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Validation of Daily Growth Increments in Otoliths of Milkfish Larvae by Oxytetracycline Labeling

W.-N. TZENG AND S.-Y. YU

Department of Zoology, National Taiwan University
Taipei, Taiwan 10764, Republic of China

Abstract.—The formation of growth increments in the otoliths of wild-captured larvae of milkfish *Chanos chanos* was validated by the oxytetracycline-labeling method. Immersion of the fish in 400–500 mg oxytetracycline/L for 24 h is recommended for marking the otoliths of milkfish larvae. A golden-yellow band and the newly deposited growth increments in the otolith were both discernible after marking. Otolith growth increments were deposited at the rate of about one per day, and their formation rate was unaffected by the growth rate of the fish. However, increment width varied with the growth rate of fish. Therefore, otolith growth increments are a valid characteristic for determining daily age and for studying the growth history of wild milkfish larvae.

Milkfish *Chanos chanos,* one of the most important fish species cultured in brackish water in the southeast Asian region, have been cultured for over 300 years in Taiwan. The annual demand for milkfish young for culture is about 200 million individuals, and the supply is still largely dependent on naturally recruited stock. The continued development of the milkfish-culture industry has been constrained by the fluctuation of recruitment, and the number of captured young is insufficient to meet the demands. Increased knowledge of the early life history of milkfish is considered to be important for improving the catch efficiency of wild young and for developing artificial propagation and larval rearing techniques (Villaluz et al. 1982).

Since the daily formation of discernible growth increments in fish otoliths was first reported (Pannella 1971), aging of larval and juvenile fish by counting daily growth increments has been widespread (Brothers et al. 1976; Taubert and Coble 1977; Wilson and Larkin 1980; Neilson and Geen 1982; Campana and Neilson 1985). However, daily increments are not universally observed in teleosts (Beamish and McFarlane 1983). Environmental and physiological variables, such as photoperiod, temperature, feeding, and growth, may fluctuate cyclically and play an important role in the process of otolith deposition (Campana and Neilson 1985). Therefore, validation of the relationship between otolith increment and age and an understanding of the factors that affect increment formation are essential to the study of wild fish populations.

Three basic techniques have been used to validate otolith growth increments in teleosts: examination of otoliths from laboratory-reared larvae of known age, examination of the changes in the mean number of increments over the defined period in captivity, and examination of the newly deposited increments after the growing margin of calcified structures has been marked with chemicals.

We examined the daily growth increments of otoliths of hatchery-reared milkfish larvae (Tzeng and Yu 1988). The direct 1:1 correlation of increment counts to days of age was confirmed by use of the oxytetracycline (OTC)-labeling technique to mark the growth margins of otoliths of wild-captured milkfish larvae. Tetracycline has been used widely as a temporal marker for age validation and growth studies (Weber and Ridgway 1967; Wild and Foreman 1980; Hettler 1984; Tsukamoto 1985). It is incorporated into calcifying tissue during growth and accurately marks the date of application by depositing a fluorescent band that is easily discernible under ultraviolet light. This technique has been applied successfully to several species (Weber and Ridgway 1967; Wild and Foreman 1980; Campana and Neilson 1982; Simoneaux and Warlen 1987). Therefore, we used the OTC-labeling method to mark the otoliths of wild-captured milkfish larvae to validate daily growth increments of otoliths and to examine the effects of fish growth on otolith increment formation.

Methods

Milkfish larvae used in this study were captured with a skimming net in the coastal waters off Ilan Prefecture, northern Taiwan, on August 8 and 25, 1987. The skimming net filtered milkfish larvae from the water while it was pushed forward by the operator (Villaluz et al. 1982). The size of cap-

tured milkfish larvae ranged from 11.2 to 14.5 mm and averaged 13.5 mm total length (TL). Larvae were acclimated for a few days under a natural photoperiod at a room temperature of about 28°C; they were kept in 16.5‰ natural seawater and fed artificial eel-larva feed until the marking was attempted.

Oxytetracycline hydrochloride dissolved in NaCl solution was used to mark the otoliths of milkfish larvae by immersion. Sodium chloride solution was used instead of seawater because OTC chelates with calcium and magnesium in natural seawater before it binds with calcium in bone-forming tissue (Hettler 1984). Circumvention of this chelation problem was the greatest difficulty we experienced with the OTC-marking procedure.

