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# Vertebral cartilage of the clearnose skate, Raja eglanteria: Development, structure, ageing, and hormonal regulation of growth

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# VERTEBRAL CARTILAGE OF THE CLEARNOSE SKATE, *RAJA EGLANTERIA*: DEVELOPMENT, STRUCTURE, AGEING, AND HORMONAL REGULATION OF GROWTH

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Presented to

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In Partial Fulfillment

Of the Requirements for the Degree of

Doctor of Philosophy

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by

James J. Gelsleichter

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APPROVAL SHEET

This dissertation is submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

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## ABSTRACT

Incremental marks formed in the vertebral cartilage of most sharks, skates and rays are widely used as indicators of age in elasmobranch growth studies. Such information is essential for fisheries management, thus vertebral ageing has become an invaluable tool for investigating elasmobranch life history. Unfortunately, lack of information on the processes that regulate vertebral growth and mineralization limit efforts to correlate episodic stimuli with increment production. To address this research need, this dissertation investigated these processes through a detailed study on the vertebral cartilage of the clearnose skate, *Raja eglanteria*.

Histologic observations indicated that changes in appositional cartilage growth are the catalyst for increment formation. Observations by scanning electron microscopy supported this conclusion by demonstrating that growth increments differ in the proportion of cells to mineralized matrix. In contrast, elemental analysis of vertebrae using energy-dispersive spectrophotometry demonstrated no change in mineral concentration between seasonal growth layers. This observation was advantageous, because uptake of the radiotracer $^{45}Ca$ was ineffective in estimating the rates of vertebral calcification in captive *R. eglanteria*. Failure of this standard method appeared to reflect the free exchange of calcium between skeletal and serologic reservoirs.

Seasonally formed vertebral increments were used to estimate growth rate in *R. eglanteria*. Estimates of von Bertalanffy growth parameters were as follows, males: $DW_\infty = 584.07$ mm, $K = 0.19\ y^{-1}$, $t_0 = -0.86$; females: $DW_\infty = 633.24$ mm, $K = 0.17\ y^{-1}$, $t_0 = -0.89$; combined sexes: $DW_\infty = 617.62$ mm, $K = 0.17\ y^{-1}$, $t_0 = -0.88$. The results of this analysis indicated that *R. eglanteria* grows rapidly over a relatively short life span (5 - 7 y). Studies on captive *R. eglanteria* injected with fluorescent skeletal markers indicated that somatic growth and vertebral growth are highly coupled. In most vertebrates, these processes are under strict control by the endocrine system. Based upon this, studies were undertaken to investigate the hormonal regulation of vertebral growth.

An *in vitro* method for measuring vertebral growth was developed using incorporation of $^{35}S$-sulfate as a marker for cartilage matrix synthesis. Certain physiological mechanisms responsible for governing skeletal growth appear to be conserved among elasmobranchs and higher vertebrates. The growth hormone-dependent serum factor insulin-like growth factor-I (IGF-I) increased vertebral matrix synthesis, suggesting an important role for this hormone in regulating elasmobranch skeletal growth. In contrast, corticosterone reduced $^{35}S$-sulfate uptake in vertebral cartilage, suggesting an inhibitory role for glucocorticosteroids in elasmobranch chondrogenesis. Calcitonin also inhibited vertebral matrix synthesis and, *in vivo*, may play some role in skeletal development or mineral homeostasis. Finally, nutritional status also appeared to influence vertebral growth *in vitro*, perhaps indirectly through effects on hormone production.

In sum, this dissertation identified changes in cartilage growth as the impetus for vertebral growth zone production in *R. eglanteria*. Physiological mechanisms that likely regulate vertebral growth are described in this pioneer study on elasmobranch skeletal growth.

# VERTEBRAL CARTILAGE OF THE CLEARNOSE SKATE, *RAJA EGLANTERIA:* DEVELOPMENT, STRUCTURE, AGEING, AND HORMONAL REGULATION OF GROWTH

## GENERAL INTRODUCTION

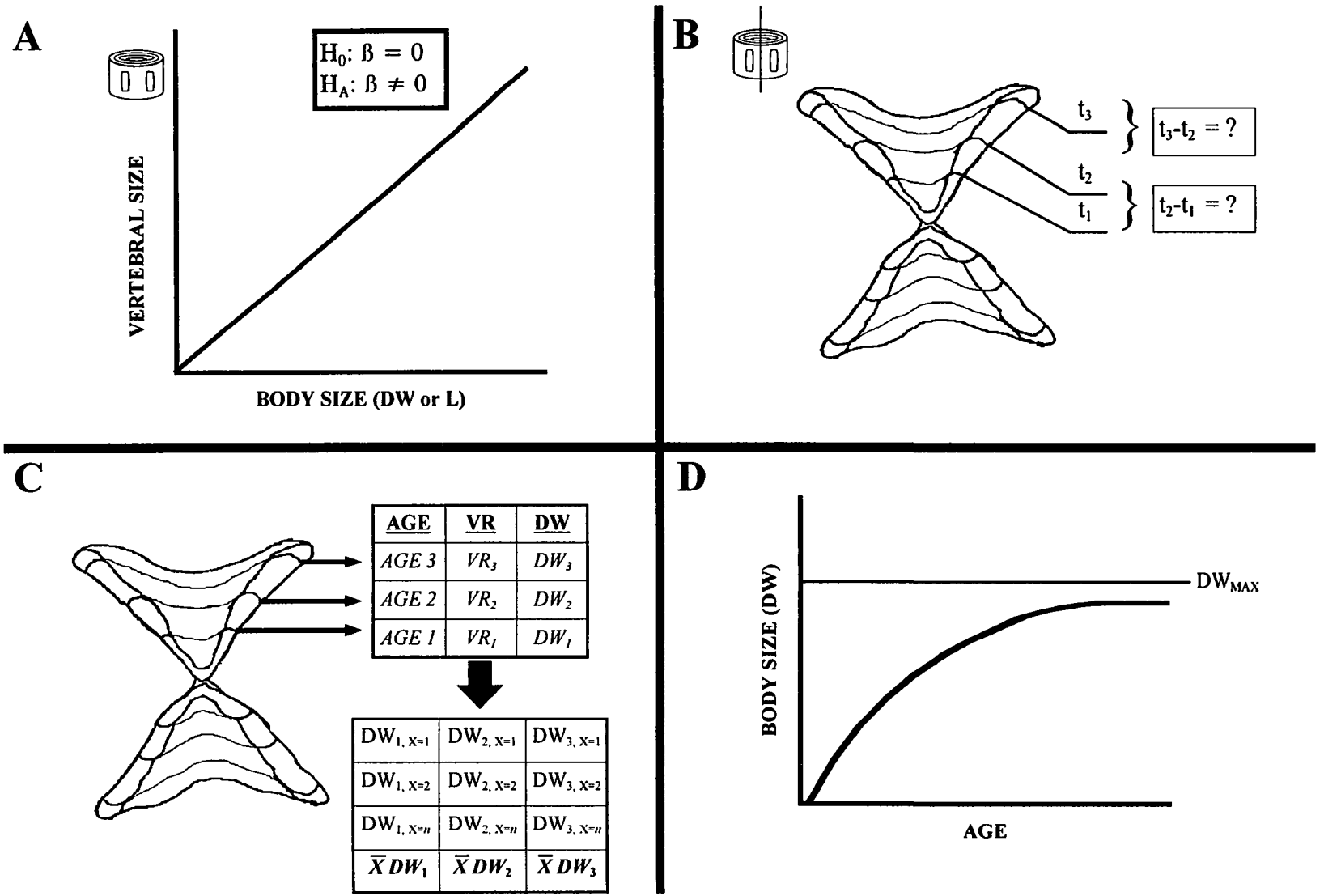
The vertebral column of elasmobranchs is composed of disc-shaped units of hyaline cartilage, termed centra, which surround and constrict the embryonic notochord (Ridewood, 1921; Applegate, 1967). Deposition of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] occurs within the extracellular matrix of these skeletal elements in a unique, pervasive manner which Ørvig (1951) termed "areolar mineralization." In most elasmobranchs, this process appears to proceed incrementally and results in the production of rhythmic mineral discontinuities or growth bands (Ridewood, 1921). Such features are also observed in the calcified structures of many invertebrate and vertebrate species, and have proven useful as indicators of age and growth rate (Simkiss and Wilbur, 1989). Predictably, a similar use was suggested for elasmobranch vertebrae soon after its detailed description in Ridewood (1921).

Due to the lack of practical alternatives (i.e. otolith or scale ageing), analysis of vertebral growth patterns has evolved into the most widely used method for determining elasmobranch age and growth (Table 1). In such studies, allometric relationships between vertebral size and body size are used to determine average size-at-age, which is defined by enumerating vertebral annuli (Fig. 1). Afterwards, estimates of mean size-at-age are used to approximate growth rate using selected growth functions. Several studies have facilitated this process by providing new methods of age

**Table 1. Elasmobranch age and growth studies that utilized vertebral band analysis. IR: In review, IP: In progress.**

| Species | Reference |
|---|---|
| *Orectolobus japonicus* | Tanaka, 1990 |
| *Ginglymostoma cirratum* | Carrier and Radtke, 1988 |
| | Gelsleichter *et al.*, 1997 |
| | Gelsleichter *et al.*, 1998a |
| *Rhincodon typus* | Cailliet *et al.*, 1986 |
| *Carcharias taurus* | Branstetter and Musick, 1994 |
| *Alopias vulpinus* | Cailliet *et al.*, 1983 |
| | Cailliet and Radtke, 1987 |
| *Cetorhinus maximus* | Parker and Stott, 1965 |
| *Carcharodon carcharias* | Cailliet *et al.*, 1985 |
| *Isurus oxyrinchus* | Cailliet *et al.*, 1983 |
| | Pratt and Casey, 1985 |
| *Lamna ditropis* | Tanaka, 1980 |
| | Goldman, IP |
| *Cephaloscyllium umbratile* | Tanaka, 1990 |
| *Carcharhinus acronotus* | Cortes, pers. comm. |
| *C. altimus* | Jensen *et al.*, 1996 |
| *C. amblyrhynchos* | Cailliet and Radtke, 1987 |
| *C. brachyurus* | Walter and Ebert, 1991 |
| *C. brevipinna* | Branstetter, 1987a |
| *C. falciformis* | Branstetter, 1987c |
| | Bonfil *et al.*, 1993 |
| *C. leucas* | Thornson and Lacey, 1982 |
| | Branstetter and Stiles, 1987 |
| *C. limbatus* | Branstetter, 1987a |
| | Killam and Parsons, 1989 |
| | Wintner and Cliff, 1996 |
| *C. obscurus* | Schwartz, 1983 |
| | Natanson *et al.*, 1995 |
| | Natanson and Kohler, 1996 |
| *C. plumbeus* | Lawler, 1976 |
| | Casey *et al.*, 1985 |
| | Branstetter, 1987b |
| | Casey and Natanson, 1992 |
| | Sminkey and Musick, 1996 |
| *C. longimanus* | Seki *et al.*, 1998 |
| *C. sorrah* | Davenport and Stevens, 1988 |
| *C. tilstoni* | Davenport and Stevens, 1988 |
| *Galeocerdo cuvier* | Branstetter *et al.*, 1987 |
| *Galeorhinus japonicus* | Tanaka et al., 1978 |
| *Galeorhinus galeus* | Ferreira and Vooren, 1991 |
| | Officer *et al.*, 1996 |
| *Mustelus antarcticus* | Walker *et al.*, 1995 |
| | Officer *et al.*, 1996, 1997 |
| *M. californicus* | Yudin and Cailliet, 1990 |
| *M. canis* | Conrath, IP |
| *M. griseus* | Wang and Chen, 1982 |
| *M. henlei* | Yudin and Cailliet, 1990 |
| *M. manazo* | Tanaka and Mizue, 1979 |
| | Cailliet *et al.*, 1990 |
| | Yamaguchi *et. al.*, 1996 |
| *Negaprion brevirostris* | Gruber and Stout, 1983 |
| | Brown and Gruber, 1988 |
| | Pike, 1988 |
| *Prionace glauca* | Stevens, 1975 |
| | Cailliet *et al.*, 1983 |
| | Tanaka *et al.*, 1990 |
| | Skomal, 1990 |
| *Rhizoprionodon taylori* | Simpfendorfer, 1993 |
| *R. terranovae* | Parsons, 1985 |
| | Branstetter, 1987b |
| *Triakis semifasciata* | Smith, 1984 |
| | Kusher *et al.*, 1992 |
| *Sphyrna lewini* | Schwartz, 1983 |
| | Branstetter, 1987c |
| | Chen *et al.*, 1990 |
| *S. tiburo* | Parsons, 1993 |
| | Carlson, pers. comm. |
| *Squatina californica* | Natanson and Cailliet, 1990 |
| *Rhinobatos annulatus* | Roussouw, 1984 |
| *Torpedo californica* | Neer, 1998 |
| *Raja batis* | DuBuit, 1972 |
| *R. binoculata* | Zeiner and Wolf, 1993 |
| *R. clavata* | Taylor and Holden, 1964 |
| | Holden and Vince, 1973 |
| | Ryland and Ajayi, 1984 |
| *R. eglanteria* | Daiber, 1960 |
| | Berry, 1965 |
| | Gelsleichter, this study |
| *R. erinacea* | Johnson, 1979 |
| | Waring, 1984 |
| *R. fusca* | Ishiyama, 1951 |
| *R. hollandi* | Ishiyama, 1958 |
| *R. kenojei* | Ishiyama, 1958 |
| *R. microocellata* | Ryland and Ajayi, 1984 |
| *R. montagui* | Ryland and Ajayi, 1984 |
| *R. naevus* | Du Buit, 1972 |
| *R. rhina* | Zeiner and Wolf, 1993 |
| *Dasyatis garouensis* | Tanaka, 1987 |
| *D. sabina* | Schmid, 1987 |
| *D. sayi* | Gelsleichter and Musick, IP |
| *Gymnura altavela* | Gelsleichter *et al.*, IP |
| *Myliobatis californica* | Martin and Cailliet, 1988 |
| *Narcine entenedor* | Villavicercio and Cailliet, IP |
| *Rhinoptera bonasus* | Smith and Merriner, 1987 |
| *Urolophus paucimaculatus* | Edwards, 1980 |

**Figure G.1.** Components of a typical elasmobranch age and growth study using vertebral band analysis. Determination of (A) relationship between vertebral and somatic growth; (B) temporal periodicity of vertebral band formation; (C) average size-at-age (to age *n*) using back-calculation; and (D) growth rate.



validating growth band periodicity (Cailliet, 1990). Other studies have addressed the importance of measuring precision in age estimates (Cailliet *et al.*, 1990; Hoenig *et al.*, 1995), the location of vertebral sampling (Officer *et al.*, 1996), and the potential effects of fluorescent "validation" markers on growth rate and animal health (Tanaka, 1990; Gelsleichter *et al.*, 1997; 1998a). Clearly, vertebral ageing is essential research and, consequently, it has been the subject of numerous symposia and critical reviews (Cailliet *et al.*, 1986; Cailliet, 1990; Natanson and Gelsleichter, in review).

Despite widespread use, recurrent problems have complicated the analysis of vertebral growth patterns and continue to hamper progress in elasmobranch ageing biotechnology. The temporal periodicity associated with growth zone production, in particular, appears to vary in certain species (Parker and Stott, 1964; Natanson and Cailliet, 1990; Branstetter and Musick, 1994) without any clearly defined origin. In addition, efforts to age vertebrae using radiometric dating have been unsuccessful, perhaps due to dynamic remodeling of vertebral mineral (Welden *et al.*, 1987). Also, the vertebral centra of several deep-sea species are poorly calcified and provide no advantage in age determination (Cailliet *et al.*, 1983). Such problems clearly illustrate the need for studies on the poorly understood process of elasmobranch vertebral growth and calcification. This need has been stressed repeatedly in contemporary scientific literature (Cailliet, 1990; Clement, 1992).

This dissertation reports on aspects of the growth and calcification of vertebral centra in the clearnose skate, *Raja eglanteria*. In Chapter 1, the embryonic development of these structures is addressed. Chapter 2 discusses seasonal cycles of

vertebral growth and mineralization, and the interactions between mineral and matrix constituents. In Chapter 3, growth rate of *R. eglanteria* is estimated using vertebral increments, and the effectiveness of fluorescent markers in age validation is explored. Chapter 4 describes *in vivo* uptake of $^{45}Ca$ as a means to study rates of vertebral calcification. Finally, a new method for investigating the growth of vertebral cartilage is introduced in Chapter 5. This method is used to investigate the hormonal regulation of vertebral growth, and the effects of nutrition on this process.

Collectively, this dissertation represents the first comprehensive study on elasmobranch vertebral physiology. As suggested by Cailliet (1990), such information is intended to:

> *"...help us understand the interplay between ecological and physiological factors and how they sculpt the features of calcified structures which may reflect the age and growth patterns of elasmobranch fishes"*

## IMPORTANCE OF THE STUDY

Commercial and recreational exploitation of elasmobranchs has increased dramatically in several regions of the world (Bonfil, 1994). As an example, the shark resources of the northwest Atlantic Ocean had been increasingly exploited by commercial and recreational fisheries since the early 1970's (Musick *et al.*, 1993). In many regions, the management of valuable elasmobranch resources under increasing fishery pressure became a matter of regional and/or national concern (NMFS, 1993). Accurate biological data are essential for the production and effectiveness of such

management efforts and, thus, remain a high-priority research need for the majority of elasmobranch species.

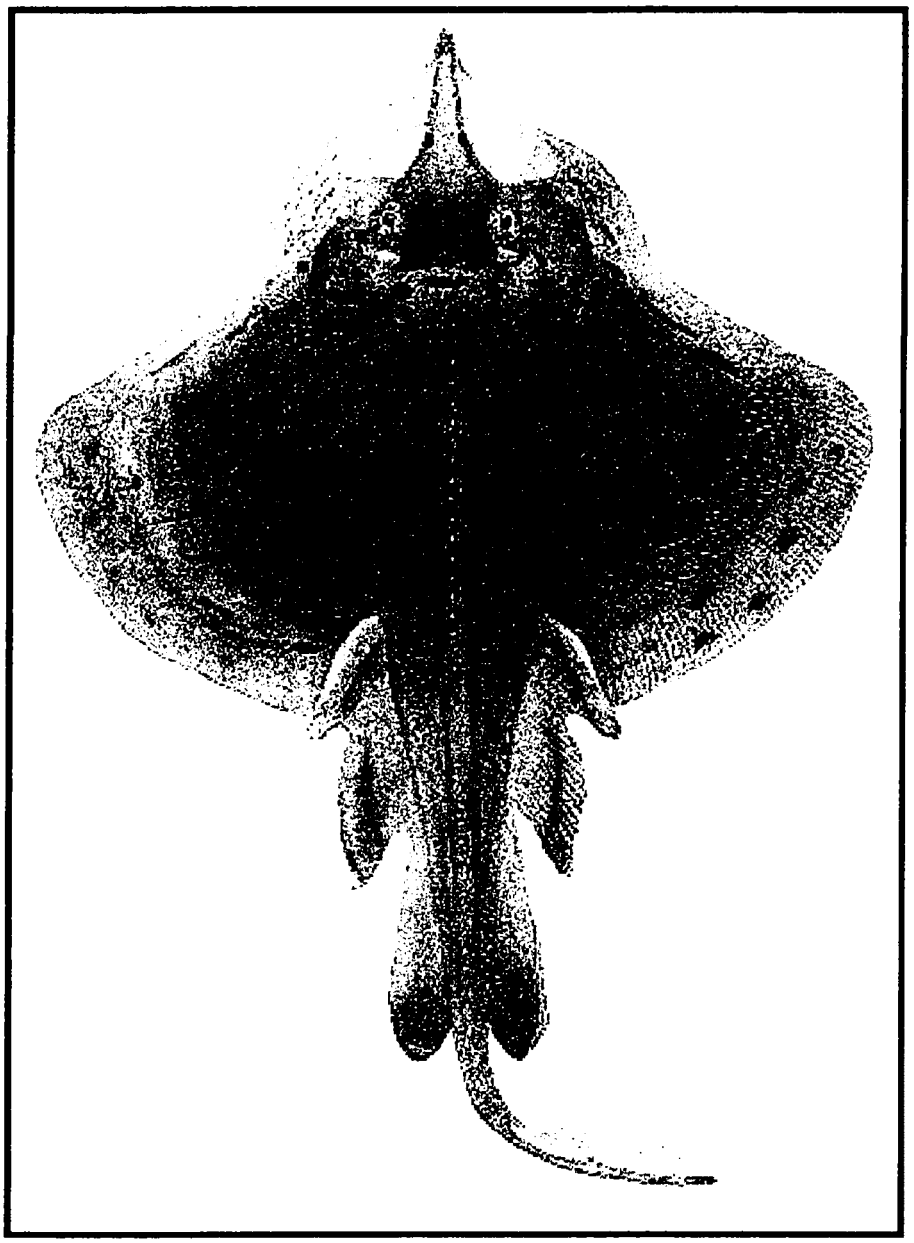
Among other factors, accurate age and growth data are necessary for estimating the population parameters that define a species' biological resilience to fishing pressure (Cailliet *et al.*, 1986). Unfortunately, lack of this information has severely hampered the management of exploited elasmobranch resources. For example, the federal Fishery Management Plan (FMP) for sharks of the U. S. Atlantic Ocean, Gulf of Mexico and Caribbean Sea specifically identified shark age and growth data as a priority research deficiency and requirement (NMFS, 1993). Information on the factors that regulate annulus formation in vertebral centra has obvious implications for improving elasmobranch ageing biotechnology. Clement (1992) stressed this notion eloquently, by stating:

> *"What is the nature of the systematic physiological disturbances that give rise to the growth rings in shark vertebrae? If this simple question could be answered, better correlations between seasonal or other episodic events and particular growth rings could be made."*

## EXPERIMENTAL SPECIES

The clearnose skate, *Raja eglanteria* Bosc (Fig. 2), is a common inhabitant of coastal waters along the eastern United States. In the Atlantic Ocean, *R. eglanteria* ranges from Cape Cod, MA to southern Florida and is commonly observed in estuaries, including the Chesapeake (McEachran and Musick, 1973) and Delaware Bays (Fitz and

**Figure G.2.** Clearnose skate, *Raja eglanteria* (Murdy *et al.*, 1997).



Daiber, 1963). In the Gulf of Mexico, its range extends from mid-Florida to Texas (Luer and Gilbert, 1985).

The life history of *R. eglanteria* has been the subject of numerous studies (Breder and Nichols, 1937; Breder and Atz, 1938; Libby and Gilbert, 1960; Fitz and Daiber, 1963; Luer and Gilbert, 1985; Luer *et al.*, 1994). Due to its small size, *R. eglanteria* has little commercial importance and is typically discarded or used for bait by recreational anglers (Luer and Gilbert, 1985). However, certain studies indicate that it is important as prey (Stillwell and Kohler, 1993; Gelsleichter *et al.*, in press) or competition (Fitz and Daiber, 1963) for more commercially desirable species. In addition, *R. eglanteria* has become useful as an experimental animal for biomedical research (Luer, 1989). Researchers at the Mote Marine Laboratory (MML) in Sarasota, Florida have been highly successful in maintaining a captive breeding population of this species for use in these critical studies.

*Raja eglanteria* was chosen as an experimental species for this dissertation due to its abundance in the Chesapeake Bay and coastal waters of Virginia (McEachran and Musick, 1973). In addition, access to MML's skate-rearing facility ensured the availability of newly hatched individuals for laboratory studies. Finally, previous studies indicated that vertebral calcification is rhythmic in *R. eglanteria*, and thus produces the growth zones that are the focus of this dissertation (Daiber, 1960; Berry, 1965).

# Chapter 1

## Development of vertebral cartilage in *Raja eglanteria*

# INTRODUCTION

In all vertebrates, the embryonic notochord is ultimately replaced partially or in whole by cartilaginous or bony vertebral bodies (Torrey, 1971). These structures originate from embryonic connective tissue termed mesenchyme, which aggregates on each side of the notochord and neural tube during development. Despite this common origin, many variations in vertebral morphogenesis exist. Consequently, patterns in vertebral development have often been described for individual vertebrate groups rather than as a generalized process (Williams, 1959).

Histogenic factors leading to the development of elasmobranch vertebrae have previously been described by Ridewood (1921). Unfortunately, these observations were limited to early developmental stages only. No study has investigated the morphogenesis of vertebrae in a growth series extending from young embryos to full-term progeny for an individual elasmobranch species. Such information may be useful for characterizing changes in vertebral growth and mineralization that appear to occur during this period. For example, several elasmobranchs develop vertebral growth discontinuities prior to and during parturition (Casey *et al.*, 1985; Branstetter, 1987a; Branstetter and Stiles, 1987; Walter and Ebert, 1991). These "pre-birth" and "birth" growth marks are often structurally similar to the seasonal increments used in age and growth studies. Thus, knowledge about the development of these embryonic

phenomena may provide much-needed information on the processes that give rise to annual changes in vertebral morphology.

The present chapter describes the development of vertebral cartilage of the clearnose skate, *Raja eglanteria*. This species reproduces well in captivity and lays eggs that develop over a 12-week period (Luer, 1989). This study provides a description of the histogenic changes that occur in the radial growth of vertebrae during embryogenesis.

## MATERIALS AND METHODS

Embryonic (3-12 weeks) and juvenile (1-2 week hatchlings) *R. eglanteria* were obtained from Mote Marine Laboratory's skate-rearing facility, where they were incubated under constant temperature (20-22°) and photoperiod (12L: 12D) regimes (Luer, 1989). Specimens were processed by routine methods for paraffin histology (Luna, 1968). Cross-sections of mid-trunk vertebrae were fixed in Bouin's solution, decalcified with buffered formic acid, washed in running tap water followed by multiple rinses in 50% ethanol saturated with lithium carbonate, dehydrated in a graded series of ethanol, and embedded in paraffin. Transverse sections of trunk and caudal vertebrae were cut at 5 $\mu$m using a rotary microtome, stained with haemotoxylin-eosin (H&E), and coverslipped with synthetic mounting media. Slides were examined using a compound microscope.

# RESULTS

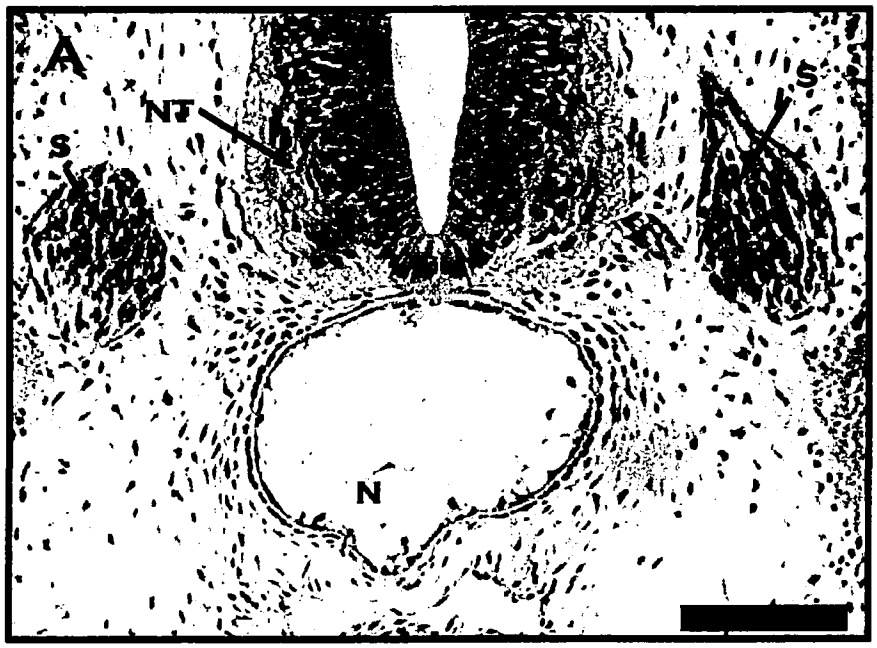
### *Stage 1. Sclerotome (Week 2-3)*

Aggregations of vertebral mesenchyme, termed sclerotomes, developed from mesoderm prior to the observations in this study. At 19 d, these cell masses had begun to migrate towards the notochord and neural tube (Fig. 1a). The notochord was surrounded by a multi-layered sheath that appeared intimately associated with the ventral portion of the neural tube (Fig. 1b).

### *Stage 2. Invasion of the notochordal sheath and development of pre-cartilage (Week 3-6)*

After 3 weeks, sclerotomal cells penetrated the notochordal sheath on its left and right borders (Fig. 2). These cells migrated to surround the entire notochord and separate the internal and external layers of the notochordal sheath. At the same time, sclerotomal cells assembled around the neural tube in preparation to form the cartilaginous neural arch. Both of these processes continued until week 6 (Figs. 3-5), when precursors of the neural arch and vertebral centrum were fully discernible. Lateral processes termed basiventrals (Ridewood, 1921) also began to develop during this period (Fig. 5).

**Figure 1.1.** (**A**) Cross-section of mid-trunk vertebrae from a 19 d *R. eglanteria* embryo, demonstrating initial migration of sclerotomes (S) toward the neural tube (NT) and notochord (N). Bar: 100 μm. (**B**) Higher magnification demonstrates the close structural relationship between the neural tube and the notochordal sheath (NS). Bar: 100 μm.



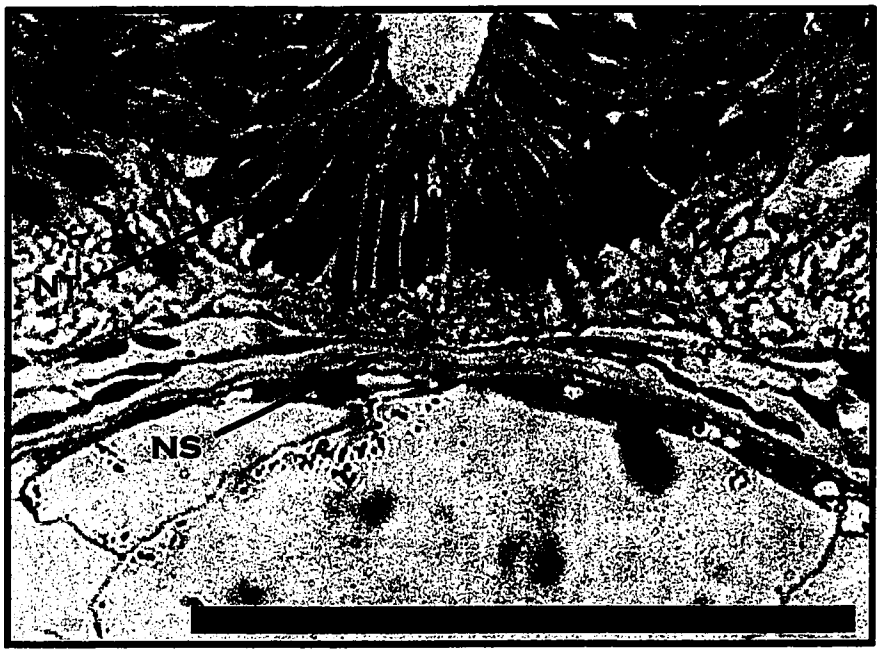
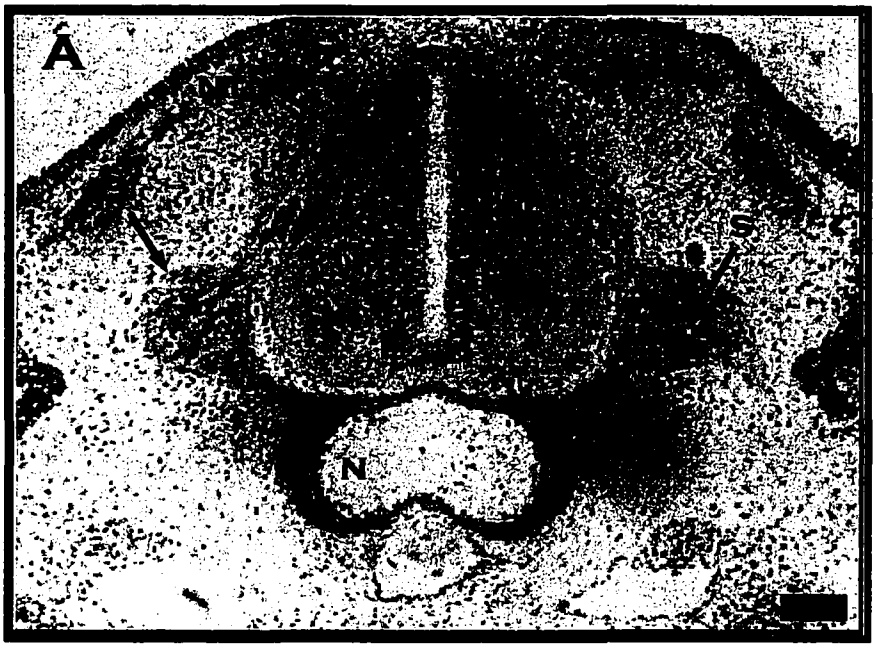



**Figure 1.2.** **(A)** Cross-section of mid-trunk vertebrae from 23 d *R. eglanteria* embryo, demonstrating initial migration of free sclerotomal cells around the neural tube (NT) in preparation to form cartilaginous arches. Cells derived from the sclerotomes (S) also penetrate the notochordal sheath. Bar: 100 μm. **(B)** Higher magnification of notochord (N) from the same animal, demonstrating sclerotomal invasion of the notochordal sheath in preparation to form vertebral centra. Bar: 100 μm.



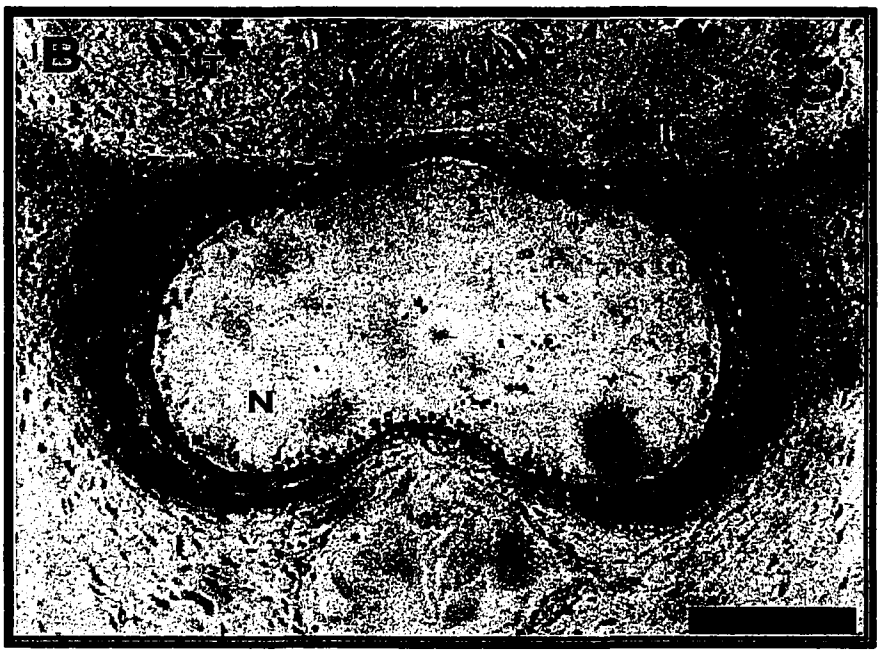



**Figure 1.3.** **(A)** Cross-section of mid-trunk vertebrae from a 27 d *R. eglanteria* embryo, depicting further migration of free sclerotomal (S) cells around the neural tube (NT). Bar: 100 μm. **(B)** Sclerotomal invasion of the notochordal sheath also continues as cells surround the notochord (N) and produce vertebral pre-cartilage (PC). Bar: 100 μm.

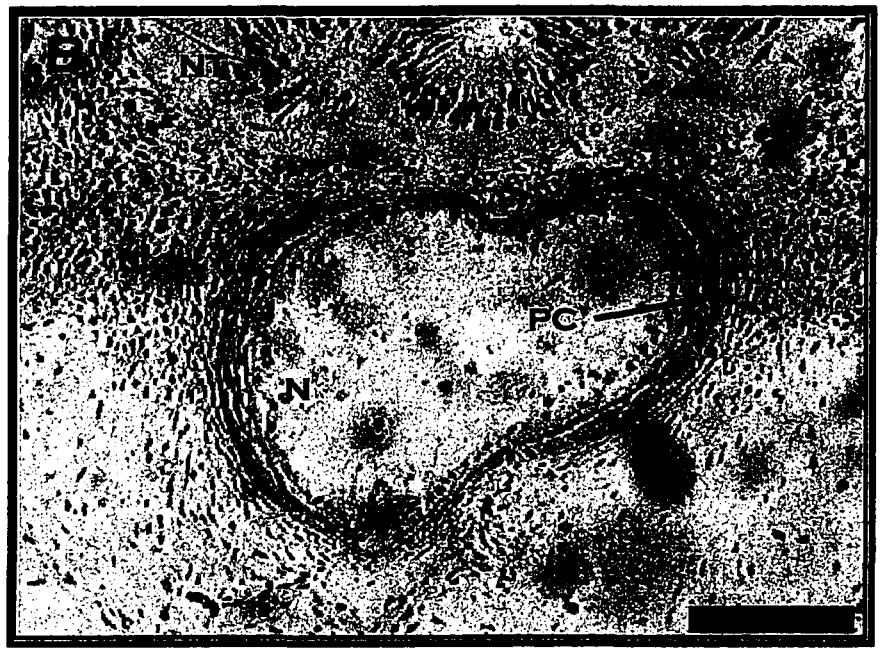
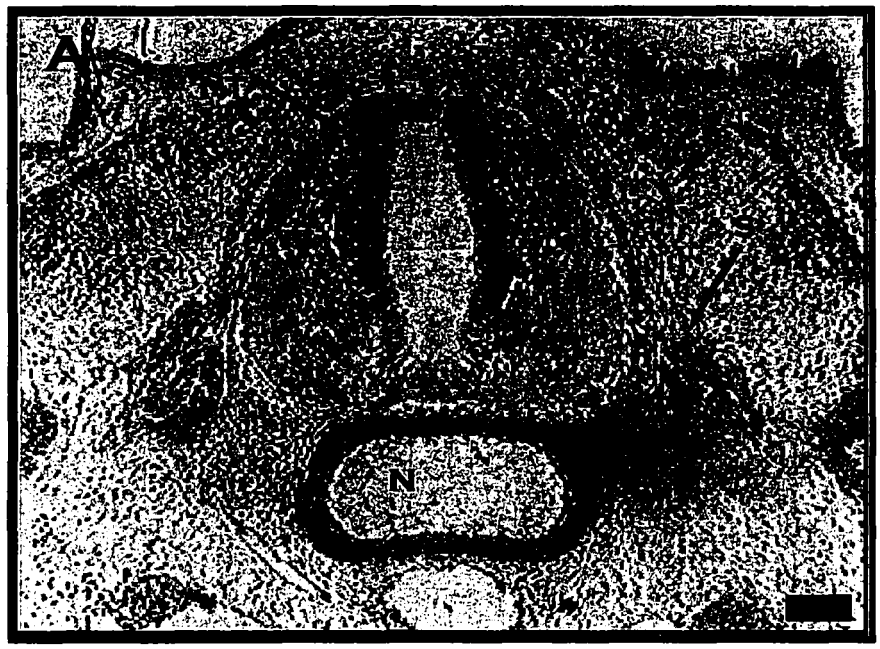


**Figure 1.4.** **(A)** Cross-section of mid-trunk vertebrae from 30 d *R. eglanteria* embryo. At this stage, sclerotomal (S) cells have virtually surrounded the neural tube (NT). Bar: 100 μm. **(B)** At the same time, sclerotomal cells have completely surrounded the notochord (N), and additional migration gives rise to radial expansion of vertebral pre-cartilage (PC). Bar: 100 μm.



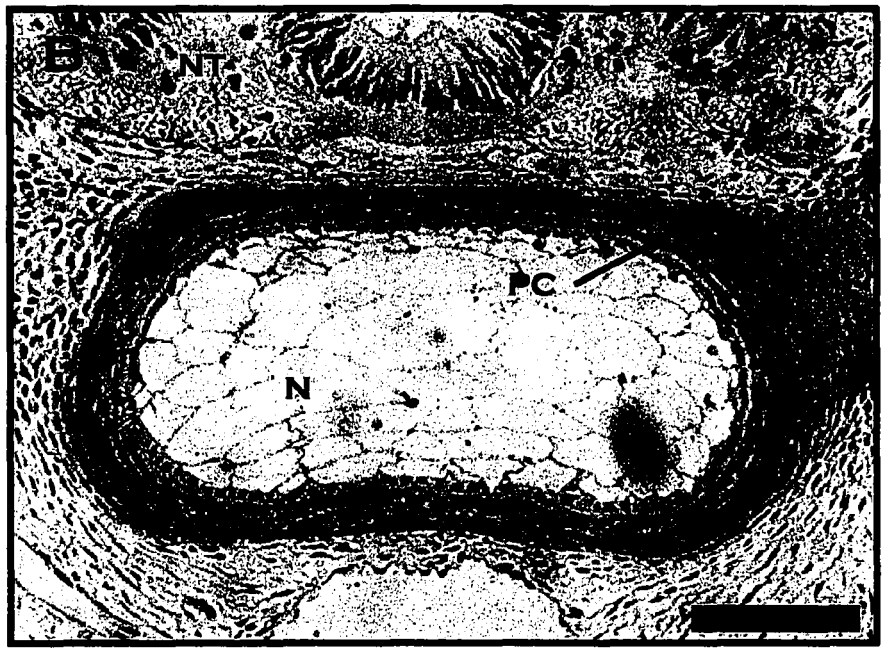
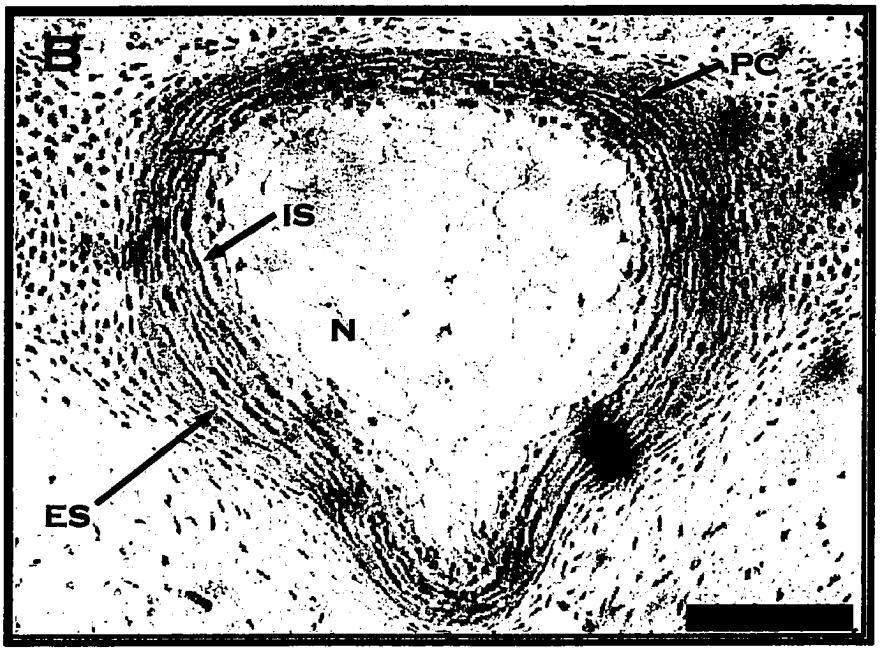



**Figure 1.5.** (A) Cross-section of mid-trunk vertebrae from 35 d *R. eglanteria* embryo, demonstrating further development of the neural arch (NA) cartilage from sclerotomal (S) cells. Lateral processes (LP) also begin to develop in trunk vertebrae at this stage. Bar: 100 μm. (B) Continued invasion of sclerotomal cells in the notochordal sheath have produced a thick layer of pre-cartilage (PC), surrounded by external (ES) and internal (IS) layers of the sheath. Bar: 100 μm.

