NOTES

MARKING GROWTH INCREMENTS IN OTOLITHS OF LARVAL AND JUVENILE FISH BY IMMERSION IN TETRACYCLINE TO EXAMINE THE RATE OF INCREMENT FORMATION

Age determination of fishes by counting daily growth increments in their otoliths is becoming a widely used technique in growth and population studies. Daily formation of otolith increments was first reported by Pannella (1971) for three species of temperate fish. Since then a number of workers, using three basic techniques for confirming the periodicity of increment formation, have reported the presence of daily increments in larval or adult otoliths of at least 15 species of marine and freshwater fishes. Laboratory rearing from eggs to larvae of known age was used to confirm daily increments by brothers et al. (1976), Taubert and Coble (1977), Barkman (1978), Tanaka et al. (1981), and Laroche et al. (1982). The change in the mean number of increments over time in fish captured in the wild and held in captivity was used to validate daily increments by Struhsaker and Uchiyama (1976), Wilson and Larkin (1980), and Uchiyama and Struhsaker (1981). The third method makes use of chemical agents to mark the growing margin of calcified structures in order to examine their rate of growth (Harris 1960). Tetracycline is one of the best chemical markers because it is relatively nontoxic and produces a fluorescent mark which is easily viewed in ultraviolet light (Harris 1960; Weber and Ridgway 1962). It has been administered to fish by feeding (Choate 1964; Weber and Ridgway 1967; Trojnar 1973; Odense and Logan 1974) and by injection (Kobayashi et al. 1964 and others below). Tetracycline has been used in two studies to determine the rate of increment formation in otoliths. Wild and Foreman (1980) injected the drug into large juveniles and adult skipjack tuna, *Katsuwonus pelamis*, and yellowfin tuna, *Thunnus albacares*, in a mark-recapture program in the tropical eastern Pacific. They found that otoliths of yellowfin tuna of 40-110 cm FL showed daily average increment formation, but that skipjack tuna of 42-64 cm FL showed <1 increment/d. Campana and Neilson (1982) injected tetracycline into juvenile starry flounders, *Platichthys stellatus*, and found that daily increments were subsequently produced in both field and laboratory conditions. These authors briefly mentioned obtaining similar marking results by immersion, but did not detail their procedure.

This paper presents a technique for marking otolith increments by immersing larval and juvenile fish in a solution of tetracycline in seawater, and reports the rate of increment formation under laboratory conditions for two species from the Great Barrier Reef, Australia: *Hypoatherina tropicalis* (Altherinidae) and *Spratelloides dellicatulus* (Dussumeriidae).

Materials and Methods

The experiments were conducted between July 1980 and February 1982 at One Tree Island Field Station and Lizard Island Research Station, during a field study of the population dynamics of these species.

Achromycin (a brand of tetracycline HCl[1]) was used in all experiments. The concentration that would mark the otoliths but not kill the fish was determined by testing three concentrations (400 mg/l, 250 mg/l, and 40 mg/l) using *H. tropicalis* from 12.8 to 23.0 mm SL. The otoliths of survivors were compared with untreated specimens to assess the effectiveness of the mark.

The appropriate concentration, 250 mg/l, was then used in a series of similar experiments to determine the rate of increment formation (Table 1). The experiment number (I-IV) designates a group of fish collected at the same time. In each experiment, fish were killed at two different times, designated as A or B, to compare the number of increments in fish held for different time periods. In experiment IV, the treatment times also differed, but in all other experiments the treatment time was the same for both groups A and B.

Both species are small (adults <7 cm SL), midwater, reef-associated, schooling fishes which do not undergo a marked metamorphosis between larval and juvenile stages (pers. observ.). Both attain their full complement of fin elements and begin to form scales and adult pigmentation at a standard length of 17-19 mm. Following the convention of Ahlstrom (1968), I consider this to be the size at which larvae become juveniles. *Hypoatherina tropicalis* used in the rate-determination experiments ranged from 12.8 to 27.2 mm SL, with 10 of 21 fish classed as larvae (<17.0 mm SL). *Spratelloides delicatulus* ranged from 15.5 to 22.9 mm SL, with 2 of 29 being larvae (Table 1).

[1]Manufactured by Lederle Labs, a division of Cyanamid Australia Pty. Ltd. References to trade names do not imply endorsement by the National Marine Fisheries Service, NOAA.

The fish were collected at night with a light and a dip net, and placed in 25 l aquaria without aeration or running seawater as soon as possible after collection. The aquaria were located outdoors under an awning, and therefore were exposed to the ambient diel light cycle, but not to direct sunlight. The fish were allowed to acclimatize for 12-24 h before treatment. Usually there was mortality during this period, but the proportion was not determined. All dead fish were removed prior to treatment.

