Contribution to Guidelines for Age Determination of Chondrichthyes fish from the Mediterranean Sea
(application to selected species)

Report of the MedSudMed Training Course on Age Determination of Selacean Fish

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Report of the MedSudMed Training Course on
Age Determination of Selacean Fish

Mazara del Vallo, Italy, 22 November–01 December 2004

by

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Preface

The Regional Project "Assessment and Monitoring of the Fishery Resources and the Ecosystems in the Straits of Sicily" (MedSudMed) is executed by the Food and Agriculture Organization of the United Nations (FAO) and funded by the Italian Ministry of Agriculture Food and Forestry Policies (MiPAAF).

MedSudMed promotes scientific cooperation among research institutions of the four participating countries (republics of Italy, Libya, Malta and Tunisia) for continuous and dynamic assessment and monitoring of the status of the fisheries resources and the ecosystems in this area of the Mediterranean Sea.

Research activities and training are supported to increase and use knowledge of fisheries ecology and ecosystems and to create a regional network of expertise. Particular attention is given to technical coordination of the research activities among the countries, which should contribute to implementation of the Ecosystem Approach to Fisheries. Consideration is also given to development of an appropriate tool for managing and processing data related to fisheries and their ecosystems.

GCP/RER/010/ITA Publications

MedSudMed Project publications are issued as series of Technical Documents (GCP/RER/010/ITA/MSM-TD-00) related to meetings, missions and research organized by or conducted within the framework of the Project.

Comments on this document would be welcomed and should be sent to the Project headquarters:

Preparation of this document

This document is the final version of the Report of the MedSudMed Training Course on Age Determination of Selacean Fish, organized by the FAO-MedSudMed Project (*Assessment and Monitoring of the Fishery Resources and the Ecosystems in the Straits of Sicily*) in Mazara del Vallo, Italy, from 22 November to 1 December 2004.

Acknowledgements

ABSTRACT

The MedSudMed workshop on age determination of selacean fish was held in Mazara del Vallo, Italy, from 22 November to 1 December 2004. It was attended by participants from Tunisia, Italy and Malta. The main objective of the workshop was to provide an overview of techniques currently used in the MedSudMed Project area for preparation and reading of spines and vertebrae of a series of cartilaginous fish species (*Centroscymnus coelolepis, Cetorhinus maximus, Carcharhinus plumbeus, Galeus melastomus, Scyliorhinus canicula, Mustelus mustelus, Rhinobatos cemiculus, Rhinobatos rhinobatos, Raja clavata, Squalus blainvillei, Chimaera monstrosa*). The course provided training and joint preparation and interpretation of samples provided by the participating institutes. Participants had agreed on a common protocol for preparation of the samples prior to the course. Direct observation of vertebrae and spines was conducted, along with tests of two methods for enhancing the appearance of the growth bands (red alizarin and cobalt nitrate). On the basis of the laboratory work and of the relevant bibliography available, trials were performed to identify the most relevant techniques for each species studied. This document details the work done during the workshop and provides general guidelines for cartilaginous fish age determination, as well as the limits of the methods currently used and improvements that could be made in the near future. It appeared that direct observation gave good results for all species except *C. coelolepis*. Red alizarin and cobalt nitrate staining procedures also improved the appearance of growth patterns except for C. *plumbeus, Centroscymnus coelolepis and Scyliorhinus canicula and Galeus melastomus*. Good results were obtained for some species (*C. plumbeus*, and *G. melastomus*), according to the size of the observed individuals. Further investigations are needed; in particular, larger samples should be examined to confirm the methods used and to provide detailed guidelines to be used at the regional level.

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1. Background information

The Expert Consultation on the "Spatial Distribution of Demersal Resources in the Straits of Sicily and the Influence of Environmental Factors and Fishery Characteristics" held in Gzira, Malta (10-12 December 2002), suggested that a programme be developed to implement common methodology, propose a pilot study and identify gaps in national and regional expertise within the framework of the MedSudMed Project. Among other issues, the experts of the region highlighted the importance of developing standard methodology for research activities in order to standardize spatiotemporal data analysis. As a result of the importance assigned by the MedSudMed Coordination Committee to building capability, the project organized a regional training course on age determination of selacean fish that aimed to standardize the methodology.

The "MedSudMed Training Course on Age Determination of Selacean Fish" was held in Mazara del Vallo (Italy) at IRMA-CNR (22 November – 1 December) and was supervised by scientists of the institute. Six experts from the region attended.

The course, which lasted eight days, consisted mainly of practical manipulation of cartilaginous fish vertebrae that trainees brought with them from their own institutes. During the course, the following topics were touched on: a) collection and comparison of bibliographic material on the species to be examined (biological and reproductive aspects, age at first sexual maturity, age and growth); b) use of cobalt nitrate (Hoenig and Brown, 1988) and red alizarin (LaMarca, 1966) to stain vertebrae; c) production of sections of vertebrae and spines by embedding in resin, sectioning, mounting on glass slides, grinding and polishing; d) interpretation of growth bands; e) comparison of readings among the various techniques and "readers"; f) analysis of the marginal increments.

As a result of this training activity and in collaboration with the supervising scientists, the participating experts prepared this "Guidelines for Age Determination of Chondrichthyes Fish From the Mediterranean Sea". This paper also represents a contribution of the MedSudMed Project to standardize methodologies as well as to promote scientific exchanges among the countries and institutions involved in project activities.

2. Introduction

In order to study the problems associated with population dynamics, exploitation of fishery resources and fishing activity, it is necessary to know the age composition of the populations. Stock assessment of any fish species requires estimates of growth rates, maximum age and age at maturity. Elasmobranchs (Chondrichthyes) are at the top of marine food webs and have k-strategy in their life history (slow growth rates, long longevity, late maturation, low fecundities and long gestation periods) (Holden, 1977).

Age and growth of living fish is estimated by interpreting the growth zones in some calcified structures. Otoliths and other skeletal parts are typically used for age estimation of teleost fishes (Casselman, 1983), but because of the lack of these hard parts in elasmobranchs, vertebral centra (Cailliet *et al*. 1983), spines (Holden and Meadows, 1962), caudal thorns (Callagher and Nolan, 1999) and neural arches (McFarlane *et al*., 2002) are used to gather information on age estimation and growth rate.

