Preparation and ageing of otoliths from the ICCAT Atlantic-Wide research programme on Bluefin Tuna (Contracts ICCAT GBYP 03/2019 & 17/2019) during ICCAT GBYP Phases 8 & 9

Fish Ageing Services Pty Ltd

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Preparation and ageing of otoliths from the ICCAT Atlantic-Wide research programme on Bluefin Tuna (Contracts ICCAT GBYP 03/2019 & 17/2019) during ICCAT GBYP Phases 8 & 9

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Non-Technical Summary

Preparation and ageing of otoliths from the ICCAT Atlantic-Wide research programme on Bluefin Tuna (Contracts ICCAT GBYP 03/2019 & 17/2019) during ICCAT GBYP Phases 8 & 9

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This report details the work carried out under contracts 03/2019 & 17/2019 during ICCAT GBYP Phases 8 & 9. Specifically, the objectives are as follows:

Objectives:

- Receive otoliths shipped by the ICCAT GBYP (c/o AZTI).
- Register the otoliths and associated biological data according to the Fish Ageing Services (FAS) internal coding system, ensuring at all times that the reference to the original sample ID is retained.
- Weigh one otolith from each sample (only if the otolith is complete).
- Prior to the cutting of the otoliths, take images and measurements from each otolith, following the procedures described in the ABFT Direct Age_2019 Manual (Rodrigues-Marin *et al, 2019)*
- Prepare one otolith from each sample in line with methods outlined in the ABFT Direct Age_2019 Manual.
  - For samples greater than two years old, the otoliths are to be prepared for age reading by performing a double section cut on low speed saw to keep the nucleus section useful for further micro-chemical analyses.
  - For samples two-year-old or less the otolith will be prepared for age reading by performing a single section cut on low speed saw, which includes the primordium
- Select a sub-sample of 10 individual and cut serial sections throughout the entirety of the otolith to determine the effect that section position has on the distance to the first annuli from the primordium and also the potential to bias age.
- Use a two-reader process for the age determination. Reader one is to complete the 1st read, reader two the 2nd read and then the first reader completes the final "agreed zone count". Ageing is to be carried out in line with the GBYP Protocol for ageing as outlined in the ABFT Direct Age Manual.
- For each sample, capture an annotated image of each sample and measure the increment widths along a standardized transect starting at the primordium.
- Obtain a measure of inter-laboratory precision by engagement an additional reader pertaining to another laboratory to complete age reading on 10% of the otoliths.
- Prepare five samples from young of year (YOY) fish to determine the utility of daily ageing methods for this species.

- Provide a spreadsheet with age reading data and biological data with reference to the FAS code and the original ID code for each sample.
- Ship back all the sections, unprocessed otoliths and all the original vial at the completion of the project or when advised.

Non-Technical Summary:

On the 31st March 2019, Fish Ageing Services (FAS) was contracted to provide the services outlined above. In total, 1984 Atlantic Bluefin Tuna (ABFT) otolith samples were sent to FAS. The package of otoliths for this contract was received and all samples were registered according to the FAS internal numbering system. Otoliths were provided in individual vials containing the corresponding original sample reference number. Each individual otolith was prepared as a transverse thin section in a process that allowed annual ageing to be completed and ensured that for samples greater than 90 cm SFL two otolith sections per otolith were cut following the new ageing and preparation protocols for ABFT.

The ageing error estimated by the average percent error (APE) calculated from the age estimates between the primary reader and the agreed estimate was 1.15% and the APE between the secondary reader and the agreed estimate was 3.62%. The APE between the primary and the secondary reader was 4.23%. These values are within the acceptable limits of error used within our ageing programme. We did note that the APE between readers was higher than in the last study (4.23% compared to 2.80% in the previous study) and that the otolith sections were noticeably more difficult to interpret than last time. This is likely due to the fact that in this study the section that we aged was the section adjacent to the section containing the primordium , rather than the one containing the primordium as was the case in the last ageing project. This suggests that sections cut through the primordium is still preferable if the goal is to try and reduce ageing error and ensure that the age estimates are as precise as possible. The majority of the samples were classified with an age reading score of 3 or above, indicating that on average the otolith sections were relatively straight forward to interpret and there is a reasonable degree of confidence on the age estimates assigned. The inter-laboratory testing on a 10% sub-sample of slides has not been completed due to the current COVID-19 travel and work restrictions. Once those restrictions are lifted then the additional reading work will be completed.

Age estimates could be provided for 1908 of the 1984 samples received and ranged from 0 to 22 years. Examination of the age composition by collection year indicated that there was evidence of modal progressions of stronger age classes between years. Modes found from these estimates were also present in the ABFT ageing undertaken in Phase 7 in their respective years.

To determine the effect of section position has on the zone radius between each zone and the primordium and also the potential for the sectioning position to bias the zone counts ten samples were selected for serial sectioning. The otoliths were serially sectioned from the primordium to the post rostrum and for each section the thickness was measured. This work allowed us to determine the effect that section position has on the distance to the first and second annuli from the primordium and if by what effect the position of the section may have on the potential to bias the age. It also allowed us to approximate how far away from the primordium that the otolith sections taken for ageing were taken. Through this process we determined that most sections taken using the new protocol (used for the main ageing component of this study) were located approximately between 1.2 to 1.8mm away from the primordium. While this did increase the readability (visually and also confirmed by readability score) of the section compared to the primordial section, the "secondary section" used for age estimation should not lead to a bias in the age estimation as long as the ageing section is cut within a maximum of 1.8 mm from the core.

Once the annual ageing process was completed, 10 samples estimated to in the 0+ age class were selected for daily age determination. Samples were selected to ensure that there was good representation of size ranges within the age class and also covered as many months as possible within the first year of life. Samples ranged from 41.5 to 63.8cm SFL and the resulting microincrement counts ranged from age from 106 to 239 days. The otolith microstructure pattern within thin transverse sections of ATBFT otoliths was similar to that of other tuna species that we have examined. For all but one of the samples, the back calculated hatch dates were considered plausible for this species based on the known spawning duration in the Mediterranean Sea. This provides some weight of evidence that counts of microincrements within the otoliths of this species can provide an accurate estimation of age for samples up to at least 6 months of age.

Keywords: Atlantic Bluefin Tuna, otolith, ageing, zone count, microincrement, transverse section, serial section, precision

Methods

In total, 1,984 otoliths sampled from six different years (2013-2018) were received and registered by FAS (Table 1). Otoliths were sampled from all months in the calendar year (Table 2**Error! Reference source not found.**), however most of the samples were collected between September and December (87.9% of the samples). From the 1,984 samples received, only 1,036 samples had a recorded date-of- sampling. Where samples had no date-of-sample information, the month and year of date-of- capture was used. From the 1,984 samples received, 1,610 samples had date-of-sampling information. No samples were missing both date-of-capture, date-of-sampling, and harvest date. Samples had been previously assigned a size category. These are presented in Table 3.

Of the 1,984 otolith samples received, 191 samples had been previously embedded and sectioned. These were supplied as unmounted sections (Table 4). The whole otoliths were provided in individual vials containing the corresponding original sample reference number and from each sample only one otolith provided. Samples were batched into nine batches indexed at eleven, which is the next number on from the previous batch of Atlantic Bluefin Tuna FAS has aged (Table 1). Each box supplied was given a unique batch number. Individual samples within each batch were assigned a sequential number and placed in individual envelopes containing that number.

Table 1. Number of Bluefin Tuna registered by Batch at FAS.

		Year						
Box number	**Batch**	**2013**	**2014**	**2015**	**2016**	**2017**	**2018**	**Total**
1a	11	15			9	57		81
2a	12					90		90
3a	13	1		24	39	35		99
4a	14				81			81
5a	15				31	308		339
6a	16				253	61		314
7a	17	74	24	12	39	111	50	310
8a	18	89	63	30	114	31	7	334
9a	19	2		3	2	299	30	336
Grand Total		**181**	**87**	**69**	**568**	**992**	**87**	**1984**

Table 2. Number of otoliths registered by FAS by year and month.

						Month							
Year	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**Total**
2013				2		1	10	11	40	100	15	2	**181**
2014	5	1	5		5				2	59	10		**87**
2015					3	2	2	7	4	49		2	**69**
2016	3		3					1	2	296	245	18	**568**
2017	41					25	5	21	145	237	310	208	**992**
2018	29		34	16		8							**87**
Total	**78**	**1**	**42**	**18**	**8**	**36**	**17**	**40**	**193**	**741**	**580**	**230**	**1984**

Table 3. Number of otoliths supplied to FAS by year and size range.

	Size Range				
Year	**Unknown**	**Juvenile**	**Medium**	**Large**	**Total**
2013	1	29	91	60	**181**
2014	14		73		**87**
2015	1	3	60	5	**69**
2016	16	3	528	21	**568**
2017	14	24	778	176	**992**
2018	16	1	57	13	**87**
Total	**62**	**60**	**1587**	**275**	**1984**

Table 4. Numbers of sample vials which contained either thick otolith sections, no sample in vial, or as the whole otolith.

	Supplied sample			
Year	**No sample**	**Otolith**	**Slice**	**Total**
2013	1	97	83	**181**
2014		39	48	**87**
2015	1	58	10	**69**
2016	3	565		**568**
2017	3	989		**992**
2018		37	50	**87**
Total	**8**	**1785**	**191**	**1984**

All otoliths received by FAS were registered into a database. Otolith samples arriving at FAS are batched by species and sampling location. In this case, we assigned a batch number to each box of otoliths. Each box number was given a sequential batch number and is detailed in Table 5. Each sample was assigned a unique identification code (Sample ID) used by FAS for our data systems. The sample consists of a 15-digit code, which is comprised of five sets of triplets, with each triplet being a category. An example of this would 082003300019042. This comprised of

1. The client code is the first three digits (082), ICCAT/GBYP client code.
2. The job identification (003), the first job for this client
3. Species code (300), the species code for *T.thynnus*
4. The batch ID (019), the third batch of *T.thynnus* that we have processed at FAS
5. The fish ID, of fish number (042), fish number 42 from the first batch.

Using this method, all samples can be identified quickly and uniquely identified. Throughout this report, the client code and job code may be omitted for brevity, and underscores may be used between placeholders. Images (jpg's) collected automatically, as part of the ageing process, use this code and are also appended by the reader and then reading number. Therefore, the image name 082001300019042_1_1.jpg would be the above sample, aged by reader one, for the first time. After the ageing was completed duplicate images were named using the original otolith ID so that ICCAT could archive the images and data according to their internal numbering system.

The biological and sampling data that was supplied was entered into a database ensuring that the original otolith ID code was retained at all stages. Each unbroken otolith was weighed on an electronic balance to the nearest 0.0001g.

Image collection

Whole otoliths were imaged against a black background and illuminated with reflected light. The collection of images, otolith measurements (length and width) and image related data was completed using an inhouse imaging program and followed the specifications outlined in the *Report of the ICCAT GBYP International Workshop on Atlantic Bluefin Tuna Growth (2019) (Appendix 3 – Protocol for the age reading of Atlantic Bluefin Tuna otoliths). (*Rodriguez-Marin et al*, 2019)*

Otolith preparation for annual age estimation

Otoliths were embedded in slow cure Epoxy Resin (Aka-Resin slow cure) in individually numbered silicon moulds. The resin was allowed to dry for at least 24 hours prior to sectioning.

For samples up to two years old (approximately 90 cm SFL), a single section (approximately 360um) was cut through the nucleus (**Figure 1**). Care was taken to ensure that this section contained the primordium. The sister otolith was retained to be used for stock identification analyses. For samples greater than 90cm, two individual sections were taken from each whole otolith. The first section was cut through the nucleus at a thickness of approximately 1.5 millimetres and reserved for future micro-milling and elemental isotope analysis. The location of the cuts for this section were positioned so that the resulting sectioned contained the V and Y type section in each extreme, or at least the tip of the antirostrum appearing as a satellite in the section as per Rodriguez-Marin et al, 2019). The second section (to be used for ageing) was cut at approximately 380-micron thickness (Figure 2).

Figure 1. Sample AZTI-BB-J-768 (61.8 cm SFL) indicating the position of the single section taken for annual ageing. The yellow mark indicates the primordium, while the numbers indicates the position of each cut in order.

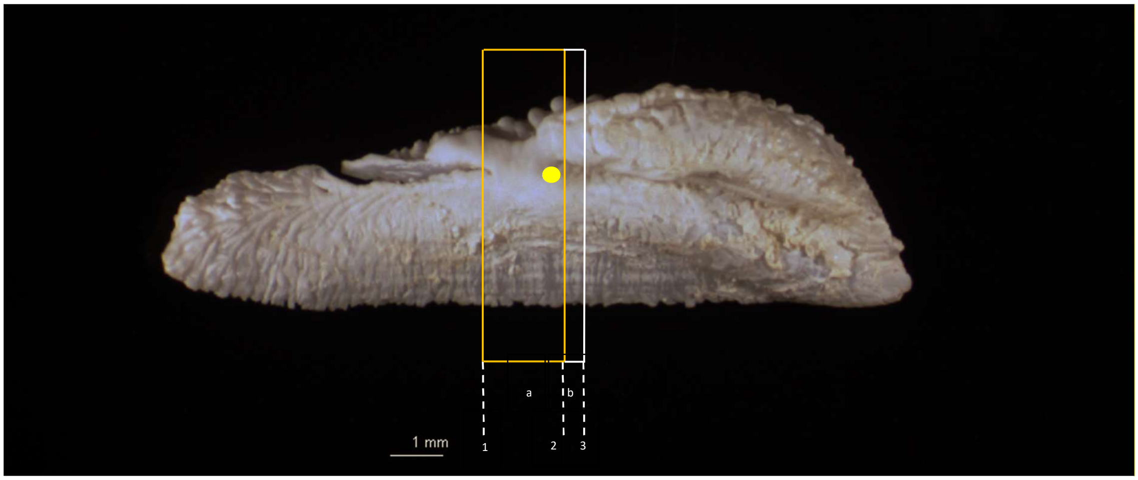


Figure 2. Sample ABFT_UNIC-SA-M-385 indicating the position of the sections taken. The section reserved for micro-milling is marked in orange box (a) and the section prepared for ageing is represented in the white box (b).

All cuts on the otolith blocks were made using a Buehler Isomet 1000 running a single four-inch diamond wafering blade (Isomet 15HC – 4 x 0.12in - Arbor Size:0.5in) set at a speed of 450 RPM. The sections that were cut for micro-milling were cleaned with distilled water, dried, and placed along with the remaining portions of the block back into the corresponding envelope. Each section that required further preparation for ageing was dried and placed in a vial that contained the sample number.

To prepare the ageing section for age reading, each section was ground down manually to a thickness between 320-340µm using increasing grades of wet/dry paper (600, 800 and 1200grit) lubricated with distilled water and finally polished dry with 8µm and 5µm aluminium oxide lapping film. Before grinding, the section was inspected and the side of the section that contained otolith material that was furthest away from the primordium was chosen for grinding. This was to ensure (where possible) that the finished preparation still contained the primordium. During the grinding process, the otolith preparation was continuously checked for the appropriate thickness (320-340µm) using digital Vernier callipers. Once the appropriate thickness was reached the section was given a final polish and was secured on a microscope slide with Crystalbond© (509). Three sections were placed on each slide and were numbered with the original sample IDs for those three samples.

To investigate the effect that section position has on the age estimates, section readability and the distances between the primordium and the first and second opaque zones, a sub-sample of ten samples were selected for serial sectioning and examination. The samples for this component of the project were sister otoliths of a set of otoliths to be aged in this project. The samples were selected because their sister otoliths showed relatively clear zone structure on the distal surface of the otolith when observed under reflected light. These particular samples were identified during the otolith imaging process. Samples were serially sectioned using a modified highspeed lapidary saw with up to 29 sections taken from each sample. The benefit of using the highspeed lapidary saw is that the diamond blades used are only 200µm thick and remove only a minimal amount of otolith material each cut compared to the thicker Isomet© blades.

The first cut was positioned at the tip of the post rostrum and for each sample up to 29 serial sections were cut through the otolith block. The thickness of each section was measured using Vernier callipers and for each sample the first 20 sections were mounted on a 50x76 mm glass slide in section order. One sample (300011034 – BALF-BA-L-282) was sectioned in its entirety and mounted on two slides. Descriptive statistics were taken from each set of serial sections. A blank block (containing no samples) was cut 13 times to determine the mean of the amount of material removed by the saw.

For each sample, the section containing the primordium was identified and the section number which contained the section that most closely resembled the section cut for the annual ageing process was identified. This then allowed us to approximate the position on the otolith from which the ageing section was likely to have come from (i.e. distance away from the primordium).

To determine what effect that sectioning position has on the potential to bias the age estimate, the section containing the primordium was firstly identified and aged. Age estimates were then made on the seven serial sections predating this section working backwards from the primordia to the posterior tip of the otolith, i.e. if the primordium section was identified as section 16, then sections 15 through to 8 were aged.

To determine the effect that different sectioning positions have on the position of the inner increments in the otoliths sections the distance from the beginning of the ageing transect to the first two zones was measured. The measurements were made along a transect parallel to the sulcus towards the post rostrum until the section became unreadable; this was between six and nine sections. The readability of each section was recorded and with the zone distances and associated metadata for each section.

