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CALIBRATION OF ATLANTIC BLUEFIN TUNA OTOLITH READING CONDUCTED BY AN INDEPENDENT FISH AGEING LABORATORY CONTRACTED BY THE ICCAT RESEARCH PROGRAMME GBYP

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SUMMARY

A calibration exercise was carried out with the objective of ensuring that age readings provided by the Fish Ageing Services laboratory (FAS) follow the ICCAT reviewed reading protocol. There were found differences in band counts between ICCAT expert readers and FAS readings. These differences start from specimens with more than 10 bands and are more pronounced for older specimens. The results of the present calibration are very similar to those of the previous one. These differences in readings appear to be due to the fact that FAS uses the entire section of the otolith to count annual bands, whereas ICCAT readers focus on the inner part of the ventral arm. Analyses conducted to establish which reading is more appropriate: growth function estimation and cohort follow-up analysis, seem to indicate that ICCAT readers are more accurate than FAS readers. We suggest applying a correction vector to otoliths read by FAS and with more than 10 annual bands, in order to incorporate the FAS readings into the GBYP age database. This ageing bias vector has been obtained from the current and previous calibrations.

RÉSUMÉ

Un exercice de calibrage a été réalisé afin de s'assurer que les lectures des âges fournies par le laboratoire Fish Ageing Services (FAS) sont conformes au protocole de lecture révisé de l'ICCAT. On a constaté des différences dans le nombre d'anneaux entre les lecteurs experts de l'ICCAT et les lectures du FAS. Ces différences commencent à partir des spécimens ayant plus de 10 anneaux et sont plus prononcées pour les spécimens plus âgés. Les résultats de la présente calibration sont très similaires à ceux de la précédente. Ces différences de lecture semblent être dues au fait que le FAS utilise la section entière de l'otolithe pour compter les anneaux annuels, alors que les lecteurs de l'ICCAT se concentrent sur la partie interne du bras ventral. Les analyses menées pour établir quelle lecture est la plus appropriée (l'estimation de la fonction de croissance et l'analyse du suivi des cohortes) semblent indiquer que les lecteurs de l'ICCAT sont plus précis que ceux du FAS. Nous suggérons d'appliquer un vecteur de correction aux otolithes lus par FAS et ayant plus de 10 anneaux annuels afin d'incorporer les lectures de FAS dans la base de données des âges du GBYP. Ce vecteur de biais de la détermination de l'âge a été obtenu à partir des calibrations actuelles et précédentes.

RESUMEN

Se realizó un ejercicio de calibración para garantizar que las lecturas de edad proporcionadas por el laboratorio de Servicios de lectura de edades de Peces (FAS) siguen el protocolo de lectura revisado por la ICCAT. Se encontraron diferencias en los recuentos de bandas, entre los lectores expertos de ICCAT y las lecturas de FAS, a partir de los especímenes con más de 10 bandas y son más pronunciadas para atunes más mayores. Los resultados de la presente calibración son muy similares a los de la anterior. Estas diferencias en las lecturas parecen deberse a que FAS utiliza toda la sección del otolito para contar las bandas anuales, mientras que los lectores del ICCAT se centran en la parte interna del brazo ventral. Los análisis realizados para establecer qué lectura es más adecuada: la estimación de la función de

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crecimiento y el análisis de seguimiento de cohortes, parecen indicar que los lectores de la ICCAT son más precisos que los del FAS. Se sugiere aplicar un vector de corrección a los otolitos leídos por FAS y con más de 10 bandas anuales, con el fin de incorporar las lecturas de FAS a la base de datos de edad del GBYP. Este vector de sesgo de la determinación de la edad ha sido obtenido a partir de las calibraciones actuales y precedentes.

KEYWORDS

Age estimation, otolith, standardization, Thunnus thynnus.

1. Introduction

The Fish Ageing Services laboratory (FAS) was contracted in Phase 7 and Phase 9 of the ICCAT bluefin tuna research programme for the whole Atlantic (GBYP) to provide age estimates from 4000 Atlantic bluefin tuna otolith samples. A first calibration exercise was carried out with the objective of ensuring that there was no systemic bias in the FAS age readings in ICCAT GBYP Phase 7, compared to age estimates made by laboratories performing direct age readings for ICCAT. In this first calibration, a one-year bias in the count of bands in older specimens was found, with a lower count by FAS compared to the rest of the laboratories starting from 10-13 years of age. This bias appears to be due to the fact that FAS counts the bands in a different area of the ventral arm of the otolith (Rodriguez-Marin *et al*. 2020a).

Methodology standardization of age interpretation from ABFT calcified structures has been a priority within the GBYP (Busawon *et al*., 2020). With this purpose, an international ICCAT GBYP workshop on ABFT direct ageing was held at the beginning of 2019 (Rodriguez-Marin *et al*., 2020b). This workshop had the participation of most of researchers currently involved in direct ageing of ABFT. In addition, a scientist from FAS, with extensive experience in the field of fish ageing, was also involved. The workshop reviewed the current protocols for otolith preparation and age reading criteria, which allowed for standardized ageing methodology.

A second calibration exercise was planned with FAS age readings carried out after the workshop and within GBYP Phase 9, with the objective of testing ageing consistency and precision between ICCAT laboratories and FAS. Another aim is to use the findings of the current and previous calibrations to be able to incorporate the FAS readings into the GBYP age database. In addition, the samples used in the calibration will enlarge the new reference collection.

2. Material and methods

Seven research centers have participated in this task, four from Europe (AZTI; University of Cagliari, UNICA; University of Genoa, UNIGE and Spanish Institute of Oceanography, CNIEO-CSIC), one from Turkey (Istanbul University, IU), one from Canada (St. Andrews Biological Station, SABS) and one from USA (National Marine Fisheries Service, NMFS). The collaboration of these laboratories is necessary for the participation of researchers from both sides of the Atlantic. The researchers involved have experience in direct ageing of Atlantic bluefin tuna and contribute with age-length data to the assessment of this species. Furthermore, to assess the effectiveness of the new reading protocols, two inexperienced readers participated in the calibration.

Laboratories read a sub-sample of 10% of the otoliths aged by FAS in Phase 9 GBYP, to determine a measure of inter-laboratory precision. Based on the 1st calibrations results (Rodriguez-Marin *et al*., 2020a), this subsample favored larger specimens (>200 cm straight fork length) however all sizes were still represented (**Figure 1**). In the ageing precision analysis, CNIEO-CSIC and AZTI aged using the physical otolith sections while the rest of the laboratories involved used digital images as they are easier to share among partners, and allowed, to a limited extent, the comparison between both sets of readings. Additionally, both sets, physical sections and digital images will be added to the new reference collection using the consensus age obtained from expert readers.

A modal reading has been used: "Mode Experts" (Mod_E) for the readings of all laboratories including the readings of both physical otolith sections and digital images but not including the readings of the two inexperienced readers. FAS used live readings and for the rest of the reader the reading mode is indicated at the end of the name (reader acronym name_L for live and reader name_P for pictures).