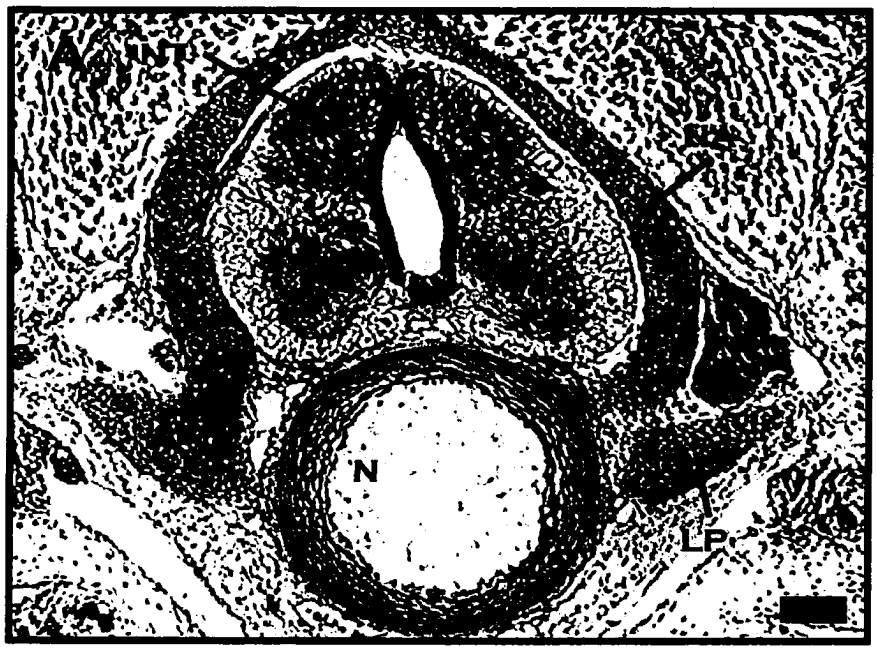



***Stage 3. Chondrogenesis, perichondrial development, and cell differentiation (Weeks 6-8)***

Rudiments of the neural arch and centrum were fully formed by the sixth week of development. Initially, these structures were separated by the persistence of the outer layer of the notochordal sheath (Fig. 6). By the end of week 6, this layer was no longer discernible, and the centrum and neural arch was continuous at certain sites (Fig. 7). Cells from the neural arch began to immigrate into the centrum at these junctions and initiated chondrification. Dorsal and ventral regions of the centrum not contiguous with arch cartilage were bordered by the fibrous perichondrium, which initially was poorly developed (Fig. 7b). Rapid increases in the complexity and activity of this proliferative layer occurred after cell invasion (Fig. 8), likely due to the action of cartilage matrices, which encourage chondrocyte differentiation (Alberts *et al.*, 1983).

By week 8, the pre-cartilage stage had passed and vertebral centra attained the characteristics of true cartilage. During this period, multiple stages of cytodifferentiation similar to those observed in mammalian growth plate cartilage (Oohira *et al.*, 1974) became visible. Proliferating chondrocytes were round and typically displayed high nucleocytoplasmic ratios (Fig. 9b). As these cells matured, they became flattened in appearance and were characteristic of active glycosaminoglycan synthesis (Fig. 9c). Afterwards, cells became ovoid in shape and appeared to shift their biosynthetic activity to collagen production (9d). In most

**Figure 1.6.** **(A)** Cross-section of mid-trunk vertebrae from 43 d *R. eglanteria* embryo, depicting full development of neural arch cartilage (NA), lateral processes (LP), and the vertebral centrum. Bar: 100 μm. **(B)** Vertebral centrum remains in the "pre-cartilage" stage, and is still surrounded by the external layer (ES) of the notochordal sheath. Bar: 100 μm.



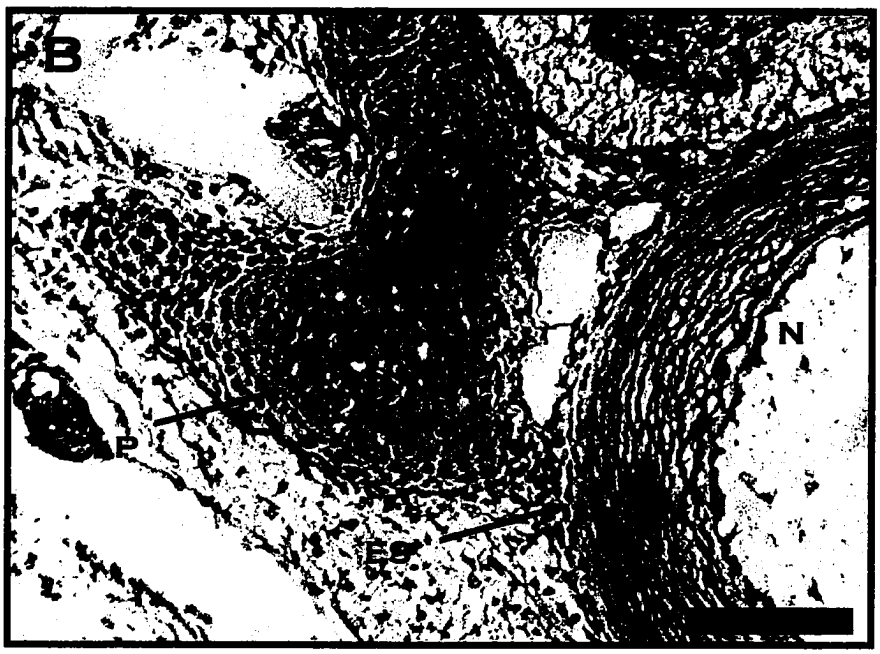
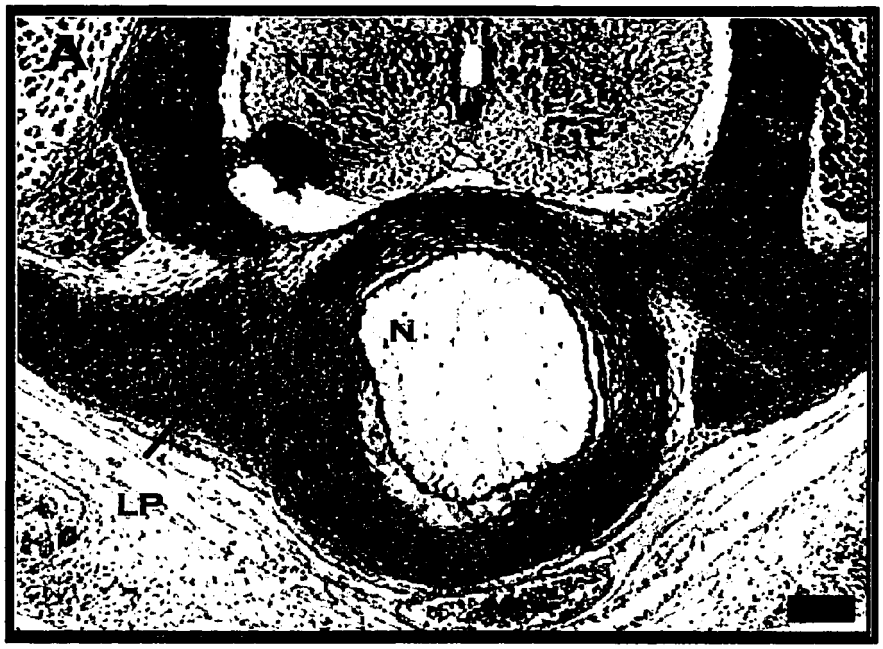



**Figure 1.7.** (**A**) Cross-section of mid-trunk vertebrae from 6 w *R. eglanteria* embryo. Centrum remains in the pre-cartilage stage. The notochordal sheath is no longer discernible. Bar: 100 μm. (**B**) Removal of the sheath has allowed the centrum and arch cartilage to join at centrolateral junctions (Jx). Regions of the centrum not bordered by arch cartilage are surrounded by the perichondrium (P). N: notochord; LP: lateral process; NT: neural tube. Bar: 100 μm.



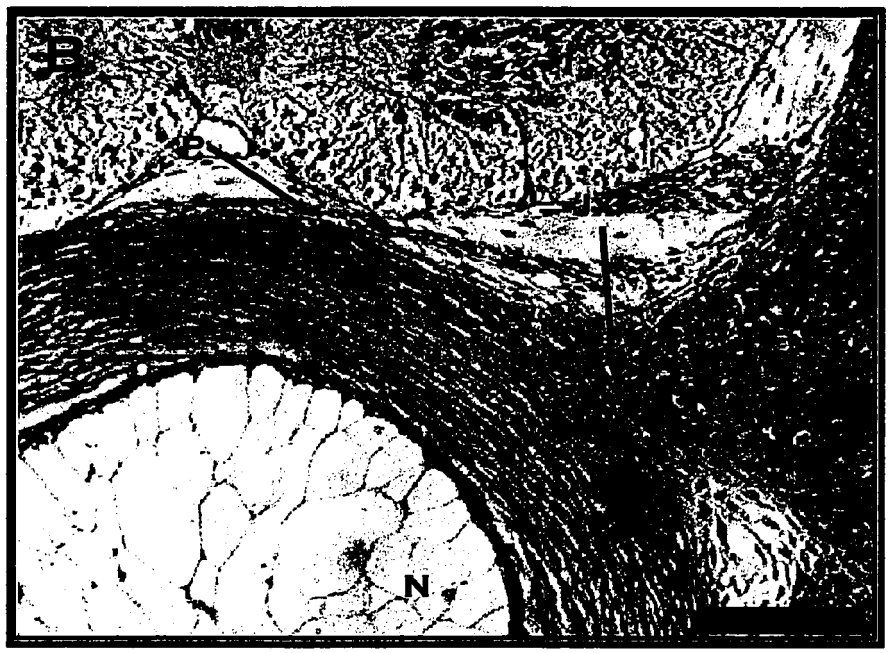
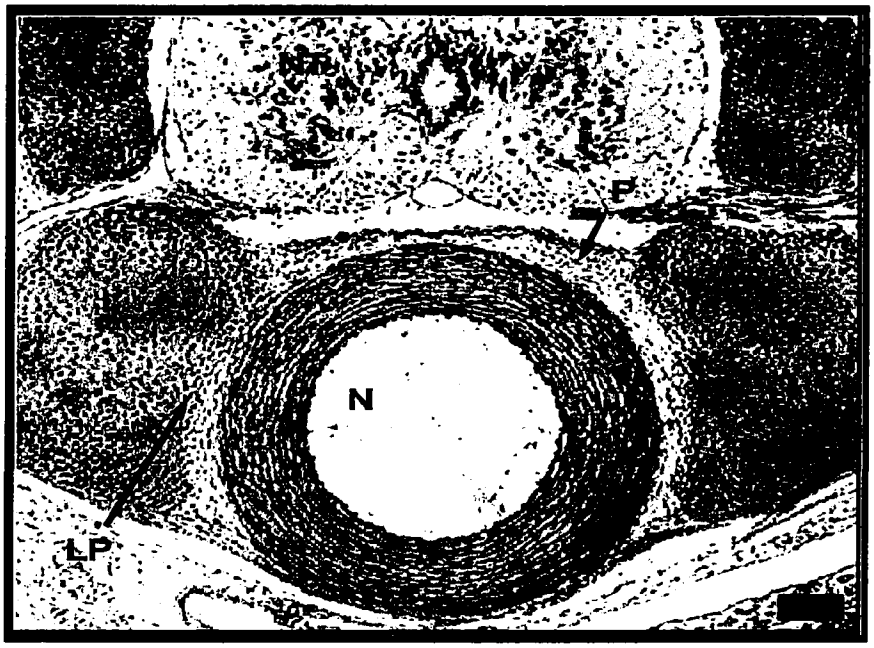



**Figure 1.8.** (**A**) Cross-section of rear-trunk vertebrae from a 7 w *R. eglanteria* embryo. Vertebral centrum remains in pre-cartilage phase. Bar: 100 μm. (**B**) Perichondrium has developed considerably since initial cellular invasion at the centrolateral junctions. Bar: 100 μm. N: notochord; NT: neural tube; LP: lateral process.



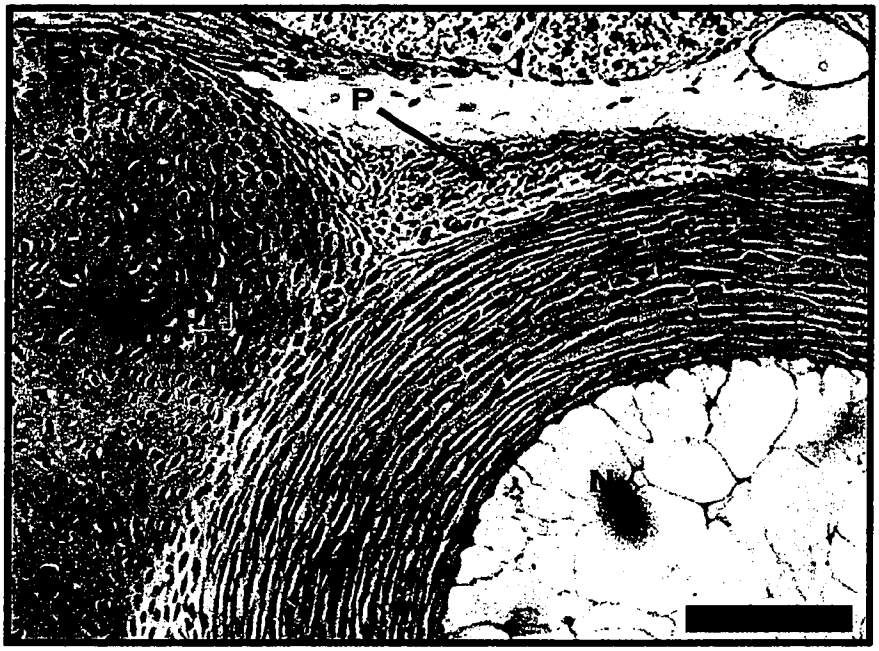
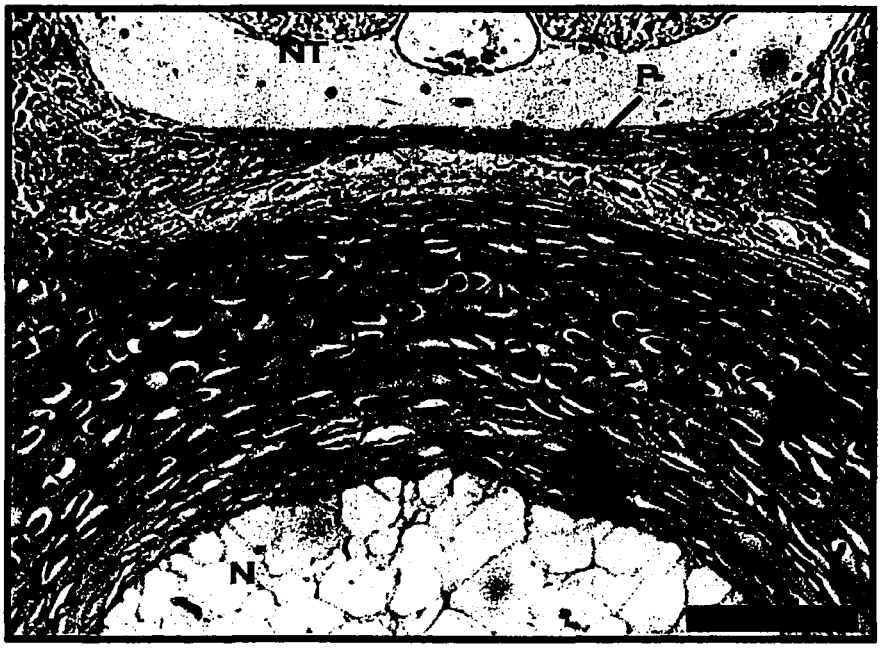
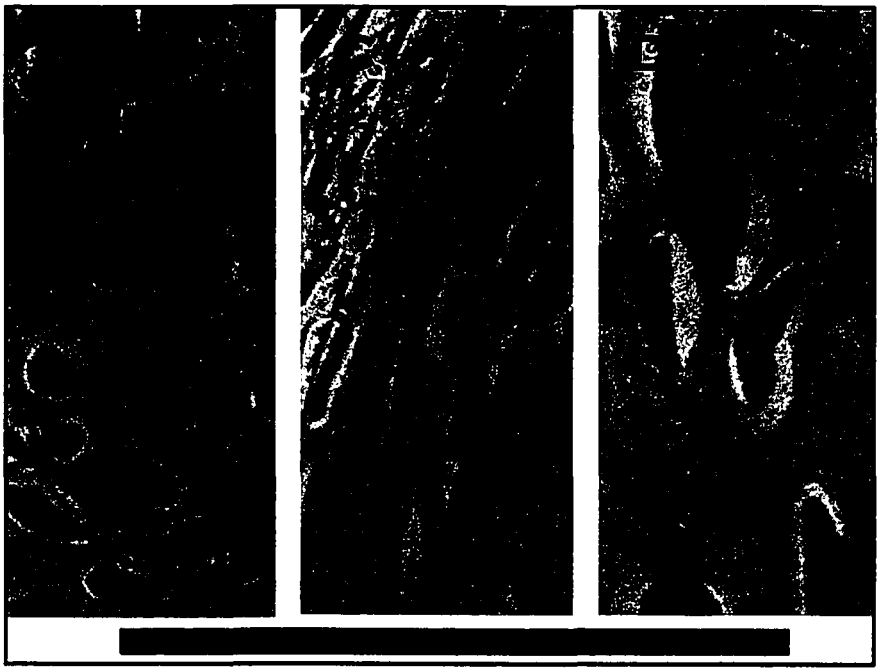



**Figure 1.9.** (**A**) Cross-section of rear-trunk vertebrae from 8 w *R. eglanteria* embryo. Pre-cartilage stage is completed and vertebral cartilage is fully differentiated. Perichondrium (P) continues to develop and begins chondrocyte proliferation. Bar: 100 μm. Three stages of cytodifferentiation are present: (**B**) proliferating chondrocytes, which appear round with high nuclear content; (**C**) maturing chondrocytes, which are flattened in appearance; and (**D**) mature chondrocytes, which are ovoid with higher cytoplasmic content. Bar (**B-D**): 100 μm. NT: neural tube N: notochord.



histologic preparations, an acidophilic "tidemark" separated this latter stage from other cell types (Fig. 9a).

*Stage 4. Rapid expansion of embryonic vertebral cartilage (Weeks 9-11)*

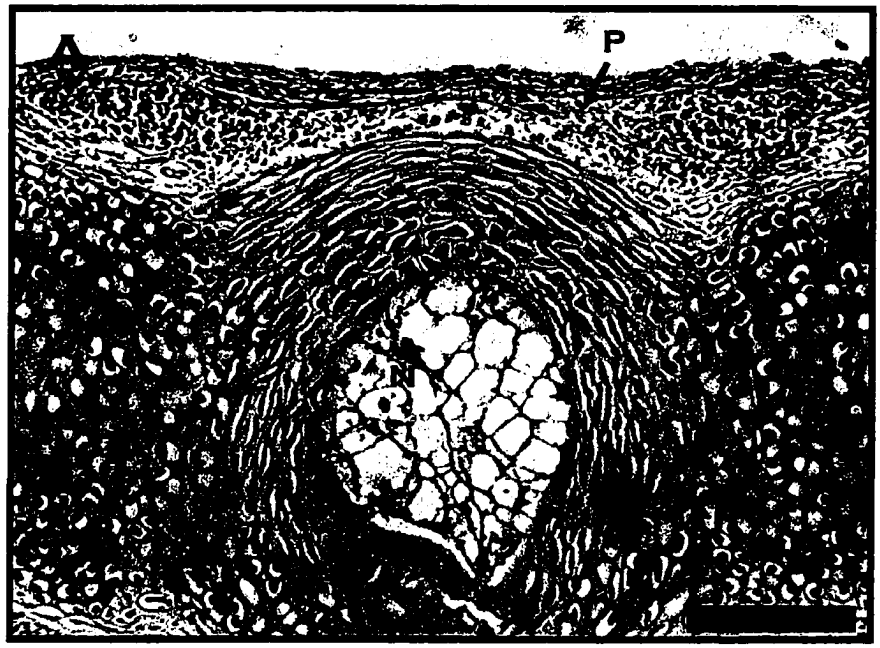
From week 9 until hatching, perichondrial development and radial cartilage growth occurred at a rapid pace (Figs. 10-12). Chondrocytes matured soon after proliferation and quickly developed the capacity for matrix biosynthesis (Figs. 10a, 11a). Rapid cartilage growth also occurred in other skeletal tissues, as demonstrated by intense basophilia in sub-perichondrial regions (Figs. 10b, 11b). Accelerated vertebral growth continued into the final week of embryogenesis, when the primary growth layer (composed of stage II chondrocytes) remained 6-7 cells in width (Fig. 12).

*Stage 5. Hatching*

Immediately after hatching, vertebral growth appeared to continue at a relatively rapid pace (Fig. 13a). No visible changes in cell proliferation/maturation or matrix synthesis appeared to occur during this initial period. The primary layer of vertebral growth remained similar to that in 9-11 week embryos; 6-7 cells in width.

One week after hatching, there was some evidence of reduced vertebral growth and matrix synthesis (Fig. 13b). The primary layer of radial growth appeared reduced to approximately 2-3 cells in overall width. At the same time, a wide layer of basophilia was present and likely reflected the deposition of vertebral mineral deposits (Clement, 1992).

**Figure 1.10.** (**A**) Cross-section of rear-trunk vertebrae from 9 w *R. eglanteria* embryo. Perichondrium (P) is well developed and gives rise to rapid centrum growth, as demonstrated by accelerated cell maturation. Primary layer of appositional growth (consisting of flattened chondrocytes) is 6-7 cells in width. Bar: 100 μm. (**B**) Neural arch cartilage of the same animal, demonstrating rapid matrix production (RMP) in other skeletal tissues. P: perichondrium. Bar: 100 μm.



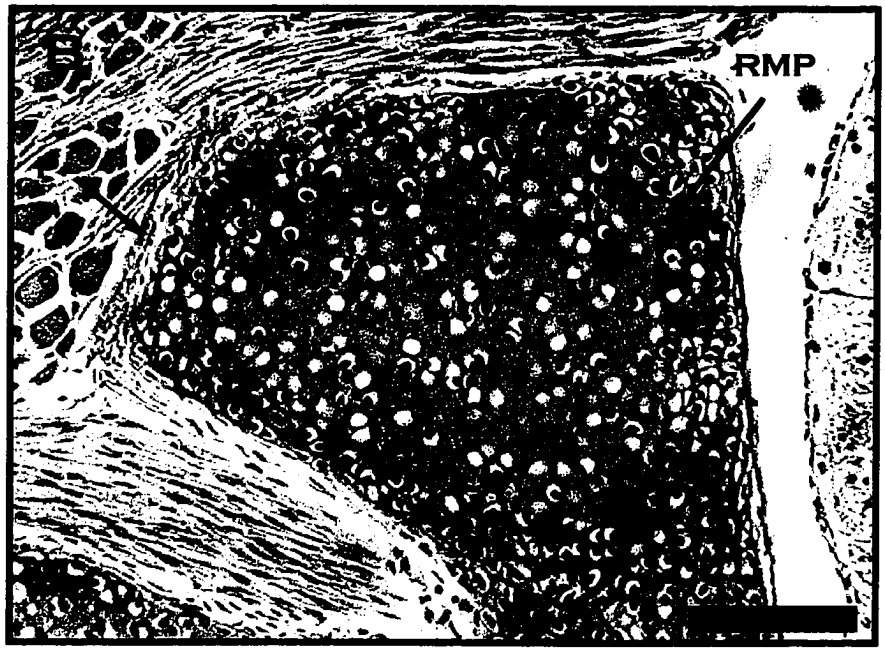



**Figure 1.11.** (**A**) Cross-section of rear-trunk vertebrae from 10 w *R. eglanteria* embryo. Sub-perichondrial growth remains rapid, as demonstrated by regions of intense basophilia and rapid cell maturation. Bar: 100 μm. (**B**) Neural arch cartilage of the same animal, demonstrating rapid matrix production (RMP) in other skeletal tissues. P: perichondrium. Bar: 100 μm.

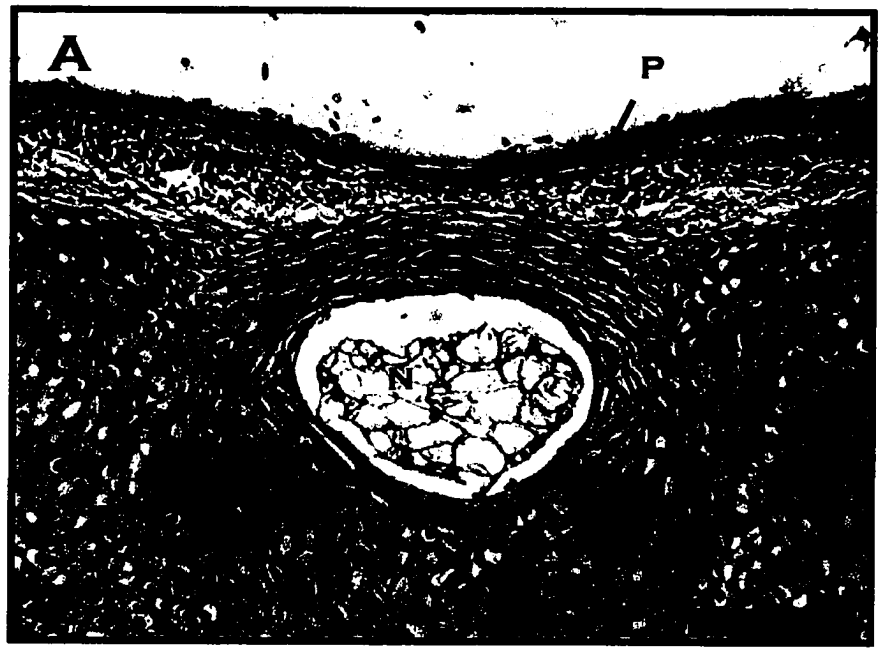



**Figure 1.12.** **(A)** Cross-section of rear-trunk vertebrae from a *R. eglanteria* embryo, during the last week of embryonic development. The primary region of appositional growth is 5-6 cells in width, indicating that radial growth remains rapid. P: perichondrium. Bar: 100 μm.

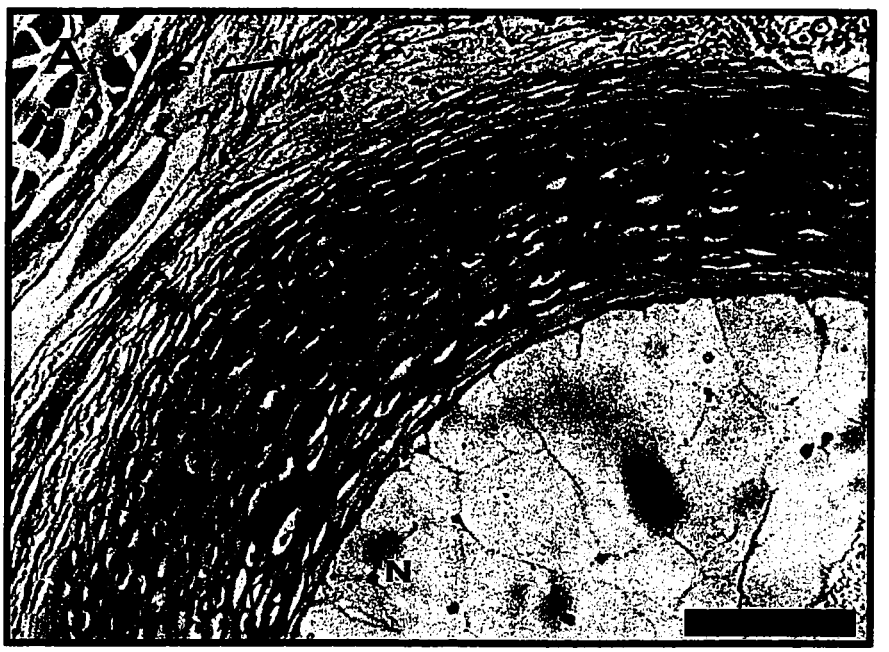
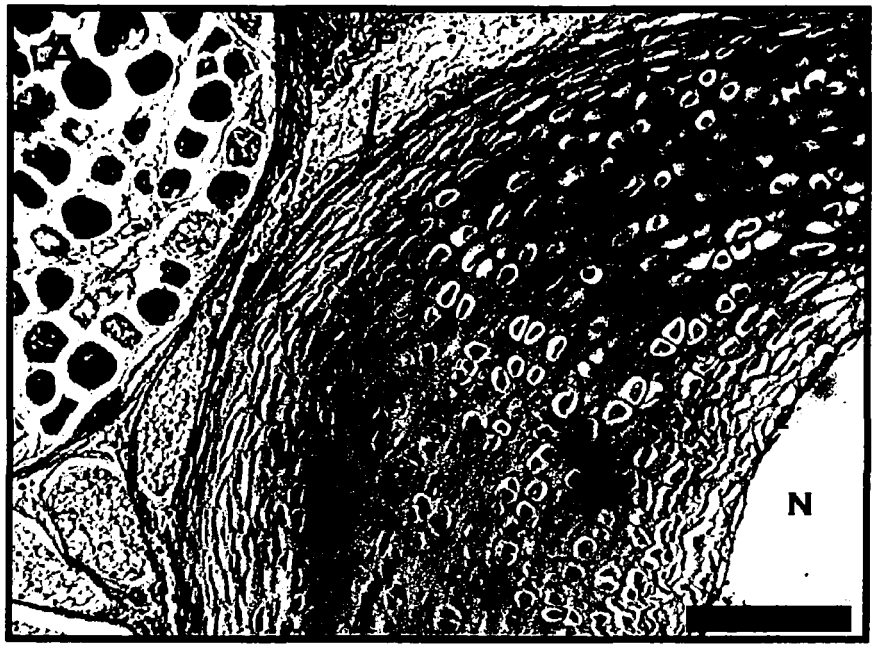
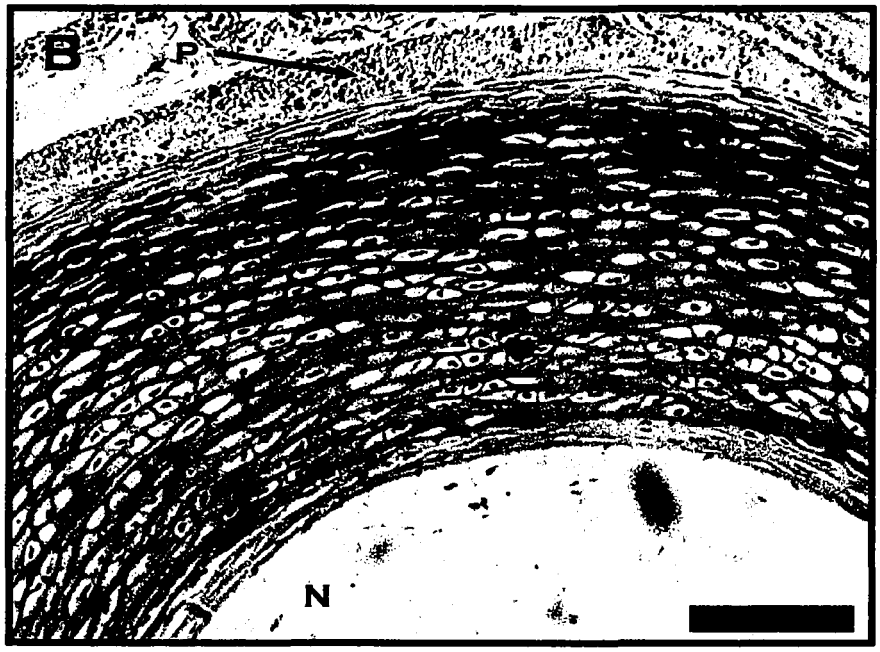


**Figure 1.13.** (**A**) Cross-section of rear-trunk vertebrae from juvenile *R. eglanteria*, during the first week after hatching. Appositional vertebral growth remains accelerated, as indicated by rapid cell maturation in the sub-perichondrial region (5-6 cells in width). Bar: 100 μm. (**B**) Cross-section of rear-trunk vertebrae from juvenile *R. eglanteria*, during the second week after hatching. Appositional growth is reduced in the sub-perichondrial region, which is only 2-3 cells in width. Bar: 100 μm. Perichondrium: P.



# DISCUSSION

Development of vertebral centra in *R. eglanteria* follows the pattern described by Ridewood (1921), in which mesenchymal tissue penetrates the embryonic notochordal sheath and initiates chondrification. This pattern sets elasmobranchs apart from most other vertebrates, in which the centra are formed external to the notochordal sheath (Williams, 1959; Torrey, 1971). Once the basic architecture of the vertebral centrum is established, the remainder of development involves radial and longitudinal cartilaginous growth and calcification. This, too, differs from other vertebrates, in which the remainder of embryonic centrum development is given over to ossification shortly following cartilage calcification.

Cartilage of the neural and hemal arches, which originates independently from mesenchyme, appears to play an important role in centrum development. Immigration of chondrocytes from these structures into centra occurs at arch bases soon after sclerotomal cells have surrounded the notochord. Portions of the centrum that are not bordered by the arches are surrounded by perichondrial tissue instead. Historically, this arrangement has led to confusion as to whether the perichondrium or immigrant chondrocytes give rise to centrum expansion. Results from the present study indicate that perichondrial activity is increased shortly following the immigration of chondrocytes at arch bases. Secretory activity of these immigrant cells probably acts as a positive feedback mechanism, prompting chondrogenesis at the perichondrial borders. Such a process is consistent with the action of cartilage proteoglycans (Alberts *et al.*,

1983) and supports Ridewood's (1921) suggestion that "it is cellular activity that spreads, not the cellular tissue."

No changes in vertebral growth occurred in concert with hatching, suggesting that the production of vertebral discontinuities termed "birth marks" is not dependent upon this event. However, evidence of reduced growth was visible in vertebral sections taken from juvenile *R. eglanteria* one week after hatching. At this stage, internal yolk resources have been fully depleted (Luer, 1989), and animal survival depends upon initiation of feeding. It is reasonable to suggest that loss of these metabolic resources may be related to disruptions in vertebral (and somatic) growth, yet more evidence is needed. Nevertheless, this hypothesis provides a rational explanation for the production of disruptive vertebral marks.

The production of "pre-birth" and "birth" marks in vertebrae of other elasmobranch species also appear to be associated with changes in metabolic supply. In placental sharks, "pre-birth" marks appear to form at placentation, when embryonic nourishment shifts from lecithotrophy to matrotrophy (Branstetter, 1987). Such marks also form in the vertebrae of rays (Martin and Cailliet, 1988), which shift from lecithotrophy to trophonematal matrotrophy *in utero*. Finally, "birth marks" appear to occur in the vertebrae of many elasmobranchs, and are likely associated with the overall loss of embryonic fuel provisions. This may suggest that temporal disruptions in vertebral development may reflect seasonal changes in energy supply (i.e. nutrition) or metabolism. Because such changes certainly occur in many vertebrates, this suggestion warrants further investigation.

Additional aspects of vertebral formation may be clarified using *R. eglanteria* as an experimental model. In particular, the roles of the neural tube and notochord in centrum development require further investigation. Extirpation of these structures in vertebrate embryos results in abnormal development of the vertebral column, suggesting a developmental relationship (Torrey, 1971). In addition, grafts of the neural tube or notochordal tissue can produce vertebral cartilage in experimental cultures (Torrey, 1971; Monsoro-Burq *et al.*, 1994). Similar experiments on elasmobranchs are necessary to elucidate patterns of skeletal formation in these fishes.

In describing patterns of vertebral development in *R. eglanteria*, the present chapter provides an important basis for further observations on the growth and calcification of these structures. In particular, the potential relationship between nutrition and vertebral growth may clarify the processes involved in seasonal growth zone formation.

# Chapter 2

## Aspects of vertebral structure in *Raja eglanteria*

# INTRODUCTION

Following parturition, the vertebral centra of elasmobranchs continue to expand radially and longitudinally through appositional growth from the perichondrium (Clement, 1992). Chondroblasts derived from this germinative layer differentiate into chondrocytes, which secrete organic matrices to form mature, unmineralized cartilage (Ferreira and Vooren, 1991). Afterwards, mineralization occurs in association with these cells, which are eventually incorporated into a consolidated mineralizing front (Clement, 1992). In most elasmobranchs, these events proceed incrementally thereby forming periodic growth discontinuities typically used in age and growth studies (Clement, 1992; Cailliet, 1990). Perceptible variations in the structural content (e.g. mineral-to-matrix ratio) of these growth layers are often used to qualitatively describe them and determine their temporal periodicity (Cailliet, 1990).

In general, the production of growth discontinuities in calcified structures is poorly understood (Simkiss and Wilbur, 1989). In some tissues, these zones may reflect periods of slow or arrested accretion, which have become compressed by increased growth (Albrechtsen, 1968; Guarino *et al.*, 1998). In others, these regions may be comprised of changes in mineral content or crystalline structure (Gauldie, 1987; 1988). Finally, such features may incorporate both of these conditions. In all cases,

characterizing the structural nature of growth increments is an essential prerequisite for understanding the processes that ultimately lead to their formation.

A common aspect of biomineralization that may have some importance in incremental growth processes is the relationship between organic matrices and mineral deposition. In most calcified structures, organic matrices appear to act as mediators of mineralization by either initiating or inhibiting crystal formation (Simkiss and Wilbur, 1989). In cartilage, collagen and proteoglycans comprise the major organic elements that may influence skeletal development. Proteoglycans, in particular, are suggested to play a central role in cartilage calcification, yet the nature of this role remains a controversial topic (Shepard, 1992). However, proteoglycan aggregates from mammalian cartilage have been shown to effectively inhibit hydroxyapatite formation *in vitro* in several studies. In addition, ultrastructural (Takagi *et al.*, 1984) and mineralogical studies (Edmonds *et al.*, 1996) on elasmobranch jaw cartilage indicate a progressive decrease in proteoglycan content and size between uncalcified and calcified tissues. These observations suggest that partial degradation of proteoglycans may be necessary for the calcification of cartilage to occur (Kemp, 1984). If so, spatial differences in proteoglycan content may be associated with the fluctuations in hydroxyapatite concentration that are characteristic of mineral-variable growth layers. Thus, temporal changes in the synthesis and/or enzymatic degradation of these matrices may permit the production of these phenomena.

The present chapter outlines investigations on the structural aspects of vertebral growth in *R. eglanteria*. Seasonal changes in centrum growth and mineralization were

examined to determine the nature of vertebral growth discontinuities in this species. Spatial variations in hydroxyapatite concentration and proteoglycan content were analyzed to further characterize vertebral growth zones. These data were also used to examine the relationship between organic matrices and cartilage calcification. Finally, the effects of proteoglycan extracts from vertebral centra on hydroxyapatite formation *in vitro* were assessed.

## MATERIALS AND METHODS

### *Seasonal variations in vertebral growth*

Seasonal changes in the radial growth of vertebral centra were examined by histology. Vertebral samples were removed from clearnose skates collected by bottom trawl in the Chesapeake Bay and off the eastern shore of Virginia. Centra were fixed in Bouin's solution, decalcified with buffered formic acid, washed in running tap water followed by multiple rinses in 50% ethanol saturated with lithium carbonate, dehydrated in a graded series of ethanol, and embedded in paraffin. Longitudinal sections of vertebrae were cut at 5 $\mu$m using a rotary microtome, stained with haemotoxylin-eosin (H&E), and coverslipped with synthetic mounting media. Slides were examined using a compound microscope. Due to sampling constraints (i.e., weather, migratory patterns), few animals were collected during the months of December ($n$ = 4), January ($n$ =2), and April ($n$ = 2). Because of this, only

generalized features of vertebral growth were examined when comparing these samples to those collected in May ($n$ = 10), June ($n$ = 12), and August ($n$ = 10).

### *Proteoglycan content*

Spatial variations in proteoglycan content were also analyzed in histologic preparations, using the high-iron diamine (HID) method for localization of sulfated glycosaminoglycans (Takagi *et al.*, 1984). Vertebral sections were stained for 18 h in HID working solution, which was prepared by adding 1.4 ml of 40% $FeCl_3$ to a solution containing 120 mg of N,N-dimethyl-m-phenylenediamine dihydrochloride and 20 mg of N,N-dimethyl-p-phenylenediamine dihydrochloride in 50 ml of distilled water. Interactions between glycosaminoglycan sulfate esters and cationic HID produces a black stain reaction for the localization of these macromolecules.

### *Patterns of mineralization*

Characteristics of vertebral growth were further examined by scanning electron microscopy. At the same time, patterns of vertebral mineralization were examined by energy-dispersive spectrophotometry (EDS). Vertebral centra from *R. eglanteria* ($n$ = 30) were longitudinally sectioned (1.0 mm) using an Isomet rotary saw, cleaned with distilled water, and air-dried. Dried specimens were mounted on aluminum stubs with colloidal graphite and coated with carbon. Samples were examined using an Advanced Metals Research (AMR) Corporation model 1000 scanning electron microscope outfitted with an IXRF Systems, Inc. Quantitative EDS system. The following

elemental analyses were performed: 256-point line scans across the central radii of vertebral sections, point analyses of vertebral outer margins, and multiple point analyses and spectral X-ray mapping of alternating growth layers.

***Effect of proteoglycan extracts on hydroxyapatite formation***

The effects of proteoglycan extracts from vertebral cartilage on hydroxyapatite formation were examined *in vitro*. Vertebral samples were removed from freshly sacrificed *R. eglanteria*, cleared of extraneous tissue, and stored at -70°C. Subsamples of vertebral cartilage (1 g) were crushed to powder form with a mortar and pestle. Proteoglycans were extracted with 2 ml of a 3 *M* guanidine hydrochloride/10% EDTA solution (*p*H 7.6) containing the protease-inhibitor phenylmethylsulphonyl fluoride ($2 \cdot 10^{-3}$ *M*), following Michelacci and Horton (1989). The extraction process took place for 72 h at 4°C. Afterwards, extracts were separated from cartilage remnants by centrifugation at 10,000*g* for 20 min. Supernatant was collected and subjected to exhaustive dialysis (Spectra/Por 3, MW cutoff 3,500) against distilled water. Proteoglycans were precipitated by dropwise addition of ethanol. They were separated from ethanol by centrifugation at 10,000*g* for 20 min and redissolved in 2 ml of distilled water. Proteoglycan content was measured with Alcian blue as described in Gold (1979) using chondroitin-6-sulphate as a standard. Extracts were stored at 4°C for a maximum of 2 weeks.

The effect of proteoglycan solutions on the rate of hydroxyapatite formation was determined by monitoring *p*H following the rapid addition of 5 ml of 10 m*M* $CaCl_2$ to a

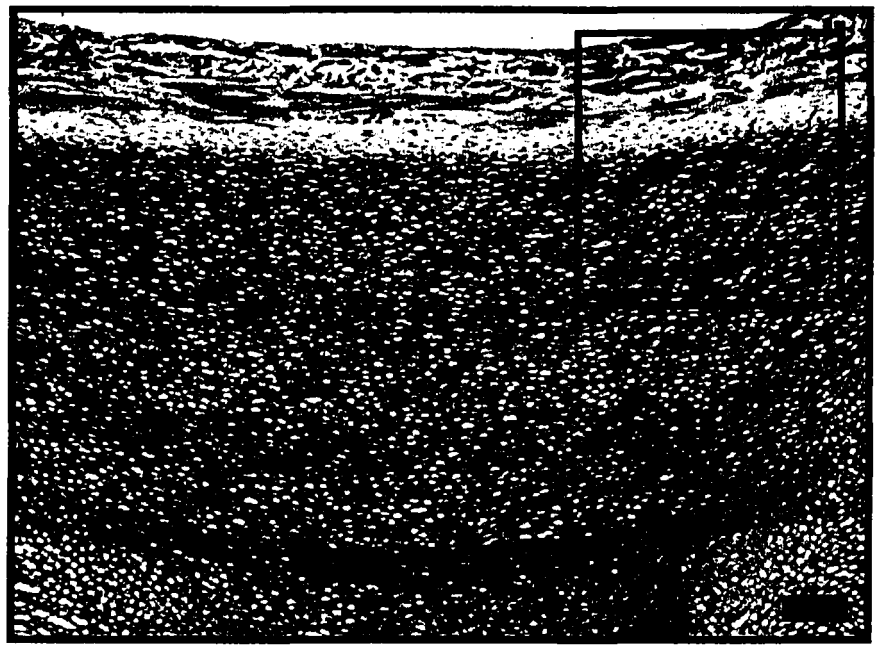
solution containing 3 ml of 10 m*M* $Na_2HPO_4$ and 2 ml of 12 $\mu$g $ml^{-1}$ proteoglycan solution (experimental) or 2 ml distilled water (control). Five replicates were performed for each treatment. At *p*H values above 6.0, the solubility of calcium phosphate is governed by the $K_{sp}$ of brushite ($CaHPO_4 \cdot H_2O$), which undergoes spontaneous hydrolysis leading to an equilibrium state in which hydroxyapatite crystals are formed (Kemp, 1984). Crystal formation is indicated by a rapid decrease in *p*H reflecting acidosis (Simkiss and Wilbur, 1989). The decrease in *p*H was followed with a *p*H meter (Hanna) while the reaction medium was stirred continuously. The initial *p*H of the sodium phosphate solution was above 8.3, giving precipitation with 2 ml distilled water added at 24°C in approximately 20 min. Hydroxyapatite precipitation was observed between *p*H 7.0-6.5. Treatment effects were compared by two-way ANOVA.

