FAO-GFCM Geographical Sub-Areas (GSA; GFCM, 2009). The main evidence from this workshop have been presented during the first day of the WKARA3; no differences in otoliths preparation techniques were found among laboratories, but the main discrepancy was the adoption of the species birthdate. The attendees of this Italian workshop give the opportunity to participate at an extensive exercise of otolith readings and jointly discussions on the different otolith interpretations. Finally, a common protocol adopted for all Italian areas was showed to all the WKARA3 attendees. Similarly, at local level, an age calibration analyses for anchovy in Division 9.a was held in 2018 among readers of the IEO and the IPMA and results were showed during the WKARA3. The readers appeared in agreement among them and with the proposed WKARA2 suggestions, but some discrepancy among the lab of Division 9.a was detectable in the assignment of age 2, due to the misinterpretation of the spawning check.

Furthermore, the meeting goes ahead with presentations of age validation studies proposed in different areas, since the traditional, direct validation methods (e.g. mark-recapture, rearing, radiochemical dating) are difficult to be applied on short living species, as anchovy. Particularly, preliminary results on validation study conducted on European anchovy inhibiting in the Gulf of Cádiz was presented, showing to use the marginal increment analysis (MIA) and the nature of the edge analysis (EA) as age corroboration studies. Similarly, in the Strait of Sicily, a statistical modelling approach assessed the coherence of MIA and EA methods, to validate the first annulus formation in European anchovy. Both methodologies converged toward the same result, thus confirming the annulus identification for the first year class. In the central-southern Tyrrhenian Sea, the comparison between back-calculated sizes and average sizes of the mode of a winter survey was used; moreover, the results have been corroborated by the comparison of the growth curves from back-calculation, direct reading, LFDA and otolith reading. These observation and results about validations were discussed by attendees during the first and the second day of the WKARA3.

During the second day, the main results from the otolith exchange analyses for anchovy coordinated by B. Villamor, A. Uriarte, and G. Basilone were presented. This exchange programme was held in 2018 with the aims to evaluate if the age reading protocol of WKARA2 was adopted by all readers, and to evaluate if the accuracy and precision in otolith age reading among readers of fishery and survey samples improved. The readers come from European labs, in Atlantic and Mediterranean areas, representing an especially important starting point for further exchange exercises and highlighting difficult linked to consider the birthdate in July. At the conclusion of this presentation, the WKARA3 attendees suggested to recommend new otolith exchange analysis, applying a common protocol but also to explore the aging variability linked to the birthdate on 1 January or 1 July, which resulted main in disagreement in the identifications of the first ring and number of false rings, as emerged during the presentation of preliminary analysis as well as by the joined discussion.

The last day of the workshop proceeded afterwards with a more detailed and joint discussion on the growth patterns shown by otoliths from the different areas. While doing this, each participant has voluntarily shared the image of some otolith, which required particularly attention or determined big doubts during the analysis. Readers had been asked in advance to bring with them images of otoliths. This common examination and discussion of otoliths by areas allowed finding out the major the reasons for discrepancies in age determination among readers (ToR c). At the same time allowed a better understanding the pattern of otolith growth increments by areas (ToR b) to improve the guidelines for their interpretation. In summary, a direct common discussion on the interpretations was joined. Such an exercise served to propose a set of otoliths for the collections of agreed aged otoliths (ToR d).

The last day of the meeting was devoted to a comparative debate, by sharing pictures of otoliths, which generated special doubts on reader. Then, the general discussion proceeded on structure of the report, proposal of next meeting and new chairs, and further recommendations for future research and work to do

The group was satisfied with the discussions conducted on their respective otoliths by regions and there was a general feeling that progress had been achieved towards a common understanding of the growth pattern of anchovy otoliths across all regions as reflected in the report.

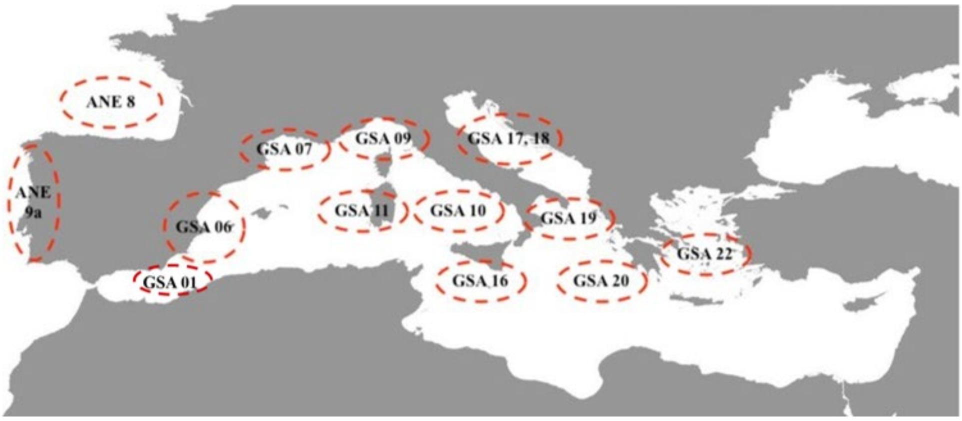


Figure 1.1. Map of the different areas from which participated to the current workshop.

2 ToR a: Review information on anchovy age estimations, otolith exchanges, workshops, and validation work

2.1 Background information on age determination, otolith exchanges, and workshops on age reading of European anchovy conducted from the last meeting in 2016

European anchovy (*Engraulis encrasicolus*) is a small pelagic species of high commercial importance in European waters, in both the Atlantic and the Mediterranean Sea, and is assessed in most of the stocks that are distributed in these areas. The assessments are conducted within the framework of ICES for stocks in the Atlantic area and in the GFCM for stocks in the Mediterranean Sea.

Age reading on anchovy are important input data for the assessment and conducted by number of laboratories using international aging criteria. There is an international age reading protocol and a consensual age reading criteria for Atlantic and Mediterranean areas from the Workshop on Anchovy age reading in 2009 (ICES, 2010) and upgraded in 2016 (ICES, 2017).

In the past, since the 1990s, exchanges, workshops and cross-checking of the procedures for age determination of European anchovy otoliths in Atlantic areas have been made in the Bay of Biscay (Astudillo *et al*., 1990; Villamor and Uriarte, 1996; Uriarte *et al*., 2002, 2006 and 2007) and in the Gulf of Cádiz (Garcia Santamaría, 1998; Uriarte *et al.*, 2002). However, no proper exchanges or workshops on reading procedures of European anchovy otoliths had been held in Mediterranean areas until 2009 (ICES, 2010).

Since 2009, there have been three workshops and two exchanges on anchovy otoliths taking into accounts the Atlantic and the Mediterranean areas together (Table 2.1).

Comparing the results of last Exchange 2018 with that of 2014 for all readers, there has been a small decrease of the overall level of agreement and a decrease of CV in those areas that were analysed in the two exchanges. For the Bay of Biscay stock readers there is no variation from one exchange to another with a high PA and low CV in the two exchanges. However, for the anchovy of the Strait of Sicily there is no improvement for the expert's readers. Restricting the comparison to those who participated in the 2014 exchange (and in WKARA) no improvement is seen either (similar PA for the case of the Bay of Biscay and some decline of agreement in GSA16), with a bit greater variability—CVs—in the two areas. This leads to conclude that no improvement can be noticed in general in agreement and precision, neither for all readers nor for the WKARA readers.

The last exchange 2018 recommended that "in view of the current results and that there are new readers a new workshop might be considered for 2021".

WGBIOP 2021 (ICES, 2021) recommends the realization of a Workshop on Age Reading of Anchovy for all European countries in 2021 (WKARA3). This workshop can be considered as a follow-up to WKSTCON held in Palma de Mallorca, Spain, in April 2018; WKBECOSS held in Santander, Spain, in September 2019; and the June 2020 RCG meetings.

This report represents the results of this workshop.

Table 2.1. Summary of the ICES anchovy otolith workshops and exchanges conducted on European anchovy.

	Date	Venue	Charis	Reference
WKARA 2009	9–13 November 2009	Sicily, Italy	Gualtiero Basilone (Italy), Begoña Villamor (Spain), and Mario la Mesa (Italy)	ICES, 2010
Exchange 2014		Online meeting	Andrés Uriarte (Spain), Begoña Villamor (Spain)	Villamor and Uriarte, 2015
WKARA2 2016	28 November–2 December 2016	Pasaia, Spain	Andrés Uriarte (Spain), Begoña Villamor (Spain), and Gualtiero Basilone (Italy)	ICES, 2017
Exchange 2018		Online meeting	Andrés Uriarte (Spain), Begoña Villamor (Spain), and Gualtiero Basilone (Italy)	Villamor *et al.*, 2018
WKARA3 2021	23–25 November 2021	Online meeting	Gualtiero Basilone (Italy)	ICES, 2022 (Present report)

2.1.1 Age calibration exercise in Division 9.a

In November 2018, a new acoustic survey (IBERAS) coordinated by IEO and IPMA was conducted to estimate the strength of sardine and anchovy recruitment in the Atlantic waters of the Iberian Peninsula (ICES Division 9.a) and to map its distribution area. In January-February 2019, an age calibration exercise was conducted among the anchovy readers of the IEO and the IPMA. The overall result of the age calibration exercise (Villamor *et al.*, 2019) was that there were high agreements, low CV and without biases; the three readers apply well the current age determination criteria updated in the last workshop of the anchovy age (WKARA2; ICES, 2017). The three readers have achieved higher agreements and lower CVs in this Calibration than in the last International Exchange of anchovy in the Bay of Biscay in 2018 (Villamor *et al.*, 2018), especially noted the improvement of the IPMA reader. The weighted average percentage agreement (PA) based on modal ages for all readers and samples were 93.4%, with the weighted average CV of 8.4%. In 2018 Exchange, the two readers of the IEO had a PA above 90% (91 and 92% respectively with the modal age) and CV of 15% and the IPMA reader had a PA of 76% and CV 21%. The biggest discrepancies found in this Calibration were in age 2. This is mainly since in some cases the false spawn ring that deposits the anchovy in summer is confused with the annual winter ring. It is recommended to continue and follow the protocols and criteria for the interpretation of anchovy age in all areas proposed in WKARA2.

2.1.2 Italian Workshop on Age estimation of European anchovy (*Engraulis encrasicolus*)–WKAGESP

During November 2019, an Italian national workshop on Age estimation of the European anchovy (WKAGESP) was held in Capo Granitola (Trapani, Italy). The workshop, chaired by Gualtiero Basilone and Pierluigi Carbonara, hosted eight researchers that came from five different Italian laboratories involved in the national data collection framework. All the seven Italian FAO-GFCM Geographical Sub-Areas (GSA) were involved. The WK aimed in reviewing the existing information on anchovy age estimation, proposing new otoliths reference collection (on national basis) and, finally, standardizing age assignments also improving the coherence of age estimation between each laboratory. Each laboratory shared its adopted protocol. While no major differences in otoliths preparation techniques were found, the main discrepancy between labs was represented by the adoption of 1 January or 1 July as the species birthdate. To reduce assignment discrepancies and to improve standardization among Italian areas in age assignment the

WKAGESP group adopted 1 January as the conventional birthdate for anchovy. Each laboratory shared its adopted protocol. While no major differences in otoliths preparation techniques were found, the main discrepancy between labs was represented by the adoption of 1 January or 1 July as the species birthdate. To reduce assignment discrepancies and to improve standardization among Italian areas in age assignment the WKAGESP group adopted 1 January as the conventional birthdate for anchovy. The working group also produced a new aging scheme slightly modifying the one introduced in the WKARA2 to also consider the possibility of the presence of a "not expected" otoliths edge type. Extensive group exercise of otolith age readings was organized, with sessions with all attendees jointly discussing the different otolith interpretations. At the end of the exercise, attendees had the impression that differences in growth patterns among areas were not as relevant as originally presumed. Finally, to standardize the anchovy age assignments and to improve the coherence of the age estimates, during the workshop a common protocol was adopted for all Italian areas.

Table 2.2. WKARA2 upgraded summary of age validation methods published for anchovy in Atlantic and Mediterranean areas.

Validation method	Area	Method	Time-series	Age/size Range	References
Marginal Increment Analysis/ Edge Analysis	Bay of Biscay	Qualitative	1984–1992	Ages 0–3+	Uriarte *et al.*, 2016
	Gulf of Cádiz		2005–2008	Ages 1–4	Millan and Tornero, 2009
	Alboran Sea		Oct. 1989–Dec. 1992	All ages together	Giraldez and Torres, 2009
	North Adriatic Sea		Jan. to Dec. 2007	All ages together/ 10.5–16.5 cm	Donato and La Mesa, 2009
	Strait of Sicily	Validation of first annulus	Feb. 2015 to November 2016	Ages 1–3/Size 9–17 cm	Basilone *et al.*, 2020
Progression of strong year classes	Bay of Biscay	Successive modal lengths in the catches	1983–1986	Age 1–4	Uriarte and Astudillo, 1987; Uriarte, 2002b
		Tracking abundance indices by age in surveys and catches	1987–2013	Age 1–3	Uriarte *et al.*, 2016
Daily increments between annuli	Bay of Biscay	Validation of first annulus	October 2012–April 2013	Age 1/8.5–13.6 cm	Aldanondo *et al.*, 2016
		Corroboration of first check	2010–2011	Age 1/11.7–20.5 cm	Hernández *et al.*, 2013
Length frequency analysis	NW Mediterranean Sea		April 1984–Oct. 1985	Age 0–4/ 5–18.5 cm	Pertierra, 1987
			Jan. 1987–Jun. 1989	Age 0–4/ 6.5–20 cm	Morales-Nin and Pertierra, 1990

2.2 Validation studies (upgrading the list with new works already published on ISI journal)

In the present workshop a new validation study already published on ISI journal from the Strait of Sicily (GSA16) was presented and discussed: *Basilone et al. First annulus formation in the European anchovy; a two-stage approach for robust validation. Sci Rep 10, 1079 (2020).*

Several methods exist for validation of age readings of calcified structures (Campana, 2001). A summary of age validation methods used for anchovy in European waters was provided in the Table 2.2.

3 Review of aging techniques adopted by each lab/area (protocols included)

3.1 Bay of Biscay

3.1.1 Bay of Biscay (Spain)

The aging criteria for anchovy in the Bay of Biscay is the one adopted in WKARA (ICES, 2010) and WKARA2 (ICES, 2017) according by Uriarte (2002b) and Uriarte *et al.* (2016).

The method is based on the knowledge of the annual growth pattern of the anchovy otoliths, of the seasonal growth of otolith edge by ages and of the most typical checks.

- A set of an opaque and hyaline zone corresponds to an annual growth zone (annulus).
- The date of birth is conventionally assumed to be 1 January and the fish is assigned to a year class on this basis (if an otolith is collected during the first semester the age group correspond to the number of hyaline zones, if the otolith is collected from a fish caught during the second semester, the hyaline edge will not be considered).
- Maximum otolith growth (opaque ring formation) takes place in summer, and it decreases in winter (hyaline ring formation).
- Typical checks occur before and after the first winter ring is formed, during age 0 and age 1 of anchovy.
    - The checks before the true hyaline winter ring are generally present around the nucleus (central check), these checks are named C05 and C08.
    - The most typical ring formed after the first true hyaline ring is formed during June/July in many of 1 years old anchovy at the peak of their first spawning period (spawning check), these checks are named C15 or C18

Advice about some methods and guidelines to consider estimating age was also presented.

3.1.2 Bay of Biscay (France)

The aging criteria for anchovy in Bay of Biscay in France is the same protocol previously described (see section 3.1.1) for the PELGAS survey and we take all otoliths in picture. Since 2018, for all otolith except PELGAS survey, we start to include otolith in water instead of eukitt resin. The advantage is that otolith can be store in tube and it is dry. Problem is the preparation time and the otolith's hydration. In fact, this hydration does not permit age reading because the otoliths, after 5 minutes in water, become all hyaline.

3.2 Division 9.a

3.2.1 Subdivision 9.a North

The same age interpretation criteria previously described for the Bay of Biscay (Spain; see section 3.1.1.) are followed for Subdivision 9.a north.

3.2.2 Subdivision 9.a South (Gulf of Cádiz)

The same age interpretation criteria previously described for the Bay of Biscay are followed also for Subdivision 9.a south. The difficulty involved in reading these otoliths is due to several factors mainly related to the biology of the species in this area and was widely described in WKARA2 (ICES, 2017).

This is a summary of the Working Document presented in WKARA3: Extracting, mounting and reading anchovy otoliths from the Gulf of Cádiz (ICES Subdivision 9.a-South).

Based on the experience at Spanish Institute of Oceanography Cádiz laboratory, the WD focuses on the practical aspects of the otolith extraction and reading describing custom tools, preparation and reading procedures used at that facility for anchovy otoliths and the improvements and experience gathered so far. Moreover, the document provides information about the design and testing of new small pelagic fish otolith storing slide for improved otolith extraction and preservation.

