Supplementary Materials

GOLIATH GROUPER AGE STRUCTURE TRAINING GUIDE

Goliath Grouper *Epinephelus itajara*

Highlights

- Sagittal otoliths are the preferred structure for ageing, but are rarely collected due to fishing moratorium enacted in 1990.
- Non-lethal ageing has been used successfully with dorsal spines and dorsal fin rays, but spines are the preferred structure.
- Common issues of spine and ray ageing include occlusion of inner growth zones, checks, compaction of latter annuli.
- Otolith deposition has been directly validated using OTC, and indirectly using marginal increment analysis (Bullock et al. 1992).
- Maximum age is reported to be 37 (Bullock et al. 1992), but is likely to be longer.

Description

Otolith
Like most of the groupers, the sagittae are relatively large, laterally compressed and have an arrow shape (Figure 1). The rostrum, anterostrum, and sulcus are easy to distinguish and locate. It is common to see protrusions or irregularities along the ventral edge of the sagitta. Smaller or younger fish otoliths can be more fragile. Unlike other grouper species, Goliath Grouper otoliths are traditionally embedded and thin sectioned, not read whole.

Spine
Fin spines are formed as a single fused element of bone with a central lumen that is connected to the inner support system (pterygiophores) within the body cavity of a fish. Spines are rigid, unsegmented and they articulate from the base (condyle) (Figure 2). The central lumen of the spine is typically filled with vascular tissue, which can obscure growth rings. Goliath Grouper spines often have protrusions and growths on the exterior surface, but these do not typically impede sectioning or ageing.

Figure 1. Whole otolith of Goliath Grouper distal view with core marked (top) and proximal view (bottom).

Fin Ray

Fin rays are comprised of two parallel elements (hemitrichia) and are segmented, allowing them to have flexibility (Figure 2). Unlike a fin spine, a fin ray does not have a central lumen completely surrounded by bone. Rather, the vascular tissue lies between the hemitrichia and is offset from the core (lies in a medial groove). There is a pterygiophore, or knuckle of cartilage or bone, at the base of each fin ray pair that articulates with the dorsal skeletal elements, or the pelvic/pectoral girdles.

Figure 2. Goliath grouper spine (top) and fin ray (bottom). The fin ray has been separated into the two hemitrich elements, and the distal tips have broken off and are not pictured.

Extraction and Cleaning

Otolith

Otolith extraction in Goliath Grouper can be difficult due to the size of the fish and the fragility of the otoliths. The otic capsule in Goliath Grouper is relatively thick and is located directly behind and under the brain (Figure 3). The preferred method of extraction is to cut through the upper neurocranium (top method, Figure 3). It is common

practice to remove grouper otoliths using the gill-extraction technique, this is not preferable for Goliath Grouper due to their body size.

While a hacksaw might be sufficient for smaller individuals, a battery powered reciprocating saw is preferable for larger adults. Blades with more teeth per inch, such as metal-cutting blades work the best. A dorso-ventral first cut can be made at a point in line with the preopercle followed by a second transecting cut from just above the eyesocket towards the gills (Figures 3 and 4).

Otoliths can be rinsed with water and stored dried in a vial or envelope.

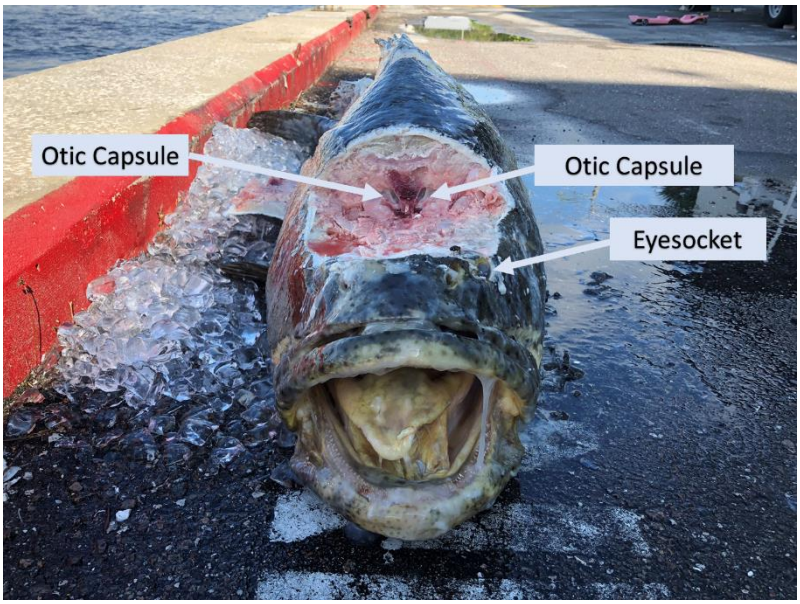

Figure 3. Goliath Grouper carcass with otoliths removed, demonstrating location of otic capsules and eyesocket.

Spine

Non-lethal sampling of spines requires a cut as close to the surface of the body as possible. The membrane between the desired spine and the subsequent spines should be cut (using a knife or scalpel), and the spine removed as close to the body as possible with a pair of wire cutters, pruning shears, etc. If the fish is deceased, a knife or reciprocating saw can be used to cut the entire spine including the condyle and surrounding muscle tissue from the sample (Figure 4). The preferred spine for Goliath Grouper ageing is the second dorsal. If the second dorsal is damaged, the third spine can be used.

Removed spines can be cleaned of the surrounding muscle tissue with a knife or scalpel and boiled in a beaker of water. After a couple minutes of boiling, the skin will peel off the spine. Any remaining tissues should be manually removed, and the spine air dried until ready for processing.

