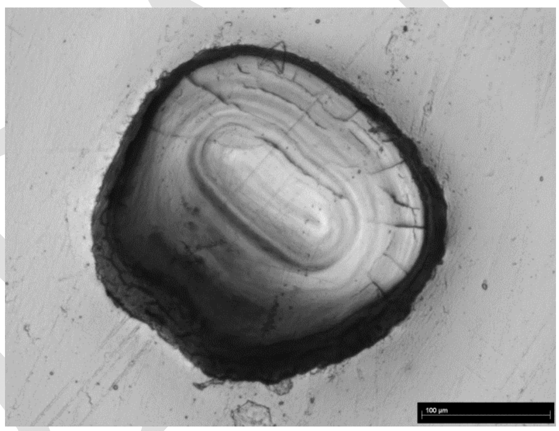
Report of the

Workshop on Age Estimation of Angler (*Lophius piscatorius*) in the Bay of Biscay

by

J. Landa, C. M. Villanueva, A. Antolínez



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1 Introduction

The state of the angler (*Lophius piscatorius*) stocks are annually assessed using models different from those structured by age (such as VPA-XSA) and annual age estimates are not taken into account in the stock assessment process. That kind of models are not used to these stocks since 2008 because the traditional age estimation criterion based on illicia was found as inaccurate.

The most recent studies on age in angler based on illicia of Jónsson (2007), Landa *et al*., (2013) and Ofstad *et al*. (2013) presented similar growth patterns (~29 and 65 cm for the ages 1 and 5, respectively) in several northern European areas (Iceland, Porcupine Bank and Faroese waters, respectively). These results are also consistent with growth estimates from length frequency analyses and tagging-recapture results. Thus, the advances in the research of the angler growth, corroborating the illicia age estimates could allow a forthcoming use of illicia in the stock assessment process.

A detailed description of the steps taken in the study of the age and growth of angler is being prepared in the Handbook of fish age (ICES, in prep.) and a summary of them is detailed below. Several age estimation workshops and exchanges for both European anglerfish species (*Lophius piscatorius* and *L. budegassa*) took place and, in general, illicia showed better precision (CV and APE), agreement and relative accuracy among readers than otoliths. The presence of multi-checks in the otolith, the increasing its opacity with age (Crozier, 1989) and the lack of a standardized age estimation criterion for the readers (Duarte *et al*., 2005) hindered that the age estimation based on otoliths to be used in the stock assessment process. So, illicia were used for stock assessment and a standardized age estimation criterion was established (Duarte *et al*., 2002). However, inconsistencies in cohort tracking of catch at age time series were found using illicia age estimates, evidencing that the traditional criterion based on illicia was not accurate for the two southern shelf stocks of each anglerfish species (Azevedo *et al*., 2008). Since then both stock/species have not been assessed using age estimates based on that criterion and a greater effort has been made to provide more accurate and corroborated growth patterns.

There has been no direct validation of age estimation for white anglerfish, but semi-direct validation has been performed in illicia and otoliths using marginal increment analysis (Woodroffe *et al*., 2003) and edge state analysis (Dupouy *et al*., 1986; Crozier, 1989; Woodroffe *et al*., 2003; Ofstad *et al*., 2013). Growth corroboration studies, such as tagging-recapture (Laurenson *et al*., 2005; Landa *et al*., 2008), micro-increment analyses (Wright *et al*., 2002) and length frequency distributions of catches (Fulton, 1903; Landa and Duarte, 2005; Jónsson, 2007), presented a faster growth rate and were basic to prove that the growth pattern estimated using the traditional criterion based on illicia (~19 and 50 cm for the ages 1 and 5, respectively) was not accurate (Landa *et al*., 2008) and showed inconsistencies in the cohort tracking (Azevedo *et al*., 2008). With the recent modifications in the methodology of illicia preparation and in the traditional age estimation criterion, a faster growth has been estimated, enabling a good cohort tracking of the catch at age data (Landa *et al*., 2013).