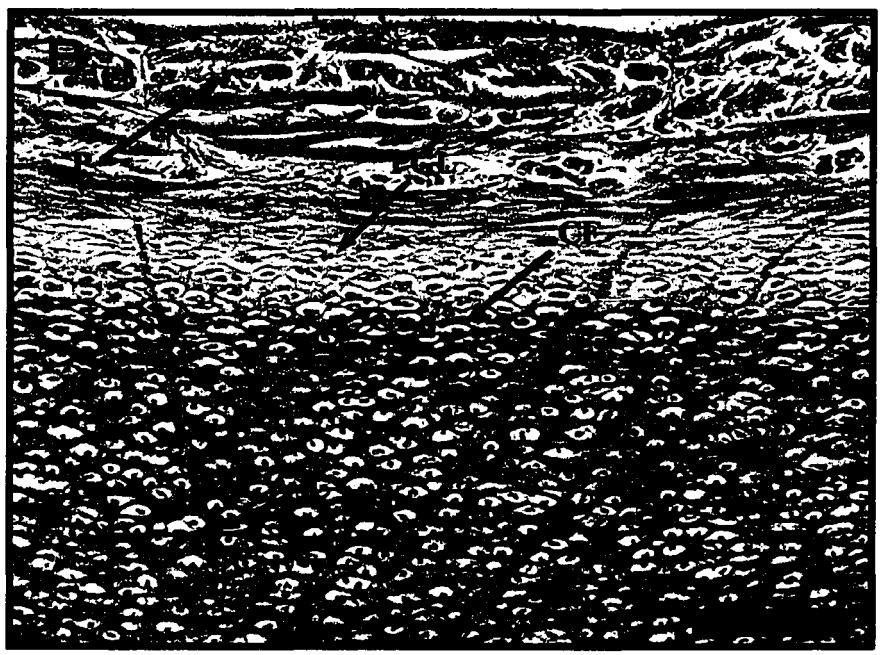
## RESULTS

### *Histologic features of vertebral growth*

As previously described by Clement (1992), histologic preparations of vertebral centra are characterized by multiple zones of localized activity (Fig. 1). The marginal edge of the centrum was bordered by a fibrous perichondrium, which gives rise to appositional growth. Below this envelope was a layer of round or flattened cells surrounded by an unmineralized matrix, which appeared to comprise the primary zone of cell proliferation and growth. Further in, cells were ovoid in shape and eventually

**Figure 2.1.** (**A**) Longitudinal section of vertebral cartilage from adult (400 mm disk width) *R. eglanteria*. Bar: 100 μm. (**B**) Higher magnification demonstrates the proliferation of chondrocytes from the perichondrium (P), forming the primary growth layer (PGL) where appositional growth occurs. Cells from this region are eventually incorporated into a consolidated calcifying front (CF). Several cartilage canals (C) extend from the perichondrium into the vertebral mass. Bar: 100 μm.





were incorporated into the mineralizing front. Once in this region, cells appeared shrunken with pyknotic nuclei, likely due to inadequate fixation of these densely mineralized areas (Clement, 1992). Small canals extended from the perichondrial tissue deep into the cartilaginous mass in a seemingly randomized manner.

Despite limited collection of winter samples, seasonal changes in vertebral growth were observed in histologic sections (Fig. 2). From late spring through summer, perichondrial growth appeared rapid based upon cellular activity of the proliferative zone. During this period, the zone contained densely packed, flattened cells, characteristic of active proteoglycan synthesis. In winter samples, sub-perichondrial growth was greatly reduced and largely comprised of small, round chondrocytes. These cells contained high nucleocytoplasmic ratios and appeared to be in the earliest stage of cytodifferentiation. Typically, cells in this stage have yet to express high capacities for matrix biosynthesis (Oohira *et al.*, 1974).

***Proteoglycan content***

Proteoglycan content was high in the proliferative zone, which was thoroughly stained black by the HID method (Fig 3a). Below this layer, proteoglycan concentration decreased considerably and was largely restricted to pericellular regions (Fig. 3b). A "layered" distribution of proteoglycans, akin to rhythmic growth zones, was observed in some preparations (Fig. 3c), and appeared to be related to cell concentration.

**Figure 2.2.** Appositional growth of the vertebral margin (i.e. primary growth layer) in adult (400-420 mm disk width) *R. eglanteria*, collected in (**A**) January, (**B**) April, (**C**) May, (**D**) June (**E**) August and (**F**) December. Cellular activity and marginal growth (bracket) is high during late spring-late summer, yet decreases considerably from winter-early spring. Arrow denotes calcifying front. H&E stain. Bar: 100 μm.

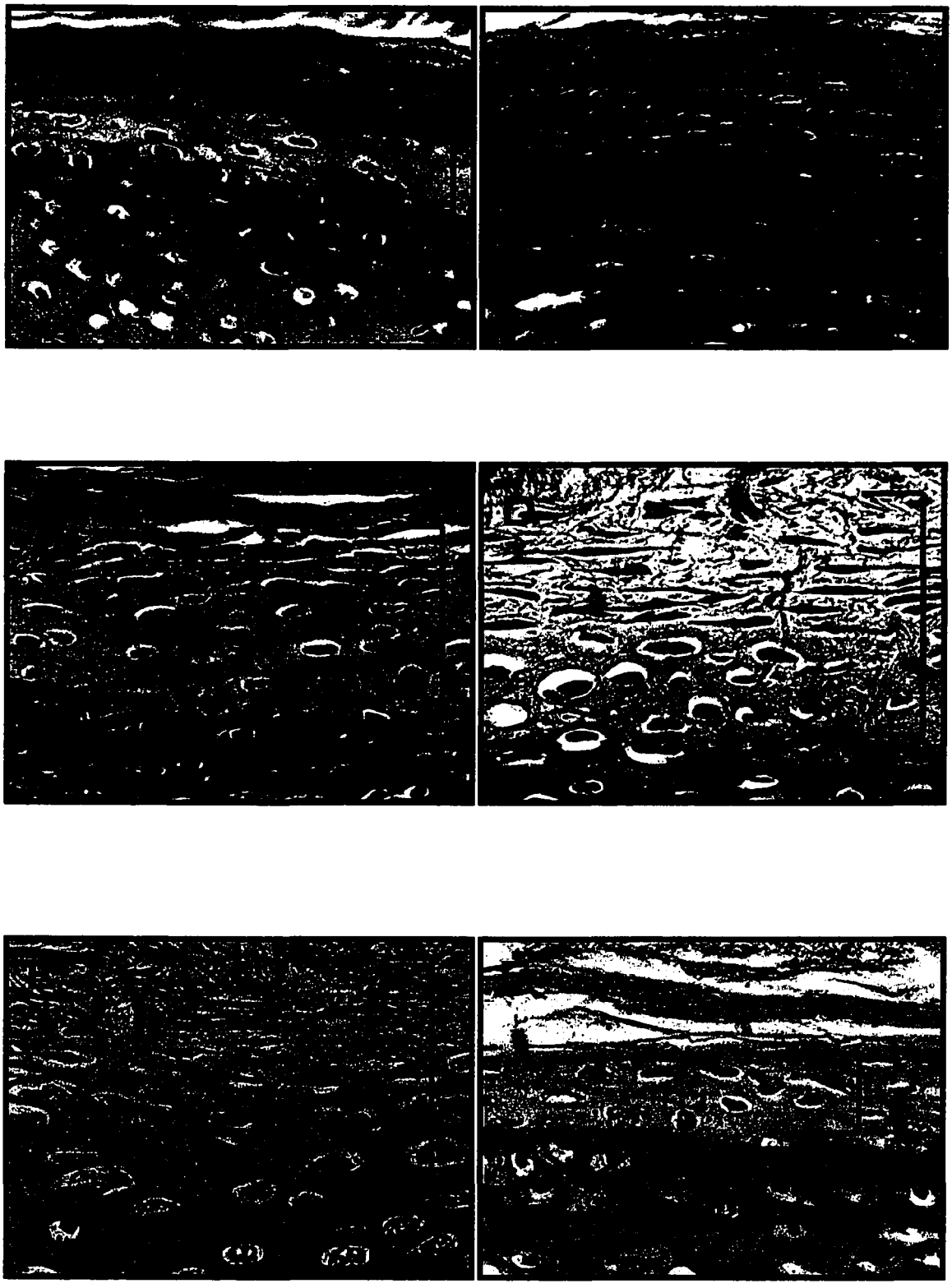
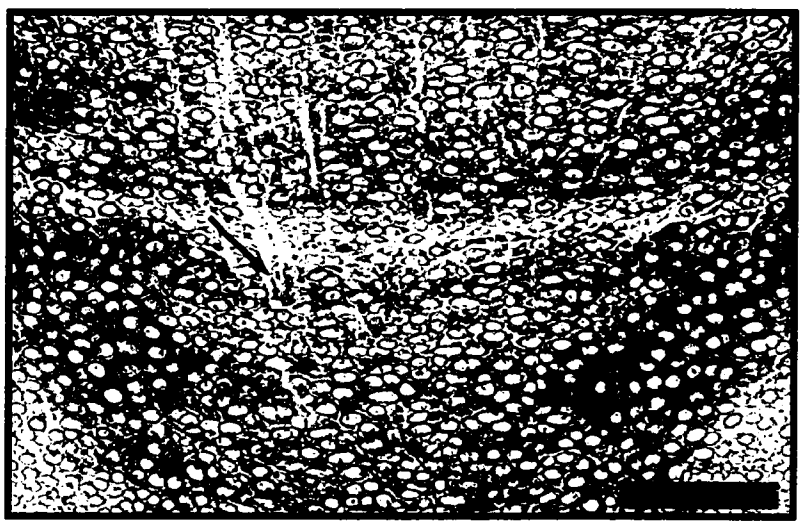


**Figure 2.3.** (**A**) Longitudinal section of vertebral margin from adult (410 mm disk width) *R. eglanteria*, demonstrating high regional concentration of proteoglycan matrices (arrow). P: perichondrium. Bar: 100 μm. (**B**) Higher magnification demonstrates the pericellular localization of proteoglycan matrices (arrow) in mature, calcified regions of vertebral cartilage. Bar: 100 μm. (**C**) Differential staining of vertebral growth layers illustrates the decline of proteoglycans in vertebral growth discontinuities, apparently due to decreased cell concentration. Bar: 100 μm. High-iron diamine stain.

***Scanning electron microscopy and energy-dispersive spectrophotometry***

Examination of vertebral centra by scanning electron microscopy demonstrates a continuous, mineralized matrix perforated by cellular lacunae that previously contained individual chondrocytes (Fig. 4a, b). Growth discontinuities are characterized by low cellular concentrations within this mineralized framework (Fig 4c). These zones are easily distinguished from neighboring growth layers, which contain substantially higher proportions of cells to matrix.

Elemental microanalyses conducted by EDS revealed that the chemical composition of vertebral centra was largely comprised of calcium, phosphorous, and oxygen (Fig. 5). Other elements including magnesium, sodium, chlorine and sulfur were also observed in low concentrations. Subsequent analyses focused on the distribution and concentration of the three major elements, yet sulfur was also examined in depth as it reflected proteoglycan content.

Temporal differences in the mineral concentration of vertebral margins were not significant (Fig. 6; nested ANOVA, $P > 0.05$). Therefore, seasonal changes in vertebral composition did not appear to reflect shifts in mineralization. Spatial variations in mineral composition across vertebral radii also demonstrated a relatively homogenous distribution (Fig. 7). Spot analyses of alternating growth zones clearly indicated that neither region could be empirically classified by mineral concentration (Fig. 8). However, spectral X-ray mapping of calcium indicated that regions with lower cell concentrations (i.e., growth discontinuities) may have more free space for mineral deposition (Fig. 9). Therefore, these increments appear to reflect changes in

**Figure 2.4.** (**A**) Longitudinal section of vertebral cartilage from mature (410 mm disk width) *R. eglanteria,* viewed using scanning electron microscopy. 70x. (**B**) Higher magnification of vertebral cartilage demonstrates a continuously mineralized matrix perforated by cellular lacunae (arrows). 1,000X. (**C**) Comparison of vertebral growth layers indicates that vertebral discontinuities (left) are characterized by low cellular concentration in comparison with wide growth increments (right). 500X.

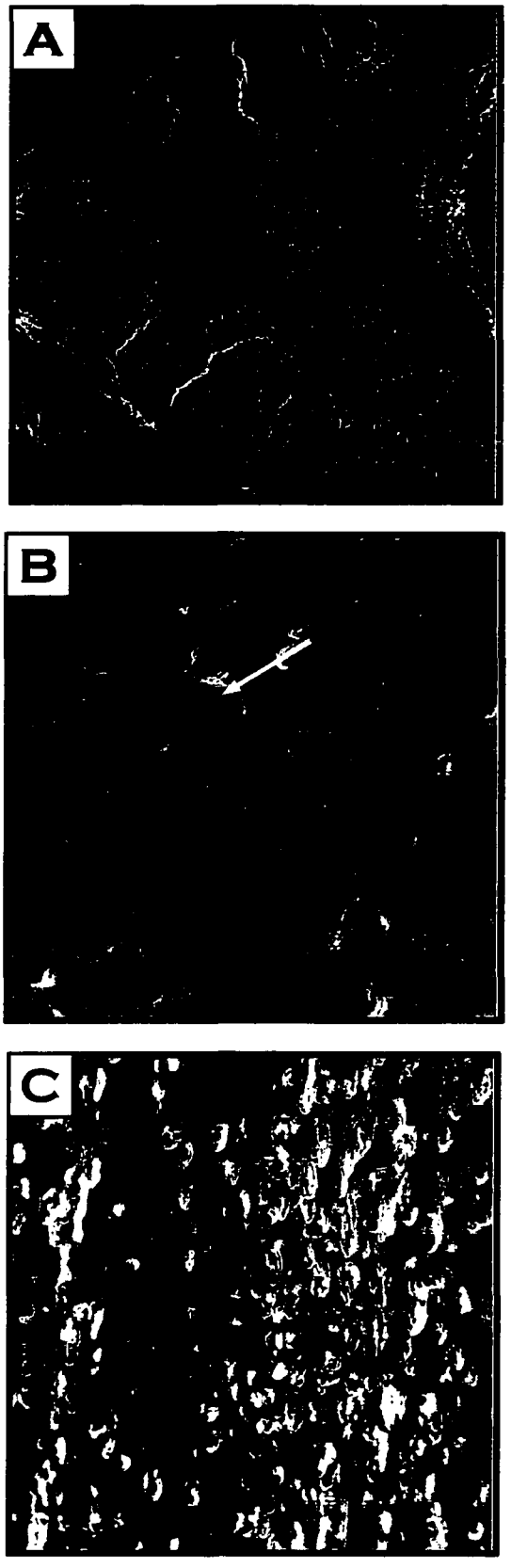


**Figure 2.5.** Sample energy spectrum of elements observed in vertebral sections of *R. eglanteria* using energy-dispersive spectrophotometry. High concentrations of oxygen (O), phosphorus (P), and calcium (Ca) reflect the distribution of hydroxyapatite. Carbon (C) and sulfur (S) spectra are also labeled.

**Figure 2.6.** Concentration of calcium (% weight) in the outer margin of vertebral centra from mature *R. eglanteria,* collected between April and December. Points represent means of 2-5 individuals each (10 scan points per vertebrae) $\pm$SEM. No differences in calcium concentration were detected by month of capture (nested ANOVA, $P<0.05$). Inset demonstrates the relative position of points (**X**) examined by energy-dispersive spectrophotometry. 500X.

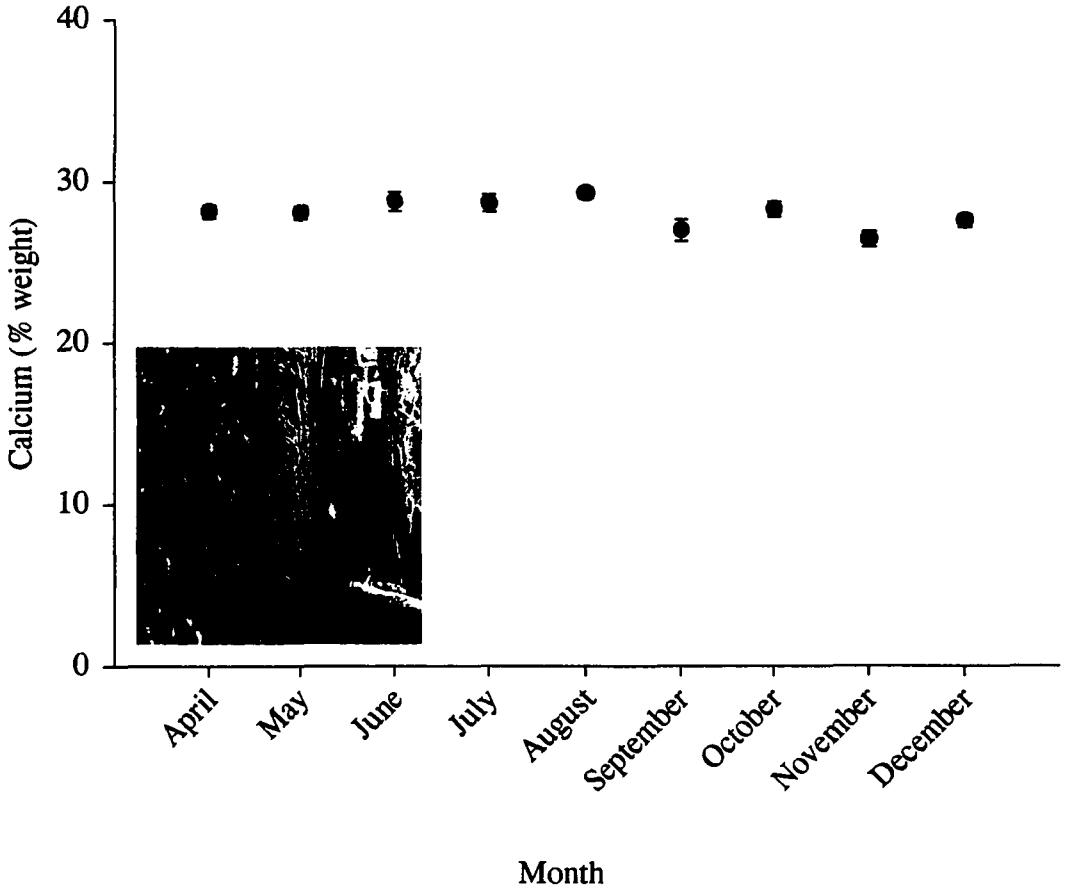
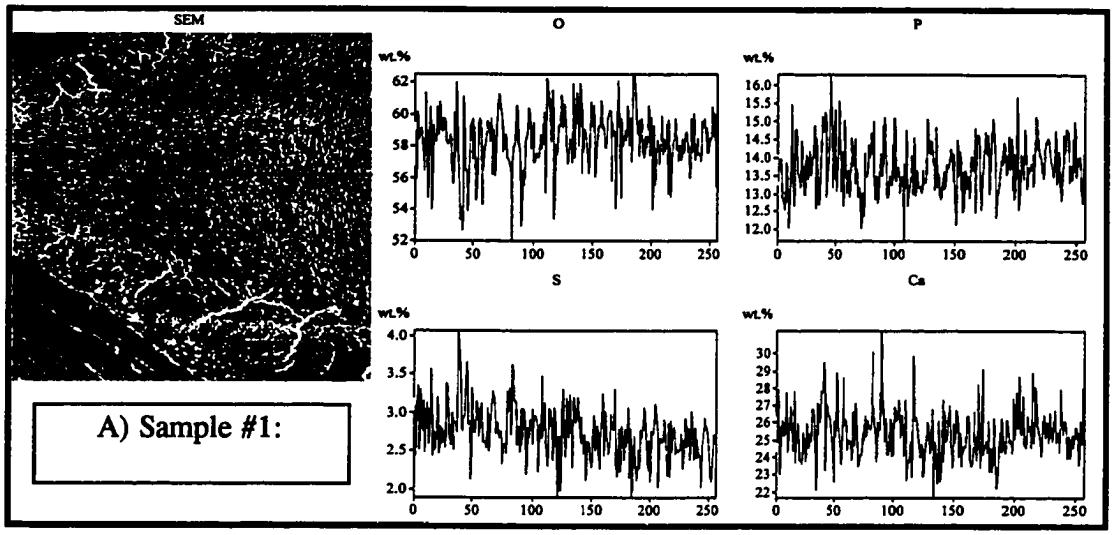
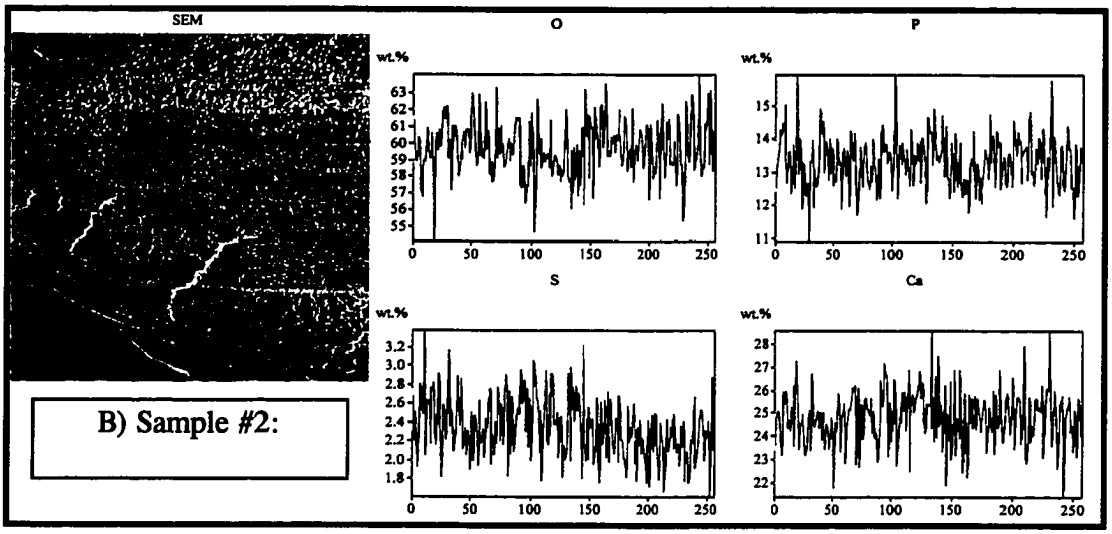


**Figure 2.7.** Three examples (**A-C**) of 256-point line scans across the radii of vertebral sections from mature *R. eglanteria*. Relative position of line scans are indicated by a solid black line crossing the central radius of each section (inset; 100X). Spatial concentrations (% weight) of calcium (Ca), phosphorus (P), oxygen (O), and sulfur (S) are relatively homogeneous, except for small disturbances due to sample topography (**C**, arrows). Line scans did not extend beyond the vertebral margin so that fine changes in elemental concentration could be detected.



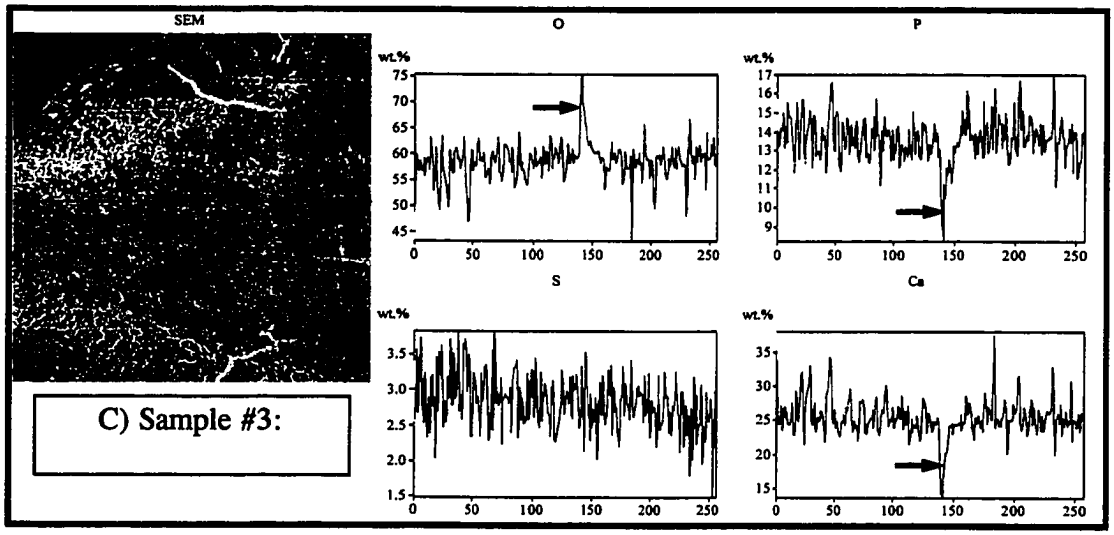



**Figure 2.8.** Qualitative comparison of sulfur (open circles) and calcium (closed circles) concentrations (% weight) in growth layers of three *R. eglanteria* vertebrae (**A-C**). Dissimilar patterns of mineral or matrix concentration were not detected between cell-poor (blue line) or cell-rich (red line) growth layers. Colored lines on graphs correspond to the position of individual scan points on micrographs (500X).

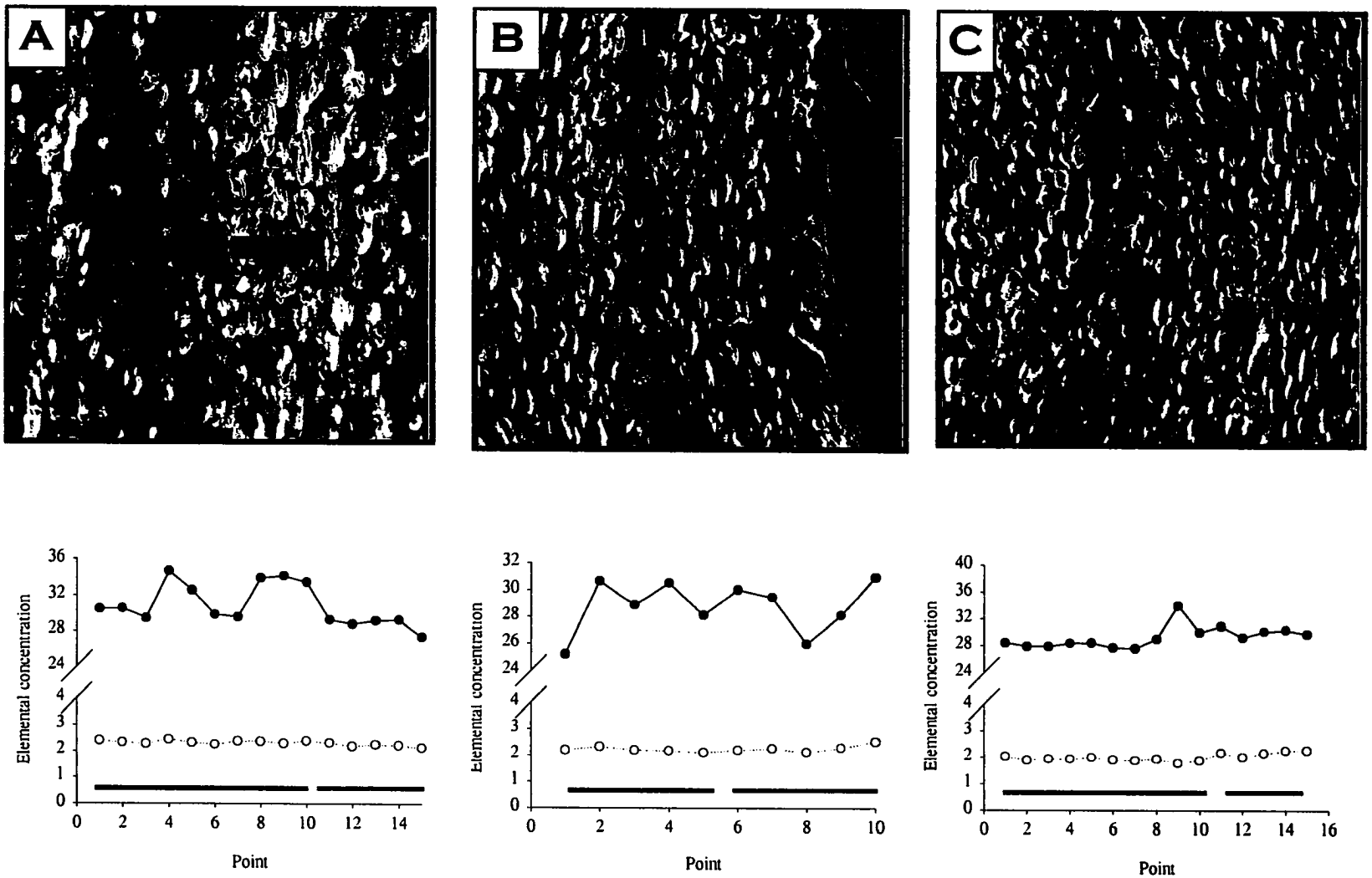
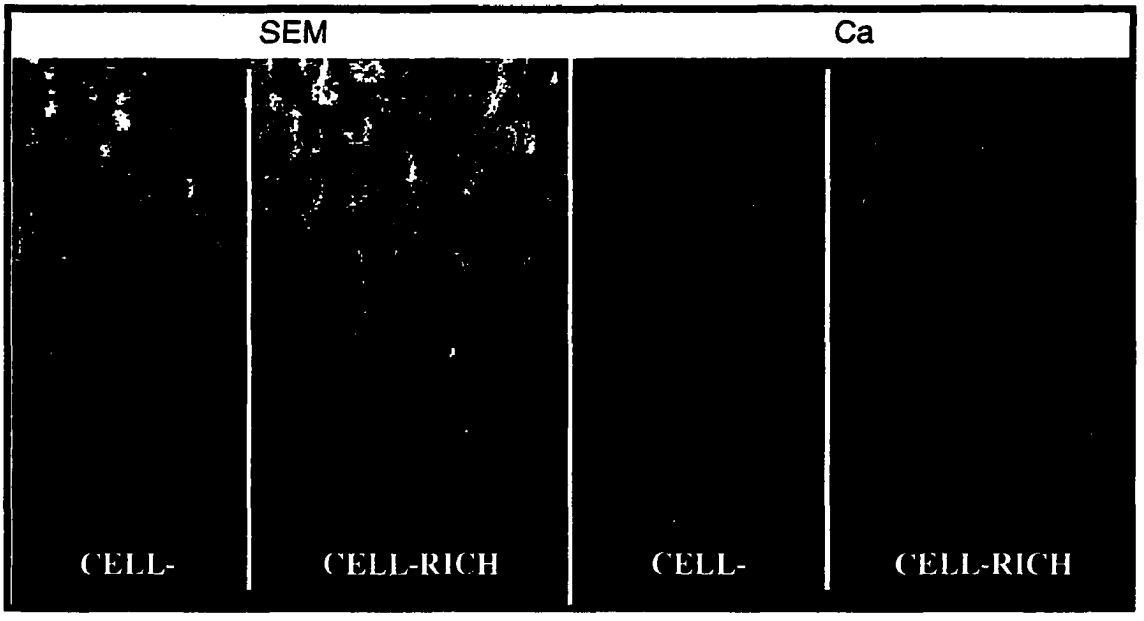


**Figure 2.9.** Spectral X-ray map (Ca) of calcium concentration and distribution in cell-poor (left) and cell-rich (right) growth layers of *R. eglanteria* vertebrae. No differences in calcium concentration are detected, but mineral distribution in cell-rich growth layers appears relatively patchy (arrows). SEM: image observed using scanning electron microscopy. 500X.



cellular content rather than in the degree of cartilage calcification. In all analyses, sulfur content also appeared relatively stable.

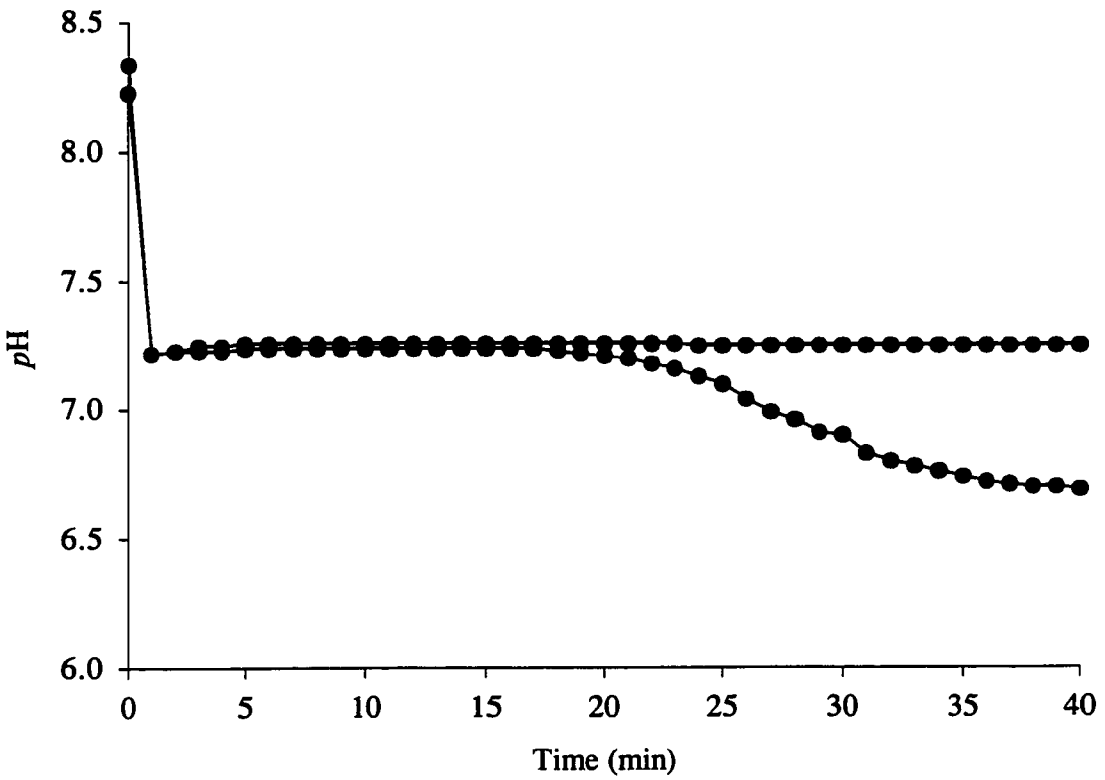
### *Effect of proteoglycan extracts on hydroxyapatite formation*

Crystal formation occurred at approximately 20 min in all control replicates, as indicated by a rapid decline in *p*H (Fig. 10). Precipitation continued for approximately 40 min after the addition of $CaCl_2$. Proteoglycan extracts decreased the rate of crystal formation and inhibited hydroxyapatite formation, as indicated by relatively stable *p*H values for the duration of the experimental period. Crystal formation was delayed for several hours in all experimental trials. However, small amounts of amorphous precipitate were observed under experimental conditions.

## DISCUSSION

The results of the present study suggest that alternating growth zones in the vertebral centra of *R. eglanteria* are a consequence of seasonal changes in appositional growth. In summer, growth of the vertebral outer margin is rapid and can be expected to produce a wide cartilaginous increment over the duration of this period. In contrast, growth of vertebrae during the winter appears reduced and is likely to result in the production of thinner strata. Observations by scanning electron microscopy support this conclusion by demonstrating that neighboring growth increments differ primarily in the proportion of cells to mineralized matrix. Clement (1992) observed similar

**Figure 2.10.** Mean decrease in *p*H with time associated with hydroxyapatite formation in control (black) replicates containing 2 ml distilled water or in experimental (red) replicates containing 2 ml of 12 $\mu$g/ml proteoglycan solution. Calcium chloride added at time 0. Treatment differs significantly from control (two-way ANOVA, $P<0.05$).



characteristics in centra of *Pristis pectinata*, thus this may be a common pattern of growth in elasmobranch vertebrae.

Significant differences in mineral concentration were not detected between the vertebral growth increments of *R. eglanteria*. This finding is contrary to common perceptions regarding the nature of vertebral calcification in elasmobranchs, and to mineralogical studies on certain species. For example, Cailliet and Radtke (1987) observed well-defined fluctuations in calcium and phosphorus across vertebral radii, which corresponded to visibly detectable growth bands in centra of *Alopias vulpinus* and *Carcharhinus amblyrhynchos*. Similarly, Clement (1992) found that mineralization in vertebrae of *Squatina squatina angelus* was restricted to discrete concentric lamellae, in between which lay rings of unmineralized tissue. However, as Clement (1992) has stressed, this represents only one extreme in the patterns of mineralization of elasmobranch vertebrae. In other species, mineralization may be continuous throughout the tissues of the centrum, as observed in *R. eglanteria*.

Despite the relative stability in calcium concentration across vertebral sections, it is likely that growth increments may rather be described on basis of mineral ***content***. As demonstrated by spectral X-ray mapping, mineral deposition in cell-rich growth zones appears patchy in distribution. A similar appearance may be expected in vertebral preparations following the use of staining techniques for the visualization of mineral deposits. Thus, it is reasonable to characterize alternating growth layers in the vertebrae of *R. eglanteria* as "highly" or "poorly" calcified, but only in regards to net

mineral deposition. As previously stressed, these differences appear to result from changes in cellular density.

The homogenous distribution of vertebral mineral precluded attempts to examine the spatial relationship between sulfur and calcium by EDS. However, histochemical analyses demonstrated that sulfated proteoglycans are largely restricted to unmineralized portions of centra, especially the proliferative zone. In mineralized tissue, proteoglycan staining is reduced and generally confined to discrete pericellular aggregations. Similar observations have been described for elasmobranch jaw cartilage (Takagi *et al.*, 1984; Edmonds *et al.*, 1996) and mammalian growth plate cartilage (Baylink *et al.*, 1972; Poole and Rosenberg, 1987), suggesting the necessary removal of proteoglycans for mineral deposition. In contrast, other studies have failed to detect a loss or, in fact, observed an increase in proteoglycan content in calcifying cartilage (Poole *et al.*, 1982; Shepard, 1992). In either case, both scenarios clearly suggest a role, albeit unclear, for proteoglycans in tissue mineralization.

In the current study, proteoglycan extracts from vertebral cartilage were effective inhibitors of hydroxyapatite formation *in vitro*. This effect was similar to that displayed by mammalian cartilage proteoglycans, as reported in earlier studies (Cuervo *et al.*, 1973; Blumenthal *et al.*, 1979; Dziewiatkowski and Majnerski, 1985). Although these findings suggest that proteoglycans may play an inhibitory role in cartilage calcification, there is substantial evidence to suggest that this conclusion may need to be re-evaluated. Such evidence is largely associated with the processes that lead to the

formation of hydroxyapatite from inorganic solutions, which has long been a topic of intense debate (Termine and Eanes, 1974).

The formation of hydroxyapatite crystals from high concentration calcium phosphate solutions typically occurs through a precursor phase (Termine and Eanes, 1974). In this process, an amorphous calcium phosphate precursor forms and then dissolves to allow mineral ions into solution (Blumenthal *et al.*, 1979). Afterwards, ions interact to form embryonic hydroxyapatite nuclei, which grow to a critical size through accretion. Through additional accretion, critical nuclei eventually begin to form hydroxyapatite crystals.

Previous studies indicate that proteoglycans do not interfere with formation of the precursor stage and, in fact, may encourage it through calcium binding (Cuervo *et al.*, 1973; Blumenthal *et al.*, 1979). This is also supported by the presence of precipitate in the current study. Rather, proteoglycans appear to hinder the phase transformation from amorphous calcium phosphate to hydroxyapatite (Blumenthal *et al.*, 1979). This may occur through proteoglycan binding of (1) the amorphous phase, thus reducing the rate of dissolution and ion supply, or (2) embryonic hydroxyapatite nuclei, thus reducing growth to a critical size necessary for crystal formation (Cuervo *et al.*, 1973). In both cases, adsorption to hydroxyapatite precursors increases the activation energy barrier to the processes involved in mineral crystallization (Robertson, 1973).

Based upon this information, proteoglycans in elasmobranch vertebrae may initiate crystal formation by binding calcium and permitting the growth of amorphous

calcium phosphate (Poole and Rosenberg, 1987). Once mineral precursors form, they are kept in stasis through matrix binding. As chondrocytes mature, a reduction in matrix synthesis reduces local proteoglycan content and allows crystal formation to proceed. Alternatively, an increase in matrix degradation by proteolytic enzymes may also allow mineral precursors to undergo crystalline conversion. Thus, as observed in *R. eglanteria*, vertebral tissue is destined to calcify once it approaches the mineralizing front. In this model, proteoglycans are mainly responsible for guiding appositional growth and preparing conditions that will encourage mineralization. However, in species with mineral-variable growth layers (i.e. *Squatina squatina angelus*), persistence of widespread matrix synthesis may restrict calcification to discrete concentric lamellae.

In summary, results from the present study suggest that vertebral growth layers in *R. eglanteria* form due to seasonal changes in appositional growth rather than mineralization. This observation has implications for investigations on the physiological factors that influence these processes. Clearly, such studies should utilize methods that reflect vertebral growth, such as rates of cell proliferation or synthesis of proteoglycan matrices. Chapter 5 discusses the use of such methods, as a means to examine hormonal regulation of vertebral growth.

# Chapter 3

## Vertebral ageing and age validation in *Raja eglanteria*

# INTRODUCTION

As suggested in Chapter 2, seasonal changes in appositional growth produce increments in the vertebrae of *R. eglanteria*, which are often perceived on basis of mineral content. Daiber (1960) first described these increments as "opaque white rings alternating with gray translucent rings", and suggested their utility in age determination. Later, Berry (1965) estimated growth rate of this species based upon vertebral rings in an unpublished (M. S.) Thesis. Nonetheless, little published information is available for the growth of *R. eglanteria*.

A limitation of both Daiber's (1960) and Berry's (1965) investigations is the lack of age validation, an essential component of all age and growth studies (Beamish and McFarlane, 1983). One way of accomplishing this process is by administering chemical markers, which deposit at sites of active calcification and produce fluorescent or pigmented marks in calcified structures. The location of these marks can then be examined to determine if the structure in question accurately records age. A variety of chemicals including oxytetracycline (OTC; Yamada, 1971; Hettler, 1984), calcein (2,4-bis-[*N*,*N*'-di(carbomethyl)-aminomethyl] fluorescein; Yamada, 1971; Wilson *et al.*, 1987; Hales and Hurley, 1991; Monaghan, 1993; Brooks *et al.*, 1994; Bumguardner and King, 1996), calcein blue (Brooks *et al.*, 1994), and alizarine complexone (Lang and Buxton, 1993; Ahrenholz *et al.*, 1994) have proven effective in validating age estimates derived from teleost otoliths. At the beginning of this study, only OTC had

been used to validate growth zone periodicity in elasmobranch vertebrae (Holden and Vince, 1973; Smith, 1984; Branstetter, 1987b; Brown and Gruber, 1988; Tanaka, 1990; Kusher *et al.*, 1992; Natanson, 1993). No published studies had examined the effectiveness of alternative chemical markers in elasmobranch age validation until recent observations by Gelsleichter *et al.* (1997) and Officer *et al.* (1997).

