ICES Planning Group on Commercial Catch, Discards and Biological Sampling (PGCCDBS)

Report of the Sand Eel Otolith Ageing Workshop

DIFRES
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1. Introduction

The distribution of the sand eel stretches in the Northeast Atlantic from 49^0N to 73^0N (Reay, 1970) with a tendency to inhabit in well-defined areas on shallow banks (Macer, 1966). The substrate must be conductive to burrowing (Meyer et al., 1979) with the choice of habitat probably determined by the type of sediment and the hydrodynamic processes affecting the sediment (Pinto et al., 1984). Wright et al., 1998 found that the sand eel was absent from sediment with a silt content less than 10% which together with depth and tide influenced habitat choice. The depth maximum for sand eel abundance is about 100m (Pedersen et al., 1999), and the water flow must be above 0.6 ms-1 (Wright et al., 1998). These constraints leads to a very restricted distribution of sand eels, i.e. they are found in sandy areas where tides and wave action is expected to scour the bottom giving rise to coarse sand and well-aerated substrate. These areas appear to coincide with the areas of the North Sea influenced by tidal front activity. The distribution of fishing areas clearly matches the san eel preferred habitat (figure 1).

Due to the stationary habit of post-settled sand eels, a patchy distribution of the sand eel habitat, and a limited interchange of the planktonic stages between the spawning areas the sand eel stock in IV consist of a number of sub-populations (Proctor et al. 1998, Pedersen et al. 1999). Due to a to coarse spatial aggregation level of the fisheries data that is used in the sand eel assessment and a lack of biological information for defining the limits of each of the reproductively isolated population units, it is presently not possible to make an assessment that take account of the sub-population structure of sand eels (ICES 2006).

The catches of sand eels in area IV consist mainly of the lesser sand eel *Ammodytes marinus*. However, other species of sand eels is also caught. At some of the grounds in the Dogger Bank area the smooth sand eel *Gymnammodytes semisquamatus* can be important, and in the catches from more coastal grounds the other *Ammodytes* species *Ammodytes tobianus* can be important. The greater sand eel *Hyperoplus lanceolatus* appears in the catches from all grounds, but usually in insignificant numbers compared to *A. marinus*. The population dynamics of *A. tobianus, G. semisquamatus,* and *H. lanceolatus* are largely unknown, and so are the possible effects on these species of commercial fisheries. Figure 2 shows the total catch of sand eel over the past 50 years in the North Sea area (ICES 2006).

ICES expert group PGCCDBS have recommended a further investigation of the age estimation of sand eel (ICES 2005) and the present report gives the results from a workshop hosted by DIFRES aiming at securing consistent age readings at different laboratories. Prior to the workshop an exchange was carried out between Denmark, Norway and Scotland, results indicating a limited inconsistency among international sand eel age readers (discussed in a separate section of the present report).

The objectives of the workshop were manifold; apart from the overall goal of securing consistency in age estimation of sand eel; updating and assembling age readers from all national laboratories handling sand eel from the North Sea to exchanged views on methods and experiences was among the objectives. This had not been done for more than a decade among the participating laboratories. The aim of such an exchange of experiences was to start the process of the creation of a training manual/DVD to be used as both quality assurance for trained readers and as a guide for new readers.
The workshop had one day of calibration exercise where a traditional age calibration exercise was combined with an image analysis system approach.

2. Participants

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3. Materials and Methods

3.1. Otolith exchange programme

The otolith exchange set consisted of 920 otoliths selected from commercial catches taken from the major Danish fishing areas in the North Sea during 2004. Sampling dates were evenly distributed over the months April through June. There were no differences in the length distribution by month and area except for samples from Jyske Rev in June, which had a length distribution shifted towards higher lengths than the remaining areas (figure 3).
As the otoliths in the exchange set were extracted from DIFRES archives, the exchange set was comprised of otoliths fixed in nail vanish on a microscope slide, which is the standard preparation at DIFRES. Readers were instructed to view the otoliths under a dissection microscope with the glass slide covered in water.

The analysis of the results was performed using an Excel ad-hoc Workbook "AGE COMPARATIONS.XLS" from A.T.G.W. Eltink from RIVO following the recommendations of EFAN (Eltink et al., 2000). This analysis is based on a reference age when there are no validated ages available, which is the case for sand eel. As reference age the modal age was chosen as the experience level and 'reading school' of the participants varied to some extent and choosing one reader or another as reference age was not an obvious task. However, the most experienced readers were weighed higher in the modal age and were used as 'true age' when no modal age could be reached, though this only were the case in 3 out of the 920 otoliths.

3.2. Traditional age calibration/Image analysis set-up

The calibration otolith set consisted of 102 otoliths selected from Danish commercial samples from the Dogger Bank and Jyske Rev areas in the North Sea in April, May and June 2004. The length distribution was not as even as in the exchange set as the number of individuals was much less and thus covering the same distribution by month and area was not possible (figure 4).
The otoliths were selected from archives at DIFRES and were removed from the nail varnish, cleaned in water and then digitized using a dissection microscope with reflected light (Leica MZ12) connected with a camera (Leica DC 300F) to an image analysis system (Leica IM50 ™). The pictures were used in the image analysis age calibration part of the exercise.

During the calibration exercise the otoliths were read submerged in water under dissection microscopes by all participants.

The analysis of the results was performed using an Excel ad-hoc Workbook "AGE COMPARATIONS.XLS" from A.T.G.W. Eltink from RIVO following the recommendations of EFAN (Eltink et al., 2000). Modal age was reached for all otoliths in the calibration set.

The image analysis age calibration was performed using both 'live' otoliths under the stereomicroscope and digitized images of the corresponding otoliths. The readers had the otolith exposed under the stereomicroscope while pointing at the age structures on the picture using the image analysis system tool and could consult the 'live' otolith if the pictures did not reveal all the desired otolith structures clearly. The image analysis system tool makes use of XY-coordinates corresponding to the points, the reader marks as age structures on the digitised image of the otoliths. Prior to the exercise the readers agreed on one axis from the centre and towards the edge along the rostrum along which all points should be placed. All reading on the digitised images were done by marking the first age structure as the first point and then marking all identified age structures along the agreed axis. All points logged on each individual otolith were then transferred into an Excel spreadsheet with the correct ID (otolith number, picture number and reader ID). The readers agreed to mark the outer edge of each translucent ring identified as an annual structure.

