ICES COOPERATIVE RESEARCH REPORT (CRR) ON FISH AGEING

CHAPTER 5: Small and Medium Pelagic Species

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1. Introduction

The knowledge of age and growth of fish populations is essential to understand their life cycle (life span, age of recruitment, age of sexual maturity, spawning season, migrations, mortality). In fishery biology it is also important for the study of the demographic structure and its dynamics, which is fundamental to the assessment and management of fish populations subject to fishery exploitation, when age-structured evaluation models are used (Panfili et al., 2002).

Over the years, the importance of the study of age and growth in fisheries biology has grown in parallel with the increase in the number of stocks subject to assessment and management (Panfili et al., 2002). However, the study of growth remains a complex issue. In various international commissions of fisheries management, especially in ICES (International Council for the Exploration of the Sea) and GFCM (General Fisheries Commission for the Mediterranean), it has been shown the need to review the protocols of age interpretation of many species. Age determination requires a continuous and never-ending process of maintenance of the consistency of the age estimation criterion of the readers of each laboratory and of those of different laboratories. The ultimate aim of all the work of age interpretation is clear: the establishment of accurate, precise and practical methods, so that they can be routinely used in the age interpretation of all the samples needed for the assessment of exploited species (Panfili et al., 2002) in the context of DCF (Data Collection Framework). The common protocol could be an important tools to decrease the relative/absolute bias and to improve the precision (reduce CV) of age determinations (their reproducibility) between age readers of the different age reading laboratories (ICES, 2011a).

This chapter presents a summary of the age estimation procedures used in European waters (Atlantic and Mediterranean areas) for some of the main commercial small and medium pelagic species anchovy (*Engraulis encrasicolus*), sardine (*Sardina pichardus*), herring (*Clupea arengus*), sprat (*Sprattus sprattus*) mackerel (*Scomber scombrus*), chuck mackerel (*Scomber colias*), horse mackerel (*Trachurus trachurus*) and Mediterranean horse mackerel (*Trachurus mediterraneus*). It provides information about the age estimation criteria and interpretation difficulties. A summary of the information related to the age accuracy, validation and corroboration of each species is also presented, as well as that related to the age precision, quality control and verification. The procedures included in this chapter are derived from the age estimation protocols of the some European Institutes and from the cooperation among institutions through the ICES exchanges and workshops about age interpretation; and others are obtained from the more relevant literature.

2. Summary of age estimation methodologies

Considerable efforts are made by international committees to standardize the age interpretation in European waters. During these exchanges and workshops, the samples used were not validated therefore the « true age » is not known. In this way, the work groups demonstrate the precision of age estimation between readers but not the accuracy (Secor *et al.*, 1995; Panfili *et al*., 2002). For small and median pelagic species in ICES and GFCM waters there are over 30 reports available in the repository of the ICES PGCCDBS (http://www.ices.dk/community/Pages/PGCCDBS-doc-repository.aspx). A summary of the results from the lasts workshops or exchanges for determination of annual age (ICES, 2008a, 2008b, 2009, 2010, 2011b, 2012, 2012; Martins *et al*., 2014) and daily age (Villamor *et a*l., 2013; WKMIAS, 2013) can be found in **Tables 2.1 & 2.2.**

Table 2.1. Summary of the last annual growth workshops and exchanges by species

Species	WK/Exchange	Area	Mode of preparation	% Agreement	CV	APE
Anchovy	WKARA 2009	Bay of Biscay (All readers/B&B readers)	Whole otolith, in resin	86.2/92.5	41.4/8.1	-
		Alboran Sea		75	66.40	-
		Strait of Sicily		61.9	67.30	-
	Exchange 2009	Gulf of Cadiz IXa		58.30	68.1	-
		North Adriatic Sea		55.60	72.2	-
		Gulf of Lyon		71.50	37.40	-
		North Adriatic Sea	Whole otolith, in alcohol	60.30	63.3	-
Sardine	WKARAS 2011	Bay of Biscay North (VIIIa)	Whole otolith, in resin	73.1	17.3	-
		Portuguese Coast (IXa)		76.5	18.1	-
		Gulf of Cadiz (IXa)		77.4	10.9	-
	Exchange 2010	Bay of Biscay South (VIIIb)		77.4	10.9	-
		Cantabrian Sea (VIIIc)		79.20	16.8	-
Herring	WKARBH2008	Baltic Sea	Whole otolith, in resin	86.9	6.4	-
	Exchange 2008		stained otolith slices			

	Exchange 2005	Atlantic Sea	Whole otolith, in resin	-	-	-
Spratt	WKARBS 2008	Baltic Sea	Whole otolith, with nail polish	76.10	17.1	-
	WK 2004	North Sea and Coastal Skagerrak	Whole otolith, in resin	85.8	12.0	-
Mackerel	WKARMAC 2010	Northeast Atlantic (IV, VI, VII, VIII and IX)	Whole otolith, in resin (only images)	78.10	11.60	Per reader
Chub mackerel	Exchange 2013	Northeast Atlantic (VIIIc, IXa) and Mediterranean Sea (Southwest)	Whole otolith, in resin	60.4	22.7	-
Horse Mackerel	Exchange2012	Ireland waters (VII)	Sectioned otolith	36.4	26.9	-
		North of Spain (VIIIc, IXa)		53.2	42.3	-
		Portugal waters (IXa)		44.7	54.7	-
		South of Spain (IXa South)		43.9	43.8	-
		Western Ireland		46.4	21	-
Mediterranean horse mackerel	Exchange2012	Mediterranean Sea (Italy waters)	Whole otolith	56.6	28.7	-
		North of Spain (VIIIc, IXa)		57.5	30.5	-

Table 2.2. Summary of daily growth workshops and exchanges by species

Species	WK/Exchange	Area	Live Stage	Mode of preparation	% Agreement	CV	APE
Anchovy	Exchange2013	Bay of Biscay (VIII)	Larvae & juvenile	Whole and sectioning otolith	-	15.3	10.4
		Western Mediterranean	Larvae	Whole otolith	-	18.6	14.1
		Strait of Sicily	Juvenile	Sectioning otolith	-	34.9	23.0
		Adriatic Sea	Larvae & juvenile	Whole and sectioning otolith	-	24.0	18.9
		North Aegean Sea	Larvae & juvenile	Whole and sectioning otolith	-	9.0	7.8
		Overall	Larvae & juvenile	Whole and sectioning otolith	-	18.9	13.3
Sardine	Exchange2013	Bay of Biscay (VIII)	Larvae	Whole otolith	-	14.5	10.7
		Atlantic Iberian	Juveniles	Sectioning otolith	-	18.0	13.5
		Western Mediterranean	Larvae	Whole otolith	-	11.7	8.6
		Adriatic Sea	Larvae & juvenile	Whole and sectioning otolith	-	14.3	11.6
		North Aegean Sea	Larvae &	Whole and	-	9.4	7.4

			juvenile	sectioning otolith			
		Aquaculture	Juveniles	Sectioning otolith	-	24.6	12.2
		Overall	Larvae & juvenile	Whole and sectioning otolith	-	13.7	10.6

2.1. Summary of general age estimation methods and problems

2.1.1 Anchovy (*Engraulis encrasicolus*)

Annual age estimation criteria: European anchovy age estimation criteria were recommended by ICES 2009. The method was adopted explicitly in the Workshop of anchovy age in 2002 (Uriarte et al., 2002) based in the validation study of Uriarte (2002). Otoliths of Anchovy are at present aged on whole immersed in the clarified medium (sea water, glycerin, alcohol) with reflected light against the black background. The otoliths also be analyzed, mounted on individual cavities on black plastic slides glued with a transparent resin. The orientation for the analysis is with the distal surface up and the proximal surface (sulcus acusticus) down. The method is based on the knowledge of the standard pattern of annual growth in anchovy otoliths, the process of edge formation, and the most common false growth increments (checks) which are expected to be found. A set of an opaque and hyaline zone corresponds to an annual growth zone (annulus) as is reported in Giráldez and Torres, 2009 (**Figure 2.1.1.1**). The date of birth is conventionally assumed to be the 1st of January in all Atlantic areas (Bay of Biscay and Iberian Atlantic coast) and in Gulf of Lyon, and the fish is assigned to a year class on this basis. In the **Table 2.1.1.1** is reported the scheme to assign the age: in the otolith collected during the first semester with the transparent edge the age corresponds to the number of hyaline zones included that one on the edge. In the first semester the pattern of deposition ring show that in the late spring early summer the probability to found the opaque ring is around 50% (Giráldez and Torres, 2009) in this case the age is equal to the number of completed annulus (n). If the otolith is collected from a fish caught during the second semester, the hyaline edge will not be considered, while if the otolith shows the opaque edge the age is equal to number of annulus (n).

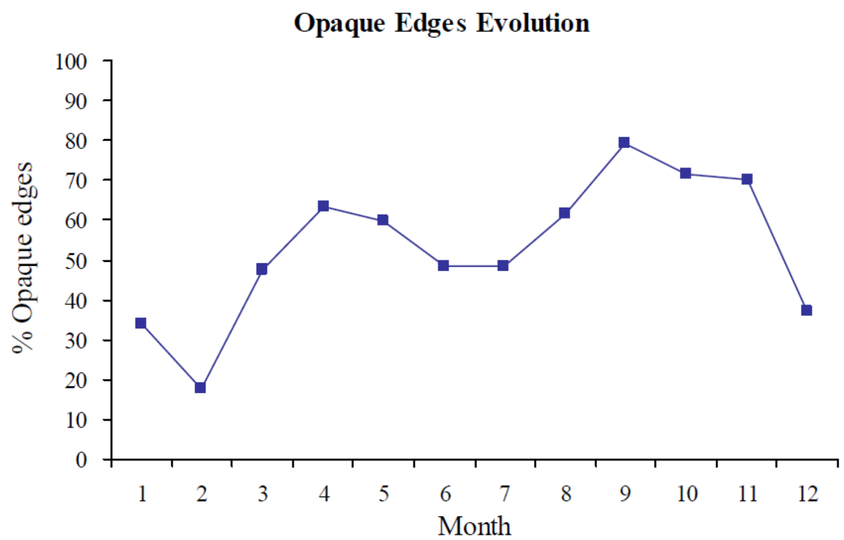

Figure 2.1.1.1 – Annual pattern of opaque edge formation in Alboran Sea in Giráldez and Torres, (2009)

Table 2.1.1.1 - The ageing scheme for *E. encrasicolus* with birthday 1st Jannuary. The n is the number of annulus (opaque zone and transparent ring); N is the number of transparent ring included the edge.

Date Capture	Otholiths edge	Age
1 January-30 June	Transparent	N
	Opaque	n
1 July-31 December	Transparent	N-1
	Opaque	n

In Mediterranean Sea the date of birth is 1st of June or 1 July based on reproductive traits such as gonad development cycle and gonado-somatic index followed throughout the year. In this case the scheme to assigned the age is reported in the **Table 2.1.1.2**

Table 2.1.1.2 - The ageing scheme for *E. encrasicolus* with birthday 1st July. The n is the number of annulus (opaque zone and transparent ring); N is the number of transparent ring included the edge.

Date Capture	Otholiths edge	Age
1 January-30 June	Transparent	N-1
	Opaque	n-1
1 July-31 December	Transparent	N-1
	Opaque	n

Annual age interpretation difficulties: These difficulties could be explained by: 1) the first annulus position; 2) the otolith edge identification (opaque or hyaline); 3) the presence of false growth increments (checks). In general, anchovy otoliths are not difficult to interpret, although there are geographic areas where growth patterns structures are more difficult. In all areas / stocks, the main problem is to determine the position of the true first annual ring. Being a short-lived species, this may give some erroneous ages and therefore influence on the determination and quantification of recruitment, so important to determine the status of these stocks, since the populations of this species is based mainly in ages 1.

Except for anchovy in the Bay of Biscay, the precision in determining the age of anchovy in other areas / stocks is quite low (Table 2.1). Probably the success of the readers of the Bay of Biscay, compared with the other areas, is reasonably because exchanges and workshops have been conducted since 1990 in this area, and there are sufficient criteria for the interpretation of anchovy otoliths. However in the other areas there have been only one or two exchanges and workshops. Furthermore otoliths from the Bay of Biscay had the clearest structure with high percentage of good otoliths (ICES, 2009).

Generally, a well-defined true hyaline ring accomplishes the following features (ICES, 2009):

a) to be continuous all around the otolith;

b) it remains clearly visible changing the focus;

c) the relative distances between adjacent rings is proportional to the expected growth pattern in the otoliths from each area.

Moreover the first annulus (opaque and transparent ring) consist in a wide opaque zone (some time interrupted by the false ring) in comparison to the successive annuli. However the distances between the ring decrease with the age (**Figures 2.1.1.3** and **2.1.1.4**).

Around the nucleus before the first winter ring (ICES, 2009) is laid down a false with a distances to the core about 0.8 mm. This ring can be distinguish because is less market in comparison to the winter one (Figure 2.1.1.3).