Annual Age Estimation

Standard FAS protocol requires that before the reading of a set of otoliths begins, for each given species, a sub sample of otoliths is selected out from the reference set. The reference set consists of a sub-sample of previously read otoliths from that particular species. These samples are read and then compared against the originally assigned ages and ageing of the current sample only commences if an acceptable level of precision and accuracy has been achieved (species-specific indices). If acceptable levels are not achieved, then readers are retrained. Calibration was done on the previously aged Atlantic Bluefin Tuna images from images. Ages were estimated from unmarked images. Experience with otolith reading of other Tuna species also proved invaluable in the calibration process. Information in the literature (Rodríguez-Marín1 *et al*., 2014, Rodríguez-Marín *et al*., 2007, Secor *et al*., 2014, Neilsen and Campana, 2008) was also used as a basis to aid interpretation. Images supplied by Naomi Clear (CSIRO Oceans and Atmosphere Research) that were previously examined at the 2006 age reading workshop also were viewed to ensure that our age protocols provided similar age estimates to those for the images. All estimates from each reader involved in this project used the protocol to ensure that readers followed the same interpretation and methods of marking the images.

All preparations were aged using a two-reader method; two readers each reading the total sample once. A third and final age reading was completed by the first age reader to produce the agreed "final" age estimate. All age readings were made without knowledge of fish size, otolith weight, sex and location. The final "agreed" age estimate was made with knowledge of the first two readings.

Age estimation procedures followed those detailed in the *Report of the ICCAT GBYP International Workshop on Atlantic Bluefin Tuna Growth (2019) (Appendix 3 – Protocol for the age reading of Atlantic Bluefin Tuna otoliths)*. Sections were viewed under transmitted light using a Leica M80 stereo microscope with 20x magnification. Opaque zones were counted along a transect that ran from the distal margin of the otolith section to the proximal margin adjacent and parallel to the sulcus on the ventral side of the otolith (Figure 3). 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. A word document was developed to aid in the interpretation of otolith structure. This document contains various images of clear to read examples and notes regarding various aspects of the interpretation process. A copy of this document is provided in Appendix 3.

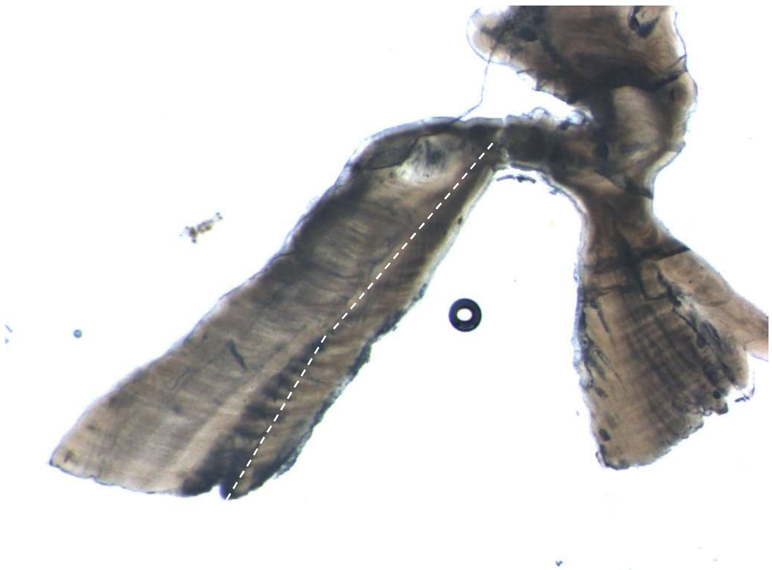


Figure 3. Atlantic Bluefin Tuna section viewed at 20x magnification using transmitted light. Dashed line is the approximate transect used in the counting and marking of zones.

Data acquisition

A custom image analysis system was used to measure zone distance between the distal edge of each of the manually marked opaque zones from the first inflection. This system counts and measures manually marked increments and collects an image from each sample aged.

A CCD digital camera mounted onto the dissecting microscope (Leica MZ80) displays the live image on the monitor. Using the image analysis system, the positions of the opaque zones and the otolith edge was marked with a screen cursor along the preferred ageing path. The number of zones marked and the measurement from the primordium to each subsequent mark along the transect was automatically exported to a Microsoft database. The otolith image was automatically captured and exported into the database.

In addition, the sample was assigned a readability score which rates the otoliths on a sale of 0 to 5 (Table 1) and the marginal edge type of the otolith section was recorded. The marginal edge type was subjectively assigned by the reader as 1, 2 or 3 and is based on the optical properties of the edge using the following categories:

1. WT (translucent material past last opaque zone is generally greater than 1/3 of previously completed zone)
2. NT (translucent material past last opaque zone is generally less than 1/3 of previously completed zone)
3. O (opaque visible on edge)

The edge is an indicator of the likelihood of zone formation in the next period of deposition. An edge type 1 indicates that an opaque zone is likely to form soon, an edge type coded 2 indicates that the it was likely that the opaque zone had only recently formed and an edge type 3 indicates that the opaque zone on the otolith margin is still to be completed. The confidence of the edge type (1-3) was also recorded with (1) being not confident on edge assignment to being confident (3).

Other information collected during the reading process included whether the section was 'Y' or 'V' shaped, which is an indicator of whether the section is on the primordium ('V') or slightly away from the primordium ('Y').

Table 5. Readability Index

Score	Interpretation
0	No sample provided or sample unable to be prepared
1	Sample unreadable due to poor preparation or zone structure
2	Difficult to interpret. Age estimate maybe +/- 1 or more from true age
3	Reasonable zone structure and pattern. Confident that estimate is no more than 1 different from the true age
4	Good zone structure, confident age estimate
5	Excellent zone structure, easy to interpret

Protocol for adjusting zone counts into age estimates followed the methods outlined in the *Report of the ICCAT GBYP International Workshop on Atlantic Bluefin Tuna Growth (2019) (Appendix 3 – Protocol for the age reading of Atlantic Bluefin Tuna otoliths).* Specifically:

- no adjustment to zone counts for samples with wide translucent edge type for all months
- samples with narrow translucent edge types were adjusted between April and June to zone count -1; all other time periods were unadjusted.
- samples with opaque edge types were adjusted to zone count-1 for the period July to October. All other time periods were unadjusted.

Table 6. Zone count adjustment criteria to account for zone formation

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 zone counts had been adjusted for zone formation the age estimates were also adjusted to account for biological age and a calendar age. In Table 7 and Table 8 below, A = age adjusted for zone formation.

Table 7. Table for biological adjustment criteria

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

Table 8. Table for calendar year adjustment criteria

Catch Month	January to June	July to December
Calendar year	A + 1	A

Precision and accuracy

Currently the accepted protocol for ageing tuna within Australia is to complete a double bind read (can be completed by the same reader or by a second reader) and then provide a final "agreed" zone count/age estimate. This final read is not a blind reading and can access the data from the previous two reads and the biological data. This also provides the necessary data to determine the precision within the readings and to test for reader drift. Beamish and Fournier (1981) have developed an index of average percent error (IAPE), which has become a common method for quantifying this variation. The IAPE is calculated as:

$$IAPE = \frac{100}{N}\sum_{j=1}^{N}\left[\frac{1}{R}\sum_{i=1}^{R}\frac{|X_{ij}-X_j|}{X_j}\right]$$

Where N is the number of fish aged, R is the number of times the fish are aged, X_{ij} is the ith determination for the *j*th fish, and X_j is the average estimated age of the *j*th fish. The index has the property that differences in age estimates for younger fish will contribute more to the final value than will the same absolute error for older fish (Anderson *et al.* 1992). The IAPE was converted to CV following methods in Kimura and Anderl (2005).

The IAPE was estimated, and the precision investigated by examining age difference tables and age bias plots. Re-reads do not validate the assigned ages but provide an indication of the magnitude of error expected within the set of age estimates. These differences are due to variations in interpretation of the otolith zones.

To provide an additional measure of quality control, a 10% sub-sample of otoliths (198) was selected to be read by an additional reader experienced with tuna ageing from another laboratory. This will allow for a measure of inter-laboratory accuracy to be calculated. Unfortunately, due to the current COVID-19 travel and work restrictions that are currently in place, this task has not been completed. It is expected, that as soon as restrictions are eased this will be completed as a priority.

Daily Age estimation

Daily ageing of tunas can be very useful for determining the accurate age of otoliths from either larvae of juvenile samples, to help describe growth in young-of-the-year (YOY) fish and to develop reference scales that aid in detecting the first annual growth increment. To determine the utility of daily ageing in the process of ABFT age and growth estimation, a sub sample of 10 otoliths that had, based on annual ageing already been assigned to the 0+ age class, were selected for daily analysis. Originally only five samples were to be prepared and analysed, however that number was increased to 11 to allow for a better representation of the size range observed within the 0+ age class and also include samples caught in as many months as possible within the first year of life. Each otolith section was removed from the original side of three samples and was fixed to individual microscope slides using crystalbond. The top surface of the section was ground further using 1200 grit wet/dry paper until the primordium was visible. The section was then flipped, and the other side of the section was ground down until the preparation thickness was approximately 60 - 80µm and the microincrements could be clearly identified.

Microincrements were counted using a transmitted light microscope at various magnifications ranging between 250x and 1000x depending on the area of the otolith being interpreted. Microincrements were counted from the presumed hatch mark (approx. 10um from the primordium) out to the otolith margin along a transect running through the ventral arm of the transverse section. The number of microincrements observed between the primordium ranged between 5-8, however they were not included in the total count. The reading path for the estimation of the micro-increments is shown in Figure 4. The interpretation of otolith microstructure in tuna otoliths can be difficult and subjective and it can be difficult to distinguish sub-micro-increments from the assumed daily increments. However, for the most part the sub-increments were characterized by faint and incomplete rings. As with other tuna species we have examined, the internal zones closer to the sulcus were more regularly spaced and did not show much overlapping, splitting, or merging of zones compared to the zones closer to the distal and ventral edge. Unfortunately, after a count of between 130-150 micro-increments the internal structure became increasingly difficult to interpret so the reading path was required to shift closer to the outer edge of the ventral arm. To be consistent in our methodology for reading microstructure in tuna otoliths, the reading of the outer increments was directed along the axis of maximum concavity of increments (Figure 5). In many of the samples there were sub-sections of the otolith along the preferred reading path where the zone structure was either difficult to interpret or was not present. If the zone structure adjacent to these areas showed a clearer pattern, then this alternate count path was used until increments could be again interpreted along the preferred ageing path. If the adjacent area was also unclear, then an interpolation method which used the zone pattern immediately before and after the difficult area was applied (Figure 6). This type of interpolation method is considered reasonable in cases where the number of interpolated micro-increments is relatively small in comparison to the total increment count (Campana, 1992).

The otoliths were read several times by the one reader until a consensus age was reached. A readability scale was not applied, however any relevant comments regarding the interpretation were recorded. Estimated daily age was plotted against fish length and used to back-calculate hatch date (assuming the first zone counted represented hatching).

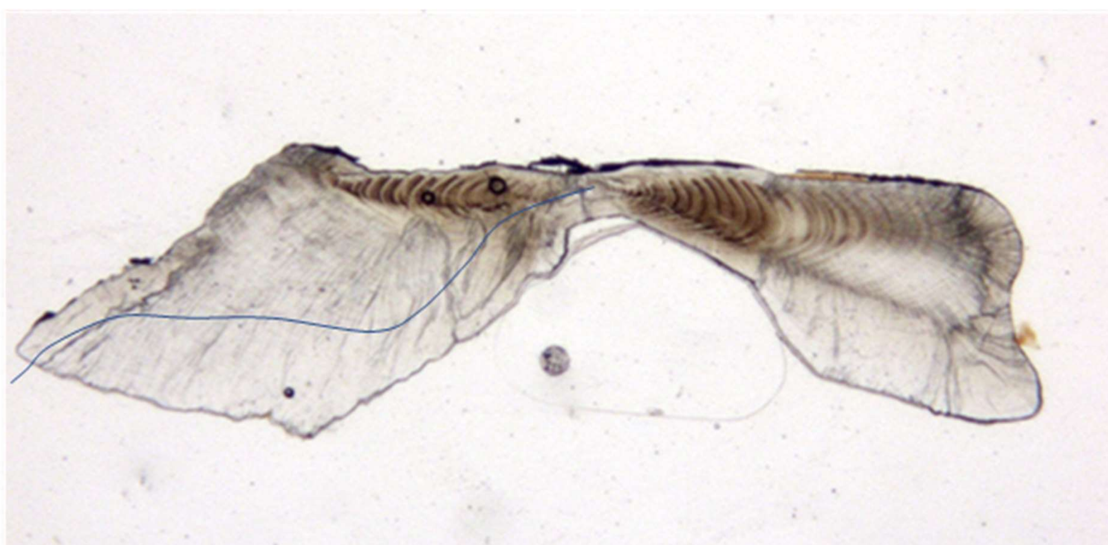


Figure 4. Transverse section prepared for daily age estimation. Blue line indicates approximate ageing transect.

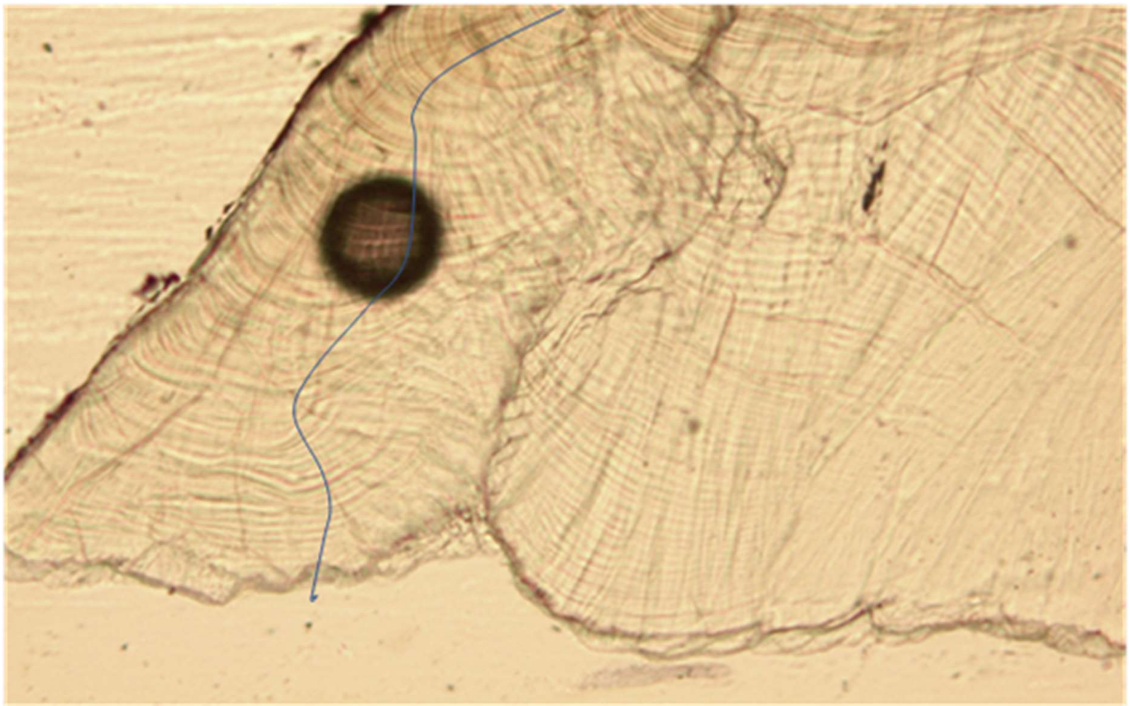


Figure 5. Higher magnification image showing the counting path going through the maximum concavity of increments (maximal growth axis)

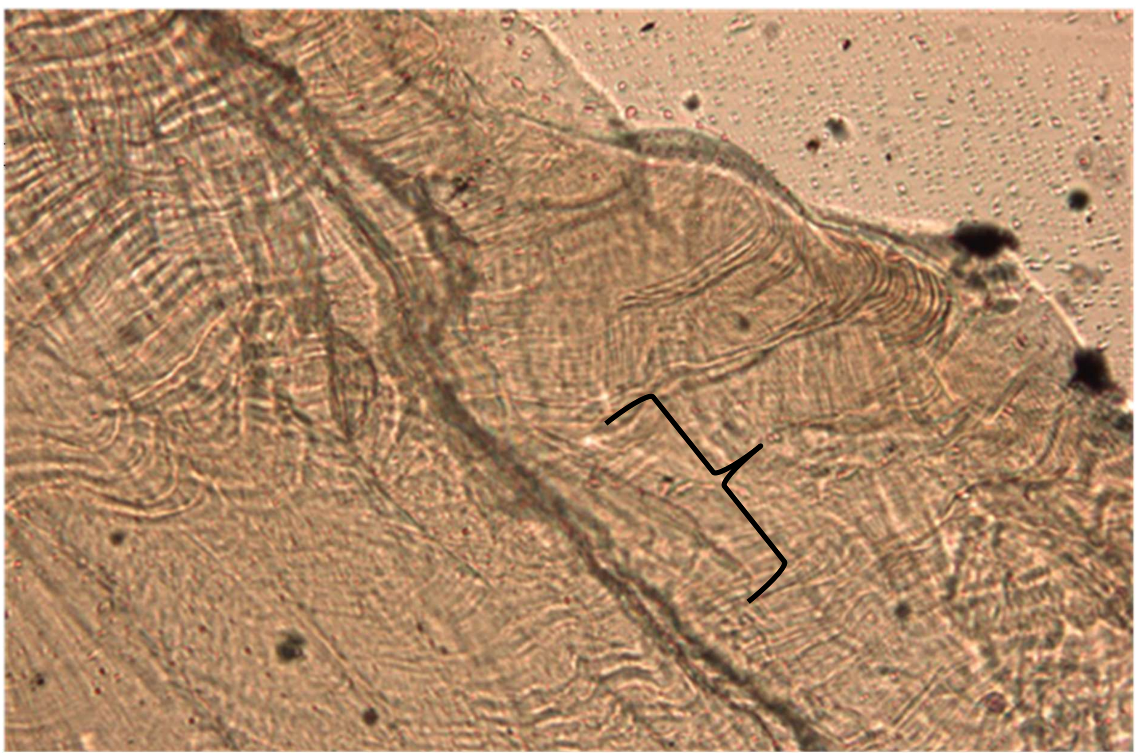


Figure 6. Higher magnification of the otolith section one area where the interpretation is considered subjective. The black brace indicates an area where the microincrements needed to be interpolated.