The fish were exposed to 250 mg tetracycline/l seawater for 12 h from sunset to sunrise, except in experiment IVB when the immersion period was from sunrise to sunset (Table 1). After an immersion period, the aquarium was flushed with 90% water changes until no visible color remained. The tetracycline-seawater solution is yellow until exposed to sunlight for more than ~3 h, when it turns pink, due to oxidative photolysis. Following the treatment, fish were maintained in clean seawater for 2-6 d by feeding either fresh wild plankton >125 mm diameter once a day (experiment I) or *Artemia salina* nauplii 3-4 times/d (all subsequent experiments). *Artemia* nauplii were more convenient for frequent feedings than fresh wild plankton. Ninety percent of the water in each aquarium was changed each morning by siphoning, to minimize handling the fish. Tank water temperatures were measured over the diel cycle during February 1982 (summer) at One Tree Island. The temperature ranged from 25°C at 0630 h to 30°C at 1800 h. Replacement water, added at 0700 h from the surface of the lagoon, measured 27°C.

Larvae were killed at the end of each experiment by placing them into 70% ethanol. Fish were subsequently measured to the nearest 0.1 mm SL. Their otoliths (both sagittae and lapilli) were removed and mounted whole on glass slides without coverslips, using Protexx.

The following terms are used in this report for the concentric rings seen in otoliths. A growth zone is a wide ring which appears light or hyaline under transmitted light. A discontinuous zone is the narrower ring between two growth zones, often called the opaque zone because it appears dark under transmitted light. A growth increment, or simply an increment, is a growth zone plus a discontinuous zone.

Otoliths were examined at 250-1,000× magnification with a combination of incident ultraviolet light to reveal the fluorescent tetracycline-marked rings, and polarized transmitted light to count the rings. The fluorescence microscope used ultraviolet light from a 50W mercury lamp. Excitation wavelength was limited by a band pass filter (450-490 nm) and a long pass suppression filter (515 nm).

In most cases, one sagitta from each fish was read, although occasionally the lapillus was used if its rings were clearer. The area to be counted was selected by scanning the margin of each otolith to find the place where the greatest number of distinct rings could be seen between the innermost fluorescent increment and the edge. A datum was considered valid only if identical counts were obtained in at least two out of three blind readings. No other otoliths were considered in the analysis. Of 21 *H. tropicalis* otoliths

TABLE 1.—Summary of tetracycline-marking experiments to determine the rate of increment formation in *H. tropicalis* and *S. delicatulus*.

Experiment	N	Standard length (mm) Mean (range)	Treatment period	Date and time of killing	Predicted no. of discontinuous zones	No. of fish with various deviations from the predicted number		
						−1	0	+1−
Hypoatherina tropicalis								
IA	2[1]	14.0 (13.6-14.4)	2130, 8 July to 0830, 9 July 1980	0830, 12 July	2+1		1	1
IB	4	13.7 (12.8-14.7)	2130, 8 July to 0830, 9 July 1980	1730, 14 July	5		2	2
IIA	6[2]1)	20.5 (16.2-27.2)	1830, 31 Oct. to 0630, 1 Nov. 1980	0730, 6 Nov.	4+1		5	
IIB	6	18.9 (16.8-20.7)	1830, 31 Oct. to 0630, 1 Nov. 1980	0600, 7 Nov.	5+1		6	
IIIA	3	16.1 (15.4-17.2)	2000, 6 Nov. to 0700, 7 Nov. 1981	0545, 12 Nov.	4+1		3	
Total	21[2]1)					0	17	3
Spratelloides delicatulus								
IIIA	6	17.5 (15.5-19.1)	2000, 6 Nov. to 0700, 7 Nov. 1981	0545, 12 Nov.	4+1		3	3
IIIB	5[2]2)	17.9 (17.6-18.2)	2000, 6 Nov. to 0700, 7 Nov. 1981	1800, 9 Nov.	2			3
IVA	9	19.9 (18.8-22.8)	1800, 31 Jan. to 0630, 1 Feb. 1982	1800, 6 Feb.	5	5	3	1
IVB	9[2]1)	20.5 (17.9-22.9)	0600 to 1800 31 Jun. 1982	0715, 6 Feb.	4+1	2	6	
Total	29[2]3)					7	12	7

[1]Otoliths of two treated fish were destroyed by poor preservation.
[2]Number of fish discarded because of inconsistency between otolith readings.

examined, 1 (4.8%) was discarded. Of 29 *S. delicatulus*, 3 (10.3%) were discarded (Table 1).

