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NEW PROTOCOL TO AVOID BIAS IN OTOLITH READINGS OF ATLANTIC BLUEFIN TUNA JUVENILES

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SUMMARY

Identification of the first annual increments in otoliths of Atlantic bluefin tuna juveniles is difficult due to the frequent appearance of numerous sub-annual bands which can be easily misinterpreted. Instead, the pattern of deposition of the annuli on the first dorsal fin rays (spines) is very distinct during the first years. Furthermore, the bias vector for correcting otoliths age, and developed from paired otolith-spine samples, allowed a better tracking of cohorts in the 2017 assessment. With this background, 262 paired structures with identical age estimates were used to revise reading criteria for annual increments of juvenile bluefin otoliths and to create a template. A table with first annuli measurements was built to serve as a reference and help identifying the first otolith annual bands. For this, it was necessary to establish the location of origin of the measurements (anchor point) and define them. In addition, otoliths from this set of paired structures can be used as a collection that will serve to test the new reading criteria and template.

RÉSUMÉ

Il est difficile d'identifier les premiers incréments annuels dans les otolithes de juvéniles de thon rouge de l'Atlantique en raison de l'apparition fréquente de nombreuses bandes infra-annuelles qui peuvent facilement être mal interprétées. Au contraire, le schéma de dépôt des anneaux sur les premiers rayons de la nageoire dorsale (épines) varie très fortement pendant les premières années. En outre, le vecteur de biais pour corriger l'âge des otolithes, développé à partir d'échantillons appariés d'épine-otolithe, a permis de mieux suivre les cohortes dans l'évaluation de 2017. Dans ce contexte, 262 structures appariées avec des estimations d'âge identiques ont été utilisées pour réviser les critères de lecture des incréments annuels d'otolithes de thons rouges juvéniles et pour créer un modèle. Un tableau incluant les premières mesures des anneaux a été élaboré pour servir de référence et aider à identifier les premières bandes annuelles d'otolithes. Pour ce faire, il a été nécessaire d'établir le lieu d'origine des mesures (point d'ancrage) et de les définir. De plus, les otolithes de ce jeu de structures appariées peuvent être utilisés comme une collection qui servira à tester les nouveaux critères de lecture et le modèle.

RESUMEN

La identificación de los primeros incrementos anuales en el otolito de juveniles de atún rojo del Atlántico, es difícil debido a la frecuente aparición de numerosas bandas sub-anuales que pueden malinterpretarse fácilmente. En cambio, el patrón de deposición de los anillos en el primer radio de la aleta dorsal (espina) es muy nítido durante los primeros años. Además, el vector de tendencia para corregir la edad de los otolitos, desarrollado a partir de muestras pareadas de otolitos y espinas, permitió un mejor seguimiento de las cohortes en la evaluación de 2017. Con estos antecedentes, se usaron 262 estructuras pareadas con estimaciones de edad idénticas para revisar los criterios de lectura de los incrementos anuales y para crear una plantilla. Se construyó una tabla de referencia con las primeras medidas de los anillos para ayudar a identificar las primeras bandas anuales. Fue necesario establecer la ubicación de origen de las mediciones y definirlas. Además, los otolitos de este conjunto de estructuras pareadas se pueden usar como una colección que servirá para valorar los nuevos criterios de lectura y la plantilla.

KEYWORDS

Age estimation, Reading criteria, Otolith, spine, Thunnus thynnus

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1. Introduction

Otoliths of Atlantic bluefin tuna (*Thunnus thynnus*) are the best calcified structure to estimate the age of this species throughout its long life cycle. However, age estimation using this structure is problematic as the identification of the first annual increments is difficult due to the frequent appearance of numerous sub-annual bands (Rodriguez-Marin *et al.* 2007; Busawon *et al.* 2015). In the area close to the core region of the ventral arm, the first 5 annuli are broad and diffused making identification difficult, while in later areas bands are more distinct. This difficulty in reading the otolith sections has also been described for other species of the same genus such as *T. maccoyii* and *T. orientalis* (Clear *et al*. 2002; Shimose *et al.* 2009).

An opaque sub-annual mark or false first annulus occurs frequently on bluefin tuna otoliths and can be easily misinterpreted as the first annual band. The identification of first annual increment has been assisted by a reference scale (Busawon *et al*. 2015); however, the remaining annual increments in the first area of the ventral arm are still difficult to interpret. In contrast, the first annuli are comparatively easy to identify in fin spine sections, so it might be possible to obtain more robust age assignments by using both calcified structures coming from the same fish (Anon. 2014).

The estimation of absolute age by reading otoliths has been validated by the bomb radiocarbon method (Neilson and Campana 2008) and the periodicity of the formation of the annual increments by measuring otolith strontium: calcium ratio (Siskey *et al.* 2016). In contrast, growth increments of spine sections have only been indirectly validated by cohort tracking, marginal increment and edge type analysis for juveniles and young adults (Rodriguez-Marin *et al.* 2009; Luque *et al.* 2014). The use of the spines to estimate the age of bluefin is affected by the disappearance of the early growth marks, which are replaced by vascular tissue as the fish grows (Santamaria 2009; Luque *et al.* 2014). However, the pattern of deposition of the annuli on the spines is very distinct during the first years. Furthermore, it is easy to distinguish the formation of the translucent and opaque bands at the edge of the spine throughout the year, while in the otoliths the pattern of deposition of both types of bands at the edge of the section is hardly distinguishable (Rodriguez-Marin *et al*. 2014; 2016b; Busawon *et al.* 2015).

When comparing otolith and fin spine readings from the same bluefin, the agreement between otolith and spine age estimates is good for bluefin tuna younger than 14 years old with less than one year difference (Rodriguez-Marin *et al.* 2016a). But by analyzing this comparison data in more detail, we have found that there is a significant overestimation of age from otolith readings at the first ages (group of ages 1 to 7, chi-squared test $p < 0.001$) and that these differences are no longer significant in the 8 to 14 years group (**Figure 1**). This is because the difference of a year in the reading of a 5-year-old specimen is not the same as in a 13-year-old tuna. However, although the latter difference is not significant, it may imply that the 13-year-old is assigned to an erroneous year class.

