Ageing Laboratory/Fisheries Management Division

Protocol

Preparation of Otolith Transverse Cross-Sections for Age Estimation

October 2, 2023

Contents

Introduction

In this protocol we will mainly introduce two methods (hot glue vs. epoxy resin method) which we use to prepare sagittal otolith transverse cross-section (hereafter referred to as "thin-section") from the species we age in the VMRC Ageing Lab. The protocol starts with a brief introduction on the structure of whole sagittal otoliths using Spotted Seatrout *Cynoscion nebulosus* as an example, and then describe the hot glue method and epoxy resin method in detail on how to mount and section the otoliths. The protocol ends with a brief description on how the completed thin-sections are mounted on slides and stored in the VMRC Ageing Lab.

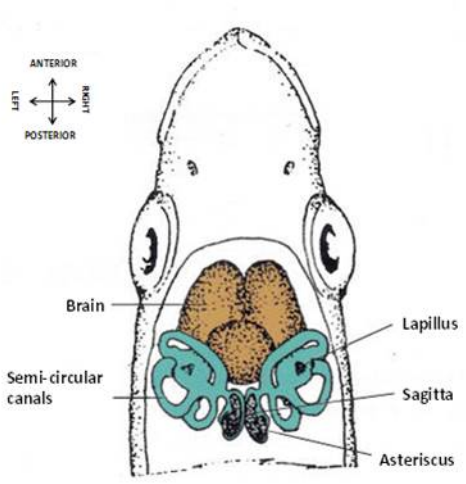

Figure 1: Position of otoliths within the vestibular apparatus of typical Teleost species, e.g. spotted seatrout.

Structure of Whole Sagittal Otoliths

Three pairs of otoliths (sagitta, lapillus, and asteriscus) are located within the vestibluar apparatus of typical Teleost species (Figure 1). They all play important roles in the sensory systems of these fishes for mechanoreception and maintenance of equilibrium in their environment. The sagitta is the largest of the three and used for ageing finfish.

Spotted Seatrout have extremely large sagittal otoliths relative to their body size. Their saggitae have a unique, tadpole-shaped sulcus acusticus (sulcus or sulcal groove) with an enlarged ostium anterior and tail-like cauda posterior (Figure 2). For purposes of this protocol, the sagittal otoliths will be referred to simply as "otoliths".

As in all finfishes, Spotted Seatrout otoliths are formed through biomineralization: specifically, the extracellular crystallization of calcium carbonate (primarily aragonite) onto an organic matrix which is composed of a keratin-like protein called "otolin" (Secor et al. 1992; 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, i.e., colder seasons of the year, are characterized by densely-packed precipitate. The core and opaque layers of the otolith, visible under transmitted light, represent such growth. Periods of faster growth in the fish, i.e., 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 referred to as "thin-section") is removed from the core region (Figure 3) and viewed through a stereomicroscope. Each

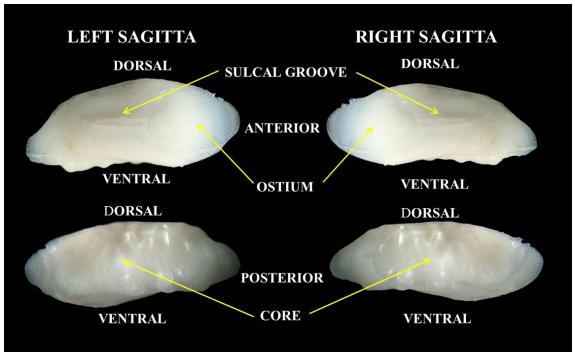

Figure 2: Extracted left and right sagittal otoliths of spotted seatrout labeled to illustrate orientation and basic structure (The otoliths are positioned at the proximal facing front).

of the opaque and translucent layers within the otolith constitutes an annulus, which occurs once per year (Murphy and Willis 1996).

