A Comparison of Ageing Methods in Red Snapper *Lutjanus campechanus*

by

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Abstract

Sectioning otoliths and counting annuli is an accepted method for ageing of red snapper (*Lutjanus campechanus*). To reduce effort, time, and costs, whole otolith increment counts were compared to sectioned otolith increment counts to investigate the accuracy of whole otolith age estimations. Increments on whole otoliths were counted for all fish (n = 1743), and subsets were randomly selected across all whole increment counts for sectioning and counting. A linear regression of whole on sectioned otolith increment counts had a slope of 0.85 (n = 694, R^2 = 0.91, p < 0.01). Mean differences between whole and sectioned otoliths showed that estimated ages of older, larger fish were underestimated by whole otolith counts. Therefore, an adjusted count was derived by removing large and unreadable otoliths, which increased the regression slope to 0.89, but the slope was still significantly different from 1 (n = 534, R^2 = 0.92, p < 0.01). Mortalities and von Bertalanffy growth curves were compared between methods to determine if the adjusted count method was accurate relative to the sectioned count method. Similar mortality estimates and growth curves were produced. Furthermore, shape analysis performed on age-0, age-1, and age-2 fish supported the whole count method with an 89% agreement rate between classified ages from the discriminate function analysis and whole increment counts. Marginal increment analysis was performed on the sectioned otoliths in conjunction with an oxytetracycline study to determine increment formation. The marginal increment analysis indicated opaque formation from May through September, which is most likely due to reproductive stress. Most (69%) OTC-marked fish

also supported a Summer-Fall formation of opaque increments. This study provides a new method for otolith ageing in red snapper that could increase efficiency, reduce costs, and remove much of the inherent reader based bias typical of traditional otolith counting.

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INTRODUCTION

Red snapper (*Lutjanus campechanus*) is a highly exploited species of the northern Gulf of Mexico (GOM), supporting a fishery vital to coastal communities. In 2011, the commercial red snapper fishery generated $11.4 million for the Gulf States (NMFS, 2012). In addition, the sport fishery attracts anglers who spend money directly on fishing gear and boat expenditures and indirectly on amenities supporting their stay (e.g., lodging, food). In 2011, the sport fishery harvested more than 500,000 fish and contributed to the $9.8 billion spent for all recreational marine fishing activities in the GOM (NMFS, 2012). Red snapper supports a lucrative fishing industry, and the stock has only recently been removed from the overfishing list. However, the stock is still considered overfished because it remains beneath a prescribed threshold (SEDAR, 2013). Federal management remains in the rebuilding phase for red snapper, and monitoring of the population continues to be a critical agenda.

Not only are red snapper an important economic stock, but they are an essential component of reef communities. For example, large adult red snapper have a significant effect on the abundance and species richness of small reef fishes and the abundance of age-0 and age-1 red snapper (Mudrak and Szedlmayer, 2012). Red snapper inhabit hard structure with vertical relief (Ouzts and Szedlmayer, 2003; Szedlmayer and Lee, 2004; Piko and Szedlmayer, 2007; Gallaway et al., 2009). However, the benthos of the waters off Alabama, along with much of the northern GOM, is dominated by mud and sand substrate, and natural rocky reef habitats are rare (Schroeder et al., 1988; Dufrene, 2005). To increase habitat of economically important species like red snapper, an artificial reef program was initiated in 1953. Presently, the general permit zones where artificial reefs are allowed to be deployed amount to over 1930 km^2 (Minton and Heath, 1998).

Present management goals are to monitor red snapper stocks and determine directional trends, which frequently relies on estimations of growth rate, mortality rate, and productivity. These values are generated in large part from accurate age data based on otolith opaque increment counts (Reibisch, 1899; Campana, 2001; Jackson, 2007). Typically, thin transverse sections are cut from sagittal otoliths, and opaque increments are counted as annuli (Nelson and Manooch, 1982; Szedlmayer and Shipp, 1994; Patterson et al., 2001; Wilson and Nieland, 2001; Fischer, 2002; White and Palmer, 2004; Allman et al., 2005; Szedlmayer and Beyer, 2011). However, whole otolith counts have been used successfully for red snapper < 7 y (Szedlmayer, 2007; Syc and Szedlmayer, 2012), and whole otolith counts are shown to be as accurate as sectioned otoliths of young fish in Atlantic herring (*Clupea harengus*) (Peltonen et al., 2002), Arctic grayling (*Thymallus arcticus*) (Gettel et al., 1997), and yellowtail flounder (*Limanda ferruginea*) (Dwyer et al., 2003). Also, whole otoliths were shown to be a reliable cost effective alternative to sectioned otoliths for ageing Pacific red snapper (*Lutjanus peru*), a Lutjanidae ranging off northern Australia (Rocha-Olivares, 1998).

Even though whole otoliths of Red Snapper have been used successfully, no previously published study has validated the accuracy of whole otolith increment counts as compared to sectioned otolith increment counts. Sectioning of otoliths increases processing time and labor, equipment, and supply costs. If whole otoliths can provide an accurate age similar to sectioned otoliths in red snapper, such a method would substantially reduce processing time by eliminating the labor intensive task of cutting and polishing otoliths and significantly increase the number of fish that could be aged in a given time period.

The accuracy of age data is assessed through validation of the frequency of the formation of opaque zones and of absolute ages (Campana, 2001). Annulus formation has been documented for adult red snapper, but questions remain concerning the timing of opaque zone formation (Patterson et al., 2001; Wilson and Nieland, 2001; Allman et al., 2005; Szedlmayer and Beyer, 2011). A previous marginal increment analysis (MIA) suggested that opaque zones were formed from January through May (n = 1,676, Patterson et al., 2001). Wilson and Nieland (2001) performed MIA on red snapper collected over an 8 y period from off Louisiana (n = 3,791). Those fish formed opaque zones from December through June. Allman et al. (2005) reported that opaque zone formation (n = 259) was sudden and brief occurring April through July. Using scales, which are generally less accurate (Campana, 2001), Nelson and Manooch (1982) showed a summer timing of opaque band formation. In another approach, Szedlmayer and Beyer (2011) released oxytetracycline (OTC) injected red snapper and showed opaque formation from August through December. Thus, every month of the year has been identified as a month of opaque zone formation among these studies.

For red snapper, basic questions remain concerning ageing methods, and it is critical that the timing of opaque zone formation be resolved for future life history studies and improved management of red snapper. The differences in timing of opaque zone formation may be accurate and caused by differences in collection seasons, fish sizes, locations, and duration. However, they are most likely an artifact of methods. For example, MIA can be a subjective evaluation (Campana, 2001) and may need to be used in conjunction with OTC marking studies to verify timing.

Otolith morphometrics is another method that can be used to validate ages of young red snapper. This is an alternative technique that has been used to distinguish between age classes of a few fish species, e.g., Atlantic Cod (*Gadus morhua*) (Doering-Arjes et al., 2008) and gray angelfish (*Pomacanthus arcuatus*) (Steward et al., 2009). Beyer and Szedlmayer (2010) determined that otolith shape analysis coupled with otolith weight successfully predicted ages of age-0, age-1, and age-2 red snapper. In addition to being objective, shape analysis is less time consuming.

In many instances, the primary objective of ageing individual fish is to determine growth and mortality estimates of a population or stock (Francis and Campana, 2004). Therefore, to determine if age estimation based upon whole otolith increment counts are accurate relative to those based upon sectioned otoliths, mortalities and von Bertalanffy growth curves were compared among methods. In addition, the present study also applied a MIA of sectioned otoliths and made comparisons to recently recaptured OTC marked red snapper. Finally, determination of age classes from otolith shape analysis of age-0, age-1, and age-2 were compared to whole otolith counts in an attempt to validate whole otolith counting for these younger age classes.

MATERIAL AND METHODS

Otolith Collection: Whole verses Sectioned Otolith Increment Counts

Red snapper were collected from December 2011 through October 2013. A total of 1753 fish were collected from unpublished artificial reefs (geographic coordinates unknown to the public), published artificial reefs (geographic coordinates reported by the Alabama Department of Conservation of Natural Resources), and one unpublished natural reef for a total of 59 sites (Fig. 1). Most sites were within the artificial reef permit zones off Alabama. Reefs were randomly chosen through a stratified sampling design based on depth and distance from shore, and unpublished reef sites were located with a side scan sonar (EdgeTech, 4125 –Dual, 400/900 Hz, EdgeTech, West Wareham, MA).

