VMRC Cobia otolith preparation protocol

VMRC

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Equipment

Item	Model/Source	Number/Use
• Leica MZ 9$_5$ or Leica MZ12 Stereo-microscope with transmitted light source and polarizing filter		1
• Buehler ® Isomet ™ low-speed saw	Model number 11-1280-160	1
• Flanges, steel	6.03cm diameter	2
• Spacer, steel	0.4mm thickness, 6.03cm diameter	1
• Norton® Diamond Grinding Wheel	1A1 3 x 0.006 x ½" ME120928, M3D220-N75M99-1/8, UPC/Cup: 69014192342	2
• Allen wrench	1/8in	1
• 25 gram weights	Buehler/ 1180S33	1-2
• Water for IsoMet™ lubricant tray		approx: 300mL, unfiltered water
• Ceramic top hot plate	Model number VWR 82026-752 115V, 1000W, 7 x 7"	1
• Barnstead 1400 Thermolyne furnace	VWR 30605-022	1

Supplies

• Micro slide [1inch x 3 inches x 1.2mm]	VWR 48318-0	1 per otolith plus 4-6 extras for "sectioning slide"
• Micro slide storage box	VWR 28511-012	1 per 100 otoliths
• Aluminum slide tray	VWR 48467	1 per 20 otoliths
• Porcelain Color Plate	VWR 53636-105	1 per 12 otoliths
• Barnes glass eye dropper and dropper bottle	VWR 14216-246	1
• Stender dish with lid	VWR WLS26155-C	1
• Dissecting forceps	VWR 82027-398	1 fine point, 1 broad tip
• Diamond etcher	VWR 52865-122	1 for etching hardpart information on microslides
• Metal Spatula	General store brand	1
• Crystalbond 509-S	AREMCO Products Inc.	1/4 stick
• Applicator stick		1
• Flo-Texx ® liquid cover slip	Lerner Laboratories	1 quart
• Kimwipes ® Delicate Task Wipers or VWR Light-Duty Tissue Wipers		1 box per work station for anyspilled or Flo-Texx ®
• Razor blace		1 for removing remnants of Crystal Bond from micro slides post-sectioning
• CQFE red drum otolith storage box, cardboard		enclosed with labeled coin envelopes and microtubes containing individual fish's otoliths
• Sharpie® Ultra Fine Point Permanent Marker		1
• Hard-Part Processing Log		lists selected hard-parts to be processed, categorized alphabetically by species

Introduction

This protocol is for the preparation of sagittal otolith transverse cross-sections from cobia (*Rachycentron canadum*) for age determination. Otoliths are obtained following the proceedures outlined in the Protocol for collecting biometric data and extracting whole sagittal otoliths.

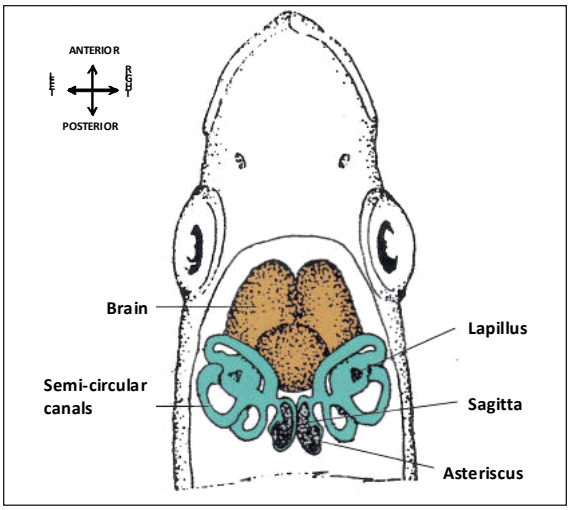

Figure 1A
Position of otoliths within the vestibular apparatus of typical Teleost species, e.g. tautog (Secor et al., 1992)

Structure of whole sagittal otoliths from tautog

Cobia have small otoliths relative to their body size. Unlike sciaenids, their saggitae do not have a unique, tadpole-shaped sulcus acusticus (sulcus or sulcal groove), but, rather a sulcal groove that extends from the posterior edge to the anterior edge (*Figure 1A & 1B*). These otoliths, along with the smaller lapilli and asteriscii, play an important role in the sensory systems of these fishes, namely, mechanoreception and maintenance of equilibrium in their environment.

For the sake of simplification, whole sagittal otoliths will hereafter be referred to as "otoliths" in this protocol.