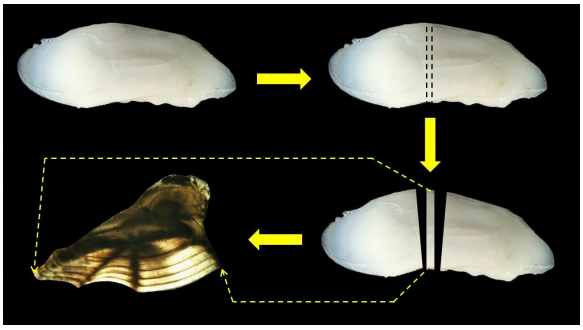

Figure 3: Spotted seatrout otolith, thin-section removal, and visible annuli within the thin-section under transmitted light.

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

Prepare Otoliths for Sectioning

In the VMRC Ageing Lab we use two methods, hot glue and epoxy resin method, to prepare otoliths for sectioning depending on their size and fragility. We will use Spotted Seatrout and Striped Bass *Morone saxatilis* as examples to introduce the hot glue and epoxy resin method, respectively.

Hot glue method

In Family Sciaenidae (aka Drum Family) for many species, such as Atlantic Croaker *Micropogonias undulatus*, Black Drum *Pogonias cromis*, Red Drum *Sciaenops ocellatus*, Spotted Seatrout, and Weakfish *Cynoscion regalis*, their otoliths are large and strong enough to withstand the vibrations from the sectioning process without breaking. Therefore, we section their otoliths using the hot glue method which is less time/labor consuming than Epoxy resin method.

1. Find the species and hardpart that has been selected for processing from the Hardpart Processing Log;
2. Remove the selected otolith from the coin envelop and mark the core using an ultra-fine point Sharpie®;
3. Cut a piece of water resistant paper (Rite in the Rain®) into 1x2 inch pieces (hereafter referred to as “tag”);
4. In the upper right corner of the tag write the fish ID of the selected otolith.;
5. On the right side of the tag place 3 dots of hot glue with the middle dot being smaller than the left and right dots (Figure 4). The total width of the dots will depend on the size of the otolith;
6. While the glue is still hot, place the otolith on top of the dots so that the core mark is on top of the middle dot (Figure 5);
7. Once the otolith is set on the hot glue, cover both ends of the otolith, ensuring each end is covered in hot glue that is touching both the otolith and the tag (Figure 6);
8. Once the hot glue has cooled off, you are ready to start sectioning the otolith. To set up the saw, you will need to place an empty resin block (hereafter referred to as "backer

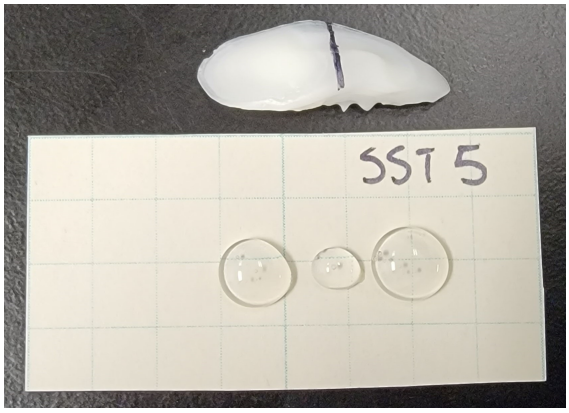

Figure 4: Three glue dots are placed on a tag based on the size of an otolith.

Figure 5: A marked otolith is placed on the tops of three glue dots.

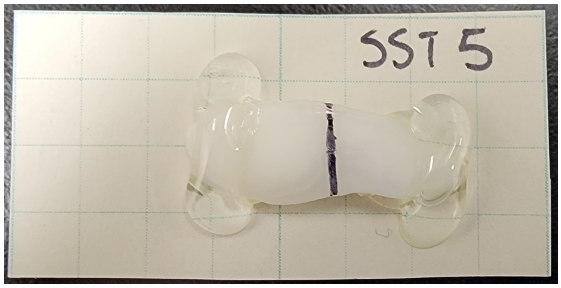

Figure 6: A marked otolith is glued to a tag at its bottom and both ends.

