Abstract.–A model was proposed for validation studies of the periodicity and timing of growth checks on fish otolith sections, based on measurements of otolith radii around tetracycline (OTC) marks. Continuous variables were obtained by expressing measurements of the marginal increment and distance between the OTC mark and the subsequent opaque zone as "fractions" of the width of a completed increment cycle within an otolith. The sum of these fractions and the counts of whole cycles completed outside the OTC mark, divided by the known time at liberty, produced estimates of the periodicity of opaque zone completion. Given this rate of completion and known dates of OTC marking and sacrifice, the marginal increment was used to estimate a date on which the outermost opaque zone was completed. The model was applied to 82 marked fish of 11 *Lutjanus* species recovered after 6–22 months at liberty, and an hypothesis of annual periodicity, about a mean of 0.96 ±0.32 cycles/yr, was retained for the pooled species along the best, ventral reading axis. Model estimates for *L. erythropterus*, *L. johnii*, *L. malabaricus*, and *L. sebae* were in the range of 0.78 ±0.22 cycles/yr to 1.03 ±0.29 cycles/yr along this axis. Median ages of these fish were 3+ for *L. erythropterus* and *L. sebae* and 5+ for *L. johnii*. A two-fold difference in somatic and otolith growth detected between field-tagged and captive fish did not affect periodicity. Extension of the model suggested false annuli were induced by changes in salinity or adverse weather. On average, annuli were completed within 1–3 months after the minima in water temperature, in the austral spring–early summer, around early September for *L. erythropterus* and *L. johnii*, late September for *L. malabaricus* and late October for *L. sebae.* Results from the model were only preliminary for the small samples of *L. argentimaculatus, L. bohar, L. carponotatus, L. monostigma, L. rivulatus,* and *L. vitta,* although the common validation approach indicated annual periodicity of opaque zone completion for these species.

A new approach to validation of periodicity and timing of opaque zone formation in the otoliths of eleven species of *Lutjanus* from the central Great Barrier Reef*

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There have been recent developments in the use of thin sections of otoliths for counting annual increments in otolith macrostructure as accurate and precise indicators of age for a variety of tropical reef species (see Fowler, 1995 for review). Lifespans of 15–30 years were estimated for tropical snappers in Mexico (Rocha-Olivares, 1998) and the central Great Barrier Reef (GBR) region (Sheaves, 1995; Newman et al., 1996). In contrast, Milton et al. (1995) applied a radiometric aging technique and estimated longevities of <10 years for the "red snappers" *Lutjanus erythropterus*, *L. malabaricus* and *L. sebae* in the Gulf of Carpentaria (lat. <14°S). These results were one third to one half of the maximum ages estimated by Newman et al. (in press) from sectioned otoliths for the same species in the central GBR (lat. 18–20°S).

These differences have produced uncertainty about the nature of potential development of northern Australian fisheries and raised important questions regarding intraspecific, latitudinal variation in otolith interpretation and demographic parameters. There is a clear need to validate age estimates from both regions.

Francis (1995) proposed that validation is the process of estimating the accuracy of an aging method and that a first stage in the validation involves confirming the temporal meaning of the zones. A three-step conceptual approach to direct age validation studies with tetracycline (OTC) marking and recapture has also been used (Fowler, 1990, 1995; Fowler and Short, 1998), but no corresponding protocols have been published for the statistical analysis

and reporting of the periodicity and timing of annulus formation outside OTC marks.

The most common analysis of periodicity has been that of informally comparing the observed number and position of annuli distal to the OTC mark with an expected number of annuli chosen from the whole number of years or seasons elapsed between marking and recapture. The most extensive studies, comprising large numbers of long-term recoveries of marked fish, reported the results as either percentage agreement (Mac-Lellan and Fargo, 1995) or as a *t*-test (McFarlane and Beamish, 1995). Both approaches could not include fish recovered at liberty for less than one year and did not allow exploration of the sources of error evident in the comparisons. Low recovery rates of fish at liberty for more than 2–3 years are a feature of such tagging programs (Campana and Jones, 1998) and sample sizes are usually small. A median of only 21 fish was reported by Francis et al. (1992) from 14 earlier OTC mark-recapture studies. Such characteristics prevent the use of linear regressions, such as those used in validating daily microincrements (e.g. Foreman, 1996). The timing of opaque zone formation is most commonly inferred by informally noting the relative position of opaque zones, OTC marks, and the otolith margins on diagrams and photomicrographs (Choat and Axe, 1996; Ferreira and Russ, 1992, 1994; Fowler and Doherty, 1992; Murphy et al., 1998).

We propose that validation can be formalized into models to develop separate estimators of both the periodicity and timing of formation of opaque zones based on ratios of increment measurements around OTC marks. Our primary goals were to determine directly if the distinct opaque zones in sectioned otoliths of 11 species of *Lutjanus* from the central GBR were formed once per year and to estimate the dates of completion of these zones. Long-term recovery of useful sample sizes of GBR lutjanids marked with OTC is difficult because of ontogenetic changes in habitat of many species, the low levels of fishing effort, and tag shedding. Therefore we held captive fish to complement recoveries from a field-tagging program.

Our objectives were to develop a model for otolith growth past OTC marks that would allow tests of the null hypothesis of annual periodicity of opaque zone formation and that would provide mean estimates and measures of variation of the date of completion of these zones for comparison with environmental variables. These objectives required that we compare interpretations of otoliths and otolith growth between captive and wild fish, that we compare different reading axes and readers, and that we compare model estimates with the earlier validation approaches.
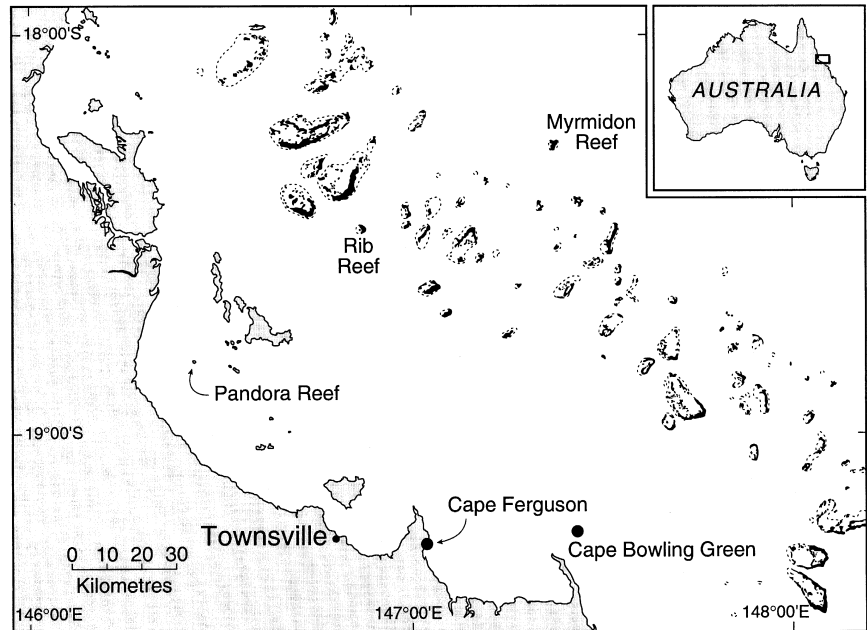

Figure 1
Central section of Great Barrier Reef Marine Park, showing sampling locations.

Materials and methods

Field techniques

Fish were collected with baited lines or traps (see Newman and Williams, 1995) in shallow water (<10 m) around the wharf at Cape Ferguson and Pandora Reef, and in deeper water (<37 m) in the GBR lagoon off Cape Bowling Green and the bases of Rib and Myrmidon Reefs (Fig. 1). There were two separate groups of fish used in the study. One group (n=170) of seven species were tagged and released in the shallow locations, where commercial and recreational fishing was prohibited, and repeated visits were made under research permits to recapture tagged fish. A second group (n=251) of 12 species were transported from the deeper locations and housed in two sea cages at Cape Ferguson wharf. The cages had a volume of 17.7 m^3 (cage dimensions: 2.95 m long × 2.45 m deep × 2.45 m wide) and were constructed from 25-mm galvanized steel tube covered in square, galvanized weldmesh (50 mm or 25 mm). Fish in each cage were fed with 2.5 kg of chopped pilchards (*Sardinops sagax*) each night.

Tetracycline was administered to both groups of fish in the form of Terramycin/MA injectable solution (oxytetracycline hydrochloride at 100 mg/mL) into the coelomic cavity at a dosage rate of 50 millilitres of Terramycin/MA per kilogram of estimated fish weight. In the field-tagging program, the fork length (FL mm) of all fish was measured and they were double-tagged below the spinous dorsal fin with numbered "T-bar" anchor tags or nylon-tipped dart tags. Tag legends advised retaining the whole fish upon recapture, but some fish recaptured after short times at liberty were injected and released again. Batches of captive fish were measured and injected once with OTC. Some *L. erythropterus* were injected

Table 1

Details of release, numbers of fish recovered (*n*), and ranges in age, length at recovery (FL mm) and time at liberty (months) of fish with visible OTC marks. Field = fish released in shallow locations where commercial and recreational fishing was prohibited; cage = fish held captive in sea cages.