According to the reviewed protocol, age estimates consisted in the counting of opaque bands using transmitted light. The preparations, consisting of sections of physical otoliths, were read twice. A third and final band count was completed if the first two band estimations differ, to produce the final band estimate. All band counts were performed blindly without knowledge of fish size or catch date. The final band estimate was done with knowledge of the first two counts. Band counts were transformed to ages by applying the procedure described in Rodriguez-Marin *et al*. (2020b). The experts mode (Mod_E) and FAS age estimates were fitted to the Von Bertalanffy equation and plotted together with growth functions from the eastern stock and western stock (Cort, 1991; Ailloud *et al*., 2017). Residual plots were made comparing the growth curves of both stocks predicted ages to readers age estimates.

A reading form was provided and the following information was recorded for each sample: number of annual bands (opaque), ventral arm edge type (wide translucent, narrow translucent or opaque), edge confidence (1= no confident; 2= confident in completeness and not with the type and 3= confident), sample 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), reading date and notes with observations about the sample. This form also included the measurement of the first five annual bands to see if there are differences between readers and to have a check of the readers' reading criteria. Annual band measurements during otolith reading was done following the "measurement line" defined in Rodriguez-Marin *et al*. (2020b). Annotated images and annual band measurements were produced for control quality of age estimates. A survey on the use of each reading area within the otolith section was conducted for each reader.

The readings from each reader and laboratory were compared with FAS readings. Precision was estimated through Coefficient of Variation (CV), Average Percent Error (APE), Evans-Hoenig and Bowker symmetry tests, age bias plots and age difference distributions between readers (Campana *et al*., 1995; McBride, 2015). FSA, R package version 0.8.20 (Ogle 2018) was used for the analysis.

3. Results and discussion.

In terms of precision, age estimates from FAS and readers were within the acceptable limits (CV<10) except for two expert readers. Despite the good precision, the symmetry test indicated a bias for all expert readers. Interestingly, the non-expert readers showed no bias and were within the acceptable level of precision (**Table 1**).

Marginal edge agreement was high between readers and FAS, reaching an average of 50% when all three edge types (O, NT and WT) are used, and 75% when only opaque and translucent edge types are taken into account (**Table 1** and **Figure 2**). The high level of agreement supports the use of transmitted light for band counting of otolith sections (Rodriguez-Marin *et al*., 2020b). Readers rated the readability of images as good and had high confidence in edge type assignment (**Table 1**).

The use of transmitted light, as compared to reflected light, not only improves agreement on the marginal edge type, but also allows a clearer view of the entire otolith section, including the dorsal arm. However, readers who have used reflected light to read the sections state that in the inner part, at the end of the ventral arm, the bands are very clearly visible in old specimens, whereas with transmitted light this final area of the ventral arm sometimes appears hard to interpret, while the bands are visible in the outer area.

The distribution plots of differences between band counts and bias plots show that in general expert readers count more bands than FAS from 10 annual bands onwards (**Figure 3**, **Figure 4**, **Figure 5** and **Figure 6**). There are only three readers that show practically no bias up to specimens with more than 13 bands and two of them are precisely the inexperienced ones.

The measurements of the first annual bands show that there are two readers that are interpreting the bands differently from the rest of the readers and they are precisely the two readers with the lowest accuracy and CV higher than 10 (**Table 1** and **Figure 7**). The misinterpretation of the first bands influences the total band count of the sample and final age estimation.

The influence of sample quality (with respect to readability code and edge confidence) on the differences between the band counting of FAS and the other readers was analyzed for specimens with more than 10 bands. No trend was observed with either the best or the worst quality samples. The differences in band counts occur for samples with an average readability code of around 2.6. These differences are mainly due to a different band count at the end of the ventral arm, with a higher band count in the inner part of the ventral arm compared to the outer part (**Figure 8**).

A survey on the use of each reading Zone within the otolith section was conducted for each reader (**Figure 9**). Results show that all readers mainly use Zone 2 of the ventral arm (agreed in the reading protocol). They use Zone 3 to corroborate or check the continuity of the opaque bands over the entire width of the ventral arm, especially in the first 3-4 first annual bands. Zone 1 is used to double-check when the bands are not seen very clearly in Zone 2 or to check the total number of bands, taking into account that in this Zone 1 the count is underestimated.

Five readers (PLL, RAL, DBU, SKA and FGA) follow the same reading pattern; using Zone 1 as double-checking of the number of bands in Zone 2; FGA corroborates the number of bands in Zone 1, especially in large specimens; PLL considers the number of bands counted in Zone 1 as the minimum number it expects to count in Zones 2 and 3; RAL uses the Zone 2 reading for the first 4-5 bands and tries to find continuity in Zone 3, thereafter he counts in Zone 2.

ABE and PAD use Zone 1 only to double check the other two Zones, Zone 2 for reading and measuring and Zone 3 as the main double-checking zone. ICG almost never uses Zone 1, only if it has a lot of doubts on the reading done in Zone 2. APL uses Zone 1 when Zone 2 is unclear when reading the last bands. ERM almost never uses Zone 3, except for the first annuli.

FAS uses all zones of the otolith section and uses Zone 2 to take the third reading when there is a disagreement of more than 1 band between the first and second readings.

In order to compare the age estimates obtained in the present and previous calibrations with the growth curves of both stocks, data from the experts mode (Mod_E) and FAS age estimates were fitted to the Von Bertalanffy equation and plotted together with growth functions from the eastern stock, Cort (1991) based on 1st dorsal fin spine reading and length frequency analysis, and from the western stock, Ailloud *et al.*, (2017) based on otolith readings and tagging. Using the growth models of both stocks at ages 0 to 20 years, which are the ages covered in both calibrations, and where there are practically no differences between the both stocks growth models, it is observed that the fit of the expert mode (Mod_E) is coincident with the growth functions of both stocks, while the fit of the FAS readings, diverges from 10 years and shows from this age onwards higher length-at-age values than those of the stock growth functions (**Figure 10**). These results are not entirely conclusive, since the fit to the length-at-age data is sensitive to the extreme values of the oldest specimens being well represented, which is not the case.

To try to determine which of the two readings is more appropriate, the strong 2003 cohort (ICCAT, 2018) was used to see which of the two readings would identify it better. For this purpose, the abundance per year class of the batch 1 and batch 2 specimens (4000 samples) read by FAS in GBYP Phases 7 and 9 was obtained. The abundance of these samples was also obtained by applying a correction vector from the Mod_E readings. The ageing bias vector was produced using data from present and previous calibrations. A vector of bias-corrected aged otoliths was created by taking the weighted average of the FAS band counts associated with each band count group of the corresponding Mod_E. This vector was applied only to otoliths with more than 10 bands (**Table 2**). The cohort tracking analysis showed that the FAS readings identify the 2004 cohort as the most abundant, while the Mod_E readings identify the 2003 and 2004 year classes as the most abundant, with the year classes shifting towards one year less. The year classes before and after the most abundant ones estimated by FAS and Mod_E also showed some abundance (**Figure 11**). These results indicate that Mod_E readings are more appropriate. We suggest applying this correction vector to otoliths read by FAS and with more than 10 annual bands, in order to incorporate the FAS readings into the GBYP age database.

Residual plots were made comparing the growth curves of both stocks predicted ages to readers age estimates. A bias in the residual plots for fish measuring more than 250 cm SFL was observed for all readers, including FAS. This bias is slightly more pronounced with the Richards model than with the Von Bertalanffy model (**Figure 12**, **Figure 13** and **Figure 14**). A similar divergence from the validated samples from Neilson and Campana (2008) vas found with a bias at older ages compared to the growth curves from Aillod *et al*. (2017), specifically with the Richards model (**Figure 15**).

