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# Report on an

# OTOLITH WORKSHOP

# in

# Bergen, January 18-29, 1982

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# PREFACE

Since the publication of Pannella's paper on daily growth rings in fish otoliths in 1971, there has been a growing interest in the use of these rings for ageing. For many species, this method has been verified and is used routinely. For other species there is evidence that considering the primary growth rings as daily marks is too simplistic, and that growth also is an important regulator of the ring formation. Therefore, it seems necessary to verify the reliability of the method for each new species and probably also for different environments.

To discuss the problems involved in ageing fish by means of daily growth rings and to try to verify the method for some selected species, a workshop was arranged. As the participants were mainly interested in larval and juvenile cod and herring, and in adult sardinella, the work was concentrated on these species.

The following questions were put forward for discussion:

1. Can primary growth rings be used as a practical tool for ageing the species in question?
2. How should the otoliths be prepared and read to give optimal results?
3. What kind of research should be undertaken to improve the methods?

The workshop held in Bergen on January 18-29, 1982, was organized by the Department of Fisheries Biology, University of Bergen, in cooperation with the Institute of Marine Research and with help from the Zoological Laboratory, University of Bergen. The Norwegian Fisheries Research Council gave financial support to one of the participants, and one participant holds a NORAD fellowship.

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Jakob Gjøsæter

## INTRODUCTION

It was discovered, in the early seventies, that the otoliths of some tropical and temperate fishes contained primary growth increments which seemed to be formed daily. (Pannella 1971, 1974). Since then, several research workers have studied the potential use of counting these increments as a routine ageing method. As far as temperate species are concerned, the work has concentrated on larvae and juveniles. The number of species found to have primary otolith increments has steadily increased. Since the list includes species from very different environments and geographical regions, it seems reasonable to assume that an incremental growth is a universal phenomenon in fish otoliths.

Before being accepted for routine ageing, the method should be verified for each new species under consideration, especially if this species seems to differ from earlier studied species in terms of behaviour, habitat, growth etc.
Ring deposition of one ring/day has been established for some species by reading otoliths from fishes of known age. However, it is not known whether the results of these laboratory studies are applicable to field studies. The mechanisms involved in increment formation are as yet unknown. Therefore it is difficult to assess which factors (environmental or internal) influence the deposition of zones. Light, temperature and feeding cycles have been proposed as possible rhytmic stimuli that could function as trigger factors for the zone deposition.
So far, no experiments undertaken to study the influence of these environmental stimuli have given conclusive support for one or the other. The influence of these factors on ring deposition could differ among species and in different environments.

A difficulty with starting to use the method for ageing wild fish, even where a daily deposition of increments has been confirmed on "known-age" material, is the possibility that fluctuations in growth rate could affect the deposition rate, slowing it down or even stopping it.

In addition to these theoretical problems, the participants in this workshop had previously encountered various practical problems in preparing and reading otoliths. Even though the counts of each person seem quite consistent, comparison of different counts of otoliths prior to this workshop has shown that there can be a substantial variation between readers. Thus there appeared to be an obvious need for reaching a point where all counters agree on a certain definition of an increment.

It was therefore decided to organize a workshop, where in addition to an intercalibration of counters, the more basic problems concerning this ageing method and the literature could be discussed.

It was decided to concentrate the practical work on larval material of the two temperate species cod (Gadus morhua) and herring (Clupea harengus), in addition to adults of a tropical Sardinella species (Clupeoid).

SHORT COMMENTS ON PREVIOUS WORK ON OTOLITHS OF COD, HERRING AND SARDINELLA.

Cod.

The amount of work done prior to this workshop is rather preliminary if the motivation is to establish a practical ageing method for larval and juvenile cod. Radtke and Waiwood (1980) presented results of a study on otoliths from lab-reared larvae up to an age of four days using SEM. They found that one zone was formed daily from the day after hatching and onwards. T. Dale (pers. comm.) came to the same conclusion after TEM - studies of laboratory reared yolk sac larvae. He could detect faint zones prior to hatching in some larvae, but a prominent hatch-line was formed within 24 hous after hatching. The distinctness of the zones formed daily after hatching was variable. H. Gjøsæter (1981), using a light microscope, found a good correlation between the number of increments and age up to an age of about 40 days. He concluded that one zone was formed per day from 4-5 days after hatching and onwards.

