Manual for spawning type determination based on herring otolith microstructure

Introduction

Otolith microstructure formed during the herring's larval period is retained as the central part of the adult otolith. Primary increment formation is found to be diurnal and increment width is assumed to indicate environmental conditions, primarily temperature. Analysis of daily increment patterns in larvae and the larval features of adult otoliths may be used to discriminate between spawning types at the individual level and identify groups of herring from different spawning grounds. Herring populations with different spawning times mix in the North Sea and ICES Division IIIa. For stock assessment, otolith microstructure analysis is used to determine the hatching season of individuals from this area and classify them into hatch type spring, autumn, or winter.

Preparation

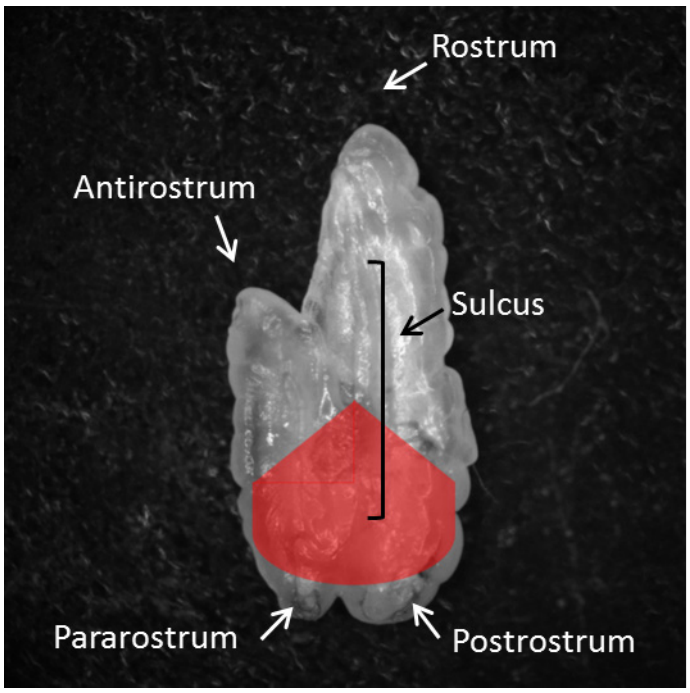
Before any spawning type determination can be done, the otoliths must be mounted on glass slides. There are various types of resin for this purpose, the main important feature is that the resin can be re-heated allowing for reposition of the otolith. The following section provides a list of the steps to go through when preparing the otoliths for polishing. The materials mentioned are examples and alternatives may be applied as long as they possess the same features (Table 1).

Table 1. Materials required including examples

Inventory	**Example (DTU Aqua)**	**Example (SLU)**
Glass slide	Thermo Menzel Glässer	Menzel Glässer superfrost med skrivfält
Resin	Buelher Thermoplastic Cement	Buehler Crystalbond
Labelling machine	Zebra GKU20t	Human (handwritten)
Hotplate	Präzitherm	Ikatherm HCT
Preparation box	VWR Cat No. 631-1517	Kaiser microslide box
Polishing machine	Buehler Phoenix Beta	Buehler Phoenix Beta
Grinding paper	Buehler SiC P1200	Buehler P 600
Polishing paper	Buelher 3M	Buehler 3M
Microscope	Leica DMLB	Leica DMLB
Stereo microscope	Leica MZ6	Wild M3B
Software	IC Capture and Image J	IM500
Camera	Imaging Source DMK 23U274	Leica DC 300

1. Microscope glass slides are marked with journal ID and otolith number, either by handwriting, or by using a label-printer. Ensure that the labelling ties with the ID of the individual herring in the database.
2. A thermostatically controlled hot plate is turned on with the thermostat set to the temperature which will melt the thermoplastic resin. Please note that some types of resin require air-ventilation. It is useful to cover the hot plate with aluminum foil to protect it from waste thermoplastic resin.
3. While the hot plate is heating up, the otoliths in the black tray are arranged in such a way, that the sulcus side facing up (Figure 1A).
4. When the hot plate has reached the required temperature, the microscope glass slides are placed on it and are heated up to the temperature required to melt the resin upon them.

5. An appropriate amount (about 1 cm^2) thermo-plastic resin is melted onto each glass slide and the otoliths are mounted sulcus up in the resin. Some laboratories chose to mount both otoliths on one slide; in this case, the label should be positioned in the middle of the slide (Figure 1B).
6. When mounting the otolith it is important to ensure that that the otolith is positioned completely plane on the slide. Air bubbles must be avoided as much as possible. Several 'tricks' ensuring that the otoliths are not lost in the transfer from the tray to the glass slide have been developed, e.g. using a wet matchstick; it is recommended that this part of the process is just developed for the specific laboratory.
7. Once the glass slides have cooled, they must be placed in labelled boxes and stored until the analyses of the microstructure.

A 

B 

Figure 1 A) Sagitta otolith facing sulcus up, the red area indicates the best viewing area for spawning type determination, B) Positioning of otoliths on the glass slide

First glance

Before polishing/grinding have a look at the nature of the larval centre in the stereo microscope; if it is large and transparent, this indicates an autumn or a winter spawned individual. A check of the size of the otolith before the first wintering will also give pointers to which spawning type you are looking at. The range of size at first winter for autumn/winter spawners is larger than for spring spawners.