Results and Discussion

Marking Technique

In the experiment to determine an effective tetracycline-marking concentration, all fish ($n = 17$) in 400 mg/l died during the 12-h immersion period. Of 10 fish treated with 250 mg/l, 1 died during treatment, and 1 died during the subsequent holding period. Of 10 fish treated with 50 mg/l, 1 died during treatment.

Otoliths of untreated specimens showed faint fluorescence around the edge and occasionally along cracks and surface irregularities (Fig. 1A); this is a naturally occurring autofluorescence (Campana and Neilson 1982). Otoliths of fish in 50 mg/l were indistinguishable from those of untreated specimens. Otoliths of fish in 250 mg/l showed a strong fluorescent band medial to the edge, in addition to the weak fluorescence at the edge (Fig. 1B, C). This strong band consisted of two growth zones and one discontinuous zone (Fig. 2).

It is not known how long it takes for tetracycline to be incorporated into the growing otoliths when administered by immersion. Campana and Neilson (1982) reported that after injection, 50% of fish showed fluorescent otoliths after 10 h, and 100% after 24 h. If one assumes similar or slightly longer incorporation times in the present study, then the inner fluorescent growth zone was probably formed the day after the immersion period. The subsequent discontinuous zone and growth zone were formed while there was residual tetracycline in the water or fish. Another possible explanation is that the appearance of fluorescence in two growth zones is an artifact of viewing whole otoliths.

The results of this experiment indicate that immersion in a concentration of 250 mg Achromycin/l of seawater for 12 h is adequate to mark one or more growth increments in *H. tropicalis* and *S. delicatulus* larvae and juveniles. The overall mortality rate in experiments I, II, and III (total $n = 37$), was 5.4% during treatment and 2.7% during the holding phase.

To determine whether fluorescent marking would occur if the tetracycline immersion period was during daylight hours, an experiment was conducted using *S. delicatulus* from 17.9 to 22.9 mm SL (experiment IV). The fish were collected and divided between two tanks. One tank received tetracycline from 1800 h to 0630 h, the other from 0600 to 1800 h. Mortality due to treatment was not monitored. After 6 d, the fish were killed and examined. The fluorescent bands medial to the edges were similar in width and intensity to those in previous experiments, and showed no difference between the two treatments. This indicates that tetracycline is incorporated into growing otoliths and produces fluorescent increments equally well during the day and night, regardless of whether the solution is yellow or has oxidized to pink.

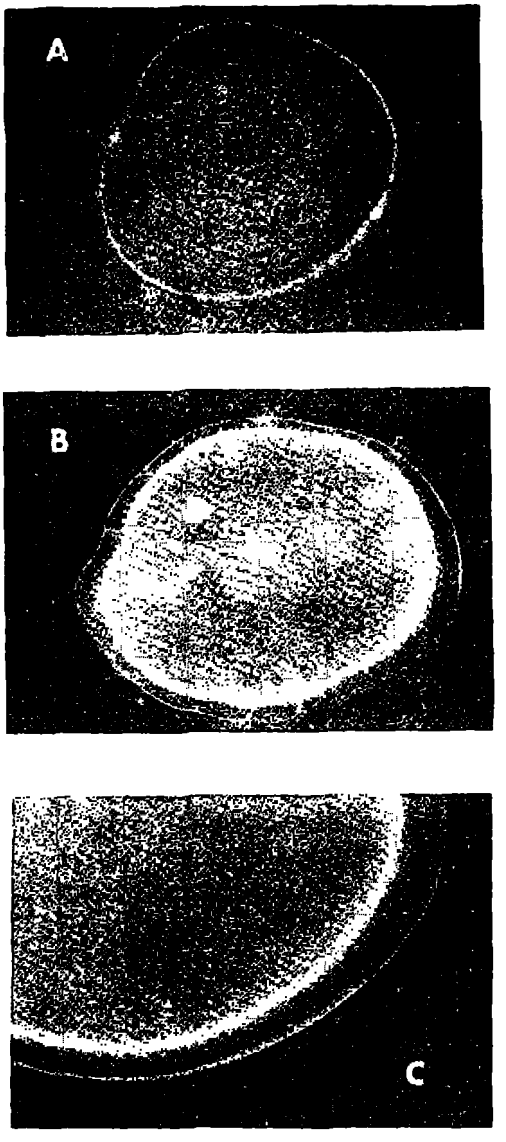

FIGURE 1.—Flourescence photomicrographs of sagittae of larval *Hypoatherina tropicalis*. A. Untreated otoliths, showing autofluorescence around the edge (10.1 mm SL). B. Tetracycline-marked otolith, showing fluorescent band medial to the edge (16.2 mm SL). C. Marked otolith under higher magnificaton (17.6 mm SL).

