Report on Age Determination and Reproductive Classification Workshops for Gray Triggerfish (*Balistes capriscus*), September 2011 and October 2012

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SEDAR32-DW03
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Report of a Workshop on Age Determination and Histological Staging of Reproductive Tissues for Gray Triggerfish (*Balistes capriscus*)

Workshop Date: September 26-28, 2011 and follow-up on Oct. 15-18, 2012
Workshop Locations:
September 26-28, 2011
Marine Resources Research Institute
South Carolina Department of Natural Resources
217 Fort Johnson Road
Charleston, SC 29412

October 15-18, 2012
NOAA SEFSC-Beaufort Laboratory
101 Pivers Island Road
Beaufort, NC 28516

Participants:
September 26-28, 2011
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October 15-18, 2012
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Executive Summary:

Gray triggerfish from the US South Atlantic are undergoing a benchmark stock assessment through the SEDAR process in 2013 (SEDAR 32), with expected life history data providers including SCDNR/MARMAP/SEAMAP-SA, SEFIS, and SEFSC-Beaufort, NC lab. As with other species, a need exists to standardize techniques of processing life history samples, age determination methods, and methods of assessing reproductive parameters to provide consistency in age and reproductive analyses among labs. Further, inclusion of expertise from the Gulf of Mexico, given their assessment history in the region, helped ensure that samples from the separate regions are treated and analyzed in a consistent manner.

The objectives of the workshop were to (1) compare sample preparation, reading methods and data analysis of the first dorsal spine of gray triggerfish and (2) compare reproductive histological assessments with the goal of providing recommendations on the processing and examination of spines and reproductive tissues to laboratory staff and the SEDAR Data Workshop. During the initial two-day workshop held September 26-28, 2011, we discussed spine structure of gray triggerfish, sample processing (cutting and mounting samples for age analysis), age determination methods, data analysis, and the revised criteria for interpreting histological sections of reproductive tissue. An additional one-day discussion regarding aging procedures was held on October 15-18, 2012 to finalize aging methodology. Criteria for reproductive analysis were finalized in fall, 2012. **Recommendations included cleaning of spines prior to processing, sectioning using multiple blades, collecting edge information, and inclusion of both distinct increment formations and diffuse formations.**

After the initial workshop, an inter-laboratory calibration set was exchanged between the two age data providers (SCDNR and NMFS-Beaufort) in the U.S. South Atlantic region to assess the precision and bias of age readings within and between laboratories. Each lab provided 100 randomly chosen spines for the inter-laboratory calibration study. While initial reads indicated an acceptable level of precision between the two labs, there was an indication of possible bias in readings between the labs with regards to the NMFS-Beaufort calibration set, with SCDNR on average assigning older counts (1 to 2 increments) to fish with fewer than 7 increments. The source of this bias did not appear to be due to systematic differences in spine interpretation, but rather stemming from differences in spine section morphology between the SCDNR and NMFS-Beaufort calibration set. Both labs recognized the differences in morphology and agreed that additional discussions were needed to standardize interpretation of spine sections produced by the NMFS-Beaufort laboratory. As such, one of the goals of the 2nd age workshop and subsequent discussions were to address the differences.

After the second age workshop, the SCDNR age readers re-examined the NMFS-Beaufort calibration set individually (without prior knowledge as to the sampling date, fish size and prior age estimates) and developed an SCDNR consensus read. Increment counts and calendar ages determined from these reads were used to finalize the inter-laboratory calibration study. Results of these analyses suggest that within and between labs precision were within acceptable limits, as defined during the 1st age workshop, and there was no biologically significant bias in age readings within or between laboratories. Given these results, there appears to be no concern with combining the age data sets between the two primary age laboratories aging gray triggerfish captured in the U.S. South Atlantic region. Both laboratories are interpreting spine structures in a similar manner.

Currently, the SCDNR MARMAP laboratory is the only source for reproductive data from fishery-independent surveys. Workshop participants emphasized the importance of having testes, sperm ducts and accessory glands present in all samples to accurately assign maturity stages since male staging is based strongly on spermatozoa densities within the ducts**. Most of the descriptors for reproductive classes are based on Moore (2001), Wyanski (2006) and Brown-Peterson et al. (2011), with the**

inclusion of subclasses for male staging to more accurately define reproductive seasonality. It was decided that all reproductive samples would be independently assessed by two readers and reevaluated if assessments differ. SCDNR/MARMAP also collected samples during the 2012 season. Only twelve samples were collected, so this effort will continue in future sampling seasons to better understand fecundity for gray triggerfish. **Workshop participants recommend using gonad weight as a proxy for fecundity analysis.**

Introduction:

Gray triggerfish (*Balistes capriscus*) is a marine species in the family Balistidae that occurs in the tropical and temperate zones across the entire Atlantic Ocean, including the Mediterranean Sea (Bernardes 2002, Robins and Ray 1986). Gray triggerfish occur in coastal waters of the western Atlantic from Nova Scotia (Canada) to Argentina, including the Gulf of Mexico and off Bermuda (Bernardes 2002, Robins and Ray 1986). Throughout this distribution gray triggerfish generally are found at depths of 0-100 m (Harmelin-Vivien and Quéro 1990). In the Gulf of Mexico, they are found commonly at depths between 12 and 42 m among reefs and hard bottom habitat (Harper and McClellan 1997).

Previous research on the age and growth of gray triggerfish has been derived predominately from fish outside the jurisdiction of the South Atlantic Fisheries Management Council (SAFMC). Peer-reviewed and unpublished studies in other regions, using the first dorsal spine as the aging structure, include the southern coast of Africa (Caveriviere et al. 1981, Ofori-Danson 1989, Aggrey-Fynn 2009), Brazil (Bernardes 2002), and the Gulf of Mexico (Johnson and Saloman 1984, Wilson et al. 1995, Hood and Johnson 1997, Ingram 2001, Fioramonti 2012). Along the US South Atlantic, only two of these have focused on the age and growth of gray triggerfish in coastal waters (Escorriola 1991, Moore 2001). Moore (2001) found that gray triggerfish collected among reefs and hard bottom habitat from Cape Fear, North Carolina to Cape Canaveral, Florida ranged in age from 0 to 10 years old, with a maximum observed fork length (FL) of 560 mm. Moore (2001) also found that males were significantly larger than females.

Gray triggerfish from the US South Atlantic are undergoing an inaugural benchmark stock assessment through the SouthEast Data, Assessment, and Review (SEDAR) process in 2013 (SEDAR 32). This assessment will include data through 2011. Life history data providers for the region include the South Carolina Department of Natural Resources (SCDNR) Marine Resources Monitoring, Assessment and Prediction (MARMAP) program, the Southeastern Fishery Independent Survey (SEFIS), and the National Oceanographic and Atmospheric Administration's (NOAA) Fisheries Service Southeast Fisheries Science Center, Beaufort, NC (SEFSC-Beaufort).

Despite the lack of assessment history in the US South Atlantic region, an assessment of the Gulf of Mexico population via the SEDAR process was completed in 2006, with an update assessment in 2011 (SEDAR 9). Dr. Gary Fitzhugh and Carrie Fioramonti from Gulf of Mexico SEDAR (NOAA Fisheries Service Southeast Fisheries Science Center-Panama City, FL [NMFS-Panama City] and University of West Florida) participated in a gray triggerfish age/growth workshop on September 26-28, 2011, given their expertise in standardized techniques to process and analyze spines, determining ages, and assessing reproductive parameters.

Representatives from Florida Fish and Wildlife Research Institute (FWRI), University of West Florida (UWF) and University of South Carolina-Aiken (USC-Aiken) were asked to participate in the workshop because of their current activities and past experience with gray triggerfish life history and ecology. Staff from FWRI and UWF have relevant experience with age and growth of gray triggerfish from Gulf of Mexico. FWRI and UWF staff worked closely with SEFSC-Panama City and other state agencies in preparation for the SEDAR stock assessments in the Gulf of Mexico. MARMAP staff were involved because they have been investigating the life history of gray triggerfish through their long-term

reef fish monitoring and research program in preparation for SEDAR assessments. Dr. Virginia Shervette from USC-Aiken was a participant due to her current fishery-dependent and independent research on gray triggerfish, including an age validation study. Dr. Shervette's graduate student, Amanda Kelly, is conducting life history research on this species within the MARMAP laboratory.

As with other species (such as black sea bass, red snapper, golden tilefish, red porgy) recently assessed through the SEDAR process in the US South Atlantic region, a need exists to standardize techniques of processing life history samples, age determination methods, and methods of assessing reproductive parameters. With the potential number of data providers for SEDAR 32, consistency in age and reproductive analyses among labs is essential and should be ensured through standardization. Further, inclusion of expertise from the Gulf of Mexico helps ensure that data collected from the separate regions are consistent.

The goals of the workshop were to (1) compare sample preparation, reading methods and data analysis of the first dorsal spine of gray triggerfish, with an emphasis on addressing difficulties and issues previously encountered by Gulf of Mexico and Atlantic labs and (2) compare reproductive histological assessments and finalize methodology and analyses. During the initial two-day workshop held September 26-28, 2011, we discussed spine structure of gray triggerfish, sample processing (cutting and mounting samples for age analysis), age determination methods, data analysis, and the revised criteria for interpreting histological sections of reproductive tissue. Two MARMAP triggerfish age readers (Betsy Laban and Tracey Smart) and SEFSC-Beaufort triggerfish age readers (Jennifer Potts, Mike Burton, Mike Cooper) conducted an additional one-day discussion regarding aging procedures at the SEFSC-Beaufort Laboratory on October 15-18, 2012.

The classification scheme Moore (2001) used for reproductive analysis was addressed and discussed. We wanted to clearly and definitively describe, and possibly modify, the criteria to improve the accuracy of histological assessments of reproductive state. Female gray triggerfish are nest-builders (Ingram 2001) and males have structurally unique characteristics related to their ability to store spermatozoa in the common spermatic duct and surrounding accessory gland (Moore 2001). Initial discussions occurred during the workshop in September 2011, with participation by Gary Fitzhugh, David Wyanski, Kevin Kolmos, Nicole Kozlowski, and Amanda Kelly. The resulting criteria were finalized by David Wyanski, Kevin Kolmos, and Amanda Kelly in fall 2012.

Age Analysis:

<u>Aging Structure</u>

To our knowledge, all previous studies conducted on the age and growth of gray triggerfish utilized the first dorsal spine as the primary aging structure. The spine is used rather than the otoliths due to the extremely small size and irregular shape of gray triggerfish otoliths. This makes routine extraction and examination of otoliths in this species difficult and time consuming compared to other species. Currently, no published documentation exists of comparisons among potential aging structures (spines, otoliths, vertebrae, etc.) in gray triggerfish. All laboratories involved in aging gray triggerfish in the Gulf of Mexico use the first dorsal spine as the primary aging structure. Personnel from MARMAP and SEFSC-Beaufort Laboratory have also utilized the first dorsal spine as the primary aging structure. Thus, consistency exists across US South Atlantic concerning the aging structure used for gray triggerfish.

The workshop participants recommend that gray triggerfish spines be used to determine age for use in age based analyses for stock assessments.

Since no peer-reviewed literature exists validating the annual formation of increments on the first dorsal spine, Dr. Virginia Shervette and Betsy Laban are investigating multiple aging structures from gray

triggerfish, including the first dorsal spine, fin rays, vertebrae and otoliths (sagittae and lapilli), in an effort to corroborate comparable increment formation on each structure. This study has not been completed at time of submission for thisreport.

Spine Processing

All laboratories utilize transverse sections of the dorsal spine just distal to the condyle groove for age determination (Figure 1). Noticeable differences existed within and between laboratories concerning the specific technique used to obtain the transverse section (i.e., use of high speed grinders and low speed sectioning saw with single or multiple cutting blades). Several issues regarding different processing techniques were discussed, with emphasis on how and if these differences were expected to affect final spine section quality and increment counts.

1: How much and what type of pre-processing cleaning of the dorsal spine should be conducted prior to spine sectioning?

We agreed during the workshop that cleaning superficial skin tissues from the dorsal spine prior to sectioning can result in overall improved section quality. It is difficult to determine exact edge of sample sections that have not been cleaned of excess tissue prior to sectioning. Also, not removing excess tissue impedes the determination of increments near the spine edge. Further, identification of the condyle groove is difficult when excess tissue is not removed. We agreed that obvious excess muscle and skin tissue should be removed from around and just above the condyle groove.

Each laboratory (SCDNR/MARMAP, SEFSC-Beaufort, and SEFSC-Panama City) independently determined the importance of removing excess tissue prior to sectioning. However, the method and extent of tissue removal differs among laboratories. We attribute these differences to the balance between time needed to clean a spine, number of samples to process, time allotted for processing, and improvement in overall section quality. The SEFSC-Panama City lab's pre-processing cleaning is extensive; they boil all spines and then use a toothbrush and forceps to remove as much excess tissue from the spine as possible. Staff from the SCDNR/MARMAP lab use tweezers and a nylon brush to scrape away excess tissue from the spine prior to sectioning, focusing on and distal to the condyle groove. In the past, staff of the SCDNR/MARMAP laboratory used scissors and tweezers to remove some tissue covering the condyle groove prior to sectioning, but not to the current extent. This is analogous to cleaning performed by staff of SEFSC-Beaufort laboratory.

The workshop participants recommended cleaning the spine to a degree that surplus skin and muscle tissue is removed prior to sectioning the spine.

2: What type of sectioning equipment should be used to obtain the spine section for age interpretation?

SEFSC-Beaufort and SEFSC-Panama City laboratories have experimented with using two different machines for obtaining spine sections: a Hilquist saw and a low-speed Isomet saw. The labs identified several issues with the Hilquist saw including limitations with obtaining multiple sections within a single spine and degradation of spine section margin quality to the extent that interpretation of margin type is compromised. Because of these limitations, all participants agreed that the preferred sectioning equipment is the low-speed Isomet saw equipped with diamond wafering blades. Spine sections produced by SCDNR/MARMAP for age interpretation for SEDAR 32 are sectioned with the low-speed Isomet saw. The SEFSC-Beaufort Laboratory also uses a low-speed Isomet saw for the majority of their spine sections, but approximately 100 spine sections were produced by a Hilquist saw. Historically, SCDNR/MARMAP used a high speed Isomet saw prior to preparation for SEDAR 32.

Overall, workshop participants recommended the use of an Isomet low speed saw to section gray triggerfish spines.

3: Given the use of an Isomet saw to obtain a spine section, what blade system (single, double, triple, etc.) should be used and how are you mounting the spine to the saw for sectioning?

SCDNR/MARMAP Historically:

For the work contained in Moore (2001) and any other spines sectioned prior to preparation for SEDAR 32, SCDNR/MARMAP used a single blade. In this configuration, spines were directly mounted in the cutting chuck. The spine initially was cut distal to the condyle groove, shifted approximately 0.7 mm closer to the condyle groove, and cut again to obtain the 1st spine section of approximately 0.5-0.7 mm thickness. The spine was shifted again approximately 0.7 mm to obtain a 2nd and sometimes a 3rd spine section.

Workshop participants felt the single blade system and mounting the spine directly in the cutting chuck negatively impacted quality of the resulting spine sections. Specific concerns included shifting of the spine in the chuck during sectioning due to improper fit and lack of chuck tightness. This method produces sections of uneven thickness. Additionally, the single blade method was more likely to cause breakage of the section edge as the sections were removed during cutting and increased the potential for breakage of the cutting blade.

