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Measurements on ground or sectioned otoliths: possibilities of bias

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Back-calculation usually requires measurements of growth marks revealed on otoliths by specific preparations. The standardization of the grinding (or sectioning) plane is necessary, but difficult, especially along the antero–posterior axis. In order to show the importance of the grinding plane, tetracycline labelling of eel otoliths (*Anguilla anguilla* L.) has been used. This marking has a calcio-traumatic effect on otoliths, which can be revealed with staining techniques. Unless the grinding plane is incorrect, the tetracycline labelling and the staining of the ground surface are then superposed.

Key words: otoliths; sectioning; measurement bias; growth rings; *Anguilla anguilla*.

I. INTRODUCTION

Otoliths are commonly used for age determination in fish. Apart from the direct age estimates given by the number of annual rings or micro-increments, measurements can be taken and used for instance in back-calculation of growth. Generally, preliminary preparations of the otoliths are necessary before observations. One of the most common preparation technique, burning and cracking the otolith, developed first by Christensen (1964), reveals growth marks but does not permit standardized measurements, because of the random alignment of the fractured plane. The staining technique, developed by Albrechtsen (1968), offers the possibility of measuring narrow marks. These measurements can be standardized, because the preparation passes through a sectioning or grinding stage (Richter & McDermott, 1990), which allows a constant ' reference plane ' to be selected. After the grinding and polishing stages, acid etching of the calcified parts of the otolith brings out some surface structures which can be underlined with many specific histological stains (Bouain & Siau, 1988; Richter & McDermott, 1990). Otolith preparations, for observation by scanning electron microscope or for acetate replicas (Brothers, 1987), have to pass through the same stages as with staining (grinding or sectioning and acid etching), and also offer the possibility of working on a standardized plane (Liew, 1974; Karakiri *et al.*, 1989; Morales-Nin & Ralston, 1990).

Back-calculation models have been discussed by several authors (see Francis, 1990, for review), but the problem of obtaining unbiased data, e.g. measurements of growth marks, has not been considered. The aim of this study is to show the importance of the otolith grinding or sectioning stages. The choice of the section plane, for the location and measurements of the marks, is important, because of the otolith morphology, ' elliptical, laterally compressed, distally concave bodies with

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their long axis oriented in an antero-posterior direction' (Pannella, 1980), with a succession of concentric layers around a nucleus.

The otolith of the European eel (*Anguilla anguilla* L.), which is very small (about 5 mm maximum), is taken as an example and it is assumed that generalizations can be made for all types of otoliths.

II. MATERIALS AND METHODS

Eels, captured from coastal lagoons of the south of France, were injected with tetracycline hydrochloride (75 mg kg^{-1} of fish) in order to label the otoliths. They were then released (50 kg ha^{-1}) in a 2 ha natural pond (Les Garcines, Camargue). Fifteen months later, they were recaptured and the otoliths extracted.

The otoliths were stored frozen, and were then embedded in a polyester resin, as described in Panfili *et al.* (1992), the convex side uppermost, in order to permit grinding of the proximal face. A standard grinding plane has been defined, which has to: (1) retain the nucleus dimensions (in the eel otolith, the nucleus is always surrounded by a thin highly stained ring with a constant diameter of 300 µm); (2) retain the otolith edge on the maximum growth axis. The same otoliths were ground by hand several times in their antero–posterior axis, with different angles and grinding planes, which were either the same or different from the standard plane (with grinding papers of 240 to 1200 grit). The grinding level was carefully checked with a binocular microscope, as the grinding progressed, in order to accentuate or reduce angles. The exact value of angles was difficult to estimate with accuracy. The otoliths were then polished under water with aluminium powder (3 µm), and the surface was etched with 5% EDTA (ethylenediaminetetraacetic acid) for 3–5 min to eliminate the superficial calcium particles. Toluidine blue (1% solution for 3–5 min) was chosen for staining; it is known as the best for many fish species (Richter & McDermott, 1990). Stains specifically colour the collagen component of otolin, the protein part of otoliths, and the toluidine blue reacts as a metachromic nuclear stain (Richter & McDermott, 1990).

After each stage, the stained otolith was photographed under reflected light against a dark background. Incident fluorescent light was also used on several examples: it reveals the tetracycline deposit, which emits a yellow-green fluorescence under UVB (490 nm exciter filter). The final result was a succession of photomicrographs of stained and tetracycline labelled preparations for the same otolith.

