Validation of Annual Growth Zone Formation in Gray Triggerfish *Balistes capriscus* Dorsal Spines, Vertebrae, and Otoliths

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ABSTRACT

Uncertainty in age estimates from dorsal spines has been a persistent issue in stock assessments of gray triggerfish, *Balistes capriscus*. This study sought to validate the annual deposition of growth zones on dorsal spines, vertebrae, and otoliths of gray triggerfish through chemical marking. Fish (n=101) were collected from offshore habitats and held in an aquaculture facility. 74 adult fish were chemically marked with a 50 mg/kg body weight injection of calcein, and reared for an average of 527 days post-marking. At intervals, fish were sacrificed and first dorsal spines, vertebrae, and otoliths were extracted and sectioned. Annuli , were enumerated for spines (n=96), vertebrae (n=94), and otoliths (n=48) and ranged from 0-11 annuli for spines and vertebrae, and 1-12 annuli for otoliths. Age bias plots showed strong agreement between spine and vertebra annuli counts for all observed ages, while counts from spines and vertebrae appeared to underage beginning at age 5 when compared to otolith annuli counts. Tests of symmetry indicated that the annuli counts between paired age structures were not biased ($p > 0.05$). Analysis of growth zones observed distal to calcein marks in all of the age structures confirmed that these zones were deposited annually, and the expected number of these zones, or annuli, were observed in 91% of spine, 90% of vertebrae, and 100% of otolith sections. Marginal increment analysis of ageing structures indicated that annuli form during summer months. Percentages of annuli deposited on the margins peaked in June for spines (58%) and otoliths (29%), and August for vertebrae (30%).. Results from this study validate the annual deposition of growth zones but further consideration needs to be taken when ageing older than age-4.

Keywords: Gray triggerfish, *Balistes capriscus*, age validation, otoliths, dorsal spines.

1. Introduction

Triggerfishes, Balistidae, are distributed throughout the Atlantic, Pacific, and Indian Oceans, and are most abundant in tropical and sub-tropical waters (Matsuura 2015). Species in this family are targeted in recreational and commercial fisheries across the planet (FAO 2021). Age and growth studies have been conducted for various Balistid species, including Picasso triggerfish, *Rhinecanthus aculeatus* (Ziadi-Künzli and Tachihara 2012), black triggerfish, *Melichthys niger* (Kavanagh and Olney 2006), finscale triggerfish, *Balistes polylepis* (Barroso-Soto et al. 2007), and queen triggerfish, *Balistes vetula* (Manooch and Drennon 1987; Albuquerque et al. 2011; Shervette and Hernández 2021). The most studied species in the Balistid family has been the gray triggerfish *Balistes capriscus* Gmelin, 1789 (Table 1). This moderately long-lived species supports fisheries in the eastern and western Atlantic Ocean, and increased exploitation has been reported in stocks throughout its native range since the 1980's (Johnson and Salomon 1984; Bernardes 2002; Aggrey-Flynn 2009; Burton et al. 2015; Kacem et al. 2015; Shervette et al. 2020).

To support proper management of gray triggerfish recreational and commercial fisheries, stock assessments are necessary. The most robust stock assessments utilize reliable fish age data to estimate age structure, growth rates, and mortality in a given stock (Brander 1974; Yin and Sampson 2004). Ageing error can lead to the over-exploitation of a stock, especially if the ages are underestimated (Campana 2001). To obtain accurate age estimates in teleost fish, sagittal otoliths are generally the preferred ageing structure versus scales or structures such as fin spines, fin rays, and vertebrae (Campana 2001). However, for practical reasons, gray triggerfish sagittal otoliths are not routinely used to obtain ages used for stock assessments, as they are small, fragile, and difficult to extract (Hood & Johnson 1997; Bernardes 2002; Milazzo et al. 2004; Allman et al. 2016). Besides, otolith removal generally requires mutilation of gray triggerfish carcasses, which is considered unacceptable by fish vendors. Therefore, port agents on the docks or in fish houses cannot efficiently provide otoliths as age samples for production ageing laboratories (Burton et al., 2015). Additionally, sagittal otoliths of gray triggerfish have a unique shape, making it difficult to determine the best spatial plane on which to section and read samples for age estimation (Shervette et al. 2020). Compared to otoliths, dorsal spines are easier to remove and process for age determination. For these reasons, the first dorsal spine is used as the primary ageing structure for gray triggerfish stock assessments (Burton et al. 2015; SEDAR 2016).

Several gray triggerfish age and growth studies have noted difficulty in interpreting the growth zones on dorsal spines (Bernardes 2002; Burton et al. 2015; Allman et al. 2017), and the lack of validation of growth zone formation has left age readings in question. As fish grow, bony tissue in spines can be resorbed or remodeled, causing difficulty in age determination (Panfili et al. 2002). Shervette et al. (2020) suggested that ages read from spines were generally underestimates compared to otolith ages. Bernardes (2002) and Allman et al. (2016) reported that two translucent zones may be formed each year, while other studies reported just one translucent zone per year. Jefferson et al. (2019), Ingram (2001), and Caverieri et al. (1981) also discussed the formation of the first annulus, but did not agree specifically on which translucent zone was the first annulus or how close to the focus it was formed. Due to these concerns regarding dorsal spines as ageing structures, and their potential effects on management of the

U.S. South Atlantic stock of gray triggerfish (SEDAR 2016), fishery managers determined that an age validation study for gray triggerfish was necessary.

A previous study in the Gulf of Mexico attempted to validate growth zone deposition on the spines of gray triggerfish (Allman et al. 2016). In this study, eight fish were captured from offshore habitats in the northern Gulf of Mexico, injected with oxytetracycyline (OTC), and held in an aquaculture facility with ambient light and mean seasonal bottom water temperatures from the capture area. Four out of the eight fish survived for a period of 262 days from October to July, and dorsal spines, fin rays, and vertebrae sections from each fish showed one translucent zone (annulus) forming in the late winter months. Though the results of the OTC experiment indicated one annulus formed per year, marginal increment analysis (MIA) of 2,411 spine samples and found that a second translucent zone appeared in the fall, around September. Hence, further examination of growth zone formation on gray triggerfish age structures was warranted.