Acknowledgements

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Table 1. Diagnosis of paired band counting agreement for all data (n = 200). Precision indices: CV = Coefficient of Variation, APE = Average Percent Error, Evans-Hoenig and Bowker symmetry tests, symmetry bias (*, ** = significant differences in one or both symmetry tests, p < 0.01), marginal edge type agreement with FAS (%) using three edge types (O, NT and WT) and two edge types (O, T), and mean readability score and mean edge type confidence by reader. Readers acronyms are explained in material and methods section.

Readers comparison	CV	APE	Evans-Hoenig (p)	Bowker (p)	Symmetry bias	Edge 3 type agreement with FAS (%)	Edge 2 type agreement with FAS (%)	Mean sample readability by reader	Mean edge type confidence by reader
FAS - ModE	5,8	4,1	0,0005	0,0921	*	49	74	2,9	2,5
FAS - ERM_L	6,5	4,6	0,0000	0,0098	* *	40	71	2,4	2,5
FAS - PLL_L	7,2	5,1	0,0025	0,1449	*	44	83	2,6	2,6
FAS - ABE_P	8,9	6,3	0,0053	0,0621	*	51	88	3,0	2,6
FAS - PAD_P	16,6	11,7	0,0000	0,0001	* *	49	79	2,9	2,4
FAS - DBU_P	7,1	5,0	0,0044	0,0859	*	45	74	2,6	2,4
FAS - SKA_P	11,5	8,1	0,0033	0,1292	*	21	49	3,1	2,4
FAS - RAL_P	6,8	4,8	0,0000	0,0429	*	50	79	3,0	2,2
FAS - FGA_P	7,5	5,3	0,0005	0,0624	*	47	88	3,0	2,5
FAS - ICG_P (non expert)	7,8	5,5	0,0127	0,1044		36	72	3,0	2,7
FAS - APL_P (non expert)	7,8	5,5	0,7137	0,4196		47	69	3,1	2,5

Table 2. Band counts from present and previous calibrations obtained from 423 otolith and resulting differences in bands counting between FAS and Mod_E. The ageing bias vector applied to otoliths with more than 10 bands appears framed (A.b.v.).

Band counting from Mod_E \ Band counting from otoliths aged by FAS	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	No Otol.	A.b.v.
0	0,00																	32	**0,00**
1		0,77	0,46															26	**1,23**
2		0,03	1,17	1,14														29	**2,34**
3			0,20	2,50	0,27													30	**2,97**
4					2,00	2,33	0,20											30	**4,53**
5					0,57	3,81	0,57											21	**4,95**
6						1,19	3,43	1,00	0,38									21	**6,00**
7						0,36	1,29	4,00	1,14									14	**6,79**
8								1,75	3,60	0,90	2,00							20	**8,25**
9								0,61	2,43	4,70	0,87							23	**8,61**
10									0,33	3,38	3,33	1,83	1,00					24	**9,88**
11								0,21	0,48	1,09	2,12	4,33	1,45	0,79				33	**10,48**
12										0,41	1,14	2,75	5,45	1,48	0,32			44	**11,55**
13											0,61	1,33	2,91	5,91	1,27	0,45		33	**12,48**
14												1,05	2,86	6,81	2,00			21	**12,71**
15											0,91		1,09	2,36	5,09	1,36	2,91	11	**13,73**
16														2,17	2,33	5,00	5,33	6	**14,83**
17															3,50	3,75	8,00	4	**15,25**
18																	16,00	1	**16,00**
No Otol.	32	21	26	36	20	36	18	19	22	29	29	34	40	36	13	5	7	423	

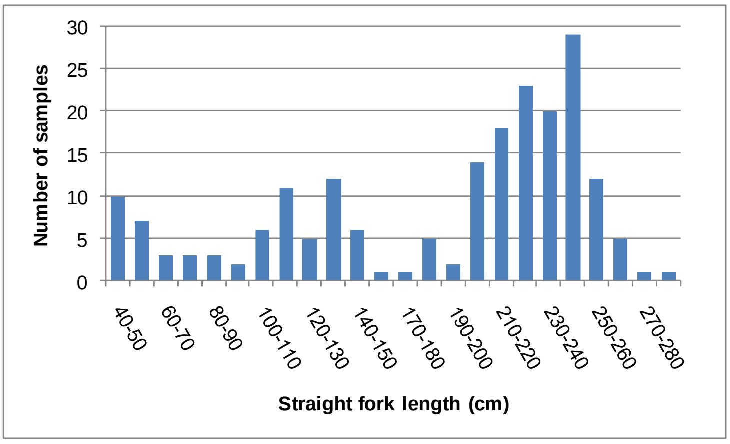


Figure 1. Length distribution of analyzed specimens by 10 cm size bin.

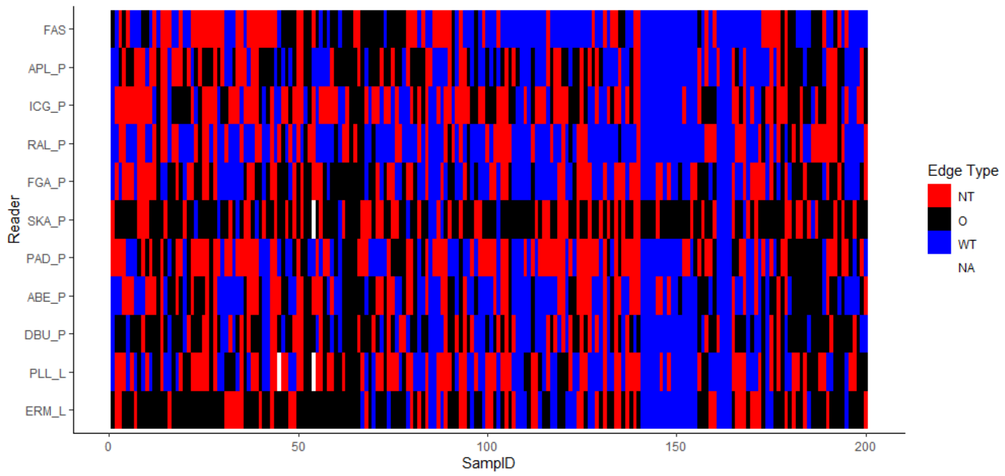


Figure 2. Tile plot showing otolith marginal edge type assignment (NT= narrow translucent, Opaque= O, WT= wide translucent, NA= missing data) by sample for each reader.