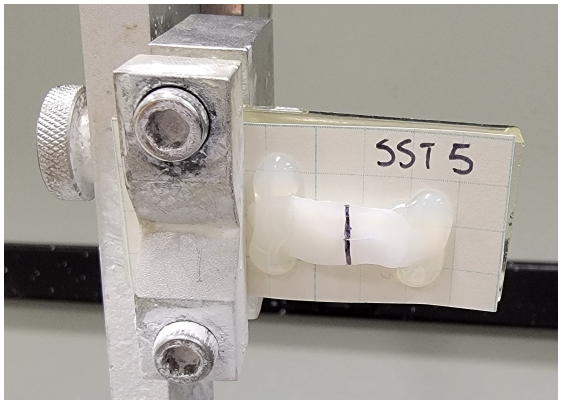

Figure 7: A resin block with a marked otolith is mounted on the chuck but the right end of the block and tag are not clamped together, yet.

block") in the chuck of the saw. Ensure the flat (bottom) side of the backer block is facing forward;

9. Then slide your set tag in the chuck on top of the backer block and tighten the chuck all the way (Figure 7). This will ensure the tag is secured on the left side, providing the majority of the stability of the tag;

10. To secure the right side of the tag onto the backer block, use a large binder clip to clamp the tag and backer block together (Figure 8);

11. With the saw turned off ensure your core mark is lined up with the spacer in between the blades;

12. Now your otolith is ready for sectioning. Please go to Section Section otoliths which demonstrates how to operate the slow speed saw to section the otoliths in detail (using Striped Bass otoliths as an example);

13. Once the section is done, the thin-section will either fall off the tag or remain attached to the tag in the middle dot of hot glue;

14. Loosen the chuck just enough to remove the tag and then place the next prepared otolith in the chuck;

15. Mount the thin-section on a glass slide. Please go to Section Mount Otolith Thin-section on Slide which demonstrates how to mount a thin-section on a glass slide in detail (using Striped Bass otoliths as an example);

16. Store the thin-section slides in a slide box. Please go to Section Store Otolith Thin-section Slides which demonstrates how we store thin-sections in detail (using Striped Bass otoliths as an example).

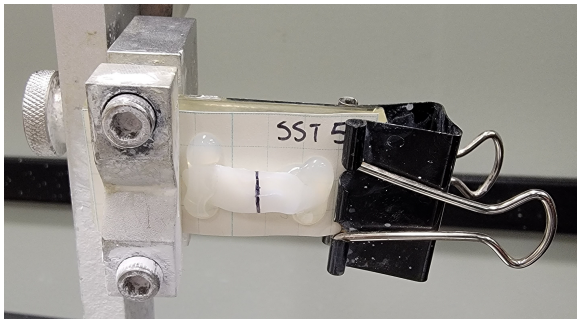

Figure 8: A resin block with a marked otolith is completely fixed on the chuck and ready to be sectioned.

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Epoxy resin method

We section otoliths of nine species in epoxy resin blocks because their otoliths are either small, fragile, or both. They are Bluefish *Pomatomus saltatrix*, Cobia *Rachycentron canadum*, Sheepshead *Archosargus probatocephalus*, Spadefish *Chaetodipterus faber*, Spanish Mackerel *Scomberomorous maculatus*, Spot *Leiostomus xanthurus*, Striped Bass, Summer Flounder *Paralichthys dentatus*, and Tautog *Tautoga onitis*.

Make fish ID labels for epoxy resin blocks

Before putting an otolith in a silicone well, make sure that the well is labeled with the fish ID of the otoliths. In the VMRC Ageing Lab we make the labels on a spreadsheet using an Excel macro which connects to the Lab data files. Print, cut, and put the labels in the silicone wells.

Put otoliths in silicone wells

To put otoliths in silicone wells, please follow the steps below:

1. After the fish ID labels are ready, put them in the silicone tray wells (Figure 9);
2. From the otolith storage box, search for the coin envelopes with the fish IDs matching those in the silicone wells. The fish IDs are found on the lower right-hand corner of each envelope;

Figure 9: Silicone tray with fish ID labels in wells.

3. Remove one otolith from the microtube in the coin envelope and place it in the well that corresponds to the fish ID. Do this until the silicone tray is full or all the selected otoliths are placed (Figure 9).