4. Results

4.1. Otolith exchange programme

The results from the traditional age calibration exercise displayed that the differences in perception of otolith structures between the participating age readers was not that alarming. The overall agreement was 83.5 % with a precision of 19.7% CV and in 53% of the otoliths the agreement was larger then 90%. Figure 5 shows the relative bias by modal age indicating any trends in over-or under estimation of ages by all readers combined. The relative bias were not skewed for any ages, although there were a slight, but not significant, tendency to overestimate the younger ages and overestimate the older compared to modal age.

Month and location showed to have an effect on percent agreement as shown in figure 6. Fisher Bank and month 4 proved to be the combination which gave the lowest agreement, however removing Fisher Bank from the data did not change the significant effect of sampling month and location on the percent agreement (Table 1). The modal age was significantly higher at the location Firth of Forth (figure 6), but did not have a significant effect on the percentage agreement (ANOVA, F=0.34. p=0.51).

Though the bias and disagreement in general in the present calibration may be considered as low, a difference in perception of age structures is clearly present. The two most experienced readers in the exchange had a consequent pattern of disagreement, where one reader (R1) were interpreting the age 1 year younger than the other in 42% of the individuals compared to the other reader (R2). These discrepancies in interpretation of age structures in the otolith were further explored in the image analysis calibration described below.

4.2. Traditional age calibration/Image analysis

4.2.1. Traditional age calibration

The overall agreement in the calibration exercise was somewhat lower than in the exchange (72.5% with a CV of 21.2%) The distribution of the age reading errors in percentage by modal age as observed from the whole group of age readers compared to modal age is shown in figure 7. The achieved precision in age reading by modal age group decreased as the spread of the age readings errors have increased when compared to the exchange. This however should be taken with some caution as the participants in the exercise counted two new readers who did not participate in the exchange and then the two experienced readers, who did participate in the exchange. Thus an additional comparison of the results between the exchange and the calibration exercise only including the two experienced readers was performed and that showed an increase in percent agreement from 52% to 67% just between the two readers. The pattern in disagreement was persistent as R1 was identifying fewer age structures in the otoliths compared to R2.

Location did not have an effect on the percent agreement in the calibration set (ANOVA, F=1.93, p=1.97) which most likely was an effect of fewer locations represented only covering Dogger and Jyske Rev, which did not seem to be the more difficult areas in the exchange. The collection month did have a significant effect on the percent agreement (ANOVA, F=4.81, p=0.01), the latest collection month (June) being the most difficult one to reach agreement in.

4.2.2. Image analysis age calibration

The spreadsheet program, which combined image analysis and plots, made it possible to demonstrate where the individual age readers interpreted the rings directly on the digitised images of the otoliths.

The omission of age structures by the individual readers did have a pattern, thus it was possible to direct the discussion of which age structures to count towards the conception of ‘false rings’. Some otoliths showed to be very difficult to reach a common interpretation of the age and the points counted as age structures were scattered along the otolith (figure 8), however some trends were obvious and figures 9 and 10 shows the most typical patterns of the selective interpretation of age structures. The ‘false’ ring most frequently appeared when a second opaque zone had been formed during the first summer by some individuals, thus the definition of the first annual structure showed to be of high importance for reaching agreement on the age of the individual fish. However, also between the first and second year of growth the appearance of a ‘split-ring’ structure were the cause of discrepancy between readers.

The most frequent argument for omitting a ring as ‘false’ was the width of the structure, if it appeared less wide than the remaining transparent structures it was considered false by some readers.

A dissection microscope with reflected light (Leica MZ12) connected with a camera (Leica DC 300F) to an image analysis system (Leica IM50 ™) was used to facilitate the discussion of individual otoliths on a computer screen and ultimately for measurements of various features, e.g. the distance from the centre to the first winter ring.

In order to examine possible ‘false’ rings the otoliths in question were mounted on glass slides using thermoplastic resin (Buehler Thermoplastic Cement no. 40-8100) at 150^{o}C to facilitate grinding and polishing on both sides. The otoliths were polished using a series of grinding and polishing films with decreasing grain sizes from 30 µm to 0.3 µm to optimise the visual resolution at a focal plane through the otolith. Possible ‘false’ winter rings were examined through examination of the daily ring structure facing up to and in the translucent zone continuing the examinations of daily increments in sand eel otoliths described in the former report on age calibration of sand eel (ICES 1995). The characteristics of the winter ring are decreasing width of the daily rings prior to the winter ring, no daily ring formation during the winter ring formation and then progressively wider daily rings after the winter ring, whereas the ‘false’ rings can be identified by the continuing daily increments almost unchanged in width through the translucent band. These features were examined using the same camera and image analysis system, though using transmitted light microscopy using a 20x and/or 40xobjective lens. Figures 11a-c shows a typical example of a ring omitted by R1 as 'false' because of the macroscopic features (width and 'sharpness' of the false ring). The structure was validated as a 'false' ring as the daily increments continued through the translucent zone.

5. General guidelines for ageing of sand eel

Sand eel species should be separated at the time of capture (scientific sample) or at the time of receiving the sample (commercial sample). This is to prevent the collection of A.marinus otoliths being contaminated with otoliths from other eel species. In particular A.tobianus & G.semisquamatus. Although H.lanceolatus and H.immaculatus may often be present in samples it is unlikely that these would be mistaken for A.marinus due to their size and physical features. Experienced readers will be able to distinguish between eel species from otoliths, but inexperienced readers may not, so the above separation practice can be useful and thus prevent age data from non marinus species entering the assessment process.