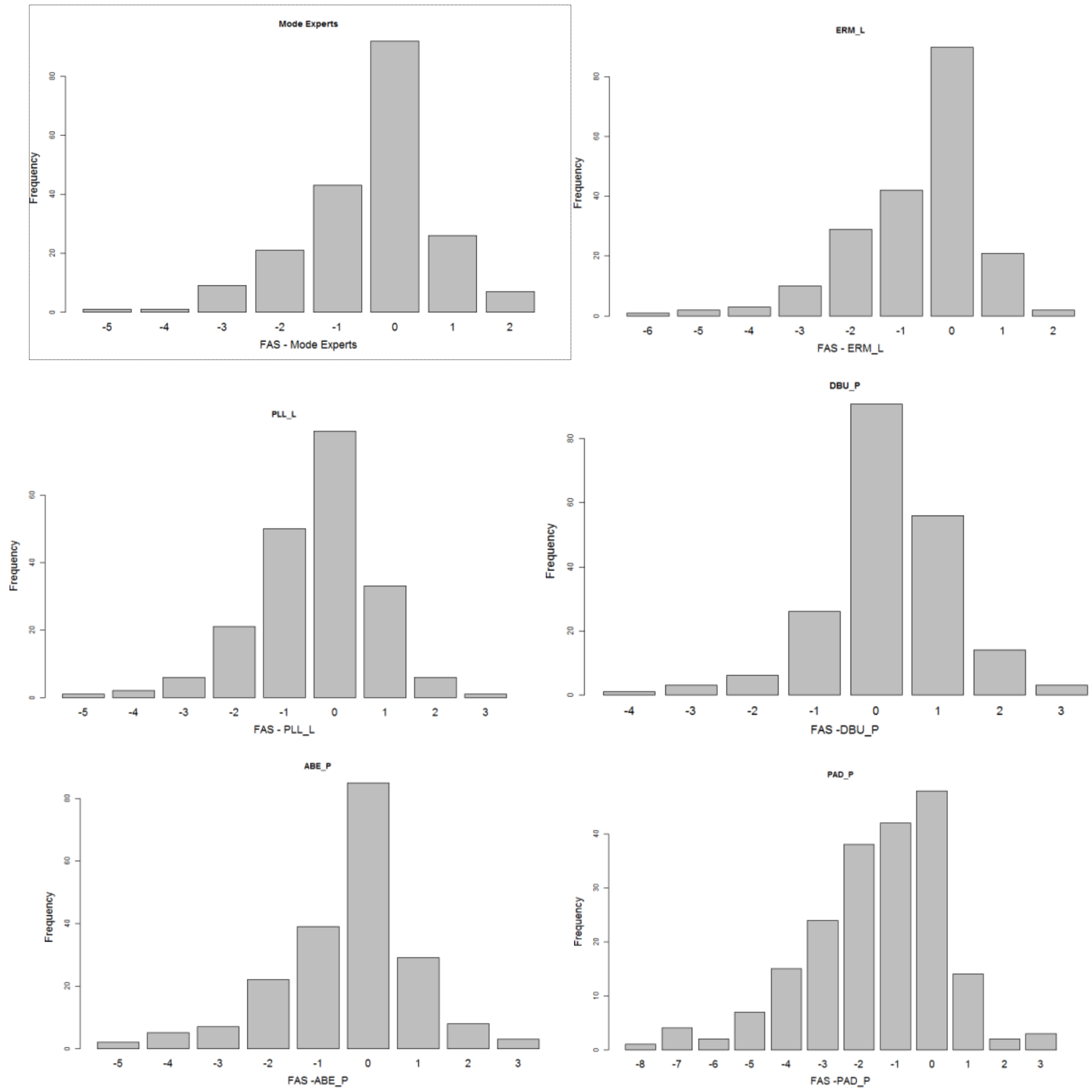


Figure 3. Band count difference distributions between FAS and experts mode (boxed figure), and between FAS and each reader.

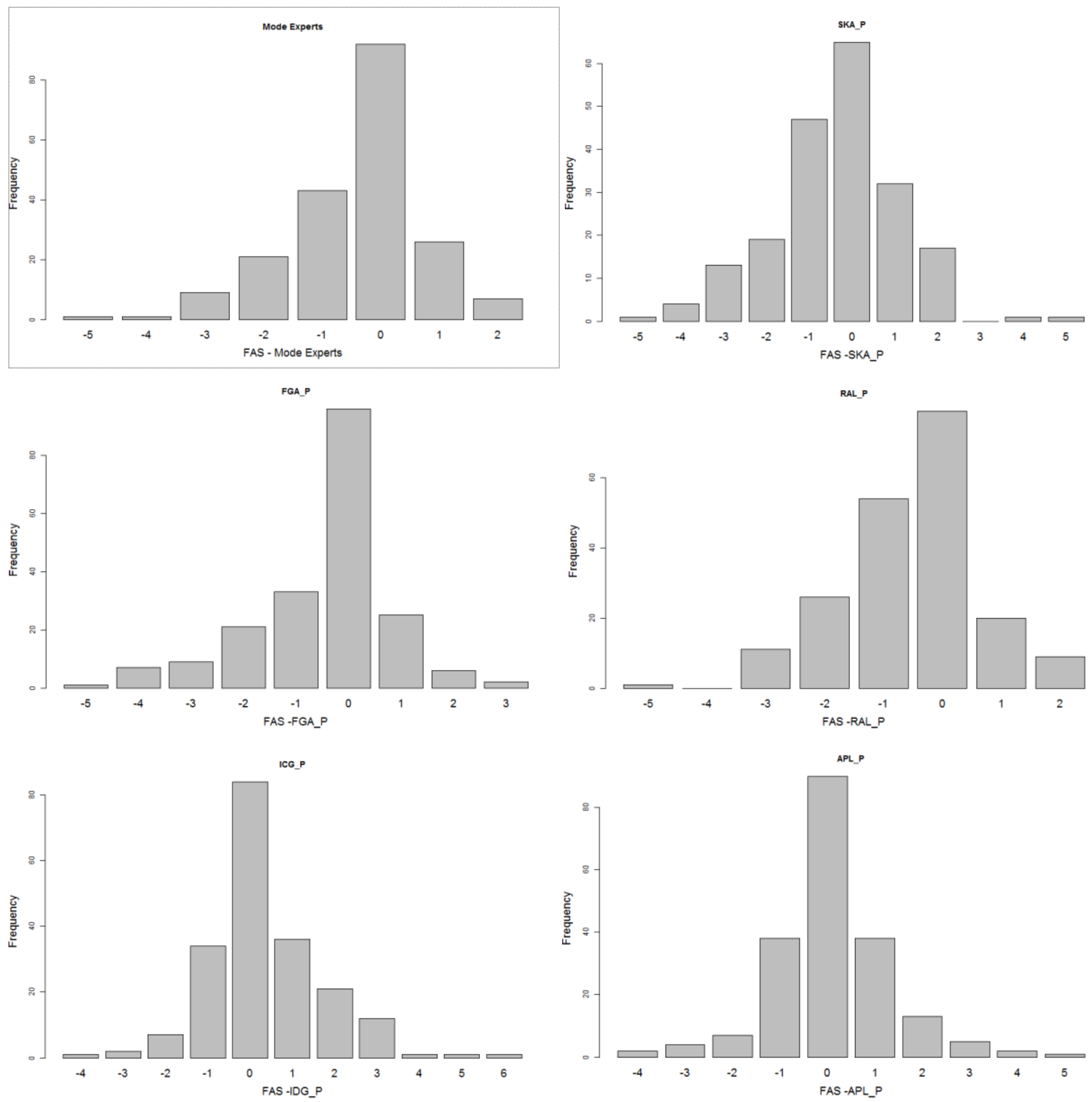


Figure 4. Band count difference distributions between FAS and experts mode (boxed figure), and between FAS and each reader. The last two figures at the bottom correspond to inexperienced readers.

