Confidence of otolith ageing through the juvenile stage for Atlantic menhaden, *Brevoortia tyrannus*

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Abstract.—The periodicity of increment formation and our ability to enumerate increments in sagittal otoliths of Atlantic menhaden are evaluated from hatching through a nine-month period. We studied otoliths from one group of field-collected larvae that was marked by immersion in oxytetracycline (OTC) and from a second group that was marked by immersion in alizarin complexone (ALC). Additionally, otoliths from known-age juveniles resulting from an Atlantic menhaden laboratory spawning and rearing experiment were examined. We determined that, on the average, larval and juvenile Atlantic menhaden form one growth increment per day. We were able to age juvenile menhaden reliably up to 200 days old within a confidence interval (CI) of about 7 days and up to 250 days old within a CI of about 16 days. We hypothesized that growth rates may have impacted the periodicity of increment formation, as well as our ability to count them accurately. The statistically strongest results were obtained from the ALC-marked fish, which were reared outdoors and displayed growth rates (0.67 to 0.95 mm·day^{-1}) similar to higher rates observed for juveniles captured from estuarine nursery areas. The periodicity of increment counts for the ALC-marked fish was less than one per day when growth rates were observed to be less than 0.3 mm·day^{-1}. Increments in otoliths from the known-age and OTC-marked fish, which were reared indoors, had lower contrast than their outdoor-reared counterparts. Otoliths were sectioned for enumeration on both a transverse and oblique-transverse plane. With minor exception, no differences in age estimation could be attributed to the orientation of the sections.

Manuscript accepted 21 September 1994.
Fishery Bulletin 93:209–216 (1995).

The daily age information obtained from larval and juvenile fish otoliths is a valuable tool for studies of early life history and factors affecting recruitment (Jones, 1992). Daily age information is necessary for backcalculating cohort-specific spawning dates and is the best approach for estimating mortality and growth rates in young fish (Essig and Cole, 1986; Pepin, 1989; Jones, 1992). A prerequisite, however, is the validation of the temporal periodicity of otolith increment formation (Geffen, 1992). While the otolith approach to determination of vital rates has successfully been applied to larval fish, use of otoliths for the juvenile stage has been more controversial, often because of additional requirements of otolith preparation, including sectioning and polishing of otoliths, and because of increased uncertainty in age estimations and back-calculations of size at age (cf. Rice, 1987; Mosegaard, 1990; Jones, 1992).

Many validation studies have determined that increments are, on average, daily in periodicity (cf. Jones, 1986), but conditions affecting a low growth rate, for example, can result in increment periodicity other than a daily one (Geffen, 1992) or can result in difficulty in the detection of daily increments (Campana, 1992). Therefore ageing error commonly increases with age and otolith size as increment widths decrease with decreasing growth rates, resulting in greater uncertainty of ages, growth rates, and birthdates (Rice et al., 1985; Rice, 1987; Campana and Jones, 1992). We examined conditions where confidence about the assumption of daily ring deposition may be low and what the consequence would be of increased ageing error on age estimation for Atlantic menhaden, *Brevoortia tyrannus*.

Larger, older otoliths are more difficult to prepare and read. To address this issue, we also exam-

ined the efficacy of sectioning and polishing juvenile menhaden otoliths on two orientations of transverse planes. Greater attention to otolith preparation can significantly improve ageing accuracy (Campana and Moksness, 1991), and section orientation can affect the ability to read otoliths (Secor et al., 1992).

Previous menhaden validation studies have used only a minor amount of otolith processing, and the material has been examined on the sagittal plane. Maillet and Checkley (1990) and Warlen (1992) used known-age, lab-spawned and reared larvae, whereas Simoneaux and Warlen (1987) examined the outermost growth increments of juvenile Atlantic menhaden injected with oxytetracycline (OTC). Maillet and Checkley (1990) examined growth/increment formation from hatching through 36 days of age, Warlen (1992) through 41 days of age, and Simoneaux and Warlen (1987) used juveniles (63–98 mm in fork length) with an experimental duration of 7–14 days after marking with OTC. With the exception of one test group (Maillet and Checkley, 1990), results of all studies indicated that, on average, one growth increment was formed daily.

