Validation of annual periodicity in otoliths of red snapper, *Lutjanus campechanus*

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SEDAR31-RD20

13 August 2012

Environ Biol Fish (2011) 91:219–230
DOI 10.1007/s10641-011-9774-6

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Received: 1 October 2009 / Accepted: 16 February 2011 / Published online: 5 March 2011

Abstract The periodicity of otolith growth increments (opaque and translucent zones) from adult red snapper (*Lutjanus campechanus*) was examined through a mark and recapture study (2005–2010), and laboratory holding of hatchery reared red snapper over a 2 year period (2002–2004). Wild red snapper (n=295) were caught hook-and-line, marked with anchor tags, injected with oxytetracycline dihydrate (OTC), and released in the Gulf of Mexico 15–40 km south of Dauphin Island, Alabama. Marked fish were recaptured up to 2.8 years after release (n=35) and sagittal otoliths were dissected, sectioned and examined under white and blue-violet light. The number of opaque growth zones past the OTC mark was compared to time at liberty for each fish and supported an annual periodicity of growth increment formation. Also, most (87%) of the hatchery reared fish showed two opaque zones that supported an annual increment formation rate. However, an unusual timing of opaque zone formation was shown for mark-recaptured fish. Based on known timing of OTC marking, otoliths from mark-recapture fish showed opaque zone formation from late summer (August) to early winter (December). This fall formation of opaque zones is in contrast to previous studies and its timing may relate to the end of spawning for this species.

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Keywords Otolith aging · Mark-recapture · Growth rate · Opaque zones

Introduction

Red snapper (*Lutjanus campechanus*) is an important marine fish species to both 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 and McFarlane 1983).

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 and one translucent zone. Age estimation typically occurs by counting the number of opaque zones viewed on the otolith, with consideration of the date of capture. Opaque zones usually show as thinner dark zones in sectioned otoliths when viewed with transmitted white light and indicate periods of slower growth in the fish. Translucent zones are usually wider and lighter compared to opaque zones when viewed with transmitted white light and indicate periods of faster growth. Counting the number of opaque zones in sectioned otoliths is the presently accepted method to age red snapper (Nelson and Manooch 1982; Szedlmayer and Shipp 1994; Patterson et al. 2001; Wilson and Nieland 2001; Rooker et al. 2004; Allman et al. 2005). Even though age determination with sectioned otoliths is widely used for red snapper, it is critical to validate all ageing methods (Beamish and McFarlane 1983; Campana 2001). In addition, age must be validated for all age classes for each species. A misunderstanding of life history and subsequent mismanagement of Pacific ocean perch (*Sebastes alutus*) was attributed to lack of age validation in older fish (Beamish and McFarlane 1983). To date, direct validation of ageing methods for red snapper is lacking, but indirect methods have indicated that red snapper were accruing one full opaque and translucent zone per year, at least for the first 10 years. However, after the first 10 years, age validation is particularly difficult due to low sample sizes and increasingly small growth increments at the edge of the otolith (Nelson and 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 increments suggested that red snapper can routinely reach ages of 40 or 50 years (Szedlmayer and Shipp 1994; Patterson et al. 2001; Wilson and Nieland 2001).

Three indirect methods have been applied in attempts 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 that samples fish throughout the year and plots the width or percentage of the outermost translucent or opaque zones by month of capture. With this method, Wilson and Nieland (2001) indicated opaque zone formation in red snapper otoliths was between the months of December and June, and translucent zones 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 zone. Patterson et al. (2001) also indicated that opaque zones 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 zone formation from April to August, peaking in May.

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 et al. (2001) showed that some of those assumptions were violated in red snapper otoliths. Radiometric ageing was also shown to overestimate ages compared to sectioned otoliths in orange roughy (*Hoplostethus atlanticus*) (Smith et al. 1995) and was inaccurate for elasmobranchs (Welden et al. 1987). Campana (2001) further suggested that radiometric ageing 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 increase in oceanic ^{14}C is detectable in the hard structures of fish and coral, and has been used to verify fish ages when compared to otolith growth increment counts. This method of age validation requires that particular species of interest must have 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 that red snapper could reach at least 30 to 38 years of age. 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, although this particular fish was at least 38 years old, otoliths increment counts estimated its total age at 55 years, which still leaves many years un-validated. Bomb radiocarbon dating can estimate the minimum longevity of a fish, but the method does not actually validate annual periodicity of increment formation in otoliths. Although the above methods all indicated that red snapper reach ages close to the estimated maximum around 40 years first suggested by Nelson and Manooch (1982), questions remain concerning the validity of the maximum age estimation and the periodicity of growth increment formation in red snapper otoliths.

