Direct validation of annual increments in the otoliths of juvenile southern bluefin tuna, *Thunnus maccoyii*, by means of a large-scale mark-recapture experiment with strontium chloride

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Abstract.—From 1990 to 1996, during a large-scale tag-and-release program in the Great Australian Bight, 20,204 southern bluefin tuna (SBT), *Thunnus maccoyii*, were injected with strontium chloride ($SrCl_2$). The objectives of the marking experiment were to examine the efficacy of $SrCl_2$ as an otolith marker and to determine the periodicity of increment formation in SBT otoliths. Nine-hundred and sixty-one Sr-injected fish were recaptured and 616 otoliths were sampled from these; the high level of sampling success was attributable to a major liaison effort throughout the multinational SBT fishery. The same tag return rates for fish that were tagged and injected and for fish that were tagged only, indicated that the injection of strontium did not affect the survival rate of tagged fish. Strontium marks were detected with a Robinson detector or an energy dispersive spectrometer (EDS) (or with both) linked to a scanning electron microscope; 59 of the 67 otoliths from injected fish examined had discernible marks. The intensity of the strontium mark and the dosage rates were linked; a dosage of 100 mg Sr/kg fish weight is recommended to ensure easy identi fication of the strontium mark. Using the strontium marks, we established that in SBT with 1 to 6 increments in their otoliths, one increment is laid down per year at liberty. In the 59 marked fish that were examined, there was 100% agreement between the expected and observed number of increments after marking. These results, and the data from two supplementary tag returns from unmarked fish recaptured after long times at liberty, provide unambiguous evidence that increments on the otoliths of SBT are formed annually, to at least the age of 13 years. In addition, a recent study that used bomb-radiocarbon levels to estimate ages of older SBT has provided strong evidence that annual increments are deposited in the sagittal otoliths of SBT throughout life.

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Southern bluefin tuna (SBT), *Thunnus maccoyii* Castelnau, 1872, is a large, long-lived, migratory, pelagic fish with a circumglobal distribution between 30°S and 50°S (Caton, 1991). Its only known spawning ground is in the Indian Ocean south of Java, between 7°S and 20°S (Caton, 1991). Since it was first exploited in the 1950s the stock has declined dramatically to between 16% and 25% of its initial level (Sainsbury[1]). The fishery is currently managed by individual transferable quotas (ITQ) and the total allowable catch (TAC) is assessed each year.

Virtual population analysis (VPA) has been the main method of assessing the condition of SBT stock since 1980 (Murphy and Majkowski, 1981). The age structue of the population, a major input to VPA, has been estimated by converting lengths and weights to ages, using growth curves derived from tagging data (Hampton, 1991; Polacheck et al.[2]). To reduce the unmeasurable uncertainties that the estimates introduce into VPA assessments, a validated direct method for determining age was required.

In 1992 we began a study to develop reliable techniques for determining ages of SBT. Validation of assigned ages is critical in age estimation studies (Beamish and McFarlane, 1983; Smith, 1992; Secor et al., 1995); therefore a large-scale mark-recapture experiment was initiated to provide the basis for validating the age estimates. From the validation study we aimed to confirm the periodicity of the zones that were counted on the hard parts collected from SBT. The overall objective of these two studies was to develop a validated length-at-age key for the entire size range of the SBT population. We present details of the mark-recapture experiment and our evidence that increments in otoliths are formed annually.

[1] Sainsbury, K. 1993. What is happening to the southern bluefin stock? *In* W. Whitelaw and V. Mawson (eds.), Proceedings of the inaugural southern bluefish tuna science-industry-management workshop, Port Lincoln, Australia, p. 5–19. Commonwealth Scientific and Industrial Research Organization (CSIRO) Marine Research, GPO Box 1538 Hobart, 7001 Australia.

[2] Polacheck, T., K. Sainsbury, and N. Klaer. 1995. Assessment of the status of the southern bluefin tuna stock using virtual population analysis—1995. Paper SBFWS/95/17. First scientific meeting of the Commission for the Conservation of Southern Bluefin Tuna (CCSBT), Shimizu, Japan, 70 p. Commonwealth Scientific and Industrial Research Organization (CSIRO) Marine Research, GPO Box 1538, Hobart, Tasmania, 7001, Australia.

Previous attempts to estimate SBT ages directly, either did not attempt validation, or attempted it for only a few age classes. Hynd (1965) used scales to estimate ages of fish up to 80 cm fork length (FL) but did not attempt to validate his age estimates. Yukinawa (1970) counted up to eight rings on scales, using marginal increment analysis, to show that the rings form at the same time each year. Thorogood (1987) used otoliths to estimate age in fish from 42 to 167 cm FL and, using marginal increment analysis, was able to show seasonal band formation in what he called ages 2 to 4. Jenkins and Davis (1990) examined microincrements in the otoliths of SBT larvae between 3.5 and 12 mm standard length (SL) collected from the same cohort over consecutive days. From these microincrements, they validated daily increment formation and assigned approximate ages of 7 to 18 days to their samples.

In many age determination studies of other species of tuna, tetracycline has been used in marking experiments to validate daily increment formation in wild and captive tunas: e.g. yellowfin tuna, *Thunnus albacares* in the wild (Wild and Foreman, 1980; Wild et al., 1995) and in captivity (Yamanaka[3]); skipjack tuna, *Katsuwonus pelamis,* in the wild (Wild et al., 1995); black skipjack tuna, *Euthynnus lineatus,* in captivity (Wexler, 1993); and Atlantic bluefin tuna, *Thunnus thynnus,* in the wild (Inter-Am. Trop. Tuna Comm.[4]).

However, similar experiments using oxytetracycline (OTC) as a marker in SBT in the 1980s were less successful. In high proportion of OTC-injected fish, a mark failed to show up in the otoliths (Gunn[5]). Given this previous failure, and public health concerns over the use of tetracycline (in the USA, the Federal Drug Administration [US FDA] prohibits its use in wild fisheries), we selected strontium chloride ($SrCl_2$) as an alternative marker.

Strontium chloride is a nontoxic salt that occurs naturally in sea water. It is a component of some foods and is considered to be benign at the concentrations used as a marking agent (Sax and Lewis, 1987). Strontium is readily incorporated into the bloodstream of fish and, although not used previously on scombrids, it has been used successfully with other fish species to mark vertebrae (by introduction into food or in the surrounding water [Behrens et al., 1990]), and otoliths (by immersion and injection [Brothers, 1990]). Strontium is chemically and biologically similar to calcium. Because calcium and strontium ions have the same valency (2+) and a similar ionic radius (Ca, 0.099 nm; Sr, 0.113 nm), strontium readily substitutes for calcium during deposition of calcium carbonate.

The first two objectives of this study were 1) to evaluate whether intramuscular injection of strontium chloride resulted in effective and reliable marking of otoliths, and 2) to determine if strontium chloride injections increased mortality and, hence, affected recapture rates.

If successful and benign marking was demonstrated, we planned to use the strontium chloride marks to verify the accuracy of direct aging techniques by determining the periodicity of increment formation for as many year classes of SBT as possible.

Materials and methods

Tagging and marking

From 1990 to 1996, a total of 64,497 juvenile SBT in the Great Australian Bight were tagged and released. Of these, 20,204 tagged SBT were injected with $SrCl_2$ (Table 1). All fish were double-tagged (in case of "tag shedding") (Williams, 1992): strontium-injected fish were tagged with orange tags, fish that were not injected were tagged with yellow tags. When both orange and yellow tags were being deployed, an equal number of fish from targeted schools were chosen at random for injecting or not injecting. The smallest fish caught during the tagging program was 40 cm fork length (FL); we did not tag and inject fish smaller than this size because they were considered prerecruits, i.e. were not caught in the fishery. The lengths of orange-tagged fish ranged from 41 to 120 cm in FL. The return rates of yellow-tagged and orange-tagged fish were compared by using a chi-squared test to determine if they were significantly different.

