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JUVENILE ATLANTIC BLUEFIN TUNA OTOLITHS EXCHANGE

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SUMMARY

Otolith reading bias for juvenile Atlantic bluefin tuna was detected at its 2017 assessment. To try to reduce this bias, the current standardized reading protocol was revised. The new protocol uses age estimates from the first dorsal fin ray (spine) to identify the growth increments in the otoliths removed from the same young specimen. An exchange involving 14 experienced otolith readers was conducted to verify if the new reading procedure minimized the difference between otolith and spine readings. The results showed that there is a good agreement in the first five years, but from age 6, otoliths ages tended to be higher than spine age. Some readers had difficulty following the new protocol despite being experts, which indicates that it is necessary to improve it. The use of annual band measurements has shown to be a good tool for the control of the quality of age estimates. The results indicate that we are progressing in the recognition of the deposition pattern of the first annuli. It is recommended to carry out an ageing workshop to achieve the tasks identified as necessary.

RÉSUMÉ

Un biais de lecture des otolithes des thons rouges de l'Atlantique juvéniles a été détecté lors de l'évaluation de cette espèce en 2017. Pour tenter de réduire ce biais, le protocole de lecture standardisé actuel a été révisé. Le nouveau protocole utilise des estimations d'âge du rayon de la première nageoire dorsale (épine) pour identifier les augmentations de croissance des otolithes prélevés sur le même jeune spécimen. Un échange impliquant 14 lecteurs d'otolithes expérimentés a été mené pour vérifier si la nouvelle procédure de lecture minimisait la différence entre les lectures d'otolithes et d'épine. Les résultats ont montré qu'il y avait une bonne concordance en ce qui concerne les cinq premières années, mais qu'à partir de l'âge de 6 ans, l'âge des otolithes avait tendance à être supérieur à celui de l'épine. Certains lecteurs ont eu du mal à appliquer le nouveau protocole alors qu'ils étaient expérimentés, ce qui indique qu'il est nécessaire de l'améliorer. L'utilisation de mesures de bandes annuelles était un outil efficace pour contrôler la qualité des estimations d'âge. Les résultats indiquent que des progrès ont été accomplis pour reconnaître le schéma de dépôt des premiers anneaux. Il est recommandé de réaliser un atelier sur la détermination de l'âge pour remplir les tâches considérées nécessaires.

RESUMEN

En la evaluación de atún rojo de 2017 se detectaron sesgos en la lectura de otolitos de juveniles de atún rojo del Atlántico. Para intentar reducir este sesgo, se revisó el actual protocolo estandarizado de lectura. El nuevo protocolo utiliza estimaciones de edad del radio (espina) de la primera aleta dorsal para identificar los aumentos de crecimiento en los otolitos extraídos del mismo ejemplar joven. Se llevó a cabo un intercambio en el que participaron 14 lectores de otolitos experimentados para verificar si el nuevo procedimiento de lectura minimizaba las diferencias entre las lecturas de espinas y las de otolitos. Los resultados demostraron que existe acuerdo en los primeros cinco años, pero desde la edad 6, las edades de los otolitos tendían a ser más elevadas que las edades de las espinas. Algunos lectores encontraron dificultades en seguir el nuevo protocolo a pesar de ser expertos, lo que indica que es necesario mejorarlo. El uso de mediciones de la banda anual ha demostrado ser una buena herramienta para el control de calidad de las estimaciones de edad. Los resultados indican que estamos progresando en el reconocimiento del patrón de deposición de los primeros anillos. Se recomienda celebrar un taller de determinación de la edad para lograr las tareas identificadas como necesarias.

KEYWORDS

Age estimation, Otolith, Spine, Precision, Thunnus thynnus

1. Introduction

Estimating the age of Atlantic bluefin tuna *(Thunnus thynnus*, BFT) from counts of annuli in otoliths has been validated and age interpretation protocols along with inter-calibration experiences have been carried out (Neilson and Campana 2008; Secor *et al*. 2014; Rodriguez-Marin *et al.* 2014; Busawon *et al*. 2015). However, otolith reading bias was detected at the 2017 Atlantic bluefin tuna assessment meeting (Anon. 2017). This bias indicated an overestimation of the age due to the presence of sub-annual bands in the otoliths during the first years of life of this species. A revised protocol for reading otoliths has been prepared to avoid this bias in determining age (SCRS/2018/126). This protocol aims to improve the reading criteria for the first few annuli, which are the most difficult to interpret.

The revised protocol uses age estimates from the first dorsal fin radius (spine) to identify the growth bands in otoliths of the same specimen (SCRS/2018/126). The spine has been used because the age of juvenile BFT is easier to estimate using this calcified structure and because it allows the tracking of the cohorts better than by using the otolith (Anon. 2017). This report presents the results of an inter-laboratory aging exchange conducted to determine if the revised protocol eliminates the positive age bias and provides similar age from otoliths and spines in juvenile BFT.

2. Material and Methods

To test the new ageing protocol, a collection of 129 digital images of otoliths sections was prepared. All images had a scale bar for magnification reference and used Tiff-format to allow raster layers to be added so that each reader could annotate the image. The straight fork length (SFL) of the specimens ranged from 50 to 200 cm with the majority being 50 to 140 cm SFL (**Figure 1**). Specimens were captured in the East Atlantic management area, including the Mediterranean. Otoliths were prepared following the protocol described in Busawon *et al.* (2015). Otolith opaque bands were counted and only Y-type sections were used. Otolith images were prepared using reflected light. A template, created to assist reading (SCRS/2018/126), was included in some images (n = 27). It was recommended to read these images first ("as a pattern recognition essay"). Readers were then instructed to read all the images without the template but to use it when there was low confidence with the age estimation.

A reading form was designed to record the following information for each sample: age, ventral arm edge type, 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), reader code, reading date, notes with observations about the sample and size of the first 5 annual bands, including the innermost false annulus. To obtain measurements of the annual bands, the anchor point and each annulus was marked on the otolith image following the methodology described in the new reading protocol (SCRS/2018/126).