Vertebrae and dorsal spines, in particular the second dorsal spine in some elasmobranchs, are the most frequently used structures and are good indicators of age because the banding pattern in vertebrae and the annuli formed in second dorsal spines are influenced by changes in environmental parameters (McFarlane and Beamish, 1987; Cailliet *et al.*, 1983).

In vertebral centra, the growth pattern consists of a series of concentric incremental zones on the vertebra; the zones are the result of two kinds of concentric marks. Cailliet *et al.* (1983) defined a "ring" as the narrowest kind of concentric mark and used the term "band" to refer to wider concentric marks that may be composed of groups of rings.

Usually bands formed during the summer period, called opaque bands, have a higher rate of calcium and phosphorus across vertebral centra than those formed during the winter-spring period, called translucent bands (Cailliet *et al.*, 1986)

However, the opacity and translucency of these bands varies considerably, depending upon species, light source and enhancing methodology (Cailliet *et al.*, 1983). In general, the opaque bands (wide bands) represent faster growth during the summer period, and the translucent bands (narrow bands) represent slower growth during the winter-spring period.

In any case, it is essential to evaluate the temporal periodicity with which the growth zones are deposited. It is generally accepted that these growth zones are deposited annually, but few studies have been attempted to validate the temporal periodicity of growth increment formation (Cailliet, 1990).

Validation of the frequency of growth increment formation should substantiate that age estimation is accurate. This should be done for all available ages, with validation of the first growth increment and incremental analysis of the edge.

In these guidelines, two staining methods are explained that enhance the appearance of the growth bands in vertebral centra: the red alizarin method (LaMarca, 1966; Gruber and Stout, 1983) and the cobalt nitrate and ammonium sulphide method (Hoenig and Brown, 1988). A non-staining method, involving a thick section of vertebral centra, was also used (Kusher *et al.*, 1992).

Samples of vertebrae from the following species were contributed by course participants:

Species		Catch Area
Centroscymnus coelolepis	(Bocage & Capello, 1864)	Balearic island
Cetorhinus maximus	(Gunnerus, 1765)	Thyrrenian Sea (Tuscany)
Carcharhinus plumbeus	(Nardo, 1827)	Tunisia (Gulf of Gabes)
Galeus melastomus	(Rafinesque, 1810)	Mediterranean Sea (Sicily)
Scyliorhinus canicula	(Linnaeus, 1758)	Mediterranean Sea (Sicily) Thyrrenian Sea (Tuscany)
Mustelus mustelus	(Linnaeus, 1758)	Tunisia (Gulf of Gabes)
Rhinobatos cemiculus	(Geoffroy Saint-Hilaire, 1817)	Tunisia (Gulf of Gabes)
Rhinobatos rhinobatos	(Linnaeus, 1758)	Tunisia (Gulf of Gabes)
Raja clavata	(Linnaeus, 1758)	Tunisia (Gulf of Gabes) Thyrrenian Sea (Tuscany) Mediterranean Sea (Sicily)

A cross-section was taken at different levels of dentine of the second dorsal spine (Holden and Meadows, 1962) and in the spine of the first dorsal fin in holocephali species.

Spines were collected from the following species:

Species		Catch Area
Squalus blanvillei	(Risso, 1827)	Tunisia (Gule of Gabes)
		Ionian Sea
Chimaera monstrosa	(Linnaeus, 1758)	Thyrrenian Sea (Tuscany)

3. Methodological approach

3.1 Collection, description and preparation of vertebra and fin spines

After collection of biometric data from the sampled animals (i.e., precaudal, fork and total length, weight and sex), the first eight to ten cervical vertebrae were removed. The vertebrae were stored in a deep freezer at -18°C. Vertebrae can also be stored in ethanol, dried or fixed in 10% formalin for 24 h and preserved in alcohol (Goldman, 2004).

3.1.1. Vertebral centra

Vertebrae can contain large amounts of calcium (calcium phosphate) (Moss, 1977), and variations in calcium and phosphorous occur across the face of vertebral centra at regular intervals. Staining the calcium phosphate enhances the growth bands.

Before preparation and observation, the haemal arch, the neural arch, the spinal cord and most of the connective tissue must be removed with a scalpel to expose the surface of the vertebral centrum (also referred to simply as vertebra). The centrum must be intact and must be perfectly cleaned, the two opposed cavities must be concentric, and the cleaning must not damage the edge.

The vertebrae are then immersed in bleach to remove all connective tissue and residual flesh. Longer soaking time in bleach is needed for larger centra. Immersion time ranges from 15 to 30 minutes. It is recommended that a magnifying lens or an eyepiece be used during this process because excessive bleaching can decalcify the vertebrae, thus affecting future observation and discrimination of growth bands. On the other hand, a bleaching time that is too short can result in incomplete removal of residual tissue, thus leading to non-uniform staining. The cleaned vertebrae are washed under running tap water. Vertebrae < 4 mm in diameter can be stained without bleaching (Figure 1).
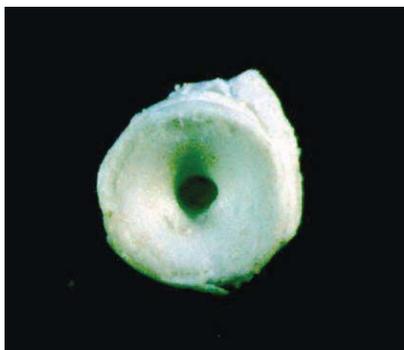

Figure 1. Cleaned whole vertebral centrum, actual diameter 2.8 mm (0.8×)

Figure 2. Cleaned half vertebral centrum, diameter 84 mm (0.8×)

If vertebrae are > 4mm, it is advisable to grind them transversally with progressively finer grinding paper. Grinding is done by hand or with grinder/polisher equipment, holding the vertebra with the fingers, starting with the coarser paper and ending with the finest paper available (400, 600, 800 grit) (Figure 2).

When grinding, care must be taken to hold vertebrae firmly and to make the section as smooth as possible. It is particularly important not to grind beyond the focus of the vertebra, which would obliterate growth bands and might result in incorrect band counts and subsequent measurements.