After the initial age workshop, SCDNR/MARMAP staff adopted a modified version of the SEFSC-Panama City laboratory technique. Post-cleaning, staff mark a cut-line on the spine perpendicular to the dorsal tip of the condyle groove to assist in aligning the spine when mounting and cutting (Figure 3). The marked spine is mounted to a piece of heavy-gauge plastic notebook cover (or heavy-gauge 3-ring notebook dividers; plastic cutting plate) cut to the size of the metal cutting plate. During the mounting process, the plastic cutting plate is aligned on top of a piece of craft foam that has guidelines clearly marked (Figure 4). The spine is mounted to the plastic cutting plate using hot glue, ensuring that the mark on the spine is aligned with the craft foam (Figure 4). This ensures proper placement of the spine for sectioning. Finally, we use a cushion rest to achieve a level, perpendicular cut through the spine, since the spine tapers from the base to the tip (Figure 4). The use of the cushion rest eliminates the need to remove the condyle prior to sectioning. The entire assembly of spine, plastic cutting plate and foam is mounted to the metal cutting plate with three binder clips (Figure 5). We section the spine using a three blade system (with approximately 0.5 mm spacers) which produces two sections (approximately 0.4 mm) just dorsal to the condyle groove. This method trades the time to remove the condyle with the time to remove spines from the mounting assembly to allow for its re-use.

SEFSC-Beaufort Laboratory:

The SEFSC-Beaufort lab mounted a majority of their spines directly to a low-speed Isomet saw using a v-shaped cutting chuck. Once mounted, they used a double blade system with an approximately 0.7 mm spacer between the blades. Proper positioning of the chuck in relation to the diamond wafering blade resulted in a single transverse section of the spine just distal to the condyle groove. This double blade system reduces the issue of variable spine thickness demonstrated with the single blade system. However, as indicated above, the spine may shift in the chuck due to its non-uniform shape.

SEFSC-Panama City Laboratory:

Staff from SEFSC-Panama City use a technique developed by FWRI and SEFSC-Panama City staff strictly for sectioning gray triggerfish dorsal spines. This technique secures the mounted spine to a fabricated metal plate that is then mounted to the cutting chuck (Figure 2). Prior to mounting, staff remove the condyle using a Hilquist saw and then attach the remainder of the spine to card stock with hot glue. Through this process, the spine is oriented perpendicular on the card stock ensuring true transverse sections. Once mounted to card stock, the spine is clipped to the metal plate mounted in the

cutting chuck with cardboard placed between the cardstock and plate to provide a cushion during cutting. The saw is equipped with a four blade system, resulting in three transverse spine sections immediately distal to the condyle groove.

This technique prevents the spine shifting in the chuck during the sectioning process. This provides higher quality sections that are easier to read and determine margin type relative to other methods. However, this method does increase the time it takes to process each spine.

Participants agreed that mounting improves average quality. However, in a production aging lab a balance exists between processing time and quality. Therefore, the consensus was that SCDNR/MARMAP will continue to utilize the mounting assembly and SEFSC-Beaufort will mount spines directly in the cutting chuck. We do not expect that this will lead to major differences in age estimates between the two mounting methods. However, it may lead to higher variability in quality of sections between labs. Individual labs are responsible for deciding what mounting technique is most appropriate for their needs and available supplies and equipment.

All labs agreed that a multi-blade system should be used in the future for cutting. However, the differences in the number of sectioned obtained (and blades used) does not produce sections of different quality and therefore does not warrant a standard number of blades and sections collected.

4: How many sections should be obtained from each dorsal spine?

All participants agreed that the most basal section (i.e., section closest to the condyle groove) is optimal for aging when multiple sections are available for a spine. As distance from the condyle groove increases along the spine, the increments tend to be more compacted and more difficult to distinguish, especially in older fish. The labs that obtain multiple sections indicated that the additional sections are usually for confirmation of an initial age estimate derived from the basal most section. Multiple sections provide opportunities to obtain age when the basal most section is compromised and unreadable. Several participants acknowledged that obtaining and reading multiple sections adds to the overall processing time when hundreds to thousands of spines are processed at a time.

Currently, both labs involved in data preparation for SEDAR 32 make a single cut for each spine using multiple blades. SCDNR/MARMAP obtains 2 sections with their triple blade system and SEFSC-Beaufort obtains 1 section with their double blade system. In the case of SEFSC-Beaufort, the choice was made to produce a single section due to the high volume of specimens to process and costs associated with producing and storing multiple sections for each fish.

The workshop recommended the use of a section taken close to the condyle to be used as the primary ageing structure for gray triggerfish, and that multiple section may be useful, but not critical for determining the age of the fish.

5: What is an appropriate thickness for the resulting spine sections?

Section thickness can influence accuracy in aging from a hard structure. Several thicknesses were examined and participants agreed 0.4 - 0.7 mm is optimal for age determination. Thicker or thinner sections may impact the ability to discern difficult to read increments. All labs currently employ sectioning techniques that result in sections within the desired range.

The workshop recommended preparation of sections with a thickness between 0.4 and 0.7 mm for optimal reading of gray triggerfish spines.

6: How should spine sections be mounted to slides? Are there differences in techniques between labs that are expected to affect age determination?

Based on previous experience with other species, workshop participants were not concerned that different techniques for mounting sections to slides would impact age interpretation. SEFSC-

Beaufort lab will continue to mount sections to slides using crystal bond, and then covering the section with DePeX or GURR, a liquid coverslip, to improve the optical properties of the section. SCDNR/MARMAP will continue mounting sections to slides using Cytoseal, also a liquid coverslip, which serves a dual purpose of mounting and improving optical properties of the sections under the microscope. All participants agreed employing techniques that limit the number of trapped air bubbles between the sample and slide is important because air bubbles can complicate the readability of the sample.

As the mounting technique does not seem to affect the quality of the preparations, the workshop did not have a recommendation as to the mounting technique of slides, as long as care is taken to limit the number of air bubbles in the preparation.

Age Determination

During the initial age workshop (September 26-28, 2011), staff from UWF gave a presentation regarding protocols for age determination from gray triggerfish dorsal spine sections in the Gulf of Mexico region. In general, workshop participants accepted their recommendations for age determination in US South Atlantic region. Following is a list of identified possible issues and workshop consensus on addressing these issues.

1: What light source should be used for age determination of gray triggerfish spines?

In general, two different light sources (transmitted light and reflected light) can be used during age determination for all hard structures, including gray triggerfish spines. All workshop participants felt that transmitted light provided the best readability of samples for age determination. However, staff from SCDNR/MARMAP and SEFSC-Beaufort indicated that they do sometimes use reflected light for confirmation of increment counts. A suggestion was made that polarized light can, at times, improve the optical quality of spine sections under a microscope. However, workshop participants did not recommend that polarized light be used for all aging. Instead, the use of polarized light was left to reader preference.

W**orkshop participants recommended that transmitted light be used as a primary light source when analyzing gray triggerfish spines, although the use of reflected light and polarized light in certain circumstances can/should be used to confirm increment counts.**

2: What type of microscope should be used for age determination in gray triggerfish spines? What magnification should be used?

Many researchers commonly use stereomicroscopes when reading spine sections for age determination including scientists at SCDNR/MARMAP, SEFSC-Beaufort, and SEFSC-Panama City. Some participants at the workshop who have extensive experience with using hard parts as aging structures emphasized that using compound microscopes provides higher resolution. Scope type was left to individual reader preference with the caveat that each reader consistently utilizes the same type of scope for all age determinations. We also discussed magnification. All readers agreed that using too high a magnification is problematic because of the abundance of micro-structural features in the spine. This could lead to over-estimation of increment counts. Given this concern, workshop participants suggested restricting magnification to 20-40X. We also recommended that readers maintain a consistent magnification (based on reader preference) for most spines. By assuring this spine to spine consistency in magnification and experience, individual readers develop the ability to recognize patterns for triggerfish spines.

The workshop concluded that the choice of microscope type did not affect the quality of the readings, but recommended the use of magnifications of 20X to 40X as optimal for accurate age estimates.

3: How is the 1st increment location determined in the spine section?

Staff from SEFSC-Panama City provided guidance in identifying the first translucent zone. They suggested that, under transmitted light, the first translucent zone appears as a translucent band similar in width and contour to subsequent increments and it exhibits a distinct contrast to adjacent, opaque areas. If all quadrants are clearly visible, the first increment should encircle the entire focus, or the vascularized center of the spine. Based on initial measurements, they estimated that mean distance from the focus to the distal edge of the first translucent zone is approximately 1.6 mm.

Building upon this initial discussion, SCDNR/MARMAP staff noted the presence of what they call a "core ring" (the dimension of the spine at egg hatch) located in close proximity to the vascularized center of the spine. They used the presence of the core ring, when it is present, to aid in identification of the 1st translucent zone. In cases where the core ring could be identified, the first clearly discernible translucent band after is counted as the first translucent zone or increment.

Workshop participants accepted these criteria as general rules to identify the first increment. Additionally, we noted that readers at the workshop experienced higher rates of agreement in identifying the first increment position in gray triggerfish compared to many other species in the snapper-grouper species complex.

The vascularization in the center of the spine can lead to erosion of initial spine material in older individuals. Staff from SEFSC-Panama City, FWRI, SCDNR/MARMAP, and SEFSC-Beaufort laboratories indicated they had seen no evidence of this occurring with gray triggerfish. Furthermore, there is no mention of this occurring in the published literature. Re-absorption was removed as a possible concern when determining increment counts from older gray triggerfish for these reasons.

The workshop concluded that readers for SEDAR 32 are using the same criteria for determining the 1st increment.

4: What criteria are used to define increments? How do we deal with "double" and "triple" increments?

Based on conversations amongst workshop participants and examinations of spines during the workshop, we concluded that the greatest source of increment count variability between readers stemmed from interpretations of translucent bands along each lobe of a spine section. Some samples examined during the workshop had a high occurrence of double and triple ring patterns (called doublets or triplets: two or more "increments" exhibiting consistent spacing between each other as a grouping; but inconsistent spacing between groupings for each to be considered a singular formation or an individual increment). Because of this, workshop participants established criteria to aid in distinguishing true increments from split increments (such as doublets and triplets). These are analogous to the criteria developed by the SEFSC-Panama City lab for Gulf of Mexico fish.

First, they noted that increments can vary in the visual properties within an individual spine section and among spines. Increments may appear as distinct translucent zones in sharp contrast to surrounding opaque zones or may appear as diffuse areas of slightly different translucence compared to surrounding opaque areas. Given this variability in increment pattern, particularly in the lobes, workshop participants recommend tracing probable increments around the margin. If the probable increment does not remain distinct from other probable increments or if it disappears, it is not counted as an increment. This tracing of probable increments should be attempted for all quadrants of the spine, unless it is physically impossible to age along that axis due to other issues such as damage or tissue remnants.

Second, workshop participants noted a high rate of occurrence of doublets. As with many fish otoliths, the prevalence of doublets seems to occur more frequently in younger and older fish in the most internal increments. In particular, they noted a high prevalence of doublets in the 2nd and 3rd increments as seen in Ingram (2001). Doublets appear most prominent in the lobes of the spine section. Participants agreed great care must be taken when treating suspected doublets, as this is likely the greatest source of between reader variability in increment counts. In addition to tracing increments, readers are encouraged to look at the overall pattern and spacing of increments within a spine section. Although spacing of increments in spines is more variable relative to otoliths, general spacing patterns throughout the spine are apparent. Increments that greatly deviate from the observed spacing patterns observed for other neighboring increments should be viewed cautiously.

Third, the workshop participants noted a high degree of compaction of increments in older fish along the edge of the spine, and more so in the anterior-dorsal region (closest to the focus) due to the spicule growth nature of the spine. This makes interpretation of increments difficult for older fish. Workshop participants suggested the degree of distinction between probable increments along different aging axes should be used to help identify individual increments along the margin. This was easiest to discern along the dorsal half of each lobe of the section. Another line of evidence used to discern individual increments was whether the increment could be traced around the lobes and into the interior groove of the spine section. If distinction was maintained, they were considered separate increments.

Finally, the workshop participants noted a sizable edge effect due to the three dimensional structure of the spine. This often caused the presence of a shadow along the edge. This problem seemed to be exaggerated by the presence of skin tissue along the margin of the spine section. This is one of the main reasons that cleaning tissue from the spine prior to sectioning is important. Another reason for the edge effect is inadequate use of the liquid coverslip. A sufficient amount of liquid coverslip needs to be used to cover the section and not just "coat" the section, so that a shadowed edge is prevented. When the shadowed edge is present, determination of individual increments along the edge is difficult, and may lead to variability in increment counts among readers. Edge type determination also is difficult in these situations. One potential fix suggested by SEFSC-Panama City was to tilt the section when viewing on a stereomicroscope.

The workshop concluded that, provided the use of procedures and criteria outlined above, all readers are interpreting the spine structure in a similar manner. However, examination of a number "calibration" slides should be conducted to provide data for analysis of reading error and possible bias.

5: Is determination of edge type from spine sections feasible? If so, what edge type classification system should be used?

Workshop participants discussed their ability to determine edge type from gray triggerfish spine sections so that raw increment counts can be converted to calendar ages for use in stock assessments as has become a common practice in most recent SEDARs. Where possible, SCDNR/MARMAP and SEFSC-Beaufort staffs try to determine an edge type for all individuals. However, concerns existed at the aging workshop that edge type determination for gray triggerfish may not be possible due to the compaction of increments near the margin in older gray triggerfish. A further complication, leading to questioning of the validity of edge types, was higher variability in increment widths relative to spines.

Despite these concerns, the **workshop participants recommended an edge type be assigned to all aged gray triggerfish.** SEFSC-Panama City laboratory uses three edge type categories (the 2-4-6 system), but participants noted that the four categories used by SCDNR/MARMAP and SEFSC-Beaufort can be collapsed to the three-stage classification that is consistent with the Panama City laboratory system by combining edge types 3 and 4 into a single category (equivalent to 6 in the 2-4-6 system). By assigning four edge types, we will have the greatest amount of flexibility in converting increment counts

to calendar ages. **Staffs from SCDNR/MARMAP and SEFSC-Beaufort are using a four stage classification system for edge type:**

1 – Translucent zone on the spine edge
2 – Small opaque zone on spine edge equivalent to <30% of the previous opaque zone
3 – Moderate opaque zone on spine edge equivalent to 30-60% of the previous opaque zone
4 – Wide opaque zone on spine edge equivalent to >60% of the previous opaque zone.

6: When is the period of increment formation? Is a single increment formed per year?

A definitive period for increment formation in gray triggerfish captured in the US South Atlantic has not been determined yet. Further, no definitive direct age validation of gray triggerfish from any region exists in the current published literature. Increment estimation from spines has been accepted as a proxy for ages in the US (Gulf of Mexico – see SEDAR 9 and subsequent updates) and other parts of the world (Brazil and South Africa).

The justification for using increment counts as a measure of fish age is based on several marginal increment analysis studies conducted in Brazil (Bernardes 2002), the Gulf of Mexico (Ingram 2001, Fioramonti 2012), and the US South Atlantic (Moore 2001). The frequency and period of increment formation has varied among regions. Bernardes (2002) reported that two increments develop per year in gray triggerfish caught off Sao Paulo, Brazil. Increment formation occurred during the months of April/May and Aug/Sept (Figure 6). In the Gulf of Mexico, Ingram (2001) and Fioramonti (2012) concluded that a single increment is formed per year, but they differed in their conclusions about the timing of increment formation. Ingram (2001) suggested that the increment formed during December and January in fish captured off the Alabama coast (Figure 7), while Fioramonti (2012) suggested that increment formation occurred during the month of May from fish captured off the Alabama and Florida coast (Figure 8). Finally, Moore (2001) determined that increments formed only once a year, during the month of June for gray triggerfish captured in the US South (Figure 9).