After putting all the otoliths in the silicone tray, we have two ways to continue the preparation depending on species. For Cobia, Sheepshead, Spanish Mackerel, and Spot, we don't bake their otoliths before embedding them in epoxy resin, therefore, please skip the next section *Bake otoliths* and directly go to Section *Embed otoliths with epoxy resin*. To increase their readability, we bake the otoliths of Bluefish, Spadefish, Striped Bass, Summer Flounder, and Tautog before embedding their otoliths in epoxy resion (Please see the next section *Bake otoliths*).

Bake otoliths

Begin by turning on the Barnstead/Thermolyne 1400 Small Benchtop Muffle Furnace (hereafter referred to as "oven"). The temperature setting should be set to 400 °C (Figure 10).

To set the temperature:

1. Depress the black button that reads "Push to Set Temp" above it;
2. Turn the "Tempurature" knob until the display reads 400 °C;

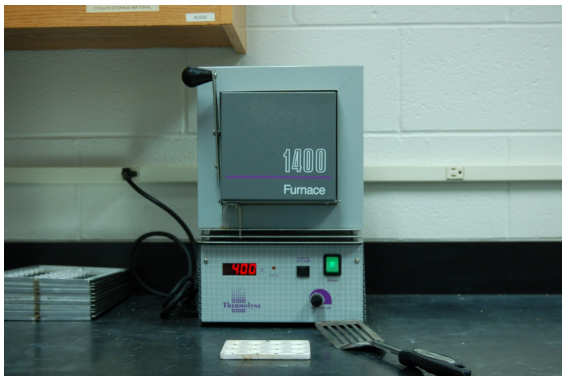

Figure 10: Barnstead/Thermolyne 1400 Small Benchtop Muffle Furnace.

3. Release the black button.

While the oven is warming, transfer the unbaked otoliths one at a time to the CoorsTek® Spot Plate (hereafter referred to as "ceramic plate"). **Carefully track the transferring of the otoliths from the silicone tray to the ceramic plate using the fish IDs in the silicone tray and the well numbers marked on the ceramic plate, ensuring that the otoliths will be correctly transferred back to their original wells in the silicone tray from the ceramic plate after they are baked** (Figure 11).

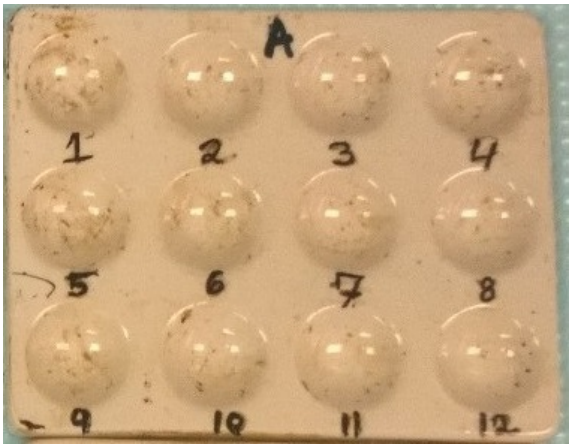

Figure 11: A ceramic plate marked with numbers.

Once all of the wells in the ceramic plate have been filled, use the metal spatula to pick up the filled ceramic plate and place it inside the oven. Close the oven door and set the timer for one minute. When the timer goes off, remove the ceramic plate with the metal spatula. Examine the otoliths' color. A light caramel color is ideal for aging. If the color is not caramel enough, put the ceramic plate back into the oven for 30 seconds. Sometimes it only takes one additional 30-second bake; sometimes it takes three to four additional bakes. There should not be any charring or large black spots on the otoliths, indicating the otoliths have been overbaked. In some situations the charring can be removed by scraping it with tweezers. When the light caramel color is achieved, transfer the baked otoliths from the ceramic plate to the silicone tray in the same order as from the silicone tray to the ceramic plate so that each otolith will be placed back to its original well, matching its fish ID.

