REPORT OF THE ICCAT GBYP INTERNATIONAL WORKSHOP ON ATLANTIC BLUEFIN TUNA GROWTH

E. Rodríguez-Marín[1], P. Quelle[1], P. Addis[2], F. Alemany[3], A. Bellodi[2], D. Busawon[4], O. Carnevali[5], J.L. Cort[1], A. Di Natale[6], J. Farley[7], F. Garibaldi[8], S. Karakulak[9], K. Krusic-Golub[10], P.L. Luque[11], M. Ruiz[1]

SUMMARY

In the last Atlantic bluefin tuna assessment, an age-length database coming from direct ageing was presented for the first time. It was observed that otolith age estimates for fish younger than 8 years old had a smaller size at age compared to spine (first dorsal fin radius) age estimates. This difference, although small, was enough to misallocate the year class. This misallocation was solved when introducing a vector of bias corrected aged otoliths based on paired otolith-spine samples. We have identified two possible causes for over-estimating age in the otolith age-length data: the current age adjustment criterion (to convert the bands counting into ages) and a reading bias in age estimations from some laboratories. Otolith preparation and reading protocols have been reviewed. The edge type and marginal increment analysis showed that the formation of opaque zones would seem likely to occur primarily between December through to June, contrary to what was thought until now, for which a new criterion for age adjustment has been proposed.

RÉSUMÉ

Dans le cadre de la dernière évaluation du thon rouge de l'Atlantique, une base de données âge-taille basée sur la lecture de structures calcifiées a été présentée pour la première fois. Il a été observé que lorsque l'âge des poissons de moins de 8 ans était estimé à partir d'otolithes, ceux-ci avaient une taille par âge plus petite que lorsque les épines (premier rayon de la nageoire dorsale) étaient utilisées. Cette différence, bien que faible, était suffisante pour attribuer de manière erronée les classes d'âge. Cette répartition erronée a été corrigée en appliquant un vecteur de correction basé sur les lectures des épines. Nous avons identifié deux causes possibles de surestimation de l'âge dans la base de données âge-taille des otolithes : le critère d'ajustement actuel de l'âge (pour convertir le comptage des bandes en âges) et un biais dans les estimations de l'âge de certains laboratoires. Les protocoles de préparation et de lecture des otolithes ont été revus. Les analyses du type de bord et de l'incrément marginal ont montré que la formation de zones opaques semble se produire principalement entre décembre et juin, contrairement à ce que l'on pensait jusqu'à présent, c'est pourquoi un nouveau critère d'ajustement de l'âge a été proposé.

RESUMEN

En la última evaluación de atún rojo del Atlántico se presentó por primera vez una base de datos talla-edad basada en la lectura de estructuras calcificadas. Se observó que cuando se estimaba la edad de los peces menores de 8 años a partir de otolitos, estos tenían una menor talla por edad que cuando se utilizaban espinas (primer radio de la aleta dorsal). Esta diferencia, aunque pequeña, era suficiente para asignar erróneamente las cohortes, y se corregía cuando se aplicaba un vector de corrección basado en las lecturas de espinas. Hemos identificado dos

[1] Spanish Institute of Oceanography. Santander Oceanographic Centre, Santander (Spain). E-mail: enrique.rmarin@ieo.es
[2] Department of Environmental Life and Science, University of Cagliari (Italy).
[3] GBYP, ICCAT Secretariat (Spain).
[4] Large Pelagics Group, St. Andrews Biological Station, St.Andrews (Canada).
[5] Department of Environment and Life Science, Universita Politecnica Delle Marche - Ancona, (Italy).
[6] Aquastudio Research Institute, Messina (Italy).
[7] CSIRO Marine and Atmospheric Research, Hobart, Tasmania (Australia).
[8] Dipartimento di Scienze della Terra, dell'Ambiente e della Vita, University of Genova (Italy).
[9] Department of Fisheries Technology, Faculty of Aquatic Sciences, Istanbul University (Turkey).
[10] Fish Ageing Services, Portarlington, Victoria (Australia).
[11] AZTI - Tecnalia, Pasaia, Gipuzkoa (Spain).

causas posibles para la sobrestimación de la edad en la base de datos talla-edad de otolitos: el actual criterio de ajuste de la edad (para convertir el contaje de bandas en edades) y un sesgo en las estimaciones de edad procedente de algunos laboratorios. Se han revisado los protocolos de preparación y de lectura de otolitos. Los análisis de tipo de borde y de incremento marginal mostraron que es probable que la formación de zonas opacas ocurra principalmente entre diciembre y junio, contrariamente a lo que se pensaba hasta ahora, por lo que se ha propuesto un nuevo criterio de ajuste de la edad.

KEYWORDS

Age estimation, otolith, standardization, Thunnus thynnus

1. Opening, adoption of the agenda and meeting arrangements

The ICCAT GBYP International Workshop on Atlantic bluefin tuna growth was held from 4-8 February 2019, at the Oceanographic Center of the Spanish Institute of Oceanography in Santander, Spain.

The Workshop was opened by Dr. Alicia Lavín, Director of the Oceanographic Center of Santander, who welcomed the participants to the oldest marine biology research center in Spain and one of the first marine laboratories in Europe. She wished a fruitful meeting. Drs. Francisco Alemany and Enrique Rodriguez-Marin, the GBYP Coordinator and workshop Chairman, respectively, welcomed the participants and indicated the purpose of the workshop regarding the need of the standardization of the otoliths preparation and reading methodology to minimize bias in age estimations of young bluefin tuna.

The Chair proceeded to review the Agenda, which was adopted with no changes (**Appendix 1**). The List of Participants is included in **Appendix 2**. The following served as Rapporteurs: Patricia Lastra, Jessica Farley, Dheeraj Busawon and Enrique Rodriguez-Marin.

2. Presentations and discussions

Background of the workshop and findings from the otoliths exchange of Atlantic bluefin tuna juveniles. Enrique Rodriguez Marin

The presentation reviewed the background of what has been achieved in Atlantic bluefin tuna (ABFT) ageing using otoliths, giving main reasons that support the need of organizing the current workshop. A bias in juvenile age estimates was detected at the 2017 ABFT stock assessment. Age estimations for younger ages remain uncertain due to the frequent appearance of numerous sub-annual bands. To try to resolve this issue, following SCRS general recommendations to the Commission and conclusions from the recent calibration exchange, a direct ageing workshop was scheduled to minimize bias in age estimations of young BFT using otoliths.

The difference between spine and otolith ages was discussed briefly, given that the age bias plots for the otolith exchange generally indicated older ages from otoliths compared to spines after age 4 years. Spine ages 1-8 are considered accurate and easier to read compared to otoliths, but the ages have not been directly validated. Concerns were expressed on the large range of ages obtained from otoliths for some length classes (e.g., 105-135 cm) from previous eastern ABFT age length keys. In fact, there was discussion concerning the age range of younger fish, as some thought it was too wide, which could be indicating reader bias due to sub annual structures. It was suggested that false annuli may be being counted. Spines could aid correcting this ageing bias. The reviewed otolith ageing protocol (Rodriguez- Marin *et al.*, 2019) that include a template (of the first five annual increment measurement) indicate that annual increment measurement was useful to identify the first annuli and detect age bias. Other issues of concern refer to otolith preparation protocol including: location of ageing section, section thickness, type of light, where and how to measure annual measurements.

It was noted that tuna growth is variable between individuals, and since cohorts (e.g. 2003) can be tracked in the data, it is believed that the age must be relatively accurate. It was suggested that the growth rates of fish in captivity could be examined to determine the level of individual growth variability. Tagging data could also be examined and it was noted that the ICCAT tag database has been “cleaned” so that data only with high confidence in fish length measurements and recapture dates can be analyzed. Could sexual differences in growth explain the bias?

The variability in observed length at age may also be due to sex differences in growth. Difference in growth happens about age 10 and it would be very difficult to split the catch by sex.

Evaluation of Atlantic Bluefin tuna otolith ageing protocols. Dheeraj Busawon

The presentation showed results from the exercise to evaluate standardized and revised reading criteria age estimates that were compared to reading and bomb radiocarbon age estimates used to validate age estimates of ABFT (Neilson and Campana 2008). Evaluation of the Yardstick and Template scale images age estimates showed no systematic bias compared to radiocarbon age estimates for most readers. When age estimates using images with Yardstick and images with Template were compared among readers, there was an acceptable level of precision and overall lack of significant age bias, except for some readers that seems to show higher ages using the Yardstick. The false innermost annulus was identified more frequently using the Template scale.

Overall, the level of accuracy in the radiocarbon ages was noted that it could be 2-3 years higher (older), but not lower (younger). The presenter noted that there was little difference in age assignment by section type (standard versus non-standard) but it does have a slight influence on the measures of annual increments, especially when using the yardstick scale. It was noted that the use of the Yardstick allowed readers to be less guided in identifying zones to count than using the Template, and it was suggested to use the Template scale only for hard to interpret juvenile otoliths. Also, it was noted that the otolith edge type can be difficult to classify. New image capture software with wider focus depth is now available. These advances in imaging technology may help with the edge type classification by enabling better images with clearer edges.

Standardized protocol on the preparation and reading of Atlantic bluefin tuna. Pablo Quelle

Standardized protocols that have been used so far for the preparation and reading of otoliths, along with recent updates, were examined in order to agree on a new standardized protocol (Secor et al, 2014; Busawon *et al.* 2015; Rodriguez-Marin *et al.* 2019). The otolith preparation protocol used until now had not been published in a referenced document and was only available as a working document (Busawon *et al.* 2018). The presentation reviewed methodological aspects in which there was controversy or that were carried out in slightly different ways according to the laboratory (thickness of the section, location of the sectioning, where to mark growth bands, scale / reference measurements, images vs. physical samples, edge assignment criteria, etc.).

There was some discussion on the use of the age adjustment criterion, and it was confirmed that all ages were adjusted in the same way as information was not collected on the otolith edge type to determine if an increment had formed (or not) just prior to capture. It was noted that it should be applied in a flexible way to account for individual variability in zone formation time to avoid introducing error. But it was clarified that if the age adjustment criterion was not applied, the age length key looked odd. It was suggested that a criterion on edge type formation should be applied before making an age assignment. The participants confirmed that the collection of edge type data was needed for each otolith reading. It was also noted that opaque zones may form at different times of the year for different age classes, and this needs to be investigated further inasmuch as marginal increment analysis (MIA) for otoliths has not been reported up to date. It was also suggested to use fractional age using an assumed birth date (June 1). For assessment purposes, January 1 may need to be used (calendar year) to keep cohorts together, which does not appear to be truly happening using the current age adjustment method.

There was a general discussion on the need to keep the otolith section containing the "primordium" for ageing or for microchemistry analysis to determine the stock of origin. It was noted that the count of annual bands becomes more difficult in sections that are taken further away from the otolith "primordium". The first opaque zone is easier to identify in the otolith section containing this core area, and it may disappear from some sections if they are too far away from the "primordium". The current otolith preparation protocol uses this core area for stock identification analyses; therefore, the “primordium” section is unavailable for ageing. It was mentioned that this issue would be solved if both otoliths from the specimen were used, one for each analysis, instead of taking two sections from the same otolith, as section location seems to have a big effect on age estimation. It was also suggested whether we could explore changing the milling zone (using the bridge area before 1st inflection or sample of the entire otolith in the primordium zone). Modern techniques require less otolith material and it may now be possible to use the same section for both purposes. The Group also discussed section thickness required for the microchemistry and ageing work. It was suggested that the section used for otolith chemistry could be re-polished after the microchemistry work was complete for ageing purposes, as it is cut at ~2mm thick.

There was some discussion on changes in the yardstick size (reference scale used to help first annulus identification) depending on the section location (along the otolith) employed. It was recognized that section thickness would vary depending on the type of light, with transmitted light requiring thinner sections (~ 300 µm). All these discussions and the accorded protocols have been added to the **Appendix 3**.

Methods used by Fish Ageing Services for the preparation and age reading of Atlantic Bluefin Tuna. Kyne Krusic-Golub

Presenter provided details about Fish Ageing Services (FAS) ageing experience in several tuna species and described the procedure, preparation of samples and ageing criteria, they followed to age ABFT. They used sections containing the "primordium" which resulted in clear image sections for reading (sections of 370 microns were polished to 320 um, using 800 grit wet /dry paper). Opaque zones were counted and marginal opaque zone was only counted once it was fully complete (i.e. when there was translucent material between the last opaque and the edge). They used the following edge type assignment: Edge type: wide translucent (WT) narrow translucent (NT), and opaque. According to presenter, opaque is forming through May-July and translucent in July to March. They use a customized image analysis system to mark, count and measure the distance from first inflection to the start of each subsequent opaque zone. A 10% re-read was completed by an age reader outside of FAS, to provide a measure of inter-laboratory precision and bias.

Overall there was some discussion on the edge type, timing of increment formation and whether the opaque zones form during periods of fast growth when the fish are feeding. It was also discussed the light source used when reading otoliths (transmitted or reflected). The presenter recommended using transmitted light as less sub-annual bands are visible when compared to reflected light. Section thickness was also considered and it was recommended that for transmitted light the thinner the section the better. A question was raised about how they differentiated between first and false annulus. The presenter answered that they examined good images to get an idea on where the first annulus should be, and measurements were consistent with yardstick and template scales (scales used in standardized protocols for ABFT, around 750 microns).

Atlantic bluefin tuna otolith measurements. Enrique Rodriguez-Marin

In this presentation, different options for measuring otoliths were reviewed. Annual band measurements during otolith reading are useful for quality control of age estimates. Reference was made to: where to measure, type of measurement (a straight line, line segments or parallel lines distance) and starting and end point of the measurement.

There was a discussion on how to take consistent annual band measurements along the ventral arm and various suggestions were considered and discussed. The Group agreed that the ventral groove is the best area for aging, especially from the 7-8th annual band (a little before the second inflection, going from the primordium to the edge of the ventral arm).

Agreement was reached on using a “measurement line” to standardize otolith growth measurement; this can facilitate detection of ageing bias and enable analyses such as back calculation. This "measurement line" is equidistant between 2 sets of parallel lines. In the first set, the lines are drawn parallel to the sulcus margin of the ventral arm and also through the ventral groove between the 1st and second inflection point (**Figure 3** from **Appendix 4**). In the second set, the lines are drawn similarly but between the 2nd inflection point and the end of the otolith. Note that if the measurements of the annual bands after the second inflection are not needed, defining the second segment is not required. The origin of the "measurement line" or anchor point is where the “measurement line” crosses the bridge. Note: care should be taken in placing the anchor point due to the 3 dimensional aspects of the image (i.e. there is an inner edge and an external edge at the bridge area due to the thickness of the section), the anchor point is located on the inner edge of the bridge area (i.e. where the section has been polished). To quantify the distance between annual bands, measure within the "measurement line", from the anchor point to where the opaque bands of each presumed annual growth band are most marked. The most marked areas (or most opaque) have been chosen as the end points of the measurement because in the first 7-8 annual bands it is difficult to establish the edge of the opaque band. The end point may be modified to conform with an edge of the opaque band when measuring growth bands as in case of MIA.

