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Evaluation of staining techniques for the observation of growth bands in tropical elasmobranch vertebrae

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Summary: The aim of this study was to assess the suitability of different vertebrae staining techniques for the visualization and counting of growth bands in tropical species of batoids (*Narcine leoparda*, *Urotrygon aspidura*, *Hypanus longus*, *Potamotrygon magdalenae*) and sharks (*Alopias pelagicus*, *Carcharhinus falciformis*, *Sphyrna lewini*, *Sphyrna corona* and *Mustelus lunulatus*). Different cutting thicknesses and staining protocols were tested, analysing the precision and bias of each combination to identify the most accurate technique for estimating age. Vertebral sections of 0.4 mm were more suitable for batoids, except for *Narcine leoparda*; for this species and for all the shark species assessed, sections of 0.5 mm are recommended. Different combinations of stain and exposure time were required to achieve the best visualizations of vertebral growth band pair for the shark and ray species. Intraspecific variation occurred among vertebrae size of batoids. Our results confirm the importance of defining a suitable species-specific protocol for sectioning and staining hard structures before carrying out an age and growth study to improve the reliability of the age estimates.

Keywords: age; growth; sharks; batoids; freshwater stingray; precision; bias.

Evaluación de técnicas de tinción para la observación de bandas de crecimiento en vértebras de elasmobranquios tropicales

Resumen: El objetivo de este estudio fue evaluar la efectividad de diferentes técnicas de tinción de vértebras en la visualización y el conteo de bandas de crecimiento en especies tropicales de batoideos (*Narcine leoparda*, *Urotrygon aspidura*, *Hypanus longus*, *Potamotrygon magdalenae*) y tiburones (*Alopias pelagicus*, *Carcharhinus falciformis*, *Sphyrna lewini*, *Sphyrna corona* y *Mustelus lunulatus*). Se probaron diferentes espesores de corte y protocolos de tinción, analizando la precisión y el sesgo de cada combinación para identificar la técnica más precisa para estimar la edad. Las secciones vertebrales de 0,4 mm fueron más adecuadas para batoideos, excepto para *Narcine leoparda*; para esta especie y para todas las especies de tiburones evaluadas, se recomiendan secciones de 0,5 mm. Se identificaron diferentes combinaciones de tinción y tiempo de exposición para lograr las mejores visualizaciones de las bandas de crecimiento vertebral en las especies de tiburones y rayas. En los batoideos se identificó variación intraespecífica de acuerdo con el tamaño de las vértebras. Nuestros resultados confirman la importancia de definir un protocolo especie-específico adecuado para cortar y teñir las estructuras duras antes de realizar un estudio de edad y crecimiento y así incrementar la confiabilidad de las estimaciones de edad.

Palabras clave: edad; crecimiento; tiburones; rayas; raya de agua dulce; precisión; sesgo.

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INTRODUCTION

Due to their life history characteristics (Cortés et al. 2012) and the increase in fishing pressure, elasmobranchs are currently one of the most vulnerable fish groups, with high threat levels worldwide (Stevens et al. 2000, Dulvy et al. 2014). Age estimation for these species is therefore important to suggest fishery management measures. This information is also relevant for the estimation of growth and mortality rates, maturity age and longevity, among other parameters, as they are primary inputs in demographic studies that allow the vulnerability and productivity of the populations to be established (e.g. Campana 2001, Goldman et al. 2012). Despite their importance, the number of age and growth publications in tropical elasmobranchs is relatively low (14.1%; 25 of 177 reviewed papers; e.g. Harry et al. 2010, O'Shea et al. 2013, Mejía-Falla et al. 2014) compared with those in cold and temperate waters (133 papers; e.g. Duman and Başusta 2013, James et al. 2014, Kadri et al. 2013) (Supplementary material Table S1).

Age studies in elasmobranchs are based on counting pairs of growth bands in hard structures (e.g. vertebrae, spines) which are formed periodically. As the visualization of these band pairs in some cases is not simple, over time several methods, such as X-rays (Natanson and Cailliet 1990), staining (Goldman et al. 2012) and histological sections (Natanson et al. 2007), have been suggested and tested to enhance the visualization of the growth bands and therefore improve the accuracy of age estimation (Goldman et al. 2012).

Of the aforementioned processes, staining has been the most widely used, mainly because it is the least complex and least expensive procedure, and it is even suggested as a first step before applying more demanding and expensive methods (Goldman et al. 2012). Staining techniques have been used in age studies of several elasmobranch species (e.g. Neer and Cailliet 2001, Fernández-Carvalho et al. 2011, Torres-Palacios et al. 2019). The result of each technique has been found to be species-specific (Goldman et al. 2012), so it is not possible to define a standard protocol for the use of these techniques in elasmobranchs.

Furthermore, few studies have reported in detail the procedures applied to select the most appropriate staining method for each species (e.g. Fernández-Carvalho et al. 2011, Huveneers et al. 2013, Torres-Palacios et al. 2019), and a large number of studies have applied methods previously described in similar species without evaluating their effectiveness in the particular study species (e.g. Aversa et al. 2011, Sánchez de Ita et al. 2011, Chin et al. 2013). Given that there is evidence that the results of these techniques are species-specific, the need to assess and test how these tools influence the visualization of the growth bands before carrying out age and growth studies is highlighted. Additionally, recent studies have shown that band formation in some elasmobranch species occurs bi-annually (Wells et al. 2013) or irregularly (Huveneers et al. 2013), so their age may be underestimated (Hamady et al. 2014, Harry 2018, Natanson et al. 2018). Therefore, improving the visualization of the bands and facilitating their counting would reduce the sources of error and increase the precision of the readings.

For the above reasons, and because studies based on radiocarbon dating, histology and X-rays are more restrictive in tropical countries, this study aimed to evaluate different staining techniques in vertebrae of nine tropical elasmobranch species to determine those that facilitate the visualization and counting of the growth bands, contributing to future age estimates and population assessments of the evaluated tropical species.

MATERIALS AND METHODS

Vertebrae processing

A total of 428 individuals were collected between 2007 ans 2015 from fisheries of the Colombian Pacific region (79°44′W, 5°45′N; 77°12′W, 2°15′N). These individuals belong to four batoid species, *Narcine leoparda* (n=90 individuals), *Urotrygon aspidura* (n=90 individuals), *Hypanus longus* (n=90 individuals) and *Potamotrygon magdalenae* (n=24 individuals); and five shark species, *Alopias pelagicus* (n=27 individuals), *Carcharhinus falciformis* (n=21 individuals), *Sphyrna lewini* (n=53 individuals), *Sphyrna corona* (n=18 individuals) and *Mustelus lunulatus* (n=15 individuals).

Once each specimen was identified at the species level, its sex was determined and total length of sharks or disc width of rays was recorded. From each individual, a section of 14 to 16 vertebrae was extracted from under the first dorsal fin (in sharks) and from the abdominal region (in rays), which were labelled and frozen until laboratory analysis. The excess tissue in the vertebrae was removed using a scalpel. Because affinity of the stains and visualization of the growth bands could be affected by the individual size, vertebrae were separated into three size intervals: small, medium and large diameter. This was done for the species whose sample size allowed it.

As the thickness of the vertebrae sections influences the visualization and reading of the growth bands, size ranges of the vertebrae with different section thicknesses (0.3 to 0.7 mm) were combined per individual. For this purpose, each vertebra was fixed to a slide with Crystalbond 509 and cut sagittally with an Isomet Buehler low-speed saw cutter with two diamond head blades (Buehler, Lake Bluff, IL, USA) to obtain bow-tie sections (Cailliet and Goldman 2004). The thickness of the section that best allowed the visualization of the bands was based on a qualitative evaluation carried out by expert readers.

In order to assess the effect of the stains used, sections were randomized and stained with alizarin red (0.05%), methylene blue (0.001%), crystal violet (0.001%), basic fuchsin (0.001%), acid fuchsin (0.001%), Bismarck brown (0.05%), light green (0.05%), silver nitrate (1%) and the Dahl staining (alizarin red 0.01% and light green 0.05%). Each dye was applied in successive intervals of one minute until reaching its saturation point in each structure. This was

done as a preliminary test for a sub-sample of each species (n=5).

Subsequently, the best three staining times for each dye were qualitatively established and applied to the vertebrae of the individuals selected for the analysis. Biases given by individual variations were avoided using several vertebrae per individual to apply the treatments (stain + staining time). Sections (with and without staining) were mounted on a slide, observed under a microscope using transmitted light and photographed for each treatment. Additionally, vertebral sections without any staining were used to evaluate the effect of immersion oil and distilled water (imbibing each in a drop of the substance) in the visualization of the growth bands. The images obtained were analysed with the Image Pro Plus 7.0 software (Media Cybernetics) in which two skilled readers performed the quantitative counting of the growth bands independently in each sample. Readings were carried out twice per reader, who did not know the details of the sex, size or previous reading of each vertebra. Based on this information, analyses were carried out to establish the accuracy and bias among readers and subsequently to establish the most efficient technique for observing and counting growth bands per species.

Data analysis

In order to assess the degree of precision in the vertebral band readings among readers, the index of average percentage error, the coefficient of variation, the percentage of agreement between readers, Bowker's symmetry test and the percentage of vertebrae read were calculated and analysed as follows.

The index of average percentage error (IAPE) provided information on the accuracy of age estimations among readers; small values indicated more precise readings (Beamish and Fournier 1981). The IAPE was calculated as follows:

$$IAPE = \left[\frac{1}{n} \left(\frac{1}{R} \sum_{i=1}^{R} \frac{\left| x_{ij} - \overline{x}_j \right|}{\overline{x}_j} \right) \right] \times 100$$

where n is the number of samples, x_{ij} is the i^{th} age estimation for individual j, R is the number of readings and X_j is the average age calculated for individual j.

Coefficient of variation (CV) measured reading accuracy (Campana 2001) expressed as the proportion of the mean and standard deviation, as follows:

$$CV = \left[\frac{1}{n} \left(\frac{\sqrt{\sum_{i=1}^{R} \frac{\left(x_{ij} - \overline{x}_j \right)^2}{R}}}{\overline{x}_j} \right) \right] \times 100$$

where x_{ij} is the i^{th} age for the individual j, is the average age of individual j and R is the number of readers.

Percentage of agreement between readers (PA) allowed us to establish the variation in the reading of bands among readers (Goldman 2002), using the following equation that was applied to each vertebra size range (diameter) and considering differences between bands (0, ±1, ±2).

$$PA = \frac{\text{number of agreements in the reading}}{\text{total number of reading}} \; 100$$

Bowker's symmetry test determined, using a chi-square test, whether differences between the readers were systematic ($p<0.05$) or random ($p>0.05$; Hoenig et al. 1995), the latter being the expected result because random errors indicate that there is no bias among readers.