3.2.3 Subdivision 9.a (Portugal)

The anchovy stock in the ICES division 27.9.a includes two components of the same stock: west (ICES subdivisions 9.a.N, 9.aCN and 9.aCS) and south (ICES subdivisions 9.a.S). Scientific advice for this stock started in 2018 after a Benchmark WKPELA 2018 (ICES, 2018). The western component had a significant increase in biomass since 2016, when biological sampling of fisheries and surveys was intensified. The quantity and quality of data required for a rigorous analytical assessment of a given stock is a function of the species' life cycle parameters. Short-lived species such as anchovy require higher data frequency than long-lived ones, to follow cohorts and fluctuations in recruitment strength over time. Age represents an important data for most analytical assessment models, so it is important to try to minimize age reading errors, as these errors can have very adverse effects on the quality of the advice. In 2020, the experienced age reader of anchovy at IPMA retired. This researcher regularly participated at international intercalibration exercises and showed excellent age agreement with its international peers (Villamor *et al*., 2019). Before retiring, he trained two colleagues that recently participating in the anchovy age workshop (WKARA2) and described the protocol for anchovy age reading in Portugal. Sagittae otoliths are extracted, then washed to remove the organic material, dried and stored in Eppendorf tubes. Some of the otoliths are mounted in black plates and covered with resin (Entellan). For age determination, the otoliths mounted are read in a stereomicroscope with reflected light (magnification x20). The age reading criteria follows the WKARA (ICES, 2010) and WKARA2 (ICES, 2017). It is planned that the two readers can first intercalibrate with the Spanish peers, who conduct age readings of anchovy from the Iberian stock, and then with the international peers within the scope of WKARA3.

3.3 Alboran Sea and Northern Spain (GSAs 1 and 6)

During the MEDIAS survey conducted in the Spanish Mediterranean Sea, around 900 pairs of anchovy otoliths were collected. These otoliths are mounted on black plates and fixed with resin. One of the limitations of the old resins such as “Eukitt” is their toxicity, which is why we are currently looking for substitutes for this that first, do not harm health and second, do not imply a change in the legibility of the otoliths.

On the other hand, we present the greatest difficulty that we face when determining the age of the otoliths from the MEDIAS surveys. This survey is conducted during June–July, coinciding with the change of semester, therefore, with the change of age assignation criteria. This fact makes it difficult to assign the age of the specimens when their otoliths have a hyaline or semi-hyaline edge. For readers who only deal with otoliths collected in this survey, this is an open question on which we should work together to achieve robust results and continue to improve the management of sardine populations in the Mediterranean Sea.

3.4 Gulf of Lion (GSA 7)

For age reading in Gulf of Lion, we use the same preparation that section 3.1.2. All otoliths put into water on black background and reflected light. Readers always take picture for every otolith in laboratory or MEDIAS survey on stereomicroscope with camera. Readers observe a significant reduction of length since approximately the year 2010 onward. Length values range between 9.0 cm TL and 15.0 TL cm, with a modal value of 12.0 TL cm. We cannot see differences on otoliths with these ages' differences.

3.5 Ligurian and Northern Tyrrhenian Sea (GSA 9)

In terms of landings, anchovy represent one of the most important fish species in GSA9; it is caught mainly by purse-seine and occasionally by bottom trawl fisheries.

In the last 5 years, a positive trend can be observed for landings; moreover, no variation in length frequencies distribution of landing is observed. Length values range between 9.0 cm TL and 15.0 TL cm, with a modal value of 12.0 TL cm. Regarding biomass data from MEDITS and MEDIAS survey in GSA 9, show a gradual increase in the last 10 years. In GSA 9, anchovy show a limited spawning season in late spring/summer, and it reach first maturity about 11.6 cm (sex combined).

Regarding otoliths preparation, they are extracted through a transversal cut of cranium, rinsed in water to remove organic material, dried and stored in Eppendorf. Otoliths do not require pre-treatment and whole otoliths are photographed using stereomicroscope connected to camera, in seawater or alcohol with the distal side up, reflected light and a black background

Age assignment follow aging scheme proposed during WKAGESP 2019 (Basilone *et al*., 2019).

3.6 Western Ionian Sea (GSA19)

The samples were collected within the Italian Work Plan for data collection in the fisheries and aquaculture sectors, in the GSA 19, from boat using purse-seine, driftnet and midwater otter trawl. The most important harbours for this fishery are Cirò, Crotone and Catania. The adopted aging criteria are that presented in the "Handbook on fish age determination: a Mediterranean experience" published in the Studies and Reviews FAO-GFCM. The extraction is done true a transversal section slice in correspondence of the preopercula. After the extraction, the otoliths are cleaned, dried and stored in labelled plastic tubes. Two dates of theoretical birthday are applied in base of the purpose of the research. To the Growth parameters of von Bertalanffy growth curve (VBGC), according with the spawning period in spring month (June–July), the first of July is used. To estimate the Age at Length Key (ALK), according to the last otolith exchange, the first of January is used. The otoliths were analysed whole, by the binocular microscope, rinsed with seawater, with reflected light against a black background, with the distal surface turned up and the proximal surface (sulcus acusticus) turned down. One of the pair of otoliths (usually the left) are placed in immersion in seawater; otoliths do not be clarified before the analysis. Annuli are counted on the antirostrum area (radius) as translucent growth rings (slow growth). The opaque zone (white—fast growth) plus a transparent ring is considered as an annual increment (annulus). Five specimens per sex and quarter each 0.5 cm of total length are collected. The age is assigned with a resolution of 0.5 years, counting the transparent (winter) ring and considering the quarter as capture date. Translucent true rings should be visible around the whole otolith to be considered as annual rings. The increments between the consecutive annuli should be decreasing with age. The first winter ring is laid down at 1.03 ± 0.06 mm from the nucleus. Before the first winter ring a false one is laid down with a distance to the core about 0.8 mm. This ring can be distinguished because is less marked compared with the winter one. The main questions and difficulties are the identification of the first true ring, the identification of the edge, and the identification of false rings.

3.7 Sardinian Sea (GSA 11)

Traditionally in Sardinian seas, small pelagic fish were never really considered as valuable target species. For this reason, in GSA 11 few fishing vessels are registered as purse-seiners. Consequently, within the national data collection framework DCF the Purse-seiner métier was not assigned to the GSA 11 to be monitored. In this regard all age data on anchovy were obtained almost exclusively by occasional catches of bottom trawl hauls conducted by both OTB_DES and OTB_MDD métiers. Otoliths are extracted through a transversal cut of the fish cranium, after the extraction they are washed, dried and stored in Eppendorf. Otoliths, with the distal side up, are observed under a stereomicroscope equipped with reflected light and a black background. Seawater is used as clarifying medium. If needed, images are often quickly post-produced increasing their contrast and sharpness following the available literature. Since 2020, fish age is assigned applying the aging scheme proposed during the Italian national workshop on age estimation of the European anchovy WKAGESP 2019 (Basilone *et al.*, 2019) considering 1 January as the species birthdate.

3.8 Strait of Sicily (GSA 16)

In GSA 16 two different gears exploited small pelagic resources: the purse-seiner and the mid water pelagic trawl. The main target species for the fishery is the Anchovy which supply a well-established local canning industry. Otolith are obtained by sampling the landings on fortnightly intervals. Sagittae, are routinely extracted and stored dry in black plastic mould ready to be analysed. For reading purposes the otoliths are immersed in 70% alcohol under a stereomicroscope equipped with reflected light and an image analysis system to measure, save, annotate, catalogue and archive the image in high resolution. Each annulus consists of one opaque zone plus one hyaline ring; The age is equals to the number of true winter complete hyaline zones, corresponding to the expected annual growth pattern of the otoliths, excluding the marginal edge development of the year. The conventional birthdate and the date of capture to be used together with a common adopted otolith reading scheme from WKARA2. Since 2020 onward according to WKARA2 and WKAGESP 2019 recommendations, the conventional birthdate was moved from 1 July to 1 January.

3.9 Eastern Adriatic Sea (Croatia, GSA17)

Anchovy (*Engraulis encrasicolus*, L.) stock in the Adriatic Sea is shared between five countries: Italy, Slovenia, Croatia, Montenegro and Albania.

Regarding the conventional birthday date for anchovy, AdriaMed SG-OTH group has decided to move it since 2019, for stock assessment purposes (according to WKARA2; ICES, 2017), from 1 June to 1 January. The year is divided into two 6-month semesters, the first semester from 1 January to the 30 June and the second semester from 1 July to 31 December.

Samples are collected on boat while otolith extraction is conducted at the laboratory. After the extraction, otoliths are cleaned in freshwater and stored dry in vials till age reading occurs. Age reading is performed with Dino-Lite digital microscope. Otoliths are immersed in 70% alcohol in black Petri dish and illuminated with light. For each otolith we take picture and store data like age, edge, annulus, radius, diameter and quality of reading.

3.10 Western Adriatic Sea (Italy, GSAs 17 and 18)

Anchovy (*Engraulis encrasicolus*, L.) is one of the most important commercial species of the Adriatic Sea. The stock is shared between five countries: Italy, Slovenia, Croatia, Montenegro and Albania.

An official stock assessment is conducted every year by GFCM during specific Working Group on Stock Assessment of Small Pelagic Species (WGSASP; Basilone *et al*., 2019) to assess the status of the stock, using mandatory data both from commercial activities and from acoustic scientific surveys.

Only Italy, Slovenia and Croatia are included in the Data Collection Framework (DCF, EC, 2008) and for this reason, to overcome some discrepancies in otolith readings among Adriatic experts, FAO-AdriaMed Project has organized several Study Groups (SG-OTH) on the intercalibration of the reading of anchovy otoliths during last years. Adriatic experts on age reading agreed on new criteria and the protocol to be followed, considering ICES ones (ICES, 2010, 2017), obviously considering the specificity of Adriatic Sea.

Regarding the conventional birthday date for anchovy, AdriaMed SG-OTH group has decided to move it since 2019, for stock assessment purposes (ICES, 2017), from 1 June to 1 January. The year is divided into two 6-month semesters, the first semester from 1 January to 30 June and the second semester from 1 July to the 31 December.

Regarding laboratory procedures, the sagittae extraction is made by the posterior section. After the extraction, the otoliths are washed to remove the organic material and then dried and stored in cuvettes. Age determination is performed with the aid of a transmitted-light stereomicroscope, with a magnification between 16x and 25x. For the readings, otoliths are immersed in a 70% alcohol solution and illuminated with reflected light on a black background, to better identify the different growth bands. The orientation of the otolith for analysis is performed with the distal surface turned up and the proximal surface (acoustic sulcus) down. The measurements are taken on the major axis passing through the core on the posterior area (post-rostrum). Finally, through the installation of a video camera connected to the computer and with the aid of image analysis software (Image Pro Plus) it is possible to measure, save, annotate, catalogue and archive the image in high resolution.

3.11 Aegean Sea and Eastern Ionian Sea (GSAs 22 and 20)

The European anchovy (*Engraulis encrasicolus*) in northern of the GSA 22 and 20 is the most important small-sized pelagic species in Greek waters and together with sardine comprise about one third of the total fisheries catch, making up 30% of the total Greek landings and 59% of the total purse-seine landings. The main anchovy distribution grounds in the Greek Seas are located within the continental shelf and the semi-closed areas with enrichment processes associated with the outflow from large rivers or the Black Seawater (BSW) in the north Aegean. The abundance estimates of anchovy ranged between 40 000 and 45 000 tin the north Aegean and between 11 000 and 15 000 tin the central Aegean and Ionian Seas. The spawning period of anchovy in the Greek Seas occurs from May to September, but some spawning activity can be observed up to December in the central Aegean Sea. Spawning peaks at around June in all areas studied so far, north

Aegean Sea, central Aegean Sea, central Ionian Sea, NW Aegean Sea. The samples collected experimentally through acoustic and/or egg surveys have been obtained in recent years in the framework of various EU and national projects. During the years 2019–2020 the length of the anchovies sampled ranged between 60–160 mm with a peak at 100–120 mm. The length at first maturity was estimated at approximately 105 mm total length. The conventional birth date is assumed to be 1 July. Spawning season goes from spring to autumn with a peak in June-July. Season of recruitment-at-age 0 is assumed to be in March. Length frequency distribution was obtained on a semester basis and on average 20 to 25 otoliths (sagittae) from each sample and per each length class were removed and used for age determination. Age groups range from 0 to 3, with age 0 being less than 1% of the total catch and age 3 being around 0.5% of the total catch due to the limited presence of this age group in the anchovy population.

In total, 2100 otoliths were used for age reading, around 2000 from Aegean and 100 from Ionian Seas. The sagittae otoliths were removed using the ‘up through the gills’ method. After the extraction, the otoliths are cleaned from adhering tissues and then dried and mounted (with the sulcus down) on black plastic plaques and stored in the freeze. The otoliths are immersed in seawater (clarification medium) and analysed under the binocular microscope with reflected light against a black background (25x magnification). In this way, the dark rings can be counted in the antirostrum area (radius) as translucent growth rings (slow growth). The opaque zone (white—fast growth) with a dark ring is considered an annual increment (annulus). Thus, the age scheme of anchovy otoliths considers the number of translucent rings (winter rings), the pattern of annulus formation (generally translucent ring during winter/spring months; opaque ring during summer/autumn months).

Table 3.1. Summary of anchovy otolith sampling methods and preparation techniques of each participant laboratory.

Country/Institute	Laboratory	Stock/area	Sample	Periodicity	Otolith sampling	Total N/Year	Otolith preparation
Spain-AZTI	Pasaia (Basque Country)	Bay of Biscay anchovy: VIIIc, VIIIab	Egg Surveys	Annual-Spring	Stratified by length class, 10 otolith pairs by length class (0.5 cm)	2000	Black plastic moulds and polyester resin (Eukitt).
			Acoustic Surveys	Annual-Autumn		2500	
			Landings	Monthly		2000	
Spain-IEO	Santander/Vigo	Bay of Biscay anchovy: VIIIc, VIIIb	Acoustic Surveys	Annual-Spring	Random, 40 by fishing haul	261	Black plastic moulds and polyester resin (Eukitt). One plaque of each sample (10 pairs of otoliths) was stored dry for other studies (daily growth or microchemical studies) and inmersed in water for observations
			Botom trawl survey	Annual Autumn		301	
			Landings	Monthly (fortnightly in spring)	Random, 100 by sample	1500	
		Anchoy IXa: Galician waters-Subdiv. IXa North	Acoustic Surveys	Annual-Spring	Random, 40 by fishing haul	50	
			Landings	Monthly	Random, 100 by sample	400	
	Cadiz	Anchovy IXa: Gulf of Cadiz-Subdiv. IXa South	Acoustic Surveys	Annual Summer & Autumn	50 by haul, plus tails	about 2000	Black plastic moulds and polyester resin (Eukitt).
			Landings	Fortnighly	100 random	about 2000	
		Morocco: North Atlantic area (North of 34°18′00″9)	Landings	All year but close season (February-March)	Stratified by length class, 5 otolith pairs by length class (0.5 cm)		
	Málaga	GSA01: Northern Alboran Sea	Acoustic Surveys	Annual Summer			
			Landings	Monthly			
		GSA06: Western Mediterranean Sea	Acoustic Surveys	Annual Summer			
			Landings	Monthly			
Italy-IAS-CNR	U.O.S. Capo Granitola (Sicily)	GSA16: Strait of Sicily	Acoustic Surveys	Summer	Stratified by length class, 10 otolith pairs by length class (0.5 cm)	~1500	Black plastic moulds and dried for observation
		GSA09: Northern Tyrrhenian Sea				~1500	
		GSA10: Southern Tyrrhenian Sea				~1500	
Italy-IRBIM-CNR	U.O.S. Ancona (Marche)	GSA17: Northern Adriatic Sea	Acoustic Surveys	Summer	Stratified by length class, 5 otolith pairs by length class (0.5 cm)	about 1500	Stored dried in vials and immersed in alcohol for observation by transmitted-light stereo-microscope on petri dish
			Landings	Annual			
		GSA18: Southern Adriatic Sea	Acoustic Surveys	Summer		about 1500	
			Landings	Annual			
Italy-UNICA	Cagliari	GSA11: Sardinian Sae	On bord observers and Market	Monthly	Stratified by length class, 5 otolith pairs by length class (0.5 cm)	About 100	Stored dried in vials and immersed in water for observation
Italy-COISPA	Bari	GSA19: Western Ionian	Landings	Monthly	Stratified by length class, 5 otolith pairs by length class (0.5 cm), sex and quarter	About 500	Stored dried in labeled plastic tubes and immersed in sea water for observation
Greece - HCMR	Heraklion	GSA 22: North Aegean Sea	Acoustic Surveys	Summer/Automn	Stratified by length class, 20 otolith pairs by length class (0.5 cm)	~1000	Stored dried in plastic plaques in freeze and immersed in seawater for observation
		GSA 20: Eastern Ionian Sea				~100	
Greece - HCMR	Athens	GSA22: Aegean Sea	Landings	Summer/autumn	Random 100 by sample	~250	Stored dried in plastic cases and immersed in water for observation
				Monthly	Stratified by length class, 10 otolith pairs by length class (0.5 cm)	~1200	
Croatia-IOF	Split	GSA 17: Northern and Central Adriatic Sea	Acoustic Surveys	Annual	Stratified by length class, 5 otolith pairs by length class (0.5 cm)	1000/1500	Stored dried in vials and immersed in alcohol for observation

Table 3.2. Summary of the otolith image processing techniques used by WKARA3 participant laboratories

Country/Institute	Laboratory	Image capture software	Type of camera	Magnification	Otoliths measures	Image format	Image uses
Spain-AZTI	Pasaia (Basque Country)	Olympus Cell Sens Dimension 1.6 Micro	Imaging Micropublisher 5.0	X20	Otolith total length; radius	JPEG/TIFF	Exchanges, otolith images collection
Spain-IEO	Santander						
	Cadiz	NIS-Elements AR 3.2	NIKON DXM1200C	X20	None as routine. Otolith diameter, and radii (post rostrum, antirostrum and A1) for specific purposes	JPEG2000, jpg (preferred)	Exchanges, validation studies, otolith images bank
	Malaga	Leica Application Suite (LAS) 4.1	Leica DC200	X25	Otolith diameter/radius annual ring radius	JPEG	Exchanges, thesis, validation studies, otolith images collection, training
Italy-IAS-CNR	U.O.S. Capo Granitola (Sicily)	Leica Application Suite (LAS) 4.1	Color digital Camera LEICA DC200	X25, x32	Otolith diameter/radius annual ring radius, checks radii	TIFF	Exchanges, thesis, validation studies, otolith images collection, image bank
Italy-COISPA	Bari						Exchanges
Italy-UNICA	Cagliari	EOS Utility 2	Canon EOS 650D	X20	None as routine. Otolith total length and radius length, annual rings radius length	JPEG	Exchanges, otolith images collection
Croatia-IOF	Split	Dino-lite software	Dino-Lite Digital microscope	/	Otolith total length and radius length, annual rings radius length	JPEG	Exchanges, otolith images collection
Greece - HCMR	Heraklion	Leica Application Suite X	Leica Flexcam C1	x2	Otoliths annual ring radius	TIFF	Exchanges otolith images bank
	Athens	Image Pro Plus	SONY WILD	x20, x25, x32	Otolith diameter/radius annual ring radius	TIFF	Exchanges, otolith images bank

4 Validation studies presented during the workshop

During the workshop, a summary of works recently conducted on the validation of age determination for anchovy stocks were presented by three labs from both Atlantic (i.e. Gulf of Cádiz) and Mediterranean (i.e. Strait of Sicily and South Tyrrhenian Sea).