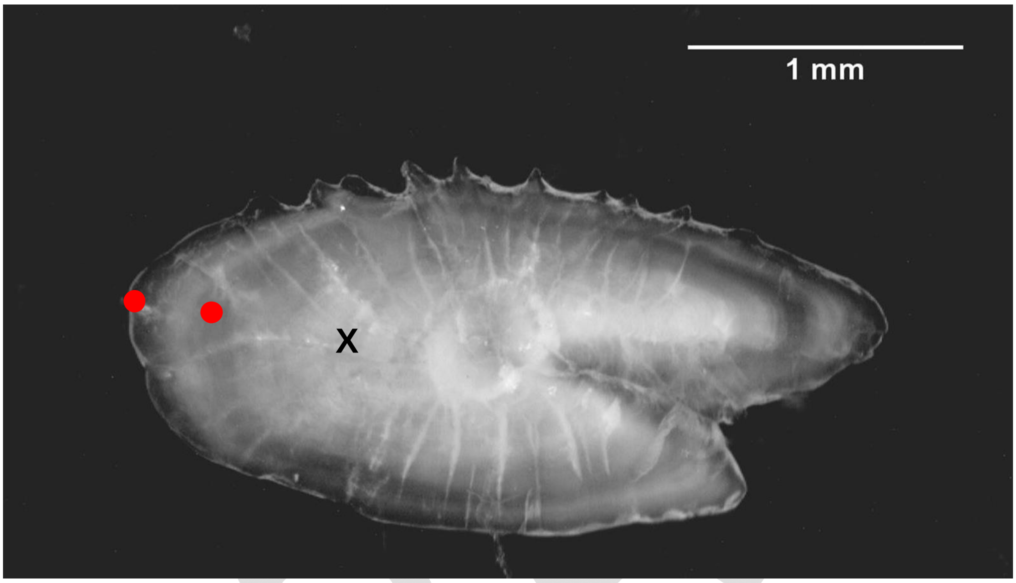

Figure 2.1.1.3 - Otolith of E. encrasicolus (TL: 13.5 cm, Sex: F, month of capture: May). Red dots winter ring; X check.

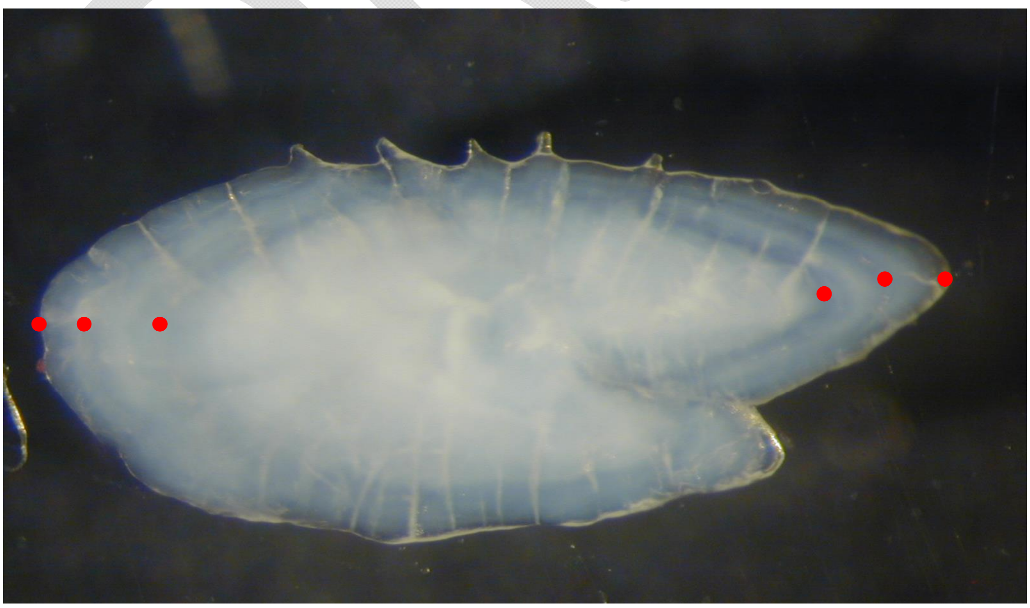

Figure 2.1.1.4 - Otolith of E. encrasicolus (age: 3; TL: 13 cm, Sex: F, month of capture: 9). Red dots winter ring.

Daily age estimation criteria: For daily increment interpretation, two different criteria have been suggested: using the known as group band reading (GBR) criterion, the reader counts every repetitive cyclic set of growth bands (usually two, but occasionally more) as single daily increment, assuming that they are sub-daily marks. And, in the other criterion, known as individual mark reading (IMR), each increment, regardless of its appearance, is counted as single daily increment. According to Cermeño *et al*. (2008), the GBR criterion is the most reliable method for ageing European anchovy. In the Bay of Biscay, Western Mediterranean and North Aegean Sea the agreement was to apply this methodology (GBR) for anchovy, irrespective of the season and geographical area (Morales-Nin *et al*., 2010; SARDONE protocol, 2010; ICES, 2013). However, there was not an agreement in reading interpretation criteria in the Strait of Sicily and Adriatic Sea (ICES, 2013).

Daily age Interpretation difficulties: These difficulties could be explain by: 1) difficulties in the interpretation of sub-daily increments, double structures or band zones; 2) unclear images, in which is difficult to interpret correctly the daily growth pattern due to under-or over-polishing, poor image acquisition or calibration problems. It is very important to obtain clear images to interpret properly daily micro increments in this species.

2.1.2 Sardine (*Sardina pilchardus*)

Annual age estimation criteria: The sardine age estimation criteria were recommended in last ICES sardine age reading workshop (ICES, 2011b). The method was adopted explicitly in FAO (1979) and can be summarized as follows: i)a set of an opaque and hyaline zone corresponds to an annual growth zone (annulus).ii) the date of birth is conventionally assumed to be the 1st of January and the fish is aged on this basis (if an otolith is collected during the first semester the age correspond to the number of hyaline zones, if the otolith is collected from a fish caught during the second semester, the hyaline edge will not be considered). In the case the edge is opaque in the first semester, mostly around the June when could start the deposition of the opaque zone, the age correspond at the number of annulus (opaque zone and transparent ring). The assignment of age for the otolith with opaque edge collected in the second semester follow the same role and it is equal to the number of the annulus (see Table 2.1.1.1 in anchovy)

Annual age interpretation difficulties: The main discrepancies in sardine age determination are the identification of the otolith edge type and the first annulus. Two problems related to the edge type were discussed at the last workshop: 1) difficulty in identifying the edge type (hyaline or opaque); 2) variation in the seasonality of the edge type

Also for the sardina before the first winter is often laid down one false ring with a distances from the core about 0.5 mm on the postrostrum side(**Figure 2.1.2.1**). The sardina otolith have the similar growth pattern of the anchovy with a big opaque zone in the first annulus. Moreover to recognize the winter ring is important to follow around all otolith the ring in order to distinguish the true one (**Figure 2.1.2.2** and **2.1.2.3**).

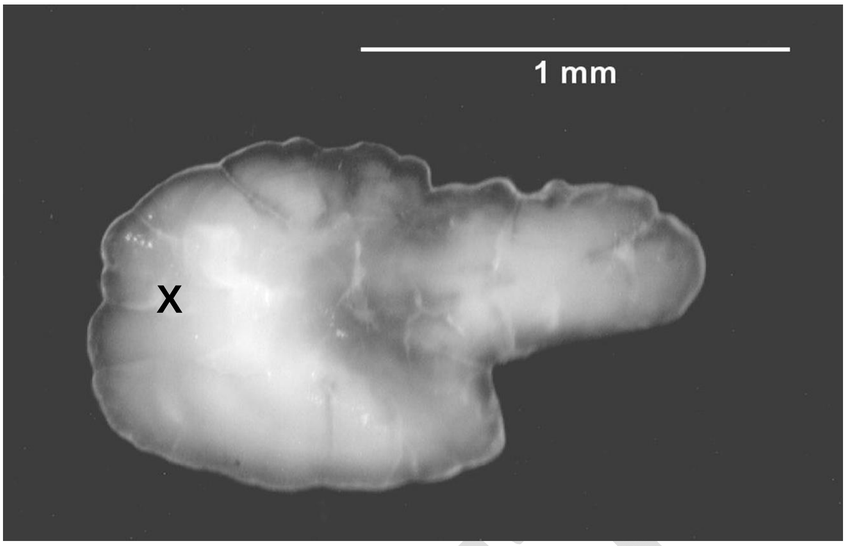

Figure 2.1.2.1 - Otolith of *S. pilchardus* (TL: 8 cm, Sex: F, month of capture: June). X indicates the false ring.

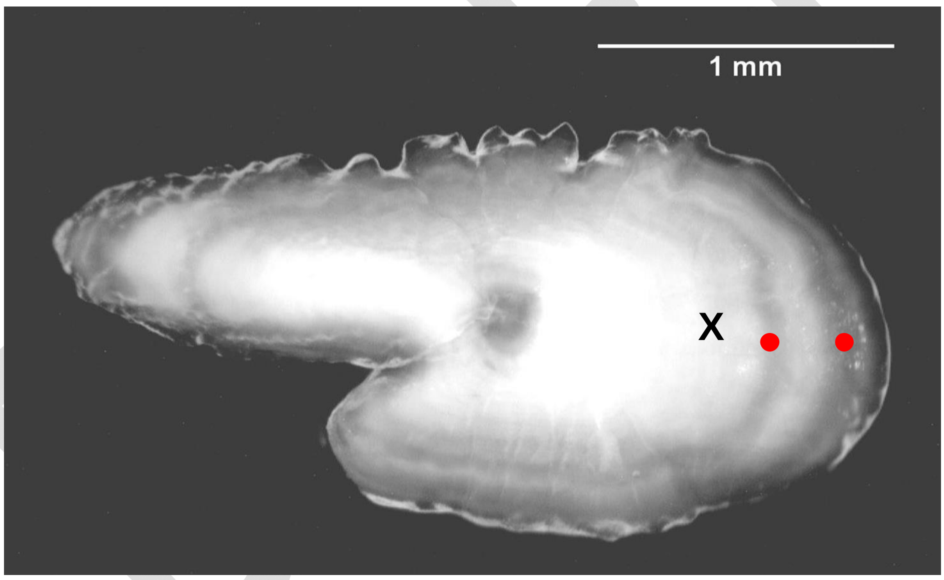

Figure 2.1.2.2 - Otolith of *S. pilchardus* (TL: 17 cm, Sex: F, month of capture: August). The red dots indicate the winter ring; X indicates the false ring.

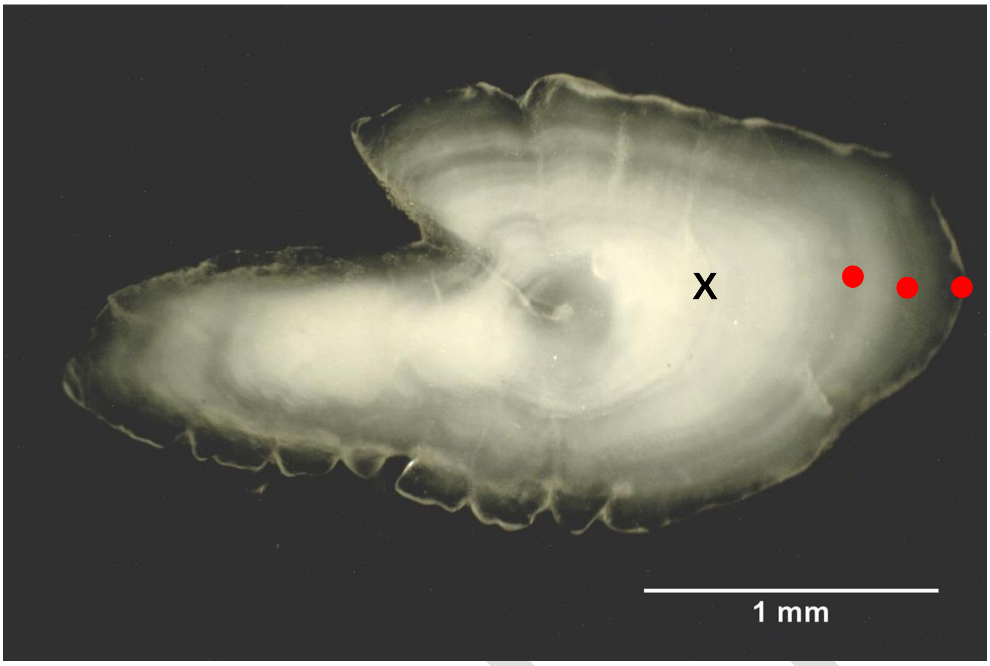

Figure 2.1.2.3 - Otolith of *S. pilchardus* (TL: 18 cm, Sex: F, month of capture: January). The red dots indicate the winter ring; X indicates the false ring.

Daily age estimation criteria: For daily increment interpretation of sardine otoliths, the same recommendations suggested for anchovy by ICES 2013a (ICES WKMIAS) are followed (See section 2.1.1). In the Bay of Biscay, Atlantic Iberian Peninsula, Western Mediterranean and North Aegean Sea the agreement was to apply the GBR criteria for sardine, irrespective of the season and geographical area (Morales-Nin et al., 2010; SARDONE protocol, 2010; ICES, 2013), except for the Adriatic Sea where IMR is adopted (ICES, 2013).

Daily age interpretation difficulties: These difficulties could be explain by: 1) difficulties in the interpretation of sub-daily increments, double structures or band zones; 2) unclear images, in which is difficult to interpret correctly the daily growth pattern due to under-or over-polishing, poor image acquisition or calibration problems. It is very important to obtain clear images to interpret properly daily micro-increments in this species.

2.1.3 Herring (*Clupea harengus*)

Age estimation criteria: The herring age estimation criteria were recommended in the last workshops, for Atlantic and Baltic stocks (Raitaniemi and Halling, 2005; ICES 2008a). A 1 January birth date is used. The date of capture must always be available. One year's growth ring consists of one opaque zone and one hyaline zone. For younger fish the winter zones are usually well visible on the whole otolith, while for older herring they are best seen on the rostrum and this part of otolith should be preferred for age determination.