III. RESULTS

On the surface of the ground otolith, the staining reveals (Fig. 1), from the centre to the edge: (1) a circular and highly coloured nucleus, corresponding to the larval stage; (2) thin, more or less coloured rings, appearing with various environmental stresses which have a calcio-traumatic action, such as winter time, starvation, temperature variations etc. (Liew, 1974; Deelder, 1976; Gauldie *et al.*, 1990); (3) a large deposit of stain on the ground surface limit, between the resin and the otolith. If eels are stressed, otolith calcification is modified, and acid-etching reveals a wide deep groove, containing almost no aragonite crystals (Liew, 1974): the toluidine blue is deposited preferentially at this site. Under additional incident ultra-violet light, the tetracycline deposit fluoresces (Fig. 1). The antero–posterior grinding axis was chosen as this permits observation of the maximum growth. Staining only reveals the location of marks on the grinding surface, whereas the incident fluorescent light shows their location deep inside the otolith.

The superposition of the stained and fluorescent rings, on the left side of Fig. 1(a) and the lower part of Fig. 1(b), indicates that the labelling disrupted otolith calcification. When these two marks diverge [right side of Figs 1(a) and (b)], the surface

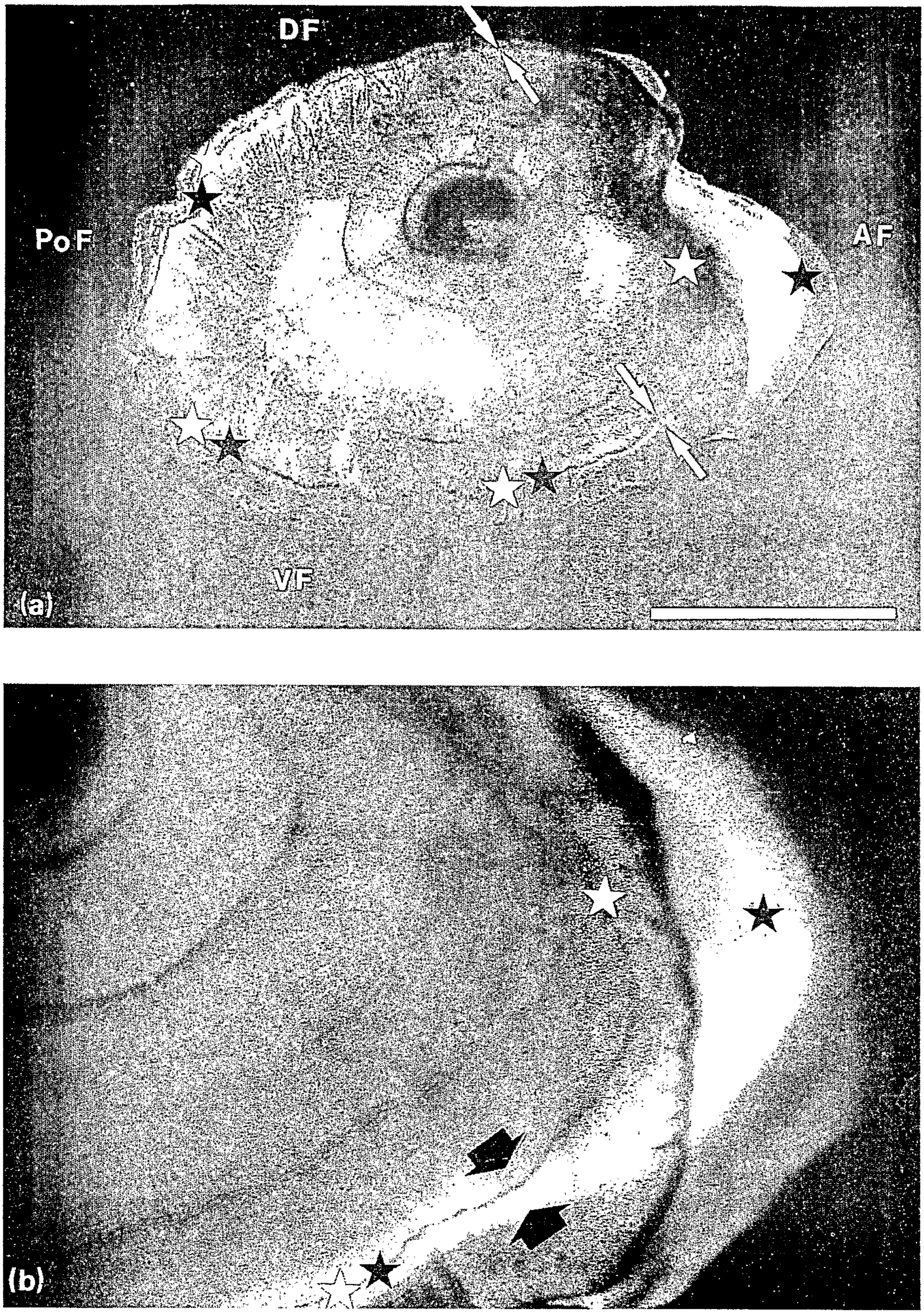


FIG. 1. Ground, stained and tetracycline labelled otolith observed under reflected light, a dark background and incident ultraviolet light. This view shows the location of the stained rings (☆) and the tetracycline deposit (★). The arrows indicate the separation of the two rings (stain and tetracycline) on the right side. The grinding plane corresponds with Fig. 2(a). AF, anterior face; DF, dorsal face; PoF, posterior face; VF, ventral face. Scale bar = 0·5 mm. (a) Whole otolith prepared and observed on the convex side. (b) Detail of the same otolith.

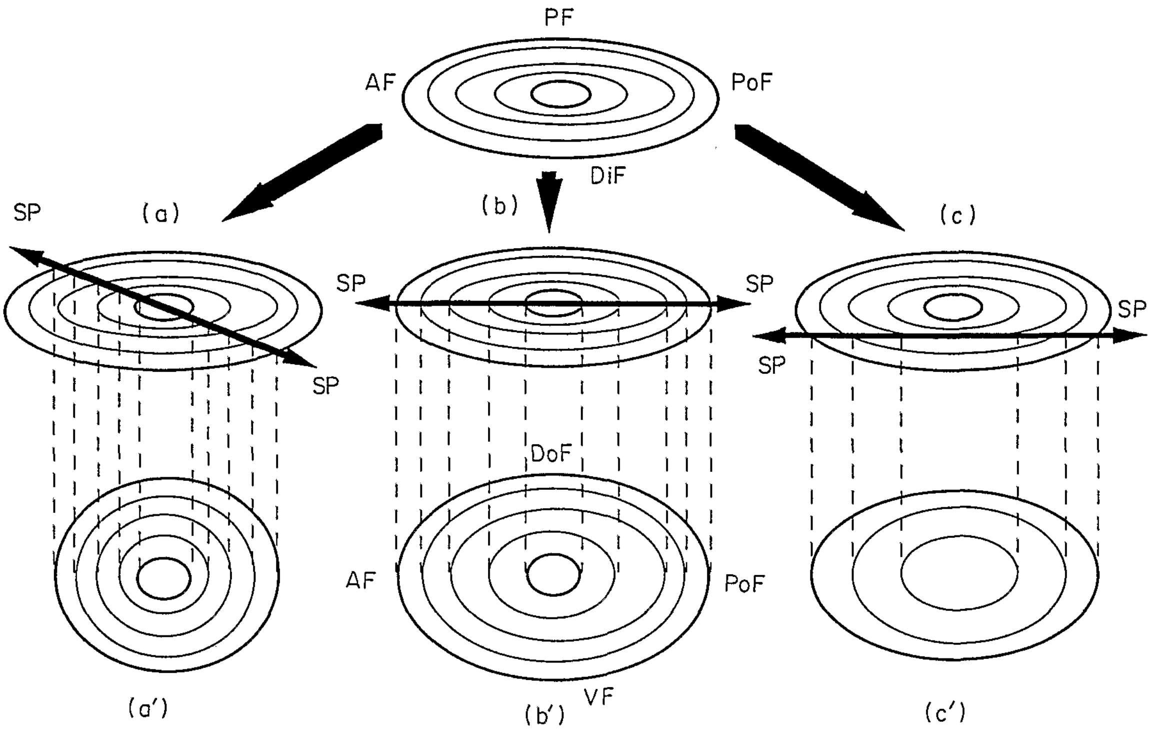


FIG. 2. Diagram of a theoretical otolith with growth rings and the effect of different grinding planes. AF, anterior face; DiF, distal face; DoF, dorsal face; PF, proximal face; PoF, posterior face; SP, sectioning plane; VF, ventral face. (a), (b), (c). Lateral view of the otolith on which the double arrows indicate the grinding or sectioning plane (SP). (a′), (b′), (c′). Proximal face of the otolith; the apparent location of growth rings on the different grinding planes is shown.

