

Age estimation and validation for South Pacific albacore *Thunnus alalunga*

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(Received 23 July 2012, Accepted 28 January 2013)

Validated estimates of age are presented for albacore *Thunnus alalunga*, sampled from a large part of the south-western Pacific Ocean, based on counts of annual opaque growth zones from transverse sections of otoliths. Counts of daily increments were used to estimate the location of the first opaque growth zone, which was completed before the first assumed birthday. The periodicity of opaque zones was estimated by marginal increment analysis and an oxytetracycline mark–recapture experiment. Both validation methods indicated that opaque zones formed over the austral summer and were completed by autumn to winter (April to August). The direct comparison of age estimates obtained from otoliths and dorsal-fin spines of the same fish indicated bias, which was assumed to be due to poor increment clarity and resorption of early growth zones in spines, resulting in imprecise age estimates. As such, age estimates from otoliths are considered to be more accurate than those from spines for *T. alalunga*. This is consistent with results for a growing number of tropical and temperate tuna *Thunnini* species. It is recommended that validated counts of annual growth zones from sectioned otoliths is used as the preferred method for estimating age-based parameters for assessment and management advice for these important stocks.

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Key words: ageing protocols; annual; marginal increment analysis; otoliths; spines.

INTRODUCTION

Tuna (*Thunnini*) support several of the largest pelagic fisheries in the world (Majkowski, 2007). To determine the status of these stocks and provide management advice, routine assessments of the target species are undertaken periodically. Many of these assessments use age-structured models requiring estimates of length-at-age, age-at-maturity and age-specific natural mortality (FAO, 2001), yet validated age estimation methods across the full age range of many of these important species are not available. Many tuna stock assessments use analysis of length–frequency and tagging data to estimate growth parameters. There is considerable uncertainty in growth parameter estimates using these methods because of the limited size range of fishes included and the inadequacies inherent in some of the methods for old,

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slow-growing species (Farley *et al.*, 2006). Studies over the past decade, however, have shown that tuna otoliths exhibit annual increments that can be consistently and unambiguously interpreted and provide a valuable source of size-at-age data (Clear *et al.*, 2000; Farley *et al.*, 2003; Neilson & Campana, 2008; Griffiths *et al.*, 2009) as a basis for estimation of population parameters.

Albacore *Thunnus alalunga* (Bonnaterre 1788) are distributed in waters between 10–50° N and 5–45° S globally. In the Pacific Ocean, discrete northern and southern stocks are assumed to exist based on their spatial distribution and different spawning times and locations (Nishikawa *et al.*, 1985; Ramon & Bailey, 1996; Hampton, 1999). In the South Pacific Ocean, *T. alalunga* have been targeted by longline fleets since the early 1950s, and recent catches have increased from 20 000 to 30 000 t year⁻¹ in the mid-1980s to 60 000 to 70 000 t year⁻¹ in the mid-2000s (Hoyle, 2011). Current regional stock assessments for South Pacific *T. alalunga* indicate that the stock is not overfished and that overfishing is not occurring (Hoyle, 2011). The stock assessment, however, uses many biological variables that are either uncertain or assumed. Growth parameters are estimated from length–frequency data as part of the assessment, while the maturity schedule is based on a study of North Pacific *T. alalunga* from the 1950s (Hoyle, 2011).

Validated age estimates for South Pacific *T. alalunga* are required for estimating growth curves by sex, sex ratio-at-age, age-at-maturity, life span and determining the extent of regional variation in these variables. The direct age of *T. alalunga* has been estimated using a variety of hard parts including scales, vertebrae, otoliths and fin spines; the last two are most commonly used to estimate daily and annual age, respectively (Labelle *et al.*, 1993; Megalofonou, 2000; Santiago & Arrizabalaga, 2005; Lee & Yeh, 2007; Davies *et al.*, 2008; Karakulak *et al.*, 2011; Quelle *et al.*, 2011). A difficulty of using fin spines for estimating annual age is that vascularization and resorption of the core can obscure the early growth zones in older fishes. This loss of growth zones has been overcome in some species by estimating the positions of these zones in younger fishes (Hill *et al.*, 1989), although this method is yet to be validated for *T. alalunga*.

In this study, otolith-based protocols developed for other tuna species are refined and applied to *T. alalunga* to estimate annual ages from counts of opaque zones. Marginal increment analysis (MIA) and an oxytetracycline (OTC) mark–recapture experiment were used to validate the periodicity of opaque zone formation. Age estimates from otoliths and fin spines from the same fish were compared to examine the relative bias.

MATERIALS AND METHODS

SAMPLE COLLECTION

A total of 2120 sagittal otoliths were extracted from *T. alalunga* sampled from the south-western Pacific Ocean between January 2009 and December 2010 (43–133 cm fork length; L_F) (Table I). To compare age estimates from different hard parts of the same fish, fin spines were selected from a sub-sample of 278 fish across a wide size range (45–120 cm L_F), both sexes and from several sampling locations (Table I).

The fish were caught over a broad area between 130° E and 130° W from waters off Australia, New Zealand, New Caledonia, Fiji, Tonga, American Samoa, Cook Islands, French

TABLE I. Number of *Thunnus alalunga* otoliths and fin spines sampled in each location that were selected for annual and daily age estimation. International waters 1 refers to the waters between the Australian and New Caledonian Exclusive Economic Zones (EEZ) and international waters 2 refers to the waters south of the Pitcairn Islands

Location	Otolith annual age	Fin spine annual age	Otolith daily age
American Samoa	208	2	
Australia	709	132	50
Cook Islands	168	3	
Fiji	121		
French Polynesia	248		
International waters 1	66	13	
International waters 2	74	53	
New Caledonia	218	16	
New Zealand	184	43	18
Tonga	124	21	
Total	2120	283	68

Polynesia and in international waters west of New Caledonia and south of the Pitcairn Islands (Fig. 1). Within Australia, fish were sampled from the Eastern Tuna and Billfish longline fishery (ETBF) and the recreational fishery on the east coast. Sampling in the ETBF was predominantly undertaken in port, from fish caught along most of the mainland coast of Australia between 14° and 37° S. Fish sampled from the recreational fishery were caught between 37° and 44° S. In New Zealand, *T. alalunga* were sampled in port from the domestic troll fishery as well as at sea during chartered tagging operations. From all other regions across the south-western Pacific Ocean, *T. alalunga* samples were collected either by observers on longline fishing vessels or directly by the fishing crew of longline fishing vessels.

ANNUAL AGE ESTIMATION

Otoliths

The left or right sagitta was selected randomly from each fish (with preference given to undamaged otoliths) and weighed to the nearest 0.1 mg if undamaged. No significant difference in otolith mass was detected between left and right otoliths (paired *t*-test, d.f. = 151, *P* > 0.05), suggesting that either otolith could be used for age estimation.

All otoliths were embedded in clear casting polyester resin. Four serial transverse sections were cut from 2002 otoliths (one section including the primordium) while a single transverse section, incorporating the primordium, was cut from the remaining 118 otoliths. All sections were mounted on glass slides with resin and polished to 400 µm following the protocols developed for southern bluefin tuna *Thunnus maccoyii* (Castelnau 1872) (Anon, 2002). Opaque (dark) and translucent (light) zones were visible along the ventral 'long' arm of each otolith section viewed under transmitted light on a Leica Wild stereo microscope (www.leica-microsystems.com) (Fig. 2). The number of opaque zones was counted using the techniques developed for *T. maccoyii* and bigeye tuna *Thunnus obesus* (Lowe 1839) (Anon, 2002; Farley *et al.*, 2006). All sections prepared from an otolith were read before a count was assigned. Opaque zones at the distal edge of the otolith were counted only if a translucent edge was visible after the opaque zones. A confidence score of 0 (low confidence) to 5 (high confidence) was assigned to each reading.