Once the otolith has been removed from the sand eel and 'dip' cleaned in H2O or EtOH, it should be placed in a suitable container for reading and subsequent storage. Otoliths should be allowed to dry completely before attempting to read. One method is to read them whole, immersed in EtOH, however, at present some countries/institutes mount or fix the sand eel otolith onto glass microscope slides, using a form of varnish. This does however eliminate the opportunity to move and turn over the otolith during the reading process and may as such is seen as less desirable as the loose collection and storage method. It is envisaged that all countries will resort to the loose collection and storage method in the near future, after a period of trial.

The otoliths are examined under microscope, using variable power reflected light source and magnification of X20 to X25, with the subject placed in front of a black background. Individual readers will have personal preferences as to the light level and magnification that they feel best suits them and the environment in which they are reading the otoliths. For example, some experienced scleroclinologists prefer to assess otoliths in a darkened room and other equally experienced otolith readers do not require such conditions

The majority of readers focus a narrow beam of light perpendicular to the anterior – posterior axis. It is common for this to be done on both sides of the otolith and also for a steep angle to be used so as to illuminate the otolith surface effectively. It is worth noting that the use of very high magnification should be avoided, as this may reveal the presence of secondary features and lead to confusion; particularly in the inexperienced reader.

Under these conditions, the winter growth rings (zones) on the otolith appear translucent. It is these rings (zones) which are counted to determine the age of the sand eel.

In the early years of the life of the sand eel, the opaque zone, laid down during the period of the year when the sand eel growth is rapid (summer), is usually wider than the corresponding translucent zone which is laid down at the time when there is little or no growth (winter). As the sand eel grows older, the opaque and translucent zones become progressively narrower. This process continues until the outermost rings in old fish become extremely narrow, regular and are of generally equal width. The deposition of alternate opaque and translucent zones is a regular annual occurrence, which has been confirmed by examining otoliths from samples taken throughout the year and observing the type of growth present at the extreme outside edge of the otolith. In this way, the time of year at which each zone begins to grow has been determined.

To simplify ageing, age estimates are usually made in terms of age groups with a reference to a designated birthday. For stock assessment purposes, this does not have to coincide with the biological time of birth. As there is an internationally accepted convention to use January the 1st for most North Atlantic demersal species, there can be no ambiguity about the year class to which a fish belongs and thus all data produced by different institutes are compatible.

Age estimations are usually made by counting the translucent zones from the centre to the edge of the otolith. The first annulus is rarely located very close to the nucleus. Age estimations can be made on several areas of the otolith, however reading from the nucleus to the point of the distal/ventral surfaces is the predominant method. Rings can also be well defined on the dorsal end, but an age reader should carefully follow the rings around to the distal and proximal sides, to ensure no splitting has occurred. A ring which splits at some point on the otolith but then emerges again in another part such as the sulcus should be counted as a single annulus.
In the case of very old sand eel, it may be necessary to count the number of rings in several places on the otolith surface before arriving at a consistent number.

The appearance of opaque zones can be very variable and it is seldom that they are of uniform opacity either within an individual otolith or from ring to ring within the whole otolith. Differences in opacity in a single opaque zone can be either gradual, with very dense areas slowly giving way to less dense areas, or abrupt with a narrow band of completely translucent material being deposited within the overall boundaries of the opaque zone. These narrow translucent rings are termed ‘splits’ or ‘checks’ and their presence in the structure is the main source of difficulty in the interpretation of the otolith as a whole.
Some translucent ‘split’ rings can be of such width and so positioned within a narrow opaque zone, that there appear to be two narrow rings instead of one wide one. In such cases the accuracy of the age estimation depends largely upon the skill and experience of the age reader. This skill is not easily acquired and will require the examination of many thousands of otoliths. When an otoliths with ‘split’ or ‘check’ rings are encountered, it is often beneficial to slightly focus out the microscope or interfere (partly obscure) the beam of light. This causes only the dominant and darkest translucent rings to become more visible and reduces the prominence of the ‘split’ rings and false internal structures. A validation of true and false rings by the use of otolith microstructure, as described in section 4.4.2, may ultimately be the solution in otoliths, where there is considerable doubt about which rings to count as age structures.

For many species, the fish length is not a reliable indicator of its likely age. This is true for sand eel. It is not uncommon for the growth rate of the same species, from the same area to show an extremely wide variation (Williams & Bedford). It is therefore important to avoid the error of first noting the length of the fish from which the otolith came, deciding upon it’s probable age, and then attempting to make the visible ring structure fit that age. Either ring structures observed on an otolith adhere to a recognisable time scale or they do not, and must be so interpreted. A simple basis rule which otolith readers should bear in mind is that although a length is often a useful indicator of likely age, that it is not necessarily a function of age.

6. Discussion

The overall result of the workshop exercise is that there is a general high agreement between readers; however, discrepancies and bias were experienced both between laboratories and individual readers. The discrepancy was related to both collection month and area which reiterates the

importance of familiarity with area and the typical appearance of otoliths from individuals from the specific area.
The image analysis exercise clarified that the lack of agreement can be referred to two reasons, the first being the position of the first ring where a secondary period of growth has been taken place during summer. This is often seen in the younger individuals as the otolith is thinner and thus the structures more clear. The second reason to disagreement arose where some readers choose to leave out specific rings identified by other readers as true annual rings where the rings successive to the 2^{nd} ring were split rings.
Validation of annual structures by otolith microstructure appearance showed to be very useful for reaching agreement in the majority of the otoliths, where the readers did disagree. Inclusion of this method in the routine work with sand eel otoliths when a reader is in doubt of the character of the age structures would be desirable. It will be a part of the standard set up in one of the ageing labs that participated in the workshop.

The two most experienced readers (R1 and R2) reached a high agreement through the course of the workshop and the training of the new readers would be done following the agreements from the workshop thus facilitating a continued high agreement between ageing labs despite the change of personnel.