Variance from annual increment periodicity in fish otoliths and spines has been documented in some fish. For example, two opaque zones per year were formed in dorsal spines of gray triggerfish (*Balistes capriscus*) in the northeastern Gulf of Mexico (Ingram 2001), and 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 and showed distinct slow growth increments in both the winter and the summer months. These two periods of slow growth per year within the spines corresponded with minimum water temperatures along with reduced food supply in the winter, and with spawning in the summer months (Ingram 2001; Bernardes 2002). During the spawning season, both female and male gray triggerfish invest considerable energy into reproduction through elaborate displays of courtship behavior, nest building and parental care of the eggs (MacKichan and Szedlmayer 2007). Adult tilapia (*Oreochromis niloticus*), from Lake Awassa in the Ethiopian Rift Valley also showed two opaque zones per year (Admassu and Casselman 2000). In that study, otolith increment formation 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 et al. 2001). Although opaque zone formation in red snapper otoliths has been suggested for winter to early spring months by indirect methods (Patterson et al. 2001; Wilson and Nieland 2001), there is also a possibility that red snapper may form an opaque zone during the summer spawning period when fish divert greater amounts of energy to reproductive growth rather than somatic growth. Also, there is a possibility that increment formation rates may change with increasing age considering the 50 plus increments on the oldest red snapper. For example, younger fish may form annual growth increments but older fish may show rates other than annual (e.g., 2 increments per year), especially if older fish are investing greater energy into reproductive output through multiple spawning events and higher batch fecundity.

The consequences of incorrect age determination of red snapper were first pointed out by Rothschild et al. (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 10 years of life and fecundity increases with age (Szedlmayer and Shipp 1994; Collins et al. 2001; Woods et al. 2003). For example, batch fecundity ranged from 13 eggs in an age-4 to 3.4 million eggs in an age-11 red snapper (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 age-3, 4, or 5 females (Collins et al. 2001). The inconsistency between red snapper life history and that of other long-lived species, the greater than annual increment rates for other long lived species (Beamish and McFarlane 2000), and the lack of direct mark recapture studies, showed a need for further validation of annual increment 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 zones in otoliths from known-age fish. Oxytetracycline marking for the purpose of validating otolith annual increments has been applied to many fish species, including yellowtail rock fish (*Sebastes flavidus*) (Leaman and Nagtegaal 1987), inshore coral trout (*Plectropomus maculates*) (Ferreira and Russ 1992), lemon damselfish (*Pomacentrus moluccensis*) and Ward's damselfish (*P. wardi*) (Fowler and Doherty 1992), orange roughy (*H. atlanticus*) (Smith et al. 1995), several acanthurid fishes (Choat and Axe 1996), tropical gobies (Gobiidae) (Hernaman et al. 2000) and sablefish (*Anoplopoma fimbria*) (Beamish and 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. Otolith growth increments were also counted on sectioned otoliths from known-age (age-2) hatchery-reared red snapper for additional annual increment validation, but also because of the difficulty in identification of the first increment in red snapper.

Materials and methods

Mark-and-recapture

The study site for mark-and-recapture of wild adult red snapper was located in the Gulf of Mexico approximately 15 to 40 km south of Mobile Bay, Alabama in an artificial reef area with gas platforms, submerged liberty ships, army tanks, car bodies and pipelines, which all serve as habitat for red snapper (Szedlmayer and Shipp 1994; Gallaway et al. 2009). 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 et al. 1983).

Red snapper were caught by 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 (SL; mm) and weight (kg) were recorded for each marked fish. Red snapper were injected intramuscularly (epaxial muscle) with OTC at a dosage of 100 mg kg^{-1} body weight (Francis et al. 1992). Fish were also marked with individually numbered Floy anchor tags inserted into the peritoneal cavity. Fish were released at the capture site by lowering fish to the bottom on weighted line with an inverted barbless hook that was attached to the fish's lower jaw. Upon retrieval of the weighted line the fish was released at depth near the reef site. Initially in 2005 a reward of $50 was offered to fishers for returning the tags and fish carcasses, this was increased to $150 in 2008.