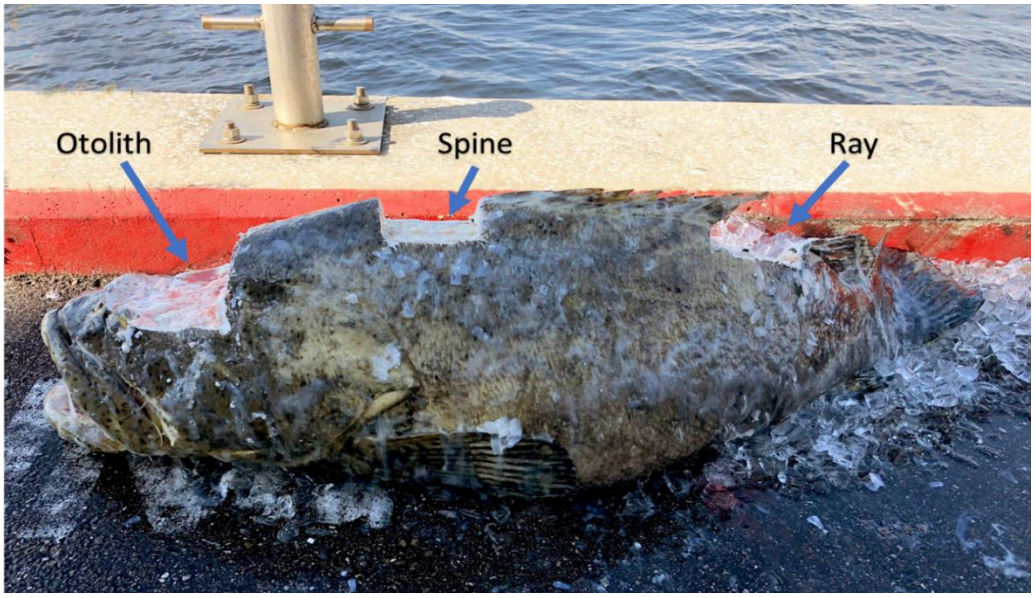

Figure 4. Goliath Grouper carcass with removed ageing structures. Otoliths removed from head (left), spines removed from dorsal fin (middle) and rays removed from dorsal fin (right). Note: this image is intended to demonstrate the regions in which the different ageing structures are located. This was a carcass, and image is *not* demonstrative of non-lethal structure removal.

Fin Rays

Fin rays must be removed as close to the surface of the body as possible to make sure that all annuli (especially the first) are present in the base of the fin ray. On dead fish, the rays can be removed down to their base (knuckles), which extends into the muscle of the fish. As with spines, for non-lethal removal, the fin membranes between the rays are cut down to the back of the fish and then the fin rays are cut off as close to the back of the fish as possible, usually using heavy duty lab scissors or pruning shears. Goliath Grouper have been aged using rays 3-7, depending on sampling program and project (Figure 4).

Fin rays can be cleaned of the surrounding muscle tissue with a knife or scalpel and simmered in water. A water bath using a cake pan, or something of similar size, is preferable, given the length of the rays (Figure 5). After a few minutes of simmering, the skin will start to fall off the ray, and the vascular tissue in the medial groove will soften. This allows for the manual removal of this tissue and separation of the hemitrichia with a knife or similar tool. Each hemitrich should be cleaned and all tissue removed from the medial groove, particularly around the base. This can be accomplished using alternating simmering and manual tissue removal.

Figure 5. (A) Simmering adult Goliath Grouper fin rays and (B) manual removal of vascular tissue from the medial groove of a Goliath Grouper hemitrich.

Processing

The technique used for processing all Goliath Grouper structures is sectioning, detailed in the manuscript, as well as Chapter 3.0, Section 3.2.6. of VanderKooy et al. 2020. While the basic principle is the same as with otoliths of other species, the size of the Goliath Grouper structures necessitates some specificity in processing techniques.

Otolith

Sectioning Goliath Grouper otoliths is most successful when otoliths are embedded. Embedding is necessary due to the depth of the sulcal groove and overall rarity of otolith samples. Embedding will enable thin sectioning and reduce breakage of the section. Embedding techniques are detailed in Chapter 3.0, Section 3.2.5.2. of VanderKooy et al. 2020. Any embedding material that has successfully been used for otoliths can also be used for Goliath Grouper. Care must be taken to ensure that the embedding mold is large enough to fully encapsulate the otolith. Common otolith embedding molds are likely too small. Silicone molds for other industries, such as chocolate molds, can be used in this circumstance.

While any processing technique in VanderKooy et al. 2020, Section 3.2.6 is likely to be successful, the most common technique utilized for otoliths is multi-blade sectioning using an Isomet low-speed saw with the embedded otolith mounted on cardstock using hot-melt glue (Section 3.2.6.2.3). This sectioning yields three transverse sections, including the core of the otolith (Figure 6). Thin sections should be ~400µm.

Figure 6. A sectioned age-3 Goliath Grouper sagittal otolith. Note the lines above and below the otolith indicating the edges of the embedding material.

Spine

Spine processing is detailed in the manuscript. The size of adult Goliath Grouper spines can be an impediment to processing, so care must be maintained to ensure the spine, saw blades or saw arm do not get broken during the procedure. Processing adult Goliath Grouper spines can be accomplished on any type of saw used for processing otoliths, but have most commonly been processed on Isomet low speed saws using the multiblade and hot-melt glue technique described in Section 3.2.6.2.3. of VanderKooy et al. 2020. While this saw is likely less efficient than other equipment to accomplish the task, it has nonetheless been successful. The main limitation of a low speed saw in this instance is the maximum blade size. Adult Goliath Grouper spines can nearly exceed the cutting radius on the 4" blades of these saws. While challenging, it is possible to safely process adult Goliath Grouper spines on low speed saws using the multiblade protocol, given the following steps are followed:

- The preferred processing zone of Goliath Grouper spines is distal of the condyle base (Figure 7, image A). The zone is marked by a symmetry of the spine, and the resultant sections should be nearly round (as opposed to oblong if sectioned at the base). The zone should be marked with a pencil to align the structure for a transverse cut.

- The condyle base, if present, may need to be removed for the saw blades to access the cutting zone (Figure 7, image B). To determine if this is necessary, the spine can be placed on the mounting chuck and blades adjusted to see if they will align. If the base needs to be removed, that can be accomplished on the saw, and the spine remounted on another piece of cardstock.

- It is critical to place the spine on the cardstock, and the saw, perpendicularly so that the resultant cut will yield transverse sections of the structure (Figure 8). If the spine is not perpendicular, the annuli on the sections will be skewed, which could result in inaccurate ageing.

- For the multiblade hot-melt technique, the cardstock should be cut to a size that enables the spine to hold firm on the saw arm during cutting. Ideally, about half of the spine should be glued to the cardstock (Figures 7 and 8). Glue should be applied liberally to the cardstock, in a shape resembling a dog bone, to ensure a firm adhesion of spine to paper. The thinner part of the dog bone should align with where the sections will be removed. Given the size of the spines, this amount of gluing beyond the processing zone enables the spine to hold firm through the resistance placed on it during sectioning, and keeps the structure from wobbling (which would create wedge cuts). The spine should be mounted on the cardstock with the base to the left; this enables the spine to fit on the saw.

