AGE DETERMINATION THROUGH SHAPE ANALYSIS AND VALIDATION OF OTOLITH ANNULAR INCREMENTS IN RED SNAPPER, *LUTJANUS CAMPECHANUS*

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AGE DETERMINATION THROUGH SHAPE ANALYSIS AND VALIDATION OF OTOLITH ANNULAR INCREMENTS IN RED SNAPPER, *LUTJANUS CAMPECHANUS*

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THESIS ABSTRACT

AGE DETERMINATION THROUGH SHAPE ANALYSIS AND VALIDATION OF OTOLITH ANNULAR INCREMENTS IN RED SNAPPER, *LUTJANUS CAMPECHANUS*

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The periodicity and timing of otolith growth rings in red snapper was examined through a mark-and-recapture study and with known-age, hatchery-reared fish. In 2005 through 2008, red snapper (n = 265) were caught hook-and-line, injected with oxytetracycline, and released 15 to 20 km south of Dauphin Island, Alabama. Fish were recaptured approximately one year after release (n = 6). From recaptured fish, sagittal otoliths were sectioned and the number of opaque growth rings past the OTC mark was compared to time at liberty of the fish. Otoliths from hatchery-reared, age-2 red snapper were sectioned and the number of growth rings visible on the otolith compared to known age. An annual periodicity of growth ring formation was shown in both adult and young red snapper. However, if the reading transect was slightly altered from a radius immediately next to the sulcus, false increments were detected and showed two rings formed during one year at liberty. The timing of annulus formation was in the summer to early fall months which correlated with the summer spawning season of red snapper. This was in contrast to previous literature that indicated late winter mark formation. All adult recaptured fish were less than six years of age and questions remain as to the validity of annual ring formation in older red snapper.

In younger (age-0, 1 and 2) hatchery-reared red snapper, a new method of age determination was attempted independent of counting otolith growth rings. Otolith shape analysis was applied to whole sagittal otoliths and morphological shape indices were able to distinguish among age-0, age-1, and age-2 otoliths. Significant differences in the aspect ratio, box x/y, and radius ratio showed juvenile red snapper otoliths grew faster along the anterior-posterior axis compared to the dorsal-ventral axis. A discriminant function analysis and cross-validation showed an age classification success of 70% based on shape variables alone. The addition of otolith weight to the discriminant function increased classification success to 93%. This method is especially significant considering that age determination in young red snapper by ring counts is problematic, especially with identification of the first opaque ring.

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INTRODUCTION

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Red snapper, *Lutjanus campechanus* (Poey), is an important marine fish species to both the sport and commercial fisheries in the northern Gulf of Mexico. Accurate biological data on red snapper is critical for the development of management strategies to regulate the current fishery. Models of sustainable yield and stock assessment by virtual population assessment (VPA) rely heavily on mortality data, which are typically derived from age frequency distributions or catch curves. Therefore, accurate age determination is vital to understanding the life history and biology of this species, and important for any management strategy (Beamish & McFarlane 1987).

Numerous methods are used to age fish, and most rely on the counting of growth increments on scales, spines, and otoliths. One complete otolith growth increment is composed of one opaque ring and one translucent ring. Age estimation typically occurs by counting the number of opaque rings viewed on the otolith, with consideration of the date of capture. Opaque rings appear visually as thinner, dark bands in sectioned otoliths when viewed with transmitted light, and indicate periods of slower growth in the fish. Translucent rings are comparatively wider bands appearing lighter when viewed with transmitted light and indicate periods of faster growth. Counting the number of opaque rings in sectioned otoliths is the presently accepted method to age red snapper (Nelson & Manooch 1982, Szedlmayer & Shipp 1994, Patterson, Cowan, Wilson & Shipp 2001, Wilson & Nieland 2001, Rooker, Landry, Geary & Harper 2004, Allman, Fitzhugh, Starzinger & Farsky 2005). Even though age determination with sectioned otoliths is widely used for red snapper, it is critical to validate all ageing methods (Beamish & McFarlane 1983, Campana 2001). In addition, age must be validated for all age classes of a fish. Only 17 out of 500 studies involving fish age estimates published between 1907 and 1980 validated all age classes. A misunderstanding of life history and subsequent mismanagement of Pacific ocean perch *Sebastes alutus* (Gilbert)*,* was attributed to lack of age validation in older fish (Beamish & McFarlane 1983). To date, direct validation of age determination methods for red snapper is lacking, but indirect methods have indicated that red snapper were accruing one full opaque and translucent ring per year, at least for the first ten years. However, after the first ten years, age validation is particularly difficult due to low sample sizes and increasingly small growth increments at the edge of the otolith (Nelson & Manooch 1982). This leaves substantial potential for error, considering that red snapper are believed to live much longer than 10 years. For example, studies that used sectioned otoliths to count growth rings suggested that red snapper can routinely reach ages of 40 or 50 years (Szedlmayer & Shipp 1994, Patterson *et al.* 2001, Wilson & Nieland 2001).

Three indirect methods have been applied in attempt to validate annual formation of growth increments in red snapper otoliths: marginal increment analysis, radiometric testing, and bomb radiocarbon dating. The most widely used method is marginal increment analysis, which begins with the sampling of fish throughout the year, then plotting the condition (opaque verses translucent) and width of the outermost increment by month of capture. With this method, Wilson and Nieland (2001) indicated opaque ring formation in red snapper otoliths was between the months of December and June, and a translucent zone formed between the months of June and November. However, edge condition analysis also showed that 10% of fish sampled in the month of September had an opaque edge. Patterson *et al.* (2001) also indicated that opaque rings in red snapper otoliths were formed in winter (January through May), but were unable to validate the age of red snapper past 8 years due to small sample sizes of older fish. In contrast, Allman *et al.* (2005) suggested opaque ring formation from April to August, peaking in May. In that study, image analysis and light intensity curves were used to aid in the identification of opaque rings. Presently, ageing methods of red snapper assume that the annulus is formed in the winter and the fish is advanced one year of age after January 1 (Patterson *et al.* 2001, Wilson & Nieland 2001).

A second age validation method applied to red snapper was radiometric testing, which measured the chemical disequilibria of ^{210}Pb (lead) to ^{226}Ra (radium) within the otolith. The accuracy of radiometric ageing is based on the validity of three assumptions: (1) there is a constant uptake of ^{226}Ra in the otolith throughout the life of the fish, (2) the otolith is a chemically closed system, and (3) the initial ratio of ^{210}Pb to ^{226}Ra is zero. Baker, Wilson & VanGent (2001) showed that some of those assumptions were violated in red snapper otoliths. Furthermore, radiometric ageing overestimated ages determined by sectioned otoliths in orange roughy, *Hoplostethus atlanticus* (Collett) (Smith, Fenton, Robertson & Short 1995) and was also shown to be inaccurate in elasmobranchs (Welden, Cailliet & Flegal 1987). Campana (2001) further suggested that radiometric age determination was better suited for discerning short-lived from long-lived species, and not for validating the periodicity of annual growth in fish otoliths.

Bomb radiocarbon dating was a third method of indirect age validation applied to red snapper. Bomb radiocarbon dating exploits a jump in levels of oceanic ^{14}C during the 1950's and 1960's due to atmospheric nuclear bomb testing. This is detectable in the hard structures of fish and coral, and is used to verify fish ages when compared to otolith growth ring counts. This method of age validation requires fish specimens with hatch dates during or before the nuclear bomb testing period. Baker and Wilson (2001) were the first to apply bomb radiocarbon dating to fish from the northern Gulf of Mexico, and estimated longevity of red snapper with this method. That study indicated that red snapper could reach at least 30 to 38 years of age based on the bomb radiocarbon dating method. For example, the oldest fish in that study was caught in 1998 and the otolith core did not contain bomb radiocarbon, suggesting that the fish hatched sometime before 1960, the year of first detectable ^{14}C in otoliths, and was therefore at least 38 years old. However, even though bomb radiocarbon dating indicated that this particular fish was at least 38 years old, the fish was aged at 55 years based on the number of opaque rings viewed on the sectioned otolith. Bomb radiocarbon dating estimates longevity of a fish, but the method does not actually validate annual periodicity of ring formation in otoliths. Although the above methods all indicated that red snapper reach ages above the 16 year maximum first suggested by Nelson and Manooch (1982), questions remain concerning the validity of the maximum age estimation and the periodicity of growth ring formation in red snapper otoliths.

