

A METHOD FOR DATING TETRACYCLINE BIOMARKERS IN BLACK BEAR CEMENTUM

GARY M. MATSON, Matson's Laboratory, P.O. Box 308, Milltown, MT 59851, USA, email: gjmatson@montana.com

KEN D. KERR, Minnesota Department of Natural Resources, Forest Wildlife Research, 1201 East Highway 2, Grand Rapids, MN 55744, USA, email: ken.kerr@dnr.state.mn.us

Abstract: Dating of the tetracycline biomarker in a tooth section of a black bear (*Ursus americanus*) can be accomplished by noting its position relative to that of the cementum annuli which indicate age. However, annuli and biomarkers are best demonstrated by different laboratory techniques. The technique used for the biomarker, calcified sections examined using epi-fluorescence microscopy, does not clearly demonstrate annuli. Conversely, the histological staining technique that is best for viewing annuli must be preceded by decalcification, which destroys the tetracycline biomarker. We combined the methods by first using epi-fluorescent photomicrography to record the biomarker's position in a calcified section. Next, the section was decalcified and stained to demonstrate cementum annuli and photographed using bright-field illumination. The resulting 2 photographs were compared to date the biomarker to the nearest year by determining its exact position relative to annuli.

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Tetracycline and its derivatives have been used in wildlife studies to produce time-specific biomarkers in calcified body tissues. When thin sections of calcified tooth or bone are examined using epi-fluorescence microscopy, the incorporated tetracycline appears as a bright yellow fluorescent tissue layer. Thin sectioning of calcified tissues is described by Weaker and Richardson (1978). The general use of tetracycline biomarkers was reviewed by Johnston et al. (1987). Johnston (1975) used the method to label bait-vaccinated foxes (*Vulpes* sp.), and Day and Carrel (1986) used it to calibrate cementum growth layers of javelina (*Tayassu tajacu*). Garshelis and Visser (1997) estimated the statewide population of black bears in Minnesota by using tetracycline biomarkers. Van Brackle et al. (1994) tested acceptance and effectiveness of tetracycline as an orally administered biomarker in white-tailed deer (*Odocoileus virginianus*).

The position of the fluorescent biomarker relative to the cementum annuli indicates the age at the time of tetracycline administration. Unfortunately, annuli of calcified tooth sections are not always readily visible under epi-fluorescent microscopy. Conversely, the biomarker is not visible in decalcified tooth sections. To clearly view both the tetracycline biomarker and cementum annuli, we devised a method that successively employs 2 techniques on the same tooth section.

METHODS

Tetracycline Biomarker Detection

First premolar teeth from hunter-killed Minnesota black bears were thoroughly cleaned to remove all soft

tissues. A Buehler low-speed saw (Buehler Ltd., 41 Waukegan Road, Lake Bluff, Ill.) equipped with two 4 x 0.012 inch (10 cm x 0.30 mm) diamond blades (Streurs RS-70308, 230CA, VWR Scientific, P.O. Box 66929, Chicago, Ill.) was used to cut a longitudinal section of the tooth root. The blades were separated by 2 spacers 2.5 inches (6.4 cm) in diameter made from acetate film (PP2500 Transparency Film, 3M Company, St. Paul, Minn.) to achieve an approximate section thickness of 100 microns (within 20 microns). A 5–10 mm section of the tooth root was cut at a blade speed of 300 rpm while the tooth was held rigidly in the specimen holder. The section was mounted on a microscope slide using either dilute glycerol or glycerol jelly with a glass coverslip applied on top, and examined with an epi-fluorescence microscope (Leitz Laborlux S, Bartels and Stout, Inc., P.O. Box 1994, Bellevue, Wash.). The epi-fluorescence microscope was a compound microscope with a beam of ultraviolet light that first passes through filters, then through the objective to light the specimen from above the microscope stage. The filter combination providing the best visual contrast was a 405/10 nm excitation filter, a 430 nm dichroic mirror, and a 435 nm barrier filter.

Biomarker Documentation

The location of the biomarker in the tooth section was documented with 1 or more color epi-fluorescent illumination photomicrographs. Fluorescence photomicrography was done with Kodacolor Gold 100 color print film (Eastman Kodak Company, Rochester, N.Y.) exposed for 30 seconds. Interference from ambient light was eliminated by completely darkening the room. (This

film-exposure method may vary greatly among different microscope-illumination-camera setup combinations).

We confirmed the usability of the photograph before proceeding with the next step. The exact orientation of the tooth section and morphological landmarks were sketched so the section could be removed for subsequent processing and re-mounted with an identical orientation.

Histological Staining

After photographing the biomarker, the section was decalcified and stained as follows. The section was rinsed in water to remove mountant, then decalcified with 1 normal hydrochloric acid (HCl) for 20 minutes. After it was rinsed in distilled or deionized water for 15 minutes, the section was stained in modified Harris hematoxylin (Cat.# HHS, Sigma Chemical Co., St Louis, Mo.) for 10 minutes and rinsed again in distilled water for 10 minutes. Next, the section was rinsed in mildly basic water (approximately 0.1 g of baking soda/500 ml) for 5 minutes to blue the hematoxylin, remounted with its original orientation using reference notes and sketches, and excess water was removed from around the section. Dilute glycerine or glycerol jelly was placed on the section before it dried and a coverslip applied.

