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Evaluating otolith increment deposition rates in bigeye tuna (*Thunnus obesus*) and yellowfin tuna (*T. albacares*) tagged in the Atlantic Ocean

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Abstract—A recent Atlantic-wide tag-recapture experiment run by the International Commission for the Conservation of Atlantic Tunas was an opportunity to directly validate otolith increment deposition rates for bigeye tuna (*Thunnus obesus*) and yellowfin tuna (*T. albacares*) in the region. Age and time at liberty were estimated by using annual and daily increment counts for sectioned otoliths from sampled fish previously injected with oxytetracycline and later recaptured. The use of annual increment counts resulted in greater age estimates than those from daily increment counts for fish >55 cm straight fork length (SFL). Use of daily increment counts led to underestimation of time at liberty for fish >55 cm SFL at recovery, compared with known times at liberty. In contrast, predictions based on annual increment counts are accurate across the entire size range of sampled fish, validating the notion that increments are deposited annually. We therefore recommend that counting annual increments be the preferred method for aging yellowfin and bigeye tuna from the Atlantic Ocean and that the use of daily increments for aging be limited to young of the year. Aging fish accurately is important for stock assessments in which data on age and growth play an increasingly essential role in examining population dynamics. It is crucial that otolith reading practices and analyses based on age data reflect the most up-to-date recommendations for age estimation.

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Fish otoliths (or ear stones) are metabolically inert structures whose concentric growth rings have been widely used as indicators of fish age. Although the exact physiological and biochemical processes of increment formation in otoliths are not well understood, it is generally accepted that the rate of deposition is regulated by variations in both biotic (e.g., growth, feeding, reproduction, and stress) and abiotic (e.g., light and water temperature) factors (Morales-Nin, 2000). For many species, the otolith increment deposition rate has been validated. However, for tropical bigeye tuna (*Thunnus obesus*) and yellowfin tuna (*T. albacares*), validation studies have been limited (Table 1), and the protocols accepted for age determination are not the same across stocks and are applied inconsistently across the size spectrums of stocks.

The results of many studies indicate that the technique of using daily increment counts is not suitable for species that are medium- to long-lived. The outer daily increments become very narrow once fish reach a certain size, making them difficult to discern (Campana, 1992; Jones, 1992). In addition, otolith increments may not be deposited daily after fish reach a certain age or size (Neilson and Campana, 2008). Annual increments, on the other hand, are generally visible throughout the life of a fish; therefore, counts of these increments are often deemed more accurate than daily increment counts, especially once a fish reaches a certain age or size (Casselman, 1983; Francis et al., 1992; Williams et al., 2013). However, until recently, scientists thought that fish inhabiting tropical environments could not be aged reliably by using this technique because their environment lacks clear seasonal signals. Increased expertise and results of recent studies validating the rate of deposition of annual increments in otoliths of fish in tropical environments have shifted that notion (Newman et al., 1996; Cappo et al., 2000; Pilling et al., 2000; Morales-Nin and Panfili, 2005; Fowler, 2009).

Annual growth increments in otolith sections have successfully been

Table 1

Summary of studies, adapted from Williams et al. (2013), in which the periodicity of the formation of growth increments in otoliths was directly validated for yellowfin tuna (YFT) (*Thunnus albacares*) and bigeye tuna (BET) (*T. obesus*) sampled in the Atlantic, Pacific, and Indian Oceans. The age validation methods used in these studies to determine the deposition rate of daily and annual increments in otoliths included oxytetracycline (OTC) mark-recapture experiments, captive experiments (captive), bomb radiocarbon dating (^{14}C), and strontium chloride ($SrCl_2$) mark-recapture experiments. LM=light microscope; SEM=scanning electron microscope; SFL=straight fork length.

Region (ocean)	Species	Sample size	Validation method	Increment type	Reading method	Time at liberty (d)	Length range (cm SFL)	Age range (years)	Source
Eastern Pacific	YFT	53	OTC	Daily	Whole otolith, LM	3–389	40–110	–	Wild and Foreman, 1980
Eastern Pacific	YFT	74	OTC	Daily	Whole otolith, LM	<515	<148	–	Wild et al., 1995
Western Pacific	YFT	12	Captive	Daily	Whole otolith, LM	3–39	25–40	–	Yamanaka, 1990[a]
Western Pacific	YFT	3	OTC	Daily	Sectioned otolith, SEM and LM	21–175	39–91	–	Lehodey and Leroy[6]
Eastern Atlantic	BET	83	OTC	Daily	Sectioned otolith, LM	10–412	44–95	–	Hallier et al., 2005
North Pacific	YFT	2	Captive	Daily	Whole otolith, LM	24–30	52	–	Uchiyama and Struhsaker, 1981
Eastern Pacific	BET	70	OTC	Daily	Sectioned otolith, LM	15–551	38–135	–	Schaefer and Fuller, 2006
Western Pacific	BET	10	$SrCl_2$	Annual	Sectioned otolith, SEM	207–2420	79–159	2–9	Farley et al., 2006
Western Indian	BET	116	OTC	Daily	Sectioned otolith, LM	3–1166	46–142	–	Sardenne et al., 2015
Western Indian	YFT	112	OTC	Daily	Sectioned otolith, LM	8–969	48–135	–	Sardenne et al., 2015
Western Atlantic	BET	12	^{14}C	Annual	Sectioned otolith, LM	–	130–175	3–17	Andrews et al., 2020
Western Atlantic	YFT	34	^{14}C	Annual	Sectioned otolith, LM	–	100–180	2–18	Andrews et al., 2020

[a] Yamanaka, K. L. 1990. Age, growth and spawning of yellowfin tuna in the southern Philippines. Indo-Pac. Dev. Manag. Program., IPTP Work. Pap. 21, 87 p.

used to age a number of tuna and billfish species (Gunn et al., 2008; Griffiths et al., 2010; Farley et al., 2013; Wells et al., 2013; Secor et al., 2014; Farley et al.[1]; Lang et al., 2017; Murua et al., 2017; Farley et al.[2]; Pacicco et al., 2021). The annual rate of increment deposition has been recently validated for bigeye and yellowfin tuna in the Atlantic Ocean by using bomb radiocarbon dating (Table 1) (Andrews et al., 2020; Pacicco et al., 2021) and for bigeye tuna in the western Pacific Ocean by using strontium chloride in mark-recapture experiments (Farley et al.[3]). Direct comparison of ages based on annual increment counts versus daily increment counts have also been carried out for tropical tuna species, and the results indicate that age estimates from daily increment counts are negatively biased compared with age estimates from annuli (Griffiths et al., 2010; Williams et al., 2013).

In the eastern Pacific Ocean, daily increment counts are used for aging yellowfin and bigeye tuna up to ~150 cm in straight fork length (SFL) (Wild and Foreman, 1980; Schaefer and Fuller, 2006; Minte-Vera et al., 2020; Xu et al., 2020). No direct aging is carried out for fish larger than 150 cm SFL, even though the longevity for bigeye tuna has been estimated to be at least 15–16 years on the basis of tagging data (Langley et al., 2008).

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[1] Farley, J., N. Clear, D. Kolody, K. Krusic-Golub, P. Eveson, and J. Young. 2016. Determination of swordfish growth and maturity relevant to the southwest Pacific stock. West. Cent. Pac. Fish. Comm. WCPFC-SC12-2016/SA-WP-11, 90 p. [Available from website.]

[2] Farley, J., K. Krusic-Golub, P. Eveson, N. Clear, F. Roupsard, C. Sanchez, S. Nicol, and J. Hampton. 2020. Age and growth of yellowfin and bigeye tuna in the western and central Pacific Ocean from otoliths. West. Cent. Pac. Fish. Comm. WCPFC-SC16-2020/SA-WP-02, 27 p. [Available from website.]

[3] Farley, J., K. Krusic-Golub, N. Clear, P. Eveson, N. Smith, and J. Hampton. 2019. Project 94: workshop on yellowfin and bigeye age and growth. West. Cent. Pac. Fish. Comm. WCPFC-SC15-2019/SA-WP-02, 14 p. [Available from website.]

In the western and central Pacific Ocean, Indian Ocean, and Atlantic Ocean, the ages of yellowfin and bigeye tuna are now routinely estimated by using a combination of counting daily increments, hereafter referred to as *daily aging* (restricted to small fish <80 cm SFL or at age 1), and counting annual increments, hereafter referred to as *annual aging* (Farley et al.[4]; Farley et al.[2]; Allman et al., 2020; Pacicco et al., 2021).

