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DAILY GROWTH INCREMENTS IN OTOLITHS OF JUVENILE BLACK ROCKFISH, *SEBASTES MELANOPS*: AN EVALUATION OF AUTORADIOGRAPHY AS A NEW METHOD OF VALIDATION

Investigations into the temporal periodicity of growth increment formation in otoliths of larval and juvenile fishes have produced conflicting accounts. Taubert and Coble (1977), Barkman (1978), Wild and Foreman (1980), and Campana and Neilson (1982), among others, have confirmed daily increment formation in otoliths from various species of larval and juvenile fishes. There have been a few studies, however, in which increment counts were not representative of actual age of the fish (Wild and Foreman 1980; Geffen 1982; Neilson and Geen 1982). Nondaily increment formation has been explained by the inclusion of subdaily rings in age estimates as well as by methodological errors in preparing and viewing the otoliths (Campana 1983a; Campana and Neilson 1985). Since size and age of fish, food limitations, and environmental conditions have been suggested to affect increment formation, validation is necessary in each study where fish age is estimated.

Several techniques have been used to validate daily growth increments in larval and juvenile fish otoliths. Fish of known age, raised from fertilization or birth under controlled laboratory conditions, provide the best material to determine frequency of increment formation (Taubert and Coble 1977; Barkman 1978; Tanaka et al. 1981; Miller and Storck 1982). For many species, however, rearing the larvae from birth through the juvenile stage is difficult or impossible. An alternate method of age validation introduces a chemical mark onto those calcified structures which exhibit periodic growth zones, such as otoliths, scales, and spines. The antibiotic oxytetracycline hydrochloride (OTC) has been used most successfully in this manner (Wild and Foreman 1980; Campana and Neilson 1982; Ralston and Miyamoto 1983; Dabrowski and Tsukamoto 1986). The OTC is taken up at the site of calcification and fluoresces bright yellow under ultraviolet light, compared with the blue autofluorescence of normal tissue. Most recently, stable strontium has been used to demonstrate daily increment formation in squid statoliths (Hurley et al. 1985) and in mass marking of coho salmon (Yamada et al. 1979). For some species, a timemark may also be induced on the otolith by stress,

such as cold shock (Mugiya and Muramatsu 1982), or by simply bringing field-captured fish into the laboratory (Boehlert and Yoklavich 1985). Comparing increment counts with number of days following the time-mark accurately estimates frequency of occurrence of the growth increments. Our study evaluates the commonly used OTC and an alternate chemical, the radioisotope calcium-45, in terms of their success as time-markers to validate daily growth increment formation in the otoliths of juvenile black rockfish, *Sebastes melanops*.

Materials and Methods

Young-of-the-year black rockfish, *Sebastes melanops*, ranging from about 2 to 5 g wet body weight and 47 to 64 mm standard length (SL), were collected from a rocky, intertidal area 8 km south of Newport, OR, in July 1982 and from Yaquina Bay, OR, in July 1983. Fish were held in 200 L tanks under ambient water temperature conditions which fluctuated between 13° and 17°C; a ration of ground squid and shrimp was offered ad libitum and photoperiod was maintained at 13 h light and 11 h darkness. After at least 10 days of acclimation to laboratory conditions, fish were anesthetized with MS-222 and injected intramuscularly (midbody below dorsal fin) with a solution of either OTC or calcium-45. Fish continued to feed immediately following injection and handling.

Calcium-45

Fish were injected with a solution of low-calcium physiological saline and calcium-45 ($^{45}CaCl_2$ dissolved in HCl; New England Nuclear).[1] Through preliminary experiments, a dose of 0.1 μCi ^{45}Ca/g wet body weight proved to be optimum for isotope uptake and retention. Four fish each were sacrificed at 1, 4, 12, 24, 48, 72, 96, 120, 144, 168 hours and at subsequent 4-d intervals for 63 days following injection. Three fish were sacrificed after having maintained good health and growth for 1 year following injection. Four nonradioactive fish were sacrificed on the first day of the experiment and used as blanks or controls in determining activity levels of the injected fish.