Species	Release group	Place of capture	*n*	Age (yr)	FL	Liberty
L. argentimaculatus	field Sep 92–Mar 93	Cape Ferguson	3	6–7+	435–520	13.2–19.3
L. bohar	cage Mar 94	Myrmidon Reef	1	6+	386	11.6
L. carponotatus	cage Jul 93	Rib Reef	1	7+	326	19.9
L. carponotatus	cage Mar 94	Pandora Reef	1	4+	288	11.6
L. erythropterus	cage Aug 93	Cape Bowling Green	21[1]	2–5+	335–496	9.0–18.5
L. erythropterus	cage Nov 93	Cape Bowling Green	13[1]	3–4+	427–481	15.4
L. gibbus	cage Mar 94	Myrmidon Reef	1	4+	296	11.6
L. johnii	cage Feb 94	Cape Ferguson	10	4–7+	422–598	12.7
L. johnii	field Feb 93–Apr 94	Cape Ferguson	10[1]	3–7+	376–740	6.0–21.7
L. malabaricus	cage Mar 94	Pandora Reef	4	3+	402–466	11.2–11.6
L. monostigma	cage Mar 94	Pandora Reef	1	4+	306	11.6
L. rivulatus	cage Feb 94	Cape Ferguson	1	4+	312	12.7
L. rivulatus	cage Mar 94	Cape Ferguson	1	5+	401	12.0
L. sebae	field Mar 93	Pandora Reef	2	3+	280–296	12.3
L. sebae	cage Aug 93	Cape Bowling Green	3	2–4+	371–446	18.5
L. sebae	cage Mar 94	Pandora Reef	8	2–5+	331–444	11.6
L. vitta	cage Aug 93	Cape Bowling Green	2	4–6+	284–315	18.5

[1] Five of these fish were injected twice.

again about 3 months later. Most individuals were identified before release into the cages by tagging with anchor tags or by clipping of fin spines or rays. Some rarer species were not identified this way to reduce handling stress, and other individuals shed tags before recapture. The histories of these captive fish were recognizable from unique combinations of cage and species or, for some *L. erythropterus,* from twin OTC marks visible on otolith sections (Table 1).

Monthly measurements of surface seawater temperature and salinity recorded next to the cages and continuous records from the Australian Institute of Marine Science weather stations nearby on the GBR were used to derive information on environmental stressors and wind speed and direction. Caged fish were subject to extremes of wave energy, water temperature, turbidity, and salinity that are characteristic of the inshore habitat in the cyclone belt of the dry tropics. Water temperatures rose by 11°C from the austral winter to summer, and freshwater plumes from cyclonic rains caused fluctuations in salinity and turbidity. These influences extended out to Pandora Reef, but not to Rib and Myrmidon Reefs where there were clear waters and only a 6°C seasonal change in water temperature.

Laboratory techniques

For each fish after death, the FL was measured where possible and both sagittae (hereafter referred to as the otoliths) were removed, weighed, and measured. One otolith from each fish was randomly selected and embedded in soft epoxy resin. Three transverse sections were cut with a low-speed saw and diamond wafering blade in the vicinity of the primordium. The sections were 0.25–0.50 mm thick, depending on width of the otolith, and were lightly polished on wet ebony paper (1000 grade) and lapping film (9 and 3 μm). The sections were mounted on microscope slides under cover slips in a colorless epoxy casting resin and stored in darkness between short periods of examination.

The counts and measurements of otolith features made at the Australian Central Ageing Facility (CAF) were used in the analyses in our study to avoid possible bias from familiarity with the treatment of the individual fish. These data were compared with counts made by the senior author to assess precision and bias in otolith interpretation. Opaque zones were counted under magnifications from 7.5× to 25.2× with a stereo-dissecting microscope with transmitted white light, or under reflected light against a dark background (Newman et al., 1996). The position of the OTC mark was then measured by using epifluorescence illumination with a compound microscope at 20× to 40× magnification.

Video images were obtained, enhanced, and digitized with Bioscan's Optimas[TM] and Optimate[TM] software by using customized macros (Morison et al., 1998) along two planes (Fig. 2), from the primordium to the ventral apex of the otolith (ventral L1 axis), and from the primordium to the proximal edge parallel to the sulcus on the christa inferior (sulcal L3 axis). To define the position of the opaque
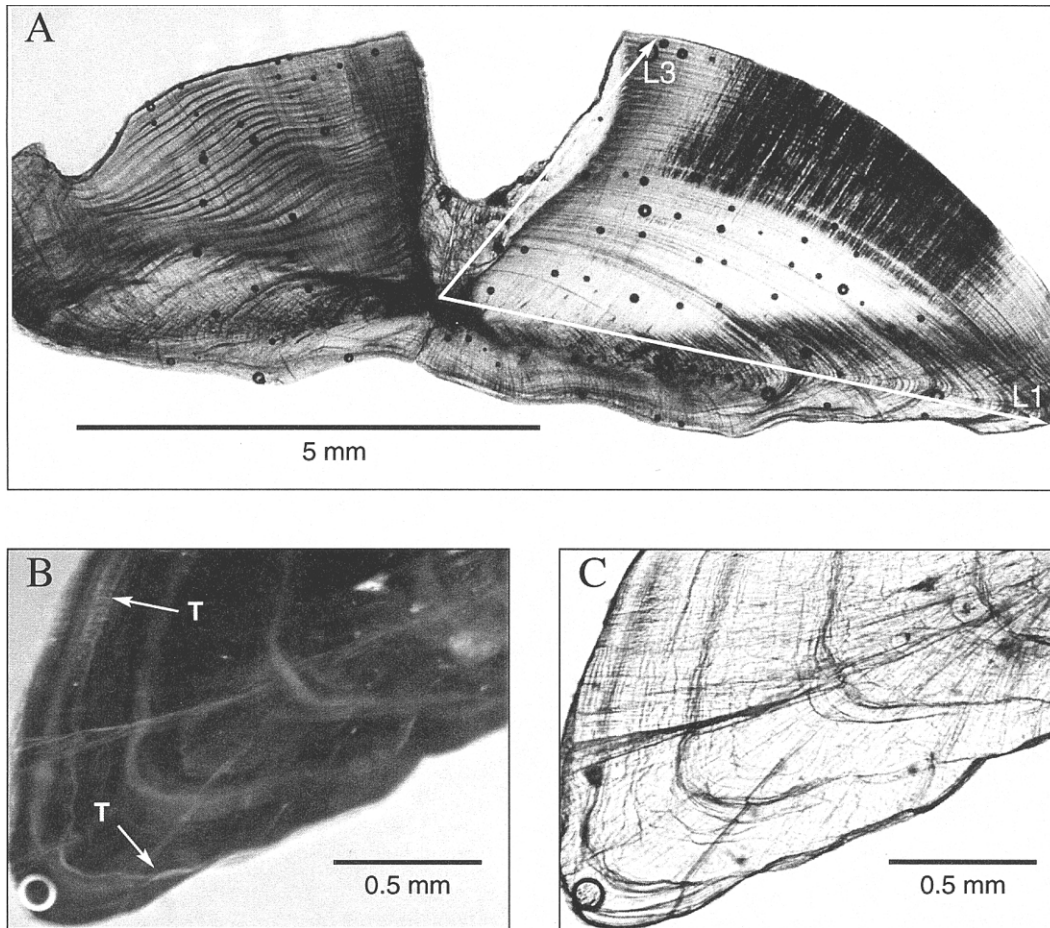
Figure 2

Photomicrographs of transverse sections of the sagittae of (**A**) *L. erythropterus* s1799 (21+yr) by transmitted white light showing the ventral L1 and sulcal L3 axes used for counting opaque zones; (**B**) the outer L1 axis of *L rivulatus* (s1996) showing the position of an OTC mark (T) by reflected ultraviolet light; (**C**) the same outer L1 axis of *L rivulatus* (s1996) showing narrow opaque zones by transmitted white light.
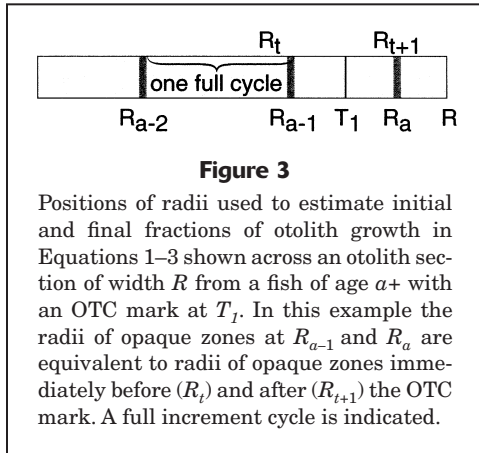
zones along these axes we recognized sharply defined "structural check rings" (sensu Gauldie, 1988) that could be followed from the ventral leading edge of the otolith around to the sulcus. These check rings were evident on the outermost edges of the opaque zones and were counted and measured after adjustment for aspect ratios. The position of the OTC mark was measured from the primordium to the outside edge of the mark and from there to the edge of the otolith along the L1 and L3 axes. The distance between twin OTC marks was measured from the distal edge of the first mark to the proximal edge of the second.