Differences in aging methods result in divergent assumptions regarding the life history of fish of the same species. The oldest aged specimens of yellowfin tuna from the Atlantic Ocean and from the western and central Pacific Ocean have been estimated to be 18 years old (Andrews et al., 2020) and 15 years old (Farley et al.[2]), respectively, and both ages are based on annual aging. In contrast, the oldest aged specimen of yellowfin tuna from the eastern Pacific Ocean is estimated to be 4 years old, an age based on daily aging (Wild, 1986). Although differences in maximum age could result from geographical differences in historical fishing pressure (i.e., age truncation of exploited populations caused by sustained, size-selective fishing), in this case, they are more likely the result of differences in aging protocols (i.e., using annual versus daily increment counts). With fish aging underpinning estimates of age composition, growth, and natural mortality in stock assessments, it is particularly important that precise and unbiased aging protocols be used across all stocks.

Age validation and comparison studies for tuna species are limited in the Atlantic Ocean. Our goals in this study were to provide additional evidence for the periodicity of daily and annual increment formation in yellowfin and bigeye tuna in the Atlantic Ocean and to provide guidance regarding the utility of daily and annual increment counts for studies of the age and growth of tropical tuna. We analyzed a subset of otoliths sourced from a large-scale tropical tuna tagging campaign that was carried out from 26 June 2015 through 28 February 2021. As part of the Atlantic Ocean Tropical tuna Tagging Program (AOTTP) of the International Commission for the Conservation of Atlantic Tunas (ICCAT) (AOTTP Coordination Team[5]), over 9000 tropical tuna were injected with oxytetracycline (OTC), a chemical marker commonly used to validate the rate of deposition of daily and annual increments in otoliths (Campana, 2001).

Once a fish is injected with OTC, the chemical is quickly incorporated into the otolith structure and leaves a permanent mark on the increment that formed at the time of tagging. Upon recovery, the mark can be detected by using fluorescence microscopy. Comparing the number of increments present beyond the OTC mark with the known time at liberty of a fish allows one to validate the rate of deposition of individual increments or growth bands. Our specific objectives therefore were as follows: 1) to test the frequency of deposition of daily and annual increments in the sagittal otoliths of yellowfin and bigeye tuna marked with OTC, 2) to compare age estimates based on daily versus annual increment counts for otolith thin sections taken from the same fish that were OTC marked and not, and 3) to test whether the sectioning plane used influenced total counts of daily increments and counts of increments after the OTC mark.

Material and methods

Otolith sampling

As part of the AOTTP, 3146 yellowfin tuna and 1967 bigeye tuna were injected with OTC. Of those injected fish, 498 yellowfin tuna and 384 bigeye tuna have been physically recovered to date (Suppl. Fig. 1). The OTC-marked fish were measured and dissected to obtain biological data (e.g., length, weight, and sex), and hard parts were extracted, cleaned, and stored for further analysis. A subsample of fish that included the most valuable samples (i.e., the largest fish and fish with times at liberty sufficient for increments to be detected beyond the OTC mark) were selected for the age analyses (Fig. 1). The subsample comprised 31 bigeye tuna and 38 yellowfin tuna marked or recaptured across the tropical and subtropical Atlantic Ocean, including waters off Brazil, Azores, Canary Islands, West Africa, St. Helena, and South Africa. Additional otoliths from large (127–172 cm SFL) yellowfin tuna not marked with OTC were donated to the project, 2 otoliths by the Centre for Environment Fisheries and Aquaculture Science (St. Helena, UK) and 11 otoliths by the University of Cape Town in South Africa (Fig. 1).

Otolith preparation

Otoliths were imaged prior to sectioning and weighed to the nearest milligram if unbroken. The most complete otolith from each pair was selected for preparation, and the core was marked prior to it being embedded in Polyplex Clear Ortho Casting Resin (Allnex, Frankfurt, Germany). Otoliths were then set in resin blocks, oriented to allow a transverse section to be cut from the center of the otolith (Suppl. Fig. 2A). Sectioning otoliths on the transverse plane allowed results and methods to be directly compared to those of previous studies on yellowfin tuna in the Atlantic Ocean (Stéquert et al., 1996; Shuford et al., 2007) and the western and central Pacific Ocean (Farley et al.[2]) and on yellowfin and bigeye tuna in the Indian Ocean (Stéquert and Conand, 2000; Sardenne et al., 2015). It also enabled us to conduct annual and daily aging on the same otolith because, once annual increment counts and OTC-mark examination were completed, the otolith could be ground thinner for daily increment analysis, leaving the remaining otolith to be retained for other purposes.

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[4] Farley, J., K. Krusic-Golub, P. Eveson, N. Clear, P. L. Luque, I. Artetxe-Arrate, I. Fraile, I. Zudaire, A. Vidot, R. Govinden, et al. 2021. Estimating the age and growth of bigeye tuna (*Thunnus obesus*) in the Indian Ocean from counts of daily and annual increments in otoliths. Indian Ocean Tuna Comm. IOTC-2021-WPTT23-18_Rev1, 28 p. [Available from website.]

[5] AOTTP Coordination Team. 2021. ICCAT Atlantic Ocean Tropical tuna Tagging Programme (AOTTP)—final narrative report, 57 p. Int. Comm. Conserv. Atl. Tunas Secr., Madrid, Spain. [Available from website.]

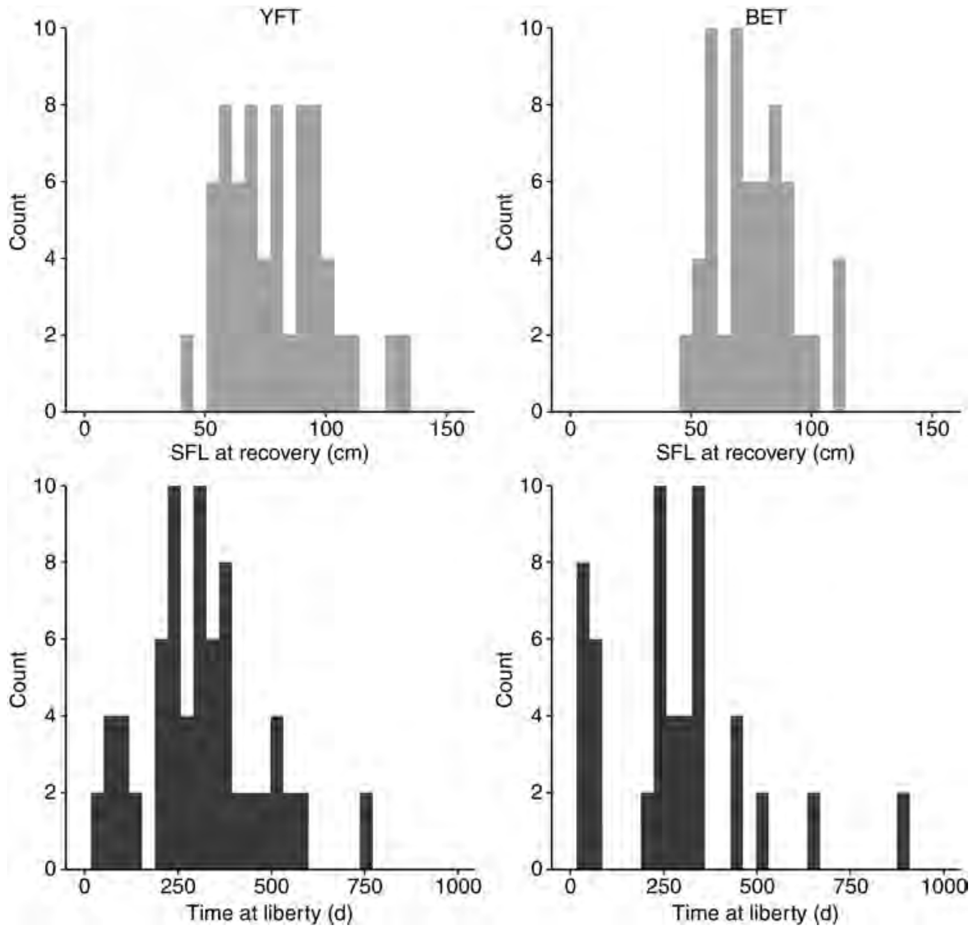

Figure 1

Frequency distributions for straight fork lengths (SFLs) at time of recovery and for times at liberty of yellowfin tuna (YFT) (*Thunnus albacares*) and bigeye tuna (BET) (*T. obesus*) sampled in the tropical and subtropical Atlantic Ocean from 2015 through 2021. Most fish (31 bigeye tuna and 38 yellowfin tuna) were injected with oxytetracycline (OTC) and recovered as part of the Atlantic Ocean Tropical tuna Tagging Program. An additional 11 otoliths from bigeye tuna and 2 otoliths from yellowfin tuna (127–172 cm SFL) were not marked with OTC.