The strontium chloride solution for injection was prepared in the laboratory. A stock solution of 1 g $SrCl_2.6H_2O$/mL was made up by dissolving 1 kg of analytical grade $SrCl_2.6H_2O$ crystals in 1 liter of distilled water, resulting in a 0.21 g/mL solution of Sr^{2+}. The solution was buffered to pH 7.0 with KOH and stored between 0°C and 4°C.

For rapid injections into large numbers of fish, a 5-mL automatic vaccinator fitted with a 0.2-mm needle was used. Flexible tubing connected the vaccinator to a plastic storage container that was either worn as a

[3] Yamanaka, K. L. 1990. Age, growth and spawning of yellowfin tuna in the southern Philippines. FAO, Indo-Pacif. Tuna Dev. Man. Prog. Working paper 90/WP/21, 87 p.

[4] Inter–Am. Trop. Tuna Comm. 1982. Annual Rep. for 1981, 303 p.

[5] Gunn, J. S. 1992. Progress report on strontium chloride marking of SBT during 1990–92 CSIRO–JAMARC tagging programs. Paper MWS4/WP–3. Fourth workshop of the southern bluefin tuna recruitment monitoring and tagging programs, Hobart, Australia, 9 p. Commonwealth Scientific and Industrial Research Organization (CSIRO) Marine Research, GPO Box 1538 Hobart, 7001, Australia.

Table 1

Numbers of southern bluefin tuna released with yellow tags and numbers of SBT injected with $SrCl_2$ and released with orange tags, and a summary of yellow and orange tag returns from 1990 to 1996. The number of returned yellow and orange tags released in each year of the tagging program, and the number of returned tags as a percentage of the total number released in the year (shown in parentheses) are shown. (The years of release and recapture are from October of one year to September of the next.) NS = not significant.

	1990–91		1991–92		1992–93		1993–94		1994–95		1995–96		1990–96 All release years	
	yellow tags *n*=6909	orange tags *n*=835	yellow tags *n*=4543	orange tags *n*=3595	yellow tags *n*=5907	orange tags *n*=5304	yellow tags *n*=8253	orange tags *n*=8251	yellow tags *n*=15,683	orange tags *n*=2219	yellow tags *n*=2998	orange tags *n*=0	yellow tags *n*=44,293	orange tags *n*=20,204
Fish recaptured														
1990–91	183 (2.65)	25 (2.99)	—	—	—	—	—	—	—	—	—	—	183	25
1991–92	180 (2.61)	19 (2.28)	84 (1.8)	55 (1.53)	—	—	—	—	—	—	—	—	264	74
1992–93	111 (1.61)	11 (1.32)	116 (2.6)	86 (2.39)	56 (0.95)	63 (1.19)	—	—	—	—	—	—	283	160
1993–94	62 (0.90)	6 (0.72)	102 (2.2)	76 (2.11)	130 (2.20)	90 (1.70)	50 (0.61)	47 (0.57)	—	—	—	—	344	219
1994–95	29 (0.42)	6 (0.72)	43 (0.95)	36 (1.00)	177 (3.00)	116 (2.19)	171 (2.07)	168 (2.04)	67 (0.43)	20 (0.90)	—	—	487	346
1995–96	2 (0.03)	0	6 (0.13)	6 (0.17)	29 (0.49)	17 (0.32)	86 (1.04)	89 (1.08)	94 0.60)	25 (1.13)	21 (0.70)	—	217	137
1990–96 All recapture years	567 (8.22)	67 (8.03)	351 (7.68)	259 (7.20)	392 (6.64)	286 (5.40)	307 (3.72)	304 (3.69)	161 (1.03)	45 (2.03)			1,779 (4.01)	961 (4.76)
Difference between yellow and orange tag returns	5.02 (0.83)		3.17 (0.94)		1.44 (0.61)		3.22 (0.40)		1.68 (0.39)				2.10 (0.56)	
($\chi^2(P)$)	NS		NS		NS		NS		NS				NS	

back-pack or attached to the tagging cradle (Williams, 1992).

We injected the fish about 2 cm below the dorsal midline, in line with the center of the first dorsal fin. We occasionally observed a loss of the solution from the muscle, especially in larger fish. When this happened, we noted the loss and injected the fish a second time. To minimize loss of solution we carefully expelled all the air from the vaccinator so that air bubbles were not injected into the muscle.

To determine a suitable dosage rate for SBT, initial trials with strontium were made on three nontuna species in captivity: silver trevally, *Pseudocaranx dentex*, sand flathead, *Platycephalus bassensis*, and purple wrasse, *Pseudolabrus fucicola*, the largest fish weighing 500 g. The dosages were based on Brothers' (1990) immersion and injection trials with strontium on trout—100 mg/kg—and Wild and Foreman's (1980) tetracycline experiments on tuna—0.3 mL/kg or 27.5 mg/kg of fish. Dosages between 50 and 200 mg Sr/kg resulted in clear Sr marks on otolith sections and no mortalities (CSIRO, unpubl. data[6]). In the field, the length of the fish was measured to the nearest centimeter, but weight could only be estimated. We therefore chose the required $SrCl_2$ dose according to length (Table 2). These dosages were increased for fish longer than 90 cm when the marks on the first otoliths recovered from SBT of this size showed up faintly, indicating low strontium absorption. The dosages were adjusted so that at least 80 mg of Sr was injected per kilogram of fish.

Tag collection and otolith sampling

A critical factor in the experiment was the sustained effort, over many years, to recover otoliths from recaptured fish. An extensive campaign was conducted to explain the objectives of the marking experiment to the SBT fishing industry and to develop a protocol for

[6] CSIRO (Commonwealth Scientific and Industrial Research Organization) unpublished data. 1989. CSIRO Marine Research, GPO Box 1538, Hobart, Tasmania, 7001, Australia.

Table 2
Dosages for $SrCl_2$-injected fish, based on length and weight of fish. FL = fork length.

FL (cm)	Approx. wt (kg)	Dose 1990–92			Dose 1993–96		
		$SrCl_2$ (mL)	Sr (mg)	mg Sr/kg fish	$SrCl_2$ (mL)	Sr (mg)	mg Sr/kg fish
40–50	1.5–3.0	2	400	130–270	2	400	130–270
51–70	3.0–7.0	3	600	86–200	3	600	86–200
71–80	7.0–10	4	800	80-114	4	800	80–114
81–90	10–15	5	1000	67–100	5	1000	67–100
91–95	15–18	6	1200	67–80	7	1400	78–93
96–100	18–21	7	1400	67–78	9	1800	86–100
>100	>21	8	1600	76	12	2400	114

collecting the samples. Posters and information notes, in Japanese and English, were distributed throughout the fishery. Rewards were offered for the return of tags and a substantial bonus for allowing scientists to sample otoliths from orange-tagged fish. To aid in otolith recovery, kits containing large orange disks were distributed to the crew of Japanese vessels; when an orange-tagged fish was caught, the disks were attached to the fish to clearly identify it in the freezers.

