Validation of age estimates from otoliths of larval and juvenile spotted seatrout, *Cynoscion nebulosus*

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Otolith microstructure analysis has been shown to be valuable for relating biotic and abiotic factors to growth and survival (Crecco and Savoy, 1985; Thorrold and Williams, 1989; Maillet and Checkley, 1991; Jenkins et al., 1993) and determining size-specific mortality (Gleason and Bengtson, 1996; Sabo and Orth, 1996; Hare and Cowen, 1997) for the early life history stages of fishes. Prior to using otolith microstructure analysis, it is important that otolith validation studies be undertaken to examine the rate of increment formation and the timing of initial increment formation, and to check the interpretive skills of the otolith reader (Geffen, 1992).

Our validation study dealt with spotted seatrout, *Cynoscion nebulosus*, an economically important sciaenid. Otolith increment deposition rates from spotted seatrout have been validated by using tetracycline marking of wild-caught fish larvae and juveniles collected in Tampa Bay, Florida (McMichael and Peters, 1989). Because known-age larvae were not used, the timing of initial increment formation was not determined. The purpose of our study was to determine the rate of increment formation and timing of initial increment formation as well as to provide confidence to otolith readers by using known-age larvae and wild-caught juveniles. Our study is ancillary to spotted seatrout early life history studies in Florida Bay, Everglades National Park.

Materials and methods

Spotted seatrout eggs were obtained from the Texas Parks and Wildlife GCCA/CPL Marine Development Center hatchery from adults collected from upper Laguna Madre, Texas. Eggs were transported to the University of Texas Marine Science Institute (UTMSI), Port Aransas, Texas, in May 1997. Eggs were incubated in 600-L tanks at egg densities of 100 eggs/L. Tanks were held in greenhouses, illuminated with natural sunlight and 40-W overhead flourescent lights at a 24-h (15L:9D) photoperiod. A closed system was used, but water was continuously recirculated through a biofilter. During the rearing process, ambient temperatures ranged from 25.0 to 28.0°C; salinities, from 32 to 33 ppt. Larvae to age 12 d were fed rotifers (5/mL), which in turn were provided the algae *Isochrysis*. At age 13 d, brine shrimp nauplii, enriched with oil emulsion (Leger et al., 1986), were introduced (4 rotifers/mL; 2 nauplii/mL on a daily basis), and from age 15 d until the end of the experiment, 2–3 nauplii/mL were maintained on a daily basis. Food density was estimated twice a day and food added to the required density. Frozen adult brine shrimp and red drum, *Sciaenops ocellatus*, eggs were added on a daily basis from age 23 d to the end of the experiment. Otoliths of reared larvae were marked with alizarin complexone (ALC) at concentrations of 50 mg/L for four hours at age 9 and 16 d. Fifty larvae were sacrificed at ages 6, 9, 14, 20, 23, and 32 d and preserved in 95% ethyl alcohol. Larvae sacrificed at ages 6 and 9 d were not immersed in ALC.

Wild-caught juveniles were collected in Aransas Bay, Texas, in August 1997 with a 6-m bag seine (4.5-mm mesh in both the wings and the bag). Juveniles were placed in 45-L tanks and transported to the rearing facility at UTMSI, Port Aransas, Texas. Juveniles were held in 3-m raceways in 1100-L water at temperatures 27.0–28.0°C and a salinity of 36 ppt. Juveniles were segregated by size to avoid cannibalism. Two days after capture, otoliths were marked with ALC (100 mg/L for 4 h). Otoliths were similarly remarked 7 d later (9 d after capture). Mortality associated with the marking process was nil. All fish were sacrificed and preserved in 95% ethyl alcohol at 21 d after capture.

Otoliths were removed with probes and fine-tipped forceps. Polarized light was used to facilitate location of otoliths from fish <6 mm standard length (SL). All otoliths, except for the right sagitta, were placed on a slide, covered with mounting media and archived. The right sagittal otolith was embedded for transverse sectioning or polishing (or for both procedures). The left sagitta was embedded for transverse sectioning if the right was damaged during preparation.