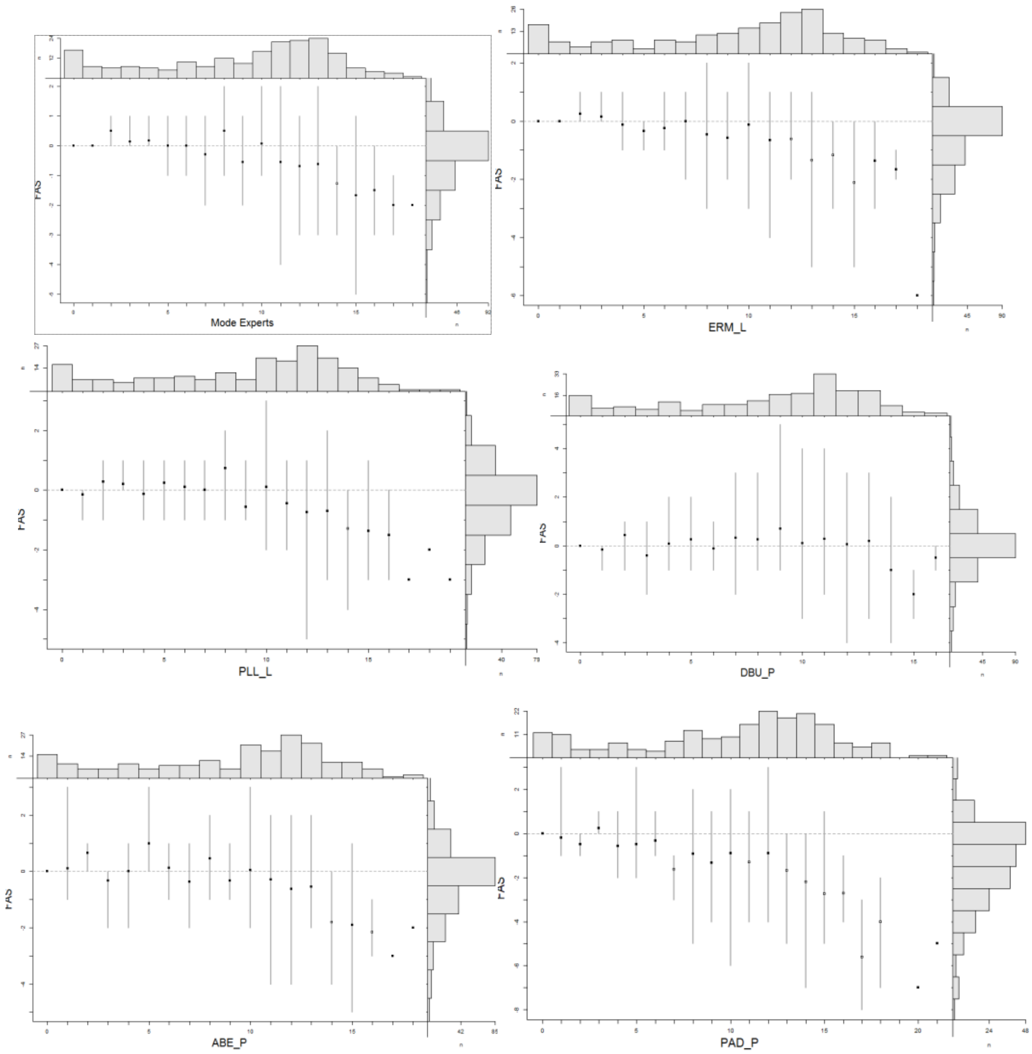


Figure 5. Band count bias graphs between FAS and each reader (experts mode in the boxed figure) (FAS band count minus reader band count). The number of samples per band count and band differences in the readings appears at the top and right side of each graph.

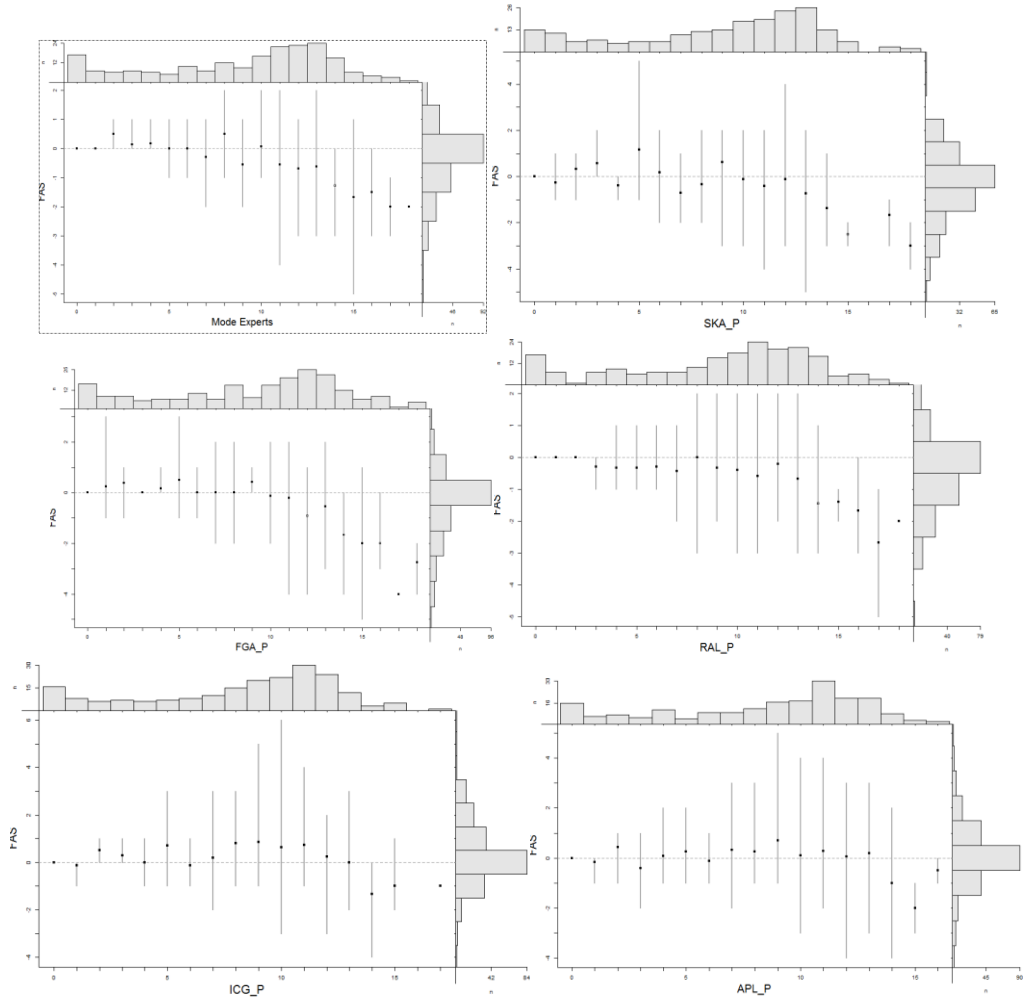


Figure 6. Band count bias graphs between FAS and each reader (experts mode in the boxed figure) (FAS band count minus reader band count). The number of samples per band count and band differences in the readings appears at the top and right side of each graph.