The exchange of images between the participants was possible thanks to the GBYP Program as it facilitated cloud storage space. Participants performed a blind age reading (after doing a warm-up reading of the reference collection) (Busawon *et al*. 2015), completed the reading form and produced annotated images with the position of each annual band marked using a raster layer and a reader assigned color. A survey was distributed among the participants to determine the degree of satisfaction and use of the new reading protocol, as well as to seek suggest improvements (Appendix).

The otoliths used in the exchange were removed together with the spines from the same specimen (paired hard parts). The spines were sectioned and read using standard protocols (Luque *et al*. 2014) and were considered "accurate" for young fish (Rodriguez-Marin *et al*. 2009; Luque *et al*. 2014). The spine ages were compared to the age estimates from otolith using the new ageing protocol. The spines belong to the set that was used to create an ageing bias vector to correct otolith readings that were used in the last BFT assessment (Anon. 2017). The majority of selected spine samples were from fish aged 1 to 5 years with very few specimens aged 6 to 9 years.

Final age estimates of both structures were adjusted to account for the date of harvesting and the timing of bands formation throughout the year. Otolith final age was adjusted by adding 1 year to the age when the fish was caught between January 1 and the assumed time of the opaque band formation (June 1) (Rodriguez-Marin *et al.* 2016). Spines final age was adjusted by subtracting 1 year to the age when the fish was caught between June 1 and December 31 and the edge of the structure was translucent (Luque *et al*. 2014).

Diagnosis of paired age agreement was evaluated by precision indices through age bias graphs, Coefficient of Variation (CV), Average Percent Error (APE) and Evans-Hoenig and Bowker tests of symmetry (Campana *et al*. 1995, McBride 2015). FSA, R package version 0.8.20 (Ogle 2018) was used for the analysis.

3. Results

The exchange involved 14 experienced otolith readers from 10 institutions in Australia, Canada, Italy, Japan, Spain, Turkey and USA. Precision and symmetry tests were estimated by reader for all data (n = 129) and for spines aged up to 5 years (n = 115). CV ranged between 8.1 and 23.9. APE values range from 5.7 and 16.9 (**Table 1**). These values show moderate precision as only one third of the readers had CV values lower than 10. The precision of the samples aged up to 5 years is slightly lower than that obtained from using all the samples. On the other hand, it slightly improves the symmetry ($p < 0.01$). The mode of each sample age from all readers was called the "modal age" and was represented against spines age. CV and APE values of this modal age were precise and paired readings did not show bias (**Table 1**). Most of the readers showed no bias or slight bias in the agreement between paired ages (**Table 1**).

The age bias graphs by reader showed that as age increased, otoliths ages tended to be higher than spine age (**Figure 2**). This occurs mostly from age 6, where the number of samples is very small. This difference is more pronounced in readers that showed paired age bias. Only the two readers with the lowest precision showed systematic higher age estimates from spines compared to otoliths (**Figure 2**). The age bias graph of the modal age from otoliths showed good agreement with the age from the spine in age classes with sufficient sample size, i.e. between 1 and 5 years (from age 5, most ages have less than 5 samples). The biggest disagreement occurred at age 5, but it was less than half a year (**Figure 3**).

The confidence in readings, measured as readability code, showed no relationship with precision (**Table 1**). Reading confidence increased from age 1 to 5 and then dropped at higher ages (**Figure 4**).

The measurements of otolith annuli by reader showed that most located the annuli in the same locations along the ventral arm of the otolith section, showing a consistency in the measurements of the first five increments (**Figure 5**). Extreme values were also seen in some readers, and other readers displayed great variability in the measurements of the bands. As an example, the frequency distribution graph of the size of the annuli for the agers with highest and lowest CV was constructed (**Figure 6**), which indicates that the reader with the lowest precision had bimodal distributions for some of the annual bands.

The boxplot of the first bands measurements by spine age by all readers showed similarity in the size of the first annuli among age groups (**Figure 7**). Monthly formation of edge type showed an increasing trend of the opaque edge percentage in summer months, reaching values slightly above 50% in September (**Figure 8**).

4. Discussion

The purpose of this otolith exchange was determine if the new reading protocol reduced the bias in the otolith reading for juveniles BFT detected in the 2017 stock assessment (Anon. 2017). The bias was only for approximately 1 year, but it is important for the correct tracking of cohorts and, therefore, it is vital for the use of age-structured population models.

The CV values of the readers are moderately precise, which indicates the difficulty of reading otoliths of BFT juveniles, as has already been described (Clear *et al*. 2002; Rodriguez-Marin *et al.* 2007; Busawon *et al*. 2015). The age bias graphs by reader showed a higher age estimated from otoliths compared to that from spines, but this only occurs at ages over 5 years, where samples sizes are very low. This finding is unexpected since once the first annual bands are determined, the remaining bands are easier to identify and, therefore, this bias should not be present. It is possible that it is simply because these samples are not easy to read (poor preparations), and in fact the readability score of these samples over 5 years decreases drastically. In addition, symmetry tests, which are very sensitive to bias (McBride 2015), do not detect paired age bias in many of the readers for paired ages up to 5 years. Therefore, older ages agreement should be interpreted with caution. An enlarge of compared ages should be considered by reading a sufficient number of paired samples.

Some agers had difficulty following the new protocol despite being experts, which indicates that it is necessary to improve it. The type of light used to obtain the images can also influence, since several readers were more accustomed to transmitted light than to reflected. Thus, for example, both readers with less precision showed a systematic overestimation of spine age (**Figure 2**). This was probably due to the fact that both readers were mis-assigning the innermost false annulus as the first annulus. Indeed, when we analyze the measurements of the annual bands per reader, we can see that both gave exceptionally large measurements to the false and the first annulus (**Figure 5**). In the case of these two readers, it was because they had difficulty identifying the anchor point from which the measurements were taken, and that they did not usually use the scale bar for the identification of the first annual band. The mistake in the allocation of the first bands, resulted in errors in the counting of the following bands.