However, these approaches have not resulted in a method that will permit precise and accurate ageing of older juvenile Atlantic menhaden otoliths. While Simoneaux and Warlen (1987) were able to validate the daily periodicity of increment formation for a short period of time, their otolith processing method could not be used to determine the actual age of the juveniles examined. The periodicity of increment formation in otoliths should be validated over the ranges in age and size that can potentially be encountered with unknown-age, field-collected material.

We use two of the preferred validation methods, known-age and otolith-marking, to bridge the gap in age and size among the studies of Maillet and Checkley (1990), Warlen (1992), and Simoneaux and Warlen (1987) and to provide an estimate of the uncertainty in deriving age from older juveniles. Our known-age study provides a continuous validation from first feeding through metamorphosis, to juveniles up to 9 months old.

Materials and methods

Known-age fish

Atlantic menhaden brood stock were held in the laboratory and induced to spawn in February 1987 by Hettler's (1981) methods. Eggs were hatched and larvae were reared under laboratory conditions as described by Warlen (1992). Samples of postlarvae and later juveniles were sampled periodically from 52 to 136 days after hatching, then preserved in 70% ethyl alcohol.

Oxytetracycline (OTC) marked fish

In 1988, larval Atlantic menhaden collected at Pivers Island, Beaufort, North Carolina, were acclimated to 100-L indoor laboratory tanks and immersed on 7 April in OTC by a procedure modified from Hettler (1984). Salinity was slowly reduced to 0‰ by adding tap (well) water over several hours. A premixed, buffered (sodium bicarbonate) stock solution of OTC was added to the tank. The resulting treatment conditions were 300 $mg \cdot L^{-1}$ OTC at pH 6.3. After four hours of immersion, ambient seawater flow was restored; the test solution was thus diluted within an hour and salinity slowly increased. The fish remained in this tank for the duration of the study. Samples of postlarvae and juveniles were taken periodically, 13 to 147 days following treatment, and preserved in 95% ethyl alcohol.

Alizarin complexone (ALC) marked fish

In 1992 we conducted validation trials with marked fish under high and low feeding rations to examine further the effect of growth rate on increment deposition. Larval menhaden were captured with a neuston net at Pivers Island, NC, on 1 April 1992 and held at ambient temperatures and salinities until 21 April, then immersed for 14 hours in 100 $mg \cdot L^{-1}$ ALC buffered with sodium bicarbonate to pH 6.5. After immersion, 1,026 larval menhaden were divided between two 2,100-L outdoor holding tanks and sampled monthly, May through December. The larvae were fed cultured, live *Artemia franciscana* and increasing additions of dry food until 29 May, when only dry food (Ziegler salmon starter) was added in a ratio of 3 (high food tank) to 1 (low food tank). The amount of dry food initially added for the low food treatment was 25 $mL \cdot day^{-1}$ in April; this amount was increased to approximately 90 $mL \cdot day^{-1}$ by July and held constant after this period.

Otolith preparation and increment counting

Some otoliths from late larvae were mounted whole in a mounting medium (Flo-tex) on glass microscope slides. After increments were counted on the sagittal plane, many were removed from the mounting medium and sectioned on one of two planes as described below.

We generally followed the sectioning techniques described in Epperly et al. (1991) and Secor et al. (1992). Our processing techniques for the ALC-

marked group were altered: otoliths were dissected from each fish without bleaching (i.e. without a 5% sodium hypochlorite solution to remove tissue) and serial sectioning was used for the transverse sections rather than single cuts with dual blades. Two sectioning orientations were used: a transverse section, taken with the primordium (and focus) as the centerline, on a proximal-distal plane 90° to the anterior-posterior axis, and an oblique-transverse section on a proximal-distal plane from a posterior and dorsal position through the focus to the anterior ventral(most) portion (Fig. 1). (Some otoliths were also examined on an unsectioned, sagittal plane.) Transverse sections were taken on the right sagitta and oblique sections were taken on the left, with the exception of the 1992 ALC-marked material for which selection of the right or left sagitta was randomized. Resulting sections were then ground and polished according to the methods of Epperly et al. (1991) and Secor et al. (1992). For otolith terminology and increment interpretation we followed Pannella (1980) and Campana (1992).