Percentage of vertebrae read (RV) estimated the proportion of vertebrae that could be read successfully considering the entire sample; higher values indicated better performance of the assessed technique.

An evaluation of the results of all the tests applied was carried out in order to determine the most efficient technique for observing and estimating the age in each species studied. Subsequently, the final decision per species was taken by comparing the results of each of the treatments as a whole and by vertebrae size.

RESULTS

Section thickness

The qualitative evaluation of the section thickness for batoids showed that sections of 0.4 mm were most suitable, except for *Narcine leoparda*, for which, as for all the shark species assessed, sections of 0.5 mm are recommended. These thicknesses allowed the growth bands to be visualized and counted more easily in the three size classes assessed (large, medium and small) for *Narcine leoparda*, *Urotrygon aspidura* and *Hypanus longus*. In addition to the visibility of the bands, this thickness showed the lowest proportion of fractured vertebrae during the sectioning process. The other thicknesses evaluated did not allow the clear observation of the growth bands because of the low contrast or excess light passage (0.3 mm) or, on the contrary, because of too little light passage through the sections (0.6 and 0.7 mm).

Batoids

Narcine leoparda (n=90 individuals)

For the leopard electric ray, treatments were applied for three vertebrae size intervals: small (diameters of 1.30 to 2.13 mm), medium (2.14 to 2.99 mm) and large (3.00 to 4.84 mm). There was no systematic bias in any of the treatments used for visualization of growth bands, except for small vertebrae stained with alizarin red for 14 min (Supplementary material Table S2).

Treatment analysis by vertebrae size showed that the highest PA (±0 bands) values were found in unstained large vertebrae (91.3%), medium vertebrae stained with basic fuchsin for 7 min (73.1%), and small vertebrae stained with methylene blue for 1 min (75%) followed by alizarin red for 16 min (73.1%) (Table S2). The three vertebrae sizes evaluated showed percentages of read vertebrae (RV) higher than 70% in all treatments. The

Table 1. – Results of the precision and bias tests between readers for selected treatments for small, medium and large vertebrae of *Narcine leoparda, Urotrygon aspidura* and *Hypanus longus*, and for all vertebrae of *Potamotrygon magdalenae, Alopias pelagicus, Carcharhinus falciformis, Sphyrna lewini, Sphyrna corona* and *Mustelus lunulatus*. IAPE, index of average error percentage; CV, coefficient of variation; PA (± 0 bands), percentage of total agreement; RV, percentage of read vertebrae; p, p-values of Bowker's symmetry test. Dash indicates that data were not available because the treatment was not used.

Species	Vertebra size	Treatment (stain + staining time)	IAPE	CV	PA	RV	p
Narcine leoparda	Small	Alizarin red 16′	4.9	7.0	73.1	86.7	0.14
	Medium	Alizarin red 16′	3.3	4.6	64.0	83.3	0.22
	Large	No staining	0.7	1.7	91.3	76.7	0.09
Urotrygon aspidura	Small	Light green 5′	5.1	7.3	64.7	76.5	0.16
	Medium	Methylene blue 20′	1.2	1.7	84.2	89.5	0.32
	Large	Methylene blue 10′	2.2	3.1	80.0	100.0	0.39
Hypanus longus	Small	Bismarck brown 1′	2.2	3.1	93.3	100	0.32
		Bismarck brown 2.0′	2.2	3.1	93.3	100	0.32
		Light green 0.5′	2.2	3.1	93.3	100	0.32
		Light green 1′	2.2	3.1	93.3	100	0.32
	Medium	Basic fuchsin 2′	2.2	3.1	93.3	100%	0.32
	Large	Light green 7′	0.0	0.0	100.0	100.0	–
Potamotrygon magdalenae	All	Alizarin red 7′	0.1	1.3	95.5	91.7	0.16
Alopias pelagicus	All	Bismarck brown 15′	1.8	2.5	57.1	77.8	0.39
Carcharhinus falciformis	All	Crystal violet 35′	0.5	0.7	85.7	63.6	0.42
Sphyrna lewini	All	Immersion oil	7.0	2.4	89.8	94.2	0.14
Sphyrna corona	All	Crystal violet 20′	0.0	0.0	100.0	55.6	0.16
Mustelus lunulatus	All	Light green 40′	0.0	0.0	100.0	78.6	0.30

highest RV values were found for large vertebrae stained with methylene blue and basic fuchsin for 3 min (90%); for medium vertebrae without staining or immersed in oil, basic fuchsin for 7 min or alizarin red for 14 min (86.7%); and for small vertebrae immersed in distilled water or in alizarin red for 14 and 16 min (86.7%) (Table S2). IAPE and CV showed relatively low values in large and medium vertebrae and high values in small vertebrae, except in those stained with alizarin red for 16 min, which showed the best values for small and medium intervals.

In conclusion, unstained large vertebrae and the medium and small vertebrae treated with alizarin red for 16 min (Fig. 1A-C) showed the best combination of values in the precision and bias tests between readers. Further, these treatments obtained the lowest IAPE and CV, the highest total agreement percentage and a high percentage of read vertebrae (Table 1, S2).

Urotrygon aspidura (n=90 individuals)

For the Panamic stingray, treatments were also applied to three vertebrae size: small (diameters of 0.70 to 1.59 mm), medium (1.60 to 2.49 mm) and large (2.50 to 3.30 mm). Only the treatment with basic fuchsin for 1 min in medium vertebrae showed bias in the reading; for all others, the differences were due to random errors (Table S3).

The percentage of read vertebrae was higher than 70% in all treatments for the three size interval assessed, reaching a maximum of 100% with methylene blue for 10 min and alcohol in small vertebrae, with alizarin red for 15 min in medium vertebrae, and with almost all the treatments in the large vertebrae, except with crystal violet (Table S3). The highest PA (±0 bands) between readers occurred with alizarin red for 15 min in small vertebrae, methylene blue for 20 min in medium vertebrae, and methylene blue for 10 min in large vertebrae.

The lowest CV and IAPE values were found with methylene blue for 10 min in large vertebrae (Fig. 1D), with methylene blue for 20 min in medium vertebrae (Fig. 1E), and with light green for 5 min in small vertebrae (Fig. 1F). These stains also showed a high percentage of total agreement and a high percentage of read vertebrae, being chosen as the best treatment for each vertebra size (Tables 1, S3).

Hypanus longus (n=90 individuals)

Vertebra sections of *H. longus* were also separated in three size intervals: small (diameters of 3.00 to 5.15 mm), medium (5.16 to 8.39 mm) and large (8.40 to 12.34 mm). For this species, four stains were discarded (methylene blue, crystal violet, immersion oil and acid fuchsin) as the vertebral growth bands showed no clear delimitation among them, and this would increase the reading errors.

PA values varied between 80.0% (no staining, basic fuchsin for 1 min and light green for 2 min) and 100% (light green for 7 min) in large vertebrae; between 53.9% (basic fuchsin for 3 min) and 93.3% (basic fuchsin for 2 min) in medium vertebrae, and from 73.3% (basic fuchsin for 1 and 3 min) to 93.3% (Bismarck brown for 1 and 2 min, light green for 0.5 and 1 min) in small vertebrae (Table S4). Previously selected stains showed variable IAPE and CV values within and among vertebrae sizes; the lowest values were found for small vertebrae stained with Bismarck brown for 1 and 2 min and light green for 0.5 and 1 min, for medium vertebrae stained with Bismarck brown for 2 and 3 min, and for large vertebrae stained with light green for 7 min (Table S4). In general, vertebrae with no treatment showed higher IAPE and CV values than vertebrae that received staining. All vertebrae sections of this species were read (100% RV in all cases).

Considering all the results, the best treatment was light green for 7 min for large vertebrae of *H. longus*, basic fuchsin for 2 min for medium vertebrae (although Bismarck brown for 2 and 3 min were also good treatments), and Bismarck brown for 1 and 2 min and light green for 0.5 and 1 min for small vertebrae (Table S4).

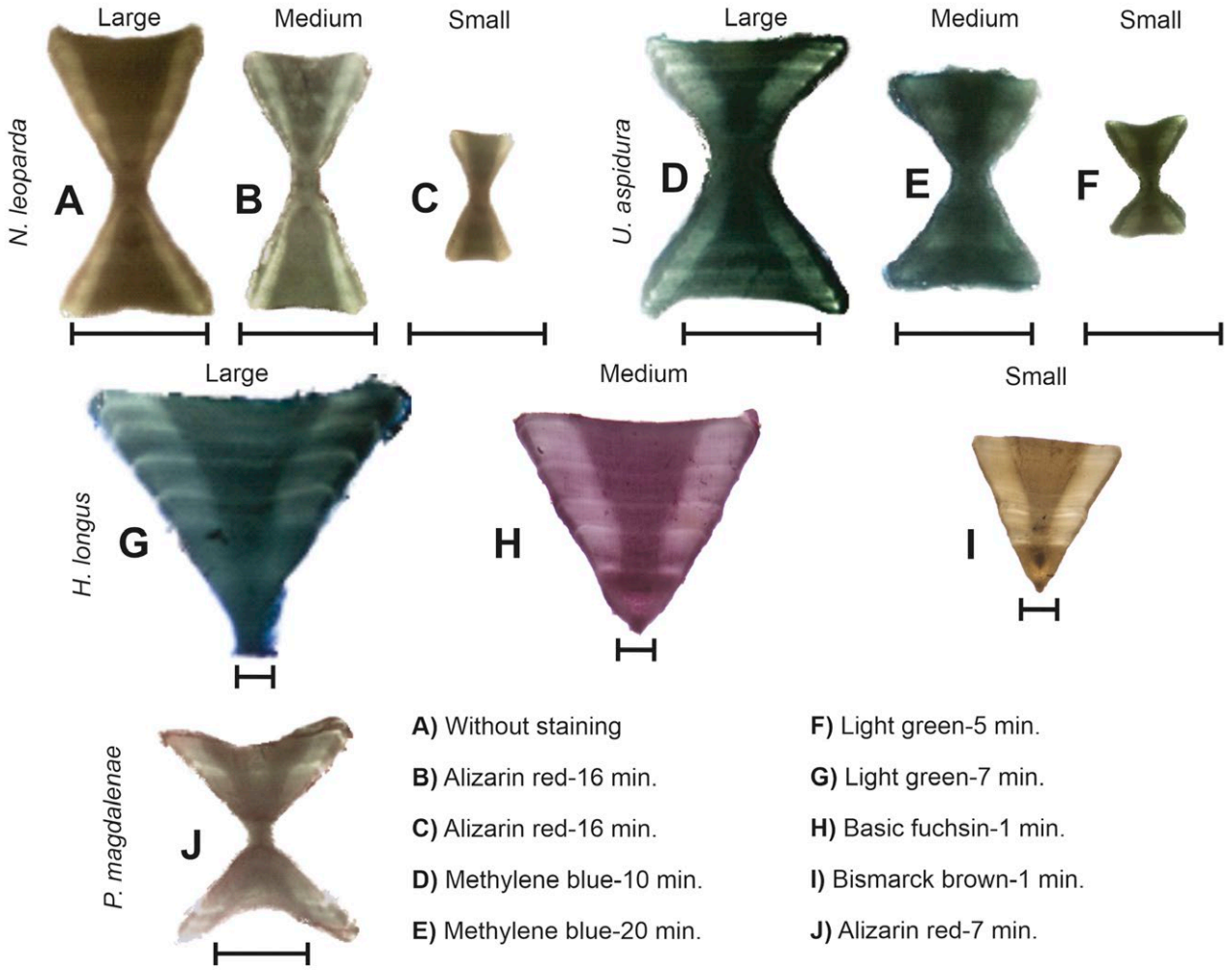

Fig. 1. – Vertebra sections showing the best treatment (stain and staining time) for each batoid species. The scale bar corresponds to 1 mm.