Embed otoliths with epoxy resin

Now the unbaked (such as Cobia's) and baked (such as Striped Bass's) otoliths are ready to be embedded in West System® 2-part epoxy resin (hereafter referred to as "resin"), part 1 is the 105-B epoxy resin (hereafter "epoxy") and part 2 is the 206-B slow hardener (hereafter referred to as "hardener"). The resin is a 5:1 epoxy to hardener ratio. We use West System® 306-25 Metering Pump (Figure 12) to generate a mixture of expoxy and hardener at a ratio of 5:1 in a small measuring cup. Be sure to mix the epoxy and hardener well but stir slowly to minimize air bubbles in the mixture. Fill the silicone wells until the resin is just above the top of the otolith, be sure to pop or move any air bubbles around and underneath the otolith. Leave the resin to cure for 24 hours.

When the resin blocks are completely cured, remove them from the wells, and use a dissecting microscope to mark the core of each otolith using a fine-tipped Sharpie® (Figure 13). Now the otoliths embedded in the resin blocks are ready to be sectioned.

Figure 12: The pump for mixing Epoxy resin and hardener at a ratio of 5:1.

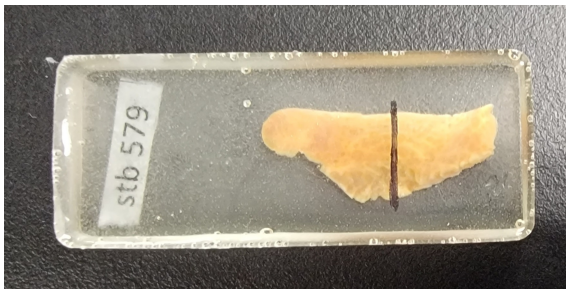

Figure 13: Otolith embedded in resin block with its core marked by Sharpie.

Section otoliths

Before sectioning a resin block with an embedded otolith (unbaked or baked), make sure that the Buehler® IsoMet™ low speed saw (hereafter referred to as " 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 MTI Corp® diamond grinding wheel (hereafter referred to as "blade", Figure 14), a 0.5 mm spacer, a 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 15). The saw's lubricant pan should be filled with water and the specimen basket should be in place (Figure 16).

Place the marked resin block into the chuck of the saw's support arm (Figure 17), and secure it using the allen wrench. Once the resin block

Figure 14: MTI Corp® Diamond Grinding Wheel.

is secured within the chuck, use the micrometer (Figure 18) to align the support arm and bring the cut line marked on the resin block into position between the two blades. The line should run completely parallel to both blades, and fall directly within the 0.5 mm space between them (Figure 19).

When all of the saw specifications have been met, it is safe to start sectioning the otolith. Before moving the support arm and placing the resin block on 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, bringing the resin block into contact with the blades. Allow them to section at this speed for several seconds. Once the blades have established a groove, bring the saw speed up to 7 or 8. The blades should now be close to sectioning through the otolith, separating the thin-section from the matrix. It should take 3 to 5 minutes to complete the section. In some cases the section will take longer, maybe over 10 minutes.

The sectioning 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. Resin is dense and can withstand higher speeds than the 7 or 8 setting.

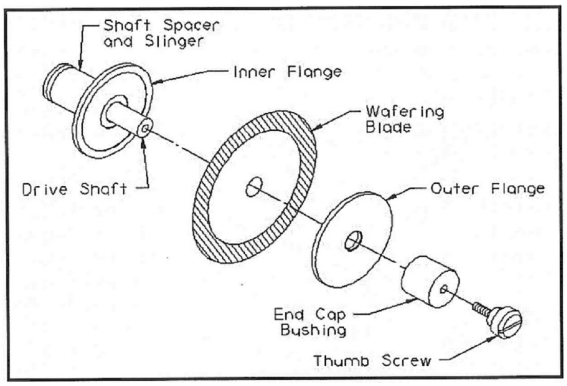

Figure 15: IsoMet^{TM} low speed saw blade installation diagram, showing the order flange and MTI Corp® Diamond Grinding Wheel placement on the drive shaft. Note, that in our procedure we use a 0.5 mm spacer between two MTI Corp® Diamond Grinding Wheels (modified from Buehler® IsoMet^{TM} Low Speed Saw Manual).