Oxytetracycline and alizarin complexone marks were located with blue light epifluoresence on prepared otolith sections and viewed directly on a compound microscope or on a video image analysis system. The OTC-marked increment(s) fluoresced yellow-green when illuminated with blue light (Fig. 2) and the ALC-marked zone fluoresced orange. The position of each fluorescing mark was fixed with the aid of an ocular micrometer (scope viewing) or with a pointer on the viewing screen; increment counts were made with white or polarized light. Otolith sections viewed on a video monitor were magnified to 1,500×. Since only a fraction of a section would fill the screen at this magnification, increment counting was done stepwise between distinguishable features or "landmarks," and counts were summed when interpretation was complete. An additional series of increment counts was performed with the microscope at 1,000× and by tallying counts blindly on a handheld counter. Agreement between the two methods on enumeration generally was better than 95%. Final counts were means from the two enumeration

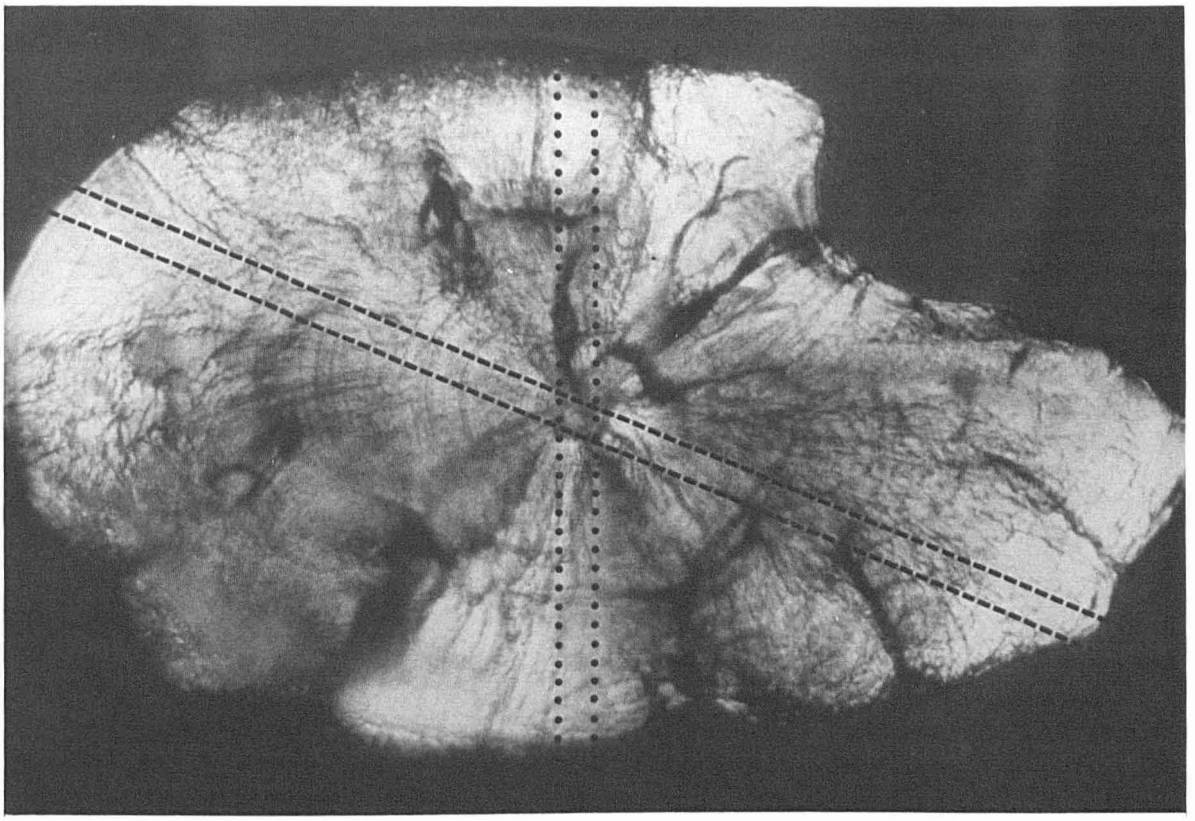

Figure 1

A whole sagittal otolith from a 39.0-mm (0.481-g) juvenile Atlantic menhaden, *Brevoortia tyrannus*, is shown to demonstrate the orientation of sectioning for this study. Transverse (dotted lines) and oblique-transverse (dashed lines) sections are displayed. The otolith is oriented with the dorsal edge up and the anterior to the right.

methods. Mean counts from the known-age fish were increased by five to estimate time from spawning rather than from first feeding (Warlen, 1992). ALC-marked material was viewed under a microscope at 400–1000×, counted with a hand-held counter, and the median of five serial counts was taken.

