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Otolith Ageing of Age-0 Splittail: Techniques, Validations, and Limitations

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Introduction

Otoliths are an important tool for understanding fish life history and population dynamics. One of the many useful "markers" that can be obtained from otoliths is that growth increments are deposited daily by most young fish, allowing the determination of age and growth rates. As part of a study on the early life history of splittail, we are using otolith microstructural analysis to determine the growth rates of age-0 fish collected from different habitats throughout their entire distribution. Together with data on diet and environmental conditions, our ultimate goal is to test hypotheses that will improve our understanding of factors controlling the recruitment of young splittail.

Although the formation of daily increments in otoliths is widespread among young fishes, validation is still necessary for unstudied species. Further, the time of first increment formation can vary substantially among species, potentially adding significant error to age estimations. We have previously reported a preliminary validation of daily growth increments in the otoliths of age-0 splittail using marked fish (Feyrer and others 2001, 14:3:15). However, at that time we still did not know when the first increment was formed. We have since been able to examine the otoliths of

known-age fish to further validate daily growth increments, as well as demonstrate that the first increment forms at hatch. The primary purpose of this paper is to document these recent findings. We also want to share our techniques for preparing young splittail otoliths for examination, as well as the problems we encountered while developing these techniques. In addition to expanding our knowledge of an important native species, we hope that this account will help other researchers who are considering otolith studies on young cyprinids.

Otolith Function and Structure

Teleost fishes have three pairs of otoliths that function in balance and hearing. The three pairs are the lapillus (lapilli, pl.), sagitta (sagittae, pl), and asteriscus (asterisci, pl), and they differ in location, function, and morphology. Otoliths are in the vestibular apparatus (inner ear structure) of fishes. The inner ear structure in most bony fishes is basically made up of an upper section (pars superior) and a lower section (pars inferior). Typically, the pars superior regulates balance and equilibrium and contains the lapilli, while the pars inferior contributes to hearing and contains the sagittae and asterisci. Sagittae are usually the largest otoliths and are commonly used for aging studies. However, the otolith structure of ostariophysans (minnows, catfishes, and characins) differs substantially from that of other teleosts because these fishes have a special structure called the Weberian apparatus. In the simplest sense, the Weberian apparatus connects the inner ear structure of a fish to the air bladder, and is known to assist with high frequency hearing. For splittail, the result is a unique otolith morphology in relation to non-ostariophysans (Figure 1).

Lapilli are the largest otoliths in splittail and were considered the most suitable for our ageing studies. In the young splittail we have examined, the lapilli are typically shaped like small round stones that take on a somewhat heart-like shape in older fish. Sagittae, typically the largest otoliths in most bony fishes, are much reduced in size in splittail. Early sagittae are nearly round. Two projections, a rostrum and prorostrum, form in older fish and extend outward from an inner kernel. In the young fish we have examined, these projections are extremely delicate and fragile; it is nearly impossible not to break them. Asterisci are shaped like irregular little stone pancakes. They are flat on one side with a convex pattern of irregular growth on the other side. We have found that sagittae and asterisci are unsuitable for our ageing studies of age-0 splittail because of their delicate nature and apparent irregular growth patterns.



Figure 1 Otoliths of juvenile splittail.

Otolith Extraction and Mounting

Extraction and mounting of lapilli otoliths from juvenile splittail is quite easy and simple, with a little practice. We have obtained the best results for extraction with a modified open-the-hatch technique (Figure 2). With a sharp scalpel, we cut through the fish laterally, just above the eye extending to beyond the operculum. This cut piece can then be removed to expose a dorsal view of the brain. With the brain tissue removed, lapilli are located in vestibules on either side of the brain cavity, just where it bottlenecks. The otoliths can be easily removed from the vestibules with fine forceps. We move the otoliths directly into a drop of 10% sodium hypochlorate solution (bleach) to clean off any attached tissues. The otoliths are then transferred from the bleach into a drop of water to rinse. Prior to placing the otoliths on a dry section of the dissecting tray for air drying, we rinse them in ethanol because it is highly volatile and evaporates very quickly, minimizing the drying time. We mount the otoliths whole in CrystalBond wax mounting media on glass microscope slides (one otolith per slide). We place a small piece of the mounting media on a slide and heat it on a hot plate until it is soft and easily manipulated. It is important not to overheat the mounting media because it will bubble and eventually burn. It is also important to use the minimum amount of mounting media necessary to cover the otolith. This will prevent spending an excessive amount of time later during the polishing stage trying to grind through excessive overburden to reach the otolith. While the mounting media is still workable and gummy, the otolith is carefully placed into it so that it is positioned flat (exposing a sagittal plane) on the slide and is completely covered. Which side of the otolith is placed is down (left or right) does not seem to matter for our studies. Small dry otoliths have an amazing ten-