3.1.2. Spines

 In Squalidae species, the spines are shaped by cartilaginous interior, stem and mantle (Figure 3). The stem surrounds the pulp tissue and is the main body of the spine. The stem consists of three layers of dentine and is produced on the internal and external surfaces of the stem; the mantle originates at the enamel gland. Growth increments can be seen on the surface of the mantle (Beamish and McFarlane, 1985), but alternating growth bands are also observed in cross-sections of the inner dentine (Chilton and Beamish, 1982). Sections of the dorsal spine exhibit a series of rings surrounding a central pulp cavity.

Figure 3. Second dorsal spine of *Squalus blainvillei* (68.0 cm TL, female. Reflected light (0.8×)

Usually the second dorsal spine is used: it is cleaned and air-dried (Beamish and McFarlane, 1985). Spines are removed by cutting horizontally above the notochord, ensuring that the spine base and stem are intact.

In *Chimaera monstrosa* (Linnaeus, 1758), there are two distinct dorsal fins. The first, which originates over the gill openings, is supported by a stout spine at its anterior margin and is free along the terminal part, with the rear surface of the free part double-saw-edged. The higher spine is removed and stored dry.

3.2 Methods for enhancing growth bands

A variety of stains and techniques have been used to enhance the zonation normally visible in spine sections, such as alizarin red, crystal violet, silver nitrate and cobalt nitrate (Cailliet *et al*., 1983, Goldman, 2004). The success of the techniques is often species-specific; modification would change the results. Interpretation of the birth mark and the first growth ring is particularly difficult.

In addition to examining unstained sections of vertebral centra, two staining methods, the red alizarin method (La Marca, 1966; Gruber and Stout, 1983) and the cobalt nitrate and ammonium sulphide method (Hoenig and Brown, 1988), were applied to samples of different species to enhance the growth bands.

3.2.1 Sections

3.2.1.1. Vertebrae

The cleaned vertebrae, prepared as above (3.1), were embedded in an epoxy resin (Inplex® transparent-Remet S.a.s., Italy) in a plastic mould (Figure 4) and sectioned, using an Isomet™ low-speed saw (Buehler® Ltd) with double blades, to obtain a sagittal section (longitudinal section) 0.5 mm thick (Figure 5). The section was then glued (Figure 6) to a glass slide with thermoplastic glue (Lakeside® No. 70C thermoplastic quartz cement, HUGH COURTRIGHT & Co., Ltd) and polished with grinder/polisher equipment (LS2 Remet S.a.s., Italy) (Figure 7).
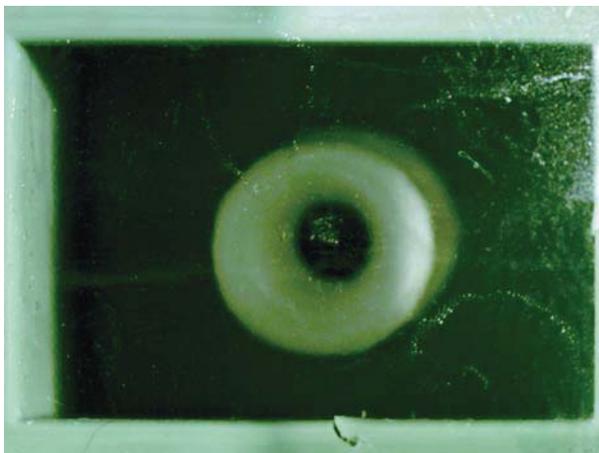

Figure 4. Cleaned whole vertebral centrum embedded in plastic mould

Figure 5. Sectioning apparatus

Figure 6. Slide heated on a hot plate

Figure 7. Polishing with grinder/polisher equipment

The last step was to polish the surface of the sample, using grit paper with a 0.3-µm alumina, to remove scratches. A small suction cup was used to hold the glass slide during the grinding/polishing process (Secor *et al*., 1991) (Figure 8).

Figure 8. Section polished with finer grit paper to remove scratches

Figure 9. Dissecting microscope workstation

When sectioning, grinding and polishing are completed, the intermedial zone, the corpus calcareum and the focus of the vertebra should be visible when viewed through a dissecting microscope under transmitted or reflected light (Figure 9).

Depending upon the size of the vertebra, growth bands should be visible in the intermedial region and particularly along the corpus calcareum. The birth mark is detected by a change in direction, or angle (Goldman, 2004) (Figure 10).

Figure 10. Section of vertebral centrum of *Carcharhinus plumbeus* (202.5 cm TL). Reflected light (0.8×)

This method can be used for vertebrae at least 5 mm in diameter, since smaller vertebra are difficult to section.

3.2.1.2 Spines

The procedure used for spine sections was the same as that used for vertebra sections. Cleaned spines were embedded in a plastic mould in Inplex® resin and cross-sectioned with a double-bladed Buehler Low Speed Saw.

Numerous sections were made at different levels, from the base to the end of the pulp cavity, to find the best region for evaluating growth zones. The section started near the base in order not to miss the first few annual increments (Figure 11).

Figure 11. Several sections of spine of *Squalus blainvillei* (71.0 cm TL).
Reflected light (0.8×)

All sections (0.5 mm thick) were observed under the dissecting microscope with transmitted and reflected light at different magnifications (Figures 12a and 12b).
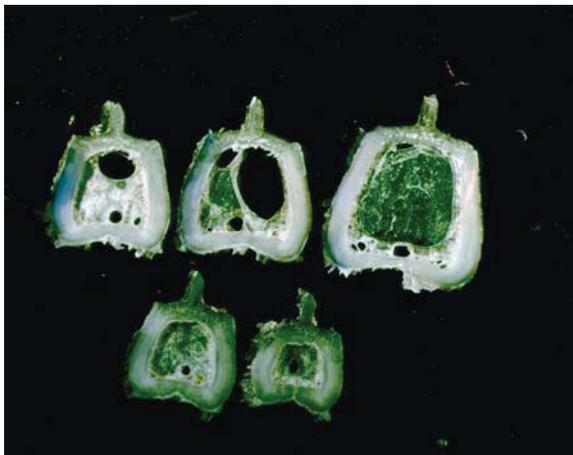

Figure 12a. Sections of first dorsal spine of *Chimaera monstrosa* (84.0 cm TL) with reflected light (0.8×)
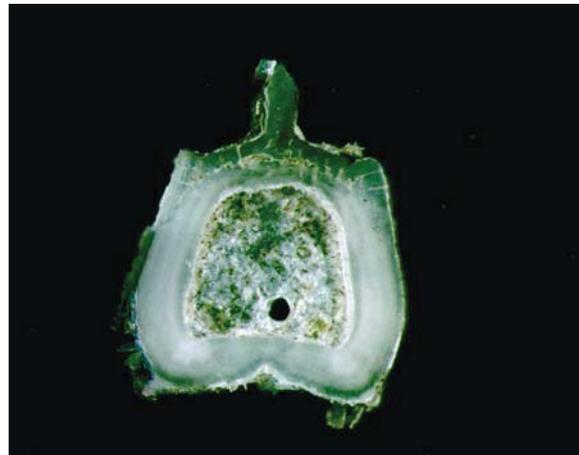
Figure 12b. One section of the same dorsal spine with greater reflected light magnification (2.0×)

3.2.2. Staining methods

3.2.2.1. Red alizarin stain

The red alizarin staining technique used by LaMarca (1966) and Gruber and Stout (1983) is simpler, less expensive and more time-efficient than other staining techniques using crystal violet and silver nitrate.

