ICES J. mar. Sci., 50: 213–217. 1993

A chemical way of thinning otoliths of adult Atlantic herring (*Clupea harengus*) to expose the microstructure in the nucleus region

Z. Zhang, and E. Moksness

Zhang, Z., and Moksness, E. 1993. A chemical way of thinning otoliths of adult Atlantic herring (*Clupea harengus*) to expose the microstructure in the nucleus region. – ICES J. mar. Sci., 50: 213–217.

The thickness of adult herring (*Clupea harengus*) otoliths was reduced chemically through selective decalcification in contrast to physical grinding. Microstructure around the nucleus was clearly visible under a light microscope after the preparation. The process of this technique included three steps: first, the two ridges along either side of the sulcus were roughly ground; second, the otolith exterior side was brought down to the midplane by decalcification; finally, the decalcification of the exterior side was prevented while the interior side was further decalcified to produce a thin section, which was then mounted on a glass slide and studied.

Key words: herring, otolith, microstructure, decalcification.

Received 16 July 1992; accepted 28 October 1992.

Z. Zhang, and E. Moksness: Institute of Marine Research, Flødevigen Marine Research Station, N-4817 His, Norway. Z. Zhang present address: Pacific Biological Station, Nanaimo, British Columbia, Canada V9R 5K6. E. Moksness: to whom correspondence should be addressed.

Introduction

Both spring- and autumn-spawned herring (*Clupea harengus* L.) inhabit the northern Atlantic. Distinguishing one from the other is important for studying the dynamic structure of herring stocks and for establishing a proper management plan, as in the North Sea area (Anon., 1991). Recently, Moksness and Fossum (1991) suggested a new method to distinguish these two herring stocks by using otolith microstructure around the nucleus region.

The method described by Moksness and Fossum (1991) has only been applied to larval herring, the otoliths of which are sufficiently thin that the microstructure can be studied with light microscopy without grinding. To study the microstructure of adult herring otoliths, otolith preparation is essential. The conventional method, to grind or cut and then grind the otolith until the microstructure is visible, is time-consuming and tedious and may not consistently produce clear increments. Zhang *et al.* (1991) used a solution of glutaraldehyde and EDTA to fix and decalcify otoliths simultaneously. Completely decalcified otoliths were sectioned on a microtome and then stained with toluidine blue. Daily increments were clear in sections of *Oreochromis niloticus* otoliths as well as young herring otoliths. However, using this technique, no clear pattern of daily increments was produced in sections of adult herring otoliths, although annual checks (checks formed during the season of slow growth) were well retained (Zhang and Moksness, 1992).

In the present study we used selective decalcification, not to decalcify the otolith completely, but to remove the calcareous material from both the exterior and interior sides until it became thin enough for examination of the microstructure in the nucleus region.

Materials and methods

Otoliths (sagittae) from adult herring (21–36 cm in total length), caught in the northern North Sea (61°27′N, 0°15′E) and in the Skagerrak off Arendal at the southern coast of Norway, were used in the present study. The otoliths were removed from either fresh or frozen herring and attached tissues were removed in distilled water or 0.14% sodium hypochlorite before being prepared.

Step 1: removal of the ridges parallel to the sulcus

The sulcus (groove) on the interior side of an adult herring otolith extends from the *excisura major* nearly to the posterior end, and ridges occur on either side of the sulcus. The frontal part of the sulcus (from the *excisura major* to