Given the high value of SBT on the Japanese sashimi market, it was essential that otoliths could be sampled without affecting the external appearance of the fish. Using a modification of the technique described by Thorogood (1986), we removed from the skull 35–44 mm cores that contained the semicircular canals and sagittal otoliths with a holesaw attached to a drill. The points of entry for the cores were over the basioccipital plates, which are anterior to the first vertebra and immediately lateral to the junction of the skull and first vertebra; this area was exposed when the tuna were cleaned and dressed—a process which removes the gill-filaments and surrounding tissue and most of the opercular flaps. By drilling through each of the plates in the direction of the back of the opposite eye, the cores coalesced at a point beyond the sagittal otoliths and could be removed easily from the skull, leaving no external mark on the fish (Fig. 1) Sagittal otoliths were removed from the cores, cleaned in distilled water, and dried at 28°C.

Age estimates

An age estimate was made from the otoliths before we attempted to identify a Sr mark. Increments on the whole sagittal otoliths comprised two zones: an opaque (assumed to be fast growth) and a narrower translucent zone (assumed to be slow growth) that appeared dark under a dissecting microscope with reflected lighting and a black background. Following Thorogood's (1987) method, one of each pair of sagittae was burned on a 400°C hot plate until it turned golden brown. The color change was greater in the translucent zones, making them more visible (Fig. 2A). A digital image of the burnt otolith was taken (using the public domain "NIH Image" program[7] and a video camera mounted on a Wild M5A dissecting microscope) and measurements were made of the otolith length and of the distance between the primordium and the inside of the translucent zones along both the postrostral (PR) and transverse axes. We use terminology that is currently widely accepted (Secor et al. [1992]; Kalish et al. [1995]; Stequert et al. [1996]). For each specimen, the reader made three independent age estimates by counting the number of increments obvious on the distal surface of the sagitta. These were made without reference to either the length of the fish or the time that the fish was at liberty after tagging and injection.

Detection of strontium marks

Scanning electron microscope (SEM) with a Robinson backscatter detector In the early stages of the project we used a Robinson backscatter detector coupled to a Philips 515 SEM for detecting the strontium-rich band in the otoliths (which we refer to as "the strontium mark"). The Robinson detector visualizes differences in the total atomic weight (Z) across a specimen. Because a strontium mark within the calcium carbonate contains a higher concentration of Sr, and hence a higher Z than surrounding uncontaminated calcium carbonate, it appears as a weak to intense bright band across the growth axis of the section (Figs. 2B and 3). The

[7] Rasband, W. 1994. NIH Image, vers. 1.54. U.S. National Institutes of Health. [Available from the Internet by anonymous ftp from zippy.nimh.nih.gov or as a floppy disk from NTIS, 5285 Port Royal Rd., Springfield, VA 22161, part number PB93-504868.]

intensity of the band depends on the magnitude of the difference in Z between the two portions of the otolith.

This kind of analysis requires a flat, polished surface; therefore we sectioned the sagittal otoliths either along the postrostral axis to produce an oblique longitudinal section (LS), or along a transverse axis. The rostral axis often shows clear increments, but we did not find distinct Sr marks in this part of the otolith. The sections were ground and polished following Gunn et al.'s (1992) methods and an evaporated carbon coat (25–30 nm thick) was applied to the sections to minimize charging in the SEM. The position of the Sr mark along the axis was measured with the vernier attached to the SEM stage drives (Fig. 2B).

Figure 1

The drilling technique used to extract otoliths from southern bluefin tuna destined to be sold as "whole" fish.

Energy-dispersive spectroscopy (EDS) In the later stages, an EDS x-ray microanalysis system became available and we used it to confirm the presence of Sr in the bright bands, and also to detect Sr marks in unsectioned otoliths (the use of unsectioned otoliths decreased the preparation time required for SEM analysis). The system consisted of a Link 133 eV Si(Li) detector with light element capability and a Thomson Scientific "WinEDS" PC-based analyzer attached to a Philips 515 SEM. Before x-ray analysis, whole (unsectioned) otoliths were acid-etched along the postrostral axis from the surface with 1 N and 3.5 N HCl, to expose the growth plane, then rinsed in bleach and distilled water, and dried. To minimize charging in the SEM, the otoliths were dipped in a dilute carbon DAG solution (approximately 1:50 with dichloroethane) immediately before analysis. Strontium marks were detected by operating the SEM in "spot" mode and searching for a point or zone where a significant Sr signal was detected on the x-ray microanalyzer (typically, a strong peak at 1.81 keV in the spectrum, corresponding to emission of Sr Lα x-rays). When a strontium mark was detected, its position along the PR axis was measured and the mark photographed either with conventional SEM photography (Fig. 4) or with a rapid, low-grade video print, which also showed the features of interest. To confirm that the suspected mark was strontium-rich, two plots of the x-ray spectra from the otolith were taken: one on the strontium mark and one just before the mark. Acceptable evidence of the correct identification of a strontium mark was considered to be the presence of an enhanced Sr level in the area analyzed, together with an absence of Sr (except for background levels) immediately before the area (Fig. 5).

The measurements of increments on the whole otoliths, and the strontium mark in sections or whole otoliths, were made along the same axes without reference to the other. This procedure enabled us to compare the number of increments observed after the strontium mark with the number expected, calculated from the known time at liberty after tagging.

Quantitative EDS analyses for linescans were carried out on carbon-coated polished sections in the Philips 515 SEM operated at an accelerating voltage of 20 kV, by using a focused electron beam of 0.15 µm diameter and analysis times of 60–200 seconds. The effective area analyzed by the beam was larger than the diameter of the beam itself because the beam spreads within the specimen after entry; examination of superficial beam damage to specimens after analysis suggested that the area analyzed by the beam is in the order of 2 µm diameter. Elemental concentrations were calculated by reference to appropriate standards

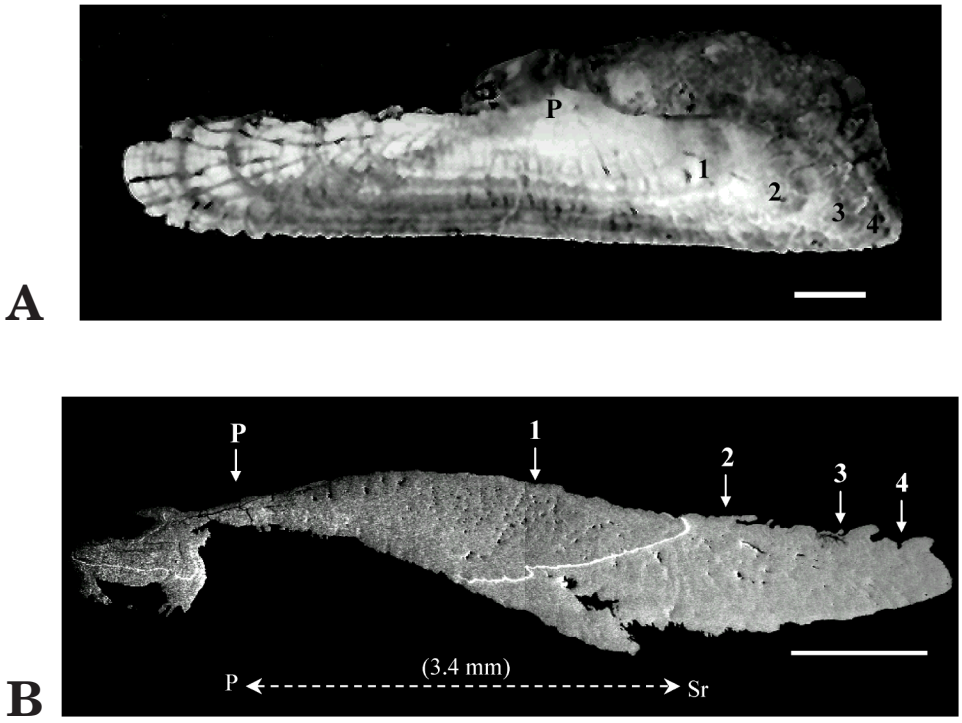

Figure 2

Sagitta (specimen OB 764) from a fish tagged and injected in March 1994 at 57 cm FL and recaptured in February 1997 at 111 cm FL. (**A**) Four increments were counted on the whole, burnt otolith and measured from the primordium (P) to the beginning of each translucent zone apparent on the distal surface along the postrostral axis at 2.2 mm, 3.6 mm, 4.4 mm, and 4.9 mm. (**B**) The sagitta was sectioned through the postrostral axis and viewed in the SEM: the micrograph shows the strontium mark which was located 3.4 mm from the primordium and the positions of the translucent zones measured on the otolith before it was sectioned. Scale bars: 1 mm

(calcite and celestite for Ca and Sr, respectively), with the "WinEDS" software.

