Sources of variation in counts of growth increments in vertebrae from gummy shark, *Mustelus antarcticus*, and school shark, *Galeorhinus galeus*: implications for age determination

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Abstract: Sources of variation in counts of vertebral growth increments from gummy shark, *Mustelus antarcticus*, and school shark, *Galeorhinus galeus*, were examined in vertebrae taken from the cervical, thoracic, and precaudal regions of the vertebral columns. Vertebrae from school shark were more difficult to read than those from gummy shark. The number of increments an experienced reader counted on the external surface of alizarin-stained whole vertebral centra was consistent with the number of major hypermineralized increments counted on microradiographs of vertebral sections. Differences between increment counts obtained by four different readers were significant. Increment counts by experienced readers were more precise and less biased between repeated readings. Increment counts from vertebrae sampled in the same region of the vertebral columns were not significantly different. Increment counts from vertebrae sampled in different regions were significantly different and were highest for vertebrae from the thoracic region. Differences in increment counts between regions of the vertebral column may distort von Bertalanffy growth curves and therefore affect the reliability of fisheries stock assessments based on age-structured population models.

Résumé : Les sources de variation du nombre de zones de croissance sur les vertèbres de l'émissole gommée (*Mustelus antarcticus*) et du requin-hâ (*Galeorhinus galeus*) ont été examinées sur des vertèbres prélevées dans les régions cervicale, thoracique et pré-caudale de la colonne vertébrale. Les vertèbres du requin-hâ étaient plus difficiles à lire que celles de l'émissole gommée. Le nombre de zones de croissance comptées par un lecteur expérimenté sur la surface externe des centres vertébraux complets colorés à l'alizarine correspondait au nombre de zones de croissance hyperminéralisées bien définies dénombrées sur des microradiographies de coupes de vertèbres. Les écarts entre le nombre de zones de croissance obtenu par quatre lecteurs étaient importants. Les nombres obtenus par les lecteurs expérimentés étaient plus précis et les lectures répétées moins biaisées. Le nombre de zones de croissance sur les vertèbres prélevées dans la même région de la colonne vertébrale ne différait pas de façon significative. Par contre, dans le cas de vertèbres provenant de régions différentes, il variait considérablement et était plus élevé sur les vertèbres de la région thoracique. Des écarts au niveau du nombre de zones de croissance entre les différentes régions de la colonne vertébrale peuvent déformer les courbes de croissance de von Bertalanffy et donc affecter la fiabilité des évaluations des stocks de poissons fondées sur des modèles de population établis selon la structure par âge.
[Traduit par la Rédaction]

Introduction

Gummy shark, *Mustelus antarcticus* Günther, and school shark, *Galeorhinus galeus* (Linnaeus), off southern Australia provide most of the catch in a shark fishery that requires careful monitoring and reliable stock assessments (Walker 1983, 1992, 1993). As part of a stock assessment program for the fishery, Moulton et al. (1992) attempted to verify, but did not validate, ages estimated from increment counts of whole vertebrae stained with alizarin red S solution (Walker 1983). Moulton et al. (1992) compared von Bertalanffy growth curves derived from length-at-age data with those derived from length-increment data available from tag release–recapture studies and concluded that although the ages estimated for gummy sharks of all lengths and school sharks of less than 1300 mm total length were reliable, those for school sharks longer than 1300 mm were underestimates. Moulton et al. (1992) also suggested that the microradiographic method (where increment patterns are interpreted on X-radiographs of sectioned vertebrae) (Cailliet et al. 1983; Ferreira and Vooren 1991; Kusher et al. 1992) might yield more reliable increment counts for larger sharks.

Whichever method is used, the results need to be validated

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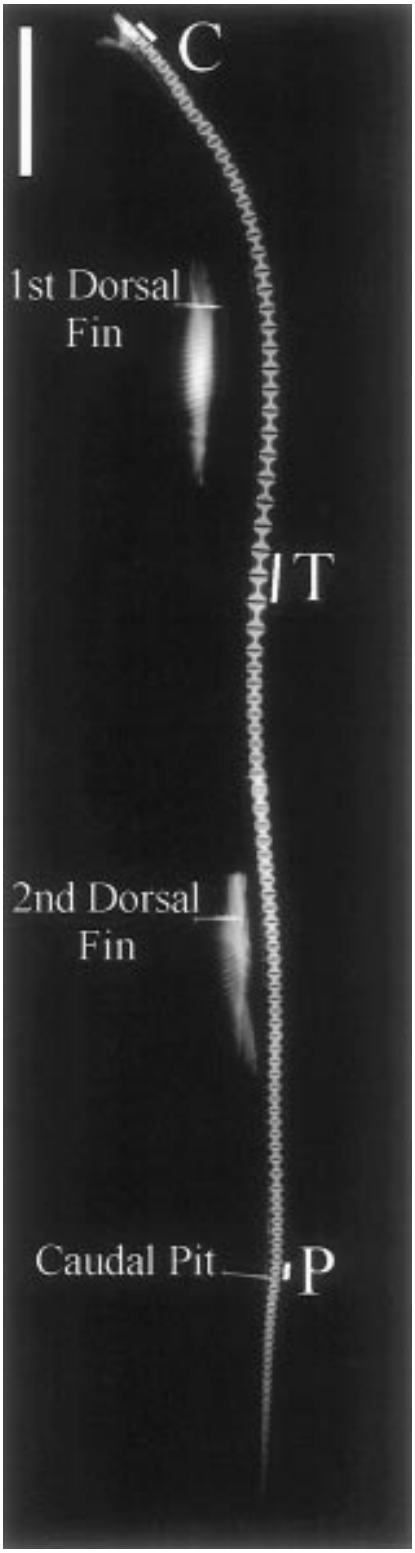
Fig. 1. Radiograph of the entire vertebral column from a male gummy shark 625 mm in total length. The locations of the origins of the first and second dorsal fins and the caudal pit were marked with steel pins before radiography. The three regions from which two vertebrae were sampled are labelled C (cervical), T (thoracic), and P (precaudal). Scale bar = 50 mm.



or at least verified (Cailliet 1990; Beamish 1992). Such validation requires an appreciation of the variability inherent in each method. Both the microradiographic and the alizarin staining method involve the counting of growth increments in vertebrae. However, in elasmobranchs not all of the vertebrae in a vertebral column have the same number of growth increments (Brown and Gruber 1988; Natanson and Cailliet 1990). Such differences in increment counts may result from biological differences in vertebral development or simply from the differing resolution of the methods used to examine increments.

The study we describe here was designed to assess how much the variation in increment counts from gummy and school shark vertebrae depends on (*i*) the method used to count growth increments, (*ii*) the effect of within-reader variability on precision and bias in repeated increment counts derived for the staining and microradiographic methods, (*iii*) the effect of between-reader variability on precision and bias in the staining method, (*iv*) the variation in increment counts from vertebrae taken within the same region of the vertebral column, and (*v*) the variation in increment counts between vertebrae taken from different regions of the vertebral column. Knowledge of the significant sources of variation in increment counts is valuable because it may indicate which sources of variation could be minimized or eliminated, and thereby improve the precision of age determinations based on increment counts.