4.1 Division 9.a (Subdivision 9.a South, Gulf of Cádiz)

The Spanish waters of the Gulf of Cádiz (GoC, ICES Subdivision 9.a South) is one of the core areas of the distribution of anchovy in the Division 9.a. The spawning season extends from late winter to early autumn with a peak spawning time occurring from June to August. GoC anchovy shows a protracted spawning season, which could cause that anchovy from different spawning batches (say spring, summer and autumn-born) could show different growth patterns. For this reason, it is necessary to conduct growth validation studies. The validation of the annual deposition of seasonal zones and the checks in the otolith represent the focal point to improve the precision in the anchovy age determination. Previous works attempting to validate annuli of anchovy in the GoC apply the qualitative method (Millán and Tornero, 2009; Tornero, 2016). But more validation age studies should be conducted for all anchovy stocks, and especially for those ones, such as this stock, which are assessed analytically.

The objective of this study is to validate the periodicity of the otolith increment formation in European anchovy through the otolith marginal increment (MIA) and the otolith edge analysis (EA), and furthermore to evaluate the temporal link between the reproductive cycle (GSI, gonadosomatic index) and the somatic conditions (CF, condition factor) with the otolith edge formation and the patterns of monthly increments.

A total of 6359 *E. encrasicolus* from the GoC were analysed. The total length, total weight, gutted weight, gonad weight and the otoliths were collected monthly from landings between 2015–2016, from scientific acoustic surveys "ECOCADIZ" during July-August and "ECOCADIZ-RECLUTAS" during October. Otoliths were aged following standardized criteria (WKARA; ICES, 2010; WKARA2; ICES, 2017). A total of 3038 age group 1 individuals were selected for this study. The MIA was analysed by calculating the total radius minus the radius of the last complete hyaline zone in a random selection of ten one-year-old fishes per month and the nature of the edge was estimated by month as the percentage of hyaline (H) and opaque (O) edge. The gonadosomatic index (GSI, only for females) and the condition factor (CF) for the age group 1 were computed.

Otolith marginal increment analysis (MIA)

Analysis of the MIA variability through the year revealed that the minimum marginal width, which is associated to the beginning of the new annulus formation, was recorded in April. In August, the highest value is reached, and a decrease is observed in the following months (Figure 4.1)

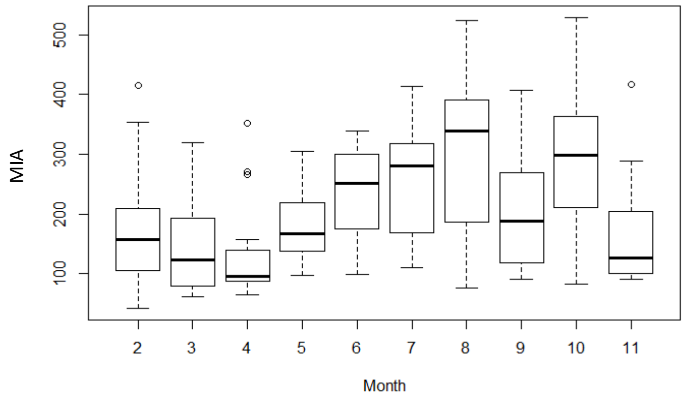


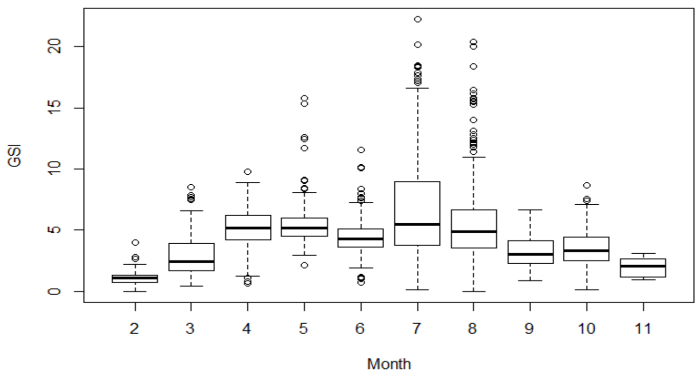
Figure 4.1. Boxplot of monthly trend of otolith marginal increments (MIA).

Otolith edge seasonal growth pattern (EA)

A preliminary analysis of the nature of the edge was done but given the complexity of its determination in the otoliths of this area, a new classification needs to be done to properly address this issue (Uriarte *et al.*, 2016, Basilone *et al.*, 2020). As these authors indicate, the nature of the edge must be reclassified as opaque (O), semihyaline (OH) or hyaline (H). The criterion used to differentiate OH and H is the presence of the same edge around the whole otolith otherwise the OH is to be considered as opaque and thus merged with O.

Reproductive cycle and somatic condition (GSI, FC)

The boxplot of monthly GSI from age group 1 fish for 2015–2016, showed that the reproductive season extends from April to August, with a spawning peak in July, with the index decreasing later and recording the lowest values in autumn and winter. The CF monthly pattern obtained for the age group 1 fish showed a maximum in April, decreasing progressively in the following months and showing the lowest values in winter, as in GSI.



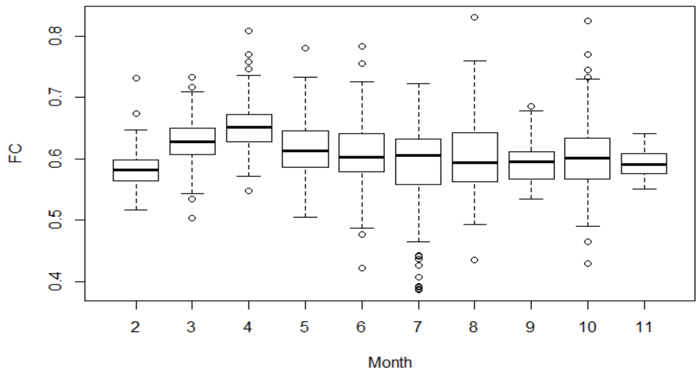


Figure 4.2. Boxplot of monthly trend of gonadosomatic index (GSI) and condition factor (FC).

Conclusion

The otolith study for the two analysed years shows that the annulus formation resulted mostly complete for this species in the GoC by April. A noticeable minimal marginal increment on otoliths occurred only once per these years, the annulus was accepted as valid indicator of age, supporting the consistency in the age estimation criteria. The GSI show a spawning peak in July, according to the spawning season. The spawning peak in the Bay of Biscay occurs between May and June. Uriarte *et al.* (2016) showed for the Bay of Biscay anchovy similarities to that of the Atlantic waters studied here, for age 1, growth resumes usually during March. The results obtained here corroborated temporally coherent patterns.

Further analyses must be addressed to analyse the data separately by years, reclassify the nature of the edge, analyse the otolith edge seasonal growth pattern and evaluate the presence of significant effects on otolith structure of month, GSI and CF using Generalized Additive Models (GAM). General additive models using marginal increments as dependent variable could show the effect of the month, highlighting the presence of minima, as well as specific relationships with condition factor and gonadosomatic index.

4.2 Strait of Sicily (GSA 16)

First annulus formation in the European anchovy; a two-stage approach for robust validation (Basilone *et al*., 2020; integrally reported below)

Introduction

Otolith annual growth increments, composed of alternating opaque and translucent zones, are commonly used to determine the age of fishes since 1899[1]. However, counting annuli was sometime misleading in estimating the age of tropical or deep-sea adult fishes, due to a more stable environment leading to more constant growth, thus resulting in less certain annulus formation[2]. Erroneous age estimates can negatively affect management of marine resources, since several biological variables such as growth rate, mortality rate and productivity are obtained by the age structure of the population[3,4,5]. Age determination in short living species, such as anchovies, is further complicated because of the predominance of young fishes (0, 1, or 2 years old), which often show false rings or checks[6,7,8,9]. Correct age assignment mainly relies on a correct identification of year classes. However, the year-classes separation is difficult because anchovies have a long spawning season, which in the central Mediterranean Sea extends from April to October[10,11]. Although most fish age-estimation studies have assumed that growth increments occur annually, a substantial part of these did not test this assumption[12,13,14]. Even if not validated, these information are currently heavily used for anchovy stock assessment in Mediterranean and Atlantic waters[15,16]. There are various approaches for verifying age estimation methods[4,17]; edge analysis (EA) and marginal increment analysis (MIA) are among the most frequently methods employed[4,5]. These methods focus on incremental patterns of growth-band pairs throughout the year. They assume that, if growth bands are formed annually, the width and the density of the outermost increment will exhibit a yearly sinusoidal cycle when plotted against the month of capture. The EA is a qualitative assessment of the relative opacity and translucency of the edge of the otolith, whereas MIA typically compares the width of the last developing band to the width of a complete annulus. Both techniques are characterized by comparable properties and, when used as validation method, provide valuables results, especially for fish species presenting annual periodicity in bands formation[4].

Past studies have reported age validation based on MIA of young fish, but noted that the same aging structure and/or approach provided incorrect ages mainly in older fish, suggesting that MIA is one of the few validation methods which is restricted to young, fast-growing fish[18,19,20]. Moreover, since proper age validation studies are still lacking for many species and study areas, age reading workgroups, specially addressed on European anchovy (*Engraulis encrasicolus*) age validation, recommended to seize upon any available method, which can corroborate the age interpretations as well as the dynamics of otolith formation (checks and true annual rings) by ages[13]. Although these techniques have sometimes been questioned[4], their combined use, adopting a robust statistical framework and following rigorous criteria, may permit to evidence biases or inconsistencies between the different methods, and represents a useful tool for corroborating the correct annulus identification[4]. The use of combined information from different sources (fishery-dependent or not) into a single holistic approach to corroborate or validate the annulus formation was already tested on different fish species, e.g. for Atlantic horse mackerel (*Trachurus trachurus*)[21] and for red mullet (*Mullus barbatus*)[22], as well as for both wild and reared anchovy in the waters of the Bay of Biscay[14,23,24]. The habitat conditions of the Bay of Biscay are characterized by higher riverine input nutrients and higher productivity[25] compared to the oligotrophic Mediterranean waters[26,27]. Otherwise, the strong influence of habitat condition (e.g. primary production and temperature variability) in shaping otolith and growth rate was already detected for anchovy both in the Strait of Sicily[28,29] and in the Bay of Biscay[24,30]. Therefore, validation studies for a single species should also consider the ecosystem variability, since it has been observed that the growth pattern could change among different habitat conditions[12,13]. In Mediterranean Sea, studies aiming at anchovy age validation are very scarce and pertain only to the NW area[31]. Indeed, the NW Mediterranean is among the most productive areas in the Mediterranean Sea[32,33], thus not easily comparable with the oligotrophic nature of the study area (Strait of Sicily)[34,35]. Furthermore, none of the previous investigations used physiological information to support the results of the otolith analyses. Although two studies already validated the first annulus formation[14,24], they were conducted through mesocosm experiments or by monitoring existing long time-series of year classes in catches. However both studies are not easy to be replicated in other areas (specially in Mediterranean Sea), since they rely on the availability of specific and complex infrastructures or long time-series characterized by strong year classes in successive yearly catches. In this study, we aimed to provide a different validation framework based on the combination of known methods coupled with robust statistical analysis, for areas/stocks where long time-series of data or infrastructure facilities for rearing experiments are not available.

Methods

Sampling

Otoliths of European anchovy were collected from fishes of commercial catches in the Strait of Sicily from February 2015 to November 2016 at monthly intervals (Table below). A random sample of a minimum of fifty fish per month was processed fresh in the CNR laboratory. For each fish, total length (TL, ±1mm), total weight (TW, ±0.01g), somatic weight (i.e. ovary-free weight, SW, ±0.01g), and gonadic weight only for females (GW, ±0.01g) were measured. Then, fishes were dissected to determine sex and reproductive phase, according to a six-phases scale[36].

No use of live animals has been required for this study and no specific permissions were needed for the sampling activities in all the investigated areas because the species of interest is commercially harvested (neither endangered nor protected) and it was caught in areas where fishing is allowed.

According to ICES guidelines, otoliths (*sagittae*) were removed from a subsample of 5 -10 individuals per size class at 0.5-cm length intervals[37], cleaned, dried, and stored in black-plastic labelled moulds. The observations of entire otoliths were made under reflected light against a black background and using dissection microscopes with 25X magnification. Magnification has been increased (40X) near the otolith margin to improve the discrimination power of edge type morphology. For each specimen, both sagittal otoliths were laid in parallel with the sulcus facing down[12,13].

Sampling period		Total body length (cm)																	n° of fishes sampled
Year	month	9	9.5	10	10.5	11	11.5	12	12.5	13	13.5	14	14.5	15	15.5	16	16.5	17	
2015	February			1		5	5	5	5	5	5	5	5	2	2				45
	March		1	5	5	5	5	5	5	5	5	5	1	1					48
	April								1		8	9	6	4	7	6	2	1	44
	May						1	6	10	10	10	10	10	10	10	1	2		80
	June					2	6	10	10	10	10	10	10	2	1				71
	July						6	10	9	11	9	4							49
	August			2	9	10	10	6	7	10	10	10	10	1					85
	September					2	9	5	4	7	6	6	3						42
	October				3	4	10	10	10	9	8	9	10	4	1	1			79
	November								1	5	10	10	10	9	10	9	3		67
	December	1		10	10	10	10	10	10	5	4								70
2016	January	2	2	10	10	10	10	10	10	10	10	7	6			1			98
	February	1	2	3	10	10	10	10	10	10	9	7	3	3	1				89
	March				3	10	10	10	10	9	9	10	8	1					80
	April				7	14	20	20	20	12	13	10	9	9	1	1			136
	May			2	5	10	10	9	11	10	7	1	1						66
	June					4	10	10	10	10	10	2	1						57
	July				1	1	2	10	10	19	20	20	17	11	10				121
	August			2	2	9	10	10	10	9	3	1							56
	September				6	10	10	10	11	10	1								58
	October		2	2		1	9	13	21	28	30	30	23	15	12	6	1		193
	November			1		1	7	10	10	10	10	10	10	8	4				81
	Total	*4*	*7*	*38*	*71*	*118*	*170*	*189*	*205*	*214*	*207*	*176*	*143*	*80*	*59*	*25*	*8*	*1*	*1715*

Table 1. Number of samples obtained by commercial landings in the Strait of Sicily during 2015 and 2016.

Age assignment

The method of age determination is based first on the interpretation of otoliths according to the prior biological knowledge of the annual growth pattern of the anchovy otoliths[13]. The basic information required for annual growth pattern identification and for age assignment are the date of capture and the conventional birthdate, which in the study area is set to 1 July[13].

The hyaline zones are usually formed in winter but are not necessarily present from the beginning of the year[14,24]; therefore, the discrimination of true winter rings from checks is based on the knowledge of the typical annual growth pattern, seasonal growth of the edge (by ages), and of the most typical checks[13]. According to the typical annual growth of the otoliths, annulus width during the first, second and third year of life (corresponding to 0, 1, and 2 years old groups) decreases progressively.

Therefore, according to the previous observations the adopted aging criteria can be summarized as follows: each annulus consists of one opaque zone plus one hyaline ring; the age is equals to the number of true winter complete hyaline zones, corresponding to the expected annual growth pattern of the otoliths, excluding the marginal edge development of the year (Figure 4.3).

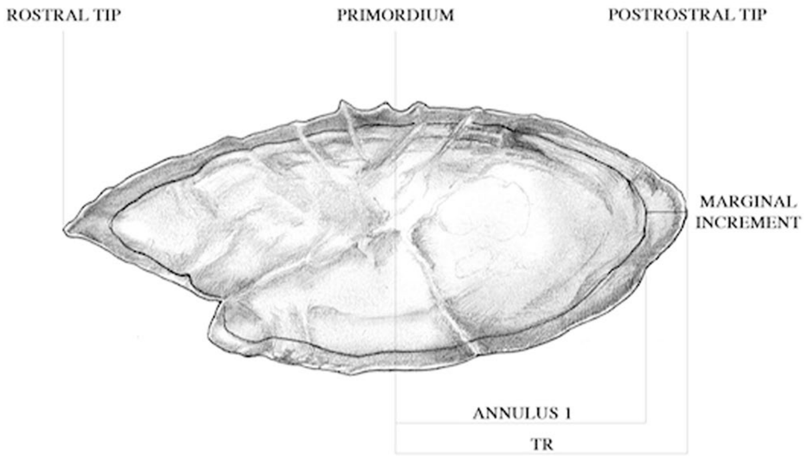


Figure 4.3. Schematic representation of the European anchovy whole otolith sagitta: TR = otolith total radius, and annulus 1 is the combination of one opaque plus one true hyaline zone.