Results

Otoliths

A total of 1,984 otolith samples were received and recorded in the FAS data entry database. These samples were collected between 2013 and 2018. Of these, 1,908 annual estimates were made. The minimum age class in the samples was zero while the oldest was 22 years. The mode readability was 3. No samples were considered readability of 5 (Table 9). The readability of the edge type was also recorded using a scale of one to three, with one being uncertain of edge type to three being confident of the edge type (Table 10).

Table 9. Sample readability score by index.

Readability	N
0	56
1	20
2	556
3	1216
4	136
5	0
N	**1984**

Table 10. Edge readability score by index.

Readability	N
1	68
2	727
3	1113
N	**1908**

Estimates of precision

The age difference distribution and the first age against the second age plots for the two age readers from FAS are shown in Figure 7. Age difference distribution and age bias plots for Reader 1 vs Reader 2 (APE 4.23%), Agreed age vs Reader 2 (APE 3.62%) and Agreed age vs Reader 1 (APE 1.15%). The IAPE between Reader 1 and Reader 2 was 4.23%, the IAPE between Reader 2 and the agreed age was 3.62%, the IAPE between Reader 1 and the agreed age was 1.15%. Examination of the age bias plots suggested no systematic bias between age difference distributions between Reader 1, Reader 2, and the agreed age with a mode of zero in all comparisons.

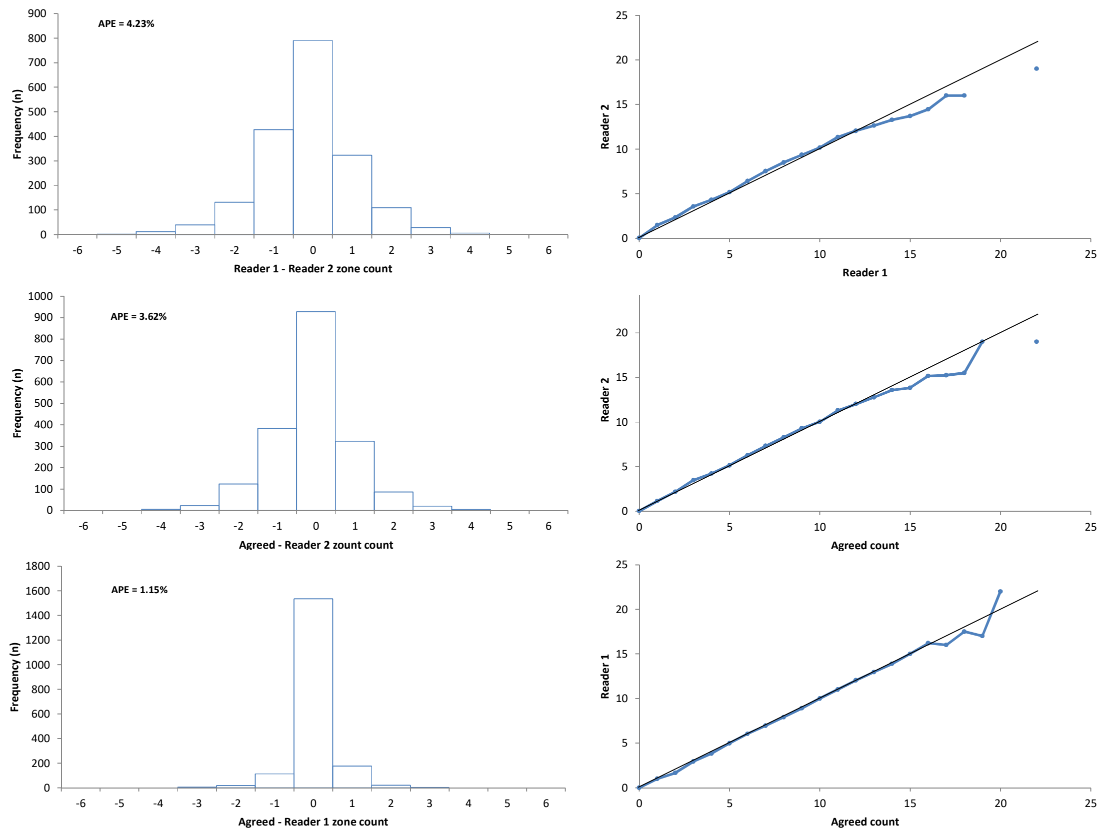


Figure 7. Age difference distribution and age bias plots for Reader 1 vs Reader 2 (APE 4.23%), Agreed age vs Reader 2 (APE 3.62%) and Agreed age vs Reader 1 (APE 1.15%).

The comparison of the age estimates from the 10% subsampled for re-reading by an experienced reader from another laboratory has yet to be completed. The age difference distribution between the “Final” age and the 10% re-read and the age bias will be completed when available.

Age estimation

Age estimates were obtained for 1908 from the 1984 samples. For the adjusted age estimates, ages ranged from 0 to 22 years for all samples (Figure 8). The modal age for all samples combined was 12 years. The composition for each year of capture indicates some differences with the age-classes being mainly older in years 2014-2018. Samples from 2013 also contain younger samples. The zero plus age-class was present in all years and evidence of modal progression is apparent in all years except 2018, with a mode of nine-year old’s in 2013 progressing by one year with each successive year up to 2017. The mode is not present in 2018 (Figure 9). The age-otolith weight relationship, the age-length relationship and the length-otolith weight relationship are shown in Figure 10 and the age-length key for all years is shown in Table 11. Total age composition, age composition by year and the ALK’s for the zone data adjusted for biological year and calendar year are shown in Appendix 4 and 5, respectively.

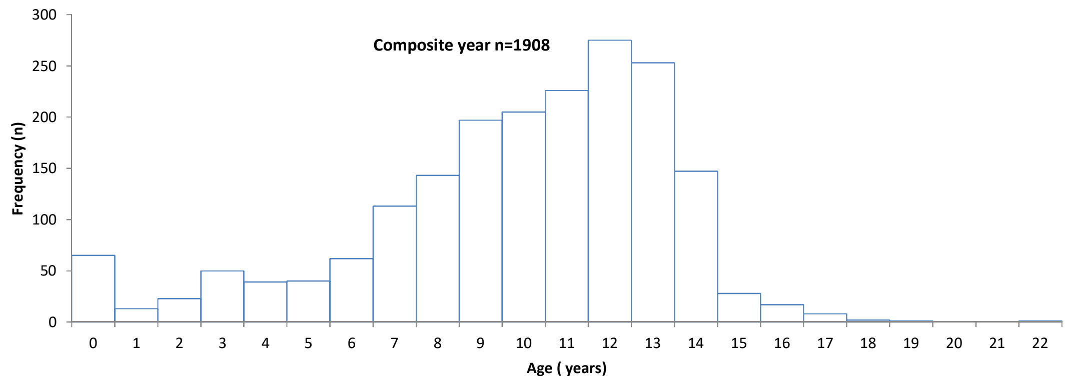


Figure 8. Age composition for combined years (adjusted age)

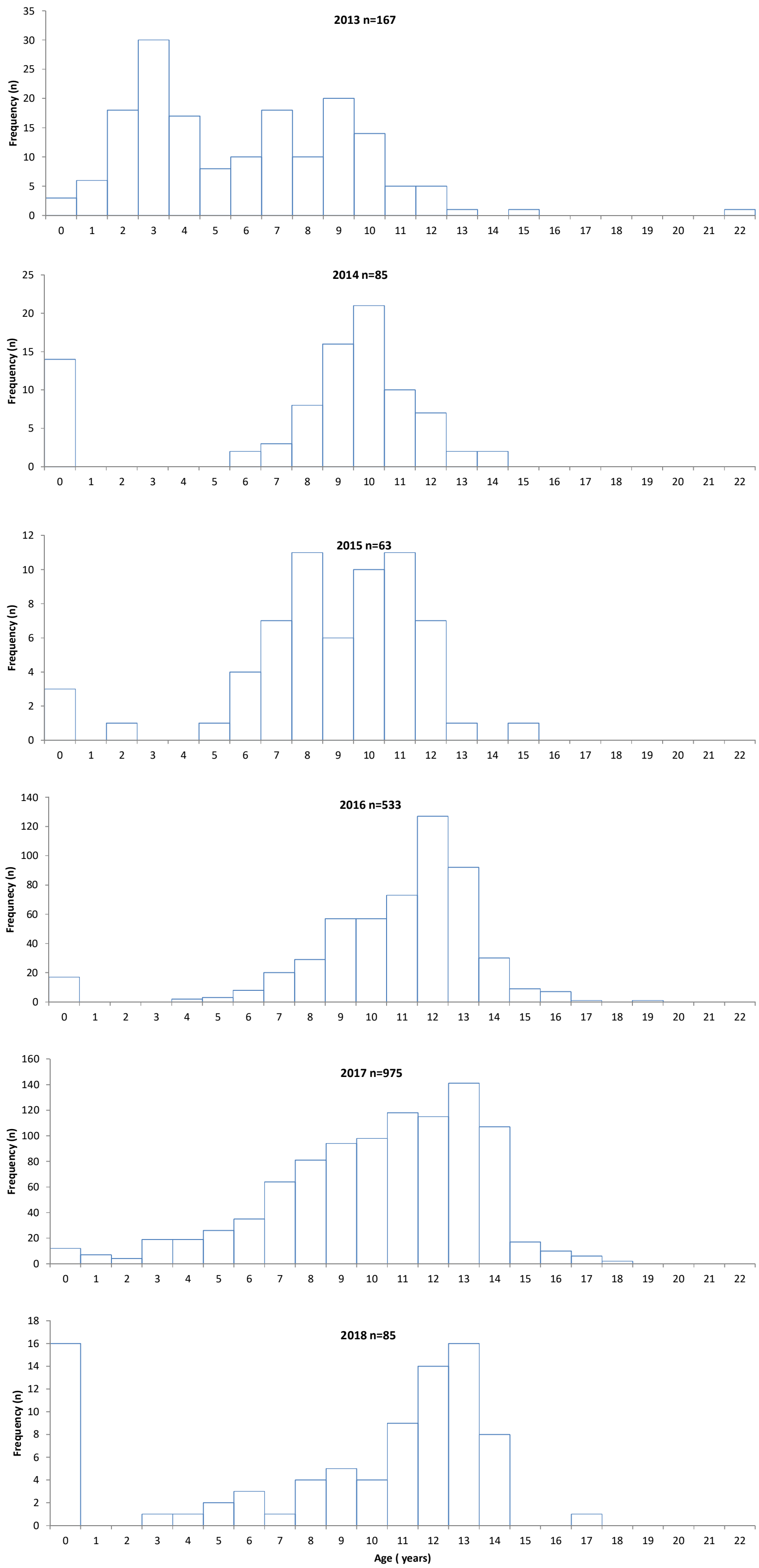
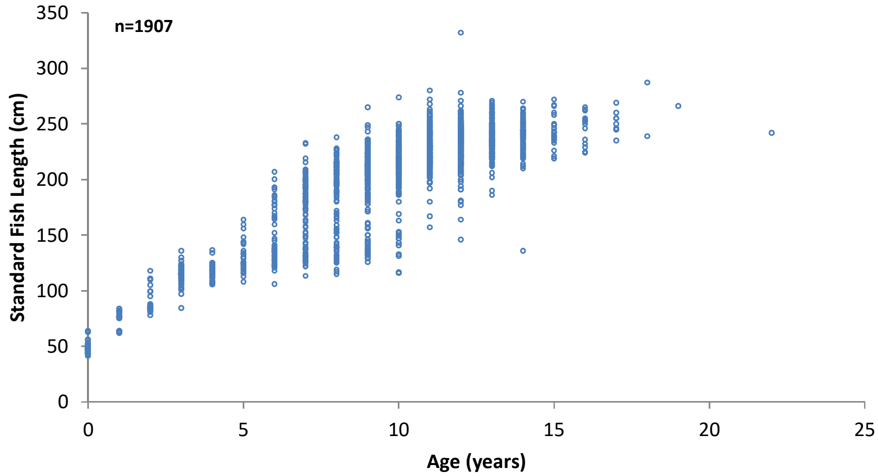
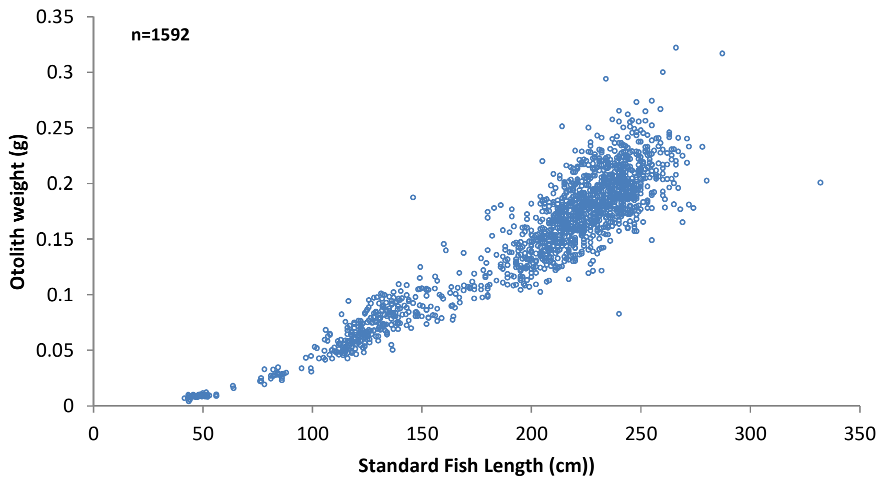


Figure 9. Age composition by year of capture





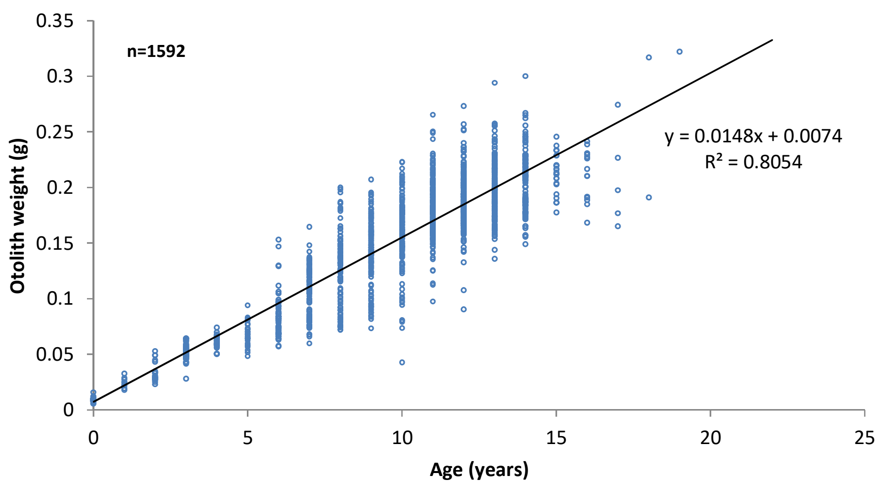


Figure 10. Age / length, length/otolith weight and age/otolith weight relationships for *T. thynnus* samples used in this study.

