Food and Agriculture Organization of the United Nations

General Fisheries Commission for the Mediterranean
Commission générale des pêches pour la Méditerranée

BlackSea4Fish Project

Workshop on age reading of select Black Sea species (anchovy and rapa whelk)

Trabzon, Turkey, 28 January–1 February 2019

Report

ANCHOVY WORKSHOP

EXECUTIVE SUMMARY

In the Black Sea anchovy stock assessment conducted during the first Subregional group on stock assessment in the Black Sea (SGSABS), held in November 2014, internal inconsistency was found to be high, suggesting that this could be due to methodological differences in the ages estimated by different specialists. Thereupon, an otoliths exchange exercise was arranged with the participation of experts who determined the ages of the anchovy data used in the stock assessment, and the results, which displayed very low percentage agreement were presented in the second meeting of the SGSABS. The group agreed that the inconsistency observed in the assessment results might be caused by age reading, and requested the General Fisheries Commission for the Mediterranean (GFCM) to hold a reading workshop.

The workshop[1], organized between 28 January and 1 February 2019 in Trabzon, Turkey, was held as an activity of BlackSea4Fish (BS4Fish) project which aims to facilitate the implementation of tasks of the Working Group on the Black Sea (WGBS). The workshop was held at the premises of the Central Fisheries Research Institute (SUMAE) of the Turkish Ministry of Agriculture and Forestry and was attended by 12 experts from all six Black Sea riparian countries.

The main objectives of the workshop were: i) to demonstrate the difference in age determination among country experts; ii) to assess the applicability of the protocols prepared by GFCM and ICES to eliminate these differences; iii) to modify the existing protocols by taking into account the biology of the species, the structure of the anchovy stock in the Black Sea, and the temporal differences in the exploitation patterns in different countries; iv) to verify that the new protocol to be established responds to the needs of the stock assessment requirements.

[1] This meeting was supported by the European Union under grant agreement no SI2.795396.

Workshop description

On the first day, the workshop was opened by the welcome speech of İlhan Aydın, Deputy General Director of the Agricultural Research and Policy of the General Directorate. This was followed by the presentation of the BS4Fish project coordinator outlining the purpose, content, importance and place of age reading workshop in the ongoing stock assessments. After that, Alexander Chashchin, chairperson of the workshop, and, the rapporteur Gizem Akkuş, a research assistant of the METU Marine Sciences Institute who prepared the material, coordinated the hands-on practical sessions before and during the workshop, and made introductory presentations.

Presentations in anchovy session

The participants were invited to read the ages of 27 otoliths selected to represent different seasons and regions in the Black Sea. The agreement (%) with the reference reader, between-reader bias and variability in the age-determination among different readers are presented below (Figures 1 and 2).

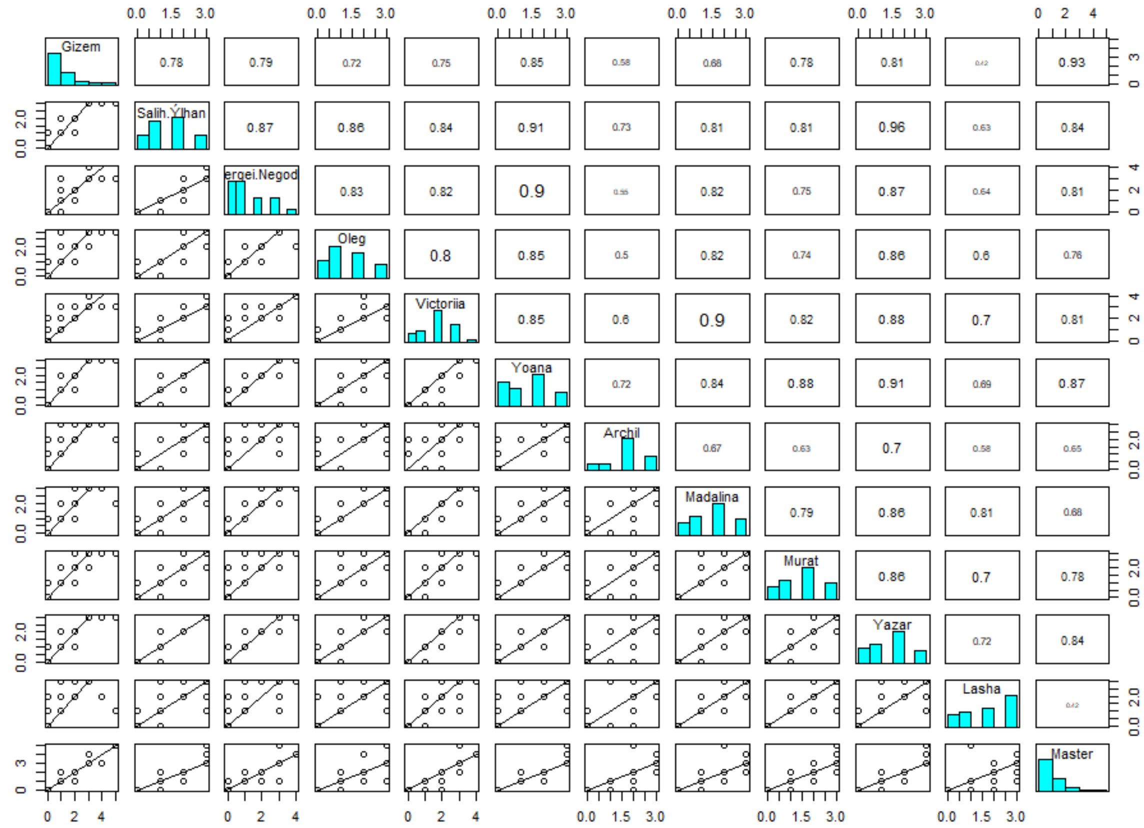


Figure 1. Correlation between readers in the first exercise

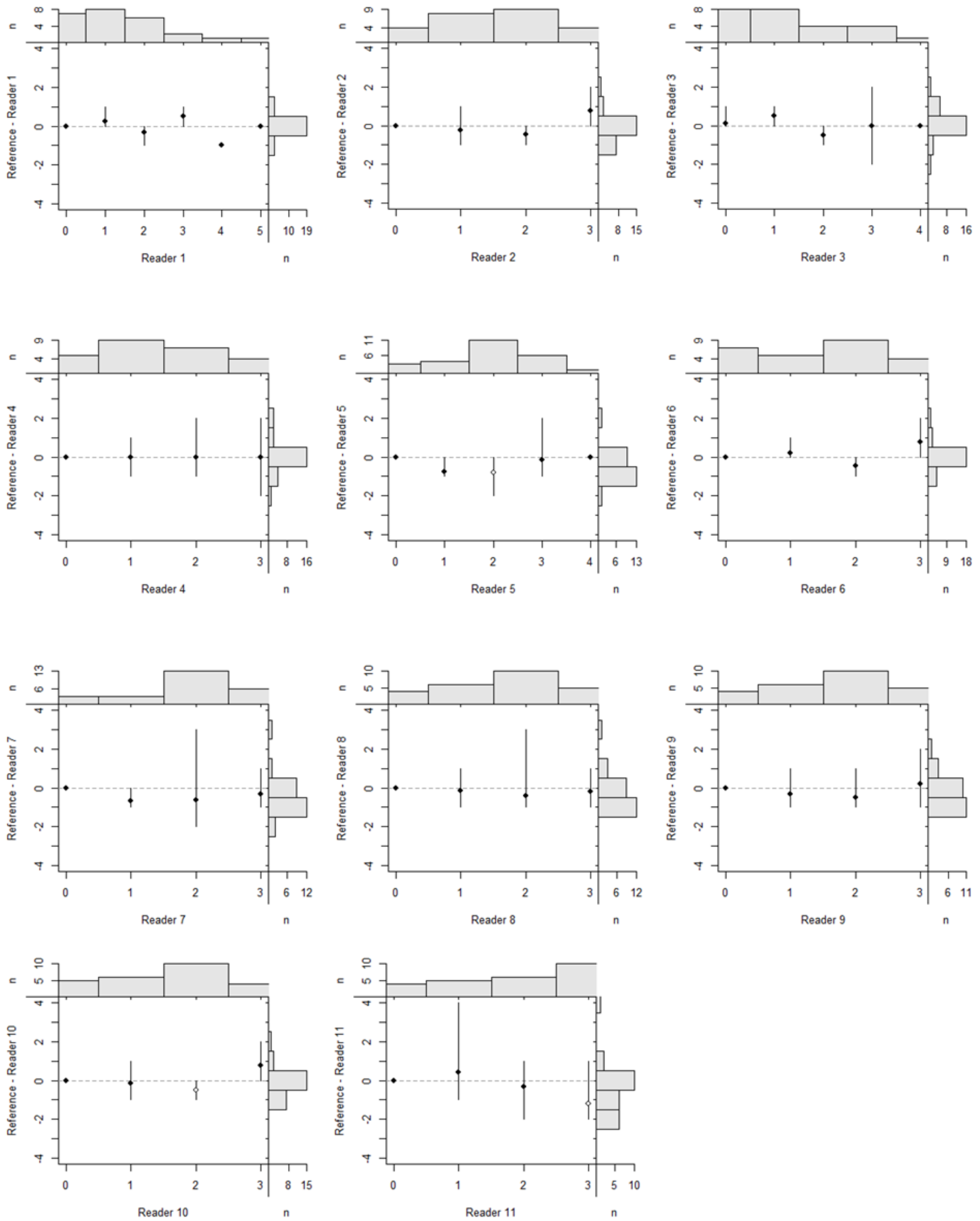
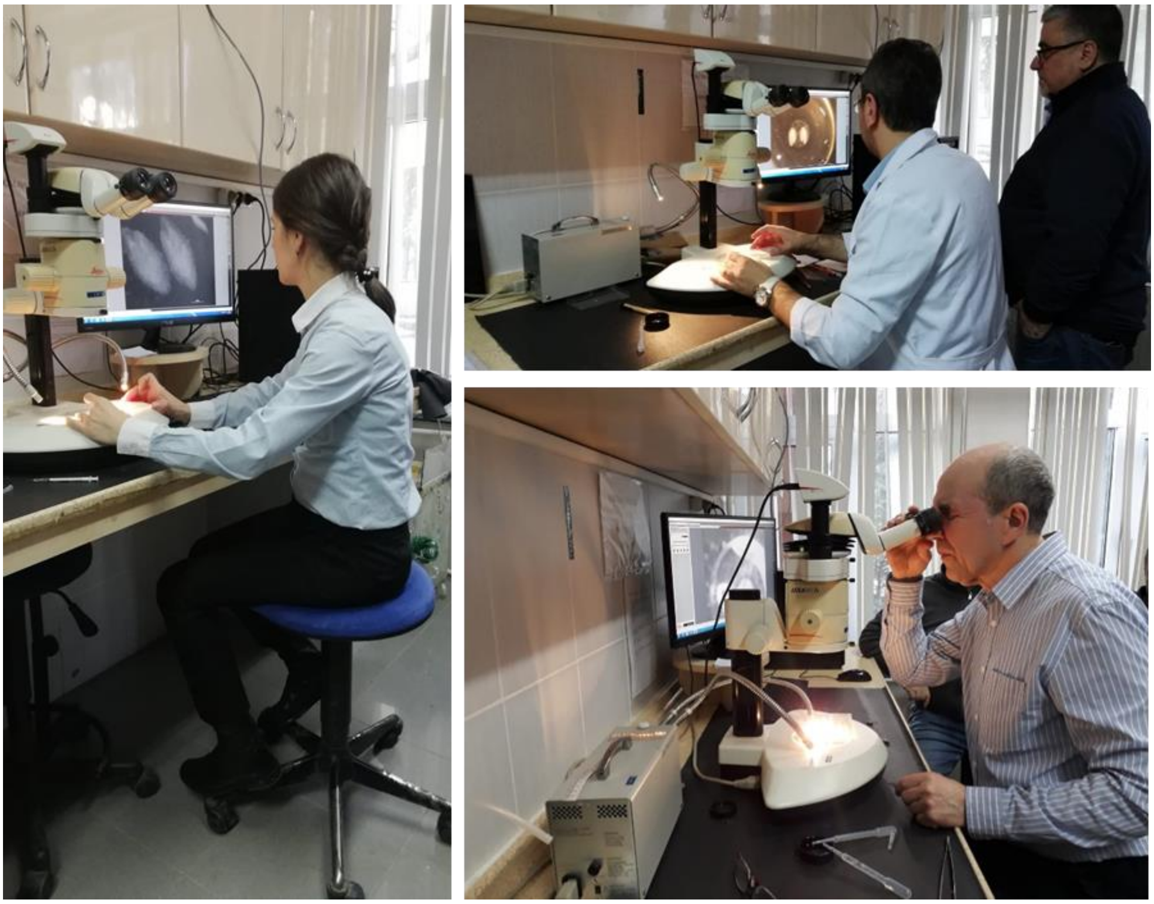


Figure 2. Age reading bias in the first exercise

Comparison of the results indicated that the age readings of the participants were not consistent with each other during the first exercise made at the onset of the workshop. The average agreement of the group with the true age (reference reader's estimate) was 53 percent, and the highest and the lowest agreements observed were 76 and 36 percent, respectively. The within group agreement with reference to mean and modal age was 68 percent. The correlation among the readings and the age bias were mutually examined to see whether there is systematic deviation in the age readings originating from the technique used, i.e. whether some readers systematically under- or over- age the samples. However, no such pattern was observed. It was therefore decided that the disagreement in the reading largely resulted from the interpretation of the rings.