Otoliths were read at least two times by the same reader (OR1) without reference to the previous reading, size of fish or capture date. If the successive readings were in agreement, this estimate was used as the final count for the otolith. If the readings differed, however, a further reading was made with knowledge of the previous readings to decide on a final count

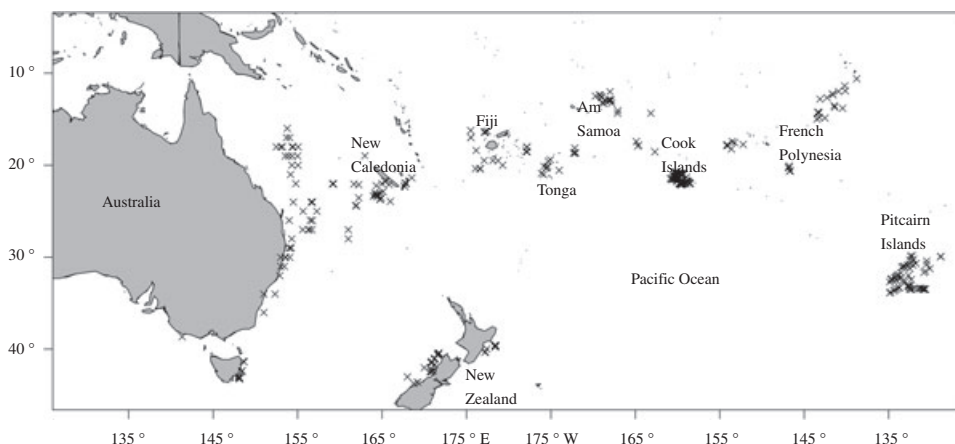


FIG. 1. Map of the South Pacific Ocean indicating locations where samples of *Thunnus alalunga* were collected (x).

and confidence score. If no obvious pattern could be seen in the otolith section, a confidence score of 0 was assigned to the sample and a count was not made.

Marginal increment (MI) measurements were made to determine the relative state of completion of the most distal opaque zones. Measurements were made on sectioned otoliths along the same axis as the counts. The distance from the distal edge of the two most recently completed opaque zones to the edge of the otolith was measured. From these measurements, the MI ratio (R_{MI}) was calculated as the state of completion of the MI as a percent of the previous increment (*i.e.* relative MI). For otoliths with only one opaque zone, the absolute MI was measured. These measurements were taken for all otoliths with clear opaque zones at the distal edge, although the measurements were probably most accurate for age classes one to five where the width of individual opaque zones is relatively large. Measurements were made using an image analysis system; images were acquired with an Olympus F-view II digital camera (www.olympus-global.com/en) mounted on the Leica Wild stereo microscope connected to a computer. AnalySIS 3.2 (Soft Imaging System; www.soft-imaging.net) was used to take the measurements. An edge classification was assigned as either narrow (MI < 30% complete) or wide (MI > 30% complete) for otoliths with opaque zone counts ≥ 2 .

To assess the consistency of counts and edge classification, a second reader (OR2) read 10% of the otoliths. OR2 was experienced in reading sectioned *T. maccoyii* otoliths, but not *T. alalunga* otoliths. OR2 received limited training in otolith interpretation *via* annotated otolith images and a learning set of 85 otoliths. Each otolith was then read twice by OR2, a final opaque zone count was estimated and the edge was classified as narrow or wide.

Fin spines

The spines were sectioned using the protocols of Rodríguez-Marín *et al.* (2007). Initially, the location and diameter of the fin spine immediately above the hallows near the base of the spine were determined. The first cut of the spine was then made along the spine at a distance half the diameter above the hallows (Rodríguez-Marín *et al.*, 2007). Several serial cross sections 500 μm thick were made from this position. The sections were embedded in clear casting polyester resin and mounted on glass slides with resin.

As with otoliths, opaque (dark) and translucent (light) zones were visible across the sectioned spine when viewed under transmitted light at a low magnification on an Olympus BX51 compound microscope (Fig. 3). The translucent zones were observed in different forms: fine or thick single zones, and double or triple translucent rings that were separated by narrow opaque rings. Multiple translucent rings were considered to belong to the same translucent zone if the distance between them was less than the distance to the preceding and subsequent

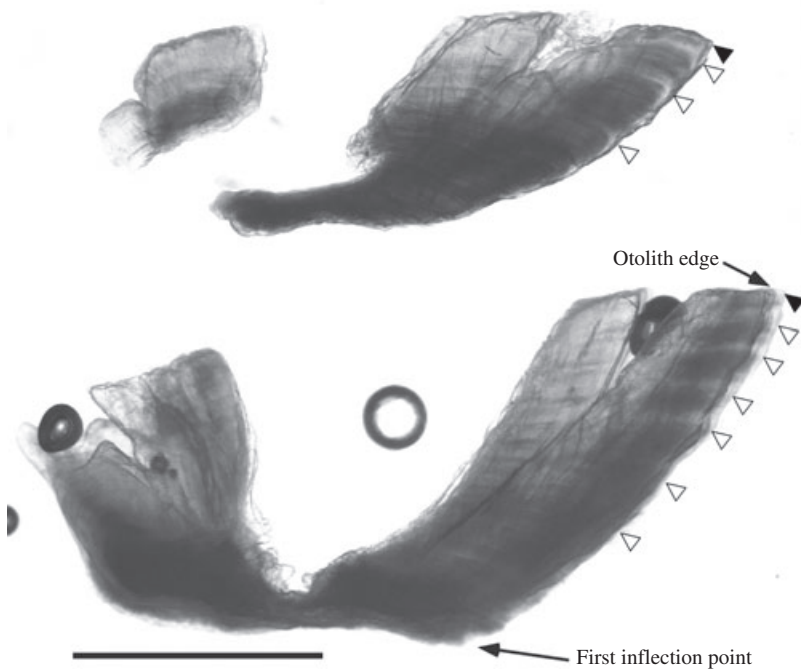


FIG. 2. Images of transverse sections of two *Thunnus alalunga* sagittal otoliths under transmitted light showing clear opaque and translucent growth zones along the ventral arm. Locations of the distal edges of the opaque zones counted are shown (\triangleright) with a partially completed opaque zone at the margin of both otoliths (\blacktriangleright). Scale bar = 1 mm.

translucent zone, and the rings converged at the vertex of the spine (Megalofonou, 2000; Santiago & Arrizabalaga, 2005). To be consistent with otolith reading, the number of opaque zones observed in spines was counted only if a translucent edge was visible after the zone.

A large proportion of spines showed some level of resorption or vascularization of the core, even in fish as small as 60 cm L_F . A stepped statistical replacement method modified from the study of Hill *et al.* (1989) was used to account for inner opaque zones that may have been obscured by the vascularization and resorption processes. Initially, the appearance and location of the first opaque zone were identified in spines of the smallest fish sampled (<50 cm L_F) with an estimated age of *c.* 1 year based on daily ageing ($n = 11$) and annual ageing ($n = 16$) of otoliths. The maximum diameter of this zone was then used to identify the first opaque zone (if present) in the spines of older fish ($n = 20$) (Table II). Second, 1 s.d. of the mean diameter of the second opaque zone (if present) in these later spines was used to identify the second opaque zone in spines where the first zone was judged to be obscured ($n = 92$). Finally, the diameter of the third and subsequent opaque zones in this pool of 112 spines was used to estimate the number of missing opaque zones in the remaining spines (Table II). An age was assigned to the first opaque zone present after the obscured area if its diameter fell within 1 s.d. of the mean diameter of a 'known age' opaque zone in the data from the pooled spines. If the diameters of the first apparent opaque zone did not fall within the ranges calculated from the s.d., they were excluded from further analysis. R_{MI} measurements and calculations were made using the same protocols as for otoliths. The diameter of the section to the outer edge of the last two opaque zones and to the edge of the spine was measured.

