Southern Shark Age Validation:
Part 1 - Project Overview, Vertebral Structure and Formation of Growth-Increment Bands Used for Age Determination

Final Report to
Fisheries Research and Development Corporation
(FRDC Project 91/037)

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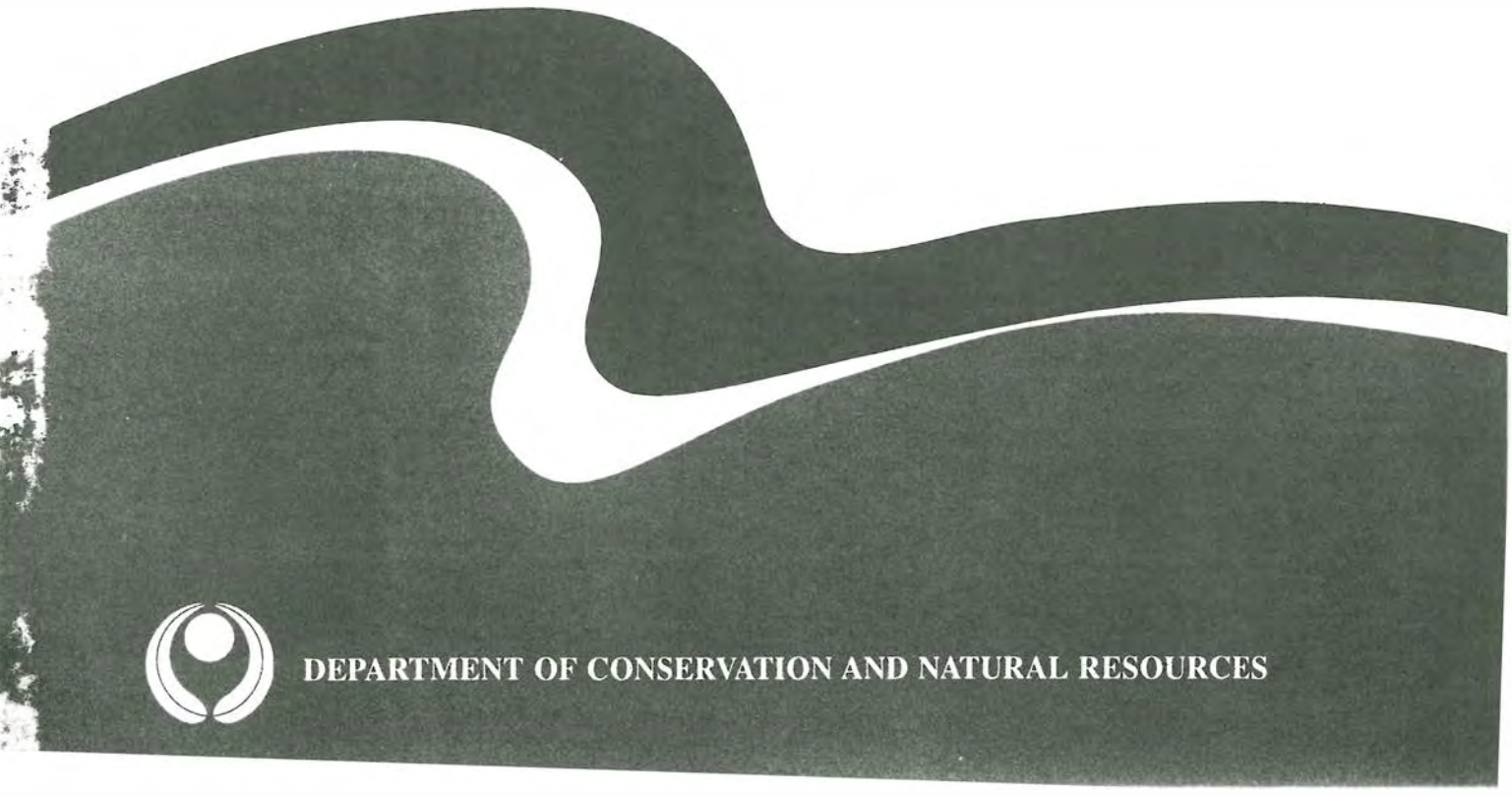

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Non Technical Summary

- Gummy sharks and school sharks are aged by counting growth-increment bands on the articular faces of vertebrae stained with alizarin red. The age data are required for application of age-structured fishery assessment models used by the Southern Shark Fishery Assessment Group for stock advice to the Southern Shark Fishery Management Advisory Committee. The Southern Shark Age Validation Project had five successfully completed tasks and the three following objectives.

- Objective 1 was to validate the assumption that the bands of alizarin red stain are annual and provide reliable estimates of age. This objective could not be met completely by the proposed method of injecting captive sharks with vertebra-marking tissue-dyes. The objective is being further addressed as part of the current FRDC funded Southern Shark Tagging Project.

- Objective 2 was to investigate the feasibility of adopting a microradiographic method as a more accurate and more cost-effective method than the alizarin red staining method. This objective was met completely.

- Objective 3 was to investigate whether growth rates of gummy sharks have changed in response to fishing by testing the hypothesis for the Phenomenon of Apparent Change in Growth Rate. This objective was met completely.

- The project demonstrated that gummy sharks can be held captive in land-based tanks for long periods, that survival rates in captive sharks can be improved with appropriate feeding regimes and treatment with antibiotics, and that growth rates of captive sharks can be increased over those found in the wild. The study also demonstrated that school sharks are difficult to hold in captivity.

- Application of light microscopy, microradiography, electron microscopy and injection of live sharks (held captive or tagged and released in the wild) with vertebra-marking tissue-dyes showed that growth-increment bands stained with alizarin red on the articular face of a whole vertebra centrum and visible on microradiographs of a sectioned centrum arise from patterns of varying mineral density.

- It is the bands of high mineral density that stain with alizarin red on whole vertebrae and give radio-opaque bands in microradiographs of sectioned vertebrae. These bands tend to be deposited during the winter months when overall growth of the sharks is lowest.

- Additional growth-increment bands, 'disturbance check marks', were found to form in captive sharks. These are assumed to be caused by the trauma of initial capture of the shark from the wild and unavoidable handling in captivity.

- Age estimates made from the alizarin staining method are similar to those made from the microradiographic method. Whilst there is no bias between the methods, the microradiographic method gives marginally better precision. Experienced readers using the alizarin red method provide more precise and less biased age estimates than do inexperienced readers.

- Observed differences in von Bertalanffy growth curves for gummy shark between two separate periods and two separate regions have been explained by the Phenomenon of Apparent Change of Growth Rate caused by length-selective fishing mortality. Three pieces of evidence are presented to support this conjecture.

Technical Summary

- A vertebra from a gummy shark or school shark comprises a centrum, haemal arch and neural arch. The centum has two articular cups and four struts (intermedialia) arranged laterally and dorso-ventrally as buttresses between the articular cups. These structures are comprised of heavily mineralized cartilage which provides strength to the vertebra whilst the cavities behind the articular cups and between the struts are filled with non-calcified hyaline cartilage. The vertebra is enclosed, along with the other vertebrae, in a fibrous tissue sheath (perichondrium) which extends the full length of the vertebral column. The perichondrium enables the vertebrae to grow by producing cells (chondroblasts) which mature into cartilage cells (chondrocytes) and secrete extracellular matrix which depending on location on the surface or marginal edge of the vertebra is either mineralized (i.e. becomes cemented) or non-mineralized.

- Application of techniques in light microscopy, microradiography, electron microscopy and injection of vertebra-marking tissue-dyes showed that growth-increment bands visible on microradiographs of sectioned vertebrae (longitudinally through opposite intermedialia) arise from patterns of hyper-mineralized (radio-opaque) and hypo-mineralized (radio-translucent) bands across a section. It was found that growth-increment bands result from changes over time in the ratio of extracellular matrix volume to cell volume. When cells grow or proliferate slowly prior to cementation by the extracellular matrix, the ratio is high and results in a hyper-mineralized growth-increment band. Conversely, when cells grow or proliferate quickly prior to cementation, the ratio is low and results in a hypo-mineralized growth-increment band. Mineralization of cartilage involves deposition of mineral salts, primarily of calcium, in the extracellular matrix.

- Sharks inhabit a calcium-rich environment and unlike terrestrial vertebrates do not have to store calcium for their metabolic requirements. Hence, the growth processes in mineralized tissues of sharks differs from those of other vertebrates by not having to resorb calcium. Once cemented, the mineralized tissue of sharks remains permanently deposited and subsequent growth can only occur by apposition, where new layers of cartilage are deposited over existing cartilage.

- Temporal variation in ambient phosphorus may affect the rates at which sharks can deposit calcium phosphate in the mineralizing cartilages. High availability of calcium phosphate could facilitate rapid mineralization allowing the formation of hyper-mineralized growth-increment bands; whereas low availability of calcium phosphate could retard mineralization allowing formation of hypo-mineralized growth-increment bands.

- Simulated three-dimensional reconstruction of vertebrae by computer-imaging corroborated results from light microscopy, microradiography and electron microscopy that growth was appositional on some parts of the vertebra surface.

- Husbandry methods were developed to hold sharks captive to enable serial injection with fluorescent tissue-dyes which mark vertebrae in the regions of growth at the time of injection to determine rates at which growth-increment bands form. With appropriate feeding regimes and procedures for care, survival rates in a 6.25-m diameter tank (27,000 litres) with flow-through natural seawater (temperature range 10-21°C) were found to be satisfactory. Survival rates were poor in smaller tanks.

- School sharks were more difficult to hold than gummy sharks. School sharks need to continually swim to ventilate their gills, whereas gummy sharks can ventilate their gills while remaining stationary. Large school sharks developed snout damage, leading to necrosis of the snout and eventually death as a result of swimming into the sides of the tank. Hence only school sharks of 520-610 mm total length were introduced into the tank. A total of 53 gummy shark and 31 school shark surviving the first month in captivity were held for varying periods during the 32-month period from August 1992 to March 1995.

- Growth rates of gummy sharks held captive exceeded growth rates of those of sharks in the wild and growth rates slowed during the winter months when water temperatures were lowest.

- Several tissue-dyes (alizarin red, alizarin complexone, calcein, oxytetracycline and xylenol orange) injected into captive and tagged sharks in the wild were found to give distinct fluorescent marks in the vertebrae without affecting the growth rates and health of gummy sharks and school sharks.

- The density of mineralized cartilage formed during winter, when overall growth of gummy shark is slowest, was found to be significantly higher than the density of mineralized cartilage formed during the other seasons.

- Bands of alizarin red on the stained surfaces of the articular faces of whole centra were found to correlate with layers of hyper-mineralized bands deposited during winter in microradiographs of vertebral sections.

- The number of hyper-mineralized bands formed in tagged gummy sharks while at liberty was found to be less than the number formed in sharks held captive for comparable periods. This difference and the positions of the bands indicated that the formation of growth-increment bands resulting from disturbance is not as common in wild sharks as it is in captive sharks. Inevitable formation of 'disturbance check marks' from unavoidable handling of captive sharks reduces the reliability of methods involving captive sharks for determining the periodicity of formation of growth-increment bands.

- Hence given the problem of 'disturbance check marks' detected in gummy sharks and difficulties holding school sharks captive, validation of the assumption that alizarin red stained bands (or hyper-mineralized growth-increments bands in microradiographs) are annual is being further addressed by injecting a large proportion of the sharks tagged and released as part of the current FRDC funded Southern Shark Tagging Project with oxytetracycline. Although available vertebrae from several recaptured tagged sharks injected with oxytetracycline provided evidence that the bands are annual, there is a need to test sharks from a range of sizes and a range of periods at liberty.

- Growth-increment bands in the vertebrae of school shark for both the alizarin staining method and microradiographic method are more difficult to count than those in the vertebrae of gummy shark.

- The readability of a vertebra for both the alizarin red method and the microradiographic method tends to be no better after repeated examination of growth-increment bands.

- The readability of vertebrae from the same region of the vertebral column of a shark for both the alizarin red method and the microradiographic method are not significantly different (i.e. readability is dependent on the shark).

- Experienced readers using the alizarin red method provide more precise and less biased counts of growth-increment bands than do inexperienced readers.

- Counts of vertebral growth-increment bands made by both the alizarin red method and the microradiographic method from the largest vertebrae (i.e. occurring in the region of the vertebral column near the first dorsal fin) are statistically higher than counts in vertebrae from the regions near the head and the tail. The magnitude of the difference in the counts is being determined and whether the difference is large enough to have practical implications for age determination is being addressed separately.

- Vertebral growth-increment bands visible on microradiographs of sectioned vertebrae for the microradiographic method can be classified as minor and major bands.

- Counts of alizarin stained bands on whole vertebrae are similar to counts of major microradiographic bands on sectioned vertebrae. While there is no bias between the alizarin red method and microradiographic method, the microradiographic method gives marginally better precision.

- Overall the alizarin red method gives similar results to the microradiographic method, indicating that there are no benefits in changing from the alizarin red method to the microradiographic method for routine ageing of sharks.

- Three types of evidence are presented to support the hypothesis of the 'Phenomenon of Apparent Change in Growth Rate' caused by length-selective fishing mortality to explain observed differences in von Bertalanffy growth curves published previously for gummy shark between 1973-76 and 1986-87 in Bass Strait and between Bass Strait and South Australia during 1986-87.

  (1) The mean lengths of sharks within each of the recruited ages-classes of 3-7 years are shown to be markedly different between the two periods and two regions, whilst the mean lengths of sharks within the less fully recruited age-class of 2-years are shown not to be different between the two periods and only slightly different between the two regions.

  (2) Avoiding 'back-calculation' of length of shark, Rosa Lee's Phenomenon was detected by directly comparing the radii of growth-increment bands visible on the surfaces of vertebrae from sharks of various ages caught in the two periods and the two regions.

  (3) An appropriate model was developed to simulate the effects of length-selective fishing mortality on the mean length of sharks in the population for ages 2-16 years for a range of levels of hook and gill-net fishing effort, with separate mesh-sizes of 6 and 7 inches for the gill-nets. The simulated changes among the recruited age-classes, although not as large as observed differences in the published growth curves, were generally consistent with the observed trends. The simulations demonstrated how the von Bertalanffy growth parameters L_∞ and t_0 tend to increase and K tends to decrease as gill-net fishing effort increases, and hence explains how these types of biases, commonly reported in the scientific literature for gill-net shark fisheries, can occur.

Introduction

This final report for the FRDC funded Southern Shark Age Validation Project consolidates material presented in the first milestone report (Walker *et al.* 1992) and the second milestone report (Walker *et al.* 1994) as well as presenting material not previously reported. Preparation of this report, along with several other reports and scientific papers, is the result of successful scientific collaboration between the Victorian Fisheries Research Institute (VFRI), the School of Dental Science at the University of Melbourne, and the Zoology Department at the University of Melbourne. This collaboration brought together VFRI facilities for collecting and holding sharks live; VFRI skills in ageing sharks, fisheries population dynamics, statistical analysis and data modelling; and the School of Dental Science facilities and skills in histology (white and UV light microscopy), scanning electron microscopy, microradiography and three-dimensional computer-imaging.

Details of the methods, results and interpretation of the results associated with Tasks 1, 2 and 3 are presented in Appendices 1, 2 and 3, respectively, which appear at the end of this report. Tasks 4 and 5 are referred to briefly in Appendices 4 and 5, respectively, whilst details are printed separately in Part 2 (Officer *et al.* 1995) and Part 3 (Walker *et al.* 1995) of this report as manuscripts prepared to be submitted for publication in the *Canadian Journal of Fisheries and Aquatic Sciences*. Two additional manuscripts resulting from work associated with the project which have been published already in scientific journals are reprinted as Appendix 6 (Officer *et al.* 1995) and Appendix 7 (Clement *et al.* 1992). One report (Brown in press) and a Ph.D. thesis (Officer 1995) are printed separately from this final report. The following list of works resulting from the project includes the milestone reports, thesis, publications and unpublished reports.

Brown, L. P. (in press). Rearing and vertebra-marking captive gummy shark and school shark for age validation. *Victorian Fisheries Research Rep*ort. (Victorian Fisheries Research Institute, Department of Conservation and Natural Resources: Queenscliff).

Clement, J. G., Officer, R. A., and Dykes, E. (1992). Three-dimensional reconstruction of shark vertebrae: a technique with applications to age and growth studies. In 'Age Determination and Growth in Fish and Other Aquatic Animals'. (Ed. D. C. Smith.) *Australian Journal of Marine and Freshwater Research.* **43**, 923-933.

Officer, R. A. (1995). Vertebral mineralisation patterns in gummy and school sharks and their utility in age determination. Ph.D. Thesis. 299 pp. (University of Melbourne: Melbourne.)

Officer R. A., Clement J. G., and Rowler, D. K. (1995). Vertebral deformities in a school shark, *Galeorhinus galeus*: circumstantial evidence of endoskeletal resorption? *Journal of Fish Biology* **46**, 85-98.

Officer, R. A., Gason, A. S., Walker, T. I., and Clement, J. G. (1995). Southern Shark Age Validation Project: Part 2 - Sources of variation in growth increment counts from vertebrae of gummy shark, *Mustelus antarcticus* Günther, and school shark, *Galeorhinus galeus* (Linnaeus). Final Report to Fisheries Research and Development Corporation (FRDC Project 91/037). 24 pp. (Victorian Fisheries Research Institute, Department of Conservation and Natural Resources: Queenscliff).

Walker, T. I., Clement, J. G., Officer, R. A., and Brown, L. P. (1993). Southern Shark Age Validation Project - Progress Report: No 1, Report to Fisheries Research and Development Corporation February 1993. *Marine Science Laboratories Internal Report No.* 207. 15pp. (Victorian Fisheries Research Institute, Department of Conservation and Natural Resources: Queenscliff).

Walker, T. I., Taylor, B. L., Hudson, R. J., and Cottier, J. P. (1995). Southern Shark Age Validation Project: Part 2 - The phenomenon of apparent change of growth rate in gummy shark, *Mustelus antarcticus* Günther, harvested by gill-net and hooks off southern Australia. 50 pp. Final Report to Fisheries Research and Development Corporation (FRDC Project 91/037). (Victorian Fisheries Research Institute, Department of Conservation and Natural Resources: Queenscliff).

Walker, T. I., Officer, R. A., Clement, J. G., and Brown, L. P. (1994). Southern Shark Research Program: Age Validation Project - Report to Fisheries Research and Development Corporation Milestone Report No 2. *Marine Science Laboratories Internal Report No.* 210. 15pp. (Victorian Fisheries Research Institute, Department of Conservation and Natural Resources: Queenscliff).

Background

The southern shark fishery produces annually nearly 4000 tonnes, carcass weight, of shark valued at $15.6 million to fishers based in Victoria, Tasmania and South Australia during 1993 (Walker *et al.* 1994b).

Biological data were collected on school shark by the CSIRO during the 1940s and early 1950s and on gummy shark by the former Victorian Fisheries Division [research function currently adopted by the Victorian Fisheries Research Institute (VFRI)] during 1973-76. Stock assessments had been inconclusive and in 1984 the former Southern Shark Fishery Task Force (predecessor to the current Southern Shark Fishery Management Advisory Committee) recommended that further research be undertaken.

FIRTA funds were subsequently made available to establish the Southern Shark Assessment Group, a regional research group based at VFRI in Queenscliff for the purpose of continuing investigation of the southern shark with the aim of providing resource assessments to guide and assist management of the fishery. The Group is currently undertaking the Southern Shark Tagging Project, the Victorian component of the Southern Shark Nursery Project, and the Southern Shark 'Drop-Out' Project funded by FRDC and is undertaking the ongoing Southern Shark Monitoring Project funded by the Australian Fisheries Management Authority from the Fisheries Resources Research Fund and an industry levy collected from shark fishers. Other southern shark studies include nursery, genetic and fishery modelling projects conducted by the CSIRO Marine Laboratories, nursery and trawl projects conducted by the Tasmanian Marine Resources Division and a general biological project on sharks conducted by the Western Australian Marine Research Laboratories.

Gummy sharks were first aged by Walker (1983) after adapting a method developed for staining growth-increment bands in the vertebrae of tuna, *Thunnus thynnus,* by Galtsoff (1952) and of elasmobranches by LaMarca (1966). The method was subsequently adopted by Moulton *et al.* (1992) and by the Central Ageing Facility based at VFRI in Queenscliff (Walker *et al.* 1994a) for both gummy shark and school shark.

The need to routinely collect length-at-age data and the need to validate the ageing method was repeatedly emphasised during a series of scientific workshops (1983-92) attended by fisheries scientists from Australia and New Zealand with special expertise in the fields of fishery population dynamics and shark biology. The recommendation for validation of the ageing method was supported by the Southern Shark Fishery Management Advisory Committee, and FRDC subsequently funded the 'Southern Shark Age Validation Project' for the 3-year period from July 1991 to June 1994.

Need

Up-to-date length-at-age data are essential for the age-structured models currently used for stock assessment of gummy shark and school shark. These assessments are reviewed annually by the Southern Shark Fishery Assessment Group which provides stock assessment advice to the Southern Shark Fishery Management Advisory Committee.

To produce the required data for the assessments, several vertebrae are routinely collected annually from each of about 2000 commercially caught gummy sharks and school sharks, as part of the Southern Shark Monitoring Project. Following subsequent laboratory preparation, the vertebrae are used by the Central Ageing Facility for estimating the ages of the sharks. The adopted method of ageing requires manually separating the vertebrae and removing soft tissues, and then bleaching, staining with alizarin red solutions and counting concentric growth-increment bands visible on the faces of the 'articular cups' of the vertebrae.

Need to Validate Method Adopted for Routine Ageing

Moulton *et al.* (1992) attempted to verify age estimates of gummy shark and school shark by comparing von Bertalanffy growth curves determined from length-at-age data with von Bertalanffy growth curves determined from length-increment data available from tag release-recapture studies. They found reasonable agreement between the von Bertalanffy growth curves from the two types of data for gummy sharks of all lengths and for school sharks of less than 1300 mm total length. Moulton *et al.* (1992) concluded, however, that the alizarin staining method could not reliably estimate the age of school sharks longer than 1300 mm and that the method underestimated the ages of these larger sharks.

To validate the method adopted for routine ageing of gummy sharks of all lengths and school sharks of length less than 1300 mm there is a need to establish that the alizarin stained concentric growth-increment bands visible on the faces of vertebra centra are deposited annually. This is most reliably achieved by counting growth-increment bands in sharks of known age or by counting growth-increment bands outside a vertebra-mark induced by injecting tissues-dyes into sharks held captive or sharks tagged and released in wild.

Need to Evaluate Microradiography as an Alternative Method for Ageing Shark

The microradiographic method of ageing sharks from sectioned vertebrae adopted by various authors (Cailliet *et al.* 1983, 1985, 1986, 1990; Cailliet and Radtke 1987; Ferreira and Vooren 1991; Yudin and Cailliet 1990) might be better than the alizarin staining method from whole vertebrae adopted for gummy shark and school shark. Ferreira and

Vooren (1991), for example, obtained age estimates up to 41 years for school shark from waters off southern Brazil which is about double the highest age estimate obtained for a school shark from waters off southern Australia using the alizarin staining method. For these reasons and to develop a method for ageing school sharks of length longer than 1300 mm there is a need to explore microradiography as a more accurate and possibly a more cost-effective method of age determination.

Need to Explain Apparent Changes in Growth of Gummy Sharks

Moulton *et al.* (1992) presented different von Bertalanffy age-length growth curves from counting growth-increment bands on the vertebrae of gummy sharks captured during two separate sampling periods and captured from two separate regions in southern Australia. Comparison of the growth curves for sharks captured in Bass Strait during 1986-87 with those captured in Bass Strait during 1973-76 suggests that growth rates slowed between the two periods. Similarly, comparison of growth curves for sharks captured in Bass Strait during 1986-87 with curves for sharks captured in waters off South Australia during 1986-87 suggests that growth rates of gummy sharks between the two regions are different.

These observed differences in the growth curves comprise either 'real differences in growth' or 'apparent differences in growth' or a combination of both. Moulton *et al.* (1992) advance seven hypotheses which can explain differences in growth curves.

(1) Growth curves change in response to changes in stock density through growth rates decreasing when biomass is high and, conversely, increasing when biomass is low.

(2) Growth curves change in response to environmental conditions through growth rates decreasing when conditions are unfavourable for growth and, conversely, increasing when conditions are favourable for growth.

(3) Growth curves change in response to evolutionary adaptation through growth rates decreasing where artificial selection by the fishing gear deployed in a fishery provides for a higher probability of survival to slow-growing individuals than to fast-growing individuals, or, conversely, through growth rates increasing where artificial selection by the fishing gear provides for a higher probability of survival to fast-growing individuals than to slow-growing individuals.

(4) Growth curves determined from length-at-age data are affected by the limitations of the von Bertalanffy growth function to describe the relationship between length and age of shark over the full age-range of a species, particularly when the data points are not distributed adequately over the age-range.

(5) Growth curves are affected by sampling bias caused by the length-selective characteristics of the fishing gear deployed to sample the wild population through the length-frequency composition of sharks collected for age determination in any age-class not being representative of the length-frequency composition of sharks in that age-class in the wild population.

(6) Growth curves are affected by length-selective immigration of sharks into a region or length-selective emigration of sharks out of a region through migrating sharks altering the length-frequency composition of sharks in particular age-classes of the wild population.

(7) Growth curves are affected by gill-nets or hooks selectively removing the longest sharks from the population among young age-classes and selectively removing the shortest sharks from the population among old age-classes by altering the length-frequency composition of sharks in particular age-classes of the shark population.

Moulton *et al.* (1992) presented arguments against Hypotheses 1-5, suggested Hypothesis 6 might have contributed to some of the observed differences between Bass Strait and South Australia, but favoured Hypothesis 7 for explaining most of the difference observed between the growth curves. By accepting this hypothesis they were postulating that there was an apparent change in growth rate rather than a real change in growth rate and that the apparent change was caused by length-selective fishing mortality. Because the choice of growth curve affects the results of stock assessment, it is important to establish whether the observed differences in the growth curves are apparent or real.

Objectives

The project had three objectives.

(1) Validate the method of staining whole vertebrae with alizarin red currently adopted for routine ageing of gummy sharks and school sharks.

(2) Investigate the feasibility of adopting a microradiographic method as a more accurate and a more cost-effective method than the currently adopted alizarin red staining method for routine ageing of sharks.

(3) Investigate whether growth rates of gummy sharks have changed in response to fishing by testing for the Phenomenon of Apparent Change in Growth Rate using vertebrae samples and data available for 1973-76 and 1986-87.

Objectives (2) and (3) were met completely but Objective 1 could not be met completely by the proposed method of injecting captive sharks with vertebra-marking tissue-dyes. The objective is being further addressed as part of the current FRDC funded Southern Shark Tagging Project (see section on Further Development).

Methods

This section of the report provides only a brief description of methods. Full details of methods are presented separately with details of results and interpretation of the results as they address each of the five tasks. Tasks 1, 2 and 3 are presented in Appendices 1, 2 and 3, respectively. Brief summaries of Tasks 4 and 5 are presented in Appendices 4 and 5, respectively, and full details are presented in Parts 2 and 3 of this report, respectively.

Task 1: Vertebra-marking in vivo with fluorescent tissue-dyes

This task involved testing the assumption that bands of alizarin red stain on the articular cups of whole vertebrae of gummy shark and school shark are annual. The assumption was tested by injecting fluorescent vertebra-marking tissue-dyes, designed to stain the actively growing areas of vertebrae *in vivo,* into the coelomic cavities of live sharks held captive for extended periods and of sharks recaptured after being opportunistically tagged, injected and released in the wild. To undertake appropriate experiments on captive sharks husbandry methods were developed to hold gummy sharks and school sharks in a 6.25-m diameter by 1.2 metre high tank filled with 27,000 litres of flow-through seawater from

Port Phillip Bay. During the 32-month period from August 1992 to March 1995 for the work annual cycles of water temperatures ranged 10-21 °C.

Task 2: Determining microstructure of shark vertebrae

A vertebra from a gummy shark or school shark comprises a centrum, haemal arch and neural arch. The centum has two articular cups and four struts (intermedialia) arranged laterally and dorso-ventrally as buttresses between the articular cups. These structures are comprised of heavily mineralized cartilage which provides strength to the vertebra whilst the cavities behind the articular cups and between the struts are filled with non-calcified hyaline cartilage. The vertebra is enclosed, along with the other vertebrae, in a fibrous tissue sheath (perichondrium) which extends the full length of the vertebral column. The perichondrium enables the vertebrae to grow by producing cells (chondroblasts) which mature into cartilage cells (chondrocytes) and secrete extracellular matrix which depending on location on the surface or marginal edge of the vertebra is either mineralized (i.e. becomes cemented) or non-mineralized.

Describing the microstructure of shark vertebrae involved application of techniques in light microscopy, microradiography, and electron microscopy. Emphasis was given to describing how the spatial orientation of the microstructure of the calcified tissues, non-calcified tissues and the organic matrices of shark vertebrae relate to alizarin red stained bands visible on the surface of the centra of whole vertebrae and to other growth-increment bands visible on longitudinally sectioned vertebrae. The task also involved describing how these structures might form and how they vary with increasing size of shark and, in the marginal region of vertebrae, time of the year.

Task 3: Reconstructing shark vertebrae by computer imaging

This task involved providing a computer-imaging three-dimensional reconstruction of gummy shark and school shark vertebrae. The purpose of the task was to objectively model three-dimensional growth by adapting an available computer package initially developed for reconstruction of bone and tooth organs. Reconstruction of vertebral growing structures at various stages of life, from data of fluorescent tissue-marking dyes collected under Task 1, enabled the study of the regions of a vertebra undergoing active growth.

The three-dimensional reconstructions presented in this report are of trunk vertebrae from a male gummy shark and a male school shark kept captive in tanks at VFRI from August 1992 to August 1993, and from May 1993 to May 1994, respectively. During captivity the gummy shark grew from 1190 mm to 1230 mm total length and the school shark grew from 580 mm to 830 mm total length. While in captivity the sharks were injected with vertebra-marking tissue-dyes (oxytetracycline, calcein, alizarin complexone and oxytetracycline) on each of three and four occasions, respectively.

Following each animal's death, vertebrae were removed from the trunk region of the vertebral column, immediately posterior to the first dorsal fin. These vertebrae were chosen because they were amongst the largest where presumably the mineral accretion rates are fastest and provide the best resolution between fluorescent bands.

The vertebrae were embedded in polyester resin and a sawing microtome was used to saw 100 μm sections from squared resin blocks containing the vertebrae. As the blade of this machine was 300 μm thick, the average spacing between adjacent sections was 400 μm. By digitising the positions of various structures and the fluorescent tissue-dyes, gummy shark and school shark vertebrae were reconstructed by applying the surface rendering and three-dimensional volume rendering techniques, respectively.

Task 4: Comparing alizarin stain and microradiographic age-reading methods

This task involved evaluating the currently adopted method of reading alizarin stained whole vertebrae and comparing this method with the method of reading microradiographs of sectioned vertebrae adopted for ageing sharks in other parts of the world. Evaluation and comparison of the methods required assessing how much the variation in counts of growth-increment bands from gummy shark and school shark vertebrae depends on (a) the method used to count growth-increment bands, (b) the effect of within-reader variability on precision and bias in repeated counts of growth-increment bands for the two methods, (c) the effect of between-reader variability on precision and bias in the alizarin staining method, (d) the variation in the counts of growth-increment bands between vertebrae taken within the same region of the vertebral column, and (e) the variation in counts of the growth-increment bands between vertebrae taken from different regions of the vertebral column. Most of the work was undertaken on 24 sharks where 2 gummy sharks and 2 school sharks collected from within each of 3 length-classes for each sex (i.e. 2 species × 2 sexes × 3 length-classes × 2 sharks = a total of 24 sharks), using two vertebrae from within each of three regions (post-cranial, trunk and caudal) of the vertebral column of each shark. In addition, counts of growth-increment bands from reading alizarin stained whole vertebrae were compared with counts of major bands of hyper-mineralization on microradiographs of sectioned vertebrae on post-cranial vertebrae collected from an additional 39 school sharks of length exceeding 1400 mm.

Task 5: Testing for Phenomenon of Apparent Change in Growth Rate

This task involved adopting three approaches to explore the feasibility that the differences in the growth curves reported by Moulton *et al.* (1992) were apparent differences rather than real differences in growth rate. (1) The length-frequency distributions of sharks sampled between the two sampling periods of 1973-76 and 1986-87 in Bass Strait and between the two regions of Bass Strait and South Australia during 1986-87 for each of the age-classes 2-7 years were compared by re-examining the length-at-age data produced from vertebral ageing by Moulton *et al.* (1992). (2) Available archived vertebrae were prepared and stained with an alizarin red solution by procedures similar to those adopted by Moulton *et al.* (1992), and then, for the purpose of testing for Rosa Lee's Phenomenon (Lee 1912; Ricker 1969, 1975), statistically tested for differences with age in the radius of the growth-increment bands on the surface of the vertebral centra. (3) An appropriate model was developed and used to computer simulate the effects of length-selective fishing mortality on the mean length of shark in the population by varying fishing effort of gill-nets and hooks and mesh-size of gill-nets in the fishery.

Results

This section contains only a summary of results. Details of three of the five tasks (Tasks 1-3) are presented at the end of the report in Appendices 1-3 whereas details of Tasks 4 and 5 are presented separately in Part 2 (Officer *et al.* 1995) and Part 3 (Walker *et al.* 1995) of this report, respectively.

Task 1: Vertebra-marking in vivo with fluorescent tissue-dyes

Appropriate feeding regimes and procedures for care were successfully developed to hold gummy sharks and school sharks in the 6.25-m diameter above-ground tank but survival rates were poor in smaller tanks. School sharks were more difficult to hold than gummy sharks. School sharks need to continually swim to ventilate their gills, whereas gummy sharks can ventilate their gills while remaining stationary. Large school sharks developed snout damage, leading to necrosis of the snout and eventually death as a result of swimming into the sides of the tank. Hence only school sharks of 520-610 mm total length were introduced into the tank. A total of 53 gummy shark and 31 school shark surviving the first month in captivity were held for varying periods during the 32-month period from August 1992 to March 1995.

Growth rates of gummy sharks held captive exceeded growth rates of sharks in the wild. Growth rates of captive gummy sharks and school sharks slowed during the winter months when water temperatures were lowest.

Several tissue-dyes (alizarin red, alizarin complexone, calcein, oxytetracycline and xylenol orange) injected into captive and tagged sharks in the wild were found to give distinct fluorescent marks in the vertebrae without having appreciable deleterious effects on the growth rates and health of gummy sharks and school sharks. After the sharks had died, by comparing the positions of vertebra-marks resulting from quarterly injections of the series of different tissue-dyes with the positions of growth-increment bands on the articular faces of whole vertebrae stained with alizarin red and with regions of hyper-mineralization and hypo-mineralization in microradiographs of vertebral sections, several important conclusions can be drawn.

(1) The density of mineralized cartilages that had formed during the winter was significantly higher than the density of mineralized cartilage formed during the other seasons. Overall growth of gummy shark was slowest during winter.

(2) Bands of alizarin red on the stained surfaces of the articular faces of whole vertebrae were found to correlate with regions of hyper-mineralized tissue visible in microradiographs of sections of vertebrae deposited during winter months.

(3) The number of hyper-mineralized bands formed in tagged gummy sharks while at liberty was found to be less than the number formed in sharks held captive for comparable periods. This difference and the positions of the bands indicated that the formation of growth-increment bands resulting from disturbance is not as common in wild sharks as it is in captive sharks. Inevitable formation of 'disturbance check marks' from unavoidable handling of captive sharks reduces the reliability of methods involving captive sharks for determining the periodicity of formation of growth-increment bands.

(4) Hence given the problem of 'disturbance check marks' detected in gummy sharks and difficulties holding school sharks captive, validation of the assumption that alizarin red stained bands (or hyper-mineralized growth-increments bands in microradiographs) are annual is being further addressed by injecting a large proportion of the sharks tagged and released as part of the current FRDC funded Southern Shark Tagging Project with oxytetracycline. Although available vertebrae from several recaptured tagged sharks injected with oxytetracycline provided evidence that the bands are annual, there is a need to test sharks from a range of sizes and a range of periods at liberty.

Task 2: Determining microstructure of shark vertebrae

Application of techniques in light microscopy, microradiography and electron microscopy, in conjunction with injection of vertebra-marking tissue-dyes, showed that growth-increment bands visible on microradiographs of sectioned vertebrae arise from patterns of hyper-mineralized (radio-opaque) and hypo-mineralized (radio-translucent) bands across a section. Statistically significant differences were detected in the mean area of chondrocyte lacunae (cartilage cell cavity) between hypo-mineralized and hyper-mineralized bands; the size of chondrocyte lacunae were larger and more variable in hypo-mineralized bands than in hyper-mineralized bands. No differences were detected in mean area or in standard deviation of chondrocyte lacunae between repeated measures within hypo-mineralized band or within hyper-mineralized bands. The greater average area of the lacunae in hypo-mineralized bands was reflected in the significantly greater proportion of the area occupied by lacunae. Also more cells per unit area were detected in hypo-mineralized bands than in hyper-mineralized bands. No differences were detected in the number of cells per unit area between repeated measures in separate quadrats within hypo-mineralized band or within hyper-mineralized bands.

Heavily mineralized growth-increments bands visible on microradiographs were found to be associated with regions containing relatively high volumes of dense extracellular matrix, surrounding small cells. It is concluded that growth-increment bands result from changes over time in the ratio of extracellular matrix volume to cell volume. When cells grow or proliferate slowly prior to cementation of the extracellular matrix, the ratio is high and results in a hyper-mineralized growth-increment band. Conversely, when cells grow or proliferate quickly prior to cementation, the ratio is low and results in a hypo-mineralized growth increment band.

Vertebrae increase in size when chondrocyte lacunae are engulfed by the consolidated mineralizing front through coalescence and fusion between spheroidic beads of mineral. On the margin of the articular face, chondrocytes become incorporated into the mineralizing front. Sometimes existing growth-increment bands on the articular face appear to be grown over and this probably explains why some sharks are difficult to age when using the current alizarin red staining method and why growth-increment bands are better resolved near the centre of the articular face than near the margin. When vertebral growth is slow, as in older animals, the obscuring of growth-increment bands through overgrowth could be more pronounced.

Mineralization of cartilage occurs when mineral salts, primarily of calcium, are laid down in the extracellular matrix. Although mineral deposits in mineralized elasmobranch

cartilages eventually become crystalline and closely resemble apatite, the first deposits of mineral are thought to be amorphous calcium phosphate, $Ca_3(PO_4)_2$.

Sharks inhabit a calcium-rich environment and unlike terrestrial vertebrates do not have to store calcium for their metabolic requirements. Hence, the growth processes in mineralized tissues of sharks differs from those of other vertebrates by not having to resorb calcium. Once cemented, the mineralized tissue of sharks remains permanently deposited and subsequent growth can only occur by apposition. It is this sequential, and periodic, incremental growth of shark vertebrae that makes these tissues a record of the growth history that can be used as a basis for age estimation.

Temporal variation in ambient phosphorus may affect the rates at which calcium phosphate can be deposited in the mineralizing cartilages. High availability of calcium phosphate would facilitate rapid mineralization of the extracellular matrix allowing the formation of hyper-mineralized growth-increment bands; whereas low availability of calcium phosphate would retard mineralization of the extracellular matrix allowing the formation of hypo-mineralized growth-increment bands.

Task 3: Reconstructing shark vertebrae by computer imaging

Three-dimensional reconstruction of vertebrae by computer-imaging was successfully simulated. This work applied data produced from Task 1 and corroborated results from Task 2 that growth was appositional but not evident on all surfaces of the vertebra. Although the method proved valuable, several shortcomings in the method were found.

(a) Since reconstructed bands represented previous growth sites, discontinuities in the sites of growth meant that reconstruction produced by either surface rendered reconstruction or volume rendered reconstruction were incomplete.

(b) Surface areas and volumes could not be measured accurately due to the incomplete reconstructions.

Other imaging methods that reconstruct the periphery of a vertebra directly rather than natural or induced bands may prove more useful. Further technological advancements may allow the application of previously unavailable imaging modalities to future vertebral growth studies.

Task 4: Comparing alizarin stain and microradiographic age-reading methods

Several important conclusions were drawn from this comparison. (1) Growth-increment bands in the vertebrae of school shark for both the alizarin stain method and the microradiographic method are more difficult to count than those in the vertebrae of gummy shark. (2) The readability of a vertebra for both the alizarin stain method and the microradiographic method tends to be no better after repeated reading for counting growth-increment bands. (3) The readability of vertebrae from the same region of the vertebral column of a shark for both the alizarin stain method and the microradiographic method are not significantly different (i.e. their readability is dependent on the shark). (4) Experienced readers using the alizarin stain method provide more precise and less biased counts of growth-increment bands than do inexperienced readers. (5) Counts of vertebral growth-increment bands made by both the alizarin stain method and microradiographic method

from the largest vertebrae which occur in the region of the vertebral column near the first dorsal fin are statistically higher than counts in vertebrae from the regions near the head and the tail. (6) Vertebral growth-increment bands visible on microradiographs of sectioned vertebrae can be classified as minor and major bands. (7) Counts of alizarin stained growth-increment bands on whole vertebrae are similar to counts of major growth-increment bands of sectioned vertebrae.

While there is no bias between the alizarin stain method and microradiographic method, the microradiographic method gives marginally better precision. Given the similarity in results between the alizarin stain method and microradiographic method, there are insufficient benefits to justify changing from the alizarin stain method to the microradiographic method for routine ageing of sharks.

Task 5: Testing for Phenomenon of Apparent Change in Growth Rate

Three types of evidence were found to support the hypothesis of the 'Phenomenon of Apparent Change in Growth Rate' caused by length-selective fishing mortality to explain observed differences in published von Bertalanffy growth curves determined from length-at-age data for gummy shark between 1973-76 and 1986-87 in Bass Strait and between Bass Strait and South Australia during 1986-87. (1) Mean length of the sharks in each of ages-classes 3-7 years were shown to be different between the two periods and the two regions, but not different or less different for the less fully recruited 2-year age-class. (2) Avoiding 'back-calculation' of length of shark, Rosa Lee's Phenomenon was detected by directly comparing the radii of growth-increment bands visible on the surfaces of vertebrae from sharks of various ages caught in the two periods and the two regions. (3) Developing an appropriate model, the effects of length-selective fishing mortality on the mean length of sharks in the population for ages 2-16 years were simulated for a range of levels of hook and gill-net fishing effort, with separate mesh-sizes of 6 and 7 inches for the gill-nets. Simulated changes in mean length for sharks older than 2 years tended not to be as large as the differences observed in the published von Bertalanffy growth curves but they were generally consistent with the observed trends. The simulations demonstrated how the von Bertalanffy growth parameters L_∞ and t_0 tend to increase and K tends to decrease as gill-net fishing effort increases, and hence explains how these types of biases, commonly reported in the scientific literature for gill-net shark fisheries, can occur.

Benefits

The shark fishing industry of southern Australia and the general community will benefit directly from a better managed fishery through improved methods of ageing sharks. Because management depends on the application of age-structured models for stock assessment of the fishery, benefits from the project will be effected though more reliable stock assessments resulting from better determined von Bertalanffy growth equations and from better quality age-composition data for the commercial catch. The benefits are allocated as 60% Commonwealth, 10% Victoria, 10% Tasmania, 10% South Australia and 10% Western Australia.

In addition, benefits will flow to other shark and non-shark fisheries from the evaluation and comparison of the alizarin red stain and microradiographic age-reading methods. Benefits will also flow through contributions to fisheries science theory by demonstrating

new approaches to statistical analysis of data for evaluating ageing methods and by demonstrating how the Phenomenon of Apparent Change of Growth Rate can occur in fisheries deploying length-selective fishing gear.

Benefits may eventually flow to the mariculture, tourist and educational industries. The project demonstrated that gummy sharks can be held captive in land-based tanks for long periods, that survival rates of captive sharks can be improved with appropriate husbandry, and that growth rates of captive sharks can be increased over those found in the wild.

Intellectual Property

No intellectual property has arisen from the research that is likely to lead to significant commercial benefits, patents or licences. Intellectual property associated with data produced from the project will be shared equally by the Fisheries Research and Development Corporation, the Victorian Department of Conservation and Natural Resources, and the University of Melbourne.

Further Development

(1) A major proportion of the sharks tagged and released as part of the FRDC funded Southern Shark Tagging Project are injected with oxytetracycline to further validate that alizarin stained bands on whole vertebrae and major microradiographic growth-increment bands in sections of vertebrae are formed annually. Shark fishers are being actively encouraged to return several vertebrae from each recaptured tagged shark. Although there are no funds currently allocated for processing the vertebrae, the vertebrae are being stored for future examination.

The Southern Shark Age Validation Project demonstrated that various tissue-dyes can (a) effectively mark the sharks' vertebrae *in vivo* to produce a reference mark (i.e. a 'date stamp') against which subsequent growth of vertebrae during a known period can be quantified, and (b) provide a basis for determining seasonal variation in the degree of mineralization associated with the growth-increment bands used for age estimation (Task 1). However the project also showed that handling sharks in captivity can cause hyper-mineralization in the vertebrae and form bands (i.e. 'stress-induced growth-increment bands'). Hence validation that growth-increment bands are deposited annual needs to be done by injecting sharks tagged and released in the wild.

(2) Counts of vertebral growth-increment bands made by both the alizarin stain method and the microradiographic method from the largest vertebrae which occur in the region of the vertebral column near the first dorsal fin are statistically higher than counts in vertebrae from the regions near the head (used for routine sampling of commercial catches) and the tail.

Additional samples of vertebral columns have been collected to determine the magnitude of the difference in the counts between these three regions. The task of will be completed by VFRI by December 1995 using available resources.

Staff

Organisation, position, period on project and percentage of time while on project are listed for each staff member.

Victorian Fisheries Research Institute, Department of Conservation & Natural Resources

Mr Terry Walker	Principal Investigator	1 Jul 91-30 Jun 95	15%
Dr Patrick Coutin	Marine Scientist	1 Jul 91-31 Dec 91	25%
Ms Lauren Brown	Technical Officer	1 Jul 91-31 Dec 91	25%
	Marine Scientist	1 Jan 92-30 Jun 94	25%
Mr Russell Hudson	Technical Officer	1 Jan 92-31 Jan 94	25%
Ms Natalie Bridge	Technical Officer	1 Feb 94-30 Jun 94	25%

School of Dental Science, University of Melbourne

Assoc. Prof. John Clement	Co-investigator Academic Supervisor	1 Jan 92-30 Jun 95	10%
Mr Dennis Rowler	Laboratory Manager	1 Jan 92-30 Jun 95	5%
Mr Rickard Officer	Stipendiary Student	1 Jan 92-30 Jun 95	100%

Zoology Department, University of Melbourne

Dr Robert Day	Academic Supervisor	1 Jan 92-30 Jun 95	5%

Final Cost

Details of grant and expenditure are in the following tables. Expenditure on the project was $328 less than total grant.

Budget item	Grant ($)			
	1991/92	1992/93	1993/94	Total
Project grant				
Salaries	34,380	34,890	35,370	104,640
Operating expenses	12,658	12,658	12,658	37,974
Travelling expenses	2,000	2,000	2,000	6,000
Capital items	0	0	0	0
Total	49,038	49,548	50,028	148,614
Expenditure				
Salaries	33,966	31,621	37,146	102,733
Operating expenses	12,450	14,575	15,268	42,294
Travelling expenses	1,702	387	1,170	3,259
Capital items	0	0	0	
Total	48,118	46,583	53,584	148,286

Distribution

This report is being distributed to researchers who have an interest in shark research, to several libraries and to each of the following organisations.

Australian Bureau of Agricultural and Resource Economics
Macarthur House, Macarthur Avenue, Lyneham ACT 2602

Australian Fisheries Management Authority
Burns Centre, 28 National Circuit, Forrest ACT 2603

Australian Institute of Marine Science
PMB No 3, Townsville QLD

Australian Maritime College
Beauty Point, TAS 7270

Bureau of Resource Sciences, Fisheries Resources Branch
Curtin House, 22 Brisbane Avenue, Barton ACT 2601

CSIRO Marine Laboratories, Division of Fisheries
Castray Esplanade, Hobart TAS 7001

Marine Resources Division, Department of Primary Industry and Fisheries
GPO Box 619F, Hobart TAS 7001

National Fishing Industry Council
Unit 1, 6 Phipps Place, Deakin ACT 2600

New South Wales Fisheries Research Institute
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Primary Industries (Fisheries) South Australia
GPO Box 1625, Adelaide SA 5001

Shark Tri-State Industry Body
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South Australian Fishing Industry Council
c/o Adrian Fletcher, 9 Angas Street, Port Lincoln SA 5606

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Southern Shark Fishery Assessment Group
(Distributed to all members: Horst Fischer, Kevin McLoughlin, Jeremy Prince, Andre Punt, Peter Riseley, John Stevens, Trysh Stone, Bruce Taylor, Terry Walker, John Wallace, and Yongshun Xiao)

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APPENDIX 1

TASK 1: VERTEBRA-MARKING *IN VIVO* WITH FLUORESCENT TISSUE-DYES

Introduction

This task was designed to test the assumption made by Walker (1983), Moulton *et al.* (1992) and Walker et al. (1994) that growth-increment bands stained by alizarin red on the centra of whole vertebrae in gummy shark and school shark are formed annually. The assumption was tested by injecting fluorescent vertebra-marking tissue-dyes into live sharks to stain for the actively growing areas in the vertebrae of sharks held captive for extended periods. The assumption was also tested on several sharks recovered after being captured, injected with oxytetracycline, tagged and released into the wild. When the captive sharks subsequently died or when tagged sharks were subsequently recaptured, growth-increment bands laid down at the margins of the vertebrae outside the tissue-dye marks could be related to period elapsed since injection.