The primary objective of our study was to validate the annual deposition of growth zones on the first dorsal spines, vertebrae, and otoliths of gray triggerfish by capturing juvenile fish from surface and adults from bottom habitats, chemically marking them, and holding them in an aquaculture facility for intervallic durations. The resulting estimates of age, and interpretation of the micro-structure obtained from dorsal spines, vertebrae, and otoliths were compared to elucidate differences among the three ageing structures and aid in determining the most effective age structure for production ageing in support of stock assessments.

2. Materials and methods

2.1. Fish collection and rearing

Gray triggerfish were collected off the coast of North Carolina with hook and line (n=69), traps (n=19) and dip net (n=13) during December 2014 – Spring 2016 and transported to the NOAA Beaufort Laboratory marine aquaculture facilities. To eliminate potential protozoan parasites, all fish were treated for 24 hours with 30ppm formalin immersion, then 14 days with a $CuSO_4$ (0.15 ppm) immersion. Adult fish were reared in two recirculating aquaculture (RAS) systems, each consisting of three 2.3 m^3 (600 gallon) round fiberglass tanks. Juvenile fish were reared in a RAS system consisting of three 0.5 m^3 (130 gallon) semi-round polyethylene tanks. All RAS systems were equipped with mechanical filtration and bio-filtration along with an UV sterilizer. These systems were housed in a climate-controlled facility and had inline heat pumps and chillers to control water temperature to replicate offshore bottom temperature data collected from long-term monitoring programs conducted at the NOAA Beaufort laboratory. Skylights were installed in the aquaculture facility to allow ambient light into the rearing tanks, ensuring natural diurnal light cycles. Water temperature, salinity, alkalinity, dissolved oxygen, pH, total ammonia, nitrites, and nitrates were measured daily and adjusted to replicate ambient seawater parameters. Gray triggerfish were fed a daily diet of cut squid and commercial marine finfish pellets, and uneaten food was removed from the bottom of tanks after feeding. Rearing tanks were scrubbed daily with abrasive pads, and fish waste was removed via siphon.

2.2. Marking

Adult gray triggerfish were chemically marked on March 25, 2015 (n = 47), March 1, 2016 (n = 2), June 16, 2016 (n = 12), and October 11, 2016 (n = 13). Live fish were anesthetized by submersion in a 75 mg/L solution of Tricaine-S, and a sterile syringe was used to inject a 50 mg/kg body weight dose of calcein into muscle tissue below the dorsal fin rays (Monaghan, 1993). To make the injectable solution, calcein was mixed into a 0.9% bacteriostatic solution buffered to a pH of 7.3 at a concentration of 60mg/mL. Following injection, the fish were returned to rearing tanks for recovery.

2.3. Processing

All fish in this experiment were sampled to obtain the spines, vertebrae and otoliths from the same fish. Fish that died before the end of the experiment were measured for fork length (FL, cm), weighed (g), and frozen whole in plastic bags for subsequent processing. Upon completion of the rearing period, fish were euthanized using a lethal dose of Tricaine-S and placed in an ice slurry according to Institutional Animal Care and Use Committee (IACUC) guidelines. After length and weight measurements were taken, first dorsal spines were removed using a knife to create identical incisions in the flesh immediately anterior and posterior to the spine. Once the incisions were made, the spine was released from the fish with the condyle intact. The vertebral column was excised from each fish using heavy-duty snips, and a strand of wire was run through the notochord opening to hold the column together. The vertebral column was then boiled to remove attached soft tissue, and the second and third anterior vertebrae were detached for ageing purposes. The otoliths were extracted by using a serrated knife to cut through the cranium approximately 20 mm behind the eye and in line with the operculum opening to expose the brain cavity and otic capsule. The entire vestibular process containing the lapilli, asteriscii, and sagitta was extracted with fine-tipped forceps then stored in microcentrifuge vials with ethanol for further processing. Sagittal otoliths were dissected from the vestibular process, rinsed in water, and allowed to dry.

Dorsal spines, vertebrae, and sagittal otoliths were sectioned using a low-speed saw equipped with two 10-cm diamond-edged wafering blades separated by a 0.5 mm spacer. Spines were processed following the methods of Burton et al. (2015), taking a single transverse section cut immediately distal to the condyle groove of each spine (Figure 1a). Following the methods from Ziadi-Künzli and Tachihara (2012), the third abdominal vertebrae were sectioned along the median (sagittal) plane (Figure 1b). Because the sagittal otoliths were very fragile, they were placed in silicone bullet molds and embedded in a two-part liquid epoxy prior to mounting them for sectioning. Once the epoxy cured, the embedded otoliths were removed from the mold and affixed to microscope slides using thermal adhesive. Embedded otoliths were sectioned along the dorso-ventral plane (Figure 1c). Resulting spine, vertebra, and otolith sections (0.4-0.5 mm thick) were attached to a microscope slide using a thermal adhesive and covered with mounting medium.

2.4. Analyses of Ageing Structures

Dorsal spine, vertebra, and otolith sections were aged by two independent readers without reference to the other structures, size of the fish, date of death, or date of chemical marking. Spines were read using the protocol established during an inter-agency age determination workshop (Kolmos et al. 2013). Sections from each of the age structures were viewed primarily under reflected light using a stereomicroscope at 15-40x magnification, though some sections were viewed using both reflected and transmitted light. When illuminated using an LED epi-fluorescent light source, calcein marks were revealed as neon yellow bands on sections (Figure 2). Images for each section were captured using a digital camera attached to the stereomicroscope. Translucent growth zones on spine and vertebra sections, and opaque growth zones otolith sections, were enumerated as annuli to estimate the age of the fish. Consensus annuli counts and margin types were recorded after both readers simultaneously re-examined samples for which annuli counts differed. Age bias plots were created to visualize differences in annuli counts between the three structures. Based on the recommendation by McBride (2015), Evans and Hoenig (1998) and Bowker (1948) tests of symmetry were performed to examine bias between annuli counts from different structures.