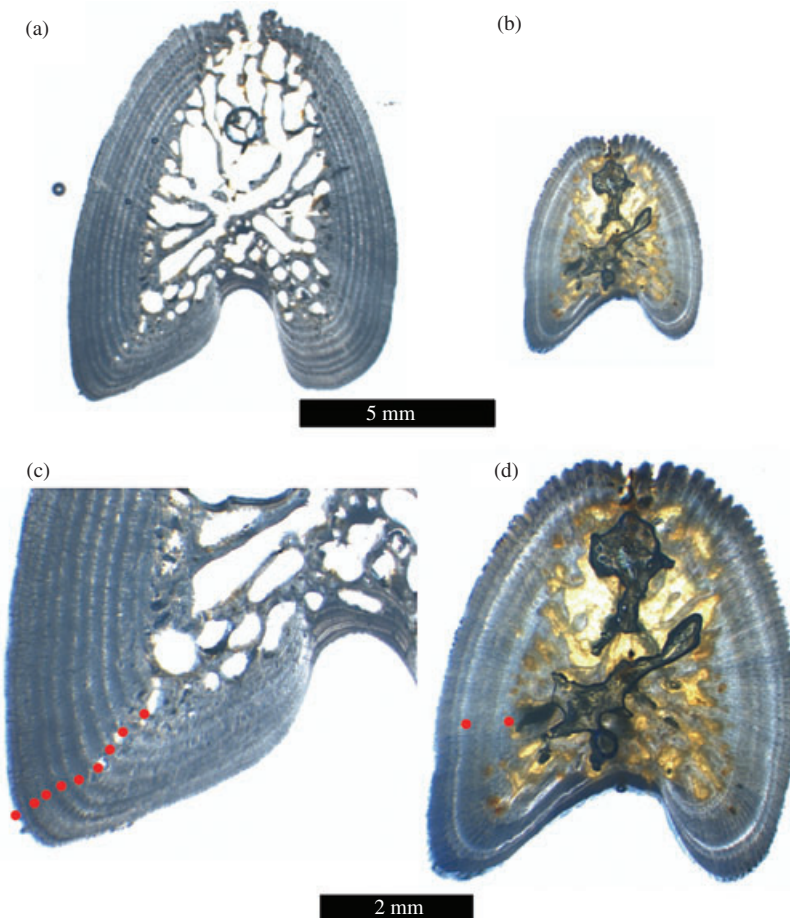


FIG. 3. Images of transverse sections of two *Thunnus alalunga* dorsal-fin spines under transmitted light, (a, b) spines from different specimens and (c, d) at higher magnification indicating (●) the zones counted at the transition between opaque (dark) and translucent (light) zones.

Precision

The precision of readings (intra- and inter-reader consistency) was assessed using the index of average percent error (I_{APE}) (Beamish & Fournier, 1981). Age-bias and age difference plots were used to detect systematic disagreement between age estimates from different readers and structures (Campana *et al.*, 1995), and a paired *t*-test was used to compare the age estimates obtained from otoliths and spines of the same fish.

AGE VALIDATION

Location of the first opaque zone

To estimate the location of the first opaque growth zone in otoliths, micro-increment analysis and annual ageing were performed for 68 individuals ranging in size from 43 to 56 cm L_F sampled in the months of January to April (Table I). Initially, otolith pairs were selected from 18 fish and one transverse section incorporating the primordium was prepared from each otolith ($n = 36$). One otolith from each pair was prepared for micro-increment

TABLE II. Diameter measurements of opaque growth zones in *Thunnus alalunga* fin spines used to assign an age to the first observed zone in sections with the core obscured

Opaque growth zone	Count	Mean diameter (mm)	S.D.	Range
1	27	1.69	NA	1.32–2.06
2	20	2.52	0.231	2.29–2.75
3	92	3.13	0.196	2.94–3.33
4	75	3.66	0.280	3.39–3.94

NA, not applicable as the full range of measurements was used rather than the S.D. of the mean.

analysis; the sections were attached to microscope slides using Crystalbond 509 mounting adhesive (www.crystalbond.com) and ground with wet 1200 grit sandpaper and polished with water and aluminium powder ($0.3\ \mu\text{m}$) until the primordium was reached and the section was c. $75\text{--}100\ \mu\text{m}$ thick. The number of (assumed) daily increments was counted by OR2 under high magnification on a dissecting microscope. Counts were made from the primordium to the distal edge of the otolith along the ventral arm of the section. Measurements were taken from the first inflection point to the 365th increment (age 1 year; Y1) and to the edge of the otolith (Fig. 2). The second otolith in each pair was prepared for annual ageing as detailed above, but was polished to a thickness of $400\ \mu\text{m}$. A measurement was then made from the first inflection point to the outer edge of the first opaque zone and to the edge of the otolith by OR1. Comparisons were made between the location of the 365th micro-increment and the end of the first opaque (presumably first annual) zone on the other (sister) otolith in the pair.

In addition, transverse sections of one otolith were prepared for micro-increment analysis from a further 50 fish. The number of (assumed) daily increments visible in each section was counted and the distance from the first inflection point to the otolith edge was measured by OR2. Each section was then examined by OR1 and if an opaque zone was visible, the distances from the first inflection point to the outer edge of that zone and to the edge of the otolith were measured.

Direct validation

A total of 1457 *T. alalunga* (44–100 cm L_F) were captured by troll fishing in New Zealand waters between January and March 2009. A further 92 (52–97 cm L_F) were captured by long-line fishing in April and May 2010. Each fish was given an injection of OTC ($200\ \text{mg ml}^{-1}$) at an approximate dose of $25\text{--}50\ \text{mg kg}^{-1}$ body mass injected deeply within the muscle tissue under the first dorsal fin using a 40 mm 18 gauge needle in an automatic syringe. To identify the fish, they were tagged with a conventional plastic-tipped 140 mm PDAT Hallprint dart tag (www.hallprint.com).

Three OTC-marked fish were recaptured, from which the otoliths and fin spines were collected. Four or five serial transverse sections were prepared for annual age estimation for one otolith from each pair using the methods described above. Five serial sections were prepared for each spine using the methods described above. Without the knowledge of time at liberty, the total number of opaque zones and the number of opaque zones after the OTC mark were counted for each otolith and spine.

Indirect validation

MIA was undertaken to examine the periodicity of opaque zone formation in otoliths. R_{MI} data were divided into a western region (west of 180° E) and eastern region (east of 180° E) for comparison of broad spatial scale variation in opaque zone formation. Mean R_{MI} for each region, age class (opaque zone count) and month were estimated to examine annual, spatial and age-specific patterns in opaque zone formation. There were several months in 2009 and 2010 when sample sizes were low or when no samples were collected, particularly in the eastern region. A single, orthogonal analysis of region \times year \times month \times age was not possible

because of this uneven distribution of the data. Accordingly, separate fixed factor ANOVA was used to test for the effects of year, region and age on R_{MI} , and their interaction with month. The first ANOVA tested for the effect of year (2009 and 2010) and age for age classes 4, 5 and 6 years in the western region to determine whether the timing of opaque zone formation varied between years and age classes within that region. A second ANOVA tested for the effect of region for both years and all age classes combined to determine whether the timing of opaque zone formation varied between regions. Significant interactions between any factor and month were explored using Bonferroni-adjusted pair-wise comparisons to determine in which months differences in R_{MI} occurred.

RESULTS

ANNUAL AGE ESTIMATION

Otolith reading and precision

The clarity of growth zones in *T. alalunga* otoliths varied between individuals. Many otoliths contained opaque and translucent zones that were distinct and regularly spaced while others were more difficult to interpret. Opaque zones were generally wide with alternating narrow translucent zones. The distal edge of the first opaque zone was often the most difficult to locate because it was less distinct than subsequent zones. Small bumps along the medio-ventral ridge were occasionally associated with the opaque zones and assisted in their identification. Paired opaque zones were often visible at the edge of large otoliths and if joined at the margin they were counted as a single zone (Gunn *et al.*, 2008). The preparation of multiple sections for most otoliths had the advantage of increasing the likelihood of at least one section being clear enough to interpret; often the section not including the primordium was easier to interpret.

A final opaque zone count, ranging from 1 to 14, was assigned to 1969 otoliths and the remaining 151 (6.8%) otoliths were considered either unreadable or the R_{MI} could not be measured. The I_{APE} between readings by OR1 was 4.77%, indicating a reasonable level of precision given their 14 year range in estimated age. Overall, 74.3% of the otoliths were given a confidence score of 3–5 (45 were given a score of 5). The mean \pm s.d. confidence score was 2.86 ± 1.09 . There was no significant correlation between final count and mean confidence score ($P > 0.05$), indicating that the variation in confidence cannot be explained by variation in age (approximate otolith size).