Therefore, taking into account the aforementioned recent advances in the knowledge of the growth pattern of angler in northern European waters based on illicia, the possibility of carrying out a Workshop on Age Estimation of Angler in the Bay of Biscay was raised, where mainly the advances made were presented, and the age estimation criterion used in IEO was described and shown to the current IFREMER colleague involved in the growth and stock assessment of angler.

2 Objectives

The objectives of this workshop are:

- Describe the current state of the art on age and growth of angler.
- Detail and show, using samples from IEO, the methodologies of preparation and age estimation using illicia that have allowed to estimate, in recent works, accurate growth patterns and fitted to the validated growth.
- Check the possible particularities of the samples from IFREMER.

The development of the workshop was agreed to be interactive, asking and solving doubts constantly. During all the days of the workshop a more theoretical-descriptive part of the methodologies of preparation and age estimation using illicia was combined with a more practical part.

3 Participants

Jorge Landa (coordinator)	IEO. Santander, Spain	jorge.landa@ieo.es
Ching Maria Villanueva	IFREMER. Brest, France	Ching.Villanueva@ifremer.fr
Ana Antolínez	IEO. Santander, Spain	ana.antolinez@ieo.es

4 Description of methodologies of preparation and age estimation in angler using illicia (theoretical)

A summary based on the manual "Age determination procedures for pelagic and benthic species from ICES area in Spanish Institute of Oceanography (IEO)" (Villamor et al., 2015) is shown below. A more detailed description and figures are available in that manual.

4.1 Sampling program and biological parameters

Biological samplings of both European anglerfish species are performed in IEO throughout the year for monitoring the state of their stocks. Samples usually come from the fish market (fish landed from the commercial fleet) and the sampling unit planned is the quarter. Also, biological samplings are performed during autumn research surveys.

Both biological parameters (length, weights, sex, sexual maturity, age, illicia edge, age estimation quality) and catch information (sampling date, fishing harbor, origin, institution, vessel name, fishing gear, species, category, etc) of each sampling are recorded in a database.

After removing, the angler illicia are stored in paper envelopes, including data of the specimen printed on their front (code, species, fish length, sex, sexual maturity, date / haul number, etc). These envelopes are stored in cardboard boxes. The skin is no retired from the illicium.

4.2 Preparation of illicia sections for age estimation

This process is also performed in IEO (C.O. Santander) for black-bellied anglerfish.

4.2.1 Material and previous preparations

The material used for the mounting of illicia in plates is the following: polyester resin, universal black colouring, catalyst / hardener, vaseline, acetone (solvent), container for the mixture, metal bar, metal plates (mould), tweezers, punch, scissors, rule paper (to place the illicia), sellotape, labels, spaghetti, microscope slides, slicing machine (essential a machine with a cutting speed of 2000 rpm or higher, a diamond sectioning blade and a cooling system). A mounting station for CS processing can be also useful (consists of: mounting jig with X-Y positioning, video camera and monitor with fixing jig, extractor hood).

<u>Preparation of the moulds</u>. A metal plate (mould) is greased with vaseline and two markers (e.g. spaghetti) are placed at an angle of 45º to indicate the number or position of each cut. The mould code is also written on its edge.

<u>Preparation of the fixing medium (resin)</u>. A jet of previously stirred black colouring is poured into a cup. Then, 100 g of polyester resin is added and all is well mixed using a metal bar, avoiding the bubbles formation. Finally, 1 ml of catalyst/hardener is added to accelerate the reaction and to harden the resin faster, and all is mixed again. The variation in the amount of catalyst to add is no so important since the mixing is already pre-accelerated and the reaction will depend mainly on environmental factors (as temperature or humidity) in the lab.

Afterwards, the mixture is poured inside the mould placed on the mounting jig and under the extractor hood, until approximately half its capacity. The mould is placed on a holder, which is adapted to the video camera, and it is left to dry until the mixture is solid enough to place the otoliths there without sinking. The mixture must to be spongy but not sticky. This usually takes 30-60 minutes, depending of factors such as the temperature or the resin viscosity.