The workshop achieved quite a lot in terms of ironing out, through discussion and calibration, some of the major problems in ageing otoliths of sand eel. The group reached agreement on an outline of an ageing protocol/guidelines as described in section 5 of the present report and the aim is to produce a DVD training package, including extensive photo-documentation of otoliths with agreed and validated age structures by area and sampling month. This would be part of a reference collection for each area where actual otoliths and digitized images are available for training and future workshops.

7. Recommendations

The present workshop was the first one to be held in decade and this does call for a far more regular intercalibration of the age readers to prevent drift and keep track of interpretation of age structures so that the agreements and conclusions from the present workshop will continue to guide the age readers. The group therefore recommend an exchange to be established during 2008 followed by a workshop in 2009 to finalize the manual commenced in the present report. The set of agreed age otoliths which is a product of the present report should be included in an upcoming calibration.

The use of otolith microstructure as a tool for validation of age structures when necessary in the age estimation of sand eel is strongly recommended by the group and further exploration of the method is suggested. Additionally the group recommends all ageing labs to use measurement scales (e.p.u) and note down distances between age-structures in ‘typical’ individuals specific for their stock. It is the intention to compile a dataset consisting of measurements on distances between age-structures from all stocks and areas from which the groups get samples of sand eel. This would facilitate the definition of area specific otolith patterns which then would be included in the discussed training DVD manual.

In relation to the area specific otolith patterns, there may be a need for population structure projects within these particular areas (migration, drift of larvae, etc) and the group encourage the inclusion of such knowledge (in a condensed format) from already ongoing projects and upcoming projects in the training package.

Knowledge of area specific environmental conditions by year should be available for readers as the formation of the annual structures and potential secondary growth structures are closely related to the environmental conditions. If some years stand out otoliths from individuals originating from these may be used as reference-age-classes.

Finally the group strongly recommends further research into the description of species specific otolith patterns as some sand eel species are not easily recognized (*A. marinus* and *A. tobianus*).

8. References

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Figures

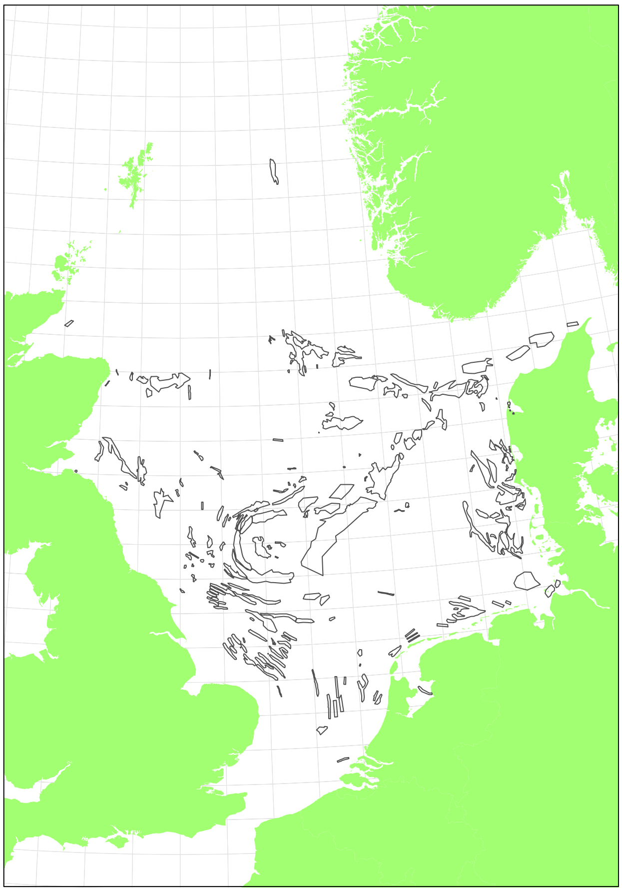

Figure 1. Distribution of sand eel fishing grounds in the North Sea (Jensen 2004)

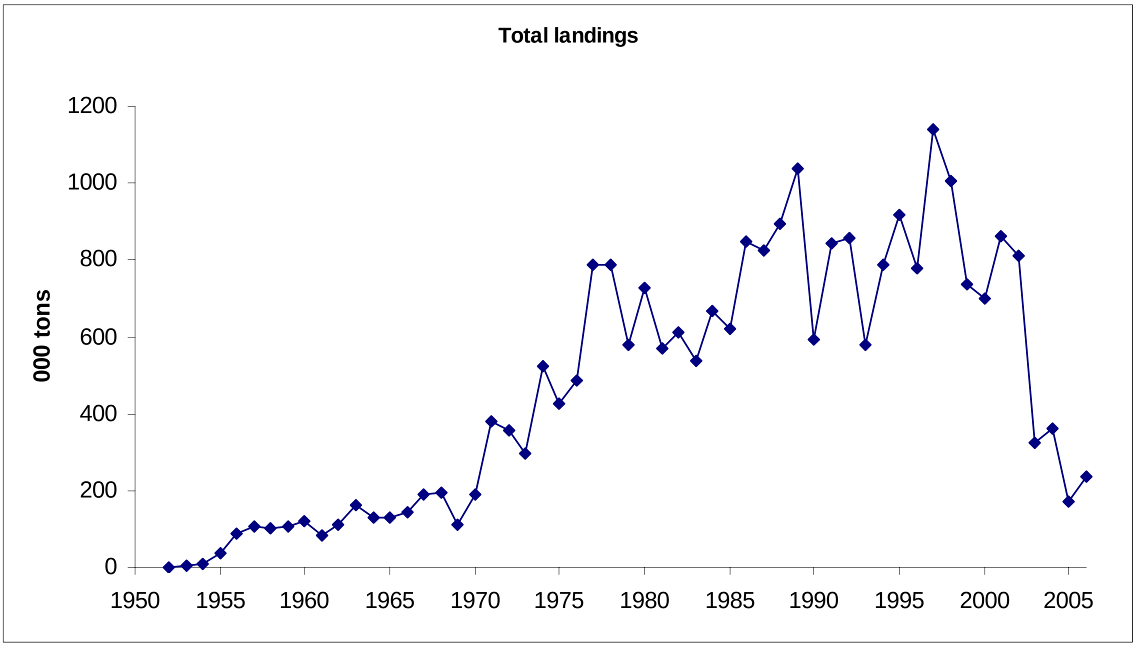

Figure 2. Total catches of sand eel since 1950 in the North Sea. Catches in 2006 only represent the first 6 months of 2006. (ICES 2006)

