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Evaluation of Age Determination Methods for Rex Sole (*Errex zachirus*)

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NOTICE

EVALUATION OF AGE DETERMINATION METHODS FOR REX SOLE (*Errex zachirus*)

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Preface

Rex sole (Errex zachirus) otoliths from the Gulf of Alaska were exposed to a variety of staining and laboratory techniques by the Resource Ecology and Fisheries Management Division's Age and Growth Task as part of an evaluation of current ageing procedures. In the past, the interpretation of rex sole otoliths has been sporadic and inconsistent. Because the annuli of rex sole otoliths are faint, missing, or hard to interpret, a means of enhancing the visibility of the annuli was sought. A method was sought to reliably determine the age of an individual fish using otoliths.

To measure the "ageability" a technique provided, we implemented a system that quantified our observations. Two readers provided independent age readings of the same otolith, thus allowing for the calculation of a percent agreement for each technique. In general, a high percent agreement indicated that more precise ages were determined for a given method. The validation of ages was outside the realm of our study, therefore the accuracy between the two readers was not calculated.

Ageing methods were evaluated by percent agreement, time spent completing a technique, complexity of the technique, and any problems encountered with a technique. Examination of whole, cut, and thin-sectioned otoliths was done using a Wild[1]

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[1] Reference to trade names does not imply endorsement by the National Marine Fisheries Services, NOAA.

stereomicroscope using transmitted and refracted light. The chemical methods evaluated included: decalcification using disodium ethylene diamine tetraacetate (EDTA), calcium staining alizarine red S, protein staining rhodamine-B, and ninhydrin. The other methods evaluated were muffle furnace, polarized light, and ultraviolet light.

The best preparation method for the muffle furnace was cut otolith halves baked at a temperature of 270°C for 7 minutes and then examined under reflected light conditions. The agreement achieved between two readers was 66.7%. In contrast, the traditional break-and-burn technique used for otoliths yielded a 24.2% agreement between two readers. The amount of time to prepare samples for age determination work was minimal (1 day), and no problems were encountered in using the muffle furnace technique. In the future, the muffle furnace method will be used to examine otoliths of the many fish species which are considered unsuitable for the break-and-burn method.

Age composition data for rex sole (Errex zachirus) populations along the Pacific coast and Gulf of Alaska have been very limited. In previous studies, otoliths have been used to determine the age of rex sole (Hosie and Horton 1977, Needler 1953). The purpose of our study was to develop a method of age-determination that would generate highly precise age composition information.

Rex sole have oval to glove-shaped otoliths with an average diameter of approximately 6 mm. The otoliths are relatively flat except for the nucleus region. The area around the nucleus through the second year projects upward and has the greatest thickness compared to the rest of the otolith. Interpretation of the first year's growth, which falls within this boundary, is very difficult because less light is refracted due to the nucleus's thickness.

The difficulty with reading rex sole otoliths lies in the faintness and fragmentation of their annuli, particularly those produced during the first year. Another difference in the otoliths of rex sole and those of other fish species lies in their chemical composition.

The chemical and structural composition of otoliths from many different species have been documented (Degens et al. 1969). A fibrous protein, called otolin, has been found in the otoliths of all species. Although the composition of otolin varied in the amount of certain amino acids found in otoliths of different species, it had the same core constituency of amino acids. However, the amount of protein in an otolith varies from species

to species. In general, a large portion of the protein content is found in the annulus (Richter and McDermott 1990). In rex sole the protein content might be much lower over the entire otolith or it might be lower in just the annuli, compared with a species like yellowfin sole (Pleuronectes asper). The yellowfin sole have well-formed, distinct annuli that are easily visible after the otolith has been exposed to the break-and-burn technique (Christensen 1964). In contrast, the otoliths of rex sole do not show well-formed annuli when the break-and-burn technique is used. The use of stains in age-determination work could possibly be useful in successfully identifying annuli of otoliths where the traditional break-and-burn method is not effective. While researching the various staining techniques, we kept in consideration that the organic components of the otolith react to stains (Albrechtsen 1968).

Since Christensen (1964) discovered the break-and-burn method, it has been the technique of choice for ageing fish otoliths because it is easy to apply and efficient at ageing otoliths on a production basis. Nevertheless, for some species of fish the results generated with the break-and-burn technique are not reliable. Subsequently, those species with otoliths that are difficult to interpret are set aside and no microscopic examination of otoliths is done. However, use of stains in age determination work has recently been discovered as a useful, practical tool (Gauldie et al. 1990, Richter and McDermott 1990,

Bouain and Siau 1988, Cailliet et al. 1983, Schneppenheim and Freytag 1980).

