

## A SIMPLE TECHNIQUE FOR STAINING GROWTH BANDS IN ELASMOBRANCH VERTEBRAE

*J. M. Hoenig and C. A. Brown*

A variety of techniques have been developed to stain or otherwise clarify growth bands in the vertebral centra of elasmobranchs (Hoenig, 1979; Schwartz, 1983; Cailliet et al., 1983; 1986). Most recent studies have used variations of the alizarin red S staining technique of LaMarca (1966), the silver nitrate technique of Stevens (1975), or the cedarwood oil immersion procedure of Cailliet et al. (1983). These procedures are among the easiest to apply and seemingly provide good resolution of growth bands. However, several authors have concluded that additional efforts should be made to develop and improve aging methods to increase resolution of the crowded bands at the edges of the centra.

This note presents a simple, sensitive, and rapid method for staining elasmobranch vertebrae that have been previously frozen, air dried, or stored in ethanol. The technique uses cobalt nitrate and ammonium sulfide to stain the phosphate in the calcified bands. The staining procedure can be used with minor modifications on whole, split, or sectioned vertebrae.

## PROCEDURE

The procedure can conveniently be divided into six steps, as follows: (1) preparation of vertebral centra, (2) immersion in cobalt nitrate, (3) wash in acid alcohol, (4) immersion in ammonium sulfide, (5) wash in acid alcohol, and (6) preservation of stained specimens. Between each step the centra are washed in distilled water for 30 seconds. These steps are described in detail below.

(1) Dried segments of vertebral columns are soaked in running tap water overnight so that the centra can be easily separated with a knife. Vertebrae that were stored in 70% ethanol are washed in tap water overnight to remove the preservative.

If whole centra are to be stained, the tough layer of loose connective tissue on the faces of the centra must be removed prior to staining. This can be accomplished by soaking the vertebrae in full strength chlorine bleach until the surface is free of connective tissue (approximately 15 to 30 min). Following use of bleach, the centra are washed in tap water for at least 20 min.

If split vertebrae are to be stained, the centra should be cut in half (frontally) with a saw, or ground to one half their original size using a grinding wheel. The exposed face is then polished under tap water using a series of grits ranging from 180 to 600 until the surface is smooth.

Vertebral sections can also be prepared by cutting, grinding and polishing.

(2) The centra are immersed and agitated gently in a 5% solution of cobalt nitrate for 15 min.

(3) Whole centra are washed in an agitated solution of acid alcohol for 1-5 min according to the size of the vertebrae and the species. Split vertebrae are washed for up to 30 min. This step appears to be the most critical in determining the quality of the results. If differentiation is incomplete (poor contrast, everything dark) the wash time should be increased. If the rings are too faint and have poor contrast, the wash time should be reduced. The acid alcohol is prepared by adding concentrated hydrochloric acid to 70% ethanol in a ratio of 1:10.

(4) Centra are immersed in a 5% solution of ammonium sulfide for 2 to 3 min until rings are dark.

(5) Centra are again washed in acid alcohol, as in step 3. This stops the reaction and prevents the centra from becoming progressively darker.

(6) Whole and split centra can be stored in 70% ethanol or air dried. Sections of centra are best stored by mounting on a glass slide with any appropriate mounting medium.