The marks left by the tissue-dyes in mineralizing tissues are discrete lines laid down at the time of injection, and provide a 'date stamp' from which any growth after the injection can be measured. Hence, injecting live sharks with vertebra-marking tissue-dyes is an appropriate method for determining:

(a) the frequency and widths of growth-increment bands formed in vertebrae beyond a single fluorescent mark during the known period of time after injecting the tissue-dye into a captive shark or a tagged shark in the wild,

(b) the frequency and widths of growth-increment bands formed in vertebrae between two or more fluorescent marks during the known period of time between successive injections of the tissue-dye into a captive shark, and

(c) whether vertebrae in different regions of the vertebral column grow synchronously throughout the life of a shark and add the same number of growth increment bands in a given period (the tissue-dyes should be visible in all vertebrae of injected sharks if this is true).

Comparing the pattern of tissue-dyes in whole and sectioned vertebrae with the various patterns of growth-increment bands seen on stained whole vertebrae using light microscopy and sectioned vertebrae using microradiography provides a way of investigating the periodicity of the deposition of growth-increment bands. The method provides a basis for validating, or perhaps reinterpreting, age determinations derived by the current method of counting bands of alizarin red stain on whole vertebrae, described by Walker (1983) and Moulton *et al.* (1992), and under Task 4 (see Part 2 of this report), or, perhaps, a basis for developing an alternative method of ageing. Material presented here for Task 1 is reproduced from Officer (1995) and Brown (in press).

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Methods

Holding Sharks Captive

Initially gummy sharks and school sharks were held in an oval tank (6 m long x 2 m wide x 1 m high) available at VFRI, but none survived, probably because the tank was too small to allow the sharks to swim and glide. In a larger tank (8 m long x 3 m wide x 1 m deep) at Port Fairy survival rates were higher but still inadequate. Survival rates were best in the much larger (27 000 L) above ground 'Aqualok AFT6' tank (6.25 m diameter x 1.2 m high) erected at VFRI. The tank had a flow through system (10 000 L h^{-1}) with sea water pumped from the Queenscliff Pier in Port Phillip Bay.

The tank's inlet, located above the water surface, acted as a water aerator and created an anti-clockwise current when viewed from above. The outlet, centred at the bottom of the tank drew water from the floor of the tank and thereby produced greater mixing of water within the tank. The tank was lined with black plastic and light intensity within the tank was reduced to about 5% of the light intensity outside the tank by adding two layers of 60% shade cloth to the roof 1 m above the water and one layer of 60% shade cloth to the walls. The tank's floor was vacuumed each week and its walls and floor were scrubbed every 3 months. Two 4-m diameter tanks were available nearby for temporary holding of sharks.

Stocking density in the tank was 1 kg of shark per 350 L of sea water and the captive sharks were fed a mixture of frozen squid tails and pilchards, with an occasional prawn, mussel or scallop. The mixture, thawed as needed, was cut into mouth-sized portions and given to the sharks 5 days a week which equated to 15% of the sharks body weight. Food not consumed was removed daily. While thawed food reduces the possibility of disease transmission it introduces the problem of vitamin loss due to freezing. From March 1993 the sharks were given a vitamin and mineral supplement specially formulated for sharks, in the form of a capsule inserted into a squid tail. One capsule per shark was twice weekly.

The sharks were captured by bottom-set long-lines, otter board trawls or gill-nets of 17-mm mesh-size within 2 hours travelling time of Queenscliff. Most of the sharks captured were immediately placed in a 500 L (1.2 m long x 600 mm wide x 600 mm high) padded holding tanks and supplied with continuously flowing sea water via a deck hose; a vessel's wet well was used when available. One of the keys to survival was to minimise physical damage to the sharks and the time the sharks are kept in holding tanks. Gummy sharks, being able to remain in a resting state, presented fewer problems when transported than did school shark which have to continually move to ventilate their gills. Whenever a shark was handled its body was completely supported and the shark was immobilised by holding it belly up, head down, to reduce thrashing. Clark's (1963) suggestion that a shark should never be lifted by the tail was implemented (Brown in press).

Injection of Captive Sharks with Vertebra-marking Tissue-dyes

Trials to determine dose rates that produce detectable marks in the vertebrae and to determine the toxicity of various tissue-dyes were undertaken with Port Jackson sharks, *Heterodontus portusjacksoni* (Meyer), and whitefin swell sharks, *Cephaloscyllium sp.*, before tissue-dyes were tried on gummy sharks and school sharks. Within a few days of

being caught and at approximately 3-month intervals all the sharks were synchronously injected with one of several vertebra-marking tissue-dyes (alizarin complexone, alizarin red, calcein, oxytetracycline or xylenol orange). Oxytetracycline was injected first to utilize its ability to act as an antibiotic as well as a vertebra-marking tissue-dye.

Prior to injecting, each shark was anaesthetised in a bath of 'MS-222' prepared at a concentration of 50 mg L^{-1} (Gudkovs 1984). The anaesthetic which is absorbed through the gills, took effect within 5-15 min of the shark being placed in fresh sea water.

Each of the vertebra-marking tissue-dyes was injected as a saline solution intra-peritoneally at a dose of 25 mg/kg. Doses were calculated as a proportion of the animals' estimated total body weight using established length-weight relationships (Gason and Walker 1991). Injection was carried out in a measuring tray, which supported the animal along its entire dorsal side, allowing for the total length to be measured, and for intra-peritoneal injection with the selected vertebra-marking tissue-dye. The tissue-dye was injected anterior to the cloaca. The skin at the injection site was pinched between the thumb and forefinger and the needle pushed through the middle of the skin fold. The needle was pushed through at an acute angle until it could be felt to have passed into the peritoneal cavity. Injecting at the posterior end of the body cavity, lifting the skin and angling the needle minimised the chances of rupturing internal organs or injecting into them.

After each injection a shark was measured, weighed and identified from a 'Trovan Passive Implantable Transponder (Model ID 100)' implanted subcutaneously 5 cm laterally and posterior to the right pectoral fin. The condition of the shark was assessed and recorded before and after injection. Malachite green dissolved in alcohol was applied to all the sharks' lesions and open wounds, including those caused by hooks and injections. When a shark died or was sacrificed, the entire vertebral column, jaws, mouth floor, skin from several sites, cartilage from the cranium, the pectoral girdle and, in male sharks, the claspers were removed and stored at -25°C.

Injection of Sharks Tagged and Released with Vertebra-marking Tissue-dyes

Between December 1991 and December 1992, about 400 gummy and school sharks were captured by gill-nets, injected, tagged and returned to the wild in the bays and inlets of south-eastern Tasmania by the CSIRO Division of Fisheries as part of a project investigating abundance of juvenile shark (John Stevens, CSIRO Marine Laboratories, personal communication). Most of these sharks were juvenile fish in their first or second year and were tagged with a numbered dart tag inserted through the skin on the right lateral side or with a numbered cow ear tag clipped through the first dorsal fin.

The vertebra-marking tissue-dye injected was a saline solution of oxytetracycline hydrochloride. All animals were released at the location where they were caught after only a few minutes out of the water. The vertebral columns of recaptured tagged sharks, when made available, were removed from the sharks and stored at -8°C.

Analysis of Ultra Violet Light Fluorescence of Vertebra-marking Tissue-dyes

Once returned to the laboratory, intact vertebral columns were stored at -25°C until required for examination. The pieces of vertebral column were initially examined

macroscopically under incident UV light to determine the extent of fluorescent marking by the tissue-dyes along the length of the vertebral column.

Vertebrae were then prepared for analysis of growth-increment bands and fluorescent marks of the tissue-dyes. The mean growth rate of the vertebrae of a captive shark during the period between when the shark was injected and when it died, or of a recaptured tagged shark during the period at liberty, was calculated as the distance between the fluorescent mark and the vertebral margin divided by the period. Similarly, the mean growth rate of the vertebrae of a captive shark for the period between subsequent injections was calculated as the distance between the fluorescent marks divided by the period between the injections.

Vertebrae were extracted from the post-cranial and trunk regions of the vertebral column of each of the selected sharks. Longitudinal sections (100 μm thick) were sawn through the lateral intermedialia of each vertebra and stored in the dark in 70% alcohol to prevent loss of fluorescence.

For gummy shark, variation in the relative mineral density across sections of vertebrae was statistically analysed to test for the effects of season using a multi-variate analysis of variance with repeated measures ('Systat for Windows Version 5.0', Systat Inc., IL, USA). The relative mineral density was treated as a repeated measure because one measure was made for each gummy shark in each of the four seasons, providing a total of four measurements of relative mineral density for each gummy shark. The region of the vertebral column from which vertebrae were sampled was used as a grouping factor to compare the mineral density between regions of the vertebral column for each season.

An insufficient number of school sharks survived long enough in captivity to provide enough samples for a repeated measures analysis of variance. A measure of the mean mineral density for each season was expressed for gummy and school sharks as the grand mean for each species of the relative mineral density during each season.

Results

Holding Sharks Captive

A total of 53 gummy shark and 31 school shark surviving the first month in captivity were held for varying periods during the 33-month period from August 1992 to March 1995 in the 27,000 L tank. During this period, 87%, 47%, 43%, 20%, 15% and 6% of these gummy sharks survived 3, 6, 9, 12, 15 and 18 months, respectively, and 29%, 13%, 10% and 6% of the school sharks survived 3, 6, 9 and 12 months, respectively.

School sharks were more difficult to hold than gummy sharks. School sharks need to continually swim to ventilate their gills, whereas gummy shark can ventilate their gills lying stationary on the substrate. Large school shark developed snout damage, leading to necrosis of the snout and eventually death as a result of swimming into the sides of the tank. Hence only school sharks 520-610 mm total length at capture were kept in the tank.

Injection of Captive Sharks with Vertebra-marking Tissue-dyes

The project established procedures for administering several different vertebra-marking tissue-dyes, determined the suitability of each tissue-dye, and determined appropriate dose-rates of each tissue-dye (high doses can have deleterious effects on the growth and health of a shark whereas low doses give indistinct markings).

Vertebral growth-increment bands were highly variable between individuals and between the seasons for both gummy shark (Figure 1.1a) and school shark (Figure 1.1b). Despite this variability there was a trend for growth rates to be lowest during winter which correspond with the lowest water temperatures in the VFRI tank (Figure 1.2). The photomicrographs in Figure 1.3 show two vertebral preparations from a male gummy shark held captive in the tank for 463 days from 9 December 1992 to 17 March 1994 when it was sacrificed. During this period the shark grew from 750 mm to 1090 mm total length. The images are of the margin of the same longitudinal section through a post-cranial vertebra seen as a fluorescence photomicrograph (left) and as a microradiograph (right). This animal was injected with one of several fluorochrome marking tissue-dyes on five separate occasions. In the fluorescence photomicrograph, Band 1 (feint red) marks the first injection with alizarin complexone and is followed by an additional four bands.

Since the tissue-dyes are incorporated into mineralizing tissues on the date of injection they allow growth rates to be calculated between the dates of injection. This is done by dividing measured differences in the radius of the centrum between fluorescent bands (or between the last fluorescent band and the margin) by the number of days between injections (or between the last injection to the date of death). In this specimen, in common with many others, it is clear that some fluorescent bands are closer than others, despite being incorporated a similar number of days apart. This indicates that the vertebral growth rate varied over the course of the experiment, and was least during the winter months.

This approach can be used for comparing the positions of hyper-mineralized bands visible in microradiographs of sectioned vertebrae. The positions of fluorescent marks are used as calibration marks from which the approximate dates of deposition of growth-increment bands can be back-calculated. In the specimen pictured in Figure 1.3, many fine hyper-mineralized bands were visible on the microradiographs for the 463 days that this animal was in captivity; hence, indicating that fine bands are not useful in determining the age of the animal. It is more appropriate to examine broader bands of hyper-mineralization on the basis that these represent slow growth periods (as corroborated by Task 2). Such slow growth periods could be expected to occur seasonally. For this case, in common with others, the highest degree of mineralization, and the slowest growth, occurred during the winter. Handling, transportation and injection appears to interfere with the normal growth of the vertebrae. The unusual disturbance marks (marked D) observed in many specimens appear to have been deposited immediately following dates of handling. The narrow hyper-mineralized bands associated with each disturbance mark could have resulted from the animal growing slowly whilst recovering from handling stress.

To confirm that the mineralized tissues formed during winter are hyper-mineralized, additional gummy shark and school shark were selected for examination from the sharks held in captivity at the VFRI. To allow for comparison between animals, only sharks of each species that had shared the same year in captivity were selected for this analysis.

Whilst 10 gummy sharks satisfied this criterion, only 2 school sharks shared a full year in captivity. Variations in the vertebral growth rate during the year were examined for approximately quarterly intervals. The beginning and the end of each of these seasonal growth intervals were delineated by the positions of fluorescent bands of vertebra-marking tissue-dyes.

For gummy shark, the relative mineral density of vertebral sections did not differ significantly between regions of the vertebral column (Table 1.1). The interaction between the region of the vertebral column and season was significant and it was therefore necessary to use the Greenhouse-Geiser method to determine the probability value. Differences in mineral density between seasons were highly significant. For gummy shark and school shark, the mean mineral density of the vertebral sections was highest during winter. The gummy shark vertebrae were least mineralized during summer. School shark vertebrae were least mineralized during autumn growth periods. Whilst the general trend for higher mineral densities during winter was common to both species, the results for school shark must be treated with some caution due to the very small sample size (Table 1.2). Using the same statistical procedures, growth rates of vertebrae were found to be least during the winter and highest during the autumn and trunk vertebrae grow faster than post-cranial vertebrae (Tables 1.3 and 1.4).

Table 1.1. Effects of region within the vertebral column of the gummy sharks and season on the relative log mineral density of vertebral sections (df = degrees of freedom, MS = mean square, F = F-test ratio, P = probability value, G-G = Greenhouse-Geiser statistic which adjusts for unequal covariance, na = not applicable).

Source of variation	df	MS	F	P	G-G
Region	1	0.47	0.41	0.537	na
Error	9	1.14			
Season	3	67.03	9.92	<0.001	0.001
Error	27	6.76			
Season x region	3	6.14	4.68	0.009	0.021
Error	54	4.03			

Table 1.2. Seasonal mean mineral density in sections of vertebrae of gummy sharks and school sharks held captive in the VFRI tank during Autumn 1993 -Autumn 1994 (n = sample size, nd = no data).

Species of shark	Region column	n	Mean mineral density (with standard deviation)[A]				
			Autumn	Winter	Spring	Summer	Autumn
Gummy	Post-cranial	10	101.2 (1.6)	102.5 (2.0)	101.0 (1.5)	97.7 (1.4)	nd
	Trunk	10	99.8 (1.8)	102.7 (1.2)	101.7 (2.9)	98.8 (2.3)	nd
School	Post-cranial	2	nd	101.0 (0.2)	100.0 (0.3)	100.6 (0.2)	97.8 (1.3)
	Trunk	2	nd	101.8 (0.1)	101.2 (0.5)	100.1 (0.6)	95.8 (1.8)

[A]Density measured as grey shading on computer images of section

Table 1.3. Effects of region within the vertebral column of the gummy sharks and season on the vertebral growth rate (df = degrees of freedom, MS = mean square, F = F-test ratio, P = probability value, G-G = Greenhouse-Geiser statistic which adjusts for unequal covariance, na = not applicable).

Source of variation	df	MS	F	P	G-G
Region	1	58.68	69.40	<0.001	na
Error	9	0.85			
Season	3	40.06	13.34	<0.001	<0.001
Error	27	3.00			
Season x region	3	3.02	13.54	0.009	<0.001
Error	27	0.22			

Table 1.4. Seasonal vertebral growth rate determined from sections of vertebrae of gummy shark and school sharks held captive in the VFRI tank during Autumn 1993 -Autumn 1994 (n = sample size, nd = no data).

Species of shark	Region column	n	Mean vertebral growth rate (μm/day) (with standard deviation)				
			Autumn	Winter	Spring	Summer	Autumn
Gummy	Post-cranial	10	3.3 (1.6)	1.0 (0.8)	1.9 (1.3)	3.1 (1.9)	nd
	Trunk	10	6.1 (2.3)	2.2 (1.2)	2.9 (1.9)	4.8 (2.7)	nd
School	Post-cranial	2	nd	2.2 (0.3)	4.7 (0.4)	6.4 (0.1)	6.1 (0.5)
	Trunk	2	nd	3.2 (0.0)	6.5 (0.5)	9.0 (0.2)	8.2 (1.4)

Injection of Tagged Sharks with Vertebra-marking Tissue-dyes

Vertebrae from eleven recaptured tagged sharks injected with oxytetracycline, six gummy sharks and five school sharks, have been examined. Most of these sharks were at liberty for only brief periods; all of the school sharks were at liberty for less than 84 days and two of the gummy sharks were at liberty for less than 222 days. Four of the six gummy sharks re-captured were at liberty for periods in excess of one year, with two of these sharks at liberty for more than two years. These animals were re-captured within short distances of their release location, with the exception of one that moved 149 nautical miles.

All of the available vertebral columns displayed oxytetracycline fluorescence along their entire lengths. This suggests that all vertebrae in the column incorporated the tissue-dye and were therefore growing during the time the tissue-dye was available in the animals' circulation. The quick uptake of the tissue-dye into mineralizing tissues was confirmed by its presence in the centra in those sharks re-captured soon after initial release and injection.

The fluorescent bands in vertebrae from animals re-captured after extended periods at liberty did not appear to be any less intense than those bands in vertebrae from animals re-captured soon after marking. Vertebrae from animals at liberty for longer periods did not display any of the diffuse background fluorescence that was characteristic of vertebrae from animals re-captured soon after marking.

For those gummy sharks at liberty for more than a year at liberty, the number of growth-increment bands deposited was compared with the number of months at liberty. Some correlation is evident between the number of years at liberty and the number of bands observed. In one case, the shark was at liberty for less than two years, and yet two major increments were deposited. However, the period at liberty included two winters, the periods when increment deposition was calculated to have occurred for this animal.

Whilst the calculated date of deposition of most major increments visualised using the microradiographic method fell during the winter months, the calculated dates of deposition of all of these increments did not fall strictly within a single period or season. Minor bands, when formed, were calculated to have formed at all times of year. Estimations of the periods during which alizarin stained increments formed were also variable. In one case, alizarin stained increments were indistinguishable and no deposition dates could be calculated. Accurate estimation of the period during which growth-increment bands were deposited was complicated by the fact that the growth rates used in this estimation represented average growth rates for extended periods at liberty. Hence, these growth rates were insensitive to seasonal variations in the vertebral growth rate.

The number of growth-increment bands formed in recaptured wild gummy sharks was less than the number formed in captive gummy and school shark. This result suggests that the formation of 'disturbance check marks' is not as common in wild sharks as it is in captive sharks, and hence indicates the advantages of injecting tagged sharks in the wild over injecting captive sharks for validating the assumption that growth increment bands are deposited annually.

The number of major hyper-mineralized bands formed in the post-cranial vertebrae of the four gummy sharks recaptured after over one year at liberty is shown in Figure 1.4. The

general agreement between increment counts obtained using the alizarin staining method and the microradiographic method (corroborated by Task 4 which is reported in Part 2 of this report) is illustrated by the superimposition of some data points obtained using each reading method. However, the plot also illustrates the advantage of using both reading methods to obtain an increment count for a vertebra. A count of three increments was obtained for one vertebra using the microradiographic method compared with a count of 2 obtained when using the alizarin stain method. This difference may be due to the higher resolution of the microradiographic method and its ability to resolve a third, possibly minor growth increment. In another case the vertebra was unreadable using the alizarin stain method (increment count = 0) but the microradiographic method obtained a count of 2. The higher resolution of the microradiographic method might also explain this difference. Whilst this shark was at liberty for less than 2 years, its period at liberty included 2 winters, possibly explaining the formation of 2 hyper-mineralized bands.

The sample size is too small to draw a firm conclusion from the present study about the relationship between increment count and time at liberty. Determination of this relationship will be further addressed following the recovery of an adequate sample size of vertebrae from sharks injected with oxytetracycline, tagged and released of as part of the FRDC funded Southern Shark Tagging Project.

Discussion

Seasonal Changes in Mineral Density

Changes in the mechanisms of cellular growth at the growing margins of vertebrae are directly involved in the production of discrete hyper-mineralized growth increment bands (see discussion for Task 2). Whilst the cellular processes associated with the deposition of discrete growth increments have parallels with periods of slow growth in mammals (Breur *et al.* 1991, 1992), it appears that episodes of slow growth occur throughout the year in gummy and school shark. Discrete growth increments deposited in captive gummy and school sharks in the present studies were deposited at all times of year. This suggests that normal vertebral growth in these sharks was punctuated by episodic disturbances to the normal process of vertebral growth. The formation of narrow bands of hyper-mineralized tissue in the vertebrae may represent the episodic slowing of vertebral growth in response to these disturbances.

The fact that discrete growth increments may sometimes be induced by disturbance, and therefore not always periodic, means that these growth increments may confuse the reader attempting to determine the age of a shark based on a count of its vertebral growth increments. Any periodic fluctuations in the mineralization pattern visible in the vertebrae may be obscured by a super-imposed pattern of episodic growth increments.

Seasonal changes in the overall growth rate may be accompanied by seasonal changes in the degree of vertebral mineralization (Ishiyama 1951; Holden and Vince 1973; Stevens 1975; Tanaka *et al.* 1978; Cailliet *et al.* 1983; Cailliet and Radtke 1987; Ferreira and Vooren 1991). In the gummy shark, cells exhibiting the characteristics of a slow growth rate were associated with narrow bands of hyper-mineralized tissue (see Appendix 3). It follows that longer periods of slow growth might induce broader bands of hyper-

mineralization. Longer periods of slow growth are likely to be seasonal because factors such as food availability and water temperature that limit the growth capacity are seasonal.

In this study, vertebra-marking tissue-dyes were used to examine seasonal variations in the growth rate and in the degree of vertebral mineralization. The quarterly injection of tissue-dyes into captive sharks provided a means of delineating the beginning and end of each season. Differences in the degree of mineralization of the extracellular matrix were examined by comparing the mineral density during periods of slow growth and during periods of rapid growth.

Hyper-mineralization of the vertebrae has been described as a winter phenomenon in several other species of sharks (Ishiyama 1951; Tanaka *et al.* 1978; Killam and Parsons 1989; Ferreira and Vooren 1991; Parsons 1993). Despite the fact that only a small number of gummy sharks and school sharks were available for this study, the results indicate that both these species share the trend for hyper-mineralization of vertebral growth zones during the winter months.

The regions of the vertebrae with the highest mineral density were related to periods of slow growth, indicating a parallel between the process of vertebral growth in gummy and school shark and the process of bone growth in mammals. During normal bone growth in mammals the degree of mineralization in newly forming tissues is highest during slow-growth periods (Hunziker *et al.* 1987; Breur *et al.* 1991; Hunziker 1994). Parallels in the nature of mammalian bone growth and vertebral growth in gummy sharks and school sharks may help improve understanding of the factors that induce slow bone growth at the cellular level in mammals may also improve our understanding of the regulation of vertebral growth in gummy shark and school shark.

The results of electron microprobe analyses of the vertebrae from the grey reef shark, *Carcharhinus amblyrhynchos* (Bleeker), and thintail thresher shark, *Alopias vulpinus* (Bonnaterre), by Cailliet and Radtke (1987) do differ from the results for gummy shark, school shark, and other studies (Ishiyama 1951; Jones and Geen 1977; Tanaka *et al.* 1978; Killam and Parsons 1989; Ferreira and Vooren 1991; Parsons 1993). Cailliet and Radtke (1987) found that more calcium and phosphorus was deposited during the summer in the vertebrae of the reef and thresher sharks than during the winter. However, it can be inferred that vertebral tissues formed during the winter in the reef and thresher sharks were hyper-mineralized when compared to tissues formed during summer since Cailliet and Radtke (1987) reported that winter growth zones in the reef and thresher shark were translucent to transmitted light; a characteristic of hyper-mineralized zones in the vertebrae of other species (Ishiyama 1951; Kusakari 1969; Tanaka *et al.* 1978; Killam and Parsons 1989; Ferreira and Vooren 1991; Parsons 1993).

Cailliet and Radtke's (1987) observed reduction in the amount of calcium and phosphorus deposited during winter vertebral growth runs contrary to the growth hypothesis of Jones and Geen (1977). Jones and Geen (1977) suggested that the observed heavier mineralization in winter growth zones of the vertebral tissues in the white spotted spurdog, *Squalus acanthias* Linneaus, was explained by the greater amount of phosphorus available during winter. Phosphorus is a necessary ingredient of hydroxy-apatite, the principal component of the shark calcified cartilage matrix (Clement 1986). The secretion of alkaline phosphatase is required to precipitate mineral salts into the extracellular matrix that

surrounds each new cell and eventually cement these cells into the calcified bulk of the vertebral centrum to facilitate appositional growth (Bancroft and Stevens 1990). Temporary increases in the abundance of phosphorus may be accompanied by temporary increases in a shark's capacity to form more heavily mineralized vertebral tissues.

Cailliet and Radtke's (1987) results suggests that alternative mechanisms may induce vertebral hyper-mineralization and that heightened phosphorus availability in winter is only indirectly related to the increased mineral density in vertebral growth zones that formed during winter. The importance of nitrogen in the formation of hypo-mineralized zones in the otoliths of some fish (Mugiya 1965) suggests an alternative mechanism that may be associated with temporary hyper-mineralization in the growth zones of shark vertebrae. Whilst elucidating which factors influence vertebral hyper-mineralization is an important and interesting area of study, the present incomplete understanding of these factors does not preclude one from interpreting hyper-mineralization patterns as a record of the life history of a shark or from using these patterns to estimate the age of a shark.

The general pattern of distinct winter hyper-mineralization of vertebral growth zones in the gummy shark and school shark vertebrae examined in this study suggests that interpreting finer (or minor bands visible in microradiographs) of discrete growth increments as age-related marks may be inappropriate. Periodic changes in mineral density are more likely to be displayed microradiographically as broad radio-opaque bands, and not as narrow, discrete growth-increment bands. However, since the episodic disturbances that give rise to discrete growth increments are more likely to occur during winter, clusters of discrete growth increments may represent a winter growth period. Back calculation of the dates of formation of discrete growth increments in captive gummy shark and school shark held during the course of tissue marking experiments of the present study indicated that some sharks formed several heavily mineralized growth increments during the course of a year and that, during winter, hyper-mineralized growth increments could be formed only a few weeks apart.

Deposition of fine (or minor) growth increment bands is probably not as common in wild sharks as it is in captive sharks because the opportunities for unnatural interferences in the growth of wild sharks are probably minimal compared to captive animals. More major hyper-mineralized vertebral growth increments and fine check marks were formed in the vertebrae of sharks kept in captivity during the course of tissue marking experiments of the present study than in the vertebrae from returned tagged wild sharks. Whilst minor growth increment formation might occur to a lesser extent in wild sharks, the fact that minor increments can form at all is a cause of concern when attempting to assign an age estimate to a vertebral section based upon an interpretation of its mineralization pattern. Minor increments have been reported in the vertebrae of other species of shark caught in the wild (Cailliet and Radtke 1987).

Increment counts made by methods reading whole stained vertebrae which are unable to resolve individual minor increments may serendipitously reflect the age of a shark since clusters of such minor bands may instead be interpreted as separate broad zones of seasonally hyper-mineralized tissue. The alizarin staining method currently in use in the routine ageing of vertebrae in the southern shark fishery is unable to resolve fine increments and these are instead read as broad stained bands. This consolidation of

increments may explain the reasonable agreement between von Bertalanffy growth curves produced from tag length-increment data and curves produced from increment counts from stained whole vertebrae (Moulton *et al.* 1992). The agreement between these growth curves is further evidence that major hyper-mineralized bands are formed annually.

The better resolution of the microradiographic method developed in the present study makes the fine vertebral increment patterns that are indistinguishable using the alizarin stain method readily visible. Counting all of these fine increment bands might produce an inflated age estimate for a shark. However, with an understanding of how and when they are formed, a reader can be trained to subjectively exclude certain minor increments and instead count only the major bands of hyper-mineralized tissue which are distinct using the microradiographic method, and are more probably age-related because they represent winter hyper-mineralization. Use of the microradiographic method may also allow estimation of the age of vertebrae from large school sharks which are difficult to age using the alizarin stain method (Moulton *et al.* 1992). The increment patterns visible in large school shark vertebrae using the microradiographic method are distinct even at the outer margins of the vertebrae; the region most difficult to interpret using surface reading methods (Holden and Vince 1973).

Conclusions

(1) Gummy shark and school shark can be held captive in large tanks for extended periods but school sharks are more difficult to hold than gummy sharks. School sharks need to continually swim to ventilate their gills, whereas gummy shark can ventilate their gills lying still on the substrate. The growth rates of gummy sharks held captive appeared to exceed the growth rates of those in the wild.

(2) Several vertebra-marking tissue-dyes can be administered to give highly distinct fluorescent marks in the vertebrae without having deleterious effects on the growth and health of gummy sharks and school sharks.

(3) Several vertebra-marking tissue-dyes, once deposited, persist as a permanent record of growth, unlike bone tissue in mammals.

(4) Density of mineralization of vertebrae is significantly higher during the winter, when overall growth of gummy shark was slowest, than during the other seasons.

(5) Growth-increment bands apparent on the surfaces of the articular cups and in sections of vertebrae correlated with bands of hyper-mineralized tissue formed during the winter.

(6) The number of growth-increment bands formed in recaptured tagged gummy sharks at liberty is less than the number formed in captive gummy sharks and school shark suggesting that the formation of growth-increment bands resulting from disturbance (i.e. 'disturbance check marks') is not as common in wild sharks as it is in captive sharks. Hence, inevitable formation of 'disturbance check marks' from unavoidable handling of captive sharks diminishes the value of using captive sharks for validating the assumption that growth-increment bands are deposited annually.

(7) Validation that growth-increment bands used for ageing sharks are annual is best done by injecting tagged sharks released in the wild.

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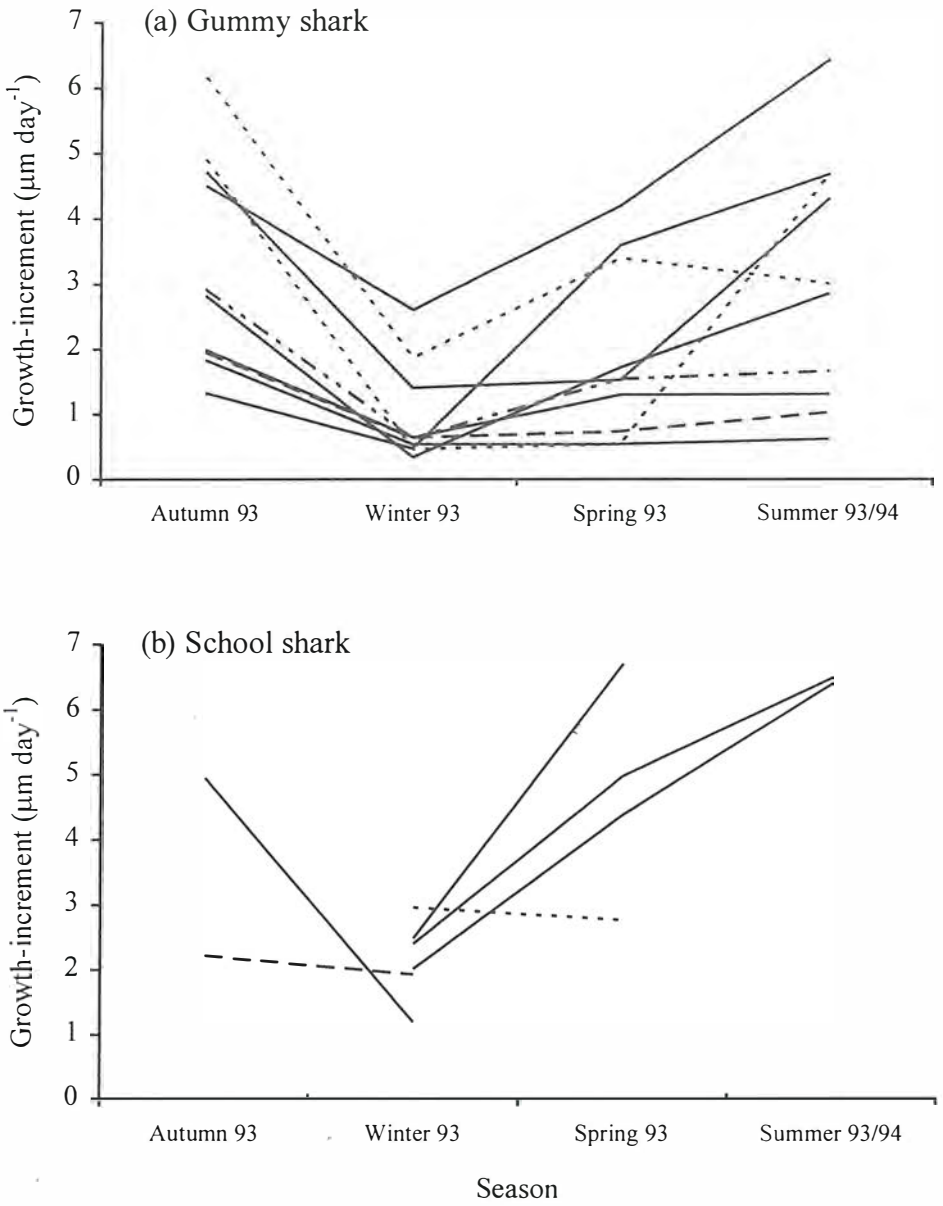

Figure 1.1. Vertebral growth increments for (a) ten gummy sharks and (six school sharks during each season.

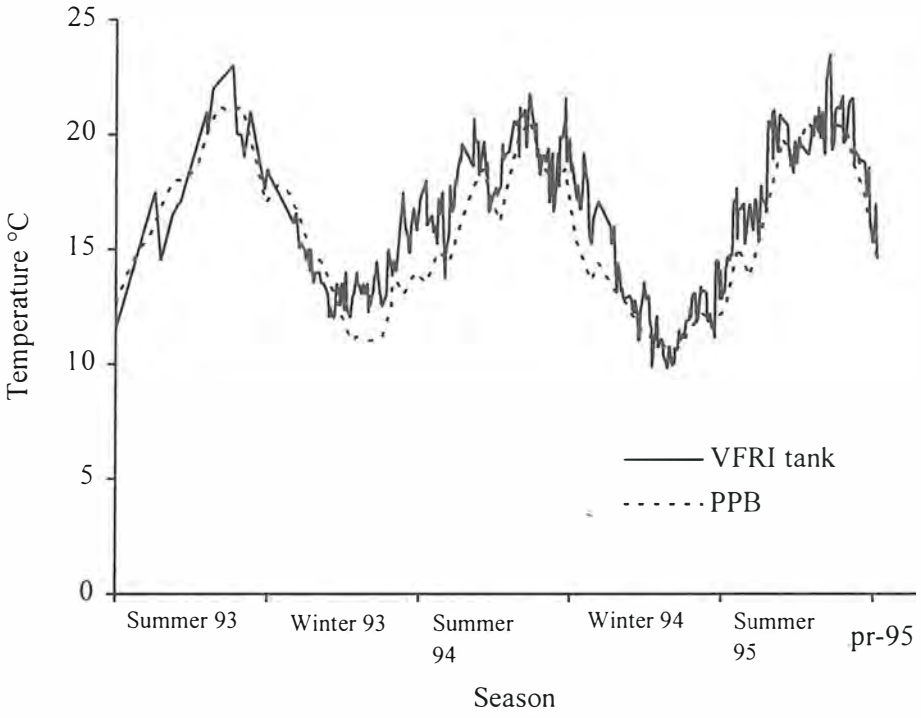

Figure 1.2. Water temperatures in the shark tank at the Victorian Fisheries Research Institute (VFRI) and in Port Phillip Bay (near Blairgowrie) (PPB) during October 1992 -April 1995.

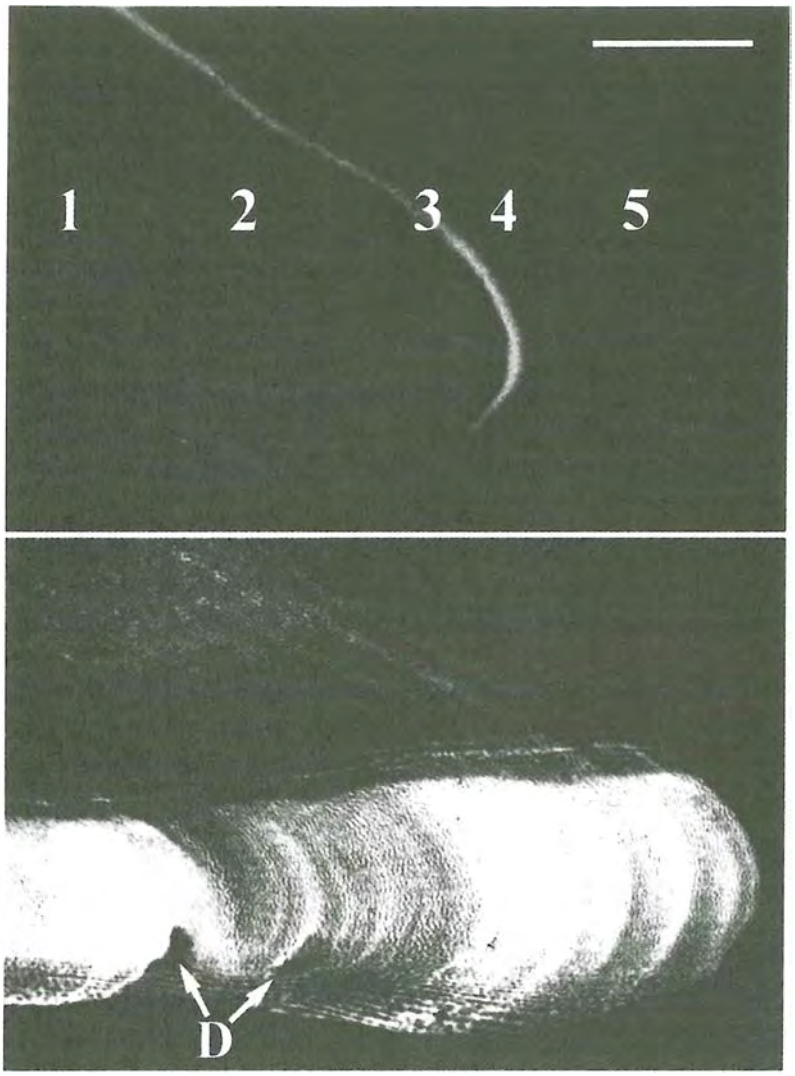

Figure 1.3. Fluorescence photomicrograph (top), and microradiograph (bottom) of the margin of a vertebral section from a captive gummy shark held 463 days show five bands resulting from five separate injections with vertebra-marking tissue-dyes. Disturbance marks (marked D), possibly caused by handling, are easily confused with growth-increment bands. (Scale bar: 0.5 mm)

Band	Injection date	Vertebra-marking tissue-dye	Dose (mg/kg)	Total length (mm)
1	9 Dec 1992	Alizarin complexone	25	750
2	23 Feb 1993	Alizarin complexone	25	830
3	1 Jun 1993	Calcein	25	940
4	7 Sep 1993	Oxytetracycline	25	980
5	21 Dec 1993	Xylenol orange	65	1040

Figure 1.4. Number of growth-increment bands versus period at liberty for four gummy sharks where the bands were identified by the alizarin stain method (o) and the microradiographic method (□) beyond the oxytetracycline (OTC) mark.

APPENDIX 2

TASK 2: DETERMINING MICROSTRUCTURE OF SHARK VERTEBRAE

Introduction

A vertebra from a gummy shark or school shark comprises a centrum, haemal arch and neural arch. The centum has two articular cups and four struts arranged as buttresses (intermedialia) between the articular cups. These structures are comprised of heavily mineralized cartilage which provides strength to the vertebra whilst the cavities behind the articular cups and between the struts are filled with non-calcified cartilage. The vertebra is enclosed, along with the other vertebrae, in a fibrous tissue sheath (perichondrium) which extends the full length of the vertebral column. The perichondrium enables the vertebrae to grow by producing cells (chondroblasts) which mature into cartilage cells (chondrocytes) and secrete extracellular matrix which depending on location on the surface or marginal edge of the vertebra is either mineralized (i.e. becomes cemented) or non-mineralized.

This task was designed to describe the microstructure of gummy shark and school shark vertebrae through application of light microscopy, microradiography and electron microscopy. The task involved determining (a) the spatial orientation of the structure of the calcified tissues, non-calcified tissues and the organic matrices of shark vertebrae in relation to alizarin red stained bands visible on whole vertebrae and hyper-mineralized and hypo-mineralized growth growth-increment bands on microradiographs of sectioned vertebrae, (b) how these structures are formed, and (c) how these structures might vary with increasing length of shark and, in the marginal region of vertebrae, time of the year.

Observations of mineralization processes inevitably have to be reconciled with physiological processes because it is these processes that govern the formation of mineralization patterns (Clement 1992). Hence this task involved describing the cells and cellular processes associated with the growth and mineralization of skeletal tissues of gummy shark and school shark, and how variations in mechanisms of mineralization can result in the growth-increment bands visible on the surface of whole vertebrae and in sectioned vertebrae that are used for age determination. Material presented for Task 2 is reproduced from Officer (1995).

Officer, R. A. (1995). Vertebral mineralisation patterns in gummy and school sharks and their utility in age determination. Ph.D. Thesis. 299 pp. (University of Melbourne: Melbourne.)

Methods

Histology for light microscopy

Initial sectioning of vertebrae from sharks stored chilled or frozen before laboratory processing indicated that the vertebral tissues dehydrate, shrink and become distorted, and cellular breakdown through autolysis causes inaccurate staining for complex chemicals because the target substances became denatured. These problems were avoided later by fixing vertebral tissues in formalin within 1 h of a shark dying. Stains tested to target specific cellular structures include haematoxylin and eosin for cell nuclei, toluidine blue

for cartilage, Giemsa (pH stain) and Periodic Acid Schiffs for muco-polysaccharides, Masson's trichrome for connective tissue, and picrocirus red for collagen orientation.

Longitudinal histological sections of vertebrae from eight gummy sharks were sectioned at 5 μm and stained in Azan. The position of hyper-mineralized growth-increment bands visible in each microradiograph was noted for slight irregularities in the morphology of the section. These irregularities were used as 'reference positions' from which the position of hyper-mineralized increments could be located in corresponding histological sections.

The eight histological sections chosen for analysis displayed particularly distinct hyper-mineralized increments in their microradiographs. These were easily related to the 'reference positions' that assisted in locating the hyper-mineralized increments in the histological section made from each of the neighbouring vertebrae.

Each section was examined microscopically (x 40 magnification). A colour video ('Sony 3-CCD (Model DXC-930P)', Sony Corp., Japan) fitted to the microscope was used to capture images of regions of interest within each section. Captured images were analysed using an image analysis system ('Optimas Version 4.1.1', Bioscan Inc., WA, USA). The software was used to draw a smaller rectangular region of interest or 'sampling quadrat' within each captured image.

Chondrocyte lacunae were identified using a feature of the software that shaded all areas of the sampling quadrat below a given grey level (threshold). This process was an objective means of identifying chondrocyte lacunae which capitalised on the distinctly lighter appearance of the chondrocyte lacunae compared with the surrounding extracellular matrix. Counts of the number of areas below the colour threshold provided a count of the number of chondrocyte lacunae within each sampling quadrat. Summation of the areas below the colour threshold provided a measure of the total area of the sampling quadrat occupied by the chondrocyte lacunae. Subtraction of this area from the total area of the sampling quadrat gave the area occupied by mineralized extracellular matrix. The analysis of each sampling quadrat also returned the mean area of the chondrocyte lacunae within the sampling quadrat, and the standard deviation about this mean size. The amount of extracellular matrix per chondrocyte was calculated by dividing the area of each sampling quadrat occupied by extracellular matrix by the number of chondrocytes within the sampling quadrat. The number of chondrocyte lacunae per unit area (1 mm^2) was calculated from the data.

The process was repeated a total of four times in several locations within the hyper-mineralized increment where a quadrat of identical dimensions was used. The parameters used to define a cell lacunae were kept constant by applying the same threshold prior to gathering the data. Once data were extracted from four quadrats in the hyper-mineralized increment, an identical process was used to extract data from four quadrats placed within an adjacent hypo-mineralized increment. For each parameter, the variability due to the degree of mineralization of the sampled quadrat, and between repeated samples within the same increment, was assessed using a multi-variate analysis of variance with repeated measures ('Systat for Windows Version 5', Systat Inc., IL, USA).

Microradiography

Besides providing information on the microstructure and development of the vertebrae, microradiography was evaluated as an alternative method to the one currently used for routine ageing of sharks from the southern shark fishery. Ferreira and Vooren (1991) applied microradiographic methods to age and growth of vertebrae in school shark from southern Brazil and several other authors have applied them to several other species of shark in various parts of the world (Cailliet *et al.* 1983, 1985, 1986, 1990; Cailliet and Radtke 1987; Yudin and Cailliet 1990).

For the initial work on gummy shark (Officer 1991), four to six anterior vertebrae were collected from each shark and stored at -22^{o}C. In the laboratory, the vertebrae were thawed, separated, trimmed of connective tissue, including the neural and haemal arches, and soaked in a 0.001% sodium hypochlorite solution until the fascia material could be removed effectively and the less mineralized hyaline cartilage between the lateral and dorso-ventral support struts (intermedialia) had dissolved. Small vertebrae (3-5 mm diameter) could be cleaned effectively within 12 h, but larger vertebrae (>15 mm diameter) required soaking for up to 3 days. To avoid over-exposure to sodium hypochlorite which can produce chalky or partly decalcified vertebrae, the concentration of sodium hypochlorite used in this preparation is lower than that used in other studies (Moulton *et al.* 1992; Gruber and Stout 1983). When clean, the treated vertebrae were placed under a flow of tap-water for about 30 min to remove traces of bleach and then left to air dry.

Up to 16 cleaned vertebrae were each placed in a compartment of a divided plastic basket for dehydration through a graded series of acetone solutions of 50% acetone and 50% distilled water for 24 h, 75% acetone and 25% distilled water for 24 h, and 100% acetone for 24 h. The vertebrae were then infiltrated with non-catalysed polyester through a graded resin series of 50% resin and 50% acetone for 24 h, 75% resin and 25% acetone for 24 h, and 100% resin for 24 h. Finally the vertebrae were removed from the plastic baskets, placed in individual reusable moulds and embedded in catalysed resin. To hasten the polymerisation process the embedded vertebrae were placed in an incubator overnight at 45°C.

To speed up subsequent sectioning, excess polymerized resin on the embedded vertebrae was removed on a water-cooled grinding wheel. Each embedded vertebra, attached by 'Kerr-Type 1' dental impression compound to a chuck mounted on a 'Leitz 1600' sawing microtome, was then sectioned longitudinally at a thickness of 100 µm. The most central section through the vertebra 'focus', and including both lateral intermedialia, was selected and flattened between two microscope slides held by spring-loaded clips.

The selected section from each vertebra was placed on top of a light-safe bag containing a 'Kodak Industrex SR' sheet of film (up to 35 sections per sheet) and radiographed in a 'Hewlett-Packard, Faxitron Series X-ray System (Model No. 43805N)'. The bag was positioned 48 cm from the focus of the instrument and exposed at 20 kV and 2 mA for 1 min 55 sec. After using standard X-ray development chemicals and procedures, the radiograph of each section, with a calibrating graduate marked in mm, was mounted in a 35-mm slide mount.

The number of growth-increment bands and their calibrated distances from the vertebra 'focus' were recorded from images of the radiographs, scanned at 1850 dots per inch by a 'Microtek Scan Maker (model MTS-1850)' 35-mm slide scanner and cropped to include only one side of the articular face of the vertebra and the mm graduate. The recording of data from these images, the down loading of these data for subsequent manipulation by the 'Excel 5.0' (Microsoft Corporation) spreadsheet program, and the computer disk storage of these scanned images stored in Tagged Image File Format were effected through application of the image analysis program 'Optimas Version 3.10' (BioScan Inc.).

Scanning Electron Microscopy

Vertebrae were removed from captive sharks that had been sacrificed, or had died in captivity during the tank age validation experiments. Animals of a variety of sizes were used and, in most cases, the 10th, 11th and 12th vertebrae were removed from the vertebral columns.

The extracted vertebrae were separated and the fascia material was removed by immersion in a bleaching solution of sodium hypochlorite (10-14 % available chlorine) for up to a week. Because the strength of the solution decreases rapidly, which reduces its ability to dissolve soft tissue, the bulk of the solution was decanted and replaced daily with fresh solution. The process was stopped once none of the residual soft tissue could be seen on the bleached skeletal remains. At this stage the vertebrae were washed carefully in running tap water to remove traces of the sodium hypochlorite and sodium chloride crystals which formed as a result of the treatment. The vertebrae were then dehydrated by placing the vertebrae for 24 h in each solution of a graded alcohol (LR Grade) series of 25% alcohol: 75% distilled water, 50% alcohol: 50% distilled water, 75% alcohol: 25% distilled water, 100% alcohol, and 100% alcohol.

The first of the three vertebrae extracted from each animal was placed on filter paper in a petri dish and dried under vacuum (30 mm mercury pressure). The specimens were then shattered with a scalpel blade to expose surfaces for study that would otherwise remain hidden in the intact vertebra. Although the vertebrae usually break along natural lines of weakness, care was taken to avoid their disintegration. The various pieces from each shattered vertebra were then mounted on scanning electron microscope stubs using conductive carbon dag and sputter coated with gold. Once coated all specimens were stored in a desiccator under vacuum.

The specimens were examined under a 'Phillips SEM505' scanning electron microscope operated in 2° mode to reveal surface topography. A series of surfaces was examined in each specimen including the articular face, the surface behind the articular face, the side of the intermedialia, the back of the intermedialia, and the top, side and back of the margin. Electron micrographs were taken of each surface at low power (x 78-163 magnification) and of a smaller area, within each of these frames, at higher power (x 312-655 magnification). The electron micrographs were examined for evidence of cellular growth and dormancy in each region.