Age Interpretation difficulties: In general, herring otoliths are relatively easy to read and the annuli can easily be recognized. Misleading false rings, caused by resorption, hunger, diseases or spawning etc. are not very pronounced and are therefore do not have a great effect on the quality of age reading process. By contrast, problems are encountered by mixing of stocks, which spawn at different times of the year. For this reason it may happen that in the western Baltic typical spring spawner are found, which have a hyaline nucleus, together with some autumn spawning herring which lack a hyaline nucleus.

Herring spawning later in the second half of the year produce larvae, which by definition should be one year old, once they have grown into January of the following year. These larvae would therefore be of the same age as young fish which have been spawned early in the year and are considerably larger. Irrespective of this the herring are therefore classified as "ringer", i.e. the total number of annuli is not set equal to years but to the number of rings. For the same reason the herring of the North Sea are attributed to their stocks as "winter-ringer" or "summer-ringer". The allocation of herring to either a spring or autumn-spawning stock could in principle be a problem in the Western Baltic. However, praxis has shown that this is not the case since the herring are clearly attributable to the spawning stock, i.e. the spring spawning herring.

2.1.4 Sprat (*Sprattus sprattus*)

Age estimation criteria: The spratt age estimation criteria were recommended in last ICES spratt age reading workshops, for Atlantic and Baltic stocks (Torstensen *et al*., 2004; ICES, 2008b). For age determination, hyaline zones (winter rings) are counted. For sprat older than 2 years at least the first two inner annuli should be traceable throughout the whole otolith. The reading of the otolith is done primarily in the rostrum, however, the rest of the otolith is not be neglected.

Age Interpretation difficulties: In general sprat otoliths are not easy to read and the annuli cannot be easily recognized. Misleading false rings, caused by resorption, hunger, diseases or spawning etc. are not very pronounced and therefore do not have a great effect on the quality of age reading process. By contrast, as in herring of the same region, problems are encountered by mixing of sprat stocks and locally occurring fast growing sub-populations in Subdivisions 22-32. Mixing of fast growing sprat from the west with slower growing specimens from the east occurs in Subdivision 24 (Arkona Basin), due to the decreasing salinity from west and the subsequent decrease of annual growth. The otoliths of sprat from the east are generally more difficult to read, as a result of the slower growth and the higher age at a given size.

2.1.5 Mackerel (*Scomber scombrus*)

Annual Age estimation criteria: Mackerel age estimation criteria were recommended by ICES (2010). The method was adopted explicitly in the Workshop of mackerel age in 1995 (ICES, 1995) based in the age validation of this species, by reading otoliths of known age (obtained from a tagging program). The date of birth is conventionally assumed to be the 1st of January and the fish is aged on this basis (as described in anchovy and sardine, see Table 2.1.1.1)

Otoliths of Mackerel are aged on whole immersed in the clarified medium (sea water, glycerin, alcohol) with reflected light against the black background. The otoliths also are analyzed, mounted on individual cavities on black plastic slides glued with a transparent resin. The orientation for the analysis is with the distal surface up and the proximal surface (sulcus acusticus) down. The area where the ring appear more clear is the post-rostrum than in the rest of the otolith, even if ring continuity should be checked on the anterior part of the otolith (rostrum and antirostrum) and where possible on the dorso lateral edge.

<u>Annual Age Interpretation difficulties</u>: These difficulties could be explained by: 1) different length of time for the opaque zone formation of the otolith between the different areas during the first year; 2) during first year some false ring are laid down (**Figures 2.1.5.1; 2.1.5.3**); 3) otolith edge interpretation; 4) possible presence of false annuli associated with the first maturity (**Figure 2.1.5.2**); 4) slowdown growth in older fish to such an extent that the opaque and translucent (hyaline) zones become confused and are more difficult to distinguish (**Figure 2.1.5.3**).

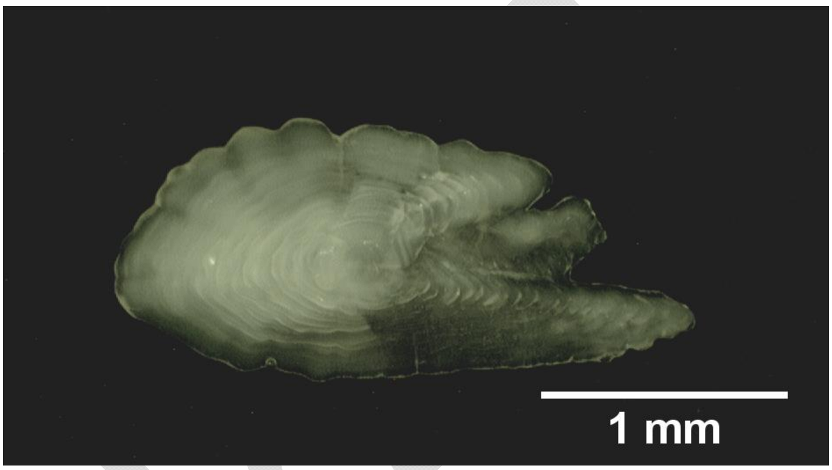

Figure 2.1.5.1 - Otolith of *S. scombrus* (TL: 17 cm, Sex: F, month of capture: November) age 0 year.

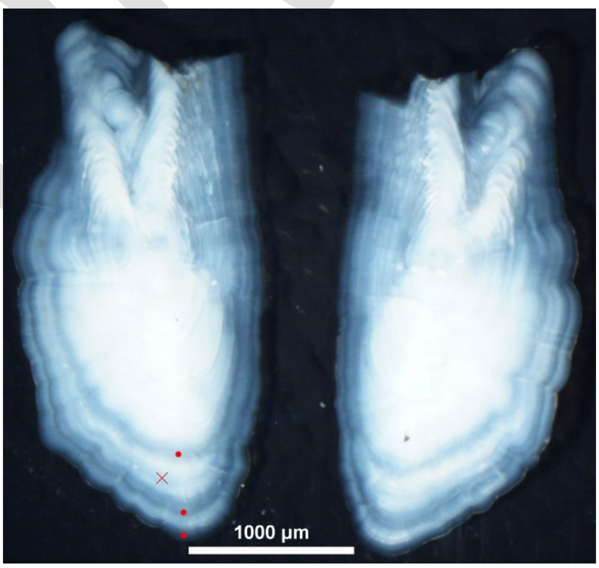

Figure 2.1.5.2 – Otolith of *S. Scombrus* (TL: 32 cm; Sex: F, month of capture: January) age 3 years. The red dots indicate the winter ring; X indicates the false ring.

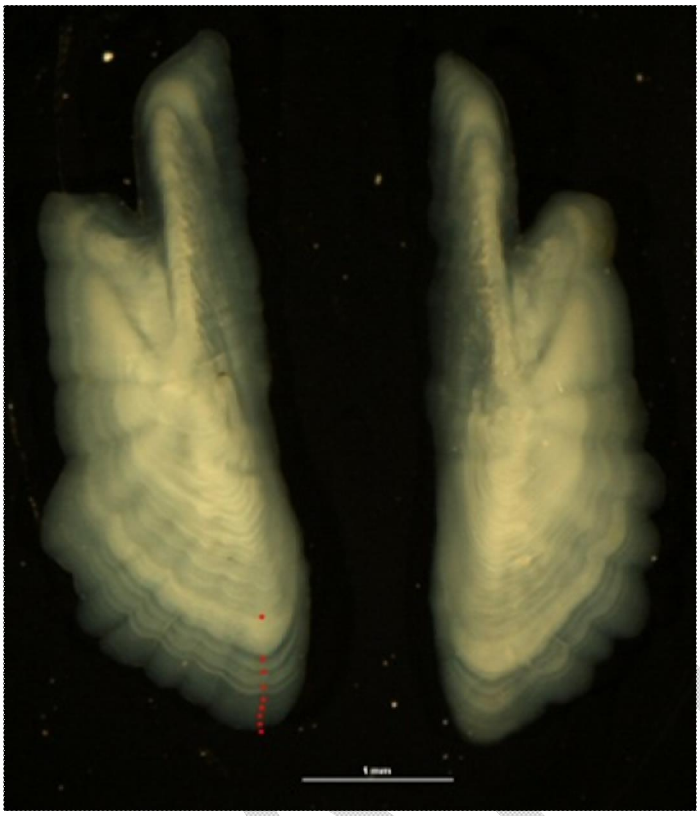

Figure 2.1.5.3 – Otolith of *S. scombrus* (TL: 40cm; Sex: F, month of capture: March) age 9 years. The red dots indicate the winter ring

<ins>*Daily Age estimation criteria*</ins>: The numbering of otolith growth increments begins at the hatch check. The last increment is omitted because it is considered incomplete since it does not represent a full day. The deposition of daily growth increments in mackerel larvae, post-larvae and juveniles has been validated by Migoya (1989) and D'Amours *et al*. (1990) in the Northwest Atlantic, and by Mendiola and Alvarez (2008) in the Northeast Atlantic.

<ins>*Daily Age Interpretation difficulties*</ins>: These difficulties could be explained by: 1) difficulties in the interpretation of subdaily increments, double structures or band zones; 2) difficulties in the interpretation of intermediate areas without growth increments in juvenile otoliths; 3) unclear images, in which is difficult to interpret correctly the daily growth pattern, due to under- or over-polishing, poor image acquisition or calibration problems. It is very important to obtain clear images to interpret properly daily micro-increments in this species.

2.1.6 Chub mackerel (*Scomber Colias*)

<ins>*Age estimation criteria*</ins>: The criteria for the age determination of *Scomber colias* are still developing in European waters (Martins *et al*., 2014). Due to the similarity between both species, and while the criteria for the age determination of chub mackerel are not fully defined, to follow the reading criteria applied to mackerel is recommended (see section 2.1.5).

Although certain peculiarities are observed, such as: 1) higher presence of false growth increments; 2) priority is given to the post-rostrum in the age interpretation, as annuli tend to be better determined in this area than in the rest of the otolith, even if ring continuity should be checked on the anterior part of the otolith (rostrum) and where possible on the dorso lateral edge; 3) usually, the otolith point provides little help in the age interpretation in chub mackerel, since the annuli are very crowded in this area and are difficult to determine, especially in otoliths of older specimens.

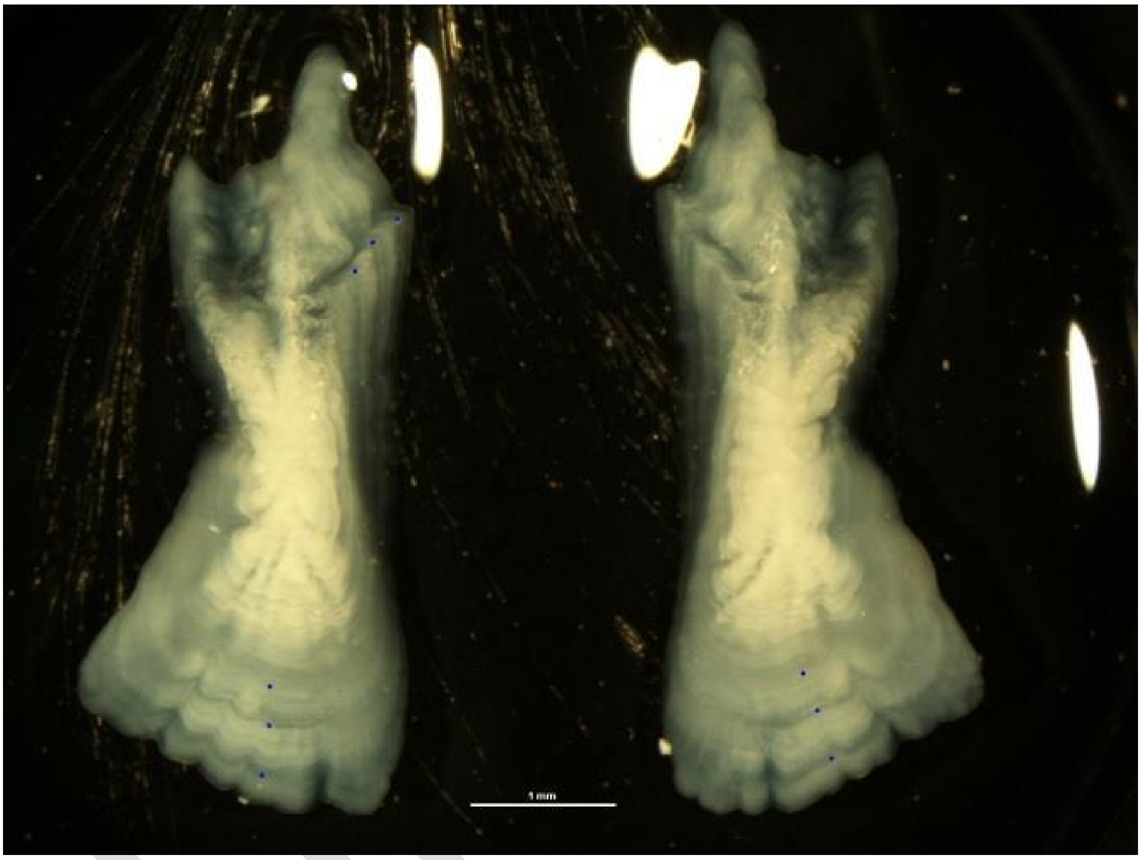

Figure 2.1.6.1 - Three years old chub mackerel from Atlantic (Martins et al., 2014). The winter ring are visible both in the post rostrum and rostrum area.