Despite widespread use, chemical marking may have detrimental effects on the health or growth of treated organisms. If so, the value of these chemical probes in age validation may be severely compromised. Chemical toxicity associated with OTC treatment, in particular, has been well documented in several ageing studies. For example, injection of or immersion in OTC at recommended dosage levels (Beamish and McFarlane, 1983) have been reported to produce lethargy, behavioral abnormalities, and mortality in certain teleosts (Schmitt, 1984; Wilson *et al.*, 1987; Marking *et al.*, 1988; Monaghan, 1993; Bumguardner and King, 1996). In contrast, Tanaka (1990) reported that various dosage levels (20-80 mg/kg body weight) of OTC did not significantly affect growth rates of injected Japanese wobbegongs (*Orectolobus japonicus*) or swell sharks (*Cephaloscyllium umbratile*). Unfortunately, no other studies have investigated the possible implications of chemical marking in elasmobranchs and, thus, they remain largely unclear.

In the present chapter growth rate of *R. eglanteria* was re-evaluated, based upon verified age estimates derived from vertebral analyses. In addition, the effectiveness of calcein as a chemical marker for elasmobranch age validation was determined. Finally, the toxicity of calcein and OTC on the growth rate of captive *R. eglanteria* was

investigated. These studies were conducted in conjunction with additional observations in the nurse shark, *Ginglymostoma cirratum*, which were recently described in Gelsleichter *et al.* (1997; 1998).

## MATERIALS AND METHODS

### *Age and growth*

Clearnose skates were collected from various sites in Chesapeake Bay and northwest Atlantic Ocean by longline (Musick *et al.*, 1993) and bottom trawl. Each specimen was sexed and measured in disk width (DW) and total length (TL) shortly following capture. Vertebral centra were sampled from the central region of the disk, where DW is at its maximum. Centra were frozen or preserved in 70% ethanol (ETOH), and stored until further processing.

Several vertebrae from each specimen were prepared for age determination. Centra were cleared of extraneous tissue, fixed in 70% ETOH for a maximum of 24 h (if frozen), and air-dried. Dried vertebrae were mounted on cardboard with thermoplastic cement and sectioned through the central longitudinal axis with a high-speed rotary saw. Vertebral sections were affixed to microscope slides using Permount, and polished with wet sandpaper (250-600 grit) to a thickness of approximately 0.5-1.0 mm. Vertebral growth patterns were examined under a binocular dissecting microscope using transmitted light.

Vertebral radius (VR) was measured as a straight line from the central focus to the outer margin of the corpus calcareum (Fig. 1). The relationship between VR and DW was characterized by regression to determine if VR grew with body size and could be used for estimating growth rate.

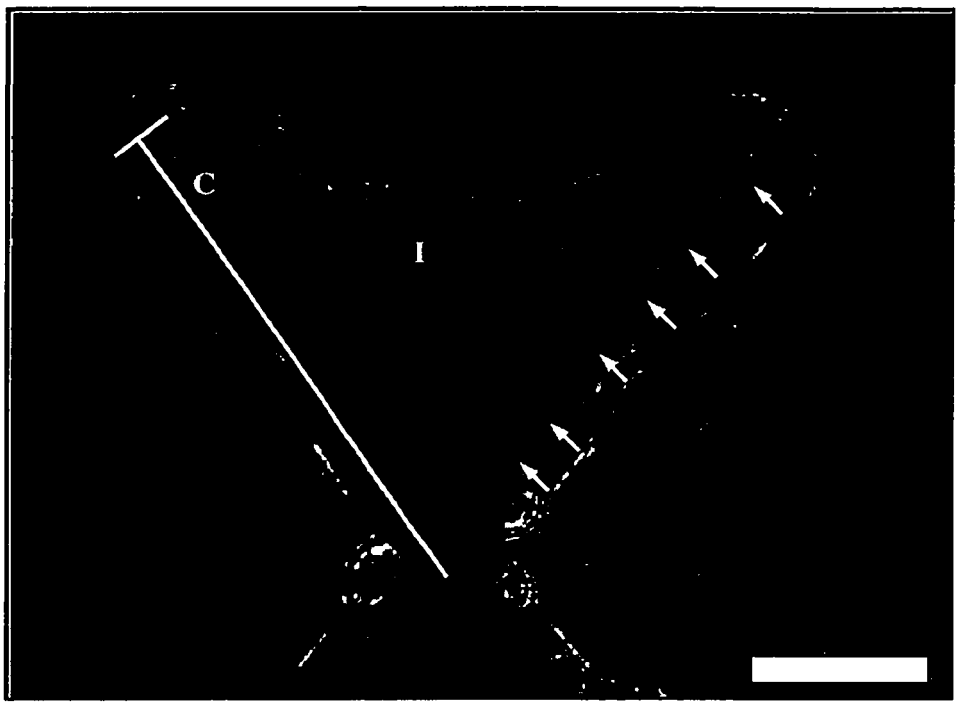
Vertebral growth patterns were similar to those described in Daiber (1960) and Berry (1965). Observation of thin sections revealed two types of vertebral increments: broad bands, which appeared to represent periods of rapid accretion, followed by narrow growth layers (Fig. 1). Narrow layers (hereafter called "growth rings") were enumerated to determine the age of individual skates. Growth rings were counted several times until consensus was reached. Results of the first two ageing trials, which occurred 6 months apart, were compared to assess reader precision using the index of average percent error (IAPE):

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

where $N$ = the number of fish aged; $R$ = the number of times each fish is aged; $X_{ij}$ = the $i$th age determination for the $j$th fish; and $X_j$ = the average age calculated for the $j$th fish (Beamish and Fournier, 1981).

Verification of age estimates derived from vertebral counts was accomplished by examining vertebral growth and increment formation in *R. eglanteria* of known age (7-10 months), which were obtained from Mote Marine Laboratory's skate-rearing facility. In addition, centrum growth was investigated in young-of-the-year (YOY) *R.*

**Figure 3.1.** Longitudinal section of vertebral cartilage from a male, 390 mm DW skate. Line demonstrates the measurement of vertebral radius along the surface of the corpus calcareum (C). Six vertebral rings are labeled on the right margin. The first ring corresponds to a hatching mark. I: intermedialia. Bar: 1.0 mm.



*eglanteria* injected with fluorescent chemical markers, in conjunction with studies on the effectiveness and toxicity of these compounds discussed later in this chapter.

Because certain size groups were not well represented in the sample, back-calculations were performed to estimate size at age. Like VR, the distance between the centrum focus to individual growth rings was measured along the corpus calcareum (Fig. 1). Back-calculations were performed by the Dahl-Lea method (Carlander, 1969):

$$DW_i = r_i(DW / VR)$$

where $DW_i$ = disk width at ring $i$ ($r_i$), $DW$ = observed disk width at capture, and $VR$ = vertebral radius. Mean back-calculations for DW at age were determined for males and females separately, and for sexes combined.

von Bertalanffy growth functions (VBGF) were fitted to size-at-age data for male, female, and combined sexes data sets using the software program *Sigmaplot* (Jandel Scientific), which implements Marquardt's (1963) algorithm for least-square estimation of non-linear parameters. The modified model (von Bertalanffy, 1938) is:

$$DW_t = DW_\infty\left(1 - e^{-k(t-t_0)}\right)$$

where $DW_t$ = predicted disk width at time $t$; $DW_\infty$ = mean asymptotic disk width; $K$ = VBGF growth rate constant; and $t_0$ = theoretical age at which the fish would have been zero DW.

***Effectiveness and toxicity of chemical markers***

The effectiveness and toxicity of calcein were examined in captive, newly hatched *R. eglanteria*. The toxicity of OTC, the standard chemical marker for elasmobranch age validation, was also assessed. Twelve juvenile (1-3 weeks old) *R. eglanteria* were sexed, measured, marked with identification, and partitioned into three groups: a control group ($N$ = 3, mean DW ($\pm$SE) = 102 $\pm$ 1.53 mm), an OTC group ($N$ = 5, mean DW ($\pm$SE) = 99.72 $\pm$ 1.16 mm), and a calcein group ($N$ = 4, mean DW ($\pm$SE) = 102.75 $\pm$ 2.56 mm). Control group skates received injections of 50 $\mu$l elasmobranch Ringer's solution (e-Ringers; Forster *et al.*, 1972) in the dorsal musculature. Tréatment group skates received injections containing either 5 mg calcein or 25 mg OTC per kg body weight (BW). Dosage levels for chemical markers were selected based upon Beamish and McFarlane's (1983) recommendations, and earlier observations on calcein by Gelsleichter *et al.* (1997). Each group was maintained for 12 weeks in closed-system aquaria, subjected to constant temperature (19°C) and photoperiod regimes (12L: 12D). Skates were fed three times per week *ad libitum* with shrimp or miscellaneous cutfish.

During the experimental period, all skates were measured on a weekly basis and examined for external lesions. Specimens that survived to the end of the experimental period were eventually anesthetized with 1 g/l tricaine methanesulfonate (MS-222) and sacrificed by severing the spinal cord. Necropsies were performed to observe pathological abnormalities. Vertebral centra from marked animals were sectioned longitudinally and examined for localization of chemical markers using blue (470 nm)

and short-wave ultraviolet (UV; 254 nm) light to detect fluorescence. Growth of chemically marked skates (%DW $d^{-1}$) was compared with the control group and a historical database for *R. eglanteria* maintained in the same aquarium system.

## RESULTS

### *Age and growth*

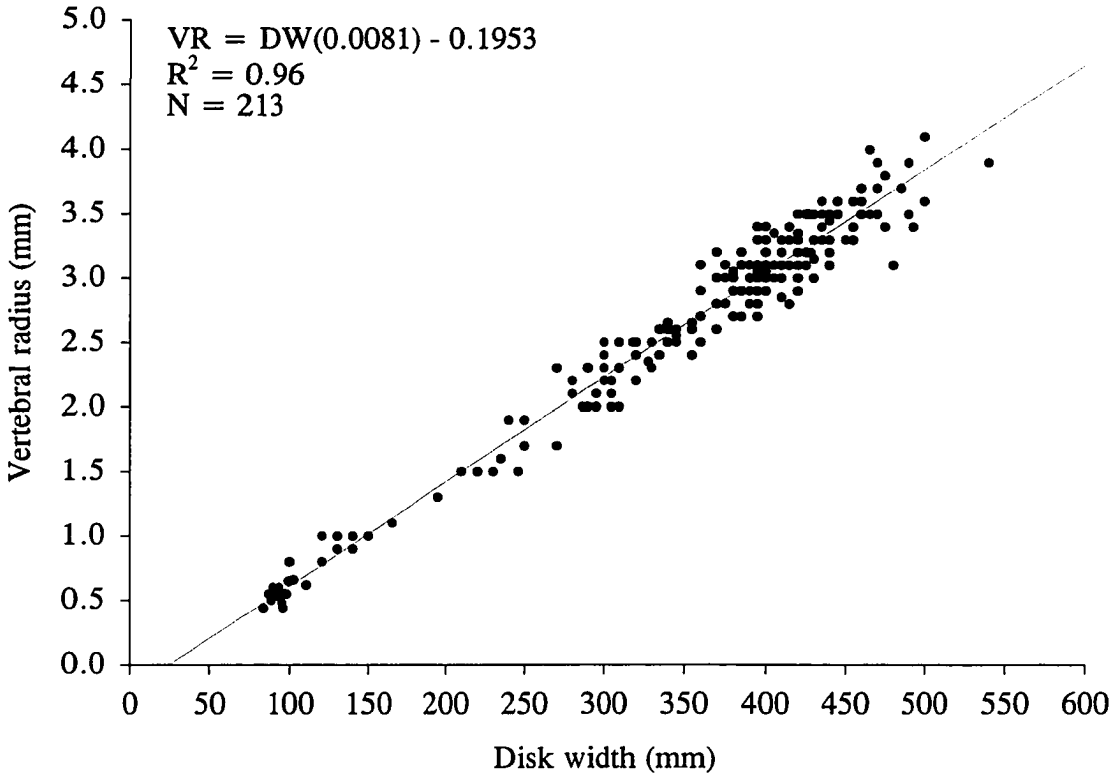
Vertebral centra were sampled from 104 male and 109 female *R. eglanteria*, ranging in size from 83-460 and 86.4-540 mm DW, respectively. Females larger than 500 mm DW have not been reported in earlier ageing studies (Fitz and Daiber, 1963; Berry, 1965), thus this range appeared to encompass most possible size and age groups. As in Berry's (1965) study, skates between 150-200 mm DW were rarely collected and only comprised a small portion of the overall sample. Despite this, there were no obvious gaps in the data that would suggest the absence of any single age class.

The relationship between VR and DW was linear and significant (Fig. 2), thus supporting the use of vertebrae for estimating growth rate. Linear relationships for separate sexes are described by the following equations:

$$\text{Males: } VR = 0.00817 \times DW - 0.20 \ (N = 104, \ r^2 = 0.96, \ P < 0.0001)$$
$$\text{Females: } VR = 0.00804 \times DW - 0.18 \ (N = 109, \ r^2 = 0.96, \ P < 0.0001)$$

There were no significant differences between sexes (ANCOVA, $P < 0.05$), thus data were combined to yield the relationship:

**Figure 3.2.** Relationship between vertebral radius (VR) and disk width (DW) for 213 (combined sexes) *R. eglanteria*.



$$VR = 0.0081 \times DW - 0.19 \quad (N = 213, r^2 = 0.96, P < 0.0001)$$

Examination of vertebrae from year-old *R. eglanteria* demonstrated the presence of two narrow increments or growth rings (Fig. 3). The first of these rings occurred at approximately 0.6 mm from the focus and was present in nearly all specimens, including young-of-the-year. Back-calculation of size at this ring (80.5 mm DW male, 80.8 mm DW female) indicated that it formed close to the time of hatching (late spring-summer). Comparison of vertebrae from hatchling and yearling skates indicated that the second ring formed between late fall and late winter, at approximately 140-150 mm DW. Thus, this ring appears to represent arrested growth of vertebral centra during this period. A skate hatchling that was maintained in captivity until January of the following year (6 months) grew to 145 mm DW, supporting this proposed scenario. No other growth rings were observed in vertebrae of yearling skates, thus narrow increments appear to form on an annual basis. Hereafter, narrow growth rings used for age determination are termed "annuli."

Annulus counts were relatively consistent as demonstrated by an IAPE of 2.5. The same age was determined in 79% of paired readings and 100% agreed within one year. As observed by Berry (1965), vertebrae from mid-sized specimens (280-350 mm DW) were often unclear and required additional reading. Nevertheless, consensus was reached for all within four ageing trials.

*Raja eglanteria* appears to grow rapidly over a relatively short life span (Tables 1-3). Estimated sizes for younger fish were slightly smaller when calculated from older

**Figure 3.3. (A)** Longitudinal section of vertebral cartilage from a 10 month old, male *R. eglanteria*. Two narrow increments (growth rings, annuli) are present at 0.6 and 1.0 mm (arrows). Bar: 0.5 mm. **(B)** Higher magnification demonstrates the first winter annulus (arrow). Bar: 0.25 mm.

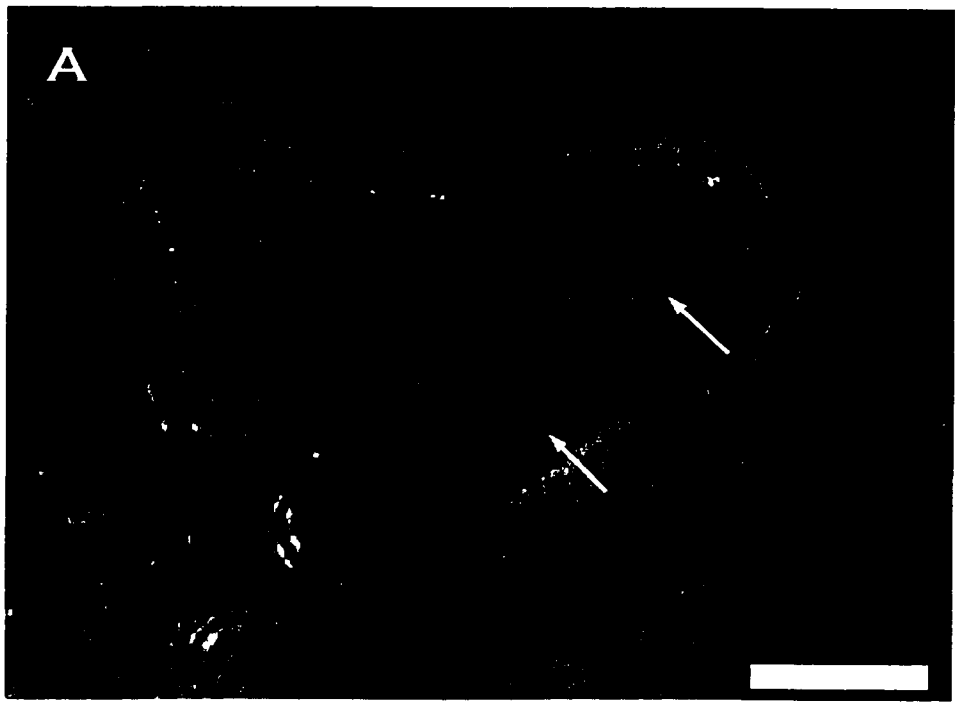



**Table 1. Back-calculated disk width (mm) of male clearnose skates H: hatching ring.**

| | | Mean back-calculated disk width at successive bands | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Age group | *N* | H | 1 | 2 | 3 | 4 | 5 | 6 |
| 0 | 14 | 85.02 | | | | | | |
| 1 | 1 | 88.0 | 161.33 | | | | | |
| 2 | 1 | 86.1 | 143.5 | 229.6 | | | | |
| 3 | 12 | 85.71 | 147.45 | 222.49 | 285.54 | | | |
| 4 | 20 | 80.21 | 144.21 | 209.81 | 275.29 | 330.55 | | |
| 5 | 49 | 78.40 | 143.88 | 204.89 | 267.26 | 325.88 | 375.17 | |
| 6 | 7 | 76.78 | 144.29 | 206.59 | 261.34 | 321.63 | 376.48 | 413.93 |
| Mean | 104 | 80.54 | 144.65 | 208.78 | 271.11 | 326.72 | 375.33 | 413.93 |

**Table 2. Back-calculated disk width (mm) of female clearnose skates. H: hatching ring.**

| | | Mean back-calculated disk width at successive bands | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Age group | *N* | H | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 0 | 12 | 86.55 | | | | | | | | |
| 1 | 2 | 87.0 | 152.0 | | | | | | | |
| 2 | 7 | 88.11 | 142.12 | 217.42 | | | | | | |
| 3 | 18 | 82.01 | 141.65 | 208.22 | 271.51 | | | | | |
| 4 | 6 | 82.01 | 141.11 | 207.24 | 275.33 | 337.03 | | | | |
| 5 | 21 | 80.65 | 140.08 | 205.46 | 273.33 | 332.88 | 383.71 | | | |
| 6 | 30 | 77.94 | 139.16 | 199.51 | 264.42 | 326.44 | 373.04 | 412.34 | | |
| 7 | 11 | 76.80 | 139.36 | 199.04 | 261.85 | 320.12 | 368.09 | 408.65 | 445.85 | |
| 8 | 2 | 78.12 | 143.23 | 195.31 | 260.41 | 325.52 | 377.60 | 416.66 | 455.72 | 484.81 |
| Mean | 109 | 80.80 | 140.53 | 204.14 | 268.33 | 328.26 | 375.83 | 411.60 | 447.37 | 484.81 |

**Table 3. Back-calculated disk width (mm) of clearnose skates (sexes combined). H: hatching mark.**

| | | Mean back-calculated disk width at successive bands | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Age group | *N* | H | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 0 | 26 | 85.73 | | | | | | | | |
| 1 | 3 | 87.33 | 155.11 | | | | | | | |
| 2 | 8 | 87.86 | 142.29 | 218.95 | | | | | | |
| 3 | 30 | 82.69 | 143.97 | 213.93 | 277.12 | | | | | |
| 4 | 26 | 80.63 | 143.49 | 209.22 | 275.30 | 332.05 | | | | |
| 5 | 70 | 79.07 | 142.74 | 205.06 | 269.08 | 327.98 | 377.73 | | | |
| 6 | 37 | 77.72 | 140.13 | 200.85 | 263.83 | 325.53 | 373.69 | 412.64 | | |
| 7 | 11 | 76.80 | 139.36 | 199.04 | 261.85 | 320.12 | 368.09 | 408.65 | 445.85 | |
| 8 | 2 | 78.12 | 143.23 | 195.31 | 260.41 | 325.52 | 377.60 | 416.66 | 455.72 | 484.81 |
| Mean | 213 | 80.67 | 142.51 | 206.38 | 269.72 | 327.46 | 375.60 | 411.93 | 447.37 | 484.81 |

age groups, indicating the presence of a slight Lee's phenomenon. Initial growth was rapid, with both males and females growing approximately 60 mm in DW within the first six months (first winter annulus). Growth averaged 60 mm per year for the subsequent three years, then decreased to approximately 50 mm in the following year. Afterwards, growth slowed to approximately 35 mm $y^{-1}$ for the oldest age groups examined. The oldest male was aged to 5+ years and the oldest female, 7+ years.

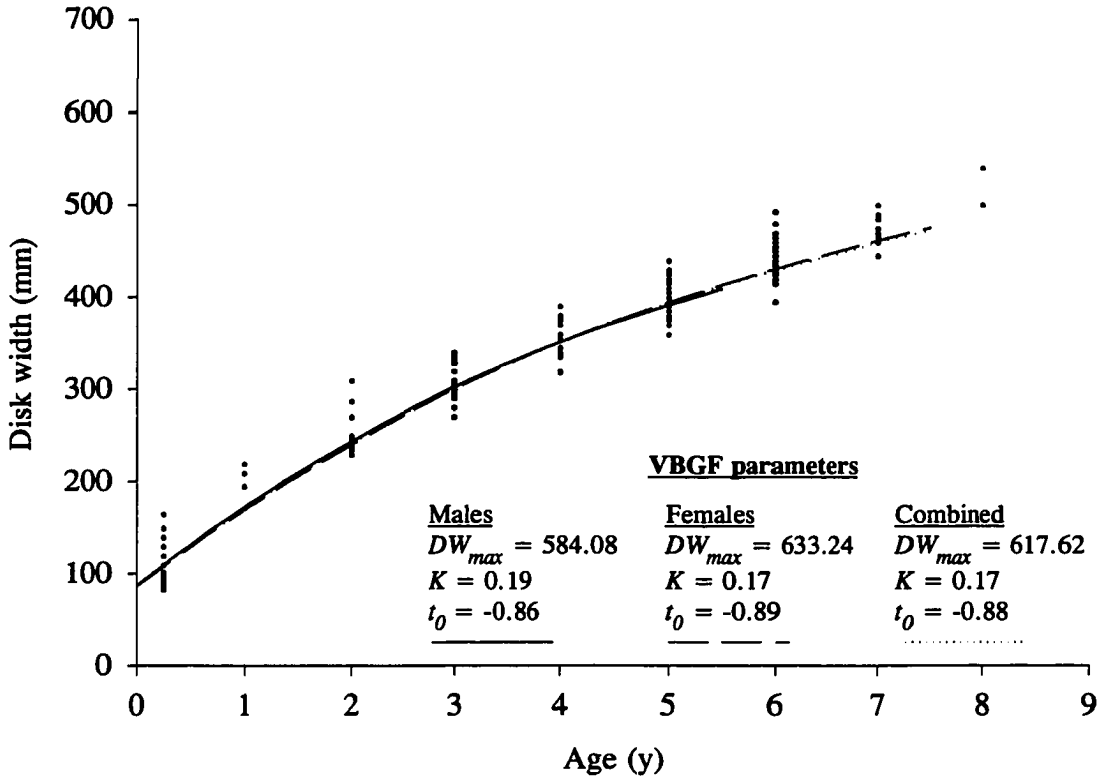
The von Bertalanffy growth function provided a reasonable fit to male, female, and combined data sets (Fig. 4). Estimates of VBGF parameters were as follows, males: $DW_\infty$ = 584.07 mm, $K$ = 0.19 $y^{-1}$, $t_0$ = -0.86; females: $DW_\infty$ = 633.24 mm, $K$ = 0.17 $y^{-1}$, $t_0$ = -0.89; combined sexes: $DW_\infty$ = 617.62 mm, $K$ = 0.17 $y^{-1}$, $t_0$ = -0.88.

***Effectiveness and toxicity of chemical markers***

Chemical treatment was associated with animal mortality during the experimental period. Two calcein-injected skates died within three weeks of treatment. Two OTC-injected skates also died rapidly, 5 d following injection. A third OTC-treated animal died approximately 50 d later. In all, 2 calcein- and 2 OTC-injected skates survived throughout the initial 12 week period. There was no mortality in the control group.

Lack of feeding typically preceded death in chemically treated specimens. No other behavioral abnormalities were observed in treatment or control group skates. No external lesions or abnormalities were apparent on any specimens. However, calcein

**Figure 3.4.** von Bertalanffy growth curves fitted to mean back-calculated size at age data for *R. eglanteria* (male, female and combined sex data sets). Points represent observed data. Estimates for parameters of the von Bertalanffy growth function (VBGF) are summarized.



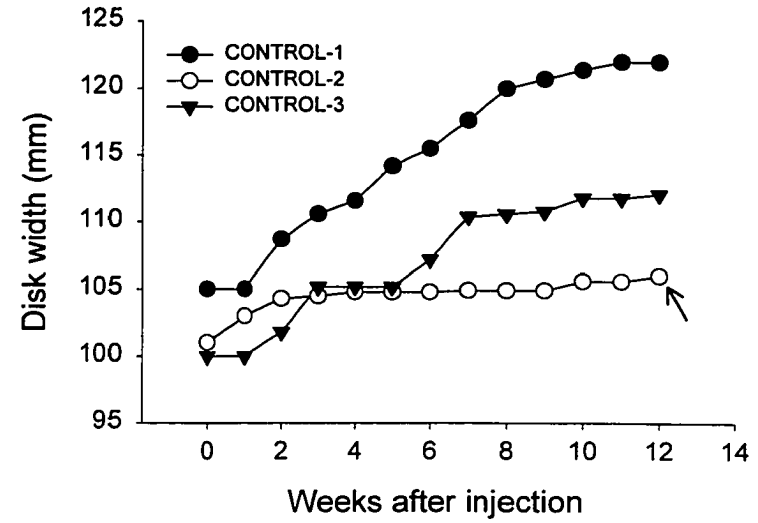
deposition occurred in the dermal spines of calcein-treated animals, which fluoresced bright green under exposure to UV light. Calcein was also detected in teeth of a calcein-treated skate that died 9 d after injection.

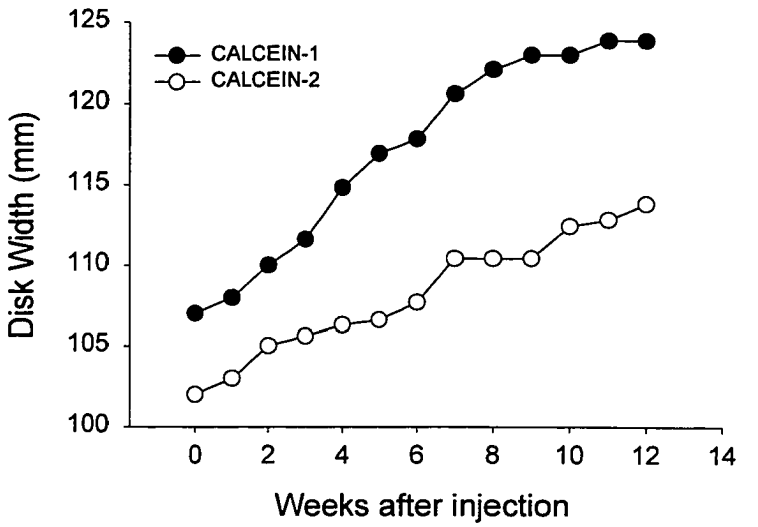
All skates grew in disk width during the experimental period (Fig. 5). One-way ANOVA revealed that there were no significant differences in growth rates between control and treatment groups ($P = 0.525$; Table 4). Growth of chemically treated individuals was also similar to unpublished data recorded for male and female *R. eglanteria* reared and maintained in the same aquarium system (Fig. 6). One OTC- and one control specimen grew significantly less than all other skates (t-test, $P<0.05$), yet this appeared to be unrelated to experimental manipulation. Natural differences in animal condition and survivorship commonly occur in the experimental aquarium system and may be expressed in certain individuals (C. A. Luer, Mote Marine Laboratory, pers. comm.).

Both chemicals produced distinct fluorescent marks in the vertebral centra of *R. eglanteria* (Fig. 7). As observed in earlier studies, marks produced by OTC were white under UV excitation. Marks produced by calcein were bright green, under exposure to blue or UV light. Fluorescent marks produced by both chemicals were suitable for age validation, yet calcein marks were substantially brighter and more persistent despite repeated viewing.

Although no chemically treated specimens survived beyond 3 months, fluorescent markers were still useful for characterizing the initial (summer) growth of vertebral cartilage. All specimens that survived past 2 weeks had dense, white bands

**Figure 3.5.** Growth of control and chemically-marked skates during a 12-week experimental period. Chemically-marked individuals received intramuscular injections of 50 $\mu$l elasmobranch Ringer's solution containing 5 mg calcein or 25 mg oxytetracycline (OTC) per kg body weight. Arrows denote two individuals that grew poorly during the experimental period.



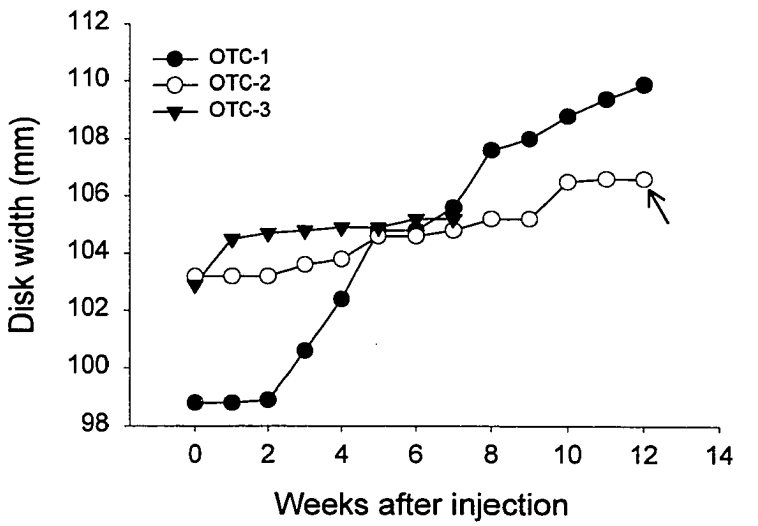
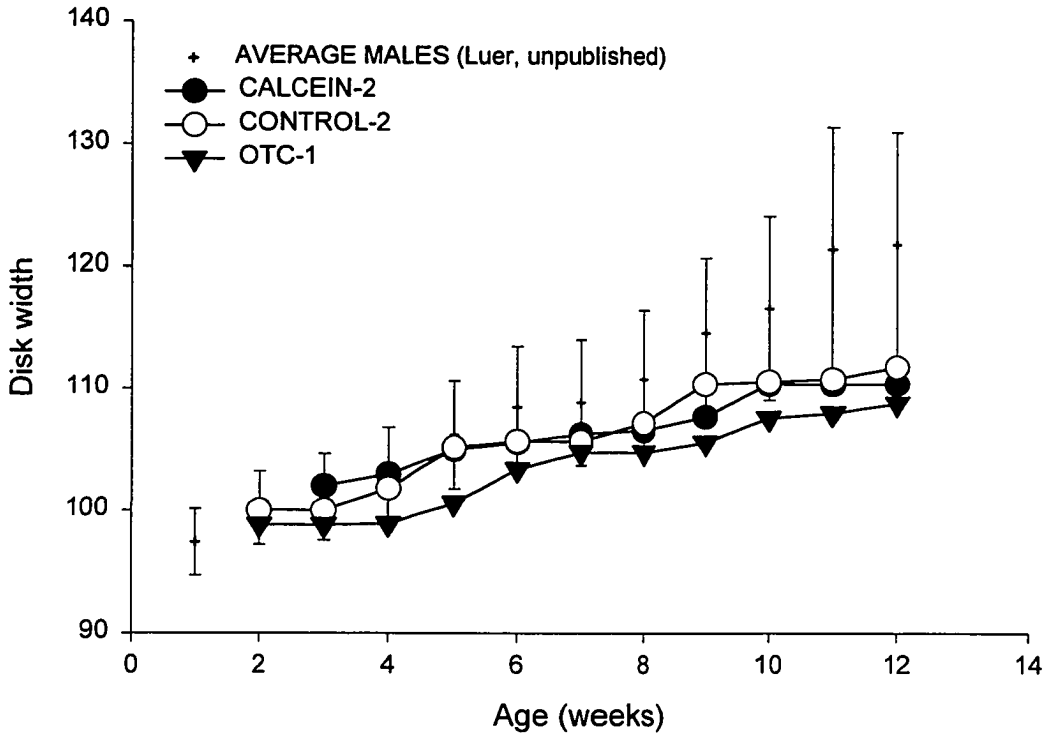



**Table 4. Growth rates (expressed as %DW $d^{-1}$) of clearnose skates in control and treatment groups. Disk width (DW) are in mm.**

| Group | *N* | Initial DW (Mean ±SE) | Final DW (Mean ±SE) | Growth rate (Mean ±SE) |
|---|---|---|---|---|
| Control | 3 | 102.0 ± 1.5 | 113.4 ± 4.7 | 0.12 ± 0.04 |
| Calcein | 2 | 104.5 ± 2.5 | 118.8 ± 5.0 | 0.15 ± 0.02 |
| OTC | 2 | 100.6 ± 2.6 | 108.2 ± 1.6 | 0.08 ± 0.05 |

**Figure 3.6.** Growth of control and chemically-marked skates compared with unpublished data on growth from the experimental aquarium system. Unpublished data is presented as means ± SD (obtained from C. A. Luer, Mote Marine Laboratory).



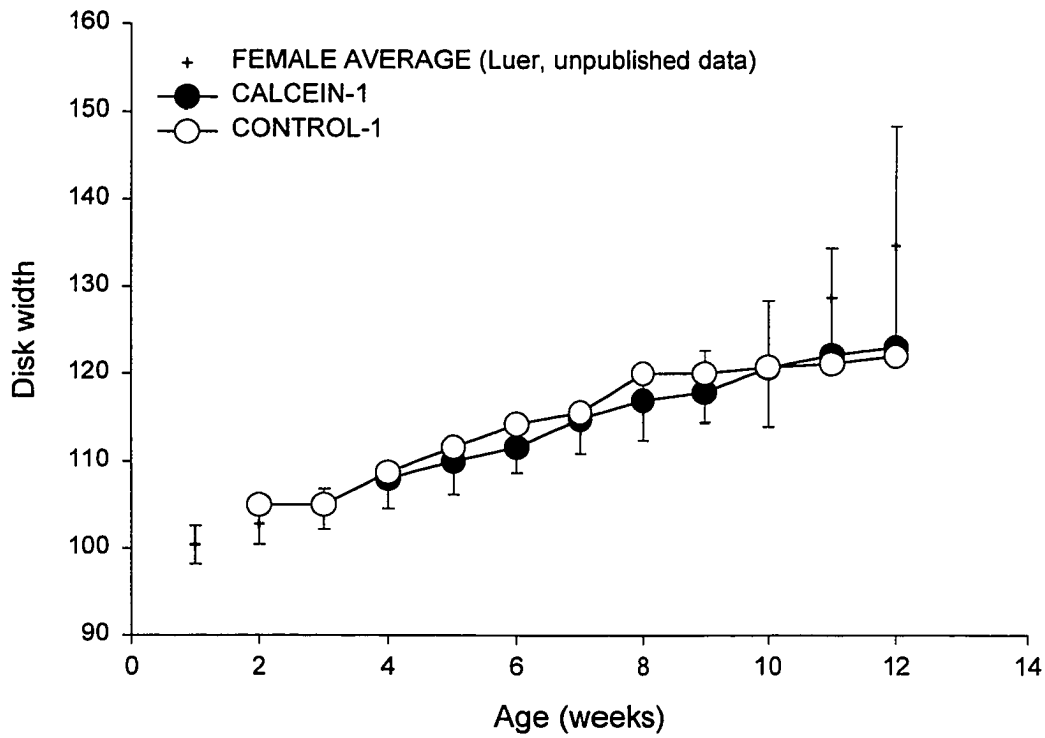



**Figure 3.7.** Longitudinal sections of vertebral centra from chemically-marked *R. eglanteria*. **(A)** Vertebrae from skate injected with 5 mg calcein per kg body weight (BW). Blue light illumination. Bar: 0.1 mm. **(B)** Vertebrae from skate injected with 25 mg OTC per kg BW. Short-wave UV illumination. Bar: 0.1 mm. Arrows denote fluorescent chemical marks.


deposited between fluorescent marks and the outer vertebral margin. No growth rings (narrow increments) appeared to form during the experimental period. These observations supported the belief that vertebral annuli form between fall and winter. The OTC-injected specimen that grew poorly during the experimental period showed little vertebral growth beyond the OTC mark. Thus, the relationship between somatic and vertebral growth appeared highly coupled.

## DISCUSSION

As in most elasmobranchs, vertebral growth patterns appeared to provide a reliable indicator of age and growth in *R. eglanteria*. Vertebral growth significantly increased with somatic growth, thus establishing the functional relationship necessary for successful application of these methods. In fact, changes in somatic and vertebral growth were highly coupled, as shown by the lack of both in stunted captive specimens. The presence of this relationship make vertebral centra the only skeletal structures appropriate for ageing elasmobranchs, and indispensable tools for understanding the life history of these fishes.

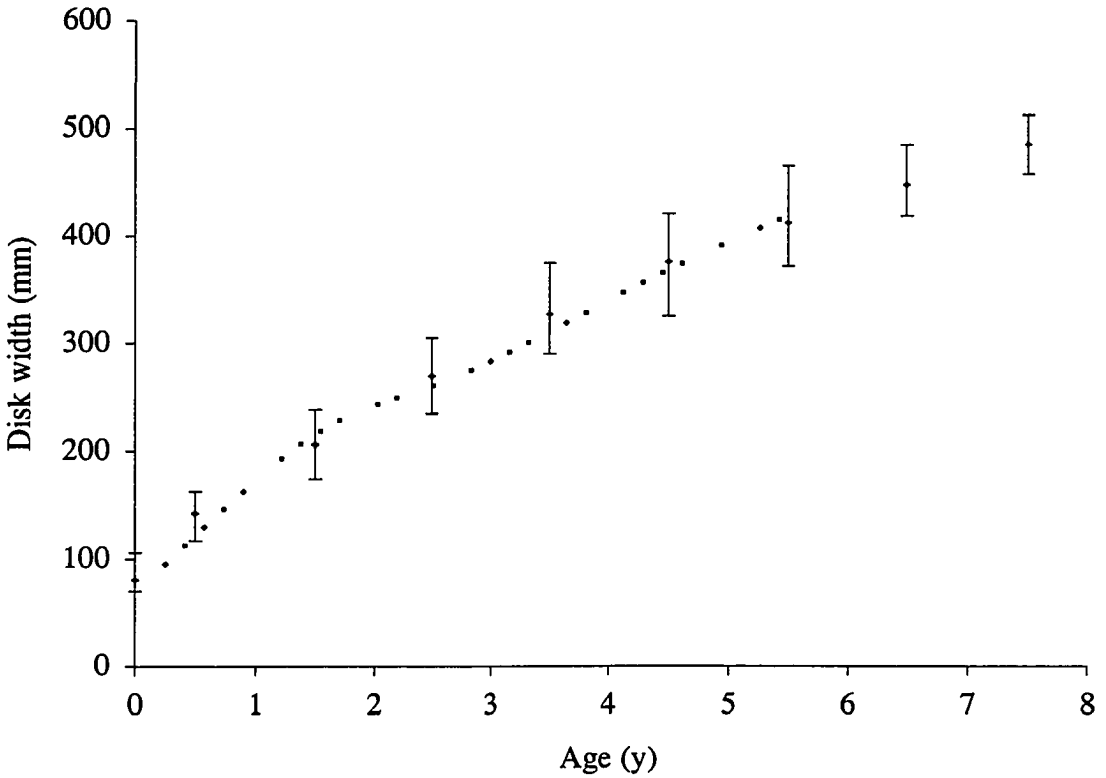
Substantial evidence suggested that narrow lines of arrested growth form in the vertebrae of *R. eglanteria* on an annual basis. Comparison of vertebral growth patterns in YOY and yearling skates indicated that these rings are likely produced between fall and winter. In contrast, dense, wide increments develop between spring and early fall, as demonstrated in OTC- and calcein-injected hatchlings. These observations agree

with earlier reports on *R. eglanteria*, despite their lack of age verification. For example, Figure 1 in Daiber (1960) clearly demonstrates the production of a wide band in vertebrae from a clearnose skate collected in July. Similar rhythms have been suggested or confirmed for several elasmobranchs (Cailliet, 1990), and likely reflect seasonal changes in cartilage growth due to environmental constraints or endogenous cycles.

Although vertebral growth layers typically form with predictable regularity, minor increments may develop at any time of the year due to short-term changes in cartilage growth (Officer *et al.*, 1997). These "check marks" have been observed in the vertebrae of most elasmobranchs and routinely complicate age determination. Such marks were also noted in the present study, indicating that vertebral growth in *R. eglanteria* is a sum of both seasonal and irregular events. These observations formed the basis for experiments on the effects of "non-cycling" hormones (i.e. corticosteroids) on vertebral growth, which are discussed in Chapter 5.

Results of the present study corresponded closely with published data on the life history of *R. eglanteria*. Back-calculated size at hatching agreed with Luer and Gilbert's (1985) observed range of 8-10 cm DW. Growth data were similar to that described in Fitz and Daiber (1963), which were estimated using length-frequency distributions and Daiber's (1960) vertebral age readings (Fig. 8). All reports indicate that *R. eglanteria* grows rapidly over a relatively short life span. Growth rate did not appear to differ between sexes, yet males appear to attain a smaller maximum size ($L_\infty$ = 584.08) than females ($L_\infty$ = 633.62). Estimates of $L_\infty$ are close to maximum

**Figure 3.8.** Mean back-calculated size at age data (combined sexes) for *R. eglanteria* compared with Fitz and Daiber's (1963) estimates of growth (dotted line). Error bars represent ranges around mean DW at age. Fitz and Daiber's (1963) estimates were derived from length-frequency distributions and Daiber's (1960) vertebral age readings.



reported size of 620 mm DW (Fischer, 1978). Asymptotic DW and maximum observed age were slightly greater than those reported in Fitz and Daiber (1963), yet likely reflected the presence of larger individuals in the present study. Reported size at maturity for female and male *R. eglanteria* ranges from 330-380 mm DW (Fitz and Daiber, 1963), which correspond to 3-4 years of life.

Estimates of the Brody growth coefficient for male and female *R. eglanteria* were comparable to those reported for other rajiids (Table 5). In general, Holden (1974) theorized that batoids have values of $K$ ranging from 0.2-0.3, which reflect faster growth than most sharks ($K$ = 0.1-0.2). This theorem appears to be accurate for skates, many of which expressed values of $K$ greater than 0.19. Along with early maturity and short longevity, rapid growth is probably related to high levels of predation (Branstetter, 1990). Since *R. eglanteria* is a common prey item of several coastal shark species (Gelsleichter *et al.*, in press), selective pressures have likely favored the development of resilient life history characteristics.