Results

Of the 20,204 fish injected with $SrCl_2$ between 1990 and 1996, 9614 had been recaptured and 616 sets of sagittal otoliths were recovered from these by 1 January 1996. Seventy sets of otoliths were chosen for the validation study, selected from the range of size classes in the recaptures—fish of 45 to 102 cm FL at release and 57 to 133 cm FL at recapture—and from the range of times at liberty. Age estimates were made from 67 of the 70 otoliths; three sets of otoliths were excluded from the experiment because the increments on the whole otoliths were either ambiguous or uninterpretable and the reader could not give an age estimate with confidence.

Recapture rates of orange-tagged fish and recovery of otoliths

There were no statistically significant differences between the return rates of yellow tags (from fish not injected with $SrCl_2$) and orange tags (from fish injected with $SrCl_2$) released in all years of the program (χ^2=2.10, P=0.56) nor between the return rates of yellow tags and orange tags for any of the release years (Table 1). The number of otoliths recovered, as a percentage of orange tags recaptured, varied between 20%, in the first year of the tagging program, and 88% in the final year (Table 3); overall, otoliths were recovered from 65% of the orange-tagged fish that were recaptured.

Detection of Sr marks in the otoliths of orange-tagged fish

The otoliths removed from fish injected with strontium chloride typically showed a bright band in back-

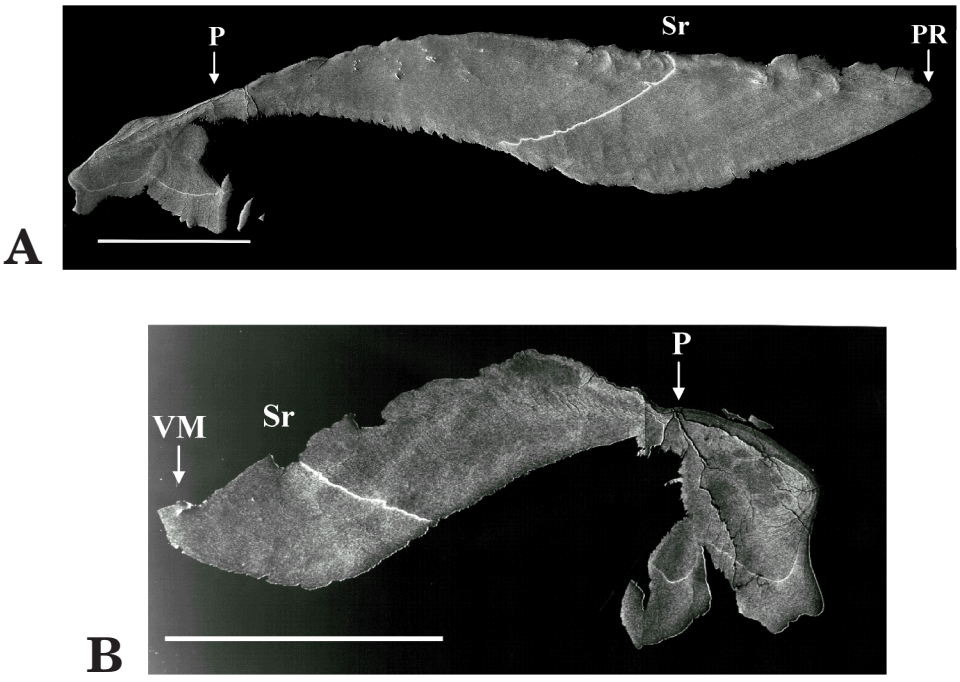

Figure 3

SEM micrographs showing strontium marks apparent as bright bands on sections taken from sister otoliths of specimen OB 102. (**A**) An oblique longitudinal section through the postrostral axis on which the primordium (P) and postrostrum (PR) are marked. (**B**) A transverse section on which the primordium and ventral margin (VM) are marked. Scale bars: 1 mm

Table 3

The number of tagged fish recaptured, and the number of otoliths recovered from orange-tagged fish during each year of the tagging program. (Data to 16 February 1996. The years of release and recapture are from October of one year to September of the next.)

Recapture year	Yellow-tagged fish recaptured	Orange-tagged fish recaptured	Otoliths recovered (otoliths recovered as a percentage of the orange-tagged fish recaptured)
1990–91	183	25	5 (20)
1991–92	264	74	49 (66)
1992–93	283	160	94 (59)
1993–94	344	219	107 (49)
1994–95	487	346	248 (72)
1995–96	238	137	120 (88)
Total	1799	961	623 (65)

scattered electron images of polished sections through appropriate growth planes (e.g. the oblique longitudinal section along the PR axis). The visibility of the Sr mark (brightness in the backscattered electron image) was highest in fish that had been relatively small at the time of injection (e.g. 50–55 cm FL). An example is specimen OB 102 (Fig. 3), which measured 49 cm at time of release and was injected with 2 mL $SrCl_2$ solution. This bright band was frequently associated with a local growth interruption immediately before the band, presumed to be a tagging check. This check was sometimes seen on the whole, etched otoliths (Fig. 4) but, although apparent in the SEM at high magnifications, it was not discernible from the other surface