At each site fish were collected with hook-and-line or fish trap (1.2 × 1.5 × 0.6 m; Collins, 1990). To standardize the methods, hook-and-line was carried out by two fishers for 30 min, and the trap was fished for four 15 min intervals. Hook-and-line terminal gear included two 7/0 j-hooks that were baited with Atlantic menhaden (*Brevoortia tyrannus*) or gulf menhaden (*B. patronus*). The trap was also baited with Atlantic or gulf menhaden. All collected fish were separated by fishing method, stored on ice, and returned to the laboratory for measurements and otolith extraction.

Fish were brought back to the laboratory and processed within 72-h. Fish sizes (SL, FL, TL) were measured to the nearest mm. Total wet weight (g) of each fish was measured with an Ohaus Trooper digital scale (Parsippany, NJ). To extract otoliths, a dorsal cut (sagittal plane) was made through the head just posterior to the preopercle.

With larger (> 200 mm SL) red snapper, a Bosch (Stuttgart, Baden-Württemberg) fine cut electric saw was used to remove the otoliths, while a small knife was sufficient on smaller fish (< 200 mm). For the largest red snapper (> 9 kg), a lateral cut underneath the operculum was used to minimize the risk of breaking the otolith. After removal, all otoliths were cleaned, dried, and placed into pre-labeled vials.

Otolith Collection: OTC Study

Otoliths from red snapper marked by OTC were obtained from laboratory reared fish and a mark-recapture study. In the laboratory rearing study, red snapper were caught and marked on 15 June 2010 and reared in the laboratory for > 1 y. For the mark recapture study, fish were captured and released on artificial reefs beginning in 2007 (Szedlmayer and Beyer, 2011) and continuing through 2013 (Topping and Szedlmayer, 2011; Piraino and Szedlmayer, in press). All recaptures were examined for OTC mark retention.

For the laboratory study, red snapper (n = 18) were collected from an artificial reef located off the coast of Alabama on 15 June 2010 and held in a 11000 L recirculating seawater system at constant salinity (35-40 ppt) and temperature (19-22 °C). Light periods were natural daylight seasonal cycles. On 16 June 2010, fish lengths (SL, FL, TL mm) and weights (g) were measured, and fish were tagged with internal PIT tags. Each fish then received an intramuscular injection of OTC (10 mg OTC kg^{-1} body weight) in the epaxial musculature with an 18 gauge hypodermic needle. Fish were fed every two days to satiation with white, brown, or pink shrimp (*Litopenaeus* spp.). Final weights, lengths, and otoliths were collected for all fish that died (n = 7) in captivity. On 15 Sept

2011, the remaining fish (*n* = 11) received a second injection of OTC. The purpose of the second injection was to document any time delay in fluorescent band formation after injection. All captive fish were sacrificed on 8 Nov 2011, identified by the PIT tags, weighed, measured, and otoliths removed.

Three previous studies (Szedlmayer and Beyer, 2011; Topping and Szedlmayer, 2011; Piraino and Szedlmayer, in press) and one study in progress (Williams and Szedlmayer unpublished data) have marked red snapper with OTC and released them on artificial reefs in the northern GOM. Any recaptures from these studies were used in the present study to examine otolith increment formation in relation to time of OTC marking. These fish were caught by hook-and-line using a 6/0 or 7/0 circle hook baited with Gulf or Atlantic menhaden. Fish were anesthetized on the research vessel with MS-222 (150 mg tricaine methanesulfonate L^{-1} seawater) in a 70 L container of seawater. All fish were weighed, measured, and injected with OTC. For visual identification, all fish were also marked with individually numbered internal anchor tags (FLOY TAG Inc., Seattle, WA). The tagged fish were observed for a short recovery period (about 2 min) in 70 L container of seawater. When gill and fin movements became active, the fish was released at the bottom with a quick release drop weight or using cage release methods (Szedlmayer and Beyer, 2011; Topping and Szedlmayer, 2011; Piraino and Szedlmayer, in press). Recaptures were dependent on fisher reported captures.

All private fisher recaptures were filleted; therefore, final weight was unavailable. Lengths (SL, FL, TL) were measured to the nearest mm. Several fish scales were collected from each fish and examined under blue-violet light to quickly assess that the fish was an OTC marked fish. The otoliths were removed laterally from underneath the operculum, with a small knife, to reduce the incidence of breaking. The otoliths were cleaned, dried, and stored in the dark in pre-labeled vials.

Otolith Collection: Shape Analysis

Age-0 through age-2 otoliths (*n* = 556) used in shape analysis were taken from the hook-and-line and trap samples (mentioned above), and from fish collected by SCUBA diver drop nets on small (1 m^2) recruitment reefs off Alabama (Mudrak and Szedlmayer, 2012). Drop net samples were collected on 15 and 20 November 2011. Rotenone was used to euthanize the fish trapped under the drop net. All drop netted fish were stored on ice and brought back to the laboratory for processing. Within 72-h, lengths and weights were measured for all fish. Red snapper otoliths were removed with a small knife with a dorsal cut. The otoliths were cleaned, dried, and placed in pre-labeled vials.

Otolith Imaging and Ageing

Once collected, digital images of both otoliths from each fish were captured by a Lumenera Infinity 3-1 (Ottawa, Ontario) camera attached to a Leica MZ6 stereoscope (Wetzlar, Germany). Each image recorded (Infinity Analyze version 6.1, Lumenera, Ottawa, Ontario) was standardized by maintaining the magnification at 20x. All otoliths were placed in a petri dish and covered with water before images were recorded to minimize the diffraction of light caused by the irregular surface of the otolith. Otoliths were illuminated with fiber optic transmitted light (Ace One, Schott Fostec, Elmsford, NY).

The opaque increments of whole otoliths were counted on the captured images independently by two individual readers a minimum of 3 times. The count was accepted as final if the counts were the same between readers. If the counts did not agree, the otolith was reexamined together by the two readers. If agreement could still not be reached, than the otolith was removed from further analyses.

Sectioning Otoliths

Otoliths were selected for sectioning based on a stratified random sample of whole otolith counts. This was necessary due to decreasing samples with increasing whole otolith counts. Also, all otoliths were sectioned when there were > 6 increments or if declared unreadable by the two independent readers. An unreadable whole otolith was defined as the inability to discern opaque increments from the center to the edge. Sectioning of otoliths with > 6 increments was based partly on previous red snapper otolith ageing (Syc and Szedlmayer, 2012).

To begin the sectioning process the core of the otoliths was marked with a pencil under the stereoscope. The marked otoliths were attached to a glass slide using crystal bond cement with the proximal side facing up. Once attached, a transverse section through the primordium was cut using a Buehler Isomet low speed saw. The section was checked underneath the stereoscope to ensure that the cut was centered. If the cut was off center, then another transverse section was taken. The second otolith from an individual fish was sectioned if cuts on the first otolith failed to include the primordium. All sections were stored in pre-labeled vials separate from the whole otoliths. Image capture and ageing of the sections followed the protocol of whole otoliths. In addition, the margin of the sectioned otoliths was identified as either opaque or translucent without prior knowledge of capture date.

Sectioning of OTC Marked Red Snapper

Sectioning and imaging of the OTC otoliths was performed as described above; however, sections were mounted on a glass slide with crystal bond. To view the OTC marks, the sections were viewed with an Olympus (Shinjuku, Tokyo) BH-2 microscope at 40x magnification. The microscope was equipped with blue-violet and ultraviolet light that excites the OTC mark and makes it visible. Images were taken with a Lumenera Infinity 3-1 microscope camera. Otolith images were measured from the OTC mark to the edge on the ventral side of the sulcus with Image Pro Plus V 4.5 (Media Cybernetics, Rockville, MD).

Shape Analysis

Imaging and counting of increments of the whole otoliths (age-0 to 2) used the same methods as above. In addition, these otoliths were further analyzed using shape analysis. For this procedure, the otolith was weighed with the Ohaus scale to the nearest 0.01 g and an additional image was recorded. For this image, the otolith was placed on a black background, proximal surface down, and with the rostrum pointing to the left for standardization. The otolith was illuminated with reflected fiber optic light and the image was recorded at a standardized distance (20x magnification). Any broken or damaged otoliths were not used in this analysis.

The image was further processed with Image-Pro Plus version 4.5. This imaging program automatically traced the whole otolith and generated different shape measures. Shape variables measured were area, perimeter, aspect ratio, rectangularity, roundness, box x/y, radius ratio, and perimeter ratio. Explanations of these variables are given in Table 1.