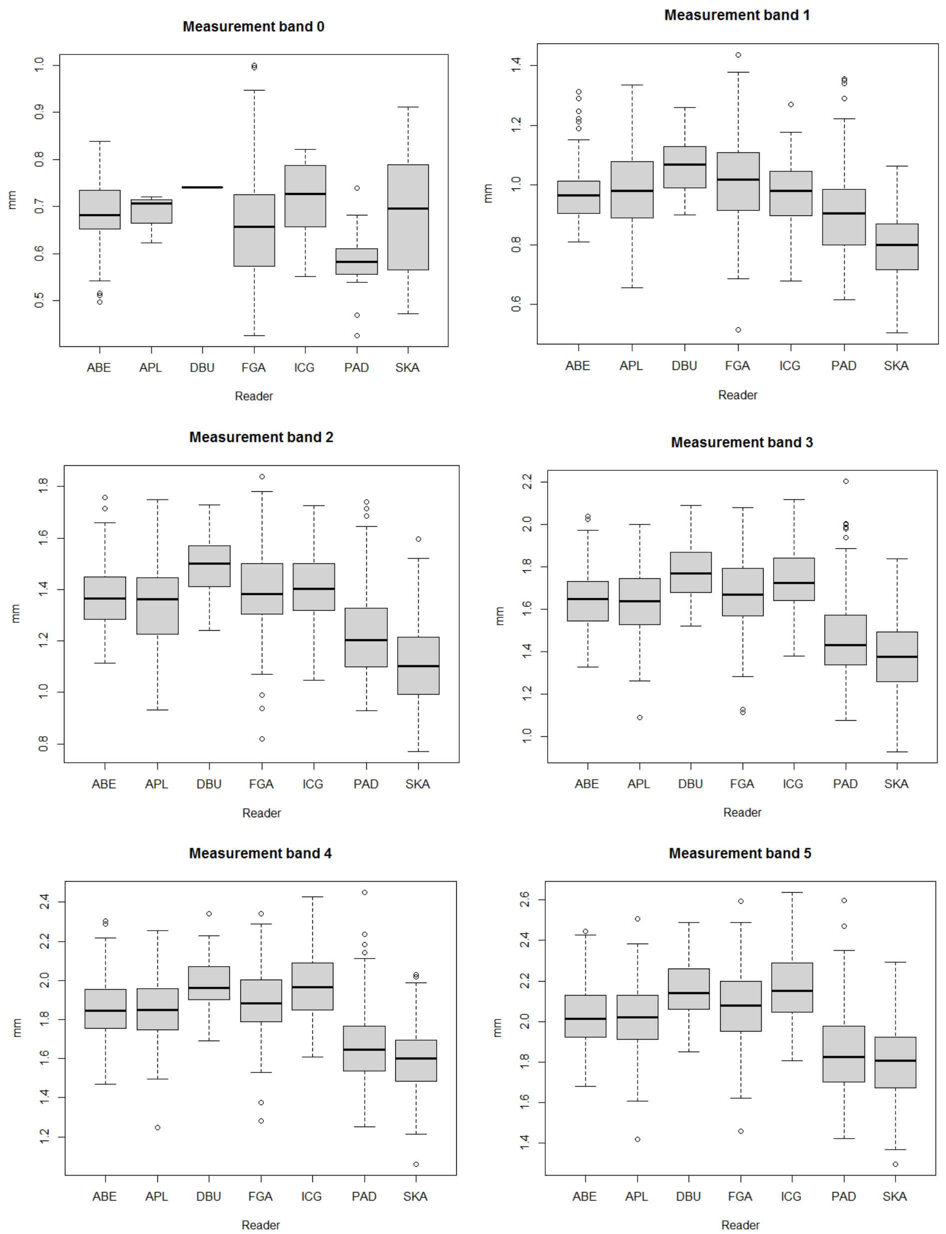


Figure 7. Box-plot of the measurements of the first five annual and innermost sub-annual bands by some readers.

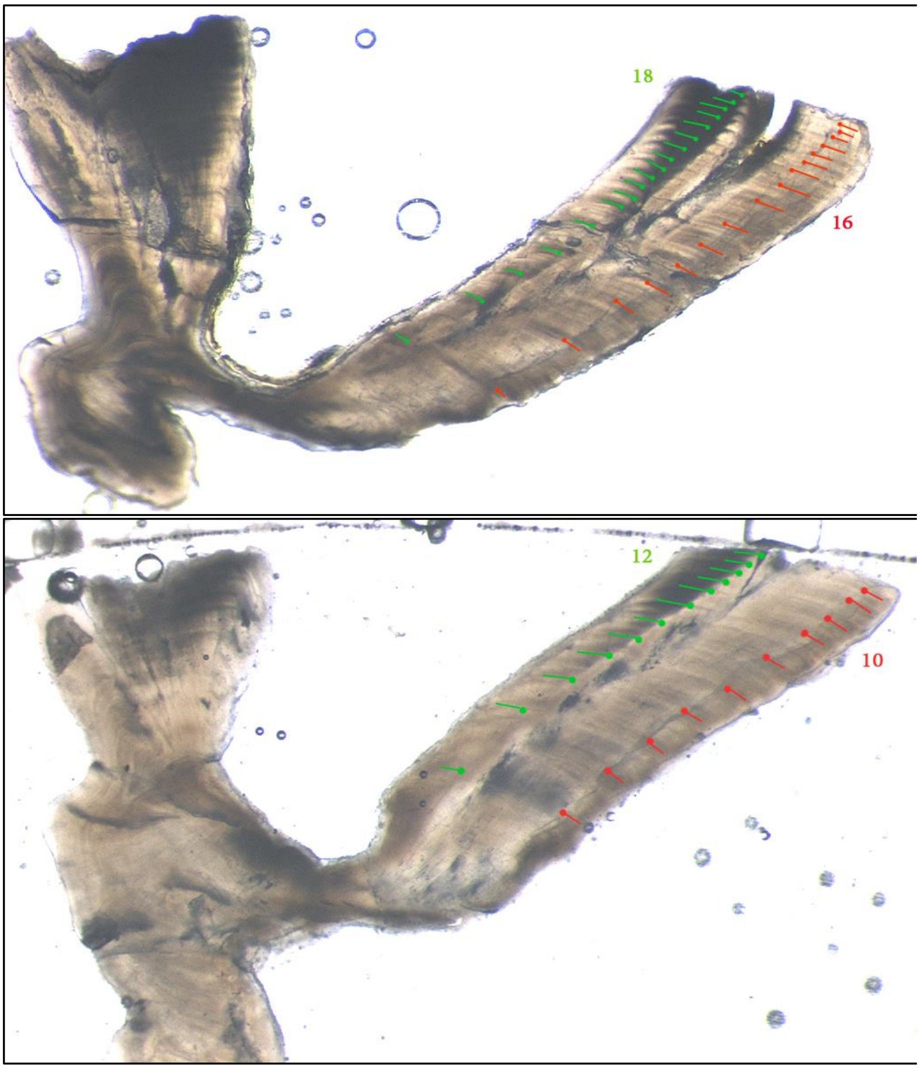
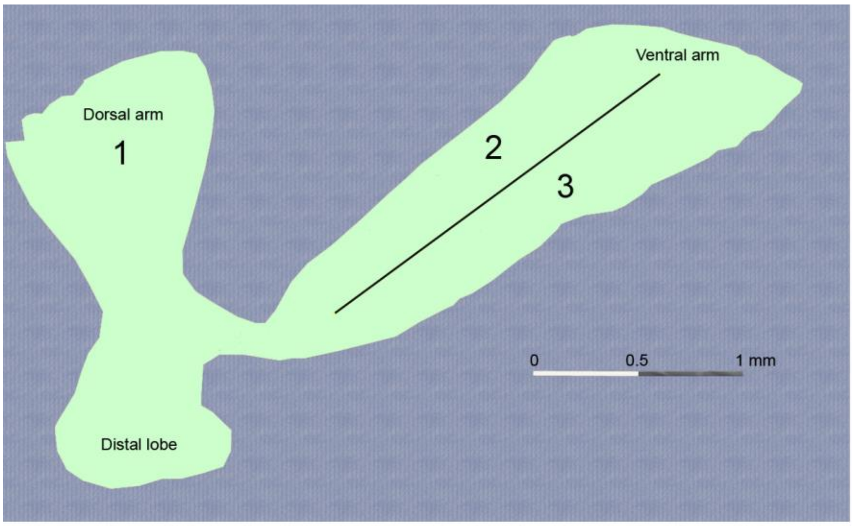