Fish were recaptured by hook-and-line by the present study and from private fishers. Recaptures by this study were stored on ice for return to the laboratory for measurement of SL (mm) and weight (g), and otoliths dissected within 24 h of capture. Sagittal otoliths were dissected, cleaned and stored in darkness in dry plastic vials.

Sagittal otoliths were mounted to a wood block (5×15×60 mm) with thermoplastic cement (Crystal bond, SPI Supplies) and 1 mm transverse sections were cut with a diamond blade on a Buehler Isomet low speed saw. Sections were then fixed to glass slides with Crystal bond, polished with a 9 μm aluminum oxide abrasive lapping film, and finished with 0.3 μm type-A-alumina on a micropolishing cloth (Buehler, Inc). Thin sections were viewed with an Olympus BH-2 microscope at 40 X magnification under blue-violet light to locate the fluorescent OTC mark, and with transmitted white light to view the growth increments. Images were captured with a Nikon D200 digital camera, and analyzed in Image-Pro Plus V 4.5. Otolith sections were measured (mm) from the OTC mark to the otolith edge along the ventral side of the sulcus. The second measure was from the OTC mark to the start of the first opaque zone, the third measure was to the distal end of the first opaque zone. These measurements were repeated for each opaque zone. The timing of opaque zone formation was based on the relation of known days at liberty to maximum radius measured from the OTC mark to the edge of the otolith (Ferreira and Russ 1992; Fowler and Doherty 1992; Choat and Axe 1996; Cappo et al. 2000). If the OTC mark was in the middle of an opaque zone only the distance from OTC mark to the end of the opaque zone was estimated (Fig. 1). We assumed constant otolith growth of total increment widths over the time from OTC marking to recapture (Cappo et al. 2000). Growth rates of OTC marked fish were determined by change in SL (mm) over time at liberty for each individual fish.

Hatchery age-2 fish

Otoliths from known-age, hatchery-reared red snapper were examined for growth increment periodicity in age-2 fish. Red snapper were spawned in May 2002 at the Claude Peteet Mariculture Center in Gulf Shores, Alabama and reared for 2 years (n=108) in circular tanks (1.5 m diameter, 0.7 m depth) within an 11 000 L recirculating seawater system (Chapin et al. 2009). A random sample of red snapper (n=31) were selected for otolith samples in 2004. Sagittal otolith pairs were dissected, dried and stored dry in plastic vials. One otolith per fish was sectioned and examined by methods described above. Opaque zones were identified and counted along the ventral edge of the sulcus in the sectioned otolith. Opaque zones were measured from the center to the middle of each zone along the ventral edge of the sulcus.

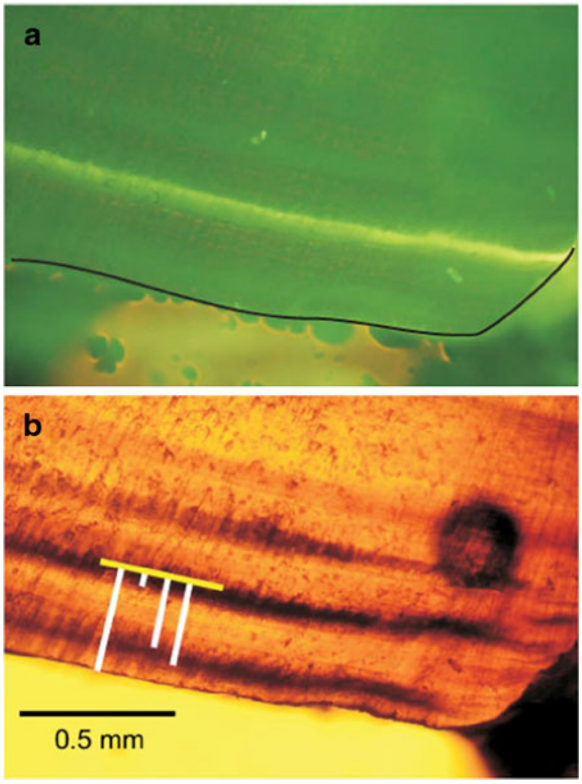


Fig. 1 Otolith sections showing a OTC mark from a recaptured red snapper (fish 7; Table 1; Fig 3), viewed under blue-violet light (**a**) and under white light (**b**). The location of the OTC mark (21 Aug 06) is shown by the yellow line under white light, and the measurement transects from the OTC mark to the opaque zones are shown in *white*. Edge of the otolith was 20 Nov 07