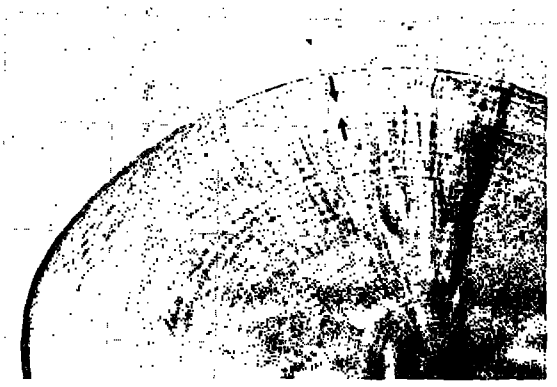

FIGURE 2.—Tetracycline-marked otolith from *H. tropicalis* (17.6 mm SL), photographed with a combination of fluorescent and transmitted polarized light. Arrows indicate the fluorescent band produced by the marking technique. This individual is from experiment IIB, and shows six discontinuous zones between the innermost fluorescent growth zone and the edge. The edge appears to be a growth zone.

In summary, tetracycline can be administered by three techniques: feeding, injection, and immersion. Feeding has apparently not been used in otolith studies. The immersion technique presented here has advantages over injection in some situations. It can be used on fish which are too small or fragile for injection. The fluorescent mark obtained is relatively narrow, covering only two increments, compared with the wider mark resulting from injection (Kobayashi et al. 1964; Campana and Neilson 1982). Therefore, it is distinguishable from edge autofluorescence after a shorter period of time, and allows finer resolution of increment formation, which may be useful in some experimental situations. Also, immersion requires minimum equipment, facilities, and handling of fish.

Rate of Increment Formation

In interpreting the results of my experiments, the number of discontinuous zones between the innermost fluorescent growth zone and the edge was compared with the number predicted if one discontinuous zone formed every day from ca. 0700 to 1000 h. Tanaka et al. (1981) found that growth zones in juvenile *Tilapia nilotica* held under various photoperiods started forming a few hours after lights-on, continued through the dark period, and stopped or slowed down about the time of the following lights-on. The discontinuous zone was formed in the few hours after lights-on. Mugiya et al. (1981) demonstrated that the deposition of calcium in goldfish, *Carassius auratus*, slowed down or stopped at sunrise and resumed in 3 h. Since otoliths are made of a matrix of organic fibers, which are calcified in the growth zones and not calcified in the discontinuous zones (Panella 1980; Watabe et al. 1982), the findings of Mugiya et al. (1981) support Tanaka et al. (1981). Whether this rhythm of increment formation is found in most fish remains to be investigated.

The results for all experiments are presented in Table 1. For fish that were killed between 0545 and 0830 h, the predicted number includes an additional discontinuous zone that should have been forming at the time of death, although this ring was probably not always sufficiently formed to be counted. In these cases, an otolith was considered to show daily increment formation even if the number of discontinuous zones was one less than predicted.

One growth increment was formed each day in 85% of *H. tropicalis* ($n = 20$); the rest had one more than the predicted number of increments. In *S. delicatulus*, 46% ($n = 26$) showed daily formation of growth increments; 27% showed one less, and 27% showed one more, than expected if increments form daily. Thus, the variability in rate of increment formation was greater in *S. delicatulus* than in *H. tropicalis*, but the average rate for *S. delicatulus* was still 1 increment/d.

This apparent difference in the rate of increment formation between species may be partially due to a difference between larvae and juveniles. Almost all (93%) of the *S. delicatulus* treated were juveniles, but only about half (52%) of the *H. tropicalis* were juveniles. However, no conclusion can be drawn from these data because the experiments were not designed to examine this factor, and the numbers are too small to compare larvae with juveniles.

It is possible that tetracycline may affect the rate of increment formation. Some workers have reported that tetracycline inhibits mineralization in scales and bone (Harris 1960; Kobayashi et al. 1964), although others note neither growth promotion, retardation, nor structural weakness in bone as a result of tetracycline administration (Weber and Ridgway 1967). The possibility that the tetracycline treatment interferes with growth of otoliths or fish was not considered in this study, but should be examined before further use is made of this technique.

