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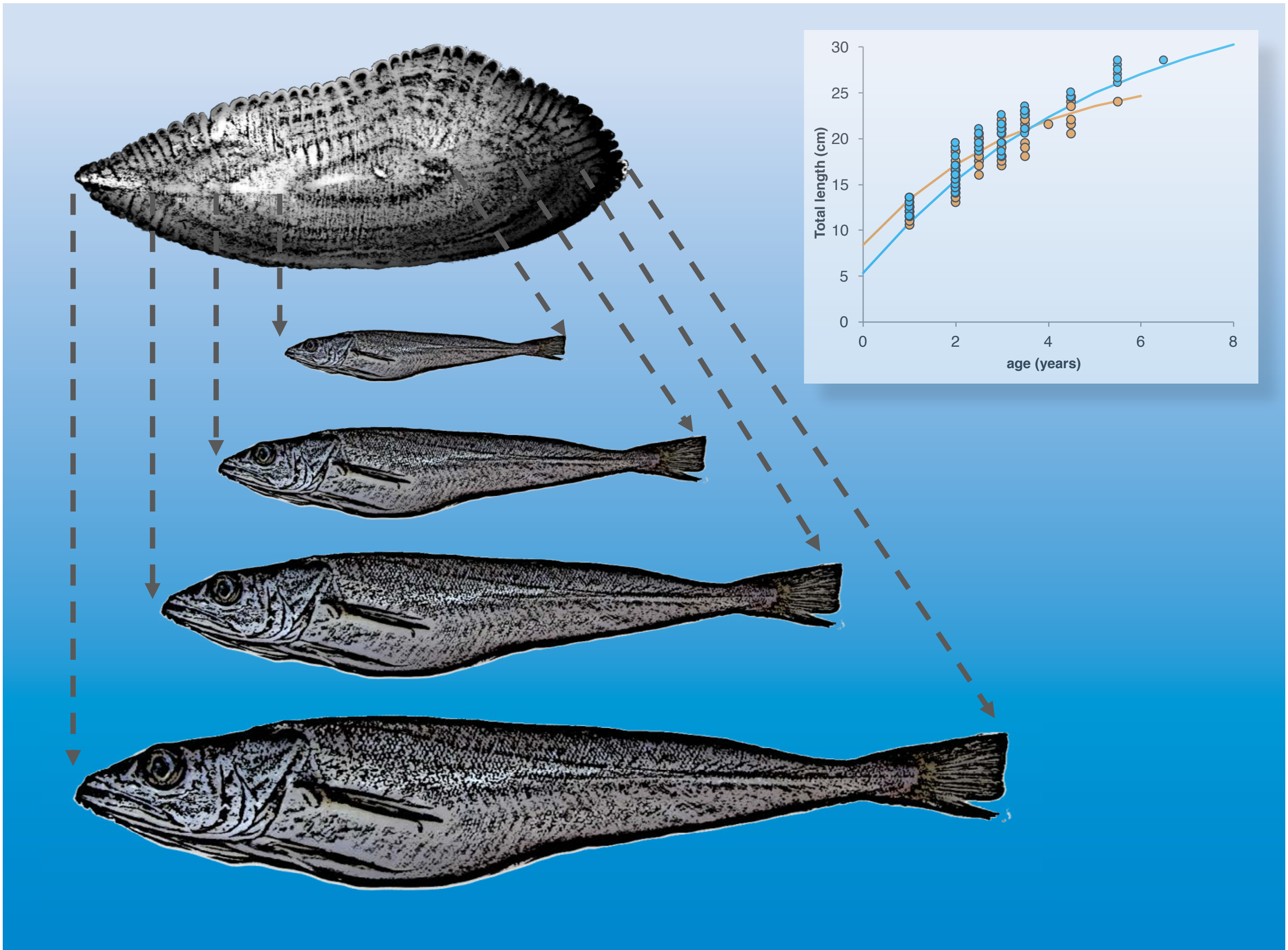
HANDBOOK ON FISH AGE DETERMINATION
a Mediterranean experience



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GENERAL FISHERIES COMMISSION FOR THE MEDITERRANEAN

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HANDBOOK ON FISH AGE DETERMINATION

a Mediterranean experience

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Preparation of this document

This publication is part of the Studies and Reviews series of the General Fisheries Commission for the Mediterranean (GFCM), which focuses on specific aspects of scientific interest for Mediterranean and Black Sea fisheries. This handbook is the fruit of the coordinated work by 23 researchers from different institutes and stems from an experience on fish ageing analysis carried out at the Mediterranean level. It falls within one of the main targets of the mid-term strategy (2017–2020) towards the sustainability of Mediterranean and Black Sea fisheries implemented by the GFCM, which aims, among other things, to enhance knowledge and expertise on fisheries namely by strengthening data collection and information.

Since 1984, several institutes in Italy have been involved in national and international scientific programmes on fisheries, focused on the study of the biology, ecology, population dynamics and assessment of the most important fishery resources, such as *Merluccius merluccius*, *Mullus barbatus*, *Sardina pilchardus* and *Engraulis encrasicolus*. In accordance with regulations, (including within the GFCM Data Collection Reference Framework [DCRF] and, at the European level, the Data Collection Framework [DCF]), biological, environmental, technical and socio-economic data have been regularly collected for the fishing and processing sectors. Since the beginning of data collection, the need for coordination was present and there was general agreement that regional coordination would greatly increase the efficiency of national programmes. In light of this, a working group on fish ageing analysis was created in 2014 under the supervision of the Italian Society of Marine Biology (SIBM). The present volume reports the main results of this working group.

This document was produced with the financial support of the European Union. The views expressed herein can in no way be taken to reflect the official opinion of the European Union.

Abstract

Fish age, among other biological parameters, is one of the most relevant pieces of data in reaching sustainable exploitation of fishery resources. Indeed, most analytical methods used in stock assessment require knowledge of demographic structure according to the age of stocks, as well as to recruitment, growth, maturity, natural mortality, etc., which are strictly linked to information on age and age structure.

The literature on ageing analysis shows some gaps regarding ageing schemes, criteria and methodologies used in preparing calcified structures. These aspects affect both the precision and accuracy of age estimation. One action that could be taken to overcome this gap was to formalize a handbook that clarified approaches to ageing schemes, criteria and preparation methods. Having a common protocol is fundamental to decreasing relative/absolute bias associated with the activities of age determination and to improving the precision (reproducibility and reduction of the coefficient of variation) of age readers from the various laboratories. In the light of these considerations, this handbook aims to be a guideline to standardizing the methods used in fish ageing studies. The document is focused on a description of the general principles on which age analysis relies (assignment of birth date, preparation methods, aging scheme reading and identification of true and false rings). Moreover, common shared analysis methods can enable a high level of calibration among the diverse institutes involved, thus improving the quality and reliability of results.

The volume is subdivided into five main sections: small pelagic species, demersal species, cartilaginous species, large pelagic species and diadromous species. For each section, information on extraction and storage, preparation method, interpretation of age (age scheme) and ageing criteria are provided by species. In total, 30 species were analysed: 6 small pelagic, 12 demersal, 5 cartilaginous, 6 large pelagic and the European eel. These species represent some of the most important fish from an economic and ecological point of view. Thus this volume represents one of the most complete outlooks for fish ageing analysis in the Mediterranean context.

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Acronyms

AA.VV.	autori vari/various authors
AL	anus length
ALK	age–length key
APE	average percent error
BM	birthmark
CAS	catch at size
CFL	curve fork length
CMSY	catch maximum sustainable yield
CS	calcified structure
CV	coefficient of variation
DCF	Data Collection Framework
DCRF	Data Collection Reference Framework
DL	disc length
DW	disc width
EC	European Commission
EDTA	ethylenediamine tetra-acetic acid
EMU	Eel Management Unit
EU	European Union
FAD	fish-aggregating device
FAO	Food and Agriculture Organization of the United Nations
FL	fork length
GFCM	General Fisheries Commission for the Mediterranean
GSA	geographical subarea, sensu FAO-GFCM
G1	group one: including species under EU management or recovery plans or EU long-term multiannual plans or EU action plans for conservation and management and for which assessment is regularly carried out
G2	group two: including species that are important in terms of landing and/or economic values, and for which assessment is not regularly carried out
IA	integrated analysis
IAPE	index of the average percentage error
ICCAT	International Commission for the Conservation of Atlantic Tunas
ICES	International Council for the Exploration of the Sea
LD1	length to first dorsal fin
LJFL	lower jaw – fork length
L/F	length/frequency
LFD	length/frequency distribution analysis
LHead	length of head
LV	length of vertebrae
MEDIAS	Mediterranean Acoustic Survey
MEDITS	Mediterranean International Bottom Trawl Survey

PA	percentage of agreement
PGCCDBS	Planning Group on Commercial Catch, Discards and Biological Sampling (ICES)
RV	radius of vertebrae
SCAA	statistical catch at age
SFL	straight fork length
SIBM	Italian Society of Marine Biology
SPF	small pelagic fish(es)
SS3	stock synthesis
STECF	Scientific Technical and Economic Committee for Fisheries
TL	total length
TW	total weight
VBGF	Von Bertalanffy growth formula
VPA	virtual population analysis
XSA	eXtended survival analysis
YOY	young of the year

1. Introduction

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Since 1984, several institutes in Italy have been involved in national and international scientific programmes on fisheries, focused on the study of the biology, ecology, population dynamics and assessment of the most important fisheries resources, such as European hake (*Merluccius merluccius*), red mullet (*Mullus barbatus*), deep-water rose shrimp (*Parapenaeus longirostris*), giant red shrimp (*Aristaeomorpha foliacea*), blue and red shrimp (*Aristeus antennatus*), sardine (*Sardina pilchardus*), European anchovy (*Engraulis encrasicolus*), swordfish (*Xiphias gladius*), etc. (Relini, 2000; Relini, Carpentieri and Murenu, 2008).

Most analytical methods used in stock assessment – such as virtual population analysis (VPA) and statistical catch at age (SCAA) – require knowledge of demographic structure according to the age of stocks. Moreover, they require information on other biological parameters and processes, such as recruitment, growth, maturity, natural mortality, etc., that are strictly linked to information on age and age structure. Thus it is clear that the availability of accurate information provided by age determination analysis – on age, age structure and growth parameters of exploited stocks – is fundamental to the reliability of scientific advice and the efficacy of the resulting management measures (GFCM, 2017; STECF, 2017).

Fisheries data are commonly collected by length. Length data are generally much easier and cheaper to collect than age data (Hoggarth *et al.*, 2006; Froese, Thorson and Reyes, 2014). The conversion of the length structure of a stock to the age structure required by VPA- and SCAA-based stock assessment models is usually performed by means of age slicing procedures using growth parameters (i.e. the Von Bertalanffy growth formula [VBGF]), or by age–length keys (ALKs) to convert size distribution into age distribution. Despite the availability of models able to perform this conversion internally (e.g. integrated analysis [IA]), this process is usually carried out during data preparation for stock assessment. The use of inappropriate growth parameters or ALKs to convert length distribution into age structure can lead to assessment outputs providing unreliable stock status figures (STECF, 2017). In light of these considerations, it is fundamental that accurate and reliable information on age and growth is made available to end-users and experts involved in stock assessment working groups, such as those carried out under the European Union's Scientific Technical and Economic Committee for Fisheries (STECF) and FAO's General Fisheries Commission for the Mediterranean (GFCM).

It is well known that fish have several calcified structures (CSs) (as do other organisms, such as cnidarians, molluscs, crustaceans, etc. [Panfili *et al.*, 2002]) that can be used for age determination and growth parameter estimation, with the aim of obtaining the age composition of exploited fish populations and stocks. Fish ageing analysis relies on the presence on those CSs with a structural pattern of growth rings in terms of a succession of opaque and translucent zones, as well as on knowledge of the periodicity of that deposition and growth pattern.

In fish, there are several CSs that can be used for ageing analysis: otoliths (sagittae, lapilli, asterisci), scales, vertebrae, spines and opercular bones (Panfili *et al.*, 2002).

So far, about 26 species and taxa are subject to ageing analysis by Italian institutes involved in fisheries data collection and research (i.e. the Data Collection Framework [DCF] in the context of European Union [EU] Council Regulation 1004/2017; GFCM Data Collection Reference Framework DCRF [GFCM, 2018]) (Table 1): bogue (*Boops boops*), tub gurnard *(Chelidonichthys lucerna*), *E. encrasicolus,* grey gurnard (*Eutrigla gurnardus*), blackbellied angler *(Lophius budegassa*), angler (*Lophius piscatorius*), *M. merluccius,* blue whiting *(Micromesistius poutassou*), *M. barbatus,* surmullet *(Mullus surmuletus*), common pandora (*Pagellus erythrinus*), *S. pilchardus,* Atlantic mackerel *(Scomber scombrus*), Atlantic chub mackerel (*Scomber colias*), common sole (*Solea solea*), picarel (*Spicara smaris*), Mediterranean horse mackerel (*Trachurus mediterraneus*), Atlantic horse mackerel (*Trachurus trachurus*), European eel (*Anguilla anguilla*), skates (*Raja* spp.*),* dogfishes nei *(Squalus* spp.*),* Atlantic bluefin tuna *(Thunnus thynnus*), albacore *(Thunnus alalunga*), swordfish (*Xiphias gladius*), Atlantic bonito (*Sarda sarda*), common dolphinfish (*Coryphaena hippurus*).

In bony fish, otoliths (sagittae, in particular) are generally used for age determination of demersal species, with the exception of anglerfish, *L. budegassa* and *L. piscatorius*, in which the thin transverse section of the illicium (first transformed spine of the dorsal fin) is preferred (Landa *et al.*, 2002; Duarte *et al.,* 2005. In large pelagic species (i.e. tuna, swordfish), several CSs can be used, such as otoliths, vertebrae and spines, while in elasmobranchs, a section of vertebrae and/or spines are usually used.

Stock assessment and management need information on an annual basis on fishing effort, total production, size distribution and age composition of catches, etc. from all GSAs in the Mediterranean. The Italian institutes (Table 1) involved in fisheries data collection and research, collect and process biological data, both from commercial landings (EU DCF and GFCM DCRF) and scientific surveys (e.g. the Mediterranean International Bottom Trawl Survey

TABLE 1 – Italian Institutes involved in fish ageing analysis for the Data Collection Framework

Institutes	Area covered	Group(s) of species studied
Centro Interuniversitario di Biologia Marina ed Ecologia Applicata "G. Bacci" – Livorno	GSA 9 – Ligurian Sea and northern Tyrrhenian Sea	Demersal and small pelagic species
Università di Cagliari – Dipartimento di Scienze della Vita e dell'Ambiente	GSAs 11.1 and 11.2 – Sardinian Sea	Demersal and small pelagic species
COISPA Tecnologia & Ricerca – Bari	GSA 10 – southern and central Tyrrhenian Sea GSA 18 – southern Adriatic Sea GSA 19 – western Ionian Sea	Demersal and small pelagic species
Consiglio Nazionale delle Ricerche (CNR) – Istituto per l'Ambiente Marino Costiero (IAMC) – Mazara del Vallo, Trapani	GSA 16 – southern Sicily	Demersal and small pelagic species
Consiglio Nazionale delle Ricerche (CNR) – Istituto di Scienze Marine (ISMAR) – Ancona	GSA 17 – northern Adriatic Sea GSA 18 – southern Adriatic Sea	Demersal and small pelagic species
Laboratorio di Biologia Marina e Pesca – Fano	GSA 17 – northern Adriatic Sea	Demersal and small pelagic species
Università di Bari – Dipartimento di Zoologia	GSA 19 – western Ionian Sea	Demersal and small pelagic species
Università di Roma "Tor Vergata" – Dipartimento di Biologia	All GSAs	European eel
UNIMAR – Rome	All GSAs	Large pelagic species

[MEDITS – AA.VV., 2017b]), covering seven GSAs. Each year, more than 70 000 calcified structures are analysed. In this context, thorough methodological standardization in extracting, preparing and reading CSs is crucial.

Having a common protocol is fundamental in decreasing relative/absolute bias associated with the activities of age determination and in improving the precision (reproducibility and reduction of the coefficient of variation [CV]) of age readers from the various laboratories (PGCCDBS, 2011). In the light of these considerations, this handbook aims to be a guideline to standardizing the methods used in fish ageing studies. The document focuses on a description of the general principles on which age analysis relies (assignment of birth date, preparation methods, ageing scheme reading and identification of true and false rings). Moreover, common shared analysis methods can enable a high level of calibration among the diverse institutes involved, thus improving the quality and reliability of results.

1.1 Sampling methods

Knowledge of the age structure of fish populations can be used to estimate mortality, growth rates, gear selectivity, cohort strength, and other demographic and population dynamics parameters. However, age information is often costly to obtain. High costs force many management programmes to limit the number of fish age-analysed directly, and to rely on ALKs or on age slicing from growth parameters to estimate the age composition of fish stocks.

Proportional subsampling of the catch is desirable as it is based on multiple statistical properties. However, fixed subsampling is frequently used because of improved efficiency in field operations. Instructing field and laboratory staff to collect CSs from a fixed number of fish by length class is much easier than sampling fish by length in proportion to the abundance of each length-class in the catch. The use of ALKs or of age-slicing procedures to provide unbiased age composition of a stock requires that the analysed fish are representative of the whole population. This implies that they are taken with the same gear and in the same season and spatial location as the unanalysed fish (Ricker, 1975; Kimura, 1977). The ability of ALKs to accurately represent the actual age structure of the entire population depends on many factors, such as the sampling strategy (fixed versus proportional subsampling), life span (i.e. short- or long-lived species), exploitation status and recruitment strength (Coggins, Gwinn and Allen, 2013).

The optimum number of otoliths per length class cannot always be provided *a priori*. A description of the optimum sample size for age reading and length measurements dependent on a universal cost function is given in Oeberst (2000). Moreover, according to Mandado and Vásquez (2011), a sample of 20 otoliths per length class is considered the optimum for a species with 30–40 length classes. Coggins, Gwinn and Allen (2013) showed that ten specimens aged per length class (500–1000 fish in total) provide unbiased ALK for both short- and long-lived fish. Negligible benefits were achieved collecting more than ten fish per length class (Coggins, Gwinn and Allen, 2013).

Experiences gathered from the samplings of commercial catches in Italian GSAs evidenced an acceptable coefficient of variation (about 5 percent) when five otoliths per length class (0.5 or 1 cm depending on the species), sex and quarter are collected. The following criteria are taken into account to set the sample size:
- For the smallest size groups, which presumably contain only one age group, the number of otoliths per length class may be reduced.
- In contrast, more otoliths per length are required for the largest length classes (Table 2 provides general criteria).

The combination of data from surveys and landings/discards sampling can contribute to better coverage of a population at sea for growth estimation.

Biological samplings are carried out both at sea, during scientific trawl surveys (i.e. the Mediterranean Acoustic Survey [MEDIAS] and MEDITS), on board commercial vessels, and at landing points (AA.VV., 2017a; GFCM, 2018). Biological sampling of commercial fisheries covers all four quarters of the year, while scientific surveys are usually performed in one season.

1.1.1 MEDITS sampling

In the case of the MEDITS survey, otolith collection and age determination are mandatory for the following species: *M. merluccius*, *M. barbatus* and *M. surmuletus*. Otolith sampling and age determination address several objectives:
- estimate indices of abundance-at-age and monitoring of stock age structure over time;
- monitor spatial distribution of age groups;
- use length-at-age data to estimate growth curves;
- estimate age-based survey indices to be used as tuning information in stock assessment models (i.e. VPA, SCAA, IA); and
- use age data to estimate ecosystem indicators (Barot *et al.*, 2004).

The sampling design adopted is a stratified sampling based on fish size (total length [TL]), in which a fixed number of individuals are randomly collected by length class and sex (Table 2) (AA.VV., 2017b). To avoid samples deriving from only a few hauls, the stratification scheme also includes the haul factor (maximum two pairs of otoliths by length class, sex and haul).

TABLE 2 – MEDITS survey otolith sampling scheme

Species	Length class (cm)	Sample size	Sex
Merluccius merluccius	1	5	M≤14 cm TL
		10	M≥15 cm TL
		5	F≤25 cm TL
		10	F≥26 cm TL
Mullus barbatus	0.5	5	M≤9 cm TL
		10	M≥9.5 cm TL
		5	F≤9 cm TL
		10	F≥9.5 cm TL
Mullus surmuletus	0.5	5	M≤9 cm TL
		10	M≥9.5 cm TL
		5	F≤9 cm TL
		10	F≥9.5 cm TL

Note: M = male, F = female.

For each fish sample, the CSs are usually stored in labelled plastic vials. The code reported on the labels is a combination of both biological (i.e. species name, individual size, sex) and sampling information (i.e. date and haul code).

1.1.2 Sampling of landings/discards of demersal and small pelagic species

The main objective of fisheries data collection is to obtain the demographic structure (by length and age) of the catch (i.e. landings and discards) of each stock. This represents the most important input data for most stock assessment methods (e.g. VPA- and SCAA-based models) for assessing the state of exploitation of the stocks. In each GSA, the sampling design adopted is represented by a stratified random sampling with quarter and *métier* (i.e. fishing technique, such as bottom trawl, pelagic trawl, longline, gill net, trammel net, purse seine, etc.) considered as strata. Species are divided into two main groups: G1 species – which drive the international management process, including species under EU management or recovery plans or EU long-term multiannual plans or EU action plans for conservation and management and for which assessment is regularly carried out – and G2 species – which are important in terms of landings and/or economic values, and for which assessment is not regularly carried out. G1 species are: *M. merluccius*, *M. barbatus*, *M. surmuletus*, *S. solea*, *E. encrasicolus*, *S. pilchardus* and Elasmobranchii. For these species, a fixed number of CSs are randomly collected to achieve a total number of eight CSs (four by sex) for each length class. Length classes are by 1 cm for *M. merluccius*, *S. solea* and Elasmobranchii, and 0.5 cm for *E. encrasicolus*, *S. pilchardus* and *M. barbatus*. G2 species sampling is based on the same protocol with the exception of the *métier* level, which is not considered in the stratification scheme. G2 species are: *B. boops*, *C. lucerna*, *E. gurnardus*, *L. budegassa*, *L. piscatorius*, *M. poutassou*, *P. erythrinus*, *S. scombrus*, *S. colias*, *S. smaris*, *T. mediterraneus* and *T. trachurus*.

Stock management requires information annually owing to the interannual variation in recruitment, which ultimately influences population abundance and age structure. For G1 stocks, collection and analysis of otoliths on an annual basis is mandatory (AA.VV., 2017a; AA.VV., 2017b; ICES, 2015a; GFCM, 2018).

An example of the frequency of sampling for age determination is reported in Table 3 in accordance with the protocol planned in the Italian national programme (AA.VV., 2017a). Data are collected each year, but are provided on an annual basis only for G1 stocks, and every three years for G2 species.

TABLE 3 – Long-term planning of sampling for stock-based variables

MS	Species	Region	RF MO/ RF O/IO	Area/Stock	Frequency	AGE		
						2017	2018	2019
ITA	*Boop boops*	Mediterranean Sea and Black Sea	GFCM	GSAs 9, 10, 11, 16, 17, 18, 19	Q			X
ITA	*Engraulis encrasicolus*	Mediterranean Sea and Black Sea	GFCM	GSAs 9, 10, 11, 16, 17, 18, 19	Q	X	X	X
ITA	*Merluccius merluccius*	Mediterranean Sea and Black Sea	GFCM	GSAs 9, 10, 11, 16, 17, 18, 19	Q	X	X	X
ITA	*Mullus barbatus*	Mediterranean Sea and Black Sea	GFCM	GSAs 9, 10, 11, 16, 17, 18, 19	Q	X	X	X
ITA	*Mullus surmuletus*	Mediterranean Sea and Black Sea	GFCM	GSAs 9, 10, 11, 16, 17, 18, 19	Q	X	X	X
ITA	*Pagellus erythrinus*	Mediterranean Sea and Black Sea	GFCM	GSAs 9, 10, 11, 16, 17, 18, 19	Q			X
ITA	*Sardina pilchardus*	Mediterranean Sea and Black Sea	GFCM	GSAs 9, 10, 11, 16, 17, 18, 19	Q	X	X	X
ITA	*Trachurus mediterraneus*	Mediterranean Sea and Black Sea	GFCM	GSAs 9, 10, 11, 16, 17, 18, 19	Q			X
ITA	*Trachurus trachurus*	Mediterranean Sea and Black Sea	GFCM	GSAs 9, 10, 11, 16, 17, 18, 19	Q			X
ITA	*Micromesistius poutassou*	Mediterranean Sea and Black Sea	GFCM	GSAs 11, 18, 9	Q			X
ITA	*Diplodus annularis*	Mediterranean Sea and Black Sea	GFCM	GSAs 16, 9	Q			X
ITA	*Lophius budegassa*	Mediterranean Sea and Black Sea	GFCM	GSAs 9, 16, 18, 19	Q			X
ITA	*Scomber japonicus*	Mediterranean Sea and Black Sea	GFCM	GSAs 9, 16, 17, 18, 19	Q			X
ITA	*Scomber scombrus*	Mediterranean Sea and Black Sea	GFCM	GSAs 9, 16, 17, 18	Q			X
ITA	*Solea vulgaris*	Mediterranean Sea and Black Sea	GFCM	GSA 17	Q			X
ITA	*Spicara smaris*	Mediterranean Sea and Black Sea	GFCM	GSAs 17, 18	Q			X
ITA	*Thunnus alalunga*	Mediterranean Sea and Black Sea	ICCAT	all areas	Q			X
ITA	*Thunnus thynnus*	Mediterranean Sea and Black Sea	ICCAT	all areas	Q	X	X	X
ITA	*Xiphias gladius*	Mediterranean Sea and Black Sea	ICCAT	all areas	Q			X
ITA	*Galeus melastomus*	Mediterranean Sea and Black Sea	ICCAT, GFCM	GSAs 9, 10, 11	Q			X
ITA	*Raja asterias*	Mediterranean Sea and Black Sea	ICCAT, GFCM	GSAs 9,11	Q			X
ITA	*Raja clavata*	Mediterranean Sea and Black Sea	ICCAT, GFCM	GSAs 9, 11, 16, 18	Q			X
ITA	*Raja miraletus*	Mediterranean Sea and Black Sea	ICCAT, GFCM	GSA 16	Q			X
ITA	*Anguilla anguilla*	Mediterranean Sea and Black Sea	GFCM	all areas	Q			X

Note: Q = quarterly; A = annual.

1.1.3 European eel sampling

Pilot surveys have been carried out under the DCF national programme since 2009–2010, and then on a regular basis since 2011–2013. Currently biological samplings are foreseen for the triennial DCF programme 2017–2019 (AA.VV., 2017a). Samplings are planned for every Eel Management Unit (EMU) – regional administrations in the case of Italy.

Triennial biological surveys are carried out for every EMU in a specific site for each stratum, representative in that EMU in terms of habitat extent and/or amount of eel landings. Eel fishery is still allowed only in the nine regions that presented a management plan (Table 4) and sampling programmes are carried out only in those EMUs.

In each EMU, about 100 individuals for each eel life stage (yellow and silver eel) are randomly sampled every three years from cumulative catches of some days to assess stage composition (reconfirm yellow or silver stage), sex ratio, length and age frequency distributions. Sampling usually takes place in autumn, when eel catches consist of both yellow and silver eels.

From the spatial point of view, for each of the nine EMUs in which eel fishery continues, biological samplings are carried out considering the most relevant sites in terms of eel annual yields (e.g. Comacchio Lagoon in Emilia Romagna, Lake Garda in Lombardia, etc.).

TABLE 4 – Italian administrative regions designated as EMUs (eel fishery is still present in only nine of them)

Administrative region	Code			Stratum	
		River	Lake	Open Lagoon	Managed Lagoon
Valle d'Aosta	VDA			Eel fishery forbidden	
Piemonte	PIE			Eel fishery forbidden	
Lombardia	LOM	np	Y	np	np
Trentino Alto Adige	TAA			Eel fishery forbidden	
Friuli Venezia Giulia	FVG	Y	np	Y	Y
Veneto	VEN	Y	Y	Y	Y
Liguria	LIG			Eel fishery forbidden	
Emilia Romagna	EMR	Y	np	Y	Y
Toscana	TOS	Y	Y	np	Y
Marche	MAR			Eel fishery forbidden	
Umbria	UMB	np	Y	np	np
Lazio	LAZ	Y	Y	Y	Y
Abruzzo	ABR			Eel fishery forbidden	
Molise	MOL			Eel fishery forbidden	
Campagna	CAM			Eel fishery forbidden	
Basilicata	BAS			Eel fishery forbidden	
Puglia	PUG	np	np	Y	Y
Calabria	CAL			Eel fishery forbidden	
Sicilia	SIC			Eel fishery forbidden	
Sardegna	SAR	Y	np	Y	Y

Note: Y = fishery present; np = fishery not present.

Sample processing foresees different procedures depending on the data to be obtained. Annually, length and weight are directly measured on anaesthetized eels, and digital pictures for subsequent specific morphometric measurements are obtained. Samples are released if no other observations are due, or else frozen for further analyses (maturity, ageing analysis, etc.). Every three years, otoliths are collected, but only the left ones are processed for age determination.

1.2 Calcified structure extraction and storage

As the otoliths are located in the saccule (also known as utricle) of the inner ear, specifically in the vestibular labyrinth, their extraction requires the cranium of the animal to be exposed. In order to access the cavities in which the otoliths are enclosed, different cutting methods can be used: i) posterior section ("open the hatch" method); ii) transverse section (guillotine method); iii) longitudinal section ("right between the eyes" method); and iv) "up through the gills" method. In general, the first two methods are used in demersal species. The slicing and cutting tools vary according to the cranium size and robustness, but in general consist of razor blades, scissors and knives. The section must be done with care to avoid damage to the inner ear or to the otoliths. After making the appropriate cut, the otoliths can be removed with stainless steel tweezers.

1.2.1 Posterior section

Holding the fish's head between your thumb and forefinger, a cut at about 30° grade is made on the posterior part of the head (Plate 1).

PLATE 1
Posterior section cut (blue line) in *M. merluccius*, relative to the otolith position (white ellipse)

Once the skull is opened and the brain moved forward to the anterior part of the fish head, the two largest otoliths (sagittae) are easily detected and can be removed with stainless steel tweezers (Plate 2).

1.2.2 Transverse section

The transverse section cut is performed on the dorsal side of the fish head in correspondence with the preoperculum (Plate 3). Once the skull is opened, the sagittae can be extracted from the posterior part of the head (Plates 4 and 5). This extraction technique is generally used in *M. barbatus*, *M. surmuletus*, *T. trachurus*, *T. mediterraneus* and *A. anguilla*.

PLATE 2
Otolith extraction via posterior section in *M. merluccius*

PLATE 3
Position of transverse section cut (blue line) in *M. surmuletus*, relative to the otolith position (white circle)

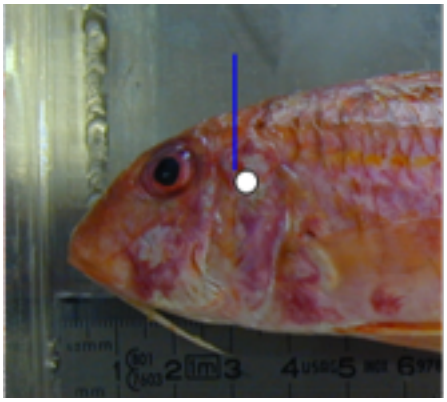
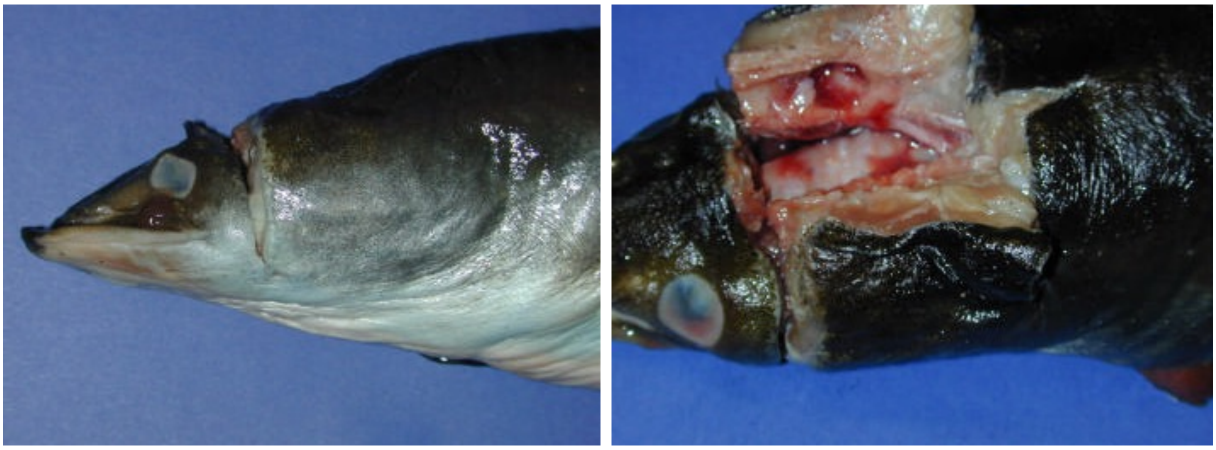


PLATE 4
Extraction of otolith via transverse head section in *T. trachurus*

PLATE 5
Extraction of otolith via transverse head section in *A. anguilla*



1.2.3 Removal of illicium

In anglerfish, in addition to the otoliths, the first dorsal transformed spine (fishing filament), also called illicium, is removed with a knife. The illicium is cut at the level of its base and cleaned of soft tissue before storage (Plates 6 and 7). A section of 7–8 cm in length from the base is enough. It is then stored in a plastic vial or an envelope.

PLATE 6
Removal of the illicium in anglerfish (slice in red)

1.2.4 Storage

After extraction, the CSs (i.e. otoliths, illicium) must be cleaned of any residual organic tissue, then washed and dried with paper, and stored in plastic vials or envelopes (subsection 1.2). Plastic tubes (Plate 8) have the advantage of being sufficiently rigid to protect CSs from damage due to handling. When a CS is sampled to estimate its age, it is important to label the vial or envelope with a univocal code to link the CS to a specific specimen.

PLATE 7
Removal of the illicium in anglerfish and storage in envelope

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PLATE 8
Diverse kinds of plastic vial used to store CSsa

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1.3 Ageing scheme

An important point for good practice in ageing analysis is a standardized ageing scheme (ICES, 2013a). This is generally based on several elements: number of translucent rings, theoretical birth date, the pattern of annulus deposition (generally translucent ring during winter/spring months, opaque area during summer/autumn months), date of capture, age resolution (year or half-year) and the edge type (opaque or transparent). A theoretical birth date is set for each species following the reproductive data available in the literature: 1 January for species with the bulk of spawning concentrated during late autumn/winter/early spring, and 1 July for species with a spawning period concentrated in late spring/summer/early autumn.

The age is calculated in year or half-year depending on the lifespan of the species and the possibility of edge discrimination (opaque and transparent).

1.3.1 Species with birth date 1 January

For species with a birth date set at 1 January (i.e. *T. trachurus*, *S. scombrus*, *S. pilchardus, B. boops, C. lucerna*), the translucent rings should be counted, with a 0.5-year resolution for age determination following the ageing scheme reported in Table 5.

TABLE 5 – Ageing scheme for species with a birth date of 1 January

Date capture	Otolith edge	Age
1 January-30 June	Transparent	N
1 July-31 December	Opaque	N + 0.5

Note: N is the number of translucent rings, including those that might be visible on the edge.

Following the scheme in Table 5, specimens caught in the first part of the year (winter/spring) usually have a translucent ring on the otolith edge; this translucent ring is counted as an annual ring and the age is equal to the number of translucent rings (including the edge). In the case of specimens caught in the second part of the year (summer/autumn), with an opaque edge, the age corresponds to the number of translucent rings plus 0.5, which represents the half year already passed.

In some particular cases, this general scheme (Table 5) is not applicable. In fact, an opaque edge can be also present at the beginning and/or the end of the first part of the year (Table 6). The presence of an opaque edge at the beginning of the first part of the year could be due to the fact that formation of a translucent ring has not started yet. In those cases, the age is equal to the number of translucent rings plus 1, because the theoretical birth date has already passed. In contrast, an opaque ring can be present on the otolith edge at the end of the first part of the year owing to an anticipated start of deposition of the opaque ring (Table 6). In those cases, the age is equal to the number of translucent rings (N).

TABLE 6 – Ageing scheme for species with a birth date of 1 January

Months	Jan	Feb	Mar	Apr	May	Jun	July	Aug	Sep	Oct	Nov	Dec
Deposition pattern	T/O	T	T	T	T	O/T	O/T	O	O	O	O	T/O
Capture date												
Age with edge T	N	N	N	N	N	N	N+0.5					N-0.5
Age with edge O	N+1					N	N+0.5	N+0.5	N+0.5	N+0.5	N+0.5	N+0.5

Note: N is the number of winter rings (translucent); T = transparent edge, O = opaque edge.

In the second part of the year, specimens with a translucent ring at the edge could also be present. As with the presence of an opaque edge in the first part of the year, the presence of a transparent edge in the second part of the year could occur in specimens that have not yet started depositing an opaque ring in early summer (July) or that have already started translucent ring deposition at the end of autumn or early winter (i.e. December) (Table 6). In the first case, age will be equal to the number of translucent rings, including the edge, plus 0.5, which represents the half year already passed. Indeed, the presence or lack of an opaque edge is irrelevant, because the transparent one is counted in the age calculation. If a translucent ring is present at the edge at the end of autumn or early winter (i.e. December), age will be equal to the number of translucent rings, including the edge, minus 0.5, because counting the translucent ring on the edge may overestimate the age by one year, the birth date (1 January) not yet being passed.

1.3.2 Species with birth date 1 July

For *M. barbatus, S. smaris, S. colias* and *E. encrasicolus*, the birth date is set at 1 July. It is commonly accepted that only the translucent rings should be counted, with a 0.5-year resolution. The ageing scheme is reported in Table 7.

Specimens caught in the first part of the year (winter/spring) usually have a translucent ring on the edge, but, according to the scheme, this is not counted as an annual ring as the birth date has not yet passed. Thus the age is equal to the number of translucent rings, including the edge, minus 0.5.

In the specimens caught in the second part of the year (summer/autumn), when an opaque ring is present on the otolith edge, the age is equal to the number of translucent rings (Table 8).

TABLE 7 – Ageing scheme for species with a birth date of 1 July

Date capture	Otolith edge	Age
1 January-30 June	Transparent	N - 0.5
1 July-31 December	Opaque	N

Note: N is the number of translucent rings, including those that might be visible on the edge.

TABLE 8 – Ageing scheme for species with a birth date of 1 July

Months	Jan	Feb	Mar	Apr	May	Jun	July	Aug	Sep	Oct	Nov	Dec
Deposition pattern	T/O	T	T	T	T	O/T	O/T	O	O	O	O	T/O
Capture date												
Age with edge T	N-0.5	N-0.5	N-0.5	N-0.5	N-0.5	N-0.5	N					N-1
Age with edge O	N+0.5					N-0.5	N	N	N	N	N	N

Note: N is the number of winter rings (translucent); T = transparent edge, O = opaque edge.

The general scheme reported in Table 7 is not applicable when an opaque edge is present at the beginning and/or end of the first part of the year (Table 8), or a transparent edge is present in the second part of the year. An opaque edge can be present in the first part of the year in specimens that have not yet started deposition of a translucent ring or have already started formation of an opaque edge in early summer. In contrast, a transparent edge can be present in the second part of the year in specimens that have not yet started formation of the opaque ring (July), or have already started formation of a translucent ring at the end of autumn or early winter (i.e. December) (Table 8).

When a transparent edge is present after 1 July, the age is equal to the number of winter rings, including the edge (N); when an opaque edge is present before 1 July, the age is equal to the number of winter rings minus 0.5.

When a translucent ring is present in early winter (i.e. before 1 January), the age is equal to the number of translucent rings minus 1, because despite the presence of the winter ring on the edge, the birth date has not yet been reached (Table 8). When an opaque edge is present in the early winter (i.e. January), the age is equal to the number of translucent rings plus 0.5, because, although deposition of the winter ring has not yet started, the birth date has passed. The 0.5 represents the half year that has passed since the birth date.

1.3.3 Ageing scheme for *M. merluccius* and in Elasmobranchii

For Elasmobranchii and *M. merluccius*, the birth date is usually set at 1 January, counting the translucent rings and using a 1-year resolution. The ageing scheme is reported in Table 9.

Following this scheme (Table 9), specimens caught in the first part of the year (winter/spring) usually have a translucent ring on at the edge. This is counted as an annual ring and age will be equal to the number of translucent rings, including the edge. In specimens caught in the second part of the year (summer/autumn) with an opaque edge, the age also corresponds to the number of translucent rings (Table 10).

Table 9 is not always applicable. Indeed, as mentioned in subsection 1.3.1, an opaque edge could be present mostly at the beginning and/or end of the first part of the year (Table 10), as could a transparent edge in the second part of the year (at the beginning and end) (see subsections 3.1 and 3.2). In the second part of the year, specimens with a transparent edge could be present. This could occur in specimens that have already started translucent ring deposition at the end of autumn or early winter (i.e. December) (Table 10). In this case, age will be equal to the number of translucent rings, including the edge, minus 1,

TABLE 9 – Ageing scheme for species with a birth date of 1 January

Date capture	Otolith edge	Age
1 January-30 June	Opaque	N
1 July-31 December	Transparent	N

Note: N is the number of translucent rings, including those that might be visible on the edge.

TABLE 10 – Ageing scheme for species with a birth date of 1 July

Months	Jan	Feb	Mar	Apr	May	Jun	July	Aug	Sep	Oct	Nov	Dec
Deposition pattern	T/O	T	T	T	T	O/T	O/T	O	O	O	O	T/O
Capture date												
Age with edge T	N	N	N	N	N	N	N					N-1
Age with edge O	N+1					N	N	N	N	N	N	N

Note: N is the number of winter rings (translucent); T = transparent edge, O = opaque edge.

because counting the transparent edge may overestimate the age by one year, the birth date (1 January) not yet having passed.

In the case of an opaque edge present at the beginning of the first part of the year, this may occur in a specimen that has not yet started deposition of a translucent ring. In this case, age will be equal to the number of translucent rings plus 1, because the theoretical birth date has already passed.

1.4 Precision, accordance of readings and preconditioning

In order to minimize the risk of systematic errors due to preconditioning, CS readings should be performed by at least two independent operators with no information on the specimen (i.e. size, sex, etc.). Moreover, readings should be performed at least twice by each reader at an interval of 10–15 days. When readings are in disagreement, the CS should be reanalysed. If no agreement is reached, the CS must be discarded (Goldman, 2005).

The main methods for determining the degree of accuracy of CS readings are: the CV, the index of the average percentage error (IAPE) (Beamish and Founier, 1981; Chang, 1982); and the percentage of agreement (PA), calculated as follows:

$$CV_j = \frac{\sqrt{\sum_{i=1}^{R} \frac{(X_{ij} - X_j)^2}{R-1}}}{X_j} \times 100$$

$$IAPE = \frac{1}{N} \sum \left(\frac{1}{R} \sum \frac{(|X_{ij} - X_j|)}{X_j} \right) \times 100$$

$$PA = \left(\frac{No.agreed}{No.read} \right) \times 100$$

where N is the number of samples read; R represents the number of readings; X_{ij} is the i^{th} reading of the j^{th} individual; and X_j is equivalent to the average age calculated for the j^{th} individual.

For elasmobranchs and bony fish, CV values of about 10 percent are usually considered acceptable (Bell, 2001). It should, however, be noted that the value of CV is commonly higher by about 40 percent compared to that of IAPE.

As concerns PA, the value for new readers can be considered acceptable when it reaches at least an 80 percent agreement with expert readers. At that point, a new reader can be included in the list of expert readers for a given species.

2. Small pelagic species

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The assemblage of small pelagic fish includes several species belonging to four families: Clupeidae, Engraulidae, Scombridae and Carangidae. The most important in terms of biomass and commercial interest include: *S. pilchardus*, *E. encrasicolus*, *S. scombrus*, *S. colias*, *T. trachurus* and *T. mediterraneus*. This group of fish is characterized by pelagic, schooling and migratory behaviour, ichthyoplanktonic diet and relatively small size.

Small pelagic fish are predominantly confined to coastal regions, with the largest populations occurring in regions of upwelling. The spatial heterogeneity of the physical characteristics of the coastal pelagic environment, and the high mobility of small pelagic fish, generally leads to their distribution being concentrated within areas that they find most favourable (Bellido *et al.*, 2008).

Small pelagic fish are important elements of marine ecosystems due to their relevant biomasses at intermediate levels in the food web, notably contributing to canalizing the energy connecting the lower (plankton) and upper trophic levels (predators). These species, in fact, represent the prey of the other pelagic (i.e. large pelagic species, cetaceans) and benthopelagic (i.e. hake, Sparidae, bluefish) species. Fluctuations in small pelagic fish populations due to fishing or to environmental factors can contribute to modifying the structure and functioning of marine ecosystems (Cury *et al.*, 2000; Duarte and García, 2004; Albo-Puigserver *et al.*, 2016).

Small pelagic species are very important to fisheries activity along the Italian coast. In 2011, as in previous years, *E. Encrasicolus* and *S. pilchardus* were the main species landed by the Italian fleet (*E. Encrasicolus*: 46 237 tonnes, approximately 22 percent of total landings; *S. pilchardus* 14 377 tonnes, approximately 6.8 percent of total landings). The principal fishing gear for small pelagic species are: bottom pair trawl, beam trawl, purse seine, lampara nets and small-scale driftnet (IREPA, 2012).

Growth and age determination studies are essential in fish population dynamics and in fishery ecology for their link to mortality estimates. This is particularly true for small pelagic species, where natural mortality is very often higher than fishing mortality, implying that assumptions regarding growth and natural mortality can substantially affect the level of exploitation of these resources (Arneri *et al.*, 2011). There is still much disagreement regarding age determination (ICES, 2015a, 2015b, 2017a) and further investigation is needed, because the ecological implications of differing assumptions regarding growth are substantial (e.g. for recruitment studies). Moreover, the recent use in stock assessment of changes in the "natural mortality vector" seems to be particularly vulnerable to these assumptions regarding growth. For these reasons, this document on the methodology and criteria for age analysis represents an important tool in the standardization and reduction of absolute and/or relative bias.

Within Italian marine institutes, the age of small pelagic fish is determined on the basis of calcified structures, mainly otoliths (sagittae). Currently, the following pelagic fish species are dealt with: *E. Encrasicolus*, *S. pilchardus*, *S. scombrus*, *S. colias*, *T. trachurus* and *T. mediterraneus*.

2.1 *Engraulis encrasicolus*

E. encrasicolus is an important species in the Mediterranean basin, both from an ecological and socio-economic point of view. The main characteristics of this small pelagic species are a short life span, seasonal migrations, high growth rates, early maturity, long spawning period and schooling behaviour. The otolith reading is the common method of determining the ageing of this species (ICES, 2017a).

E. encrasicolus spawning season is broad, extending from April–May to September with a peak in June–July (Kada *et al.*, 2009; Basilone *et al.*, 2006; Zupa *et al.*, 2013). The main factor determining the reproductive cycle is water temperature. Indeed, the spawning season starts with the water warming and ends with its cooling (Basilone *et al.*, 2013).

2.1.1 Extraction and storage

Sagittae extraction is made through the posterior section of the head. After extraction, the otoliths are washed to remove organic material and then dried and stored in rigid plastic vials.

2.1.2 Preparation and interpretation

One otolith from each pair (usually the left one) is immersed in 70 percent alcohol or in seawater (clarification medium) to be analysed (ICES, 2017a). Otoliths of *E. encrasicolus* don't need the clarification phase before analysis. The otolith is approximately oval with an outer concave face. Its major axis is oriented in an anterior-posterior direction (Plate 9). The dorsal and ventral edges converge towards the front, forming the rostrum. The dorsal edge is slightly curved, while the ventral one possesses quite regular saw-like teeth, which become more marked with the increasing age of the animal.

Otoliths are analysed under the binocular microscope, rinsed with seawater (clarification medium), with reflected light against a black background. The best otolith orientation for analysis is with the distal surface up and the proximal surface (sulcus acusticus) down (Plates 9 and 10). In this way, the dark rings can be counted in the antirostrum area (radius) as translucent growth rings (slow growth). The opaque zone (white – fast growth) with a dark ring is considered an annual increment (annulus). The main magnification used ranged from 20 to 40x.

Moreover, for every otolith the edge quality is noted (opaque or translucent), while the measurements from the core to each translucent ring (at the end of the ring) on the postrostrum

PLATE 9
Both *E. encrasicolus* otoliths, one with proximal face up (top) and one with distal face up (bottom)

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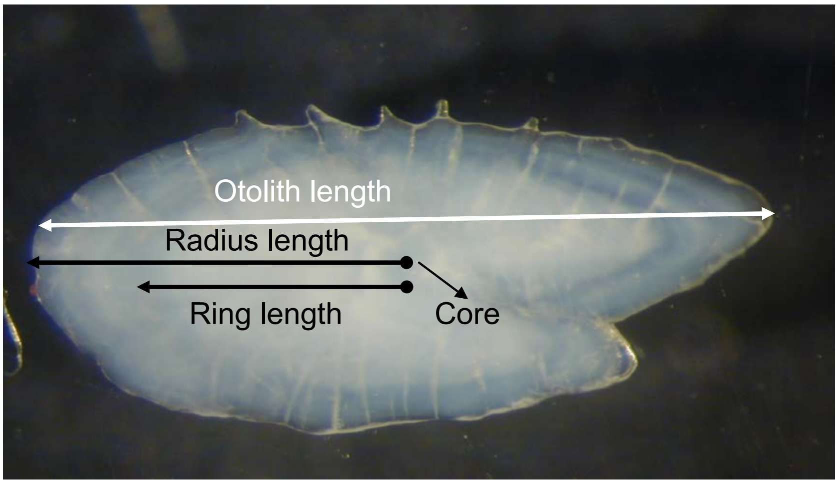
Note: Female, TL = 13.5 cm, captured in December.

PLATE 10
E. encrasicolus otolith indicating where morphometric measures are taken



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area (Plate 9) and the radius and otolith lengths are taken on a subsample of otolith. The measurements are taken on the major axis passing through the core (Giannetti and Donato, 2003; ICES, 2017a) on the posterior area of the otolith (postrostrum).

In accordance with the peak spawning period in the summer (Sinovčić and Zorica, 2006), and May–September with a peak in July–August (Regner, 1996), the birth date is considered 1 July (Gianetti and Donato, 2003; ICES 2010b). In agreement with this spawning period, with a birth date June–July and with analysis carried out of the marginal edge (Ana Giraldez and Pedro Torres, working documents; ICES, 2010b), the first band deposited is the opaque band.

The age scheme of *E. encrasicolus* otoliths is reported in section 1.3.2 (ageing scheme with birth date 1 July) and takes into account the number of translucent rings (winter rings), the pattern of annulus formation (generally translucent ring during winter/spring months; opaque ring during summer/autumn months), the capture date, otolith edge and spawning period. Moreover, the age is assigned with a half-year resolution.

2.1.3 Difficulties in interpretation

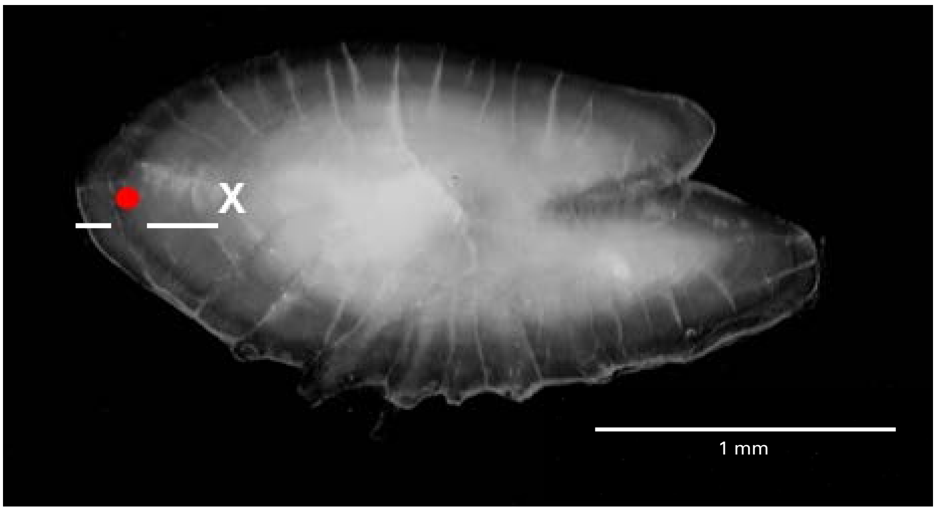
Around the nucleus before the first winter ring (ICES, 2010b), a false one is laid down with a distance to the core of about 0.8 mm. This ring can be distinguished because it is less marked in comparison with the winter one (Plates 11, 12, 13 and 16).

Moreover the first annulus consists of a wide opaque zone (sometimes interrupted by a false ring) in comparison with the successive annuli. However, distances between the rings decrease with age (Plates 13, 14 and 15). Other false rings could be laid down after the first winter ring as well as the spawning ring (Plate 13) (ICES, 2010b).

Another aspect to take into account in reading the *E. encrasicolus* otolith is to follow the ring around the whole otolith, because sometimes the rings are more visible in the rostrum area than in the posterior area (Plates 14 and 15).

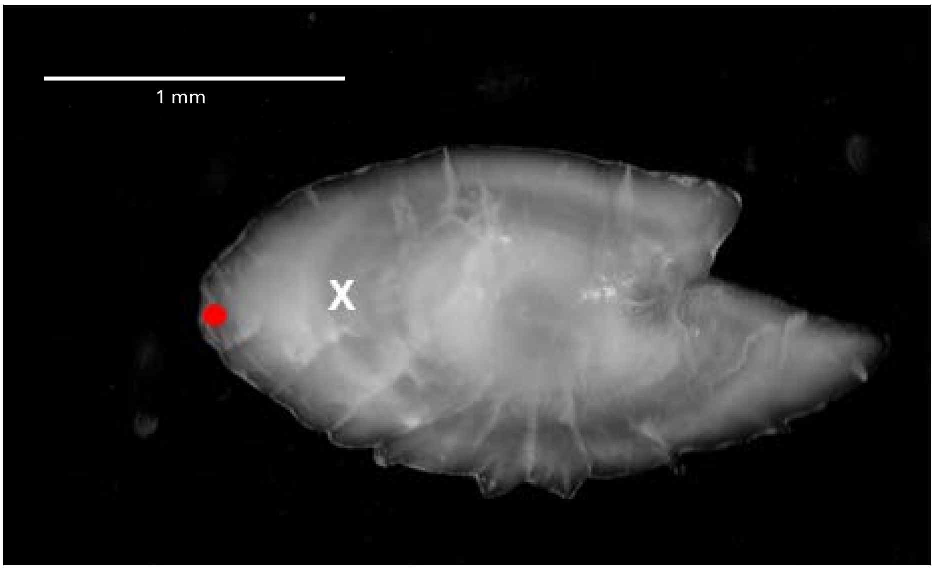
The ICES WKARA2 workshop (ICES, 2017a) recognized the following sources of bias in ageing analysis:
– difficulties in differentiating between true annual winter hyaline rings and false rings (or checks), mainly the first annual winter ring;
– insufficient typical annual growth pattern recognition and insufficient criteria regarding the otolith edge that can be expected to be seen during the year;
– difficulties in recognizing the opaque or hyaline nature of the edge, which may affect age determination. Identifying hyaline edges seems to be a difficult issue, as the edge continuously changes and no clear unambiguous definition exists;
– difficulties in application of the rule.

PLATE 11
E. encrasicolus otolith annulus formation



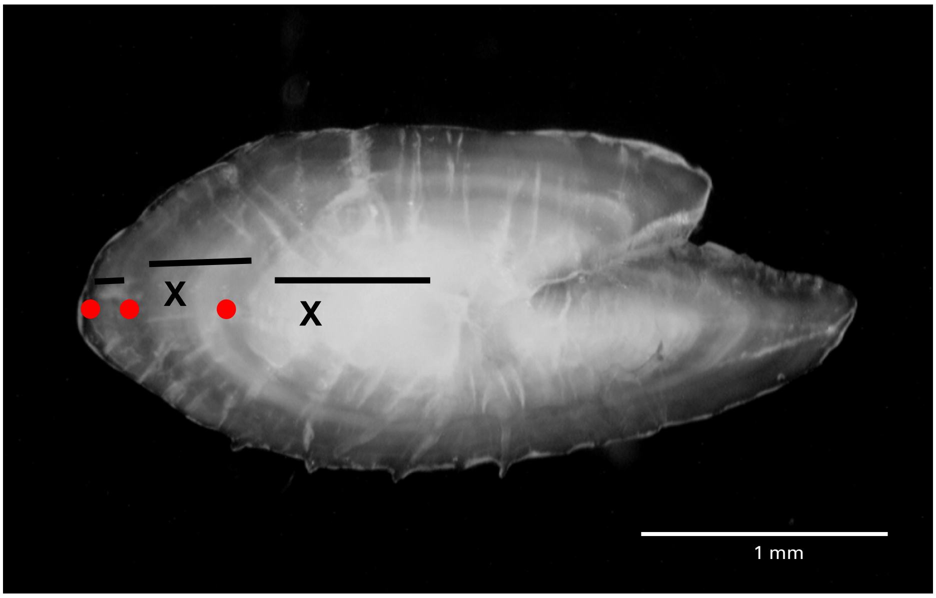
Note: Red dot = winter ring, X = check, horizontal with line = opaque area. A 'check' is a discontinuity (e.g. a stress-induced mark) in a pattern of opaque and translucent zones, or microincrements (see Glossary).

PLATE 12
Otolith of *E. encrasicolus* age determination



Note: Age 0.5 years, female, TL = 10 cm, captured in April, red dot = winter ring, X = check.

PLATE 13
Otolith of *E. encrasicolus*



Note: Age 3 years, female, TL = 14 cm, captured in July, red dots = winter ring, black lines = opaque area, X = check.

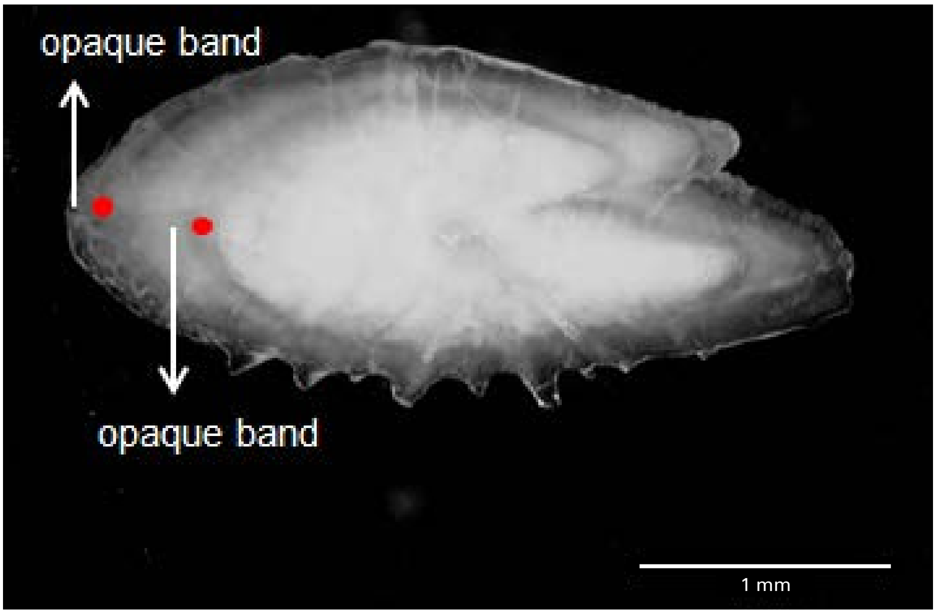
In any case, some age criteria may be useful in overcoming these difficulties:

- considering as true winter rings those that can be followed around the whole otolith, with a decreasing distance between them;
- analysing the nature of the edge as soon as the otolith is immersed in the clarification medium, before the thin edge becomes transparent;
  and
- considering an aging scheme that includes all possible combinations (edge nature, birth date, date of capture).

In the example reported in Plate 12, the opaque nucleus and the beginning of winter deposition appear (translucent ring on the edge). According to the scheme, it's a specimen of age 0.5 year with a translucent edge (0 opaque ring), captured in March (first half of semester). The annulus is not yet complete (N), so the sample has about 9 months (1 July–April).

In the example reported in Plate 13, the otolith shows three winter rings and a transparent edge. The date of capture is July (second half of the year); according to the scheme of age assignment, the age is three years (N+1). Indeed, this specimen has spent three winters and shows two complete annuli, but, despite the fact that the deposition of the summer ring has not yet started, the capture date is after the birth date, so the age is three years.

PLATE 14
Otolith of *E. encrasicolus*



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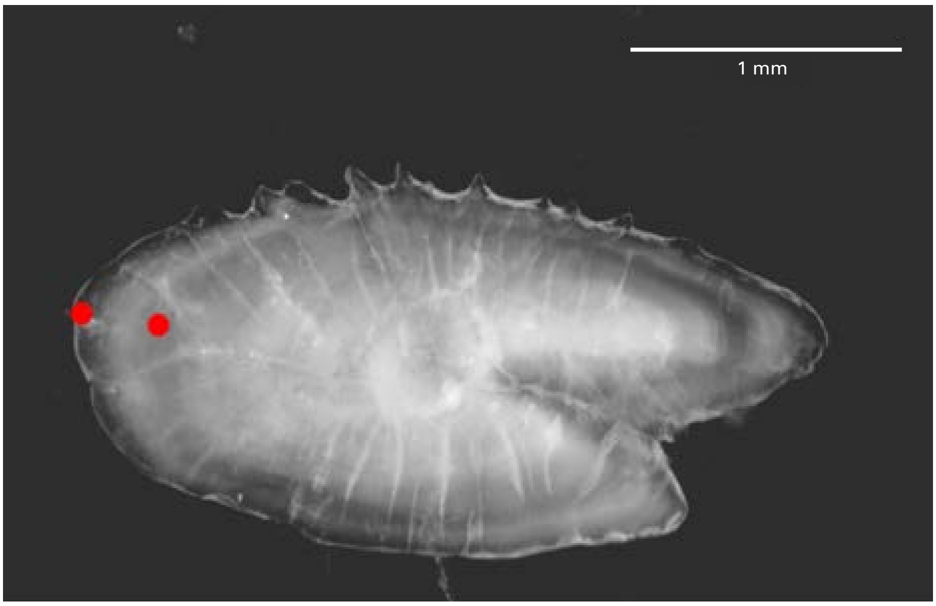
Note: Age 2 years, female, TL = 13 cm, captured in September, red dots = winter rings, white arrows = opaque rings.

PLATE 15
Otolith of *E. encrasicolus*



© P. Carbonara

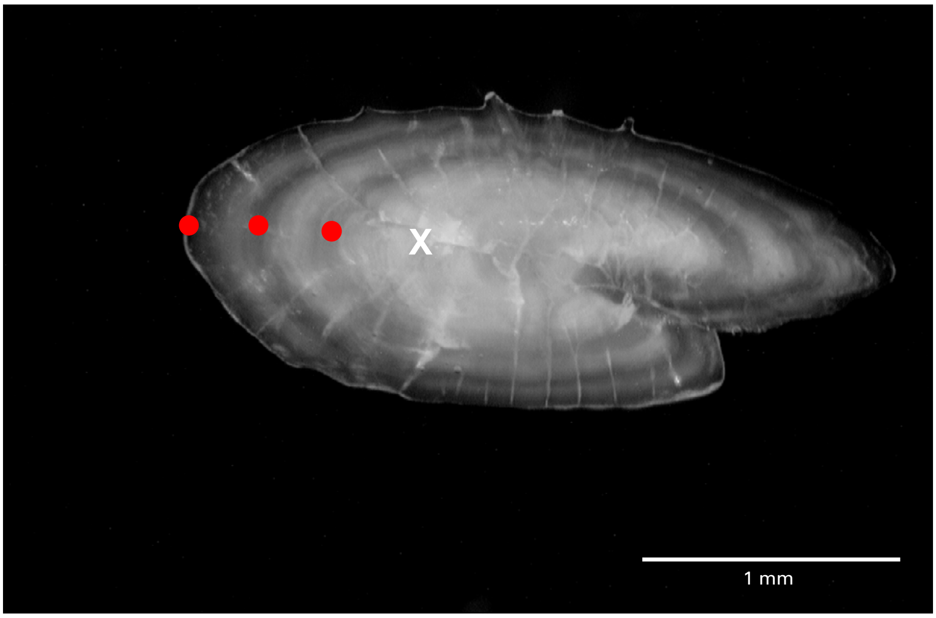
Note: Age reading = 2 winter rings, age 1.5 years, female, TL = 12 cm, captured in March, red dots = winter ring.