Periodicity of strontium: calcium across annuli further validates otolith-ageing for Atlantic bluefin tuna (Thunnus thynnus). Siskey et al. (2016)

The Group discussed the findings of the paper that suggested opaque bands in otoliths are formed during the winter months (higher Sr/Ca in opaque zones). This is the opposite of what happens in southern bluefin tuna (*Thunnus maccoyii*) and what has been described for ABFT using several calcified structures such as otoliths, spines and vertebrae, including edge type frequency analysis, where translucent edges appear more frequently in winter months (Clear *et al.*, 2000; Cort *et al.*, 2014; Luque *et al.*, 2014). Several explanations were explored such as whether there was a delay in when the bands are formed and seen in the otoliths. It was noted that the timing of band formation may also change with fish age. It was mentioned that a general conclusion on time of formation of annulus might be erroneous as there are so many variables that come into play. Hence, MIA is needed to resolve this issue.

The opposite deposition pattern was found in spines of Atlantic bluefin tuna, where strontium was significantly higher in the translucent bands in the second year (Luque *et al*., 2017). Nevertheless, we should consider that otolith and fin spines are different hard structures in terms of their chemical composition and the higher amount of organic material present in spine bone very likely might be affecting the results. Overall participants noted that there was a lack of knowledge on the mineralization process of otoliths and the factors affecting band formation.

Annual ageing of southern bluefin tuna, albacore and bigeye tuna using otoliths. Jessica Farley

Presenter provided an overview on methodology used for preparing and reading otoliths, and age assignment (converting zone count to age) for several tuna species sp: southern bluefin tuna (*Thunnus maccoyii*, SBT), albacore (*Thunnus alalunga,* ALB) and bigeye (*Thunnus obesus*, BET). Also there were showed some validation studies achieved including: 1) bomb radiocarbon analysis for SBT, that indicated a close agreement between bomb radio carbon ages with otolith increment counts for ages 23-34y. 2) Mark-recapture analysis for SBT, ALB and BET ($SrCl^2$ and OTC) getting success in validating growth zones. No success was achieved when using OTC in fin spines, where the mark is not such clear. 3) MIA for SBT, ALB and BET; and daily ageing to locate first growth band for ALB and BET. Presenter also showed the usefulness of otolith weight information. Using otolith weight as a proxy of age you can infer differences in growth between geographical areas.

Presenter also showed how to convert annual counts to fractional age using an age adjustment algorithm, that accounts for: birth date, timing of year that opaque zones form, otolith edge type (narrow, intermediate or wide) and capture date. The participants largely discussed that we should revise our edge assignments before assigning a final age. Applying similar edge categories as were used for BET, it was also discussed to develop a similar algorithm for a final age adjustment. Still pending some discussion on what to do with historic samples and absence of information on edge type.

Effects of age on growth in Atlantic Bluefin tuna (Thunnus thynnus). OlianaCarnevali

The presentation showed results from a pilot study using ABFT cages with the aim to evaluate the expression of the insulin-like growth factor (IGF) system genes to gain information on the growth process at different age and possible gender differences. Results showed that there is an evidence of sexual dimorphism in length-at-age and weight-at-age in female and male of ABFT. Male individuals tended to have a higher size (weight and length) with respect to females. Molecular analysis of genes belonging to IGF system revealed that genes involved in growth were differentially expressed in relation with ABFT age. Also a progressive increase in lipid concentration in liver among groups and the onset of hepatic steatosis in 8 years old tuna, indicated that the metabolic energies start to be used for fattening associated with lower growth.

There were questions concerning extrapolation of results from farmed fish to wild fish. And it was recalled that provenance of the fish would be relevant for the interpretation of the results. Previous growth studies have also shown that males reach greater size than females at a given age, with these differences becoming apparent by age 8 to 10 years.

Preliminary edge type and marginal increment analysis on 2000 ABFT otoliths samples aged by Fish Ageing Services. Kyne Krusic-Golub

Edge type analysis and marginal increment analysis (MIA) was conducted using data from 2000 ABFT samples aged at Fish Ageing Services (FAS) in 2018. Samples were collected from 9 months of the year. No samples were available for January, February, and April (**Table 1**). The distance between the first inflection and the outer edge of each opaque zone (up to a maximum of 15 zones) was measured. Additionally, the distance from the first inflection to the edge was measured and the edge type was classified either as wide translucent (WT), narrow translucent (NT) or opaque (O). Because there was a high proportion of otoliths classified as 0+ within the total sample, in order for these to be useful we decided to divide the distance from the last opaque zone to the edge (margin distance) by the average distance for that corresponding completed annulus. The average width of each completed annuli was estimated from the measurements taken during the ageing process. The mean percentage of completion was estimated for each month and the marginal state was plotted as a percentage against each month. According to the preliminary analysis based on MIA and marginal type (**Figure 1**), opaque zones finish forming and are less frequent in July and August, while translucent zones are present from March to November but are more frequent between July and August. Because there were only limited otolith samples collected between November through to March, it was difficult to be able to determine the exact time of opaque zone formation. If we assume that the months with the highest proportion of wide translucent zones (Nov/Dec) corresponds with the start of the opaque zone formation, and that the months corresponding to the lowest MIA and the highest proportion of Narrow Translucent edge types indicate opaque zone completion, then opaque zone formation would seem likely to occur primarily between December through to June. This finding is relatively consistent with Siskey *et al.* (2016), who suggested that opaque bands in otoliths are formed during the months were the water temperature is the lowest (higher Sr/Ca in opaque zones). It should be noted again that in order to properly investigate the zone deposition within otoliths of this species, samples from each month of the year need to be collected and a dedicated MIA and edge type study be conducted.

Analysis of the age-length ICCAT database for Atlantic bluefin tuna. Pablo Quelle and Enrique Rodriguez-Marin

The age-length data used in the 2017 bluefin tuna stock assessment was examined. Age data comprised nearly 14000 records, of which 70% are from the Eastern stock. In the western data practically all the readings come from otoliths, while in the East, they are formed by otoliths and first dorsal fin radius (spine) in a proportion of 10% and 90%, respectively. For the analysis, all records had the same type of length measurement (straight fork length, SFL). Eastern age-length data contains predominantly small fish, while western data contains predominantly large fish and better covers the age range over the last decade. In the last bluefin tuna assessment in 2017, it was observed that for western Atlantic bluefin tuna stock, the strong cohort apparent in the catch at age derived from the combined forward-inverse key was being assigned to the 2002 year class instead of the 2003, when it is the latter which has been identified as a strong cohort. Introducing an otolith ageing bias vector in the assessment model, derived from spine ages from paired otolith-spine readings, this sharpened the estimate of the 2003 cohort rather than blurring it between 2002 and 2003. This bias vector was only applied in the first 7 years of life, since previous studies showed that it is at these ages that age estimates from spines and otoliths from the same specimen differ (Rodriguez-Marin *et al.* 2019).

With the intention of identifying which factor may be influencing these differences in the age-length relationship of the ICCAT bluefin tuna database, the average length by age of the first 7 years was analyzed, according to the following factors: type of structure (otolith vs. spine), management area (East vs. West), age assignment criteria (bands counting vs. adjusted age, both data were only available for otoliths), reading laboratory and reading protocol (old vs. reviewed).

The average size by age of the specimens aged by spines is generally longer than that coming from the otolith readings from both eastern and western stocks, and this difference is greater with western stock otoliths. In addition, a smaller size by age in otoliths is obtained when applying the ICCAT age adjustment (Anon. 2017) compared to actual band counts (**Figure 2**). These differences in the average size cannot be attributed to the sampling throughout the year, since in general there is less than a month of difference in the sampling between calcified structures of both stocks (**Table 2**). Sampling is, logically, better represented in the months in which the fisheries occur, which are mainly from May to October, with June to September representing around 80% of the sample.

In the comparison of the average size by age and by laboratory obtained by reading otoliths, it is observed that the laboratory 18 produces a low average size by age from age 3, indicating that from this age an overestimation of one year is being produced. This age overestimation is also observed in laboratory 13 at ages 5 and 6. By contrast, laboratory 15 is underestimating one year at ages 6 and 7 (**Figure 3**). In **Figure 3** the size obtained by reading spines is not directly comparable with that obtained by otoliths, since the first is adjusted age, while the second represents the band counts. The sampling months among groups (age and laboratory) were similar with sampling being performed in the summer, except for ages 6 and 7 from laboratory 16, whose samples were from late spring, and ages 6 and 7 from laboratory 15, whose samples were collected at the beginning of autumn (**Table 3**). The samples collected at the end of the year from laboratory 15 can partially explain the larger mean size of their 6 and 7 year estimates.

We also compared the ages of the ICCAT database obtained following the protocol adopted in 2014 (Busawon *et al.* 2015) with those obtained following the reviewed protocol (Rodriguez-Marin *et al.* 2019). Otolith sections from 61 samples from 1 to 7 years old, which were read by both protocols, were used (**Table 4**). The precision between protocols gives a Coefficient of Variation and an Average Percent Error of 5.2 and 3.7, respectively. The histogram of differences between the estimated age with both protocols shows that the ages obtained applying the 2014 protocol tend to be higher than with the reviewed one (**Figure 4**). Analyzing the differences by age, the old protocol estimates slightly younger ages in specimens under 4 years, and older ages in fishes aged 4 to 7 years. This difference is small, less than a year, but enough to misallocate the year class.

Findings showed that the there are two possible causes for age overestimation in the otolith age-length data: the current age adjustment criterion and a reading bias in age estimations from some laboratories. This last bias seems caused by the false growth bands that appear in the otoliths of juvenile bluefin tuna.

3. Otoliths preparation methodology review

The protocol for the preparation of Atlantic bluefin tuna otoliths for direct ageing was revised from the working document of Busawon *et al.* (2018). During the workshop, the text was partially reviewed, but the final version was adopted by correspondence (**Appendix 3**). The most important issues reviewed have been the thickness of the section according to the type of light used for reading, and the location of sectioning.

Section location affects age estimates because the count of annual bands becomes more difficult and may introduce an ageing bias in sections that are taken further away from the otolith primordium. This core area of the otolith is being used to obtain a section for stock identification analyses (SI). After having consulted the experts who carry out isotopic analysis for stock identification, and with the aim of using the same otolith to obtain the sections for ageing and SI, leaving the other one for other possible studies, the primordium remains inaccessible for direct aging. The SI section will continue to contain the primordium, and the ageing section will be adjacent to it. This is because the methodology applied so far to obtain the section for SI cannot be changed without modifying the isotope signature baseline.

Two new sections have been included: an otolith cleaning and storing description and an examination of samples section, with pros and cons of reading otolith sections physically or in the form of digital images, including image enhancing to improve reading precision.

4. Otoliths reading protocol review

The previous reading protocols from Busawon *et al.* (2015) and Rodriguez-Marin *et al.* (2019) were used as reference documents. The new contributions refer to:

- The recommendation to use transmitted light instead of reflected, since it allows to better distinguish the section edge type.
- A new criterion for the counting of opaque bands, they are only counted if completely formed.
- A procedure to measure otolith sections is proposed, which will serve as a reference measurement for otolith metrics and as a quality control of the age estimates.
- A new classification of otolith edge type is proposed
- A new adjustment criterion is established to convert the count of bands into ages.

Final version was adopted by correspondence (**Appendix 4**).

5. Practical exercise to compare readings and edge type identification using transmitted and reflected light

Edge type assignment was identified as a source of ageing error due to lack of agreement between readers in previous ageing exercises. In addition, conversion of annuli counts to age, in which edge type is taken into consideration, was also identified as a source of error. A new criterion, in which edge width and confidence in edge type is recorded, was suggested to aid with edge type assignment and age adjustment. In order to test this new criteria, readers were asked to do a blind reading of 30 images sampled along the whole year, taken under reflected and transmitted light.

It was agreed to count opaque bands, but only those completely formed. Therefore, the marginal opaque zone was only counted once translucent otolith material could be seen between the outer edge of the opaque zone and the edge margin. The reading form designed for this exercise recorded: readability code (1= Pattern present-no meaning, 2= Pattern present-unsure with age estimate, 3= Good pattern present-slightly unsure in some areas, 4= Good pattern-confident with age estimate), edge type (translucent/opaque), edge thickness (narrow/wide) and edge confidence (1= not confident, 2= confident in completeness and not with the type, 3= confident).

The results showed that edge type assignment, both within reader and between readers differed by light type. Readers showed better agreement on edge type using transmitted light (**Figure 5**). However, this could be the result of image quality as the images taken under transmitted light appeared to be better. There were no clear patterns in edge type assignment by month, therefore no clear indication on timing of formation of opaque or translucent bands (**Figure 6**). Most readers showed no systematic bias compared to the modal age (**Figure 7**).

The influence of the light type, transmitted or reflected, on a better identification of the type of marginal edge, translucent or opaque, was discussed by the Group. It was accepted that the use of transmitted light produced, in general, a better agreement in the type of marginal edge among readers. Image quality (readability) is also perceived a little better with the use of transmitted light. Busawon *et al.* (2015) also found a slight improvement in agreement on edge type and readability using transmitted light.

6. Adopt a correction procedure to enable the use of the age-length keys developed so far

The analyzes performed from the 2017 ABFT stock assessment (Rodriguez-Marin *et al.* 2019) and during the present workshop (**Figure 2** and **Figure 4**) show that the length at age keys of juveniles made up so far from otoliths, present a lower average size by age than that obtained by reading spines or applying the reviewed otolith reading criterion, and this is observed mainly at the ages of 4, 5 and 6 years. This lower size at age is small, less than a year, but enough to misallocate the year class. Three possible reasons have been found for these differences in the average size at age: age adjustment criterion used up to now, laboratory ageing bias, and the difficulty in reading otoliths of juveniles due to the appearance of false or double annual bands, which has motivated the reading protocol review. What solutions are there to solve these three sources of bias?

6.1 Age adjustment performed so far

Findings show that opaque bands are formed in other months than previously thought. Therefore, the current criterion for adjusting the age of the otoliths is not correct and produces an overestimation of age. The participants decided not to use the adjustment criterion that has been used until now to convert the bands counting into ages and a new one has been proposed.