Table 11. Age-length-key for all years combined, binned into 5cm length classes

	Age Class																					
Length (cm)	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**22**	**Total**
40	13																					**13**
45	32																					**32**
50	14																					**14**
55	4																					**4**
60	1	6																				**7**
75		7	1																			**8**
80		1	13																			**14**
85			6																			**6**
95			3	2																		**5**
100				3																		**3**
105			1	5	5		2															**13**
110			2	11	6	1		1														**21**
115			1	22	11	6	1	1	2		1											**45**
120				9	10	9	7	3														**38**
125				1	2	4	7	10	6	1												**31**
130				1	1	7	7	9	7	4	2											**38**
135				1	1		12	9	6	5	1				1							**36**
140						4	3	4	4	5	2											**22**
145						1	2	4	3	1	3		1									**15**
150							1	3	5	1	3											**13**
155							3	2	5	2		1										**13**
160						1	2	1	2	2	1		1									**10**
165							4	1	4		1	1										**11**
170							2	6	2	2												**12**
175							4	3	1	1			1									**10**
180							4	11	4	3	2		2									**26**
185							2	9	7	4	3	1		1								**27**
190							3	9	8	6	5	2	3									**36**
195								8	18	15	4	4										**49**
200								10	10	20	12	3	5	1								**61**
205							1	8	19	26	19	10	7	2								**92**
210									18	36	35	17	11	6	2	1						**126**
215								1	9	25	30	30	18	9	4							**126**
220								1	4	26	25	34	36	16	10	1						**153**
225									5	10	14	40	26	24	8	1	2					**130**
230								1	2	10	15	39	30	41	7	1	1					**147**
235									1	4	9	18	41	27	15	4	1	1	1			**122**
240										2	10	27	41	40	21	3					1	**145**
245									1	1	3	19	37	19	12	4	1					**97**
250											2	11	10	15	9	3	4					**54**
255												6	8	12	4	2	1	1				**34**
260												3	5	10	3	2	1	1				**25**
265										1		1	1	5		3	1			1		**13**
270										1		1	1	1	2							**6**
275													1									**1**
280												1										**1**
285																		1				**1**
330													1									**1**
Total	**64**	**14**	**27**	**55**	**36**	**33**	**67**	**115**	**153**	**214**	**202**	**269**	**287**	**229**	**98**	**25**	**12**	**4**	**1**	**1**	**1**	**1907**

Serial Sections

The serial sections were measured and the descriptive statistics for these are presented below (Table 12). Thirteen cuts were made on a blank block and measured to estimate the distance between the serial sections (μ=0.279 mm, SD = 0.0085 mm). The distance from the section containing the primordium to each subsequent serial section could then be calculated by the sum of each individual section width and the mean width of the cut. The distances from the section containing the primordium to each section that was selected for examining age estimation and inner zone distances in the serial sections is shown in Table 13.

Table 12. Summary statistics for the samples selected to examine zone distances as a function of distance from the primordium.

Sample	FAS ID	Mean	S.E	Median	Mode	S.D.	Variance	Kurtosis	Skewness	Range	Min	Max	Sum	Count
BALF-BA-L-282	*300-011-034*	0.3054	0.0032	0.31	0.31	0.01688	0.00029	8.26779	-2.47799	0.09	0.24	0.33	8.55	28
BALF-BA-L-308	*300-011-048*	0.3204	0.0064	0.31	0.31	0.03345	0.00112	21.78472	4.47230	0.18	0.3	0.48	8.65	27
BALF-BA-L-309	*300-011-049*	0.2973	0.0029	0.3	0.31	0.01485	0.00022	-0.67233	-0.44978	0.05	0.27	0.32	7.73	26
BALF-BA-L-321	*300-011-058*	0.3000	0.0038	0.3	0.29	0.02000	0.00040	-0.62981	-0.03115	0.08	0.26	0.34	8.1	27
BALF-BA-L-390	*300-012-030*	0.3011	0.0026	0.3	0.3	0.01340	0.00018	2.23303	-1.15034	0.06	0.26	0.32	8.13	27
BALF-BA-L-462	*300-013-001*	0.3069	0.0029	0.31	0.31	0.01490	0.00022	0.46713	-0.75874	0.06	0.27	0.33	7.98	26
ABTL-MA-L-43	*300-013-092*	0.3036	0.0031	0.3	0.3	0.01551	0.00024	4.97410	-1.75342	0.07	0.25	0.32	7.59	25
ABTL-MA-L-51	*300-013-098*	0.3045	0.0026	0.31	0.3	0.01378	0.00019	2.56965	-1.24772	0.06	0.26	0.32	8.83	29
ABTL-MA-L-121	*300-014-058*	0.3017	0.0038	0.3	0.29	0.02071	0.00043	0.22914	0.16708	0.09	0.26	0.35	8.75	29
ABTL-MA-L-871	*300-015-151*	0.3031	0.0041	0.3	0.3	0.02074	0.00043	2.55031	0.36903	0.11	0.25	0.36	7.88	26

Table 13.Distances from primordium to the last section used for examining age estimation and inner zone distances in the serial sections. Section containing the primordium (PS), the section number to which the first two zones are confidently identified (AgeTo), the width of each section from the primordium (N-ps), the width of the space (S) and the distance from the primordium to where the first and second zone were identifiable, which is the sum of the section widths and the space to the last readable section (RFP).

Sample_ID	FAS_D	PS	AgeTo	N-1	S	N-2	S	N-3	S	N-4	S	N-5	S	N-6	S	N-7	RFP
BALF-BA-L-282	300-011-034	15	7	0.30	0.297	0.32	0.297	0.31	0.297	0.30	0.297	0.31	0.297	0.31	0.297	0.31	**3.94**
BALF-BA-L-308	300-011-048	14	5	0.30	0.297	0.31	0.297	0.31	0.297	0.32	0.297	0.33					**2.76**
BALF-BA-L-309	300-011-049	15	7	0.29	0.297	0.29	0.297	0.32	0.297	0.30	0.297	0.27	0.297	0.31	0.297	0.32	**3.88**
BALF-BA-L-321	300-011-058	13	5	0.34	0.297	0.29	0.297	0.31	0.297	0.29	0.297	0.31					**2.73**
BALF-BA-L-390	300-012-030	15	6	0.31	0.297	0.29	0.297	0.31	0.297	0.30	0.297	0.32	0.297	0.31			**3.33**
BALF-BA-L-462	300-013-001	14	7	0.30	0.297	0.32	0.297	0.31	0.297	0.32	0.297	0.28	0.297	0.29	0.297	0.30	**3.90**
ABTL-MA-L-43	300-013-092	13	6	0.32	0.297	0.32	0.297	0.32	0.297	0.30	0.297	0.30	0.297	0.32			**3.37**
ABTL-MA-L-51	300-013-098	16	6	0.32	0.297	0.30	0.297	0.29	0.297	0.29	0.297	0.32	0.297	0.31			**3.32**
ABTL-MA-L-121	300-014-058	16	6	0.30	0.297	0.29	0.297	0.30	0.297	0.29	0.297	0.27	0.297	0.35			**3.29**
ABTL-MA-L-871	300-015-151	15	7	0.30	0.297	0.33	0.297	0.29	0.297	0.29	0.297	0.30	0.297	0.31	0.297	0.29	**3.89**

As the serial sections were sister otoliths to sections which had been previously aged, the serial section which most resembled the section that was aged was identified and the distance from the primordium calculated for the aged section. The distance was calculated as:

$$\boldsymbol{Distance = Space + S_{n-1} + Space + S_{n-2} + Space + \cdots +}$$

Where $\boldsymbol{Distance}$ is the distance from the primordium where the section was read, $\boldsymbol{Space}$ is the mean width of the cut (0.297mm), and $\boldsymbol{S_{n-1}}$ is the section number width. For example, $\boldsymbol{S_{n-1}}$ is the width of the 15th section if the primordium was in the 16th section. The distances from the primordium for each sample is shown in Table 14. Most sections cut for the ageing work matched up to a position corresponding between two and three serial sections away from the primordium (≈1.2 to 1.8mm). One section was close to the next section from the primordium (≈0.6 mm). Images of the sections used in the comparative measurement estimation and the actual ageing process are shown in Figures 11 through to 40. The samples that were provided as LAMINA sections (pre-cut) were compared to those sections taken from the serially section otoliths. It was determined that these sections usually corresponded to a position on the otolith at least 2mm or more away from the primordium. An example of one of the supplied sections is shown in Figure 41 and is compared to a similar section (S9) taken from an otolith used in the serial sectioning analysis. It was estimated that in this case, section 9 (S9) would provide a zone count this is at least 1 or 2 less than the zone count from the primordia section (Table 15).

Table 14. The distances from the primordium to each of the ageing sections. PS is the section which contained the primordium, AS is the section which most resembled the ageing section, SFP is the number of sections from the primordium, Distance is the distance from the primordium to the aged section.

Sample_ID	FAS_ID	PS	AS	DFP	Distance	Figures
BALF-BA-L-282	300-011-034	15	13	2	1.204	11-13
BALF-BA-L-308	300-011-048	14	12	2	1.204	14 -16
BALF-BA-L-309	300-011-049	15	12	3/2	1.791	17 - 19
BALF-BA-L-321	300-011-058	13	12	1/2	1.224	20 - 22
BALF-BA-L-390	300-012-030	15	13	2	1.194	23 - 25
BALF-BA-L-462	300-013-001	14	11	3	1.821	26 -28
ABTL-MA-L-43	300-013-092	13	10	3	1.851	29 -31
ABTL-MA-L-121	300-014-058	16	14	2	1.184	32- 34
ABTL-MA-L-871	300-015-151	16	13	3/2	1.811	35 - 37
ABTL-MA-L-51	300-013-098	15	13	2	1.214	38 - 40

Age estimates for each of the serial sections selected for ageing are shown in Table 15. Results indicate that for 8 of the 10 samples, the first three sections after the primordia (P) produced age estimates consistent with the section containing the primordia, however the remaining four sections showed various amounts of under-estimation. In one of the other samples, the age difference started in the 2nd section from the P section while in the remaining sample the age difference started in the 3rd section from the P section. The readability of each section became more difficult as the position of the section moved further away from the primordium (Table 16).

The measured distances between the primordium out to the first and second opaque zone for each of the serial sections analysed are shown in Table 17Table 18, respectively. The measured distances showed little variability between sections. The assumed first and second opaque zones were generally reasonably clear in three to four sections taken after the section containing the primordium, however similar to the age estimation, the readability or clarity of the first two zones reduced the further away the section was from the primordium (Table 19). The mean distances for the first and second zones between samples for the serial sections was 1.01 mm and 1.44mm, respectively. These values are consistent with the measured distances for the first 2 zones in the otoliths from the larger ageing sample set (N= 1984).

Table 15. Zone count data from eight serial sections for 10 sections that were selected for serial sectioning. The first zone count was made using the primordium section (P) and the last was made on the 7th most posterior section (P-7). Numbers in the table are the number opaque zones visible in that particular section for that sample. Green shaded cells represent estimates that were equal to the estimate from the P section, yellow shaded cells indicate that the estimate was one less than the P section and red shaded cells indicate that the estimates were two or more less than the P section. Blank cells represent sections where no estimate could be made. The boarded cells represent the approximate section which was aged in the annual ageing process.

Sample_ID	FAS_ID	P	P-1	P-2	P-3	P-4	P-5	P-6	P-7
BALF-BA-L-282	300-011-034	12	12	12	12	11	11	10	10
BALF-BA-L-308	300-011-048	9	9	8	7	6	6	5	5
BALF-BA-L-309	300-011-049	10	10	10	10	9	9	8	8
BALF-BA-L-321	300-011-058	12	12	12	11	10	9	8	8
BALF-BA-L-390	300-012-030	14	14	14	14	13	13	12	11
BALF-BA-L-462	300-013-001	9	9	9	9	9	8	7	6
ABTL-MA-L-43	300-013-090	9	9	9	9	8	6	-	-
ABTL-MA-L-51	300-013-098	9	9	9	9	8	7	7	5
ABTL-MA-L-121	300-014-058	12	12	11	11	10	-	9	9
ABTL-MA-L-871	300-015-151	9	9	9	9	8	8	7	6

Table 16. Readability of sections by index from the primordium (P). Green shaded cells represent sections from which the age was estimated with confidence, yellow shaded cells indicate that an estimate could be made with lower confidence in, orange shaded cells represent sections where ages were estimated with low confidence. Red shaded cells represent sections where no estimate could be made.

Sample_ID	FAS_ID	P	P-1	P-2	P-3	P-4	P-5	P-6	P-7
BALF-BA-L-282	300-011-034	4	4	4	4	3	3	2	2
BALF-BA-L-308	300-011-048	3	2	2	2	2	2	2	2
BALF-BA-L-309	300-011-049	3	3	3	3	3	2	2	2
BALF-BA-L-321	300-011-058	4	4	3	3	2	2	2	2
BALF-BA-L-390	300-012-030	4	4	4	4	3	3	2	2
BALF-BA-L-462	300-013-001	3	3	3	3	2	2	2	2
ABTL-MA-L-43	300-013-090	3	3	3	3	2	2	1	1
ABTL-MA-L-51	300-013-098	3	3	3	2	2	2	2	2
ABTL-MA-L-121	300-014-058	4	3	3	2	2	0	2	2
ABTL-MA-L-871	300-015-151	3	4	3	3	2	2	2	2

Table 17. Distance (mm) between the primordium to the outer edge of the first opaque zone on serially sectioned otolith samples.

Sample_ID	FAS_ID	P	P-1	P-2	P-3	P-4	P-5	P-6	P-7
BALF-BA-L-282	300-011-034	1.01	1.05	0.98	0.92	0.82	0.72	0.92	
BALF-BA-L-308	300-011-048	1.03	0.97	1.01	0.98				
BALF-BA-L-309	300-011-049	1.03	1.03	0.88	0.84	0.87	1.01		
BALF-BA-L-321	300-011-058	0.93	1.07	1.11	0.96	1.14	1.01		
BALF-BA-L-390	300-012-030	1.06	1.28	1.04	1.36	1.19	1.01		
BALF-BA-L-462	300-013-001	0.96	1.08	1.15	1.07	1.07	1.01		
ABTL-MA-L-43	300-013-090	1.09	1.03	1.01	0.92	0.83			
ABTL-MA-L-121	300-014-058	0.99	1.19	0.89	1.07	1.17	0.99		
ABTL-MA-L-51	300-013-098	1.05	0.97	1.02	1.13	1.13	0.85	1.01	1.08
ABTL-MA-L-871	300-015-151	1.09	1.04	1.14	1.08	1.14			

Table 18. Distance (mm) between the primordium to the outer edge of the 2nd opaque zone on serially sectioned otolith samples.

Sample_ID	FAS_ID	P	P-1	P-2	P-3	P-4	P-5	P-6	P-7
BALF-BA-L-282	300-011-034	1.49	1.43	1.42	1.34	1.35	1.23	1.26	
BALF-BA-L-308	300-011-048	1.46	1.39	1.48	1.40				
BALF-BA-L-309	300-011-049	1.40	1.43	1.45	1.36	1.34	1.34		
BALF-BA-L-321	300-011-058	1.29	1.51	1.56	1.37	1.44	1.34		
BALF-BA-L-390	300-012-030	1.43	1.71	1.52	1.84	1.58	1.40		
BALF-BA-L-462	300-013-001	1.32	1.57	1.65	1.68	1.60	1.56		
ABTL-MA-L-43	300-013-090	1.55	1.41	1.43	1.38	1.27			
ABTL-MA-L-121	300-014-058	1.45	1.55	1.40	1.48	1.60	1.30		
ABTL-MA-L-51	300-013-098	1.39	1.30	1.47	1.64	1.54	1.29	1.48	1.38
ABTL-MA-L-871	300-015-151	1.47	1.43	1.54	1.54	1.54			

Table 19. Readability of sections by index from the primordium (N). Green shaded cells represent sections from which an estimate could be readily made, yellow shaded cells indicate that an estimate could be made with lower confidence in, orange shaded cells represent sections where an estimate could be made. Red shaded cells represent sections where no estimate could be made. The boarded cell represents the approximate section which was aged in the annual ageing process.

Sample_ID	FAS_ID	N-1	N-2	N-3	N-4	N-5	N-6	N-7	N-8
BALF-BA-L-282	300-011-034	4	4	3	3	3	2	2	1
BALF-BA-L-308	300-011-048	4	4	3	2	1	1	1	1
BALF-BA-L-309	300-011-049	4	4	3	2	2	2	1	1
BALF-BA-L-321	300-011-058	4	4	3	3	2	2	1	1
BALF-BA-L-390	300-012-030	4	4	3	3	2	2	1	1
BALF-BA-L-462	300-013-001	4	4	3	3	1	1	1	1
ABTL-MA-L-43	300-013-090	4	4	3	2	2	1	1	1
ABTL-MA-L-121	300-014-058	4	4	3	3	2	2	1	1
ABTL-MA-L-871	300-015-151	4	4	3	3	2	1	1	1
ABTL-MA-L-51	300-013-098	4	4	3	3	3	3	1	1

Figure 11. BALF-BA-L- 282 (300011034) The section used for ageing.

Figure 12. BALF-BA-L-282 (300011034) Section S13 from the sister otolith, which was closest to the aged sample, distance from the primordium was 1.204 mm.

Figure 13. BALF-BA-L-282 (300011034) Section S15 from the sister otolith which contains the primordium.