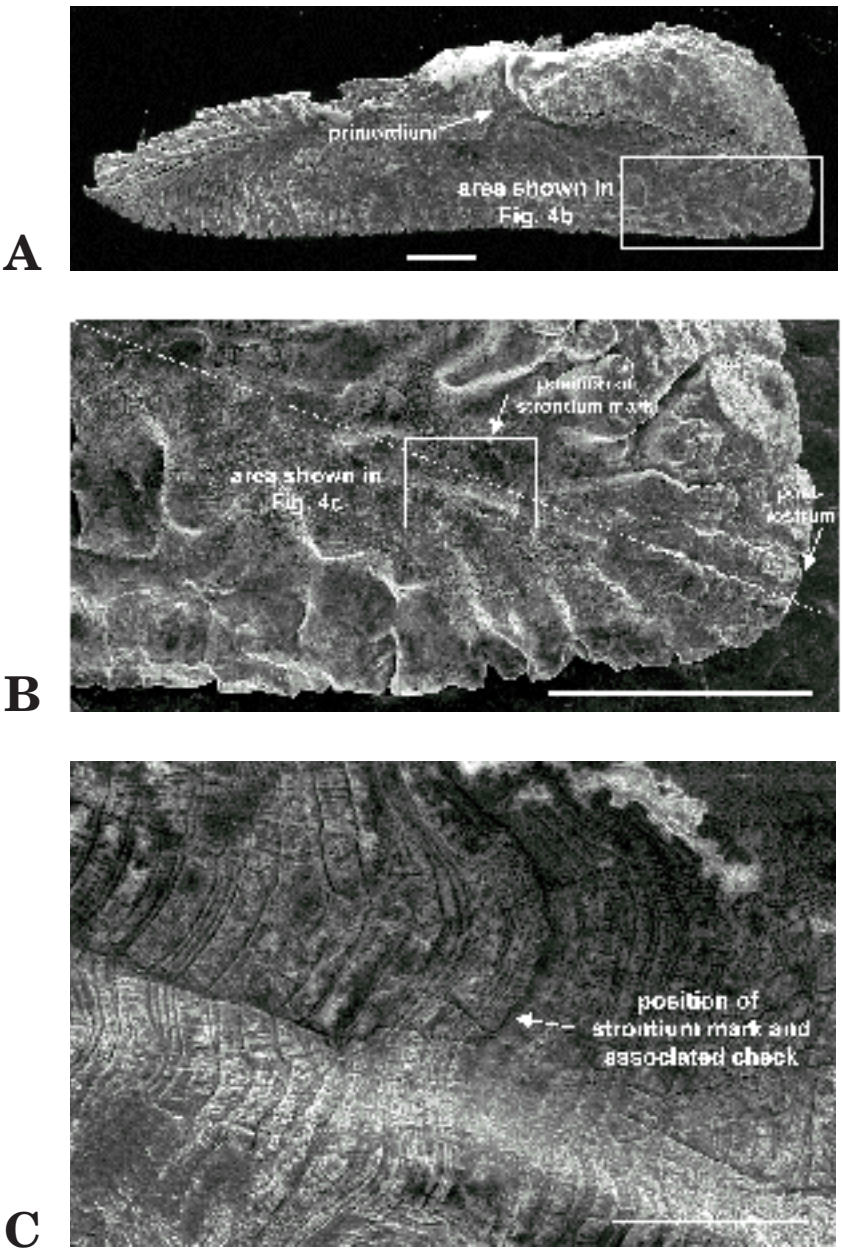

Figure 4

SEM micrographs of a carbon-coated whole etched otolith of specimen OB 127, showing the position of the strontium mark and the check associated with tagging and injection. The dotted line (B) indicates the axis along which the position of the strontium mark was measured in relation to the primordium and the postrostrum. Scale bars: 1 mm (**A** and **B**), 0.1 mm (**C**).

features on the whole otolith when increments were counted. We could not identify Sr marks on vertebral sections from fish injected with $SrCl_2$.

The presence of strontium in the bright bands was demonstrated by EDS spectra, which showed a strong peak of strontium Lα x-rays when the electron beam was directed to the Sr mark, in contrast with very low (background) levels in the regions of the otolith preceding the mark (Fig. 5). The relative levels of strontium and calcium in the bright band were further confirmed by inspection of the difference spectrum obtained by subtracting a spectrum acquired in the area

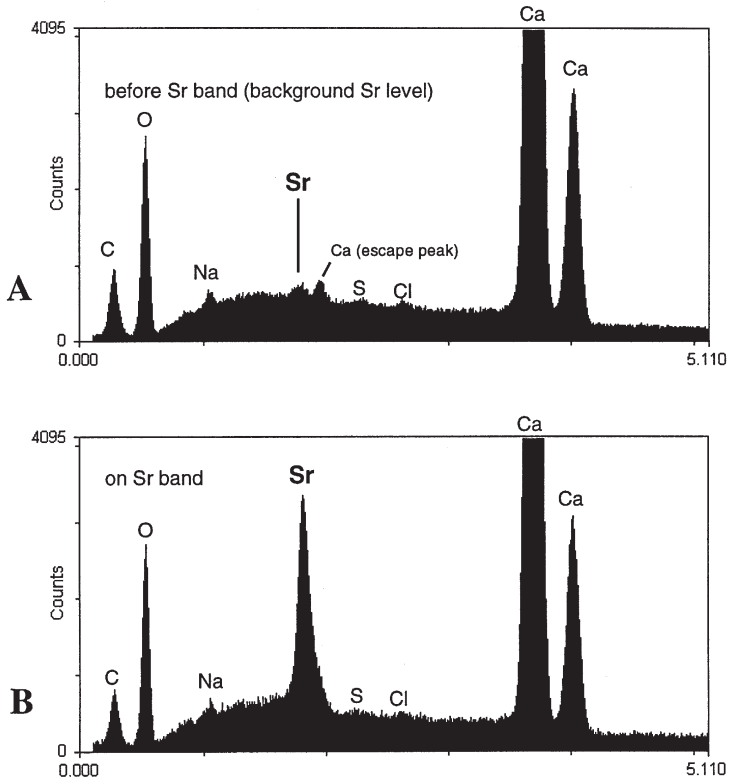

Figure 5

Examples of EDS spectra from a sectioned SBT otolith showing peaks due to background levels of Sr (**A**) and enhanced Sr levels associated with the strontium mark (**B**). Spectra similar to that shown in Figure 6B were used to positively identify the location of strontium marks in sections and etched whole otoliths (see text for further details).

preceding the mark (e.g. Fig. 5A) from one acquired on the bright band (Fig 5B), which demonstrated that strontium levels were enhanced and calcium was reduced in the bright band; however, no increase in chlorine was apparent. A quantitative EDS linescan across a bright band in one specimen, SBT OB 96, sectioned in oblique LS along the PR axis and analyzed along the direction of maximum observed growth, revealed "background" levels of 0.1% to 0.25% Sr by weight up to 5 microns before the band and a measured peak of 7.1% Sr on the band, falling to 3.5% (50% of peak level) 6 µm after the start of the band, and 0.7% (10% of peak level) approximately 15 µm after the start of the band. There is some indication of continuing slightly elevated Sr levels out to around 50 µm beyond the band, although visibility of these levels is at the limits of the EDS technique (Fig. 6).

Accompanying the measured maximum 7.1% increase in Sr level in the bright band is a fall in measured Ca concentration from 39% to 40% before the band to a minimum of 35.5% on the band—a decrease of 3.5–4.5% in absolute value or 10% relative value. Within the limits of accuracy of the EDS technique, this decrease in calcium concentration supports the theory that Ca atoms are being replaced by Sr atoms in the atomic structure on a 1:1 basis, each Sr atom being approximately twice as heavy as a Ca atom. Calculation of the increase in mean atomic number of the specimen resulting from a 7% increase in Sr and a 3.5% decrease in Ca gives a value of approximately 104 for the Sr-enriched zone. This value compares with 100 for the unaltered $CaCO_3$—a difference resolvable with backscattered electron imaging on the SEM on a suitably polished and coated specimen. The extent of the visible bright band in this specimen (OB 96) coincided with measured Sr levels in the range of 5–7%; thus it is possible that elevated Sr concentrations in the range of 0.5–5% may not be detectable by backscattered imaging although they should still be detectable by EDS. The EDS system is also essential for testing the identity of weak bright bands in sectioned specimens when it is not clear from

