STAINING TECHNIQUES FOR AGEING TOPE SHARK, *Galeorhinus galeus* (Linnaeus, 1758), FROM THE AZORES: A COMPARISON BASED ON PRECISION ANALYSIS

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A vertebrae sample of tope shark, *Galeorhinus galeus* (Linnaeus, 1758), stratified by size-classes, was used to test between different staining techniques of enhancing vertebral growth ring visibility. Four techniques were tested: alizarin red, silver nitrate and cobalt nitrate staining, and the "deep-coned vertebrae" technique. The latter was discarded due to its unsuitability for tope shark vertebrae. The cobalt nitrate original protocol was modified. Upon staining, each vertebra was subject to three replicated independent readings, by a single reader. Within-reader consistency and bias of growth ring counts determination were evaluated using four different precision indices: percent of agreement (PA), average percent error (APE), coefficient of variation (V) and index of precision (D). Results indicated that the vertebrae stained with the cobalt nitrate technique showed both better optic enhancement of growth rings and higher degree of count consistency, comparatively to the alizarin red and silver stained vertebrae. Conclusively, the cobalt nitrate was the most efficient staining technique upon tope shark vertebrae, among the methods tested herein.

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INTRODUCTION

Ageing of elasmobranch fishes is usually achieved by interpreting concentric growth rings that are periodically deposited on their skeletal parts, particularly vertebrae. Yet, the methods employed for enhancing vertebral calcified rings are numerous and diverse, as reviewed by CAILLIET et al. (1986) and CAILLIET (1990). Staining techniques are among the most commonly used, due to their simplicity and cost-effectiveness. There is, however, no staining methodology that can be uniformly adopted for all species with the same rate of success. On the contrary, specific patterns and degree of calcification are aspects that need to be taken in account when selecting the appropriate technique for each case. CAILLIET et al. (1983) underlined the usefulness of preliminary assays in comparing and deciding upon different staining methodologies.

Age determination is often regarded as a matter of routine, yet counting growth rings is subject to bias (variability) from various sources. Such bias might result either from the methods used, the readers involved or even the actual growth pattern of the fish (EKLUND et al. 2000). The quantification and elimination of ring-count variability both play an important role in the consistency of age assessment, and yet are often neglected (CAILLIET & TANAKA 1990). Ageing variability can be mathematically expressed in terms of precision, and comparative analysis between different methods, readers and/or skeletal structures, may be used to evaluate or even to increase the consistency of age determination. The so-called precision indices are most commonly employed in such cases (BEAMISH & MCFARLANE 1987).

The tope shark, *Galeorhinus galeus*, is a highly abundant species in Azorean coastal waters. Its role as a top predator in the demersal

fish community has already addressed to by GOMES et al. (1998). It is furthermore believed that the Azores represent important tope shark nursery areas in the Northeastern Atantic. Aside from its ecological relevance, the tope shark is also an important by-catch in the demersal longline fishery, nowadays the most profitable fishing activity in the Azores. The status of the tope shark population in these waters, however, remains virtually unknown, and basic information for stock assessment, such as age and growth, is still lacking.

This study provides a first step onto the investigation of age and growth of the tope shark in Azorean waters. Techniques for enhancing the visibility of growth rings in *G. galeus* vertebrae were tested and compared herein. The purpose was to experimentally determine which technique most effectively enhances growth rings, and to substantiate its effectiveness using indices of precision as an indicator of ring-counting consistency.

MATERIAL AND METHODS

Between March and April of 1995 to 1997, tope sharks were caught with bottom longline, during cruise surveys onboard R/V ARQUIPÉLAGO. The study site comprised most of the coastal areas and major offshore fishing grounds within the Azorean EEZ. Those surveys were conducted on an annual basis, as part of a more comprehensive research program focusing on the Azorean demersal fish community (MENEZES et al. 1998, 1999).

Each specimen was measured (total length) and sexed. A segment of approximately 10 *centra* was extracted from the vertebral column, just anterior to the region beneath the first dorsal fin. These vertebral segments were then labelled and frozen. This sampling of vertebrae was carried out as part of a broader study on the age and growth of tope shark from Azorean waters.