The previous ICCAT age adjustment criterion adopted for otoliths consisted of adding 1 year to the opaque bands counting, when the fish was caught between January 1 and June 1. The adjustments described below have to be applied to the bands counting and not to the ICCAT adjusted age. Therefore, it is necessary to subtract one year to the ICCAT adjusted age in the samples coming from fish captured between January 1 and June 1, to obtain the opaque bands counting.

New age adjustment for age readings performed so far. This adjustment should be applied to band counting performed before the ICCAT GBYP international workshop on Atlantic bluefin tuna growth, February 2019.

Previous age reading protocols required the age reader to count opaque zones on the otolith margin as soon as they are observed, even if not complete. Taking into account the growth band forming periods and in order to place each fish into its correct year class, a two-step age adjustment process is required. This is necessary to firstly account for zone formation and secondly to then align the age estimates to a further adjustment for biological or fisheries management requirements.

6.1.1 Zone formation adjustment

Edge type information was recorded for previously aged samples as: translucent, opaque or not available. Accordingly, to convert the count of opaque bands (N) into age estimates (A), since the previous age reading protocol stated that opaque bands at the otolith edge were counted, even if not complete, the following adjustment based on the otolith edge (margin) type, adjustment date of the 1st July and catch date should be used:

Catch month	*January to June*	*July to December*
Translucent, (T)	N	N
Opaque, (O)	N-1	N
No edge type information	N-1	N

Additional adjustment option 1- Biological age adjustment

June 1 is the universal birth date assumed for both ABFT management units based on the bluefin tuna reproductive cycle, where spawning occurs from May to June in the western Atlantic (Gulf of Mexico) and eastern Mediterranean (Levantine Sea), or from June to July in the western and central Mediterranean (Balearic Islands waters, South of Tyrrhenian Sea and Sea of Sicily) (Rooker *et al.*, 2007). Given this, the following adjustment table should be used:

Catch month	*January to May*	*June*	*July to December*
Biological year	A	A+1	A

Additional adjustment option 2- Calendar year adjustment

To convert the zone counts into calendar year the following adjustment should be used. Note: According to the largest edge change occurring on 1st July (adjustment date), the same limiting date needs to be used to make the adjustment:

Catch month	*January to June*	*July to December*
Calendar year	A+1	A

Thus, a bluefin tuna caught at the beginning of the year is interpreted as being 1 year older, despite being 5 or 6 months prior to the assumed date of birth, which occurs mid-year based on the reproductive cycle

The result of applying the new adjustment criterion is that the otoliths have the same age as that obtained directly from the counting of opaque bands following the previous reading criterion (opaque bands at the otolith edge were counted, even if not complete). Therefore, there is still a lower mean size at age than estimated by the spine and there is still the bias of the laboratory where the otoliths were read (otolith band counting in **Figure 2** and **Figure 3**). This bias occurs mainly in the ages of 4 5 and 6 years.

6.2 Laboratory ageing bias

The analysis of the mean size at age by laboratory (**Figure 3**), shows that the laboratory 18 (Panama City Lab) is biased towards over-aging for ages 4 to 7, as well as the laboratory 13 (Santander Lab) for ages 5 and 6; while laboratory 15 (St. Andrews Lab) is biased towards under-aging for ages 6 and 7. This bias occurs mainly in juvenile specimens, because when the otolith comes from a larger specimen, the size of the otolith (e.g. the appearance of the second inflection) prevents the reading errors that are committed when reading smaller otoliths from juveniles. It would be necessary to re-read the samples of specimens under 10 years old or if there are many, read a sufficient number to obtain an age bias vector.

6.3 Difficulty in reading juvenile otoliths and reading protocol review

The reading protocol has been revised and methods have been suggested to facilitate reading such as: the use of transmitted light that allows to better identify the type of edge, make the sections as close as possible to the nucleus, use measurements and reference scales for the identification of the first annuli.

During the workshop it was raised whether the correction of the age adjustment criterion would be enough to test the corrected age database and re-estimate the catch at age matrix from the combined forward-inverse key and verify whether the combined key gives results similar to cohort slicing. It does not seem that this correction is sufficient, since a reading bias by laboratory has also been detected. There were also e-mail exchanges with Dr. Lisa Ailloud to address the issue of the correction of the ABFT age database by incorporating a bias vector and the need to have annual bias vectors. Unfortunately, Dr. Ailloud could not participate in the workshop and the resolution of this issue was postponed for the near future.

7. Otoliths reference collection

The reference collection that has been used so far is based on 100 images of otoliths read with both types of lights, reflected and transmitted, following the protocol of Busawon *et al.* (2015). It is advisable to reread this collection following the most up-to-date reading protocol (**Appendix 4**) and trying to minimize the possible bias in age estimations of young BFT, due to the presence of sub-annual bands during the first years of life of this species (Rodriguez-Marin *et al.* 2019).

It was argued that it would be appropriate to extend this reference collection, since in addition to serving to monitor ageing consistency, it should also serve for training purposes, and for this second role the current number of one hundred is scarce to get a subsample. Among the factors that should be represented in this reference collection, in addition to span the entire length range, emphasis was placed on a good seasonal coverage in light of the effect on the appearance of the marginal edge of otolith sections.

It was also suggested to use otolith sections obtained as close as possible to the otolith primordium, since section location affects age readability and it is more difficult to read sections that are taken further away from the primordium. For imaging this reference collection, it was recommended to use the same scale of magnification for the whole collection, regardless of the size of the otolith and, of course, to include a reference scale of measurement (burned within the image).

8. Conclusions

1. Section location affects age readability as the count of annual bands becomes more difficult in sections taken further away from the otolith primordium and this may result in an ageing bias.

2. The Group recommends using transmitted light instead of reflected for otolith sections reading, since it allows to better identification the section marginal edge type.

3. A new criterion for the counting of opaque bands was proposed, in which they are only counted if completely formed.

4. Otolith opaque zone formation would seem likely to occur primarily between December through to June. This finding, based on edge type and marginal increment analysis, is relatively consistent with Siskey *et al.* (2016), who suggest that opaque bands are formed in the months with lower temperature and, therefore, have a high strontium: calcium ratio. This time of formation of the opaque band in winter and spring, is different from that previously thought for ABFT otoliths.

5. The current ICCAT criterion for adjusting the age of the otoliths, to convert the count bands into age estimates, is not correct and produces an overestimation of age. A new adjustment criterion has been established, which accounts for new time information of band forming.

6. A procedure to measure otolith sections is proposed, which will serve as a reference measurement for otolith metrics and as a quality control of the age estimates.

7. Findings suggest two possible causes for age overestimation in the current ICCAT otolith age-length data: the existing age adjustment criterion and a reading bias in juvenile age estimations from some laboratories. The first bias can be corrected by applying a revised adjustment procedure, but for the reading bias from some laboratories it will be necessary to perform a re-reading of a sample selection by applying the reviewed reading protocol.

8. A new reference collection is needed that takes into account the revisions and recommendations of the new otolith preparation and reading protocols.

9. Recommendations

- The Group recommends to stop applying the current criterion of adjustment of age and use another criterion, developed in the workshop, that meet the reviewed reading protocol.

- Since new technologies require less sample tissue to perform the analysis, the Group recommends that options are explored on whether both analysis, ageing and those aiming for stock identification (SI), can be obtained from the same primordium section from the single otolith. For example, the section currently used for studies of SI is 2 mm and as thick section may not be necessary. Another option could be to take the SI sample of the entire otolith in the primordium zone.

If the above is not possible, then the Group recommends using one otolith for the age estimation process and the other otolith of the pair for stock identification (SI), particularly for juvenile bluefin tuna (ages 0-3). This is because the count of annual bands becomes more difficult and may introduce an ageing bias in sections that are taken further away from the otolith primordium. The current otolith preparation protocol prevents the use of the primordium area for reading ages, since this area is used for stock identification (SI) analyses. In the case of juvenile tunas, when making the 2 mm section for SI, the remaining otolith section is practically useless for ageing.

- The Group recommends regular inter-laboratory exchanges/checks (e.g. small subsample) to prevent laboratory bias and ensure that correct methods are being applied.

- The Group recommends exploring the possibility of collaboration with tuna farms to carry out direct ageing studies. Bluefin tuna can be individually tagged to track calcified structures growth. This growth is affected by the conditions of captivity but it can be useful to identify, for example, the deposition of annual bands in specimens marked with OTC. Reared age 0 fish are being used in daily growth studies.

- The Group recommends performing age validation studies, for example tag-mark and recapture studies or daily growth analysis on wild caught fish to validate the formation of the first annual band. Other indirect validation techniques should be undertaken.

- The Group recommends conducting a survey to gather information on collections of calcified structures (otoliths and first dorsal fin spines) collected by national or international research programs. As well as to find out if these structures have been read and what method of reading has been used.

- The Group recommends identifying agencies that are currently working on direct ageing of Atlantic bluefin tuna, in order to coordinate future research and share important information as it arises.

10. Other matters

An inter-laboratory calibration exercise for GBYP samples read by an external ageing agency was scheduled.

11. Adoption of the report and closure

The Chairman thanked participants for their hard work. The report was adopted by correspondence. The meeting was adjourned.

References

Anon. 2017. Appendix 4 Otolith and fin spine age estimation protocols. *In:* Report of the 2017 ICCAT bluefin tuna Data Preparatory Meeting. Collect. Vol. Sci. Pap. ICCAT, 74(6): 2268-2371

Busawon, D.S., Rodriguez-Marin, E., Luque, P.L., Allman, R., Gahagan, B., Golet, W., Koob, E., Siskey, M., Ruiz, M., Quelle, P., Neilson, J.D., Secor, D.H. 2015. Evaluation of an Atlantic bluefin tuna otolith reference collection. Collect. Vol. Sci. Pap. ICCAT 71, 960-982.

Busawon D., Rodriguez-Marin E., Quelle P. 2018. Protocol for the preparation of Atlantic bluefin tuna otoliths. SABS, IEO Working doc.

Clear, N. P., Gunn, J. S., and Rees, A. J. 2000. Direct validation of annual increments in the otoliths of juvenile southern bluefin tuna, *Thunnus maccoyii*, by means of a large-scale mark-recapture experiment with strontium chloride. Fish. Bull. 98, 25-40.

Cort, J. L., Arregui, I., Estruch, V. and Deguara, S. 2014.Validation of the growth equation applicable to the eastern Atlantic bluefin tuna, *Thunnus thynnus* (L.), using Lmax, tag-recapture and first dorsal spine analysis. Rev. Fish. Sci. Aquac. 22: 3, 239–255

Neilson, J. and Campana, S. 2008. A validated description of age and growth of western Atlantic bluefin tuna (*Thunnus thynnus*). Can. J. Fish. Aquat. Sci. 65: 1523-1527.

Luque, P., Rodriguez-Marin, E., Landa, J., Ruiz, M., Quelle, P., Macias, D. and Ortiz De Urbina, J. 2014. Direct ageing of *Thunnus thynnus* from the eastern Atlantic Ocean and western Mediterranean Sea using dorsal fin spines. J. Fish Biol. 84(6), 1876-1903.

Luque, P.L., Zhang, S., Rooker, J.R., Bidegain, G. and Rodriguez-Marin, E. 2017. Dorsal fin spines as a non-invasive alternative calcified structure for microelemental studies in Atlantic bluefin tuna. J. Exp. Mar. Biol. Ecol. 486, 127-133. doi:http://dx.doi.org/10.1016/j.jembe.2016.09.016

Rodriguez-Marin E., Quelle P., Busawon, D., and Hanke, A. 2019. New protocol to avoid bias in otolith readings of Atlantic bluefin tuna juveniles. Collect. Vol. Sci. Pap. ICCAT, 75(6): 1301-1314.

Rooker, J.R., Bremer, J.R.A., Block, B.A., Dewar, H., De Metrio, G., Corriero, A., Kraus, R.T., Prince, E.D., Rodriguez-Marin, E., Secor, D.H., 2007. Life history and stock structure of Atlantic bluenfin tuna (*Thunnus thynnus*). Reviews in Fisheries Science 15, 2365-2310.

Secor, D.H., Allman, R., Busawon, D., Gahagan, B., Golet, W., Koob, E., Luque, P.L., Siskey, M. 2014. Standardization of otolith-based ageing protocols for Atlantic bluefin tuna. Collect Vol Sci Pap ICCAT. 70: 357-363.

Siskey, M., Lyubchich, V., Liang, D., Piccoli, P., Secor, D. 2016. Periodicity of strontium: Calcium across annuli further validates otolith-ageing for Atlantic bluefin tuna (*Thunnus thynnus*). Fish. Res. 177, 13-17.

Table 1. Number of samples available for edge type and/or MIA analysis separated by zone count and month.

	Zone count																				
Month	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	20	N
Jan																					
Feb																					
Mar									1	15	14	28	22	7	2		1			1	91
Apr																					
May	1	14	15	9	22	20	12	19	41	84	102	123	171	68	24	15	6	3	2		751
Jun		5	33	28	28	30	6	5	11	10	15	9	8	3		1					192
Jul	1	67	29	23	15	12	5	4	3	1	2	5	7	1							175
Aug	5	27	16	32	16	22	10	1	4	4	1	3	2								143
Sep	11		4	13	13	13	1	4	4	2	5	8	2	1	1	1			2		85
Oct	32	6	6	6	2	6	9	38	79	76	77	72	23	5	2	1					440
Nov	34	1	1	2	1	3	1	2	7	4	2	6	2		3	2					71
Dec	26			1			1														28
N	110	120	104	114	97	106	45	73	150	196	218	254	237	85	32	20	7	3	4	1	1976

Table 2. Age-length ICCAT data base analyses. Number of samples (Num), average month (Aver. mo) and standard deviation month (SD mo) of sampling, separated by age class and management area.

	Eastern stock						Western stock		
	Otolith			Spine			Otolith		
Age	Num.	Aver. mo	SD mo	Num.	Aver. mo	SD mo	Num.	Aver. mo	SD mo
1	41	7.7	1.2	1510	8.5	1.4	35	7.7	0.8
2	38	7.1	1.5	2069	7.9	1.4	128	7.4	1.0
3	53	6.5	1.8	1655	7.9	1.5	229	7.0	1.0
4	53	7.4	2.1	1078	7.8	1.3	347	7.1	1.0
5	72	8.1	1.9	641	7.5	1.4	253	7.2	1.2
6	51	7.9	2.0	338	7.4	1.7	148	6.8	2.3
7	81	7.9	2.0	264	7.4	1.9	188	6.5	2.8

Table 3. Age-length ICCAT data base analyses. Number of samples (Num), average month (Aver. mo) and standard deviation month (SD mo) of sampling, separated by age class and reading laboratory.