At the time intervals specified above, fish were anesthetized, blotted dry, measured (nearest mm, SL), and weighed (nearest 0.01 g). Both sagittal otoliths were removed from each fish, rinsed thoroughly in water to remove surface contamination of calcium-45, and stored dry for liquid scintillation counting (LSC) or autoradiography. One otolith from each fish, with the exception of the three 1-yr-olds, was weighed, dissolved in 0.1 mL concentrated HCl, diluted with 10 mL of Beckman Ready-solv EP liquid scintillation cocktail, and assayed for calcium-45 activity in a Beckman LS 8000 liquid scintillation counter. Activity was corrected for decay and quench and expressed as disintegrations per minute (DPM) per mg of sample. The perceived decrease in radioactivity due to the increase in weight of otolith over the experimental period was corrected using the following equation:

$$\text{Corrected activity} = \frac{\text{DPM}}{\text{mg tissue}_{t_f}} \times \frac{\text{weight tissue}_{t_f}}{\text{mean weight tissue}_{t_i}}$$

where t_f is time at sacrifice and t_i is time of experiment initiation. Mean weight of otolith at t_i was obtained from the 4 fish sacrificed prior to injection; since all fish were of similar length at the onset of experimentation, these 4 fish adequately represented size of injected fish.

Four otoliths from time interval 1 hour and two otoliths from each of intervals 4 and 12 hours and 1, 4, 11, 19, 39, 55, and 63 days were prepared for autoradiography. The right otolith of each pair was affixed to a microscope slide with histological mounting medium. The proximal surface of the otolith was ground with 600 grit carborundum paper on a rotating wheel until the focus was just visible and most of the curvature of the otolith was removed. The mounting medium was gently heated and the otolith was turned to expose the distal surface. Grinding was continued until most of the mounting medium was removed from the margins of the otolith. The external surface was polished using jeweler's rouge (3 μm) and the whole slide was immersed in an ultrasonic cleaner to remove all loose particles from the otolith surface. The resulting sagittal section was coated with Kodak NTB3 nuclear emulsion and exposed in a light-tight box for 8 days at 4°C. The autoradiographs were developed in Kodak D-19 developer for 2 minutes, fixed for 5 minutes in

[1]References to trade names do not imply endorsement by the National Marine Fisheries Service, NOAA.

Kodak Fixer, rinsed for 20 minutes in distilled water, and viewed under transmitted light with a compound microscope at 400× magnification to determine presence and location of exposed silver grains. Growth increments were enumerated from the time-mark to the otolith margin. In most of the otoliths, a check (or exceptionally dense band) was noted prior to the deposition of the radioactive mark. The location of this check, in terms of numbers of increments from the time-mark, was also determined.

Oxytetracycline

A stock solution was prepared using 25 mg OTC (Sigma Chemicals Co.) in 5 mL of physiological saline. Each fish received a dosage of 0.5 mg OTC or 0.1 mL of stock solution. This approximates the dosage reported by Mugiya and Muramatsu (1982) for goldfish and Weber and Ridgway (1962) for sockeye salmon smolts. Fish were sacrificed 21 days after injection, weighed and measured, and both sagittal otoliths were removed, cleaned, and stored dry in the dark.

A sagittal section of the right otolith was prepared as previously described. Sections were viewed at 160× magnification, using a compound light microscope equipped with ultraviolet illumination. The fluorescent mark was located with an ocular marker. Increments were enumerated from this mark to the outer margins of the otolith using visible light.

Results and Discussion

Calcium-45

One hundred and three black rockfish were injected with the radioisotope, calcium-45; there were no mortalities during the 63-d postinjection sampling period. Over the course of the experiment, average fish length increased from 52.5 mm (SD = 1.29, $N = 4$) on day zero to 70.5 mm (SD = 8.23, $N = 4$) on day 63; average total body weight increased from 2.3 g (SD = 0.13, $N = 4$) to 8.10 g (SD = 2.60, $N = 4$).