As found in previous studies (Gelsleichter *et al.*, 1997; Officer *et al.*, 1997), calcein appeared to be an effective chemical marker for elasmobranch age validation. Present observations support the use of low dosage levels rather than those used for teleosts (25 mg/kg BW; Monaghan, 1993), which appeared to be toxic in the nurse shark, *Ginglymostoma cirratum* (Gelsleichter *et al.*, 1997). In fact, reduced dosage levels may be more appropriate for teleosts as well, since Yamada (1971) observed that the concentration of calcein required to produce a suitable mark was one-quarter that of OTC. No effects of calcein on growth rate of captive *R. eglanteria* were observed, and

**Table 5. Summary of Brody growth coefficients estimated for males (♂), females (♀), and combined sexes (C) of various skate species. [1]logistic growth coefficient.**

| Species | K | Method | Reference |
|---|---|---|---|
| *Raja batis* | 0.057C | vertebral bands | Du Buit, 1972 |
| *R. binoculata* | 0.43♂, 0.37♀[1] | vertebral bands | Zeiner and Wolf, 1993 |
| *R. brachyura* | 0.19C | tagging | Holden, 1972 |
| *R. clavata* | 0.22♂, 0.10♀ | vertebral bands | Taylor and Holden, 1964 |
| | 0.21♂, 0.13♀ | tagging | Holden, 1972 |
| | 0.21C | length-frequency | Brander and Palmer, 1985 |
| ***R. eglanteria*** | **0.19♂, 0.17♀** | **vertebral bands** | **present study** |
| *R. erinacea* | 0.34♂, 0.28♀ | vertebral bands | Johnson, 1979 |
| | 0.35C | vertebral bands | Waring, 1984 |
| *R. microocellata* | 0.086C | vertebral bands | Ryland and Ajayi, 1984 |
| *R. miraletus* | 0.19♂, 0.17♀ | vertebral bands | Abdel-Aziz, 1992 |
| *R. montagui* | 0.19♂, 0.18♀ | tagging | Holden, 1972 |
| | 0.15C | vertebral bands | Ryland and Ajayi, 1984 |
| *R. naevus* | 0.11C | vertebral bands | Du Buit, 1972 |
| *R. rhina* | 0.25♂, 0.16♀ | vertebral bands | Zeiner and Wolf, 1993 |

associated mortality was arguably due to natural differences in animal condition (Luer, pers. comm.). Admittedly, further studies on the dose-related toxicity of calcein in elasmobranchs need to be completed prior to its recommended use as a vertebral marker. Nevertheless, calcein may provide a suitable alternative to OTC use. More importantly, it may be used in conjunction with OTC to facilitate the validation of multiple-year patterns of vertebral growth, as Officer *et al.* (1997) did for the gummy shark, *Mustelus antarcticus*.

Results from the present study indicated that OTC did not adversely affect short-term growth of *R. eglanteria*. Similar results have been reported for Japanese wobbegongs and swell sharks (Tanaka, 1990) and, more recently, captive *G. cirratum* (Gelsleichter *et al.*, 1998). Therefore, continued use of OTC injection is still supported as an effective, non-toxic method for determining vertebral ring periodicity. However, Gelsleichter *et al.* (1998) observed high levels of lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) in the serum of OTC-injected *G. cirratum*, which may have reflected hepatotoxicity. High activities of LDH and AST are often used to diagnose toxicant stress and hepatocellular injury, which would allow leakage of these enzymes from damaged liver cells (Krajnovic-Ozretic and Ozretic, 1987). Unfortunately, the diagnosis of toxicant stress in elasmobranchs using serum enzyme levels has not been well investigated. Nevertheless, these observations recommend future studies on OTC toxicity that investigate other characteristics of health less conspicuous that animal growth or survival.

In summary, the present chapter describes rapid growth of *R. eglanteria* based upon analysis of vertebral growth patterns. These patterns are significantly related to somatic growth, thus establishing the need to investigate physiological links between these two processes. This need forms the basis for studies on the hormonal regulation of vertebral growth, which are detailed in Chapter 5. In addition, the present chapter confirms the effectiveness and relative safety of OTC and low-dosage calcein injections. Both chemicals are useful for validating growth estimates derived from vertebral ageing, thus this information has value for elasmobranch growth studies.

# Chapter 4

## Vertebral calcification in *Raja eglanteria*

# INTRODUCTION

Alternating growth increments in the vertebral cartilage of elasmobranchs are traditionally perceived as spatial differences in mineral content (Cailliet, 1990). The present study suggests that this is not the case in *R. eglanteria*, yet such differences have been confirmed in vertebrae of *Carcharhinus amblyrhynchos* and *Alopias vulpinus*, using electron microprobe spectrophotometry (Cailliet and Radtke, 1987). In these species, vertebral mineralization may be considered similar to that which occurs in teleost otoliths and scales; the successive deposition of mineral-rich and mineral-poor growth layers corresponding to a predictable temporal schedule. The processes that control band deposition in these structures are not completely understood, yet models developed to study them may be applicable for elasmobranch fishes. If so, they may provide useful means for investigating the mechanism(s) involved in the incremental growth of elasmobranch vertebrae.

Uptake of the radioisotope $^{45}Ca$, in particular, has been used as a reliable index of otolith calcification in several studies. Using such methods, Mugiya *et al.* (1981) demonstrated a diurnal rhythm in otolith formation in goldfish, *Carassius auratus*, which was associated with photoperiod. Wright *et al.* (1992) observed a similar rhythm for otolith calcification in Atlantic salmon parr, *Salmo salar*, in which calcium uptake was largely restricted to the light phase of daily photoperiod cycles. Thus, both

studies provided strong evidence that growth zone formation in teleost otoliths is affected by light. Photoperiod has also been suggested to be an important environmental cue for growth zone formation in elasmobranch vertebrae, yet no studies have investigated this hypothesis. Among other factors, lack of an appropriate method to measure vertebral calcification has clearly hindered such investigations.

Initially, the goal of this dissertation research was to investigate seasonal changes in vertebral calcification in *R. eglanteria*. Although present data suggest that such changes do not exist, preliminary attempts were made to characterize the calcification of centra in live clearnose skates using $^{45}Ca$. This chapter describes these efforts, which were conducted to investigate the direct relationship between vertebral calcification and environmental photoperiod.

## MATERIALS AND METHODS

Twenty-four clearnose skate hatchlings were obtained from Mote Marine Laboratory's skate-rearing facility for this study. Skates were transferred to an 80 L aquarium containing $^{45}Ca$ (ICN Biomedical) at a concentration of approximately 10 $\mu$Ci/L. Animals were maintained under constant temperature (19°C) and photoperiod (12L: 12D) regimes, and were not fed during the experimental period. Four skates were sacrificed at 4-h intervals over a 24-h period, which began at 1200 hours. Animals were euthanized by severing the spinal cord, following anesthesia with 1 g/l

tricaine methanesulfonate (MS-222). Afterwards, ten vertebral centra were sampled from each skate for $^{45}Ca$ analyses.

Vertebral centra were rinsed several times in elasmobranch Ringer's solution (Forster *et al.*, 1972) and dried overnight at 90°C. Dried samples were weighed and placed into 20-ml scintillation vials for solubilization with 60% perchloric acid and 30% hydrogen peroxide warmed to 75°C for 2 h (Mahin and Lofberg, 1966). Afterwards, the commercial scintillant ScintiSafe Plus 50% (Fisher Scientific) was added and radioactivity counted by liquid scintillation. Data were expressed as counts per minute (cpm) per mg dry weight. Vertebral uptake of $^{45}Ca$ over time was described by linear regression. Differences in vertebral radioactivity were compared using a Kruskal-Wallis test.

## RESULTS

Radioactivity was detected in vertebral cartilage as early as 4 h following introduction into $^{45}Ca$-labeled water (Fig. 1). Afterwards, uptake of $^{45}Ca$ appeared moderately time-dependent (ANOVA; $P<0.05$), yet much of the variation in vertebral radioactivity was unassociated with temporal exposure to isotopic water ($R^2 = 0.34$). Uptake of $^{45}Ca$ also appeared unrelated to photoperiod (Fig. 2), and there were no significant differences between levels of vertebral radioactivity (Kruskal-Wallis; $P<0.05$) that occurred during the full experimental period.

**Figure 4.1.** Uptake of $^{45}Ca$ in vertebral cartilage of *R. eglanteria* hatchlings over a 24-h experimental period. Points represent vertebral radioactivity of four skates sampled every four hours. Relationship between time and radioisotope incorporation is significant ($P<0.05$), yet most of the variation in vertebral radioactivity is unassociated with time ($R^2 = 0.34$). Point at time 0 represents mean vertebral radioactivity of 2 skates sampled prior to introduction of the radioisotope.

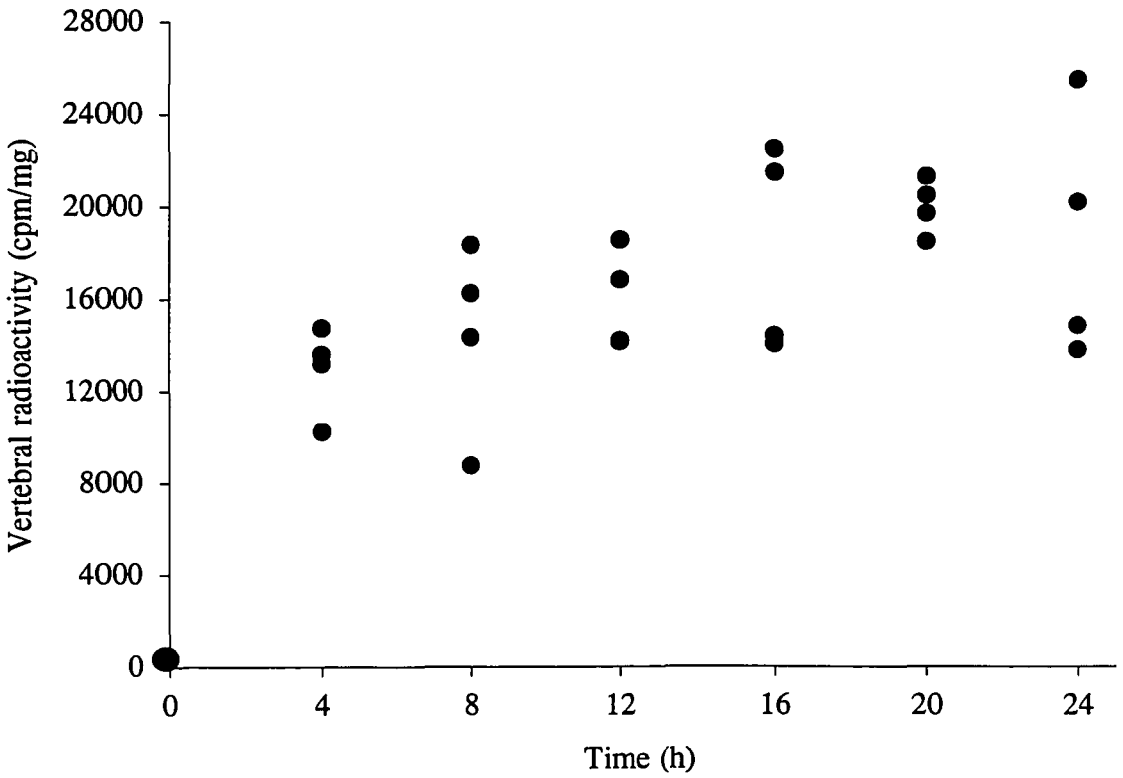
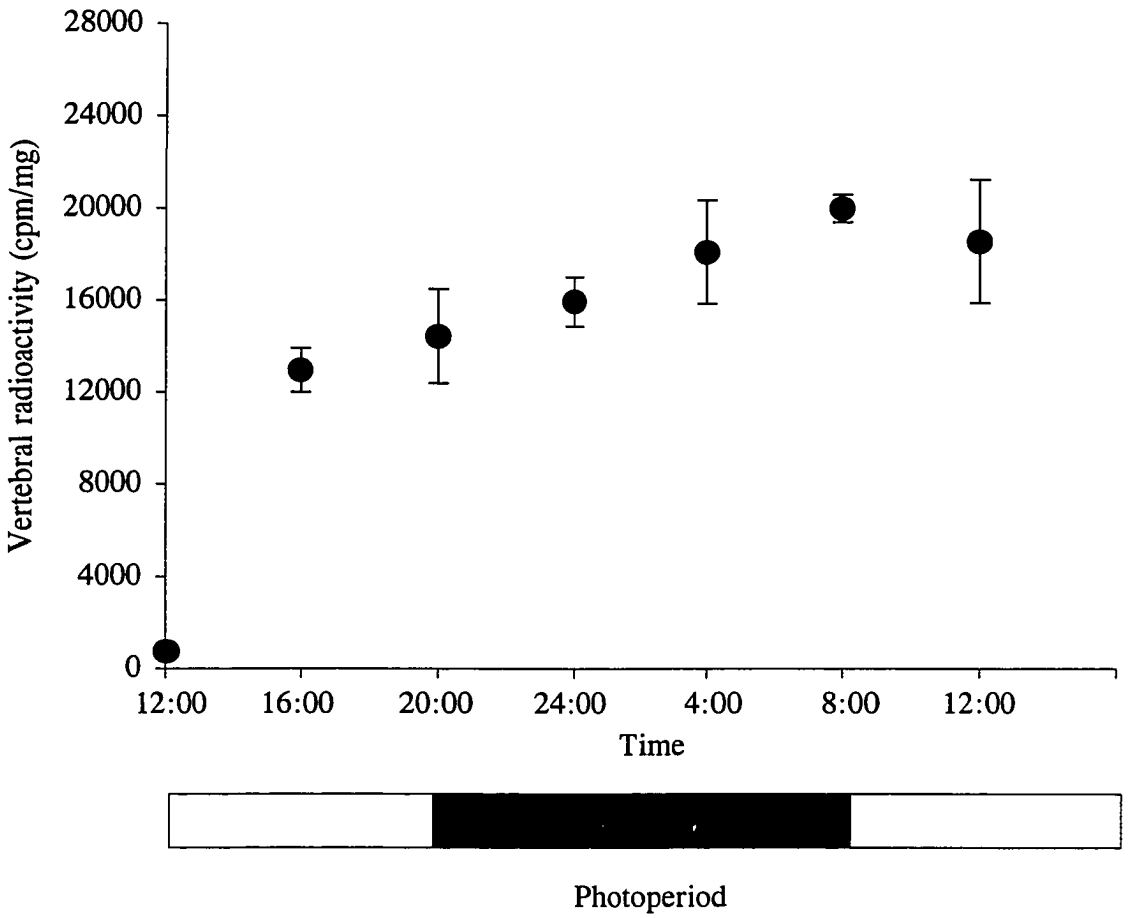


**Figure 4.2.** Mean vertebral radioactivity (±SE) in *R. eglanteria* hatchlings observed over time and in relation to photoperiod. No significant differences between mean values obtained at each sampling ($P<0.05$) except for the first observation ($t = 0$), which represents vertebral radioactivity in skates sampled prior to addition of $^{45}Ca$. Bar represents light (❑) and dark (■) photoperiod cycles.



# DISCUSSION

The results of this experiment demonstrate that uptake of the radioisotope $^{45}Ca$ in vertebral cartilage of *R. eglanteria* is highest following initial exposure to radiolabeled water. Afterwards, no significant changes in vertebral radioactivity appeared to occur over the 24-h experimental period. These results suggest that the uptake of $^{45}Ca$ may not reflect the rate of vertebral calcification in this species. Rather, $^{45}Ca$ appears to rapidly label vertebrae, supporting Urist's (1964) assertion that most of skeletal mineral in elasmobranchs is exchangeable and in equilibrium with the blood. Similar results have been observed following the injection of $^{45}Ca$ into elasmobranchs (Urist, 1964), yet direct evidence of mineral resorption is lacking. Nonetheless, these data do not support the use of $^{45}Ca$ in studies on vertebral mineralization.

Although no evidence of mineral resorption from elasmobranch cartilage has been documented, direct routes of calcium exchange between the vertebrae and blood are present. As demonstrated in Chapter 2, numerous channels are present in vertebral cartilage, and may provide an important boundary layer in the "blood-skeleton continuum." Such channels, often termed "cartilage canals", have been observed in several elasmobranch species (Hoenig and Walsh, 1982), and likely play a role in supplying nutrients to entombed chondocytes. In the present study, these canals probably permitted the rapid uptake of $^{45}Ca$ into vertebral centra.

Efforts to determine the effect of light on vertebral mineralization were precluded by the rapid, nonspecific uptake of $^{45}Ca$. Thus, the role of photoperiod as an

environmental cue for vertebral growth zone formation remains unclear. Similar attempts to investigate the effect of dissimilar photoperiod regimes (18L: 6D; 6L: 18D) on vertebral calcification also failed, due to mortality of experimental animals (data not presented). Soon after, it was confirmed that vertebral growth bands in *R. eglanteria* vary in cellular content rather than mineral concentration. Thus, research efforts were re-directed towards investigating the physiological modulators of vertebral growth, as outlined in Chapter 5.

Although the focus of this dissertation shifted to the hormonal regulation of vertebral growth, environmental factors are still likely to play a role in growth band formation. Indeed, factors such as photoperiod, temperature, or nutrition may act as important external "triggers" that coordinate temporal shifts in endocrine activity and cartilage growth. To date, only temperature has been studied as an environmental influence of vertebral band deposition in elasmobranchs. Natanson (1993) determined that temperature did not affect vertebral band formation in little skate, *Raja erinacea*, yet suggested that it may be influential in other elasmobranch species. Thus, as Natanson (1993) stressed, additional studies must be conducted to better understand the abiotic factors that promote increment formation.

In summary, uptake of $^{45}Ca$ in centra of *R. eglanteria* does not appear to reflect the rate of vertebral mineralization. Therefore, other methods are necessary to characterize this process, and examine factors that may influence it. It is perhaps appropriate that this method is not useful for elasmobranchs, due to the unavoidable compromise between radiation safety and experimental precision. In general,

elasmobranchs typically require large aquaria for captive maintenance, yet large volumes of radiolabeled water are a substantial health risk and unlikely to be compatible with institutional policies. Thus, an investigator must limit sample size in order to comply with federal and state regulations on radioactive materials. In addition, the relatively short half-life (145 d) of $^{45}Ca$ makes it inappropriate to use in characterizing seasonal patterns of vertebral calcification.

# Chapter 5

## Vertebral growth in *Raja eglanteria*

## Part I. An *in vitro* method for studying growth of elasmobranch vertebral cartilage.

### INTRODUCTION

As discussed in Chapter 2, observations on vertebral centra from *R. eglanteria* suggest that seasonal growth increments form due to changes in cartilage growth. Thus, efforts to determine causative factors that produce these phenomena must directly examine the components of this process, which include chondrocyte proliferation and glycosaminoglycan (GAG) synthesis. Unfortunately, no study to date has described reliable methods for investigating these processes in elasmobranch vertebrae. In fact, no study has examined these generalized features of growth in any form (e.g. chondrocranial, branchial, etc.) of elasmobranch cartilage.

Organ culture of cartilaginous structures has been effective in assessing cartilage growth in most vertebrates. To date, such techniques have been described for teleosts, amphibians, reptiles, birds, and mammals. Typically, these procedures involve the use of $^{3}$H-methyl thymidine and $^{35}$S-sulfate as markers for chondrocyte proliferation and GAG synthesis respectively (Takagi and Björnsson, 1996). Alone, or combined with histomorphometric analyses (Takagi *et al.*, 1994), these methods are useful in defining the physiological factors that regulate cartilage development and growth.

The present study describes an *in vitro* culture method for investigating the physiology of vertebral cartilage from *R. eglanteria*. The uptake of $^{35}S$-sulfate by isolated vertebral centra was measured as a marker for GAG synthesis. In addition, uptake of $^{45}Ca$ was measured to determine its utility as a marker for vertebral calcification. This study provides the first logical stage in characterizing the hormonal regulation of elasmobranch skeletal growth.

## MATERIALS AND METHODS

### *Animals*

Juvenile *R. eglanteria* (8–10 cm disk width) were obtained following captive breeding of wild-caught animals at Mote Marine Laboratory (Sarasota, FL). Skates were maintained in recirculating seawater aquaria, under conditions previously described in Luer and Gilbert (1985) and Luer (1989).

### *Solutions and hormones*

Elasmobranch dissecting medium (EDM) was prepared as described in DuBois and Callard (1991) and contained the following: D-glucose (5.5 mM), KCl (5.3 mM), $KH_2PO_4$ (0.44 mM), $NaHCO_3$ (0.18 mM), $Na_2HPO_4 \cdot 7H_2O$ (0.33 mM), NaCl (280 mM), urea (350 mM), penicillin (150 U/ml), and streptomycin (150 $\mu$g/ml). Basal elasmobranch culture medium (ECM) was prepared with Leibovitz L-15 medium adjusted for urea (350 mM) and NaCl (280 mM) and contained 150 U penicillin and 150 $\mu$g streptomycin per ml (DuBois and Callard, 1993). The radiochemicals $Na_2{}^{35}SO_4$

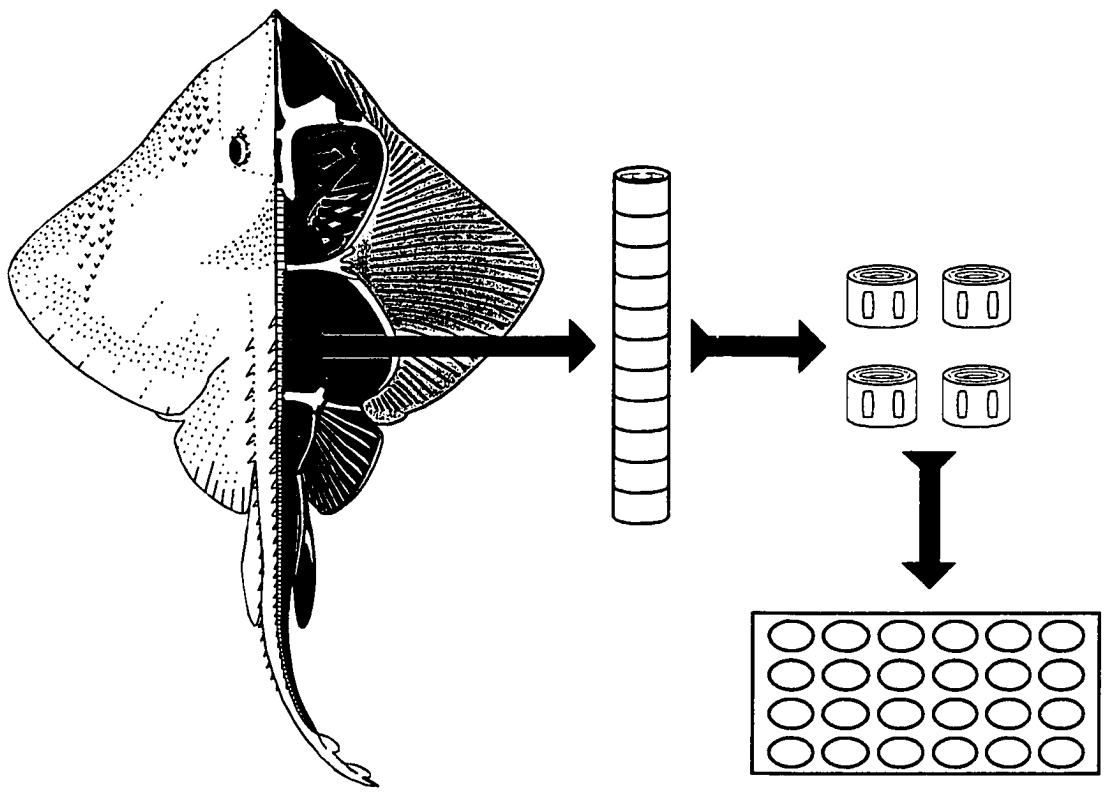
and $^{45}CaCl_2$ (ICN Biomedicals) were dissolved in ECM at a concentration of 20 $\mu$Ci/ml and stored at 4°C.

*In vitro assay of $^{35}SO_4$ and $^{45}Ca$ uptake*

The methods used to determine radioisotope uptake in skate vertebrae were modified from those described for teleost cartilage (Duan and Inui, 1990; Gray and Kelley, 1991). Skates were anesthetized with 1 g/L tricaine methanesulfonate (MS-222) and sacrificed by decapitation. Vertebral cartilage was removed, cleared of extraneous tissue, and dissected into individual centra in EDM (Fig. 1). Some centra were boiled in EDM for 30 min to heat-kill vertebral chondrocytes and thereby determine non-specific $^{35}SO_4$ and $^{45}Ca$ uptake. Afterwards, live and heat-killed explants were cultured in ECM containing 1 $\mu$Ci $^{35}SO_4$ or $^{45}Ca$ per ml in separate wells of 24-well microplates. All cultures were incubated for 72 h at 19°C under air.

Following culture, vertebral centra were removed and placed into vials containing either saturated $Na_2SO_4$ ($^{35}SO_4$) or $CaCl_2$ ($^{45}Ca$) solution for at least 6 h. Centra were then rinsed in distilled water overnight to remove excess free radioactivity. Rinsed tissue was dried to a constant weight at 60°C, weighed to the nearest $\mu$g, and placed in scintillation vials containing 0.5 ml 90% formic acid heated to 60°C overnight. After addition of scintillation cocktail (ScintiSafe Plus 50%; Fisher Scientific), samples were analyzed for radioactivity in a Beckman 5000 scintillation counter. Results were expressed as counts per minute (cpm) per $\mu$g dry tissue weight.

**Figure 5.1.** General description of the *in vitro* method for culturing vertebral cartilage of *R. eglanteria*.



*Statistical analysis*

To determine if $^{35}SO_4$ uptake differed significantly between boiled and non-boiled tissue, a Mann-Whitney U test was applied. A t-test for independent samples was used to compare $^{45}Ca$ uptake in boiled and non-boiled units. Significance was set at $P<0.05$. All results are presented as means $\pm$ SEM.

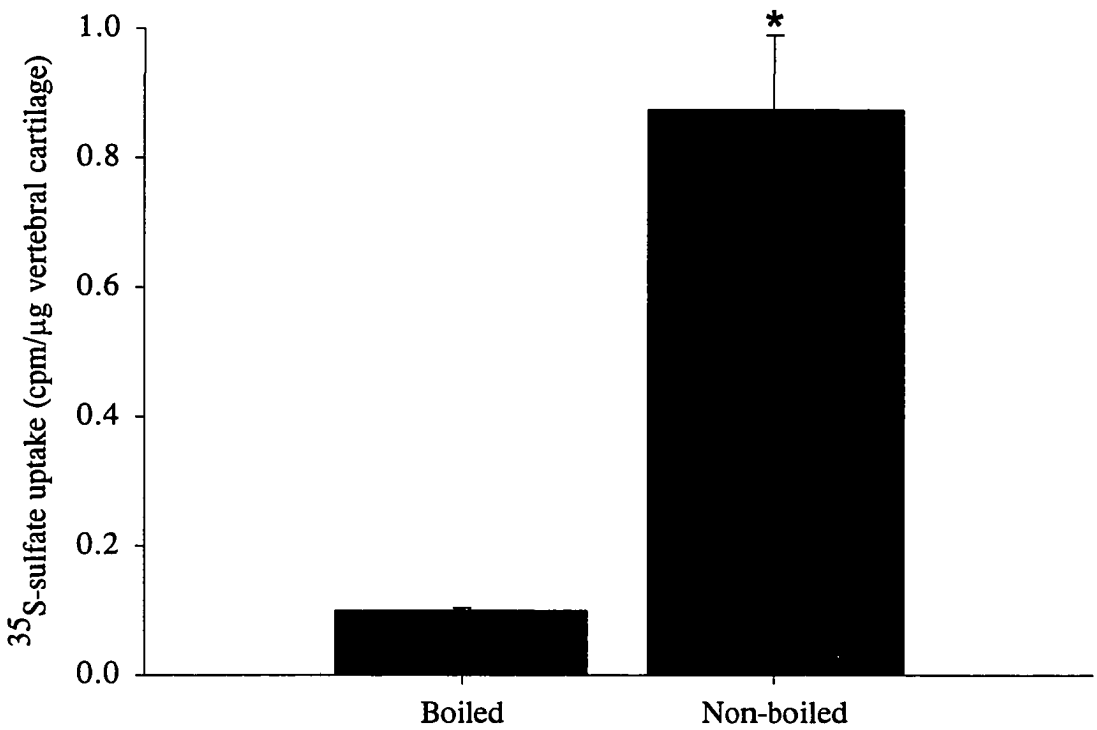
*Histological features of in vitro growth*

To examine the histological characteristics of vertebral cartilage associated with *in vitro* culture, additional cultures were prepared without the addition of radioisotopes. Following culture, centra were fixed in Bouin's solution for 48 h, decalcified, and embedded in paraffin. Transverse sections were cut at 5 $\mu$m, deparaffinized, and stained with hematoxylin-eosin (H&E). Slides were evaluated using a compound microscope using transmitted light.

## RESULTS

Uptake of $^{35}SO_4$ in boiled vertebral cartilage was significantly lower than that observed in non-boiled tissue (Mann-Whitney U, $P<0.05$). Radioactivity of heat-killed cartilage was approximately 11% of that in live centra (Fig. 2). These values were suggested to reflect the non-specific uptake (NSU) of $^{35}SO_4$, which occurs through non-cellular activities (i.e. adsorption).

**Figure 5.2.** *In vitro* uptake of $^{35}$S-sulfate in boiled and non-boiled vertebral cartilage from *R. eglanteria*. Asterisk (*) denotes significant difference from boiled tissue, indicating effective measurement of glycosaminoglycan synthesis and cartilage growth (Mann-Whitney $U$, $P<0.05$).



The uptake of $^{45}Ca$ in vertebral cartilage is graphically summarized in Figure 3. Mean radioactivity of boiled units did not differ significantly from that of live cartilage (t-test, $P = 0.9$). In fact, values obtained for both groups were virtually indistinguishable.

Non-boiled vertebral cartilage incubated in ECM had the histologic appearance of viable tissue (Fig. 4). Chondrocytes retained the characteristics previously described (in Chapter 2) for *in vivo* cartilage. Cells were surrounded by pericellular aggregations of GAG matrices. Multiple stages of cytodifferentiation were present and corresponded to cellular maturation.

## DISCUSSION

Vertebral cartilage from juvenile *R. eglanteria* remains vital for at least 72 h *in vitro*, providing a simple method for investigating the growth of elasmobranch cartilage. Examination of non-boiled vertebral cartilage confirmed that it had the histologic appearance of living tissue. In addition, uptake of $^{35}SO_4$ in live cartilage was significantly greater than that of boiled units, suggesting that it occurred partially due to an active, cell-mediated process. Interestingly, the values obtained for non-specific uptake of $^{35}SO_4$ using a 15 min boiling period (Gelsleichter and Musick, 1997) were moderately greater than those reported in the present and similar (Marchant and Moroz, 1993; Takagi and Björnsson, 1996; 1997) experiments. Initially, these observations suggested that elasmobranch vertebral cartilage is either greatly absorptive or requires

**Figure 5.3.** *In vitro* uptake of $^{45}Ca$ in boiled and non-boiled vertebral cartilage from *R. eglanteria*. No difference observed between groups (*t*-test, $P<0.05$, indicating lack of effectiveness as a marker for vertebral calcification.

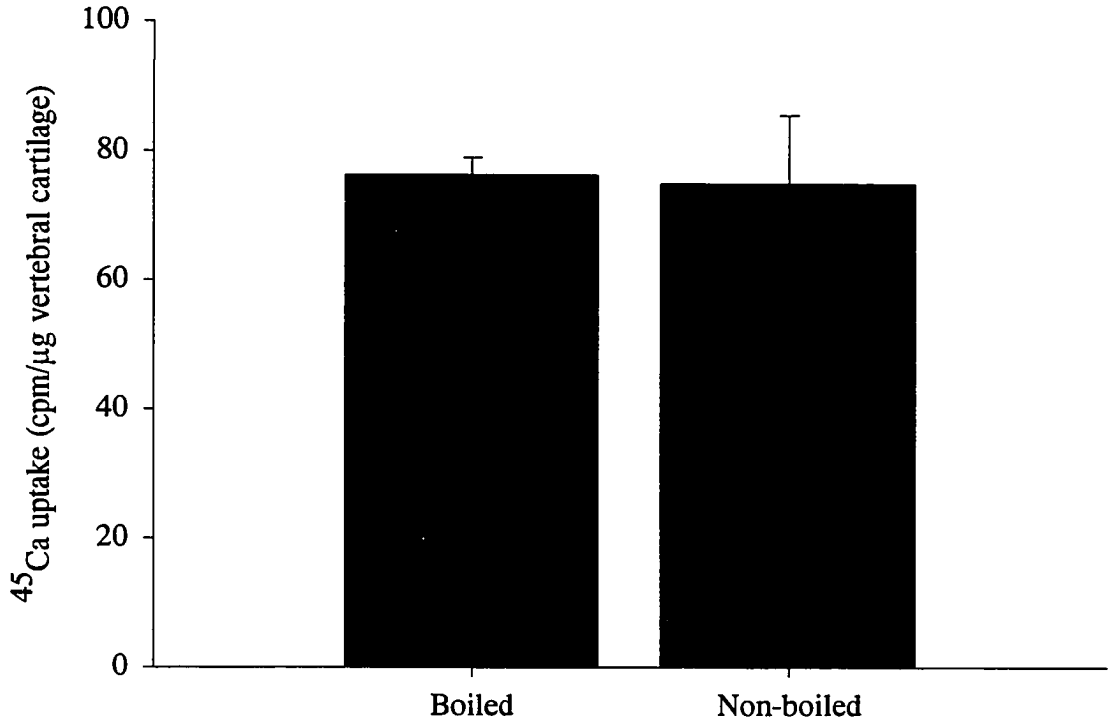
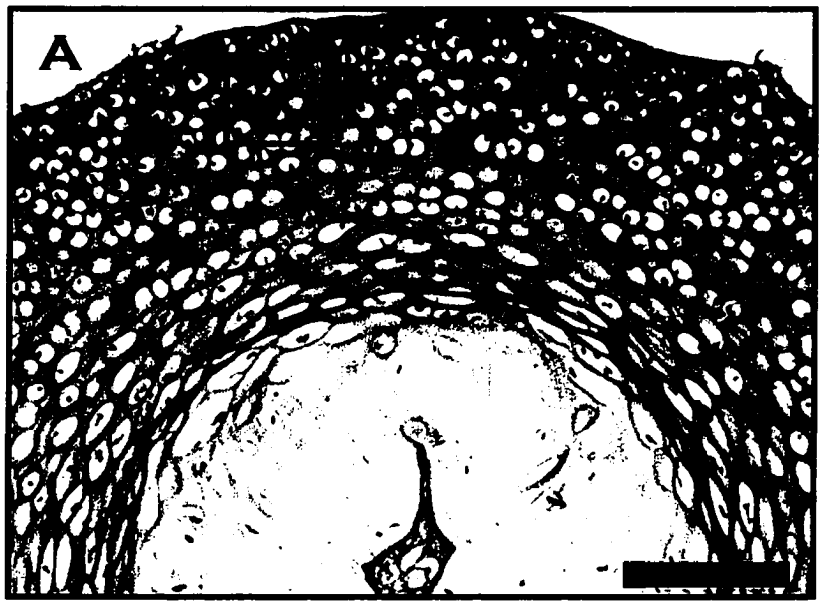
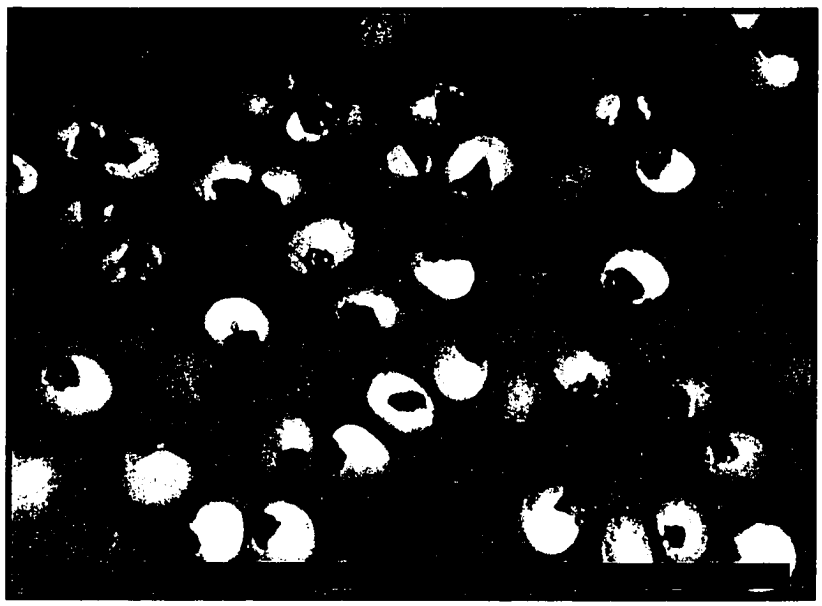


**Figure 5.4.** **(A)** Cross section of vertebral cartilage from *R. eglanteria* hatchling following 72 h *in vitro* culture. Bar: 100 $\mu$m. **(B)** Higher magnification demonstrates viable chondrocytes surrounded by pericellular aggregations of glycosaminoglycan matrix. Bar: 100 $\mu$m.



substantial boiling to heat-kill chondrocytes. Observations presented herein support the latter explanation, thus, longer periods of tissue disruption (i.e., 30 min) are recommended.

Results of the present study indicate that uptake of $^{45}Ca$ by cultured vertebral cartilage is not an effective measure of vertebral calcification. Incorporation of $^{45}Ca$ was similar in live and heat-killed centra, demonstrating that this process is not dependent on cellular activity. Rather, it appears likely that vertebral radioactivity reflected the free exchange of calcium between vertebrae and their surrounding medium. Like the results of Chapter 4, this supports Urist's (1961) suggestion that most of the mineral in elasmobranch skeletons is in equilibrium with the blood and fully exchangeable. This differs significantly from most vertebrate bone, in which 98-99% of hydroxyapatite is contained within large, nonexchangeable "diffusion-locked" crystals (Norris, 1997).

Although not employed in this study, alternative methods may be useful for measuring the calcification of elasmobranch vertebrae. The activity of alkaline phosphatase (ALPase), in particular, is an effective indicator of this process in mammalian growth plate cartilage (Kato *et al.*, 1990; Jikko *et al.*, 1996). Following its production by chondrocytes, ALPase directly induces calcification by affecting the transport and supply of mineral ions (Iwamoto *et al.*, 1989). Future studies are necessary to determine if this enzyme plays a similar role in elasmobranch skeletal tissues, and if it can be measured using the experimental culture system.

Other modifications to the *in vitro* culture method may also provide a more comprehensive view of elasmobranch cartilage physiology. For example, use of $^{3}$H-methyl thymidine as an indicator of chondrocyte proliferation is well supported by previous studies on teleost skeletogenesis (Takagi and Björnsson, 1996). In addition, histomorphometric studies are necessary to correlate cellular events with specific stages of chondrocyte differentiation. Nevertheless, the current technique provides a reliable tool for investigating a major component of elasmobranch cartilage growth; GAG synthesis. Future improvements of this method, and its use in characterizing the development of other skeletal structures (e.g. claspers, fin supports), are planned.

In summary, an effective method for measuring matrix synthesis in elasmobranch vertebral cartilage is presented. This method will be used to investigate the physiological and environmental factors that influence the growth of these structures. By doing so, these studies will provide the first investigation on elasmobranch skeletal physiology.

## Part II. Studies on the hormonal regulation of vertebral growth.

### INTRODUCTION

In most vertebrates, skeletal growth is an orderly process under precise control of the endocrine system. This process depends largely upon the growth of cartilage, thus several hormones play important roles in regulating chondrocyte proliferation and matrix synthesis. Multiple interactions between these hormones and other metabolic factors ensure proper development of the vertebrate skeletal system. In addition, hormones involved in mineral homeostasis may also influence skeletal physiology in order to maintain plasma and interstitial levels of essential ions.

#### *Growth hormone and insulin-like growth factor-I*

Pituitary growth hormone (GH) exerts a major influence on cartilage growth through the action of GH-dependent serum factors, particularly insulin-like growth factor-I (IGF-I). GH stimulates hepatic and extrahepatic production of IGF-I, which increases cartilage matrix synthesis, protein, DNA and RNA synthesis and chondrocyte proliferation (Duan and Hirano, 1990; Gray and Kelley, 1991; McCormick *et al.*, 1992). In addition, GH may directly accelerate cartilage growth by promoting differentiation of chondrocyte progenitor cells, thereby making them more responsive to IGF-I (Gray and Kelley, 1991; Cheng and Chen, 1995). The synergistic relationship

between GH and IGF-I in regulating skeletal growth has been termed the "dual effector theory" (Green *et al.*, 1985), and has been shown to be active in virtually all major vertebrate groups.

Few studies have been conducted on elasmobranch GH (Lewis *et al.*, 1972; Hayashida and Lewis, 1978; Yamaguchi *et al.*, 1989), yet it has been shown to actively promote skeletal growth using the rat tibia bioassay (Hayashida and Lewis, 1978). In addition, the presence of IGF-I has been confirmed in elasmobranchs (Daughady *et al.*, 1985; Duguay *et al.*, 1995), although no definite link to GH has been established. Nevertheless, it is likely that these hormones play important roles in regulating cartilage growth in elasmobranchs as they do in other vertebrates. Direct evidence of such roles is lacking, thus, studies on the effects of GH and IGF-I on elasmobranch cartilage growth remain necessary.

***Thyroid hormones***

Thyroid hormones, particularly 3,3´,5-tri-ido-L-thyronine ($T_3$), are required for normal skeletal growth of most vertebrates. In studies on teleost, avian, and mammalian cartilage, $T_3$ has been shown to directly stimulate chondrocyte proliferation and maturation, and overall cartilage growth (Burch and Leibovitz, 1982a; b; Burch and McCarty, 1984; Takagi *et al.*, 1994; Takagi and Björnsson, 1996). In addition, $T_3$ indirectly promotes cartilage growth by stimulating GH secretion and the production of IGF-I (Hervas *et al.*, 1975; Vale *et al.*, 1983; Ikeda *et al.*, 1989). Stimulative actions

of $T_3$ and the GH-IGF-I axis appear additive (Takagi and Björnsson, 1996), thus stressing the complexity of the hormonal regulation of skeletal growth.

Only limited information is available on thyroid function in elasmobranchs (Leary *et al.*, in review). In general, studies have demonstrated that $T_3$ is active in these fishes, and may play roles in reproduction (Volkoff *et al.*, in review), neural differentiation (Norris, 1997), or other physiological processes. However, no studies have investigated the role of $T_3$ in the skeletal development of cartilaginous fishes.

### *Corticosteroids*

Corticosteroids, produced by the mammalian adrenal cortex and non-mammlian interrenal tissue, appear to be greatly involved in regulating cartilage development. In human children, excess glucocorticosteroids occurring naturally (i.e., in Cushing's syndrome) or due to therapy can severely retard somatic and skeletal growth (Preece, 1976; Peck *et al.*, 1984). These hormones bind at cytoplasmic receptors within chondrocytes (Lee *et al.*, 1978; Blondelon *et al.*, 1980; Kan *et al.*, 1983), and have been shown to inhibit matrix synthesis in teleost (Takagi and Björnsson, 1997), avian (Kilgore et al., 1979) and mammalian (Tessler and Salmon, 1975; Hill, 1981; Weiss *et al.*, 1988) cartilage. However, inconsistencies in the effect of corticosteroids on cartilage growth have been observed in several studies (Kato and Gospodarowicz, 1985; Takano *et al.*, 1985; Takigawa *et al.*, 1988). Thus, the role of these hormones in skeletal physiology may require further clarification.

Elasmobranchs produce several corticosteroids including the unique 1α-hydroxycorticosterone (1α-OHB), which appears to function as both mineralo- and glucocorticosteroid in these fishes (Nunez and Trant, in review). There is considerable evidence that 1α-OHB acts in both the stress response (Nunez and Trant, in review) and osmoregulation (Idler *et al.*, 1967; Truscott and Idler, 1972; Hazon *et al.*, in review) of elasmobranchs, yet no studies have investigated its role in skeletal growth. However, similarities between the regulation and function of corticosteroids in elasmobranchs and other vertebrates suggest that such a role is likely and warrants investigation.

### *Calcitonin*

Calcitonin (CT) is produced by the C cells of the thyroid gland in mammals and the ultimobranchial bodies of non-mammalian vertebrates (Dacke, 1979). In terrestrial vertebrates, its principal role is to evoke hypocalcemia, largely through the inhibition of osteoclastic bone resorption (Aliapoulias *et al.*, 1966; Stevenson, 1979). In addition, CT may regulate calcium homeostasis in tetrapods by reducing renal tubular resorption of monovalent and divalent ions (Norris, 1997; Marx *et al.*, 1972). In fish, the effects of CT are inconsistent and its role remains largely unclear (Pang, 1971). However, a calcitropic role is likely since it has been shown to alter branchial calcium flux (Milet *et al.*, 1979), stimulate growth and mineralization of bone and scales (Lopez *et al.*, 1976; Wendelaar Bonga and Lammers, 1982), and produce hypo- (Mathur,

1979; Chakrabarti and Mukherjee, 1991; Singh and Srivastav, 1993) or hypercalcemia (Fouchereau-Peron *et al.*, 1987; Oughterson *et al.*, 1995) in certain teleosts.