	Reading laboratory								
	13						15		
	Otolith			Spine			Otolith		
Age	Num.	Aver. mo	SD mo	Num.	Aver. mo	SD mo	Num.	Aver. mo	SD mo
1	41	7.7	1.2	1510	8.5	1.4			
2	38	7.1	1.5	2069	7.9	1.4			
3	53	6.5	1.8	1655	7.9	1.5			
4	53	7.4	2.1	1078	7.8	1.3			
5	72	8.1	1.9	641	7.5	1.4			
6	51	7.9	2.0	338	7.4	1.7	6	9.2	0.8
7	83	7.9	2.0	266	7.4	1.9	34	9.0	0.7

	16			17			18		
	Otolith			Otolith			Otolith		
Age	Num.	Aver. mo	SD mo	Num.	Aver. mo	SD mo	Num.	Aver. mo	SD mo
1	35	7.7	0.8						
2	84	7.6	1.0				44	7.0	1.0
3	48	7.2	0.9				181	6.9	1.0
4	44	6.9	0.8	9	8.6	1.2	294	7.1	1.0
5	40	6.4	1.5	13	8.6	1.0	200	7.2	1.0
6	47	4.8	2.6	15	8.3	1.0	80	7.4	1.5
7	77	4.2	2.6	40	7.9	1.2	35	7.6	2.1

Table 4. Age-length ICCAT data base analyses. Number of samples (Num), average month (Aver. mo) and standard deviation month (SD mo) of sampling used for the ageing protocol comparison.

	Reading prot. compar.		
	Otolith		
Age	Num.	Aver. mo	SD mo
1	7	7.1	0.4
2	5	6.8	1.6
3	17	6.6	1.9
4	12	6.3	2.9
5	13	7.1	1.8
6	4	7.5	2.4
7	3	6.3	0.6

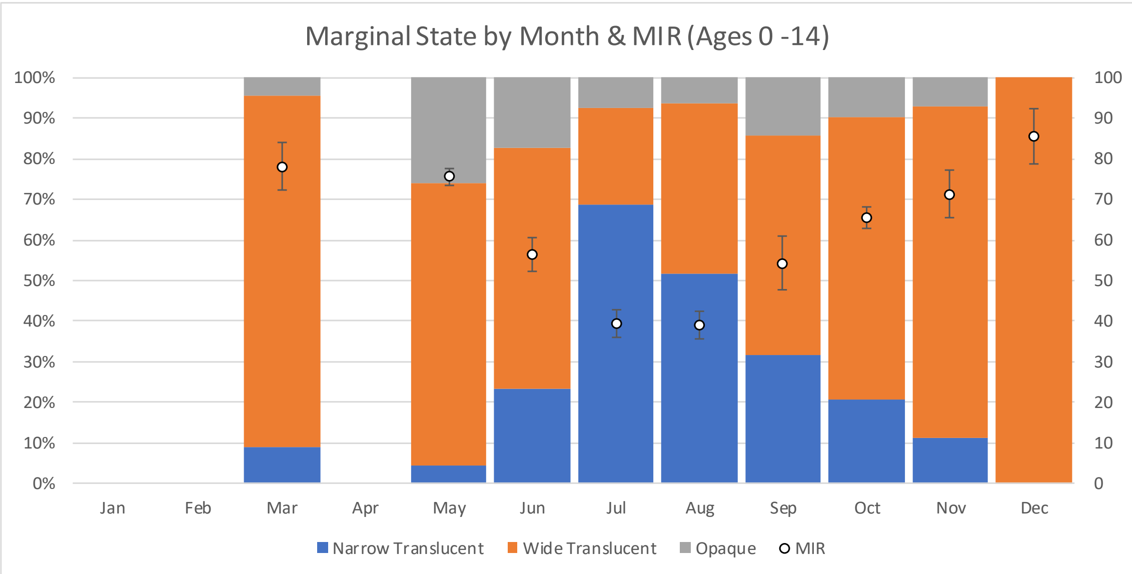


Figure 1. Marginal state and Marginal Increment Analysis (MIR or MIA) for age classes 0 to 15 years of eastern Atlantic bluefin tuna otoliths plotted against month.

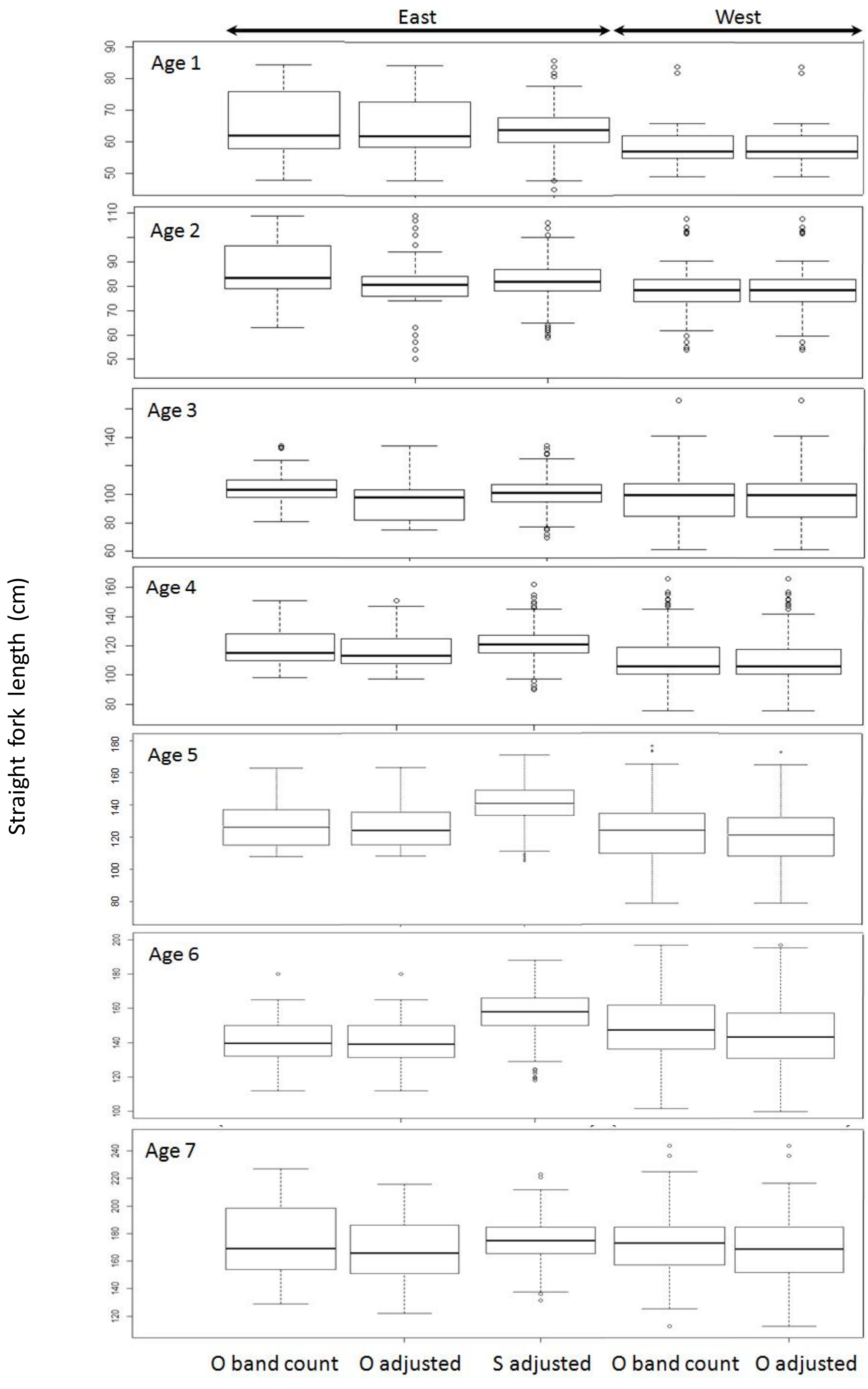


Figure 2. Box plot of straight fork length by age class and management area obtained from calcified structures interpretation. Otolith band counting (O band count), otolith age adjusted (O adjusted) and spine age adjusted (S adjusted).

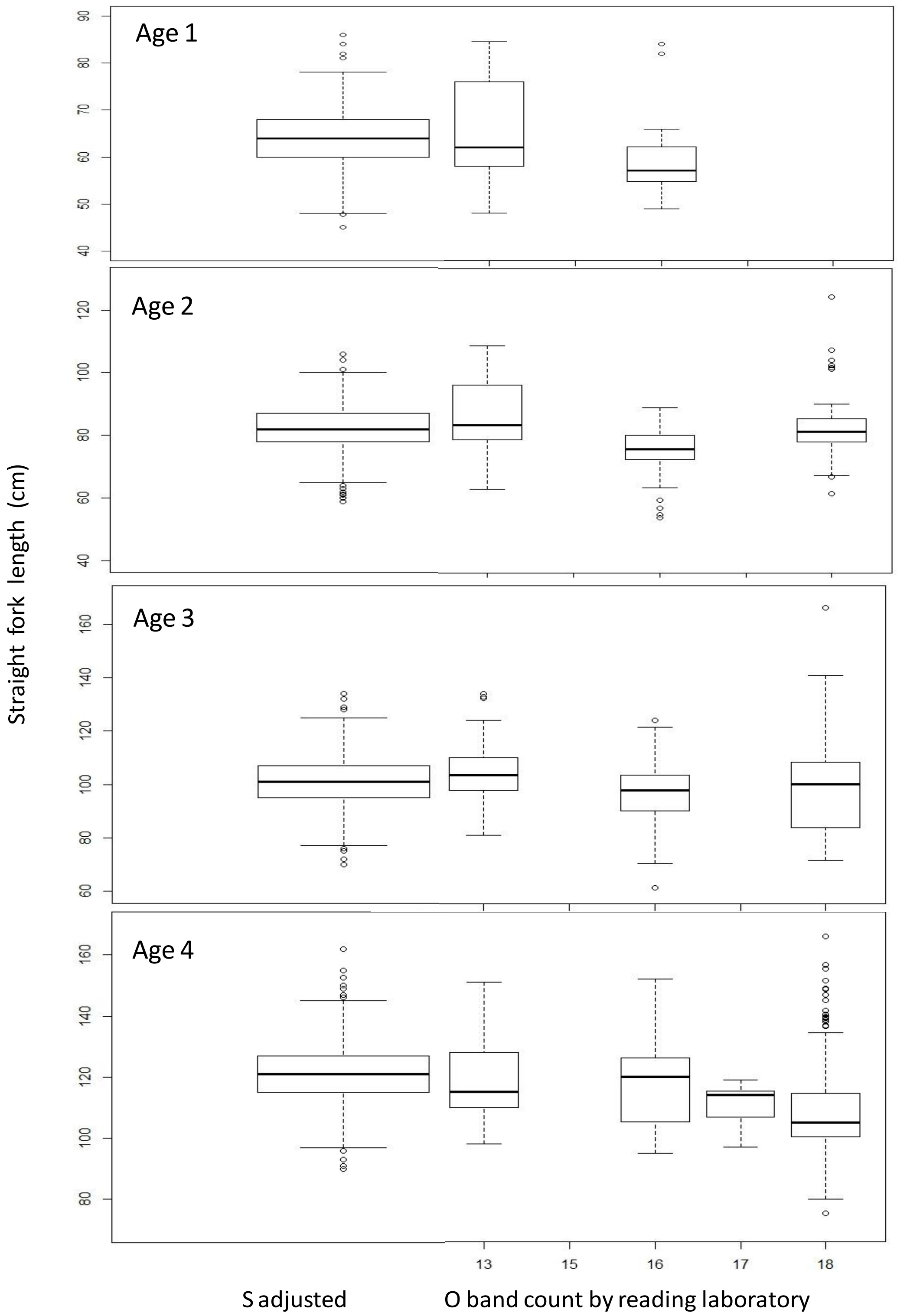


Figure 3. Box plot of straight fork length by age class (1 to 4) and reading laboratory obtained from calcified structures interpretation. Spine age adjusted (S adjusted) and otolith band counting (O band count). Numbers on the X axis represent laboratories.