One section approximately 500 µm thick was cut through the center of each embedded otolith, ensuring that the primordia and core area remained within the otolith section. Cuts were made by using an IsoMet 1000 precision cutting machine (Buehler Ltd., Lake Bluff, IL) and a single diamond wafering blade (102 × 0.3 mm) set to a cutting speed of 7.5 Hz (450 rpm). To allow detection of OTC marks and annual aging, sections were mounted on microscope slides (76.2 × 25.4 mm) by using thermoplastic resin (Cystalbond 509, Electron Microscopy Sciences, Hatfield, PA), with the side of the section farthest away from the core facing up. Each section was ground to a thickness between 320 and 350 µm with wet-and-dry sandpaper (800 and 1200 grit), lubricated with distilled water. Once annual increment counts and the OTC-mark examination were completed, the transverse sections were further ground down, by using 1200-grit wet-and-dry sandpaper, to a thickness of approximately 60–90 µm to reveal the daily increment structure. The sections were polished with 2-µm aluminum oxide slurry against a felt pad, rinsed, and dried.

For the donated otoliths sampled from large fish, slides were prepared only for use in annual aging for the following reasons: the discrepancy between annual and daily increment counts already reported for otoliths from large specimens caught in the Pacific Ocean (Williams et al., 2013); the value of preserving prepared slides for annual age estimation training purposes, especially considering that otoliths from very large fish are difficult to source; and the need to ensure that the otoliths remaining from each pair are still available for future analyses or validation work (e.g., age estimation through near-infrared spectroscopy or age validation through the use of bomb radiocarbon dating).

Age estimation with otoliths

Annual increment counts The methods used for the annual aging of bigeye tuna and yellowfin tuna follow those developed for other tuna species that are routinely aged at Fish Ageing Services, in Queenscliff, Australia (Farley et al., 2006; Gunn et al., 2008; Farley et al., 2013), and those in information available in the literature at the time (i.e., Lang et al., 2017). Information on aging bluefin tuna sampled in the Atlantic Ocean was also used as a basis to aid interpretation of what may constitute an annual growth zone in bigeye tuna and yellowfin tuna from the Atlantic Ocean (Rodríguez-Marín et al., 2007; Neilson and Campana, 2008; Rodríguez-Marín et al., 2014; Secor et al., 2014). Before reading each section, the ground surface of the otolith was covered in a thin layer of low-viscosity immersion oil (Type A, Cargille-Sacher Laboratories Inc., Cedar Grove, NJ) to fill in any residual scratches and aid in the imaging process. Sections were examined at 25× magnification under transmitted light with a Leica M125 C routine laboratory stereo microscope (Leica Microsystems, Wetzlar, Germany).

The annual age of each section was estimated by counting opaque growth zones (which appear dark under transmitted light). The last fully completed opaque zone before the otolith edge was counted only if translucent otolith material was detected between the outer edge of the last opaque zone and the otolith edge. All age readings were made without knowledge of fish size, otolith weight, sex, location of capture, or time at liberty. A single TIFF image was captured, and the distances between the primordium and the outer edge of each opaque zone was measured. For each section, a readability score of 1–5 was assigned, with 1 meaning a zone pattern could not be interpreted and 5 meaning there was a clear pattern of alternating opaque and translucent zones. Also recorded were any relevant comments related to the otolith structure or interpretation of the zones.

Daily increment counts Daily increments were counted by using a Leitz Diaplan compound microscope (Leica Microsystems) with transmitted light at various magnifications ranging between 400× and 1000×, depending on the area of the otolith being interpreted. The interpretation of the otolith microstructure in tuna can be subjective, and it can often be difficult to distinguish sub-daily increments from the assumed daily increments. These difficulties were recognized by Shuford et al. (2007) and Sardenne et al. (2015), and, as per their methods, the sub-daily increments were characterized by faint and incomplete rings. Further complicating the daily increment count was the presence of subsections within some otoliths for which little or no increment pattern could be detected. Because daily increment widths tend to be autocorrelated (Campana, 1992), the counts of micro-increments within these subsections were interpolated from the surrounding areas. The method used for the interpretation of daily increments is consistent with those methods published for reading transverse sections (Lehodey and Leroy[6]; Shuford et al., 2007; Sardenne et al., 2015) and frontal sections (Schaefer and Fuller, 2006). The otolith sections were read at least 2 times by the single reader before a final count was completed and the number of daily increments was recorded.

Validation of increment deposition rate: oxytetracycline-mark detection

Each otolith section from a fish that was injected with OTC was examined for the presence and position of the OTC mark by using a Leitz Diaplan compound microscope fitted with a 100-W incident ultraviolet light source and a Leitz D filter block (Leica Microsystems, with excitation filter of 450–520 nm) to suit the fluorescent properties of the OTC. Images were taken at magnifications of 25× and 40×, and, if the OTC mark was very faint, at 100× magnification with a DFK 31AU03 digital camera (The Imaging Source, Charlotte, NC) attached to the microscope and the camera's corresponding image analysis software (IC Measure, vers. 2.0.0.245; The Imaging Source). Scale bars appropriate to the magnification were included on each of the images. Images were collected for 2 reasons: 1) to capture an image of the section when the OTC mark was at its brightest, given that continual exposure to UV and the further processing of sections for daily increment counts can result in the OTC mark becoming very faint or even disappearing within the section, and 2) to detect, through the use of measured distances, the approximate position of the OTC mark in the otolith section, when not viewing the section under ultraviolet light. To make this detection, the distances between the OTC mark and the otolith margin on both the inside and outside of the ventral arm were measured by using the image capture software.

The number of annual increments observed between the position of the OTC mark (based on the measured distances taken in the previous step) and the otolith edge were recorded following the annual aging protocols detailed in the previous section. With annual aging, the direct comparison between the count of annual increments after the OTC mark and the time at liberty can be misleading because, unlike with daily increment counts, age estimates based on counts of opaque zones do not provide a fractional age (e.g., a fish at liberty for only 4 months could have an opaque zone between the OTC mark and the edge if the opaque zone completed formation shortly after tagging). It is possible to estimate a fractional age if the timing of annual zone formation is well known. However, for yellowfin and bigeye tuna sampled in the Atlantic Ocean, annual aging is only in its infancy, and the timing of zone formation for fish caught in the equatorial and southeast Atlantic Ocean is currently unknown. Therefore, to

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[6] Lehodey, P., and B. Leroy. 1999. Age and growth of yellowfin tuna (*Thunnus albacares*) from the western and central Pacific Ocean as indicated by daily growth increments and tagging data. Standing Comm. Tuna Billfish 12, Work. Pap. YFT-2, 21 p. Ocean. Fish. Progr., Secr. Pac. Community, Noumea, New Caledonia. [Available from website.]

provide an alternative, yet practical, comparison between estimated time at liberty based on annual aging and the true time at liberty, we calculated an adjusted estimate (in fractional years) for each of the OTC-marked sections using methods modified from those used by Cappo et al. (2000). This adjusted count was based on the following: 1) the position of the first opaque zone after the OTC mark, relative to the OTC mark, 2) the number of annual zones observed after the mark, and 3) the relative distance between the last assumed annual opaque zone and the otolith edge—assuming that one translucent and one opaque zone are formed each year.

The sections prepared for daily increment counts were reexamined, and increment counts were made from the OTC mark to the otolith edge along a count path similar to that used for the total daily increment counts. For the sections in which the OTC mark had become undetectable because of additional otolith processing, the positions of the OTC marks were determined from the measurements taken on each of the otolith sections during the imaging procedure.

Longitudinal sections

To examine whether the sectioning plane that was used influenced increment counts, longitudinal (frontal) sections of the remaining otolith for a subset of fish (6 bigeye tuna and 5 yellowfin tuna) were prepared following methods used by Schaefer and Fuller (2006) (Suppl. Fig. 2B). These longitudinal sections were used to age the subset of fish following methods used by Williams et al. (2013), in which opaque zones were counted from the primordia to the otolith margin along the clearest count path in the area of the section adjacent to the proximal edge. The total count of daily increments and the number of daily increments between the OTC mark and the edge were recorded.