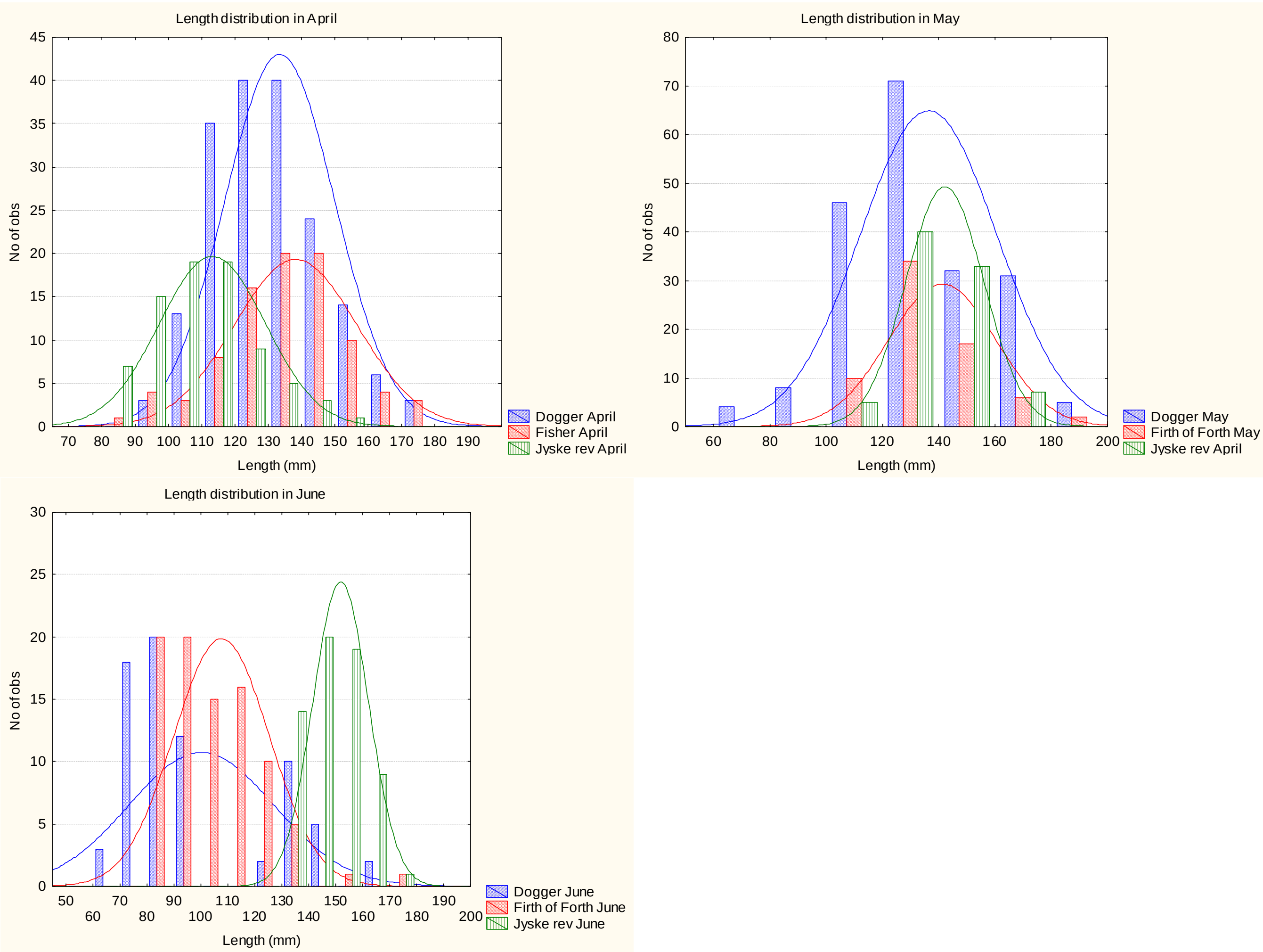

Figure 3. Length distribution of the selected individuals in the traditional exchange. All individuals were selected from the DIFRES archive of otoliths from sand eel catches in the North Sea during 2004, covering as many areas and seasons as possible.

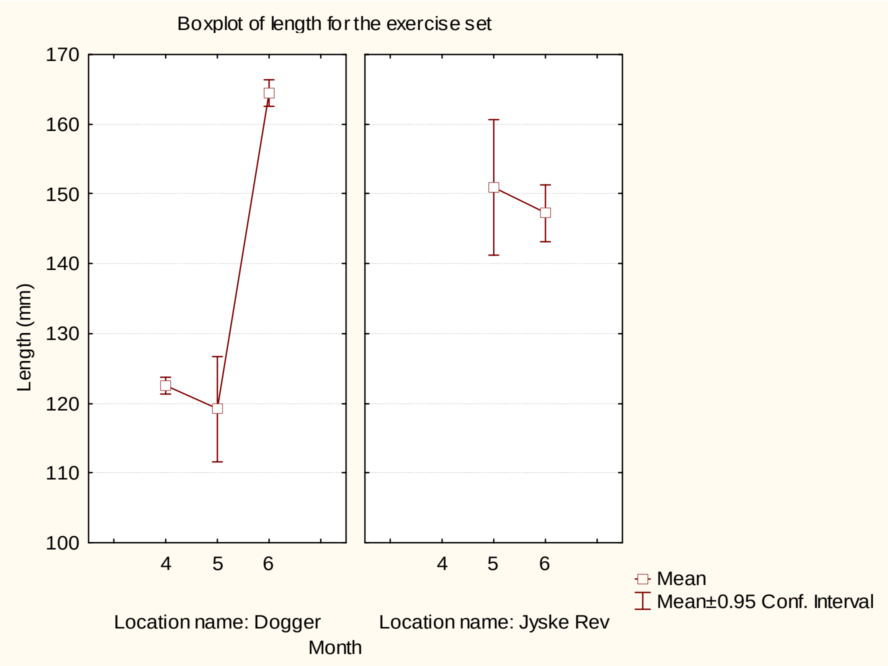

Figure 4. Box plot of the length distribution by month and area in the exercise set. All individuals were selected from DIFRES archives from sand eel catches in the North Sea during 2004, covering as many areas and seasons as possible.

