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The goal of this study was to validate annual growth-zone formation in the gray triggerfish *Balistes capriscus* dorsal spines, fin rays, and vertebrae. Adult gray triggerfish (n = 4) were chemically marked by injecting with 50 mg of oxytetracycline (OTC) per kilogram body mass and reared in a 2,300-liter aquaculture tank. Fish were exposed to ambient light and water temperature mimicked bottom temperatures observed at an approximately 30-m depth in the northern Gulf of Mexico. Fish died after 262 d and their first dorsal spines, pectoral fin rays, and vertebrae were extracted and sectioned. One translucent zone formed distal to the OTC mark in all hardpart types during the study period. Additional fin rays and vertebrae with corresponding dorsal spines were sampled from fish (n = 27 and 59, respectively) during fishery-independent surveys to compare translucent zone counts between hardparts. There was a significant difference between translucent zone counts between fin ray and dorsal spine sections ($t_{df = 1,25} = -3.15$, $P = 0.004$). Fin ray counts on average were one zone greater than dorsal spine counts. Translucent zone counts in vertebrae were similar to those counted in dorsal spines with no significant difference between structures ($t_{df = 1,57} = 1.90$, $P = 0.062$). The percentage of dorsal spines with translucent margins increased in winter months, peaking in February, and dropped to the lowest values in summer. The combined results of this study validate annual translucent zone formation in gray triggerfish hardparts, with dorsal spines being the preferred ageing structure for production ageing.

The gray triggerfish, *Balistes capriscus*, is a conspicuous member of the northern Gulf of Mexico (GOM) reef fish community (Dance et al., 2011; Patterson et al., 2014). Historically, gray triggerfish were not heavily targeted or considered an important food resource. However, due to increased regulations on other reef fishes, such as snappers and groupers, they have become increasingly targeted both commercially and recreationally (Valle et al., 2001; Bernardes, 2002). Landings for gray triggerfish in the GOM increased substantially from the mid-1980s to the late 1990s and declined thereafter due to stock depletion (SEDAR, 2006, 2012). The most recent stock assessment determined that gray triggerfish were overfished and experiencing overfishing. In 2013 commercial and recreational catch limits were reduced and a fixed closed season was established (SEDAR, 2015).

Recent stock assessments for gray triggerfish have been performed using age-based statistical catch at age models (SEDAR, 2006, 2015), with triggerfish ages estimated via counts of translucent zones in dorsal spines (Johnson and Saloman, 1984; Ofori-Danson, 1989; Ingram, 2001; Burton et al., 2015). However, this is problematic, owing to the fact that annual growth-zone formation has not been directly validated for gray triggerfish spines or any other hardpart (e.g., otoliths, fin rays, vertebrae). The first dorsal spine has been the preferred ageing structure for gray triggerfish because otoliths are difficult to locate, extract, and process due to their small (< 5 mm) size and irregular shape (Moore, 2001; Bernardes, 2002), while dorsal spines are relatively easy to sample and process for ageing. Consequently, age estimation has been accomplished by counting translucent zones in sectioned dorsal spines that have been presumed to be formed annually. As is well established, validation of annual growth-zone formation in fish hardparts is critical to ensure accurate estimation of growth rates and for estimating catch at age as data inputs for stock assessments (Beamish and McFarlane, 1983; Campana, 2001).

Validation can be accomplished by counting growth zones in hardparts of known-aged fish or by chemically marking hardparts with calcium-binding compounds, such as oxytetracycline (OTC), calcein, or alizarin (Campana, 2001). Chemical marking requires examination of growth-zone formation following some period of growth, either in sacrificed captive-reared fish or recaptured tagged fish (Beamish and McFarlane, 1983). Hood and Johnson (1997) attempted to validate the periodicity of translucent zone formation in the first dorsal spine of gray triggerfish by injecting fish with OTC and rearing them in an indoor aquaculture facility under constant light and temperature. Dorsal

spine sections of those animals did not show translucent zone deposition following OTC marks, but deviation from natural light and temperature fluctuations may have altered normal physiological processes. Outdoor enclosures or tanks may be a better approach to replicating natural conditions, particularly light cycles (Natanson, 1993; Campana, 2001).