Data analyses

Analyses of the results for periodicity and timing of increment formation each comprised both an informal approach analogous to those used in earlier validation studies and a model of otolith growth. The informal validation of periodicity assumed a calendar date of 1 October for completion of opaque zones, based on previous Australian studies (Fowler, 1995), to report frequency distributions of the observed number of zones minus the expected number of zones formed outside OTC marks. The informal, "method of best fit" approach to estimate timing of completion of opaque zones was to assume that formation was completed annually on a particular day of the year. For each otolith section, and for every single day of the year, the number of opaque zones expected to be completed during time at liberty was calculated from the dates elapsed between marking and sacrifice. Only those dates for which the expected number of opaque zones formed past the OTC mark equalled the number actually observed were retained for analysis. The mean dates (MD) from frequency distributions of these possible calendar dates were selected as best informal estimates of time of completion of opaque zones.

We defined an "increment cycle" as the complete formation of adjacent translucent and opaque zones visible on an otolith section (Fig. 3). Two models were developed in a "direct method" to estimate the periodicity and timing

Figure 3

Positions of radii used to estimate initial and final fractions of otolith growth in Equations 1–3 shown across an otolith section of width R from a fish of age $a+$ with an OTC mark at T_1. In this example the radii of opaque zones at R_{a-1} and R_a are equivalent to radii of opaque zones immediately before (R_t) and after (R_{t+1}) the OTC mark. A full increment cycle is indicated.

of opaque zone completion based on the following observations. First, otoliths grow at a measurable rate that allows both counts of annuli and measurements of otolith weight to be converted to an estimate of age, with an associated error (Worthington et al., 1995). Second, this is a continuous process best represented by a continuous variable (*sensu* Zar 1996)—not a discrete variable such as the (whole) number of opaque zones. Third, the widths of outer increment cycles become similar for older fish (see figures in Fowler, 1995). We therefore proposed that ratios of otolith widths could be equated to ratios of corresponding time intervals in a model if two approximations were assumed:

1 the rate of otolith growth throughout the formation of an increment cycle was constant; and
2 the widths of increment cycles on outer margins of otoliths were the same.

We obtained continuous variables by expressing measurements of the marginal increment and distance between the OTC mark and subsequent opaque zone as "fractions" of widths of a completed increment cycle within an otolith. The sum of these fractions and the counts of whole cycles completed outside the OTC mark, divided by the known time at liberty, produced estimates of the rate, or periodicity, of opaque zone completion. Given this rate of completion, and known dates of OTC marking and sacrifice, the marginal increment could be used to estimate a date on which the outermost opaque zone was completed.

The number of increment cycles completed per year was the "cycle frequency" (V) and its inverse was the "cycle period" in units of years. The "closing date" (CD) of an increment cycle was defined as the date on which the formation of the opaque zone was completed. The time elapsed between OTC injection and the day on which the fish was sacrificed was referred to as the "time at liberty" (L) measured in decimal units of years.

The "initial fraction" (IF) and the "final fraction" (FF) of otolith growth were the two ratios of widths modelled to estimate time intervals with this direct method. Following

the measurements shown in Figure 3, these two values were calculated as

$$IF = (R_{t+1} - T_1) / (R_{t+1} - R_t), \qquad (1)$$

where R_t = the radius to the opaque zone formed immediately before OTC injection; and
R_{t+1} = the radius to the opaque zone formed immediately after OTC injection;

and as

$$FF = (R - R_a) / (R_a - R_{a-1}), \qquad (2)$$

where a = age of the fish;
R_a = the radius to the final opaque zone; and
R_{a-1} = the radius to the penultimate opaque zone.

The terms R_a and R_{t+1} were equivalent, and R_{a-1} and R_t were equivalent, where there was only a single opaque zone measured past the OTC mark.

The cycle frequency (V) was estimated for all sections with at least one opaque zone past the OTC mark as

$$V = (IF + N + FF) / L, \qquad (3)$$

where N = the number of full increment cycles visible outside the OTC mark; and
L = the time at liberty; the term N = an integer and $N = 0$ in Figure 3.

The "liberty fraction" (LF) was calculated as the sum of the full cycles and partial cycles that formed during time L, and was expressed in decimal fractions of cycles as

$$LF = IF + N + FF. \qquad (4)$$

The cycle frequency was investigated using linear regression (SAS Institute Inc., 1989a) of the model

$$LF = \beta L. \qquad (5)$$

This assumed an intercept value of zero (there was no time for otolith growth to occur when $L=0$) and the slope $\beta = 1$ when cycles had an annual periodicity. Confidence intervals for β were calculated from t distributions (Montgomery, 1991).

The second model, to estimate closing dates (CD) of the last cycle fully completed, assumed that for each otolith section the cycles that finished during time L had equal cycle periods. Closing dates were calculated as

$$CD = K - 365(FF/V), \qquad (6)$$

where K = the date on which the fish was sacrificed, coded by SAS in units of days (SAS Institute Inc., 1989b).

The calendar day of the year on which the cycle ended (calendar closing date CCD) was derived from CD by

using SAS date functions as an integer between 1 and 365. The closing dates of anomalous extra cycles for comparison with environmental stressors were estimated by using

$$CD_{t+i} = TET_1IN +$$
$$365\,(i - 1 + IF)\,/\,V, \qquad (7)$$

where TET_1IN = the date of the first OTC injection coded in SAS internal format in units of days; and

$t + i$ = the i_{th} opaque zone completed after the OTC mark.

The mean of the *CCD* estimates could be used as an estimate of the calendar date (*MCCD*) on which the opaque zones were most likely to have been completed. Days or months of the year are circular scales of measurement and best expressed in Polar coordinates as angles and radius unity. Means, angular deviations (analogous to the linear standard deviation), and confidence intervals were therefore calculated for circular von Mises distributions of *CCD* for each species by using the methods of Zar (1996). The Hotelling paired sample test was used to determine the significance of differences in angular *CCD* data between reading axes.

Figure 4

Measurements of the position of opaque zones and the OTC marks (unbroken or extended vertical bar) on the ventral L1 axis from the primordium to the otolith edge for *L. malabaricus* and *L. sebae*, identified by slide numbers. Bars representing field-tagged recaptures are shaded darker than those for captives. Dates of injection and sacrifice are shown. Otoliths marked on the same date are arranged with OTC marks aligned vertically, with a subjective horizontal scale for the spread of injection dates.

Results

Recoveries of marked fish

A total of 82 individuals from 11 species were recovered with OTC marks visible on sections of their otoliths (Table 1), equating to a recovery rate of less than 20% from the field-tagged and captive groups. Times at liberty were less than 22 months and the marked fish were all in younger year classes, less than 8+, in relation to the longevities estimated in some current studies (e.g. Williams et al.[1]). The median ages estimated for the most numerous species were only 3+ for *L. erythropterus*, *L. malabaricus,* and *L. sebae,* and 5+ for *L. johnii*. Apart from a few early recoveries of fatalities in the cages, all captive fish were sacrificed in the beginning of March 1995. Fish were recaptured in the field mainly over the summer period between September 1994 and April 1995.