Vertebrae preparation for staining followed the general guidelines given by CAILLIET et al. (1983). After defrosting, the vertebral section was separated into individual *centra*, which were then washed in tap water for a minimum period of 30 min. Afterwards, each *centrum* was soaked between 3 and 10 minutes in sodium hypoclorite (chlorine bleach) at an actual concentration of 10%, to remove the tough layer of connective tissue on both faces of vertebra, exposing the conical surfaces of the *corpus calcareum*. This step was carefully monitored to prevent an overexposure of the *corpus calcareum* to the bleach, which may compromise the subsequent assimilation of staining reagents (SILVA et al. 1997). Finally, each *centrum* was rinsed overnight under running tap water, in order to remove all traces of bleach. Finally, the vertebrae were briefly soaked in distilled water and air-dried.

For the purpose of this particular study, a subsample of 30 specimens was randomly selected from the available main sample of topes. To ensure coverage of the entire specimens' size range, this subsample was stratified by three size-classes of 10 individuals each: Small (TL < 80cm), Medium (80cm < TL < 120cm) and Large (TL > 120cm). Tope shark size-class limits were defined according to OFFICER et al. (1996). The overall sample was also considered for a size-independent analysis.

Four different staining techniques were considered for testing: alizarin red S staining (LAMARCA 1966), silver nitrate staining (STEVENS 1975; CAILLIET et al. 1983), cobalt nitrate staining (HOENIG & BROWN 1988), and the "deep-coned vertebrae" method (CORREIA & FIGUEIREDO 1997). The first three techniques involve reagents with high affinity to calcium salts, hence producing a chromatic enhancement of mineral-rich rings on the *corpus calcareum* (MEUNIER 1992). The "deep-coned vertebrae" technique does not include a staining phase, as it is based on direct observation of growth rings in the *corpus calcareum*, under transmitted light.

From each individual in the subsample, four *centra* were randomly selected, each technique being applied to its corresponding *centrum*. Overall, 3 (classes) x 10 (individuals) x 4 (techniques) = 120 *centra* were selected for processing.

Preliminary testing was carried out to enable calibration of technique protocol routines. All techniques were applied following the original protocols compiled in SILVA et al. (1997). Strongly colour-enhanced bands were identified as growth rings and counted. Three independent

readings – replicates – were performed on each *centrum*, based on which counting precision was determined. Replicates were separated from one another by 15 day-periods, and made without prior knowledge of individual size, sex, or previous readings. The senior author was the only reader involved.

Reproducibility of growth ring counts, i.e. between-replicate consistency, was quantified on each technique using four different precision indices: the percent of agreement (PA) and the average percent error (APE), as shown by BEAMISH & FOURNIER (1981); the coefficient of variation (V) and the index of precision (D), proposed by CHANG (1982). Mathematical expressions for the calculation of APE, V and D indices were used as follows:

(1) $$APE = \frac{1}{N}\sum_{j=1}^{N}\left[\frac{1}{R}\sum_{j=1}^{R}\frac{\left|X_{ij}-X_j\right|}{X_j}\right]$$

(2) $$V = \frac{\sqrt{\frac{1}{R}\sum_{i=1}^{R}\frac{\left(X_{ij}-X_j\right)^2}{R-1}}}{X_j}$$

(3) $$D = \frac{V}{\sqrt{R}}$$

where N is the number of fish in the sample; R is the number of replicate readings; X_{ij} is the ith replicate ring count for the jth fish; and Xj is the average ring count, between replicates, for the jth fish.

The APE, V and D indices provide direct measurements of count consistency, as they express variability within a set of readings (CASSELMAN 1983). In other words, the lower the value of such an index, the more consistent (reproducible) and precise the estimates are for that set.

Technique efficiency was evaluated using both visual enhancement and precision analysis results.

RESULTS

The instantaneous reactivity of cobalt nitrate staining technique originally proposed by HOENIG & BROWN (1988) proved unsatisfactory to this experiment, when applied to tope shark vertebrae. To prevent rejection of this technique, a variation of the original staining protocol was used herein (Table I of the Appendix).