Figure 8. Images of bluefin tuna otolith sections with different band counts on the inner and outer part of the ventral arm.



	Use of each reading zone		
Reader	**Zone 1**	**Zone 2**	**Zone 3**
ERM_L	4	1	5
PLL_L	2	1	3
RAL_P	2	1	3
DBU_P	2	1	3
ABE_P	2	1	2
PAD_P	2	1	2
FGA_P	2	1	3
SKA_P	2	1	3
ICG_P	5	1	3
APL_P	4	1	3
FAS	1	1	1

1. Always. The main zone used for reading
2. To double check the band counting in zone 2
3. Very often to corroborate the nº bands throughout the ventral arm
4. Eventually. If bands are not clearly visible in zone 2
5. Hardly ever

Figure 9. Survey on the use of the different zones of an otolith section for band counting. Top: reading zones. Middle: survey results. Below: coding of the frequency of zones use.

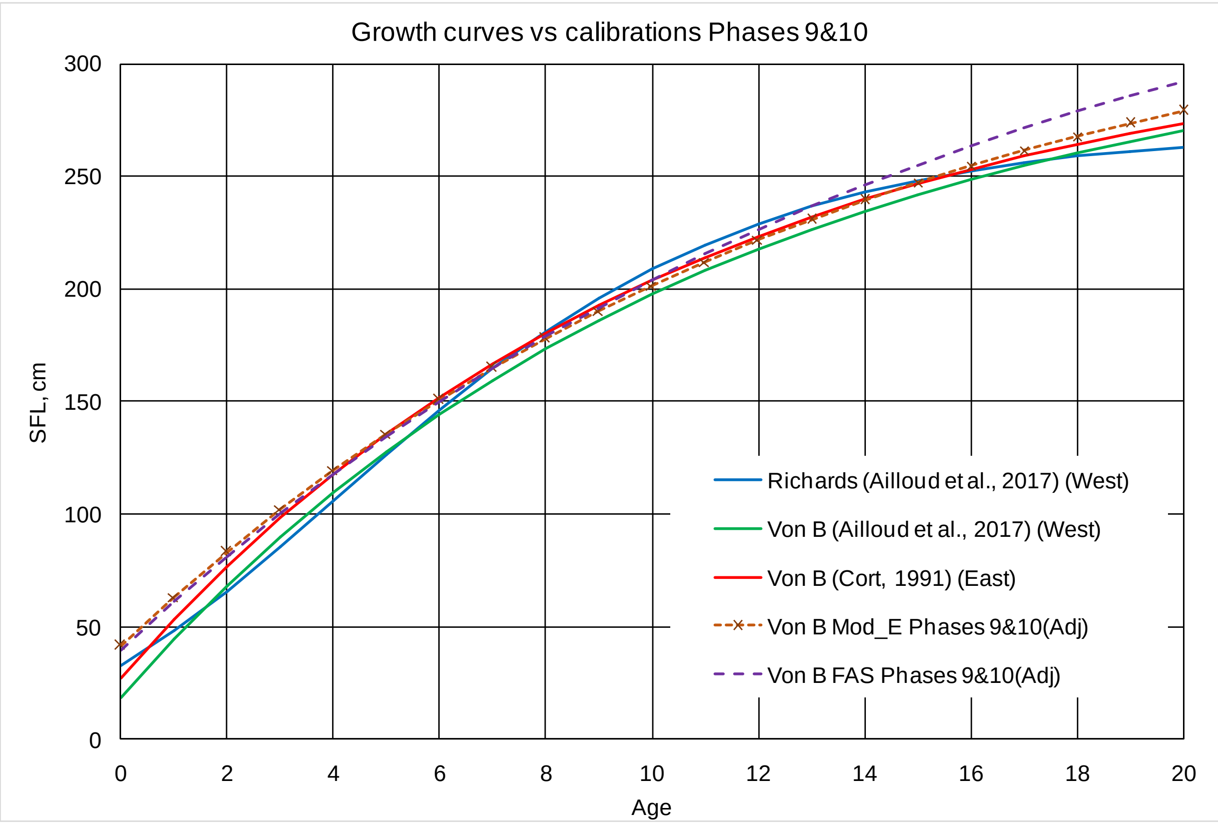


Figure 10. Comparison of age estimates obtained in the present and previous calibrations (GBYP Phases 9&10) with the growth curves of both stocks.

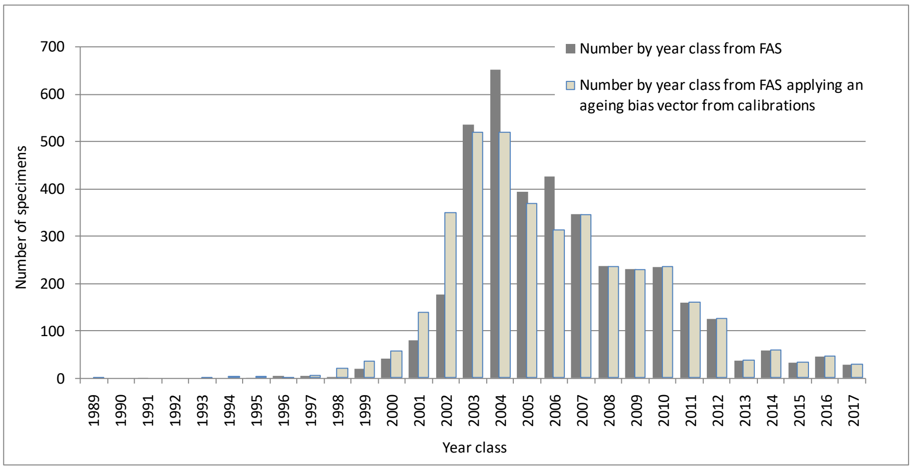


Figure 11. Abundance per year class of samples read by FAS and by FAS corrected by applying an ageing bias vector obtained from present and previous calibrations.