Alizarin red S (C.L. 58005) was substituted for pure alizarin (C.L. 58000, 1-2-dihydroxyanthraquinone) because it shortens the staining time by half. To prepare the solution, alizarin red S is diluted to 1% with distilled water and 10% ammonia solution is added. Cleaned whole or half vertebra were stained by immersing them in the solution for a variable length of time (from 10 sec. to 1 min.). Immersion time is related to both the size of sample and the species to be examined, but for most samples in the present study, 20 seconds was sufficient. (Figure 13).


Figure 13. Red alizarin solution
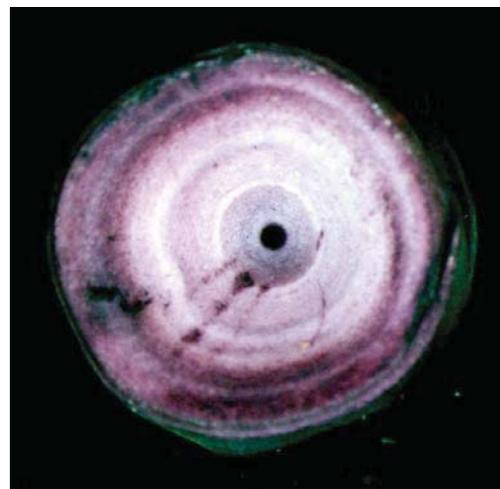
Figure 14. Whole vertebra of *Rhinobatos cemiculus* (103.0 cm TL) stained with red alizarin. Reflected light (0.8×)

When the sample is immersed, it turns a bright red colour and must be rinsed well in running water before being placed under the dissecting microscope. This process should be repeated until the growth patterns are clearly visible. If vertebrae are over-dyed, stain can be removed by immersing them in 20% hydrogen peroxide, the length of time depending on the size of the vertebra and the resolution of the growth bands, usually between 1 and 1½ min. (Figure 14).

3.2.2.2. Cobalt nitrate stain

The cobalt nitrate and ammonium sulphide staining method (Hoenig and Brown, 1988) is easy to use and time-efficient but is much longer and more delicate than the alizarin staining method. Cleaned whole, half or sectioned vertebra can be stained by putting them in solutions prepared beforehand. Unlike the alizarin procedure, the same sample cannot be restained with this technique.

The following solutions must be prepared in advance: 5% cobalt nitrate, 5% ammonium sulphide and 70% ethanol. All solutions are diluted with distilled water. A 10% solution of hydrochloric acid in ethanol (70%) must also be prepared. This solution is called acid alcohol.

Staining was performed by successively dipping the samples into different solutions in a precise order and with carefully controlled immersion times. Small glass containers, previously aligned, were filled with the above solutions (Figure 15) in the following sequence: cobalt nitrate, acid alcohol, ammonium sulphide and acid alcohol. Specimens were immersed and rinsed in distilled water between each solution.

Figure 15. Preparation of cobalt nitrate and ammonium sulphide stain

Figure 16. Cleaned vertebra immersed in cobalt nitrate solution

The cleaned samples were first immersed in cobalt nitrate for not less than 15 minutes and shaken carefully (Figure 16).

The samples were then washed in distilled water and immersed in acid alcohol (estimated time 1-5 min.). This is the most delicate and important step of the stain procedure and fixes the cobalt nitrate; subsequent immersion in ammonium sulphide stains the phosphate in the calcified bands.

Samples stained rather quickly in ammonium sulphide (estimated time 30-60 sec. for average-sized vertebra). This does not always work, since much depends on the success of the preceding steps. The sample may not stain uniformly, or it may stain in a way that does not allow easy identification of growth bands; therefore, staining must be carefully monitored with a magnifying lens. The procedure can thus be stopped either when the sample starts to darken too much or when it doesn't become any darker with further immersion. Unfortunately, it is only at this stage that one can perceive whether the step preceding immersion in ammonium sulphide was suitable.

All the preceding steps must be repeated if the time of immersion in acid alcohol has to be better tuned. Then the sample is immersed in acid alcohol and finally in distilled water.

Except for immersion in distilled water, which is always for 30 seconds, the acid alcohol immersion time is difficult to standardize, and unfortunately, it is the most important variable of the entire staining process. Immersion time varies according to sample size and species. Generally, average immersion time is between 1 and 3 min.; larger samples should remain in acid alcohol for less time than smaller samples (Figure 17).
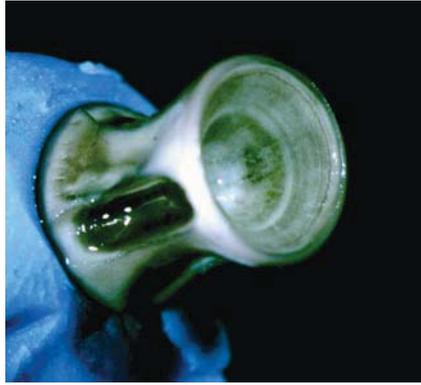
Figure 17. Whole vertebra of *Mustelus mustelus* (62.0 cm TL) stained with cobalt nitrate and ammonium sulphide. Reflected light (1.25×)