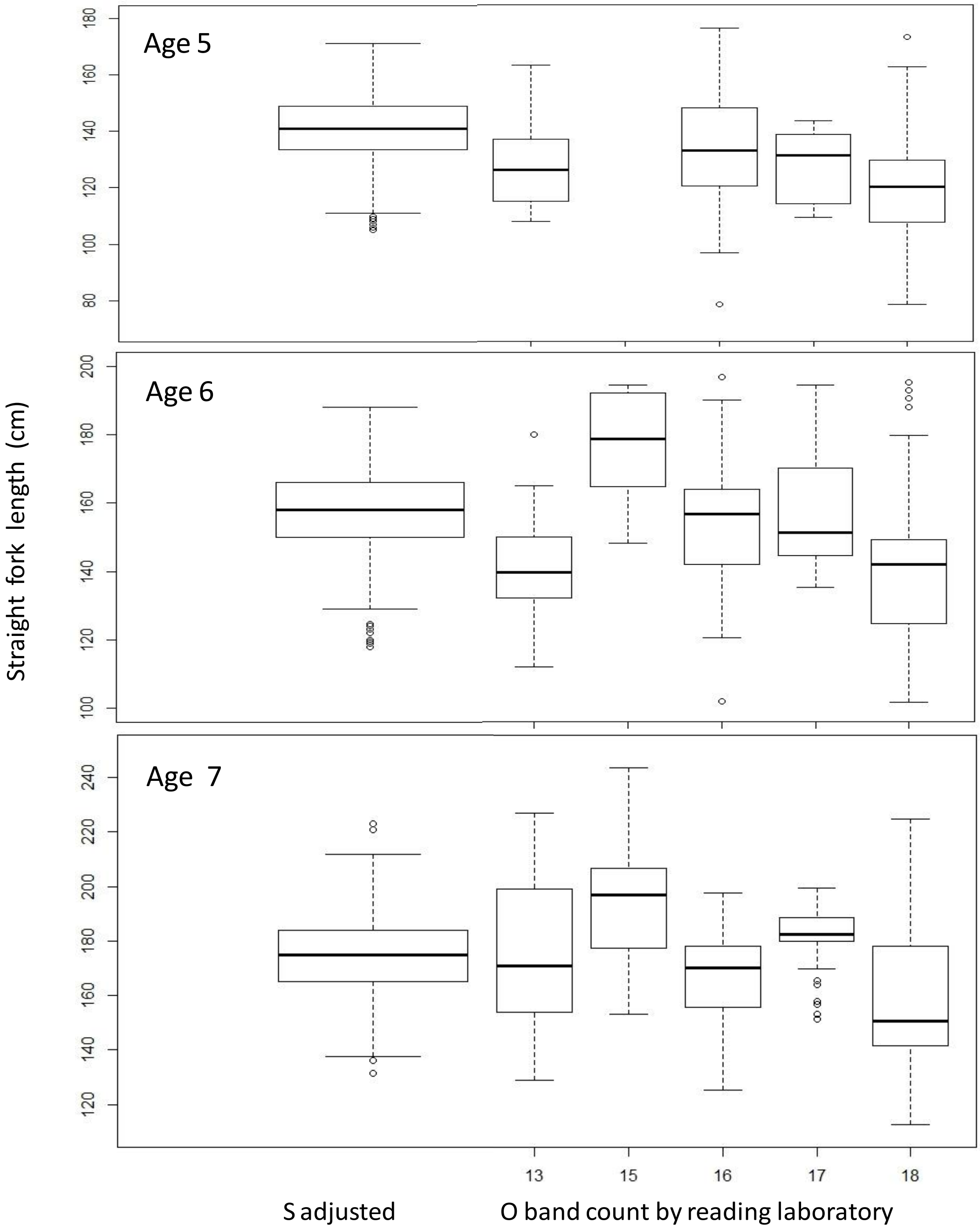
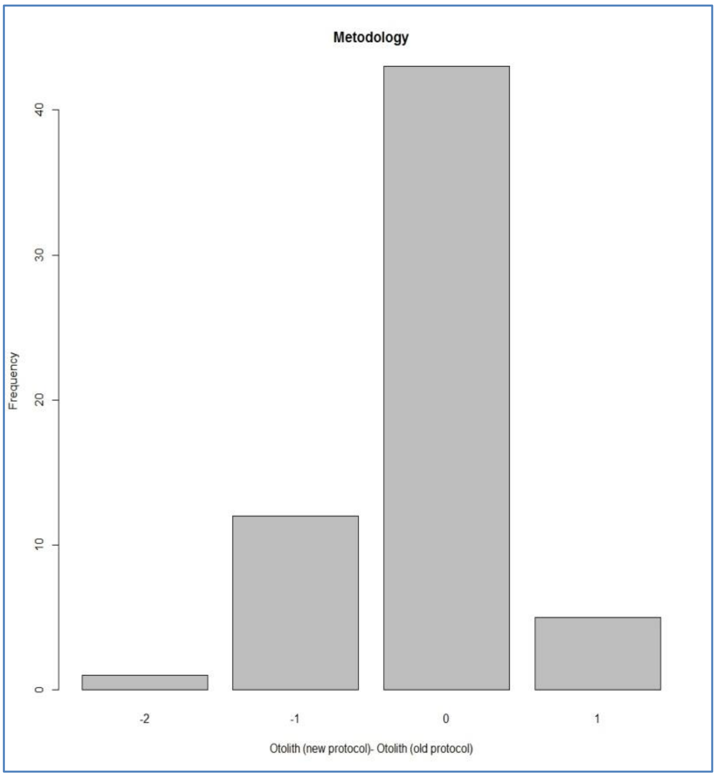


Figure 3 cont. Box plot of straight fork length by age class (5 to 7) and reading laboratory obtained from calcified structures interpretation. Spine age adjusted (S adjusted) and otolith band counting (O band count). Numbers on the X axis represent laboratories.



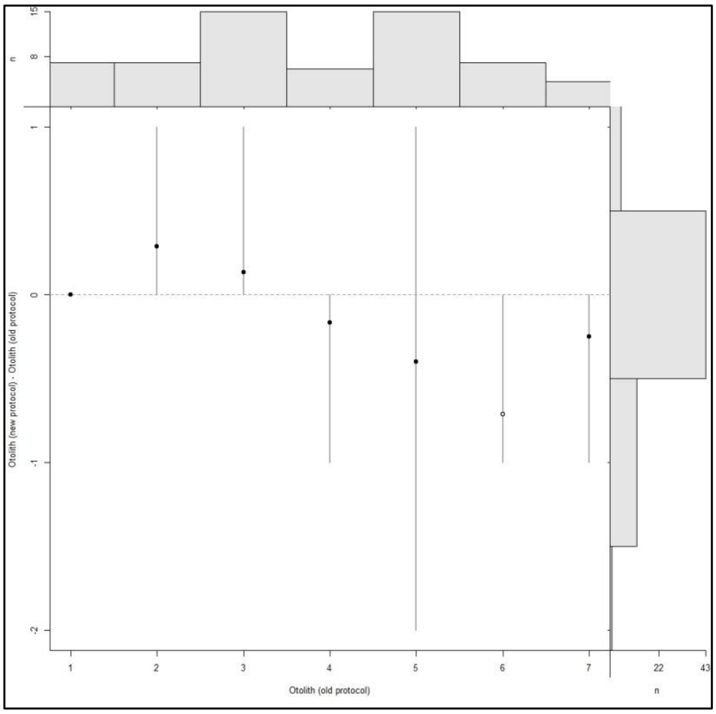


Figure 4. Histogram of differences (top) and age bias graph (bottom) between reading protocols: 2014 (old) and 2019 (new).

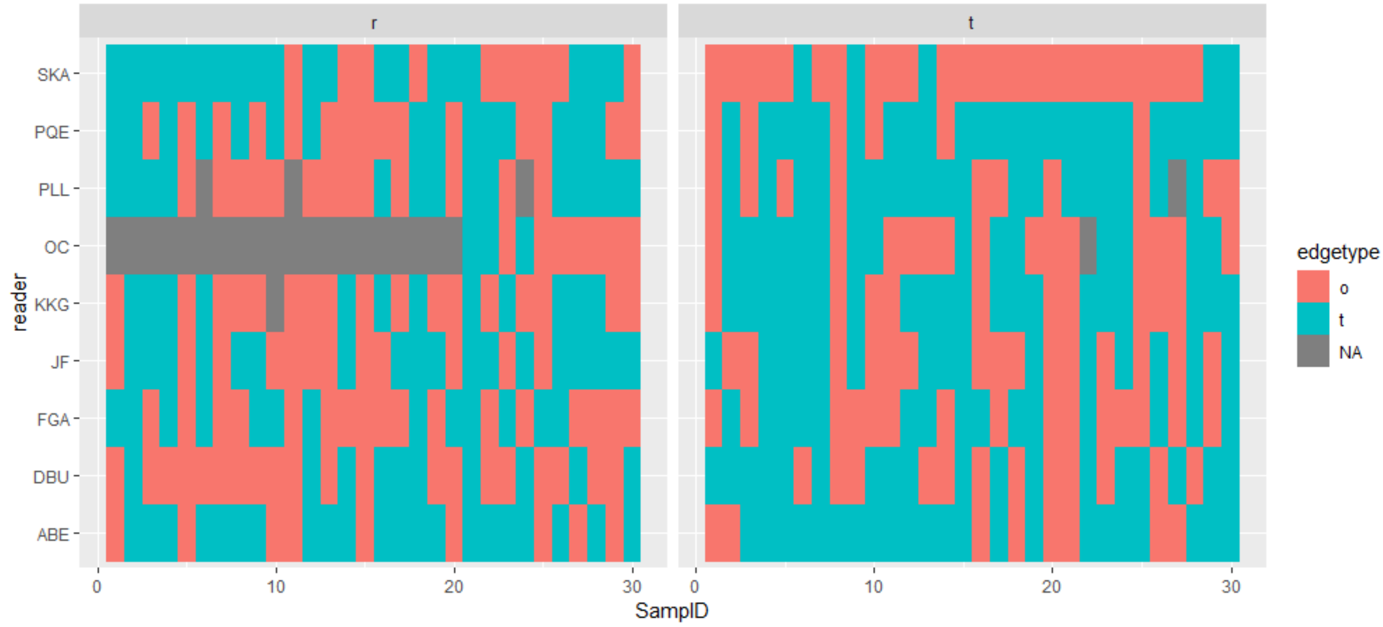


Figure 5. Tile plot showing edge type assignment (o= opaque and t= translucent) by sample for each reader and type of light used, left panel (r) for reflected light and right panel (t) for transmitted light.

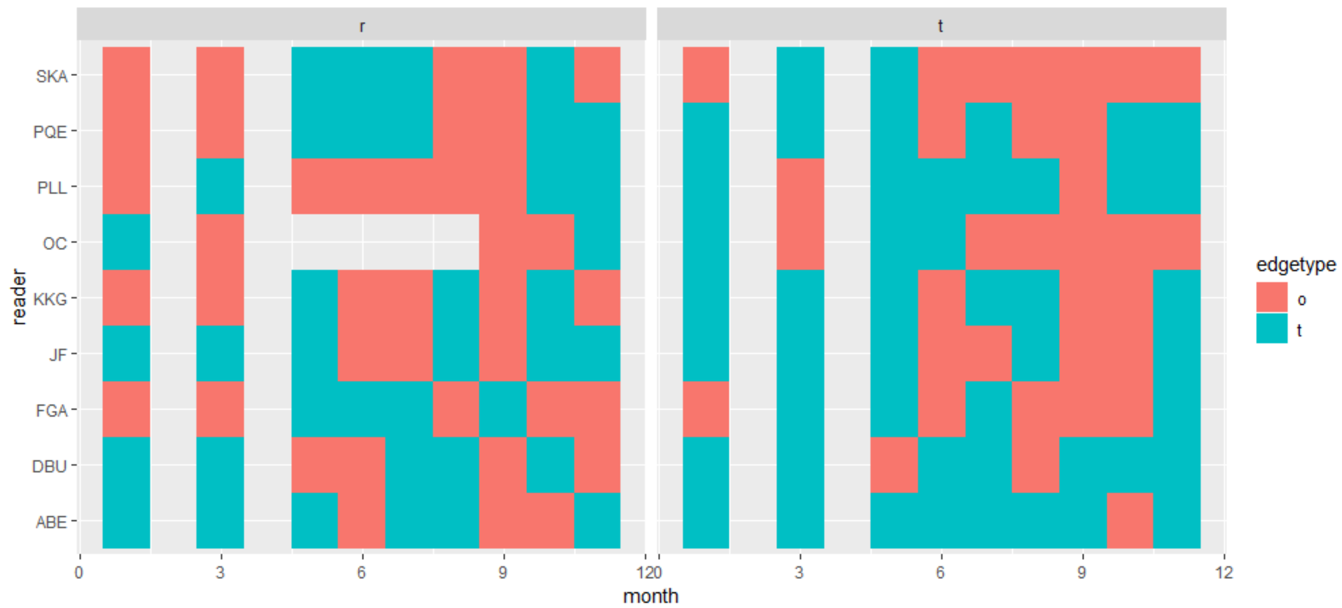


Figure 6. Tile plot showing edge type assignment (o= opaque and t= translucent) by month for each reader and type of light used, left panel (r) for reflected light and right panel (t) for transmitted light.

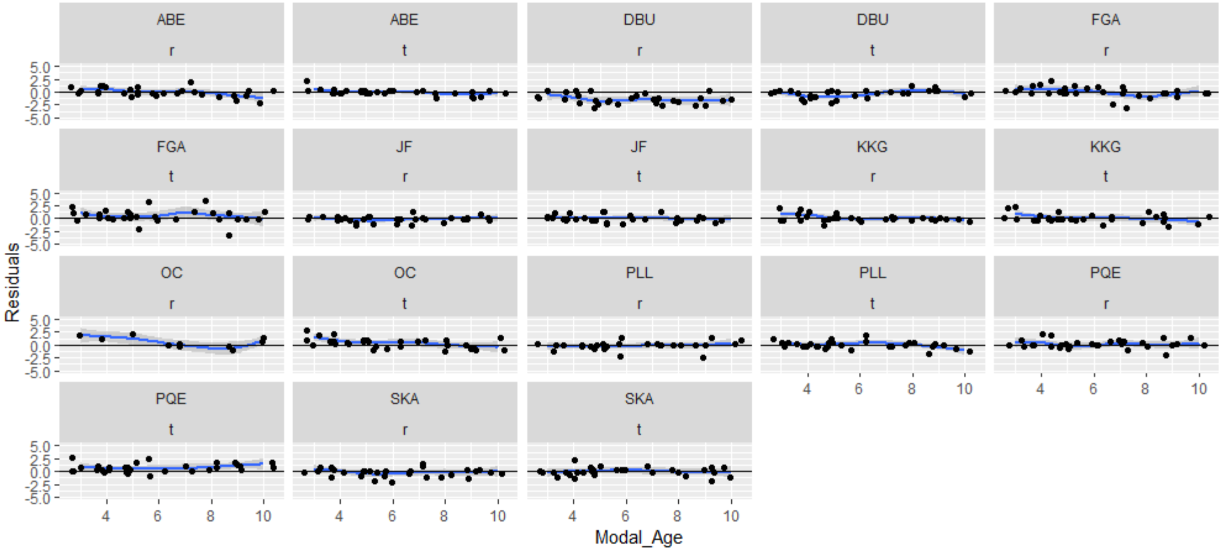


Figure 7. Residual plots (Residuals = Age estimates – Modal Age estimates, loess smooth line (α =0.9)) of individual readers by light type (r= reflected, t= transmitted) compared to modal age estimates.

Appendix 1

Tentative Agenda

1. Opening
2. Adoption of Agenda
3. Nomination of the rapporteurs
4. Review of available presentations
5. Otoliths preparation methodology revision (thickness of the section, location of the sectioning, ...)
6. Otoliths reading protocol revision (where to mark bands, reference measurements, images vs. physical samples, edge assignment criteria...)
7. Practical exercise by reading 30 samples, to compare readings of images vs. readings physical samples (edge assignment criteria).
8. Adoption of a revised protocol for the bluefin tuna otoliths preparation and reading, based in the agreement on bullets 5 an 6.
9. Practical exercise by reading 50 samples from the "old reference collection" using the revised protocol.
10. Agree on the method to quantify differences in the readings by using the old protocol and the new one, and adopt a correction procedure to enable the use of the age length keys developed so far.
11. Agree on the selection of samples, likely by enlarging the current one, to set up a new reference collection.
12. Adoption of the report.