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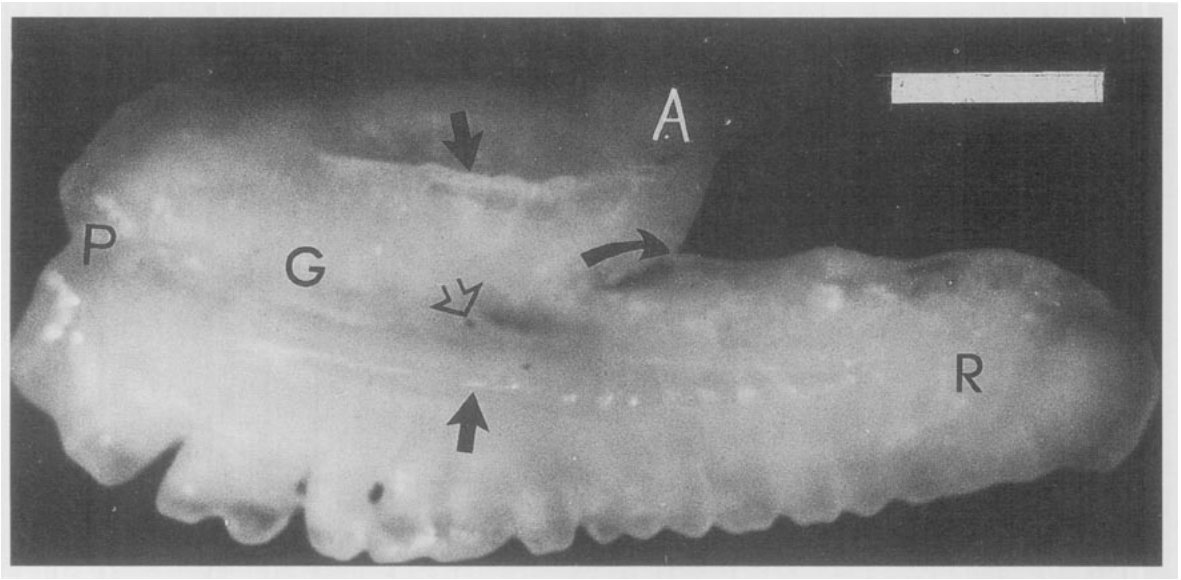

Figure 1. The interior surface of an adult herring otolith, showing the gross structure. A groove (G) between two ridges (straight arrows) extends from the *excisura major* (curved arrow) nearly to the posterior end (P). Rostrum (R) and antirostrum (A) are well developed. Note the short distance between the *excisura major* and the nucleus (open arrow). Scale bar = 1000 μm.

the nucleus) is very deep (Fig. 1). The two ridges were roughly ground down on a piece of lapping film (grade 30 μm) using gentle finger pressure.

Step 2: decalcification of the exterior side down to the midplane

Prior to decalcification otoliths were individually embedded in 2% agar in a polythene bottle top with a diameter of about 16 mm and a depth of 1.5 mm. Agar-embedded otoliths are easier to handle when they become smaller. In addition, the decalcification rate of the peripheral regions of the otolith is reduced, providing more time for decalcification of the exterior and interior sides. The procedure of embedding was as follows. Each otolith (from herring of less than 32 cm in total length) was put into a bottle top with the exterior surface down. Warm liquid agar was dripped into the top until it was filled. To reduce the amount of decalcification in the frontal part of the sulcus, which is very thin due to the presence of the deep groove there, it was covered with a small piece of waste otolith, making sure that the nucleus was still exposed. After the agar had set, that part of it above the height of the bottle top was sliced off and the block remaining was removed from the top.

All decalcifications in this study were carried out in a 40-ml beaker with a 1.5 cm high, close-fitting, polyethylene ring at the bottom supporting a close-fitting metal mesh. Resting on the mesh, the agar-embedded otoliths were completely immersed in the EDTA solution, which was stirred by a magnetic stirrer at the bottom of the beaker. It took 1 to 4 h to bring the exterior side down to the midplane in 6% EDTA after decalcification in 0.8% EDTA overnight, depending upon the size of the otolith and the position of the nucleus in it. Four to eight agar-embedded otoliths were placed overnight (about 17 h) in 0.8% EDTA, adjusted to a pH level of about 8.10 using NaOH. The next day they were transferred to 6% EDTA, also adjusted to a pH level of about 8.10, for further decalcification. (The alkaline solution minimizes the production of bubbles of CO_2.) EDTA solution penetrated the agar and decalcified all sides of the otolith, but the decalcification rate varied depending upon the thickness of agar over each side. Due to the above way of mounting, the exterior surface was overlaid by a thin layer of agar and the interior surface by a thick one. Thus, the decalcification rate was faster from the exterior side than from the interior side. In addition, the distance between the nucleus and the exterior surface is always shorter than that between the nucleus and the interior surface in adult herring otoliths. The midplane was therefore always reached from the exterior side. It took 1–4 h to process the exterior side down to the midplane in 6% EDTA after decalcification in 0.8% EDTA overnight, depending upon the size of the otolith and the position of the nucleus in it. Preliminary studies showed that the position of the otolith nucleus in relation to the interior and exterior surfaces varied even among otoliths of the same size. It is therefore impossible to set a narrower range of time required to decalcify the exterior side to the midplane.