Sagittae were read with a light microscope at 1000× magnification under oil immersion with blue light epifluorescence to detect ALC marks that fluoresce as reddish orange. The first increment was determined as that following the core increment, the latter a well-defined dark increment surrounding the core. Sagittae were read by one

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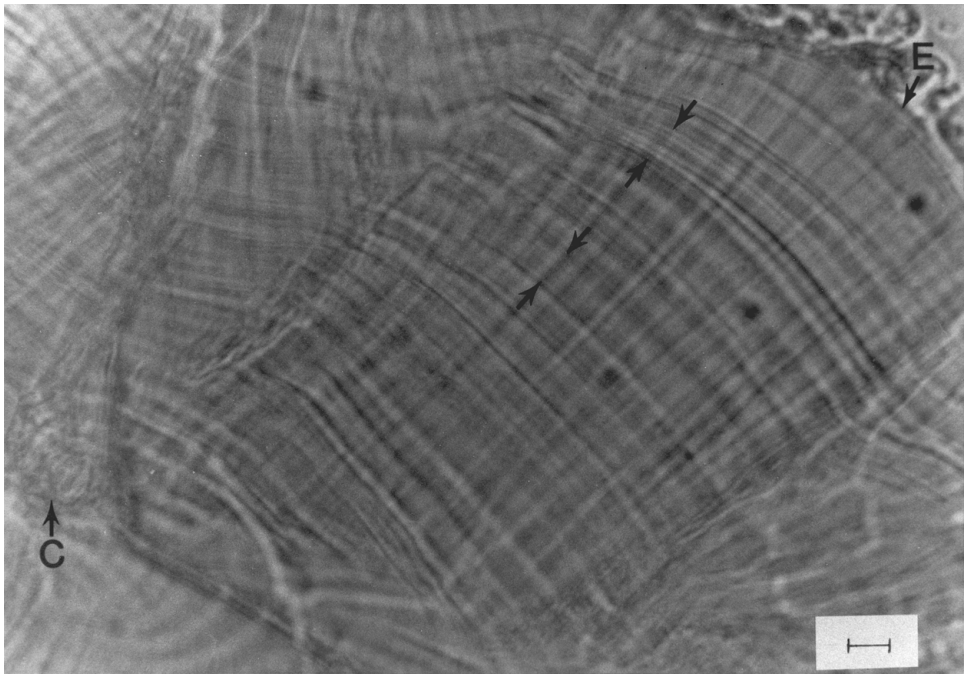

Figure 1

Photomicrograph of a transverse section of a spotted seatrout otolith from a 32-d-old laboratory-reared fish (SL=23.6 mm). C = the core; E = edge. The arrows point to daily increments that were determined as those rings that maintained relatively equal spacing as the fine focus was manipulated. The rings between the arrows were considered subdaily rings or optical artifacts. These rings did not maintain equal spacing when the fine focus was manipulated, were not continuous, and generally were poorly defined. Scale bar = 10 µm.

reader without knowledge of age of fish. Two to three readings were made and the counts averaged. Following Rice et al. (1985) and Ahrenholz et al. (1995), counts were regressed on age (SAS Institute, Inc., 1985), and Student's *t*-test used to determine if the slope was significantly different (α=0.05) than one. The null hypothesis is that the slope of increment counts on known age equals one. The null hypothesis is rejected if the slope is significantly different from one. A summary of the increment count data from known-age fish is shown in Table 1.