The otolith in Plate 14 shows an opaque edge, so the age assignment is two years, because the specimen was captured in September, in the second half of the year, with two completed annuli.

In Plate 15, the edge appears transparent and the age is 1.5 years, considering the three elements for assignment of age (birth date, capture date and the edge).

Plate 16 shows an example of an otolith with three winter rings (age 2.5 years).

PLATE 16
Otolith of *E. encrasicolus*



© P. Carbonara

Note: Age reading = 3 winter rings, age 2.5 years, female, TL = 14.5 cm, captured in March, red dots = winter ring, X = check.

2.2 *Sardina pilchardus*

S. pilchardus is a small pelagic Clupeidae distributed in the northeastern Atlantic from the North Sea to Senegal and throughout most of the Mediterranean Sea. It has high commercial importance, being targeted by purse-seine fisheries across most of its distribution area and by pelagic trawlers mainly. As with *E. encrasicolus*, this species is characterized by high growth rates in the first two years (Morales-Nin and Pertierra, 1990; Erdogan *et al.*, 2010; Bedairia and Djebar, 2009); early maturity at one year old (Tsikliras and Koutrakis, 2013; Sinovčić, Keč and Zorica, 2008; Ganias *et al.*, 2003), with a relatively short life span of about 8–12 years – although, in the Mediterranean basin, the maximum longevity observed was eight years (Sinovčić, 2000). *S. pilchardus*, as with most clupeids is a batch-spawner, with a long spawning period from autumn to spring and a peak in winter during the colder months (Ganias *et al.*, 2007; Tsikliras and Koutrakis, 2013; Pešić *et al.*, 2010). *S. pilchardus* is a gregarious fish, with a schooling behaviour and gregarism based on size, as it involves the aggregation of specimens of similar size (Donato, La Mesa and Santojanni, 2017). For *S. pilchardus*, as well, otolith analysis is the method most used to determine ageing (ICES, 2011a; Soares, Silva and Morais, 2005).

2.2.1 Extraction and storage

Sagittae extraction is made through the posterior section of the head. After extraction, the otoliths are washed to remove organic material and then dried and stored in rigid plastic vials.

2.2.2 Preparation and interpretation

One otolith from each pair (usually the left one) is immersed in alcohol 70 percent or in seawater to be analysed. Otoliths of *S. pilchardus* don't need the clarification phase before analysis, being so small and thin. They are analysed under a binocular microscope, with reflected light, against a black background. The best otolith orientation for analysis is with the distal surface up and the proximal surface (sulcus acusticus) down (Plates 17 and 18).

PLATE 17
Both *S. pilchardus* otoliths, one with proximal face up (top) and one with distal face up (bottom)

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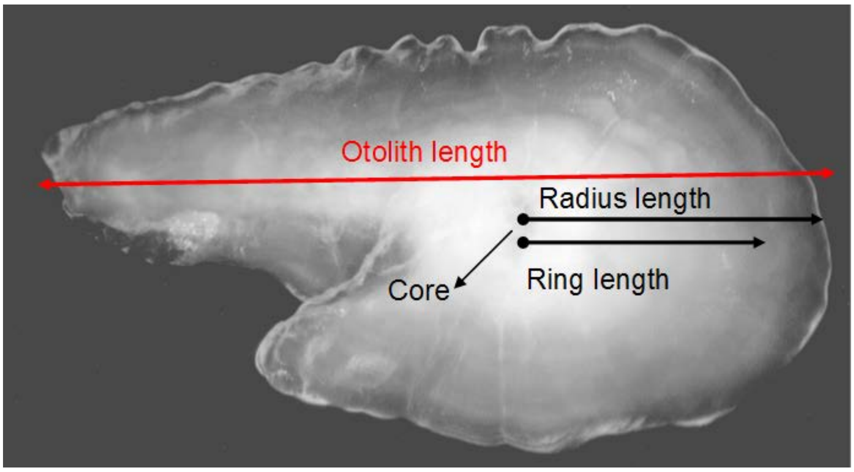
Note: Female, TL = 17.5 cm, captured in October.

PLATE 18
S. pilchardus otolith indicating where morphometric measures are taken



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In this way, the translucent rings are counted in the antirostrum area (radius) as translucent growth rings (slow growth). The opaque zone (white – fast growth) plus a translucent ring is considered an annual increment (annulus). For every otolith, the edge quality is noted (opaque or translucent), while the measurements from the core to each translucent ring (at the end of the ring) on the postrostrum area (Plates 17 and 18) and the radius and otolith lengths are taken on a subsample of otoliths. Measurements are taken on the major axis passing through the core on the posterior area of the otolith (postrostrum), while the nature of the edge (opaque or translucent) is always noted.

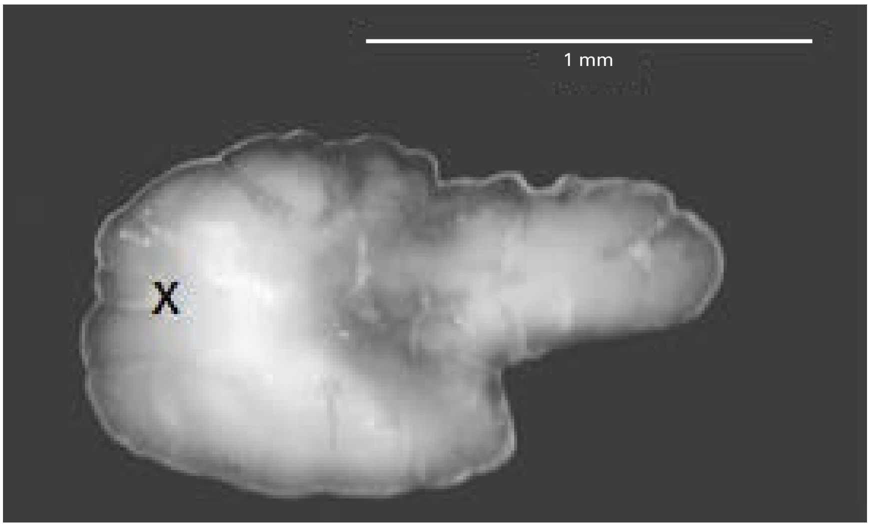
In accordance with a peak spawning period during the winter months, the birth date is set at 1 January (Panfili *et al.*, 2002). The age scheme of *S. pilchardus* otoliths is reported in section 1.3.1 (birth date 1 January). *S. pilchardus* being a species with winter reproduction, the core is usually translucent (Plates 16, 17 and 18). The opaque area, corresponding to the summer/autumn months, is laid down around the core. The first annulus is completed after deposition of the first winter ring.

2.2.3 Difficulties in interpretation

For *S. pilchardus*, as well, before the first winter, one false ring is often laid down with a distance from the core of about 0.5–0.8 mm (Plates 19, 20 and 21). In some cases, it was observed that *S. pilchardus* of TL 6–11 cm exhibits from two to five translucent false rings, indicating an age of 60–220 days by means of microincrement counts (FAO, 2002). *S. pilchardus* otoliths have a growth pattern similar to *E. encrasicolus*, with a large opaque zone in the first annulus. Moreover, to recognize the winter ring, it is important to follow the ring around the whole otolith to distinguish the true one.

Other discrepancies in *S. pilchardus* age determination are identification of the otolith edge type and the first annulus (ICES, 1997, 2011a; Soares *et al.*, 2009). According to the age reading scheme (section 1.3.1), a hyaline edge is counted as an annual ring in the first semester and in the third quarter. Indeed, the hyaline growth visible at the edge is assumed to be deposited during the past winter, while in the fourth quarter it is assumed to correspond to the coming winter – meaning that the opaque growth has already taken place. In any case, the *S. pilchardus*

PLATE 19
Otolith of *S. pilchardus*



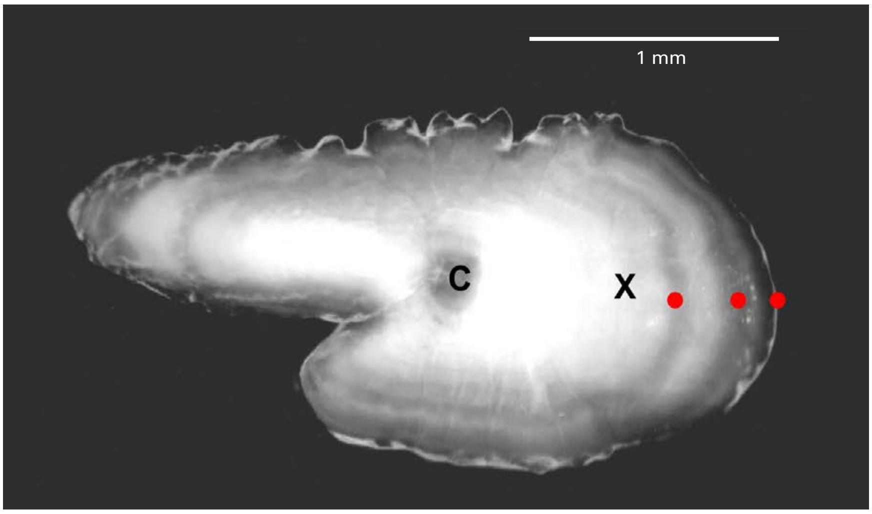
Note: Age 0, female, TL = 8 cm, captured in June, X = check.

edge can often appear hyaline owing to the thinness of the *S. pilchardus* otolith and/or to a drastic narrowing of the rings, mainly of the opaque bands, reflecting the deceleration of growth in older specimens (ICES, 2011a). Moreover, the clarity of the edge varies between years and area. Thus it is difficult to describe a general pattern of ring deposition for the Mediterranean basin. For each area, specific analysis (marginal analysis) is required. In this context, where specific studies on deposition rings are not available, it is possible to consider that the translucent ring is laid down in the first semester and the opaque one in the second semester.

In this case (Plate 19), the core is translucent because the *S. pilchardus* individual was born in winter. Then there is an opaque part and, on the edge, the start of translucent deposition. But the capture date is June, so this individual has just 5 months of age.

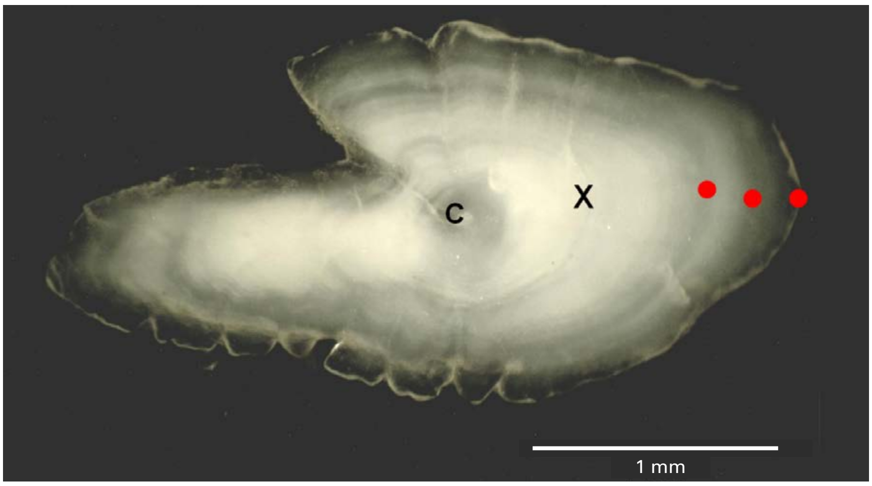
In Plate 20, two annuli are visible, with a transparent edge. The age reading corresponds to two complete winter rings, and the age determination is 2.5, because the month of capture is August, in accordance with the scheme of age assignment. In this case, the specimen has not yet begun deposition of the opaque ring.

PLATE 20
Otolith of *S. pilchardus*



Note: Age 2.5 years, female, TL = 17 cm, captured in August, red dots = winter ring, X = check, C = core.

PLATE 21
Otolith of *S. pilchardus*



Note: Age 3 years = January, female, TL = 18 cm, red dots = winter ring, X = check, C = core.

In Plate 21, two complete annuli are also visible, with one translucent ring on the edge. The age reading is three winter rings and the age determination is three years, because the month of capture is January, in accordance with the scheme of age assignment.

2.3 *Scomber scombrus*

S. scombrus is a schooling, migratory and ichthyoplanktonic species abundant in cold and temperate waters of the northern Atlantic Ocean and Mediterranean. Several fleets and gear exploit the *S. scombrus* fishery in Mediterranean waters: purse seine, midwater trawls, longlines, etc. Landings of *S. scombrus* presently peak in the late spring/early summer. Along the Italian coast, the Central-North Adriatic Sea is the most important area for *S. scombrus* fishing (IREPA, 2012).

Most studies show a high growth rate during the first year of life and a deceleration after the species reach the length of first maturity (Meneghesso *et al.*, 2013; Sinovčić, 2001; Jabeur *et al.*, 2013). In the Atlantic area, many more age classes can be found (Villamor *et al.*, 2004) compared with the Mediterranean basin (Sinovčić, 2001; Jabeur *et al.*, 2013; Meneghesso *et al.*, 2013).

Females had asynchronous ovaries, with different stages of vitellogenic oocytes during the spawning season, which extends all winter to early spring (Meneghesso *et al.*, 2013), even if some interannual variation may occur (Jansen and Gislason, 2011).

2.3.1 Extraction and storage

Sagittae extraction is made through the posterior section of the head. After extraction, the otoliths are washed to remove organic material and then dried and stored in rigid plastic vials.

2.3.2 Preparation and interpretation

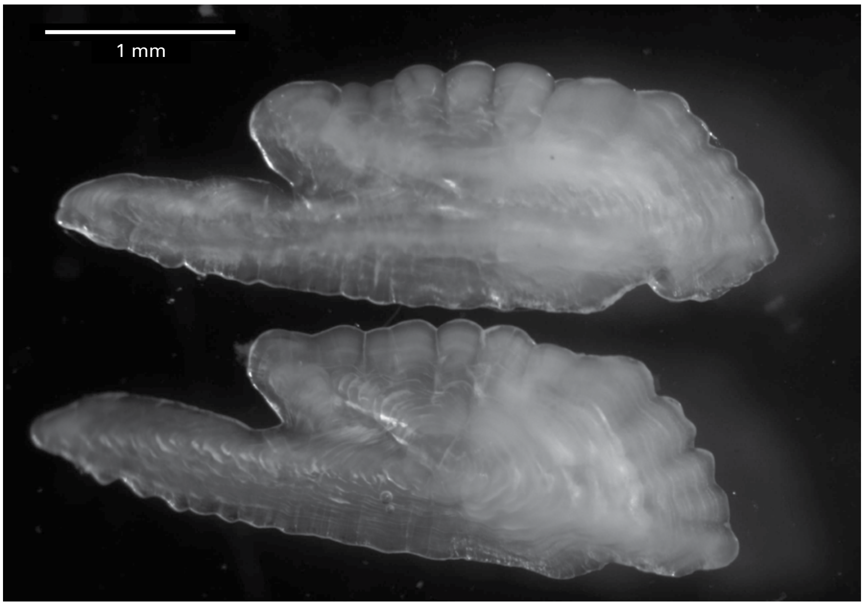
One otolith from each pair (usually the left one) is immersed in seawater to be clarified before analysis. The otoliths are analysed under the binocular microscope, in seawater, with reflected light, against a black background. The best orientation for analysis is with the distal surface up and the proximal surface (sulcus acusticus) down.

In whole otoliths, annuli are counted on the posterior part of the otolith (postrostrum). However, ring continuity should be checked on the anterior part of the otolith (rostrum) and, wherever possible, on the dorso lateral edge.

Dark rings are counted as the translucent growth zone (slow growth). The opaque zone (white – fast growth) with a dark ring is considered an annual increment (annulus). The ageing of *S. scombrus* is performed considering 1 January as the birth date, in accordance with the spawning period (Watson *et al.*, 1992), which is prolonged, from January to April (Meneghesso *et al.*, 2013). *S. scombrus* being a species that is born in the winter period (birth date: 1 January), the core is translucent (Plates 22, 23 and 24).

The opaque area, corresponding to the summer/autumn months, is laid down around the core. The first annulus is formed after the deposition of the first winter. Moreover, age is assigned in terms of 0.5 years, following the scheme reported in section 1.3.1. It counts the number of translucent rings, starting at the first winter ring and taking into account several elements: birth date, number of translucent rings, date of capture and edge type.

PLATE 22
S. scombrus otoliths, one with proximal face up (top) and one with distal face up (bottom)



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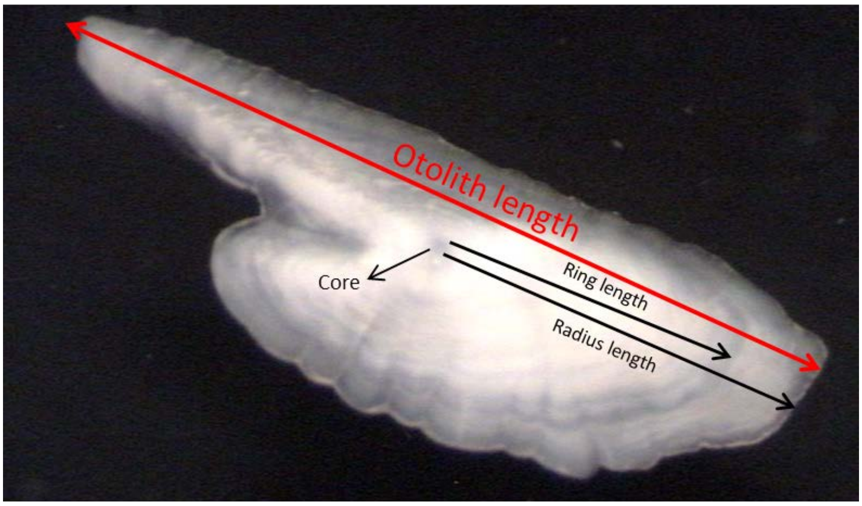
Note: Female, TL = 25 cm, captured in October.

PLATE 23
Otolith of *S. scombrus* indicating where morphometric measures are taken



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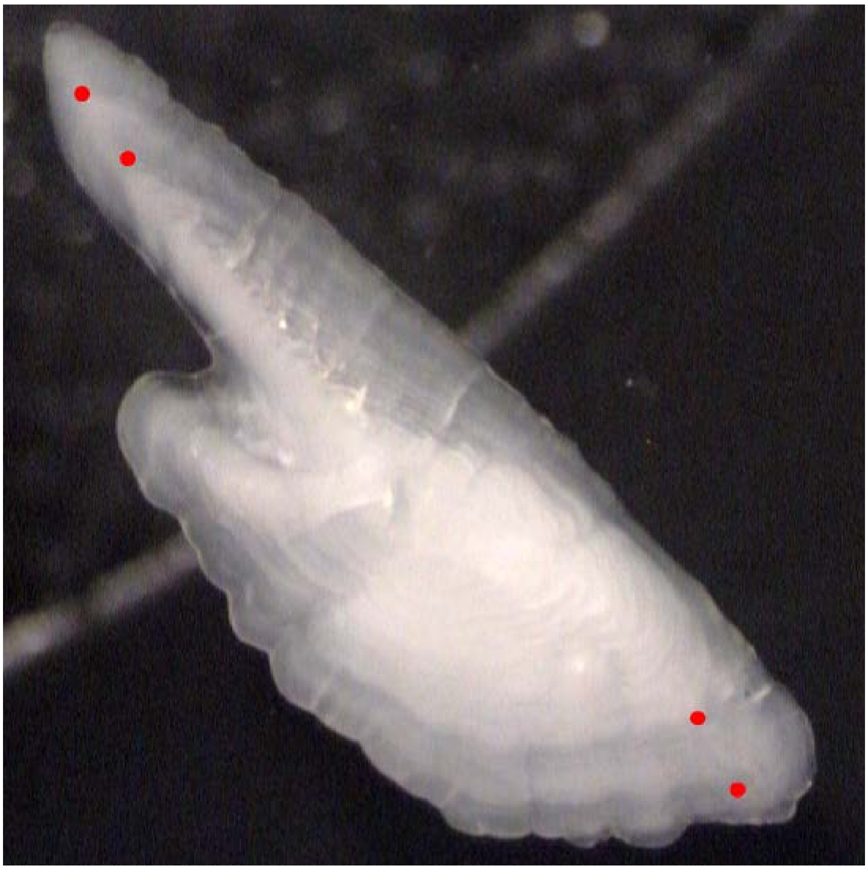
2.3.3 Difficulties in interpretation

The main age interpretation difficulties for the *S. scombrus* are (ICES, 2010c):

- differing length of time for opaque zone formation of the otolith between the different areas during the first year;
- during the first year, some false rings are laid down (Plates 25 and 27), but usually the first winter ring is more pronounced and/or can be identified following it around the whole otolith;
- otolith edge interpretation (Plate 24). In this case, the edge is established observing the margin around the whole otolith;
- possible presence of false annulus associated with the first maturity (Plate 26). It is possible to distinguish this kind of ring because it is less marked and is not visible around the whole otolith;

– slowed growth in older fish – to such an extent that the opaque and translucent zones become overlapped and are more difficult to distinguish (Plate 27).

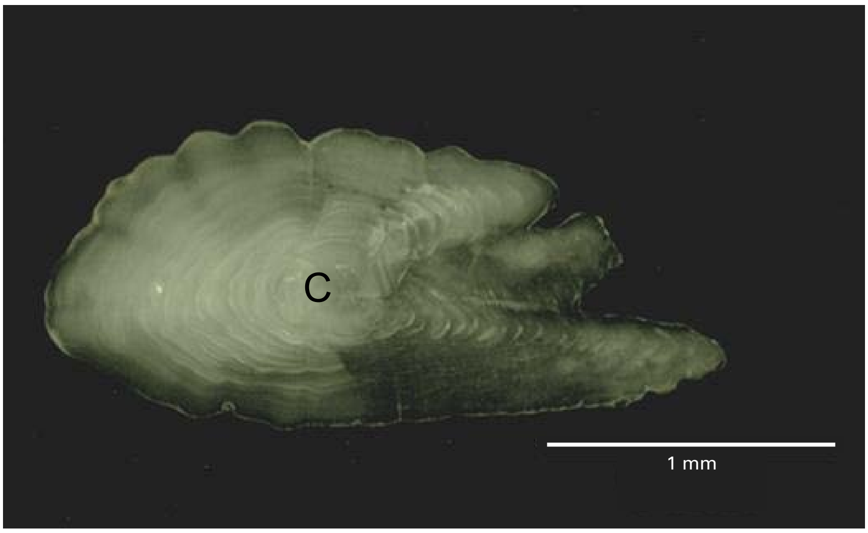
PLATE 24
Otolith *S. scombrus*



© F. Donato

Note: Age 2 years, male, TL = 23.5 cm, opaque edge, captured in March, red dots = winter rings.

PLATE 25
Otolith of *S. scombrus*



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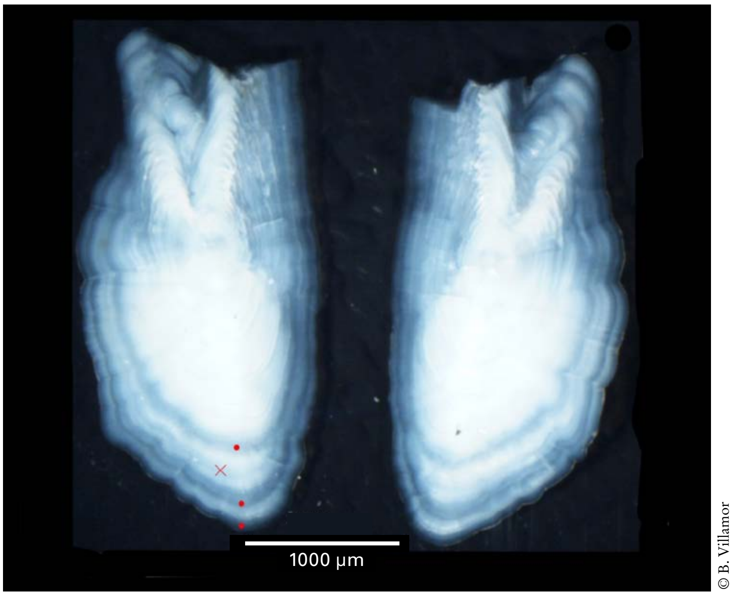
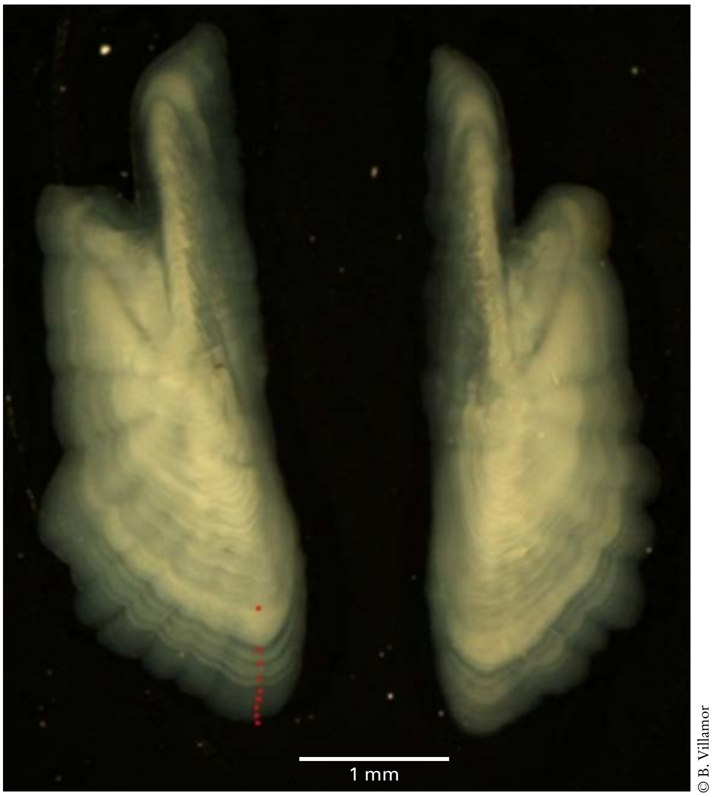
Note: Age 0.5 years, female, TL = 17 cm, captured in November, C = core (South Adriatic Sea).

PLATE 26
Otolith of *S. scombrus*



Note: Age 3 years, female, TL = 32 cm, captured in January, red dots = winter ring, X = false ring (Atlantic Ocean).

PLATE 27
Otolith of *S. scombrus*



Note: Age 9 years, female, TL = 40 cm, captured in March, red dots = winter ring (Atlantic Ocean).

2.4 *Scomber colias*

S. colias, formerly named *Scomber japonicus*, is a cosmopolitan species inhabiting temperate and subtropical waters worldwide at depths ranging from near the surface down to 300 m. *S. colias* is primarily a coastal pelagic species and, to a lesser extent, epipelagic or mesopelagic over the continental slope. *S. colias* has a very wide distribution in the Atlantic, Mediterranean and Black Sea. It lives in shoals based on size, as with other small pelagic species (Orsi Relini, 2017). Growth studies show high growth in the first years until the first maturity (Orsi Relini, 2017). In the Adriatic Sea, the maximum age was nine years in a sample of more than 4 000 specimens caught by purse seine (Čikeš Keč and Zorica, 2013). The Adriatic and North Aegean Seas seem to present a single spawning period from May to September, with a peak in June–July (Čikeš Keč and Zorica, 2013; Cengiz, 2012).

2.4.1 Extraction and storage

Sagittae extraction is made through the posterior section of the head. After extraction, the otoliths are washed to remove organic material and then dried and stored in rigid plastic vials.

2.4.2 Preparation and interpretation

One otolith from each pair (usually the left one) is immersed in seawater to be analysed. Otoliths of *S. colias* don't need a clarification phase before analysis.

The otoliths are analysed under a binocular microscope, rinsed with seawater (clarification medium), with reflected light, against a black background (ICES, 2015c). The best otolith orientation for analysis is with the distal surface up and the proximal surface (sulcus acusticus) down (Plates 28 and 29). In this way, the dark rings can be counted in the antirostrum area (radius) as translucent growth rings (slow growth). The opaque zone (white – fast growth) with a dark ring is considered an annual increment (annulus). Moreover, for every otolith, the edge quality is noted (opaque or transparent), while the measurements from the core to each translucent ring (at the end of the ring) on the postrostrum area (Plate 30) and the radius and otolith lengths are taken on a subsample of otolith.

PLATE 28
Both *S. colias* otoliths, one with proximal face up (top) and one with distal face up (bottom)

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Note: Female, TL = 24 cm, captured in July.

PLATE 29
Otolith of *S. colias* indicating where morphometric measures are taken

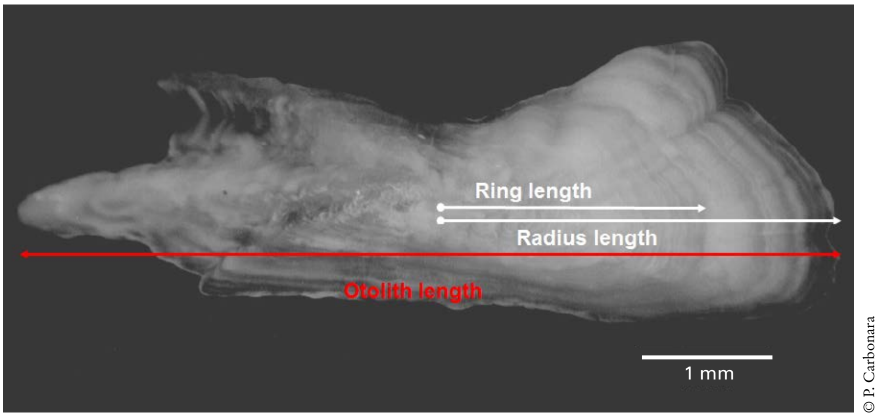
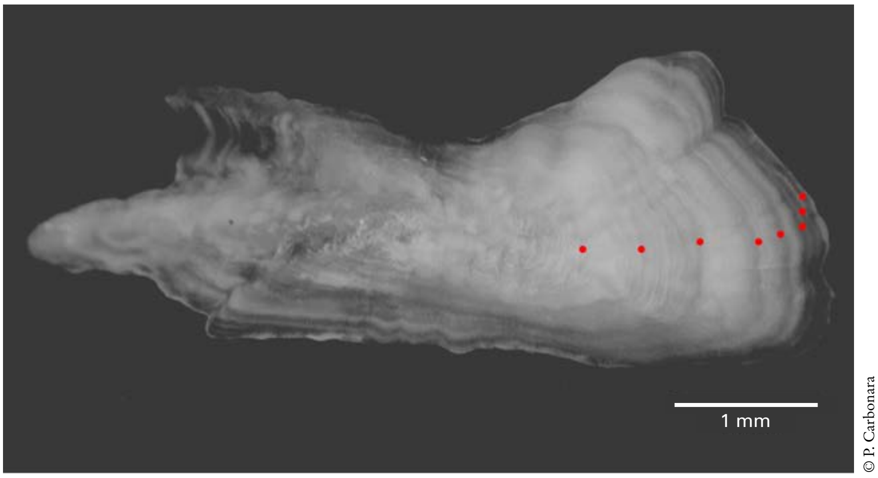


PLATE 30
Otholith of *S. colias*



Note: Age = 8 years, male, TL = 39 cm, captured in July, with opaque edge, red dots = winter rings.

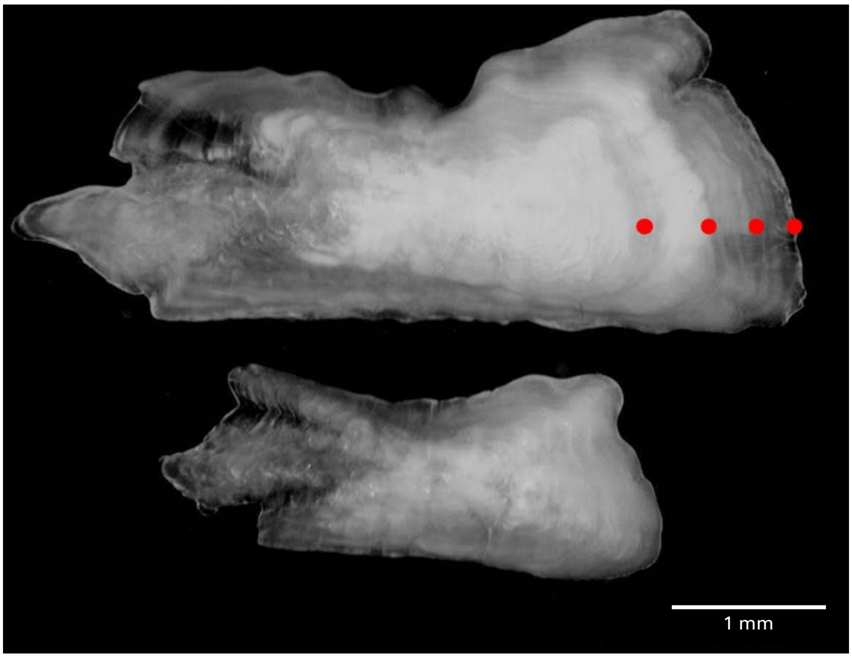
In accordance with the spawning period in late spring/early summer (Cengiz, 2012; Čikeš Keč and Zorica, 2012), the birth date is set at 1 July (ICES, 2015c). The criteria for determining the age of otoliths, reported in section 1.3.2, take into account the time of annulus formation (generally translucent rings during winter and spring months; opaque area during summer and autumn months), capture date, otolith edge and spawning period (birth date 1 July). Moreover, the age is assigned with a resolution of 0.5 years.

2.4.3 Difficulties in interpretation

The main interpretation difficulties for the *S. colias* otolith are linked to those in identifying the first annulus (Plate 31), owing to the presence of double rings and the overlapping of translucent rings on the margin (Plate 30).

The otoliths of *S. scombrus* in Plate 26 show the start of translucent ring deposition (Velasco *et al.*, 2011). The distance from the core to the postrostrum edge (~ 1.4 mm) of the specimen with 0 age (lower) is comparable to the distance to the first true winter ring on the older specimen (upper). So this distance could be taken into account in recognizing the first winter ring (ICES, 2015c).

PLATE 31
Otoliths of *S. colias* caught in November in the Adriatic Sea (central Mediterranean)



© P. Carbonara

Note: Upper specimen – age 3.5 years, TL = 28.5 cm; lower specimen – age 0, TL = 16 cm.

Otoliths of *S. colias* present a growth pattern with a large first annulus (ICES, 2015c) and decreased distances between the other annuli, but overlapping one another in the older specimens, making it difficult to determine the age of these otoliths (Plate 30).

2.5 *Trachurus mediterraneus*

T. mediterraneus is a semi-pelagic species, distributed in the Mediterranean and Black Seas and along the eastern coasts of the Atlantic from the English Channel to Morocco. In the Mediterranean, the genus includes two other very similar species, *T. trachurus* and *T. picturatus*.

T. mediterraneus is caught commercially by pelagic and bottom trawls, longlines and purse seines (using light).

At present, age and growth data on *T. mediterraneus* are very limited for the Mediterranean basin (Profeta *et al.*, 2017). Generally, horse mackerel (*Trachurus* spp.) otoliths are very difficult to read in older fish because they become thick with age (ICES, 1991). Because of these difficulties, several otolith exchange programmes and workshops have taken place in recent years attempting to reach a common agreement on assigning age (ICES, 1991, 2015b). This species shows a high growth rate in the first years (Zupa *et al.*, 2006; Karlou-Riga, 2000; Belcari *et al.*, 2007). The maximum age observed in the Mediterranean basin for *T. mediterraneus* is 12 years (Nobile *et al.*, 2008).

T. mediterraneus spawning season is quite long – from spring to early autumn (Viette, Giulianini and Ferrero, 1997; Karlou-Riga *et al.*, 2000; Šantič, Pallaoro and Jardas, 2006).

2.5.1 Extraction and storage

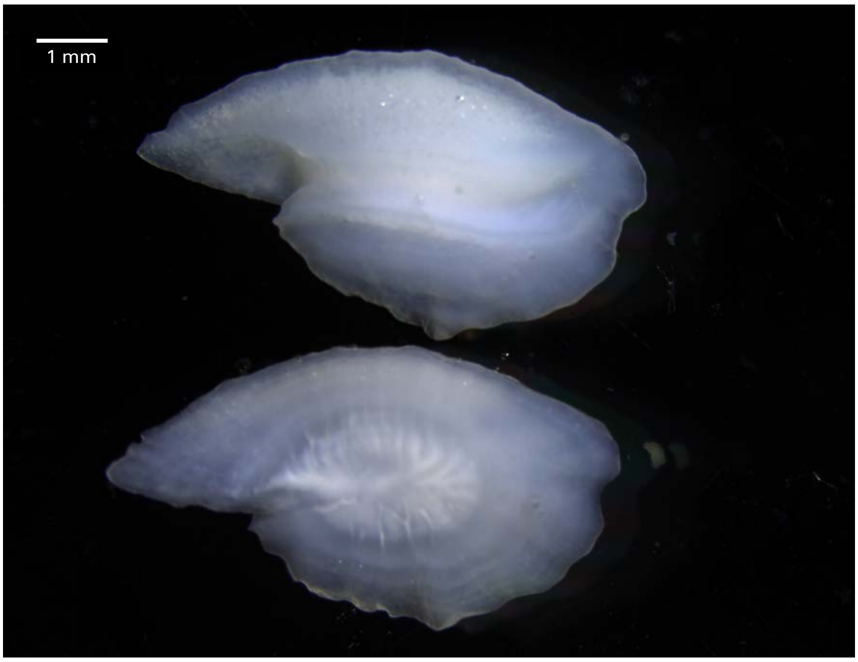
Sagittae extraction is made through the transverse section of the skull. After extraction, the otoliths are washed to remove organic material and then dried and stored in rigid plastic vials.

2.5.2 Preparation and interpretation

T. mediterraneus otoliths don't need a clarification phase before analysis, except for the larger specimens (> 30 cm), where a very short permanence in seawater (5–10 minutes) could be necessary (ICES, 2015b).

Otoliths are analysed under a binocular microscope, rinsed with seawater (clarification medium), with reflected light, against a black background. The best results will be obtained with the otolith placed with the distal surface up and the proximal surface (sulcus acusticus) down (Plates 32 and 33). In this way, dark rings will be counted in the antirostrum area (radius) as translucent growth rings (slow growth) (ICES, 2015b).

PLATE 32
Both *T. mediterraneus* otoliths, one with proximal face up (top) and one with distal face up (bottom)



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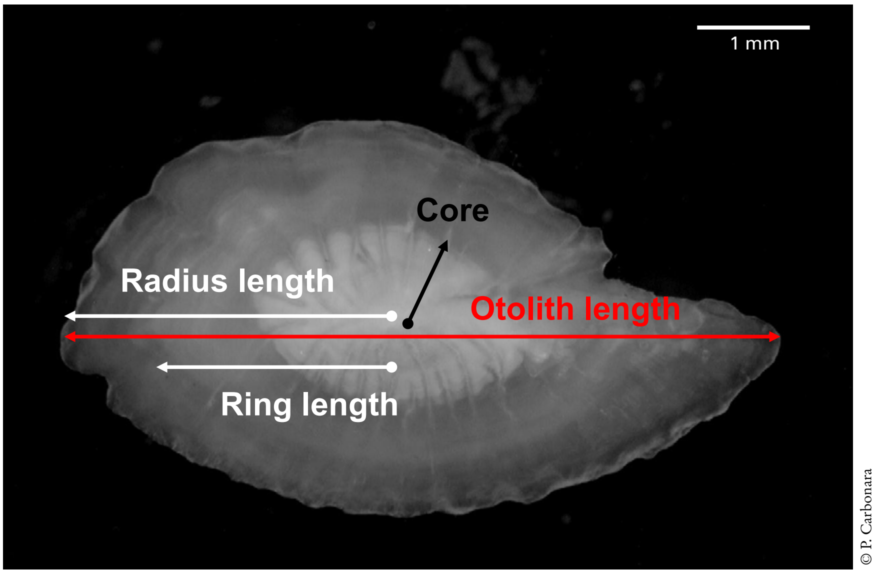
Note: Male, TL = 22.5 cm, captured in July.

The opaque zone (white – fast growth) with a dark ring is considered an annual increment (annulus). For each otolith, edge transparency will be noted (opaque or translucent ring) and a measurement of ring radius done. Ring radius will be measured along the postrostrum area from the core to each translucent ring (up to the edge) (Plates 34 through 39); radius and otolith lengths will be measured on an otolith subsample.

In accordance with *T. mediterraneus* spawning period (Viette, Giulianini and Ferrero, 1997; Karlou-Riga *et al.*, 2000; Šantič, Pallaoro and Jardas, 2006), the birth date is set at 1 July (ICES, 2015b).

PLATE 33
Otolith (sagitta) of *T. mediterraneus* with indication of main measures (ring, radius and otolith lengths)



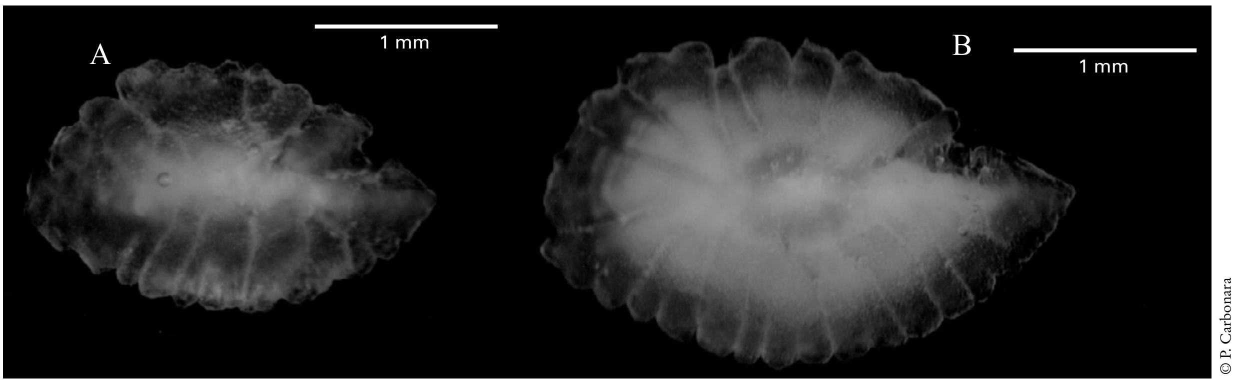
The ageing criteria are reported in section 2.3.2 and take into account the time of annulus formation (generally a translucent ring during winter and spring months and an opaque area during summer and autumn months), number of translucent rings, capture date, otolith edge and spawning period (birth date). Moreover, a 0.5-year time resolution is applied.

2.5.3 Difficulties in interpretation

As reported by Karlou-Riga (2000), before the first winter ring, some false rings are laid down. Indeed, the small specimens – "juveniles", TL 5–8 cm – born during spring/summer spawning and caught during summer/autumn months, present a transparent edge (Plate 34). This is a false ring, probably laid down when juveniles changed their environment and diet behaviour (from the pelagic to the benthopelagic phase). The diameter and length of these otoliths are about 2 mm (0.95 mm radius), and false ring marks are visible also in otoliths of older specimens with similar measurements (Plate 35).

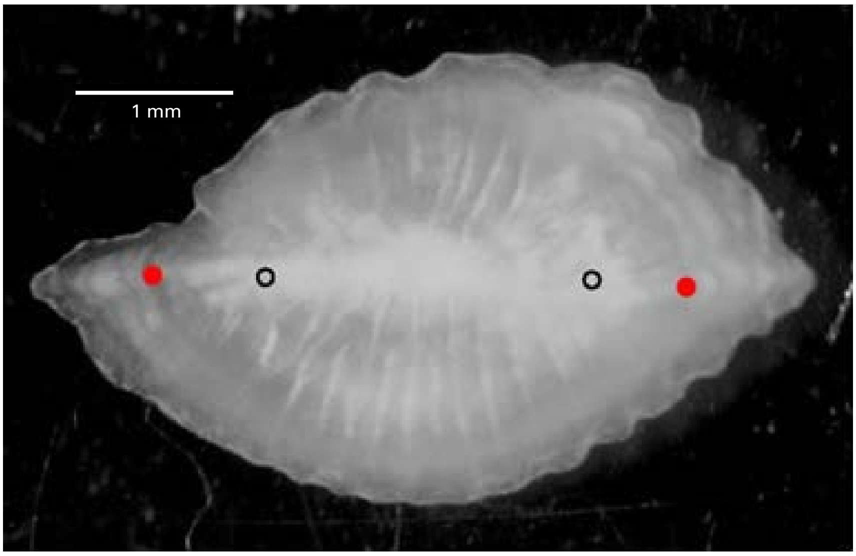
The first winter ring (true ring) is laid down later. Consequently, specimens caught during winter and early spring months (TL 12–14 cm) show a translucent ring more evident on the edge,

PLATE 34
Otoliths of juvenile specimens



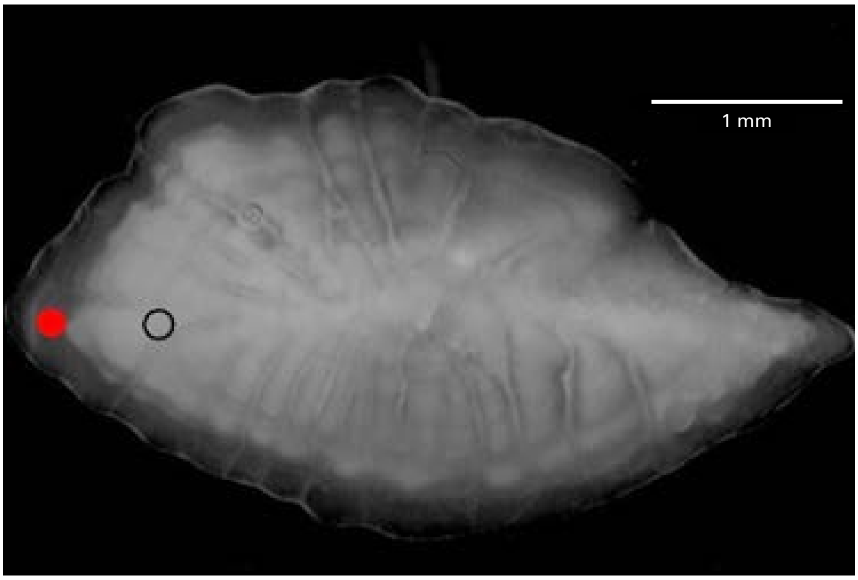
Note: TL = (A) 5 cm, (B) 7.5 cm, caught in (A) summer (29/07/2011) and (B) autumn (06/10/2011).

PLATE 35
Specimen caught in summer (28/07/2011)



Note: Age 1 year, TL = 14.5 cm, open black circles = false rings, red dots = first winter ring.

PLATE 36
Specimen caught in spring (12/05/2011)



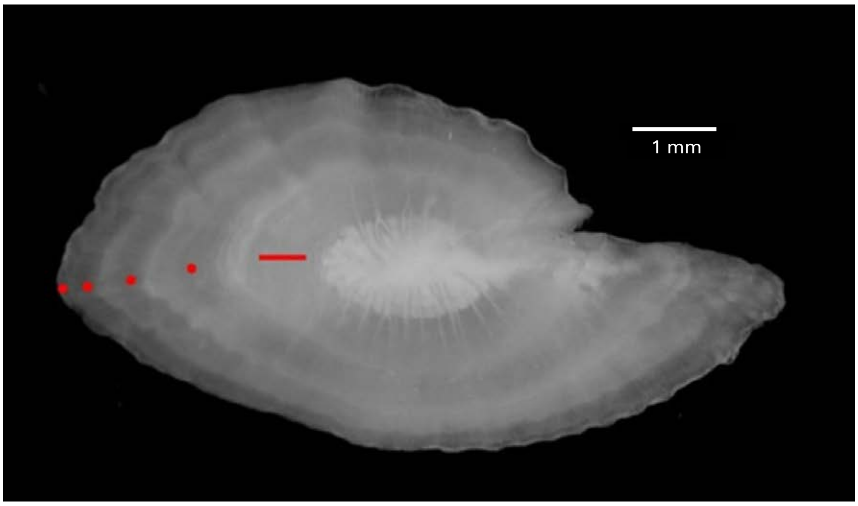
Note: Age 0.5 year, TL = 12.5 cm, open black circle = false ring, red dot = first winter ring.

with a radius of about 1.5 mm (about 3.5 mm total diameter) and a false ring close to the edge (Plate 36).

Sometimes, the first true ring appears not exclusively as a single ring, but as a more translucent area. Indeed, Karlou-Riga *et al.* (2000) distinguished four types of otoliths based on the morphology of the first winter ring (Plate 37).

After the first winter ring, other false rings could be laid down during the second year of life (Plate 38). This could be a mark laid down at the age of first maturity. For the North Adriatic Sea, Viette, Giulianini and Ferrero (1997) report the age at first maturity at two years, with a smallest mature specimen length of TL 15.6 cm and 16 cm, respectively, for a male and female.

PLATE 37
Specimen caught in spring (15/05/2011), with first winter ring as broad band (translucent zone) that includes some false rings



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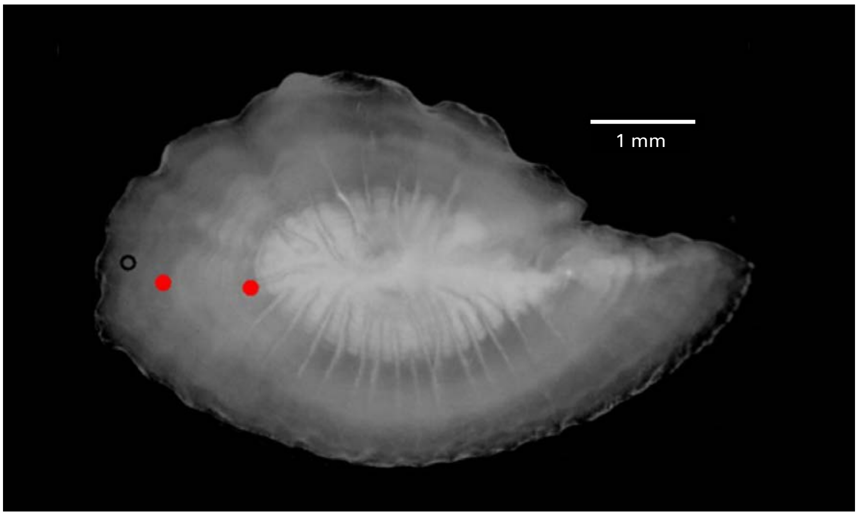
Note: Age 4.5 years, female, TL = 29 cm, red dots = winter rings, red line = first winter ring.

PLATE 38
Specimen caught in late autumn (15/10/2011), presenting gonads in post-reproductive stage



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Note: Age 2 years, female, TL = 20.5 cm, open black circle = false ring, red dots = true winter rings.

After the second winter ring, the winter band pattern appears more regular and the distance between rings becomes smaller. Rings in the older specimens overlap, and it will be more difficult to detect winter rings (Plate 39).

2.6 *Trachurus trachurus*

T. mediterraneus is widely distributed in the Atlantic Ocean from eastern Norway to South Africa, and is present in the Mediterranean and the Black Sea.

T. trachurus is a semi-pelagic species living in a wide depth range, from 10 to 500 m. It is caught by various kinds of gear (purse seine, midwater and bottom trawl, nets and longlines), and, in terms of landings, represents an important fishery species in Italian seas (IREPA, 2012).

PLATE 39
Specimen caught in March

Note: Age 9.5 years, male, TL = 35.5 cm, open black dots = false rings, red dots = true winter rings.

Despite the wide diffusion and its presence in commercial landings, significant gaps still exist in knowledge of the age and growth of this species for the Mediterranean area. The age reading for *T. trachurus*, as well for *T. mediterraneus* and *T. picturatus*, is generally considered difficult owing to the presence of false rings and to the fact that, in older fish, the annuli become thick (ICES, 1991, 2015b). Indeed, the deposition pattern of the opaque band and translucent ring is often interrupted by the presence of a double ring. These characteristics of the *T. trachurus* otolith could be at the origin of discrepancies in the growth data for the Mediterranean basin (Karlou-Riga and Sinis, 1997; Matarrese *et al.*, 1998; Nobile *et al.*, 2008).

The spawning season is long – from late autumn to early summer – with a peak in early spring (Carbonara *et al.*, 2012; Abaunza *et al.*, 2003; Karlou-Riga and Economidis, 1996).

2.6.1 Extraction and storage

The sagittae extraction is made through the transverse section of the skull. After extraction, the otoliths are washed to remove organic material and then dried and stored in rigid plastic vials.

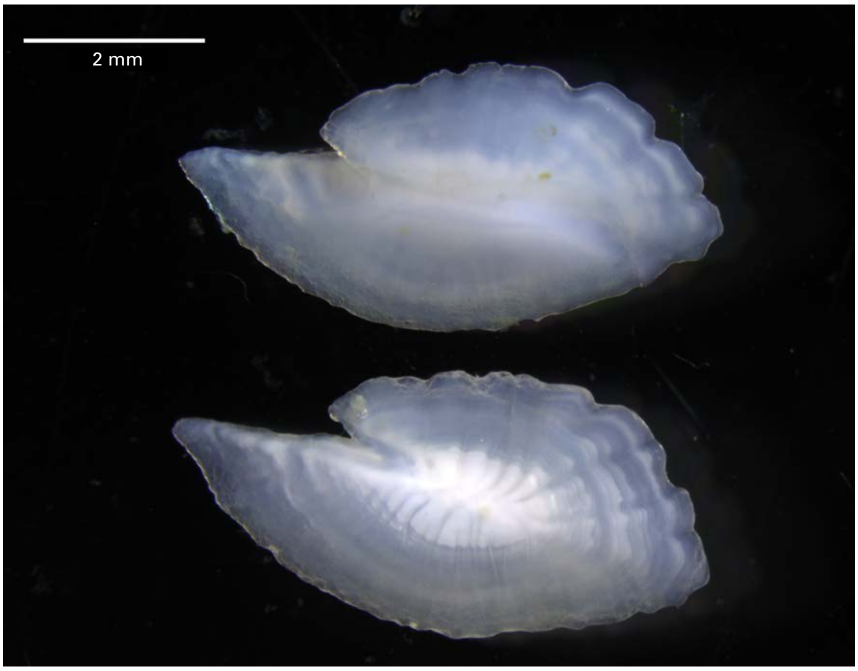
2.6.2 Preparation and interpretation

One otolith from each pair (usually the left one) is placed in immersion in seawater to be clarified before analysis. The time of immersion depends on the size of the fish: one or two minutes for juvenile specimens (TL < 20 cm), no more than two hours for specimens of TL 20–30 cm and about four hours for specimens of TL > 30 cm (ICES, 2015b).

The otoliths are analysed under a binocular microscope, in seawater (clarification medium), with reflected light, against a black background. The best orientation of the otolith is with the distal surface up and the proximal surface (sulcus acusticus) down (Plates 40 and 41).

Edge transparency will be noted for each otolith (opaque or translucent). Otolith metrics from the core to each translucent ring (at the end of the ring) on the postrostrum area, the radius and otolith lengths will be taken on a subsample.

PLATE 40
Both *T. trachurus* otoliths, one with proximal face up (top) and one with distal face up (bottom)



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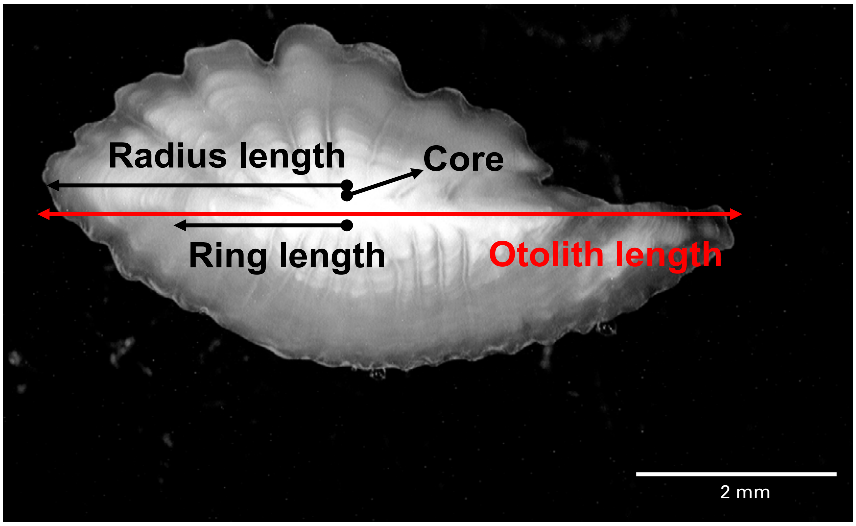
Note: Male, TL = 17 cm, captured in July.

PLATE 41
T. trachurus otolith (sagitta) with indication of main measures (ring, radius and otolith lengths)



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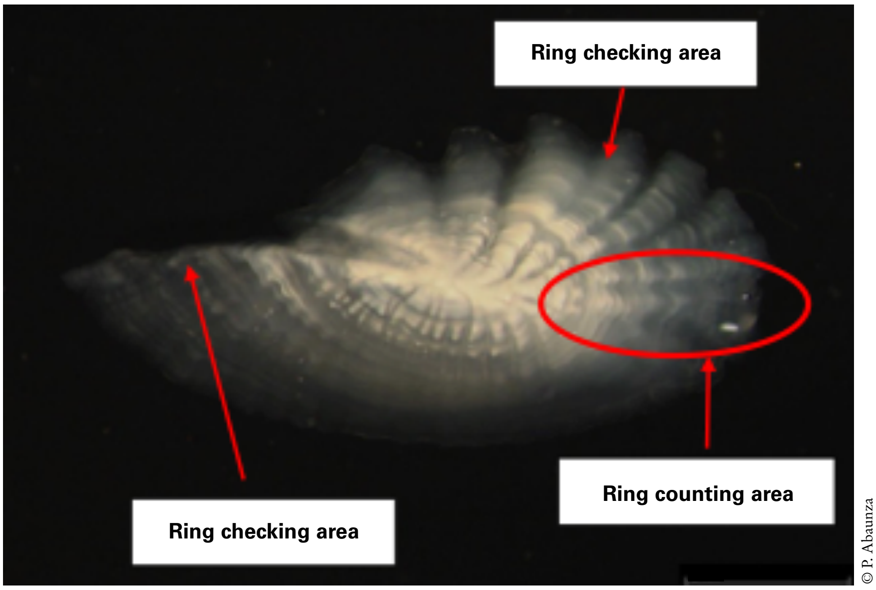
In the whole otolith, annuli are counted on the posterior area (postrostrum). However, ring continuity should be checked on the anterior part of the otolith (rostrum) and, wherever possible, on the dorso-lateral edge (Plate 42).

The dark rings are counted as the translucent growth zone (slow growth). The opaque zone (white – fast growth) with a dark ring is considered annual growth (annulus).

In accordance with a spawning period extending almost all year, with a peak during winter months (Abaunza *et al.*, 2003; Carbonara *et al.*, 2012), *T. trachurus* ageing sets 1 January as the birth date (ICES, 2015b). Ageing is performed counting translucent rings from the first winter ring, with a resolution of 0.5 years (see scheme reported in section 2.3.1).

PLATE 42
Whole otolith of *T. trachurus* immersed in seawater, showing main areas for counting and checking translucent rings



For larger fish (TL > 30 cm), it will be useful to also analyse the dorsal-ventral thin sections (about 550–650 microns [µm]). After having embedded the otolith in an epoxy resin, thin sections will be made through the core by a cutting machine (i.e. Buehler Isomet low speed, Struers Minitom). The blade mounted on the cutting machine has a continuous rim with a high concentration of diamond powder (i.e. Buehler series 15HC n.11–4244, diameter 100 mm, thickness 0.25 mm). The sections are analysed both with reflected light (black background) and transmitted light (Plates 43 and 44).

PLATE 43
Thin section of *T. trachurus* caught in September, analysed with reflected light

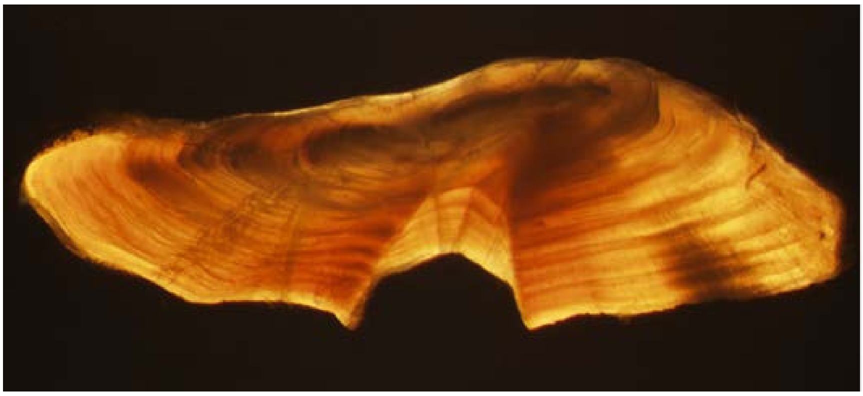
Note: Age 7.5 years, TL = 34.5 cm.

As there are no statistical differences in terms of agreement and CV in the results obtained with both techniques (whole and thin section) applied to the same sample, it is not possible to indicate a best ageing preparation technique (ICES, 2015b).

2.6.3 Difficulties in interpretation

The determination of annual increments is difficult owing to the presence of false rings, which can mislead interpretation of the annuli formation pattern. According to ICES (2015b), two major types of age-reading errors can be distinguished:

PLATE 44
Thin section of *T. trachurus* caught in October, analysed with transmitted light



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Note: Age 8.5 years, TL = 38.5 cm.

- False rings appear as translucent zones within an opaque zone. They are common in the first year of life of the fish and, in many cases, are easily confused with the first annual increment.
- Split rings, double structures, are composed of two unusually thin translucent bands separated by a very thin opaque band.

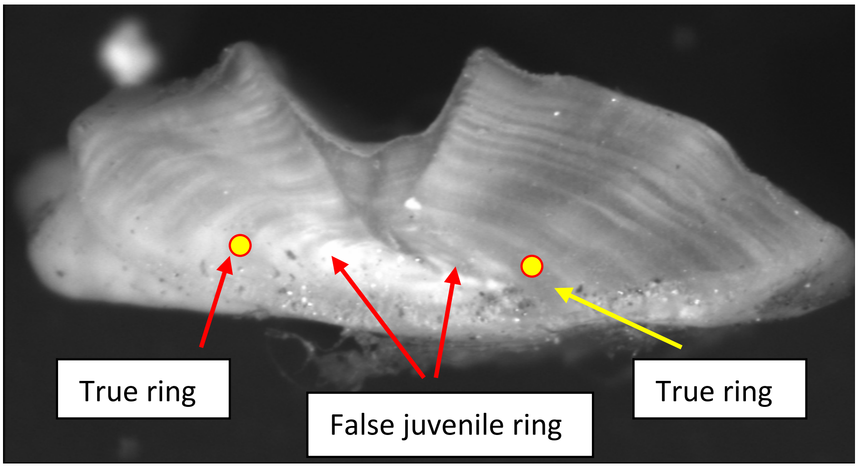
The causes of their formation aren't clear, although some factors, such as temperature, food intake, environmental conditions and development, have been suggested.

Due to difficulties in distinguishing false juvenile rings from true seasonal marks (Plates 45 and 46), identification of the first annulus is a matter of discussion at present. A comparison between whole otolith and sliced otolith views (of the same specimen) can be useful in distinguishing the common juvenile false rings.

In addition, sometimes the slices are not made through the core exactly, leading to a modified perception of the distance from the core to the first translucent mark (true ring). This can be a cause of difficulty in interpreting the first true mark (annulus) in the otolith.