Variance from annual growth ring periodicity in fish otoliths has been documented in some fish, for example, two opaque rings were formed per year in gray triggerfish, *Balistes capriscus* (Gmelin), in the northeastern Gulf of Mexico (Ingram 2001), and for the same species off the coast of Sao Paulo, Brazil (Bernardes 2002). Marginal increment analysis of sectioned dorsal spines was used in age and growth studies of gray triggerfish that showed distinct slow growth rings in both the winter and the summer months. These two periods of slow growth per year within the otoliths corresponded with minimum water temperatures along with reduced food supply in the winter, and with spawning in the summer months (Bernardes 2002, Ingram 2001). During the spawning season, both female and male gray triggerfish invest considerable energy into reproduction through elaborate displays of courtship behavior, nest building and biparental care of the eggs (MacKichan & Szedlmayer 2007). Adult tilapia, *Oreochromis niloticus* (Linnaeus), from Lake Awassa in the Ethiopian Rift Valley also showed two opaque rings formed per year (Admassu & Casselman 2000). In that study, the formation of otolith growth rings was correlated with minimum water temperature in the lake and weakened condition of the fish during spawning, along with a reduction of the quantity and quality of food during the spawning season.

Red snapper have an extended spawning season from May through October with large females spawning more frequently compared to smaller females (Collins, Fitzhugh, Mourand, Lombardi, Walling, Fable, Burnett & Allman 2001). Although opaque ring formation in red snapper otoliths was suggested for winter to early spring months by indirect methods (Patterson *et al.* 2001, Wilson & Nieland 2001), there is a possibility that red snapper may accrue an opaque ring during the summer spawning period as well, especially if a protracted spawning season results in greater amounts of energy diverted to reproductive growth rather than to somatic growth. Also, changes in the pattern of otolith growth increment formation may be shown as a fish ages. For example, younger fish may form annual growth rings but older fish could show greater than annual growth ring formation (i.e. 2 rings per year), especially if older fish are investing greater energy into reproductive output through multiple spawning events and higher batch fecundity. The possible appearance of opaque rings during the spawning season may suggest that two opaque rings could be formed per year in older fish, thus leading to incorrect ageing by current methods.

The consequences of incorrect age determination of red snapper were first pointed out by Rothschild, Sharov & Bobyrev (1997) in their review of the red snapper management plan. In particular, some life history aspects of red snapper appeared to differ from typical long-lived marine species, such as their rapid growth and early maturity, which were more consistent with short-lived species. Red snapper in the Gulf of Mexico mature as early as age two and show rapid growth within the first ten years of life (Collins *et al.* 2001, Woods, Fischer, Cowan & Nieland 2003). Fecundity increases with age of the fish. In one study, batch fecundity ranged from 13 eggs in a 4 year old red snapper to 3.4 million eggs in an 11 year old fish (Collins *et al.* 2001). Red snapper have a spawning season ranging from May to October and spawning frequency was shown to be 50% greater in older fish as compared to 3, 4, or 5 year old females (Collins *et al.* 2001). The inconsistency between red snapper life history and that of other long-lived species indicated a critical need for a direct validation method of annual growth ring formation in otoliths of red snapper, especially for older, larger fish.

The best direct method to validate annual otolith increment formation is through a mark-and-recapture study in which otoliths are marked with the antibiotic oxytetracycline (OTC), or by counting growth rings in otoliths from known-age fish. Oxytetracyline marking for the purpose of validating otolith annual increments has been applied to many fish species, including yellowtail rock fish, *Sebastes flavidus* (Ayres) (Leaman & Nagtegaal 1987), orange roughy, *H. atlanticus* (Smith *et al.* 1995), tropical gobies (Gobiidae) (Hernaman, Munday & Schläppy 2000) and sablefish, *Anoplopoma fimbria* (Pallas) (Beamish & McFarlane 2000). In the present study OTC mark-and-recapture methods were applied to red snapper with the objective of validating annual increment formation in otoliths. Growth rings were also counted on sectioned otoliths from known-age (age-2), hatchery-reared red snapper.

Otolith shape analysis

In addition to problems with ageing older fish, there has been difficulty with age determination of juvenile red snapper, particularly age-0 through age-2 fish. An average of 22.1 million juvenile red snapper per year were taken as shrimp trawl by-catch between the years of 1987 and 1995 (Ortiz, Legault & Ehrhardt 2000). Accurate age determination of red snapper from shrimp trawl by-catch is critical for population assessment models and management. The present method of age determination in young red snapper is by reading growth increments on sectioned otoliths. This method can be inaccurate due to reader error particularly with identification of the first growth ring (Holt & Arnold 1982, Wilson & Nieland 2001, Rooker *et al.* 2004, Allman *et al.* 2005, Morales-Nin & Panfili 2005). Therefore, newer, more accurate methods of age determination in young red snapper are needed.

Otolith shape analysis is a method previously used to identify fish stocks and separate fish species but may be applied for ageing purposes. For example, otolith shape analysis was applied in the identification and discrimination of Atlantic cod, *Gadus morhua* (Linnaeus) into regional spawning stocks based on differences in otolith morphometric variables and Fourier descriptors (Cardinale, Doering-Arjes, Kastowsky & Mosegaard 2004). Otolith shape analysis also was used to separate Atlantic and Mediterranean stocks of comber, *Serranus cabrilla* (Linnaeus) (Tuset, Lozano, González, Pertusa & García-Díaz 2003). The Atlantic stock had irregular shaped otoliths compared to fish from the Mediterranean stock that had rounder otoliths.

For species identification, otolith shape analysis was applied to differentiate similar looking species, walleye pollock, *Theragra chalcogramma* (Pallas) and Arctic cod, *Boreogadus saida* (Lepechin) from the Bering Sea (Short, Gburski & Kimura 2006). Combinations of otolith characteristics were used in a discriminant function analysis, followed by a classification success matrix to assess which variables best distinguished between otoliths of the two species. The discriminant function that gave the best classification accuracy (99%) for a known-specimen mixed sample, included the variables otolith area, number of scallops, and fish length. The number of scallops was the best distinguishing variable, with otoliths from walleye pollock, *T. chalcogramma* having a greater number of scallops than Arctic cod, *B. saida*.

Applying otolith shape analysis for the purpose of ageing fish has not been tested. However, significant differences in otolith morphology among age classes of fish was shown for queen corvina, *Cynoscion albus* (Günther) (Mug-Villanueva, Gallucci & Lai 1994), long rough dab, *Hippoglossoides platessoides* (Fabricius) (Fossen, Albert & Nilssen 2003) and Atlantic cod, *G. morhua* (Galley, Wright & Gibb 2006). If otolith shape analysis can be used to age juvenile fish, it may eliminate a time-consuming process of cutting and polishing otoliths, and the subjective counting of growth rings.

In addition to shape variables, otolith weight has been a successful proxy for age. The otolith weight measure was shown to increase accuracy of proportions-at-age estimates over estimates derived from age-length keys for known-age *G. morhua* samples (Francis, Harley, Campana & Doering-Arjes 2005). Otolith weight was positively correlated with age in plaice, *Pleuronectes platessa* (Linnaeus) (Cardinale, Arrhenius & Johnsson 2000), lane snapper, *Lutjanus synagris* (Linnaeus) (Luckhurst, Dean & Reichert 2000) and emperor fish, *Lethrinus mahsena* (Forsskål) (Pilling, Grandcourt & Kirkwood 2003). Otolith weight in plaice, *P. platessa* was a better predictor of fish age compared to estimates based on fish length, and age structure estimated by otolith weight was shown to not significantly differ from age structure estimated by reading otolith increments (Cardinale *et al.* 2000).

Research objectives

The objectives of this study were threefold: 1) to directly validate annual periodicity of growth increment formation in otoliths of red snapper through a mark-and-recapture study, 2) to identify growth increments in known-age (age-2), hatchery-reared red snapper, and 3) to assess the use of otolith shape analysis for the discrimination of age-0, 1, and 2 known-age, hatchery-reared red snapper.

MATERIALS AND METHODS

Otolith annual increment validation

Mark-and-recapture - The study site for mark-and-recapture of wild adult red snapper was located approximately 20 to 40 km south of Mobile Bay, Alabama in an area of artificial reefs such as gas platforms, sunken liberty ships, army tanks, car bodies and pipelines, which all serve as habitat for red snapper (Szedlmayer and Shipp 1994). Natural reefs are rare in the northeastern Gulf of Mexico, making up only 3.3% of the bottom area from Pensacola, Florida to Pass Cavallo, Texas (Parker, Colby & Willis. 1983).

Red snapper were caught hook-and-line from artificial reef sites and anesthetized in a tricaine methanesulfonate (MS-222; 150 mg MS-222 L^{-1} seawater) bath for 3 min or until sedated. Standard length (mm), fork length (mm), and total length (mm) were recorded along with fish weight (kg). For visual identification, individually numbered Floy anchor tags were inserted into the peritoneal cavity of all fish. Red snapper were injected intramuscularly (epaxial muscle) with oxytetracycline dihydrate (OTC) at a dosage of 100 mg kg^{-1} body weight (Francis, Paul & Mulligan 1992). A reward of $50 was offered to fishers for returning the tags and fish carcasses.