Analytical methods

For the comparison of age estimates, daily and annual increment counts from the same individuals were plotted against each other to help visualize differences. For the exercise of validating the increment deposition rate, daily increment, annual increment, and adjusted annual increment counts after the OTC mark were plotted against time at liberty to aid visualization of differences between estimated and true times at liberty. Age-agreement tables and age-bias plots (Ogle, 2016) were constructed, and an Evans–Hoenig test of symmetry (Evans and Hoenig, 1998) was applied to evaluate bias in the times at liberty estimated by using daily increment and adjusted annual increment counts. The Evans–Hoenig test is designed to detect bias in paired-age data and, in our study, was applied to paired data for estimated and true times at liberty. A significant P-value (≤ 0.05) indicates that differences between estimated times at liberty and true times at liberty were due to bias and not random error, implying that daily and annual increments were not strictly deposited on a daily and annual basis. For these calculations, times at liberty were converted to integer month to reduce the number of categories being compared.

To determine whether the sectioning plane that was used influenced total and post-OTC-mark counts of daily increments, the increment counts from transversely sectioned otoliths were compared with counts from frontal sections of otoliths. A Bland–Altman plot (Bland and Altman, 1999) was used to evaluate bias and define the interval of agreement between reads (total counts) from otoliths sections made with the 2 cutting planes.

Results

Age estimation: daily versus annual increment counts

Detailed results pertaining to each otolith analyzed for daily and annual increment counts are presented in Supplementary Tables 1 and 2. The annual increments in the form of one opaque and one translucent zone, although often difficult to interpret from sub-annual marks (also known as *check marks* or *split zones*), were apparent in all transverse sections examined, except in sections of otoliths from certain young-of-the-year samples for which the first zone had not yet formed. Zone structure was observed on both the dorsal and ventral side of otolith sections, although our experience in aging otoliths from samples of other tuna species indicates that the zone structure within the ventral arm is generally easier to interpret in comparison to that within the dorsal arm and that counts of assumed annual increments on the ventral side are likely to provide for more accurate age estimates. Annual increment counts ranged from 0 to 3 for bigeye tuna and from 0 to 10 for yellowfin tuna. A transverse section of an otolith from one of the oldest yellowfin tuna examined in this study (not OTC marked) appears in Figure 2, which shows the positions of each of the opaque zones used to assign age based on counts of annual increments.

Clear daily increments, consisting of alternating opaque and translucent zones, were observed throughout most of the ventral arms of the otolith sections examined. However, for some areas in many of the otolith sections examined, interpretation of the daily increment pattern was subjective. Overlapping and split increments often made interpretation difficult, and no increments were visible in some regions within the otolith sections. The number of regions that were difficult to interpret within an otolith generally increased with an increase in relative otolith size. Similar to that observed in otoliths from yellowfin tuna caught in the Pacific Ocean (Lehodey and Leroy[6]), the core area in otoliths examined in this study consisted of a primordium and approximately 8–10 fine daily increments (with widths of ≈2 µm) followed by a check mark. Increment widths became progressively wider, and at approximately the fifteenth increment, growth increments became very optically dense and their thickness increased up to 20 µm at their maximum concavity. Counts from the first obvious zone to the first apex averaged between 30–35 increments for both species. The internal zones closer to

Figure 2

Image of a section of an otolith from the oldest individual aged in this study: an Atlantic Ocean yellowfin tuna (*Thunnus albacares*) captured on 18 October 2018, measuring 161 cm straight fork length and weighing 80.3 kg. The white circles indicate the positions of the opaque zones used to assign age based on counting of annual increments. The letter *N* indicates the position of the nucleus. The opaque zone on the otolith margin (black arrow) is incomplete and was not included in estimation of integer age. This otolith was donated to the study by the Centre for Environment Fisheries and Aquaculture Science and was not marked with oxytetracycline.

the sulcus were more regularly spaced and did not have as much overlapping, splitting, or merging of zones compared with the zones close to the distal and ventral edges. Given these observations, it is our recommendation that the initial count path should include the clearer internal increments rather than run close to the distal and ventral edges of the otolith.

Unfortunately, for bigeye and yellowfin tuna, after approximately 150–180 daily increments, the internal structure of otoliths becomes difficult to interpret and a count path closer to the outer edge of the ventral arm needs to be used. As per the method of Lehodey and Leroy[6], the reading of the outer increments was directed along the axis of maximum concavity of increments. In many of the otoliths examined, subsections along the preferred reading path had a zone structure that was either difficult to interpret or not present. These areas were relatively common in otoliths from individuals of both species and often occurred with a change in the otolith growth plane, and in visual comparisons made with the otolith images captured during preparation for aging, the positions of these areas often corresponded to the positions of the opaque zones marked on images. In these cases, the zone structure adjacent to these areas usually had a clearer pattern of alternating opaque and translucent zones. For these cases, daily increments were counted in the adjacent area until increments could again be interpreted clearly along the preferred aging path. If the adjacent area was also unclear, interpolation was used (Suppl. Fig. 3). Daily increments were detectable close to the outer edge even in the largest otoliths from individuals of both species (114 and 159 cm SFL for bigeye and yellowfin tuna, respectively). These increments were at least 1.5–2 µm wide and well above the minimum resolution of light microscopy.

Age estimates, both daily increment counts and raw annual increment counts, are presented in Figure 3 and in Supplementary Tables 1 and 2. In the count comparison exercise, sizes of yellowfin tuna at recapture ranged from 45 to 159 cm SFL, daily increment counts ranged from 247 to 1168, and annual increment counts ranged from 0 to 8. For bigeye tuna, recapture sizes ranged from 50 to 114 cm SFL, daily increment counts ranged from 248 to 642, and annual increment counts ranged from 0 to 3. Age estimates of bigeye tuna with no annual zones were strongly negatively biased compared with the corresponding daily increment counts (Fig. 3). However, the edge type for all 6 otolith sections with no annual increments was classified as opaque, indicating that the fish was likely closer to age 1 than to age 0. Although it is difficult to objectively compare raw annual increment counts to fractional ages obtained from daily increment counts, for fish at sizes beyond 55 cm SFL (~age 1), age estimates based on annual aging tended to be higher than estimates based on daily aging for both species, with differences generally more pronounced in fish greater than 100 cm SFL for which age estimates from annual aging were consistently higher than estimates from daily aging (Fig. 3). The most drastic difference between age estimates was observed for a yellowfin tuna of 159 cm SFL: it had a daily increment count of 753 and an annual increment count of 8.

Validation with oxytetracycline marking

Oxytetracycline marks were detected in 32 of the 38 otoliths from yellowfin tuna and 20 of the 31 otoliths from bigeye tuna that were injected as part of the AOTTP and included in this study. For most examined otoliths, the OTC mark was clear and easy to detect from the background fluorescence of the otolith section (Fig. 4). Lengths at recapture of the fish successfully marked with OTC ranged from 45 to 159 cm SFL for yellowfin tuna and from 50 to 114 cm SFL for bigeye tuna (although the length ranges for fish with OTC marks on their otoliths are the same as those for all recaptured fish, not all recaptured fish had an OTC mark on their otolith). Known times at liberty ranged from 7 to 995 d for yellowfin tuna and from 18 to 879 d for bigeye tuna.

The estimated times at liberty (i.e., counts of increments after the OTC mark) were plotted against the known