A comparison of counts by independent readers found that 53.9% were in agreement and 93.8% were within ± 1 . Counts by OR2 were slightly (but significantly) higher on average than OR1 (paired *t*-test, d.f. = 195, $P < 0.001$) [Fig. 4(a)]. The classification of the otolith edge was also compared between readers and found to be different for 33.5% of otoliths. In the majority (92.1%) of these cases, OR2 classified the edge as narrow (the distal opaque zone was complete) while OR1 classified the edge as wide (the distal opaque zone was incomplete). These differences in edge classification are likely to have contributed to the bias in counts if, for example, OR2 counted an additional opaque zone near the otolith edge (*i.e.* count +1 and a narrow MI) while OR1 did not (*i.e.* count and a wide MI). To determine if this was the case, 1 was subtracted from OR2's counts when the edge was classified as narrow

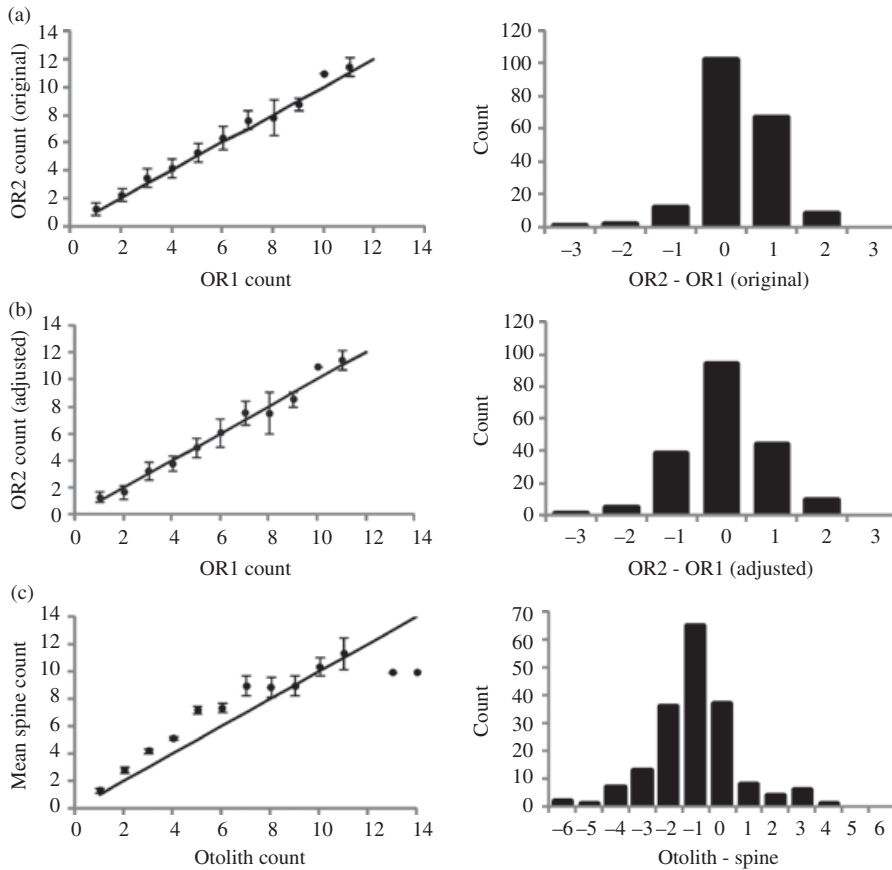


FIG. 4. Age-bias (mean \pm s.e.) and age difference plots for *Thunnus alalunga* for comparisons of (a) counts by otolith reader 1 (OR1) and otolith reader 2 (OR2) (unadjusted for edge classification errors, $n = 192$), (b) counts by OR1 and OR2 (adjusted for edge classification errors) and (c) counts from otoliths and spines ($n = 180$).

by OR2 and wide by OR1. Similarly, 1 was added to OR2's counts when the classification difference was reversed. The resulting comparison of counts showed that 92.1% were within ± 1 , the age bias was no longer evident (paired t -test, $P > 0.05$) [Fig. 4(b)] and the overall I_{APE} was similar to the intra-reader I_{APE} at 6.82%.

Fin spine reading and comparison of structures

The clarity of growth zones in cross sections of fin spines varied between fish. Of the 273 sections examined, 23 were considered unreadable and not given a final count. Only 31.0% of the spines were given a confidence score of 3 or 4 (none were given a score of 5). The mean \pm s.d. confidence score was 2.02 ± 1.02 . Unlike otoliths, there was a significant negative correlation between count and mean confidence score ($P < 0.01$), suggesting that spines became more difficult to interpret with increasing age.

A direct comparison of counts from otoliths and spines could be made for 180 fish. Spines with opaque growth zones that were outside the diameter ranges determined for each age could not be assigned a count. The age estimate from spines agreed with the estimate from otoliths in 37.0% of cases and 61.1% were within ± 1 of each other. The overall I_{APE} for counts between structures was 12.8%, however, and there was a significant difference in counts between structures (paired t -test, d.f. = 179, $P < 0.001$). The age-bias plot illustrates the ages at which differences in counts occurred [Fig. 4(c)] and shows that the bias was non-linear (Campana *et al.*, 1995). A bias was apparent for age classes 2–7 years where spine-based age estimates were higher on average than otolith-based age estimates. A bias was also apparent for age classes > 11 years where spine-based estimates were lower than otolith-based estimates.

AGE VALIDATION

Location of the first opaque growth zone

Counts of assumed daily increments were obtained for 16 of the 18 otoliths examined for micro-increment analysis and ranged from 185 to 552 days (mean \pm s.d. = 392 ± 81). Measurements from the first inflection point to the 365th micro-increment (Y1) were obtained for the 15 otoliths with counts > 365 and for one additional otolith for which a final count could not be resolved, but a measurement to the 365th micro-increment was obtained. The position of Y1 on the otolith occurred after the first opaque zone in 100% of sister otoliths. The average distance from the first inflection point to the outer edge of the first opaque zone was $660 \mu\text{m}$, to the 356th micro-increment (Y1) was $759 \mu\text{m}$ and to the otolith edge was $944 \mu\text{m}$. This confirms that the first opaque zone was being successfully identified in sectioned otoliths.

Counts of assumed daily increments were obtained for 47 of the subsequent 50 otoliths from juvenile fish examined and ranged from 258 to 445 days (mean \pm s.d. = 352 ± 41). Of these, an opaque zone was visible in 41 otoliths; the other sections were too thin to clearly locate the opaque zones assumed to be annuli. Similarly, the average distance from the first inflection point to the outer edge of the first opaque zone was $668 \mu\text{m}$ and to the edge of the otolith was $926 \mu\text{m}$, again supporting the conclusion that the first opaque zone is being correctly identified in these fish.

Direct validation

Examination of otoliths from the three recaptured OTC-injected fish supported the hypothesis of annual formation of opaque zones in *T. alalunga*. For all recaptured fish, an OTC mark was clearly visible in the otolith when viewed under ultraviolet (UV) light (Fig. 5). Fish number 1 was injected with OTC on 28 February 2009 at 61 cm L_F and recaptured on 16 February 2010 at 68 cm L_F . Fish number 2 was injected with OTC on 10 May 2010 at 78 cm L_F and recaptured on 28 March 2011 at 83 cm L_F . Fish number 3 was injected with OTC on 15 March 2009 at 51 cm L_F and recaptured on 23 March 2012 at 82 cm L_F . The OTC marks in the otoliths of fish numbers 1 and 2 were adjacent to the distal edge of the second last opaque zone suggesting that these zones are deposited over summer. The amount of otolith

growth in the 11–12 months subsequent to the OTC injection was consistent with the expected otolith growth if zones are deposited annually, as a thin translucent zone is present after the OTC mark and an additional opaque zone is almost complete at the edge of both otoliths (Fig. 5). The final opaque zone counts for these two injected fish were two and four with R_{MI} values of 81.3 and 66.7%. The high R_{MI} values indicate that the third and fifth opaque zones were forming at the otolith margin when the fish were recaptured. The OTC mark in the otolith of fish number 3 was adjacent to the distal edge of the first opaque zone, again supporting a summer deposition time for these zones. The overall clarity of growth zones in this otolith was not as good as for fish numbers 1 and 2 [Fig. 5(c)]. Despite this, the amount of otolith growth in the 3 years subsequent to the OTC injection was consistent with the expected otolith growth if zones are deposited annually. Three opaque zones were identified after the OTC mark, although the last zone was unclear and may not have fully formed, and the total count for this fish was 4.