As in all finfishes, cobia otoliths are formed through the process of biomineralization. Namely, the otoliths are formed through

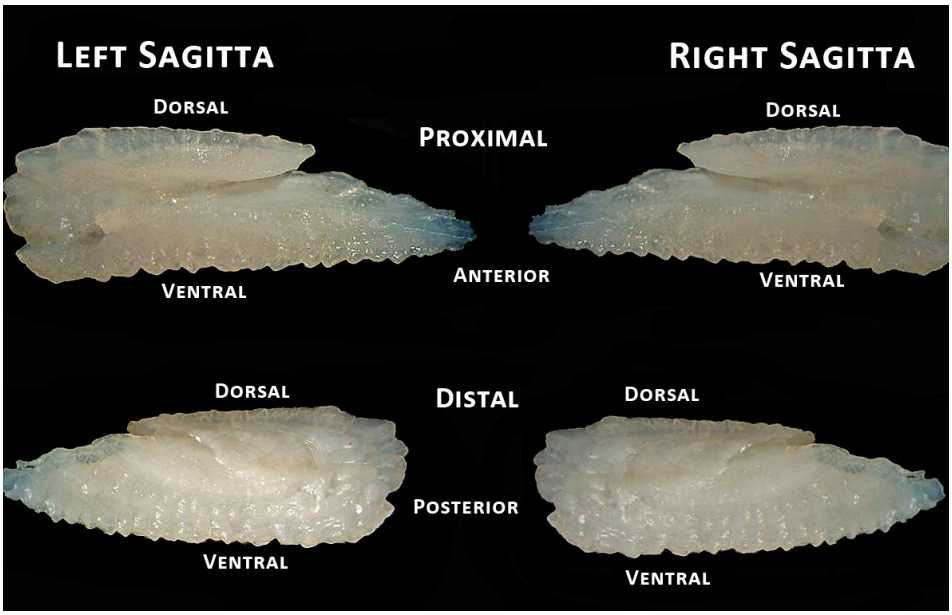

Figure 1B
Extracted left and right sagittal otoliths of cobia labeled to illustrate orientation and basic structure

the extracellular crystallization of calcium carbonate (primarily aragonite) onto an matrix template, composed of a keratin-like protein called "otolin" (Panfili et al. 2002).

Otolith formation begins early in the development of a fish, typically at the hatch-date of the larvae. The initial structure that is mineralizied is called the primordium, or primordia, which fuses to form the otolith core. This core is the foundation on which all new otolith growth occurs. Concentric layers of the protein and calcium carbonate matrix accrete, or grow, outward from the core throughout the lifetime of the fish. This results in a structure that is comparable to that of an onion.

Within the otolith matrix, aragonite is precipitated at varying rates throughout each year. Periods of slower growth in the fish, e.g. colder seasons of the year, are characterized by densely-packed precipitate. The core and opaque layers of the otolith, visible under transmitted light, respresent such growth. Periods of faster growth in the fish, e.g. warmer seasons of the year, involve less-densely compacted mineralization of the precipitate and are seen as translucent layers of the matrix when viewed in transmitted light.

The collection of successive opaque and translucent layers within the otolith can be made fully visible when a transverse cross-section (hereafter thin-section) is removed from the core region (*Figure 2*) and viewed through a stereomicroscope. Each of the opaque and translucent layers within the otolith constitutes an annulus, which occurs once per year.

For the purposes of age determination, only the opaque layers encircling the core are called annuli. They are counted outwards from the core towards the outer-edge of the otolith thin-section.

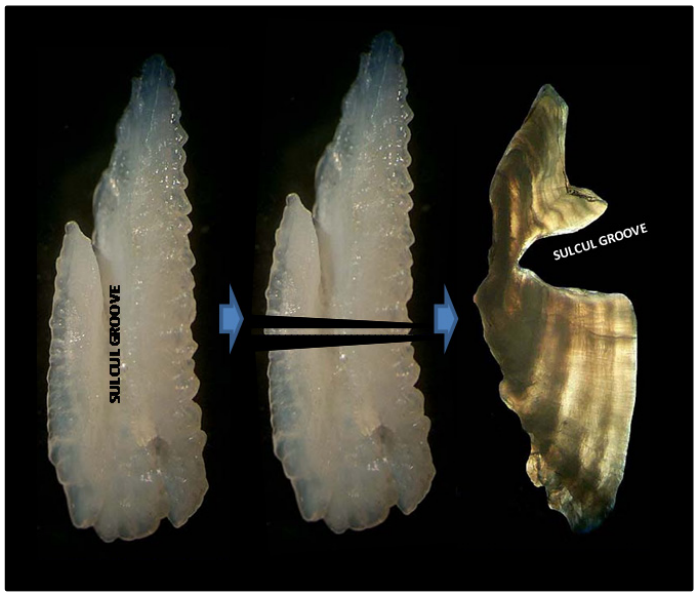

Figure 2
Cobia otolith, thin-section removal, and visible annuli within the section under transmitted light.