Results

Mark-and-recapture

A total of 295 adult red snapper were captured by hook-and-line, marked with OTC and anchor tags, and released back into the wild. The mean ± SD fish size of released fish was 462±82 mm SL ranging from 302 to 720 mm SL, and mean weight was 3.32± 1.9 kg, ranging from 0.91 to 13.0 kg. Recaptures by this study for fish at liberty for less than 4 months (n=13) were measured, re-marked with OTC and re-released. A total of 35 red snapper were recaptured for fish at liberty for more than 4 months. Ten fish were at liberty for 0.42 to 0.95 years, 16 fish were at liberty for 1.01 to 1.92 years, and 9 fish were at liberty for 2.02 to 2.79 years. Most fish (97%) were recaptured at the same location as released, while only one fish was recaptured by a private fisher at a reported different site (a gas platform, location unknown, Table 1). Age of recaptured red snapper ranged from 2 to 10 years, based on total opaque zone counts. All 35 fish recaptured by the present study showed a clear OTC time mark in the otoliths similar to that shown for fish-7 (Fig. 1). An annual increment deposition rate was supported for all otoliths after the OTC mark (Table 1). Mean ± SD growth rate for recaptured fish was 71.5±33.0 mm SL yr^{-1} based on individual rates from all recaptures (n=35), with a range of 33.8 to 233.8 mm SL yr^{-1}. Mean ± SD growth in weight was 1.2±0.4 kg yr^{-1} based on recaptured fish (n=13) measured by the present study (Table 1).

Opaque zones in OTC marked red snapper were formed from late summer (August) to early winter (December), while translucent zones were formed in later winter and spring. The time periods for opaque and translucent zone formations were based on known dates of OTC marking and recapture (Fig. 2). We also counted all otolith sections for a particular month, and determined the number cases with any opaque zone showing. For example, for fish 1 there were three January periods (2006, 2007 and 2008) that this fish was at liberty and examined for the presence of an opaque zone (Fig. 2). Fish 1 showed no opaque zones during January. All opaque zones were then counted over all fish otoliths that were at liberty in January, which resulted in 15 opaque zones and 40 translucent zones, for a frequency of opaque zones = 27% (n=55 total sections for January over all years). There was little evidence of winter opaque formation with the lowest percent (7%) of opaque zones in April, and the highest percent (82%) of opaque zones in October, with most formed from August to December (Fig. 3).

Hatchery age-2 fish

Hatchery-reared red snapper were exposed to less variable environmental conditions in the laboratory from 2002 to 2004 as compared to their natural environment (Fig. 4). Most (87%; n=27) of the age-2 hatchery-reared fish showed two opaque growth zones on their otoliths, while 12% (n=4) showed only one visible opaque zone. The mean ± SD distance from the otolith core to the first opaque zone was 1.2±0.21 mm, to the second was 1.7± 0.21 mm, and to the otolith edge was 1.9±0.16 mm (Fig. 5).

Table 1 Recaptured red snapper *Lutjanus campechanus*, from the Northern Gulf of Mexico