Examination of the spines of the three OTC-injected fish was ambiguous. An apparent OTC mark was present on the fin spine of fish numbers 1 and 3, but not of fish number 2 (Fig. 6). There was a lot of background fluorescence associated with spine number 1, so it is possible that the apparent OTC mark for this fish is background fluorescence and that the OTC did not produce a fluorescent mark. In fish number 3, resorption and vascularization obscured both the mark and growth zones in some places, but the OTC mark was clear in other areas. Three opaque zones were obvious after the OTC mark [Fig. 6(d)]. The diameter of the first apparent opaque zone fell within the diameter range for second annual increments established using the stepped statistical replacement method (Table II), so one inner increment was assumed to have been obscured, producing a count of 4 for this fish.

Indirect validation

ANOVA indicated no significant differences in monthly R_{MI} between age classes 4, 5 and 6 years (Table III). There was, however, a significant year \times month interaction indicating that monthly R_{MI} varied between years. This result is most probably due to lower R_{MI} values in June, July and August in 2010 compared to 2009 (Fig. 7). To compare the pattern of monthly R_{MI} between regions, it was necessary to pool data across years as there were only 3 months of data for 2009 in the eastern region. Age classes were also pooled for each region based on the inference that R_{MI} did not differ significantly among age classes other than ages 4–6 years. ANOVA for these pooled data indicated that R_{MI} differed significantly between the western and eastern regions for at least some months (region \times month interaction) for all age classes combined (Table IV). Pair-wise comparisons, however, using the Bonferroni-adjusted significance value of 0.007 (0.05/7) revealed no significant differences in the monthly R_{MI} between regions. This result is probably due to slightly higher R_{MI} values in February and lower R_{MI} values in June in the eastern region compared to the western region (Fig. 8). For all other months, when data were available, the R_{MI} values were similar between the western and eastern regions. R_{MI} data were available for all months in the western region where there was a strong seasonal cycle in R_{MI} , indicating that one opaque zone forms per year, between April and September. During these months, the R_{MI} changes from being very large (opaque zone almost fully formed) to very small (new translucent zone forming).

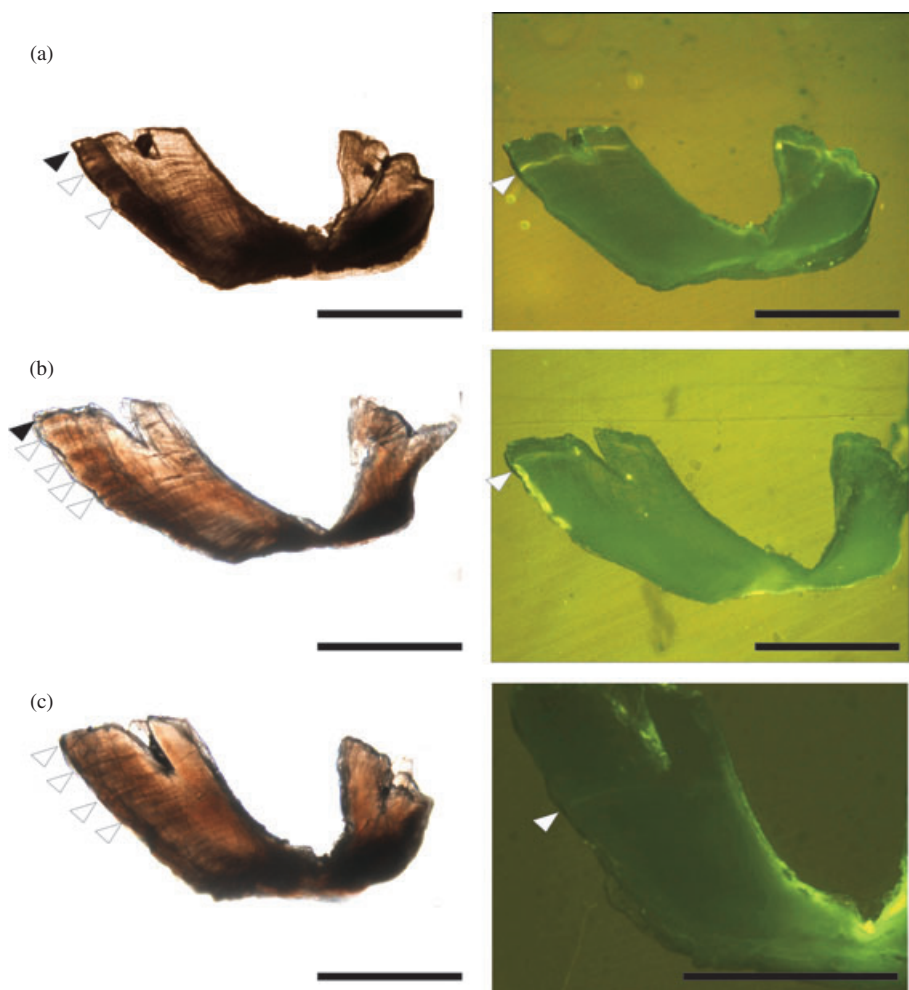


FIG. 5. Transverse sections of *Thunnus alalunga* otoliths injected with oxytetracycline (OTC) of fish number (a) 1, (b) 2 and (c) 3 showing the location of the distal edges of the opaque zones (▶) counted under transmitted light and partially completed opaque zone at the margin (▷) on the left side, and the OTC mark visible under ultraviolet light (▷) on the right side. Scale bar = 1 mm.

Decimal age

A decimal age was calculated for each fish using the count of opaque zones, an assumed birth date, the R_{MI} and date of capture. A birth date of 1 December was assigned based on the middle of the spawning season for *T. alalunga* in the South Pacific Ocean (Otsu & Hansen, 1962). It was then assumed that fish caught in December to March (classified as pre-increment fish) had not yet completed an opaque zone since their last birthday while fish caught in September to November (classified as post-increment) had completed an opaque zone. Given the variability in R_{MI} for fish caught between April and August (*i.e.* the period when opaque zones are completed) (Fig. 9), it was not known whether a fish with an R_{MI} in the range 40–70% had completed an opaque zone since their last birthday (in which case, it