Grinding and polishing

Using a machine with two rotating plates fit for grinding and polishing paper facilitates an even and fast preparation of the otolith. While grinding and polishing it is vital to apply water on the paper throughout the entire process and to keep an even light pressure upon the glass slide. The process is initiated by using the coarser paper (grit P1200), grinding the top layers of the otolith away. The otoliths must be regularly controlled in the stereo-microscope in order to see, how much of the otolith material, that has been removed. The grinding proceeds until the centre appears clear/translucent, and the nucleus is a little dot (about a couple of microns) above a translucent groove, which is the centre. Also at this point a few rings are visible around nucleus.

Once the centre appears visible, the otolith must be polished using the finer paper (3 micron) to remove grinding tracks. The reading is done in the microscope using at least a 20 times magnification. It is seldom

the case that the otolith is completely ready for hatch-month determination after the grinding process alone, and it must often be further prepared:

- If the otolith is over-grinded, the centre is without any structures at all. In other words is it impossible to retrieve any ring structure at any focal plane.
- If the centre is not completely visible, but can be faintly seen when the focal plane is below the surface of the otolith, the otolith has to be polished further, until the centre is just below the surface.
- If the otolith is completely dark and non-translucent regardless of any light setting and focal plane, it has to be flipped over, and ground on the other side. The glass slide is heated, and the otolith is carefully flipped over. After this, the grinding and polishing process is repeated but carried out with caution while constantly checking under the microscope until the centre is completely visible. The otolith should then also be viewed through the glass slide to check where the increments are clearest.

Viewing

When viewing the otolith in the microscope the following steps should be followed:

1. Examine the whole area around the larval centre in a diameter of 200-250 microns. It is necessary to change light settings and the focal plane frequently when analysing the otoliths in order to get the best view of the centre.
2. Firstly, identify the true daily ring structures, these may not be complete and cannot be followed in a complete circle. A minimum of 10 clear successive increments must be identified for the signal to be reliable.
3. The final determination should be based on a minimum of 3 clear successive increment patterns located at 3 different distances from the centre (turning and polishing of the otolith may be required to achieve this number). Figure 2
4. The most reliable reading direction is at 90 degree angle from the centre towards the para- and postrostrum. Figure 1A
5. It is recommendable to apply a ‘readability scale’ when determining the spawning type to rate the certainty of the visual inspections. The most commonly used is shown here:

Readability	Code
Easy to read with clear structures, no doubt about age provision	A
Not so easy to read, but clear enough that the age determination is reliable	B
Unreadable, no clear structures, age provision omitted / guessed	C