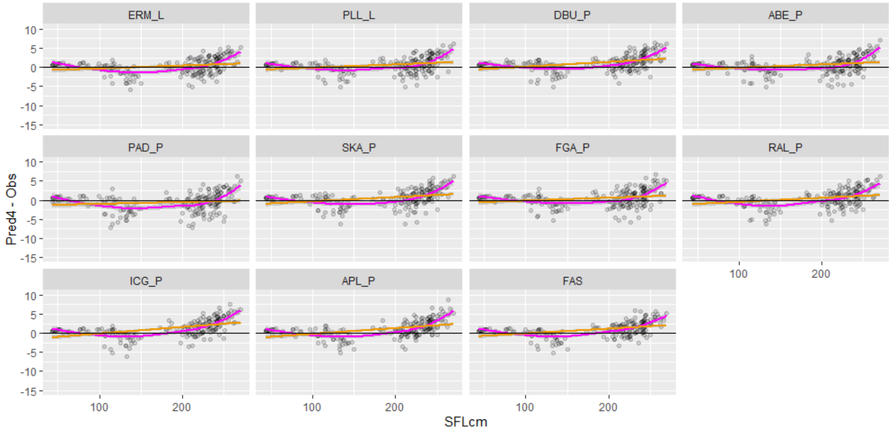


Figure 12. Residual plots comparing Cort *et al*. (1991) bluefin tuna Von Bertalanffy predicted ages to reader age estimates (residuals= predicted – observed). Orange lines represent linear regressions and purple lines represent smoothers (Loess) fit to the data using the geom_smooth function in ggplot2.

Figure 13. Residual plots comparing Ailloud *et al*. (2017) bluefin tuna Richards predicted ages to reader age estimates (residuals= predicted – observed). Orange lines represent linear regressions and purple lines represent smoothers (Loess) fit to the data using the geom_smooth function in ggplot2.

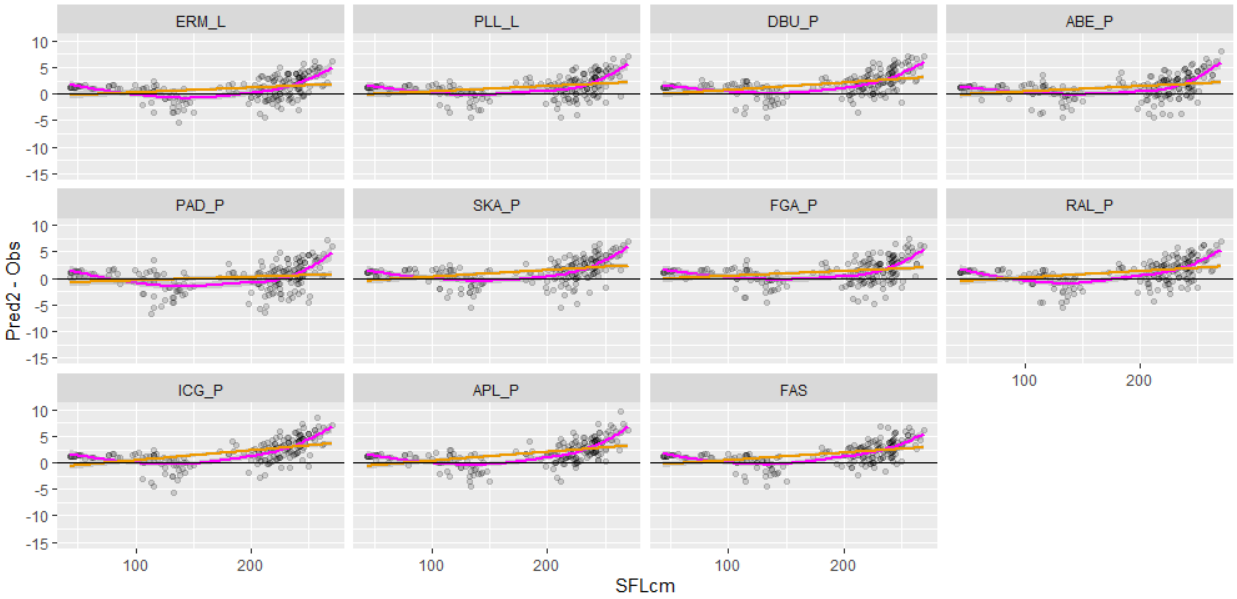


Figure 14. Residual plots comparing Ailloud *et al*. (2017) bluefin tuna Von Bertalanffypredicted ages to reader age estimates (residuals= predicted – observed). Orange lines represent linear regressions and purple lines represent smoothers (Loess) fit to the data using the geom_smooth function in ggplot2.

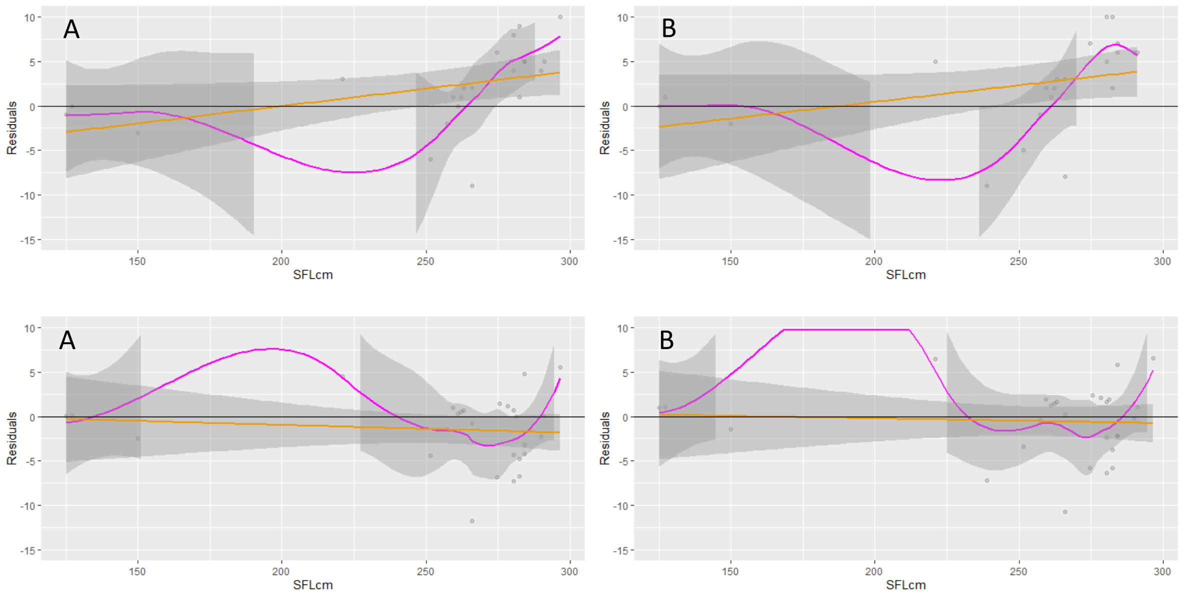


Figure 15. Residual plots comparing Ailloud *et al*. (2017) bluefin tuna Richards (top row) and Von Bertalanffy (bottom row) predicted ages to primary reader age estimates (A) and radiocarbon age estimates(B) (residuals= predicted – observed). Orange lines represent linear regressions and purple lines represent smoothers (Loess) fit to the data using the geom_smooth function in ggplot2.