The otoliths are analyzed whole immersed in clarification medium by the binocular microscope with reflected light against a black background. The otoliths can also be analyzed, mounted on individual cavities on black plastic slides glued with a transparent resin. The best otolith orientation for the analysis is with the distal surface turned up and the proximal surface (sulcus acusticus) down (Figure 2.1.6.1; 2.1.6.2). In this way the dark rings can be counted in the post-rostrum area (radius) as translucent growth rings (slow growth) (Figure 2.1.6.1)

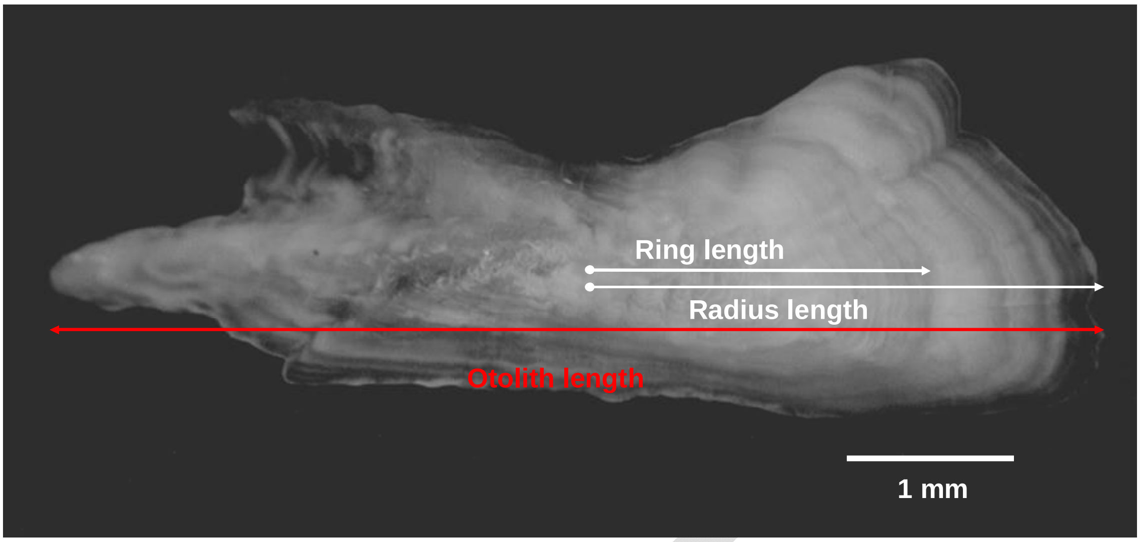

Figure 2.1.6.2 - The otolith of Atlantic chub mackerel from Adriatic (central Mediterranean)

The date of birth is conventionally assumed to be the 1st of January in all Atlantic areas (Bay of Biscay and Iberian Atlantic coast) and the fish age is assigned on this basis (as described in anchovy and sardine, see Table 2.1.1.1).

In Mediterranean Sea the date of birth is 1st of June or 1 July based on reproductive traits (Cengiz, 2012). In this case the scheme to assigned the age as described in anchovy (see Table 2.1.1.2).

Interpretation difficulties: These difficulties could be explained by: 1) difficulties in identifying the first annulus (**Figure 2.1.6.3**); 2) difficulties in differentiating between true annual rings (annuli) and false rings (checks); 3) insufficient annual growth pattern recognition; and 4) insufficient criterion regarding the otolith edge that can be expected to be seen along the year.

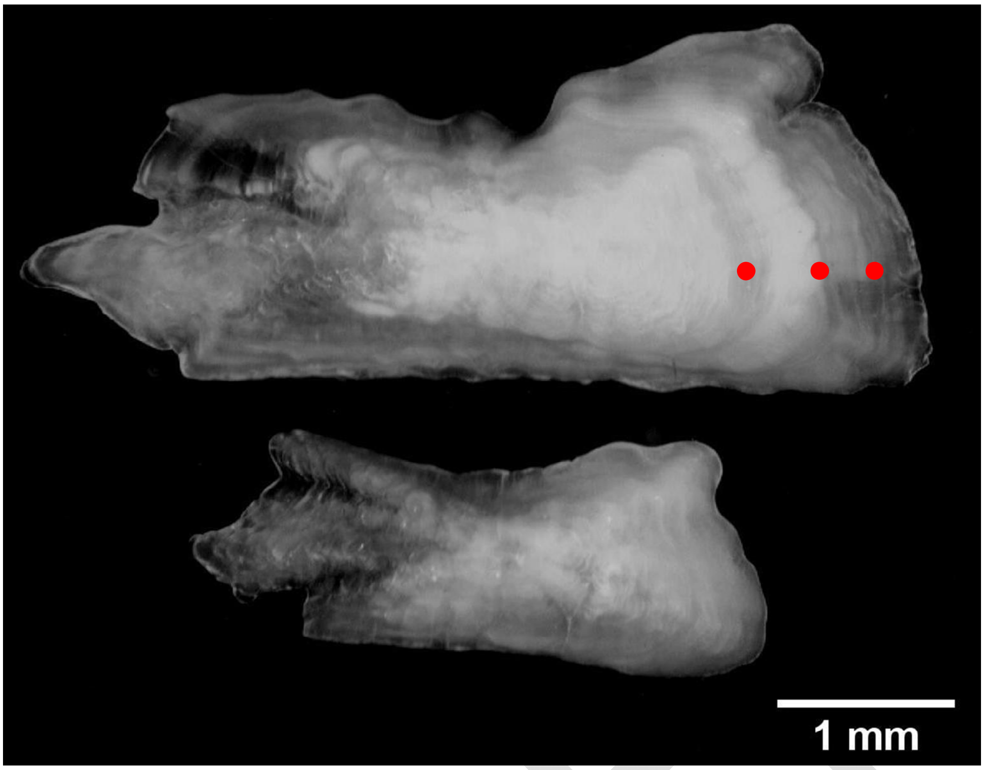

Figure 2.1.6.3 - The otoliths of Atlantic chub mackerel caught in November in Adriatic sea (central Mediterranean). Upper specimen 28.5 cm TL age class 3+; down specimen 16 cm TL 0+.

The otolith in the figure 2.1.6.3 are come from a period when start the transparent ring deposition in the Gulf of Cadiz (Velasco *et al*., 2011) and the distance from the core to the post rostrum edge (~ 1.4 mm) of the specimen with 0 age (down) is comparable with distance of the first true winter ring on the older specimens (up).

The otoliths of *S. colias* present a growth pattern with a large first annulus and the other annulus more with a decrease distances, but in the older specimens pile up with one another, making it difficult to ageing the otoliths from old specimens (**Figure 2.1.6.4**)

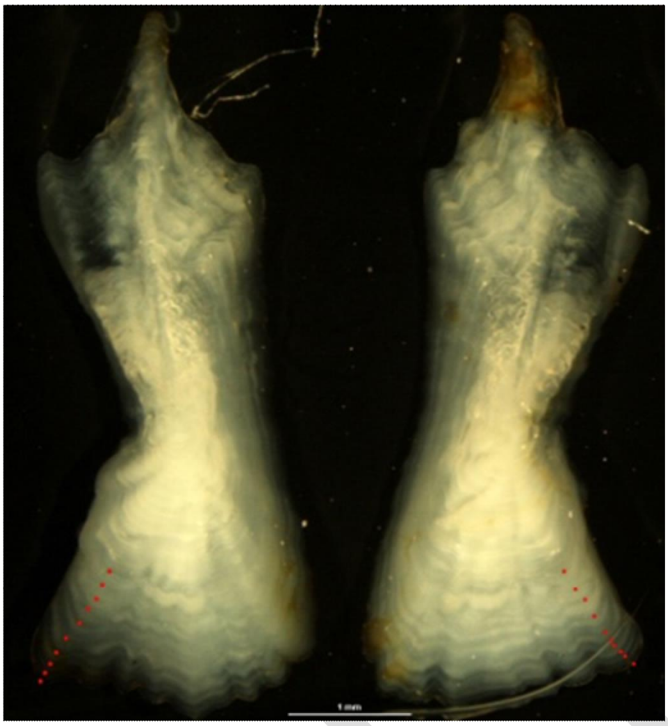

Figure 2.1.6.4 - Otholith of S. colias TL 38 cm; age 10 years caught in June. The red dots represent the winter rings.

2.1.7 Horse Mackerel (*Trachurus trachurus*)

Age estimation criteria: Horse mackerel age estimation criteria were established by ICES (1999; and 2012), based on direct age validation studies (Kerstan and Waldrom, 1995) and on indirect validation studies (ICES, 1999; Waldron and Kerstan, 2001; Abaunza et al., 2003). Otoliths of Horse Mackerel are at present aged on sectioned, broken and burned or whole. The interpretation of growth zones varies between readers. Despite of efforts to standardize preparation techniques, interpretation of growth zones with numerous workshops and otolith exchanges, precision in age estimates is still very low. This may negatively influence the quality of the assessment.

Regarding the ageing of the whole otolith: one of the pair of *sagittae* (usually the left) are placed in immersion in sea water or alcohol and glycerin as clarification medium before the analysis. The otoliths are analyzed by the binocular microscope immersed in the clarification medium with reflected light against the black background. The orientation for the analysis is with the distal surface up and the proximal surface (sulcus acusticus) down (**Figure 2.1.7.1**).

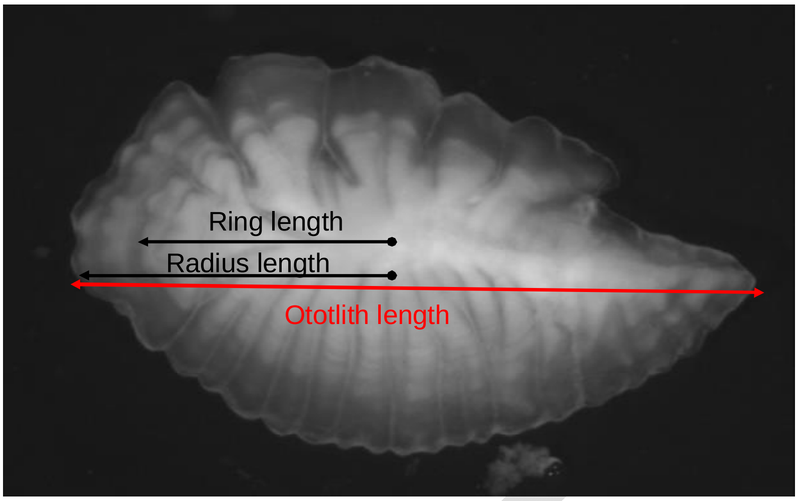

Figure 2.1.7.1 - The otolith of Atlantic horse mackerel

In whole otoliths annuli are counted on the posterior part of the otolith (post rostrum). However, ring continuity should be checked on the anterior part of the otolith (rostrum) and wherever possible on the dorso lateral edge (**Figure 2.1.7.2**)

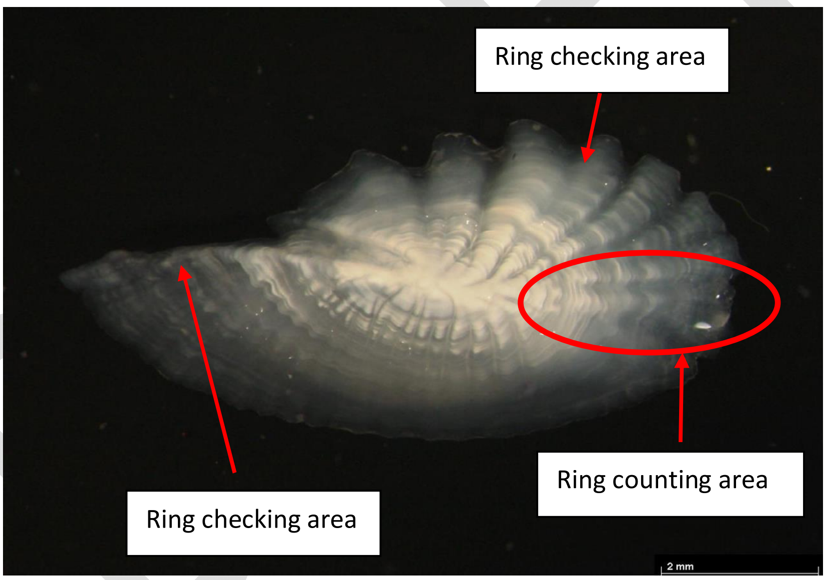

Figure 2.1.7.2 - Whole otolith of *T. trachurus* immersed in sea water in which the preferred sites for counting rings are showed.

So the dark ring are counted as the translucent growth zone (slow growth). The opaque (white – fast growth) zone plus a dark ring is considered as an annual growth (annulus). The ageing of Atlantic horse mackerel are performed considering 1st of January as the conventional birthday according with the spawning period (Abaunza et al., 2003; Carbonara et al. 2012) that is prolonged almost all year with a peak during the winter month. Moreover the age is following as the other species described above (see Table 2.1.1.1). For the specimens caught in the first part of the years if a transparent ring is observed at the edge of the otolith, is counted as annual ring. If a transparent ring is observed at the edge of the otolith at the second semester of the year, it is not considered as annual ring and the age is equal to the number of transparent ring excluded the edge (N-1). For the specimens with opaque edge caught in the first part of the years the age correspond to the number of the annulus (n), because mostly around the June the opaque zone could begin to laid down. For otolith collected from the specimens caught in the second part of the year with the opaque edge the age correspond to the completed annulus (n).