Otolith Edge state analysis (EA)

To assess the seasonal development of opaque and translucent zone within the population, the marginal growth of all the whole otoliths, collected from February 2015 to November 2016 (two complete cycles; 1715 otoliths), was examined also blinding the sampling date to the examiner.

The edge was classified as opaque (O), semihyaline (OH) or hyaline (H), according to the criteria described in literature[14] and as shown in Figure 4.4. Although the morphology of OH and H are quite similar, the criterion used to differentiate between them was the presence of the same edge around the whole otolith otherwise the OH is to be considered as opaque and thus merged with O[14]. According to this, the percentage of individuals showing different kind of edge were applied in the age 1group, for opaque (O), hyaline (H) and merged opaque and semihyaline (O+OH)/(O+OH+H) groups per month and sampling year.

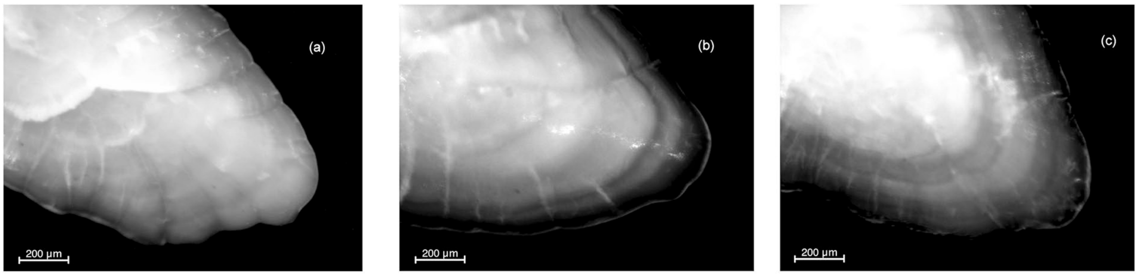


Figure 4.4. Morphology of the otolith edge for each type: (a) opaque margin (O); (b) hyaline margin (H); (c) semihyaline margin (OH).

Translucent completion analysis (TCA)

To obtain information on the monthly distribution of the first annulus as well as on the translucent zones laid down before the first annulus (false ring or checks), the completion date of each translucent zone was reconstructed by back-calculating the fish length at the outer margin of each translucent zone, and then applying the von Bertalanffy growth function with L_{inf}, k and t_0 parameters specific for the study area [28].

The translucent zones were labelled sequentially from T1 to T3 where the latter represents the zone identified as first annulus by readers, while T1 and T2 are those rings laid down in the inner zone of the otolith, which are generally one or two clear zones. Starting from length at capture (Lc) and radius measurement, the length of fishes at complete formation (i.e. the outer margin) of each translucent zone (L*i*) were obtained by backcalculation[38]:

$$Li = \frac{(a+bTi)}{(a+bTR)}Lc \; Li = \frac{(a+bTi)}{(a+bTR)}Lc$$

where a and b are the parameters of the linear relationship between otolith radius (TR) and fish length at capture (TL), while Ti represents the radius from the core to the outer margin of *i*-th translucent zone.

Starting from back calculated Li for each translucent zone, the corresponding ages at completion were then obtained by using the growth model estimated for the whole anchovy population in the study area[28].The use of the latter published model is justified by the narrow size range of the present dataset, preventing a robust estimation of L_{inf}, t_0 and k parameters. Finally, the completion date in each fish (F*i*) was computed according to the following formula:

$$Fi = Capture\_date - (Ac - Ai) \; Fi = Capture\_date - (Ac - Ai)$$

where A*c* is the age at capture (in days) and A*i* is the age (in days) at *i*-th translucent zone completion.

The otolith Marginal Increment Analysis (MIA)

Individual data on the seasonal development of the marginal zone were obtained according to the procedure previously described for the translucent analysis. Despite digital measurement is often used in MI studies, in the present study it was preferred to take the radius measurements, just after the annulus zone has been identified on the whole otolith, by using a micrometre fitted in the eyepiece[39]. This procedure may ensure to take the measurement of the checks or true annuli just after the identification by readers, therefore potentially reducing sources of bias (e.g. misidentification of the annual zone on digital system). According to the adopted age reading protocol[12,13], the whole otolith under a dissecting microscope was used to assign properly the true annual ring. The total radius length (TR) of the whole otolith was measured along the longest axis from the core to the post-rostral outer edge of the otolith. The intermediate radii (R*i*) at each complete translucent zone corresponding to annulus formation were also measured (Figure 4.3). The MIA technique[3,40] was applied by calculating the total radius (TR) minus the radius of the last complete hyaline zone (R*i*) in a selection of one-year-old fishes and MI was expressed as a proportion of the measurement of the previous last complete annulus, i.e. marginal increment ratio (MIR) (Figure 4.3).

In order to give more robustness to MIA, four prerequisite were satisfied[4]: 1) blinded sampling date to the examiner; 2) a minimum of two complete cycles (years) have been examined, in accordance with accepted methods for detecting cycles; 3) the results were objectively interpreted by means of a statistical test, which may show significant differences among some or all of the seasonal groups in each of the cycles examined; 4) MIA has been restricted to age group 1, avoiding age 0 and older ages (age 2 and age 3). Therefore, the validation results should be age-specific.

Reproductive and body condition cycle

To evaluate the temporal link among the reproductive cycle, somatic conditions otolith edge and microincrement formation monthly patterns, gonadosomatic (GSI) and a condition factor (CF) were computed per age group. Although some authors questioned about the usefulness of the gonadosomatic index as a proxy of reproductive potential, because it can be influenced by fish length[41,42,43], several studies validated its applicability for such investigation[44,45], highlighting also its validity in batch spawning species[10,46]. In the present work, GSI was calculated according to the equation described by Bougis[47]:

$$GSI = \frac{GW}{SW} * 100; GSI = \frac{GW}{SW} * 100;$$

To investigate the otolith growth pattern formation, with respect to the annual cycle of fish condition, the Fulton's equation was used for condition factor:

$$CF = \frac{SW}{TL^3} \quad CF = \frac{SW}{TL^3}$$

In both equations, the SW was preferred to the TW to reduce the effect of GW seasonal variability[10].

Statistical analysis

Generalized Additive Models (GAM)[48] were used to evaluate the presence of significant effects on otolith structure of month, GSI and CF. GAMs represent a generalized form of linear models that allow dealing with complex relationships. Due to the differences in the number of observations between the two years, models were fitted separately by years. In particular, the probability to find a hyaline band, as well as MIR values, were modelled as function of month, GSI and CF.

In modelling the probability to find a hyaline band, the categorical variable related to the classification of otoliths bands (H, O, OH) was transformed in a binary variable by assigning 1 to the H bands and 0 in all other cases (O and OH) and a binomial error distribution family with logit link was used. On the contrary, a Gaussian family with identity link was adopted to model the MIA values. The best model was selected according to Wood's guidelines[49] and adopting a backward strategy. All final models were checked for residuals autocorrelation and concurvity.

All statistical analyses were conducted in R statistical environment[50]. GAMs were fitted by using "mgcv" package[51].

Results

Age structure

In the two years considered in the present study, otolith readings and age assignment of the anchovy stock displayed a quite different age structure where the age classes 1 and 2 accounted for most part of the population (Figure 4.5a). Most likely, the age 0 is not fully representative of the recruitment, because size classes of age 0 fishes were mostly below the minimum legal size and the samples came from commercial landings. Despite the dissimilarities recorded in the age structure by year, the length frequency distribution between the two analysed years (Figure 4.5b) is not significantly different (Kolmogorov Smirnov test p>0.1).

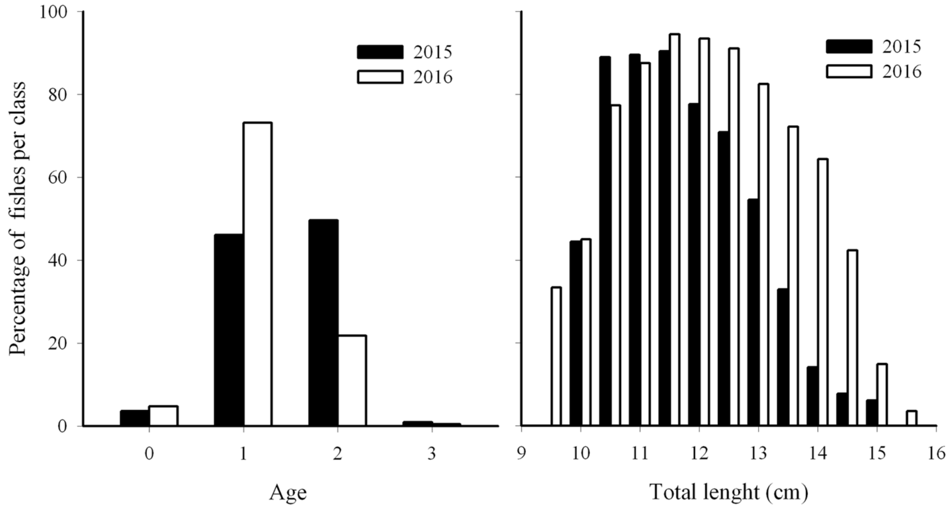


Figure 4.5. Age structure of European anchovy for each analysed year from the GSA 16 stock: (a) proportion for age class; (b) proportion of age 1 fishes for length class in 2015 (n 313) and 2016 (n 757).

Reproductive cycle and somatic condition

The monthly boxplot of GSI from age 1 class showed that the reproductive season extends mainly from May to July-August and started decreasing along autumn and winter (Figure 4.6a). The GSI values suggested an earlier seasonal resume of the reproductive investment, since GSI values at the start of reproductive season in May were already similar to those estimated in August; while in 2016 the highest value was recorded in July. The visual inspection of the CF monthly pattern obtained for the age 1 class showed a trend similar to GSI with lower values between October and March, and maxima between June and August (Figure 4.6b).

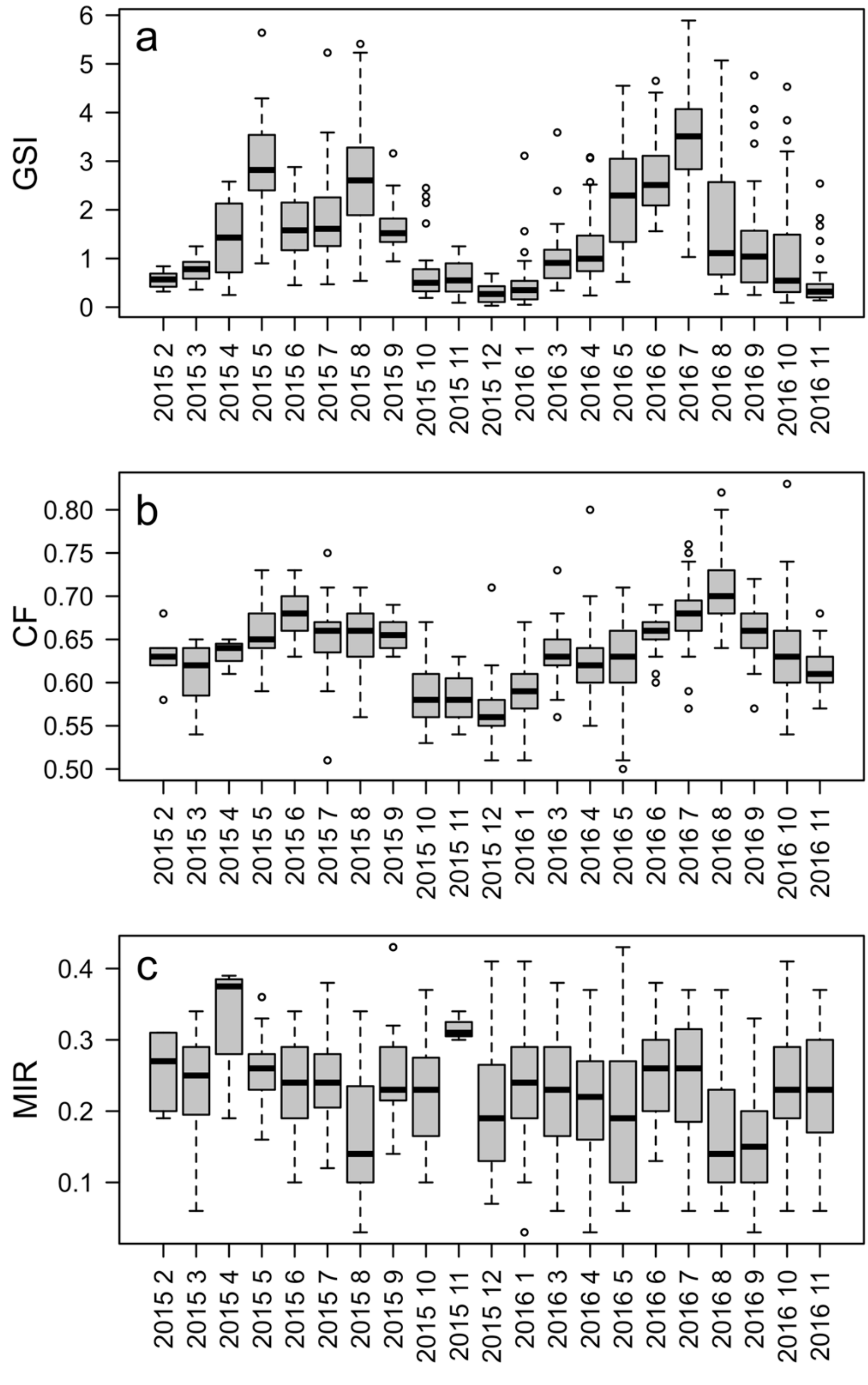


Figure 4.6. Boxplot of temporal trend (monthly) for fishes of age class 1: (a) gonadosomatic index (GSI); (b) condition factor (CF); (c) and otolith marginal increments (MI).

Otoliths edge seasonal growth pattern (EA)

The inspection of the morphology of the otolith edge in 1 year old fishes showed a clear yearly cycle (Figure 4.7). In both years, the opaque growth (O+OH) resumes in March, and until September–October in above 90% individuals. The application of GAM to model the probability to find a hyaline band using CF, GSI and month as predictor corroborated a significant effect ($p < 0.001$) for month only in both years. Explained deviance was 43.6% in 2015 and 18.6% in 2016. Model residuals were checked for autocorrelation and analysis results corroborated the presence of a weak autocorrelation for specific lags. Anyway, the maximum observed significant correlation values were respectively 0.25 in 2015 and 0.23 in 2016 model and were thus considered as negligible. Opposite (but coherently) to the opaque growth pattern, the shape of the relation of hyaline margin occurrence showed in both years a common temporal pattern (Figure 4.8), with a clear minimum in July/August, and higher values between November and January.

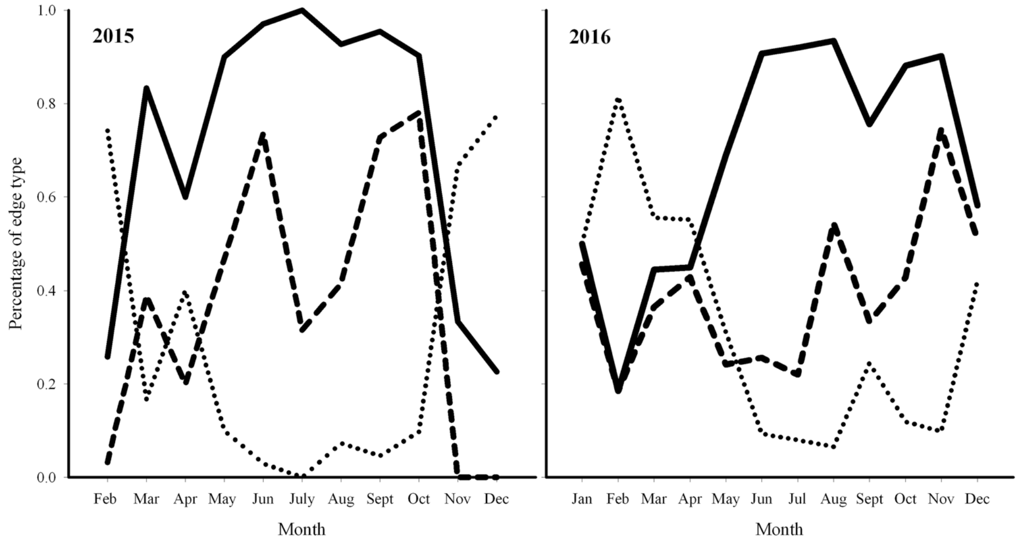


Figure 4.7. Monthly evolution (%) of edge morphology in 2015 (left panel) and 2016 (right panel) for fishes of age class 1: opaque plus semihyaline edges (O + OH, merged according literature[14], solid line); hyaline edge (H; dotted line); opaque edge (O; dashed line).