Materials and methods

Collection of sharks

Gummy shark and school shark were collected during January–July 1992 from the catches of commercial fishers. Most of the sharks were caught in gill nets of 6-inch (1 inch = 25.4 mm) mesh size in Bass Strait, near the coast of Victoria, Australia; some small sharks were caught on long lines and by otter board trawlers in Port Phillip Bay, Australia. We collected two sharks within each of three length-classes (<900, 900–1200, and ≥1200 mm total length) for each species and sex (i.e., 2 species × 2 sexes × 3 length-classes × 2 sharks = 24 sharks). The total length (TL) of each shark was measured to the nearest millimetre after the tail had been allowed to take a natural position and after the top caudal lobe had been placed parallel to the body axis. Whole carcasses were stored at –20°C until processed in the laboratory.

Selection and preparation of vertebrae

The shark carcasses were thawed and filleted such that the cranium, dorsal fins, and tail remained attached to the vertebral column. Steel pins were inserted at the anterior origins of the first and second dorsal fins, and at the precaudal pit (Fig. 1) so that when the vertebral columns were radiographed the positions of external anatomical features could be related to the region of the vertebral column beneath them. For radiography we used an Ultrays full-wave rectified radiation generator (Watson & Sons (Electro-Medical) Ltd., U.K.) and a Machlett Dynamax 54 X-ray tube (Machlett Laboratories Inc., U.S.A.) with a 1.0-mm focal spot; the focus to film distance was 200 cm. The vertebral columns were placed in a plastic dish containing water to a depth of 3 cm to provide iso-density to the exposure. This dish was placed on a film cassette fitted with one fine-detail intensifying screen and containing Ortho G film (Agfa-Gevaert N.V., Belgium). The film was exposed at 50 kV and 150 mA for 1.5 s, and developed according to the manufacturer's instructions.

Three regions of the vertebral columns, each with two vertebrae, were clearly delineated by images of the steel pins (Fig. 1). The first two postcranial vertebrae were designated as the cervical region,

which is routinely sampled from commercial catches. The 12th and 13th vertebrae posterior to the origin of the first dorsal fin (the 32nd and 33rd vertebrae in gummy shark, the 38th and 39th vertebrae in school shark) were designated the thoracic region, and included some of the largest vertebrae in the vertebral columns. The two vertebrae anterior to the caudal pit (the 82nd and 83rd vertebrae in both species) were designated the precaudal region. The precaudal area was selected for analysis because this region is an alternative region for routine vertebral sampling from commercial catches, since removal of the tail does not damage the carcass and hence maintains the carcass's higher commercial value. Hence, 6 vertebrae were taken from identical locations in each of the 24 vertebral columns, providing a total of 144 vertebrae for analysis (i.e., 2 species × 2 sexes × 2 sharks × 3 length-classes × 3 regions × 2 vertebrae = 144 vertebrae).

Each vertebra was separated and trimmed of connective tissue, including the haemal and neural arches, and then cleaned of remaining soft tissue by immersion in sodium hypochlorite solution (Aquachlor liquid pool chlorine, Aquaswim P/L, Australia). We used a 1% solution rather than the 4% solution used by Gruber and Stout (1983) to minimize the possibility of overbleaching, which can damage the vertebral centra. The bleaching process was stopped once all the fascia material had been removed and the less-mineralized hyaline cartilage between the lateral and dorso-ventral support struts (intermedialia) had been dissolved. The vertebrae were washed carefully in running tap water to remove traces of bleach and sodium chloride crystals (a breakdown product of the hypochlorite treatment) and allowed to air dry at ambient room temperature. Small centra (3–5 mm diameter) were clean within 30–40 min; larger centra were clean within 1–2 h.

Alizarin staining method

Four readers were engaged to read the alizarin-stained vertebrae. Of the readers, one (A1) had extensive experience, having produced age determinations from several hundred gummy and school shark vertebrae (Moulton et al. 1992). A second reader (A2) had limited experience in reading alizarin-stained shark vertebrae, a third reader (A3) had extensive experience reading otoliths of teleosts, and the fourth reader (A4) had no experience reading alizarin-stained shark vertebrae or otoliths. Prior to beginning the experiment the readers were briefed on the characteristics used to classify the readability and count the increments on a set of vertebrae from sharks representing all length-classes in the population.

The 144 vertebrae were read on two occasions (round 1 and round 2) by each of the four readers. During round 1 each of the four readers took, at random, one vertebra and stained it by immersing it for 1–2 min in a solution of alizarin red S (Sigma-Aldrich P/L, Australia) prepared from a concentrated aqueous solution of alizarin red S and a 0.1% aqueous solution of potassium hydroxide (1:9) (LaMarca 1966; Gruber and Stout 1983). A view of an alizarin-stained vertebra is shown in Fig. 2A. Each reader then viewed the stained vertebra under a stereomicroscope (6.4× to 10× magnification), assigned it an increment count and a subjective readability score (see Table 1), and then passed the vertebra to each of the other three readers who independently assigned an increment count and readability score to the vertebra. To complete round 1 this cycling procedure was repeated until, after 36 cycles of 4 vertebrae, all 144 vertebrae had been examined by each of the four readers. All possible orders of passing the vertebrae between the four readers were used so that reader precision and bias were affected as little as possible by the stain drying and degrading during a cycle.

At the end of round 1, stained vertebrae were rinsed in tap water in preparation for round 2 in which vertebrae were passed among readers in the same order as that adopted in round 1. Neither the readers nor the person administering the transfer of vertebrae had any prior knowledge of the species, sex, length-class, or region of the vertebral column from which the vertebrae under examination were taken. Hence, the readings were undertaken according to the principles of a double blind study.

Table 1. Description of readability scores assigned to readings of vertebrae.

Readability score	Description
1	Increment count unambiguous with exceptionally clear increments
2	Increment count unambiguous but increments of diminished clarity
3	Two increment counts possible but indicated increment count is most likely
4	More than two interpretations possible; increment count is best estimate
5	No increment count possible; specimen abnormal or otherwise unreadable
6	Specimen missing or broken

Microradiographic method

To allow for comparison between reading methods, 72 vertebrae used during the staining method were prepared for analysis by the microradiographic method. These vertebrae included two vertebrae from each of the three designated regions of the vertebral columns of 12 sharks (i.e., 2 species × 2 sexes × 1 shark × 3 length-classes × 3 regions × 2 vertebrae = 72 vertebrae).