These reports support the assumption that zone formation is rhythmic during periods of larval life, but the conclusions which can be drawn are limited by several factors e.g. the limited age range examined, the small sample sizes and the unusual rearing conditions. In addition there have been problems with finding a standardized way of reading cod otoliths. O.A. Bergstad (pers. comm.) has undertaken a test where different readers counted the same otoliths. He detected a considerable variation between readers, especially for the older larvae.

Herring.

Lough et al. (1980) report a study of otoliths from herring larvae. They kept the larvae for 18 days in the laboratory, and by then the larvae had deposited only three increments in their otoliths. The first increment formed soon after yolk sac absorbtion. Then there was a delay of about one week before

the second increment appeared, and when the rest of the larvae died after 18 days they had just deposited a third increment.
H. Gjøsæter (1981) found that the increment deposition was daily for herring kept in a large out-door basin starting at an age of 3-4 days when the larvae were released in the basin counting to the end of the experiment at a larval age of 135 days.
A. Geffen (pers. comm.) has studied the otoliths of herring larval groups with different growth rates. Her findings indicated that the ring deposition rate is dependent on the growth rate. Larger larvae had more increments than smaller ones of the same age, this applied both for within rearing groups and between rearing groups. Only the fastest growing larvae, with a mean growth rate of 0.4 mm/day had a ring deposition rate of nearly one ring/day. These results indicate that primary ring counting is probably not applicable for ageing herring larvae in the field.
There have also been some problems with defining the rings in herring in a standard way to get indentical counts for different readers, but the problem is not as severe as in cod.

Sardinella.

H. Gjøsæter (1981) found primary growth increments in the otoliths of adult Sardinella longiceps caught in the Gulf of Aden which were relatively easy to count. Assuming a daily deposition rate, the age corresponded more or less to what was previously known about the growth of this species and the spawning season in the area.
P. Dayaratne (unpubl. mat.), working with adult S. sirm from Sri Lankan waters, has found increments, the number of which corresponds well with the age deduced from length frequency analysis and from the known spawning time of the species.
Even though the otoliths of these species are more easy to read than the temperate ones, there has been a variation between different counters in test runs. There is also a tendency for the otoliths to become more difficult to read when the preparations get older, which could be caused by the preparation technique in use.

## RESULTS OF THE WORKSHOP

### Definitions of terms.

As a result of discussion during the workshop several definitions were formulated as a quide for the interpretation of zones to be counted for the purpose of ageing. On the basis of the work with cod, herring and Sardinella the following counting procedure was designed for light microscope examinations.
One ring (zone, or increment) was defined as the distance from one discountinuity layer to the next. This is visible as the distance from the beginning of one dark band to the beginning of the next dark band. The dark bands were used for counting rather than the light bands.

It was sometimes necessary to alter the focus while counting the rings, but care must be taken in interpreting the "movement" of the rings when the focus is changed and of the optical phenomena that this produces at the edge of the otolith. The fact that a ring was not visibly countinous around the entire otolith was not a criteria for excluding these rings from the counts.

In herring and cod the first ring was usually a very prominent band. This ring was chosen as the first ring for counting purposes. Rings could sometimes be seen inside the prominent ring but since their presence and number was not consistent these rings were not included in the ring counts. They should be excluded until their significance is investigated more closely. If no prominent ring is visible on the otolith, counting should begin at a speciefied distance from the nucleus which is approximately equal to the size of the otolith at hatching (herring: 10.8 µm: cod: 6 µm). In juveniles where it is often difficult to grind the otolith to see the innermost rings, a measured distance could be taken from the nucleus once this distance has been calibrated to equal a constant number of rings.

The edge of the otolith was included in the ring counts if the beginning of the next increment was visible at the edge. Optical phenomena produced at the otolith edge often made the interpretation of the last band difficult.
The last rings or the group of rings closest to the edge of the otoliths of Sardinella and juvenile cod were very narrow and it was important to make the transition between counting wide rings and narrow rings correctly. This could be done by carefully following the gradual reduction in zone width approaching the edge.
The otolith of the oldest herring and cod larvae, and of the juvenile cod and the Sardinella were best counted at the lowest magnification possible. In these otoliths many of the rings that were visible at 1000x could not be included in the ring counts for the purposes of ageing. The interpretation of these rings and the selection of "daily" rings was accomplished more successfully, with better agreement between readers and with age, when done under lower magnification (400x) (see Table 1.). Before going to lower magnification as a standard method a thorough calibration must be complete for these counts on known-age material.