Statistical Analysis

Whole verses Sectioned Otolith Increment Counts

Three approaches were used to determine if whole otolith increment counts were significantly different from sectioned otolith increment counts. First, a linear regression was used for a direct comparison using whole otolith counts as the dependent variable and sectioned otolith increment counts as the independent variable. Second, von Bertalanffy growth curves were independently fitted to whole otolith counts, sectioned otolith increment counts, and an adjusted method with Fisheries Analysis and Simulation Tools (FAST) software version 2.0 (Slipke and Maceina, 2000) . The adjusted method used counts from whole otoliths < 7 increments and sectioned counts for whole otoliths with > 6 increments and those declared unreadable. The model is as follows:

$$TL_t = L_\infty (1 - e^{(-k(t - t_0))}),$$

Where TL_t = total length at age t, L_∞ = the total asymptotic length, k = growth coefficient, t = age in y; and t_0 = a hypothetical age when TL is zero. These functions were log transformed and the slope and intercept were analyzed with analysis of covariance (ANCOVA). Third, whole otolith increment counts were compared to sectioned increment counts through estimations of mortality (Z) based on a weighted catch curve analysis. Mortalities were estimated using FAST.

Discriminate function analysis method

A discriminate function analysis was used to validate the accuracy of whole otolith reading for younger age red snapper (age-0, 1, and 2). A previous study using a known age data set of hatchery otoliths classified the shape variables and otolith weight for age 0 to 2 (Beyer and Szedlmayer, 2010). In the present study, this shape analysis was applied to unknown young red snapper otoliths to estimate ages. A discriminate function analysis was then used to cross-validate whole otolith age estimates with shape analysis age estimates which generated a percent agreement between the two methods. All statistics were analyzed with SAS 9.2. (Cary, NC) unless otherwise noted.

RESULTS

Whole versus Sectioned Otolith Increment Counts

A total of 1758 red snapper were collected from December 2011 to October 2013. Among these, 15 fish were not aged: seven were eliminated because both otoliths were broken during removal, three could not be agreed upon, and five were eliminated due to errors in labeling or other difficulties. There were 709 otoliths selected for sectioning. Among these, 694 were successfully sectioned and counted. An example of a whole and sectioned otolith, each with 4 increments, is shown in Fig. 2.

The average percent error (APE) quantifies the disagreement between readers as described by Beamish and Fournier (1981). Comparisons within methods showed an APE = 9.2 % for all whole otoliths, and an APE = 4.6 % for sectioned otoliths. Comparisons across methods showed an APE = 3.2 % between the sectioned and the adjusted count method.

Overall, 184 out of 694 (27%) whole otolith increment counts did not agree with their sectioned increment count. This 27 % disagreement was based on all sectioned otoliths, including opaque otoliths (declared unreadable) and the larger, older fish (> 6 increments). When the unreadable and larger otoliths were eliminated, disagreements were reduced to 18%. Among the remaining disagreements (n = 98), 93% differed by one increment, 5% differed by two increments, and 2% differed by three increments. The mean difference between sectioned otolith increment counts and whole otolith increment counts for red snapper was < 1 increment until fish reached the 800 mm size class (Fig. 3).

For all otoliths, the relation of whole increment counts to sectioned increment counts showed a slope = 0.85 (linear regression: $n = 694$, $R^2 = 0.91$, $p < 0.01$; Fig. 4). The adjusted method increased the slope to 0.89, but the slope was still significantly different from $b = 1$ ($n = 539$, $R^2 = 0.92$, $p < 0.01$; Fig. 5). However, the mean difference between sectioned otolith increment counts and the adjusted method was < 0.2 for all size classes (Fig. 6).

Von Bertalanffy growth curves showed significant differences in slope between whole and sectioned otolith methods (ANCOVA $F_{1,196} = 4.15$, $p = 0.04$). However, the sectioned growth curve was indistinguishable from the adjusted growth curve (Fig. 7). No significant differences were detected between slopes (ANCOVA $F_{1,196} = 0.14$, $p = 0.71$; Power = 0.89) or among the y-intercepts (ANCOVA $F_{1,197} = 0.01$; $p = 0.96$; Power = 0.88). The model parameters L_∞, k, and t_0 are shown in Table 2.

Mortality estimates were significantly different between whole increment counts and adjusted method when all fish were included ($n = 1743$, ANCOVA, $F_{1,140} = 110.23$, $p < 0.01$). A weighted catch curve regression estimated mortality for whole increment counts at $Z = 0.62$, while the adjusted method estimated $Z = 0.50$. Mortality estimates for randomly selected otoliths were not significantly different between sectioned and the adjusted method ($n = 694$, ANCOVA, $F_{1,140} = 0.36$, $p = 0.548$, power > 0.99). For randomly selected sectioned otoliths, mortality ($Z = 0.35$) was similar to the adjusted method ($Z = 0.36$). In contrast, mortality estimates of randomly selected otoliths were significantly different between sectioned (Z = 0.35) and whole otoliths (Z = 0.29) (n = 694, ANCOVA, $F_{1, 140} = 18.26$, $p < 0.01$).

Marginal Increment Analysis and OTC

There were 694 sectioned otoliths used for the marginal increment analysis. Every month of the year was sampled except February, because poor weather prevented offshore sampling during this month. Opaque zone formation mostly (93 %) occurred from May through September with a peak in July. A low percentage (7 %) showed formation in March, April, and October. No otoliths showed opaque zone formation from November through January (Fig. 8).

In 2012 and 2013, there were 17 recaptures of OTC marked fish. One fish did not have a mark on the otolith or scale and was discarded from further analyses. Most likely the returned fish was not the actual tagged fish. At times the Floy and internal tags can be separated from the fish by the fisherman, and the fish becomes indistinct from the rest of the catch. The assumption of one increment per year was verified by the other 16 otoliths. One fish remained at liberty for 6.2 y and displayed 6 increments after the OTC mark.

All captive fish showed a distinct OTC mark. The captive fish that were marked twice displayed OTC absorption on the otolith in < 54 d. In addition, a marked fish that was quickly (41 d) recaptured in the field displayed a clear OTC mark. In a study defining OTC absorption rate, 100 % of marked fish displayed an OTC mark within 24 h (Campana and Neilson, 1982).

Most (69 %, 11 of 16) OTC marked recaptures supported a summer to fall opaque zone formation. Six showed opaque increments formed from June through December, and five showed translucent zones from January through April. A few (19 %, 3 of 16) supported an early spring opaque formation in April, and two showed translucent zones in July and August (opaque zone timing indeterminate).

The mean ± SD otolith growth rate for fish held in the laboratory was 0.77 ± 0.14 mm y^{-1} and 0.36 ± 0.08 mm y^{-1} for OTC marked fish at liberty. For comparison, the otolith growth in mm y^{-1} was plotted against the change in SL mm y^{-1} for captive fish and for fish at liberty (Fig. 9). Otolith growth was evident for all fish even with negative growth of the standard length. The negative SL growth in captive fish occurred in fish that died prior to the end of laboratory study.

Shape Analysis

The discriminate analysis showed an 89% agreement between shape analysis derived ages and whole otolith count derived ages (Table 3). Percent agreements analyzed for each age class was 100% agreement for age-0, an 80 % agreement for age-1, and an 88 % agreement for age-2.

DISCUSSION

Whole versus Sectioned Otolith Increment Counts

Whole otolith increment counts were similar to sectioned otolith increment counts. Once the variance caused by larger fish and opaque otoliths were removed, the agreement between the two methods reached 82%. The majority of the discrepancies (93%) differed by one increment. For the most part these differences resulted from difficulty with ageing of red snapper near the core and identification of the first annulus. The first annulus can be adjacent to the core making the core appear relatively large and masking the presence of the annulus. Two studies described the first increment of red snapper as broad and diffuse with a variation in opaqueness and position (Wilson and Nieland, 2001; Allman et al., 2005). Other *Lutjanus* spp. (n = 11, from Australia) have also displayed the same difficulty in interpretation of the first annulus which caused higher estimates in age (Cappo et al., 2000). Another difficulty with the core is that it can also consist of multiple increments that are actually false annuli. This same pattern of varying opaqueness was also noted in Atlantic croaker (*Micropogonias undulatus*) (Barbieri et al., 1994). Another difficulty with ageing of red snapper is distinguishing the presence of an opaque increment on the marginal edge especially towards the end of opaque increment formation (Allman et al, 2005). This problem has also been described in silver seabream, *Pagrus auratus,* (Francis et al., 1992), and *Lutjanus* spp. (from Australia) (Cappo et al., 2000).