The goal of this study was to determine whether annual growth-zone formation in three different ageing structures could be validated. The specific objectives were to 1) evaluate dorsal spines, fin rays, and vertebrae as ageing structures for gray triggerfish by rearing OTC-marked fish, 2) examine marginal condition of dorsal spine sections of wild-caught gray triggerfish to verify annual translucent zone formation, and 3) compare translucent zone counts among dorsal spines, fin rays, and vertebrae. Fin rays were of particular interest as potential nonlethal ageing structures (Cass and Beamish, 1983; Koch and Quist, 2007; Murie et al., 2008).

Materials and Methods

Age validation experiment.—In October 2009 gray triggerfish (n = 8) were collected with fish traps off the coast of Panama City, FL. Fish were transferred to and reared in a 2,300-liter aquaculture tank with a recirculating bio-filtration system. The tank was housed in a building constructed with a translucent vinyl covering that allowed natural light to penetrate. After a 14-d acclimation period, each fish was tagged with a Floy FM-95 stainless steel internal anchor tag and chemically marked by injecting into the muscle with 50 mg of OTC per kilogram body mass. Fish were exposed to ambient light and diurnal rhythms. Water temperature ranged from 19.1 to 27.5°C and was maintained with heaters during the winter months to replicate mean bottom temperature observed in the northern GOM at an approximately 30-m depth. Salinity was monitored and maintained at 32–34 practical salinity units. Gray triggerfish were fed approximately 25 g of cut squid, shrimp, or fish every other day throughout their captivity. We chose these foods since they were consistently available and were similar to the diet of wild fish (Vose and Nelson, 1994). To prevent fouling of the tank, uneaten food was promptly removed. Following their death, fish carcasses were placed in plastic bags and frozen whole until processing.

Hardparts were extracted from thawed fish in a darkened room to prevent degradation of OTC marks due to light exposure. First dorsal spines were extracted by inserting a knife just posterior to a spine and cutting medially approximately 2.5 cm into the fish. Another identical cut anterior to the spine effectively cut out a notch of flesh that included the entire condyle of the dorsal spine. Dorsal spines were prepared for sectioning by boiling in water for 1 min to remove soft tissue and scraping the posterior groove free of tissue. Each spine was glued to cardstock and three transverse sections (0.5–0.7 mm thick) were cut simultaneously distal to the condyle with four 10-cm diamond-coated blades on an Isomet low-speed saw. Prepared sections were fixed to microscope slides with Cytoseal 60® mounting medium.

Dorsal and pectoral fin rays were extracted by cutting below the pterygiophores of each fin with a scalpel. Fin rays were cleaned of tissue by submerging the basal portion of rays in boiling water for up to 20 sec. Soft tissue was removed with forceps and a soft-bristled brush and then laid flat to dry. Once dry, fin rays were embedded in a commercial epoxy for sectioning. Embedded fin rays were sectioned using a single 5-cm blade on an Isomet saw. Each fin ray was sectioned through the basal region between 0.5 and 0.7 mm thickness. Sections were mounted on microscopic slides with mounting medium.

The three anterior-most abdominal vertebrae were dissected from each fish for ageing, as Künzli and Tachihara (2012) reported that translucent bands were more apparent in anterior vertebrae than in more posterior vertebrae in Picasso triggerfish, *Rhinecanthus aculeatus*. Vertebrae were boiled for 3–5 min to remove soft tissue. Each was sectioned with an Isomet low-speed saw equipped with two 10-cm blades with a 0.5-mm spacer. Vertebrae were sectioned in the sagittal plane through the focus and then sections were mounted on microscope slides with mounting medium. A few sections were stained with a solution of crystal violet in an attempt to improve resolution between opaque and translucent zones. This step did not improve readability and was abandoned.

Prior to age determination for dorsal spines, an ageing protocol was established through workshops with Gulf States Marine Fisheries Commission and a set of dorsal spine section digital images (n = 115) was established to train readers (Fioramonti and Allman, 2012). Dorsal spine, fin ray, and vertebral sections were aged by counting the number of translucent zones present. Dorsal spine and vertebral sections were viewed with a dissecting microscope under ×10–40 magnification with transmitted light, and fin ray sections were viewed with a compound microscope under ×100 magnification using transmitted light and a green filter to enhance contrast between opaque and translucent zones (Murie et al.,

2008). For all three structures, opaque zones representing faster growth are relatively wide, and zones corresponding to slow growth periods are narrow and appear translucent under transmitted light (Lessa and Duarte-Neto, 2004; Brusher and Shull, 2009; Künzli and Tachihara, 2012). Broad opaque zones in vertebrae often contained faint translucent zones, which were considered checks and were not counted for ageing (Künzli and Tachihara, 2012). OTC marks in dorsal spine, fin ray, and vertebral sections were examined as described above but with transmitted ultraviolet light.