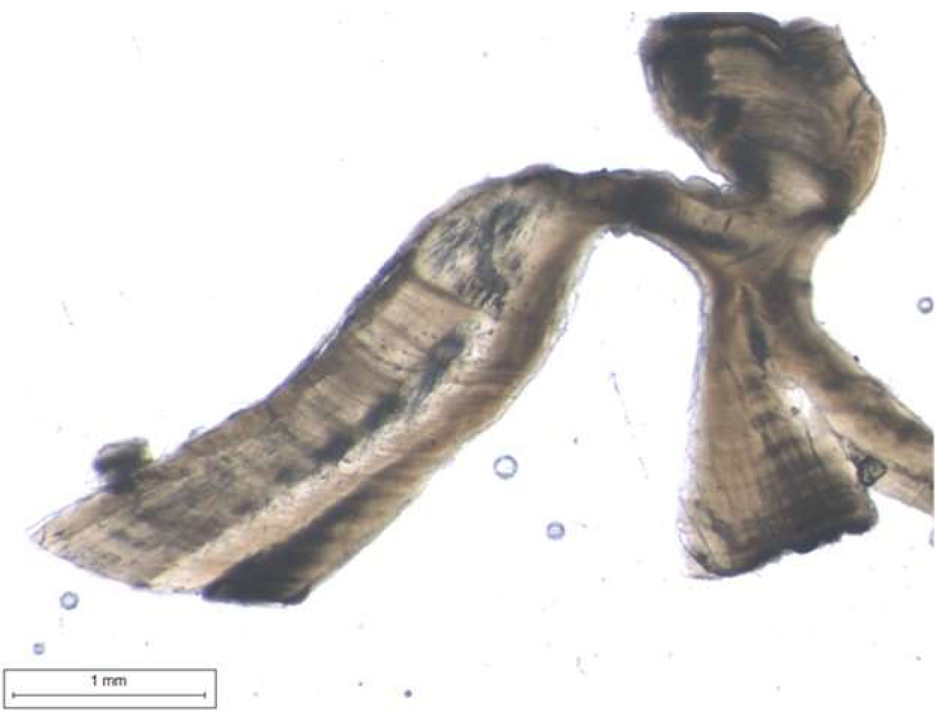


Figure 14. BALF-BA-L - 308 (300011048) The section used for ageing.

Figure 15. BALF-BA-L-308 (300011048) Section S12 from the sister otolith, which was closest to the aged sample, distance from the primordium was 1.204 mm.

Figure 16. BALF-BA-L-308 (300011048) Section S14 from the sister otolith which contains the primordium.

Figure 17. BALF-BA-L - 309 (300011049) The section used for ageing.

Figure 18. BALF-BA-L-309 (300011049) Section S12 from the sister otolith, which was closest to the aged sample, distance from the primordium was 1.174 mm.

Figure 19. BALF-BA-L-309 (300011049) Section S14 from the sister otolith which contains the primordium.

Figure 20. BALF-BA-L - 321 (300011058) The section used for ageing.

Figure 21. BALF-BA-L-321 (300011058) Section S12 from the sister otolith, which was closest to the aged sample, distance from the primordium was 1.220 mm.

Figure 22. BALF-BA-L-321 (300011058) Section S14 from the sister otolith which contains the primordium.

Figure 23. BALF-BA-L-390 (300012030), the section used for ageing.

Figure 24. BALF-BA-L-390 (300012030) Section S13 from the sister otolith, which was closest to the aged sample, distance from the primordium was 1.190 mm.

Figure 25. BALF-BA-L-390 (300012030) Section S15 from the sister otolith which contains the primordium.

Figure 26. BALF-BA-L-462 (300013001), the section used for ageing.

Figure 27. BALF-BA-L-462 (300013001) Section S11 from the sister otolith, which was closest to the aged sample, distance from the primordium was 1.821 mm.

Figure 28. BALF-BA-L-462 (300012031) Section S14 from the sister otolith which contains the primordium.

Figure 29. ABTL-MA-L-43 (300013092), the section used for ageing.

Figure 30. ABTL-MA-L-43 (300013092) Section S13 from the sister otolith, which was closest to the aged sample, distance from the primordium was 1.850 mm.

Figure 31. ABTL-MA-L-43 (300013092) Section S10 from the sister otolith which contains the primordium.

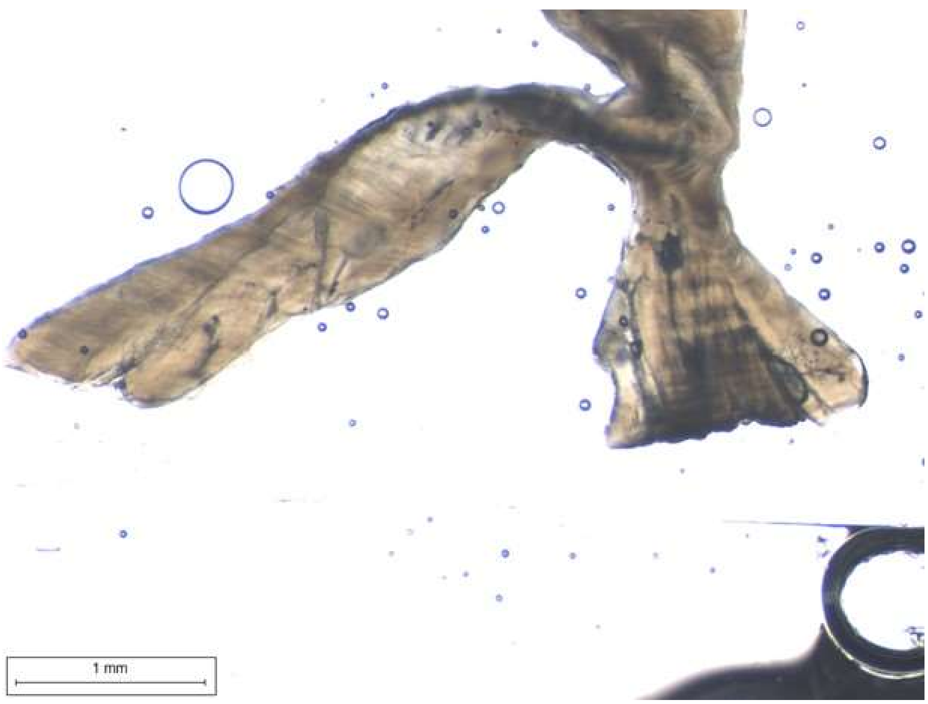


Figure 32. ABTL-MA-L-51 (300013098), the section used for ageing.

Figure 33. ABTL-MA-L-51 (300013098) Section S14 from the sister otolith, which was closest to the aged sample, distance from the primordium was 1.210 mm.

Figure 34. ABTL-MA-L-51 (300013098) Section S16 from the sister otolith which contains the primordium.

Figure 35. ABTL-MA-L-121 (300014058), the section used for ageing.

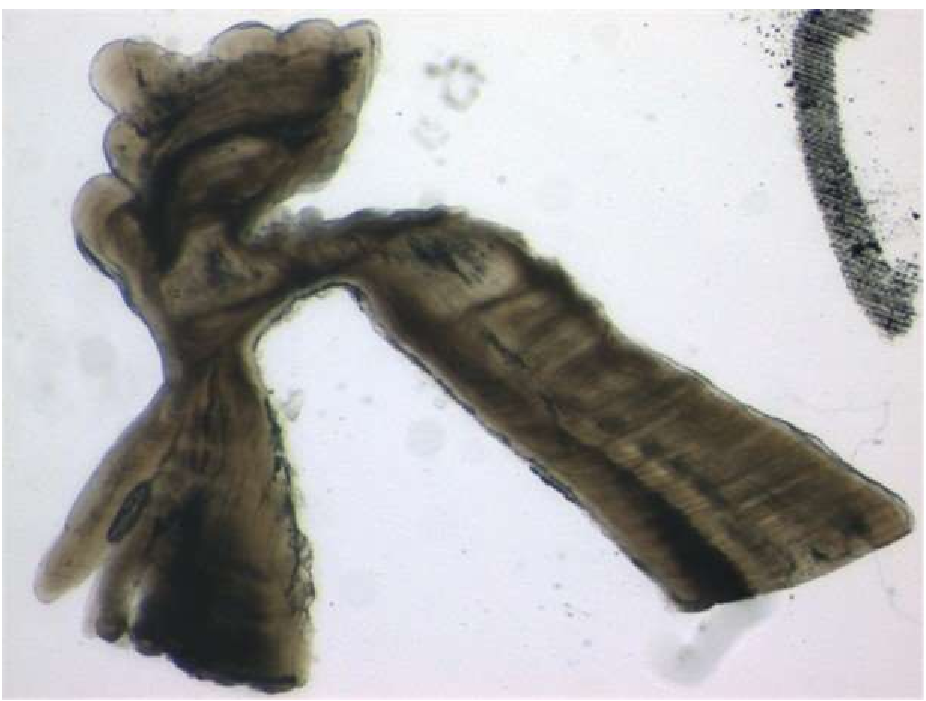


Figure 36. ABTL-MA-L-121 (300014058), section S13 from the sister otolith, which was closest to the aged sample, distance from the primordium was 1.180 mm.

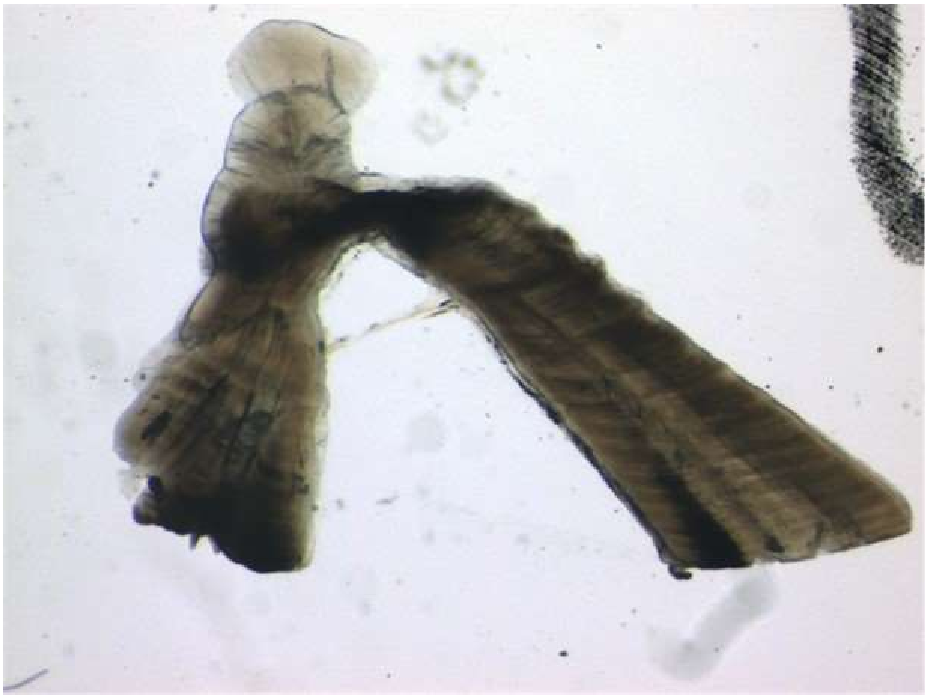


Figure 37. ABTL-MA-L-121 (300014058), Section S15 from the sister otolith which contains the primordium.

Figure 38. ABTL-MA-L-871 (300015151), the section used for ageing.

Figure 39. ABTL-MA-L-871 (300015151), section S13 from the sister otolith, which was closest to the aged sample, distance from the primordium was 1.220 mm.

Figure 40.ABTL-MA-L-871 (300015151), section S15 from the sister otolith which contains the primordium.

Figure 41. A) Example of one of the LAMINA sections (pre-cut) supplied and B) a similar section taken from sample 301013090 (ABTL-MA-L-43) used in the serial section experiment. The section in B was 4 cuts away from the section containing the primordia and estimated to be at least 2mm away from the primordia.

Daily Estimates

The otolith microstructure in ABFTR was consistent with other species of tunas examined. Split and overlapping zones made interpretation of the otolith microstructure difficult at times but for most part the pattern of translucent and opaque zones was relatively clear. Assuming that one microincrement represents one day then the resulting counts ranged from 106 to 239 days from otoliths from fish ranging in size from 41.5 to 63.8cm SFL. The age verses fish length relationship (Figure 42) showed a linear increase in age as the fish length increased. Details for the samples selected for daily ageing are shown in Table 20. The back calculated hatch dates ranged from 15th June to the 21st December, however if the estimates from the largest individual was excluded the latest hatch date was estimated to be the 13th September. A hatch date between June to September is not unreasonable when considering the known spawning duration of this species in the Mediterranean Sea occurs largely during the summer months (F. Alemany *pers comm*).

Table 20. Sample details for the 11 samples selected for daily age estimation

Sample ID	Daily age	Annual Zone count	Annual edge_type	Catch Date [dd/mm/yy]	Estimated Hatch date	SFL (cm)	Fish Weight [kg]	Otolith weight (g)
AZTI-BB-J-920	239	1	NT	17-Aug-13	21-Dec-12	63.8	4.9	
CYPR-LS-0-401	106	0	WT	29-Sep-15	15-Jun-15	41.5	1.54	0.0069
NECT-SI-0-11	129	0	WT	27-Oct-17	20-Jun-17	48	2.3	
IEO-BA-0-395	120	0	WT	17-Nov-16	20-Jul-16	44		0.0057
MVAL-SI-0-42	118	0	WT	27-Nov-14	01-Aug-14	48.5	2.42	0.0103
NECT-TY-J-15	137	0	WT	22-Jan-16	07-Sep-15	56	3.2	0.0089
NECT-TY-J-16	133	0	WT	22-Jan-16	11-Sep-15	53	3.15	
NECT-SI-0-39	134	0	WT	12-Jan-18	31-Aug-17	48	2.3	
NECT-SI-0-41	121	0	WT	12-Jan-18	13-Sep-17	46.5	2.2	
NECT-SI-0-43	136	0	WT	12-Jan-18	29-Aug-17	46	2.1	
NGBFT-SIE-0-45	192	0	WT	30-Jan-18	22-Jul-17	52	2.5	0.0104

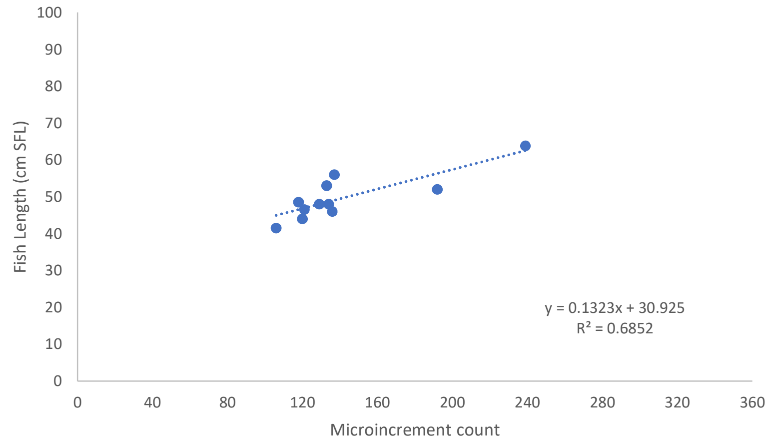


Figure 42. Estimated daily age compared to straight fork length for 10 samples selected for daily ageing.

Discussion and Conclusions

This report details the results from the second iteration of Atlantic Bluefin Tuna otoliths aged by FAS. As previously reported, when compared to the otoliths from other tuna species that we have previously examined, Atlantic Bluefin Tuna otoliths were relatively easy to interpret. The internal structure of the otoliths showed good contrast between opaque and translucent rings and while sub-annual zones were present these were generally easy to distinguish from the annual zones.

The age composition was determined from samples collected between 2013 and 2018 and is consistent with the previous study. The transect used to measure the increment widths differed from the original study and is now consistent with *Report of the ICCAT GBYP International Workshop on Atlantic Bluefin Tuna Growth (2019) (Appendix 3 – Protocol for the age reading of Atlantic Bluefin Tuna otoliths)*. The difference in protocol is primarily with the start position used for the measurement of increments which was set on the 1st apex rather than the nucleus close to the primordium. The ageing/measurement transect used in this study is more medial to the transect used in the previous ageing project, however in both studies the increment structure on all parts of the otolith section was considered when interpreting age estimates.

We also undertook a small study, using a number of serial sectioned otoliths to allow us to determine the approximate position within the otolith, relative to the primordium, that the new preparation protocols required the ageing section to be cut from within the otolith. This work also allowed us to determine the effect that section position has on the distance to the first and second annuli from the primordium and what effect the sectioning position may have on the potential to bias the age estimate. Through this process we determined that most sections taken using the new protocol (used for the main ageing component of this study) were located approximately between 1.2 to 1.8mm away from the primordium. While this did decrease the readability (visually and also confirmed by readability score) when compared to the primordial section, the "secondary section" used for age estimation should not lead to a bias in the age estimation as long as the ageing section is cut within a maximum of 1.8 mm from the core. Unfortunately, most samples provided to us as thin sections did not appear to be taken as close to the primordium as the new preparation protocols allowed. By visually comparing the shape and morphology of the sections provided with those serially sectioned, it was evident that the majority were sectioned at a position in the otolith between 1.6 -2.5mm away from the primordium. It is likely for some of these samples the first annuli (and perhaps even the second) have not been included in the otolith section and that a bias of negative one or two years in the resultant age estimates is possible. Interestingly, the distances to the first and section zones on the serial sections examined did not show much variability and the section position (for at least the 4 to 5 sections that were cut prior to the primordium section) didn't seem to change the distance between the first and second observed opaque and the primordium. The ageing and measurement data from the serial sections suggests that for the sections that are more than 2mm away from the primordium, the first opaque zone was completely missed, and it was the 2nd zone that was measured as the first.