When the modal age of all readers is used, a good agreement is found between the ages of the otoliths and the spines up to five years. This modal age represents, to some extent, the reading criterion of the new protocol. Therefore, this exercise represents a step forward in getting reliable age estimations of young BFT using otoliths, raising the importance of having a common criterion in the interpretation of the bands deposited between the first and second inflections of the otolith section.

The measurements of otolith annulus have not been used until now in the age reading exchanges for this species, and are a useful tool to quantify differences in the interpretation of calcified structures. **Figure 5** shows that there were some readers with extremely low or high values, or great variability in the measurements of annuli. This may be due to the application of a different interpretation criterion or to methodological differences such as the location of the anchor point or the way that bands were measured. These extreme measurements were mainly due to these last two reasons. Annuli measurement also help determine if there are age interpretation errors, thus, when representing the annuli size frequency distribution of the first and last ager in order of lower to higher CV, it was appreciated that the reader with higher CV showed a bimodal distributions in some annuli, which should not happen (**Figure 6**).

The size of the annual bands should be the same regardless of the age of the individual, and this is what was seen, in general, in the whole set of readers (**Figure 7**). This finding increases confidence in the ageing procedure.

Present results in monthly formation of edge type, translucent or opaque, showed an increasing opaque edge presence in summer months (**Figure 8**). Values greater than 50% for opaque edges were also found by Rodriguez-Marin *et al*. (2016), with a much larger sampling. In any case, these percentages remain inconclusive and winter sampling is still lacking. Sampling in winter is difficult as most fisheries of this species operate from spring to autumn. The interpretation of the edge type has been addressed in this new protocol, but it is clear that it has not made a contribution that improves its identification, and this has been recognized by the exchange participants in the survey on the new protocol. A better criteria for and improvement in the assignment of edge type (opaque or translucent) is needed. It was suggested to use the same criterion as for southern bluefin tuna (*Thunnus maccoyii*), where an opaque zone is only counted if it is completed (translucent material is visible at the edge) and edge type is recorded by noting the state of completion of the last zone in the edge. Using images as examples can also help.

The results of the survey show that readers value the new protocol in a satisfactory way, including aspects such as the use of the selected location for counting and marking the annual bands and performing measurements of the annual bands. The anchor point that serves as a starting point for taking measurements has generally been easy to locate for most readers, but other options have been suggested for locating the anchor point and for measuring annuli in other way. The results show that in spite of the difficulty in measuring bands in the otolith sections, it is possible to do, and the option used in the new protocol has been easy to implement. The use of a reference scale measurement to identify the first annual band is still an important aid in reading otoliths, however, it may not be necessary for all readers.

The use of reference measurements to identify the annual bands should not be considered a constriction when reading, but the truth is that is not the same to read otoliths from juveniles than from big adults. In the small BFT otoliths there are so few references in the shape of the otolith (for example 2nd inflection), that it is difficult to know what to count when there are multiple bands. Whereas in adults, there is a frame of reference (2nd inflection) and clear annual bands. The new protocol template with the reference measurements of the first bands establish the deposition pattern of the first annuli, including the gradual decrease of the distance between them. Therefore, it can assist annuli assignment in difficult-to-read samples.

Conclusions

1. This exchange represents a step forward in minimizing bias in age estimations of young BFT using otoliths. The bias identified in ages >5 needs to be further investigated.

2. Annual band reference measurements are a useful tool to help identify the first few annuli (including the false innermost one).

3. Obtaining annual band measurements during otolith reading is useful for control quality of age estimates. A consensus is needed to improve the definition of the measurement of the annual bands in the otolith sections (including the location of the anchor point or origin of the measurement).

4. It is necessary to improve the description / completion state of the edge type on the ventral arm of the otolith section. It is necessary to increase the winter samples so that the annual deposition cycle of the translucent and opaque bands can be established.

5. A new reference collection incorporating samples aged with the revised reading protocol is needed.

6. It is necessary to quantify the differences in reading between the old protocol (Busawon *et al.* 2015) and the new one (SCRS/2018/126), this will allow the calculation of correction factors to enable the use of the age length keys developed so far.

7. To achieve the subjects identified above, it is advisable to conduct an ageing workshop.

Acknowledgements

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References

Anonymous. 2017. Report of the 2017 ICCAT bluefin stock assessment meeting (Madrid, July 2017). SCRS/2017/010.

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

Campana, S.E.; Annand, M.C.; Mcmillan, J.I. 1995. Graphical and statistical methods for determining the consisteny of age determinations. Transactions of the American Fishery Society. 124:131-138.

Clear, N., Francis, M., Tsuji, S., Krusic-Golub, K., Itoh, T., Tzeng, W., Sutton, C., Findlay, J., Hirai, A., Shiao, J., Omote, K., An, D. 2002. A manual for age determination of southern bluefin tuna *Thunnus maccoyii*, Otolith sampling, preparation and interpretation, Direct Age Estimation Workshop CCSBT, Aust.

Luque, P.L., Rodriguez-Marin, E., Ruiz, M., Quelle, P., Landa, J., Macias, D., Ortiz de Urbina, J.M. 2014. Direct ageing of *Thunnus thynnus* from the east Atlantic and western Mediterranean using dorsal fin spines. J Fish Biol 84, 1876-1903.

McBride. R.S. 2015. Diagnosis of paired age agreement: a simulation of accuracy and precision effects. ICES J Mar Sci; doi:10.1093/icesjms/fsv047.

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.

Ogle, D.H. 2018. FSA: Fisheries Stock Analysis. R package version 0.8.20.