In conclusion, the rate of increment formation has been examined in only a small number of species under a limited range of conditions. Recent evidence suggests that increment formation may be affected in some species by temperature, food availability and feeding frequency, photoperiod, and developmental stage (Taubert and Coble 1976; Brothers 1978; Pannella 1980; Wild and Foreman 1980; Geffen 1982;

Lough et al. 1982; Neilson and Geen 1982). It is therefore desirable to examine the rate of increment formation under various conditions before using otoliths for age determination (Brothers 1979). The technique presented here is a tool for studying increment formation in otoliths of young fish under laboratory and possibly field conditions. It can be used for reef and nearshore benthic species which can be captured while larvae or juveniles and kept in containers or enclosures.

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TAG-RECAPTURE VALIDATION OF MOLT AND EGG EXTRUSION PREDICTIONS BASED UPON PLEOPOD EXAMINATION IN THE AMERICAN LOBSTER, *HOMARUS AMERICANUS*

Techniques for molt prediction based upon epidermal and setal development in pleopods (Aiken 1973) and for egg extrusion prediction based upon pleopod cement gland development (Waddy and Aiken 1980; Aiken and Waddy 1982) provide opportunities for more comprehensive studies of growth and reproductive potential in natural American lobster, *Homarus americanus*, populations than have previously been possible. These laboratory-developed techniques have only recently been applied to field samples from a number of areas of Atlantic Canada (Robinson 1979; Campbell and Robinson 1983; Ennis 1984). Although the methodologies are fairly straightforward and may be applied in field studies quite readily, in practice the investigator will sometimes be faced with specimens for which predictions can only be made with some degree of uncertainty. A study of Newfoundland lobsters using these techniques has included the tagging of animals from which pleopods were obtained. This paper presents results from observations on recaptured lobsters which validate the predictions that were made at the time of tagging that molting or egg extrusion would or would not occur during the current molting/spawning period.

Materials and Methods

Pleopods were obtained from American lobsters (ranging from 33 mm to 130 mm CL (carapace length)) caught in traps and by scuba divers near Arnold's Cove, Placentia Bay, Newfoundland, between 24 June and 17 July 1981. These were examined for molt and cement gland stages according to the methodologies of Aiken (1973), Waddy and Aiken (1980), and Aiken and Waddy (1982).

It is clear from Aiken (1973) that one can predict with considerable confidence that lobsters with pleopod stages 3.0 and higher just prior to or early in the molting season will molt that year. It is also clear, however, that for animals with pleopod stages 1.0-2.5 one cannot predict with confidence that molting will or will not occur. Molt prediction for these stages is not reliable because of development plateaus that occur during D_0 (i.e., molt stages 1.0-2.5). However, most such plateaus occur at stages 1.5-2.0, and a lobster will rarely remain at stage 2.5 for more than 2 wk. Once an animal has passed beyond stage 2.5, there will be no further plateaus, and proecdysis will proceed at a rate that is regulated by temperature (Aiken 1973). Aiken (1980) also stated that at stage 2.5, the epidermis in the general integument begins to show signs of activity, indicating imminent transition from indecisive D_0 into the irreversible premolt development of D_1. Considering that animals with stage 2.5 pleopods should molt in 48-52 d at 10°C (Aiken 1973) plus the fact that at Arnold's Cove the July-August temperatures on the lobster grounds average in excess of 10°C (mean daily temperatures from 24 June to 31 August averaged 12.1°C in 1981), it appeared more likely that lobsters with stage 2.5 pleopods during the 24 June-17 July sampling at Arnold's Cove would molt. As a working hypothesis, it was decided to predict that lobsters with pleopod stages 2.5 and higher would molt during the 1981 molting season at Arnold's Cove and that those with pleopod stages 0-2.0 would not molt.

Cement glands were initially staged according to the classification scheme of Waddy and Aiken (1980). These stages were subsequently converted to their more recent scheme (Aiken and Waddy 1982). It is clear from these papers that for lobsters with stage 0 or stage 1 cement glands just prior to or early in the spawning season one can confidently predict that egg extrusion will not occur that year, whereas for those with stage 2 or higher cement glands one can confidently predict that egg extrusion will occur.

During the sampling at Arnold's Cove, 356 of the lobsters from which pleopods were removed for molt and cement gland staging were tagged with "sphyrion" tags, which are designed to remain attached through ecdysis (Scarratt and Elson 1965), and released within a few minutes of being taken from the water very close to where they were captured. Observations on 171 of these lobsters recaptured subsequent to the molting/spawning period (mainly during the 1982 fishing season, 20 April-30 June)