To be effective as an accurate marker, OTC must be incorporated into the otolith tissue soon after application. To determine the efficiency of OTC incorporation, milkfish larvae were subjected to 35 combinations of immersion time (1.5, 3.0, 6.0, 12.0, and 24.0 h) and OTC concentration (50, 100, 200, 300, 400, 500, and 600 mg/L); there were 10 larvae per combination. At the end of each treatment, the larvae were immediately transferred to natural seawater (16.5‰) and fed with artificial eel-larva feed four times a day. During the 3 weeks when marking treatments were applied, the milkfish grew from 13.5 to 30.6 mm mean TL. After the experiment, otoliths were removed, and the fish were preserved immediately in absolute ethyl alcohol. The sagittal otoliths were used to determine the best treatment method (i.e., optimum OTC concentration and treatment duration).

Two groups of marked milkfish with different growth rates were established by rearing the fish at stocking densities of 15 fish/L and 1.5 fish/L and water temperatures of 25–26°C and 28–30°C, respectively. The fish were maintained under a natural photoperiod. The quantity of artificial diet provided to the fish also was adjusted to enhance the difference of growth rate. Then, fish from each treatment group were killed and preserved after 7, 14, and 21 d for slow-growing fish and after 10, 20, and 30 d for fast-growing fish. The total lengths of the preserved fish were measured to the nearest 0.1 mm after 1 week in absolute ethyl alcohol. Both sagittal otoliths from each fish were removed and mounted with permount on a microscope slide with the proximal surface down.

A fluorescence microscope (Nikon Optiphot) with incident ultraviolet light from a 50-W mercury lamp was used to detect the fluorescent OTC-marked ring on the otoliths; transmitted visible light was used to count daily growth increments. Excitation wavelength of the incident ultraviolet light was limited by a band-pass filter (400–440 nm) and a long-pass barrier filter (470 nm). A single growth increment comprised of an incremental zone and a discontinuous zone was presumed to represent 1 d of age (Tzeng and Yu 1988). The number of newly deposited otolith growth increments were counted, and the increment widths from the fluorescent band to the periphery along the long axis of the sagittal otolith were measured from the fluorophotographs at a magnification of 250–500×. In addition, the otolith radius along the long axis of the sagitta was measured with a video-microscope–IBM PC image analysis system (Visionetics, VFG-512) at a magnification of 400–1,000×.

A linear regression of otolith radius on fish length after log transformation of data and linear regressions of increment width on increment count and increment count on age were calculated. These relationships were compared between slow- and fast-growing fish by analysis of covariance (Snedecor and Cochran 1967).

Results

Optimum Treatment

The effectiveness of OTC marking of the hard tissue of fish was dependent on OTC concentration, treatment time, and fish size. The sizes of milkfish used in this experiment were similar, averaging 13.5 mm TL, which was the size of newly recruited milkfish in coastal waters. No OTC marks were found on the otoliths when milkfish were immersed in the OTC solution for 12 h or less at any of the seven concentrations examined. However, when treatment time was increased to 24 h, the marking rate was increased to 20% at OTC concentrations of 200 mg/L, 90% at 300 mg/L, and 100% at concentrations of 400 mg/L and higher. Therefore, immersion in a solution of 400–500 mg OTC/L for 24 h is recommended for marking the otoliths of milkfish larvae.

Discrimination of Marks

Slow-growing and fast-growing milkfish larvae were immersed in a 500-mg OTC/L solution for 24 h and then reared in natural seawater for 14 and 10 d, respectively. Afterward, otoliths from each treatment group were examined under fluorescent light illumination, and a golden-yellow band was detectable in otoliths from both treatments (Figure 1A, B). These bands were indicative