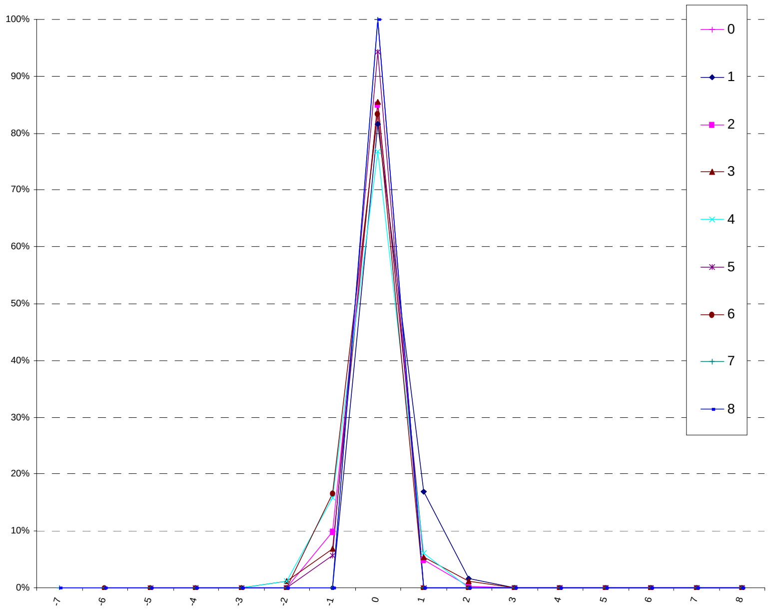

Figure 5. The distribution of the age reading errors in percentage by modal age as observed from the whole group of age readers in an age reading comparison to modal age. The achieved precision in age reading by modal age group is relatively high as the spread of the age readings errors is narrow. There appears to be no relative bias, as the age reading errors are normally distributed.

Effect	SS	df	MF	F	p
Month	1.988	2	0.99	36.42	0.000*
Location	1.768	3	0.59	21.60	0.000*
Month (excl. FB)	2.312	2	1.16	36.87	0.000*
Location (-)	3.244	2	1.66	53.67	0.000*

Table 1. The effect of month and location on the percent agreement in the exchange set. Removing Fisher Bank from the data did not change the significant effect of sampling month and location on percent agreement.

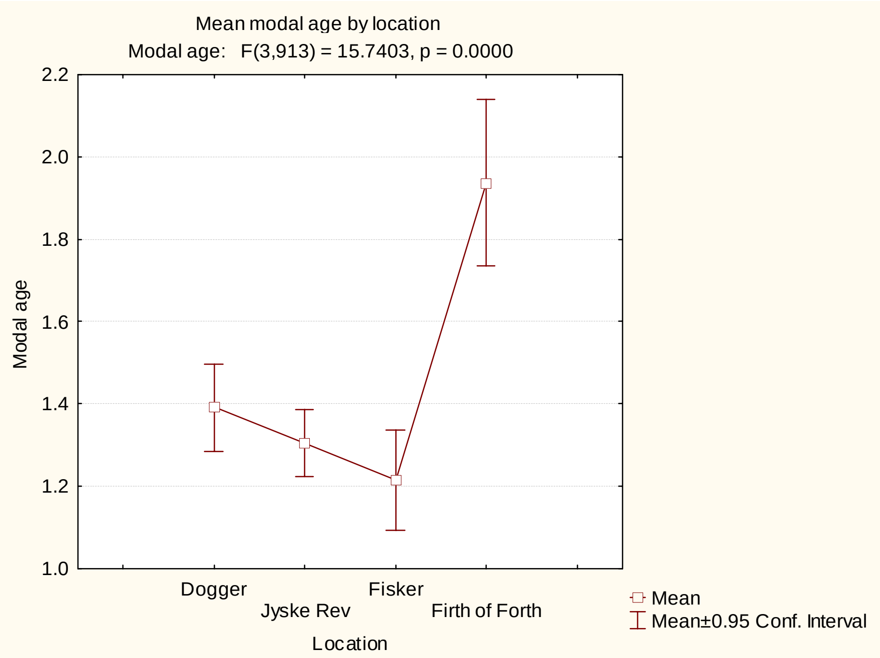

Figure 6. Mean modal age by sampling location. The modal age was significantly higher at the Firth of Forth location (p<0.00001).

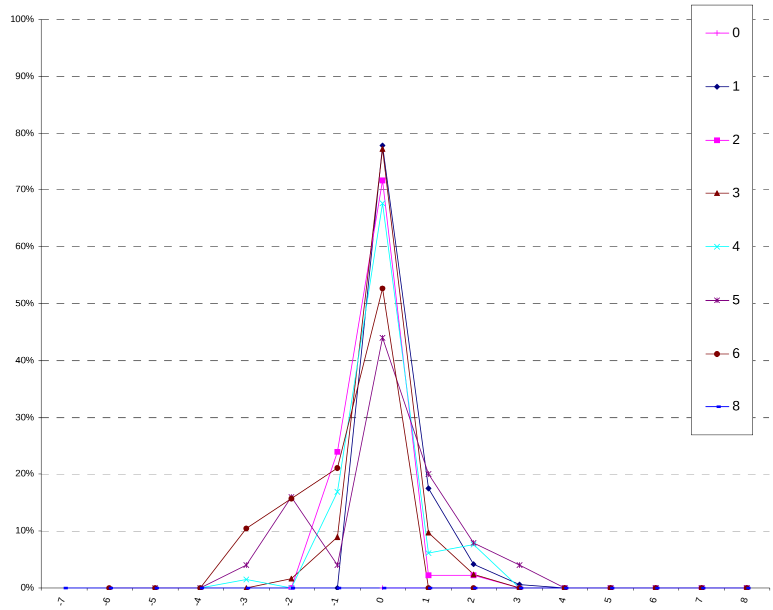

Figure 7. The distribution of the age reading errors in percentage by modal age as observed from the whole group of age readers in an age reading comparison to modal age. The achieved precision in age reading by modal age group is somewhat lower than achieved in the exchange; however, new inexperienced readers were included. There appears to be a higher tendency to under-estimate the ages as the age reading errors are shifted towards the left.