The "deep-coned vertebrae" technique was discarded, as it proved unsuitable due to the thickness and opacity of the highly mineralised *corpus calcareum* of *G. galeus* vertebrae (FERREIRA & VOOREN 1991), even after prolonged exposure of the *centra* to decalcifying agents. The remaining three techniques – alizarin red, silver nitrate and cobalt nitrate – were able to provide identifiable and countable rings for all stained vertebrae.

Overall, counts ranged from 2 to 11 growth rings (Table II of the Appendix). The absolute difference between replicates never exceeded two rings for any of the techniques tested and on any of the size-classes. Vertebrae stained with cobalt nitrate had the highest consistency between replicate readings, whereas silver nitrate staining resulted in the lowest count consistency, irrespective of size-class. Cobalt nitrate staining was also the only method where the three replicates where never in full disagreement in any of the size-classes, meaning that at least two out of three replicates were always consensual using cobalt nitrate staining. In the remaining two techniques no agreement between all three replicates sometimes occurred.

With combined size-classes, replicates differed by either zero (50.0%) or one growth ring (50.0%) using vertebrae stained with cobalt nitrate, whereas 26.7% and 63.3% of full and partial replicate agreement, respectively, were obtained using alizarin red, and 20.0% and 63.3% using silver nitrate (Fig. 1). Furthermore, full disagreement between replicates occurred using both alizarin red and silver nitrate staining, in 10.0% of the *centra* for the former and in 16.7% for the latter. For all techniques, the absolute difference between replicates seemed to correlate with fish size, increasing from size-classes S to L.

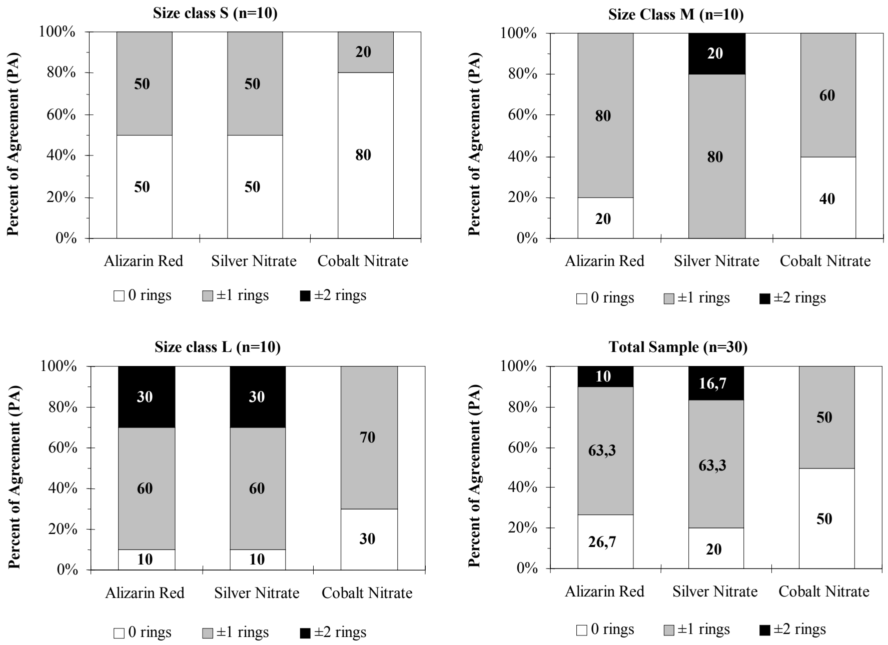

Fig. 1. Percent of agreement (PA) between counts using different staining techinques. Histograms are shown separately for each of the size-classes (S) TL<80cm, (M) 80cm<TL<120cm and (L) TL>120cm, as well for the overall sample.