The linear regression of whole increment counts on sectioned increment counts shows that as fish age the variance increased. For example, whole otoliths with two increments ranged between one and three increments in sectioned counts, while whole otoliths with six increments ranged between four and nine increments in sectioned otoliths. An increase in variation with age between sectioned counts and whole increment counts was also evident in Atlantic herring (Peltonen et al., 2002). The mean difference plot also reveals that larger individuals display lower increment counts with whole otoliths as compared to sectioned otoliths (Fig. 3). This was most evident starting at 700 mm. As a fish grows older, the otolith becomes larger and more opaque obscuring the increments. For example, the oldest red snapper had 18 increments with sectioned otoliths, but only 12 increments with whole otolith counting. Age estimations of Atlantic herring produced a similar trend of under estimation of whole otolith counts as compared to sectioned otoliths in older fish (Peltonen et al., 2002). Yellowtail flounder also had similar age estimates from whole and sectioned otoliths up to age 7, while older fish were underestimated by whole increment counts (Dwyer et al., 2003). Furthermore, whole increment counts for Pacific red snapper were similar to sectioned increment counts up to a certain size (> 500 mm) after which they also underestimated counts (Rocha-Olivares, 1998). This increased difference in larger, older otoliths in the present study led to the development of the adjusted method. This method used whole otolith counts for fish with < 7 increments and sectioned counts for fish with > 6 increments and improved the slope between sectioned and whole otoliths from 0.85 to 0.89.

Many ageing studies are interested in growth rate comparisons and not the individual age of a particular fish (Powers, 1983; Worthington et al., 1995; Campana, 2001; Jackson, 2007; Doering-Arjes et al., 2008). Thus, the comparisons of von Bertalanffy growth curves and mortality estimates are important in the evaluation of the different count methods. The growth curves show a rapid rate of growth until about age-10 after which the rate decreases and levels out as it approaches L_∞. Similar growth curves were fitted in previous studies for red snapper (Szedlmayer and Shipp, 1994; Patterson et al., 2001; Wilson and Nieland, 2001; Fig. 10). The whole otolith method estimated a greater L_∞ than the section or adjusted methods. This arises because larger whole otoliths were consistently under-aged which was most extreme for fish > age-10. In contrast, the adjusted method curve was indistinguishable from the sectioned otolith growth curve (Fig. 7). In the present study, the majority of fish (97%) were aged whole because they had < 7 increments. This is typical of many red snapper ageing studies (Patterson et. al., 2001; Wilson and Nieland, 2001; Fischer et. al., 2004; White and Palmer, 2004). Thus, using the adjusted method could save a great deal of time and effort in future red snapper ageing studies.

Mortality is also an important estimate used in describing and managing populations. An underestimation of age leads to an overestimate of mortality (Z) and increase in growth allowing for a potential overexploitation of a population (Campana, 2001). In the present study the adjusted ageing method of all fish (n = 1743) estimated Z = 0.50 which was consistent with past estimates of Z = 0.54 (Gitschlag, et. al, 2003; Szedlmayer 2007). The mortality estimates derived from the randomly selected sectioned (n = 694, Z = 0.35) and adjusted method (Z = 0.36) were < estimates from all otoliths (Z = 0.50) due to the stratified random sampling that skewed the catch curves to the older age fish and caused lower estimates. Importantly, Z estimates were not significantly different between sectioned and the adjusted method for the randomly selected otoliths suggesting that mortality rates for the adjusted method were accurate. Moreover, two different studies found that increasing sample size was more important in reducing error in mortality estimates than an alternative more precise method that reduced sample size (Powers, 1983; Worthington et al., 1995).

Whole ageing substantially reduces the effort and time spent ageing red snapper. During this study about 1 h was needed to section 8 otoliths depending on their size. This 1 h period did not include the imaging or ageing aspect of the process. In that same 1 h period, 30 whole otoliths could be imaged and aged by one reader. This substantial savings in time and effort indicates that if whole otolith ageing can work it should be the preferred method in future studies.

Marginal Increment Analysis and OTC

Marginal Increment Analysis showed a peak occurrence of opaque zone formation from June through August with a maximum of 63% during July. This time period of peak opaque formation was not as sharp as shown in other studies that reported percentages. Allman et al. (2005) showed a peak of 85% in May, and Wilson and Nieland (2001) also showed a peak in May near 100%. It is difficult to explain these differences, but they may simply be due to annual environmental fluctuations. The present finding of opaque zone formation during the summer months may be explained by linkages to reproductive stress. Spawning in red snapper occurs from April through October peaking in June and July (Bradley and Bryan, 1975; Collins et al., 2001, Fitzhugh et al., 2004). This link to spawning was supported by Campana and Neilson (1985) which described otolith increment formation as tightly linked to endocrine rhythm. The present timing of opaque formation differs from some previous studies using similar MIA methods on red snapper (Patterson et al., 2001; Wilson and Nieland, 2001), but was supported by other studies that suggested later formation of opaque zone formation (Nelson and Manooch, 1982; Goodyear, 1995; White and Palmer, 2004; Allman et al. 2005). In addition, other species have shown similar opaque zone formation related to spawning season, e.g., Vermilion Snapper (*Rhomboplites aurorubens*) (Zhao et al., 1997) and Yellowtail Snapper (*Ocyurus chrysurus*) (Johnson, 1983).

The OTC method is more accurate compared to MIA due to difficulty in defining opaque zones on the edge of sectioned otoliths (Campana, 2001). For the fish at liberty, 68 % of the OTC otoliths supported summer to fall opaque zone formation similar to the present MIA results and to a previous OTC mark recapture study (Szedlmayer and Beyer, 2011). This continuation into fall is more likely a better estimate because the OTC method is more accurate in defining the end of opaque formation. In red snapper (Allman et al., 2005) and other *Lutjanus* species (Cappo et al., 2000), identifying the edge was found to be most ambiguous soon after the completion of opaque increment formation.

Shape Analysis

The 89% agreement rate between shape analysis and whole otolith counts further supports the use of whole otolith counting methods. This classification success was similar to comparisons of the same shape analysis method with known age hatchery fish (87 %; Beyer and Szedlmayer, 2010). Otolith growth continued independent of somatic growth further supporting the use of shape analysis. Shape analysis is an objective, unbiased, and independent method that provides a permanent record (i.e., results will not change due to reader variance). In addition, it is a faster method than whole increment counts or the section method. Similarly, in gray angelfish (*Pomocanthus arcuatus*) otolith thickness was found to be effective in determining age and a useful method in ageing a large quantity of fish in a more economic manner (Steward et al., 2009). Atlantic cod (*Gadus morhua*) otoliths were also successfully aged using shape analysis and otolith weight; a method deemed cost-effective by the authors (Doering-Arjes et al., 2008).

In contrast, a major disadvantage is that a source of known age fish are needed for each age class as a basis for the discriminate function (Doering-Arjes et al., 2008; Szedlmayer and Beyer, 2010). Also, the startup costs are more expensive than whole otolith ageing because of the need for an imaging system and software. These additional costs might be offset over time because the method is more efficient and requires fewer individuals to complete the ageing process, but, clearly, whole otolith counts are fast and relatively inexpensive and may be the method of choice for many laboratories (Rocha-Olivares, 1998; Peltonen et. al., 2002; Dwyer et. al., 2003).

Conclusion

Ageing is an important aspect of fisheries research. Population parameters like growth rate, mortality rate, age at maturity, and maximum age are all important in fitting predicative models used to make management decisions about the fishery. Many marine and coastal fishes are affected by exploitation either through direct fishing activities or as by-catch. Effective monitoring of these fish stocks requires age and growth determination studies (including aforementioned otolith analyses), which aids to determine if a stock is remaining constant, declining, or improving.

For red snapper, the labor intensive and costly method of sectioning otoliths has been a common process for decades. The adjusted method proposed in this study showed that increment counts derived from whole otoliths for ages < 7 combined with sectioned counts of ages > 6 provided an accurate method for ageing red snapper. This new method is preferred over previous methods that sectioned all age classes, because it greatly reduces the effort, time, and cost of ageing for this species. The accuracy of the method is also supported by the objective shape and weight analysis of age-0 to age-2 fish. The most ideal method may be a combination of shape analyses for the youngest ages, whole otolith increment counts for intermediate ages, and sectioned increment counts for older age fish. Future studies should consider implementing the adjusted method as well as shape analysis.