Analysis of the ageing data available at the 2017 assessment meeting showed that mean size at age of spine samples was in general larger than the mean size at age in the otolith samples, these differences being greater with western otoliths (**Figure 2**) (Anon. 2017). This difference could be due to an overestimation of the age in the otolith readings and would explain why when the otolith-based age-length key is used to estimate the catch at age for the western stock, the strong cohort of 2003 is displaced in one year and assigned to the 2002 year class (**Figure 3**) (Anon. 2017). Introducing a vector of bias corrected aged otoliths using data from paired otolith-spine samples and assuming spine readings are correct for fish up to age 7, the Stock Synthesis model was able to sharpen the estimate of the 2003 cohort rather than blurring it between 2002 and 2003 (Anon. 2017). It is therefore important that the age allocation of the first annuli is correct so that there are no errors in the ageing of otoliths.

The purpose of this protocol is to determine if the use of spines to identify growth bands in the otoliths of juvenile Atlantic bluefin tuna will lead to more robust age assignments. Spines and otoliths from the same specimen will be used and the assumption will be that the spine estimated age is correct up to 8 years. Paired structures with identical age estimates will be used to create a template and establish criteria for reading annual increments of juvenile bluefin otoliths. In addition, otoliths from this set of paired structures will constitute a collection that will serve to test the new criteria and template.

2. Material and methods

A total of 262 calcified structures, otoliths and dorsal fin rays (spines) from the same bluefin tuna were used. The straight fork length (SFL) of these specimens was 50 to 200 cm, the majority being 50 to 140 cm SFL (**Figure 4**). Specimens were captured in the East Atlantic management area, including the Mediterranean. Both structures were prepared and read following the protocol described in Busawon *et al.* (2015) and Luque *et al.* (2014).

Otolith opaque bands were counted and only Y-type sections were used. Otoliths were read once by two expert readers. Samples with low confidence on age estimates were read by a second reader and determined a final consensus increment count. Readings were conducted with reference to the aged spine from the same specimen. The age interpretation of both structures was carried out using digitally enhanced images.

When it comes to counting the annual increments in the otolith sections, it has been necessary to adopt the following assumptions in view of the difficulty in interpreting the edge type throughout the year:

1. The opaque band of the annual bipartite structure is formed in winter (Siskey *et al.* 2016), but is shown or appreciated later at the edge of the section, about 6 months later.

2. The edge of the otolith section is considered translucent or opaque if the aspect is 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%.

3. The edge is considered of one type or another (translucent or opaque) when it is a clear band (around 0.01 mm width) and will not be considered if it appears just as a thin line.

Measurements of annual increments in otoliths

Annuli (annual increments) need to be measured using a standardized procedure. There is a consensus measurement for otolith reading that uses a reference scale for the allocation of the first annual increment (Busawon *et al.* 2015). This measurement is taken from the bottom centre of the bridge between the two arms and extends up the inner ventral arm to the end of the first *translucent* band at the "sulcus accusticus" margin (Secor *et al.* 2014) (**Figure 5**). We propose two additional measurements based on the current one and a new one to help in the allocation of the first five annual increments. The new measurements have a clearly defined anchoring point and better describe the location of annuli.

Anchor point. To locate the anchor point three lines are drawn: a first line parallel to the upper part of the bridge between the ventral and dorsal arms; a second line parallel to the lower edge of the bridge between the two arms; and a third line that bisects the first line, and which is perpendicular to the second line. The point where the third line intersects the second is the anchor point (**Figure 6**).

- *Annual increment measurement (Ai)*. It is the distance from the anchor point to the center of the width of the *opaque* band (or where it is most marked, but not on the upper or lower edge of the opaque band), measured in the upper area of the ventral groove of the ventral arm (**Figure 7**).

The area of the ventral groove is usually where bands are clearer and it is preferable to the "sulcus acusticus" border of the ventral arm because the convexity of bands in this border can produce unrealistic distances between annuli.

- *Reference scale measurement (Rs)*. It is the distance from the anchor point to the center of the width of the *opaque* band (or where it is most marked, but not on the upper or lower edge of the opaque band), measured at the sulcus acusticus border of the ventral arm (**Figure 7**).

This measurement was chosen to compare it with the reference scale used in assigning the first annual increment which was established by measuring the distance to the end of translucent band instead of to the middle of the opaque one. This measurement was only used to measure the false and first annual increments.

- *1st inflection measurement (1sti)*. It is the distance from the first inflection to the center of the width of the *opaque* band (or where it is most marked, but not on the upper or lower edge of the opaque band), measured at the external margin of the ventral arm (**Figure 7**). As the starting point of this measurement can be subjective, it was only used to establish a ratio of distances for the false and first annual increments.

The annual increment measurements of the first 5 annuli were compared among otoliths of different ages in order to determine if the size of each annulus varies with the age of the specimen. The Shapiro–Wilk test was used to test the normality of measurements distribution by each annulus and age. A one-way analysis of variance (ANOVA) was used to detect significant differences among the size of the first five annuli in otoliths at different ages (Penha *et al.* 2004; Quelle *et al.* 2014).

3. Results and Discussion

Results of measurements

The measurements of the first 5 annual increments and the sub-annual first one, according to the different types of measurements described above, are shown in **Table 1**. This table includes the mean and standard deviation (SD) of the annuli by age. Annuli measurements, SD and the reference template are shown in **Figure 8**. This template layer was developed as a reading aid for otolith section photos (**Figure 8** bottom).

The frequency distribution and boxplot of the first five annual increment measurements of each presumed annulus are represented in **Figure 9 and Figure 10**. The ANOVA did not detect significant difference in the size/location of the first five annuli among age groups (**Figure 11**), which increases confidence in the ageing procedure.

Reading criteria

These criteria (highlighted in cursive) are simply a guide that seeks to complement the criteria defined by Busawon *et al.* (2015), whose instructions are also included (normal font).

- *Ageing of Atlantic bluefin tuna*

- Prior to production ageing, readers should read the reference set one time single blind under their preferred light type. 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.