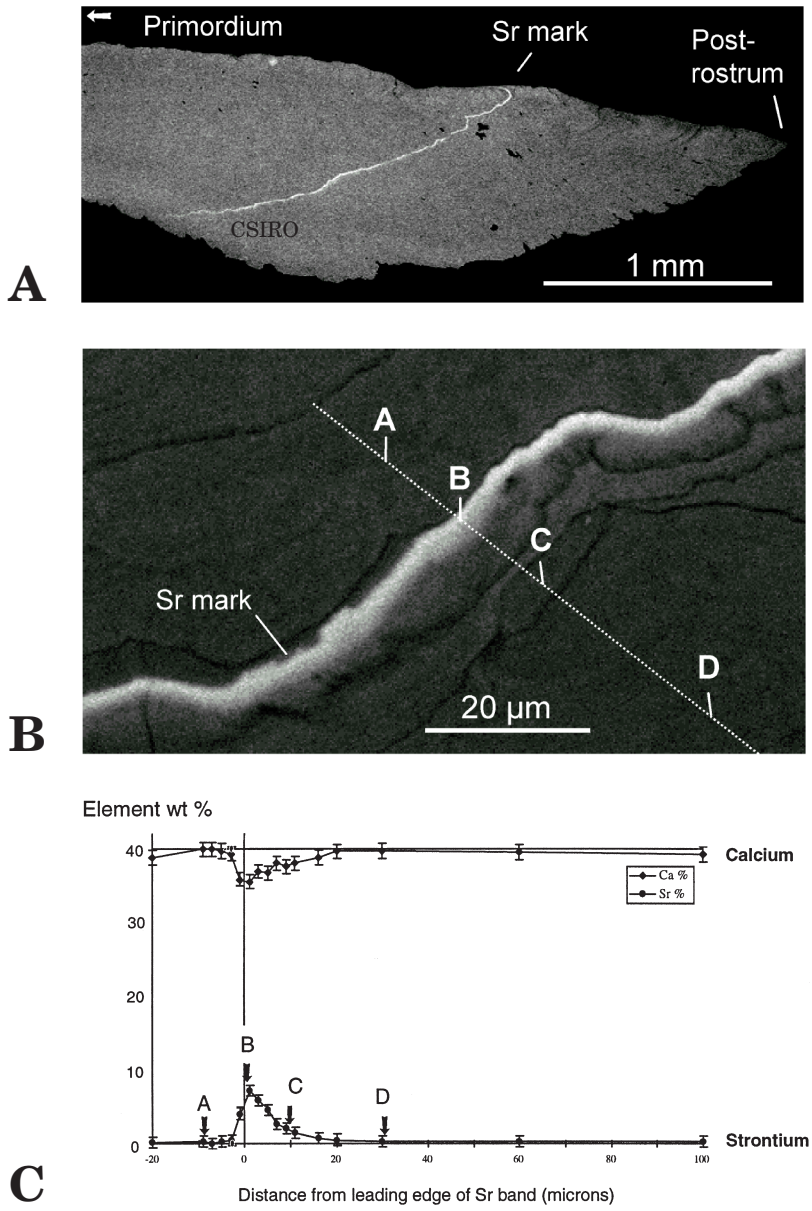

Figure 6

Details of the strontium mark on polished LS of specimen OB 96. Backscattered SEM images showing overall location of mark (**A**) and detail of region analyzed for Sr and Ca levels (**B**). Dashed line indicates transect followed for x-ray microanalysis (**C**): Measured variation in Sr and Ca levels along transect shown in B; points A–D indicate representative positions along the transect to enable comparison of sections B and C.

the backscattered imaging which band, if any, is a strontium mark.

Reliability of marking through injection of strontium

Of the 67 otoliths from which an age was estimated, strontium marks were detected with the Robinson detector in 19 of the 20 sectioned otoliths (95% detection rate) and with EDS in 40 out of 47 whole otoliths (85% detection rate). Strontium marks were as visible in the oblique longitudinal section (postrostral axis) as in the transverse section (Fig. 3). However, as increments are more widely spaced along the postrostral axis, we generally used the postrostral axis on the whole otolith for measuring the

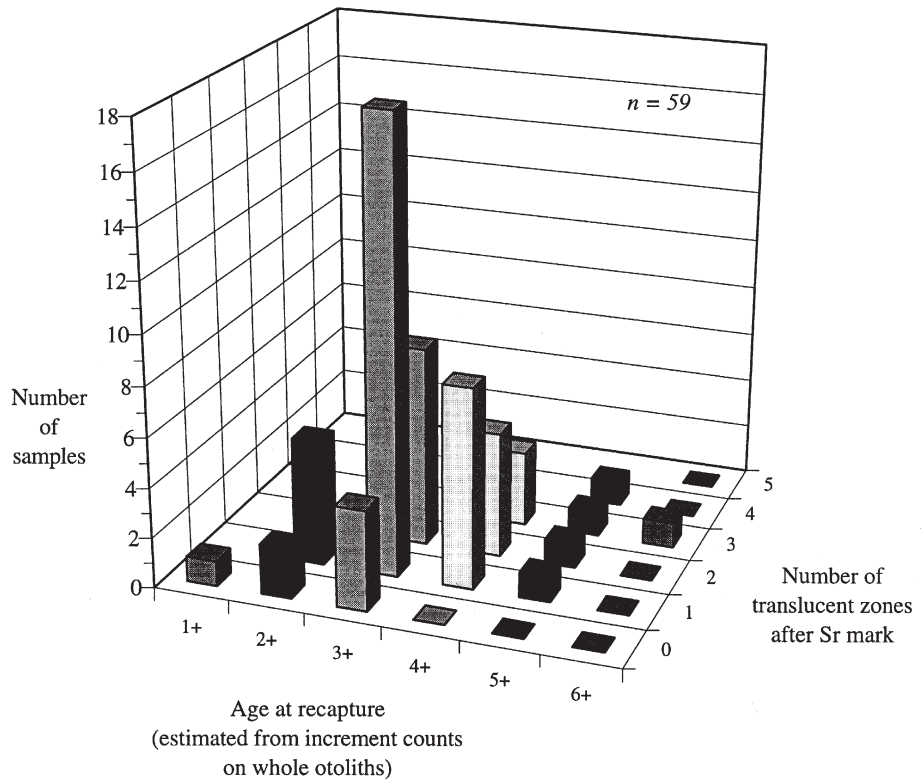

Figure 7

The number of strontium-marked otoliths used to validate age estimates that were made by counting increments on whole otoliths. The number of translucent zones observed after the strontium mark is shown for each age class. For all otoliths analyzed, the number of translucent zones counted after the Sr mark equalled the number that were expected from the period at liberty after fish were tagged and injected with $SrCl_2$.

position of translucent zones and for locating the strontium mark, either on a section (on the Robinson detector), or from the whole, etched otolith (with EDS).

Early in the experiment we found that the Sr marks in fish tagged and injected when they were 90 cm FL or larger were consistently fainter than in smaller fish. The concentration of Sr in the Sr marks of large fish was also significantly lower than in smaller fish caused, possibly, by loss of some of the solution during injection. To overcome this, dosages for large fish were increased in 1993 (Table 2), after which the bands were markedly more intense and easier to detect.

There was no apparent correlation between the time at liberty (i.e. time between Sr injection and recapture) and the intensity of the Sr marks; the longest time at liberty for a fish from which otoliths were analyzed for Sr was 1638 days. There was also no correlation between the intensity of the Sr marks and the delay between otolith recovery and analysis. Unlike tetracycline, which is photosensitive, the strontium mark did not fade after exposure to light.

Validation of annual increment formation from Sr marks

The 59 fish in which Sr marks were located ranged from 45 to 102 cm FL at the time of release, which corresponds to estimated ages of 1 to 4 (Gunn et al.[8]). Times at liberty ranged from 8 to 1638 days. The oldest recaptured fish was estimated to be 6+ years old; it had been at liberty for 1242 days (over 3 years).