The APE, V and D indices reflected the results obtained with PA. Overall analysis (size-classes combined) indicated that cobalt nitrate technique again provided the most precise counts, showing the lowest incidence of error between replicates – 3.5%, 4.5% and 2.6%, for APE, V and D, respectively (Fig. 2). The alizarin red technique followed, with 6.1%, 8.0% and 4.6%; and then silver nitrate with 6.8%, 9.1% and 5.3%. Nevertheless, none of the techniques has ever exceeded the 10% error limit.

In opposition to the size-correlated increase of discrepancy between replicates obtained with the PA index, no trends between body size and precision were observed APE, V and D. The three indices varied proportionally to each other, but behaved differently from technique to technique. In the alizarin red technique, APE, CD and D values dropped with size-class, indicating higher precision in larger-sized specimens. Seemingly, silver and cobalt nitrate staining did not show this same decreasing trend, as APE, V and D values peaked in the M size-class. The reasons for such tendencies are further analysed in discussion.

The overall qualitative results agreed well with the quantitative analysis. Of all three techniques, cobalt nitrate staining provided the best optical resolution, with clearer and more contrasted growth rings, throughout the entire size range of the sample (Fig. 3). The alizarin red and the silver nitrate staining provided lower optical resolution of enhanced rings.

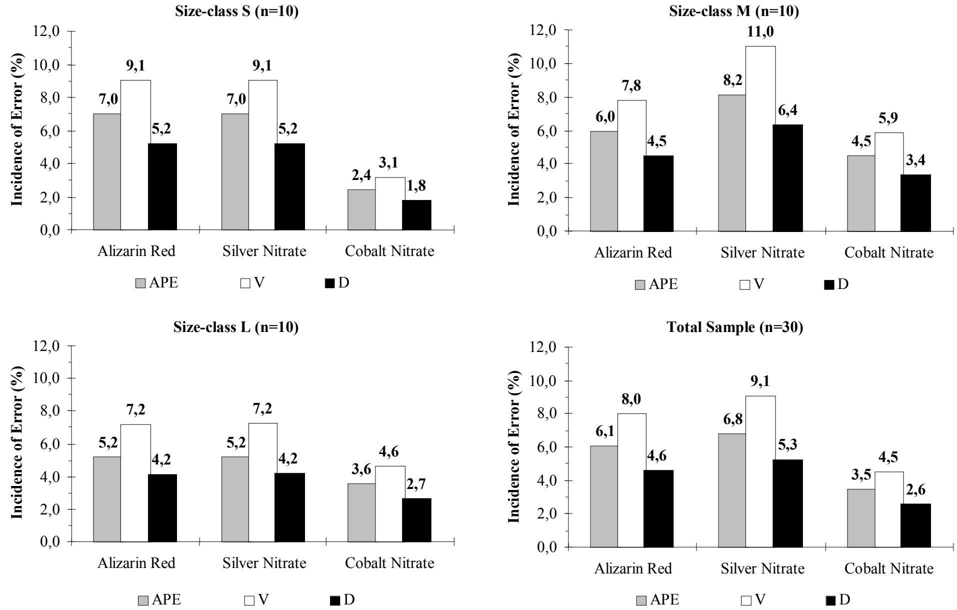

Fig. 2. Average percent error (APE), coefficient of variation (V) and index of precision (D) of counts using different staining techniques. Histograms are shown separately for each of the size-classes (S) TL < 80 cm, (M) 80 cm < TL < 120 cm and (L) TL > 120 cm, as well for the overall sample.