- Identification of annuli on otolith section.
  - View sections (or images) under either transmitted or reflected light.
  - Annuli are a bipartite structure consisting of a translucent and opaque zone.
  - Count opaque growth zones. These appear dark under transmitted light and white under reflected light.
  - The ventral (long) arm is used for age estimates. The dorsal (short) arm can be used as check; however it important to remember that the dorsal arm tends to underestimate age.
  - There are no annual growth increments between the "primordium" and the 1st inflection.
  - Start reading at the "primordium" and proceed towards the edge of the ventral arm. It is recommended to count annual increments at the upper part of the ventral groove (**Figure 5**).
  - 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). *These annual growth zones are broad and contain multiple sub-annual translucent and opaque 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 (grooves along the margins) may aid in the identification of growth zones. The distance between the first five annuli is greater than in the rest.*
    - 2nd region: Annuli are less broad and closer together (~5-10 annuli). *From the fifth, the distance between annuli decreases and from the eighth or ninth annual deposition, and especially after the second inflection, the deposition of the annual bands is distinct and regular.*
    - 3rd region: Annuli appear clearer and are regular in width (~ 10+ annuli).

- *The section of the ventral arm between the first and second inflection is difficult to interpret. The first 10 annual bands are located in this section and sub-annual marks are frequently observed. Here are some guidelines to help interpretation*:

    - *The appearance of a false sub-annual increment or annulus 0, which can even present crenulation, 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.*

    - Use the reference scale as a guide to identify the first annulus. *The first annual deposition should be within this distance of 1 mm, measured as annual increment measurement.*

    - *The second annulus is about one third the distance of the first one (annual increment measurement). The recognition of the first two annuli is important to establish the deposition pattern of the first 5 annual bands, including the gradual decrease of the distance between them (**Figure 8**).*

    - *A template has been developed to help locate the first five annuli.*

- Edge type assignment. *Follow these guidelines to help identify the type of edge of the ventral arm (edge of the last annulus), opaque or translucent, since it is difficult to determine and therefore, whether it is counted or not*.

    - View both the enhanced and un-enhanced version of the image. *Keep in mind that the thickness of the section and the diffraction of light can influence the perception of the type of edge.*

    - Edge type should only be noted if more than 50% of the edge is visible 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 edge is considered of one type or another (translucent or opaque) when it is a clear band (around 0.01 mm width) and will not be considered if it appears just as a thin line.*

    - *The opaque band of the annual bipartite structure is formed in winter (Siskey et al. 2016), but is shown or appreciated later at the edge of the section, about 6 months later.*

- Converting annuli counts to Age estimates.

- *The adopted rule for otoliths is that when counting opaque bands: if the fish is caught between January 1 and June 1, then 1 year is added to the age. When counting translucent bands: if the fish is caught between June 1 and 31 of December, then 1 year is subtracted to the age (Rodriguez-Marin et al. 2016b).*

This protocol needs to be tested and agreed among the different laboratories which perform direct otolith ageing of Atlantic bluefin tuna. For this purpose, the collection of otolith images used in this study may be useful.

Acknowledgements

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Table 1. Measurements of false (0) and first five annuli (1 to 5) according to Annual increment measurement (Ai), Reference scale measurement (Rs) and 1st inflection measurement (1sti).

	Annual increment measurement (mm)						Reference scale measurement (mm)		1st inflection measurement (mm)	
	Ai 0	Ai 1	Ai 2	Ai 3	Ai 4	Ai 5	Rs 0	Rs 1	1sti 0	1sti 1
Aritmetic Mean	**0.84**	**1.13**	**1.54**	**1.81**	**2.02**	**2.17**	**0.60**	**0.85**	**0.30**	**0.62**
Number	54	129	99	64	46	30	33	88	33	88
SD	0.08	0.08	0.10	0.11	0.12	0.14	0.07	0.12	0.12	0.13
Mean - SD	0.76	1.05	1.44	1.70	1.91	2.03	0.53	0.74	0.18	0.49
Mean + SD	0.91	1.21	1.64	1.93	2.14	2.31	0.67	0.97	0.41	0.76