METHODS AND MATERIALS

Procedure For Evaluation

Six laboratory and staining techniques were selected for evaluation after a literature search was conducted. Our procedure for implementing each technique will be described in detail. Some procedures have been slightly modified from the original published methods.

All rex sole otoliths were collected during the Resource Ecology and Fisheries Management Division's (REFM) 1990 triannual fishing survey of the Gulf of Alaska in International North Pacific Fisheries Commission (INPFC) statistical areas 610, 620, 630, and 640.

To evaluate each stain or laboratory method, a minimum sample size of 9 otoliths and a maximum sample size of 102 otoliths were selected. The otoliths were examined using a Wild stereomicroscope at powers ranging from 60X to 250X. Surface axis readings of the otoliths were taken at 60X and again at 180X for cut halves before any of the staining procedures were implemented. This procedure was followed to ensure that the stained annuli were the same as the unstained annuli. The unstained annuli that were observed and the corresponding annuli

on the stained otoliths were the same. However, it was much harder to identify the annuli of the unstained otoliths.

Some of the staining techniques were used on cut halves of a specimen and the thin sections from the same specimen depending on the procedure. Stained, cut otolith halves were examined to determine how well the stain was taken up by the otolith. If the staining technique was successful on the cut halves, then a thin section was taken of a specimen and it was also stained. The muffle furnace was used to bake whole otoliths. The whole otoliths were then sectioned into 0.25 mm and 0.5 mm sections. Cut halves were also read after baking in the muffle furnace. An evaluation of the different techniques was completed at the time the technique was employed. All prepared otoliths were read independently by two readers, and the results were compared for each technique. The percentage of otoliths for which the two readers gave the same age indicated the "ageability" of otoliths prepared by a particular method (Kimura and Lyons 1991). Results of these comparisons are summarized as the percent agreement in Table 1. Our study appears to be the first to apply the percent agreement method to evaluate otolith preparation methods.

EDTA Method

The use of Disodium Ethylene Diamine Tetraacetate (EDTA) has been commonly used in the preparation of otoliths for examination under the scanning electron microscope (SEM) (Brothers 1987).

The compound is used to decalcify otoliths by removing the calcium ions. A 0.1M EDTA solution has been shown to etch check rings into otoliths, of which the most deeply etched correspond to opaque zones (Gauldie and Radke 1990).

Opaque zones alternate with hyaline zones forming annuli on all species. The opaque zone is usually much wider then the corresponding hyaline zone in the early years of the fish's life, but decreases in size for the older years (Williams and Bedford 1974). By etching the opaque zone with EDTA, we hoped the hyaline zone would become more visible.

Rhodamine-B Method

Rhodamine-b is a fluorochrome dye which is highly visible under ultraviolet light by giving off an orange glow. The reactivity of rhodamine-b with various proteins has been documented (Metcalf and Patton 1944). Rhodamine-b chemically bonds with fibrous proteins such as collagen. Otolin, the protein present in otoliths, has a similar chemical structure to that of collagen. It was this similarity that encouraged us to experiment with rhodamine-b. No previous use of rhodamine-b as a staining technique for age determination was encountered in our search of the literature.

Ninhydrin Method

The use of ninhydrin has been effective in staining purple the annuli of ice fish, Notothenia rossii marmorata, with good agreement between ages of stained otoliths and other structures such as scales and bones (Schneppenheim and Freytag 1980). The

process has been described as the reaction of a neutral solution of amino acid with ninhydrin (triketohydrindene hydrate) by heat to cause oxidative decarboxylation. The central carbonyl of the triketone is reduced to an alcohol. The alcohol further reacts with the ammonia formed from the amino acid and causes a reddish-purplish color. Since the reaction is a quantitative measurement of the optical density, the color produced is an indication of the amino acids present and their concentration. Amino acids such as hydroxyproline and proline develop a yellow color in the same type of reaction (Considine 1976). Identification of different amino acids such as alanine, serine, lysine, and many others have been documented in the otoliths of several species (Degens et al. 1969). The reaction of the amino acids with ninhydrin has been commonly used by chemists working for the Utilization Research Division of the Northwest Fisheries Science Center. The use of ninhydrin offered us a possible new technique for identifying the annuli in rex sole otoliths.