Snapshots from individual age readings

On the second day of the workshop, Dr Chashschin read the ages of the otoliths and the participants discussed the differences case by case until they all reached an agreement. The group witnessed deterioration of the otoliths exposed to different chemicals (alcohol +glycerine) during the course of the exercise.

Later, Gizem Akkuş presented the existing age reading protocols prepared by ICES (WKARA2 2016 REPORT _Report of the Workshop on Age Estimation of European anchovy (*Engraulis encrasicolus*) and GFCM (Handbook on fish age determination: a Mediterranean experience) to the participants. The pros and cons of these guidelines were discussed item by item in terms of their applicability to the Black Sea anchovy.

On the third day of the workshop, the group drafted a new guideline for the age reading of the anchovies found in the Black Sea (Appendix 1) based on this discussion held during the second day. The participants were then invited to read the ages of a new set of otoliths extracted from fresh fish based on the new protocol under the supervision of the reference reader.

Snapshots from the extraction of anchovy otoliths from the fresh fish

Some photos from the age reading session with the group discussions

On the fourth day of the workshop, two more issues were discussed. The first topic was whether the newly created protocol could respond to the needs of the stock assessment. The problem arises from the fishing season of anchovy in the Black sea which differs from country to country. To solve this problem SGSABS has decided not to use the official landings statistics as they were reported for the calendar year (January-December), but instead, to use the quantity of anchovy landed within a fishing season (October-April). This approach is applied to the case of Georgia and Turkey. For the other countries whose catch is negligibly low, the official landings are used as they were reported. Given that the fishing is the time when the samples for age determination are collected, and considering the data collation method applied for Black Sea anchovy stock assessment, the group agreed that the protocol applied for age reading would perfectly matches the data requirements.

Table 1. Monthly distribution of the Black Sea (upper panel) and Azov (lower panel) anchovy by countries (in %)

Black Sea anchovy	**Jan**	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Jul**	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**
Bg					20	50	25	5				
Ro					15	40	35	5	5			
Ua					20	50	15	10	5			
Ru					20	50	15	10	5			
Ge	32	24	9	4							3	28
Tr	10	2								13	51	24

Azov anchovy	**Jan**	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Jul**	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**
Bg												
Ro												
Ua									40	55	5	
Ru	25	15	8	2						10	20	20
Ge												
Tr												

The group also discussed the possible ways to test the accuracy of age estimates through complementary validation methods. In this respect, marginal increment reading and length frequency distributions were taken into consideration.

The marginal increase analysis was not considered as a feasible method due to the seasonal availability of anchovy associated with its migratory behaviour. However, the experts, based on their observations, schematized the timing of hyaline and opaque ring formation in the Black Sea and Azov anchovy (Figure 3).

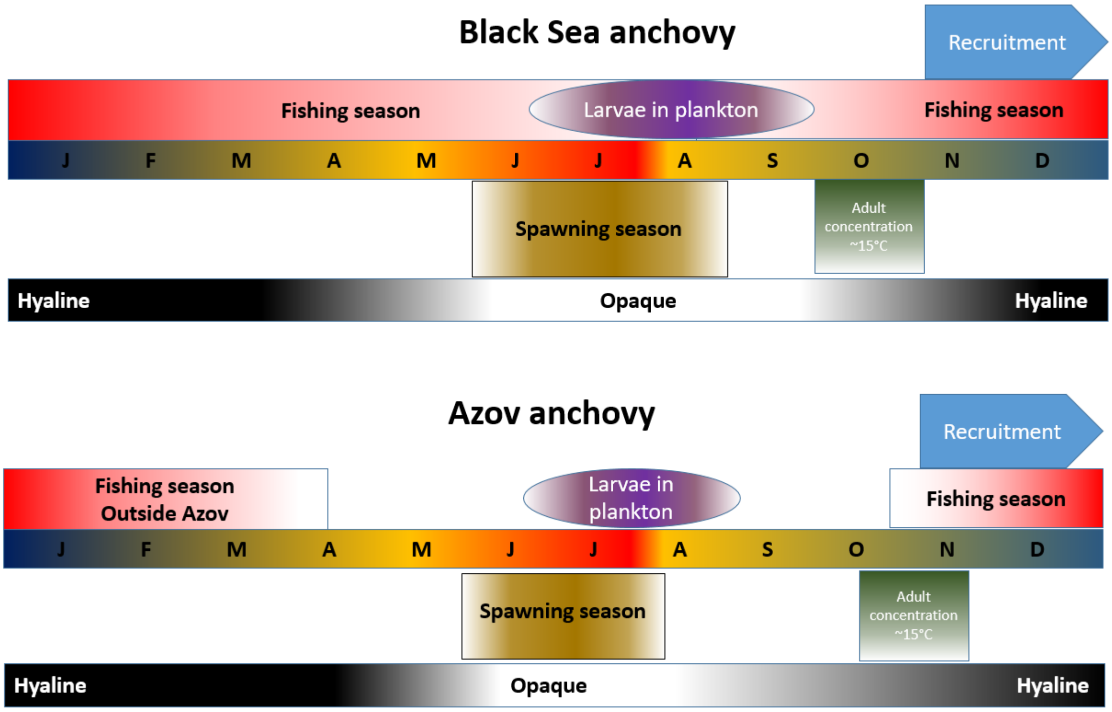


Figure 3. Timing of hyaline and opaque ring formation in the Black Sea and Azov anchovy

Moreover, the importance and the necessity of a collective study in which the riparian countries will contribute by providing samples for the months when anchovy is present in their waters were emphasized.

It was also agreed that the fitting a Von Bertalanffy growth formula (VBGF) curve estimated using the age readings, to the length-frequency distribution is an appropriate technique to validate the accuracy of the age readings.

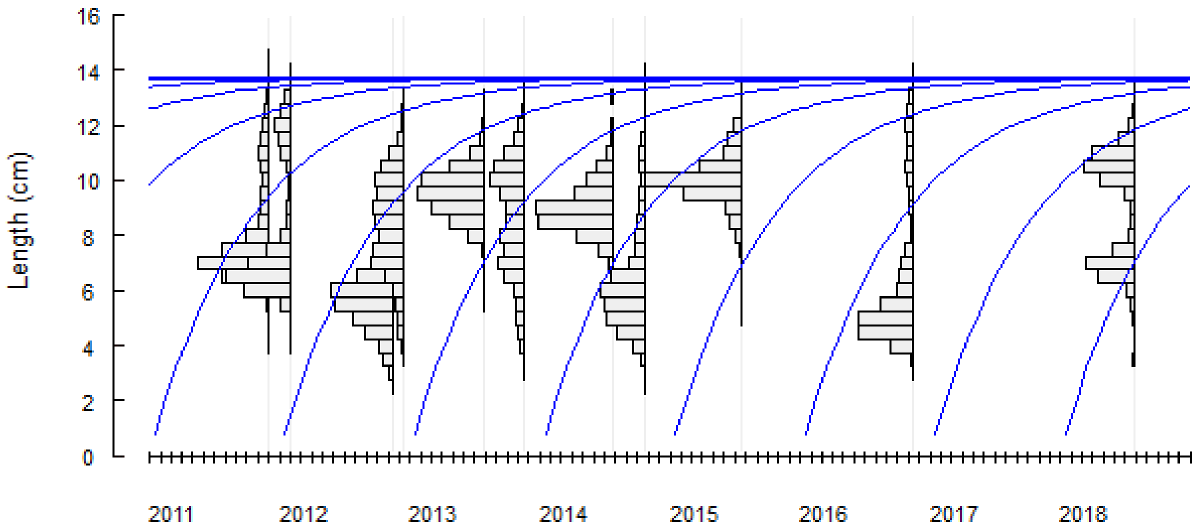


Figure 4. VBGF curve and length frequency distribution of anchovy

In the last day of the workshop, participants were asked to read the age of a new set of otoliths. The results were analysed to evaluate the success of the workshop (Figures 5 and 6). Disregarding one of the otoliths which was classified as AQ-III (very difficult to read) by the reference reader, the average agreement has increased to 92 percent, and 7 out of 10 readers achieved 100 percent agreement.

