RESEARCH PAPER

Comparison of Staining Techniques for Age Determination of Some Chondrichthyan Species

Nuri Başusta[1,*], Sefa Ayhan Demirhan[2], Erdoğan Çiçek[3], Asiye Başusta[1]

[1] Fırat University, Faculty of Fisheries, 23119, Elazığ, Turkey.
[2] Iskenderun Technical University, Faculty of Marine Science, 31200, Iskenderun, Hatay, Turkey.
[3] Nevşehir Hacı Bektaş Veli University, Department of Biology, Faculty of Art and Science, 50300, Nevşehir, Turkey.

Abstract

In this study, six chondrichthyan species (*Raja clavata, Raja miraletus, Raja asterias, Torpedo marmorata, Rhinobatos rhinobatos and Gymnura altavela*) were captured for age determination in Iskenderun Bay (North eastern Mediterranean Sea) between May 2010 and January 2012. Age was determined from vertebral sectioned, using four staining techniques, namely Crystal Violet, Silver Nitrate, Safranin O and Alcian Blue. The results from these four techniques were compared to each other, with respect to visibility success on growth rings. Of all four staining techniques, Silver Nitrate staining provided the best optical resolution for growth rings of *R. clavata, R. miraletus* and *R. rhinobatos*. For, *R. asterias, T. marmorata* and *G. altavela*, however, it provided a lower optical resolution. In fact, four staining technique was also efficient for *R. clavata*. The most efficient staining techniques were Safranin O in *T. marmorata* and Alcian Blue in *R. rhinobatos*. The order of average readability scores for staining techniques was as follows: Silver Nitrate = Safranin O > Crystal Violet = Alcian Blue. A standardized staining methodology that can be used for all species studied, however, has not been achieved.

Keywords: Age determination, Cartilaginous Fish, Crystal Violet, Silver Nitrate, Alcian Blue, Safranin O.

Bazı Kıkırdaklı Balık Türlerinde Yaş Tayininde Kullanılan Boyama Yöntemlerinin Karşılaştırılması

Özet

Bu çalışmada altı kıkırdaklı balık türü (*Raja clavata, Raja miraletus, Raja asterias, Torpedo marmorata, Rhinobatos rhinobatos* and *Gymnura altavela*) yaş tayini amacıyla Mayıs 2010 – Ocak 2012 tarihleri arasında İskenderun Körfezi'nde yakalanmıştır. Yaş tayini Kristal Viyole, Gümüş Nitrat, Safranin O ve Alsian Mavisi boyama yöntemleri kullanılarak omur kesitlerinden yapılmıştır. Bu dört teknik büyüme halkalarındaki görünürlülük başarısı açısından birbirleriyle karşılaştırılmıştır. Bu dört boyama tekniğinden gümüş nitrat, *R. clavata, R. miraletus* ve *R. rhinobatos* türlerinin büyüme halkaları için en iyi görüntü çözünürlüğünü sağlamıştır. *R. asterias, T. marmorata* ve *G. altavela* türleri için ise daha düşük görüntü çözünürlüğü vermiştir. Aslında, *R. clavata* için dört boyama tekniği de etkili olmuştur. *R. rhinobatos* omurlarındaki büyüme halkalarını belirginleştirmek için en etkili boyama tekniği Alsian Mavisi ve *T. marmorata* için ise Safranin O olmuştur. Boyama teknikleri için ortalama okunabilirlik puanları sırası: Gümüş Nitrat = Safranin O > Kristal Viyole = Alsian Mavisi. Bununla birlikte, türlerin hepsinde kullanılabilecek standart bir boyama tekniği bulunamamıştır.

Anahtar Kelimeler: Yaş tayini, Kıkırdaklı balıklar, Kristal Viyole, Gümüş Nitrat, Alsian Mavisi, Safranin O.

Introduction

Most chondrichthyan fishes exhibit slow growth; late age at maturity; low fecundity and productivity (small, infrequent litters); long gestation periods; high natural survivorship for all age classes; and a long lifespan compared to teleost fishes (Cailliet *et al.,* 2005; Cavanagh and Gibson, 2007). In many parts of the world, some chondrichthyans are fished commercially; thus, in order to ensure proper management of the stocks, age and growth data must be obtained. Because of low productivity, both target and non-target some chondrichthyan species are adversely affected by commercial fisheries (Camhi *et al.,* 1998; Stevens *et al.,* 2000, 2005; Cavanagh and Gibson, 2007). For this reason, many chondrichthyan species are listed in the IUCN Red Data Book (Cavanagh and Gibson, 2007; Serena, 2010). The impact of commercial fisheries on chondrichthyan fish populations around the world is currently the focus of considerable international concern, on the part of both academic and non-governmental

organisations working for the conservation and management of stocks (Camhi *et al.,* 1998; Fowler and Cavanagh, 2005; Cavanagh and Gibson, 2007).

One of the most important tasks in fish and fisheries biology is the age determination. Age information forms the basis for calculations of growth rate, mortality rate and productivity, ranking it among the most influential of biological variables. Many calcified structures produce periodic growth increments useful for age determination on an annual or daily scale (Campana, 2001). There are some difficulties in accurately ageing chondrichthyans, which, unlike teleost fish, do not possess otoliths or conventional scales (Holden, 1974). Chondrichthyan ageing studies, therefore, have been based on a variety of different techniques, including tooth replacement rates (Moss, 1972), eye lens weight (Siezen, 1989), X-radiography (Ferreira and Vooren, 1991; Officer *et al.*, 1996), dorsal spines (Holden and Meadows, 1962) and caudal thorns (Gallagher and Nolan, 1999). Most chondrichthyan ageing studies, however, are based on the analysis of periodic growth increments within vertebral centra (Cailliet *et al.*, 1986).