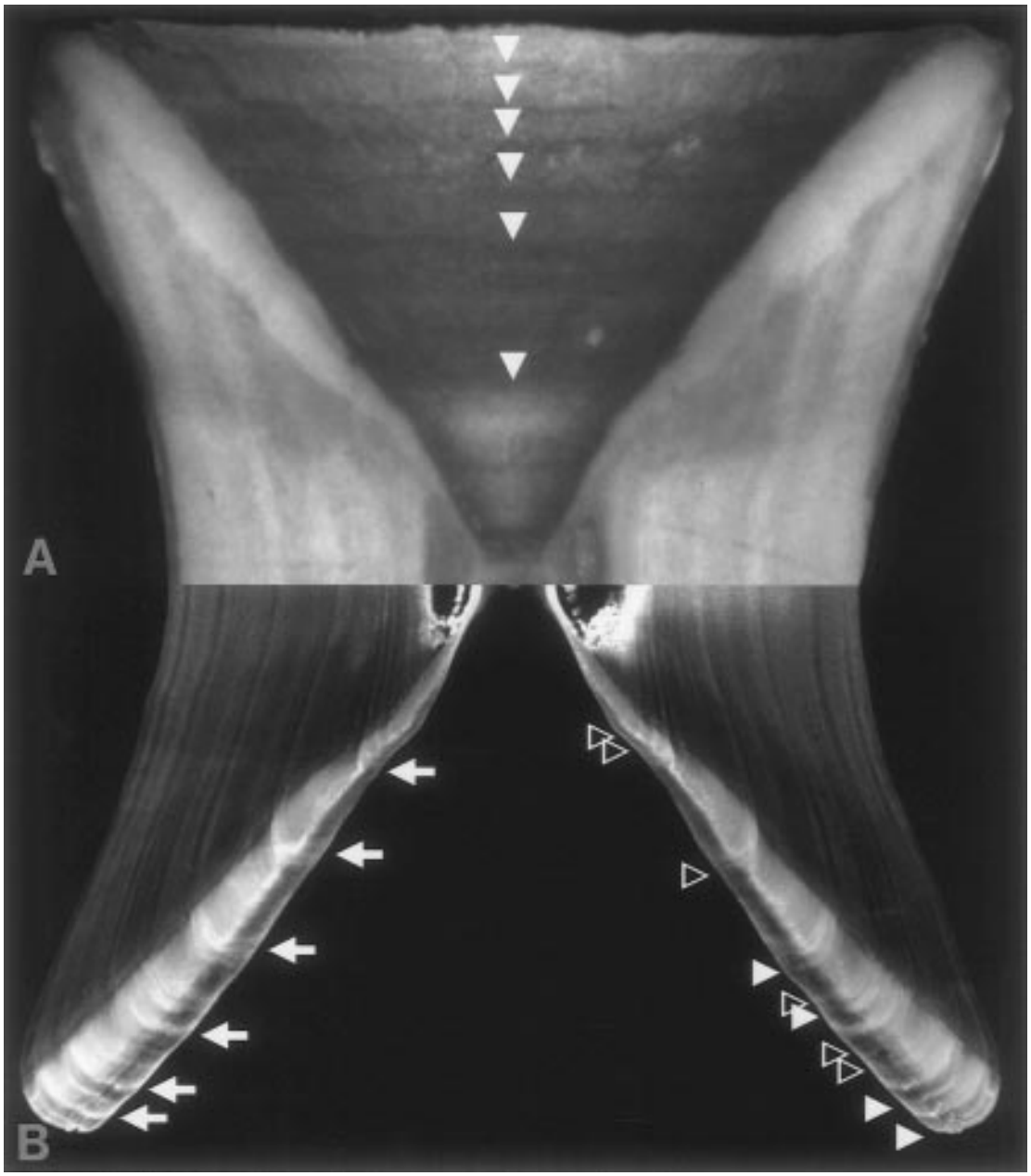
The vertebrae were dehydrated with acetone (LR grade) and embedded in polyester resin (Servic clear embedding resin, RF Services P/L, Australia). Embedded centra were mounted in a Leitz 1600 sawing microtome (Leica Instruments P/L, Australia) and 100-μm longitudinal sections were sawn through the focus, so as to include both lateral intermedialia. The most central section from each vertebra was radiographed (Faxitron Series X-ray system, model No. 43805N, Hewlett-Packard Co., U.S.A.). Sections were placed on top of a light-safe bag containing Industrex SR film (Eastman Kodak Co., U.S.A.) The film was positioned 48 cm from the focus, exposed at 20 kV and 2 mA for 1 min and 55 s, and then developed with standard radiograph development chemicals and procedures.

Each microradiograph was scanned at high resolution and its image was stored on a computer file. An interactive subprogram was custom written for the Optimas 3.10 image analysis program (BioScan Inc., U.S.A.) to allow an increment count to be assigned to the scanned image of each microradiograph. One reader experienced in reading microradiographs of vertebral centra (but inexperienced in reading alizarin-stained vertebrae or otoliths) read the scanned images of microradiographs in a random order as a double blind study. The reader counted all increments seen adjacent to the articular face and characterized these increments as belonging to one of three categories: major increments (well-defined, hypermineralized growth increments), uncertain increments (hypermineralized growth increments with an irregular spacing), or minor increments (fine check marks). The characteristics of each type of increment are shown in Fig. 2B. For each vertebra, increment counts were assigned for three types of increments: type M1 included major increments only; type M2 included major and uncertain increments; and type M3 included major, uncertain, and minor increments. Each vertebra was assigned a readability score in the same way as were vertebrae read by the staining method. The microradiographs of each vertebral section were read a second time in the same random order as that used in the first round.

Data analysis

Variability in increment counts was assessed from the results of six statistical analyses: three involved pairwise comparisons to assess within-reader variability for the staining method and for each of the three increment types for the microradiographic method; two

Fig. 2. Growth increments on and within a thoracic vertebra from a male gummy shark 1115 mm in total length. (A) Alizarin-stained preparation showing stained increments on the articular face (arrowheads). In the corresponding microradiographic preparation (B) from the same vertebra the classification of increments is shown. Arrows indicate well-defined hypermineralized growth increments that were defined in the present study as major (M1). Hypermineralized increments with an irregular spacing were classified as uncertain (M2) (solid arrowheads) and fine hypermineralized growth increments were classified as minor (M3) (open arrowheads).



involved pairwise comparisons to assess between-reader variability for the staining method; and one involved a nested analysis of variance (ANOVA) to assess effects of sex and length of shark, position of vertebrae within a region of the vertebral column, region of vertebral column, and reading method on variability in increment counts. The analyses for within-reader variability involved a test for within-reader bias (analysis 1) and tests for within-reader precision (analyses 2 and 3). The two analyses for between-reader variability involved tests for between-reader bias (analysis 4) and tests for between-reader precision (analysis 5) using the readings of the most experienced reader as a standard. These analyses, as well as the ANOVA (analysis 6), were undertaken for gummy and school shark separately. Before undertaking the analyses, the increment counts were evaluated according to their readability score.

Readability

Readings having high readability scores did not yield an increment count and were therefore excluded from statistical tests. The percentage of readings given each readability score was calculated for each staining method reader and each type of increment count from the microradiographic method. Readings assigned high readability scores were rejected from subsequent analyses when data from each reader were sufficient to allow comparison of increment counts between rounds 1 and 2.