Fish were recaptured by hook-and-line, spear-fishing and returns from fishers. Recaptures by this study were stored on ice for return to the laboratory, and within 24 h length and weight measured and otoliths dissected. Sagittal otoliths were dissected, cleaned and stored dry in plastic vials with individual labels identifying the otolith along with date of OTC marking, date of recapture and fish measurements. Otoliths were stored in darkness to prevent exposure of the OTC mark to light.

Sagittal otoliths were mounted to a wood block (5 x 15 x 60 mm) with thermoplastic glue (Crystal bond, SPI Supplies) and sectioned along the transverse plane twice through the core to a thickness of 1 mm with a Buehler Isomet low speed saw with a diamond blade. Sections were then adhered to glass slides with Crystal bond and polished first with a 9 μm aluminum oxide abrasive lapping film and second with 0.3 μm type-A-alumina on a micropolishing cloth (Buehler, Inc). Thin sections were viewed with an Olympus BH-2 light microscope at 20 X magnification with blue-violet light to locate the flourescent OTC mark and with transmitted white light to view the growth increments. Images were captured with a Sony CCD video camera and Flashpoint 128-4M digitizing board (Integral Technologies, Inc), and analyzed in ImagePro Plus V 4.5. Calibrations with a micrometer were taken for each image in both the vertical and horizontal axes to correct for any distortions of the camera or microscope. Distance measurements (mm) from the otolith core to the OTC mark and to growth rings were taken from digitized images.

<u>Data analysis</u> - A linear regression was used to show growth increment periodicity by comparing the number of increment cycles (one whole opaque plus one whole

translucent ring) formed during the time at liberty against the time at liberty of the fish. For fish that formed an opaque ring while at liberty, the count of whole plus fractional increments was based on the number of whole increments formed past the OTC mark plus the initial fractional increment formed from the OTC mark to the following opaque ring and the final fractional increment formed from the outermost opaque ring to the edge of the otolith (Cappo, Eden, Newman. & Robertson 2000). For fish that did not form an opaque ring during the time at liberty, a ratio of the width (mm) of the otolith margin (OTC mark to edge) to the width of the proceeding whole growth increment was calculated to find the fractional increment formed during the time at liberty for the fish.

Growth rates of OTC marked fish were assessed by a linear regression of change in fish length (mm) against time at liberty for all recaptured fish. Growth rates of OTC marked fish were compared to published red snapper growth rates to assess any possible marking effects on growth.

Hatchery age-2 fish

Sample collection - Otoliths from known-age, hatchery-reared red snapper were examined for growth increment periodicity in age-2 fish. Otolith samples were taken from juvenile red snapper spawned in May 2002 and 2003 at the Claude Peteet Mariculture Center in Gulf Shores, Alabama. Fish were reared in circular tanks (1.5 m diameter, 0.7 m depth) within an 11,000 L recirculating seawater system. Fish were sampled at age-0, 1, and 2 years of age in 2002, 2003 and 2004. Sagittal otolith pairs were dissected, dried and stored in individual plastic vials. A total of 750 otolith pairs were taken from hatchery-reared red snapper, including 518 pairs from age-0, 124 pairs from age-1 and 108 pairs from age-2 (Chapman, Szedlmayer & Phelps, In press).

For this study, a total of 31 otolith pairs from age-2 fish were randomly selected and one otolith per pair was sectioned by methods described above for adult fish. Otoliths were viewed under an Olympus BH-2 light microscope and images imported into the ImagePro Plus V 4.5 image analysis computer program. Distance measurements (mm) were taken from the otolith core to the outer edge of each growth ring and to the otolith edge along the ventral edge of the sulcus in the sectioned otolith.

Data analysis - The number of growth rings observed on sectioned otoliths was compared to the known age of the fish (2 years) and presented as the percentage of otoliths showing the two growth rings. The mean distances (mm) ± SD from the otolith core to the first and second growth rings, and otolith edge were calculated.

Otolith shape analysis

For otolith shape analysis, 50 otoliths (left side only) were randomly selected from each age-0, age-1, and age-2 classes of hatchery-reared red snapper. Only whole otoliths in good condition without chips or damage were used in the analysis. Whole otolith images were recorded with the same methods described for sectioned otoliths but viewed under a dissecting microscope. Otoliths were placed on a dark background, sulcus down and rostrum positioned to the left for standardization. Magnification was varied from 1 X to 4 X to ensure detailed photographs among varying otolith sizes. An automated trace function of the otolith perimeter collected size and shape data from the otolith image. Descriptions of size and shape indices from ImagePro are listed in Table 1. Dry weights of otoliths were measured with an Ohaus balance to the nearest 2 mg.

To assess structural differences on the otolith surface, 90 whole otoliths (30 age-0, 30 age-1, 30 age-2) were mounted to individual glass microscope slides with Crystal bond adhesive and photographed at a 45° angle under a dissecting microscope to count the number of finger-like projections emanating from the core on the concave surface of the otolith.

Data Analysis - All variables were tested for normality with the Shapiro-Wilk (W) statistic and multivariate homogeneity of the within-group variance-covariance matrices was assessed with the Bartlett's modification of the likelihood ratio test. A multivariate analysis of variance (MANOVA) was used to test shape differences among age-0, age-1, and age-2 otoliths. The shape variables used in the MANOVA were aspect ratio, rectangularity, box x/y, radius ratio, roundness, perimeter ratio, and counts of finger-like projections (Table 1). Multiple a posteriori analysis of variance's (ANOVA's) tested individual shape variables against age class to further define the MANOVA results, followed by a Tukey HSD test to better identify patterns in otolith growth with age. Significant differences in otolith weight and fish length among age classes also were tested by individual ANOVA. Bonferroni adjustments to the significance level for multiple statistical tests were not made since ANOVA tests were a posteriori and applied for the purpose of identifying trends already reported as significant by the MANOVA.

Linear discriminant function analysis created a linear equation to separate otoliths into age groups based on shape variable characteristics. Cross-validation classification procedures created an unbiased matrix of age classification success. Cross-validation procedures applied the linear discriminant function from the calibration data set while omitting the individual object being classified (i. e. n-1) to classify each otolith into an age class (Friedman 1989).

Multidimensional scaling based on a Bray-Curtis dissimilarity matrix further illustrated otolith shape differences among age classes through a non-parametric test (Clarke & Warwick 2001). Mulitdimensional scaling (MDS) does not require the same assumptions of normality and homogeneity as the MANOVA since it is based on rankings of dissimilarities among age groups. A non-parametric analysis of similarity (ANOSIM) tested significant shape differences among age classes with the Global R statistic. A significance level of $P < 0.05$ was used for all statistical tests unless specified.

RESULTS

Otolith annual increment validation

Mark-and-recapture - A total of 265 adult red snapper were captured by hook-and-line, marked with oxytetracycline, and released back into the wild. The mean ± SD fish total length was 571 ± 100 mm, ranging from 389 mm to 860 mm. The mean ± SD fish weight was 3.28 ± 2.0 kg, ranging from 0.91 kg to 13.0 kg. Of all OTC marked fish, 23 were reportedly caught by private fishers, but only 4 carcasses returned. Recaptures by this study for fish at liberty for less than six months (n = 9) were measured, re-marked with OTC and released. Recaptures by this study for fish at liberty for more than six months (n = 6) included five from hook-and-line and one from spear-fishing. Linear regression of change in fish length against time at liberty for recaptured fish, excluding fisher return data, showed growth of OTC marked fish at 95 mm yr^{-1} (r^2 = 0.93, n = 15, P < 0.0001, Fig.1).

All six fish recaptured by the present study showed a clear OTC time mark in the otoliths similar to that shown for fish-4 in Figure 2; whereas, none of the returns from private fishers showed an OTC mark. Thus, a linear regression of whole plus partial increment cycles formed after the OTC mark, against the time at liberty of the fish only included data from recaptured fish. Fisher returns were excluded. The slope of the linear regression showed a formation rate of 1.1 increment cycles year^{-1} ($r^2 = 0.96$, $n = 6$, $P < 0.0001$, Fig. 3). Age ranged from two to five years for recaptured fish.

Opaque rings in OTC marked red snapper were formed in summer and early fall, while translucent rings were formed in winter and spring. The time period of both opaque and translucent zone formation was based on known dates of OTC marking and recapture. Fish-1 and fish-2 were OTC marked in August 2006 (Fig. 4). Fish-1 was recaptured in February 2007 and fish-2 was recaptured in July 2007. Both fish-1 and fish-2 were at liberty over the winter months but only showed translucent zone formation in their otoliths. Fish-3 and fish-4 were OTC marked in August 2006 and both showed the OTC mark either in an opaque ring or just past an opaque ring (Fig. 4). Fish-5 was OTC marked in February 2007 and this mark was in the middle of a translucent zone. This same fish was recaptured, OTC marked again and released for a second time in July 2007 and this second OTC mark was at the beginning of a new opaque ring. An opaque ring was not formed between the two OTC marks (February through July), however, an opaque ring was formed in the time at liberty between the 2nd OTC marking and final recapture (July through November). The same pattern was shown for fish-6. Fish-6 was OTC marked in November 2006 and showed that an opaque ring had formed just prior to the OTC mark. That fish was at liberty for just over one year and showed formation of one opaque ring during the time at liberty. All data from OTC marked otoliths supported validation of one opaque ring formed per year when read along an axis parallel to the sulcus, however, fish-4 showed two opaque rings formed during the year at liberty when the otolith was read along an axis 30° of the sulcus (Figs. 2 & 4).