Fish	Mark date	Recapture date	Years out	Opaque zones	Move	Start wt (kg)	End wt (kg)	Growth (kg/yr)	Start SL (mm)	End SL (mm/yr)	Growth (mm/yr)
1	12-Jan-06	1-Jul-08	2.47	2.00	no	1.87			410	570	64.82
2	11-Jul-06	21-Jul-08	2.03	2.00	no	3.70			500	620	59.11
3	11-Aug-06	25-Jul-07	0.95	1.00	no	1.96			410	515	110.1
4	14-Aug-06	16-Jul-08	1.92	2.00	no	2.25	5.64	1.76	399	543	74.87
5	21-Aug-06	12-Jul-08	1.89	2.00	no	1.50	2.69	0.63	334	471	72.37
6	21-Aug-06	16-Jul-08	1.90	2.00	no	2.00	5.27	1.72	379	544	86.65
7	21-Aug-06	20-Nov-07	1.25	2.00	no	1.75	2.78	0.82	359	454	76.04
8	24-Aug-06	14-Nov-07	1.22	2.00	no	1.25			340	423	67.77
9	24-Aug-06	6-Jun-09	2.79	3.00	yes	0.94			318	553	84.34
10	28-Aug-06	12-Feb-07	0.46	1.00	no	1.50	1.75	0.54	309	347	82.56
11	28-Aug-06	8-Jul-08	1.86	2.00	no	2.00			376	509	71.39
12	10-Nov-06	15-Jan-08	1.18	2.00	no	1.13			330	442	94.85
13	7-Feb-07	26-Jun-09	2.38	2.00	no	1.75			360	528	70.48
14	12-Feb-07	20-Jul-08	1.44	1.00	no	2.25			393	495	71.05
15	12-Feb-07	29-Nov-07	0.79	1.00	no	2.00	2.90	1.13	398	465	84.33
16	3-Apr-07	26-Jun-09	2.23	2.00	no	2.50			430	584	68.97
17	3-Apr-07	8-Jun-09	2.18	2.00	no	2.25			410	558	67.78
18	21-May-07	6-Mar-10	2.79	3.00	no	2.50	6.22	1.33	435	605	60.83
19	9-Jul-07	14-Jul-09	2.02	2.00	no	2.00	5.23	1.60	405	568	80.84
20	13-Jul-07	2-Aug-08	1.06	1.00	no	3.25			490	573	78.48
21	25-Jul-07	12-Aug-09	2.05	2.00	no	1.75			408	524	56.53
22	29-Aug-07	2-Aug-08	0.93	1.00	no	3.80			515	557	45.22
23	29-Aug-07	2-Aug-08	0.93	1.00	no	3.80			510	568	62.45
24	5-Sep-07	2-Aug-08	0.91	1.00	no	3.50			500	559	64.86
25	13-Nov-07	3-Jul-09	1.64	1.00	no	5.00	7.57	1.57	565	642	47
26	14-Nov-07	18-Jul-09	1.68	1.00	no	3.00			469	579	65.6
27	20-Nov-07	12-Jun-09	1.56	2.00	no	2.80			460	585	80.04
28	18-Dec-07	19-May-08	0.42		no	2.00	2.39	0.93	406	504	233.8
29	8-Feb-08	6-Jul-09	1.41	1.00	no	3.25			490	540	35.51
30	8-Feb-08	16-Jul-09	1.44	1.00	no	2.50			465	545	55.73
31	10-Jun-08	30-Jun-09	1.05	1.00	no	7.75	9.09	1.27	655	692	35.08
32	21-Oct-08	1-Aug-09	0.78	1.00	no	4.75			543	596	68.12
33	21-Oct-08	19-Jul-09	0.74	1.00	no	3.70			535	564	39.06
34	21-Oct-08	26-Feb-10	1.35	2.00	no	2.70	4.42	1.27	460	528	50.34
35	26-Nov-08	19-Jun-09	0.56		no	5.00	5.45	0.80	577	596	33.83

Discussion

Mark recapture

Past estimates of growth rate in red snapper appeared similar to present estimates. Szedlmayer and Shipp (1994) reported growth of tagged red snapper at 80.3 mm TL yr^{-1}, and 98.6 mm TL yr^{-1} from a length-at-age regression of fish less than 10 years of age. Patterson et al. (2001) reported red snapper growth at 86.9 mm TL yr^{-1} for tagged fish, and 87.6 mm TL yr^{-1} from length-at-age regression. These previous rates were similar to the present rate (72 mm SL yr^{-1}) after adjusting for differences

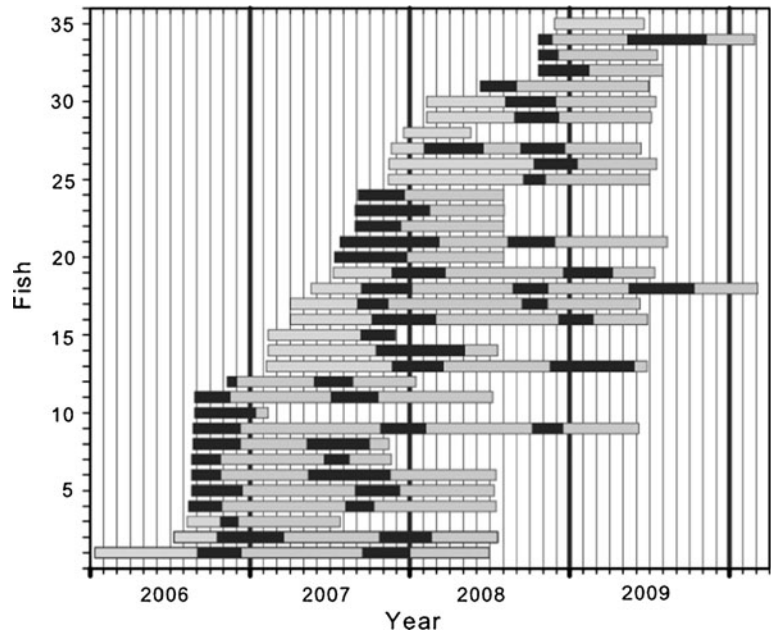


Fig. 2 Timing of opaque zone (*black bars*) and translucent zone (*light grey bars*) formation for all recaptured red snapper