2.5 Timing of Annulus Formation

Marginal increment analysis was conducted to estimate periodicity of annulus formation in spines, vertebrae, and otoliths. The margin of each section was recorded as translucent or opaque. Additionally, an index of margin type was developed to describe completeness of annulus formation on the margin. This index categorized annulus formation by comparing accretion of material on the margin to that of the previously formed annulus. The codes for the relative marginal index were noted as follows:

1 = Complete annulus on margin;

2 = Growth zone < 1/3 the width of the previously formed annulus;

3 = Growth zone 1/3 - 2/3 the width of previously formed annulus;

4 = Growth zone > 2/3 the width of the previously formed annulus.

Percentage of sections with translucent or opaque margins and marginal index (1-4) were plotted against month of sacrifice to create a timeline of annulus formation.

The location of calcein marks within spine sections was also analyzed to estimate periodicity of annulus formation. Since both the dates of marking and sacrifice were known, a chronology of annulus formation could be determined by examining the accretion of material in the spines after the mark was applied (Figure 3). Similar to the codes for the marginal condition index, location of the calcein marks on a given spine section were coded as follows:

1 = Mark lies immediately distal to translucent zone/proximal to opaque zone.

2 = Mark lies 1/3 of the distance between two translucent zones (annuli);

3 = Mark lies 1/3 to 2/3 of the distance between two translucent zones (annuli);

4 = Mark lies more than 2/3 of the distance between two translucent zones (annuli), and/or immediately proximal to the subsequent translucent zone.

The percentage of sections with each mark code (1-4) was plotted against month of marking.

2.6 Estimating Growth

Following the MIA, a calendar and fractional ages were calculated for each sample. The calendar age was based on the annuli count, the marginal index, and the month of capture. The annuli count was advanced by one for samples that were collected in the months prior to complete annulus formation, and exhibited a margin type of 3 or 4. For all other samples, the annuli count equaled the calendar age.

Fractional age (A_f) was calculated based on the calendar age (A_c), month of capture (M_c) and the month of peak spawning (M_s) in the U.S. South Atlantic, which is July (SEDAR 2016), using the following equation:

$$A_f = A_c + \left(\frac{M_c - M_s}{12}\right)$$

Von Bertalanffy growth parameters were calculated from length-at-fractional age data for each structure, both individually and for only those samples that had paired structures. These parameters were then compared to the parameters calculated for the most recent South Atlantic gray triggerfish stock assessment, SEDAR 41 (SEDAR, 2014). The resulting formula was:

$$L_t = L_\infty \left(1 - e^{-k(t-t_0)}\right)$$

where L_t = fish length at age, L_∞ = theoretical asymptotic length, *k* = growth coefficient, and t_0= theoretical age at length of zero.

3. Results

A total of 101 gray triggerfish were successfully held in tanks during the study, 74 of which were marked with calcein. Fish ranged in size from 31 – 498 mm fork length (FL) (Figure 4). Fish survived from 5 days to 29 months after calcein marking (Figure 5). Those marked in March 2015 (n=47) were held for an average of 24 months, while fish marked in March 2016 (n=2) were held for 16 months; marked in June 2016 were held for 1.5 months (n = 12; tank mortality); and fish marked in October 2016 were held for an average of 9 months (n = 13). The remaining fish (n = 27), which were not marked with calcein, were some of the smallest fish in the study that were captured in traps or dipnets at opportunistic times.

Translucent and opaque growth zones were observed in all ageing structures from the captive rearing period. Narrow translucent zones were counted as annuli on spine and vertebra sections, and narrow opaque zones were counted as annuli on otolith sections. In some of the spine and vertebrae sections, thin and discontinuous translucent zones appeared to form in close proximity to more distinct translucent zones. These “doublets” did not appear in all of the sections and were not enumerated as annuli. Annuli counts for spines (n=96), vertebrae (n=94), and otoliths (n=48) ranged from 0-11 for spines and vertebrae, and 1-12 for otoliths. Fewer otolith samples were examined due to difficulties in the extraction and sectioning processes. The average percent error (APE) between independent readers was 8.5% for spines, 13.0% for vertebrae, and 13.7 % for otoliths.

Age bias plots showed strong agreement and low bias between spine and vertebra annuli counts for all observed ages. Counts of spines and vertebrae appeared to under-age beginning at age 4 when compared to otolith annuli counts (Figure 6). Annuli counts agreed for 57.3% of spine-vertebra pairs, 52.2% of spine-otolith pairs, and 53.2% of vertebra-otolith pairs. Respectively, 93.3%, 82.6%, and 85.1% of annuli counts for these pairs were within one year of each other (Table 2a). The Evans and Hoenig (1998) and Bowker (1948) tests indicated that the paired age data were not biased (p >0.05) (Table 2b).

Marginal increment analysis of the of ageing structures indicated that annuli form in summer months. The highest percent of translucent margins occurred in June for spines (58%) and in August for vertebrae (30%). In otoliths, the highest percentage of opaque margins occurred in June (29%) (Figure 7).

Calcein marks were observed in spine (n=68), vertebrae (n=72), and otolith (n=37) sections. In some of the spine and vertebrae sections, calcein marks that were applied in March generally appeared at the beginning of wide opaque zones while marks applied in October appeared within or at the end of these zones. This suggests that narrow translucent zones could begin to form during the late fall/early winter (Figure 8).

The expected number of post-mark annuli was present in 62 of 68 spine sections, 65 of 72 vertebrae sections, and in all of the otolith sections. Analysis of calcein mark location revealed that the majority of March and June marks were coded as 1 or 2 (narrow), while the majority of the October marks were coded as 3 or 4 (wide; Table 3).

Based on the MIA, the annuli count from each spine and otolith was converted to calendar age. To generate a calendar age, annuli counts were advanced by one for fish sacrificed between January and June whose age structure had a margin type of 3 or 4. If the margin type was 1 or 2 in the same span of months, annuli count was set equal to the calendar age. If the fish was sacrificed during July through December, regardless of margin type, then the annuli count was set equal to the calendar age for that fish. These calendar ages were used to calculate fractional ages for estimation of growth.