Hatchery age-2 fish

Hatchery-reared young red snapper were exposed to static environmental conditions in the laboratory from 2002 to 2004 as compared to their natural environment (Fig. 5). Most (87%) of the age-2 hatchery-reared fish showed two opaque growth rings on their otoliths similar to the otolith shown in Figure 6. Three otoliths showed only one visible growth ring, and one showed four to five rather indistinct growth rings. The mean ± SD distance from the otolith core to the first growth ring was 1.2 ± 0.21 mm, to the second growth ring was 1.7 ± 0.21 mm, and to the otolith edge was 1.9 ± 0.16 mm.

Otolith shape analysis

The variables roundness, perimeter ratio, otolith weight and fish length all had at least one age class with a non-normal distribution according to the Shapiro-Wilk statistic. The test for multivariate heterogeneity of covariance was significant at the 0.05 level so the within-group covariance matrices were used in the discriminant function analysis ($\chi^2 = 77.48$, d.f. = 56, $P < 0.0303$). Transformation of the data did not improve normality. However, frequency distribution plots of the shape variables showed all variables having uni-modal, bell-shaped distributions, with minimal skewness or kurtosis. Also, the MANOVA and ANOVA are robust statistical tests with respect to violations of normality and homogeneity especially with equal sample sizes across groups. Violations of these two assumptions have been shown to have little effect on Type I error rates, hence all variables were kept in the analysis (Mardia 1971, Olson 1974).

Significant differences were found in fish length and otolith weight among all ages (Table 2). Otolith shape analysis showed significant differences in shape variables among age classes (MANOVA Wilks' $\lambda = 0.31$, $F = 7.6$, d.f.= 14, 134, $P < 0.0001$). Separated into component parts, a posteriori ANOVA showed significant differences among ages for all shape variables except for the number of finger-like projections (Table 2). The shape variables aspect ratio, box x/y and radius ratio significantly increased across all three of the age classes.

Cross-validation classification success of otoliths into correct age classes was 61.2% when including the finger-like projections (FP) variable. Excluding FP increased classification success to 70%. The addition of otolith weight (OW) to the discriminant function further increased classification success to 93.3% (Table 3).

A two-dimensional MDS plot showed separation of the age classes based on shape indices, however, the plot also showed considerable overlap among the age classes (Fig. 7). The MDS test had a stress of 0.05, considered an excellent representation of the Bray-Curtis dissimilarity rankings in the MDS ordination plot (Clarke and Warwick 2001). The ANOSIM, which analyzes the rankings data from the Bray-Curtis dissimilarity matrix, reported a Global R statistic of 0.298 with a 0.1% or 1 in 1000 chance of the null hypothesis, of no shape difference among age classes being true. The Global R statistic ranges from 0 to 1. A value of 1 indicates all within group replicates are more similar to each other than any between group replicates, and a value of 0 indicates similarities within a group are, on average, the same as similarities between groups (Clarke and Warwick 2001). The significant results of the ANOSIM, a non-parametric test, further supported the findings of the MANOVA.

DISCUSSION

Otolith annual increment validation

Mark-and-recapture - Growth of OTC marked red snapper in this study showed similar growth rates compared to those in other red snapper studies. Szedlmayer and Shipp (1994) reported growth of tagged red snapper at 0.22 mm d^{-1} (80.3 mm y^{-1}), and 0.27 mm d^{-1} (98.6 mm y^{-1}) from a length-at-age regression of fish less than 10 years of age. Patterson *et al.* (2001) reported red snapper growth at 0.238 mm d^{-1} (86.9 mm y^{-1}) for tagged fish, and 0.240 mm d^{-1} (87.6 mm y^{-1}) for fish aged by otoliths. These rates are comparable to the growth rate of 95 mm y^{-1} from this study, therefore, the OTC injection appeared to have little or no effect on fish growth.

The OTC mark was clearly visible on all otoliths in fish recaptured by the present study, however, OTC marks were absent on otoliths from four fish returned by private fishers. Two of these fish returned by private fishers were at liberty for less than 60 days. It is possible that the OTC mark was too close to the edge of the otolith for detection since the mounting glue used to adhere the otolith to the microscope slide showed a slight fluorescence under blue-violet light. The private fishers returning the other two recaptures were fishing from for-hire charter boats, and it is highly possible there was a mix up of fish returned, especially after fish had been gutted and cleaned for clients.

There is little possibility that the OTC failed to leave a mark in recaptures by private fishers since recaptures from the present study all showed OTC marks, and the high success of OTC marking in other studies (Hernaman *et al.* 2000).

This study showed opaque rings in sectioned red snapper otoliths are formed annually in fish at least until age five thus validating this method of ageing up to age five. The data from this study support the findings from other studies showing annual periodicity of otolith ring formation; however, timing of opaque ring formation in the present study differed considerably from other estimates (Patterson *et al.* 2001, Wilson & Nieland 2001, Allman *et al.* 2005). Winter to spring opaque ring formation was indicated in two studies by marginal increment analysis (Patterson *et al.* 2001, Wilson & Nieland 2001), and spring to summer formation indicated in a third study also by marginal increment analysis, however, that study was aided in the identification of opaque rings by computer-generated light intensity curves (Allman *et al.* 2005). Red snapper are presently aged by assuming the opaque ring is formed during the winter months beginning January 1 (Patterson *et al.* 2001, Wilson & Nieland 2001).

An opaque ring was formed once per year during the summer to fall months in the present OTC mark-and-recapture study, which corresponded with the red snapper spawning season (May to October). None of the otoliths from recaptured fish showed a winter or spring opaque ring. The methods of this study allowed for direct assessment of periodicity and timing of opaque ring formation in red snapper otoliths unlike the indirect methods described above. These results are similar to findings from Nelson and Manooch (1982) whom found summer formation (June and July) of red snapper scale annuli with marginal increment analysis. Marginal increment analysis on red snapper scales was only completed for 2 to 6 year old fish in that study.

There are numerous explanations for the discrepancy in timing of ring formation found in the present study as compared to other studies. For example, the method of marginal increment analysis has several weaknesses pointed out by Campana (2001). The recognition of edge condition (opaque verses translucent), which is the foundation of marginal increment analysis is not always clear in red snapper otoliths and especially dependent on the quality of the sectioning and polishing of each otolith. Also, the edge condition may be opaque when read along either the dorsal or ventral side of the sulcus, yet appear translucent on an axis further away from the sulcus. Therefore, the axis chosen to analyze otolith edge condition could affect results. Variability in timing of opaque ring formation among individuals and across regions is another explanation and was shown for *Lutjanus* species from the Great Barrier Reef (Cappo *et al.* 2000).

A possible criticism of the present study is that the process of OTC marking may have affected growth in tagged fish. However, OTC marked fish grew at a rate similar to that of other studies on red snapper growth, and the OTC marking did not appear to change the pattern of ring formation in the otoliths of recaptures. For example fish-6 was OTC marked in November 2006 and had a clear translucent zone following the OTC mark indicating a period of faster growth just after being marked (Fig. 4). Also, one opaque ring was formed in that otolith during the year at liberty. The two fish (fish-1 and fish-2), OTC marked in August 2006 and recaptured February and July 2007 did not show opaque rings formed during the time at liberty and these fish were at liberty during winter to spring, the months where opaque ring formation was reported by previous studies with marginal increment analysis. The OTC marking left a clear time mark in the otoliths that allowed for direct evaluation of the periodicity and timing of opaque ring formation and there is little evidence that the OTC marking process affected growth of the fish.

A final explanation for the discrepancy in timing of opaque ring formation is that opaque rings may be formed in both winter and summer months resulting in two opaque rings formed per year. This has been shown in grey triggerfish, *B. capriscus*, and tilapia, *O. niloticus*. One fish in my study (fish-4) showed a distinct pattern of false annuli formed just prior to the annulus when the otolith was read along an axis 30º of the sulcus (Fig. 2). This otolith actually showed two rings formed during the year of liberty for this fish when read along this alternative axis. These secondary rings were distinguishable from the annulus based on increment width and that they did not extend entirely to the sulcus. The timing of formation for these secondary rings was not possible by methods of this study, however, it is important to be able to distinguish these secondary rings from annual rings for accurate age determination and clearly continues to raise questions concerning present age determination methods.