[1] Williams, D. McB., S. J. Newman, M. Cappo, and P. J. Doherty. 1995. Recent advances in the ageing of coral reef fishes. South Pacific Commission, Forum Fisheries Agency Workshop on the Management of South Pacific Inshore Fisheries. South Pacific Commission Integrated Coastal Fish Management Project Technical Document 12. Forum Fisheries Agency, P.O. Box 629, Honiara, Guadalcanal, Solomon Islands.
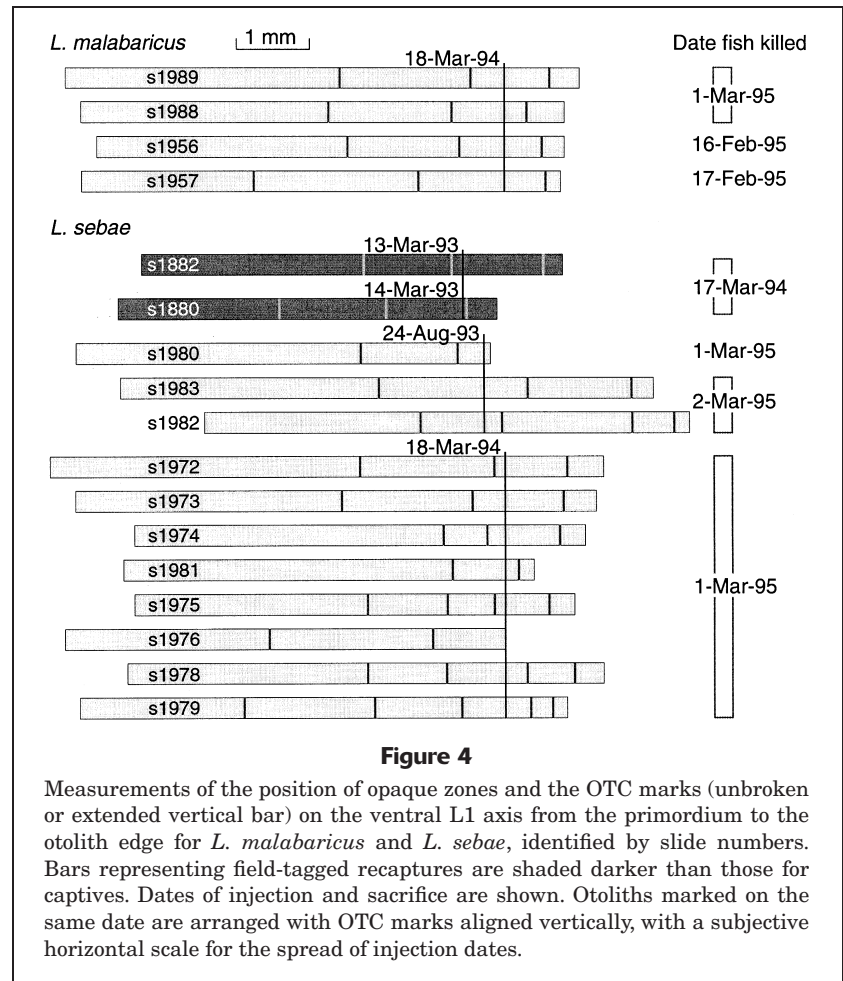
Recoveries of field-tagged *L. johnii*, *L. argentimaculatus* and *L. sebae* were low (only 8–10% of the initial number released were recovered, and tag loss was evident (60% of field recoveries had lost one tag and three others, not included in our study, had an OTC mark but no tags). Of the captive group, only 60 fish from 10 species, including six dead fish, were recovered. None of the smallest species, *L. fulviflamma* and *L. kasmira,* and only 5% of the numerous *L. vitta* remained at the end of captivity. Only eight captive fish had retained anchor tags, and individual lengths at release for captive fish without tags were generally not distinguishable. One field-tagged *L. johnii* and four captive *L. erythropterus* had two OTC marks visible on their otoliths.

Otolith growth and interpretation near OTC marks

The strength of the working assumptions used in the models developed in our study depended on the sources of variability apparent in rate of growth of the otoliths outside the OTC mark amongst species, age classes, field-tagged and captive groups, and individuals in Figures 4–7. This region generally comprised only a small proportion of the otolith width, especially in the oldest fish, *L. johnii* (Fig. 6),

L. argentimaculatus, L. gibbus, and *L. bohar* (Fig. 7). There were no opaque zones visible past the OTC mark on both axes of one *L. gibbus*, and *L. monostigma* had no OTC mark visible on the ventral axis. An opaque zone was coincident with the OTC mark on the ventral axis, but not the sulcal axis, of *L. bohar*.

The ease of interpretation of opaque zones in the otolith preparations was tested by comparing the pairs of counts made by the two otolith readers. Nonparametric, Wilcoxon signed rank tests showed significant differences between readers in estimates of fish age along both reading axes (ventral axis $n=82$ $S=285.0$ $P > |S| = 0.0001$; sulcal axis $n=82$ $S=285.5$ $P > |S| = 0.0001$) but not in interpretation of the number of opaque zones past the OTC mark (ventral axis $n=61$ $S=21.0$ $P > |S| = 0.5034$; sulcal axis $n=62$ $S=15.0$ $P > |S| = 0.6476$). An age bias plot (Campana et al., 1995) showed that the mean and standard deviation of the CAF estimates were 2.9 ±0.5 for 2+ fish, 3.5 ±0.6 for 3+ fish and 4.2 ±0.4 for 4+ fish aged by the senior author. We inferred that there was similar interpretation amongst readers of the area of interest outside the OTC mark and that the definition of the first opaque zone was a source of bias for youngest fish.

Sections from *Lutjanus* otoliths have been read along the sulcal L3 axis in a protocol developed by Newman et al. (1996), but opaque zones may be closest together in this area and measurements of growth past OTC marks might best be made elsewhere. To justify our choice of measurement axes for subsequent analyses we compared the mean growth and variance from the two axes in Table 2. Growth rates along the ventral axis were consistently twice as high as those along the sulcal axis; therefore we judged that errors in measurement of zone radii would be least, and interpretation best, along the ventral axis. The coefficients of variation were similar for both axes, with the exception of those for *L. sebae*.

Lutjanus sebae showed the highest mean growth rates and variability along both reading axes (Table 2) which led us to investigate artifacts of captivity. The growth rates of otoliths of *L. johnii* and *L. sebae* in the field were much lower than those of captives (Figs. 4 and 6). Captive *L. johnii* had mean otolith growth rates of 0.78 mm/yr along the ventral axis—twice as high as those tagged in the field (0.36 mm/yr). These differences were highly significant ($n=20$ ndf=1 ddf=18 $F=22.49$ $P>F=0.0002$), but no significant difference in otolith weight was detected when fish length was used as a covariate ($n=20$, ndf=1 ddf=18 $F=0.19$ $P>F=0.6704$). This latter finding indicated that faster otolith growth rates were correlated with the faster somatic growth of captive fish.

Further evidence of this correlation and the effects of captivity on somatic growth were an increase in mean
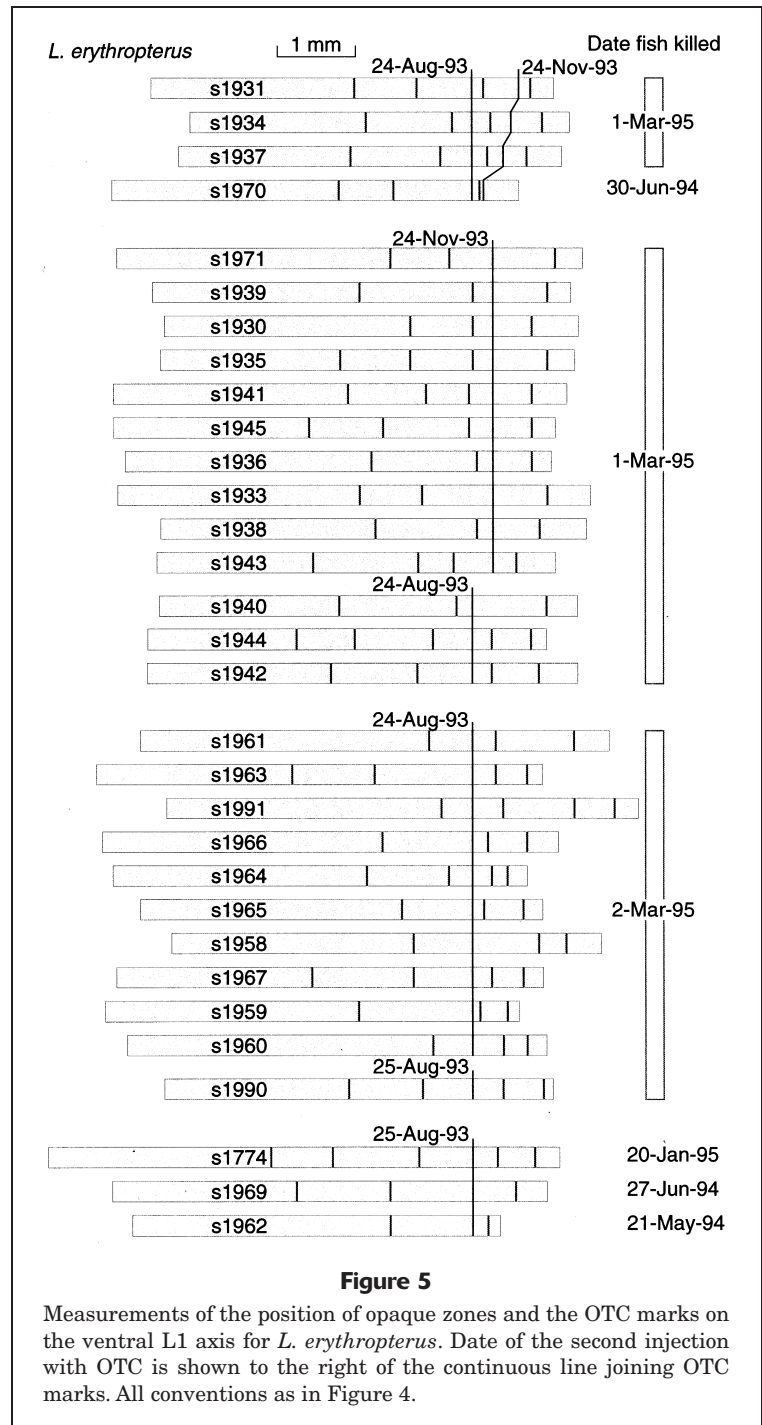

Figure 5

Measurements of the position of opaque zones and the OTC marks on the ventral L1 axis for *L. erythropterus*. Date of the second injection with OTC is shown to the right of the continuous line joining OTC marks. All conventions as in Figure 4.