The von Bertalanffy growth parameters were calculated based on the spine and otolith length-at-fractional age for all data available for each structure and for the data from paired readings (limited to samples that had both a spine and otolith from the same fish). The resulting von Bertalanffy growth equations for all available data were:

$$L_{t(\mathrm{spines})} = 492.4\left(1 - e^{-0.31(t+0.50)}\right)$$

$$L_{t(\mathrm{otoliths})} = 425.1\left(1 - e^{-0.55(t+0.5)}\right)$$

For paired readings, the equations were:

$$L_{t(\mathrm{spines})} = 484.2\left(1 - e^{-0.33(t+0.69)}\right)$$

$$L_{t(\mathrm{otoliths})} = 434.7\left(1 - e^{-0.55(t+0.06)}\right)$$

Parameter values are summarized in Table 4 and Figure 9.

4. Discussion

The annual deposition of growth zones on dorsal spines, vertebrae, and otoliths of gray triggerfish was successfully validated. In aggregate, fish survived in tanks for an average of 527 days after chemical marking, nearly double the duration of previous studies. Water temperature and diurnal light cycles were successfully maintained to replicate ambient conditions. The expected number of growth zones, or annuli, distal to the chemical mark was present on >90% of spines and vertebrae, and on 100% of the otoliths.

Marginal increment analysis of growth zones in spines indicated that one annulus formed between June and August. These findings agree with previous studies of this species showing annulus formation in late spring to summer in South Atlantic (Burton et al. 2015;

Moore 2001) and Gulf of Mexico (Johnson and Saloman 1984). According to these studies, annulus formation coincided with the spawning/nesting season. In the south Atlantic and Gulf of Mexico, gray triggerfish generally spawn in the spring and summer months, with peaks occurring in June and July (Kelley-Stormer et al. 2017, Wilson et al. 1995). During these periods, fish devote considerable energy to reproduction versus somatic growth. Therefore, it makes sense that slow-growth zones would form concurrent with spring/summer spawning (Moore 2001, Ingram 2001, Bernardes 2002).

Other studies have reported divergent patterns of annulus formation in gray triggerfish spines. In the Gulf of Mexico, Allman et al. (2016) reported that peak translucent zone formation in spines occurred in February and March, with a smaller peak occurring in September. Bernardes (2002) reported a similar bimodal pattern of translucent zone formation in fish from the Brazilian coast, with peaks occurring in both winter and summer months. These studies postulate that decreases in temperature and food supply in winter, and reproductive activity during summer, contribute to translucent zone formation. The presence of faint and discontinuous “doublets” in our study corroborate the findings from Allman et al. (2016) and Bernardes (2002) where thinner translucent growth zones were observed to have inconsistent spacing when compared to true translucent annuli. These secondary translucent zones, or doublets, should not be counted as annuli.

In the majority of the spine sections from fish marked in March, calcein marks appeared distal to narrow translucent zones, while marks applied in October appeared proximal to the translucent zones (Figure 8). These observations support the assertion that a translucent zone, or annulus, may begin to form in late winter to early spring months, as was reported in MIA results from Burton et al. (2015). Because the majority of the fish in our study (90%) died between May and September, it was difficult to report exactly when the annulus on the spines began to form. Both our study and Burton et al (2015) agree that annulI on dorsal spines essentially finish forming by the end of June.

The ages estimated from paired otoliths and spine sections showed strong agreement to age-4, after which spine readings began to underestimate age compared to otoliths. Spines are the preferred ageing structure used to obtain direct age data used for fisheries stock assessments; therefore, it is important to examine potential ageing inconsistencies in these structures. Systematic under-ageing can lead to biased estimates of spawning stock biomass, mortality, recruitment, and growth rates among other parameters used in stock assessments (Mills and Beamish 1980, Reeves 2003, Yule et al. 2008, Henriquez et al. 2016). The effects of under-ageing on growth models were of particular concern in our study. The age data obtained from spines caused the Von Bertalanffy growth model to have a larger asymptotic length (L_∞) and a smaller growth coefficient (k) than what was estimated from otolith age data (Figure 9). However, both growth models (all available data and paired readings) showed that asymptotic growth was generally achieved by age-5. The population growth model used in the previous U.S. Atlantic stock assessment (SEDAR 2016) estimated growth parameters intermediate to those estimated in our study, though caution should be taken when comparing growth models from our study to those used in stock assessments due to discrepancies in sample sizes (Table 4).

All analyses presented in this report were based upon consensus ages among readers, but upon closer inspection, some spine sections exhibited unusually wide translucent zones as seen at lower magnification (15x - 20x). Examination of these spine sections under higher magnification (up to 40x) revealed that wide translucent areas contained compacted growth zones (Figure 10). Carroll (2022) observed a similar phenomenon in goliath grouper spine sections where “stacked” annuli were observed on the margin at increased magnification, especially in sections from older fish. In our study, once these compacted growth zones were enumerated as annuli, age readings of spines matched more closely to otolith readings for older fish. This suggests that increasing magnification when viewing spine sections, and counting compacted growth zones within wide translucent zones, may alleviate under-ageing errors. We strongly recommend that production ageing laboratories conduct ageing workshops to explore this new ageing methodology.

Additional work was done, as a result of this study, to develop new age reading methodology and re-read a subset of spine sections used for age data in the SEDAR 41 stock assessment (SEDAR 2016). The new age reading methodology is detailed as follows:

1. Sections should initially be examined at 15x – 20x magnification.
2. The first annulus should be a distinct translucent zone that is lobate in shape and continuous all the way around the section.
3. Any faint and/or discontinuous translucent zone located close in proximity to a more distinct translucent zone should be considered a doublet and not counted.
4. For all samples with five or more annuli, zoom in on the margins or any wide translucent zone up to 40x magnification to look for additional growth zones.

Compared to the age readings using the new methodology, the original ages recorded for the SEDAR 41 stock assessment appeared to be underestimates, specifically for samples aged 5 or older (Figure 11). We feel that using the new methodology produces annuli counts that more closely reflect the true age of the fish.