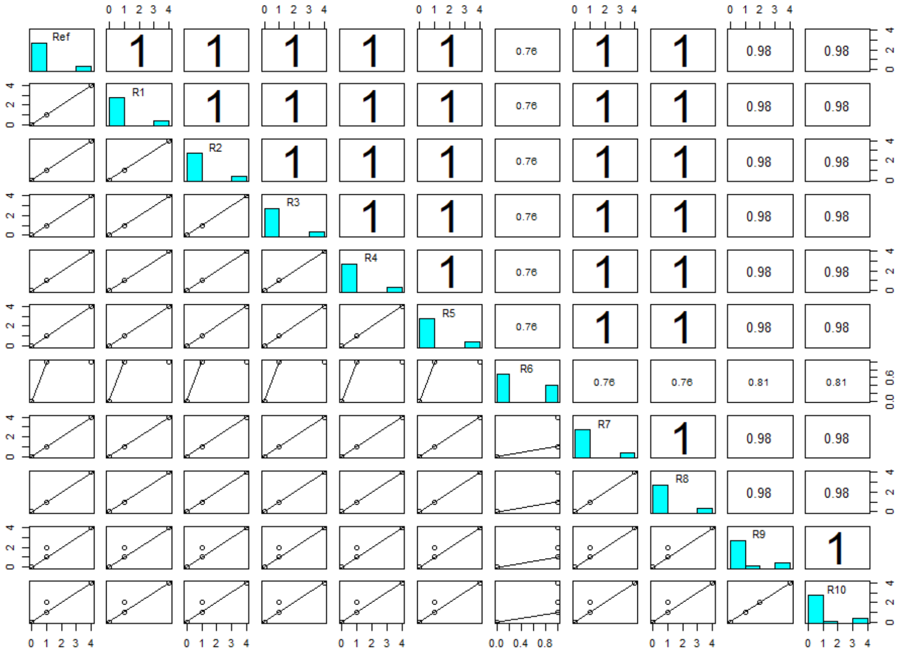


Figure 5. Correlation between readers in the final exercise

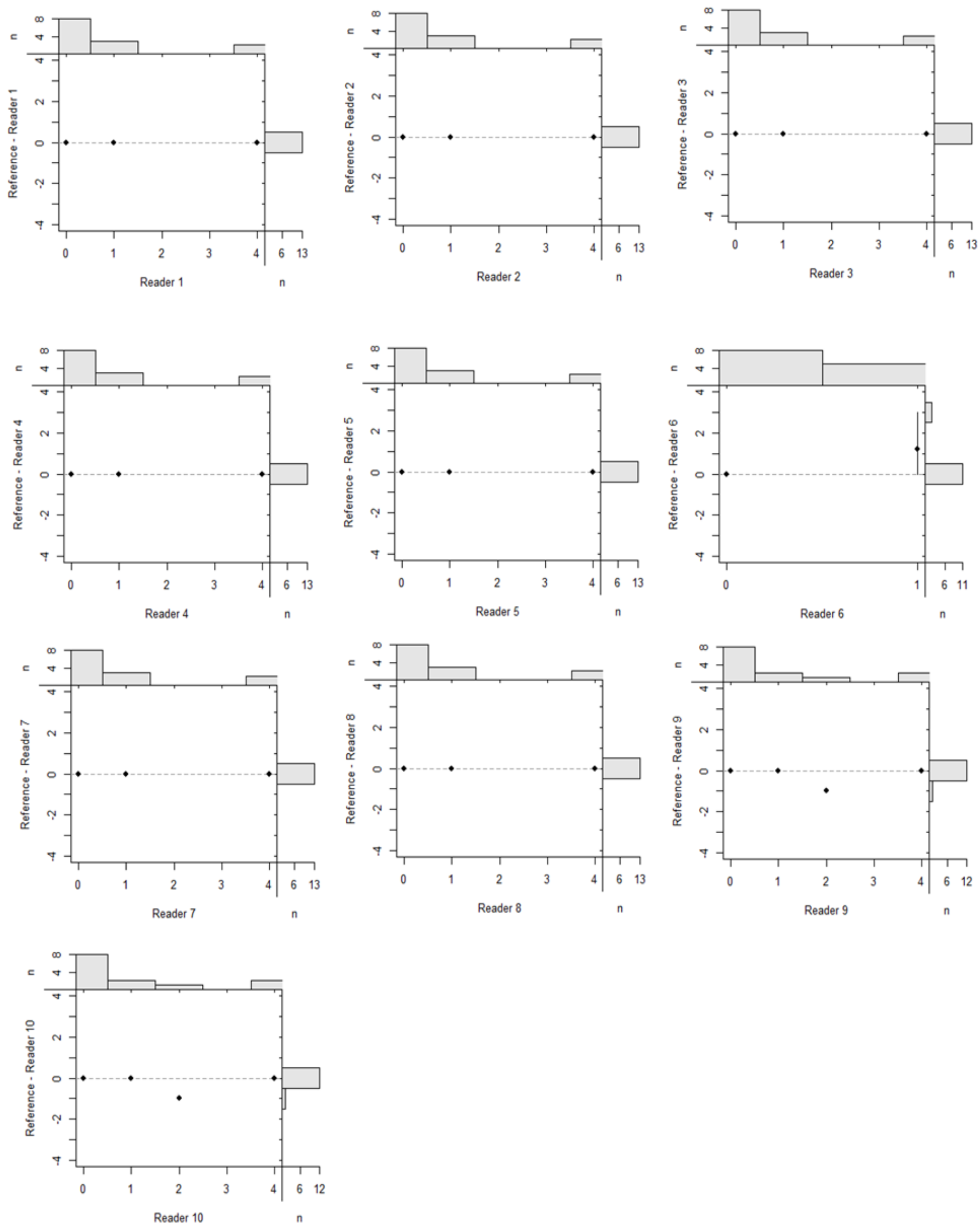


Figure 6. Age reading bias in the final exercise

RAPA WHELK WORKSHOP

EXECUTIVE SUMMARY

In the Black Sea, there is no method that can be considered as a reference for the reading of the age of Rapa whelk, except in a few studies dated back to 1960s. However, there are various studies describing age determination in the Gastropods, or even age reading of the same species in the areas other than the Black Sea. Two of these methods were presented by Mr Bohdan Hulak in SGSABS 2018, but a consensus could not be reached on the suitability of the method for the whole Black Sea region. The issue was also discussed in small group meetings held during the GFCM Fish Forum in Rome between 11 and 14 December 2018. As a result of the discussions, it was iterated that one of the major problems limiting the scientists to develop accurate and precise population age structure estimates for stock assessments is the lack of a reliable age determination method. It was emphasised that this is a significant shortcoming in the stock assessment studies and should be overcome in the nearest occasion. In this context, it was also decided to bring together the local experts and to reveal the current experience and difficulties encountered in the reading of age in a workshop. After the issue was discussed at the first workshop, it was decided to use the help of an external expert outside the Black Sea, if deemed necessary. This workshop, held between 28 January and 1 February 2019, was the first stage of the decisions taken during afore mentioned meetings.

Background on the workshop

People who have some experience about the species and who are identified as national experts responsible of reading the ages of the species within the scope of EU's Rapa whelk research project were invited to the workshop. In addition, a Turkish professor who has experience in age determination on hard structures of marine organisms was invited to chair the workshop. The workshop aimed to focus on five different gastropod age determination methods and to reveal their pros and cons.

On the first day of the workshop, presentations were made on the biological and anatomical features of the species, such as location of the statoliths that can be used in age reading. The preparatory work done before the workshop was also presented by Prof. Başusta. The participants were then invited to discuss interpretation of annuli formation based on ready-made samples of sectioned shells, operculum and a statolith. In a nutshell, these discussions included the following topics:

Age reading on the operculum

The first method, which is commonly used to determine the age in gastropods and so that discussed within the framework of the rapa whelk session of the workshop was the reading growth rings on the operculum, an organic “shield” that is used to protect the shell aperture when the animal withdraws into its shell. The participants expressed their views about the method and agreed that the rings formed on the operculum are not easy to interpret as, not all of them were in line with the biological growth cycle of the species, and often the operculum is subjected to erosion. In conclusion, the group has decided not to consider this method during the workshop.

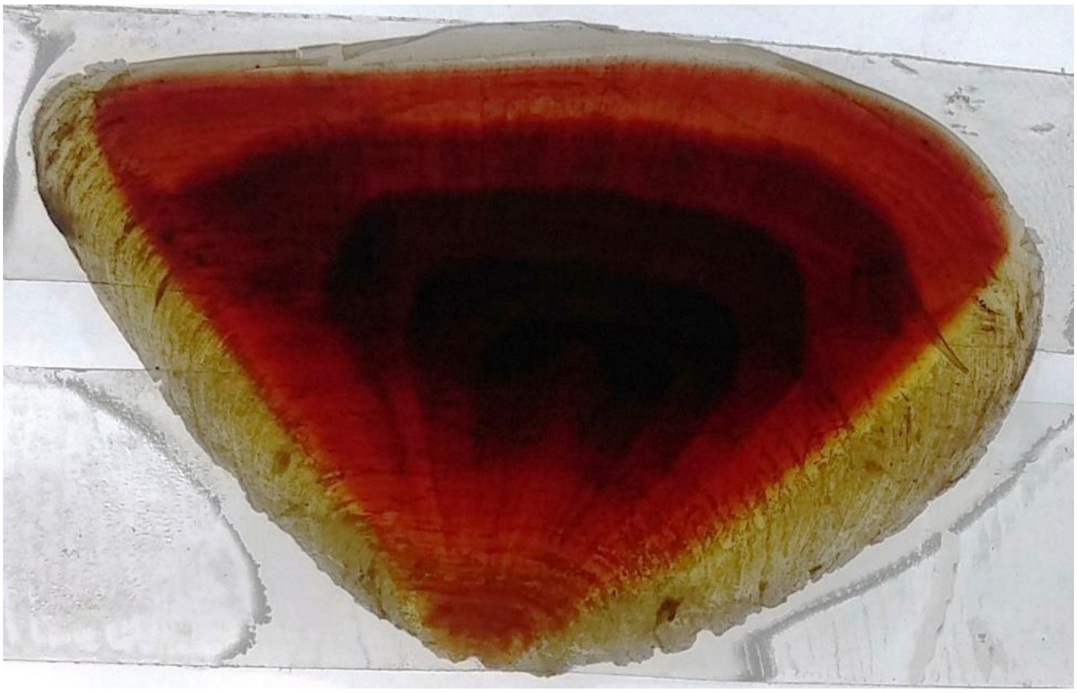


Figure 7. Operculum of *Rapana venosa* and the ring formations (Photo by Prof. Nuri Başusta)

Surface spawning marks/lips

The method was explained to the participants by Bogdan Hulak. Basically, growth in rapa whelk reaches a standstill in the spawning period, and thickening of the shell occurs during this period. The number of these thickenings is used to determine the number of times the individual spawns. It is reported that reproduction in rapa whelk takes place only once in a year in the Black Sea and the species begins to spawn in the third year of life (Chukhchin, 1961). Accordingly, it is possible to estimate the age of the Rapa whelk by adding 2 to the total number of thickenings on the outer surface of the shell.

A snapshot from the discussion on Rapa whelk age reading

The group discussed on the issue of age before spawning, considering that there might be some regional differences associated with temperature, and decided to leave the issues related to age of first maturity aside, and focus on the determination of the age after first spawning. It was, however, strongly underlined that estimation of first maturity age is a critical question to be further discussed.

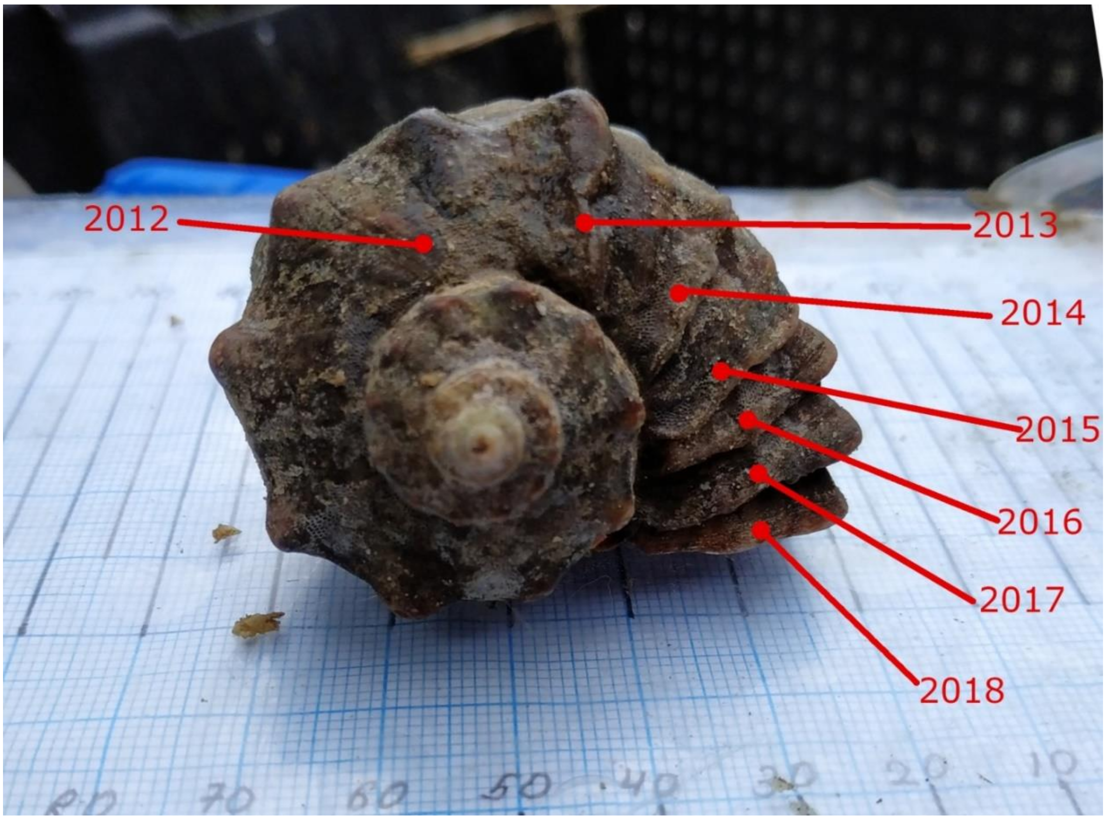


Figure 8. Spawning lips on the surface of the shell (Photo by Bogdan Hulak)

Following the discussions, all the participants involved in the rapa whelk session were invited to count the spawning lips on the surface of the rapa whelk shells of 88 freshly collected specimens. In this very first exercise, pairwise comparison of age readings of 9 readers displayed high correlations (Pearson correlation) some of which were as high as R=0.84 (see Figure 9); however, it was also noted that some users are tend to under- or over-estimate the number of external spawning lips. This mainly resulted from the difficulties in determining the first spawning lip.