Periodic growth increments and ring formations on these parts are not easily visible. Dyeing techniques, therefore, are useful in aiding age determination and are particularly helpful if there is poor ring formation, calcification, etc. Numerous staining techniques involving calcium-binding chemicals (e.g. Alizarin Red S, Crystal Violet and Silver Nitrate), as well as soaking techniques using chemicals such as ethanol, have been used in an effort to enhance band resolution in chondrichthyan vertebrae. The success of each technique, however, is often species specific, and slight modifications in technique may enhance the results (Goldman, 2005). Unfortunately, most chondrichthyans have not yet been reliably aged. For some species, using the current scientific techniques on calcified structures may not even be possible (Cailliet *et al.* 2005).

Obtaining a small sample size from cartilaginous fish leads to poor parameter estimation. It is very important, therefore, to conduct an efficient age determination of the specimens. Moreover, it is of utmost importance to complete the most appropriate method of age determination, in terms of time and financial costs; the methods used for age determination of cartilaginous fish are a lot more expensive, require more chemicals, and take longer to process in laboratory studies, compared to the methods applied on osteichthyes (Goldman, 2005).

It is known that spotting and precisely determining the age rings depend on the dyeing technique used. The purpose of this study, therefore, was to test the efficiency of four different staining techniques, namely Crystal Violet, Silver Nitrate, Safranin O and Alcian Blue, in enhancing the clarity of bands in the vertebrae of six batoid species: *Raja clavata*, *Raja miraletus*, *Raja asterias*, *Torpedo marmorata*, *Rhinobatos rhinobatos* and *Gymnura altavela*. The determination of their efficiency was based on a comparative analysis of readability.

Materials and Methods

This study was conducted on the north-eastern Mediterranean coast of Turkey (36° 43' 990" E 35° 47' 075" N; 36° 37' 920" E 35° 38' 804" N) (Figure 1) between May 2010 and January 2012. A total of 144 specimens (*R. clavata*, 25; *R. miraletus*, 22; *R. asterias*, 15; *T. marmorata*, 22; *G. altavela*, 28; *R. rhinobatos*, 32) were collected using commercial gill net (44 mm mesh size), trawling (44 mm stretch length) and longline fishing. Specimens were initially preserved in a plastic box with ice and kept so for approximately 6–8 h. A segment of the vertebral column containing the first 10–12 vertebrae immediately posterior to the scapular origin was removed on board, labelled and then stored in ice until the laboratory studies.

Figure 1. The sampling area Iskenderun Bay (North-eastern Mediterranean Sea).

Processing of Vertebrae and Centra Preparation

In the laboratory, soft tissue was removed from the frozen vertebral segments using a scalpel and fine forceps. The individual vertebrae were then cut apart from each other and soaked in warm distilled water. Hypochlorite (6%) was used to remove the last remaining bits of connective tissue from the vertebrae. Hypochlorite, however, can decalcify cartilage when overused, so soak times were kept to less than 10 minutes. The vertebrae were then air-dried for at least 48 hours, and later they were sectioned using a Ray Tech Gem Saw with two diamond blades separated by a 0.6 mm spacer (Başusta and Sulikowski, 2012). Smaller centra were sanded with a Dremell™ tool to replicate a sagittal cut. Processed vertebrae were mounted horizontally on glass microscope slides and ground with successively finer-grit (no. 400, no. 600) wet-dry sandpaper. Each vertebra was then remounted, and the sides were ground to produce a thin (0.4–0.5 mm) sample (Başusta *et al.,* 2008).

Staining Methods

The following four staining techniques for enhancing the clarity of the bands were tested on vertebral sections of the specimens: Crystal Violet, Silver Nitrate, Safranin O and Alcian Blue.

Safranin O

The Safranin O staining method has been used to detect the cartilage of rabbit and mouse embryos. This staining method was modified by Kahveci *et al.* (2000) and Tran *et al.* (2000) and was used for enhancing growth bands in *Torpedo marmorata* (Duman and Başusta, 2013).

Vertebral sections were stained with Weigert's iron hematoxylin working solution for 10 minutes and washed in running tap water for 10 minutes. They were stained with the Fast Green (FCF) solution for 5 minutes and rinsed quickly with acetic acid solution for no more than 6 seconds. Again, vertebral sections were stained in Safranin O solution for 5 minutes and dehydrated and cleared with ethyl alcohol (95%) and absolute ethyl alcohol, using 2 changes for each, with 2 minutes between each change. The cartilage stains varied from orange to red.

Alcian Blue

In this method, Alcian Blue dyeing techniques were used to enhance the visibility of the band on vertebral sections. Vertebral sections were soaked in Alcian Blue solution (16 ml 100% ethanol, 2 mg Alcian Blue and 4 ml glacial acetic acid in 0.8 ml distilled water) for 12 h (Başusta *et al.,* 2008).

Crystal Violet

Vertebral sections were soaked in a 0.01% solution of crystal violet (Schwartz, 1983). The staining interval was 10 minutes in this study.

Silver Nitrate

Vertebral sections were placed in a 1% silver nitrate solution for 1–3 minutes and simultaneously illuminated with an ultraviolet light source for anywhere between 2 and 4 minutes, depending on the species and size of the vertebral sections (Stevens, 1975; Schwartz, 1983; Cailliet *et al.,*1983).

Band Counts

In order to identify the growth bands, vertebral longitudinal sections were viewed and digitally photographed under reflected light, against a black background. A total of 10–12 vertebral sections were taken for each fish examined and read independently by two readers. All images were taken using a Leica M40 dissecting microscope with a high-resolution (2084*2048 pixels) Diagnostic Instruments CCD digital camera and Leica IM1000 image analysis software. Vertebral images were then enhanced using Adobe Photoshop CS2 to improve sharpness and clarity.