Similarly, only increment counts having acceptable readability scores from round 1 were used to compare increment counts from the three regions of the vertebral column. Hence, various separate selections of increment counts with acceptable readability scores had to be made for gummy and school shark separately when we examined variability in increment counts within reader, between readers, within region and between regions of the column, and between the staining and microradiographic methods.

Within-reader bias (analysis 1)

To compare the mean and standard deviation of the differences in increment counts between rounds 1 and 2 for each staining method reader and for each of the three microradiographic increment types we used pairwise *t* tests to test the following null hypothesis:

$$H_0: \quad \frac{(Rd_1 - Rd_2)}{(Rd_1 + Rd_2)/2} = 0$$

where Rd_1 and Rd_2 are the increment counts obtained for a vertebra by a reader during rounds 1 and 2, respectively. The differences in increment counts between rounds 1 and 2 were adjusted by dividing by the mean increment count for the two rounds to avoid distortion caused by high increment counts in either round.

The mean and standard deviation of the differences between rounds 1 and 2 were calculated to indicate any positive or negative trends in the differences between rounds. Within-reader bias was also assessed by calculating the number and percentage of readings that were in agreement between rounds 1 and 2 within zero, one, two, or more than two increments.

Within-reader precision; index of average percent error (analysis 2)

The index of average percent error (IAPE) (Beamish and Fournier 1981) was used in the first of two analyses of within-reader precision. The IAPE was used because it provided estimates of precision that were not independent of the increment counts obtained for each reader. IAPE values may also be compared between studies. The IAPE values calculated in this experiment were equivalent to those calculated using Chang's (1982) measure of precision (*D*) because only two readings were compared. Therefore, only IAPE values are quoted. Sets of readings with the smallest IAPE indicate the greatest precision. Calculations of IAPE did not include the increment counts of zero value from vertebrae with acceptable readability scores because such values can distort the IAPE.

Within-reader precision; sign rank test (analysis 3)

For the second analysis of the within-reader precision in increment counts between rounds 1 and 2 for each of the four staining method readers and for each of the three microradiographic increment types we used a sign rank test that tested the following null hypothesis:

$$H_0: \quad |Rd_1 - Rd_2| = 0$$

where Rd_1 and Rd_2 are the increment counts obtained for a vertebra by a reader during rounds 1 and 2, respectively.

A sign rank test was used because the absolute differences between pairs of increment counts did not have a normal distribution. The mean and standard deviation of the differences between rounds 1 and 2 were calculated to indicate the magnitude of any differences between rounds.

Between-reader bias (analysis 4)

To test the bias in increment counts between pairs of staining method readers we used pairwise *t* tests to test the following null hypothesis:

$$H_0: \quad \frac{Rr_i - Rr_j}{(Rr_i + Rr_j)/2} = 0$$

where Rr_i and Rr_j are the increment counts obtained for a vertebra by the *i*th and *j*th readers during round 1. To avoid distortion caused by high increment counts the differences in increment counts between each pair of readers were adjusted by dividing by the mean increment counts for the two readers.

The mean and standard deviation of the differences were calculated for each pair of readers to indicate any positive or negative trends in the differences between readers.

Between-reader precision (analysis 5)

A sign rank test was used to test precision in increment counts between pairs of staining method readers. The differences in increment counts between a pair of readers during round 1 were adjusted by dividing by the mean increment count for the two readers. Thus, distortion caused by high increment counts was avoided in this test. The null hypothesis for this test was

$$H_0: \quad \frac{|Rr_i - Rr_j|}{(Rr_i + Rr_j)/2} = 0$$

where Rr_i and Rr_j are the increment counts obtained for a vertebra by the *i*th and *j*th readers during round 1. The mean and standard deviation of the differences were calculated for each pair of readers to indicate the magnitude of any differences between readers.

Analysis of variance in increment counts owing to other sources of variation (analysis 6)

We used a nested-design ANOVA to assess variation in increment counts between the staining and microradiographic methods and within and between regions of the vertebral column. Results from readers whose increment counts showed significant bias or poor precision between rounds 1 and 2, or between readers, were excluded from the analysis of differences in increment counts between reading methods and between regions of the vertebral column. In our analysis of increment counts from the three microradiographic increment types M1, M2, and M3 we treated each type as a separate reading method, because each represents a different way of estimating an increment count from a vertebra sectioned for the microradiographic method. The primary model statement used for this analysis tested for the effects of sex, length-class, position of the vertebra within a region of the vertebral column, region within the vertebral column, reading method, and interactions between these main effects.

Variation owing to each effect was tested by using the error term appropriate to each analysis. Where increment counts were significantly different owing to interactions between length-class or sex with reading method or region, the ANOVA was performed separately for appropriate subgroups of the data.

Tukey's studentized range test of comparison of means was used to compare increment counts obtained by each reading method and in each region. These planned comparisons were made to determine which microradiographic increment type yielded the most similar increment count to those obtained using the staining method and to determine which regions yielded the highest and lowest increment counts.

Variation between increment counts for vertebrae from the three regions of the vertebral column was also assessed by calculating the adjusted mean difference (given by the equation below) and standard deviation of increment counts for all vertebrae within each region and comparing that to the mean for all regions combined:

$$\text{adjusted mean difference} = \sum_{i=1}^{n} \left(\frac{\overline{Sk_i} - \overline{Rg_i}}{\overline{Sk_i}} \right) \Big/ n$$

where Sk is shark, Rg is region, *i* is the *i*th shark or region, and *n* is the number of sharks in which at least one vertebra from each region was used.

To indicate the relative readability of vertebrae selected from each region, we calculated the percentage of vertebrae used from those available in each region.

Table 2. Percentage of vertebrae showing differences in readability score for each reading method between readings made in rounds 1 and 2 (comparison A) and between round 1 readings of vertebrae 1 and 2 within a region (comparison B).

Comparison	Difference in readability score	Percentage of vertebrae differing in readability score	
		Alizarin staining method	Microradiographic method
A[a]	0	54	79
	1	42	20
	2	4	1
B[b]	0	37	62
	1	46	29
	2	17	9

Note: Data for the alizarin staining method were calculated from readability scores of only the most experienced reader (A1).

[a] $n = 71$ for alizarin staining method and 70 for microradiographic method.

[b] $n = 35$ for alizarin staining method and 34 for microradiographic method.

Results

Readability

Even though we reached agreement beforehand on what characteristics were to be used to classify the readability of the vertebrae, post-hoc comparison of readability scores showed that vertebrae assigned a readability score of 4 by reader A2 and the microradiographic reader were generally assigned a score of 3 by the other readers. This suggested that reader A2 and the microradiographic reader were simply taking a conservative approach and that it was legitimate to group their readings assigned a readability score of 4 with those assigned a score of 3 by the other readers.