A similar OTC mark-and-recapture study on sablefish, *A. fimbria*, first reported annular growth ring validation from recaptures a few years after initial mark and release, but recaptures 20 years after initial release indicated more than one ring formed per year in sectioned otoliths (Beamish & McFarlane 2000). Winter annulus formation was reported for sablefish, and it was hypothesized that spawning, or other growth checks on the otolith, easily identifiable during the first 10 years of growth, were mistaken as annuli in older fish. It was observed that as fish growth slowed with age, annual growth rings on the otolith became thinner and closer together, making it easier to mistake spawning checks as annuli. This resulted in an overestimation of age, although sablefish are clearly still a long-lived and slow growing species. The OTC mark, and known years at liberty, allowed researchers to identify growth patterns in the otoliths of sablefish that otherwise would have been mistaken for annuli.

For the majority of fish, winter formation of opaque rings is generally assumed, however, the present study and a number of other studies have correlated opaque ring formation with the spawning period. Opaque rings were formed in April through June for yellowtail snapper, *Ocyurus chrysurus* (Bloch) from south Florida, which corresponded with their peak spawning period of May through July (Johnson 1983). Spring formation of opaque rings in otoliths of damselfish, *Pomacentrus mollucensis* (Bleeker), from the Great Barrier Reef during the onset of the reproductive period was shown by OTC mark-and-recapture methods (Fowler 1990). Carpenter, *Argyrozona argyrozona* (Valenciennes), found in warm-temperate waters off South Africa showed opaque ring formation during the spawning season by a combination of OTC mark-and-recapture and marginal increment analysis methods (Brouwer & Griffiths 2004). Water temperature was not correlated with opaque ring formation in that study, although both photoperiod and gonadosomatic index (GSI) were positively correlated with opaque ring deposition.

Many questions remain about what causes annual growth increment patterns in otoliths. Considering the recaptures in my study were all relatively young adults (< six years old), it would be beneficial to expand and continue this study to validate timing and periodicity of otolith growth increment formation for red snapper greater than 10 years, and to identify growth ring patterns in otoliths of older fish to be sure false annuli are not mistaken as annuli.

Hatchery age-2 fish

The age-2 otolith increment validation study showed that hatchery-reared young red snapper formed one opaque ring per year. This study validates the method of reading sectioned otoliths for age determination of juvenile red snapper up to age two. The fact that otoliths were from fish of known-age aided in the identification of the first and second annulus. The first annulus was not as distinct as later growth rings because it was a more diffuse, broad opaque zone compared to thinner, later growth rings. The first opaque ring was most clearly identified along the ventral edge of the sulcus in the sectioned otolith. The outer edge of the first growth ring was located approximately 1.2 mm from the core. This measurement is similar to the location of the outer edge of the first annulus for wild red snapper otoliths, although measures on those otoliths were taken along the dorsal edge of the sulcus (Allman *et al.* 2005). Wild juvenile red snapper in that study were sampled from February to November 2002, and otoliths were measured from the core to the otolith edge to verify the position of the first annulus with a mean ± SD distance of 1.05 mm ± 0.11.

It was hypothesized that age-2 hatchery-reared fish would not show two growth increments because these fish were never exposed to a natural fluctuating environment and were sexually immature. Therefore, it was surprising that two growth rings were visible for fish reared under seemingly static conditions. Although otoliths have been used in many age and growth studies, the physiology behind otolith annular incremental growth is surprisingly not well understood (Campana 2005). It is often reported that the slow growth (opaque) rings on the otolith are formed during winter months, corresponding to periods of lower water temperature, and periods of diminished food supply (Admassu & Casselman 2000, Beamish & McFarlane 2000, Bernardes 2002, Ingram 2001). Spawning periods also have been correlated with the timing of opaque ring deposition (Admassu & Casselman 2000, Bernardes 2002, Ingram 2001). However, hatchery-reared fish in our study were sexually immature, held in a static environment and had unlimited food supply throughout the year. Temperatures in the holding tanks ranged from 18 to 24.3 °C showing substantially less variation compared to sea surface water temperature of 11.4 to 32.9 °C from January 2002 to December 2004 (Fig. 5). One possible explanation is that photoperiod was kept as close to natural as possible throughout the time the fish were in the lab. Photoperiod was correlated with opaque ring formation in carpenter, *A. argyrozona* (Brouwer & Griffiths 2004) and has been shown to affect daily growth increment formation (Mugiya 1987).

<u>Otolith shape analysis</u>

Otolith shape analysis was successful at identifying differences among age-0, age-1, and age-2 red snapper otoliths based on differences in shape variables. This study differs from others that have focused on otolith size variables, such as length, in their analysis (Petursdottir, Begg & Marteinsdottir 2006). It is well understood that otolith size increases with age and size of the fish, in contrast, the present study showed changes in otolith shape that were independent of size. Three shape indices, aspect ratio, box x/y and radius ratio all showed significant differences among all three age groups. These three shape indices were related, in that all independently measured some aspect of the otolith along both the horizontal and vertical axes. The juvenile red snapper otoliths in this study grew faster along the anterior-posterior axis compared to the dorsal-ventral axis (Fig.8). The red snapper otolith growth patterns were similar to patterns shown for long rough dab, *H. platessoides*, (Fossen *et al.* 2003), and Atlantic cod, *G. morhua* (Galley *et al.* 2006) that both showed otoliths became more elongate with age.

A 70% classification success of otoliths into their correct age classes based solely on shape variables was similar to other studies using this technique to separate fish stocks (Tuset *et al.* 2003, Cardinale *et al.* 2004, Stransky 2005). The addition of otolith weight in the linear discriminant function increased classification success to 93%, supporting evidence that otolith weight is useful for age estimation in fish.

This study shows exciting potential for using otolith shape analysis to age wild caught juvenile red snapper. One advantage is that classification error is clearly defined as compared to visual reading error that can vary substantially among different readers. This method of shape analysis is considerably less time consuming and less expensive than traditional techniques, negating the costs of sectioning and reading individual otoliths. Reading growth rings on sectioned otoliths is subjective to reader experience and has been especially difficult in juvenile red snapper otoliths (Buckmeier 2002, Allman *et al.* 2005). Otolith shape analysis reduces human-induced age determination error through automated computer image analysis and could benefit large-scale population studies involving young red snapper. However, otolith shape analysis for age determination has not been applied to any species of wild caught fish. For application of this method, future studies should first sub-sample otoliths to create a calibration data set where ages have been determined by validated otolith annular increments. This is critical since otolith shape can be affected by environmental and genetic factors and significant shape differences have been shown between otoliths from wild and captive fish (DeVries *et al.* 2002, Cardinale *et al.* 2004, Petursdottir *et al.* 2006).

CONCLUSION

Annual periodicity of growth increments in red snapper otoliths up to age five was validated with an OTC mark-and-recapture study, including validation for age-2 hatchery-reared fish. The timing of opaque ring formation in otoliths of both wild adult fish and young hatchery fish did not correlate with water temperature minimums, and opaque rings were formed during summer to fall months in wild adult fish.

Otolith shape analysis in this study proved to be a simple and straightforward method that was effective in correct age determination of red snapper age-0 to age-2. Otoliths were found to grow faster along the anterior-posterior axis as compared to the dorsal-ventral axis. Shape analysis is more cost effective than the traditional technique of visually reading sectioned otoliths and better quantifies error in age estimation since age estimations are based on automated computer image analysis rather than individual reader variance.

The process of otolith annular growth increment formation is an important area of future research, especially since the majority of fish species are currently aged with otoliths, and with consideration that the management of red snapper and many fish species relies heavily on accurate ageing practices.

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TABLES

Table 1. Description of measures used in otolith image analysis.

Name	Description
Size measurements	
Area	Area of object
Perimeter	Length of the object's outline
Length	Feret diameter along major axis of object
Width	Feret diameter along minor axis of object
Shape indices	
Aspect ratio	Ratio of major axis and minor axis of an ellipse equivalent to the object
Rectangularity	Ratio of object area and area of its bounding box
Box X/Y	Ratio of width and height of object's bounding box
Radius ratio	Ratio of maximum radius and minimum radius
Roundness	$\text{Perimeter}^2 / (4 * \Pi * \text{area})$
Perimeter ratio	Ratio of convex perimeter to perimeter
Other	
Otolith weight	Weight to the nearest 2 mg
Fish length	Total length (mm)
Finger-like projections	Number of structural projections emanating from the core on the concave surface of the otolith

Table 2. Comparison of fish size and otolith shape variables (mean ± SD) across age classes of otoliths from known-age, hatchery-reared red snapper. Significant differences were based on a posteriori ANOVA and Tukey HSD following MANOVA. Lower case letters represent significant differences.