In addition, annual periodicity in increment formation was investigated by examining vertebrae from each of 48 sharks (two male gummy sharks, two female gummy sharks, two

male school sharks, and two female school sharks caught on the same day on each of six separate occasions, 2 months apart).

To avoid damage to the margins of vertebrae though bleaching, fascia material was removed by immersion in an enzyme digestion solution of Papain - *Carica papaya* (Sigma Chemical Company) dissolved at the rate of 3 g per 100 ml distilled water - at an incubation temperature of 43°C for 24 h. Specimens were then mounted whole on scanning electron microscopy stubs using conductive carbon dag and sputter coated with gold, and stored in a desiccator under vacuum.

Results and Discussion

Understanding how normal skeletal structures of sharks, other chondrichthyans and the bony vertebrates are formed and maintained provides a basis for understanding how variations in the mechanisms of mineralization produce the growth-increment bands visible on the surfaces of shark vertebrae and in various planes of section through vertebrae. Mineralization processes in bone are well understood while there is lack of even the most basic knowledge of the skeletal structures, histology and cellular processes in most genera of chondrichthyans.

Function of Skeletal Tissues

Consideration of the functions of the skeleton helps understand its structure and histology. Calcification of the skeleton is necessary to achieve locomotion through hardening of bones to form a rigid framework on which to anchor muscles and within which to protect vital organs. However it is a physiological role of the mammalian skeleton that serves to contrast a major difference between mammals and sharks.

Mammalian bones are constantly reworked, being resorbed internally and replaced by new osteoblasts. In this way, calcium and other essential mineral metabolites are released. The bony skeleton acts as a reservoir of minerals and reference standard around which the serum level of mineral metabolites can be regulated hormonally. Resorption is a necessary and characteristic process of animals living in environments depleted in calcium.

Sharks are different, they inhabit a calcium rich environment and do not need to store calcium to meet their metabolic requirements. Sharks have lower serum calcium concentrations than their surrounding environment (Holmes and Donaldson 1969; Urist 1976) and need to prevent excess calcium from entering their bodies. Hence, resorption of mineralized tissues and the liberation of calcium into circulation from their skeletal structures would exacerbate the problem of excess calcium. It is logical, therefore, that the growth processes of mineralized tissues in sharks differ from those in mammals by stopping before resorption and interstitial replacement occurs. Once cemented, the mineralized tissue of sharks remains rigid and subsequent growth can only occur by apposition. It is this sequential, and, as shown in this study, periodic incremental growth of skeletal structures in sharks that forms the basis for studying these tissues as a record of the growth history, which can be used as a basis for age estimation.

Growth-increment bands result from disturbances in an otherwise continuous process of vertebral growth (Clement 1992), the understanding of which requires investigating the

interactions between vertebrae and their functional environment. In some species of shark, changes in the normal mineralization pattern occur quite abruptly; e.g. in the angel shark, *Squatina squatina* (Linnaeus), growth increments occur as distinct, discontinuous bulkheads (Clement 1986, 1992; Natanson and Cailliet 1990). In other species of shark, growth increments are visible as changes in the degree of mineralization in what is otherwise a continuously mineralized structure; e.g. in the wide sawfish, *Pristis pectinata* Latham (Clement 1992), and, as indicated by the present study, in gummy shark and school shark. Hence, an understanding of the means by which the degree of mineralization of vertebrae might vary is central to understanding the formation of growth-increment bands.

Structure of Skeletal Tissues

In sharks the process of mineralization is one of mineralizing cartilage which is a specialised form of connective tissue consisting of cells, chondrocytes and extracellular collagenic fibres embedded in an amorphous, gel-like matrix. Like all connective tissues, cartilage is composed of living cells and a non-living amorphous ground substance in which the cells are embedded. The way in which these components combine makes cartilage a specialised form of connective tissue that can be sufficiently rigid to provide the skeletal support necessary for locomotion and the protection of vital organs, and yet deformable enough at other locations to allow flexion and growth.

Except where it is exposed to the synovial fluid in joints, cartilage is enclosed in a membranous sheath called the perichondrium. The perichondrium is derived from mesenchyme and eventually develops two layers. The cells in the outer layer differentiate into fibroblasts and produce collagen to form a connective tissue sheath which is supplied with nutrients by blood vessels. In the inner layer, mesenchymal cells remain relatively undifferentiated and retain the potential to form precursors to cartilage forming cells (chondroblasts), and, eventually, mature cartilage cells (chondrocytes). It is this inner chondrogenic layer which allows the cartilage to grow by apposition, the addition of new matrix onto a pre-existing surface. Chondroblasts recruited at the chondrogenic layer may also contribute to the interstitial growth of the cartilage through their division and the secretion of extracellular matrix. Because the perichondrium is continuous over the surfaces of mineralized and unmineralized cartilage along the axis of the vertebral column in gummy and school sharks, vertebral growth-increment bands, which may result from cellular processes at work in the perichondrium, should share the same histological appearance in all the vertebrae of the vertebral column. The mechanisms of appositional and interstitial growth, and their relative importance in the formation of vertebral growth-increment bands, are discussed in greater detail below.

The principal types of cartilage are distinguished on the basis of the amount of amorphous extracellular matrix and the relative abundance of the collagenous and elastic fibres embedded within the extracellular matrix. The types of cartilage found in the elasmobranch skeleton include elastic cartilage, fibro-cartilage, hyaline cartilage and mineralized cartilage.

Elastic cartilage is the most cellular of the three cartilage types. The extracellular matrix of elastic cartilage is permeated with numerous branching elastin fibres and is a suitable material for the construction of shaped and flexible tissues. Examples of such tissues include the pinna of the ear and the walls of the eustachian tubes in humans.

Notwithstanding its functional importance in mammals, in the present study, it is not important to the formation of mineralized growth-increment bands in shark vertebrae.

Fibro-cartilage is a more important tissue as it forms tissues of the 'articular ball' for the articulation between adjacent vertebrae of gummy sharks and school sharks. The abundance of inelastic, yet flexible, collagen fibres in the extracellular matrix of fibro-cartilage allows articulation between attached hard tissues and explains the utility of this tissue in the formation of joints. Chondrocytes within fibro-cartilage are often arranged in parallel rows sandwiched between collagen bundles and orientated parallel to the stresses placed upon them. The fibre bundles are orientated parallel to the stresses placed upon them and are of great functional importance in the movement of the vertebral column. Fibro-cartilage can generally remain unmineralized throughout life and therefore may be removed during de-organification in preparation of the mineralized vertebral centra for age determination. When fibro-cartilage becomes mineralized it may contribute to the formation of the grow-increment bands visible in each of the vertebrae of the shark vertebral column.

Hyaline cartilage is the most prevalent form of cartilage in the vertebral columns of gummy sharks and school sharks. Macroscopically, hyaline cartilage has a semi-transparent, opalescent appearance. In gummy sharks and school sharks, hyaline cartilage (a in Figure 2.1) fills the cavities between the buttresses of mineralized cartilage (intermedialia) that are located dorsally, ventrally and laterally (b in Figure 2.1). The intermedialia separate and support the articular cups of the mineralized centrum. Hyaline cartilage is also the principal component of the haemal and neural arches (c in Figure 2.1).

The extracellular matrix of hyaline cartilage appears homogenous. Fine collagenic fibres are embedded within the extracellular matrix with a random orientation. The extracellular matrix stains heavily for complex carbohydrates (evidenced by positive staining during the periodic acid Schiff's reaction) and shows a marked affinity for basic dyes such as toluidine blue (Figure 2.2). These staining properties reflect the content of the extracellular matrix. The principal components of the ground substance are glycosaminoglycans and proteoglycans; polymers including the highly acidic sulphate groups chondroitin sulphate A and C (Takagi *et al.* 1984). The high concentration of these sulphate groups is thought to prevent the mineralization of hyaline cartilage (Takagi *et al.* 1984) and the penetration of blood vessels into the hyaline cartilage (Lee and Langer 1983; Lee *et al.* 1984). In the absence of blood vessels, the nutrients required for the nourishment of embedded chondrocytes (arrowed in Figure 2.2) must diffuse passively through the extracellular matrix.

The mineralized cartilage that makes up the vertebral centra of the gummy shark and school shark vertebral column represents a variant of fibro-cartilage and hyaline cartilage in which the density of the extracellular matrix is greatly increased through the deposition of mineral salts. Sufficient evidence has accumulated to substantiate the thesis that the location of these skeletal elements within the embryo is determined by interactions between the skeleton-forming mesenchyme and the epithelia with which the mesenchyme interacts (Hall 1978).

The mineralized articular cups of the articular face are made up by layers of mineralized fibro-cartilage, arranged with two distinct morphologies in a way that seems to provide

strength and rigidity to the vertebra. Chondrocyte lacunae in the articular cup and in the deeper packing cartilage have markedly different orientations. The surface of the articular cup (a in Figure 2.3) is comprised of near parallel layers of chondrocytes (b in Figure 2.3) disposed radially from the focus of the centrum to the margins at a slight angle to the articular cup. Between the layers of chondrocytes lay bundles of collagen fibres which run from deep in the articular cup, through the fibro-cartilage to join the adjacent vertebra. The parallel layers of chondrocytes on the articular cup are thickest at the margin of the centra and might partly explain the better resolution of growth-increment bands near the focus than near the vertebral margin where they are more obscured.

Beneath the radially disposed chondrocyte lacunae of the articular cups lay small chondrocyte lacunae which are embedded in a densely mineralized extracellular matrix (c in Figure 2.3) The chondrocyte lacunae are arranged circumferentially in layers, at right angles to the parallel rows of chondrocyte lacunae in the articular cup. The mineralized extracellular matrix that surrounds the chondrocyte lacunae in the articular cup is the most heavily mineralized of any extracellular matrix in an entire vertebra. It is this layering of tissues in the articular cup that may provide the vertebra with its great strength and rigidity.

In contrast, the chondrocyte lacunae within the intermedialia are arranged amorphously, and, together with their extracellular matrix, act as packing cartilage. In bony vertebrates, chondrocytes in the mineralized tissues appear to become starved of nutrients and die. The extracellular matrix previously secreted by the now seemingly necrotic chondrocytes is sometimes resorbed and then replaced by bone. The development of mineralized tissues in elasmobranchs differs from that in mammals in that chondrocytes incorporated into the mineralized tissues of elasmobranches remain vital. The vitality of the chondrocytes in the intermedialia is even evident in the oldest chondrocytes; i.e. those near the focus of the centrum (arrowed in Figure 2.4). Because these chondrocytes are vital, they must be supplied with nutrients by some source. In some species of elasmobranch, large blood vessels penetrate into the vertebral intermedialia (Hoenig and Walsh 1982; Clement 1986, 1992). For example, small channels (canaliculi) penetrating the mineralized matrix and connecting neighbouring chondrocytes have been described in the vertebrae of the Brazilian school shark (Ferreira and Vooren 1991). However, canaliculi were not evident in the present study of gummy shark and Australian school shark. The lack of canaliculi in the vertebrae of these species suggests that the vital chondrocytes buried deep within the mineralized vertebral matrix must be receiving their nutrients by diffusion of metabolites through the mineralized extracellular matrix, or the cells are so densely packed that they just touch each other.

Experiments with vertebra-marking tissue-dyes provide supporting evidence for this hypothesis. Whilst vertebra-marking tissue-dyes are incorporated specifically into sites of active growth, they initially appear diffusely throughout all the mineralized extracellular matrix. This diffuse marking does not mean that permanent deposition of mineral is occurring within the already mineralized extracellular matrix. Rather, dyes initially circulate dissolved in the sera that normally flows through the extracellular matrix. Examination of vertebral sections taken from sharks several years after injection show the fluorescent tissue-dyes are confined to discrete narrow bands (Smith 1984; Kusher *et al.* 1992). Either the diffuse fluorescence seen previously throughout the extracellular matrix has been washed out with time or there is a very slight passive physico-chemical uptake

similar to passive ionic exchange of minerals on all free surfaces. The flux of apparently unbound vertebra-marking tissue-dye through the mineralized matrix and the permanent binding of tissue-dye at sites of incorporation suggest that the underlying fibrous matrix is not replaced even if a small proportion of the mineral within it is (Clement 1992). This is further evidence that the fundamental structure of the mineralized tissues in elasmobranchs are permanent and physically and chemically unreactive. As such, the growth-increment band that are formed within shark vertebrae may be confidently used as a record of some aspects of a shark's life history.

Growth of the Vertebral Cartilages

The appositional growth of the persistently hyaline and eventually mineralized derivatives of such cartilages are essentially similar. Appositional growth occurs through the proliferation and differentiation of undifferentiated mesenchymal cells into cartilage forming cells (chondroblasts) (a in Figure 2.5 and 2.6) at the inner chondrogenic layer of the perichondrium (b in Figure 2.5 and 2.6). As the chondroblasts mature they differentiate into chondrocytes (c in Figure 2.5 and 2.6) and secrete extracellular matrix about themselves and eventually completely enclose themselves in extracellular matrix. Once enclosed in extracellular matrix the cartilage cells (chondrocytes) reside in small cavities (lacunae) (d in Figure 2.5). In this way chondroblasts contribute new extracellular matrix onto the pre-existing surface of the cartilage and contribute to the appositional growth of the pre-existing cartilage. Appositional growth of the hyaline and mineralized cartilages can continue throughout adult life in elasmobranchs.

Interstitial growth can occur within the mass of cartilage through the continued secretory activity and growth (hypertrophy) of the chondrocytes. Whilst the interstitial growth may occur at depth within hyaline cartilages it can only occur in the eventually mineralized cartilages within the newly formed but as yet unmineralized band of extracellular matrix (d in Figure 2.6) secreted by chondrocytes just recruited from the chondrogenic layer of the perichondrium. Interstitial growth is not possible once the cartilage mineralizes because the rigidity of the mineralized extracellular matrix prevents the continuing hypertrophy of the chondrocyte lacunae and any separation of chondocytes through the secretion of further extracellular matrix.

Chondrocytes may continue to divide by mitosis in the hyaline cartilages and in the unmineralized periphery of the eventually mineralized cartilages. When this occurs in hyaline cartilage, the tendency is for daughter cells to reside temporarily in the same lacuna (e in Figure 2.5), with only a thin partition of extracellular matrix formed between them. Such groups of two to four chondrocytes are called isogenous groups because each group represents the progeny of a single chondrocyte that has undergone mitotic divisions. Hypertrophy of isolated chondrocytes and chondrocytes in isogenous groups in conjunction with the continued secretion of extracellular matrix separates individual chondrocytes to a greater extent and adds volume to the pre-existing mass of hyaline cartilage.

The extent to which interstitial growth contributes to the overall growth of the mineralized centrum is dependent on the level of activity of the chondrocytes in the peripheral as yet unmineralized band. The volume of extracellular matrix secreted by chondrocytes prior to

mineralization and their degree of hypertrophy are of particular importance. If mineralization of the extracellular matrix in this band proceeds rapidly, the chondrocytes cannot undergo a significant amount of hypertrophy and remain relatively small as they are cemented into the mineralized extracellular matrix. Conversely, if mineralization proceeds more slowly, the chondrocytes can undergo hypertrophy for longer and attain a larger size before being cemented into the mineralized extracellular matrix.

Mineralization of Cartilage

The processes by which extracellular cartilage matrix becomes mineralized through the impregnation of mineral salts are not fully understood (Ham and Cormack 1979), and determining these processes is beyond the scope of this study. Nevertheless it is important to understand the nature of mineralization.

Mineralization of cartilage occurs when mineral salts, primarily of calcium, are laid down in the extracellular matrix. Although mineral deposits in mineralized elasmobranch cartilages eventually become crystalline and closely resemble apatite (Urist 1961; Moss 1977), the first deposits of mineral are thought to be amorphous calcium phosphate, $Ca_3(PO_4)_2$. Determining how crystals of apatite form from amorphous calcium phosphate is a separate problem from determining why mineral salts are initially deposited in the extracellular cartilage matrix. Hence, only the initial process of mineral deposition is considered here.

Mineral salts deposited in the cartilage have to come from blood via the circulating extracellular fluid. Most of the calcium in the blood is bound to protein and does not directly combine with phosphate ions (Urist 1961). However, the remainder of calcium in blood is ionised and can combine with phosphate ions to produce calcium phosphate. In elasmobranchs, the circulating sera are normally saturated with calcium and phosphate ions (Urist 1961). Since calcium phosphate is very insoluble, regulatory mechanisms must operate to prevent an increase in calcium phosphate levels that would cause it to precipitate in the blood. In the mineralizing cartilages, however, the precipitation of calcium phosphate is necessary. It follows that there must be a mechanism operating in mineralizing cartilage that increases the local concentration of calcium phosphate in the extracellular fluid sufficiently to cause calcium phosphate to precipitate locally. Alternatively, newly formed extracellular matrix may possess some chemical or physical property causing it to take up calcium phosphate selectively from the extracellular fluid in which the calcium and phosphate might ordinarily stay in solution.

It is known that precipitation of calcium phosphate normally occurs in mammalian tissue culture at physiological concentrations of calcium and phosphorus ions (Ham and Cormack 1979). These findings have turned attention away from determining why mineralization occurs in the organic matrix of cartilage and bone, to ascertaining why the extracellular substances of ordinary connective tissues do not also become mineralized. It is now believed that mineralization inhibitors act normally to prevent mineral deposits from forming in ordinary connective tissues (Ham and Cormack 1979).

It seems that several processes may work concurrently to permit the mineralization of cartilage and bone. Increases in the concentration of phosphate ions may occur locally, accompanied by a reduction in the normal ability of mineralization inhibitors to prevent

mineralization from occurring. Both processes may be mediated by the chemical and physical properties of the local chondrocytes and extracellular matrix.

Mineralization of Gummy Shark and School Shark Vertebral Cartilage

Significant differences were detected between the mean area, and hence by inference volume, of cell lacunae measured in hypo-mineralized increments and those measured in hyper-mineralized increments. Cell lacunae were larger in hypo-mineralized increments (Table 2.2), and had a more variable size (i.e. higher standard deviation) than those measured in hyper-mineralized increments. No difference in mean area or in standard deviation of cell size was detected between repeated measures of mean area in the quadrats (Tables 2.1 and 2.2).

Table 2.1. Comparison of mean measurements of selected cellular parameters between hyper-mineralized and hypo-mineralized growth increments (n = sample size, SD = standard deviation).

Parameter measured	n	Type of growth-increment			
		Hyper-mineralized		Hypo-mineralized	
		Mean	SD	Mean	SD
Mean area of cells (μm^2)	32	15.4	5.0	29.0	10.0
SD in cell area (μm^2)	32	8.3	4.4	17.3	7.6
Matrix area per cell (μm^2)	32	164.8	50.7	115.6	16.7
Cells per mm^2	32	5.5	1.2	6.6	0.7

Table 2.2. Results of analyses of variance of repeated measures of cellular parameter differences between hyper-mineralized and hypo-mineralized growth increments (df = degrees of freedom; MS = mean square; F = F ratio, P = probability value).

Parameter measured	Source of variation	df	MS	F	P
Log mean area of cell lacunae	Increment type	1	6.17	18.82	<0.01
	Error	7	0.33		
SD in cell lacunae	Increment type	1	1304.84	11.91	0.011
	Error	7	109.55		
Log matrix area per cell lacuna	Increment type	1	1.68	16.59	<0.01
	Error	7	0.10		
Cell lacunae per unit area	Increment type	1	17.94	7.65	0.028
	Error	7	2.35		

The greater average area of the lacunae in hypo-mineralized increments was reflected in the significantly greater proportion of the area occupied by lacunae in these increments (Table 2.1 and Table 2.2). No difference was detected between repeated measures of the proportion of the area occupied by lacunae.

More cells per unit area were detected in hypo-mineralized increments than in hyper-mineralized increments (Table 2.1 and Table 2.2). No statistically significant difference was detected between the repeated measures made of cells per unit area.

The hyaline cartilages remain unmineralized except where they occur in close proximity to the heavily mineralized extracellular matrix of the vertebral centra. This close proximity of mineralization sites in the hyaline cartilage to the mineralized extracellular matrix of the centra suggests that initiation of mineralization may be mediated by chemical or physical properties of the neighbouring mineralized extracellular matrix. Pioneer mineralization (a in Figure 2.7) of the hyaline extracellular matrix initially occurs at a distance from chondrocytes (b in Figure 2.7). These chondrocytes appear to inhibit mineralization of the extracellular matrix in the perilacunar envelope that closely surrounds their lacunae (c in Figure 2.7).

Chondrocytes in the hyaline cartilage seem to lose the ability to inhibit mineralization of the extracellular matrix. Eventually the mineralizing extracellular matrix encroaches further into the peri-lacunar territory once secreted by each chondrocyte until the lacunae are completely engulfed in mineralized extracellular matrix (Figure 2.8). Appositional growth of the mineralized centrum is achieved when individual chondrocyte lacunae (a in Figure 2.8) are engulfed by the consolidated mineralizing front (b in Figure 2.8) through coalescence and fusion between the spheritic beads of mineral deposited in the extracellular matrix. Through this process the mineralization of the hyaline extracellular matrix of chondrocytes becomes continuous with the heavily mineralized extracellular matrix of the centrum. This is the process by which mineralization of cartilage is achieved in other species of elasmobranch (Clement 1992) and mammal (Boyde and Jones 1983).

Mineral accretion occurs in this way at interfaces between the hyaline cartilage and the mineralized centrum. These surfaces include the interfaces between the hyaline cartilage and the surfaces beneath the articular cups of the centrum and between the hyaline cartilage and the sides of the intermedialia (interface between a and b in Figure 2.1). Since hyaline cartilage in the cavities between the intermedialia penetrates right down to the focus of the vertebral centra (d in Figure 2.1), active accretion of mineral occurs near the focus. Accretion of mineral from the hyaline cartilage onto the sides of the intermedialia results in a thickening of these margins and a slow constriction of the space between the intermedialia.

Mineral accretion on the outer margins of the intermedialia (Figure 2.6) occurs through a process which is essentially similar to that outlined above for hyaline cartilage. However, at the outer margins of the intermedialia the extracellular matrix into which mineral salts are deposited (d in Figure 2.6) is markedly different from the extracellular matrix of the hyaline cartilage. The extracellular matrix at the outer margins of the intermedialia contains less amorphous ground substance than the extracellular matrix of the hyaline cartilage. At

the outer margins of the intermedialia the mineralizing front (e in Figure 2.6) runs parallel to, but inside, the perichondrium. It is the incremental, and possibly periodic, growth of the mineralized parts of the vertebral centra of elasmobranch skeletons forms the basis of the study of these tissues as records of the growth history of the elasmobranch.

On the articular cup, chondrocytes becoming incorporated into the mineralizing front indicate that some appositional growth takes place over the articular surface (Figure 2.9), but most occurs at the margins of the vertebrae. The perichondrium extends just inside the lip of the articular cup and active accretion takes place at this site. Accretion of mineral over the surface of the articular cup was reported by Clement (1986, 1992) who describes 'igloos of mineral engulfing chondrocyte lacunae' which may represent the forming walls of chondrocyte lacunae being incorporated into the articular cup. Vertebra-marking tissue-dyes were rarely seen on the articular cup except at the periphery of the structure which suggests that the appositional accretion of mineralized matrix onto the face of the articular cup is likely to be only slight and a very slow process.

Nevertheless, any accretion of mineral onto the articular cup does have implications for the alizarin stain age-reading method because growth-increments bands being counted on the articular cup are being overgrown. When vertebral growth is slow, as it is in older animals, the obscuring of growth-increment bands through overgrowth is likely to be more pronounced.

The Formation of Growth-Increment Bands

Whilst the degree of mineralization of the extracellular matrix in the mineralized cartilages is normally homogenous, occasionally increment bands can be seen in histological sections of gummy and school shark vertebrae (large arrows in Figure 2.10). In histological sections of Brazilian school shark, vertebral growth-increment bands were apparently observed frequently (Ferreira and Vooren 1991). Growth-increments seen in histological sections form bands which traverse the intermedialia, running parallel to the peripheral perichondrium (small arrows in Figure 2.10).

The differential histological staining of increments indicates that they are associated with bands of localised variation in the amount of muco-polysaccharides in the mineralized extracellular matrix (Figure 2.10). Such changes in the proportions of muco-polysaccharides in the extracellular matrix may indicate that the overall mineral density of the mineralized cartilage is moderated simply through the deposition of extra mineral salts in the matrix at some times.

The parallel orientation of growth-increment bands to the perichondrium suggests that the cellular growth processes at work in the perichondrium contribute to the formation of these bands. The staining reactions of the increments suggest that their formation is related to cycles of mineralization which results in variations in the proportions of muco-polysaccharides and fibrous tissues incorporated into the extracellular matrix. Hence, the formation of some growth-increment bands may be directly related to the cellular metabolism of the mineralized cartilage.

Whilst the growth-increments bands seen in histological sections can sometimes be seen in microradiographs of vertebral sections as zones of differing mineral density (a in Figure

2.11) to that of the surrounding extracellular matrix, it is important to recognise that such radiographic representations of mineral density are a reflection of the overall mineral density of the mineralized cartilage and not just the density of its mineral extracellular matrix. Therefore growth-increment bands are best considered as zones of variation in the overall degree of mineralization of the mineralized tissue. As such, their formation can be understood through a consideration of the factors that might contribute to fluctuations in the degree of mineralization of the extracellular matrix. This approach considers the cellular processes that may cause local variations in the mineral density of the whole tissue, as well as the important contribution to mineral density made by the density of substances within the extracellular matrix.

In taking this approach to understanding the mechanisms of formation of growth-increment bands it is useful to compare the contributions made to the overall density of the mineralized cartilage by the ground substance of the extracellular matrix itself, and by the chondrocyte lacunae within it. Chondrocyte lacunae (b in Figure 2.11) are radiolucent compared with the dense, radio-opaque extracellular matrix (c in Figure 2.11). Therefore, the lacunae can be considered as 'voids' within the mineralized extracellular matrix. Changes in the volume occupied by chondrocyte lacunae within a given volume of extracellular matrix will therefore effect the overall mineral density of the tissue. Hence, local variation in the overall mineral density of the mineralized tissue could result from variation in the volume occupied by chondrocyte lacunae within the extracellular matrix, or variation in the density of the mineralized extracellular matrix, or a combination of the two effects.

The cellular processes involved in the appositional and interstitial growth of the centrum could all contribute to a variation in the volume occupied by the chondrocyte lacunae within the matrix. Temporal changes in the number of chondroblasts recruited from the chondrogenic layer, and the extent to which they proliferate, may directly affect the number of chondrocytes that subsequently differentiate from the chondroblasts. The subsequent hypertrophy of the chondrocytes would result in a hypo-mineralized increment if the amount of extracellular matrix secreted was independent of the increased number of chondrocytes. However, the amount of extracellular matrix secreted may not be independent of the number of chondrocytes. Therefore the hypo-mineralizing effect of having an increased number of chondrocytes incorporated in the matrix might be countered by the hyper-mineralizing effect of the secretion of more extracellular matrix.

The extent to which chondrocyte lacunae are able to undergo hypertrophy before becoming mineralized may have a more significant effect on the overall mineral density of newly formed mineralized cartilage. If individual chondrocyte lacunae are each able to grow larger before mineralization of the surrounding extracellular matrix prevents their continued growth they will each occupy a larger volume within the extracellular matrix and could therefore create a relatively hypo-mineralized band in the newly formed mineralized cartilage. Alternatively, if the extracellular matrix becomes mineralized before the chondrocyte lacunae have undergone any significant degree of hypertrophy the chondrocyte lacunae will each occupy a smaller volume within the extracellular matrix and could therefore create a relatively hyper-mineralized band.

Hence, the speed with which the extracellular matrix is mineralized may affect the overall mineral density of newly mineralized cartilage by moderating the extent to which chondrocytes can undergo hypertrophy. Factors which might affect the speed with which the extracellular matrix can be mineralized might include variation in the activity of inhibitors of mineralization and limitation in the availability of the mineral salts required for deposition in the extracellular matrix during mineralization.

Despite the limited availability of phosphorus in seawater (Urist 1964), elasmobranchs are able to saturate their sera with phosphate ions that are readily available for metabolic processes (Urist 1961). It follows that temporal variation in the environmental availability of phosphorus might affect the level to which elasmobranchs can saturate their sera with phosphate. Consequently such variation in the environmental availability of phosphorus might affect the amount of phosphate available for deposition as calcium phosphate in the mineralizing cartilages (Halstead 1974). If greater amounts of calcium phosphate permit the more rapid mineralization of the extracellular matrix at some times, then hypertrophy of the chondrocyte lacunae might be prevented sooner. In this situation the 'voids' occupied by newly incorporated lacunae will be smaller and the cartilage will appear relatively hyper-mineralized.

The present review describes some of the mechanisms by which growth-increment bands used as the basis of age determination studies may form in shark vertebrae. Whilst these mechanisms have been discussed separately, it must be stressed that some, or all of them, probably work in concert to produce growth-increment bands in elasmobranch vertebrae. The explanations of formation of increment presented here support the hypothesis of Clement (1986, 1992) that external growth increments are the manifestations of internal changes in mineralization. It has been suggested that growth-increment bands in elasmobranch vertebrae probably form in response to the changing proportions of cells to extracellular matrix rather than any significant variation in the degree of mineralization of the extracellular matrix itself (Clement 1992). The mechanisms by which the proportions of cells to extracellular matrix might alter were not suggested by Clement (1992).

Conclusions

(1) Parallels exist in the processes of growth of the mineralizing tissues between sharks and mammals, and, as such, improved understanding of the mineralization processes in higher vertebrates will provide improved understanding of the mineralization processes in sharks.

(2) Whilst both appositional and interstitial growth occur in the shark vertebrae, interstitial growth occurs only in the soft hyaline cartilages and not in the calcified cartilage of the articular cups and intermedialia.

(3) Growth processes occurring in the perichondrium results in the formation of growth-increment bands.

(4) During periods of hypo-mineralization chondrocyte lacunae attain a greater size.

(5) Hypertrophy of chondrocyte lacunae has a more significant effect on vertebral growth than the rate of recruitment and the degree of hypertrophy is greatest during periods of fast growth.

(6) Variations in the degree of mineralization of the extracellular matrix probably correlate with the formation of growth increments.

(7) The importance of the formation of extracellular matrix to hypertrophic activity and extracellular mineralization means that environmental factors, such as phosphate availability, which affect the ability of chondrocytes to secrete and mineralize the extracellular matrix may induce the formation of growth-increment bands.

(8) Periodic fluctuations in phosphate availability may correlate with the periodic formation of regions of differing mineral density in the extracellular matrix of shark vertebrae.

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Figure 2.1. (Left.) Photomicrograph of a transverse section of a gummy shark vertebra. (Section is caudal to the equatorial plane). (Picrosirius red stain; scale bar: 5 mm).

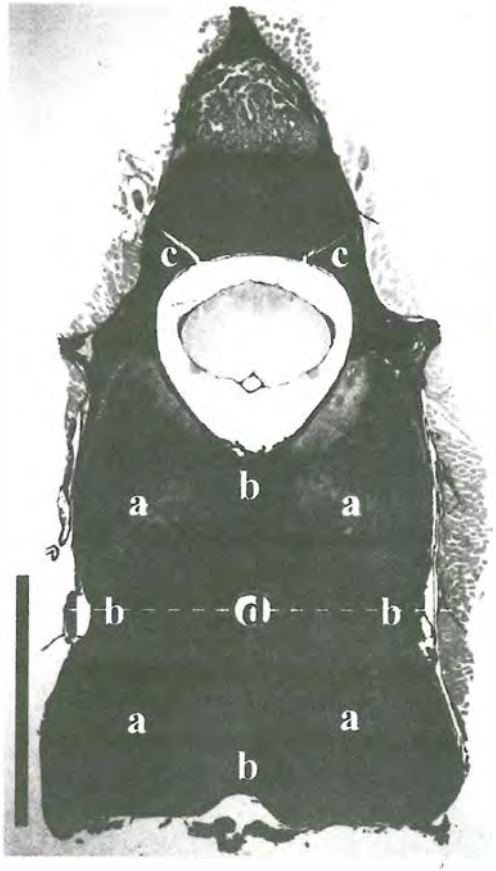
The centrum is embedded in hyaline cartilage (a). The intermedialia (b) form lateral and dorso-ventral buttresses within the mineralised centrum which is surmounted by the neural arches (c). The site of the primitive notochord now forms the focus (d) of the centrum. Dashed line indicates the plane of section used in Figure 2.11.

Figure 2.2. (Top right.) Photomicrographs showing the staining reactions of hyaline cartilage from a gummy shark to the periodic acid Schiff's reaction (top) and to toluidine blue (bottom). (Scale bar: 5 μm).

The high concentration of acidic sulphate groups in the extracellular matrix may prevent the penetration of blood vessels. Embedded chondrocytes (examples arrowed) are vital and must be supplied with nutrients by the diffusion of nutrients through the extracellular matrix.

Figure 2.3. (Bottom right.) Scanning electron micrograph of a side view through the articular cup of a gummy shark vertebra. (Focus of vertebrae is left; vertebral margin is right). (10 kV; scale bar graduations: 0.1 mm).

The inorganic vertebra was fractured in a longitudinal plane to show the appearance of the tissue beneath the articular face (a). Chondrocyte lacunae are arranged in radially disposed parallel layers in the mineralised tissues of the articular face (b), and circumferentially in the packing cartilage of the intermedialia (c) beneath the articular cup. This structure could give the centra great strength. Collagen fibres in neighbouring fibro-cartilage have been removed in this preparation but would have inserted into the spaces between the radiating layers of chondrocytes and their lacunae.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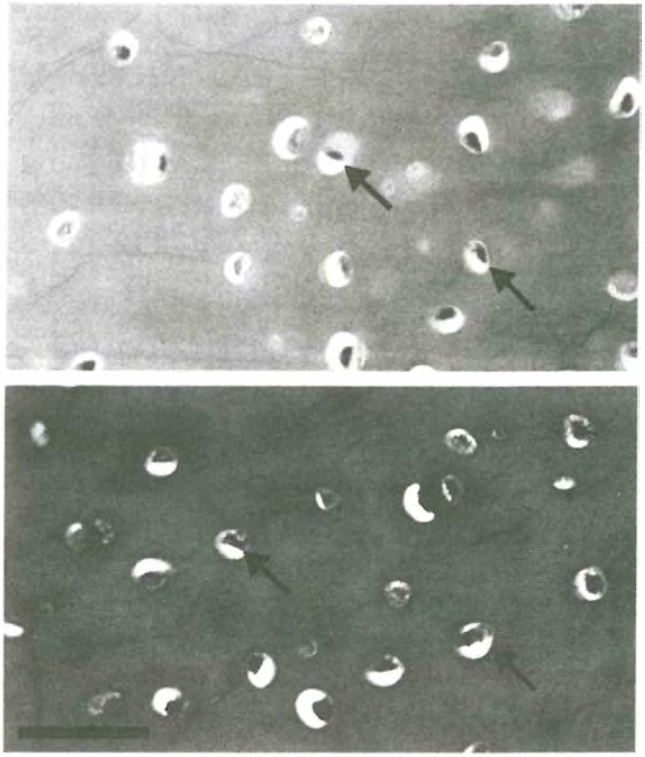

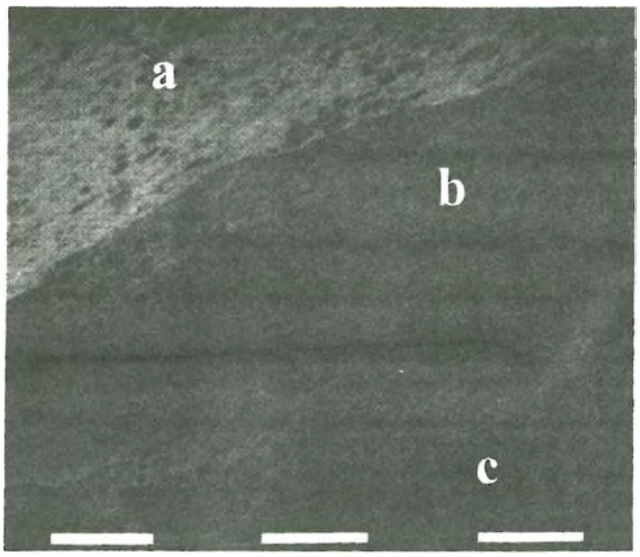
a
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Figure 2.4. (Top left.) Photomicrograph of the mineralised cartilage in the intermedialia close to the focus of a school shark vertebral centrum. (Haematoxylin and eosin stain; scale bar: 5 μm).

Even at this deepest level the embedded chondrocytes (examples arrowed) remain vital. The lack of canaliculi between neighbouring lacunae suggests that each chondrocyte must be nourished by the diffusion of nutrients through the mineralised matrix or the cells touch one another.

Figure 2.5. (Bottom left.) Photomicrograph showing the growth mechanisms of hyaline cartilage. (Haematoxylin and eosin stain; scale bar: 5 μm).

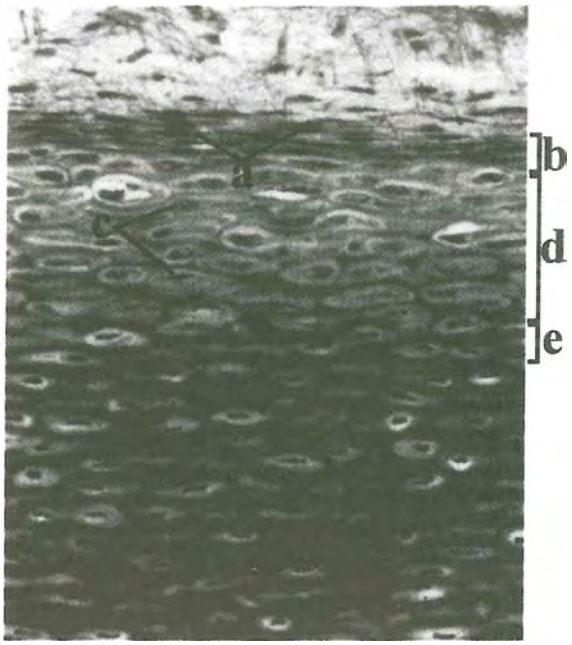
Chondroblasts (a) are formed from the differentiation of mesenchymal cells in the chondrogenic layer (b) of the perichondrium. Their secretion of an extracellular matrix incorporates the chondroblasts onto the hyaline cartilage resulting in its appositional growth. Incorporated chondroblasts are termed chondrocytes (c) and each occupy a lacuna (d). Hypertrophy and mitotic division of chondrocytes results in the interstitial growth of the hyaline cartilage. Groups of chondrocytes within the one lacuna (e) are called isogenic groups because they represent the progeny of a single chondrocyte that underwent mitotic division.

Figure 2.6. (Top right.) Photomicrograph showing the growth mechanisms of mineralised cartilage. (Azan stain; scale bar: 10 μm).

Chondroblasts (a) are formed from the differentiation of mesenchymal cells in the chondrogenic layer (b) of the perichondrium and incorporated onto the cartilage in a similar fashion to the appositional growth of hyaline cartilage. Mineralisation of the extracellular matrix prevents interstitial growth within the mineralised cartilage. However, mitotic division of chondrocytes (c) and hypertrophy of their lacunae is possible within the unmineralized band of extracellular matrix (d) peripheral to the encroaching mineralisation front (e).

Figure 2.7. (Bottom right.) Photomicrograph of longitudinal section of gummy shark vertebra showing mineralisation of the extracellular matrix in hyaline cartilage. (Azan stain; scale bar: 5 μm).

Mineral deposition (a) occurs at a distance from the chondrocytes (b) and conforms to the shape of the peri-lacunar envelopes of unmineralized extracellular matrix (c) that surround each chondrocyte. Individual chondrocytes may actively inhibit mineralisation of the extracellular matrix in close proximity to themselves but either allow, or cannot prevent pioneer mineralisation of the extracellular matrix occurring at a distance.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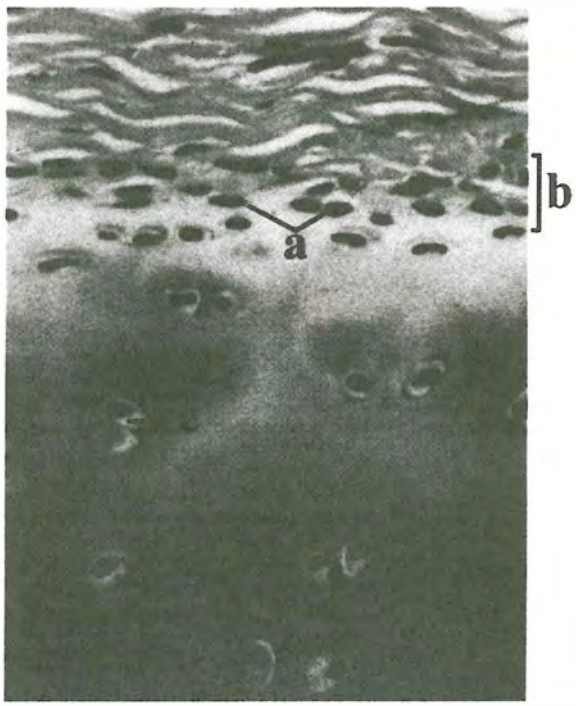
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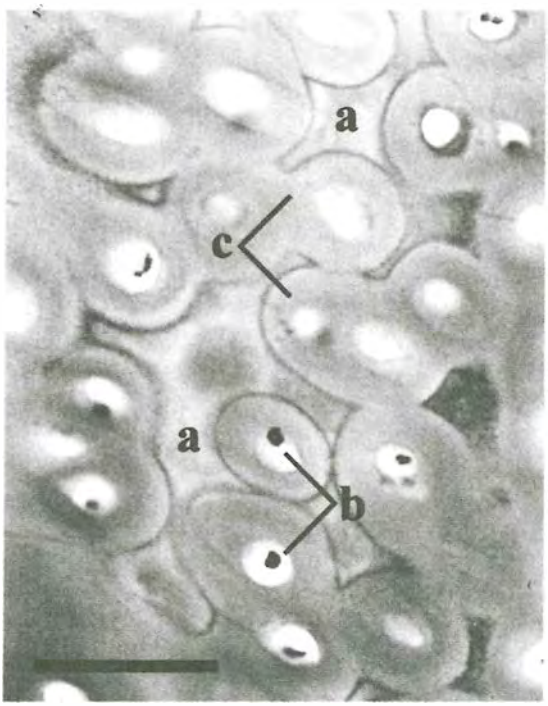
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Figure 2.8. (Top left.) Photomicrograph of transverse section of gummy shark vertebra showing mechanism of appositional growth. (Azan stain; scale bar: 5 μm).

Appositional growth of the mineralized centrum (bottom) is achieved through the incorporation of chondrocytes (a) from surrounding hyaline cartilage (top). Mineralization of the extracellular matrix (b) initially occurs remotely from chondrocytes which remain in peri-lunar envelopes of unmineralized matrix (c). The chondrocytes lose the ability to inhibit proximal mineralisation and, in the mineralizing front (d), mineralization of the extracellular matrix encroaches right up to the cell membrane of each chondrocyte. Mineralized chondrocytes (e) remain vital despite being entombed in a lacuna space smaller than their original peri-lacunar envelope.

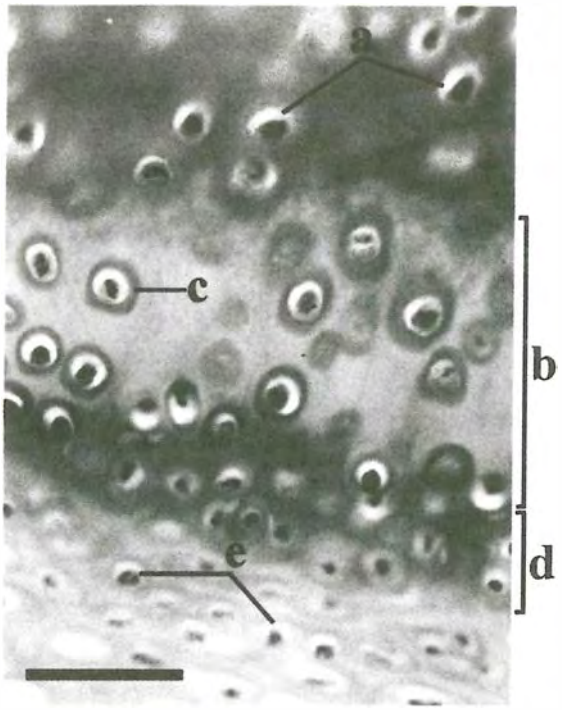
Figure 2.9. (Bottom left.) Scanning electron micrograph of an organic preparation of the surface of the articular face just inside the margin. (20 kV; scale bar: 0.1 mm).

The spheritic beads of mineral (a) that form the lacunar walls (b) around each chondrocyte (c) are visible. The accretion of mineral inside the margin of the articular face results in a thickening of the layered columnar chondrocyte lacunae in the articular face with growth of the centra. This thickening covers heavily mineralised regions of the extracellular matrix deeper within the centrum and may explain why alizarin staining at the margins is poor compared to that nearer the focus.

Figure 2.10. (Top right.) Photomicrograph of mineralised cartilage in a gummy shark vertebra showing a growth-increment 'band' appearing at this magnification as a band (large arrows) in the mineralised extracellular matrix running parallel to the perichondrium (small arrows). (Alcian blue/periodic acid Schiff's stain; scale bar: 20 μm).

Figure 2.11. (Bottom right.) Microradiograph of a longitudinal section through the lateral intermedialia of a gummy shark vertebra showing growth-increment bands (a) as bands of tissue with a higher overall mineral density. (Scale bar: 1 mm.) (Plane of section: dotted line in Figure 2.1.)

Incorporated chondrocyte lacunae are relatively radiolucent compared to the extracellular matrix in which they are embedded. As such, variation in the volume occupied by lacunae could effect the overall mineral density of the cartilage. Since growth-increment bands represent localised variations in overall mineral density their formation could not only result from variation in the density of the extracellular matrix, but from variation in the distribution, abundance and volume of incorporated lacunae.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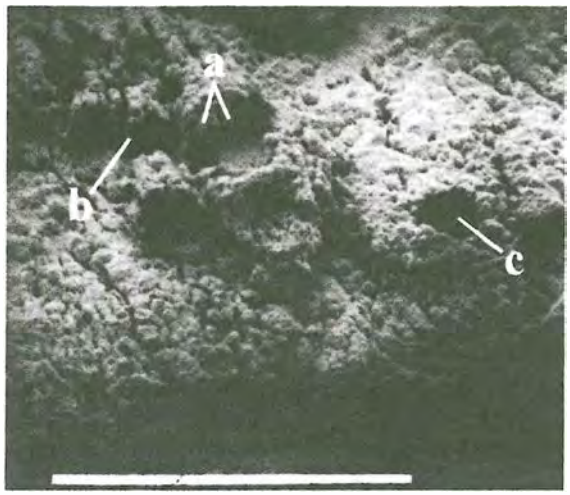
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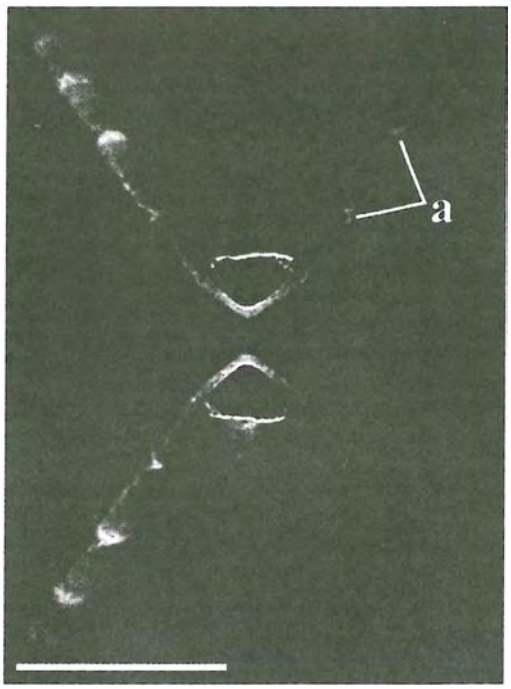
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APPENDIX 3

TASK 3: RECONSTRUCTING SHARK VERTEBRAE THROUGH COMPUTER IMAGING

Introduction

This task was designed to provide an objective means of modelling appositional growth of a vertebra by using a computer-based three-dimensional reconstruction of gummy shark and school shark vertebrae. This involved adapting available computer software, originally developed for reconstruction of bone and tooth structures. Such a model can improve understanding of the dynamics of the growth of vertebrae at both macroscopic and microscopic scales. More importantly, the reconstruction provides an objective means of modelling vertebral growth which does not rely on the interpretation of patterns of observed growth increment bands. A method to enable the three-dimensional reconstruction of shark vertebrae from serial histological sections is described by Clement *et al.* (1992) (see Appendix 6) and examples of images prepared by this method for gummy shark were presented in the First (Walker *et al.* 1993) and Second (Walker *et al.* 1994) Milestone Reports to FRDC and Officer (1995).

Methods

The three-dimensional reconstructions presented here are of trunk vertebrae from a male gummy shark and a male school shark. These animals were kept captive in tanks at VFRI from 1 August 1992 to 13 August 1993, and from 6 May 1993 to 6 May 1994, respectively. During captivity the gummy shark grew from 1190 mm to 1230 mm total length and the school shark grew from 580 mm to 830 mm total length. While in captivity the sharks were injected with vertebra-marking tissue-dyes on each of three and four occasions, respectively. These procedures were conducted as part of the captive shark experiments described for Task 1.

The gummy shark was injected with the vertebra-marking tissue-dye oxytetracycline in August 1992, xylenol orange in late October 1992, and calcein in June 1993. The school shark was injected with oxytetracycline in May 1993, alizarin complexone in September 1993, calcein in December 1993, and oxytetracycline in March 1994. Following each animal's death, vertebrae were excised from the trunk region of the vertebral column, immediately posterior to the first dorsal fin. These vertebrae were chosen because they were amongst the largest where presumably the mineral accretion rates are fastest and provide the best resolution between fluorescent bands.