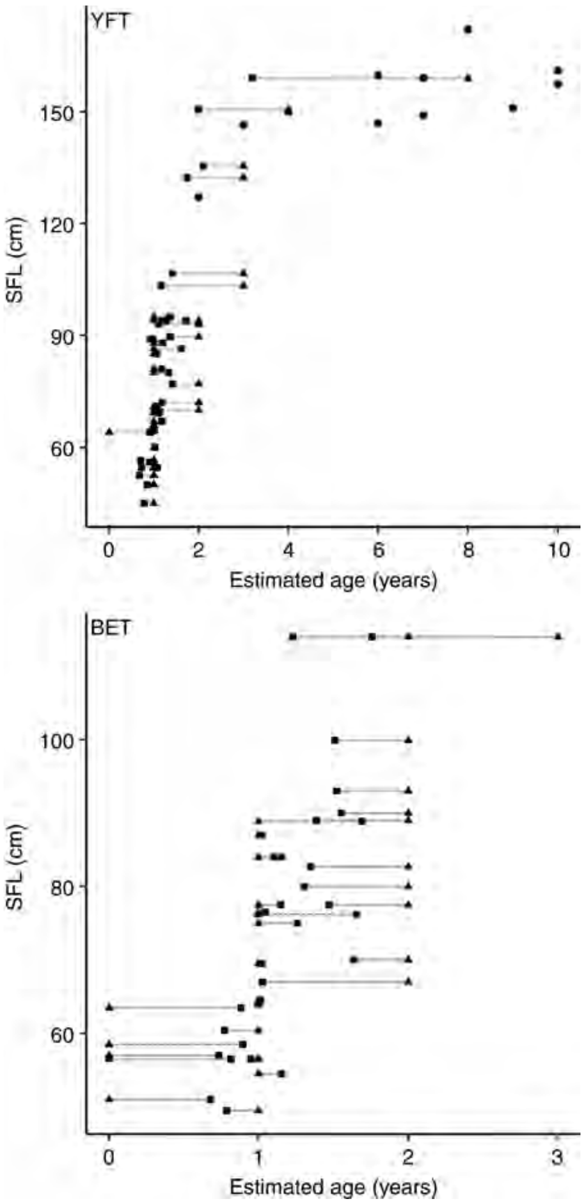

Figure 3

Comparison of age estimates based on daily (squares) and raw annual (triangles) increment counts on the same otolith, plotted by straight fork length (SFL), for yellowfin tuna (YFT) (*Thunnus albacares*) and bigeye tuna (BET) (*T. obesus*) sampled in the tropical and subtropical Atlantic Ocean between 2015 and 2021. Circles in the top panel indicate the increment counts for the 11 YFT for which only annual increment counts were taken.

times at liberty (Figs. 5 and 6). If the increments were truly deposited on a daily basis, and assuming there was no interpretation error, the points in each plot should fall directly on the solid black line that represents the 1:1 ratio of increment counts to times at liberty (Figs. 5A and 6A). Except for the readings of otoliths from one bigeye tuna and one yellowfin tuna, the daily increment counts fell below the 1:1 line, indicating that counts were lower than the true times at liberty. Results also indicate that the larger the fish size, the greater the discrepancy between the age assumed based on daily increment count and the true time at liberty. The raw counts of annual increments indicate a high level of correspondence between the number of opaque zones observed after the OTC mark and the actual time at liberty (Figs. 5B and 6B). The otolith from one yellowfin tuna at liberty for just over 2 years had only one visible opaque zone after the OTC mark, but the otolith had a wide translucent edge, indicating that a second opaque zone was due for deposition on the margin (Fig. 6). When the reader estimated times at liberty on the basis of the position of the OTC mark, the number of annual zones observed after the mark, and the relative distance between the last assumed annual opaque zone and the otolith margin, the estimated time at liberty agreed with the 1:1 line more closely and no directional bias was apparent (Figs. 5C and 6C).

Age-bias plots (Figs. 7 and 8) and results of the Evans–Hoenig test confirm our observation that daily increment counts are biased in that they are low in comparison to true times at liberty (Suppl. Tables 3–6). With the test, we detected a significant difference between the daily increment counts and the known time at liberty in both yellowfin tuna (χ^2=32, df=11, P=0.0008) and bigeye tuna (χ^2=18, df=10, P=0.05), indicating that increments were not systematically deposited on a daily basis for either species. The results from the Evans–Hoenig test of symmetry between the times at liberty estimated from annual increment counts and the actual times at liberty indicate that there was no systematic bias in times at liberty estimated from annual increment counts for both yellowfin tuna (χ^2=3.33, df=6, P=0.77) and bigeye tuna (χ^2=5.81, df=5, P=0.33).

Transverse versus longitudinal sections

When directly compared, the counts of increments in transverse versus longitudinal sections of otoliths did not differ greatly, nor did they indicate an obvious bias due to the preparation method. The Bland–Altman plot indicates a high degree of agreement between the counts made with the 2 reading methods, and there was no systematic difference between the paired counts (Fig. 9). There was a small negative bias in mean difference in counts with the 2 methods (−13 increments), a difference that is negligible in the context of age estimation (Fig. 9). All data points that were plotted fell within the 95% limits of agreement (−83, +57). This range indicates how far apart paired counts were most likely to be for most individuals, and the range is considered acceptable given the inter- and intra-reader variability typically observed in aging of tropical tuna. We did note, however, that daily increments within the longitudinal sections were easier to interpret than those within the transverse sections and that this difference in readability was particularly true for sections of otoliths from bigeye tuna.

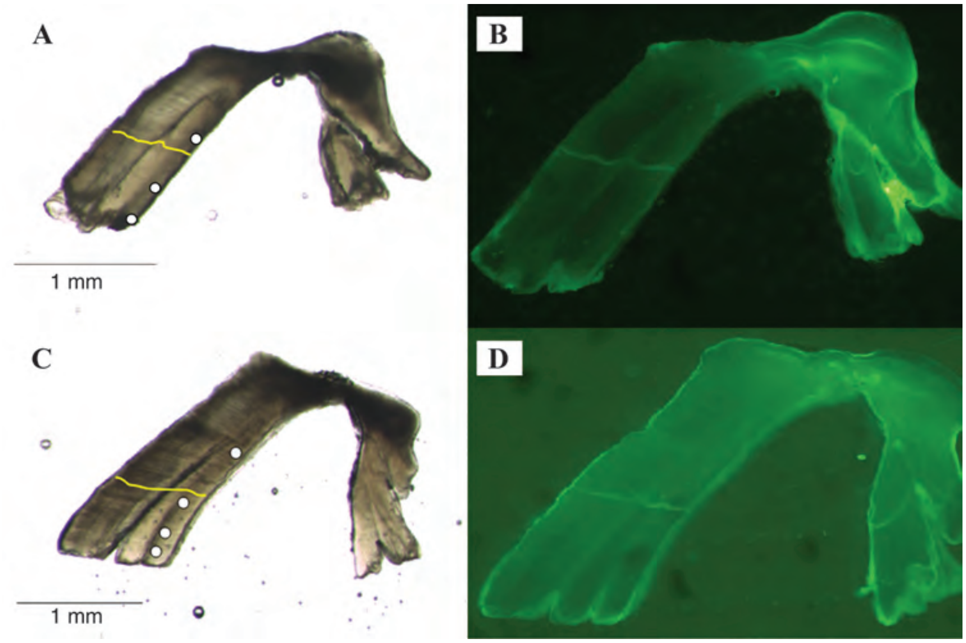

Figure 4

Examples of otolith sections from (**A** and **B**) a bigeye tuna (*Thunnus obesus*) and (**C** and **D**) a yellowfin tuna (*T. albacares*) sampled in the Atlantic Ocean. The images in panels A and C were illuminated with transmitted light, and the yellow line indicates the approximate position of the oxytetracycline (OTC) mark on each section. In panels B and D, the OTC marks are clearly visible in the corresponding sections illuminated with ultraviolet light. The white circles indicate the positions of the annual opaque zones used for aging. The bigeye tuna was recaptured on 29 April 2000 after 879 d at liberty, and its lengths at release and recapture were 45 and 114 cm straight fork length (SFL), respectively. The times at liberty estimated for this fish from daily and annual increment counts were 494 and 821 d, respectively. The yellowfin tuna was recaptured on 2 February 2020 after 995 d at liberty, and its lengths at release and recapture were 77 and 154 cm SFL, respectively. The times at liberty estimated for this fish from daily and annual increment counts were 357 and 912 d, respectively.

Discussion

In this study, reading otolith thin sections with transmitted light proved to be an appropriate method for obtaining age estimates based on annual increment counts for bigeye and yellowfin tuna sampled in the Atlantic Ocean. The age-at-length data presented in Figure 3 are similar to those data reported from other studies of tropical tunas that also are based on counts of annual increments over the age range examined, namely studies on yellowfin tuna in the Atlantic Ocean (Lang et al., 2017; Pacicco et al., 2021) and on both bigeye tuna and yellowfin tuna in the western Pacific Ocean (Farley et al., 2006; Farley et al.[2]). The strong relationship between the estimated time at liberty and the true time at liberty and the lack of systematic bias confirm that counting annual growth zones on otoliths from individuals of these 2 species is a valid method for age estimation. Furthermore, there were no instances of multiple annual rings being detected in a single year, as have been detected for little tunny (*Euthynnus alletteratus*), skipjack tuna (*Katsuwonus pelamis*), and blackfin tuna (*T. atlanticus*) from the Atlantic Ocean (Adams and Kerstetter, 2014).