In the herring larvae examined differences in the ring structure of reared and wild larvae were observed. The first prominent ring described for reared cod and herring larvae was not as common in the wild material, although ring deposition started from the same distance from the nucleus. The rings of wild larvae were generally more distinct (clearer) and more evenly spaced than in reared larvae.

Standard Methodology.

A standard method for the collection, preparation and examination of otolith material was designed. This standardization is a necessary part of future larval studies utilizing otoliths. These methods expand on the manual for otolith work produced at La Jolla, California (1977).

TABLE 1.

RESULTS OF INTERCALIBRATION FOR 40 D. OLD COD LARVAE

| | Counts obtained by counter: | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | $\bar{X}$ | SD | C.V. |
| | 50.3 | 68 | 59.6 | 45.7 | - | - | 55.9 | 9.9 | 17.7 |
| Group 1. | 38.0 | 47 | 45.4 | 45.0 | - | - | 43.6 | 4.0 | 9.1 |
| | 54.3 | 65 | 58.6 | 44.7 | - | - | 55.6 | 8.5 | 15.3 |
| | 27 | 18.5 | 37.0 | 29 | - | - | 27.9 | 7.6 | 27.2 |
| | - | 40.7 | 48 | 33 | - | 36.2 | 39.5 | 6.5 | 16.4 |
| Group 2. | - | 38 | 44 | 36.7 | - | 37.8 | 39.1 | 3.3 | 8.4 |
| | - | 39 | 41.5 | 36 | - | - | 38.8 | 2.7 | 6.9 |
| | - | 27 | 36 | 28 | - | - | 30.3 | 4.9 | 16.2 |
| | - | 38.3 | 47.5 | 34.7 | - | 40.2 | 40.2 | 5.4 | 13.4 |

1. Group: Counts were made without giving any instructions to the counters.

2. Group: All counters used 400x magnification and counted the zones which appeared most prominent.

## I. Collecting specimens

Field samples should be preserved in buffered 95% alcohol. Buffered formalin may be acceptable for periods of 1-2 months. Freezing or drying the larvae are also possible preservation methods. Any other preservation methods should be tested first for their effects on otoliths.

A measure of the shrinkage in length, weight or other alterations in the morphology of the larva caused by the preservation should be made.

Relevant information should be recorded for each haul, such as depth, time of day, haul duration, location, water temperature, light levels. It is also valuable to record an estimate of abundance of food organisms if possible.

Lab samples. All calibration work to establish the deposition rate and time of first ring formation must use larvae hatched within one 24 h period, preferably during the peak of hatching. There should be intensive sampling around the stages of hatching, yolk aborption and first feeding. Later samples should be frequent (every 2-7 days) and cover the entire larval period, but this depends both on the hypothesis being tested and the time and resouces available. The number of larvae required for a good sample should be determined by first using at least 10 larvae of each age to check the amount of variation in ring number, for each size group at each age. This will determine how many larvae are needed to give a representative sample in the future. If there is little variation in ring number among size groups, or little variation in size among individuals of the same age, then three larvae at each age could be sufficient for a representative sample the entire size range of larvae. Lab. samples should be frozen or dried (if the dry weight is to be taken). Larvae preserved in this way can be stored on microscope slides untill dissection. Measurements made on lab. samples should be taken from fresh material whenever possible to give an additional measure of shrinkage or alterations due to preservation. A detailed measure of feeding condition is more of a possibilty when working with lab. samples.

II. Measurements for the larvae

The measurements to be made on each larvae should consist of 1) the standard (notochord) length (taking care consistently measure the upper jaw), 2) some measure of larval condition such as dry weight, myotome height, eye height, distance between the eyes, feeding incidence, and 3) the developmental stage. The staging should be done using a standard, published method whenever possible. The staging system used should always be reported. 4) The time of day sampled.

III. Removing the otoliths

Larval otoliths should be removed working under binocular microscope at about 50x magnification with fine needles (insect needles or fine glass needles). The larvae should be soaked in distilled water to make them flexible for easier dissection. All the otoliths should be removed from each larva until it has been determined which pair is the sagittae. Only the sagittae should be used for ageing. Both sagittae should be used as a safeguard in case one of the pair is not clear. It is possible to try mounting one of the pair convex-side up and the other convex-side down on the slide.
A circle should be marked on the underside of the slide and the otoliths placed within it. All markings should be done with a xylene-proof marker. The otolith should be dried for approximately ½ hour before mounting.