- The mounting chuck on the saw arm must be large enough to hold the spine firm during processing. A 2" square mount on a saddle chuck is preferable. There will be a lot of resistance against the spine during processing, so securing the cardstock in at least two places prior to cutting will enable the spine to remain in place for the duration (Figure 8). The region marked for sectioning should be aligned with the left blade.

- Water is the only lubrication necessary for processing the spine. The spine is softer material than an otolith, so the saw will not be as loud as when cutting through otoliths. However, because the spine extends from the mount, when the spine is halfway cut, water will start to flow off the distal tip and not cycle back to the water bath. Sponges are recommended to pick up the flowing water, which can then be squeezed back into the bath. If there is significant wobble in the blades that transfers to the spine during processing, the distal portion of the ray can be manually held in place on the mounting chuck during sectioning. It takes ~1–2 minutes to cut an adult Goliath Grouper spine, and resultant sections should be ~500µm thick.

- Sectioning of the spine is complete when the area of the spine along the backside of blade rotation starts to pile up with glue (this indicates that the spine has been fully sectioned and that the glue is the only remaining material being sectioned). At this time, the spine should remain on the saw arm, but the saw arm should be lifted, and the sections raked over with a finger. If the sections readily move or wiggle, they are fully cut. If they don't move, they need more sectioning (so the saw can be turned on and the arm slowly lowered into place again).

- The fully sectioned spine can be removed from the mount and sections carefully fanned out and removed from the glue. They should be rinsed in a clean water bath, dried and placed on a slide. Care must be taken to ensure that the back side of the spine is as dry as possible because if it is not, the water will react to the liquid mounting medium and create bubbles.

- Processed adult Goliath Grouper spine sections should be covered with a liquid coverslip within 10 minutes of processing. If left to air dry for longer than 10 minutes, the sections will start to curl up and will not lay flat on the slide. The coverslip will clear up scratches on the processing surface and hold the section in place on the slide (Figure 8).

- The vascularization of adult Goliath Grouper spines can be considerable, but care must be taken to remove as many air bubbles from the coverslip as possible (Figure 9). Too many bubbles will impede ageing.

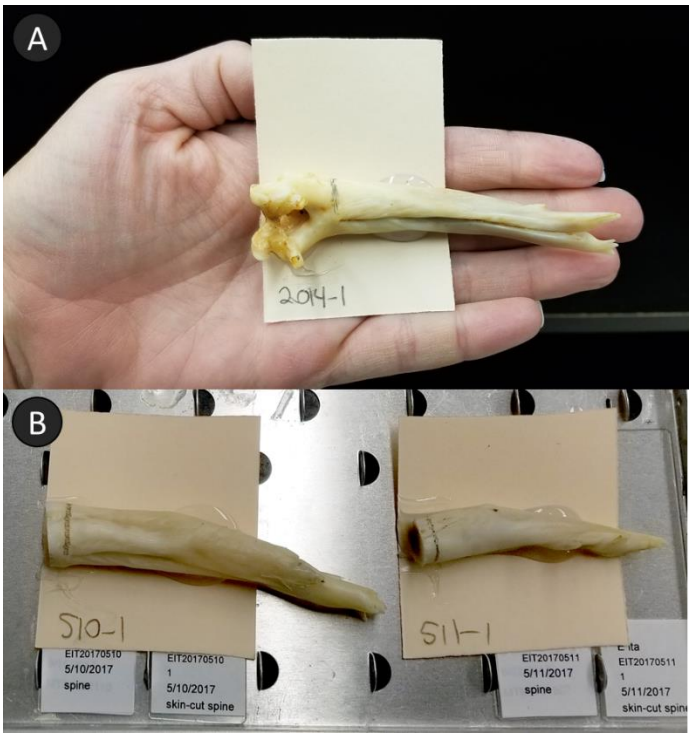

Figure 7. Image of mounted adult Goliath Grouper spine. (A) Whole spine, including the condyle base, (B) two spines with bases previously removed. Note the width of the cardstock relative to the spine, as well as the amount of glue and transverse sectioning mark.

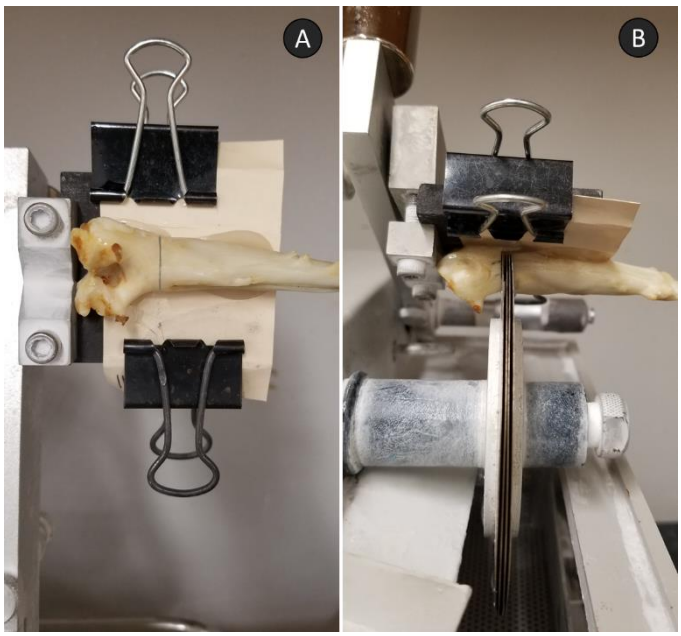

Figure 8. Isomet low speed saw with mounted Goliath Grouper dorsal spine. (A) Close-up of saddle chuck, 2” mount and binder clips for securing cardstock in place. (B) Spine during sectioning on multiblade setup.