Biomarker Dating

With the fluorescence photomicrographs for reference, identical views of the stained section were photographed through the microscope under bright-field illumination (Kodacolor Gold 100 film, blue filter No. 80A). We determined the age at biomarking by measuring between corresponding reference points on the epi-fluorescent and bright-field photomicrographs and determining the biomarker position relative to cementum annuli age indicators.

RESULTS AND DISCUSSION

The comparison of the epi-fluorescence photomicrograph of the calcified section with the bright-field photomicrograph of the decalcified, stained section successfully dated the biomarker (Figs. 1, 2). When biomarker location could not be clearly determined in the tooth section because of closely spaced annuli, visibility was improved by increasing microscope magnification, photographic enlargement, or both.

Annuli were more clearly identifiable and decalcification caused less shrinkage when section thickness was near 100 microns. Minimizing section shrinkage permitted better correlation of measured reference points when

comparing photographs of the same view of the section before and after decalcification. However, when sections were cut thicker than 100 microns, the greater thickness interfered with microscopic detail.

Teeth from which sections have been cut by the low speed saw can be subsequently processed for cementum aging using standard methods of laboratory tooth sectioning. The tooth can be decalcified, sectioned with a microtome at right angles to the plane of the saw cut, mounted, and stained. Sections prepared in this way are more suitable for detailed study of the cementum than thicker sections cut with the saw. They also show the slot where the section was removed. Correlating annuli locations between sections cut with the microtome and those cut with the saw can facilitate aging and biomarker dating when there is a need to increase the reliability of a biomarker location.

Inability to date a biomarker may occur when the point of highest biomarker visibility in the calcified tooth section corresponds with a point of poor histological detail in the same section after it is decalcified and stained. To maximize the chance of a clearly demonstrable correlation between biomarker and annuli, photomicrographs should be taken of several areas of the section. Otherwise, the opportunity to relocate the biomarker is lost because it is removed by decalcification.

Correct biomarker dating depends upon unequivocal cementum annuli identification throughout the entire cementum thickness. Errors may occur in teeth of older bears because annuli at the outer root surface in tooth sections may be too closely spaced to identify individually. The upper third incisor tooth (UI3) is larger and has a thicker cementum layer than the more commonly used first premolar tooth and is a better choice for tetracycline biomarker studies of material from dead animals. We do not recommend extraction of the UI3 from live bears because of animal welfare considerations.

This method should be confirmed on teeth with no other research value before important study specimens are processed. Although most researchers seem to prefer longitudinal sections, transverse sectioning can work. Because the tooth length is greater than its breadth, a greater number of transverse sections can be cut for viewing and staining to compare the biomarker position with adjacent annuli. However, the longitudinal section permits more certain identification of the first annulus, which is not clearly visible in all transverse sections because of its highly variable separation from the dentine-cementum junction.

Mechanically marking the fluorescing layer in the calcified tooth section during fluorescent microscopic ex-