In larger otoliths (from herring of more than 32 cm in total length) too much excess material on the interior side prevented the nucleus region from being clearly observed

when the midplane was reached from the exterior side. A variation of the process was therefore necessary to remove more material from the interior side. Each large otolith was mounted in a bottle top in 2% agar with the interior surface down and a fraction of an otolith covering the *excisura major*. They were then kept overnight in 1.5% EDTA, where heavier decalcification took place from the interior side than the exterior side. Next morning the otoliths were removed from the agar blocks and cleaned in distilled water and 0.14% sodium hypochlorite. The midplane had not yet been reached from either side. They were re-mounted in the bottle tops in 2% agar, exterior side down, with a fraction of an otolith covering the frontal part of the sulcus, before being decalcified in 6% EDTA. The time needed to expose the midplane in 6% EDTA varied from 2 to 6 h.

As agar blocks appear translucent in EDTA solution or water, otoliths in them can be clearly seen. Observations of all processed otoliths were made under a low-power light microscope to monitor the progress of the decalcification. Once the hatching check was visible, the otolith was removed from the agar block, cleaned in distilled water and then immersed in 0.14% sodium hypochlorite for about 5 min to remove any attached organic residues derived from the decalcified part of the otolith.

Step 3: further removal of excess material on the interior side

The otolith was dried and mounted on a small piece of a broken slide-cover slip with a small drop of nail varnish as an adhesive. The central part of the exterior surface (midplane) was put on top of the drop, the amount of which was so small that the varnish would not overflow on to the top of the otolith, i.e. on to the interior surface. The mounted otolith was then embedded in 2% agar with the interior surface downwards. After the agar had set, a block containing the otolith was cut and either decalcified in 0.8% EDTA overnight and then transferred to 6% EDTA (if there was still considerable amount of excess material left on the interior side) or simply decalcified in 6% EDTA if this material could be removed within normal working hours. The presence of the varnish prevented any further decalcification in the central part of the exterior surface. The decalcification was terminated when the otolith became a thin section. The cover slip, together with the otolith section, was removed from the block, washed in distilled water to remove any EDTA and transferred to 0.14% sodium hypochlorite to remove attached protein residues. The otolith section, together with the attached varnish, was gently removed from the cover slip and transferred to a glass slide. Acetone was added to dissolve the varnish, before fresh varnish was used to mount the otolith.

Summary of the main procedure of the otolith preparation

1. Grind down the two ridges on either side of the sulcus on a piece of lapping film using gentle finger pressure. If the otolith is from an adult herring less than 32 cm in total length, then:
2. (i) embed the otolith, the exterior side down, in 2% agar in a bottle top;
   (ii) decalcify the otolith in 0.8% EDTA overnight and then in 6% EDTA until the midplane is reached, i.e. the hatching check is visible.
   If the otolith is from an adult herring more than 32 cm in total length, then:
   (a) embed the otolith, the interior side down, in 2% agar in a bottle top;
   (b) decalcify the otolith in 1.5% EDTA overnight;
   (c) re-embed the otolith, the exterior side down in 2% agar in a bottle top;
   (d) decalcify the otolith in 6% EDTA until the midplane is reached, i.e. the hatching check is visible;
3. remove the otolith from the agar block and clean it in distilled water and 0.14% sodium hypochlorite;
4. mount the otolith, the midplane down, on a piece of cover slip using nail varnish, making sure that the varnish does not overflow on the other side of the otolith;
5. embed the mounted otolith, the midplane up, in 2% agar;
6. decalcified the agar-embedded otolith in 6% EDTA or in 0.8% EDTA and then in 6% EDTA until it becomes a thin section;
7. remove the otolith section from the agar block and clean it in distilled water and 0.14% sodium hypochlorite;
8. mount the otolith section on a glass slide using fresh varnish.

Results and discussion

The otolith became thinner and smaller as decalcification progressed. Meanwhile, the increments became clearer from the peripheral regions towards the nucleus. Thus, the clearance of the otolith increments under a light microscope indicated the progress of the decalcification procedure. When the midplane was reached the hatching check and surrounding increments became visible (Fig. 2).