As the true age of the samples were not known, and as there were no reference reader in the group, the accuracy of the results could not be tested. The precision were estimated based on the modal age of the specimens determined by the readings of the participants. The variation in age estimates of 9 readers were not extremely high (coefficient of variation [CV] = 23 percent) and readers' agreement (%) with the modal age was 45 percent. It was noted that the percentage agreement was the highest (50 percent<) in the readers who has some past experience in Rapa age reading.

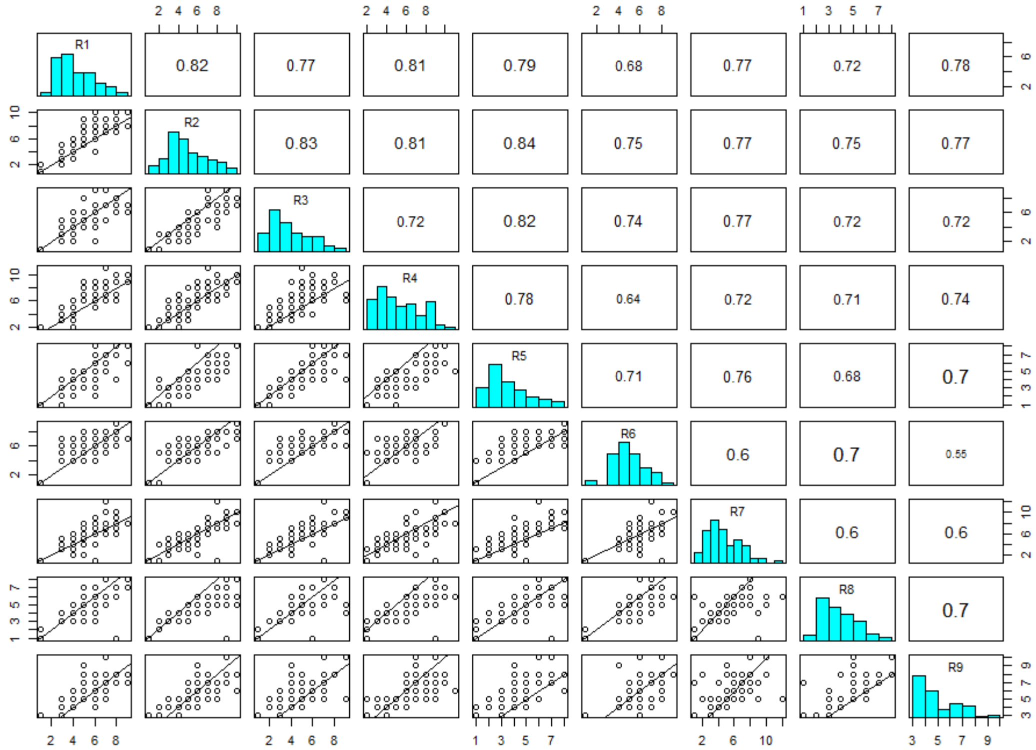
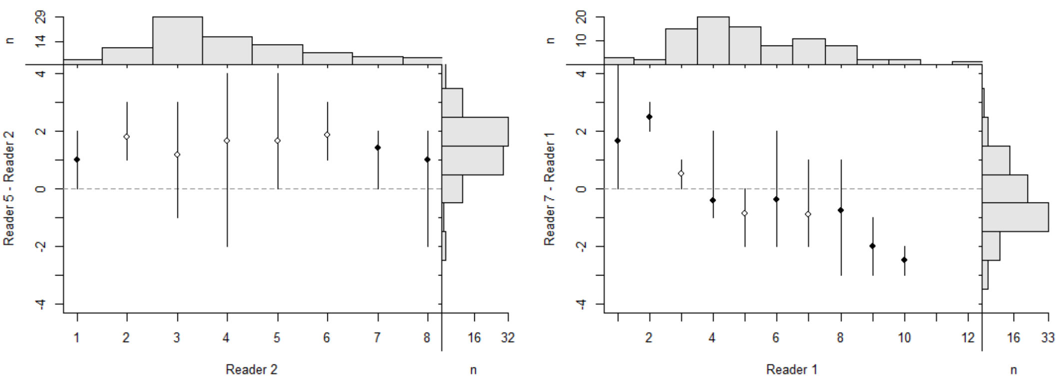


Figure 9. Age (number of spawning lips on the shell) distribution of samples (histograms), correlations between readers (upper panel) and comparison of age estimates provided by each reader taking part in the first rapa whelk age reading exercise.

The bias in the age estimates of the readers having the highest agreement was also evaluated (Figure 10). It was noted that age bias is high and is not systematic, indicating that the method is not straightforward and more efforts are needed to harmonize the interpretation of the lips on the rapa whelk shells.



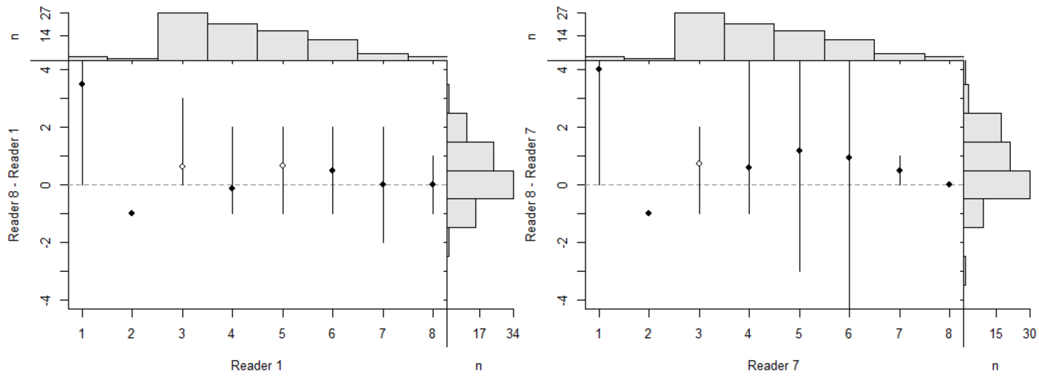


Figure 10. Age bias plots of some selected readers having high percentage agreement

The group also estimated the VBGF parameters plotting modal and mean age estimates of the group (Figure 11 and 12) and compared them with the growth estimates used in the preciously held SGSABS sessions. The resulting curves were meaningful in a sense that the estimated ages were internally consistent with regard to the corresponding body size, although they estimated slower growth than those used in the assessment of the species.

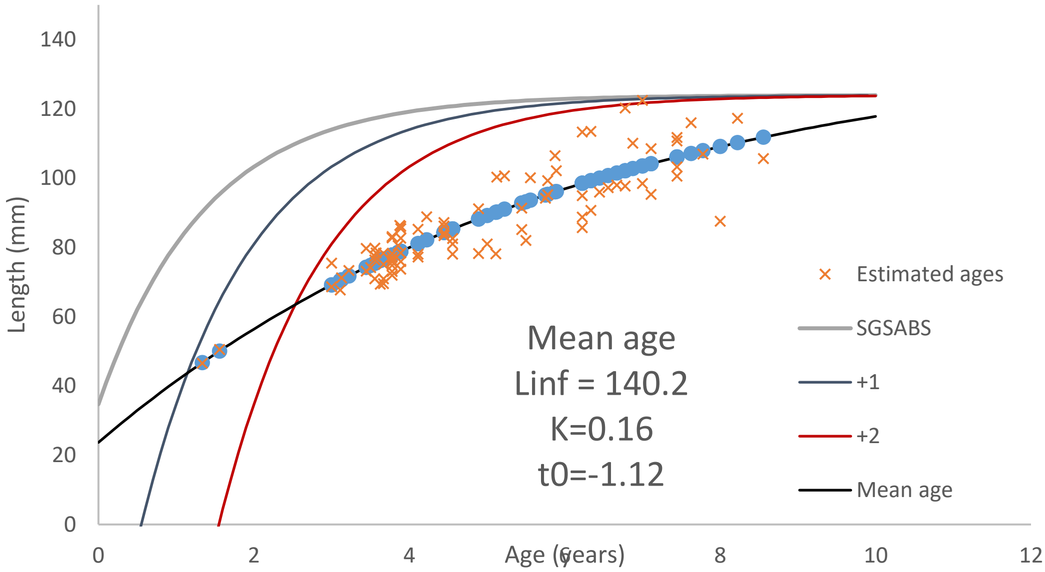


Figure 11. Von Bertalanffy growth curve (blue dots) fitted to the mean age estimated based on external spawning lips and comparison with the growth parameters used in SGSABS with different age at first maturity assumptions

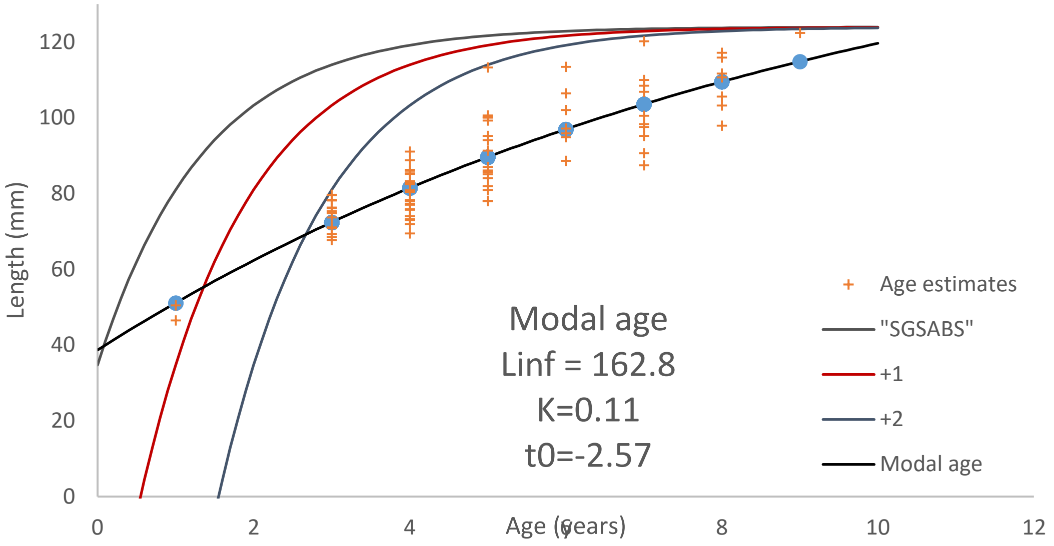


Figure 12. Von Bertalanffy growth curve (blue dots) fitted to the modal age estimated based on external spawning lips and comparison with the growth parameters used in SGSABS with different age at first maturity assumptions

Vertical and horizontal shell cross-sections

Another exercise was to cut the samples horizontally and vertically, and to the read the ages from the spawning and growth libs on the edge of the shell. Participants read the age of 29 individual prepared in this way. They considered, i) the number of orange layers on the shell, and ii) sharp lips (projecting edge) on the sectioned surface of the shells.