The only additional line of evidence suggesting the timing of increment formation is the marking of four fish with oxytetracycline (OTC) reared by SEFSC-Panama City (Fioramanti 2012). These fish were marked with OTC in October 2008 and then held in an outside tank at the SEFSC-Panama City laboratory until death. Of the original four, one fish survived until July 2009. Upon examination of a dorsal spine from this individual, the OTC mark was clearly visible, with one additional translucent zone formed after the OTC mark (Figure 10). This suggested that only a single increment formed during the nine months following OTC marking.

Given the high degree of variability in the estimated periodicity and timing of increment formation across different studies, the age workshop participants were hesitant to declare the periodicity of increment formation. Instead, we opted to continue collecting edge type data on specimens and then look at the pattern of edge type distribution during the data workshop to determine if a period of increment formation can be established.

The workshop concluded that the increments as identified by the workshop participants can be considered annuli, and as such, be used to determine the age of gray triggerfish.

7: How do we convert from increment counts to calendar ages and from there to fractional ages?

Given that the period of increment formation has yet to be determined, which is needed to facilitate conversion to calendar ages, no rubric for increment count to calendar age formation has been formulated yet for the US South Atlantic. This precludes the conversion of calendar age to fractional age. As mentioned above, workshop participants suggested that the period of increment formation should be investigated at the SEDAR 32 data workshop and formal recommendations made there. Only at that point can increment counts be possibly converted to calendar ages and fractional ages. In the

Gulf of Mexico, Fioramanti (2012) calculated fractional age and birth dates from consensus increment counts, capture date, and edge type.

After the 2nd workshop, analysis of NMFS-Beaufort edge type distribution gave sufficient evidence to "bump" to calendar ages (Figure 11). Moore (2001) showed similar results, increasing confidence in these plots. The SCDNR/MARMAP edge type distribution did not have high n-values for months outside April-October making it difficult to discern any true pattern. Further analysis of spawning season showed a peak in June and July suggesting a July 1st birthday.

The workshop recommended the use of the following criteria to convert increment counts to annual ages: any fish captured prior to July 1st with an assigned edge type of 3 or 4 were assigned a calendar age of increment count + one, otherwise calendar age = increment count.

8: What do workshop participants consider an acceptable average percent error in age readings for gray triggerfish?

Campana (2001) suggested there is no *a priori* value of precision that can be designated as a target level for aging studies. Workshop participants anticipated that an average percent error (APE) in the 10-15% range based on the moderate difficulty associated with aging gray triggerfish spines. This is in-line with APEs calculated for several other snapper-grouper complex species that are considered moderate to difficult to age. An inter-laboratory calibration study based on exchanged slides was used to determine the APE and if bias in aging exists.

Inter-laboratory Calibration:

Based on the conclusions and recommendations of the 1st workshop, SEFSC-Beaufort and SC-DNR staff conducted inter-laboratory calibration of gray triggerfish spines. NMFS-Beaufort and SCDNR each provided 100 randomly chosen spines for the inter-laboratory calibration study. Five readers (2 NMFS-Beaufort and 3 SCDNR) currently aging gray triggerfish for SEDAR 32 participated. Initial calibration reads by all readers on both sets were completed after the first gray triggerfish aging workshop. Results suggested precision in age readings was higher within a laboratory (APE: 8.61-14.66%) than across laboratories (14.72-20.45%). Further, a bias was evident in the age readings between laboratories, with Bowker's symmetry test (Hoenig et al. 1995) suggesting a significant bias in all pairwise comparisons of readers between laboratories. It appeared that SCDNR readers were assigning older ages than NMFS readers for gray triggerfish under approximately 7 years of age.

Closer investigation of the source of the bias revealed that it was due to interpretation differences on the NMFS-Beaufort calibration set only. Thus, the bias did not arise from an across-the-board difference in spine structure interpretation (e.g. difference in 1st annulus determination or problem with determination of last annulus along edge of the spine). Both laboratories noted differences in spine structure in this calibration set compared to the SCDNR calibration set. Specifically, it was noted the NMFS-Beaufort spines had a different morphology and the diameter of the spine for a given age fish was larger than that observed in the SCDNR calibration set, possibly resulting in SCDNR readers assigning older ages on average to fish in this calibration set than NMFS readers. Plausible reasons for the spine morphology differences in the NMFS-Beaufort calibration set includes true regional differences in spine morphology (NMFS-Beaufort calibration set was primarily from fish captured off North Carolina, while South Carolina calibration set included fish captured further south) and differences in initial spine sectioning techniques (NMFS-Beaufort obtaining sections closer to the condyle groove). This issue was discussed in the second age workshop. At that time all readers had gained a significant level of experience and the SCDNR age readers re-examined the NMFS-Beaufort calibration set. Examination was done without prior knowledge of size, date of collection and previously determined number increments. Increment counts and ages determined from these reads were used to finalize the inter-laboratory calibration study. For the final analysis, individual reads by both NMFS-

Beaufort age readers were combined with the SCDNR consensus reads. Such a comparison is valid given that ages provided for SEDAR 32 by NMFS-Beaufort staff represents only a single age read performed by one of the two age readers while ages provided by SCDNR represents a final age read determined via consensus between the three SCDNR age readers.

Final analyses were performed using both raw increment count and calendar age. Calendar age was determined based on a July 1st date of increment formation (see above). The APE between all readers on data from both calibration sets combined was 13.4% and 12.3% and 14.4% on the SCDNR and NMFS-Beaufort calibration sets, respectively (Table 2). This APE between all readers was comparable to that calculated based on readers from individual labs (11.3-14.6%; Table 4) and within the acceptable 10-15% APE values recommended by the initial workshop.

In pairwise comparisons, APE ranged from 8.3% to 11.6% (Table 5), which is well within the acceptable 10-15% APE. Indicating that gray triggerfish spines are difficult structure to age, absolute percent agreement between readers ranged from 35.4-52.6%, though percent agreement within ±2 years was 89.0-91.2% (Table 3). The only indication of a bias arose from a comparison of the two NMFS-Beaufort readers, as Bowker's symmetry test (p= 0.0149) suggested a slight bias (Table 3, Figure 12) with NMFS reader 2 aging slightly older than NMFS reader 1 for fish between the ages of 2 through 4, though the bias is likely biologically insignificant as it is less than one year on average for all ages. In comparisons of the individual NMFS-Beaufort readers to the SCDNR consensus read, no bias was evident (Table 3, Figure 13-14).

Calendar Age Analysis

Some differences between readers can be ascribed to edge type differences that disappear after adjustments for calendar age are made. For instance, one reader assigns a fish age 3 with edge type 1, while another reader assigns an age 2 with edge type 4. When edge codes are used to assign a calendar age, this fish may in fact be bumped to age 3, regardless of reader. However, the analysis indicated that the calendar age APE was not much different from the one based on increments. The APE between all readers on data from both calibration sets combined was 14.0% and 12.4% and 14.0% for the SCDNR and NMFS-Beaufort calibration sets, respectively (Table 2). This APE between all readers was comparable to that calculated based on readers from individual labs (11.1-14.8%; Table 2) and within the acceptable 10-15% APE values desired by the workshop participants.

In pair-wise comparisons, APE ranged from 8.0% to 11.4% (Table 4), which is well within the acceptable 10-15% APE set during the initial workshop. As gray triggerfish spines are difficult to age, absolute percent agreement between readers ranged from 37.9 to 52.6%, though percent agreement within ±2 years was 89.0-90.6% (Table 4). Once again, the only indication of a bias arose from a comparison of the two NMFS-Beaufort readers, as Bowker's symmetry test (p= 0.0400) suggested a slight bias (Table 4) with NMFS reader 2 aging slightly older than NMFS reader 1 for fish ages of 2 through 4 (Figure 15). As is the case based on the raw increment count analysis, the bias is likely biologically insignificant. In comparisons of the individual NMFS-Beaufort readers to the SCDNR consensus read, no bias was evident (Table 3, Figure 16-17).

Age Workshop Conclusion:

Though an initial bias was evident in the initial reads of the calibration sets by the NMFS-Beaufort and SCDNR gray triggerfish age readers, the 2nd age workshop and follow-up discussions rectified the issue. The initial discrepancies were not across-the-board issues with structure interpretation, but rather stemmed from individual spine preparation techniques used at the two aging

laboratories and/or geographical differences in gray triggerfish spine morphology. Once the differences were noted and accounted for by SCDNR readers, all bias in age determination between laboratories disappeared in the final calibration set reads. Further, precision of age readings between laboratories was well within workshop agreed upon acceptable levels.

Given these results, the workshop concluded that all readers are interpreting the spine structures in a similar and consistent manner, allowing for combining the age data sets between the two primary age laboratories aging gray triggerfish captured in the U.S. South Atlantic region.

Reproduction Analysis:

Gray triggerfish are iteroparous gonochorists that are nest builders and exhibit bi-parental care (Mackican and Szedlemayer 2007). Early life stages include demersal eggs and pelagic larvae (Richards and Lindeman 1987). Eggs may not fully hydrate or exhibit the degree of yolk fusion observed in pelagic eggs (Moore 2001). Postovulatory follicles (POFs) are rare in collections possibly due to reduced feeding by spawning females, thereby reducing the chances of females foraging, accepting bait and interacting with collection gear at this phase of the reproductive cycle (Moore 2001). It is unknown if fecundity is determinate or indeterminate. Thus, we know little about female reproductive potential, spawning frequency, and overall ovarian organization.

Male gray triggerfish have separate, small, oval-shaped testes that lie close together on the ventral side of the swim bladder (Figure 18). The common spermatic duct is lined with columnar secretory epithelial cells and surrounded by an accessory gland that may function to secrete substances that maintain spermatozoa while they are stored. Spermatic ducts act as a storage system for spermatozoa before release; therefore, both the testes and the spermatic duct/accessory gland complex are needed to accurately assess reproductive condition. A sample from the testes or duct/gland alone is usually only useful to assess sexual maturity (i.e. juveniles vs. adult).

Based on work by Moore (2001), female gray triggerfish in US South Atlantic waters are in spawning condition from April through August, with a spawning peak in June-July. Ovaries with vitellogenic oocytes were observed April through August and regressing females were observed May through November in the US South Atlantic (Moore 2001). Moore (2001) observed group synchronous oocyte development with females carrying no more than 3 to 4 batches of oocytes in the ovary. Male gray triggerfish were found in spawning condition throughout the year; however, there was a peak in activity during May through September (Moore 2001). Mature females from fishery-independent samples were found in 0% of age-0, 98% of age-1, and 100% of older fish (Moore 2001). Mature males from fishery-independent samples were present in 63% of age-1, 91% of age-2, 98% of age-3, 99% of age-4 and age 5, and 100% of older age fish (Moore 2001). Females first reached maturity at 142 mm FL, with a length at 50% maturity (L_{50}) of 158 mm FL. Males first matured at 170 mm FL, with a L_{50} of 180 mm FL. The sex ratio was not significantly different from 1:1 for fishery-dependent samples (Moore 2001). The sex ratio for fishery-independent samples was 1.2:1 in favor of females (Moore 2001).

Gonad Processing

Samples for the determination of gray triggerfish reproductive parameters are provided by SCDNR/MARMAP from fishery-independent surveys. Following capture and dissection, the posterior portion of the gonads are fixed for 7–14 d in 11% seawater–formalin solution buffered with marble chips and transferred to 50% isopropanol for an additional 7–14 d. Male gray triggerfish are unique in that both testes and the spermatic duct/accessory gland must be collected for complete analysis. For this reason, two different sections of the spermatic duct/accessory gland are taken along with a sample of the testes to ensure accurate staging. Reproductive tissue is processed in an automated and self-

enclosed tissue processor and blocked in paraffin. Three transverse sections (6–8 um thick) are cut from each sample with a rotary microtome, mounted on glass slides, stained with double-strength Gill hematoxylin, and counterstained with eosin-y. Sections are viewed under a compound microscope at 20-400X magnification, and sex and reproductive class are determined without knowledge of capture date, specimen length, or specimen age.

Reproductive Class Determination

Two hundred samples from 2002-2004 were selected randomly and used as a training set to ensure accuracy and consistency between the three readers; Kevin Kolmos, David Wyanski, and Amanda Kelly. Samples were divided into thirds randomly and each reader independently assigned sex and reproductive class via the criteria outlined in Table 7 and Table 8. Amanda K.'s samples were also read by David W. and Kevin K. since she had no prior experience examining histological sections of fish reproductive tissue. If the assessments differed, the slide was viewed simultaneously by both readers and omitted from analyses if disagreement persisted.

Most of the descriptors for reproductive classes are based on Moore (2001), Wyanski (2006) and Brown-Peterson et al. (2011), with the inclusion of subclasses for male staging. Specimens with developing, spawning capable, regressing, or regenerating gonads are considered sexually mature. For females, this definition of maturity includes specimens with oocyte development at or beyond the cortical alveoli stage and specimens with beta, gamma, or delta stages of atresia (Hunter and Macewicz 1985). We are aware that beta-stage atresia cannot always be distinguished from medium to old POFs. Photographs of the female classes can be found in Figure 19 and Figure 20 for identification purposes.

Male staging is based strongly on spermatozoa densities within the ducts as well as the ratio of structural tissue to sinus space. The inclusion of subclasses within male classes will allow us to more accurately describe reproductive seasonality. The modified definitions of reproductive classes for males will not affect any maturity aspect of previously analyzed reproductive data because the classes with modified definitions all represent mature specimens. However, the new classes may have an effect on seasonality data but we are assessing this issue as we continue to stage unread samples. Photographs for male stages are seen in Figure 21, Figure 22 and Figure 23.

Individuals with only enough tissue to confirm maturity status but not specific reproductive class (Class 8) are used for maturity analysis only. These samples are excluded from analyses of reproductive seasonality data.

SCDNR/MARMAP has developed a fecundity collection procedure which was implemented during the 2012 season. Only twelve samples were collected, so this effort will continue in future sampling seasons to better understand fecundity for gray triggerfish.

The workshop participants recommend use of the descriptive criteria for reproductive classes with the inclusion of subclasses for male staging listed in Table 5 and Table 6. Workshop participants also recommended using gonad weight as a proxy for fecundity analysis.

The use of trade names in this report does not imply endorsement by workshop participants or their affiliates NOAA Fisheries, SC-DNR, or other organizations.

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Table 1: Calculated mean CVs, APEs, percent agreement and Bowker's symmetry test results from final inter-laboratory calibration reads performed by current age readers after the 2nd age workshop. Analyses based on calendar ages. Raw increment counts were converted to calendar age based on month of capture and edge type. If month of capture < July and edge type > 2, then calendar age = increment count +1, otherwise calendar age = increment count. This calendar age conversion was suggested by SEDAR 32 data workshop panelists prior to the SEDAR 32 Data Workshop.

					% Agreement				Symmetry Test
Reader 1	**Reader 2**	**n**	**CV**	**APE**	**+/- 0 years**	**+/- 1 year**	**+/- 2 years**	**+/- 3 years**	**p-value**
NMFS-1	NMFS-2	198	16.09%	11.38%	37.88%	70.20%	89.90%	97.47%	0.0400
NMFS-1	SCDNR Consensus	190	11.32%	8.00%	52.63%	82.11%	88.95%	94.21%	0.7018
NMFS-2	SCDNR Consensus	192	15.17%	10.73%	40.63%	72.40%	90.63%	95.83%	0.1405

Table 2: Calculated APEs from final inter-laboratory calibration reads performed by current age readers after the 2nd age workshop.