Rodríguez-Marín, E., Clear, N., Cort Basilio, J.L., Megalofonou, P., Neilson, J.D., Neves dos Santos, M., Olafsdottir, D., Rodríguez-Cabello, C., Ruiz, M., Valeiras, J. 2007. Report of the 2006 ICCAT. Workshop for bluefin tuna direct ageing. Collect. Vol. Sci. Pap. ICCAT 60, 1349-1392.

Rodriguez-Marin E., Di Natale A., Quelle P., Ruiz M., Allman R. , Bellodi A., Busawon D., Farley J., Garibaldi F., Ishihara T., Koob E., Lanteri L., Luque P.L., Marcone A., Megalofonou P., Milatou N., Pacicco A., Russo E., Sardenne F., Stagioni M., Tserpes G. and Vittori S. 2014. Report of the age calibration exchange within the Atlantic Wide Research Programme for bluefin tuna (GBYP). SCRS/2014/150.

Rodriguez-Marin, E., Ortiz de Urbina, J.M., Alot, E., Cort, J.L., De la Serna, J.M., Macias, D., Rodríguez-Cabello, C., Ruiz, M., Valeiras, J. 2009. Tracking bluefin tuna cohorts from east Atlantic Spanish fisheries since the 1980s. Collect. Vol. Sci. Pap. ICCAT 63, 121-132.

Rodriguez-Marin, E., Quelle, P., Ruiz, M., Luque, P. 2016. Standardized age-length key for east Atlantic and Mediterranean bluefin tuna based on otoliths readings. Collect. Vol. Sci. Pap. ICCAT 72: 1365-1375.

Rodriguez-Marin E., Quelle P., Busawon D. and Hanke, A. 2018. New protocol to avoid bias in otolith readings of Atlantic bluefin tuna juveniles. SCRS/2018/127.

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.

Table 1. Diagnosis of paired age agreement for all data (n = 129) and for spines aged up to 5 years (n = 115). Precision indices: CV = Coefficient of Variation, APE = Average Percent Error, readability score and Symmetry tests: bias = significant differences in both Evans-Hoenig and Bowker tests, slight bias = significant differences in at least one of the tests, no bias = no significant differences, $p < 0.01$. Readers were ordered according to the CV.

	All data (n = 129)				Only spines aged up to 5 years (n = 115)			
Reader	CV	APE	Readability score	Symmetry test	CV	APE	Readability score	Symmetry test
14	8.1	5.7	2.7	slight bias	8.1	5.7	2.7	no bias
1	8.6	6.1	2.8	slight bias	9.0	6.4	2.9	slight bias
6	8.6	6.1	1.9	no bias	8.8	6.2	1.9	no bias
7	8.9	6.3	2.8	no bias	9.2	6.5	2.8	no bias
9	9.6	6.8	2.2	no bias	9.3	6.6	2.2	no bias
12	10.4	7.4	2.9	no bias	10.3	7.3	2.9	no bias
2	10.6	7.4	2.3	slight bias	11.1	7.8	2.3	no bias
3	10.6	7.5	2.9	no bias	10.9	7.7	2.9	no bias
10	12.8	9.0	3.0	bias	13.5	9.5	3.0	bias
11	14.5	10.3	2.6	no bias	14.1	10.0	2.7	no bias
13	15.1	10.7	2.9	bias	15.9	11.2	2.9	bias
8	18.3	12.9	2.3	slight bias	19.7	14.0	2.2	slight bias
4	21.4	15.1	2.5	bias	22.4	15.9	2.5	bias
5	23.1	16.4	2.4	bias	23.9	16.9	2.4	bias
Average	12.9	9.1	2.6		13.3	9.4	2.6	
Modal age all readers	4.5	3.2	3	no bias	4.5	3.1	3	no bias

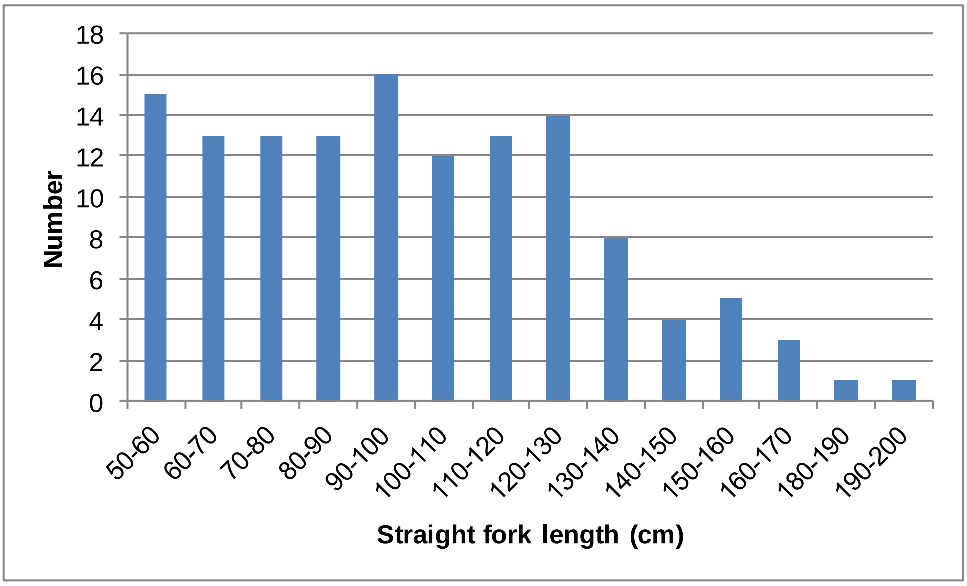

Figure 1. Size distribution of the Atlantic bluefin tuna used for this study.