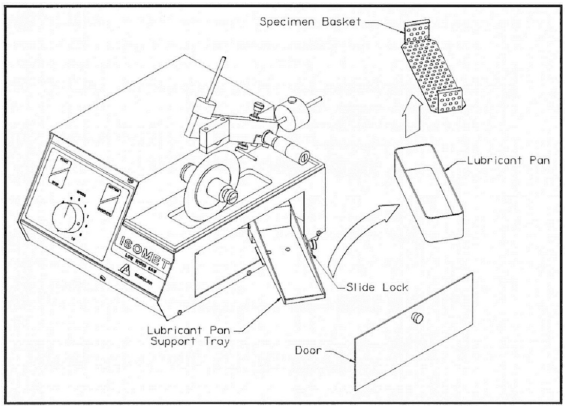

Figure 16: IsoMet^{TM} saw lubrication pan diagram (Buehler® IsoMet^{TM} Low Speed Saw Manual).

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

While the blades are still sectioning the resin block, using a cordless precision engraver, engrave a clean microscope slide (hereafter referred to as "slide") on the left end with VMRC and the year abbreviation (e.g. 22 for 2022). Using a fine point black Sharpie®, write the species name abbreviation (e.g. STB for

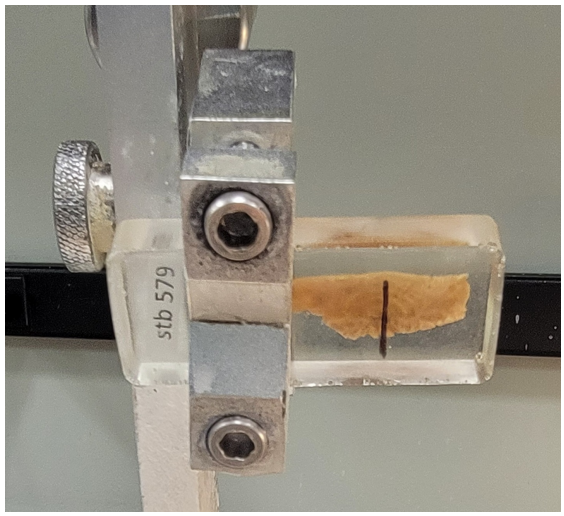

Figure 17: Marked resin with otolith embedded is mounted in the chuck of the saw arm.

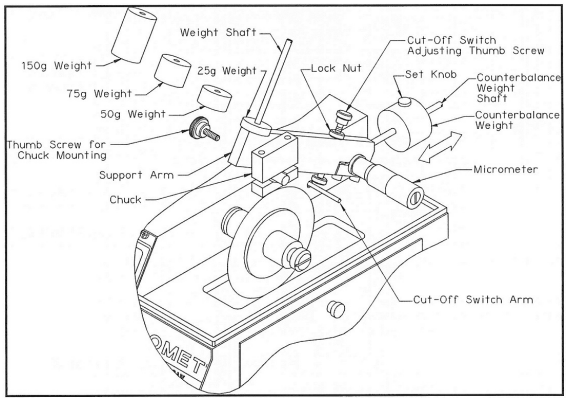

Figure 18: IsoMet^{TM} saw weight-balanced diagram (Buehler® IsoMet^{TM} Low Speed Saw Manual).

Striped Bass) and fish ID (Here is 095) in the upper right-hand corner (opposite to the engraved end) on the long side of the slide edge. This slide will be used for mounting the thin-section being sectioned.

Monitor the sectioning progress and the advancement of the blades through the resin block and otolith. From the back of the resin block, you can view the otolith matrix becoming thinner as the blades near the back of the resin block. Once the blades section through the entire resin block, stop the saw. Collect the large piece of resin remaining in the chuck and the small piece of resin that has fallen in the lubricant water, and put them back in the

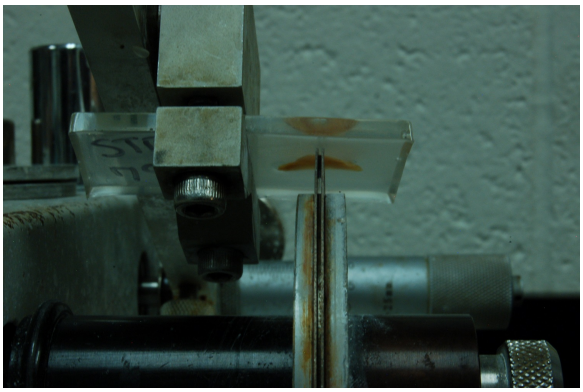

Figure 19: The black Sharpie® mark is lined up right between 2 blades.

coin envelope from which they came.