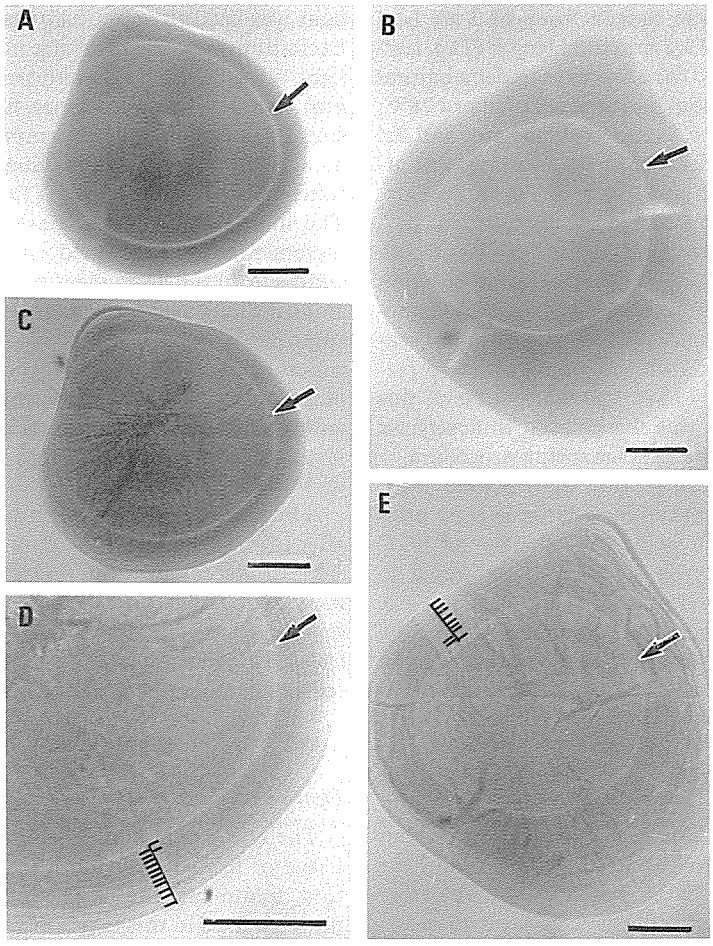

FIGURE 1.—Fluorophotographs showing otoliths of two milkfish larvae reared for 14 d (14.1 mm total length, TL; **A**, **C**, and **D**) and 10 d (19.1 mm TL; **B**, **E**) after a 24-h immersion in oxytetracycline (OTC). **A** and **B** were photographed with ultraviolet (UV) light to reveal OTC marks. **C**, **D**, and **E** were photographed with UV and transmitted light to reveal newly deposited growth increments (**D** shows an enlarged portion of **C**). Arrows indicate OTC marks; bars = 50 μm.

of OTC in the calcium-rich growth increment zone of the otolith; OTC was incorporated as the otolith growth increments were cyclically deposited during immersion in OTC solution. About one or two growth increments were deposited with OTC marks. These were discernible under both reflected fluorescent and transmitted light (Figure 1C–E).

Otolith increments beyond the OTC mark of fast-growing milkfish reared for 10 d were wider than those of slow-growing fish reared for 14 d (Figure 1A, B). The number of growth increments beyond the OTC mark approximately corresponded to the number of reared days for fast- and slow-growing milkfish (Figure 1D, E). This suggested that the OTC-marking technique was a useful tool for validation of otolith growth increments and growth rate of milkfish larvae.

Body and Otolith Growth

Milkfish larvae reared at two different stocking densities and water temperatures and fed two different food rations showed obvious differences in body and otolith growth (Figures 2, 3) after OTC marking of otoliths. At the time of marking, sizes of milkfish larvae were similar for fast- and slow-growing groups. Average total lengths were 13.8 and 13.0 mm, respectively, and the size distributions overlapped. However, the size distributions of the two groups gradually separated during the postmarking rearing period, and growth rates were estimated at 0.37–0.44 mm TL/d and 0.07–

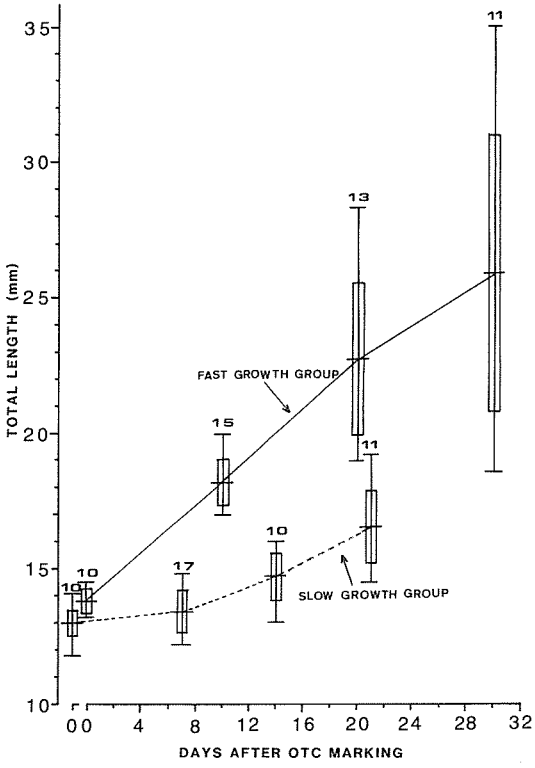

FIGURE 2.—Comparison of total lengths of milkfish larvae reared under two growth regimes after otolith marking with oxytetracycline. Numerals indicate sample sizes. Boxes indicate means ± SEs; vertical lines indicate ranges.