A snapshot from the cross sectioning of the rapa whelk

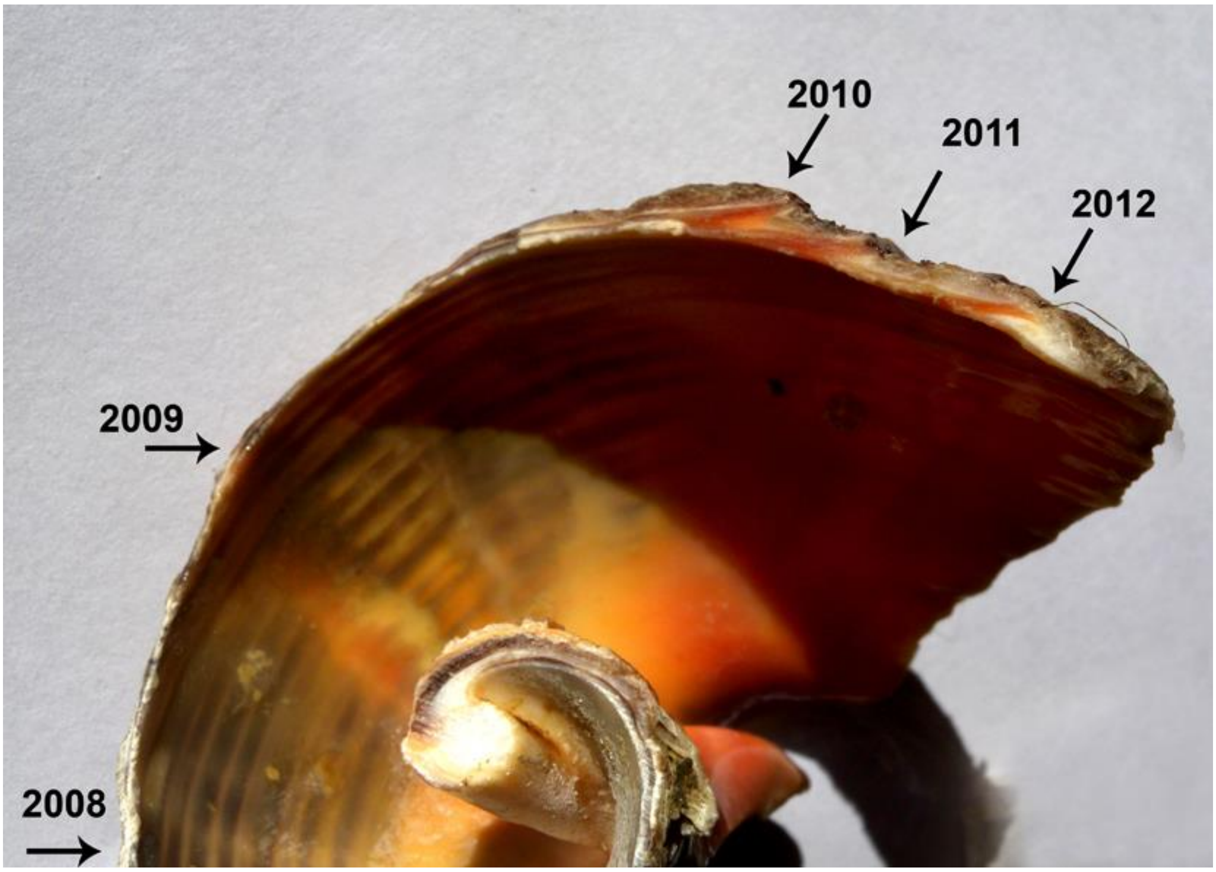


Figure 13. Spawning marks on the shell cuts (Photo by Dr. Oleg Kovtun)

The results of the exercise were evaluated by the group. The overall CV was 23.7 percent and percentage agreement was estimated as 47 percent, indicating that the agreement among the readers increased slightly with respect to the previous method. The correlations between readers were as presented in the following figure (Figure 14). The group noted very high correlations observed between some experienced readers and decided that this score when associated with low percentage agreement, could be, to a certain extent, due to disagreement in the determination of the first spawning marks (lips on the outer surface of the shell).

Evaluating the results of the exercise

The growth curve estimated by the modal and mean length estimates based on shell cross-sectioning method were closer to the growth parameters used for assessment in the SGSABS. The group also noted that the curve which assumes the first age at maturity as 2 was closer to the SGSABS.

Figure 14. Correlation between readers - shell cutting exercise

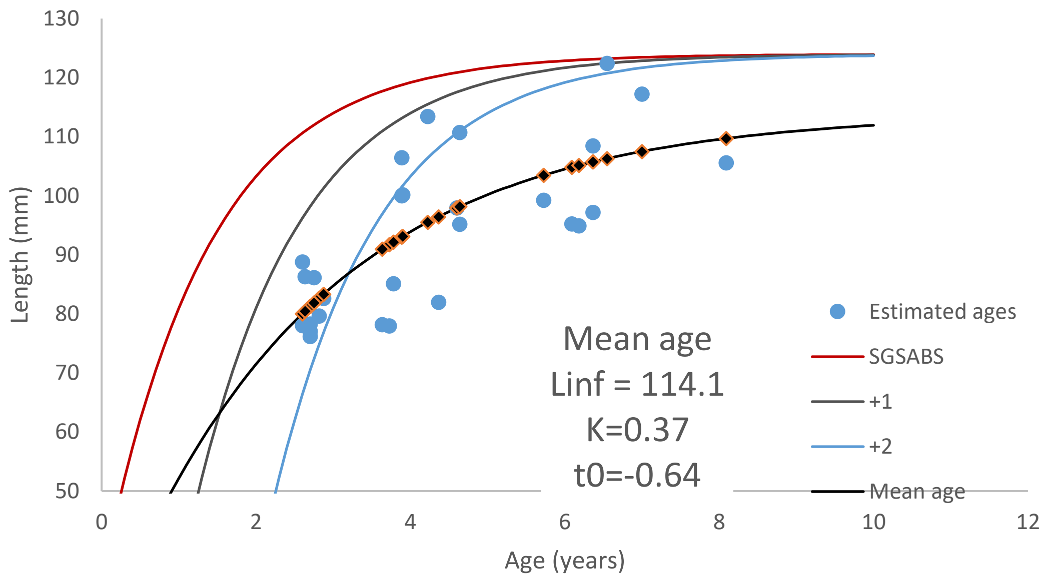


Figure 15. Growth curve estimated based on mean age (shell cross-sectioning method). The three lines presented on the graph displays the growth curve drawn based on VBGF used in the SGSABS assessment with maturity ages of 2 and 3.

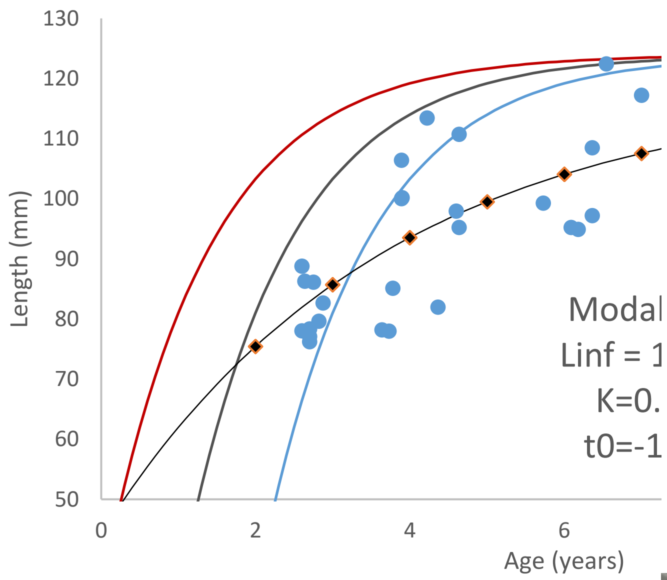


Figure 16. Growth curve estimated based on modal age (shell cross-sectioning method). The three lines presented on the graph displays the growth curve drawn based on VBGF used in the SGSABS assessment with maturity age of 2 and 3.

After the exercises, the participants discussed their views on the application of the methods based on marks on the shells. The difficult aspects of the method underlined by the participants are, i) the determination of first spawning mark on the shell surface; ii) separation of the true spawning thickenings from other thickenings occurring, due to slowing in growth in winter or environmental changes in different seasons of the year (hypoxia, sharp fluctuations in water temperature), and iii) the age before the formation of the first spawning mark.

The advantageous aspect of the method, particularly the one based on projected lips on the shell surface is that it is possible to determine the age over a large number of samples in a short time in the field. In addition, when mean and model age are taken into consideration, the results indicate that even this very first exercise which is not yet harmonized among the readers, has a remarkable potential to produce meaningful findings.

Some additional remarks that the group thinks important for the development of the method in the future are listed below

- Participants agreed that rapa whelk in the Black Sea displays two different forms. These two forms, separated by their "dark" and "light" colour shells, have differences in shell morphology and this imposes some inconsistency in the identification of first spawning marks. The "dark" forms, are thought to be found on rocky grounds. The group, therefore, proposed to use only the "light" coloured forms inhabiting the soft substrate.

- The crustal growth is fast after the first reproduction. Consequently, the most intensive growth occurs in the first years of life and then the growth slows down noticeably. Accordingly, it is underlined that the distance between the successive spawning marks should also be taken into consideration in the determination of the first spawning mark. Moving from the margin of the shell to the centre, the first spawning mark should be expected to occur after a long interval following successive narrowly spaced markings.

- The discrepancy in the determination of the first spawning mark along the edge of the cut shell could partly be due to technical reasons. The shells were cut by large-diameter abrasive discs (10-12 cm), which made it difficult to cut in the area of the first spawning mark visible on the surface of the shell. In the case of small shells, this problem did not occur. In larger shells however, the coiled morphology makes it difficult to obtain a single clear growth axis using sectioning. This observation emphasizes the importance of determining the axis to be cross-sectioned. It also shows that it is crucial to standardize the axis of the cut to ensure harmonization among readers.

- Not all the shells have a clear structure for the determination of age. In this case, discarding the problematic shells may be considered.

- Some of the individuals displayed larger distances between spawning marks (several times larger than the following marks). On the shell of such individuals, these broad areas between the lips are thinner; on the inner surface the orange pigmentation indicating the spawning does not exist, and the colour is not different than that of outer surface. Given that the orange colour is associated with accelerated reproductive metabolism, one possible explanation, which needs to be further studied, is that such zones may occur in the years when the individual was not involved in spawning. In this case, rapa whelk actively feeds, grows fast, and no thickening that would indicate slowdown in growth, is formed on the surface of the shell. Based on this assumption, such zones of accelerated growth may be taken as the annual mark, in the event that the length of the zone of accelerated growth is more than twice as long as the distance between the two previous marks on the shell.

- Regarding the maturity age of the rapa whelk in the Black Sea, several proposals, such as monitoring the growth of rapa whelk in a controlled environment and tagging; isotope ageing were discussed. One of the proposals, which could be tested during the workshop was to read the age of individuals at the size of first maturity given in the literature and some individuals around 4 cm in length (Sağlam et al., 2009) were examined. The results showed that while some individuals smaller than 4 cm were maturing; some others larger than 4 cm had not reached sexual maturity yet. Consequently, the group has agreed that it is crucially important to carry out studies for the determination of the first reproductive age taking into account the regional differences.

Figure 17. Suggested sectioning axis on Rapa Whelk

Age determination based on statoliths

The last two days of the workshop were devoted to the Rapa Whelk statoliths which are located near the pedal ganglia and known to display rings that are deposited annually (Barroso et al., 2005). The group followed the procedure described for channelled rapa whelk, *Busycotypus canaliculatus*, by Fisher (2018). With that regard, the statocysts were transferred to a Petri dish and the statoliths removed using a hypodermic needle. Each statolith was cleaned of any adhering tissue by immersion in 1M sodium hydroxide (NaOH) for one hour and rinsed in pure water and then stored in 85 percent ETOH. Once the statoliths had air-dried they were mounted on a microscope slide using Crystalbond™ 509 thermoplastic resin and imaged under a microscope. Examing the samples, participants managed to extract four pairs of statoliths.

The maximum magnification of the microscope used in the exercise was not high enough to interpret the age rings located at the edge of the statoliths (Figure 18). Therefore no further exercise could be performed to test the consistency among the readers. Nevertheless, this method was found more accurate than the other methods tested during the workshop as the statoliths are not affected by the physical factors in their surroundings.

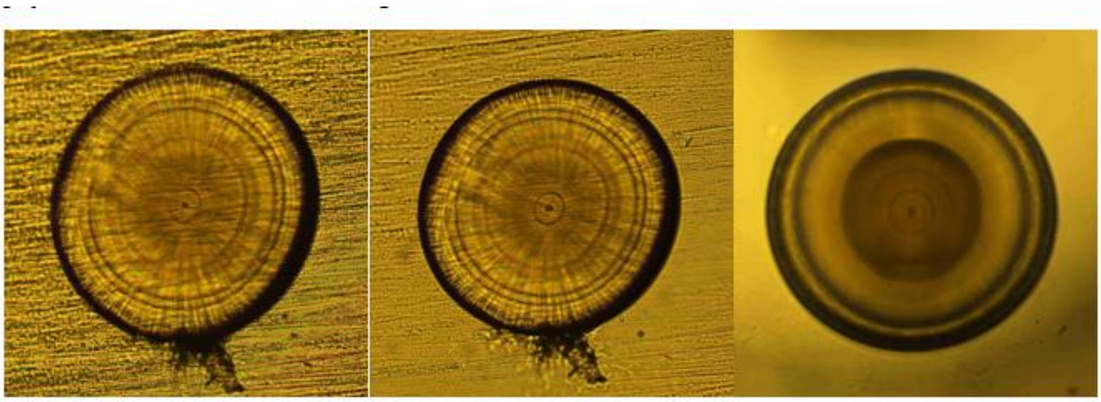


Figure 18. Statoliths extracted by the participants and blurred visibility at the edges

Based on this experience the participants listed the following remarks with regards to the method. Despite its accuracy, the method is associated with a number of difficulties:

- The extraction of statoliths and the preparation of the statoliths for microscopy require a lot of time and effort.
- Interpretation of the result directly depends on the quality of polishing the surface of the statoliths buried in a media, like resin.
- The microscope and camera used for age reading from the statoliths requires very high quality.
- The method benefits greatly from image processing, which would allow more accurate identification of annual marks.
- Some critical issues like, interpretation of rings formed on the statoliths, presence of false rings and determination of first age ring, requires further attention.