Each section was scored subjectively for readability on a five-point scale: 1, excellent; 2, good; 3, acceptable (some increments not clear, or some uncertainty in distinguishing 'true' increments from growth checks); 4, poor (many increments not clearly defined, and alternative counts possible); 5, virtually unreadable, as used by Paul and Horn (2009).

In addition, to evaluate the efficiency of each technique, two independent readings temporally spaced by a minimum of 30 days were made by a single reader and several precision indices calculated, namely the percent agreement, the index of average percent error (IAPE) proposed by Beamish and Fornier (1981), the coefficient of variation (CV) and precision index (D) by Chang (1982) and Coelho and Erzini (2002). The equations used for IAPE, CV and D were as follows:

$$IAPE$$

where *N* is the number of animals aged, *R* is the number of readings, *Xij* is the count from the *j*th animal at the *i*th reading and *Xj* is the mean age of the *j*th animal from *i* readings.

$$CV = 100\% \times \frac{\sqrt{\sum_{i=1}^{R}(Xij-Xj)^2 / (R-1)}}{Xj}$$

where CV is the age precision estimate for the jth fish, Xij is the age determination of the jth fish by the ith reader, Xj is the mean age of the jth fish and R is the number of readings.

Index of precision (D) was calculated using $D = CV/\sqrt{Z}$ where Z = the number of readers. The hatch mark (age zero) was defined as the first distinct mark distal to the focus that coincided with a change in the angle of the corpus calcareum (Sulikowski *et al.*, 2003).

Results and Discussion

Images, after sanding and polishing, of thin vertebral sections of each species stained with Crystal Violet, Silver Nitrate, Safranin O and Alcian Blue to enhance growth patterns are presented in Figure 2–7.

Average readability scores for each species for each staining technique are presented in Table 1. The order of scores for staining techniques was Silver Nitrate = Safranin O > Crystal Violet = Alcian Blue. Of all four staining techniques, Silver Nitrate staining provided the best optical resolution for growth rings of *R. miraletus* and *R. rhinobatos*. For, *R. asterias* and *G. altavela*, however, it provided a lower optical resolution. For *R. clavata*, all of the four staining techniques were considered efficient. The most efficient staining techniques were Safranin O in *T. marmorata* and Alcian Blue in *R. rhinobatos*.

Although Crystal Violet was used successfully to enhance the growth bands of *R. clavata*, Safranin O was used for *R. asterias*, *G. altavela* and *T. marmorata*, and Alcian Blue was used for *R. mirelatus* and *R. rhinobatos* (Table 2).

The preservation of vertebrae before dyeing is important. We recommend putting them into deep freeze. Otherwise, they can become more fragile, and age rings can be lost. If so, they should be held in distilled water for 24 h or buffer for longer preservation. In addition, the thickness of a longitudinal section is very important; it should be 0.4–0.6 mm. The glass slides on which vertebrae sections will be stacked should be clean and dry. In order to have clear reading of age rings, sectioned vertebrae should be bow-tie or butterfly shaped because it makes it possible to see bands on both sides of the sectioned vertebrae.

Numerous techniques have been employed to enhance the visibility of possible microstructures within hard parts of chondrichthyans. The staining methods examined in this study are known to enhance growth increments in vertebral centra of various chondrichthyan species (Ismen, 2003; Gallagher *et al.*, 2004; Başusta *et al.*, 2008; Başusta *et al.*, 2010). No standardised ageing methodology for these groups, however, has been developed. The success of each staining technique is species dependent, and slight modification of an individual technique may be required to enhance the results for a given species (Wischniowski, 2008). It is thought that some chondrichthyan species/specimens may have less calcium in their cartilaginous structures, possibly due to a more nutrient-poor environment (Cailliet, 1990). Staining techniques, therefore, are generally inefficient for some species/specimens. In addition, when we look at Figures from 3 to 7, different ages are seen in vertebrae. This difference may change to the vertebrae series of specimen. In this case, the best choice of vertebrae for age determination should be identified. It was found that using cross sections of vertebrae is very important, and it should be taken from the centre of the vertebra (Başusta *et al.*, 2013).

No standardised staining methodology has been developed for age determination in the species examined in this study. Wischniowski (2008) indicated that the success of each staining technique is species dependent and that slight modifications may be applied to enhance growth rings. Furthermore, the modified staining methods could be used to enhance the visibility of the growth increments in the vertebrae in chondrichthyans. In a future study, therefore, staining techniques should be applied with some modifications to enhance vertebral sections to provide higher precision in ring counts in species whose age is difficult to determine. In addition, the best vertebra for age determination should be identified according to young and old individuals, and sex of species.

Table 1. Distribution of average readability scores for each species in each staining technique

Species	Alcian Blue	Chrystal Violet	Silver Nitrate	Safranin O
Raja clavata	2	2	1	2
Raja miraletus	4	4	1	4
Raja asterias	4	4	5	4
Torpedo marmorata	4	2	4	1
Gymnura altavela	3	3	4	3
Rhinobatos rhinobatos	1	3	1	2

Five-point scale: 1, excellent; 2, good; 3, acceptable; 4, poor and 5, virtually unreadable