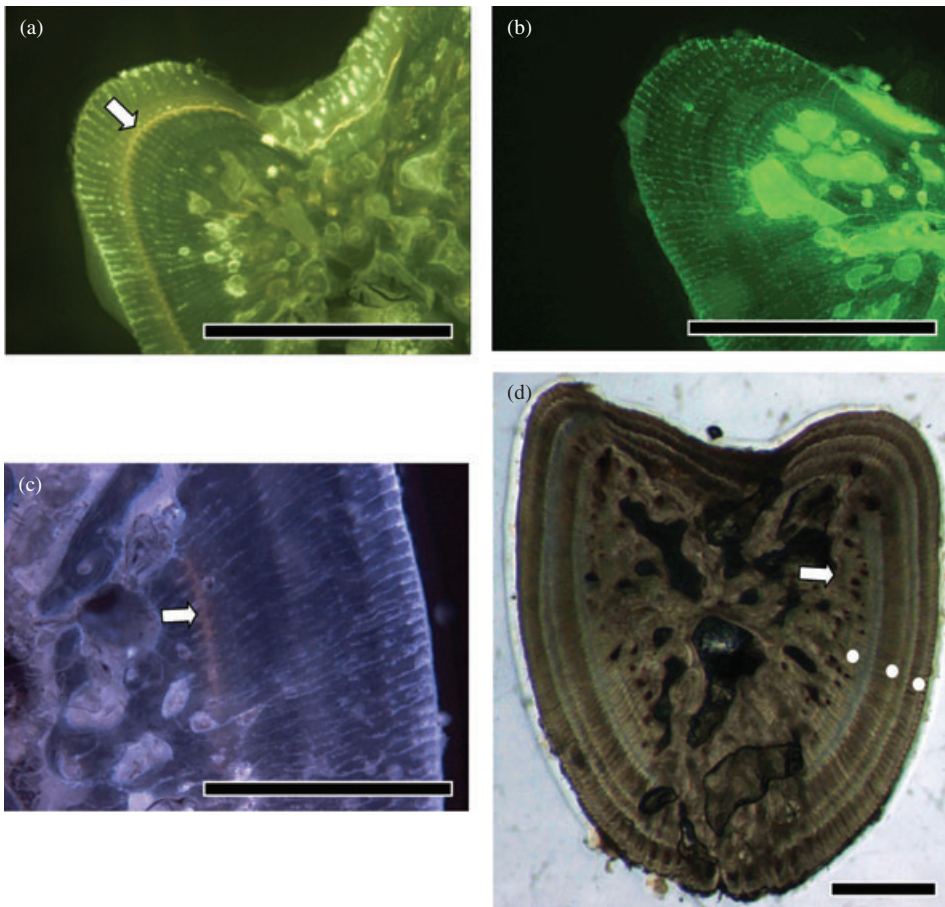


FIG. 6. Transverse sections of *Thunnus alalunga* fin spines injected with oxytetracycline (OTC). (a) Fish number 1 with possible location of the OTC mark visible under ultraviolet (UV) light (\rightarrow), (b) fish number 2 with no apparent OTC mark visible under UV light, (c) fish number 3 with OTC mark visible under dark field (\rightarrow) and (d) fish number 3 with location of the distal edges of the opaque zones (O) counted after the OTC mark under transmitted light. Scale bar = 1 mm.

should be classified as post-increment) or not (in which case, it should be classified as pre-increment). To assign these fish into their probable age class, an R_{MI} value was specified for each of the five uncertain months above which otoliths were classified as pre-increment, and below which they were classified as post-increment. The R_{MI} values were specified based on calculating the mean $R_{MI} - 2$ s.d. for December to March and the mean $R_{MI} + 2$ s.d. for September to November, and then fitting a regression line through these points (Fig. 9). Then, an otolith from a fish caught in April to August was classified as pre-increment if its R_{MI} value was above the regression line, and as post-increment if its R_{MI} value was below the line.

The decimal age for *T. alalunga* was calculated using the following algorithms described by Eveson *et al.* (2004): for fish classified as pre-increment: $a = n + r \cdot 365^{-1}$ and for fish classified as post-increment: $a = n - 1 + r \cdot 365^{-1}$, where a is the

TABLE III. ANOVA comparing marginal increment ratios between 2009 and 2010 and among the 4, 5 and 6 year age classes in the western region

	d.f.	SS	RSS	<i>F</i>	<i>P</i>
Year	1	587	152 134	1.75	>0.05
Age	2	1661	153 208	2.48	>0.05
Month	9	10 314	161 861	3.42	<0.001
Year × age	2	1876	153 422	2.80	>0.05
Year × month	8	6141	157 688	2.29	<0.05
Age × month	16	5729	157 276	1.07	>0.05
Year × age × month	14	5224	156 771	1.11	>0.05

decimal age, n the count and r is the day of capture (expressed as number of days since the assumed birth day of 1 December).

The maximum age obtained was 14.3 years. The relationship between otolith mass and estimated age was curvilinear (Fig. 10) with an r^2 value of 0.83 (cubic polynomial fit). The relatively high coefficient of determination provides some evidence that fish were assigned into their correct age classes.

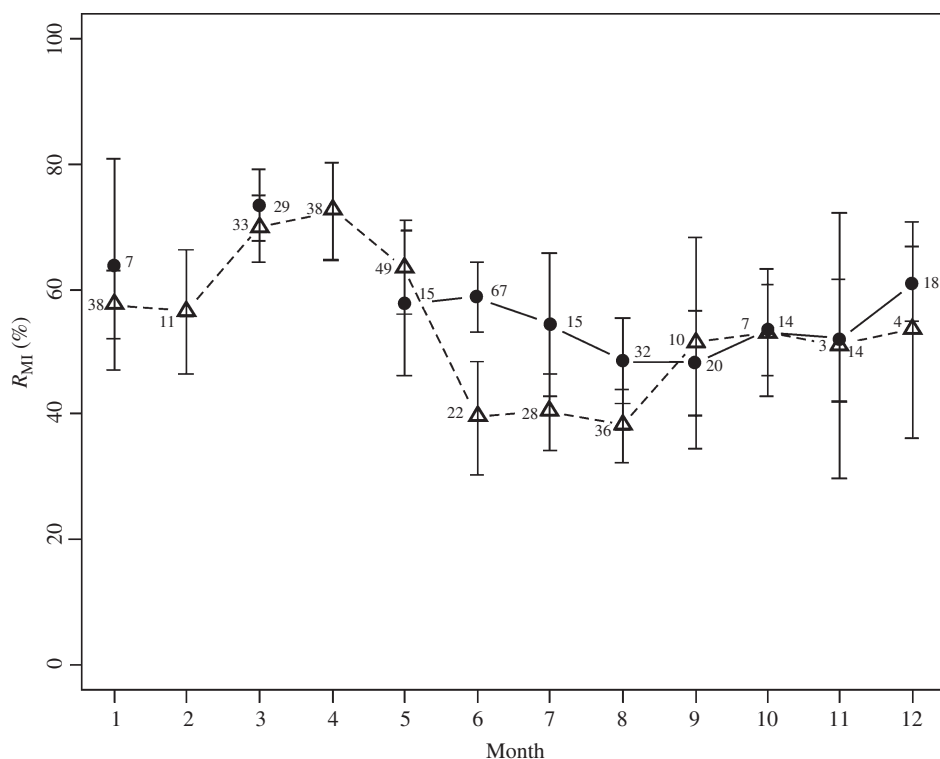


FIG. 7. Mean (\pm 95% c.i.) monthly marginal increment ratio (R_{Mi}) for 2009 (●) and 2010 (△) in the western region. Data were pooled across age classes 4–6 years. Numbers on lines are sample size.

TABLE IV. ANOVA comparing marginal increment ratios between the western and eastern regions

	d.f.	SS	RSS	<i>F</i>	<i>P</i>
Region	1	1649	543 124	3.85	0.05
Month	6	13 230	554 705	5.15	<0.001
Region × month	6	7500	548 975	2.92	<0.01

DISCUSSION

Validated annual age estimates using sectioned otoliths have been obtained for many fish species, including tunas (Clear *et al.*, 2000; Farley *et al.*, 2006; Gunn *et al.*, 2008; Neilson & Campana, 2008; Green *et al.*, 2009; Griffiths *et al.*, 2009). The results presented here support a growing body of evidence that sectioned otoliths can be used for routine ageing of tropical tuna and *T. alalunga*, in particular. The results demonstrate that otoliths provide accurate age estimates and are considered to be the preferred calcified structure for age determination as their growth is continuous and they are not subject to the resorption or vascularization

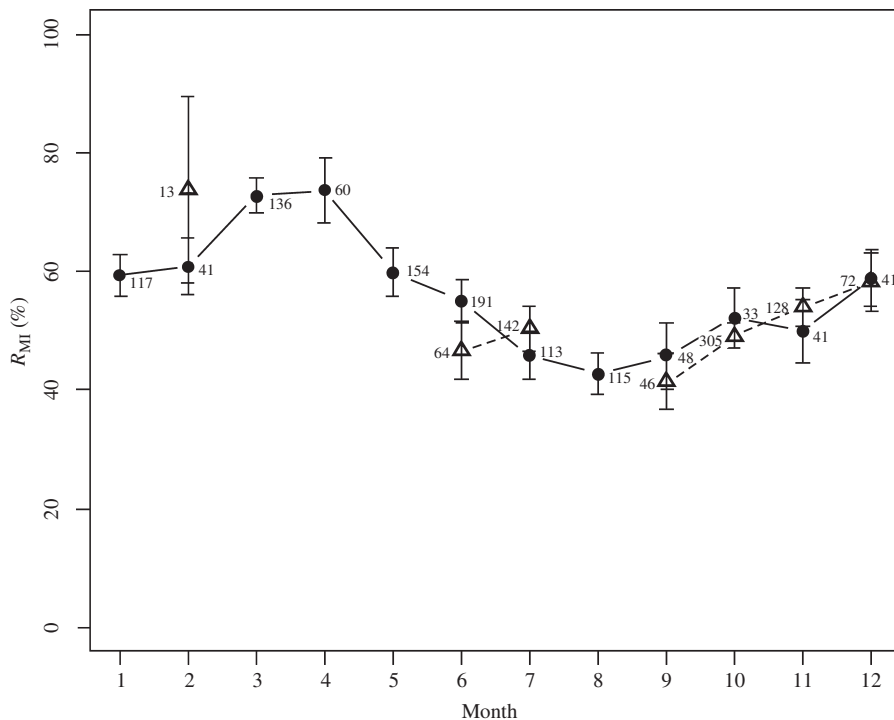


FIG. 8. Mean (\pm 95% c.i.) monthly marginal increment ratio (R_{MI}) for the western (●) and eastern (Δ) regions. Data were pooled across years and age classes. Numbers on lines are sample size.