Preparation for Sectionion Cobia Otoliths

Begin by filling ¼ of each cavity of the embedding mold with Loctite® 349 (Loctite); place the mold under the UV lamp (*Figure 3*).

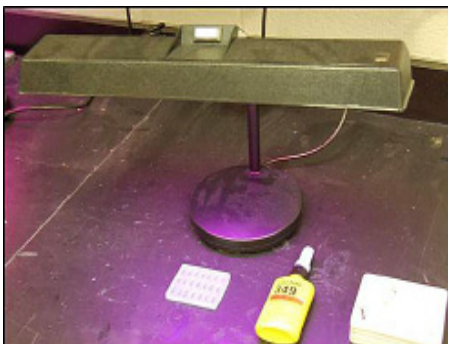

Figure 3
Embedding mold with Loctite curing under UV lamp.

Find the species and hard-part that has been selected for processing from the Hard-Part Processing Log. Search for the coin envelope with the selected Age and Growth ID number (AGID) within the CQFE Cobia otolith storage box. The AGIDs are found on the lower right-hand corner of each envelope.

Place each whole otolith, proximal side up, in the embedding mold's cavities. The Loctite does not have to be cured completely; it only needs to be solid enough to keep the otolith off the bottom of the cavity. This will ensure Loctite covers the entire otolith surface. Fill each of the cavities with Loctite; reposition the otolith if a bubble appears (a bubble makes the Loctite weak and provides a better chance for the thin-section to break). When all otoliths have been covered with Loctite, place the embedding tray under the UV light to cure (Figure 3). It usually takes 3-5 hours for the cavities to cure thoroughly.

Mounting Cobia Otoliths for Sectioning

Begin by turning on the hotplate. Each of the hotplates have a recommended mark where the temperature setting should be fixed; the VWR brand hotplate setting is ~1.5 and the Corning is ~3.5. Place ¼ stick of Crystalbond® 509 (hereafter "Crystalbond") into a stender dish. Put the dish on the hotplate surface. While the hotplate is warming up to its recommended temperature, mark each otolith core with a fine-tipped Sharpie® under a dissecting microscope, (*Figure 4*).

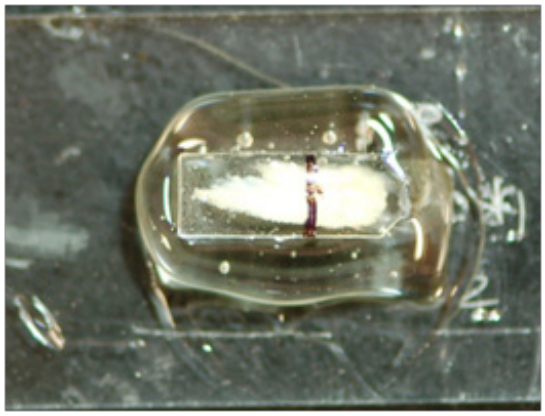

Figure4
The otolith core, marked with a Sharpie®

When the hotplate has reached its proper temperature and the crystalbond has become easy to manipulate, lay one of the "extra" micro slides on the hotplate surface. Using an applicator stick, evenly spread the semi-liquid Crystalbond onto the micro slide. Place another "extra" micro slide on top of the Crystalbond-covered slide; es-

sentially, make a micro slide-Crystalbond-micro slide sandwich. Press the micro slides together with the applicator stick to evenly distribute the Crystalbond. Hereafter, this will be referred to as a "sectioning slide". Use tweezers to set the sectioning slide on the table to cool to room temperature. Remove an otolith from the cavity from within the embedding mold. If the sectioning slide is cool to the touch, use the applicator stick to place an amount of Crystalbond on the center of the third quarter of the sectioning slide. The amount of Crystalbond needed is roughly 0.5 cubic cm. Create a platform of Crystalbond and mount the otolith proximal-side up on the micro slide. Make sure that the dorsal and ventral sides of the otolith lie parallel to the long edge of the slide and perpendicular to the 25-mm edges. Push Crystalbond up the sides of the cavity to secure it in place. Set the sectioning slide (with mounted otolith) on the table to cool until the otolith is firmly locked in the Crystalbond (*Figure 5)*. When sectioning, the saw blades should fall on either side of the line.