Both vertebrae were embedded in polyester resin as described above under Task 2. A sawing microtome was used to cut 100 μm thick sections from squared resin blocks containing the vertebrae. As the blade of this machine was 300 μm thick, the average spacing between adjacent sections was 400 μm. By digitising the positions of the fluorescent tissues-dyes and the vertebral margin, gummy shark and school shark vertebrae were reconstructed the surface rendering and three-dimensional volume rendering techniques, respectively

Surface Rendered Reconstruction of Gummy Shark Vertebra

A gummy shark vertebra was prepared for surface rendered reconstruction following the method of Clement *et al.* (1992) with several modifications described below. The parallel and diagonal grooves, milled on the face of the block to locate each section in three dimensions, were filled with a mixture of pigmented polyester resin and the powdered fluorescent tissue-dye calcein. Filling the grooves in this way made the grooves visible in sections, under both white and ultraviolet light. The outlines of the external surface of the vertebra were digitised from traced projections using a translucent, back-lit digitising tablet (Type 5A, GTCO Corp., USA). Since the fluorescent tissue-dyes used in this animal were not visible in projections, the outlines of the fluorescent lines visible within the sections were digitised on a microscope adapted to observe fluorescent images as described Clement *et al.* (1992).

In the gummy shark, the innermost oxytetracycline band and the band resulting from injection of xylenol orange were extremely close to each other. This indicated that little vertebral growth had occurred in the interval between the injection of each tissue-dye. Unfortunately the proximity of these bands made it difficult to resolve them individually, and hence, digitise them separately. Therefore, only the innermost oxytetracycline band and the peripheral calcein band were digitised in the gummy shark vertebra. These tissue-dyes had been injected 291 days apart and the spacing between these bands in the vertebra represented almost ten months of vertebral growth. The fluorescent bands were intense and distinct only in the intermedialia (lateral buttresses separating the articular faces). The four sockets that separate the intermedialia, normally filled with hyaline cartilage in life, showed only feint and discontinuous fluorescence at their margins. These discontinuities in the fluorescent outlines meant that the outlines could not be traced completely around the structure. The digitised data collected from the digitising tablet and digitising microscope were combined and processed using a suite of three-dimensional reconstruction programs developed by Cookson *et al.* (1987). The features of the software described by Clement *et al.* (1992) were then used to create various views of the reconstructed vertebra.

Volume Rendered Reconstruction of School Shark Vertebra

A school shark vertebra was prepared for volume rendered reconstruction. Serial 100 μm thick sections through the vertebra were scanned using a high resolution 35-mm slide scanner ('ScanMaker MTS 1850', Microtek Intl. Inc., Hinschu, Taiwan). Their images were saved to a computer file. The same area was scanned in every section to ensure alignment and stacking of sections during subsequent computer three-dimensional image reconstruction.

Photomicrographs were then made of each section under a fluorescence microscope using a filtering combination which excited the emission of fluorescence from all the vertebra-marking tissue-dyes incorporated into the vertebral centrum. The photomicrographs were then scanned and images of each section were analysed by an image processing program ('Aldus Photostyler Version 1.1', Aldus Corp., WA, USA). The area of the centrum visible in each photomicrograph was re-sized and rotated to match its area in the original scan of the section. The images of the fluorescent bands were then superimposed upon the original scan, replacing the original silhouette of the section with the coloured image of the fluorescence within each section.

The computer images produced for the series of sections were edited. Areas showing fluorescence on each image were recoloured such that each fluorescent band was given a different shade of grey. The unstained areas of the section, and the resin surrounding them, were similarly selected and recoloured. Stored in 8-bit format, the images from the series of sections were then processed in a volume rendering three-dimensional reconstruction computer program ('Spyglass Slicer Version 1.0', Spyglass Inc., IL, USA) to create various views of the three-dimensional reconstructed vertebra.

Results

Images of the surface rendered three-dimensional reconstructions of a gummy shark vertebra are shown in Figure 3.1. The views are of the lateral side of the reconstructed vertebra image of (a) the oxytetracycline band, (b) the calcein band, (c) the external surface, and (d) all three structures in their correct three-dimensional orientation.

Calcein fluorescence appeared as broad green staining in the tissues close to the margins of the articular face. This may have been due to the relatively short-time interval between the injection of calcein and the animal's death (73 days) and the inability of the animal to clear the tissue-dye from its circulation in this time. However, in the articular faces the fluorescent marking resulting from the other tissue-dyes was also diffuse and not confined to a distinct band. It is likely that the plane of section through the fluorescent bands was cut obliquely at the margins and causing their outlines to be broad and diffuse. The lack of clearly defined fluorescent outlines in the tissues directly apposed to the articular faces meant that fluorescent outlines could not be traced in these areas. Incomplete or diffuse fluorescent marking meant that fluorescent outlines could only be accurately traced in sections taken near the middle of the structure. Discontinuity in the fluorescent lines around the entire structure meant that the fluorescent bands visible in each intermedialia had to be reconstructed as separate structures.

Images of volume rendered reconstructions of the school shark vertebra are shown in Figure 3.2. The view is the same as that in Figure 3.1. The reconstructions shown are of (a) the innermost oxytetracycline band, (b) the closely apposed alizarin complexone band, (c) the calcein band, and (d) the outermost oxytetracycline band. A view of the external surface of the reconstruction is shown in (e).

In both reconstructions, the internal fluorescent bands are approximately parallel to one another and to the external surface of the vertebra. The parallel relationship of successive growth bands confirms that the growth of the intermedialia is appositional and, hence, that growth-increment bands are only added at the peripheral margins of the intermedialia. The innermost bands in the reconstruction clearly define the margins of smaller structures than those confined within subsequent bands, and the external surface of the vertebra at the time of the animal's death.

Discussion

Shark vertebral centra show no histological evidence of resorption at any time in the animal's life. De-organification of centra always reveals a large, residual, stable skeleton (Clement 1992). In contrast, the mineralized parts of other organs such as claspers and jaws crumble into their individual mineralized sub-units, the tesserae, upon de-organification.

Appositional growth of the basic 'double-cone' shape of shark vertebrae enables increase in body length and girth. Once the stages of formation of initial shape of the mineralized portion of a vertebral centrum is understood, then relatively simple mathematical models can be developed to describe growth patterns.

The utility of creating three-dimensional reconstructions of solid objects for growth studies was suggested by Gillings and Buncore (1961). Reconstructing a growing structure at various stages of life allows enables the study of areas actively undergoing growth and the application of measurements related to the growth of the structure, such as volume and surface area.

Sullivan (1972) applied the three-dimensional reconstruction technique suggested by Gillings and Buncore (1961) to a study of bone growth in the dog mandible. The study was unique at the time because it involved the use of fluorescent marking tissue-marking dyes. However, technical improvements were necessary to make Sullivan's techniques efficient. Advances made through the establishment of dedicated data gathering systems and three-dimensional reconstruction computer packages (Dykes and Clement 1980; Dykes *et al.* 1981; Clement and Dykes 1982; Cookson *et al.* 1987) have recently enabled three-dimensional reconstruction of shark vertebrae to be undertaken (Clement 1986; Clement *et al.* 1992).

Clement *et al.* (1992) developed a method that could accurately record the size and shape of complex three-dimensional anatomical structures. They described a technique that could record and measure the size and shape of shark vertebra, and model subsequent growth. They proposed that three-dimensional reconstruction would provide a better understanding of how organs with a complex three-dimensional morphology might continue to grow over time. Thus, the value of three-dimensional reconstruction as a useful tool for predicting the growth shark vertebrae lay in treating the vertebra as a whole. This enabled change in size and shape to be measured in three dimensions, rather than through measuring and interpreting increment patterns on two-dimensional surfaces such as the articular face and vertebral sections.

The microscope system used by Clement *et al.* (1992) permitted the outlines of structures to be traced using transmitted white light and then, after changing the illumination to ultraviolet light, allowed the digitisation of fluorescent outlines within the structure to be traced, without moving the specimen of study from the microscope stage. Hence, fluorescent labels on a vertebra resulting from injection of hard tissue-marking dyes, such as oxytetracycline, could be digitised under the microscope, and then related to other, non-fluorescent, anatomical features. The tracing of outlines or fluorescent bands visible in vertebra following the injection of hard tissue-marking dyes would make three-dimensional reconstruction an objective method for measuring the size and shape of the vertebra at earlier stages in a shark's life. Clement *et al.* (1992) proposed that the ability to compare growth outlines inside a single vertebra with its external surface had great potential application for tagged, oxytetracycline injected, released and recaptured animals. Increasing the objectivity of techniques directed towards age and growth studies is a well recognised priority in fisheries biology (Beamish and MacFarlane 1987; Cailliet and Radtke 1987; Cailliet and Tanaka 1990).

The proposal by Clement *et al.* (1992) was based on the assumption that the fluorescent outlines used to create reconstructions were a true representation of the size and shape of the vertebra at specific earlier stages in the animals life. Differences in the morphology of a reconstructed structure, between a vertebra at different ages, might only be of proportion, and not absolute differences in the basic structural model describing the vertebra. The three-dimensional reconstruction would enable the experimenter to see if indeed there was any difference in accretion rate at different sites.

Based on this premise the technique of three-dimensional reconstruction was applied in this study to vertebrae from gummy shark and school shark. The sharks under study had been injected with vertebra-marking tissue-dyes on several occasions (see Task 1). A three-dimensional reconstruction of the size and shape of the vertebra at the time of each injection was made through tracing the fluorescent outlines resulting from injection of each tissue-dyes. Using fluorescent vertebra-marking tissue-dyes was an attractive proposition because the tissue-dyes effectively colour coded the structures to be reconstructed.

The lack of fluorescence in some parts of the vertebrae suggests that these areas were not mineralizing during the time the tissue-dye was available in the animals body for incorporation into mineralizing tissues. Alternatively, these areas may have been growing at such a slow rate that too little of the tissue-dye was incorporated to be visible. It may be that the surfaces between the intermedialia never undergo new mineralization once deposited. If this is the case, then reconstructions based on fluorescent outlines should assume that there had been no accretion between the intermedialia, and hence, follow the outside edge of the vertebra between the intermedialia as part of the 'increment'. In the absence of knowledge as to whether slow or sporadic growth occurs on these surfaces, one can not legitimately build a reconstruction following these outlines.

It is known that parts of shark vertebrae, like teleost otoliths, do not all grow proportionally (Gruber and Stout 1983; Smith 1984; Beamish and McFarlane 1987). However, it has been suggested that vertebral growth is isometric; i.e. relative increases in size may vary, but shape is maintained (Clement 1986; Clement *et al.* 1992). The exact nature by which this growth occurs has not been fully explained. Despite the lack of evidence for growth on all surfaces of the vertebra in this reconstruction, it is evident that the fluorescent outlines depict a structure of the same shape, but smaller size, than the reconstruction based on the external outline of the vertebra. This reconstruction provides evidence that the shape of the vertebra is maintained not by adding mineralized tissue to all surfaces, including the radiating sides of the intermedialia, but by progressively adding a wider band of tissue to the circumferential margin of the intermedialia.

The diffuse marking of the fluorescent tissue-dyes in the articular faces meant that outlines of the tissue-dyes could not be digitised using the surface rendering technique in sections of the articular face. Whilst reconstructions of the vertebrae created using the surface rendering technique were extremely life-like, the inability to digitise outlines in every section made them incomplete.

The volume rendering technique was applied to the reconstruction to overcome some of the limitations of the surface rendering method but the approach was only partially successful. The stepped nature of the reconstructions made volume rendered reconstructions look unrealistic. The use of thinner sections would overcome some of these difficulties, but

producing thinner sections is technically difficult. Decalcification of the vertebrae would allow thinner sectioning, but this would remove the calcium and hence the fluorescent tissue-dyes.

Inaccuracies were introduced with the volume rendering method because of the need to use scanned images. Scanning the fluorescent outlines directly is not possible without somehow exciting the fluorescence. Capturing the images via a video camera overcomes the need to scan the images but this is not possible for fluorescent outlines without the use of expensive, super-cooled video equipment. In this study the images of the fluorescent outlines were imaged through first photographing each specimen under ultraviolet illumination, and then through scanning each photograph.

The difficulties experienced in the preparation of sections for volume rendered reconstruction are reminiscent of the technical limitations experienced by Sullivan (1972, 1975). The main reason for development of the digitising microscope (Dykes and Clement 1980) was to subject all parts of the section to identical optical treatment. The data were recorded directly by the operator from the real object of study, and hence, no additional intermediate optical imperfections arising from photography were incorporated. In the preparation for volume rendered reconstruction, inaccuracies were introduced by the need to photograph fluorescent outlines, scan, re-size and then superimpose them upon the original scanned images of non-fluorescent anatomical features. The inevitable optical imperfections in the lenses and mirrors in the microscope and camera meant that viewing conditions were sub-optimal. The periphery of the field of view exhibits the most marked chromatic and spherical aberrations, distorting some parts of the outline with respect to other parts of the same outline. This prohibited making an accurate calculation of the surface areas and volumes of the scanned vertebrae.

Reconstructions produced by either the surface rendered reconstruction method or volume rendered reconstruction method could be 'sectioned' on screen, in any selected new plane, and the distances between increments measured. Such a process would yield the growth coefficients of surfaces in each section, in each of the three dimensions. However, using a single coefficient for each dimension, to 'grow' the vertebra on the computer, as a means of predicting future vertebral growth, would be fallible. The apparent lack of growth on all surfaces means that growth coefficients would alter with the depth of the section within the structure, and the position of the measurement within each section. Hence, no single coefficient would adequately describe the overall growth of the structure in any of the three dimensions. Instead, coefficients would need to be calculated with regard to the position of points within the vertebra, and the degree of growth normally undertaken by the surfaces they constitute. Computer programming to facilitate these calculations is a feasible future addition to the present three-dimensional reconstruction program.

Estimation of changes in the entire vertebral volume may be more useful to growth studies than estimation of differences in the spacing between the increments seen in two-dimensional sections (Clement *et al.* 1992). Measurement of the entire vertebral volume is a means of monitoring the growth process directly. Its avoidance of the process of interpreting and counting growth-increment bands satisfies the need to develop an objective means of examining growth (Beamish and McFarlane 1987).

Unfortunately, the nature of the growth of the vertebrae meant that volumes could not be estimated for the three-dimensional reconstructions produced by either imaging method. Fluorescent outlines did not follow the outline of the vertebra continuously. Hence, the utility of a reconstruction prepared in this manner as a means of objectively comparing measurements of surface area and volume is lost. Should an imaging method with sufficient spatial resolution become available that is able to trace the true outline of a vertebra at various stages in a shark's life, then calculations of volume and surface area would be possible.

The application of new technologies could avoid the reliance on vertebra-marking tissue-dyes, and the problems associated with their administration and discontinuous incorporation. In captive animals it would be possible to generate serial sections through the vertebrae using magnetic resonance imaging, computed axial tomography, or another imaging modality. Reconstruction of selected vertebrae at various stages in a shark's life could then be made directly from the scanned films. Improvements in the quality and processing of these images (Allardice *et al.* 1993; Deverell *et al.* 1993) means that the size, shape and volume of the imaged vertebrae could then be monitored as part of a longitudinal growth study.

Whilst the main obstacle to this approach is gaining access to the necessary equipment, recent advances in the application of these technologies to elasmobranchs (Waller *et al.* 1994) show the possibilities of completing such studies. The increasing reliance of the medical fraternity on three-dimensional imaging (Koltai and Wood 1986; Anderson and Svartz 1988; Sarver *et al.* 1988; Mahoney 1990) can only serve to bring costs down and facilitate the wider biological application of objective three-dimensional imaging methods to be fully explored.

Conclusions

Simulated three-dimensional reconstruction of vertebrae corroborated results from Task 2 that growth was appositional but not evident on all surfaces of the vertebra. Although the method proved valuable several shortcoming in the method were found.

(a) Since reconstructed bands represented previous growth sites, discontinuities in the sites of growth meant that reconstruction produced by either surface rendered reconstruction or volume rendered reconstruction were incomplete.

(b) Surface areas and volumes could not be measured accurately due to the incomplete reconstructions.

Other imaging methods that reconstruct the periphery of a vertebra directly rather than natural or induced bands may prove more useful. Further technological advancements may allow the application of previously unavailable imaging modalities to future vertebral growth studies.

(1) The appositional mode of vertebral growth determined as part of Task 2 was corroborated through simulated three-dimensional reconstruction.

(2) Lack of growth on some surfaces of a vertebra determined as part of Task 2 was also corroborated.

(3) Because reconstructed bands represented previous growth sites, discontinuities in the sites of growth meant that reconstruction produced by either surface rendered reconstruction or volume rendered reconstruction were incomplete.

(4) Surface areas and volumes could not be measured accurately due to the incomplete nature of the reconstructions.

(5) Other imaging methods that reconstruct the periphery of the vertebra directly rather than natural or induced bands may prove more useful.

(6) Further technological advancements may allow the application of previously unavailable imaging modalities to future vertebral growth studies.

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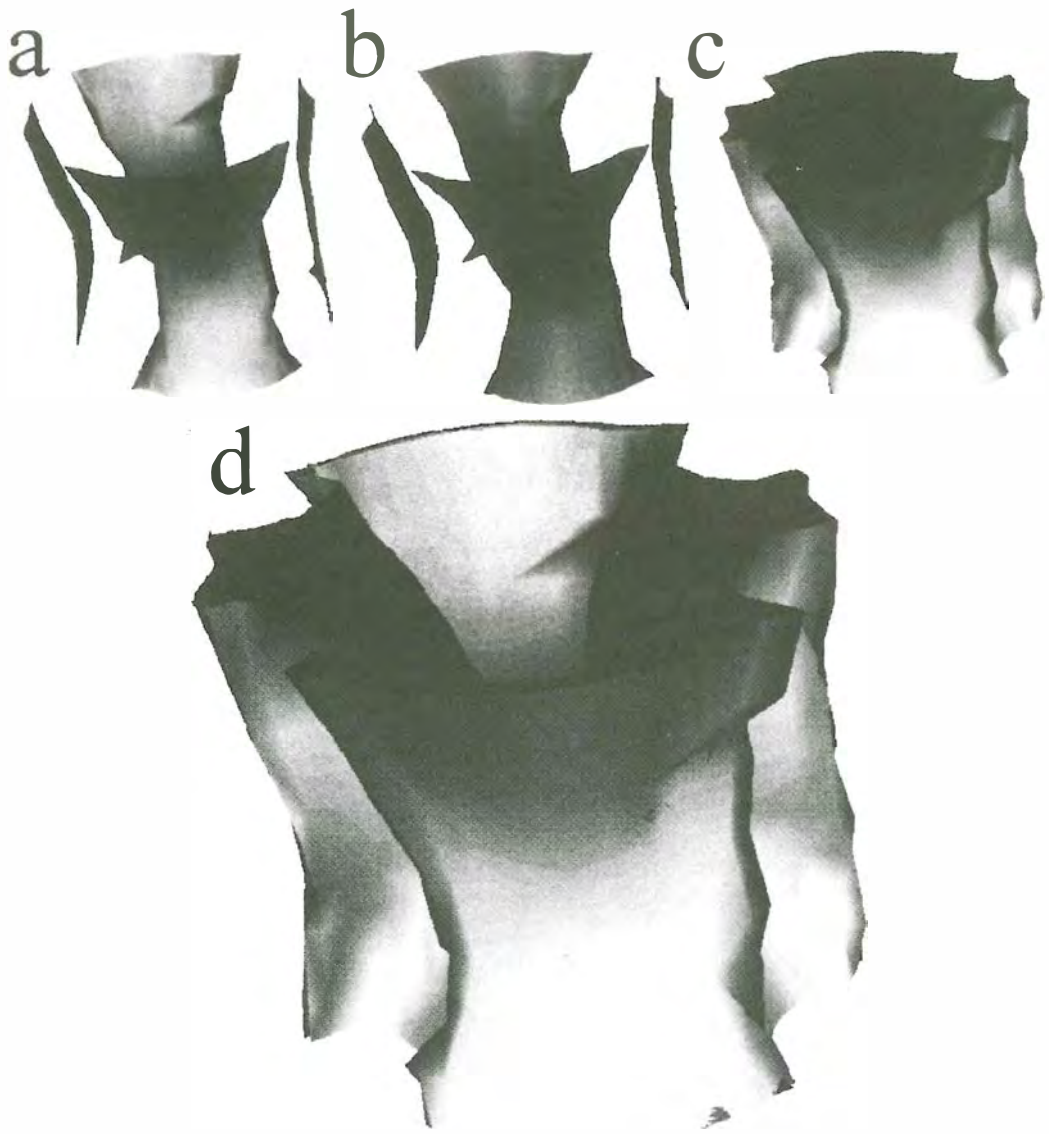

Figure 3.1. Coloured and shaded surface rendered three -dimensional reconstructions of a gummy shark vertebra. The view is from the lateral side of the reconstructed structure. The reconstructions represent (a) the oxytetracycline band, (b) the calcein band, (c) the external surface, and (d) all three structures in their correct three-dimentional orientation

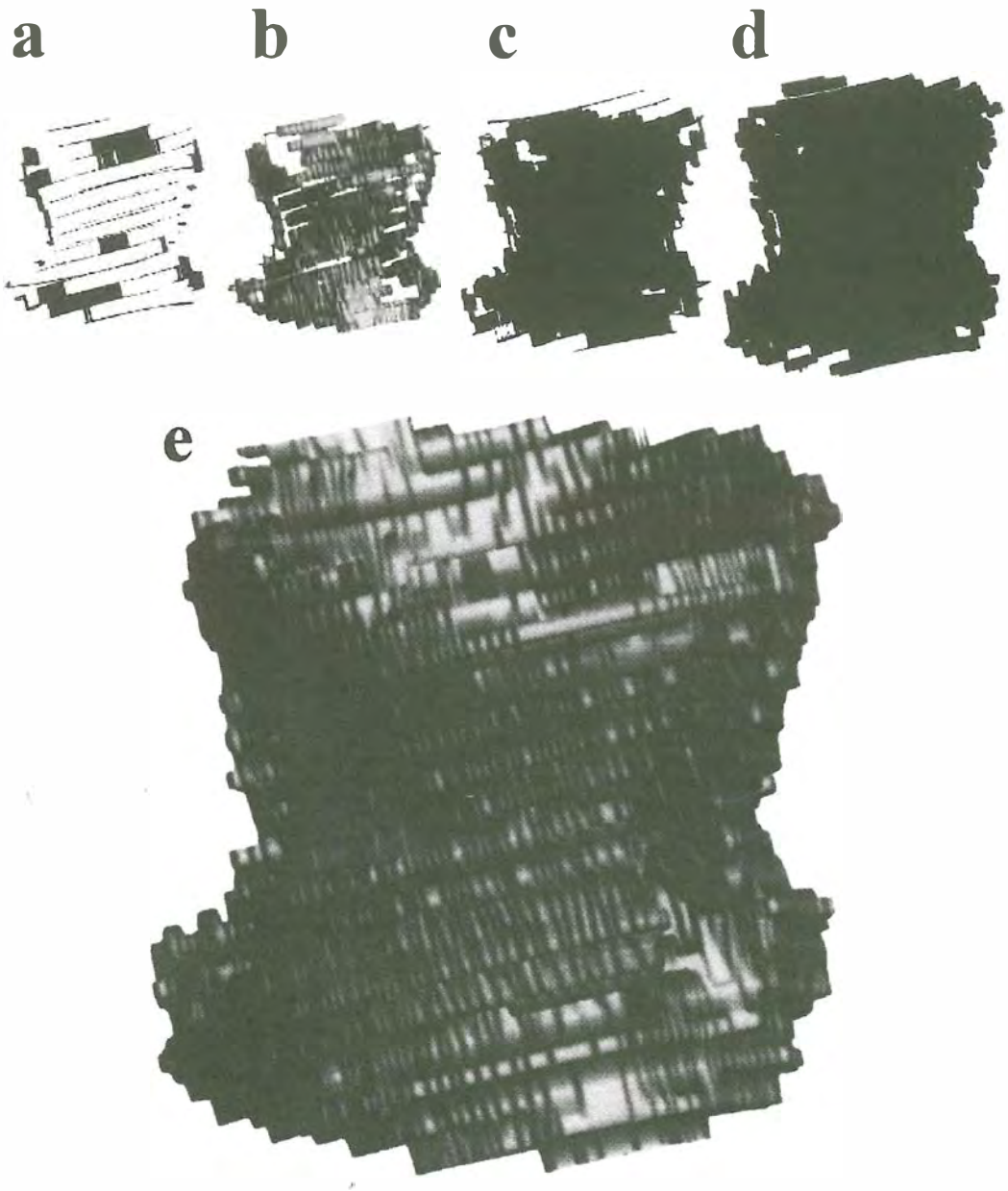

Figure 3.2. Volume rendered three-dimensional reconstructions of a school shark vertebra. The view is from the lateral side of the reconstructed structure. The reconstructions represent the (a) alizarin complexone band, (b) the first oxytetracycline band, (c) the calcein band, (d) the second oxytetracycline band, and (e) the external surface of the vertebra at the time of the animals death.

APPENDIX 4

TASK 4: COMPARING THE ALIZARIN STAIN AND MICRORADIOGRAPHIC METHODS

This task had two purposes. One was to evaluate the age-determination method of reading alizarin stained whole vertebrae, which is the method presently used for routine ageing of sharks in the southern shark fishery. The other purpose was to compare counts of growth-increment bands from this method with the method of reading microradiographs of sectioned vertebrae, which is the method adopted for ageing sharks in some other parts of the world. The work was undertaken on 24 sharks, such that 2 gummy sharks and 2 school sharks were collected from within each of 3 length-classes for each sex (i.e. 2 species × 2 sexes × 3 length-classes × 2 sharks = a total of 24 sharks), using 2 vertebrae from within each of 3 regions of the vertebral column (post-cranial, trunk and caudal) of each shark.

Most of the work associated with this task is fully documented in a separate report which forms the basis of a manuscript prepared for the *Canadian Journal of Fisheries and Aquatic Sciences*.

Officer, R. A., Gason, A. S., Walker, T. I., and Clement, J. G. (1995). Southern Shark Age Validation Project: Part 2 - Sources of variation in growth increment counts from vertebrae of gummy shark, *Mustelus antarcticus* Günther, and school shark, *Galeorhinus galeus* (Linnaeus). Final Report to Fisheries Research and Development Corporation (FRDC Project 91/037). 24 pp. (Victorian Fisheries Research Institute, Department of Conservation and Natural Resources: Queenscliff).

A small component of additional work associated with this task is described below.

After comparing von Bertalanffy growth curves determined from length-at-age data with growth curves determined from tag release-recapture growth-increment data, Moulton *et al.* (1992) concluded that the method of staining whole or sectioned vertebrae with alizarin red under-estimates the age of school sharks longer than about 1300 mm total length. Hence, to determine whether the microradiographic method might be a better method for resolving the growth-increment bands in large school sharks, in additional to the experiment described by Officer *et al.* (1995), vertebrae from the post-cranial region of 39 school sharks longer than 1400 mm total length were collected to evaluate these differences.

A whole vertebra from each of the 39 sharks was cleaned of fascia material, bleached in a solution of sodium hypochlorite, stained with a solution of alizarin red and the growth-increment bands on one of the two articular cups of the centrum were examined under a microscope and counted by a reader experienced with the alizarin stain method according to the procedures described by Moulton *et al.* (1992). The same vertebra of each shark was subsequently sectioned, microradiographed and the growth-increment bands of hyper-mineralization were counted by a reader experienced with the microradiographic method according to the procedures described by Officer *et al.* (1995). For the latter method, the reader characterised the growth-increment bands as belonging to one of three categories: major increments (well defined hyper-mineralized growth-increment bands), uncertain increments (hyper-mineralized growth-increment bands with an irregular spacing), and minor increments (fine check marks). The preparations and readings were undertaken according to the principles of a 'double blind' experiment as neither reader had any prior

knowledge of the sex or exact length of shark or increment count obtained from the other method.

For statistical analysis of the data, one type of increment was adopted for the alizarin stain method (Type A1) whereas three types of increments were adopted for the microradiographic method: Type M1 included major increments only, Type 2 included major and uncertain increments, and Type 3 included major, uncertain and minor increments. Differences in the increment counts between the two methods were tested by applying three separate pairwise t-tests (i.e. M1-A1, M2-A1 and M3-A1) (Table 4.1).

Table 4.1. Results of pairwise t-tests between counts of Type A1 growth-increment bands from the alizarin stain method and counts of each of Type M1, M2 and M3 growth-increment bands from the microradiographic method obtained from 39 school sharks of total length exceeding 1400 mm (df = degrees of freedom, SD = standard deviation, F = F ratio, P = probability value).

Pairwise t-test	Mean difference	SD difference	t-calc	df	P
M1-A1	0.54	2.72	1.24	38	0.2244
M2-A1	0.79	2.70	1.84	38	0.0735
M3-A1	2.15	2.87	4.69	38	0.0000

The similarity between growth-increment counts from the alizarin stain method and the growth-increment counts of major bands from the microradiographic method is indicated by the lack of statistical significance between counts of Type A1 and Type M1 growth-increment bands. This is consistent with the results presented in Part 2 of the report (Officer et al. 1995).

Conclusions

(1) Growth-increment bands in the vertebrae of school shark for both alizarin stain method and microradiographic method are more difficult to count than those in the vertebrae of gummy shark.

(2) The readability of a vertebra for both the alizarin stain method and the microradiographic method tends to be no better after repeated examination of growth-increment bands.

(3) The readability of vertebrae from the same region of the vertebral column of a shark for both the alizarin stain method and the microradiographic method are not significantly different (i.e. their readability is dependent on the shark).

(4) Experienced readers using the alizarin stain method provide more precise and less biased counts of growth-increment bands than do inexperienced readers.

(5) Counts of vertebral growth-increment bands made by both the alizarin stain method and microradiographic method from the largest vertebrae which occur in the region of the vertebral column near the first dorsal fin are statistically higher than counts in vertebrae from the regions near the head and the tail.

(6) Vertebral growth-increment bands visible on microradiographs of sectioned vertebrae for the microradiographic method can be classified as minor and major bands.

(7) Counts of alizarin stained growth-increment bands on whole vertebrae for the alizarin stain method are similar to counts of major growth-increment bands of sectioned vertebrae for the microradiographic method. While there is no bias between the alizarin stain method and microradiographic method, the microradiographic method gives better precision.

(8) Overall the alizarin stain method gives similar results to the microradiographic method, indicating that there are no immediate benefits in changing from the alizarin stain method to the microradiographic method for routine ageing of sharks.

APPENDIX 5

TASK 5 TESTING FOR PHENOMENON OF APPARENT CHANGE OF GROWTH RATE

Three types of evidence are presented to support the hypothesis of the 'Phenomenon of Apparent Change in Growth Rate' caused by length-selective fishing mortality to explain observed differences in von Bertalanffy growth curves published previously for gummy shark between 1973-76 and 1986-87 in Bass Strait and between Bass Strait and South Australia during 1986-87.

The work associated with this task is completely documented in a separate report which forms the basis of a manuscript prepared for the *Canadian Journal of Fisheries and Aquatic Sciences*.

Walker, T. I., Taylor, B. L., Hudson, R. J., and Cottier, J. P. (1995). Southern Shark Age Validation Project: Part 2 - The phenomenon of apparent change of growth rate in gummy shark, *Mustelus antarcticus* Günther, harvested by gill-net and hooks off southern Australia. 50 pp. Final Report to Fisheries Research and Development Corporation (FRDC Project 91/037). (Victorian Fisheries Research Institute, Department of Conservation and Natural Resources: Queenscliff).

Conclusions

(1) The mean lengths of sharks within each of the recruited ages-classes of 3-7 years are shown to be markedly different between 1973-76 and 1986-87 in Bass Strait and between Bass Strait and waters off South Australia during 1986-87, whilst the mean lengths of sharks within the less fully recruited age-class of 2-years are shown not to be different between the two periods and only slightly different between the two regions.

(2) Avoiding 'back-calculation' of length of shark, Rosa Lee's Phenomenon was detected by directly comparing the radii of growth-increment bands visible on the surfaces of vertebrae from sharks of various ages caught in the two periods and the two regions.

(3) Developing an appropriate model, the effects of length-selective fishing mortality on the mean length of sharks in the population for ages 2-16 years were simulated for a range of levels of hook and gill-net fishing effort, with separate mesh-sizes of 6 and 7 inches for the gill-nets. The simulated changes among the recruited age-classes, although not as large as observed differences in the published growth curves, were generally consistent with the observed trends. The simulations demonstrated how the von Bertalanffy growth parameters L_∞ and t_0 tend to increase and K tends to decrease as gill-net fishing effort increases, and hence explains how these types of biases, commonly reported in the scientific literature for gill-net shark fisheries, can occur.

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Vertebral deformities in a school shark, *Galeorhinus galeus*: circumstantial evidence for endoskeletal resorption?

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The appearance of deformed vertebrae from a single mature female school shark, *Galeorhinus galeus*, are described. Two unusual, pronounced bumps were noticed in the caudal region of this shark. There were no scars in the skin over these protrusions, suggesting that the lesions had arisen internally. Radiographic and histologic investigation of these lesions showed that mineralized tissue had probably been lost following an injury to the tail. Histological observations provided circumstantial evidence that mineralized tissue had been removed by internal processes, but did not reveal the agency by which skeletal tissue had been resorbed. Since the capacity to resorb mineralized tissue is characteristic of animals possessing bone, the apparent loss of mineralized tissue seen in this shark provides circumstantial evidence for the existence of bone cell lineages in school sharks. This evidence is discussed in relation to the possible implications for evolutionary and fisheries biology.

Key words: chondrichthyan; deformity; endoskeleton; *Galeorhinus galeus*; resorption.

INTRODUCTION

Sharks and rays are grouped together as cartilaginous fish (Chondrichthyes) on the basis that they possess a completely cartilaginous skeleton at all stages in their life history. In other vertebrates, including man and bony fishes, most of the cartilage in the embryo is replaced by bone in the adult (Hall, 1982). A supposition of the embryological argument is that the transition from cartilage to bone never evolved in the Chondrichthyans. Adherence to the view that ontogeny reflects phylogeny placed the Chondrichthyans as the evolutionary predecessors to all the other fish and amphibians (De Beer, 1947).

In addition to its structural function, the osseous skeleton acts as a reservoir of minerals, such as calcium and phosphorus, which are essential to maintain mineral homeostasis (Urist, 1964). Utilizing the skeleton as a mineral store and internal reference for the electrolyte balance is particularly important in animals, such as terrestrial tetrapods, that live in environments depleted in calcium. Constantly reworking the skeleton, through alternately resorbing and replacing bone, helps to maintain a level of calcium in the blood sera and yet allows growth. Hence, resorption is a feature characteristic of osseous skeletons.

Since sharks live in an environment abundant in calcium, they should not need to utilize the endoskeleton as a calcium store, nor to resorb their skeletons, in order to regulate their calcium metabolism, or to grow. For these reasons, resorption, a feature peculiar to bony skeletons may not have evolved in the Chondrichthyans. However, the theory that sharks are ancestral forms is countered by the fact that these animals show the appearance of calcium

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regulating hormones, such as calcitonin, that are found in higher vertebrates (Dacke, 1979). Dacke maintained that calcitonin in sharks had no role in calcium regulation, suggesting rather that the hormone might regulate other ions, or have a role in their water balance. Another of Dacke's suggestions was to challenge the positioning of sharks as ancestral forms to teleosts. He speculated that calcitonin may be vestigial in sharks, and hence that they may have evolved from bony ancestors.

The relatively recent appearance of the elasmobranchs in the fossil record confirms that they are not primitive forms (Urist, 1964). Examination of the paleohistological record shows that some of the earliest bony fishes engaged in active endoskeletal resorption (Bystrow, 1942, 1959). If the elasmobranchs are derived from these bony ancestors, then they possibly were once able to resorb their skeletons, and have since lost that ability. Moss (1977) and Clement (1992) have provided reviews of this classical argument concerning the phylogenetic significance of elasmobranch cartilage.

Glowacki *et al.* (1985) were able to demonstrate that calcitonin could produce hypercalcemia in a shark. This suggests that the hormone might still be involved in calcium regulation. Glowacki *et al.* also concluded that ' because shark skeleton is composed of cartilage, this hypercalcemic effect of calcitonin does not require a bony skeleton '. An alternative explanation, not explored by Glowacki *et al.*, was that the shark skeleton may contain vestiges of bone, which require these calcium regulating hormones.

Other authors have explored this alternative. Peignoux-Deville *et al.* described bone in the neural arches of the dogfish, *Scyliorhinus canicula* Linnaeus (Peignoux-Deville *et al.*, 1981, 1982, 1984, 1985). These discoveries of vestiges of bone provided ' a new element for the analysis of the phyletic position of the Chondrichthyes ' (Peignoux-Deville *et al.*, 1985). The findings led Peignoux-Deville *et al.* to pose a most important question: ' is bone a primitive feature that persists during all vertebrate evolution, from the Placoderms, or has it disappeared in some groups and reappeared? ' (Peignoux-Deville *et al.*, 1985).

Later work by Bordat (1987) described bands of lamellar and non-lamellar bone in the neural arches of the dogfish, *S. canicula*. Bordat went further, describing areas of resorption by mononucleated cells at the borders of the bone. Whilst Bordat was later to question the presence of bone in the tesserae of sharks, he added further support to the argument that sharks were capable of calcified tissue ' degeneration ', and described degeneration of the ' double calcified cone ' (vertebral centra) (Bordat, 1988). Whilst fusion, extra deposition and erosion of calcified material in shark vertebral centra have previously been described (Hoenig, 1979; Hoenig & Walsh, 1983), Hoenig and Walsh overlooked the significance of their observations. Their findings suggested that endoskeletal resorption was a reasonable hypothesis to explain the removal of mineralized tissues.

Attempts to initiate resorption experimentally have been unsuccessful. In an effort to provoke resorption Peignoux-Deville *et al.* (1989) implanted eel bone into dogfish (*S. canicula*). Whilst the multinucleated giant cells which surrounded the implanted bone had some of the histological features of mammalian osteoclasts, the suggestion given by Peignoux-Deville *et al.* that these multinucleated cells were involved in resorption remains unconvincing. The study was

unable to show that mineral was actually removed from the implanted bone fragments. Also, since no non-osseous material was implanted in the experiment, it is not possible to determine whether the cellular aggregations around the implanted bone were part of the normal inflammatory response that might have occurred following the implantation of any foreign body. Other authors were also unable to provoke resorption (Glowacki *et al.*, 1982, 1986; Clement, 1986) when performing similar implantation experiments.

To date, the question of the presence of bone in Chondrichthyans remains problematic. Hence, the presence of resorptive capabilities, which normally accompany the presence of bone, is also still at issue. The lack of a clearly understood mechanism by which resorption may occur does not help resolve the debate. The demonstration of bone in elasmobranchs, or the unequivocal presence of resorption, would have important evolutionary implications. Such a discovery may indicate the retention of ancestral cell lineages in sharks, placing them as the highly evolved descendants of the bony fishes.

MATERIALS AND METHODS

The animal documented here was a mature female school shark, *Galeorhinus galeus* L., caught off Cape Schank, Victoria, Australia in April 1992. At capture, it measured 1380 mm (total length) and had a total weight of 9.6 kg. The animal was stored frozen, awaiting further study for a separate project devoted to age determination and validation. Therefore, the unusual nature of the caudal region was discovered serendipitously, during routine radiography of the entire vertebral column. At this stage two pronounced bumps were noticed in the caudal region of the animal. The skin covering the caudal vertebrae was removed, and the specimen was laid on its right side for radiography. Following this initial radiography of the entire caudal region, the two skeletal lesions visible in the initial radiographs were excised from the column, and re-radiographed to improve the radiographic clarity. All radiographs were taken in a Hewlett-Packard Faxitron Series X-ray system (model 43805N), with a focus to film distance of 48 cm, using Kodak Industrex SR film.

Following radiography, the excised skeletal deformities were transferred to 10% formalin. The tissues were then decalcified in a buffered formic acid solution, and processed to paraffin wax for histological sectioning at 4 μm. Sagittal sections were cut through the anterior lesion, and those from the posterior lesion were also cut longitudinally, but in the dorso-ventral plane. A block of three normally articulated vertebrae from an apparently normal area proximal to the anterior deformity was also taken. This piece of tissue was sectioned in the same plane as the anterior deformity, to allow comparison of the appearance of deformed and apparently normal tissue. All sections were stained with haematoxylin and eosin.

RESULTS

Two unusual, pronounced bumps were noticed in the caudal region of this shark. There was no scarring in the skin over these protrusions. Radiographs revealed that each bump in the vertebral column was associated with skeletal lesions (marked a and p in Fig. 1). The anterior lesion showed a large, unusual radiolucency (arrowed in Fig. 2). In neighbouring vertebrae, the regular shape and orientation of the vertebrae was lost. In the area of discontinuity the densely mineralized arms of the articular faces of the corpus calcareum were not present. In contrast, above the radiolucency, in a position that might normally be

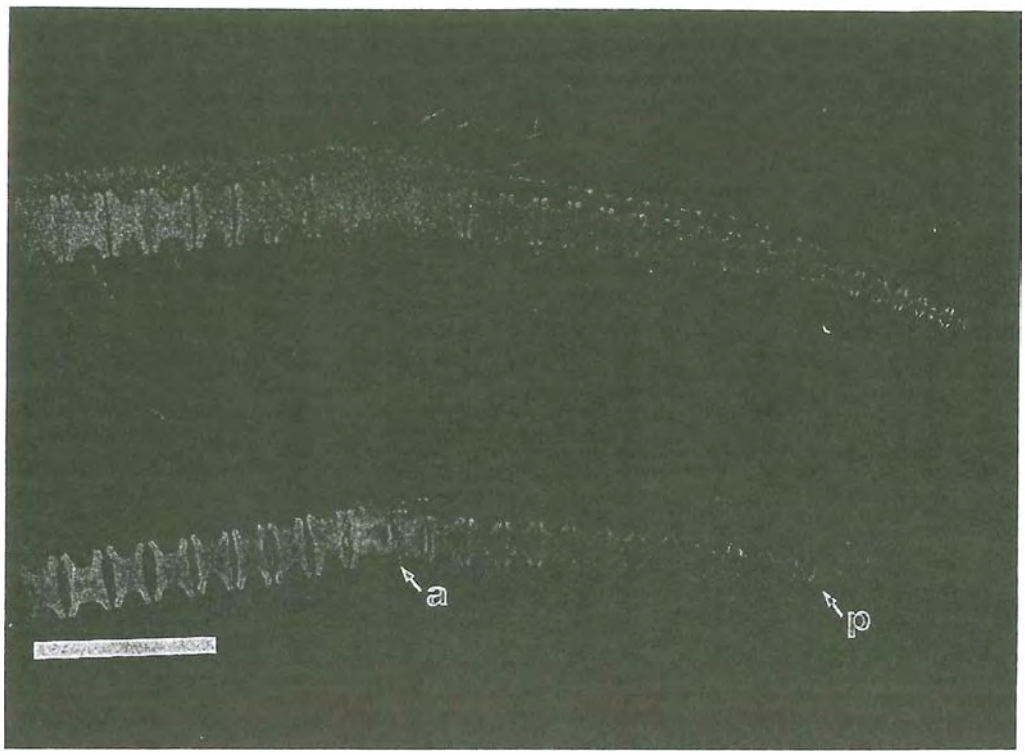

FIG. 1. Two radiographs of the tail showing the orientation of the deformities within it (upper), and detail of the skeletal lesions (lower). Specimen viewed from left lateral side (left of frame anterior, top of frame dorsal). Both the anterior (a) and posterior (p) lesions are characterized by irregularly shaped and sized vertebrae surrounding areas of radiolucency. Radiolucent areas show a loss of mineralized tissue. Exposure: 28 kV, 2·25 mA, 3 min (upper), and 5 min (lower). Scale bar = 25 mm.

occupied by the four articular faces of just two vertebrae, there appeared to be eight articular faces. The arrangement of the articular faces was both crowded and distorted.

The soft tissues surrounding the vertebral column showed no inflammatory cellular infiltrate. Severe displacement and distortion in the mineralized arms of the articular faces of the corpus calcareum was seen in histological sections of the anterior deformity [area bound by large box in Fig. 3(a), at higher power in Fig. 4]. The arrangement of tissues in this area was irregular, when compared to the regular symmetry and discrete nature of vertebrae from an unaffected part of the vertebral column [Fig. 3(b)]. The darkly stained tissue seen in radiolucent areas [marked h in Fig. 3(a)] resembled hyaline cartilage. The site of calcified tissue deposition, the perichondrium, was absent at the junction between the two articular faces (marked j in Fig. 4). The mineralized tissue in the intermedialia showed curvature in its growth (marked c in Fig. 4). The mineralized tissue within the other intermedialia (marked r in Fig. 4) occupied an unusually small area, its growth having been restricted to the space between the displaced articular faces that surround it.

Extensive fusion was seen between neighbouring vertebrae [marked f and with arrows in Fig. 3(a), at higher power in Fig. 5(b)]. Deeper sections revealed that the apparently homogenous area labelled f in Fig. 3(a) was, in fact, two vertebrae which were fused at this level of sectioning. In a normal junction, the perichondrium (site of active calcification) was evident at the edge of each

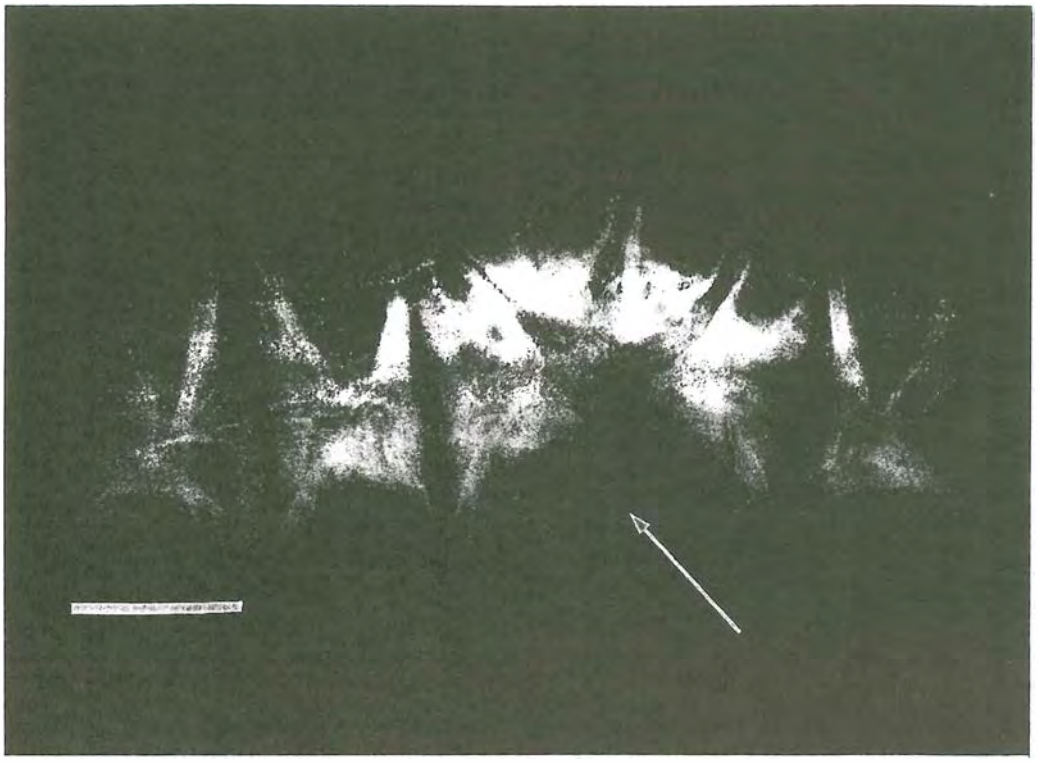

FIG. 2. Radiograph of the anterior lesion. Specimen is viewed from dorsal side (left of frame anterior, top of frame animal's right side). A large radiolucency (arrowed) shows no evidence of the densely mineralized arms of the articular faces of the corpus calcareum. Supernumerary articular faces appear above the radiolucency. Crowded neighbouring vertebrae have lost their symmetry and orientation and are distorted. Exposure: 45 kV, 2·5 mA, 6 min. Scale bar = 5 mm.

articular face [arrowed in Fig. 5(a)]. The normal junction was simply formed by a discontinuity in the mineralization of the extracellular matrix. Between the vertebrae the tissue was fibrillar and elastic, allowing articulation between the vertebrae.

In contrast, the fused junction [Fig. 5(b)] had lost flexibility and was characterized by a continuity in the mineralization of the extracellular matrix across the junction. Whilst the perichondrium was still distinct and continuous, it was no longer at the termination of the junction of the vertebrae, but rather at the periphery of the mineralized structure [arrowed in Fig. 5(b)]. The banding in the mineralized tissue beyond the junction represented incremental growth lines [marked i in Fig. 5(b)].

The appearance of a physical fracture was maintained in sections taken at various depths in the tissue, and therefore the crack could not possibly be wrongly identified as a tubule. The crack in the vertebrae contained exogenous tissue [marked e in Fig. 6(a)], and hence, could not be the result of physical damage to the tissue, resulting from poor histological technique, or trauma during excision. Incremental lines running across the crack [arrowed in Fig. 6(a)] would meet perfectly if they were re-apposed. The surfaces of these fractures were not eroded [Fig. 6(b)] and would also meet in perfect alignment if the sides were re-apposed.