These juvenile rings may be separate from the first translucent ring or may join with it, forming a broad translucent zone. Completion of the first translucent zone is usually detected on the

PLATE 45
True and false juvenile rings in a thin section of *T. trachurus*



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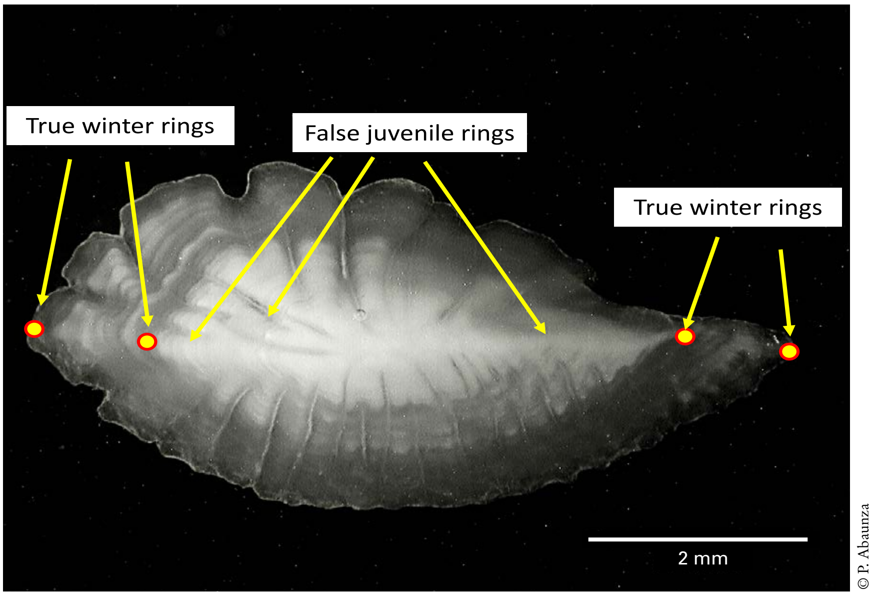
Note: TL = 36 cm, captured in February.

rostrum (Karlou-Riga and Sinis, 1997) (Plate 47). ICES (2012) provides some criteria to help with identification of these secondary structures (false rings):

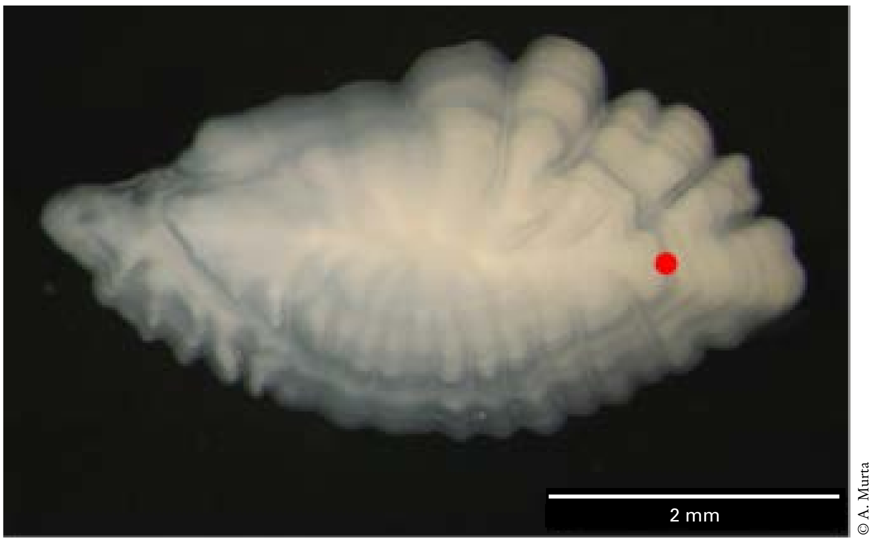
– Annulus extension in the otolith: in general, a true annulus, ring or mark should be traceable around the whole otolith (Plate 47) or section (ICES, 1999). Difficulties in detecting the last annuli increase when a fish is older and its otolith thicker.
– Distance between annuli: the width of consecutive annual growth zones should decrease with increasing age (Plates 48 and 49). In *T. trachurus*, the decrease in width is clear from one to five years. From five years on, the rates of decrease are slower, albeit rather constant (Plate 49).
– Contrast between seasonal marks: annual growth zones (annuli) can be distinguished from false rings by their sharper images and high contrast to the subsequent opaque (= white) increment of the next annual growth zone. Thus it can be distinguished by the brightest contrast between the preceding translucent and the subsequent opaque zones.

PLATE 46
True and false juvenile rings in whole otolith of *T. trachurus*



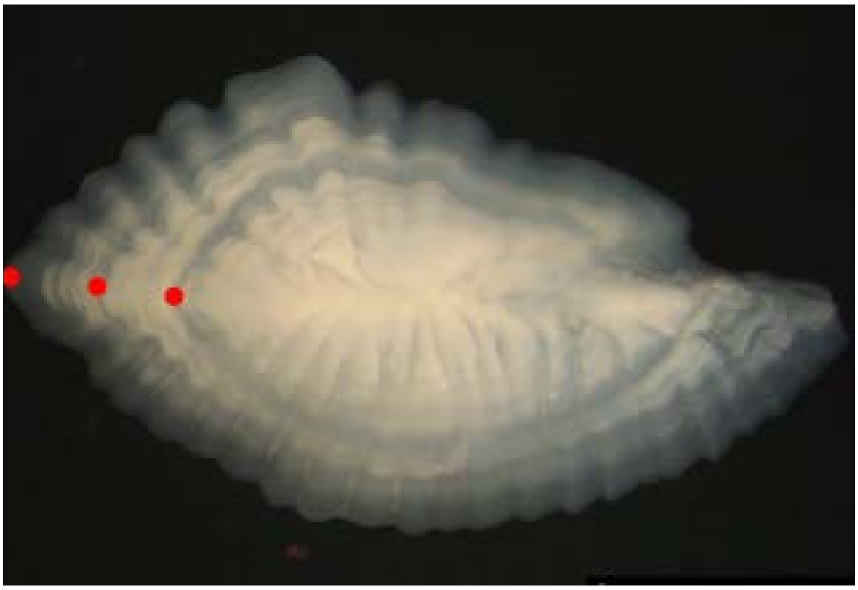
Note: TL = 19 cm, captured in December.

PLATE 47
Specimen caught in summer (August)



Note: Age 1.5 years, male, TL = 15.5 cm, red dots = true winter rings.

PLATE 48
Specimen caught in winter (March)



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Note: Age 3 years, male, TL = 22.5 cm, red dots = true winter rings.

PLATE 49
Specimen caught in autumn (October)

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Note: Age 7.5 years, female, TL = 33.5 cm, red dots = true winter rings.

3. Demersal species

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Demersal resources are represented by species that live on the bottom or directly related to it. Many of these species also live a pelagic phase, generally in the early months of their life. In the Italian seas, these species are mainly exploited, excluding bivalves, by otter trawlers, with minor importance in terms of total landings. They are also exploited by small-scale fisheries, especially with set nets (i.e. trammel and gill nets).

Demersal bony fish are represented by many species. The most important in terms of landings are: *M. merluccius*, *M. barbatus*, *M. surmuletus*, *B. boops*, *P. erythrinus*, *L. piscatorious*, *L. budegassa*, *S. solea*, some Triglidae such as *E. gurnardus*, and *C. lucerna*, some Gadidae such as *M. poutassou*, Greater forkbeard (*Phycis blennoides)* and *Spicara* spp.

Total landings of fisheries in Italian waters were about 172 000 tonnes in 2013. Otter trawlers account for about 69 650 tonnes (about 40 percent of total production) and small-scale fisheries about 26 570 tonnes (15 percent of total). Total income was about 839.01 million euros; otter trawlers account for about 53 percent and small-scale fisheries about 23 percent. Of 172 000 tonnes of total production, about 65 percent was represented by bony fish, 20 percent by molluscs, 13 percent by crustaceans and less than 1 percent by cartilaginous fish (sharks and rays). Demersal resources represent about 45 percent of total national production (33 percent caught by otter trawlers and 12 percent by small-scale fisheries). Bony fish represent about 50 percent, molluscs, mainly represented by cephalopods, about 25 percent, crustaceans about 24 percent and cartilaginous species about 1 percent (Mannini and Sabatella, 2015).

Otter trawlers mainly exploit resources living on sandy and muddy bottoms, while fixed nets are often set near the rocky substrate. As main target species, the trawlers have: *M. barbatus*, *M. merluccius*, *Lophius* spp., mixed soup fishes (Sparidae, Triglidae, etc.), *S. solea* caught by beam trawl (*rapido*) in the Adriatic Sea, squid (mainly broadtail shortfin squid [*Illex coindetii*], European squid [*Loligo vulgaris*]), octopus (common octopus [*Octopus vulgaris*], horned octopus [*Eledone cirrhosa*], musky octopus [*Eledone moschata*]) and crustaceans (*P. longirostris*, Norway lobster [*Nephrops norvegicus*], Spottail mantis squillid [*Squilla mantis*], *A. antennatus*, *A. foliacea*, caramote prawn (*Penaeus kerathurus*) Fixed nets mainly catch: *M. barbatus*, larger individuals of *M. Merluccius*, common cuttlefish (*Sepia officinalis*), in some areas *S. solea* and, again, mixed soup fishes.

Growth and age determination studies are essential in setting up appropriate management measures. Indeed, stock assessment analytical models require growth parameters as important input data. Even today, there are many uncertainties regarding age estimation of key species such as hake and monkfish. A common protocol could be an important tool to decrease relative/absolute bias and to improve the precision of age determination among age readers at diverse laboratories or institutes.

3.1 *Merluccius merluccius*

M. merluccius is one of the most important commercial species in the Mediterranean. As with many species, for *M. merluccius*, as well, assessment includes several areas of uncertainty, such as growth, population structure and stock definition, which in some cases represent limiting factors in evaluating the state of exploitation of this important resource. Many of the models used to evaluate the health status of the exploited population are based on age models, so growth becomes a crucial point to investigate. In *M. merluccius*, sagittal otoliths (sagittae) are routinely used for age determination. Annual rings (annuli) have proven difficult to interpret (ICES, 2009a) owing to the complexity of the otolith macrostructure. Interpretation of otolith growth marks is thus often a difficult task, in which subjectivity increases with the complexity of the structural pattern of the otoliths. Visible macrostructures on *M. merluccius* otoliths may have different origins. Identification of these macrostructures and establishing a link between them and any event in the life cycle represent fundamental steps in accurate age estimation. The *M. merluccius* long spawning season is a main cause of complexity in interpreting the growth pattern. Piñeiro (2000) has noted that the major difficulties in otolith age estimation are:

- location of the first annual ring (first annulus);
- classification of rings as growth ring with annual periodicity or false ring (or checks) due to phenomenon other than growth;
- interpretation of the edge nature.

Identification of the first annual ring is problematic owing to the presence of several checks (ICES, 2010a).

3.1.1 Extraction and storage

Sagittae extraction is made through a posterior section (see subsection 1.2.1) or inferior portions of the neurocranium. In the latter case, sagittae are extracted cutting through the gill isthmus. Gill arches are cut away and tissue is removed from inferior portions of the neurocranium before exposing the prootic bulla. Once extracted, the otoliths are washed to remove organic material and then dried and stored.

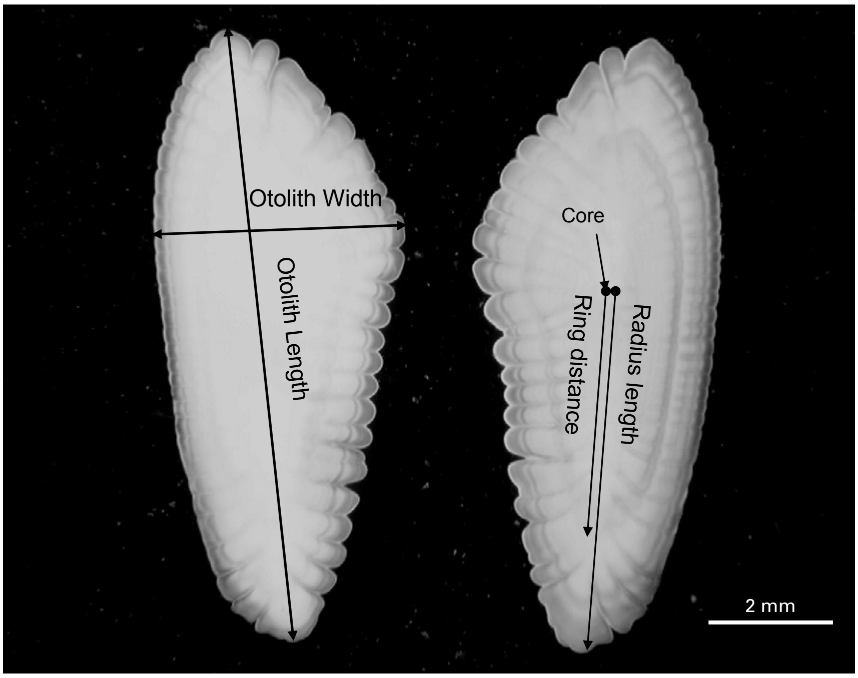
3.1.2 Preparation and interpretation

M. merluccius sagittae need to be clarified and ground/polished before analysis. Generally, only one otolith from each pair (usually the left one) is clarified by permanence in seawater for some hours (5–10 h). The proximal surface of the otoliths is then ground/polished by sandpapers in successively finer steps (from 180 to 600 µm grain size). The otoliths are analysed under a binocular microscope, immersed in seawater, with reflected light, against a black background. Analysis should be carried out with the otolith oriented with the proximal surface up and the distal one down (Plate 50).

In this way, the dark rings can be counted in the rostrum area (radius) as translucent growth rings (slow growth). The opaque zone (white – fast growth) with a dark ring is considered an annual increment. Morphometric measures of otoliths and ring distances are routinely taken, as is shown in Plate 50.

As otoliths of larger specimens (> 40 cm TL) are in some cases difficult to read whole, they are embedded in epoxy resin and dorsal-ventral thin sections made (about 550–650 µm) through the nucleus by a cutting machine (i.e. Buehler Isomet low speed, Struers Minitom). The blade mounted on the cutting machine has a continuous rim with a high concentration of diamond powder (i.e. Buehler series 15HC n.11-4244, diameter 100 mm, thickness 0.25 mm).

PLATE 50
Pair of *M. merluccius* otoliths – right otolith is clarified and ground/polished



Note: Male, TL = 20.5 cm, main morphometric measures shown.

An epoxy resin (i.e. Buehle EPO-KWICK, Prochima E-30) with two components (resin and catalyst) is used to embed the otoliths, while a cold resin (i.e. Entellan Merck™) is used to mount the thin sections on glass slides. The mounted slices are analysed under a binocular microscope, with reflected light (translucent rings appear dark and opaque zones in white), and/or an optical microscope, with transmitted light (translucent rings appear white and opaque zones appear dark). Annual translucent rings are counted, preferably on the ventral region, and used to estimate age (Plate 51).

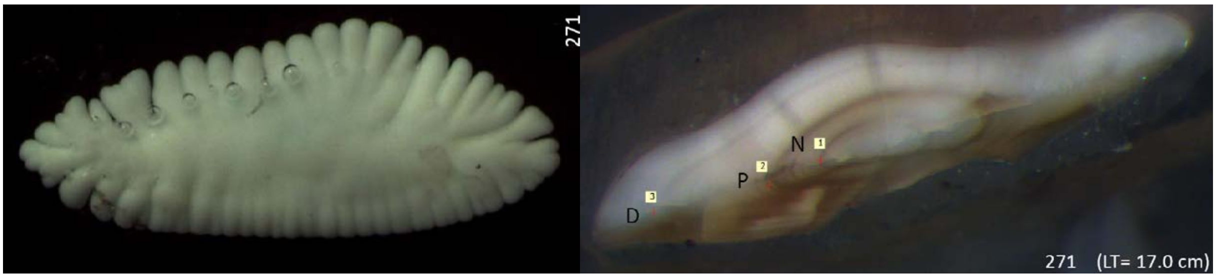
PLATE 51
Whole otolith (left image) and its thin transverse section (right image) of *M. merluccius*

© P. Carbonara

Note: Female, TL = 49.5cm.

Another preparation method consists in burning the otolith before realizing the transverse section. In this method, saggitae are put in an oven for 20 minutes at 350 °C, then embedded in resin, transversely sectioned, mounted on glass slides and finally analysed under a binocular microscope, with reflected light (Plates 52 and 53).

PLATE 52
Whole (left image) and burned/sectioned (right image) otolith of *M. merluccius*



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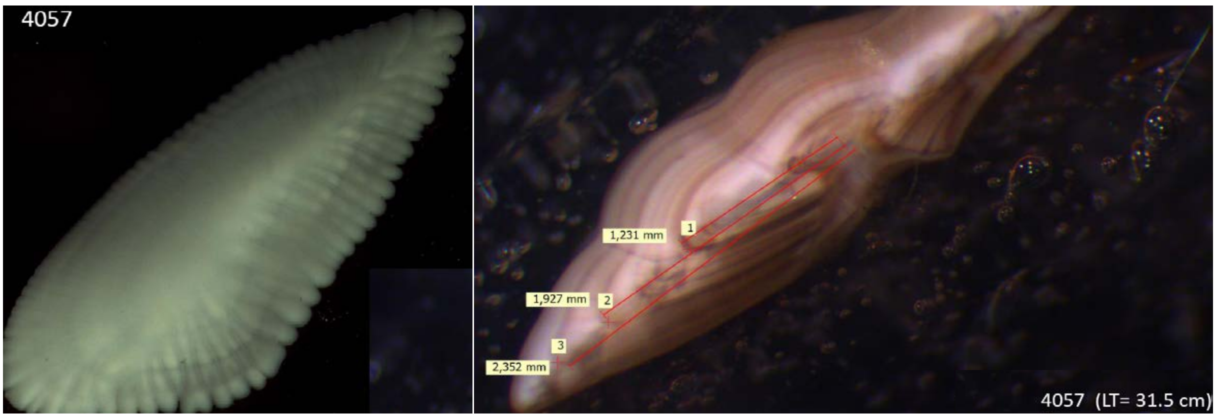
Note: Female, TL = 17 cm, month of capture = June.

PLATE 53
Whole (left image) and burned/sectioned (right image) otolith of *M. merluccius*



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Note: Female, TL = 31.5 cm, month of capture = July.

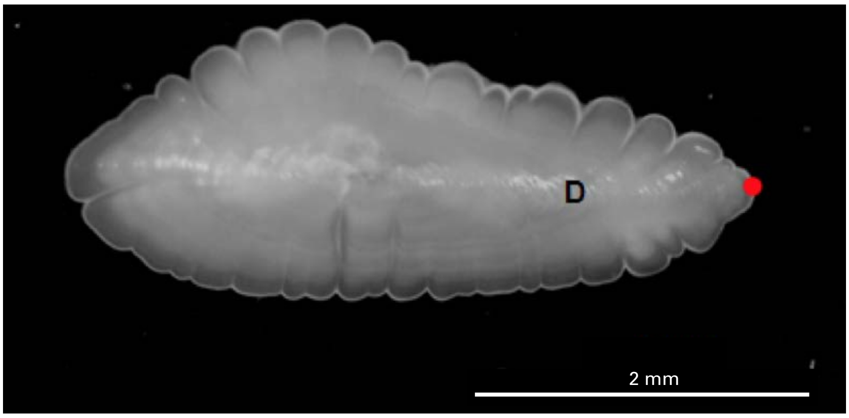
For the ageing criteria, the birth date is set at 1 January, with resolution of one year (see subsection 1.3.3).

3.1.3 False rings and true growth annuli

In reading hake otoliths, one important problem is identification of the first growth ring (ICES, 2009a). Indeed, before the first growth ring, the presence of checks is common (usually three pelagic and one demersal) around the nucleus (Piñeiro *et al.*, 2009) (Plates 52 and 54).

Moreover, the spawning period of *M. merluccius* extends throughout the year, with two peaks in the winter and spring/summer months (Recasens, Chiericoni and Belcari, 2008; Belcari, Ligas and Viva, 2006). In this way, it is possible to recognize two cohorts (specimens born in winter and specimens born in spring/summer) through observation of the otolith morphometric measures, considering the distances from the nucleus to the first growth ring. Indeed, the first growth ring in specimens born in winter months will present a greater distance than specimens born in summer months (Plate 55). So, the first growth ring could have a distance from the core of from 1.5 to 4.5 mm (ICES, 2013b).

PLATE 54
Otoliths from specimen caught on 21/01/2010



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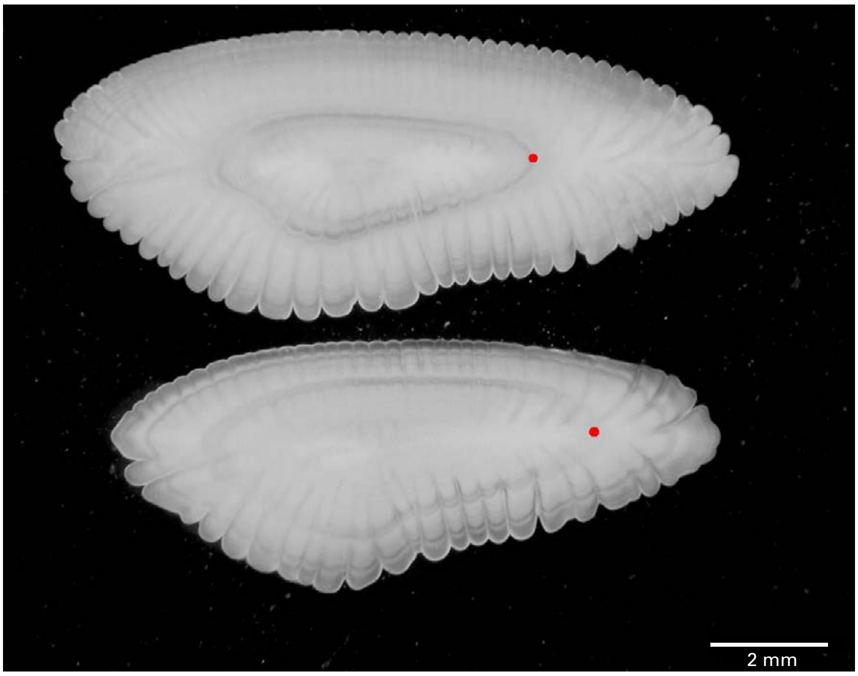
Note: TL = 9 cm, red dot = growth ring, D = potential demersal check.

Growth during the first and second years is greater compared with subsequent annual increments. Moreover, a check is frequently found after both the first and second growth rings (Plates 56 and 57).

During the last workshop on *M. merluccius* ageing (ICES, 2010a), some criteria were defined for recognizing the growth ring:

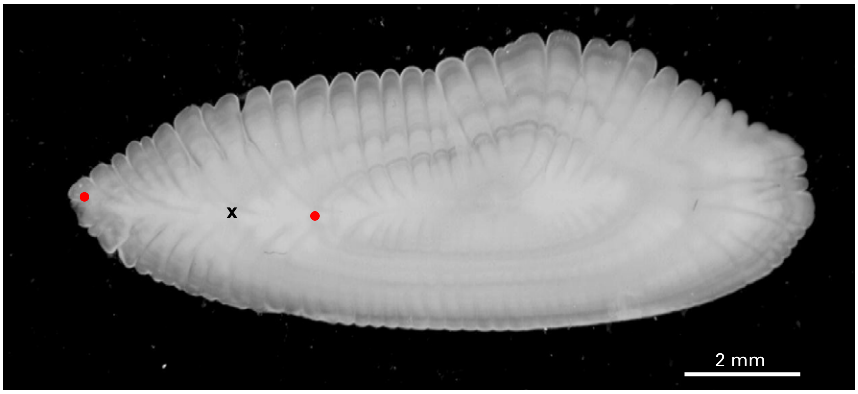
- The growth pattern should be considered based on growth bands around the whole transverse section or the whole otolith, including both ventral and dorsal axes, so as to be able to distinguish the splitting into two or more translucent rings and thus to better discriminate between possible false rings and the annulus.
- Each annulus (annual translucent zone) consists of bands of several thinner translucent rings. Interpreting these individual components of the annuli as complete annuli is a potential cause of overestimation of age when reading *M. merluccius* otoliths.
- Using different magnifications helps reveal the pattern of translucent and opaque bands. Changing the light source from transmitted to reflected light can also help in interpretation of the otolith.
- Growth rings follow a pattern of decreasing distance between the rings (Plates 58 and 59).

PLATE 55
Otoliths from specimens born in summer (top) and winter (bottom)



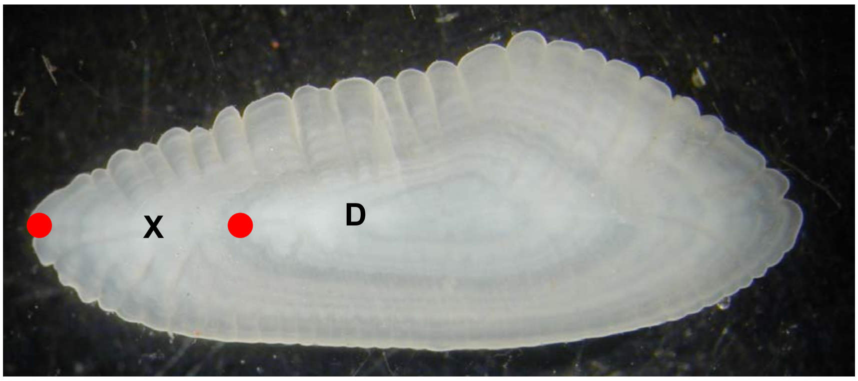
Note: Top – male, TL = 23, bottom – male, TL 20.5, red dots = first growth ring.

PLATE 56
Otoliths from male specimen caught on 21/01/2010



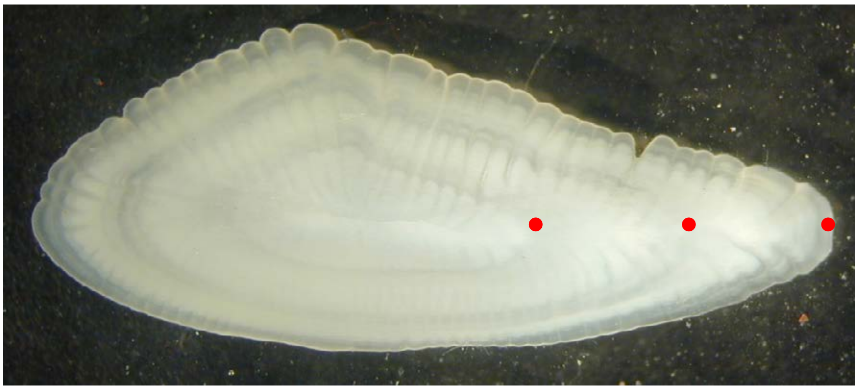
Note: Age 2 years, TL = 25 cm, red dots = growth rings, X = check.

PLATE 57
Otoliths from male specimen caught on 30/03/2010



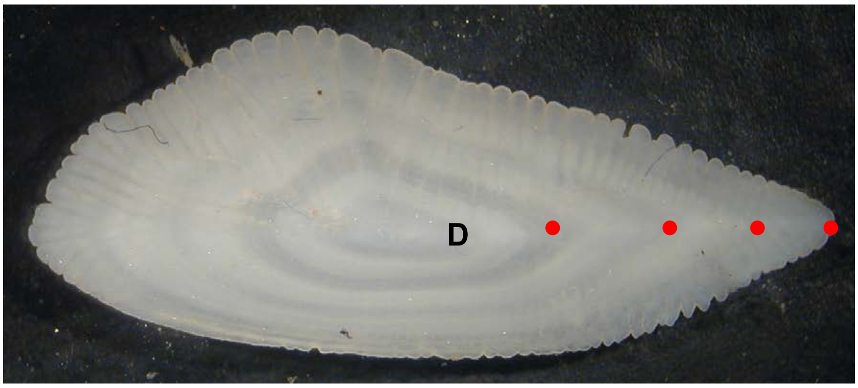
Note: Age 2 years, TL = 19 cm, red dots = growth ring, X = check and D = potential demersal ring.

PLATE 58
M. merluccius otoliths with a reduction of distances between growth rings



Note: Age 3 years, male, TL = 32 cm, date of capture = 3/05/2010.

PLATE 59
M. merluccius otolith of female specimen caught on 31/03/2010



Note: Age 4 years, TL = 46 cm, red dots = growth rings, D = potential demersal ring.

3.2 *Mullus barbatus*

M. barbatus represents one of the most important resources for shelf fisheries. Indeed, during 2011 it constituted about 27 percent (4 167 tonnes) of landings of the demersal species (IREPA, 2012) along the Italian coast. Given its economic relevance in both small- and large-scale fisheries, *M. barbatus* is among the most-investigated fish of the Mediterranean basin (Bianchini and Ragonese, 2011), but some gaps remain, for example regarding growth (ICES, 2009a, 2012, 2017b). Scales and otoliths are both used to estimate the age of *M. barbatus* and *M. surmuletus*, but the latter are considered the most suitable structure (ICES, 2012); in fact, scale reading seems to underestimate older ages.

The last otolith exchanges (Mahé *et al.*, 2012a, 2016) and follow-up workshops (ICES, 2012, 2017b) identified two very important sources of bias:
– disagreement regarding identification of the first annual ring;
  and
– in older specimens, last rings too close, creating great difficulty differentiating them (Plates 64 and 65).

Regarding the first annual growth ring, some authors indicate only one check before the first growth ring (Carbonara *et al.*, 2018; Sonnin *et al.*, 2007; Tursi *et al.*, 1997; Livadas, 1989), while others (Sieli *et al.*, 2011; Fiorentino *et al.*, 1998; Vrantzas *et al.*, 1992) don't count the first two translucent rings in age estimation, considering them as pelagic and demersal checks.

3.2.1 Extraction and storage

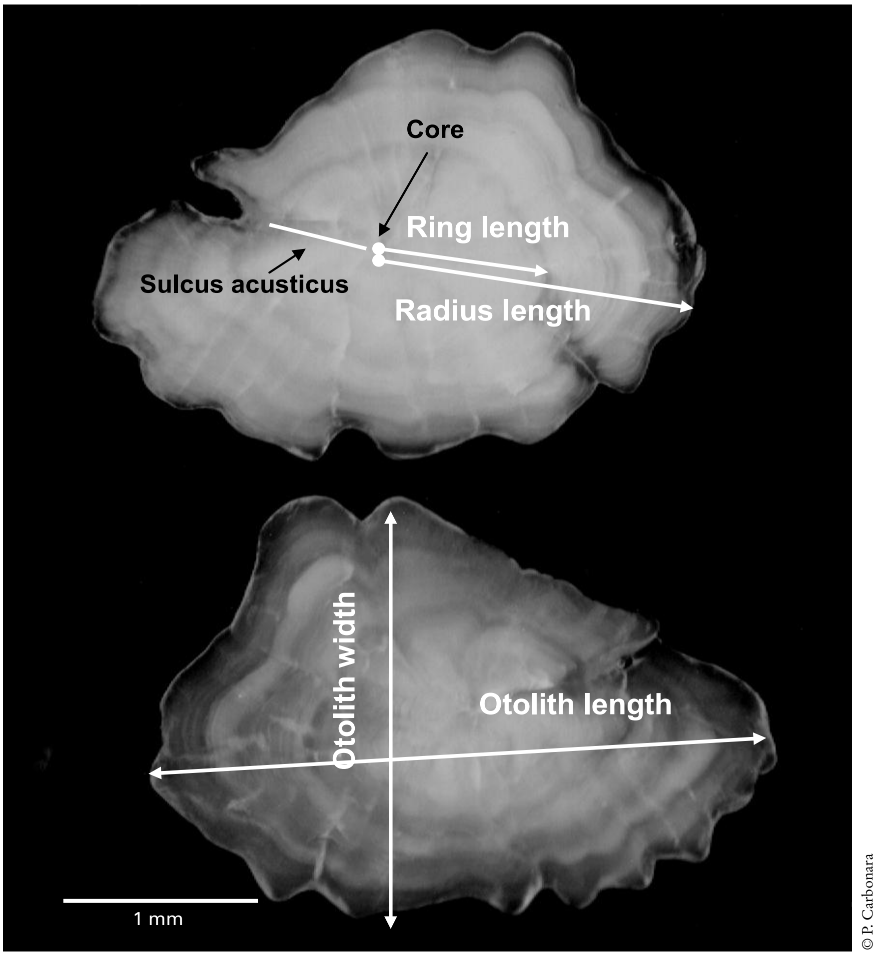
Sagittae extraction is made through the transverse section of the head. After extraction, the otoliths are washed to remove organic material and then dried and stored.

3.2.2 Preparation and interpretation

One otolith from each pair (usually the left one) is immersed in seawater to be analysed. Otoliths of *M. barbatus* don't need the clarification phase before analysis, except for larger specimens (TL > 20 cm), where a very short permanence in seawater (2–4 minutes) could be necessary. A too-long permanence in seawater of the *M. barbatus* otolith, being very thin, could render it too translucent and thus barely readable (Plate 60).

The otoliths are analysed under a binocular microscope, rinsed with seawater (clarification medium), with reflected light, against a black background. The best otolith orientation for analysis is with the distal surface up and the proximal surface (sulcus acusticus) down (Plate 60). The opaque zone (white – fast growth) with a translucent zone (dark – slow growth) are considered an annual increment (annulus).

PLATE 60
Otoliths of *M. barbatus* (bottom – after a long permanence in sea water)



Note: Male, TL = 17 cm, axes of morphometric measures shown (otolith width and length).

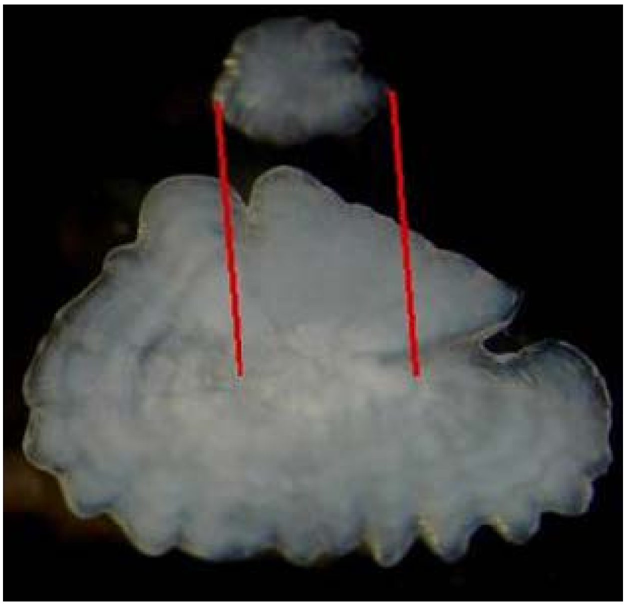
On a subsample of otoliths, morphometric measures and growth ring distances are routinely taken on the postrostrum area along the axis joining the sulcus and the nucleus (ICES, 2009a; Plate 60). The nature of the edge (opaque or translucent) is always noted.

In the Mediterranean basin, the spawning period for *M. barbatus* is reported as April to August (Ezzat, Hosny and Osman, 1997; Menini *et al.*, 2001; Cherif *et al.*, 2007; Carbonara *et al.*, 2015), with a peak in June–July, so the birth date is set at 1 July (ICES, 2012; ICES, 2017b). Criteria for age determination of *M. barbatus* are reported in subsection 1.3.2 and take into account the time of annulus formation, capture date, otolith edge and spawning period. Moreover, age is assigned with a resolution of 0.5 year.

3.2.3 False rings and true growth annuli

Before the first true growth ring, a potential demersal ring is laid down, in correspondence with the change in behaviour from pelagic (blue phase) to demersal (Sieli *et al.*, 2011). This check (demersal ring) corresponds to the first ring that appears on the otolith. The measure of the demersal ring is about TL 0.6 cm, corresponding to the back-calculated TL of 4–5 cm, the size at settlement on the bottom (Carbonara *et al.*, 2018). Indeed, the specimens of about TL 4 cm caught in July and August present a translucent edge (demersal check) (Carbonara *et al.*, 2018) (Plate 61).

PLATE 61
Otoliths of *M. barbatus*



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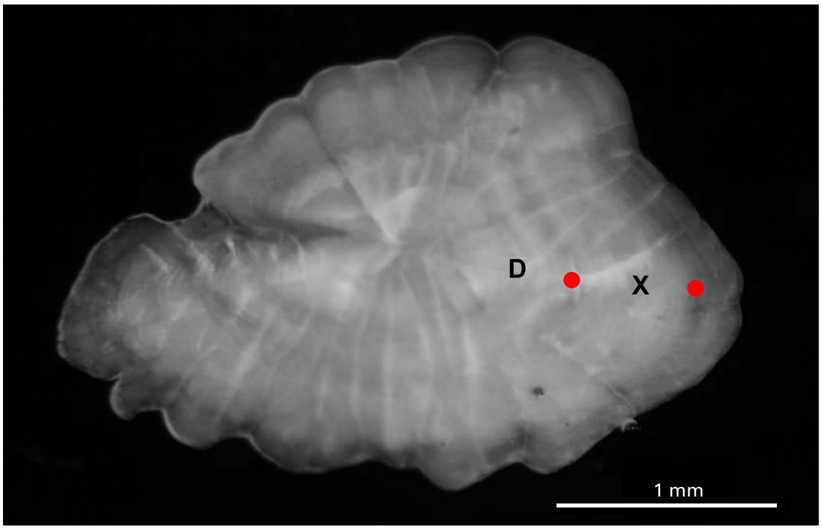
Note: Bottom –TL = 28 cm, month of capture = May; top – TL = 4 cm, month of capture = August.

The otolith ring deposition pattern in juvenile specimens (TL 3.5–8 cm) shows that it was laid down only one check before the growth ring (ICES, 2017b; Carbonara *et al.*, 2018). So it is possible to consider only one false ring before the first growth ring as an ageing criterion.

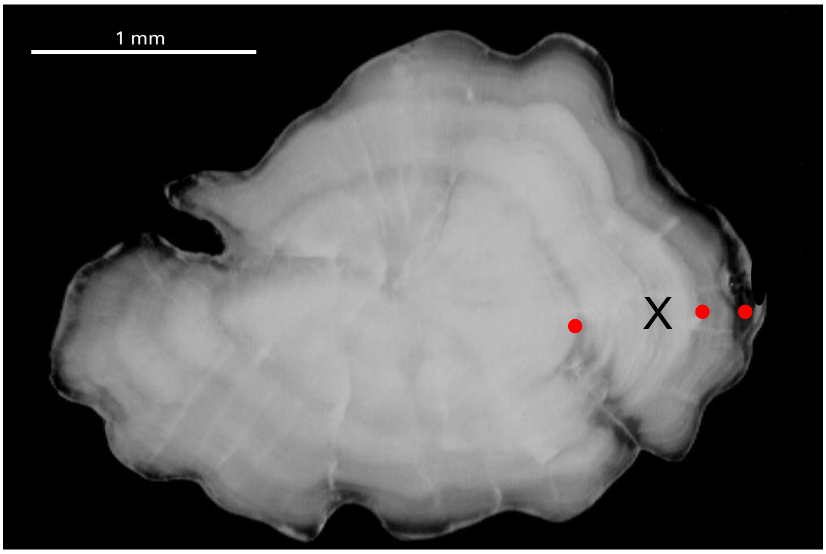
During the first year, growth of otoliths is rapid compared with subsequent annual increments. Moreover, a check could also be found after the first growth ring (Plates 62 and 63). This false ring could be due to the first sexual maturity. This check usually appears less marked in comparison with growth rings. It is often interrupted and isn't visible around the entire otolith circumference (Plates 62 and 63). Sometimes the first growth ring appears not exclusively as a single ring, but as a translucent area (Plate 64). This could happen if the demersal ring merges with the first growth ring.

PLATE 62
Otolith of *M. barbatus*



Note: Age 2 years, female, TL = 17 cm, month of capture = September, red dot = growth ring, D = demeral ring, X = check.

PLATE 63
Otolith of *M. barbatus*

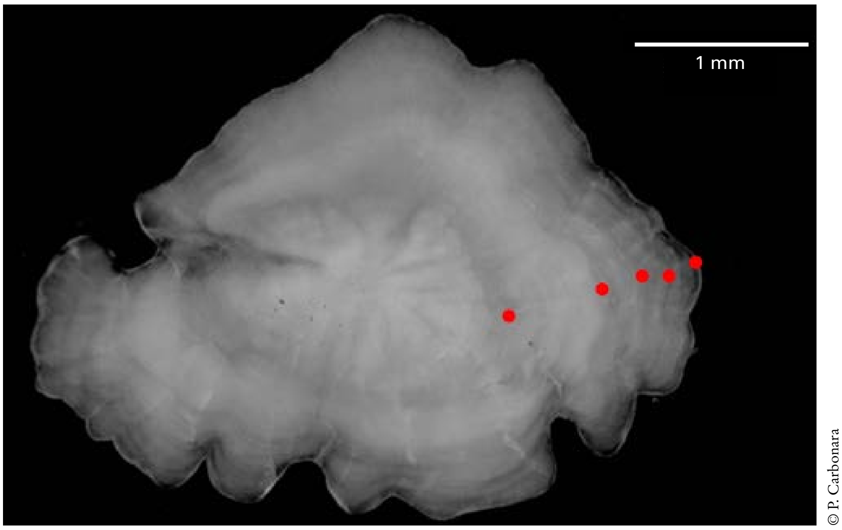


Note: Age 2.5 years, male, TL = 16.5 cm, month of capture = March, red dot = growth ring, X = check

Some criteria were defined to recognize the true growth ring during the last workshops on red mullet ageing (ICES, 2009a, 2017b; Carbonara *et al.*, 2018):

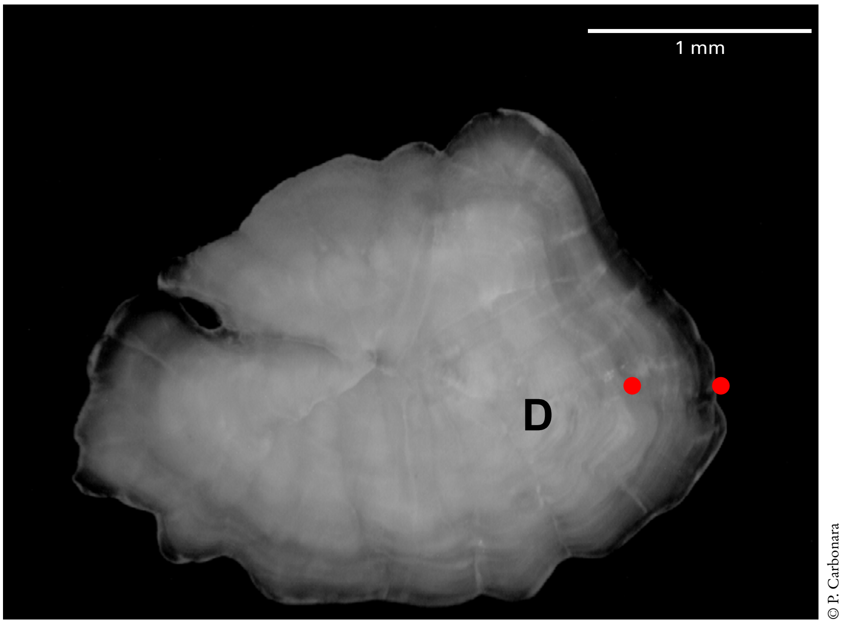
- To be considered as annual rings, translucent true rings should be visible more or less around the whole otolith.
- Before the first growth ring is laid down, only one false ring (demersal) occurs.
- The increments between the consecutive annuli should decrease with age (Plates 64, 65 and 66).

PLATE 64
Otolith of *M. barbatus*



Note: Age 4.5 years, female, TL = 24 cm, month of capture = March, red dot = growth ring.

PLATE 65
Otolith of *M. barbatus*



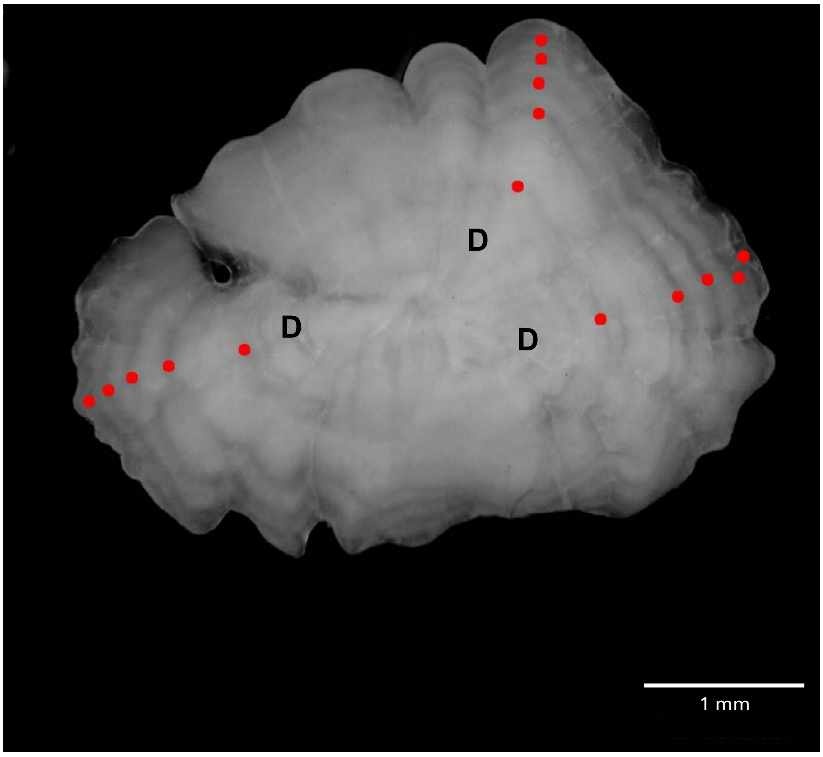
Note: Age 1.5 years, female, TL = 14.5 cm, month of capture = March, red dot = growth ring, D = demersal ring.

3.3 *Mullus surmuletus*

Information on the aging of *M. surmuletus* (striped red mullet) in the Mediterranean is scarcer than that on *M. barbatus*. However, many of the methods and ageing criteria applied to *M. barbatus* and discussed above seem to be equally useful for *M. surmuletus* (ICES, 2012). In particular, the CS used to determine age used to determine age (otoliths), extraction techniques, otolith preparation before reading, measures, birth date (e.g. 1 July) (ICES, 2017b) and, finally, interpretation criteria are the same applied to *M. barbatus* (subsection 3.2). Moreover, in otoliths of *M. surmuletus*, one check (demersal) is laid down before the first growth ring at a distance of about 0.6 mm from the core. The same criteria are used to distinguish false and true rings.

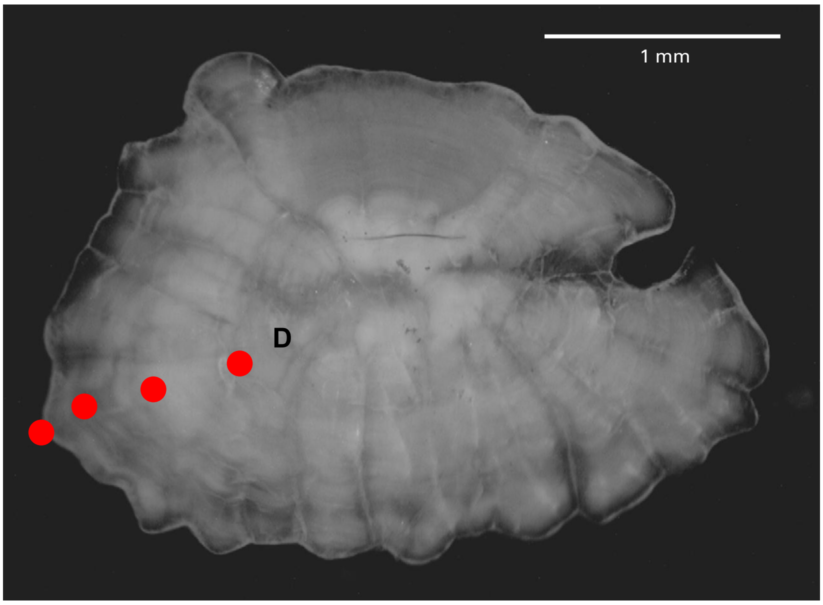
However, there are specific considerations regarding ageing analysis of *M. surmuletus*. Indeed, the two species present a different growth pattern, generally faster in *M. surmuletus* (Plates 67–70; Reñones, Messuti and Morales-Nin, 1995; Mehanna, 2009).

PLATE 66
Otolith of *M. barbatus*



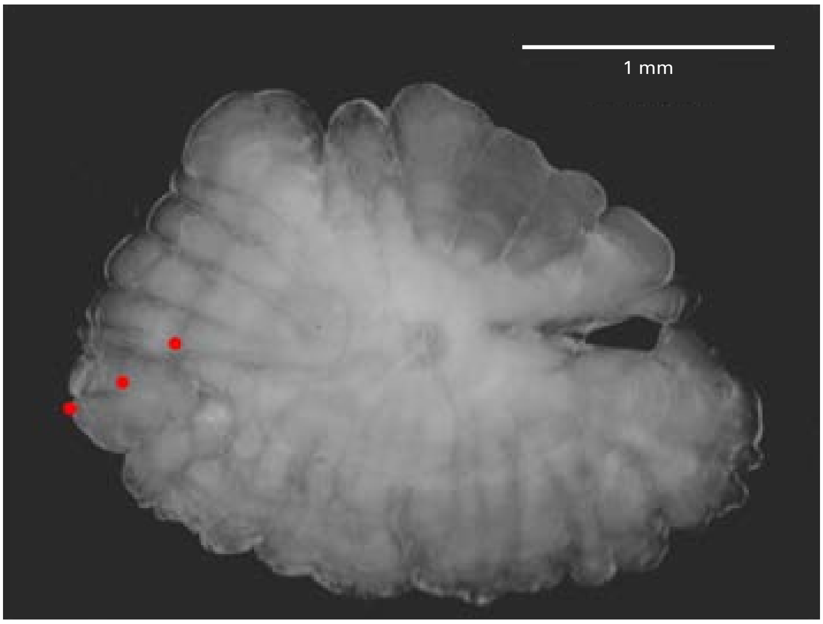
Note: Age 5 years, female, TL = 26 cm, month of capture = September, red dot = growth ring, D = demersal ring.

PLATE 67
Otolith of *M. surmuletus*



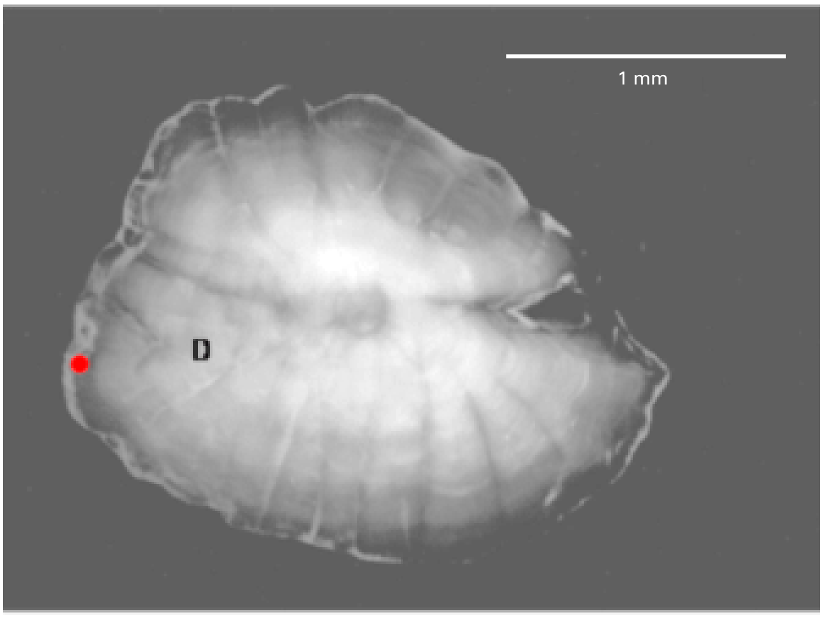
Note: Age 3.5 years, female, TL = 22 cm, month of capture = March, red dot = growth ring, D = demersal ring.

PLATE 68
Otolith of *M. surmuletus*



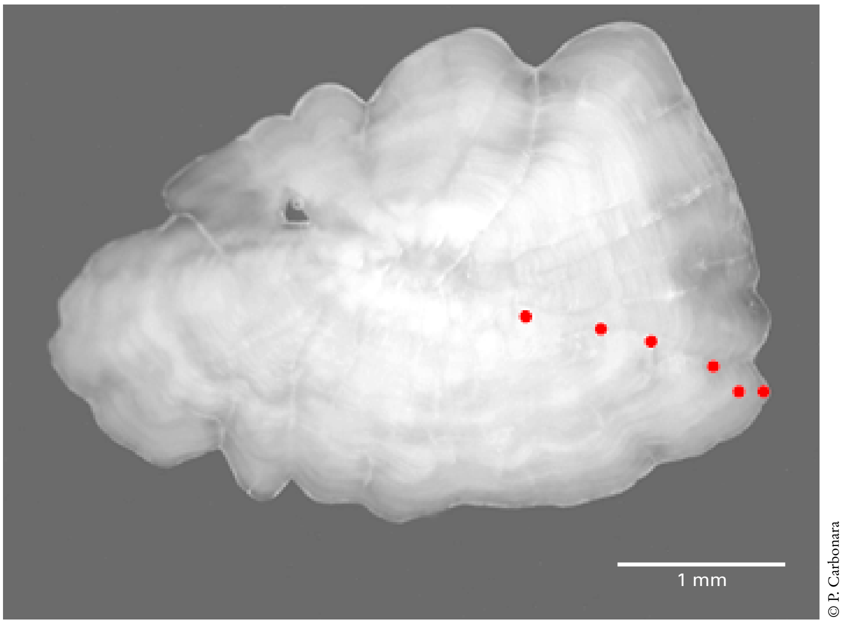
Note: Age 2.5 years, male, TL = 18 cm, month of capture = May, red dots = growth ring.

PLATE 69
Otolith of *M. surmuletus*



Note: Age 0.5 year, male, TL = 10 cm, month of capture = February, red dot = growth ring, D = demersal ring.

PLATE 70
Otolith of *M. surmuletus*



Note: Age 5.5 years, male, TL = 28.5 cm, month of capture = May, red dot = growth ring.

3.4 *Lophius budegassa*

L. budegassa is caught mainly with bottom trawls or gill nets and longlines. In Italian waters, this species is rarely the main target species, but is usually a bycatch with target species such as hake, squid and prawns. *L. budegassa* has a high commercial value, with an average price among the highest of the species landed.

Age estimation for the stock assessment of *L. budegassa* in the Mediterranean area has been traditionally based on two CSs: the illicium (used in most European countries) and the sagittal otolith. Otoliths from *Lophius* spp. have confusing false rings or multichecks (Landa, 2002) and an increase in their opacity with age, which makes them more difficult in age estimation than illicia, where the growth pattern is easier to distinguish, as they exhibit fewer secondary structures (Duarte *et al.*, 2005). However, both calcified structures are sampled routinely.

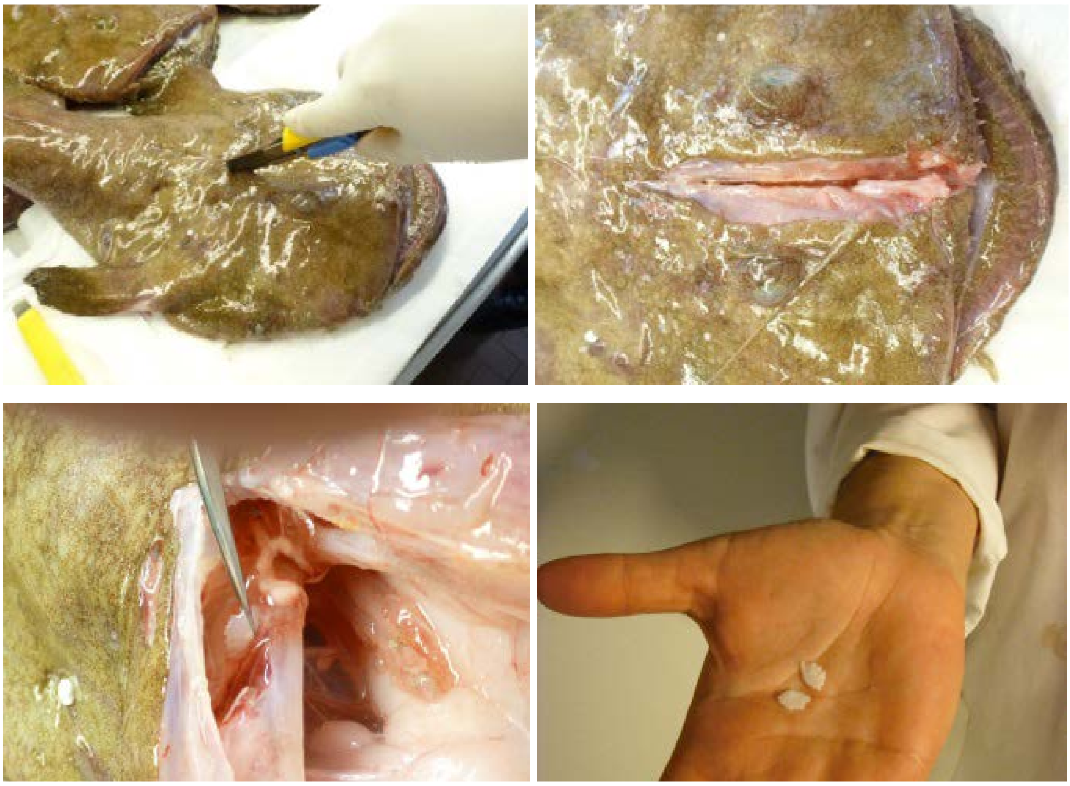
3.4.1 Extraction and storage

To extract the otoliths, a dorsal ventral cut (parallel to the margin of the orbit) is made using a sharp knife, thus exposing the brain, which is carefully extracted. Through close observation, the semicircular canals are located along the lateral walls of the brain cavity, and the otoliths are found at the confluence of the canals (Secor, Dean and Laban, 1991).

The otoliths are removed using tweezers, and cleaned and stored in black plastic plates labelled with the appropriate sample information (Plate 71).

The illicium is cut at its base, lifting its apical end to facilitate cutting (Plate 72). The cut illicium of each *Lophius* is stored in an envelope, in which the data of the sampled specimen are recorded. Just the last part of the illicium (6–7 cm) is sufficient for placing in the envelope.

PLATE 71
Macroscopic dissection technique used on *Lophius* to extract otoliths



© F. Donato

Note: Age 5.5 years, male, TL = 28.5 cm, month of capture = May, red dot = growth ring.

PLATE 72
Cutting illicium of *Lophius* at its base; then a section for storage in an envelope

© F. Donato

3.4.2 Preparation and interpretation

The traditional methodology for mounting illicia in resin plates was originally described by Dupouy, Pajot and Kergoat (1986). After several European age estimation workshops (Anon., 1997; Anon., 1999; Landa *et al.*, 2002, Duarte *et al.*, 2005), the method was standardized and included in an age estimation guide for *Lophius* (Duarte *et al.*, 2005). This methodology was used in most growth studies using illicia (Duarte, Azevedo and Pereda, 1997; Quincoces, Lucio and Santurun, 1998; Landa *et al.*, 2001; Landa, Barrado and Velasco, 2013). However, several modifications in the traditional methodology of Dupouy, Pajot and Kergoat (1986) have recently been carried out for illicia preparation, observation and age interpretation (Landa, Barrado and Velasco, 2013). These methodological modifications have been performed to allow clearer observation of the growth pattern for distinguishing the annuli.

The new method embeds the illicia in resin to realize transverse sectioning. Usually, a two-component epoxy resin is used for the embedding, with a cold resin to mount the thin slices on glass slides. Thin sections are made through the core by a cutting machine (i.e. Buehler Isomet low speed, Struers Minitom). The blade mounted on the cutting machine has a continuous rim with a high concentration of diamond powder (i.e. Buehler series 15HC n.11-4244, diameter 100 mm, thickness 0.25 mm). The transverse sections of illicium are made in the area from 0.5 cm from the base to a maximum of 6 cm. Transverse sections have a thickness of 0.50–0.55 mm. This width allows observation of the clearest marked annual increments. Use of sections thinner than 0.50 mm could produce some false rings (Duarte *et al.*, 2005; Landa, Barrado and Velasco, 2013).

The thin sections are analysed under an optical microscope with transmitted light, so the translucent ring represents a slow growth zone, while the black one represents a fast growth zone (Plates 75 through 78). The sum of these areas is considered an annual growth increment (annulus). The use of high magnification (> 10x), which was the standard observation methodology used by Duarte *et al.* (2002) and subsequent studies, emphasized the presence of false annual rings (Landa, Barrado and Velasco, 2013; Duarte *et al.*, 2005). *L. budegassa* ageing analysis using illicia consists of identifying dark and light rings (Plates 75 through 78). For age determination, only the dark rings are counted. For this we assume that one dark ring represents one year of growth. The growth rings are often duplicated and not well defined. Their interpretation is often very difficult.

Ageing analysis of *L. budegassa* is done considering 1 January as the birth date, according to the spawning period during the winter months (Carbonara, Zupa and Spedicato, 2005; Carlucci *et al.*, 2009). Moreover, age is assigned with yearly resolution following the scheme reported in Table 1. For the specimens caught in the first semester of the year, if a translucent ring is observed on the edge of the illicia, it is counted as an annual growth ring and age will be equal to the number of dark rings (opaque rings). For the specimens with an opaque edge, caught in the first semester of the year, age corresponds to the number of annuli (growth ring) minus 1, because if the new opaque ring on the edge is counted, age would be overestimated, as the specimen has not yet passed the theoretical birth date (1 January).

This general scheme (Table 11) is not applicable in some cases. Indeed, an opaque edge could also be present mostly at the beginning and/or end of the first semester of the year (Plate 72). If an opaque edge is present at the beginning of the first semester of the year, it could be because deposition of a translucent ring has not yet started. Whereas, if an opaque ring is present at the edge at the end of the first semester (June), it could be because deposition of the opaque ring has already started, being near the summer season, when this kind of ring is usually laid down (Table 12). In the case of a specimen caught in early winter (i.e. January) with an opaque edge, age will be equal to the number of dark rings, because the theoretical birth date has already passed. If the opaque edge is present at the end of spring (i.e. June), age will still be equal to the number of dark rings (N) minus 1, because counting the new opaque ring on the edge could overestimate the age, as the theoretical birth date (1 January) hasn't yet passed. In

TABLE 11 – Interpretation scheme for assigning age to *L. budegassa*

Date capture	Otolith edge	Age
1 January-30 June	Transparent	N
1 July-31 December	Opaque	N-1

Date capture	Otolith edge	Age
1 January-30 June	Opaque	N
1 July-31 December	Transparent	N-1

Note: N is the number of translucent rings, including those that might be visible on the edge.

TABLE 12 – Interpretation scheme for assigning age to *Lophius budegassa*

Months	Jan	Feb	Mar	Apr	May	Jun	July	Aug	Sep	Oct	Nov	Dec
Deposition pattern	T/O	T	T	T	T	O/T	O/T	O	O	O	O	T/O
Capture date												
Age with edge T	N	N	N	N	N	N	N					N-1
Age with edge O	N					N-1	N-1	N-1	N-1	N-1	N-1	N-1

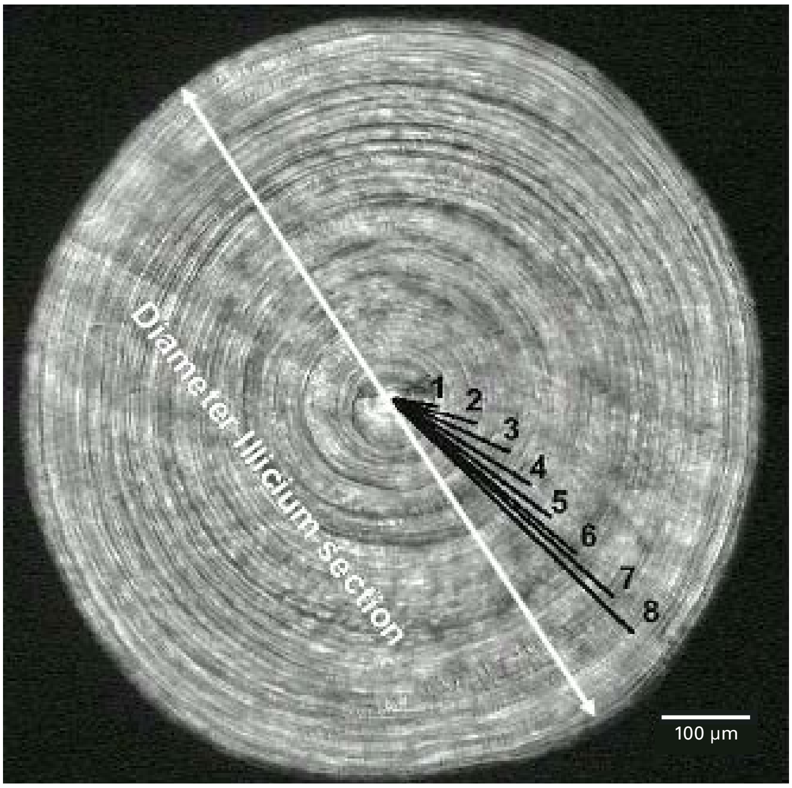
Months	Jan	Feb	Mar	Apr	May	Jun	July	Aug	Sep	Oct	Nov	Dec
Deposition pattern	T/O	O	O	O	O	O/T	O/T	T	T	T	T	T/O
Capture date												
Age with edge T	N	N	N	N	N	N	N					N-1
Age with edge O	N					N-1	N-1	N-1	N-1	N-1	N-1	N-1

Note: N = number of opaque rings (dark rings), T = translucent edge, O = opaque edge.

the case of the presence of a translucent edge at the beginning of winter (i.e. December), age will be equal to the number of dark rings (N) minus 1, because the theoretical birth date (1 January) hasn't yet passed.

For every illicia section, the edge quality is noted (opaque or translucent), while the measurements from the core (or nucleus) to each translucent ring (Plate 73), are taken on a subsample of sections.