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Tables

Table 1. Shape variables used in the shape analysis.

Shape Variable	Description
Area	Area of object
Perimeter	Length of the outline of object
Aspect ratio	Length major axis/length minor axis (of an ellipse equivalent to object)
Rectangularity	Object area/area of enclosing box
Roundness	$Perimeter^2 / (4*\pi*area)$
Box x/y	Width/height (enclosing box)
Radius ratio	Max radius/min radius
Perimeter ratio	Convex portion of perimeter/actual perimeter

Table 2. Von Bertalanffy parameters for 4 ageing methods: 1) whole increment counts only (*n* = 1743), 2) adjusted method (*n* = 1743), 3) sectioned increment counts only (*n* = 694), 4) adjusted method (*n* = 694). The adjusted method refers to using whole otolith counts for readable otoliths with < 7 increments and sectioned counts for the larger, older fish (> 6 increments) and opaque otoliths.

Method	*n*	$L\infty$	k	t_0
Whole counts	1743	1019.21	0.165	-0.349
Adjusted method	1743	963.46	0.174	-0.402
Sectioned counts (randomly selected)	694	973.09	0.164	-0.544
Adjusted method (randomly selected)	694	963.67	0.173	-0.439

Table 3. Classified ages from the shape analysis compared to whole otolith counts. The total number of otoliths is followed by the percentage agreement for each age group. Classified age is the age generated by the discriminate function, and otolith counts are the ages generated from whole otolith readings.

	Shape Classified Age			
Otolith Counts	0	1	2	Total
0	117 (100)	0	0	
1	20 (7)	221 (80)	35 (13)	89%
2	0	18 (12)	136 (88)	

Figures

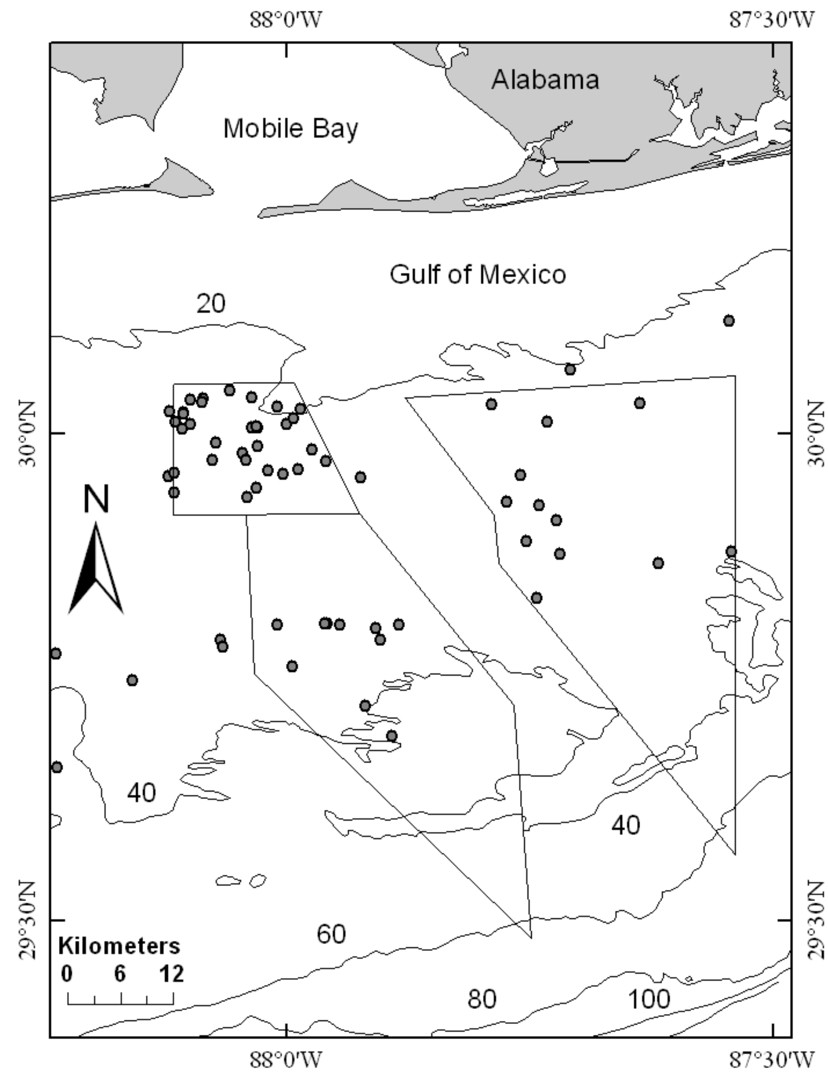

Fig. 1. Red snapper collection sites (n = 59) in the northern GOM. Each gray circle represents one site. Polygons are perimeters of the artificial reef permit zones. Depths are in meters.

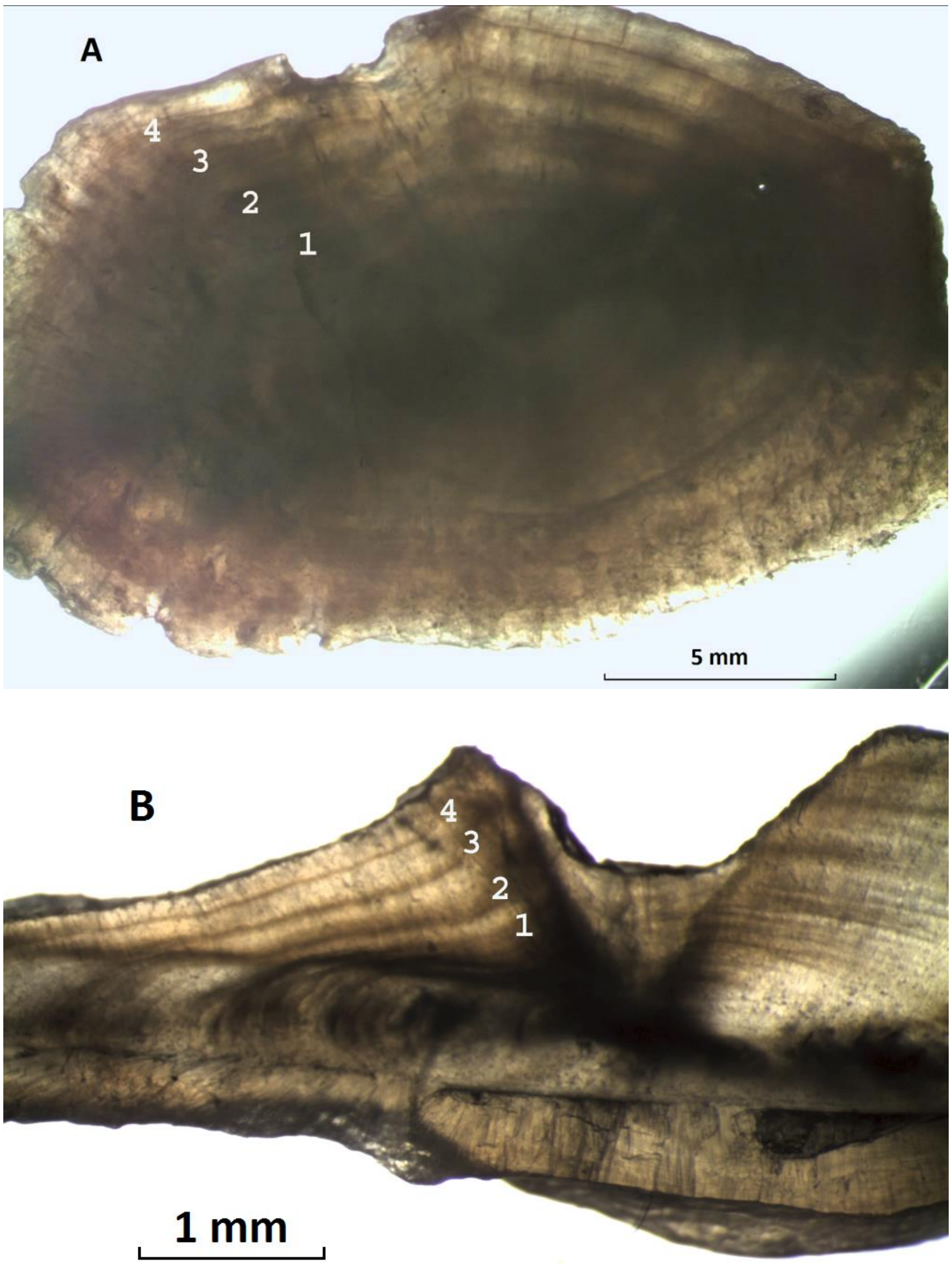

Fig. 2. Red snapper whole otolith (A) and the transverse section (B). Each method showed four increments.