Hardpart comparison.—Dorsal spines, fin rays, and vertebrae were extracted from gray triggerfish sampled during fishery-independent surveys and all hardparts were processed for ageing as described above. Ageing of all three structures was conducted independently by two readers without knowledge of fork length (mm) to prevent bias. Average percent error (APE; Beamish and Fournier, 1981) was used to estimate precision between reader estimates of ages. Any disagreement in ages was resolved by reader consensus. If a consensus could not be reached, the hardpart was rejected. Translucent zone counts of dorsal spine sections were compared to fin-ray and vertebrae-section counts using paired t-tests ($\alpha = 0.05$). Bias plots were constructed to detect any systematic differences in translucent zone counts between ageing structures (Campana et al., 1995).

Timing of translucent zone formation.—To estimate the periodicity of translucent zone formation, dorsal spine sections were selected for marginal increment analysis from archived gray triggerfish samples collected 2003–10. The margin of each spine section was recorded as translucent or opaque and assigned a readability code of good, fair, poor, or unreadable. Analysis was restricted to sections that were assigned readability codes of fair to good. The percentage of dorsal spines with a translucent margin was plotted vs month to examine the temporal progression of translucent zone formation.

Results

Age validation experiment.—Of the eight fish captured, only four survived until the end of the experiment. Gray triggerfish were marked with OTC on 31 Oct. 2009 and removed from the tank on 20 July 2010 (262 d). Fork lengths were 178, 204, 249, and 271 mm at the beginning of the experiment and 213, 244, 266, and 266 at the end of the experiment, respectively. Second lengths were taken after fish had been frozen and thawed. The original intent was to rear fish for at least 1 yr but an unexpected pump failure resulted in low dissolved oxygen levels and the four remaining fish died. Nevertheless, results of the OTC marking experiment indicate that one translucent zone formed in dorsal spines, fin rays, and vertebral sections during winter months (Fig. 1). An opaque margin on each structure also indicated opaque zone formation had begun prior to experiment termination in July.

Hardpart comparison.—Gray triggerfish selected for hardpart comparisons included the most common sizes seen in the fishery and ranged from 75 to 450 mm fork length for fin ray samples (n = 27) and from 108 to 481 mm fork length for vertebrae samples (n = 59). Translucent and opaque zones were apparent in all structures and translucent zone counts were the same as dorsal section counts for 37% of fin rays and 51% of vertebrae. APE between readers was 10.8% for dorsal spines, 12.3% for fin rays, and 18.8% for vertebrae. A paired t-test indicated a significant difference existed between translucent zone counts in fin ray vs dorsal spine sections ($t_{df = 1,25} = -3.15$, $P = 0.004$). On average, one more translucent zone was counted in rays than in spine sections, which is apparent in the bias plot for those two structures (Fig. 2A). Translucent zone counts in abdominal vertebrae were similar to those counted in dorsal spines, and no significant difference in counts existed between those structures ($t_{df = 1,57} = 1.90$, $P = 0.062$) (Fig. 2B).

Timing of translucent zone formation.—Marginal-condition analysis of dorsal spine sections indicated that translucent zones began forming in fall, with the highest percentage of translucent margins occurring in winter (February and March) and a secondary peak in September (Fig. 3). Fish sampled in summer months (June and July) had the lowest percentage of translucent dorsal spine margins, hence most margins in summer were opaque.

Discussion

This study represents the first attempt to directly validate annual translucent zone formation in gray triggerfish dorsal spines, as well as in fin rays and vertebrae. All hardparts of experimental fish that were injected with OTC in fall 2009 demonstrated translucent zone formation during winter, followed by opaque zone formation in spring and summer. Rearing experimental fish for a second year likely would have provided even more robust results, as would have