PLATE 73
L. budegassa with 42 cm total length



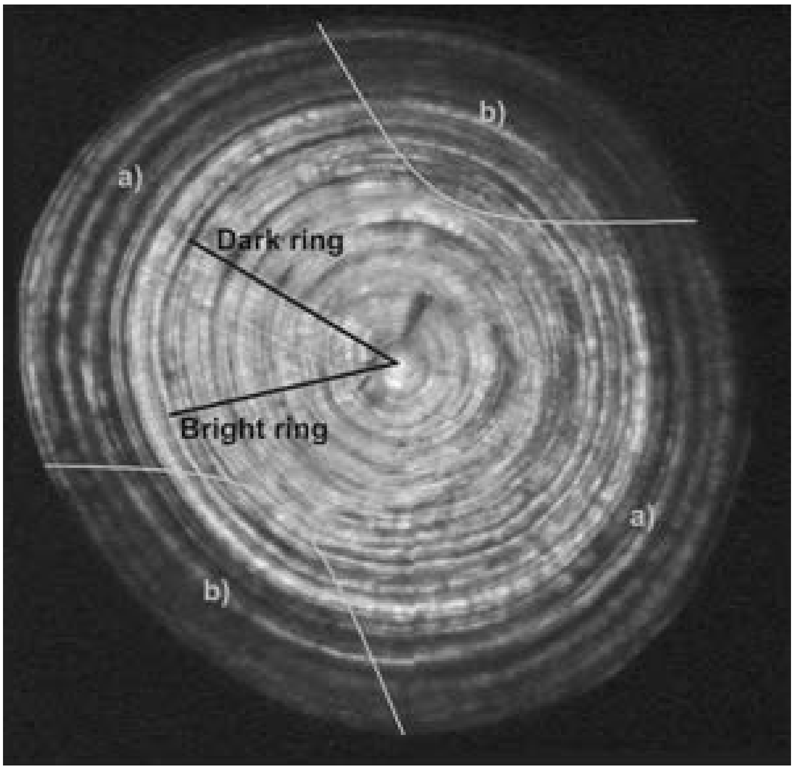
Note: Eight annual growth rings are visible, with indication of where morphometric measures are taken.

3.4.3 False rings and true growth annuli

Sometimes these rings are well defined and clearly visible, but most of the time, growth rings are duplicated and not well defined. Consequently, they present some difficulties. The workshop on European anglerfish in 2002 (Landa *et al.,* 2002) established criteria to help identify growth rings:

- It is important to play with and adjust the light and focus of the microscope, to identify the pairs of opaque and translucent rings, and to try to find a general pattern of growth. In illicia, growth rings remain a similar width apart throughout the transverse section. Growth rings close to the edge may be wider apart than those close to the nucleus.
- Growth rings may not be visible in all axes of the transverse section. Defined rings, which are clearly visible in one part of a section, may be less defined or even appear double in another part. Counting should be based on an area showing good contrast between the rings (Plate 74).
- To confirm the position of the first annual growth ring, measure its distance from the core and its diameter. The first well-marked growth ring observed is usually considered a consequence of a change in the life cycle (from planktonic to benthic) and is thus designated as the benthic ring. The next ring is considered to be the first annual ring. For *L. budegassa*, the first growth ring tends to be circular in shape and its mean diameter tends to be about 80 µm (from 60 to 100 µm).
- The diameter of the benthic ring can be a help in identifying the first annual growth ring. The distance of the first annual ring from the benthic ring is usually not greater than half the diameter of the benthic ring (Plates 75 and 76).

PLATE 74
L. budegassa, 64 cm in length. Region a) of illicium with good contrast between growth rings, and region b) with low contrast

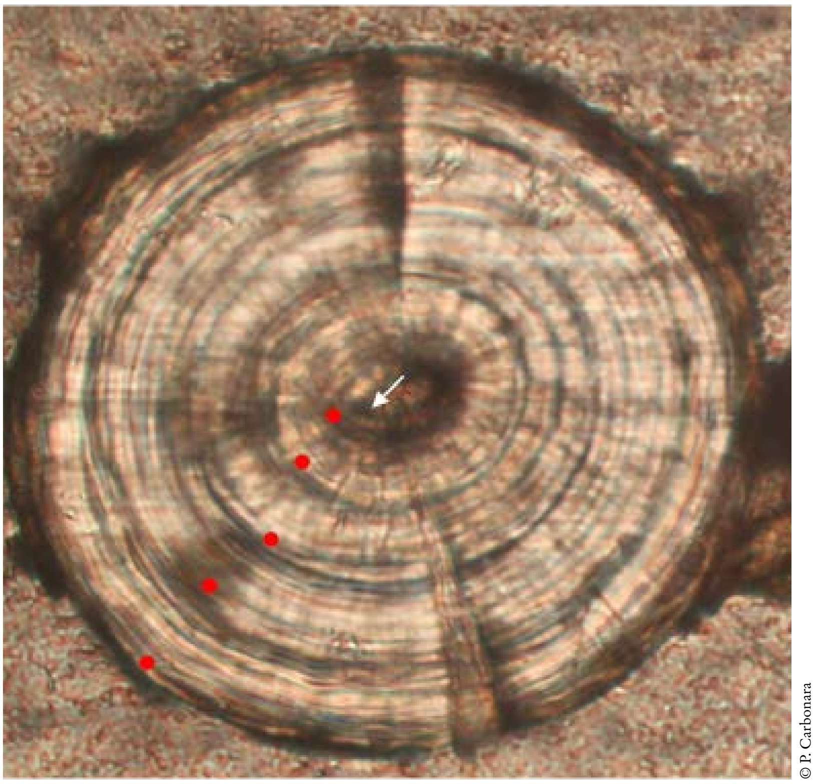


© Landa *et al.*, 2002

Note: Some growth rings visible in region a) are not distinguishable in region b). Relative to the ring coloration, there are two well-marked dark rings in one region of the illicium, and in another region of the cut, the dark part disappears and a bright part is very visible and easy to count.

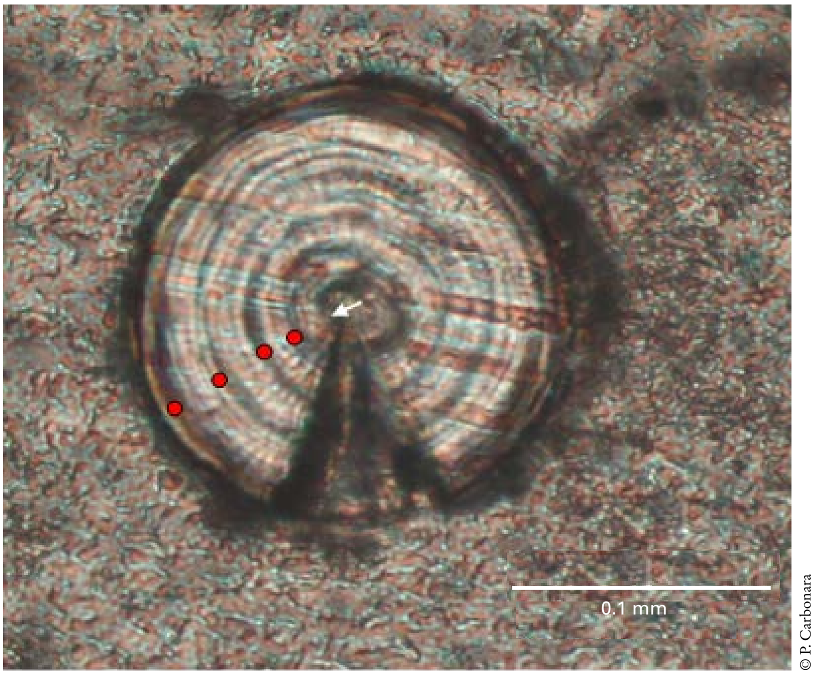
– Confusion after some years (at about age six) may be related to the first sexual maturity or any other unidentified life history event that causes changes in the growth pattern (Plates 77 and 78).

PLATE 75
L. budegassa captured in March



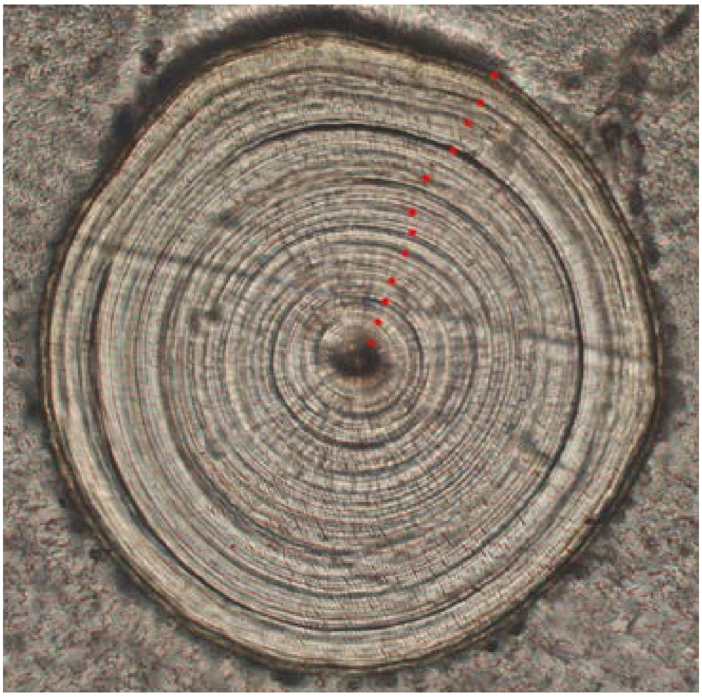
Note: Age 5 years, male, TL = 29.5 cm, red dots = annuli, white arrow = false ring.

PLATE 76
L. budegassa captured in March



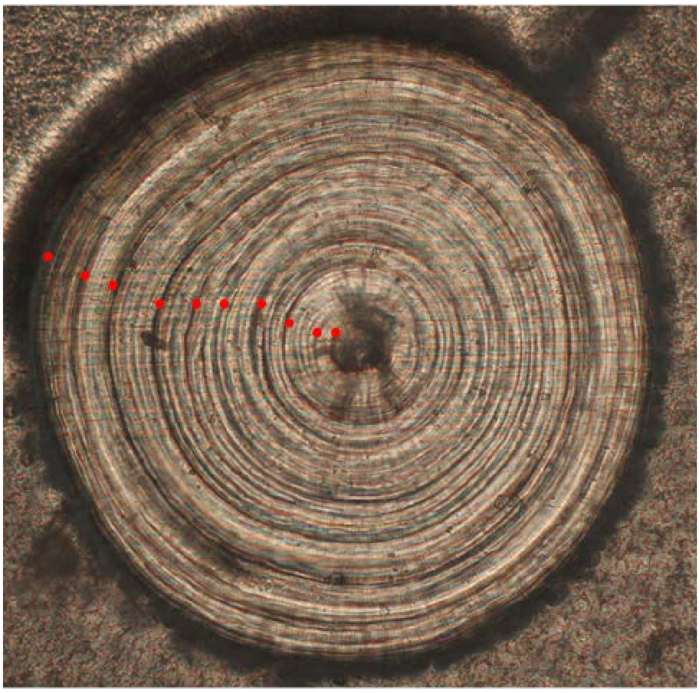
Note: Age 4 years, female, TL = 33 cm, red dots = annuli, white arrow = false ring.

PLATE 77
L. budegassa captured in March



Note: Age 12 years, female, TM = 53.5 cm, red dots = annuli.

PLATE 78
L. budegassa captured in September

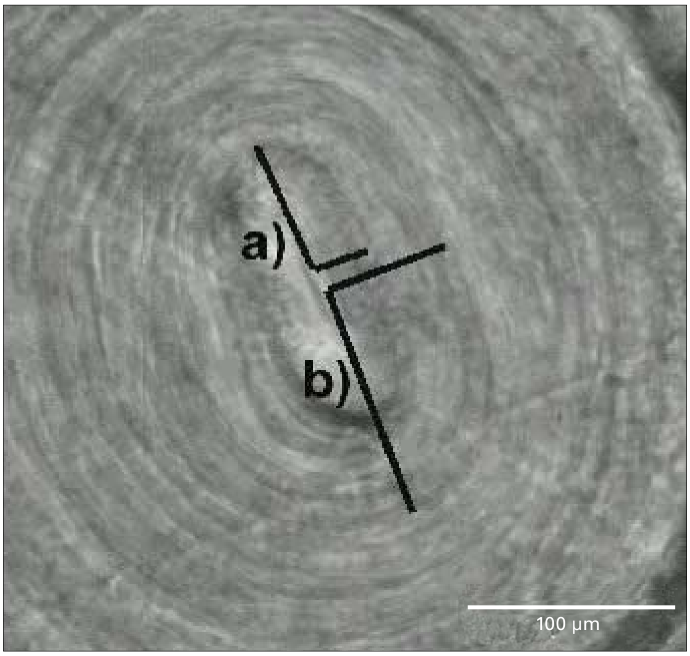


Note: Age 9 years, female, TL = 45.5 cm, red dots = annuli.

3.5 *Lophius piscatorius*

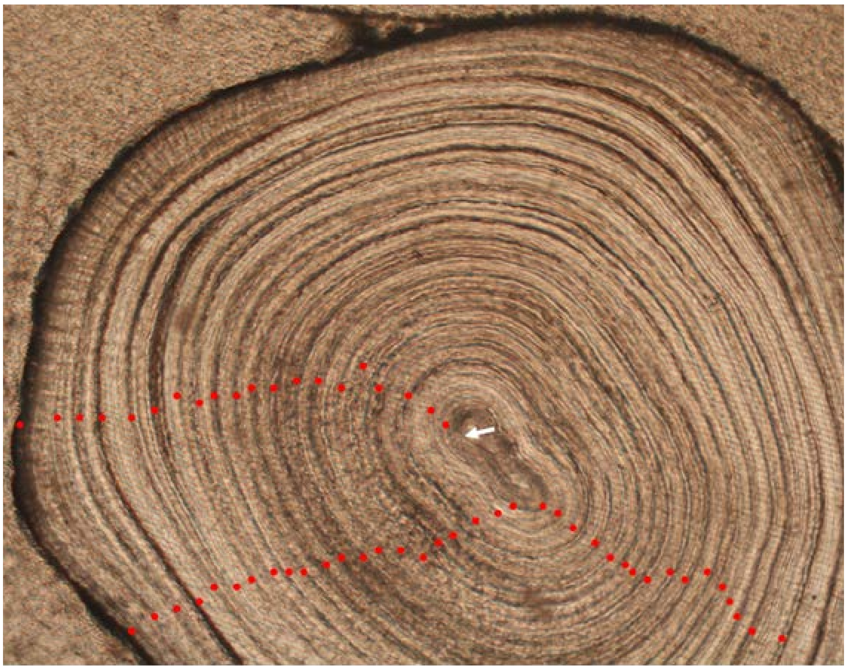
Information on aging of *L. piscatorius* in the Mediterranean Sea is limited. However, many of the methods and ageing criteria applied to *L. budegassa* and discussed here, seem to be equally useful for *L. piscatorius* (Landa, 2012; Landa, Barrado and Velasco 2013). In particular, the calcified structures (illicium) used, extraction techniques, preparation, measures and interpretation criteria (birth date 1 January) are the same as those of *L. budegassa* (subsection 3.4). Moreover, the same criteria are used to distinguish false and growth rings. In *L. piscatorius*, unlike *L. budegassa*, the first ring tends to be oblong in shape and the mean horizontal diameter of the first ring tends to be from 200 to 300 µm (Plate 79). Moreover the first ring visible is considered false (benthic ring) (Landa *et al.*, 2002) (Plates 80 and 81).

PLATE 79
L. piscatorius shows the oval shape of first growth ring characteristic of this species



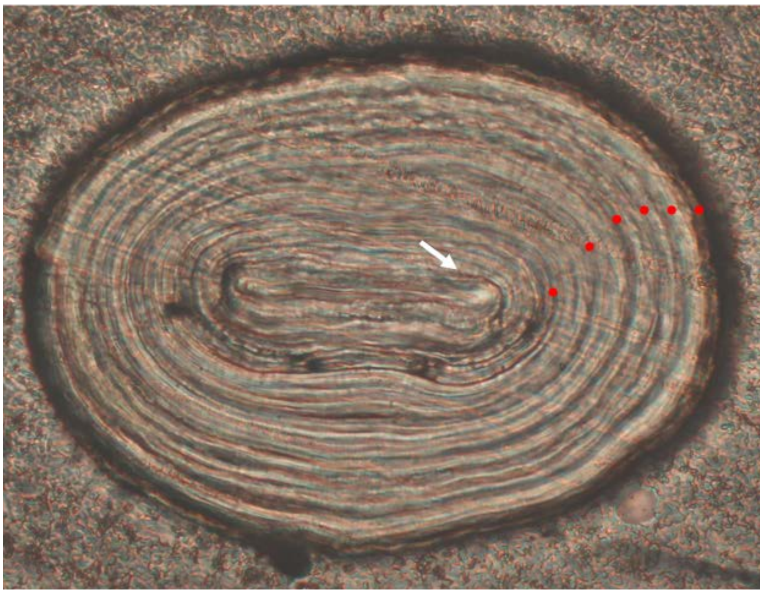
Note: Benthic ring is marked as a) and first annual ring as b).

PLATE 80
Female *L. piscatorius* captured in March



Note: Age 20 years, TL = 97 cm, red dots = annuli, white arrow = false ring.

PLATE 81
Female *L. piscatorius* captured in October



© P. Carbonara

Note: Age 5 years, TL = 45 cm, red dots = annuli, white arrow = false ring.

3.6 *Boops boops*

B. boops is a very common seabream (Sparidae family) in Italian coastal waters. This species inhabits the eastern Atlantic, from Norway (where it is considered occasional) to Angola, and throughout the Mediterranean Sea, including the Black Sea. It also occurs in the western Atlantic, the Gulf of Mexico and the Caribbean Sea (Bauchot and Hureau, 1986). It is a gregarious and benthopelagic species, and can generally be found down to 100 m (Mediterranean Sea) or 300 m (Atlantic Ocean), on different types of bottom (e.g. sand, mud, rocks and Posidonia seagrass beds) (Tortonese, 1975; Bauchot and Hureau, 1986).

In Italy, *B. boops* has limited commercial value and is frequently discarded at sea. It represents a bycatch of bottom trawl and midwater trawl fisheries. The species is also caught by purse seines and trammel and gill nets (Cannizzaro *et al.*, 2001). In Italy, landings of *B. boops* accounted for 1 158 tonnes in 2013 (Busalacchi *et al.*, 2017), representing about 0.7 percent (Mannini and Sabatella, 2015) of total landings for the fishery in Italian waters.

3.6.1 Extraction and storage

In *B. boops*, sagittae extraction is made through the transverse section – that is, a median-sagittal cut starting from the mouth up to the back of the head area, in correspondence with the rear edge of the opercular bones (Plate 82). In this way, otoliths are easily visible in the vestibular apparatus and can be extracted using stainless steel tweezers. Otoliths are then cleaned of any remnants of membranes and organic parts and stored dry in an Eppendorf vial.

3.6.2 Preparation and interpretation

Age determination is performed by observing the whole otolith under a binocular microscope (magnification 6.4x or 16x), with the distal side up and the proximal surface down, in seawater (clarification medium), using reflected light, on a black background (translucent rings appear black and opaque ones white). The otolith reading is conducted assuming an annual cycle that corresponds to deposition of an annulus formed by a translucent ring and an opaque ring. Translucent rings are counted starting from the central region of the otolith (core) to the margin.

Rings are counted on the antirostrum area (Plates 83 through 86). Otoliths can also be burned (Christensen, 1964) to achieve a stronger contrast between translucent and opaque rings, thus facilitating their reading. Translucent rings acquire a darker, caramelized tone when burned. The otoliths can be burned in an oven at 300 °C, with the time depending on their size (Plates 85 and 86).

Age is determined considering 1 January as the birth date, according to the biology of the species (i.e. spawning period) (Bottari *et al.*, 2014; Dobroslavić *et al.*, 2017; El-Agami *et al.*, 2004), and is assigned with a 0.5-year resolution (see subsection 1.3.1).

PLATE 82

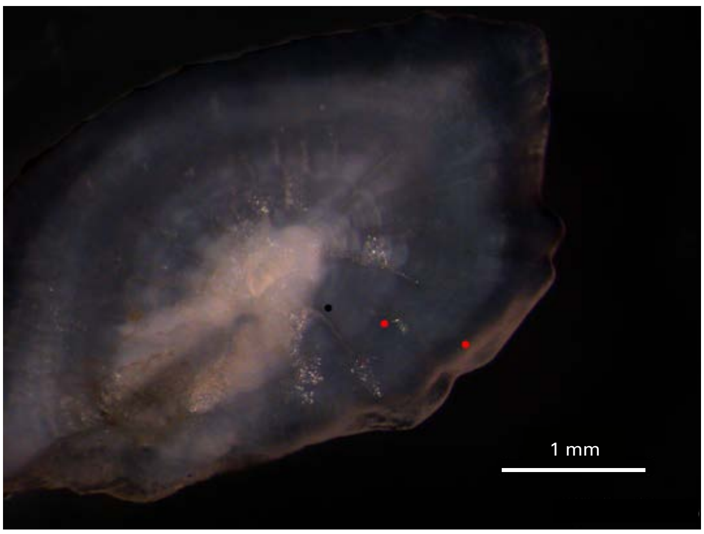
Extraction of otoliths by means of a transverse section in *B. boops*

© A. Massaro

Note: Female, TL = 11 cm, northern Tyrrhenian Sea.

PLATE 83

Otolith of *B. boops* captured in March, northern Tyrrhenian Sea



© A. Massaro

Note: Age 2 years, male, TL = 18 cm, red dots = growth rings, black dot = false ring.

PLATE 84
Otoliths of *B. boops*, northern Tyrrhenian Sea

© A. Massaro

Note: Age 3 years, female, TL = 22.5 cm, captured in March, red dots = translucent rings, black dots = false rings.

PLATE 85
Burned otolith of *B. boops*, northern Tyrrhenian Sea

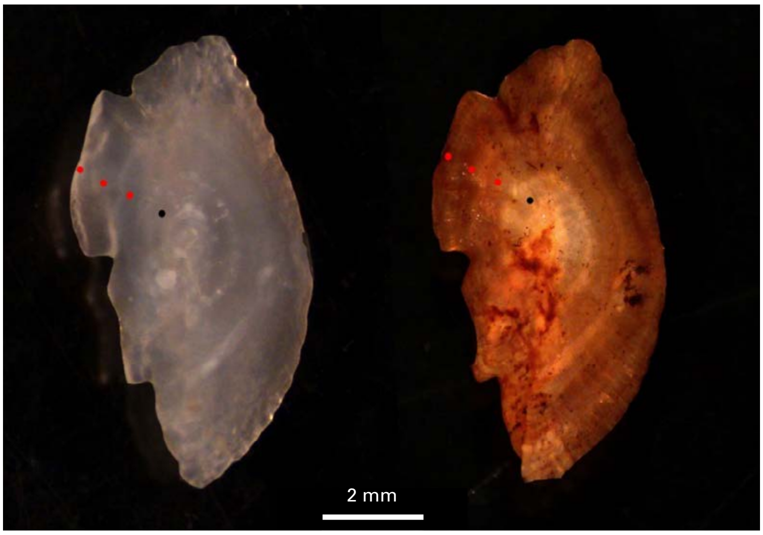
© A. Massaro

Note: Age 2.5 years, female, TL = 20 cm, captured in September, red dots = translucent rings, black dot = false ring.

3.6.3 False rings and true growth annuli

Interpretation of the growth pattern and identification of the first annual ring in *B. boops* are often misled by the presence of false and multiple rings (Plate 86). Before the first growth ring, it is often possible to observe the presence of one or two false rings (Plates 88 and 89) (Khemiri *et al.*, 2005). These false rings are considered migratory rings and represent the changing of habitat and diet during the first months of life (Zuñiga, 1967; Khemiri *et al.*, 2005). The first false ring appears as a translucent band, usually well evident around the core (Plate 87). Sometimes, it is possible to identify a second check, close to the first translucent ring, which is thin and not continuous. The presence of one or two false rings may depend on the merging of the second false ring with the first growth ring. In fact, the first growth ring often appears as a translucent area (Plates 85 and 88) rather than a well-defined ring. In any case, the first growth ring is laid down at a distance from the core (posterior area) ranging from 1.0 to 1.5 mm.

PLATE 86
Otolith of *B. boops* before burning (left) and after (right)



Note: Age 2.5 years, male, TL = 20.5 cm, northern Tyrrhenian Sea, captured in November, red dots = translucent rings, black dot = false ring.

PLATE 87
Whole otolith of *B. boops* (left) and particular of core (right)

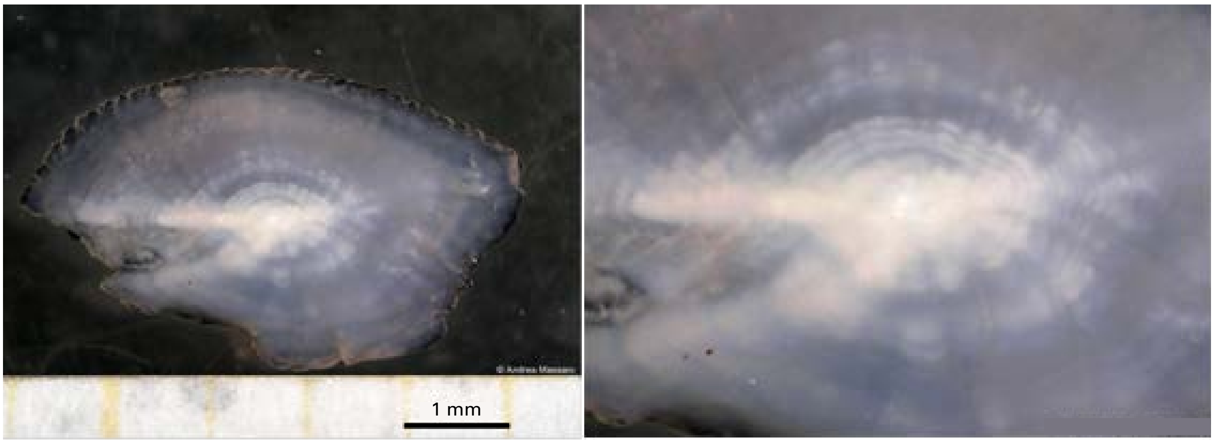
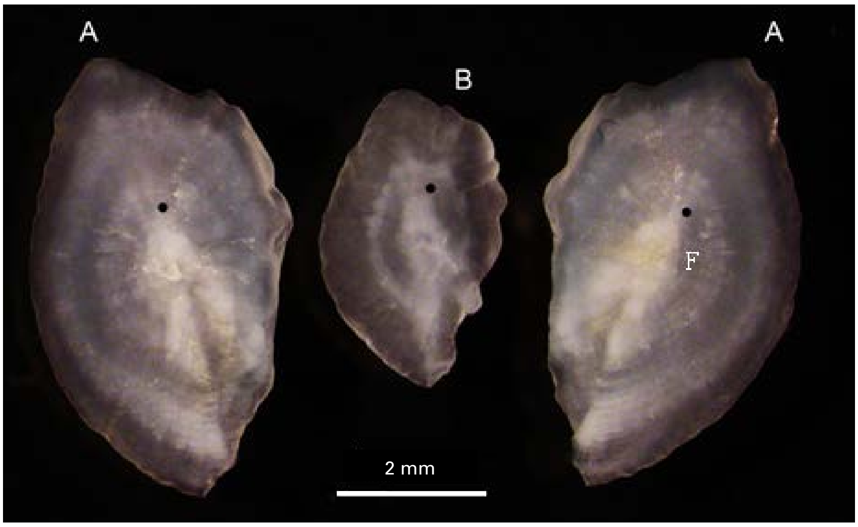
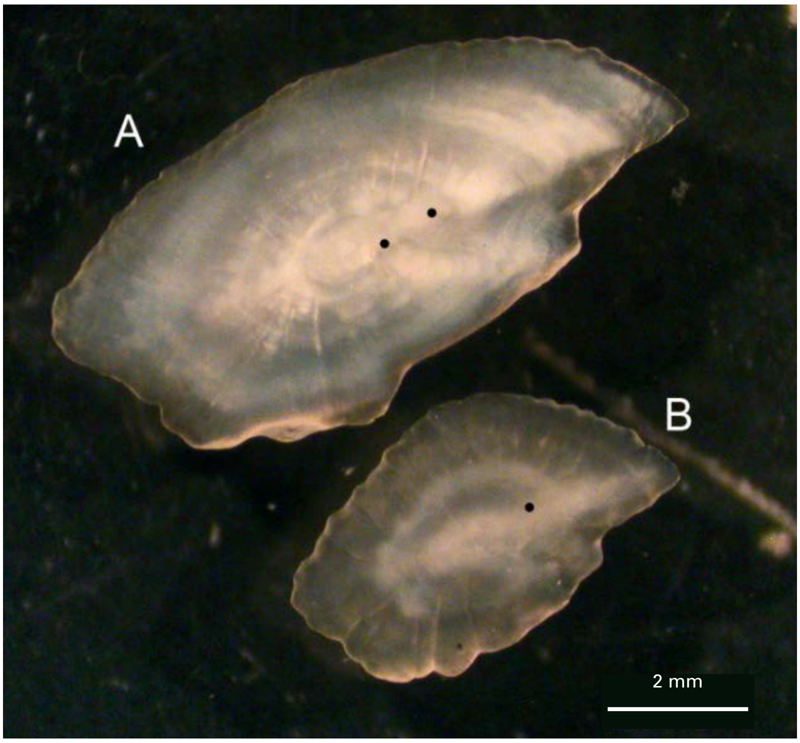


PLATE 88
First false ring (black dots) in otoliths of *B. boops*



Note: A – age 1.5 years, male, TL = 17.5 cm, captured in November; B – age 0.5 year, male, TL = 9.5 cm, captured in December, northern Tyrrhenian Sea.

PLATE 89
False rings (black dots) in otoliths of *B. boops*



© A. Massaro

Note: A – age 3 years, male, TL = 20.5 cm, captured in April; B – age 0.5 year, male, TL = 9.5 cm, captured in December, northern Tyrrhenian Sea.

3.7 *Spicara smaris*

S. smaris is a marine fish species inhabiting seagrass beds and muddy bottoms at about 15–100 m. It is distributed in the Mediterranean and Black Seas, and in the Atlantic from Portugal to Morocco and the Canary Islands. *S. smaris* is caught mostly by bottom trawl and gill and trammel nets, and represents an important resource especially for the small-scale fishery. It is a multiple spawner with peak spawning in April–May (Karlou-Riga *et al.*, 2007). In general, for the Mediterranean basin, information on its growth is very limited. For the first two years of its life, *S. smaris* shows relatively rapid growth. Maximum life span is five years for females and seven for males (Vidalis and Tsimenidis, 1996).

3.7.1 Extraction and storage

Sagittae extraction is made through the transverse section. After extraction, the otoliths are washed to remove organic material, then dried and stored.

3.7.2 Preparation and interpretation

One otolith from each pair (the left one when possible) is analysed under a binocular microscope, in seawater (clarification medium), with reflected light, against a black background. Orientation for analysis is with the distal surface up and the proximal surface (sulcus acusticus) down.

In whole otoliths, annuli are counted on the posterior part of the otolith (postrostrum). However, ring continuity should be checked on the anterior part of the otolith (rostrum area) and, wherever possible, on the dorso-lateral edge.

Age is determined considering 1 July as the birth date, in accordance with the spawning period in late spring/early summer (Karlou-Riga *et al.*, 2007; Karlou-Riga and Petza, 2010), and is assigned with a resolution of 0.5 year, counting the translucent (winter) rings (see subsection 1.3.2).

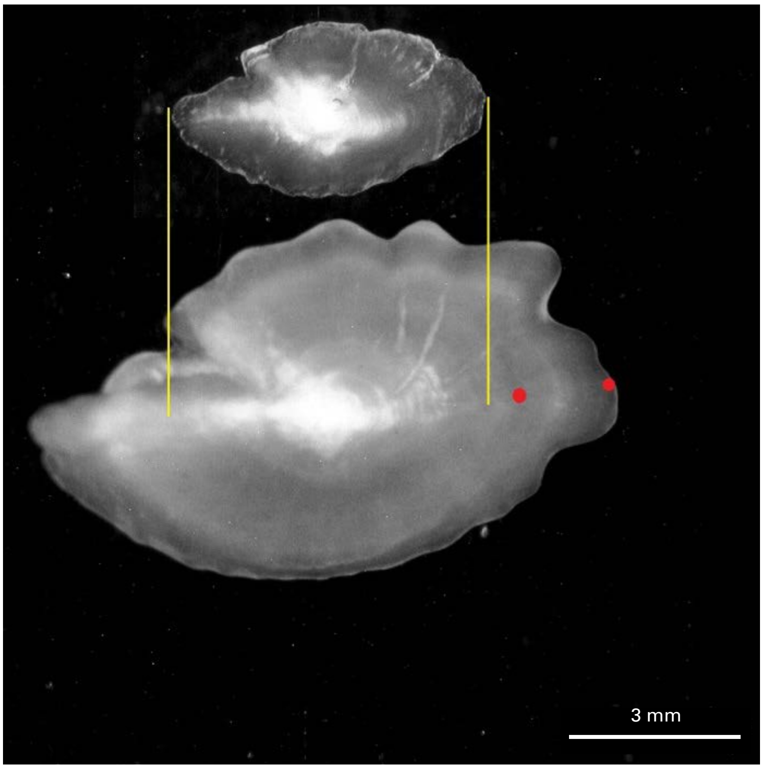
3.7.3 False rings and true growth annuli

Identification of the first annual ring is an important source of bias in the age determination process. Before the first growth ring, a false ring can be laid down. In fact, very small specimens, around TL 6 cm, caught in late summer, could present a translucent edge (Plate 90). The distance from the core of this ring is about 1 mm (0.8–1.1 mm). After this false ring is laid down, the first growth ring is at a distance from the core (postrostrum area) of from 1.2 to 1.7 mm (Plate 91). Sometimes the first growth ring is jointed with this false ring; in this case, the first true ring appears as a wide translucent area (Plates 90 and 91).

Another source of bias is due to a ring deposition pattern that is poorly differentiated between the opaque and translucent rings. Moreover, false rings could be laid down for reproductive periods and/or other environmental stresses. Criteria for recognizing the true growth ring include:

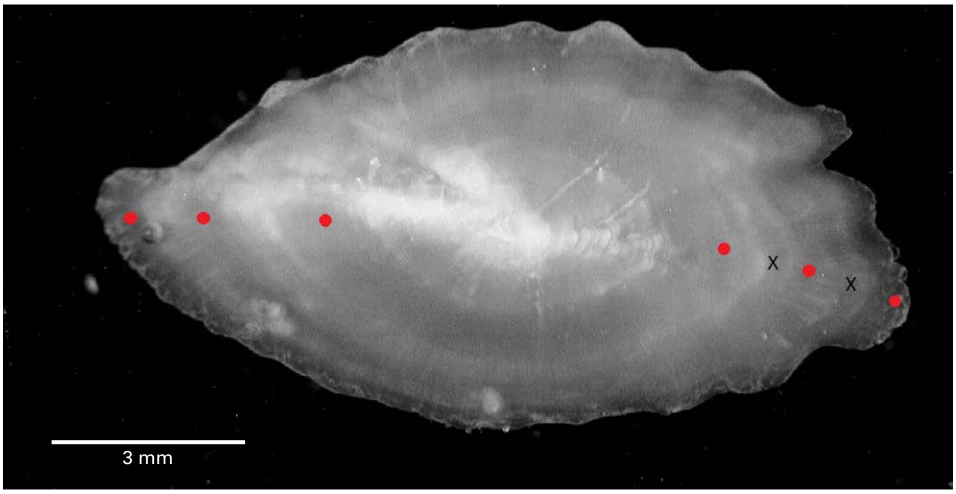
– To be considered annual growth rings, translucent rings should be visible more or less around the whole otolith (Plate 91).
– The increment between the consecutive annuli should decrease with age (Plates 90 and 91).

PLATE 90
Otoliths of *S. smaris*



Note: Top –TL = 5.5 cm, captured in September; bottom –TL = 12.5 cm, captured in November.

PLATE 91
Otoliths of *S. smaris* captured in November



© L. Casciaro

Note: Age 2 years, TL = 16.5 cm, red dots = growth rings (visible around whole otolith), X = false ring.

3.8 *Solea solea*

S. solea represents one of the most important species owing to its high commercial value. In Italy, this resource is targeted by artisanal fishing fleets using set nets and demersal fishing fleets using various bottom trawl gears, such as beam trawl (*rapido*) and the Italian commercial trawl net (*tartana*). It is a demersal and sedentary species, living on sandy and muddy bottoms, most frequently caught from coastal waters to a depth of 100 m in the Adriatic Sea (Vrgoč *et al.*, 2004). Along the Italian coast, 1 839 tonnes (IREPA, 2012) of *S. solea* were landed in 2011, and, in particular, in the Adriatic Sea, about 77 percent of total production of the commercial fleet was landed. Considering that the quantities landed in Apulia represent only 1 percent of the total catch landed in the Adriatic Sea, this value can be referred to GSA 17 (northern Adriatic Sea).

3.8.1 Extraction and storage

In flatfish, an incision is made above the ventral eye along the anteroposterior axis, and with the aid of a scalpel or cutter, the skull is dissected and the brain removed, as shown in Plate 92. Sagittae, which are easily visible in the vestibular apparatus, are removed with forceps, cleaned in tap water, dried and stored in plastic tubes (MedSudMed, 2005).

3.8.2 Preparation and interpretation

Otoliths (sagittae) are the most common and widespread hard structure used for the age determination of flatfish. The Planning Group on Commercial Catch, Discards and Biological Sampling (PGCCDBS, 2011) recommended an otolith exchange for Bay of Biscay *S. solea* (Mahé *et al.*, 2012b). To implement this exchange, only images of thin staining sections of otoliths were used and two main criteria for age determination of *S. solea* were defined:

- Date of birth is conventionally attributed to 1 January, as for every species for which the reproductive period is autumn/winter.
- One annulus consists of one opaque and one translucent zone. For age estimation, the deeply stained zones in the transverse sections are counted (corresponding to translucent zones of whole otoliths). Thus, one annulus is interpreted as one year of life.

PLATE 92
Extraction of otoliths in *S. solea*

© S. Colella

Note: Female, TL = 26.5 cm, Port of Ancona, northern Adriatic Sea – GSA17.

Nevertheless the thickness of the otoliths in all flatfish make them easy to age by inspecting the whole otolith, immersed in a clarifying liquid such as alcohol 70, glycerin, distilled or fresh water, under reflected light, against a dark background, or under transmitted light (Scarcella *et al.*, 2014; Mehanna, El-Regal and Aid, 2015).

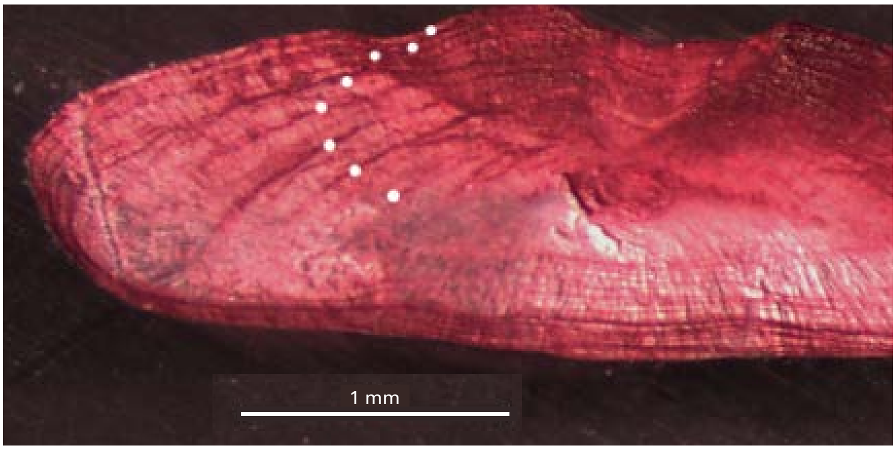
The seasonal appearance of opaque and translucent rings in the whole otolith shows a reversal pattern for otoliths of *S. solea* as regards fishes of temperate and cold water. In fact, opaque zone deposition in winter is associated with intense fish growth, while the translucent zone corresponds to reduced growth in summer (Froglia and Giannetti, 1985, 1986; Arneri, Colella and Giannetti, 2001). An opaque (fast growth) zone plus a translucent ring (slow growth) is considered an annual growth (annulus).

On the basis of these assumptions, it is considered appropriate to count opaque zones of whole otoliths for age estimation, and the first annulus consists of the opaque and translucent zones subsequent to the core of the otolith, which appears opaque.

The best orientation of the otolith is with the distal surface up and the proximal surface (sulcus acusticus) down. In whole otoliths, annuli are counted on the posterior part (postrostrum). However, ring continuity should be checked on the anterior part of the otolith (rostrum) and, wherever possible, on the dorso-lateral edge.

For specimens greater than TL 28–30 cm and for all samples for which the age determination is doubtful, a more suitable method is the sectioning and staining of the transverse section of the otolith (Plate 93) (Arneri, Colella and Giannetti, 2001; Easey and Millner, 2008; Mahé *et al.*, 2012b). With this methodology, the overlapping rings on the edge are highlighted. One otolith (always the same, when possible) is embedded in epoxy resin using semi-rigid plastic moulds sprayed lightly with a silicone spray to facilitate subsequent extraction. Transverse sections, 0.7–0.8 mm thick through the core, are obtained using a low-speed saw (i.e. a Remet Micromet) equipped with two diamond blades separated by a metallic spacer. Otolith sections are then stained for about 30 minutes with the histological stain Neutral Red Solution (Sigma), with the addition of 1 percent sodium chloride acidified with 0.5 percent acetic acid (Arneri, Colella and Giannetti, 2001; Easey and Millner, 2008). Stained sections are rinsed in tap-water, dried, and then observed with a dissecting microscope at low magnification (10–25x) under reflected light. The mechanism of the staining, investigated by Richter and McDermott (1990), consists in the reaction of the calcium carbonate on the surface of transverse sections of otoliths with the acetic acid, causing mild decalcification of this surface and leaving the protein bands (otolin) exposed of the translucent zones that the histological stain reacts with. Translucent zones are generally considered to have a higher relative protein content than opaque zones and thus become more-deeply stained.

PLATE 93
Transverse stained section of otolith of *S. solea*



© S. Colella

Note: Age 8.5 years, female, TL = 34 cm, Ancona, northern Adriatic Sea – GSA17, captured in October.

Alternatively, if it is not possible to perform the staining protocol, it is advisable to embed the whole otolith in the epoxy resin to obtain a thinner dorso-ventral section (0.5–0.6 mm) through the core. The thin slice, mounted on a glass slide in a mounting medium, is analysed under an optical microscope at a resolution of 10x. The growth rings are counted on the dorsal side with reflected light (Plates 94 and 95) (growth rings appear white) or with transmitted light (Plate 96) (growth rings appear dark).

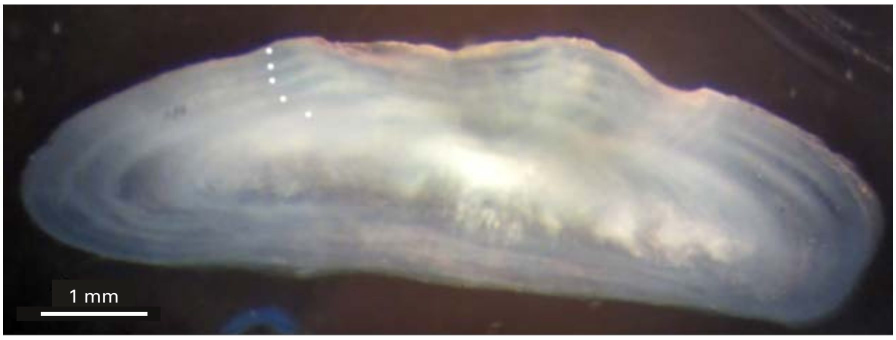
Whole otoliths – in a Petri dish, immersed in 70 percent ethyl alcohol or distilled water as clarification media – are observed under a microscope at low magnifications (10–25x), with reflected light against a dark background. An alternating pattern of white and dark zones is shown (Plates 97 and 98): the dark zones correspond to translucent zones and can thus be considered summer growth zones. Opaque rings are counted from the core of the otolith to the margin, up to the outer edge.

PLATE 94
Transverse section of otolith of *S. solea* observed under reflected light

© A. Massaro

Note: Age: 5 years, female, TL = 32 cm, central Tyrrhenian Sea, captured in June.

PLATE 95
Transverse section of otolith of *S. solea* observed under reflected light



© A. Massaro

Note: Age 4.5 years, female, TL = 33 cm, central Tyrrhenian Sea, captured in November.

PLATE 96
Transverse section of otolith of *S. solea* observed under transmitted light

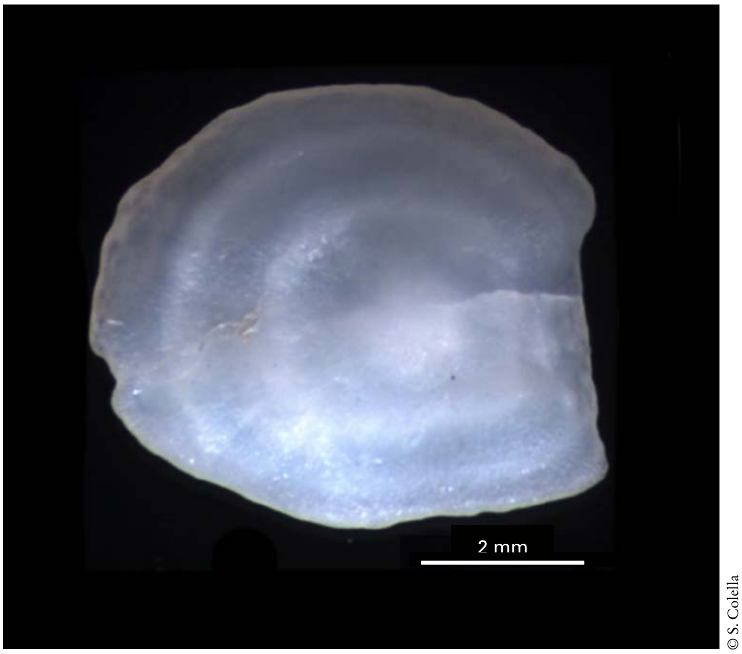
© Carbonara *et al.*, 2006

Note: Age 14 years, female, TL = 42.5 cm, Castellamare Gulf, southern Tyrrhenian Sea, captured in June, dark rings correspond to opaque rings.

PLATE 97
Otolith of *S. solea*

Note: Age 2 years, female, TL = 31.5 cm, Ancona, nothern Adriatic Sea – GSA17, captured in April.

PLATE 98
Otolith of *S. solea*



Note: Age 3 years, female, TL = 31.5cm, Ancona, nothern Adriatic Sea – GSA17, captured in April.

Age is assigned considering 1 January as the theoretical birth date, with a resolution of 0.5 year, counting the opaque rings (growth rings) (Table 13).

For specimens caught in the first part of the year, an opaque ring on the edge of the otolith is counted as an annual ring. For specimens with the translucent ring on the edge in the second semester, age will be equal to the number of growth rings (opaque) plus 0.5, which represents the additional half-year lived.

In some particular cases, the general scheme in Table 13 is not applicable. Indeed, a translucent edge could be also present at the beginning and/or end of the first part of the year (Plate 100). When a translucent edge is present at the beginning of the first part of the year (i.e. January), it may be that the specimen has not yet started the deposition of an opaque-growth ring. When a translucent ring is present at the edge at the end of the first part of the year (i.e. June), it could appear in a specimen that has already started deposition of the translucent ring, being near the summer season, when this kind of ring is usually laid down (Table 14). In specimens caught in early winter (i.e. January) with a translucent edge, age will be equal to the number of opaque rings plus one, because the theoretical birth date has already passed, even if the growth ring (opaque) has not yet appeared on the edge. If a translucent edge is present at the end of spring (i.e. June), age will be equal to the number of growth rings (opaque).

TABLE 13 – Interpretation scheme of *S. solea* for determining age

Date capture	Otolith edge	Age
1 January-30 June	Opaque	N
1 July-31 December	Transparent	N+0.5

Date capture	Otolith edge	Age
1 January-30 June	Opaque	N
1 July-31 December	Transparent	N+0.5

Note: N is the number of opaque rings, including those that might be visible on the edge.

TABLE 14 – Age scheme for *S. solea*

Months	Jan	Feb	Mar	Apr	May	Jun	July	Aug	Sep	Oct	Nov	Dec
Deposition pattern	T/O	O	O	O	O	O/T	O/T	T	T	T	T	T/O
Capture date												
Age with edge T	N	N	N	N	N	N	N					N-0.5
Age with edge O	N+1					N-1	N+0.5	N+0.5	N+0.5	N+0.5	N+0.5	N-0.5

Months	Jan	Feb	Mar	Apr	May	Jun	July	Aug	Sep	Oct	Nov	Dec
Deposition pattern	T/O	T	T	T	T	O/T	O/T	O	O	O	O	T/O
Capture date												
Age with edge T	N	N	N	N	N	N	N+0.5					N-0.5
Age with edge O	N+1					N-1	N+0.5	N+0.5	N+0.5	N+0.5	N+0.5	N

Note: N = number of opaque rings typically laid down in winter/spring months, T = translucent edge, O = opaque edge.

An opaque edge could appear also in the second part of the year, mostly at the beginning and/or end of the second part (Plate 100). When an opaque edge is present at the beginning of the summer period, age will be equal to the number of growth rings (opaque) plus 0.5, which represents the additional half-year lived. Indeed, in this case, the specimens have not yet started deposition of the translucent ring typical of the summer months. However, the opaque edge could also appear in late autumn/early winter (Plate 100). In this case, the specimens have started deposition of the growth ring (opaque), being close to the season when this kind of ring is laid down (winter months). Age will be equal to the number of growth rings (opaque) up to the edge, minus 0.5, because the theoretical birth date has not yet passed.

3.8.3 False rings and true growth annuli

Although the scientific literature is very scarce regarding this species, the problem of false rings has not been reported and the deposition pattern usually appears clear.

3.9 *Micromesistius poutassou*

M. poutassou is a mesopelagic gadoid widely distributed in the eastern North Atlantic and the Mediterranean Sea. It carries out extensive migration throughout its wide distribution area, which involves a very diverse fishery. In the Mediterranean basin, the fishery is mainly done by trawlers. Along the Italian coast, the areas with greater landings are: the Ligurian Sea, northern Adriatic Sea and northwest Ionian Sea (IREPA, 2012).

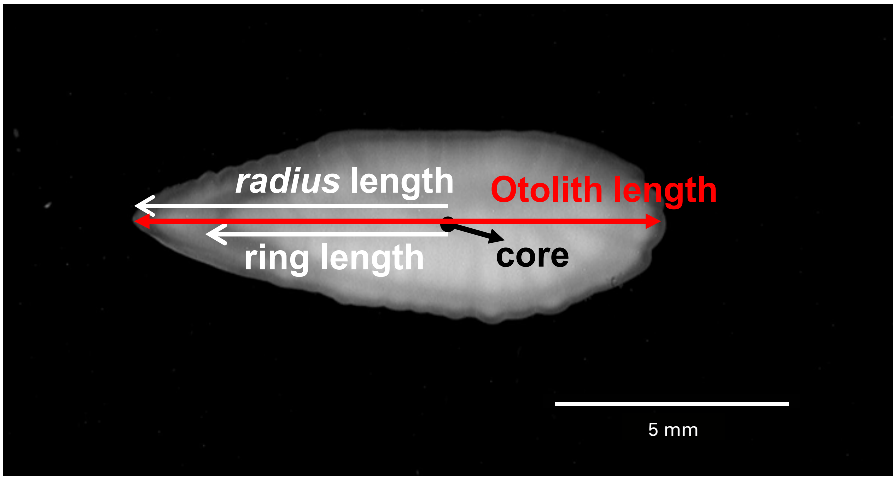
3.9.1 Extraction and storage

The sagittal otolith extraction is made through the transverse section. After extraction, the otoliths are washed to remove organic material, then dried and stored.

3.9.2 Preparation and interpretation

If stored dry, the otoliths (usually the left) are placed in immersion in seawater to be clarified 24 hours before analysis. They are analysed under a binocular microscope, in seawater (clarification medium), with reflected light, against a black background. The best orientation of the otolith is with the distal surface up and the proximal surface (sulcus acusticus) down. However, handling the otolith, turning it in various directions, may be a way of assuring the estimated age. Under reflected light with the black background, winter translucent rings appear dark (Plate 99). The otolith is interpreted by reading the postrostrum area. However, ring continuity should be checked on the anterior part of the otolith (rostrum area) and, wherever possible, on the dorso-lateral edge. Morphometric measures of otoliths and ring distances are taken routinely, as is shown in Plate 99, on subsamples of the *sagittae*, while the nature of the edge (opaque or translucent) is always noted.

PLATE 99
Otoliths of *M. poutassou*



© P. Carbonara

Note: Male, TL = 19 cm, axes of morphometric measures shown.

In older individuals, the first annulus may be difficult to define due to overlaying otolith growth. Before analysis, the proximal surface of the otolith of larger specimens (TL > 35 cm) is ground/polished by sandpapers in successively finer steps (from 180 to 400 µm grain size). Usually, the characteristic of the first ring in older individuals is a more-undefined wavy appearance (Plate 101).

Determination of the age of *M. poutassou* considers 1 January as the birth date, in accordance with a spawning period during winter months (Sbrana, Chiericoni and Biagi, 1998). Age is assigned with a resolution of 0.5 year, counting the translucent (winter) ring (see subsection 1.3.1).

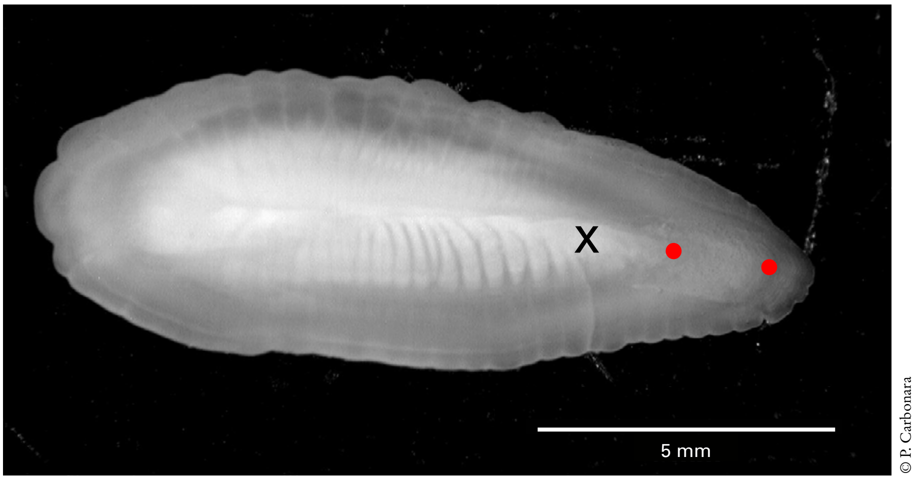
3.9.3 False rings and true growth annuli

Since 1977, when the first otolith exchange was initiated (Anon., 1978), several attempts have been made to improve determination of ageing for *M. poutassou* in the ICES context, including otolith exchanges and otolith reading workshops (ICES, 1992, 2005).

The basic problems in reading age from *M. poutassou* otoliths can be divided into the following (ICES, 1992, 2005):
- identification of the first annual growth ring;
- interpretation of the edge; and
- overlapping of growth rings in older specimens owing to decreasing distance between rings.

A false ring known as the Bailey's zone may appear inside the first growth ring (ICES, 2005); confusion can be eliminated by referring to the measurement. This false ring has been linked to a change in behaviour from pelagic to demersal feeding and distribution in the first year of life. If a ring is less than 3.5 mm, it is probably a Bailey's zone. Moreover, the first growth ring usually appears clearer than a false ring (Plate 100).

PLATE 100
Otoliths of *M. poutassou*

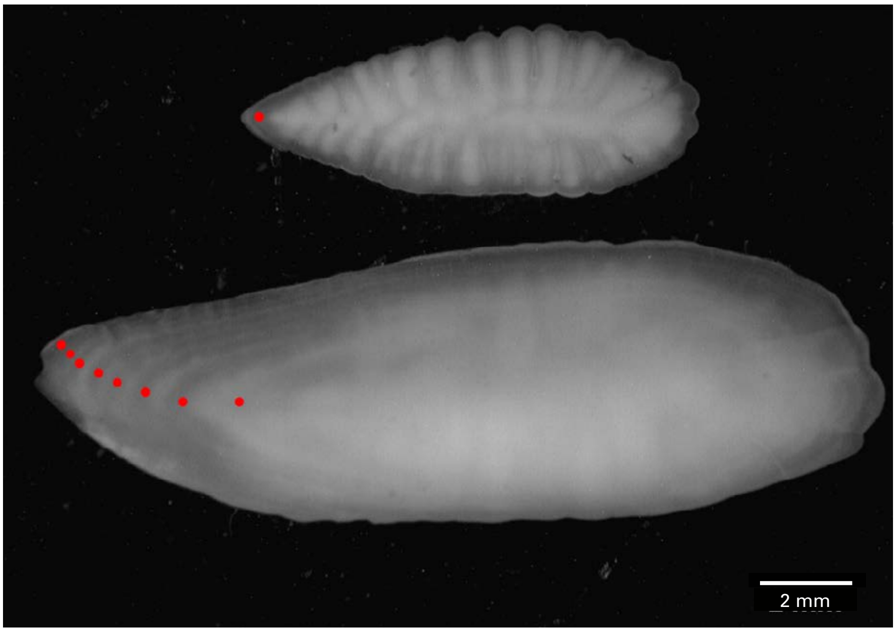


Note: Age 2.5 years, female, TL = 28 cm, captured in August.

Regarding interpretation of the edge, a correct reading must consider the edge around the whole otolith, evaluating what kind of edge (opaque or translucent) is present on most (3/4) of the otolith perimeter .

The pattern of otolith growth in *M. poutassou* presents a wide area in the first year, which is greatly reduced in subsequent years (Plate 101). In older specimens (TL > 30 cm), overlapping of the rings can make age analysis difficult. In this case, a higher magnification can be useful to discriminate the rings on the edge (Plate 101).

PLATE 101
Otoliths of *M. poutassou*



Note: Top – age 1 year, male, TL = 17 cm, captured in June; bottom – age 8.5 years, female, TL = 36 cm, captured in September.

3.10 *Chelidonichthys lucerna*

In the Mediterranean, eight species of gurnards have been identified and *C. lucerna* is one of the major species landed. *C. lucerna* lives mostly on sand or gravel bottoms at depths ranging from 80 to 200 m. Migratory movements within its overall depth range during the year range from shallow depths in spring/summer and to deeper waters in winter. Information on the biology of this species in the Mediterranean Sea is scarce, especially with regard to age and growth.

3.10.1 Extraction and storage

The sagittal otolith extraction is made through the transverse section. After extraction, the otoliths are washed to remove organic material and then dried and stored.

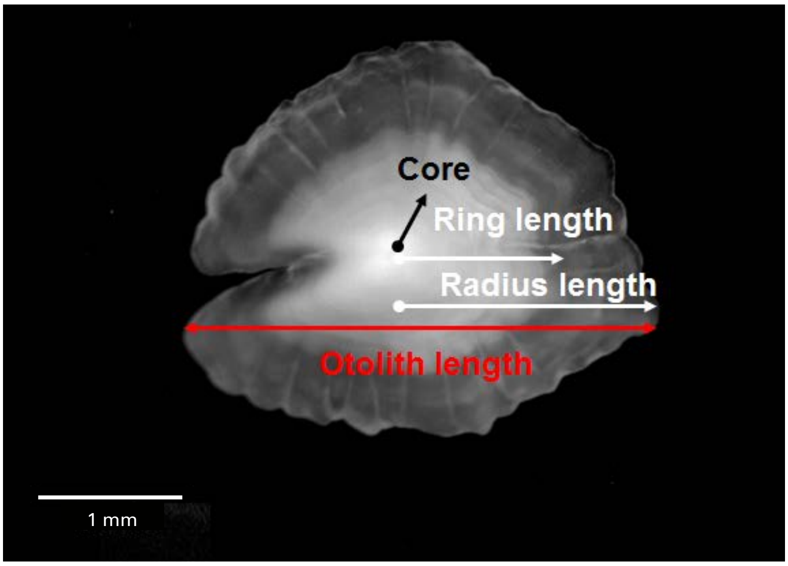
3.10.2 Preparation and interpretation

One otolith from each pair (usually the left one) is placed in immersion in seawater to be clarified before analysis. The otoliths are analysed under a binocular microscope in seawater (clarification medium), with reflected light, against a black background (Plate 102). The best orientation of the otolith is with the distal surface up and the proximal surface (sulcus acusticus) down.

In whole otoliths, annuli are counted on the posterior part of the otolith (postrostrum) (Plate 102). However, ring continuity should be checked on the anterior part of the otolith (rostrum) and, wherever possible, on the dorso-lateral edge. Dark rings are counted as the translucent growth zone (slow growth). The opaque zone (white – fast growth) with a dark ring is considered annual growth (annulus).

For larger specimens (TL > 35 cm), it will be useful to analyse the thin section; in this way the overlapped rings on the edge are highlighted. One otolith (always the same, when possible) is embedded in epoxy resin and transverse sections (0.7–0.8 mm thick) are cut through the core by a low-speed saw equipped with diamond blades. The thin sections are analysed with reflected light, on a black background (Plate 103).

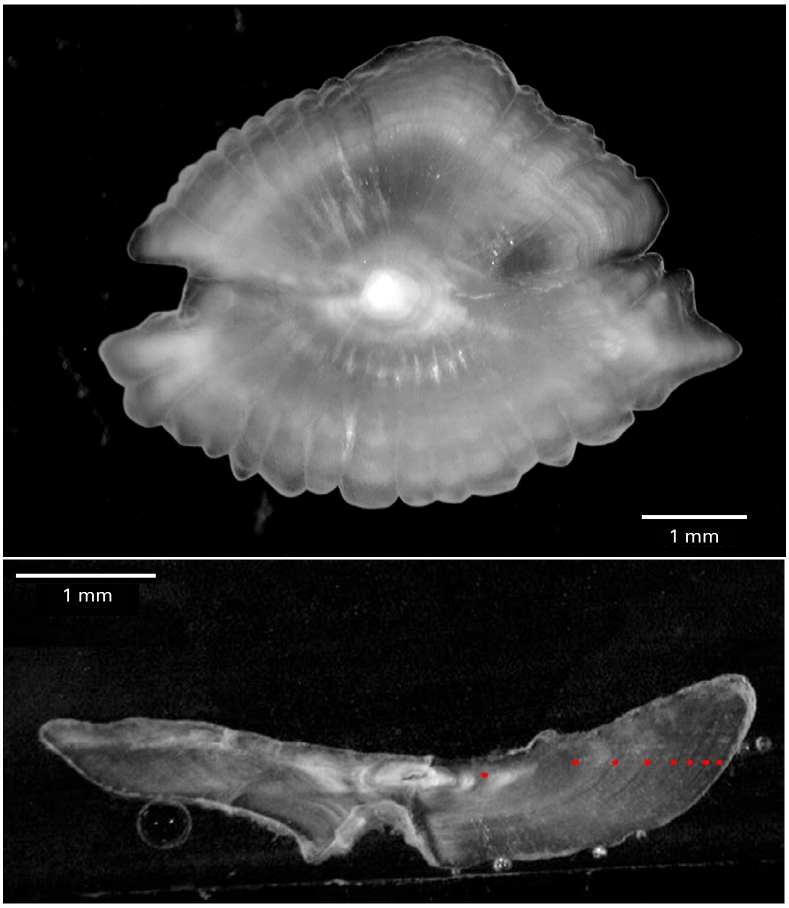
PLATE 102
Otoliths of *C. lucerna*



© P. Carbonara

Note: Female, TL = 20 cm, axes of morphometric measures shown.

PLATE 103
Otoliths of *C. lucerna*



© P. Carbonara

Note: Top – whole otolith, female, TL = 44.5 cm, caught in September; bottom – thin section, red dot = growth ring.

Age determination for *C. lucerna* considers 1 January as the birth date, in accordance with the spawning period (Boudaya *et al.*, 2008), with a peak in January–February. Age is assigned with a resolution of 0.5 year, counting the translucent (winter) ring (see subsection 1.3.1). Moreover, for every otolith the edge quality is noted (opaque or translucent), while measurements from the core to each translucent ring (at the end of the ring) on the posterior, radius length and otolith length are taken on a subsample of otolith.