	Fish age class		
Variable	Age-0	Age-1	Age-2
Fish total length (mm)	68 ± 30 (a)	219 ± 29 (b)	284 ± 36 (c)
Otolith weight (mg)	13 ± 17 (a)	167 ± 47 (b)	302 ± 57 (c)
Aspect ratio	1.47 ± .06 (a)	1.54 ± .06(b)	1.61 ± .07 (c)
Rectangularity	0.71 ± .01 (a)	0.72 ± .01 (ab)	0.72 ± .02 (b)
Box x/y	1.43 ± .05 (a)	1.50 ± .06 (b)	1.60 ± .08(c)
Radius ratio	1.59 ± .06 (a)	1.66 ± .07 (b)	1.76 ± .10 (c)
Roundness	1.15 ± .04 (a)	1.15 ± .03 (a)	1.18 ± .04 (b)
Perimeter ratio	0.98 ± .01 (a)	0.99 ± .01 (b)	0.98 ± .01(ab)
Finger-like projections	4.8 ± 3.2 (ns)	5.1 ± 2.5 (ns)	3.8 ± 1.4 (ns)

Table 3. Cross-validation classification of the linear discriminant function analysis among age classes using shape descriptors for known-age, hatchery-reared red snapper otoliths.

	Number classified (percent)			
Group	Age-0	Age-1	Age-2	total classification success (%)
Shape variables (with finger-like projections)				
Age-0	20 (74.1)	5 (18.5)	2 (7.4)	61.2
Age-1	4 (15.4)	16 (61.5)	6 (23.1)	
Age-2	3 (13)	9 (39.1)	11 (47.8)	
Shape variables (without finger-like projections)				
Age-0	43 (86)	6 (12)	1 (2)	70.0
Age-1	7 (14)	30 (60)	13 (26)	
Age-2	2 (4)	16 (32)	32 (64)	
Shape variables (with finger-like projections and otolith weight)				
Age-0	27 (100)	0 (0)	0 (0)	93.3
Age-1	1 (3.9)	23 (88.5)	2 (7.7)	
Age-2	0 (0)	2 (8.7)	21 (91.3)	

FIGURES

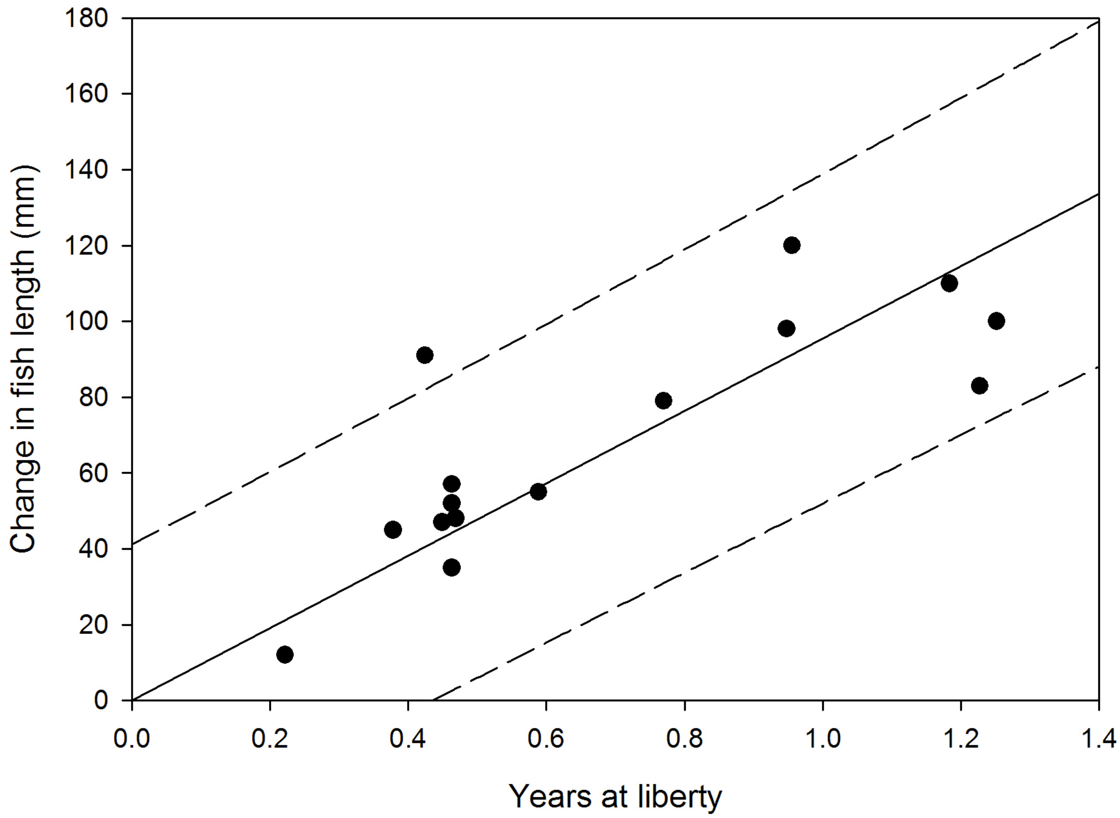

Figure 1. Linear regression with 95% confidence intervals for the change in fish length against the time at liberty of tagged red snapper ($r^2 = 0.93$, $n = 15$, $P < 0.0001$).

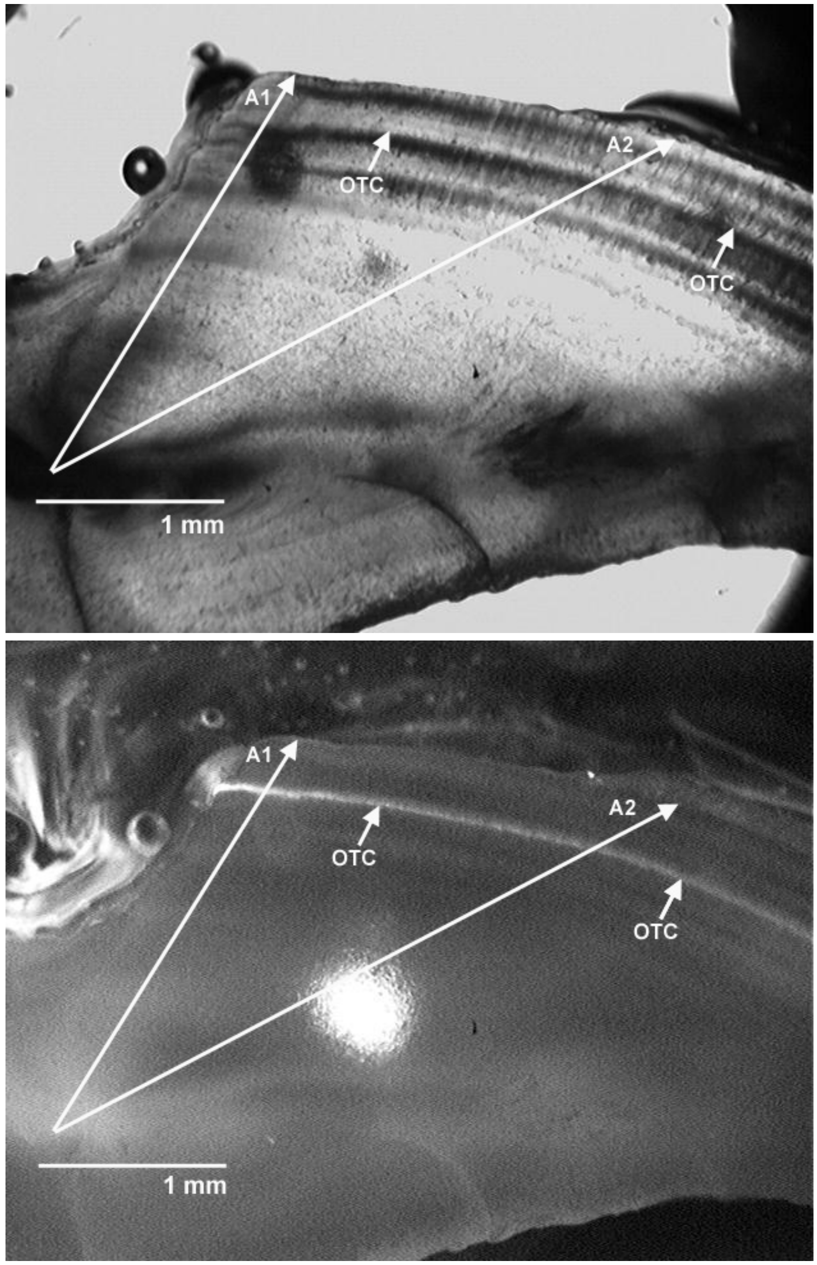

Figure 2. Sectioned otolith (fish-4) viewed with white light (top) and with blue-violet light (bottom). Arrows indicate location of OTC mark, A1 is the axis parallel to the ventral edge of the sulcus and A2 is the axis 30º of Al.