Potamotrygon magdalenae (n=24 individuals)

Age bias analysis of Magdalena river stingray showed no systematic bias in readings or treatments (Table S5). The percentage of read vertebrae varied between 52.9% for light green for 40 min and 95% for methylene blue for 30 and 40 min, the latter being followed by alizarin red for 7 min, with 91.7%. The lowest IAPE and CV values and the highest PA (±0 bands) values between readers were found with crystal violet for 60 min and light green for 40 min (0 IAPE, 0 CV, 100% PA each), followed by alizarin red for 7 min (0.1 IAPE, 1.3 CV, 95.5% PA). The group analysis of the treatments showed that vertebrae treated with light green and crystal violet require too much time for staining (≥40 min), while alizarin red showed the second-best values, with only 7 min of staining (Tables 1, S5; Fig. 1J).

Sharks

Alopias pelagicus (n=27 individuals)

Vertebrae of the pelagic thresher shark involved high difficulty in observing and counting a growth band pattern, generating high variation in the precision tests and percentages of read vertebrae (Table S5). None of the treatments showed systematic bias in their readings (P>0.05 in all cases). Vertebrae stained with alizarin red for 5 and 7 min showed the best results in the precision analyses (IAPE, CV and PA), but the percentages of RV with these treatments were very low. Conversely, basic fuchsin for 45 min showed the highest RV (91.7%) but a low PA value (±0 bands=36.4%). Considering the values of all the tests, staining with Bismarck brown for 15 min showed a good performance, occupying the third place in IAPE, CV, and RV and the fourth place in PA values (Tables 1, S5; Fig. 2A).

Carcharhinus falciformis (n=21 individuals)

None of the treatments analysed for the silky shark showed systematic bias in the readings (P>0.05 for all cases). The highest PA (±0 bands) values between readers were found with silver nitrate for 2 and 3 min (100%) and crystal violet for 35 min (85.7%). Similarly, the lowest IAPE and CV values were obtained with silver nitrate for 2 and 3 min, crystal violet for 35 min and acid fuchsin for 50 min. However, the percentage

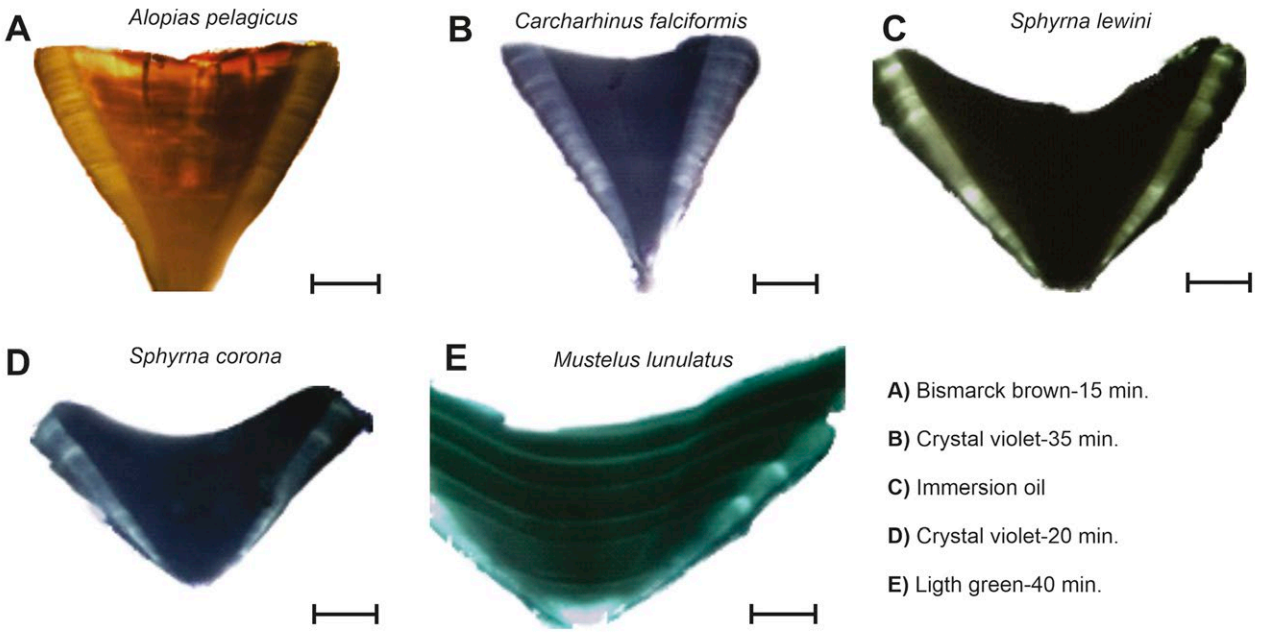

Fig. 2. – Vertebra sections showing the best treatment (stain and staining time) for each shark species. The scale bar corresponds to 1 mm.

of RV ranged from a very low value (16.7%) for silver nitrate for 2-3 min to 100% for light green for 35 min (Table S5). Vertebrae stained with crystal violet for 35 min showed the second-best values of IAPE (0.5), CV (0.7) and PA (85.7%), as well as an acceptable RV value (63.6%), being considered the most appropriate treatment for this species (Table 1, Fig. 2B). The vertebrae without staining showed the lowest performance in the precision tests, followed by methylene blue for 25 min and basic fuchsin for 40 min (Table S5).

Sphyrna lewini (n=52 individuals)

For the scalloped hammerhead none of the treatments analysed showed systematic bias in the readings (except using Bismarck's brown for 25 min, P=0.02; Table S6), and all showed a high percentage of RV (>84%) but also relatively high IAPE and CV values. The best values of PA (±0 bands), IAPE and CV were found with immersion oil and methylene blue for 35 and 30 min, which was the best treatments for the species. Therefore, immersion oil was suggested as the reagent for treating the vertebrae of *S. lewini* (Table 1, Fig. 2C).

Sphyrna corona (n=18 individuals)

For the scalloped bonnethead none of the treatments analysed showed systematic bias in the readings (P>0.05 in all cases) and all had a percentage of RV higher than 50%, reaching a maximum of 77.8% (with immersion oil and light green for 35 min). The highest PA values (±0 bands) were found with crystal violet for 20 min (100%), methylene blue for 25 min (81.8%) and crystal violet for 25 min (80.0%; Table S6); similarly, IAPE and CV were lower with crystal violet for 20 and 25 min, as well as with methylene blue for 25 min. Vertebrae treated with crystal violet for 20 min showed the best combination of precision and bias values, establishing it as the best treatment for this species (Table 1, Fig. 2D).

Mustelus lunulatus (n=15 individuals)

For the smooth-hound shark, none of the treatments analysed showed systematic bias in the readings (P>0.05) and the percentage of RV was higher than 60% in all treatments, reaching a maximum of 100% with crystal violet for 30 and 40 min. The highest PA values (±0 bands) and lowest IAPE and CV values were found with light green for 40 min (100%; 0 and 0, respectively) and 30 min (91.7%; 1.2 and 1.7, respectively), followed by crystal violet for 20 min (71.4%; 3.2 and 4.5; Table S6). From the group analysis of the treatments assessed, light green for 40 min showed the best combination of values and is therefore the most appropriate treatment for the species (Table 1, Fig. 2E).

DISCUSSION

There is sufficient evidence that the visualization of the growth bands in hard structures of elasmobranchs varies substantially among species (Duarte et al. 2001, Goldman et al. 2012, this study). Therefore, it is essential to identify and apply species-specific techniques that enhance this visualization and consequently increase the accuracy of age and growth estimations. In this study, the results support this statement for the marine species analysed, which come from a single geographical region, revealing that alizarin red works better for *N. leoparda,* light green for *M. lunulatus,* immersion oil for *S. lewini*, Bismarck brown for *A. pelagicus*, crystal violet for *S. corona* and *C. falciformis*, methylene blue and light green for *U. aspidura* and

light green, basic fuchsin and Bismarck brown for *H. longus*, Furthermore, our research documents intraspecific variation according to the size of the vertebrae in *U. aspidura* and *H. longus* (see Supplementary material, Species comments section). Similarly, vertebrae staining with alizarin red works better for the freshwater stingray *P. magdalenae.*

Additionally, systematic development of sectioning and staining of the vertebrae and visualizing and counting the growth bands with different arrangements to establish the best combination is a great training exercise for the researchers, which will result in a higher precision in identifying and performing growth band counts and therefore a better estimation of the age of an individual.

Despite the evidence on the importance of developing this procedure by species to establish age and growth of elasmobranchs, many papers do not include detailed information on the procedures performed. This may be due to limited word constraints in publications (usually limiting them to one sentence) or to the fact that these procedures were not performed (and a thickness-stain-staining time combination already established in other studies was chosen; e.g. Kadri et al. 2013, O'Shea et al. 2013, Drew et al. 2015). A review of publications on elasmobranch age and growth (n=177) showed that only 39% used staining techniques; of these, 40 were carried out on rays and 29 on sharks (Table S1). Furthermore, the number of studies regarding precision and bias evaluations to compare treatments is even smaller.