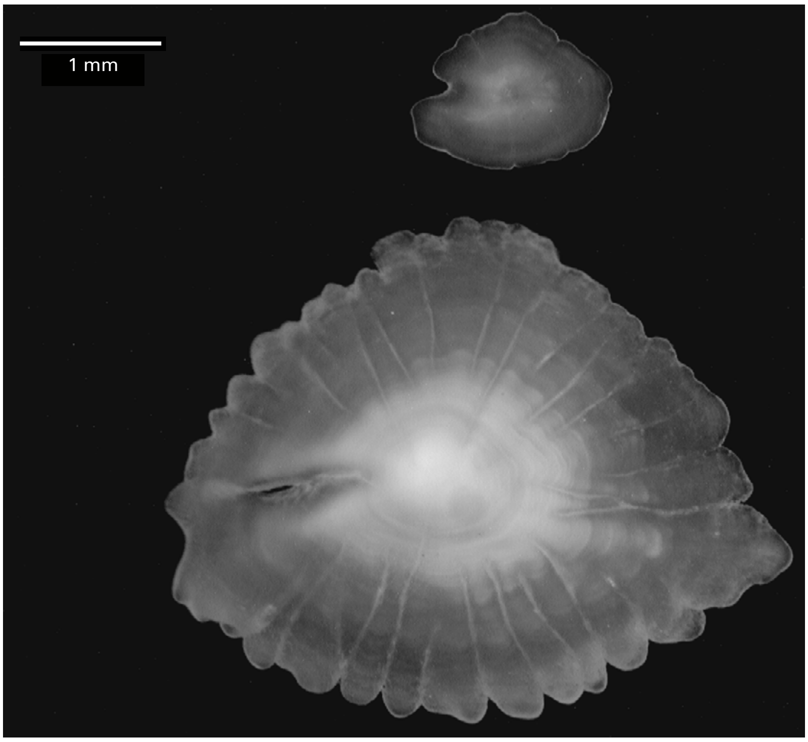
3.10.3 False rings and true growth annuli

The presence of false rings could be a main source of error in ageing analysis. Indeed, close to the core a ring is often present that could be considered a false ring. The distance between two growth rings seems be decreasing from the nucleus to the edge. Moreover, the otoliths of very small specimens (TL 5 cm) caught in spring present a translucent edge of a size that corresponds to about the first translucent growth ring. So, this first ring could represent the passage from the pelagic to the demersal life.

Another source of bias occurs when the ring deposition pattern is poorly differentiated between the opaque and translucent rings. Moreover, a false ring can be created by environmental stress. The criteria for recognizing true growth rings are:

- Translucent growth rings should be visible more or less around the whole otolith to be considered annual rings (Plate 104).
- The increment between the consecutive annuli should decrease with age (Plates 105, 106 and 107).

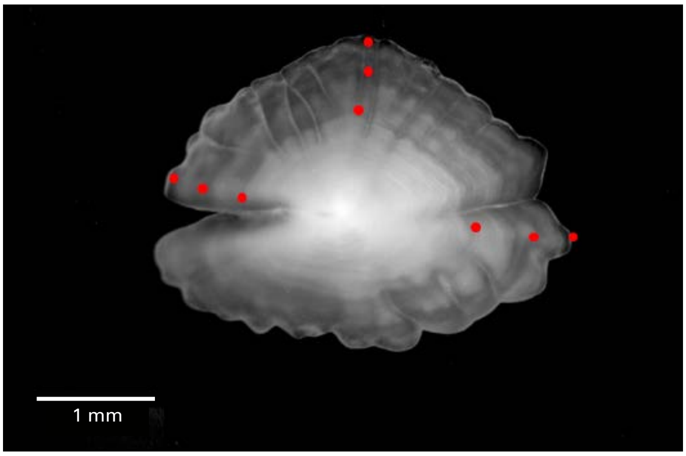
PLATE 104
Otoliths of *C. lucerna*



© P. Carbonara

Note: Top –TL = 5 cm, captured in April; bottom: male, TL = 29 cm, captured in December.

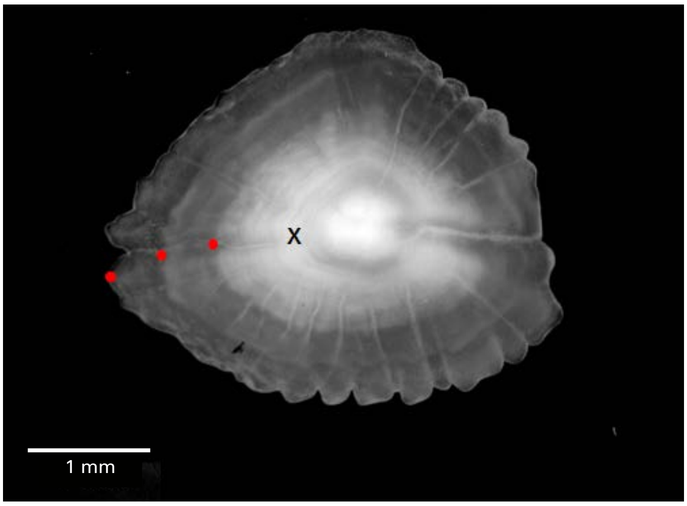
PLATE 105
Otoliths of *C. lucerna*



© P. Carbonara

Note: Age: 2.5 years, female, TL = 23 cm, captured in December, red dot = growth ring.

PLATE 106
Otoliths of *C. lucerna*



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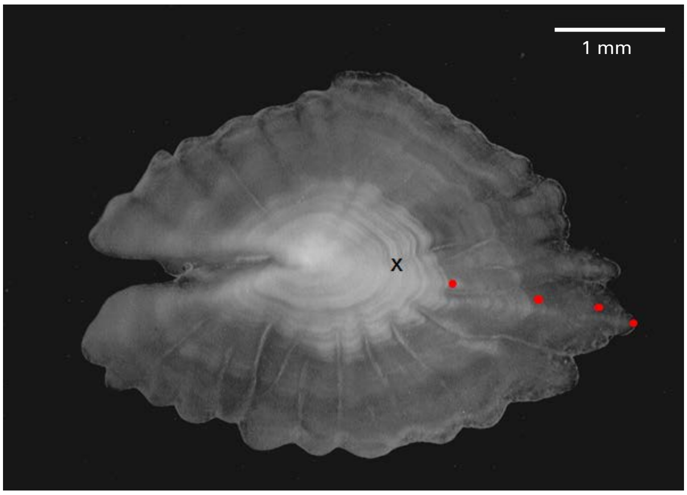
Note: Age: 3 years, male, TL = 29 cm, captured in March, red dot = growth ring, X = false ring.

PLATE 107
Otoliths of *C. lucerna*



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Note: Age: 4 years, male, TL = 33.5 cm, captured in June, red dot = growth ring, X = false ring.

3.11 *Pagellus erythrinus*

P. erythrinus is a highly valued demersal species distributed in the Mediterranean and Black Seas and along the European and African coasts of the Atlantic Ocean. It is a protogynous hermaphrodite species, with batch spawner reproduction behaviour.

3.11.1 Extraction and storage

Sagittae extraction is made through the transverse section. After extraction, the otoliths are washed to remove organic material and then dried and stored.

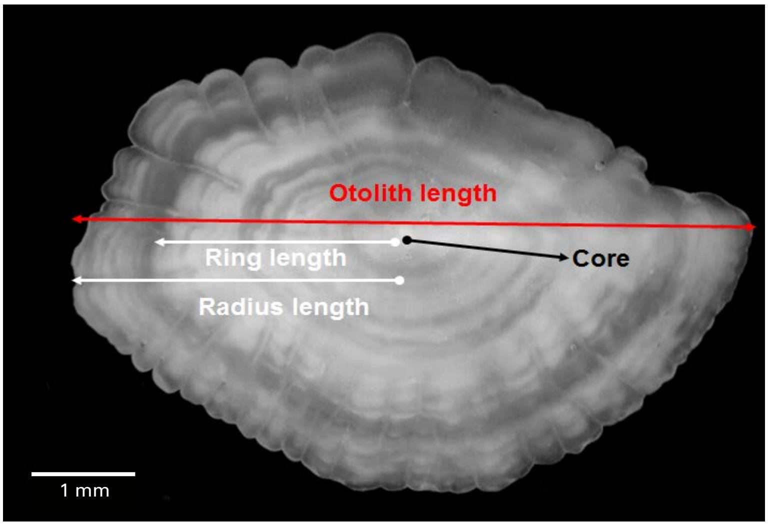
3.11.2 Preparation and interpretation

One otolith from each pair (usually the left one) is immersed whole for 3–4 minutes in seawater to be clarified before analysis. The otoliths are analysed under a binocular microscope, in seawater (clarification medium), with reflected light, against a black background. The best orientation for the otolith is with the distal surface up and the proximal surface (sulcus acusticus) down (Plate 108).

In whole otoliths, annuli are counted on the posterior part. However, ring continuity should be checked on the anterior part (rostrum) and, wherever possible, on the dorso-lateral edge (Plates 109 and 110).

Dark rings are counted as the translucent growth zone (slow growth). The opaque (white – fast growth) zone with a dark ring is considered an annual growth (annulus). Age determination for *P. erythrinus* considers 1 July as the birth date, in accordance with the spawning period (Valdes *et al.*, 2004; Metin *et al.*, 2011) in spring/early summer. Age is assigned with a resolution of 0.5 year, counting the translucent (winter) rings (see subsection 1.3.2).

PLATE 108
Otoliths of *P. erythrinus*

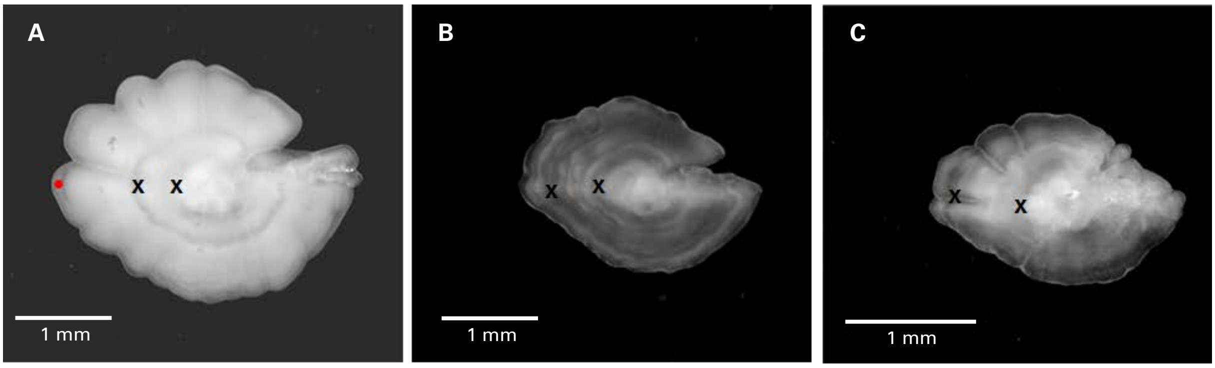


Note: Male, TL = 15.5 cm, axes of morphometric measures shown.

3.11.3 False rings and true growth annuli

Some false rings may be laid down before the first growth ring. Plate 109 shows examples of otoliths extracted from juvenile specimens (TL 3–6.5 cm) – caught in the fall/winter and born in the previous spawning season (spring/summer) – that show one or two checks. These false rings can be laid down during the pelagic phase and during passage from pelagic to benthopelagic life. When these two false rings are present, distances from the core (posterior area) are respectively about 0.4 and 0.85 mm. Juveniles captured in the winter months present a translucent edge with a radius length of about 1.8 mm (Plates 109A and 110).

PLATE 109
Three examples of otoliths from juvenile *P. erythrinus*

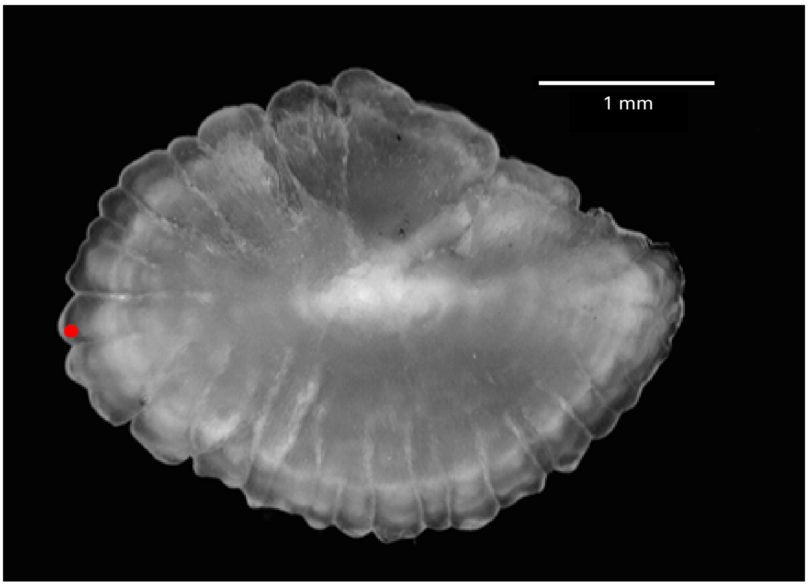


Note: A – TL = 6.5 cm, captured in March; B – TL = 3.5 cm, captured in October; C – TL = 3 cm, captured in August, red dot = growth ring, X = false rings.

Sometimes the first true rings appear not as a single ring (Plate 110), but as a translucent area. After the first growth ring, other false rings could be laid down during the second year of life (Plate 113) owing to reproductive periods and/or other environmental stresses; however, they are recognizable because they are less marked (Plate 113). In any case, the following criteria can be used to recognize true growth rings also for *P. erythrinus*:

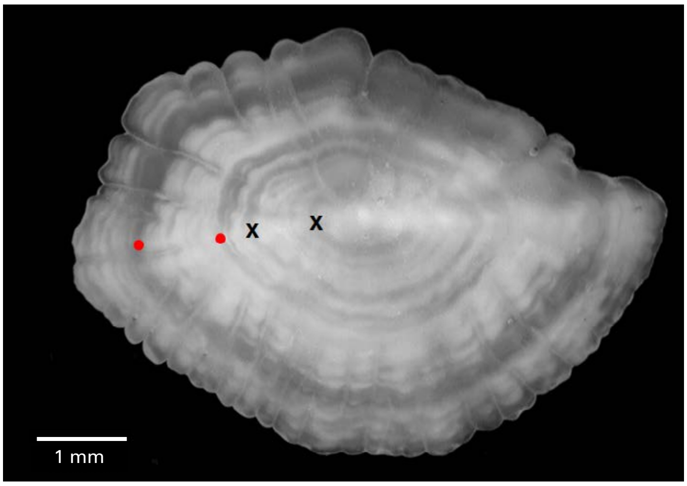
– Translucent true rings should be visible more or less around the whole otolith (Plates 112 and 113).
– The increment between consecutive annuli should decrease with age (Plates 111, 112 and 113).

PLATE 110
Otoliths of *P. erythrinus*



Note: Age 0.5 year, TL = 7.5 cm, captured in January, red dot = growth ring.

PLATE 111
Otolith of *P. erythrinus*



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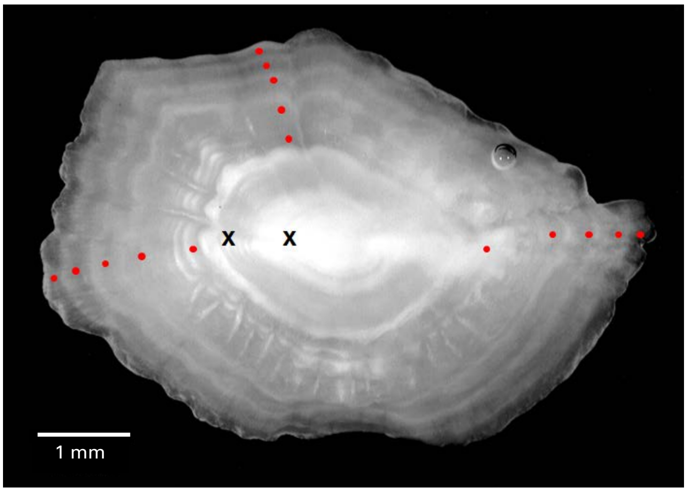
Note: Age 2.5 years, male, TL = 15.5 cm, captured in November, red dots = growth rings, X = false rings.

PLATE 112
Otolith of *P. erythrinus*



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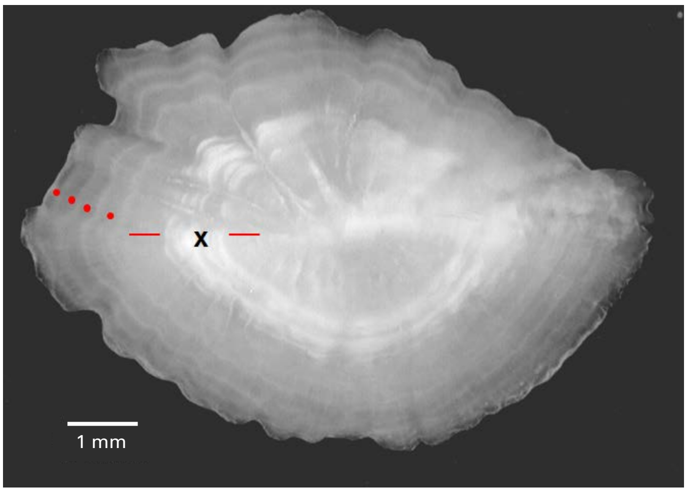
Note: Age 5 years, female, TL = 20.5 cm, captured in November, red dots = growth rings, X = false rings.

PLATE 113
Otolith of *P. erythrinus*



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Note: Age 6 years, male, TL = 22.5 cm, captured in November, red dots = growth rings, red line = winter area, X = false rings.

3.12 *Eutrigla gurnardus*

E. gurnardus may be considered a Lusitanian-Boreal species that is widespread in the eastern Atlantic, occurring from Iceland, Norway, the southern Baltic, via the North Sea to southern Morocco and Madeira. The species is also found in the Mediterranean and the Black Sea. Most common on sandy bottoms, but also on mud, shell and rocky bottoms in shallow water (10–250 m). The distribution of *E. gurnardus* overlaps with other species of Triglidae: *C. lucerna* and red gurnard (*Aspitrigla cuculus*). Information on the biology of this species in the Mediterranean Sea is very scarce, especially with regard to age and growth.

3.12.1 Extraction and storage

Sagittae extraction is made through the transverse section. After extraction, the otoliths are washed to remove organic material, and then dried and stored.

3.12.2 Preparation and interpretation

One otolith from each pair (usually the left one) is immersed in seawater to be analysed. Otoliths of *E. gurnardus* don't need the clarification phase before analysis.

The otoliths are analysed under a binocular microscope, rinsed with sea water (clarification medium), with reflected light, against a black background. The best otolith orientation for analysis is with the distal surface up and the proximal surface (sulcus acusticus) down. In this way, the dark rings can be counted in the antirostrum area (radius) as translucent growth rings (slow growth). The opaque zone (white – fast growth) with a dark ring is considered an annual increment (annulus). Moreover, the edge quality is noted (opaque or translucent) for every otolith, while measurements from the core to each translucent ring (at the end of the ring) in the postrostrum area, radius length and otolith length are taken on a subsample of otolith.

Age determination for *E. gurnardus* sets the birth date at 1 July, in accordance with the spawning period in summer/early autumn (Baron, 1985). Age is assigned with a resolution of 0.5 year, counting the translucent (winter) rings (see subsection 1.3.2).

4. Cartilaginous species

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Knowledge of fish age is a fundamental tool in estimation of the main biological parameters of species and in correct fishery stock management (Goldman, 2005). Estimation of growth, mortality and longevity rates is, in fact, essential to resource evaluation, and it requires careful studies on the ageing of individuals. Erroneous evaluation may affect stock assessment and ultimately lead to inadequate management measures (Hoenig and Gruber, 1990; Goldman, 2005). Knowledge of the age and growth of cartilaginous fish in the Mediterranean Sea is rather scarce and little information is available for most species living in the basin.

Unlike teleosts, which have scales and otoliths, in elasmobranchs age determination proves to be more complex (Campana, 2014), both for the absence of these hard structures, and for the low level of calcification of the cartilaginous parts. Thus additional methods are often required to improve the visibility of the growth bands. In many cases, these techniques are species-specific.

The structures suitable for age reading in cartilaginous fishes are the vertebral centra (analysed in toto or sectioned), neural arches, spines and dermal denticles (Campana, 2014). Among these, the most widely used are vertebrae and spines. In the first case, the most common method is preparation of thin sagittal sections of the vertebral centra.

Vertebrae are usually extracted from the thoracic region in batoids and from the thoracic-predorsal region in shark-like species and then stored frozen.

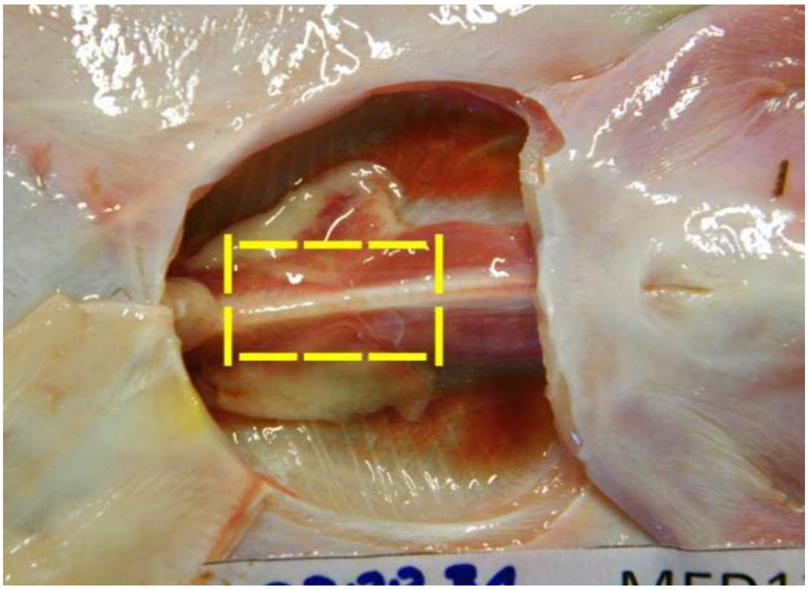
4.1 Vertebrae extraction and storage

Sampling should consider the population structure by analysing a number of individuals for size class, proportional to their distribution in the population and to the sex ratio. For each individual, the main biometrics in cm, total weight (TW) in grams, sex and maturity stage (AA.VV., 2016a) should be registered.

For shark-like species, total length (TL) and anus length (AL). For batoids, TL, disc width (DW) and disc length (DL) must be recorded. In some families, such as Dasyatidae and Myliobatidae, the filamentous tail could be easily broken. This makes TL unreliable. Thus, in these families, DW should be the main measure.

For each sampled individual, a section of at least ten vertebrae should be extracted from the thoracic region for batoids and from the thoracic-predorsal region for shark-like species. In these anatomical zones, which are immediately posterior to the scapular origin of the vertebral column, vertebral centra are bigger and thus easier to analyse (Plate 114).

PLATE 114
Thoracic cavity of *Raja brachyura*



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Note: Dotted line = zone from which vertebral centra should be extracted.

Vertebral sections should be frozen dry. Temperatures of at least -18 °C are recommended. Table 15 lists selected species with main and secondary biometrical measures.

TABLE 15 – List of selected species

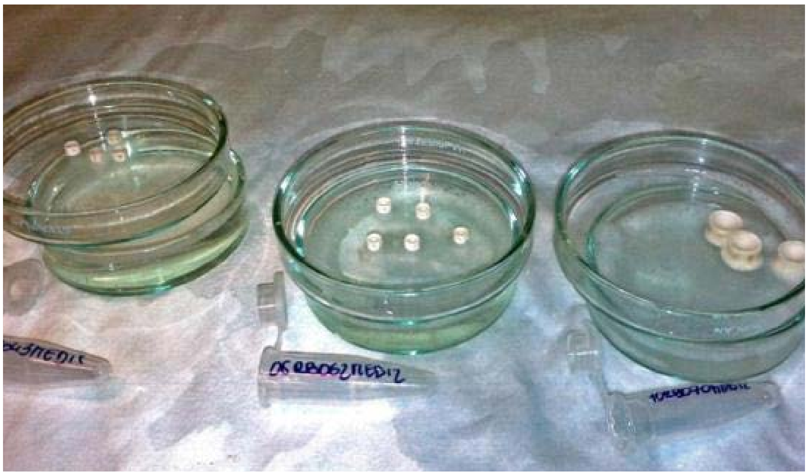
Species	Main measure	Secondary measure
Dipturus oxyrinchus	TL	DW
Etmopterus spinax	TL	AL
Raja brachyura	TL	DW
Raja clavata	TL	DW
Raja polystigma	TL	DW
Scyliorhinus canicula	TL	AL

4.1.1 Cleaning and embedding

In order to obtain a thin section suitable for reading, the vertebral centra must be embedded in epoxy resin. Before embedding, the vertebrae should be separated and cleaned of residues of muscle tissue, and both neural and hematic arches should be cut away. A part of the sample should be preserved for further analysis. To better remove excess tissue, the vertebral centra are immersed in a solution of 5-percent sodium hypochlorite, for a variable time depending on size, taking care to stop the operation before the solution begins to deteriorate them (Plate 115). Vertebral centra should then be immerged in distilled water for a time ranging from 30 to 45 minutes and subsequently dried to eliminate excess moisture.

The cleaned vertebral centra should be photographed through a stereomicroscope under reflected light with the aid of an integrated camera. A reference in the picture should be included. The main measures of the vertebrae should be recorded, such as radius (RV, in mm) and length (LV, in mm) (Plates 126, 133, 140, 144 and 150).

PLATE 115
Cleaning of the vertebral centra (*R. brachyura*)



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Embedding is done using special teflon moulds and highly transparent epoxy resins (Plates 116 and 117). As an example, the Struers Caldo-Fix 2 bicomponent resin could be used. The advantage of this kind of resin is that it hardens directly in the oven (at approximately 75 °C for a variable time from 1.5 to 4 hours), shortening the processing time. The mixture of resin and hardener should be poured onto the centra filling the mould, making sure to remove any air bubbles with tweezers.

PLATE 116
Teflon moulds

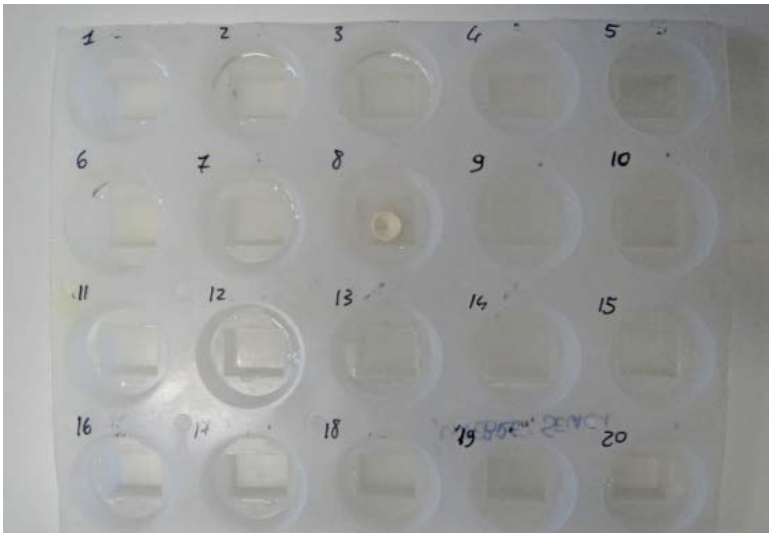

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PLATE 117
Vertebral centra embedded in epoxy resin
(*Oxynotus centrina*)

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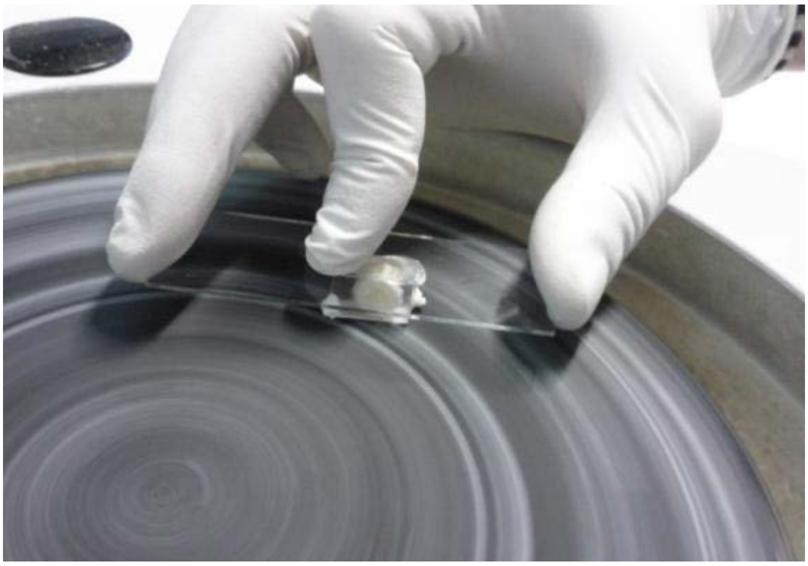
4.1.2 Sectioning

Sections, from 0.3 to 0.5 mm in width, can be obtained through two methods:
- by cutting, using special low-speed double-bladed saws, which allow regulating the width of the cut (e.g. the IsoMet 1000 precision saw); or
- through grinding.

In the latter case, the resin block must be mounted (with hot glue) on a microscope slide (Plate 118). Grinding can be done either manually, through progressively lower-grain abrasive pastes (such as Logitech Silicon Carbide Powder), or mechanically, with a lapping machine (e.g. Struers Dap-V) with abrasive discs in descending grits (320, 500, 800) (Plate 118).

PLATE 118
Grinding (*R. brachyura*)



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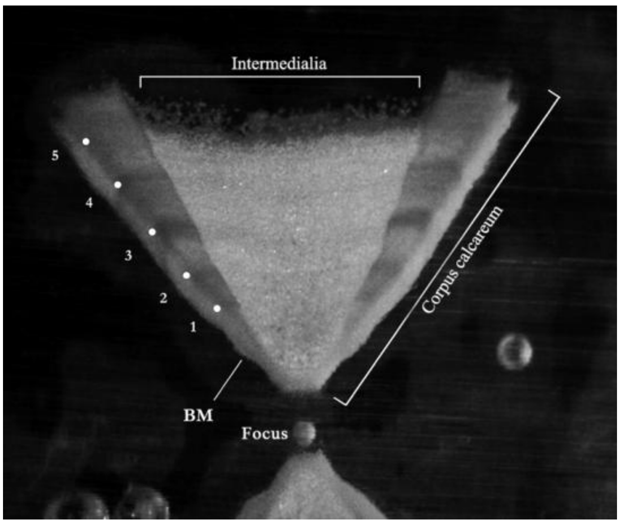
The section must include the vertebral focus, so that the four arms of the corpus calcareum are fully visible and thus no bandwidth is lost (Plate 119). To do this, the block must be filed until the focus, then mounted backwards so that the operation can be repeated.

PLATE 119
Vertebral section of a 5-year-old *R. brachyura*



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Note: TL = 51.4 cm, BM = birthmark.

4.1.3 Staining

Before proceeding to age reading, it is often necessary to improve growth band visibility through the use of staining techniques. Some of the most commonly employed are:
- alizarin red (La Marca, 1966)
- crystal violet (Johnson, 1979)
- silver nitrate (Stevens, 1975)
- cobalt nitrate and ammonium sulfide (Hoenig and Brown, 1988).

The effectiveness of each technique depends on the species examined. In the literature, modifications of these techniques are common. The vertebral centra can be coloured in toto, before inclusion, or when the section is completed. In this case, as well, effectiveness is species specific, even if the last solution (colouring at a later time) appears to be more rapid and allows good results (Table 16).

TABLE 16 – Staining methods for selected species

Species	Staining methods
Dipturus oxyrinchus	NS; AR
Etmopterus spinax	CNit+AS
Raja brachyura	NS; AR; CrV
Raja clavata	NS; AR; SN
Raja polystigma	NS; AR; SN
Scyliorhinus canicula	CrV; NS

Note: NS: no staining; AR: alizarin red; CrV: crystal violet; SN: silver nitrate; CNit+AS: cobalt nitrate+ammonium sulfide.

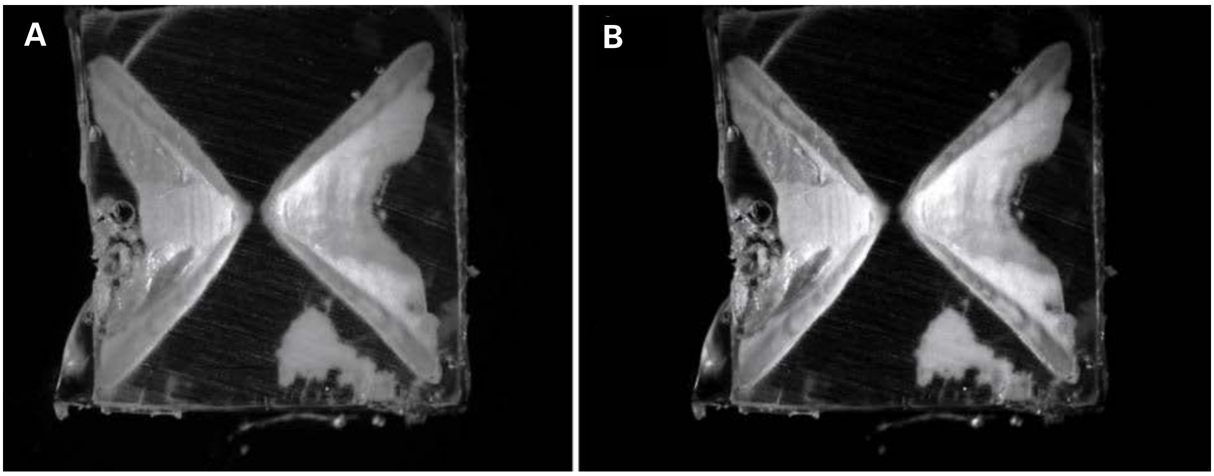
4.1.4 Image capture and post-production

Sections should be observed and photographed with a stereomicroscope under reflected light with the aid of integrated cameras. To ensure good results, photos should have a resolution of at least 3 megapixels, equal to 2048 x 1536 pixels. A clarifier liquid such as ethanol or, more simply, water should be used on the section. As far as possible, magnification for all samples belonging to the same species should be standardized.

Before analysing the images, a slight post-production process is needed to emphasize the growth bands (Campana, 2014). This can be done using any software that can regulate the main image parameters, such as contrast, brightness and colour balance.

First, colour should be deleted, converting the image to greyscale. Then work with contrast and, for a finer result, sharpness should be enhanced (Plate 120a and b).

PLATE 120
Vertebral centrum (*D. oxyrinchus*) a: before and b: after the post-production process



4.1.5 Section interpretation

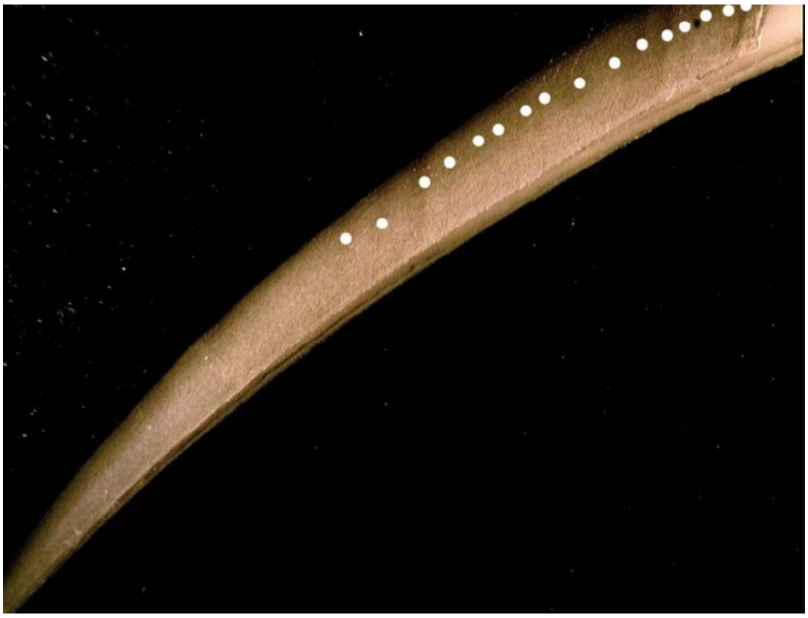
Correct interpretation of growth bands is of fundamental importance in age estimation of the analysed specimen. Reading should be done along the corpus calcareum, starting from the first annulus after the birthmark (BM), which is sometimes associated with a variation in the angle of the corpus calcareum (Plate 119) (Casey, Pratt and Stillwell, 1985; Sulikowski *et al.*, 2003). As a general rule, the better-defined annulus – meant as the union between the winter/spring band (translucent) and the summer/autumn one (opaque), or vice versa, after the BM – should be considered the first year. Generally, age is determined with a resolution of one year.

4.2 Dorsal spine

4.2.1 Imaging and interpretation

Elasmobranchs with fin spines sometimes show distinct growth-band patterns. These growth bands have been validated to form annually in species such as picked dogfish (*Squalus acanthias*) (Campana *et al.*, 2006), where the second dorsal spine is generally used for age determination, as it tends to be less worn (Plate 121). Fin spines are generally too large to image under a dissecting microscope, but growth bands can sometimes be counted visually under the microscope. The best approach is to capture a digital image of the spine with a standard digital camera, preferably mounted on a camera stand, and then enhance the image prior to age determination. Growth bands are usually most clear if the spine is held with the convex side of the curve facing upwards, but offset slightly from the vertical. As was the case with whole vertebrae, proper lighting is important, and is best provided from a fibre optic light source, although at relatively low levels.

PLATE 121
Spine of *S. acanthias* with annotated (white dots) growth bands



Source: Campana, 2014.

4.2.2 Worn dorsal spine

Fin spines grow conically from the base, implying that the tip of the spine is the oldest part. As a result, and combined with other sources of wear throughout life, the spine enamel often becomes so eroded that any growth bands that were present become lost (Plate 122). Without correcting for the eroded growth bands, any age determined from the remaining growth bands will underestimate the true age.

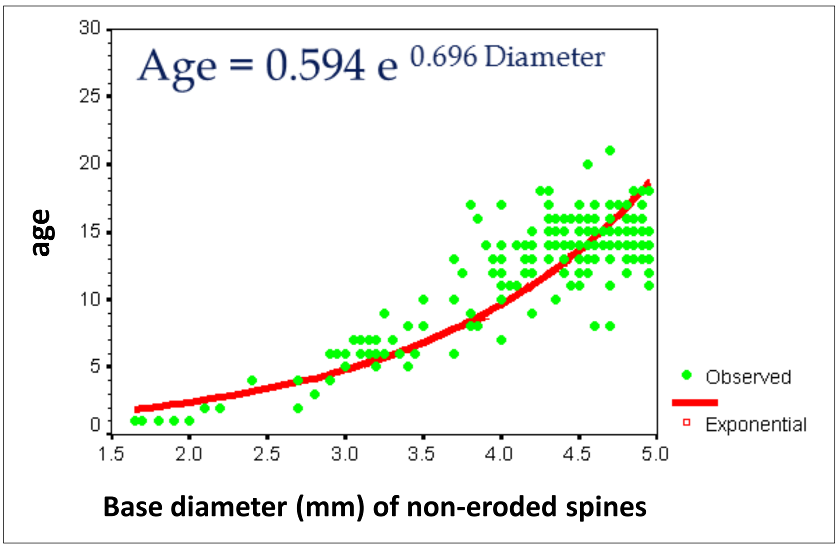
The relationship between number of growth bands and basal diameter of the spine needs to be determined from unworn spines. Unworn spines will usually be seen only in relatively young elasmobranchs, but that is acceptable, as growth bands formed in younger ages are usually the ones worn away.

PLATE 122
Spine of *S. acanthias* indicating growth bands (black dots), and the anterior portion in which growth bands have been eroded

Source: Campana, 2014.

Any curvilinear equation can be fitted to the band count/basal diameter data. Exponential equations are often used, but as long as the fitted curve represents the data well, any equation can be used (Ketchen, 1975; Campana, Joyce and Kulka, 2009) (Plate 123). It is important to note that the relationship between band count and basal diameter in unworn spines will vary with the growth rate of the population; published equations from one population cannot be applied to a different population unless their growth rate is similar.

PLATE 123
Relationship between the number of growth bands in unworn spines and the diameter of the spine base



Source: Campana, 2014.

Note: Used to estimate the number of missing growth bands in worn spines.

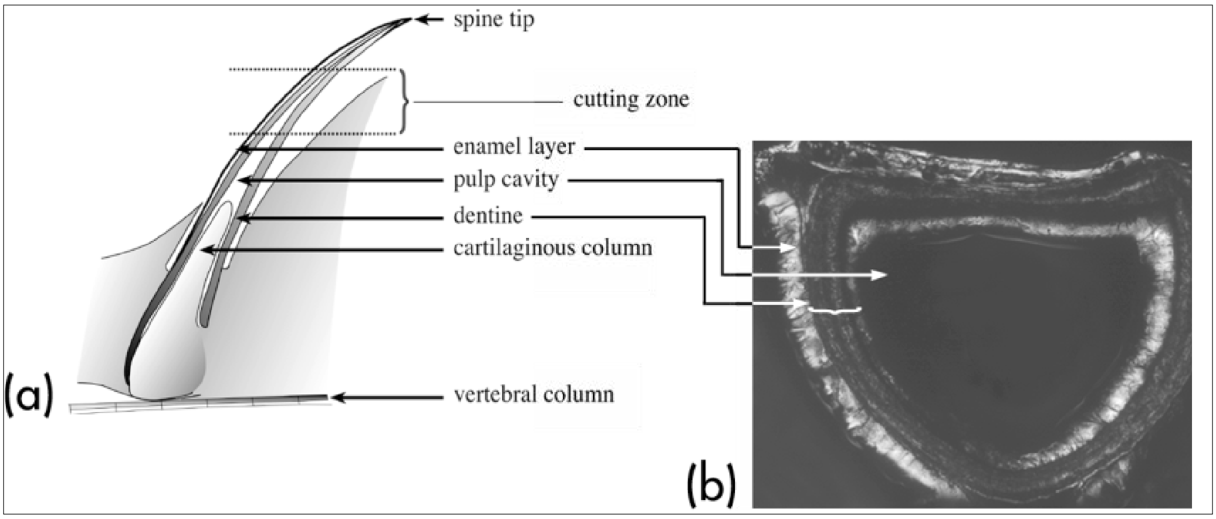
Once the relationship between band count and basal diameter has been determined in unworn spines, the same relationship can be used to estimate the number of missing growth bands in worn spines. The spine diameter in the worn spine is measured at the most distal point at which growth bands are still visible. This measurement is then inserted into the equation calculated from the unworn spines to estimate the number of missing growth bands. That number can then be added to the observed number of growth bands in the worn spine to derive the estimated age of the elasmobranch.

4.2.3 Section of dorsal spine

Cross sections of the dorsal fin spine are often used to observe patterns of growth band deposition. The structure of the spine of velvet belly (*E. Spinax)* seems to be comparable to that of other sharks (Holden and Meadows, 1962; Ketchen, 1975; Beamish and McFarlane, 1985), although no marks are evident on the mantle. *E. spinax* provides good enhancement of growth marks on cross sections of the dorsal spine. Moreover, it does not require staining or the use of chemical reagents, and thus is not time-consuming. However, many spines are subject to rejection, due to their small size and fragility.

As reported by Beamish and McFarlane (1985), the spine of *E. spinax* also appears approximately triangular in section and consists of three main structural components: the cartilaginous interior, the stem and the mantle. The interior of the spine is filled with cartilage and surrounded by pulp tissue. The cartilage and pulp tissue degenerate towards the spine tip, leaving a central cavity that decreases from the cartilaginous column to the tip (Plate 124a). In the sections, the central cavity, the dentine stem and the enamel layer are clearly evident (Plate 124b).

PLATE 124
Structure (a) and cross section (b) of the second dorsal spine of *E. spinax*



Source: Sion, D'Onghia and Carlucci, 2002.

The spines of velvet belly do not show banding on the mantle, in contrast to that observed in other shark species (Ketchen, 1975; Beamish and McFarlane, 1985; Tanaka, 1990).

4.2.4 Collection and storage

Prior to the collection and subsequent storage of structures, each specimen was measured for total length (TL in mm) weighed (in grams), the sex identified and the maturity stage of gonads determined.

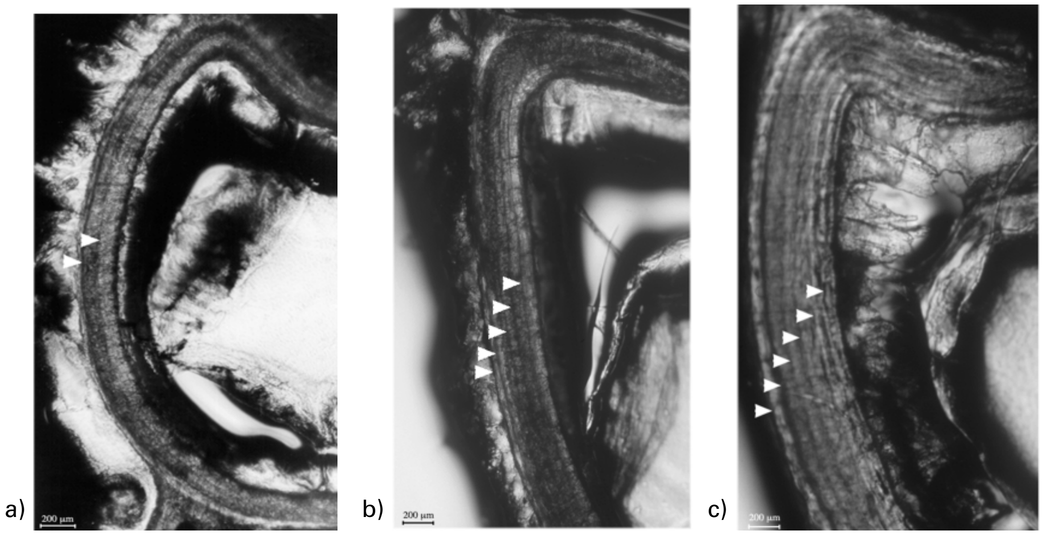
Following the indications of Holden and Meadows (1962), Ketchen (1975) and Beamish and McFarlane (1985), the whole second dorsal spine was removed by cutting horizontally just above the notochord to ensure that the spine base and stem were intact. Each spine was cleaned of excessive muscle and connective tissue by immersing the structure in a solution of 5-percent sodium hypochlorite for a variable time, depending on the spine size, then immersed in distilled water (30–40 minutes) and finally stored frozen to -20 °C.

4.2.5 Preparation of sections

The spines were sectioned transversely using an IsoMet 1000 precision low-speed saw with a diamond blade. Cross sections 450–500 µm thick were made from different regions of the spine (proximal, central and distal). The distal cuts were the only ones used, as they provided a better optical resolution of growth marks. The sections were mounted on microscope glass slides in a colourless transparent resin. Growth marks in the dentine stem were observed with a binocular microscope at 5x under low-intensity transmitted light. According to McFarlane and Beamish (1987), dark bands were assumed as annuli, and thus counted in estimating the age of *E. spinax*.

Dark annuli counts of the second dorsal spine cross section of *E. spinax* are shown in Plate 125 (Sion, D'Onghia and Carlucci, 2002).

PLATE 125
Particular of the second dorsal spine cross section of E. *spinax*



Source: Sion, D'Onghia and Carlucci, 2002.

Note: a) 2 years old (TL = 222 mm), b) 5 years old (TL = 348 mm), c) 6 years old (TL = 372 mm), arrows = annuli.

Afterwards, the study of age determination should be compared with analysis of other skeletal structures, for example in *E. spinax* by readings of vertebrae centra, but should be corroborated especially through the indirect method by analysis of modal components.

4.3 Species

4.3.1 *Dipturus oxyrinchus*

The longnosed skate (*Dipturus oxyrinchus*) can be considered one of the largest skates of the Rajidae family, reaching a maximum total length of 150 cm, though TL from 60 to 100 cm is more common (Serena, 2005). It can usually be found on sandy and muddy bottoms at depths of from 90 to 900 m, although it is mostly concentrated from 200 to 500 m (Serena, 2005; Ebert and Stehmann, 2013).

Some authors reported a spawning period for the species from February to April in the Mediterranean basin (Stehmann and Burkel, 1984; Notarbartolo di Sciara and Bianchi, 1998), while others reported from February to May (Serena, 2005). However, in Sardinian seas, females are reported to spawn throughout the whole year (Cabiddu *et al.*, 2012). So, for ageing purposes, it is difficult to define the birth date.

Because of its low commercial value, the longnosed skate is not directly targeted. Nevertheless, it represents a relatively common bycatch species in trawl and long-line fisheries (Relini *et al.*, 2010; Ebert and Stehmann, 2013). In the Mediterranean, the species is considered moderately abundant (Ungaro *et al.*, 2007) and, particularly in Italian waters, the population seems stable (Relini *et al.,* 2000, 2010; Follesa *et al.,* 2013) or even increasing (Marongiu *et al.*, 2017).

Unfortunately, it is still a poorly studied species and the lack of biological and ecological information, together with poor fishery statistics, as for most skates (Robinson, Cailliet and Ebert, 2007), makes stock assessment and development of effective management strategies difficult (Ungaro *et al.*, 2007).

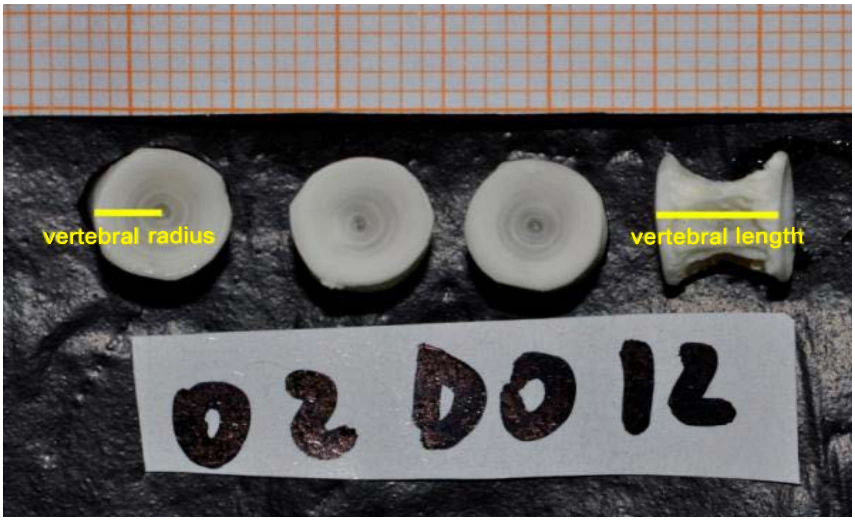
4.3.1.1 Extraction and storage

A portion of at least ten vertebrae is extracted from the thoracic region and stored frozen at -19 °C.

4.3.1.2 Preparation and interpretation

After separation and cleaning, vertebral centra are photographed and measured with a camera integrated into a binocular microscope (Plate 126).

PLATE 126
Main measurements on vertebral centra of *D. oxyrinchus*



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Note: Male, TL = 90.1 cm.

Centra are then embedded in epoxy resin. The resin blocks are ground to obtain thin sections and are analysed unstained, stained with alizarin red, silver nitrate and decalcified with ethylenediamine tetra-acetic acid (EDTA) (Plates 127, 128 and 129) (Bellodi, 2015). Although

Yigin and Ismen (2010) suggested staining the vertebral centra with silver nitrate, growth-band visibility was good even without staining (Plates 127, 128 and 129) (Kadri *et al.*, 2014; Bellodi *et al.*, 2017). Sections were photographed under reflected light, using water as the clarification medium.

PLATE 127
Vertebral centrum of *D. oxyrinchus* stained with alizarin red

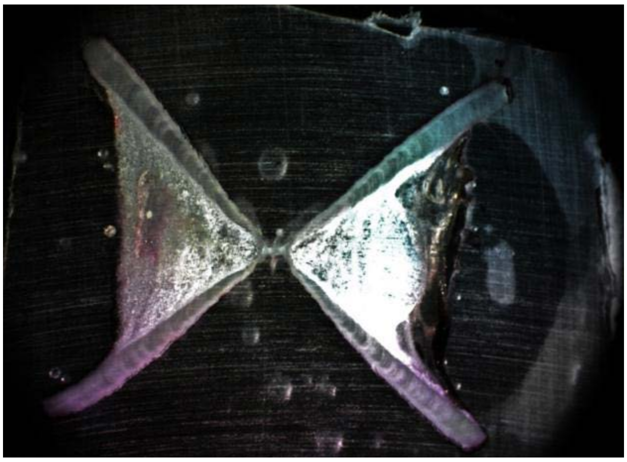
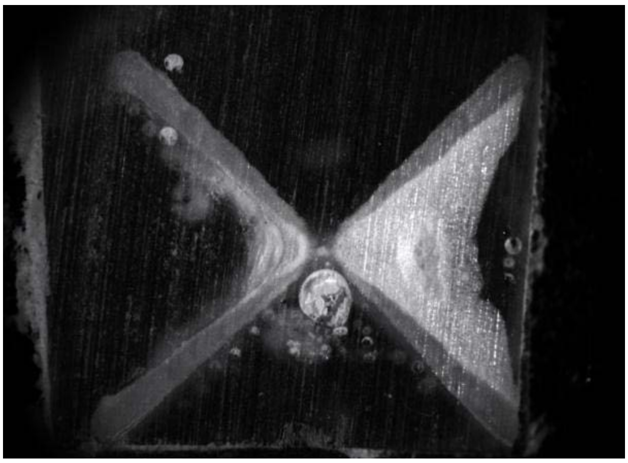


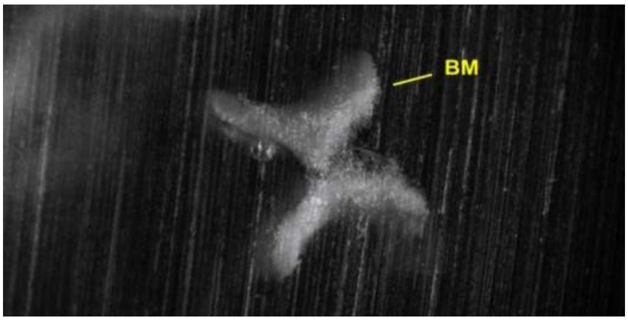
PLATE 128
Vertebral centrum of *D. oxyrinchus* stained with silver nitrate

PLATE 129
Vertebral centrum of *D. oxyrinchus* decalcified with EDTA



The BM was always easily identifiable as a clear variation in the angle of the corpus calcareum (Plate 130).

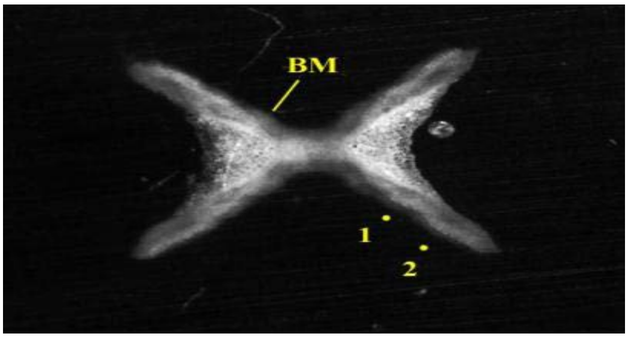
PLATE 130
Clear angle variation in the corpus calcareum of *D. oxyrinchus*



Note: Age 0, female, TL = 18 cm.

The better-defined annulus – meant as the union between the winter/spring band (translucent) and the summer/autumn one (opaque), or vice versa, after the BM – should be considered the first year. Age is determined with a resolution of one year (Plate 131).

PLATE 131
BM identification in a 2-year-old *D. oxyrinchus*



Note: Female, TL = 33.1 cm, vertebral radius = 0.89 mm.

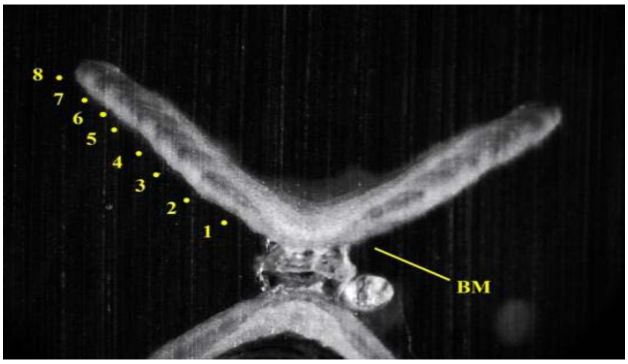
4.3.1.3 False and true annuli

During the first years, growth is very fast. In this phase, it is important to recognize the first annulus after the BM.

The presence of annuli with strongly closed, multiple bands represents the main source of bias in age interpretation of older individuals. This problem can be easily solved by paying particular attention to the frequency with which the bands are repeated (Plate 132).

Only bands that were clearly visible in all arms of the corpus calcareum were included in the reading.

PLATE 132
Closed multiple bands in an 8-year-old *D. oxyrinchus*



Note: Female, TL = 63.5, vertebral radius = 1.77 mm.

4.3.2 *Raja brachyura*

The blonde ray (*Raja brachyura)* is a large benthic species with a preference for sandy bottoms in the superior continental shelf to about 100 m (Serena, 2005).

Although *R. brachyura* is considered uncommon in the Mediterranean Sea (Matallanas, 1974; Serena, 2005), some observations (Follesa *et al.*, 2003, 2010; Relini *et al.*, 2010; Catalano *et al.*, 2007; Ragonese *et al.*, 2003) show that Sardinia and western Sicily represent zones of the basin where *R. brachyura* is relatively abundant. *R. brachyura* is a commercial species, usually landed across its distribution range (Catchpole, Enever and Doran, 2007). It is sometimes targeted in areas where it is locally abundant, but generally represents a bycatch species in mixed demersal fisheries using trawls, gill nets and longlines (Gibson *et al.*, 2006).

The population abundance in Sardinian waters seems on the increase (Follesa *et al.*, 2013; Marongiu *et al.*, 2017). In these seas, mature females are found between late May and September (Porcu *et al.*, 2015). Considering the long embryonic development typical of cartilaginous fishes, for this species, as well, determination of birth date is difficult.

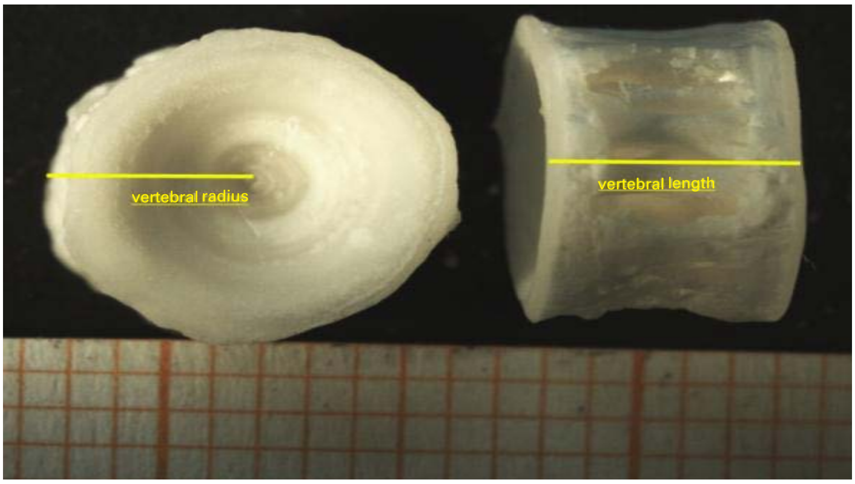
4.3.2.1 Extraction and storage

A portion of at least ten vertebrae is extracted from the thoracic region and stored frozen at -19 °C.

4.3.2.2 Preparation and interpretation

After separation and cleaning, vertebral centra are photographed and measured with a camera integrated into a binocular microscope (Plate 133). Centra are then embedded in epoxy resin and the resin blocks ground to obtain thin sections.

PLATE 133
Main measurements on vertebral centra of *R. brachyura*



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Note: Male, TL = 93.3 cm.

Gallagher, Nolan and Jeal (2005) suggested a modification of the crystal violet technique (Gallagher, 2000) as a staining method to enhance growth-band visibility. Nevertheless, this technique is expensive in terms of time (it requires 36 hours).

Improvement in growth-band visibility was analysed using silver nitrate, alizarin red and in non-stained vertebral centra. No clear increase in band visibility after staining was found (Plates 134 and 135) (Porcu *et al.*, 2015). Another enhancing process, decalcification with EDTA, worsened readability of the vertebral centra (Plate 136) (Bellodi, 2015).

PLATE 134
Vertebral centrum of *R. brachyura* stained with alizarin red

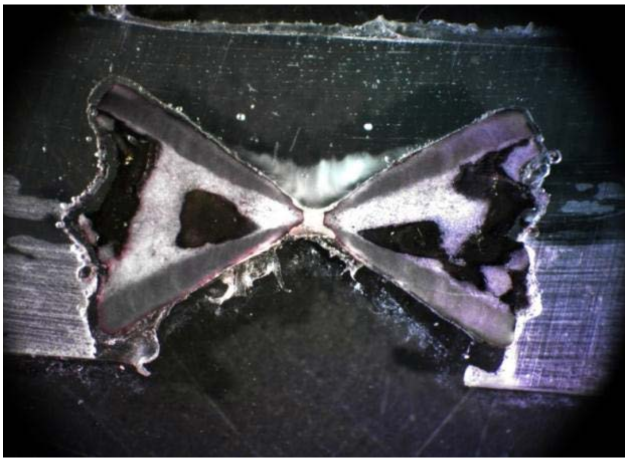


PLATE 135
Vertebral centrum of *R. brachyura* stained with silver nitrate

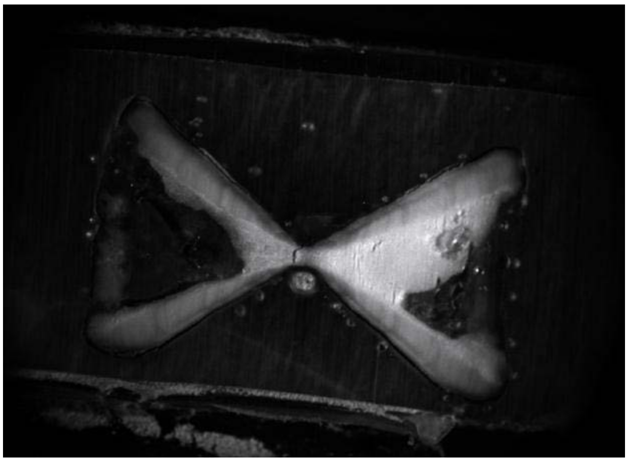
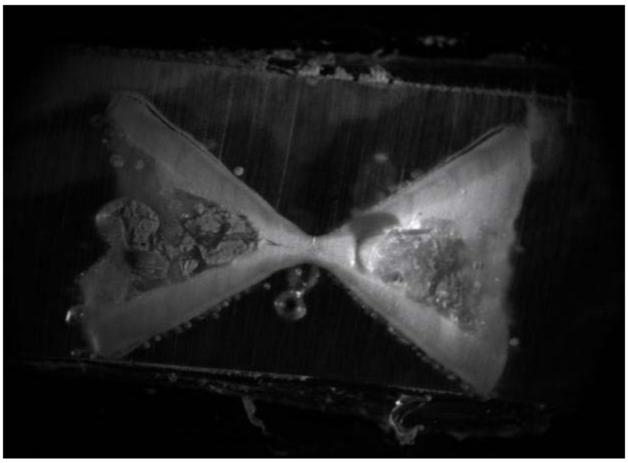


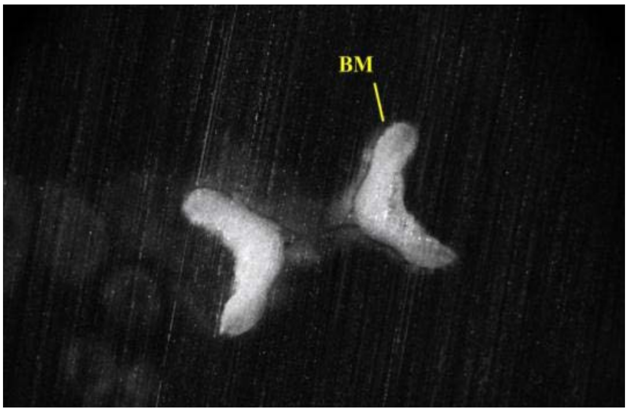
PLATE 136
Vertebral centrum of *R. brachyura* decalcified with EDTA



Non-stained sections were analysed and photographed with the same binocular under reflected light, using water as the clarification medium.

The better-defined annulus after the BM is considered the first year (Plate 137). Age is determined with a resolution of one year.