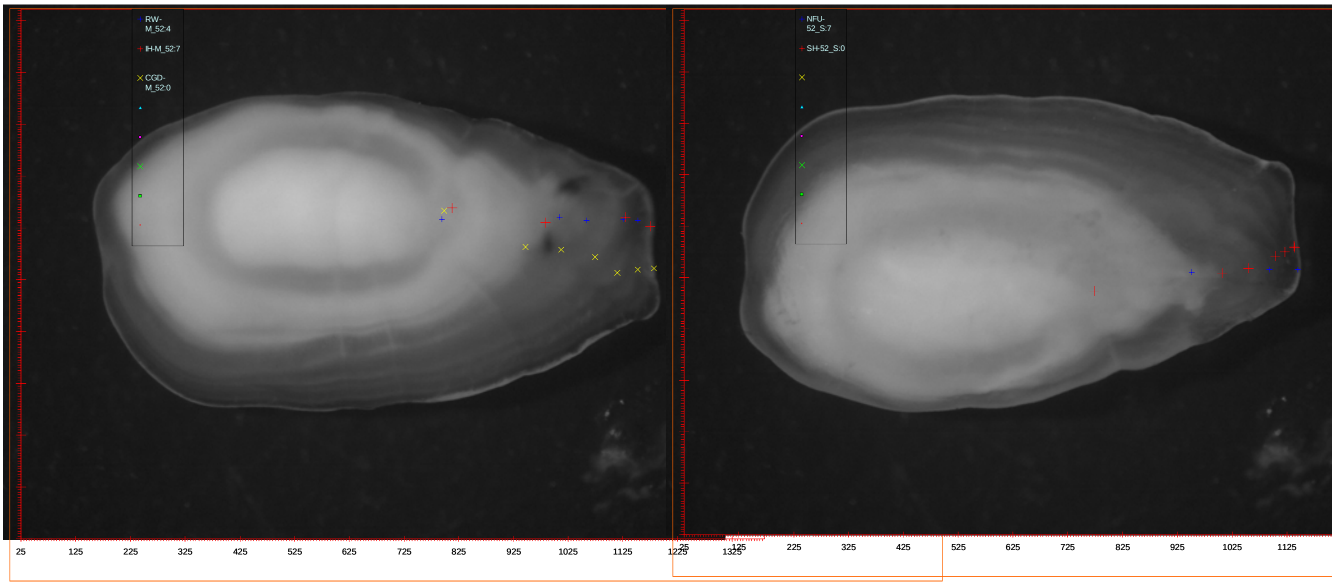

Figure 8. Individual nr. 52: From May, location 36F6; Ages: 2 – 3 – 5 – 6 – 7

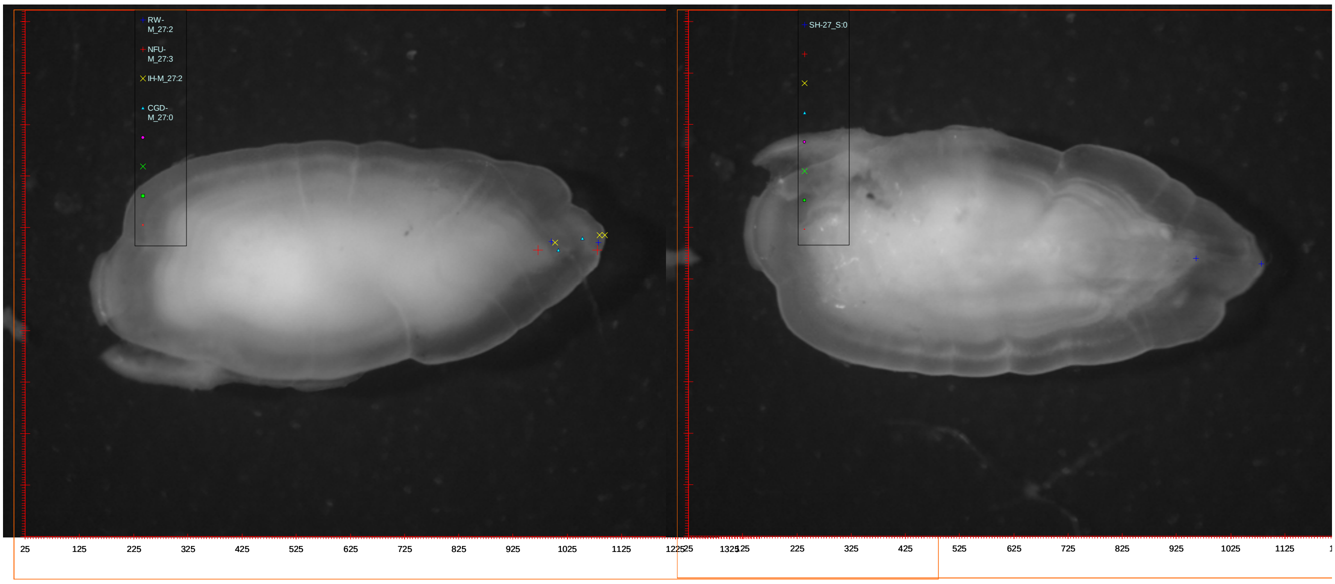

Figure 9. Individual nr. 27. From June, location 39F7; ages set as either 1 or 2-