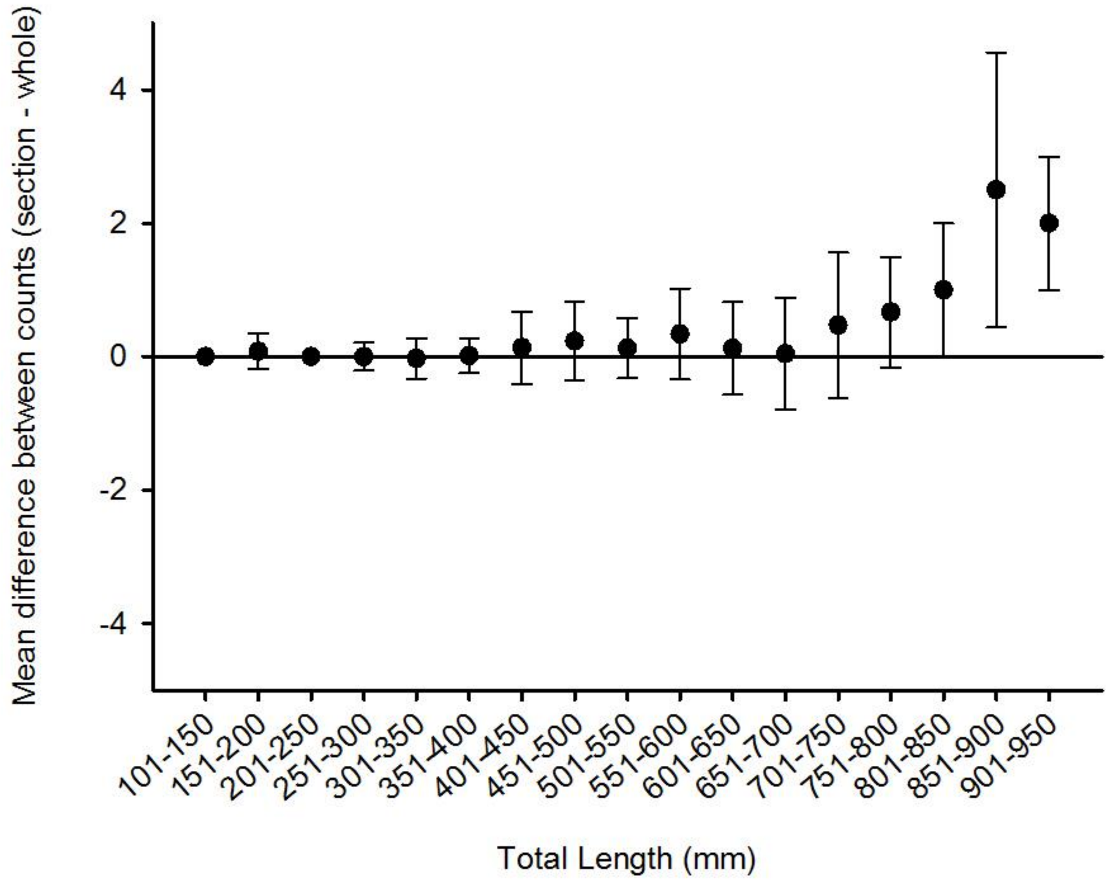

Fig. 3. Mean difference between sectioned and whole otolith counts for red snapper in 50 mm size class intervals. Error bars represent standard error.

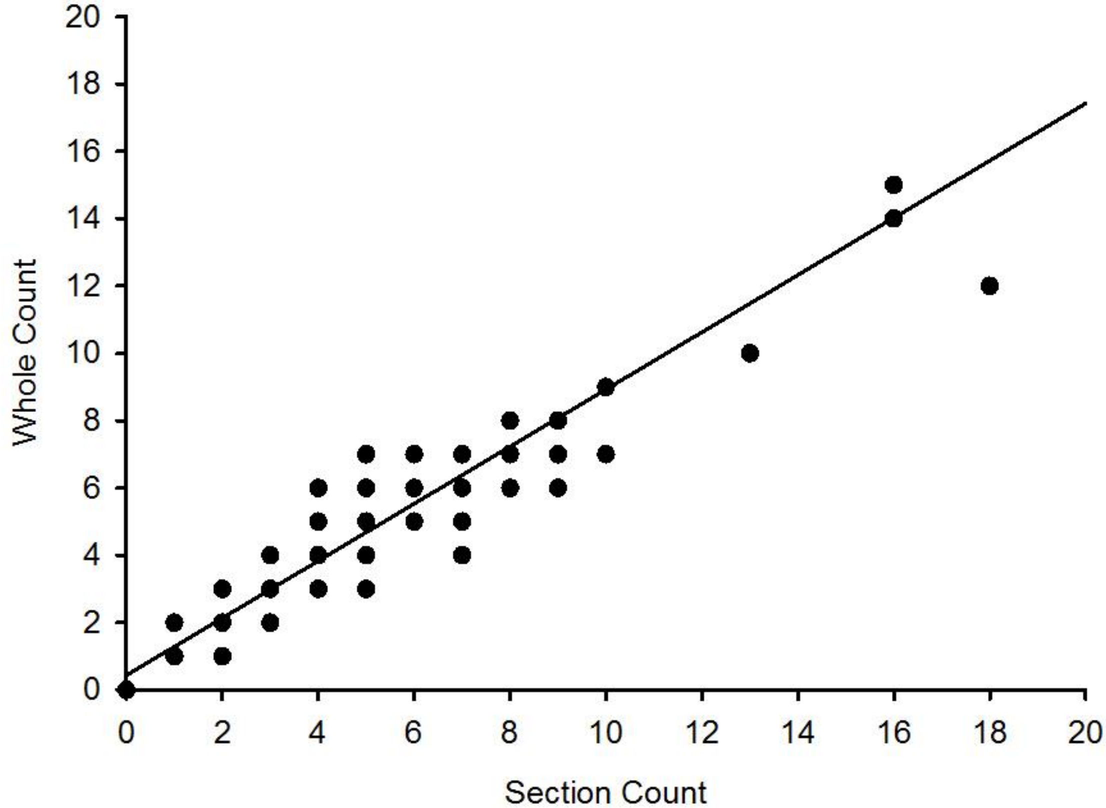

Fig. 4. Red snapper whole otolith increment counts on sectioned otolith increment counts. Linear regression = solid line (n = 694, slope = 0.85, R^2 = 0.91, $p < 0.01$).