4.2.2 Obtaining illicia sections

4.2.2.1 Placing the illicia on the plates

Illicia preparation: The illicia for sectioning are selected and located by their code. The spare parts of each illicium, both the base and the tip, are removed just leaving the main part of the illicium to be cut (~2.5 cm of illicium). It is important to be careful with the skin of the illicia and it is recommended not to remove it from the illicium or take it away but without touching the skeletal part, so the external growth increments do not disappear.

Each illicium is placed on a laminated sheet marked with groups of lines. The illicia are stuck with sellotape to avoid their movement, so they are placed in the cutting area. This cutting area is placed to a certain distance of the top of the illicia, and this distance depends on the fish length. So, for illicia of small anglerfish, the cutting area will be located e illicium top; and for larger fish, it will be placed to a higher distance (~5 mm from the illicium top)

Placing the otoliths/illicia on the plates: This process is performed using the mounting station for CS processing: on the mounting jig with X-Y positioning, using the video camera and monitor with fixing jig, and under an extractor hood. The side marks of the mould are aligned with the mark showed in the monitor. The illicia are placed one by one following the line of the monitor, so that the painted mark over the illicia coincides with the monitor line.

When all the illicia are placed on a mould, the process continues in the same way with the next one. Other mixture of polyester resin is prepared in the same way as above and very carefully poured over the illicia to prevent their displacement. Enough quantity of mixture has to be added to cover all the mould capacity. It is left to dry until is completely hard.

Marking of the cutting lines on the resin blocks: Once the resin is dry, the cutting line is marked on it coincide with the mould marks.

Removing the polyester blocks from their moulds: The resin blocks are removed from their moulds unscrewing the pieces of the moulds. When the blocks are released, the mould is cleaned with acetone.

4.2.2.2 Sectioning

After the slicing guide is lifted and the resin block are placed matching the machine pointer, the slicing machine, power cutting head and the coolant buttons are turned on to wet the base, where the resin block is placed. The lever is lowered with a light pressure until the cut is completely done. Then, the first section is removed. The resin block is moved towards the metal guide and re-adjusted with the nut. The process is repeated until obtaining several sections by block. As they are being extracted, the resin sections are cleaned using absorbent paper.

It is recommended to clean the new slides with acetone, so the sections can stick to them easily. The resin sections are impregnated with Eukitt (a transparent and fast-drying fixing medium) and placed on a slide. When they are dry, the slides with the illicia sections are stored in cardboard boxes.

4.3 Observation and age estimation

The microscope is used for the age estimation of illicia sections of both anglerfish species in Santander laboratory. A digital camera is attached to the microscope. The analogical output signal is digitized by an acquisition card connected to a computer, which carries adapted an images analysis system (TNPC 4.1, VISILOG 6.4) that integrates the image capture, the data administration and the analysis.

The age estimation criterion used in IEO and described in Landa et al. (2013) and Villamor et al. (2015) is showed below:

The traditional methodology of *illicia* mounting in resin plates was originally described by Dupouy *et al*. (1986) and, after several European age estimation workshops of anglerfish (Anon, 1997; Anon, 1999; Landa *et al*., 2002), it was standardized and was included in an age estimation guide for anglerfish (Duarte *et al*., 2002). That methodology was used in most of the growth studies using *illicia* (Duarte *et al*., 1997; Quincoces *et al*., 1998; Landa *et al*., 2001; Ofstad and Laurenson, 2007). However, several modifications in the traditional methodology of Dupouy *et al*. (1986) have been recently carried out for *illicia* preparation, observation and age interpretation (Landa *et al*., 2013). Those methodological modifications have been performed to allow a more clear observation of the growth pattern, showing mainly the most apparent growth marks, in order to allow the distinction of the annuli:

<u>Section thickness</u>. More annual increments are observed with thin illicia sections than with thicker ones. Transverse sections ~0.50–0.55 mm thick allow the observation of the most clearly marked increments, probably those that are annual. However, the observation of sections thinner than 0.5 mm (~0.4 mm) can show some false increments that can be wrongly counted as true annuli (Landa *et al*., 2013).

<u>Magnification</u>. Both, the use of a profile projector at 50 × (as it was initially used by Dupouy *et al*., 1986), or the use of a microscope at 40x, allow a better observation of the annuli. However, the use of higher magnification (100 x), which was the standard observation methodology used by Duarte *et al*. (2002) and subsequent studies, involves the observation and counting of both, true annuli and some false increments (Landa *et al*., 2013).