	Raw Increment Count						Calendar Age					
	All Sets		**NMFS-Beaufort Set**		**SCDNR Set**		**All Sets**		**NMFS-Beaufort Set**		**SCDNR Set**	
Group	**n**	**APE**	**n**	**APE**	**n**	**APE**	**n**	**APE**	**N**	**APE**	**N**	**APE**
All Readers	200	13.37%	100	14.44%	100	12.30%	200	14.00%	100	14.00%	100	12.37%
NMFS-Beaufort Readers	198	11.56%	100	11.33%	98	11.80%	198	11.38%	100	11.08%	98	11.67%
SCDNR Readers	197	14.56%	97	14.48%	100	14.64%	197	14.60%	97	14.42%	100	14.77%

Table 3: Calculated mean CVs, APEs, percent agreement and Bowker's symmetry test results from final inter-laboratory calibration reads performed by current age readers after the 2nd age workshop. Analyses based on raw increment counts.

					% Agreement				Symmetry Test
Reader 1	**Reader 2**	**n**	**Mean CV**	**Mean APE**	**+/- 0 years**	**+/- 1 year**	**+/- 2 years**	**+/- 3 years**	**p-value**
NMFS-1	NMFS-2	198	16.35%	11.56%	35.35%	71.72%	90.40%	97.47%	0.0149
NMFS-1	SCDNR Consensus	190	11.67%	8.25%	52.63%	82.11%	88.95%	94.74%	0.8173
NMFS-2	SCDNR Consensus	192	15.69%	11.09%	38.54%	71.35%	91.15%	95.31%	0.1176

Table 4: Calculated mean CVs, APEs, percent agreement and Bowker's symmetry test results from final inter-laboratory calibration reads performed by current age readers after the 2nd age workshop. Analyses based on calendar ages. Raw increment counts were converted to calendar age based on month of capture and edge type. If month of capture < July and edge type > 2, then calendar age = increment count +1, otherwise calendar age = increment count. This calendar age conversion was suggested by SEDAR 32 data workshop panelists prior to the SEDAR 32 Data Workshop.

					% Agreement				Symmetry Test
Reader 1	**Reader 2**	**n**	**CV**	**APE**	**+/- 0 years**	**+/- 1 year**	**+/- 2 years**	**+/- 3 years**	**p-value**
NMFS-1	NMFS-2	198	16.09%	11.38%	37.88%	70.20%	89.90%	97.47%	0.0400
NMFS-1	SCDNR Consensus	190	11.32%	8.00%	52.63%	82.11%	88.95%	94.21%	0.7018
NMFS-2	SCDNR Consensus	192	15.17%	10.73%	40.63%	72.40%	90.63%	95.83%	0.1405

Table 5: Histological interpretation of female gray triggerfish. Most descriptors based on Moore (2001), Wyanski (2006) and Brown-Peterson et al. (2011).

Maturity Class	Description
Uncertain Maturity (Class 0)	Inactive ovaries, primary growth oocytes only; unable to assess maturity
Immature (Class 1)	Primary growth oocytes 20-60 micron diameter (Moore 2001); no evidence of atresia. In comparison to regenerating female, transverse section of ovary is smaller, lamellae lack muscle and connective tissue bundles and are not as elongate, oogonia abundant along margin of lamellae, ovarian wall is thinner
Cortical alveolar oocytes (Class E)	Early Developing; Previtellogenic; cortical alveolar oocytes 140-200 micron diameter
Yolked oocytes (Class F)	Vitellogenic; Most advanced oocytes in yolk-granule or yolk-globule stage; oocyte 170-400 micron diameter
Migratory nucleus oocytes (Class G)	Oocyte maturation; partial coalescence of yolk globules possible; Oocytes 385-500 micron diameter
Postovulatory follicles (POFs): early (Class B), intermediate (Class C), late (Class D)	Vitellogenic oocytes and POFs; Evidence of recent spawn; note that beta-stage atresia cannot always be distinguished from medium to old postovulatory follicles (Hunter and Macewicz 1985)
Regressing (Class 4)	>50% of yolked oocytes undergoing alpha or beta stage of atresia
Regenerating (Class 5)	Primary growth oocytes > 60 micron diameter, with traces of atresia possible. In comparison to immature female, transverse section of ovary is larger, lamellae have muscle and connective tissue bundles and are more elongate and convoluted, oogonia less abundant along margin of lamellae, ovarian wall is thicker and exhibits varying degrees of expansion due to previous spawning
Mature specimen (Class 8)	Mature, but postmortem histolysis or inadequate quantity of tissue prevent assessment of reproductive class
Unknown (Class 9)	Postmortem histolysis or inadequate quantity of tissue prevent assessment of reproductive state

Table 6: Histological interpretation of male gray triggerfish. Most descriptors based on Moore (2001), Wyanski (2006) and Brown-Peterson et al. (2011) with the inclusion of sub-classes.

Maturity Class	Sub-Class	Description
Uncertain Maturity (Class 0)		Inactive testes; unable to assess maturity
Immature (Class 1)		Small transverse section compared to regenerating male; little or no spermatocyte development
Developing (Class 2)		Limited spermatogenesis in testes; elongation of lobules and some accumulation of spermatozoa (SZ) in testes BUT no accumulation in lobules, efferent ducts (within testes), and spermatic ducts
Spawning Capable (3 sub-classes)	Early Spawning Capable (Subclass ESC)	Spermatozoa evident in ducts; amount of spermatogenesis in testes ranges from limited to extensive; in ducts, greater area of structural tissue compared to sinuses
	Storage (Subclass H)	Spermatozoa storage within expanding ducts; >50% of area of sinuses densely packed with spermatozoa; amount of spermatogenesis in testes ranges from limited to extensive
	Recent Spawn (Subclass 7)	Large, expanded ducts not as densely packed with spermatozoa; area of sinuses greater than that of structural tissue; usually has empty lobules toward center of testes
Regressing (Class 4)		Limited spermatogenesis in testes; shrinking ducts/lobules with residual spermatozoa present; overall number of ducts containing spermatozoa also small; increase of connective tissue in testes, proliferating from center; may have enlarged cells lining sinuses
Regenerating (Class 5)		Larger transverse section compared to immature male; very limited or no spermatogenesis in testes; little or no residual spermatozoa in ducts
Mature Specimen (Class 8)		Postmortem histolysis or inadequate quantity of tissue prevent assessment of reproductive class
Unknown (Class 9)		Postmortem histolysis or inadequate quantity of tissue prevent assessment of reproductive state

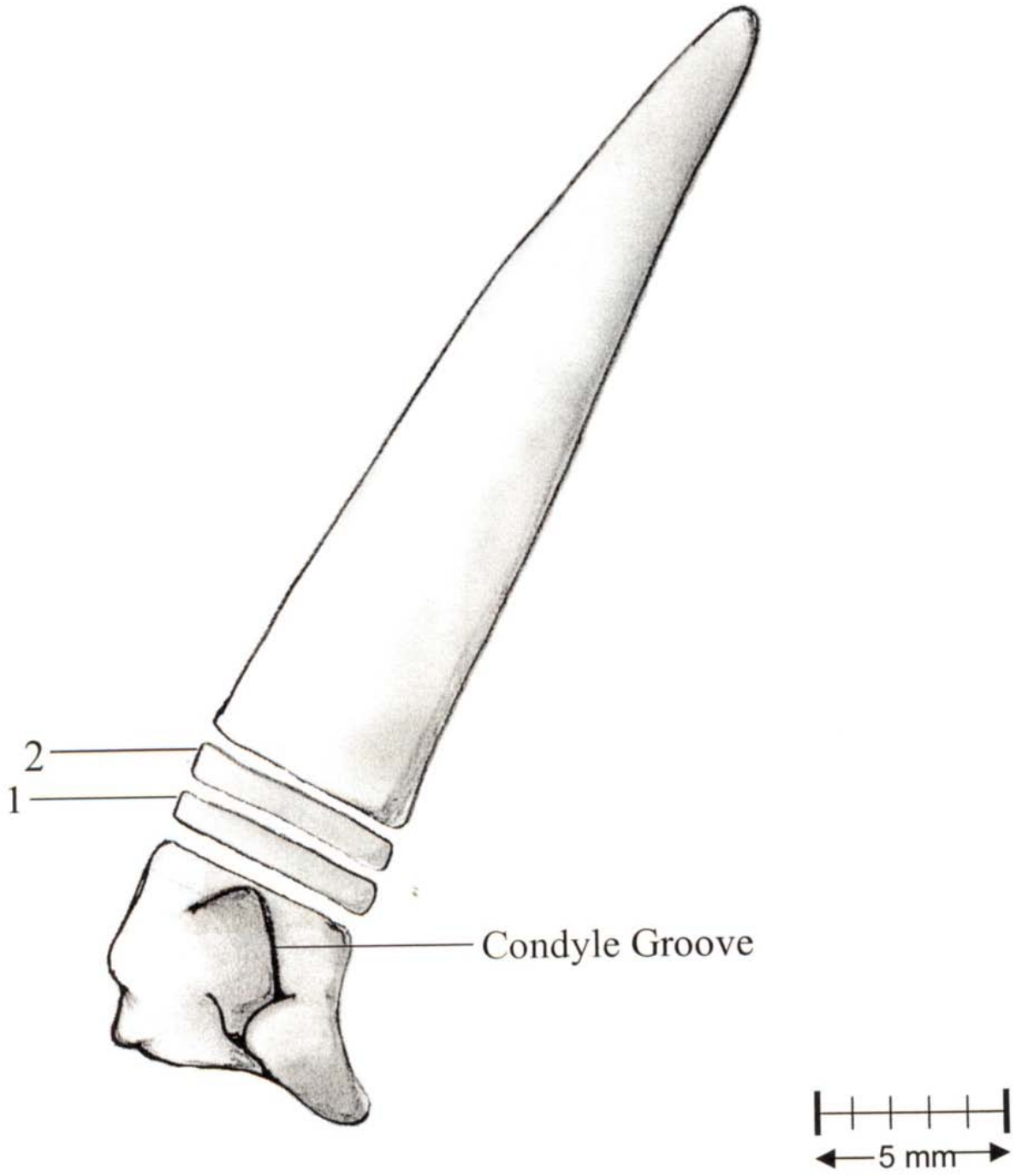

Figure 1: Schematic lateral view of the first dorsal spine from a gray triggerfish, showing location of transverse sections obtained in relation to the entire spine. Figure was extracted from Moore (2001).

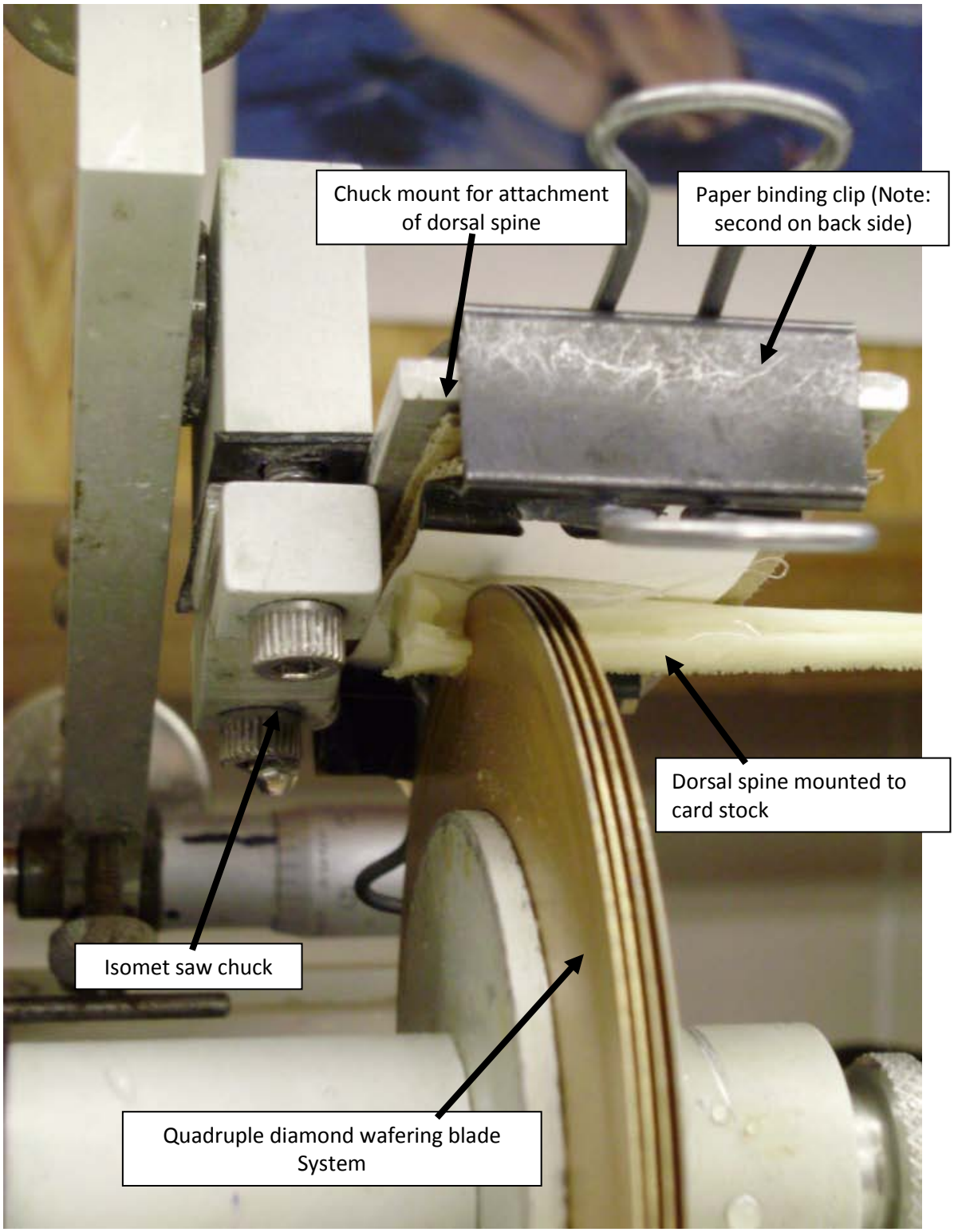

Figure 2: Illustration of sectioning method employed by the Gulf Coast laboratories. Note that the condyle has been removed prior to mounting to the saw. The dorsal spine has been mounted to card stock paper using hot glue and then clipped to the metal chuck mount using binder clips. Cardboard is placed between the cardstock paper and the metal chuck mount to provide a spacer when cutting. Image extracted from Fiorimanti PowerPoint presentation from the September 2011 workshop.