4. Results of laboratory work

4.1 Direct observations

Before any treatment (staining or sectioning), all cleaned vertebrae were observed under a dissecting microscope with transmitted light or with oblique lateral illumination (Francis and O'Maolagàn, 2000), (Figure 18).
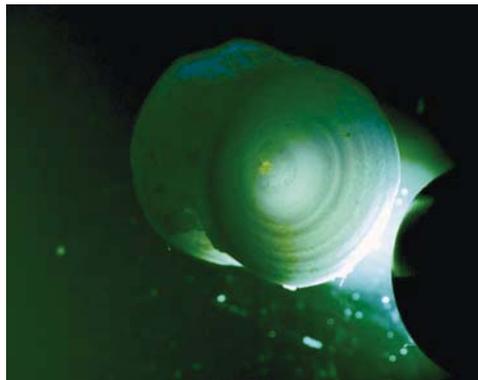

Figure 18. Cleaned vertebra (*Rhinobatos cemiculus)* under dissecting microscope with oblique lateral illumination (1.0×)

Alternating translucent and opaque bands were clearly visible in vertebrae from *Cetorhinus maximus*, *Carcharhinus plumbeus*, *Mustelus mustelus*, *Rhinobatos cemiculus* and *Rhinobatos rhinobatos*. In vertebrae from other species, such alternate banding was less evident because the change in angle representing the birth mark was not evident and the bands were not visible.

4.1.1 Vertebrae

The four species belonging to the families Rhinobatidae, Rajidae and Triakidae showed very good results with thick sections of vertebrae. The angle change was easily identified, and both intermedialia and corpus calcareum showed distinct bands (Figure 19, 20, 21, 22).
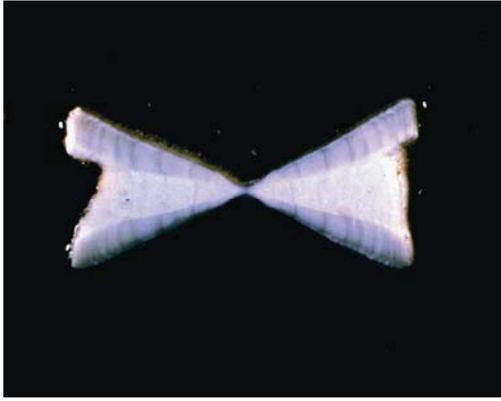
Figure 19. Section of vertebra of *Rhinobatos cemiculus* (164.5 cm TL, female). Reflected light (0.8×)
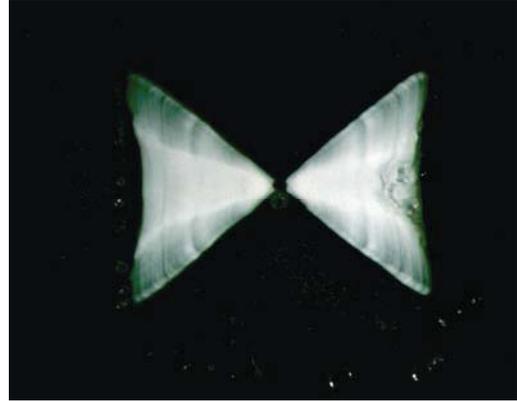

Figure 20. Section of vertebra of *Rhinobatos rhinobatos* (101.0 cm TL, female). Reflected light (0.8×)
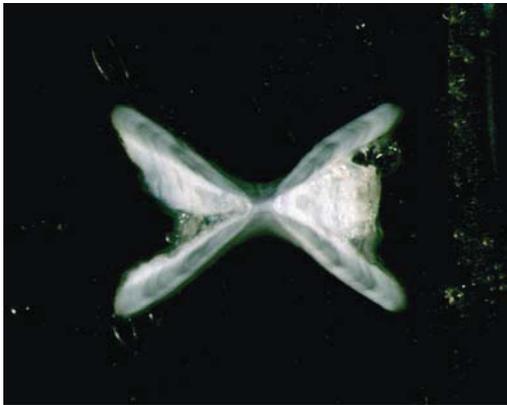
Figure 21. Section of vertebra of *Raja clavata* (73.0 cm TL, male). Reflected light (1.25×)
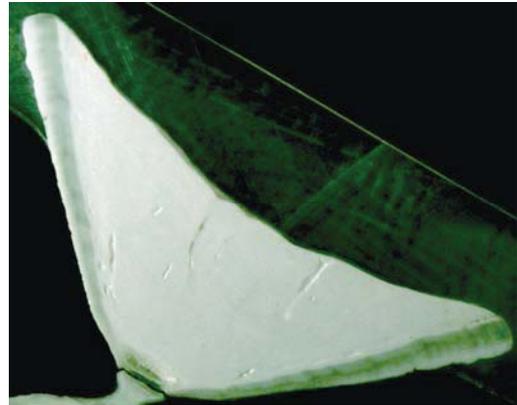
Figure 22. Part of a section of vertebra of *Mustelus mustelus* (147.0 cm TL, female). Reflected light (0.8×)

Banding was less clear in samples of Carcharhinidae and Scyliorhinidae (Figures 23 and 24). Perhaps this pattern would have been more visible if more samples had been analysed.

Figure 23. Part of a section of vertebra of *Carcharhinus plumbeus* (202.5 cm TL, female). Reflected light (1.0×)
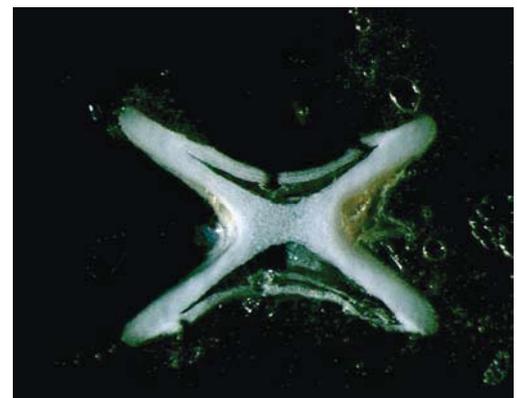

Figure 24. Section of vertebra of *Scyliorhinus canicula* (43.0 cm TL, female). Reflected light (1.0×)

The Squalidae samples showed some traces of bands (Figure 25), but it was not possible to establish whether they were a true banding pattern. Moreover, the shape of the vertebrae

presented problems. It was difficult to detect the change in angle, since the focus of the centrum is very large and open and the corpus calcareum is rather difficult to see.


Figure 25. Section of vertebra of *Centroscymnus coelolepis* (44.0 cm TL, female). Reflected light (1.6×)

Small vertebrae were more difficult to interpret than larger ones. Growth bands of deep-dwelling species are affected by the fact that there is little seasonal variation in the environment, which leads to continuous deposition of phosphorus and calcium, producing a homogeneous banding pattern (Cailliet, 1990).