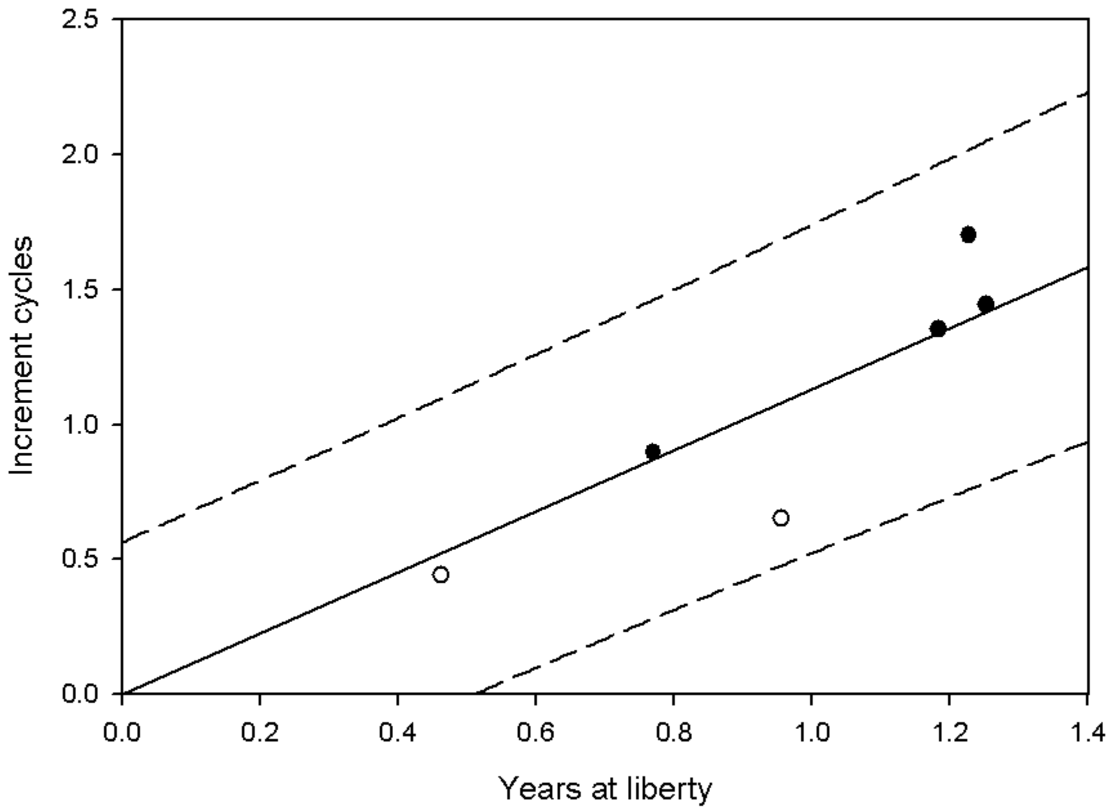

Figure 3. Linear regression with 95% confidence intervals for the number of increment cycles formed during time at liberty against the time at liberty of the fish ($r^2 = 0.96$, $n = 6$, $P < 0.0001$). Closed circles indicate otoliths with an opaque ring formed during time at liberty. Open circles indicate otoliths without an opaque ring formed during time at liberty. A whole increment cycle is complete formation of one opaque plus one translucent ring.

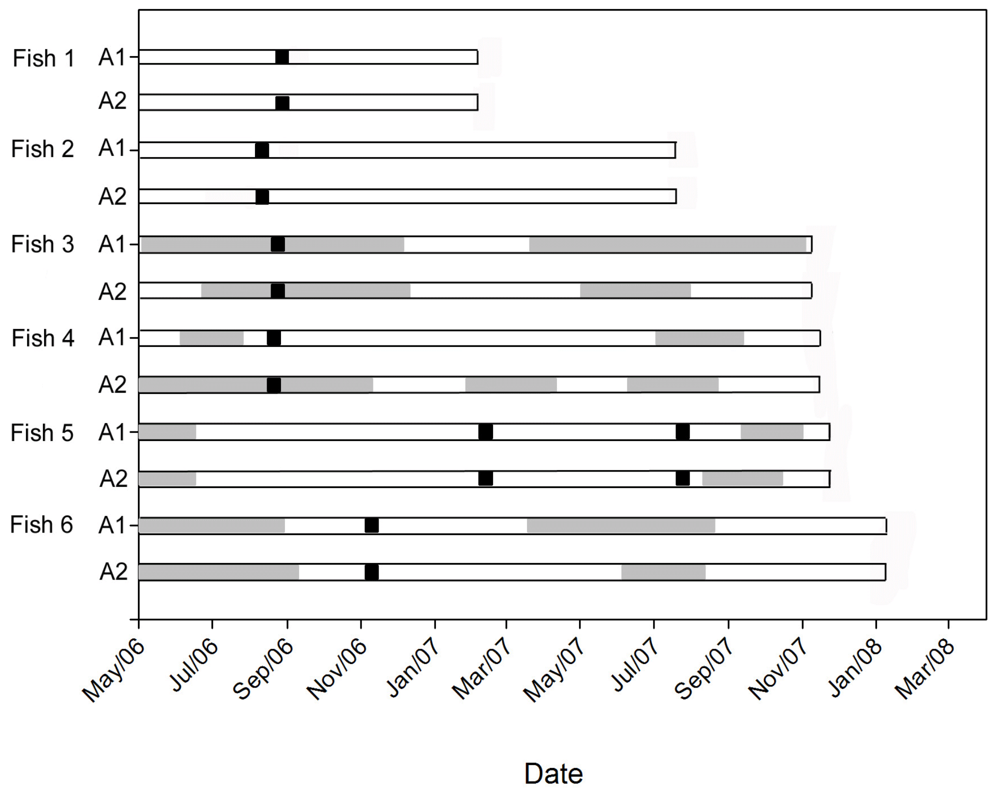

Figure 4. Graphical representation of OTC marked otoliths. Black bars represent the OTC mark, gray bars indicate opaque rings and white bars indicate translucent rings. Location of opaque rings was estimated based on OTC marking date, recapture date and otolith radii measurements. Otoliths were read from the core to the edge along the A1 and A2 axes.

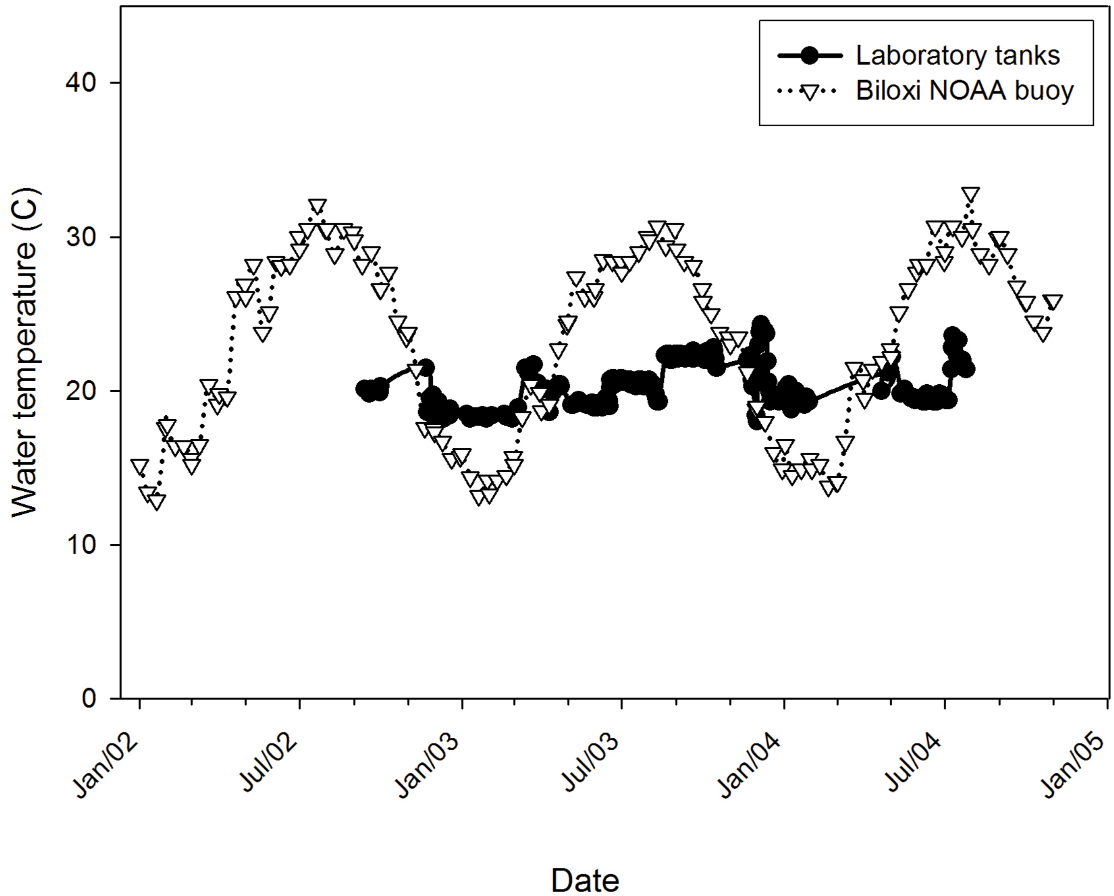

Figure 5. Laboratory tank water temperature for hatchery-reared red snapper and sea surface water temperatures reported from a NOAA marine weather buoy, 22 nm south-southeast of Biloxi, Mississippi for the years 2002 to 2004.