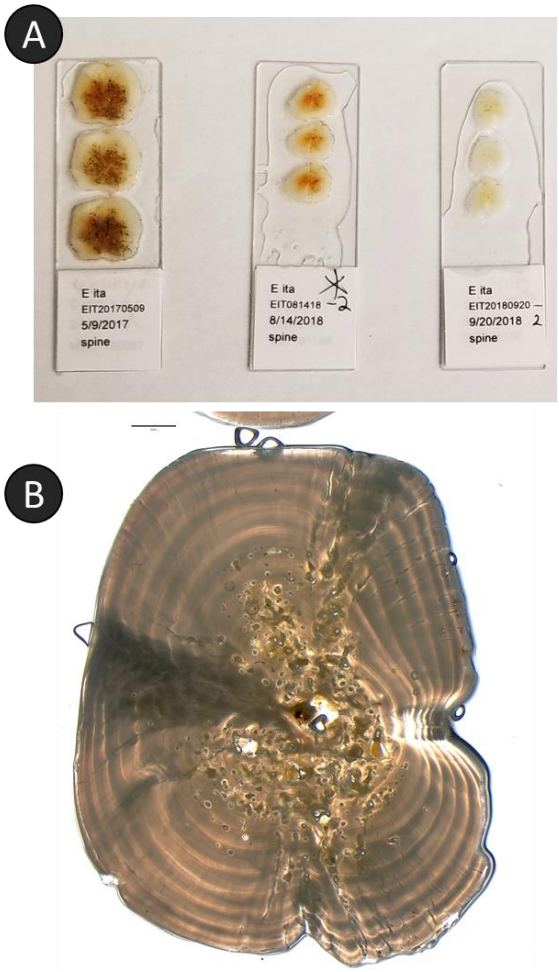

Figure 9. (A) Sectioned and mounted Goliath Grouper spines. Note the difference in coloration of the center based on the size of vascularization, which is highly individualized. Slides are paired with Figure 13 for comparison of structure size within fish. (B) Section of an age-10 dorsal spine. Some air bubbles are visible, but have largely been removed, which is ideal.

Fin Rays

Fin ray processing is detailed in the manuscript. The limitations of the saw as experienced with adult Goliath Grouper spines, on the whole, do not occur with fin rays. This is mostly due to the width of the rays. The processing protocol presented herein calls for the processing of a single hemitrich, which is (even in adult Goliath Grouper) nearly equivalent to the cutting depth of a large otolith. While the length of the Goliath Grouper hemitrich is substantial, the depth through which the saw must cut for a transverse section is not abnormally large. Several steps are necessary to process an unembedded, single Goliath Grouper hemitrich using the multiblade processing technique using a hotmelt glue mount:

- As with otoliths, it is preferable to process the same structure (i.e. left or right) for consistency. For this processing protocol, the left hemitrich from the 4th dorsal fin ray is chosen for processing. If this hemitrich is damaged, the 4th right hemitrich or 3rd fin ray can be used. The left and right hemitrichia can be identified after cleaning by viewing laterally, with the medial groove facing away; in this orientation, the posterior basal process of the left hemitrich will point to the right (Figure 10). The general posterior curvature of the hemitrichia (for articulation within the dorsal fin) is also a characteristic that can be used to identify the left and right, and this can be used when the base is not available for identification.

- The preferred processing zone of Goliath Grouper fin rays starts at the base of the structure (Figure 11, cut line A). This zone incorporates the convolutions of the fin ray, but provides the clearest view of the earliest annuli. The processing zone should be marked with a pencil to align the structure for a series of transverse

cuts. With the multiblade technique, typically no more than three sequential cuts (which yields nine individual ray sections) are necessary to provide enough sections to identify all annuli (Figure 11, cut line B).

- It is critical to place the ray on the cardstock, and the saw, perpendicularly so that the resultant sections will be transverse cross sections of the structure (Figure 12). If the spine is not perpendicular, the annuli on the sections will be skewed, which could result in inaccurate ageing.

- For the multiblade hot-melt technique, the cardstock should be cut to a size that enables the ray to hold firm on the saw arm during cutting. Ideally, the cardstock should be about 2" wide. Glue should be applied liberally to the cardstock, in a shape resembling a dog bone, to ensure a firm adhesion of the ray to paper. The thinner part of the dog bone should align with where the sections will be removed. Given the length of the rays, this amount of gluing beyond the processing zone keeps the structure from wobbling during processing. The ray should be mounted on the cardstock with the base to the left; this enables the ray to fit on the saw.

- The mounting chuck on the saw arm does not need to be altered from the mount used for otolith processing. However, it is recommended to secure the cardstock in at least two places prior to cutting, which will enable the ray to remain in place for the duration. The region marked for sectioning should be aligned with the left blade.

- Water is the only lubrication necessary for processing the ray. The ray is softer material than an otolith, so the saw will not be as loud as when cutting through otoliths. Because the ray extends from the mount, when it is halfway cut, water will start to flow off the distal tip and not cycle back to the water bath. Sponges are recommended to pick up the flowing water, which can then be squeezed back into the bath. A significant wobble in the blades will transfer to the ray during processing, so to offset this wobble, the distal portion of the ray can be manually held in place on the mounting chuck during sectioning. It takes ~45 seconds to cut an adult Goliath Grouper ray, and resultant sections should be ~500µm thick.

- Sectioning of the ray is complete when the area of the ray along the backside of blade rotation starts to pile up with glue (this indicates that the spine has been fully sectioned and that the glue is the only remaining material being sectioned). At this time, the ray should remain on the saw arm, but the saw arm should be lifted, and the sections raked over with a finger. If the sections readily move or wiggle, they are fully cut. If they don't move, they need more sectioning (so the saw can be turned on and the arm slowly lowered into place again).

- Once the basal cut is complete, the ray should remain on the saw and the saw micrometer used to move the ray over for the next sequential cut (align the previously-sectioned end of the ray with the outer edge of the blades). If sections have fallen off the glue into the blades or water bath, those should be removed prior to continuing with sectioning.

- The fully sectioned ray can be removed from the mount and sections carefully fanned out and removed from the glue. They should be rinsed in a clean water bath, dried and placed on a slide. Care must be taken to ensure that the back side of the ray is as dry as possible because if it is not, the water will react to the liquid mounting medium and create air bubbles.

- Processed adult Goliath Grouper ray sections should be covered with a liquid coverslip after processing. This will clear up scratches on the processing surface and hold the section in place on the slide (Figure 3).

Figure 10. Lateral view of paired left and right hemitrichia from a Goliath Grouper dorsal fin ray. Note the position of the posterior basal process, which aids in identifying the left and right hemitrich.