<u>Age interpretation</u> in *illicia* consists of identifying dark and light annual increments; although for age estimation only the dark annuli are counted (Figure 1). The annuli in some *illicia* are clearly visible because they are well defined, but the increments appear doubled in others, which makes age estimation difficult.

The age estimation guide of anglerfish (Duarte *et al*., 2002) also included some characteristics inherent to the age interpretation using *illicia* that must be considered:

It is important to adjust and play with the <u>light and focus</u> of the microscope, to identify an overall pattern of growth. Unlike otoliths, where the annuli widths tend to decrease when approaching the edge, in *illicia*, the annuli remain with a similar width throughout all the section. Annuli close to the edge may even be wider than those closer to the nucleus (Duarte *et al*., 2002).

Annuli in *illicia* differ in composition. As a result, the surface appears rippled, alternating between high and low ridges. The differences in these levels relate directly to the dark and light rings. This characteristic is very apparent from research carried out using scanning electron microscopy (Duarte *et al*., 2002).

Annuli may not be visible in all the axes of the section. Defined annuli, which are clearly visible in one part of a section may be less defined or even appear to double in another part of the section. The counting should be based upon the area where good contrast between annuli exists (Duarte *et al*., 2002).

The next step in the age estimation process is the identification of the first annulus position, and its confirmation by measuring its diameter. The first well-marked growth increment observed is considered to be a consequence of a change in the life cycle of the fish (from planktonic to benthic living), and is therefore designated as the benthic growth increment. Although the next growth increment has been traditionally considered to be the first annulus, following the age estimation guide (Duarte *et al*., 2002), the study of Wright *et al*. (2002) based on micro-increment analysis of *L. piscatorius*, concluded that that growth increment (that oval shaped in Figure 1) should not be considered as an annulus. The following growth increment, with a diameter that tends to be around 300-380 µm (Figure 1), is then considered as a real annulus (Landa *et al*., 2013), while the named benthic growth increment (not an annual increment) tends to be around 160-220 µm. Although, there is not yet validation studies on the diameters of the first growth increments for *L. budegassa*.

To identify the outer annulus, it is very important to observe the *illicium* edge. For this, it is essential to know the quarter (or month) in which the sample was taken. This will determine whether or not the annulus at the edge is to be counted in the age estimation process. If the outer annulus is not visible throughout the whole *illicium* section, this may be because the section has not been cut perpendicularly and not because it is not a true annulus. When a dark annulus appears at the edge in Q1, it should be counted and included in the age estimation. If a similar annulus appears in Q4 it should not be counted or included in the age estimation.

To estimate the age of the *illicia* of a fish group with a similar length altogether is recommended, starting first with the clearest *illicia* sections. This is a good exercise to help train the eye in identifying the typical growth pattern of the *illicia*. Since the first annuli in young fish are often difficult to define it is easier to begin estimating the age of the *illicia* of a medium-size length fish to establish the growth pattern of these first annuli (Duarte *et al.,* 2002).

Fish length can be a useful piece of information in the *illicia* age estimation. Doing a first estimation of the age and, afterwards, checking that this age estimation lies within the possible mean length range of the fish at that age is recommended (Duarte *et al.,* 2002).

Figure 1. *Illicium* of *L. piscatorius* of 89 cm and an estimated age of 8 years old. The annuli are marked with numbers, and the two structures marked in the central area, lineal and oval in shape, are both considered checks (false annual increment) (Landa *et al.*, 2013).

Difficulties in age interpretation: The age estimation of anglerfish is not easy, mainly because annuli appear doubled or are not well defined in some *illicia* sections and false annual increments may also occur (Duarte *et al.*, 2002). As an example we can see that within the *illicia* exchange of *L. piscatorius* in 2011, the "medium" credibility level was the most frequent for most readers (50%). The "high" and "low" credibility levels were estimated in a similar proportion (around 21-25%) (Landa, 2012).