between TL (past studies) and SL (present study). Therefore the OTC injection and marking appeared to have little or no effect on red snapper growth. One aspect of growth rate for red snapper in the present study that was unique was the large range (33. 8 to 233.8 mm SL yr^{-1}). This range was based on actual measures with little variance by the present study rather than estimates usually based on otoliths aging. This nearly 7-fold difference in growth rate clearly indicates that caution should be applied when converting length measures to ages as commonly practiced in stock assessments.

The OTC mark was clearly visible on all otoliths in fish recaptured by the present study, however, OTC marks were absent on some otoliths (n=4) returned by private fishers. These 4 fish were not part of the 35 recaptures considered in this study. 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

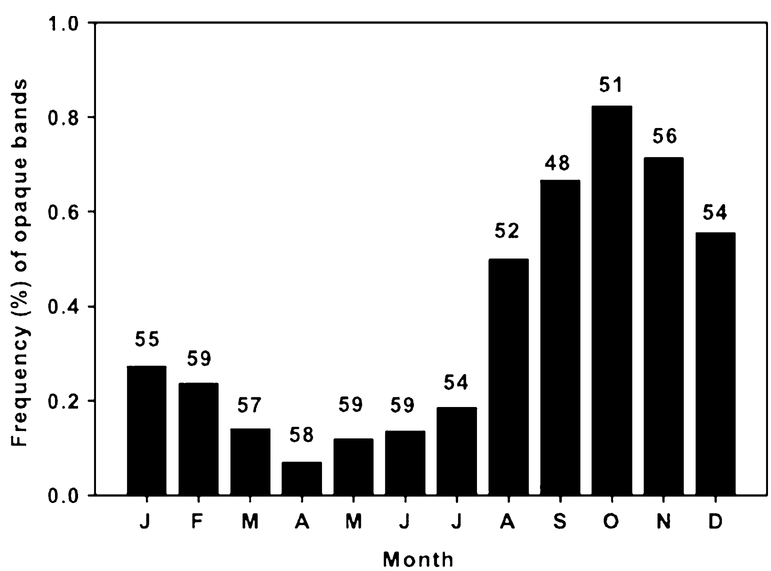
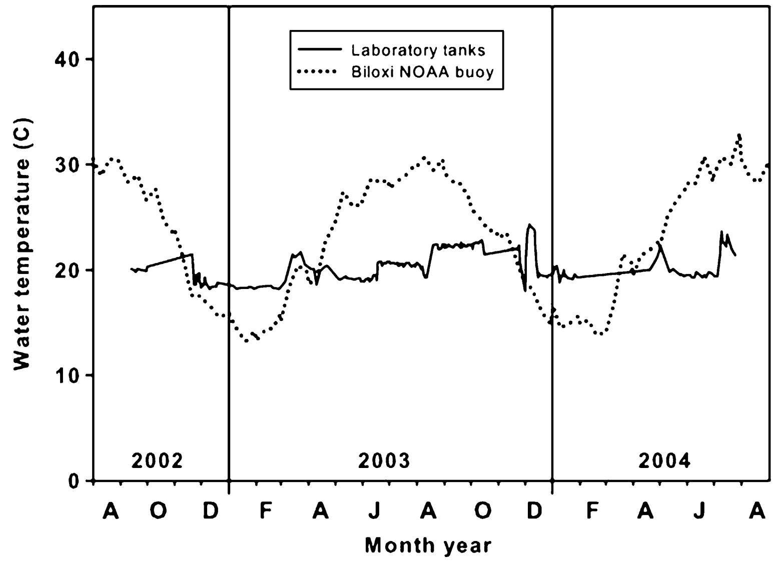


Fig. 3 Percent frequency of opaque zones for all recaptured red snapper for each month, pooled over years. Numbers above bars are the total number of instances that a fish was at liberty for each month