LSC demonstrated that calcium-45 was taken up and retained in the sagittal otoliths of all fish. Incorporation of calcium-45 into the otolith occurred as early as 1 hour following injection, which was the initial sampling interval; mean activity at this time was 1,377 (SE = 329) DPM/mg otolith (Fig. 1). Similar activity values and uptake patterns of calcium-45, up to 72 hours

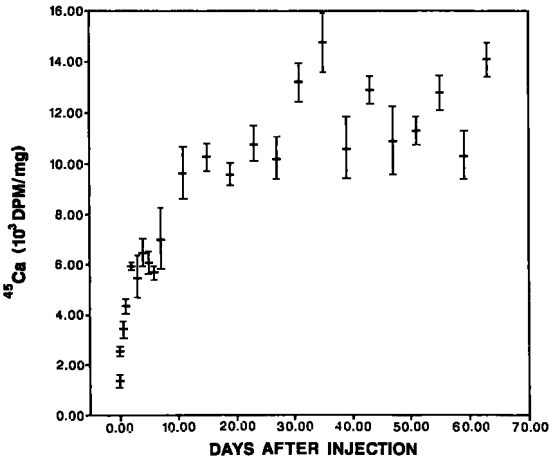

FIGURE 1.—Accumulation and retention of ^{45}Ca in the otolith of black rockfish. Mean activity and 1 SE are indicated. $N = 2$ to 4 fish per time interval.

postinjection, were observed in the otoliths of rainbow trout, although times of maximum incorporation and retention were not assessed over longer periods (Mugiya 1974). Radioactivity in the rockfish otoliths increased sharply for the first 15 days (up to an average of 10,279 DPM/mg, SE = 581), followed by a gradual increase to an apparent asymptote (Fig. 1). The rapid uptake and retention of calcium-45 in liver, muscle, and epidermis of *S. melanops*, and the gradual elimination of the isotope from these tissues (Yoklavich and Boehlert unpubl. data), could contribute to the increase in otolith activity over time; presumably, calcium-45 is transported from these tissues to the otolith via the blood (Mugiya 1974). The lack of decrease in activity in the otolith substantiates the findings of Ichii and Mugiya (1983) and Campana (1983b), which suggest that calcium deposited in otoliths of goldfish and stressed coho salmon, respectively, remains immobilized and is not resorbed. Although no data were presented, it had been suggested earlier by Pannella (1980) that resorption of calcium occurs in the otoliths of some tropical fish species, possibly invalidating ages based on increment counts. Our data show no evidence of resorption, lending support to the usefulness of increment counts in reliably estimating age.

Scattered exposed silver grains were evident along the interface of otolith section and mounting resin in the autoradiographs of otoliths from the earliest sampling periods (1 hour to 1 day), although positive association of the grains with a

distinct site of isotope incorporation into the otolith was not discernible. Since the radioactive mark was on the edge of the otoliths from these early sampling periods, it was more difficult to identify than the mark left on otoliths of fish sacrificed later in the experiment. Distinct bands of silver grains, designating the site of isotope uptake, were evident in all but four otoliths sampled from day 4 through day 63 (Fig. 2). A less dense background of grains spanned 7 to 10 bands around the site of uptake; postinjection increment counts were made from the site of densest grain