Although the bias in the band reading was almost null, the greatest difficulty in the counts was related to the definition of the birthmark and the reading of the bands located towards the edges of the vertebrae. Consequently, the greatest differences between readers occurred in areas near the focus and on the edge of the vertebral sections. Other studies that used similar methods in sagittal vertebrae sections have experienced problems with sharpness in these same areas (e.g. Licandeo et al. 2006, McFarlane and King 2006). Similarly, Ainsley et al. (2011) recorded difficulties for identifying band pairs close to the focus in about 20% of the individuals of *Amblyraja radiata*, *Malacoraja senta* and *Bathyraja interrupta.* Band reading problems in the distal region of the vertebrae, especially in older specimens, could be due to the proximity of the last and penultimate band, or to the fact that growth zone periodicity changes or ceases later in life, potentially after the onset of sexual maturity (Harry 2018, Natanson et al. 2018). The cited authors proposed several effects generated by those underestimates and the techniques available to address them.

There are also contrasting results with those found in this study regarding cut thickness, since wider or thinner thicknesses have been proposed as the most suitable for counting growth bands in other species such as *Mustelus canis, Mustelus asterias, Prionace glauca* and *Pristis pectinata* (e.g. Conrath et al. 2002, Farrell et al. 2010). This implies that the suitable visualization of bands with a certain section thickness is not a shared attribute in all elasmobranch species, even in species of the same genus, as was the case in *Bathytoshia centroura* (formerly *Dasyatis centroura*) and *Dasyatis pastinaca* (Yigin and Ismen 2012). In these species, the section thicknesses chosen by the authors were 0.6 mm and 0.5 mm, respectively, which differ from the optimum found for *Hypanus longus* (formerly *Dasyatis longa*) in this study (0.4 mm).

These procedures that require few logistical resources and significant investment of time, substantially improve the results obtained in terms of experience, learning and quality of the data. However, as seen in this study, this is not a general rule, and some shark and ray species have vertebral structures that can be easily visualized without staining procedures (e.g. large vertebrae of *N. leoparda*), while for others staining are good treatment (e.g. *C. falciformis*). As another example, Geraghty et al. (2013) found no differences in the accuracy of the age estimate of *Carcharhinus brevipinna*, *C. obscurus* and *C. plumbeus* among unstained vertebrae and those stained with alizarin red and crystal violet. Whatever the case may be, it is suggested that the usefulness of the staining tests should be tested and evaluated through specific quantitative analyses, as shown in this study.

From the results obtained in this study we conclude that it is essential to consider several precision indexes (PA, IAPE, and CV) to define, with the best possible criteria, the combination of section thickness, stain and staining time that is most suitable for the species of interest (Supplementary material, Species comments section). It is a mistake to make this decision based on a single index and to assess the performance of a single technique without contrasting or comparing treatments. In this regard, most studies that used staining techniques have found better performance with stained vertebrae than without staining. Duarte et al. (2001) found better precision and bias values in age estimation of *Galeorhinus galeus* using cobalt nitrate; Girgin and Başusta (2016) found better results for *Dasyatis pastinaca* using safranin-O than using crystal violet, silver nitrate and alcian blue. Furthermore, these authors found differences in the sizes within the age groups estimated for the species from those found by Ismen (2003), assigning these differences to the staining technique used. Additionally, Başusta et al. (2017) suggested the use of crystal violet to improve visualization in *Raja clavata*, safranin-O in *Raja asterias*, *Gymnura altavela* and *Torpedo marmorata* and alcian blue in *Raja miraletus* and *Rhinobatos rhinobatos*, also finding differences among genera.

All the examples mentioned above illustrate how the implementation of techniques to enhance the visualization of growth bands significantly influences the accuracy of the age estimation, thus supporting our results. Even the results obtained versus the bibliographic references reviewed show how studies carried out within a same species in different geographical locations (latitudinal differences) identified different techniques as the most appropriate. Furthermore, considering only tropical species, a great variation is identified in the techniques selected for the visualization of the growth bands. This reaffirms the importance

Table 2. – Age and growth studies of tropical distribution elasmobranch species, indicating the techniques used to visualize the vertebral growth bands and the technique selected for each species. *Studies that include tropical and subtropical areas.

Species	Study area	Techniques used	Stainings used	Technique defined	Reference
Batoids					
Dasyatis lata	Kane'ohe Bay on Oahu, Hawai'i, USA	Sagittal sectioning, OTC	None	Sagittal sectioning	Dale and Holland (2012)
Glaucostegus typus	Cleveland Bay, Great Barrier Reef, Australia	Sagittal sectioning	None	0.4 - 0.6 mm sagittal sectioning	White et al. (2014)
Himantura uarnak	Ningaloo Reef Marine Park, Australia	Sagittal sectioning	None	0.35 mm sagittal sectioning	O'Shea et al. (2013)
Hypanus guttatus	Eastern Atlantic coast, Rio Grande do Norte, Brazil	Sagittal sectioning	None	0.2 mm sagittal sectioning	Gianeti et al. (2019)
Neotrygon annotata	North-east Australia	Sagittal sectioning	None	0.2 - 0.3 mm sagittal sectioning.	Jacobsen and Bennett (2010)
Neotrygon kuhlii	Ningaloo Reef Marine Park, Australia	Sagittal sectioning	None	0.35 mm sagittal sectioning	O'Shea et al. (2013)
	North-east Australia	Sagittal sectioning	None	0.2 - 0.3 mm sagittal sectioning.	Jacobsen and Bennett (2010)
Neotrygon picta	North-east Australia	Sagittal sectioning	None	0.2 - 0.3 mm sagittal sectioning	Jacobsen and Bennett (2010)
Pastinachus atrus	Ningaloo Reef Marine Park, Australia	Sagittal sectioning	None	0.35 mm sagittal sectioning	O'Shea et al. (2013)
Rhynchobatus australiae	Cleveland Bay, Australia	Sagittal sectioning	None	0.4 - 0.6 mm sagittal sectioning	White et al. (2014)
Rhynchobatus laevis	Cleveland Bay, Australia	Sagittal sectioning	None	0.4 - 0.6 mm sagittal sectioning	White et al. (2014)
Rhynchobatus palpebratus	Cleveland Bay, Australia	Sagittal sectioning	None	0.4 - 0.6 mm sagittal sectioning	White et al. (2014)
Taeniura lymma	Ningaloo Reef Marine Park, Australia	Sagittal sectioning	None	0.35 mm sagittal sectioning	O'Shea et al. (2013)
Urotrygon aspidura	Central Pacific coast, Colombia	Sagittal sectioning and staining	Light green (0.05%) methylene blue (0.001%)	0.4 mm sagittal sectioning staining using light green for small vertebrae and methylene blue for medium and large vertebrae	Torres-Palacios et al. (2019)
Urotrygon chilensis	Tehuantepec Gulf, Southeast Pacific, Mexico	Sagittal sectioning	None	0.3 - 0.5 mm sagittal sectioning	Guzmán-Castellanos (2015)
Urotrygon microphthalmum	Eastern Atlantic, Pernambuco, Brazil	Sagittal sectioning	None	0.3 mm sagittal sectioning	Santander-Neto (2015)
Urotrygon rogersi	Central Pacific coast, Colombia	Sagittal sectioning	Several (not indicated)	0.4 mm sagittal sectioning	Mejía-Falla et al. (2014)
Sharks					
Alopias pelagicus	Java Sea, Indonesia	Sagittal sectioning	None	0.3 mm sagittal sectioning	Drew et al. (2015)
Alopias superciliosus	Northeastern Taiwan	X-ray radiography and staining	Silver nitrate	X-ray radiography	Liu et al. (1998)
	Atlantic Ocean*	Sagittal sectioning and staining	Crystal violet	0.5 mm sagittal sectioning and staining	Fernández-Carvalho et al. (2015)
Carcharhinus coatesi	Queensland, Australia*	Sagittal sectioning	None	0.4 mm sagittal sectioning	Smart et al. (2012)
Carcharhinus fitzroyensis	Queensland, Australia*	Sagittal sectioning	None	0.4 mm sagittal sectioning	Smart et al. (2012)
Carcharhinus leucas	Veracruz and Campeche, Mexico	Sagittal sectioning	None	0.3 - 0.6 mm sagittal sectioning	Cruz-Martínez et al. (2005)
Carcharhinus limbatus	Eastern Lombok, Indonesia	Sagittal sectioning	None	0.4 mm sagittal sectioning	Smart et al. (2015)
Carcharhinus macloti	Queensland, Australia*	Sagittal sectioning	None	0.4 mm sagittal sectioning	Smart et al. (2012)
Carcharhinus plumbeus	Northeastern Taiwan	Staining of sagittal and longitudinal sectioning, X-radiography of sagittal and longitudinal sectioning	Eosin, haematoxylin	X-radiography	Joung et al. (2004)
Carcharhinus porosus	Eastern Atlantic coast, Maranhão, Brazil	Sagittal sectioning, staining and cedarwood oil	Alizarin red cedarwood oil	Sagittal sectioning and staining	Lessa and Santana (1998)
Carcharhinus sorrah	Northern Australia	Staining. Protein stains mercurochrome and ninhydrin. Histology, Radiography X-ray, spectrometry, image analysis and examination of sectioned vertebrae under transmitted, reflected, interference and polarized light OTC	Silver nitrate, Alizarin Red S, crystal violet cobalt nitrate, ammonium sulphide, mercurochrome ninhydrin	Staining with ninhydrin	Davenport and Stevens (1988)

Table 2 (Cont.). – Age and growth studies of tropical distribution elasmobranch species, indicating the techniques used to visualize the vertebral growth bands and the technique selected for each species. *Studies that include tropical and subtropical areas.