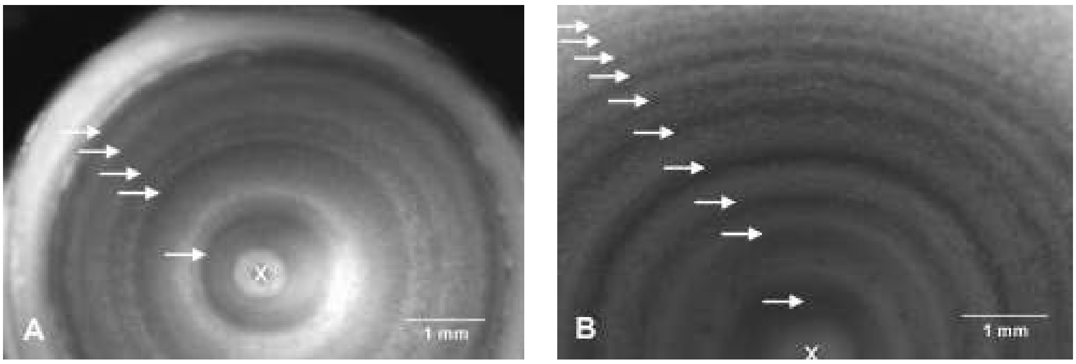

Fig. 3. *Galeorhinus galeus* vertebrae stained with cobalt nitrate. Arrows indicate enhanced growth rings; (×) marks the vertebral *focus*. A. Female specimen of 84cm TL; B. Male specimen of 131cm TL.

DISCUSSION

According to CARLANDER (1987), there are two main sources of bias inherently associated with ageing fish from vertebral growth rings, which are: 1) reduction of the spacing between successive growth rings towards the edge of the *centra* with the increase of body size; 2) experienced *versus* novice readers interpreting and counting rings; WALKER et al. (1995) and OFFICER et al. (1996) also suggest two additional potential sources of error: 3) variation in the number of rings *versus* the position that the *centra* occupies in the vertebral column; and 4) technique used to optically enhance the growth rings.

Bias source (1) refers to fact that the gradual decrease in growth rate with increasing size leads to an accumulation of narrow-spaced rings towards the outer zone of the *corpus calcareum*, ultimately resulting in illegibility of this zone and thus underestimation of the number of peripheral rings (TANAKA et al. 1990). Therefore, a negative correlation between ring count consistency and

fish size is expected, at least in theory. The results obtained in this study agree with this to some extent, since a clear size-correlated increase of discrepancy between replicates was recorded with the PA index. APE, V and D indices would be expected to respond similarly, by evidencing a drop in precision from size-classes S to L, however no such trend between body size and precision was observed with any of the three indices. The reason for this is simple, though: whereas PA only expresses the absolute difference between a given set of ring counts; the APE, V and D indices also incorporate ring count means, expressing precision in terms of total deviation to the mean, thus taking fish size into account (BEAMISH & FOURNIER 1981; CHANG 1982). As the mean ring counts increase considerably with size (from 3 rings in S class to 9 rings in the L class), and the absolute difference between replicate counts increased only from ±1 ring to ±2 rings, it is logical to think the deviation to the mean counts decrease with size. CHANG (1982) demonstrates this, obtaining lower APE, V and D values for fish with higher number of rings. It is therefore explained the decreasing tendency of APE, V and D with fish size. Exceptions to such trend were observed with cobalt nitrate and silver nitrate, where values peaked in the M size-class. With cobalt nitrate, this was due to very low discrepancy between replicates in the S-class, resulting in index values lower than expected. With silver nitrate, it was due to higher discrepancy in the M size-class, resulting in higher index values.

Overall, the main goal was achieved: when compared to the alizarin red and the silver nitrate, the cobalt nitrate staining has always provided higher precision in ring counts, no matter the vertebral sample is analysed in separate size-classes or considered as a whole.

According to CAILLIET et al. (1985), decreasing count consistency with increasing size is a common problem arising from the interpretation of growth patterns in skeletal parts for age determination purposes, particularly when dealing with species of great longevity – a life history trait for the majority of elasmobranchs (CAILLIET et al. 1983). The tope shark is a typical case of a slow-growing and long-lived elasmobranch species, attaining a maximum total length of 200 cm during a lifespan of over 50 years (COMPAGNO 1984), and slower growth has been demonstrated for tope sharks older than 10-11 years and larger than 130 cm TL (GRANT et al. 1979). In specimens larger than 130 cm, a gradual decrease in optical resolution between growth marks is observed towards the peripheral region on vertebrae, particularly when staining techniques are used (MOULTON et al. 1992). In this study, the maximum count of growth rings was 11, corresponding to specimens of nearly 150 cm TL, hence it is plausible to consider the occurrence underestimation in ring count, particularly with larger-sized tope.