Our study represents the most comprehensive effort to date using chemical marking to directly validate annulus formation in gray triggerfish ageing structures. Two full years of fish growth were observed in some samples, allowing for a robust documentation of annulus formation. Previous studies were limited by sample size and experimental duration. While marginal increment analysis has the ability to verify annulus formation, direct validation of age estimates using chemical marking is the “gold standard” for these studies (Beamish and McFarlane 1983; Campana 2001, Allman et al. 2016). This validation is crucial in producing robust age-based stock assessments. The results of this study reinforce the utility of the first dorsal spine as the practical ageing structure for gray triggerfish; however, increased care needs to be taken when examining sections from older fish. Work is underway to build a larger catalogue of paired spine and otolith samples, the analysis of which will create a more complete picture of gray triggerfish ageing.

Declarations

Compliance with Ethical Standards

Fish were handled and cared for according to the guidelines of the Animal Welfare Act (AWA) and with the U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training (USGP) OSTP CFR, May 20, 1985, Vol. 50, No. 97

Data Availability Statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Tables

Table 1 Published life history studies of gray triggerfish, *Balistes capriscus,* with reported maximum ages from spines. Otolith ages were reported in one study (Shervette et al. 2020).

Study	Location	Age Structure	Validation method	Max age (yrs) reported
Johnson and Saloman, 1984	U.S. Gulf of Mexico – NW Florida	First dorsal spine	Monthly frequency of annulus (translucent zone) on margin	13
Burton et al., 2015	U.S. Southeastern Atlantic	First dorsal spine	Marginal Increment Analysis	15
Kelly-Stormer et al., 2017	U.S. Southeastern Atlantic	First dorsal spine	Monthly frequency of annulus (translucent zone) on margin	12
Allman et al.,2017	U.S. Gulf of Mexico – NW Florida	First dorsal spine	Chemical marking, monthly frequency of annulus (translucent zone) on margin.	None reported
Shervette et al., 2020	U.S. South Atlantic – North Carolina and South Carolina	First dorsal spine and whole sagittal otoliths	None reported	Spines (sp)= 11; Otoliths (ot) = 13
Shervette et al., 2020	West Africa - Ghana	First dorsal spine	None reported	9
Caveriviere et al., 1981	West Africa – Senegal and Ivory Coast	First dorsal spine	Monthly frequency of annulus (translucent zone) on margin	Senegal (S) = 6 Ivory Coast (IC) = 7
Ofori-Danson, 1989	West Africa - Ghana	First dorsal spine	None reported	4
Aggrey-Fynn, 2001	West Africa – Western Gulf of Guinea	First dorsal spine	None reported	11
Bernardes, 2002	Brazil	First dorsal spine	Monthly frequency of annulus (translucent zone) on margin	11
İşmen et al., 2004	Mediterranean Sea İskenderun Bau	First dorsal spine	None reported	3
Milazzo et al., 2004	Mediterranean Sea – Strait of Sicily	First dorsal spine and whole otolith	None reported	7
Kacem et al., 2015	Mediterranean Sea – Gulf of Gabes	First dorsal spine	Marginal increment analysis	13

Table 2a Percent agreement of age readings (annuli counts) from gray triggerfish, *Balistes capriscus,* spine-vertebra, spine-otolith, and vertebra-otolith pairs. The first row depicts the percentage of structure pairs from the same fish that had the exact same age reading (annuli count). The second row depicts the percentage of structure pairs from the same fish that had age readings that were within one year of one another.

	Spine-Vert	Spine-Oto	Vert-Oto
Agreement (%)	56.82	52.17	53.19
Agreement ±1 (%)	93.26	82.61	85.11

Table 2b Tests of symmetry between age readings (annuli counts) from gray triggerfish,*Balistes capriscus*, spine-vertebra, spine-otolith, and vertebra-otolith pairs.

Tests of Symmetry	Spine-Vert	Spine-Oto	Vert-Oto
Evans Hoenig (chi square, p)	5.79, 0.55	4.34, 0.50	7.6, 0.80
Bowker (chi square, p)	16.34, 0.29	11.33, 0.50	12.33, 0.26

Table 3 Relative location of chemical mark on spine sections of gray triggerfish, *Balistes capriscus,* by month of chemical marking. 1 = mark lies immediately distal to translucent zone/proximal to opaque zone; 2 = mark lies 1/3 of the distance between two translucent zones (annuli); 3 = mark lies 1/3 to 2/3 of the distance between two translucent zones (annuli); 4 = mark lies more than 2/3 of the distance between two translucent zones (annuli), and/or immediately proximal to the subsequent translucent zone.

	Mar.	June	Oct.
% Mark Code 1	41.25	4.76	0
% Mark Code 2	33.75	57.14	13.04
% Mark Code 3	5.00	28.57	17.39
% Mark Code 4	20.00	9.52	69.56

Table 4 Von Bertalanffy growth parameters (±1 standard error) estimated from gray triggerfish, *Balistes capriscus*, spine and otolith age data from the age validation study. Data from all specimens were used for separate spine and otolith models. Data from specimens that had pairs of spines and otoliths were included in the paired comparison models. Population growth model parameters from the most recent stock assessment, SEDAR 41, are included for reference (SEDAR, 2016).

	Age Structure	L_∞	k	t_0
All data available (present study)	Spine (n = 96)	492.4 (28.3)	0.31 (0.05)	-0.50 (0.18)
	Otolith (n = 48)	435.1 (20.4)	0.55 (0.15)	-0.05 (0.36)
Paired spine and otolith readings (present study)	Spine (n = 46)	484.2 (51.7)	0.33 (0.14)	-0.69 (0.67)
	Otolith (n = 46)	434.7 (21.3)	0.55 (0.15)	-0.06 (0.37)
SEDAR 41	n = 8,102	453.2 (23.3)	0.34 (0.12)	-0.98 (0.66)

Figure Captions

Fig. 1. Image of a gray triggerfish, *Balistes capriscus,* a. dorsal spine, b. vertebra, and c. sagittal otolith. Red arrows indicate the orientation of the two blades of the low-speed wafering saw used to section the samples. A spacer was placed between the two blades to produce sections that were 0.4-0.5 mm thick.