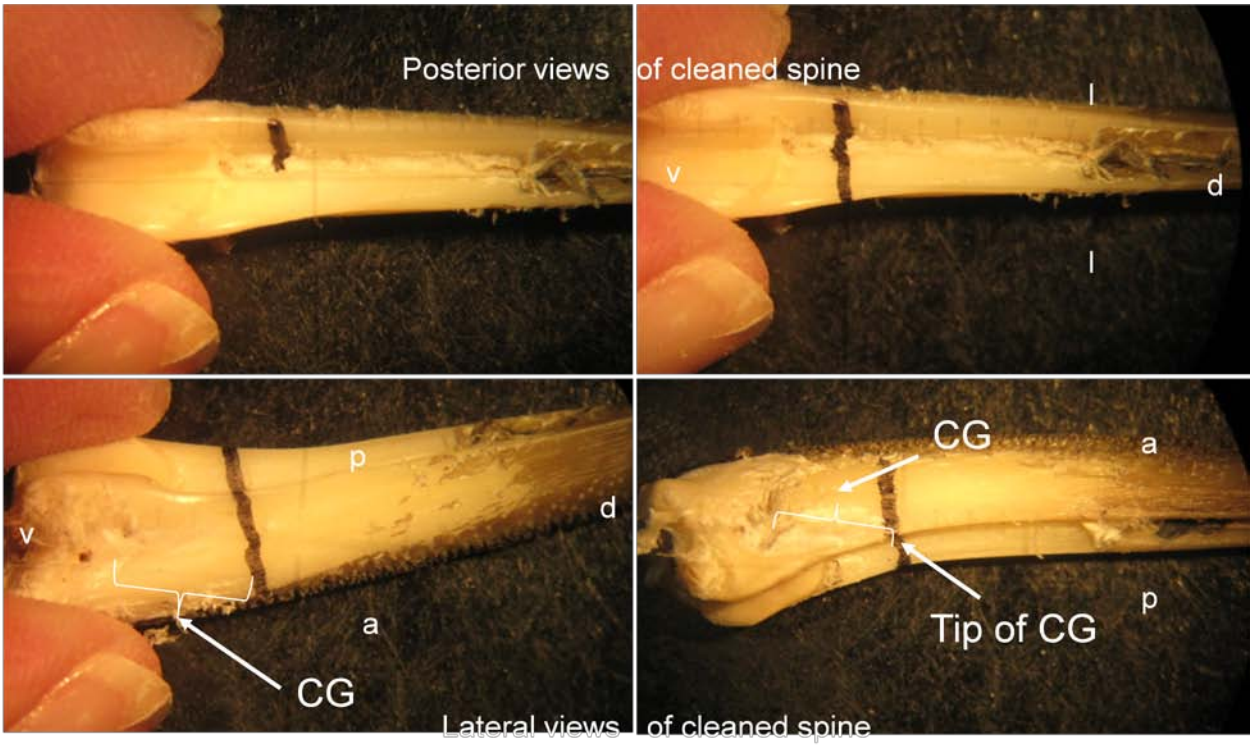

Figure 3: Posterior and lateral views of a cleaned spine being prepared for sectioning. a = anterior, p = posterior, d = dorsal, v = ventral, l = lateral, CG = condyle groove.

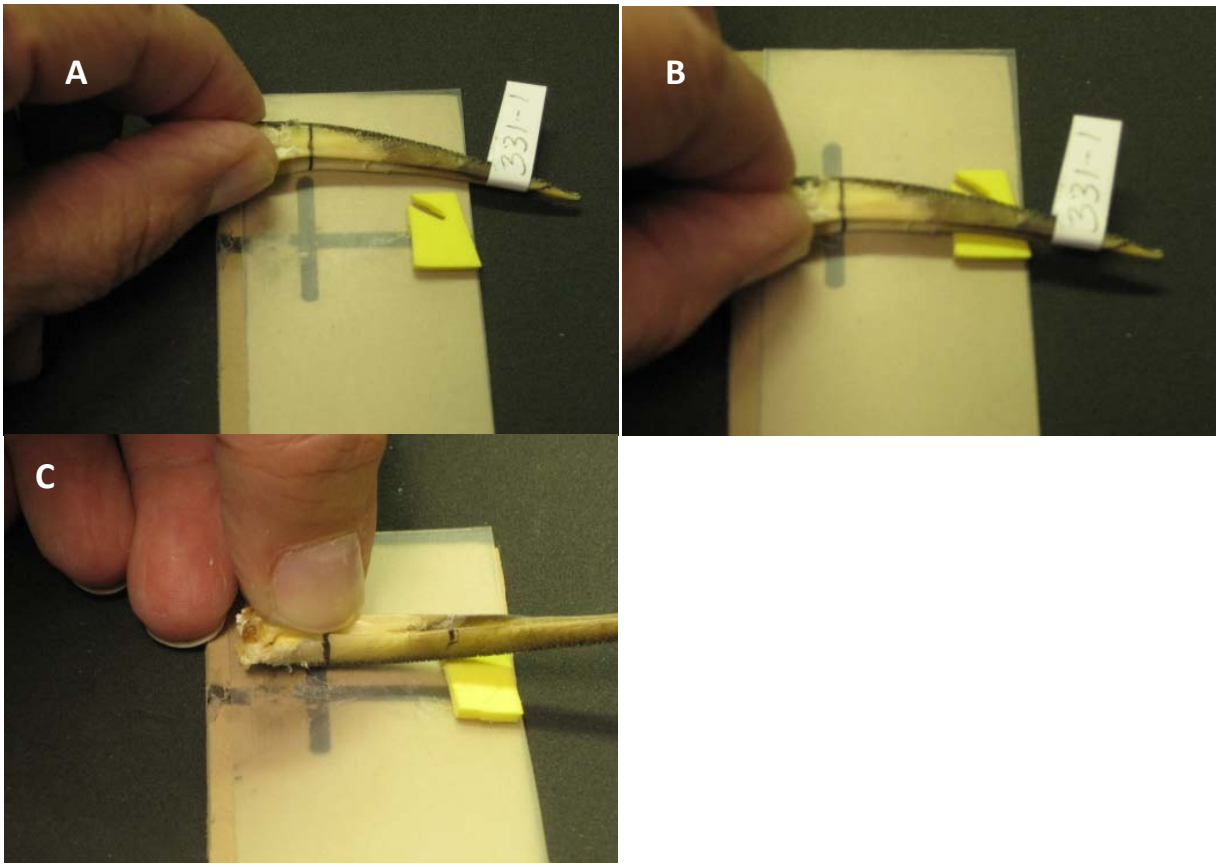

Figure 4 A-C: Figure illustrating the procedure of mounting a marked dorsal spine for sectioning. A) Prior to mounting, the plastic cutting plate is placed on top of a piece of craft foam that has been marked with two alignment lines. The alignment lines should be clearly visible through the plastic cutting plate. B) The marked on the dorsal spine is aligned with the vertical line and the spine is place above the horizontal line on the craft foam, ensuring proper placement of the spine for sectioning. C) Hot glue is used to attach the spine in the proper position. The "cushion rest" is an additional piece of craft foam secured to the plastic cutting plate with double sided tape. The purpose of the "cushion rest" is to aid in achieving a level, or perpendicular, cut through the spine, since the spine tapers from the base to the tip.

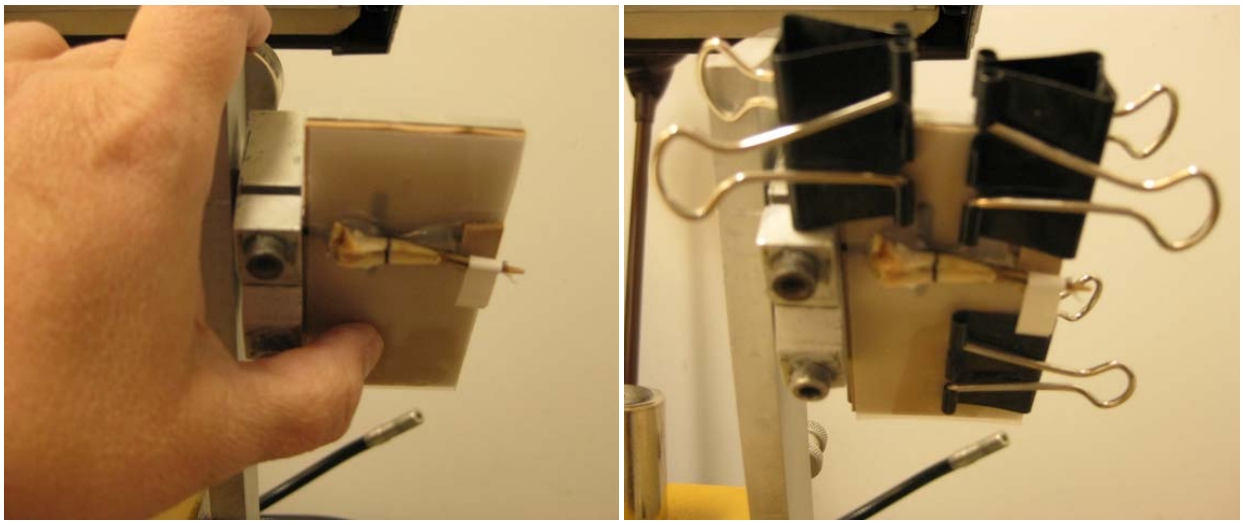

Figure 5: Illustration of how the plastic cutting plate and craft foam cushion are attached to the metal cutting plate firmly secured to the Isomet saw chuck. A) Prior to attachment ensure proper alignment. B) Illustration of a spine mounted to Isomet saw and prepared for sectioning.

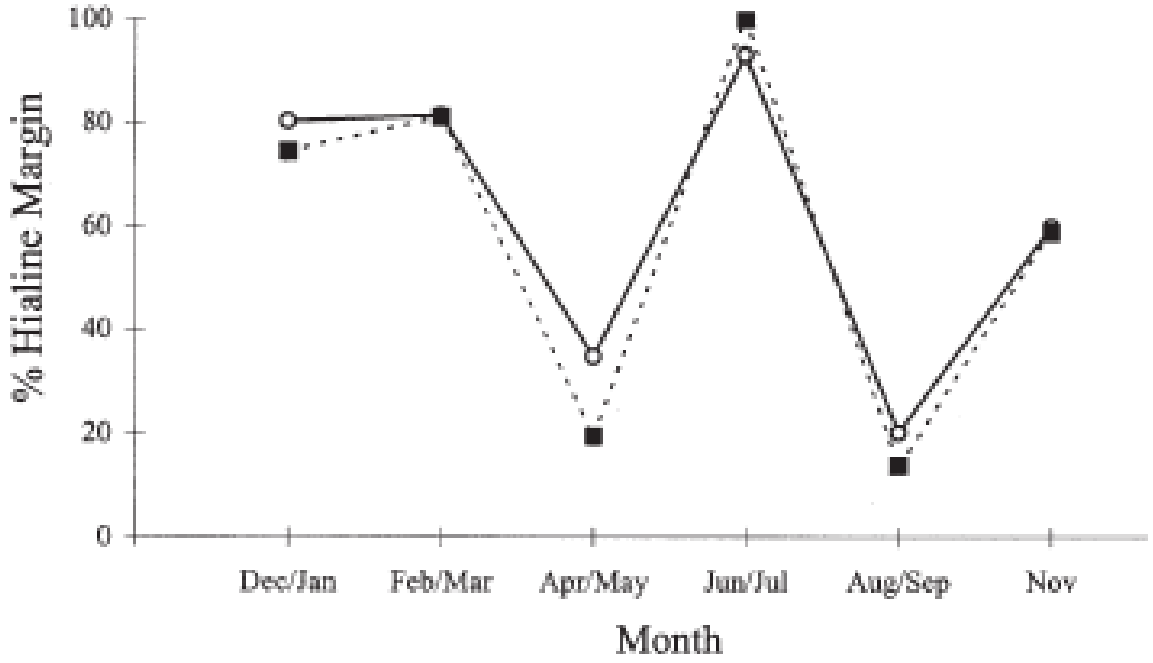

Figure 6: Seasonal variation of percentage of hialine margin on dorsal spine and vertebrae of *Balistes capriscus*, captured off Sao Paulo. Figure extracted from Bernardes (2002).

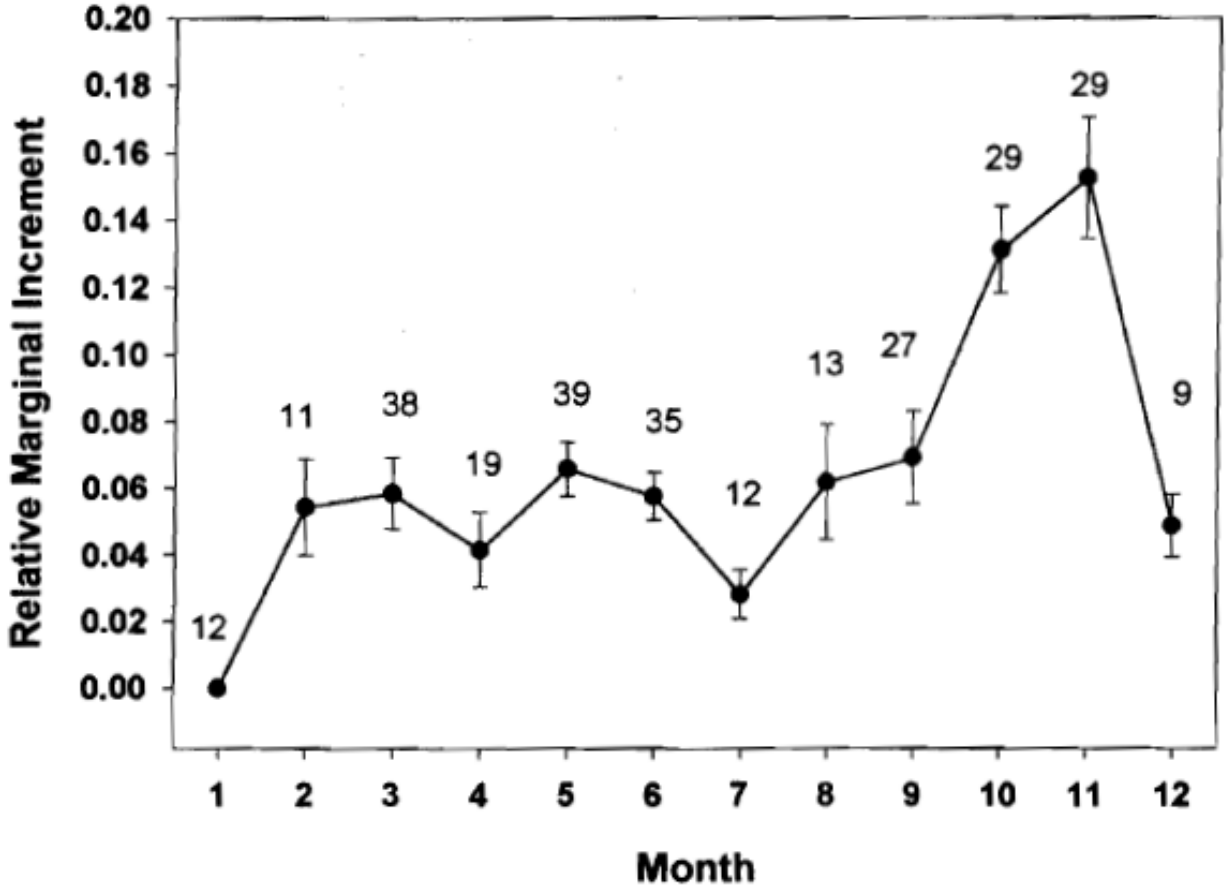

Figure 7: Average relative marginal increment of the first dorsal spine of gray triggerfish collected off Alabama from 1996-2000. Error bars represent standard error, and numbers above symbols represent sample size. Figure extracted from Ingram (2001).