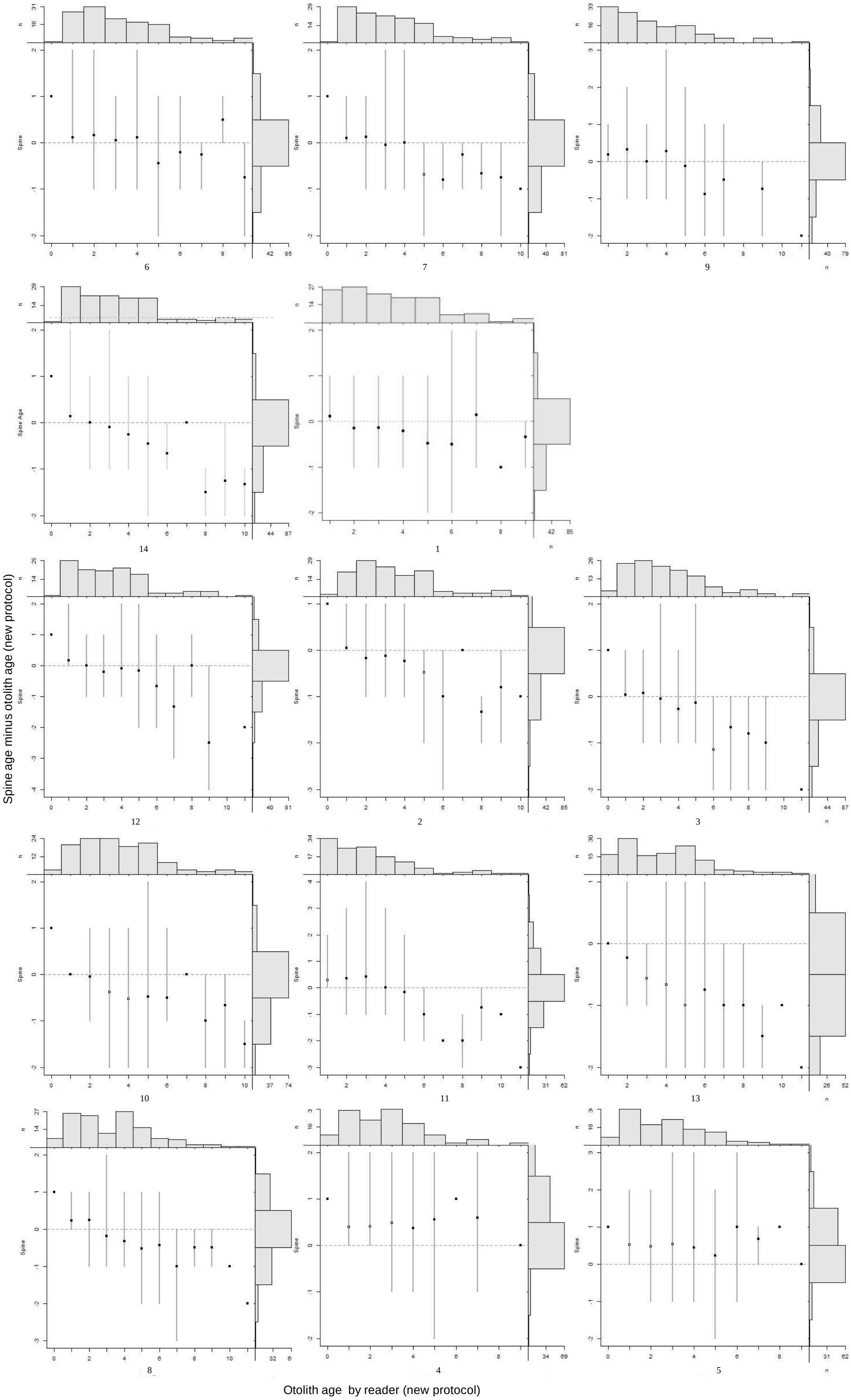

Figure 2. Age bias graphs (spine age minus otolith age) by reader. The number of samples per age class appears at the top and right of the graph. Graphs were ordered from top to bottom by increasing CV value.