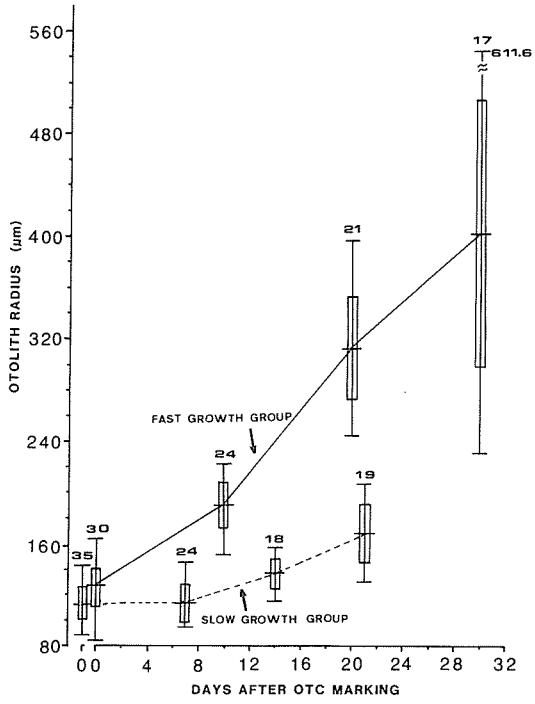

FIGURE 3.—Comparison of otolith radii of milkfish larvae reared under two growth regimes after otolith marking with oxytetracycline. Numerals indicate sample sizes. Boxes indicate means ± SEs; vertical lines indicate ranges.

0.26 mm TL/d for fast- and slow-growing fish, respectively (Figure 2). Similarly, otolith growth rates were obviously different for fast- and slow-growing fish. The initial size of otoliths before marking averaged 127 and 114 μm for fast- and slow-growing fish, respectively, and their size distributions overlapped. The ranges of otolith radius of fish in the two groups became increasingly divergent during the rearing period (Figure 3).

The relationship between otolith radius (Y) and total length (X) of milkfish larvae was expressed by the allometric formula $Y = aX^b$ or $\log_e Y = \log_e a + b\log_e X$. This was calculated for each treatment group (Figure 4). A significant difference in the otolith radius–fish length relationship between fast- and slow-growing fish was indicated by analysis of covariance (F_o, NS; F_b and F_a, $P < 0.01$; F_o, F_b, and F_a indicate the F-test difference between residual mean squares, slopes, and adjusted means of regression lines, respectively). The slope of the regression line for the fast-growing fish was smaller than that of the slow-growing fish, which indicated that the growth rate of otoliths decreased as the fish grew.

Relationship between Increment Count and Age

The relationships between increment widths from the proximal edge of the OTC band to the periphery of the otolith after OTC marking and the number of newly deposited growth increments were plotted and fitted with linear regressions for both fast- and slow-growing fish (Figure 5). A significant difference between increment width–increment count relationships for fast- and slow-growing fish was indicated by analysis of covariance (F_o, NS; F_a, F_b, $P < 0.01$). Both the slope and the adjusted mean for fast-growing fish were significantly larger than those of slow-growing fish. This indicated that the difference between the growth of otoliths of fast- and slow-growing fish was reflected in the change in width of otolith increments.

When the number of growth increments counted from the proximal edge of the OTC band to the periphery of the otolith were regressed on the