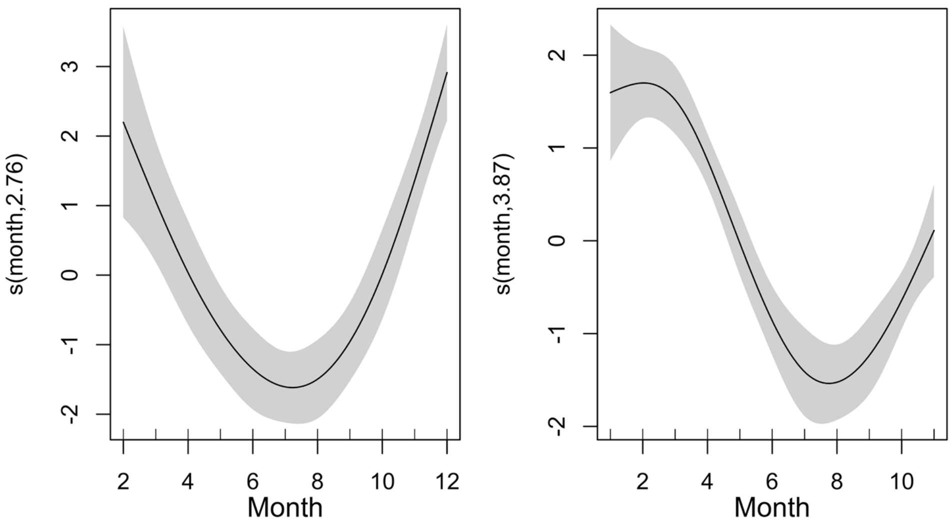


Figure 4.8. Plots of the fit (GAM) between the probability to find a hyaline margin and month. Shaded (light grey) regions represent the confidence bands (2 SE) for smooths. Model-predicted presence of translucent otolith edges in 2015 (left panel) and 2016 (right panel).

Translucent zone completion analysis (TCA)

The back-calculation estimated parameters, used to obtain the fish length at hyaline complete formation, were computed by fitting a linear model for each year (to avoid bias due to the larger number of fishes in 2016 dataset). The obtained relationships were TL = 69.66 * TR + 20.94 (r^2 = 0.74) in 2015 and TL = 71.22 * TR + 19.99 (r^2 = 0.67) in 2016.

The completion date frequency distribution of the three translucent zones (T1, T2 and T3) identified two different cohorts in both 2015 and 2016, which present quite similar monthly distribution in the two years. Namely, the ring closest to otolith core (T1) spanned from May–June to October-November with clear peaks in June, July and August (Figure 4.9a). The second translucent zone (T2) completion spanned mainly between September and April of the subsequent year, with maximum in December–January (Figure 4.9b). Finally, the third translucent zone (T3) distribution showed starts to be completed not before May, while ending in October until February of the following year. Larger proportions of T3 completion were recorded in July–August (Figure 4.9c).

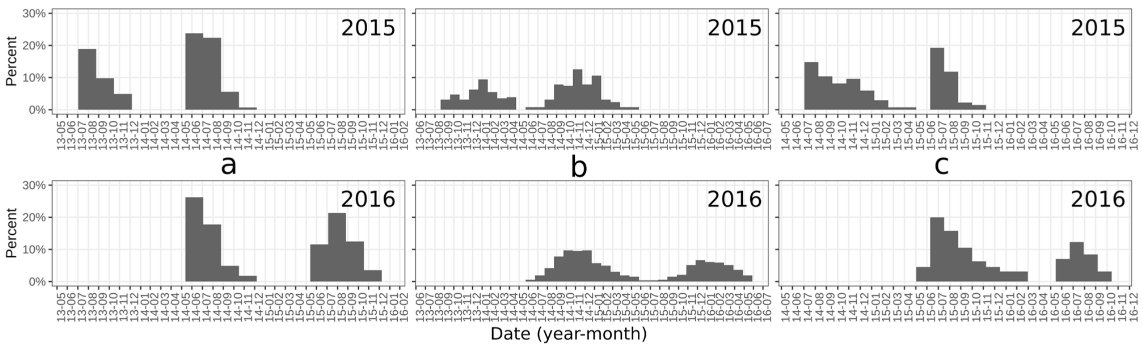


Figure 4.9. Completion date frequency distribution (TCA) of the analysed translucent zones showing two cohorts per sampling year respectively in 2015 top panel, and 2016 bottom panel. The three different translucent zones were plotted respectively T1 in panel (a); T2 in panel (b); and T3 in panel (c).

Marginal increment width seasonal variability (MIA)

Marginal increment analysis of otolith measurements from the two analysed years was obtained only for age 1 class anchovy, according to the recommendations to obtain a robust application of MIR to validate annuli[3,4,52]. Analysis of the MI variability through the year revealed that the minimum of marginal width, which is associated to the beginning of the new annulus formation, was recorded in August in both years (2015 and 2016), even if in September 2016 the minimum of MI values still persist (Figure 4.6c and 4.8).

The application of GAM confirmed a significant ($p < 0.05$) effect of month, GSI and CF in both years. Indeed, the best model was in both years the one considering all the terms, while all the reduced models (i.e. the ones considering a smaller number of explicative terms) were characterized by a strong decrease in explained deviance. Both final models were checked for residuals autocorrelation and concurvity. Significant maximum absolute autocorrelation values for residuals were 0.17 in 2015 and 0.1 in 2016 and were considered as negligible. Estimated concurvity ranged between 0.24 and 0.28 for the 2015 model and 0.2 and 0.25 for 2016. The deviance explained by the models was 19.3% in 2015 and 16.7% in 2016, highlighting a certain amount of intrinsic variability due to the large extension of the anchovy spawning period (about 6 months). The shape of relationships was quite consistent between the two years (Figure 4.10), clearly confirming the presence of a well-defined minimum of the MI values in august. CF also showed a consistent pattern with respect to the MIR, highlighting a positive relationship up to CF value between 0.60 and 0.70 where a plateau was reached, while an almost linear positive relationship was observed for GSI.

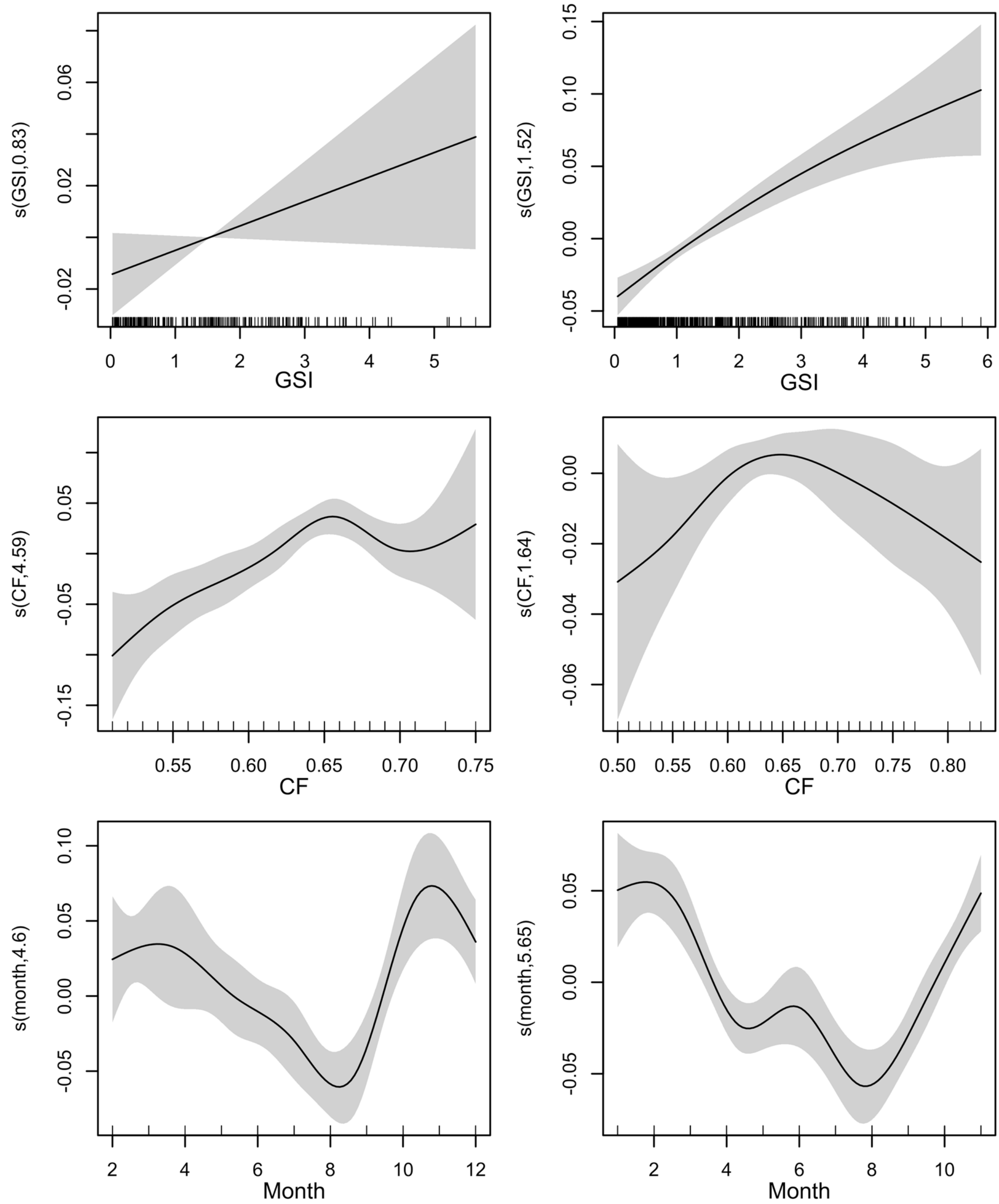


Figure 4.10. Plots of fitted GAMs in 2015 (left panels) and 2016 (right panels) of the marginal increment ratios (MIR) considered as dependent variable and the three considered factors: gonadosomatic index (GSI), condition factor (CF), and month. Shaded (light-grey) regions represent the confidence bands (2 SE) for smooths.

Discussion

In this study, EA and MIA were used to validate the periodicity of otolith increment formation in European anchovy. Annulus formation for this species in the Strait of Sicily occurred in August for both years, even if in 2016 insisted one month more, due to a shift in the spawning period in 2016 compared to 2015, as indicated by the GSI monthly evolution (Figure 4.6a) and by the slightly different position of the minimum in GAMs (Figure 4.8). Since minimal marginal increments on otoliths occurred only once per year (Figure 4.10), the annulus was accepted as valid indicator of age. Moreover, the change in relative frequency of each edge zone (EA) plotted against months, as with MIA, confirmed that the frequency was one cycle per year (Figure 4.7).

The time completion of the first translucent zone showed that in most part of samples T1 is already laid down around summer, which corresponds to the peak of spawning period for this species in the study area[10]. This first translucent zone could be associated to the metamorphosis, which occurs between 40 and 60 days after hatch[29]. This evidence agrees with the time elapsed from the beginning of the spawning period (April) in the study area[10].

The TCA for T2 suggested that its formation would start in spring–summer, since it is completed mostly from September–October (Figure 4.9b). Consequently, this should not be considered a true winter ring (annulus).

The completion distribution of the T3 showed a time shift compared to Bay of Biscay anchovy[24], where the hyaline growth in anchovy juveniles was observed ending in April. Anyway, it is worth noting that the spawning peak of anchovy in the Bay of Biscay occurs between May and June[53]; thus the first annulus formation is completed (T3) one month before the spawning peak. In our study area a similar pattern was observed, since the first annulus start to be completed in May/July, with the spawning peak occurring in late July/August. Numerous studies have reported large variations in seasonality of otolith band formation between species[54], as well as between populations within the same species[55,56], mainly linked to somatic growth[57], reproduction[58], photoperiod[57], and temperature[54,59,60]. In this context, the observed time shift in the completion date of the first winter ring with respect to the one observed in Bay of Biscay anchovy could be due to the combined effect of temperature characterized by higher minima (SST estimates in the study area was never lower than 14°C[10], also during the study period; data not shown) and the shift in the spawning season. Such temporal lag is also indicated by EA, showing the resume of opaque growth zone to begin in March and above 90% of specimens with opaque margin in July (Figure 4.7). Accordingly, the MIA indicated that the annulus formation resulted mostly complete in August (Figure 4.10c), as also suggested by the hyaline edge monthly pattern (Figure 4.8).

A minimal marginal increment on otoliths occurring only once per year has been observed in literature also for anchovy of the Chesapeake Bay[61]. Although different from the European anchovy, such species in the Chesapeake Bay presents its birthdate on mid-July and the annulus formation has been established one month apart (May–June) of the following year. Other studies dealing with MIA on other pelagic species (*Sardinops sagax* and *Trachurus trachurus*), conducted in South African waters, showed the use of marginal increment technique as useful tool for annulus validation[62,63]. A proper validation study would necessitate several evidences from different methodologies and, if possible, by a direct approach (e.g. mark-recapture, modal progression analysis of strong year classes in the catches, etc.). For European anchovy, Aldanondo *et al.*[24] focused their study on validation by means of daily ring analysis in juveniles. Another important result for this species was obtained by Uriarte *et al.*[14] that adopted a specific approach, based on the opportunity to detect a particular abundant year class along the time-series of landings data, which allowed the authors to follow this cohort for the lifespan of the species. The validation framework, proposed in the present study, based on the combined use of MIA and EA, and supported by statistical modelling and translucent zones completion frequency distribution, permits to obtain a robust corroboration of first annulus formation, even when data like those proposed by previous studies are not available (i.e. daily rings and/or long time-series of landings data with a particularly abundant cohort).

Although previous studies exist for European anchovy, such investigations are only conducted on fishes from the Bay of Biscay[14,23,24] and from the NW Mediterranean area[31]. In both these areas the habitat conditions are characterized by higher riverine input nutrients and higher productivity[25,31] than the oligotrophic waters of the study area and of the most part of the Mediterranean Sea[26]. The Strait of Sicily is considered an oligotrophic area[34,35,64], where the enrichment of the upper water layers is associated with upwelling phenomena, which allow nutrient inputs from deeper waters[27,65]. Literature widely reported the variability of somatic growth linked to habitat variability e.g.[24,28,29,64,66,67]. The growth rate of the anchovy in the Strait of Sicily was at the low end of the range observed for this species among different areas, also including the Bay of Biscay and NW Mediterranean[24,31]. Taking into account such aspects, validation studies should be carried out not only at species level but also on different populations of the same species inhabiting areas with different environmental conditions.

Each age validation method has advantages and disadvantages which would be expected to affect the results. In the present study, the analyses were conducted according to the recommendations to obtain sufficient rigor for the validation of the first annulus formation cycle[4]. Both methodologies (EA and MIA) in the two different years converged toward the same result, thus confirming the annulus identification to be correct at least for the first annulus formation. The convergence of independent methods toward the same result, supported by a statistical approach, represents in this context a good indicator to determine the robustness of the age validation. The relative scarcity of validation studies on wild fish therefore suggests that all available data and methods should be used to provide valuable support for age validation in the meantime that new validation studies are designed and implemented to confirm the accuracy of an age estimate[4,13,14].

For references see the manuscript here[1].

4.3 Tyrrhenian Sea South (GSA 10)

Verification, Corroboration and Validation of the Anchovy (*Engraulis encrasicolus* (Linnaeus, 1758)) age analysis in the Central-Southern Tyrrhenian Sea (West Mediterranean). Pierluigi Carbonara, Loredana Casciaro, Michele Palmisano.

The validation of growth of European Anchovy (*Engraulis encrasicolus* (Linnaeus, 1758)) presents several gaps, despite the age and growth has been widely studied using different methods. The uncertainty in estimating the age of anchovy by otolith reading is linked to identification of the first growth ring; ii) a certain number of false increments; iii) disagreement considering the age scheme (e.g. theoretical birthdate); and iv) the overlapping of the annulus in the older specimens. The analyses of the otolith margin type elucidate the deposition patterns of the annuli. The modal components of the length–frequency distribution analysis (LFDA) was identified in the winter survey (Bhattacharya methods), and they did not show significant differences from the length back-calculation of the winter annuli. Moreover, no significant differences were found between the growth curves calculated by otolith reading (back calculation and direct otolith reading) and the LFDA. The agreement between the length–frequency results and the otolith age estimation either corroborated or indirectly validated the growth pattern estimated in the otoliths of European anchovy, mainly when the direct validation methods (e.g. mark and recapture, captivity, radiochemical) were difficult to implement, like the case of this species. The comparison and discussion of the results of the present work to previous Mediterranean studies was conducted.

[1] https://www.nature.com/articles/s41598-020-58174-5

5 ToR c: Estimate (relative) accuracy and precision of anchovy age determination in the main European fishing areas

The main results of the exchange of 2018 are resumed in this part of the report. A complete report is available online here[2].

5.1 Exchange 2018 results

During the WKARA2 (ICES, 2017) a significant effort of standardization of procedures among different labs and groups for age determination was conducted. These efforts produced a general agreement on anchovy growth patterns among areas (both Mediterranean and Atlantic waters) and common reading criteria were adopted. According to all these new insights, along the meeting it was proposed to assess if the mentioned efforts finally produced an increase of agreement among readers and labs compared to the previous exchange. The aim of such exchange was 1) Evaluate if the updated Age reading protocol in WKARA2 have been adopted by all readers (at least the participants in WKARA2). 2) Evaluate if the accuracy and precision in otolith age reading of anchovy among readers of fishery and surveys samples throughout the year has improved. The achieved results have been presented at WGBIOP 2018 (ICES, 2018b).

The exchange, held from April to September 2018, involved 25 readers from 15 labs and 10 Countries (Denmark, Germany, England, France, Spain; Portugal, Tunisia, Italy, Croatia, and Greece). From all readers fourteen readers have a long time experience reading anchovy otoliths (experts); seven was intermediate and four trainees. Thirteen of the 25 readers also took part in the last anchovy workshop (WKARA2; ICES, 2017), representing the 52% of the total readers of this Exchange, and twelve readers attended the exchange directly without participating in the WKARA2 (48%).