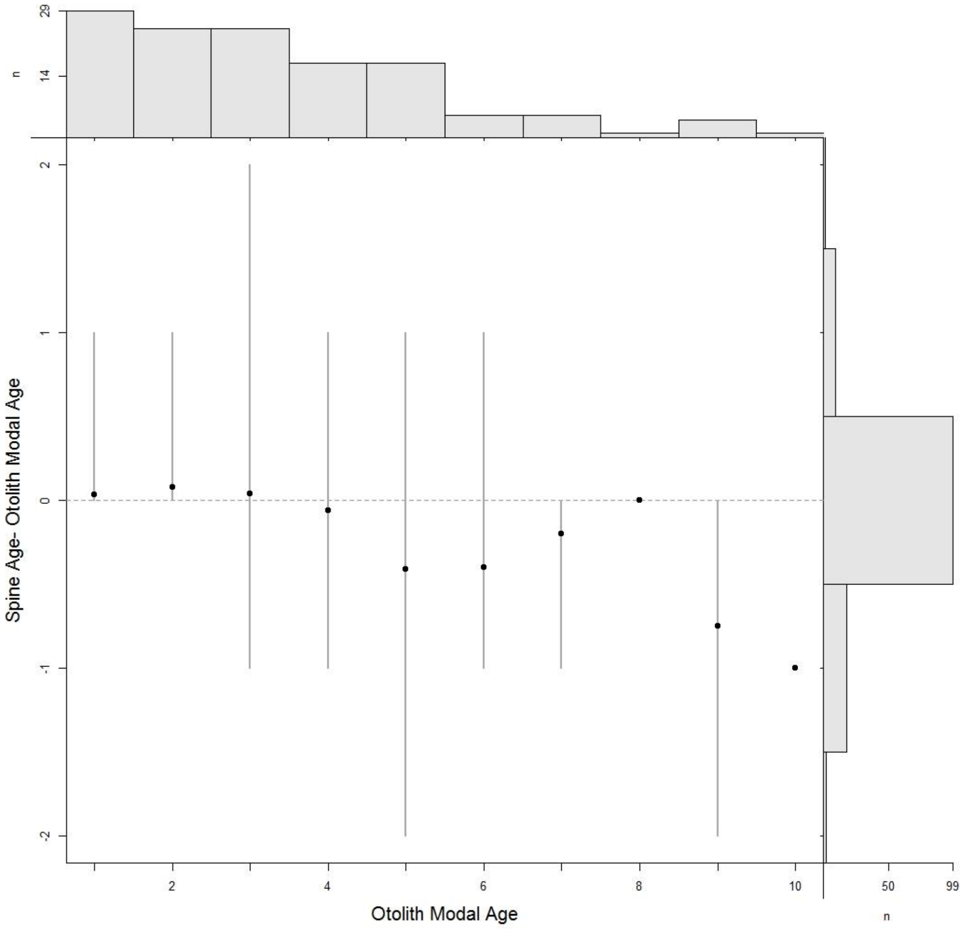

Figure 3. Age bias graphs. Age of the spine minus otolith modal age from all readers. The number of samples per class appears at the top and right of the graph.

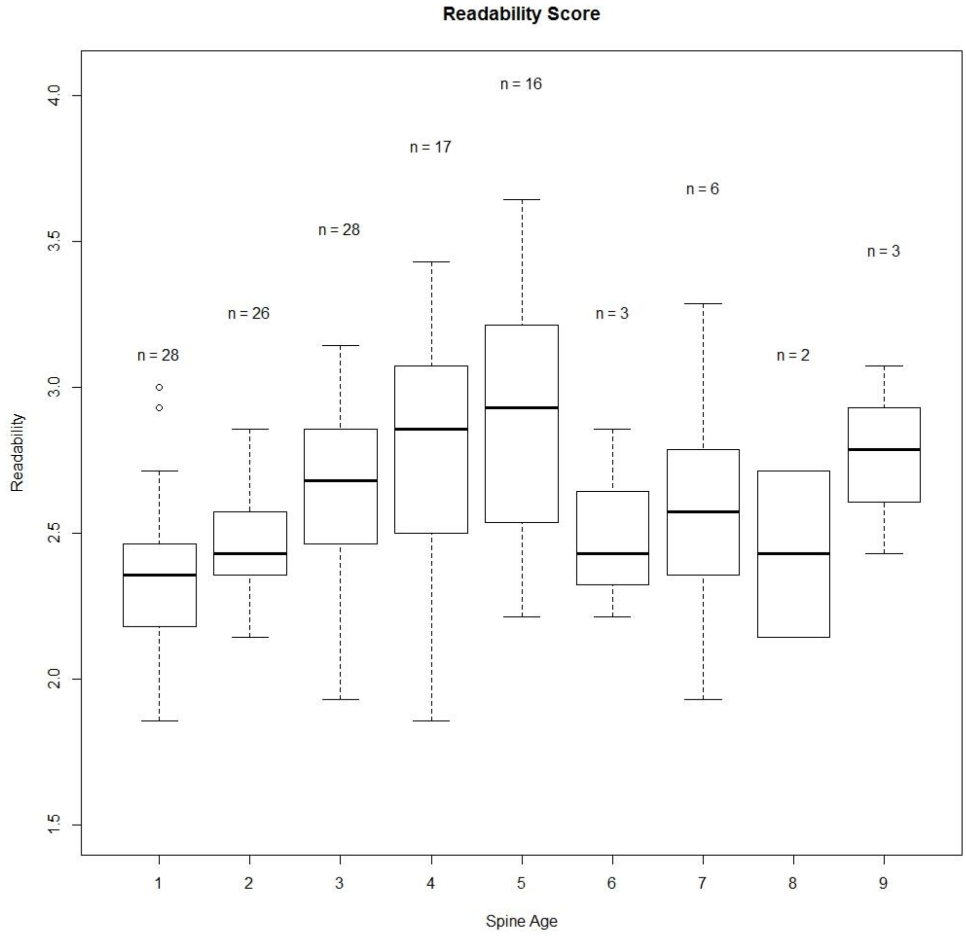

Figure 4. Mean readability score obtained from all the readers by spine age.