PLATE 137
Clear angle variation in the corpus calcareum of *R. brachyura*



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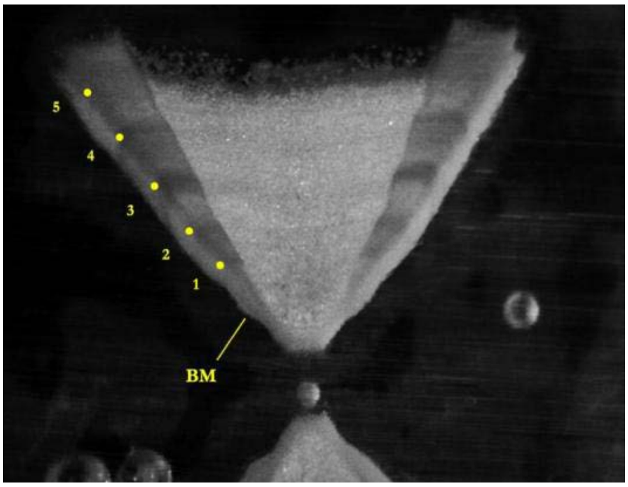
Note: Age 0, female, TL = 18 cm.

4.3.2.3 False and true annuli

The BM was always easily identifiable and growth bands relatively visible and unambiguous (Porcu *et al.*, 2015) (Plates 138 and 139).

Only bands clearly visible in all arms of the corpus calcareum were included in the reading.

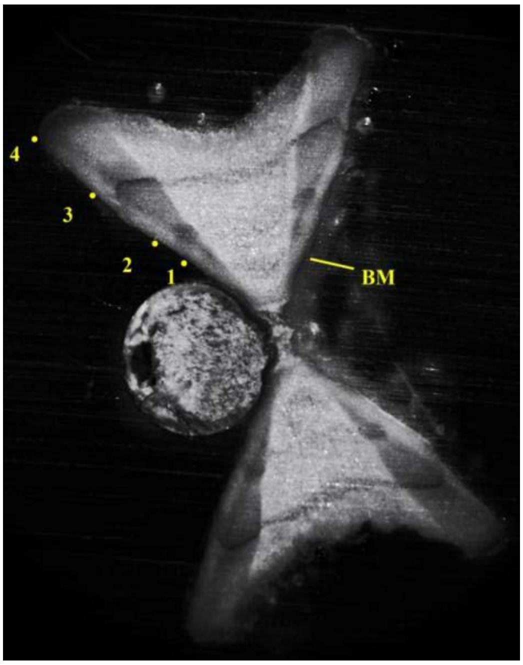
PLATE 138
BM identification and growth bands visibility in 5-year-old *R. brachyura*



© A. Bellodi, from Porcu *et al.*, 2015.

Note: Female, TL = 51.7 cm, vertebral radius = 2.21 mm.

PLATE 139
BM identification and growth bands visibility in a 4-year-old *R. brachyura*



© A. Bellodi

Note: Female, TL = 47.4 cm, vertebral radius = 1.88 mm.

4.3.3 *Raja clavata*

The thornback ray (*Raja clavata)* is a benthic species inhabiting sandy and muddy bottoms, but also rocky areas at depths from 20 to 700 m (Serena, 2005). It represents one of the most important components of demersal fisheries in most European waters and is taken by trawl and gillnet, particularly as bycatch (Ellis, 2005). Due to its large size (TL 110 cm) and wide distribution, it can be considered a common bycatch species of demersal fishery, with a moderate commercial interest also in the Mediterranean Sea (Serena, 2005; Relini *et al.*, 2010). In Italian waters, and particularly in Sardinia and in the Strait of Sicily, it represents, together with the small-spotted catshark (*Scyliorhinus canicula*), one of the most abundant elasmobranchs in terms of both density and biomass (Relini *et al.*, 2010).

Age and growth studies on this species through the count of growth bands have been conducted both on vertebral centra (Holden and Vince, 1973; Ryland and Ajayi, 1984; Fahy, 1991; Gallagher, Nolan and Jeal, 2005; Serra-Pereira *et al.*, 2008; Kadri *et al.*, 2014 among others) and caudal thorns (Serra-Pereira *et al.*, 2008), although the former are the most used.

The greatest source of bias for age estimation of this species is represented by the presence of a high number of annuli with strongly closed multiple bands, particularly marked in older individuals. Considering the spawning period (from winter to late spring, Holden, 1975) and the development of the embryo (about five months, Ellis and Shackley, 1995), for ageing purposes it is not possible to determine a sure birth date.

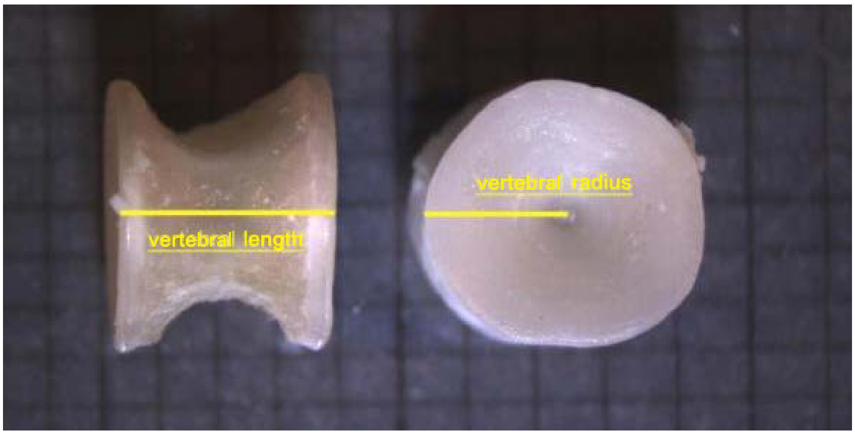
4.3.3.1 Extraction and storage

A portion of at least ten vertebrae is extracted from the thoracic region and frozen.

4.3.3.2 Preparation and interpretation

After separation and cleaning, vertebral centra are photographed and measured with a camera integrated in a binocular microscope, under reflected light (Plate 140).

PLATE 140
Main measurements on vertebral centra of *R. clavata*



© A. Mulas

Note: Male, TL = 71 cm.

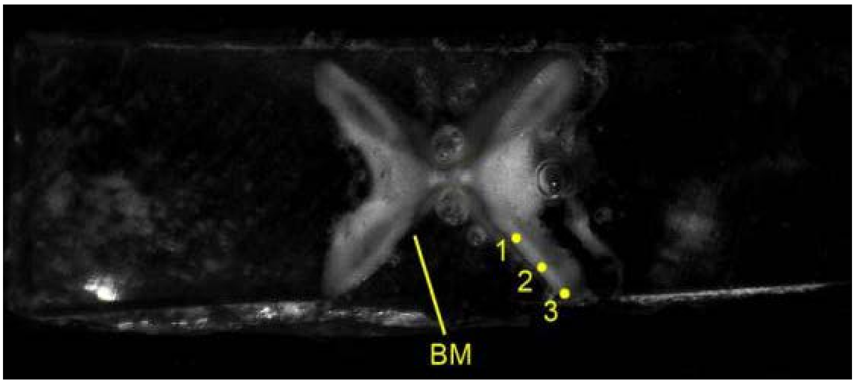
Some techniques have been suggested to enhance growth-band visibility. Gallagher, Nolan and Jeal (2005) used a modification of the crystal violet technique (Gallagher, 2000) as the staining method. Nevertheless, this technique is expensive in terms of time (it requires 36 hours). Serra-

Pereira *et al.* (2008) suggested immersion of the vertebral centra in a 5-percent EDTA solution (Gallagher and Nolan, 1999). This technique needs to be used with caution, because the opaque bands near the edge easily became translucent (Serra-Pereira *et al.*, 2008). Başusta *et al.* (2017), after comparing several staining techniques, reported silver nitrate as the most suitable.

Centra were embedded in epoxy resin, ground to obtain thin sections and then analysed and photographed unstained under reflected light, using water as the clarification medium.

The BM was always easily identifiable as a clear variation in the angle of the corpus calcareum (Plate 141).

PLATE 141
Clear angle variation in the corpus calcareum of *R. clavata*



© A. Mulas

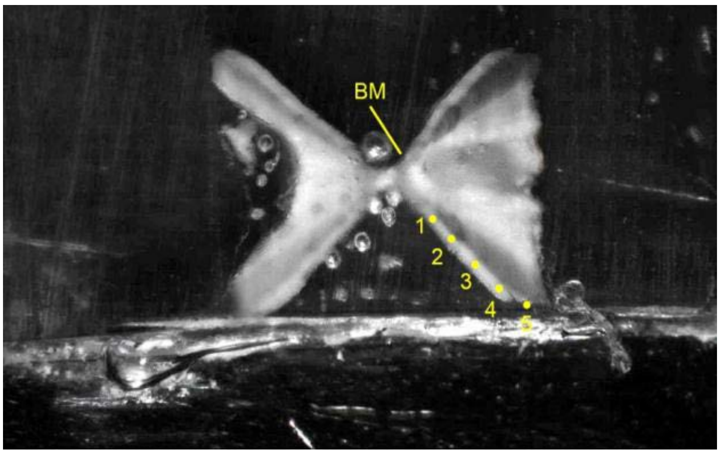
Note: Age 3 years, male, TL = 28.4 cm, vertebral radius = 0.67.

The better-defined annulus after the BM is considered the first year. Age is determined with a resolution of one year.

4.3.3.3 False and true annuli

The main source of bias for age interpretation of older individuals is represented by the presence of annuli with very close multiple bands (Plates 142 and 143). This problem can be easily solved by paying particular attention to the frequency with which the bands are repeated.

PLATE 142
Close growth bands in a 5-year-old *R. clavata*



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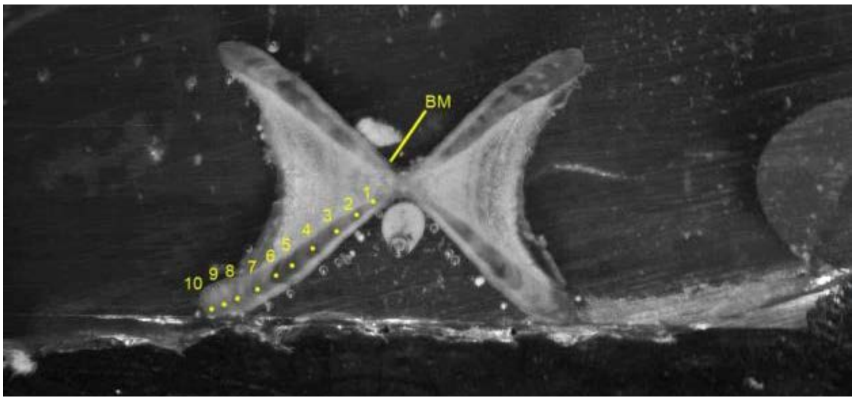
Note: Female, TL = 42.8 cm, vertebral radius = 0.86 mm.

PLATE 143
Close growth bands in a 10-year-old *R. clavata*



© A. Mulas

Note: Female, TL = 69 cm, vertebral radius = 2.41 mm.

4.3.4 *Raja polystigma*

The speckled ray (*Raja polystigma*) is a relatively small Mediterranean endemic batoid (TL 60 cm maximum) (Serena, 2005). It represents a moderately common species, widely distributed on the soft bottom of the continental shelves of the basin (although most concentrated in the central western part) (Serena, 2005). Because of its small size, *R. polystigma* is not directly targeted by fisheries (except along the African coasts), but represents a bycatch species in trawl nets (Ungaro *et al.*, 2009). Despite its wide distribution and relative abundance, it still represents, as do most elasmobranchs, a poorly studied species, particularly concerning age and growth.

Given that mature females are found mostly in autumn (Serena, 2005) and that knowledge of the time of embryonic development is scarce, a birth date is difficult to assess.

As with other elasmobranchs, the most important source of uncertainty for age reading is finding an effective staining method to improve growth-band visibility.

4.3.4.1 Extraction and storage

A portion of at least ten vertebrae is extracted from the thoracic region and stored frozen.

4.3.4.2 Preparation and interpretation

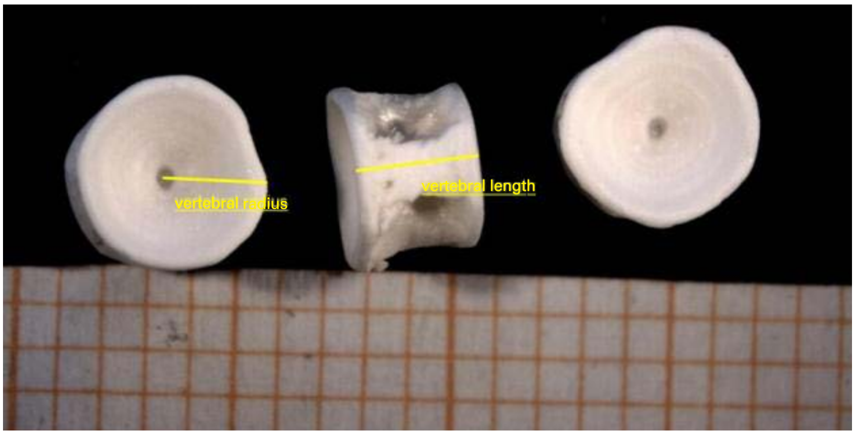
After separation and cleaning, vertebral centra are photographed and measured with a camera integrated into a binocular microscope, under reflected light (Plate 144).

Centra are embedded in epoxy resin and ground to obtain thin sections.

Comparisons of the EDTA decalcification process, staining with silver nitrate, alizarin red and the analysis of unstained samples showed that the latter two methods were the most suitable for enhancing growth-band visibility (Plates 145, 146 and 147) (Bellodi, 2015). Nevertheless, the alizarin red technique was the most effective in improving growth-band readability in older specimens (Bellodi, 2015). In many elasmobranchs, these bands represent the most important source of bias in age determination.

The silver nitrate technique offered no advantage, while the EDTA decalcification process even worsened band visibility (Bellodi, 2015).

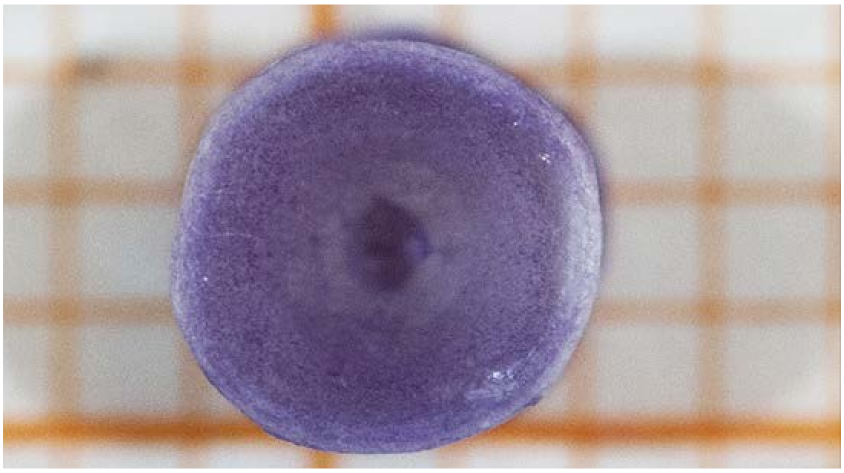
PLATE 144
Main measurements on vertebral centra of *R. polystigma*



© A. Bellodi

Note: Female, TL = 57.7 cm.

PLATE 145
Vertebral centrum of *R. polystigma* stained with alizarin red

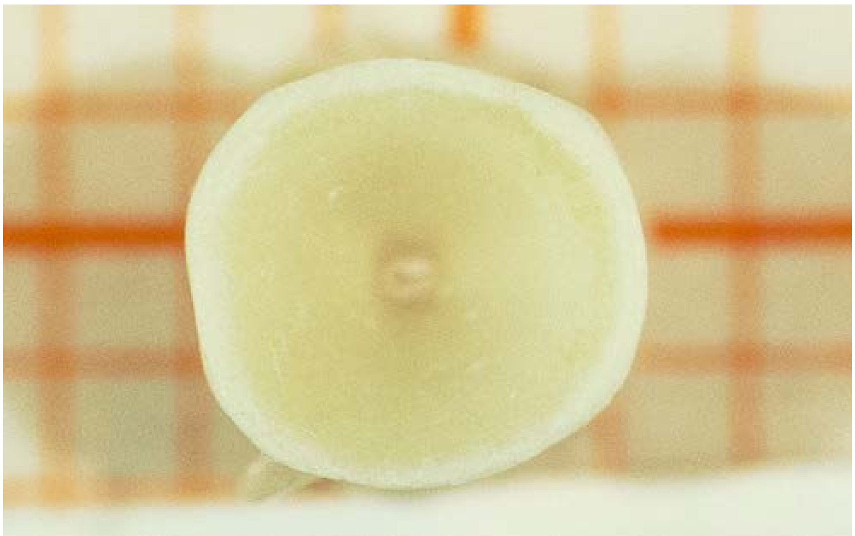


© A. Bellodi, from Bellodi *et al*., 2014

PLATE 146
Vertebral centrum of *R. polystigma* stained with silver nitrate

© A. Bellodi, from Bellodi *et al*., 2014

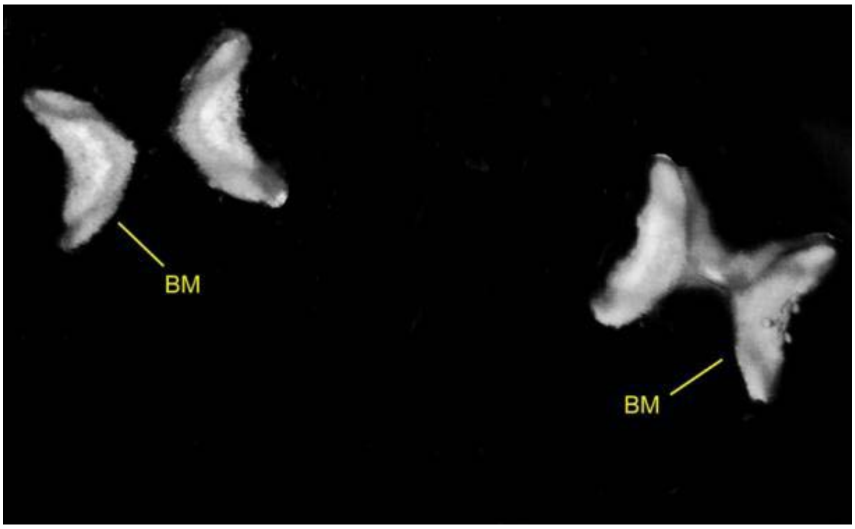
PLATE 147
Vertebral centrum of *R. polystigma* decalcified with EDTA



Sections were analysed stained with alizarin red and photographed with the same binocular microscope, under reflected light, using water as the clarification medium.

The better-defined annulus after the BM is considered the first year (Plate 148). Age is determined with a resolution of one year.

PLATE 148
BM identification in a 0-year-old *R. polystigma*

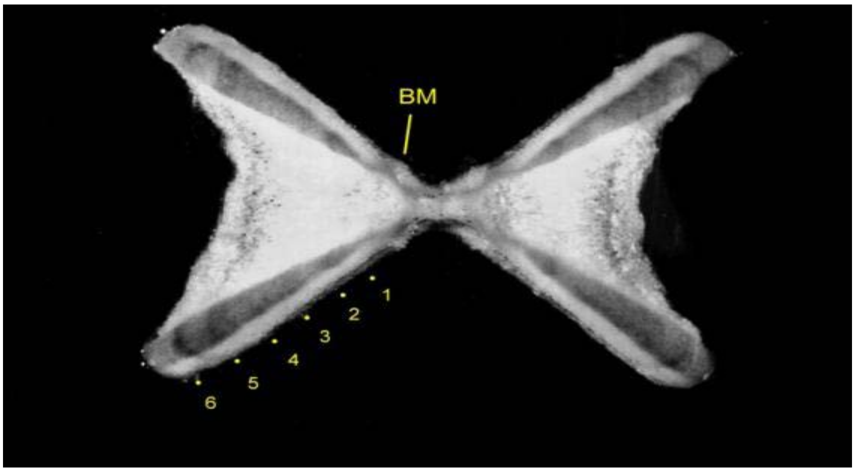


Note: Female, TL = 16 cm, vertebral radius = 0.57 mm.

4.3.4.3 False and true annuli

As with *D. oxyrinchus*, unlike other species, the significant presence of closed multiple bands was not registered. The BM was always easily identifiable and growth bands relatively visible and unambiguous (Plate 149) (Bellodi, 2015).

PLATE 149
BM identification and growth visibility in a 6-year-old *R. polystigma*



Note: Female, TL = 47.8 cm, vertebral radius = 1.75mm.

4.3.5 *Scyliorhinus canicula*

S. canicula is one of the most common elasmobranchs in the northeast Atlantic and Mediterranean Sea (Ellis *et al.*, 2009). It is a benthic small species (to about TL 50 cm) inhabiting sandy, muddy and gravelly bottoms from shallow water up to 550 m, although mainly present from 50 to 250 m (Serena, 2005). It can commonly be found in the bottom trawl fishery bycatch, representing an important part of chondrichthyan landings in Europe (Serena, 2005). In Italian waters, and particularly in Sardinia and in the Strait of Sicily, it represents, together with *Raja clavata*, one of the most abundant cartilaginous fish, both in terms of biomass and density (Relini *et al.*, 2010).

Taking into account that *S. canicula* spawns all year round, and that the incubation period lasts 9–11 months (Serena, 2005), it appears very difficult to assess a birth date useful for ageing purposes.

The greatest source of bias for age estimation of this species is the need to enhance growth-band visibility through staining techniques.

4.3.5.1 Extraction and storage

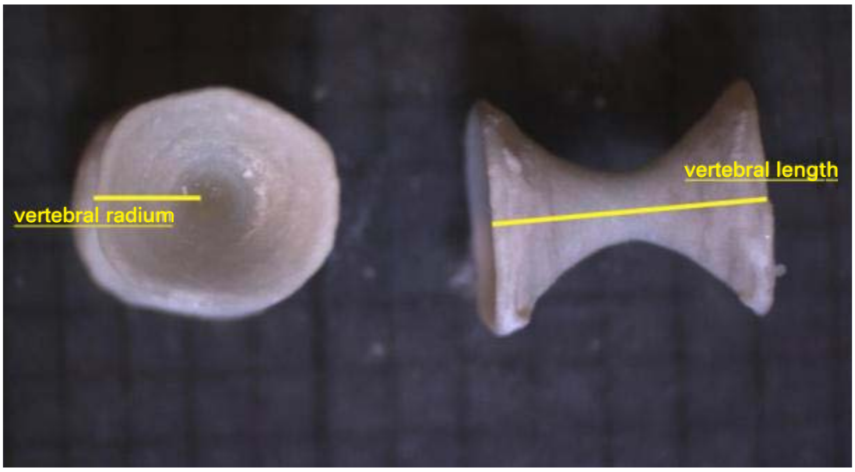
A portion of at least ten vertebrae is extracted from the thoracic region and stored frozen.

4.3.5.2 Preparation and interpretation

After separation and cleaning, vertebral centra are photographed and measured with a camera integrated into a binocular microscope, under reflected light (Plate 150).

Some techniques have been suggested for improving growth-band visibility (Ivory, Jeal and Nolan, 2004), while other authors have worked on unstained vertebral centra (Henderson and Casey, 2001). Ivory, Jeal and Nolan (2004), in particular, had very good results applying the crystal violet staining technique (Schwartz, 1983) modified (Ivory, 1999).

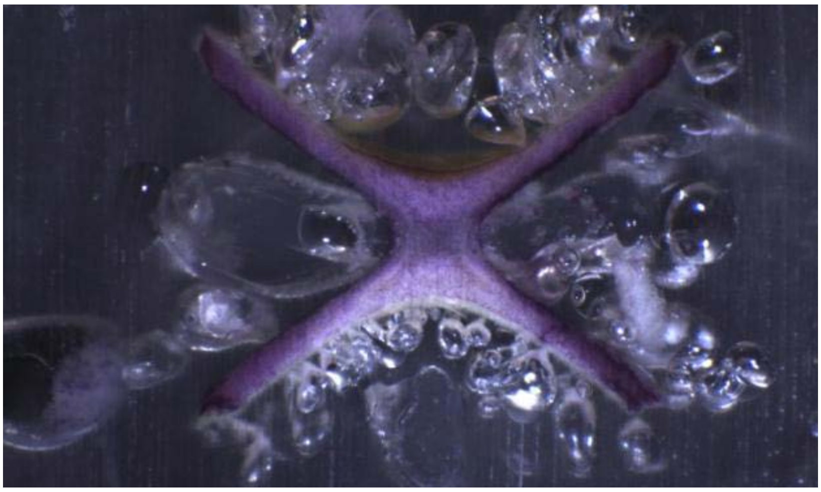
PLATE 150
Main measurements on vertebral centra of *S. canicula*



Note: Male, TL = 44 cm.

Centra are embedded in epoxy resin, ground to obtain thin sections and then photographed under reflected light, using water as the clarification medium.

PLATE 151
Vertebral centrum of *S. canicula* stained with alizarin red



Samples were analysed unstained, while a part was stained with alizarin red to test the effectiveness of this relatively inexpensive technique (Plate 151).

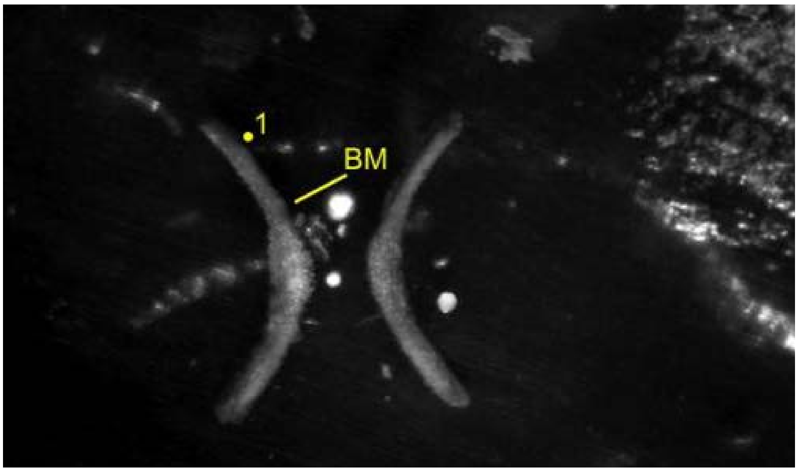
While unstained samples were relatively easy to read, those stained with alizarin red were confusing and unreadable (Plate 152).

Annual growth increment was verified for this species by Ivory, Jeal and Nolan (2004). The better-defined annulus after the BM is considered the first year (Plate 152). Age is determined with a resolution of one year.

4.3.5.3 False and true annuli

The significant presence of close, multiple growth bands was not registered. The BM was always easily identifiable and growth bands relatively visible and unambiguous (Plate 153).

PLATE 152
BM identification in a 1-year-old *S. canicula*



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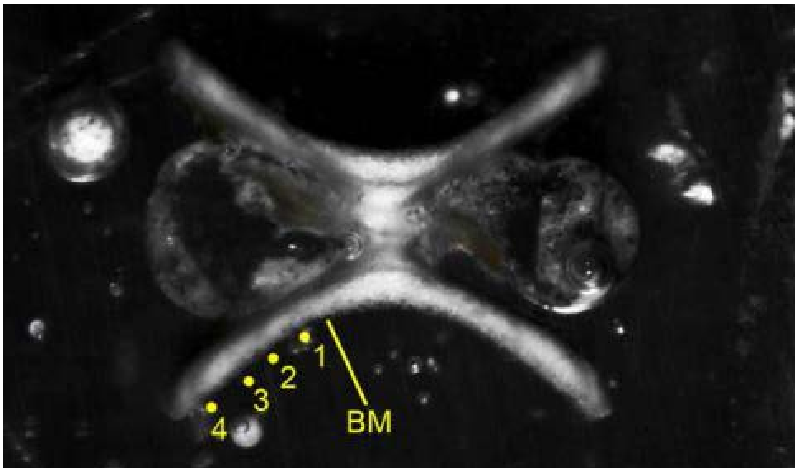
Note: Male, TL = 23.2 cm, vertebral radius = 0.7 mm.

PLATE 153
BM identification and growth band visibility in a 4-year-old *S.canicula*



© A. Mulas

Note: Male, TL = 29 cm, vertebral radius = 1 mm.

5. Large pelagic species

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The evaluation of other fishing resources – such as small pelagic, demersal species or bivalve molluscs – is carried out at a national or regional level (i.e. GSAs). Given that large pelagic fish stocks are exploited by diverse countries over a wider area, stock assessments and management measures are conducted under the umbrella of the International Commission for the Conservation of Atlantic Tunas (ICCAT), whose decisions are then implemented by all contracting parties.

The ICCAT Scientific Committee uses several stock assessment models: virtual population analysis (VPA); extended survival analysis (XSA); and catch-based models: catch maximum sustainable yield (CMSY); stock synthesis (SS3) based on catch; catch at size (CAS); and statistical catch at age (SCAA) data. These are coupled with biological parameters, such as length at first maturity (L_{50}), growth, natural mortality, etc. In this context, collection of data on age and growth becomes fundamental (i.e. for EU countries, it is part of the obligations of the DCF (Council Regulation [EC] No. 199/2008 and Regulation [EC] No. 665/2008).

This section will refer mainly to the most-important large pelagic species exploited in the Mediterranean: *T. thynnus*, *X. gladius*, *T. alalunga*, *S. sarda* and *C. hippurus*. Samples are collected monitoring the fishing activities of the following main gear:

1 drifting longline
2 purse seine
3 trap
4 fish-aggregating device (FAD)
5 harpoon, where this fishery is still active

5.1 Sampling

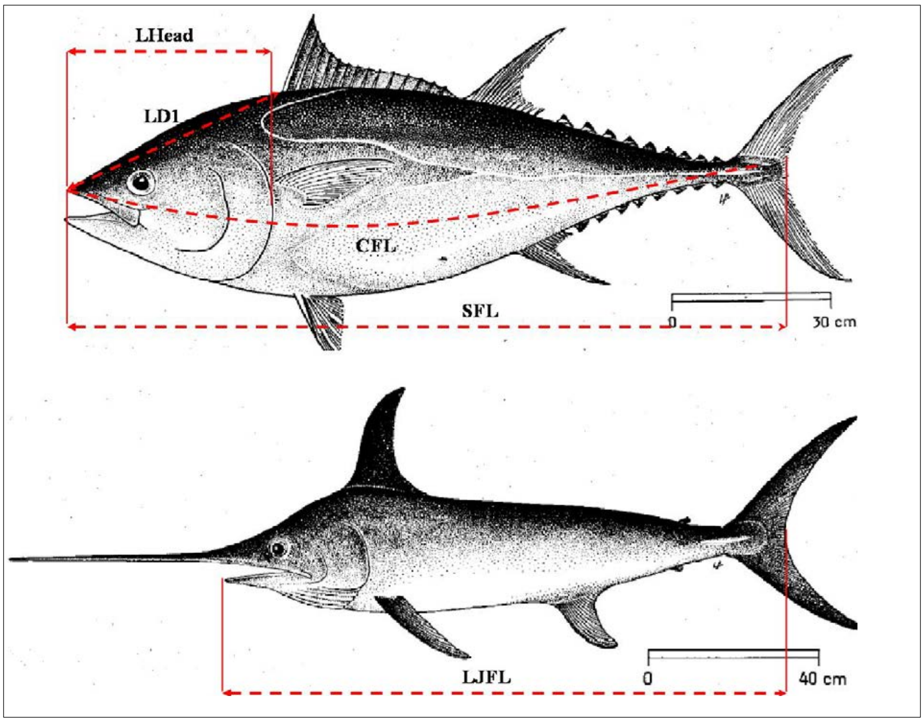
5.1.1 Data collection

The standard length measurement used in ICCAT is the fork length (FL), which is the distance from the tip of the upper jaw to the fork of the caudal fin. For Istiophoridae and *X. gladius*, this measurement is taken from the lower jaw to the caudal fork (lower jaw – fork length [LJFL]). FL and LJFL should always be measured following a straight line (i.e. straight fork length [SFL]) (Plate 154).

ICCAT conversion factors provide the opportunity to collect measurements alternative to the SFL. For instance, for *T. thynnus* it is feasible to convert to SFL from curve fork length (CFL, taken following the fish body curvature), length to the first dorsal fin (LD1) or length of the head (LHead) (Rodríguez-Marín *et al.*, 2015). If possible, it's always better to collect SFL (Plate 154).

Length/frequency (L/F) data are grouped in 5-cm intervals for *T. thynnus* and *X. gladius* and in 2-cm intervals for *T. alalunga*, *S. sarda*, *C. hippurus* and Istiophoridae.

PLATE 154
Indication of the main length measurements to be taken for large pelagic species



In addition to size, the essential data to be collected for age analysis are catch date (day/month/year) and sex, especially for species with a marked sexual dimorphism (e.g. *X. gladius*).

Less important for age determination, but to be collected when possible, are: weight (round or gutted), fishing area and gear, and port of landing.

The following paragraphs describe the main and more-appropriate methodologies adopted for direct age determination in large pelagics, with particular reference to the two most-studied species, *T. thynnus* and *X. gladius*.

In addition to the instructions derived from ICCAT manuals, other methods reported in the scientific literature could be adopted for large pelagic species, such as length/frequency distribution (LFD) analysis. This last method will not be presented in detail in the text, given that this is an operational manual on hard structures. For completeness, however, we will refer to papers in the literature in which the analysis of cohorts was used, both to estimate the age classes of diverse species and for validation of studies carried out on skeletal structures.

5.1.2 Structures to be sampled

The main calcified structures used in age analysis of the target species of this study are four: otoliths, fin spines, vertebrae and scales (Campana, 2001; Rodríguez-Marín *et al.*, 2007). In Table 17, the advantages or disadvantages related to sampling, preparation and age interpretation of the various structures are reported schematically.

Despite the fact that scales could easily be removed without killing the specimen (non-lethal sampling), they are less-reliable structures for age interpretation, in particular in long-lived species. In fact, they are not always present in all species (e.g. adult *X. gladius*) and are commonly subject to regeneration. These structures have been adopted in past studies carried out on *C. hippurus* and *T. alalunga* (Arena, Potoschi and Cefali, 1980; Cefali *et al.*, 1986; Megalofonou, 1990; Megalofonou, Yannopoulos and Dean, 2003; Massutí, Morales-Nin and Moranta, 1999; Morales-Nin *et al.*, 1999), but the technique is not taken into account in ICCAT sampling protocols (Ruiz, Rodríguez-Marín and Landa, 2005; Rodríguez-Marín *et al.*, 2007, 2012; Ortiz de Zárate, Valeiras and Ruiz, 2007). For this reason, it will be discussed only marginally in the following paragraphs.

5.1.3 Sampling, extraction and storage

The structures traditionally sampled in large pelagic species are otoliths, fin spines and vertebrae. The first difficulty is in obtaining samples. It is possible to sample fish on board or at landing, but, when possible, collection of all the structures from each specimen is always recommended. This can be useful in comparing results obtained from the various hard parts (Ruiz, Rodríguez-Marín and Landa, 2005; Ortiz de Zárate, Valeiras and Ruiz, 2007; Rodríguez-Marín *et al.*, 2012).

TABLE 17 – Advantages and disadvantages of diverse calcified structures

Structure	Otoliths	Spines	Vertebrae	Scales
Used	Yes, useful in all species	Yes	Yes	Not used in all species (see text)
Sampling	Difficult to acquire the sample (necessary to buy whole fish or, at least, head)	Not necessary to buy whole fish and minimal damage to individual	Damage to fish; difficult to acquire sample (necessary to buy whole specimen)	Not necessary to buy whole fish and non-lethal
Extraction	Difficult to extract in some species (*Xiphias gladius, Coryphaena hippurus*)	Easy to extract	Easy to extract	Easy to extract and not lethal to fish
Preparation	Time consuming and expensive preparation, especially for species with small otoliths (i.e. Istiophoridae*, Coryphaena hippurus*)	Easy to prepare for age reading	Time-consuming preparation for staining; attention to conservation	Easy to prepare for age reading
Interpretation	Difficult interpretation of growth bands (false rings, etc.) and possible overestimation	Resorption of inner growth bands in some species (underestimation); presence of false rings (overestimation)	Difficult interpretation of growth bands (false rings, etc.)	Subject to regeneration and loss (possible underestimation)
Advantage	Good representation of the entire life-cycle (birth to death); not exposed to natural external factors (breakage, consumption); ideal composition for other kinds of analysis (microelements, isotopes, etc.)	Easy to sample, extract and prepare; good for short-lived species	Good alternative when other structures not available; good for short-lived and fast-growing species	Easy to sample, extract and prepare; good for short-lived species
Disadvantage	Difficult to sample, extract and interpret, in particular in Istiophoridae and *Coryphaena hippurus*	Not good for long-lived species (possible underestimation); possible damage to the sample (broken condyle)	Not good for long-lived species (possible underestimation)	Not useful for all species (i.e. *Xiphias gladius*); variability depending on body area sampled; less representative for long-lived and slow-growing species

5.1.3.1 Otoliths

Otoliths are the most reliable structures, because they represent the entire life cycle of a specimen and are not exposed to natural external factors (breakage, consumption, regeneration, etc.). However, these advantages are sometimes overcome by the problems encountered in sampling, extraction, preparation and interpretation of the bands, which lead to increased time and costs required for age interpretation.

It is useful to remember that otoliths in large pelagic fishes are generally small (or very small), so extraction methods can be different depending on species and size. For large individuals, given that buying a whole fish is really expensive, its processing can be followed to recover the entire head or directly the otoliths.

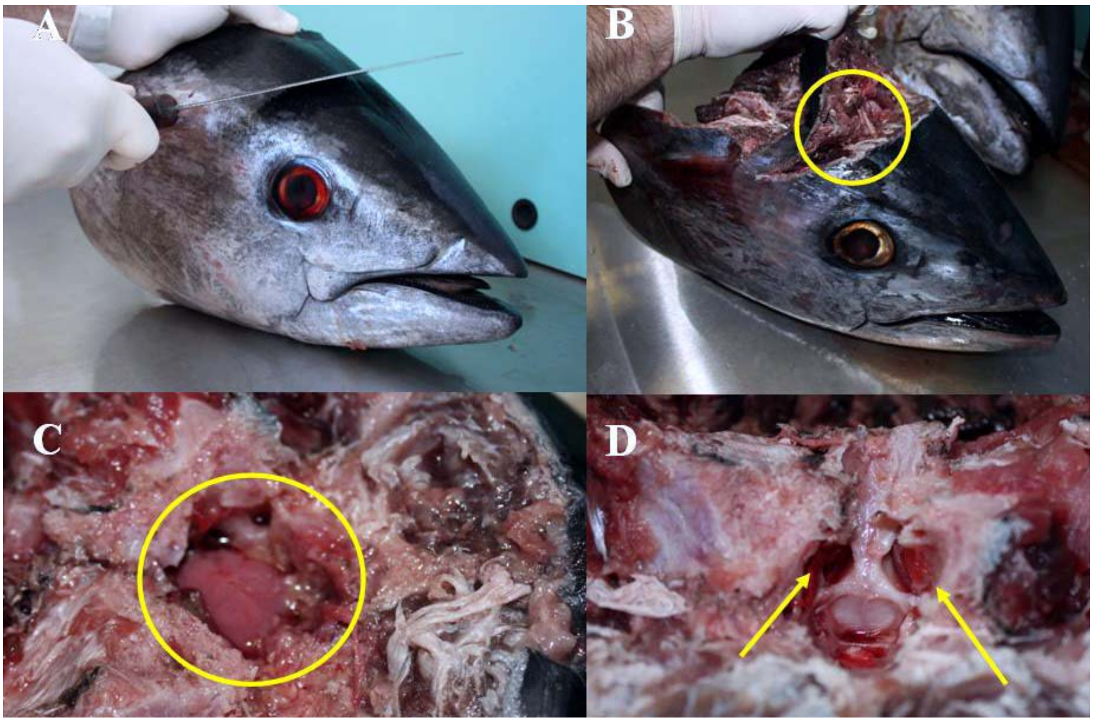
Otolith extraction is performed more easily on a fresh sample. If the samples have been preserved frozen, they must be completely thawed before extraction to avoid risk of damage to the otoliths.

The methods described below can all be applied with success, depending on the experience of the single samplers and the size of the fish.

The head can be cut on a longitudinal plane (horizontally or vertically) or on a transverse plane.

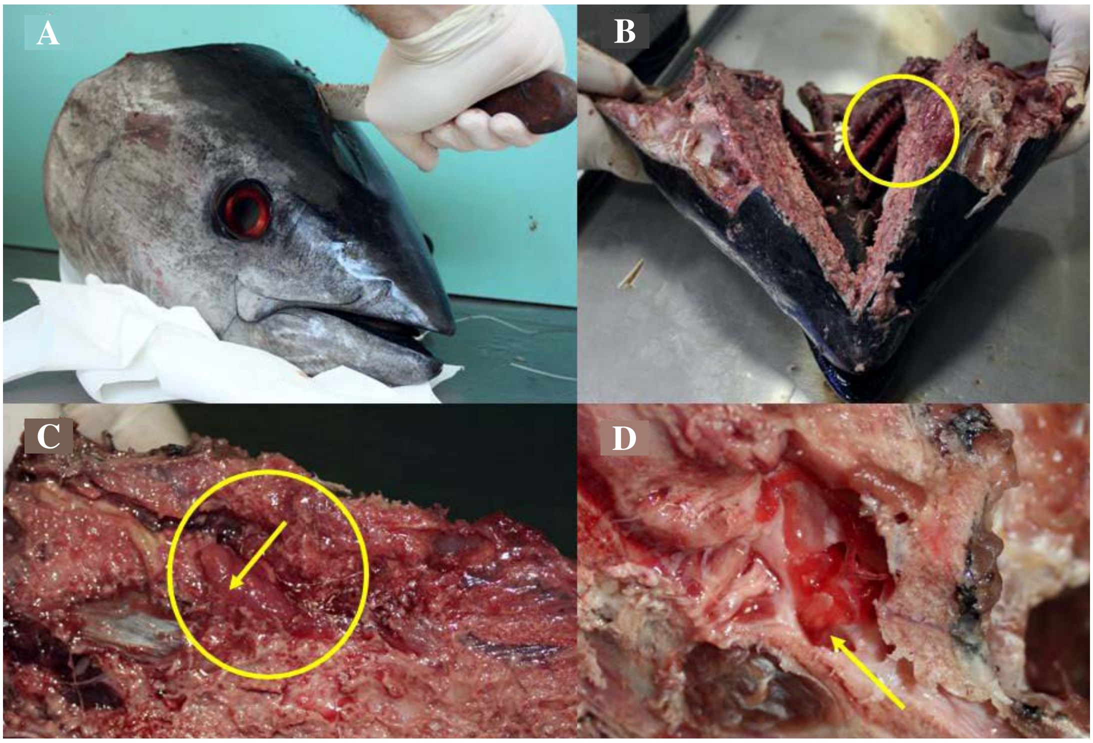
For *T. thynnus* and all other tuna-like species, the most common method involves cutting the head on a horizontal-longitudinal plane, above the orbital ridge (Plate 155), taking as a reference point half the distance from the posterior margin of the orbit to the preoperculum. Once the skull is uncovered (Plate 155B) and the brain carefully removed (Plate 155C), the semicircular canals will be immediately exposed. Generally, in all tunas (large and small), direct extraction of the sagittae is preferred. These are found in the two lateral cavities at the base of the skull (Plate 155C) and are visible to the naked eye (Plate 155D).

PLATE 155
A – *T. thynnus* otolith extraction cutting on a longitudinal plane (horizontally); B – exposure of the brain cavity; C – individuation of semicircular canals and the two cavities at the base of the skull; D – sagittae visible inside the two cavities



An equally effective technique consists in making the cut vertically on the longitudinal plane (sagittal plane), exactly at the midpoint of the head, between the eyes. This exposes the two sides of the cranium, and the otoliths can be easily identified in the two cavities (Plate 156).

PLATE 156
T. thynnus otolith extraction cutting on the sagittal plane. Yellow circles indicate the area where otoliths are placed; arrows indicate the precise location of the sagittae



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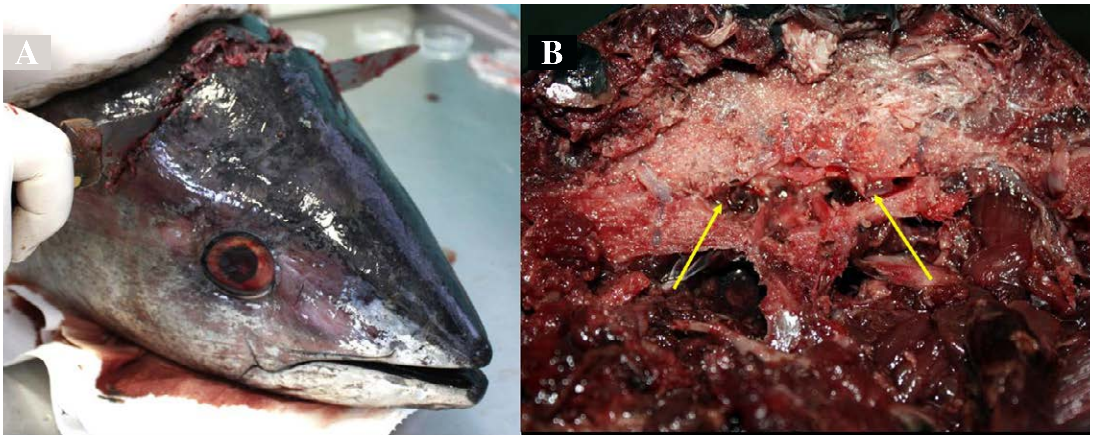
A third method consists in cutting the head along a transverse plane, halfway between the posterior margin of the eye and the preoperculum, reaching the cavities from behind. It is advisable to employ this technique for larger specimens (Plate 157).

PLATE 157
T. thynnus otolith extraction cutting on a transverse plane; arrows indicate the two sagittae inside the cavities



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Another method, exclusively adopted when the head has to be preserved intact due to its high commercial value (e.g. in the Japanese market), consists in removing a core containing the sagitta from the cranium using an electric drill provided with a hole-saw (Anon., 2002). This technique is less reliable and more subject to otolith damage, especially when carried out by inexperienced samplers.

The same methods can generally be applied to billfish. Unlike in tuna, in billfish (*X. gladius*, Mediterranean spearfish [*Tetrapturus belone*]) and *C. hippurus*, otolith extraction can, preferably, be made according to the 'classic' cut (i.e. horizontal plane) above the orbital ridge. Extraction is done by completely removing the semicircular canals, due to the extremely small size of the otoliths, which are not visible to the naked eye (Plate 158). This is fairly easy in *X. gladius*, where the canals can be clearly identified and carefully extracted. In other Istiophoridae and *C. hippurus*, the canals are fully embedded inside the surrounding connective tissues, and the membrane containing the otoliths (sacculus) has a tendency to break, with the risk of losing the otoliths. Thus it is advisable, for these species, especially for small individuals, to perform the extraction under a stereomicroscope (or using a binocular lens magnifier or a magnifying lamp).

After extraction, the otoliths will be placed immediately in a Petri dish containing deionized water. Immersion in deionized water is necessary to facilitate the removal of organic materials attached to the otoliths surface and to avoid contamination with elements contained in running water, in case the otoliths will be used for microchemistry studies.

At this point, they must be perfectly cleaned of any organic matter, through immersion for a few minutes in 0.1 percent nitric acid or hydrogen peroxide, and then rinsed again in deionized water to remove any traces of acid (Rooker *et al.*, 2008).

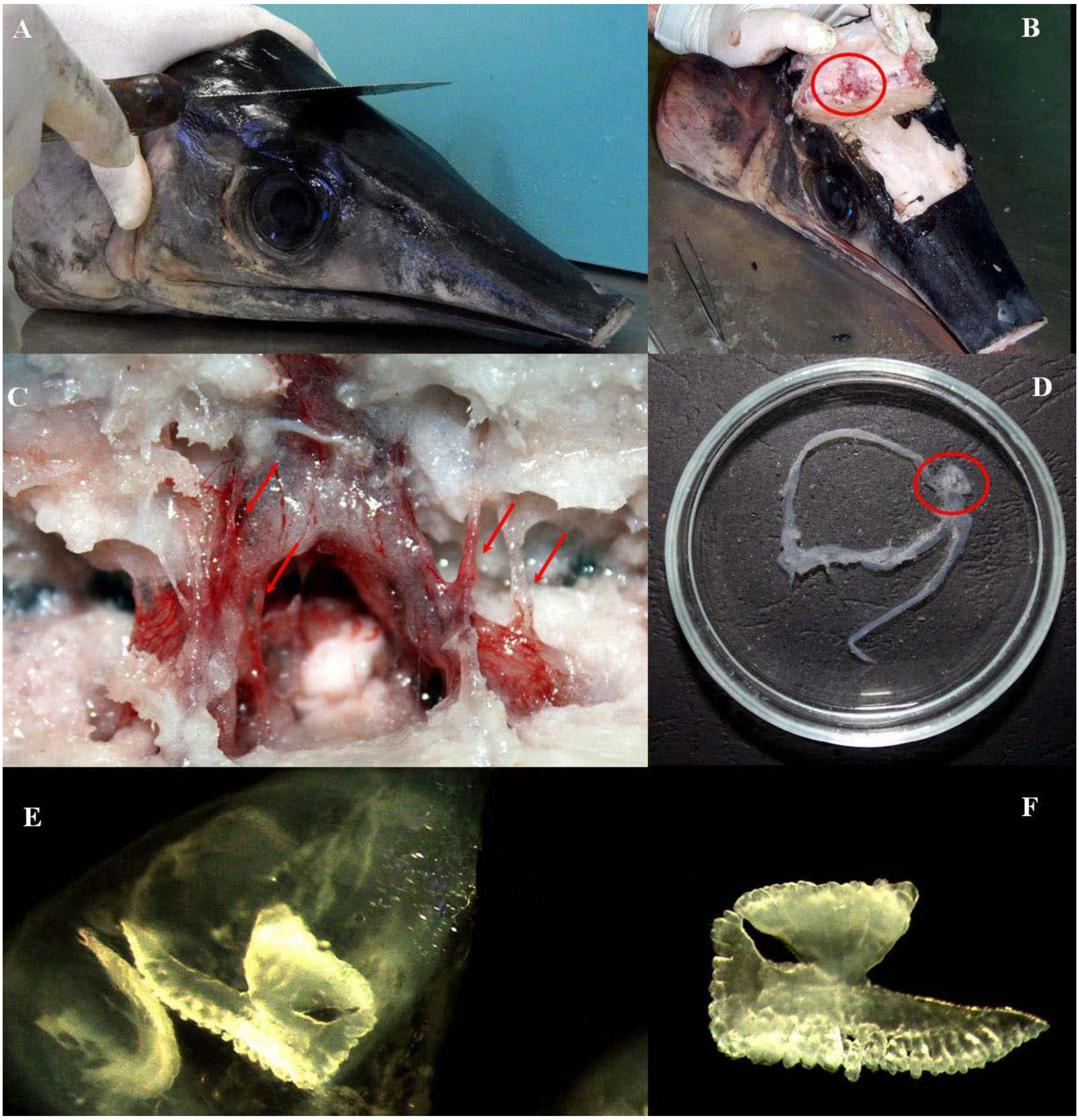
Once cleaned, the otoliths should be perfectly dried under a fume hood for 24 hours, to avoid the formation of a browning film on the surface of the structure, before being stored dry in a plastic tube, with a label reporting the ID code of the specimen, date of capture, length, sex, etc.) (Ruiz, Rodríguez-Marín and Landa, 2005; Rodríguez-Marín *et al.*, 2007; Rooker *et al.*, 2008).

5.1.3.2 Spines

Spines (fin rays) are the most used and easiest to sample structures for a large pelagic fish; their removal will only partially damage the specimens, in comparison with other structures (otoliths and vertebrae).

The spines usually used for ageing analysis differ depending on the species:
- For tunas (*T. thynnus, T. alalunga, S. sarda*), the structure to be taken is the first ray of the first dorsal fin.
- For *X. gladius*, it is the second ray (or the last unbranched ray) of the anal fin.
- For other Istiophoridae, as a protocol hasn't been established, either the dorsal fin ray or the anal fin ray can be used (see subsection 5.4.3); specifically, the first three spines of the dorsal fin (D 1–3) or the second/third spines of the anal fin (A 2–3) should be collected.
- For *C. hippurus*, the sampling of otoliths or scales is preferred, instead of spines (Massutí, Morales-Nin and Moranta, 1999; Morales-Nin *et al.*, 1999).

PLATE 158
Otolith extraction in *X. gladius*: B – brain cavity (red circle); C – semicircular canals exposed (red arrows); D, E, F – semicircular canals in Petri dish: the red circle indicates sacculus and lagena containing, respectively, sagitta and asteriscus

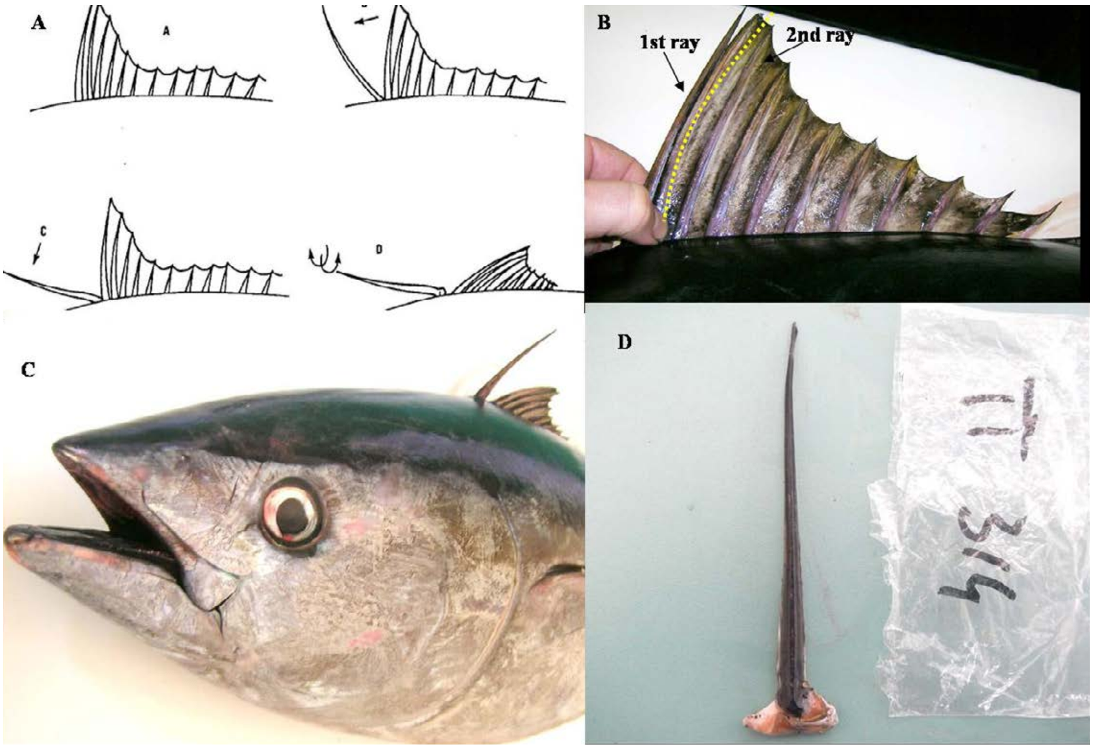


It is important to extract the whole spine (dorsal or anal), including the condyle at the base, which is an essential reference point for preparation of the sections (see subsection 5.2.2).

In tuna-like fish, a scalpel or a knife can be used to cut the membrane joining the first and second dorsal fin rays. Then the ray must be carefully pushed forward, rotating it alternately left and right, until complete breakage of the ligaments (tendons) supporting the ray (Plate 159) (Compean-Jiménez and Bard, 1980; Ruiz, Rodríguez-Marín and Landa, 2005; Ortiz de Zárate, Valeiras and Ruiz, 2007).

PLATE 159

T. thynnus fin ray or spine extraction: A – scheme of the sampling procedure (from Compean-Jimenez, 1980); B – *T. thynnus* dorsal fin; C – spine isolated; D – spine immediately after removal



The second anal fin ray is currently considered the best structure for direct ageing analysis of *X. gladius* and other Istiophoridae, as demonstrated by many studies carried out globally, including of the Mediterranean Sea (Berkeley and Houde, 1983; Estevez *et al.*, 1995, Tserpes and Tsimenides, 1995; Ehrhardt, Robbins and Arocha, 1996; Orsi Relini *et al.*, 1996a, 1999; Aliçli and Oray, 2001; Sun, Wang and Yeh, 2002; Rollandi *et al.,* 2004; De Martini *et al.*, 2007; Valeiras *et al.*, 2008a; Kopf, Drew and Humphreys, 2010; Quelle *et al.*, 2014). It is advisable to remove the entire anal fin in the field (taking care not to damage the condyles at the base) and, later on, carefully extract the second ray in the lab, given the particular shape and morphology of the anal fin (Plate 160). In fact, the best structure to use doesn't always correspond to the second ray, counted in the cephalocaudal direction, for the possible presence/absence of a first small ray; it is always considered good practice to sample the last unbranched ray (Quelle *et al.*, 2014). Otherwise, the anterior half of the fin could be sampled in the field, using a technique similar to that used for extracting tuna spines.

Once sampled, the ray should be cleaned as soon as possible, when the connective tissues and soft parts are still fresh and can be easily removed. If this is not possible and samples are stored frozen or dry, it is advisable to rehydrate them before processing with a quick passage in warm water. During cleaning, avoid removing the most peripheral part of the bone tissue, scraping too deeply into the surface, which could result in loss of the outer margin of the spine.

Once cleaned, rays must be dried for at least 24–48 hours, making sure they are completely dry before being placed with the identification codes (date, length, sex, etc.) in a paper or perforated plastic bag, to prevent mould from appearing.

PLATE 160
Extraction of the 2nd anal fin spine of *X. gladius*

5.1.3.3 Vertebrae

Vertebrae are considered an alternative calcified structure for ageing analysis; caudal vertebrae are generally sampled, because the tail is often discarded in individuals dressed for market.

T. thynnus has 39 vertebrae, including 18 precaudal and 21 caudal; sampling must be done on the 35th and 36th vertebrae. This sampling protocol, based on the study carried out on the 35th vertebra by Berry, Lee and Bertolino (1977) and Farber and Lee (1981), has been proposed for the ICCAT sampling protocols by Ruiz, Rodríguez-Marín and Landa (2005).

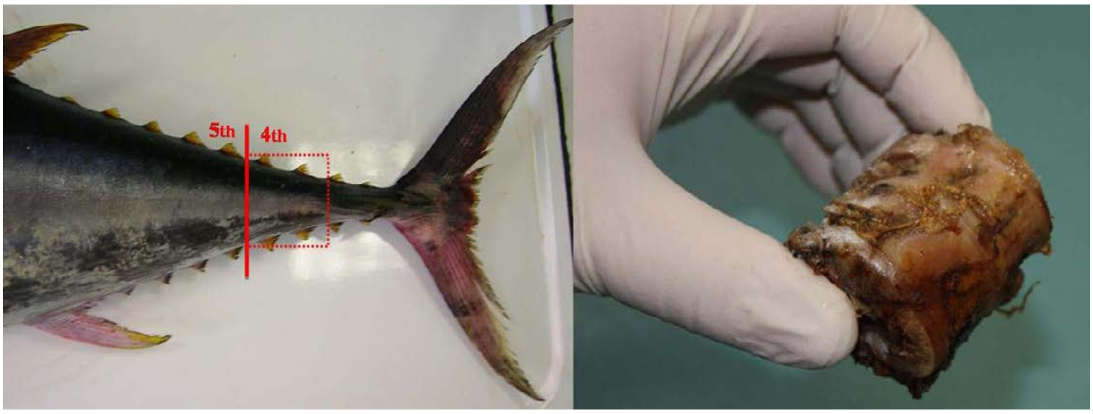
To find the 35th vertebra, a cross-cut should be done between the fourth and the fifth finlet of the caudal area, starting from the tail (Plate 161). It is advisable to process them within 48 hours and to not separate the two vertebrae until preparation, in order to avoid the tissue drying out (Berry, Lee and Bertolino, 1977, Farber and Lee, 1981; Ruiz, Rodríguez-Marín and Landa, 2005; Rodríguez-Marín *et al.*, 2007). If this is not possible, the vertebrae should be stored frozen.

For *T. alalunga*, Fernandez (1992) indicated the 27th vertebra as the best choice, while Lee and Yeh (1993, 2007) removed the 38th vertebra for their studies.

As no official protocol has been established for sampling the vertebrae for *S. sarda* and *C. hippurus*, the removal of at least the last five caudal vertebrae is recommended.

Studies on age determination of billfish (*X. gladius* or other Istiophoridae) using vertebrae are rare. Estevez *et al.* (1995) found that 56 percent of the first cervical vertebra for *X. gladius* were readable, but only 28 percent (APE [average percent error]) of the readings provided reliable results.

PLATE 161
Cutting area to find the 35th and 36th vertebrae in *T. thynnus*



5.2 Preparation of structures

5.2.1 Otoliths

Age estimation can be done analysing the whole otolith, mainly under reflected light, or a thin section of the otolith under either reflected or transmitted light. As stated before, otoliths of large pelagics are very different in size, thus affecting the choice of the most appropriate technique. When the growth marks represent age in years, they are referred to as annual marks or annuli, and when they represent age in days they are referred to as daily increments.

Age estimation based on observation of the whole otolith gives the best results when applied to juvenile *T. thynnus* (up to five to seven years) (Rodríguez-Marín *et al.*, 2007), *X. gladius* and *S. sarda* (Ateş, Deval and Bok, 2008), given that the small size of the sagitta in these two latter species could create difficulties in preparing good readable sections. Nevertheless, some studies were performed on thin sections of otoliths also for larvae (Brothers *et al.*, 1983; García *et al.*, 2013; Malca *et al.*, 2017) and for analysis of daily increments in very young *X. gladius* (Megalofonou, Dean and De Metrio, 1991, 1995), *T. alalunga* (Laurs, Nishimoto and Wetherall, 1985; Farley *et al.*, 2013), *T. thynnus* (Radtke and Morales-Nin, 1984; La Mesa, Sinopoli and Andaloro, 2005) and *S. sarda* (Santamaría, Deflorio and De Metrio, 2005). Alternatively, for *X. gladius*, analysis may also be performed on the whole otolith, as described by Estevez *et al.* (1995). The whole otolith can be immersed in water, oil or glycerol for clarification or, alternatively for *T. thynnus* and other tuna-like fish, burned until golden brown, to improve the contrast between the translucent and opaque (browner) bands.

Otolith sectioning, transverse or oblique, is preferred for large *T. thynnus* (> five years). The following otolith preparation is thus based mainly on the methodology described in the ICCAT report of the workshop on *T. thynnus* direct ageing analysis (Rodríguez-Marín *et al.*, 2007) and can also be applied for *T. alalunga* (Chen and Holmes, 2015).

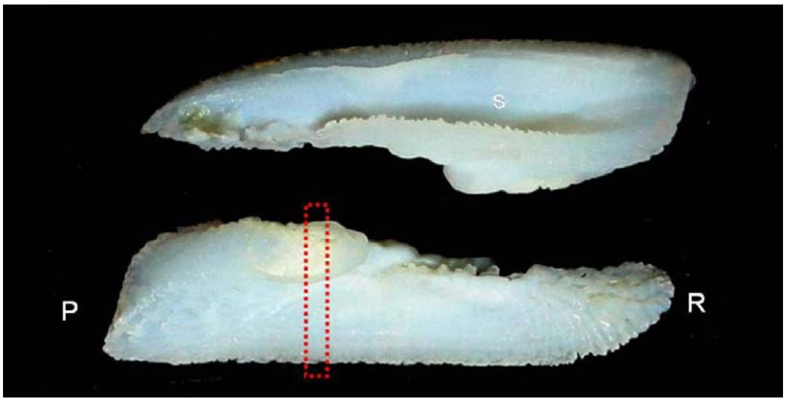
The otolith is embedded in a solution of epoxy resin and hardener, in different proportions (e.g. 5:1 or 5:2) depending on the factory/producer. Once mixed, the solution is left to rest for five to ten minutes. A reduced amount of compound should be prepared (50–100 ml from time to time) to prevent the possible formation of air bubbles in the mixture, which could negatively affect the success of the preparation, and for better handling and management of the solution. Generally, a first layer of the mixture is laid on silicon moulds (alternatively, a light coat of releasing agent can be applied, for example paraffin wax), on which the otoliths are gently laid down, keeping the sulcus acusticus side up. Before covering the otoliths with a second layer, it is good practice to insert a label with the ID code of the specimen inside. The preparation is then left under a fume hood until completely hardened (24–72 hours).

The section is performed along a transverse plane using a low-speed saw with a diamond blade, passing through the central core (primordium) of the otolith (Plate 162). The section located in this area shows its typical Y shape (in *T. thynnus* and *T. alalunga*) (Plate 163 – upper). One to two serial Y-shaped sections should be produced for readings, not moving too much along the rostrum. Sectioning out of the primordium produces V-shaped sections (Plate 163 – lower), which are not suitable for readings. In fact, they could lead to a possible loss of information, resulting in underestimation of age (Secor *et al.*, 2014).