The radiograph of the posterior lesion (Fig. 7) showed irregularly shaped and sized vertebrae around a radiolucent discontinuity in the vertebral column. The

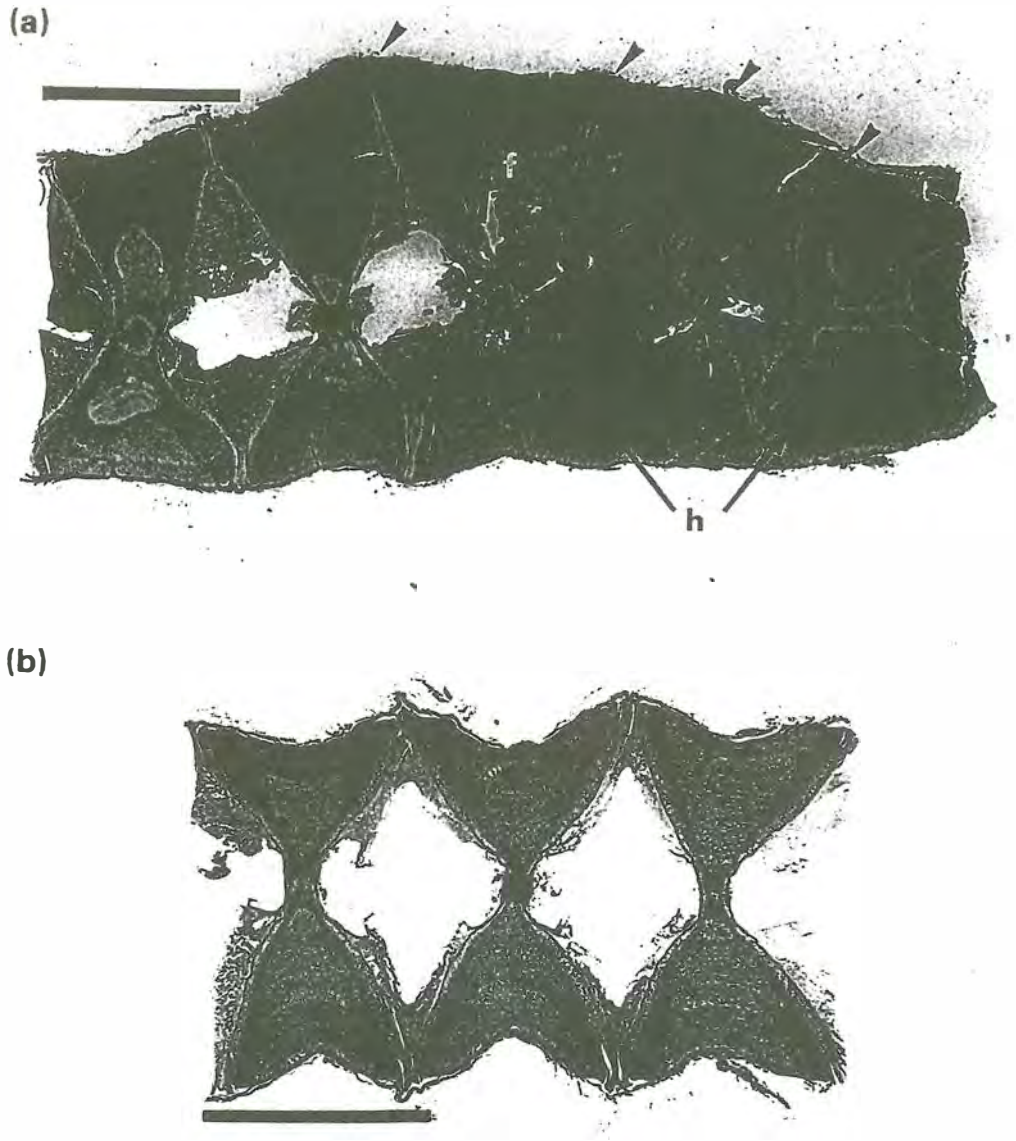

FIG. 3. Photomicrographs of sagittal sections from (a) the anterior deformity (same plane as Fig. 2), and (b) from normal tissue proximal to the anterior lesion. In the deformity the heavily mineralized arms of the articular faces of the corpus calcareum are distorted (large box, see Fig. 4 for detail). Fusion between neighbouring vertebrae is extensive (marked with arrows), and pronounced (f). Cracks (small box) contain exogenous tissue and are not eroded (see Fig. 6 for detail). Dark stained tissue (h) in radiolucent areas resembles hyaline cartilage. In normal tissue (b) the symmetry in the centra and discrete nature of each centrum is evident. Scale bars=5 mm.

mineralized tissue in the opposing articular faces of the middle vertebrae was absent (marked with a large arrow in Fig. 7). Neighbouring vertebrae were unusual in that their articular faces were elongated and curved towards each other (marked with small arrows in Fig. 7).

In histological sections, the posterior lesion [Fig. 8(a)] showed a similar curvature in the arms of the articular faces as that seen in the proximal lesion [marked with small arrows in Fig. 8(a) and (b)]. The site of missing mineralized tissue was also evident [marked with a large arrow in Fig. 8(a)]. Dark stained tissue [marked n in Fig. 8(b)] was unidentifiable. Hyaline cartilage [marked h in Fig. 8(b)] filled the space between the deformed vertebrae.

FIG. 4. Photomicrograph from sagittal section of the anterior deformity showing the severely displaced articular face [large box in Fig. 3(a)], and loss of the perichondrium at the termination of the junction (j). Growth of the mineralized tissue in the intermedialia shows curvature (c) and restriction (r). Scale bar=0·5 mm.

DISCUSSION

The cause of these deformities is difficult to deduce from the appearance of the tissues presented. It is possible that the deformities resulted from localized tumours or infection, or from past physical damage to the vertebral column. However, the point at issue is not how the lesions were sustained, but rather, the response processes used by the animal to heal the lesions, and hence create the deformities. In addition to the present study, there is apparently only one other study (Hoenig, 1979), that has described such extensive removal of mineralized tissue from the vertebral centra of a shark.

The removal of large amounts of mineralized tissue from the vertebral column of this shark, as evident in radiographs, and the lack of scarring in the skin adjacent to each wound, suggests that the mineralized tissue was most probably removed by internal cellular processes. Fusion of vertebrae and curvature in remnant mineralized tissues, suggests that unusual compensatory growth may have occurred, thereby restoring function to the vertebral column. The appearance of incremental growth lines in fused tissues laid down since the initial resolution of the lesions, indicates that a considerable amount of time has elapsed since the causative event. It appears that successive growth then occurred, overtaking the joints between vertebrae, and resulting in the observed deformities. The lack of any inflammatory cellular infiltrate in the histological sections also suggests that a considerable amount of time has elapsed since the lesions arose and that irregular cellular activity had ceased around the lesions at the time of the animal's death.

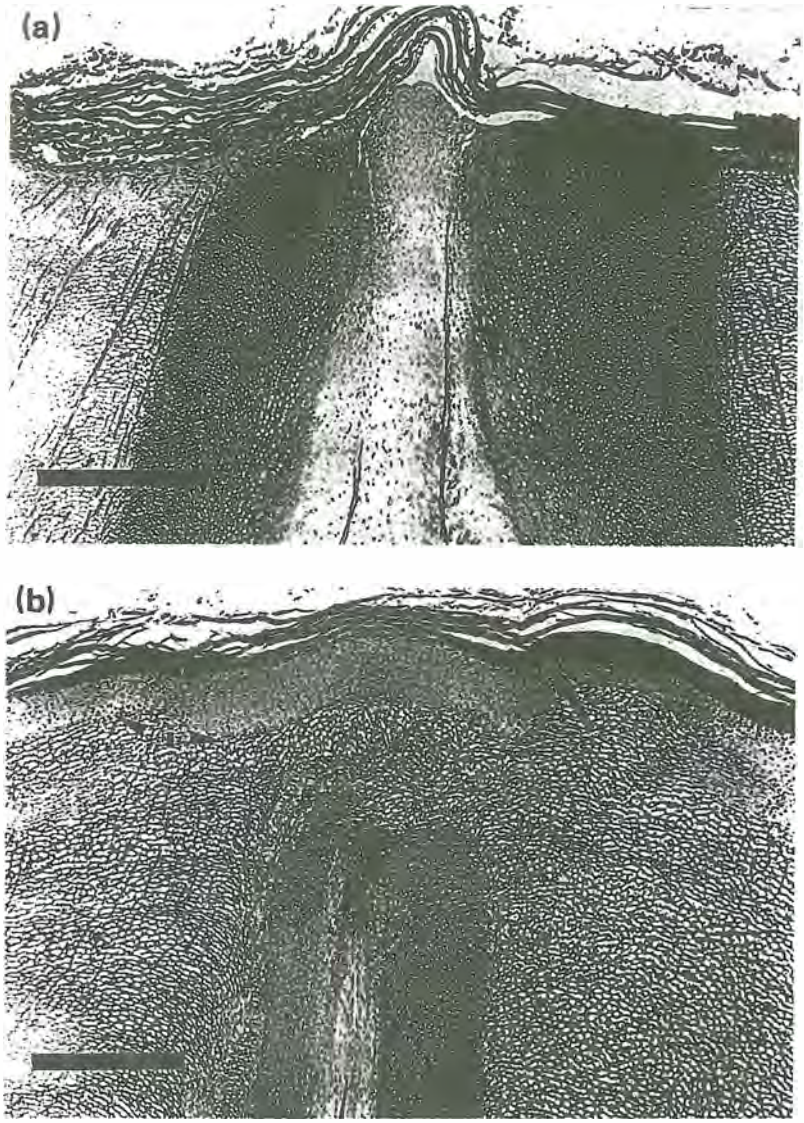

FIG. 5. Photomicrographs from sagittal sections of the anterior lesion showing normal (a), and fused (b) junctions between vertebrae. In the normal junction the perichondrium is located at the edge of each articular face (arrowed). Discontinuity in the heavily mineralized tissues of the intermedialia and articular faces separates the vertebrae. Elastic, fibrillar tissue between the vertebrae allows articulation. In the fused junction, continuity in the heavily mineralized cells across the junction has resulted in inflexibility. The perichondrium is no longer at the termination of the junction, but at the periphery of the mineralized structure (arrowed). Incremental growth lines [i in (b)] in the tissue beyond the junction indicate that fusion occurred by successive growth, the joint being overtaken, and growth then continuing. Scale bars = 0·5 mm.

Evidence of physical fracture suggests that the origin of the lesions was unlikely to have been developmental. The crack has a superficially similar appearance to a longitudinally cut cartilage canal, as described by Hoenig and Walsh (1982). However, the crack was evident in sections taken at various depths in the tissue, and therefore the crack could not possibly be tubular. Incremental lines running across the crack would meet perfectly if re-apposed, indicating that there was no lateral displacement in the crack. The lack of resorption in these areas does not represent an optimal recovery from physical trauma, but resorptive processes may have ceased once function was restored.

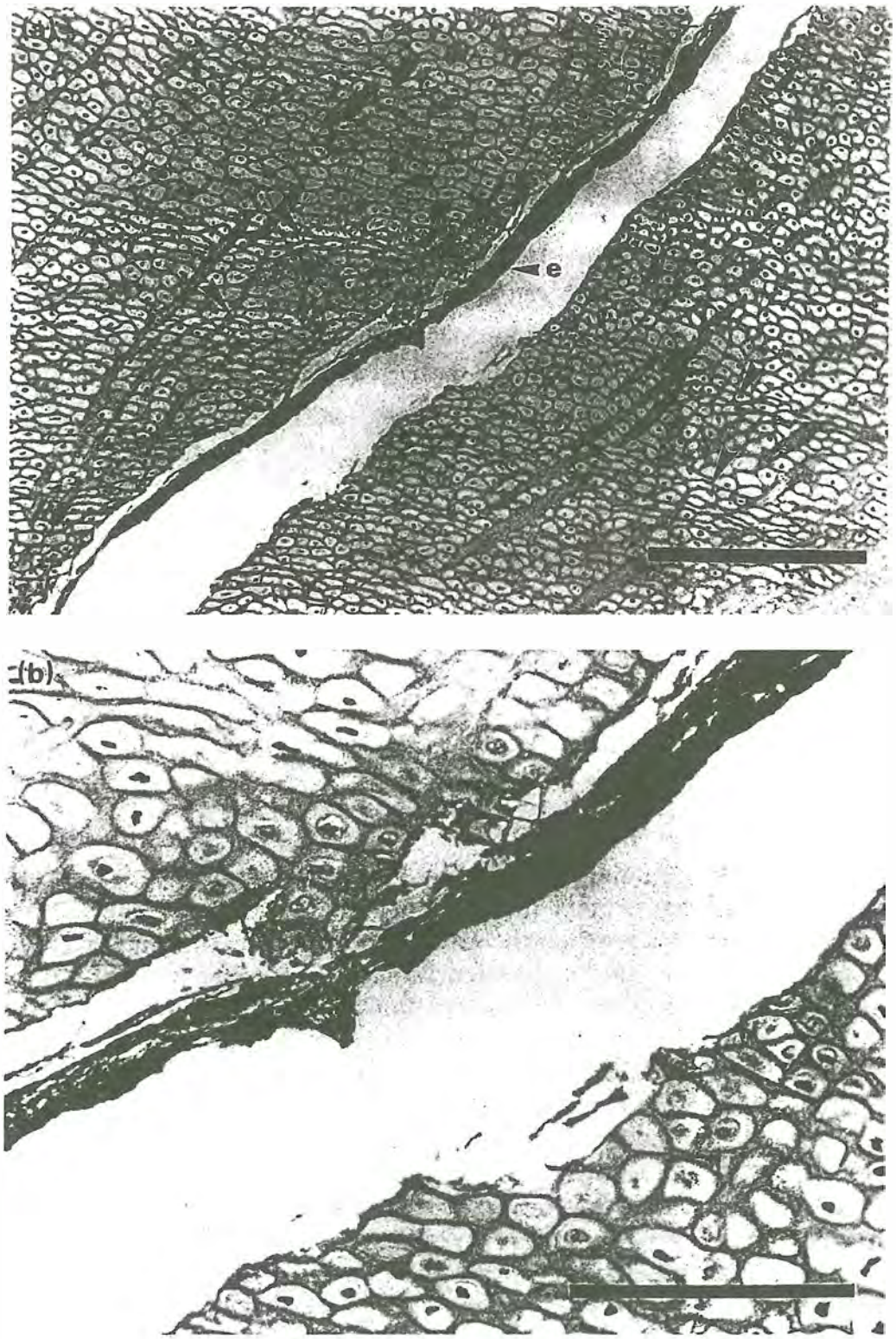

FIG. 6. Photomicrographs from a sagittal section of the anterior lesion showing a crack in a vertebra [small box in Fig. 3(a)]. The crack displays exogenous tissue [e in (a)], and therefore cannot be artefactual. Incremental lines (arrowed) running across the crack would meet perfectly, if re-apposed. Higher magnification (b) reveals that the sides of the crack are not eroded and would also meet if re-apposed. Scale bars: (a)=0·25 mm; (b)=0·1 mm.

This might explain why resorption did not occur in this area, despite the considerable time since the injuries were sustained.

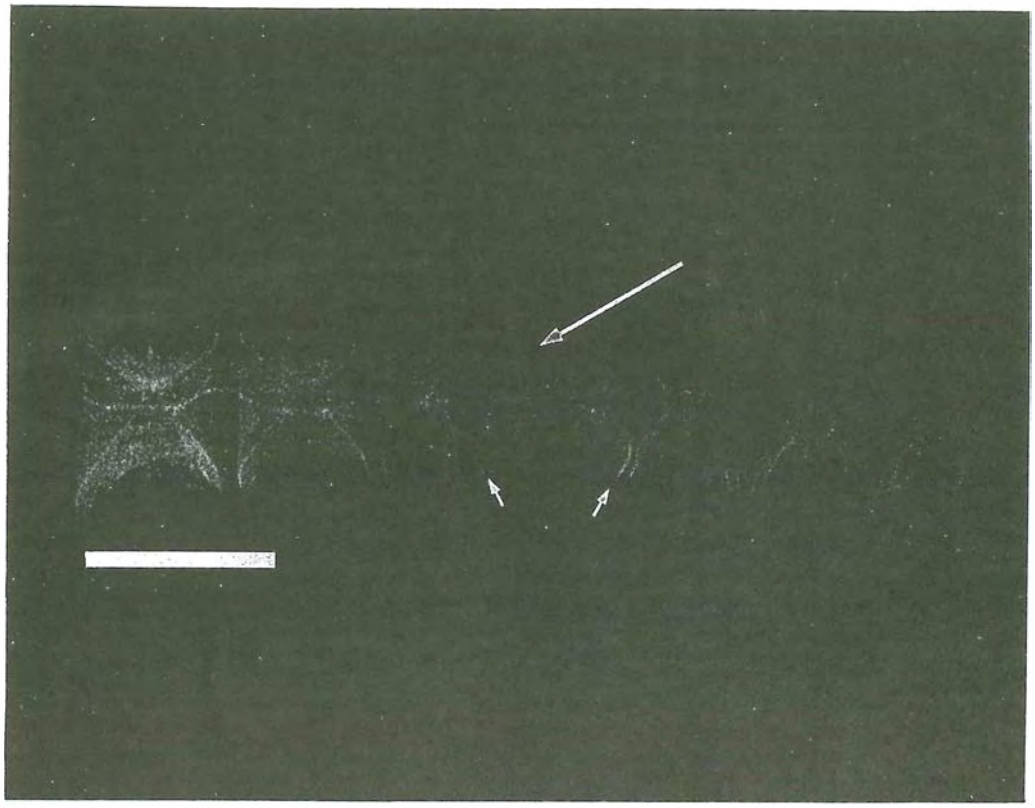

FIG. 7. Radiograph of the posterior lesion. Specimen viewed from dorsal side (left side of anterior, top of frame animal's right side). The deformity shows a radiolucent discontinuity in the vertebral column (large arrow), and distortion in the orientation of the arms of the articular faces of the vertebrae adjacent to the radiolucency (small arrows). Exposure: 45 kV, 2·5 mA, 5 min. Scale bar = 5 mm.

Incomplete healing of the lesions supports a limited resorption hypothesis. The remnant structures present in the deformities documented here may have resulted from irregular growth in the residual portions of the mineralized tissue. Such unusual growth could have served to stabilize the damaged region of the vertebral column, restoring near normal function to the column, despite the injury.

The probability that mineralized tissue was removed by internal processes is important because previous authors have concluded that the sharks they studied lacked the cellular or humoral factors necessary for the recruitment or activation of osteoclasts, or other resorptive cells (Glowacki *et al.*, 1986; Clement, 1992). These authors were unable to provoke detectable resorption in elasmobranchs by the implantation of mineralized foreign bodies (Clement, 1986; Glowacki *et al.*, 1986), or by fracture of dermal denticles and claspers (Clement, 1986).

Examination of histological sections from this study revealed no cellular evidence for continuing resorption of mineralized components at the time of the animal's death. However, it is possible that we may have viewed this animal too long after the initial lesions arose to see any resorptive cells. Active cellular resorption could have occurred closer to the time of injury but this activity may have since ceased with partial resolution of the lesions. Localized resorption could be activated temporarily, as part of an inflammatory response, with the resorbing cells disappearing once resorption of irreparably damaged skeletal tissue has been achieved. Similarly, some resorption in mammals has been

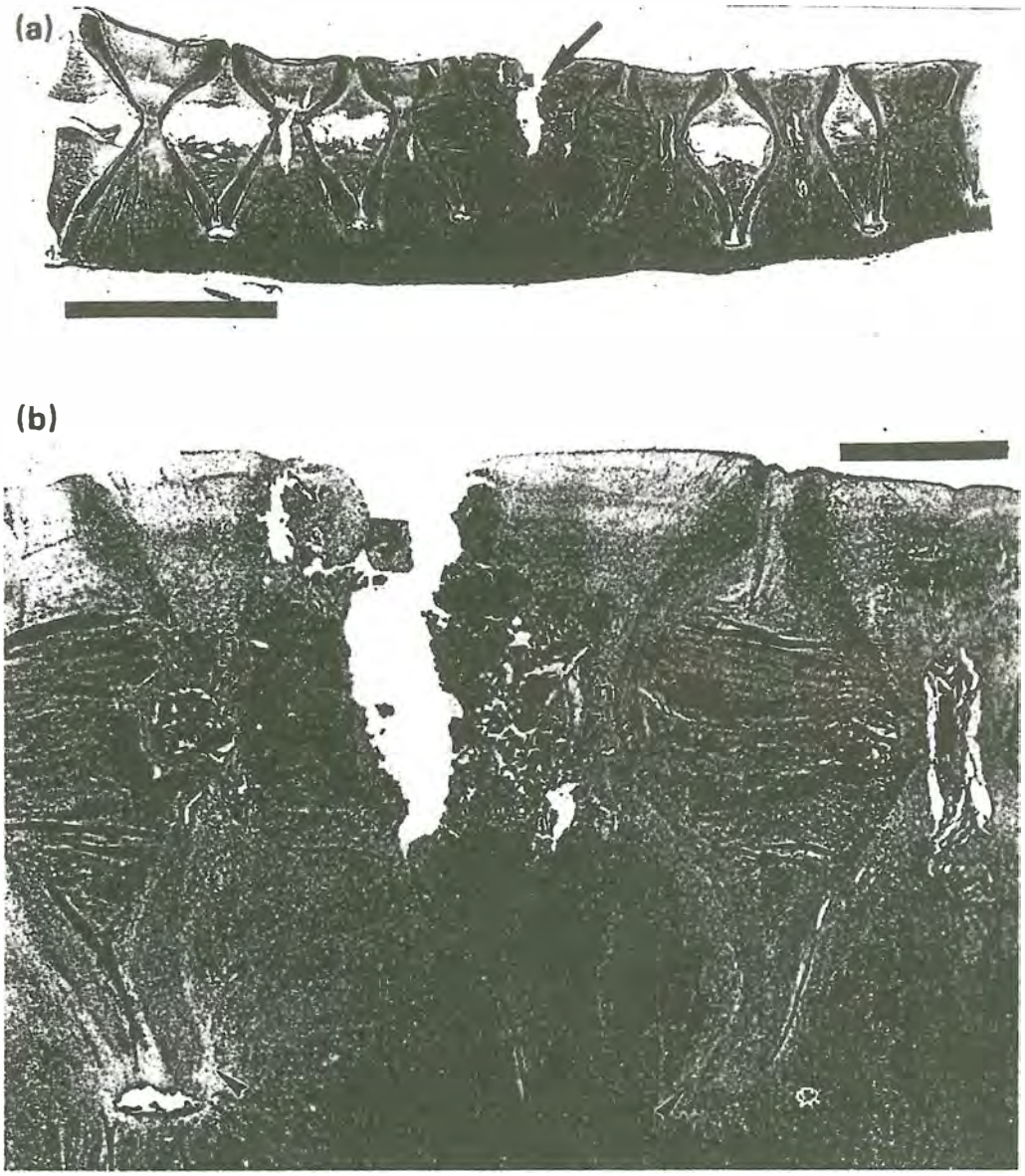

FIG. 8. Photomicrographs from a longitudinal section of the posterior lesion taken in the same plane as Fig. 7. Removal of mineralized tissue is evident [large arrow in (a)]. Curvature in the arms of the articular faces [small arrows in (a) and (b)] indicates compensatory growth. Higher magnification (b) reveals unidentified tissue (n), possibly resulting from prolapse of the notochord. Hyaline cartilage (h) fills the space between deformed vertebrae. Scale bars: (a)=5 mm; (b)=1 mm.

reported to be under local control (Mundy, 1992). Whilst other authors have suggested various cellular mechanisms by which resorption might occur (Holtrup *et al.*, 1982; Glowacki & Cox, 1986; Mundy, 1992), these explanations only provide a starting point from which to begin an examination of endoskeletal resorption in sharks. The processes described may not necessarily apply to other taxa, and therefore make extrapolation difficult. Even in mammals, resorption can occur by alternative mechanisms, including mononuclear phagocytosis (Holtrup *et al.*, 1982), highlighting the need to consider other cellular agencies of resorption. The absence of cellular evidence for continuing resorption in this study means we can only speculate as to how the lesions were initially resolved.

Studies by Peignoux-Deville *et al.* (1981, 1982) described osteoblasts and osteocytes in the neural arches, but only typical calcified cartilage in the centra

and haemal arches of sharks. Whilst no evidence was found for osteoclasts or resorption in these earlier studies, Peignoux-Deville *et al.* suggested mononucleated cells as potential resorptive cells (Peignoux-Deville *et al.*, 1982). In a subsequent study, Peignoux-Deville *et al.* (1989) suggested that both mononucleated and multinucleated giant cells might play a role in endoskeletal resorption.

Other cellular agencies of resorption could be considered. One possibility is that metaplasia could transform injured cartilage into fibrous tissue, which is then removed by fibroclastic resorption. This differs markedly from the situation in animals with an osseous skeleton, where the mineral and the collagen template are removed almost simultaneously by the same resorptive cell. A second alternative, drawn from Bordat's (1988) description of degeneration of chondrocytes in the dogfish, *S. canicula*, suggests that chondroclastic resorption may exist in the dogfish, acting independently of any bone, or bone-resorbing cells. These alternative mechanisms do not discount the possibility that the mechanisms themselves may be the phylogenetic relics of osteoclastic resorption in bony shark ancestors. Molecular biological techniques which probe for, and label the cells actively engaged in a resorptive role, would be more useful than descriptions of the morphology of the cells associated with areas of active resorption. Such primary methods of cellular identification would help settle the discourse over what might characterize an ' osteoclast ' in sharks.

The possibility that Chondrichythyans may resorb mineralized tissue, whether or not this occurs independently, or in the presence of bone, has important implications for the appropriate management of the world's shark fisheries. Growth increments seen in vertebral centra are often used as a means of ageing individual fish in the fishery (Cailliet *et al.*, 1986; Cailliet, 1990). Permanent physical stability of the vertebral centra, once formed, is an essential prerequisite to using the growth increments seen in the centra to determine the chronological age of the animal. If shark vertebral centra do not only grow in a strictly appositional manner, but rather undergo resorption at any stage, then their display of growth increments may not be chronological. It follows that any estimates of age and growth obtained from these structures will be flawed.

Ageing is necessary to calculate the age at which fish populations reach sexual maturity, and their longevity, mortality, reproductive rates and growth rates (Beamish, 1992). Since these parameters are needed to calculate the abundance of the stock available to a fishery, accurately determining the age of commercially exploited species is essential to good fisheries management (Ricker, 1977; Beamish & McFarlane, 1983). It follows that special care must be taken in age determination, otherwise calculations of stock abundance may be incorrect.

Despite the loss of mineralized tissue observed in this study, the present authors would claim the use of vertebral centra as a means of ageing school sharks is still valid. Resorption of the centra in this study may well be a process peculiarly invoked following damage to the affected vertebrae, and not part of the animal's normal physiological growth or calcium regulating processes. Therefore, the incremental pattern in other, unaffected centra would be undisturbed, remaining in a chronological order, and therefore still providing a valid pattern from which the animal could be aged.

This chance observation might also contribute to the debate over the evolutionary position of the cartilaginous fishes. The suggestion that resorptive processes might be present in a contemporary shark implies that bone cell lineages may still exist in the elasmobranchs, and consequently may help resolve the evolutionary debate (Hall, 1975, 1982). If it can be established that resorption exists in sharks, then sharks may be legitimately placed as highly evolved animals, in which their ability to form bone is now vestigial, or usually latent and unexpressed. Such a position contrasts markedly with the more traditional view that sharks are primitive forms, ancestral to all other fishes and amphibians (De Beer, 1947).

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Each photographic slide was projected at × 10 magnification onto a special digitizing table, where a cursor was moved around chosen outlines in order to define shapes as a series of X–Y coordinates recorded on punched paper tape. The tape was later used as input for computer manipulation of the digitized data. The relative positions of each section in three dimensions (X, Y and Z coordinates) were also digitized. This allowed correction orientation and spacing of each section with respect to its neighbours.

With this method, Sullivan was able not only to visualize the sites of bone deposition but also to calculate the volume of bone laid down during known time intervals. Kimura *et al.* (1977) improved Sullivan's technique by extending the computer programming to allow accurate calculation of surface areas and stereoscopic three-dimensional visualization of the computer reconstructions in the form of 'wire' models (Fig. 1). By manipulation of the program, the computer reconstruction could be viewed from any chosen direction. Where tracings were made of objects or spaces contained within larger objects, the option of removing or including lines hidden in any selected projection proved useful (Figs 1*B*–1*D*).

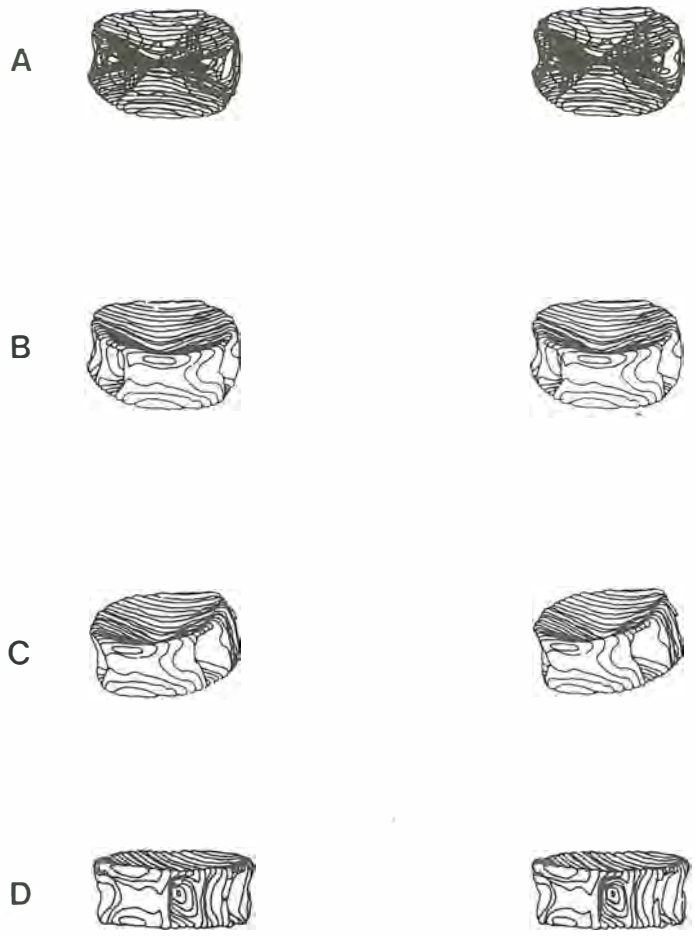

Fig. 1. Four computer-generated stereo-pair views of one longitudinally sectioned vertebra, drawn as wire models. Pair *A* reproduces all of the digitized information. The value of suppressing hidden lines is shown in Pairs *B*–*D*.

It was believed that the procedure of photographing and projecting at a desired magnification onto a digitizing table could lead to errors. This was particularly important when two photographic slides of the same object, but made with different modes of illumination, had to be employed to view fluorescent lines within bones. Furthermore, the projection magnification was restricted by the absolute necessity of being able to view the markers and the whole section simultaneously. Sullivan also had problems with insufficient light intensity when fluorescent outlines were projected. It therefore became necessary to radically improve aspects of the microscopy if the full potential of the method was to be exploited. A description of these improvements follows.

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Three-dimensional Reconstruction of Shark Vertebrae: A Technique with Applications to Age and Growth Studies

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Abstract

Shark vertebral centra show no histological evidence of resorption at any time in the animals' life. Deorganification of centra always reveals a large, residual, stable, three-dimensional skeleton. In contrast, the mineralized parts of other organs (e.g. claspers and jaws) crumble into their individual mineralized subunits, the tesserae, upon deorganification. In both cases, only appositional growth of cartilage on the pre-existing mineralized template is possible. The basic 'double-cone' shape of the vertebrae facilitates increases in body length simultaneously with an accompanying increase in girth. Once the initial shape of the mineralized portion of a vertebral centrum is fully established and hence can be described, then relatively simple mathematical models might be devised to predict future growth patterns. To advance this hypothesis, it has first been necessary to develop a method that can accurately record the sizes and shapes of complex three-dimensional anatomical structures. This paper describes a technique that is capable not only of recording and measuring the size and shape of shark vertebrae but also of predicting their subsequent growth. Furthermore, the technique enables reproduction of three-dimensional coloured and shaded stereoscopic images of vertebral structures, facilitating a better understanding of their intricate morphology. Three-dimensional coordinate data gathered from any shark vertebra can be manipulated mathematically to model future vertebral growth. Producing realistic images of vertebrae transformed in this way may allow the exploration of possibly unrealized taxonomic affinities.

Introduction

A method to enable the three-dimensional reconstruction of solid objects from serial sections was proposed by Gillings and Buncore (1961). At that time, the method, although theoretically possible, required the manipulation of such large amounts of data that it was rendered practically useless. A decade later, Sullivan (1972) developed a very similar principle, but, as had been predicted by Gillings and Buncore (1961), his method was a computer-based one, which he used to study jaw growth in dogs. In that study, dogs were treated with fluorescent dyes that were selectively taken up at the mineralizing fronts of growing bone and tooth surfaces. Growing dogs were injected at known intervals, killed, and their hemimandibles embedded in blocks of polyester resin. The blocks were then sectioned in such a way that the orientation of each section with respect to its neighbours, and within the depth of the block, could be noted.

In Sullivan's (1972) study, it was necessary to photograph every section, first in white light to reveal anatomical details and then under ultraviolet radiation to excite the fluorescence from the bone-marking dyes selectively incorporated within the tissues.

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Table 1. Digitization data obtained from British Standard gauge blocks and used to determine the accuracy of the prototype coordinate-plotting microscope and its micrometer-driven stage

Quoted distance between parallel faces of gauge block (mm)	Line No.	No. of points defining line	Calculated slope	Standard error
15·000	1	47	−5·0258	0·0002
(±2 μm)	2	27	−5·0213	0·0003
	3	60	−1·3072	0·0002
2·005	4	64	−1·3072	0·0003
(±2 μm)				

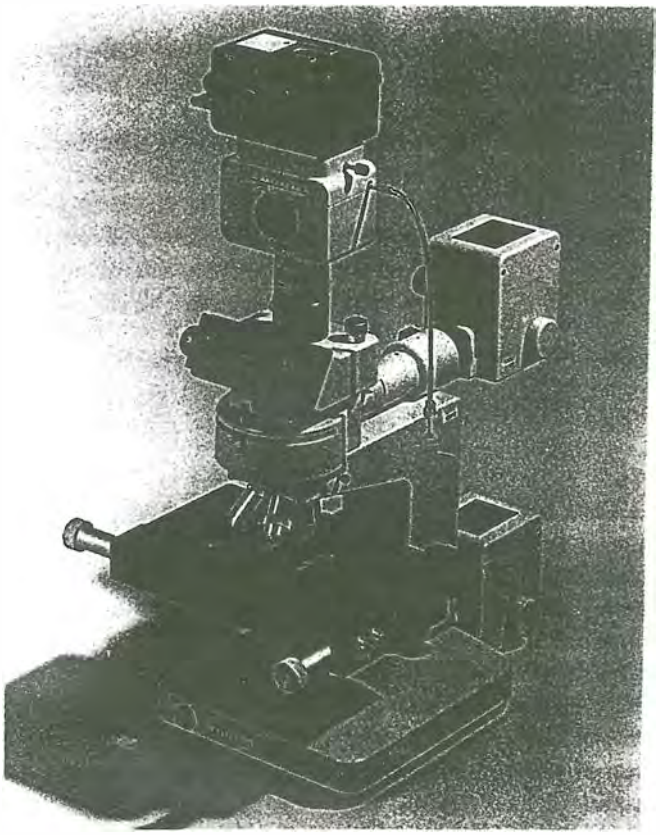

Fig. 3. Modified Leitz Dialux 20 microscope equipped with a stepper-motor-driven stage, fluorescent and white light sources, and a photomicrographic recording system.

MPS 11) and an exposure meter (Wild MPS 15). The standard fixed stage of the microscope was removed and replaced by a stepper-motor-driven Mäerhauzer EK32 stage capable of 75- and 50-mm movements in the X and Y directions, respectively, and of accurate, reproducible stage increments as small as 10 μm.

Potentiometers within a Lang Elektronik MCC12 JS-RS232 positioning system are coupled to the stage stepper motors. Altering the resistance in the potentiometers induces a precise movement of the stage. The positioning system is driven by a Logitech Trackman stationary mouse. Rolling the trackball of the mouse alters the resistance within the potentiometers and consequently the position of the stage.

In the eyepiece of the microscope there is a fixed cross-wire. Objects or sections to be digitized are securely fixed to the stage of the microscope. Manipulation of the trackball driving the stage brings any point chosen to define an outline under the eyepiece cross-wire. Depression of the buttons on the mouse records the X and Y values of the stage and hence of the feature of interest in the section

attached to the stage. The stage is then moved to the next selected point defining the chosen outline, and the new X and Y coordinates are recorded in an identical way. Operation of the system in this manner becomes a quick, one-handed process. This procedure is repeated until the whole outline is digitized.

It was discovered that blunting of the tip of the milling cutters leaves the fiducial markers with slightly rounded points that are visible through the microscope. To overcome this problem, each sloping face of a marker is digitized as a series of points. The best straight line fitting these points is calculated, and the theoretical intercept of both sloping faces is taken as the 'infinitely sharp' point of the marker (Clement and Dykes 1982). Sections can be automatically aligned to common axes and a common origin by using these reference points.

The number of points needed to define any outline varies, but complex shapes can be accurately recorded only by using many points close together. The maximum size of specimens that can be digitized is limited only by the range of the stepper motors driving the stage. Once the coordinates of the fiducial markers have been digitized, they can be moved out of the optical field of view. This allows different magnifications to be used as appropriate to the detail of the object being traced.

Large photographic sheets or tracings obtained from magnetic resonance imaging (MRI), computed axial tomography (CAT), or any other imaging method can be digitized with an appropriate digitizing tablet. In such cases, in which the use of a digitizing microscope is not advisable, a digitizing tablet can be a feasible alternative to either of the above digitizing microscopes. The present system utilizes a translucent, backlit digitizing tablet (GTCO Corporation, Type 5A) with an active area of $60 \times 42{\cdot}5$ cm (Fig. 4). Each point in the outline is recorded by depressing buttons on a cross-hair cursor as the outline is traced. The quoted accuracy of the tablet is $\pm 0{\cdot}25$ mm, with a resolution of $0{\cdot}025$ mm.

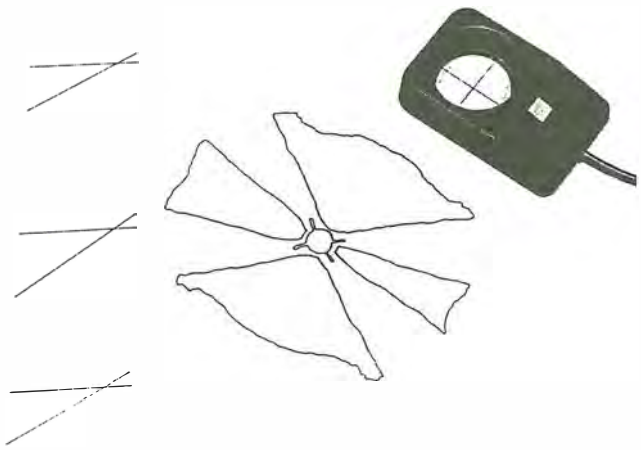

Fig. 4. Projected outline on a digitizing tablet, with (*left*) the three reference markers used to align sections and (*right*) the four-button cursor used to trace projected outlines or images on sheet film.

Results

Detail of one transverse section from a blue shark vertebra is shown in Fig. 5. A comparison is made between a photomicrograph of the section and an image of the same structure as drawn by the computer from the X–Y coordinates recorded with the digitizing microscope. The four rectangular-based, pyramidal, tapering holes entering the centrum can be seen to converge at a capstan-shaped structure at the centre of the vertebra.

Digitized sections are aligned by computer to common axes and a common origin (see Appendix for details). The computer drawings of all of the sections from the original vertebra can then be superimposed in their correct two-dimensional relationship (Fig. 6). The utility of incorporating a diagonal fiducial marker is clearly illustrated in Fig. 1, where all of the section outlines have been given their correct separation in addition to their accurate alignment in two dimensions. This three-dimensional alignment of all of the outlines can be perceived only when the coordinates of all of the outlines are rotated by the

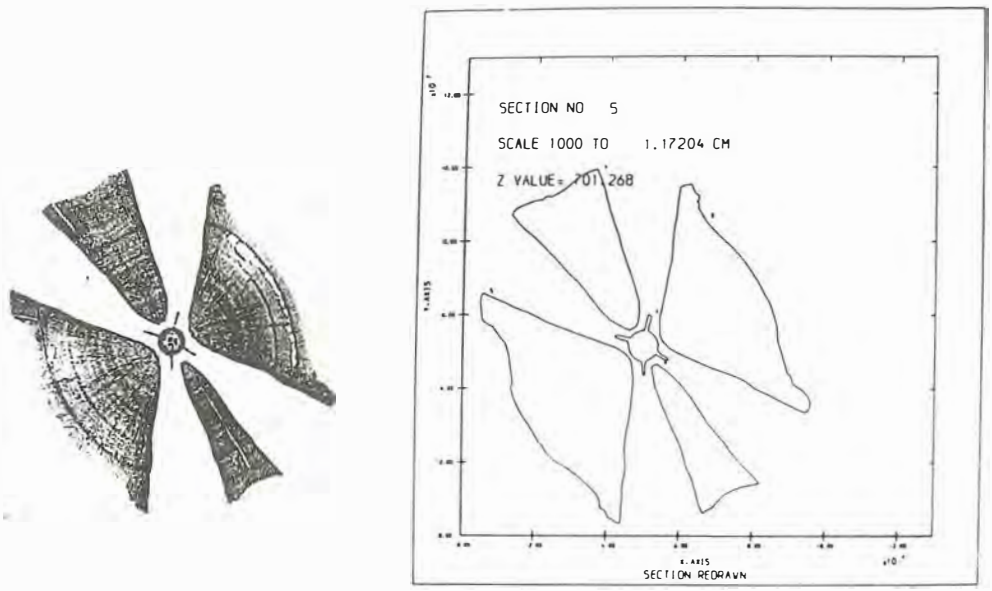

Fig. 5. (*left*) Photomicrograph of the centre section of a transversely sawn vertebra, and (*right*) the corresponding computer-generated drawing oriented according to the X–Y coordinates gathered by the digitizing microscope.

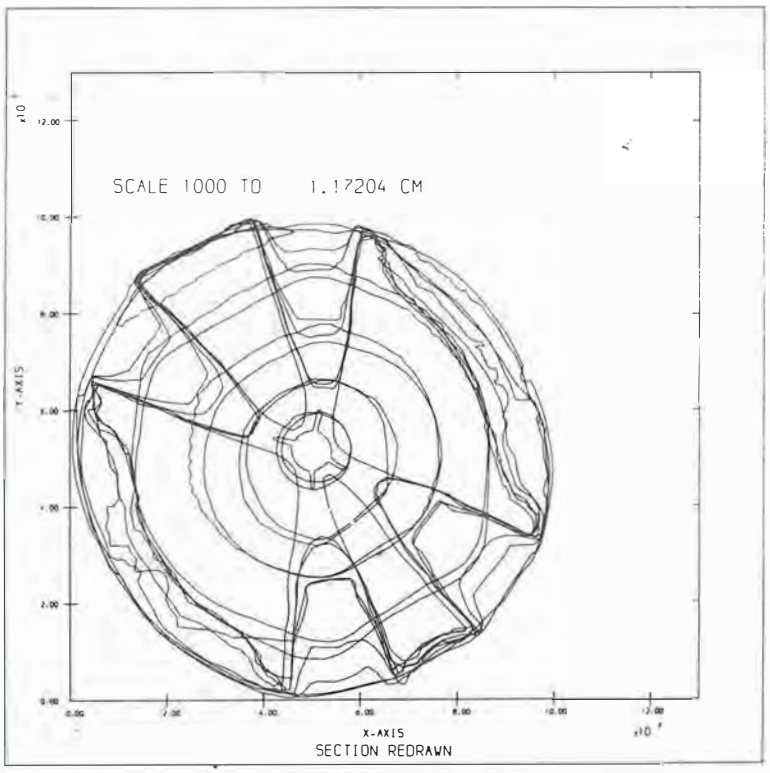

Fig. 6. Ten computer-drawn transverse sections of a complete vertebra superimposed on a single frame. The two fiducial markers common to all sections have been used to orient the outlines to a common origin and common axes.

computer. The three-dimensional reconstruction is coloured, shaded and lit by the computer and compares very well with a photograph of the original specimen under oblique lighting (Figs 7 and 8).

The ability to recalculate the three-dimensional coordinates means that it is possible to view the original structure from a variety of directions. This can be exploited to produce stereoscopic pairs by rotating the image for one eye 10° from the image for the other eye.

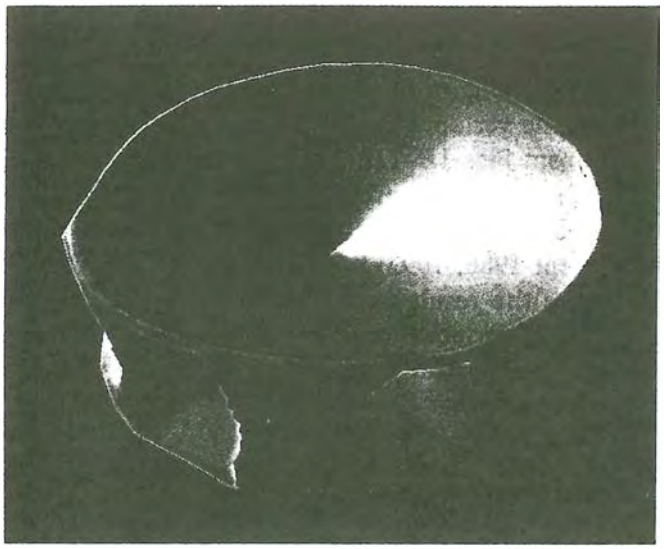

Fig. 7. Coloured and shaded computer-reconstructed image of a vertebra from a blue shark (*Prionace glauca*).

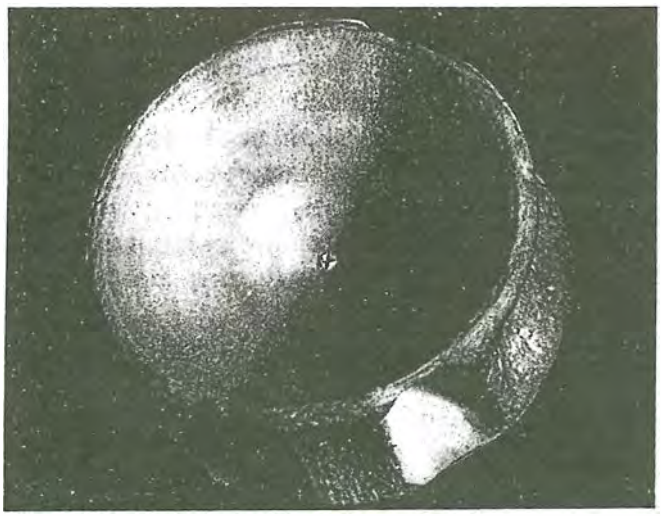

Fig. 8. Photograph of an original anorganic vertebra from a blue shark following treatment in a sodium hypochlorite solution.

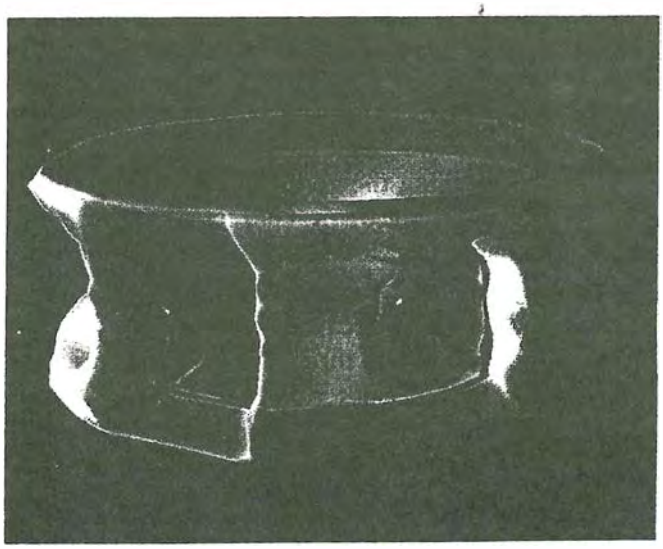

Fig. 9. Simple mathematical manipulation creates an image of a vertebra differing in proportion from the original reconstruction. The enclosed vertebra depicts a vertebra from the same animal at a younger age.

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Appendix. Description of Computer Programs

A suite of nine computer programs written by Cookson *et al.* (1987) in the language C for maximum portability have been developed to implement (*a*) data acquisition and section alignment (three programs), (*b*) surface modelling (three programs), (*c*) image generation (one program), and (*d*) calculation of surface area and volume (two programs).

Data Acquisition

The output from the preferred digitizing device is recorded and displayed on the computer screen. The digitization procedure is menu-driven and allows for editing of points. Each digitized structure is treated individually to produce a separate data file. These contours are then scaled and rotated to their correct alignment relative to each other. Multiple structures forming a single scene are then oriented relative to an appropriate origin to ensure the correct three-dimensional alignment of the separate structures.

Surface Modelling

The missing surface between adjacent sections is reconstructed in the form of a series of triangles generated from the X–Y contour points defining each outline. Although this process can be achieved automatically, the facility exists to force correct alignment between equivalent features in adjacent sections. The surface-modelling programs produce two output files for each structure that contain information regarding the triangle vertices and triangle normals.

Image Generation

The image-generation program produces shaded images from the data created by the surface-modelling program. To select a view, the user specifies a target point within the object that is to appear at the centre of the image on the computer screen. The position of the observer (distance from target point, bearing, and elevation) is then selected relative to this target point. The user may also define the magnification of the image and the size and shape of the viewpoint on the screen in which the image is to be generated. The triangles that describe the surface model are then transformed to take account of these viewing parameters and are mapped onto the two-dimensional computer display with a perspective projection for maximum realism. Hidden-surface removal and smoothing of the image can be selected to produce realistic solid and shaded images.