In elasmobranchs, the presence of CT is especially puzzling since these fishes lack bony skeletons. Most studies conducted on elasmobranchs have demonstrated that CT has little or no effect on plasma calcium (Urist, 1967; Louw *et al.*, 1969; Copp *et al.*, 1970; Hayslett *et al.*, 1971) or renal function (Hayslett *et al.*, 1972). However, Glowacki *et al.* (1985) observed CT-induced hypercalcemia in leopard sharks (*Triakis semifasciata*), yet the origin and significance of this effect were unclear. More recently, Srivastav *et al.* (1998) found that salmon CT produced hypocalcemia and hyperphosphatemia in the stingray *Dasyatis akajei*. Although conflicting, these two studies suggest that CT may be involved in homeostatic processes unrelated to bone calcium metabolism. Furthermore, recent studies that have demonstrated a relationship between CT production and estrogen in *D. akajei* imply that such roles may be related to reproduction or development (Takagi *et al.*, 1993; Yamamoto *et al.*, 1996).

Although an osseous role for CT in elasmobranchs is not feasible, it may still influence mineral homeostasis in these fishes through effects on cartilage growth and calcification. To date, such effects have been demonstrated in the cartilage of reptiles (Bélanger *et al.*, 1973), birds (Suzuki *et al.*, 1976; Kawashima *et al.*, 1980a; b), and mammals (Baxter *et al.*, 1968; Martin *et al.*, 1970; Burch and Corda, 1985; Eguchi *et al.*, 1986; Kato *et al.*, 1990). Changes in the growth and mineralization of elasmobranch vertebral cartilage, in particular, may play some role in calcium or phosphate metabolism since these structures represent a rich store of hydroxyapatite

biomineral (Clement, 1992). However, no studies have investigated the potential role of CT in elasmobranch skeletal growth.

***Parathyroid hormone-related protein***

Parathyroid hormone-related protein (PTHrP) is a factor that shares amino-terminal sequence homology with parathyroid hormone (PTH), the primary hypercalcemic agent of terrestrial vertebrates. This protein was initially identified as the primary mediator of humoral hypercalcemia associated with human malignancy (HHM), a condition in which tumor-produced PTHrP acts on osseous and renal tissues to pathologically disrupt calcium homeostasis (Mosley *et al.*, 1987; Suva *et al.* 1987). In addition to tumor cells, PTHrP is also produced by several non-malignant cell types, including keratinocytes (Danks *et al.*, 1989), lactating mammary tissue (Thiede and Rodan, 1988), endothelial cells, smooth muscle, bone, and embryonic tissues (Mosley *et al.*, 1991). Its actions are expressed through the receptor for PTH, and it is believed to act in an autocrine or paracrine manner (Orloff *et al.*, 1989). Although its role remains partially unclear, PTHrP may function in calcium transport, relaxation of smooth muscle, and regulation of growth and differentiation in fetal and adult tissues (Norris, 1997).

Recently, PTHrP has been shown to play a critical role in mammalian skeletogenesis. In human Jansen metaphyseal chondrodystrophy, mutation of PTH/PTHrP receptors render them constitutively active and produce skeletal abnormalities, such as delayed mineralization in growth plate cartilage (Schipani *et al.*, 1995). Similarly, overexpression of PTHrP in cartilage of newborn mice causes

dramatic slowing of chondrocyte differentiation and endochondral bone formation (Weir *et al.*, 1996). Mice lacking both copies of the PTHrP gene die at birth due to gross malformation of the skeletal system (Karaplis *et al.*, 1994). In a detailed review, Kronenberg *et al.* (1997) indicated that PTHrP regulates skeletal formation by ensuring the normal pace of chondrocyte differentiation in growth plate cartilage. In this process, PTHrP produced by the perichondrium acts to slow cell maturation and facilitate the proper development of bones by endochondral replacement.

In recent studies, PTHrP has been determined to be a normal factor in both cartilaginous and bony fish. In the chain dogfish, *Scyliorhinus canicula*, PTHrP was detected in plasma and several tissues, including brain neurons, kidney tubules, rectal gland, vascular epithelia, and cells of the pituitary pars distalis (Ingleton *et al.*, 1995). More recently, Trivett *et al.* (in review) localized PTHrP in kidney, muscle, gill, rectal gland, nerve cord, pituitary, and dermal denticles of several elasmobranch species. In addition, Trivett (pers. comm.) has also detected PTHrP in the perichondrium of elasmobranch vertebral cartilage, suggesting that it may play a role in vertebral chondrogenesis. Given the similarities between elasmobranch vertebrae and mammalian growth plate cartilage, this role appears likely and requires investigation.

### *Introductory summary*

Clearly, several hormones have the potential to play critical roles in the growth and mineralization of elasmobranch vertebral cartilage. Therefore, the goal of this chapter was to determine the effects of GH, IGF-I, $T_3$, corticosteroids, CT, and PTHrP

on growth of vertebral cartilage of the clearnose skate, *Raja eglanteria*. This study represents the first investigation on the hormonal regulation of skeletal growth in elasmobranch fishes.

## MATERIALS AND METHODS

### *Animals*

Juvenile *R. eglanteria* were obtained following captive breeding of adults at Mote Marine Laboratory (Sarasota, FL). Animals were maintained in recirculating seawater aquaria, under conditions previously described in Luer and Gilbert (1985) and Luer (1989).

### *Solutions and hormones*

Elasmobranch dissecting medium (EDM) was prepared as described in DuBois and Callard (1991), and contained the following: D-glucose (5.5 m*M*), KCl (5.3 m*M*), $KH_2PO_4$ (0.44 m*M*), $NaHCO_3$ (0.18 m*M*), $Na_2HPO_4 \cdot 7H_2O$ (0.33 m*M*), NaCl (280 m*M*), urea (350 m*M*), penicillin (150 U/ml), and streptomycin (150 $\mu$g/ml). Basal elasmobranch culture medium (ECM) was prepared with Leibovitz L-15 medium adjusted for urea (350 m*M*) and NaCl (280 m*M*) and contained 150 U penicillin and 150 $\mu$g streptomycin per ml (DuBois and Callard, 1993). The radiochemical $Na_2{}^{35}SO_4$ (ICN Biomedicals,) was dissolved in ECM at concentrations ranging from 20 - 40 $\mu$Ci/ml and stored at 4°C. Human growth hormone (hGH; Sigma Chemical Co., St.

Louis, MO) was dissolved in distilled water at a concentration of 0.5 mg/ml. Recombinant human IGF-I (rhIGF-I; Sigma) and fowl CT (fCT; Sigma) were dissolved in ECM at concentrations of 5000 ng/ml and 1.5 $\mu M$, respectively. $T_3$ (Sigma) was dissolved in 0.9% NaCl solution containing 0.001 *M* NaOH at a concentration of 75 m*M*. Human PTHrP (fragment 1 – 34 amide, Sigma) was dissolved in distilled water at a concentration of 20 $\mu$g/ml. Due to the lack of 1$\alpha$-OHB, corticosterone (a gift from J. Trant) was dissolved in ethanol at a concentration of 10 m*M*. All stock solutions were diluted with ECM to appropriate working concentrations and stored at -20°C.

***In vitro assay of glycosaminoglycan synthesis***

Glycosaminoglycan (GAG) synthesis in skate vertebrae was measured as a marker for cartilage growth. Skates were anesthetized with 1 g/l tricaine methanesulfonate (MS-222) and sacrificed by decapitation. Vertebral cartilage was removed, cleared of extraneous tissue, and dissected into individual centra in EDM. Some centra were boiled in EDM for 30 min to heat-kill vertebral chondrocytes and thereby determine non-specific $^{35}SO_4$ uptake. Afterwards, live and heat-killed explants were cultured in ECM containing 1 – 2 $\mu$Ci $^{35}SO_4$ per ml in separate wells of 24-well microplates. Treatment group cultures contained hormones at multiple concentrations to determine dose-specific responses. Cultures were incubated for 48 – 72 h at 19°C under air.

Following culture, vertebral centra were removed and placed into vials containing saturated $Na_2SO_4$ solution for at least 6 h. Centra were then rinsed in

distilled water overnight to remove excess free $^{35}SO_4$. Rinsed tissue was dried to a constant weight at 60°C. Dried cartilage was weighed to the nearest $\mu$g and placed in scintillation vials containing 0.5 ml 90% formic acid heated to 60°C overnight. After addition of scintillation cocktail (ScintiSafe Plue 50%, Fisher Scientific), samples were analyzed for radioactivity in a Beckman 5000 liquid scintillation counter. Results were expressed as counts per minute (cpm) per $\mu$g dry tissue weight and specific incorporation was calculated by subtracting the average value obtained for non-specific uptake from that of each individual culture.

### *Statistical analyses*

For data sets that were normally distributed, t-tests and one-way ANOVA were used to investigate the differences between control and treatment groups. When this assumption was not satisfied, Kruskal-Wallis tests and Wilcoxon signed rank tests were applied to determine if sulfate uptake differed significantly between control and treatment cultures. Significance was set at $P<0.05$. All results are presented as means $\pm$ SEM.

### *Histologic observations*

The effect of IGF-I on vertebral metabolism was also examined in histologic preparations. Additional cultures were prepared without the use of $^{35}$S-sulfate. Treatment group cartilage was cultured in the presence of IGF-I at a concentration of 100 ng/ml. Control tissues were cultured without the addition of exogenous hormone.

Following 48-h incubation, centra were fixed in Bouin's solution, decalcified, and embedded in paraffin. Transverse sections were cut at 5 $\mu$m, deparaffinized, and stained with hematoxylin-eosin (H&E). Slides were evaluated using a compound microscope.

## RESULTS

### *Growth hormone and insulin-like factor-I*

The effect of IGF-I on vertebral incorporation of $^{35}$S-sulfate was investigated in 3 separate experiments. In the first two experiments, IGF-I significantly increased vertebral GAG synthesis when concentrations of 100 ng/ml were present in culture media (t-test, $P<0.05$, Figs. 1a - b). In the third experiment, vertebral sulfation showed a parabolic response to varying levels of IGF-I, with maximal incorporation of $^{35}$S-sulfate at 10 - 100 ng/ml and a reduced effect at 1000 ng/ml (Fig. 1c). In this latter trial, vertebral GAG synthesis was significantly enhanced by the lower concentrations of IGF-I (t-test, $P<0.05$), yet not by the highest level (t-test, $P = 0.9$).

Vertebral centra treated with IGF-I also demonstrated histologic evidence of increased growth and enhanced cellular activity (Fig. 2). In comparison with control tissue, chondrocytes exposed to IGF-I appeared larger and more differentiated. Nucleocytoplasmic ratios also decreased with IGF-I treatment, suggesting the heightened activity of intracellular membrane systems.

**Figure 5.2.1.** *In vitro* effects of IGF-I on $^{35}$S-sulfate uptake in vertebral cartilage from *R. eglanteria*. Results from 3 experiments are demonstrated. Graphs (**A**) and (**B**) depict effects of IGF-I at the concentration of 100 ng/ml. Graph (**C**) displays dose-specific response of vertebral GAG synthesis to variable concentrations of IGF-I. Values are means ±SEM ($n$ = 7 - 8). Asterisks denote statistical difference from control tissues ($P<0.05$).

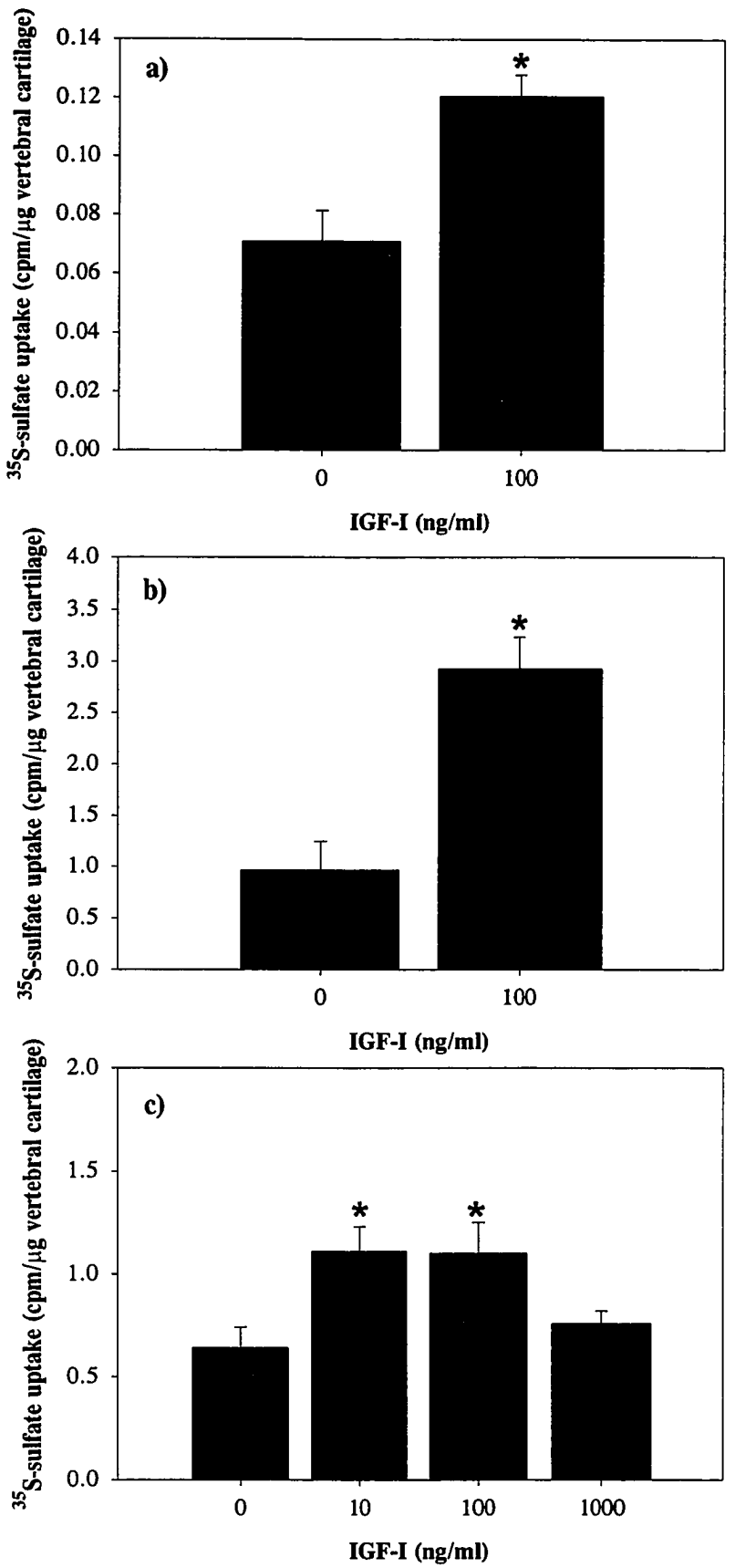
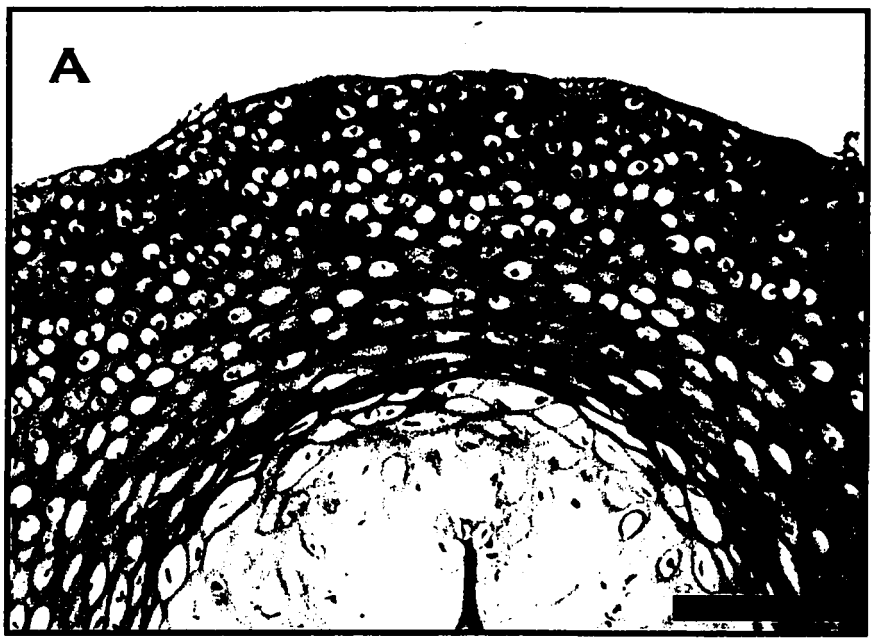
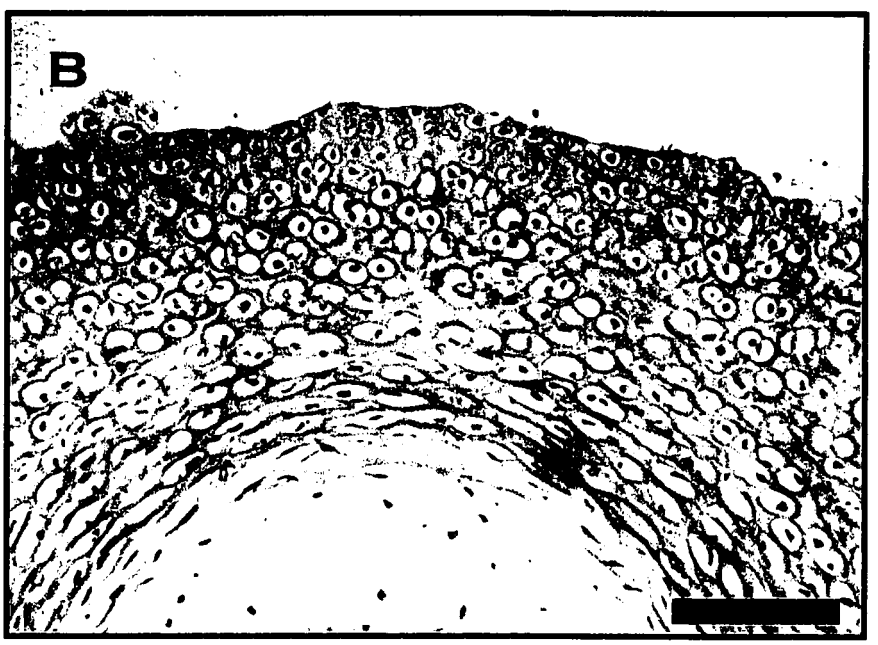


**Figure 5.2.2.** Transverse sections of vertebral cartilage from juvenile *R. eglanteria*, cultured in basal ECM alone **(A)** or in presence of IGF-I at a concentration of 100 ng/ml **(B)**. Chondrocytes in cartilage treated with IGF-I are larger and appear more differentiated. Matrix synthesis appears to be enhanced by hormone treatment. Bar: 100 $\mu$m. H&E stain.



Unlike IGF-I, GH had no effect on $^{35}$S-sulfate uptake in vertebral cartilage from *R. eglanteria* (Fig. 3). Vertebral radioactivity was statistically indistinguishable in explants cultured in the presence of GH, at concentrations of 10, 100, and 1000 ng/ml (ANOVA, $P = 0.37$).

Combined presence of IGF-I and GH in the experimental culture system did not significantly alter GAG synthesis in vertebral cartilage (ANOVA, $P = 0.243$, Fig. 4). Surprisingly, basal stimulation of vertebral sulfation by IGF-I alone was not significantly greater than that observed in the control group (t-test, $P < 0.05$). However, the same hormone solution used in this experiment was effective in stimulating $^{35}$S-sulfate uptake in cartilage from other specimens. This may suggest that the cartilage used for this trial may have been less responsive to hormonal stimulation for reasons unclear.

***Thyroid hormone***

Vertebral GAG synthesis was not influenced by the addition of $T_3$ into the culture system (Fig. 5). Concentrations of 0.75, 7.5, and 75.0 n*M* $T_3$ had no significant effect on sulfate uptake in comparison with control tissues (ANOVA, $P < 0.05$).

***Corticosteroids***

Sulfate uptake was significantly inhibited in centra incubated with corticosterone (Wilcoxon signed rank test, $P<0.05$, Fig. 6). At concentrations of 1 and 10 n*M*,

**Figure 5.2.3.** Lack of effect of GH on $^{35}$S-sulfate uptake in vertebral cartilage of *R. eglanteria*. Values are means ±SEM ($n$ = 7 - 8). No values differ significantly from control tissues.

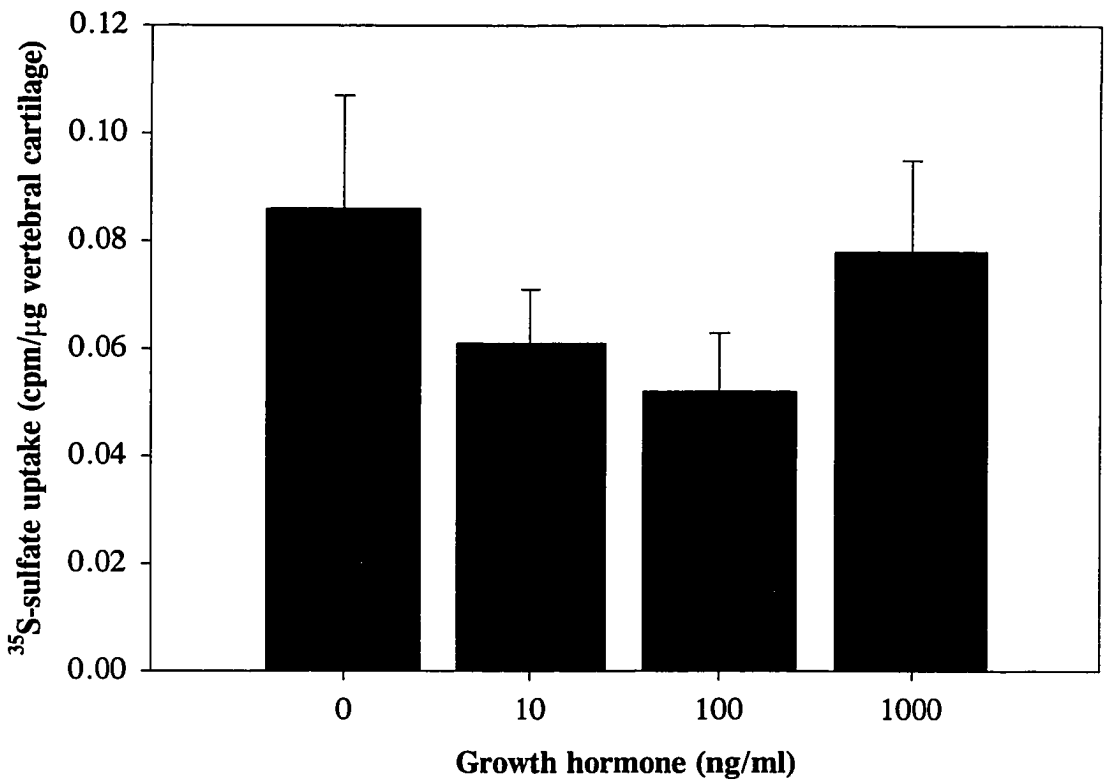


**Figure 5.2.4.** *In vitro* uptake of $^{35}$S-sulfate in vertebral cartilage from *R. eglanteria* exposed to IGF-I alone (100 ng/ml), and in combination with GH (100 and 1000 ng/ml). Values are means ±SEM ($n$ = 7 - 8). No values differ significantly from control tissues.

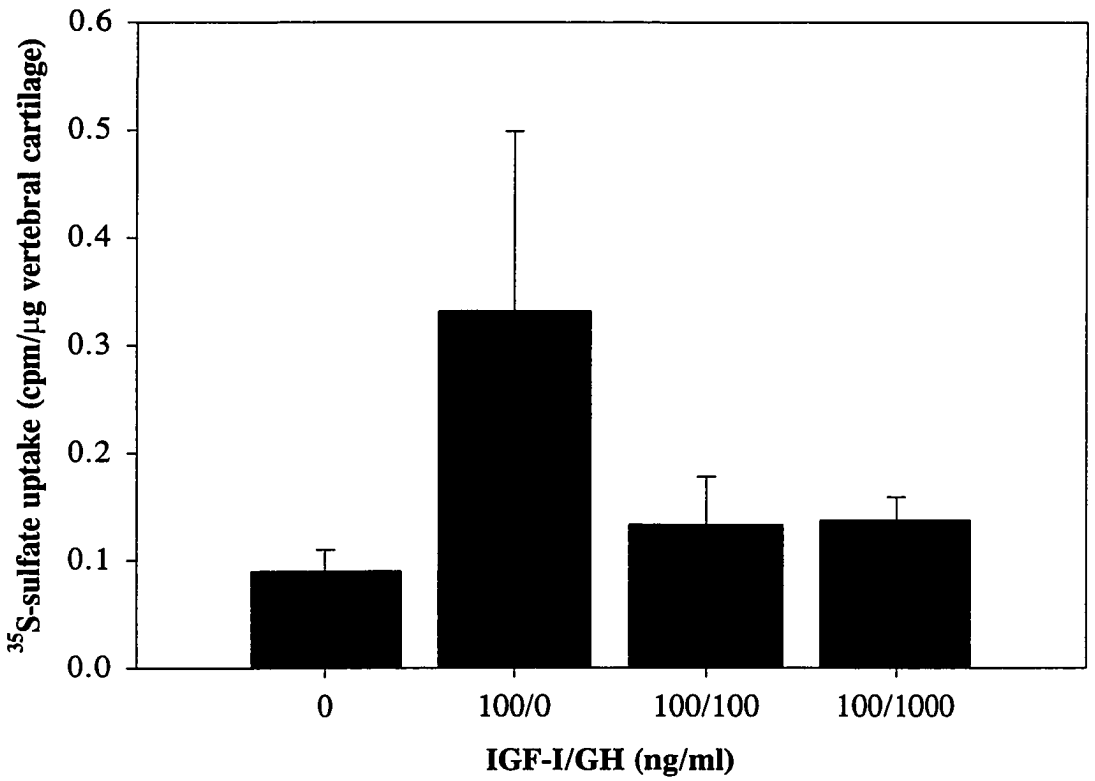


**Figure 5.2.5.** Lack of effect of $T_3$ on $^{35}S$-sulfate uptake in vertebral cartilage from *R. eglanteria*. Values are means ±SEM ($n$ = 7 - 8). No values differ significantly from control tissues.

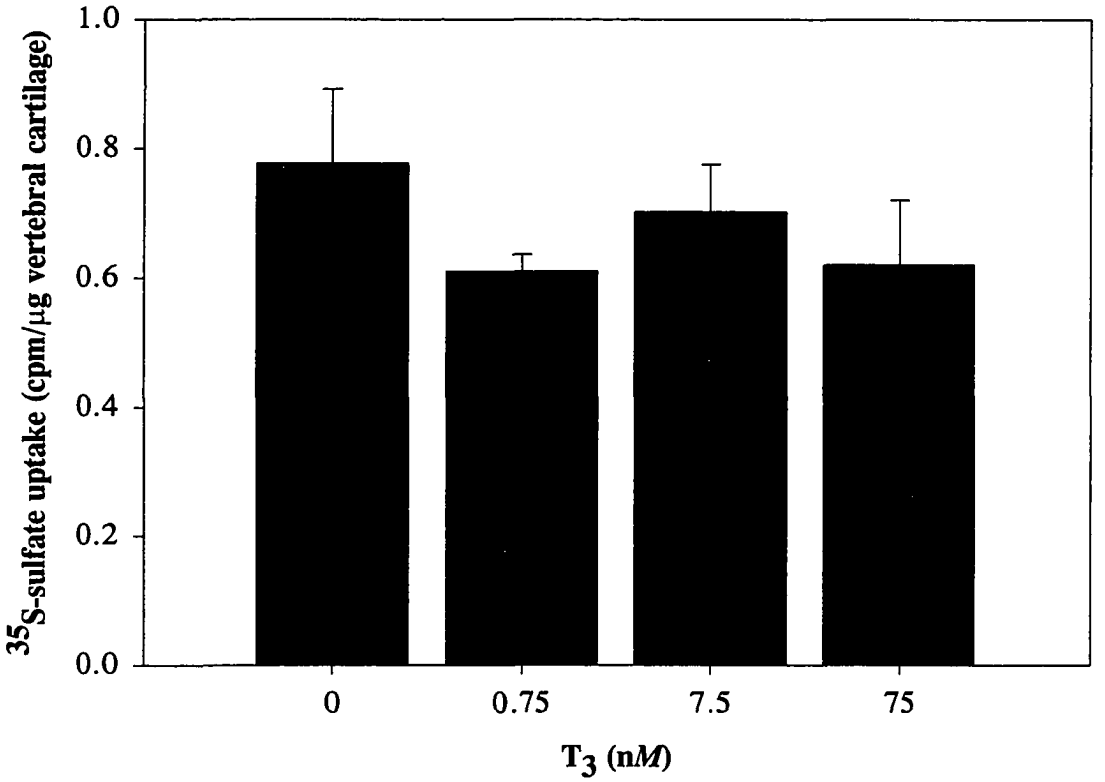
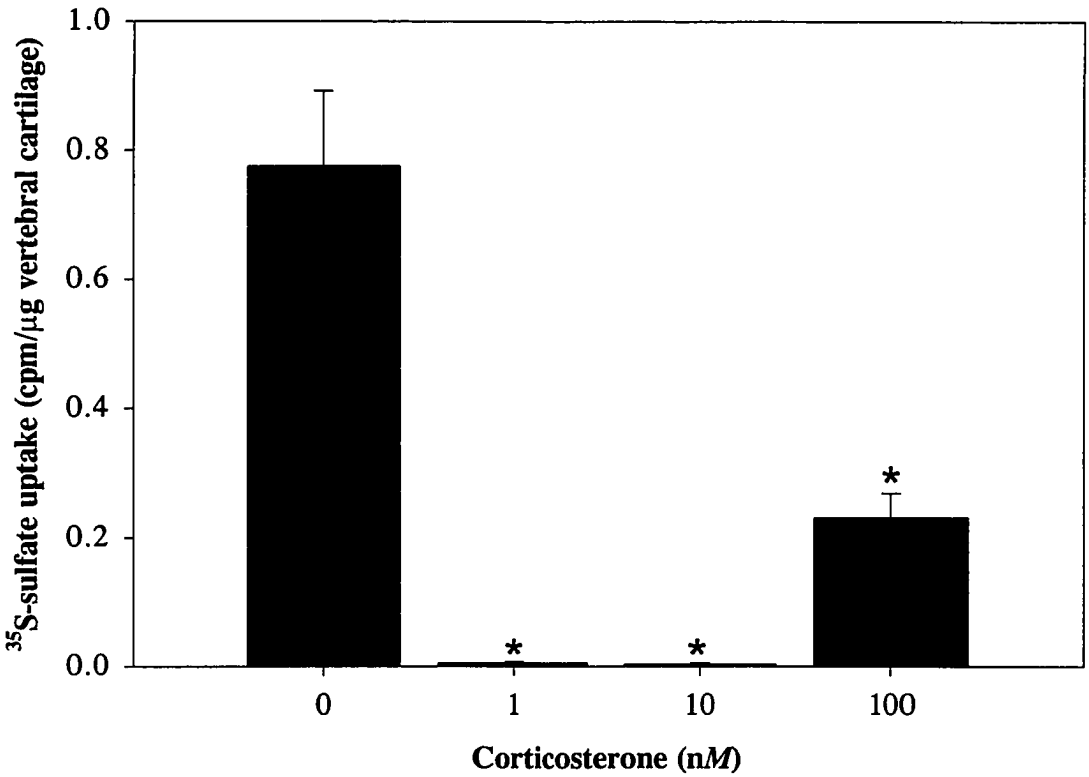


**Figure 5.2.6.** Effect of corticosterone on *in vitro* uptake of $^{35}$S-sulfate in vertebral cartilage from *R. eglanteria*. Values are means ±SEM ($n = 8$). Asterisks represent statistical difference from control tissues ($P < 0.05$).



corticosterone virtually ceased vertebral GAG synthesis. At the higher concentration of 100 n*M*, sulfate uptake was less reduced, yet still significantly lower than that in control tissues.

### *Calcitonin*

At 0.3 n*M*, fCT had no significant effect on GAG synthesis (Wilcoxon signed rank test, $P<0.05$), yet at the higher concentrations of 3.0 and 30.0 n*M*, fCT dose-dependently inhibited vertebral uptake of $^{35}SO_4$ (Wilcoxon signed rank test, $P<0.05$). Sulfate uptake in cartilage treated with 3.0 and 30.0 n*M* fCT was approximately 40% and 2% of that in control cartilage, respectively.

### *PTHrP*

Parathyroid hormone-related protein had no measurable effect on vertebral GAG synthesis (Fig. 8). Concentrations of 0.4 - 4 ng/ml PTHrP did not significantly alter $^{35}$S-sulfate uptake in experimental cultures (One-way ANOVA, $P = 0.6$).

## DISCUSSION

### *Growth hormone and IGF-I*

The results of the present study indicate that IGF-I increases $^{35}$S-sulfate uptake in the vertebral cartilage of *R. eglanteria*. Histologic observations provide evidence that this effect is consistent with increased matrix synthesis and cartilage growth. This

**Figure 5.2.7.** Effect of calcitonin on *in vitro* uptake of $^{35}$S-sulfate in vertebral cartilage from *R. eglanteria*. Values are means ±SEM ($n$ = 8). Asterisks represent statistical difference from control tissues ($P<0.05$).

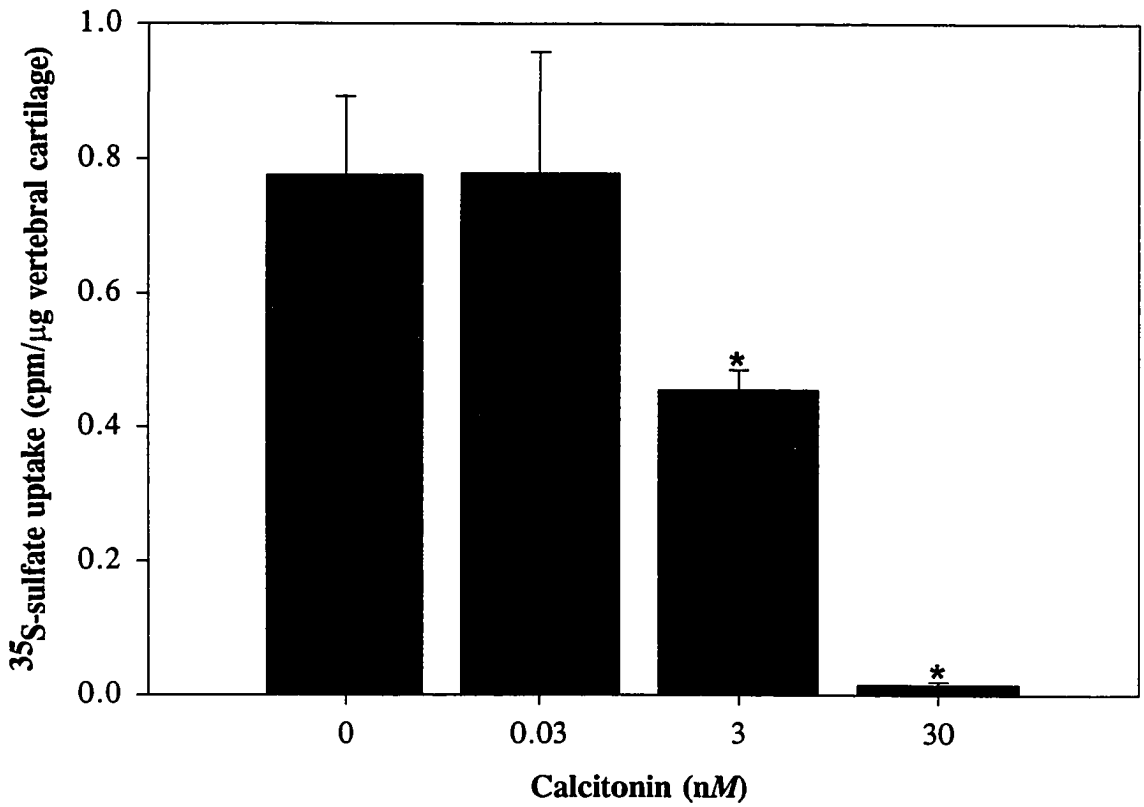
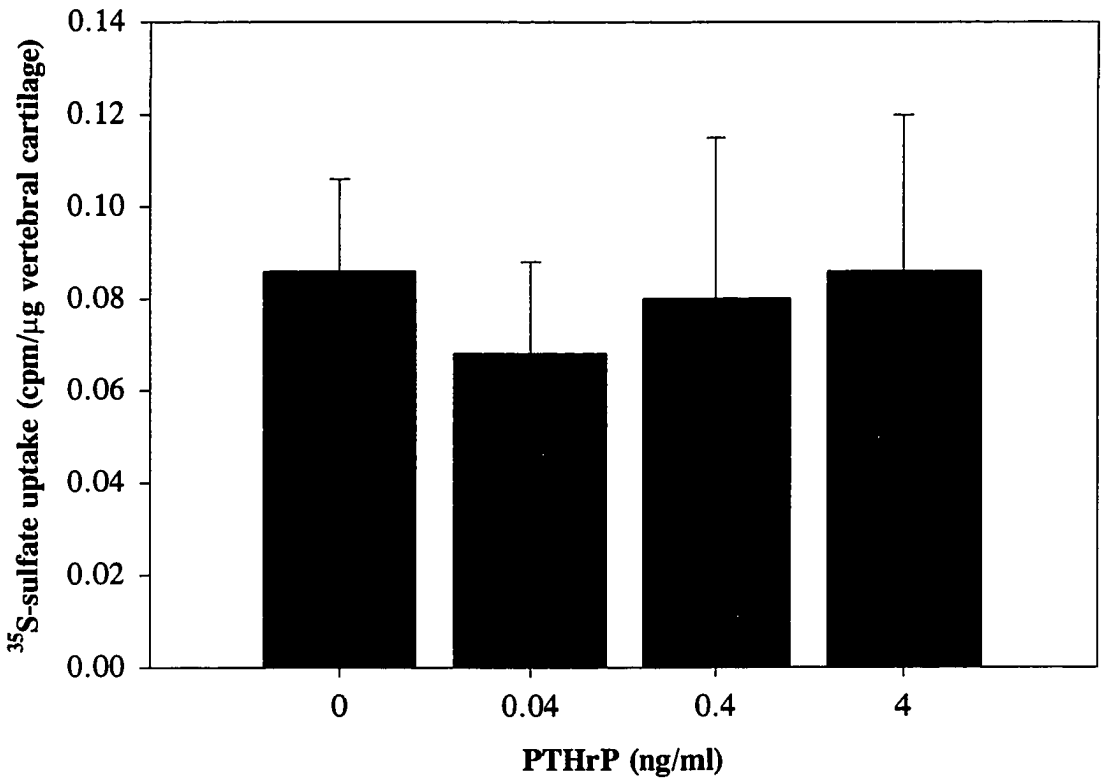


**Figure 5.2.8.** Effect of PTHrP on *in vitro* uptake of $^{35}$S-sulfate in vertebral cartilage from *R. eglanteria*. Values are means ±SEM ($n$ = 8). No values differ significantly from control tissues.



is the first study that has demonstrated the effect of this serum factor on elasmobranch cartilage, and has suggested its importance in the skeletal metabolism of this group. Similar studies have demonstrated that IGF-I also stimulates cartilage growth in teleosts (Duan and Hirano, 1990; Gray and Kelley, 1991; McCormick *et al.*, 1992; Marchant and Moroz, 1993; Cheng and Chen, 1995; Takagi and Björnsson, 1996), amphibians (Schneider and Hanke, 1996), birds (Burch and McCarty, 1983, Böhme *et al.*, 1992), and mammals (Daughaday and Rotwein, 1989). Thus, this physiological mechanism appears to have been highly conserved throughout vertebrate evolution.

To date, no information is available on the serum levels of IGF-I in elasmobranch fishes. Despite this, the present results indicate that the concentrations required to stimulate cartilage growth in elasmobranchs are similar to circulating levels of IGF-I in teleost serum (20 - 100 ng/ml; Niu *et al.*, 1993). In addition, these levels are also similar to those that prompt a similar response in teleost (Cheng and Chen, 1995) and amphibian (Schneider and Hanke, 1996) cartilage. Thus, it is likely that the effect of IGF-I on skate cartilage is of physiological relevance. Nonetheless, information on the serum levels of IGF-I in elasmobranchs and on *in vivo* effects of this growth factor are necessary to confirm its role in elasmobranch skeletal growth.

As demonstrated in Figure 1c, the dose-response curve for the effect of IGF-I on vertebral sulfation appears parabolic in nature. This phenomenon occurs commonly in IGF-I-treated cartilage, and appears to be a consequence of the nutritional and/or hormonal status of experimental animals prior to cartilage removal. For example, Duan and Hirano (1990) observed a reduced effect in cartilage of non-

hypophysectomized Japanese eels (*Anguilla japonica*), when exposed to IGF-I at concentrations greater than 250 ng/ml. In contrast, the dose-response curve for cartilage of hypophysectomized eels exposed to the same dosage levels was linear (Duan and Hirano, 1990). Gray and Kelley (1991) also observed similar discrepancies between cartilage of starved and fed long-jawed mudsuckers, *Gillichthys mirabilis*, treated with 1000 ng IGF-I per ml culture media. In such cases, reduction of serum IGF-I levels through hypophysectomy or short-term food deprivation appears to decrease basal cartilage growth and make chondrocytes more responsive to IGF-I *in vitro* (Gray and Kelley, 1991). Skates used in the present study were not starved, thus a similar relationship between nutritional status and cartilage metabolism likely occurs in elasmobranchs.

In the present study, GH had no direct effect on GAG synthesis in skate vertebral cartilage. Thus, these results support a "somatomedin hypothesis" for the regulation of elasmobranch cartilage growth, in which IGF-I mediates the anabolic effects of GH. The presence of this mechanism in other vertebrates has been well established through similar experiments on cartilage sulfation (Duan and Inui, 1990; McCormick *et al.*, 1992; Cheng and Chen, 1995). In addition, Duan and Inui (1990) observed that GH stimulated growth of coho salmon (*Oncorhynchus kisutch*) cartilage *in vivo*, despite its lack of effect *in vitro*. Similarly, Komourdjian and Idler (1978) determined that GH increased $^{35}$S-sulfate uptake in teleost bone *in vitro* only when liver slices were present in experimental cultures. The liver appears to be the primary site for GH-dependent IGF-I production. Injection of GH increases hepatic IGF-I mRNA in

coho salmon (Cao *et al.*, 1989), and plasma immunoreactivity to mammalian IGF-I in sea bream, *Sparus auratus* (Funkenstein *et al.*, 1990). Furthermore, Pérez-Sánchez *et al.* (1994) recently correlated circulating levels of immunoreactive IGF-I in *S. auratus* with seasonal changes in serum GH and hepatic GH-binding. Similar work needs to be completed in elasmobranchs to better characterize the GH-IGF-I axis in this group.

A widely accepted model termed the "dual effector theory" suggests that GH and IGF-I act synergistically to promote skeletal growth in most vertebrates (Isaksson *et al.*, 1991). Prior treatment of mammals and fish with GH *in vivo* has been shown to enhance the effect of IGF-I on *in vitro* cartilage growth (Lindahl *et al.*, 1987; Gray and Kelley, 1991; Tsai *et al.*, 1994). In addition, Cheng and Chen (1995) found that the combined effect of GH and IGF-I on *in vitro* growth of carp (*Cyprinus carpio*) cartilage was significantly greater than that produced by IGF-I alone. Results from the present study did not provide evidence for a synergistic effect of GH and IGF-I on skate cartilage growth, yet more information is necessary. McCormick *et al.* (1992) also observed no difference in the sulfation activity of salmon cartilage incubated with IGF-I alone or in combination with GH. Cheng and Chen (1995) suggested that this discrepancy may have been associated with the concentration of IGF-I (100 ng/ml) used by McCormick *et al.* (1992), since they observed that the addition of GH to such levels does not cause any appreciable increase in cartilage sulfation. Since the present study used concentrations of IGF-I similar to those described in McCormick *et al.* (1992), presence of the "dual effector theory" in elasmobranch skeletogenesis requires further investigation.