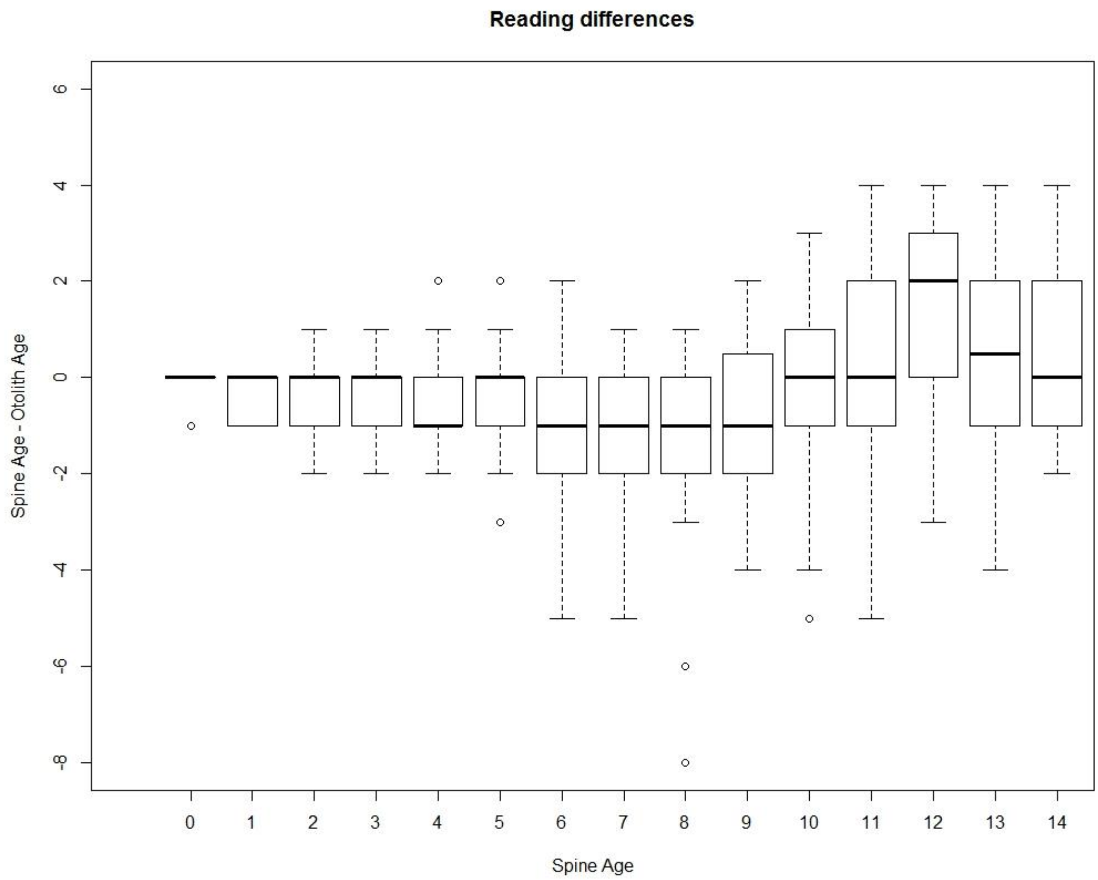

Figure 1. Boxplot indicating differences in age estimates from otoliths and spines collected from the same fish (n=503). Data from Rodriguez-Marin *et al.* (2016a).

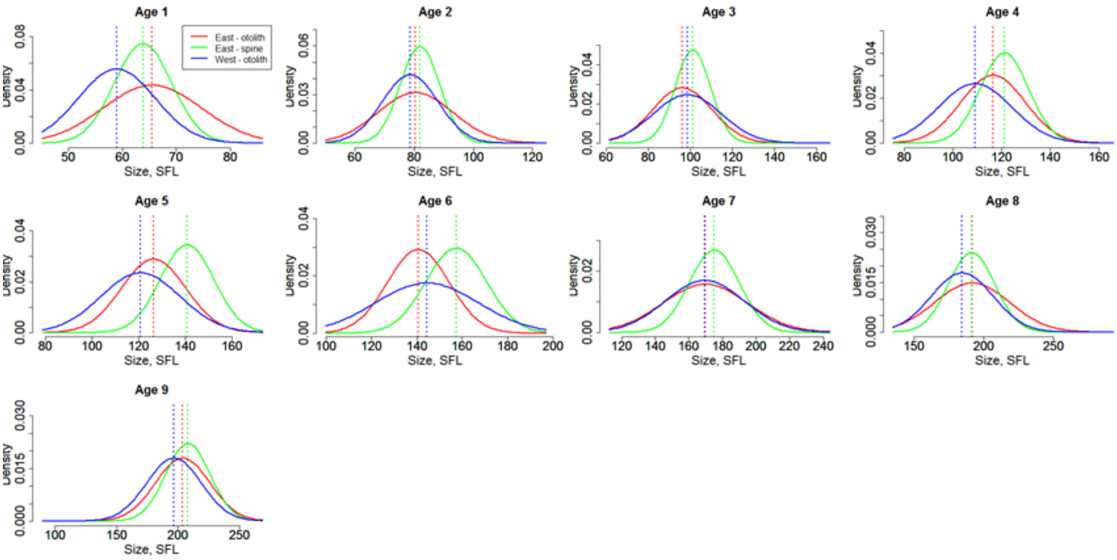

Figure 2. Normal distributions fitted to the eastern and western otolith and spine samples used in the combined forward inverse age-length key. From Anon. (2017).

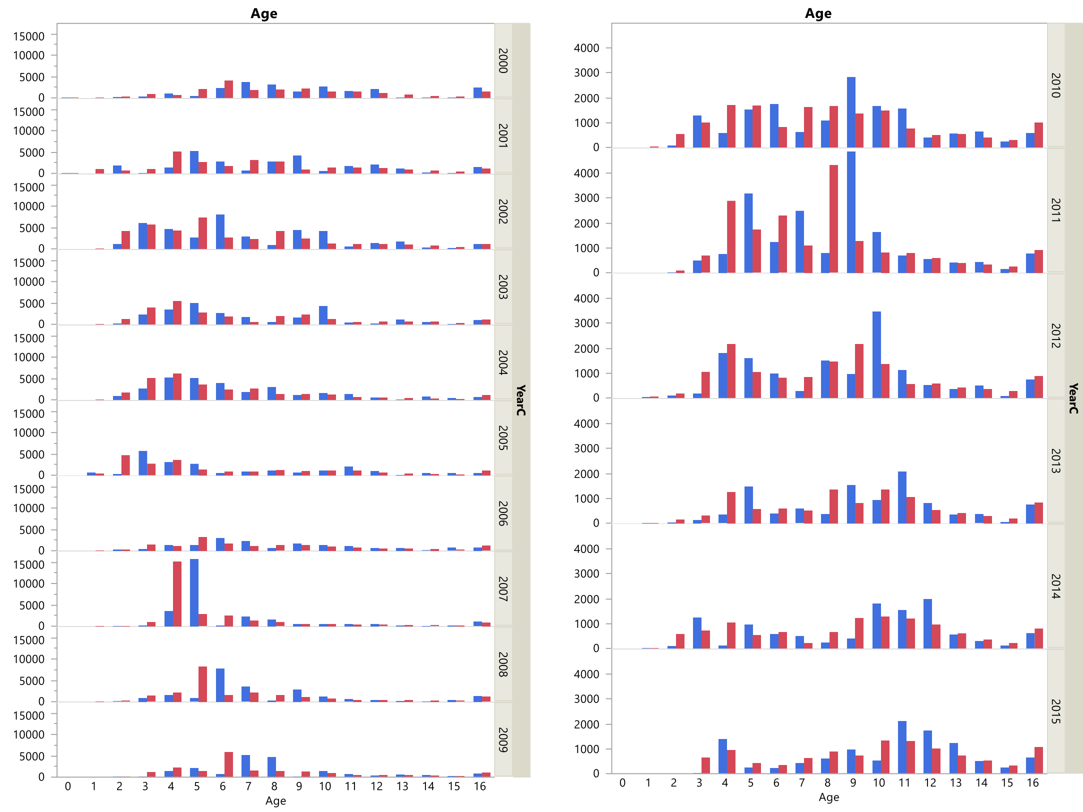

Figure 3. Histograms comparing the catch at age estimates from cohort slicing (red) and the combined forward inverse age-length key (blue) for western bluefin tuna stock. From Anon. (2017).