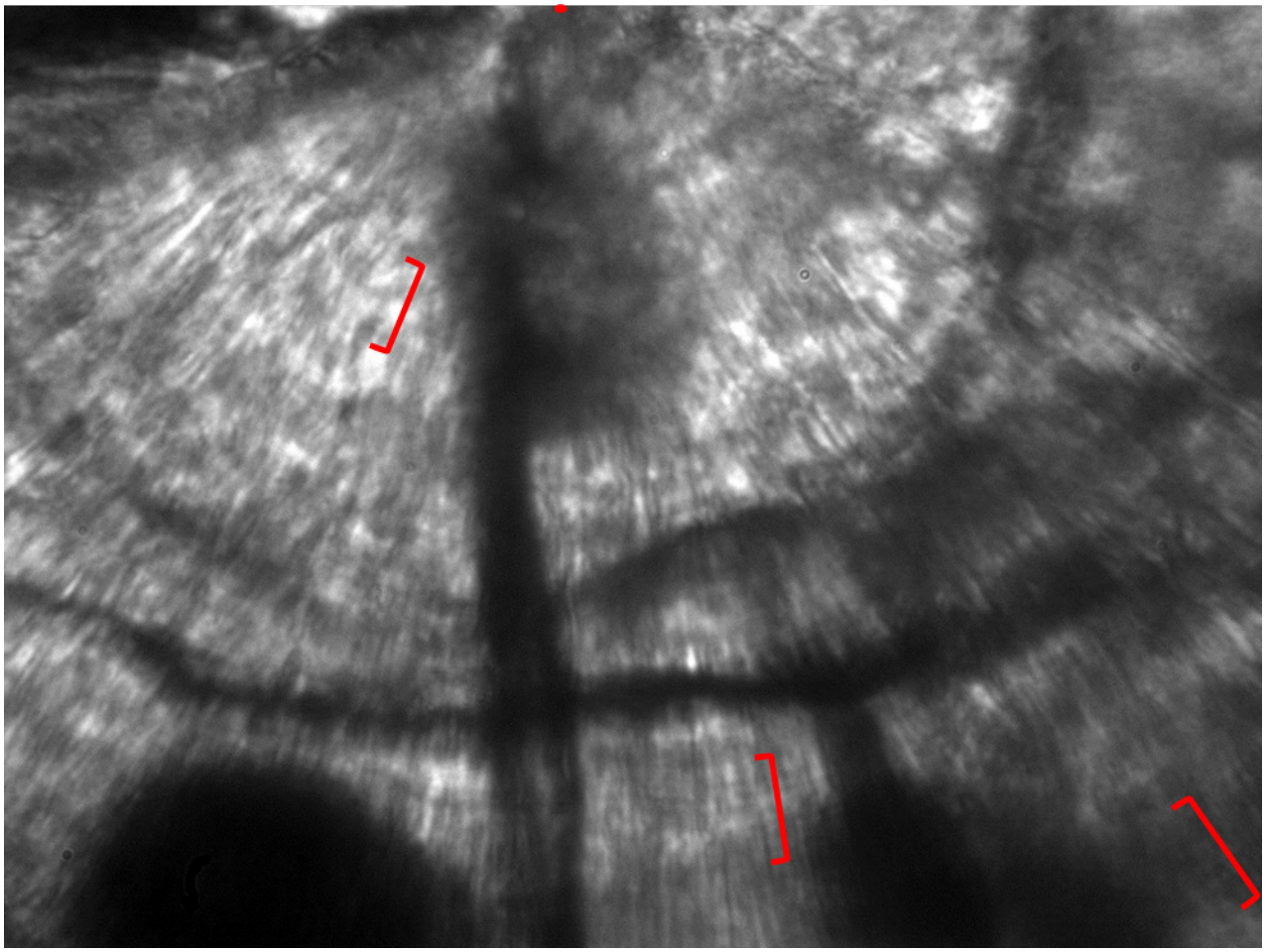


Figure 2 Image of the optimal reading direction, the red dot indicates the centre of the otolith and the red brackets indicating 3 areas with clear successive daily rings

Determination of spawning types
Herring in the North Sea is a complex mixture of different stocks. In the deeper parts of the eastern North Sea, Skagerrak and Kattegat, Western Baltic spring spawners mix with North Sea autumn and Downs winter spawners during summer migrations.

Autumn spawners:
Otolith increments less than 2.5 µm wide are found more than 200 µm from the center. Primary increments will often not be visible from the nucleus to the end of the larval zone but become clearer around 150 microns from the centre, after which the increment widths appear relatively constant (Figure 3). The true spring growth (increments widths of 4 or more microns) is not seen before a distance of XXX from the centre which is equivalent to roughly 4 months (+ 120 daily rings).

If the otolith during the grinding and polishing process appears to have a wide transparent central area with an abrupt change to less transparent otolith material, it is most probably an autumn spawner.

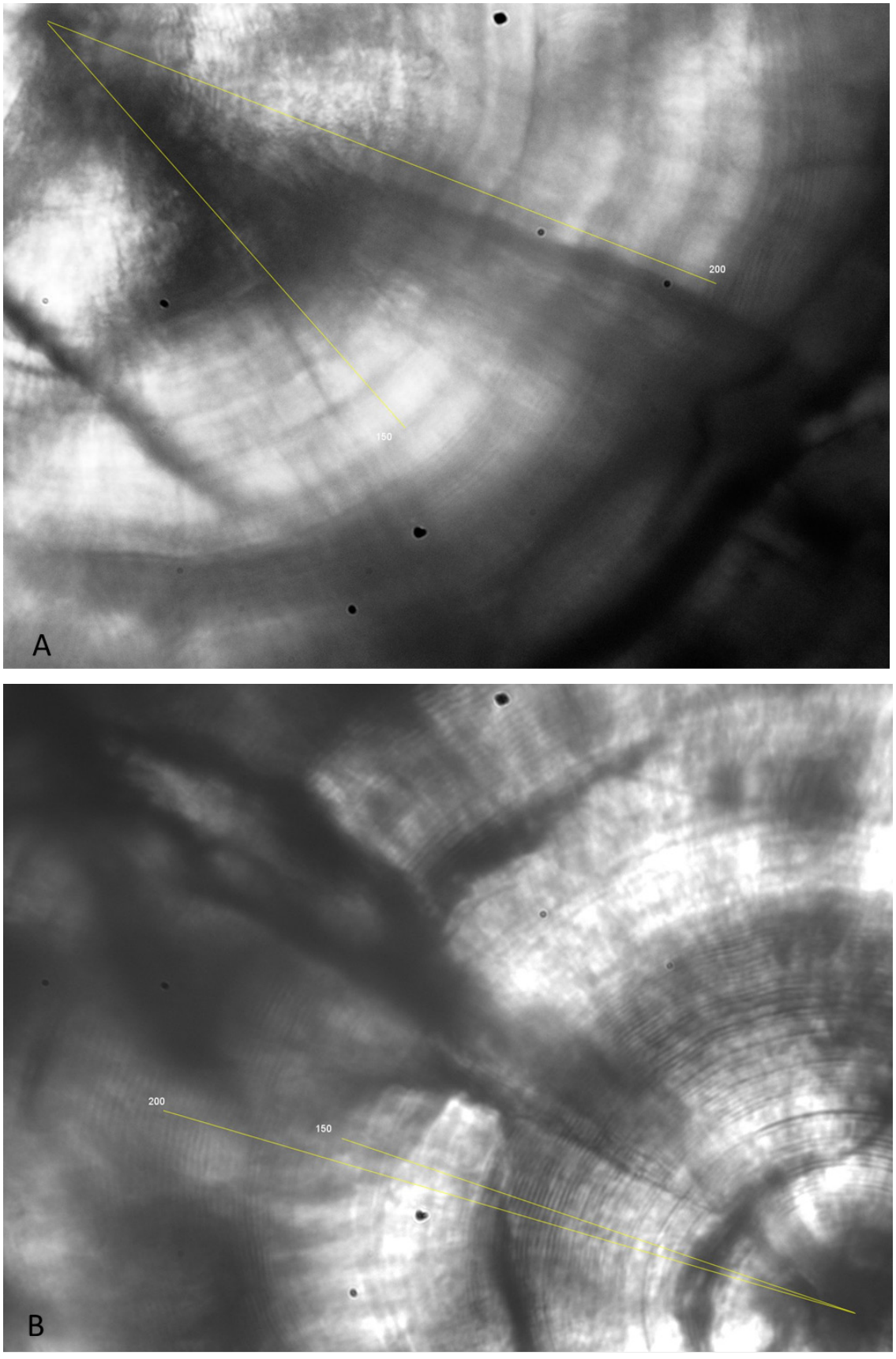


Figure 3 A) Poor example of an autumn spawner, B) Good example of an autumn spawner