Species	Study area	Techniques used	Stainings used	Technique defined	Reference
Carcharhinus tilstoni	Northern Australia	Staining. Protein stains mercurochrome and ninhydrin. Histology, Radiography X-ray, spectrometry, image analysis and examination of sectioned vertebrae under transmitted, reflected, interference and polarized light OTC	Silver nitrate, Alizarin Red S, crystal violet, cobalt nitrate, ammonium sulphide, mercurochrome and ninhydrin	Staining with ninhydrin	Davenport and Stevens (1988)
Eusphyra blochii	Queensland, Australia*	Sagittal sectioning	None	0.4 mm sagittal sectioning	Smart et al. (2012)
Galeocerdo cuvier	Australian east coast*	Sagittal sectioning and staining	Unstained, crystal violet, silver nitrate	0.15 mm sagittal sectioning	Holmes et al. (2015)
Hemipristis elongata	Queensland, Australia*	Sagittal sectioning	None	0.4 mm sagittal sectioning	Smart et al. (2012)
Isogomphodon oxyrhynchus	Eastern Atlantic coast, Maranhão, Brazil	Sagittal sectioning and staining	Alizarin Red S	Sagittal sectioning and staining	Lessa et al. (2000)
Isurus oxyrinchus	Western and central North Pacific Ocean*	Shadowing (half-cut centra) staining, soft X-radiography (whole or half-cut centra)	Alizarin red, silver nitrate	Shadowing on half-cut centra	Semba et al. (2009)
	Western and central Atlantic*	Sagittal sectioning	None	0.3 mm sagittal sectioning	Barreto et al. (2016)
	Southern Indian Ocean*	Sagittal sectioning and soft X-ray	None	1-1.44 mm sagittal sectioning and soft X-ray	Liu et al. (2018)
Prionace glauca	Eastern Atlantic, Brazil	Sagittal sectioning	None	1 mm sagittal sectioning	Lessa et al. (2004)
	Baja California Peninsula, Mexico	Sagittal sectioning and staining	Silver nitrate	Whole vertebra stained with silver nitrate and 0.5 mm sagittal sectioning	Blanco-Parra et al. (2008)
	South Pacific Ocean*	Transversal sectioning and soft X-ray radiograph	None	Transversal sectioning and soft X-ray radiograph	Joung et al. (2018)
*Rhincodon typus**	Northwestern Pacific, Taiwan*	Transversal sectioning and X-ray radiograph	None	Transversal sectioning and X-ray radiograph	Hsu et al. (2014)
	Northwestern Pacific, Taiwan and Pakistan*	Sagittal sectioning Bomb radiocarbon	None	Sagittal sectioning Bomb radiocarbon	Ong et al. (2020)
Rhizoprionodon acutus	Northeastern coast, Australia	Sagittal sectioning	None	0.4-0.6 mm sagittal sectioning	Harry et al. (2010)
	Western Atlantic, Senegal	Sagittal sectioning and staining	Acetic acid + Toluidine blue	Sagittal sectioning and staining	Ba et al. (2015)
Rhizoprionodon lalandii	Eastern Atlantic coast, Maranhão, Brazil	Sagittal sectioning	None	0.3 mm sagittal sectioning	Lessa et al. (2009)
Rhizoprionodon porosus	Eastern Atlantic coast, Maranhão, Brazil	Sagittal sectioning	None	0.3 mm sagittal sectioning	Lessa et al. (2009)
Rhizoprionodon taylor	Cleveland Bay, Australia	Vertebrae grounding	None	0.2-0.4 mm grounding	Simpfendorfer (1993)
Sphyrna lewini	Northeastern Taiwan	Sagittal sectioning	None	0.2 mm sagittal sectioning	Chen et al. (1990)
	Michoacan, Mexico	Sagittal sectioning and staining	Crystal violet (0.01%)	Sagittal sectioning and staining	Anislado-Tolentino and Robinson-Mendoza (2001)
	Java Sea, Indonesia	Sagittal sectioning	None	0.3 mm sagittal sectioning	Drew et al. (2015)

of evaluating different combinations (thickness, stain, staining time) for each species to be studied (Table 2). The conditions that produce these intraspecific variations are unknown, but may involve environmental factors (temperature and/or productivity) or be related to physiological changes induced by the consumption of food and starvation periods, which cause variation in salt deposition and consequently in the formation of pairs of growth bands (Goldman 2005).

This variation could determine the calcification of the vertebrae and the affinity of the stains to these structures (based on the carbonate and/or phosphate concentrations), influencing the nature of the dye (basophilic or acidophilic) on the quality of the dye and hence the visualization of the growth bands. However, it is recommended to further this analysis in order to establish whether there is any influence of the type of dye on its ability to improve the visibility of the growth bands. In this regard, it is necessary to carry out a study on the possible causes of the accumulation and type of compounds that make possible changes in birthmarks, e.g. ontogenetic changes in the diet, temperature or reabsorption of materials accumulated in the vertebrae (Licandeo et al. 2006).

Parameter estimation from age and growth studies has profound implications in population assessments based on demography, directly affecting the estimation of demographic parameters and thus the potential management of the species based on their life history traits. Hence, an incorrect specification of the bands and their deposition frequency could lead to the under- or overestimation of the growth coefficient and the asymptotic lengths of the populations. Therefore, any effort made to reduce the bias in the visualization, counting and analysis of the growth bands is an indirect but essential contribution to the management and subsequent conservation of elasmobranch species.

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SUPPLEMENTARY MATERIAL

The following supplementary material is available through the online version of this article and at the following link: http://scimar.icm.csic.es/scimar/supplm/sm05045esm.pdf

Table S1. – Age and growth publications in tropical elasmobranchs (Spreadsheet in MS Excel format available at: http://scimar.icm.csic.es/scimar/supplm/sm05045TableS1.xlsx).

Table S2. – Results of precision and bias tests between readers, for each of the treatments applied to the small, medium and large vertebrae of *Narcine leoparda*. IAPE, index of average error percentage; CV, coefficient of variation; PA (±0 bands), percentage of total agreement; RV, percentage of read vertebrae; P, p-values of the Bowker symmetry test. Best results of each test for each vertebrae size are highlighted in bold and the selected treatments are shaded in gray.

Table S3. – Results of precision and bias tests between readers, for each of the treatments applied to the small, medium and large vertebrae of *Urotrygon aspidura*. IAPE, index of average error percentage; CV, coefficient of variation; PA (±0 bands), percentage of total agreement; RV, percentage of read vertebrae; P, p-values of the Bowker symmetry test. Best results of each test for each vertebrae size are highlighted in bold and the selected treatments are shaded in gray.

Table S4. – Results of precision and bias tests between readers, for each of the treatments applied to the small, medium and large vertebrae of *Hypanus longus*. IAPE, index of average error percentage; CV, coefficient of variation; PA (±0 bands), percentage of total agreement; RV, percentage of read vertebrae; P, p-values of the Bowker symmetry test. Best results of each test for each vertebrae size are highlighted in bold and the selected treatments are shaded in gray.

Table S5. – Results of precision and bias tests between readers for each of the treatments applied to *Potamotrygon magdalenae*, *Alopias pelagicus* and *Carcharhinus falciformis* vertebrae. IAPE, index of average error percentage; CV, coefficient of variation; PA (±0 bands), percentage of total agreement; RV, percentage of read vertebrae; P, p-values of the Bowker symmetry test. Best results of each test are highlighted in bold and the selected treatment for each species.is shaded in gray.

Table S6. – Results of precision and bias tests between readers for each of the treatments applied to *Sphyrna lewini*, *Sphyrna corona* and *Mustelus lunulatus* vertebrae. IAPE, index of average error percentage; CV, coefficient of variation; PA (±0 bands), percentage of total agreement; RV, percentage of read vertebrae; P, p-values of the Bowker symmetry test. Best results of each test are highlighted in bold and the selected treatment for each species.is shaded in gray.

Species comments.

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Evaluation of staining techniques for the observation of growth bands in tropical elasmobranch vertebrae

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Supplementary material

Table S1. – Age and growth publications in tropical elasmobranchs (Spreadsheet in MS Excel format available at: http://scimar.icm.csic.es/scimar/supplm/sm05045TableS1.xlsx).

Table S2. – Results of precision and bias tests between readers, for each of the treatments applied to the small, medium and large vertebrae of *Narcine leoparda*. IAPE, index of average error percentage; CV, coefficient of variation; PA (± 0 bands), percentage of total agreement; RV, percentage of read vertebrae; P, p-values of the Bowker symmetry test. Best results of each test for each vertebrae size are highlighted in bold and the selected treatments are shaded in gray.

	Small (30)					Medium (30)					Large (30)				
Treatment (Stain+Time)	PA	RV	IAPE	CV	P	PA	RV	IAPE	CV	P	PA	RV	IAPE	CV	P
No staining	21.7	76.7	27.4	38.8	0.39	65.4	**86.7**	8.6	12.1	0.25	**91.3**	76.7	**0.7**	**1.6**	0.09
Immersion oil	38.5	70.0	19.3	41.7	0.19	57.7	**86.7**	4.5	6.4	0.14	37.5	76.7	8.3	13.9	0.28
Distilled water	27.8	**86.7**	23.4	33.1	0.27	68.0	83.3	7.8	11.1	0.16	39.1	76.7	5.4	7.6	0.33
Methylene blue 1′	**75.0**	80.0	8.1	11.4	0.41	50.0	80.0	5.8	8.2	0.10	73.9	76.7	1.6	2.3	0.37
Methylene blue 2′	65.2	76.7	6.8	9.6	0.81	60.9	76.7	4.3	8.1	0.50	62.5	80.0	2.4	4.7	0.16
Methylene blue 3′	63.6	73.3	8.3	14.3	0.54	68.2	73.3	3.8	11.0	0.50	70.4	**90.0**	2.6	5.3	0.22
Crystal violet 1′	69.6	76.7	5.5	7.8	0.11	58.3	80.0	5.2	7.3	0.33	64.0	83.3	2.7	3.8	0.32
Crystal violet 3′	62.5	80.0	10.3	14.6	0.12	44.4	60.0	4.9	7.0	0.52	58.3	80.0	2.8	3.9	0.17
Crystal violet 7′	65.2	76.7	6.7	9.4	0.16	57.1	70.0	3.9	5.5	0.12	65.2	76.7	2.4	3.4	0.09
Basic fuchsin 3′	47.8	76.7	16.9	23.9	0.12	64.0	83.3	4.3	6.1	0.42	63.0	**90.0**	2.8	4.0	0.16
Basic fuchsin 5′	56.5	76.7	14.8	20.9	0.08	60.0	83.3	4.6	6.5	0.29	46.2	86.7	4.5	6.4	0.19
Basic fuchsin 7′	50.0	73.3	12.0	16.9	0.34	**73.1**	**86.7**	3.4	4.8	0.42	54.2	80.0	4.8	6.7	0.09
Alizarin red 12′	52.0	83.3	13.1	18.5	0.21	52.0	83.3	8.2	11.6	0.43	56.0	83.3	4.2	5.9	0.10
Alizarin red 14′	65.4	**86.7**	8.8	17.9	0.04	61.5	**86.7**	2.4	5.4	0.39	43.5	76.7	6.1	8.6	0.51
Alizarin red 16 ‘	73.1	**86.7**	**4.9**	**7.0**	0.14	64.0	83.3	**3.3**	**4.6**	0.22	68.2	73.3	3.2	4.5	0.41

Table S3. – Results of precision and bias tests between readers, for each of the treatments applied to the small, medium and large vertebrae of *Urotrygon aspidura*. IAPE, index of average error percentage; CV, coefficient of variation; PA (± 0 bands), percentage of total agreement; RV, percentage of read vertebrae; P, p-values of the Bowker symmetry test. Best results of each test for each vertebrae size are highlighted in bold and the selected treatments are shaded in gray.