The otoliths of juveniles need to be ground in order to see the inner rings. The otoliths should be scraped clear of all the adhering tissue (working under a dissecting scope) either in water or very dilute (0.19%) acid. After drying thoroughly (½ h) the otoliths can be mounted with superglue or mounting medium onto a slide. Grinding should be done using very fine carborundum (wet-dry) paper placed in water in a petri-dish. A dentists drill can also be used for grinding. The grinding process should be done at the dissecting microscope. If the grinding paper is fine enough, no further polishing is necessary to clear the surface.

Commercial metal polish is suitable for polishing if needed. The otoliths can be mounted with medium under coverslips, or viewed under a drop of water or immersion oil.

## IV. Clearing and staining techniques

There are no staining methods in common use for small otoliths. Some techniques were tried at the workshop, but without improvement in ring clarity. These methods could not be investigated adequately in the time available, and it is possible that clearing, staining, or other methods of microscopy could increase the clearness and visibility of the rings.

## V. Mounting the otoliths

Of the different mounting media discussed, Pro-texx, Microkitt, or immersion oil were considered to produce the best results for permanent otolith preparations. Canada-balsam is not recommended since it disrupts the ring structure after a period of one month. This may be due to a reaction between the medium and the otolith itself or because the otoliths were not properly dried before mounting. Canada balsam is acidic and probably does cause some dissolution of the otolith. Neutralized Canada balsam is available, but its use as a permanent mountant has not been tested. In most cases the coverslips should be sealed with nail varnish to prevent evaporation of the medium. Evaporation can cause the coverslip to be sucked down onto the otoliths and crush them, or it can pull them apart as the medium shrinks. Nail varnish also prevents the coverslips from sliding when using immersion oil.

## VI. Examining the otoliths

Several measurements should be made when examining each otolith. The diameter and radius should be measured using the longest diameter and radius in larval otoliths and a radius from the nucleus to the posterior edge in juvenile otoliths. The exact radius used for juveniles should be that which gives the most simple form to the relationship between otolith size and fish size.

The measurements made on each otolith should include the diameter, radius, radius of the first prominent ring and subsequent prominent rings, individual zone width if possible, and information on the stage of completion of the last increment.

VII. Counting rings

The counts of ring number should be made at least three times, recording the mean and standard deviation of the counts. These counts should be separated in time so that the decisions made in one counting do not influence the results of the next. The limit of acceptable error in counts should be set before the examinations are begun. Ring counts should be checked independently by at least one other experienced reader. Each reader should count with a tally-counter for ring numbers higher than 20. This makes the counting easier and avoids biasing one count with the results of another.

A Counting procedure was developed for cod, herring and sardinella otoliths as a guide for interpreting rings.

1. If a prominent first ring is present in all larvae this seems to be a good place to start counting, making a separate record of the number of rings visible inside this band. There is evidence for earlier rings from EM work but these are not consistently visible in the light microscope. There is also the possibility of using the radius of the otolith at first feeding or hatching (if this is constant) and beginning to count the first ring vislible outside that distance.

2. In small otoliths it is important to count all the rings, focusing several times to verify the counts. The edge is counted as a ring if it appears as a dark band.

3. In larger otoliths it is likely that counting every ring gives counts much higher than the age. If only the heaviest bands are counted there is better agreement with

age and also between readers. This is best accomplished by focusing so that the outer bands are thick and fuzzy and not devided into smaller rings.

4. The width of the outer rings in older larvae + juveniles tends to decrease towards the edge and all of these narrow rings should be counted as separate rings.

## VIII. Suggested equipment for otolith work

Freezer for storing samples

Dissecting microscope (with x50 mag.)
Compound microscope (with x1000 mag.)
Photographic equipment

Tally counter
Fine needles
Grinding papers
Mounting media
Nail varnish

Extra equipment helpful in otolith work

Microscope attachments:

- video vamera + monitor
- viewing screen
- polaroid camera
- large-format negative camera
- discussion tube
- drawing tube

Dental drill and grinding discs
Digitizing table
Desk-top computer

EVALUATION OF TECHNIQUE FOR AGEING OF ADULT, JUVENILE AND LARVAL FISHES.

The use of observed number of primary growth increments for ageing fishes will depend on several factors. There seems to be no basis for the general assumption that increment formation is a daily event in most fish species unaffected by other factors, either genetic or external. At this point it seems impossible to draw conclusions from results obtained for one species to validate the use of increment counts as an ageing method for another species.