Fig. 4 Laboratory temperature over time for laboratory held red snapper compared to offshore surface temperatures from a NOAA data buoy



cement used to attach the otolith to the microscope slide showed a slight fluorescence under blue-violet light. Another difficulty was that the private fishers returning the other two recaptures were fishing from for-hire charter boats, and it is likely that the tagged fish were lost among the many other fish on the vessel, especially after fish had been dressed for clients. It was unlikely that the OTC failed to leave a mark after injection 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 growth increments in sectioned red snapper otoliths were formed annually in fish at least up to age-10 and supports past studies showing annual periodicity of otolith increment formation; however, the timing of opaque zone formation in the present study differed considerably from other estimates. Marginal increment analysis was used to show winter to spring opaque zone formation by Patterson et al. (2001) and Wilson and Nieland (2001), and spring to summer formation by Allman et al. (2005). An opaque zone was formed once per year during the fall months in the present OTC mark-and-recapture study, which occurred at the end of the red snapper spawning season (May to October). Some of the otoliths from recaptured fish showed an early winter opaque zone but none showed a late winter-spring opaque zone formation. The advantage of the present study was that the methods allowed for direct assessment of periodicity and timing of opaque zone formation in red snapper otoliths unlike the indirect methods described above. Thus, a late summer—fall opaque zone formation is a more accurate assessment of red snapper otolith growth patterns.

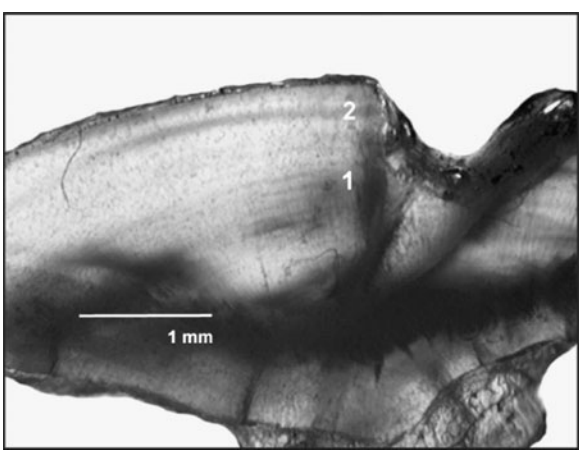


Fig. 5 Otolith section from a red snapper that was hatchery reared for 2 years in captivity. Numbers refer to the first and second opaque zones

There are several explanations for the discrepancy in timing of opaque zone 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 is especially dependent on the quality of the sectioning and polishing of each otolith. Also, the edge condition may be opaque when examined 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 zone 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 similar rates compared to other studies on red snapper growth, and otoliths showed no apparent differences in increment patterns. Another point of possible contention is that opaque and translucent zones grow at different rates within a year. We agree that this occurs, but this rate variation would not change the mid points of the opaque zones. For example, if the translucent zone was growing faster compared to the opaque zone, then the time period estimates of opaque zone formation would actually be longer and expand in both directions, but not change the general conclusion of late summer—fall formation.

Another possible difficulty with the present study is that otolith increment deposition rates change over years with younger fish usually showing much wider increments compared to older fish. Thus, with rates changing over years it may be difficult to estimate the time of opaque zone formation for any time periods other than at mark and recapture. We agree that differences are substantial for young age red snapper (approximately first 2 years) but for older (>2 years) fish used in this study increment widths become very similar, for example, little difference was apparent between years for fish at liberty for two or more years (Fig. 2). Also, even if younger increments showed faster growth, the result of accounting for this would be a shift of the opaque zones towards earlier in the late summer months not towards the winter months.

The most compelling evidence of a late summer early fall opaque zone formation was based on an examination of the time of OTC marking and recapture and the occurrence of opaque zone formation. Most (89%) fish that were released from July to October (n=18) showed an opaque zone exactly on the OTC time mark and a translucent zone that usually started to form before January. In contrast, fish showed a translucent zone when they were released at other times of the year, especially in the winter when they were "supposed to" be forming an opaque zone (n=13) (Fig. 2).

A final explanation for the discrepancy in timing of opaque zone formation is that opaque zones may be formed in both winter and summer months resulting in two opaque zones formed per year. This has been shown in grey triggerfish (*B. capriscus*) (Ingram 2001) and tilapia (*O. niloticus*) (Admassu and Casselman 2000). One fish in this study (fish-7) showed a distinct pattern of false annuli formed just prior to the annulus when the otolith was read along an axis 30° from the sulcus (Fig. 6). This otolith actually showed two opaque zones formed during the year of liberty for this fish when read along this alternative axis. These secondary zones were distinguishable from the annulus based on increment width and that they did not extend entirely to the sulcus. It is important to be able to distinguish these secondary zones from annual increments for accurate ageing of red snapper.