When a sufficient amount of calcareous material had been removed from the exterior and interior sides, the otolith became a thin section around the nucleus. Although the decalcified otolith surface was very smooth, the microstructure could not be clearly observed as long as the otolith section remained embedded in the agar. After the section was mounted in fresh varnish, the increments around the nucleus were clearly visible (Fig. 3), and could be counted and measured.

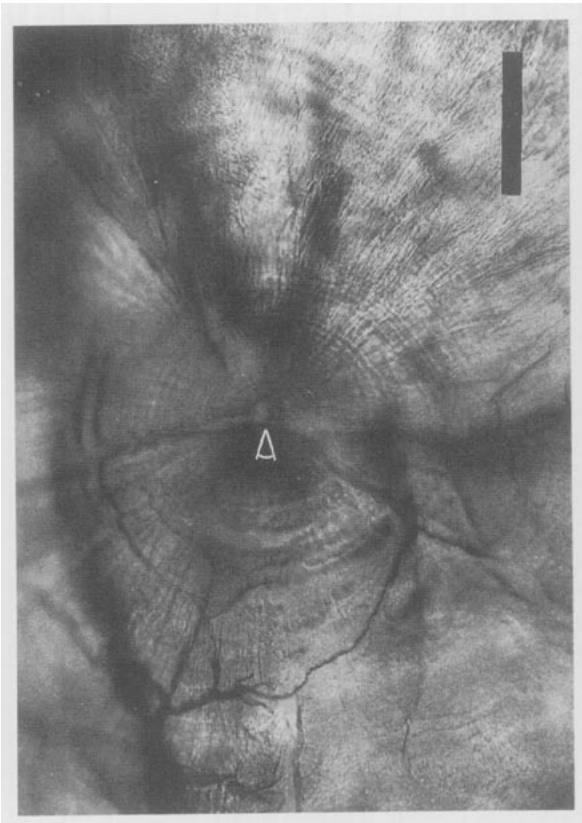

Figure 2. The exterior surface, which has been decalcified down to the midplane, showing the hatching check (arrow) and surrounding increments. Scale bar = 85 μm.

Figure 3. An otolith after decalcification from both sides, showing 56 clear increments in the central region. Scale bar = 45 μm.

The decalcified otoliths from 25 herring caught in the North Sea and 55 herring caught in the Skagerrak were examined. Of these, three otoliths were discarded due to loss of their microstructure. A few otoliths did not exhibit clear increments in the fast- or slow-growth section, probably because the plane of the otolith, as mounted on the slide, did not intersect these increments at right angles. Obviously, increments will be most clear, if they are perpendicular to this plane. As increments are generally not deposited in a symmetrical and uniform fashion, it is often impossible to find a single plane intersecting all increments at the optimum level (Brothers, 1987). The size of the increments are unlikely to be altered by this preparation, since no shrinkage or expansion of the remaining calcified part of the otolith was noticed.

Juvenile herring otoliths can be easily prepared using this technique. These otoliths are comparatively thin and there is little difference between the distance from the nucleus to the exterior and interior surfaces. A few hours of decalcification of juvenile otoliths in 6% EDTA reveal the hatching check and surrounding increments. After being mounted, the otoliths exhibit clear microstructure in the central area.

At the expense of sacrificing the periperal regions of the otolith, this technique allows a group of otoliths to be processed at the same time, while physical grinding normally requires otoliths to be individually processed by hand. About 40 otoliths of adult herring can be processed each day using this technique, while only two such otoliths can be prepared by grinding. As far as discrimination of spring- and autumn-spawned herring is concerned, the peripheral regions have no value. Furthermore, in adult fish from temperate areas the growth of otolith increments becomes so compressed during seasons of slow growth that the increments are difficult to resolve under a light microscope (Campana and Neilson, 1985). Combining our approach with a second grinding stage may also produce a good preparation. After the exterior side of the otolith is decalcified down to the midplane, the otolith can be mounted with the midplane attached to a glass slide and then ground to produce a thin section. Such grinding should be very easy, as there is less danger of over-grinding past the midplane.

Acknowledgements

We thank Inger Henriksen at the Institute of Marine Research, Flødevigen Marine Research Station, for her

help in the laboratory. We are grateful to Prof. J. H. S. Blaxter for his helpful comments and correction of the English text. We also appreciate the valuable comments by two anonymous referees. This project was funded by the Norwegian Fisheries Research Council.

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