Winter spawners:

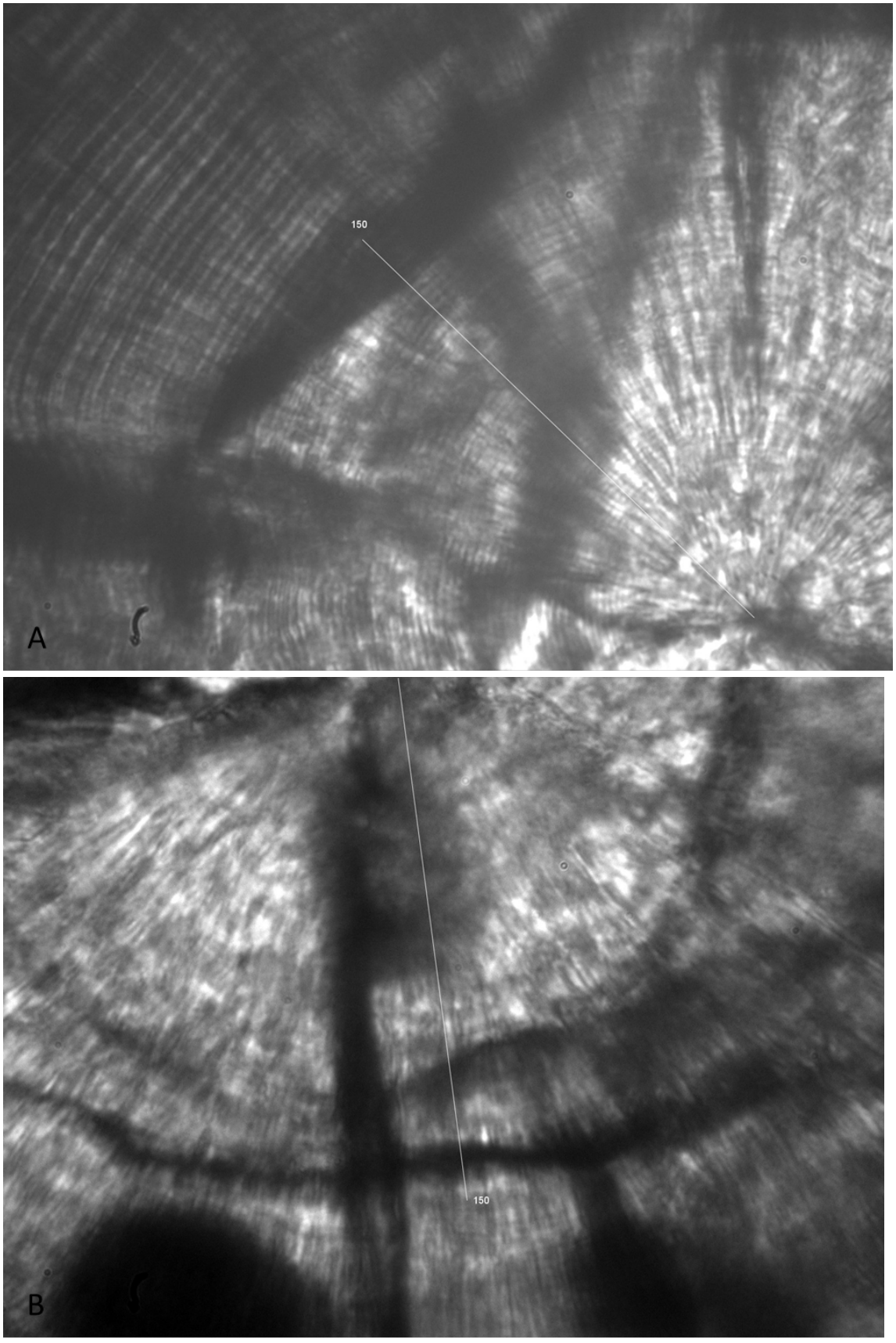
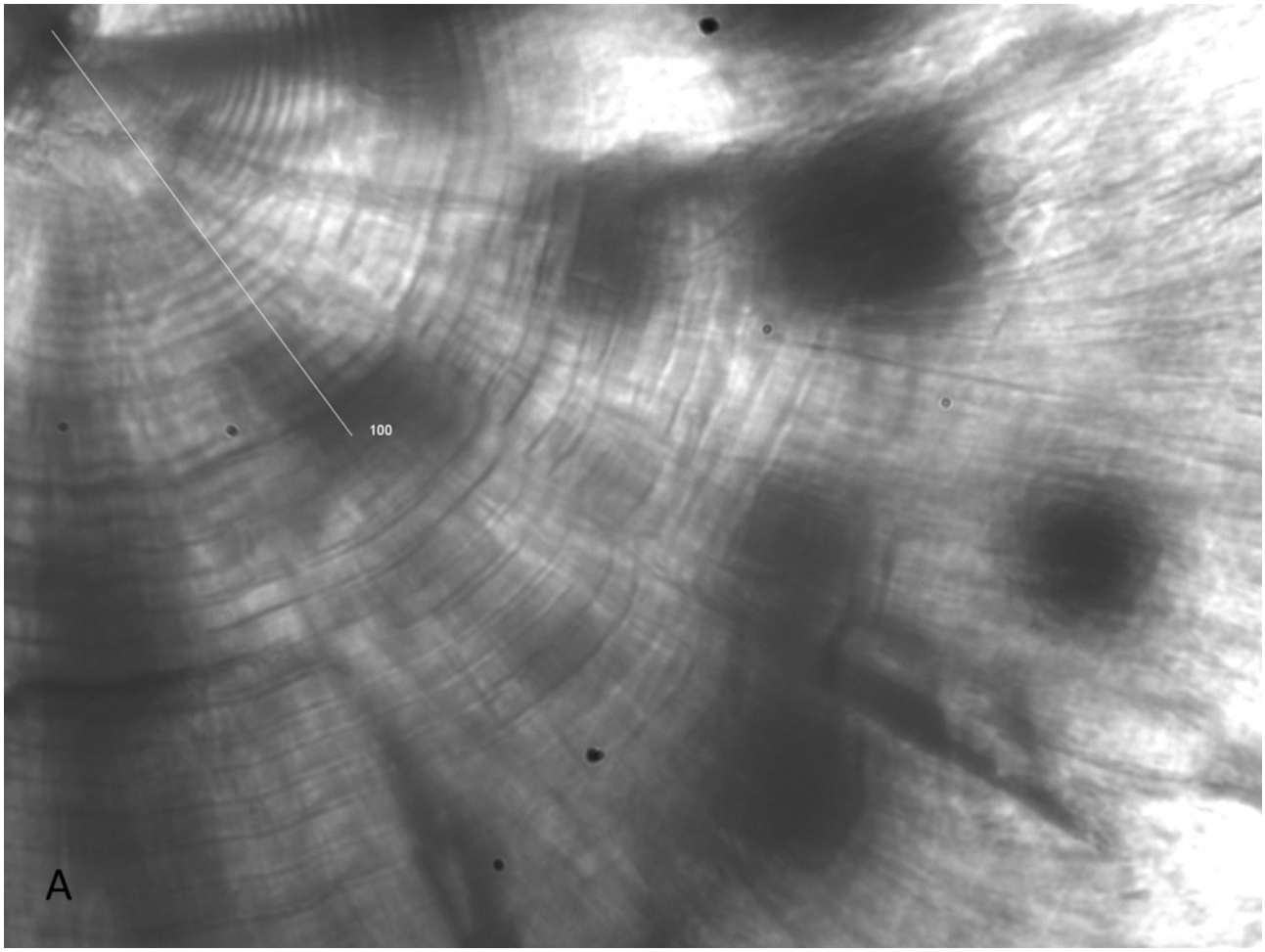