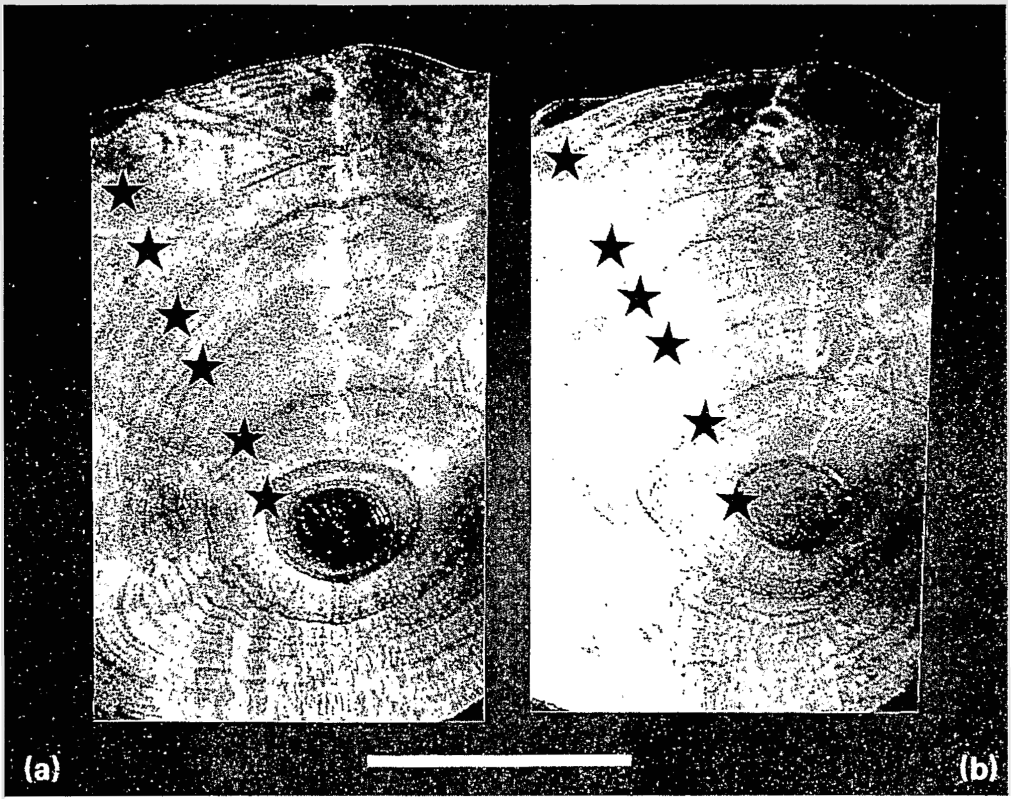
structure (staining) does not correspond to the interior structure (labelling). This divergence results from an inappropriate axis of the grinding plane, as shown on the diagram of the effect of some grinding planes on the otolith concentric rings [Figs 2(a) and (a′)].

The diagram [Figs 2(c) and (c′)] also shows another possibility of bias. When the grinding plane is correct, but the grinding is excessive, underestimations will occur in measurements. This phenomenon is represented clearly on Fig. 3, where successive grindings in the same plane have been stained and photographed: as the grinding progresses, the nucleus is reduced in size, the coloured ring patterns change and the distances between the nucleus and the edge, or between the rings, vary. Some marks can even disappear [Fig. 3(c)].

The interpretative diagram [Figs 2(b) and (b′)] points out that the best grinding plane crosses the nucleus and reaches the edge on the radius of maximum growth.

IV. DISCUSSION

The results presented above are obviously extreme, and, in practice, the preparations are often made with more appropriate grinding planes. Because of the otolith morphology, it is difficult to grind in the ideal plane [Figs 2(b) and (b′)], which passes through the middle of the nucleus and contains the edge on the largest otolith axis. This problem had already been mentioned by Lucio (1986), Brothers (1987), Radtke (1987), Maceina & Betsill (1987) and Panfili *et al.* (1990). Maceina & Betsill (1987) showed that the correlation between fish size and otolith size is