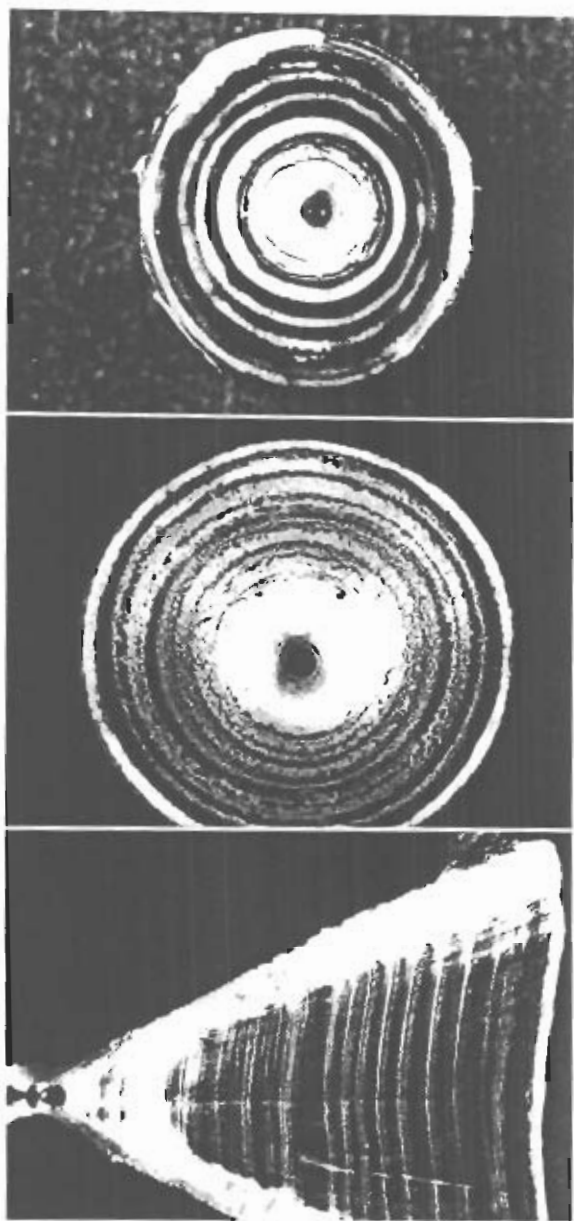


Figure 1. a. Stained whole centrum of an 83.5-cm fork length male smooth dogfish (*Mustelus canis*) from Rhode Island. Diameter = 8 mm. b. Whole centrum from a 116.3-cm fork length male lemon shark (*Negaprion brevirostris*) from Southeast Florida. Diameter = 12 mm. c. Split (frontal cut), polished and stained centrum from a 225-cm fork length male lemon shark (*N. brevirostris*) from Southeast Florida. Vertebra measured 29 mm in diameter.

## RESULTS AND DISCUSSION

Stoeltzner (1905) used cobalt nitrate and ammonium sulfide to stain mammal bones for pathology studies. Wallin (1957) stained teleost scales with these reagents and suggested that this staining procedure might provide a sensitive indicator of recent calcification processes. Keeton (1965) evaluated this suggestion in detail and finally abandoned the idea of using Stoeltzner's reagents to detect recent calcification. Since shark vertebrae can contain large amounts of calcium in the form of apatite (calcium phosphate) (Moss, 1977), and cyclical variations in calcium and phosphorous occur across the face of shark vertebral centra (Cailliet et al., 1986), we felt it would be worth evaluating this combination of reagents for staining phosphate in elasmobranch vertebrae.

We used cobalt nitrate and ammonium sulfide to stain growth bands in whole dried vertebrae from a smooth hammerhead shark (*Sphyrna zygaena*, 282 cm total length, from Northwest Atlantic), several whole and split vertebrae from lemon sharks (*Negaprion brevirostris*, 60–225 cm fork length, from Southeast Florida) which had been previously frozen or stored in ethanol, and on whole vertebrae stored in ethanol from three other species: the smooth dogfish (*Mustelus canis*, various sizes, from Rhode Island), blue shark (*Prionace glauca*, various sizes, from northwest Atlantic), and sandbar shark (*Carcharhinus plumbeus*, various sizes, from northwest Atlantic). Results are shown in Figure 1.

The procedure described here provided as good or better results than those we obtained with silver nitrate or alizarin red S, particularly at the edges of the centra where bands are crowded and resolution tends to be a problem. The use of an acid alcohol wash to stop the reactions may also result in slight etching of the exposed surface. However, it appears that the dominant effect is due to staining rather than etching.

The cobalt nitrate/ammonium sulfide technique is simple, rapid, versatile, and inexpensive. In contrast, silver nitrate is rather expensive and its use requires a source of ultraviolet light. We have found that the silver nitrate method tends to produce large silver grains which can decrease the resolution of closely spaced bands. Alizarin red S stain must be applied over a period of several hours. We conclude that the use of cobalt nitrate and ammonium sulfide deserves further evaluation as a method for enhancing growth bands in elasmobranch vertebrae.

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ADDRESS: Rosenstiel School of Marine and Atmospheric Science, University of Miami, 4600 Rickenbacker Causeway, Miami, Florida 33149-1098.