FL of captives by about 7 mm/month for *L. johnii* and about 12 mm/month for *L. sebae*, compared with field growth rates of 0.2–6.2 mm/month for *L. johnii* and only 2.6–3.9 mm/month for *L. sebae*. One field-tagged *L. johnii* (s6667, Fig. 6) showed both an apparent decline in FL and no otolith growth outside the OTC mark on the ventral axis.

Plots of increment cycle width (W) along the ventral axis against cycle number (a) were made to examine

Table 2

Mean growth rates of otoliths (mm/yr) outside OTC marks and measures of dispersion from sample size (n) of the most numerous species along the ventral L1 and sulcal L3 axes. Captive = fish held in sea cages; field = fish released in shallow locations where commercial and recreational fishing was prohibited.

Group	Age range (yr)	Axis	n	Growth rate	SD	CV (%)
L. johnii—captive	4–7+	L1	10	0.78	0.21	27
		L3	10	0.31	0.08	25
L. johnii—field	3–7+	L1	10	0.36	0.18	51
		L3	10	0.21	0.09	45
L. sebae—captive	2–5+	L1	11	1.00	0.61	61
		L3	11	0.43	0.15	36
L. sebae—field	3+	L1	2	0.91	0.62	69
		L3	2	0.52	0.17	33
L. erythropterus—captive	2–5+	L1	27	0.77	0.22	28
		L3	27	0.33	0.08	24
L. malabaricus—captive	3+	L1	2	0.94	0.15	16
		L3	2	0.36	0.07	20

our second working approximation that outer cycles had equal widths on the marked otoliths. The plots were fitted best by exponential curves: $W = 0.38 + 4.92e^{-0.81a}$ for species pooled; $W = 0.70 + 11.44e^{-1.68a}$ for *L. erythropterus*; $W=0.30 + 3.21e^{-0.5a}$ for *L. johnii*; and $W = 0.75 + 9.12e^{-1.26a}$ for *L. sebae*. On average, there was a small decrease in cycle width from the third to the fourth cycle and then successive cycles were similar; however captivity or tagging may have caused anomalous growth of some otoliths. Two *L. sebae* showed no (s1976), or insignificant (s1980), growth along the ventral axis (Fig. 4) but their otoliths did continue to thicken. The largest *L. sebae* and *L. erythropterus* showed substantial growth along the ventral axis in relation to other individuals (Figs. 4 and 5), contributing to the high coefficients of variation shown in Table 2. For some captives this phenomenon led to a progressive or irregular increase in cycle width, most notable for *L. erythropterus* marked in August 1993 (eg. s1937, s1943, s1954, and s1958 in Fig. 5), that violated the second approximation of our model.
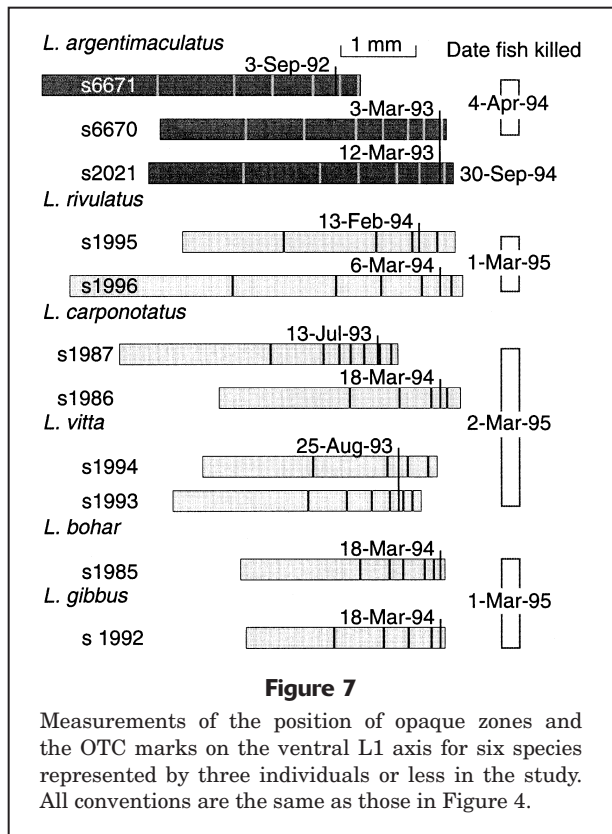
Periodicity of zone formation

Using the informal validation approach, and assuming a springtime completion date of 1 October, we estimated the frequency distributions shown in Table 3 that indicated an annual periodicity of deposition of opaque zones in the majority of otolith sections. However, there was a small minority of fish with more or less zones than expected, and information about the opaque zones counted for these individuals was available by applying the "direct method" developed in our study by rewriting Equation 3 as

$$V = (IF + FF)/L + N/L. \qquad (8)$$
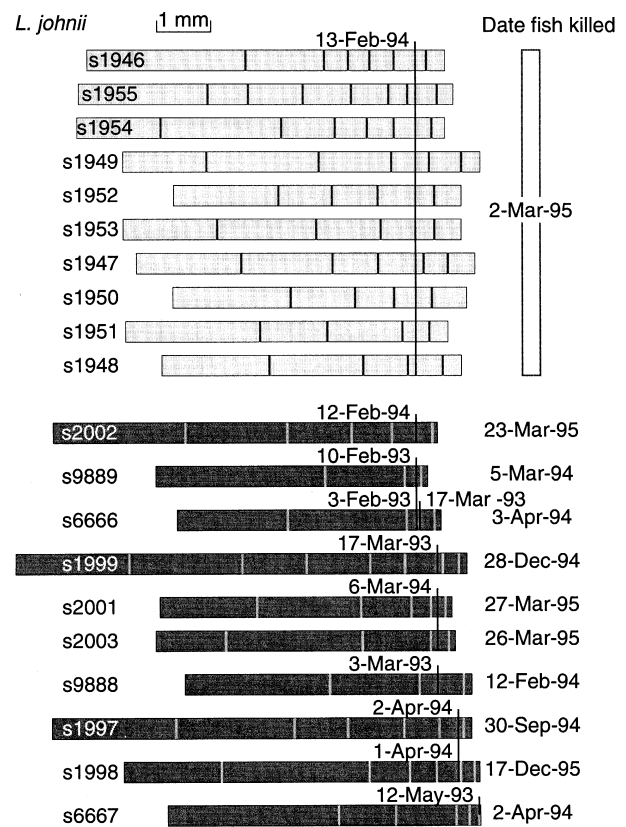
Figure 6

Measurements of the position of opaque zones and the OTC marks on the ventral L1 axis for *L. johnii*. All conventions are the same as those in Figures 4 and 5.