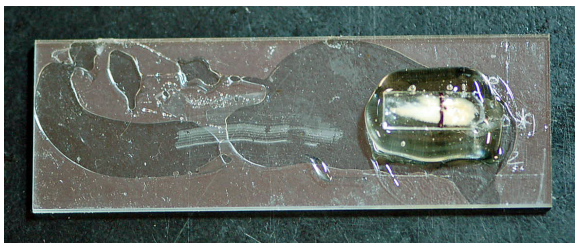

Figure 5
Cobia otolith embedded in loctite, marked and mounted on a sectioning slide.

Before cutting the otolith, make sure that the Buehler® IsoMet™ low speed saw (hereafter IsoMet™ saw) is set-up correctly. From left to right on the drive-shaft there should be a shaft spacer and slinger followed by an inner flange, a Norton® diamond blade (*Figure 6*), a 0.4mm spacer, an additional Norton® diamond blade, an outer flange, and an end cap bushing. The previous items are fixed to the drive shaft by a hand-tightened thumb screw (*Figure 7A*). The saw's lubricant pan should be filled with unfiltered water and the specimen basket should be in place (*Figure 7B & 7C*).

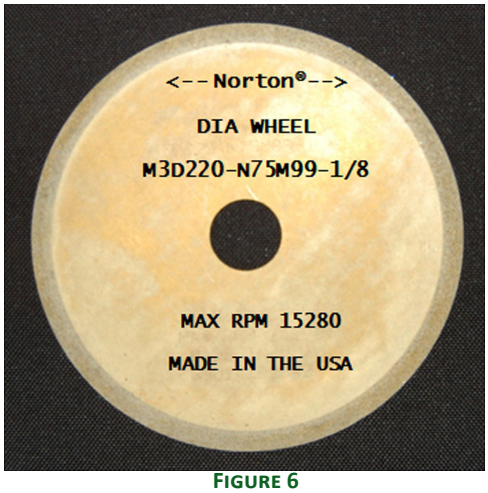

Figure 6
Norton® Diamond Grinding Wheel

Mount the sectioning slide with the marked otolith into the chuck of the saw's support arm (*Figure 8*). Secure it using the allen wrench.

Once the microslide is secured within the chuck, use the micrometer (*Figure 7C*) to align the support arm and bring the cut line marked on the otolith into position between the two Norton® Diamond Grinding Wheels (hereafter blades). The line should run completely parallel to both blades, and

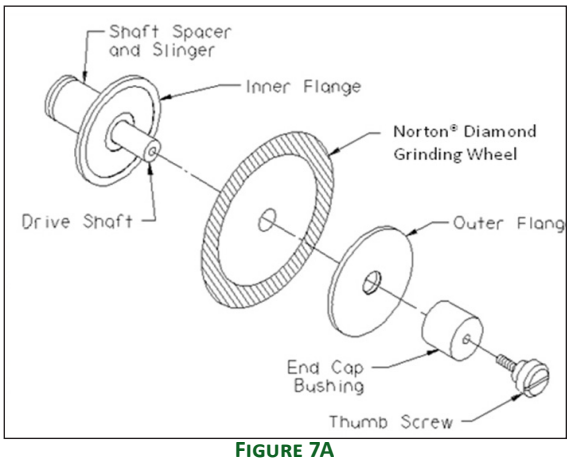

Figure 7A

IsoMet™ low speed saw blade installation diagram, showing the order of flange and Norton® Diamond Grinding Wheel placement on the drive shaft.

Note: CQFE uses a 0.4mm spacer between two Norton® Diamond Grinding Wheels (modified from the Buehler® IsoMet™ Low Speed Saw Manual).