Conclusions

Following the examination of the statoliths, it was decided that the most accurate age reading in rapa whelk could be done on statoliths. However, due to the difficulty and length of the preparation process, this method was not found practical for routine age determination needed for stock assessment purpose. The group also underlined the importance of conducting an additional comparative study targeting standardization of a more practical method easily applicable in the field. To this end, all the participants expressed their preference towards “external spawning marks”. The main advantages of this method over the rest tested during the workshop are its simplicity, speed and applicability in the field. These advantages would help age readers to reach much higher sample sizes in the age determination, and this help compensating the possible biases. The use of external structures for age determination requires that the problems identified by the participants (see above) are solved and well defined guideline is made available to the readers to ensure harmonization within the region. The group, therefore, requested for an additional comparative study, which will utilize the accuracy of the statoliths to rectify the uncertainties encountered in the external spawning marks method. This study should be based on statoliths of at least 10 Rapa whelk in each 0.5 cm size range between 2 and 10 cm (~200 individual in total) and should aim at interpreting the external formations on the shells of Rapa Whelks based on their ages determined using their statolith.

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Appendix 1

Agenda

Opening and arrangements of the meeting

- Welcome addresses and introduction of participants
- Adoption of the agenda

General overview of objectives and expected outcomes

Introductory presentations

- Rapana age reading (Nuri Başusta)
- Anchovy age reading (Alexander Chashchin and Gizem Akkuş)

Parallel sessions

Anchovy	Rapana
Hands-on session Ageing of a set of otoliths by each participant and by Dr. Chashchin as reference reader)	Whelk anatomy and biology in relation to ageing (Nuri Başusta)

Anchovy	Rapana
Analysing the results of the ageing exercise, Discussions on the results of age reading exercise Review of the existing ageing procedures, including Handbook on fish age determination: a Mediterranean experience and ICES (WKARA) by Gizem Akkuş, Discussions on stock assessment related age reading issues, Demonstration on the problems associated with 1.False rings 2.Check rings 3.Origin of the samples 4.Hybridization Presentation of proposed protocol (Alexander Chashchin and Gizem Akkuş) Discussions	Whelk ageing methods in fresh samples (Nuri Başusta)- Operculum ageing Shell cutting and ageing Statolith recovery and ageing, statolith sections surface reading, and sectioning with emphasis on embedding and sectioning.

Anchovy	Rapana
Hands-on age reading exercise utilizing the proposed age reading protocol (10X2 Azak; 10X2 Yaz Tr, 10X2 Kış Tr, 10X2 Ge, 10X2 Ro, 10X2 Ua) Discussion	Instructions for image analysis and enhancement. Age interpretation and methods for confirming ageing accuracy

Anchovy	Rapana
Drafting the first age reading protocol of anchovy Discussions on validation techniques	Discussion on data gaps and possibilities to fill these gap Analysis of age data including age comparisons, Index of APE Growth

Joint Session

Concluding the outcomes and suggestions on the draft age reading guidelines

Re-evaluation of the anchovy otoliths by each participant with the adopted methodology and practising Rapana ageing

Appendix 2

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Workshop on Age Reading of selected Black Sea species participants

Appendix 3

Age reading Protocol for Black Sea Anchovy (*Enghraulis encrasicolus ponticus*, Alexandrov 1927) and Azov Sea Anchovy (*Engraulis encrasicolus maeticus*, Pusanov, 1936)

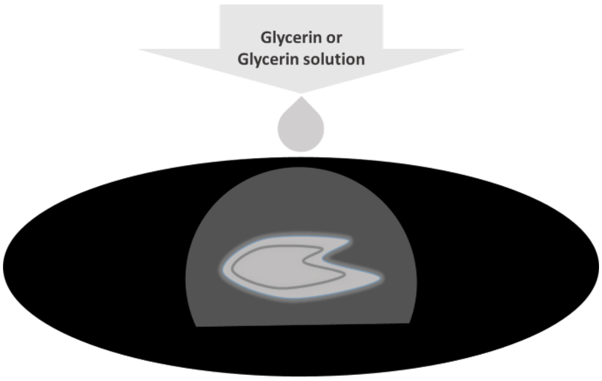
Prepared by: Gizem Akkus, Alexander Chashchin, and Ali Cemal Gücü
Contributors: Murat Erbay, Madalina Galatchi, Yasar Genç, Yoana Georgieva, Archil Guchmanidze, Victoria Hetmanenko, Salih Ilhan, Sergei Negoda, Oleg Perevalov and Lasha Salukvadze**Assumptions**

Birth Date	Annuli	First ring determination
1st June	A "true ring" should be observed "*at least on two different areas"* around the otolith pairs	If the first ring is completed and continues all around the otolith, it is count as "*1st age ring*" disregarding its distance from the nucleus

Otolith Extraction and Storage

- Both otoliths (*sagittal*) should be extracted carefully (unbroken), cleaned and dried.
- If it is to be stored after reading, it should be kept in dry in paper or plastic envelopes.

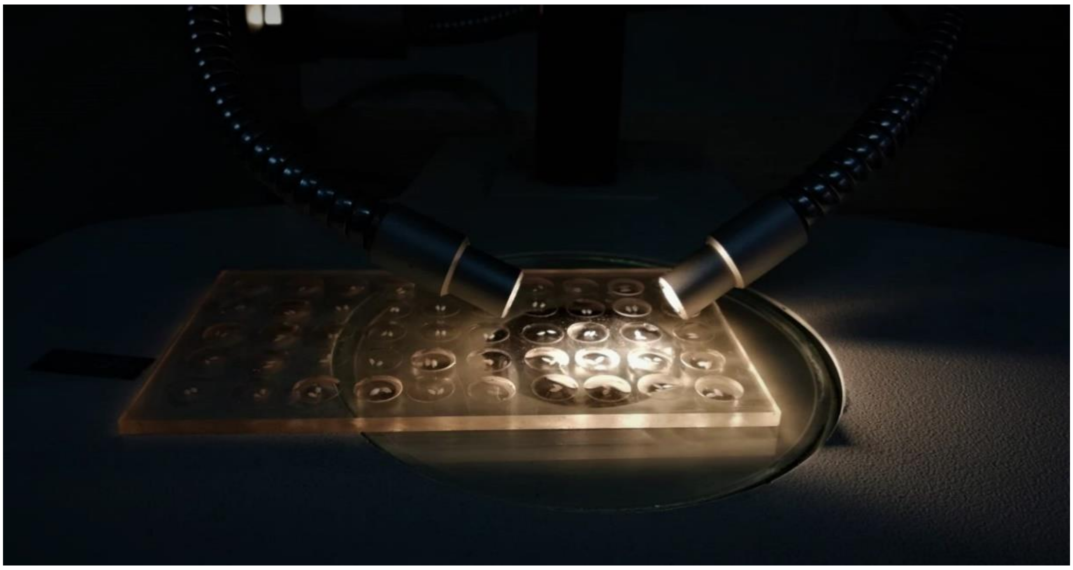
Preparation techniques



- Otolith should be immersed in Glycerin or in a solution (~70% of pure glycerin, ~20% of alcohol and ~10% of water) to make rings more visible under the reflected light (Figure 1).

Figure 1: Resolution solution for otolith reading

- The otoliths should be observed under the reflected light against a black background by using binocular microscope with the magnification of 25X. Magnification can be increased to improve the visibility of the edges and some ring formations.
- The sources of light should be oriented from sides but not from the top
- It is important to move the otolith in various angles to detect the possible ring formations. To move the otolith a thin needle should be used (to not to damage the otolith)
- Camera and screen is not recommended, however can be used for the morphological measurement.
- A plate (see the photo below) which can hold ~50 otoliths is suggested to minimize the time needed to replace the otolith while age reading



- Otolith should be oriented as the distal surface (convex side) up and the proximal surface (concave side) down while reading.

Age Reading from the Otolith

In the Black Sea anchovy each true hyaline ring (transparent zone) represents an age. Therefore the edge of the otolith should be checked first to determine whether it is ended with an opaque or hyaline ring. Care should also be taken to the beginning of opaque/hyaline formation at the edge.

If the fish is caught before 1st of June, if the edge is transparent and not completed (not followed by an opaque formation), the outer hyaline ring (at the edge) should NOT be considered as an age ring.

If the opaque formation starts in the edge of the otolith (it means hyaline ring is completed), then the number of hyaline rings corresponds to the age of the fish.

If the fish is caught after 1st of June, the number of completed hyaline zones correspond to the age of the fish. The new zone observed on the edge corresponds to the growth in the summer (denoted by +).

If the hyaline formation is observed at the edge of the otolith, then this ring should NOT be considered as an age ring as its formation is not completed.

The rostrum, post rostrum and the most importantly anti-rostrum are the areas where the age rings and formation of new zone are best distinguished (figure 2). This is particularly important for the older fish as the 3rd and later age rings densely accumulate in the dorsal and ventral zones and they might not be distinguishable around the otolith.

- Except the first hyaline ring which should be fully formed all around the otolith, the following age rings are not expected to be observable all around the otoliths. To be considered “a true age ring”, the hyaline zone should be sighted on at least two different areas of the otolith pairs.

- The “+” symbol is traditionally used in all Black Sea countries, to indicate that the fish passed its birthday (Figure 3) i.e. 2+ means that the fish is older than two years old by months.

- Considering the existence of the different forms of anchovy with different growth rate exploited on the same fishing ground, the first completed hyaline ring fully formed around the center of the otolith should be recognized as the “first age ring” without considering its distance from the core of the otolith. To avoid misinterpretation due to false rings, it is, however recommended to conduct further studies to determine the location of the first ring with respect to the center of the otolith.

- Given that growth performance of the Black Sea anchovy is largely determined by the food availability changing drastically from one year to another (negative impact of ctenophore *Mnemiopsis leidy*), the distance between the successive age rings are not necessarily reduced as the fish gets older. Therefore, while deciding on a true age ring, it is recommended sometimes NOT exactly to take the distance between the rings into consideration.

- If the first hyaline ring does not form all around the otolith it should be considered as a FALSE ring. It is also noted that in the case of Azov Anchovy, it is sometimes possible to observe a fully formed but not bright ring on the thin otoliths of the juveniles (0+). This kind of formations should be considered as a FALSE ring.

- Interrupted translucent formations, if NOT observed at least on two different regions of the otolith pairs should be noted as FALSE ring.

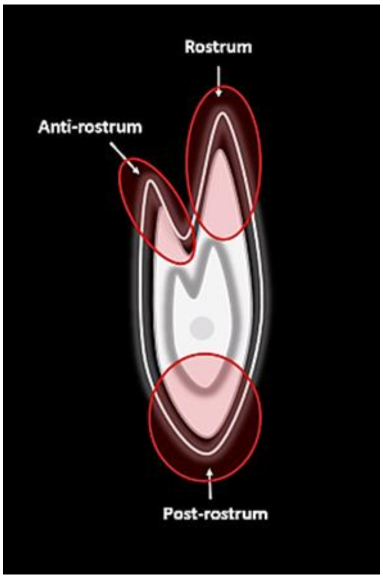


Figure 2. Best areas to check the age ring formations are the anti-rostrum, rostrum and post-rostrum

Additional recommendations

The anchovies collected in April and May should be avoided and NOT used for age reading as the transition from hyaline to opaque zones occurs during this period and interpretation of the age rings may therefore be very difficult (error-prone age reading).

The otoliths which are not easy to interpret (AQ III) should be discarded provided that total numbers of otoliths discarded do not exceed 10% of the samples.