For *Cetorhinus maximus*, growth patterns were very clear in both whole and half vertebrae. Slightly fewer bands were visible in whole vertebrae than in half vertebrae. Moreover, almost double the number of bands was observed in the intermedialia in comparison with the corpus calcareum. For this species, six prenatal bands were observed, confirming results from the literature (Parker and Boeseman, 1954; Parker and Stott, 1965; Springer and Gilbert, 1976; Izawa and Shibata, 1993). Estimation of growth patterns gave similar results when examined by different observers (Table 1).

4.1.2 Spines

Spines of *Squalus blainvillei* and *Chimaera monstrosa* were examined.

For both species, the first step was to observe the mantle of whole spine. For *Squalus blainvillei*, the second dorsal spine was observed.

For *Squalus blainvillei*, it was difficult to decide which area of the spine showed the most distinct growth pattern. Moreover, it was difficult to identify the inner dentine, where the interpretations are conducted.

Usually four sections were made. Growth bands were always visible, but interpretation was difficult. The best section appeared to come from the end of the pulp cavity; however, there is a risk of under-estimating the age of younger fish (Sion *et al.*, 2002) (Figure 26).

Figure 26. Section of dorsal spine of *Squalus blainvillei* (71.0 cm TL, female).
Transmitted light (2.0×)

In spines from small fish, growth bands were more easily visible. However, there appeared to be agreement between the estimation of growth bands and data published by Cannizzaro *et al.* (1995b), who estimated age from stained vertebral centra.

All samples of spines were observed under a dissecting microscope on a black background, using transmitted light at several magnifications.

For *Chimaera monstrosa*, several sections were made; growth bands were quite clear in all thick sections when sectioning was 2/3 of the total length of the spine.

4.2 Red alizarin stain

When applied to whole vertebrae, half sections and sections, this technique almost always gave clear observations for all species.

The four species belonging to the families Rhinobatidae, Rajidae and Triakidae showed the clearest banding pattern when treated with red alizarin (Figures 27, 28, 29).
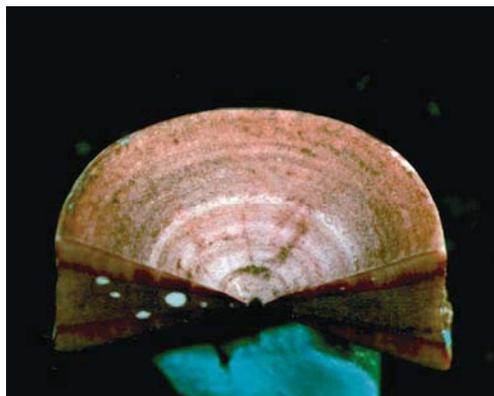
Figure 27. Half vertebra of *Rhinobatos cemiculus* (197.0 cm TL, female) stained with red alizarin.
Reflected light (1.0×)

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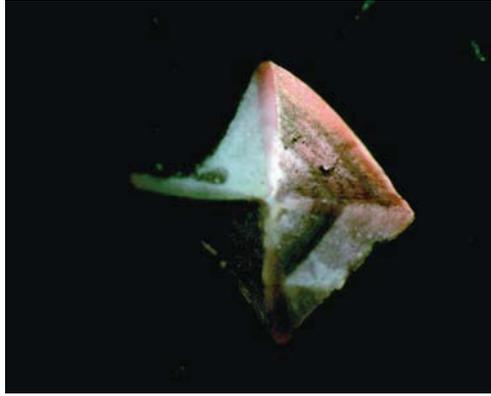
Figure 28. Half vertebra of *Raja clavata* (78.0 cm TL, male) stained with red alizarin.
Reflected light (1.25×)
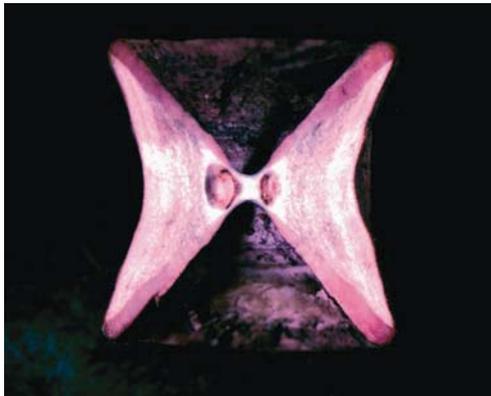
Figure 29. Half vertebra of *Mustelus mustelus* (101.5 cm TL, female) stained with red alizarin.
Reflected light (1.0×)

For *Rhinobatos cemiculus*, the techniques were applied to whole vertebrae, halves and sections, but for the other families, sections were not examined.

Results of age estimation provided by the present exercise for *Raja clavata* were similar to those obtained by Cannizzaro *et al.* (1995a) on 424 specimens that were processed with the same staining procedure. When red alizarin was applied to samples from the families Scyliorhinidae, Carcharhinidae and Squalidae, no growth-band pattern could be observed.

4.3 Cobalt nitrate stain

The cobalt nitrate staining technique was applied only to whole vertebrae and half sections but not to sections.