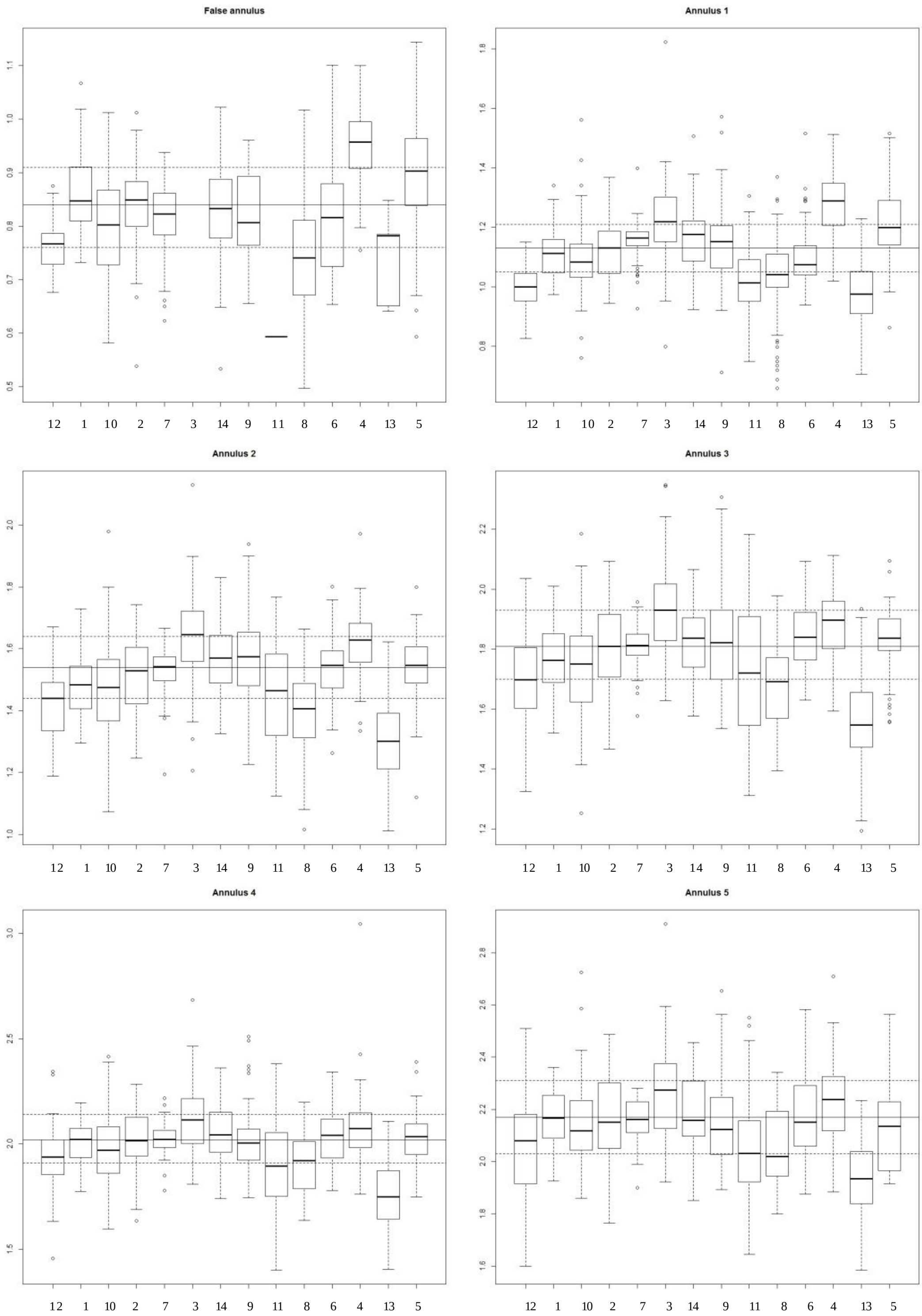

Figure 5. Box plot of first five annual and innermost sub-annual band measurements by reader. Solid and dashed horizontal lines represent the average and the standard deviation of each annulus from new protocol reference measurements table (SCRS/2018/126).

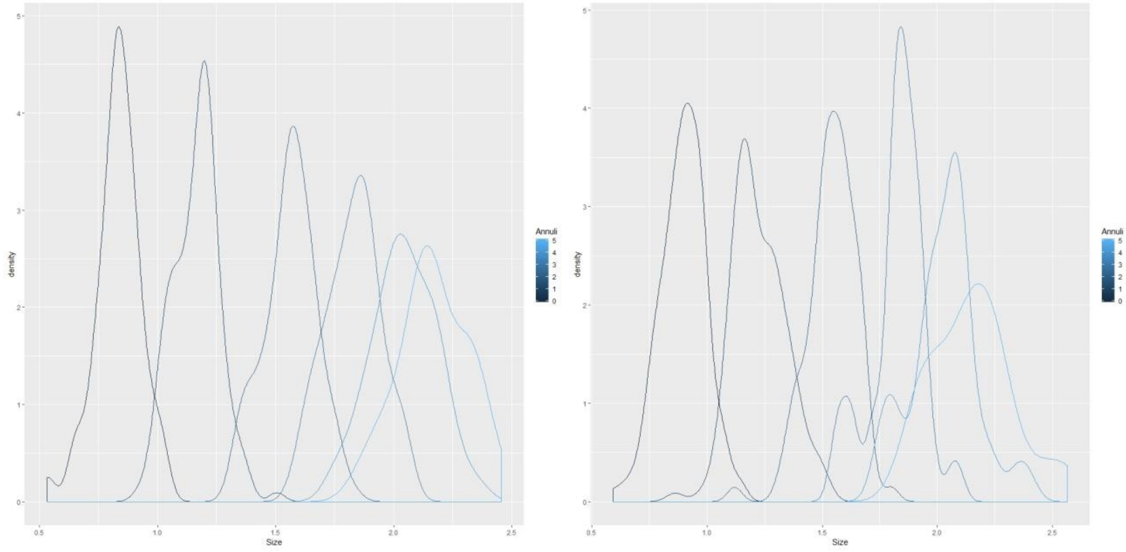

Figure 6. Frequency distribution of the first six annulus measurements, including the innermost false annulus. Left and right for the readers with the highest and lowest CV, respectively. Distance from the anchor point to the opaque band in mm.