A total 160 images of anchovy otoliths were selected from two areas, the Bay of Biscay and the Strait of Sicily. These areas have been chosen for the following reasons: 1) The Atlantic and Mediterranean areas are represented with these two stocks; 2) They have differences in the complexity of otolith interpretation: easier otoliths of the Bay of Biscay than those of the Strait of Sicily; 3) different conventional birth date are used: 1 January in the Bay of Biscay and 1 July in the Strait of Sicily and 4) by practical logistical reasons, more simple and quick to obtain the images for the exchange since the coordinators are involved in these areas. A protocol for the exchange of age readings was provided to all participants (including WKARA2 age reading protocol).

Comparing the results of Exchange 2018 with that of 2014 for all readers, there has been a small decrease of the overall level of agreement and a decrease of CV in those areas that were analysed in the two exchanges. For the Bay of Biscay stock readers there is no variation from one exchange to another with a high PA and low CV in the two exchanges. For the anchovy of the Strait of Sicily there is no improvement for the expert's readers. Restricting the comparison to those who participated in the 2014 exchange (and in WKARA) no improvement is seen either (similar PA for the case of the Bay of Biscay and some decline of agreement in GSA16), with a bit greater variability --CVs -- in the two areas. This leads to conclude that no improvement can be noticed in general in agreement and precision, nor for all readers neither for the WKARA readers.

[2] https://smartdots.ices.dk/SampleImages/2018/81/Anchovy%20Exchange%202018%20report_FINAL_20032019.pdf

Despite not having met the quality standards for age determination agreed in WKARA2, and of not having noticed any improvement vs. the 2014 exchange, it seems that many readers and mainly those who attended the WKARA2 tend to follow the same growth pattern in the otoliths of the two areas when interpreting the winter marks. This is supported by the rather high consistency achieved in the analysis of distance of winter marks from the core of otolith in both areas.

Finally, regular exchanges have been recommended to improve the agreements between readers across and within areas. Further validation studies are encouraged: research by micro-increments counting on several selected otoliths by areas to validate first annual winter mark and other validations and corroboration methods (as progression of length frequency modes throughout time to track cohorts, corroboration of inner consistency of age determination by following cohorts in catches and surveys and studies on the seasonal formation of hyaline and opaque edges).

5.2 Exchange results multiparameter analysis

Explorative analysis on *Engraulis encrasicolus* aging data variability. Pierluigi Carbonara, Walter Zupa, Walter Basilone, Begoña Villamor.

The uncertainty in age estimation by otolith reading may be at the source of the large variability of Anchovy (*Engraulis engrasicolus* (Linnaeus, 1758)) growth models in both Mediterranean and Atlantic areas. In the 2018 an exchange exercise (2018) was conducted using the ICES platform SmartDots. 160 images of entire otoliths, distributed in 2 samples from Bay of Biscay (ICES Subarea 8) and Strait of Sicily (GFCM-GSA16) and collected by IEO, AZTI and IAMC-CNR. 25 readers from 13 institutes and 10 countries (Denmark, Germany, England, France, Spain, Portugal, Tunisia, Italy, Croatia and Greece) participated to the anchovy reading exercise. Age is assigned using different interpretation schemes, including variations in theoretical birthdate and number of false ring considered before first ring, second ring, 3rd ring, in addition to differences in the experience level of readers, area of expertise (Mediterranean and Atlantic), origin of sample, distance of ring (first, second, third, fourth), sampling quarter of otolith and sex. The present work analysed the influence of these variations and the geographical location of sampling on red mullet aging using a multivariate approach (principal component analysis). Considering all data together the factors more significantly linked to the variability seem to be the distances of the winter rings and the number of false rings before the first winter. The origin of the sample and the birthday are significantly linked to the data variability more than the experience and origin area of readers. Moreover, considering only samples from each origin area (Bay of Biscay and Strait of Sicily) they were obtained the same results. Indeed, the factors significantly linked to the data variability seem to be the distances of the winter rings, the number of false rings before the first winter and among the categorical variable the sex seems the parameter more correlated to the variability. Considering these results, workshops, exchanges and the adoption of a common aging protocol based on age validation studies are considered fundamental tools for improving precision in anchovy aging procedures.

6 ToR c: Identify causes of age determination error

6.1 Intercalibration exercise conducted during WKARA3

During the workshop, no formal exercise of otolith age readings for a new evaluation of agreement, bias and precision was conducted because since the duration and nature of the meeting (virtual) it became evident that there were still discrepancies in otolith interpretation and age determination across the different areas. As a result of this, most of the work conducted during the workshop consisted in common sessions with all attendees going area by area through several otolith case examples to examine and jointly discuss the different otolith interpretations and allocation of ages which resulted in the new exchange proposal to be conducted during 2022 and 2023.

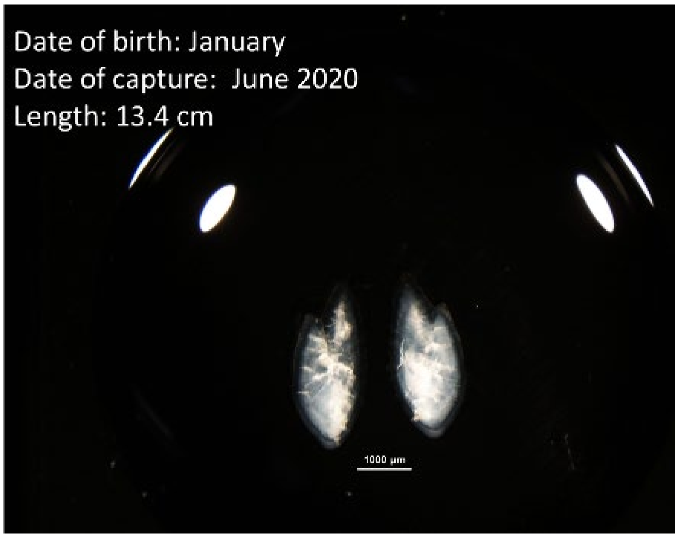
The joint exercise of otolith interpretation and age reading was considered by all workshop attendees as to be of high interest and productive in achieving a greater degree of common interpretation and assignation of ages. In summary, it was noticed that some of the differences in age allocations were due to a wrong application of rules corresponding to the birth date convention, as well as on the adopted age scheme.

However, the discussions on the analysed images during the workshop are briefly resumed as follows:

Experts: Dr Clara Dueñas-Liaño.

Title: "Otoliths images of *Engraulis encrasicolus* of the ICES zone 8c"

The pictures coming from fishes caught in the first semester of the year.



Otolith information: Length 13.4 cm, caught in June, date of birth 1 January

Otolith interpretation: A check and a clear first winter ring, approximately at. 0.8 mm. For Dr Dueñas-Liaño, is Age 1 and the edge is opaque. The distance from the nucleus is coherent. Dr Basilone aske to the other experts if all agreed with this interpretation. Dr Carbonara asks to Dr Dueñas-Liaño some information about the margin because it is not easy to identify it from the photos. In her opinion is difficult to evaluate the margin by the picture, but she confirms that is opaque. From the picture, maybe the rostrum seems hyaline, but looking at the post-rostrum it is clearly opaque. All agreed on that. Dr Basilone highlighted the problem of distinguish the ring from the margin on the border or rather, distinguish the hyaline and semi-hyaline structures. Paying attention to this distinction helps to have ready data for a validation study of marginal morphological aspects.

All experts agreed to assign Age 1 to otolith.

Age determination: 1 winter ring with opaque edge in the first semester, so Age 1

Dr Dueñas-Liaño has some problems with the presentation, so experts decided to move to the next available otoliths collection.

Experts: Dr Andrea Massaro

Title: "Otoliths images of *Engraulis encrasicolus* from GSA 9, Ligurian and Northern Thyrrenian Sea"

The picture coming from a fish caught in the second semester of the year.

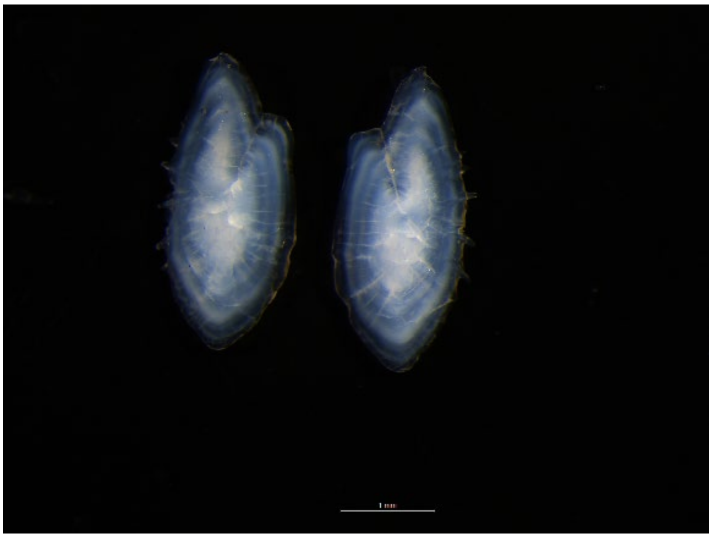
Otolith information: Length 13 cm, female, caught in October, date of birth 1 January

Otolith interpretation: Dr Massaro recognize two hyaline rings, the first one is very clear and well-formed and the second one on the edge. It is possible to identify a thin check near the nucleus. According to the WKARA2 (2016) age scheme, it belongs to Age 1. Dr Dueñas-Liaño and the other colleagues agreed with this interpretation. Dr Basilone read the otolith trying to follow the pattern in WKARA2 age scheme: first growth opaque zone that identify the first summer, after that there is the first true winter ring, a wide hyaline visible all around the otolith, after that there is the opaque growth of current year and finally the hyaline margin (spawning check or second winter ring in formation). This exercise is particularly useful to apply in the reality, and in the biology, the age scheme.

All experts agreed to assign Age 1 to the otolith.

Age determination: 1 winter ring with hyaline edge in the second semester, so Age 1

The picture coming from a fish caught in the second semester of the year.

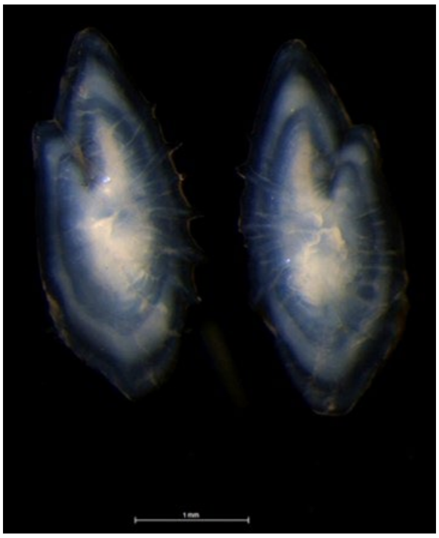


Otolith information: Length 14.5 cm, male, caught in October, date of birth 1 January

Otolith interpretation: Dr Massaro identified the first winter ring, a second winter ring and the hyaline margin, final Age 3. Dr Basilone remember the rules from WKARA2: for multiple rings could be considered as one ring. Dr Dueñas-Liaño said that in their area, they have the same otolith structures, and they consider the multiple rings as only one ring, for this reason in her opinion is Age 2. For Dr Basilone is Age 2 because he sees two clear winter rings. Dr Carbonara pointed out that it is possible to follow the rings all around the otolith and there is a coherent decrease in distance between the winter rings. For this reason, he agreed with Dr Massaro and assigned Age 3. Some bands are more hyaline than other (like in this case), due to the biological or oceanographic conditions. Dr Pulizzi identified one check, first winter ring, opaque band, second winter ring, opaque band and hyaline edge. For this reason, he assigned Age 2. The interpretation is different, but non the final age. For Dr Milhazes, the fish has 2 years, with hyaline edge. Dr Donato agreed with Dr Basilone and Dr Milhazes, so Age 2: check, first ring, opaque zone, second ring, opaque, hyaline edge. For Dr Bled Defruit it is Age 2.

Age determination: 2 winter rings with hyaline edge in the second semester, so Age 2

The picture coming from a fish caught in the second semester of the year.



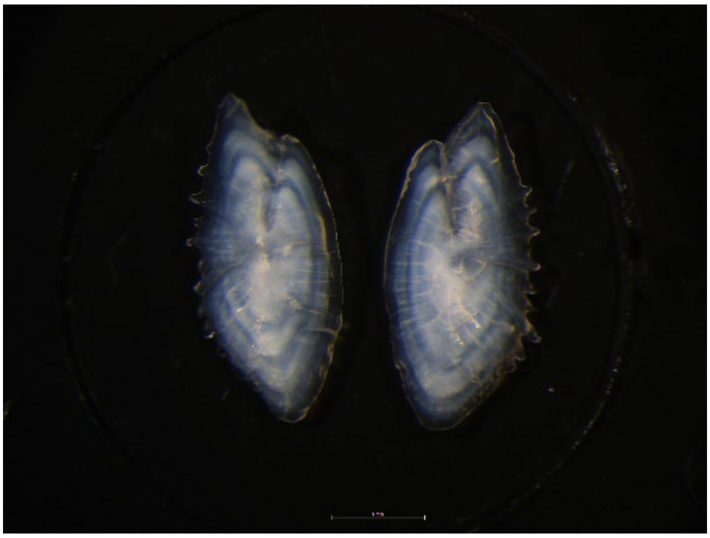
Otolith information: Length 14 cm, female, caught in July, date of birth 1 January

Otolith interpretation: Dr Massaro showed this picture because in this case, it is very clear that the multiple hyaline bands are merged in one. It is evident a first winter ring, an opaque zone, the second winter ring and the edge seems opaque. Age 2. Everybody agreed with this interpretation, but Dr Dueñas-Liaño identified hyaline edge in the rostrum and in the post-rostrum, but being in the second semester, the final age does not change.

All experts agreed to assign Age 2 to otolith.

Age determination: 2 winter rings with opaque edge in the second semester, so Age 2

The picture coming from a fish caught in the first semester of the year.



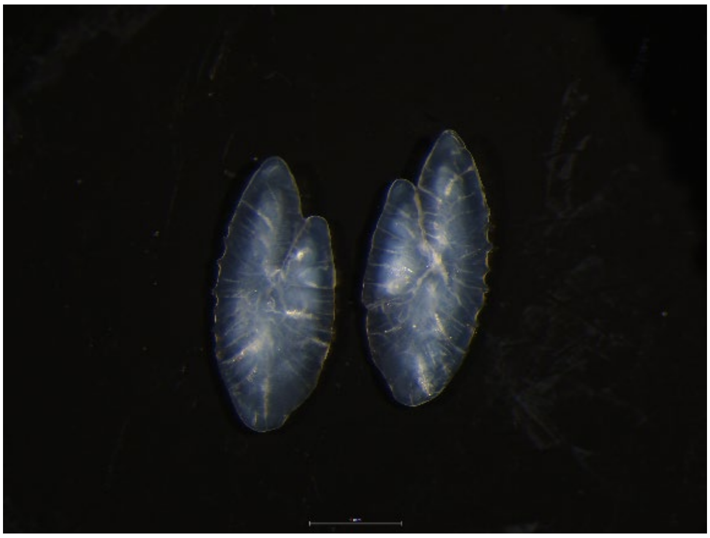
Otolith information: Length 15 cm, female, caught in March, date of birth 1 January

Otolith interpretation: Dr Massaro identified a check, first winter ring, opaque zone with a check, second winter ring, semi-opaque and hyaline edge. Age 3. Dr Donato agreed with that. Also, the distance of the check from the nucleus respects the general rules, because is less than 1 mm. Dr Basilone, after the second winter ring, expects an opaque band that is not clearly visible in this case but only hinted at. This is to consider the margin a real winter ring. At this point, is the edge a real hyaline or a semi-hyaline? Depending on this, the age should be 2 or 3. Dr Carbonara ask to Dr Basilone what the definition of semi-hyaline is. Generally, is a biological definition or rather the spawning check and can be identified inside an opaque band. Looking at the catch month, the edge should be a real hyaline. Dr Tornero use classification of semi-hyaline and semi-opaque for the readings, the same is for Dr Dueñas-Liaño. This helps to understand the otolith from a biological/otolith growth pattern point of view.

All experts agreed to assign Age 3 to otolith.

Age determination: 2 winter rings with hyaline edge in the first semester, so Age 3.

The picture coming from a fish caught in the first semester of the year.



Otolith information: Length 12 cm, male, caught in June, date of birth 1 January

Otolith interpretation: Dr Massaro assigned Age 1: one thin check, first winter ring and opaque edge.

All the experts agreed with the reading of Dr Massaro and assigned Age 1 to otolith.

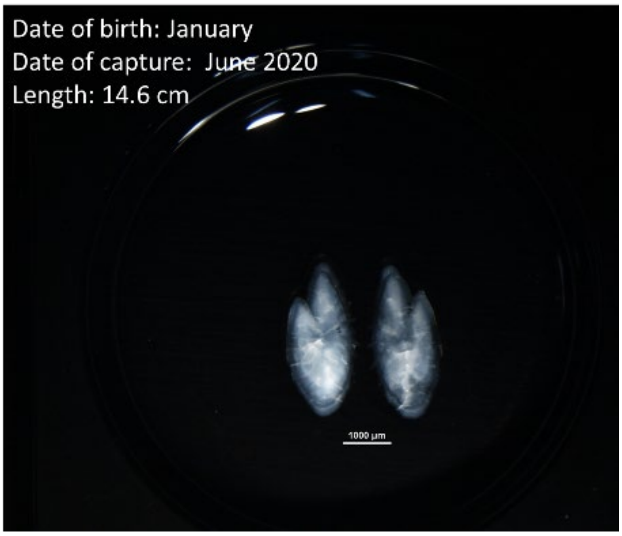
Age determination: 1 winter ring with opaque edge in the first semester, so Age 1

At this point, the pictures from ICES fishing area 27.8.c are taken again.