In our initial analysis, the slope of increment count regressed on known-age was significantly greater than one (α=0.05), but there were problems in aging the oldest juveniles (32 d)—problems that we related to variability in fish sizes (16.2 to 34.6 mm).Using linear regression, we examined the relation between increment count on size for all age groups. Only for the 32-d-old juveniles was the slope of increment count on size significantly different from zero (α=0.05), indicating that greater counts were observed as size increased. This result suggested that we interpreted subdaily rings as daily rings. We then examined otoliths from known-age juveniles in great detail to establish criteria to separate subdaily from daily rings. Although reading the older age (32-d-old) juveniles was difficult, daily rings maintained relatively equal spacing as the fine focus was manipulated. Subdaily rings or optical artifacts were not continuous, did not maintain equal spacing when focus was manipulated, and were not well defined (Fig. 1). Juvenile otoliths (23-d and 32-d-old) were reread (two blind readings per sagitta, with a hand counter). The reader avoided making mentally sequential counts, relying solely on the results of the hand counter.

Table 1

Mean, standard deviation (SD), number of fish (*n*), total number of readings (*nr*), and range of increment counts from known-age laboratory-reared *Cynoscion nebulosus*.

Age (days)	*n*	*nr*	Mean	SD	Range
6	10	25	4.7	0.7401	2–6
9	10	30	6.0	0.8383	5–8
14	10	30	12.1	0.6641	10–15
20	10	30	20.2	0.8409	17–24
23	12	24	21.9	1.3046	18–25
32	15	30	30.5	1.1568	28–33

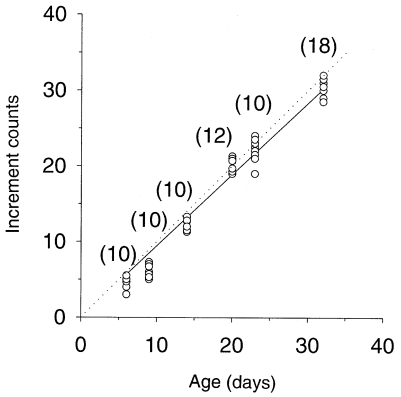

Figure 2

The relation between increment counts and known-age for spotted seatrout, *Cynoscion nebulosus*. Solid line represents regression line; dotted, 45% line. Values in parentheses indicate number of specimens.

Results

The slope of the line describing the relation between increment counts and known age was not significantly different from one (Table 2; Fig. 2). This result indicated that rings were being deposited daily and that we were correctly interpreting daily rings from subdaily rings or optical artifacts, a problem that was encountered with juveniles in our initial readings (see "Materials and methods" section).

A large percent of the laboratory-spawned and laboratory-reared fish that were marked with ALC failed to take up the stain on their otoliths. Only 15% (n=61) of the known-age fish exhibited a readable mark and only for two age groups. On the other hand, the majority (75%) of wild-caught juveniles exhibited a readable mark on their otoliths.

Increments were estimated to form at age 2–3 d. An inverse regression (counts regressed on age) yielded an intercept with a value of 2.3 d (Table 2). The intercept was significantly different from zero. Analysis of increment counts on ALC-marked otoliths from known-age larvae also revealed the first increment formed on day 2 or 3. For 14-d-old larvae marked at age 9 d, seven increments were counted; for 23-d-old larvae marked at age 9 d, six and seven were counted.

Increment counts on marked otoliths were fairly accurate. On 14-d-old larvae (n=4) marked at day 9, we counted seven increments from the core to the ALC mark, and four to six ($\bar{x}$=5.0; five expected) from the mark to the otolith edge. On 23-d larvae (n=5) that were marked on day 9 and day 16, we counted six to seven ($\bar{x}$=6.6) increments from the core to the first mark, 14 to 15 ($\bar{x}$=14.7) from the core to the second mark, and four to nine ($\bar{x}$=7.6) from the second mark (16-d-old) to the edge (23-d-old). For wild-caught larvae (n=12) (which ranged from 34.4 to 70.2 mm SL, were marked at 7-d intervals, and which were preserved 14 d after marking), we observed seven to eight ($\bar{x}$=7.0) increments between marks and 13–15 ($\bar{x}$=14.2) increments between the last mark and the edge.