Table 2. Precision of the four different staining techniques on vertebrae of six batoid. The calculated precision indexes are the average percent error (APE), the coefficient of variation (CV) and the precision index (D). The percentage of readings that did not allow the estimation of a valid age (ND) is also given

| Species	N	Technique	Precision indices			
			APE	CV	D	% ND
Raja clavata	25	Silver nitrate	4.0	5.4	3.1	0.0
		Chrystal violet	6.6	8.9	5.2	6.9
		Safranin O	8.6	9.5	5.5	4.4
		Alcian blue	7.2	10.2	6.1	12.9
Raja miraletus	22	Chrystal violet	9.2	12.4	7.2	13.7
		Silver nitrate	8.0	11.0	6.4	7.8
		Safranin O	7.9	10.6	6.2	14.9
		Alcian blue	7.6	10.3	6.0	11,5
Raja asterias	15	Chrystal violet	11.4	15.6	9.1	15.3
		Silver nitrate	-	-	-	100
		Safranin O	7.2	10.6	6.2	12.9
		Alcian blue	12.5	16.4	9.5	13.5
Torpedo marmorata	22	Chrystal violet	7.9	10.7	6.3	7.8
		Silver nitrate	9.1	12.4	7.1	13.0
		Safranin O	6.0	7.7	4.4	6.1
		Alcian blue	12.4	16.5	9.5	13.3
Gymnura altavela	28	Chrystal violet	7.5	10.2	5.9	4.5
		Silver nitrate	8.4	11.0	6.4	11.6
		Safranin O	6.2	7.6	4.5	1.0
		Alcian blue	7.2	10.2	6.1	12.9
Rhinobatos rhinobatos	32	Chrystal violet	7.9	10.6	6.2	11.5
		Silver nitrate	8.3	11.1	6.4	10.9
		Safranin O	6.6	9.0	5.1	5.5
		Alcian blue	6.0	7.5	4.4	4.5
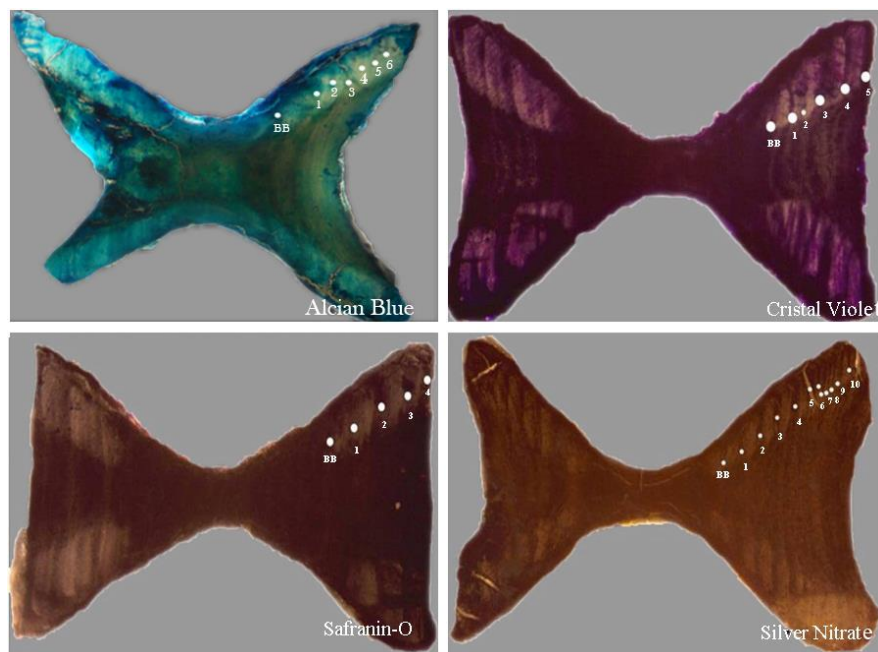
Figure 2. Mounted vertebral thin section from *Raja clavata* stained with Chrystal Violet (age -5 yr a 49.2 cm TL female), Silver Nitrate (age-10 yr a 74.2 cm TL male), Safranin O (age-4 yr a 45.9 cm TL male) and Alcian Blue (age-6 yr a 56.2 cm TL female), BB: Birth Band.
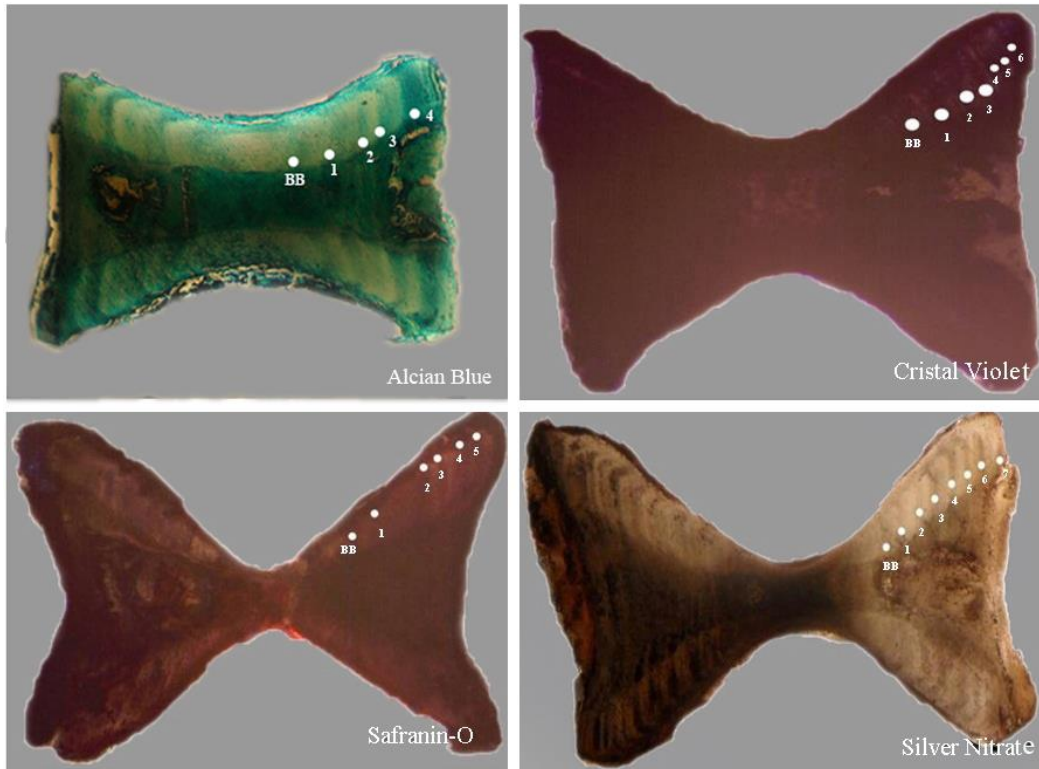
Figure 3. Mounted vertebral thin section from a 58.9 cm TL female *Raja miraletus* stained with Chrystal Violet (age-6 yr), silver nitrate (age-7 yr), Safranin O (age-5 yr) and Alcian Blue (age-4 yr), BB: Birth Band.
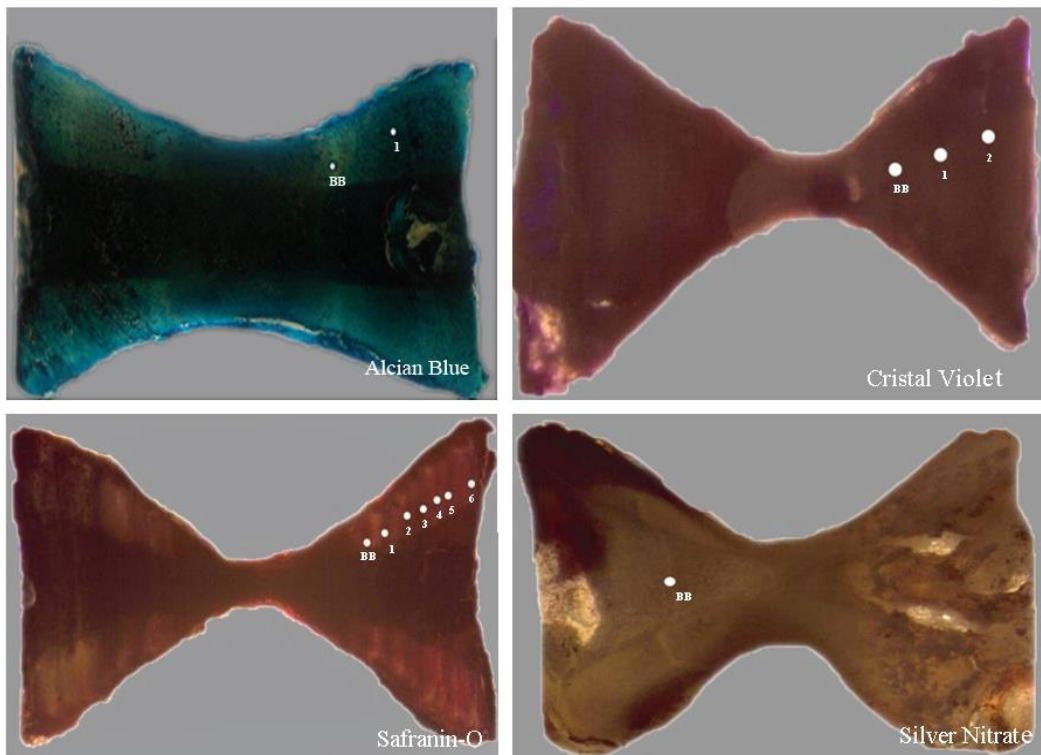