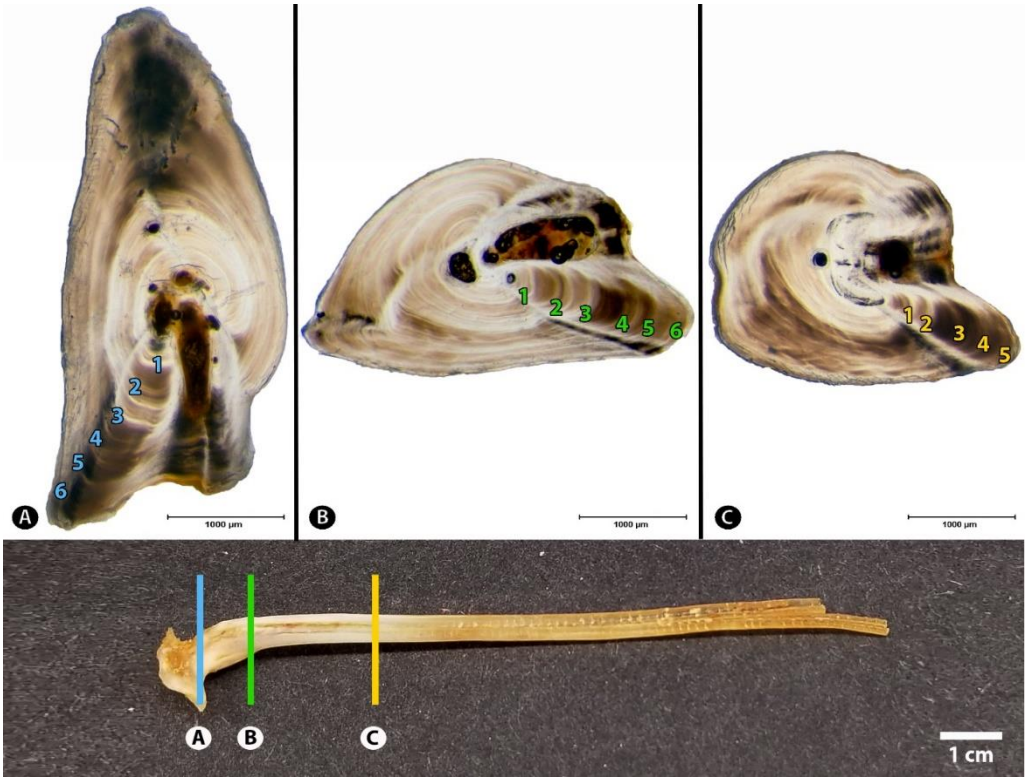

Figure 11. Cutting regions and resultant sections from a Goliath Grouper fin ray. Cut lines along the whole fin ray correspond to the labeled sections. (A) A basal cut of the fin ray, which incorporates the convolutions of the fin ray, and is the most vascularized. This is where the first transverse section should be made, (B) the region where the best section for ageing will likely originate, (C) a distal cut of the fin ray, where the true first annulus is no longer apparent, and the outer annuli have begun to compact.

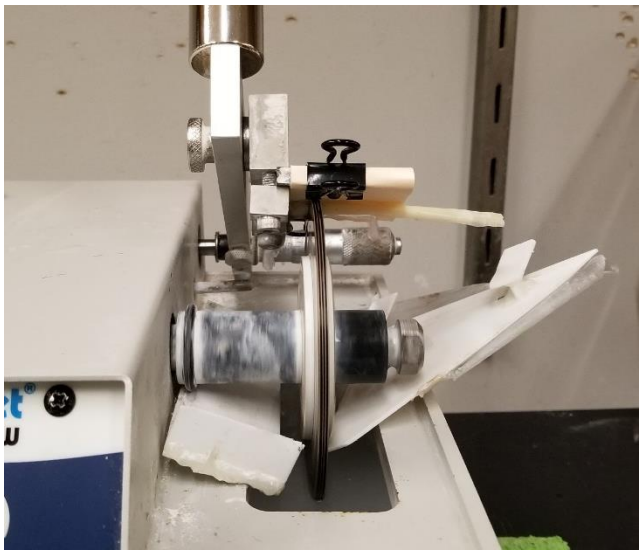

Figure 12. Isomet low speed saw with mounted Goliath Grouper dorsal fin ray using multiblade setup.

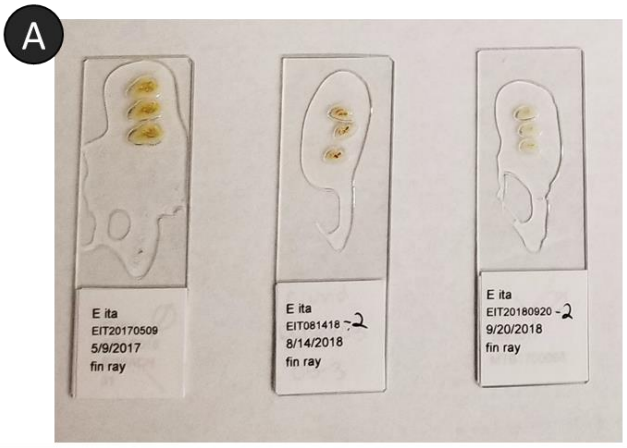

Figure 13. (A) Sectioned and mounted Goliath Grouper rays. Note: these rays are paired with the spines from Figure 9, for visual comparison of structure size within fish. (B) Section of an age-13 dorsal fin ray.

Age Determination

Goliath Grouper are assigned to age classes using annulus count, degree of marginal completion, annulus deposition period and date of collection. This method assigns fish to age classes based on the calendar year, and differs from Bullock et al. (1992) where fish were assigned into biological age groups based on timing of peak spawning. (Figure 14).

Goliath Grouper spine sections should be read using transmitted light on a dissecting microscope. The banding patterns on the cross sections of spines and rays are enumerated differently from those of otoliths due to the differential appearance of opaque and translucent bands between structures (otoliths vs. spines and rays). Otolith ageing is conducted by counting the number of opaque zones, whereby the opaque zone is enumerated as an annulus (Bullock et al. 1992). In contrast, for both spines and rays, the most definable region of the sections are the translucent zones, so these are enumerated as annuli (Brusher and Schull 2009; Murie et al. 2009).

It is important while ageing all structures of this species to ensure that the entire structure is visible within the field of view. A field of view that is too magnified will result in age overestimation.