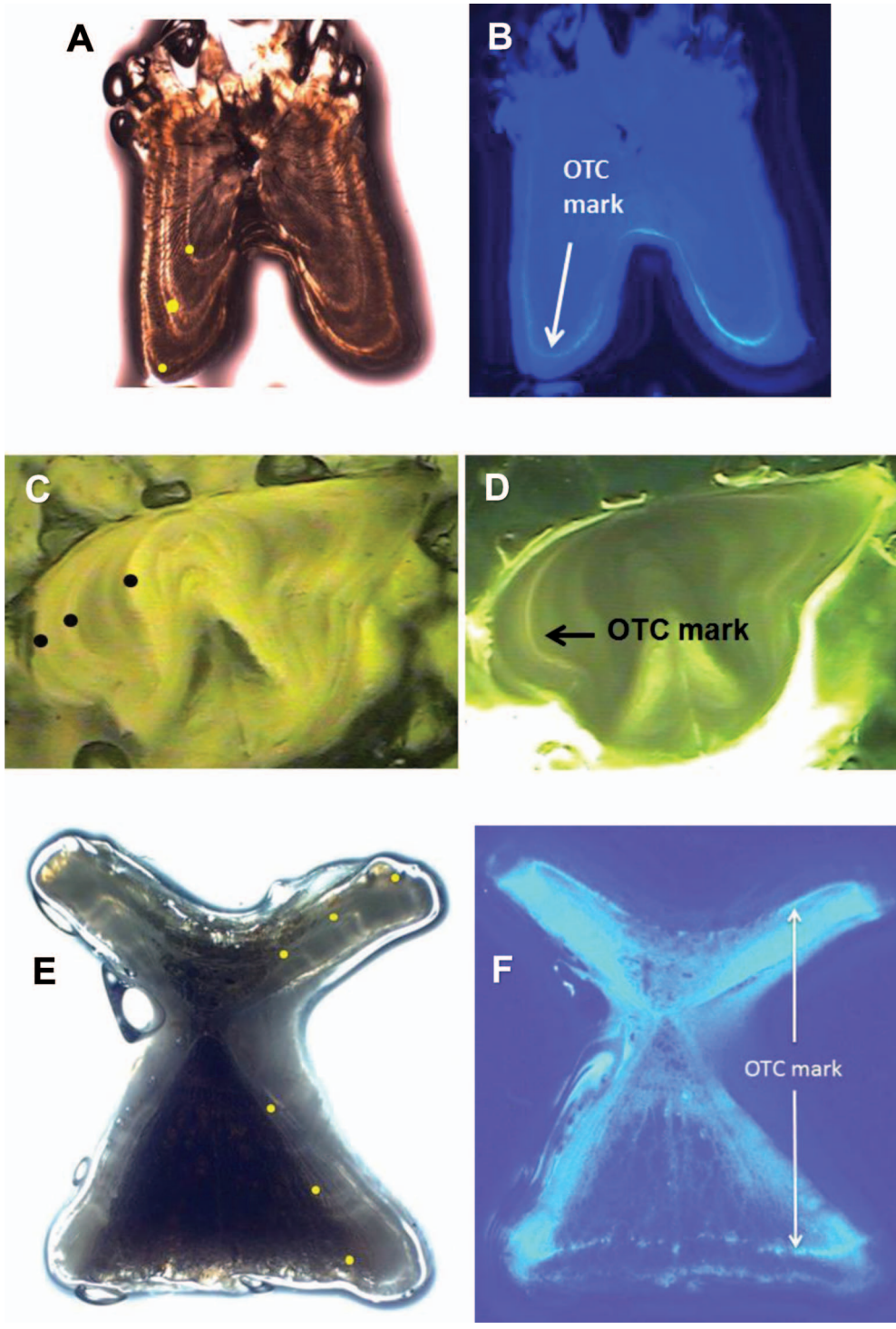

Fig. 1. Digital images of oxytetracycline-marked gray triggerfish hardparts viewed with transmitted visible (left) and ultraviolet light (right). Dorsal spine (A, B) and pectoral fin ray (C, D) sections are from a 270-mm–fork length (FL) female, while the vertebral section (E, F) is from a 243-mm FL male. The light source was covered with a green filter for fin sections. Translucent zones are marked with circles in each section.