Statistical analysis

Regression and analysis of covariance (ANCOVA) computations were conducted with SAS statistical programs (SAS Institute, Inc., 1985). Analysis of covariance was used to test for common regression parameters (ring count versus known days) for the low and high food treatments and between transverse and oblique-transverse sectioned material for each marking-validation method (Ott, 1977). Mean growth rates were estimated as the slopes of simple linear regressions of length on age.

We tested the null hypothesis that growth increments in otoliths of larval and juvenile Atlantic menhaden are formed daily. The null hypothesis is accepted if the regression of estimated increment count on known age in days since marking is significant, the slope is not significantly different from one, and the intercept is not significantly different from zero. We also calculated the appropriate statistical power to detect a relatively small difference from a slope of one (Rice, 1987). Student's-*t* test was used to test for significance of the slope and intercept. Statistical power to determine a deviation of 0.1 (α=0.05) from a slope of one was estimated for each linear regression (Rice, 1987; Neter et al., 1989).

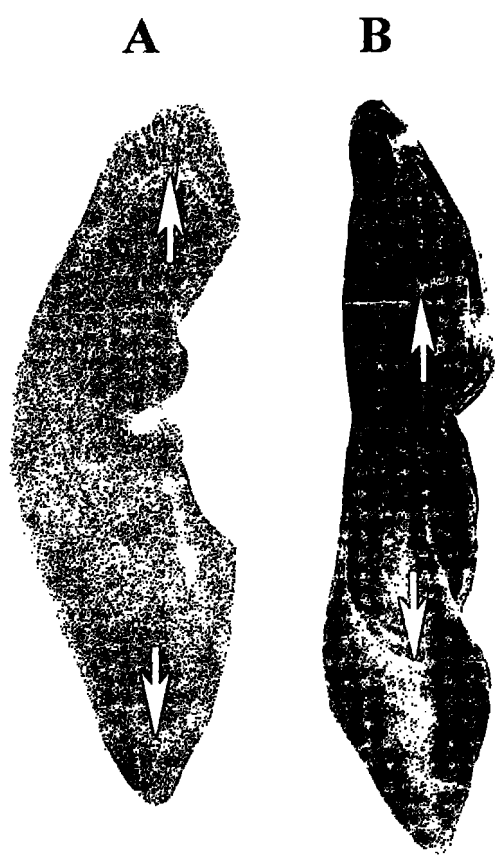

Figure 2

Photomicrographs of juvenile Atlantic menhaden, *Brevoortia tyrannus*, sagittal otolith sections showing fluorescent marks (arrows). (**A**) Transverse section of otolith from a post-larval fish 12 days after immersion in alizarin complexone solution (ALC). The maximum dimension of this section (dorsal/ventral) is 462 μm. (**B**) Oblique-transverse section of otolith from a juvenile 42 days after immersion in oxytetracycline solution (OTC). The maximum dimension of this section (posterior-dorsal/anterior-ventral) is 938 μm.

Results

Otolith preparations were generally readable for the ranges in sizes and ages for the elapsed times. Known-age fish were sampled at ages 52, 66, 81, 122, 131, 132, and 136 days; they ranged from 10 to 64 mm in fork length. Mean growth for the known age group was 0.55 mm·d^{-1}, with a standard error (SE) of 0.048 over the interval from March to June. OTC-treated fish were sampled 13, 18, 42, 110, and 147 days after treatment; they ranged from 27 to 98 mm in fork length and ranged in estimated age from 62 to 130 days with a mean age of 103 days at marking, resulting in mean growth of 0.49 mm·d^{-1} (SE=0.011) for the interval April to August. ALC-marked fish were sampled 12, 42, 71, 100, 131, 161, 190, 205, and 237 days after treatment; they ranged from 26 to 175 mm FL and were approximately 70 days old at marking. Mean growth rates of the ALC-marked fish were 0.67 and 0.95 mm·d^{-1} (SE=0.020 and 0.015) through day 131 postmarking in low and high food tanks respectively. Growth visibly declined for the interval from day 161 to day 237 postmark and was 0.29 mm·d^{-1} for both the low and high food treatments (SE=0.037 and 0.195).