The section thickness can be in the range of a mean value of 0.7 mm, which represents a good compromise for readability. Thicker sections will not be readable under transmitted light, while thinner sections can reveal too much detail (false bands, subannual bands) that could generate confusion in interpretation of the bands (Rodríguez-Marín *et al.*, 2007).

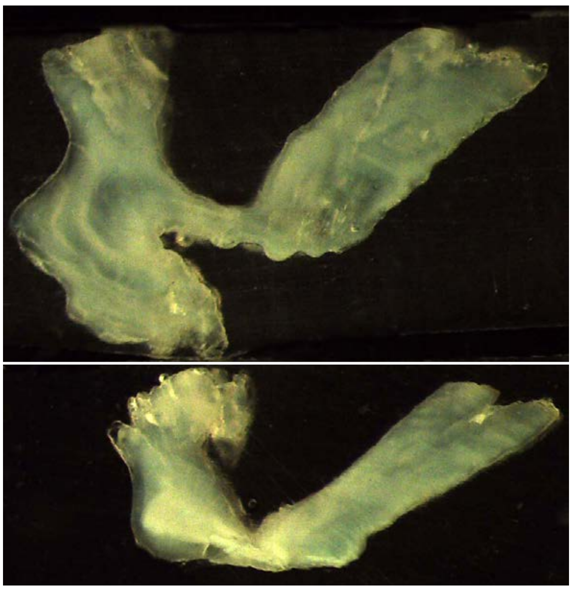
The sections can be further polished with alumina paste and mounted on a glass slide for reading under the stereomicroscope.

PLATE 162
Correct location of the cross section (in red) for a *T. thynnus* otolith



Note: R: rostrum; P: postrostrum; S: sulcus acusticus.

PLATE 163
Examples of Y-shape (upper) and V-shape (lower) sections of a *T. thynnus* otolith



For Istiophoridae and *C. hippurus*, the procedure is slightly different, given the small size of the otoliths and their consequent structural fragility. The sagitta is embedded, preferably in a temporary mounting product (resin or polyester resin) whose hardening requires a longer time. The otolith, after mounted on a slide, can then be polished (on one side and then on the other) along the sagittal plane (e.g. *C. hippurus*) or along the transverse plane (*X. gladius*, *C. hippurus*). This activity is generally carried out through a grinder/polisher, with sandpaper sheets decreasing in grain size (from 10 to 2 μm), moistened with cold water. The process is completed by performing a further polishing with a solution of alumina paste (0.3 μm) to remove abrasions (Radtke, 1983; Wilson and Dean, 1983; Secor, Dean and Laban, 1991; Moralez-Nin *et al.*, 1999; Megalofonou, Dean and De Metrio, 1995; Nishimoto, De Martini and Landgraf, 2006; Besbes Benseddik *et al.*, 2011).

However these processes are expensive and time-consuming, so other structures are often preferred for ageing analysis, such as spines (*T. thynnus*, *T. alalunga*, *S. sarda*, Istiophoridae), vertebrae (*S. sarda*, *C. hippurus*) or, alternatively, scales (*C. hippurus*, *T. alalunga*).

5.2.2 Fin rays or spines

When preparing fin ray (spine) sections, it is essential to identify the cutting location exactly. In several studies on the age of *T. thynnus*, this location near the condyle base is not clearly described (Compean-Jiménez and Bard, 1983; Cort, 1991; Megalofonou and De Metrio, 2000; Santamaría *et al.*, 2009). Other studies have given more-detailed criteria to establish a precise protocol for identification of the exact cutting location (Rodríguez-Marín *et al.*, 2007, 2012). This depends on the species and is established in relation to spine condyle base measurements (Kopf, Drew and Humphreys, 2010). For *T. thynnus*, two possible sectioning locations have been described.

- The first is located at a distance equal to half the maximum diameter of the spines (Dmax), starting from a straight line above the two hollows at the condyle base. This cutting section is named section 0.5 (0.5 Dmax) (Rodríguez-Marín *et al.*, 2012) (Plate 164).
- As we will see later, the central zone of the spines can present a different morphology, depending mainly on the extension of the vascularized area and the cutting axis position (Rodríguez-Marín *et al.*, 2007, 2012). For these reasons, Luque *et al.* (2014) have more recently proposed a new cutting location, named 1.5 Dmax, corresponding to one and a half times the maximum diameter (Dmax) as a valid alternative to the ‘classic’ 0.5, to be used as a comparison (Plate 164).

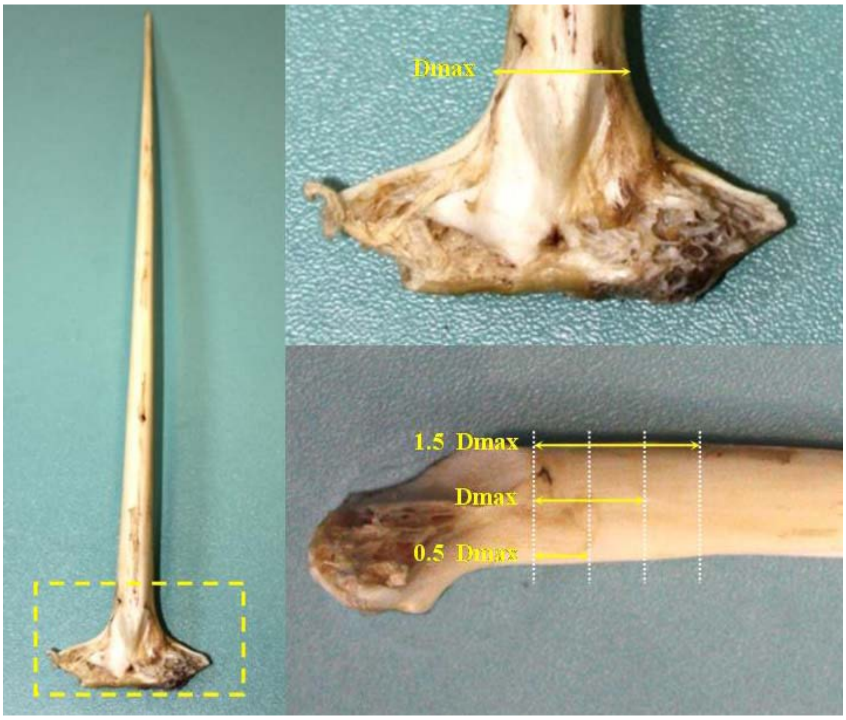
In both cases, it is advisable to produce sections (not to exceed 0.6 mm thickness) using a low-speed saw with a diamond blade, starting from the selected location and proceeding towards the apex of the spine. This will allow comparison of possible differences among sectioning locations.

For *X. gladius*, the cutting axis position is generally located at a distance equal to half the condyle width (CW) (Plate 165A, CW/2). However, several recent and past studies also proposed alternative locations (Plate 165B, CW and CW/4) that provided better results.

Considering that most ageing studies on billfish were carried out using spine sections on diverse species belonging to the genus *Tetrapturus,* for these species spine sections can be performed along two cutting axes: CW/2 and/or CW/5 (Plate 165B) (Berkley and Houde, 1983; Prince *et al.*, 1984; Riehl, 1984; Ehrhardt, Robbins and Arocha, 1996; Potoschi, 2000; Sun, Wang and Yeh, 2002; Valeiras *et al.*, 2008a; Quelle *et al.*, 2014; Drew, Die and Arocha, 2006a, 2006b; Kopf, Drew and Humphreys, 2010; Kopf *et al.*, 2011).

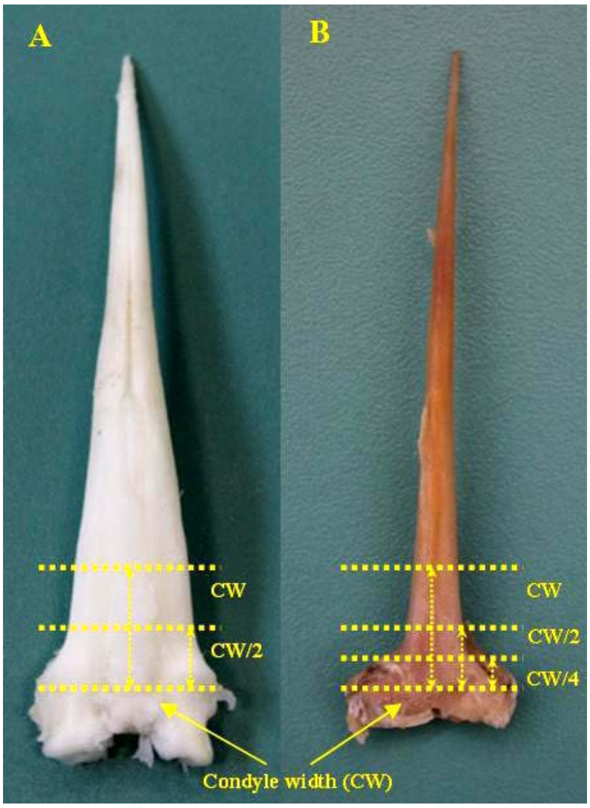
For tuna-like species, as well, it is always considered good practice to produce serial sections (0.40–0.60 mm thickness) once the sectioning location has been established.

PLATE 164
Two cutting locations (0.5 and 1.5) of the first dorsal fin of *T. thynnus*



Source: Rodríguez-Marín *et al.*, 2012.

PLATE 165
Cutting axis of an anal fin of A – *X. gladius* and
B – *T. belone*



Sections should be cleaned in ethanol or hydrogen peroxide, removing all organic matter, and then dried. It's important to verify that the sections are completely dried before storage to prevent browning and opacification of the structure. Sections can be directly observed dry, mounted on glass slides or immersed in a clarification liquid (water, oil, glycerol). In these latter cases, remember to rinse and re-dry sections before storing them again.

5.2.3 Vertebrae

Before preparation, vertebrae must be separated, taking care not to damage the inner face of the cone containing the intervertebral disc (Plate 166A). They must be carefully cleaned, removing the muscle and connective tissue, and rinsed in running water. The inner surface of the vertebra should not be allowed to dry before staining, because otherwise it will not stain properly and cracks may appear on the surface.

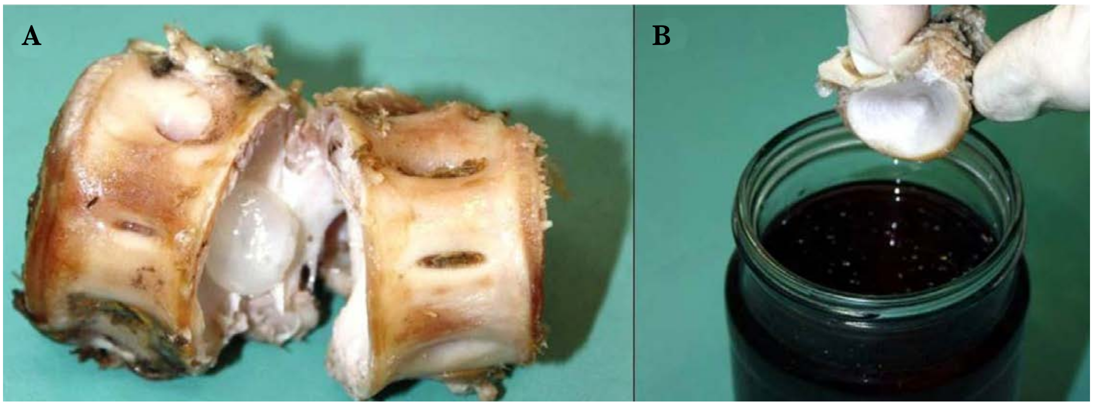
Generally, vertebrae can be analysed in two ways:
- counting growth bands present along the vertebral cone, from the focus (centrum) to the edge of the whole vertebra; or
- counting growth bands along the arms of the vertebral body in vertebral section.

If the growth bands are not clearly visible, the whole vertebra can be soaked in a solution of alizarin, glycerol and distilled water for 2–5 hours, depending on the size and proportions of the reagents in the mixture (see proportion of the stain solution in Berry, Lee and Bertolino, 1977, or Rodríguez-Marín *et al.*, 2007) (Plate 166B).

The solution should not be used more than twice, as the staining power exponentially decreases. If the vertebra was too dark, it can be immersed for 10–20 hours in a solution of distilled water. Successively, the vertebrae are rinsed in running water for 2–20 minutes and completely dried at room temperature (Rodríguez-Marín *et al.*, 2007).

The staining process with alizarin is also reported in some studies performed on vertebrae of *C. hippurus*, using different reagent ratios (Moralez-Nin *et al.*, 1999).

PLATE 166
A – cleaning and B – immersion in alizarin solution of a *T. thynnus* vertebra

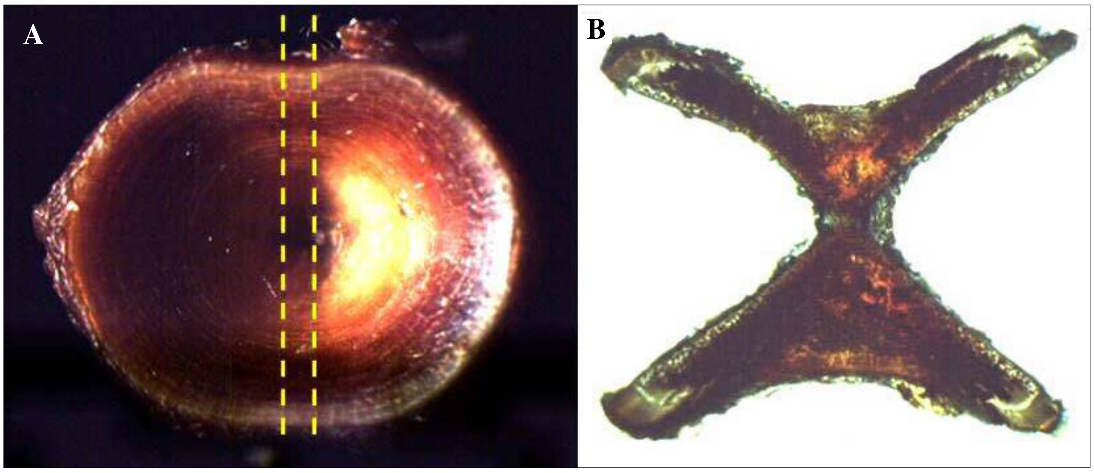


This can be considered a valid and applicable technique for other species, as well, but preference is usually given to the vertebral section (e.g. *S. sarda*) or the whole vertebra (without staining) to reduce processing time and costs.

The vertebral section (Plate 167) does not require a staining process. It is made along an anteroposterior axis (0.6–0.8 mm thickness), through the focus, using a low-speed saw equipped with a diamond blade. If the section is too thick, it can be polished to obtain the right thickness to see clear and well-defined growth bands.

The section can be mounted on a slide or directly observed in reflected or transmitted light under a stereomicroscope.

PLATE 167
A – cutting axis and B – section of *S. sarda* vertebra



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In billfish (*X. gladius* and other Istiophoridae), vertebrae are rarely used for aging estimation (Cavallaro, Cefali and Potoschi, 1998; Hill, Cailliet and Radtke, 1989; Hill and Cailliet, 1990; Estevez *et al.*, 1995). Spines (dorsal or anal fin rays) or alternatively otoliths are preferred.

5.3 Age estimation

As described above, for each species there are different possible structures and related techniques useful in the age estimation process. Comparative studies on diverse calcified structures (otoliths, vertebrae, rays) of the same species provided results not always in agreement (Lee, Prince and Crow, 1983; Rey, Alot and Ramos, 1984, 1986; Hill, Cailliet and Radtke, 1989; Estevez *et al.*, 1995; Hill and Cailliet, 1990; Rodríguez-Marín *et al.*, 2006, 2007). This could be related to intrinsic factors typical of the various hard parts (morphology, physiology, presence of false bands, etc.) or external factors (unclear preparation, experience and ability of readers, etc.).

These comparative studies are useful in estimating the agreement that exists in interpretation of the diverse structures. Different length-at-age estimates allow corroboration of age and growth and represent a valid approach to providing an indication of which structure could be the most suitable to adopt for each single species. Nevertheless, they are often incorrectly used to "validate" the age of a specimen (Campana *et al.*, 2001), but here it is necessary to remember that the validation process can only be performed having data available from other studies carried out with different techniques (radiocarbon bombs, mark recapture studies, edge-type analysis, tagging, etc.). A summary of the state of knowledge for age validation estimates is reported in Murua *et al.* (2017). A central issue in the age reading process is the development of techniques and methodologies to distinguish "false" from "real" growth bands, but there are still many uncertainties among the experts. The assignment of one year of age should be done only when the specimen has completed formation of a full translucent band, which is commonly verified through the monthly marginal increment analysis (MIA). This methodology allows "validation" not of the absolute age, but of the frequency of deposition of the growth bands by months. Generally, it was defined as a ratio (marginal increment ratio – MIR) by the following formula, adopted in various papers dealing with tuna and *X. gladius* spine sections (Prince, Lee and Berkeley, 1988; Cort, 1990, 1991; Estevez *et al.*, 1995; Tserpes and Tsimenides, 1995; Sun, Wang and Yeh, 2002; Luque *et al.*, 2011, 2014):

$$MIR = (R_{tot}-R_n) / (R_n-R_{n-1}) \text{ where}$$

R_{tot} = total radius;
R_n = ring radius measured to the band n;
R_{n-1} = ring radius measured to the band n-1.

This methodology is often "used and abused" in scientific papers. It could be biased by diverse factors (varying seasonal individual growth, age of the specimen, interpretation of marginal increments, etc.). For these reasons, MIA should be applied only to a few age groups, separating young from adults (Campana, 2001).

The main pros and cons related to the use of the three main calcified structures for ageing analysis of the target species of this section are reported in Table 18.

TABLE 18 – Strengths and weaknesses of the use of otoliths, vertebrae and spines for ageing estimation of six large pelagic species

Species	Otoliths	Spines	Vertebrae
Thunnus thynnus	Difficult interpretation of first 5 years in otolith sections; whole otolith more readable up to 6–7 years	Recommended in specimens up to 10 years; underestimation due to resorption of central area; very important to define cutting axis; presence of false and multiple bands	Recommended in specimen up to 10 years; underestimation in large specimen; difficult to recognize first annulus; presence of false and multiple bands; sections present vascularized core
Xiphias gladius	Small, fragile and difficult to prepare; difficult to recognize growth bands; particularly used for ageing analysis of juvenile species (daily increment bands)	Best structure for estimation of this species; difficult interpretation of inner growth bands in adult; presence of false and multiple bands; vascularization present	Rarely used and difficult to sample
Thunnus alalunga[a]	Difficult interpretation owing to presence of multiple bands	Underestimation due to resorption of central core; presence of false and multiple bands (double, triple)	Less used for age estimation; difficult to sample; presence of multiple bands
Sarda sarda	Difficult to define annual growth bands	Easy to sample and prepare; presence of vascularized central area; probable resorption of inner bands; underestimation in large individual	Less used, central core vascularized (in section); difficult detection of growth bands; possible underestimation
Istiophoridae	Rarely used; small otolith, fragile and difficult to prepare; difficult detection of growth bands	Best choice for aging in sailfishes; difficult to recognize first growth bands; resorption of central area; presence of false bands	Rarely used
Coryphaena hippurus[a]	Small size, fragile and difficult to prepare; used mainly in juvenile specimens); age underestimation in adults (> 1 year)	Not used	Rarely used

[a] In the literature, scales are often cited as ideal structures for ageing analysis of these species (Massutí, Morales-Nin and Moranta, 1999; Arena, Potoschi and Cefali, 1980; Cefali *et al.*, 1986; Megalofonou, 1990; Megalofonou, Yannopoulos and Dean, 2003).

5.3.1 Growth bands interpretation and age assignment

Individual ages are generally estimated using the count of opaque zones, otolith/spine edge-type analysis, catch date, assumed birth date (or spawning month) and timing of opaque zone formation (Campana, 2001).

The growth bands or annuli appearing in otoliths and spines are bipartite structures consisting of a translucent and opaque zone or, in alternative, the ridges and grooves on vertebral cones. The opaque band is dark under transmitted light and bright under reflected light; obviously it will be the inverse for the translucent band (hyaline band). The opaque band is thicker and corresponds to a period of fast growth (high food availability, intense feeding activity, high water temperature, favourable environmental conditions, i.e. summer season). The translucent area, on the contrary, is laid down during a period of slow growth (no food availability, adverse environmental conditions, i.e. winter season). As stated before, growth marks can represent age in years (annual marks or annuli) or age in days (daily increments).

5.3.2 Age adjustment

The sampling date and definition of the birth date (or birth period) of each single specimen are obviously the most important information for a correct age-determination process. In this case, tunas, for example, will be correctly assigned to their corresponding age class. Strictly coupled with this information, another key issue in age estimation is provided by examination and description of the edge type, often used for finer adjustment of the individual age assignment.

As an example, for *T. thynnus*, when a translucent band is visible on the edge in a specimen caught during winter (e.g. December–January), it should not be considered as documenting one year of growth (being six months before the date of birth). Usually, the complete deposition of the translucent band ends in late spring (April–May), when deposition of the opaque band begins (active growth), close to the birth date (1 June or 1 July). In the Mediterranean area, the spawning period for all six species has been identified as ranging from late spring until late summer:

– *T. thynnus* – from May to July in the eastern Atlantic and Mediterranean stock (Rodríguez-Roda, 1967; Piccinetti and Piccinetti-Manfrin, 1993; Susca *et al.*, 2001; Medina *et al.*, 2002; Karakulak *et al.*, 2004);
– *X. gladius* – from June to September in the Mediterranean stock, with a peak in the months of June and July and recruitment in autumn (Rey, 1988; De Metrio and Megalofonou, 1987; Megalofonou, De Metrio and Lenti, 1987, 1989; De Metrio, Giacchetta and Santamaría, 1995; Orsi Relini *et al.*, 1996a; Orsi Relini, Palandri and Garibaldi, 2003; Tserpes, Peristeraki and Somarakis, 2001; Macías *et al.*, 2005). Many authors report maximum fecundity in July off the coast of Sicily (Sella, 1911; Sanzo, 1922; Cavaliere, 1963) and in the western Mediterranean (Rey, 1987), while in the eastern Black Sea it seems to occur earlier (Artuz, 1963; Aliçli *et al.*, 2012);
– *T. alalunga, T. belone and C. hippurus* – from June to September (Sanzo, 1933 Spartà, 1953, 1960; Cavaliere, 1962; Dicenta, 1975; Lalami *et al.,* 1973; García, Alemany and Rodríguez, 2002; De Sylva, 1975; Piccinetti and Piccinetti-Manfrin, 1993; Piccinetti, Piccinetti-Manfrin and Soro, 1996; Alemany and Massutí, 1998; Massutí and Morales-Nin, 1995, 1997; Potoschi, Reñones and Cannizzaro, 1999; Potoschi, 2000);
– *S. sarda* – from May to July in a wider area of the Mediterranean Sea (Rodríguez-Roda, 1966; Rey, Alot and Ramos, 1984; Sabates and Recasens, 2001; Orsi Relini *et al.*, 2005),

while in the southern part of the western basin (Algerian coasts), it occurs between March and May (Dieuzeide, Novella and Roland, 1954).

Birth dates for all species can be placed between June and July (Table 19). For *T. thynnus*, 1 June is generally adopted, while for other species, presenting a wider spawning period, 1 July could be assumed.

TABLE 19 – Birth dates suggested for the six large pelagic species in the Mediterranean area

Species	Birth date
Thunnus thynnus	1 June
Xiphias gladius	1 June/July
Thunnus alalunga	1 July
Sarda sarda	1 June/July
Tetrapturus belone	1 July
Coryphaena hippurus	1 July

5.4 Species

5.4.1 *Thunnus thynnus* and *Thunnus alalunga*

T. thynnus and *T. alalunga* will be treated together in this subsection, given that they belong to the same genus and consequently have very similar characteristics regarding the structures considered in age determination. For these two species, especially for *T. thynnus*, there is a huge literature on age and growth based on different methods: otolith, spine, vertebra readings and, in particular for juveniles, L/F modal progression analysis (Rodríguez-Roda, 1964; Bard and Compean-Jiménez, 1980; Farrugio, 1980; Compean-Jimenez and Bard, 1983; Gonzalez-Garcez and Farina Perez, 1983; Hattour, 1984; Rey and Cort, 1984; Arena, Potoschi and Cefali, 1980; Cort, 1990, 1991; Cort *et al.*, 2014; Ortiz de Zárate *et al.*., 1996; Orsi Relini *et al.*, 1996b, 1997; Megalofonou, 2000; Farrugia and Rodríguez-Cabello, 2001; El-Kebir, Rodríguez-Cabello and Tawil, 2002; Megalofonou, Yannopoulos and Dean, 2003; Olafsdottir and Ingimundardottir, 2002; Corriero *et al.*, 2005; Ortiz de Zárate *et al.*, 2005; Santiago and Arrizabalaga, 2005; Davies *et al.*, 2008; Di Natale *et al.*, 2011; Karakulak *et al.*, 2011; Quelle *et al.*, 2011; Santamaría *et al.*, 2012; Rodríguez-Marín *et al.*, 2004, 2006, 2012, 2014; Luque *et al.*, 2011, 2014; Landa *et al.*, 2015; Santamaría *et al.*, 2015; Garibaldi *et al.*, 2017; Murua *et al.*, 2017).

5.4.1.1 Otoliths

The otolith section of *T. thynnus* and *T. alalunga* is characterized by a typical Y-shape, with a long arm on its ventral side and a short arm on its dorsal side (Plates 168, 169).

The long ventral arm is always used for age estimation. Given that the short dorsal arm generally shows a lower number of bands, it is only considered a check; in fact, an exclusive use of the dorsal arm could lead to an underestimation of age.

The reading must be performed beginning from the core (primordium) towards the edge of the ventral arm, watching the section under reflected or transmitted light (Anon., 2002; Rodríguez-Marín *et al.*, 2007; Busawon *et al.*, 2015).

The otolith section in a giant *T. thynnus* is characterized by the presence of three distinct regions, separated by two inflections (Plate 169) (Anon., 2002; Busawon *et al.*, 2015):

- region 1: where the annuli are wider and may contain multiple bands (~ 1–5 annuli);
- region 2: where the annuli are narrower and closer (~ 5–10 annuli);
- region 3: where the annuli appear clear, well defined, and at a regular distance (~ 10+ annuli).

A main problem is the identification of the first annulus, corresponding to the first year of life. A good reference point is provided by the shape of the otolith, in which it is possible to observe a first inflection of the ventral arm, beyond which the first annulus is generally completed

(Plate 170) (Rodríguez-Marín *et al.*, 2007; Busawon *et al.*, 2015; Secor *et al.*, 2014). The right position of the first annulus can also be identified by measuring along the ventral arm in young recruits of the year, in order to obtain a reference scale that would facilitate identification even in larger specimens (Plate 170) (Rodríguez-Marín *et al.*, 2007; Busawon *et al.*, 2015; Secor *et al.*, 2014). This area has been identified as ranging from 0.73 to 1 mm (average 0.86 mm) on the basis of measurements in a sample of 22 juveniles (Busawon *et al.*, 2015) (Plate 170).

Age estimation using *T. thynnus* otoliths is more difficult in juveniles up to five years, during the first years of life characterized by fast growth, before they reach the sexual maturity. In region 1, which corresponds to this period, the space between the growth bands is broad and full of details (false bands), which can confuse inexperienced readers (Plate 170) (Rodríguez-Marín *et al.*, 2007; Busawon *et al.*, 2015).

PLATE 168
T. thynnus otolith section

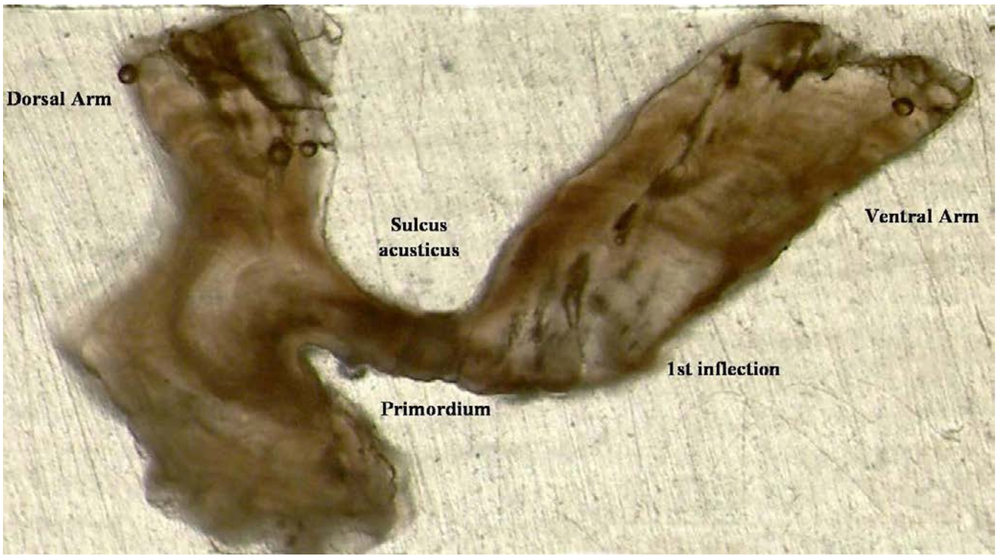

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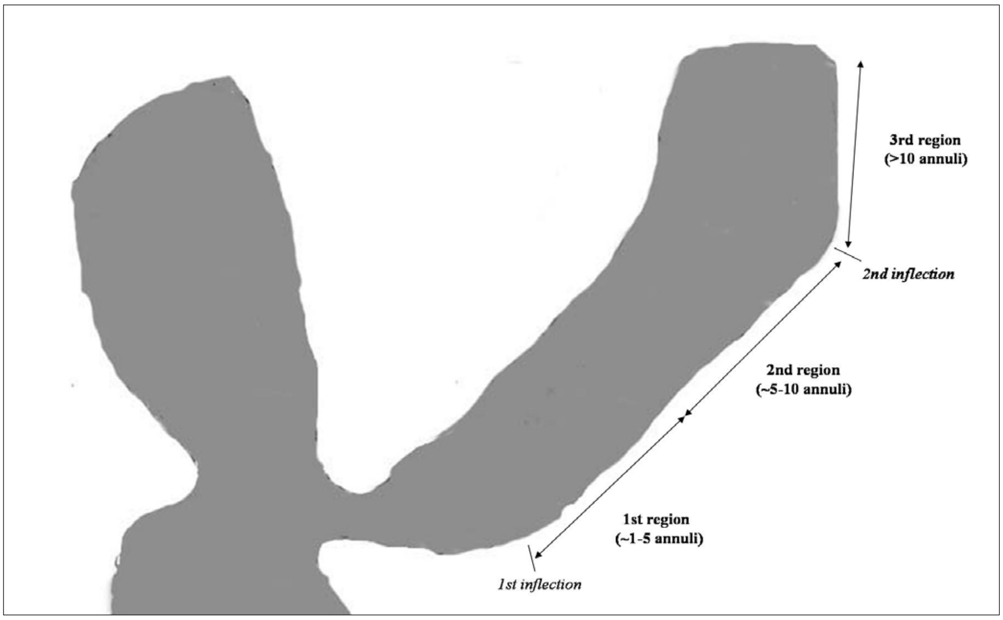
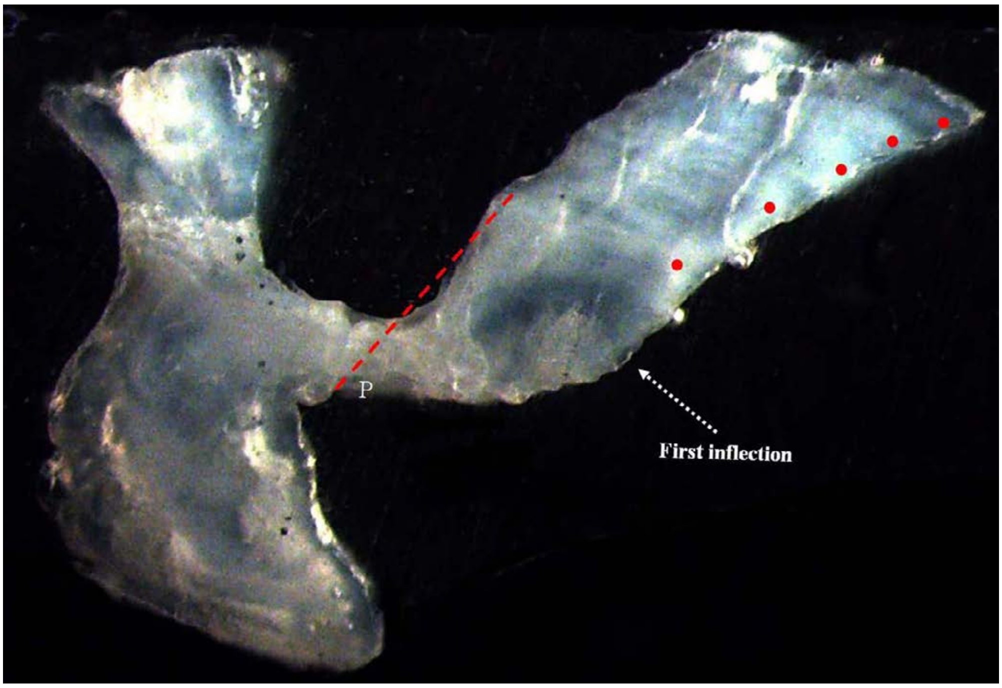
PLATE 169
Scheme of a *T. thynnus* otolith showing the three regions and two inflections



Source: Anon., 2002

PLATE 170
T. thynnus otolith section: identification of first annulus after first inflection and measure of first annulus from primordium to edge of annulus (dashed line)



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In this age range (up to five years), it can be useful to combine reading of the otolith section with that of the whole otolith, in which annual increments (up to six to seven years) are often clearly visible along the rostrum (Plate 171) (Rodríguez-Marín *et al.,* 2007; Busawon *et al.*, 2015).

In addition, analysis of the section margin can help identify growth bands. In some specimens, it is characterized by more-or-less evident crenulations that would suggest the presence of annuli (Anon., 2002; Rodríguez-Marín *et al.*, 2007; Busawon *et al.*, 2015) (Plate 172). These indentations are the result of cyclical variations in the otolith growth structure, from a slow-growing (cold season) to a fast-growing (hot season) phase (Rodríguez-Marín *et al.,* 2007; Busawon *et al.*, 2015).

The main studies carried out on *T. alalunga* in the Mediterranean Sea have been done using scales (Arena, Potoschi and Cefali, 1980; Megalofonou, 1990; Megalofonou, Yannopoulos and Dean, 2003) and spines (Megalofonou, 2000; Quelle *et al.*, 2011; Karakulak *et al.*, 2011; Garibaldi *et al.*, 2017).

Generally speaking, only a few studies were performed using otoliths for age determination in *T. alalunga* worldwide (Chen and Holmes, 2015).

For the eastern Atlantic and Mediterranean, two comparative studies are available in the literature. The first was carried out with vertebrae and rays on the basis of a sample of 21 specimens caught in the Bay of Biscay (Fernandez, 1992). The second, more-recent one concerned three structures (spines, scales and otoliths) of individuals of TL < 80 cm caught in both the Atlantic and the Mediterranean (Davies *et al.*, 2008).

Comparison of the different structures has given contrasting results: the authors refer to main sources of error in the analysis of *T. alalunga* otoliths, already described for *T. thynnus*:
– difficulty in identification of the first annual ring;
– difficulty in preparation of sections going precisely through the core;
– presence of false bands.

PLATE 171
Whole otolith of *T. thynnus* specimen (age 5 years)

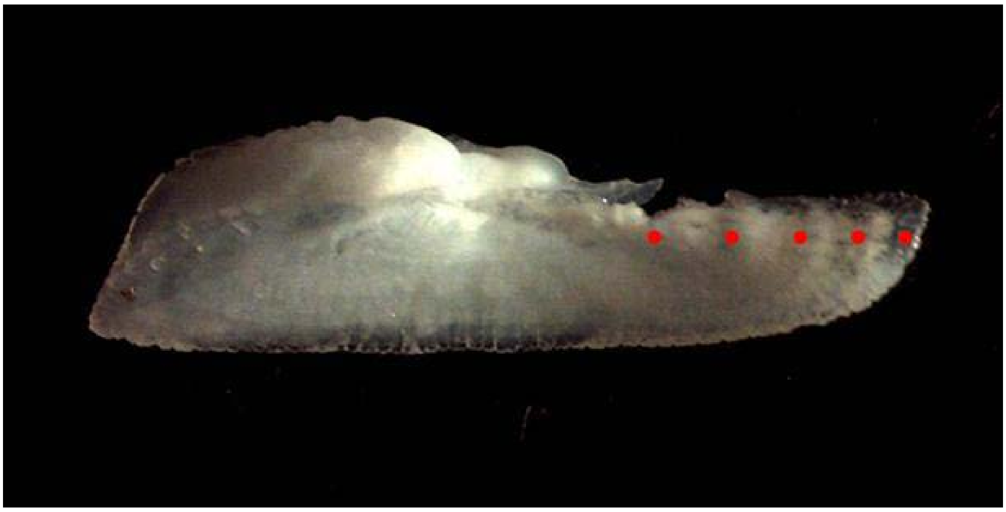
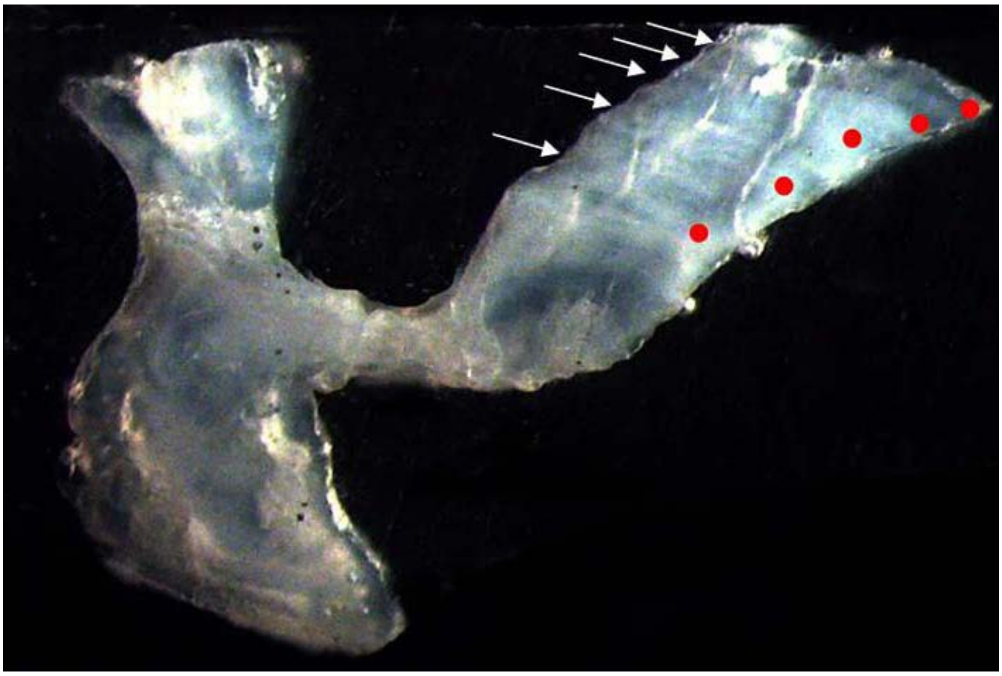


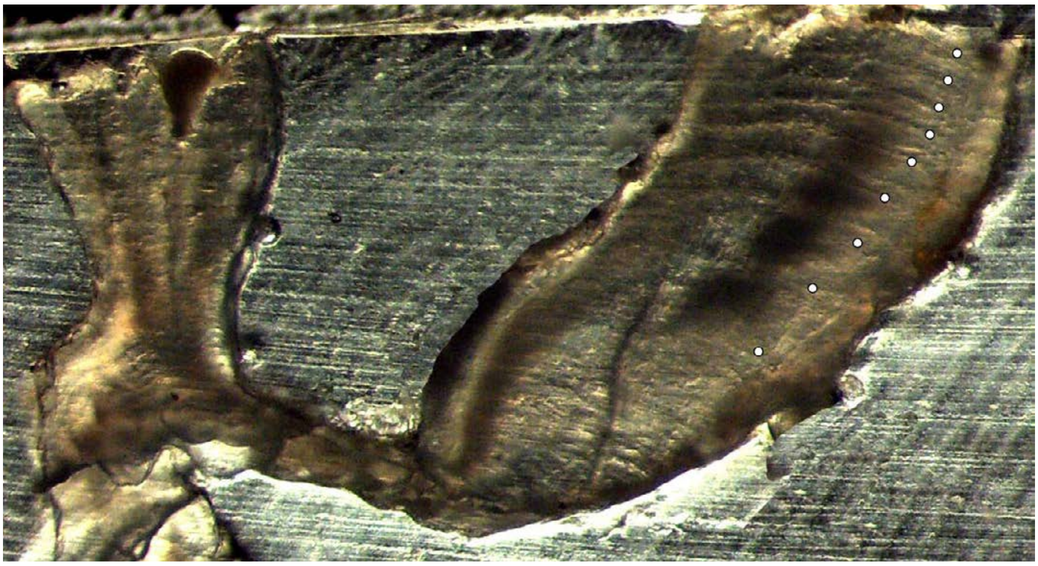
PLATE 172
Example of otolith crenulations (white arrows) in *T. thynnus*



More recently, on a total of ten specimens ranging from TL 40 to 44 cm, Lu, Ortiz de Zárate and Yeh (2006) confirm what had been described by Fernandez (1992), noting that the time of deposition of the first annulus would correspond to an age of about 200–250 days in otoliths.

Given the morphological characteristics (shape), growth and deposition patterns of bands, otolith analysis should follow the same criteria and procedures previously described for *T. thynnus* (Plate 173). Given the lack of studies in this field, improving the use of otoliths for *T. alalunga* age determination is highly recommended.

PLATE 173
Otolith section of an *T. alalunga*: estimated age 9 years

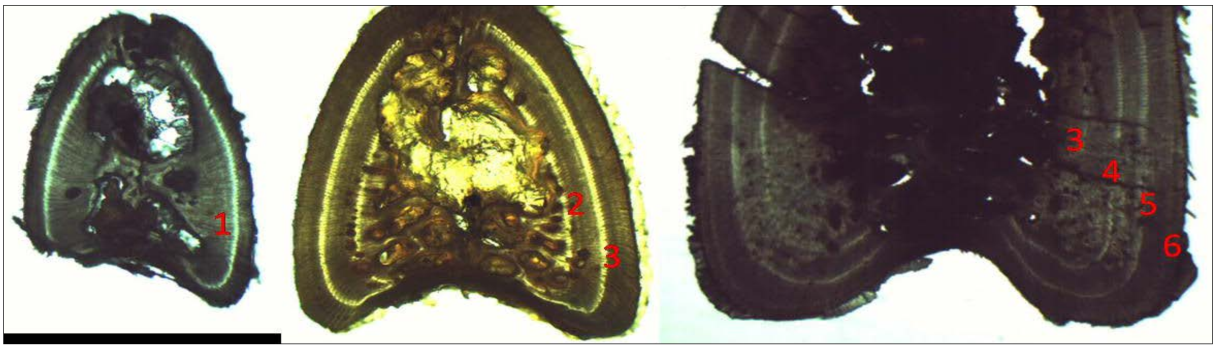


5.4.1.2 Spines

As mentioned earlier, spines (first ray of the dorsal fin) are largely used for age determination in *T. thynnus*, despite the fact that they also present some sources of possible bias.

The main problem is related to the gradual increase of the central vascularized core of spines, producing a resorption of the surrounding bony tissue and consequently a loss of the innermost bands, corresponding to the first years of life (Santamaría *et al.*, 2015). For this reason, a simple counting of the translucent visible bands would lead to an underestimation of age, in particular in *T. thynnus* older than two to three years (Plate 174).

PLATE 174
Spine sections of *T. thynnus*

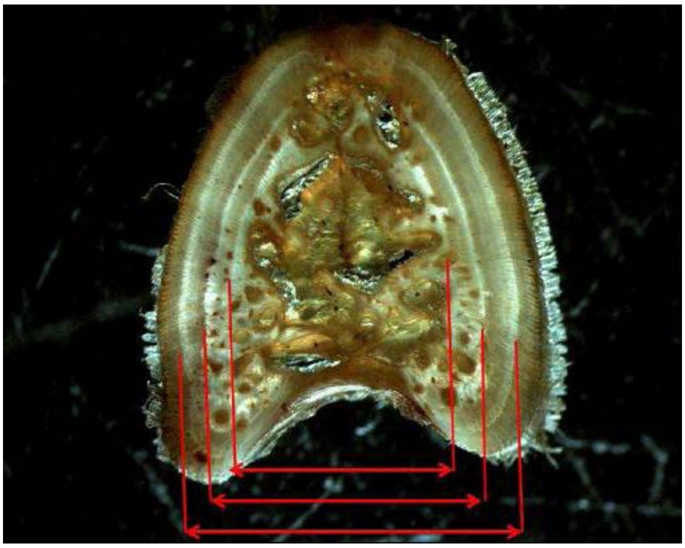


Note: Left – age 1 year (TL = 64 cm); centre – age 3 years (TL = 109 cm); right – age 6 years (TL = 156 cm).

To overcome this issue, it's useful to identify each single annulus, taking measurements of the maximum diameter of each growth band, and to reconstruct the number of inner bands reabsorbed (Rey and Cort, 1984; Cort, 1990, 1991; Rodríguez-Marín *et al.*, 2012; Luque *et al.*, 2014).

Band diameter is measured in correspondence with the outer edge of the translucent bands (Plate 175), that is, the point of transition from the winter season (slow growth) to the summer season (fast growth). If there are double/triple bands (as discussed below), the measurement is taken on the outer band.

PLATE 175
Translucent bands diameter



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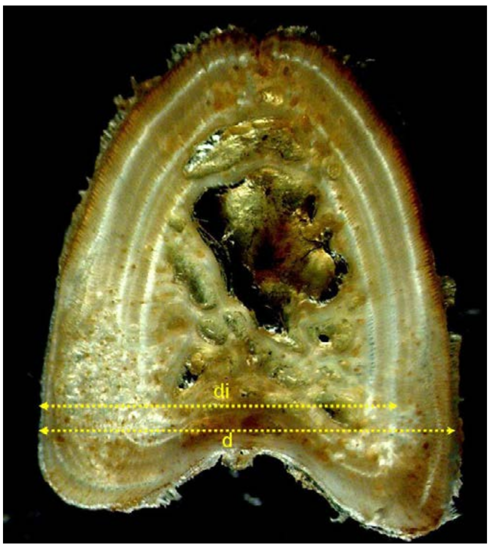
As a reference, the mean values of the band diameters obtained at two cutting levels (D 0.5 and D 1.5) in a large sample of *T. thynnus* collected over 21 years (1990–2010) are shown in Table 20 (Rodríguez-Marín *et al.*, 2012).

Given that, in some sections, the annuli could be clearly visible only on one side (Plate 176), the band diameter can be estimated applying the formula reported by Santamaría *et al.* (2009):
Di = (di-d/2) x 2

where

- *Di* is the diameter of the annulus *i*;
- *di* the distance from the outermost side of the translucent band to the opposite edge of the spine section; and
- *d* the maximum diameter of the section.

PLATE 176
Measurement of maximum diameter of two translucent bands not visible in a *T. thynnus* spine section



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The use of diameters of the translucent bands is a good and relatively easy method, especially with young and subadult *T. thynnus*, due to the fact that they have a higher growth rate and show a regular deposition of translucent bands. This results in the presence of clearly distinct and regularly spaced annuli in spines.

For larger fish (> eight to ten years), identification of the most-external bands can be more difficult, due to the slower growth rate. This physiological process determines decreased spaces between annuli, which could lead to a possible underestimation or overestimation of age.

In *T. alalunga*, considering the lower longevity of this species (up to 11 years in the Mediterranean Sea) (Megalofonou, 2000; Quelle *et al.*, 2011, Karakulak *et al.*, 2011) and the smaller size of its spines, resorption of the central nucleus is generally limited to the first annulus, identified at about 2 mm (SD 0.09) (personal observation, Plate 177).

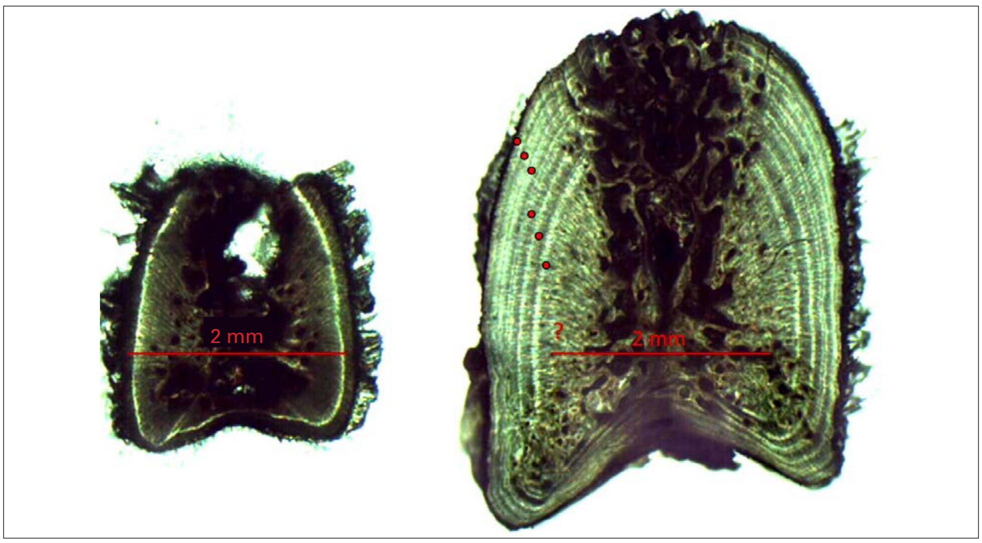
TABLE 20 – Mean value of translucent band diameter measured at 0.5 (up) and 1.5 (down) cutting sections

	S 0.5													
	Age 1	Age 2	Age 3	Age 4	Age 5	Age 6	Age 7	Age 8	Age 9	Age 10	Age 11	Age 12	Age 13	Age 14
No measurements	814	1027	635	525	529	526	477	368	266	168	91	60	25	5
Mean value	2.44	3.55	4.75	6.00	7.16	8.26	9.32	10.31	11.21	12.07	13.02	13.73	14.53	15.64
Standard deviation	0.28	0.25	0.36	0.39	0.37	0.36	0.36	0.32	0.38	0.45	0.43	0.53	0.52	0.18
Confidence interval 95%	0.02	0.02	0.03	0.03	0.03	0.03	0.03	0.03	0.05	0.07	0.09	0.13	0.20	0.16
Minimum (95%)	2.43	3.53	4.72	5.97	7.13	8.23	9.29	10.28	11.16	12.00	12.93	13.60	14.32	15.48
Maximum (95%)	2.46	3.56	4.78	6.03	7.19	8.29	9.35	10.35	11.25	12.13	13.11	13.87	14.73	15.80
	S 1.5													
	Age 1	Age 2	Age 3	Age 4	Age 5	Age 6	Age 7	Age 8	Age 9	Age 10	Age 11	Age 12	Age 13	
No measurements	42	94	106	112	149	172	158	114	77	33	15	11	4	
Mean value	2.35	3.37	4.48	5.60	6.65	7.66	8.64	9.46	10.28	11.08	12.03	12.84	13.25	
Standard deviation	0.25	0.34	0.35	0.39	0.39	0.37	0.33	0.35	0.33	0.41	0.44	0.53	0.65	
Confidence interval 95%	0.08	0.07	0.07	0.07	0.06	0.05	0.05	0.06	0.07	0.14	0.22	0.31	0.64	
Minimum (95%)	2.28	3.30	4.41	5.53	6.58	7.60	8.59	9.39	10.21	10.94	11.81	12.53	12.61	
Maximum (95%)	2.43	3.44	4.55	5.67	6.71	7.71	8.69	9.52	10.36	11.22	12.26	13.15	13.88	

Source: Rodríguez-Marín *et al.*, 2012.

PLATE 177

Spine sections of *T. alalunga* and first annulus diameter in two specimens of 1- and 7- years-old estimated



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The appearance of false bands is another possible source of error, which is more commonly found in spine than in otolith sections.

Several studies described the presence of translucent bands grouped in clusters of two or more, which must not be counted as annual rings (Bard and Compean-Jiménez, 1980; Compean-Jiménez and Bard, 1983; Rey and Cort, 1984; Cort, 1990, 1991; Cort *et al.*, 2014; Fernandez, 1992; Rodríguez-Marín *et al.*, 2007, 2012).

Three types of translucent bands are found in *T. thynnus* or *T. alalunga* spine sections:
– single band (or thin ring) laid down during the cold season (slow growth);
– thick band, with a thickness greater than the previous one; and
– multiple translucent bands grouped in two, three or, rarely, four (doublets, triplets, quadruplets), whose deposition would be associated with metabolic factors related to migration, reproductive events or active growth phases also occurring in winter.

The reader's experience plays a fundamental role in identification of false bands. Doublets and triplets are easily identified because translucent rings are very close to each other, placed at a distance lower than the previous and the following translucent band.

Plate 178 presents examples of *T. thynnus* spine sections in which different translucent band typologies are visible.

In *T. alalunga*, the double ring is the most frequent pattern observed by many authors (Bard and Compean-Jiménez, 1980; Fernandez, 1992; Megalofonou, 2000; Karakulak *et al.*, 2011) (Plates 177–179).

PLATE 178
Spine sections of *T. thynnus* and diverse patterns of translucent bands

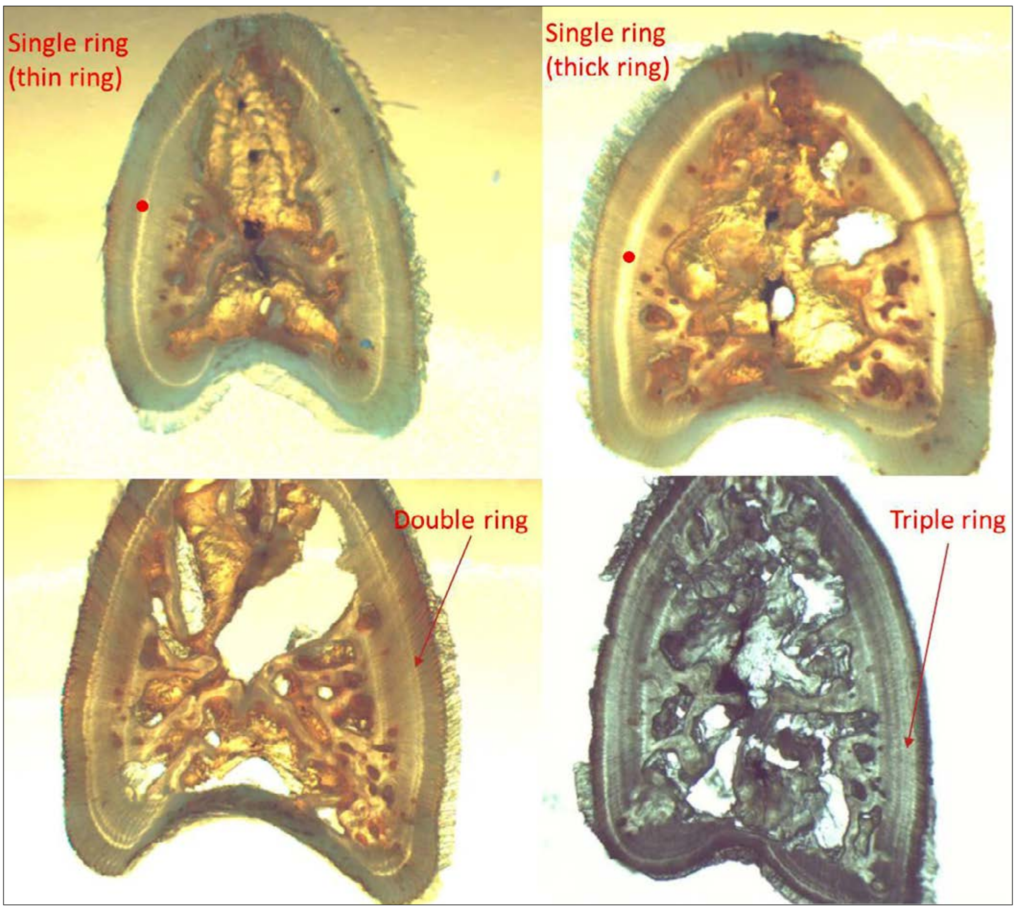
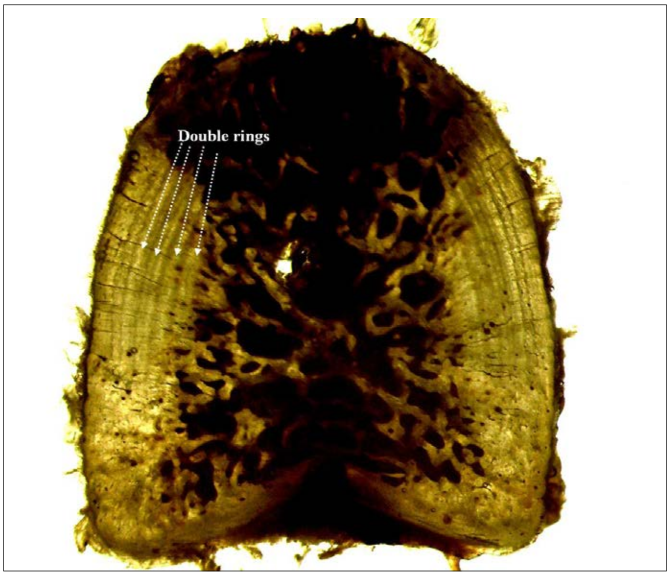


PLATE 179
Spine section of *T. alalunga* with pattern of double rings



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In *T. thynnus*, which is a long-lived species that may exceed 40 years of life, it has been demonstrated that spines give accurate results in specimens up to ten years (Rodríguez-Marín *et al.*, 2007), while they can lead to an underestimation of readings in older specimens.

5.4.1.3 Vertebrae

The vertebra of *T. thynnus* is characterized by two main growth structures (Berry, Lee and Bertolino, 1977; Rodríguez-Marín *et al.*, 2007):
- ridges, developed during the slow growth period (autumn/winter);
- grooves, developed during the fast growth period (spring/summer).

An annulus would correspond to one groove plus one ridge (Plate 180-A).

Following the alizarin staining process, two other structures are visible on the surface of the vertebral cone (Plate 180-B) (Berry, Lee and Bertolino, 1977; Rodríguez-Marín *et al.*, 2007):
- stained bands, generally placed on the focal side of the ridges, facing the centre of the vertebra;
- unstained bands, on the distal side of the ridges, facing the edge of the vertebra.

Multiple and irregular bands, analogous to the false bands found in spines and otolith sections, may be present in some specimens, making interpretation of the annuli more difficult (Plate 181). In these cases, it may be useful to:
- observe the vertebra under incident light (reflected light) from different angles (frontally or laterally) to make the details more evident;
- perform a double reading, on both sides of the vertebra (anterior and posterior cones), to compare the accuracy and precision of the results and check for differences.

Another possible source of error concerns identification of the first annulus; it can be measured from the centre of the vertebral cone (focus) to the distal edge of the first ridge in juveniles (Plate 182). Several studies indicated this position as ranging from 6 to 10 mm, calculated on the 35th vertebra (Rodríguez-Roda, 1964; Farrugio, 1980; Lee, Prince and Crow, 1983; Rodríguez-Marín *et al.*, 2007).

PLATE 180
Cone structures in an A – fresh and B – stained *T. thynnus* vertebra

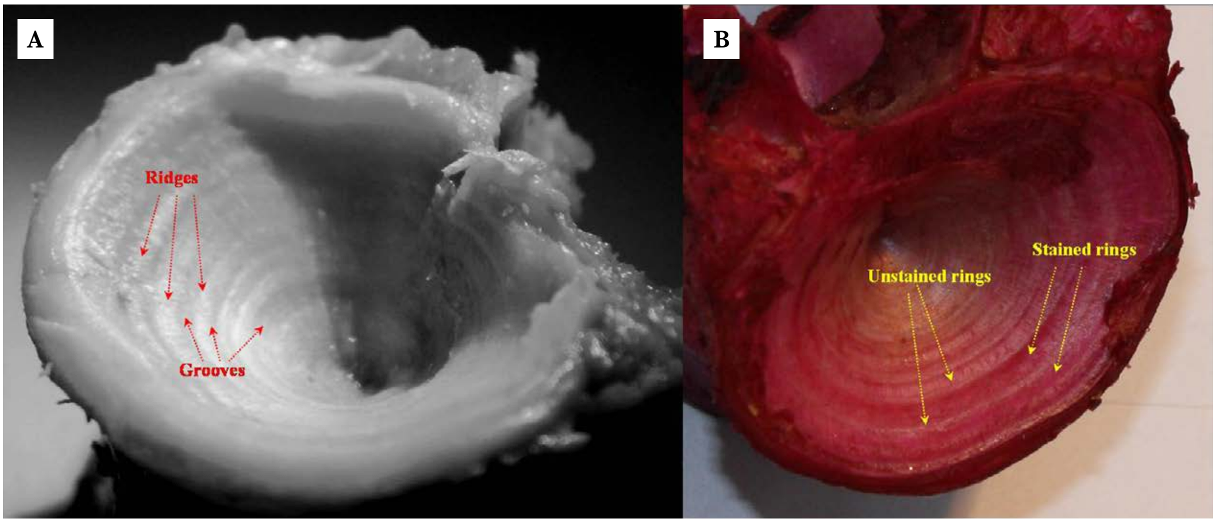
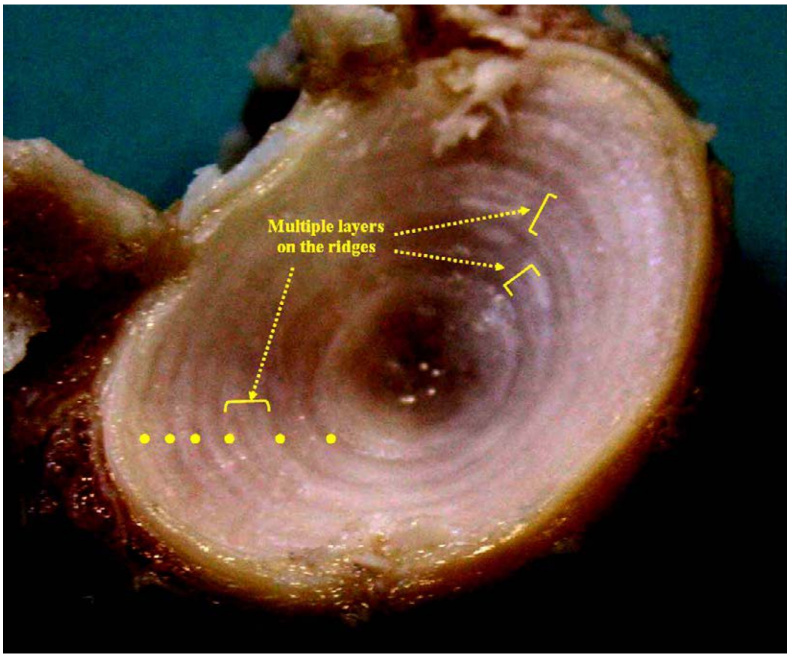


PLATE 181
Multiple bands of vertebral cone of *T. thynnus*

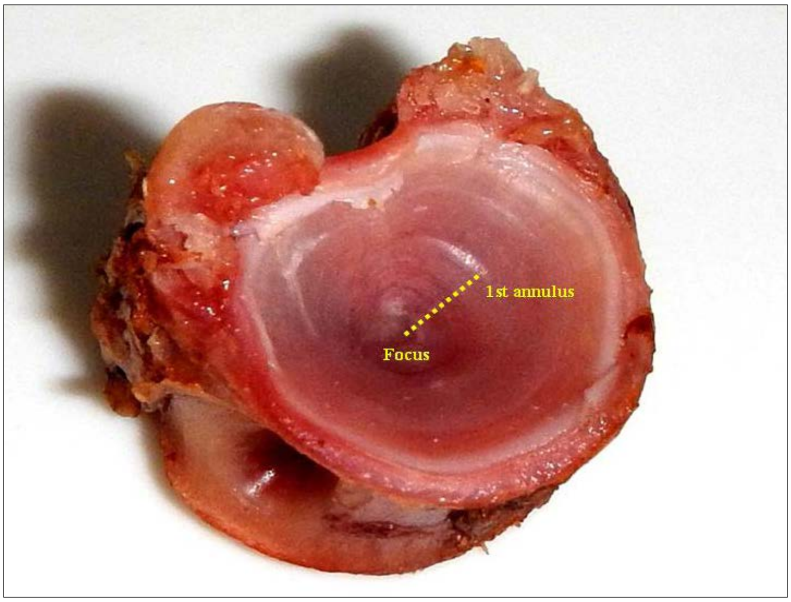


Note: Yellow dots = annuli.