*$T_3$*

In the present study, $T_3$ did not have an effect on *in vitro* GAG synthesis in vertebral cartilage from *R. eglanteria*. However, these results do not confirm that thyroid hormones play no significant role in elasmobranch skeletal growth. Indeed, other studies have also failed to demonstrate effects of both $T_3$ and thyroxine ($T_4$) on *in vitro* growth of teleost skeletal tissues (Komourdjian and Idler, 1978; McCormick *et al.*, 1992). Like the present study, these experiments used culture periods of 2 - 3 days, which Takagi and Björnsson (1996) have recently determined to be too short to properly assess hormonal activity. As evidence, Takagi and Björnsson (1996) demonstrated that $T_3$ stimulation of *in vitro* and *in vivo* cartilage growth is typically delayed, likely due to the indirect effect of $T_3$ mediated through the GH-IGF-I axis. Therefore, the effects of $T_3$ on cartilage growth may not be realized until local production of IGF-I is stimulated. This suggests that longer culture periods are necessary to adequately characterize the role of $T_3$ in elasmobranch cartilage growth. In addition, use of $^3$H-methyl thymidine may be necessary to determine if $T_3$ directly influences chondrocyte proliferation.

*Corticosterone*

Results from the present study indicate that corticosterone significantly inhibits GAG synthesis by skate vertebral chondrocytes *in vitro*. This effect is profound, whereas low levels of corticosterone may cause the near cessation of cartilage growth.

These observations provide the first evidence that corticosteroids may be involved in the skeletal metabolism of elasmobranch fish.

Corticosteroid inhibition of cartilage growth is well documented in most vertebrate groups. For example, Takagi and Björnsson (1997) found that several corticosteroids, particularly cortisol, significantly reduced GAG synthesis in cartilage of the rainbow trout, *Oncorhynchus mykiss*. Disruptions in GAG synthesis have also been observed in embryonic chick pelvic cartilage in association with cortisol treatment (Kilgore *et al.*, 1979). Finally, glucocorticosteroids have been shown to depress matrix synthesis (Tessler and Salmon, 1975; Hill, 1981) and chondrocyte proliferation and differentiation (Weiss *et al.*, 1988) in cartilage of several mammalian species. Together, these studies clearly demonstrate the importance that these hormones play in vertebrate skeletal physiology. Observations from the present study indicate that this role appears to have been highly conserved throughout vertebrate evolution.

Although corticosterone is present in elasmobranchs, 1α-OHB appears to function as the primary mineralo- and glucocorticosteroid in these fishes (Idler and Truscott, 1966; Nunez and Trant, in review). Nonetheless, circulating levels of both hormones are higher than the lowest effective concentration of corticosterone observed in this study (1 n*M*), thus the present observations are likely to be physiologically relevant. However, stimulative actions of other hormones, such as IGF-I, may mask the catabolic effects of corticosteroids, and ensure that they are only expressed when plasma levels are significantly elevated. Interactions of this nature have been observed in teleost cartilage, in which only high levels of cortisol inhibited GAG synthesis while

in the presence of IGF-I (Takagi and Björnsson, 1997). Studies on avian (Calcagno *et al.*, 1970) and mammalian (Kato and Gaspodarowicz, 1985; Takano *et al.*, 1985; Takigawa *et al.*, 1988) cartilage have also demonstrated that the effect of corticosteroids on matrix synthesis may be significantly altered by the presence of plasma growth factors. Since this present study supports a somatomedin hypothesis for elasmobranch vertebral cartilage, it is probable that a similar relationship occurs between corticosteroids and IGF-I. Thus, the role of corticosteroids in vertebral growth may only function during periods of physiological stress or when anabolic factors are low in concentration.

***Calcitonin***

The results of the present study indicate that CT significantly inhibits *in vitro* GAG synthesis in vertebral cartilage of the clearnose skate, *R. eglanteria*. This effect is dose-dependent, whereas high levels (30.0 n*M*) of CT may cause the near cessation of cartilage growth. The present study provides the first evidence that CT may be involved in the skeletal metabolism of elasmobranch fishes. In addition, this is the only study other than Glowacki *et al.* (1985) and Srivastav *et al.* (1998) which has demonstrated any effect of CT on elasmobranch physiology.

The inhibitory effect of CT on elasmobranch vertebral growth differs from that which has typically been observed for other vertebrates. For example, Bélanger *et al.* (1973) found that chronic administration of CT caused enlargement of the epiphyseal cartilage in three species of turtles. Similarly, other studies have observed that CT

increases matrix synthesis, calcification, and growth plate expansion in avian (Suzuki *et al.*, 1976; Kawashima *et al.*, 1980a; b) and mammalian (Baxter *et al.*, 1968; Burch and Corda, 1985; Eguchi *et al.*, 1986) cartilage. Kato *et al.* (1990) confirmed that these effects were directly related to CT treatment, rather than the counter-regulatory increase in parathyroid hormone (PTH) which results from CT administration. However, these effects may still be indirect through the inhibition of cellular production of local growth factors, which may influence cartilage metabolism by autocrine or paracrine mechanisms (Takagi and Björnsson, 1996).

Although the results of most research on higher vertebrates are contrary with the present observations, one study has reported a similar effect of CT treatment in mammalian cartilage. Martin *et al.* (1970) observed that although CT stimulated the synthesis of non-sulfated GAGs, it inhibited the production of sulfated forms. Unfortunately, the significance of this effect is unclear since the role of GAGs in mammalian cartilage calcification remains a controversial topic (Shepard, 1992). Nevertheless, these results suggest that modulation of GAG synthesis may be an important physiological effect of CT. Regardless of whether GAGs initiate or inhibit cartilage calcification, this effect would clearly influence the availability of ionic calcium and phosphate in a vertebrate species.

Some evidence suggests that GAGs may play an inhibitory role in the calcification of elasmobranch cartilage. Ultrastructural studies on elasmobranch jaw cartilage indicate a progressive decrease in GAG content and size between uncalcified and calcified regions (Takagi *et al.*, 1984). Mineralogical studies on jaw cartilage of

the gummy shark, *Mustelus antarcticus*, confirm the loss of sulfated compounds in calcified tissue (Edmonds *et al.*, 1996). In addition, Gelsleichter *et al.* (1995) demonstrated that GAGs extracted from the vertebral cartilage of *R. eglanteria* were effective inhibitors of hydroxyapatite formation *in vitro*. Together, these studies suggest that partial or complete degradation of GAGs by proteolytic enzymes may be necessary for cartilage calcification to occur (Kemp, 1984). If so, CT-induced changes in the synthesis or degradation of cartilage GAGs may promote calcification and produce hypocalcemia, as observed in Srivastav *et al.* (1998). However, it is unlikely that these changes would greatly affect serum calcium levels for a long duration, due to the relative availability of calcium in saltwater environments. Thus, a lack of effect as has been observed in previous studies (Urist, 1967; Louw *et al.*, 1969; Copp *et al.*, 1970; Hayslett *et al.*, 1971) may be expected using *in vivo* methods. In addition, counter-regulatory effects of CT injection may have compromised observations by Glowacki *et al.* (1985) that CT induces hypercalcemia.

As discussed in Chapter 2, mineralogical data suggests that all vertebral tissue is destined to calcify when chondrocytes mature and reduce GAG synthesis. Based upon this, a more feasible scenario for CT's action on vertebral cartilage may be proposed. The inhibition of GAG synthesis by CT may arrest the overall growth and mineralization of vertebral cartilage, making ionic calcium and phosphorus more available for physiological demands. The need for calcium is improbable due to its abundance thus changes in plasma calcium are likely to be rapidly corrected by branchial or renal flux. However, phosphorus is limiting in saltwater environments,

therefore, hyperphosphatemia (Srivastav *et al.*, 1998) may be a important physiological role of CT. Similar responses have been observed in saltwater teleosts (Chan *et al.*, 1968; Fenwick and Lam, 1988), and may mediated through actions on renal as well as skeletal metabolism (Srivastav *et al.*, 1998). Such a role supports the widely-accepted tenet that apatitic skeletons evolved in marine organisms as a means for concentrating phosphorus, an essential element for cellular structure and function (Kemp, 1984).

Recent observations that estrogen increases CT levels in elasmobranchs through interactions with ultimobranchial estrogen receptors (ERs) also suggest a role for CT in reproduction (Takagi *et al.*, 1993; Yamamoto *et al.*, 1996). In mammals, ERs have been identified in the CT-producing cells of the thyroid gland indicating a similar relationship (Naveh-Many *et al.*, 1992). Evidence for the importance of CT in reproduction and sexual maturation of teleosts is also compelling (Björnsson *et al.*, 1989; Norberg *et al.*, 1989; Fouchereau-Peron *et al.*, 1990). Wendelaar Bonga and Pang (1991) have argued that this role may be to protect the maternal skeleton during this period of high calcium demand. Alternatively, CT may be responsible for promoting skeletal mineralization in fetal and juvenile vertebrates. As evidence, Fujisawa *et al.* (1984) demonstrated that serum CT levels are highest at birth and remain elevated during the first year of growth in human subjects. Future studies on CT activity in embryonic, juvenile, and mature elasmobranchs are necessary to investigate the role of this hormone in such physiological processes.

***PTHrP***

In the present study, PTHrP had no effect on matrix synthesis in skate vertebral cartilage. Hormone concentrations used in this assay (0.04 - 4.0 ng/ml) are similar to circulating levels of PTHrP in elasmobranchs (8.5 - 10.6 p*M*; Ingleton *et al.*, 1995), thus modulation of GAG synthesis may not be a physiological function of this factor. However, localization of PTHrP in elasmobranch vertebral cartilage has been confined to the perichondrium (Trivett, pers. comm.), where cell proliferation rather than matrix synthesis is the principal cellular process. Therefore, use of markers for cell proliferation (i.e., $^{3}$H-methyl thymidine) in the culture system may prove more appropriate for investigating the role of PTHrP in elasmobranch chondrogenesis.

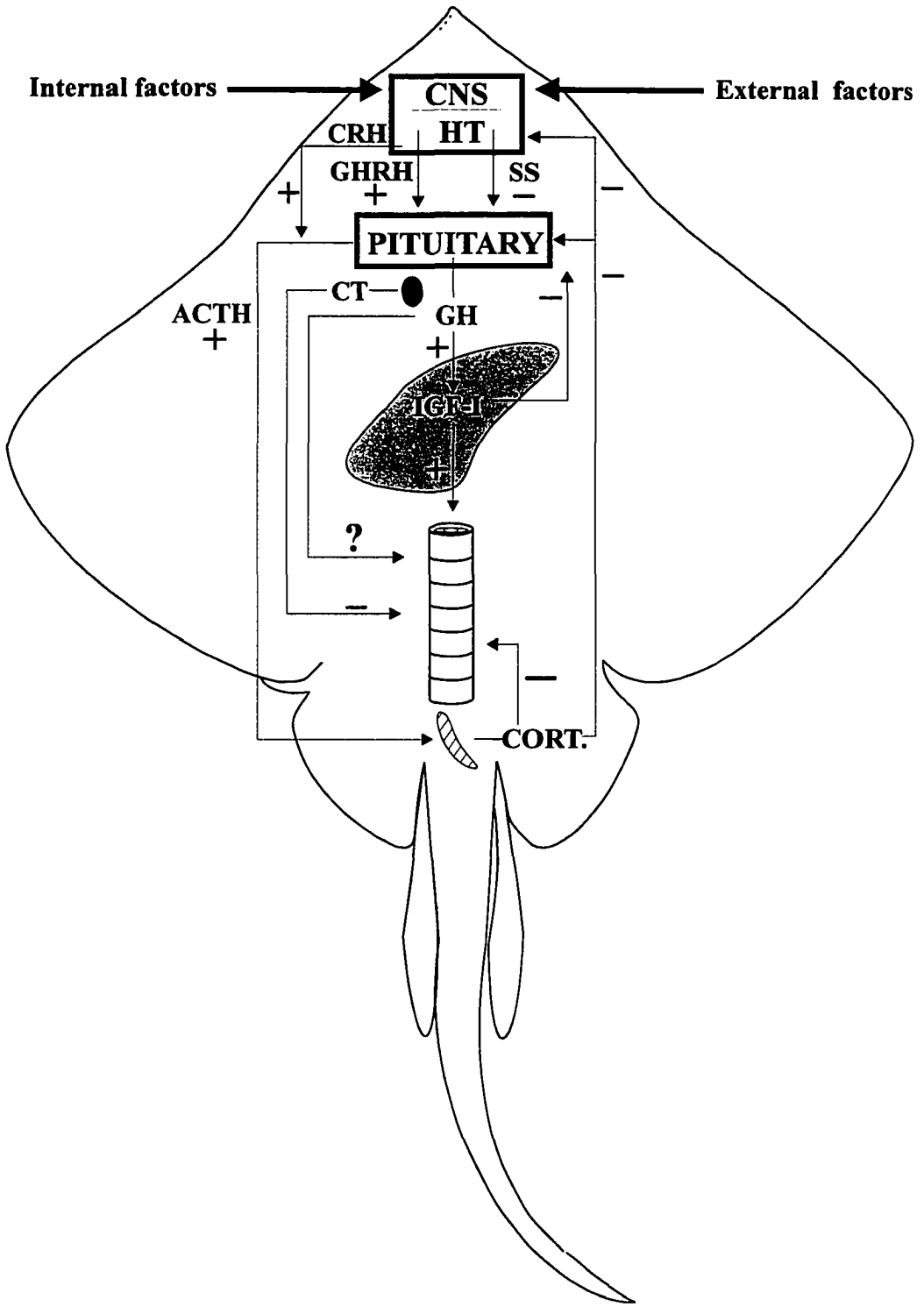
Studies on mammalian cartilage also place emphasis on PTHrP's role in regulating cell proliferation and differentiation. In such studies, PTHrP as been shown to delay these processes, presumably to ensure proper development of skeletal structures (Kronenberg *et al.*, 1997). Loss of the PTHrP gene in laboratory mice causes skeletal abnormalities due to accelerated cellular activity and premature mineralization in growth plate cartilage (Karaplis *et al.*, 1994; Lee *et al.*, 1996). Overexpression of PTHrP is also detrimental to skeletal development due to dramatic retardation of endochondral replacement (Weir *et al.*, 1996). The consequences of disruptions in PTHrP activity are perhaps best shown in humans with Jansen metaphyseal chondrodystrophy, in which persistent activity of PTH/PTHrP receptors causes widespread malformation of the skeletal system (Schipani *et al.* 1995). Clearly, these results emphasize the importance of PTHrP in mammalian skeletogenesis.

Presence of this factor in elasmobranch cartilage stresses the need for additional studies on its function.

### *Summary*

In summary, certain mechanisms responsible for governing skeletal growth appear to be conserved among elasmobranchs and higher vertebrates. Pituitary GH appears to have no direct effect on GAG synthesis in vertebral cartilage of *R. eglanteria*. The GH-dependent serum factor IGF-I promotes vertebral sulfation *in vitro*, suggesting an important role for this hormone in regulating elasmobranch cartilage growth. In contrast, corticosterone retards matrix synthesis in skate vertebral cartilage, suggesting an inhibitory role for glucocorticosteroids in elasmobranch skeletogenesis. Finally, CT also inhibits growth of skate vertebral cartilage *in vitro*. If such actions are expressed *in vivo*, CT may play an important role in skeletal development and mineral homeostasis. Thus, results from the present study suggest that elasmobranch vertebral growth is under complex control by the endocrine system (Fig. 9). Future research will enhance understanding of these control mechanisms by investigating multiple interactions between hormones and re-evaluating the potential role of $T_3$, PTHrP, and GH in regulating cartilage growth.

**Figure 5.2.9.** Proposed model for the hormonal regulation of elasmobranch vertebral growth. Internal and external stimuli convey environmental and physiological information to the central nervous system (CNS), which transmits messages to the hypothalamus (HT) via neurotransmitters and neurohormones. The hypothalamus regulates production of pituitary GH through stimulatory (growth hormone releasing hormone, GHRH) and inhibitory (somatostatin, SS) releasing factors. Growth hormone increases hepatic production of IGF-I which directly stimulates vertebral cartilage growth and decreases GH production through a negative feedback system. Growth hormone may also directly influence vertebral growth (?), yet current data do not support this process. During periods of stress, the hypothalamus produces corticotropin releasing hormone (CRH) which stimulates pituitary secretion of corticotropin (ACTH). Corticotropin stimulates production of various interrenal (shown in hatch) corticosteroids, which inhibit vertebral growth and reduce production of CRH and ACTH through negative feedback. Under certain conditions, the ultimobranchial gland (shown in black) increases production of calcitonin (CT), which inhibits vertebral growth and may influence phosphorus or calcium homeostasis.



# GENERAL DISCUSSION

The formation of incremental marks in calcified structures remains one of the most poorly understood aspects of biomineralization (Simkiss and Wilbur, 1989). In the present study, this complex topic was examined in vertebrae of the clearnose skate, *Raja eglanteria*, by first identifying the structural nature of increments to determine the processes (i.e. growth, mineralization) that lead to their formation. Afterwards, control mechanisms responsible for regulating these processes were examined. Logically, the third and final stage of these investigations requires identification of the internal and external stimuli that cause periodic changes in the physiological regulatory system that would ultimately lead to increment formation.

Results from this dissertation research indicated that increments form in *R. eglanteria* vertebrae due to temporal changes in cartilage growth. Histologic observations demonstrated that growth of vertebral centra is depressed shortly following parturition (i.e. hatching), and between winter and early spring. Such depressions likely drive the production of narrow lines of arrested growth, which were useful for assessing age and growth rate in Chapter 3. There was no evidence that suggested that increments form due to variations in cartilage mineralization. Studies using energy-dispersive spectrophotometry concluded that there were no significant differences in mineral concentration between dissimilar (i.e. narrow, wide) vertebral increments.

This observation was perhaps fortuitous because studies using the radioisotope $^{45}Ca$ were unsuccessful in developing a method to measure vertebral calcification. Efforts to characterize vertebral growth were more successful and produced the first method for *in vitro* culture of elasmobranch cartilage.

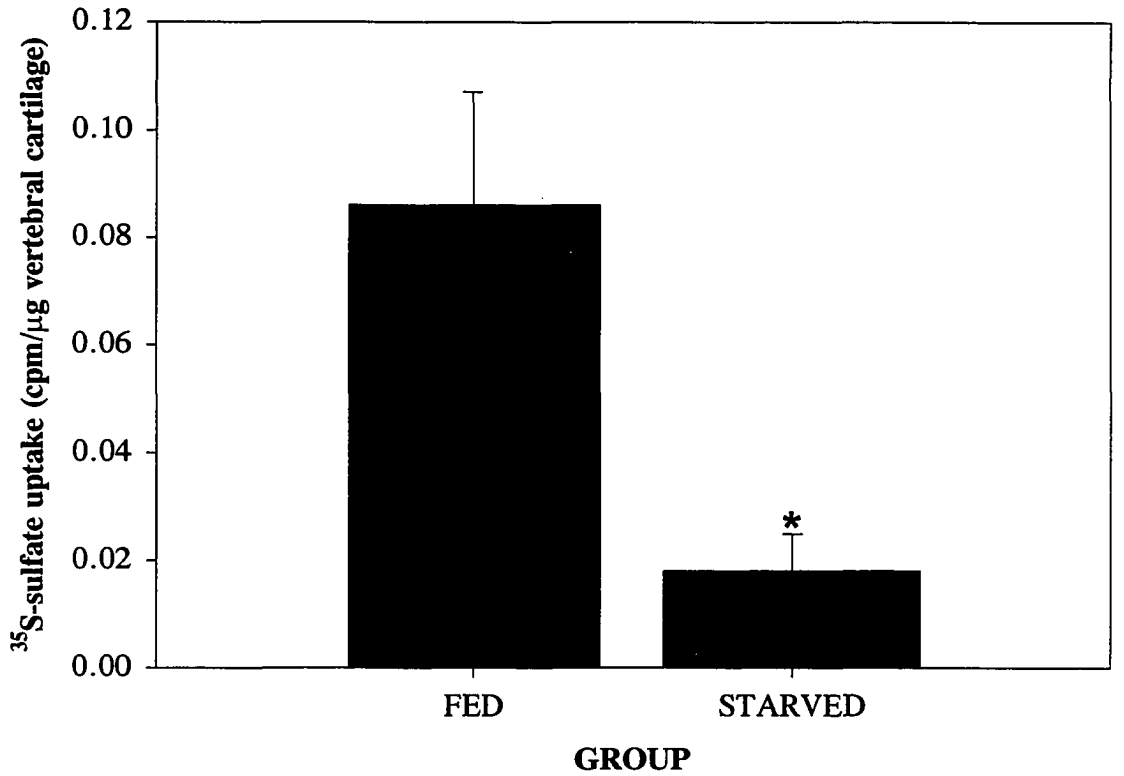
Through use of the *in vitro* culture method, vertebral growth was determined to be under complex control by the endocrine system. Hormonal factors that influence growth of vertebral cartilage appear to be similar to those that regulate skeletogenesis in higher vertebrates. The growth hormone-dependent serum factor, insulin-like growth factor-I (IGF-I), appears to exert a stimulatory effect on vertebral growth, and likely mediates the anabolic effect of growth hormone (GH) on the elasmobranch skeleton. In contrast, corticosterone inhibited vertebral growth, indicating a role for the interrenal gland in elasmobranch chondrogenesis. Calcitonin also inhibited vertebral growth, one of the first physiological effects observed for this hormone in the elasmobranch fishes. Such observations suggest that the hypothalamo-hypophyseal axis exerts the primary influence over vertebral growth by regulating the production of IGF-I and interrenal corticosteroids. Changes in hormone production that may influence vertebral growth are likely caused by environmental or internal factors acting at the level of the central nervous system.

The external and internal stimuli that alter endocrine activity and indirectly influence vertebral growth remain unclear, yet may be inferred by information on growth processes in nonmammalian vertebrates. Typically, these processes are under control of endogenous rhythms regulated by internal "biological clocks", which are

entrained to various environmental cues (e.g. temperature, photoperiod, and food availability). The timing of these processes to environmental cues has obvious implications for the growth, survival, and reproduction of these organisms. For these reasons, seasonal cycles in growth appear to be universal in fishes from temperate regions (Norris, 1997). Recent studies on teleosts have demonstrated that changes in GH secretion, hepatic GH-binding, and IGF-I production follow similarly timed cycles (Pérez-Sánchez *et al.*, 1994). If such cycles occur in elasmobranchs, they may drive the seasonal fluctuations in vertebral growth that eventually lead to increment formation. Unfortunately, no studies have measured serum levels of GH or IGF-I in elasmobranchs, thus this suggestion remains conjectural. Nonetheless, it provides a logical explanation for the seasonal production of vertebral increments in elasmobranchs, and strongly agrees with Simkiss' (1974) theory that growth increments in calcified structures may merely reflect fluctuating levels of GH.

Recent observations by the author demonstrate how cues that influence GH production, particularly nutrition, can indirectly alter vertebral growth in elasmobranchs. For example, short-term starvation (7 d) of juvenile *R. eglanteria* has been shown to significantly depress growth of vertebral cartilage *in vitro* (Fig. 1). Previous studies have also demonstrated this response, which appears to occur due to reduction of GH secretion and circulating IGF-I levels (Gray and Kelley, 1991). A similar response often follows hypophysectomy (Duan and Hirano, 1990), removal of the source of GH production. Thus, a definite link has been expressed between

**Figure D.1.** Mean *in vitro* uptake (±SE) of $^{35}$S-sulfate in vertebral cartilage from starved (7 d.) and non-starved *R. eglanteria*. Data were from 2 animals per group, pooled $n = 16$. Asterisk (*) denotes significant differences between these groups, (t-test, $P<0.05$).



external factors and elasmobranch cartilage growth. Future studies will continue to investigate additional cues that may influence this process.

As the first study on the physiological regulation of elasmobranch skeletal growth, this dissertation has provided substantial background information to be expanded upon in future studies. Methods developed in this dissertation and the observations presented herein will hopefully be useful in clarifying this poorly-understood, yet

## LITERATURE CITED

Abdel-Aziz, S. H. 1992. The use of vertebral rings of the brown ray *Raja miraletus* (Linnaeus, 1758) off Egyptian Mediterranean coast for estimation of age and growth. *Cybium* 16: 121-132.

Ahrenholz, D. W., Fitzhugh, G. R., Rice, J.A., Nixon, S.W., and Pritchard, W.C. 1994. Confidence of otolith ageing through the juvenile stage for Atlantic menhaden, *Brevoortia tyrannus*. *Fish. Bull.* 93: 209-216.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J. D. 1983. Molecular Biology of the Cell. Garland Publishing, Inc. New York.

Albrechtsen, K. A. 1968. A dyeing technique for otolith age reading. *J. Cons.* 32: 278-280.

Aliapoulis, M. A., Goldhaber, P., and Munson, P. L. 1966. Thyrocalcitonin inhibition of bone resorption induced by parathyroid hormone in tissue culture. *Science* 151: 330.

Applegate, S. P. 1967. A survey of shark hard parts. Pages 37-67 *in* P. W. Gilbert, R. F. Matthewson and D. P. Rall , editors. Sharks, Skates and Rays. John Hopkins Press, Baltimore, MD.

Baxter, E., Fraser, J. R. E., Harris, G. S., Martin, T. J., and Melick, R. A. 1968. Stimulation of glycosaminoglycan synthesis by thyrocalcitonin preparations. *Med. J. Aust.* 1: 216.

Baylink, D., Wergedal, J and Thompson, E. 1972. Loss of protein-polysaccharides at sites where bone mineralization is initiated. *J. Histochem. Cytochem.* 20: 279-292.

Beamish, R. J. and Fournier, D. A. 1981. A method for comparing the precision of a set of age determinations. *Can. J. Fish. Aquat. Sci.* 38: 982-983.

Beamish, R. J., and McFarlane, G. A. 1983. The forgotten requirement for age validation in fisheries biology. *Trans. Am. Fish. Soc.* 112: 735-743.

Bélanger, L. F., Dimond, M. T., and Copp, D. H. 1973. Histological observations

on bone and cartilage of growing turtles treated with calcitonin. *Gen. Comp. Endocrinol.* 20: 297-304.

Berry, J., Jr. 1965. The use of vertebral rings to determine age and growth of *Raja eglanteria*, the clearnose skate, in Delaware Bay. Unpublished M. S. Thesis, University of Delaware.

Björnsson, B. Th., Haux, C., Bern, H. A., and Deftos, L. J. 1989. 17ß-estradiol increases plasma calcitonin levels in salmonid fish. *Endocrinol.* 125: 1754-1760.

Blondelon, D., Adolphe, M., Zizine, L., and Lechat, P. 1980. Evidence for glucocorticoid receptors in cultured rabbit articular chondrocytes. *FEBS Lett.* 117: 195-198.

Blumenthal, N. C., Posner, A. S., Silverman, L. D. and Rosenberg, L. C. 1979. Effect of proteoglycans on *in vitro* hydroxyapatite formation. *Calcif. Tiss. Intl.* 27: 75-82.

Böhme, K., Conscience-Egli, M., Tschan, T., Winterhalter, K. H., and Bruckner, P. 1992. Induction of proliferation or hypertrophy of chondrocytes in serum-free culture: The role of insulin-like growth factor-I, insulin, or thyroxine. *J. Cell Biol.* 116: 1035-1042.

Bonfil, R. 1994. Overview of world elasmobranch fisheries. FAO Fish. Tech. Pap. No. 341. Rome, FAO. 119 p.

Bonfil, R., Mena, R., and de Anda, D. 1993. Biological parameters of commercially exploited silky sharks, *Carcharhinus falciformis*, from the Campeche Bank, Mexico. Pages 73-86 *in* S. Branstetter, editor. Conservation Biology of Elasmobranchs. NOAA Tech. Rep. NMFS 115.

Brander, R. and Palmer, D. 1985. Growth rate of *Raia clavata* in the Northeast Irish Sea. *J. Cons. int. Explor, Mer.* 42: 125-128.

Branstetter, S. 1987a. Age and growth estimates of the blacktip, *Carcharhinus limbatus*, and spinner, *Carcharhinus brevipinna*, sharks from the northwestern Gulf of Mexico. *Copeia* 1987: 964-974.

Branstetter, S. 1987b. Age and growth validation of newborn sharks held in laboratory aquaria, with comments on the life history of the Atlantic sharpnose shark, *Rhizoprionodon terranovae*. *Copeia* 1987: 291-300.

Branstetter, S. 1987c. Age, growth, and reproductive biology of the silky shark,

*Carcharhinus falciformis*, and the scalloped hammerhead, *Sphyrna lewini*, from the northwestern Gulf of Mexico. *Environ. Biol. Fish.* 19: 161-173.

Branstetter, S. 1990. Early life-history implications of selected carcharhinoid and lamnoid sharks of the northwest Atlantic. Pages 17-28 *in* H. L. Pratt, Jr., S. H. Gruber and T. Taniuchi, editors. Elasmobranchs as living resources: advances in the biology, ecology, systematics and the status of the fisheries. NOAA Tech. Rep. NMFS 90.

Branstetter, S., and Musick, J. A. 1994. Age and growth estimates for the sand tiger in the northwestern Atlantic. *Trans. Am. Fish. Soc.* 123: 242-254.

Branstetter, S., Musick, J. A., and Colvocoresses, J. A. 1987. A comparison of the age and growth of the tiger shark, *Galeocerdo cuvieri*, from off Virginia and from the northwestern Gulf of Mexico. *Fish. Bull.* 85: 269-279.

Branstetter, S. and Stiles, R. 1987. Age and growth estimates of the bull shark, *Carcharhinus leucas*, from the northern Gulf of Mexico. *Environ. Biol. Fish.* 20: 169-181.

Breder, C. M. and Atz, J. W. 1938. Further notes on the eggs of *Raja eglanteria*. *Copeia* 1938: 145-146.

Breder, C. M. and Nichols, J. T. 1937. The eggs of *Raja eglanteria* Bosc, with a key to the shells of the New York species. *Copeia* 1937: 181-184.

Brooks, R. C., Heidinger, R. C., and Kohler, C. C. 1994. Mass-marking otoliths of larval and juvenile walleyes by immersion in oxytetracycline, calcein, or calcein blue. *N. Amer. J. Fish. Mgmt.* 14: 143-150.

Brown, C. A. and Gruber, S. H. 1988. Age assessment of the lemon shark, *Negaprion brevirostris*, using tetracycline validated vertebral centra. *Copeia* 1988: 747-753.

Bumguardner, B. W., and King, T. L. 1996. Toxicity of oxytetracycline and calcein to juvenile striped bass. *Trans. Am. Fish. Soc.* 125:143-145.

Burch, W. M., and Corda, G. 1985. Calcitonin stimulates maturation of mammalian growth plate cartilage. *Endocrinol.* 116: 1724-1728.

Burch, W. M. and H. E, Leibovitz. 1982a. Triiodothyronine stimulates maturation of porcine growth-plate cartilage in vitro. *J. Clin. Invest.* 70: 496-504.

Burch, W. M. and H. E. Leibovitz. 1982b. Triiodothyroine stimulation of in vitro growth and maturation of embryonic chick cartilage. *Endocrinol.* 111: 462-468.

Burch, W. M. and K. S. McCarty. 1984. Hormonal stimulation of avian embryonic cartilage growth in vitro: Histologic and ultrastructural features. *In Vitro* 20: 329-338.

Cailliet, G. M. 1990. Elasmobranch age determination and verification: an updated review. Pages 157-165 *in* H. L. Pratt, Jr., S. H. Gruber and T. Taniuchi, editors. Elasmobranchs as living resources: advances in the biology, ecology, systematics and the status of the fisheries. NOAA Tech. Rep. NMFS 90.

Cailliet, G. M., Martin, L. K., Kusher, D., Wolf, P. and Welden, B. A. 1983. Techniques for enhancing vertebral bands in age estimation of California elasmobranchs. Pages 157-165 *in* E. D. Prince and L. M. Pulos, editors. Proceedings of the international workshop on age determination of oceanic, pelagic fishes: tunas, billfishes and sharks. NOAA Tech. Rep. NMFS 8.

Cailliet G. M., Natanson, L. J., Welden, B. A., and Ebert, D. 1985. Preliminary studies on the age and growth of the white shark, *Carcharodon carcharias*, using vertebral bands. *South. Calif. Acad. Sci. Mem.* 9: 49-60.

Cailliet, G. M. and Radtke, R. L. 1987. A progress report on the electron microprobe analysis technique for age determination and verification in elasmobranchs. Pages 359-369 *in* R.C. Summerfelt and G.E. Hall, editors. The age and growth of fish. Iowa State University Press, Iowa.

Cailliet, G. M., Radtke, R., and Welden, B. A. 1986. Elasmobranch age determination and verification: a review. Pages 345-360 *in* E. D. Prince and L. M. Pulos, editors. Proceedings of the international workshop on age determination of oceanic, pelagic fishes: tunas, billfishes and sharks. NOAA Tech. Rep. NMFS 8.

Cailliet, G. M., Uchida, S., Laurendine, W., and Brennan, J. 1986. Structure and growth zone formation of vertebral centra from captive Okinawan whale sharks (*Rhincodon typus*). (Abstr. unpubl. pap., Victoria, B.C.). Avail from author, Moss Landing Mar. Lab., P. O. Box 450, Moss Landing, CA 95039.

Cailliet, G. M. Yudin, K. G., Tanaka, S., and Taniuchi, T. 1990. Growth characteristics of two populations of *Mustelus manazo* from Japan based upon cross-readings of vertebral bands. Pages 167-176 *in* H. L. Pratt, Jr., S. H. Gruber and T. Taniuchi, editors. Elasmobranchs as living resources: advances in the biology, ecology, systematics and the status of the fisheries. NOAA Tech. Rep. NMFS 90.

Calcagno, M., Goyena, H., Arrambide, E., and de Urse, C. A. 1970. Action of cortisone and cortisol upon biosynthesis of chondroitin sulfate in femur *in vitro* cultures of chick embryo. *Exp. Cell. Res.* 63: 131-137.

Carlander, K. D. 1969. Handbook of freshwater fishery biology, volume I. Iowa State University Press, Ames.

Carrier, J. C. and Radtke, R. 1988. Preliminary evaluation of age and growth in juvenile nurse sharks (*Ginglymostoma cirratum*) using visual and electron microprobe assessment of tetracycline-labelled vertebral centra. (Abstr. unpubl. pap., Ann Arbor, MI). Avail. From author, Biol. Dep., Albion Coll., Albion, MI 49224.

Casey, J. G. and Natanson, L. J. 1992. Revised estimates of age and growth of the sandbar shark (*Carcharhinus plumbeus*) from the western North Atlantic. *Can. J. Fish. Aquat. Sci.* 49: 1474-1477.

Casey, J. G, Pratt, Jr., H. L. and Stillwell, C. E. 1985. Age and growth of the sandbar shark (*Carcharhinus plumbeus*) from the western North Atlantic. *Can. J. Fish. Aquat. Sci.* 42: 963-975.

Chakrabarti, P. and Mukherjee, D. 1993. Studies on the hypocalcemic actions of salmon calcitonin and ultimobranchial gland extracts in the freshwater teleost *Cyprinus carpio*. *Gen.Comp.Endocrinol.* 90: 267-273.

Chan, D. K. O., Chester-Jones, I., and Smith, R. N. 1968. The effect of mammalian calcitonin on plasma levels of calcium and inorganic phosphate in the European eel (*Anguilla anguilla*). *Gen. Comp. Endocrinol.* 11: 243-245.

Chen, C. -T., Leu, T. C., Joung, S. J., and Lo, N. C. H. 1990. Age and growth of scalloped hammerhead, *Sphyrna lewini*, in northeastern Taiwan waters. *Pacif. Sci.* 44: 156-170.

Cheng, C. M. and T. T. Chen. 1995. Synergism of GH and IGF-I in stimulation of sulfate uptake by teleostean branchial cartilage in vitro. *J. Endocrinol.* 147: 67-73.

Clement, J.G. 1992. Re-examination of the fine structure of endoskeletal mineralization in Chondricthyes: Implications for growth, ageing and calcium homeostasis. *Aust. J. Mar. Freshw. Res.* 43: 157-181.

Conrath, C. Age, growth, reproduction, and demography of the smooth dogfish, *Mustelus canis*. M. S. Thesis research in progress, College of William and Mary.

Copp, D. H., Brooks, C. E., Low, B. S., Newsome, F., O'Dor, R. K., Parkes, C. O., Walker, V., and Watts, E. G. 1970. Calcitonin and ultimobranchial function in lower vertebrates. Pages 281-294 *in* S. Taylor and G. Forster, Editors. Calcitonin 1969. Heinemann, London.

Cuervo, L. A., Pita, J. C. and Howell, D. S. 1973. Inhibition of calcium phosphate

mineral growth by proteoglycan aggregate fractions in a synthetic lymph. *Calcif. Tiss. Res.* 13: 1-10.

Dacke, C. G. 1979. Calcium regulation in sub-mammalian vertebrates. Academic Press, London.

Daiber, F. C. 1960. A technique for age determination in the skate, *Raja eglanteria*. *Copeia* 1960: 258-260.

Danks, J. A., Devlin, A. J., Ho, P. M. W., Diefenbach-Jagger, H., Power, D. M., Canario, A., Martin, T. J., and Ingleton, P. M. 1993. Parathyroid hormone-related protein is a factor in normal fish pituitary. *Gen. Comp. Endocrinol.* 92: 201-212.

Daughady, W. H., Kapadia, M., Yanow, C. E., Fabrick, K., and Mariz, I. K. 1985. Insulin-like growth factor I and II of non-mammalian sera. *Gen. Comp. Endocrinol.* 59: 316-325.

Davenport, S. and Stevens, J. D. 1988. Age and growth of two commercially important sharks (*Carcharhinus tilstoni* and *C. sorrah*) from northern Australia. *Aust. J. Mar. Freshwater Res.* 39: 417-433.

Duan, C. and Hirano, T. 1990. Stimulation of $^{35}$S-sulfate uptake by mammalian insulin-like growth factors I and II in cultured cartilages of the Japanese eel, *Anguilla japonica*. *J. Exp. Zool.* 256: 347-350.

Duan, C. and Inui, Y. 1990. Effects of recombinant eel growth hormone on the uptake of [$^{35}$S]-sulfate by ceratobranchial cartilages of the Japanese eel, *Anguilla japonica*. *Gen. Comp. Endocrinol.* 79: 320-325.

Dubois, W. and Callard, G. V. 1991. Culture of intact sertoli/germ cell units and isolated sertoli cells from *Squalus* testis: I. Evidences of stage-related functions in vitro. *J. Exp. Zool.* 258: 359-372.

Dubois, W. and Callard, G. V. 1993. Culture of intact sertoli/germ cell units and isolated sertoli cells from *Squalus* testis. II. Stimulatory effects of insulin and IGF-I on DNA synthesis in premeiotic stages. *J. Exp. Zool.* 267: 233-244.

Du Buit, M. H. 1972. Age et croissance de *Raja batis* et de *Raja naevus* en Mer Celtique. *J. Cons. int. Explor. Mer.* 37: 261-265.

Duguay, S. J., Chan, S. J., Mommsen, T. P., and Steiner, D. F. 1995. Divergence of insulin-like growth factors I and II in the elasmobranch, *Squalus acanthias*. *FEBS Lett.* 371: 69-72.

Dziewiatkowski, D. D. and Majznerski, L. L. 1985. Role of proteoglycans in endochondral ossification: inhibition of calcification. *Calcif. Tiss. Intl.* 37: 560-564.

Edmonds, J. S., Shibata, Y., Lenanton, R. C. J., Caputi, N., and Morita, M. 1996. Elemental composition of jaw cartilage of gummy shark *Mustelus antarcticus* Günther. *Sci. Total Envirn.* 192: 151-161.

Edwards, R. R. C. 1980. Aspects of the population dynamics and ecology of the white spotted stingaree, *Urolophus paucimaculatus* Dixon, in Port Philip Bay, Victoria. *Aust. J. Mar. Freshwater Res.* 31: 459-467.

Eguchi, M., Shibata, K., Wada, F., Kawamura, H., Shimauchi, T., Shiota, E., and Sugioka, Y. 1986. The calcium antagonist diltiazem inhibits calcification enhanced by calcitonin in growth cartilage of rats in ethane-1-hydroxy-1,1-diphosphonate (EHDP)-induced rickets. *Acta Endocrinol.* 113: 73-81.

Fenwick, J. C. and Lam, T. J. 1988. Effects of calcitonin on plasma calcium and phosphate in the mudskipper, *Periophthalmodon schlosseri* (Teleostei), in water and during exposure to air. *Gen. Comp. Endocrinol.* 70: 224-230.

Ferreira, B. P. and Vooren, C. M. 1991. Age, growth, and structure of vertebra in the school shark *Galeorhinus galeus* (Linnaeus, 1758) from southern Brazil. *Fish. Bull.* 89: 19-31.

Fischer, W. (Ed.) 1978. FAO species identification sheets for fishery purposes. Western central Atlantic (fishing area 31). Volume V. FAO, Rome.

Fitz, E. S., Jr. and F. C. Daiber. 1963. An introduction to the biology of *Raja eglanteria* Bosc 1802 and *Raja erinacea* Mitchell 1925 as they occur in Delaware Bay. *Bull. Bingham Oceanogr. Coll.* 18: 69-97.

Forster, R. P., Goldstein, L., and Rosen, J. K. 1972. Intrarenal control of urea reabsorption by renal tubules of the marine elasmobranch *Squalus acanthias*. *Comp. Biochem. Physiol.* 42A: 2-12.

Fouchereau-Peron, M., Arlot-Bonnemains, Y., Moukhtar, M. S. and Milhaud, G. 1987. Calcitonin induces hypercalcemia in grey mullet and immature freshwater and sea-water adapted rainbow trout. *Comp. Biochem. Physiol.* 87A: 1051-1053.

Fouchereau-Peron, M., Arlot-Bonnemains, Y., Maubras, L., Milhaud, G., and Moukhtar, M. S. 1990. Calcitonin variations in male and female trout, *Salmo gairdneri*, during the annual cycle. *Gen. Comp. Endocrinol.* 78: 159-163.

Fugisawa, Y., Kida, K., Matusuda, H. 1984. Role of change in vitamin D

metabolism with age in calcium and phosphorus metabolism in normal human subjects. *J. Clin. Endocrinol. Metabol.* 59: 719.

Funkenstein, B., Silbergeld, A., Cavari, B., and Laron, Z. 1990. Growth hormone increases plasma levels of insulin-like growth factor-I (IGF-I) in a teleost, the gilthead seabream (*Sparus auratus*). *J. Endocrinol.* 120: R19-R21.

Gauldie, R. W. 1987. The fine structure of check rings in the otolith of the New England roughy (*Hoplostetethus atlanticus*). *New Zealand J. Mar. Freshwater Res.* 21: 267-274.

Gauldie, R. W. 1988. Similarities in the fine structure of annual and non-annual check rings in the otolith of the New Zealand snapper (*Chrysophrys auratus*). *New Zealand J. Mar. Freshwater Res.* 22: 273-278.

Gelsleichter, J., Cortés, E., Manire, C. A., Hueter. R. E., and Musick, J. A. 1997. Use of calcein as a fluorescent marker for elasmobranch vertebral cartilage. *Trans. Am. Fish. Soc.* 126: 862-865.

Gelsleichter, J., Cortés, E., Manire, C. A., Hueter. R. E., and Musick, J. A. 1998. Evaluation of toxicity of oxytetracycline on growth of captive nurse sharks, *Ginglymostoma cirratum. Fish. Bull.* 96: *pages to be determined.*

Gelsleichter, J. and Musick, J. A. 1997. Hormonal regulation of proteoglycan synthesis in vertebral cartilage of the clearnose skate, *Raja eglanteria*. Paper presented at 13th annual meeting of the American Elasmobranch Society, University of Seattle, Seattle, WA.

Gelsleichter, J., Musick, J. A., and Nichols, S. In review. Food habits of the smooth dogfish (*Mustelus canis*), dusky shark (*Carcharhinus obscurus*), Atlantic sharpnose shark (*Rhizoprionodon terraenovae)*, and the sand tiger (*Carcharias taurus*) from the northwest Atlantic Ocean. Manuscript in review, *Environ. Biol. Fish.*

Gelsleichter, J., Musick, J. A. and Van Veld, P. 1995. Proteoglycans from the vertebral cartilage of the clearnose skate, *Raja eglanteria*: Inhibition of hydroxyapatite formation. *Fish Physiol. Biochem.* 14: 247-251.