It was readily apparent from scatter plots that the ALC growth-increment count beyond day 131 (day 161 to day 237) postmark was less than one per day and the variance about an individual sampling date substantially greater. We pooled data up through day 131 postmark from the ALC high and low food treatments because tests for homogeneity of the resulting slopes (P=0.667) and intercepts (P=0.831) for age-

increment count regressions (transverse sections) revealed that these parameters were not significantly different between treatments.

We tested for the homogeneity of slopes for the increment count-age regressions between sectioning orientations separately for the known-age experiment and both of the marking experiments. None of the slopes were significantly different (P for known age=0.0583, for OTC=0.188, and for ALC=0.667). Tests for differences in intercepts for the same experimental sets revealed none (P for known age=0.345, for OTC=0.082, and for ALC=0.526). Therefore we pooled the increment count results within each experiment.

We used regressions to compare the results for the known-age and chemically marked otoliths for about the same time duration (i.e. 136 days for known-age fish, 147 days for the OTC group, and 131 days for the ALC group; Table 1, Fig. 3). The intercepts of the three increment-count regressions were not significantly different from zero, and none of the three slope estimates were significantly different from 1.0 (Table 1). The results for the ALC and the OTC groups had sufficient power (>0.80) to detect a difference in slope of 0.1 from a value of 1.0. The standard error of the slope was relatively greater for the known-age group, and thus the power estimate was less than that for the ALC and OTC groups (Table 1).

We examined the results from day 161 to 237 for the ALC experiment in parallel fashion. A test for homogeneity of slopes for increment count on days postmark revealed a (marginally) significant difference between the sectioning orientations (P=0.044). Estimates of the slopes from separate regressions for the oblique-transverse and transverse sections were significantly different from 1 (P=0.016 and P<0.001). While these observations begin to define limits for applying daily ageing techniques to juvenile Atlantic menhaden, they do not reduce the usefulness of the technique over a relatively broad time period. With the minor exception of the period when increment counts were less than daily in the ALC trial, the results for the ALC-marked and OTC-marked test groups were equivalent for either section orientation with slopes near one and with good statistical power to detect a small deviation from one (Fig. 3, Table 1).

Discussion

Atlantic menhaden, on the average, form one growth increment per day through at least an estimable age of 200 days (131 days postmark + approximately 70 days in age at marking) and a size of nearly 150 mm fork length. We could reliably age menhaden up to 200 days old to within about 7 days when juvenile growth rates were high (e.g. above 0.6 mm·d^{-1} over summer months). At moderate juvenile growth rates (approximately 0.5 mm·d^{-1}), we still detected increments at approximately one per day through a 250 day time period for the OTC fish (147 days postmark + an average of 103 days of age at marking), but the variability of an individual age estimate increased in comparison with fish with higher growth rates. For the OTC and known-age test groups respectively, 95% confidence intervals increased to approximately ±16 and 21 d for similar-aged menhaden with slower growth rates (Table 1, Fig. 3). As growth rates declined further (below 0.3 mm·d^{-1}), our increment counts declined to less than one per day, and variability in estimated age increased; this may be due to decreases in increment width or to reduced periodicity as has been found for starved larval Atlantic menhaden (Maillet and Checkley, 1990). After day 131 postmark (ALC), declining growth rates and an increment periodicity of less than one per day (Fig.

Table 1

Least squares linear regression analysis for increment counts from known-age, oxytetracycline (OTC) marked and alizarine (ALC) marked Atlantic menhaden, *Brevoortia tyrannus*, otolith sections. The null hypotheses tested are that the intercept=0 and the slope=1. (SE=standard error.)

Test group	n	r^2	Intercept	SE	P	Slope	SE	P	%Power[1]	95%CI[2]
Known-age	37	0.92	–5.853	5.149	0.263	1.036	0.053	0.501	45.1	21
Tetracycline-marked	34	0.97	1.796	2.139	0.407	0.949	0.027	0.068	94.9	16
Alizarine-marked[3]	84	0.99	0.634	0.701	0.368	0.990	0.008	0.215	>99.9	7

[1] Estimate of percent statistical power to detect a deviation of 0.1 from a slope of one at the P = 0.05 level.
[2] 95% confidence interval (±days) for an age estimate based on individual ring counts. The 95% confidence intervals for individual age estimates were constant over the range of values used to generate the regression.
[3] Through day 131 postmark.