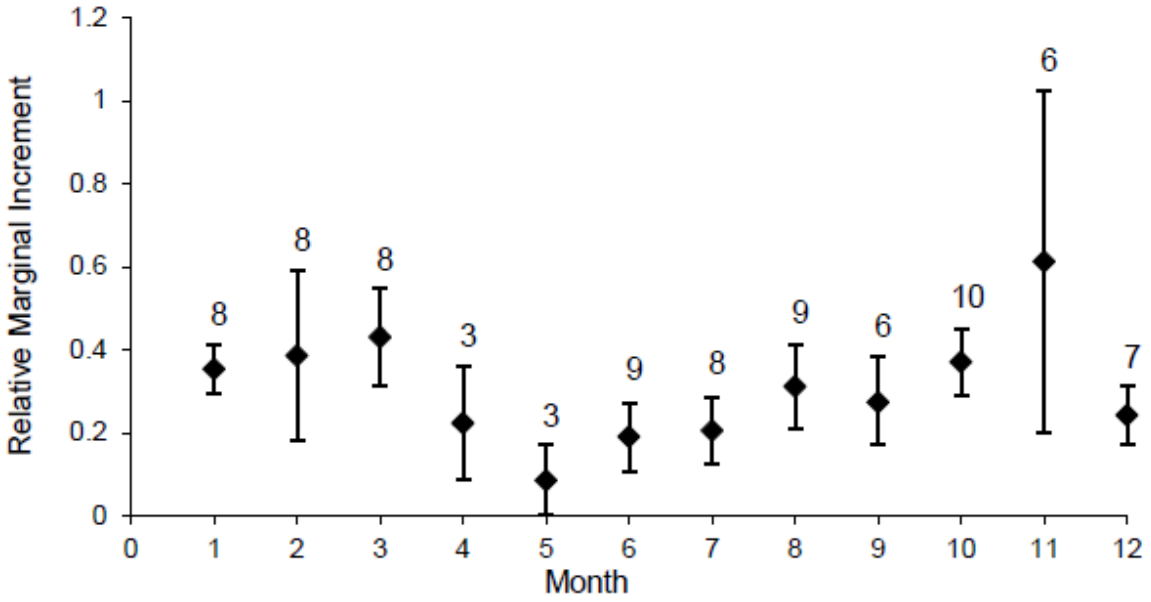

Figure 8: Mean monthly relative marginal increment (sample size for each month over data point) of age-3 gray triggerfish sampled from North West Florida study region (grids 8-10, n=85). Figure extracted (Fioramonti 2012).

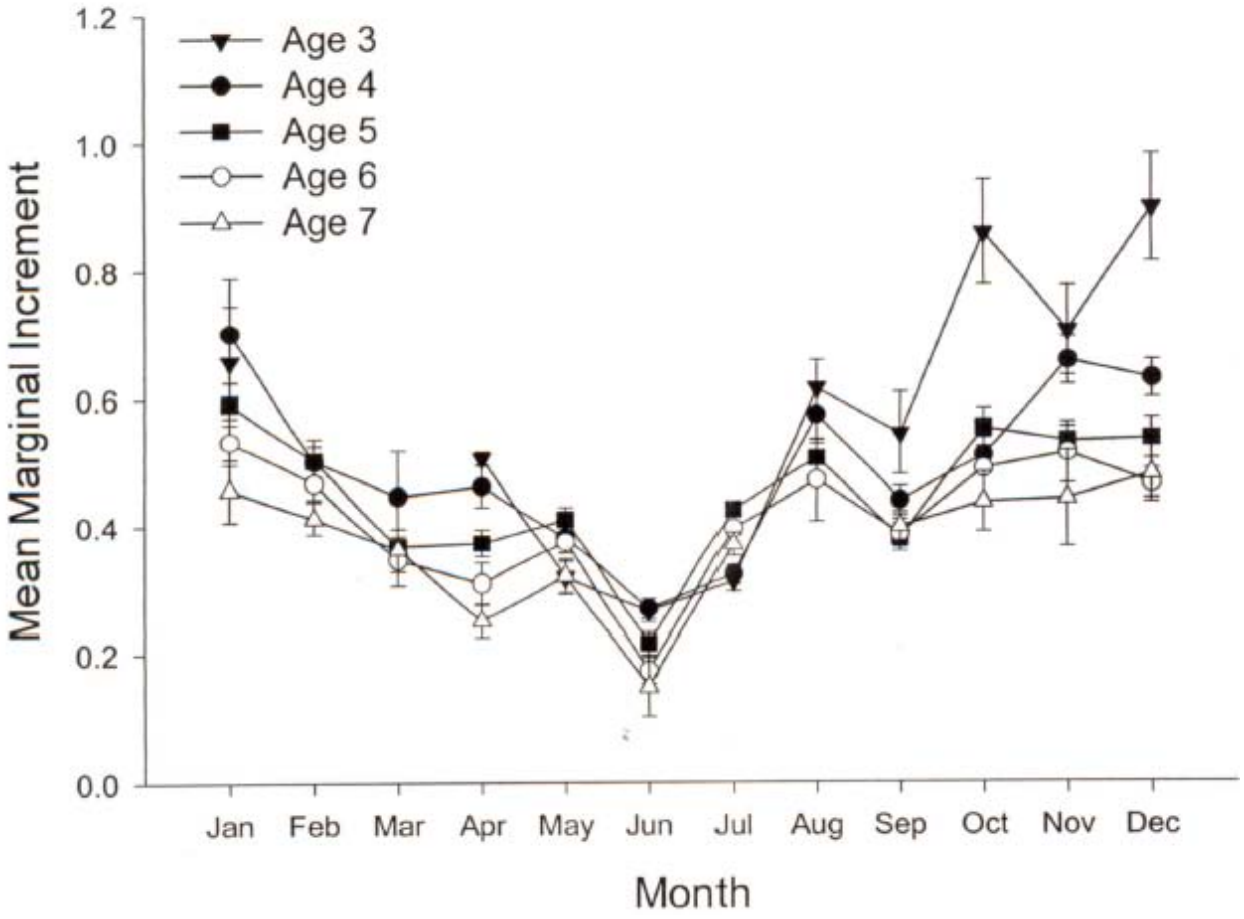

Figure 9: Mean marginal increment by month for ages 3-7 (all years combined) for gray triggerfish from fishery-independent and fishery-dependent samples from the SE region. Figure extracted from Moore (2001).

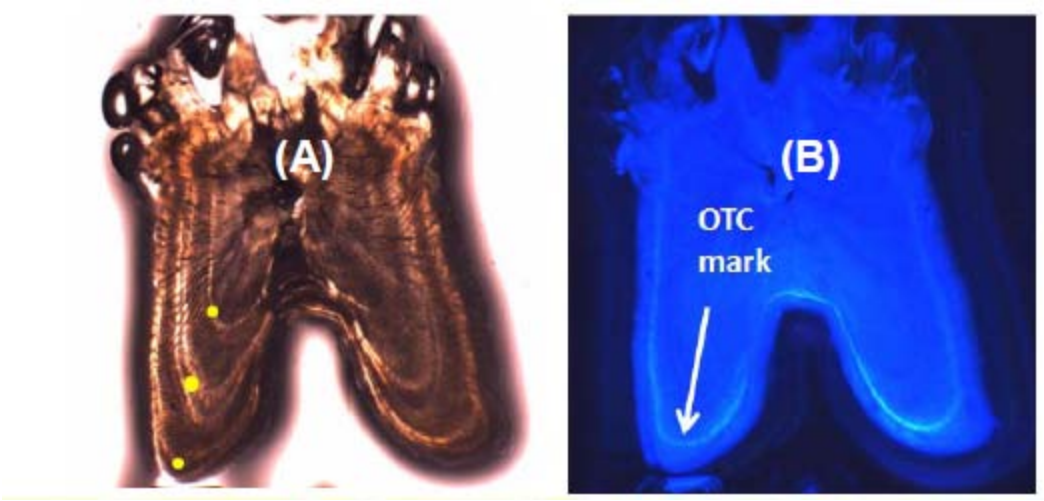

Figure 10: Spine section from a gray triggerfish, marked with OTC in October 2008, which perished in July 2009. The left figure (A) shows the specimen under transmitted light with a single, clearly discernible translucent zone. The right figure (B) is the same specimen under UV light, and clearly shows the OTC mark in the spine section being medial to the last translucent zone. Figure extracted from Fioramonti (2012).

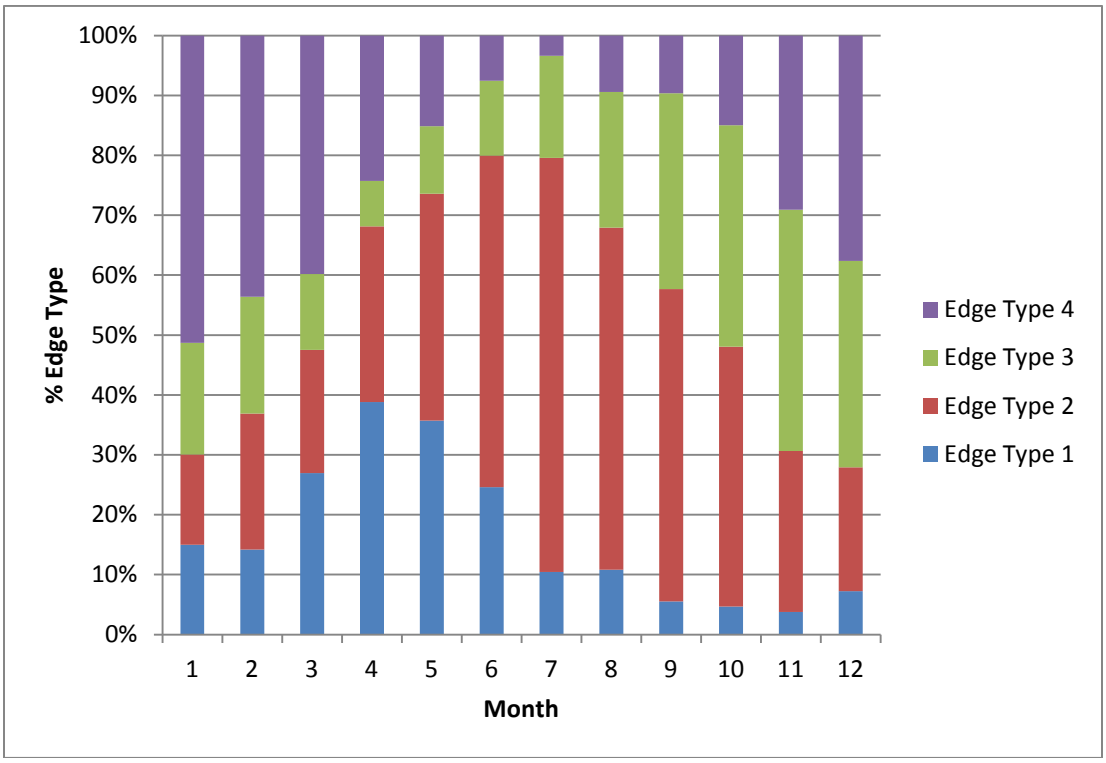

Figure 11: NMFS-Beaufort edge type distribution plot. Provided by J. Potts.

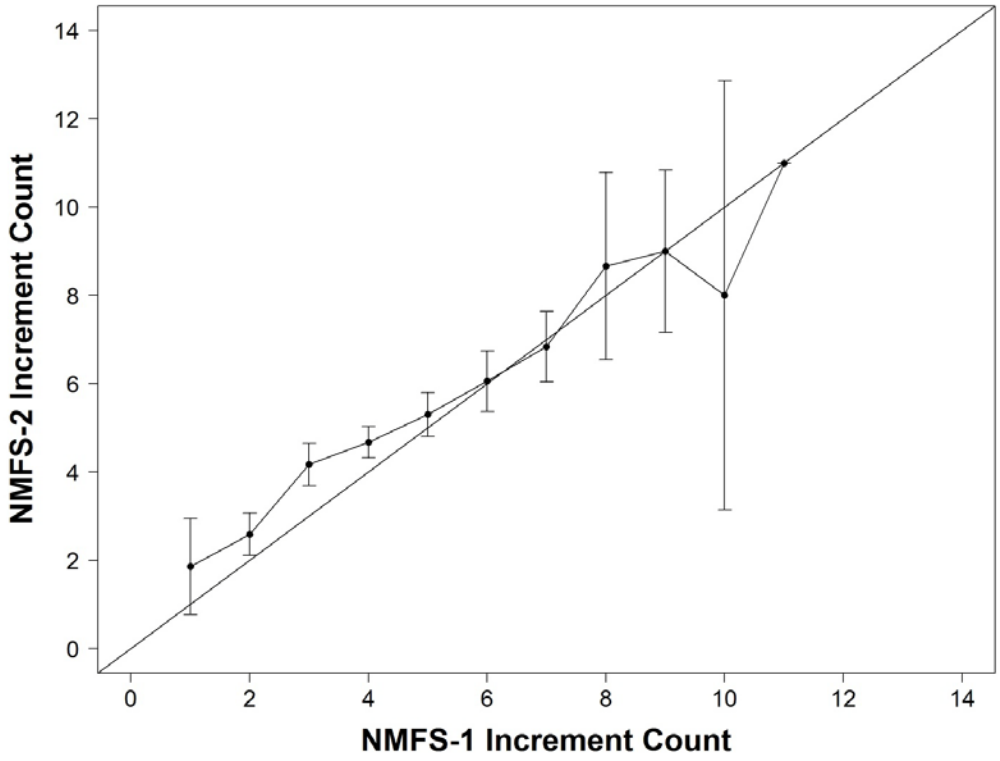

Figure 112: Bias plot for NMFS-1 vs NMFS-2 read from increment counts made for the post workshop inter-laboratory calibration study.

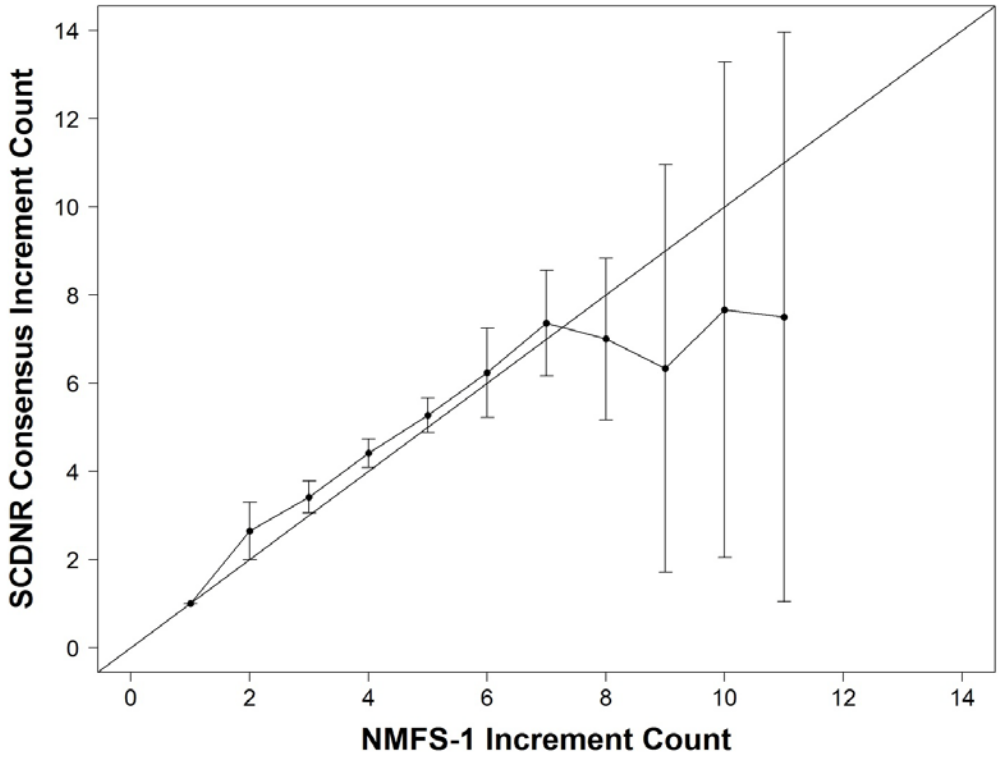

Figure 123: Bias plot for NMFS-1 vs the SCDNR consensus read from increment counts made for the post workshop inter-laboratory calibration study.