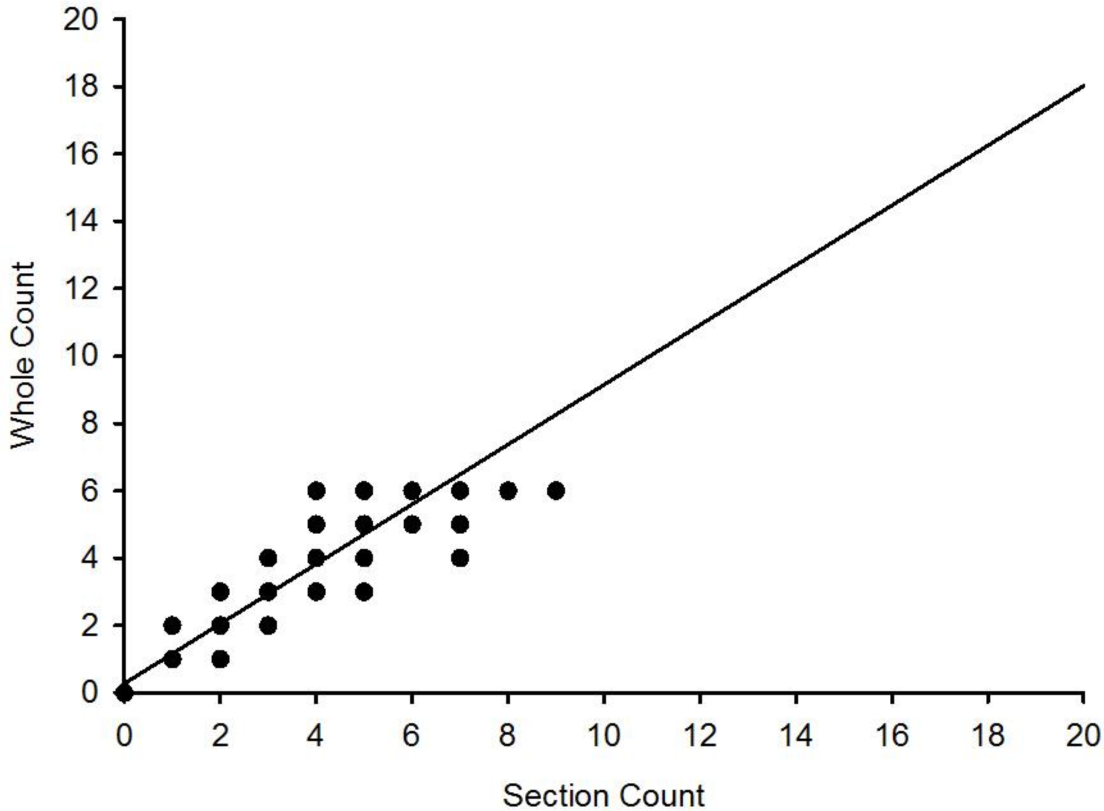

Fig. 5. Red snapper whole otolith increment counts on sectioned otolith increment counts after removal of whole increment counts > 6 increments and opaque otoliths. Linear regression = the solid line (n = 539, slope = 0.89, R^2 = 0.92, and $p < 0.01$).

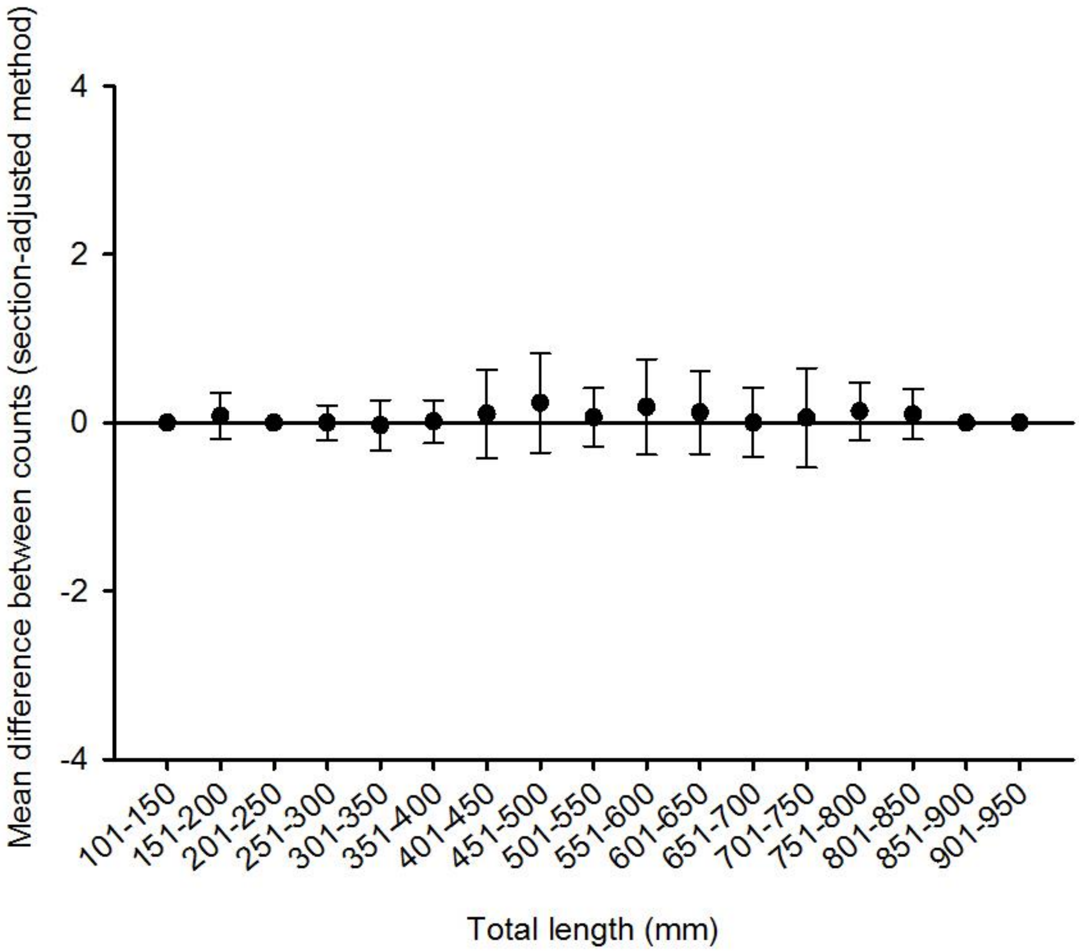

Fig. 6. Mean difference between sectioned and adjusted method counts for red snapper in 50 mm size class intervals. Error bars represent standard error.

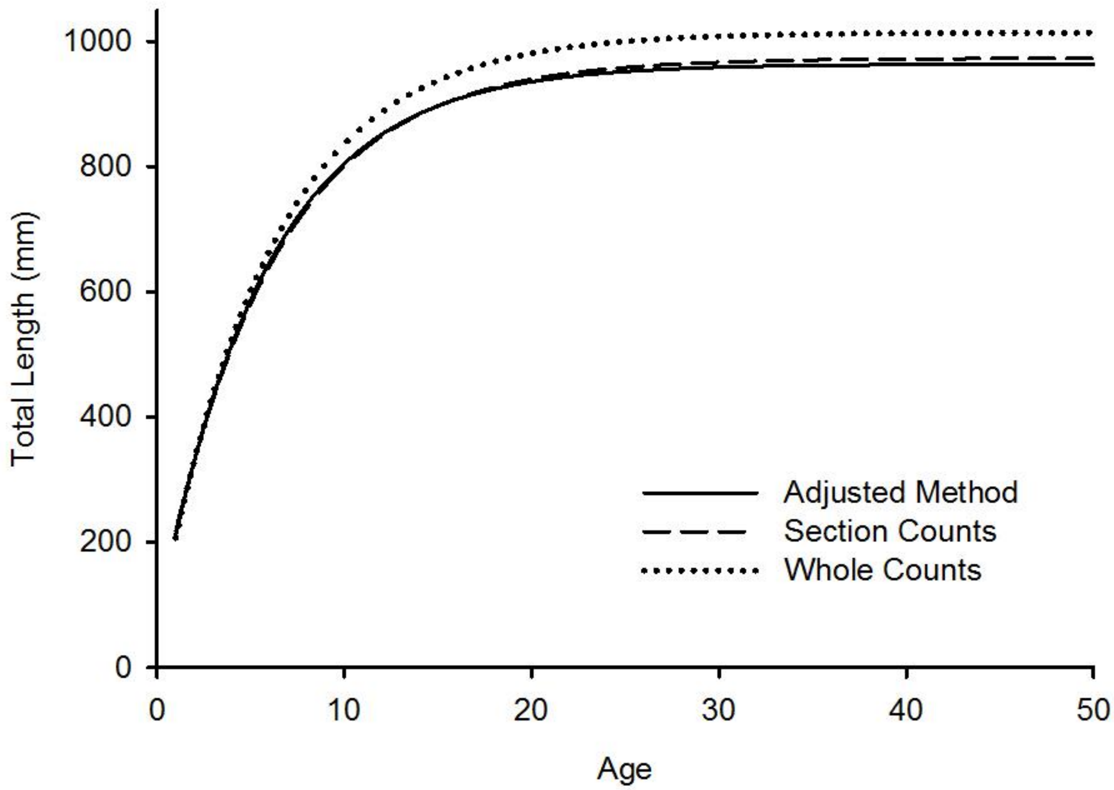

Fig. 7. Von Bertalanffy growth curves for adjusted, section, and whole otolith count methods (n = 694).