The thin-section will often stay between the blades. 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 on 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 thin-section is not between the blades, then it has fallen into the lubricant water. Pull out the lubricant pan, lift the specimen basket out of the water, search for the thin-section, and collect it using tweezers. Using a Kimwipe® gently dry the thin-section off and place on the labeled slide.

Mount Otolith Thin-section on Slide

Before permanently mounting the otolith thin-sections, look at them under the Leica stereomicroscope to make sure that the thin-section includes the core. The sulcal groove should meet the core at a precise angle such that all annuli can be seen from the origin to the edge of the thin-section, and sectioned sulcal groove has a "V" shape (Figure 20).

If the sulcal groove and the core do not come together to form a point, and the sectioned sulcal groove looks like a "tornado" shape, then, the section was placed too far from the ostium (Figure 21).

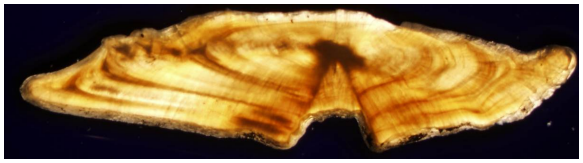

Figure 20: Completed thin-section with a "V" shape of sectioned sulcal groove (positioned as the opening of the "V" downward on the slide), indicating that the section went through the core.

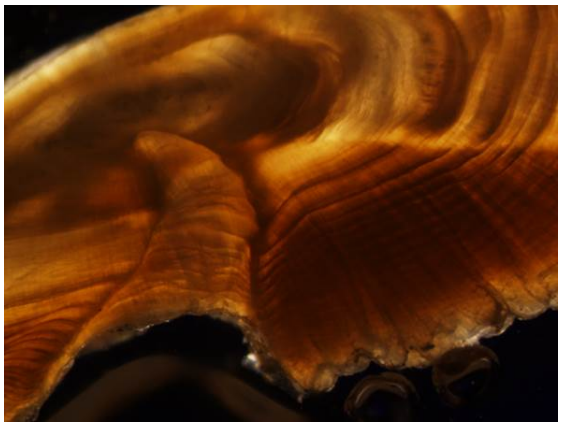

Figure 21: A thin-section with a "tornado" shape of sectioned sulcal groove (showing the opening down here), indicating that the section misses the core and is far away from the ostium.

If the sulcal groove does not come together to form a point, and the sectioned sulcal groove looks like a "dome" shape, then, the section was placed too close to the ostium (Figure 22).

A thin-section with a correctly executed section should have no chips or other imperfections that eliminate or obscure views of the core, sulcal groove, or annuli. Thin-sections not meeting these specifications must be re-sectioned. The second thin-section may be obtained from either of the two halves of the first otolith (depending on where the first section misses the core), but the second otolith may also be used if necessary.

When you have verified the thin-section's quality, place the best surface of the thin-section

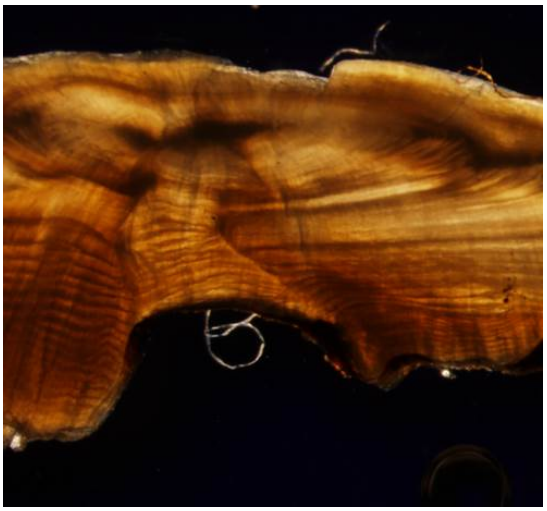

Figure 22: A thin-section with a "dome" shape of sectioned sulcal groove, indicating that the section misses the core and is too close to the ostium.

facing upwards and ensure the thin-section is sitting flat on the previously engraved slide. Make sure that the opening of the "V" shape is positioned facing the long edge of the slide while the engraved end of the slide is facing to the left of reader.