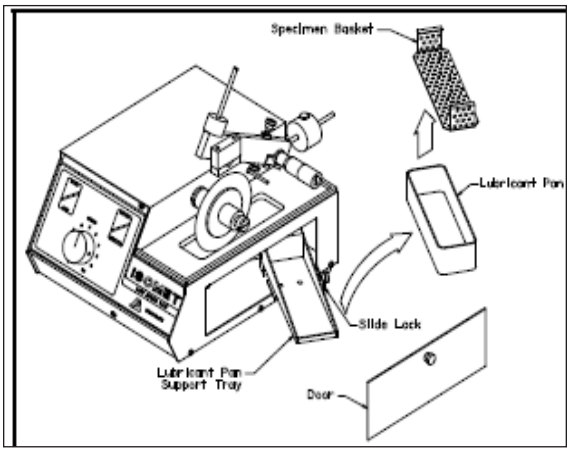

Figure 7B

IsoMet™ saw lubrication pan diagram

(Buehler® IsoMet™ Low Speed Saw Manual).

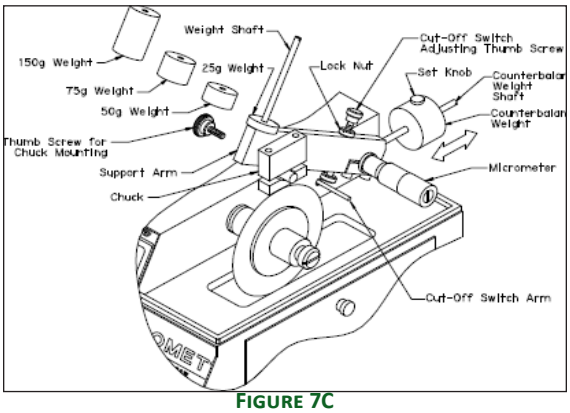

Figure 7C

IsoMet™ saw weight-balanced diagram

(Buehler® IsoMet™ Low Speed Saw Manual).

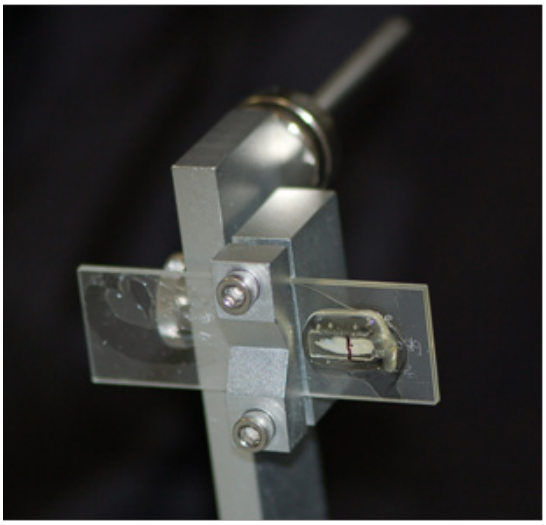

Figure 8

Cobia otolith mounted with the chuck on IsoMet™ saw arm.

fall directly within the 0.4mm space between them.

When all of the IsoMet™ saw specifications have been met, it is safe to start sectioning the otolith. Before moving the support arm and marked otolith down on to the blades, start the saw at a speed of 3 or 4. Once the blades have begun spinning, gently move the support arm downward onto the blades, bring the cured loctite into contact with the blades and allow them to cut at this speed for several seconds. Once the blades have established a groove (*Figure 9*), bring the saw speed up to 7 or 8. The blades should now be close to cutting through the otolith, separating the thin-section from the matrix. It should take 8 to 10 minutes to complete the cut. In some cases the cut will take longer, maybe over 20 minutes.

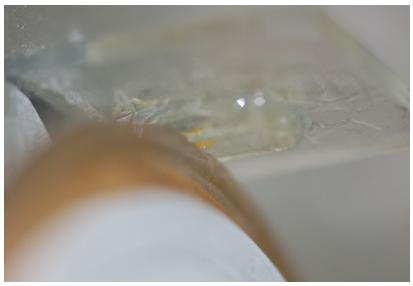

Figure 9
IsoMet™ saw with Norton® diamond blades cutting a tautog otolith.

The cutting time can be decreased by adding up to 50 grams of weight to the weight shaft at any point during the sectioning process. Increasing the saw speed will also decrease the amount of time per otolith. Loctite is dense and can withstand higher speeds than the 7 or 8 setting.

> ***Note that additional weight and speed will increase the risk of damaging the saw blades and/or the otolith sections. Technicians must use their discretion, based on personal, experience, in sectioning otoliths to maintain quality and safety.***

Monitor the cutting progress and the advancement of the blades through the Loctite and otolith. From the back of the microslide, you can view the otolith matrix becoming thinner as the blades near the crystalbond and underlying glass of the sectioning slide.