With regards to bias source (2), it should be emphasised that there was only one reader involved in this experiment, with no prior background on the interpretation of vertebral growth rings. OFFICER et al. (1996) recommend that at least two readers, preferably experienced ones, are involved in the ring counting process. Therefore, one might find pertinent to question the reliability of the estimates obtained herein by a single and inexperienced reader. However, as pointed out by MOULTON et al. (1992), the absence of more than one reader might have a positive side to it, as inter-reader bias is eliminated. Because the absolute age and size of the fish was not known when rings counts were made, any within-reader bias would to tend to appear in a systematic way and may thus be assumed negligible (KIMURA & LYONS 1991).

Bias source (3) is basically dependent upon the species' own patterns of skeletal development. OFFICER et al. (1996) presented evidence of a significant variation in the number of rings using vertebrae from different positions in the vertebral column of tope sharks. In this study, however, all *centra* were collected from the same region of the vertebral column. According to those authors, such methodological standardization should be enough to guarantee that this type of bias does not significantly affect the ring counts.

As to bias source (4), MOULTON et al. (1992), WALKER et al. (1995) and OFFICER et al. (1996) used staining techniques on tope shark vertebrae. However, the same authors point out that the

structural complexity and peculiar calcification pattern of tope shark hard parts may significantly affect staining, particularly if vertebrae from larger-sized specimens are used. Such techniques should be used mainly with smaller tope (<120 cm TL: FRANCIS & MULLIGAN 1998), which possess wider-spaced and thus more easily discernable vertebral rings. Otherwise, staining methods might be responsible for ring count underestimation in adult specimens, and the use of modern technology such as X-ray microradiography or ion spectrometry is then recommended (FERREIRA & VOOREN 1991; WALKER et al. 1995; OFFICER et al. 1996).

Although staining is generally inefficient for large tope sharks, no alternative and more robust methodology was available for this experiment. Therefore, the purpose of this study was to test between staining techniques only, and to choose the most efficient one based on comparative analysis of count consistency (precision). Moreover, precision indices showed that the discrepancy between replicates has been fairly low for all the techniques, as in no case has the 10% error limit – established by POWERS (1983) as the maximum acceptable for stock assessment purposes – been exceeded in any size-class.

Indices of precision are becoming widely used in many elasmobranch age determination studies. Some authors, however, have different opinions as to the reliability of such indices. Both BEAMISH & FOURNIER (1981) and CHANG (1982) consider the PA index to be statistically unsuitable, as it does not incorporate fish size, proportional to the number of rings. These authors suggest that indices such as APE, V and D, are much more reliable and size-dependent estimators of precision. Most recently, HOENIG et al. (1995) have demonstrated that results obtained by such indices might easily be obscured by size group-related variability, suggesting that precision analysis should be conducted separately by size-groups, thus ensuring homogeneity within each group. Such criterion not only has been met here, but also has been taken in account when choosing between the staining techniques tested: cobalt nitrate provided more consistent ring counts, independently of using separate or combined size-classes.

HOENIG et al. (1995) further suggested that tests of symmetry in contingency tables, based on χ^2 statistical analysis, provide a much more comprehensive and robust approach in determining count consistency, particularly in cases where multiple variables are being considered at the same time (e.g. two readers performing ring counts to decide upon two different skeletal structures). Such symmetry tests were not used in this experiment due to the fact that only one variable – the staining technique – was tested.

In conclusion, it has been demonstrated by the results of this experiment that the cobalt nitrate is the most efficient staining technique for enhancing growth rings in tope sharks vertebrae, when compared to alizarin red and silver nitrate staining. In addition to its higher optical resolution of growth rings, the cobalt nitrate staining also provides simplicity, versatility, speed and cost-effectiveness as major advantages. Despite their successful application in several other elasmobranch species, neither the alizarin red nor the silver nitrate staining produced results as consistent with the tope shark as did the cobalt nitrate. The former technique provided acceptable differentiation of growth patterns, it was highly time-consuming; and the latter resulted in lower optic resolution between the outer narrow-spaced growth rings, mostly due to accumulation of silver grains on the *corpus calcareum* surface (BROWN & GRUBER 1988). Furthermore, silver nitrate staining is considerably more expensive, depending on a UV-light source to enable the observation of silver-impregnated rings.