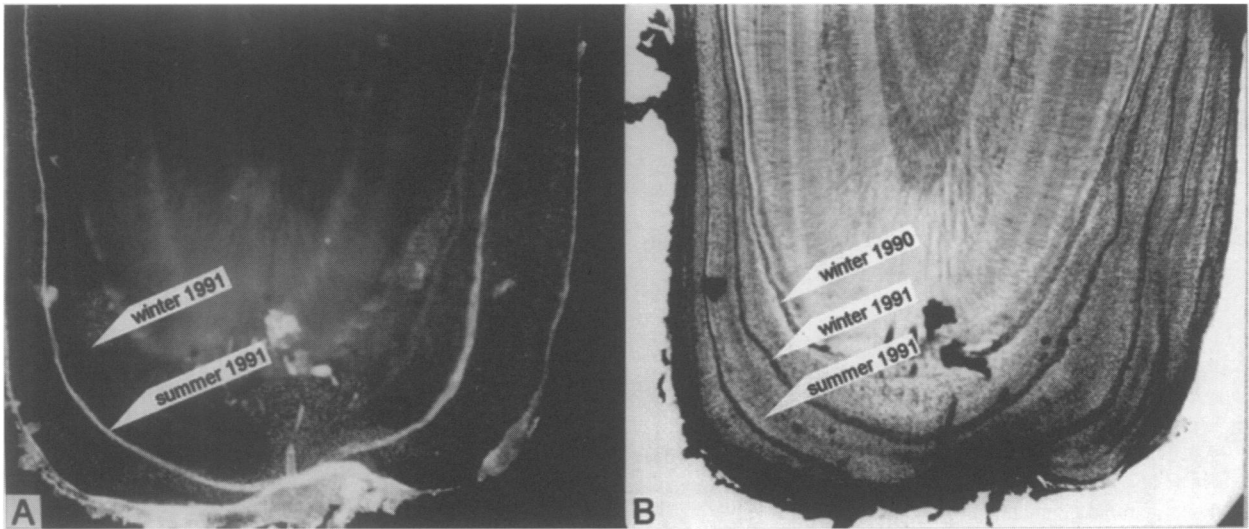


Fig. 1. A. Fluorescing tetracycline biomarker in the calcified section viewed with epi-fluorescence microscopy. B. Decalcified, stained section viewed with compound bright-field microscopy. Correlating annuli between the photographs permits accurate biomarker dating. Female Minnesota black bear #4946; born January 1990, marked July 1991. Approximately 25X.

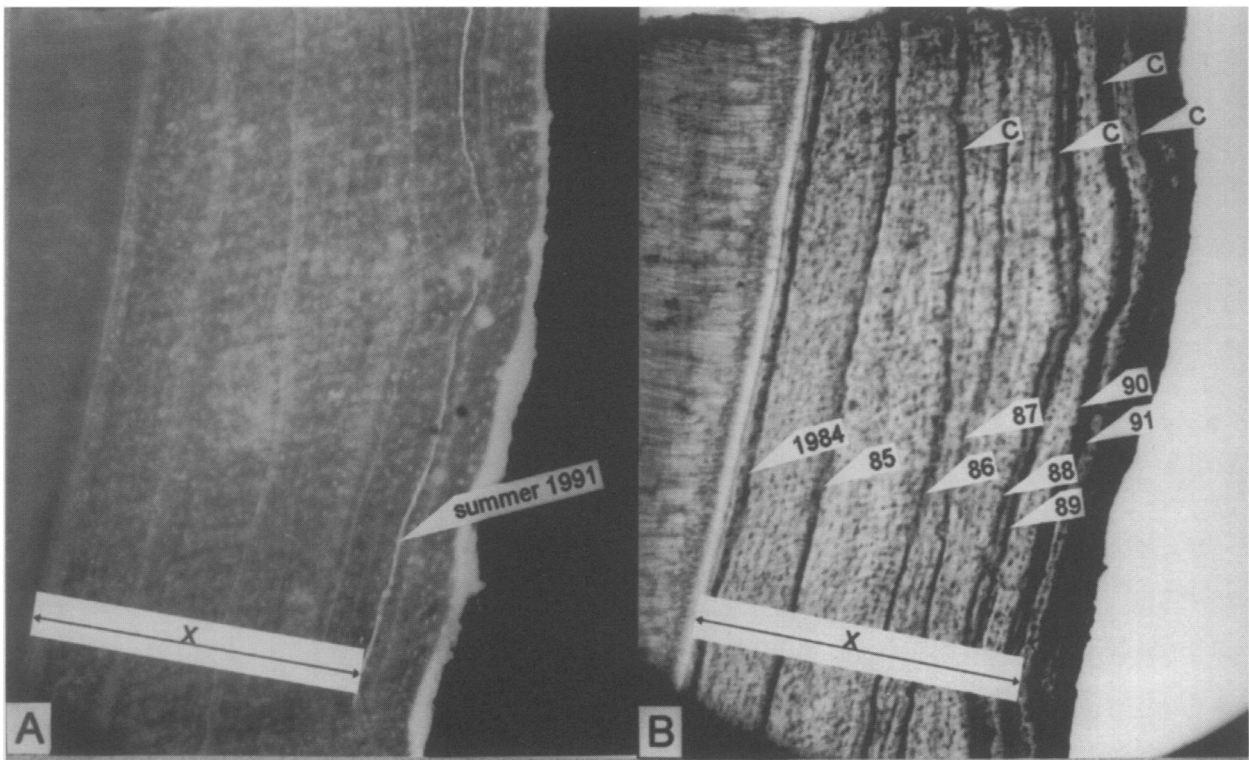


Fig. 2. A. Fluorescing tetracycline biomarker in the calcified section viewed with epi-fluorescence microscopy. B. Decalcified, stained section viewed with compound bright-field microscopy. Measurement (X) locates the biomarker and date it was formed. Female Minnesota black bear #13751; born January 1983, marked summer 1991 during 8th year of life. The ultraviolet light of the fluorescent microscope may not clearly define all of the dark cementum annuli, as can be seen by comparing the fluorescence and bright-field photomicrographs. Years in which Minnesota black bears successfully rear cubs are indicated by thinned, light cementum layers (C). This bear reared cubs in 1986, 1988, 1990, and 1992. Approximately 125X.

amination could permit dating if the marks were visible after decalcification and histological staining. Methods that may be available include the use of a micromanipulated laser. Another aid to identifying biomarker position is to use polarized or bright-field illumination to photograph sections before and after they are decalcified. Excessively thick calcified sections that lack clear biomarker visualization can be thinned by grinding on fine emery paper (Frost 1958).

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