Alizarine Red S Method

Alizarine red S has been used to stain the vertebral centra of longnose skates (Raja rhina) with variable success (Cailliet et al. 1983). The vertebrae of the sand tiger shark (Odontaspis taurus) were stained with alizarine red S with good results (LaMarca 1966). Williams (1941) showed that alizarine red S had an affinity for calcium and used it to reveal annuli. Larval fish clearly display the alizarine red S stain in their vertebrae, otoliths, and other bony structures. It currently is

a common stain used at the Larval Fish Laboratory of the Alaska Fisheries Science Center (AFSC), so its use on rex sole otoliths seemed reasonable.

Muffle Furnace Method

The manner in which otoliths react to heat has been used in ageing many species of fish (break-and-burn method) (Christensen 1964, Williams and Bedford 1974, Beamish and Chilton 1982). Also, the use of convection ovens, has shown good results in otolith studies (Pentilla and Dery 1988). A muffle furnace is capable of temperatures in excess of 270°C, and fluctuates less then 1°C after opening the door to put material inside. In contrast, the standard convection oven reaches temperatures under 270°C and fluctuates more than 1°C each time the door is opened. The muffle furnace was the instrument of choice to bake the sample otoliths because a heat source that maintained uniform temperature was needed. Annuli visibility was enhanced when the otoliths were baked. The baking process turned the hyaline zones brown in contrast to the white opaque zones. Burned otoliths may fade with time, but baked otoliths remain unchanged even after storage for several years (Penttila and Dery 1988).

A group of 15 whole otoliths and 30 cut otolith halves were baked. The whole otoliths were mounted in epoxy resin, and later thin sectioned at 0.5 mm and viewed under transmitted light. The cut otoliths were examined using reflected light immediately after baking.

All otoliths were stored in glass vials filled with a glycerol-thymol solution. Otoliths were cleaned in an ethanol-alcohol solution to remove any traces of storage medium and were dehydrated for 1 day in preparation for baking in the muffle furnace. The otoliths were placed in the furnace at variable time and temperature regimes of 5 to 9 minutes until the proper caramel color was reached. Adequate baking time was assured by first baking "test" otoliths and then baking "study" otoliths. A properly baked otolith was one in which the protein diffused throughout the otolith reacted to the heat.

Polariscopy Method

Polariscopy is the use of polarized light in histology (Lillie and Fullmer 1976). Two pieces of equipment were used to produce polarized light: a polarizer and an analyzer. The polarizer was placed over the built-in light source of the Wild stereomicroscope. The analyzer was placed over the microscope ocular. Baked thin sections of 0.25 mm and 0.5 mm were examined under polarized light. The polarizing unit uses transmitted light to operate. The transmitted light was filtered through the polarizing unit which produced a much better resolution than the unfiltered transmitted light.

RESULTS AND DISCUSSION

EDTA

The EDTA solution reacted with the annuli of the rex sole otoliths as expected. The soaking times in the EDTA solution ranged from 2.6 hours to 4.8 hours depending on the otolith involved. The annuli enhanced by the EDTA were easier to see under the microscope. The surfaces of the treated otoliths were not as readable as hoped, but the procedure was an improvement over reading untreated otoliths. After soaking the cut otolith halves in an EDTA solution, they were burned in the flame of an alcohol burner. Readings of the break-and-burn otoliths of the EDTA-treated samples resulted in a 33.6% agreement between two readers. This was higher than the percent agreement of the untreated or control otoliths of the same sample, which yielded a 24.2% agreement. The sample size tested was 102 otoliths.

The procedure demanded that the EDTA solution be mixed and transferred to individual vials for soaking each specimen which was a time-consuming task. The mixing of EDTA in solution from its crystallized form was not a complex procedure. Total time to complete the treatment of all otoliths was 1 day maximum, excluding examination of the otoliths under the microscope. The actual reading time of the samples was not estimated. No problems were encountered with the use of the EDTA technique. The EDTA technique was considered an improvement over the traditional break-and-burn method but not the best method tested.

Rhodamine B

The rhodamine-B stain was not selective toward the annuli as originally hoped. Instead, the stain indiscriminately attached to the entire otolith surface. Unfortunately, the staining procedure did not make the identification of annuli any easier than the traditional ageing methods. The dye appeared orange under ultraviolet light conditions and was almost painfully bright in some instances.

The nucleus had the greatest affinity for rhodamine-B. When viewed through the binocular microscope under ultraviolet light the nucleus appeared as a bright spot. After viewing the nucleus, it became difficult to focus one's eyes on the annular zones. The distinction between the hyaline and opaque zone was so difficult to see that no further testing was conducted.