When the thin-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 thin-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. The Flo-Texx® is used as the mounting medium to both protect the thin-section and increase its clarity.

Figure 23 shows the final otolith thin-section slide correctly labeled with abbreviation of species name (STB for Striped Bass), fish ID (095), VMRC, and collection year (2022). Place the completed slides on an aluminum slide tray and allow the Flo-Texx® to air-dry on the thin-sections for several hours (until solidified).

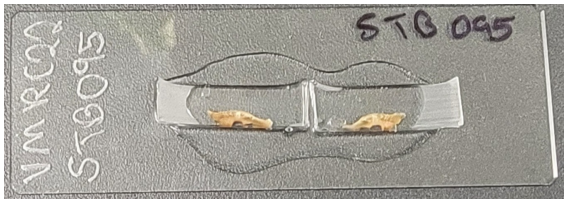

Figure 23: Completed striped bass otolith thin-section, labeled appropriately with species code and fish ID.

Store Otolith Thin-section Slides

Store the slides in a slide box labeled with VMRC, collection year (2022), species name (Striped Bass), Otoliths (indicating the box for otoliths), fish IDs, and number of boxes (Figure 24). Make sure the slide is positioned in the box with the black Sharpie® species name abbreviation and fish ID (Here is "STB 095") facing upwards for easy identification during age determination.

Figure 24: Otolith slide storage box, labeled for Striped Bass.

References

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Equipment and Supplies

Item	Specification
I. General:	
Leica MZ 95 or Leica MZ12 Stereo-microscope	Transmitted light source and polarizing filter
Buehler® IsoMet™ Low-Speed Saw	Model 11-1280-160
Steel Flanges	75 mm diameter
Steel Spacer	0.5 mm thickness, 75 mm diameter
Diamond Grinding Wheel	MTI 4"
Allen Wrench	1/8 inch
Weights	Buehler/1180S33, 25 g
Microscope Slide	Premiere 75x25x1 mm
Microscope Slide Storage Box	VWR 28511-012, 100 slides per box
Aluminum Slide Tray	VWR 48467, 20 slides per tray
Glass Eye Dropper and Dropper Bottle	VWR 14216-246, Barnes
Dissecting Forceps	VWR 82027-398, 1 fine point, 1 broad tip
Cordless Precision Engraver	General Tools and Instruments 505
Flo-Texx® Liquid Cover Slip	Lerner Laboratories, 1 quart
Wipers	Kimwipes® Delicate-Task or VWR Light-Duty
Microcentrifuge Tube with Snap Cap	Globe Scientific, 2-ml capacity (for small otoliths)
Round Plastic Container with Snap Cap	Lacons®, 0.98"x1.5" (for large otoliths)
Coin Envelopes	4.25"x2.5"
Storage Box (Cardboard)	5"x4"x10.5"
Ultra Fine Point Permanent Marker	Sharpie®
II. Hot Glue Method:	
Hot Glue Gun	AdTech 0453 2-Temp Dual Temperature
Hot Glue Stick	Magicfly 4"x0.43"
Water Resistent Paper	Rite in the Rain
Empty Resin Block "Backer Block"	
III. Epoxy Resin Method:	
Furnace	Barnstead/Thermolyne 1400
Ceramic Top Hot Plate	VWR 82026-752 115V, 1000W, 7"x7"
Resin Pump	West System 306-25 Metering
Resin Mixer	Badger Air-Brush 121
Silicone Tray	Lawei 40-well
Epoxy Resin	West Marine Pro, #105-B
Slow Hardener	West Marine Pro, #206-B