Whenever possible freshly extracted otolith should be preferred for the determination of the age. The otoliths stored more than 3 years should NOT be used for age readings as the contrast between rings fades away parallel to the storage duration.

Age Determination Diagram for the Black Sea Anchovy with the birthdate of 1st of June

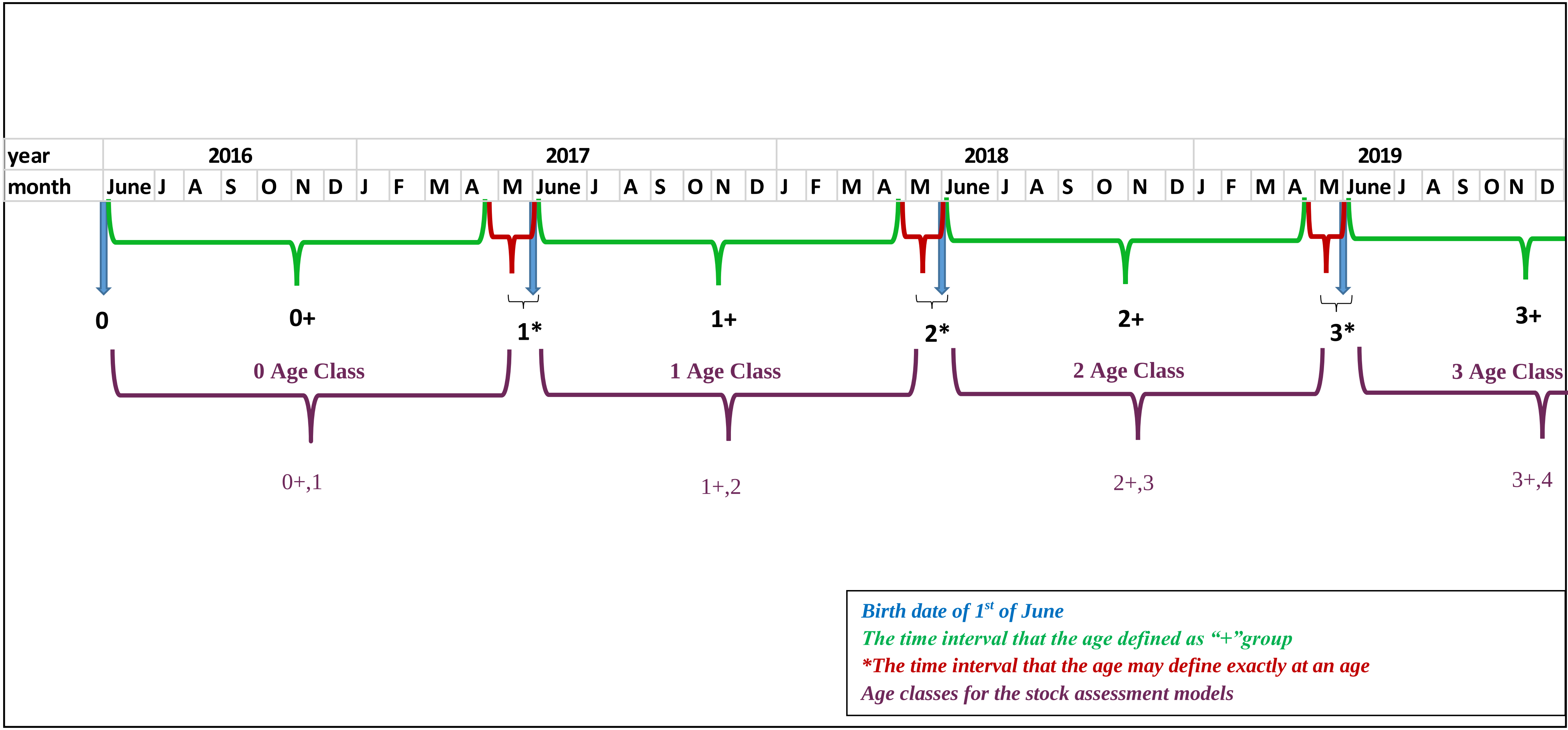


Figure 3. Demonstration of the age allocation for the Black Sea Anchovy with the birthdate of 1st of June for the biological age and for the stock age.

*** In April-May, it should be considered as having the exact age without "+". As an important note, this is a transition period and the probability to make errors in age determination is very high.**

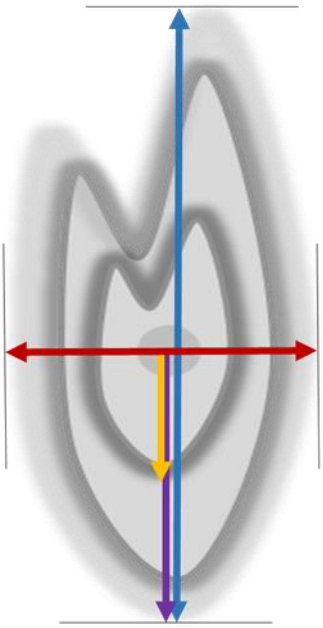
Age determination recording sheets

The group discussed on the necessity of recording the age readings in a harmonized manner and recommended that the following points should be included in the age recording sheets

- ➔ Estimated biological age of the fish
- ➔ The number of true hyaline rings (H) and true opaque rings (O),
- ➔ Edge types
  - WH for wide hyaline edge zone
  - WO for wide opaque edge zone
  - NH for narrow hyaline edge zone
  - NW for narrow opaque edge zone

- ➔ Readability of the otolith = Age quality (AQ scoring)
  - I = easy to age,
  - II = difficult to age,
  - III = very difficult to age (unreadable)
- ➔ If present, false rings (checks) are suggested to record (C0+ or C1+ or C2+) according to their location on the otolith.

It is also recommended to take of some **morphometric measurements** (Figure 4). With that regards the following measurement should be taken



- ✓ Otolith length
- ✓ Otolith width
- ✓ Radius length (from nucleus to post-rostrum)
- ✓ Ring radius length (from nucleus to end of the each ring)

The ring radius length should be measured from the center of nucleus to the end of ring/zone formation on the axis towards post-rostrum.

Figure 4. Demonstration of the morphometric measurements on the otolith

Differences between Azov and Black Sea Anchovy Otoliths

An important source of misinterpretation of otolith age in the Black Sea anchovy is the occurrence of different forms of anchovy, each displaying different growth rates. With that regards, two prominent forms Azov and Black Sea anchovies are scrutinized and the following points which may be used to recognize these forms were listed;

- In general, Azov Anchovy otoliths are more rounded with lower length/width ratio of than the Black Sea Anchovy. According to Chashchin (1996), this approximate length/weight ratio index for the Black Sea Anchovy is 2.15 and for the Azov Anchovy is 1.96.
  This morphometric property (elongated vs rounded), however, may not be very distinctive at juvenile stage.
- Most of the time, the young-of-the-year (0+) of the Black Sea anchovy reaches a larger size in the beginning of winter than the Azov anchovies (at same age). This feature, however, may not be as decisive in juvenile anchovies as in the adults. In some years and especially in the summer, it may not be possible to distinguish juvenile Black Sea anchovy from Azov sea anchovy, based solely on length. For instance, the age of an 12 cm anchovy sampled in the Black Sea was read as 1+, while the age of a same sized anchovy caught in the Sea of Azov was determined as 4+ (Figure 5).

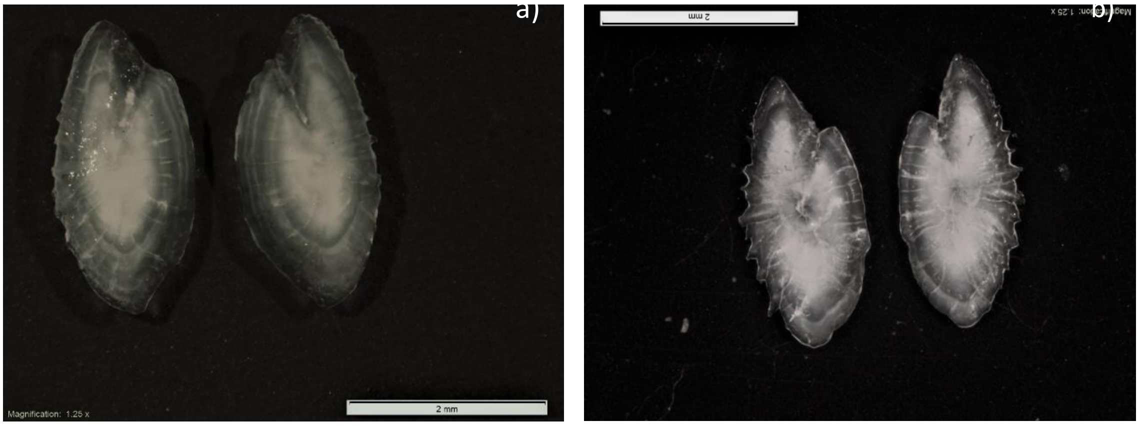


Figure 5. (a) the Azov Sea Anchovy otolith from Kerch Strait, 12cm, caught at March 2017 and the age is 4+. (b) Black Sea Anchovy otolith from South-Eastern part of the Black Sea, 12cm, caught at July 2018 and the age is 1+.

- These criteria may not be applicable for all samples and some contradicting cases may be observed. In this case one should considered hybrid forms of anchovy whose proportion in the stock is not negligible.

Reference

Chashchin, A. K. (1996). The Black Sea populations of anchovy. Scientia Marina, 60, 219-225.

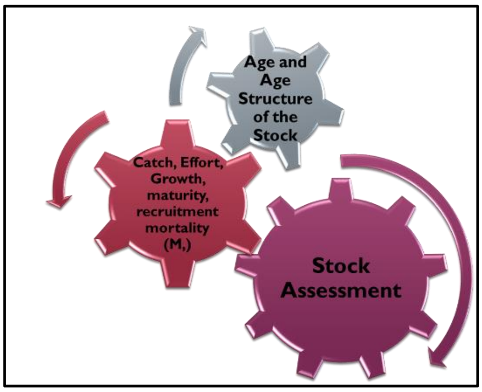
Appendix 4

Presentation summaries

by Gizem Akkuş1st presentation The Importance of a Common Age Reading Protocols for the Black Sea Anchovy Stock Management

Black Sea (*Engraulis encrasicolus ponticus*) and Azov Anchovy (*Engraulis encrasicolus maeticus*) are migratory species and shared stock for the Black Sea. Throughout their life span they are exposed to fishery by the Black Sea riparian countries in different level (in different season). Therefore, without looking at which level of fishery this fish is exposed, a scientific and sustainable assessment is very crucial for the future of both ecology and economy of the Black Sea fishery.

In the Black Sea, anchovy stock assessment have been done for decades and since last several years by the commissions of GFCM (2012-2018) and STECF (2011-2018). The main outcome after these assessments is to create a regional common age reading protocol for the Black Sea Anchovy. Because until today, after the evaluation of the data and the results of the model, the differences in age reading methods and consequently the different age results from the same stock leads to some misevaluation of the stock situation. And these less precise and low accurate age reading results create useless management plan for the stock.



Moreover, for the assessment of the stock some age-structured and VPA based models have been used. These models rely on the estimations of the growth, catch-at-age, mortality, maturity, age-based fishing selectivity, and recruitment. Moreover, estimations of these parameters are depends on the demographic information of the stock. And these information are obtained from the age reading from the calcified structures, in our case otoliths, of the fish. To make a long story short, to reach the powerful stock assessment results and management plan, it is very important to have a proper ALK of the stock which is assessed. In the current situation, the institutes which support the data for the stock assessment of the Black Sea Anchovy have their own age reading methods and consequently they have different ALKs. This situation is most probably the main source for errors and unexpected results of the assessment models.

With this requirement, it was attempted a common study between the age readers in the Black Sea to harmonize the Anchovy age reading in the region. 7 experts from four different country of the Black Sea (Turkey, Georgia, Ukraine and Romania) attended to this study and examined the 60 pairs of anchovy otoliths which collected in July, October and December. In this exercise, the consistency in the ALKs used in anchovy stock assessment was tested. And the differences in age reading among the Black Sea countries were evaluated. According to the results, overall agreement and precision in anchovy age determinations are not satisfactory as the average agreement was only 54 % and CV was 58%. The final recommendations from this exercise were (i) it is important to solve the age reading problems in the Black Sea Anchovy to make a progress in stock management, (ii) it is necessary developing a regional level common age reading protocol to collect the proper and comparable data from the countries who provide data for the stock assessment.