Figure 7

Measurements of the position of opaque zones and the OTC marks on the ventral L1 axis for six species represented by three individuals or less in the study. All conventions are the same as those in Figure 4.
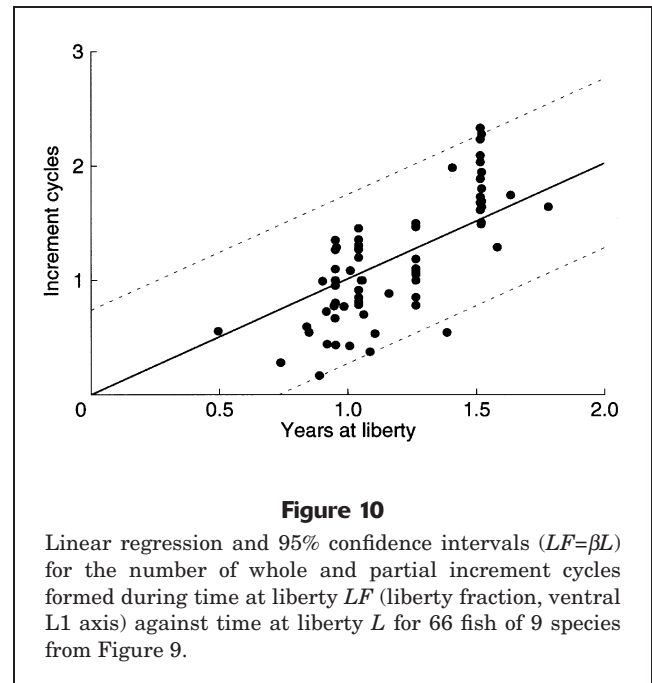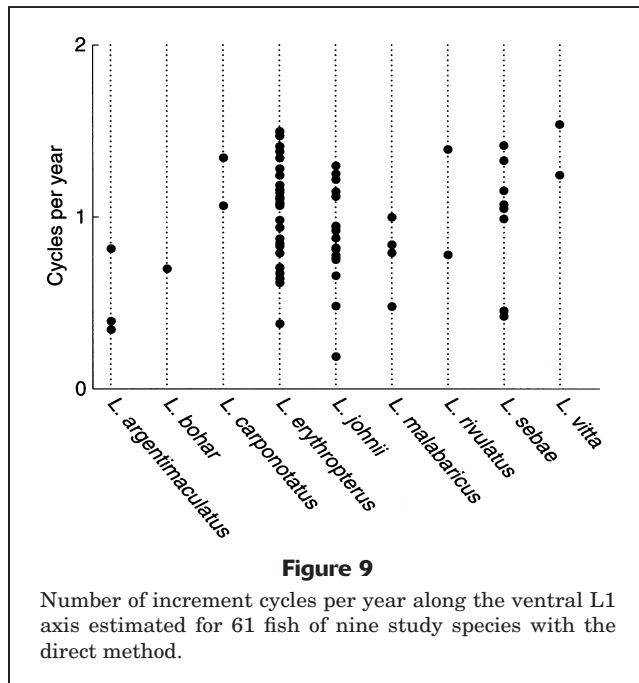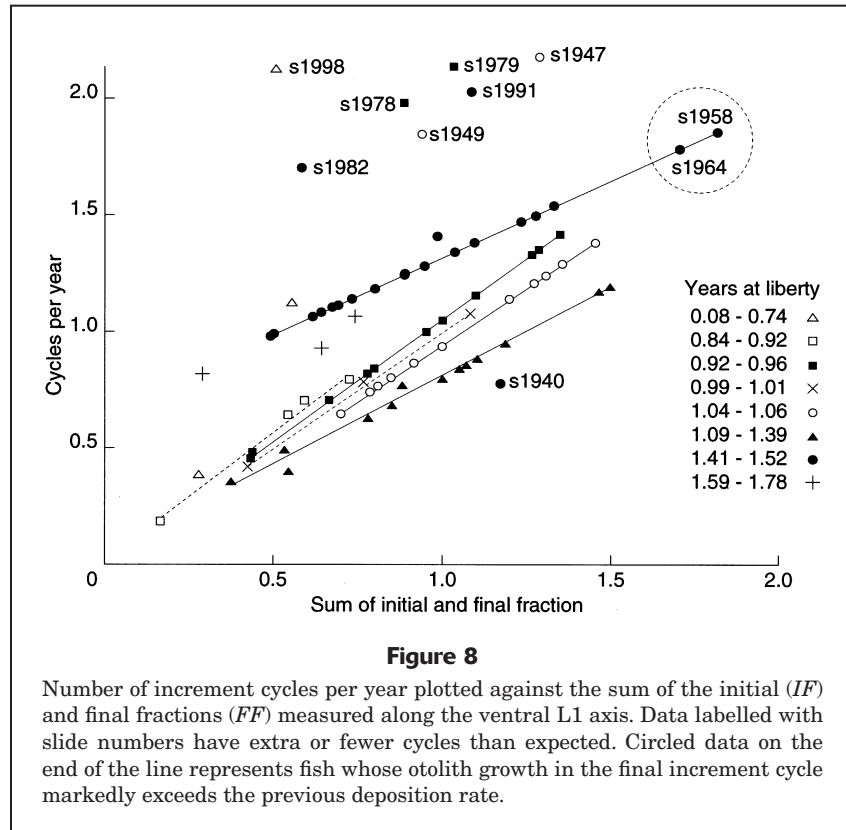
Table 3

Frequency distribution of the difference between observed (obs) and expected (exp) number of opaque zones past the first OTC mark where a nominal date of 1 October was used for completion of zone formation.

Species	n	Axis	Difference between obs and exp no. of opaque zones			
			−2	−1	0	1
L. argentimaculatus	3	L1	0	0	3	0
		L3	0	0	3	0
L. bohar	1	L1	0	1	0	0
		L3	0	0	1	0
L. carponotatus	2	L1	0	0	2	0
		L3	0	0	2	0
L. erythropterus	31	L1	0	1	29	1
		L3	0	2	27	2
L. gibbus	1	L1	0	1	0	0
		L3	0	1	0	0
L. johnii	20	L1	0	0	16	4
		L3	0	0	16	4
L. malabaricus	4	L1	0	0	4	0
		L3	0	0	4	0
L. monostigma	1	L1	—	—	—	—
		L3	0	0	1	0
L. rivulatus	2	L1	0	0	2	0
		L3	0	0	2	0
L. sebae	13	L1	1	1	8	3
		L3	1	0	8	4
L. vitta	2	L1	0	0	2	0
		L3	0	0	2	0

This procedure allowed us to determine objectively outliers in the values of N estimated for fish in groups with similar times at liberty. Fish with the same values of liberty, L, and number of complete increment cycles, N, lay on a line of slope $1/L$ in the plot of cycle frequency (V) against the sum of the initial and final fractions ($IF+FF$) in Figure 8. Fish with the same value of L and an estimate of N larger or smaller by one complete increment cycle were offset from this line by $1/L$ cycles per year.

In addition, this procedure allowed us to exclude 18 fish from the "direct method" of estimating periodicity along either reading axis, under three criteria. First, fish with anomalies in opaque zone formation, such as "false annuli" (e.g. *L. erythropterus* s1991, *L. johnii* s1947, *L. sebae* s1982), or with other errors in otolith interpretation were included only in analyses exploring the possible relationships between formation of false annuli and environmental conditions. Second, fish with $FF>1.0$ (e.g. *L. erythropterus* s1958, s1964 in Fig. 5) were excluded because they violated our second working approximation of the equal spacing of outer zones. Third, other fish were excluded because the OTC marks were absent along one axis (*L. monostigma*) or were visible outside the outermost opaque zones (*L. sebae* s1980, *L. gibbus*).

When these outliers were removed, the "direct method" produced a normal distribution of cycle frequency along the ventral axis about a mean of 0.96 ±0.32 cycles/yr (n=66 Shapiro-Wilk w=0.98 $P<w$=0.71) for all species pooled. Data from the sulcal axis produced a mean of 1.02 cycles/yr (n=64 w=0.95 $P<w$=0.04), but this was not normally distributed.

The estimated cycle frequencies were grouped loosely about one cycle/yr for *L. erythropterus*, *L johnii*, *L. rivulatus,* and *L. sebae,* with *L. malabaricus, L. argentimaculatus,* and *L. bohar* having a lower estimate and *L. carponotatus* and *L. vitta* having a higher frequency (Fig. 9). All means lay within the range 0.78–1.21 cycles/yr; apparently a lower cycle frequency was estimated when radii measured along the ventral axis (Table 4) were used. Analysis of variance showed no significant differences in cycle frequencies among these species and between reading axes, but a nonparametric Wilcoxon signed-rank test for paired data found significant differences between axes (n=61 S=–363.5 prob $>|S|$=0.008).

Simple linear regression of the number of whole and partial cycles formed after OTC marking (liberty fraction LF) on time at liberty showed significant linear relationships along both the ventral axis (n=66 ndf=1 ddf=64 F=739.37 $P>F$=0.0001) (Fig. 10) and the sulcal axis (n=64 ndf=1 ddf=62 F=913.36 $P>F$=0.0001). The slopes were β=1.01

Figure 8

Number of increment cycles per year plotted against the sum of the initial (*IF*) and final fractions (*FF*) measured along the ventral L1 axis. Data labelled with slide numbers have extra or fewer cycles than expected. Circled data on the end of the line represents fish whose otolith growth in the final increment cycle markedly exceeds the previous deposition rate.

Figure 9

Number of increment cycles per year along the ventral L1 axis estimated for 61 fish of nine study species with the direct method.