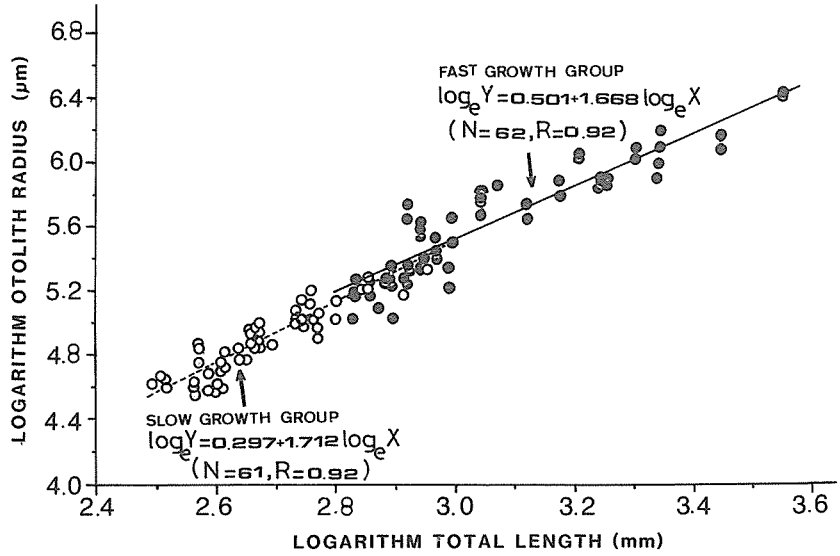

FIGURE 4.—Logarithmic plots of otolith radius (Y) versus total length (X) for milkfish larvae reared under two growth regimes after otolith marking with oxytetracycline.

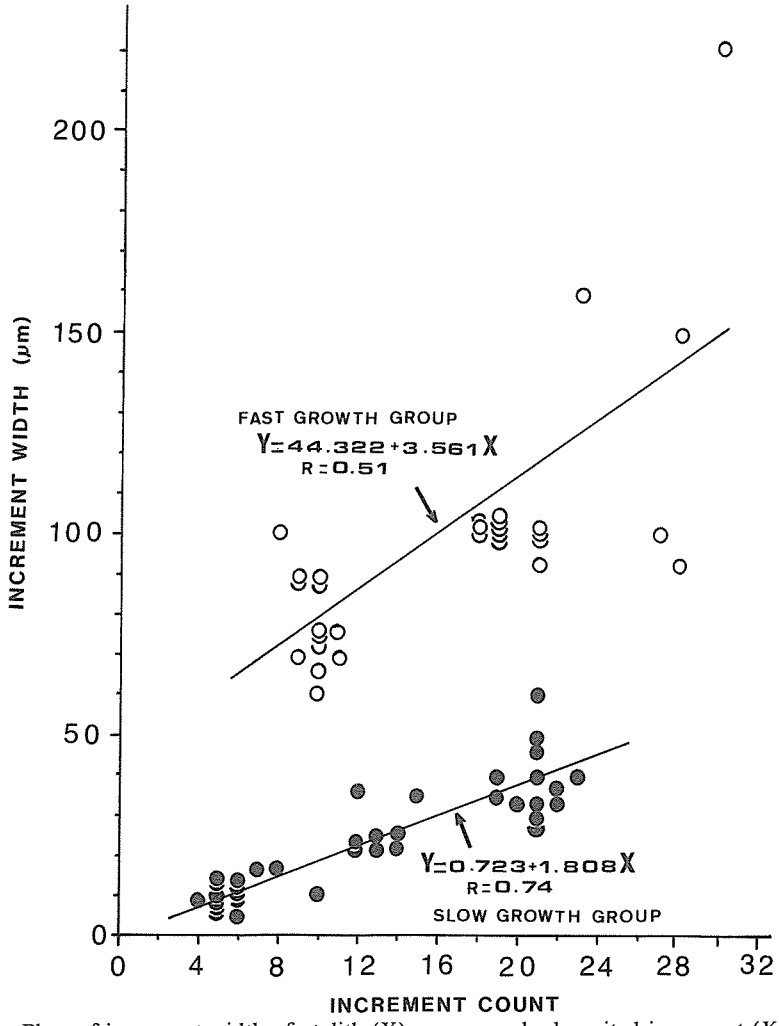

FIGURE 5.—Plots of increment width of otolith (Y) versus newly deposited increment (X) for milkfish larvae reared under two growth regimes after otolith marking with oxytetracycline.