[8] Gunn, J., N. Clear, T. Carter, A. Rees, C. Stanley, J. Kalish, and J. Johnstone. 1995. Age and growth of southern bluefin tuna. Paper SBFWS/95/8. First scientific meeting of the Commission for the Conservation of Southern Bluefin Tuna (CCSBT), Shimizu, Japan, 37 p. Commonwealth Scientific and Industrial Research Organization (CSIRO) Marine Research, GPO Box 1538 Hobart, 7001 Australia.

We counted six translucent zones on the otolith and the Sr mark occurred between the third and fourth zones (Fig. 7).

For all otoliths examined, there was agreement between the number of increments observed after the strontium mark and the number of increments expected, calculated from time at liberty. Thus, the annual periodicity in formation of increments 2 to 6 was validated for the otoliths analyzed. Because we were unable to tag young-of-the-year fish, in which the first translucent zone on the sagitta had yet to form, we could not determine when this translucent zone is laid down and when the formation of the first increment is completed. However, studies of daily microincrements (Itoh and Tsuji, 1996; Rees et al.[9]) have calculated that the approximate size at age 1 is 50 cm. We found otoliths of 50-cm fish had one increment.

Of the otolith increments counted, the first translucent zone was typically the most difficult to measure. The beginning of the first translucent zone occurred between 2.2 and 3.2 mm from the primordium along the postrostral axis, the most commonly used axis for analysis. Rees et al.[9] found microincrements in this region to be narrower than those deposited earlier, indicating a period of slow growth of the fish.

Additional validation of annual increment formation from tagged fish at liberty for extended periods

During the course of our experiment, two fish tagged by CSIRO in the 1980s were recaptured and their otoliths sampled. From lengths at first release of 45 cm and 82 cm FL, the fish had grown to 163 cm and 162 cm after being at liberty for 9 years, 7 months, and 10 years, 8 months, respectively. From the age-length key developed by Gunn et al.[8] we calculated that the 45-cm fish tagged in 1983 was one year old when tagged, whereas the 82-cm fish tagged in 1984 was two years old. The ages at recapture of these two fish were estimated from transverse sections through the primordium of the sagittal otoliths. Eleven increments (opaque and translucent zones) were counted on the otoliths from the fish released as a one-year-old and caught 9.58 years later; 13 increments were counted in the fish released as a two-year-old and recaptured 10.75 years later.

[9] Rees, A. J., J. S. Gunn, and N. P. Clear. 1996. Age determination of juvenile southern bluefin tuna, *Thunnus maccoyii*, based on scanning electron microscopy of otolith microincrements. *In* J. Gunn, N. Clear, T. Carter, J. Farley, A. Rees, and C. Stanley, Appendix 1: The direct estimation of age in southern bluefin tuna. Second scientific meeting of the Commissionfor Conservation of Southern Bluefin Tuna (CCSBT), Hobart, Australia. 26 August–5 September 1996, 22 p. Commonwealth Scientific and Industrial Research Organisation (CSIRO) Marine Research, GPO Box 1538, Hobart, Tasmania, 7001 Australia.

Discussion

Validation

This study demonstrated that, in the sagittae of SBT, the second through sixth increments, are deposited annually. This validation is independent of when the marked fish were tagged or recaptured. Because daily age estimates have been used to demonstrate that the first major increment in the sagitta forms in the first year of life (Rees et al.[9]), the annual formation of translucent zones appears to hold for the first six increments in SBT sagittae—corresponding to fish up to approximately 133 cm fork length.

The close agreement between increment counts on otoliths and the sum of age-at-tagging and time-at-liberty for two fish tagged in the 1980s and recaptured in the 1990s indicated that increment formation continues to be annual in fish up to at least 13 years old. Further evidence that increments in SBT sagittae are formed annually throughout life has been provided by a recent comparison of increment counts with age estimates derived from levels of bomb-radiocarbon in the early growth zones of sagittae (Kalish et al. 1996). This study reports close agreement between the two methods of estimating age for fish up to 34 years old.

Three sources of data—those from our marking experiment, the increment counts for two fish at liberty for over a decade, and the bomb radiocarbon data—provide strong evidence that seasonal changes in growth are expressed as clearly identifiable annual increments in the sagittal otoliths of SBT. These increments can be used to estimate the age of individual fish at any point in their lifespan.

Prior to our studies, Yukinawa (1970, using scales) and Thorogood (1987, using otoliths) used marginal increment analyses to demonstrate the annual check or translucent band deposition in fish they considered to be between 2 and 4 years old. Their results differ from ours only in the identity of year classes; their two- to four-year-olds correspond to our one- to three-year-olds. The difference in scale readings derives from Hynd's (1965) observation of two "checks" on the scales of new recruits (approximately 50 cm FL) to the Western Australian fishery. Interpretation of otolith microincrements (Itoh and Tsuji, 1996; Rees et al.[9]) indicates that these fish are only one year old. Unequivocal validation of these estimates is not possible at this stage because samples from prerecruits were not available to either Hynd or Yukinawa and we were not able to tag and mark prerecruit fish. In a number of other *Thunnus* species however, 50–60 cm fish were found to be around one year old (Uchiyama and Struhsaker, 1981; Wild, 1986; Foreman, 1996) and our counts of otolith microincrements and the data based on their inter-

pretation are consistent with this age. Therefore, we believe that the interpretation of Itoh and Tsuji (1996) and Rees et al.[9], that 50-cm-FL fish are one-year-olds, is most likely correct.

The identification of fish that we considered to be one-year-olds as two-year-olds was made by Thorogood in his 1987 study. However, we have found no evidence of two increments in the otoliths of new recruits. The early zones on all axes of otolith growth are difficult to read on some otoliths, and the increments in these areas are less distinct than those deposited later. In some fish a poorly defined "band" is also present very close to the primordium (within 2 mm along the postrostral axis). Although these two factors may confuse an inexperienced reader, Thorogood makes no mention of difficulty in reading the first increment. An alternative explanation for Thorogood's interpretations may be that his readings were influenced by the findings of Hynd (1965) and Yukinawa (1970) that were based on scales, because their estimates of age at recruitment were entrenched within the dogma of SBT population dynamics current in the 1970s and 1980s.

It has been hypothesized that more than one translucent zone forms per year in the otoliths of mature Atlantic bluefin tuna, *Thunnus thynnus* (Berry et al., 1977; Lee et al., 1983). In females, one translucent zone may correspond to a winter slow-growth period, the other to a spawning period (Lee et al., 1983). In the two large, tagged fish examined in our study, only one opaque and one translucent zone were deposited per year throughout life. The outer increments (i.e. those assumed to be deposited after sexual maturation) were consistent in both their width and optical density and were visually equivalent to the increments described by Lee et al. (1983), comprising a wide opaque region and a narrow translucent area that, on a black background, appears dark under reflected light. Occasionally, there appeared to be two translucent zones closer together than normal and, if these bands coalesced at the margin, they were counted as part of the same increment. These may be equivalent to the bands described by Berry et al. (1977) who hypothesized that a pair of these paired bands represented an annual increment. The close agreement between otolith increment counts and bomb-radiocarbon age estimates for mature SBT up to 34 years old (Kalish et al., 1996) supports our hypothesis that one increment, comprising one translucent and one opaque zone, continues to form per year, as does the consistency of the width and optical density of increments deposited after sexual maturation in the otoliths aged by Kalish et al. (1996). In summary, there is no significant evidence to suggest that mature female SBT deposit two translucent zones per year. In this regard our findings are similar to those of Hurley and Iles (1983) and Hurlbut and Clay (1988) for *T. thynnus*; they found, albeit in the absence of direct validation, that a single translucent zone is laid down per year in medium- and giant-size classes.