When you see that the blades have gone completely through the Loctite and are visible on either side of the thin-section being removed, carefully lift the support arm from the saw blades and turn off the IsoMet™ saw.

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The otolith should still contain the thin-section that was just cut, suspended in the crystalbond between the separated halves of the otolith. Use your tweezers to carefully remove it from this space. If the section was removed by the blades and the otolith reveals an empty space between the halves, remove the thumb screw and the outer flange from the drive shaft. Pull the two blades and spacer off of the drive shaft and lay them flat in your palm or on the work table. Separate the top blade from the spacer and the inner blade and search for the thin-section on the spacer-side of the blades. If the section was not caught between the blades, pull out the lubricant pan and inspect the specimen basket using tweezers.

Mounting Tautog Otolith Sections

Before permanently mounting the otolith thin-sections, look at them under the Leica stereomicroscope to make sure that the section included the core. The sulcal groove should meet the core in a precise angle (*Figure 10*) such that all annuli can be seen from the origin to the edge of the otolith.

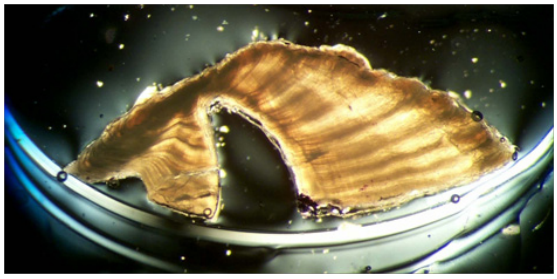

Figure 10
Completed transverse cross-section from cobia otolith used for age determination.

If the sulcal groove does not come together to form a point: the cut was placed too far from the core. A section with a correctly executed cut should also have no chips or other imperfections that eliminate or obscure views of the core, sulcal groove, or annuli. Sections not meeting these specifications must be re-cut.

The next cut may be taken from the same, mounted otolith of the fish, but the second otolith may also be used if necessary. When you have verified the section's quality, place the best surface of the thin-section facing upwards and ensure that there is no residual crystalbond causing it to sit unevenly. If residual Crystalbond is apparent on the thin-section, scrape it off using tweezers. Now that the section has been removed, use tweezers and a Kimwipe® to clean the two otolith halves so there is no crystalbond or Loctite remaining on their surfaces. Place the otolith halves back into the coin envelopes from which they came. The sectioning slide may be reused for the next selected otolith.

When the section is clean and dry, protect it with Flo-Texx®, a liquid cover slip. Use an eyedropper to put a small amount of Flo-Texx® over the section and spread it in

a circular motion. Eliminate bubbles within the Flo-Texx® by popping them or moving them away from the otolith using tweezers. Place the completed microslides on an aluminum slide-tray and allow the Flo-Texx® to air-dry on the sections for several hours (until solidified).

Storing Completed Cobia Otolith Thin-Sections

Using a fine point, black Sharpie®; write the species code (Cobia) and the AGID (e.g., 044) in the upper right-hand corner on the long side of the microslide edge (*Figure 11*). This will be at the opposite end of the etching. Store the slides in a labeled micro-slide box (*Figure 14*) with the black Sharpie® AGID facing upwards for easy identification during age determination.

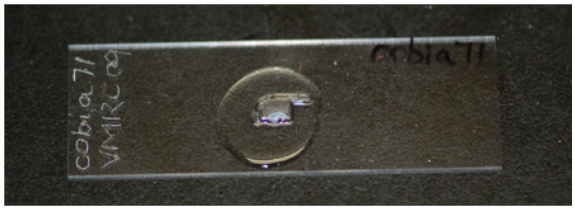

Figure 11
Completed cobia otolith thin-section, labeled appropriately with species code and AGID.

Figure 12
Otolith slide storage box, labeled for cobia.

Literature Cited:

Panfili, J., Rontual, H. Troadac, H., Wright, P. 2002. Manual of Fish Schlerochronology, pp35.

Secor, D.H., Dean, J.M. & Laban, E.H. 1992. Otolith removal and preparation for microstructural examination. In Otolith microstructure examination and analysis (Stevenson, D.K. & Campana, S.E., ed.).

Buehler® IsoMet™ Low Speed Saw Manual. Model MA111280_16.1 as of 6/8/2009

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Center for Quantitative Fisheries Ecology

Protocol

Preparation of otolith transverse cross-sections for age estimation of

Cobia

Rachycentron canadum