In some case mostly to ageing the bigger specimens (> 25 cm) the otolith are embed in the epoxy or polyester resin and dorsal-ventral thin (about 550 µm) sections through the nucleus are made. The thin slices mounted on the glass slide by the resin are analyzed under the binocular microscope with the reflected light (transparent rings dark and opache zone white) (**Figure 2.1.7.3**) and the optical microscope with transmitted light (transparent ring appear light and opaque zone darkness). The winter rings are counted usually on the dorsal side and ageing were made the interpretation scheme reported above.

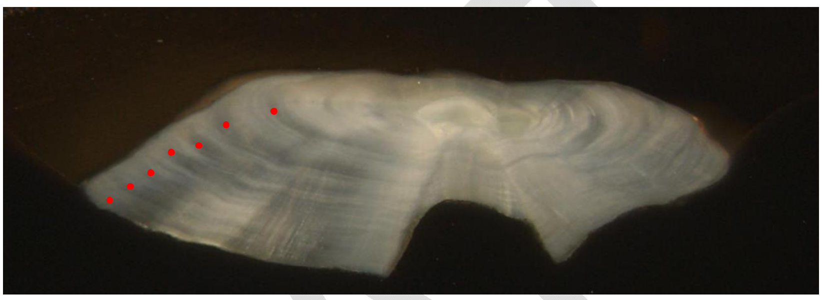

Figure 2.1.7.3 – Thin section of Atlantic horse mackerel TL 34.5 cm caught in September. The red dots represented the winter ring (age 7 year).

Regarding the broken and burn technique: before reading one of the otoliths is broken transversely across dorsal-ventral axis through the nucleus. The fractured surface of the anterior half of the broken otolith is polished using wet sandpaper, nr. P600. The rostrum is broken off and the polished part is then burnt over a Bunsen burner for a few seconds while constantly in motion. To clarify the ring structure these otoliths are carefully charred until darkish brown (Mølller Christensen, 1964). The treated otolith is mounted in plasticine and brushed with baby oil on the break. The otoliths are read under a stereo microscope using direct light, preferably an intensive cold-light source. The translucent rings in the burnt otolith are counted in the large ventral lobe near the *sulcus acusticus* (figure 2.1.7.4).

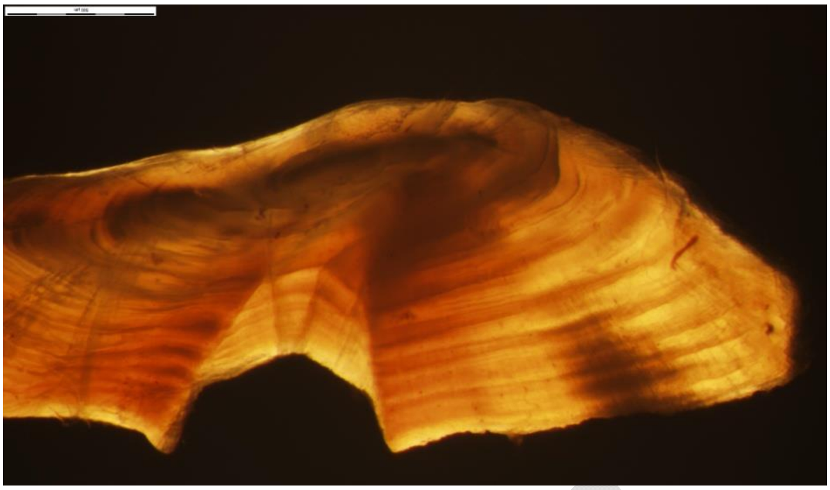

Figure 2.1.7.4 – View of broken and burned otolith from a specimens of 27.8 cm.

Interpretation difficulties: Horse mackerel (*Trachurus trachurus*) otoliths are notoriously difficult to age (Fariña Perez, 1983; Arruda, 1987; Kerstan, 1985; Abaunza *et al*., 2003), and otoliths from specimens of European waters have been shown to have particularly complicated ring structures (Karlou-Riga and Sinis, 1997). False rings may be similar in appearance to true annual rings (Karlou-Riga and Sinis, 1997) and may be erroneously interpreted as annual rings. The potential for inaccurate ageing of this species is high and can result in a wide variation of age estimates for horse mackerel (Abaunza, 2003).

In general, the age of horse mackerel otoliths is very difficult to estimate in older fishes because they become thick with age. The first annuli interpretation in both, young and older fishes appears to be the major cause of differences. The dissimilarity of the false rings and the variety of the true annuli make difficult to follow the true annuli formation. In some otoliths, problems are caused by the otoliths edge interpretation.

The determination of annual increments is difficult because the presence of false can mislead the pattern of the annuli formation. In fact, this is a major cause of age-reading errors, including the case of age determination in *Trachurus trachurus*. They are non-seasonal zones and two major types can be distinguished:

- 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, composed of two unusually thin translucent bands separated by a very thin opaque band.

It is not clear the causes of their formation although some factors such as temperature, food intake, other environmental conditions and developmental transitions have been suggested.

The interpretation of the first annulus is a matter of concern due to the difficulties in distinguishing false juvenile rings from the true seasonal mark (**Figures 2.1.7.5** and **2.1.7.6**). In the case of sliced otoliths in *T. trachurus*, the vision of the entire otolith (the untreated otolith of the pair of otoliths) besides the sliced otolith, helps in distinguish this common juvenile false annulus. In addition, sometimes the slices are not just made through the center of the otolith leading to a modification in the usual perception of the distance between the center of the otolith and the first true translucent mark. This produces a difficulty to interpret the first true mark or annual increment in the otolith.

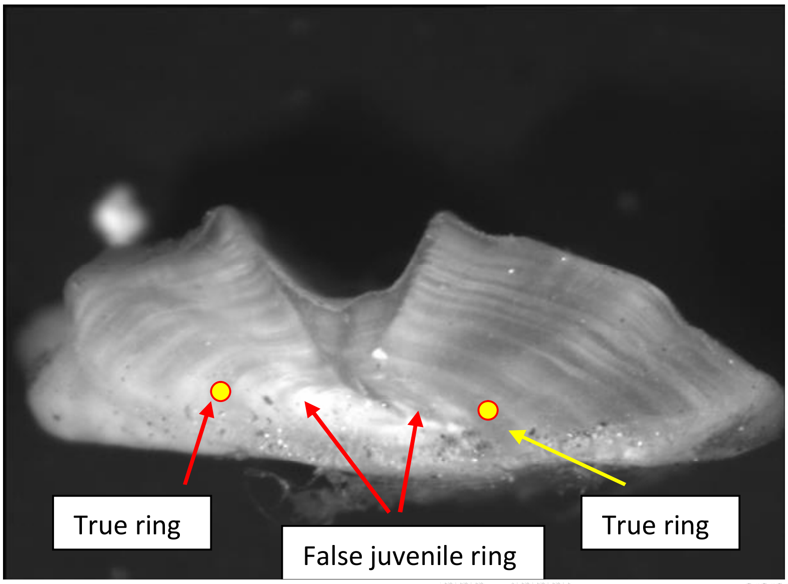

Figure 2.1.7.5 - A probable false juvenile ring in thin section from *T. trachurus*. (36. cm total length capture month: February).

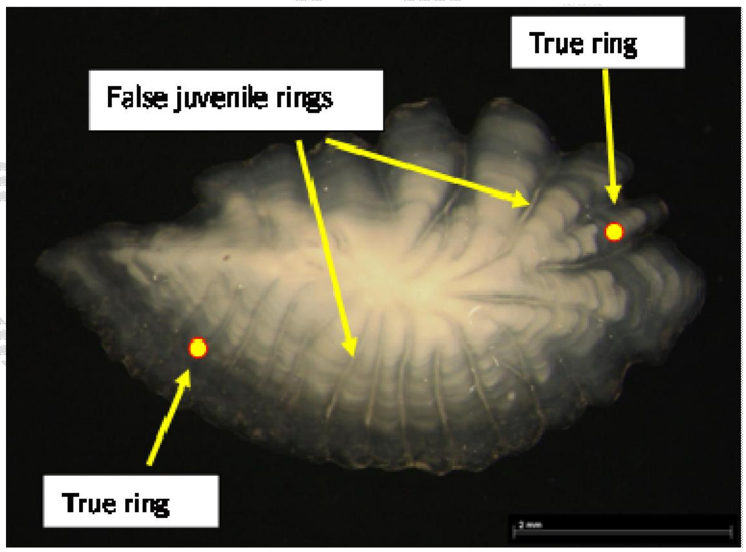

Figure 2.1.7.6 - True and false juvenile rings in a whole otolith from *T .trachurus* (18.8 cm total length caught in August)

These juvenile rings may separate from the first translucent ring or join with it forming a broad translucent zone. The completion of the first translucent zone is usually detected on the rostrum (Karlou-Riga and Sinis, 1997).

Some criteria to help the identification these secondary structures (false ring) are described in ICES (1999 &2012):

- Annulus extension in the otolith: in general, a true annulus, ring or mark should be traceable on the whole otolith or the section (ICES, 1999). This is more difficult to observe in the last annuli as fish is getting older and resulting in a thickening of the otolith.
- Distance between annuli: the widths of consecutive annual growth zones should decrease with increasing age. In horse mackerel, the decrease of the increment widths with age is most obvious between ages 1 and 5. After age 5 the rates of decrease are slow but rather constant.
- Contrast between seasonal marks: annual growth zones (annuli, marks) could be distinguished from false rings by their sharper images and the 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 zone.

2.1.8 Mediterranean horse mackerel (*Trachurus mediterraneus*)

Age estimation criteria: They were recommended by ICES (2012), as in the case of horse mackerel. Similarly to *T. tr*achurus, the interpretation of the ageing of *T. mediterraneus* otoliths is difficult, mostly for the older specimens where age determination is particularly imprecise. For the otoliths of T. mediterraneus there are specific problems to assign the age to younger specimens too and in particular to interpret the first two true annulus (Karlou-Riga, 2000), indeed, the characteristic of the detection of a ring around the otolith also on the rostrum zone is not always helpful.

The otoliths of Mediterranean horse mackerel are aged whole: one otolith from each pair (usually the left one) is immersed in sea water to be analyzed. The otoliths of *T. mediterraneus* don't need the clarification phase before the age analysis except for the bigger specimens (> 30 cm) where a very short permanence in the sea water (5-10 minutes) could be necessary.

The otoliths are analyzed to the binocular microscope rinsed with sea water (clarification medium) with reflected light against a black background. The best otolith orientation for the analysis is with the distal surface turned up and the proximal surface (*sulcus acusticus*) down (**Figure 2.1.8.1**). In this way the dark rings could be counted in the *antirostrum* area (*radius*) as translucent growth rings (slow growth). The opaque zone (white – fast growth) plus a dark ring is considered as an annual increment (*annulus*).

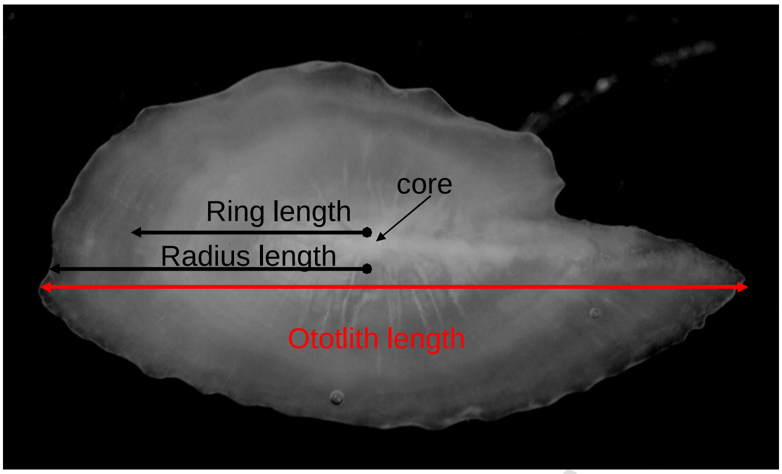

Figure 2.1.8.1 - The otolith of Mediterranean horse mackerel

About the ageing criteria, the birthday in Mediterranean is set at the 1st of July, according with the spawning period included between April and September (Vietti et al., 1997; Karlou-Riga et al., 2000). The criteria to age the otoliths, reported in **Table 2.1.8.1**, take into account the time of annulus formation (generally transparent ring during winter and spring months; opaque area during summer and autumn months), the capture date, the otolith edge and the spawning period. In the first semester in accordance with the pattern of ring deposition is highly likely to found the hyaline edge in this case the age will be the number of transparent ring exclude the edge (N-1). Also if the transparent edge appear in the second semester because the otolith could laid down a transparent ring around December the age will be N less one. In the case in the second semester the otolith edge appear hyaline around July this ring correspond to the past winter, so the age will be the number of transparent ring included the edge (N). The age in the otolith collected from a fish caught during the second semester with opaque edge will be the number of completed annulus (n). In the case the opaque edge is present in the first semester, especially around June, this opaque ring correspond to the next summer, but being before the birthday, the age is equal to the number of the annulus less one.

Table 2.1.8.1 – The ageing scheme for *T. mediterraneus* with birthday 1st July. The n is the number of transparent ring excluded the edge (annulus); N is the number of transparent ring included the edge. In bracket the case of the samples take around July.