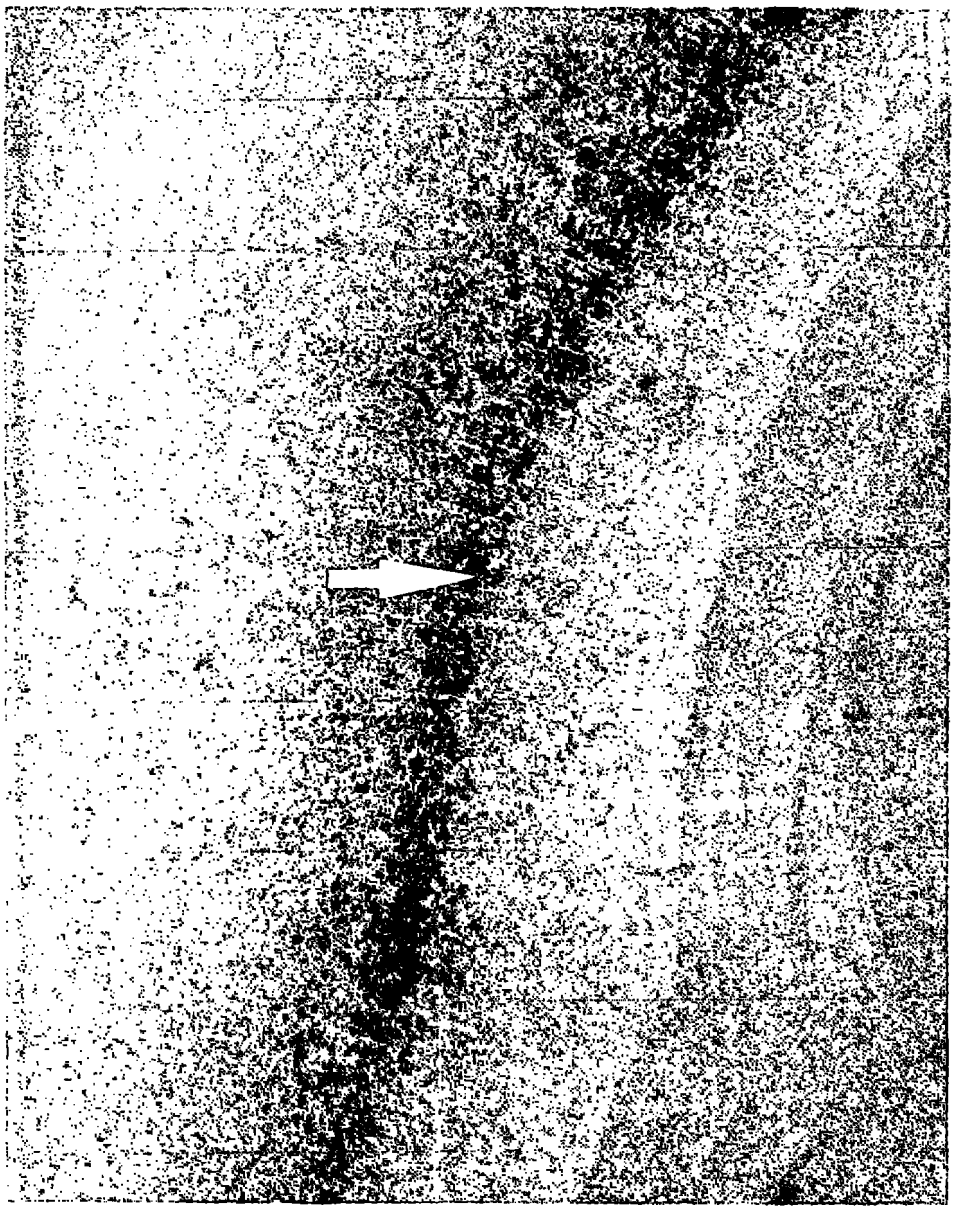

FIGURE 2.—Example of the silver grains produced by the autoradiograph of ^{45}Ca in the otolith of a 69 mm juvenile black rockfish. Arrow indicates band of densest grains; for scale, arrow = 35 μm. This photo is from the anterodorsal region of the otolith. Note the clear increments present on the left side of the figure, representing the increments distal to the time-mark.

accumulation to the edge of the otolith. The posterodorsal area of the sagittal sections showed the heaviest accumulation of the isotope and was also the easiest area in which to count increments. This observation is consistent with Irie (1960) and Mugiya (1974), who concluded that high calcium uptake occurred in the dorsal region, as well as in the anterior and posterior tips of otoliths; these are the regions of fastest otolith growth.

The number of growth increments from the band of densest accumulation of silver grains to the edge of the otolith section closely approximated the number of days the fish were held in the laboratory following injection (Table 1), thereby validating the occurrence of daily growth increments in these juvenile black rockfish. Validation of the frequency of growth increment formation, obtained from fish held under optimal growth conditions in the laboratory, and the apparent lack of otolith resorption, as demonstrated by the increasing retention of calcium-45 with time, suggest that daily increments on otoliths could provide accurate representation of age and growth for field-caught juvenile *Sebastes melanops*. Daily increments have recently been suggested to occur on otoliths of early larvae of *S. marinus* (Penny and Evans 1985). In previous work on juvenile *Sebastes*, growth increments had been counted but not validated (Moser and Ahlstrom 1978; Boehlert 1981). Our study is thus the first confirmation of daily increments on otoliths of juvenile *Sebastes*.