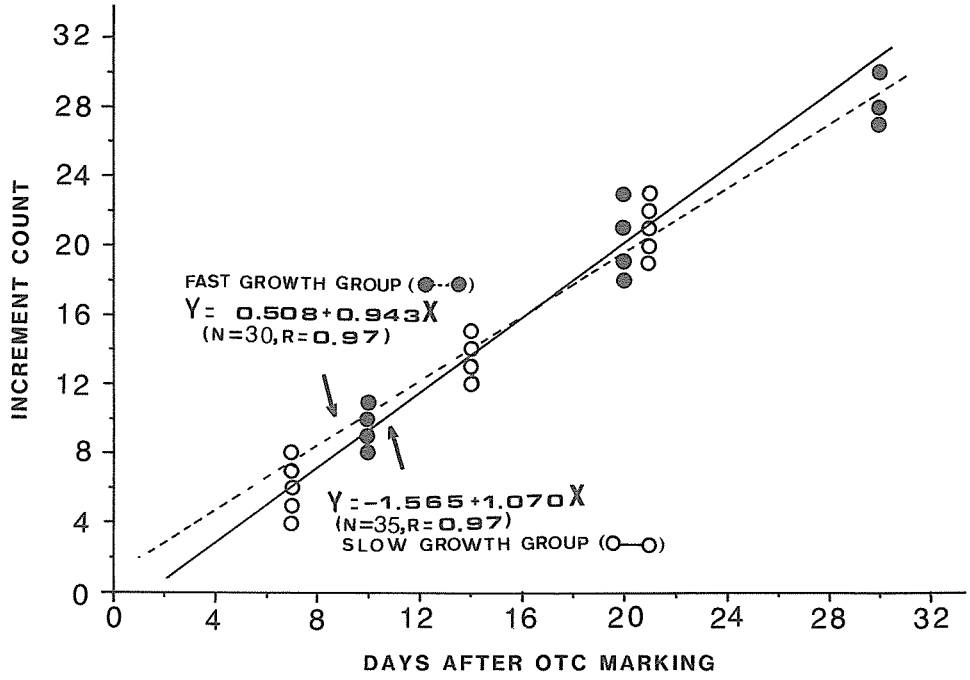

FIGURE 6.—Plots of number of otolith increments (Y) versus the number of days (X) from the date of otolith marking with oxytetracycline to the end of the experiment. The milkfish larvae were reared under two growth regimes after marking.

number of rearing days after OTC marking, the slopes of regressions were 0.94 and 1.07 for the fast- and the slow-growing fish, respectively. Neither coefficient was significantly different from 1.0 for either group (t-test, $P > 0.05$; Figure 6). This indicated that otolith growth increments formed at the rate of about one per day, irrespective of the growth rate of the fish.

Discussion

The milkfish larvae appeared to be stressed when they were immersed in the OTC solution even though the ion strength of the NaCl solution was adjusted to that of seawater. To reduce stress of the fish, we recommend that treatment time for OTC marking be minimized. The optimum treatment time for milkfish larvae appeared to be 24 h at an OTC concentration of 400–500 mg/L. The optimum treatment time seems to vary according to species and developmental stage. Hettler (1984) indicated that the larval otoliths of spot *Leiostomus xanthurus* and pinfish *Lagodon rhomboides* were marked with a band after immersion for 1–2 h in a concentration of 100–500 mg tetracycline/L in a 1% NaCl solution. In ayu *Plecoglossus altivelis,* the optimum conditions for marking were at a concentration of 200–300 mg tetracycline/L for 24–48 h for eggs and 200–300 mg/L for 3–24 h for larvae (Tsukamoto 1985). We detected no OTC mark in the otoliths of milkfish larvae when the immersion time was shorter than 24 h. Tanaka et al. (1981) also found that the deposition of otolith growth increments occurs at an interval of 24 h. Therefore, we suggest that 24-h immersion is appropriate for the effective marking of milkfish otoliths.

The OTC mark on the otoliths of milkfish larvae immersed for 24 h spanned two increments (Figure 1C–E). Tanaka et al. (1981) reported that, in groups under different photoperiods, the formation of growth increments on the otoliths of Nile tilapia *Tilapia nilotica* juveniles started a few hours after the onset of the light period and continued through the dark period. The process was stopped or slowed towards the beginning of the next light period. The milkfish in our study were immersed in OTC solution at about 1400 hours, and the treatment ended at 1400 hours on the following day (i.e., the immersion overlapped two growth increment periods). Therefore, two otolith growth increments were marked by OTC during this 24-h immersion period. This also indicated that OTC was a useful time marker for validating growth increments because it was incorporated rapidly and did not continue to be deposited in the otoliths when treatment ended. Meanwhile,

the newly deposited growth increments that formed beyond the OTC-marked band were discernible under transmitted light (Figure 1D, E). This was useful for finer resolution and validation of growth increments.

Variation in stocking densities, water temperatures, and food rations resulted in differences in body and otolith growth (Figures 2–4). The difference in growth rates of the fish did not affect the daily frequency of otolith growth increments (Figure 6) but were reflected in changes of increment width (Figures 1, 5). Therefore, otolith growth increments can be a useful characteristic for assessing the daily age of milkfish larvae and for evaluating the growth rate of wild milkfish as well.

Acknowledgments

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