Comparison of Staining Techniques for Age Determination of Some Chondrichthyan Species

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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 İskenderun 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


Anahtar Kelimeler: Yaş tayini, Kıkırdaklı balıklar, Kristal Viyole, Gümüş Nitrat, Alcian Blue, 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 chondrichthians, 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).](image-url)
Processing of Vertebræ 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 Dremel™ 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:

\[
CV = 100\% \times \sqrt{\frac{\sum_{i=1}^{R} (X_{ij} - X_j)^2}{X_j (R-1)}},
\]

where \(N\) is the number of animals aged, \(R\) is the number of readings, \(X_{ij}\) is the count from the \(j\)th animal at the \(i\)th reading and \(X_j\) is the mean age of the \(j\)th animal from \(i\) readings.
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. miraletus* 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 chondrichthians. 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; Baştura 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 vertebral 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 (Baştura 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 chondrichthians. 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 vertebrae for age determination should be identified according to young and old individuals, and sex of species.

**Acknowledgements**

The authors thank E. I. Ozer, H. Girgin, O. V. Duman, for preparation, sectioning, and staining of centra. An experimental fishing permit was granted to us by the General Directorate of Fisheries and Aquaculture, Ministry of Food, Agriculture and Livestock of the Turkish Republic to collect

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

<table>
<thead>
<tr>
<th>Species</th>
<th>Alcian Blue</th>
<th>Crystal Violet</th>
<th>Silver Nitrate</th>
<th>Safranin O</th>
</tr>
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<tr>
<td><em>Raja clavata</em></td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>2</td>
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<tr>
<td><em>Raja miraletus</em></td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
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<td>4</td>
<td>5</td>
<td>4</td>
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<tr>
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<td>3</td>
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<td>1</td>
<td>3</td>
<td>1</td>
<td>2</td>
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</table>

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.

<table>
<thead>
<tr>
<th>Species</th>
<th>N</th>
<th>Technique</th>
<th>APE</th>
<th>CV</th>
<th>D</th>
<th>% ND</th>
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<td></td>
<td></td>
<td>Chrystal violet</td>
<td>6.6</td>
<td>8.9</td>
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<td></td>
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<td>9.5</td>
<td>5.5</td>
<td>4.4</td>
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<td></td>
<td>Alcian blue</td>
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<td>10.2</td>
<td>6.1</td>
<td>12.9</td>
</tr>
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<td>12.4</td>
<td>7.2</td>
<td>13.7</td>
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<td></td>
<td></td>
<td>Silver nitrate</td>
<td>8.0</td>
<td>11.0</td>
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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.
elasmobranchs in these locations during the months of April, May and June, when these waters are closed to commercial fishing. Funding was provided by the Scientific and Technological Research Council of Turkey (TUBITAK), Project No: TOVAG 109O634.

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