dency to enter flight when held with fine-tip forceps. Therefore to keep from losing otoliths, we actually transfer them from the dissecting tray to the slide by pressing down on the otolith with a dry finger, which apparently wedges the otolith within the ridges of your fingerprint, and carefully scraping them off the finger onto the mounting media with forceps or a probe. We have not lost a single otolith using this technique. The slide is then put aside to cool before preparation for reading.



Figure 2 Basic methods for quick removal of juvenile splittail lapilli otoliths.

Extraction and mounting of otoliths from larval fish is somewhat more complicated. For extraction, what we have found to work best is to cut off the head of the larvae and then immerse it in bleach. Medicine droppers or similar devices work well for applying just enough bleach to completely immerse the head. After several minutes, the bleach

will dissolve all of the surrounding tissues and only the otoliths will be left in the solution. Because all otolith pairs will be present in the solution, it is important to know how to differentiate the lapilli. We then use fine forceps to push the otoliths out of the drop of bleach into a drop of water; the otoliths are too small to actually pick up or grasp even with fine-tip forceps. We then push the otoliths from the water into a drop of ethanol, and then ultimately onto a dry section of the dissecting dish so that they can air dry. Mounting larval otoliths is generally similar to the larger otoliths with the exception of transfer to the slide. To transfer otoliths onto the slide, we use fine forceps tipped with a small amount of the heated mounting media. The otolith is immersed in the mounting media on the forceps, and once the mounting media has cooled and slightly firmed, the otolith with the mounting media can be transferred to the slide. The slide is then slightly heated to adhere the mounting media to the slide and also so that the position of the otolith can be manipulated if needed.

Otolith Preparation and Reading

The increment structures of age-0 splittail otoliths are relatively easy to interpret. The increments of wild fish exhibit excellent contrast without any grinding or polishing. If it were not for the many cracks that obfuscate viewing planes, the otoliths would require no further preparation prior to reading; we have yet to discover lapilli otoliths from our ethanol-preserved specimens without cracks. For otoliths that require some preparation, we have found that very light polishing with 0.3 μm lapping film works well. It is important that the lapping film be adhered to a completely flat surface to ensure even polishing. We have also had success grinding the otoliths with 1200 grit wet sandpaper followed by polishing on a microcloth with 0.3 μm alumina. This method produces a very nice clean surface, but is messier and less convenient than the quick and dry lapping film technique. There is definitely a learning curve when it comes to polishing otoliths. It is very easy to over-polish the otoliths of age-0 splittail, resulting in an unreadable structure. Although it is fairly common practice with larger otoliths, polishing to the core of the otolith is not always necessary with age-0 splittail otoliths. As mentioned, the degree of polishing is largely determined by the number of cracks in the otoliths; otolith clarity and polishing intensity are inversely related. In some instances simply polishing down any mounting media overburden is all that is necessary. In addition, even small amounts of polishing seem to sometimes reduce the contrast between the increments, presumably because the increments are then viewed through

less material. We have also flipped the mounted otoliths and polished both sides, but have found that there is little, if any, improvement in readability.