A similar number of increment counts was provided for analysis by utilizing all readings assigned a readability score less than or equal to 3 for the staining method readers A1, A3, and A4 (76, 81, and 80% of readings, respectively, for gummy shark; 52, 67, and 58% of readings, respectively, for school shark) and readings assigned a readability score less than or equal to 4 for staining method reader A2 and the one reader of the three microradiographic increment types (85 and 83% of readings, respectively, for gummy shark; 61 and 99% of readings, respectively, for school shark). In most cases, reading a vertebra a second time or reading a second vertebra from within the same region of the vertebral column produced a readability score similar to the first readability score (Table 2). The microradiographic method gave more consistent readability scores than did the staining method. The results of analyses 1 to 6 are summarized in Table 3 and discussed in greater detail below.

Within-reader bias (analysis 1)

Staining method reader A1's increment counts in rounds 1 and 2 were not significantly different for either gummy shark ($P = 0.604$) or school shark ($P = 0.215$) (Table 4). However, increment counts by staining method readers A2, A3, and A4 (Table 4) indicated a bias for higher increment counts during round 2 than during round 1.

Although all readers assigned higher increment counts during round 2 than during round 1 (Fig. 3), the bias was least for A1, for whom 41% of gummy shark increment counts (Fig. 3*a*) and 49% of school shark increment counts (Fig. 3*b*) did not differ between rounds 1 and 2. Staining method readers A2, A3, and A4 did show a bias in their increment counts between rounds 1 and 2. The spread of differences in increment counts between rounds 1 and 2 for each of the microradiographic increment types M1, M2, and M3 (Fig. 3*c*) was similar to those for A2, A3, and A4 (Fig. 3*a* and 3*b*) but the spread was greater for school shark (Fig. 3*d*) than for gummy shark (Fig. 3*c*). The microradiographic reader showed no significant bias between rounds 1 and 2 for readings made by any of the three microradiographic increment types (Table 4).

Within-reader precision (analyses 2 and 3)

IAPE scores by staining method reader A1 were relatively low (gummy shark, 8.6%; school shark, 5.0%) (Tables 3 and 4). A1 was marginally less consistent than A3 for gummy shark (8.6 versus 8.1%) but was clearly more consistent for school shark (5.0 versus 10.6%). For gummy shark, IAPE scores by A1 were similar to those for each of the microradiographic increment types M1, M2, and M3 (6.6, 7.2, and 8.0%, respectively). However, for school shark, increment counts made for the three microradiographic increment types (8.9, 8.7, and 6.8%, respectively) were less consistent than the increment counts made by A1 and A2.

The sign rank test indicated that differences between increment counts in rounds 1 and 2 were highly significant for all four staining method readers and for all of the microradiographic increment types (Tables 3 and 4).

Between-reader bias (analysis 4)

For both species, staining method reader A4 tended to assign significantly lower increment counts than did reader A3 (Tables 3 and 5). Reader A3 assigned significantly higher increment counts than A2 for gummy sharks and A2 assigned significantly higher increment counts than A1 for school sharks (Table 5).

Between-reader precision (analysis 5)

The sign rank test for between-reader precision indicated that differences between all paired combinations of staining method readers were highly significant (Tables 3 and 5). However, A1 produced a mean increment count closer to the mean increment counts of A2, A3, and A4 than did A2, A3, or A4 when compared with the mean increment counts of the other three readers combined (Table 5).

Effects of other sources of variation on increment count (analysis 6)

Only the increment counts made by staining method reader A1 were included in the ANOVA. Increment counts by staining method readers A2 and A3 were excluded because of the significant within-reader bias indicated in analysis 1. Increment counts by A4 were also rejected, because of staining method reader A4's within-reader bias (analysis 1) and between-reader bias (analysis 4).

Initial analyses of variance for each species using the primary model statement revealed no significant difference in increment counts attributable to the effect of sex (gummy shark, $r^2 = 0.947$, $F_{[1,8]} = 0.04$, $P = 0.8525$; school shark, $r^2 = 0.937$, $F_{[1,8]} = 0.63$, $P = 0.4490$) or to the effect of position of a vertebra within a region of the vertebral column (gummy

Table 3. Summary of results of statistical tests.

Source of variation in IC[a]	Analysis no. and type	Test used	Alizarin staining method: Reader	Alizarin staining method: Level of significance or IAPE value: Gummy shark	Alizarin staining method: Level of significance or IAPE value: School shark	Microradiographic method: Increment type	Microradiographic method: Level of significance or IAPE value: Gummy shark	Microradiographic method: Level of significance or IAPE value: School shark
(A) Variation in increment counts within reader or increment type (analyses 1–3)								
	1. Bias	Pairwise *t* test	A1	ns	ns	M1	ns	ns
			A2	***	ns	M2	ns	ns
			A3	***	***	M3	ns	ns
			A4	ns	***			
	2. Precision	IAPE	A1	8.6	5.0	M1	6.6	8.9
			A2	9.0	6.1	M2	7.2	8.7
			A3	8.1	10.6	M3	8.0	6.8
			A4	12.5	10.5			
	3. Precision	Sign rank	*** for all readers and increment types for both species					
(B) Variation in increment counts between readers (analyses 4 and 5)								
	4. Bias	Pairwise *t* test	A1–A2	ns	ns			
			A1–A3	ns	ns			
			A2–A3	ns	ns			
			A1–A4	***	***			
			A2–A4	***	***			
			A3–A4	***	***			
	5. Precision	Sign rank	*** between all readers for both species					

(C) Analysis of variance in increment counts owing to other sources of variation (nested ANOVA, analysis 6)

	Level of statistical significance: Gummy shark	Level of statistical significance: School shark
Sx (Sk(Lc×Sx))	ns	ns
Lc (Sk(Lc×Sx))	***	***
Po (Sk×Po(Lc×Sx))	ns	ns
Rg (Sk×Rg(Lc×Sx))	***	***
Rm (Sk×Rm(Lc×Sx))	***	***
Lc×Rg (Sk×Rg(Lc×Sx))	***	ns
Lc×Rm (Sk×Rm(Lc×Sx))	*	ns
Rm×Rg (Sk×Rg×Rm(Lc×Sx))	***	ns

Note: IC, increment count(s); IAPE, index of average percent error; A1, A2, A3, and A4, alizarin readers 1–4; M1, major bands; M2, major and uncertain bands; M3, major, uncertain, and minor bands; Sx, sex; Lc, length-class; Po, position of vertebra within region; Rg, region of the vertebral column; Rm, reading method; ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

[a]Error terms used in ANOVA given in parentheses.

shark, $r^2 = 0.947$, $F_{[1,8]} = 0.57$, $P = 0.4704$; school shark, $r^2 = 0.937$, $F_{[1,8]} = 0.25$, $P = 0.6310$). However, the effects of length-class (gummy shark, $r^2 = 0.947$, $F_{[2,8]} = 16.83$, $P = 0.0014$; school shark, $r^2 = 0.937$, $F_{[2,8]} = 9.90$, $P = 0.0069$), region of the vertebral column, and reading method were statistically significant. The effects of region of the vertebral column and reading method were complicated by significant interaction effects between these two factors and other factors in the primary model (Table 3). Hence, appropriate error terms (given in Table 3) were used in altered nested ANOVA models to analyse the effects of reading method and region of the vertebral column on increment count.