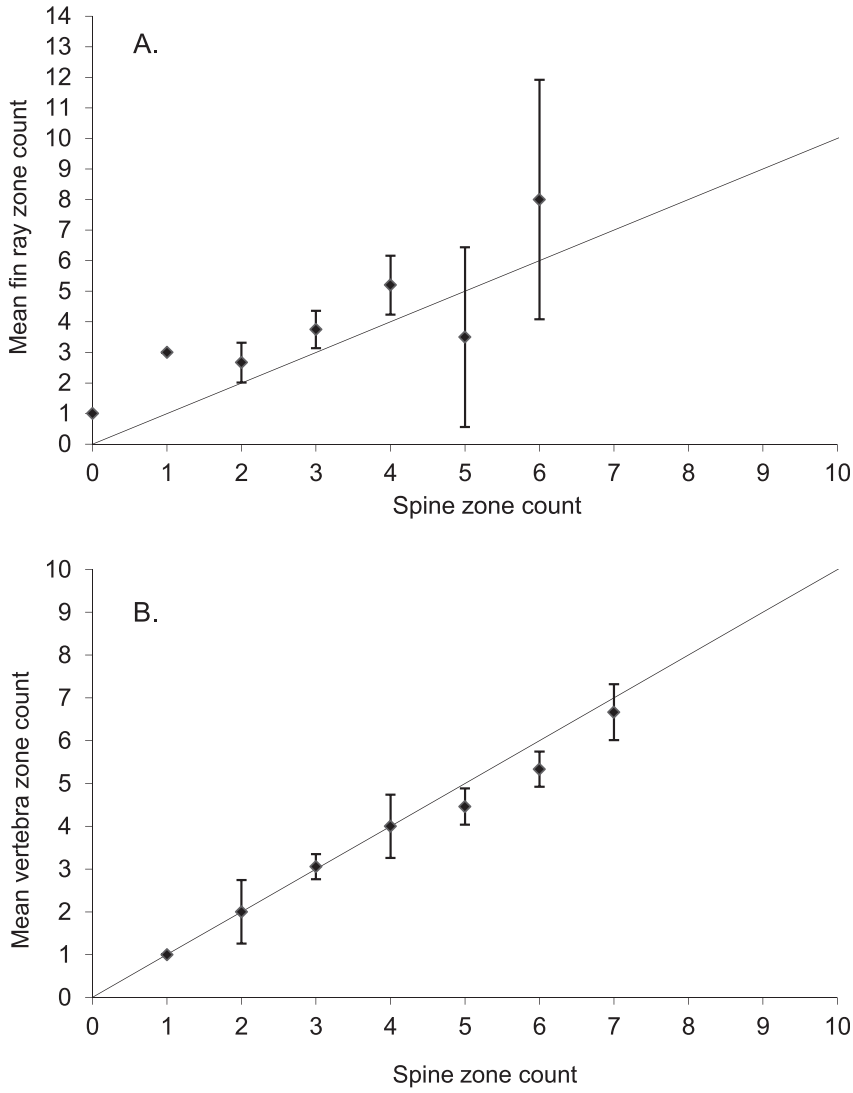

Fig. 2. Bias plots for Gulf of Mexico gray triggerfish for (A) mean fin ray and (B) mean vertebral section translucent zone counts plotted against dorsal spine counts. Error bars represent the 95% confidence interval. Line represents 1:1 relationship between counts.

tagging OTC-injected fish in the wild for subsequent recapture. Maintaining gray triggerfish in captivity proved to be difficult, with only four OTC-marked fish surviving until the conclusion of the experiment. While sample size was small, results from the OTC marking experiment, as it was conducted, clearly validate one translucent zone being formed in winter. The length of the largest fish was less at the end of the experiment than at the beginning. This fish developed a lesion at the tagging site, which may have affected overall condition and contributed to loss in length. Additionally, all fish were frozen, thawed, and measured, which has been shown to result in loss of length in Atlantic salmon, *Salmo salar* (Armstrong and Stewart, 1997).

Of the 12 species routinely aged at the Panama City laboratory, gray triggerfish dorsal spine sections are among the most difficult ageing structures to assign an age. Average percentage of reader error was 10.8% and was similar to internal reader comparisons for an annually exchanged reference set of dorsal spine sections (n = 115) and to the overall APE of 11% reported by Burton et al. (2015). In comparison, for otoliths 5% is a reference point for moderately long-lived species with relatively difficult-to-read otoliths (Morison et al., 1998; Campana, 2001). Given