	Small (30)					Medium (30)					Large (30)				
Treatment (Stain+Time)	PA	RV	IAPE	CV	P	PA	RV	IAPE	CV	P	PA	RV	IAPE	CV	P
No staining	66.7	96.7	14.7	20.6	0.56	45.7	80.0	9.5	13.4	0.18	71.4	100.0	3.3	4.7	0.29
Alcohol	70.0	**100.0**	10.9	15.4	0.37	40.6	78.1	15.6	22.1	0.47	65.2	100.0	3.9	5.5	0.41
Distilled water	56.7	96.7	20.2	28.6	0.48	43.8	75.0	9.7	13.7	0.29	76.0	100.0	2.9	4.1	0.34
Methylene blue 7′	31.3	**100.0**	34.6	48.9	0.26	–	–	–	–	–	–	–	–	–	–
Methylene blue 10′	41.2	94.1	26.3	37.1	0.06	50.0	95.8	12.7	18.0	0.80	**80.0**	**100.0**	**2.2**	**3.1**	**0.39**
Methylene blue 15′	29.4	88.2	34.7	49.0	0.41	56.0	92.0	11.3	16.0	0.50	73.3	100.0	3.2	4.5	0.56
Methylene blue 20′	–	–	–	–	–	**84.2**	**89.5**	**1.2**	**1.7**	**0.32**	60.0	100.0	4.3	6.1	0.20
Crystal violet 7′	31.3	93.8	31.3	44.3	0.29	29.2	54.2	16.3	23.1	0.31	25.0	75.0	8.2	11.7	0.31
Crystal violet 10′	18.8	87.5	34.3	48.5	0.50	25.0	58.3	20.5	29.1	0.41	50.0	75.0	4.1	5.8	0.26
Crystal violet 15′	68.8	81.3	9.2	13.1	0.37	50.0	70.8	4.7	6.7	0.39	31.3	93.8	9.5	13.4	0.59
Basic fuchsin 1′	35.3	82.4	37.1	52.5	0.26	45.0	85.0	16.5	23.3	0.03	–	–	–	–	–
Basic fuchsin 5′	29.4	94.1	47.9	67.8	0.10	40.9	95.5	19.1	27.0	0.42	50.0	100.0	8.4	11.8	0.38
Basic fuchsin 10′	46.7	93.3	27.6	39.1	0.56	45.5	95.5	21.3	30.1	0.55	68.8	100.0	4.4	6.3	0.53
Basic fuchsin 20′	–	–	–	–	–	–	–	–	–	–	50.0	100.0	7.0	9.9	0.86
Alizarin red 3′	–	–	–	–	–	–	–	–	–	–	66.7	100.0	5.1	7.3	0.08
Alizarin red 7′	61.1	77.8	7.1	10.1	0.57	52.9	82.4	14.4	20.3	0.56	60.0	100.0	6.1	8.6	0.57
Alizarin red 10′	50.0	72.2	14.4	20.3	0.22	52.9	76.5	8.8	12.4	0.51	46.7	100.0	8.8	12.4	0.55
Alizarin red 15′	**70.6**	94.1	11.7	16.5	0.26	73.3	**100.0**	5.5	7.7	0.56	–	–	–	–	–
Light green 3′	56.3	81.3	9.2	13.1	0.51	81.3	93.8	3.6	5.0	0.37	73.3	100.0	5.1	7.2	0.26
Light green 5′	**64.7**	**76.5**	**5.1**	**7.3**	**0.16**	75.0	93.8	4.5	6.4	0.39	73.3	100.0	4.7	6.7	0.51
Light green 10′	53.0	70.6	6.1	8.6	0.22	62.5	87.5	6.7	9.4	0.51	66.7	100.0	4.7	6.7	0.39

Table S4. – Results of precision and bias tests between readers, for each of the treatments applied to the small, medium and large vertebrae of *Hypanus longus*. IAPE, index of average error percentage; CV, coefficient of variation; PA (± 0 bands), percentage of total agreement; RV, percentage of read vertebrae; P, p-values of the Bowker symmetry test. Best results of each test for each vertebrae size are highlighted in bold and the selected treatments are shaded in gray.

	Small (30)					Medium (30)					Large (30)				
Treatment (Stain+Time)	PA	RV	IAPE	CV	P	PA	RV	IAPE	CV	P	PA	RV	IAPE	CV	P
No staining	86.7	100	8.9	12.6	0.37	73.3	100	3.4	4.8	0.39	80.0	100	1.6	2.3	0.57
Basic fuchsin 1′	73.3	100	8.9	12.6	0.05	66.7	100	4.4	6.2	0.29	80.0	100	1.4	1.9	0.39
Basic fuchsin 2′	80.0	100	6.7	9.4	0.08	**93.3**	**100**	**2.2**	**3.1**	**0.32**	86.7	100	0.9	1.3	0.37
Basic fuchsin 3′	73.3	100	13.3	18.9	0.14	53.9	100	7.9	11.1	0.55	86.7	100	1.2	1.7	0.16
Bismarck brown 0.5′	86.7	100	4.4	6.3	0.16	–	–	–	–	–	93.3	100	0.5	0.7	0.32
Bismarck brown 1′	**93.3**	**100**	**2.2**	**3.1**	**0.32**	–	–	–	–	–	–	–	–	–	–
Bismarck brown 1.5′	–	–	–	–	–	–	–	–	–	–	86.7	100	1.0	1.4	0.37
Bismarck brown 2.0′	**93.3**	**100**	**2.2**	**3.1**	**0.32**	86.7	100	1.9	2.7	1.00	–	–	–	–	–
Bismarck brown 3.0′	–	–	–	–	–	86.7	100	1.9	2.7	1.00	86.7	100	1.0	1.4	0.32
Bismarck brown 5.0′	–	–	–	–	–	80.0	100	3.6	5.1	0.61	–	–	–	–	–
Alizarin red 1′	–	–	–	–		–	–	–	–	–	86.7	100	1.0	1.5	1.00
Alizarin red 2′	–	–	–	–	–	–	–	–	–	–	93.3	100	0.5	0.7	0.32
Alizarin red 3′	–	–	–	–	–	–	–	–	–	–	86.7	100	0.8	1.2	0.37
Light green 0.5′	**93.3**	**100**	**2.2**	**3.1**	**0.32**	–	–	–	–	–	–	–	–	–	–
Light green 1′	**93.3**	**100**	**2.2**	**3.1**	**0.32**	80.0	100	3.2	4.6	0.22	–	–	–	–	–
Light green 2′	86.7	100	8.9	12.6	0.37	80.0	100	3.2	4.6	0.22	80.0	100	1.2	1.8	0.22
Light green 3′	–	–	–	–	–	86.7	100	2.3	3.2	0.37	–	–	–	–	–
Light green 5′	–	–	–	–	–	–	–	–	–	–	86.7	100	0.8	1.2	0.37
Light green 7′	–	–	–	–	–	–	–	–	–	–	**100**	**100**	**0.0**	**0.0**	**NA**

Table S5. – Results of precision and bias tests between readers for each of the treatments applied to *Potamotrygon magdalenae, Alopias pelagicus* and *Carcharhinus falciformis* vertebrae. IAPE, index of average error percentage; CV, coefficient of variation; PA (± 0 bands), percentage of total agreement; RV, percentage of read vertebrae; P, p-values of the Bowker symmetry test. Best results of each test are highlighted in bold and the selected treatment for each species.is shaded in gray.