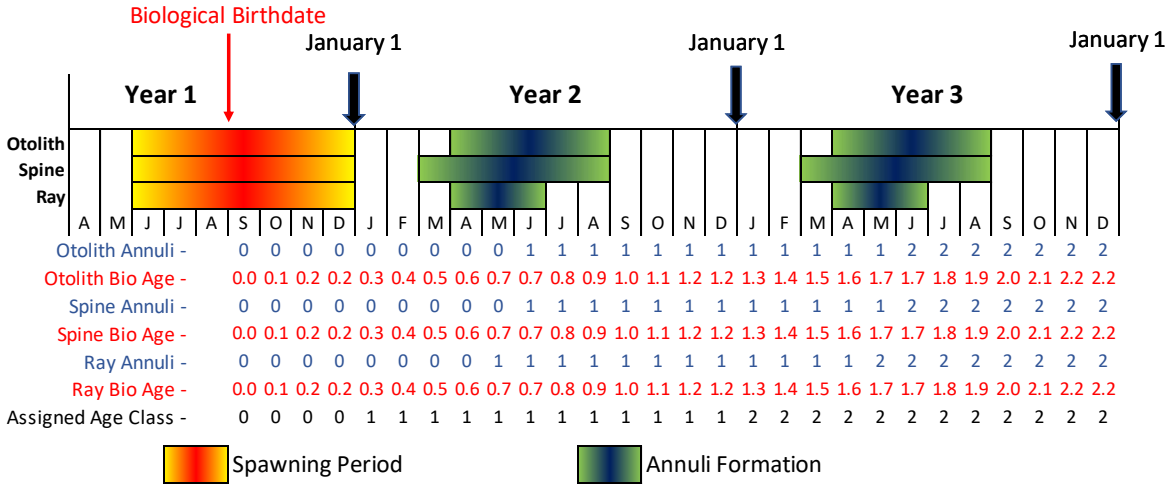

Figure 14. Timeline showing spawning period and annulus deposition ranges for Goliath Grouper hard parts. Age class assignments are based on a January-1 calendar birthdate.

Otoliths

As with other groupers, annuli should be counted on the dorsal lobe of the otolith, along a dorsomedial path (Figure 15). The ventral lobe of the sulcal groove can be used to corroborate annulus counts from the dorsal lobe, but should not be the primary counting zone due to the proclivity of annulus splitting.

Due to the protracted spawning season, the spacing between the core and first annulus can be variable. In general, the first annulus should be distinct from the core, and some measure of translucent growth is expected in between the end of the core and the beginning of the first annulus.

Bullock et al. (1992) used OTC and marginal increment analysis to validate otolith annulus formation, and determined opaque zone deposition occurred between April and August (Figure 14).

Spines

Ageing should be conducted from the core (central lumen) to the outer edge of the spine along a plane adjacent to the posterior groove of the spine (Figure 16). Marginal increment analysis of dorsal spine sections determined translucent zone deposition primarily between the months of March and August (Figure 14).

Per the attached manuscript, spines are the preferred structure for non-lethal ageing of adult Goliath Grouper, owing to the consistency of agreement between spine and otolith ages.

Fin Rays

Ageing should be conducted from the core (central lumen) to the outer edge of the fin ray along a plane adjacent to the inner groove of the spine (Figure 17). Marginal increment analysis of dorsal fin ray sections determined translucent zone deposition primarily between the months of April and June (Figure 14).