3, bottom graph) corresponded with declining tank temperatures (beginning in September; Fig. 4). However, age could still be estimated within about 3 weeks up to 300 days after hatching (230 days postmark plus about 70 days in age at marking; Fig. 3). If this relationship were consistently repeatable, it would still be a useful tool for estimating ages of older juveniles, even though the age-ring count relationship was well below 1:1. However, we suspect that this change was not so much a function of age as it was a result of reduced growth rate, possibly in conjunction with declining temperature, which affected our ability to accurately estimate ages. Savoy and Crecco (1987) also showed that reduced rearing temperatures can reduce growth rate and subsequently result in a count-age slope below 1.0 for larval American shad, *Alosa sapidissima*.

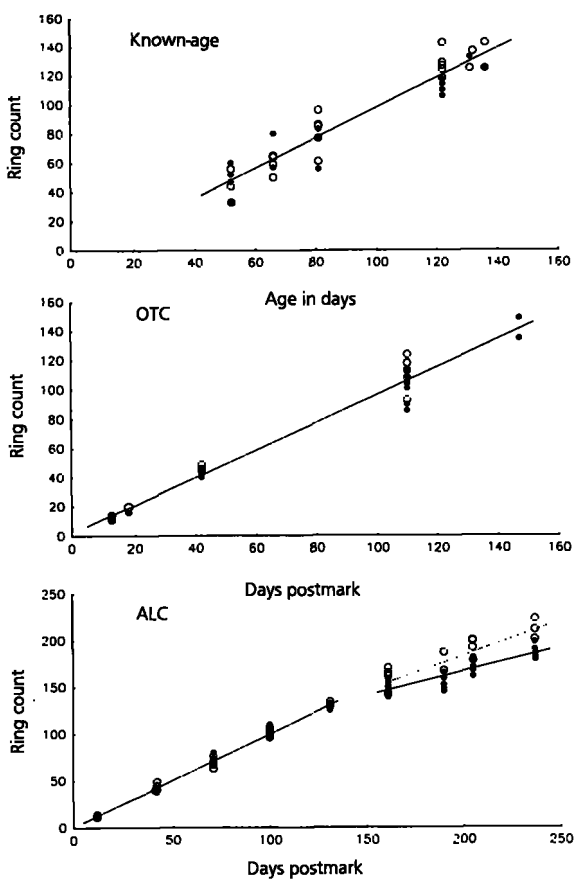

Figure 3

Estimated number of otolith growth increments against elapsed time in days (known age) or days postmark (oxytetracycline [OTC] or alizarin complexone [ALC]) of juvenile Atlantic menhaden, *Brevoortia tyrannus*. Results from oblique-transverse sections (open circles) and transverse sections (closed circles) are pooled for the regression lines shown. The regression coefficients are given in Table 1. (Results for the alizarin-complexone trial for days 161–237, where increment formation rates were less than daily, are shown on the upper right of the bottom graph. Regression parameters for the oblique-transverse (dashed line) and the transverse (solid line) data sets respectively, are r^2=0.81 and 0.79, intercept=39.62 and 62.76, and slope=0.73 and 0.52.)

The growth rates of fish treated with ALC (through day 131 post-ALC-mark) are comparable with observations for upper growth rates of juveniles in tidal creeks, spring through fall (0.7 to 0.83 mm·d^{-1}; Kroger et al., 1974). The laboratory-reared fish had lower growth rates, but their rates were still greater than those for the ALC fish following postmarking day 131. Therefore it appears that reduced growth rate was a contributing factor for the higher variance of our estimates of the slope of counts versus days for our known-age and OTC test groups. Similarly, the variances for the ALC group were highest during the period when increments displayed a less than daily periodicity (Fig. 3).