Calculation of Surface Area and Volume

The three X–Y–Z coordinates defining a particular triangle are used to calculate the area of that triangle. The sum of the areas for all of the triangles that make up the surface model of a particular object is therefore the surface area of that object. The volume enclosed by a particular structure is also derived from the triangle coordinate data. The area of each triangle normal to the X axis is multiplied by its distance from that axis. This generates a volume element for that particular triangle. Because the triangles connecting adjacent sections are ordered in a clockwise sequence in that slice, the sum of the triangle volume elements gives the total volume enclosed in that slice. This procedure is repeated for all slices that make up the whole structure, and the sum of all such slice volume data results in the total volume of that particular structure.

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Final Report to
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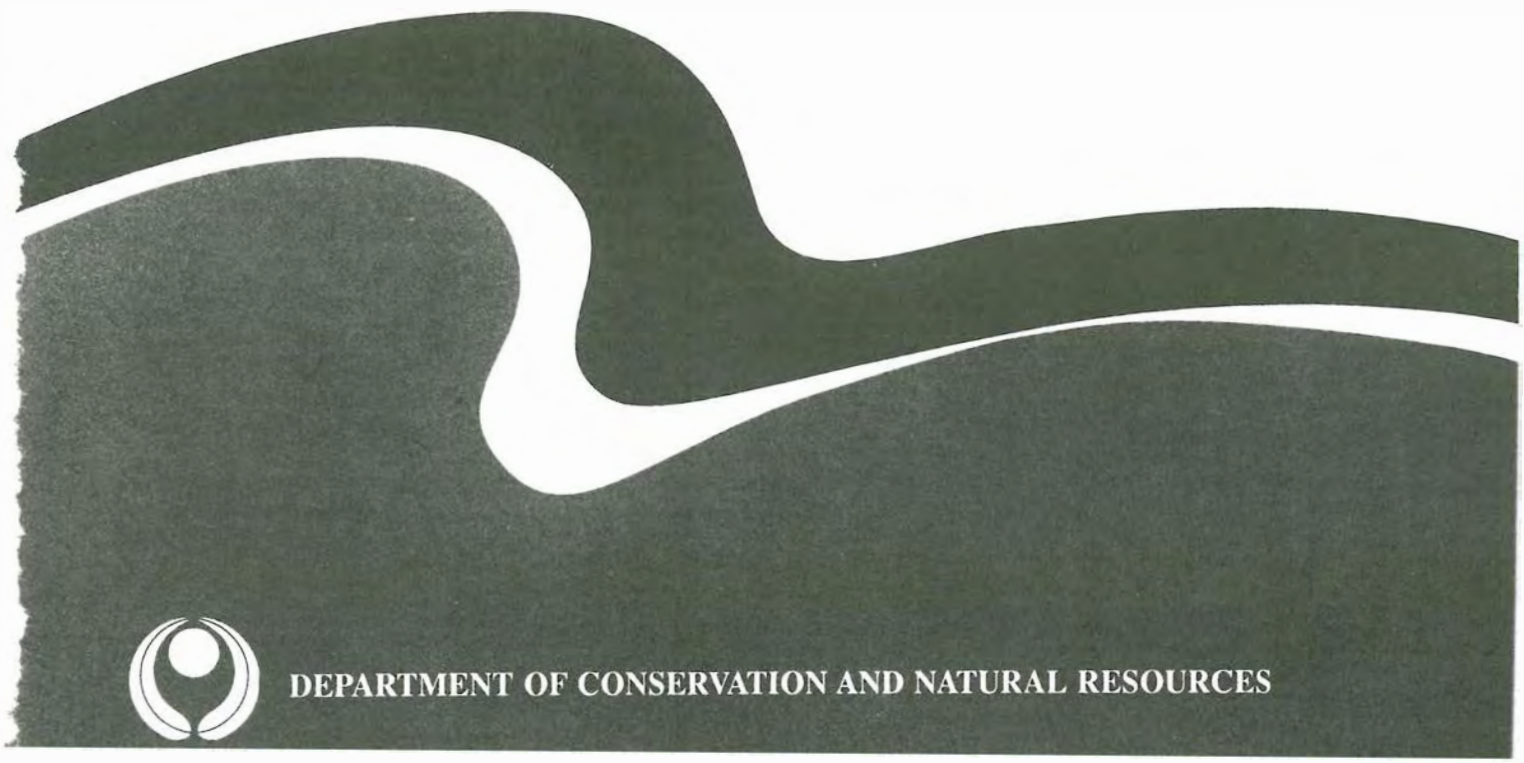

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May 1995

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Abstract

Sources of variation in counts of growth increment lines in vertebrae from gummy shark, *Mustelus antarcticus*, and school shark, *Galeorhinus galeus*, were examined in vertebrae taken from the cervical, thoracic and pre-caudal regions of the vertebral columns. Vertebrae from school shark were more difficult to read than those from gummy shark. The number of increment lines an experienced reader counted on the external surface of stained whole vertebral centra was consistent with the number of major hyper-mineralised increments counted on micro-radiographs of vertebrae. Differences between increment counts (IC) obtained by four different readers were significant. IC by experienced readers were more precise and least biased. IC from vertebrae sampled in the same region of the vertebral columns were not significantly different. IC from vertebrae sampled in different regions were significantly different and were highest for vertebrae from the thoracic region. The ramifications of such differences are discussed - especially with reference to distortions of von Bertalanffy growth curves and the reliability of stock assessments.

Introduction

Gummy shark, *Mustelus antarcticus* Günther, and school shark, *Galeorhinus galeus* (Linnaeus), off southern Australia provide most of the catch in a shark fishery which 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 (IC) of 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 under-estimates. Moulton et al. (1992) also suggested that the Micro-Radiographic Method (MRM) (Cailliet et al. 1983; Ferreira and Vooren 1991) might yield more reliable IC for larger sharks.

Whichever method is used, the results need to be validated (Cailliet 1990; Beamish 1992) and such validation requires an appreciation of the variability inherent in each method. Both the MRM and the alizarin staining method (ASM) involve the counting of growth increment lines in vertebrae. But in elasmobranchs not all the vertebrae in a vertebral column have the same number of growth increment lines (Parsons 1993). Therefore IC from vertebrae in different regions of the vertebral column will probably differ (Natanson and Cailliet 1990). Establishing whether sources of variation in IC can be either minimised or eliminated is crucial to improving the reliability of the ages estimated for gummy and school shark.

The study we describe here was designed to assess how much the variation in IC from gummy and school shark vertebrae depends on (i) the method used to count growth increment lines; (ii) the effect of within-reader variability on precision and bias in repeated IC derived for the ASM and MRM; (iii) the effect of between-reader variability on precision and bias in the ASM; (iv) the variation in IC from vertebrae taken within the

same region of the vertebral column, and (v) the variation in IC between vertebrae taken from different regions of the vertebral column.

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 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 mm, 900-1200 mm and ≥1200 mm total length) for each species and sex (i.e. 2 species × 2 sexes × 3 length-classes × 2 sharks = a total of 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. The commercial fishers made an incision at the point where they would normally have decapitated the shark so that we could estimate how many vertebrae would normally be discarded from the proximal end of the vertebral column. 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, decapitation incision, dorsal fins and tail remained attached to the vertebral column. Steel pins were inserted at the decapitation incision, at the anterior origins of the first and second dorsal fins, and at the pre-caudal 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., UK) and a 'Machlett Dynamax 54' X-ray tube (Machlett Laboratories Inc., USA) 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 kVp 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 post-cranial vertebrae were designated as the 'cervical' region, which is routinely sampled from commercial catches. The 12^{th} and 13^{th} vertebrae posterior to the origin of the first dorsal fin (the 32^{nd} and 33^{rd} vertebrae in gummy shark, the 38^{th} and 39^{th} 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 pre-caudal pit (the 82^{nd} and 83^{rd} vertebrae in both species) were designated the 'pre-caudal' region. The pre-caudal 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.

The two vertebrae from each of the three regions were prepared for staining with alizarin red S ('Sigma-Aldrich' P/L, Australia). Hence, six vertebrae were taken from each of the twenty-four 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 = a total of 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 minimise the possibility of over-bleaching which can damage the vertebral centra. The bleaching process was stopped once all the fascia material had been removed, and the less-mineralised 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 (ASM)

Four readers were engaged to read the alizarin stained vertebrae. Of the readers, one had extensive experience, having produced age determinations from several hundred gummy and school shark vertebrae (Moulton et al. 1992); one had limited experience in reading alizarin stained shark vertebrae; a third reader had extensive experience reading otoliths of teleosts; and the fourth reader had no experience of reading alizarin-stained shark vertebrae or otoliths.

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 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. 2(a). Each reader then viewed the stained vertebra under a stereo-microscope (×6.4 to ×10 magnification), assigned it an IC and a subjective readability score (see Table 1), and then passed the vertebra to each of the other three readers who independently assigned an IC and readability score to the vertebra. To complete Round 1 this cycling procedure was repeated until, after 36 cycles of four vertebrae, all 144 vertebrae had been examined by each of the four readers. All possible combinations of passing on the vertebrae among the four readers were used so that reader precision and bias were effected 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.

Micro-radiographic method (MRM)

To allow for comparison between reading methods, seventy-two vertebrae used during the ASM were prepared for analysis by the MRM. These vertebrae included two vertebrae from each of the three designated regions of the vertebral columns of twelve sharks (i.e. 2 species × 2 sexes × 1 shark × 3 length-classes × 3 regions × 2 vertebrae = a total of 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., USA). Sections were placed on top of a light-safe bag containing 'Industrex SR' film ('Eastman Kodak' Co., USA) The film was positioned 48 cm from the focus, exposed at 20 kV and 2 mA for 1 min. 55 s, and then developed with standard radiograph development chemicals and procedures.

Each micro-radiograph was scanned at high resolution and its image was stored on a computer file. An interactive sub-program was custom written for the 'Optimas 3.10' image analysis program ('BioScan' Inc., USA) to allow an IC to be assigned to the scanned image of each micro-radiograph. One reader experienced in reading micro-radiographs of vertebral centra read the scanned images of micro-radiographs in a random order as a double blind study. The reader counted all increment seen adjacent to the articular face and characterised these increments as belonging to one of three categories: major increments (well defined hyper-mineralised growth lines), uncertain increments (hyper-mineralised growth lines with an irregular spacing), or minor increments (fine check marks). The characteristics of each type of increment are shown in Fig. 2(b). For each vertebra IC were assigned for three types of increments: type M1 included major increments only; type M2 included major and uncertain increments; type M3 included major, uncertain and minor increments. Each vertebra was assigned a readability score in the same way as were vertebrae read in the ASM. The micro-radiographs 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 IC was assessed from the results of six statistical analyses: three involved pairwise comparisons to assess within-reader variability for the ASM and for each of the three increment types for the MRM; two involved pairwise comparisons to assess between-reader variability for the ASM; and one involved a nested analysis of variance 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 IC. The analyses for within-reader variability involved a test for within-reader bias (Analysis 1) and tests for within-reader precision (Analyses 2 & 3). The two analyses for between-reader variability involved tests for between-reader bias (Analysis 4) and tests for between-reader precision (Analysis 5). These analyses, as well as the analysis of variance (Analysis 6), were undertaken for gummy and school shark separately. A summary of the statistical tests

associated with these six analyses is presented in Table 2. Before undertaking the analyses, the IC were evaluated according to their readability score.

Readability

Readings having high readability scores did not yield an IC and, depending on the pairwise comparison of IC, some readings having lower readability scores produced an IC but had to be excluded from various tests. The percentage of readings given each readability score was calculated for each ASM reader and each type of IC from the MRM. Readings assigned high readability scores were rejected from subsequent analyses when data from each reader was sufficient to allow comparison of IC between Rounds 1 and 2.

Similarly, only IC having acceptable readability scores from Round 1 were used to compare IC from the three regions of the vertebral column. Hence, various separate selections of IC with acceptable readability scores had to be made for gummy and school shark separately when we examined variability in IC within-reader, between-reader, within-region, between-region of the column, and between the ASM and MRM reading methods.

Within-reader Bias (Analysis 1)

To compare the mean and standard deviation of the differences in IC between Rounds 1 and 2 for each ASM reader and for each of the three MRM increment types we used pairwise *t*-tests to test the null hypothesis of:

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

where Rd_1 and Rd_2 are the IC obtained for a vertebra by a reader during Rounds 1 and 2, respectively.

Within-reader Precision; Index of Average Percent Error (Analysis 2)

In the first of two analyses for within-reader precision we used Beamish and Fournier's (1981) formula for Index of Average Percent Error (IAPE) namely:

$$IAPE = \frac{1}{n}\sum_{j=1}^{n}\left(\frac{1}{R}\sum_{i=1}^{R}\frac{|X_{ij} - X_j|}{X_j}\right) x\,100$$

where, for the purpose of our analysis, n is the number of vertebrae read with acceptable readability scores, R is the number of times each vertebra is read (R = 2 for 2 Rounds), X_{ij} is the i^{th} IC of the j^{th} vertebra read, and X_j is the average IC calculated for the j^{th} vertebra.

Calculations of IAPE did not include the IC 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 IC between Rounds 1 and 2 for each of the four ASM readers and for each of the three MRM increment types we used a sign rank test which tested the null hypothesis:

$$H_o: \ |Rd_1 - Rd_2| = 0$$

where Rd_1 and Rd_2 are the IC 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 IC did not have a normal distribution.

Between-reader bias (Analysis 4)

To test the bias in IC between pairs of ASM readers we used pairwise *t*-tests to test the null hypothesis:

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

where Rr_i and Rr_j are the IC 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. Between-reader bias was also assessed by calculating the number and percentage of vertebrae in which pairs of IC differed by discrete amounts between Rounds 1 and 2.

Between-reader Precision (Analysis 5)

A sign rank test was used to test precision in IC between pairs of ASM readers. The differences in IC between a pair of readers during Round 1 were adjusted by dividing by the mean IC for the two readers; thus distortion caused by high IC was avoided. The null hypothesis for this test was:

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

where Rr_i and Rr_j are the IC 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.

Analysis of Variance in IC due to other Sources of Variation (Analysis 6)

We used a nested design analysis of variance to assess variation in IC between the ASM and MRM and within and between regions of the vertebral column. Results from readers presenting IC having significant bias or poor precision between Rounds 1 and 2, or between readers, were excluded from the analysis of differences in IC between reading methods and between regions of the vertebral column. In our analysis of IC from the three MRM increment types M1, M2 and M3 we treated each type as separate reading methods, because each represents a different way of estimating an IC from a vertebrae sectioned for the MRM. The primary model statement used for this analysis appears in Table 2 and 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 due to each effect was tested by using appropriate error terms, as shown in Table 3. Where IC was significantly different due to interactions between length-class or sex with reading method or region, the analysis of variance was performed separately for the subgroups comprising each of the interaction terms. In those cases, the Duncan's test of comparison of means was used to group significant results by reading method or by region. In this way, the relative magnitude of the effect detected due to region or reading method was indicated.

Variation between IC 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 IC for all vertebrae within each region and comparing that to the mean for all regions combined.

$$\textit{Adjusted Mean Difference} = \sum_{i=1}^{n} \left(\frac{\overline{Sk_i} - \overline{Rg_i}}{\overline{Sk_i}} \right) / n \Bigg\rangle$$

where Sk = shark, Rg = region, i = the i^{th} shark or region, and n = the number of sharks where 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.

Results

Readability

A similar number of IC was provided for analysis by utilising all readings assigned a readability score of less than, or equal to 3 for the ASM readers A1, A3 and A4 and readings assigned a readability score of less than, or equal to 4 for ASM reader A2 and the one reader of the three MRM increment types. The range of readability scores and readings included in the analyses for each reader are presented in Table 4. 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 5). The MRM gave more consistent readability scores than did the ASM.

Within-reader Bias (Analysis 1)

ASM reader A1's IC in Rounds 1 and 2 were not significantly different for either gummy shark (P = 0.604) or school shark (P = 0.215). However, IC by ASM readers A2, A3 and A4 (Table 6) indicated a bias for higher IC during Round 2 than during Round 1.

Although all readers assigned higher IC during Round 2 than during Round 1(Fig. 3), the bias was least for A1, for whom 41% of gummy shark IC (Fig. 3a) and 49% of school shark IC (Fig. 3b) did not differ between Rounds 1 and 2. ASM readers A2, A3 and A4 did show a bias in their IC between Rounds 1 and 2. The spread of differences in IC between Rounds 1 and 2 for each of the MRM increment types M1, M2 and M3 (Fig. 3c) was similar to those for A2, A3 and A4 (Fig. 3a and b) but the spread was greater for school shark (Fig. 3d) than for gummy shark (Fig. 3c).

Within-reader Precision (Analyses 2 and 3)

IAPE scores by ASM reader A1 were relatively low (gummy shark; 8.6%: school shark; 5.0%) (Table 7). 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 MRM increment types M1, M2 and M3 (6.6%, 7.2% and 8.0%, respectively) made by the one reader. However, for school shark, IC made for the three MRM increment types (8.9%, 8.7% and 6.8%, respectively) were less consistent than the IC made by A1 and A2. The sign rank test indicated that differences between IC in Rounds 1 and 2 were highly significant for all four ASM readers and for all the MRM increment types (Table 6).

Between-reader Bias (Analysis 4)

ASM reader A4 tended to assign significantly lower IC than did A1, A2 and A3 whereas there was no significant bias between IC by A1, A2 and A3 (Tables 7 and 8).

Between-reader Precision (Analysis 5)

The sign rank test for between-reader precision indicated that differences between all paired combinations of ASM readers were highly significant (Tables 7 and 8). However, A1 produced a mean IC closer to the mean IC of A2, A3 and A4 than did A2, A3 or A4 when compared to the mean IC of the other three readers combined (Table 8).

Effects of Other Sources of Variation on IC (Analysis 6)

IC by ASM readers A2 and A3 were not included in the analysis of variance because of the significant within-reader bias indicated in Analysis 1. IC by A4 were also rejected, because of ASM reader A4's within-reader bias (Analysis 1) and between-reader bias (Analysis 4).

Initial analysis of variance for each species using the primary model statement listed in Table 2 revealed no significant difference in IC 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 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 7). Hence, the analysis was altered to test specifically for the effect of reading method and for the effect of region of the vertebral column by applying different error terms in the nested ANOVA (Table 3).

Effects of Reading Method on Variation in IC

Interactions between reading method and length-class were significant for IC from gummy shark but not for those from school shark (Table 7). Therefore the IC for gummy shark were split into the three length-classes and the following ANOVA model statement was applied to IC for each length-class separately:

$$IC = Method + Region + Method \times Region + Position + Shark + error$$

The results of these analyses for gummy shark of each length-class are presented in Table 9 with the results from the primary nested ANOVA for school shark with all length-classes combined. IC obtained from the MRM increment types M2 and M3 are by definition higher than those obtained from the MRM increment type M1. This partly explains the differences detected between IC from these reading methods. However, IC obtained by ASM reader A1 and those obtained from MRM increment type M1 were significantly different for only small gummy shark.

Effects of Region of the Vertebral Column on IC

Interactions between region and length-class, and between region and reading method were significant for gummy shark but not for school shark. Hence, the IC for gummy shark were split by length-class and by reading method and the following ANOVA model statement was applied to each length-class for each method separately:

$$IC = Position + Region + Shark + error$$

Results from this analysis are presented in Table 10. Data presented for school shark are the results of the primary nested ANOVA. The region of the vertebral column had a significant effect on IC for large and medium-size gummy sharks but not for small gummy sharks. IC obtained from vertebrae in different regions of the vertebral column were not significantly different when MRM increment types M2 and M3 were used but were significantly different when MRM increment type M1 was used ($P < 0.05$).

Where the effects of region of the vertebral column were significant, the IC for vertebrae from the thoracic region were always higher than those for vertebrae from the cervical and the pre-caudal regions. In most cases IC for vertebrae from the cervical region were not significantly different from IC of vertebrae from the pre-caudal region. The magnitude of the difference in IC of vertebrae from the three regions of the vertebral column (Table 11) was calculated by adjusting the mean difference between the IC for each region (adjusted mean difference equation above). The overall mean difference in IC 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 ASM was used, and 0.62 higher for gummy shark and 0.43 higher for school shark when the MRM increment type M1 was used.

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

Discussion

From our results we draw seven conclusions: (1) increment lines on the vertebrae of school shark are more difficult to count than those on the vertebrae of gummy shark; (2) the readability score of a vertebra is no better after repeated reading; (3) the readability score of vertebrae from the same region of the vertebral column are not significantly different; (4) experienced readers provide more precise and less biased IC than do inexperienced readers; (5) counts of alizarin stained and major micro-radiographic growth increments are similar; (6) the position of a vertebra within a region of a vertebral column has no statistically significant effect on the IC; (7) the region of the vertebral column from which vertebrae are sampled has a statistically significant effect on the IC.

Although our results reveal that difficulties inherent in counting the growth increment lines on vertebrae from gummy and school shark contribute to the variability of IC, they also indicate ways in which several sources of variation in IC can be reduced so that IC reflect more of the natural variability within a species rather 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 IC from a vertebrae. 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). 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 due to each reader's subjective interpretation of the vertebral growth increment lines might be removed and the total variation in age determination might become more a function of the inherent variation in the sharks themselves.

IC from vertebrae in different regions of a vertebral column were significantly different. However, IC 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 IC obtained were not significantly different to those obtained when type M2 increments (major and uncertain) were counted. Such a result indicates that the MRM allows the reader who examines large vertebrae from the thoracic region to identify fine increments and 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 ASM is used.

The absence of significant differences between IC obtained by the most reliable ASM reader and those obtained by the reader of major micro-radiographic increments (type M1) is evidence that increments counted in the ASM are related to the major hyper-mineralised increments (type M1) counted in the MRM. This premise, together with the results of a study by Moulton et al. (1992), is an indication that hyper-mineralised increments are probably formed annually. Moulton et al.'s (1992) assumption that the growth increments they counted in the ASM 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 IC derived from the ASM.

Yet the von Bertalanffy growth curves constructed by Moulton et al. (1992) for school shark caught in the Southern Ocean off Australia differed greatly with the growth curves produced for school shark caught in the Atlantic Ocean off Brazil (Ferreira and Vooren 1991). Moulton et al. (1992) proposed that the differences between the growth curves might be due to real differences in the growth rates of school sharks in the two oceans, or to over-estimates of the age of sharks due to Ferreira and Vooren's (Ferreira and Vooren 1991) use of the MRM.

However, Ferreira and Vooren (1991) used only major hyper-mineralised increments (type M1) for their age determinations and our results indicate that such IC should not differ significantly from the IC obtained from the ASM used by Moulton et al. (1992). Consequently the differences between the growth curves from the two studies cannot be completely explained by an over-estimation 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 IC obtained from vertebrae from different regions of a vertebral column gave age determinations which, 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 standardising 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.

In other species of shark the annual formation of growth increments in the largest vertebrae of the vertebral column has been validated (Smith 1984; Branstetter and Stiles 1987; Brown and Gruber 1988; Parsons 1993). These validations and the differences we found between IC from vertebrae in different regions of a vertebral column suggest that large vertebrae from the thoracic region be used to age gummy and school sharks. However, verification of the hypothesis that the IC obtained from thoracic vertebrae more correctly reflect the calendar age of the shark demands an understanding of the temporal periodicity of growth increment deposition (Cailliet et al. 1986; Cailliet 1990). Without such an understanding the practice of consistent sampling from both regions of the vertebral column provides the opportunity for future correction of age estimates if IC obtained in one region is 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 IC from different regions of a vertebral column, but our results allowed us to calculate differences between the overall means of IC from vertebrae in the thoracic region and from vertebrae in the cervical region. If the differences between overall means represent consistent relationships between IC obtained from different regions then a correction factor 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 based upon IC from vertebrae in different regions of the vertebral column.

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 the 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 1994a, b). However, these models may be over-estimating length-at-age and hence, the productivity of the species, if IC 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 over-estimate of length at specific ages would lead to under-estimates of the period for which sharks are vulnerable to capture.

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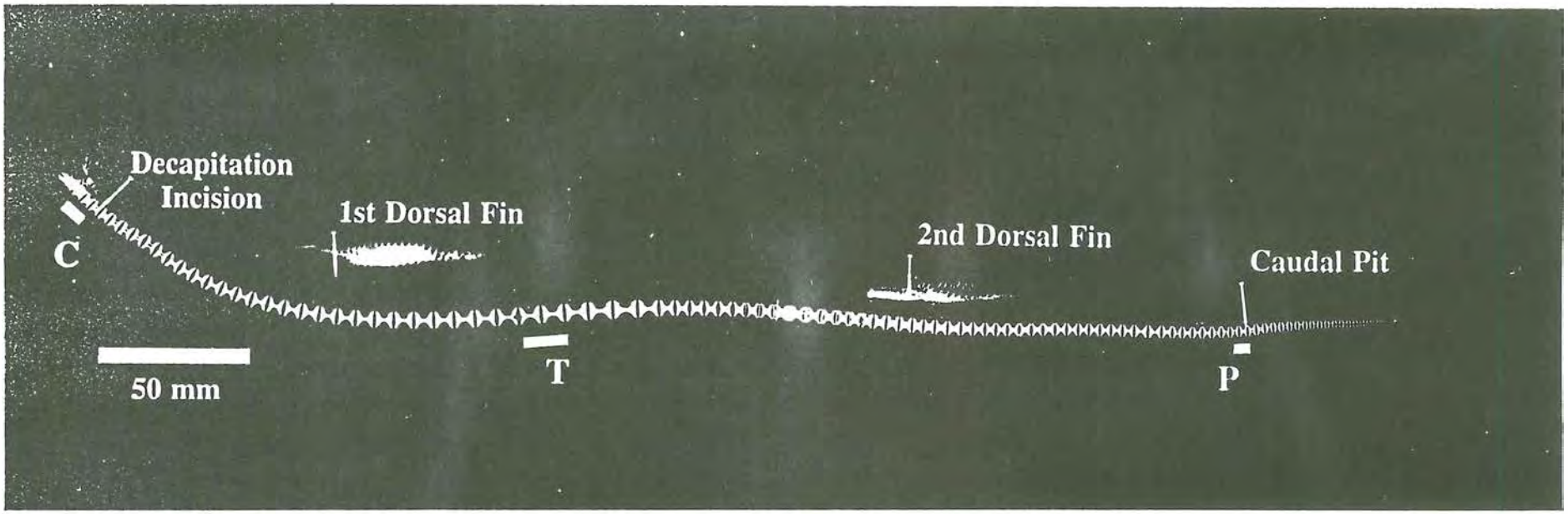

FIG. 1. Radiograph of the vertebral column from a male gummy shark 625 mm total length. The locations of the decapitation mark, the origins of the first and second dorsal fins and the pre-caudal pit were marked with steel pins before radiography. The three regions of two vertebrae are labelled C' - cervical, 'T' - thoracic and 'P' - pre-caudal.

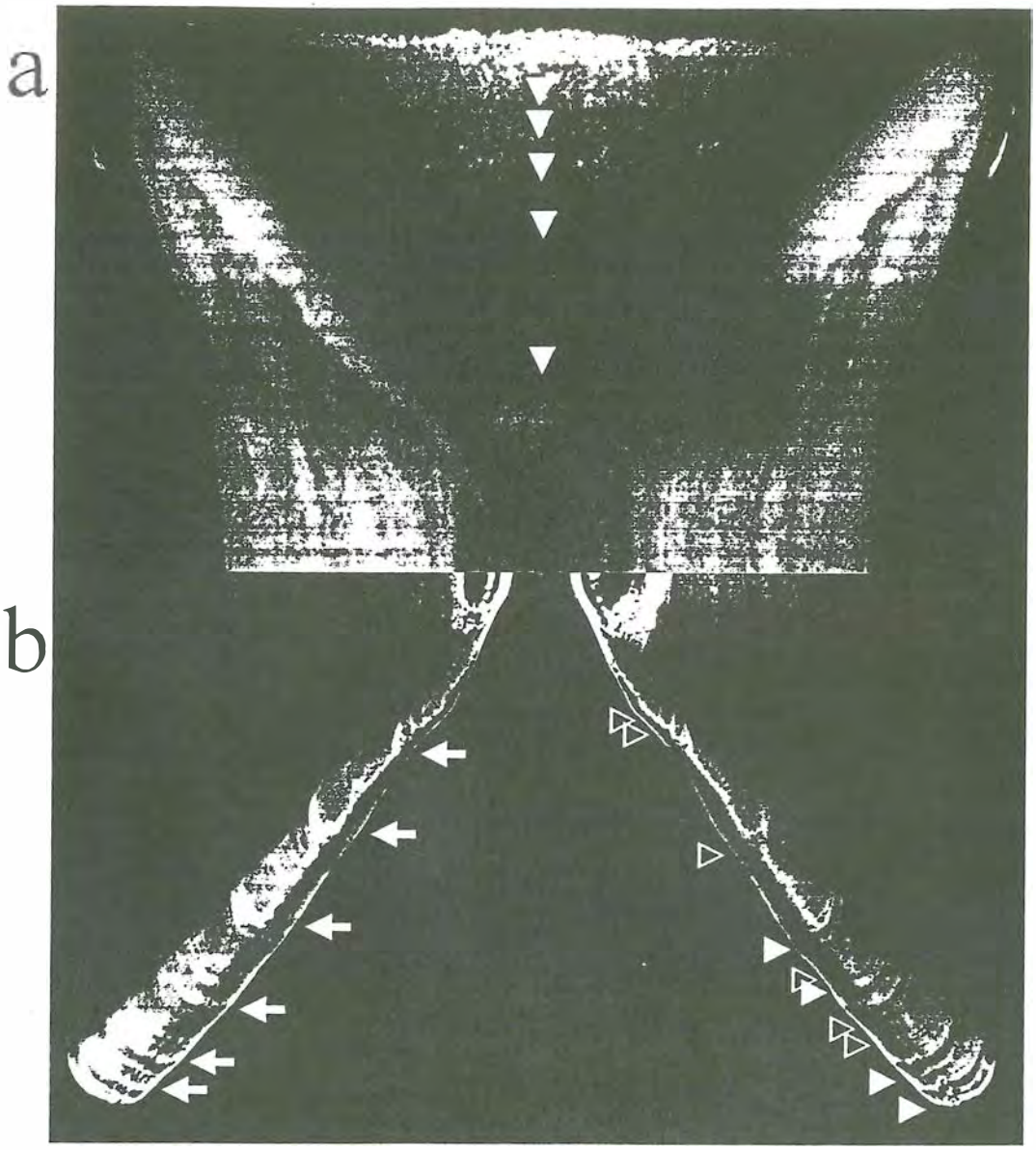

FIG. 2. Growth increments on and within a thoracic vertebra from a male gummy shark 1115 mm total length. (a) Alizarin stained preparation showing stained increments on the articular face (arrow heads). The vertebrae was sectioned longitudinally to allow comparison with the micro-radiographic preparation (b) of a section from the same vertebra. Arrows indicate major growth increments (M1). Uncertain (M2) and minor (M3) increments are indicated by filled and hollow arrow heads respectively.

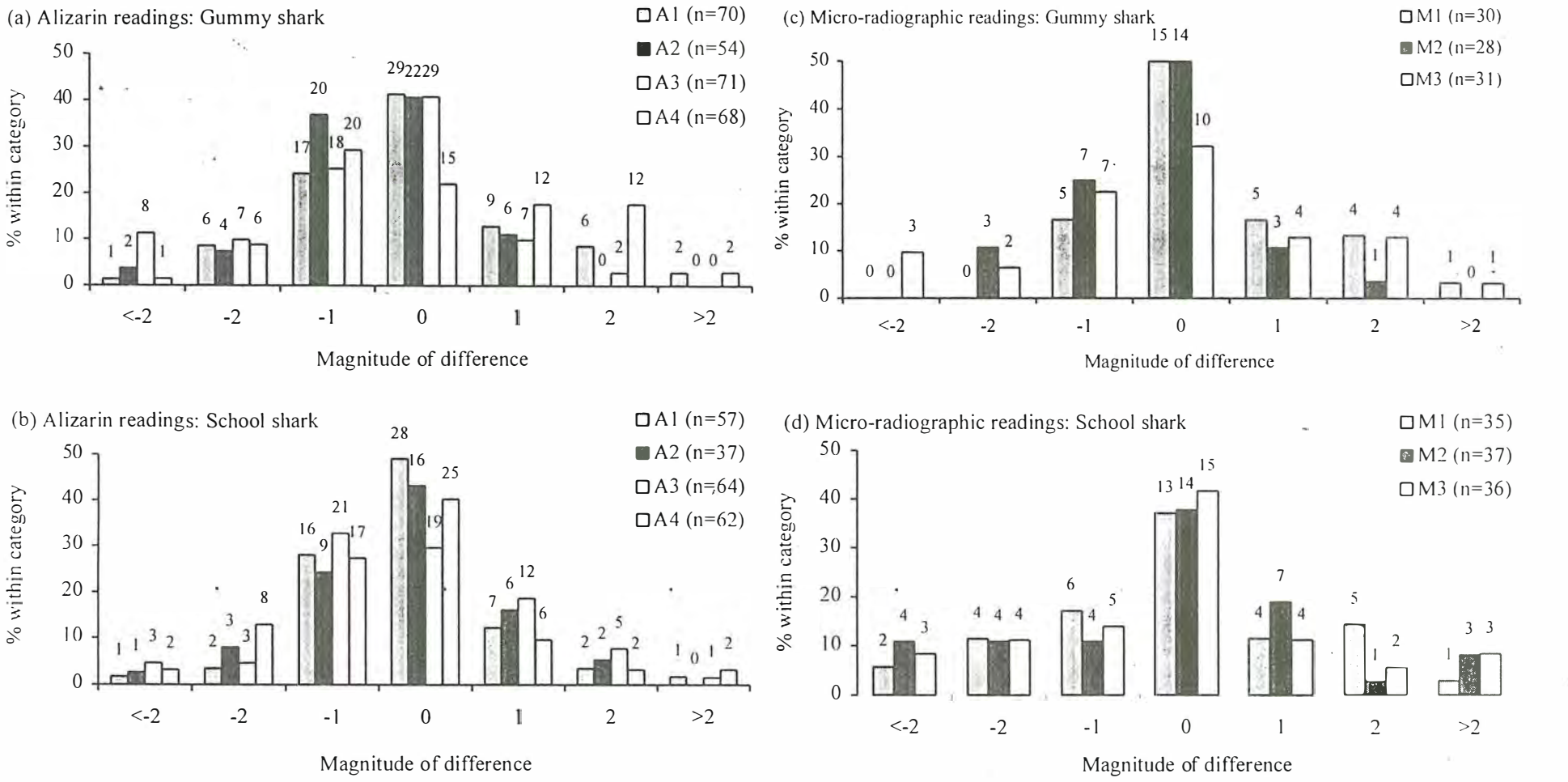

FIG. 3. Within-reader bias illustrated by differences in increment counts between Rounds 1 and 2.

TABLE 1. Description of the readability scores assigned to readings of vertebrae.

IC = Increment count(s)

Score	Description
1	IC unambiguous with exceptionally clear increments.
2	IC unambiguous but increments of diminished clarity.
3	Two IC possible but indicated IC is most likely.
4	More than two interpretations possible, IC is best estimate.
5	No IC possible, specimen abnormal or otherwise unreadable.
6	Specimen missing or broken.

TABLE 2. Summary of the six data analyses undertaken.

IC = Increment count(s); ASM = Alizarin staining method; MRM = Micro-radiographic method:
Rd = Round; Rr = Reader; Po = Position of vertebra within region; Lc = Length-class;
Sx = Sex; Sk = Shark: Rm = Reading method; Rg = Region of the vertebral column.

Source(s) of variation in IC	Analysis	Reading method	Rounds	Statistical test	Null hypothesis or model statement
Within reader[a]	1: Bias	ASM & MRM	1 & 2	Pairwise t test	$(Rd_1 - Rd_2)/(Rd_1, Rd_2)/2 = 0$
	2: Precision			IAPE[b]	Not Applicable
	3: Precision			Sign rank	$\|Rd_1 - Rd_2\| = 0$
Between reader[a]	4: Bias	ASM	1	Pairwise t test	$(Rr_i - Rr_j)/(Rr_i + Rr_j)/2 = 0$
	5: Precision			Sign rank	$\|Rr_i - Rr_j\|/(Rr_i + Rr_j)/2 = 0$
Sx[c] Lc[c] Po[c] Rg[c] Rm[c]	6: Difference in IC	ASM & MRM	1	Nested ANOVA[d]	IC = Po + Po x Lc + Po x Sx + Po x Sk (Lc x Sx) + Rm + Rm x Lc + Rm x Sx + Rm x Sk (Lc x Sx) + Rg + Rg x Lc + Rg x Sx + Rg x Sk (Lc x Sx) + Rg x Rm + Rg x Rm x Lc + Rg x Rm x Sx + Rg x Rm x Sk (Lc x Sx) + Lc + Sx + error

[a] Used only IC from vertebrae with readability scores < 4.

[b] Index of Average Percent Error.

[c] Used only IC where there was no within-reader bias or between-reader bias and used only IC from vertebrae with a readability score < 4.

[d] Error terms are listed in Table 3.

TABLE 3. Error terms used in nested ANOVA.

Sx = Sex; Sk = Shark; Lc = Length-class; Po = Position of vertebra within region; Rg = Region of vertebral column; Rm = Reading method.

Source of variation in increment count	Error term
Sx	Sk (Lc x Sx)
Lc	"
Po	Sk × Po (Lc × Sx)
Po × Lc	"
Po × Sx	"
Rg	Sk × Rg (Lc × Sx)
Rg × Lc	"
Rg × Sx	"
Rm	Sk × Rm (Lc × Sx)
Rm × Lc	"
Rm × Sx	"
Rg × Rm	Sk × Rg × Rm (Lc × Sx)
Rg × Rm × Lc	"
Rg × Rm × Sx	"

Table 4. Percentage of readings with acceptable readability scores for data analyses.

ASM = Alizarin staining method; MRM = Micro-radiographic method; A1, A2, A3 & A4 = Alizarin readers 1 to 4; M1 = major bands; M2 = major & uncertain bands; M3 = major, uncertain & minor bands.

Reading method	Species of shark	Reader or MRM Increment type	n	Acceptable readability scores	Percentage of readings accepted for analysis
ASM	Gummy	A1	144	≤ 3	76
		A2		≤ 4	85
		A3		≤ 3	81
		A4		≤ 3	80
	School	A1		≤ 3	52
		A2		≤ 4	61
		A3		≤ 3	67
		A4		≤ 3	58
MRM	Gummy	M1, M2, M3	72	≤ 4	83
	School	M1, M2, M3		≤ 4	99

TABLE 5. Percentage of verebrae showing differences in readability score for each reading method between (a) readings made in Rounds 1 and 2, and (b) Round 1 readings of vertebra 1 and 2 within a region.

ASM = Alizarin staining method; MRM = Micro-radiographic method.

Comparison	Difference in readability score	Difference in readability score (%)	
		ASM[a] (n=71)	MRM (n=70)
a	0	54	79
	1	42	20
	2	4	1
		ASM[a] (n=35)	MRM (n=34)
b	0	37	62
	1	46	29
	2	17	9

[a] Calculated from readability scores of the most experienced reader (A1) participating in the study.

Table 6. Results of analyses of within-reader variability.

ASM = Alizarin staining method; MRM = Micro-radiographic method;
Rd = Round; IAPE = Index of Average Percent Error; SD = Standard deviation;
Sign rank = Probability value returned by sign rank test; A1, A2, A3 & A4 = Alizarin readers 1 to 4;
M1 = major bands; M2 = major & uncertain bands; M3 = major, uncertain & minor bands.

Reading method	Species of shark	Reader or MRM Increment type	n	IAPE	Null hypothesis tested:					
					Ho: $\|Rd_1 - Rd_2\| = 0$			o: $(Rd_1 - Rd_2)/(Rd_{1,2})/2 = 0$		
					Mean	SD	Sign rank	Mean	SD	P
ASM	Gummy	A1	45	8.61	0.80	0.14	0.000	0.01	0.04	0.604
		A2	54	9.04	0.74	0.10	0.000	-0.14	0.03	0.000
		A3	53	8.08	0.75	0.12	0.000	-0.12	0.03	0.000
		A4	49	12.51	1.20	0.12	0.000	0.06	0.04	0.182
	School	A1	30	5.00	0.40	0.11	0.002	-0.04	0.03	0.215
		A2	37	6.07	0.76	0.13	0.000	-0.04	0.03	0.216
		A3	41	10.62	0.90	0.11	0.000	-0.13	0.04	0.004
		A4	29	10.45	0.98	0.20	0.000	-0.15	0.05	0.007
MRM	Gummy	M1	28	6.57	0.68	0.16	0.000	0.07	0.04	0.096
		M2	30	7.24	0.93	0.24	0.000	-0.02	0.05	0.616
		M3	30	7.98	1.07	0.18	0.000	-0.07	0.04	0.110
	School	M1	35	8.85	1.06	0.17	0.000	-0.03	0.04	0.550
		M2	35	8.74	1.20	0.25	0.000	-0.05	0.05	0.264
		M3	35	6.81	1.17	0.25	0.000	-0.04	0.04	0.302

Table 7. Summary of results of statistical tests.

IC = Increment count(s); IAPE = Index of average percent error; ASM = Alizarin staining method;
MRM = Micro-radiographic method; A1, A2, A3 & A4 = Alizarin readers 1 to 4;
M1 = major bands; M2 = major & uncertain bands; M3 = major, uncertain & 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; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Source of variation in IC	Analysis	Statistical test	Level of statistical significance or IAPE value					
			ASM			MRM		
			Reader	Gummy shark	School shark	Increment type	Gummy shark	School shark
Within reader/ Increment type	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 both species for readers/increment types					
				Gummy shark	School shark			
Between reader	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					
Sx	6: Difference in IC	Nested ANOVA		ns	ns			
Lc				***	***			
Po				ns	ns			
Rg				***	***			
Rm				***	***			
Lc × Rg				***	ns			
Lc × Rm				*	ns			
Rm × Rg				***	ns			

TABLE 8. Results of analyses of variability in increment count between readers who used the alizarin staining method.

Rr = Reader; SD = Standard deviation;
Sign rank = Probability value returned by sign rank test;
A1, A2, A3 & A4 = Alizarin readers 1 to 4.

Species of shark	Rr_i - Rr_j	n	Null hypothesis tested:					
			Ho: $\lvert Rr_i - Rr_j \rvert / (Rr_{i,j})/2 = 0$			Ho: $(Rr_i - Rr_j) / (Rr_{i,j})/2 = 0$		
			Mean	SD	Sign rank	Mean	SD	P
Gummy	A1-A2	53	0.16	0.03	0.000	0.05	0.04	0.205
	A1-A3	51	0.16	0.03	0.000	-0.05	0.04	0.214
	A1-A4	47	0.23	0.03	0.000	0.07	0.05	0.127
	A2-A3	58	0.21	0.03	0.000	-0.12	0.04	0.001
	A2-A4	55	0.25	0.03	0.000	0.02	0.05	0.655
	A3-A4	52	0.22	0.03	0.000	0.11	0.04	0.006
School	A1-A2	30	0.17	0.04	0.000	-0.10	0.05	0.043
	A1-A3	32	0.13	0.03	0.000	-0.04	0.04	0.350
	A1-A4	26	0.15	0.04	0.000	0.03	0.05	0.492
	A2-A3	34	0.18	0.04	0.000	0.04	0.05	0.451
	A2-A4	32	0.27	0.05	0.000	0.12	0.06	0.057
	A3-A4	29	0.23	0.04	0.000	0.11	0.05	0.049

TABLE 9. Effect of reading method on increment count.

Species of shark	Length-class	r^2	F value	P	Duncan's test grouping[a]
School	All	0.937	$F_{3,6} = 5.88$	0.0322	[A1 M1] [M1 M2] [M2 M3]
Gummy	Large	0.891	$F_{3,93} = 47.00$	0.0001	[A1 M1] [M2] [M3]
	Medium	0.751	$F_{3,85} = 10.71$	0.0001	[A1 M1] [M2] [M3]
	Small	0.663	$F_{3,67} = 7.68$	0.0002	[A1] [M1 M2 M3]

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

TABLE 10. Effect of region on increment count.

A1 = Alizarin reader 1; M1 = major bands; M2 = major & uncertain bands;
M3 = major, uncertain & minor bands.

Species of shark	Reading method	Length-class	r^2	F value	P	Duncan's test grouping[a]
School	All	All	0.937	$F_{2,11} = 19.25$	0.0003	[Pre-caudal] [Cervical] [Thoracic]
Gummy	A1	Large	0.801	$F_{2,30} = 23.66$	0.0001	[Pre-caudal] [Cervical] [Thoracic]
		Medium	0.696	$F_{2,28} = 13.04$	0.0001	[Pre-caudal Cervical] [Thoracic]
		Small	0.496	$F_{2,31} = 6.49$	0.0044	[Pre-caudal Cervical] [Thoracic]
	M1	Large	0.780	$F_{2,19} = 19.39$	0.0001	[Pre-caudal Cervical] [Thoracic]
		Medium	0.682	$F_{2,17} = 12.87$	0.0004	[Pre-caudal Cervical] [Thoracic]
		Small	0.528	$F_{1,10} = 6.00$	0.0343	[Pre-caudal] [Thoracic[b]]
	M2	Large	0.842	$F_{2,19} = 36.82$	0.0001	[Pre-caudal Cervical] [Thoracic]
		Medium	0.600	$F_{2,17} = 10.13$	0.0013	[Cervical Pre-caudal] [Thoracic]
		Small	0.635	$F_{1,10} = 3.60$	0.0870	[Pre-caudal Cervical Thoracic]
	M3	Large	0.922	$F_{2,19} = 105.80$	0.0001	[Pre-caudal] [Cervical] [Thoracic]
		Medium	0.800	$F_{2,17} = 29.77$	0.0001	[Cervical Pre-caudal] [Thoracic]
		Small	0.635	$F_{1,10} = 3.60$	0.0870	[Pre-caudal Cervical Thoracic]

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

[b] No data collected in the cervical region.

TABLE 11. Adjusted mean differences between mean increment count for each region and mean increment count for all regions

MRM = Micro-radiographic method; n = number of vertebrae used in calculation; SD = Standard deviation; A1 = Alizarin reader 1; M1 = major bands; M2 = major & uncertain bands; M3 = major, uncertain & minor bands.

Species of shark	Reader or MRM Increment type	Region	n	Percentage used	Adjusted mean difference	SD of adjusted difference
Gummy	A1	Cervical	22	61	0.07	0.12
		Thoracic	12	75	-0.24	0.12
		Pre-caudal	22	58	0.17	0.14
	M1	Cervical	14	100	0.15	0.14
		Thoracic	16	73	-0.47	0.29
		Pre-caudal	16	73	0.32	0.18
	M2	Cervical	14	100	0.22	0.13
		Thoracic	16	67	-0.49	0.27
		Pre-caudal	16	73	0.26	0.21
	M3	Cervical	14	100	0.30	0.12
		Thoracic	16	67	-0.70	0.28
		Pre-caudal	16	73	0.40	0.24
School	A1	Cervical	6	50	0.12	0.18
		Thoracic	8	80	-0.25	0.18
		Pre-caudal	10	26	0.13	0.19
	M1	Cervical	23	100	0.17	0.15
		Thoracic	24	100	-0.26	0.16
		Pre-caudal	24	100	0.10	0.14
	M2	Cervical	23	100	0.16	0.12
		Thoracic	24	100	-0.26	0.16
		Pre-caudal	24	100	0.10	0.12
	M3	Cervical	23	100	0.20	0.22
		Thoracic	24	100	-0.42	0.36
		Pre-caudal	24	100	0.22	0.19

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Final Report to
Fisheries Research and Development Corporation
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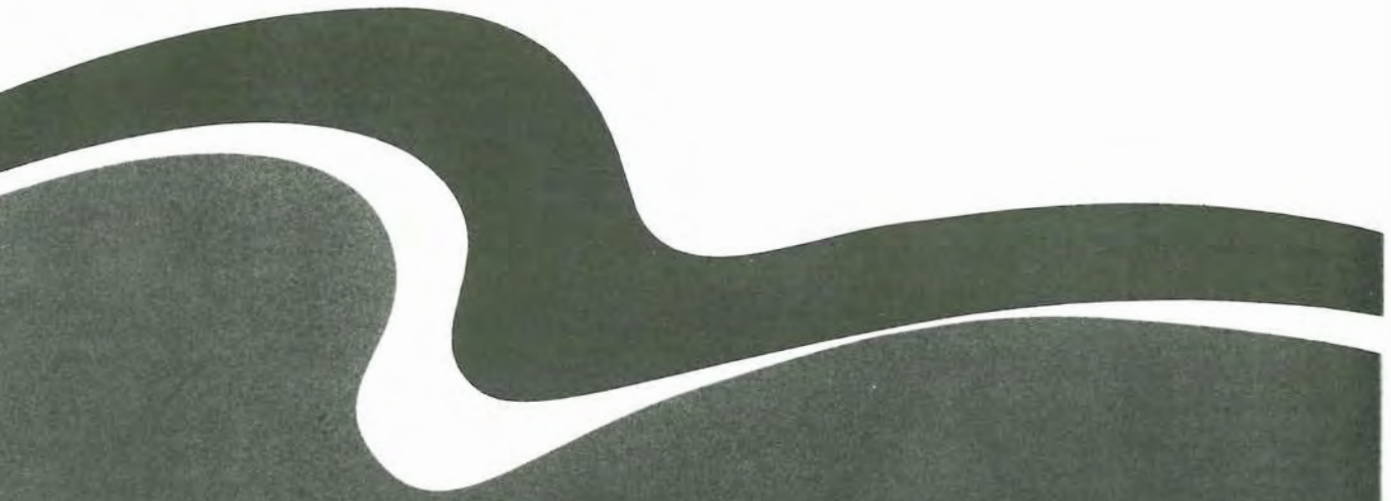

DEPARTMENT OF CONSERVATION AND NATURAL RESOURCES

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May 1995

Victorian Fisheries Research Institute
Department of Conservation and Natural Resources
PO Box 114, Queenscliff, Victoria 3225

Abstract

Three independent pieces of evidence are presented to support the hypothesis of the 'Phenomenon of Apparent Change in Growth Rate' caused by length-selective fishing mortality for explaining observed differences in published von Bertalanffy growth curves determined from length-at-age data for gummy shark (*Mustelus antarcticus*) between 1973-76 and 1986-87 in Bass Strait and between Bass Strait and South Australia during 1986-87. (1) Mean length of the sharks in each of ages-classes 3-7 years are shown to be different between the two periods and the two regions, but not different or less different for the less fully recruited 2-year age-class. (2) Avoiding the pitfalls of 'back-calculation', Rosa Lee's Phenomenon was detected by directly comparing the radii of growth-increment bands visible on the articular faces of vertebral centra from sharks of various ages caught in the two periods and the two regions. (3) Through development of an appropriate model, the effects of length-selective fishing mortality on the mean length of sharks in the population for age-classes 2-16 years were simulated for a range of levels of hook and gill-net fishing effort, with separate mesh-sizes of 6 and 7 inches for the gill-nets. Simulated changes in mean length for sharks older than 2 years tended not to be as large as the differences observed in the published von Bertalanffy growth curves but they were generally consistent with the observed trends. The simulations demonstrated how the von Bertalanffy growth parameters L_∞ and t_0 tend to increase and K tends to decrease as gill-net fishing effort increases, and hence explains how these types of biases, commonly appearing in the scientific literature for gill-net shark fisheries, can occur. It is demonstrated that hook fishing effort in conjunction with a legal minimum length would have to be very high to cause distortion of growth curves.