Experts: Dr Clara Dueñas-Liaño

Title: "Otoliths images of *Engraulis encrasicolus* of the ICES zone 8c"

The pictures coming from fishes caught in the first semester of the year.



Otolith information: Length 14.6 cm, caught in June, date of birth 1 January

Otolith interpretation: For Dr Dueñas-Liaño is clearly visible only one winter ring and opaque edge.

All experts agreed with this interpretation and agreed on assign Age 1 to otolith.

Age determination: 1 winter ring with opaque edge in the first semester, so Age 1

The pictures coming from fishes caught in the first semester of the year.

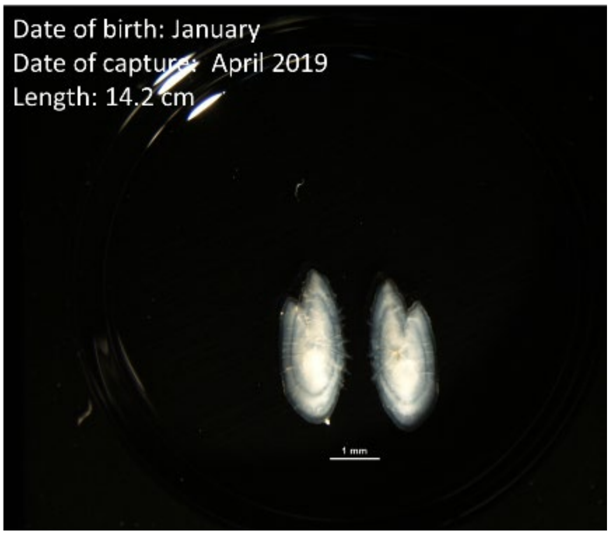
<u>Otolith information</u>: Length 15.8 cm, caught in March, date of birth 1 January

<u>Otolith interpretation</u>: In this case, for Dr Dueñas-Liaño the first winter ring is not clearly visible, only the hyaline edge as second ring. Due to catch date and the margin is Age 2.

All experts agreed to assign Age 2 to otolith.

<u>Age determination:</u> No visible ring inside the otolith, only hyaline edge which in the first semester assigns the otolith to Age 2.

The pictures coming from fishes caught in the first semester of the year.



<u>Otolith information</u>: Length 14.2 cm, caught in April, date of birth 1 January

<u>Otolith interpretation</u>: Dr Dueñas-Liaño identified first winter ring, opaque band with spawning check, second winter ring and opaque edge. Following the growth pattern is easier looking at left otolith.

All experts agreed to assign Age 2 to otolith.

<u>Age determination:</u> 2 winter rings with opaque edge in the first semester, so Age 2.

The pictures coming from fishes caught in the first semester of the year.

Otolith information: Length 14.7 cm, caught in March, date of birth 1 January

Otolith interpretation: Dr Dueñas-Liaño identified a thin check, first winter ring, opaque band with spawning check and hyaline edge as second ring, Age 2. The second check seems a real winter ring, but in the opinion of Dr Dueñas-Liaño the growth pattern of opaque band is not respected, it is too small for represent an annual growth Due to catch date and the margin is Age 2. Dr Basilone agreed with this interpretation. Dr Bellodi ask if it is possible to consider the spawning check merged with edge (or second winter ring) as the second ring, due to the catch date too. It is possible, but the final age is the same. Dr Donato, Dr Milhazes, Dr Pulizzi saw two real winter rings and opaque edge.

All experts agreed to assign Age 2 to otolith.

Age determination: 2 winter rings caught in the first semester, so Age 2.

The pictures coming from fishes caught in the first semester of the year.

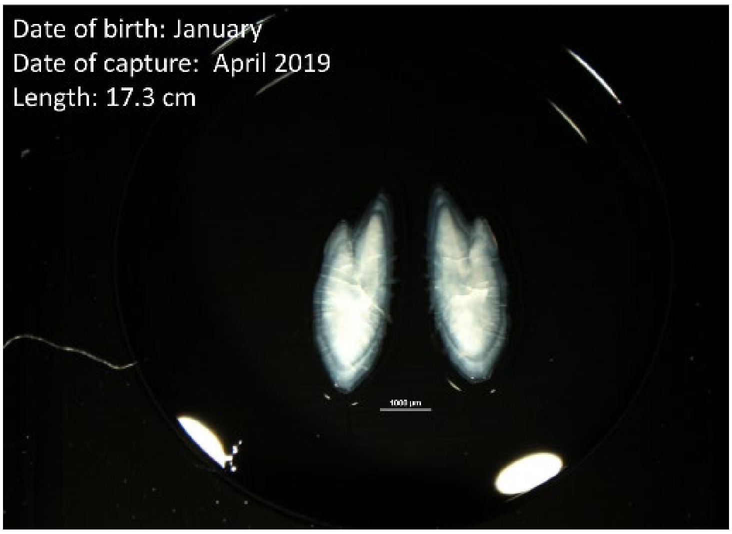
Otolith information: Length 15.7 cm, caught in April, date of birth 1 January

Otolith interpretation: Dr Dueñas-Liaño identified first winter ring, opaque band with spawning check, second winter ring and opaque edge, Age 2.

All experts agreed to assign Age 2 to otolith.

Age determination: 2 winter rings with opaque edge in the first semester, so Age 2.

The pictures coming from fishes caught in the first semester of the year.



Otolith information: Length 17.3 cm, caught in April, date of birth 1 January

Otolith interpretation: Dr Dueñas-Liaño identified first winter ring, opaque band with spawning check, second winter ring and opaque edge, Age 2. Dr Basilone said that from this picture is exceedingly difficult to follow all around the otolith the second winter ring, but it is visible in the rostrum.

All experts agreed to assign Age 2 to otolith.

Age determination: 2 winter rings with opaque edge in the first semester, so Age 2.

The pictures coming from fishes caught in the first semester of the year.

Otolith information: Length 17.6 cm, caught in May, date of birth 1 January

Otolith interpretation: Dr Dueñas-Liaño identified first winter ring, opaque band with spawning check, second winter ring, opaque band with spawning check and opaque edge and 3rd winter ring with opaque edge, so Age 3.

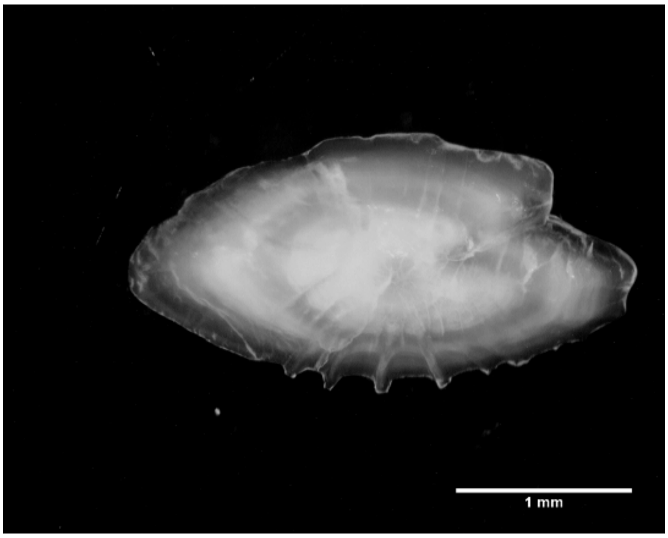
All experts agreed to assign Age 3 to otolith.

Age determination: 3 winter rings with opaque edge in the first semester, so Age 3.

Now, otolith collection from GSA19.

Experts: Dr Pierluigi Carbonara, Dr Loredana Casciaro, Dr Michele Palmisano.

The picture coming from a fish caught in the second semester of the year.



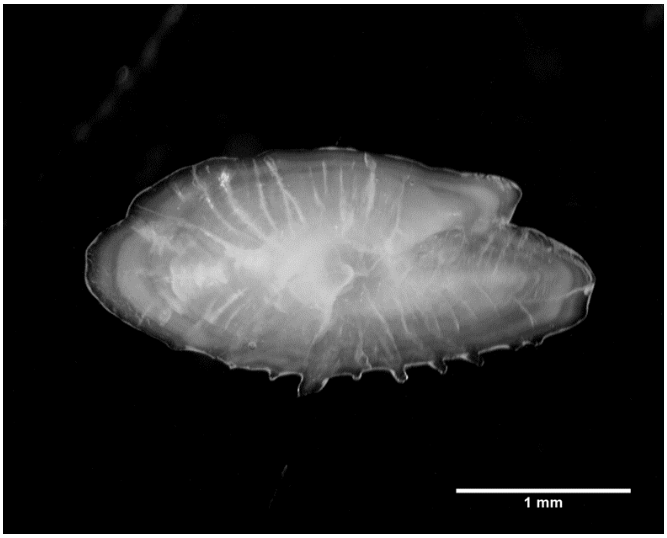
Otolith information: Length 13 cm, female, caught in May, date of birth 1 January

Otolith interpretation: Dr Casciaro identified two rings, but the first one maybe is a check. The edge seems opaque. Dr Bultó, Dr Donato, Dr Tornero, Dr Torres assign Age 1 to the otolith, a check, first real winter ring and opaque edge.

All experts agreed to assign Age 1 to otolith.

Age determination: 1 winter ring with opaque edge in the first semester, so Age 1.

The pictures coming from fishes caught in the second semester of the year.



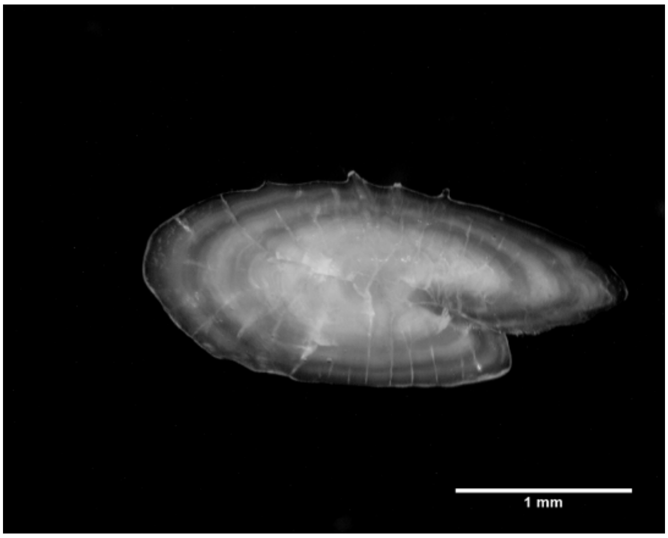
Otolith information: Length 12 cm, male, caught in August, date of birth 1 January

Otolith interpretation: Dr Casciaro saw two winter rings, hyaline edge. A clear check is visible before the first winter ring. For her is Age 3. Dr Donato identified a check, a first winter ring, opaque band with a spawning check and hyaline edge. Dr Dueñas-Liaño, Dr Basilone agreed with Dr Donato.

All experts agreed to assign Age 1 to otolith.

Age determination: 1 winter ring with hyaline edge in the second semester, so Age 1.

The pictures coming from fishes caught in the second semester of the year.



Otolith information: Length 11 cm, female, caught in August, date of birth 1 January

Otolith interpretation: Dr Casciaro identified one check, the first winter ring, opaque and hyaline edge and assigned Age 2. Dr Donato saw one check, first winter ring, opaque band and hyaline edge (spawning check), so Age 1.

All experts agreed to assign Age 1 to otolith.

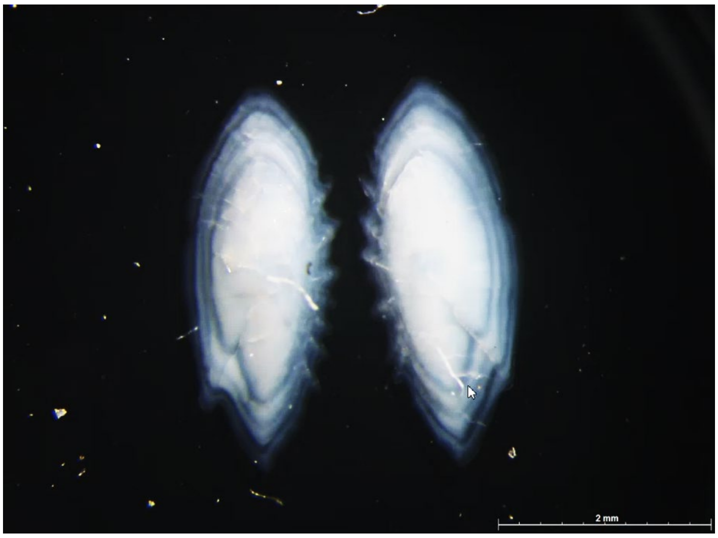
Age determination: 1 winter ring with hyaline edge in the second semester, so Age 1.

After the Italian collection, otoliths from ICES fishing area 27.9.a

Experts: Dr Dina Silva, Dr Raquel Milhazes, Dr Jorge Tornero

Title: "Otoliths of *E. encrasicolus* from ICES fishing area 27.9.a"

The picture coming from a fish caught in the first semester of the year.



Otolith information: Length 15.8 cm, caught in February, date of birth 1 January

Otolith interpretation: Dr Milhazes assigned to this otolith Age 3. Dr Donato saw first winter ring, opaque band with spawning check, second winter ring, opaque ring and hyaline edge. Due to the edge, the final Age is 2: 2 winter rings plus hyaline edge. Dr Basilone highlighted that the edge is not noticeably clear and for this reason, the perception of the final Age is not fixed. Dr Bultó ask why the spawning check is not considered as real winter ring. Dr Milhazes answered that the distance between the two bands it is too small, and the pattern is clearly related to a false ring. Dr Pulizzi identified first winter ring, opaque with false ring, second ring, opaque edge so Age 2. Dr Massaro agreed with Dr Milhazes, the edge is hyaline. Dr Carbonara and Dr Basilone assigned Age 2 to the otolith, because they did not consider the edge. Most of readers agreed with the readers that assigned Age 3 because they considered the hyaline edge.

<u>Age determination</u>: 2 winter rings and hyaline edge in the first semester, so Age 3.

The picture coming from a fish caught in the second semester of the year.

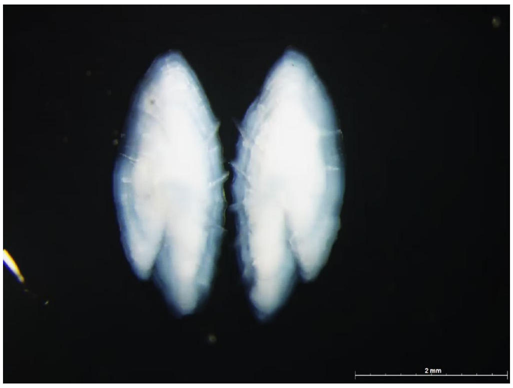
<u>Otolith information</u>: Length 15.1 cm, caught in August, date of birth 1 January

<u>Otolith interpretation</u>: For Dr Tornero is Age 0, but the length of the fish is 15.1 cm. It is exceedingly difficult to identify rings within this otolith. Dr Basilone agreed with Dr Tornero, but due to the length it should be at least Age 1. Dr Massaro identified a winter ring as Dr Milhazes, with opaque edge. The distance of the first winter ring and the nucleus is approximately 1 mm, so should be real. Dr Rico classified the otolith as "unreadable" otolith, so it is quite impossible to correctly identify the age. Dr Dueñas-Liaño saw hyaline border (spawning check) and in her opinion is Age 2 (1 winter ring+hyaline edge). Dr Torres and Dr Bultó marked this picture as unreadable. Dr Carbonara asked if the otolith is fixed in resin and the Dr Milhazes answered yes. Dr Carbonara highlighted the importance of the storage. Before including them in resin or before putting them in cuvettes, experts should be sure that the otolith is completely dried.

No agreement was reached, due to the bad condition of the otolith. Several experts marked this one as unreadable.

<u>Age determination</u>: Age 1 looking at the length, NA looking at the otolith.

The picture coming from a fish caught in the first semester of the year.



Otolith information: Length 17.1 cm, caught in February, date of birth 1 January

Otolith interpretation: Dr Silva said that this otolith is extremely hard to read, and she put a bit of water inside the resin to better identify the ring. Dr Milhazes agreed with Dr Silva and assigned Age 1. Dr Donato saw the first winter ring all around the otolith and after it a second opaque band with many thin hyaline bands inside (all checks). After that, Dr Donato identified a second winter ring and the edge that seems hyaline, at least Age 3 due to the catch data. Dr Dueñas-Liaño agreed with Dr Donato. Dr Carbonara agreed with the interpretation of Dr Donato. Dr Basilone stated that from his side the edge is opaque. Dr Rico agreed that is Age 3 and with hyaline edge. Dr Milhazes agreed with Dr Donato.

All experts agreed to assign Age 3 to otolith.

Age determination: 2 winter rings with hyaline edge in the first semester, so Age 3.