Checks, or exceptionally dense and dark bands deposited as daily increments, were observed in otoliths from fish used in the calcium-45 experiments. One check preceded the radioactive time-mark and another check was associated with the time-mark itself. Ten growth increments were noted from the earliest check to the time-mark in each otolith (Table 1). Formations of checks in otoliths have been documented for many species, including coho salmon (Campana 1983b), goldfish (Mugiya and Muramatsu 1982), and several tropical species (Brothers et al. 1983). Such checks have been attributed to periods of physiological stress to the fish due to collection, migration, change in feeding or habitat, temperature, life history stages, or anything else that disrupts growth. In the present study, the time from fish collection to injection of the isotope marker was 10 days (Table 1). It seems clear that the observed checks were produced as a consequence of stress encountered during capture and transport to laboratory conditions and can be used as additional evidence of daily deposition of growth increments. If such checks are reliably produced, they may be better than chemical time-markers for validation studies such as these.

TABLE 1.—Age validation using otoliths from black rockfish marked with calcium-45 or oxytetracycline. Number of days from capture to injection is compared with number of growth increments from capture check to time-mark; number of days from injection to sacrifice is compared with number of increments from time-mark to margin of otolith.

Treatment/ sample size	Number of days from		Mean number increments from	
	Capture-injection	Injection-sacrifice	Check-mark	Mark-margin
^{45}Ca/4	10	0.04	10	[1]0
/2	10	0.17	10	[1]0
/2	10	0.5	10	[1]0
/2	10	1	10	[1]0
/1	10	4	10	4
/1	10	11	10	11
/1	10	19	10	19
/2	10	39	10	36
/1	10	55	10	55
/2	10	63	10	63
OTC /5		21		21

[1]Scattered silver grains associated with margin.

Oxytetracycline

The OTC was incorporated into the otoliths of each of the 15 fish injected and produced a distinct fluorescent band. The growth increments following injection of OTC, however, were less distinct on most of the otoliths and consequently all otoliths could not be used to validate daily increment formation. Weak increment definition following OTC incorporation in otoliths of larval spot and pinfish has been reported by Hettler (1984). Although Hettler suggested that the lack of distinct increments resulted from experimental stress, postinjection increments were clearly visible in otoliths from the juvenile rockfish which were injected with calcium-45 and held under laboratory conditions similar to the OTC experiments. Five of the rockfish otoliths did display clear growth bands following the fluorescent time-mark; enumeration of these increments is summarized in Table 1. These otoliths show the same results as those from calcium-45 treatments, demonstrating the daily periodicity of growth increment formation in juvenile black rockfish. It is unclear, however, why 67% of the otoliths failed to produce prominent daily increments after OTC incorporation.

The fluorescent OTC mark was more intense

and easier to identify than the exposed silver grains in the autoradiographs of most otoliths. OTC is less hazardous to handle in the laboratory and can be detected in the otolith for much longer periods than calcium-45. The OTC was still visible in the otolith at least 3 years following injection and has in fact been used in studies for mass-marking of fish for identification purposes, where time at liberty may be even longer (Tsukamoto 1985). The activity of calcium-45 was evident in autoradiographs of a few otoliths which were developed 2 years following injection; amount of activity depends upon the effective half-life of the isotope (164 days for calcium-45) and the initial amount of activity in the tissue. An autoradiograph of a transverse section through the otolith of one of the fish injected and held for 1 year revealed a discontinuous band of very faint, exposed silver grains, dispersed primarily over the internal and dorsal areas of the otolith. Association of the isotope with an annular band was not observed. Autoradiographs are difficult to produce, expensive, and time consuming. On the other hand, OTC is simply observed in the otolith section under ultraviolet light. Our recommendations for validating the daily formation of growth increments in juvenile rockfishes are 1) the use of OTC, if growth increments can be routinely identified following injection, or 2) stress marks, which are induced either when transferring fish from the field to laboratory or by subjecting fish to abrupt environmental changes. Where this type of induced stress is not appropriate, as in environmentally controlled laboratory studies, and OTC marking is unsuccessful, marks could reliably be produced with the calcium-45 technique described in this paper.

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