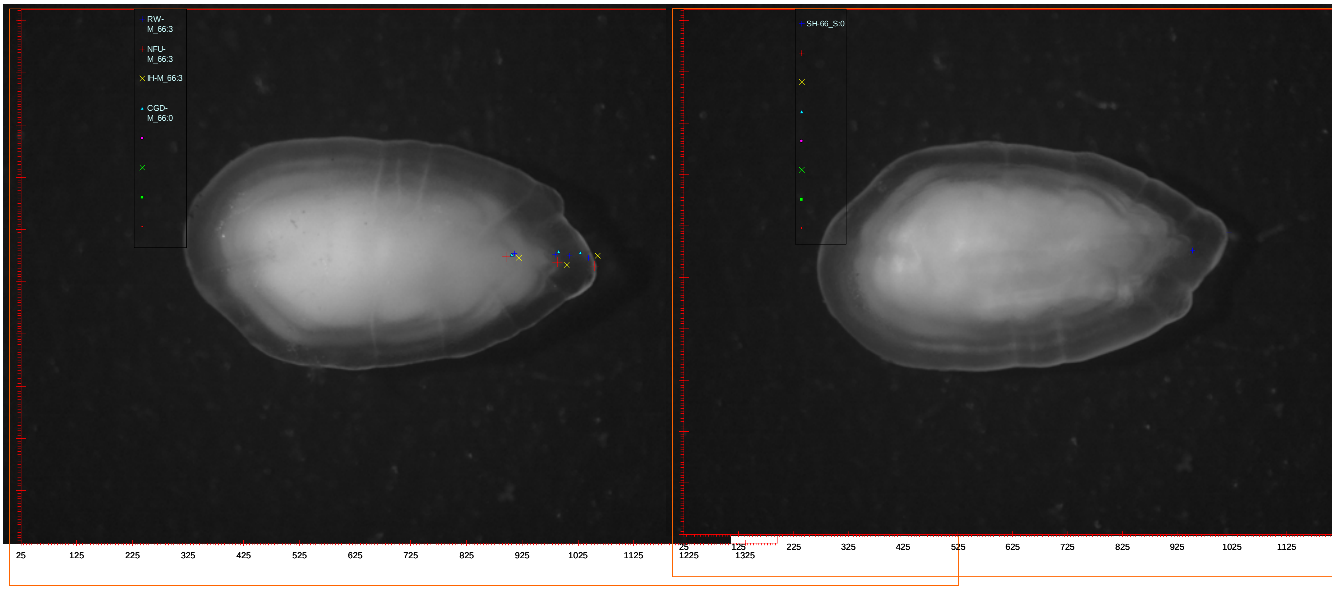

Figure 10. Individual nr. 66: From April, location 39F1; ages set as 2, 3 or 4.

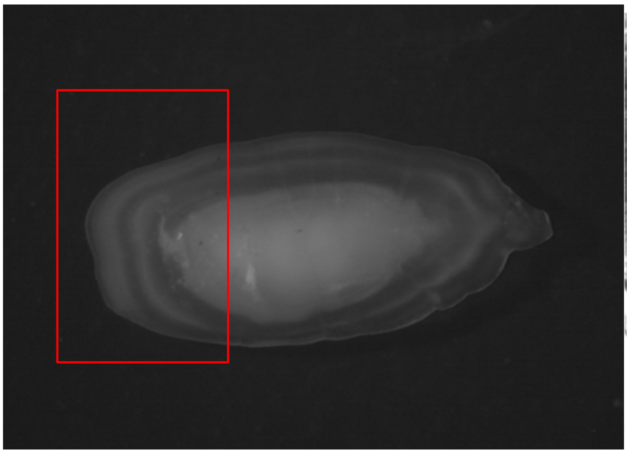

Figure 11a.

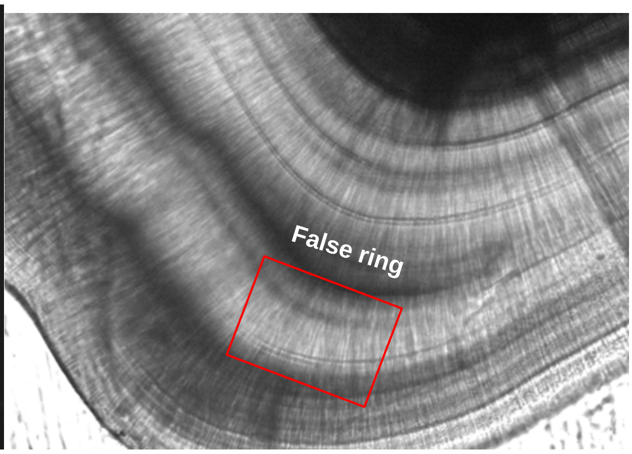

Figure 11b.

Individual caught in April 2004. The age readers discussed whether there were 2 or 3 annual structures. The otolith were polished and the 2nd translucent zone showed to have daily increments through the zone, classifying it as a false ring.

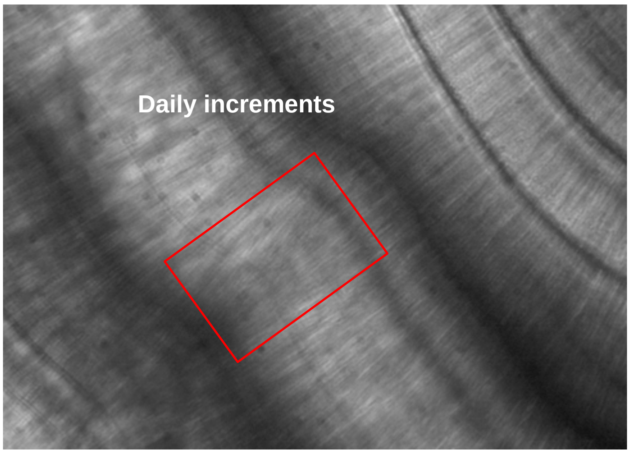

Figure 11c.