6.2 Common causes of age determination error identified during the WKARA3 meeting from the 2018 Exchange

6.2.1 A detailed analysis of the discrepancies between readers in otolith interpretation during the exchange held in 2018

In the 2018 exchange exercise there can be several reasons that might explain the agreement and discrepancies appearing in the exchange: a major reason (already mentioned in the 2016 workshop) could be the difficulty of correctly applying the age determination rule for the first half of the year to fishes with birthdate in July (GSA16Anchovy). This might have been amplified in the 2018 exercise because of the inability of SmartDots application (http://www.ices.dk/marine-data/tools/Pages/smartdots.aspx), to properly assign the age according to the number of marks of true winter rings during the first half of the year for fishes with birthdate at the middle of the year (because SmartDots presumes that birthdate is first January). In addition, the reasons already highlighted in last exchange/workshop are still appearing now, as for instance: a) Difficulties in differentiating between true annual rings and false rings (or checks), b) Insufficient typical annual growth pattern recognition and insufficient criteria regarding the otolith edge that can be expected to be seen along the year.

These evidences highlighted the need to review the convenience of setting date of birthdate at the middle of the year for anchovies in some Mediterranean areas and to consider to move it to first January, because of the difficulties perceived during the exchange on the application of a changing rule for the first and second halves of the year (as associated to birthdate first July) for the stocks in the northern hemisphere (where winter marks are laid down around January-February) and also for simplicity and coherence in naming age classes in correspondence with the year classes used in most of the assessments, based on natural calendar year (Jan-Dec). In addition, this adds difficulties when organizing exchanges and workshops because readers are familiar with one or another way of applying the age determination rules for allocating otoliths to age groups and this tend to increase the discrepancies in age determinations resulting in lower PA and greater estimates of CVs in these exercises.

Finally, the experts involved in the exchange agreed about the opportunity to obtain a good estimate of the actual age reading uncertainty in a stock assessment it is important that all age readers who supply data to the assessment participate in these exchanges. Furthermore, the exchange set should cover all ages, seasons and areas included in the assessment adequately (ICES, 2018c).

6.2.2 Main conclusions from 2018 Exchange

Overall agreement between all readers and areas is very low and remarkably similar (slightly lower) than in 2014. The results of the stock readers group are much better than the other groups of readers (including advanced and expert group), for Bay of Biscay readers and Strait of Sicily readers (91% and 96% of agreement; CV of 9% and 9%, respectively).

The analysis of distance of winter marks from the core of otolith in both areas show that globally most readers similarly place the winter rings. This means that the growth pattern is being commonly identified in both areas (with some higher discrepancies in the Bay of Biscay).

7 ToR c: Update age reading protocol for European anchovy

The result from Exchange reading exercise in 2018 and the comment during the present workshop suggests maintaining the protocol agreed during ICES WKARA2.

Also, the aging scheme was confirmed, while the next Exchange planned for 2022–2023 would provide more information on the possible adoption of the new scheme proposed from a previous working group (WKAGESP, Basilone *et al.*, 2019) and discussed during the WKARA3 meeting (Figure 7.1).

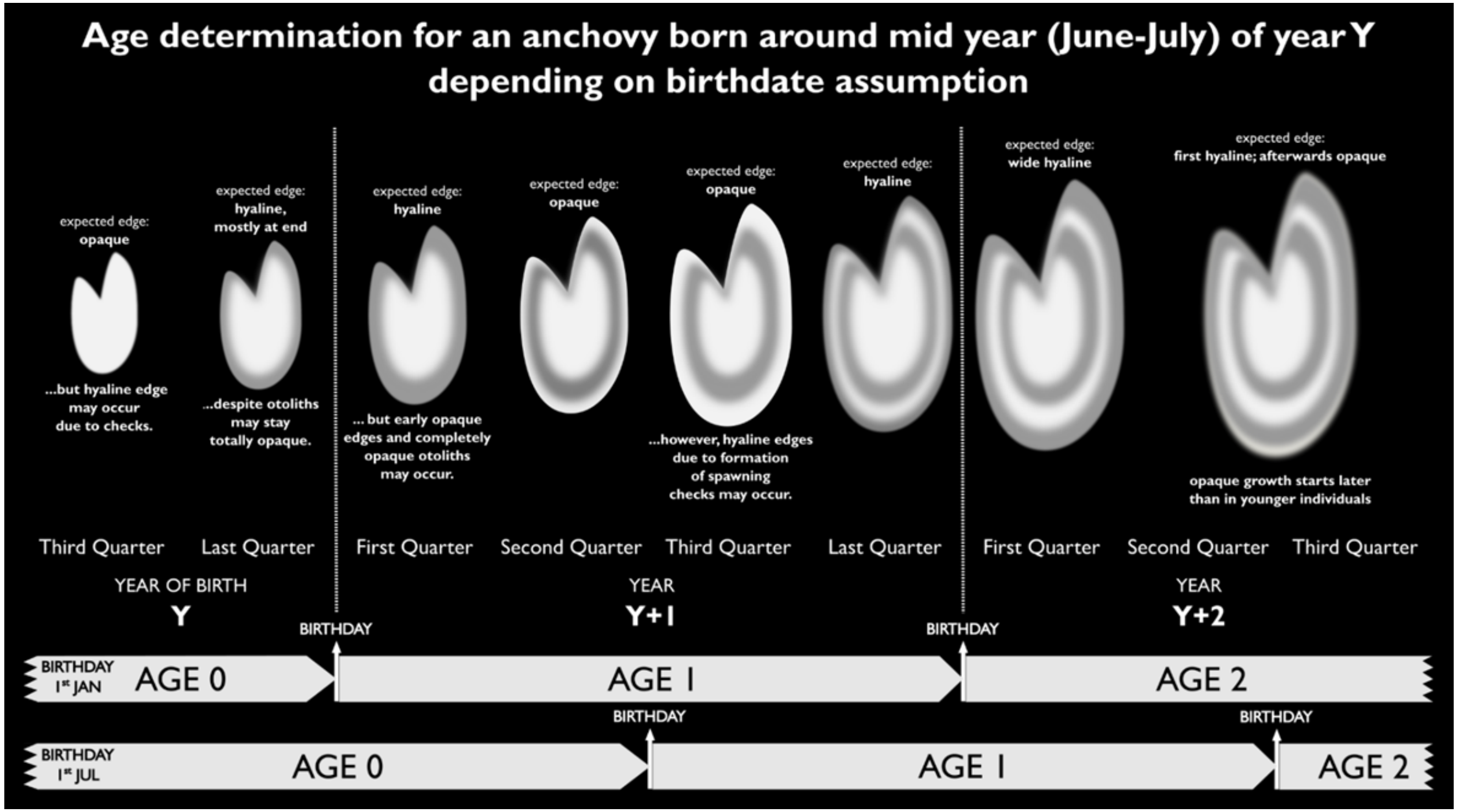


Figure 7.1. Synoptic representation of the anchovy otolith development in time and the different age allocation according to the two conventional birth dates on 1 January and on 1 July.

8 ToR d: Update otolith references collection and a database of otolith images

Given that during the meeting there were not presented new otolith the ref collection does not change.

9 Recommendations

Since a calibration exercise was not performed during the WKARA3, the WG recommend to start a new exchange to be carried out during 2022–2023 via SmartDots platform in order to see if the age reading protocol have been adopted by all readers and to see if the accuracy and precision continue to improve. Also, other aspects as the mounting method effect on age assignment variability will be carried out.

The WG recommend to the labs involved to perform at least a marginal analysis in the mean while they are collecting more data to apply more robust methods to validation/corroboration of the deposition pattern of annual rings, as well as the first annulus formation

Given that all the participants agreed on the opportunity to adopt 1 January as the conventional birthdate for anchovy, the WK recommend that for data collected for stock assessment purposes to adopt only this conventional birthdate discarding 1 July as clearly stated in the WKARA2 report. However, 1 July will maintain its value for biological studies, where the adoption of a conventional birthdate far from the real one would be misleading.

During the WKARA3 a new scheme (upgrading WKARA2 scheme) was presented and discussed for its adoption. The participants agreed to better evaluate how the adoption of the new proposed scheme may affect the age precision and accuracy during the next Exchange program (2022).

The WG recommend having a new Workshop on Age determination of Anchovy in 2023 to be held in Sicily.

Recommendations to other ICES WG and GFCM:

- Inter-calibration exercises by areas (for the different countries taking part in otolith age reading on the same stocks) are required. This becomes compulsory for regions where several countries exploit the same stock.
- It is recommended that, as far as possible, only the age readings of the most expert readers are used for the assessment inputs and second that new readers pass a training process from validated set of otoliths of the area they must work with. WKARA3 agreed on previous suggested threshold values of agreements around 80% and of CVs around 20% in the training process as a minimum for age readers to be operative to deliver inputs for assessment (ICES, 2018).
- Production of a collection of age validated otoliths by areas (or at least of agreed age determination by experts) is recommended for the purposes of helping in the training of new age readers.
- And finally, it is also recommended to have regular exchanges, both internally and externally, to learn and to improve the agreements between readers across and within areas.

10 Planning for future WKARA meetings

A new exchange after 2022 is recommended to see if the accuracy and precision continue to improve, and to fill the still present discrepancy, due to different sampling area (particularly, Atlantic and Mediterranean) as well to different embedding medium. The idea of this exchange program is to integrate and complete the previous database, obtained during the former exchange programs. A possibly dates for the exchange would be at the end of 2022 to early 2023. It is desirable that at least the same people attending the workshop take part of the exercise.

For such exchange new chairs to join the work with Gualtiero Basilone have been proposed. Particularly, Ilaria Costantini (CNR-IRBIM, Italy) and Carmen Hernàndez (IEO, Spain) expressed their interest on such role.

Following the recommendation to have regular exchanges, to improve the agreements between expert readers, as well the need of increase the validation studies across the areas and laboratories, it was agreed by the group to fix a new WKARA meeting in 2023.

Also in this case, new chairs for collaborate with the present one (i.e. Gualtiero Basilone) have been proposed. Two people expressed their agreement on acquiring this role, particularly Ilaria Costantini from Italy and Carmen Hernàndez from Spain. These new proposed entries would allow also to have, as coordinators, laboratories operating in both the Atlantic and Mediterranean areas.

11 Conclusions

Indirect validations (marginal increment analysis, length frequency analysis, progression of year classes, Daily increments between annuli) for the annual age determination of European anchovy have been applied in some new areas/stocks, but the majority of works attempting to validate annuli of anchovy are still not complete or published on a peer review ISI journal as it is expected at least this as quality indicator to be officially adopted among the studies evaluated by WKARA working group. So far, there are only three areas/stocks (Bay of Biscay, Northern Western Mediterranean and the Strait of Sicily) where more accurate validation methods have been published on ISI journal (Morales-Nin and Pertierra, 1990; Aldanondo *et al*., 2016; Uriarte *et al*., 2016; Basilone *et al*., 2020). According to discussion and presentations done in the present meeting the work Basilone *et al*. (2020) may provide a useful guideline for the adoption of a holistic approach to increase the quality of the validation conducted as well as to increase the statistical robustness and comparability of results.

During the meeting other two new corroboration/validation studies were presented for areas where not previously validation studies exist (GSA 9.a, Gulf of Cádiz and GSA 10, southern Tyrrhenian sea). Since these studies are still ongoing and not published yet, the WG agree on the promising results and strongly encouraged their publication on ISI journals to be included and accepted among the validation studies accepted by the group.

Moreover, since there are several areas/stocks for which validations for anchovy annual age determination are still lacking, for these areas the working group agree that the validation of the age determination procedures should be mandatory to assure the quality of the age structured inputs for the assessments in those areas.

Major difficulties appearing in the age assignation arise from:

- Differentiating between true annual winter hyaline rings and false rings (or checks), mainly the first annual winter ring;
- Recognizing weakly formed winter rings which masks the general pattern of growth;
- Recognizing the opaque or hyaline nature of the edge which may affect the age determination.

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Annex 2: WKARA3 agenda

	23 November 2021
9:30 – 10:30	Opening of the meeting; presentation and adoption of the agenda; brief overview of *ToRs*
10:30 – 10:45	*Coffee break*
10:45 – 13:00	Review information on age determination, otolith exchanges, workshops and validation work done so far (*ToR a)* *- Protocols used by each laboratory and conventional birthday* *- Past workshop: The first Italian National workgroup on age reading of small pelagic fishes WKAGESP 2019.*
13.00-14.30	*Lunch break*
14:30 – 16:00	Presentation of new Validation studies and growth increment patterns of the different areas. (*ToR* a and b*)*

	24 November 2021
09:00 – 11:00	Presentation and discussion of the 2018 otolith exchanges results, comparison of precision against modal age and bias; evaluation of levels of agreement among readers and institutes (*ToRs a and c*)
11:00 – 11:15	*Coffee break*
11.15 – 13:00	Analyse the results of the exchanges carried out in 2018 and the potential source of discrepancies (*ToR* c)
13:00-14:30	*Lunch break*
14:30 – 16:00	Discussing the main sources of discrepancy in age interpretation as highlighted by a multiparameter analysis. *(ToR b and c)* *Setting of a new exchange programme: date, participants, deadline, data selection etc.* New chairs candidates for next Workshop and exchanges programme
	Update age reading criteria (if needed) based on the new validation studies results, growth increment pattern and the exchange results (*ToR b*) We agree do not change.

	25 November 2021
09:00 – 11:00	Increase existing reference collections of agreed aged otoliths by stocks and areas (*ToR* d) Each lab discussing the otoliths to be added to the collection of agreed otolith based on a shared discussion in the meeting. Easy, intermediate, and difficult interpretation
11:00 – 11:15	*Coffee break*
11:15 – 13:00	Continue to increase existing reference collections of agreed aged otoliths by stocks and areas (*ToR* d)
13:00 – 14:30	*Lunch break*
14:30 – 16:00	Address the generic *ToRs* adopted for workshops on age calibration (see WGBIOP Guidelines for Workshops on Age Calibration) (Tor e)
16:00 – 16:15	*Coffee break*
16:15 – 17:00	Concluding and summing up the Workshop round off and follow up; day’s work, particularly with reference to the Final draft report elaboration. Make recommendations for next exchange programmes, and next WKARA workshop.

Annex 3: Contributions to the workshop: presentations

During the workshop 18 presentations were performed and they are deposited in the SharePoint of ICES WKARA3.

The list of presentations is the following:

- Presentation 1: G. Basilone, M. Pulizzi. Age Reading Criteria for GSA16. Presented in WKARA3 by G. Basilone.
- Presentation 2. A. Bellodi, P. Pesci, M. C. Follesa. Otolith preparation and interpretation for the Anchovy aging process in GSA11. Presented in WKARA3 by A. Bellodi.
- Presentation 3. A. Massaro. Life history and otolith preparation and interpretation for anchovy, *Engraulis encrasicolus*, in GSA 9, Ligurian and northern Tyrrhenian Sea. Presented in WKARA3 by A. Massaro.
- Presentation 4. L. Casciaro, P. Carbonara, M. Palmisano. Aging analysis of *E. encrasicolus* in the Western Ionian Sea (GSA19). Presented in WKARA3 by L. Casciaro.
- Presentation 5. I. Costantini, F. Donato. Anchovy age reading in Italian GSAs 17 and 18. Presented in WKARA3 by I. Costantini.
- Presentation 6. G. Bled Defruit, S. Telliez. Age reading protocol for European anchovy (*Engraulis encrasicolus*) in GSA 07 (MEDIAS surveys) and in ICES Subarea 8A; 8B (PELGAS survey). Presented in WKARA3 by G. Bled Defruit
- Presentation 7. C. Dueñas-Liaño, C. Hernández. Laboratory procedures and protocols for age determination in European anchovy (*Engraulis encrasicolus*) in ICES 8c and 9.a North areas. Presentation in WKARA3 by C. Hernández.
- Presentation 8. D. Silva, R. Milhazes, and S. Garrido. Sampling Protocol and Age Reading Protocol from ICES 9.a – Portugal. Presented in WKARA3 by D. Silva.
- Presentation 9. K. Markakis. Anchovy age reading in GSAs 20 and 22. Presented in WKARA3 by K. Markakis
- Presentation 10. A. Ventero and M. Iglesias. Otolith preparation and interpretation for the anchovy aging processing in GSA06 and 01. MEDIAS survey. Presented in WKARA3 by A. Ventero.
- Presentation 11. D. Gašparević. Anchovy age reading in Eastern Adriatic Sea, East (Croatia, GSA17). Presented in WKARA3 by the Chair, G. Basilone.
- Presentation 12: G. Basilone, P. Carbonara, A. Bellodi, L. Casciaro, I. Costantini, A. Massaro, M. Palmisano, M. Pulizzi. Report of the Workshop on Age estimation of European anchovy (*Engraulis encrasicolus*) WKAGESP 19–21 November 2019 Capo Granitola, Italy. Presented in WKARA3 by A. Bellodi.
- Presentation 13. C. Hernández, C. Dueñas-Liaño, J. Tornero, J. Landa, F. Ramos. Preliminary results on age validation in European anchovy (*Engraulis encrasicolus*) in Gulf of Cádiz (ICES 9.a South). Presented in WKARA3 by C. Hernández.
- Presentation 14. G. Basilone, M. Barra, R. Ferreri, S. Mangano, M. Pulizzi, G. Giacalone, I. Fontana, S. Aronica, A. Gargano, P. Rumolo, S. Genovese, A. Bonanno. First annulus formation in the European anchovy a two-stage approach for robust validation. Presented in WKARA3 by C. Dueñas-Liaño.
- Presentation 15. B. Villamor, A. Uriarte and G. Basilone. Main results from the otolith exchange analysis for anchovy. Presented in WKARA3 by G. Basilone.
- Presentation 16. P. Carbonara, W. Zupa, G. Basilone, B. Villamor. Explorative analysis on *Engraulis encrasicouls* aging data variability. Presented in WKARA3 by P. Carbonara.