Date Capture	Otholiths edge	Age
1 Jannuary-30 June	Transparent	N-1
	Opaque	n-1
1 July-31 December	Transparent	N-1 (N)
	Opaque	n

In the Atlantic areas the date of birth is 1st of January, as is described above for *T. trachurus*

<u>*Interpretation difficulties*</u>: Mediterranean horse mackerel otoliths are difficult to interpret, in a similar way as in horse mackerel otoliths, whose age determination for older individuals is particularly imprecise. However, Mediterranean horse mackerel otoliths present specific problems when assigning ages to younger individuals, related to the first annulus

interpretation.As reported in Karlou-Riga (2000), before the first winter ring some false rings are laid down. Indeed, the small specimens (5-8 cm) caught during summer and autumn months, from the spring-summer spawning, present a transparent edge (**Figure 2.1.8.2**). This is a false ring probably laid down when the juveniles changed the environment and the diet.

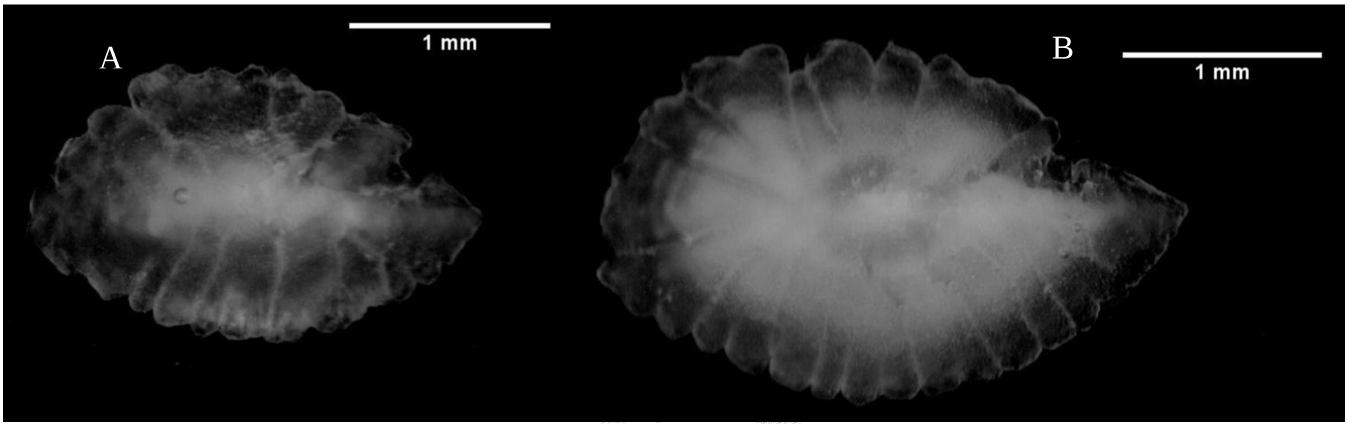

Figure 2.1.8.2 – The otoliths from small specimens (**A** 5 cm and **B** 7.5 cm total lenght) caught respectively (A) during the summer (29/07/2011) and (B) the autumn (06/10/2011).

The measure of these otoliths are about 2 mm (0.95 mm *radius*) and the trace of this false ring at the comparable measures are visible also in the otoliths of older specimens (**Figures 2.1.8.3; 2.1.8.4; 2.1.8.8**).

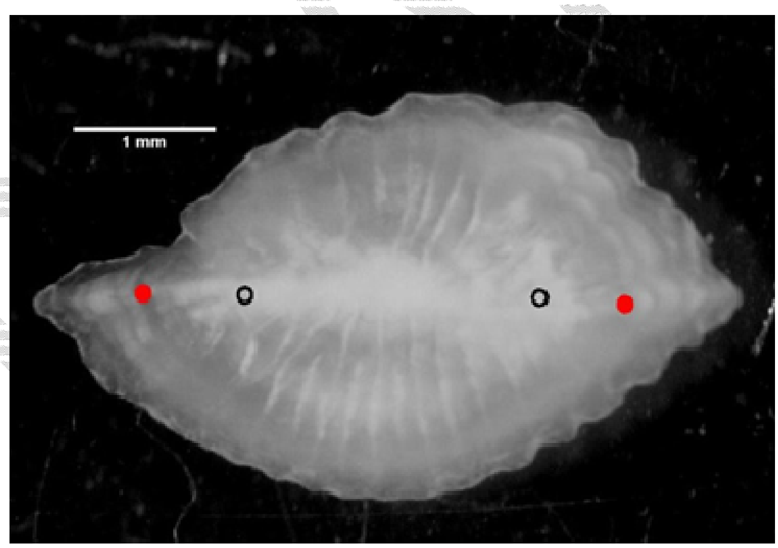

Figure 2.1.8.3 – Specimen of *T. mediterraneus* with 14.5 cm of total length caught in the summer. The open black circle is a false ring the red one the first winter ring.

The first winter true ring is laid down subsequently and specimens with total length around 12-14 cm caught in the winter and the early spring months present on the edge a transparent ring more evident than the previous false ring, with a measure on the *radius* of about 1.5 mm (whole otolith measure about 3.5 mm) (**Figure 2.1.8.4**).

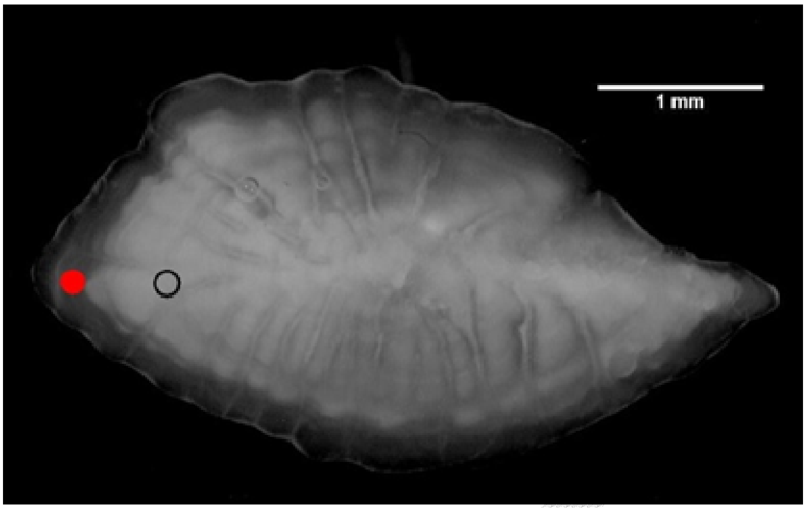

Figure 2.1.8.4 – Otolith of *T. mediterraneus* of specimen LT 12.5 cm caught in the early spring 0 year old. The open black circle is a false ring the red one the first winter ring.

Sometimes the check before the first true ring appear joined together in the one large transparent area (**Figure 2.1.8.3**).

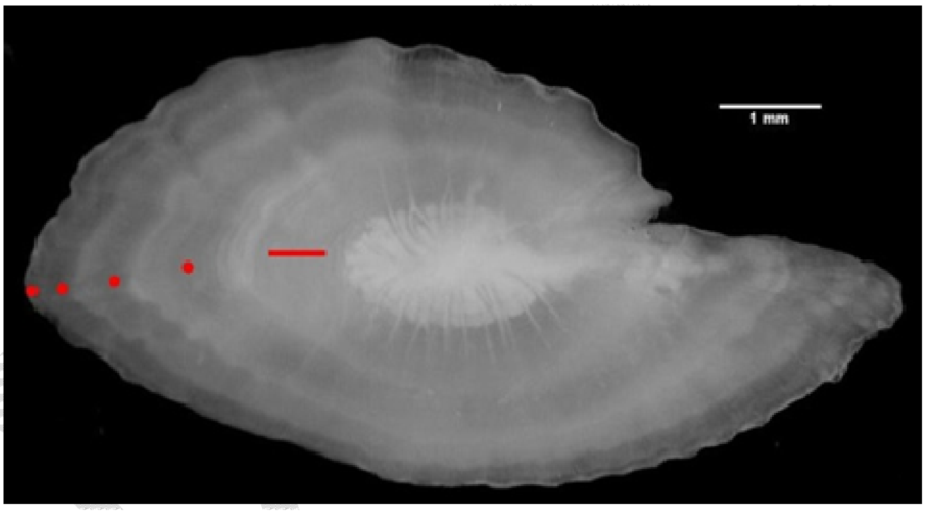

Figure 2.1.8.5 – The specimens (female TL 29 cm caught May 4 years old) with first winter ring as a transparent zone because the false rings are jointed with the first true ring. The red spot represent the winter ring; the red line represent the first winter.

After the first winter ring, another false ring could be laid down during the second year of life (**Figure 2.1.8.4**). This could be the trace of the reaching of first maturity. Indeed Vietti et al. (1997) report for the North Adriatic Sea the first maturity at 2 years old with 15.6 and 16 cm as the smaller mature specimens respectively for male and female.

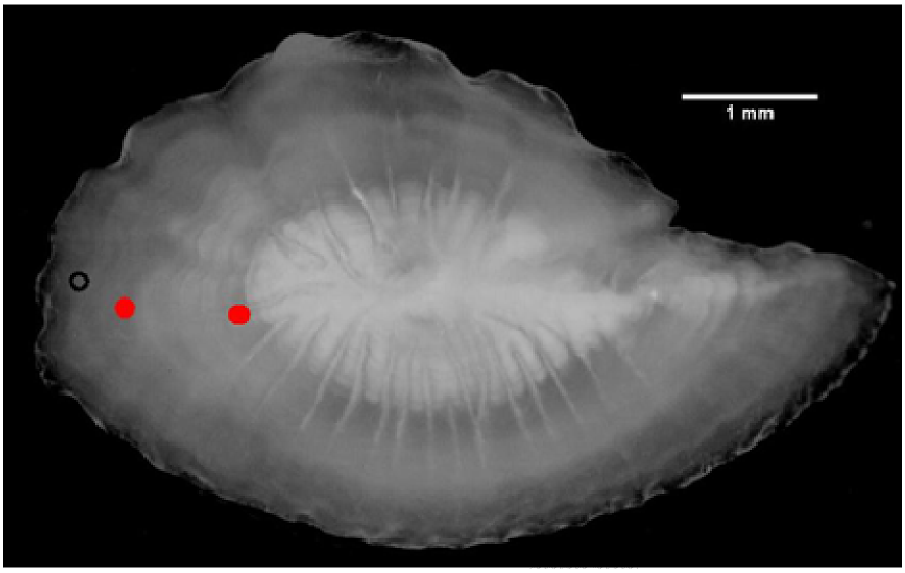

Figure 2.1.8.6 – Otolith of a female with 20.5 cm of TL and the gonads in a post reproductive stage caught during early winter age 2 years. The open black circle is a false ring the red one the true winter ring.

After the second winter ring, the deposition pattern of the winter band (transparent – black one) appear regular with a reduction of its distance (**Figures 2.1.8.5 and 2.1.8.7**).

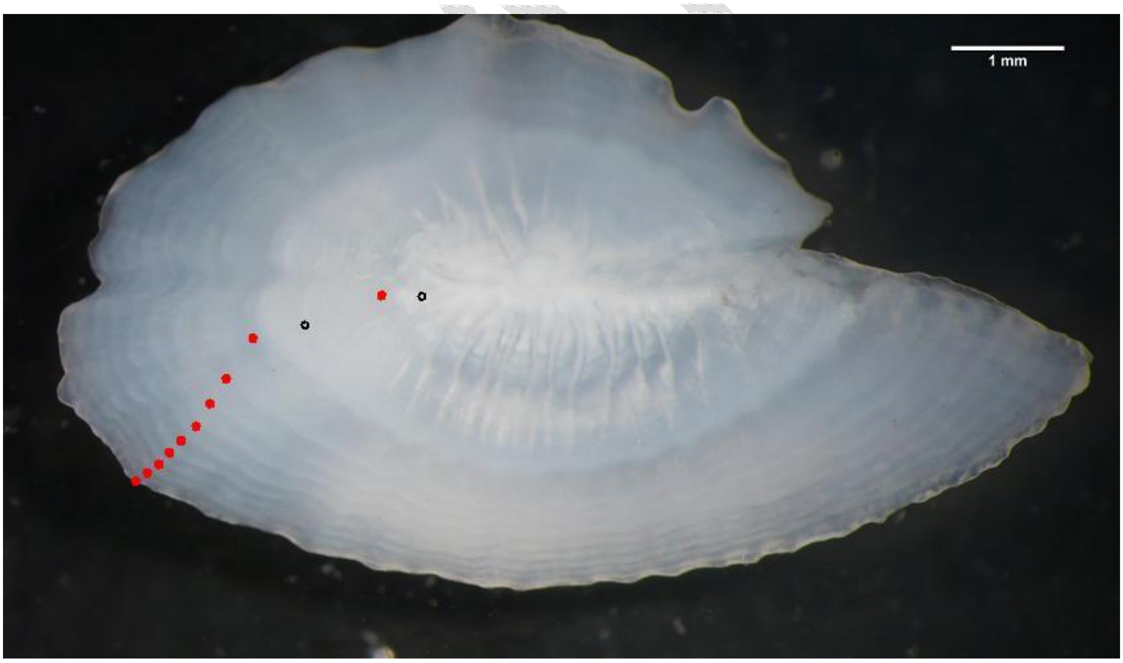

Figure 2.1.8.7 - Otholith of *T. mediterraneus* male TL 35.5 cm age 9 years caught in the winter (march). The open black circles represent the false rings while the red dots represent the true winter rings.

3. Age validation case studies

Several methods exist for validation of age readings of calcified structures (Campana, 2001). A summary of age validation methods used for small and medium pelagic species in European waters is shown in **Table 3.1**.

Table 3.1. Summary of age validation methodologies used for small and medium pelagic species in European waters.