Preparation of the rhodamine-B sample was simple. A total of three steps were needed to complete the stain procedure. Setting up the ultraviolet light source was more difficult, but it required only a few minutes for adjustments.

The staining procedure required about 1 day and 2 hours for the otoliths to be prepared for reading. Examination of the stained otoliths under ultraviolet light took about 4 hours. In total, 2 days of work was needed.

When the procedure was first tested, after a few hours no stain was visible across the entire otolith. Increasing the soaking time allowed for the entire surface to be stained uniformly. After observing the stained otolith under ultraviolet

light, we realized that the annular marks were not distinguishable. Percent agreement was not tested because the technique was considered inadequate for age determination work. Rhodamine-B is not recommended for future age determination work because of its unsatisfactory results.

Ninhydrin

We experimented with concentrations of 1% and less in solution and temperatures between 148°C and 301°C to determine the best way to use the ninhydrin stain. The dye stained the annuli purple because of the reaction with the amino acids present in the protein matrix and the temperatures encountered in the furnace. Our procedure called for baking the otoliths at 110°C. The annuli appeared very visible and were more noticeably stained than the same specimens (opposite otolith) examined with the break-and-burn technique. The percent agreement between reader and tester on the 15 samples that were treated with ninhydrin was 80%. This was a significantly higher percent agreement than had been achieved using any of the other techniques. A second test was conducted to determine the repeatability of the ages and to further evaluate the technique. The second test yielded a percent agreement of 56%, with 9 otoliths being tested. During our study, it was discovered that the ninhydrin would not permanently stain the otoliths. When the otoliths were soaked in a glycerol-thymol solution, the normal storage medium, they eventually cleared with little trace of the ninhydrin reaction remaining. We then tried to store the

otoliths dry, and placed a small amount of cedar oil on the cut portion of the otolith for examination under the microscope. The ninhydrin was cleared by the cedar oil. Cedar oil is necessary in the examination process because it provides a "window" to examine the rough surface of a cut otolith by concentrating the amount of light reflected. We tried clear nail polish as a method to seal in the ninhydrin and to also provide the needed "window" for viewing the otoliths. Unfortunately, the nail polish reacted with the ninhydrin and would not cure. All of the otoliths covered with nail polish eventually cleared, just as they had with cedar oil and glycerol-thymol.

The preparation of the solutions for the ninhydrin method included the distribution among different vials used for soaking. The soaking time for the cut otolith halves was 3 days. The time spent baking the otoliths and establishing a temperature regime added 4 hours. The total time for this procedure was 3 days and 4 hours, excluding the time for the actual age determination work.

The ninhydrin technique is not recommended for any further age determination work because of the unsatisfactory results it produced.

Alizarine Red S

The cut otolith halves appeared to absorb the alizarine red S stain very well if left to soak for a 3-week period. The final staining procedure was altered from the technique described by LaMarca (1966) by eliminating the hydrogen peroxide. We found

that the hydrogen peroxide tended to clear the stained otolith rather than preserve it. The addition of another step, which required the otoliths to soak for 1 week in 30% glycerol 70% sodium hydroxide (at 1% solution), was performed. This new procedure enhanced the visibility of the annuli. The stain did not actually penetrate the entire otolith; it penetrated the outer layers of calcium carbonate, thereby making the annuli appear dark. The stained otoliths were easier to interpret because of enhancement to the annuli.

On the first test, the percent agreement was reasonably high at 53.3% when the otoliths were examined immediately following the staining procedure. However, upon re-aging the alizarine red S stained otoliths 1 month later there was only a 27% agreement in the results. The range of 27-53.3% agreement between readers was an improvement over the break-and-burn technique and appeared to be a promising method in future age determination work. The biggest problem with the alizarine red S stain method is the time required to complete the technique. This method is not as time efficient as the break-and-burn technique and could not be used in production ageing situations. The thin sections did not appear to absorb the stain in most specimens. When the sections did absorb some of the stain, it was difficult to distinguish any recognizable pattern in the annuli. No percent agreement test was conducted on the stained thin sections because they were not effective in revealing annuli.

The method required a moderate amount of mixing of solutions

and soaking of otolith halves in glass vials. The mixing of solutions was not in itself complicated, but transferring otoliths and solutions to the various vials was time consuming. There was a total of three different soaking stages.

A total of 11 days were required to complete the staining procedure on the cut otolith halves, while 17 days were required to complete the procedure for the thin-sectioned otoliths. The cut otolith halves were soaked for a longer period of time because of the greater amount of material to be stained. The thin-sectioned otoliths were soaked for only 3 days.