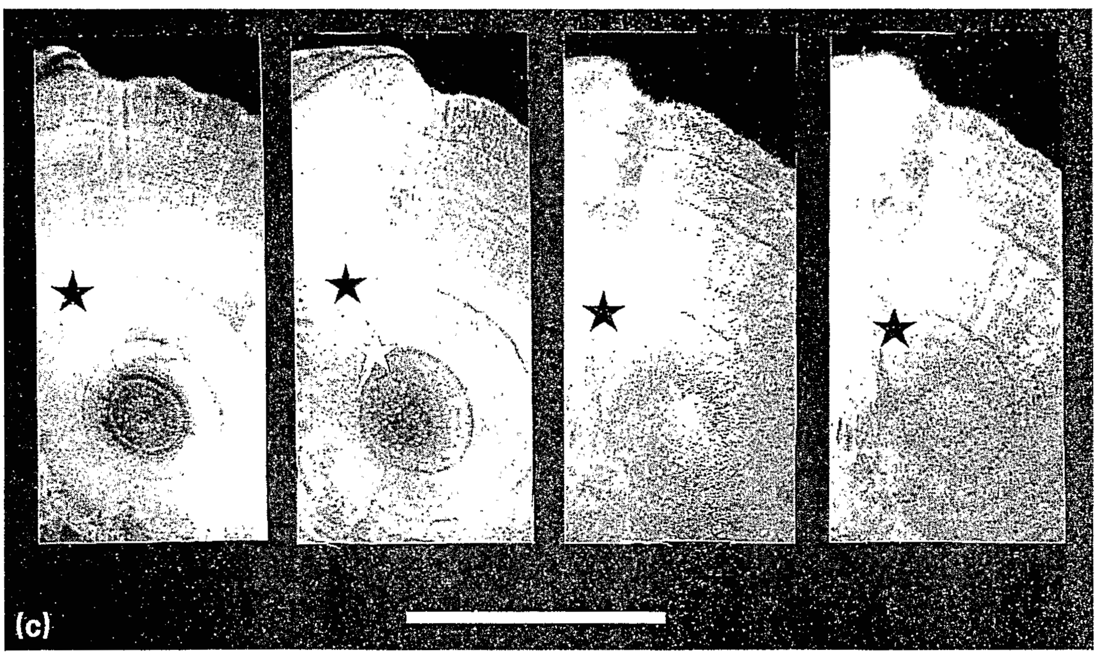


FIG. 3. Different stages of grinding and staining of two otoliths, prepared on the convex side, and observed under reflected light with a dark background. Different locations of stained rings (★, ☆) are observed on the same otolith. The grinding planes are located at different levels between Figs 2(b) and 2(c). Scale bar = 0·5 mm. (a), (b). Two grinding levels of one otolith; stage (b) is ground more than stage (a). (c). Four successive stages of one ground otolith which is ground more from the left to the right.

better for whole otoliths than for the same sectioned otoliths, which proves that the variability increases with sectioning. It is useful to find some reference marks on the otolith showing that the plane is correct. In the eel example, the nucleus diameter and the grinding level of the edge on the maximum growth axis are good criteria (see Section II above), but the latter is difficult to measure. The antero–posterior axis has been chosen for grinding, because the growth, and thus the distances between the marks, is largest. Working on the transverse dorso–ventral axis should be restricted to large otoliths, and when the growth is sufficiently rapid. In this last case, the incline of the grinding plane should not influence the mark locations excessively. However, Radtke (1987), studying a tropical fish (*Pristipomoides filamentosus*, Valenciennes 1830), found problems with the dorso–ventral axis, because the otolith nucleus ' contained several cores and the plane chosen to be sectioned for micro-increment enumeration could result in errors if more than one core were transversed '.

If only age or otolith ultrastructure are being considered, it is not necessary to obtain a very precise grinding plane, but if measurements are needed for back-calculation, a correct grinding plane is required, as well as a technique which can reveal narrow and well-defined marks. When automatic processes are used, such as sectioning of embedded otolith series (Bedford, 1983) or improved techniques, the position of the otoliths in the blocks will determine the sectioning or grinding plane; it is then difficult to work on a well-defined axis. Even with mechanical apparatus (Véró *et al.*, 1986; Karakiri & Westernhagen, 1988), constant checking of the plane is necessary during the process. When a correct grinding plane is obtained, otolith internal structures are more visible under reflected or transmitted light. Nevertheless, the growth check marks are not really clear and they are difficult to measure precisely. Staining is then an easier technique for revealing them. The scanning electron microscope method can also provide very precise measurements of the rings (Liew, 1974; Karakiri *et al.*, 1989), but on a grinding plane which cannot be determined with the same accuracy. Therefore, we think that this expensive and time-consuming method should be restricted to studying otolith ultrastructure.

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