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BRIEF COMMUNICATION

A comparison of staining techniques to improve precision of age estimation from fish otoliths

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A total of 30 pairs of sagitta otoliths of horse mackerel *Trachurus trachurus* were sampled from three length-classes, and five staining methods were applied to each pair. Ten age estimates were obtained per fish, method and length-class. Significant differences in age estimates were found between methods, due to the variable enhancement in the otolith pattern produced by each method. A method using amido-schwartz as the staining reagent produced the best results in terms of precision of the age estimates.

Key words: age estimation; horse mackerel; otoliths; staining methods.

Fish age determination is a fundamental task in fisheries biology and particularly in stock assessment, where most models used in temperate waters are age-structured (Hilborn & Walters, 1992). The success of this task may, therefore, have a strong influence on the precision and accuracy of estimated stock parameters (Gulland, 1955). The greatest difficulty in the determination of fish age is to identify annual annuli in the otolith, following objective criteria which must be validated and periodically calibrated between readers. This is helped by a clear visualization of otolith patterns. Several staining methods have been used for this purpose, some of which have been compared qualitatively by Richter & McDermott (1990). In the present study a statistical comparison of age estimates for different staining techniques, especially developed for fish otoliths, was undertaken in order to test whether staining methods significantly affect the outcome of fish age estimation.

Horse mackerel *Trachurus trachurus* (L.) otoliths were chosen for this work because of their availability and also because their structure is similar to that of many other commercial species, such as hake *Merluccius merluccius* (L.) and blue whiting *Micromesistius poutassou* (Risso). It is likely, therefore, that the best staining methods for horse mackerel would also be suitable for these other species.

Five staining methods were applied, following usual laboratory safety rules, given that these procedures do not present any particular health risk: (a) thymol, immersion in thymol 5% for 12 h; (b) burning, heating on a metal plate until turning brown (Christensen, 1964); (c) amido-schwartz, immersion in acid fuchsin 1 : 500 for 12 h and then in amido-schwartz 5% for 2 min (Bouain & Siau, 1988); (d) methyl-violet, otolith surface painted with a solution of methyl-violet B and chlorydric acid (0·05 g of methyl-violet B and 1 ml of chlorydric acid 37% for 30 ml of distilled water) (Albrechtsen, 1968); (e) neutral-red, immersion for 20 min in a solution of 1 g of sodium chloride and 0·5 ml of glacial acetic acid for 100 ml of neutral-red 0·33% (A. Eltink, pers. comm.).

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TABLE I. Mean ± S.E. age calculated for each method and length-class

Length-class (cm)	Thymol	Burning	Amido-schwartz	Methyl-violet	Neutral-red
22	3·13 ± 0·07	3·10 ± 0·05	2·98 ± 0·04	2·69 ± 0·05	2·84 ± 0·06
28	6·77 ± 0·08	6·79 ± 0·07	6·67 ± 0·07	6·23 ± 0·08	7·24 ± 0·06
35	9·90 ± 0·12	10·15 ± 0·06	9·88 ± 0.06	9·60 ± 0·07	10·32 ± 0·08

TABLE II. Average per cent error calculated for each method and length-class

Length-class (cm)	Thymol	Burning	Amido-schwartz	Methyl-violet	Neutral-red
22	2·31	3·15	1·08	4·70	5·91
28	2·77	2·14	2·10	4·79	2·78
35	2·07	1·46	1·14	5·02	1·56

Thymol was used as the control treatment in the experiment, since it does not actually stain the otoliths but temporarily enhances the pattern visualization, and is used as a routine procedure for fish age determination in some laboratories.

It is possible that some staining methods perform better on small otoliths and others on large ones, due to changes in otolith structure with growth, namely the number of annuli and their distance from each other. Therefore, all five methods were applied to fish from three length-classes of 1 cm (22, 28 and 35 cm total length, L_T), from each of which 10 pairs of otoliths were randomly sampled. In order to apply all techniques to each fish, both sagitta otoliths were cut through the nucleus, perpendicularly to the longest axis, cut surfaces were polished and the age estimations with thymol were carried out. Then, each of the other four methods were applied to a half otolith. Pairs of otoliths with malformations were excluded. This procedure has the assumption that both otoliths in a pair have the same number of annuli.

Age estimations were repeated 10 times by the same person with an interval of at least 5 days, without consulting the previous results. These 10 replicates were used to account for the variability due to the age reader and to test the agreement between age determinations within each technique. The precision of age readings was quantified by the average per cent error (APE) (Beamish & Fournier, 1981) which was calculated for each method and length-class. Differences between methods and replicates, and the significance of their first order interaction were tested with a factorial ANOVA (Montgomery, 1991) for each length-class.

The ANOVA results for all length-classes were identical: significant differences were found between methods ($P<0·01$) but not between replicates ($P>0·05$) or in the interactions between these factors ($P>0·05$). The lowest ages were on average estimated with methyl-violet for all length-classes, and the highest ages with thymol for the first length-class and with neutral-red for the second and third length-classes (Table I). The method with the lowest APE was amido-schwartz, for all length-classes (Table II).

These results show that, depending on the staining method used, the determined ages can differ significantly, due to the reader ability to distinguish more or fewer annuli. Therefore, growth modelling and the estimation of other parameters involving age are likely to be influenced by the procedure followed for age estimation. Also, the lack of significant differences between replicates and of a significant interaction between replicates and methods, suggest that consistent criteria for age estimation were followed in this work.

The precision of age estimates is most likely to be correlated with the sharpness of the annuli pattern in the stained otoliths, hence the most precise estimates in terms of APE should correspond to the staining method that performed best. This was the amido-schwartz method (Table II), which produced age estimates on average close to the highest ones (Table I). Three of the methods compared here (including amido-schwartz) follow the same staining principle: firstly an acid is used to destroy the mineral part of the otolith surface, leaving the proteins which are then stained by a protein-specific dye. The use of this type of technique for age determination was validated by the observation that the patterns produced are similar to observed micro- and macro-increments of the otoliths (Gauldie *et al.*, 1990). Differences in otolith thickness are not likely to influence these results, given that staining takes place at the surface of the otolith.

A clear visualization of the otolith pattern is important not only for an effective counting of annual annuli, but also to allow the reader to apply pre-defined criteria in distinguishing between annual and non-annual annuli, which is very important for accurate age estimation. Although the staining methods tested here are expected to act in the same way on annual and non-annual annuli, given that the chemical composition of both kind is the same (Gauldie, 1990), enhancing the visualization of the otolith pattern will help to put in practice established criteria.

Although burning is probably still the most used method to facilitate fish age determination, it performed less well than amido-schwartz in this study. Moreover, it needs to be applied by an experienced person because it is very easy to heat the otoliths for too long and turn them into ash. Burning is probably quicker to apply, but if a large batch of otoliths is processed at the same time, the amido-schwartz processing time per otolith is close to the burning one. Amido-schwartz is easier to apply and gives more precise estimates, which is a good reason to use it routinely.

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