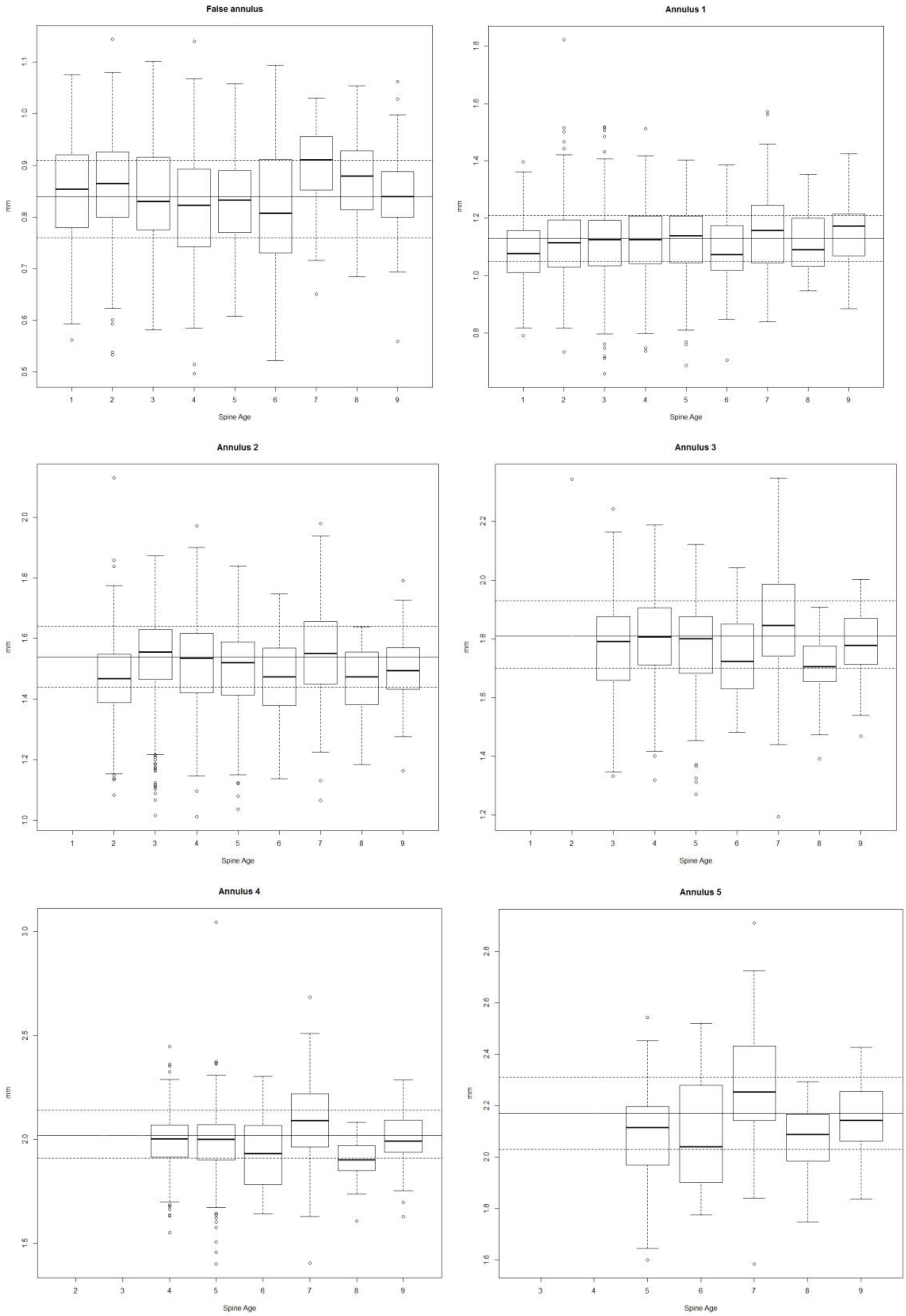

Figure 7. Box-plot of the distance of the first five annual and innermost sub-annual bands from the anchor point by spine age by all readers. Solid and dashed horizontal lines represent the average and the standard deviation of each annulus from new protocol reference measurements table (SCRS/2018/126).

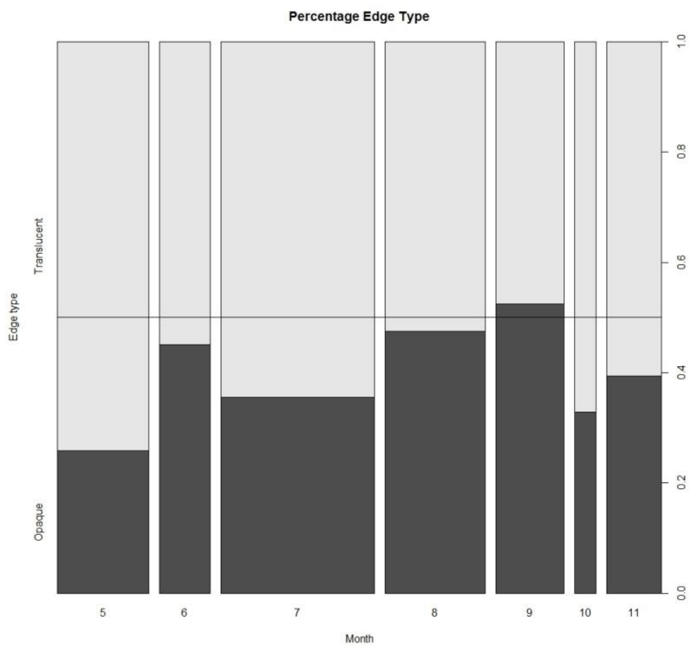

Figure 8. Monthly edge type assignment from the otoliths used in the exchange (percentage by month from all agers). The opaque edge is represented in dark gray and the translucent in light gray. The width of the columns represents the number of samples (months with less than 6 samples are not included).

Appendix

Survey for the otolith exchange of bluefin tuna juveniles

Please mark with a cross in the box for the chosen option:

1. Did you find it hard to locate the anchor point following the instructions from the new protocol?
☐ no, ☐ sometimes, ☐ frequently, ☐ always

2. If it was difficult to locate the anchor point, would you use another one? please define.

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3. Have you used the *1 mm reference / first annual band measurement* to identify the first annual mark and therefore discard the false annulus?

☐ No, ☐ Sometimes, ☐ frequently, ☐ always

4. Has it been difficult to identify the annual bands following the new protocol?

☐ No, ☐ Sometimes, ☐ frequently, ☐ always

5. Do you think that the location for counting and marking the annual bands, above the ventral groove of the ventral arm, is a good location?

☐ No, ☐ Sometimes, ☐ frequently, ☐ always

6. If you have answered negatively to the previous question, indicate another zone to read and mark the annual bands.

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7. Has it been difficult to measure the annual bands?

☐ No, ☐ Sometimes, ☐ frequently, ☐ always

8. If it has been difficult, will you use another? please define another way to measure

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9. Have you used *the template / table band measurements* to identify the annual bands

☐ No, ☐ Sometimes, ☐ frequently, ☐ always

10. Do you think the revised protocol is useful to identify the type of ventral arm margin?

☐ No, ☐ Sometimes, ☐ frequently, ☐ always

11. If you have answered negatively to the previous question, please suggest any other way or type of classification (for example thickness of the margin type)

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12. Any other comment is appreciated