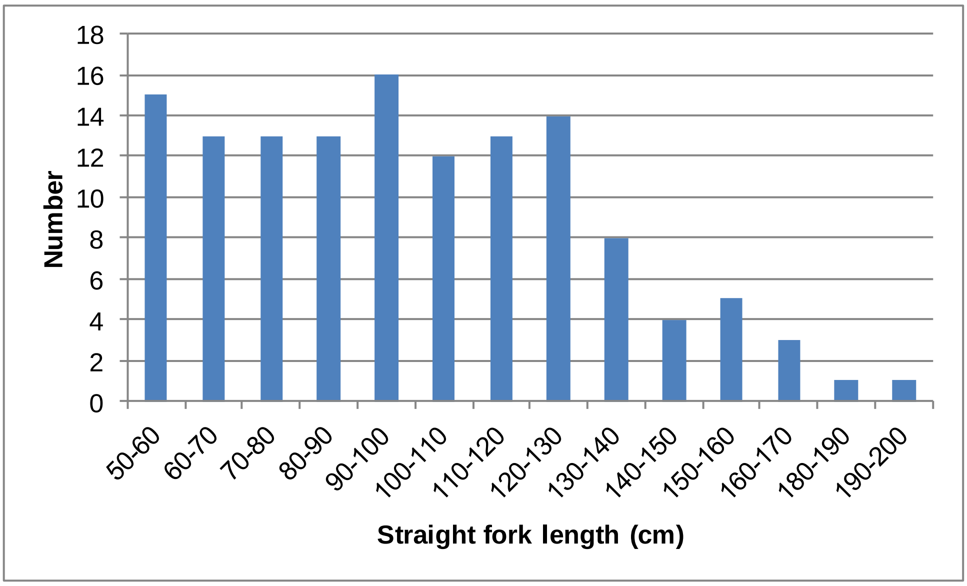

Figure 4. Size distribution of the bluefin tuna used for this study.

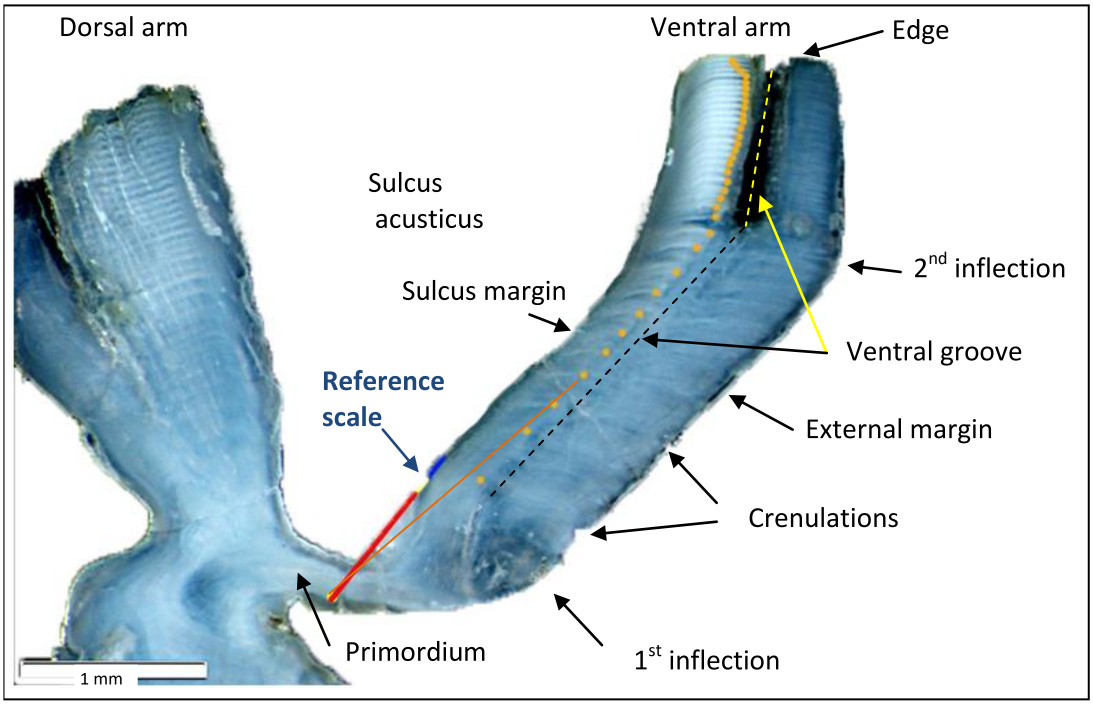

Figure 5. Otolith transverse section (through the primordium) of an Atlantic bluefin tuna. The red-yellow and blue "yardstick" is used as a reference scale to help assigning first annual increment.