Figure 4. Mounted vertebral thin section from a 49.7 cm TL male *Raja asterias* stained with Chrystal Violet (age-2 yr), Silver Nitrate (not aged), Safranin O (age-6 yr) and Alcian Blue (age-1 yr), BB: Birth Band.
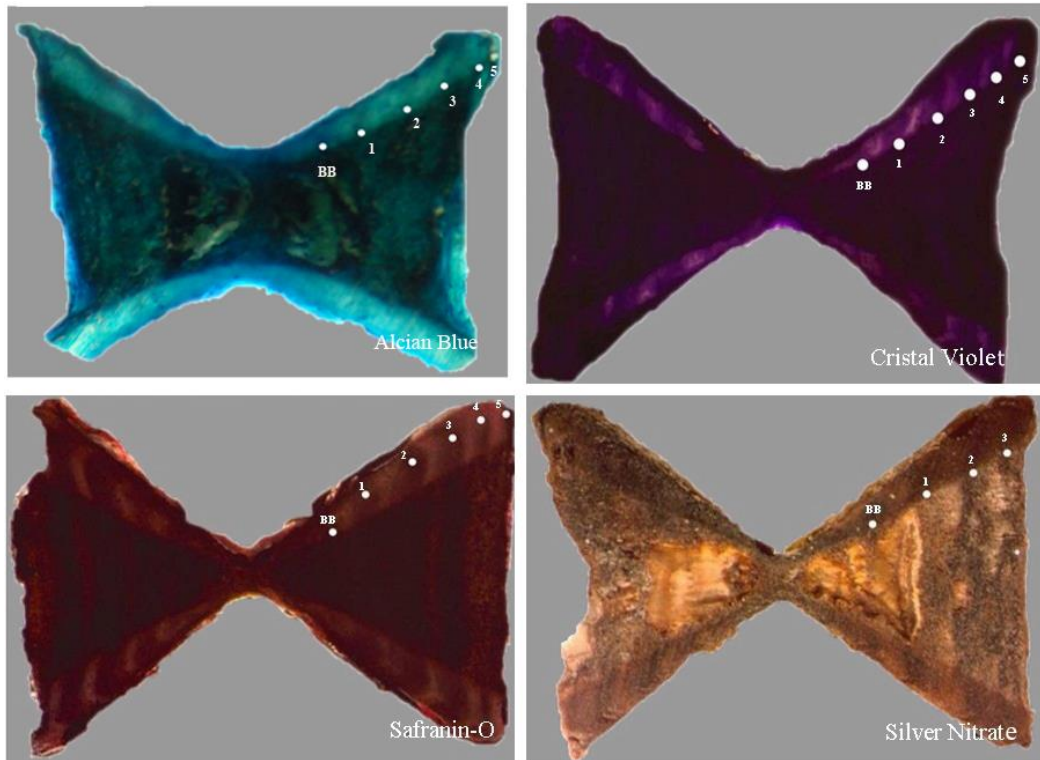
Figure 5. Mounted vertebral thin section from a 36.5 cm TL female *Torpedo marmorata* stained with Chrystal Violet (age-6 yr), Silver Nitrate (age-3 yr), Safranin O (age-6 yr) and Alcian Blue (age-5 yr), BB: Birth Band.
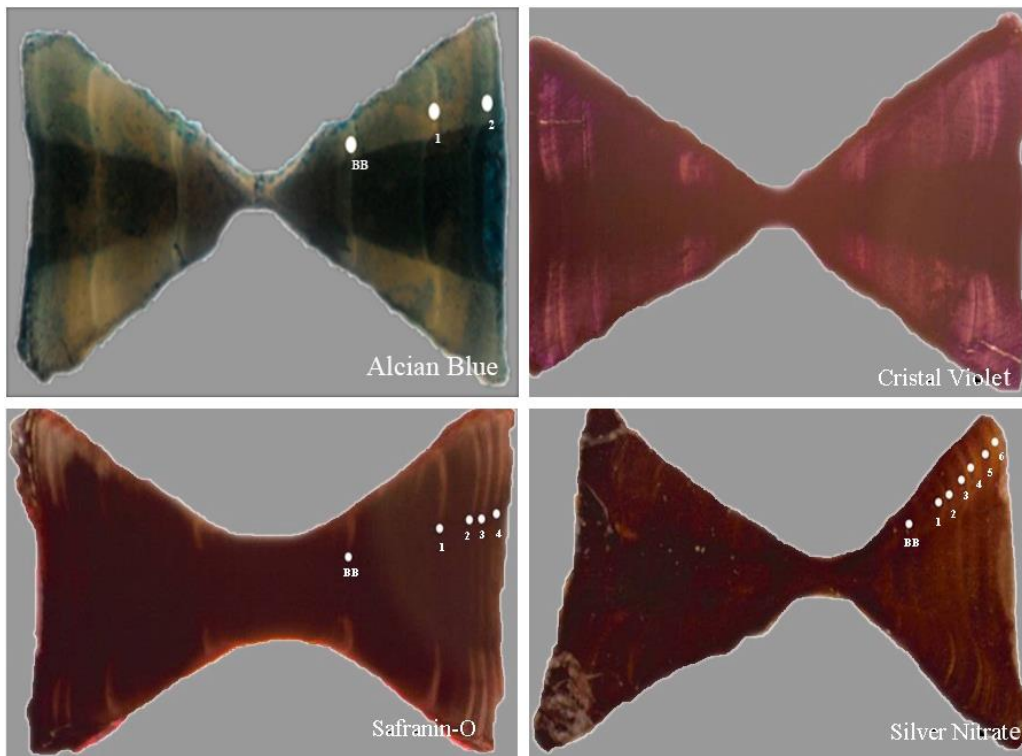