Adult tropical fishes.

For adult tropical fishes the available data support the assumption of daily increment formation. The age determinations made by increment counts show good correspondence with age as obtained by other methods i.e. length frequency modal progression and/or knowledge of spawning season. In some cases the use of otoliths appears as the only useful and reasonably precise method. When prepared in a proper manner the primary increments of tropical fishes (see Sardinella sp.) appear more distinct and easier to count than is the case for most temperate and boreal fishes. It is, however, also for these species a definite requirement that the assumption of daily increment formation is verified.

Juveniles of cold-temperate fishes.

Counting of primary increments of otoliths from juveniles of cod have been attempted, at present only from field-caught specimens. The conclusions drawn from this material rest on the same assumptions which are necessary for larval cod. In addition, a verification of a regular pattern of zone formation during the juvenile period is needed. If these requirements can be met, and if the counting pattern is standardized, the method seems promising for ageing juvenile cod.

Larvae of temperate and boreal species.

For larval fishes it has been shown that the rate of increment formation may vary considerably within the same species. If a relationship between age and number of increments is established for a stock or a species with due consideration of the possible deviations in slope and time of first zone formation, this can be used for ageing of larvae from this particular unit. Calibration of counts between counters is necessary to prove the validity and usefulness of this relationship. It is also important to base the establishment of this relationship on larvae of known age from a wide spectrum of age-groups (preferably from hatching to metamorphosis in as many steps as possible).

It should be emphasized that knowledge of the mechanisms involved in trigging or entraining zone formation must be the basis for using the established regression equation for ageing larvae from other stocks. These may live under very different conditions compared to the population for which the regression was established. Knowledge of the underlying mechanism and/or of factors which are affecting the regularity of zone formation will be a necessary guideline for what to expect when examining larvae from other conditions.

The total effort required for all steps in the routine use of the method should be considered when comparing with other available ageing methods (i.e. stageing by anatomical features, length frequency modal progression etc.). If the increase in precision of an age determination by increment counts is small, the time needed for special preservation of samples, dissection, mounting and several determination of increment number for each otolith, may cost too much to allow it for routine use. For species where the increments are rather indistinct, a fact which will always reduce the precision of the counts, this comparison is especially relevant. For other species where the counting is easy and also reasonably constant from one counter to another, ageing by some relation between the age and the number of increments still seems to by very promising.

REQUIREMENTS FOR ANSWERING BASIC QUESTIONS CONSERNING OTOLITH STUDIES - SOME RECOMMENSATIONS.

A short list of questions which at this stage seem relevant will then be:

- When does the first prominent ring seen in the light microscope form?
- Is the zone formation regular throughout the larval period?
- If so, which factors are involved in entraining the mechanism of zone formation?
- Is the rate of zone formation independent of growth rate?
- Is the step from a rearing experiment to the wild immediately acceptable?
- If a straightforward relationship exists between age and the number of zones, how precise will the age determinations by this method really be?
- Which limits are set for the amount of error acceptable for routine use? (this is likely to be governed by for what purpose the ageing is done i.e. growth studies, survival studies, studies of anatomical development etc.)
- If the method of increment counting proves unsuitable for routine ageing, can otoliths be used to gain other information about early life history?

It seems clear that the use of primary increment formation may be useful as a practical ageing method if the following requirements are met. (the list will most probably prove incomplete, but may serve as a useful starting point).

1. One species has to be considered at a time, possibly also each stock or population if growth rate or other factors are assumed to be variable between them. Special consideration should be directed to the effects of cyclic environmental stimuli.

2. The mechanism of zone formation and/or factors which influence deposition rate and the synchronization of ring deposition should be investigated.

3. The time for the formation of the first increment should be determined.

4. For each unit considered (species, stock or population) a wide spectrum of age-classes of which the exact age is known from another independent ageing method should be used.

5. The regression of number of increments vs. age must be based on results of 3 and 4.

6. The precision of the regression obtained and of age determinations made from it should be measured.

7. The precision of an age determination by this method should be tested against other available methods (if any exist) by a cost-benefit analysis (i.e. is enough precision gained by using this method to pay the costs of time and effort in preparation).

8. If the relationship between age and number of increments proves unsuitable for making age determinations the otolith may possibly provide other useful information about early life history events. The study of otoliths may prove valuable for getting information of condition, of which major stages which have been passed etc.

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