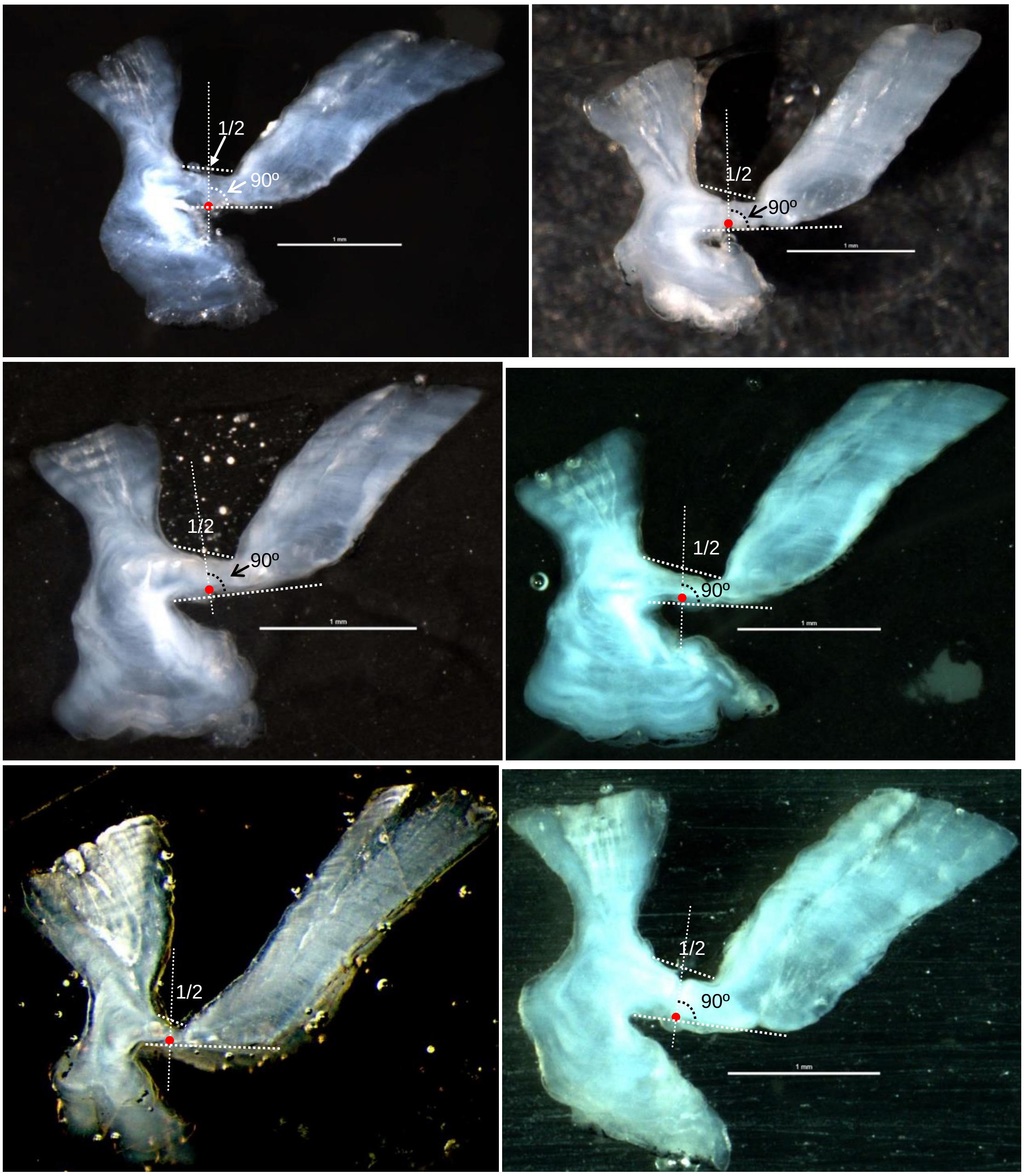

Figure 6. Several images showing how to locate the anchor point according to the shape of the otolith.

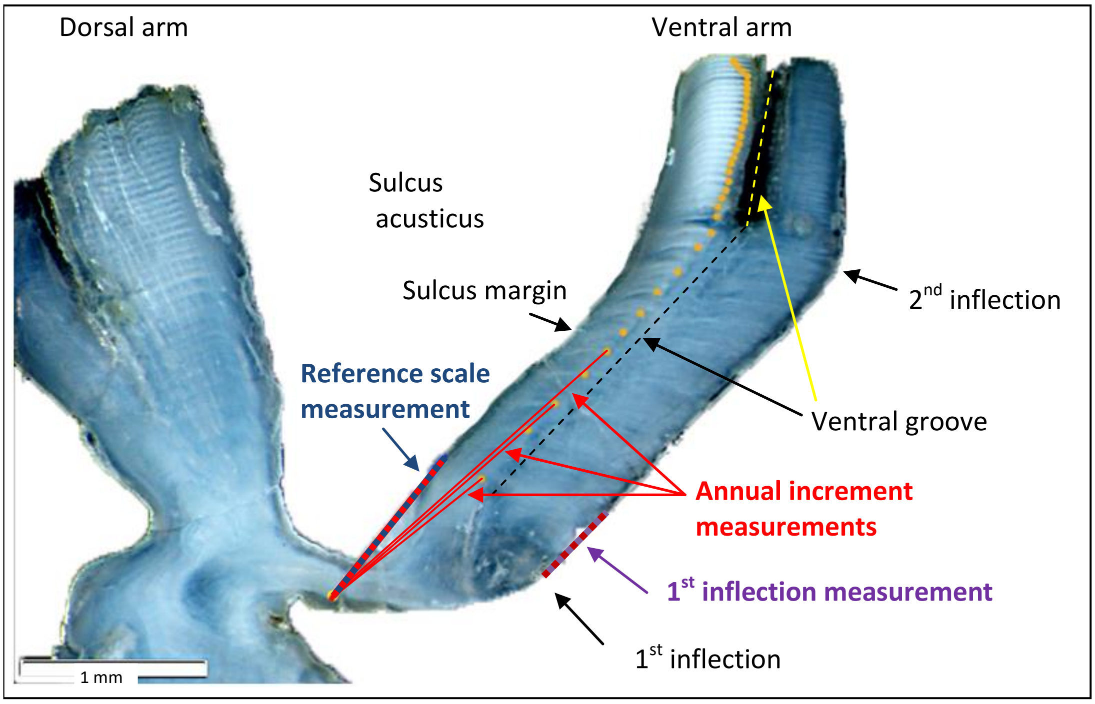

Figure 7. Otolith transverse section (through the primordium) of an Atlantic bluefin tuna. The different length measurements are shown.