Further work is needed to convert zone counts to decimal age estimates for both bigeye tuna and yellowfin tuna from the Atlantic Ocean and to increase the accuracy of age estimates for our geographically disparate fish samples. Multiple methods are currently used for assigning fractional ages for fish species. A common approach is to adjust the raw count on the basis of knowledge of the timing of band deposition and the birth date of the fish. However, neither the timing of band deposition nor the natal origin of the fish sampled were known for the otoliths used in this analysis; therefore, this approach could not be used reliably. An alternative and arguably preferable approach developed by Farley et al.[2] does not require information on spawning and timing of band formation. Instead, this approach involves developing a relationship between the age based on daily increment counts and the length of the ventral otolith arm for age 0+ fish and determining estimates of average annual increment width for each age class. Still, this approach requires large numbers of fish to

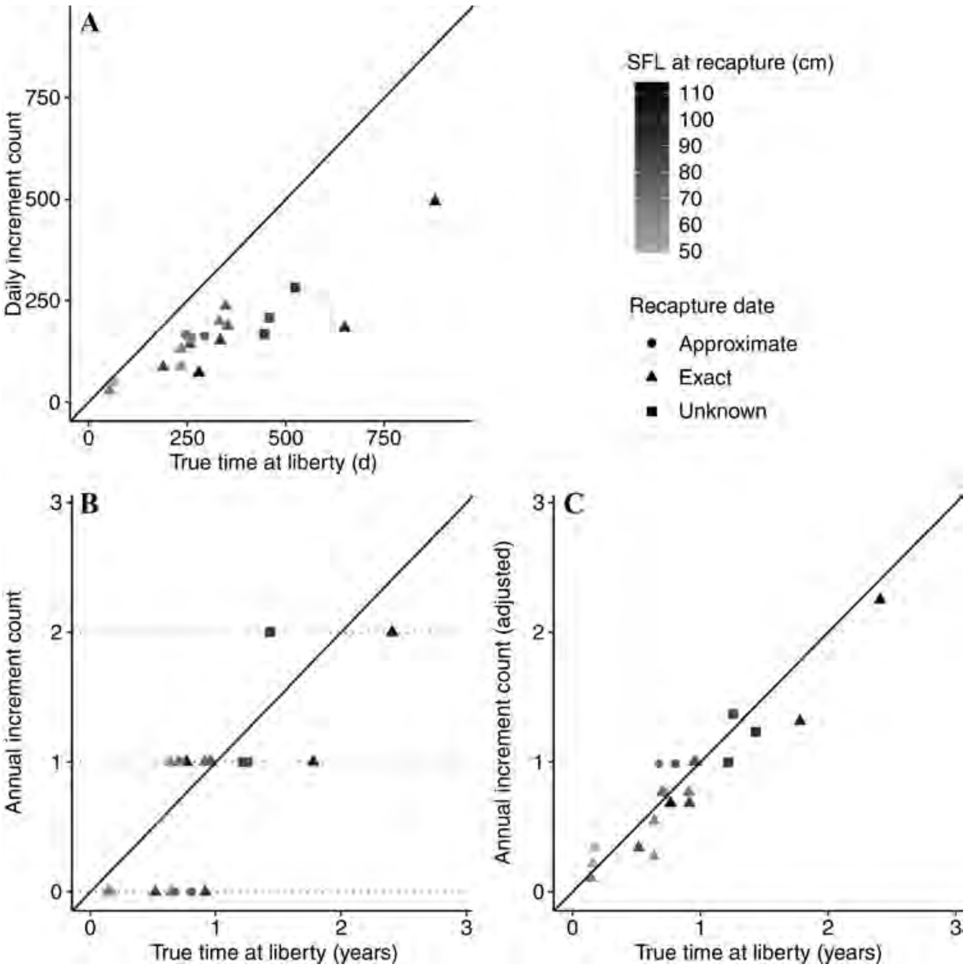

Figure 5

Comparison of (**A**) daily, (**B**) annual, and (**C**) adjusted annual increment counts, plotted with true times at liberty, for bigeye tuna (*Thunnus obesus*) marked and recaptured in the tropical and subtropical Atlantic Ocean during 2015–2021. Points that appear below the solid black line that represents the 1:1 ratio of increment counts to times at liberty indicate that the increment count is an underestimation of the true time at liberty, and points that appear above the line indicate that the increment count is an overestimation of the true time at liberty. Adjusted annual increment counts were determined on the basis of the position of the oxytetracycline mark, the number of annual zones observed after the mark, and the relative distance between the last assumed annual opaque zone and the otolith edge. Symbols relate to the quality of the recovery date: date is known only approximately and could be off by a few days, date is known exactly, and the quality of the date is unknown. Shades of gray used for symbols correspond to straight fork lengths (SFLs) of the fish at recapture according to the scale provided, with darker shades indicating longer lengths.

be sampled and to be analyzed a priori, something that could not be accomplished within the scope of our study but that could certainly be attempted in the future by using the remaining sampled fish from the AOTTP.

In the otoliths prepared for daily aging, areas with readily identifiable daily increments were observed throughout the otolith section in both transverse and longitudinal sections, albeit they were separated by areas in which the increment structure was difficult to interpret or was not present at all. Although the theory that the otoliths of older fish contain daily increments that are below the resolution limits of light microscopy is commonly held, in our study, daily increments were still detected close to the outer edge of otoliths even in the sections of the largest otoliths of both species examined. Schaefer and Fuller (2006) made the same observation for otoliths from bigeye tuna that were up to 145 cm SFL and were sampled from the eastern Pacific Ocean, although they used longitudinal sections. Similarly, Williams et al. (2013), comparing both longitudinal and transverse sections, detected daily

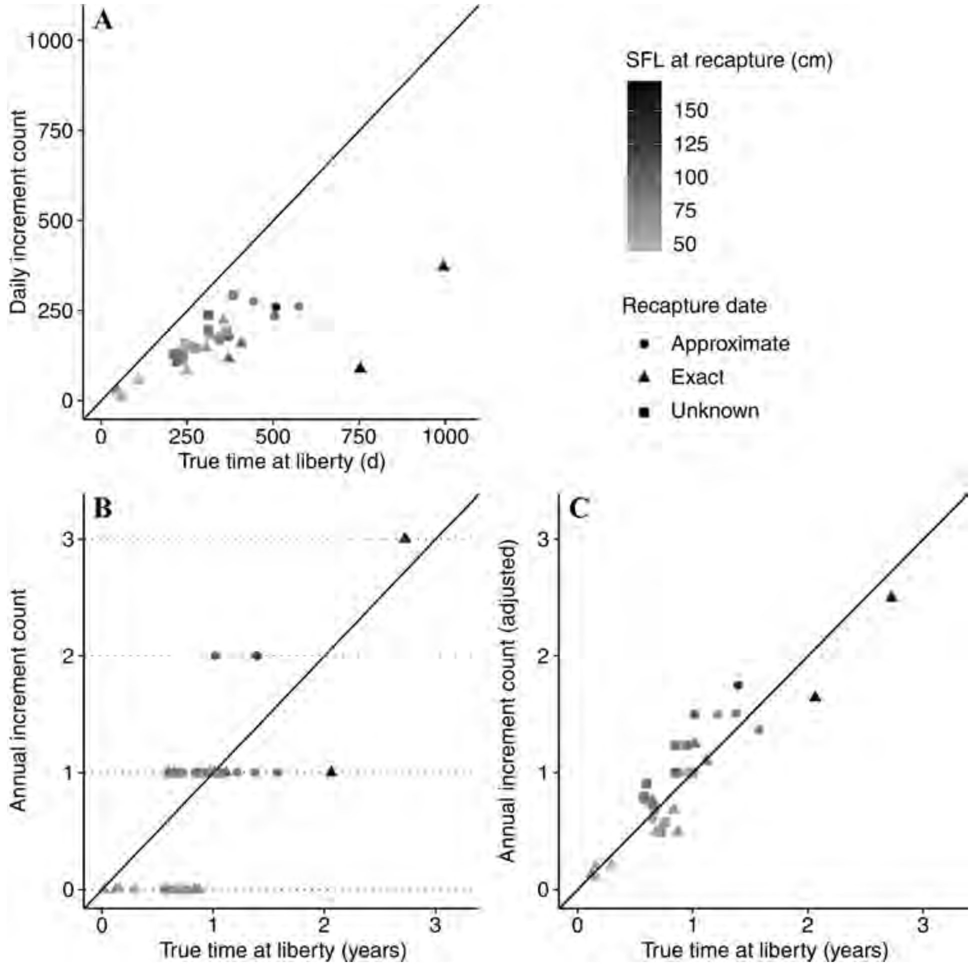

Figure 6

Comparison of (**A**) daily, (**B**) annual, and (**C**) adjusted annual increment counts, plotted with true times at liberty, for yellowfin tuna (*Thunnus albacares*) marked and recaptured in the tropical and subtropical Atlantic Ocean during 2015–2021. Points that appear below the solid black line that represents the 1:1 ratio of increment counts to times at liberty indicate that the increment count is an underestimation of the true time at liberty, and points that appear above the line indicate that the increment count is an overestimation of the true time at liberty. Adjusted annual increment counts were determined on the basis of the position of the oxytetracycline mark, the number of annual zones observed after the mark, and the relative distance between the last assumed annual opaque zone and the otolith edge. Symbols relate to the quality of the recovery date: date is known only approximately and could be off by a few days, date is known exactly, and the quality of the date is unknown. Shades of gray used for symbols correspond to straight fork lengths (SFLs) of the fish at recapture according to the scale provided, with darker shades indicating longer lengths.

increments in the outer part of the otolith section of the largest (175 cm SFL) southern bluefin tuna (*T. maccoyii*) examined in their study (senior author, personal observ.), despite the considerable divergence of the age estimates based on total annual (29 years) and daily (4 years) increment counts.