The quantitative analysis, using precision indices, was a natural reflection of the empirical results. Due to clearer rings, this method resulted in more consistent counts of growth rings, therefore being considered the most precise among the techniques tested.

It should be emphasized, however, that these results should be considered preliminary. Although the cobalt nitrate staining was successful achieved with by HOENIG & BROWN (1988) for several different species of elasmobranchs, the results obtained herein refer to a variation of the original protocol, which has not yet been test in any other elasmobranch

species. Further experimentation with this modified technique is therefore recommended, in order to extend and refine its applicability to a wider array of species.

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APPENDIX

Table I

Modified protocol of the cobalt nitrate staining technique, proposed by the authors, based on the original methodology by HOENIG & BROWN (1988). Procedures and timings were defined solely upon vertebrae of tope shark, *Galeorhinus galeus*.

Phase	Procedures	Timing
1. Immersion in 5% cobalt nitrate	• Agitate gently, until concentric rings become contrasted and pinkish coloured) • Wash in distilled water to stop reaction (agitate vigorously) • Dry with clean paper cloth	1-15 min., depending on *centrum* size
2. Immersion in 10% sodium hypocloride (chlorine bleach)	• Agitate gently, until rings are dark and contrasted (critical phase, due to the instantaneous bleach reaction; prolonging the bleach immersion will result in irreversible darkening of the entire *corpus calcareum*) • Wash in distilled water to stop reaction and prevent further darkening of *centrum* (agitate vigorously)	3-30 sec., depending on *centrum* size and darkening speed
3. Storage in 70% ethanol	• Store whole *centrum*	

Table II

Vertebral growth ring counts using different staining techniques of tope shark *Galeorhinus galeus*. Number of specimen within each size-class (S=Small, M=Medium, L=Large) and individual size (TL) and sex is shown.

Size-Class	TL (cm)	Sex	Technique / Replicate reading #								
			Alizarin Red			Silver Nitrate			Cobalt Nitrate		
			1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
S1	62	F	3	3	3	3	3	4	3	3	3
S2	73	M	3	3	3	3	3	3	3	3	3
S3	54	M	2	2	2	2	2	2	2	2	2
S4	62	M	3	3	3	3	3	3	3	3	3
S5	67	F	3	4	3	3	3	3	3	4	4
S6	69	M	4	4	3	4	3	3	4	4	4
S7	73	M	4	3	4	3	4	3	4	3	4
S8	69	M	3	3	4	4	3	3	3	3	3
S9	56	M	2	3	2	3	2	3	2	2	2
S10	63	F	3	3	3	3	3	3	3	3	3
M1	90	F	6	5	6	6	6	5	6	5	5
M2	109	F	7	6	6	7	6	6	5	6	6
M3	113	F	8	7	7	8	7	6	6	7	7
M4	81	M	5	6	5	5	4	5	5	5	5
M5	90	M	6	5	6	6	6	5	6	5	6
M6	92	F	5	6	5	5	6	6	5	6	6
M7	98	F	5	6	6	6	7	6	6	6	6
M8	100	F	6	6	6	6	7	5	6	6	6
M9	102	M	6	6	6	6	5	6	6	6	6
M10	113	F	7	6	7	7	8	7	7	6	7
L1	131	M	9	7	8	9	8	9	8	8	9
L2	147	M	10	10	11	10	9	11	10	10	10
L3	122	M	7	8	9	7	7	8	7	7	8
L4	125	M	9	9	8	8	7	9	8	7	8
L5	136	F	9	9	10	8	9	9	8	9	9
L6	138	F	8	9	10	9	9	8	9	9	9
L7	154	M	10	10	11	11	10	10	10	11	11
L8	146	M	11	11	11	10	11	11	10	10	11
L9	150	M	10	11	10	11	11	11	11	11	11
L10	141	F	9	8	9	9	8	10	9	9	10