Finally, in this workshop regional and common age reading method for the Black Sea Anchovy has been protocolled. And during the workshop, the items in this protocol have been discussed one by one. After the arrival at consensus views on protocol, hands-on session has been conducted with a set of otolith from different months. The aim of this exercise was to increase precision between the experts from different institutes in terms of interpretation of rings and decisions on controversial ring formations. Some of the issues had discussed was check/false/fake rings, uncompleted rings, expected ring location on the otolith, etc.

Common Points in European Anchovy Age Readings among the Regions

Some of the rules for age readings of the European Anchovy (*Engraulis encrasicolus*) are generally accepted and therefore common for all regions.

- The observation of the otolith is made under the reflected light against black background.
- An annulus; translucent zone (slow growth, winter) and opaque zone (fast growing, summer) represent a year for the fish.
- While determining the age, it is accepted that the number of completed translucent zones are the age of the fish.
- It is assumed that as the fish get older the distance between the annulus and the wideness of the rings get decrease.

The Main Issues needed to be solved in preparing the European Anchovy Age Reading Protocols for each Region

There are some critical points that makes the general age reading protocols controversial because of geography-specific regional peculiarities associated with growth, spawning season, etc. To make the age-reading protocol specific to the region, the following issues should be rendered a decision by the regional experts.

- The reference birth date of the anchovy.
- First true translucent ring location on the otolith, and applicability of the rule which sets distance limits on the regions where the ring is expected to occur.
- Differentiation between true age ring and the false rings and their possible locations on the otolith.
- Determining the beginning/end of the opaque/hyaline ring formation on the otolith.
- Evaluation the ring formation (edge zone) with respect to the catch date of the fish.

2nd Presentation Age Reading Protocols used for the European Anchovy &Their Applicability to the Black Sea Anchovy

Within the context of the workshop two age reading guides were evaluated with regard to their applicability to the Black Sea anchovy. These guides were

“WKARA2 2016 REPORT _Report of the Workshop on Age Estimation of European anchovy (*Engraulis encrasicolus*)” presented by the ICES and “Handbook on fish age determination: a Mediterranean experience” prepared by GFCM.

For otolith extraction, storage and preparation techniques;

In general, Sagittae is used for age reading of the anchovy. Even though there are differences in the otolith extraction method used by the Black Sea experts and although there are some suggestions in the ICES and GFCM guides for the harmonization of the methodology, the group did not see any particular concern associated with the extraction of otoliths. It was therefore decided that the expert continue with the method they have applied so far, as long as care is taken not to damage the otoliths.

In both ICES and GFCM anchovy age reading it was suggested that the otolith should be stored dry and should be examined under the reflected light against black background, the distal surface up and the proximal surface down.

- With respect to ICES protocol, otolith should preserved embedded in resin or kept dry in vials/envelops. While reading, otoliths should be embedded in resin, or immersed in water or ethanoic solution (70%) to better visualize the ring formation under the dissection microscopes with 20-25X magnification.

  On the other hand, in GFCM protocol recommends to store the otoliths as dry in rigid plastic vials. While reading, otolith should be immersed in alcohol (70%) or in seawater as clarification medium and read under the binocular microscope with the magnification of 20-40X.

  For the Black Sea, the participants agreed that; while reading, otolith should be immersed in Glycerin or in glycerin solution (~70% of pure glycerin, ~20% of alcohol and ~10% of water) to increase the resolution of the ring formation under the reflected light of binocular microscope with the magnification of 20-40 X (it can be increased to see well the edge and dense ring formations). During the workshop, alcohol (70%), glycerin and glycerin solution have been tested as immersion solution. According to the results after the third and fourth read in alcohol, some damages and loss of ring visibility were observed in otoliths. However, in glycerin, and in glycerin solution, otolith ring visibility lasted longer. Moreover, most of the institutes represented in the workshop noted that they have been using glycerin for years without noticing any damage on the otoliths. Due to these reasons it was decided to use glycerin as immersion solution.

  For the storage, otolith should be stored as dry in the envelopes (paper or plastic). The group also noted that once immersed in the glycerin, it is not easy to remove remnants from the otolith without causing damage. Also, preserving otoliths in vial tubes without removing the glycerin residuals may lead to further chemical damages and/or bacterial growth which in turn decrease the visibility of the rings. It was therefore highly recommended to clean the otoliths with paper which absorbs the residuals before storing or store in good quality paper envelopes which can absorb the glycerin remaining.

For Otolith Age Reading

- As a thumb rule in age reading for ICES, GFCM and this workshop, the number of translucent zones counted as true age ring.
- The assumed birthdate for the fish is 1st of July for the GFCM and the 0.5 year resolution is the unit for aging. If the fish had caught in the first half of the year, the translucent edge is also counted as an annual ring and the age will be the number of translucent zones (N) - 0.5. However, if the fish had caught in the second half of the year, then the edge will be opaque and the age will be equal to the number of translucent zones (N). On the other hand, ICES (2016) report is more flexible in terms of birthdate. It gives alternative birthdate (1st of January and 1st of July) and aging rules are set accordingly. The ICES protocol suggested that if the fish was caught in the first half of the year, the age will be N-1 (in here again the last translucent zone is counted_N) and if the fish caught in the second half of the year, then the age will be equal to the number of translucent zones (for the birthdate of 1st of July).

Considering the spawning season of the Black Sea Anchovy and the timing of the ring formations, the birthdate to be applied in the age readings was discussed and the group unanimous agreed to use 1st of June as the birthdate of the Black Sea Anchovy.

While aging, it was decided that the number of "completed" transparent zones are counted as age rings however if the fish was caught at the end of the year or in the first half of the year, the last transparent ring at the edge zone will not be counted as age ring. Because until the last opaque formation becomes observable, it cannot be said that the last hyaline is completed (Figure 1). Therefore the age will be N+ (N=number of completed translucent zones, + = growth after the last birthday). The + aging strategy was accepted by the working group. If the catch time is after the accepted birth date, it should be used "+" symbol (0+, 1+, 2+…) means it is not sharply at the given age but bigger than it. The applicability of the extracting 0.5 or 1 year from the counted age was found not practical to apply because it may create the error in determination of the year classes of the fish.

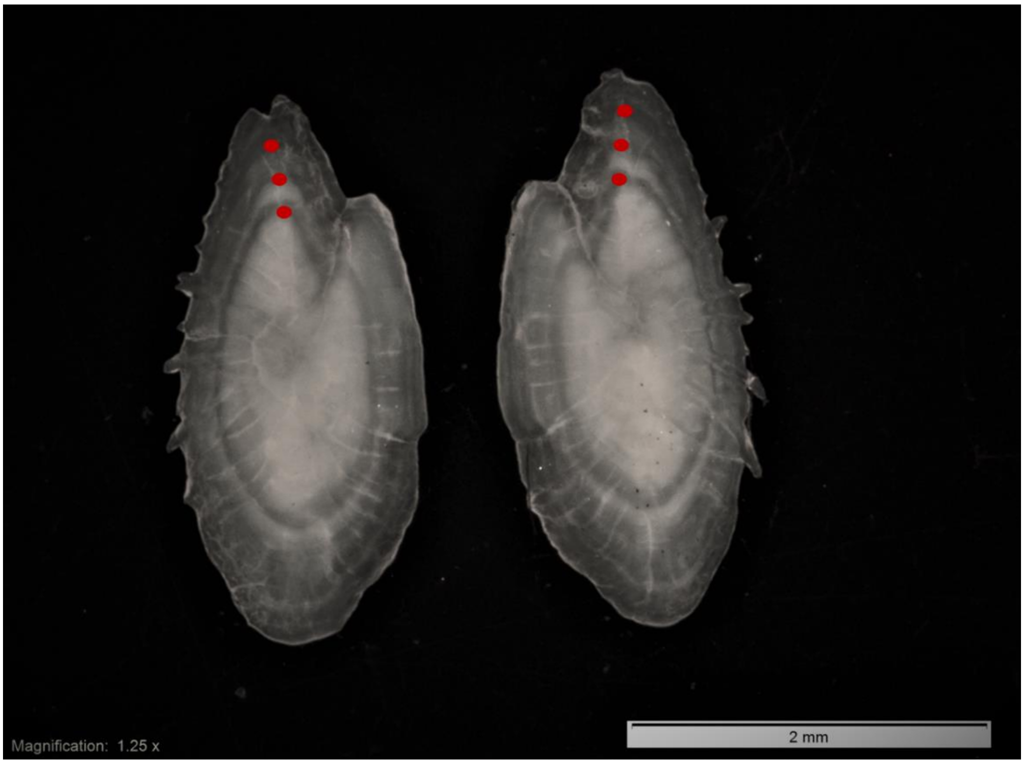


Figure 1: Catch date: November 2016 (outer hyaline formation started but not counts as age because it is not completed yet), 12.3 cm (total length), Age=3+

- According to the ICES (with some exceptions in it) and GFCM protocol, to be able to call a ring as a true age ring, it should be followable all around the whole otolith. With respect to the discussion on this item, it was decided that, except the first hyaline ring formation, it would not the case for all rings especially for the older ages. Therefore it was decided that if the ring is visible at least two different areas on the otolith, it is accepted as the true age ring.

- For the distance of the first ring from the nucleus. In ICES (2016) protocol it is used radius measurement to distinguish the check rings and the first true hyaline ring. According to Hernandez et al. (2013), if the distance from the nucleus to the first ring formation place is less than about 0.8mm then the ring is assumed as false ring. Similarly, in the GFCM report, it has been suggested same rule by referencing the ICES (2010) Report. However, the study of Hernandez et al., (2013) was done in the region (Bay of Biscay) where the anchovy growth rates is different than the Black Sea Anchovy. Therefore; before using directly same rule, similarity in growth rate should be proved first. Thus, working group decided that until further studies on the first ring distance from the center have been done for the Black Sea Anchovy, the first true 'completed' ring accepted as "first age ring", without considering how close/far to the core of the otolith.

- Except the first ring location, the other check/false/fake ring formation discussed by the working group and the decisions are same with the ICES and GFCM (which reference to ICES (2010) also). According to this, if the ring is not completed and/or shadowy/faint (especially before the first hyaline ring) and if the thin hyaline formation occur in opaque zone (especially for 1 year old fish in summer-spawning check) then these formations are assumed as "check-false-fake ring", because the occurrence of these ring are due to the environmental and/or biological stresses. Moreover, sometimes several very close rings formation can be observed (mostly in the post-rostrum part of the otolith) and should be counted as one ring. Because they are the appendences of the single ring.

- In both protocol of ICES and GFCM it was emphasized that as the fish get older the distance between annulus and also the width of the opaque and the translucent zones decreases due to the decreasing in the growth rate of the fish as it gets older. However, all experts who participates to this workshop was on the same mind about that this situation is not always the case for the Black Sea Anchovy otolith especially in last decades. The growth parameters of the Black Sea Anchovy show high variability in year to year. Due to the changes in the environmental conditions and food availability (negative effects of the ctenophore *Mnemiopsis leidy* to the Black Sea). Therefore, in Black Sea anchovy otolith, the distance between the rings and also the width of the rings can be wider or narrower than expected.

Appendix 5

Terms of reference of the age reading workshop

1. Problems associated with interpretation of the annuli (check rings, sub-species, habitat differences)

- To review the current ageing procedures, taking into account of "Handbook on fish age determination: a Mediterranean experience" and the results of the specific workshops held in the framework ICES (WKARA);
- To evaluate and elaborate the results of the otolith exchange exercise held among Black Sea institutes providing data to the SGSABS
- To review the sample processing techniques for age reading of the different laboratories and decide on standardization process to improve the quality (i.e. accuracy and precision) of otolith readings;
- To test the accuracy of age estimates through complementary validation methods (marginal increment reading, length frequency distributions, etc.);

2. Problems associated with stock assessment (birthday, incompatibility between the calendar year and fishing seasons)

- To determine the theoretical birthday that could best represent the stock, taking into consideration the different exploitation periods in different countries and biology of the anchovy.
- To agree on a standardized ageing scheme for Black Sea anchovy