The advantage of using longitudinal sections is that this sectioning plane provides the longest axis for counting daily increments, with increment spacing that is usually wider than that in corresponding transverse sections. This wider spacing can lead to fewer areas within the otolith section having daily increments that are hard to interpret or requiring interpolation. Although a detailed comparison of sectioning methods was not an objective of this study, we did compare the results between a small number of otoliths prepared by using the 2 different sectioning planes. This comparison was important to avoid potential bias in the results from using either of the otolith preparation methods and to provide insight into which sectioning method should be preferred for use in future aging work

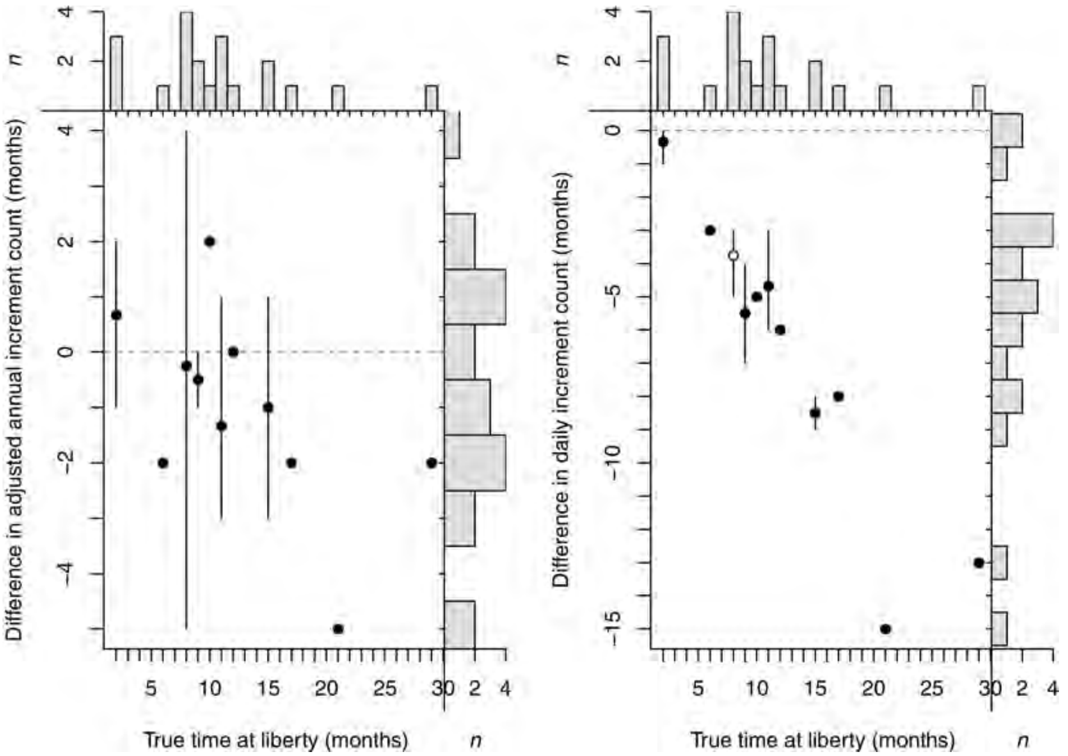

Figure 7

Age-bias plots showing the differences between annual and daily increment counts, or estimated times at liberty, against the true times at liberty for bigeye tuna (*Thunnus obesus*) marked and recaptured in the tropical and subtropical Atlantic Ocean from 2015 through 2021. In each panel, a histogram of the differences between the estimated and true times at liberty are provided in the right margin. The number of samples (*n*) for each time at liberty (in months) is provided in a histogram in the top margin. Circles indicate mean differences between the estimated and true times at liberty at each true month at liberty, and the bars stretch from the minimum to the maximum difference observed. The open circle indicates that the mean is significantly different from zero.

based on counts of daily increments for these 2 species. Preliminary results indicate no clear bias in the daily increment counts between the preparation methods used and are consistent with those of an earlier study on yellowfin tuna in the Indian Ocean for which preparation method also did not influence the age estimates (Stéquert et al., 1996). We note, however, that daily increments within the longitudinal sections in our study were easier to interpret than those within the transverse sections, and this difference in ease of detecting increments was particularly true for bigeye tuna. We therefore recommend that a more thorough comparison of the 2 methods be completed.

Results from both the comparison of annual versus daily increment counts and the validation of deposition rates of daily increments in otoliths based on examination of OTC-marked fish indicate that counting daily increments in otoliths of yellowfin and bigeye tuna may lead to underestimating age for fish larger than 55 cm SFL (greater than ~age 1). The difference between age estimates based on the 2 methods of counting can become quite large as fish grow older, as was observed for one of the yellowfin tuna examined in our study, with a difference in age of 6 years. Williams et al. (2013) counted both daily and annual increments on their largest southern bluefin tuna and found an overall difference of 20 years between the age estimates from the 2 counts. Sardenne et al. (2015) similarly found that daily increment counts in otoliths of large (>100 cm FL) bigeye tuna from the Indian Ocean resulted in underestimates of age.

One explanation for this underestimation could be that daily increments are not systematically deposited at a daily rate. It has been hypothesized that increment deposition may cease to be daily after a certain age or life history stage and that changes in environmental conditions might disrupt the regular pattern of daily increment deposition (e.g., Francis et al. 1992). Another potential explanation relates to the fact that there are areas within otoliths (even in those from relatively small fish, <75 cm SFL) where daily increments are either extremely difficult to interpret or do not appear to form at all; such areas also have been found in other studies (Shuford et al., 2007; Sardenne et al., 2015). Daily increments tend to have a merging or splitting structure in some areas, making it difficult to distinguish individual increments, and other