Figure 10

Linear regression and 95% confidence intervals (*LF*=β*L*) for the number of whole and partial increment cycles formed during time at liberty *LF* (liberty fraction, ventral L1 axis) against time at liberty *L* for 66 fish of 9 species from Figure 9.

along the ventral axis ($t_{0.025,\ 64}$=1.99; confidence interval 0.94<β<1.09) and β=1.09 along the sulcal axis ($t_{0.025,\ 62}$=1.99; confidence interval 1.02<β<1.16). The regression residuals were normally distributed only along the ventral

axis (*n*=66 *w*=0.981428 *P*<*w*=0.7105), and were influenced by a group of positive residuals around 1.5 years at liberty, corresponding to *V* > 1 cycles/yr. These were mainly *L. erythropterus*, for which Equation 6 consistently estimated

that the 1993 zones were completed later in the calendar year than those formed in 1994. The hypothesis that the frequency of zone completion, as the slope of the regression, was equal to one cycle/yr was therefore retained for the ventral axis for all species pooled.

Timing of zone formation

Four *L. erythropterus* had otoliths with two OTC marks that bracketed a fully formed opaque zone (Fig. 5), indicating that these annuli were formed in the austral spring in September–November 1993. A Hotelling paired sample test for equality of means in circular distributions showed that there were significant differences in estimates of calendar closing dates (*MCCD*) from the direct method between reading axes (*n*=60 ndf=2 ddf=58 *F*=3.41 *P*>*F*=0.04). There was also variability amongst species, with the most numerous species completing an annulus along the ventral axis in mid-August by *L. johnii*, in early September by *L. erythropterus,* and in early November by *L. sebae*.

By pooling data from the ventral and sulcal axes by taking the mean of each pair we were able to overcome problems associated with anomalous otolith growth, resolve differences in the estimates, and not significantly change the degrees of freedom in any subsequent tests for significance of timing of zone formation. This procedure tightened the estimates of closing date for *L. sebae* and generally gave a best estimate of spring–early summer for formation of the annulus in the most numerous species (Table 5). Results from the "method of best fit" were inside the bounds of the confidence limits of the direct method for *L. johnii* and *L. sebae* and were similar for *L. malabaricus*, *L. monostigma,* and *L. rivulatus* but were significantly later in the year for *L. erythropterus* (Table 5). The discrepancies between methods for *L. argentimaculatus*, *L. bohar*, *L. carponotatus,* and *L. vitta* reflected both the small number of fish and difficulties in measurement and interpretation of their smaller otoliths. The estimates for the less numerous species must be considered to be tentative.

There was a marked coincidence between the August 2nd minimum in water temperature and a peak in August for frequency of 1994 closing dates (Fig. 11). Extra opaque zones counted or deemed to be false annuli may have formed after storms and salinity fluctuations at Cape Ferguson. To investigate this possibility, we estimated closing dates using Equation 7 from the radii of anomalous "extra" opaque zones visible on both reading axes in otoliths of *L. johnii* (s1947, s1949, s1998, Fig. 6), *L. sebae* (s1979, s1982, Fig. 4), and *L. erythropterus* (s1991, Fig. 5) identified as outliers on Figure 8. All but one of these fish (s1998) were in the group of captives. Compared with salinity and sea state during the months of captivity, there appeared to be a relationship between a sharp fall and rise in salinity of at least 1.4 ppt for March–May 1994 and the completion of these "extra" zones in the following month, although periods of rough and very rough sea conditions also occurred most commonly at this time. Salinity dropped

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Table 4

Mean increment cycle frequency (increment cycles/yr) and standard deviation along the ventral L1 and sulcal L3 axes for the species represented by four or more marked individuals.

Species	L1 axis	L3 axis
L. erythropterus	1.03 ±0.29	1.15 ±0.25
L. johnii	0.88 ±0.29	0.85 ±0.22
L. malabaricus	0.78 ±0.22	0.88 ±0.15
L. sebae	0.99 ±0.37	1.21 ±0.33

Table 5

Mean calendar closing dates (*MCCD*), angular deviation (*s*) in days, and lower (*LCL*) and upper (*UCL*) 95% confidence limits pooled and averaged for the ventral and sulcal axes, from the "direct method," compared with the mean date (*MD*) estimated from the "method of best fit" for *n* fish of each study species.

Species	*n*	(*LCL* < *MCCD* <*UCL*)	*s*	*MD*
L. argentimaculatus	3	26 Oct	54	26 Dec
L. bohar	1	14 May	—	8 Sep
L. carponotatus	2	14 Aug	13	29 Oct
L. erythropterus	27	14 Aug< 2 Sep<20 Sep	42	31 Oct
L. gibbus	1	—	—	9 Mar
L. johnii	17	3 Aug< 5 Sep <8 Oct	53	16 Aug
L. malabaricus	4	30 Sep	26	3 Sep
L. monostigma	1	8 Sep	—	8 Sep
L. rivulatus	2	27 Aug	5	23 Aug
L. sebae	9	9 Sep < 19 Oct < 29 Nov	47	23 Oct
L. vitta	2	17-Sep	71	27 Nov

from 36.0 ppt on 3 February to 34.8 ppt on 1 April and rose again to 36.2 ppt on 1 May. The closing dates for these false annuli were distributed in March (*n*=1), April (*n*=2), and May (*n*=3).

Discussion

The use of increment widths on otolith sections to examine timing of opaque zone formation underpins marginal increment analysis (Fowler and Short, 1998) and informal inferences made from the relative position of OTC marks (Choat and Axe, 1996; Ferreira and Russ, 1992, 1994; Francis et al., 1992). The direct method developed in our study has essentially formalized and extended this use to allow for robust parametric tests and has three advantages over previous approaches to direct validation. First, the use of fractions of otolith growth allows linear regression of the number of whole or partially completed (or whole *and* partially completed) increment cycles on time at liberty, and both measures are expressed correctly as continuous variables. This method allows periodicity to be estimated directly from all recaptures with at least one opaque zone outside the OTC mark, including those fish at liberty for less than one year. Second, there is no need for subjective choice of a fixed date of completion of opaque zones to allow for comparisons between expected and observed counts of opaque zones. Third, the calendar date of completion of outer opaque zones can be estimated independently of the dates of marking or sacrifice for all recaptures with at least one opaque zone outside the OTC mark. Previous inferences about timing have been limited to the opaque zones that are immediately adjacent to OTC marks. Such contrasts are not always readily available, especially when tagging and recaptures cannot be spread throughout the

year because of seasonal differences in fishing effort or vulnerability of fish to capture.

Periodicity and timing of opaque zones

We tested, and retained, the hypothesis that there was an annual periodicity of formation of opaque zones for the pooled study species, around a mean of 0.96 ±0.32 increment cycles/yr. Anomalous sections and "false" annuli were identified objectively and could be related to periods of environmental stress in six cases. The model also identified interannual difference in timing of zone completion by a group of *L. erythropterus*, which clearly inflated overall estimates of periodicity. Despite this difference, estimates of periodicity in the range of 0.78–1.21 cycles/yr for both reading axes, accompanied by confidence intervals, were robust for *L. erythropterus*, *L. johnii*, *L. malabaricus,* and *L. sebae*. This result supported the use of thin sections of otoliths for accurate age estimates of lutjanids in the central GBR and supported estimates of longevity proposed for some of our study species (eg Sheaves, 1995; Newman et al., in press; Loubens[2]). The results for the other species showed similar trends, and no interspecific differences in periodicity were detected, but the numbers of marked fish were low for *L. argentimaculatus*, *L. bohar*, *L. carponotatus, L. gibbus*, *L. monostigma*, *L. rivulatus,* and *L. vitta*. Twofold differences in otolith growth rate between captive and tagged *L. johnii* and *L. sebae* did not affect the periodicity of annulus formation, further demonstrating the capacity of these otoliths to accurately reflect age under varying conditions of somatic growth.