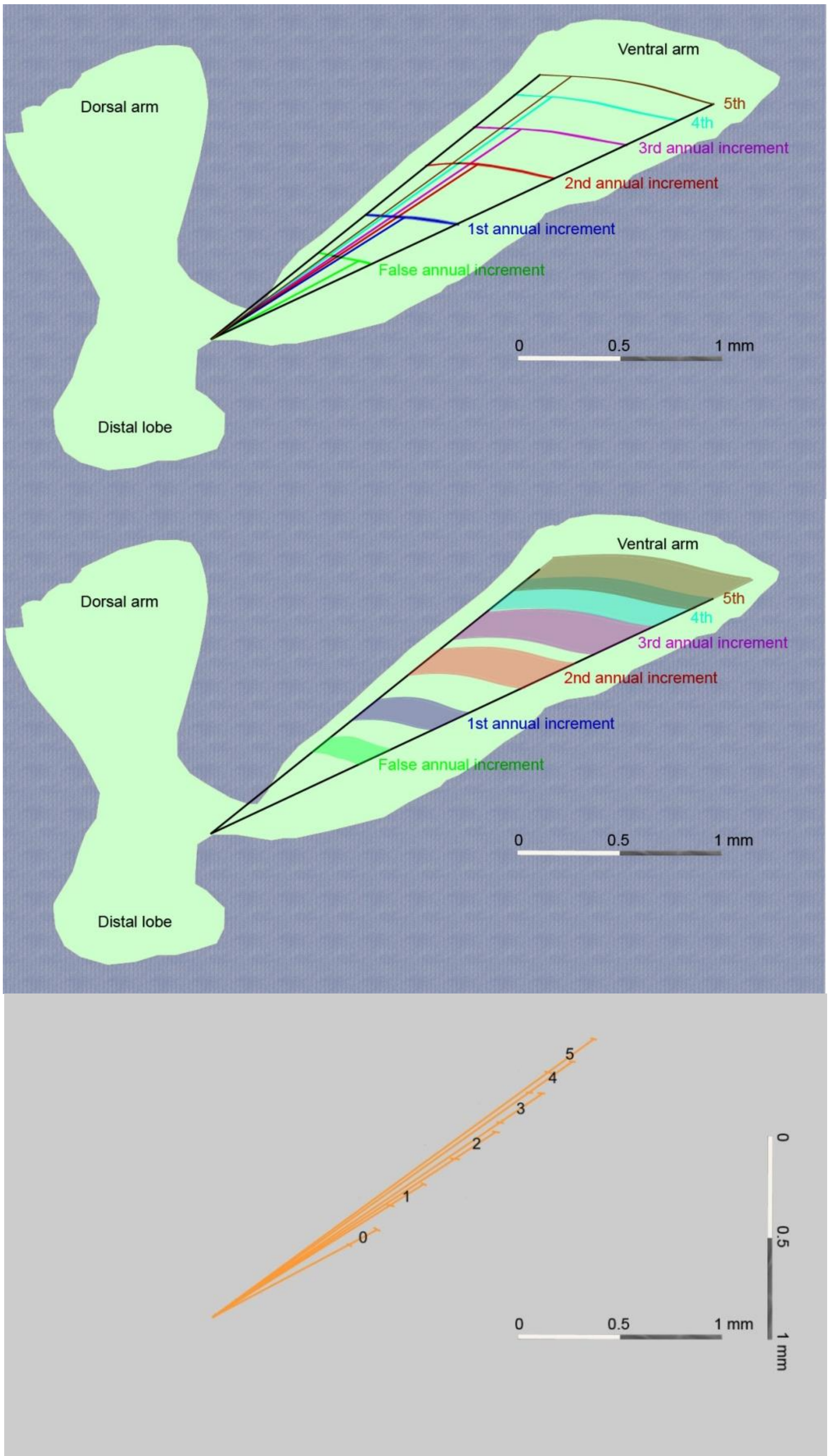

Figure 8. Otolith section shape with the measurements of annual increments. Top with the means of annual opaque marks. Middle with the mean plus-minus standard deviation. Bottom with template designed to serve as a reading aid for otolith images.

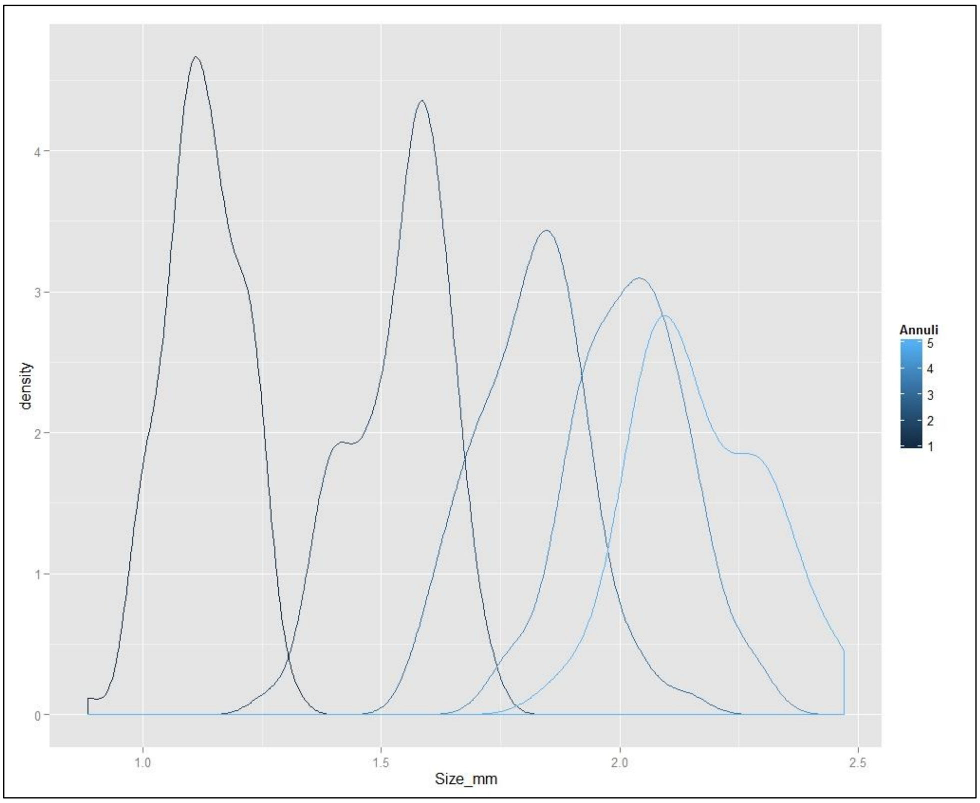

Figure 9. Measurements of the presumed first five annuli observed on the otolith section of *T. thynnus.* Distance from the anchor point to the opaque band in mm.