A similar OTC mark-and-recapture study on sablefish (*A. fimbria*) first reported annular growth increment validation from recaptures a few years after initial mark and release, but recaptures 20 years after initial release indicated more than one increment formed per year in sectioned otoliths (Beamish and 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,

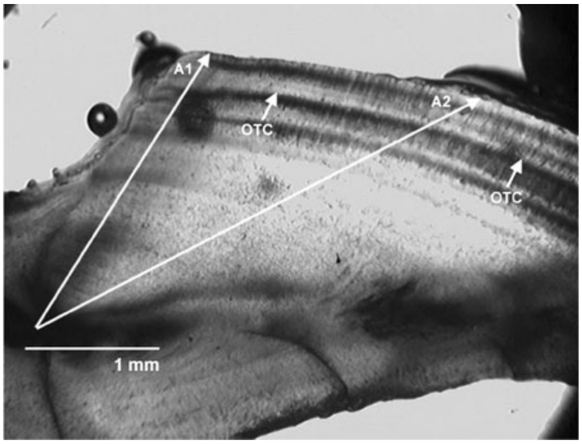


Fig. 6 Whole otolith sections showing a OTC mark location from a recaptured red snapper (fish 7; Table 1; Fig 3), viewed under white light. The OTC mark was made on 21 Aug 06. The transect A2 shows the location of false annuli (extra opaque zones). Edge of the otolith was 20 Nov 07

were mistaken as annuli in older fish. It was observed that as sablefish growth slowed with age, annual growth increments 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 zones is generally assumed; however, the present study and a number of other studies have associated opaque zone formation with the spawning period. Opaque zones were formed in April through June for yellowtail snapper (*Ocyurus chrysurus*) from south Florida, which corresponded with their peak spawning period of May through July (Johnson 1983). Spring formation of opaque zones in otoliths of damselfish (*Pomacentrus mollucensis*) 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*) found in warm-temperate waters off South Africa showed opaque zone formation during the spawning season by a combination of OTC mark-and-recapture and marginal increment analysis methods (Brouwer and Griffiths 2004). Water temperature was not correlated with opaque zone formation in that study, although both photoperiod and gonadosomatic index (GSI) were positively correlated with opaque zone deposition. Finally, the fall opaque zone formation in the present study was more consistent to early studies of red snapper that predicted summer annuli formation from scales and indicated that "annuli formation does not appear to associated with winter temperature minima" (Nelson and Manooch 1982), and both Camber (1955) and Moseley (1966) suggested that feeding cessation during spawning accounted for annulus formation.

Many questions remain about what causes annual growth increment patterns in otoliths. Considering the recaptures in this study were all relatively young adults (<11 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 increment 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 zone per year. This study validates the method of reading sectioned otoliths for ageing of juvenile red snapper up to age-2. The fact that otoliths were from fish of known-age aided in the identification of the first and second annulus. The first opaque zone was not as distinct as the second opaque zone. It was a more diffuse broad opaque zone compared to the thinner darker second opaque zone. The first opaque zone was most clearly identified along the ventral edge of the sulcus in the sectioned otolith and located approximately 1.2 mm from the core. This measurement is similar to the location 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 opaque zones 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 not well understood (Campana 2005). It is often reported that the slow growth (opaque zones) occurs during winter months, corresponding to periods of lower water temperature and diminished food supply (Beamish and McFarlane 2000), but spawning periods have also been correlated with the timing of opaque zone deposition (Admassu and Casselman 2000; Ingram 2001; Bernardes 2002). 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 August 2002 to 2004 (Fig. 4). 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 zone formation in *A. argyrozona* and has been shown to affect daily growth increment formation (Mugiya 1987; Brouwer and Griffiths 2004).

Acknowledgments We thank A Chapin, J Conti, D Miller, C Simmons and D Topping for help with laboratory and field collections. A special thanks to all the private fishers that returned tagged red snapper. This project was funded by Marine Resources Division, Alabama Department of Conservation and Natural Resources. This is a contribution of the Alabama Agricultural Experiment Station, and the Department of Fisheries and Allied Aquacultures, Auburn University. All work was carried out under Auburn University Animal Care and Use Committee approval, under Protocol 2006–0959.

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