The QA/QC results suggested that the within and between reader ageing precision was reasonably good. All distributions of age difference between Reader 1, Reader 2 and the agreed age have a mode of zero. The lowest IAPE (1.15%) was between Reader 1 and the agreed age. The maximum IAPE was 4.23% which was between Reader 1 and Reader 2. This demonstrates that the experience of Reader 1 produced less variance around repeated estimates. Morison *et al*., 1998 suggests the acceptable level of precision to be under 5.5%, this can be species specific and influenced by the age range within the samples. The index has the property that differences in age estimates for younger fish will contribute more to the final value than will the same absolute error for older fish (Anderson *et al.,* 1992). Given the distribution of age-classes represented in this study we consider the APE values to indicate high readability of the otoliths and precision of the age estimates. We did note that the APE between readers was higher than in the last study (4.23% compared to 2.80% in the previous) and suggested that the section cut through the primordia is still the preferable section to take if you are trying to reduce ageing error and ensure that the age estimates are as precise as possible. The inter-laboratory reading is yet to be completed due to COVID-19 travel on work restrictions so conclusions on the readability and precision between age readers from different laboratories is yet to be tested. It is hoped that soon the restrictions will be eased between the various states in Australia and this work can be completed.

The otolith weight-fish length relationship indicates that there were relatively fewer samples collected from fish between 150-180 cm (FL). The age-length-key was also similar to that produced in the previous study indicating further that at the very least the interpretation of the otoliths was consistent. Previous ageing has indicated that year-class strength is variable with stronger cohorts of fish present in the stock. A strong cohort of nine-year-old fish in 2013 can be tracked through each successive year through to 2018. The strong cohort in 2018 is the same as the previous year (13 years). This is most likely due to the samples coming from the first half of the year prior to the birthday. After the birthday it is assumed that most of these fish would progress to the next age-class (14) dependant on edge type. The sample size was relatively low in the 2018 sample year (n=85, compared to n=985 for 2017) which increases the variance around the relative abundance of each age-class. The evidence of cohorts moving through the data each year, which were also present in the previous ageing study, provides an internal consistency and quality control measure to this body of work.

The edge ratio of edge types was examined in the data but showed no clear pattern and were not included as a summary in this report. This result could be due to the relatively low numbers of samples collected during the period of zone formation (% of samples were collected between August and December). As we now have several thousand samples aged through various months between 2013-2018, it is recommended that this data is combined and a full diagnostic of the growth, year class strength, zone formation (edge type) etc should be completed.

Counting microincrements in thin transverse sections on a small number of YOY samples proved to be reasonably straightforward. The pattern of microstructure was similar to that observed in the transverse sections of other YOY tuna samples that we have examined. The resulting age estimates look reasonable for the given length of each sample and the back calculated hatch date from all but one sample fell within the known spawning duration of ABFT in the Mediterranean Sea. The one outlier was from a sample that was larger than the others (63.8 cm SFL) and was caught in the month of August. The annual ageing estimated this sample to be in the 1+ age class (1 with a Narrow Translucent edge). If we assume a biological birthdate for this species as the 1st June, and consider the capture month being August, then this sample should theoretically be closer to 395 days old rather than the 239 days as estimated from microincrement counts. While this is only one sample, it suggests that microincrements may not be laid down daily throughout the entirety of the first year of life. While daily ageing can be very useful for examining the age and growth of YOY samples, for this species it might be limited to samples that are less than 6 months of age.

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Appendix 1: Intellectual Property

No intellectual property has arisen from the research that is likely to lead to significant commercial benefits, patents, or licences.

Appendix 2: Staff

Mr Kyne Krusic-Golub - Principal Investigator, age estimation (Reader 1) and report

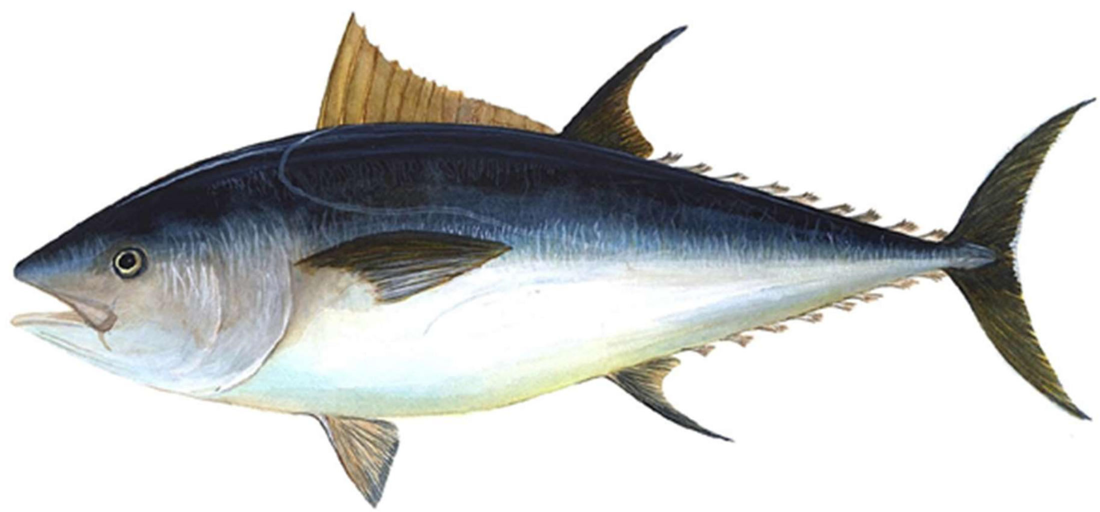
Mr Simon Robertson – Age estimation (Reader 2) and report

Admir Sutrovic – Sample Preparation

Graham Porter -Registration, sample organisation and preparation

Appendix 3: Age interpretation/document notes

Atlantic Bluefin Tuna (*Thunnus Thynnus*)



Source: Wikipedia

BACKGROUND

The Atlantic bluefin tuna (*Thunnus thynnus*) is a species of tuna in the family Scombridae. Atlantic bluefin are native to both the western and eastern Atlantic Ocean, as well as the Mediterranean Sea

BASIC BIOLOGY

This species reaches a maximum length of 300 cm TL and potentially 30 years. Spawning occurs when sea surface temperatures are between 22.6-27.5 ºC and 22.5-25.5 ºC in the Gulf of Mexico and Mediterranean Sea respectively. Spawning occurs between June and August in the Mediterranean Sea.

Source: Fishbase

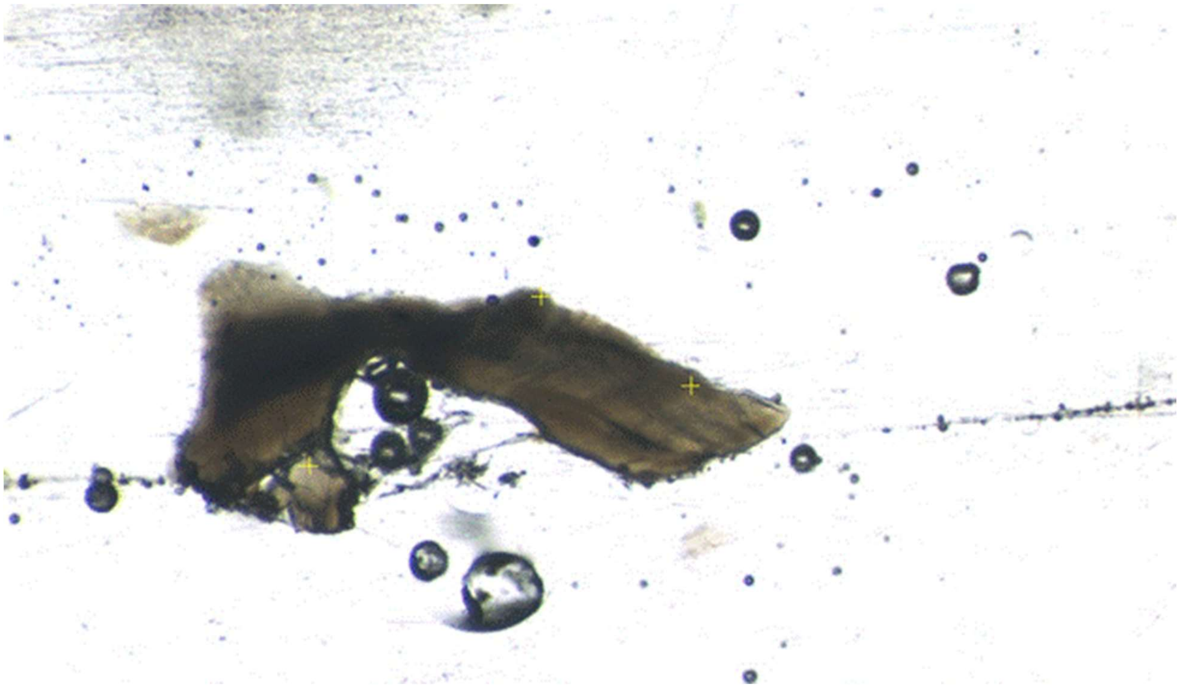
SOURCE OF SAMPLES

Samples provided by AZTI Tecnalia which were selected out of the GBYP tissue bank.

METHODS

- Weighed to the nearest 0.0001
- Individually transverse section (Isomet 1000) at approx. 380 µm
- Ground/polished down to approx. 320-340µm
- Transmitted light
- 20x magnification
- Zone counts made on both dorsal and ventral side to determine a single interpretation
- Zone marking along a transect on the ventral side from the primordia to the proximal edge
- Images taken at 20x magnification
- Zone adjustment date 1st July - See protocol document Rodrigues-Marin et al, 2019

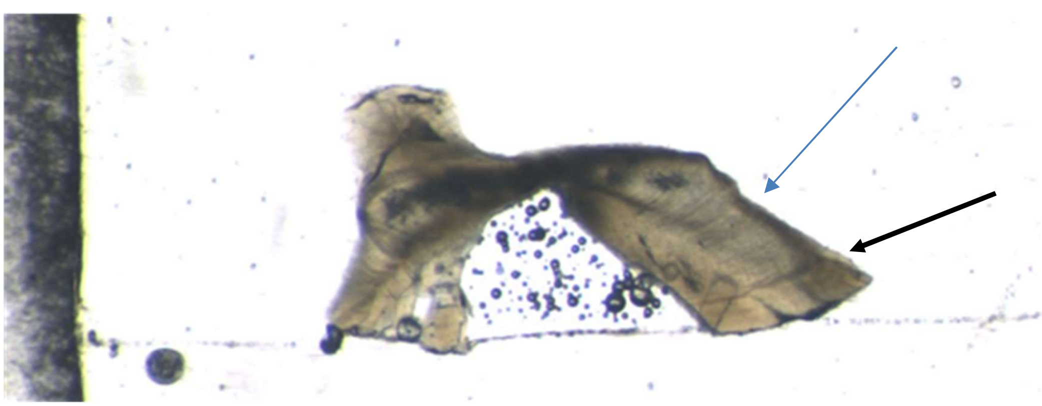
IMAGES



Example from a smaller sample. Aged at 1 WT, could be 1 O

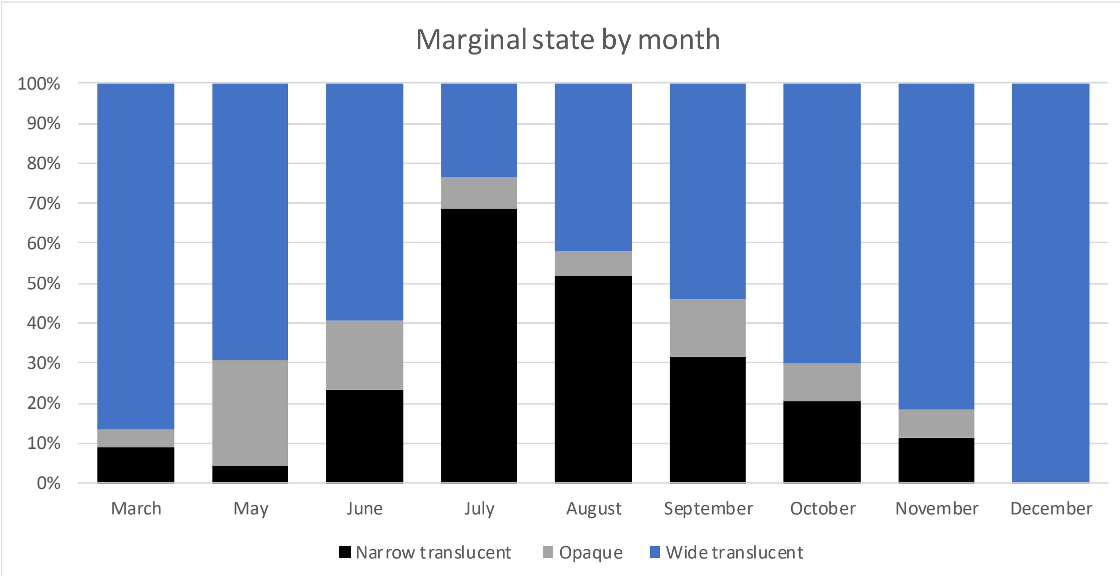
FIRST ZONE

In many of the otolith sections an opaque zone relatively close to the primordium was observed. When reviewing the literature and with our experience with other tuna species we considered that this zone was not the first annual opaque zone and was included in the age count. The average measurement from the first apex to the assumed first zone on the ventral side was 701 µm. The measurement from the primordium to the first zone was measured on the dorsal side for a few samples and the approximate distance was 700 µm.



Close inner opaque zone (arrow) not counted in the ageing protocol. Black arrow indicating assumed position of first opaque zone.

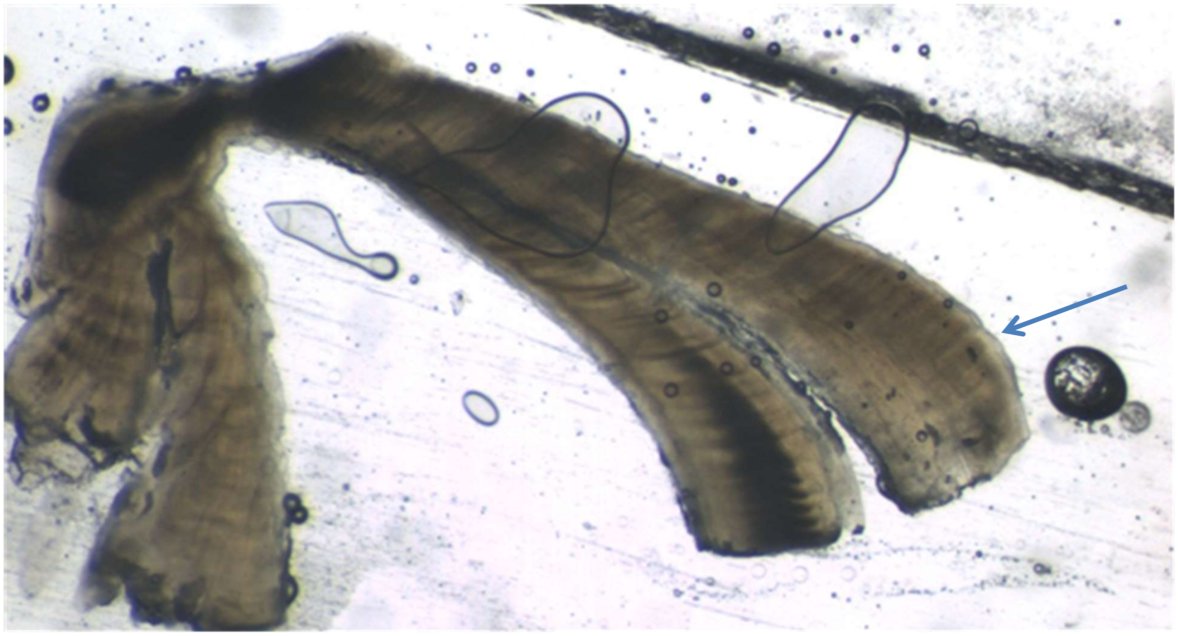
TIMING OF ZONE FORMATION/MARGINAL EDGE



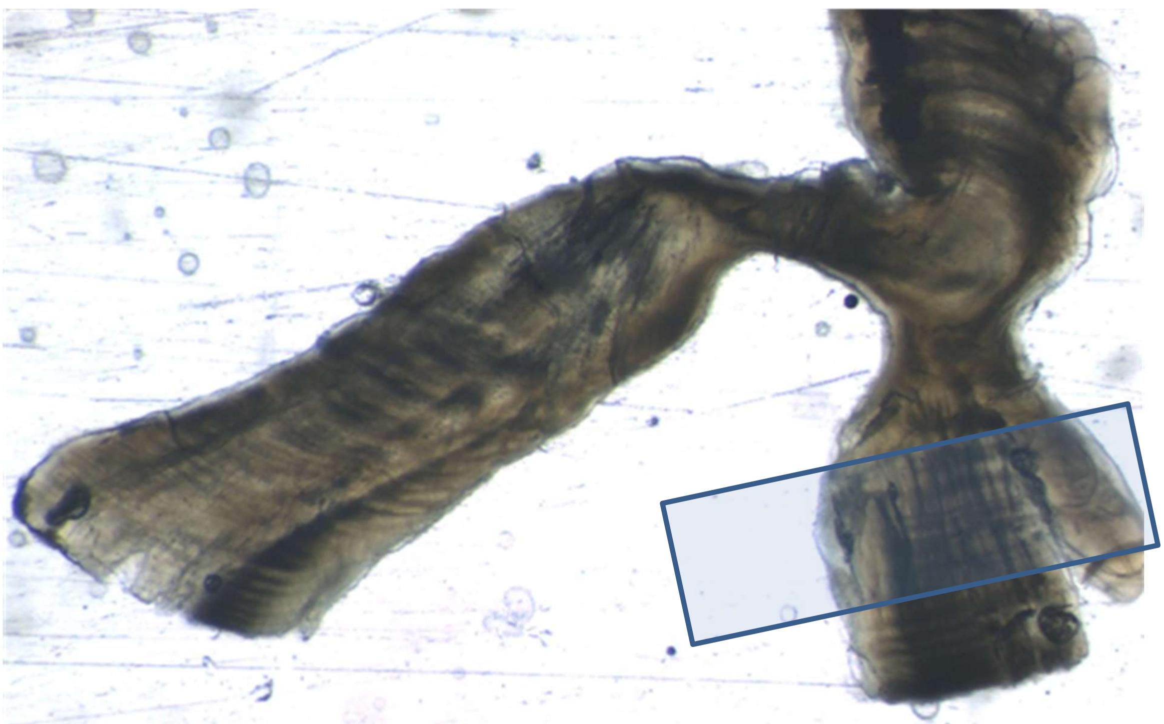
Based on the initial examination of 2000 ABFT otoliths the marginal state analysis suggests that opaque zone formation is completed by November and that the July, August and September contain the highest concentration of otoliths with opaque edges. 100% of otoliths exhibited a wide edge by December. Highest majority of Narrow translucent in May and June but evident in all months except December.