Figure 4 A) Good example of a winter spawner, B) Poor example of a winter spawner

Otolith increments are gradually increasing from about 1 µm near the end-of-yolk-sac-structure (which is about 9 to 12 µm from the nucleus), to more than 3 µm at a distance of 150 µm from the nucleus (Figure 4). The increase in increment widths accelerates at about 200 µm from the centre. The microstructure changes gradually from faint increments in an inner zone with high transparency to more pronounced increments with higher visual contrast and less transparency at about 100 µm from the centre.

This spawning type is the more difficult one to determine as it is in between the two more distinct types. If in doubt, then a good way to validate the determination is to count the number of daily rings from the centre to the onset of spring growth (daily ring widths of more than 4 micron). A winter spawner would have between 70-90 days from the centre to onset of spring growth. If all increments are not visible throughout a transect, one can extrapolate between patches of visible daily increments (Figure 2).

Spring spawners:
This is the most variable otolith type, depending on population and exact timing of hatch. Early hatched individuals form increments that rapidly increase from a width of about 2 µm to more than 4 µm less than 100 µm from the nucleus. The rapid increase following the initial relatively narrow daily increments are seen at about XX micron from the centre. Later hatched individuals have increments that are relatively wide (about 4 µm) already 20-40 µm from the nucleus. The structures have comparatively higher contrast and can appear less regular. Figures 5A and 5B show an early and late hatched spring spawned herring respectively.