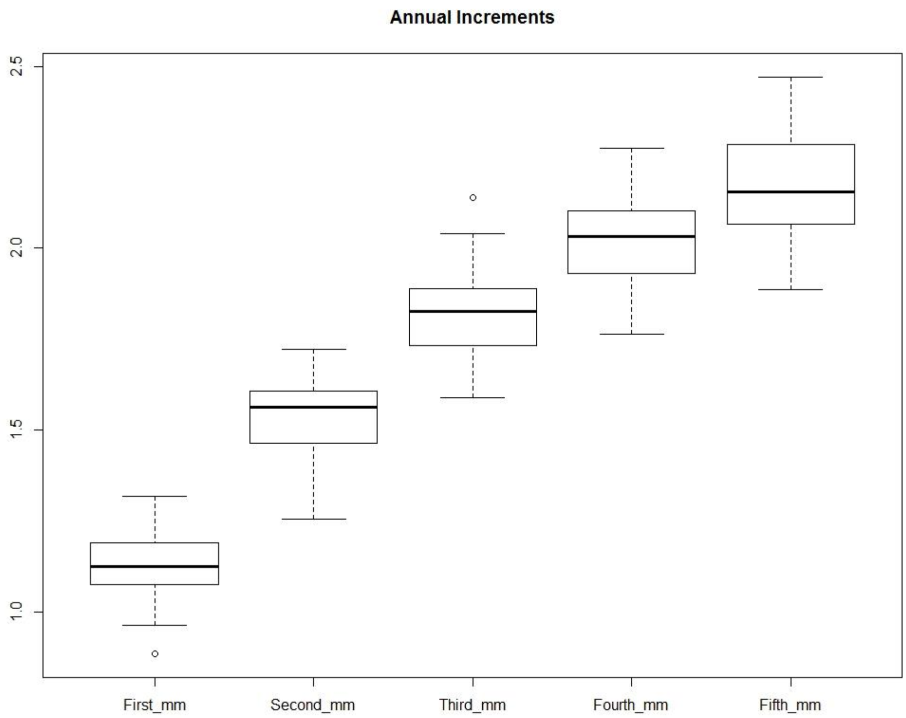

Figure 10. Boxplot of first five annual increment measurements.

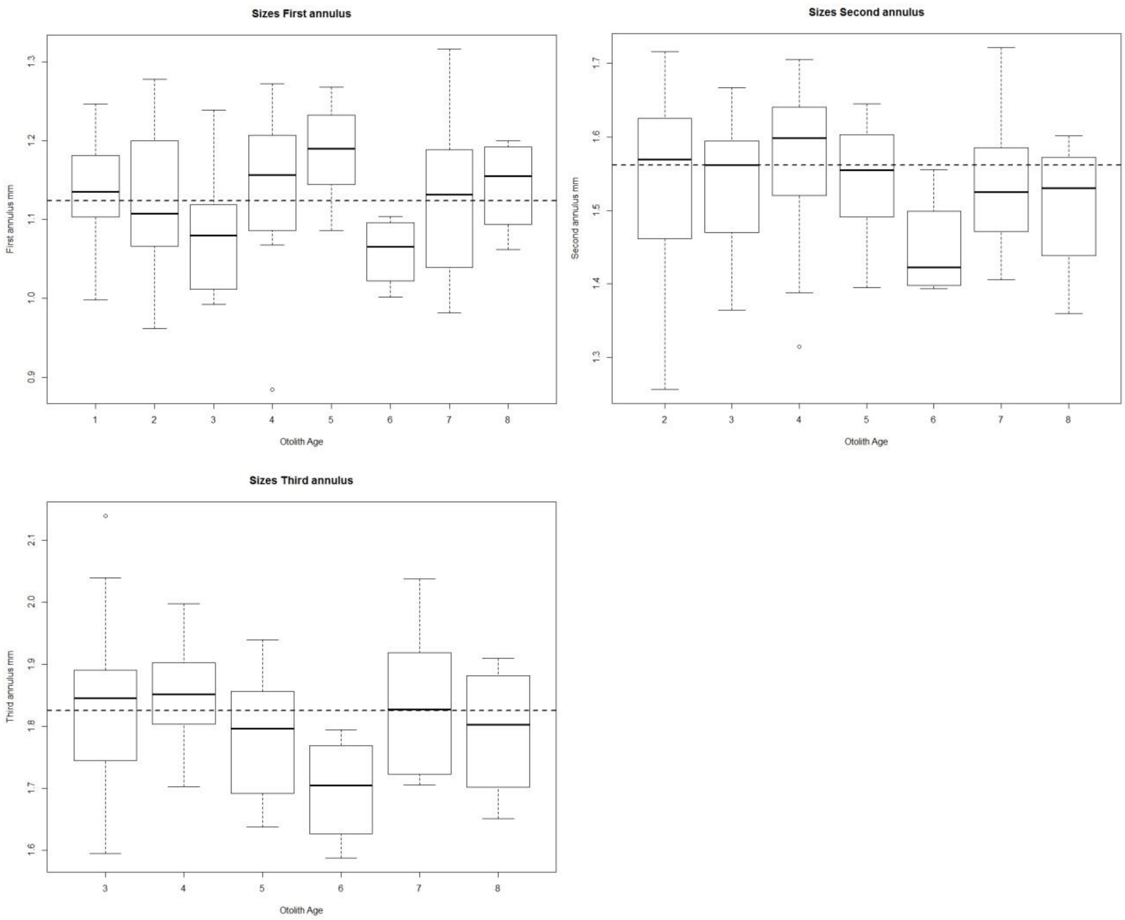

Figure 11. Box-plot showing the relationship between the median size of annual increment measurements of the first (top left), second (top right) and third (bottom left) presumed annulus and age. Dashed line represents the median size of each annulus for all ages.