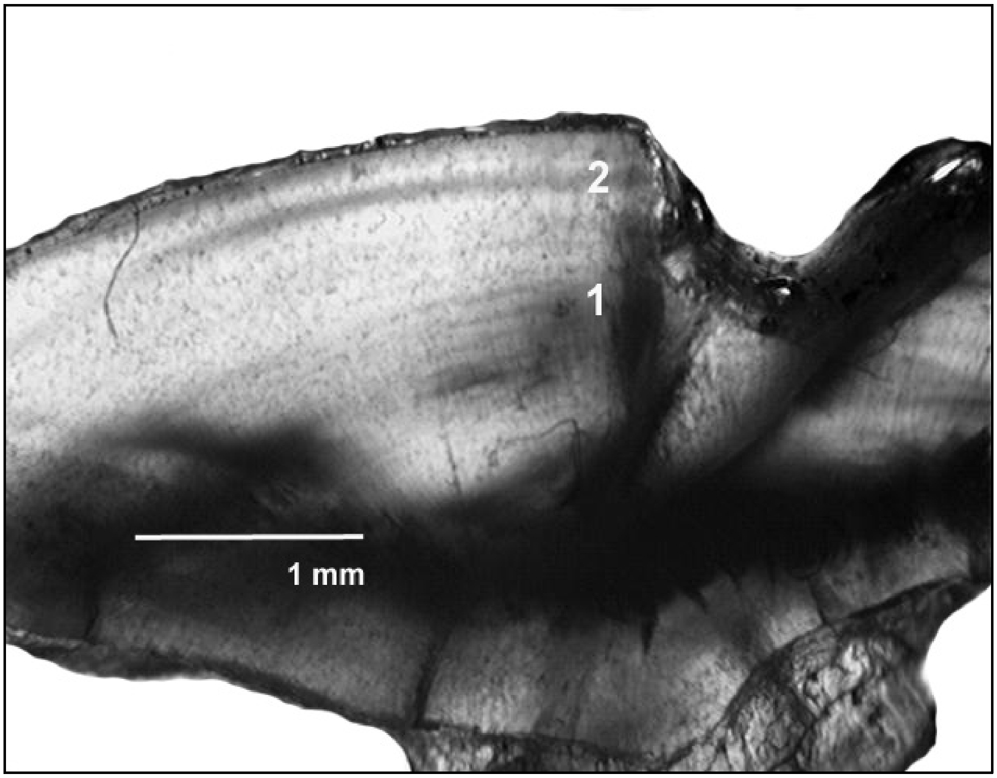

Figure 6. Sectioned otolith of an age-2 hatchery-reared red snapper. The two opaque rings are marked with numbers.

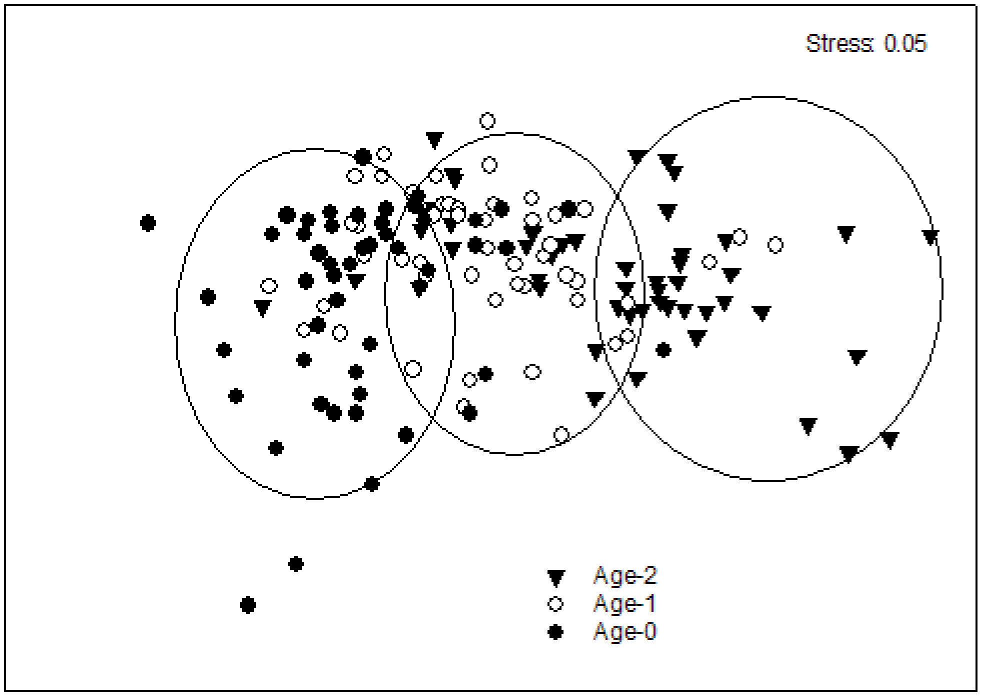

Figure 7. Multidimensional scaling ordination plot showing separation of age-0, age-1, and age-2 otoliths from hatchery-reared red snapper based on shape.

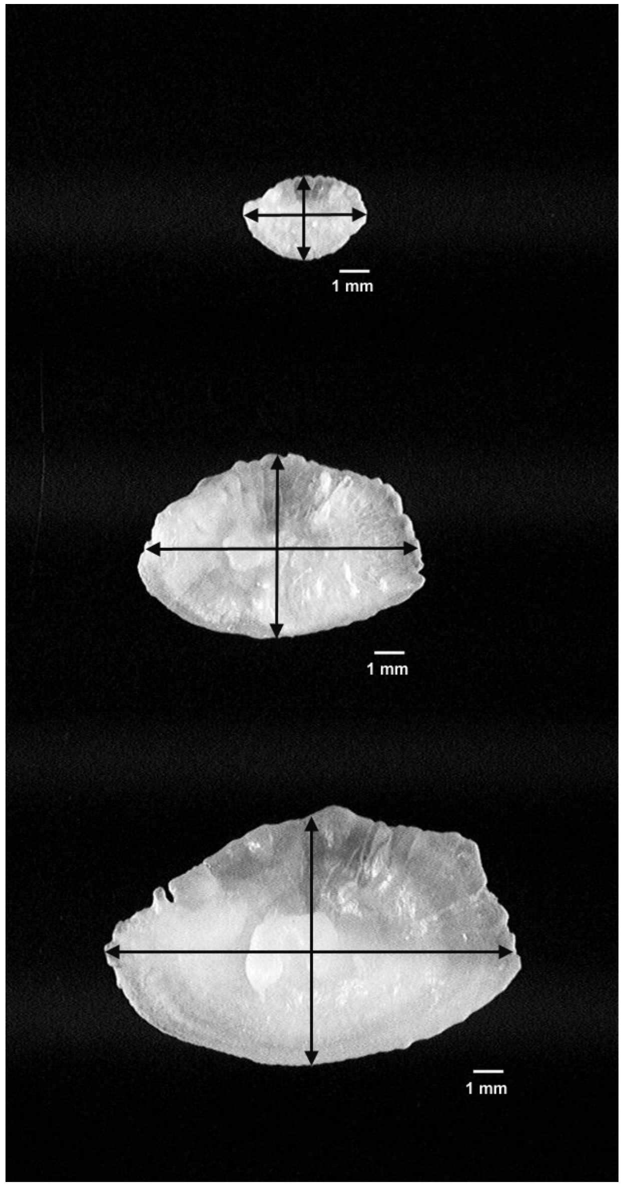

Figure 8. Whole otoliths from age-0 (top), age-1 (middle), and age-2 (bottom) hatchery-reared red snapper. Arrows show measurements used to calculate aspect ratio.

APPENDIX

Appendix 1. Example of MANOVA and a posteriori ANOVA.

MANOVA (all shape variables)

Statistic	value	*F* value	d.f. model	d.f. error	*P*
Wilks' Lambda	0.3107	7.60	14	134	<0.0001

ANOVA (aspect ratio)

Source	d.f.	sum of squares	mean square	*F* value	*P*
Model	2	0.4551	0.2275	57.61	<0.0001
Error	147	0.5806	0.0039		
Total	149	1.0356			