Effects of reading method on variation in increment count

Interactions between reading method and length-class were significant for increment counts from gummy shark but not for those from school shark (Table 3). Therefore, the increment counts for gummy shark were split into the three length-classes and the following ANOVA model statement was applied to increment counts for each length-class separately: increment count = method + region + method × region + position + shark + error.

Increment counts obtained from the microradiographic increment types M2 and M3 are by definition higher than those

Table 4. Results of within-reader variability.

Shark species	Reader or MRM increment type	*n*	Analysis 1: bias Mean ± SD	Analysis 1: bias *P*	Analysis 2: precision (IAPE)	Analysis 3: precision Mean ± SD	Analysis 3: precision Sign rank
			Alizarin staining method				
Gummy	A1	45	0.01±0.04	0.604	8.61	0.80±0.14	<0.01
	A2	54	−0.14±0.03	<0.01	9.04	0.74±0.10	<0.01
	A3	53	−0.12±0.03	<0.01	8.08	0.75±0.12	<0.01
	A4	49	0.06±0.04	0.182	12.51	1.20±0.12	<0.01
School	A1	30	−0.04±0.03	0.215	5.00	0.40±0.11	<0.01
	A2	37	−0.04±0.03	0.216	6.07	0.76±0.13	<0.01
	A3	41	−0.13±0.04	<0.01	10.62	0.90±0.11	<0.01
	A4	29	−0.15±0.05	<0.01	10.45	0.98±0.20	<0.01
			Microradiographic method				
Gummy	M1	28	0.07±0.04	0.096	6.57	0.68±0.16	<0.01
	M2	30	−0.02±0.05	0.616	7.24	0.93±0.24	<0.01
	M3	30	−0.07±0.04	0.110	7.98	1.07±0.18	<0.01
School	M1	35	−0.03±0.04	0.550	8.85	1.06±0.17	<0.01
	M2	35	−0.05±0.05	0.264	8.74	1.20±0.25	<0.01
	M3	35	−0.04±0.04	0.302	6.81	1.17±0.25	<0.01

Note: For analysis 1, the null hypothesis tested was $(Rd_1 - Rd_2)/((Rd_1 + Rd_2)/2) = 0$. For analysis 3, the null hypothesis tested was $Rd_1 - Rd_2 = 0$. Rd, round; IAPE, index of average percent error, SD, standard deviation; Sign rank, probability value returned by sign rank test; A1, A2, A3, and A4, alizarin readers 1–4; M1, major bands; M2, major and uncertain bands; M3, major, uncertain, and minor bands.

obtained from the microradiographic increment type M1. This partly explains the differences detected between increment counts from these reading methods (Table 6). However, increment counts obtained by staining method reader A1 and those obtained from microradiographic increment type M1 were significantly different only for small gummy shark.

Effects of region of vertebral column on increment count

Interactions between region and length-class, and between region and reading method, were significant for gummy shark but not for school shark (Table 3). Therefore, the data presented for school shark (Table 6) are the results of the primary nested ANOVA. Whilst interactions between reading method and region were significant for large gummy shark the analysis was not performed by different methods and regions for each length-class separately because the power of these analyses was insufficient. The overall difference in increment counts owing to region was our primary focus. Therefore, the increment counts for gummy shark were split by length-class and the following ANOVA model statement was applied to each length-class separately: increment count = position + region + position × region + region + shark + error.

The region of the vertebral column had a significant effect on increment counts for large and medium-sized gummy sharks but not for small gummy sharks (Table 6). Where the effects of region of the vertebral column were significant, the increment counts for vertebrae from the thoracic region were always higher than those for vertebrae from the cervical and the precaudal regions. In most cases increment counts for vertebrae from the cervical region were not significantly different from increment counts of vertebrae from the precaudal region. The magnitude of the difference in increment counts of vertebrae from the three regions of the vertebral column (Table 7) was calculated by adjusting the mean difference between the increment counts for each region (adjusted mean difference equation above). The overall mean difference in increment counts of vertebrae from the thoracic region and those from the cervical region was 0.31 higher for gummy shark and 0.37 higher for school shark when the staining method was used, and 0.62 higher for gummy shark and 0.43 higher for school shark when the microradiographic increment type M1 was used.

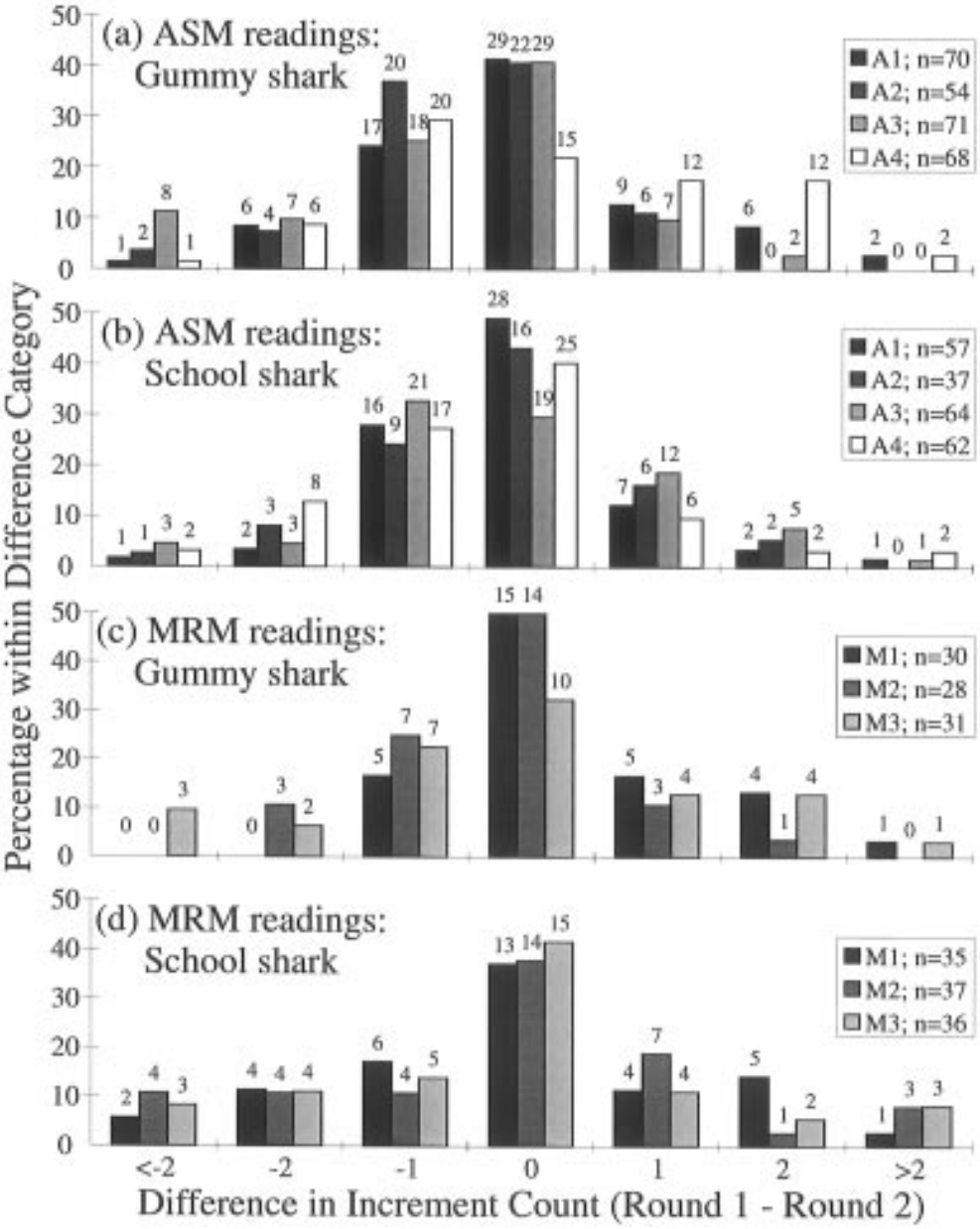
Vertebrae differed in their usefulness according to the method being used. In the microradiographic method more vertebrae from each region gave the more acceptable readability scores and for school shark all microradiographic method readings were useful. Readings obtained from vertebrae in the thoracic region gave a readability score similar to or better than those from the other two regions of the vertebral column.