Appendix 2

List of participants

Addis, Pierantonio
Senior Researcher in Ecology, University of Cagliari, Department of Life and Environment Science, Via Fiorelli 1, 09126 Cagliari, Italy. Tel: +39 070 675 8082; +34 733 67842, Fax: +39 070 675 8022, E-mail: addisp@unica.it

Alemany, Francisco
GBYP Program Coordinator, ICCAT Secretariat, Corazon de María 8, 28002 Madrid, Spain. Tel. 0034 91 4165600 direct line: 0034 91 5103706 fax 0034 91 4152612. E-mail: francisco.alemany@iccat.int

Bellodi, Andrea
University of Cagliari, Department of Life and Environment Science, Via Fiorelli 1, 09126 Cagliari, Italy. Tel: +39 070 675 8082; +34 733 67842, Fax: +39 070 675 8022, E-mail: andrea.bellodi@gmail.com

Busawon, Dheeraj
Fisheries & Oceans Canada / Pêches et Océans Canada, St. Andrews Biological Station, 125 Marine Science Drive, St. Andrews, NB E5B 0E4, Canada. Tel: (506) 529-5889, E-mail: Dheeraj.Busawon@dfo-mpo.gc.ca

Carnevali, Oliana
Universita Politecnica Delle Marche - Ancona, Department of Environment and Life Science, Italy. Tel: +39 338 264 2235; +39 71 220 4990, Fax: +39 071 220 46 50, E-mail: o.carnevali@univpm.it

Cort, José Luis
Ministerio de Ciencia, Innovación y Universidades, Instituto Español de Oceanografía, C.O. de Santander, Apartado 240; Promontorio de San Martín S/N;39004 Santander, Cantabria, Spain. Tel: +34 942 291 716, Fax: +34 942 27 5072, E-mail: jose.cort@st.ieo.es

Di Natale, Antonio
Aquastudio Research Institute, Via Trapani 6, 98121 Messina, Italy. Tel. 0039 336 333366, E-mail: adinatale@costaedutainment.it

Farley, Jessica.
CSIRO Oceans and Atmosphere, 3-4 Castray Esplanade, Hobart, Tasmania 7000, Australia. E-mail: jessica.farley@csiro.au

Garibaldi, Fulvio
Laboratorio di Biologia Marina e Ecologia Animale Univ. Degli Studi di Genova, Dipartimento di Scienze della Terra, dell'Ambiente e della Vita (DISTAV), Corso Europa, 26, 16132 Genoa, Italy. Tel: +39 010 353 8576, Fax: +39 010 357 888, E-mail: largepel@unige.it; garibaldi.f@libero.it

Karakulak, Saadet
Department of Fisheries Technology, Faculty of Aquatic Sciences, Istanbul University. Turkey. E-mail: karakul@istanbul.edu.tr

Krusic-Golub, Kyne
Fish Ageing Services, PO Box 396, Portarlington, Victoria, Australia, 3223. Tel: 0438 306 179, E-mail: kyne.krusicgolub@fishageingservices.com

Luque, Patricia L
AZTI - Tecnalia / Itsas Ikerketa Saila, Herrera Kaia Portualde z/g, 20110 Pasaia Gipuzkoa, Spain. Tel: +34 94 657 40 00; Fax: +34 94 300 48 01, E-mail: plastra@azti.es

Quelle, Pablo
Ministerio de Ciencia, Innovación y Universidades, Instituto Español de Oceanografía, C.O. de Santander, Promontorio de San Martín s/n, 39004 Santander, Cantabria, Spain. Tel: +34 942 291 716, Fax: +34 942 27 50 72, E-mail: pablo.quelle@ieo.es

Rodríguez-Marín, Enrique
Ministerio de Ciencia, Innovación y Universidades, Instituto Español de Oceanografía, C.O. de Santander, Promontorio de San Martín s/n, 39004 Santander, Cantabria, Spain. Tel: +34 942 291 716, Fax: +34 942 27 50 72, E-mail: enrique.rmarin@ieo.es

Ruiz, Marta
Ministerio de Ciencia, Innovación y Universidades, Instituto Español de Oceanografía, C.O. de Santander, Promontorio de San Martín s/n, 39004 Santander, Cantabria, Spain. Tel: +34 942 291 716, Fax: +34 942 27 50 72, E-mail: marta.ruiz@ieo.es

Appendix 3

Protocol for the preparation of Atlantic bluefin tuna otoliths for direct ageing

1. Cleaning and storing procedure

After removing the otoliths from the head of the bluefin, they should be carefully cleaned with distilled or deionized water and allow drying. It is essential that they are completely clean and free of biological residues (completely white, without yellowish remains). Cleaning otoliths immediately after collection reduces the amount of time required to prepare them for sectioning.

Once they dry and if they have not been cleaned well, it is more difficult to remove any adhering tissue. If they present organic material adhered, they should be hydrated with distilled or deionized water to remove residues with forceps, and then can be decontaminated by submerging them in 1% nitric acid for 10 seconds. Followed by cleaning with distilled water to remove the remaining nitric acid.

Finally, dry otoliths during 24 hours at room temperature or in a fume hood, and store them in plastic vials with their corresponding labels.

2. Image capture for otolith shape analysis and taking measurements

2.1 Image capture for otolith shape analysis:

A. Place clean and dry otolith on a clean, smooth black background free of dirt or discontinuities with the sulcus facing downwards so that the anti-sulcal side is photographed.

B. Orientate the otolith so that the rostrum-postrostrum axis is horizontal and the rostrum of both otoliths should be on the left to avoid parallax error when measuring with the image analysis software (see **Figure 1**).

C. View the otolith on a stereomicroscope using reflected light. Adjust the light levels to minimize distortion of the otolith margin and maximize contrast between the otolith and background (see **Figure 1**). Ensure that there is no dust or other features on the background, particularly close to the otolith edge which the edge detection software could confuse with the otolith outline. If a cross polarization filter is available, this can help to reduce such interference.

D. Capture image with a high resolution. A trade-off between resolution and image size (memory) should be selected. As guidance, should be considered a minimum resolution of 1024 x 768, settings as gain should be low and white balance correction, brightness, and sharpness should be ensured.

E. Capture the image with a <u>visible scale bar</u> and save as a TIFF file.

F. Both the left and right otolith should be photographed (in separate files) if possible (**Figure 2**).

G. Nomenclature for file name: Species_Sample#_Left/Right

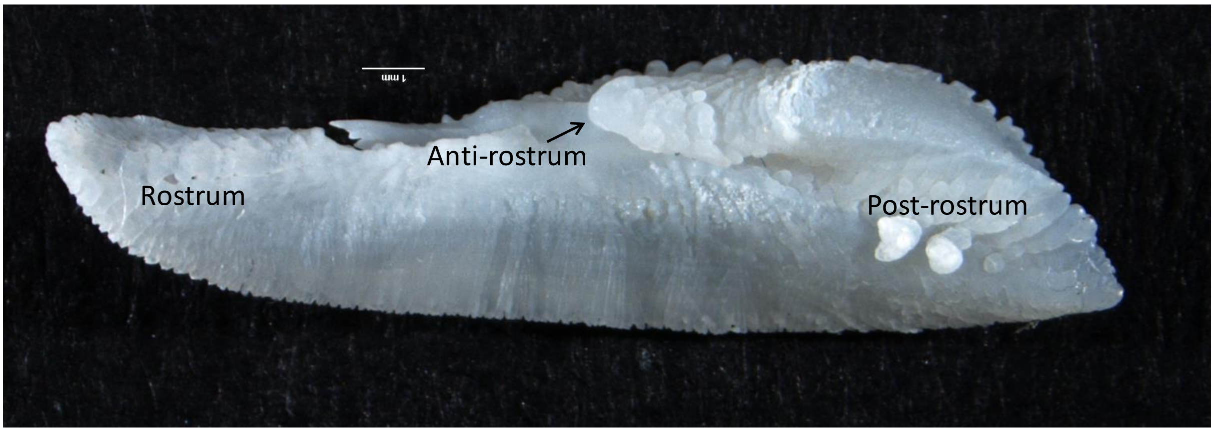


Figure 1. Photograph of a Bluefin tuna left otolith sulcus side down. The anterior part of the otolith is narrower and is called rostrum (left) and the rear part is wider and is called postrostrum (right). The protuberance in this side of the otolith is called antirostrum.

Figure 2. Photographs illustrating a right (top) and a left (bottom) otolith for Bluefin tuna.

2.2 Whole otolith measurements

Measure widest and longest side of the otolith and record measurements in a spreadsheet (**Figure 3**). The otolith is measured if it is not broken, and if both are complete, the left one is preferably measured (ShapeR package allows to easily collect otolith metrics data).

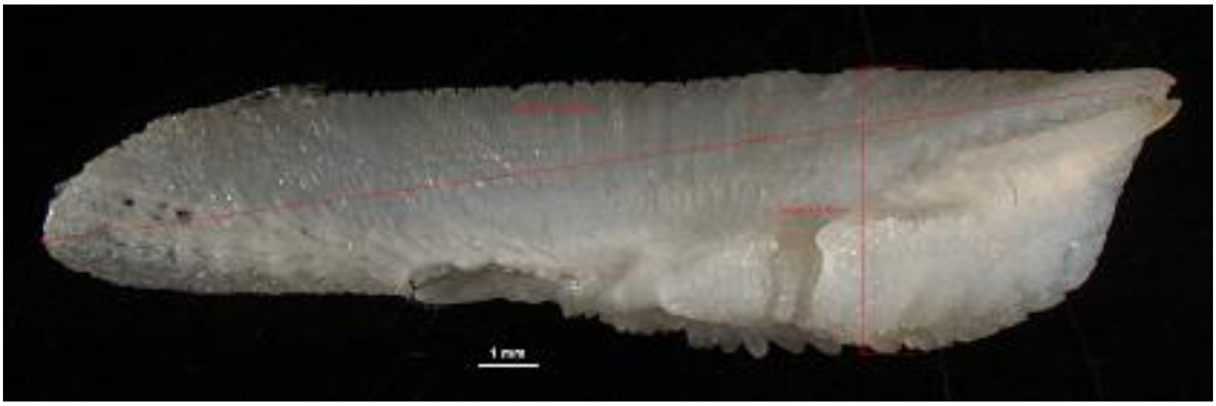


Figure 3. Right sagittal otolith of a Bluefin tuna showing whole otolith measurements

3. Weight of the otolith

Record the weight of the otolith (nearest 0.1 mg) if otolith is complete.

4. Processing of Atlantic bluefin tuna otoliths

4.1 Section location

The count of annual bands becomes more difficult and may introduce an ageing bias in sections that are taken further away from the otolith primordium. Therefore, it is recommended to perform ageing from a transverse section that includes the primordium. This section should include the tip of the antirostrum. Green rectangle in **Figure 4** illustrates optimal location of section for routine ageing. The thickness of the ageing section section is ≈ 0.5 mm; polished to 0.35 mm if reading with transmitted light or 0.40-0.45 mm if reading with reflected light.

In spite of the above, it is recommended to use the same otolith for direct aging and for stock identification analyzes (i.e. natal origin) (section for micro-milling). As such, two sections can be obtained from one otolith, but it is necessary to move the location of the ageing section and leave this area for the section used for micro-milling (i.e. stock identification analyses), **Figure 4 and Figure 5**. Thus, two sections must be cut, a micro mill section of ≈ 1.5 mm thick containing the tip of the antirostrum (section containing V and Y type in each extreme, or at least the tip of antirostrum appearing as a satellite in this section) and another section of ≈ 0.5 mm thick for ageing, adjacent to the previous one. **Figure 5** illustrates location of sections for natal origin (stable isotopes) and ageing analysis. It is recommended to use a 0.3 mm thick cutting blade to reduce the distance between both sections.

For juvenile bluefin tuna, up to 2 years old (90 cm SFL), it is recommended to use both otoliths, one for each type of analysis (this option requires increasing the sampling of juveniles of same size to keep samples obtained from the same location and day for future analysis).

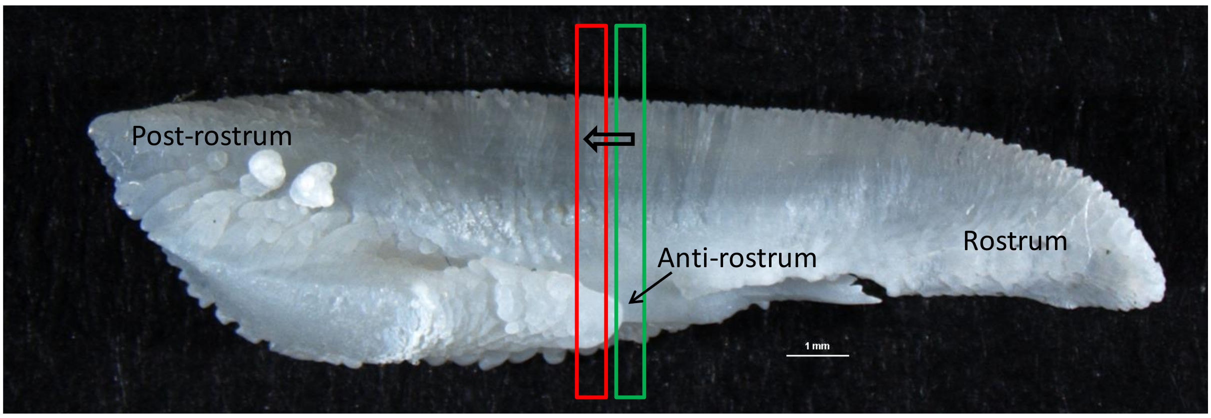


Figure 4. Optimal location of the ageing section (green rectangle) and the commitment location (red rectangle) to be able to use the same otolith for direct ageing and for stock identification analyses (section for micro-milling).

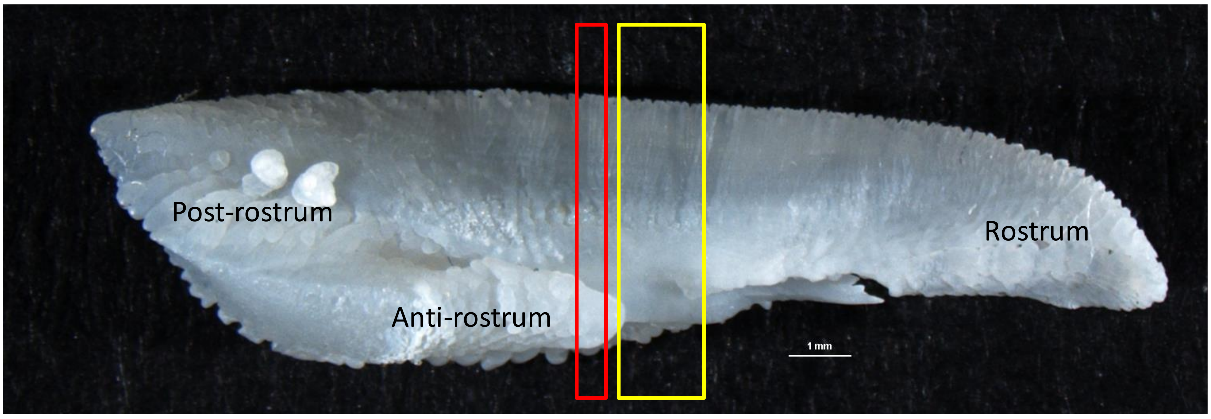


Figure 5. Commitment location of the ageing section (red line) and the micro-mill section (yellow line) on an Atlantic bluefin tuna otolith anti-sulcal side picture. The distance between both sections corresponds to a blade of 0.3 mm thick.