The four species belonging to the families Rhinobatidae, Rajidae and Triakidae showed good results for both whole vertebrae and half sections (Figures 30, 31, 32, and 33). Growth bands were considerably enhanced by this technique (Table 1).
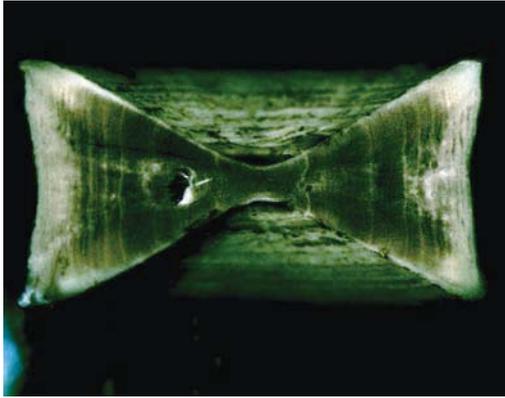
Figure 30. Half vertebra of *Rhinobatos cemiculus* (197.0 cm TL, female) stained with cobalt nitrate. Reflected light (0.8×)
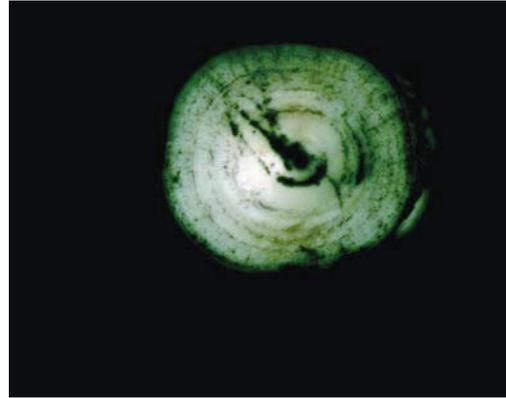
Figure 31. Whole vertebra of *Rhinobatos rhinobatos* (101.0 cm TL, female) stained with cobalt nitrate. Reflected light (1.0×)
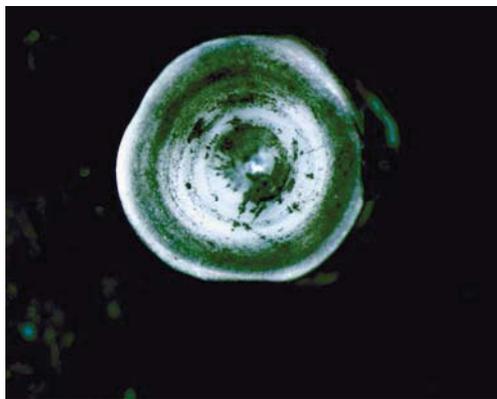
Figure 32. Whole vertebra of *Raja clavata* (73.0 cm TL, female) stained with cobalt nitrate. Reflected light (1.0×)
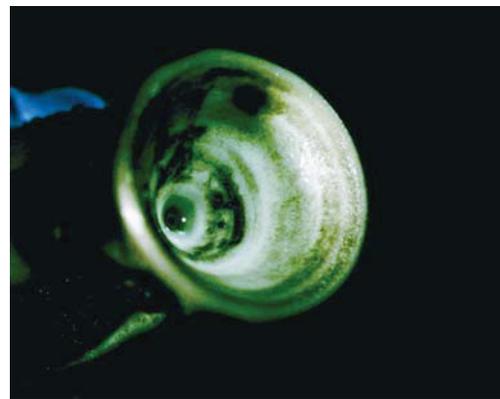
Figure 33. Whole vertebra of *Mustelus mustelus* (101.5 cm TL, female) stained with cobalt nitrate. Reflected light (1.0×)

Samples from Scyliorhinidae, Carcharhinidae and Squalidae families were difficult to interpret, since in most cases, this type of staining did not produce clear growth patterns. Cobalt nitrate staining gave good results only for Carcharhinidae (Figures 34 and 35). It is very likely that the main difficulty in the staining procedure is the amount of time necessary for the first step, which involves immersion in acid alcohol.
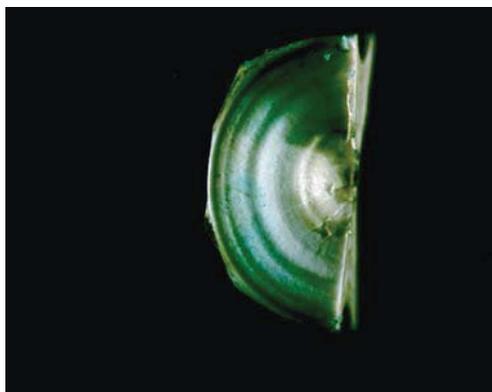
Figure 34. Half vertebra of *Carcharhinus plumbeus* (112.5cm TL, female) stained in cobalt nitrate. Reflected light (0.8×)
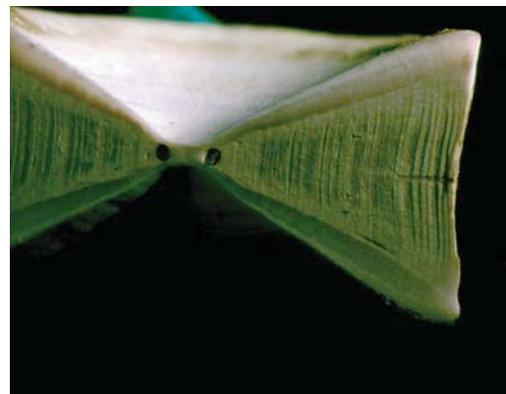
Figure 35. Half vertebra of *Carcharhinus plumbeus* (202.5 cm TL, female) stained in cobalt nitrate. Reflected light (0.8×)

Table 1: Effectiveness of the three techniques for enhancing bands on vertebra for all species examined: (++) highly effective; (+) effective; (-) unsuccessful; (NA) not tried.

Common name	Scientific name	TL (cm)	Technique			Number of growth bands
			Untreated section	Red alizarin	Cobalt nitrate	
Rhinobatidae						
Common guitarfish	*R. rhinobatos*	101.5	++	+	+	5
Blackchin guitarfish	*R. cemiculus*	103.0	++	+	+	3
	R. cemiculus	164.5	++	+	+	9 - 10
	R. cemiculus	197.0	++	+	+	16
Rajidae						
Thornback ray	*R. clavata*	46.0	++	+	+	3 - 4
	R. clavata	73.0	++	+	+	5
Triakidae						
Smoothhound	*M. mustelus*	62.0	++	+	+	3
	M. mustelus	101.5	++	+	+	4 - 5
	M. mustelus	147.0	++	+	-	14-16
Carcharinidae						
Sandbar shark	*C. plumbeus*	67.5	NA	NA	-	?
	C. plumbeus	91.0	NA	NA	-	?
	C. plumbeus	112.5	+	-	+	4 - 5
	C. plumbeus	202.5	+	-	-	20-22
Scyliorhinidae						
Smallspotted catshark	*S. canicula*	43.0	+	-	-	3-4
Blackmouth catshark	*G. melastomus*	36.5	+	-	+	3-4
	G. melastomus	44.5	NA	-	-	
Squalidae						
Portuguese dogfish	*C. coelolepis*	44.0	-	-	-	Some traces
	C. coelolepis	49.0	-	-	-	

(At least two specimens were used for all species. Only one specimen was used for *Cetorhinus maximus*, so the results are not reported in the table).