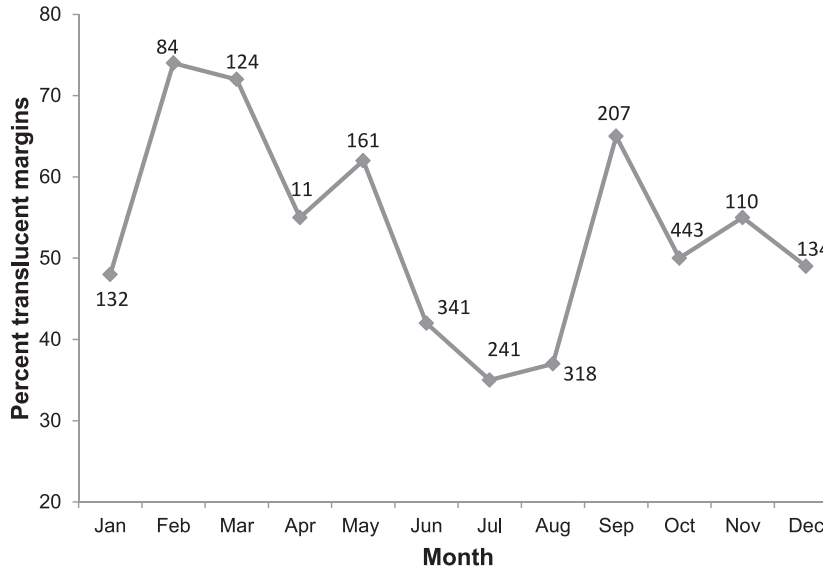

Fig. 3. Percentage of translucent margins in Gulf of Mexico gray triggerfish dorsal spines collected 2003–10 (n = 2,411). Numbers indicate monthly sample size.

the difficulty of ageing gray triggerfish spines, an APE $> 5\%$ is to be expected in a production ageing setting. Reference collections and training workshops are a crucial quality control tool, which must be used continuously to ensure that individual reader ages do not change over time and that ages from different readers remain consistent (Campana, 2001).

Verification of annual translucent zone formation in wild fish via marginal condition analysis also provided meaningful results with respect to the efficacy of ageing gray triggerfish with dorsal spines. The percentage of spines with translucent margins was greatest in the winter months and lowest in the summer, with an annual periodicity of translucent zone formation apparent in the data. Ingram (2001) reported a similar result for fish captured off Alabama in the late 1990s, whereas Moore (2001) and Burton et al. (2015) inferred from marginal condition analysis that translucent zone formation was completed by June for gray triggerfish from the South Atlantic United States. A secondary peak was detected in translucent zones in September. Ingram (2001) also noted a secondary peak in August and Johnson and Saloman (1984) recorded the highest percentage of translucent zones in June and July. It has been observed that during the spawning season both males and females appear to exhibit limited feeding due to their territoriality and resistance to capture by baited hooks and traps (Ingram, 2001; Mackichan and Szedlmayer, 2007). Ingram (2001) hypothesized that the appearance of "doublets" (two translucent bands formed close together) in spines was due to winter deposition of a translucent zone caused by lower metabolism followed by temporary fasting of spawning/nesting triggerfish during the summer. We also noted spines with this doublet pattern (Fig. 4) and since these translucent zones were closely spaced in relation to other translucent zones, we counted these as one annulus. While direct validation of annual growth-zone formation is the gold standard for ageing studies, marginal condition trends reported here provide additional evidence for annual translucent zone formation in gray triggerfish dorsal spines.

Gray triggerfish used for hardpart comparisons included the most common age classes observed in the recreational and commercial fisheries. While the maximum observed age of GOM gray triggerfish is 14 yr, older fish are rare. Gray triggerfish greater than age 10 comprised only 0.1% of recreational ages and 0.5% of commercial ages from a recent age dataset (N = 5,762) submitted for a GOM stock assessment (SEDAR, 2015). Despite validation of annual translucent zone formation in fin rays, there was considerably more variance in translucent zone counts from fin rays vs dorsal spines. Given the validation of translucent zone counts as being formed annually, the difference in counts between fin ray and dorsal spine sections indicates that fish aged with fin rays would be estimated to be 1 yr older, on average, than if aged with dorsal spines. This difference is likely due to difficulty identifying the first translucent