Method	Annual/Daily	Pelagic species in which this validation techniques has been employed
Marginal increment analysis/Edge zone analysis	A	Anchovy, Sardine, Sprat, Chub Mackerel, horse mackerel, Mediterranean horse mackerel
Progression of strong year-classes	A	Anchovy, Horse mackerel
Length Frequency analysis	A	Anchovy, Sardine, Chub Mackerel, Horse Mackerel, Mediterranean horse mackerel
Weight Frequency analysis	A	Sprat
Daily increments between annuli	A	Anchovy, Sardine
Daily increments widths	A	Herring
Tag-recapture analysis	A	Mackerel
Captive rearing	D	Anchovy, Sardine, Herring, Spratt, Mackerel

In the following, the validation studies carried out on small and medium pelagic species referred to in **Table 3.1** will be described in detail. The methods are reported according to whether they are indirect and direct validation methods (Panfili et al., 2002). The methodologies available for validating the temporal meaning of the growth increments can be grouped in direct validation methods, which take into account a precise temporal reference mark in the otolith or a known age (i.e. marking otoliths or rearing experiments); or indirect methods, which require the comparison of the age estimates with statistical age estimated from length-frequency distributions or other age data.

3.1 Indirect validation methods

3.1.1 Marginal increment analysis and Edge zone analysis

Marginal increment analysis is the most commonly used of the validation methods, and it is used for validating the periodicity of growth increment formation (Campana, 2001), and two types of studies are possible, one of which uses qualitative data and the other, quantitative data (Panfili et al., 2002). This last method consists of measuring the distances separating the latest marks at the edge of the otolith. In cases where there is low contrast between growth zones an edge zone analysis (qualitative study) may be used to achieve similar but less accurate results. Edge zone analysis does not assign a state of completion to the marginal increment, but rather records its presence as either an opaque or translucent zone (Campana, 2001), and expressing the results in percentage through time is then studied.

The majority of works attempting to validate annuli of pelagic species used qualitative method, one of the least rigorous methods, to do so (**Table 3.1.1**). Relative marginal increment analysis (quantitative method) was applied to Sprat in the Baltic Sea (Torstensen *et al*., 2004); to Chub mackerel in Madeira Islands and in Hellenic Seas (Vasconcelos, 2006; Kiparissis *et al*., 2000); to Horse Mackerel in North East Atlantic and in Hellenic Seas (Waldron and Kerstan, 2001; Karlou-Riga and Sinis, 1997) and to Mediterranean Horse Mackerel in Hellenic Seas (Karlou-Riga, 2000)

Table 3.1.1.1 Summary of species where marginal increment analysis was applied

Species	Area	Method	Time series	Age/Size Range	References
Anchovy	**Bay of Biscay**	Qualitative	1982-1989	Ages 1-3	Uriarte, 2002
	Gulf of Cadiz	Qualitative	2005-2008	Ages 1-4	Millan and Tornero, 2009
	Alboran Sea	Qualitative	Oct. 1989-Dec. 1992	All ages together	Giraldez and Torres, 2009
	North Adriatic Sea	Qualitative	Jan. to Dec. 2007	All ages together/ 10.5-16.5 cm	Donato and La Mesa, 2009
Sardine	**Bay of Biscay**	Qualitative	2006-2009	Age 1-4	Coelho and Duhamel, 2010
	Atlantic Iberian	Qualitative	2000-2008	Age 1-4	Coelho and Duhamel, 2010
		Qualitative	1979-1980	Age 1-2	Alvarez and Porteiro, 1981; Porteiro and Alvarez, 1983

			Jan to Dec 1979	Ages 1-5	Jorge and Costa Monteiro, 1980
Sprat	**Skagerrak and Kattegat**	Quantitative	Feb. 2003-Jan. 2004	Age 0-2	Torstensen *et al.*, 2004
Mackerel	**Portuguese Coast**	Qualitative	1981	All ages together	Gordo and Martins, 1982
Chub mackerel	**Portuguese Coast**	Qualitative	1981-1982	All ages together	Martins *et al.*, 1983
	Azores Islands		1996-2002	All ages together/9.6-53.5 cm	Carvalho *et al.*, 2002
	Madeira Islands	Quantitative	2002-2004	All ages together /19-41 cm	Vasconcelos, 2006
	Gulf of Cadiz	Qualitative	1977-1978	All ages together	Rodriguez-Roda, 1982
	Canary Island		March 1988-July 1990	All ages together/19.2-41.1 cm	Lorenzo *et al.*, 1995
	SW Mediterranean (Alboran Sea)		Oct. 2003-Sep. 2004	All ages together /17-40 cm	Velasco et al., 2011
	NW Mediterranean (Catalan coast)		April-July 1992 and Dec. 1997	All ages together	Perrota *et al.*, 2005
	Eastern Mediterranean (Hellenic Seas)	Quantitative	Jan – Dec 1996	Ages 1-3/-	Kiparissis *et al.*, 2000
Horse mackerel		Quantitative	Sept. 1982-	Ages 0-5	Kerstan, 1985

	North East Atlantic		Sept. 1984		
				Ages 0.6-4.3	Waldron and Kerstan, 2001
	Eastern Mediterranean (Hellenic Seas)		Oct. 1989-May 1991	Ages 1-5/ 6.5-33.9 cm	Karlou-Riga and Sinis, 1997
Mediterranean Horse mackerel	**Eastern Mediterranean (Hellenic Sea)**	Quantitative	August 1989-Nov. 1991	Age 0-3	Karlou-Riga, 2000

3.1.2 Length Frequency analysis

Length frequency analysis subsumes a variety of different length-based methods, all of which produce estimates of growth rate. The corroboration occurs when the resulting growth estimate is compared to that of the age determination method.

This method has been used for anchovy, sardine, chub mackerel, horse mackerel and Mediterranean Horse Mackerel in European waters (**Table 3.1.2.1**). In the anchovy of NW Mediterranean Sea, length frequency analysis method was applied to corroborate the otolith interpretation and growth model parameters of anchovy (Pertierra, 1987; Morales-Nin and Pertierra, 1990). In the case of sardine of NW Mediterranean Sea , Pertierra and Morales-Nin (1989) and Morales-Nin and Pertierra (1990) determined the age by means of otolith interpretation and this was validated for the younger age classes by length frequency analysis. In the Chub mackerel of Madeira Islands, the length frequency analysis method was also applied to ages 0-5 (Vasconcelos, 2006). The comparison between ageing and the length-frequency distributions of Horse mackerel in the North East Atlantic confirmed the ageing of the first years of life (up to age 4) (Letaconnoux, 1951; Ramalho and Pinto, 1956; Barraca, 1963; Polonsky, 1969; Sahrhage, 1970; Macer, 1977). In the Eastern Mediterranean area (Greek Seas), the 1st annulus formation of Horse mackerel was detected comparing the progression by month of the smaller modal fish length with the respective otolith appearance during the year (Karlou-Riga and Sinis, 1997). In the Horse mackerel of the Adriatic Sea, length frequency analysis method was applied to corroborate the otolith interpretation and growth model parameters of horse mackerel (Alegria Hernandez, 1984) and the annuli were validating until the 5th. Arneri and Tangerini (1984) studied the growth of Mediterranean Horse mackerel by otoliths and length frequency in young individuals (0-4 age groups) in the Adriatic Sea.

In the Gulf of Salermo (Southern Tyrrhenian sea), in order to validate larvae growth estimates based on otolith examination, an analisys of modal progression in the larval length frecuency distribution was used in the sardines of 20-40 mm size range (Romanelli *et al*., 2002).

Table 3.1.2.1 Summary of species where length frequency analysis was applied.

Species	Area	Annual/ Daily	Time series	Age/Size Range	References
Anchovy	**NW Mediterranean Sea**	A	April 1984- Oct. 1985	Age 0- 4/5-18.5 cm	Pertierra, 1987
			Jan. 1987- Jun. 1989	Age 0-4 /6.5-20 cm	Morales-Nin and Pertierra, 1990
Sardine	**NW Mediterranean Sea**	A	Jan. 1987- June 1989	Age 0- 5/6.5-20 cm	Pertierra and Morales- Nin,1989 ; Morales-Nin and Pertierra, 1990
	Central Mediterranean Sea (Gulf of Salerno- Western of Italy)	D	1996-1997	Age 0/ 20- 40 mm	Romanelli *et al.*, 2002
Chub mackerel	**Madeira Islands**	A	2002-2004	Ages 0- 5/13-41 cm	Vasconcelos, 2006
Horse Mackerel	**North East Atlantic**	A	-	Age 0-4	Letaconnoux, 1951
			July 1954- febr. 1955	Age 0	Ramalho and Pinto, 1956
			July 1954- Dec 1961	Age 0-2	Barraca, 1963
			1967-1970	Age 1-3	Macer, 1977
	Hellenic Seas		Oct. 1989- May 1991	Age 1	Karlou-Riga and Sinis, 1997
	Adriatic Sea		July 1980- Nov. 1981	Ages 1-5	Alegria Hernandez, 1984
Mediterranean horse mackerel	**Adriatic Sea**	A	May-Nov. 1982	Ages 0-4	Arneri and Tangerini 1984

Otolith weight frequency distribution (OWFD) was applied to sprat (Torstensen *et al*., 2004) (**Table 3.1.2.2**). This method is a variant of the length frequency distribution analysis (LFD) (Campana, 2001). This method assumes that the expected modes of the otolith weight frequency would correspond to the population age-classes.

Table 3.1.2.2 Summary of species where weight frequency analysis was applied.

Species	Area	Annual/ Daily	Time series	Age/weight Range	References
Sprat	Baltic Sea (Skagerrak and Kattegat)	A	Feb. 2003	Age 0-4/0.22—2 mg	Torstensen *et al*., 2004

3.1.3 Progression of strong year-classes

The corroboration method of tracking strong/weak year-classes compares the interval between yearly samples and the increase in the apparent modal age of a recruitment pulse as determined through annulus counts (Campana, 2001). This method, also considered as an "indirect validation" method, indicates that an age-reading method is accurate if the age composition of exceptionally good or weak year classes can be tracked over a long period of time (Panfili et al., 2002).

This method has been used only for anchovy and horse mackerel in European waters (**Table 3.1.3.1**). The age estimation criteria of Bay of Biscay anchovy were also corroborated (or indirectly validated) by tracking year-classes abundance indices 1982-1992 in research surveys in the Bay of Biscay (Uriarte, 2002). In the case of the Horse mackerel has been tested by following identifiable year classes through successive year's age compositions in the catch in number of the western horse mackerel fishery, the extremely strong 1982 year class can be followed from 1984 to 1996 (Eltink and Kuiter, 1989; Abaunza, 2003).

Table 3.1.3.1 Summary of species where progression of strong year-classes was applied.

Species	Area	Time series	Age/Size Range	References
Anchovy	Bay of Biscay	1982-1992	8-20 cm	Uriarte, 2002
Horse Mackerel	North East Atlantic	1981-1987	Age 5 and older	Eltink and Kuiter, 1989; Abaunza et al., 2003

3.1.4 Daily increments between annuli

Daily increments counts between presumed annuli can provide strong corroboration of the frequency of formation of the annuli (Campana, 2001). In this method all increments are examined and counted, presupposing knowledge of dates of hatch and annulus formation.

Based on different daily growth studies, the formation of the first annulus was validated and the position of the first false ring or check was corroborated in anchovy in the Bay of Biscay (ICES, 2013). Annual increment deposition in the otoliths of young-of-the-year European anchovy was validated (Aldanondo et al., 2013). Early anchovy juveniles were maintained in captivity from October 2012 until April 2013 and the first annulus was validated using daily increments counts. According to that, the first opaque band is completed in October-November, whereas the translucent band is formed by March-April. The position of the first check for anchovy in the Bay of Biscay was also corroborated (Hernández, C. et al., 2013). Two methods were used, in the first, age was determined by identifying and measuring growth rings formed on sagitta otoliths, and in the second, a method for age corroboration was used by means of the otolith microstructure and fish ages were determined by daily increment counts.

The position of first false ring or check formed before its first winter ring (see the section 2.1.1) was corroborated through the micro increment counts in sardine in Adriatic Sea (Panfili, 2013) and in Portuguese waters (Silva et al., 2012).

In the case of the two species the method is considered valid since validation of daily ring formation in anchovy (Cermeño et al. 2003; Aldanondo et al. 2008) and in sardine (Ré, 1984; Alemany and Alvarez, 1994) larvae and juveniles was performed.

Table 3.1.4.1 Summary of species where daily increments between annuli was applied

Species	**Area**	**Method**	**Time series**	**Age/Size Range**	**References**
Anchovy	**Bay of Biscay**	Validation of first annulus	October 2012-April 2013	Age 1/8.5-13.6 cm	Aldanondo et al., 2013
		Corroboration of first check	2010-2011	Age 1/11.7-20.5 cm	Hernandez et al., 2013
Sardine	**Atlantic Iberian**	Corroboration of first annuli	Oct 2008-April 2009	Age 1	Silva and Silva, 2010; Silva *et al.*, 2012
	North Adriatic Sea		Oct.-Dec. 2012	Age 1/11.5-13 cm	Panfili, 2013

3.1.5 Daily increments widths

The method is based on a random sample of daily increment widths along and uninterrupted growth axis of the otolith which, when integrated over the observed length of the growth radius, must yield the daily age of the otolith and fish (Campana, 2001). This can be used to identify the timing of growth zones, by linking the occurrence of translucent checks to the time of ocurrence

Studies of microstructures in sprat and herring otoliths (sagittae) have demonstrated structural differences between what are defined as true and false translucent (winter) rings (Mosegaard and Baron 1999; ICES, 2008a). When the translucent ring is deposited the width of the daily increments gradually reduces in width. This pattern can be found in true winter rings in the sagittae of sprat aged 0 – 2 years old (Torstensen *et al*., 2004). A false winter ring has no gradual reduction in the width of the daily rings in front of it, neither immediately after the translucent zone. The characteristics of the winter ring in the saggitae of herring (ICES, 2008a), decreasing width of the daily rings prior to the winter ring, no daily ring formation during the winter ring formation and then progressively wider daily rings after the winter ring, makes it possible to detect false Winter rings. These often appear within the first couple of years as narrow rings close to the centre. Thus, in otoliths where the age reader is in doubt whether a translucent zone is true or false, the validity of the ring can be examined by reading the otolith microstructure.