A summary of the average percent effectiveness of the three techniques:

Untreated section: ++ = 60%   + = 27%   - = 13%

Red alizarin: ++ = 0%   + = 56%   - = 44%

Cobalt nitrate: ++ = 0%   + = 56%   - = 44%

5. Conclusions

The techniques described in these guidelines are simple, easy to perform, time-efficient and relatively low-cost; they clearly helped to highlight and interpret growth bands in the vertebra. The study provided new insights, since very little has been published on age and growth of Chondrichthyes species in the Mediterranean Sea.

The two staining methods applied here provide only general insights because a limited number of samples were examined, given the wide variety of species; too few samples were available to perform all tests. Further investigations are necessary. Table 1 summarizes the information that was produced and can be collected. Immersion times for the staining

procedures are only approximate: further tests should be conducted to determine more precise treatment times by species and size.

However, the alizarin staining method is simpler and faster than the cobalt nitrate and ammonium sulphide staining technique. Thick sections (0.5 mm) provided the most distinct growth bands, especially in the zone of the corpus calcareous, where it is possible to make measurements.

Measurements of the radius from the focus of the vertebra towards the first and second rings, and so on, can be used to back-calculate vertebral growth and estimate body size.

Families can be divided into two general groups according to their response to the different techniques.

Samples of Rhinobatidae, Rajidae and Triakidae gave positive results (clear growth bands) with all techniques; thick sections gave especially good results.

For the second group of families, Scyliorhinidae, Carcharhinidae and Squalidae, neither the red alizarin nor the cobalt nitrate staining technique produced good growth banding. When bands were present, they were not clear enough to be distinguished properly.

Vertebrae from young individuals, whether whole, half or sectioned, were easily read even at the outer edge, where bands were more widely spaced. However, when vertebrae are large, from older individuals, it is necessary to examine sections, particularly along the corpus calcareous, since the bands near the edge are very close together.

Usually for small specimens and for some species, especially when the vertebrae do not contain a "deep cone", interpretations are difficult, and it is particularly difficult to determine the exact location of "angle change" that signifies the birth mark

The lack of knowledge concerning structure and development of the spine led to difficulty in determining the appropriate area for interpretation. As well, vertebrae were not available from the same individual, so comparative "reading" could not be done.

Although various methods have been used to estimate age in elasmobranchs, age validation needs to be conducted to confirm that growth zones are deposited annually. A number of techniques can be applied, such as size-frequency analysis, vertebra or spine-edge measurements, and tag recapture and tetracycline marking. One of the best methods involves using a tetracycline mark to confirm that growth bands are formed and deposited annually (Cailliet, 1990).

It is advisable to compare spine and vertebral centra, for those species where these structures are available, in order to better estimate the age.

Tetracycline-marked material does not exist, but could be collected in the future. Now only an indirect method of validation is possible. This involves marginal increment analysis. However, it requires intensive sampling and can help only in verification (confirming by comparison with other indirect methods) and is not true validation (Campana, 2001).

Marginal-increment analysis is a useful technique that assesses changes in growth of the margin on a seasonal basis by comparing opacity and translucency of the edge of vertebrae over time in a number of fish of the same age to quantify seasonal changes in growth. This increment can be plotted against time of capture to determine temporal periodicity of band formation. This technique should be applied only to species where banding is distinct and the species can be intensively sampled. Additional staining studies should be conducted on species for which samples were inadequate or no results were obtained.

The participants strongly recommended and encouraged that another course be conducted, since not all samples were treated.

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Appendix B: Terms of reference that were prepared for the MedSudMed Training Course on Age Determination of Selacean Fish

Activities

The course will be held in Mazara del Vallo at IRMA-CNR and will be conducted by two technicians from the institute. The trainers will prepare didactic material to be given to the trainees upon their arrival at the institute. The course will have duration of eight working days during which the programme of work will be as follows:

1) Collection and comparison of bibliographical material on the species to be examined (biological and reproductive aspects, age at first sexual maturity, age and growth)

2) Coloration of vertebrae by use of the techniques of cobalt nitrate, (Hoenig and Brown, 1988) and red alizarin (La Marca, 1966)

3) Production of thin sections of vertebrae and spines of the studied species through incorporation in resin, cutting, mounting on glass slides and possible grinding and cleaning.

4) Interpretation of the growth bands.

5) Comparison of the readings among the various techniques and among the "readers".

6) Analysis of the marginal increase.

Requirements

The trainees are expected to bring their own samples of vertebrae, prepared following some recommendations:

- The vertebrae should be brought to the laboratory already cleaned and dried.
- It is preferable to bring at least two to three vertebrae from each sampled animal and at least 15 to 20 animals of different classes of size for each species[1] (8 species x 15-20 animals x 2-3 vertebrae = 240 to 480 vertebrae).
- For *Squalus blainvillei* besides the vertebrae it is also necessary to study the spine of the first dorsal fin.

Reporting

At the end of the course, trainers and trainees shall submit a report on the activities carried out and results obtained.

- **Trainees**. The reports should describe in detail:
  - activities conducted

_____

[1] *Mustelus mustelus, Mustelus punctulatus, Carcharhinus plumbeus, Squalus blainvillei, Rhinobatos rhinobatos, Rhinobatos cemiculus, Dasyatis pastinaca, Raja clavata*

- the results obtained for each activity,
- possible problems encountered during the course,
- correspondence between the objectives of the course and its content,
- if the expectations were fulfilled,
- if the course added to their knowledge on the methods,
- if they will be able to apply the methods learned in their daily work,
- if there is a need for a follow- up to the course,
- any other comments or suggestions.

- **Trainers**. The report will be presented in the form of a manual on age determination of selacean fish to be published as a "MedSudMed Occasional Paper". The manual should contain:
  - background information on age reading of selaceans
  - a description of the activities conducted during the course
  - the methodology followed for the preparation, reading and interpretation of data
  - illustrations taken from the course highlighting the key aspects of vertebrae reading
  - definition of terms used during the course
  - relevant bibliographic references
  - information sheets on the main species with the relevant biological information.

Date and venue

The course will be held at IRMA-CNR, via L. Vaccara, 61, 91026 Mazara del Vallo (TP), Italy. Because of the involvement of the trainers in autumnal sea surveys, the most suitable period for the course would be 22 November – 1 December

Trainers:
Mr Salvatore Gancitano
Mr Pietro Rizzo