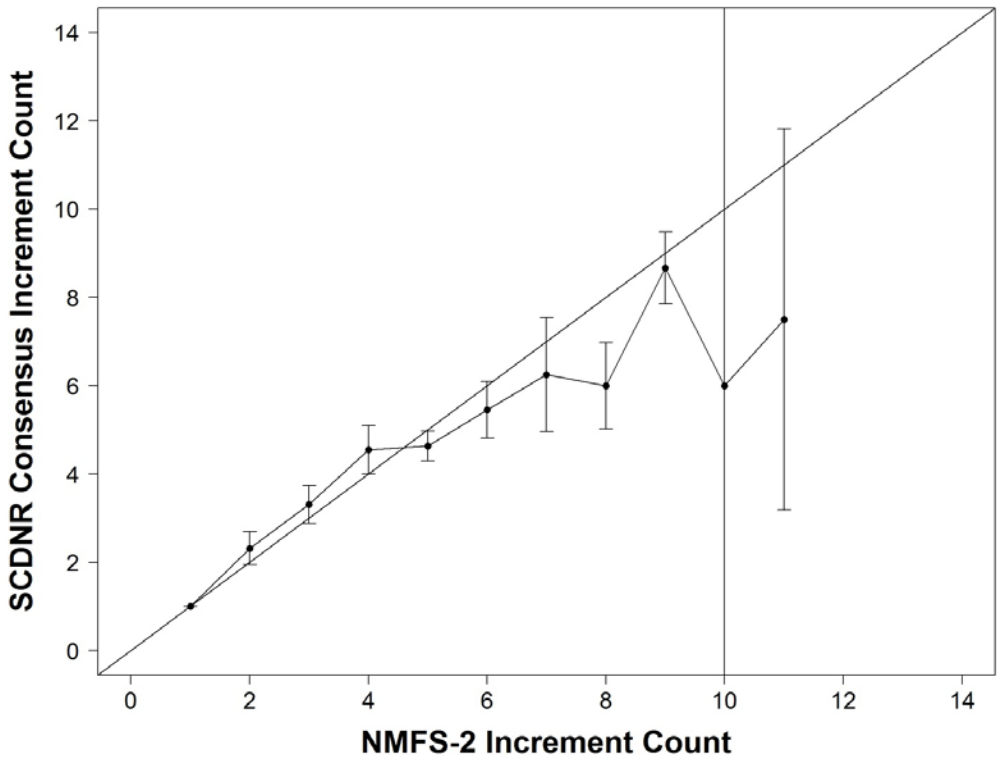

Figure 134: Bias plot for NMFS-2 vs the SCDNR consensus read from increment counts made for the post workshop inter-laboratory calibration study.

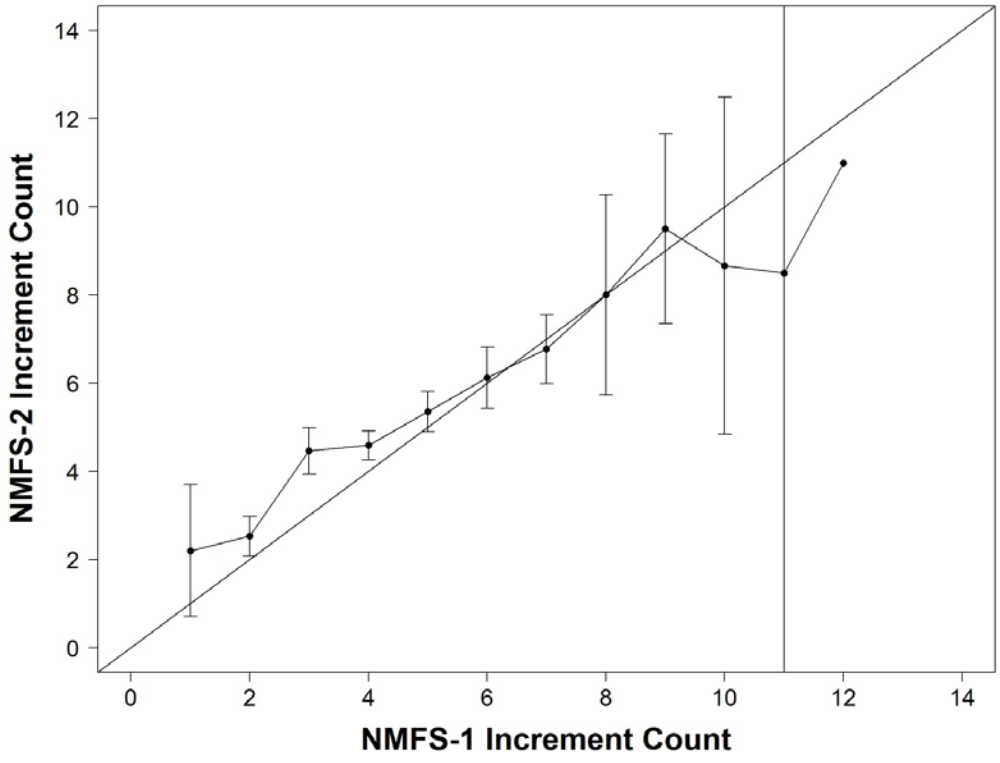

Figure 145: Bias plot for NMFS-1 vs NMFS-2 based on calendar ages determined during the post workshop inter-laboratory calibration study.

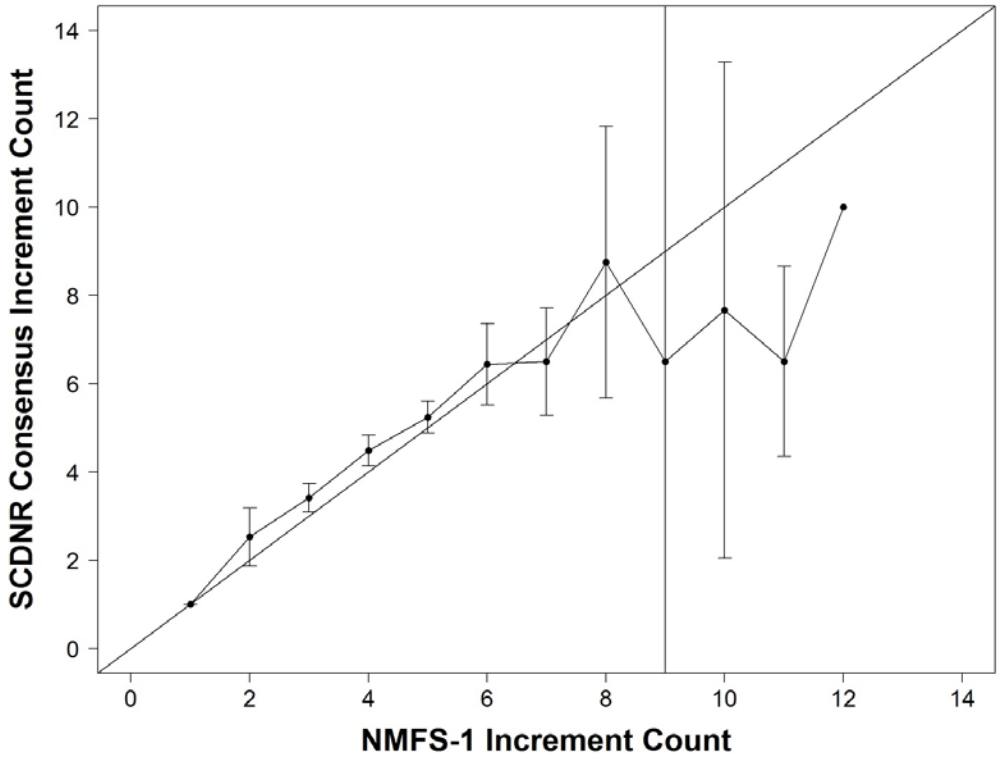

Figure 16: Bias plot for NMFS-1 vs the SCDNR consensus read using calendar ages determined during the post workshop inter-laboratory calibration study.

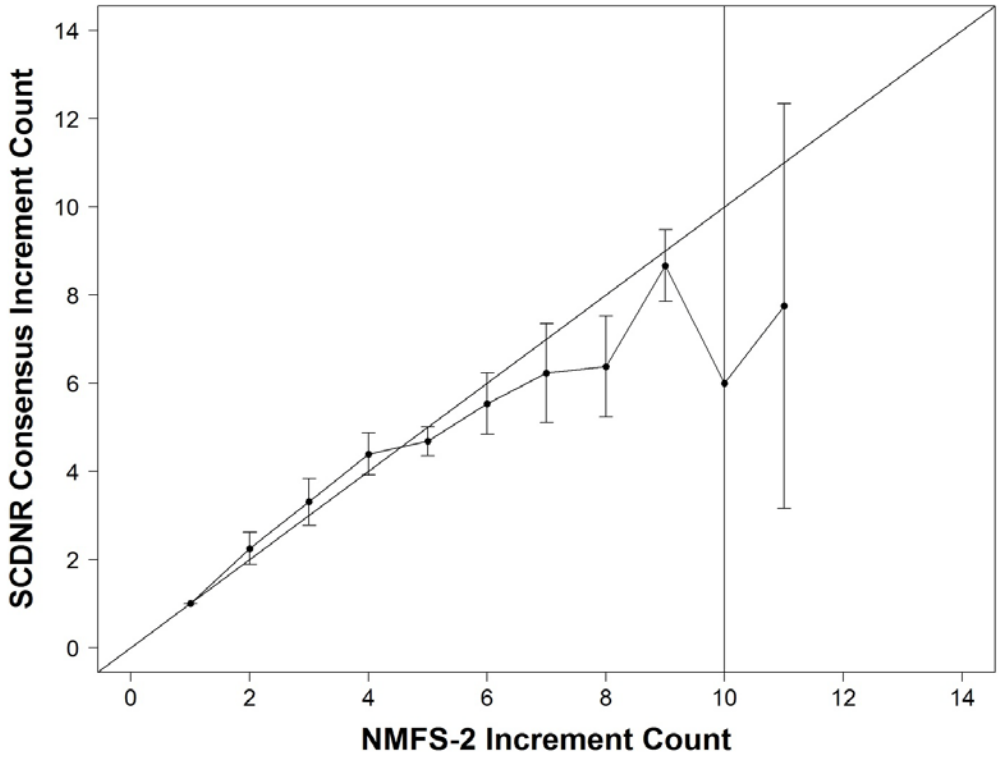

Figure 17: Bias plot for NMFS-2 vs the SCDNR consensus read using calendar ages determined during the post workshop inter-laboratory calibration study.

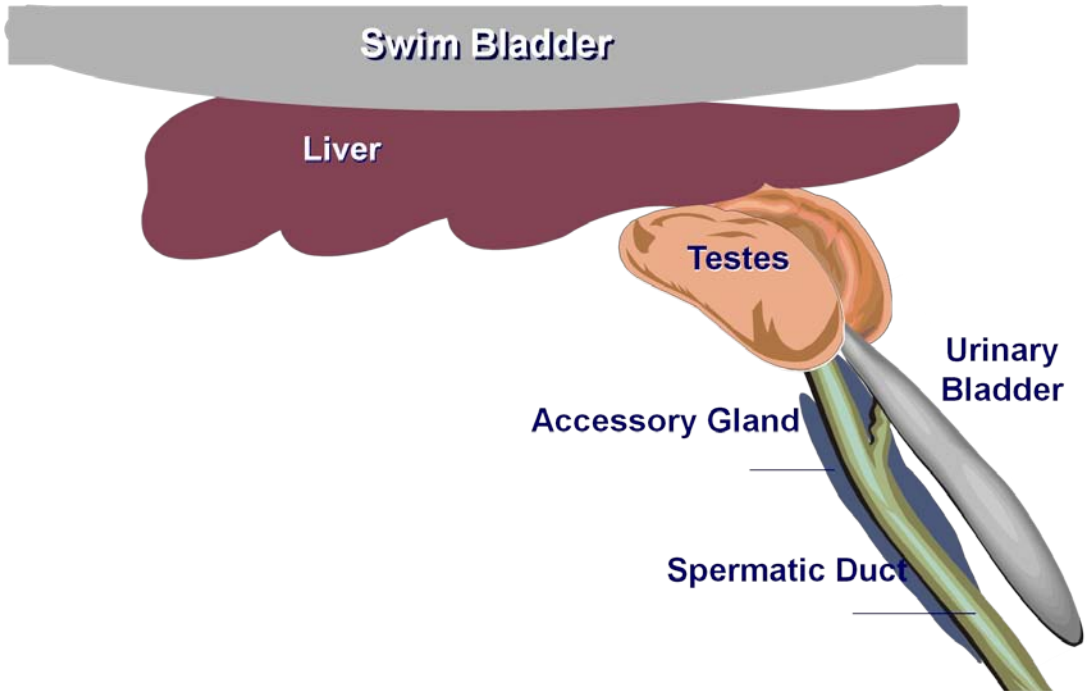

Figure18: Internal anatomy of the male gray triggerfish.

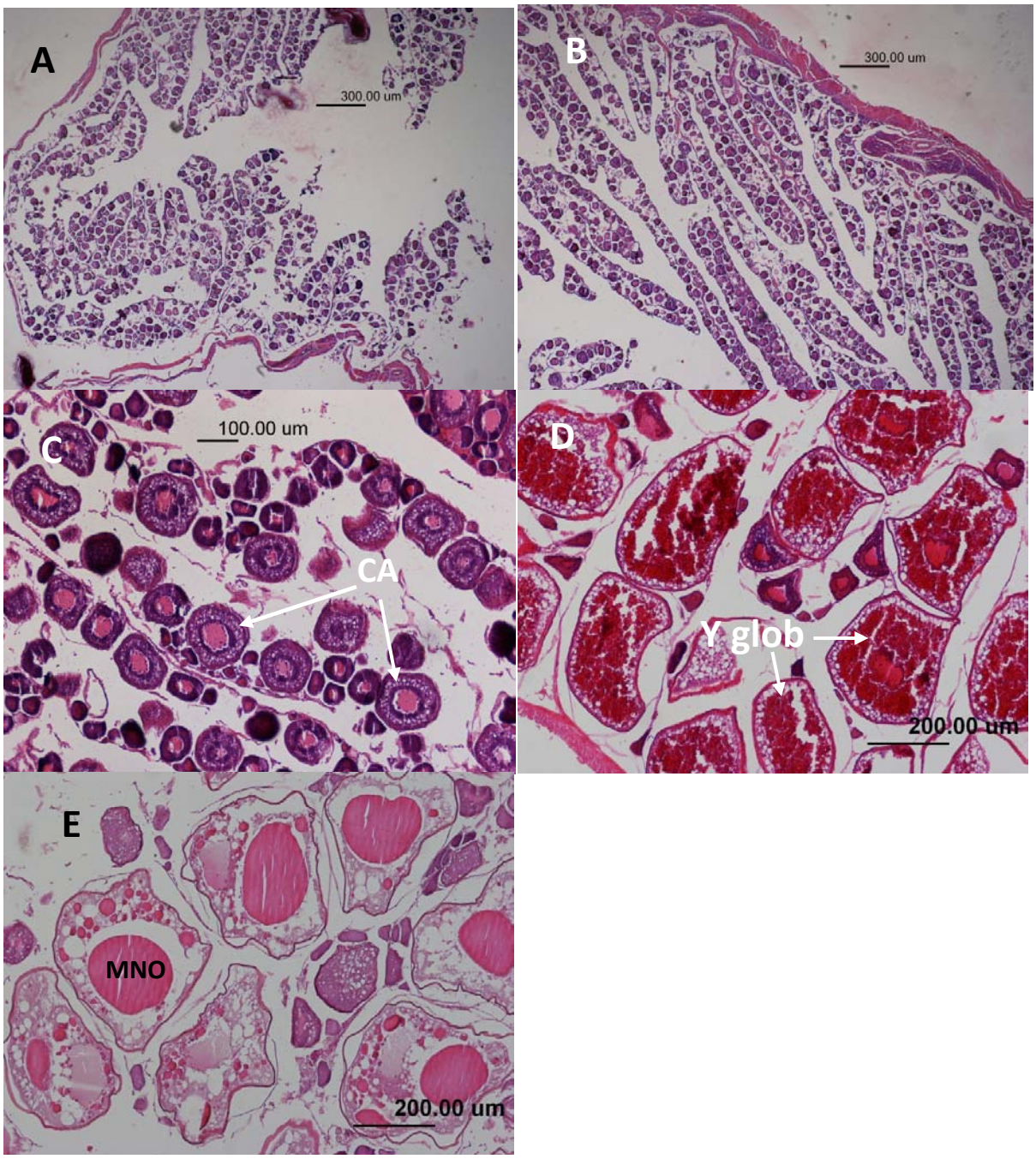

Figure 19: Female reproductive classes: A. Immature (Class 1); B. Mature, Regenerating (Class 5); C. Developing, Cortical alveolar oocytes, non-vitellogenic (Class E); D. Yolked oocytes, vitellogenic (Class F); E. Migratory nucleus oocytes (Class G). CA = cortical alveolar oocytes, MNO = migratory nucleus oocytes, Y glob = yolk globules in yolked oocytes.