Fig. 2. Image of a gray triggerfish, *Balistes capriscus*, dorsal spine section (left), vertebra section (middle), and whole sagittal otolith (right). Yellow/green bands are calcein marks that were illuminated using an LED epifluorescent light source attached to a stereomicroscope.

Fig. 3. Illustration of the codes used to assess the relative placement of the calcein mark in relation to the annulus (translucent growth zone) on the spine of a gray triggerfish, *Balistes capriscus*. 1 = mark lies immediately distal to translucent zone/proximal to opaque zone; 2 = mark lies 1/3 of the distance between two translucent zones (annuli); 3 = mark lies 1/3 to 2/3 of the distance between two translucent zones (annuli); 4 = mark lies more than 2/3 of the distance between two translucent zones (annuli), and/or immediately proximal to the subsequent translucent zone.

Fig. 4. Histogram depicting fork lengths of gray triggerfish, *Balistes capriscus*, plotted against number of individuals per 25 mm fork length bin. Fork lengths were recorded upon the completion of the experiment, concurrent with the removal of ageing structures.

Fig. 5. Histogram depicting the survival duration (month) after chemical marking of gray triggerfish, *Balistes capriscus*, in the age validation study.

Fig. 6. Bias plots of gray triggerfish, *Balistes capriscus*, age readings (years) in the age validation study. Plot a depicts the mean vertebra annuli counts (black dots) compared to spine annuli counts. Plot b depicts mean spine annuli counts (black dots) compared to otolith annuli counts. Plot c depicts the mean vertebra annuli counts (black dots) compared to otolith annuli counts.

Fig. 7. Results of marginal increment analysis of gray triggerfish, *Balistes capriscus*, a. spines, b. vertebrae, and c. otoliths. Histogram bars represent the percentage of margin types, by month, according to the relative marginal index. Black lines represent the monthly percentage of completely formed annuli on the margins of the ageing structures. Margin types: 1 = complete annulus on margin; 2 = growth zone < 1/3 the width of the previously formed annulus; 3 = growth zone 1/3 - 2/3 the width of previously formed annulus; 4 = growth zone > 2/3 the width of the previously formed annulus.

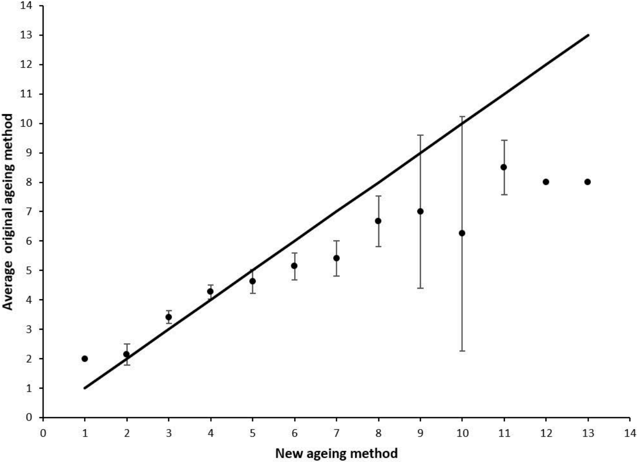
Fig. 8. Images of gray triggerfish, *Balistes capriscus*, spine sections. Black arrows point to locations on the sections that correspond with dates of calcein chemical marking and dates of sacrifice. Yellow/green bands are the calcein chemical mark.

Fig. 9. Von Bertalanffy growth models for gray triggerfish, *Balistes capriscus*, estimated from spine and otolith age readings for: a. all data available in the study (n = 96 for spines and n = 48

for otoliths) and b. Paired spine and otolith age readings: n = 46. Fractional age (years) was plotted against fork length (mm).

Fig. 10. Images of an otolith section and spine section from the same individual gray triggerfish, *Balistes capriscus*. Yellow arrows represent initial age readings (otolith age = 12 and spine age = 5). Inset is magnified area of spine containing wide translucent zone showing compacted growth zones (yellow dots). The faint yellow/green mark on the spine section is the calcein chemical mark.

Fig. 11. Gray triggerfish, *Balistes capriscus*, age bias plot of dorsal spine-based readings using the original age reading methodology compared to readings using the new methodology developed as a result of this study. The 1:1 line represents readings using the new ageing methodology in which increased magnification was used to examine wide translucent zones on the margins. The black dots represent the average age from the original readings that do not employ the new ageing methodology. Error bars represent the 95% confidence interval.