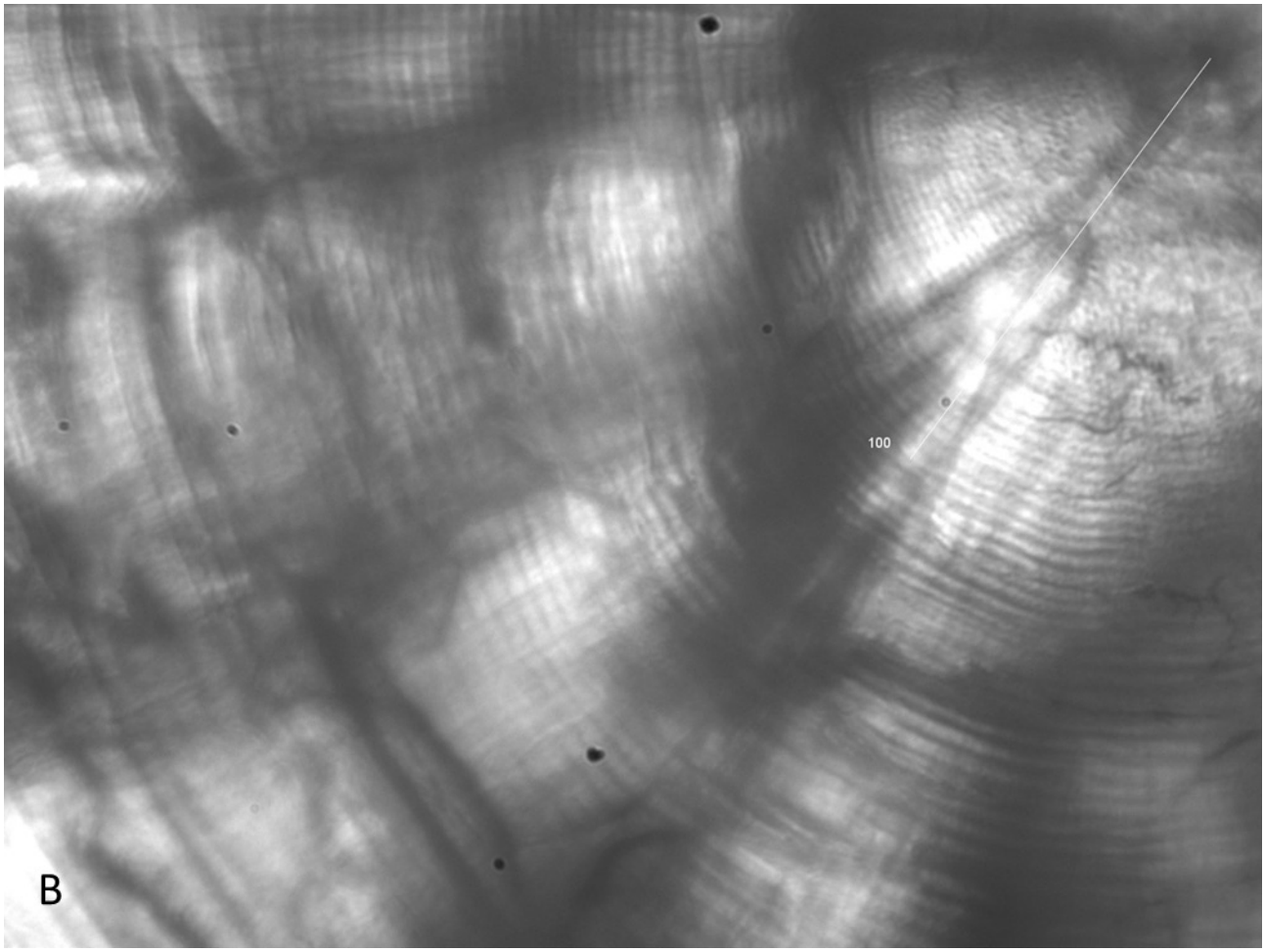


Figure 5 A) Example of an late spring spawner, B) Example of an early spring spawner

Given the high number of 'local' spring spawning herring in the Western Baltic, there are several specific features for each population. A few is described here, although the examination of these types has yet to be expanded.

- Local Skagerrak spring spawners: These spring spawners have a non-circular larval otolith (more like an oval or a circle 'compressed' in one side).
- Swedish fjord spring spawners: Initial rather narrow daily increments followed by a very rapid increase to spring growth at about 135 micron from the centre (Figure 6A).
- Rügen spring spawners: High contrast, irregular daily increments forming close to the centre (approx. 30 micron) (Figure 6B).