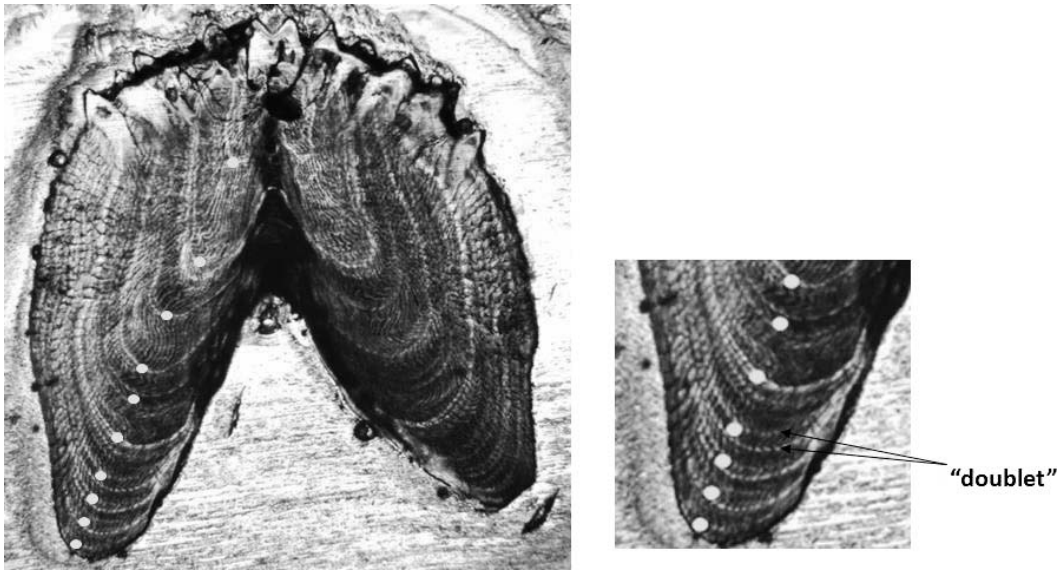

Fig. 4. Digital image of dorsal spine section from a 514-mm–fork length, age-10 gray triggerfish with "doublet" pattern magnified (right). Translacent zones are marked with circles.

zone in fin ray sections. The first counted zone looked distinctly different from others and may have been either part of the core or a settlement mark. Examination of fin ray sections from individuals of age 0 and age 1 may aid in the identification of the first annulus. Preparation and processing of the fin rays also was more laborious than for either spines or vertebrae due to their small size. Furthermore, sample extraction to produce a readable transverse section of a fin ray required rays to be removed at the insertion into the pterygiophore, thus increasing the potential for infection for the fish. Therefore, the invasive nature of fin ray extraction may preclude their use as a nonlethal means of age determination.

Experimental rearing also validated abdominal vertebrae as forming one translucent zone in winter followed by an opaque zone after the OTC mark. No significant difference was found between translucent zone counts of vertebrae sections and spine sections, and Künzli and Tachihara (2012) previously reported high (96.9%) agreement in translucent zone counts between dorsal spines and abdominal vertebrae of the Picasso triggerfish for age classes 0–14. Despite the validation of annual translucent zone formation in gray triggerfish vertebrae and the high agreement with counts in dorsal spines, vertebrae should only be viewed as complimentary ageing structures at this stage. Comparisons of translucent zone counts between vertebral and dorsal spine sections currently are lacking for fish $>$ 6 yr old; dissection of vertebrae is more labor-intensive than spines, and vertebral sections take approximately three times longer to prepare than those of dorsal spines. Therefore, even if translucent zone comparisons for older ages indicate similar numbers of zones in vertebrae vs spines of older fish, vertebrae are unlikely to replace dorsal spines as the hardpart of choice for production ageing. Further support for dorsal spines as the preferred hardpart for ageing triggerfish is the lower reader error (APE) for dorsal spines compared to both fin rays and vertebrae.

In conclusion, the most significant contribution of this study is the validation of annual translucent zone formation in gray triggerfish dorsal spines. Marginal increment analysis and hardpart comparisons corroborate these results. Dorsal spines have been employed to age triggerfish in previous studies (Johnson and Saloman, 1984; Hood and Johnson, 1997; Ingram, 2001; Moore, 2001; Burton et al., 2015), but no direct validation of annual translucent zone formation was previously conducted. Results of marginal increment or condition analysis have been cited as verification of translucent zones forming annually, but directly validating age estimates via chemical marking of hardparts is much more definitive (Beamish and McFarlane, 1983; Campana, 2001). Such validation is imperative for examining gray triggerfish population ecology, as well as for age-based stock assessment. This latter requirement is even more critical given the fact that gray triggerfish are currently estimated to be

overfished in the northern Gulf of Mexico from the results of the most recent age-based stock assessment (SEDAR, 2015). Results provided here strengthen the inference that gray triggerfish can be aged accurately based on translucent zones in dorsal spines, thus also providing validation for age-based assessment of this fishery resource.

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