Average original ageing method
New ageing method
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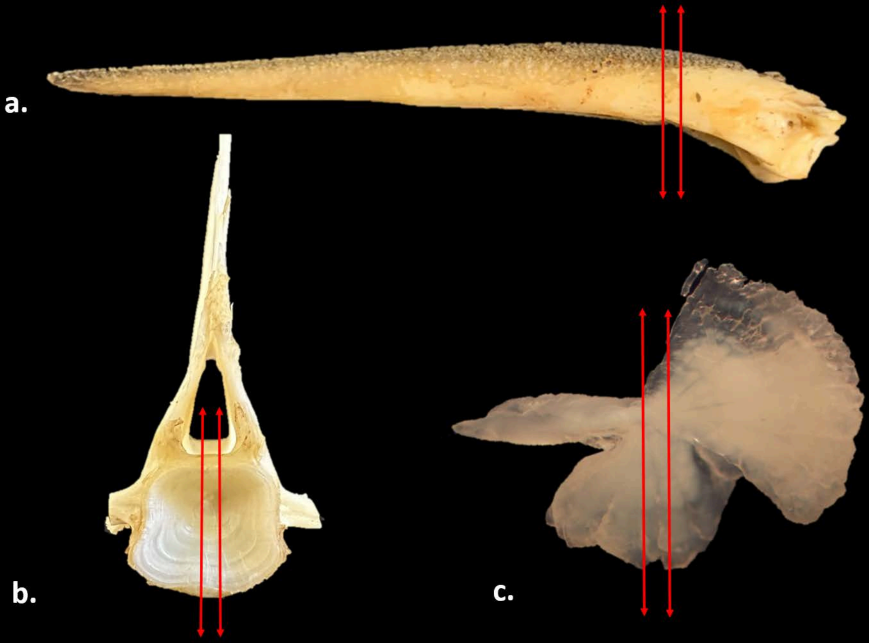
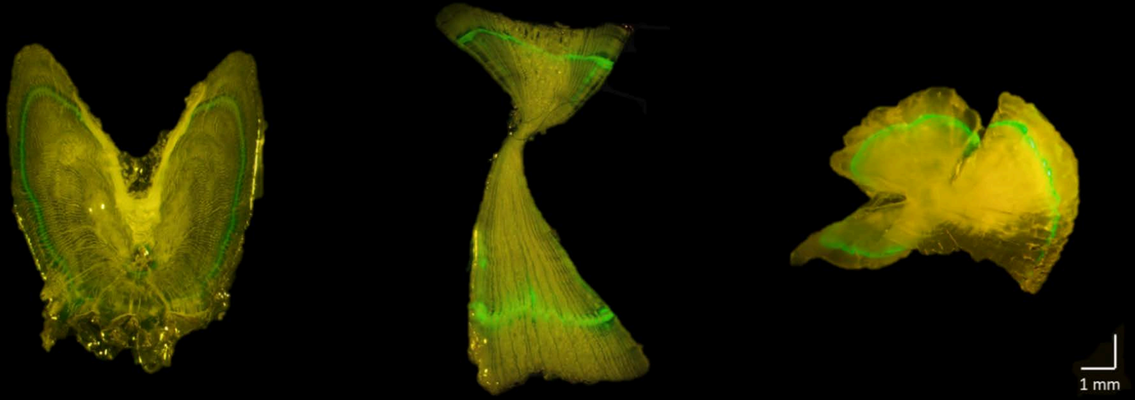
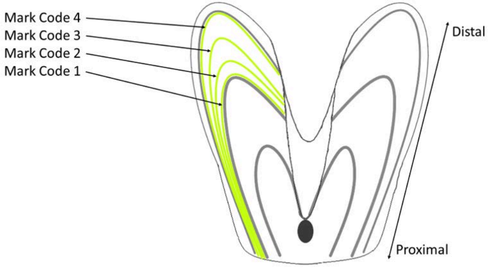
13
14


a.
b.
c.


1 mm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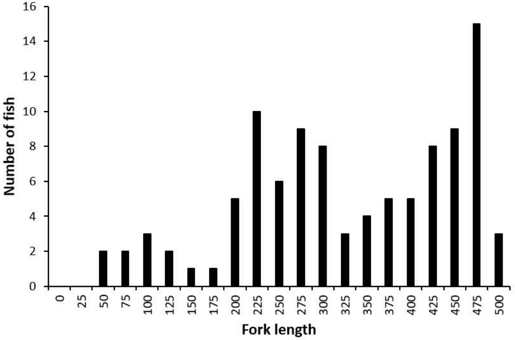
Mark Code 3
Mark Code 2
Mark Code 1
Distal
Proximal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4
6
8
10
12
14
16
Fork length
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100
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150
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200
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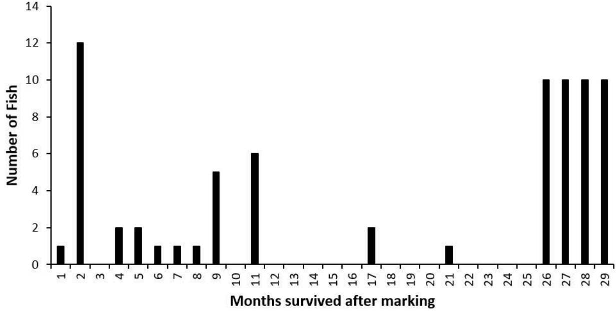
250
275
300
325
350
375
400
425
450
475
500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14
Months survived after marking
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7
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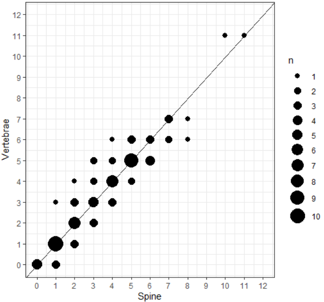
29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Spine
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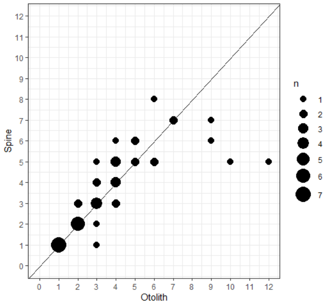
Spine
Otolith
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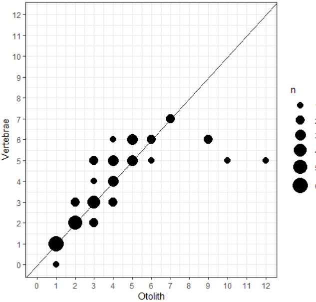
Vertebrae
Otolith
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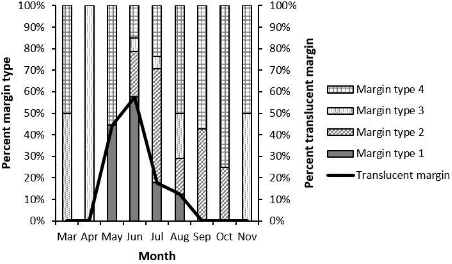
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Percent margin type
Percent translucent margin
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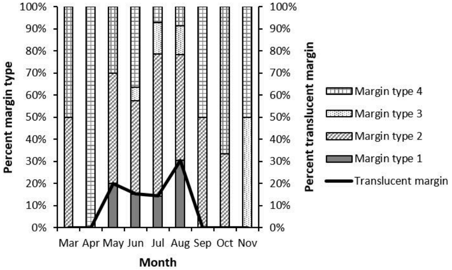
Margin type 4
Margin type 3
Margin type 2
Margin type 1
Translucent margin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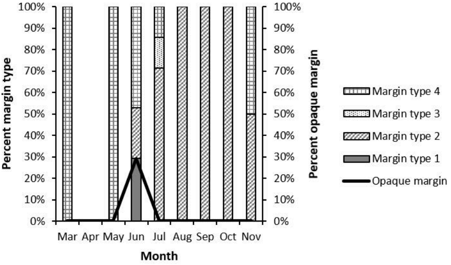
Translucent margin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Percent opaque margin
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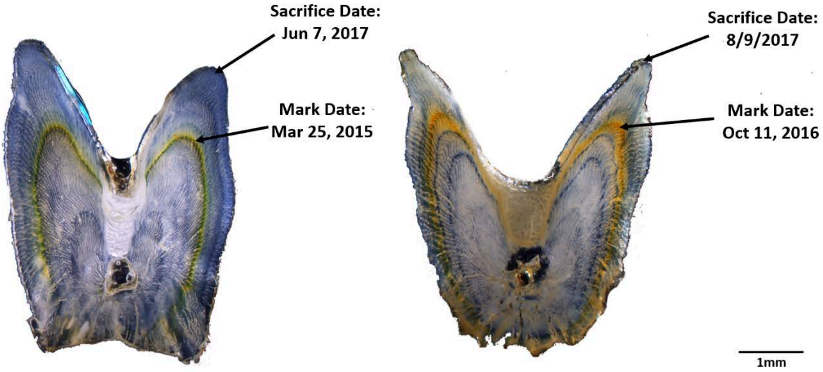
Margin type 1
Opaque margin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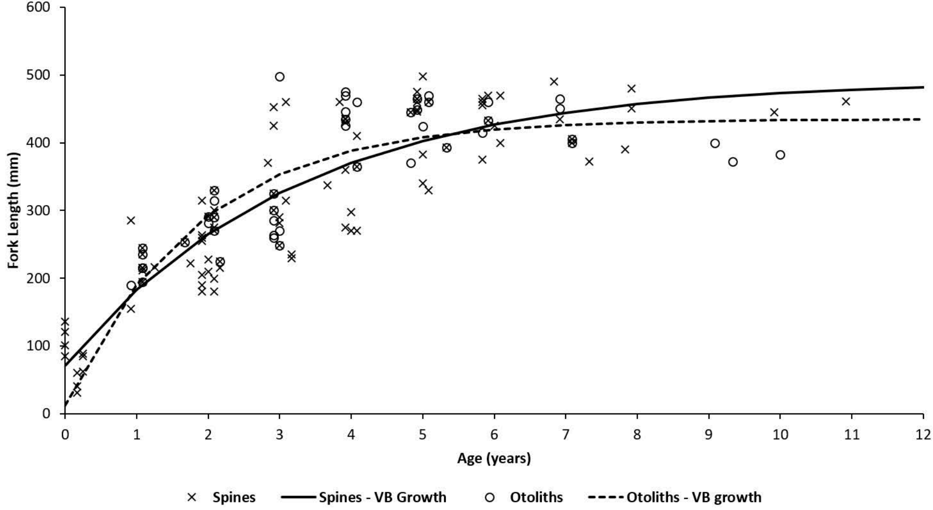