Validation of Daily Increments and Time of First Increment Formation Using Known-age Fish

Known-age study fish originated from adult splittail maintained by the US Bureau of Reclamation's (USBR) Tracy Fish Collection Facility. The adults from which the study fish were produced were collected at the facility in 1998 as juveniles. They were maintained at ambient Delta water temperature and a natural photoperiod while they were grown-out to adulthood. Adult female splittail were injected with Ovaprim in April 2003 and ovulated eggs were recovered 24 to 48 hours post injection. The eggs were mixed with sperm from three males in 1.5-gallon plastic containers and mixed with bentonite to reduce adhesiveness. Eggs were rinsed after 20 minutes, placed in Pond RidiCh (1%) for 5 minutes, and then transferred to an upwelling egg incubator using filtered Delta water. Eggs were maintained at 19 °C with a natural photoperiod (lights on timers). The eggs hatched after 5 days of incubation on 30 April and were transferred to 24-inch diameter black plastic tanks at 20 °C with a natural photoperiod. Larvae were fed rotifers and *Artemia nauplii* starting at 5 and 8 days post-hatch, respectively. Yolk sacs were completely depleted by 6 days post-hatch.

We have examined a number of these known-age fish and have found that the number of otolith increments matches age, thereby validating both daily increment deposition and that first increment forms on the day of hatch. One such example for a 15-day-old fish is given in Figure 3. However, a common problem that we encountered with these otoliths was that increment contrast was extremely poor, which made microstructural analysis very difficult. In fact, in most circumstances, we found that daily increments were very difficult to distinguish while subdaily increments were quite prevalent. Very minor focus manipulations were all that was needed to display daily versus subdaily increments (Figure 3). It should be noted that we are not completely certain if the subdaily increments, such as those shown in Figure 3, are indeed subdaily increments or simply visual artifacts. Answering such a question would require highly sophisticated techniques, such as scanning electron microscopy, to view the surface of the otolith in three dimensions. Understanding the morphological differences between daily and subdaily increments and knowing the age of the study fish was critical to our ability to examine these

particular otoliths. We believe this contrast problem stems from the fact that the fish were reared at constant temperatures. Several studies have shown that fish reared under a natural fluctuating temperature cycle have otoliths that exhibit better contrast than fish reared under constant temperature.

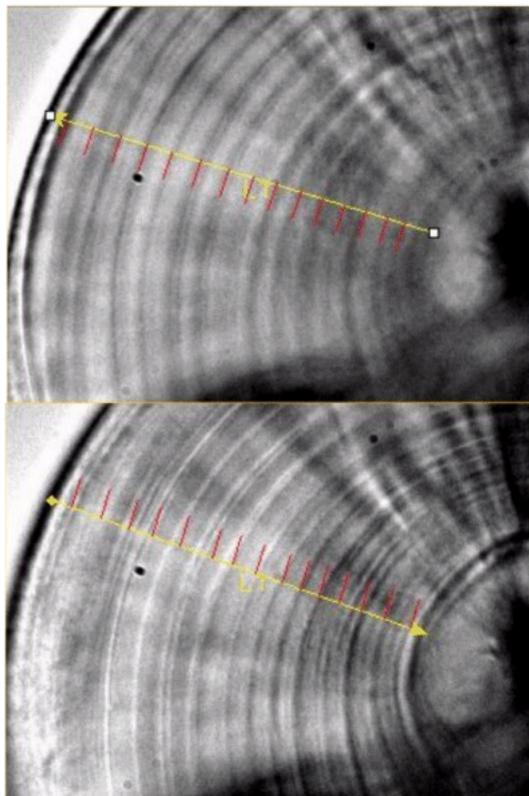


Figure 3 Photomicrographs of a lapillus otolith from a 15-day old splittail. Top panel shows daily increments. Bottom panel shows subdaily increments that appear between the daily increments when the focus is manipulated. Subdaily increments are most prominent near the core.

Conclusions

We conclude that otolith microstructure analysis is a viable method for age and growth studies of age-0 splittail. Using both marked wild fish and known-age fish, we have been able to validate daily growth increments and time of first increment formation. This information will enable us and other researchers to test hypotheses about mechanisms contributing to survival and recruitment of young splittail. Further study will determine the approximate maximum age at which daily aging becomes increasingly difficult or no longer possible.