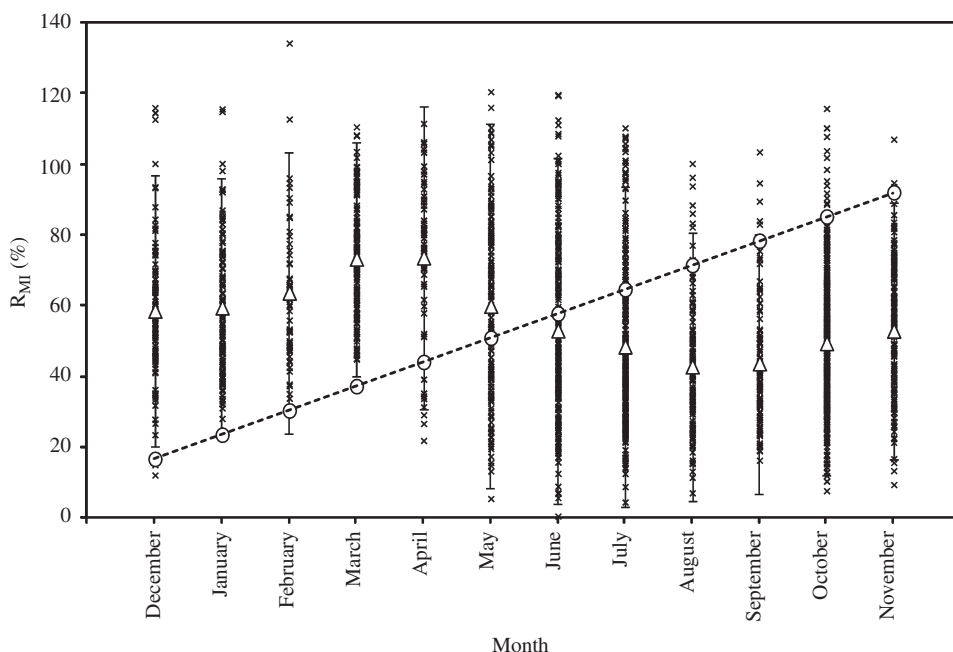


FIG. 9. Raw (x) and mean ± 2 S.D. (Δ) marginal increment ratio (R_{MI}) values by month for *Thunnus alalunga*. The regression curve is fitted to the values of -2 s.d. of the mean R_{MI} for December to March and $+2$ s.d. of the mean R_{MI} for September to November ($y = 6.85x + 16.48$). The R_{MI} values used to separate pre- and post-increment otoliths for April to August are the fitted values at each month from the regression line (\circ).

processes that commonly result in bias in counts of other fish hard parts (Secor *et al.*, 1995; Campana & Thorrold, 2001).

Otoliths in *T. alalunga* have clear growth zones that provided reproducible counts with a level of precision comparable to other tuna and temperate demersal species. To assess inter-reader precision, it was necessary to account for the edge classification assigned by each reader to their count. The resulting precision between readers was reasonable (I_{APE} of 6.82%), given the limited training provided to OR2. For some otoliths, the differences in edge classifications between readers suggested that OR1 did not count an opaque zone near the otolith margin unless a clear wider translucent zone followed it, whereas OR2 counted the opaque zone at the edge when a narrower translucent zone was evident. These differences may be the result of using different microscopes and light sources, which may have, for example, produced different refractive effects on the otolith edge, or OR1 was simply more cautious when interpreting the edge given that single opaque zones can appear as pairs. The algorithm developed to calculate decimal age for *T. alalunga* takes into account the otolith edge interpretation (*i.e.* the R_{MI}) by either reader in a consistent fashion for this study. These results indicate the importance of standardizing reading protocols and consistent training and induction of otolith readers involved in routine ageing for stock assessment and monitoring purposes.

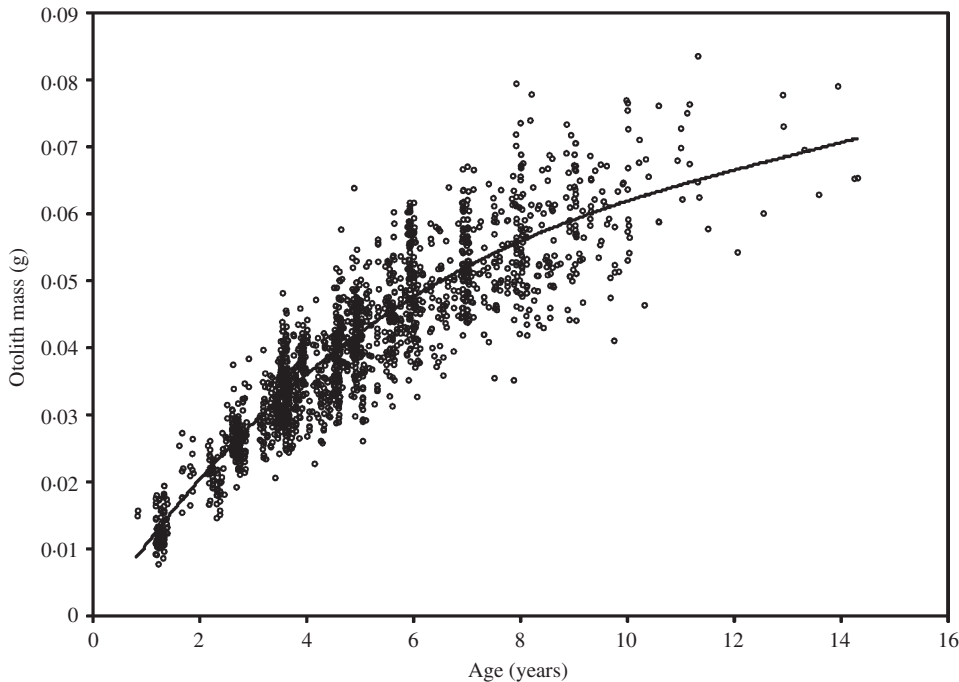


FIG. 10. Otolith mass-at-age for *Thunnus alalunga* and fitted cubic polynomial.

Counts of (assumed) daily increments were consistent with the first opaque zone being successfully identified in the annual counts and that this zone is likely to be deposited before the first birthday. This indirect validation method has been used in both tropical and temperate species but is best limited to fishes in their first year of life (Arneri *et al.*, 1998; Morales-Nin & Panfili, 2002). To use the method successfully, however, the periodicity of micro-increment formation must first be validated (Morales-Nin & Panfili, 2002). The only validation of daily increments for *T. alalunga* is from a tetracycline mark–recapture study in the North Pacific Ocean where a rate of 0.954 micro-increment per day was found from the analysis of whole otoliths (Laurs *et al.*, 1985). Despite the lack of direct validation of daily increments in the South Pacific Ocean, the age estimates obtained from daily counts are similar to those obtained for juvenile *T. alalunga* in other studies in the region (Leroy & Lehodey, 2004), and the size of estimated age 1 year fish is consistent with the first mode observed in length–frequency data for the New Zealand troll fishery (Griggs, 2005). Empirical validation of the micro-increment deposit rate in South Pacific *T. alalunga* is required to confirm the indirect validation method to locate the first opaque zone.