Introduction

Moulton et al. (1992) present different von Bertalanffy growth (VBG) curves for gummy shark (*Mustelus antarcticus*) captured from two separate two regions in southern Australia and during two separate sampling periods. Comparison of the VBG curves determined from age estimates made by counting growth-increment bands on the articular faces of vertebral centra of sharks captured in Bass Strait during 1986-87 with those of sharks captured in Bass Strait during 1973-76 suggests that growth rates have slowed. Similarly, comparison of VBG curves for sharks captured in Bass Strait (BS) during 1986-87 with those of sharks captured in waters off South Australia (SA) during 1986-87 suggests that growth rates of gummy sharks between the two regions are different (Fig. 1).

Moulton et al. (1992) verified their ageing method by demonstrating VBG curves determined from vertebral length-at-age data were similar to VBG curves determined from tag release-recapture length-increment data and Officer (1995) subsequently validated the method by injecting captive sharks and tagged sharks released in the wild with oxytetracycline and other vertebra-marking tissue stains and demonstrating that the growth-increment bands counted are annual. Hence the observed differences in the growth curves comprise either real differences in growth or apparent differences in growth or a combination of both. Moulton *et al.* (1992) advance seven hypotheses which can explain differences in growth curves.

(1) Growth curves change in response to changes in stock density through growth rates decreasing when biomass is high and, conversely, increasing when biomass is low.

(2) Growth curves change in response to environmental conditions through growth rates decreasing when conditions are unfavourable for growth and, conversely, increasing when conditions are favourable for growth.

(3) Growth curves change in response to evolutionary adaptation through growth rates decreasing where artificial selection by the fishing gear deployed in a fishery provides for a higher probability of survival to slow-growing individuals than to fast-growing individuals, or, conversely, through growth rates increasing where artificial selection by the fishing gear provides for a higher probability of survival to fast-growing individuals than to slow-growing individuals.

(4) Growth curves determined from length-at-age data are affected by the limitations of the von Bertalanffy growth function to describe the relationship between length and age of shark over the full age-range of a species, particularly when the data points are not distributed adequately over the age-range.

(5) Growth curves are affected by sampling bias caused by the length-selective characteristics of the fishing gear deployed to sample the wild population through the length-frequency composition of sharks collected for age determination in any age-class not being representative of the length-frequency composition of sharks in that age-class in the wild population.

(6) Growth curves are affected by length-selective immigration of sharks into a region or length-selective emigration of sharks out of a region through migrating sharks altering the length-frequency composition of sharks in particular age-classes of the wild population.

(7) Growth curves are affected by gill-nets or hooks selectively removing the longest sharks from the population among young age-classes and by gill-nets selectively removing the shortest sharks from the population among old age-classes by altering the length-frequency composition of sharks in particular age-classes of the shark population.

Moulton *et al.* (1992) presented arguments against Hypotheses 1-5, suggested Hypothesis 6 might have contributed to some of the observed differences between Bass Strait and South Australia, but favoured Hypothesis 7 for explaining most of the difference observed between the growth curves. By accepting this hypothesis they were postulating that there was an apparent change in growth rate rather than a real change in growth rate and that the apparent change was caused by length-selective fishing mortality.

By favouring hypotheses supporting apparent rather than real differences in their VBG curves, Moulton et al. (1992) could invoke the term 'Phenomenon of Apparent Change in Growth Rate', a term used first by Lee (1912) to explain apparent differences in growth rates inferred from comparing back-calculated mean lengths of fish at earlier ages from measurements of growth-increment bands visible on fish scales with mean lengths of younger fish in the population. Similar results from 'back-calculation', first by Sund

(1911), have been made for numerous species of fish and are usually referred to as 'Rosa Lee's Phenomenon' or Lee's Phenomenon (Ricker 1969). Moulton et al. (1992) did not attempt back-calculation from measurement of growth-increment bands on shark vertebrae and therefore did not determine whether or not Rosa Lee's Phenomenon occurred. They claim they were able to detect the Phenomenon of Apparent Change of Growth Rate in the gummy shark population directly from the VBG curves because of large differences in the level of fishing effort and differences in mesh-size of gill-nets in the fishery between 1973-76 and 1986-87 in BS and between BS and SA during 1986-87 causing length-selective fishing mortality.

Whether the observed differences are apparent or real affects the choice of growth curve used for stock assessment of gummy shark, which in 1993 provided 55% of the southern Australian shark catch of 3862 tonnes (carcass weight), valued at A$15.6m to fishers (Walker et al. 1994). Hence, in this paper, we explore the feasibility that most of the observed differences in the VBG curves are apparent rather than real. First we compare the length-frequency distributions of sharks sampled between the two sampling periods and between the two regions for each of several age-classes by re-examining the length-at-age data produced from vertebral ageing by Moulton et al. (1992). We then sample available archived vertebrae, prepare and stain the vertebrae with an alizarin red solution by procedures similar to those adopted by Moulton et al. (1992), and statistically test for differences in the radius of the growth-increment bands on the surface of the articular faces of vertebral centra. The purpose of examining band radius is to test for Rosa Lee's Phenomenon while avoiding the usual approach of back-calculation of length at age from the radii. Finally we develop an appropriate model and computer simulate the effects of length-selective fishing mortality on the mean length of shark in the population by varying fishing effort of gill-nets and hooks and mesh-size of gill-nets in the fishery. Before describing our procedures, we define what we mean by the term Rosa Lee's Phenomenon and why we avoid back-calculation to test for Rosa Lee's Phenomenon.

Materials and Methods

Back calculation

Francis (1990) defines back-calculation as a technique that uses a set of measurements made on a fish at one time to infer its length at an earlier time or times. Many authors (e.g. Jones 1958; Duncan 1980; Campana 1990; Francis 1990; Francis et al. 1993) have drawn attention to limitations of various mathematical functions underlying methods of back-calculation of fish. Francis (1990) describes the misuse of regressions of fish length against band radius as opposed to regressions of band radius against fish length, the invalid use of geometric mean regressions recommended by Ricker (1973), and the common problem of heteroscedacity of band radius versus fish length data in that back-calculation hypotheses predict increasing scatter in length of fish with increasing band radius. Campana (1990) emphasises the likelihood of bias caused by the tendency for slow-growing teleosts to have large otoliths, and Francis et al. (1993) draw attention to recent evidence of otoliths continuing to grow even when somatic growth has slowed or stopped.

Back-calculation from measurements of growth-increment bands on scales, otoliths and other hard parts of teleosts had been undertaken for many years before it had been attempted from measurements of growth-increment bands on the vertebrae or spines of

sharks. Several authors studying sharks (Casey et al 1983; Branstetter 1987; Branstetter and Stiles 1987; Killam and Parsons 1989) applied the Dahl-Lea method (Lea 1910) to back-calculate length of shark from band radius by multiplying band radius by length of shark at capture divided by centrum radius at capture, which assumes a direct proportionality between length of shark and centrum radius. All these authors, however, found from separate regression analyses that the relationship between length of shark and centrum radius can be represented by a linear function for new-born and older sharks which does not pass through the origin. They also found that a different function was required to represent the relationship during embryonic growth. Ferreira and Vooren (1991) found that parameter estimates for the linear function fitted for juvenile sharks and adult shark separately were statistically different so they assumed a curvilinear relationship by applying the power function for back-calculation. Examples of relationships commonly used in teleosts in back calculation are the constant of proportionality (Lea 1910), linear functions (Fraser 1916; Lee 1920), curvilinear functions (Fry 1943) and parabolic functions (Sheriff 1922; Hile 1936).

Hence, because of the uncertainties associated with back-calculation and because of the complexities associated with interpreting the results of statistical analysis on transformed data, we adopt the approach of Martin and Cailliet (1988). These authors, who while investigating age and growth of the bat ray (*Myliobatis californica*) off California, avoid back-calculation and present evidence of Rosa Lee's Phenomenon directly from measurements of vertebral growth-increment bands. The validity of this approach does however depend on the implicit assumption that there is a relationship, albeit undetermined, between total length of shark and centrum radius.

Rosa Lee's Phenomenon (RLP)

Gulland (1977) describes RLP as the observation that fish captured at older ages are increasingly those for which computed growth in earlier years from back-calculation was slow. Ricker (1969) explains that RLP can be caused by use of the incorrect mathematical function for back-calculation, biased sampling, length-selective natural mortality, and length-selective fishing mortality. Duncan (1980) added contraction of growth-increment bands through the passive dissolution or active uptake of calcium from hard parts as a possible cause for teleosts, and deposition of false growth-increment bands as a fish grows older. As Moulton et al. (1992) proposed that length-selective migration could cause an apparent difference in growth of gummy sharks between two regions, we propose that length-selective migration could cause RLP.

In this paper, we extend the use of the term RLP, as did Martin and Cailliet (1988), to include a detectable change in radius of any band with increasing age of fish, whereby the term 'band' is applied to any one of Band 1 (embryonic growth zone), Band 2 (birth band), Band 3 (first annual band after birth), Band 4 (second annual band after birth), and so on. We can exclude incorrect mathematical function for back-calculation and contraction of band radius (discussed later) as causes of RLP and can speculate on the relative importance of sampling bias, length-selective fishing mortality, length-selective natural mortality and length-selective migration. We also speculate on whether these causes are likely to cause positive RLP (see Ricker 1969) which would be a decline in radius of any one band with

increasing age of shark or negative RLP (see Ricker 1969) which would be a rise in band radius with increasing age of shark.

In adopting this definition of RLP, we reason, for example, that if length-selective culling of sharks varies with mesh-size and with fishing effort, then it is equally valid, if not more valid, to statistically test for detectable changes in the radius of the vertebral growth-increment bands as it is to test for detectable changes in back-calculated length of shark. For gummy sharks in the shark fishery of southern Australia, if, among the young age-classes, the longest sharks are being culled from the population at a rate faster than the shortest sharks, then among the archived vertebrae samples, the mean radius measured for any band should decline with increasing age of shark. Conversely, if, among the old age-classes, the shortest sharks are being culled from the population at a rate faster than the longest sharks, then among the archived vertebrae samples, the mean radius of any band for a sample of sharks should rise with increasing age of shark. The two effects together should produce a U-shaped trend between band radius and age of shark. Furthermore, if length-selective fishing mortality can cause mean band radius to vary with age of shark in the population, then the magnitude of the alteration relates to mesh-size of the gill-nets used in the fishery and to level of fishing effort. Given the large differences in fishing effort and mesh-size of gill-nets in the fishery between 1973-76 and 1986-87 in BS and between BS and SA during 1986-87 (Walker 1994a), then changes in band radius should be detectable. Thus, for the purpose of this paper, we refer to detectable changes in the radius of growth-increment bands between different periods, between different regions or between different bands as a 'Change in Rosa Lee's Phenomenon'.

Implicit in these conjectures is the assumption that, in the absence of fishing with length-selective fishing mortality and the absence of all other causes of RLP, the mean radius of any band with age of shark is constant. Hence, our approach to testing for RLP is to falsify this assumption and our approach to testing for a Change in RLP is to falsify any of the assumptions that band radius is not different between 1973-76 and 1986-87 in BS or between BS and SA during 1986-87 for any band.

Archived length-at-age data and vertebrae

Length-at-age data and vertebrae samples archived at the Victorian Fisheries Research Institute from two earlier studies (Walker 1983; Moulton et al. 1992) were re-examined as part of our study. Gummy sharks were caught in BS during 1973-76 by a combination of gill-nets of eight mesh-sizes ranging from 2 to 9 inches (51 to 229 mm), in steps of 1 inch (25 mm), and baited hooks attached to long-lines (Walker 1983), and were caught in BS and SA during 1986-87 by gill-nets of four mesh-sizes ranging from 5 to 8 inches (51 to 229 mm), in steps of 1 inch (Moulton et al. 1992). The sharks were measured to the nearest millimetre as total length; the tail of each shark was first allowed to take a natural position and the top caudal lobe was then placed parallel to the body axis. Several post-cranial vertebrae were collected from a sample of the sharks and used subsequently in the laboratory for estimating their ages by staining for concentric growth-increment bands with a solution of alizarin red. For statistical comparison of the growth curves for gummy sharks caught in BS during 1986-87 with the growth curves for gummy sharks caught in BS during 1973-76, Moulton et al. (1992) re-aged many of the sharks previously aged by Walker (1983) so as to standardise the method of ageing and the person undertaking the

ageing. Also, to standardise method of sampling, they included in the comparison only sharks caught by gill-nets of 5, 6, 7, or 8-inch mesh-size.

In our study, their length-at-age data were used to compare the mean lengths of shark between sharks from BS during 1973-76, BS during 1986-87, and SA during 1986-87. The comparisons were made for male sharks and female sharks separately for each age-class in the range 2-7 years; sharks of age outside this range were excluded because sample size was too small. The sample size of 90 male and 102 female sharks aged 2-7 years caught in BS during 1973-76 by gill-nets of 5, 6, 7, or 8-inch mesh-size was improved by including data from a further 27 male and 36 female sharks caught by gill-nets of 2, 3, 4, or 9-inch mesh-size and by long-lines. Pairwise t-tests, using the method described by Bailey (1964) for calculating pooled degrees of freedom, were applied to make the comparisons.

Laboratory analysis for vertebral growth-increment bands

Archived vertebrae of gummy sharks which had not been previously stained, or which had been previously stained but could be treated to remove all or most of the stain, were selected for measuring the radii of growth-increment bands visible on the articular faces of centra of stained whole vertebrae. One or two vertebrae were selected from each of a total of 66 male and 103 female gummy sharks collected from BS during 1973-76, 55 male and 67 female sharks collected for BS during 1986-87, and 65 male and 65 female sharks from SA during 1986-87. Each vertebra was coded to conceal information on the sex and length of the shark and its region and period of capture. The vertebrae from all sharks then were randomised and handled in the laboratory according to the principles of a 'double blind experiment'.

Prior to being archived, the vertebrae had been separated and trimmed of connective tissue, including the neural and haemal arches, and soaked in a solution of 2.5% sodium hypochlorite solution for various periods depending on size to remove the fascial material. Those selected for measurement of growth-increment bands were soaked again for 5-15 min depending on size.

Procedures, similar to those of Walker (1983) and Moulton et al. (1992), were adopted to stain the vertebrae to improve the definition of the concentric vertebral growth-increment bands. Each vertebra was immersed in a freshly prepared dilute alkaline solution of alizarin red prepared from a concentrated solution of alizarin red and 0.1% potassium hydroxide solution in the ratio of 1:9 (after Gruber and Stout 1983). The duration of immersion (2-5 minutes) depended on the size of the vertebra. When stained, the vertebra was washed in tap water for one min and the vertebral bands were examined immediately by viewing the surface of one articular face of the centrum, usually the clearer one, at an appropriate magnification (x4 or x6), using incident light. Each vertebra was processed by the one reader who assigned a 'readability score' of 1 to 5 based on the degree of differentiation effected by the stain and the difficulty in interpreting the arrangement of the vertebral bands. The readability scores are defined as follows: (1) bands unambiguous with exceptional clarity, (2) bands unambiguous but of diminished clarity, (3) two band counts possible but recorded count is the more likely, (4) more than two counts possible but the recorded count most likely, and (5) no count possible and recorded as 'unreadable'. If assigned a 'readability score' 1-4, an age estimate and various measurements were made on one centrum of the vertebra and recorded.

By assuming that vertebral bands were formed annually, and by selecting 1 January as an arbitrary birth date based on growth of embryos *in utero* (Walker 1983; Lenanton *et al.* 1990), ages from band counts were calculated from the formula adopted by Moulton et al. (1992),

age = number of vertebral bands

- 1 for birth band

+ 1 only if outer perimeter was stained

+ proportion of year from 1 January to capture date.

To make the measurements, the vertebra was mounted in a custom built jig and, because the articular face of the centrum is concave, only one region of it was viewed at a time; i.e. the region between the centre ('centrum focus') and the outer margin of the centrum. The region was aligned in a plane at right angles to the incident light reflected into the microscope objective. Images of the articular face were capture using a colour video camera mounted on the microscope and viewed on a 33-cm VGA colour computer screen and using the computer program 'Optimas Version 4.1.1' (Bioscan Inc., WA, USA). After calibration with a slide graticule under the microscope, a transect was drawn on the screen from the centrum focus out past the margin of the centrum using a computer mouse (Sandy Morison, Central Ageing Facility, Victorian Fisheries Research Institute, personal communication). The positions of the outer edge of each of the embryonic growth zone, the birth band, the growth bands, the outer most band where stained near the margin of the vertebra, and the margin of the vertebra were marked by the reader on the screen. The distances from the centrum focus to the various features were then measured and recorded by a customised 'Optimas' program. Repeated measurements were taken from the same articular face of the centrum after rotating the vertebra 180 degrees and re-aligning the surface of the centrum by adjusting the position of the vertebra in the jig. Finally, the diameter of the centrum was measured and recorded. When necessary the centra were also viewed down the microscope to check the number or position of growth-increment bands.

Statistical testing for RLP

Two separate analyses of variance were undertaken, using the General Linear Modelling procedure of the computer statistical package 'SAS' (SAS Institute Inc., NC., USA) to investigate the effects of period, region and age of capture of the sharks on the radius of the embryonic growth zone, the birth band, and all other growth-increment bands. For the analyses, the embryonic growth zone was numbered 'Band 1', the birth band 'Band 2', the first annual growth-increment band 'Band 3', the second annual growth-increment band 'Band 4' and so on. One analysis of variance (referred to as the 'Period ANOVA') included only the BS data for 1973-76 and the BS data for 1986-87, while the other analysis of variance (referred to as the 'Region ANOVA') included only the BS data for 1986-87 and the SA data for 1986-87.

The first step of the analysis and hypothesis testing involved addressing the question of whether RLP can be detected. RLP which, by our definition, is a change in band radius with age was tested statistically through the null hypothesis that age does not affect band radius. This hypothesis was either accepted or rejected through determining the

significance level of the F ratio from dividing the mean square value for age by the mean square value for shark (nested within period and age) in the Period ANOVA or by the mean square value for shark (nested within region and age) in the Region ANOVA. Whether RLP varied with band was tested through determining the significance level of the F ratio from dividing the mean square value for the band x age interaction term by the mean square value for band x shark (nested within period and age) interaction term in the Period ANOVA or by the mean square value for band x shark (nested within region and age) interaction term in the Region ANOVA.

The second step of the analysis and hypothesis testing involved determining whether band radius had changed with period or region. Whether or not this has occurred was tested statistically through the null hypothesis that band radius had not changed between 1973-76 and 1986-87 in Bass Strait or between Bass Strait and waters off South Australia during 1986-87. This hypothesis was then either accepted or rejected through determining the significance level of the F ratio from dividing the mean square value for period by the mean square value for shark (nested within period and age) in the Period ANOVA or from dividing the mean square value for region by the mean square value for shark (nested within region and age) in the Region ANOVA. Whether the change in band radius varied with band was tested through determining the significance level of the F ratio from dividing the mean square value for the band x period interaction term by the mean square value for band x shark (nested within period and age) interaction term in the Period ANOVA or from dividing the mean square value for the band x region interaction term by the mean square value for band x shark (nested within region and age) interaction term in the Region ANOVA.

The third step of the analysis and hypothesis testing involved testing whether the degree of RLP had changed with period or region. This was tested statistically through the null hypothesis that the effect of age on band radius had not changed between 1973-76 and 1986-87 in Bass Strait or between Bass Strait and waters off South Australia during 1986-87. This hypothesis was then either accepted or rejected through determining the significance level of the F ratio from dividing the mean square value for the period x age interaction term by the mean square value for shark (nested within period and age) in the Period ANOVA or from dividing the mean square value for the region x age interaction term by the mean square value for shark (nested within region and age) in the Region ANOVA.

Simulating effects of length-selective fishing mortality

To test the effects of length-selective fishing mortality on the mean length of shark in the population we develop a simulation model and vary fishing effort of gill-nets and hooks and mesh-size of gill-nets in the fishery.

Growth in a fish population is usually represented by the von Bertalanffy growth function

$$l_a = L_\infty \left[1 - e^{-K(a - t_0)}\right],$$

where K, L_∞ and t_0 are the von Bertalanffy parameters and l_a is the mean length of the fish at age **a**. We use the Francis growth function (Francis 1988a; Francis 1988b), derived by reparametrization of the von Bertalanffy function, where l_a is expressed as a function of a

through the Francis parameters l_ϕ, l_χ and l_ψ. These parameters are the mean lengths of fish predicted by the Francis function for the arbitrary ages of ϕ , χ and ψ , respectively, and the variances in fish length at these ages are given by σ_ϕ^2, σ_χ^2 and σ_ψ^2, respectively.

To incorporate variation in growth between individual fish into our model, several assumptions are required. Sainsbury (1980) proposed that each animal i in a fish population has its own pair of von Bertalanffy parameters (K_i and $L_{\infty i}$), which are retained throughout life, and that each parameter can be given a frequency distribution either for the entire population or for part of the population (e.g. those at a given length or age). We assume, as did Dow (1992) and Moulton et al. (1992), that each fish has its own Francis parameters $l_{\phi i}$, , $l_{\chi i}$ and $l_{\psi i}$ and that for the population at each of the ages ϕ , χ and ψ these parameters have the gamma probability density distribution about l_ϕ , l_χ and l_ψ . However, we also assume that the order of ranking from shortest to longest individual in the population at any age is maintained throughout life.

These assumptions provide a basis for grouping the population at any age into a number of length-classes and determining the Francis parameters $l_{\phi G}$, $l_{\chi G}$ and $l_{\psi G}$ of the VBG curve for each group G. The first step is to assign, on the basis its length, each fish of an arbitrary-sized population at any age (we choose the age of first recruitment, c) to one of X groups. Each of X-2 groups has a small length-range of $\delta\sigma_c$, controlled by the assigned increment value of δ , over the length range from l_c-2.5σ_c to l_c+2.5σ_c , one of the two remaining groups has the large length-range from 0 to l_c-2.5σ_c and the other has the large length range from l_c+2.5σ_c to $+\infty$. The second step is to calculate the proportion of the fish in the population at age c , p_{cG} , to group G by distributing the population at that age across the X length-ranges (i.e. G = 1, 2, ... , X) according to the gamma probability density function. The third step is to calculate the weighted mean length for each group, l_{cG} , and assign all fish in the group to this length. The fourth step is to hold the cumulative proportions of the total population associated with each l_{cG} at age c constant for all ages, in particular ages ϕ , χ and ψ (i.e. $P_{\phi G} = P_{cG}$, $P_{\chi G} = P_{cG}$ and $P_{\psi G} = P_{cG}$ for G = 1, 2, ... , X), so $l_{\phi G}$, $l_{\chi G}$ and $l_{\psi G}$ can be calculated by applying the inverse cumulative gamma probability density distribution function to the proportions p_{cG} .

The growth curve for the central group {i.e. G_0 = [(X-1)/2]+1} has the Francis parameters l_ϕ, l_χ and l_ψ which at age c includes the population in the length-range from l_c-0.5$\delta\sigma_c$ to l_c+0.5$\delta\sigma_c$. The Francis parameters of the growth curves for the two contiguous groups G = G_0-1 and G = G_0+1 have approximate values of l_ϕ-1$\delta\sigma_\phi$, l_χ-1$\delta\sigma_\chi$ and l_ψ-1$\delta\sigma_\psi$ and of l_ϕ+1$\delta\sigma_\phi$, l_χ+1$\delta\sigma_\chi$ and l_ψ+1$\delta\sigma_\psi$, respectively, and which at age c includes the population in the length-range from l_c-1.5$\delta\sigma_c$ to l_c-0.5$\delta\sigma_c$ and from l_c+0.5$\delta\sigma_c$ to l_c+1.5$\delta\sigma_c$, respectively. This pattern applies to all groups, except for the two outermost groups (i.e. G = 2, 3, ... , X-1). The Francis parameters of the growth curve for the innermost group (i.e. G = 1, the group of shortest fish) have approximate values of l_ϕ-2.6σ_ϕ , l_χ-2.6σ_χ and l_ψ-2.6σ_ψ , which at age c includes the population in the length-range from 0 to l_c-2.5σ_c . The Francis parameters of the growth curve for the outermost group (i.e. G = X, the group of longest fish) have the approximate values of l_ϕ+2.6σ_ϕ , l_χ+2.6σ_χ and l_ψ+2.6σ_ψ , which at age c includes the population in the length-range from l_c+2.5σ_c to ∞ .

We represent this variation in growth for the entire population in two ways by using two different sets of X growth curves. One set assumes variance in fish length at any age is

constant (i.e. $\sigma_\phi^2 = \sigma_\chi^2 = \sigma_\psi^2$ where each is represented by ρ_1), and the other assumes variance in fish length at any age is proportional to mean length at that age (i.e. $\sigma_\phi^2 = \rho_2 \, l_\phi$, $\sigma_\chi^2 = \rho_2 \, l_\chi$ and $\sigma_\psi^2 = \rho_2 \, l_\psi$ where ρ_2 is the constant of proportionality). Ideally these parameters are estimated from data collected before fishing begins.

For gummy sharks, given that no samples of length-at-age data are available for the period before the fishery began, l_{ϕ_g} , l_{χ_g} , l_{ψ_g} , ρ_{1_g} , and ρ_{2_g} were determined for the population of sharks of sex g (g=1 for male and g=2 for female sharks) from a sample of length-at-age data collected from BS during 1973-76, when fishing mortality was relatively low and when presumably the effects of length-selective mortality were small. These data were collected from sharks captured by gill-nets of mesh-size ranging 5-8 inches; hence we made the same corrections for sampling bias as those described by Moulton et al. (1992) and Dow (1992) using the gill-net selectivity function and parameter estimates reported by Kirkwood and Walker (1986). We followed Moulton et al. (1992) and chose ϕ , χ and ψ to represent ages 3, 7 and 11, respectively, and we assigned $\delta = 0.2$ which gave 25 groups within the length-range l_c-2.5σ_c and l_c+2.5σ_c and 2 groups outside this length-range (hence giving $X = 27$).

The gummy shark stock is assumed to be in equilibrium with, as shown by Walker (1994b), constant recruitment of 2 year-old sharks at the beginning of the fishing year (i.e. c = 2 years as it is assumed sharks of age less than 2 years are not captured by either gill-nets or hooks). An arbitrary-sized population of 2-year-old recruits, N_{cg}, is divided and distributed into the 27 separate groups according to the gamma probability density distribution function (i.e. $N_{cgG} = p_{cgG} \, N_{cg}$ for G = 1, 27), and each group is assigned appropriately to one of the 27 growth curves. Each group is then harvested with constant fishing effort by gill-nets of mesh-sizes 6 or 7 inches or by hooks depending on harvesting strategy tested until the group reaches an age of 16 years, the highest age estimated by Moulton et al. (1992).

For gill-net j, fishing mortality for recruited sharks of age a and sex g in group G, F_{jagG} , is assumed for recruited sharks to be the product of gill-net catchability, q_n , fishing effort, f_j , and the fishing gear selectivity factor, μ_{jagG} , i.e.,

$$F_{jagG} = q_n \, \mu_{jagG} \, f_j \, .$$

For hooks, which are non-selective fishing gear (Walker 1983), μ_{hagG} = 0 for pre-recruited sharks, μ_{hagG} = 1 for recruited sharks (i.e. a $\geq$ 2 years and $l_{agG} \geq$ 750 mm, the legal minimum length), and fishing mortality for sharks of age a and sex g in group G, F_{hagG} is given by

$$F_{hagG} = q_h \, \mu_{hagG} \, f_h \, ,$$

where f_h is constant hook fishing effort.

Thus the total fishing mortality, F_{agG} , from gill-nets of J separate sizes and from hooks (Walker 1992) is given by

$$F_{agG} = \sum_{j=1}^{J} q_n \mu_{jagG} f_j + q_h \mu_{hagG} f_h .$$

This process was used at 3-month intervals so the growth during the period was not greatly disproportionate to the difference between length-classes (i.e. a = 2.00, 2.25, 2.50, 2.75, ... , 16.00).

The length of shark at the middle of the 3-month period, T, is taken to be representative of length of shark for the entire 3-month period and the function derived from the function for gill-net selectivity determined by Kirkwood and Walker (1986) becomes

$$\mu_{jgaG} = (l_{a-0.5T,\,gG} / \alpha_j \beta_j)^{\alpha_j} \; e^{(\alpha_j - l_{a-0.5T,\,gG} / \beta_j)} ,$$

where α_j and β_j are parameters related to mesh size, m_j, and the length of sharks where it is assumed that the length of sharks at maximum selectivity for net j is proportional to the mesh size, i.e.,

$$\alpha_j \beta_j = \theta_1 m_j$$

and, where θ_1 is the constant of proportionality and the variance is a constant θ_2 for all mesh sizes. These assumptions lead to the following quadratic equation for positive β_j

$$\beta_j = -0.5 \left[\theta_1 m_j - (\theta_1^2 m_j^2 + 4\theta_2)^{0.5}\right] .$$

Values of θ_1 = 184.3 and θ_2 = 29739, where mesh-size is measured in inches, taken from Kirkwood and Walker (1986) were used in the model.

Survival of a shark of age a and sex g in group G is assumed to be given by

$$e^{-M_{ag} - F_{agG}} ,$$

where M_{ag} is natural mortality and the number of sharks of age a and sex g in group G at the beginning of the 3-month period, N_{gaG}, surviving to age a+T is given by

$$N_{a+1,gG} = N_{gaG} e^{-M - F_{gaG}} .$$

The mean length of sharks of age a and sex g in the entire population is given by

$$\bar{l}_{ag} = \sum_{G=1}^{X} N_{agG} l_{agG} \Big/ \sum_{G=1}^{X} N_{agG} .$$

If M_{ag} is assumed not to be length-dependent, then it is irrelevant to our model because its value cannot affect the mean value $\bar{l}_{ag}$.

In our study, the effects of length-selective fishing mortality on $\bar{l}_{ag}$ were explored by varying fishing effort with constant levels of zero, half, full and double the peak fishing effort of 240 thousand km-h for gill-nets in BS during 1987. For hooks, the length-selective effects of a legal minimum length of 750 mm on $\bar{l}_{ag}$ were explored by setting

fishing effort at zero, at 8 million hook-h (the peak level of hook effort in 1987), at 40 million hook-h (determined approximately by weighting the reported fishing effort of 8 million hook-h by the ratio of the total catch divided by the hook catch of gummy shark) and, because the effect of hooks was found to be small, at five times this level (i.e. 200 million hook-h). Adopting these values of fishing effort, the catchability parameter values of q_n = 0.00225 per thousand km-h and q_h = 0.00780 per million hook-h reported by Walker (1994b), and our estimates of the Francis parameters based on those of Moulton et al. (1992) for BS, simulations were undertaken for male and female gummy sharks separately, with either all fishing effort assumed as gill-nets of 6-inch mesh-size, as gill-nets of 7-inch mesh-size or as hooks. These procedures were undertaken for each of the two assumptions of constant variance and proportional variance with respect to length of shark separately.

Results

Length-frequency of sharks in age-classes 2-7 years.

Of a total of 1357 gummy sharks measured and aged by Moulton et al. (1992) within the age-range 2-7 years and included in our study, 117 male and 138 female sharks were caught in BS during 1973-76, 355 male and 367 female sharks were caught in BS during 1986-87, and 106 male and 274 female sharks were caught in SA during 1986-87. The number of sharks and plots for percentage frequency in 100-mm length-classes for each of the age-classes from 2 to 7 years, for male and female sharks separately, are presented in Table 1 and Fig. 2, respectively.

Results of the statistical comparison of mean length of gummy sharks in each age-class, for male and female sharks separately, between sharks caught in BS during 1973-76, in BS during 1986-87 and in SA during 1986-87, using pairwise t-tests, are presented in Table 2. At each age for male and female sharks separately, the mean length of sharks caught in BS during 1986-87 were shorter than the mean length of those caught in BS during 1973-76, except for the 2-year-old male sharks, and were shorter than the mean lengths of those caught in SA during 1986-87, except for 5 and 6-year-old female sharks. The mean lengths of sharks caught in SA during 1986-87 were shorter than the mean lengths of those caught in BS during 1973-76, except for 2 and 3 year-old sharks of both sexes and 4 year-old female sharks (Table 1).

Statistical testing for RLP

Measurements of the radius of growth-increment bands, most of which were paired, and estimates of age were made on one centrum of a vertebra from each of 66 male and 102 female sharks collected in BS during 1973-76, 55 male and 67 female sharks in BS during 1986-87 and 65 male and 65 female sharks from SA during 1986-87. These sharks were assigned a readability score 1-4; those assigned a readability score 5 (i.e. unreadable) were excluded from the analyses. Of the sharks with readable vertebrae, 5% were assigned a readability score of 1, 26% a score of 2, 28% a score of 3 and 41% a score of 4 (Table 3).

Mean radius (with standard error) for each of Bands 1-5 is plotted against age in Fig. 3 for each of the male and female sharks separately for sharks caught in BS during 1973-76, in BS during 1986-87, and in SA during 1986-87; corresponding information on the number

of sharks with measurements of radius for each band is presented in Table 4. Apart from several exceptions there is a general pattern of declining radius of band with increasing age for all bands, a trend indicative of RLP.

The results of the two separate analyses of variance and hypothesis testing indicate that RLP had occurred for gummy shark (Table 5). RLP was detected for male gummy sharks from pooling the samples collected during 1973-76 and 1986-87 from BS and for male and for female gummy sharks from pooling the samples collected from BS and SA during 1986-87. For female sharks in BS, the degree of RLP changed between 1973-76 and 1986-87. Pooling the BS and SA samples for male and for female sharks collected during 1986-87 indicated that degree of RLP varied with band and comparing the BS samples with the SA samples for female sharks collected during 1986-87 indicated that band radius varied between regions (Table 6).

From the first step of the hypothesis testing, the null hypothesis that RLP had not occurred was rejected for male gummy shark in BS because the effect of age was significant ($P<0.05$) in the Period ANOVA and was rejected for male and for female gummy sharks during 1986-87 because the effect of age was significant both for male ($P<0.05$) and for female ($P<0.01$) gummy sharks in the Region ANOVA. The effect of age was not significant for female gummy shark in the Period ANOVA. The null hypothesis that the occurrence of RLP has not varied with band was accepted both for male and for female sharks in BS because the interaction between band and age was not significant either for the male or for the female sharks in the Period ANOVA; however, this null hypothesis was accepted for male but rejected for female sharks during 1986-87 because the interaction between band and age was not significant for the male but was significant for the female sharks ($P<0.001$) in the Region ANOVA.

From the second step of the hypothesis testing, the null hypothesis that band radius has not changed with period was accepted for male but rejected for female sharks in BS because period was not significant for the male but was significant for the female sharks ($P<0.05$) in the Period ANOVA; similarly, the null hypothesis that band radius has not changed with region was accepted for male but rejected for female sharks in during 1986-87 because region was not significant for the male but was significant for the female sharks ($P<0.05$) in the Region ANOVA. The null hypothesis that change in band radius with band has not varied with period was accepted for male but rejected for female sharks in BS because the interaction between band and period was not significant for the male but was significant for the female sharks ($P<0.01$) in the Period ANOVA; however, the null hypothesis that change in band radius with band has not varied with region was rejected for both male and female sharks during 1986-87 because the effect of the interaction was significant for both the male ($P<0.001$) and the female sharks ($P<0.01$) in the Region ANOVA.

From the third step of the hypothesis testing, the null hypothesis that the degree of RLP has not changed with period was accepted both for male and for female sharks in BS because the interaction between age and period was not significant for the male or the female sharks in the Period ANOVA; and the null hypothesis that the degree of RLP has not changed with region was accepted both for male and for female sharks during 1986-87 because the effect of interaction between age and region was not significant either for the male or for the female sharks in the Region ANOVA.

Testing the effects of length-selective fishing mortality by theoretical modelling

Estimates of the Francis and von Bertalanffy parameters, where variance in length of shark at any age is assumed to be either constant or proportional to mean length at that age, are presented in Table 7. Choice of the assumption of constant variance or proportional variance has little effect on the parameter estimates for male gummy sharks but the choice did have an effect on the parameter estimates and shape of the VBG curves derived from the parameters for the female sharks. The effect of the choice of assumption on the shape of VBG curves is illustrated in Fig. 5, where, for each of male and female sharks separately for each of the two assumptions, 7 of the 27 VBG curves selected to represent growth of the 27 groups adopted for dividing the arbitrary-sized population of 2-year-old recruits are drawn (i.e. **G** = 1, 4, 9, 14, 19, 24, and 27). For the female sharks, the assumption of proportional variance in length of shark gives the appearance of straighter curves than does the assumption of constant variance.

Plots of mean length of shark in the population against age of shark are presented for each of the four fishing effort levels of zero, half, full and double the peak fishing effort of 240 thousand km-h for gill-nets in Bass Strait, assuming 6-inch mesh-size with constant variance and proportional variance (Fig. 6) and assuming 7-inch mesh-size with constant and proportional variance (Fig. 7). These plots show that at most ages the higher the fishing effort the greater is the change in the predicted mean length of shark in the harvested population from the predicted mean length of shark in an unfished population. The plots also show that the changes in mean length are affected more by the choice of mesh-size than by the choice of assumption of constant or proportional variance. When harvested with gill-nets of 6-inch mesh-size, the mean length of male sharks changes noticeably for all age-classes except for those aged <3 years for either assumption, and those aged approximately 9.5 years (i.e. where the plots intersect and mean length is approximately 1270 mm) when assuming constant variance, or those at the intersection age of approximately 10.3 years (mean length 1330 mm) when assuming proportional variance. Fishing has the effect of reducing the mean length of shark at each age in the population younger than the age at the intersection of the plots and increasing mean length of shark at each age in the population older than the intersection age. The pattern is very different for a population of male sharks harvested by gill-nets of 7-inch mesh-size; the mean length does not change noticeably for sharks aged <4 years and the mean length is reduced for all age-classes in the range 4-16 years (i.e. the plots do not intersect) for either assumption. When harvested with gill-nets of 6-inch mesh-size, the mean length of female sharks changes for all age-classes except those aged <3 years for either assumption, and those at the intersection age of 8.6 years (mean length 1410 mm) when assuming constant variance, or at the intersection age of 8.0 years (mean length 1380 mm) when assuming proportional variance. Similarly, when harvested with gill-nets of 7-inch mesh-size, the mean length of female shark changes for all age-classes except those aged <4 years for either assumption, and those at the intersection age of 11.3 years (mean length 1590 mm) when assuming constant variance, or at the intersection age of 10.9 years (mean length 1590 mm) when assuming proportional variance.

Plots of mean length of shark in the population against age of shark are presented for each of the three fishing effort levels of zero, 40 million hook-h and 200 million hook-h for hooks with constant variance and proportional variance separately (Fig. 8). Simulations

were conducted for the peak level of hook effort of 8 million hook-h but the results are not plotted because the plots cannot be visibly distinguished from zero fishing effort. Plots in Fig 8 show that, at most ages, the higher the fishing effort the greater is the change in the predicted mean length of shark; however, the hook fishing effort level had to be several times higher than the peak level of fishing effort in the fishery to produce an effect comparable with those produced by gill-net fishing effort. As with gill-nets, the choice of assumption of constant or proportional variance had negligible effects on the results.

Discussion

After detecting significant differences between VBG curves determined for gummy sharks caught in BS during 1986-87 and determined for those caught in BS during 1973-76 and between VBG curves determined for sharks caught in SA and from BS during 1986-87, Moulton et al. (1992) evaluated their VBG curves against other biological information available for gummy shark. They showed that the VBG curves determined for male and female sharks caught in BS during 1973-76 and for male sharks collected from SA during 1986-87 predict the mean length at birth (i.e. intersection between VBG curve and ordinate axis) of 335 mm (Walker 1983) well (see Fig. 1). However, they also showed that the VBG curves determined for male and female sharks caught in BS during 1986-87 and for female sharks caught in SA during 1986-87 predict the mean length at birth poorly and show that the magnitude of t_0 (i.e. intersection between the VBG curve and abscissa axis) greatly exceeds the period of gestation. Moulton et al. (1992) showed, for female sharks, that the estimates of L_∞ (asymptote of VBG curve) determined for sharks caught in BS during 1973-76, in BS during 1986-87 and in SA during 1986-87 greatly exceed the length of the longest observed length of 1750 mm for female sharks in the population, whereas, for male sharks, that the estimates of L_∞ were generally less than the longest observed length of 1451 mm for male sharks in the population.

Our results from statistical comparison of mean length of gummy sharks in each of the age classes 2-7 years, our detection of RLP from statistical analysis of measurements of growth-increment bands in shark vertebrae, and our demonstrated effects of length-selective fishing mortality on growth curves of gummy sharks by computer simulation all provide evidence of support for the hypothesis of the Phenomenon of Apparent Change of Growth Rate caused by length-selective fishing mortality advanced by Moulton et al. (1992) to explain the observed differences in the VBG curves. However, the multiplicity of causes of RLP and the Phenomenon of Apparent Change of Growth Rate, uncertainty about the most appropriate model structure to represent growth of the sharks and the possibility of real changes in growth, prevent us from fully evaluating the relative effects of all the potential causes for the observed differences in the curves. Nevertheless, it is worth speculating on the relative importance of each of the causes of apparent change in growth rates suggested from other studies and on whether these causes are likely to cause positive RLP which in our data would be a decline in band radius with age of shark or negative RLP which would be a rise in band radius with age of shark. Suggested causes of RLP and the Phenomenon of Apparent Change of Growth Rate from other studies include incorrect function for back-calculation (not applicable to our study), contraction of growth-increment bands, deposition of false growth-increment bands, sampling bias, length-selective natural mortality, length-selective migration and length-selective fishing mortality.

Contraction or incorporation of additional growth-increment bands

Contraction of bands or incorporation of additional false bands with increasing age suggested as a possibility for teleosts by Duncan (1980) is unlikely for sharks. Clement (1992) describes the chondrichthyan cartilaginous endoskeleton as a simple system in which the geometry, distribution and packing of the mineralized components permit growth by apposition, without the need for the remodelling that occurs in the bone tissue of teleosts (Simmons 1971) and other vertebrates. Because osteoclasts have never been described in chondrichthyans, and from the lack of histological evidence of resorption from mineralised cartilage and of the lack of canaliculi associated with cells either before or after entombment in mineralised cartilage, Clement (1992) concluded that growth of skeletal tissue in chondrichthyans occurred without resorption. Recent work indicates that the same is true for gummy shark (Officer 1995).

Sampling bias

The effects of sampling bias on the observed curves reported by Moulton et al. (1992) and on the curves used for computer simulations of the effects of length-selective fishing mortality should be minimal. These curves were all corrected for sampling bias by the method developed by Dow (1992), but sampling bias could affect the observations of band radius.

Sampling bias occurred in our study if the length-frequency composition of any of the age-classes of the sharks sampled for their vertebrae differed from the length-frequency composition of each age-class in the gummy shark population. Although the sharks sampled for vertebrae in our study were caught by gill-nets with a range of mesh-sizes, the overall selectivity curve for the group of nets resembles the shape of a single net with the combined nets having a much broader selectivity curve than any one of the nets forming part of the group of nets. Hence, the group of nets will selectively sample the large sharks over the small sharks among the young age-classes and the small sharks over the large sharks among the old age-classes.

The higher probability of sampling fast-growing gummy sharks than slow-growing sharks among the younger age-classes would cause band radius to decline with increasing age of shark (i.e. positive RLP). The higher probability of sampling slow-growing sharks than fast-growing sharks among the older age-classes would cause band radius to rise with increasing age of shark (i.e. negative RLP). If both these effects were present together, in the absence of other causes of RLP, band radius would initially decline and then rise with increasing age of shark. The expression of RLP through declining band radius with increasing age of shark among ages 1-4 years (i. e. positive RLP) (with the one exception of age 2 years in SA), in our data is consistent with biased sampling of fast-growing individuals among the young age-classes; however, our sample sizes are too small to determine whether negative RLP occurred among the old age-classes as a result of selectively sampling slow-growing sharks over fast-growing sharks (Table 8; Figs 3 and 4).

Length-selective natural mortality

Ricker (1975) explains that length-selective natural mortality can be higher for either the large fish or the small fish than for the intermediate-sized fish. Fast-growing fish frequently

tend to mature early and then become senile and die earlier than slow-growing fish of the same brood (Gerking 1957), whereas slow-growing fish during the early phases of life are more susceptible to predation, as shown for walleyes (*Stizostedion vitreum*) by Chevalier (1973).

Earlier senility of fast-growing gummy sharks than slow-growing sharks would be confined to the older age-classes with subtle effects that would cause band radius to decline with increasing age of shark (i.e. positive RLP). Higher natural mortality of slow-growing gummy sharks among the youngest age-classes would cause band radius to rise with increasing age of shark (i.e. negative RLP). If both these effects were present together, in the absence of other causes of RLP, band radius would initially rise and then decline with increasing age of shark.

Assuming, simultaneously, density-dependent and age-dependent natural mortality for all ages of gummy shark in an age-structured model of the fishery in Bass Strait, Walker (1994b) estimated that the annual natural mortality for 0, 1, 2, and 3 year-old age-classes were 61%, 45%, 33% and 26%, respectively, during 1973 and 47%, 33%, 24% and 18%, respectively, during 1986. If these assumptions are valid and given that predation is likely to be the main cause of natural mortality of young sharks, then the rapid decline in natural mortality with increasing age must manifest in a rapid decline in natural mortality with increasing length of shark. This should have the effect of causing negative RLP which is counter to the positive RLP expressed through declining band radius with increasing age among age-classes 1-4 years (with the one exception of age 2 in SA) (Fig. 3). Hence, any effect from length-selective natural mortality, if it occurs, has been masked in our data by other causes of RLP (Table 8).

Length-selective migration

The suggestion of Moulton et al. (1992) that the Phenomenon of Apparent Change in Growth Rate in gummy shark might be partly explained by length-selective selective migration is based on evidence of movement from BS to SA. They recount evidence from tag release-recapture data presented by Walker (1983) and draw attention to older sharks providing a much higher proportion of the population in SA than in BS in their own data.

Fast-growing gummy sharks among the older age-classes moving out of BS before slow-growing sharks would cause band radius to decline with increasing age of shark in BS (i.e. positive RLP). Conversely, fast-growing gummy sharks among the older age-classes moving into SA before slow-growing sharks would cause band radius to decline with increasing age of shark in SA (i.e. negative RLP). There is insufficient change in band radius with age of shark amongst the older age-classes in our data and insufficient data for sharks aged more than 7 years to conclude that length-selective migration is having an effect, other than to say that it might be being masked by other effects (Table 8).

Length-selective fishing mortality

Ricker (1975) explains that length-selection by a fishery is primarily the process of recruitment. The largest individuals of a year-class become vulnerable first, and it may be several years before the smallest individuals are fully vulnerable.

In the shark fishery of southern Australia, gummy sharks were mainly taken by baited hooks attached to sinking long-lines used to target school shark (*Galeorhinus galeus*) until 1964. During 1964-72 fishers gradually replaced their long-lines with bottom-set gill-nets and, following a ban in September 1972 on the sale of large school sharks in Victoria because of their mercury content (Walker 1976), gummy shark replaced school shark as the predominant species in the shark catch (Walker 1992). The change from hooks to gill-nets caused a change from knife-edge recruitment at the legal minimum length of about 750 mm to a less clearly definable length at recruitment.

Moulton et al. (1992) explain that, in a shark gill-net fishery, the probability of capture of a shark in any instant will depend on its size as well as fishing intensity and that the probability of capture throughout the shark's life-time will depend on its growth rate. Sharks of different sizes are not equally vulnerable to capture by gill-nets. Small sharks swim through gill-nets but become progressively more vulnerable to capture as they grow. After reaching the length of maximum vulnerability they then become progressively less vulnerable because their heads cannot so readily penetrate the meshes of the nets (Kirkwood and Walker 1986). The probability of a slow-growing shark being caught in a gill-net with a particular mesh-size during its life-time is higher than that of a fast-growing shark. A slow-growing shark is vulnerable to capture for a longer period than a fast-growing shark. Furthermore, at any instant, the length-frequency distribution of each age-class in the surviving population will depend on past levels of fishing effort. Length-selective mortality from gill-nets of 6-inch and 7-inch mesh-sizes have the effect of culling a greater proportion of the fast-growing sharks than the slow-growing sharks from the young age-classes and of culling a greater proportion of the slow-growing sharks than the fast-growing sharks from the old age-classes.