	Potamotrygon magdalenae (24)					*Alopias pelagicus* (27)					*Carcharhinus falciformis* (21)				
Treatment (Stain+Time)	PA	RV	IAPE	CV	P	PA	RV	IAPE	CV	P	PA	RV	IAPE	CV	P
No staining	87.5	80.0	1.2	2.5	0.16	41.7	52.2	3.6	5.1	0.54	0.0	54.6	12.6	17.8	0.42
Distilled water	53.3	80.0	2.6	3.7	0.61	41.7	52.2	4.9	6.5	0.54	60.0	45.5	4.3	5.0	0.39
Immersion oil	81.3	75.0	6.6	9.4	0.14	60.0	65.2	4.6	6.9	0.42	57.1	63.6	3.5	6.1	0.37
Methylene blue 15′	–	–	–	–	–	0.0	25.0	20.8	29.4	0.39	–	–	–	–	–
Methylene blue 20′	58.8	85.0	8.5	12.0	0.29	25.0	33.3	4.3	6.1	0.39	66.7	54.6	2.2	3.1	0.37
Methylene blue 25′	–	–	–	–	–	16.7	50.0	9.4	13.3	0.42	16.7	54.6	9.3	13.1	0.42
Methylene blue 30′	57.9	**95.0**	6.3	8.9	0.09	–	–	–	–	–	83.3	54.6	2.8	3.9	0.32
Methylene blue 35′	–	–	–	–	–	–	–	–	–	–	57.1	63.6	6.9	9.7	0.39
Methylene blue 40′	47.4	**95.0**	10.6	14.9	0.43	–	–	–	–	–	–	–	–	–	–
Crystal violet 15′	–	–	–	–	–	20.0	45.5	2.3	3.3	0.37	–	–	–	–	–
Crystal violet 20′	–	–	–	–	–	28.6	58.3	6.5	9.2	0.29	57.1	63.6	5.7	8.0	0.32
Crystal violet 25′	–	–	–	–	–	22.2	81.8	9.1	12.8	0.43	55.6	81.8	3.5	5.0	0.41
Crystal violet 30′	–	–	–	–	–	–	–	–	–	–	55.6	81.8	2.9	4.1	0.41
Crystal violet 35′	–	–	–	–	–	–	–	–	–	–	85.7	63.6	**0.5**	0.7	0.42
Crystal violet 40′	66.7	90.0	3.8	5.4	0.20	–	–	–	–	–	–	–	–	–	–
Crystal violet 50′	88.2	85.0	1.3	1.8	0.37	–	–	–	–	–	–	–	–	–	–
Crystal violet 60′	**100.0**	75.0	**0.0**	**0.0**	NA	–	–	–	–	–	–	–	–	–	–
Acid fuchsin 40′	–	–	–	–	–	–	–	–	–	–	75.0	72.7	1.4	2.0	0.37
Acid fuchsin 45′	–	–	–	–	–	–	–	–	–	–	75.0	40.0	2.8	3.2	0.32
Acid fuchsin 50′	–	–	–	–	–	–	–	–	–	–	80.0	45.5	0.7	1.0	0.37
Basic fuchsin 35′	–	–	–	–	–	33.3	75.0	4.4	6.2	0.54	–	–	–	–	–
Basic fuchsin 40′	–	–	–	–	–	22.2	75.0	6.8	9.6	0.43	20.0	45.5	6.5	9.1	0.39
Basic fuchsin 45′	–	–	–	–	–	36.4	**91.7**	4.8	6.8	0.43	66.7	54.6	2.6	3.6	0.32
Basic fuchsin 50′	–	–	–	–	–	–	–	–	–	–	75.0	36.4	1.6	2.2	0.32
Bismarck brown 10′	85.7	87.5	9.5	13.5	0.37	50.00	72.7	2.5	3.5	0.41	–	–	–	–	–
Bismarck brown 15′	–	–	–	–	–	**57.1**	**77.8**	1.8	2.5	0.39	–	–	–	–	–
Bismarck brown 20′	78.6	87.5	10.0	14.1	0.22	44.4	75.0	5.5	7.8	0.42	75.0	72.7	1.8	2.5	0.37
Bismarck brown 25′	–	–	–	–	–	–	–	–	–	–	57.1	70.0	2.7	3.9	0.39
Bismarck brown 30′	78.6	87.5	3.2	4.6	0.39	–	–	–	–	–	83.3	66.7	0.8	1.1	0.32
Bismarck brown 35′	–	–	–	–	–	–	–	–	–	–	66.7	60.0	1.4	2.0	0.32
Dahl staining10-10	–	–	–	–	–	–	–	–	–	–	50.0	40.0	3.4	4.8	0.37
Dahl staining 15-15	–	–	–	–	–	–	–	–	–	–	80.0	50.0	0.9	1.2	0.32
Dahl staining 5-5	–	–	–	–	–	–	–	–	–	–	50.0	60.0	5.5	7.8	0.39
Silver nitrate 1-1	–	–	–	–	–	–	–	–	–	–	60.0	35.7	1.8	2.5	0.39
Silver nitrate 2-3	–	–	–	–	–	–	–	–	–	–	**100.0**	16.7	0.0	**0.0**	NA
Alizarin red 3′	82.4	85.0	3.8	5.4	0.16	–	–	–	–	–	–	–	–	–	–
Alizarin red 5′	95.0	83.3	0.5	0.8	0.16	**100.0**	28.6	**0.0**	**0.0**	0.43	–	–	–	–	–
Alizarin red 7′	95.5	91.7	0.1	1.3	0.16	66.7	42.9	1.6	2.2	0.32	–	–	–	–	–
Alizarin red 9′	–	–	–	–	–	33.3	42.9	4.7	6.5	0.37	–	–	–	–	–
Alizarin red 10′	–	–	–	–	–	–	–	–	–	–	28.6	70.0	4.2	5.9	0.70
Alizarin red 15′	–	–	–	–	–	–	–	–	–	–	16.7	60.0	5.9	8.3	0.26
Alizarin red 20′	–	–	–	–	–	–	–	–	–	–	33.3	60.0	6.0	8.5	0.41
Light green 20′	–	–	–	–	–	42.9	77.8	4.6	6.5	0.41	33.3	33.3	2.6	3.6	0.37
Light green 25′	–	–	–	–	–	50.0	44.4	3.6	5.1	0.37	33.3	33.3	3.6	5.1	0.37
Light green 30′	81.8	64.7	2.12	3.0	0.37	66.7	66.7	2.1	2.9	0.37	22.2	22.2	5.2	7.4	0.31
Light green 35′	75.0	70.6	4.1	5.8	0.39	–	–	–	–	–	33.3	**100.0**	5.4	7.7	0.39
Light green 40′	**100.0**	52.9	**0.0**	**0.0**	NA	–	–	–	–	–	28.6	70.0	5.7	8.0	0.42

Table S6. – Results of precision and bias tests between readers for each of the treatments applied to *Sphyrna lewini*, *Sphyrna corona* and *Mustelus lunulatus* vertebrae. IAPE, index of average error percentage; CV, coefficient of variation; PA (± 0 bands), percentage of total agreement; RV, percentage of read vertebrae; P, p-values of the Bowker symmetry test. Best results of each test are highlighted in bold and the selected treatment for each species.is shaded in gray.

	Sphyrna lewini (52)					*Sphyrna corona* (18)					*Mustelus lunulatus* (15)				
Treatment (Stain+Time)	PA	RV	IAPE	CV	P	PA	RV	IAPE	CV	P	PA	RV	IAPE	CV	P
No staining	76.0	96.2	20.8	29.5	0.41	75.0	66.7	6.1	8.7	0.39	45.5	68.8	11.0	15.6	0.31
Immersion oil	**89.8**	94.2	**6.9**	**9.8**	0.14	78.6	**77.8**	4.0	5.2	0.57	45.5	73.3	8.6	12.1	0.67
Agua destilada	72.3	90.4	16.6	23.5	0.05	66.7	66.7	4.0	5.2	0.39	27.3	73.3	11.9	16.9	0.30
Methylene blue 20′	–	–	–	–	–	–	–	–	–	–	53.3	93.8	7.6	10.7	0.41
Methylene blue 25′	68.2	84.6	19.5	27.6	0.51	81.8	61.1	2.5	3.6	0.37	–	–	–	–	–
Methylene blue 30′	79.5	84.6	12.1	17.1	0.56	60.0	55.6	6.0	8.5	0.14	60.0	93.8	5.7	8.1	0.20
Methylene blue 35′	84.4	86.5	12.2	17.2	0.26	55.6	50.0	10.8	15.2	0.14	–	–	–	–	–
Methylene blue 40′	–	–	–	–	–	–	–	–	–	–	69.2	81.3	5.0	7.1	0.40
Crystal violet 20′	61.9	89.4	34.9	49.4	–	**100.0**	55.6	**0.0**	**0.0**	0.16	71.4	87.5	3.2	4.5	0.41
Crystal violet 25′	62.7	**98.1**	27.9	39.4	0.06	80.0	55.6	1.8	2.6	0.51	–	–	–	–	–
Crystal violet 30′	64.0	96.1	29.0	40.9	0.09	55.6	50.0	5.3	7.4	0.14	62.5	**100.0**	5.7	8.1	0.32
Crystal violet 35′	–	–	–	–	0.31	–	–	–	–	–	–	–	–	–	–
Crystal violet 40′	–	–	–	–	––	–	–	–	–	–	56.3	**100.0**	6.2	8.8	0.20
Basic fuchsin 40′	–	–	–	–	0.06	–	–	–	–	–	–	–	–	–	–
Basic fuchsin 45′	–	–	–	–	0.06	–	–	–	–	–	–	–	–	–	–
Basic fuchsin 50′	–	–	–	–	0.19	–	–	–	–	–	–	–	–	–	–
Bismarck brown 20′	71.1	91.8	23.8	33.7	0.15	50.0	66.7	8.7	12.3	0.42	66.7	64.3	6.9	9.7	0.50
Bismarck brown 25′	71.1	90.0	20.2	28.6	0.02	60.0	55.6	6.5	9.2	0.41	–	–	–	–	–
Bismarck brown 30′	65.1	87.8	22.8	32.3	0.21	76.9	72.2	5.0	7.0	0.39	55.6	64.3	9.4	13.2	0.39
Bismarck brown 40′	–	–	–	–	–	–	–	–	–	–	44.4	64.3	10.4	14.7	0.41
Alizarin red 5′	70.6	89.5	27.5	18.7	–	61.5	72.2	7.6	10.8	0.42	36.4	73.3	12.4	17.5	0.41
Alizarin red 10′	78.7	88.7	13.2	32.4	0.15	53.8	72.2	7.0	9.8	0.41	53.8	76.5	7.6	10.8	0.56
Alizarin red 15′	75.0	95.7	22.9	38.8	0.26	75.0	66.7	3.7	5.2	0.39	40.0	88.2	9.0	12.7	0.55
Alizarin red 20′	–	–	–	–	0.21	–	–	–	–	–	–	–	–	–	–
Light green 20′	–	–	–	–	–	–	–	–	–	–	69.2	92.9	3.6	5.1	0.55
Light green 25′	69.8	93.5	24.0	34.0	–	75.0	66.7	3.4	4.8	0.22	–	–	–	–	–
Light green 30′	73.9	90.2	21.7	30.7	0.17	64.3	77.8	6.1	8.7	0.39	91.7	85.7	1.2	1.7	0.61
Light green 35′	74.5	92.2	17.3	24.5	0.26	75.0	66.7	4.4	6.2	0.22	–	–	–	–	–
Light green 40′	–	–	–	–	0.12	–	–	–	–	–	**100.0**	78.6	**0.0**	**0.0**	0.30

SPECIES COMMENTS

Narcine leoparda

For this species a detailed analysis of vertebrae size effect on the growth bands visualization was performed. This is an approach rarely used, since most studies applying staining techniques do not discriminate them by size. Results showed that large vertebrae were easily read without staining, while medium and small vertebrae needed alizarin red for 16 min too enhance growth bands visualization. Thus, IAPE analysis of the assessed treatments showed an effect of the dyes on the increase of growth bands readings accuracy. There are no comparison studies for the species, however, alizarin red has shown good performance in other batoid species, even in families with significant differences in size, distribution and phylogenetic affinity, as *Raja undulata* (Coelho and Erzini 2002).

Urotrygon aspidura

Section thickness can affect the appropriate visualization of growth bands, near the focus (when defining the birthmark) and in those vertebrae with the greatest number of bands towards the edges (due to their accumulation), making difficult to count them. In the present study, appropriate thickness was chosen at 0.4 mm for best visualization of growth bands in *U. aspidura* since 0.3 mm allowed too much light to pass through the vertebra section and at 0.5 mm it was to dark, affecting the clarity of the growth bands. This result is in the range (0.3 to 0.5 mm) of age and growth studies in similar sizes species of the families Urotrygonidae, Uroplophidae and Narcinidae (White et al. 2001, Hale and Lowe 2008, Pérez-Rojas 2013, Mejía-Falla et al. 2014, Guzmán-Castellanos 2015, Santander-Neto 2015), as well as in numerous elasmobranch species (Goldman et al. 2012).