4.2 Embedding otoliths within molds for sectioning

In the case that it is not foreseen to do analysis of trace elements, and therefore the issue of contamination is not decisive, a mold with several otoliths can be prepared. The broken or incomplete otoliths can be used as long as the sectioning area is not missed. If both otoliths are equally complete, the left one should be preferably selected.

- Apply a light coat of releasing agent (see below) on the wells of the molds (**Figure 6**) under a fume hood.

- Mix transparent resin and hardener (see below) in a plastic cup under fume hood. Note: Do not mix more resin with respect hardener than recommended as it increases the number of air bubbles in the mixture. The more hardener, drying takes less time but the resin loses its transparency. Also influences the temperature and humidity in the drying time.

Resin products by laboratory:

- Instituto Español de Oceanografía (IEO) procedure: resin polyester. Releasing agent: Vaseline. Pre-accelerated resin polyester trade name Crystic 446 PALV and hardener trade name LX PEROXAN ME-50 in proportion 10 g/1ml.
- St. Andrews Biological Station, Fisheries and Oceans Canada (SABS) procedure: resin epoxy. Frekote Release agent, resin epoxy (Araldite GY 502) and hardener (Aradur 956-2), 5:1 ratio.
Note: When working with Canadian molds, it is recommended to mix enough resin for one mold at a time (60-80g) as the process of embedding is lengthy and the resin might harden.

- Stir with wooden stick for 3-4 min.

- SABS procedure: fill sonicator with distilled water. Sonify mixture for 15 min (Make sure water does not get inside the cup). When sonicator is finished, remove cup and wipe off water so that it does not drip in the wells.

- IEO procedure: Put a drop of resin in bottom of the mold and then the otolith on top and press, this procedure reduces the possible formation of bubble in the "sulcus", which will occur if first resin layer is hard and the second layer is casted from above. Finally cover all the otolith with resin, including the second upper level of the mold to obtain a surface as flat as possible or slightly convex. The latter facilitates the grip on the cutter and prevents the block from moving when cutting. Place the otolith next to the end of the mold to have place in the back of the mold to hold it with the clamp of the cutter.

- SABS procedure: Place one otolith Sulcus side up + label in each well and cover with resin. Use teasing needles to roll otolith and suction any air bubbles using disposable pipette. Once air bubbles are removed, fill the rest of the well. Pour slowly to reduce bubble formation.

- Place under fume hood and leave to set for 3 days (at least 3 days, never before).

- Clean tools with ethanol or acetone for metal tools.

Figure 6. Photograph illustrating the mold used to embed otoliths.

4.3 Sectioning using a low speed saw

It may be useful to mark the resin for the sectioning location (if the antirostrum has not been marked before). The use of 1 or 2 spacers between the blades helps to save time since it involves making fewer cuts.

- The otolith mold is clamped on the arm of the cutter; the clamping is done in the postrostrum part. The mold is placed so that the reference sectioning mark, near the end of the otolith antirostrum, is aligned with the blade.

- 1st cut: the micrometer is moved to the rostrum at a distance of 1.5 mm plus the thickness of the blade, since this material is lost when cutting according to the thickness of the blade (blade of 0.3 mm thick represents a loss of approximately its thickness, the 0.9 blade implies a loss of 0.9). Then, the cut is done. This section contains the end of the antirostrum and the contiguous zone (one end with a Y shape, the other with a V shape).

- 2nd cut: slide the micrometer back in the opposite direction, towards the postrostrum 1.5 mm + blade thickness. And the 2 cut is done. The resulting section is stored in a labeled tube and will be used for the analysis of stable isotopes and trace elements.

- 3rd cut, slide the micrometer towards the postrostrum 0.5 plus blade thickness to obtain a section of 0.5 mm for age estimation. Once cut it should be washed with 70% alcohol.

The sections are obtained by using saws with a diamond disk and using distilled water as lubricant liquid.

5. Sanding / Polishing

The sections are mounted on slides with "Crystalbond" for the sanding / polishing process. A mechanical disc sander is used. Sandpaper / polishing discs and distilled water are used. Sanding improves the visualization of the section. In general, polishing without sanding is of little use (it does not even eliminate the cut marks of the resin). Polish only after sanding to enhance the section preparation.

When using reflected light for reading the section, you may sand with a 13 micron grain (FEPA P1500), and short time, 20", without polishing. When using transmitted light, you may first sand with 9 microns (FEPA P2500) and latter with a 5 microns (FEPA P3800) sandpaper.

6. Examination of otolith sections

Otolith sections can be read physically or in the form of digital images.

Live viewing with a microscope allows to play with the focal plane and magnification which can help on growth bands and otolith edge interpretation. Light intensity and direction can also be altered, which may increase clarity in difficult to read sections (e.g. filters or tilting the otolith section). Microscopic examination is a fast system for a simple growth bands count.

Digital images can be enhanced by using image analysis systems. The improvement in the clarity of the image, for example through the use of Photoshop ©, can even be more effective than a good polish. Careful microscopy (well focused) and image enhancement with image analysis software will improve reading precision. The use of "annotated layers" allows to mark and facilitates to count growth bands on the images for later examination. Imaged otolith sections also makes it easier to take distances or measurements of the growth bands. The use of digital images facilitate training and exchanges among laboratories. A collection of prepared ageing structures of consensus derived ages, a reference collection, in the form of digital images, simplifies the training of age readers and help standardization of age interpretation among laboratories.

For section imaging, place section in a Petri dish and cover with deionized or Milli-Q water.

A high resolution for image capturing should be selected. As guidance, should be considered a resolution of 2560 x 1920, settings as gain should be low and white balance correction, brightness and sharpness should be ensured.

6.1 Capture the image with a visible scale bar

It is essential to capture the image with a reference scale "burned" in the image. In this respect, it is also convenient to use a standard magnification scale so that the same zoom is used regardless of the size of the sample. The latter is especially necessary when preparing a reference collection.

6.2 Enhancing images with Photoshop ©

Option 1
- Open image in Photoshop ©.
- Duplicate the background. This allows you to have both a regular and enhanced image.
- Go to image>Adjustments>Levels. Histogram expansion: Drag white end of the histogram into the point where the white of the otolith starts to register. Drag the black end of the histogram in until just before the edge of the otolith start to erode. Click ok.
- Go to Filter>Sharpen>Unsharp Mask. Set Amount to 150, Radius to 7-30 (15 is good for otoliths), Threshold to 2.

Nomenclature for file name: Species_sample#_year_light_type.tif.
Unsharp mask increases the contrast along the edges by detecting pixels that differ by the threshold. We choose a low threshold and then increase the contrast by 150%. We specify a radius of region to which each is compared. A large values = big edge effects.
This method can greatly improve the image, but converts the image into a very "heavy" file by duplicating the original image.

Option 2
Another simpler method than the previous one, that produces a less heavy file, is to use an Adjustment Layer
Choose Layer > New Adjustment Layer > Levels. Click OK. Introduce the name of the layer. Adjust the shadows and highlights automatically or manually. For the latter drag the black and white Input Levels sliders to the edge of the first group of pixels on either end of the histogram. To adjust midtones move the middle Input slider (grey input level) to the left or right to make the image lighter or darker.

Appendix 4

Protocol for the age reading of Atlantic bluefin tuna otoliths

1. Training or warm-up before reading

Prior to production ageing, readers should conduct a blind read of the reference collection, or a subsample, one time, under their preferred light type (transmitted or reflected). A precision level of APE and CV of 10% or lower and no bias would be acceptable to support production ageing. The reference set should also be used to monitor ageing consistency over time as well as among age readers (relative bias and precision) and for training purposes.

2. Identification of annuli on otolith section

View physical sections, or images, under either transmitted or reflected light. In live viewing tilting the otolith section may increase clarity in difficult to read samples.

Previous otolith exchanges showed a more consistent classification of edge type among readers when using transmitted light. Since edge type may have important consequences on age assignment, it is suggested to use transmitted light for otoliths direct ageing.

Annuli are a bipartite structure consisting of an opaque and translucent zone.

Count opaque growth zones. These appear dark under transmitted light and white under reflected light. Last opaque band at the marginal edge of the otolith *should only be counted if complete* and surrounded by a translucent band (once translucent otolith material could be seemed between the outer edge of the opaque zone and the edge margin). The ventral (long) arm is used for age estimates. The dorsal (short) arm can be used as check; however, it is important to remember that the dorsal arm might underestimate age (**Figure 1**).

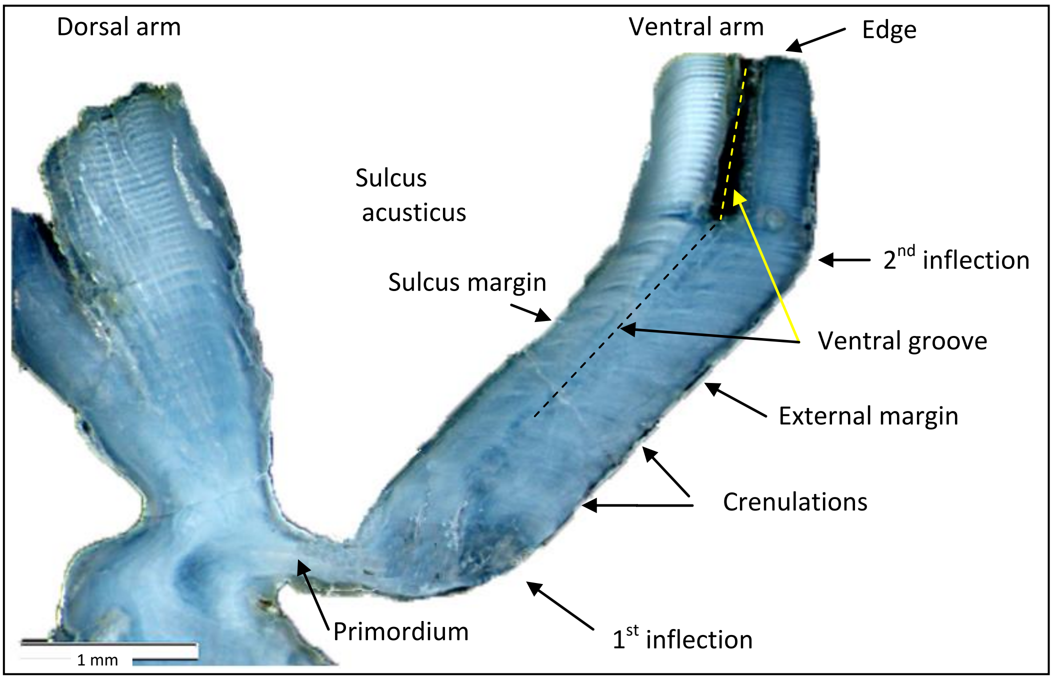


Figure 1. Otolith transverse section (through the primordium) of an Atlantic bluefin tuna.

Start reading at the "primordium" and proceed towards the edge of the ventral arm. There are no annual growth increments between the "primordium" and the 1st inflection. Opaque bands are usually more distinct at the sulcus side of the ventral groove (which is present from the third annual band approximately, **Figure 1**).

The section of the ventral arm between the first and second inflection is difficult to interpret. The first 10 annual opaque bands are located in this section and sub-annual marks are frequently observed. The ventral arm can be divided in 3 general regions as one travels from the primordium to the edge of the ventral arm:

- 1st region: Annuli are broad and diffuse and contain multiple translucent and opaque zones (~1-5 annuli).

The appearance of a false sub-annual increment or annulus 0, which can even present a crenulation (groove along the margin), is frequent (40% of the sections from a sample of n = 131). The false annulus is less marked than the first three true annual increments. The distance from the first inflection to the false annulus is less than the width of the ventral arm. The false annulus is at approximately half the distance between the 1st inflection and the 1st annual increment.

Annual growth bands in this 1st region are broad and contain multiple sub-annual opaque and translucent bands, although less marked than the annual ones, this is especially common between the first and second annulus. First 4 annual increments should cover the width of the ventral arm (marked throughout the entire arm). Crenulations may aid in the identification of growth zones. The distance among the first five annuli is greater than in the rest. The recognition of the first two annuli is important to establish the deposition pattern of the first 5 opaque annual bands, including the gradual decrease of the distance between them.

The first annulus can be identified by using a reference scale of 1 mm. The first opaque annual deposition should be within this distance of 1 mm, measured from the bottom center of the bridge between the two arms and extends up the inner ventral arm at the sulcus margin (**Figure 2**).

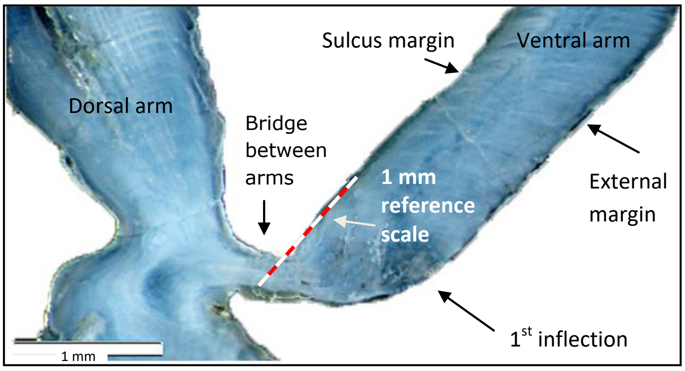


Figure 2. Otolith transverse section of an Atlantic bluefin tuna. The red and white "yardstick" is used as a reference scale to help assigning first opaque annual increment.

- 2nd region: Annuli are less broad and closer together (~5-10 annuli). From the fifth, the distance between opaque annual bands decreases and from the eighth or ninth annual deposition, and especially after the second inflection, the deposition of the opaque annual bands is distinct and regular.

- 3rd region: Annual opaque bands appear clearer and are regular in width (~ 10+ annuli).

Quality in terms of readability should be annotated on a scale from 1 to 4: 1=Pattern present-no meaning, 2=Pattern present-unsure with age estimate, 3=Good pattern present-slightly unsure in some areas, 4=Good pattern-confident with age estimate. Only otolith sections categorized as 2 to 4 should be used for further ageing analysis.

3. Annual band measurements

Obtaining annual band measurements during otolith reading is useful for control quality of age estimates. It is also essential to carry out validation studies using the marginal increment analysis (MIA) technique or other methodologies such as back-calculation. For these measurements of the size of annulus to be useful, it is necessary that they are clearly defined and agreed among readers. Here it is proposed the following otolith growth measurement, using a standardized "measurement line".