MISCELLANEOUS COMMENTS

The otolith morphology changes at approximately the 9th or 10th opaque zone where the otoliths start to curve over with reducing otolith growth in the dorsal-ventral plane and continues in the proximal plane, i.e. gets thicker not wider. Opaque zones are generally wide and diffuse for the first 3 zones and then become fine and regular thereafter. Non-annual checks are often observed between the 5th – 8th annuli.



Arrows indicate the change in otolith growth axis and the approximate position of the 9th or 10th zone.



010_099 14 O. NOTE the double zones around 5 – 10 (blue rectangle). Quite common, and I use the width of the outer zones quite a lot to tell me what type of zones I should be counting. Also, even though they are made up of split zones I often find the zones 5 to approx. 9 or 10 easier to interpret on the dorsal side.

REFERENCES/RELEVENT LITERATURE

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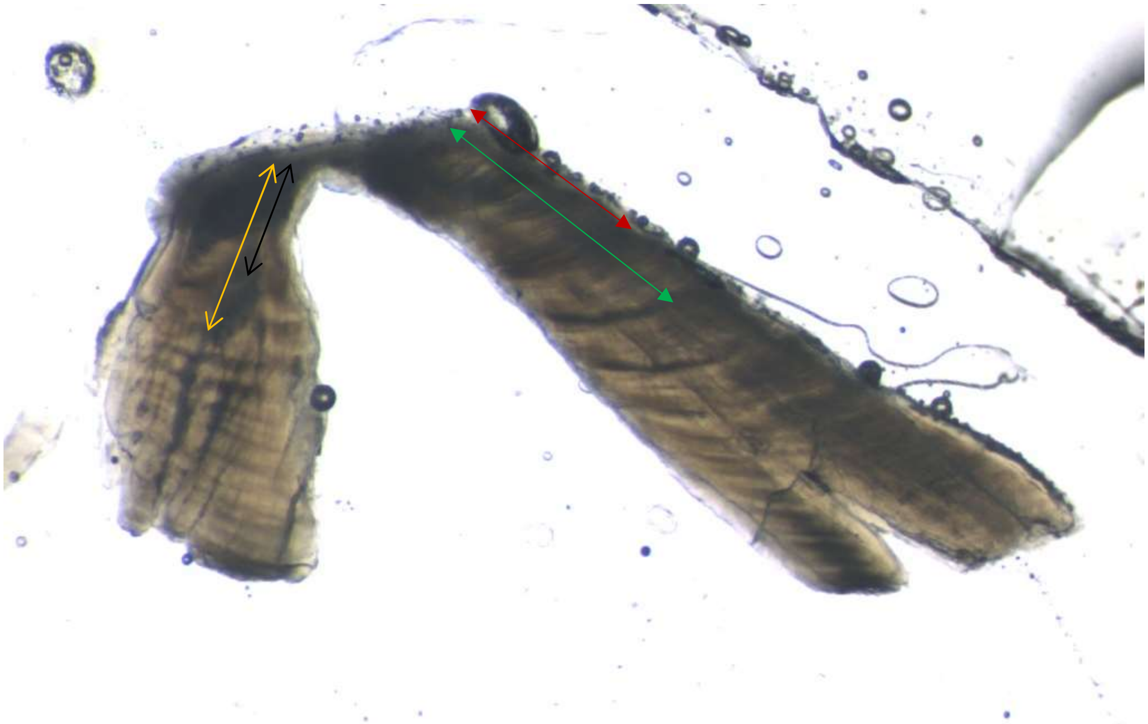
FURTHER IMAGES

003_074 - either 1 with Wide Translucent (WT) or perhaps 2nd opaque on edge (blue arrow). In this case the 2nd zone may not have finished. NOTE: 1st and 2nd opaque are generally wider and “fuzzier” than the following opaque zones

003_051 – 3 WT.   Edge maybe between Narrow Translucent (NT) and WT

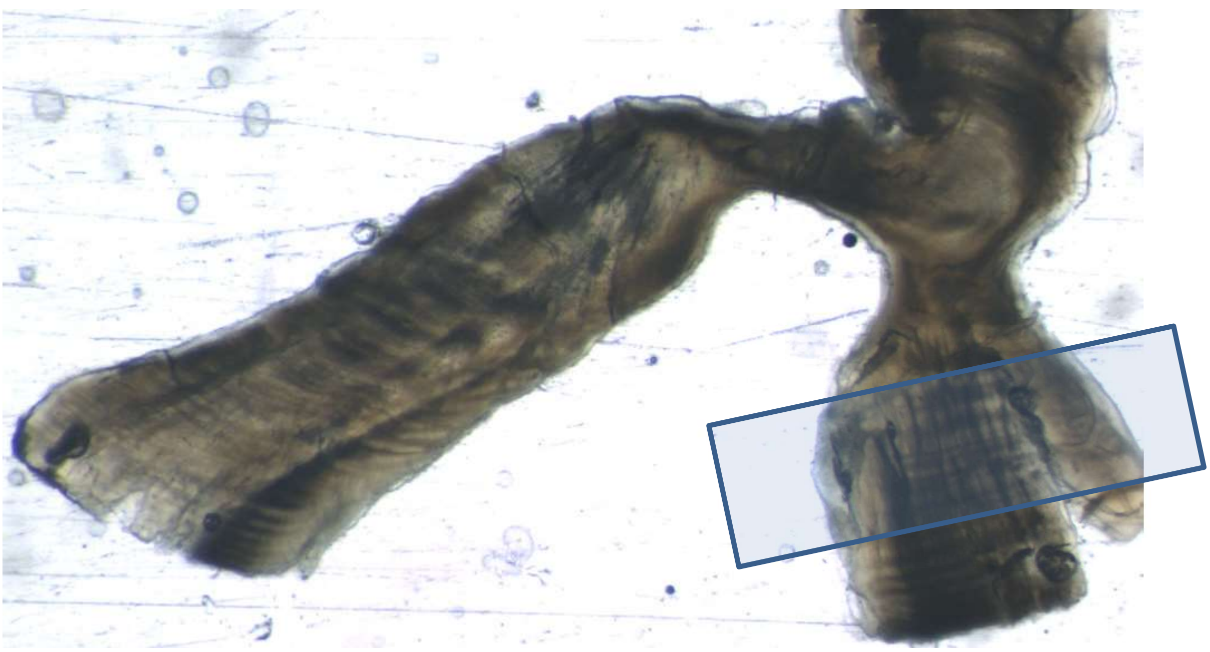
003_072 -   0+ WT, but not far off from forming fist opaque   Arrow approx. 650 µm. NOTE: from some measurements taken on various samples the first opaque looks to be complete at approx. (750-850 µm) and the second around 1000-1200 µm (ventral side) and 650-700 µm and 800-850 µm for the dorsal side



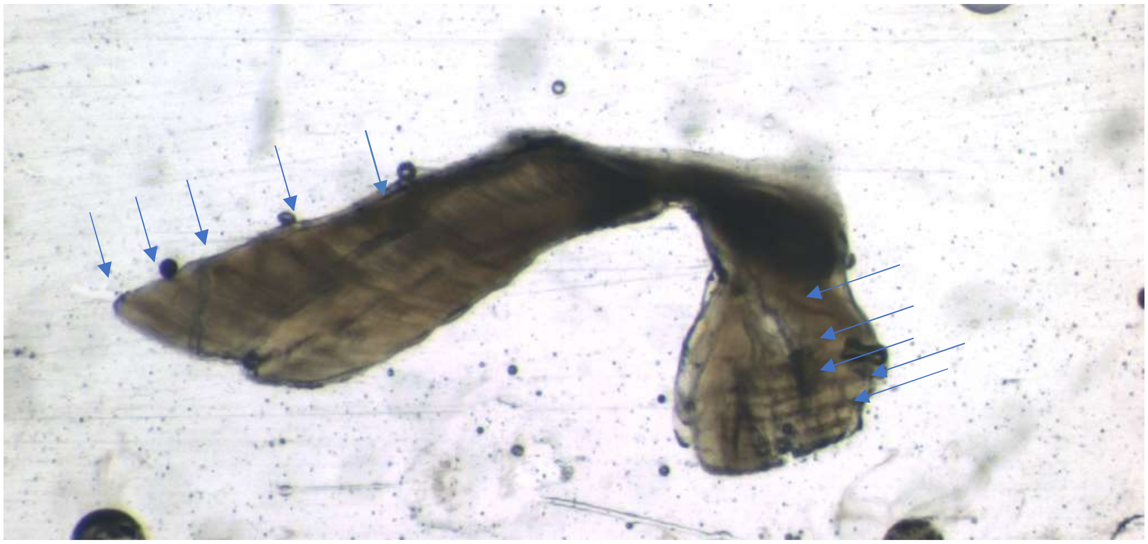
007_077 12 WT - Black arrow approx. 650-680 µm and yellow at approx. 800 µm (dorsal). Red at 850-870 µm and green at 1100-1200 um (ventral).

003_075 – large for age 2 WT or perhaps at the most 2O

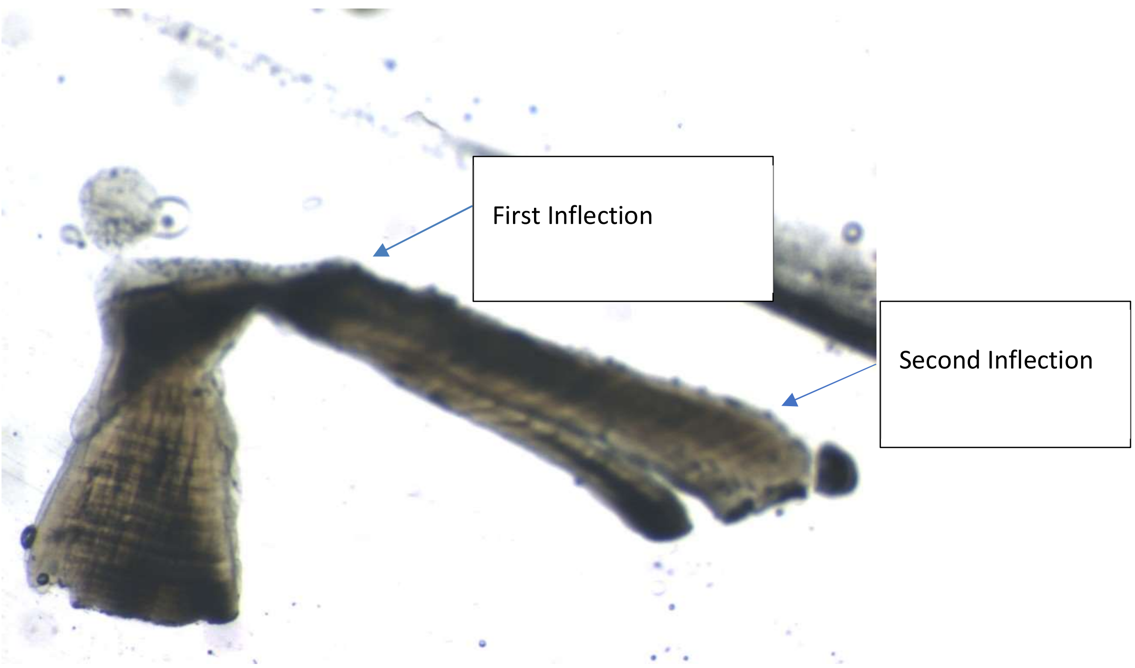
007_153 9 NT. 1st and 2nd zones clearer on the dorsal side



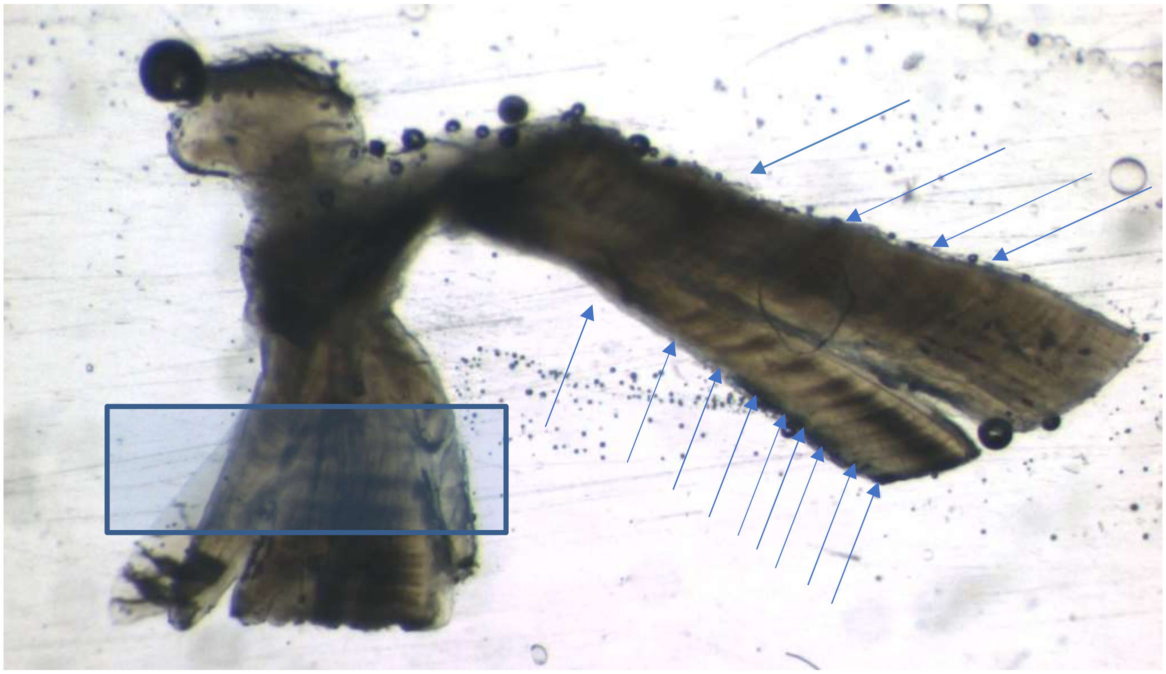
010_099 14 O. NOTE the double zones around 5 – 10 (blue rectangle). Quite common, and I use the width of the outer zones quite a lot to tell me if what type of zones I should be counting. Also, even though they are made up of split zones I often find the zones 5 to approx. 9 or 10 easier to interpret on the dorsal side.