3.1.6 Tag-recapture analysis

Tag-recapture analysis is a member of a suite of methods which provided growth rate estimates which can be compared with those derived from annulus counts (Campana, 2001).

This method only has been used in mackerel comparing known age of fish (validation collection from Norwegian tagging program) with age readings of different readers (Anon., 1995).

3.1.7 Others

Other methods used, such as back calculation lengths should not be considered as neither validation nor corroboration (Campana, 2001). The back-calculated lengths across several ageing structures merely shows consistency in the interpretation of the sequence of growth increments, independent of whether or not the interpretation is correct.

This method has been used for anchovy, sardine, chub mackerel and horse mackerel in European waters (**Table 3.1.7.1**). In The Strait of Sicily, the back-calculation method was applied of anchovy to compare results from the growth model estimation (Basilone *et al*., 2004). For sardine in the Atlantic Iberian, was also used this methods (Costa Monteiro and Jorge, 1982; Porteiro and Alvarez, 1983). In the Gulf of Cadiz, Canary Islands and Madeira Islands back-calculated length analysis methods were applied to compare the otolith interpretation and growth model parameters of chub mackerel (Rodriguez-Roda, 1982; Lorenzo *et al*., 1995; Vasconcelos, 2006). In the Eastern Mediterranean area (Greek Seas), the back-calculation method was applied of horse mackerel to compare results from the growth model estimation (Karlou-Riga and Sinis, 1997).

Table 3.1.7.1 Summary of species where back-calculated lengths analysis was applied

Species	Area	Time series	Age/Size Range	References
Anchovy	**Strait of Sicily**	May 2000-Oct. 2001	Ages 0-3/7-16 cm	Basilone *et al*., 2004
Sardine	**Atlantic Iberian**	1979-1981	Ages 0-7	Costa Monteiro and Jorge, 1982
		1979-1980	Age 0-6	Porteiro and Alvarez, 1983
Chub Mackerel	**Canary Island**	March 1988-July 1990	Ages 1-7/19.2-41.1 cm	Lorenzo *et al*., 1995
	Madeira Islands	2002-2004	Ages 1-4/20-40 cm	Vasconcelos, 2006
	Gulf of Cadiz	1977-1978	Ages 0-2	Rodriguez-Roda, 1982
Horse Mackerel	**Hellenic Seas**	Oct. 1989-May 1991	Ages 1-5/ 6.5-33.9 cm	Karlou-Riga and Sinis, 1997

3.2 Direct validation methods

3.2.1 Captive rearing

This method validates both absolute age and periodicity of growth structures (Campana, 2001).

The daily periodicity of micro-increment deposition was validated in early life stages of European anchovy, Sardine, Herring, Sprat and Mackerel (**Table 5.1.1.1**). These validations were done in culture and therefore are applicable to the species *(Engraulis encrasicolus, Sardina pilchardus, Clupea harengus, Sprattus sprattus, Scomber scombrus)*.

As far as anchovy is concerned, validation studies were carried out in individuals from Bay of Biscay. In particular, the daily increment deposition was validated in hatched eggs and larvae reared in the laboratory under different temperature conditions (Aldanondo et al., 2008). In the Bay of Biscay, as well as in wild juveniles marked by immersion in oxytetracycline hydrochloride (OTC) and reared until reaching adulthood over a period of 2 years (Cermeño et al., 2003). Furthermore, Aldanondo *et al.* (2008) demonstrated that increment deposition starts at hatching.

In the Bay of Biscay, the daily deposition was validated in sagittal otoliths of reared and wild sardine larvae from hatching to complete yolk-sac absorption (Re 1984; Alemany & Alvarez 1994). Similarly, the validation of daily otolith increment formation was carried out by a mesocosm experiment on wild sardine late larvae growing under natural environmental conditions in the Adriatic Sea (Panfili 2012).

Production of daily increments in sagittae of larval sprat has been validated from 6 to 29 days under laboratory conditions (Alshuth 1988a). Daily increments have been validated for larval herring under lab, mesocosm and field conditions (Moksness 1992, Johannessen *et al*. 2000, Fox *et al*. 2004). It has further been found that primary otolith increments are formed during the winter in both larval sprat (Alshuth 1988b) and larval herring (Moksness and Fossum 1991).

The deposition of daily growth rings in larvae, post-larvae and juveniles of mackerel (*Scomber scombrus*) was validated by Migoya (1989) and D'Amours *et al.* (1990), in Northwest Atlantic, and by Mendiola and Alvarez (2008) in Northeast Atlantic. Migoya (1989) and Mendiola and Alvarez (2008) incubated mackerel eggs in the laboratory and showed that the deposit of the first increment in the otolith occurred on the hatching day and that the increments were formed daily. In addition, D'Amours et al. (1990) performed a validation experiment on mackerel juveniles in captivity, marking their otoliths with a fluorescent substance and showing that the increments were deposited on a daily basis.

Table 5.1.1 Summary of species where captive rearing was applied

Species	Area	Rearing conditions	Age/Size Range	References
Anchovy	**Bay of Biscay**	laboratory	Age 0/ Larvae	Aldanondo et al., 2008
		laboratory / immersion in oxytetracycline	Age 0/Juveniles	Cermeño et al., 2003
Sardine	**Atlantic Iberian**	Field conditions (larval caught every hour during 20 hours)	Age 0/Larvae	Re, 1984
		laboratory	Age 0/Larvae	Alemany and Alvarez, 1994
	North Adriatic Sea	mesocosm	Age 0/Larvae	Panfili, 2012
Herring	**Norway Sea**	Laboratory and mesocosm	Age 0/larvae (from spring spawn)	Moksness 1992,
		Laboratory	Age 0/larvae (from spring and autumn spawn)	Johannessen et al. 2000
		Laboratory/ immersion in alizarin-complexone solution	Age 0/larvae	Fox et al. 2004
Sprat	**North Sea**	Laboratory	Age 0/larvae	Alshuth 1988a

Mackerel	**Bay of Biscay**	laboratory	Age 0/Larvae	Mendiola and Alvarez, 2008

4. Conclusions on age validation methods

4.1 Specific conclusions for each method

Marginal increment analysis (MIA): These methods provide a validation of the pattern deposition of the annulus. Moreover through these methods is also possible to detect the deposition of false rings in some period (reproduction, migration etc.). MIA (quantitative) is sometimes differentiated from edge analysis (qualitative), but when used as a validation method, has similar properties.

In the small and medium pelagic species listed in Table 3.1.1, the use of the MIA (qualitative and quantitative) proved a method to corroborate annual increment formation, but there are some limitations on the use of such techniques. These are as follow: the difficulty in measuring small increments accurately, over all in older individual particularly at the edge of the otolith; difficulties in interpreting opaque/translucent edge zones and the need high contrast between growth zones.

Requirements: samples from through the year. Low cost

Length Frequency analysis: Progression of length frequency modes thought time is one of the most basic of the length frequency analyses which is possible, and can be reliable form of age corroboration in young, fast-growing fish. This method is difficult to apply when an overlap between modes is present.

Otolith weight frequency distribution (OWFD) is a variant of the length frequency distribution analysis (LFD). This method assumes that the expected modes of the otolith weight frequency would correspond to the population age-classes.

The requirements and assumptions for OWFD and LFD are, however, identical. Both methods are particularly suitable for short living and fast growing species. For this reason this method has been applied to many the pelagic species (Tables 3.1.2.1 & 3.1.2.2)

Requirements: Random sampling procedure of fish is required to ensure that the samples collected are representative of the population. Low cost and takes advantage of data routinely obtained in fishery studies (length)

Progression of strong year-classes: This method can provide a strong, albeit qualitative, confirmation of growth increment periodicity. There are strong indications that the age-reading method is accurate if the age composition of exceptionally good or weak year-classes can be followed over a long period of time.

Requirements: Catch at age available, strong (or weak) year-class well-defined, no appreciable age-structured migration or mortality. Low cost if catch at age is available.

<u>*Daily increments between annuli:*</u> This method can provide strong corroboration of the frequency of formation of the annuli. This method is valuable to identify the first winter ring but pre-supposes knowledge of dates of hatch and annulus formation.

Requirements: Validation of the formation of the daily increments growth for this approach to be valid. Low cost, but time consuming work.

<u>*Daily increments widths*</u>*:* This method can be used to identify the timing of growth zones, by linking the occurrence of translucent checks to the time of occurrence. It is based on an assumption that the growth increments used to validate a macroscopic structure are formed a daily basis.

This validation method is only feasible for the 1st to the 3rd winter ring. Beyond these, the distance between the winter rings decreases making it impossible to detect the daily ring structure bordering these. For older ages, one must count on that the visible transparent zones are true winter rings, and as the occurrence and detection of false rings usually is highest within the first years of the individual of pelagic species, this assumption seems acceptable.

Requirements: validation of the formation of the daily increments growth for this approach to be valid. Low cost, but time consuming work.

<u>*Back-calculated lengths*</u>*:* This an important method of obtaining estimates of an individual's length at age prior to capture (Panfili et al., 2002). This method no validates or corroborates any age interpretation (Campana, 2001). One method of using trends in patterns of growth as verification how was applied the age criterions is to compare the length at age obtained from direct calcified structure readings and the back-calculation length at age. The results from the back-calculation (length at age) could be used in indirect age validation process if they are compared with mean length mode from length frequency distribution obtained by a survey carried out in the period of the hyaline ring formation.

Requirements: The back-calculation length at age of previous size at age is an inexpensive tool but cannot stand alone. This method should preferably be coupled to a length frequency distribution from a survey carried out in the period of the hyaline ring formation or size distributions of individual age classes.

<u>*Tag-recapture analysis*</u>*:* The existence of otoliths from Mark-Recapture experiments are potentially the golden stones and could iron out many subjective assumptions related to the age estimation. This provided a good growth comparison for age class.

Requirements: A programmers of tagging-recapture experiments. Low excluding cost of tagging survey, but rather very expensive.

Captive rearing: The only method to validate both absolute age and periodicity of growth structures. Mesocosms and outside cages provide improved and more natural rearing experiments for validation studies than indoor locations. Daily growth increments are less affected by environmental conditions than annulus formation.

Requirements: Aquaculture laboratory, mesocosms, ocean cage or outside enclosures. Expensive and may not mirror conditions in the wild.

4.2 General conclusions

- The provision of age validation studies should be carried out for all pelagic species, and especially those that are assessed analytically.
- Precision in age readings may be improved by workshops and otolith exchange, but the validation of the annual deposition of seasonal zones (opaque and transparent) and the check (i.e. the spawning ring, migration ring) in the calcified structures (CS) represent the focal point to the improve the precision in the fish age determination by the CS.
- Tagging programs or captive rearing cannot easily be applied to all species and stocks, but it could make a good practice for age determination with other techniques (marginal increment analysis, marginal analysis, following strong year classes, length back-calculation, etc) and assemble and compare them all to clarify the periodicity of CS growth and the correct interpretation of rings.

5. Future perspectives in small and medium pelagic species

Tag-recapture and use of chemical agents for otolith marking: The existence of otoliths from the Mark-Recapture experiments (i.e Norwegian programme of mackerel) are potentially the golden stones and could iron out many subjective assumptions related to the age estimation of mackerel. It is of utmost importance that the dimensions and availability of such material is clarified and that efforts are made to reach agreement on potential availability for coordinated validation studies. On the other hand, chemical marker substances could be used in the tag-recapture experiments to produce a mark in the otolith at time of capture.

Validation of Life history events: Daily ring structures have been validated in anchovy, sardine, herring , sprat and mackerel, (Alshuth 1988b; Moksness 1992; Alemany and Alvarez, 1994; Johannessen et al. 2000; Fox et al. 2004;; Aldanondo et al., 2008; Mendiola and Álvarez, 2008); and their study gives the potential for validating the first years of growth, making standards (L1, etc) and ruling out double structures in the first years of life. Knowing that the microstructure is daily, it may be possible through analysis of the combined transparency and width of the daily rings on the edge of juveniles over the season to validate the formation of the first and potentially following 2-3 age structures.

Others: Indications that correct age determination has been applied can be obtained by indirect validation techniques. For example, in the catch in numbers of mackerel fishery, the weak/strong years class can be followed in successive years (ICES, 2013). Corroborative methods for validation of annual rings, such as elemental or isotopic cycles, could potentially support the counting of rings (Campana, 2001). It is necessary to be aware that using microchemistry as a supportive tool age validation would require knowledge about the chemical environment of the species. Age verification as Bio-chronologies studies from growth-increment widths in the otoliths as supportive tool for age validation, especially for those pelagic species that live many years, such as horse mackerel and mackerel.

The methods such as LFDa, Progression of strong year-classes, Daily increments between annuli, back calculation etc. in a holistic approach can provide an alternative to method not easily applied (chemical mark and recapture, microchemistry, etc)

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