Table 2

Analysis of variance results for increment counts versus age, and inverse regression for age versus increment counts (inverse regression) for all larvae and juveniles (n=69) of known-age spotted seatrout, *Cynoscion nebulosus*. SE = standard error.

Dependent variable	Slope	SE	Intercept	SE	P
Counts	1.0296	0.0172	–1.9867	0.3574	0.0001
Age	0.9538	0.0159	2.2273	0.3135	0.0001

Discussion

Our overestimation of ages, in our initial analysis (see "Materials and methods" section), from increment counts of older juveniles is difficult to relate to environmental factors. Overestimation of age is not common, although Fives et al. (1986) reported that larger bay anchovies, *Anchoa mitchelli,* in any known age group generally had more growth increments than smaller fish. Nielson and Geen (1982, 1985) noted that for salmonids, feeding frequency, exposure to warm or cool temperature cycles twice in a 24-h period, and an enforced increase in activity increased the rate of increment formation. Fish in our study did not undergo such cyclic events.

Campana and Moksness (1991), from a detailed appraisal of accuracy and precision of age estimates derived from otoliths, concluded that accuracy of age determination from validation experiments is probably "optimistic" owing in part to the constraints and limitations of validation studies done under laboratory conditions. The marked size difference of our known-age 32-d-old juveniles (16.2–34.6 mm SL) was undoubtedly a result of laboratory rearing conditions and indicated that even with appropriate photoperiod and feeding conditions, caution should be used when interpreting rings from older laboratory-reared juveniles, especially when size ranges are highly variable as in our study. For older known-age laboratory-reared material, multiple markings should be employed to discern those areas where interpretation is difficult and to discern subdaily rings.

Previous validation studies of spotted seatrout have indicated that rings are deposited daily, but central rings are difficult to interpret (McMichael and Peters, 1989). McMichael and Peters (1989) used otoliths mounted whole from tetracycline-marked wild-caught larvae and juveniles (7–10 mm). Although they were able to observe the formation of daily rings, they had difficulty in interpreting central rings. They used the average measurement to the tenth ring (50 µm, SD=2) taken from few exception-

ally clear juveniles. Our measurements from 23-d (n=12) and 32-d-old (n=15) juveniles averaged 52.4 µm (SD=8.1). Although our data were more variable, because we did not select only clear otoliths, our measurement to the tenth increment was similar to that of McMichael and Peters (1989), indicating we were interpreting the central increments fairly accurately.

Our relative lack of success in marking known-age larvae could be due to an inadequate concentration of ALC (50 mg/L) or insufficient immersion time at that concentration. Thomas et al. (1995) produced high mark quality on 6.0–9.0 mm red drum larvae at concentrations of 100 mg/L for as little as 2-h immersions. Fish mortality was minimal (and mark quality high) for immersion times up to 24 h at that concentration. A concentration of 50 mg/L for 4 h appears to be inadequate, however, for spotted seatrout larvae. Because of the relative lack of success in marking small larvae at 50 mg/L, larger juveniles were immersed in a concentration of 100 mg /L and marking success was relatively high after 4-h immersion, but still not 100%. Thomas et al. (1995) found that mortality of larvae was fairly high (>65%) at concentrations of 250 mg/L or higher; therefore longer immersion times are probably necessary for 100% marking success. Other investigators have had difficulties in marking larvae with tetracycline immersion that were related to divalent cations in full strength seawater (Campana and Nielson, 1982; Hettler, 1984; Gleason and Recksiek, 1990).

In conclusion, we caution investigators that our interpretation of increment counts for spotted seatrout may not be valid for other studies. The skill of the otolith reader is a critical component in otolith microstructure analysis (Campana and Moksness, 1991); therefore, microstructure analysis studies on spotted seatrout should require a separate validation study for each reader.

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