Discussion

From our results we draw seven conclusions: (*i*) increments on the vertebrae of school shark are more difficult to count than those on the vertebrae of gummy shark, (*ii*) the readability score of a vertebra is no better after repeated reading, (*iii*) the readability scores of vertebrae from the same region of the vertebral column are not significantly different, (*iv*) experienced readers provide more precise and less biased increment counts than do inexperienced readers, (*v*) counts of alizarin-stained and major microradiographic growth increments are similar, (*vi*) the position of a vertebra within a region of a vertebral column has no statistically significant effect on the increment count, and (*vii*) the region of the vertebral column from which vertebrae are sampled has a statistically significant effect on the increment count.

Although our results reveal that difficulties inherent in counting the growth increments on vertebrae from gummy and school shark contribute to the variability of increment counts, they also indicate ways in which several sources of variation in increment counts can be reduced so that increment counts reflect more of the natural variability within a species rather

Fig. 3. Analysis 1: within-reader bias illustrated by the number of readings that were in agreement between rounds 1 and 2 within zero, one, two or greater than two increments. The number of vertebrae within the indicated category is given at the top of each column. ASM, alizarin staining method; MRM, microradiographic method; A1, A2, A3, and A4, alizarin readers 1–4; M1, major bands; M2, major and uncertain bands; M3, major, uncertain, and minor bands; *n, n*umber of vertebrae read.



than the combined effects of this natural variability and the variability from the age determination process itself.

Our results show that the more experienced the reader the higher the overall precision and the lower the biases between repeated increment counts from a vertebra. This result is important because lack of precision and error in age determinations have a strong, age-specific influence on growth curves (Cailliet et al. 1990; Tanaka et al. 1990). It follows that new readers must be trained carefully and their results should be calibrated against those produced by an experienced reader who gives reproducible results. Ideally, vertebrae from sharks of known age should be used for such calibrations. In this way much of the variability resulting from each reader's subjective interpretation of the vertebral growth increments might be removed and the total variation in age determination might become more a function of the inherent variation in the sharks themselves.

Increment counts from vertebrae in different regions of a vertebral column were significantly different. However, increment counts from vertebrae in the same region of a vertebral column were not significantly different. Moreover, when type M1 (major) increments were counted in vertebrae from the same vertebral region of the gummy shark the increment counts obtained were not significantly different from those obtained when type M2 increments (major and uncertain) were counted. This result indicates that the microradiographic

Table 5. Results of analyses of variability in increment count between readers who used the alizarin staining method.

Shark species	$Rr_i - Rr_j$	n	Analysis 4: bias Mean ± SD	P	Analysis 5: precision Mean ± SD	Sign rank
Gummy	A1–A2	53	0.05±0.04	0.205	0.16±0.03	<0.01
	A1–A3	51	–0.05±0.04	0.214	0.16±0.03	<0.01
	A1–A4	47	0.07±0.05	0.127	0.23±0.03	<0.01
	A2–A3	58	–0.12±0.04	<0.01	0.21±0.03	<0.01
	A2–A4	55	–0.02±0.05	0.655	0.25±0.03	<0.01
	A3–A4	52	0.11±0.04	<0.01	0.22±0.03	<0.01
School	A1–A2	30	–0.10±0.05	0.043	0.17±0.04	<0.01
	A1–A3	32	–0.04±0.04	0.350	0.13±0.03	<0.01
	A1–A4	26	0.03±0.05	0.492	0.15±0.04	<0.01
	A2–A3	34	0.04±0.05	0.451	0.18±0.04	<0.01
	A2–A4	32	0.12±0.06	0.057	0.27±0.05	<0.01
	A3–A4	29	0.11±0.05	0.049	0.23±0.04	<0.01

Note: For analysis 4, the null hypothesis tested was $(Rr_i - Rr_j)/((Rr_i + Rr_j)/2) = 0$. For analysis 5, the null hypothesis tested was $Rr_i - Rr_j/((Rr_i + Rr_j)/2) = 0$. Rr, reader; SD, standard deviation; sign rank, probability value returned by sign rank test; A1, A2, A3, and A4, alizarin readers 1–4.

method allows the reader who examines large vertebrae from the thoracic region to more easily identify fine increments and to possibly exclude them as being supernumerary, minor check marks. Because such fine increments are probably not visible on vertebrae stained with alizarin red S, they are probably not counted by readers when the staining method is used.

In a study of angel shark vertebrae, Natanson and Cailliet (1990) concluded that the observed differences in increment counts along the vertebral column were the result of differences in vertebral development and not of the methodology of examination. This conclusion was important because it shifts the focus from finding the best means of displaying increments to determining their chronological meaning. In the present study it was not possible to determine whether the observed differences in increment counts between regions were due to differences in vertebral development or due to the methodology used to display the increments. The examination of vertebral increments from recaptured, tetracycline-injected gummy and school sharks should allow the cause of the differences in increment counts between regions to be ascertained.

The absence of significant differences between increment counts obtained by the most reliable staining method reader and those obtained by the reader of major microradiographic increments (type M1) is evidence that increments counted by the staining method are related to the major hypermineralized increments (type M1) counted by the microradiographic method. The results of other studies have suggested that hypermineralized increments are formed annually in gummy and school sharks (Moulton et al. 1992; Officer 1995). Moulton et al.'s (1992) assumption that the growth increments they counted by the staining method were formed annually was supported by the agreement they found between von Bertalanffy growth curves produced from data on tag length increments and growth curves produced from increment counts derived from the staining method. Officer (1995) found that the vertebral tissues formed during winter in captive, fluorochrome-injected gummy and school sharks were hypermineralized.