Analysis of the whole vertebra is a valid methodology for *T. thynnus* specimens up to ten years (Berry, Lee and Bertolino, 1977; Hunt *et al.*, 1978; Lee, Prince and Crow, 1983; Olafsdottir and Ingimundardottir, 2002 ; Rodríguez-Marín *et al.*, 2006, 2007). In fact, in older specimens, the interpretation of growth structures may be quite difficult because bands (ridges + grooves) are less pronounced and closer to each other in proximity of the vertebral edge. This is a possible source of bias, which can lead to an underestimation of the age (Berry, Lee and Bertolino, 1977; Hunt *et al.*, 1978; Lee, Prince and Crow, 1983; Olafsdottir and Ingimundardottir, 2002; Rodríguez-Marín *et al.*, 2006, 2007).

The use of vertebral sections for age determination in *T. thynnus* is not widespread. This method seems to underestimate age in juveniles and has been recommended for ageing analysis of large specimens (giant spawners > ten years and > 200 cm) (Prince, Lee and Javech, 1985). The

PLATE 182
Identification of first annulus and measure of radius in vertebra of young *T. thynnus*



Note: Yellow dots = annuli.

vertebral core is characterized by a central part, highly vascularized, where identification of the first growth bands may be hampered by resorption.

In the Mediterranean Sea, studies on *T. alalunga* vertebrae are rare, often carried out to validate data obtained from tagging surveys, with the aid of inoculation of oxytetracycline (De Metrio *et al.*, 1997, 1999).

Fernandez (1992) considered the 27th vertebra as the best choice for *T. alalunga* age reading. This author also described a vertebral structure very similar to the *T. thynnus*, with the presence of ridges, grooves and multiple bands (double).

5.4.2 *Xiphias gladius*

Several methods have been used over the years in the study of age and growth of the swordfish *X. gladius*. Modal progression analysis (MPA) has been used in the past, applying different methods (Bhattacharya, Normsep), mainly for juveniles. This is due to the marked sexual dimorphism of this species, which makes interpretation of cohorts difficult (De Metrio and Megalofonou, 1987; Orsi Relini *et al.*, 1999; Abid *et al.*, 2013).

5.4.2.1 Otoliths

For years, the use of otoliths was one of the most applied methods of age determination for *X. gladius* (Radtke and Hurley, 1983; Wilson and Dean, 1983), particularly appropriate in estimating the age of juveniles (Li Greci, 1981; Megalofonou, Dean and De Metrio, 1990, 1991, 1995).

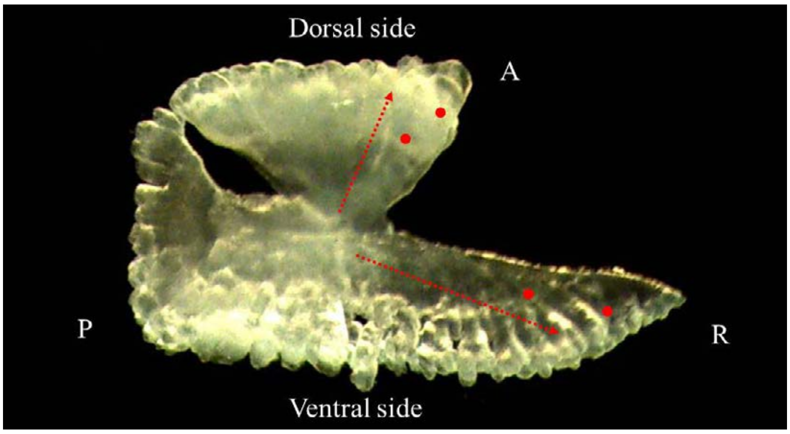
In the Mediterranean Sea, Megalofonou, Dean and De Metrio (1990, 1995) carried out a study of daily increments in young *X. gladius* (LJFL 51–74 cm). They estimated a daily growth rate of 5.7 mm/day in 87–147-day-old specimens, confirming really fast growth in the first year, as observed by modal progression analysis as well.

At present, however, otoliths of *X. gladius* are rarely used, due to difficulties in sampling and in preparation, which is time-consuming and requires advanced equipment.

In fact, the sagitta is very small, with a rostrum longer than the antirostrum and a typical, slightly concave shape (in juveniles), which tends to become deeper along the sulcus acusticus with the increase of otolith size.

The whole otolith must be examined on the proximal side (sulcus acusticus side), along two directions: from the core to the rostrum (R – ventral direction) or along the antirostrum (A – dorsal direction) (Plate 183).

PLATE 183
Sagittal otolith of *X. gladius*: R – rostrum, A – antirostrum

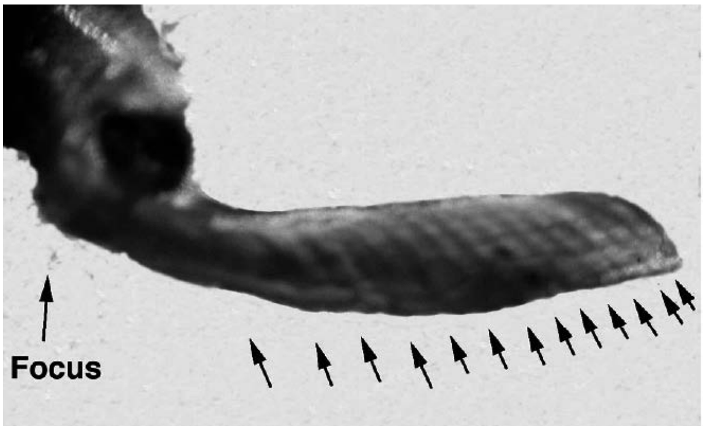


Note: Red arrows = reading directions, red dots = two visible ridges/growth bands (2-year-old specimen).

Growth bands may become more evident after polishing on the sagittal plane. However, this operation must be carried out very carefully, because it could damage the otolith owing to its morphology (slightly curved) and fragility (Nishimoto, De Martini and Landgraf, 2006).

According to many authors, analysis of the whole sagitta provides questionable results, because the annuli are not clearly visible and are difficult to recognize, even to a trained eye (Wilson and Dean, 1983; Estevez *et al.*, 1995; Nishimoto, De Martini and Landgraf, 2006).

PLATE 184
Otolith transverse section of *X. gladius* of the North Pacific: detail of the dorsal side



Preparation of a cross section, which can provide more-satisfactory results, in particular for larger specimens, is considered even more difficult and risky. An example of a transverse section of a sagitta of *X. gladius* of the North Pacific (from Nishimoto, De Martini and Landgraf, 2006) is represented in Plate 184. The alternation between the translucent and opaque zones is evident, moving from the central core (focus) towards the dorsal margin of the antirostrum.

5.4.2.2 Spines

At present, age determination in *X. gladius* is mainly carried out using sections of the second ray of the anal fin, which is more easily sampled and prepared than otoliths. Some attempts were made in the past using the dorsal fin, but this proved less suitable than the anal one (Berkeley and Houde, 1983; Radtke and Hurley, 1983; Prince, Lee and Berkeley, 1988; Tsimenides and Tserpes, 1989; Tserpes and Tsimenides, 1995; Estevez *et al.*, 1995; Ehrhardt, Robbins and Arocha, 1996; Quelle *et al.*, 2014).

A comparative study of the three structures (otoliths, rays, vertebrae) sampled from *X. gladius* has provided interesting results, demonstrating that the second ray of the anal fin is the structure to be preferred, with about 91 percent of the samples readable compared with 56 percent of the vertebral structures and 24 percent of the otoliths (Estevez *et al.*, 1995).

Consequently, most papers published for the Mediterranean have been based on analysis of sections of the second anal fin ray (Tserpes and Tsimenides, 1995; Orsi Relini *et al.*, 1996a, 1999; Aliçli and Oray, 2001; Rollandi *et al.,* 2004; Valeiras *et al.*, 2008a).

As with tunas, problems in age estimation using spines in *X. gladius* arise from the following main sources of error:
- presence of multiple bands and false bands;
- progressive disappearance of inner bands in larger specimens.

Interpretation of multiple and false bands seems to be easier than in *T. thynnus* and *T. alalunga*. Some criteria can be adopted to help identify these structures (Berkeley and Houde, 1983; Tserpes and Tsimenides, 1995):
- Identification of multiple bands is usually based on observation of the width (distance) between bands: the distance between true annuli decreases proportionally with age, slowly but rather constantly, while, in multiple bands, the distance between two or three consecutive bands is clearly reduced (Plate 185-A).
- False annuli are easily detected because they are not clearly tracked along the entire circumference of the section (Plate 185-B).

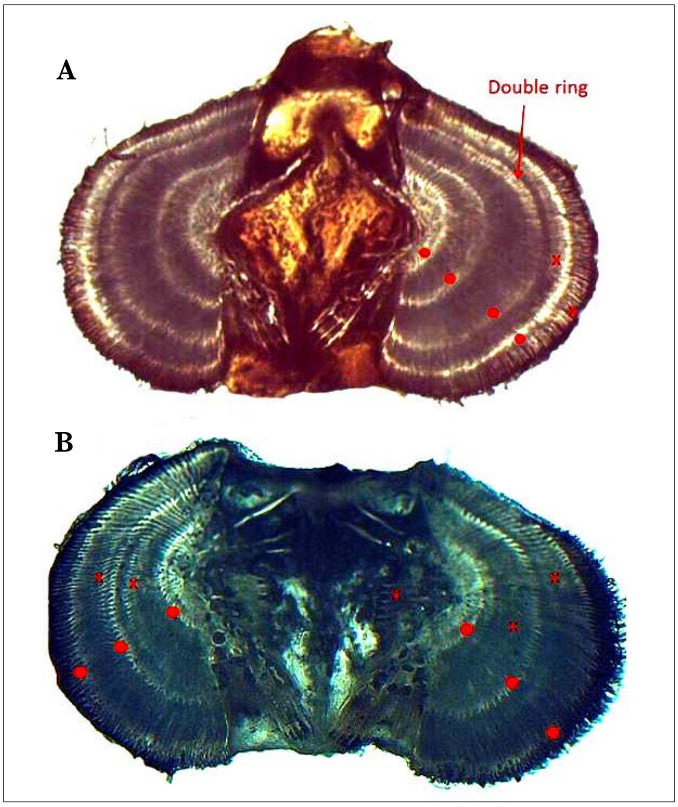
Unlike with tuna, disappearance of the inner bands in *X. gladius* is limited, probably to the first annulus in larger specimens. This is due to a different modality in the growth of *X. gladius* spines, without a true resorption of the vascularized central area, as previously described in *T. thynnus* and *T. alalunga* (Garibaldi and Lanteri, 2017) (Plates 186 and 187).

Calculation of the radius of the first annulus is fundamental in determining the exact location of the first inner ring, in larger specimens as well. The measurement can be easily made in juveniles, starting from the focus, which is placed on the edge of the vascularized area, towards the edge of the first translucent ring, along an imaginary axis passing through the diameter of the section (Plate 188) (Berkley and Houd, 1983; Tserpes and Tsimenides, 1995; Sun, Wang and Yeh, 2002). Quelle *et al.* (2014) recently proposed a new routine to identify the focus that also takes into account the irregular development and different morphology of the central vascularized area of the section.

Tserpes and Tsimenides (1995) calculated this position at a distance of 1.5 mm from the focus (standard deviation 0.05) by measuring a sample of individuals of a size from LJFL 80 to 90 cm.

An alternative methodology for identifying the exact location of the first annulus is proposed by De Martini *et al.* (2007) based on the comparative relationship between the size of the spine radius and the daily growth increments observed in otoliths. At completion of a year (365 daily increments), the radius corresponded to about 2 mm.

PLATE 185
Spine sections of *X. gladius*: A – age 4 years;
B – age 3 years



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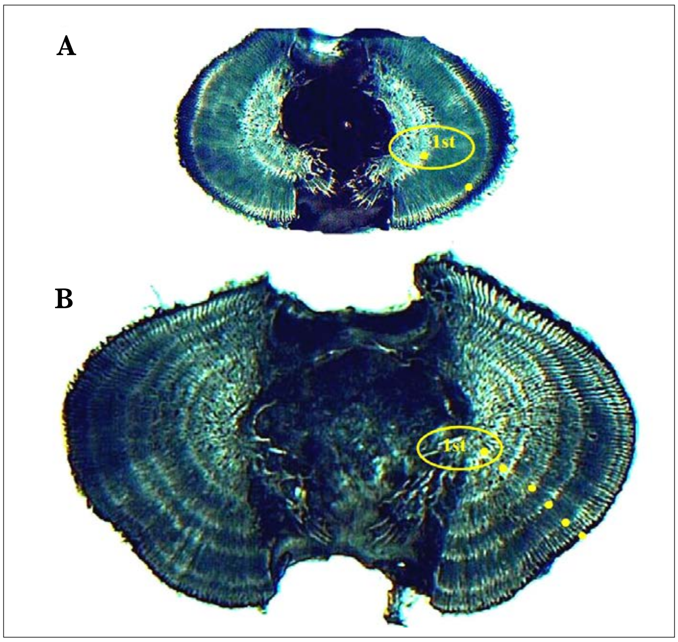
Note: Red dots = translucent bands, red crosses = false bands.

PLATE 186
Spine sections of *X. gladius*: A – age 2 years;
B – age 6 years



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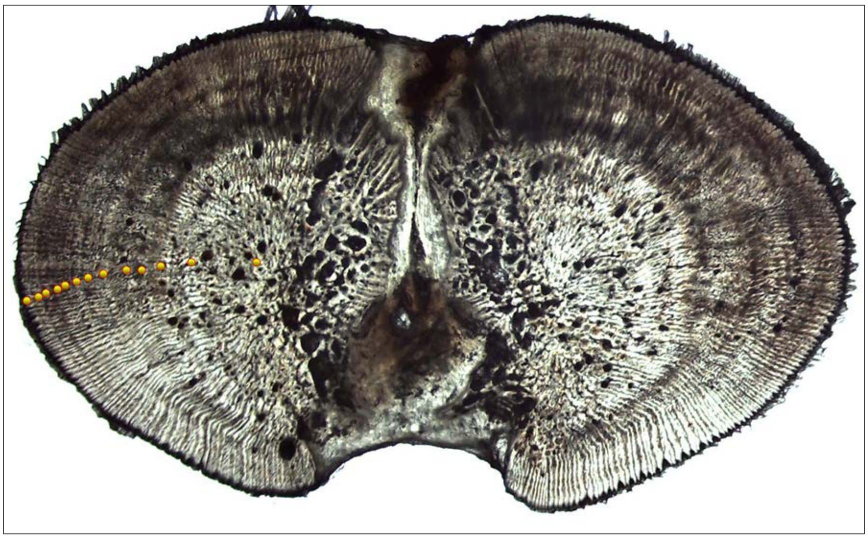
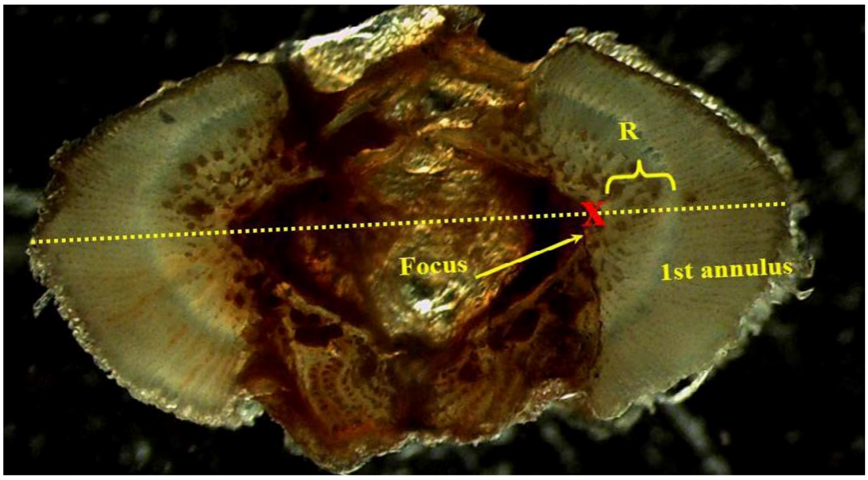
Note: First translucent band (yellow circle) is less visible in larger specimen.

PLATE 187
Spine section of a tagged and recaptured male *X. gladius* – age 13 years



Note: Inner annulus is still clearly visible.

PLATE 188
Ring radius from focus to first annulus in spine section of *X. gladius*



5.4.3 *Tetrapturus belone*

The most common species endemic to the Mediterranean Sea is *T. belone*. It is mainly caught in the southern Tyrrhenian Sea and the Strait of Messina, where it is a target species, together with *X. gladius*, *T. thynnus* and *C. hippurus*, of the harpoon fishery. It can be captured as bycatch by the pelagic longline and gillnet fisheries (Potoschi, 2000; Di Natale *et al.*, 2003, 2005).

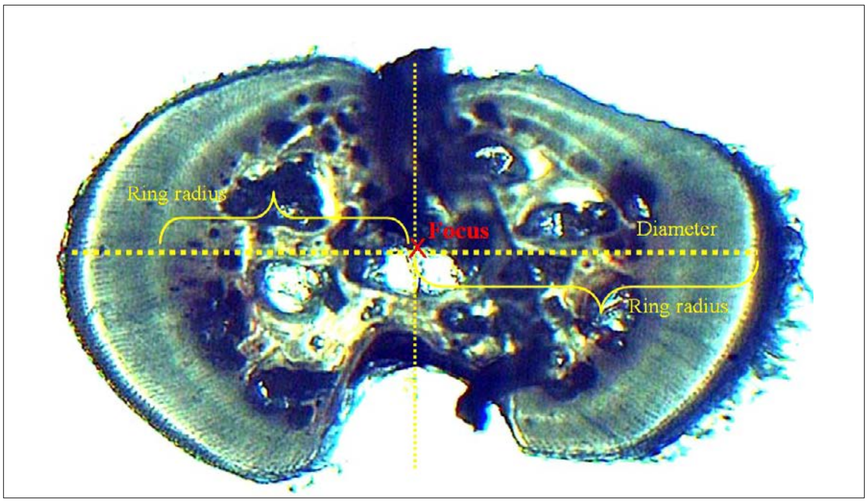
As already indicated for *X. gladius*, studies on age determination of diverse Istiophoridae were carried out in the past using also otoliths (Radtke, 1983; Prince *et al.*, 1984, 1991; Hill, Cailliet and Radtke, 1989; Kopf *et al.*, 2011), but mainly spines of the dorsal and anal fins (Berkeley and Houde, 1983; Hedgepeth and Jolley, 1983; Hill, Cailliet and Radtke, 1989; Melo-Barrera, Felix-Uraga and Quinonez-Velazquez, 2003; Chiang *et al.,* 2004; Drew, Die and Arocha, 2006a; Hoolihan, 2006; Kopf, Drew and Humphreys, 2010; Kopf *et al.*, 2011). Vertebrae provided bad results (Prince *et al.*, 1984; Hill, Cailliet and Radtke, 1989).

Given that stock assessment for the *T. belone* had not been performed by ICCAT, no official growth model was available. The only study was carried out in the area of the Strait of Messina on the basis of 457 specimens ranging from TL 97.5 to 215 cm. Analysis of the sections of the second anal fin ray showed very fast growth up to the age of four years (Potoschi, 2000).

Given that the most important issues in ageing analysis using spine sections are very similar to those described above for *X. gladius*, only some considerations for age estimation of Istiophoridae will be reported:

- False bands: verify that translucent rings are clearly visible throughout the whole section (dorsal-ventral side), as reported for *X. gladius* (subsection 5.4.2.2).
- Identification of the first growth increments: generally central rings are reabsorbed from the vascularized central part of the spine (Drew, Die and Arocha, 2006a, 2006b; Kopf, Drew and Humphreys, 2010; Kopf *et al.*, 2011).
- Measurement of ring radius: this is slightly different than in *X. gladius* due to the diverse morphology of the spine section (Plate 189) (Drew, Die and Arocha, 2006a, 2006b; Kopf, Drew and Humphreys, 2010).

PLATE 189
Anal spine section of *T. belone*: measurement of spine diameter and ring radius of translucent bands



5.4.4 *Sarda sarda*

Several methods have been used over the years in studying age and growth in the Atlantic bonito (*S. sarda*). Modal progression analysis was performed in the past, but it was helpful only in identifying the first two age classes, which represent the main component of catches in many fisheries (Dardignac, 1962; Rodríguez-Roda, 1966, 1981; Macías *et al.*, 2006; Valeiras *et al.*, 2008b; Di Natale and Mangano, 2009).

Other studies using calcified structures (otoliths, spines, vertebrae) provided results not always in agreement, so that, at present, it is not evident which could be the most suitable structure for ageing analysis of this species (Dardignac, 1962; Rodríguez-Roda, 1966, 1981; Rey, Alot and Ramos, 1984, 1986; Santamaría *et al.*, 1998; Orsi Relini *et al.*, 2005; Zaboukas and Megalofonou, 2007; Ateş, Deval and Bok, 2008; Valeiras *et al.*, 2008b; Di Natale and Mangano, 2009; Cengiz, 2013; Kahraman *et al.*, 2014).

Santamaría, Deflorio and De Metrio (2005), counting the daily increments in specimens ranging from TL 10.5 to 39.8 cm, estimated a daily growth rate of 5.83 mm/day for the first 120 days of life. This confirms significant growth during the first year, as observed by modal progression analysis as well (Rey, Alot and Ramos, 1986). In recent studies in the Black Sea, at least four age groups (zero to three years) were identified (Ateş, Deval and Bok, 2008; Cengiz, 2013; Kahraman *et al.*, 2014). Rey, Alot and Ramos (1984), comparing the three hard parts (otoliths, vertebrae and fin rays), reported a maximum age of four years for a specimen of TL 71 cm.

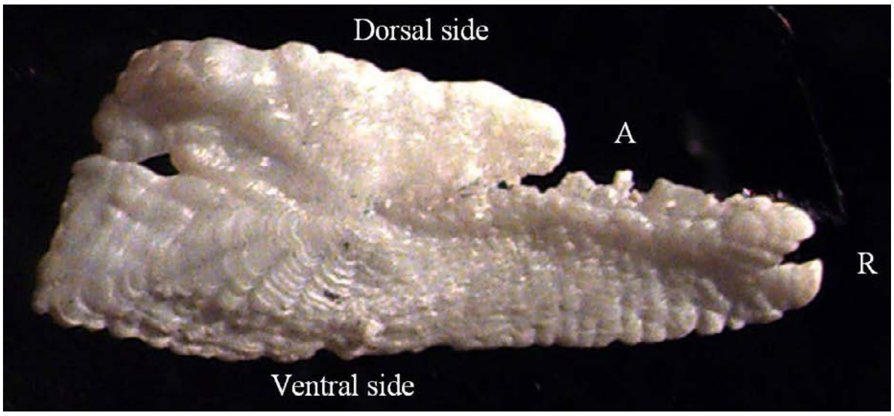
Zusser (1954), on the basis of modal progression analysis, estimated a maximum age of nine years. Recent studies based on analysis of sections of the first dorsal fin spine reported some age–length keys (Zaboukas and Megalofonou, 2007; Valeiras *et al.*, 2008b; Di Natale e Mangano, 2009), showing a maximum age of seven years (Zaboukas and Megalofonou, 2007).

In our opinion, it is advisable to collect otoliths, spines and vertebrae from each specimen to compare the three calcified structures. Some indications on analysis of these structures are reported below:

5.4.4.1 Otoliths

This otolith shape is reminiscent of a tuna-like species, but size and morphology are slightly different. In the whole otolith (Plate 190), detection of annual marks seems quite difficult, while use of a transverse section is not advisable and usually provides poor results.

PLATE 190
S. sarda whole otolith



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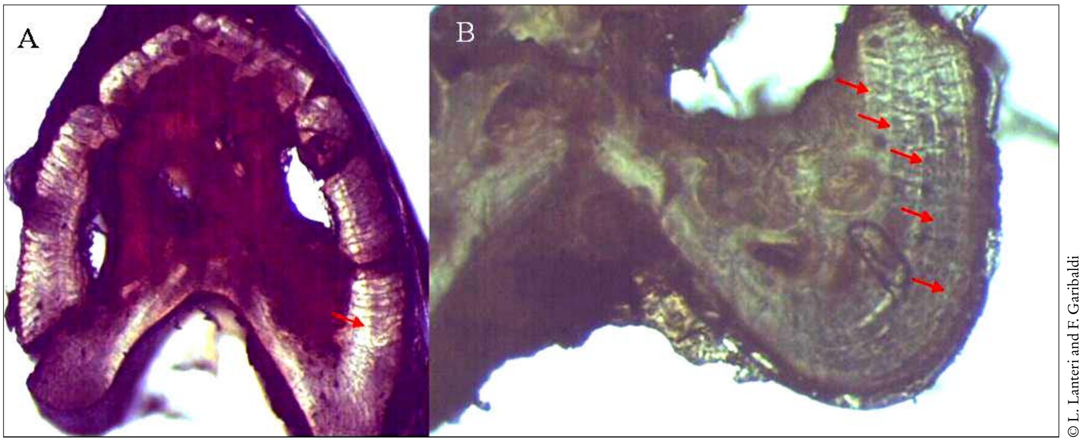
5.4.4.2 Spines

As with other species, the first dorsal fin spine section is easier to collect than otoliths and vertebrae and is considered more suitable for age determination (Zaboukas and Megalofonou, 2007; Valeiras *et al*., 2008b; Di Natale and Mangano, 2009). The same issues discussed for other tuna-like species are also true for *S. sarda*: a resorption of inner rings, caused by vascularization of the central area of the spines (Plate 191) (Zaboukas and Megalofonou, 2007) could be overcome using ring diameters, as with *T. thynnus* and *T. alalunga* (see Plate 175), to detect the lost inner rings.

PLATE 191
S. sarda spine sections



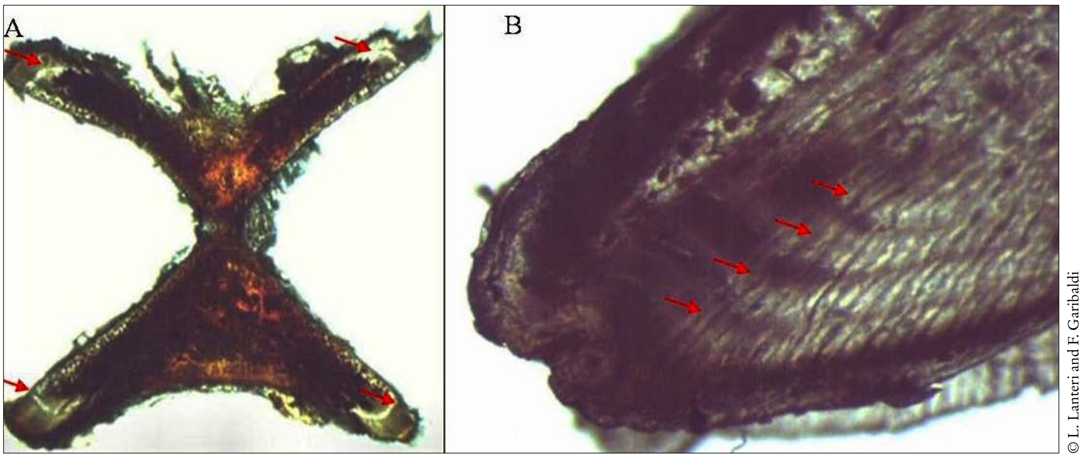
Note: A – age 1 year; B – age 5 years.

5.4.4.3 Vertebrae

Vertebrae are often adopted as a check against other structures (Rodríguez-Roda, 1966, 1981; Rey, Alot and Ramos, 1984, 1986; Santamaría *et al.*, 1998). The whole vertebra has rarely been used (Rey, Alot and Ramos, 1984), probably because it doesn't show clearly visible annual growth structures (ridges, grooves), as previously reported for *T. thynnus* (subsection 5.4.1.3).

Section readability is heavily affected by a massive vascularization of the central area (Plate 192-A), but information in the literature on this issue is scarce. Growth bands, when visible, are generally positioned on the edge of the vertebral body (Plate 192-B), with a possible underestimation of the age due to resorption of the first inner rings.

PLATE 192
S. sarda vertebral section



Note: A – age 1 year; B – age 4 years.

5.4.5 *Coryphaena hippurus*

C. hippurus is an epipelagic species exploited in some Mediterranean areas (Sicily, the Balearic Islands, Malta, Tunisia) by FADs, mainly composed of palm leaves or other floating materials, coupled with surrounding nets, traditionally called *lampughera* in Spain and *cannizzati* in Italy (Galea, 1961; Iglesias *et al.*, 1994; Massutí and Morales-Nin, 1991; Potoschi, 1998; Potoschi and

Sturiale, 1996; Bono *et al.*, 1998; Cannizzaro, D'Andrea and Pizzicori, 1998; Besbes Benseddik *et al.*, 2011). This is a seasonal fishery (from summer to late autumn) that takes advantage of the gregarious behaviour of this species during the recruitment period (juveniles tend to concentrate under floating objects) (Potoschi, 1998; Massutí and Moralez-Nin, 1995; Bono *et al.*, 1998).

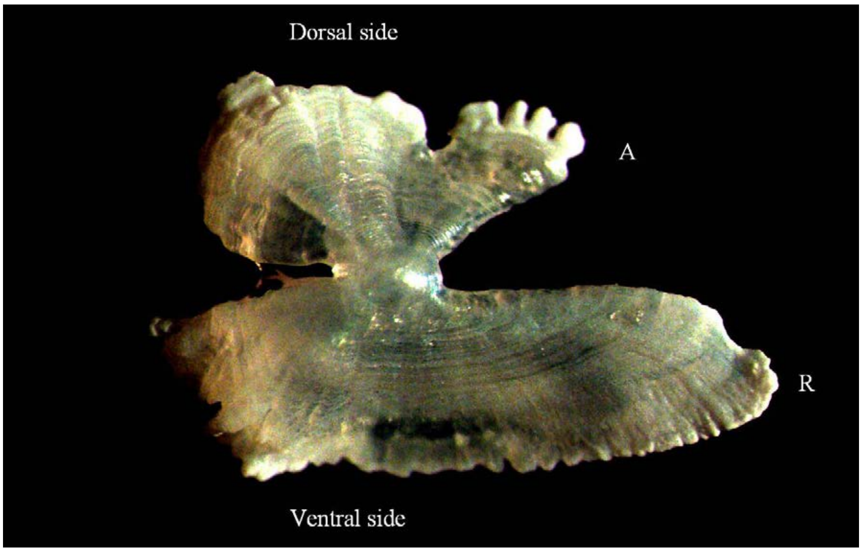
Adult specimens (> 1 year) have been frequently caught as bycatch of drifting longline, harpoon and recreational fisheries (Massutí and Morales-Nin, 1995; Massutí, Morales-Nin and Moranta, 1999; Potoschi, Reñones and Cannizzaro, 1999).

Methods used for age determination include modal progression analysis of L/F distributions and hard parts such as otoliths, vertebrae and scales (Oxenford and Hunte, 1983; Di Stefano *et al.*, 1998; Massutí, Moralez-Nin and Moranta, 1999; Moralez-Nin *et al.*, 1999; Rivera and Appeldoorn, 2000; Schwenke and Buckel, 2008; Besbes Benseddik *et al.*, 2011).

In the Mediterranean area, *C. hippurus* show very fast growth in the early life stage, reaching a length of TL 60–70 cm in 6–8 months (Di Stefano *et al.,* 1998; Massutí and Morales-Nin, 1999; Moralez-Nin *et al.*, 1999; Besbes Benseddik *et al.*, 2011) and a maximum estimated age of three to four years (~ TL 120 cm) (Potoschi, 1998; Massutí, Morales-Nin and Moranta, 1999). These studies highlight the fact that otoliths represent the best structure for identifying daily increments in young of the year (YOY) (up to ~ TL 60 cm), while scales give better results in identifying annuli in adult specimens (> TL 70 cm) (Moralez-Nin *et al.*, 1999; Massutí, Morales-Nin and Moranta, 1999).

The sagitta of *C. hippurus* looks like that of the *X. gladius* in shape and size, showing convex sides, and a pronounced rostrum (R) and antirostrum (A) (Plate 193). An attempt to use lapilli and vertebrae for juveniles provided interesting results (Di Stefano *et al.*, 1998; Morales-Nin *et al.*, 1999), but not always in agreement with what has been observed on the sagitta of the same specimens.

PLATE 193
Sagittal otolith of *C. hippurus*: R – rostrum, A – antirostrum

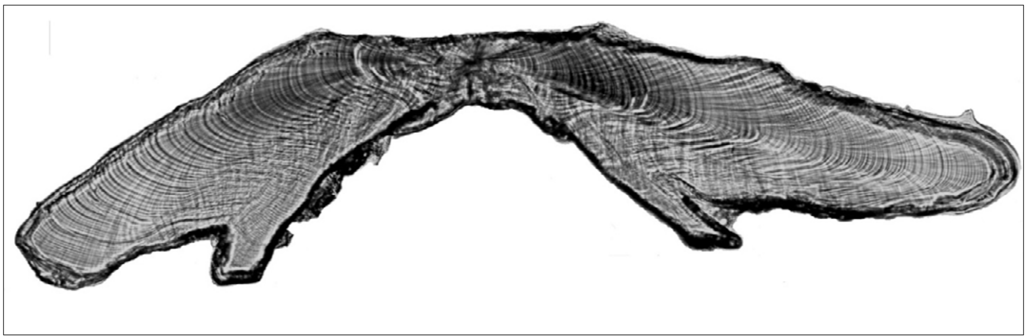


Reading of daily increments on the whole otolith (once polished on the sagittal plane) is generally performed starting from the central core, along the dorsal side of the otolith (Massutí, Morales-Nin and Moranta, 1999; Moralez-Nin *et al.*, 1999).

Plate 194 presents a sagittal otolith transverse section of a specimen with 94 daily increments (TL 38 cm; female).

C. hippurus growth has been studied almost exclusively in YOY, given that the majority of catches come from FAD fisheries (Potoschi, 1998; Di Stefano *et al.,* 1998; Massutí and Morales-Nin, 1999; Moralez-Nin *et al.*, 1999). The limited information available in the literature does not support identification of a useful protocol for age determination in larger specimens (> 1 year). In our opinion, the use of scales could lead to an underestimation of age.

PLATE 194
Sagittal otolith transverse section of *C. hippurus*



Note: Age: 94 days; TL = 38 cm.

6. Diadromous species

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6.1 *Anguilla anguilla*

A. anguilla is a diadromous panmictic species (Als *et al.*, 2011) and a shared fishery resource exploited by practically all European and Mediterranean countries. For this species, major problems exist in relation to a continent-wide decline in recruitment observed in the course of the last decades, and to a contraction in adult *A. anguilla* capture fisheries (ICES, 2001; Aalto *et al.*, 2015). *A. anguilla* shows some peculiar features compared with other shared species or other migratory fish. *A. anguilla* exploitation occurs exclusively within national boundaries, in continental waters, without any interaction between economic zones – typical *A. anguill*a fisheries being mainly small-scale. However, spawning takes place in international waters, and all oceanic life stages are unexploited.

Since 2009 the European Union established the Data Collection Framework for Eel (European Council Regulation EC No. 199/2008; EC No. 2017/1004). For the collection, management and use of data in the fisheries sector and support for scientific advice regarding the Common Fisheries Policy. The data collection concerns all *A. anguilla* fisheries in inland and coastal waters, commercial as well as recreational. Moreover the regulation EC No. 1100/2007 required that all member states adopt eel management plans aimed at progressively removing the main causes of *A. anguilla* decline. The plans would guarantee migration towards the sea of at least 40 percent of the silver eel biomass from each catchment basin, with respect to reference conditions defined by the absence of anthropogenic impacts.

The use of sagittal otolith of *A. anguilla* for ageing, rather than other structures, is the most reliable and used method in biological samplings (ICES, 2009b). Knowing age data, among other stock-related variables for a long-live diadromous species such as *A. anguilla*, is particularly important for the modelling quantification of the annual silver eel escapement (Bilotta *et al.* 2011) towards oceanic reproduction.

6.1.1 Otolith extraction and storage

The technique used for otolith extraction in *A. anguilla* is adapted from that described by Moriarty (1973). This technique minimizes loss of and damage to the otoliths and is a quick, clean and efficient method. A primary transverse incision is made behind the eye in two phases using a scissors; first, cutting the skin and flesh and, second, penetrating the cranium through the roof of the mouth and providing access to the cranial cavity.

A. anguilla otoliths, once removed from the heads, are immerged in distilled water, and the attached organic tissues cleaned with the absorbent side of lab bench paper. The otoliths are stored dry in labelled Eppendorf microtubes and left in a heater (at 70 °C) overnight. The microtubes are then closed and stored until otolith examination.

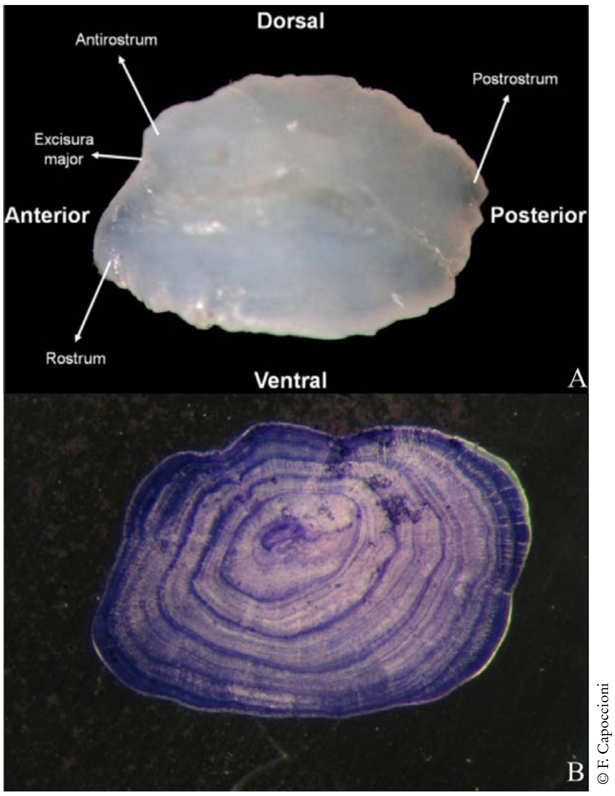
6.1.2 Preparation

The methodology described in this subsection is a modification of the one developed at the Cemagref laboratories (Bordeaux, France) (Capoccioni *et al.*, 2014). Today it is widely used in many laboratories in Italy (including at the Università degli studi di Roma "Tor Vergata"), as it is proposed as the methodology of preference in determining the age of *A. anguilla* of no more than 15 years. With respect to the burning and cracking method, results are easier to interpret and more reliable (ICES, 2009b).

The age of *A. anguilla* is assessed by counting the annuli illuminated by polarized or transmitted light after grinding and polishing (Plate 195).

Each left otolith is placed at the bottom of a numbered mould cavity, with the distal face up. Then drops of an epoxy resin are added to each cavity until the mould is completely filled. Bubbles under the otoliths are gently removed by moving the sample with a needle. Moulds are left to dry overnight until the resin becomes hard. The resin blocks with the embedded otoliths are then removed from the moulds. With the otolith's convex side up, each block is mounted with a drop of Eukitt (transparent glue) onto a histological slide with quick pressure. Slides are labelled with the appropriate code for each otolith (Plate 196).

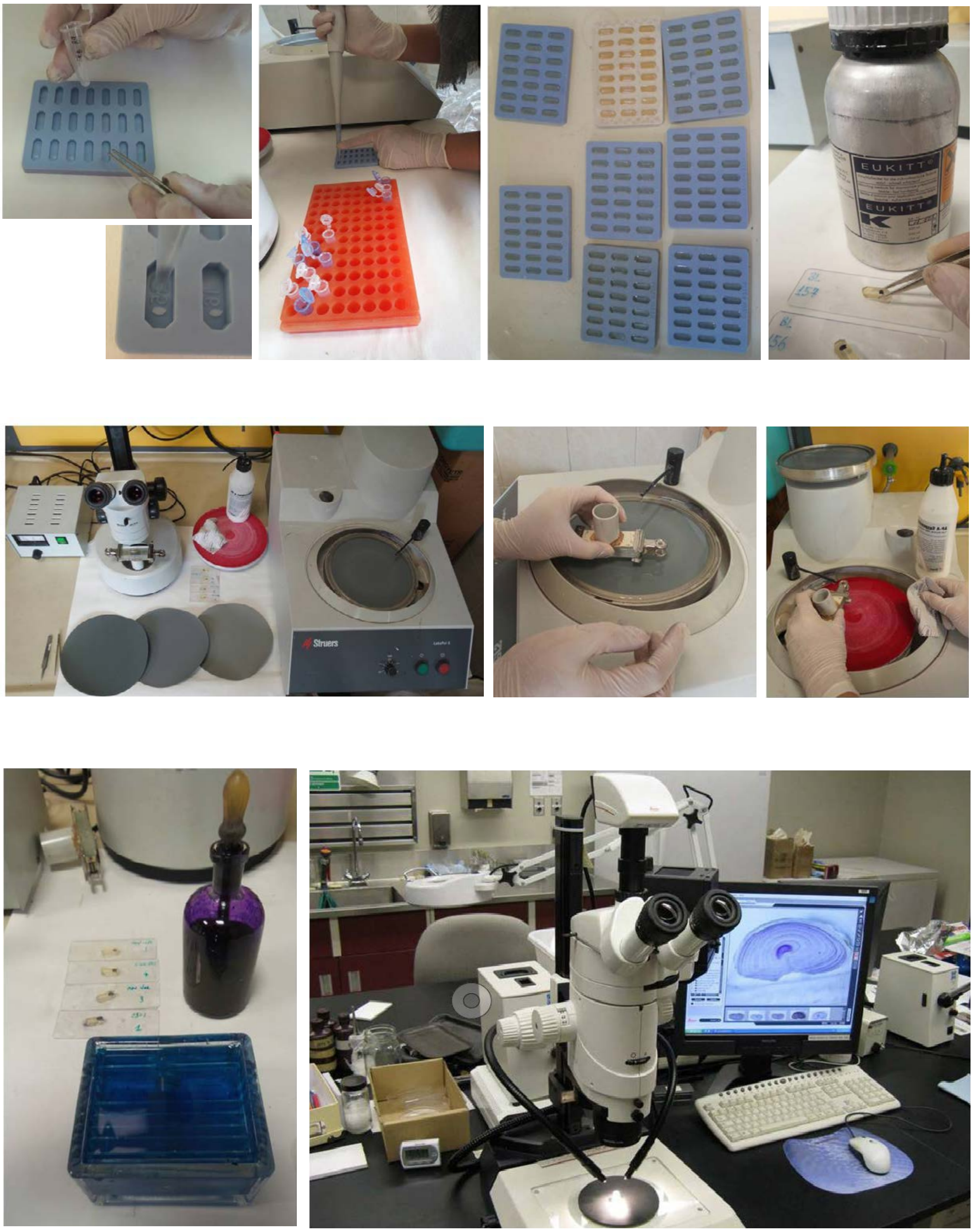
PLATE 195
Proximal face of an *A. anguilla* right otolith



Note: A – Anterior and posterior regions are aligned with the orientation of the body of *A. anguilla*; B – Example of *A. anguilla* right otolith analysed using the grinding and polishing technique.

The grinding procedure is carried out using a Struers grinding machine (LABPOL-5), beginning with 1 200-grit silicon-carbide sanding papers, and increasing to 5 000 grits until the centre and edge of the otolith are visible. Slides are continually checked to ensure that the grinding is in the right direction and with sufficient force and that the core has not been removed. When the primordium is exposed and is easily recognized as a black point inside the core, the otolith is polished with a jewellery cloth and an abrasive paste (suspension of 1μ alumina) to remove any score lines.

PLATE 196
A. anguilla otolith ageing analysis procedure, with grinding and polishing technique



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The sample is now ready for hatching with an acid preparation and then for the staining process. A drop of 5-percent EDTA is applied on each otolith for three minutes and then rinsed with distilled water. Subsequently, a drop of 5-percent toluidine blue is applied to the ground otolith surface. The stained otoliths are left to dry overnight and then immersed in distilled water for an hour.

They are now ready for observation under a binocular microscope with an image acquisition system. Results are recorded in an electronic spreadsheet program database (e.g. an Excel file).

The same reader analyses the otoliths again after three weeks and the 'second opinion' is also recorded in a file as above. After these two sessions, in the presence of contrasting evaluations of age the most frequent evaluations are accepted.

6.1.3 Interpretation

Rings are deposited on otoliths each year, alternating normally one opaque (in summer) and one translucent (in winter). Winter checks are thin and narrow, because the accretion rate varies with the growth of the fish, and during cold months *A. anguilla* metabolism is slow. A year's growth consists of both an opaque and a translucent zone.

Ageing evaluation of *A. anguill*a for the continental phase has traditionally begun from the first clearly marked band outside the nucleus. This 'zero' band (at about a 170 µm radius from the centre) is assumed to be the beginning of the continental growth of *A. anguilla*, and equates to the total length of the glass eel (Moriarty, 1983; Poole, Reynolds and Moriarty, 2004). The next annulus is considered the end of the first year of growth (Plate 197). The conventional birth date is set at 1 January, and, for ageing analysis, the capture date is crucial and must be recorded.

For yellow eel, attention should be drawn to the capture time and local conditions. The appearance of winter annuli on the otolith could vary depending on the starting point and duration of the growing season in the capture location. In the early part of the year, the outermost winter annulus might not be apparent until summer growth begins (in early spring, i.e. 1 April). This will vary by years and by location. Thus an additional year should be added to those eels sampled early in the year after 1 January.

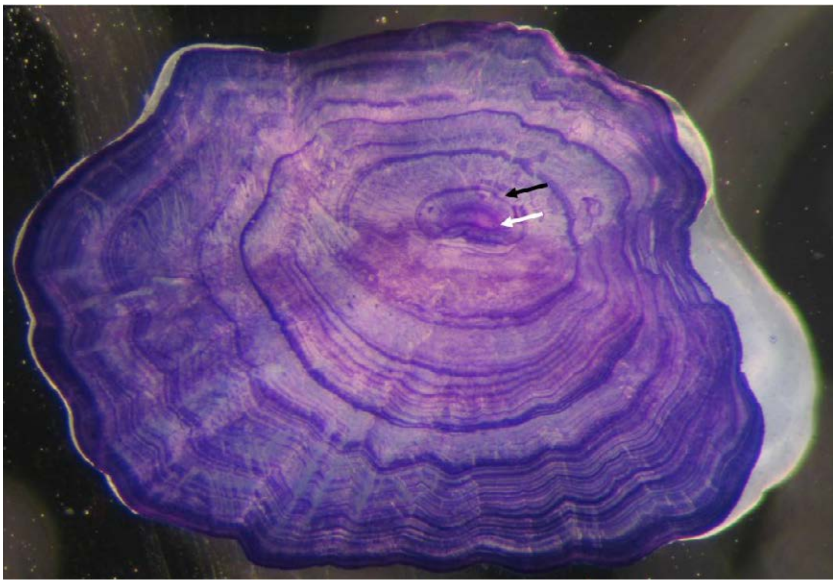
Ageing analysis of silver eel may require flexible interpretation, as their metamorphosis occurs between September and January. They then traditionally migrate to the Sargasso Sea as an annual migration cohort. This cohort receives its age from the following January, as the eels have completed their annual growth period. We assume a putative annulus on the outer margin of the otolith (e.g. silver eels migrating in December 2014 take their age from January 2015) (ICES, 2009b) (Table 21).

The presence of additional checks (false annuli) between two consecutive annual rings (one annual growth period) is also possible. Thus an overestimation of age may occur and, as a result, an underestimation of growth.

High water temperatures and associated low oxygen concentrations in summer or other stress factors in winter can result in the formation of one or more false annuli (Tzeng, Wu and Wickström, 1994; Domingos, Costa and Costa, 2006), as these stressors can produce periods of little or no growth.

The scientific literature (Table 22) and dedicated ICES workshops (Graynoth, 1999; ICES, 2009b, 2011b) have identified several guidelines for discriminating between true and false annuli:

PLATE 197
Ground, polished and stained otolith (sagittal plane) from *A. anguilla*


Source: F. Capoccioni, 2014.

Note: white arrow = nucleus, black arrow = zero band.

TABLE 21 – General ageing scheme for *A. anguilla*

	Date capture	Otolith edge	Age
Yellow eels	1 October – 31 December	Visible opaque ring	N-1
		Thick translucent zone	N
	1 January – 31 March	Visible opaque ring	N
		Thick translucent zone	N+1
Silver eels	30 September – 31 December	Visible opaque ring	N
		Thick translucent zone	N+1

Note: N is the number of opaque rings starting from the first clearly marked band outside the nucleus (0 band).

Table 22 – Criteria for separating winter annuli from summer growth bands and supernumerary checks on *A. anguilla* otoliths

Feature	Summer growth band	Winter annuli	Supernumerary check
Colour	White, often light brown in narrow bands in burnt otoliths, opaque in stained sections viewed using transmitted light	Black or dark brown, dark blue or violet in stained otoliths	Light brown, lighter stain or transppparent in stained otoliths
Width	Much wider than annuli, always >5 µm, usually >15 µm, mean 61 µm, sd 24 µm, *n*=243	Usually 4-18µm, median= 10 µm, *n*=65	Narrow <40% of annuli width, usually <4 µm mu and always < 10 µm
Fine structure	Thin dark lines or slriations occasionally seen	Multiple thin lines visible when stained	Usually one or two thin lines
Continuity	Continuous band	Continuous band	Sometimes broken and not visible on dorsal axis
Position	Uniform spacing	Uniform spacing	Often present adjacent to annulus or nucleus

Source: Graynoth, 1999.

- A clearly visible bold growth check can be considered an annulus.
- In the case of a ground sagittal plane, growth checks should be visible continuously around the otolith to be considered annuli.
- False annuli are usually of lesser strength than annuli, are discontinuous and/or merge with adjacent checks.

- *A. anguilla* growth is highly variable and thus it is difficult to predict ‘normal’ patterns of annual growth to facilitate identification of false annuli.
- Checks that appear too close to neighbouring checks may be false annuli and should be treated with caution.

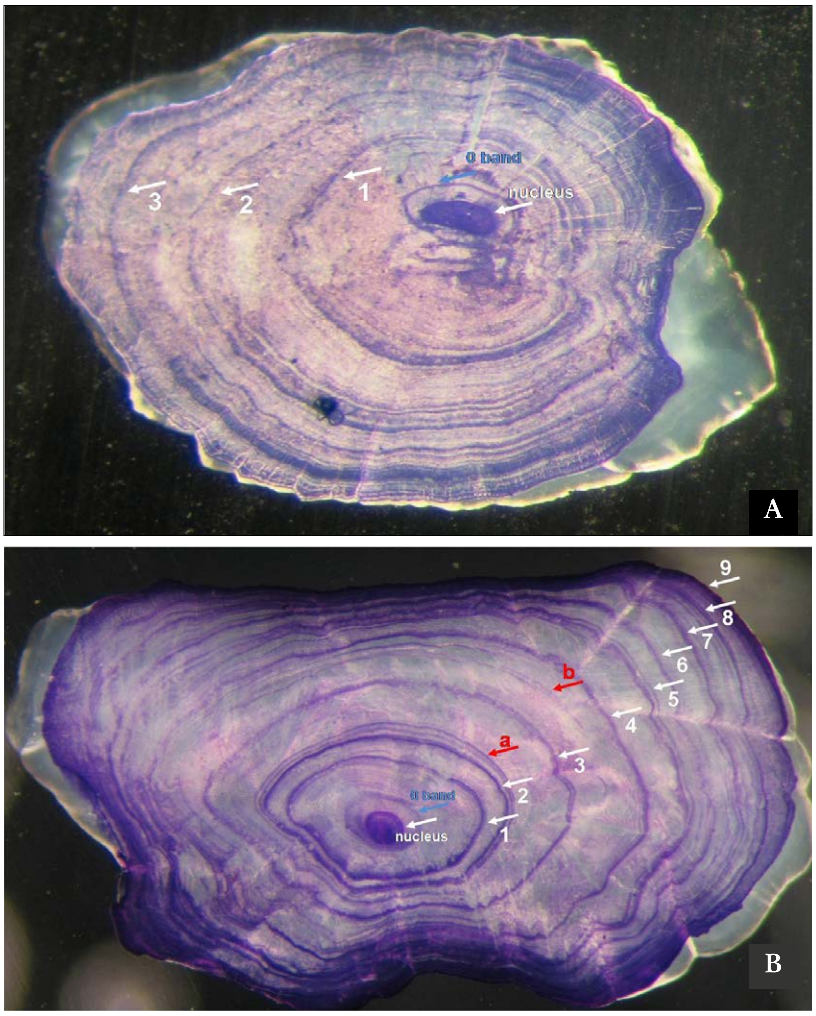
Two examples of aged *A. anguilla* otoliths are shown in Plate 198. In case A, the otolith presents three clear marks. As the specimen has been sampled in winter, the last annulus corresponding to the fourth year is not yet visible, but *A. anguilla* must be correctly aged as an individual of four years old.

In case B, a silver eel, nine years old, presents two typologies of false annulus: the red arrow “a” indicates a false check (false winter check) adjacent to the second-year ring.

The red arrow “b” indicates another kind of false annulus between the third- and fourth-year rings, probably due to a stress factor occurring in summer (false summer check). This event produced the deposition of an additional check on the otolith.

Given the relationship between stress and metabolism and the creation of annual and false check bands within otoliths, it is advisable that readers have additional information on life-cycle and on environmental data on the origin of the specimen in order to assist in correct age interpretation (ICES, 2009b).

PLATE 198
Two examples of aged *A. anguilla* otoliths



Source: F. Capoccioni, 2014.

Note: A – otolith of a 4-year old *A. anguilla* sampled in December; B – silver eel otolith presenting two false checks.

7. Glossary

The morphological description of otoliths is based on the terminology proposed by Secor, Dean and Miller (1995) and Panfili *et al.* (2002) (Plates 199 and 200).

PLATE 199
Views of right sagitta from *T. mediterraneus* with indication of basic structure

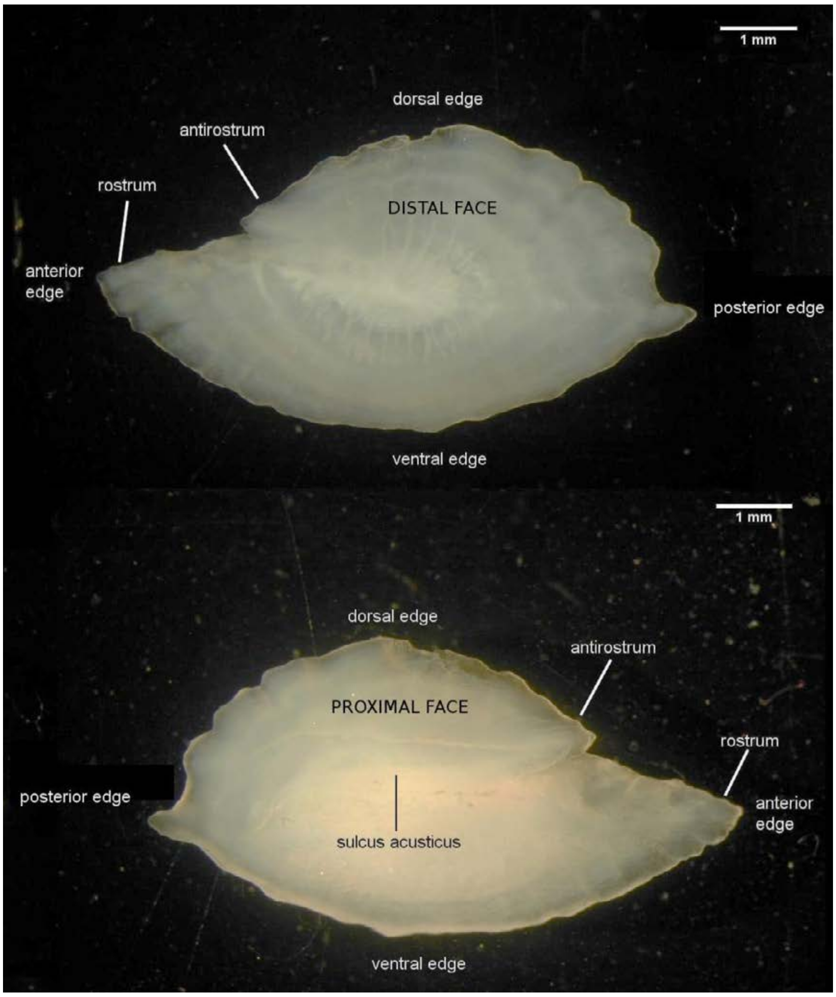


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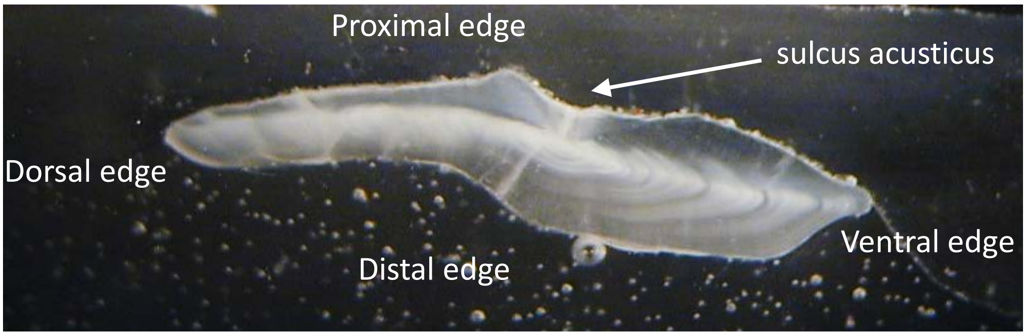
PLATE 200
Transverse thin section through core of *M. merluccius* sagitta with reflected light illumination



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Accuracy. The closeness of a quantity estimation (measured or computed value) to its true value.

Age. Age (months, half years, years) is calculated by counting the number of translucent increments, but taking into consideration the date of birth and the date of capture.

Age class. Age class corresponds to number of years. Roman numerals are used for the age class.

Age group. The age group is the number of calendar years after the birth date. The age group to which a fish will be assigned depends on the year in which it was spawned and on the date of capture. Arabic numerals are traditionally used to reflect an age group.

Annulus. One of a series of concentric zones on a calcified structure (CS) that may be interpreted in terms of age. This term usually indicates one transparent ring plus one opaque area/ring. In some cases, an annulus may not be continuous or obviously concentric. The optical appearance of these marks depends on the calcified structure and the species, and should be defined in terms of specific characteristics of the structure. This term has traditionally been used to designate 'year' marks, even though the term is derived from the Latin *anus*, meaning ring, not from *annus*, meaning year. For otoliths, the variations in microstructure that make an annulus a distinctive region are not well understood.

Back-calculation. The back-calculation procedure can be defined as estimating fish size at an earlier time (or times) on the basis of a set of measurements of CS size and fish size, made at a single point in time (usually at capture).

Birth date. The theoretical date when fish hatched; typically, 1 January is used for fish with a spawning period in autumn/winter and 1 July for a spawning period in spring/summer.

Birthmark. A clear variation in the angle of the corpus calcareum. It corresponds to a growth rate acceleration in the individual.

Centra. Central part of vertebral segments.

Check. A discontinuity (e.g. a stress-induced mark) in a pattern of opaque and translucent zones, or microincrements. Checks often appear as an abrupt change in the growth pattern.

Core. The area or areas surrounding one or more primordia and bounded by the first prominent opaque ring.

Corpus calcareum. Load-bearing axis of the vertebral centra. It represents the main structure for counting the annuli.

Corroboration. The measure of the consistency or repeatability of an age determination method.

Double band. A growth mark or check not accepted for annual age determination – also referred to as a growth check or false annulus.

Growth. The change in body or body part size between two points in time.

Hyaline zone. A zone that allows the passage of greater quantities of light than an opaque zone. However, the term hyaline should be avoided; the preferred term is translucent.

Intermedialia. Portion of cartilaginous tissue of the vertebral centra comprised between the arms of the corpus calcareum.

Marginal increment. The region beyond the last identifiable mark at the margin of a structure used for age estimation. Quantitatively, this increment is usually expressed in relative terms, that is, as a fraction or proportion of the last complete annual or daily increment.

Nucleus, kernel. Collective terms originally used to indicate the primordium and core of the otolith. These collective terms are considered ambiguous and should not be used. The preferred terms are primordium and core (see their definitions).

Opaque zone. A zone that restricts the passage of light when compared with a translucent zone. The term is relative, because a zone is determined to be opaque on the basis of the appearance of adjacent zones in the otolith (see 'translucent zone'). In untreated otoliths under transmitted light, the opaque zone appears dark and the translucent zone bright. On the contrary, under reflected light, the opaque zone appears bright and the translucent zone dark.

Precision. The closeness of repeated measurements of the same quantity. For a measurement technique that is free of bias, precision implies accuracy, but the two terms are not equivalent.

Primordium. The initial complex structure of an otolith, it consists of granular or fibrillar material surrounding one or more optically dense nuclei from 0.5 mm to 1.0 mm in diameter. In the early stages of otolith growth, if several primordia are present, they are generally fused together to form the otolith core.

Secondary structure. A term used for all macroscopic zonations that do not appear to conform to the opaque and translucent zones of an annulus. The main examples are false and split or double rings/zones.

Translucent zone. A zone that allows the passage of greater quantities of light than an opaque zone. The term is relative, because a zone is determined to be translucent on the basis of the appearance of adjacent zones in the otolith (see 'opaque zone'). An absolute value for the optical density of such a zone is not implied. In untreated otoliths under transmitted light, the translucent zone appears bright and the opaque zone dark. The term hyaline is also used, but translucent is the preferred term.

Validation. The process of estimating the accuracy of an age estimation method. The concept of validation is one of degree and should not be considered in absolute terms. If the method involves counting zones, then part of the validation process involves confirming the temporal significance of the zones being counted. Validation of an age estimation procedure indicates that the method is sound and based on fact.

Verification. The process of establishing that something is true. Individual age estimates can be verified if a validated age estimation method has been employed. Verification implies the testing of something, such as a hypothesis, that can be determined in absolute terms to be either true or false. See 'corroboration'.

Vertebral focus. The central and innermost part of the vertebral conus.

Zero band. The first growth check of *A. anguilla* outside the nucleus, from where continental age determination begins.

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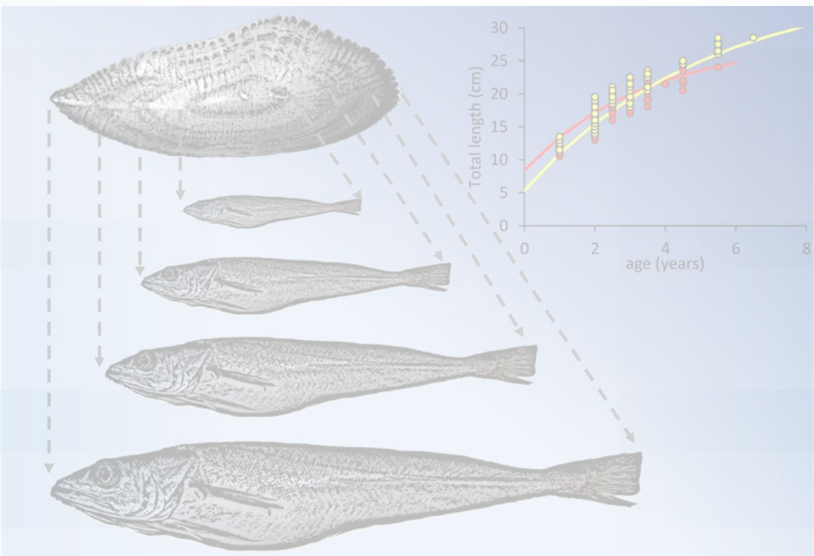
HANDBOOK ON FISH AGE DETERMINATION

This Handbook stems from an experience on fish ageing analysis carried carried out at the Mediterranean level. It aims to provide guidelines to standardize the current methods used in fish ageing studies and it gives an overview of the general principles on which age analysis relies (assignment of birth date, preparation methods, aging scheme reading and identification of true and false rings). The volume provides information on extraction and storage, preparation methods, age interpretation and ageing criteria by species, analysing a total of 30 species. As such, it represents one of the most complete outlooks on fish ageing analysis in the Mediterranean context.

Fish age, among other biological parameters, is one of the most relevant pieces of data in reaching sustainable exploitation of fisheries resources. Indeed, most analytical methods used in stock assessment require knowledge of demographic structure according to the age of stocks, as well as to recruitment, growth, maturity, natural mortality, etc., which are strictly linked to information on age and age structure.

The literature on ageing analysis shows some gaps regarding ageing schemes, criteria and methodologies used in preparing calcified structures. These aspects affect both the precision and accuracy of age estimation. One action that could be taken to overcome this gap was to formalize a handbook that clarified approaches to ageing schemes, criteria and preparation methods. Having a common protocol is indeed fundamental to decreasing bias associated with the activities of age determination and to improving precision in age reading.



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