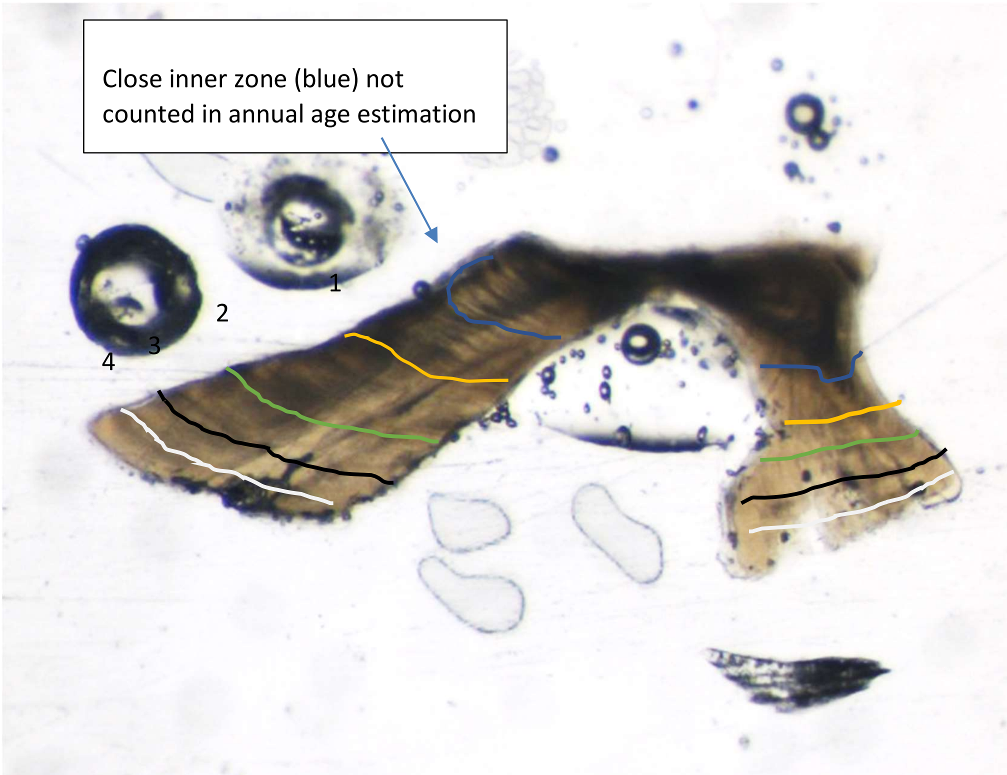
003_180. Clear 5 on both sides. Good example of distance of first and second zones on both dorsal and ventral.



007_095 - 15 WT. Tilted to show middle and outer zones on dorsal side clear. When tilted slightly differently the first 4 zones are clearer on the ventral side. For this one I got a total count using all areas and applied that count to the ventral arm. NOTE: second inflection is usually between 9-11

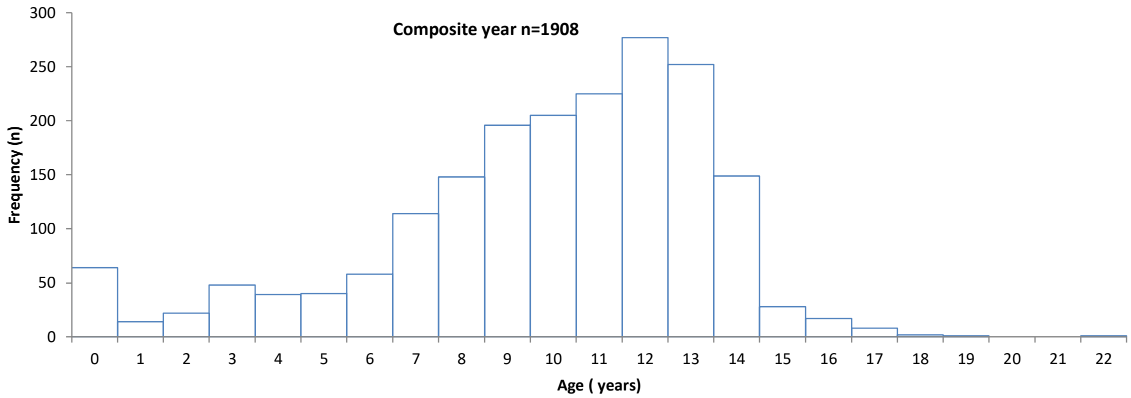


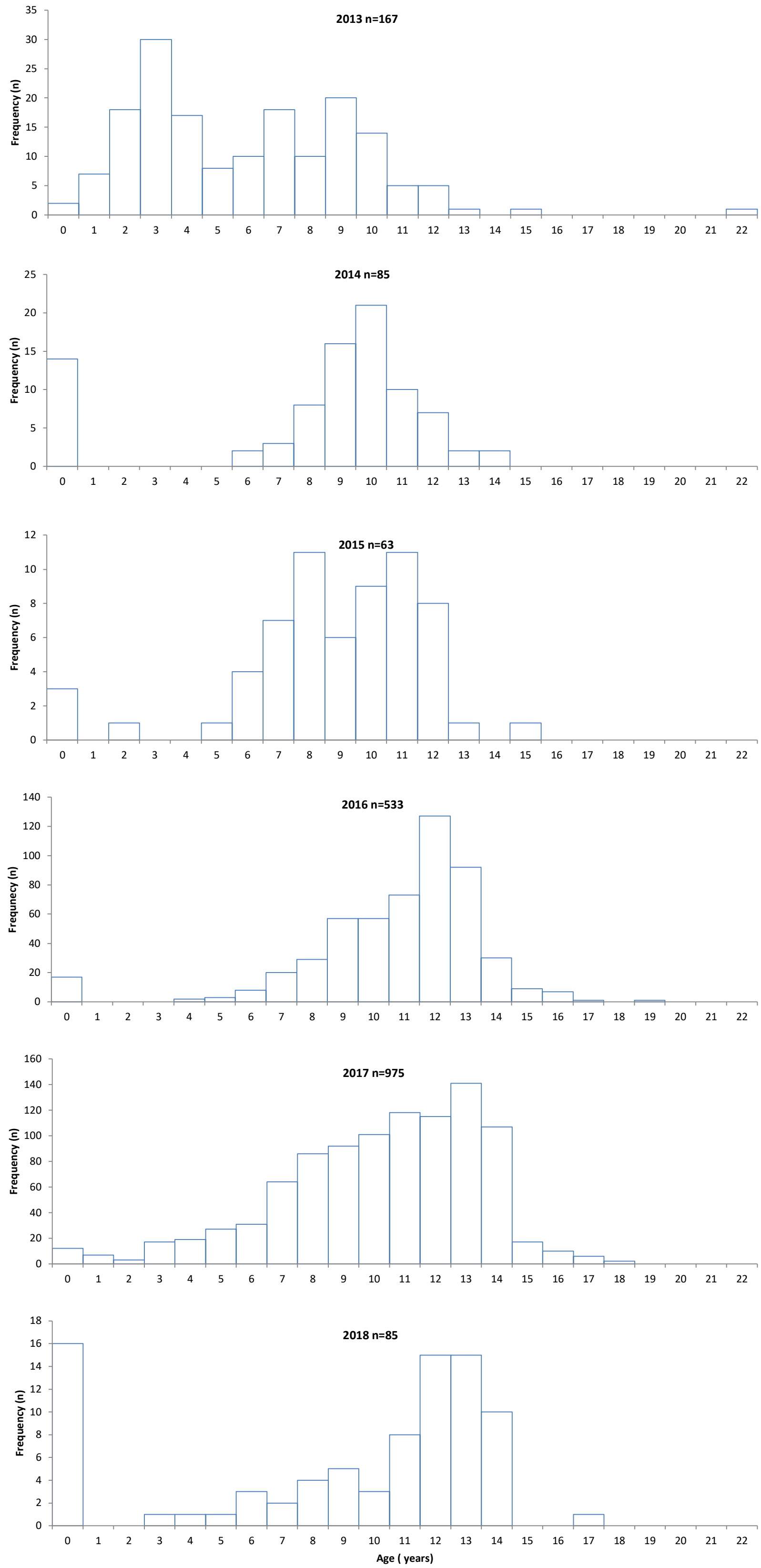
007_168. Either 9 O or 10 NT (edge difficult). Good example of split zones. In this case I have grouped them



003_054 - 4 WT. Top image an attempt to track the same features on each side. The blue is the end of close inner zone sometimes observed (probably first 50-60 days). Others are zone 1 to 4. Actual measurements in bottom image (dorsal) 1 - 0.703mm, 2 – 0.840mm, 3 – 1.003mm, 4 – 1.108mm. (Ventral) 1 – 0.689mm, 2 – 1.179mm, 3 – 1.421mm, 4 – 1.591mm

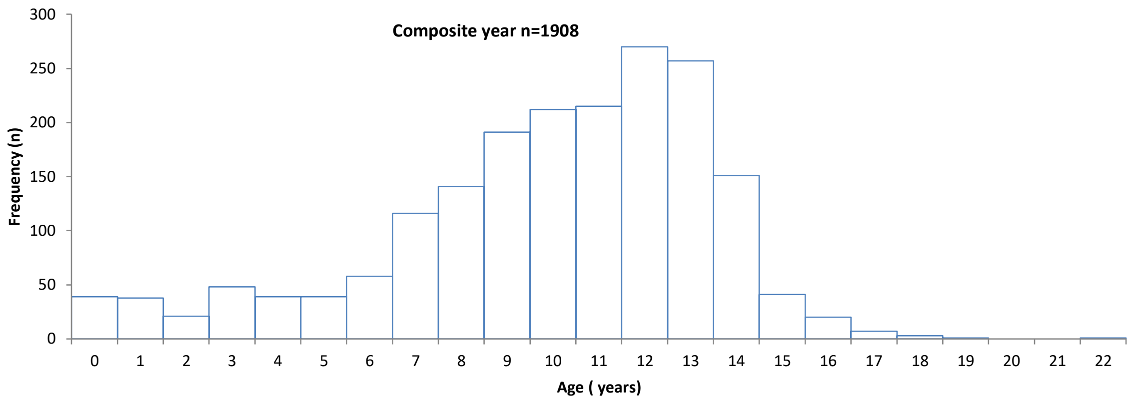
Appendix 4: Figures and tables for zone count adjusted for Biological year

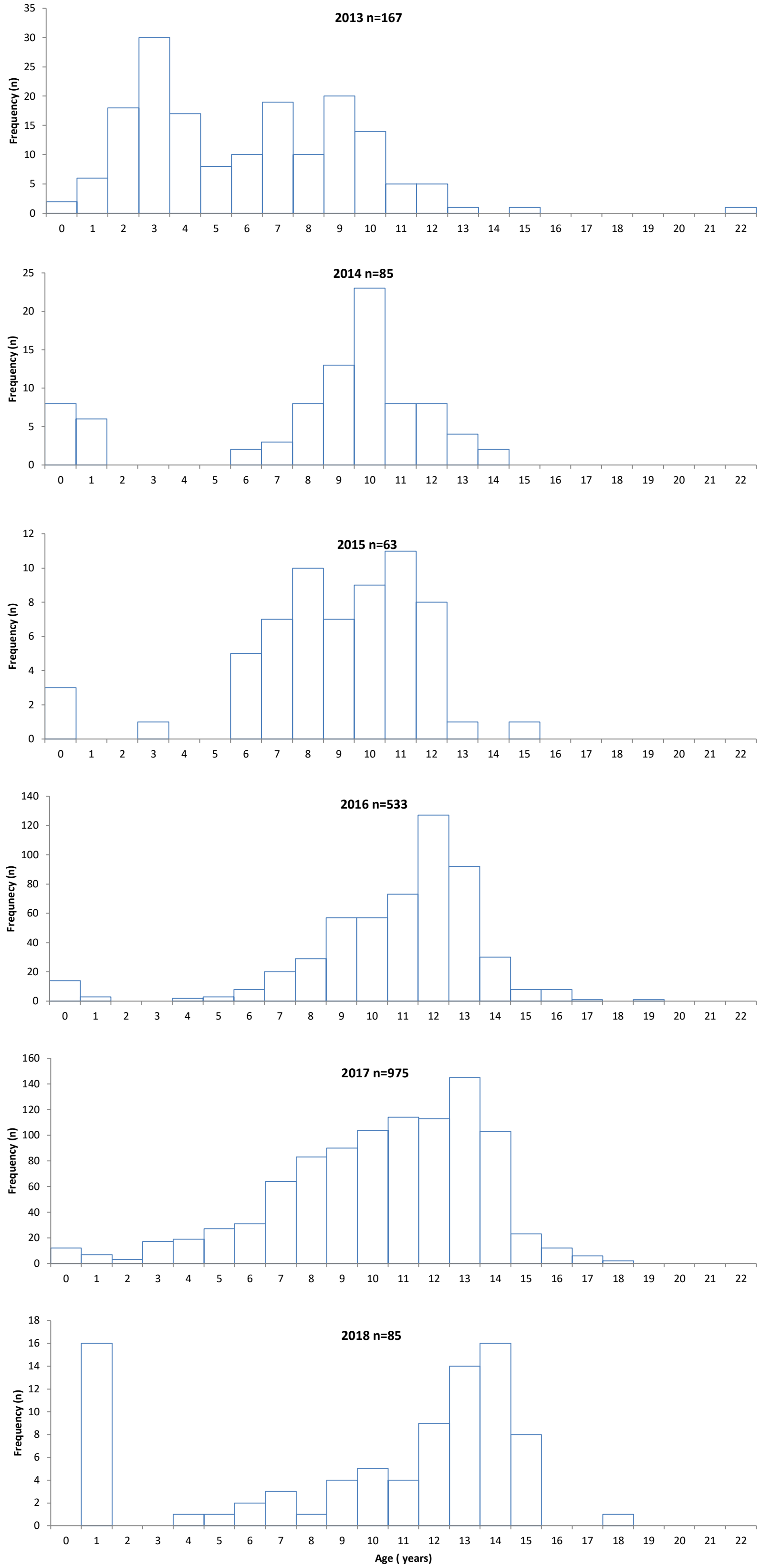



2013 n=167
2014 n=85
2015 n=63
2016 n=533
2017 n=975
2018 n=85
Frequency (n)
Frequnecy (n)
Age (years)

										Biological Age														
Length (cm SFL)	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	N
40	13																							13
45	32																							32
50	14																							14
55	4																							4
60	1	6																						7
75		7	1																					8
80		1	11	2																				14
85			4	2																				6
95			3	2																				5
100				3																				3
105			1	4	5	2		1																13
110			1	10	6	3		1																21
115			1	19	11	8	2		3		1													45
120				5	12	9	8	4																38
125					3	4	5	12	5	2														31
130				1	1	6	7	6	9	4	4													38
135					1	1	10	8	8	6		1			1									36
140						4	3	4	4	4	3													22
145						1	2	4	3	1	3		1											15
150							1	3	4	2	3													13
155						1	2	1	6	2		1												13
160						1	1	2	2	2	1		1											10
165							3	2	4		1	1												11
170							2	6	2	2														12
175							4	4		1			1											10
180							3	12	4	2	2	1	2											26
185							2	7	9	4	3	1		1										27
190							2	11	5	7	6	1	3	1										36
195								10	17	11	6	4	1											49
200								10	9	20	11	4	6	1										61
205							1	4	21	23	18	12	11	2										92
210									13	36	29	23	16	6	4									127
215								1	11	19	29	28	20	13	3	2								126
220									4	21	26	30	27	29	14	1	1							153
225									3	11	18	28	30	23	14		3							130
230								1	1	10	17	27	30	42	17	1	1							147
235									1	2	9	14	36	33	17	6	2	1	1					122
240										1	10	22	28	41	36	6							1	145
245										2	2	10	38	23	13	6	1	2						97
250											2	6	12	12	15	1	5	1						54
255												6	7	9	8	1	1	2						34
260												2	3	10	6	1	2	1						25
265										1		1	1	5		2	1	1		1				13
270											1	1	1	1	1	1								6
275													1											1
280												1												1
285																			1					1
330													1											1
N	64	14	22	48	39	40	58	114	148	196	205	225	277	252	149	28	17	8	2	1			1	1908

Appendix 5: Figures and tables for zone count data adjusted for calendar year




2013 n=167
Frequency (n)
2014 n=85
Frequency (n)
2015 n=63
Frequency (n)
2016 n=533
Frequenecy (n)
2017 n=975
Frequency (n)
2018 n=85
Frequency (n)
Age (years)

										Calendar year age													
Length (cm SFL)	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	N
40	11	2																						13
45	18	14																						32
50	7	7																						14
55	2	2																						4
60	1	5						1																7
75		7	1																					8
80		1	11	2																				14
85			4	2																				6
95			2	3																				5
100				3																				3
105			1	4	5	2		1																13
110			1	9	6	4		1																21
115			1	19	11	7	3		2	1	1													45
120				5	12	9	8	4																38
125					3	4	5	11	6	2														31
130				1	1	5	8	6	8	5	4													38
135					1	1	8	10	8	5	1	1			1									36
140						4	3	4	4	4	3													22
145						1	2	4	3	1	2	1	1											15
150							1	3	4	2	3													13
155						1	2	1	5	3			1											13
160						1	1	2	2	2	1		1											10
165							3	2	4		1	1												11
170							2	6	2	2														12
175							4	4		1			1											10
180							3	12	4	2	2	1	2											26
185							2	7	9	4	3	1		1										27
190							2	11	5	7	6	1	3	1										36
195								10	15	13	6	4	1											49
200								10	8	19	13	4	6	1										61
205							1	4	21	22	18	13	11	2										92
210									13	34	30	24	16	6	3	1								127
215								1	11	16	32	24	23	14	1	4								126
220									4	18	29	30	26	29	15	1	1							153
225									2	12	17	26	29	25	16		3							130
230								1		10	16	24	32	42	18	3	1							147
235									1	2	9	14	33	34	17	7	3	1	1					122
240										1	10	22	28	38	36	8	1						1	145
245										2	2	10	31	28	12	8	2	1	1					97
250											2	5	12	10	17	2	5	1						54
255												5	6	9	9	2	1	2						34
260												1	3	11	6	1	2	1						25
265										1		1	1	5		2	1	1		1				13
270											1	1	1	1		2								6
275													1											1
280												1												1
285																			1					1
330													1											1
N	39	38	21	48	39	39	58	116	141	191	212	215	270	257	151	41	20	7	3	1			1	1908