Moulton et al. (1992) claim they were able to detect the 'Phenomenon of Apparent Change of Growth Rate' in the gummy shark population because a large change in fishing effort and changes in mesh-size of gill-nets used in the fishery occurred between the two sampling periods for their study. From 1973-76 to 1986-87, annual fishing effort targeted at gummy shark tripled in BS, whereas in SA fishing effort increased from a very low level during 1973-76 to a level nearly as high as for BS by 1986-87. In BS, mesh-size changed from predominantly 7 inches in 1973 to mainly 6 inches in 1976, after which time mesh-size remained constant and hence was mainly 6 inches during 1986-87. In other regions, mesh-size was mainly 7 inches. The reduction of mesh-size in BS, from 7 inches, which has a maximum selectivity of 1290 mm shark length, to 6 inches, which has a maximum selectivity of 1106 mm shark length (Kirkwood and Walker 1986), increased fishing mortality for smaller and younger sharks. Moulton et al. (1992) concluded that the growth curves determined for 1986-87 are distorted by the effects of a long history of high and length-selective fishing mortality and that actual growth patterns of gummy shark are better represented by the von Bertalanffy growth equation determined for sharks caught in BS during 1973-76 when fishing mortality was much lower.

The higher probability of capture by fishers of fast-growing gummy sharks than slow-growing sharks among the younger age-classes would cause the mean band radius for a sample of shark vertebrae to decline with increasing age of shark (i.e. positive RLP). Conversely, the higher probability of capture by fishers of slow-growing sharks than fast-growing sharks among the older age-classes would cause mean band radius for a sample of

shark vertebrae to rise with increasing age of shark (i.e. negative RLP). If both these effects were present together, in the absence of other causes of RLP, band radius would initially decline and then rise with increasing age of shark. The expression of RLP through declining band radius with increasing age of shark among ages 1-4 years (i. e. positive RLP) (with the one exception of age 2 years in SA) in our data is consistent with length-selective culling by fishing of fast-growing individuals among the young age-classes; however, negative RLP among the older age-classes is less apparent (Table 8; Fig. 3).

The lack of marked negative RLP is consistent with our results from simulating the effects of varying fishing effort on mean length of shark in the population. The simulation results indicate that fishing effort causes the mean length of shark in the population to decline with increasing age over the range 0-8 years, with the exception of female gummy shark in a gill-net fishery with 6-inch mesh-size which has a range of 0-6 years. The absence of vertebral band radius data over the age range 8-16 years prevents a more thorough comparison.

The lower mean length of shark in each of the age-classes 2-7 years between 1973-76 and 1986-87 in BS (Tables 1 and 9; Fig. 2) is interpreted as being consistent with the effects of length-selective fishing mortality caused by increased fishing effort and a reduction in mesh-size from a combination of 6-inch and 7-inch mesh-sizes to mainly a 6-inch mesh size. The lower mean length of the sharks in each of most of the age-classes 4-7 years between 1973-76 in BS and 1986-87 in SA is interpreted as being consistent with the effects of length-selective fishing mortality caused by much higher fishing effort with 7-inch mesh-size during 1986-87 in SA than during 1973-76 in BS. Fishing effort was high in both BS and SA during 1986-87 but the mean length of shark in each of the age-classes 2-7 years was lower in BS than in SA. This is interpreted as being consistent with the effects of length-selective fishing mortality caused by the smaller mesh-size of mainly 6-inches in BS than the mesh-size of mainly 7 inches in SA.

It is the results of our simulation trials that show most clearly how the distortions to VBG curves identified by Moulton et al. (1992) can be effected by length-selective fishing mortality. For female sharks, sustained fishing with gill-nets of 6-inch or 7-inch mesh-size and, for male sharks, fishing with gill-nets of 6-inch mesh-size have the effects of increasing the magnitude of each of L_∞ and t_0 and decreasing the magnitude of K. For male sharks, fishing effort with gill-nets of 7-inch mesh-size has the effect of decreasing the magnitude of L_∞ and has little or no effect on t_0 (see Figs. 6 and 7). These results suggest, as did Moulton et al. (1992), that the male and female VBG curves determined for the BS samples collected during 1973-76, when fishing effort was lowest and when a mixture of 6-inch and 7-inch mesh-size was used in BS, are likely to be the most representative of actual growth rates. However, as indicated by the large proportion of sharks over the age of 8 years old predicted to have lengths larger than the longest observed lengths of 1451 mm for male and 1750 mm for female sharks (Moulton et al. 1992) (see Fig. 5) and by our tests for RLP, the BS 1973-76 VBG curves, particularly the one for female sharks, are likely to be too high at the upper end of the curves.

Conclusion

Incorrect mathematical function for back-calculation, contraction of bands or incorporation of additional false bands with increasing age are rejected as causes of RLP in gummy

shark. Length-selective natural mortality might contribute to negative RLP but, if so, its effect is masked by the combined effects of length-selective fishing mortality and sampling bias. Because both length-selective fishing mortality and sampling bias both contribute to positive RLP consistent with the patterns in our data, it is not possible to evaluate the relative contributions by these two potential causes (Table 8). However, in calculating their growth curves, Moulton et al. (1992) correct for and exclude sampling bias to explain the observed differences in their curves. Extension of the model developed in our study, and perhaps used in conjunction with vertebral band radius data, may provide a basis to develop appropriate corrections to VBG curves distorted by the effects of length-selective fishing mortality. In concluding we support the conjecture by Moulton et al. (1992) that most of the differences in the VBG curves they report for gummy shark are best explained by the Phenomenon of Apparent Change of Growth Rate caused by length-selective fishing mortality.

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Appendix A. Notation

a	Subscript integer value for age.
c	Age at first recruitment.
f_h	Fishing effort by hooks.
f_j	Fishing effort by gill-net j.
F_{agG}	Total fishing mortality for sharks of age a, sex g and group G.
F_{hagG}	Fishing mortality from hooks for sharks of age a, sex g, and group G.
F_{jagG}	Fishing mortality from gill-net j for sharks of age a, sex g and group G.
g	Subscript integer value for sex where g=1 for male and g=2 for female sharks.
G	Subscript integer value of group where G varies from 1 to X.
h	Subscript to denote hook fishing effort.
i	Subscript integer value for individual sharks.
j	Subscript integer value for gill-nets.
J	Maximum integer value of separate gill-net mesh-sizes.
K	Brody growth rate parameter in VBG function for population of fish.
K_i	Brody growth rate parameter in VBG function for individual fish i.
$\ddot{l}_{ag}$	Mean length of sharks in the population at age a for sex g predicted by our model.
l_{cgG}	Francis parameter for mean length of a population of fish in the Francis function at the age of recruitment c for sex g and group G.
$l_{\phi gG}$	Francis parameter for mean length of a population of fish in the Francis function at the arbitrary age of ϕ for sex g and group G.
$l_{\phi i}$	Francis parameter for length of an individual fish at age ϕ growing according to the Francis function.
$l_{\chi gG}$	Francis parameter for mean length of a population of fish in the Francis function at the arbitrary age of χ for sex g and group G.
$l_{\chi i}$	Francis parameter for length of an individual fish at age χ growing according to the Francis function.
$l_{\psi gG}$	Francis parameter for mean length of a population of fish in the Francis function for the arbitrary age of ψ for sex g and group G.
$l_{\psi i}$	Francis parameter for length of an individual fish at age ψ growing according to the Francis function.
L_∞	Asymptotic length of fish parameter in VBG function for a population of fish.
$L_{\infty i}$	Asymptotic length of fish parameter in VBG function for individual fish i.
m_j	Mesh size of gill-net j.
M_{ag}	Natural mortality of sharks of age a and sex g.
N_{agG}	Number of sharks of age a, sex g in group G at the beginning of year.
n	Subscript to denote gill-net fishing effort.

m_j	Mesh size of gill-net ***j***.
M_{ag}	Natural mortality of sharks of age ***a*** and sex **g**.
N_{agG}	Number of sharks of age ***a***, sex **g** in group **G** at the beginning of year.
n	Subscript to denote gill-net fishing effort.
p_{agG}	Proportion of the population of sharks of age **a** and sex **g** allocated to group ***G***.
P_{agG}	Cumulative proportion of the population of sharks of age **a** and sex **g** allocated to group ***G*** associated with weighted mean length l_{cgG}.
q_n	Catchability coefficient of gill-nets.
q_h	Catchability coefficient of hooks.
t_0	Age parameter when $l_a = 0$ in VBG function for a population of fish.
T	Time interval integer.
X	Maximum integer value of group ***G***.
α_j	Parameter of the gamma fishing gear selectivity-length function for gill-net ***j***.
β_j	Parameter of the gamma fishing gear selectivity-length function for gill-net ***j***.
δ	Increment value for defining length-classes for categorising the population at age ***c*** into groups $G = 1, X$.
θ_1	Constant of proportionality between $\alpha_j \beta_j$ and mesh size m_j for gill-net ***j***.
θ_2	Selectivity variance of length of sharks captured by gill-nets (constant for all mesh sizes).
ρ_1	Constant variance in length of fish at any age in the Francis growth function.
ρ_2	Constant of proportionality between variance in length of fish at any age and mean length at that age in the Francis growth function.
μ_{jagG}	Selectivity of gill-net ***j*** for sharks of age ***a***, sex ***g*** and group ***G***.
σ_ϕ^2	Francis parameter for variance in length of a population of fish in the Francis function at the arbitrary age of ϕ.
σ_χ^2	Francis parameter for variance in length of a population of fish in the Francis function at the arbitrary age of χ.
σ_ψ^2	Francis parameter for variance in length of a population of fish in the Francis function at the arbitrary age of ψ.
ϕ	Francis parameter to represent age at the bottom end of the data range.
χ	Francis parameter to represent age at the mid-way between ϕ and ψ.
ψ	Francis parameter to represent age at the top end of the data range..

TABLE 1. Number of gummy sharks aged from each period and each region.

Region	Period	Sex	Parameter	Parameter value for each age-class						
				2	3	4	5	6	7	Total
Bass Strait	1973-76	Male	n	18	29	39	19	6	6	117
			Mean length mm)	799	938	1053	1121	1218	1250	
			Length SE (mm)	22	21	21	23	20	30	
		Female	n	19	43	21	21	19	15	138
			Mean length mm)	825	945	1018	1174	1289	1321	
			Length SE (mm)	24	18	22	28	26	31	
Bass Strait	1986-87	Male	n	82	123	73	47	21	9	355
			Mean length mm)	816	879	959	1053	1066	1119	
			Length SE (mm)	9	7	12	15	20	46	
		Female	n	104	117	88	27	23	8	367
			Mean length mm)	812	875	965	1109	1154	1147	
			Length SE (mm)	7	8	9	18	33	54	
South Australia	1986-87	Male	n	5	15	26	20	23	17	106
			Mean length mm)	909	962	1022	1098	1111	1181	
			Length SE (mm)	40	23	24	29	16	32	
		Female	n	21	48	65	52	49	39	274
			Mean length mm)	883	984	1037	1109	1146	1218	
			Length SE (mm)	12	11	14	16	18	22	

Data after Moulton *et al.* (1992) and after Walker (1983).

TABLE 2. Statistical comparison of mean lengths within each age-class. ns = not significant; * P<0.05; ** P<0.01; *** P<0.001.

Region/period	Region/period	Sex	Significance level for each age-class (yr)					
			2	3	4	5	6	7
Bass Strait 1973-76	Bass Strait 1986-87	Female	ns	**	***	ns	**	*
		Male	ns	*	***	*	***	*
Bass Strait 1973-76	South Australia 1986-87	Female	*	ns	ns	ns	***	*
		Male	*	ns	ns	ns	**	ns
Bass Strait 1986-87	South Australia 1986-87	Female	***	***	***	ns	ns	ns
		Male	ns	**	*	ns	ns	ns

TABLE 3. Number of gummy sharks assigned each readability score.

Region	Period	Sex	No. sharks assigned each readability score				
			1	2	3	4	Total
Bass Strait	1973-76	Male	4	18	21	23	66
		Female	5	40	29	28	102
Bass Strait	1986-87	Male	1	13	17	24	55
		Female	3	16	19	29	67
South Australia	1986-87	Male	2	12	13	38	65
		Female	6	8	20	31	65

TABLE 4. Number of gummy sharks measured for each increment band.

Region/period	Band	Number of sharks in each age-class								
		1	2	3	4	5	6	7	8+	Total
Male										
	EGZ[a]	5	6	7	17	8	13	3	7	66
BS 1973-76	Birth Band	5	6	7	17	8	13	3	7	66
	Zone 1	3	6	6	17	7	13	2	7	61
	Zone 2		5	6	17	7	13	1	7	56
	Zone 3			6	16	7	13	1	7	50
	EGZ	4	12	15	8	8	2	4	2	55
BS 1986-87	Birth Band	4	11	15	7	8	2	4	2	53
	Zone 1	2	11	15	7	8	2	4	2	51
	Zone 2		7	12	7	7	1	4	2	40
	Zone 3			7	6	7	1	4	2	27
	EGZ		1	5	5	15	11	10	16	63
SA 1986-87	Birth Band		1	5	5	15	11	10	15	62
	Zone 1		1	5	5	15	10	9	14	59
	Zone 2		1	5	5	15	10	8	13	57
	Zone 3			4	5	14	10	7	12	52
Female										
	EGZ	8	2	15	13	19	7	11	22	97
BS 1973-76	Birth Band	8	2	15	12	19	7	11	22	96
	Zone 1	5	2	15	12	19	7	11	21	92
	Zone 2		1	15	12	19	6	10	21	84
	Zone 3			6	11	18	5	10	21	71
	EGZ	7	12	12	7	10	8	5	7	68
BS 1986-87	Birth Band	7	11	12	6	10	8	5	7	66
	Zone 1	4	9	12	4	10	8	5	7	59
	Zone 2		8	11	4	10	5	5	7	50
	Zone 3			8	3	10	3	5	6	35
	EGZ		2	13	8	10	9	7	16	65
SA 1986-87	Birth Band		2	13	8	10	8	7	16	64
	Zone 1		2	13	8	10	7	6	16	62
	Zone 2		2	13	8	10	7	6	16	62
	Zone 3			10	8	9	7	5	15	54

[a]Embryonic growth zone

TABLE 5. Analyses of variance testing for effects of band, age of shark, period or region of capture of shark, and various interactions on the radius of growth-increment bands of gummy shark caught in Bass Strait during 1973-76 and 1986-87 and in South Australia during 1986-87. df = degrees of freedom; MS = mean square; ns = not significant; *P<0.05; **P<0.01; ***P<0.001.

Source of variation	Male		Female	
	df	MS	df	MS
Period ANOVA (Test for age, period and band within the Bass Strait region)				
Age	11	1.557 *	15	0.856 ns
Period	1	0.016 ns	1	6.026 *
Age x period	7	1.053 ns	10	1.827 ns
Shark (age period)[a]	88	0.753	132	1.181
Band	12	67.372 ***	16	108.303 ***
Band x age	65	0.087 ns	106	0.103 ns
Band x period	8	0.048 ns	11	0.341 **
Band x shark (age period)[b]	407	0.098	791	0.291
Residual of full model	527	0.019	794	0.036
Region ANOVA (Test for age, region and band within the 1986-87 period)				
Age	11	1.169 *	14	1.950 **
Region	1	1.475 ns	1	4.624 *
Age x region	6	0.576 ns	9	1.095 ns
Shark (region age)[a]	95	0.594	102	0.789
Band	12	65.467 ***	16	104.789 ***
Band x age	65	0.089 ns	107	0.140 ***
Band x region	9	0.292 ***	10	0.181 **
Band x shark (region age)[b]	468	0.071	515	0.083
Residual of full model	563	0.020	667	0.027

[(a)]Error term used for calculating F values required to determine significance levels for age, region and age x region interaction; [(b)]Error term used for calculating F values required to determine significance levels of band, band x age interaction, and band x region interaction.

TABLE 6. Summary of results of hypothesis testing.

Does band radius vary	Period ANOVA		Region ANOVA	
	Male	Female	Male	Female
Between age[a]	Yes	No	Yes	Yes
Between age by band[a]	No	No	Yes	Yes
Between period, or between region[b]	No	Yes	No	Yes
Between period by band, or between region by band[b]	No	Yes	Yes	No
Between age by period, or between age by region[b]	No	No	No	No

[(a)]and hence indicate the occurrence of Rosa Lee's Phenomenon? [(b)]and hence indicate a change in the degree of Rosa Lee's Phenomenon?

TABLE 7. Estimates of Francis and von Bertalanffy growth parameters for gummy shark in BS during 1973-76. n = sample size; SE = standard error; SD = standard deviation; ML = maximum log-likelihood.

Sex	Variance[a]	n	Francis parameters					von Bertalanffy parameters		
			l_3 (SE, SD) (mm)	l_7 (SE, SD) (mm)	l_{11} (SE, SD) (mm)	ρ1 or ρ2[b] (SE)	ML	K (yr^{-1})	L_∞ (mm)	t_0 (yr)
Male	Constant	95	872 (21, 103)	1199 (21, 103)	1312 (48, 103)	ρ1 = 10647 (1698)	-565.6	0.266	1372	-0.80
	Proportional	95	874 (21, 80)	1196 (24, 110)	1298 (51, 119)	ρ2 = 10.902 (1.854)	-562.4	0.288	1346	-0.64
Female	Constant	134	862 (28, 107)	1310 (15, 107)	1585 (24, 107)	ρ1 = 11391 (1641)	-798.9	0.122	2019	-1.55
	Proportional	134	893 (19, 90)	1305 (15, 131)	1611 (31, 162)	ρ2 = 9.923 (1.459)	-799.4	0.074	2508	-2.99

[a]Variance in shark length at any age constant with mean lengh at age or proportional to mean length at age.
[b]Constant variance = ρ1 and proportional variance = l_a ρ2.

TABLE 8. Summary of causes and likely effects of Rosa Lee's Phenomenon (RLP) on the young age-classes and the old age-classes separately. -ve RLP is defined as increasing mean band radius with age; +ve RLP is defined as decreasing mean band with age.

Cause of Rosa Lee's Phenomenon	Effect of RLP	
	Young	Old
Length-selective natural mortality	-ve	+ve
Length-selective fishing mortality with 6 and 7-inch mesh-size	+ve	-ve
Length-selective fishing mortality with hooks	+ve	nil
Sampling bias with 5, 6, 7 and 8-inch mesh-sizes of gill-nets	+ve	-ve
Sampling bias with hooks	+ve	nil
Emigration of largest sharks in older age-classes out of BS	nil	+ve
Immigration of largest sharks in older age-classes into SA	nil	-ve

TABLE 9. Ranking of fishing effort for each of 6-inch and 7-inch gill-nets and of the mean length of shark within each age-class for male and female sharks separately across the three region-periods. H = highest; M = middle; L = lowest.

Region	Period	Ranking of fishing effort		Ranking of mean length within each age-class and sex					
		6-inch	7-inch	2 yr ♂♀	3 yr ♂♀	4 yr ♂♀	5 yr ♂♀	6 yr ♂♀	7 yr ♂♀
South Australia	1986-87	L	H	H H	H H	M H	M M	M L	M M
Bass Strait	1973-76	M	M	L M	M M	H M	H H	H H	H H
Bass Strait	1986-87	H	L	M L	L L	L L	L L	L M	L L

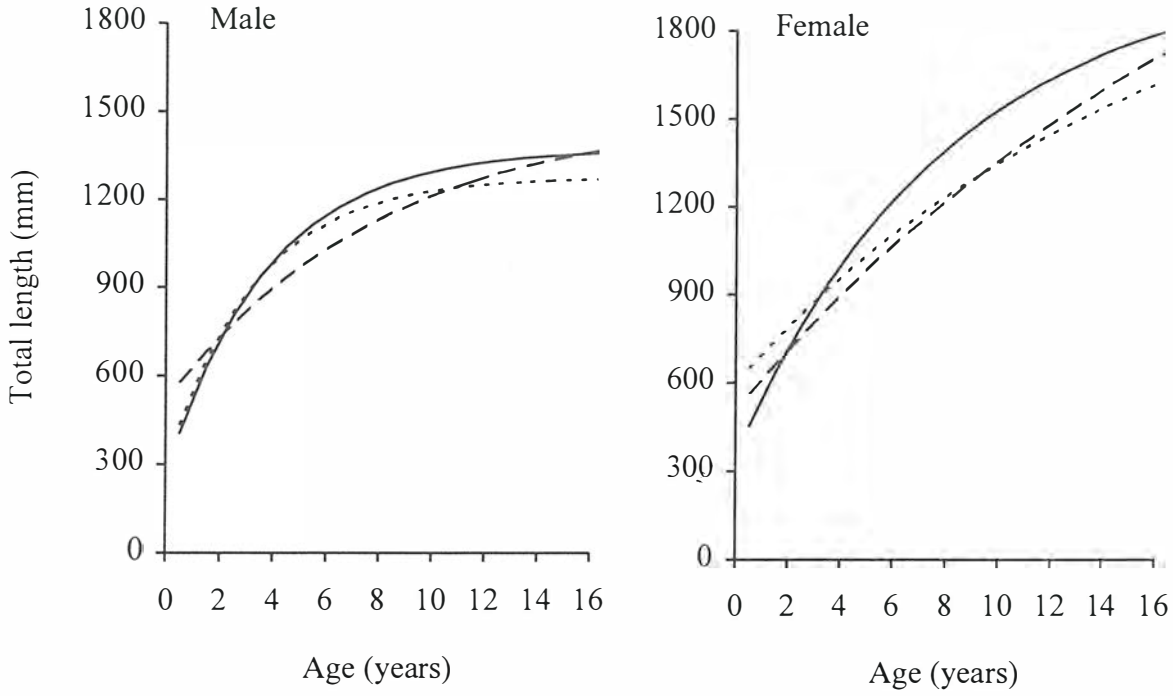

FIG. 1. Von Bertalanffy growth curves for male and female gummy sharks in Bass Strait during 1973-76 (——) and 1986-87 (– – –), and South Australia during 1986-87 (- - - - -) (after Moulton et al. 1992).

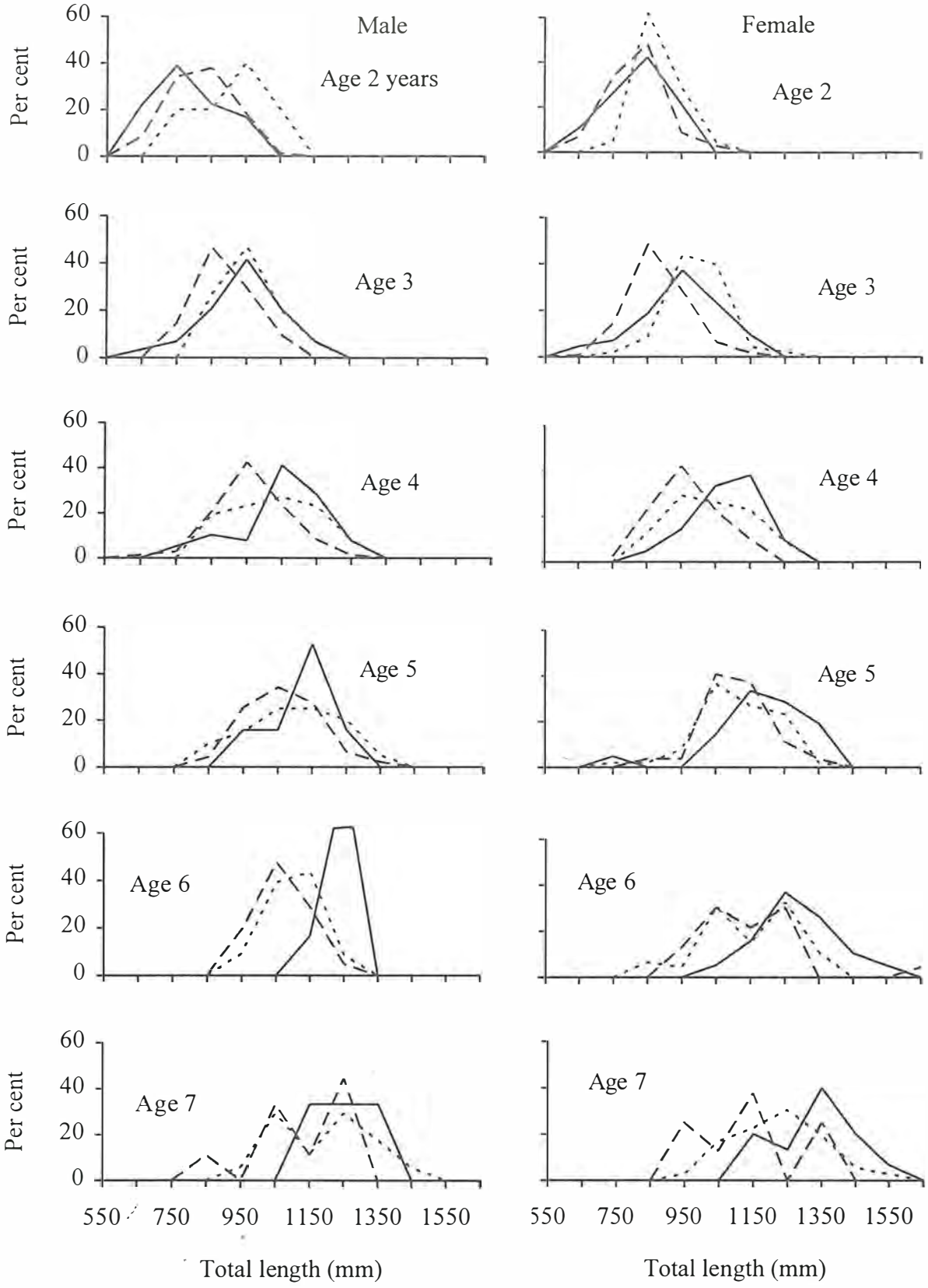

FIG. 2. Length-frequency distribution for each age in Bass Strait during 1973-76 (——), Bass Strait during 1986-87 (– – –), and South Australia during 1986-87 (- - - - - -) (see Table 1 for sample sizes).

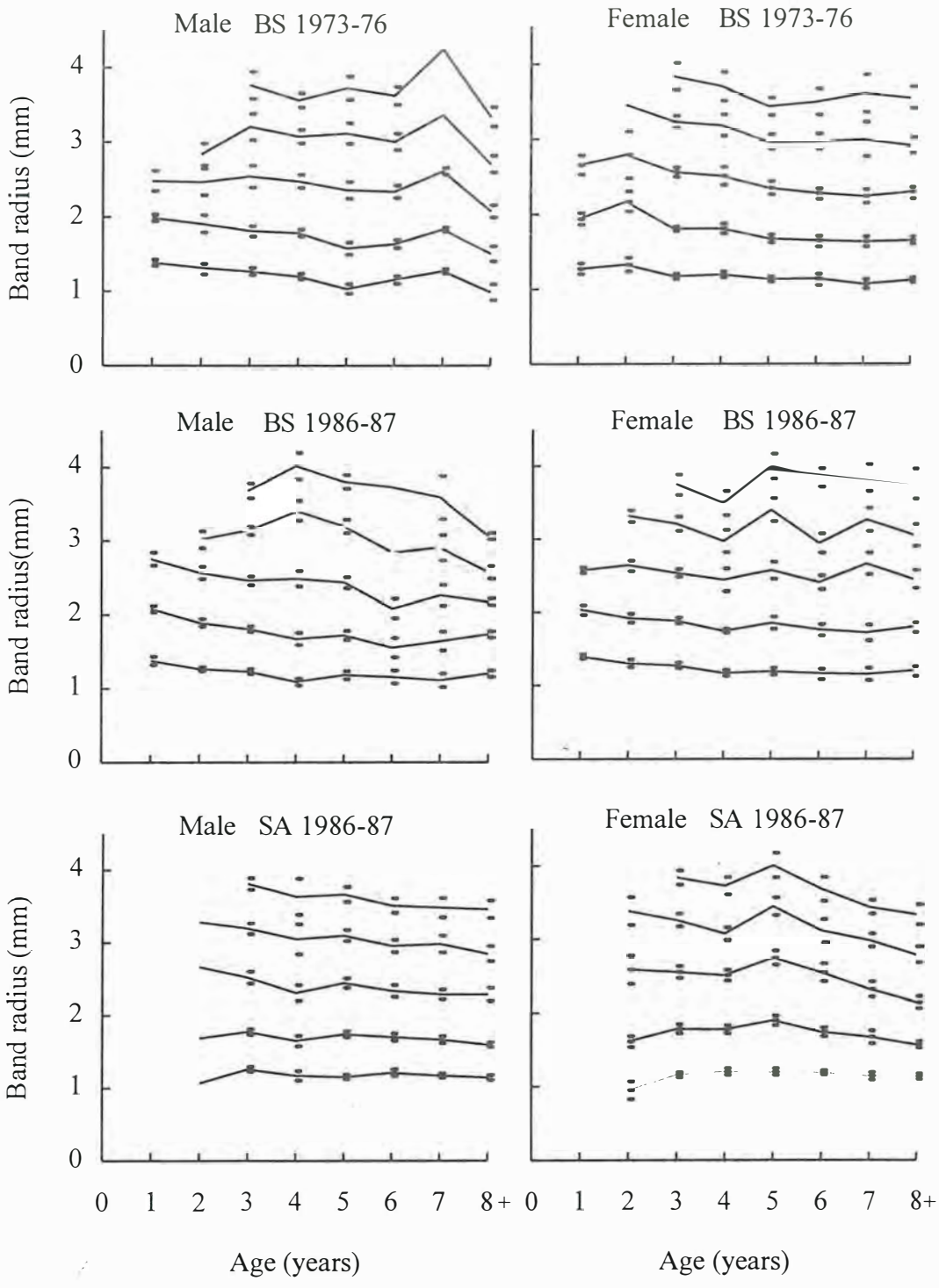

FIG. 3. Mean band radius (with SE) of Bands 1-5 for each age-class in Bass Strait (BS) during 1973-76 and 1986-87, and South Australia (SA) during 1986-87 for mal and female gummy sharks separately.

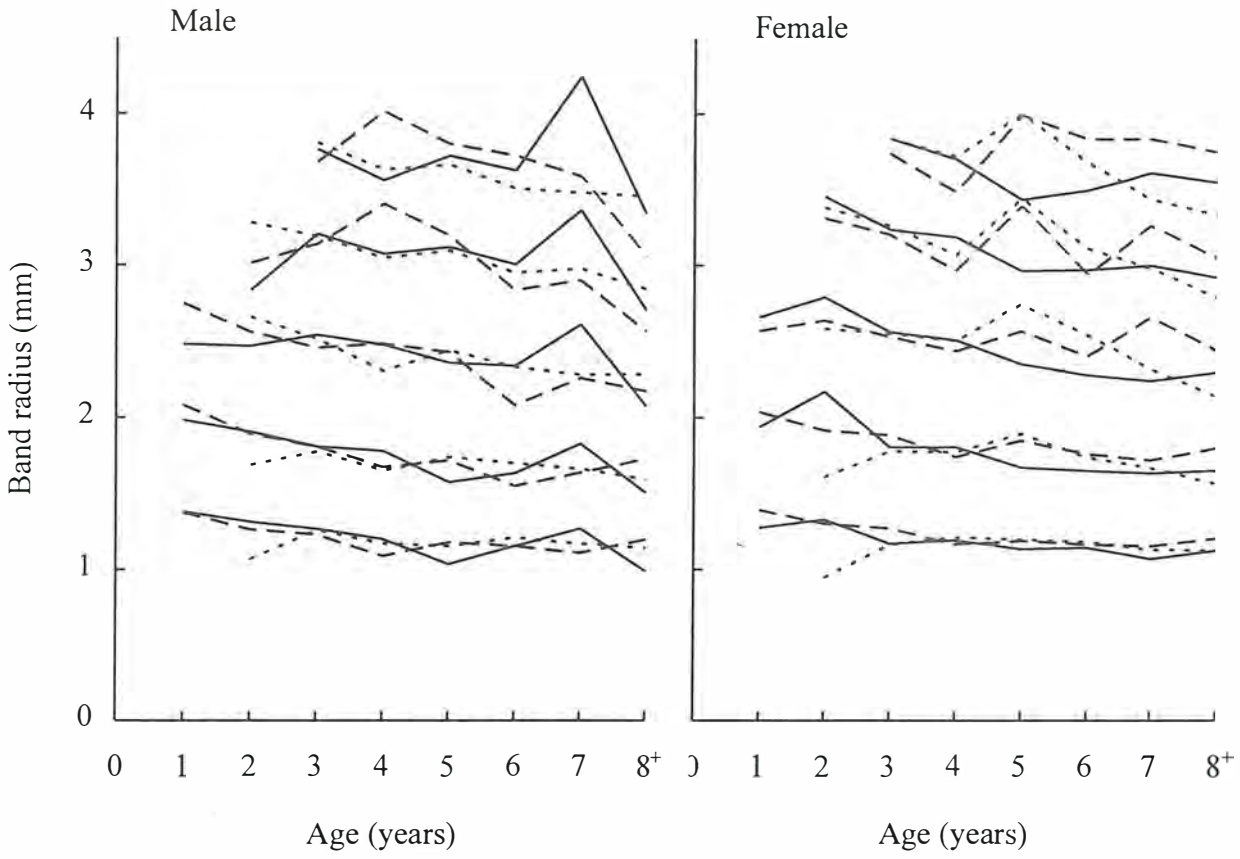

FIG. 4. Mean band radius of Bands 1-5 for each age-class in Bass Strait during 1973-76 (——) and 1986-87 (– – –), and South Australia during 1986-87 (-·- - - -) for male and female gummy sharks.

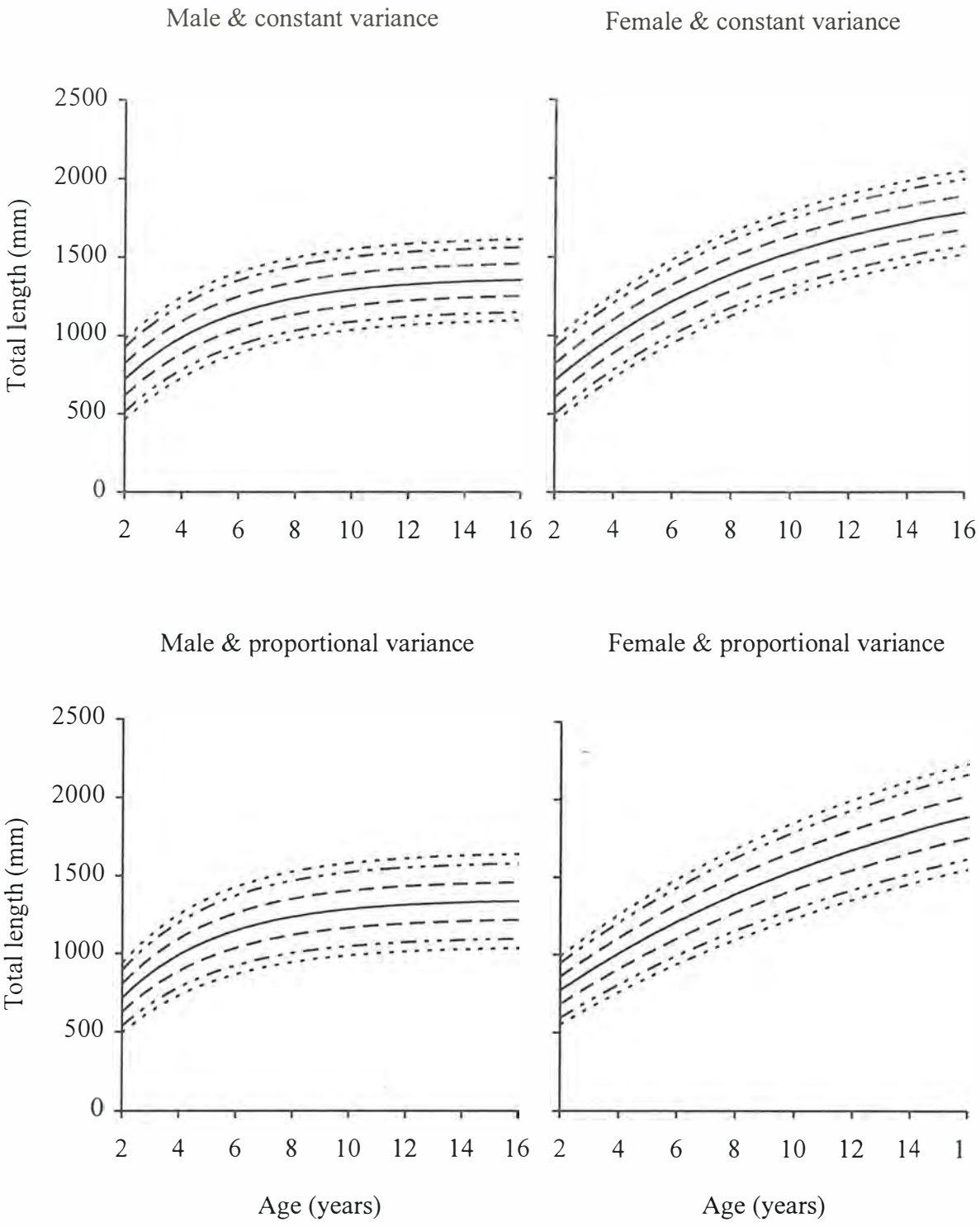

FIG. 5. Von Bertalanffy growth curves for male and female gummy sharks in Bass St during 1973-76 (———) with 1 SD (– – – –), 2 SD (– - - –), and 2.5 SD (- - - - -) assuming separately constant or proportional variance in shark length with length at ε age. [Based on the von Bertalanffy growth curves determined by Moulton et al. (199

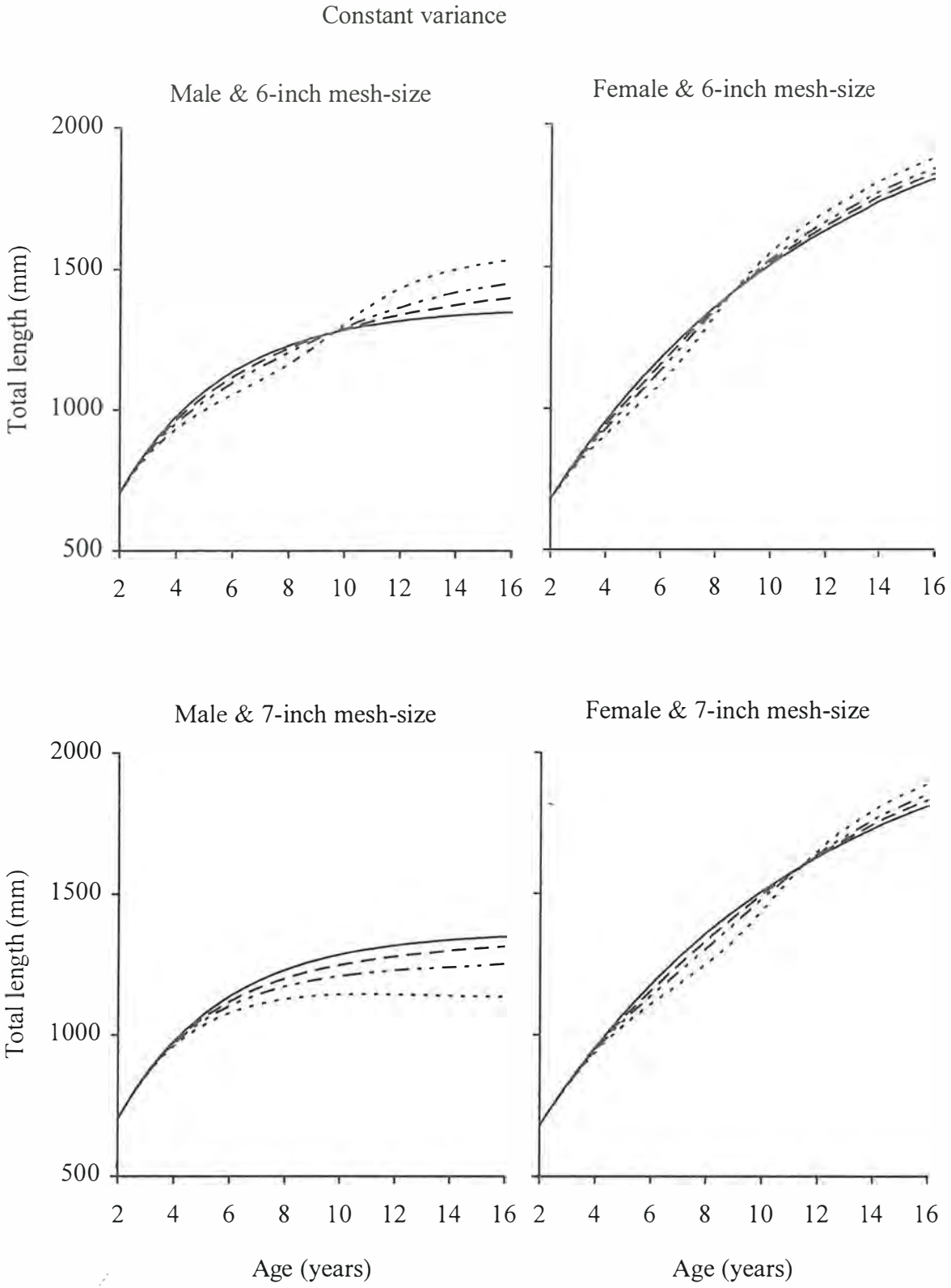

FIG. 6. Simulated mean length of population at age assuming constant fishing effort of zero (———), half (– – – –), equal (– - - –) and double (- - - - -) the 1987 peak level of fishing effort and assuming constant variance in shark length with length at any age for male and female gummy sharks in Bass Strait.

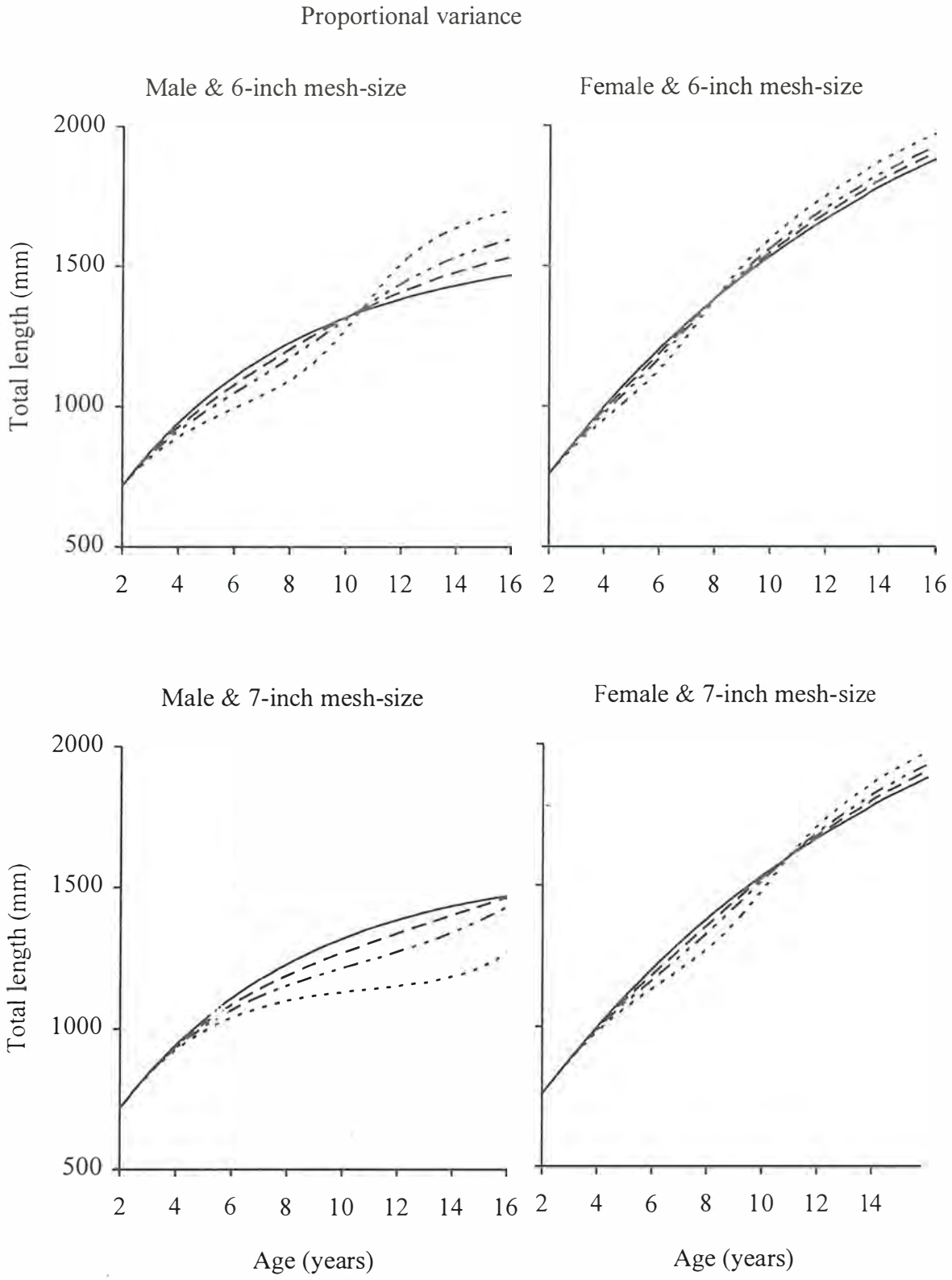

FIG. 7. Simulated mean length of population at age assuming constant fishing effort zero (———), half (– – – –), equal (– - - –) and double (- - - - -) the 1987 peak level fishing effort and assuming variance in shark length at any age is proportional to m length at that age for male and female gummy sharks in Bass Strait.

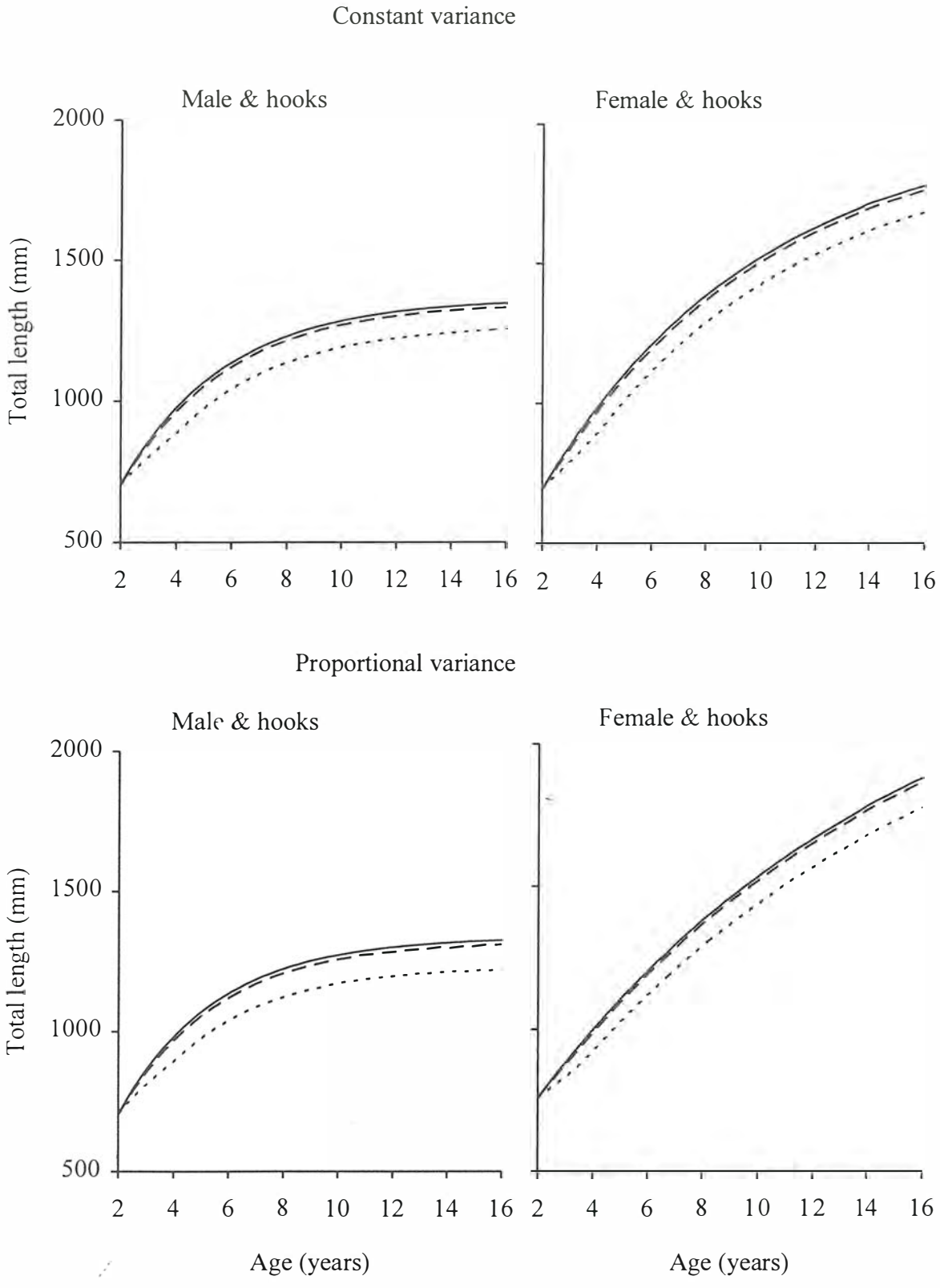

FIG. 8. Simulated mean length of population at age assuming constant fishing effort of zero (———), the peak level of equivalent hook fishing effort calculated at 40 million hook-h for 1987 (– – – –), and 200 million hook-h (arbitrarily 5 times the peak level) (- - - -) and assuming separately constant or proportional variance in shark length with length at any age for male and female gummy sharks in Bass Strait.

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