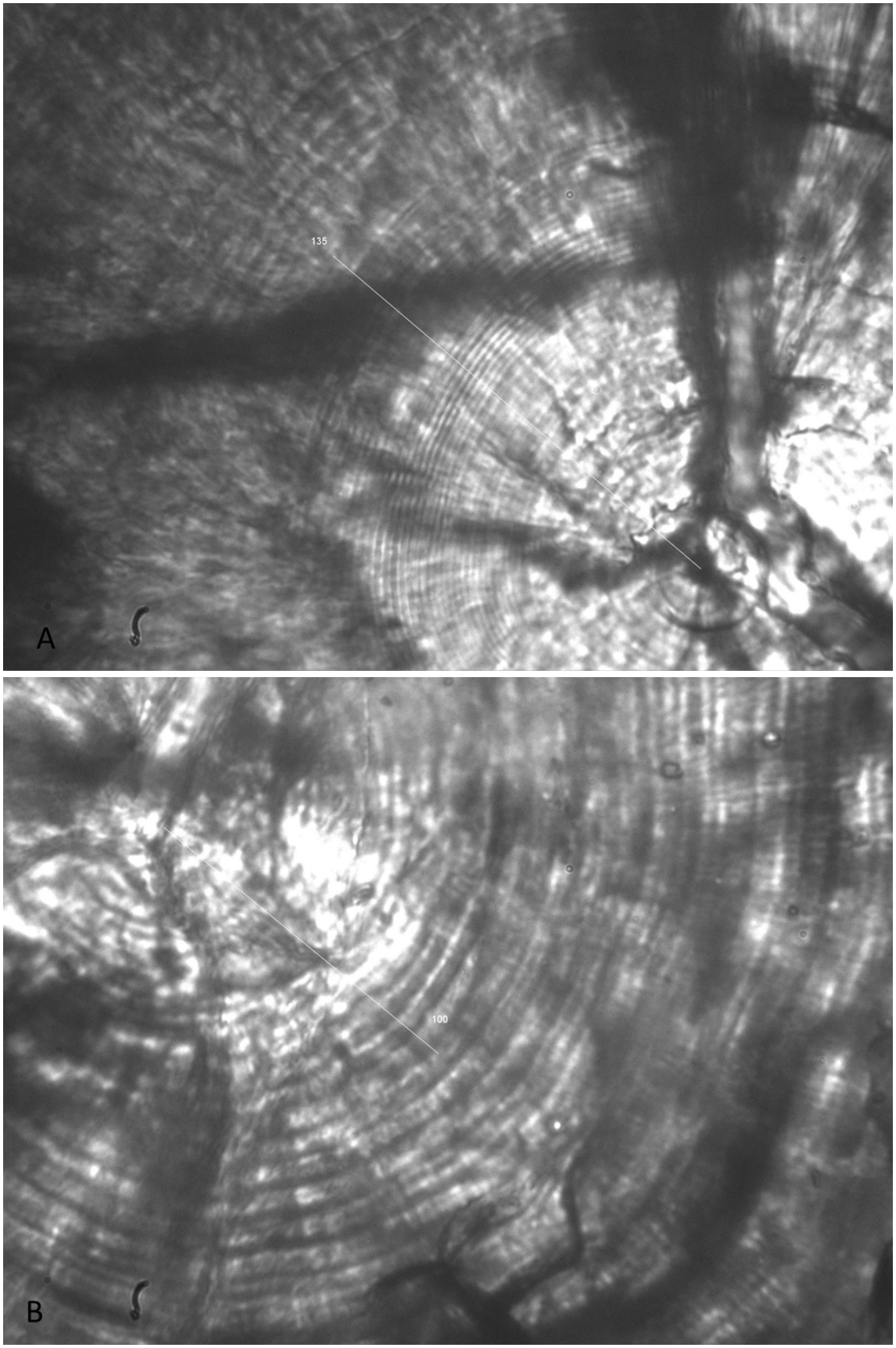


Figure 6 A) Example of a Swedish fjord spring spawner B) Example of a Rügen spring spawner

The following pages will be completed during Autumn 2017 when working on the Akustik samples

The table below gives the general guidelines in terms of daily ring width relative to the distance to the centre for each of the spawning types. *(In addition to these guidelines, the onset of rapid increase in increment width must be included in the determination (if in doubt, count the daily increments from centre to onset of summer growth* (define XX micron).)

Table 2: we need to decide on how many samples are used to make average daily ring width for the table

Type	100 micron from centre	150 micron from centre	175 micron from centre	200 micron from centre
Autumn				
Winter				
Spring (early)				
Spring (late)				

Exceptions to the rule

Problems: e.g When are measurements needed, a description of the "local" spawners with image examples, problems preparation wise and Interpretation wise

Section for readers to complete

Preparaten har vi inga problem med. Problemen är att tyda otoliterna när dom är mellan höst och vinter och mellan vinter och vår. Då behöver man mäta.

Dubbelringar kan ibland vara problem, ofta om man slipat en vårlekare för mycket.

Vissa otoliter har ingen synlig kärna, bara ett genomskinligt område även om man slipat väldigt lite.

Vi har stor hjälp av vårt mätprogram som är lätt att använda. Vi mäter så fort vi är tveksamma.

We have no problems with the preparations. The problem is to tell the otolites when they are between autumn and winter and between winter and spring. Then you need to measure.

Double rings can sometimes be a problem, often if you have a spring lover too much.

Some otolites have no visible core, only a translucent area, even though very little is sanded.

We are very helpful with our easy-to-use measuring program. We measure as fast as we are doubtful.

Supporting tools and information

Auxiliary information about the sample from which the individual herring is taken may provide support for the reader. If the sample is taken during a spawning period at a known spawning site, it is highly possible that the spawning type match the sampling date. If the sample is taken very locally (e.g. a fjord) then the likelihood of the herring belonging to this specific population is high. Information of age, length and maturity

stage can also provide support for a decision; given the link between growth, temperature and otolith microstructure appearance, knowledge of historic temperature for e.g. 1 year olds can potentially explain deviating patterns in a spawning type.

Measuring tools: There are a lot of software packages which can be used for image capture and analysis, many of which include measuring tools plus tools which can improve the quality of the image. Whichever package is used for measuring it is important to ensure that you have made a calibration image of a measuring stick which should be stored with your otolith image files.