Figure 6. Mounted vertebral thin section from a 86.5 cm TL male *Rhinobatos rhinobatos* stained with Chrystal Violet (age-4 yr), Silver Nitrate (age-6 yr), Safranin O (age-4 yr) and Alcian Blue (age-2 yr), BB: Birth Band.
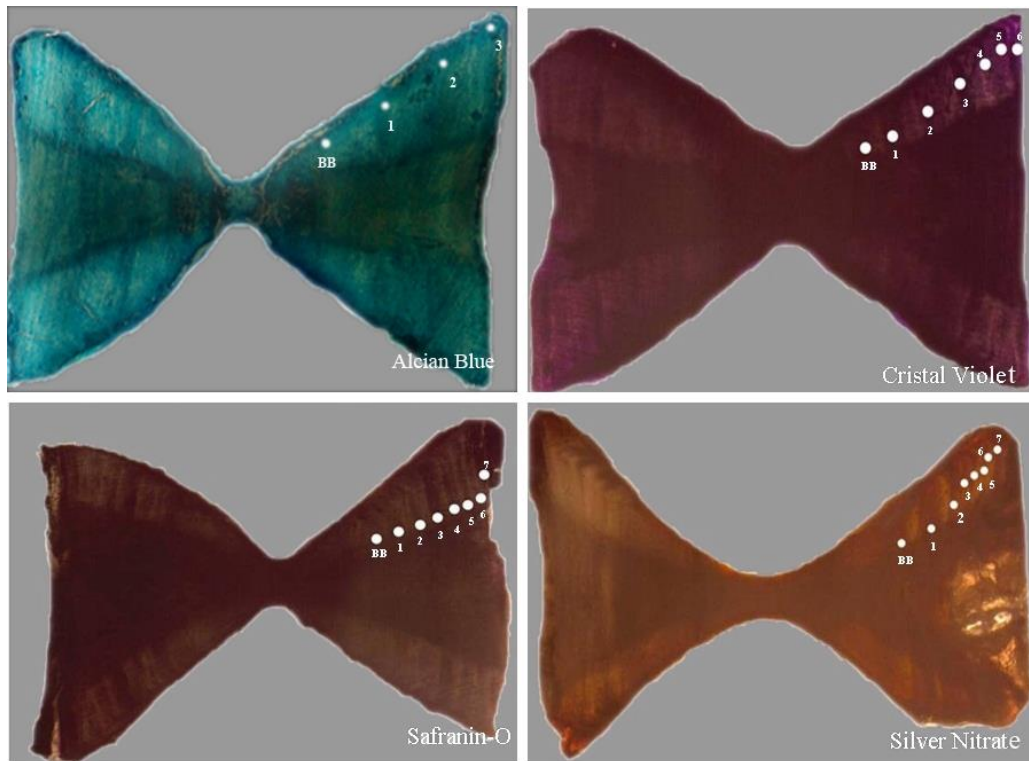
Figure 7. Mounted vertebral thin section from a 64.4 cm TL female *Gymnura altavela* stained with Chrystal Violet (age-6 yr), Silver Nitrate (age-7 yr), Safranin O (age–6 yr) and Alcian Blue (age-3yr), BB: Birth Band.