For *U. aspidura*, treatments without dyes showed a lower performance than those with it. Methylene blue for 10 min and 20 min were the most suitable for large and medium vertebrae, respectively; while light green for 5 min was the most accurate technique for small vertebrae. Mejía-Falla et al. (2014) identified that *U. rogersi* vertebrae without staining showed better results than those stained with alizarin red, crystal violet and methylene blue. Studies in other species of the family (*Urobatis halleri, Urotrygon microphthalmum, Urotrygon chilensis*) have not assessed any technique to enhance the growth bands visualization, always working on unstained vertebrae sections (Hale and Lowe 2008, Guzmán-Castellanos 2015, Santander-Neto 2015).

Hypanus longus

Sections of 0.3mm did not allow an adequate visualization of growth bands, since it increased presence of sub and pre-bands, which is related to: 1) amount of light that passed through the sample was greater, which directly affects the ability of the reader to detect the presence of the bands; 2) sections obtained with this thickness were more fragile than the other samples, which causes these structures to fracture easily and therefore lose samples, increasing the number of individuals needed for an age and growth study.

Sections of 0.5 and 0.6 mm did not facilitate the reading of growth bands in the vertebrae and, as with thin samples, light was an important factor. In these sections, the amount of light that passed through was not sufficient and, consequently, contrast between calcium deposits was hard to identify, underestimating the growth bands counting. Results of qualitative evaluation of the thickness in the present research were similar to those made in other studies with rays, where these thicknesses (0.5 and 0.6mm) were also not suitable for growth band count in *Narcine leoparda* and *Urotrygon aspidura* (Pérez-Rojas 2013, Torres-Palacios et al. 2019).

Best results were obtained using sections of 0.4 mm since light that passed through the sample was enough and allowed an adequate contrast between the calcium deposits. Additionally, vertebrae sections were not so fragile compared to 0.3 mm, reducing the loss of samples. This result is also reported for other related species (*Squalus acanthias* and *Urotrygon aspidura*), where 0.4 mm was reported as the optimum thickness for growth bands readings (e.g. Bubley et al. 2012, Torres-Palacios et al. 2019).

It is important to note that there are also contrasting results to those found in the present study, since larger or smaller section thicknesses have been proposed as the most suitable for counting growth bands in other species such as *Mustelus canis, Mustelus asterias, Prionace glauca* and *Pristis pectinata* (e.g. Conrath et al. 2002, Parra et al. 2008, Farrell et al. 2010, Scharer et al. 2012). This implies that adequate visualization of bands with a certain section thickness is not an attribute shared in all chondrichthyan species, nor even in species of the same genus, as was the case of *Dasyatis centroura* and *Dasyatis pastinaca* (Ba usta and Sulikowski 2012, Yigin and Ismen 2012), where the section thickness chosen by the authors was 0.6 mm and 0.5 mm, respectively, differing from the optimum in the present study.

Use of dyes in calcified structures has been widely used in several studies of elasmobranchs (Cailliet and Goldman 2004), where they assessed the effectiveness of stains in different species. Although for *H. longus* some treatments (methylene blue, crystal violet, acid fuchsine, immersion oil) were not adequate, it is important to highlight that these dyes allowed correct visualization in other species of rays, as *U. aspidura*, where the methylene blue treatment yielded better results for large and medium vertebrae (Torres-Palacios et al. 2019), and *Myliobatis californica* where crystal violet allowed a better visualization of growth bands (Aguirre-García 2009).

Data obtained in the present study suggest that observation and counting of growth bands in *H. longus* should be carried out using dyes treatments, since control vertebrae (without staining), belonging to the large and medium sizes, presented very low values of percentage of agreement (PA). This situation differs from other sharks and rays' studies (*Prionace glauca* and *Rhinoptera steindachneri*), which have shown high counting effectiveness of growth bands without using any dyes (e.g. Lessa et al. 2004, Pabón-Aldana 2016).

According to the results here obtained, it is recommend that age and growth studies of *H. longus* consider using light green for seven minutes in larger vertebrae ($\approx \geq 8$ 7.57 - 12.34mm), basic fuchsin (0.01%) for two minutes in medium vertebrae ($\approx$ 4.40 5 - 8.37 mm) and Bismark brown (0.05%) for one or two minutes, or light green (0.05%) for 0.5-1 min in small vertebrae ($\approx \leq$ 3.08 - 5.15 mm).

However, these treatments should not be generalized to other species of the same genus, since it has been shown that, in *Hypanus dipterurus*, the light green dye is the least suitable for growth bands observation (Carmona-Sánchez 2017). This author found that the best treatment for large vertebrae was Bismarck brown for seven minutes; for medium samples it was not necessary to apply stains, since an adequate visualization of the growth bands without staining was obtained; and finally for small vertebrae, Bismarck brown three minutes was selected as the best treatment (Carmona-Sánchez 2017).

This procedure, in spite of appearing simple and at the same time requiring an additional time, will allow that in future studies of age and growth, readings can be obtained with greater precision and less bias, and therefore estimates of growth parameters with less uncertainty. Additionally, it will reduce the waste of samples, which implies a smaller number of individuals to be slaughtered to carry out the respective studies of age and growth.

Potamotrygon magdalenae

Results obtained here showed that alizarin red for seven minutes was the technique that generated the greatest accuracy in estimating age of the species, without showing systematic bias. This treatment was followed by crystal violet. Species phylogenetically close to the Potamotrygonidae family, such as *D. parsnip*, have shown more accurate age estimates with the use of safranin (Girgin and Basusta 2016) and crystal violet (Yigin and Ismen 2012), while in *P. leopoldi* Charvet et al. (2018) used no stained treatments to assess age and growth using vertebrae sections. Also, the results of this study in *U. aspidura* identified methylene blue and light green as the best enhancement techniques, indicating that dyes and treatments can vary according to their affinity to calcium and phosphorus concentrations in each species, and therefore ratified the importance of making this type of techniques assessment for each species, before carrying out an age and growth study.

Alopias pelagicus

This species presented a high degree of difficulty for the growth bands observation and therefore the pattern definition. This influenced the final decision on the technique that was considered as the most appropriate. Alizarin red (with five and seven minutes) showed the best precision results, but a very low %R, while Bismarck brown 15 min showed an acceptable performance in precision and reading, being defined as the most suitable treatment for the species. According to this, it is highly recommended to perform different precision tests, assessing them and choosing according to what the indices show. Thus, a treatment can present high precision and very low bias, but it will not be useful if the %R is low. This situation can occur in species with poorly defined band patterns, which is generated by the low calcification of the same, as has been proposed for *Alopias superciliosus* (Fernández-Carvalho et al. 2011), who found that crystal violet stains generated the lowest values of CV and IAPE.

Drew et al. (2015) solved the problem of the vertebrae porosity of *A. pelagicus* by imbibing them in resin for later cutting. Of course, that procedure eliminated the possibility of staining. Therefore, to combine these two procedures and enhance the results, we suggest dyeing the entire vertebrae and then proceed with the cut in resin.

Carcharhinus falciformis

Age and growth studies of this species, have used undyed vertebrae (Sánchez-de Ita et al. 2011), vertebrae dye with alizarin red for 30 min (Oshitani et al. 2003) and vertebrae subjected to X-rays (Joung et al. 2008); however, none of these studies have precision tests to evaluate age estimation and therefore the results of this study compared with studies conducted for other species of the same genus. Carlson et al. (2003) estimated a higher accuracy for *Carcharhinus isodon* with vertebrae stained with 0.01% crystal violet. On the other hand, Cruz-Martínez et al. (2005) concluded that alizarin red was the best technique for improving the visualization of growth bands of *Carcharhinus leucas*. In this study, crystal for 35 min was defined as the best treatment, obtaining values of IAPE and PA (± 0 bands) similar to those obtained in the mentioned works. The low performance obtained with Dahl method, basic fuchsin and alizarin red, suggest that they can be discarded in future studies for this species.

Sphyrna lewini

Vertebrae treated with violet crystal for 25 min and immersion oil did not show significant differences between the precision indexes and the %R. Based on this, it is more efficient to use immersion oil since time investment is significantly lower. About other dyes, Zarate-Rustrián (2010) found a better bands visualization using unstained vertebrae sections, compared to vertebrae stained with alizarin red, being this similar to the present research. However, Zarate-Rustrián (2010) did not perform an analysis of precision between techniques to compare their effectiveness. In this species it has also been recorded that vertebrae stained with 0.01% crystal violet generate acceptable reading results (Anislado-Tolentino and Robinson-Mendoza 2001, Anisaldo-Tolentino et al. 2008, Piercy et al. 2007). This information shows that different techniques can generate acceptable results in the same species, and hence the need to evaluate several treatments.

Sphyrna corona

Crystal violet for 20 min showed the best visualization results for this species. Given that this is a rare and endemic species of the Tropical Eastern Pacific, there is no comparable age and growth research on it. However, close species like *S. tiburo* has estimates of age and growth (Carlson and Parsons 1993, Frazier et al. 2014), but these studies did not include techniques assessment to improve the visualization of growth bands, and the authors made age estimation using unstaining vertebrae, founding good PA and IAPE values for Florida and the western Atlantic.

Given that crystal violet was the best dye for *S. corona* and the second best for *S. lewini*, it is suggested that this dye may be a promising option for age studies in tropical species of this genus. However, and given that the use of immersion oil and unstained vertebrae have been successful in *S. lewini* and *S. corona*, it is also possible that natural configuration of the vertebrae is good enough to develop the studies without the need for stains. This, however, should be evaluated by each researcher at the time of developing his research.

Mustelus lunulatus

Assessment of precision and bias highlighted the fact that all treatments presented a %R higher than 60%, being light green for 40 min the best treatment in this species assessment. As in many other tropical species, there are no previous studies of age and growth for *M. lunulatus*. At genus level, Conrath et al. (2002) and Farrell et al. (2010) estimated age and growth of *M. canis* and *M. asterias* in the Northwest Atlantic and England, respectively, using unstained vertebrae, and defining a PA = 84% and 93%. In this study, all dyes treatments showed PA values (± 0 bands) higher than Conrath et al. (2002). On the other hand, Méndez (2008) founded a PA = 84%, IAPE = 8.5% and CV = 0.12 in vertebrae stained with violet crystal at 0.001%, for *M. henlei* in the Gulf of California, Mexico. These results show lower PA values and higher CV and IAPE in comparison with the results for *M. lunulatus* presented

in this research. However, the author did not compare the accuracy of the age estimation with other methods. Finally, Yudin and Cailliet (1990) tested different techniques in *M. californicus* and *M. henlei*, finding that, X-ray radiographs were the most successful technique to enhance visualization. However, as already mentioned, these techniques require greater logistic and economic investment.

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