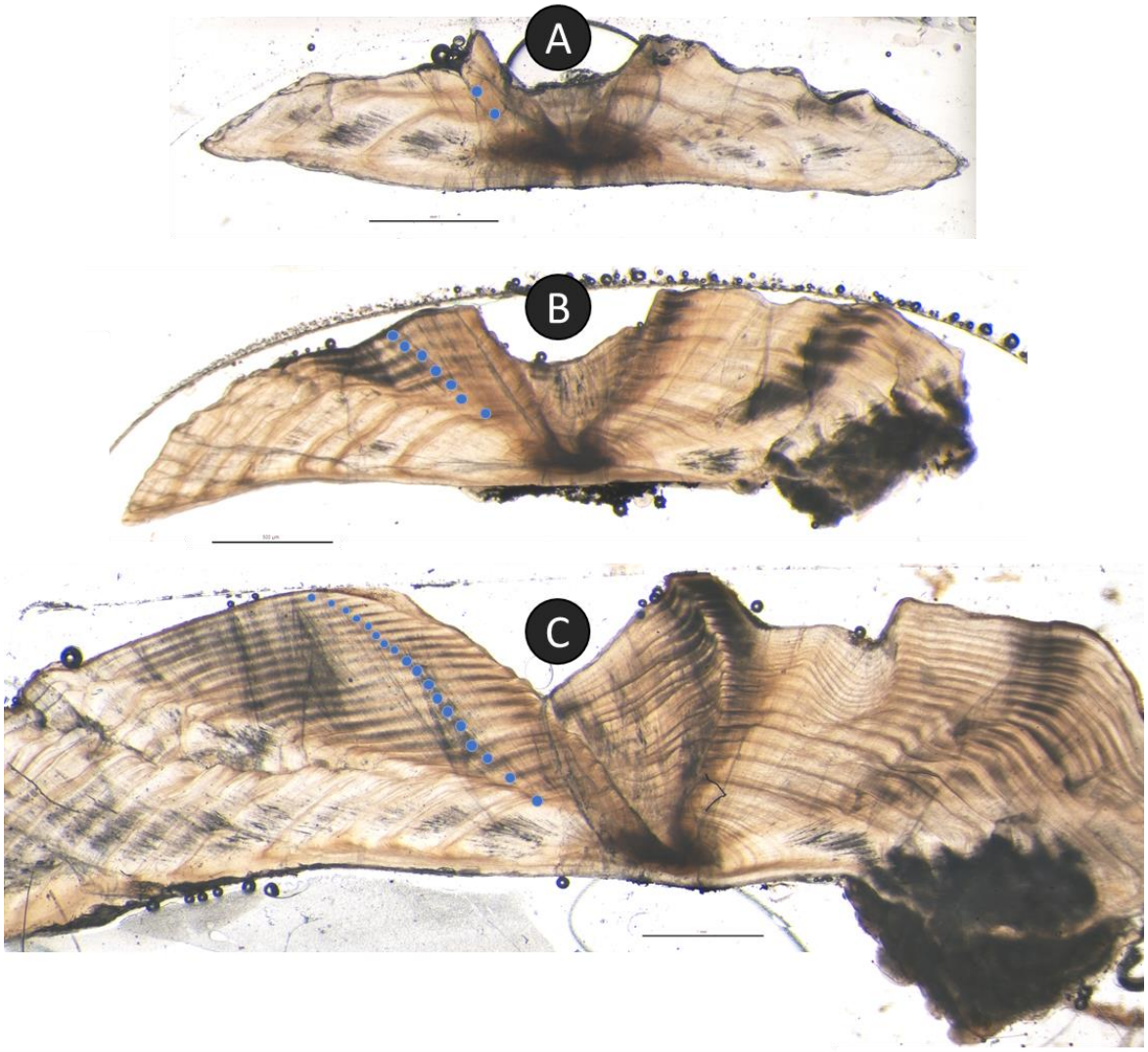

Figure 15. Annotated Goliath Grouper otolith sections from an (A) age-3, (B) age-7 and (C) age-19 fish.

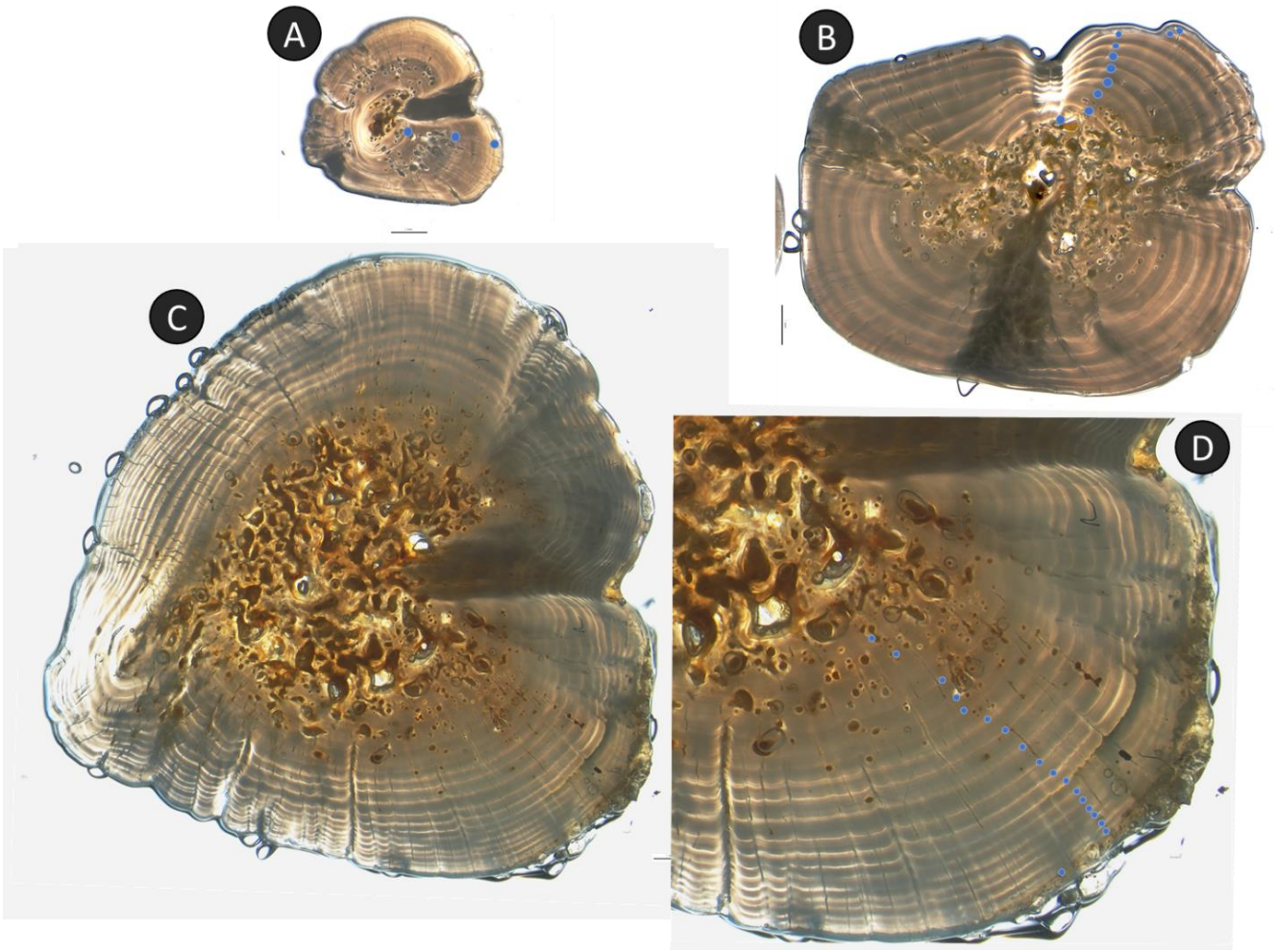

Figure 16. Annotated Goliath Grouper dorsal spine sections from (A) an age-3, (B) age-10 and (C) age-18 fish, with (D) age-18 inset annotations. Note: the annotations for D are not adjacent to the posterior groove of the spine due to the damage along the edge. Annuli can be clearly seen along the posterior groove and tracked to where the annotations are placed.