References

Başusta N, Demirhan SA, Cicek E, Başusta A, Kuleli T. 2008. Age and growth of the common guitarfish, *Rhinobatos rhinobatos* (Linnaeus, 1758), in Iskenderun Bay (northeastern Mediterranean, Turkey). Journal of Marine Biological Association of the United Kingdom, 88 (4) 837-842. doi:10.1017/S0025315408001124.

Başusta, N., Demirhan, S.A., Başusta, A., Cicek, E. 2010. Ageing with alcian blue duying techniques for some Elasmobranchs in Iskenderun bay, Eastern Mediterranean.. 39. CIESM Congress. Venice, Italy. pp 441.

Başusta, N., Sulikowski J.A. 2012. The oldest estimated age for roughtail stingray (*Dasyatis centroura*; Mitchill, 1815) from the Mediterranean Sea. Journal of Applied Ichthyology, 28: 641-642. doi: 10.1111/j.1439-0426.2012.01940.x

Başusta, N., Başusta, A., Ozer, E.I., Calta, M., Girgin, H. 2013. The most reliable vertebrae for age determination in *Dasyatis pastinaca*. 40. CIESM Congress. Marseille, France. pp 488.

Beamish, R.J., Fournier, D.A. 1981. A method for comparing the precision of a set of age determinations. Canadian Journal of Fisheries and Aquatic Sciences, 38: 982-983. doi:10.1139/f81-132.

Cailliet, G.M., Radtke, R.L., Weldon, B.A. 1986. Elasmobranch age determination and verification: a review. In: T. Uyeno, R. Arai, T. Taniuchi and K. Matsura (eds). Indo-Pacific Fish Biology: proceedings of the second international conference on Indo-Pacific Fishes, Ichthyol. Soc. Japan, Tokyo. pp. 345–360.

Cailliet, G. M., Martin, L. K., Kusher, D., Wolf, P., Weldon, B. A. 1983. Techniques for enhancing vertebral bands in age estimation of California elasmobranchs. In: E. D. Prince and L. M. Pulos (Eds), Proceedings of the International Workshop on Age Determination of Oceanic Pelagic Fishes: Tunas, Billfishes, and Sharks, 15–18 February 1982, Miami, FL, USA. NOAA Technical Report NMFS 8, pp. 157–165.

Cailliet, G.M. 1990. Elasmobranch age determination and verification: an updates review. In: W.S. Pratt, Jr., S. H. Gruber and T. Taniuchi, (Eds), elasmobranchs as living resources: advances in the biology, ecology, systematics, and the status of the fisheries, NOAA Technical Report, 90:157-165.

Cailliet, G.M., Musick, J.A., Simpfendorfer, C.A., Stevens, J.D. 2005. Ecology and life history characteristics of chondrichthyan fish. In: Sarah L. Fowler, S. L., Cavanagh, R. D., Camhi, M., Burgess, G. H., Cailliet, G. M., Fordham, S. M., Simpfendorfer, C. A., Musick, J. A. (Eds), Sharks, Rays and Chimaeras: The Status of the Chondrichthyan Fishes. IUCN-The World Conservation Union.

Chang, W.Y.B. 1982. A statistical method for evaluating the reproducibility of age determinations. Canadian Journal of Fisheries and Aquatic Sciences, 39: 1208-1210. doi:10.1139/f82-158.

Camhi, M., Fowler, S., Musick, J.A., Brautigam, A.,

Fordham, S. 1998. Sharks and their relatives-ecology and conservation. IUCN/SSC Shark Specialist Group. IUCN, Gland, Switzerland and Cambridge, UK, No: 20, iv+39p.

Campana, S.E. 2001. Accuracy, precision and quality control in age determination, including a review of the use and abuse of age validation methods. Journal of Fish Biology, 59: 197-242. doi:10.1006/jfbi.2001.1668.

Cavanagh, R.D., Gibson, C. 2007. Overview of the Conservation Status of Cartilaginous Fishes (Chondrichthyans) in the Mediterranean Sea. IUCN, Gland, Switzerland and Malaga, Spain. vi + 42 pp.

Coelho, A., Erzini, K. 2002. Age and growth of the undulate ray, *Raja undulata*, in tha Algarve (southern Portugal). Journal of Marine Biological Association of the United Kingdom, 82: 987-990. doi:10.1017/S0025315402006495.

Duman, O.V., Başusta, N. 2013. Age and growth characteristics of marbled electric ray *Torpedo marmorata* (Risso, 1810) inhabiting Iskenderun Bay, North-eastern Mediterranean Sea. Turkish Journal of Fisheries and Aquatic Sciences, 13: 551-559. DOI: 10.4194/1303-2712-v13_3_19

Ferreira, B.P., Vooren, C.M. 1991. Age, growth, and structure of vertebra in the school shark *Galeorhinus galeus* (Linnaeus, 1758) from southern Brazil. Fishery Bulletin, 89:19-31.