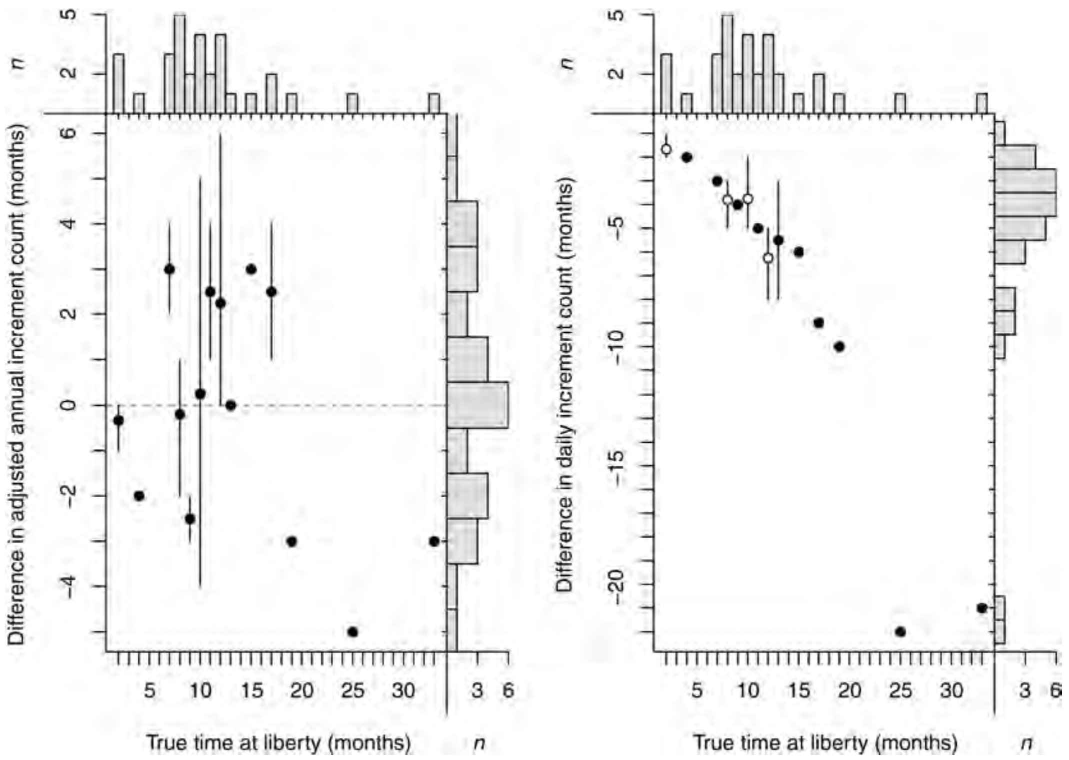

Figure 8

Age-bias plots showing the differences between the annual and daily increment counts, or estimated times at liberty, against the true times at liberty for yellowfin tuna (*Thunnus albacares*) marked and recaptured in the tropical and subtropical Atlantic Ocean from 2015 through 2021. In each panel, a histogram of the differences between the estimated and true times at liberty are provided in the right margin. The number of samples (*n*) for each time at liberty (in months) is provided in a histogram in the top margin. Circles indicate mean differences between the estimated and true times at liberty at each true month at liberty, and the bars stretch from the minimum to the maximum difference observed. Open circles indicate that the means are significantly different from zero.

areas are devoid of increments, forcing analysts to resort to interpolation to fill the missing patterns. Campana (1992) suggested that the use of interpolation is considered reasonable in cases in which the number of interpolated daily increments is relatively small in comparison to the total increment count. Unfortunately, for the otoliths of bigeye and yellowfin tuna examined in this study, these areas were relatively common and likely the main contributing factor in age underestimation.

Despite these limitations to the use of daily increment counts for aging yellowfin and bigeye tuna, daily increments do hold considerable value in the overall aging process. Daily increment counts are still important for confirming the location of the first annual increment in sectioned otoliths, as has been reported for yellowfin tuna in the Atlantic Ocean (Lang et al., 2017) and both bigeye and yellowfin tuna in the Pacific Ocean (Farley et al., 2006; Farley et al.[2]), and for providing length-at-age data during the first year of life. More recently, aging through the use of daily increments counts, particularly out to a count of 365 d, has proven to be a valuable tool in refining counts of annual zones and, therefore, in providing more accurate fractional ages and has also allowed improvement in the growth curves of yellowfin and bigeye tuna from the western and central Pacific Ocean (Farley et al.[2]). With this in mind, the challenge is determining the point at which age estimation based on daily increment counts provides a larger source of error than the use of annual zone counts. Finding that point should be considered a vital step in developing suitable aging methods for any species, let alone for tropical tunas, and the need to obtain that knowledge highlights the value of conducting a large-scale tag-recapture program such as the AOTTP.

Conclusions

On the basis of our findings, the use of annual increment counts is the best method for aging yellowfin and bigeye tuna sampled in the Atlantic Ocean, and the use of daily increment counts should be limited to aging young of the year. Additional work is needed to resolve the timing of opaque zone formation in otoliths from fish from different parts of the Atlantic Ocean and to derive fractional

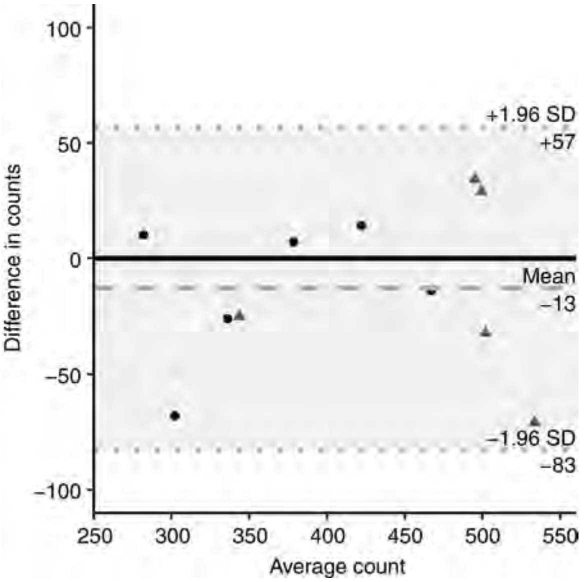

Figure 9

Bland–Altman plot of the paired readings of total daily increment counts for otoliths from bigeye tuna (*Thunnus obesus*) (circles) and yellowfin tuna (*T. albacares*) (triangles) marked and recaptured in the tropical and subtropical Atlantic Ocean between 2015 and 2021. The horizontal dashed line indicates the mean bias in the difference in counts between the transverse and longitudinal sections. The horizontal dotted lines indicate the 95% limits of agreement, calculated as the mean plus and minus 1.96 standard deviations (SDs).

ages, and we believe the AOTTP otolith collection could serve that purpose. Although the AOTTP has now ended, networks across the Atlantic Ocean are being maintained to ensure that there is a continued effort to recover OTC-marked fish and to analyze hard parts. As this effort continues, more valuable and informative samples (i.e., larger fish, with longer times at liberty) will become available, and analysis of their otoliths is likely to confirm our findings and recommendations for these 2 key tuna species.

Validating age estimation methods is particularly valuable for improving stock assessments; the importance of obtaining correct age and growth information in assessing stock status has been well documented (Maunder and Punt, 2013). Lang et al. (2017) and Pacicco et al. (2021) have suggested that yellowfin and bigeye tuna from the Atlantic Ocean can be aged by using counts of annual increments and that the maximum longevity for both species was far greater than originally determined. More importantly, the aging protocols used in those recent studies have been verified by the use of bomb radiocarbon analysis (Andrews et al., 2020). The results of that recent work, along with the results from our analysis of otoliths from OTC-marked fish in our study, support the use of annual increments for aging yellowfin and bigeye tuna sampled in the Atlantic Ocean.

The findings of our study are particularly important because the recent shift to base age estimates on annual increment counts has led to raising the estimated maximum age in the latest assessment of the Atlantic stock of yellowfin tuna from 11 to 18 years (ICCAT, 2020), which in turn has caused substantial changes in stock status (ICCAT, 2019). The ICCAT has assessed the Atlantic stocks of both the bigeye tuna and yellowfin tuna with modeling approaches of varying complexities, from simple production models to integrated statistical assessment models, and the capacity to accommodate age data in several different ways. Although age data have not yet been used to their full extent for tuna stocks in the Atlantic Ocean, we anticipate that that will change in the future, given the recent advances in the study of age and growth of tropical tuna species.

Resumen

Un experimento reciente de marcado-recaptura en todo el Atlántico llevado a cabo por la Comisión Internacional para la Conservación del Atún del Atlántico fue una oportunidad para validar directamente las tasas de incrementos de otolitos para el patudo (*Thunnus obesus*) y el atún de aleta amarilla (*T. albacares*) en la región. Se estimaron la edad y el tiempo en libertad utilizando conteos de incrementos anuales y diarios de otolitos seccionados de peces muestreados previamente inyectados con oxitetraciclina y recapturados posteriormente. Los conteos de incrementos anuales dieron lugar a mayores estimaciones de edad que las realizadas con conteos diarios, para los peces mayores de 55 cm de longitud furcal recta (SFL). El conteo de incrementos diarios condujo a una subestimación del tiempo en libertad para los peces mayores de 55 cm de SFL al ser recapturados, en comparación con los tiempos en libertad conocidos. Por el contrario, las predicciones basadas en los recuentos de incrementos anuales son precisas en todo el rango de tallas de los peces muestreados, validando así, que los incrementos se depositan anualmente. Por lo tanto, recomendamos que el conteo de incrementos anuales sea el método preferido para determinar la edad del atún de aleta amarilla y el patudo del Océano Atlántico y que el uso de incrementos diarios para determinar la edad se limite a los juveniles del año. La determinación precisa de la edad en peces es importante para la evaluación de poblaciones, en las que los datos sobre edad y crecimiento desempeñan un papel cada vez más esencial en el estudio de dinámica de poblaciones. Es crucial que las prácticas de lectura de otolitos y los análisis basados en los datos de edad reflejen las recomendaciones más actualizadas para la estimación de la edad.

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