However, the von Bertalanffy growth curves constructed by Moulton et al. (1992) for school shark caught in the Southern Ocean off Australia differed greatly from the growth curves produced for school shark caught in the Atlantic Ocean off Brazil (Ferreira and Vooren 1991). Whilst these differences could be partly explained by poor fit (Moreau 1987), Moulton et al. (1992) proposed that the differences between the growth curves were possibly due to real differences in the growth rates of school sharks in the two oceans, or to overestimates of the age of sharks owing to Ferreira and Vooren's (1991) use of the microradiographic method. However, Ferreira and Vooren (1991) used only major hypermineralized increments (type M1) for their age determinations and our results indicate that such increment counts should not differ significantly from the increment counts obtained from the staining method used by Moulton et al. (1992). Consequently, the differences between the growth curves from the two studies cannot be completely explained by an overestimation of age by Ferreira and Vooren (1991).

A more plausible explanation for the difference between the growth curves is the fact that Ferreira and Vooren (1991) used large vertebrae from under the first dorsal fin, whereas Moulton et al. (1992) used cervical vertebrae. In our study the significantly different increment counts obtained from vertebrae from different regions of a vertebral column gave age determinations that, rather than simply increasing the width of confidence intervals, would distort von Bertalanffy growth curves in one direction only, producing differences similar to those between the Brazilian and Australian school shark growth curves. Hence, this example highlights the importance of standardizing the region of vertebral sampling to enable comparison between populations. Reported differences in the parameters defining the von Bertalanffy growth function between other populations and species could well be the product of incompatible regions of the column having been used for vertebral sampling.

Verifying which region of the vertebral column produces the increment counts that best reflect the calendar age of the shark demands an understanding of the temporal periodicity of growth increment deposition (Cailliet et al. 1986; Cailliet 1990). In some species of shark the annual formation of growth increments in the largest vertebrae of the vertebral column has been validated (Smith 1984; Parsons 1993). However, the validation in another study (Brown and Gruber 1988) of a lunar cycle in increment formation illustrates the difficulties in generalizing about the periodicity of increment formation between genera and therefore the need to determine the timing of increment formation for each species. Without this understanding the practice of consistent sampling from both regions of the vertebral column provides the opportunity for future correction of age estimates if increment counts obtained in one region are later found to more accurately reflect the calendar age of the shark.

A lack of data prevented us from assessing the magnitude of differences between increment counts from different regions of a vertebral column, but our results allowed us to calculate differences between the overall means of increment counts from vertebrae in the thoracic region and from vertebrae in the cervical region. If the differences between overall means represent consistent relationships between increment counts obtained from different regions then a correction factor

Table 6. Effects of reading method and region on increment count.

Shark species	Length-class	r^2	F	P	Tukey's test grouping[a]
			Reading method		
Gummy	Large	0.934	$F_{[3,3]} = 93.64$	<0.01	[A1 M1] [M2] [M3]
	Medium	0.895	$F_{[3,3]} = 7.18$	0.069	[A1 M1 M2] [M2 M3]
	Small	0.794	$F_{[3,3]} = 20.52$	0.017	[A1] [M1 M2 M3]
School	All	0.937	$F_{[3,6]} = 5.88$	0.032	[A1 M1 M2] [M2 M3]
			Region		
Gummy	Large	0.934	$F_{[2,6]} = 27.72$	<0.01	[Precaudal Cervical] [Thoracic]
	Medium	0.895	$F_{[2,6]} = 9.97$	0.012	[Precaudal Cervical] [Thoracic]
	Small	0.794	$F_{[2,6]} = 1.49$	0.298	[Precaudal Cervical Thoracic]
School	All	0.937	$F_{[2,11]} = 19.25$	<0.01	[Precaudal Cervical] [Thoracic]

Note: A1, alizarin reader 1; M1, major bands; M2, major and uncertain bands; M3, major, uncertain, and minor bands.

[a]No significant difference was detected between bracketed methods or regions; methods and regions are listed according to the magnitude of their effect on increment count from left (lowest) to right (highest).

Table 7. Adjusted mean differences between mean increment count for each region and mean increment count for all regions.

Shark species	Reader or microradiographic method increment type	Region	n	Percentage used	Adjusted difference (mean ± SD)
Gummy	A1	Cervical	22	61	0.07±0.12
		Thoracic	12	75	–0.24±0.12
		Precaudal	22	58	0.17±0.14
	M1	Cervical	14	100	0.15±0.14
		Thoracic	16	73	–0.47±0.29
		Precaudal	16	73	0.32±0.18
	M2	Cervical	14	100	0.22±0.13
		Thoracic	16	67	–0.49±0.27
		Precaudal	16	73	0.26±0.21
	M3	Cervical	14	100	0.30±0.12
		Thoracic	16	67	–0.70±0.28
		Precaudal	16	73	0.40±0.24
School	A1	Cervical	6	50	0.12±0.18
		Thoracic	8	80	–0.25±0.18
		Precaudal	10	26	0.13±0.19
	M1	Cervical	23	100	0.17±0.15
		Thoracic	24	100	–0.26±0.16
		Precaudal	24	100	0.10±0.14
	M2	Cervical	23	100	0.16±0.12
		Thoracic	24	100	–0.26±0.16
		Precaudal	24	100	0.10±0.12
	M3	Cervical	23	100	0.20±0.22
		Thoracic	24	100	–0.42±0.36
		Precaudal	24	100	0.22±0.19

Note: SD, standard deviation; A1, alizarin reader; M1, major bands; M2, major and uncertain bands; M3, major, uncertain, and minor bands.

could be applied to the age determinations obtained in different regions of the vertebral column. Application of such a correction factor could well resolve the apparent differences in the growth curves of discrete populations in which ages were determined on the basis of increment counts from vertebrae in different regions of the vertebral column.

Validation of age estimates is required to determine which region of the vertebral column yields the increment counts that best reflect the real age of the sharks. Achieving this validation is important because the use of higher age determinations obtained from thoracic vertebrae, rather than age determinations derived from cervical vertebrae, might have important consequences for the management of Australia's southern shark fishery. Ages determined from vertebrae in the cervical region are presently being used in age-structured population models applied in the management of the shark fishery of southern Australia (Walker 1994*a*, 1994*b*). However, these models may be overestimating length-at-age and hence the productivity of the species, if increment counts from thoracic vertebrae better reflect the calendar age of these sharks. Moreover, because fishing gear used in the fishery is length selective (Kirkwood and Walker 1986), an overestimate of length at specific ages would lead to underestimates of the period for which sharks are vulnerable to capture.

The presence of these possibilities is a cause for concern. If age estimates derived from cervical vertebrae underestimate the age of these sharks then gummy and school shark populations may be under more extreme pressure than is currently thought. Ascertaining which region of the vertebral column contains the vertebrae whose increment counts provide the most accurate age estimates should be a priority of future research.

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