Doubts in age estimation of *illicia* of intermediate ages may be related to first maturation or any other unidentified life-history event, which causes changes in the growth pattern (Duarte *et al.*, 2002).

5 Preparation and age estimation of illicia (practical)

5.1 Preparation

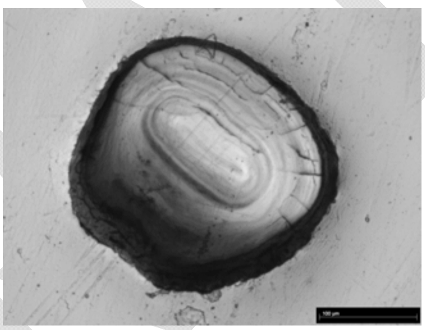
After carefully reviewing the description of the preparation methodologies, we went to the stage of putting them into practice. To this end, several illicia from ICES Div. 8c-9a collected by the IEO in the IBTS surveys were selected.
The illicia preparation was performed during the 3 days of the workshop, so that it would give enough time to a proper drying the samples before cutting them and to the subsequent observation.

5.2 Age estimation

The material for the age estimation came from:

- The samples prepared during the WK
- The images with better quality (IBTS Spain) from IEO.
- Images from IFREMER (IBTS France) (Figure 2).

In all cases, illicia from fish caught in September-October were selected in the surveys of both institutions (IEO and IFREMER). This facilitated the interpretation of the illicium edge. It is essential that in the early stages of learning a methodology the variables are minimized. In this way the workshop was more focused on the interpretation of the growth pattern.



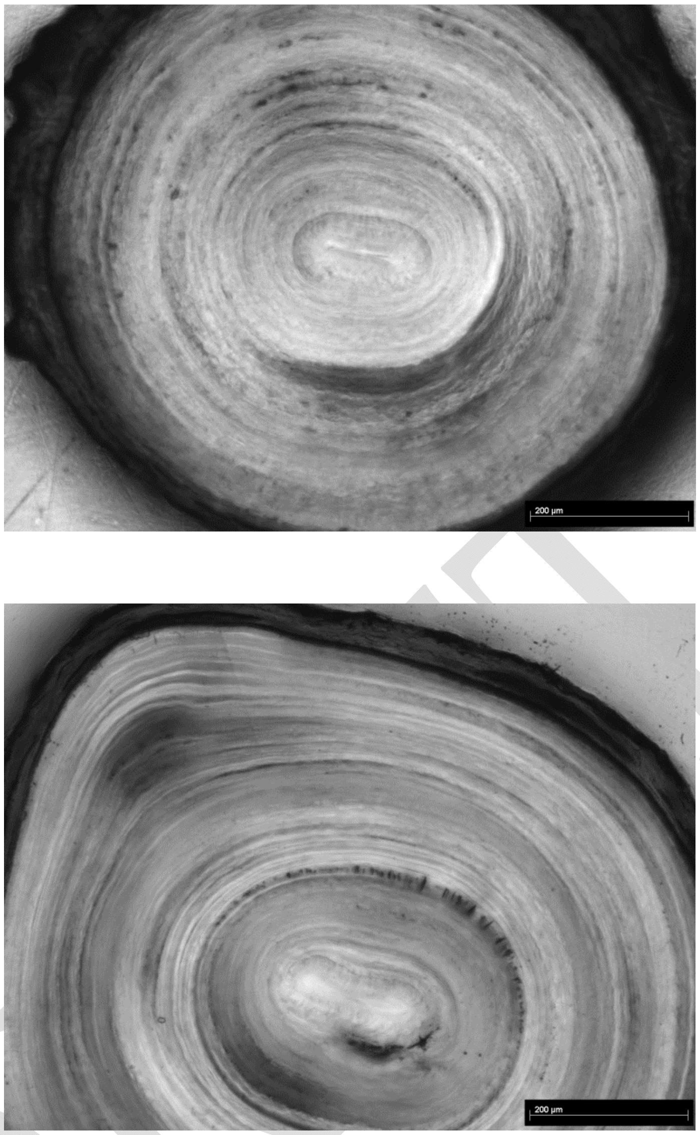


Figure 2. Images from IFREMER (IBTS France).

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