The thin-sectioned otoliths were mounted in epoxy which required 2 weeks to dry or "cure". After the otoliths were thin-sectioned, they were soaked in the alizarine red S stain. The actual sectioning of the mounted otoliths required about 2 hours. The alizarine red S method is a lengthy procedure because of the time needed to stain the otoliths. There were virtually no problems with the alizarine red S procedure after it was refined.

Although alizarine red S was not very time efficient, it might still be used in future age determination work with dorsal fin rays, opercular bones and other structures that are sensitive to baking. Alizarine red S was an improvement over the traditional break-and-burn technique and therefore warrants more testing.

Muffle Furnace

Whole otoliths and cut otolith halves were baked in a muffle furnace for 5 to 9 minutes at 270°C. The whole otoliths were then mounted in epoxy resin. After hardening, the otoliths were

thin-sectioned and observed under polarized light. Initially, the thin sections were cut at 0.5 mm in width but were later changed to 0.25 mm for easier readability. The annuli were hard to distinguish even under polarized light (transmitted light with a polarizer and analyzer). When the light could penetrate the otolith, such as in the 0.25 mm thin sections, it was difficult if not impossible to distinguish the boundary of any one annulus. This made age determination very difficult. An agreement level of 6% between reader and tester reflected the degree of difficulty. Under transmitted light the hyaline zone appeared light and the opaque zone appeared dark.

The same thin sections examined under polarized light were examined under reflected light. We found that reflected light best defined the annuli of the thin sections at 0.5 mm and at 0.25 mm. It was much easier to identify the annuli with reflected light as compared to using transmitted, polarized light. In fact, the annuli of the baked thin sections seemed much easier to identify then the annuli of the traditional break-and-burn otoliths. The heat conditions of the muffle furnace are consistent and encircle the otolith, causing protein in the annuli to react with the heat. By contrast, the heat conditions produced by an alcohol burner fluctuate and expose only a portion of the otolith's annuli to the heat. The thin sections were soaked in glycerol-thymol solution for 1 week before they were examined. The percent agreement was 42.9% for a sample of 14 otoliths examined. This was an improvement over the polarized

light technique.

A second group of otoliths were examined. The cut otolith halves that were baked and viewed under reflected light showed the best results of all the methods. A 66.7% agreement level was achieved on a sample of 30 specimens. The cut halves were much easier to work with than the thin sections and required minimal preparation. Interpretation of thin sections was difficult when determining the difference between an annulus or a check ring. In contrast, the cut halves made the identification of an annulus or a check ring much easier, as demonstrated by the high percent agreement between the two readers.

The thin section examined under polarized and reflected light was a simple procedure to implement. It was easy to attach the polarizer and analyzer to the Wild microscope, and it required no previous knowledge to operate. A total of four steps were used: baking, mounting in resin, sectioning, and examining the otoliths under polarized or reflected light.

Examining the cut halves under reflected light was a simple procedure. Glass vials were etched with sample numbers and lids to the vials were removed to bake the otoliths in the muffle furnace. The otoliths were cut before being placed in glass vials. After baking the otoliths for 5 to 9 minutes the samples were ready to be read. The time spent baking the otoliths was minimal, which makes this method excellent for production ageing work.

Preparing a thin section and completing the muffle furnace

procedure took a total of 3 weeks. In contrast, implementing the muffle furnace method on the previously cut otolith halves required only 1 day of work, not including the actual age determination work. No problems were encountered with the muffle furnace technique.

The muffle furnace method was the only method that satisfied all the criteria required by a production ageing method: good percent agreement, minimal time spent in preparation, simplicity, and minimal problems.

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Table 1.--Comparison of stain and laboratory techniques

TEST	% AGREE	SAMPLE SIZE	STRUCTURE	TIME SPENT
control (break & burn)	24.2	102	cut otolith	1 day
EDTA (break & burn)	33.6	102	cut otolith	1 day
rhodamine-B	*	15	cut otolith	1 day 2 hours
ninhydrin (1)	80.0 ♦	15	cut otolith	3 days 3 hours
ninhydrin (2)	56.0 ♦	9	cut otolith	3 days 3 hours
muffle furnace	42.9	14	thin section	21 days 2 hours
muffle furnace	66.7	39	cut otolith	1 day
muffle furnace polarized light	6.0	15	thin section	21 days 2 hours
alizarine red S (1)	53.3	15	cut otolith	11 days
alizarine red S (2)	27.0	15	cut otolith	11 days
alizarine red S	*	15	thin section	17 days

* Technique was not successful.
♦ Results not reproducible.