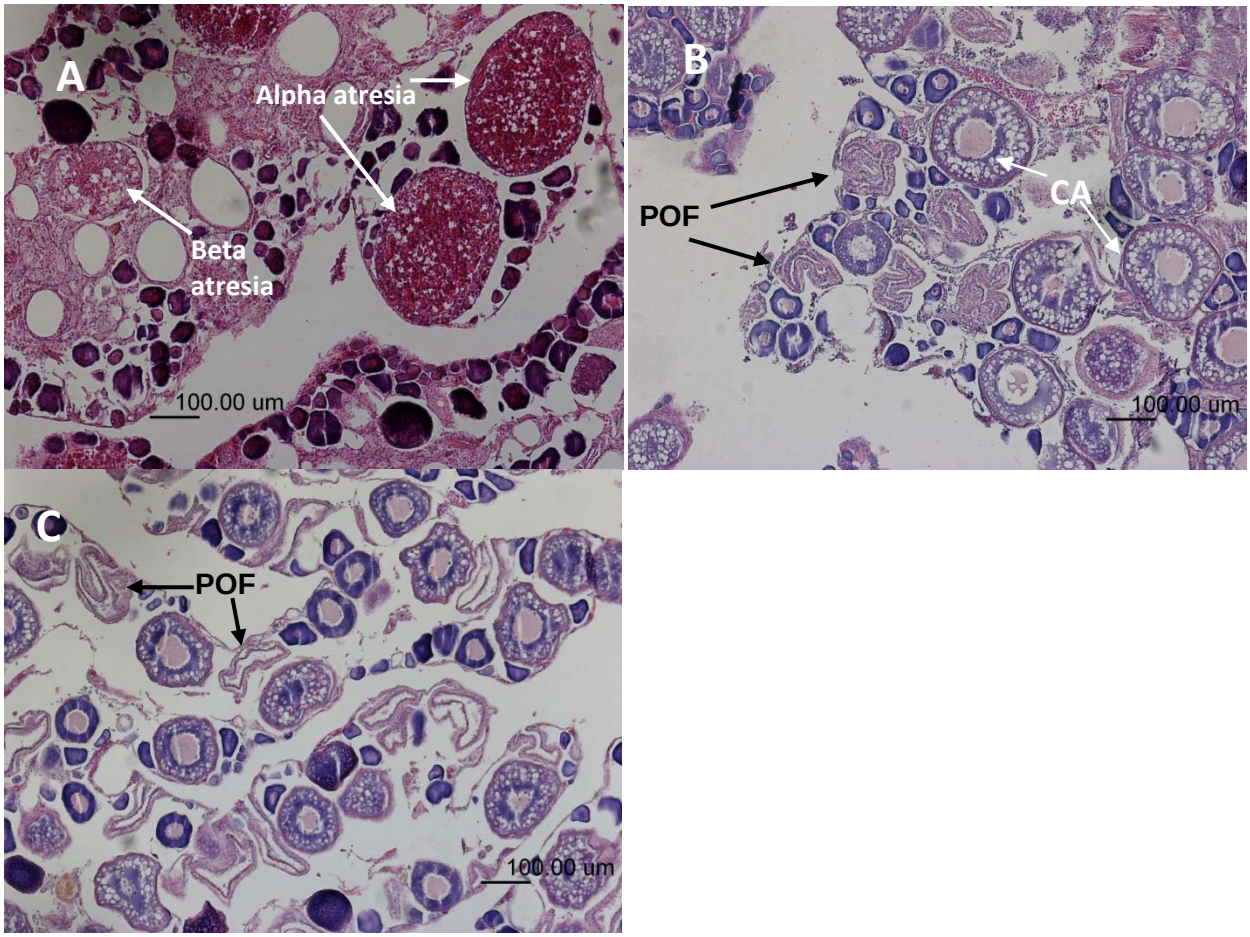

Figure 20: Female reproductive classes continues: A) alpha and beta stages of atresia in yolked oocytes (Class 4); B) new postovulatory follicles (POFs, Class B); C) intermediate POFs (Class C).

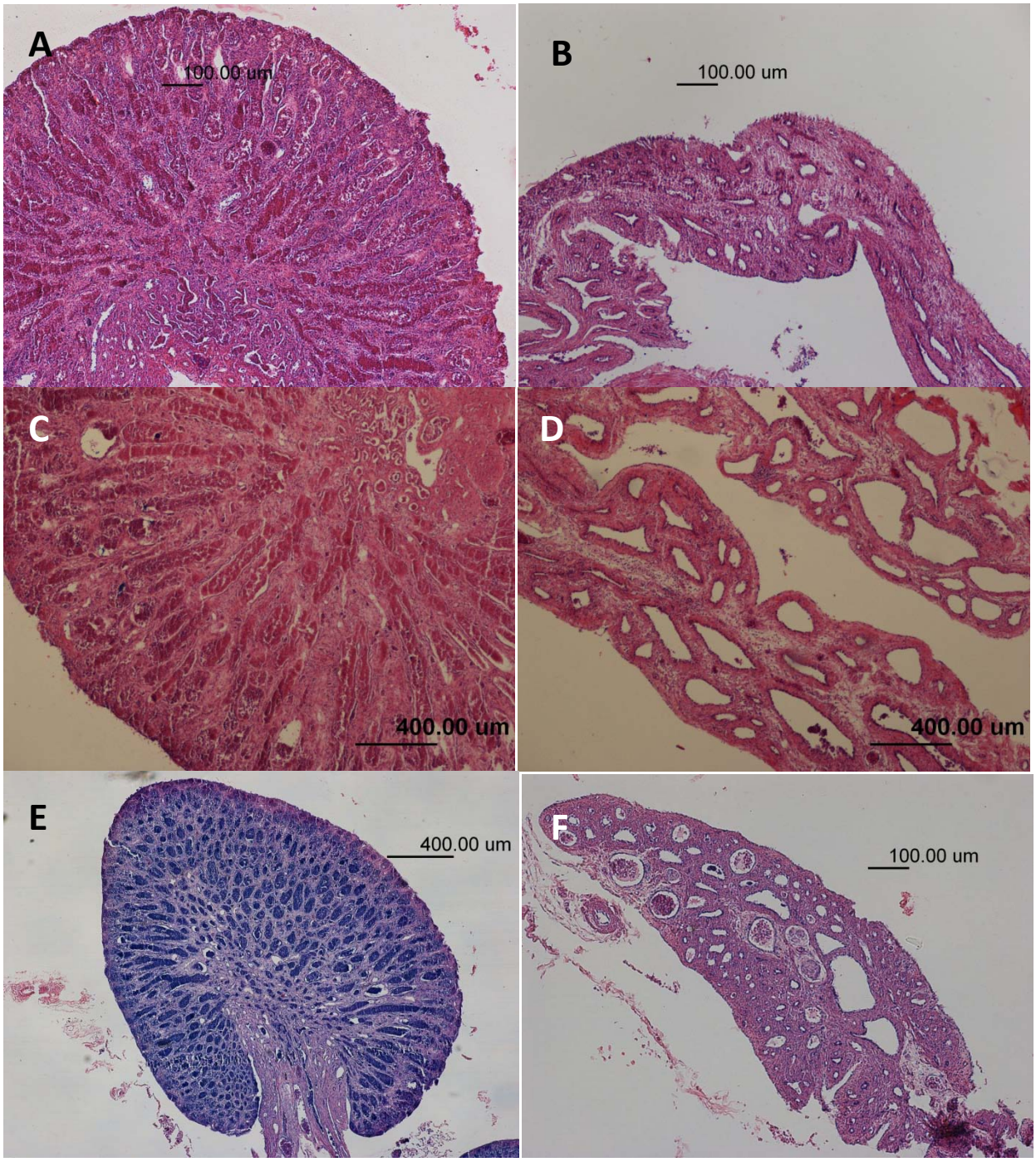

Figure 21: Male reproductive classes: A,B) transverse section of immature testes (A) and ductwork (B) (Class 1); C,D) Mature, regenerating testes (C) and ductwork (D) (Class 5); E,F) testes (E and ductwork (F) in developing class (Class 2).

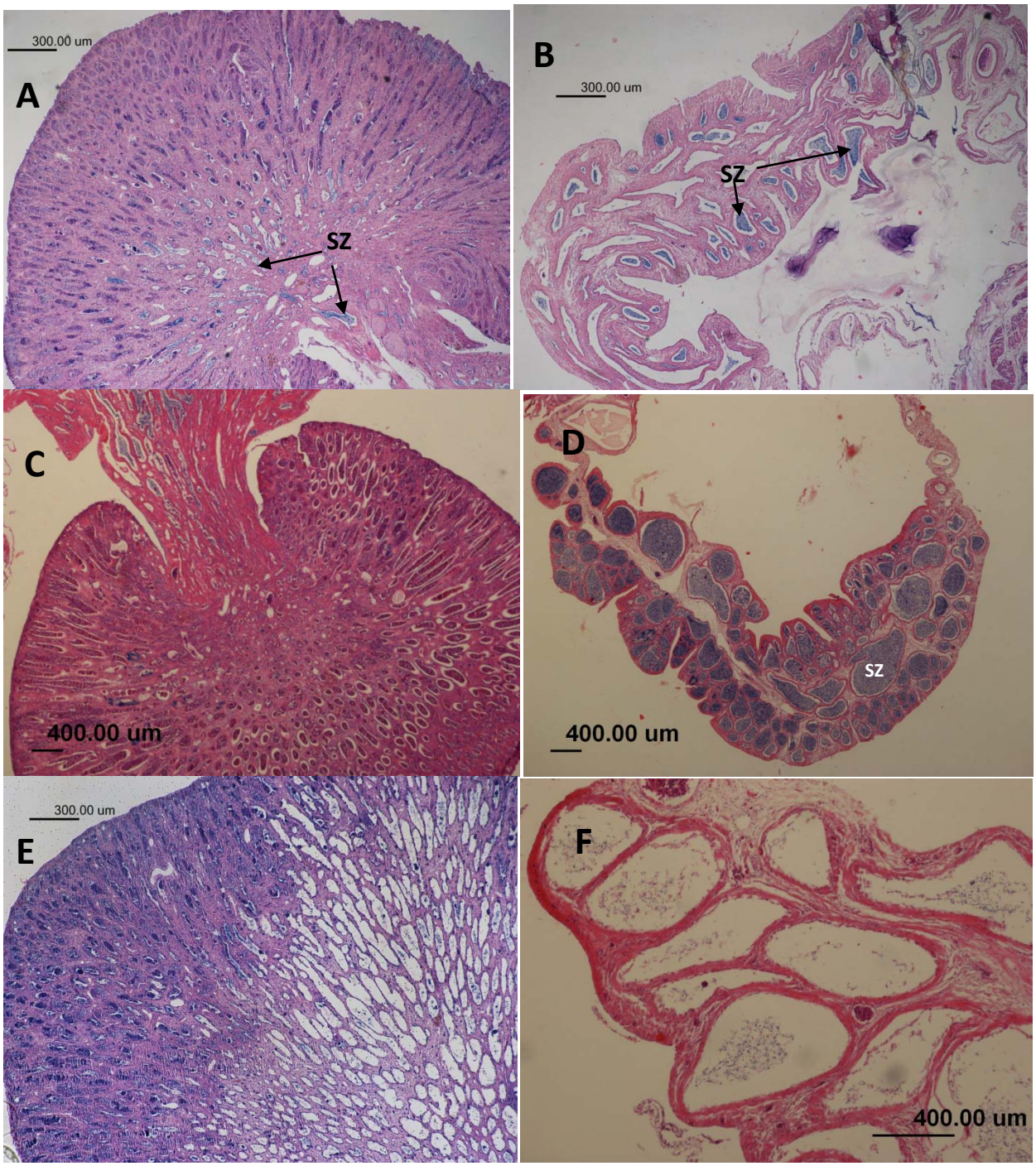

Figure 22: Male reproductive classes continued: A,B) testes (A) and ductwork (B) in early spawning capable class (Class ESC); C,D) testes (C) and ductwork (D) in storage class (Class H); E,F) testes (E and ductwork (F) in recent spawn class (Class 7). SZ = spermatozoa.

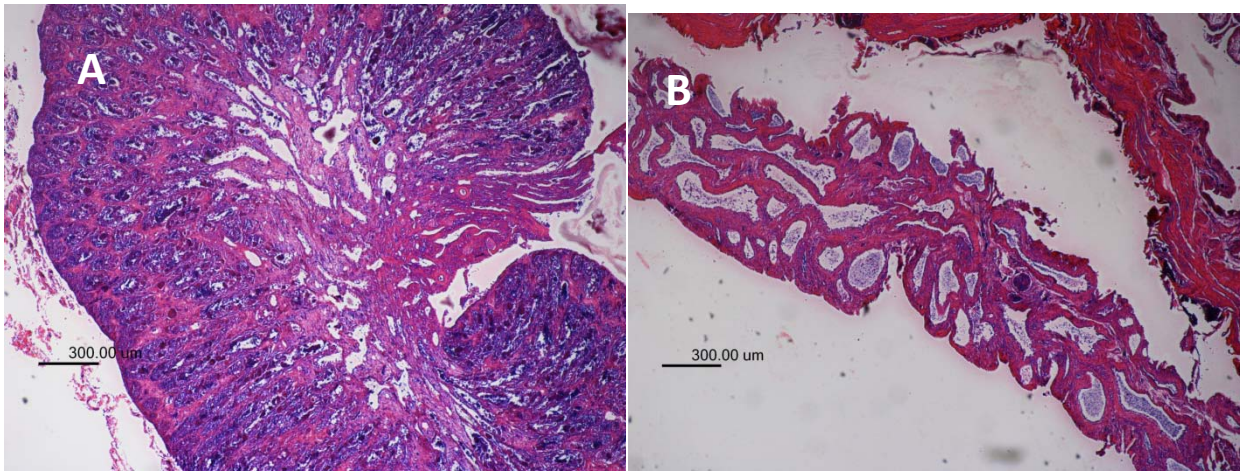

Figure 23: Male reproductive classes continued: A,B) testes (A) and ducts (B) in regressing class (Class 4).