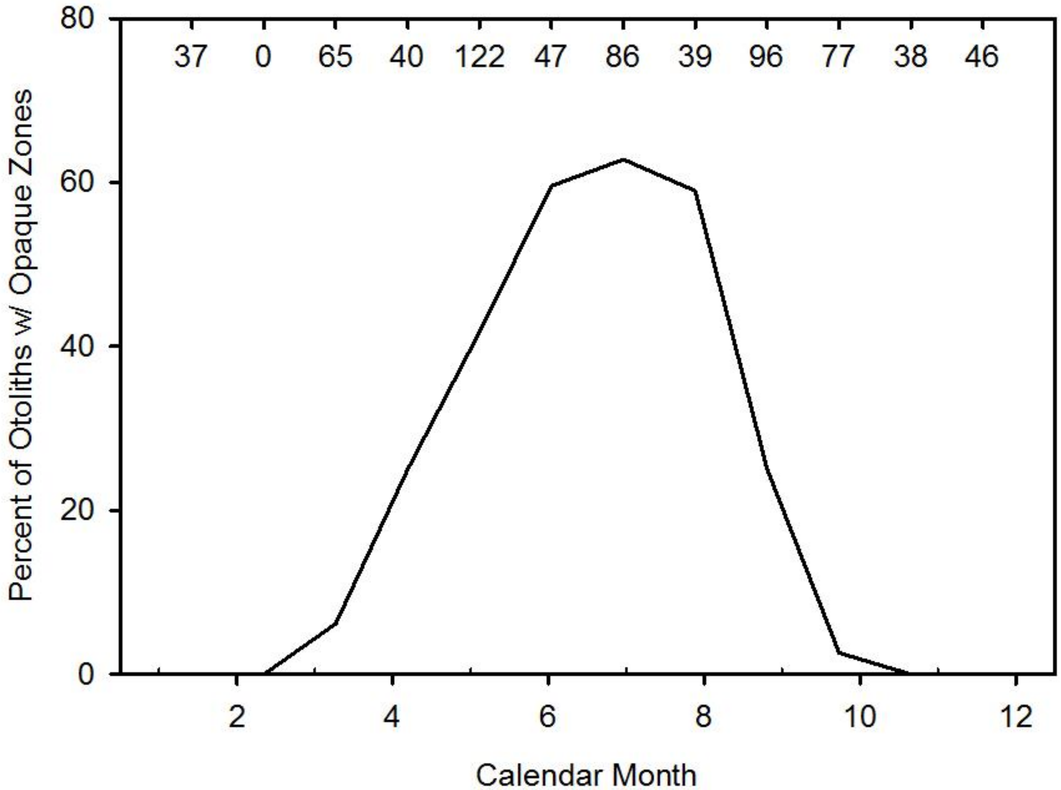

Fig. 8. Marginal increment analysis of red snapper sectioned otoliths. The line graph represents the percent of opaque margins for each month. Total number of sectioned otolith margins scored each month is shown on the top axis.

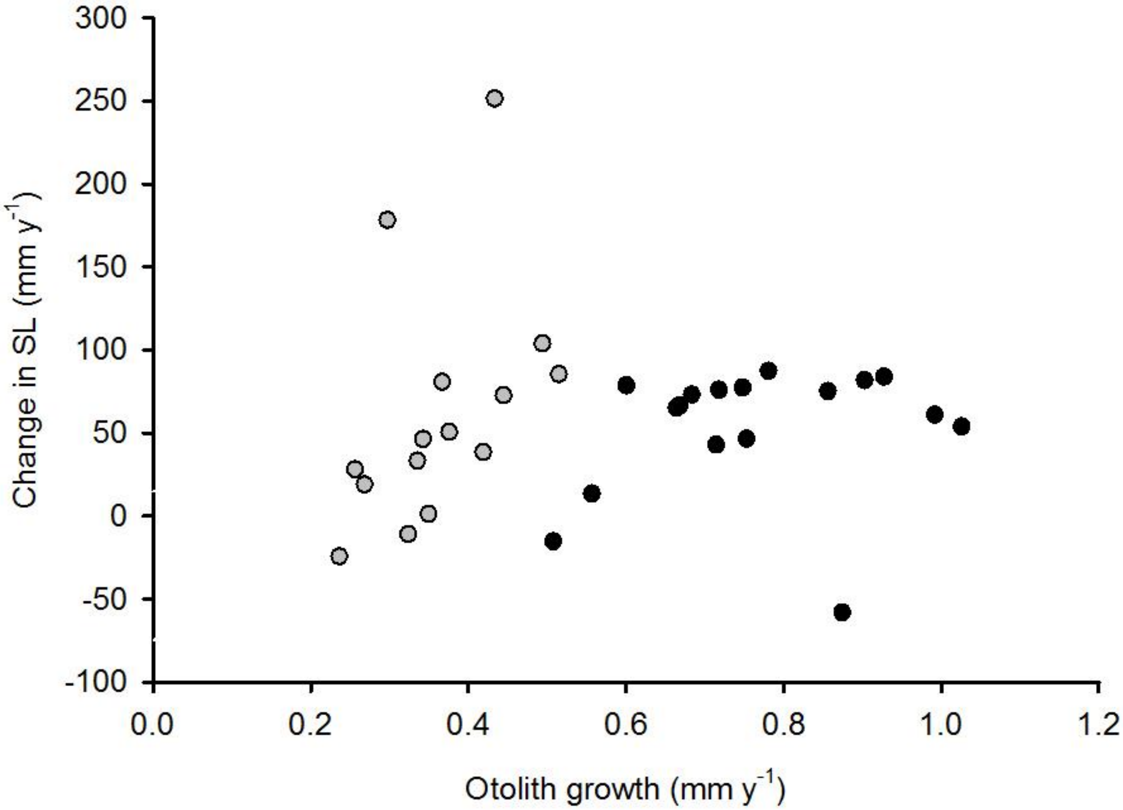

Fig. 9. Red snapper otolith growth (mm y^{-1}) compared to somatic growth rates (SL mm y^{-1}) for captive fish (black dots) and tagged fish at liberty (gray dots).

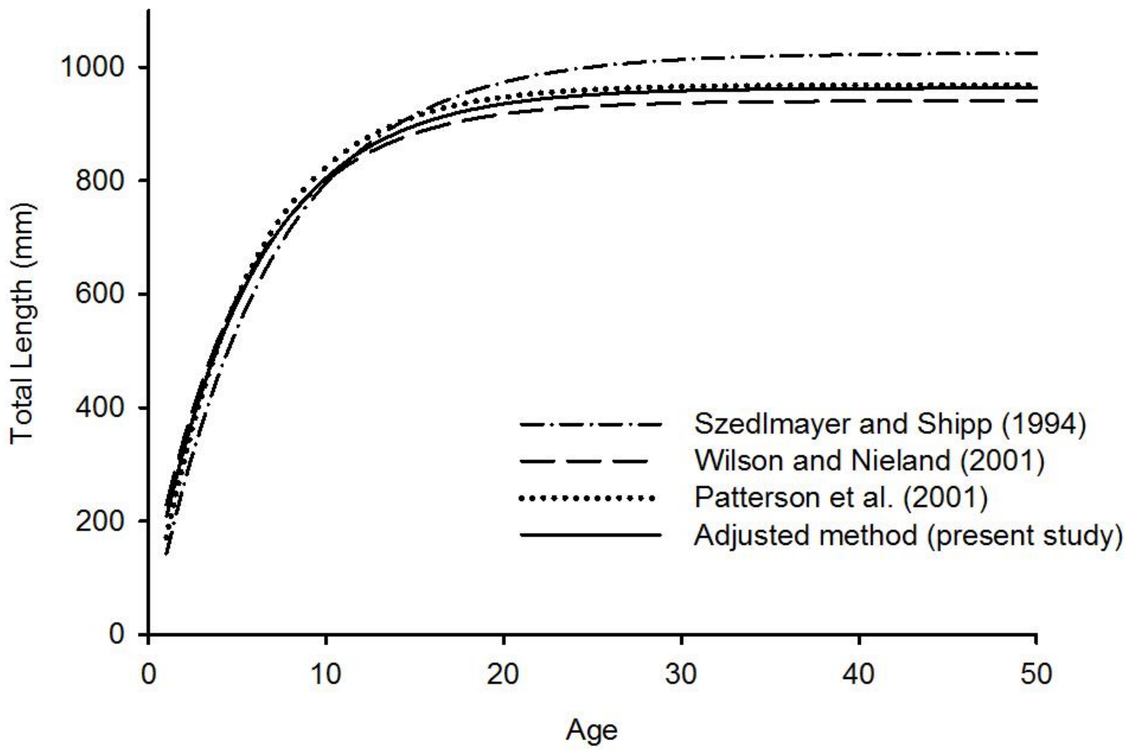

Fig. 10. Comparison of red snapper von Bertalanffy growth curves among previous studies and the present study.