The following is an example of how to calibrate and measure using Image J which can be downloaded for free from the internet:

To calibration:

- Open Image J
- From the main menu click on “File” and then “Open”, choose your calibration image from the dialogue box
- On the toolbar menu click the “line tool”
- Use the line tool to measure between 2 units on the calibration image
- From the main menu click “Analyse” and then click “Set Scale”
- In the dialogue box the “Distance in pixels” is given by the computer (do not change this)
- In the “Known distance” box enter the actual distance which you measured between 2 units on the calibration image
- In the “Unit of length” write “microns”
- Place a check in the “Global” box

To measure increment width:

- Open the image of the otolith you want to measure
- From the main menu select the “line” tool
- Click on the nucleus, move the mouse keeping your finger pressed. The distance is shown on the tool bar. When you reach the area you want to measure (100, 150 or 200 µm) and have found 5-10 consecutive visible increments take your finger off the mouse
- Press “ctrl +b” and the line will stay on the image
- You can use the “zoom” tool to zoom in/out on the image. Select the zoom tool and left click on the mouse to “zoom in”, right click on the mouse to “zoom out”
- Find an area where there are 5-10 consecutive visible increments
- Using the “line” tool place the cross at the beginning of a translucent zone, click and hold. Drag the line and count over 5 translucent zones, then take your finger off the mouse at the start of the 6th translucent zone
- Press “ctrl + m” on the keyboard to record the measurement
- A “Results” box will appear on the screen. The “length” number is the corresponding width of 5 daily growth zones (dividing this number by 5 will give you an average increment width for 1 increment in this area)

It is recommended to have a reference image of distances from centre to the measure points in Table 2 made specifically for the set-up in the laboratory as this can be very helpful when checking the otolith ‘live image’ while analyzing the otolith. This way the reader will have a clear vision of e.g. where 100 micron from the centre is in that particular set-up, see Figure 7 below for example.

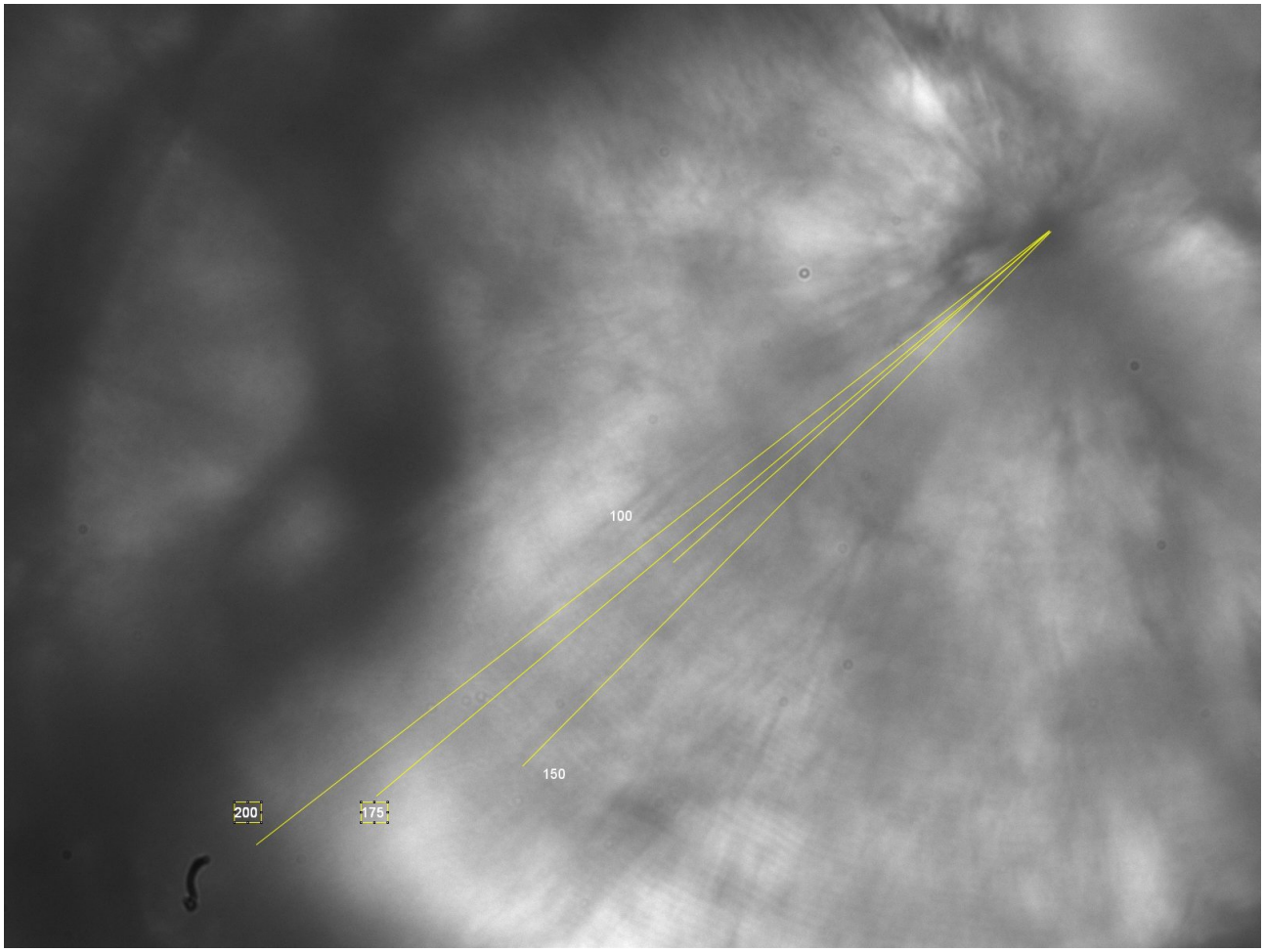


Figure 7 Example of reference image with guideline distances

Reference collections of typical spawning types by year composed of 0-group herring otoliths may provide a basis for checking spawning type typical for a given year. As updating of such databases are highly important and quite time/effort consuming, it can also be a solution to find appropriate 0-group herring in historical material and check problematic otoliths against these.