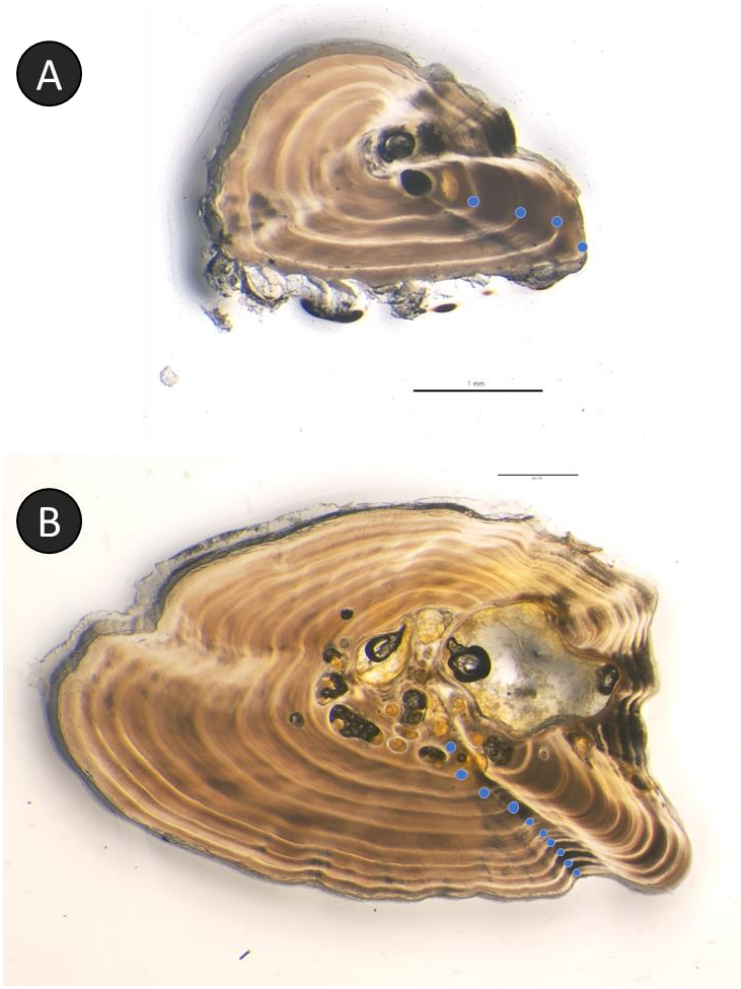

Figure 17. Annotated Goliath Grouper dorsal ray sections from an (A) age-4, (B) age-10 fish.

Spine and Fin Ray Troubleshooting

Checks or false annuli are common in fin spines and rays (Figure 18). If a suspected check is noted along the ageing plane, more investigation is necessary to examine its consistency across the entirety of the structure. Almost always, a check will merge into another translucent zone, whereas a true annulus will always remain separate (Cass and Beamish 1983; Speare 2003). If there is a translucent zone that does not merge with other translucent zones, it should be counted as an annulus regardless of spacing.

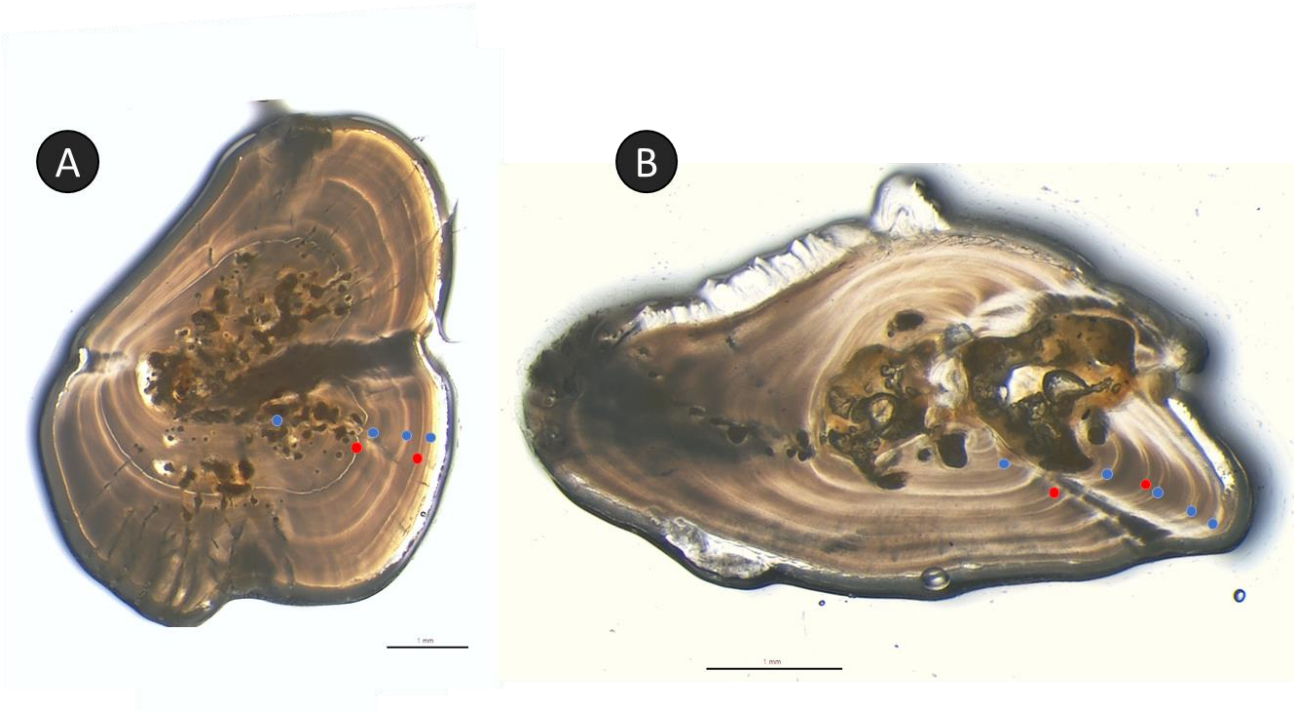

Figure 18. Goliath Grouper dorsal (A) spine and (B) ray with blue dots indicating annuli, and red dots indicating checks. The checks, while sometimes distinct, either merge back into the nearest annulus, or can't be followed around the entire structure.

As discussed in the manuscript, the central lumen of the spine is often filled with vascular tissue, and the vascularization (occlusion) can lead to the loss of the earliest growth zones on the spine. Occlusion is prevalent in nearly all spine sections, but underageing is mitigated by the visibility of early annuli from within the occlusion, even of fish from the oldest age classes, see Figure 9 in the manuscript.

In fin rays, vascularization is not as much of an issue because the vascular tissue is located primarily in the medial groove between the hemitrichia. However, resorption is apparent in Goliath Grouper fin rays, particularly in older age classes, which leads to underageing, see Figure 9 in the manuscript.

Compaction of annuli on the edge of spines and rays can occur in older individuals, which may lead to age underestimation if not properly identified. Compaction in Goliath Grouper can start around the age of 10, but is most pronounced in the oldest samples, see Figure 12 in the manuscript. It is very obvious on sections when compaction is occurring as the edge becomes almost entirely translucent when looking at a lower magnification, but focusing on the edge at a higher magnification will reveal all the annuli.