This "measurement line" is equidistant between 2 sets of parallel lines. In the first set, the lines are drawn parallel to the sulcus margin of the ventral arm and also through the ventral groove between the 1st and second inflection point (Figure 3). In the second set, the lines are drawn similarly but between the 2nd inflection point and the end of the otolith. Note that if the measurement of the annual bands after the second inflection are not needed, defining the second segment is not required. The origin of the "measurement line" or anchor point is where the "measurement line" crosses the bridge. Note: care should be taken in placing the anchor point due to the 3 dimensional aspect of the image (i.e. there is an inner edge and an external edge at the bridge area due to the thickness of the section), the anchor point is located on the inner edge of the bridge area (i.e. where the section has been polished). To quantify the distance between annual bands, measure within the "measurement line", from the anchor point to where the opaque bands of each presumed annual growth band are most marked. The most marked (or most opaque) areas have been chosen as the end points of the measurement because in the first 7-8 annual bands it is difficult to establish the edge of the opaque band. The end point may be modified to conform to an edge of the opaque band when measuring growth bands as in case of MIA.

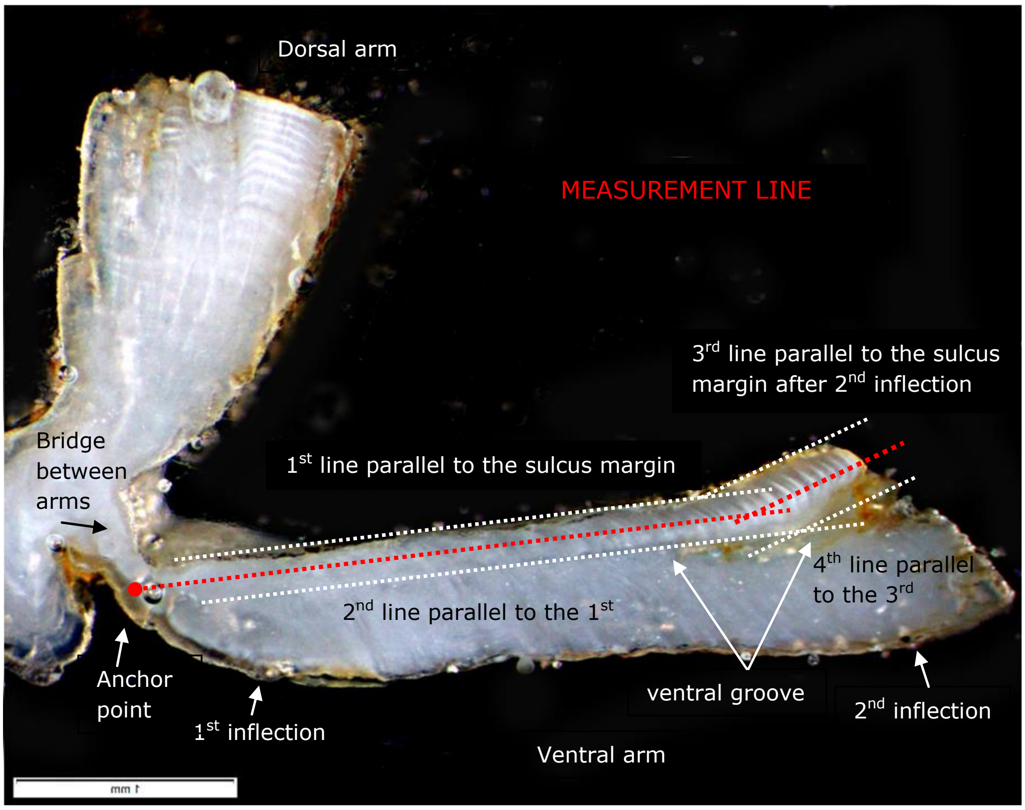


Figure 3. Image of an Atlantic bluefin tuna otolith section using reflected light. Location of the "measurement line" (in red).

Note. Previously, a template with measurements of the first 5 annuli was available (Rodriguez-Marin *et al.*, 2019). This template allowed to be a help for otoliths difficult to interpret. It would be advisable to obtain a similar reference table, with the same objective, but using this "measurement line" described above.

4. Edge type assignment

Ventral arm edge type identification, translucent or opaque, in otolith of this species is difficult. The thickness of the section and the diffraction of light can influence the perception of the type of edge. Using transmitted light for otoliths direct ageing may improve marginal edge recognition, as well as knowing the date of capture. Tilting in live viewing may help to edge classification. For images, new capture software with wide focus depth allows to obtain good images for edge recognition. It is useful to view both the enhanced and un-enhanced version of the image. Edge type must be identified as of a certain type when it occupies more than 50% of the edge across the width of the ventral arm. Dorsal arm can be used to corroborate this edge type and with the same criterion of 50%.

The marginal edge type should be assigned using the following categories (**Figure 4**):

- Wide translucent, WT (translucent material past last opaque zone is generally greater than 1/3 of previously completed translucent zone).

- Narrow translucent, NT (translucent material past last opaque zone is generally less than 1/3 of previously completed translucent zone).

- Opaque, O (opaque visible on edge).

Edge type readability should also be recorded on a scale from 1 to 3: 1= No confident; 2= Confident in completeness and not with the type and 3= confident.

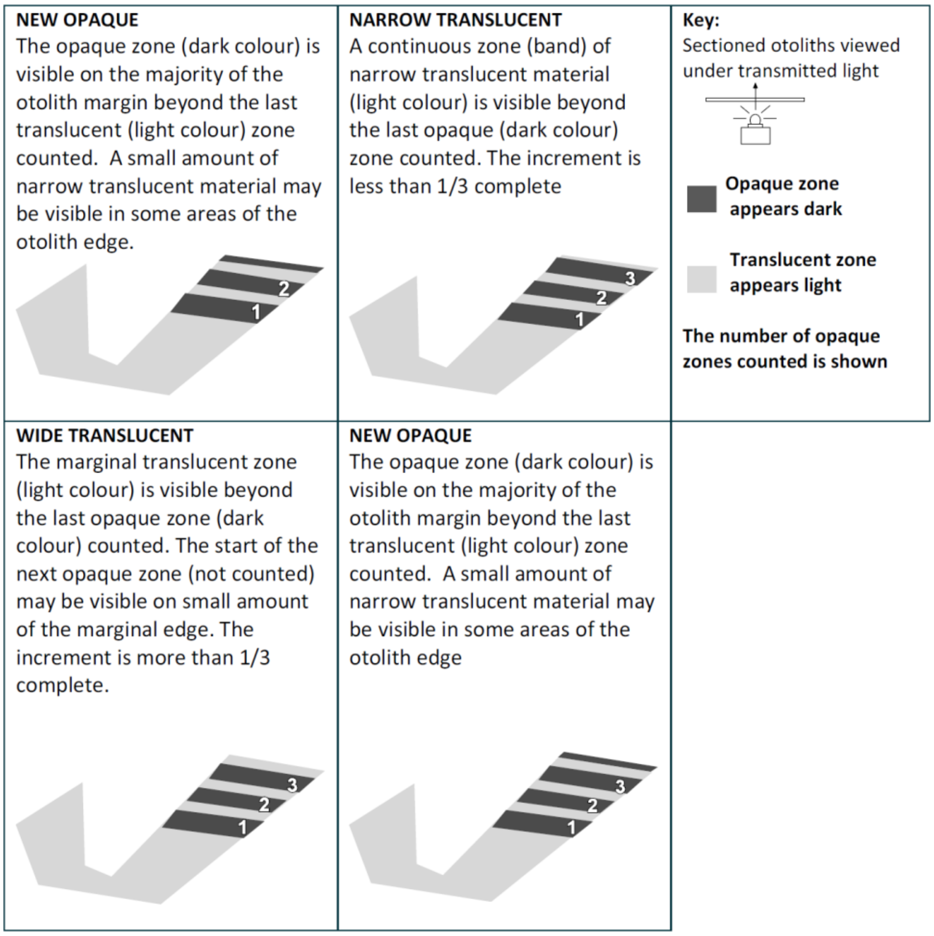


Figure 4. Type of edge assignment (figure from Farley *et al.*, 2016).

5. Ageing procedure

Each otolith should be interpreted through two independent readings with at least two weeks between readings: If the readings differ by 1 year, the 2nd reading will be used as the final age. If the readings differ by 2 or more years, conduct a 3rd reading with knowledge of the prior readings to reach a consensus final age. Otoliths where a 3rd reading is necessary should be annotated and checked for bias.

It is recommended that the reading form contain at least the following fields: Sample ID, reader ID, reading date, type of band counting (opaque or translucent), estimated age, readability code (previously described scale from 1 to 4), marginal edge type (WT, NT, O), edge type readability code (previously described scale from 1, less confident, to 3, confidence), and observations. Observations or notes field for each otolith reading is useful for writing down any useful comment about the sample as for example the appearance of certain annulus, etc.

Further information by sample, also very useful, may include: use of 1st annulus identification scale (Yes/No), section shape (V or Y), reader experience (low, medium, high), have followed standardized reading criterion (Yes/No), type of light employed (transmitted or reflected).

Band measurement information by sample. Each reader can define its own measure of the growth bands. In any case, it must be clearly indicated in which area of the otolith section this measure has been taken and the origin and end point of this measurement for each growth band. If the measurement procedure described in section 3 is followed (standardized measurement line), it is recommended to write down the following information: Sample ID, use of standardized measurement procedure (Yes or No), presumed age of the growth band, band measurement (in mm and from the anchor point) and measurement end point (most marked area or edge of the opaque band). Further information may include type of annuli (single = a single band which can be thin or thick, double = two distinct and clearly separated bands, doublet = two very close bands, triplet = three bands, multiple = more than three bands).

It may be useful to have information from the month of capture to help marginal edge type interpretation.

When reading otolith sections using digital images, it is recommended to use a tiff-format, or any other that allows to add layers for band marking. Annotated layers with the position of each annual band allows using the original image with several layers, that can be toggled off and on, for different readings or readers.

6. Converting annuli counts to age estimates

The number of growth bands counted need to be converted into age estimation by taking into account the timing of band formation, otolith marginal edge type, birth date and catch date. We will use the following **Age algorithm: a = n + adjustment**, where n is the count of opaque zones and adjustment is outlined below.

Age adjustment

Annuli are a bipartite structure consisting of one opaque and one translucent band, which form annually (Neilson and Campana, 2008). The ratio of strontium-calcium (Sr:Ca) across the bands indicates that the opaque band forms during the winter months (Siskey *et al.*, 2016). Marginal increment and edge type analysis performed by K. Krusic-Golub (presented at the ICCAT GBYP international workshop on Atlantic bluefin tuna growth, in February 2019) seem to indicate that translucent zones are present from April to November, while opaque zones would seem likely to occur primarily between December through to June. These last analyses also suggest that opaque band formation is completed between June and August, after which the subsequent translucent band starts forming. Unfortunately, due to low levels of fishing between January to April, very few samples between those months are available to provide a better indication of the opaque zone formation period.

In order to convert opaque zone counts into age, we first need to account for fish that are from the same cohort, regardless that they were allocated different zones based on whether the last opaque zone was completed and counted, or was not completed and therefore not counted. The last opaque zone is only counted when translucent material can be observed between the last opaque and the margin, i.e. narrow or wide translucent edge. Therefore, the time of year when the otolith edges are changing from opaque edge into narrow translucent needs to be the period for the initial edge type zone adjustment. The analysis of MIA and edge type suggest that this change from occurs primarily between April through to October, with the largest change occurring in July.

A two steps age adjustment protocol is proposed: firstly a zone formation adjustment and secondly a biological zone or a calendar year zone adjustment.

1) Zone formation adjustment

To convert the count of opaque bands (N) into age estimates (A), since the ageing protocol states that opaque bands are only counted if completely formed, the following adjustment based on the otolith edge (margin) type, adjustment date of the 1st July and the date of capture should be used:

Catch month	*January to March*	*April to June*	*July to October*	*November to December*
Narrow translucent, (NT)	N	N-1	N	N
Wide translucent, (WT)	N	N	N	N
Opaque, (O)	N	N	N+1	N

Once edge type has been accounted for by applying zone formation adjusted protocol, age can then be further adjusted accordingly. I.e. for biological, fisheries or management requirements. For ABFT, two options for additional adjustment are suggested: biological age (based on spawning) and calendar year (based on ICCAT annual catches reporting requirements).

2.1 Additional adjustment option 1- Biological age zone adjustment

June 1 is the universal birth date assumed for both ABFT management units based on the bluefin tuna reproductive cycle, where spawning occurs from May to June in the western Atlantic (Gulf of Mexico) and eastern Mediterranean (Levantine Sea), or from June to July in the western and central Mediterranean (Balearic Islands waters, South of Tyrrhenian Sea and Sea of Sicily) (Rooker *et al.*, 2007). Given this, the following adjustment table should be used:

Catch month	*January to May*	*June*	*July to December*
Biological year	A	A+1	A

2.2 Additional adjustment option 2- Calendar year zone adjustment

The following adjustment will be used based on catch date. Note: According to the largest edge change occurring on 1st July (adjustment date), the same limiting date needs to be used to make the adjustment:

Catch month	*January to June*	*July to December*
Calendar year	A+1	A

Thus, a bluefin tuna caught at the beginning of the year is interpreted as being 1 year older, despite being 5 or 6 months prior to the assumed date of birth, which occurs mid-year based on the reproductive cycle.

Decimal age

To convert into fractional age, sum to the previous algorithm (a), catch date in days/365.
Age algorithm: a = (n + adjustment) + catch date/365

References

Farley, J., Clear, N., Kolody, D., Krusic-Golub, K., Eveson, P., & Young, J. 2016. Determination of swordfish growth and maturity relevant to the southwest Pacific stock. WCPFC-SC12-2016/SAWP-11.

Neilson, J. and S. Campana. 2008. A validated description of age and growth of western Atlantic bluefin tuna (*Thunnus thynnus*). Can. J. Fish. Aquat. Sci. 65: 1523-1527.

Rodriguez-Marin E., Quelle P., Busawon, D., and Hanke, A. 2019. New protocol to avoid bias in otolith readings of Atlantic bluefin tuna juveniles. Collect. Vol. Sci. Pap. ICCAT, 75(6): 1301-1314

Rooker, J.R., Bremer, J.R.A., Block, B.A., Dewar, H., Metrio, G.d., Corriero, A., Kraus, R.T., Prince, E.D., Rodriguez-Marin, E., Secor, D.H., 2007. Life history and stock structure of Atlantic bluenfin tuna (*Thunnus thynnus*). Rev. Fish. Sci. 15, 2365-2310.

Siskey, M., Lyubchich, V., Liang, D., Piccoli, P., Secor, D. 2016. Periodicity of strontium: Calcium across annuli further validates otolith-ageing for Atlantic bluefin tuna (*Thunnus thynnus*). Fish. Res. 177, 13-17.