Fork Length (mm)
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200
100
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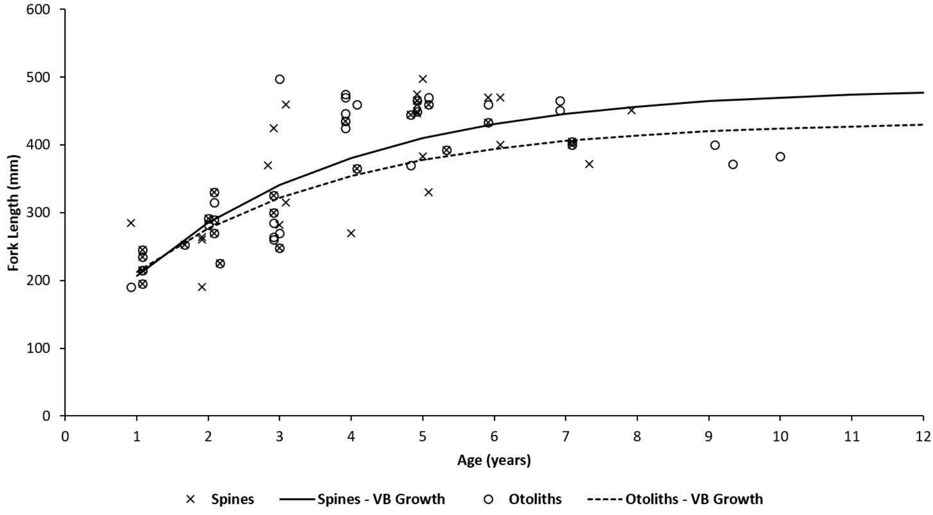
11
12
Age (years)
Spines
Spines - VB Growth
Otoliths
Otoliths - VB growth



600
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Fork Length (mm)
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Age (years)
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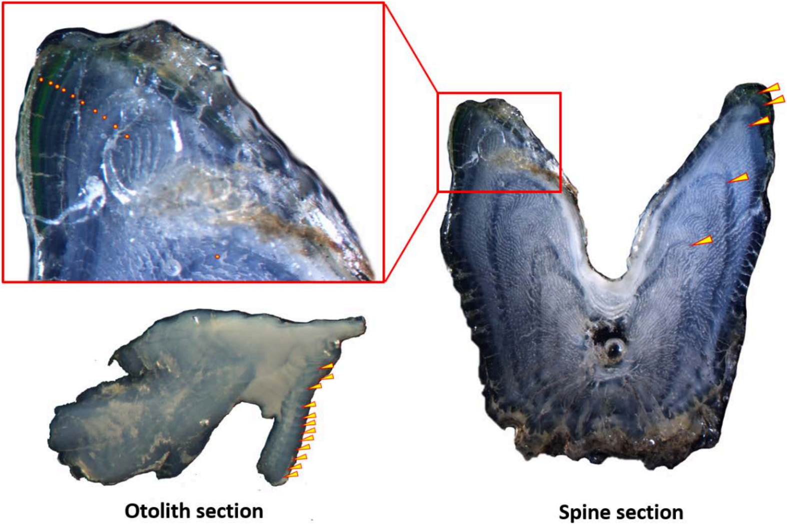
Spines - VB Growth
○ Otoliths
Otoliths - VB Growth


Otolith section
Spine section