Gelsleichter, J., Piercy, A., and Musick, J. A. Age and growth of the spiny butterfly ray, *Gymnura altavela*. Research in progress.

Glowacki, J., O' Sullivan, J., Miller, M., Wilkie, D. W. and Deftos, L. J. 1985. Calcitonin produces hypercalcemia in leopard sharks. *Endocrinol.* 116: 827-829.

Gold, E. W. 1979. A simple spectrophotometric method for estimating glycosaminoglycan concentrations. *Anal. Biochem.* 99: 183-188.

Goldman, K. Age and growth of the salmon shark, *Lamna ditropis*. Ph.D. dissertation research in progress, College of William and Mary.

Gray, E. S. and Kelley, K. M. 1991. Growth regulation in the gobiid teleost, *Gillichthyes mirabilis*: roles of growth hormone, hepatic growth hormone receptors and insulin-like growth factor-I. *J. Endocrinol.* 131: 57-66.

Green, H., Morikawa, M., and Nixon, T. 1985. A dual effector theory of growth-hormone action. *Differentiation* 29: 195-198.

Gruber, S. H. and Stout, R. G. 1983. Biological materials for the study of age and growth in a tropical marine elasmobranch the lemon shark *Negaprion brevirostris* (Poey). Pages 193-205 *in* E. D. Prince and L. M. Pulos, editors. Proceedings of the international workshop on age determination of oceanic, pelagic fishes: tunas, billfishes and sharks. NOAA Tech. Rep. NMFS 8.

Guarino, F. M., Andreone, F., and Angelini, F. 1998. Growth and longevity by skeletochronological analysis in *Mantidactylus microtympanum*, a rain-forest anuran from southern Madagascar. *Copeia* 1998: 194-198.

Hales, L. S., Jr. and Hurley, D. H. 1991. Validation of daily increment formation in the otoliths of juvenile silver perch, *Bairdiella chrysoura*. *Estuaries* 14: 199-206.

Hayashida, T. and Lewis, U. J. 1978. Immunochemical and biological studies with antiserum to shark growth hormone. *Gen. Comp. Endocrinol.* 36: 530-542.

Hayslett, J. P., Epstein, M., Spector, D., Myers, J. D., Murdaugh, H. V., and Epstein, F. H. 1971. Effect of calcitonin on sodium metabolism in *Squalus acanthias* and *Anguilla rostrata*. *Bull. Mt. Desert Is. Biol. Lab.* 11: 33-35.

Hayslett, J. P., Epstein, M., Spector, D., Myers, J. D. and Murdaugh, H. V. 1972. Lack of effect of calcitonin on renal function in the elasmobranch, *Squalus acanthias*. *Comp. Biochem.Physiol.* 43A: 223-226.

Hazon, N., Tiernay, M. L., and Takei, Y. In review. The renin-angiotensin system in elasmobranch fish: A review. *J. Exp. Zool.*

Hervas, F., Morreale de Escobar, G., and Escobar Del Ray, F. 1975. Rapid effects of single small doses of L-thyroxine and triiodo-L-thyronine on growth hormone, as studied in the rat by radioimmunoassay. *Endocrinol.* 97: 91-101.

Hettler, W. F. 1984. Marking otoliths by immersion of marine fish larvae in tetracycline. *Trans. Am. Fish. Soc.* 113: 370-373.

Hill, D. J. 1981. Effects of cortisol on cell proliferation and proteoglycan synthesis and degradation in cartilage zones of the calf costochondral growth plate in vitro with and without rat plasma somatomedin activity. *J. Endocrinol.* 88: 425-435.

Hoenig, J. M., Morgan, M. J., and Brown, C. A. 1995. Analysing differences between two age determination methods by tests of symmetry. *Can. J. Fish. Aquat. Sci.* 52: 364-368.

Hoenig, J. M. and Walsh, A. H. 1982. The occurrence of cartilage canals in shark vertebrae. *Can. J. Zool.* 60: 483-485.

Holden, M. J. 1972. The growth rates of *Raja brachyura, R. clavata* and *R. montagui* as determined from tagging data. *J. Cons. int. Explor. Mer.* 34: 161-168.

Holden, M. J. and Vince, M. R. 1973. Age validation studies on the centra of *Raja clavata* using tetracycline. *J. Cons. Int. Explor. Mer.* 35: 13-17.

Idler, D R., Freeman, H. C., and Truscott, B. 1967. Biological activity and protein-binding of 1α-hydroxycorticosterone: an interrenal steroid in elasmobranch fish. *Gen. Comp. Endocrinol.* 9: 207-213.

Idler, D. R. and Truscott, B. 1966. 1α-hydroxycorticosterone from cartilaginous fish: a new adrenal steroid in blood. *J. Fish. Res. Bd. Can.* 23: 615-619.

Ikeda, T., Fujiyama, K., Takeuchi, T., Honda, M., Mokuda, O., Tominaga, T., and Mashiba, H. 1989. Effect of thyroid hormone on somatomedin-C release from perfused rat liver. *Experientia* 45: 170-171.

Ingleton, P. M., hazon, N., Ho, P. M. W., Martin, T. J., Danks, J. A. 1995. Immunodetection of parathyroid hormone-related protein in plasma and tissues of an elasmobranch (*Scyliorhinus canicula*). *Gen. Comp. Endocrinol.* 98: 211-218.

Isaksson, O. G. P., Lindahl, A., Isgaard, J., Nilsson, A., Tornell, J., and Carlsson, B. 1991. Dual regulation of cartilage growth. Pages 121-127 *in* E. M. Spencer, editor. Modern concepts of insulin-like growth factors. Elsevier, New York.

Ishiyama, R. 1951. Studies on rays and skates belonging to the Family *Rajidae*, found in Japan and adjacent regions. 2. On the age determination of Japanese black-skate *Raja fusca* Garman (Preliminary report). *Bull. Jpn. Soc. Sci. Fish.* 16: 112-118.

Ishiyama, R. 1958. Studies on the *Rajid* fishes (*Rajidae*) found in the waters around Japan. *J. Shimonoseki Coll. Fish.* 7: 1-202.

Iwamoto, M., Sato, K., Nakashima, K., Shimazu, A., Kato, Y. 1989. Hypertrophy and calcification of rabbit permanent chondrocytes in pelleted cultures: Synthesis of alkaline phosphatase and 1,25-dihydroxycholecalciferol receptor. *Dev. Biol.* 136: 500-507.

Jensen, C. F., Natanson, L. J., and Branstetter, S. A preliminary estimate of age and growth of the bignose shark, *Carcharhinus altimus*, in the western north Atlantic Ocean. Paper presented at 12th annual meeting of the American Elasmobranch Society, University of New Orleans, New Orleans, LA.

Jikko, A., Murakami, H., Yan, W., Nakashima, K., Ohya, Y., Satakeda, H., Noshiro, M., Kawamoto, T., Nakamura, S., Okada, Y., Suzuki, F., Kato, Y. 1996. Effects of cyclic adenosine 3',5'-monophosphate on chondrocyte terminal differentiation and cartilage-matrix calcification. *Endocrinol.* 137: 122-128.

Johnson, G. F. 1979. The biology of the little skate, *Raja erinacea*, Mitchell, 1825, in Block Island Sound. Unpublished M.S. Thesis. Univeristy of Rhode Island, Kingston.

Kan, K. W., Cruess R. L., and Lacavalier, J. 1983. Zonal analysis of cytoplasmic steroid hormone receptor levels: Replication and synthetic activity in embryonic calf growth cartilage. *Calcif. Tiss. Int.* 35: 740-744.

Karalapis, A. C., Luz, A., Glowacki, J., Bronson, R. T., Tybulewicz, V. L. J., Kronenberg, H. M., and Mulligan, R. C. 1994. Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. *Genes Dev.* 8: 277-289.

Kato, Y. and Gaspodarowicz, D. 1985. Simulation by glucocortocoid of the synthesis of cartilage-matrix proteoglycans produced by rabbit costal chondrocytes in vitro. *J. Biol. Chem.* 260: 2364-2373.

Kato, Y., Shimazu, A., Nakashima, K., Suzuki, F., Jikko, A. and Iwamoto, M. 1990. Effects of parathyroid hormone and calcitonin on alkaline phsophatase activity and matrix calcification in rabbit growth-plate chondrocyte cultures. *Endocrinol.* 127: 114-118.

Kawashima, K., Iwata, S., Endo, H. 1980a. Growth stimulative effect of parathyroid hormone, calcitonin and $N^6$, $O^{2'}$-dibutyryl adenosine 3',5'-cyclic monophosphoric acid on chick embryonic cartilage cultured in serum-free medium. *Endocrinol. Jpn.* 27: 349.

Kawashima, K., Iwata, S., Endo, H. 1980b. Selective activation of diaphyseal chondrocytes by parathyroid hormone, calcitonin and $N^6$, $O^{2'}$-dibutyryl adenosine 3',5'-cyclic monophosphoric acid in proteoglycan synthesis of chick embryonic femur cultivated *in vitro*. *Endocrinol. Jpn.* 27: 357.

Kemp, N. E. 1984. Organic matrices and mineral crystallites in vertebrate scales, teeth and skeletons. *Amer. Zool.* 24: 965-976.

Kilgore, B. S., Mcnatt, M L., Meador, S., Lee, J. A., Hughes, E. R., and Elders, M. J. 1979. Alteration of cartilage glycosaminoglycan protein acceptor by somatomedin and cortisol. *Pediatr. Res.* 13: 96-99.

Killam, K. A. and Parsons, G. R. 1989. Age and growth of blacktip shark, *Carcharhinus limbatus*, near Tampa Bay, Florida. *Fish. Bull.* 87: 845-857.

Komourdjian, M. P. and Idler, D. R. 1978. Hepatic meditation of hormonal and nutritional factors influencing the *in vitro* sulfur uptake by rainbow trout bone. *Gen. Comp. Endocrinol.* 36: 33-39.

Krajnovic-Ozretic, M. and B. Ozretic. 1987. Estimation of the enzymes LDH, GOT and GPT in plasma of grey mullet *Mugil auratus* and their significance in liver intoxication. *Dis. Aquat. Org.* 3: 187-193.

Kronenberg, H. M., Lee, K., Lanske, B., and Segre, G. V. 1997. Parathyroid hormone-related protein and Indian hedgehog control the pace of cartilage differentiation. *J. Endocrinol.* 154: S39-S45.

Kusher, D. I., Smith, S. E. and Cailliet, G. M. 1992. Validated age and growth of the leopard shark, *Triakis semifasciata*, with comments on reproduction. *Environ. Biol. Fish.* 35: 187-203.

Lang, J. B. and Buxton, C. D. 1993. Validation of age estimates in sparid fish using fluorochrome marking. *S. Afr. J. Mar. Sci.* 13: 195-203.

Lawler, E. F., Jr. 1976. The biology of the sandbar shark, *Carcharhinus plumbeus* (Nardo, 1827) in the lower Chesapeake Bay and adjacent waters. Unpublished M. S. Thesis, College of William and Mary, VA. 49 p.

Leary, S., Ballantyne, J. S., and Leatherland, J. F. In review. Regulation of thyroid hormone economy in elasmobranch fishes, with measurements of hepatic 5'-monodeiodinase activity in wild dogfish. *J. Exp. Zool.*

Lee, J. A., Hicks, D. C., Hughes, E. R., Smith, D., and Elders, M. J. 1978.

Cytoplasmic dexamethasone receptors in embryonic chick cartilage: Nature and ontogeny. *J. Steroid Biochem.* 9: 23-28.

Lewis, U. J., Singh, R. N. P., Seavey, B. K., Lasker, R., and Pickford, G. E. 1972. Growth hormone- and prolactin-like proteins of the blue shark (*Prionace glauca*). Fish. Bull. 70: 933-939.

Libby, E. L. and Gilbert, P. W. 1960. Reproduction in the clear-nosed skate, *Raja eglanteria*. *Anat. Rec.* 138: 365.

Lindahl, A., Isgaard, J., and Isaksson, O. G. P. 1987. Growth hormone *in vivo* potentiates the stimulatory effect of insulin-like growth factor-I *in vitro* on colony formation of epiphyseal chondrocytes isolated from hypophysectomized rats. *Endocrinol.* 121: 1070-1075.

Lopez, E., Peignoux-Deville, J., Lallier, F., Martelly, E. and Milet, C. 1976. Effects of calcitonin and ultimobranchial (UBX) on calcium and bone metabolism in the eel, *Anguilla anguilla* L. *Calcif. Tiss. Res.* 20: 173-186.

Louw, G. N., Stahl, P. D., and Kenny, A. D. 1969. Thyrocalcitonin studies in elasmobranch fish. *Zool. Africana* 4: 95-98.

Luer, C.A. 1989. Elasmobranchs as animal models for biomedical research. Pages 121-147 *in* A. D. Woodhead, editor. Nonmammalian Animal Models for Biomedical Research. CRC Press, Florida.

Luer, C. A. and Gilbert, P. W. 1985. Mating behavior, egg deposition, incubation period, and hatching in the clearnose skate, *Raja eglanteria*. *Environ. Biol. Fishes* 13: 161-171.

Luer, C. A., Walsh, C. J., Bodine, A. B., Wyffels, J. T., and Scott, T. R. 1994. The elasmobranch thymus: Anatomical, histological, and preliminary functional characterization. *J. Exp. Zool.* 273: 342-354.

Mahin, D. T. and Lofberg, R. T. 1966. A simplified method of sample preparation for determination of tritium, carbon-14 or sulfur-35 in blood or tissue by liquid scintillation counting. *Anal. Biochem.* 16: 500-509.

Marchant, T. A. and Moroz, B. M. 1993. Hormonal influences on in vitro [$^{35}$S]-sulfate uptake by gill arches from the goldfish (*Carassius auratus* L.). *Fish Physiol. Biochem.* 11: 393-399.

Marking, L. L., Howe, G. E. and Crowther, J. R. 1988. Toxicity of erythromycin,

oxytetracycline, and tetracycline administered to lake trout in water baths, by injection, or by feeding. *Prog. Fish. Cult.* 50: 197-201.

Marquardt, D. W. 1963. An algorithm for least-squares estimation of nonlinear parameters. *J. Soc. Indus. Appl. Math.* 11: 431-441.

Martin, L. K. and Cailliet, G. M. 1988. Age and growth determination of the bat ray, *Myliobatis californica* Gill, in central California. *Copeia* 1988: 763-773.

Martin, T. J., Harris, G. S., Sallis, J. D., de Luise, M. and Melick, R. A. 1970. Effects of calcitonin on glycosaminoglycan synthesis and sulphate utilization *in vitro* and *in vivo*. Pages 281-294 *in* S. Taylor and G. Forster, Editors. Calcitonin 1969. Heinemann, London.

Marx, S. J., Woodward, C. J., and Aurbach, G. D. 1972. Calcitonin receptors of the kidney and bone. *Science* 178: 998-1001.

Mathur, R. 1979. The effect of porcine calcitonin on plasma calcium in *Channa punctatus* Bloch. *J. Fish Biol.* 15: 329-335.

McCormick, S. D., Tsai, P. I., Kelley, K. M., Nishioka, R. S. and Bern, H. A. 1992. Hormonal control of sulfate uptake by branchial cartilage of coho salmon: role of IGF-I. *J. Exp. Zool.* 262: 166-171.

McEachran, J. D. and Musick, J. A. 1975. Distribution and relative abundance of seven species of skates (Pisces: Rajidae) which occur between Nova Scotia and Cape Hatteras. *Fish. Bull.* 73: 110-136.

Michelacci, Y. M. and Horton, D. S. P. Q. 1989. Proteoglycans from the cartilage of young hammerhead shark *Sphyrna lewini*. *Comp. Biochem. Physiol.* 92B: 651-658.

Milet, C., Peignoux-Deville, J. and Martelly, E. 1979. Gill calcium fluxes in the eel, *Anguilla anguilla* (L.). Effects of Stannius corpsucles and ultimobranchial body. *Comp. Biochem. Physiol.* 63A: 63-70.

Monaghan, J. P., Jr. 1993. Comparison of calcein and tetracycline as chemical markers in summer flounder. *Trans. Am. Fish. Soc.* 122: 298-301.

Monsoro-Burq, A. -H., Bontoux, M., Teillet, M. -A., and Le Douarin, N. M. 1994. Heterogeneity in the development of the vertebra. *Proc. Natl. Acad. Sci. USA* 91: 10435-10439.

Mosley, J. M., Hayman, J. A., Danks, J. A., Alcorn, D., Grill, V., Southby, J., and

Horton, M. A. 1991. Immunohistochemical detection of parathyroid hormone-related protein in human fetal epithelia. *J. Clin. Endocrinol. Metab.* 73: 478-484.

Mosley, J. M., Kubota, M., Diefenbach-Jagger, H., Wettenhall, R. E. H., Kemp. B. E., Suva, L. J., Rodda, C. P., Ebeling, P. R., Hudson, P. J., Zajac, J. D., and Martin, T. J. 1987. Parathyroid hormone-related protein purified from a human cancer cell line. *Proc. Natl. Acad. Sci. USA* 84: 5048-5052.

Mugiya, Y., Watabe, N., Yamada, J., Dean, J. M., Dunkelberger, D. G. and Shimizu, M. 1981. Diurnal rhythm in otolith formation in the goldfish, *Carassius auratus*. *Comp. Biochem. Physiol.* 68A: 659-662.

Murdy, E. O., Birdsong, R. S., and Musick, J. A. 1997. Fishes of the Chesapeake Bay. Smithsonian Institution Press, Washington, DC.

Musick, J. A., Branstetter, S., and Colvocoresses, J. A. 1993. Trends in shark abundance from 1974 to 1991 for the Chesapeake Bight region of the U.S. mid-Atlantic coast. Pages 1-18 *in* S. Branstetter, editor. Conservation Biology of Elasmobranchs. NOAA Tech. Rep. NMFS 115.

Natanson, L J. 1993. Effect of temperature on band deposition in the little skate, *Raja erinacea*. *Copeia* 1993: 199-207.

Natanson, L. J. and Cailliet, G. M. 1990. Vertebral growth zones deposition in angel sharks. *Copeia* 1990: 1133-1145.

Natanson, L. J., Casey, J. G. and Kohler, N. E. 1995. Age and growth estimates for the dusky shark, *Carcharhinus obscurus*, in the western North Atlantic Ocean. *Fish. Bull.* 93: 116-126.

Natanson, L. J. and Kohler, N. E. 1996. A preliminary estimate of age and growth of the dusky shark *Carcharhinus obscurus* from the south-west Indian Ocean, with comparisons to the western North Atlantic population. *S. Afr. J. Mar. Sci.* 17: 217-224.

National Marine Fisheries Service (NMFS). 1993. Fishery management plan for sharks of the Atlantic Ocean. U.S. Dep. Commer. NOAA, NMFS, Silver Springs, MD.

Naveh-Many, T., Almogi, G., Livni, N., and Silver, J. 1992. Estrogen receptors and biologic response in rat parathyroid tissue and C cells. *J. Clin. Invest.* 90: 2434-2438.

Neer, J. A. 1998. Aspects of the life history of the Pacific electric ray, *Torpedo*

*californica* (Ayers), in central and southern California. Unpublished M. S. Thesis, California State University, San Jose.

Norberg, B., Björnsson, B. Th., Brown, C. L., Wichardt, U. P., Deftos, L. J., and Haux, C. 1989. Changes in plasma vitellogenin, sex steroids, calcitonin and thyroid hormones related to sexual maturation in female brown trout, (*Salmo trutta*). *Gen. Comp. Endocrinol.* 75: 316-326.

Norris, D. O. 1997. Vertebrate Endocrinology. Academic Press, San Diego.

Nunez, S. and Trant, J. M. In review. Regulation of interrenal gland steroidogenesis in the Atlantic stingray (*Dasyatis sabina*). *J. Exp. Zool.*

Officer, R. A., Gason, A. S., Walker, T. I., and Clement, J. G. 1996. Sources of variation in counts of growth increments in vertebrae from gummy shark, *Mustelus antarcticus*, and school shark, *Galeorhinus galeus*: implications for age determination. *Can. J. Fish. Aquat. Sci.* 53: 1765-1777.

Officer, R. A., Day, R. W., Clement, J. G., and Brown, L. P. 1997. Captive gummy sharks, *Mustelus antarcticus,* form hypermineralized bands in their vertebrae during winter. *Can. J. Fish. Aquat. Sci.* 54: 2677-2683.

Oohira, A., Kimata, K., Suzuki, S., Tanaka, K., Suzuki, I., and Hoshino, M. 1974. A correlation between synthetic activities for matrix macromolecules and specific stages of cytodifferentiation in developing cartilage. *J. Biol. Chem.* 249: 1637-1645.

Orloff, J. J., Wu, T. L., and Stewart, A. F. 1989. Parathyroid hormone-like proteins: Biochemical responses and receptor interactions. *Endocr. Rev.* 10: 476-495.

Ørvig, T. 1951. Histologic studies of placoderms and fossil elasmobranchs. *Ark. Zool. Ser. 2.* 2: 521-454.

Oughterson, S. M., Munoz-Chapuli, R., de Andres, V., Lawson, R., Heath, S. and Davies, D. H. 1995. The effects of calcitonin on serum calcium levels in immature brown trout, *Salmo trutta*. *Gen. Comp. Endocrinol.* 97: 42-48.

Pang, P. K. T. 1971. Calcitonin and ultimobranchial glands in fishes. *J. Exp. Zool.* 178: 89-100.

Parker, H. W. and Stott, F. C. 1965. Age, size and vertebral calcification in the basking shark, *Cetorhinus maximus* (Gunnerus). *Zool. Meded.* 40: 305-319.

Parsons, G. R. 1985. Growth and age estimation of the Atlantic sharpnose shark, *Rhizoprionodon terraenovae*: a comparison of techniques. *Copeia* 1985: 80-85.

Parsons, G. R. 1993. Age determination and growth of the bonnethead shark *Sphyrna tiburo*: a comparison of two populations. *Mar. Biol.* 117: 23-31.

Peck, W., Gennari, C., Raisz, L., Menuier, P., Rits, E., Krane, S., Nuki, G., and Avioli, L. V. 1984. Corticosteroids and bone. *Calcif. Tiss. Int.* 36: 4-7.

Pérez-Sánchez, J., Marti-Planca, H., and Le Bail, P. -Y. 1994. Seasonal changes in circulating growth hormone (GH), hepatic GH-binding and plasma insulin-like growth factor-I immunoreactivity in a marine fish, gilthead seabream, *Sparus aurata*. *Fish Physiol. Biochem.* 13: 199-208.

Preece, M. A. 1976. The effect of administered corticosteroids on the growth of children. *Postgrad. Med. J.* 52: 625-630.

Pike, C. S. 1988. Validation and verification of a temporal periodicity of circulus formation in centra of captive juvenile lemon sharks, *Negaprion brevirostris*. Unpublished M.S. Thesis, University of North Carolina, Wilmington.

Pratt, H. L., Jr. and Casey, J. G. 1983. Age and growth of the shortfin mako, *Isurus oxyrinchus*, using four methods. *Can. J. Fish. Aquat. Sci.* 40: 1944-1957.

Poole, A. R. and Rosenberg, L. C. 1987. Proteoglycans, chondrocalcin, and the calcification of cartilage matrix in endochondral ossification. Pages 187-210 *in* T. M. and R. P. Mecham, editors. Biology of Proteoglycans. Academic Press, Inc., San Diego, CA.

Poole, A. R., Reddi, A. H., and Rosenberg, L. C. 1982. Persistence of cartilage proteoglycans and link protein during matrix-induced endochondral bone development: an immunofluorescence study. *Dev. Biol.* 89: 532.

Ridewood, W. G. 1921. On the calcification of the vertebral centra in sharks and rays. *Phil. Trans. Royal Soc. London* Ser. B. 210: 311 - 407.

Robertson, W.G. 1973. Factors affecting the precipitation of calcium phosphate *in vitro*. *Calcif. Tiss. Res.* 11: 311-322.

Roussouw, G. J. 1984. Age and growth of the sand shark, *Rhinobatos annulatus*, in Algoa Bay, South Africa. *J. Fish Biol.* 25: 213-222.

Ryland, J. S. and Ajayi, T. O. 1984. Growth and population dynamics of three *Raja* species (Batoidei) in Carmarthen Bay, British Isles. *J. Cons. int. Explor. Mer.* 41: 111-120.

Schipani, E., Kruse, K., Jüppner, H. 1995. A constitutively active mutant PTH-PTHrP

receptor in Jansen-type metaphyseal chondrodysplasia. *Science* 268: 98-100.

Schmid, T. H. 1987. Age , growth, and movement patterns of the Atlantic stingray, *Dasyatis sabina*, in a Florida coastal lagoon system. Unpublished M.S. Thesis, University of Central Florida, 85 p.

Schmitt, P. D. 1984. Marking growth increments in otoliths of larval and juvenile fish by immersion in tetracycline to examine the rate of increment formation. *Fish. Bull.* 82: 237-242.

Schneider, A. and Hanke, W. 1996. Influence of insulin-like growth factors and insulin on the [$^{35}$S]sulfate uptake by cartilage of the axolotl (*Ambystoma mexicanum*). *Gen. Comp. Endocrinol.* 104: 92-102.

Schwartz, F. J. 1983. Shark ageing methods and age estimation of scalloped hammerhead, *Sphyrna lewini*, and dusky, *Carcharhinus obscurus*, sharks based on vertebral ring counts. Pages 167-174 *in* E. D. Prince and L. M. Pulos, editors. Proceedings of the international workshop on age determination of oceanic, pelagic fishes: tunas, billfishes and sharks. NOAA Tech. Rep. NMFS 8.

Seki, T., Taniuchi, T., Nakano, H., and Shimizu, M. 1998. Age, growth and reproduction of the oceanic whitetip shark from the Pacific Ocean. *Fish. Sci.* 64: 14-20.

Shepard, N. 1992. Role of proteoglycans in calcification. Pages 41-58 *in* E. Bonucci, editor. Calcification in biological systems. CRC Press, Boca Raton.

Simkiss, K. 1974. Calcium metabolism of fish in relation to ageing. Pages 1-12 *in* T. B. Bagenal editor. Ageing of fish. Unwin Brothers Limited, Surrey, England.

Simkiss, K. and Wilbur, K. M. 1989. Biomineralization: Cell Biology and Mineral Deposition. Academic Press, Inc., San Diego, CA.

Simpfendorfer, C. A. 1993. Age and growth of the Australian sharpnose shark, *Rhizoprionodon taylori*, from north Queensland, Australia. *Environ. Biol. Fish.* 36: 233-241.

Singh, S. and Srivastav, A. K. 1993. Effects of calcitonin administration on serum calcium and inorganic phosphate levels of the fish, *Heteropneustes fossilis*, maintained either in artifical freshwater, calcium-rich freshwater, or calcium-deficient freshwater. *J. Exp. Zool.* 265: 35-39.

Skomal, G. B. 1990. Age and growth of the blue shark, *Prionace glauca*, in the

North Atlantic. Unpublished M. S. Thesis, University of Rhode Island, Kingston, 82 p.

Sminkey, T. R. and Musick, J. A. 1995. Age and growth of the sandbar shark, *Carcharhinus plumbeus*, before and after population depletion. *Copeia* 1995: 871-883.

Smith, J. W. and Merriner, J. V. 1987. Age and growth, movements and distribution of the cownose ray, *Rhinoptera bonasus*, in Chesapeake Bay. *Estuaries* 10: 153-164.

Smith, S. E. 1984. Timing of vertebral band deposition in tetracycline-injected leopard sharks. *Trans. Am. Fish. Soc.* 113: 308-313.

Srivastav, A. K., Srivastav, S. K., Sasayama, Y., and Suzuki, N. 1998. Salmon calcitonin induced hypocalcemia and hyperphosphatemia in an elasmobranch, *Dasyatis akajei*. *Gen. Comp. Endocrinol.* 109: 8-12.

Stevens, J. D. 1975. Vertebral rings as a means of age determination in the blue shark (*Prionace glauca* L.). *J. Mar. Biol. Assoc. U.K.* 55: 657-665.

Stevenson, J. C., Hillyard, C. J., MacIntyre I., Cooper, H., and Whitehead, M. I. 1979. A physiological role for calcitonin: Protection of the maternal skeleton. *Lancet* 2: 769-770.

Stillwell, C. E. and Kohler, N. E. 1993. Food habits of the sandbar shark, *Carcharhinus plumbeus* off the U. S. northeast coast, with estimates of daily ration. *Fish. Bull.* 91: 138-150.

Suva, L. J., Winslow, G. A., Wettenhall, R. E. H., Kemp, V. E., Hudson, P. J., Diefenbach-Jagger, H., Mosley, J. M., Rodda, C. P., Martin, T. J., and Wood, W. I. 1987. A parathyroid hormone-related protein implicated in malignant hypercalcemia: Cloning and expression. *Science* 237: 893-896.

Suzuki, F., Yoneda, T., Shimomura, Y. 1976. Calcitonin and parathyroid hormone stimulation of acid mucopolysaccharide synthesis in cultured chondrocytes from growth cartilage. *FEBS Lett.* 70: 155.

Takagi, M., Parmley, R. T., Denys, F. R., Yagasaki, H. and Toda, Y. 1984. Ultrastructural cytochemistry of proteoglycans associated with calcification of shark cartilage. *Anat. Rec.* 208: 149-158.

Takagi, T., Suzuki, N., Sasayama, Y., and Kambegawa, A. 1993. Plasma calcitonin levels in the stingray, *Dasyatis akajei*. *Zool. Sci.* 10 (Suppl.): 134.

Takagi, Y. and Bjornsson, B. T. 1996. Regulation of cartilage glycosaminoglycan synthesis in the rainbow trout, *Oncorhynchus mykiss*, by 3,3',5-tri-iodo-L-thyronine and IGF-I. *J. Endocrinol.* 149: 357-365.

Takagi, Y. and Bjornsson, B. T. 1997 Cortisol inhibits glycosaminoglycan synthesis in cultured rainbow trout cartilage. *Gen. Comp. Endocrinol.* 108: 80-86.

Takagi, Y., Hirano, J., Tanabe, H., and Yamada, J. 1994. Stimulation of skeletal growth by thyroid hormone administrations in the rainbow trout, *Oncorhynchus mykiss. J. Exp. Zool.* 268: 229-238.

Takano, T., Takigawa, M., and Suzuki, F. 1985. Stimulation by glucocorticoids of the differentiated phenotype of chondrocytes and the proliferation of rabbit costal chondrocytes in culture. *J. Biochem.* 97: 1093-1100.

Takigawa, M., Takano, T., Nakagawa, K., Sakuda, M., and Suzuki, F. 1988. Hydrocortisone stimulation of proliferation and glycosaminoglycan synthesis in rabbit cranofacial chondrocytes in vitro. *Arch. Oral. Biol.* 33: 893-899.

Tanaka, S. 1980. Biological investigations of *Lamna ditropis* in the north western waters of the North Pacific. Report of investigation on sharks as a new marine resource. Japan Marine Fishery Resource Research Center, Tokyo.

Tanaka, S. 1987. Age determination of Garoua stingray *Dasyatis garouaensis*. Pages 45-54 *in* Studies on adaptability and evolution of freshwater elasmobranchs-III: report of the overseas scientific investigation in Africa. *Invest. Com. Freshwater Elasmobranchs*, Nagasaki University.

Tanaka, S. 1990. Age and growth studies on the calcified structures of newborn sharks in laboratory aquaria using tetracycline. Pages 189-202 *in* H. L. Pratt, Jr., S. H. Gruber and T. Taniuchi, editors. Elasmobranchs as living resources: advances in the biology, ecology, systematics and the status of the fisheries. NOAA Tech. Rep. NMFS 90.

Tanaka, S. Cailliet, G. M., and Yudin, K. G. 1990. Differences in growth of the blue shark, *Prionace glauca*: Technique or population? Pages 177-187 *in* H. L. Pratt, Jr., S. H. Gruber and T. Taniuchi, editors. Elasmobranchs as living resources: advances in the biology, ecology, systematics and the status of the fisheries. NOAA Tech. Rep. NMFS 90.

Tanaka, S., Chen, C. -T., and Mizue, K. 1978. Studies on sharks—XVI. Age and growth of Eiraku shark, *Galeorhinus japonicus* Muller et Henle. *Bull. Fac. Fish. Nagasaki University* 45: 19-28.

Tanaka, S. and Mizue, K. 1979. Studies on sharks-XV. Age and growth of Japanese dogfish, *Mustelus manazo* Bleeker in the east China Sea. *Bull. Jap. Soc. Sci. Fish.* 45: 43-50.

Taylor, A. J. and Holden, M. J. 1964. The preparation and use of vertebrae for age determination in rays. *Int. Coun. Explor. Seas, Near North Seas Comm.* No. 145: 4pp.

Termine, J.D. and Eanes, E.D. 1974. Calcium phosphate deposition from balanced salt solutions. *Calcif. Tiss. Res.* 15: 81-84.

Tessler, R. H. and Salmon, W. D. 1975. Glucocorticoid inhibition of sulfate incorporation by cartilage of normal rats. *Endocrinol.* 96: 898-902.

Thiede, M. A. and Rodan, G. A. 1988. Expression of a calcium-mobilizing parathyroid hormone-like peptide in lactating mammary tissue. *Science* 242: 278-280.

Thornson, T. L. and Lacy, E. J., Jr. 1982. Age, growth rate and longevity of *Carcharhinus leucas* estimated from tagging and vertebral rings. *Copeia* 1982: 110-116.

Torrey, T. W. 1971. Morphogenesis of the vertebrates. John Wiley and Sons, New York.

Tsai, P. I., Madsen, S. S., McCormick, S. D., and Bern, H. A. 1994. Endocrine control of cartilage growth in coho salmon - GH influence *in vivo* on the response to IGF-I *in vitro*. *Zool. Sci.* 11: 299-303.

Trivett, M. K., Officer, R. A., Clement, J. G., Walker, T. I., Joss, J. M., Ingleton, P. M., Martin, T. J., and Danks, J. A. In review. Parathyroid hormone-related protein (PTHrP) in cartilaginous and bony fishes. *J. Exp. Zool.*

Truscott, B. and Idler, D. R. 1972. Corticosteroids in the plasma of elasmobranchs. *Comp. Biochem. Physiol.* 42A: 41-50.

Urist, M. R. 1961. Calcium and phsophorus in the blood and skeleton of the Elasmobranchii. *Endocrinol.* 69: 778-801.

Urist, M. R. 1964. Further observations bearing on the bone-blood continuum. Pages 151-178 *in* E. M. Frost, editor. Bone Biodynamics. Brown, Boston.

Urist, M. R. 1967. Avian parathyroid physiology: Including a special comment on calcitonin. *Amer. Zool.* 7: 883-895.

Vale, W., Vaughan, J., Yamamoto, G., Spiess, J., and Rivier, J. 1983. Effects of

synthetic human pancreatic (tumor) GH releasing factor and somatostatin, triiodothyronine and dexamethasone on GH secretion in vitro. *Endocrinol.* 112: 1553-1555.

Villavicerio, L. and Cailliet, G. M. In progress. Age and growth of *Narcine ertenedor.*

Volkoff, H., Wourms, J. P., Amesbury, E., and Snelson, F. F. In review. Structure of the thyroid gland, serum thyroid hormones and the reproductive cycle of the Atlantic stingray, *Dasyatis sabina. J. Exp. Zool.*

von Bertalanffy, L. 1938. A quantitative theory of organic growth (inquiries of growth laws II). *Human Biol.* 10: 181-183.

Walker, T. I., Officer, R. A., Clement, J. G., and Brown, L. P. 1995. Southern age validation: Part I-Project overview, vertebral structure and formation of growth increment bands used for age determination. Final report to the Fisheries Research and Development Corporation, FDRC Project 91/037. Department of Conservation and Natural Resources. Queenscliff, Victoria, Australia.

Walter, J. P. and Ebert, D. A. 1991. Preliminary estimates of age of the bronze whaler *Carcharhinus brachyurus* (Chondrichthyes: Carcharhinidae) from southern Africa, with a review of some life history parameters. *S. Afr. J. Mar. Sci.* 10: 37-44.

Wang, T. M. and Chen, C. T. 1982. Age and growth of smooth dogfish, *Mustelus griseus*, in the northwestern Taiwan waters. *J. Fish. Soc. Taiwan* 9: 1-12.

Waring, G. T. 1984. Age, growth, and mortality of the little skate off the northeast coast of the United States. *Trans. Am. Fish. Soc.* 113: 314-321.

Weir, E. C., Philbrick, W. M., Amling, M., Neff, L. A., Baron, R. and Broadus, A. E. 1996. Targeted overexpression of parathyroid hormone-related peptide in chondrocytes causes chondrodysplasia and delayed endochondral bone formation. *Dev. Biol.* 93: 1024.

Weiss, A., Livine, E., and Silbermann, M. 1988. Glucocorticoid hormone adversely affects the growth and regeneration of cartilage in vitro. *Growth Dev. Aging* 52: 67-75.

Welden, B. A., Cailliet, G. M. and Flegal, A. R. 1987. Comparison of radiometric with vertebral band age estimates in four California elasmobranchs. Pages 301-315 *in* R. C. Summerfelt and G. E. Hall, editors. Age and Growth of Fish. Iowa State Press, Ames, Iowa.

Wendelaar Bonga, S. E., and Lammers, P. I. 1982. Effects of calcitonin on

ultrastructure and mineral content of bone and scales of the cichlid teleost *Sarotherodon mossambicus*. *Gen. Comp. Endocrinol.* 48: 60-70.

Wendelaar Bonga, S. E., and Pang, P. K. T. 1991. Control of calcium regulating hormones in the vertebrates: Parathyroid hormone, calcitonin, prolactin and stanniocalcin. *Int. Rev. Cytol.* 128: 139-213.

Williams, E. E. 1959. Gadow's arculia and the development of tetrapod vertebrae. *Quart. Rev. Biol.* 34: 1-32.

Wintner, S. P. and Cliff, G. 1996. Age and growth determination of the blacktip shark, *Carcharhinus limbatus*, from the east coast of South Africa. *Fish. Bull.* 94: 135-144.

Wilson, C. A., Beckman, D. W. and Dean, J. M. 1987. Calcein as a fluorescent marker of otoliths of larval and juvenile fish. *Trans. Am. Fish. Soc.* 116: 668-670.

Wright, P. J., Talbot, J. C. and Thorpe, J. E. 1992. Otolith calcification in Atlantic salmon parr, *Salmo salar* L., and its relation to photoperiod and calcium metabolism. *J. Fish. Biol.* 40: 779-790.

Yamada, U. 1971. Vital staining of the hard tissues of carp, *Cyprinus carpio* Linne', with tetracycline-HCL, calcein and alizarin red. *Bull. Seikai Regional Fish. Res. Lab.* 41: 107-116.

Yamaguchi, A., Taniuchi, T., and Shimizu, M. 1996. Age and growth of the starspotted dogfish *Mustelus manazo* from Tokyo Bay, Japan. *Fish. Sci.* 62: 919-922.

Yamaguchi, K., Yasuda, A., Lewis, U. J., Yokoo, Y., and Kawauchi, H. 1989. The complete amino acid sequence of growth hormone of an elasmobranch, the blue shark (*Prionace glauca*). *Gen. Comp. Endocrinol.* 73: 252-259.

Yamamoto, K., Suzuki, N., Takahashi, N., Sasayama, Y. and Kikuyama, S. 1996. Estrogen receptors in the stingray (*Dasyatis akajei*) ultimobranchial gland. *Gen. Comp. Endocrinol.* 101: 107-114.

Yudin, K. G. and Cailliet, G. M. 1990. Age and growth of the gray smoothhound, *Mustelus californicus*, and the brown smoothhound, *M. henlei*, sharks from central California. *Copeia* 1990: 191-204.

Zeiner, S. J. and Wolf, P. 1993. Growth characteristics and estimates of age at maturity of two species of skates (*Raja binoculata* and *Raja rhina*) from Monterey Bay, California. NOAA Tech. Rep. 115: 87-99.

# VITA

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# IMAGE EVALUATION
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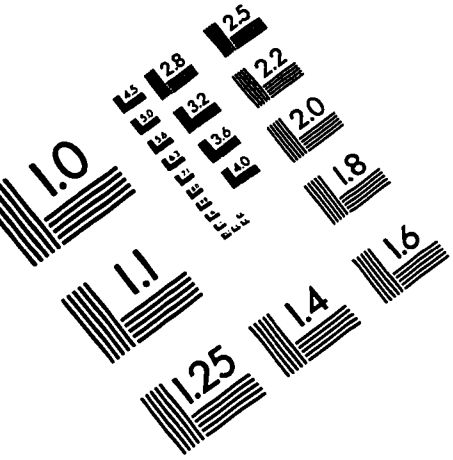


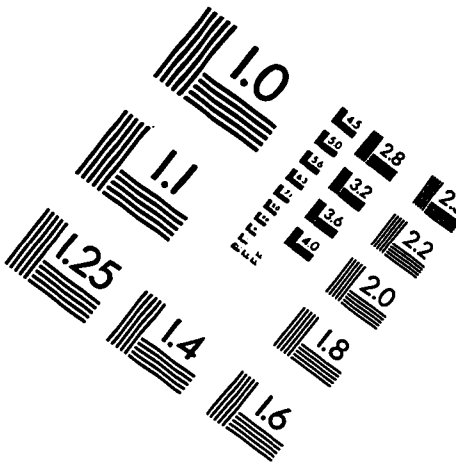


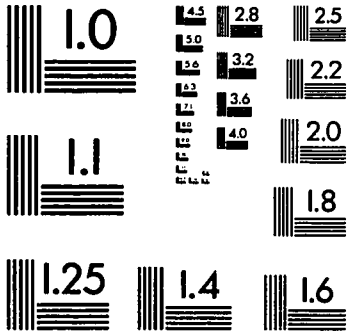


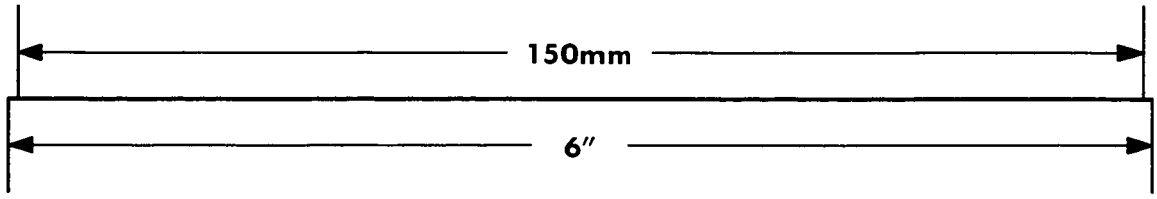


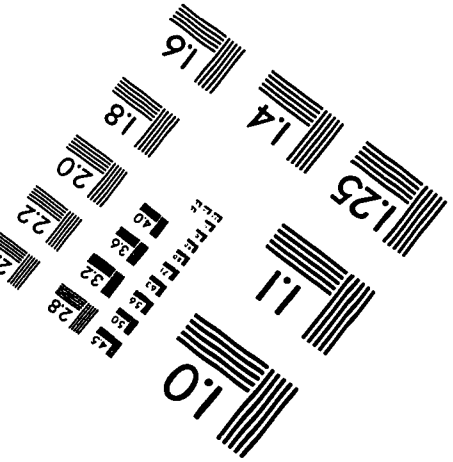


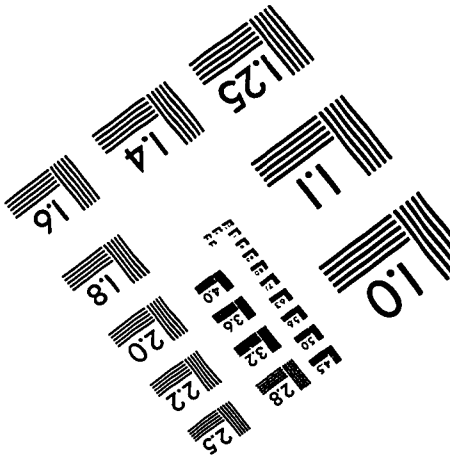


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