The use of strontium chloride to mark otoliths of large fish

This study has shown that intramuscular injection of strontium chloride leaves a distinct mark on the otoliths of SBT that is clearly visible as an SEM backscatter image in the Robinson detector. In the 20 otolith sections from Sr-injected fish that we examined, 95% had detectable marks. On this basis, we conclude that the compound is an efficient marker. Success of OTC as a marker at this rate of detection (95%) leads to high mortalities (McFarlane and Beamish, 1987). The high detection rates and lack of evidence of mortality for $SrCl_2$ are not surprising. This mineral occurs naturally in sea water, the mean concentration being 3.8–8.2 ppm (Carriker et al., 1991) or 0.09 mM/kg (Bruland, 1983), and both Sr and Cl are major constituents of the otoliths of SBT (Gunn[5]). When $SrCl_2$ is injected into the muscle it is taken up into the blood stream and incorporated in the endolymph and then the otolith, substituting for Ca within the $CaCO_3$ portion of the aragonite. The combined weight fraction of Ca and Sr (approximately 42%) within the otolith does not change as a result of the injection. However, the Ca:Sr ratio changes from 250:1 before injection to as low as 5:1 during the period over which the Sr spike induced by the injection is metabolized. At a distance of 6 μm beyond the injection mark, the Sr levels have dropped to about 50% and at 15 µm to 10% of peak values (Fig. 6). These distances correspond to time periods in the order of 2 and 5 days, respectively, based on median growth rates of around 3.0 µm/day estimated along this axis (Rees et al.[9]).

Detecting Sr marks on whole otoliths by using EDS was possible because the growth plane of tuna otoliths lies near the surface of the distal face. In otoliths of young fish, etching will expose the growth plane, so that sectioning is not required. Although this method of detection was slightly less successful (85%), it had two advantages. First, the preparation time was around half that required to prepare sections suitable for the Robinson detector. Second, the age estimate and measurements of increments were made along the postrostral axis on whole otoliths from the smaller fish (up to six years old), and the method of locating the strontium mark by EDS meant that the position of the strontium mark was measured along the axis in the same plane. With the Robinson detector, the same axis was measured but in cross section.

In fish older than about 6 years, the increments deposited on the margin can be unclear on whole oto-

liths; therefore transverse sections are used to determine ages of older fish (Gunn et al.[8]). In the future, as strontium-marked otoliths are returned from older fish that have been at liberty for longer periods, we will locate the strontium mark in transverse sections with the SEM and increase the number of increments that have been validated.

The recapture rates of orange-tagged and injected SBT were not significantly different from the recapture rates of yellow-tagged SBT; therefore the Sr injections apparently did not affect survival rate. Although the dosages of Sr varied between 65 and 250 mg/kg of fish, there is no evidence to suggest that higher doses increased mortality because Sr-injected fish with the highest doses were among those recaptured. A direct relation between the dosage and the intensity of the mark had been found in trials with three other species in 1990–91 (CSIRO, unpublished data[6]). The increased dose for large SBT resulted in much more distinct marks on their otoliths, which have a larger surface area than that in smaller fish. The less distinct marks could also be attributable in some large fish to a loss of strontium solution from the muscle after injection. Although more solution was injected if a loss was noticed, there may have been further loss of solution after the fish was returned to the water, resulting in a less effective dose. Thus, as a general guideline, we recommend a dose of 100 mg Sr/kg for marking otoliths in SBT. We note, however, that tissue area around the injection should be observed to ensure that there is no loss of injected solution from the muscle tissue.

The problem of detecting indistinct marks that result from low dosage levels are eliminated by using $SrCl_2$ as a marker. Unlike fluorescent marking, where it is very difficult to evaluate faint or ambiguous marks objectively (particularly if they are close to the outside edge of the otolith), it is possible to evaluate Sr marks objectively by x-ray analysis. Because the concentrations of the Ca and Sr on the Sr mark are high, very simple energy dispersive spectroscopy systems, which are available in many SEM facilities, can be used. Although not a trivial procedure, x-ray analysis requires preparation methods similar to those used for examining fluorescent markers and can usually be contracted to facilities at a low cost. Given the often substantial investment in tagging programs, and the common combination of low recapture rates and even lower otolith sampling rates, every sample is extremely valuable in a marking experiment. The safety net of chemical analysis is thus very advantageous.

Comparison of strontium and fluorescent markers

At the beginning of this project we chose strontium chloride over the more commonly used fluorescent markers because previous work on SBT with oxytetracycline had been unsuccessful. Although immersion in high concentrations of strontium or feeding with strontium-laced food (or both) had been used successfully for marking hard parts of larvae and juveniles of hatchery-reared salmon (Behrens Yamada and Mulligan, 1982; 1990), salmonids and a variety of tropical fish species (Brothers, 1990) and squid (Hurley et al., 1985), strontium had not previously been used to mark otoliths of large fish. On the basis of his experiments, Brothers (1990) concluded that, for mass marking of fish in captivity, detection of strontium marks was expensive and involved more difficult preparation than did fluorescent markers and other marking techniques such as thermal inducement (Volk et al., 1990).

Brothers' (1990) comment on expense is certainly pertinent but the expense of analyzing marked otoliths is often a small fraction of the cost of a marking experiment, particularly one where large numbers of fish have been tagged, injected, and released. Perhaps most important in the cost equation should be the rate of success of detecting marks in the otoliths of marked fish rather than the comparative cost of analysis. In otoliths from large tuna whose time at liberty has been long, the strontium marks are covered by a large amount of otolith material deposited after the time of injection. Sectioning is necessary for either marker; thus preparation times in these cases are much the same. For smaller tuna and those at liberty for short periods, fluorescent markers can be detected in the whole otolith, whereas detection of strontium without an EDS system would require sectioning, which would increase preparation time. The equipment for fluorescent markers is cheaper and comprises a light microscope equipped with an ultraviolet illumination source and filters to match the wave length of the fluorescence emitted from the marker when excited by the light source (see Wild and Foreman, 1980). For strontium, an SEM equipped with a Robinson detector is the minimum requirement; an EDS system is a useful extra. Although an SEM is a common apparatus in large research laboratories, hourly charges to the user can be high, although we have found that, with well prepared specimens, as many as four otoliths can be examined and analyzed per hour with an SEM.

Apart from preparation time and costs, strontium marking for age validation has clear advantages over fluorescent marking. One benefit of a technique that requires both a light microscope and an SEM is that measurements of increments and strontium marks are independent: the strontium cannot be detected in whole otoliths under the light microscope and the annual increments cannot be observed in the SEM. Allergic reactions by humans to compounds such as oxytetracycline have led the U.S. FDA to ban their use

in commercial fisheries. Strontium chloride, on the other hand, is regarded as safe for human consumption because it is a salt with a low order of toxicity (Sax and Lewis, 1987). It is even used in toothpaste by some manufacturers (e.g. "Sensodyne"). Strontium chloride, unlike fluorescent markers such as oxytetracycline, is not photosensitive. Neither the marking solution nor the marked otoliths need to be stored in the dark, and the mark does not fade with exposure to light or with time. In our study, strontium marks were as evident in fish that had been at liberty for long periods as in fish recaptured soon after release.

In summary, strontium chloride injection has proved to be a very successful way to mark the otoliths of southern bluefin tuna: 95% of those marked and recaptured in this study had detectable Sr marks in sectioned otoliths. This high "success rate," the harmless nature of $SrCl_2$ to both fish and humans, the capacity of EDS to positively identify the strontium mark, the insensitivity of the strontium mark to light, and the longevity of the strontium mark indicate that it should be seriously considered by those interested in large-scale marking experiments on commercial fishes.

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