Fowler, S.L., Cavanagh, R.D. 2005. International Conservation and Management Initiatives for Chondrichthyan Fish. In: Fowler, S.L., Cavanagh, R.D., Camhi, M., Burgess, G.H., Cailliet, G.M., Fordham, S. V., Simpfendorfer, C.A. and Musick, J.A. (Eds),Sharks, Rays and Chimaeras: The Status of the Chondrichthyan Fishes. IUCN SSC Shark Specialist Group. IUCN, Gland, Switzerland and Cambridge, UK. pp 58–69.

Gallagher, M.J., Nolan, C.P. 1999. A novel method for the estimation of age and growth in rajids using caudal thorns. Canadian Journal of Fisheries and Aquatic Sciences, 56: 1590–1599. doi:10.1139/f99-084.

Gallagher, M.J., Nolan, C.P., Jeal, F. 2004. The structure and growth processes of caudal thorns. Journal of Northwest Atlantic Fishery Science, 35: 125–129.

Goldman, J.K. 2005. Age and growth of elasmobranch fishes. In: Musick, J.A.; Bonfil, R. (eds), Management techniques for elasmobranch fisheries. FAO Fisheries Technical Paper. No. 474. Rome, FAO. 251pp.

Holden, M.J. 1974. Problems in the rational exploitation of elasmobranch populations and some suggested solutions. In: F. R. Harden-Jones (Ed.), Sea Fisheries Research, Paul Elek, London. 117–137 pp.

Holden, M.J., Meadows, P. 1962. The structure of the spine of the spur dogfish (*Squalus acanthias* L.) and its use for age determination. Journal of Marine Biological Association of the United Kingdom, 42: 179-197.

Ismen, A. 2003. Age, growth, reproduction and food of common stingray (*Dasyatis pastinaca* L., 1758) in Iskenderun Bay, the eastern Mediterranean. Fisheries Research, 60:169-176. doi: 10.1016/S01657836(02)00058-9.

Kahveci, Z., Minbay, F.Z., Cavusoglu, I., 2000. Safranin O staining using a microwave oven. Biotechnic and Histochemistry, 75(6): 264-268. doi: 10.3109/10520290009085130

Moss, S.A. 1972. Tooth replacement and body growth rates in smooth dogfish, *Mustelus canis* (Mitchill). Copeia, 4: 808-811.

Officer, R.A., Gason, A.S., Walker, T.I., Clement, J.G. 1996. Sources of variation in counts of growth increment lines in vertebrae from gummy shark, *Mustelus antarcticus*, and school shark, *Galeorhinus galeus*: implications for growth determination. Canadian Journal of Fisheries and Aquatic Sciences, 53: 1765–1777. doi: 10.1139/f96-103.

Paul, L.J., Horn, P.L. 2009. Age and growth of sea perch (*Helicolenus percoides*) from two adjacent areas off the east coast of South Island, New Zealand. Fisheries Research. 95: 169-180. doi: 10.1016/j.fishres.2008.08.011.

Schwartz, F.J. 1983. Shark ageing methods and age estimation of scalloped hammerhead, *Sphyrna lewini* and dusky *Carcharhinus obscurus* sharks based on vertebral ring counts. In: E.D. Prince, and L.M. Pulos (eds), Proceedings of the international workshop on age determination of oceanic pelagic fishes: tunas, billfishes and sharks. NOAA Tech. Rep., NMFS 8: 167-174pp.

Serena, F. 2010. Status of sharks and rays in the Mediterranean and how they are being protected. GFCM First Transversal expert meeting on Elasmobranches in the Mediterranean and Black Sea, 20-22 September 2010 Sfax, Tunisia.

Siezen, R.J. 1989. Eye lens aging in the spiny dogfish (*Squalus acanthias*) I. Age determination from lens weight. Current Eye Resesearch, 8 (7): 707-712. doi: 10.3109/02713688909025805.

Stevens, J.D., Bonfil, R., Dulvy, N.K., Walker, P.A. 2000. The effects of fishing on sharks, rays, and chimaeras (chondrichthyans), and the implications for marine ecosystems, ICES Journal of Marine Science, 57: 476–494. doi:10.1006/jmsc.2000.0724.

Stevens, J.D. 1975. Vertebral rings as a means of age determination in the blue shark (*Prionace glauca* L.). Journal of Marine Biological Association of the United Kingdom, 55: 657-665. doi:10.1017/S0025315400017318.

Stevens, J. D., Walker, T. I., Cook, S. F. and Fordham, S. V. 2005. Threats faced by chondrichthyan fish. In: Sarah L. Fowler, S. L., Cavanagh, R. D., Camhi, M., Burgess, G. H., Cailliet, G. M., Fordham, S. M., Simpfendorfer, C. A., Musick, J. A. (Eds), Sharks, Rays and Chimaeras: The Status of the Chondrichthyan Fishes. IUCN-The World Conservation Union 2005.

Sulikowski, J.A., Morin, M.D., Suk, S.H., Howell, W.H. 2003. Age and growth of the winter skate (*Leucoraja ocellata*) in the western Gulf of Maine. Fishery Bulletin, 101: 405-413.

Tran, D., Golick, M., Rabinovitz, H., Rivlin, D., Elgart, G.,Nordlow, B. 2000. Hematoxylin and safranin O staining of frozen sections. Dermatologic Surgery, 26(3): 197-199. doi: 10.1046/j.1524-4725.2000.09220.x.

Wischniowski, S. 2008. Technique development for age determination of the Pacific sleeper shark (*Somniosus pacificus*). IPHC Report of Assessment and Research Activities, 389-392.