Examining otoliths from three OTC-injected fish demonstrated the annual periodicity of the third to fifth opaque zones in those fish. The dates of OTC injection and subsequent recapture coincided with the distal edge of consecutive opaque zones in the otoliths of each fish suggesting that these zones are completed in late summer to early winter. This periodicity is also consistent with the results from the R_{MI} analysis, which showed a sinusoidal pattern consistent with an annual cycle of increment

formation (Campana, 2001). The opaque zones formed consistently during the austral summer and were completed between April and August (R_{MI} changes from highest to lowest). The formation of the subsequent, narrower, translucent zone coincides with a slowing in otolith growth and changes in the structure of the otolith matrix (Wright *et al.*, 2002). Otolith MIA for other tunas in the southern hemisphere is consistent with completion of opaque zone formation in the austral winter. In *T. maccoyii*, the MI peaked in May and was lowest in August (Gunn *et al.*, 2008) while in longtail tuna *Thunnus tonggol* (Bleeker 1851) the incidence of otoliths with a narrow translucent margin was highest in May to October (Griffiths *et al.*, 2009). The annual cycle of increment formation is less clear in *T. obesus*, which may be due to the high variability between individuals, the greater difficulty in interpreting increments in *T. obesus* otoliths, or the less marked seasonal migration of this species (Farley *et al.*, 2006). Given the low sample size of some age classes for the MIA, it is important that OTC-injected fish continue to be released and recaptured to provide a basis for more comprehensive validation and accurate estimation of decimal age.

Dorsal-fin spines have been the most commonly used hard part for ageing *T. alalunga* in previous studies, primarily due to their ease of sampling (Davies *et al.*, 2008; Boyd, 2010). Different physiological pathways, however, determine growth in otoliths and spines. Otoliths are composed of metabolically inert calcium aragonite (Sweeting *et al.*, 2004), while spines are vascularized, skeletal material and, as such, are subject to resorption (Drew *et al.*, 2006; Boyd, 2010). This study has demonstrated that estimates of annual age from otoliths and spines from *T. alalunga* in the South Pacific Ocean provided different counts, and the levels of precision and reliability of reading associated with them differ. The results indicate that age estimates from spines appear to be positively biased for ages 2–7 years, and negatively biased for fish older than 11 years, relative to age estimates from otoliths. The size of this bias is likely to be sufficiently important for estimates of population and management reference points from stock assessments. Hence, it is important to determine the extent of the generality of these results by a more comprehensive sample of regions, years and ages in the South Pacific Ocean and for other *T. alalunga* stocks.

There are several possible explanations for the disagreement between opaque zone counts in otoliths and spines in fish <8 years old, including (1) the loss of early growth zones in spines due to vascularization was overestimated by the stepped statistical replacement method, (2) double or triple rings within a single translucent zone in spines were misinterpreted as multiple rather than single zones (false annuli) or (3) one or more opaque zones in otoliths were not observed. The last is unlikely, given the age validation methods undertaken in this study and the stability of the otolith structure. In older fish (>11 years), it is unclear whether the lower age estimates from spines were due to the underestimation of vascularized inner growth zones, a discontinuity of annual opaque zone formation in spines or the difficulty in discriminating multiple zones at the spine margin due to crowding.

Vascularization at the core and crowding of growth zones at the edge have been suggested as limiting factors in the use of spines to age large *T. alalunga* accurately (Boyd, 2010). Vascularization and resorption in spines can result in the growth zones in the core area not being visible and hence the zones deposited when the fish was young may not be observable (or included in counts). Fin spine cores that are obscured are commonly observed; however, the extent and timing of vascularization

and resorption vary among individuals so that it cannot be standardized easily. Hence, the 'true' number of obscured growth zones must be estimated for each section based on direct measurements of zones in sections from other individuals in other age classes. This is likely to be one source of error contributing to the bias and lower precision for the counts from spines. It also has implications for the form of the error structure assumed for the final counts as, strictly speaking, the final counts from a large proportion of the spines (those for which early opaque zones are assumed to have been obscured) are conditional on the estimates of the position of these zones estimated from a limited sub-set of samples and age classes.

The presence of multiple translucent rings within a translucent zone has been documented previously in the spines of tunas, including *T. alalunga* (Cort, 1991; Megalofonou, 2000; Megalofonou & de Metrio, 2000), and other fish species (Wright *et al.*, 2002). The cause of multiple rings in *T. alalunga* spines is unknown, but seasonal migration has been suggested for Mediterranean *T. alalunga* (Megalofonou, 2000). Additional causes may include unusual events such as food scarcity or expected events such as life-history transitions and reproduction (Wright *et al.*, 2002).

Validation of age estimates from *T. alalunga* spines may be limited. In this study, only one of the three OTC-marked spines could be used to verify the annual periodicity of opaque zones because the OTC marks were either faint or absent in the other two fish examined. Faint marks in spines and clear marks in the matching otoliths may be due to the difference in the composition and matrix of the two structures or that the composition of spines makes the fluorescence mark more susceptible to UV exposure. Ortiz de Zárate *et al.* (1996) reported that even a few minutes of exposure during examination decreased the intensity of the mark in spines and several short exposures made the mark disappear completely. Vascularization at the core of the spines may have also obscured the OTC marks. In *T. maccoyii*, strontium marks were obvious in otoliths but not in vertebrae (Clear *et al.*, 2000).

As noted, otoliths are immune from resorption and vascularization, are generally easier and more consistently interpretable and appear to be a more reliable structure for accurately estimating the age of *T. alalunga*. This is consistent with results for *T. maccoyii* and Atlantic bluefin tuna *Thunnus thynnus* (L. 1758) where otoliths were also found to be the best structure for estimating age over their full life span (Rodríguez-Marín *et al.*, 2007; Gunn *et al.*, 2008). For *T. maccoyii*, scales were useful only up to age 4 years, while vertebrae underestimated age compared to otoliths after age 10 years. This latter age was also the limit recommended for spines and vertebrae for *T. thynnus*, after which age estimates were underestimated relative to otoliths. The longest period at liberty reported for a tagged *T. alalunga* is 11 years (Langley & Hampton, 2005), although this fish is likely to have been between 12 and 15 years old because tagged fish were between 1 and 4 years old when released. This time at liberty is consistent with the oldest fish observed in this study (14 years) and a life span of at least 14–15 years.

Bias in age estimation leads to bias in other age-related population variables such as growth, mortality and age-at-maturity. In particular, underestimation of age leads to overestimation of growth and natural mortality, which in turn leads to overestimates of sustainable harvest rates from age-based stock assessments (Campana, 2001). Currently, the majority of stock assessments for tropical tuna fisheries are length-based and use length–frequency decomposition methods and mark–recapture

data to estimate age-based population parameters from commercial catch data (Hoyle, 2011). The results from this study, together with those for *T. obesus* (Farley *et al.*, 2006), *T. tonggol* (Griffiths *et al.*, 2009) and *T. maccoyii* (Gunn *et al.*, 2008), demonstrate that it is possible to obtain accurate and reliable direct age data from routine ageing of otoliths for tropical tuna. The ageing algorithm developed for *T. alalunga* allows the decimal age of fish across their entire life span to be estimated. These age estimates will provide a sound basis for estimating age-based metrics such as growth (Williams *et al.*, 2012) and reproductive parameters and should significantly improve the accuracy of models fits to catch-at-length data.

We are especially grateful to the fishing industry members, skippers and crew and the recreational fishing clubs, who kindly allowed us to sample their catch. We also thank the many port samplers, observers and observer co-ordinators for their assistance with the biological sampling programme. In addition, we thank K. Krusic-Golub at Fish Ageing Services Pty Ltd for preparing otoliths and fin spines for reading and analysing the OTC-marked hard parts. The work was supported by grants from CSIRO Wealth from Oceans Flagship, the Australian Fisheries Research and Development Corporation, the 9th European Union Development Fund (SCIFISH project) and the Western and Central Pacific Fisheries Commission. This manuscript was improved by comments from S. Griffiths, R. Buckworth and the anonymous reviewers.

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