Substantial variability in the timing of opaque zone formation has been noted by Beckman and Wilson (1995), at the individual, population, and species levels. Our best estimates of closing dates indicated that, on average,

> opaque zones recognized as annuli in lutjanid otoliths were completed within months of the austral spring and early summer, around early September for *L. erythropterus* and *L. johnii*, late September for *L. malabaricus,* and late October for *L. sebae*. These differences were significant and indicated a timing of formation that is somewhat earlier for *L. erythropterus* and *L. johnii* than the spring–summer peak of opaque zone formation in tropical fishes identified by Fowler (1995) and Beckman and Wilson (1995). Other studies have concluded that lutjanids and serranids on the GBR form opaque zones in winter–spring (Ferreira and Russ, 1992, 1994; Newman et al., 1996).
>
> Lack of definition of the term "formation" makes it difficult to compare our results with those of previous studies. Our use of single OTC marks and measurements of only the outside edges of opaque zones allowed inferences about the timing of completion, not commencement, of annuli. We could
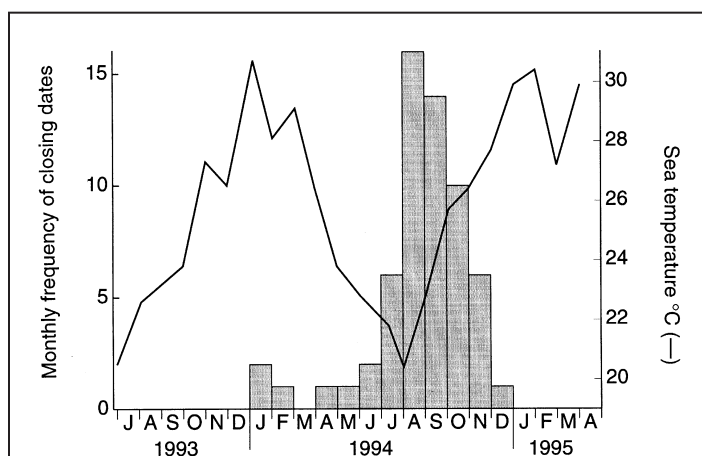

Figure 11

Monthly sea surface temperature (°C) in the cages during captivity of marked fish, plotted with the monthly frequency of closing dates (*CD*) in 1994 estimated with the "direct method" with Equation 6 for nine species from Figure 9. The estimates have been pooled and averaged for the ventral and sulcal axes for each of 61 fish.

[2] Loubens, G. 1980. Biologie de quelques de poissons du lagon Neo-Caledonian. III. Croissance. Cahiers de l'Indo-pacifique 2(2): 101–153. Foundation Singer-Polignac, 43, avenue Georges-Mandel, 75016 Paris. ISSN 0180-9954.

estimate a formation time of less than 3 months for opaque zones on otoliths from *L. erythropterus* marked twice in 1993. Those individuals clearly showed springtime formation of the opaque zones in that year, but we also concluded that completion occurred earlier in the following year, about 1–2 months after the minimum water temperature in August, indicating that commencement and formation could have occurred during winter 1994. This difference was not explained by interannual variation in the occurrence of minimum water temperatures and may have been due to changes in metabolism and physiology induced by capture, transport, handling, and captivity. These are plausible artifacts, given that opaque zone formation is under control of a poorly understood combination of environmental and endogenous factors that influence endolymph fluid chemistry (Beckman and Wilson, 1995; Romanek and Gauldie, 1996). The location of the cages may have contributed to mortality or lack of growth of the otoliths of *L. bohar*, *L. fulviflamma, L. gibbus*, *L. kasmira,* and *L. vitta* transported inshore from their reef habitats, whereas the other inshore species were expected to be more tolerant of the cage environment.

The models produced similar, but more informative, conclusions to the application of the informal approaches. Analyses following MacLellan and Fargo (1995) indicated an annual periodicity of opaque zone formation for 10 study species with few exceptions. The "method of best fit" estimated mean calendar completion dates that were inside the confidence intervals of the direct method, with the exception of *L. erythropterus,* for which the method was sensitive to the interannual variability in timing of annulus completion.

Sources of bias

Assumptions concerning otolith growth rate in the direct method were the best available approximations, given both our observations and the current understanding of otolith growth, and we were confident that their main effects were accounted for by excluding outliers and by measures of variation reported about the mean results.

Earliest models of otolith growth presumed that no net accretion occurred when fish stopped growing, and these "no-growth" horizons provided the foundation for the recognition of annual winter marks in otoliths (Romanek and Gauldie, 1996). However, otolith growth is not directly coupled to somatic growth (e.g. Mosegaard et al., 1988) and has been observed to show complexities correlated by various authors with factors such as food intake, life-history stage, temperature, and metabolic rate (Romanek and Gauldie, 1996; Schirripa and Goodyear, 1997). Most recently, Romanek and Gauldie (1996) proposed that otolith growth along the main growth axis is continuous, with the rate of deposition (that is, the microincrement width) being modulated by temperature and pH of the endolymph. This model was based on the known physiology of the endolymph and the physical chemistry of aragonite, and was tested by Payan et al. (1997) and Gauldie and Romanek (1998).

We therefore assumed a constant rate of otolith growth, while recognizing that a number of factors might be expected to vary regularly during continuous growth of the otolith, including the widths of daily micro-increments, trace element concentrations, matrix proteins, and aragonite crystal structure (Gauldie and Nelson, 1990; Gauldie et al., 1990). It remains unknown which combination of such variable characteristics integrate into the optical macrostructure of otolith sections viewed as opaque and translucent zones (Fowler, 1995).

If alternating periods of slow and fast otolith growth occurred regularly throughout each increment cycle, in violation of our working approximation, errors would tend to cancel one another during calculation of periodicity. In Equations 1–3, the initial fraction of otolith growth (*IF*) was estimated by using a numerator from the last part of a cycle, but the final fraction (*FF*) was estimated from the first part of a cycle by using the marginal increment as a numerator. If *IF* was underestimated because the otolith grew slowly at the end of a cycle, it would be countered by an overestimation of *FF* caused by faster otolith growth at the start of a cycle. Our method was most vulnerable to interannual differences in otolith growth rate, which were evident for some fish as anomalous otolith growth, possibly as a physiological response to captivity or tagging.

The widths of outer increment cycles were assumed to be the same in our second working approximation because an exponential decrease in growth along otolith reading axes and equidistant outer zones are a feature of several families from the central GBR, including lutjanids (Fowler and Doherty, 1992; Choat and Axe, 1996; Newman et al., 1996). The exponential curves presented in our study supported the approximation for the 50 marked fish that had completed their fourth increment cycle but did not support so well for the younger fish sacrificed during their fourth (*n*=28) and third cycles (*n*=4). Finally, the model assumption that the increment cycles had the same time period of formation in an individual otolith was supported for our estimation of closing dates by the determination of the annual periodicity of opaque zone formation.

There was bias in interpretation of the position of the first annulus, causing significantly higher estimates of total age by the unfamiliar reader, but not of the number of annuli past the OTC marks. The structural check rings at the outer edges of opaque zones were the best feature of the large lutjanid otoliths, and the potential for errors in interpreting the annuli on outer margins (Francis et al., 1992) was reduced by sacrificing most fish in late summer and autumn. The longer ventral axis was the most useful because of the variability in growth, measurement errors, and significant differences in estimates of closing dates from measurements along the shorter sulcal axis. However, growth in older otoliths with complex prismatic structure becomes restricted by the otic cleft (Gauldie and Nelson, 1990), and the sulcal axis may provide the only straight axis for measurement in future applications of the models.

Related studies

In contrast to our results for the "red snappers" *L. erythropterus*, *L. malabaricus,* and *L. sebae*, Milton et al. (1995)

concluded from disequilibria in ^{210}Pb to ^{226}Ra ratios of pooled otoliths that shorter lifespans obtained from whole otolith readings (*sensu* McPherson and Squire, 1992) were more accurate than the higher estimates from section counts. This independent method of age determination had previously helped differentiate between very different age interpretations of deep-water *Hoplostethus* and *Sebastes* that were thought to attain high longevities. However, Campana and Jones (1998) noted that radiochemical dating was too imprecise for detailed or individual age determinations, and West and Gauldie (1994) also concluded that the method held promise but was inadequate to validate fish ages.

Regional differences in the ease of interpretation of otolith macrostructure have been reported in tropical species separated by only four degrees of latitude (Fowler, 1995) and may partly explain the differences between our results. The lack of interpretable macrostructure in an otolith section from *L. erythropterus* from the Gulf of Carpentaria is evident when Figure 5b in Milton et al. (1995) is compared with the well-defined incremental structure for the same species in our Figure 2A. Alternatively, there may be real latitudinal differences in the demography of these species. Comparisons of the relationships between otolith weight and age estimates from the Gulf of Carpentaria and the central GBR, and further validation with the "radiocarbon bomb chronometer" (Kalish, 1995; Campana and Jones, 1998), OTC marking, or other independent techniques, may account more precisely for the differences between the validation studies.

Models with ratios of increment measurements around OTC marks in order to estimate time intervals could provide a statistical protocol to accompany the emerging definitions of various levels of validation. Francis (1995) proposed that the highest degree of validation requires demonstration that inner and outer zones are formed annually and that quantitative estimates of accuracy are provided for the process of converting a count of zones to an age estimate. This method requires knowledge of hatching dates and the age at completion of the first annulus, as well as decisions about the nominal calendar dates when opaque zones can be distinguished on otolith margins. The approach presented in our study could allow precise estimates of the timing of some of these latter events if improved theoretical models of growth of opaque zones are developed. We conclude that, for at least the three "reds" species and *L. johnii*, we have attained Francis's (1995) second level of validation—namely, that the opaque zones were formed annually and completed from spring through early summer, but there remains a need to produce quantitative estimates of the accuracy of this aging method.

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