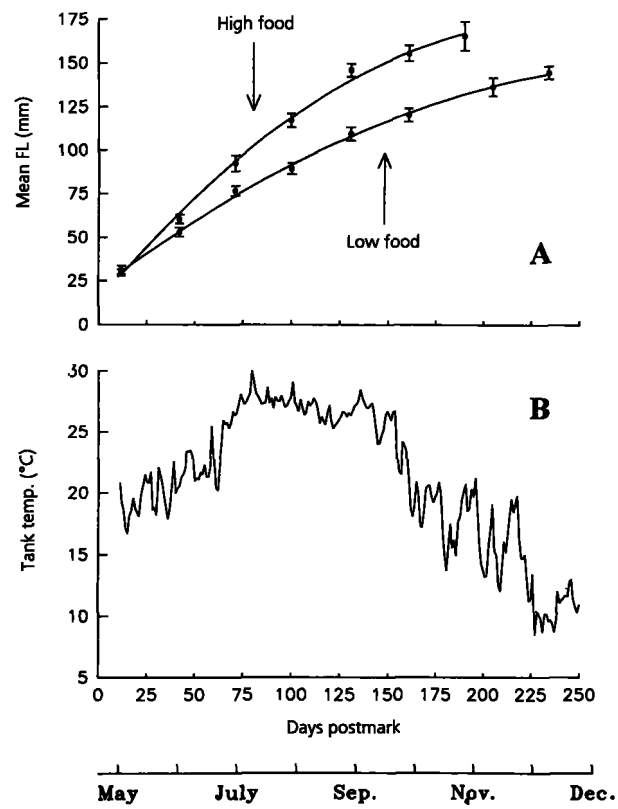

Figure 4

(A) Mean fork length of juvenile Atlantic menhaden, *Brevoortia tyrannus* (error bars represent ±1 standard deviation), and (B) tank water temperatures for the alizarin complexone rearing trial (see bottom graph on Fig. 3).

The otolith sections of the laboratory-reared material, which includes the OTC fish following marking, generally had less contrast between alternating bands than did field material. Warlen (1988) noted similar results for gulf menhaden. This was not the case for the ALC fish, where contrast more closely resembled field material. OTC and known-age specimens were raised indoors in relatively small (100 L) containers, as compared with the larger (2,100 L) outdoor containers used for the ALC fish. (All groups were reared in ambient sea water.) Problems in validating otoliths with laboratory-reared fish have been noted for other species (Campana and Moksness, 1991; Toole et al., 1993). Pannella (1980) notes that the transition between increments are unclear with respect to chemical or structural changes in some laboratory-reared material. It may be that otoliths from laboratory-reared specimens are less typical because of confounding effects from container size, growth rates, and other aspects of the rearing conditions. The poorer contrast may result in lower accuracy and precision in increment counting, and the slower growth may result in more variable increment counts for a given time period.

We obtained detectable OTC and ALC marks in viewing otoliths with the dosages used for immersing larvae. Because of the color contrast of the orange-against-blue background, ALC was visibly easier to detect under blue light fluoresence than was OTC. ALC has been used for marking eggs and hard parts in fish; it leaves a brilliant mark, does not adversely affect growth at low dosages, and does not require dilution procedures as does OTC (Tsukamoto, 1988; Kishiro and Nakazono, 1991).

Although we obtained similar results using either oblique-transverse or transverse sections for those periods when increment formation is on the average one per day, one orientation or the other may be preferred for various reasons. The oblique-transverse method of sectioning may be easier to complete in polishing because the primordium and focus can be detected from a greater distance (thickness) when the material is viewed. This reduces processing time and minimizes the number of overground, unusable preparations. On transverse sections of larger or older individuals, or both, the focus is located more by the outline shape than by early optical discovery. However, some investigators using increment measurements for size back calculation or discriminant analysis may prefer the transverse section for ease in keeping the same plane of measurement from otolith to otolith. The two section orientations were useful for cross comparisons and interpretation of certain growth zones. Therefore choice of orientation should depend upon the material being examined and the questions being addressed.

Acknowledgments

The oxytetracycline experiment was conducted by Robert M. Lewis and James F. Guthrie. The known-age fish, obtained from a spawning conducted by William F. Hettler, were kindly provided by Stanley M. Warlen, and were cared for by Sheryan P. Epperly and Ronald M. Clayton. Valerie Comparetta cared for the menhaden from the alizarin complexone rearing trial. Many of the marked otoliths were sectioned and polished by Theresa V. Henley and Robin T. Cheshire. Douglas S. Vaughan assisted us with the statistical power computations. This study was partially supported by Grant NA16RG0492 from the Coastal Ocean Program, South Atlantic Bight Recruitment Experiment (SABRE), of the National Oceanic and Atmospheric Administration to the North Carolina Sea Grant College program.

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